MOLECULAR DEFINITION OF STROMAL CELL - STEM CELL INTERACTIONS

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DEDICATION

This thesis is dedicate to the memory of my very dear brother, Robbie.
A truly wonderful brother and friend.

Robert Lawrence Dean Zannettino
(1965-1987)
"To know him, was to love him"

DECLARATION

This thesis contains no material which has been accepted as full or part requirement for the award of any other degree or diploma in any other University. To the best of my knowledge, this thesis contains no material previously published or written by any other person, except where due reference is made in the text.

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I give consent to this copy of my thesis, when deposited in the University library, being available for photocopying and loan.

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JUST A THOUGHT

"Nothing in the world can take the place of persistence
Talent will not; nothing is more common than unsuccessful men with talent.
Genius will not; unrewarded genius is almost a proverb.
Education will not; the world is full of educated derelicts.
Persistence and determination alone are omnipotent.
The slogan 'Press On' has solved and will always solve the
problems of the human race."

Calvin Coolidge (1872-1933)-Republican (Term: 1929-1933)

SUMMARY

In the adult, haemopoiesis is restricted to the extravascular compartment of the bone marrow (BM) where primitive haemopoietic stem cells (HSC) and their clonogenic progeny develop in intimate contiguity with a heterogeneous population of stromal cells that comprise the haemopoietic microenvironment (HM). Although the importance of cellular interactions between primitive haemopoietic progenitor cells (HPC) and marrow stromal cells is well established, precise definition of the nature of many of these interactions at the molecular level is lacking and remains an objective that is of fundamental importance to our understanding of the regulation of haemopoiesis. Current data suggest that a wide variety of cell surface molecules (CSMs) representing several adhesion molecule superfamilies including integrins, selectins, sialomucins and the immunoglobulin gene superfamily are involved in supporting cell-cell and cell-extracellular matrix (ECM) interactions.

The data presented in this thesis is in large part directed toward the molecular characterisation of CSMs that mediate interactions between human HPC and cells of the BM stroma. More specifically, it focuses on (i) The role of selectins in the regulation of haemopoiesis; (ii) The identification and molecular characterisation of novel structures expressed at the surface of primitive human HPC and cultured BM stromal cells utilising a newly developed retroviral expression cloning strategy; (iii) The molecular characterisation of the antigen identified by the mAb HCC-1 which delineates a subset of the $CD34^+$ cell population that exhibit multipotentiality and *in vitro* stem cell attributes, and; (iv) The molecular cloning of a novel mucin-like transmembrane glycoprotein termed MGC-24v which is expressed by both candidate HSC and elements of the bone marrow stroma.

P-, E- and L- selectin represent a family of three structurally related integral membrane glycoproteins that regulate leukocyte adhesion to platelets and/or endothelium during inflammation by means of selective protein-carbohydrate interactions mediated by

the lectin-like domain at the N-terminus of each family member. Data presented in Chapter 3 demonstrate that, in addition to the well documented role as a CAM for mature leukocytes, P-selectin also supports the adhesion of candidate human haemopoietic stem cells and of their lineage-restricted clonogenic cell progeny (CFU-GM, BFU-E). Moreover, purified platelet-derived or cell-associated P-selectin bound all precursors of committed myeloid progenitors (pre-CFU), as assayed by their ability to sustain haemopoiesis in both conventional stroma containing and stroma-free, cytokine-dependent systems. Binding of $CD34^+$ cells to P-selectin was temperature independent and shear-resistant, occurred only in the presence of divalent cations, was protease sensitive and was completely blocked by anti-P-selectin antibody. Neuraminidase treatment of $CD34^+$ cells completely abrogated their binding to P-selectin implying a prominent role for sialic acid in the structure and function of the P-selectin ligand on haemopoietic progenitors.

During the course of this study, a mucin-like glycoprotein counter-receptor for P-selectin termed P-selectin Glycoprotein Ligand (PSGL-1) was molecularly cloned from the promyelocytic leukaemia cell line HL60. Several lines of evidence demonstrate that PSGL-1 is expressed by $CD34^+$ HPC and represents the sole ligand for P-selectin on these cells, including; (i) BM-derived $CD34^+$ cells contain PSGL-1 mRNA and express PSGL-1 protein as shown by RT-PCR and flow cytometric analysis, respectively, and; (ii) Adhesion of $CD34^+$ cells to P-selectin is abolished by treatment with either an anti-PSGL-1 antibody or the highly-specific protease, mocarhagin.

The consequences of adhesion to P-selectin on the growth and development of HPC was also examined. Notably, adhesion to P-selectin was associated with a >90% inhibition of both nucleated cell number and colony formation, suggesting that ligation of PSGL-1 on $CD34^+$ cells, results in the transmission of, as yet undefined signals via the PSGL-1 cytoplasmic domain. This therefore represents the first report establishing a role for P-selectin as an adhesion molecule for human $CD34^+$ HPC. Moreover, despite the evidence for additional protein structures which may serve as P-selectin ligands on mature myeloid cells, the results presented herein strongly suggest that PSGL-1 is the sole

P-selectin ligand expressed by committed and primitive human haemopoietic progenitor cells. Finally, these studies imply a key role for PSGL-1 as a signalling molecule on primitive HPC, however, additional work is required to elucidate the nature of this signalling function.

Human L-selectin (CD62L) represents the homologue of the 90 kD murine peripheral lymph node homing receptor, originally identified by the monoclonal antibody (Mab) MEL-14. Although initially described as a lymphocyte homing receptor, it was subsequently shown to be constitutively expressed on most other peripheral blood leukocytes including neutrophils and monocytes, and shown to be involved in leukocyte traffic in the systemic microcirculation. The data presented here demonstrates that in addition to mature leukocytes, L-selectin is also expressed by haemopoietic progenitor cells. Myeloid progenitor cells (CFU-GM) were restricted to a population of $CD34^+$ cells which exhibited high level L-selectin expression ($CD34^+CD62L^{BRIGHT}$), whilst erythroid progenitors (BFU-E) were present almost entirely in the fraction which displayed low levels of L-selectin expression ($CD34^+CD62L^{DIM}$). Moreover, hierarchically more primitive progenitor cells with the capacity to initiate and sustain haemopoiesis *in vitro*, were present exclusively in $CD34^+$ cells which expressed L-selectin at low levels ($CD34^+CD62L^{DIM}$).

In addition, data presented herein demonstrates that L-selectin-specific adhesion of $CD34^+$ cells to BM stromal cells can be manifestly enhanced by prior exposure of cultured BM stromal cells to TNFα. Preliminary data suggests that this inducible ligand for L-selectin is unlikely to be the recently cloned glycosylation dependent cell adhesion molecule-1 (GlyCAM-1), however further studies are required to identify and characterise the nature of this CSM.

To identify additional molecules that participate in the regulation of haemopoiesis, a panel of mAbs reactive with tissues of haemopoietic origin were generated. The mAbs were selected due to their demonstrable effects in the regulation of haemopoiesis (refer to Chapter 6) or their unique reactivity profile with tissues of the

haemopoietic system (refer to Chapter 5). To determine the nature of the molecules identified by these mAbs, a molecular cloning approach was employed. cDNA expression cloning using retroviral vectors provides a means of stably introducing genes into target cells at efficiencies which surpass those achieved by transfection. Furthermore, retroviral vectors allow for the introduction and expression of complex cDNA libraries in a wide range of cell types, including cells of haemopoietic origin. Chapter 4 details the development of a novel method for rapidly isolating genes encoding CSMs from a human bone marrow stromal cell cDNA library constructed in the retroviral vector, pRUF*neo*. Furthermore, a highly efficient selection strategy utilising monoclonal antibodies (mAbs) and antibody-coated magnetic beads was developed and used to isolate six cDNAs encoding previously defined CSMs, including β1 integrin and Endoglin. Following the validation of such an approach, this technique was subsequently employed to isolate cDNA clones corresponding to surface (glyco)proteins expressed at the HBMSC and HSC surface recognised by mAbs of undefined specificity. The results obtained constitute the final two chapters of this thesis, and are briefly summarised below.

Monoclonal antibodies to CSM expressed on subsets of bone marrow cells have proven useful as markers of haemopoietic cell function. BM cells can be sorted according to the binding of mAbs and the haemopoietic functions of the resulting populations compared. For example, the finding that all BM progenitor cells are contained within the subset which is bound by monoclonal antibodies to the CD34 molecule, has greatly facilitated their isolation and study and has shown them to comprise a hierarchy of closely related, yet functionally heterogeneous cell populations. Antibodies which further subset the $CD34^+$ cell population are of special interest since they can provide a means to describe and isolate functional subsets found within the $CD34^+$ progenitor population at low frequency. Chapter 5 deals with a novel mAb reagent HCC-1, which exhibits limited reactivity with both peripheral blood and BM-derived cells. More significantly, HCC1 delineates a subset of the $CD34^+$ cell population which functionally exhibit multilineage engraftment potential in the chimeric SCID-hu mouse model. The cloning of a cDNA

encoding the CSM recognised by this monoclonal antibody was achieved following screening of a human BM stromal cell cDNA library and found to correspond to the CD59 molecule. CD59 is a 18-20 kD phosphoinositol glycan-linked membrane protein which protects cells against autologous complement attack. Previously published work relating to this molecule have demonstrated uniform expression of this molecule by most nucleated cells of the haemopoietic system, including all the bone marrow-derived $CD34^+$ cells. In contrast however, the HCC-1-defined epitope of CD59 was found to be differentially expressed among $CD34^+$ progenitors with the highest level present on a subset which is highly enriched for pluripotent stem cells. Studies to elucidate the physical basis for this differential expression of the HCC-1 epitope on CD59 molecules support the hypothesis that the binding of the HCC-1 mAb is physically obstructed as a result of the association of CD59 with an unrelated 80 kD (glyco)protein (gp80) whose expression through haemopoietic cell development appears to be differentiation-related. Further studies are required to define the nature of this molecule.

Mucin-like molecules represent an emerging family of glycoprotein molecules of the haemopoietic system, including CD34, CD43 (leukosialin, sialophorin), CD45RA, CD68, CD96 (Tactile), GlyCAM-1, PSGL-1, and MAdCAM-1. An adhesive function for mucins can be inferred from the recent molecular cloning of glycoprotein counter-receptors for members of the selectin family. Chapter 6 describes the isolation of a cDNA clone that encodes a novel mucin-like, transmembrane glycoprotein termed MGC-24v, expressed by both haemopoietic progenitor cells and elements of the bone marrow stroma. MGC-24v was identified using the abovementioned expression cloning strategy and 2 novel mAb reagents referred to as 9E10 and 105.A5. Both antibodies detected MGC-24v protein expression by subpopulations of the $CD34^+$ cells which include the majority of clonogenic myeloid (CFU-GM) and erythroid (BFU-E) progenitors and the hierarchically more primitive precursors (pre-CFU). Biochemical and functional characterisation of MGC-24v, revealed that this protein exists as a homodimeric molecule of 160 kD and can augment the adhesion of $CD34^+$ cells to bone marrow stroma. Finally, like PSGL-1,

signals through MGC-24v resulted in the suppression of *in vitro* haemopoiesis, a feature that appears common to mucin-like molecules.

PUBLICATIONS

A. ABSTRACTS AND CONFERENCE PRESENTATIONS

Hanson Symposium-Cellular Signalling, Adelaide, South Australia, November 1992.
Primitive Human Haemopoietic Progenitors Exhibit Multiple Adhesive Specificities: Implications For Homing, Retention, Regulation And Release From The Bone Marrow.
PJ Simmons, S Niutta, A Zannettino, LK Ashman, MA Vadas, MC Berndt and CA Juttner.

International Congress On The Regulation Of Leukocyte Production And Immune Function, Sydney, Australia, December 1993.
Use of a Retroviral cDNA expression library to clone genes encoding novel human stromal cell antigens.
ACW Zannettino, JR Rayner, LK Ashman, TJ Gonda and PJ Simmons.

International Congress On The Regulation Of Leukocyte Production And Immune Function, Sydney, Australia, December 1993.
Molecular Definition Of The Adhesive Interactions That Regulate Haemopoietic Stem Cells.
PJ Simmons, ACW Zannettino and DI Leavesley.

International Congress On The Regulation Of Leukocyte Production And Immune Function, Sydney, Australia, December 1993.
Molecular Cloning Of The cDNA Encoding a Novel Human Platelet/Megakaryocyte Surface Antigen.
S Fitter, MC Berndt, ACW Zannettino and LK Ashman.

International Society For Hematotherapy And Graft Engineering. (ISHAGE), 1994.
Isolation, Characterisation And Functional Activity Of Human Marrow Stromal Progenitors In Haemopoiesis.
PJ Simmons, S Gronthos, ACW Zannettino and S Graves.

International Leukocyte Typing Workshop V, Boston, MA., USA, 1994.
Properties Of Antibodies Directed Against Membrane-Associated SCF.
Zannettino ACW, Leavesley DI, Aylett G, Simmons PJ, Ashman LK.

Hanson Symposium, Haemopoiesis: Biology and Manipulation, Adelaide, South Australia, November 1994.
Primitive Human Haemopoietic Progenitors Adhere To P-Selectin (CD62P). ACW Zannettino, MC Berndt, MC Butcher, C Butcher, EC Butcher, MA Vadas and PJ Simmons.

Hanson Symposium, Haemopoiesis: Biology and Manipulation, Adelaide, South Australia, November 1994.
A Novel Strategy For The Molecular Cloning Of Cell Surface Antigens.
Zannettino ACW, Rayner JR, Ashman LK, Gonda TJ and Simmons PJ.

American Society Of Haematology (ASH) Conference: Annual Meeting. Seattle, Washington, December, 1995. (accepted for oral presentation).
Identification and Functional Cloning of MGC-24, A Mucin-Like Molecule Expressed By Haemopoietic Progenitors and Bone Marrow Stromal Cells: A Negative Regulator Of Haemopoiesis.
ACW Zannettino, H-J Buhring, S. Niutta, LK Ashman, L Kanz and PJ Simmons.

American Society Of Haematology (ASH) Conference: Annual Meeting. Seattle, Washington, December, 1995.
Peanut agglutinin binding protein MGC-24 is a mucin preferentially expressed on erythroid cells and a subset of $CD34^+$ bone marrow cells
H-J Bühring, ACW Zannettino, S Scheding, I Rappold, PJ Simmons and L Kanz.

B. SCIENTIFIC PUBLICATIONS

Simmons PJ, Zannettino ACW, Gronthos S, Leavesley DI (1994). Potential adhesion mechanisms for localisation of haemopoietic progenitors to bone marrow stroma. Leukaemia and Lymphoma 12: 353-363.

Simmons PJ, Gronthos S, Zannettino ACW, Ohta S, Graves S (1994). Isolation, Characterisation And Functional Activity Of Human Marrow Stromal Progenitors In Haemopoiesis. Advances in Bone Marrow Purging and Processing, Wiley-Liss Press pp 271-280.

Zannettino, A.C.W., Leavesley, D.I., Aylett, G.W., Simmons, P.J. and Ashman, L.K. (1994) Properties of antibodies directed against membrane-associated human SCF. in Leukocyte Typing V (ed S. Schlossman), Oxford University Press, Oxford, pp 17-18.

Zannettino ACW, Berndt MC, Butcher C, Butcher EC, Vadas MA and Simmons PJ . (1995) Primitive Human Haemopoietic Progenitors Adhere To P-Selectin (CD62P). Blood. 85: 3466-3477

Zannettino ACW, Aylett G, Leavesley DI, Pietsch T, Chang D, Simmons PJ, Ashman LK (1996) Specificity and Functional Effects of Antibodies to Human Stem Cell Factor. Growth Factors (in press).

Zannettino ACW, Rayner JR, Ashman LK, Gonda TJ and Simmons PJ. (1996). A Powerful New Technique For Isolating Genes Encoding Cell Surface Antigens Using Retroviral Expression Cloning. J. Immunol., 156: 611-620

Hill B, Rozler E, Travis M, Chen S, Simmons PJ, Zannettino ACW, Galy A, Chen B, Hoffman R. (1995) High Level Expression of a Novel Epitope of CD59 Identifies a Subset of $CD34^+$ Bone Marrow Cells Highly Enriched For Pluripotent Stem Cells. Exp. Hematol. 24: 936.

Fislhie RJA, Zannettino ACW, Gronthos S, Makrynikola V, Henniker AJ, Bendall LJ, Gottlieb DJ, Simmons PJS, and Bradstock KF. (1996). MUC-18, A Member Of The Immunoglobulin Superfamily Is Expressed By Bone Marrow Fibroblasts And A Subject Of Haematological Malignancies. (submitted to Exp. Hematol.).

Rappold, I., Ziegler, BL., Köhler, I., Marchetto, S., Rosnet, O., Birnbaum, D., Simmons, PJ., Zannettino, ACW., Hill, B., Neu, S., Knapp, W., Alitalo, R., Ullrich, A., Kanz, L., Buhring, H-J. (1996). Functional and phenotypic characterization of cord blood and bone marrow subsets expressing FLT3 receptor tyrosine kinase. (submitted to Blood).

Zannettino ACW, Niutta S and Simmons PJ (1996): L-Selectin Mediates the Binding of Human Long-Term Culture-Initiating Cells (LTC-IC) to a TNFα-Inducible Ligand on Marrow Stromal Cells. (In preparation).

Zannettino ACW, Swart B, Hill B, Chen B, and Simmons PJ (1996): Differential Expression of the HCC-1 epitope on CD59: Mechanism For The Preferential Expression by Normal Haemopoietic Progenitor Cells. (In preparation).

Zannettino ACW, Bühring H-J, Niutta S, Rappold, I, Benton, A, Watt, S, and Simmons PJS. (1996). Identification and Functional Cloning of MGC-24v, A Mucin-Like Molecule Expressed By Haemopoietic Progenitors and Bone Marrow Stromal Cells: A Negative Regulator Of Haemopoiesis. (In preparation).

Zannettino ACW, Levesque J-P, Haylock, DN., Berndt, MC., Niutta S, and Simmons PJS. (1996). PSGL-1 Mediated Adhesion Of Human Haemopoietic Progenitors To CD62P Results In The Suppression Of Haemopoiesis Through The Induction Of Apoptosis (In preparation).

ABBREVIATIONS

α-MEM	Alpha Modification of Eagle's Medium
A_{260}	Absorbance at 260nm
A_{280}	Absorbance at 280nm
A_{600}	Absorbance at 600nm
aa	Amino Acid
Abs	Absorbance
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloid Leukaemia
amp	Ampicillin
APS	Ammonium persulphate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
Az	Azide
BCIG	5-bromo-4-chloro-3-indolyl beta-D-galactopyranoside
bFGF	Basic Fibroblast Growth Factor
BFU-E	Burst forming unit-erythroid
BFU-Mk	Burst forming unit-megakaryocyte
bisacrylamide	N,N'-methylene-bisacrylamide
BM	Bone marrow
BMMNC	Bone Marrow Mononuclear cells
BMSC	Bone Marrow Stromal Cells
bp	Base pair(s)
BSA	Bovine Serum Albumin
CAM	Cell adhesion molecule
CD	Cluster Designation or Cluster of Differentiation
cDNA	Complimentary DNA
CFU	Colony forming unit
CFU-Bl	CFU-Blast
CFU-F	CFU-Fibroblast
CFU-GEMM	CFU-Granulocyte, Erythroid, Megakaryocyte, Macrophage
CFU-GM	CFU-Granulocyte/Macrophage
CFU-S	CFU-Spleen
Ci	Curie
CIP	Alkaline calf intestinal phosphatase
CM	Conditioned media
CML	Chronic Myeloid Leukaemia

COOH	Carboxy terminus
cpm	Counts per minute
Cr^{51}	Sodium Chromate
CSF	Colony Stimulating Factor
DAF	Decay Accelerating Factor
dATP	Deoxy-adenosine-5'-triphosphate
dCTP	Deoxy-cytidine-5'-triphosphate
DEPC	Diethyl pyrocarbonate
dGTP	Deoxy-guanosine-5'-triphosphate
DMEM	Dulbeccos modification of Eagles media
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DTT	1,4-Dithiothreitol
dTTP	Deoxy-thymidine-5'-triphosphate
E	Erythrocyte
ECD	Tandem conjugate of PE and Texas Red
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethylene Glycol-bis(β-aminoethyl ether) N,N,N',N'-Tetraacetic Acid
ELISA	Enzyme-linked immunosorbant assay
EMA	Ethidium monoazide
Eo	Eosinophil
EPO	Erythropoietin
EtBr	Ethidium Bromide
FACS	Fluorescence Activated Cell Sorting
FBS	Foetal Bovine Serum
FDC-P1	Factor-dependent cell line (Paterson Institute #1)
FITC	Fluorescein isothiocyanate
FL	Logarithmic fluorescence Intensity
FSC	Forward light scatter
G-CSF	Granulocyte-CSF
G418	Geneticin
GAG	Glycosaminoglycan
GαM	Goat-anti-mouse immunoglobulins
GM	Granulocytes and Monocytes
GM-CSF	Granulocyte/Macrophage-CSF
gp	glycoprotein

GPI	Glycosyl-phosphatidylinositol
GS	Goat serum
HA	Hyaluronic acid
HAT	Hypoxanthine-Aminopterin-Thymidine
HBSS	Hank's Balanced Salt Solution
HEPES	N-2-Hydroxyethylpiperazine N'2-ethane sulphonic acid
HEV	High endothelial venules
HHF	Hanks, Hepes, FBS
HIM	Haemopoietic Inductive Microenvironment
HLA	Human Leucocyte Antigen
HPC	Haemopoietic Progenitor Cell
HSC	Haemopoietic Stem Cell
hr	Hour
HRF	Homologous Restriction Factor
HS	Horse serum
HSPG	Heparin sulphate proteoglycan
i.u.	International Units
ICAM-1	Intercellular adhesion molecule-1
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgG1	Immunoglobulin gamma-1 isotype
IgG2A	Immunoglobulin gamma-2A isotype
IgG2B	Immunoglobulin gamma-2B isotype
IgG3	Immunoglobulin gamma-3 isotype
IgM	Immunoglobulin M
IL	Interleukin
IMDM	Iscoves Modified Dulbecco's Medium
IMVS	Institute of Medical and Veterinary Science
IPTG	Isopropyl-thiogalactoside
kb	Kilo base pairs
kD	Kilodalton
LB	Luria-Bertani broth
LECAM	Leukocyte-Endothelial Cell Adhesion Molecule
LFA	Leukocyte Function-Associated Antigen
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LTBMC	Long term bone marrow culture
LTC-IC	Long term culture-initiating cells
LTR	Long terminal repeat

M	Molar
M-CSF	Macrophage-CSF
mAb	Monoclonal Antibody
Meg	Megakaryocytes
MHC	Major Histocompatibility Complex
min	Minute
mM	Milli Molar
MMLV	Murine Moloney Leukaemia Virus
MPSV	Murine Proliferative Sarcoma Virus
mRNA	Messenger RNA
MWt	Molecular weight
NCAM	Neural Cell Adhesion Molecule
neo	Neomycin
NH_2	Amino terminus
NHS	Normal Human Serum
NP-40	Nonidet P40
O.D.	Optical density
O/N	Overnight
OPC	Oligonucleotide purification column
OPD	o-phenylenediamine.2HCl
PAGE	Polyacrylamide gel electrophoresis
PBL	peripheral blood leukocytes
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phyco-erythrin
PECAM-1	Platelet/Endothelial Cell Adhesion Molecule-1
PEG	Polyethylene glycol
pfu	Plaque forming units
PI	Phosphatidylinositol
PI-PLC	Phosphatidylinositol-specific phospholipase C
PLS	Perpendicular Light Scatter (*refer to SSC*)
PMA	phorbol myristic acid
PMN	polymorphonuclear leukocytes
PNH	Paroxysmal nocturnal haemoglobinuria
rATP	Ribo-adenosine triphosphate
RE	Restriction Endonuclease
Rh123	Rhodamine 123
rHu	Recombinant human
rIg	Rabbit Ig

rMu	Recombimant murine
RNA	Ribonucleic acid
RNase A	Ribonuclease A
RPMI	Roswell Park Memorial Institute
RT	Room temperature
s	Second
SBA	Soy bean agglutinin
SCF	Stem cell factor
SCID	Severe combined immunodeficient
SDM	Serum deprived medium
SDS	Sodium dodecyl sulphate
sIg	Sheep Ig
SSC	Side light scatter
Sulfo-NHS-Biotin	Sulfo-*N*- hydroxysuccinimide-biotin
TC	Tissue culture
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TGFβ	Transforming Growth Factor-β
TNFα	Tumour Necrosis Factor-α
TNFβ	Tumour Necrosis Factor-β
tRNA	Transfer RNA
TX-100	Triton X-100
UV	Ultraviolet light
V-CAM-1	Vascular-Cell Adhesion Molecule-1
v/v	Volume per volume
VLA	Very late acting antigen
w/v	Weight per volume

CONTENTS

CHAPTER 3: STUDIES OF THE ROLE OF SELECTINS IN HUMAN HAEMOPOIESIS

CHAPTER 4: THE DEVELOPMENT OF A NEW TECHNIQUE FOR ISOLATING GENES ENCODING CELL SURFACE MOLECULES USING RETROVIRAL EXPRESSION CLONING

CHAPTER 5: HIGH LEVEL EXPRESSION OF A NOVEL EPITOPE ON CD59 THAT IDENTIFIES A SUBSET OF CD34$^+$ BONE MARROW CELLS HIGHLY ENRICHED FOR PRIMITIVE HUMAN HAEMOPOIETIC PROGENITORS

CHAPTER 6: IDENTIFICATION & FUNCTIONAL CLONING OF MGC-24, A MUCIN-LIKE MOLECULE EXPRESSED BY HAEMOPOIETIC PROGENITORS AND BONE MARROW STROMAL CELLS: A NEGATIVE REGULATOR OF HAEMOPOIESIS

CHAPTER 1

INTRODUCTION

1.1. THE HAEMOPOIETIC SYSTEM.

1.1.1. An Overview.

In adult mammals, the continuous formation of new blood cells, a process known as *haemopoiesis*; (from the Greek-*haima* meaning blood, and *poiesis*, meaning production) occurs within the bone marrow (BM). This complex and dynamic developmental process is ultimately dependent upon the existence of a rare population of cells termed *haemopoietic stem cells* (HSC) (Williams and Nathan, 1991). These cells are characterised by their proliferative potential and capacity to give rise to each of the differentiated cell types found in the peripheral blood and tissues. In the human and murine BM, the HSC pool is estimated to represent between 0.01 and 0.05% of the nucleated cells present within the BM (Harrison *et al*, 1988; Morrison *et al*, 1995a; 1995b).

Under steady state conditions, the majority of primitive haemopoietic stem and progenitor cells (HPC) reside within the BM where they and their progeny develop in intimate contiguity with a phenotypically, and probably functionally, heterogeneous population of *stromal cells* (Dexter, 1979; Weiss, 1976; Lichtman, 1981; Bentley, 1981; 1982a; 1981b; Tavassoli and Friedenstein, 1983; Simmons *et al*, 1987; Allen *et al*, 1990; Simmons and Torok-Storb, 1991a; 1991b). The various cellular elements of the stroma, together with their associated biosynthetic products including *extracellular matrix* (ECM) components and *haemopoietic growth factors* (HGF) constitute the *haemopoietic microenvironment* (HM) of the BM (Trentin, 1970; Tavassoli, 1975; Wolf *et al*, 1968; Wolf, 1979; Dexter, 1979).

The picture that is emerging suggests that the localisation of haemopoiesis to the BM involves developmentally regulated adhesive interactions between primitive haemopoietic cells and this complex stromal cell milieu (Dexter, 1977; Tavassoli and Hardy, 1990; Clark *et al*, 1992; Long, 1992). Despite intensive study, precise definition of the nature of many of these interactions at a molecular level is lacking and remains an objective which is of fundamental importance to our understanding of the regulation of haemopoiesis. Nevertheless, at least two families of molecules have been implicated in the

maintenance of haemopoiesis, namely (i) HGFs and their corresponding receptors, and (ii) cell adhesion molecules (CAMs) and their cognate ligands.

The data presented in this thesis is in large part directed toward the examination of the nature of the molecules that participate in adhesive interactions between human HPC and cells of the BM stroma. The introduction section which follows, represents a brief overview of the literature regarding (i) the development and organisation of the haemopoietic system, (ii) the stromal tissue of the BM, (iii) the role of HGFs, and (iv) the adhesive interactions involved in the regulation of haemopoiesis.

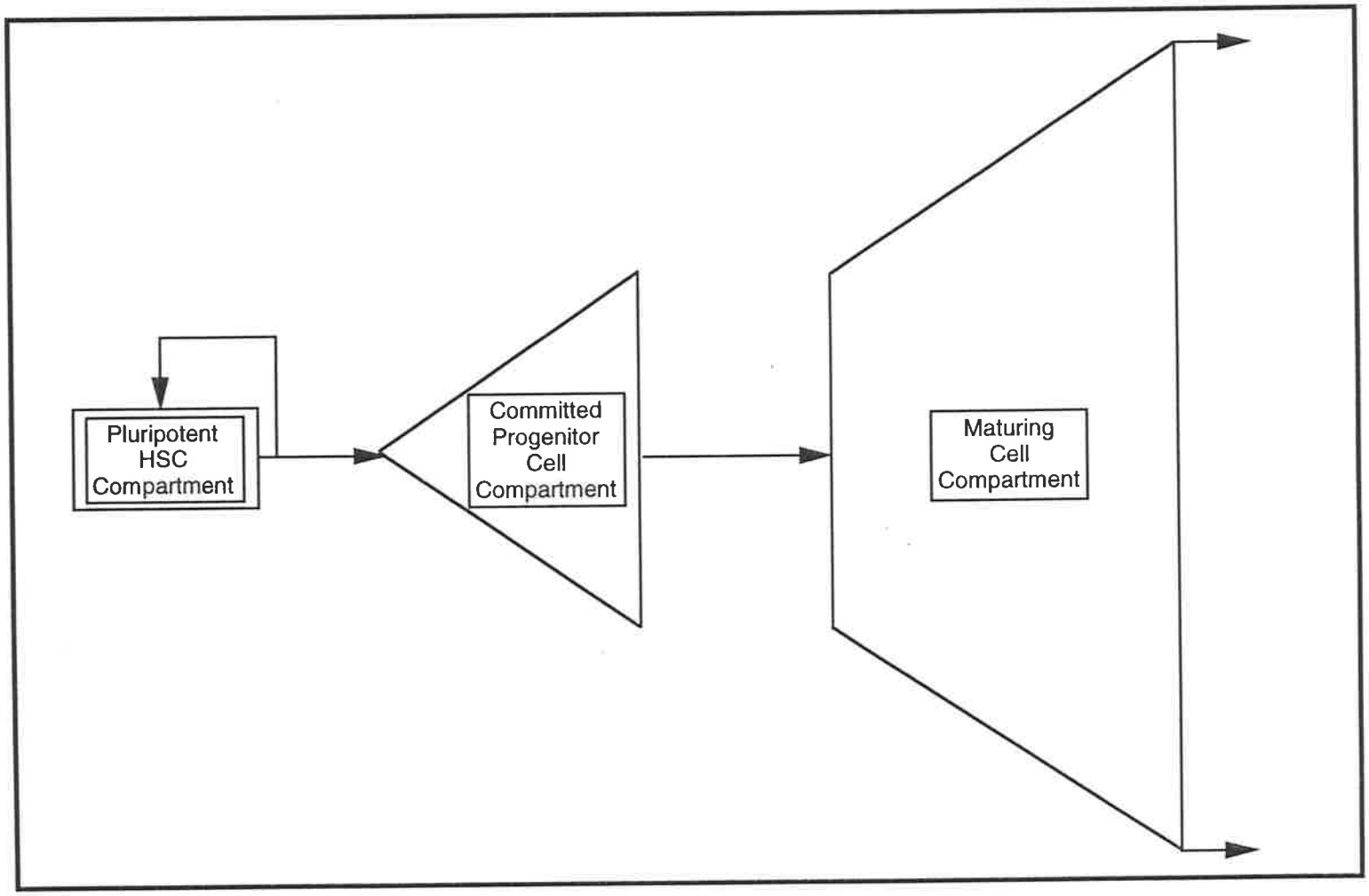
1.1.2. The Haemopoietic System : A Cellular Hierarchy.

The haemopoietic system is visualised as a cellular hierarchy consisting of sequential cell populations which are classified on the basis of differences in their capacity for self-renewal and differentiation. As illustrated in Figure 1.1., the haemopoietic system can be classified into three such sequential cell populations: the stem cells, the committed (or lineage-restricted) progenitor cells and maturing (morphologically recognisable) cells (Lewis and Trobaugh, 1964). The stem cell compartment at the apex of the system gives rise both to stem cells (by self-renewal) and to a series of progenitor cells that generate up to eight different specialised lineages (Watt *et al*, 1987a). Once a stem cell is "triggered" to proliferate, each cell division is accompanied by a progressive loss of proliferative capacity and restriction of differentiation options. Thus, in normal steady state haemopoiesis the size of the stem cell compartment is maintained at a constant level by the balance between stem cell production by self-renewal and stem cell loss by differentiation.

The probability of stem cell self-renewal at the single cell level is thought to be a random or stochastic process. Consequently, it is not possible to predict whether an individual stem cell will self-renew or differentiate (Till *et al*, 1964; Ogawa, 1993). *In vitro* studies involving replating of multipotential progenitors are consistent with a stochastic model of stem cell renewal and differentiation (Humphries *et al*, 1981; Nakahata and Ogawa, 1982). It follows that the probability of self-renewal, "p" of stem cells must be

Figure 1.1. Development Of Haemopoietic Cells: A Cellular Hierarchy.

Haemopoietic cells can be separated into three broad hierarchical compartments including (i) stem, (ii) progenitor and (iii) mature effector cells. Once a stem cell is "triggered" to proliferate, each cell division is accompanied by a progressive loss of proliferative capacity and restriction of differentiation options.


Pluripotent
HSC
Compartment
Committed
Progenitor
Cell
Compartment
Maturing
Cell
Compartment

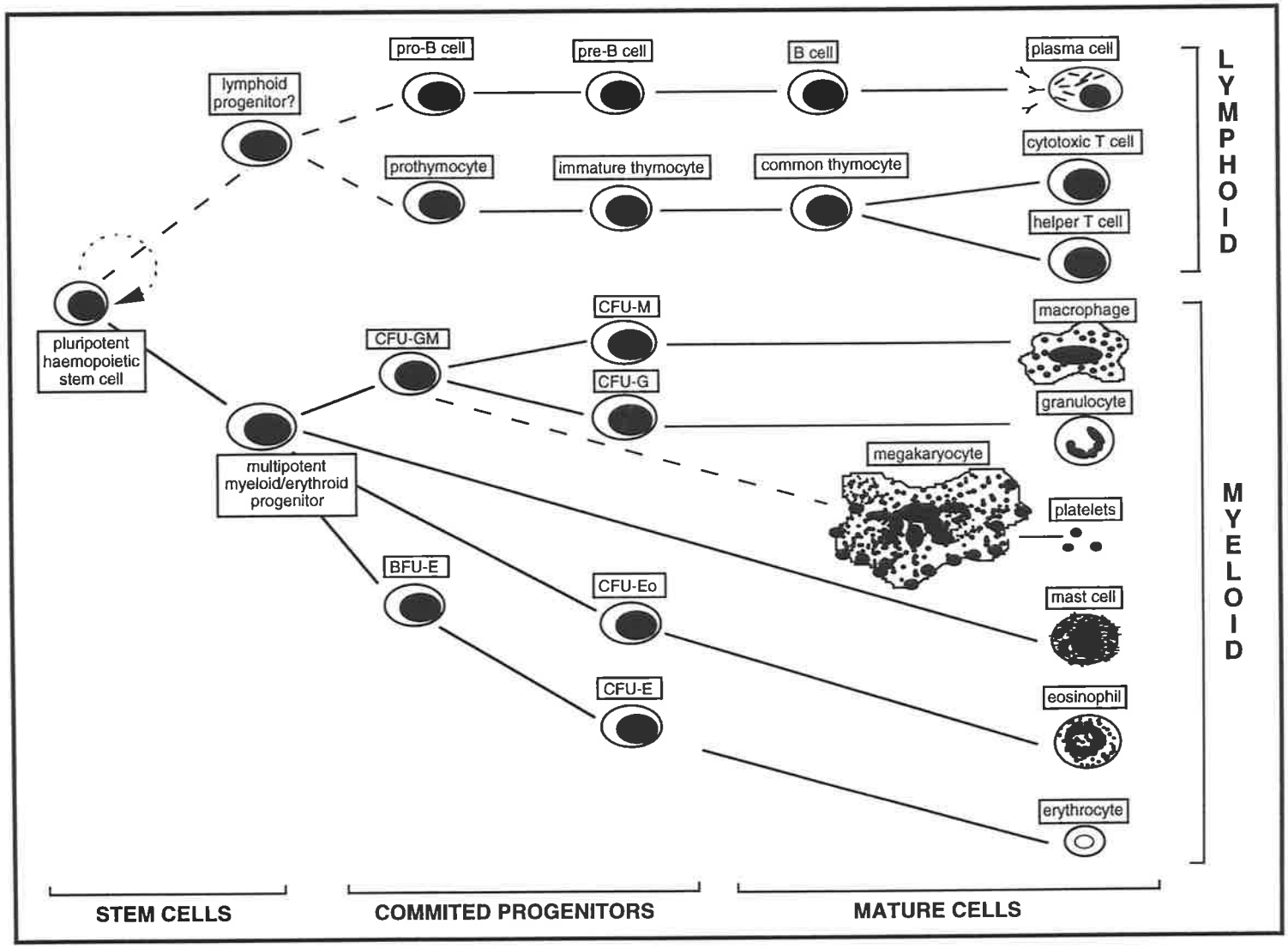
increased to more than 0.5 in order to obtain a net increase in stem cell number (Vogel *et al*, 1968; Nakahata and Ogawa, 1982). An important but unanswered question is whether "p" is fixed or is subject to extrinsic regulation. The control of stem cell proliferation is, at least in part, effected by the locality of the stem cells themselves. In the steady state, the majority of stem cells are dormant in the cell cycle, a resting state, termed G_o, by Lajtha (1979). Therefore, the self-renewal process is associated both with the triggering of cell division of quiescent stem cells and with renewed dormancy in the cell cycle.

Committed progenitor cells represent intermediate stages between the HSC and the morphologically recognisable haemopoietic cell compartment. As their name implies, these cells exhibit a more restricted developmental potential with commitment to one or few lineages. The process of commitment of HSC to a particular lineage is a poorly understood process at the molecular and cellular level. At one extreme, commitment, like self-renewal, is also regarded as a stochastic process (Ogawa, 1993), whereas others have viewed commitment as a rigidly predetermined event accompanied by a sequential loss of lineage potentials (Nicola and Johnson, 1982). Two deterministic models have been proposed to account for the mechanisms of stem cell commitment and include the "haemopoietic inductive microenvironment (H.I.M.)" model (Trentin, 1970) and the "stem cell competition model" (Van Zant and Goldwasser, 1979). The H.I.M. model proposes the existence within the haemopoietic organs of specialised anatomical niches that selectively direct the differentiation of uncommitted progenitors, whilst the stem cell competition model proposes that stem cell commitment is regulated by humoral factors such as erythropoietin and colony-stimulating factors.

The committed progenitor cell compartment in turn gives rise to the mature cell compartment comprising the bulk of morphologically recognisable cells found in the peripheral blood (PB) and tissues, which are in many cases short-lived. These cells include, erythrocytes, platelets, neutrophils, eosinophils, basophils, mast cells, monocytes/macrophages, osteoclasts, Langerhans' cells, Kupffer cells, dendritic cells and natural killer cells (Lewis and Trobaugh, 1964; Johnson and Metcalf, 1977; Nakahata and Ogawa, 1982) (refer to Figure 1.2.).

Figure 1.2. A Model Of The Differentiation Pathways Of Haemopoietic Stem Cells: The Generation Of Morphologically Recognisable Cells.

The primary site of early haemopoietic differentiation in the adult mammal is the bone marrow, which provides the appropriate microenvironment for the continued maintenance and differentiation of pluripotent haemopoietic stem cells. The stem cell compartment at the apex of the system gives rise both to stem cells (by self-renewal) and to a series of multipotent progenitor cells that give rise to lineage-restricted monopotent precursors that in turn differentiate into mature, morphologically recognisable cells. The lymphoid precursors are believed to diverge from the myeloid/erythroid precursors early in the differentiation process. Although a matter of contention, it is postulated that mature B and T lymphocytes originate from a common lymphoid precursor. Furthermore, both T and B lymphocyte differentiation is dependent upon stromal elements found in the bone marrow, thymus and spleen. Restricted myeloid and erythroid precursors are defined primarily through their ability to form colonies in *in vitro* culture (ie. CFU-GM, CFU-G, CFU-M, BFU-E, CFU-E, CFU-Eo) upon stimulation with haemopoietic growth factors.


pro-B cell
pre-B cell
B cell
plasma cell
lymphoid progenitor?
cytotoxic T cell
prothymocyte
immature thymocyte
common thymocyte
helper T cell
LYMPHOID
pluripotent haemopoietic stem cell
CFU-M
macrophage
CFU-GM
CFU-G
granulocyte
megakaryocyte
multipotent myeloid/erythroid progenitor
platelets
BFU-E
CFU-Eo
mast cell
CFU-E
eosinophil
erythrocyte
MYELOID
STEM CELLS
COMMITED PROGENITORS
MATURE CELLS

The hierarchical organisation of the haemopoietic system provides at least two important advantages for regulation. Firstly, the occurrence of a highly regulation-sensitive population (the committed progenitors) between the stem and maturing cell compartments, acts to buffer the stem cell population against depletion through excessive demands. Secondly, the inherent heterogeneity of the committed progenitor compartment provides multiple sites for the action of a variety of different regulators including cytokines and other HGF. Since the bulk of proliferation within the haemopoietic system occurs within the committed progenitor and maturing cell compartments, the haemopoietic system as a whole is able to respond rapidly, and in a highly precise manner to the particular demands imposed upon it.

1.2. THE HAEMOPOIETIC STEM CELL COMPARTMENT.

1.2.1. Haemopoietic Stem Cells: Studies In Rodents.

The notion of a common ancestral precursor or stem cell was invoked to compensate for the continual loss of mature, morphologically recognisable cells such as granulocytes and erythrocytes (Siegers *et al*, 1979). To replace these effete cells, the rate of turnover in an adult human is estimated to be approximately 1 trillion cells per day, including 200 billion erythrocytes (Erslev, 1983) and 70 billion neutrophilic leukocytes (Dancey *et al*, 1976). Although this system operates under tight homeostatic controls, it retains the flexibility to respond rapidly to a variety of perturbations by increasing the production of one or more mature cell types. Studies in rodents have provided researchers with a greater understanding of the regulation of this dynamic process.

In the early 1950's, it was recognised that animals given lethal doses of ionising radiation suffered BM failure which could be reversed by transplantation with unirradiated, histocompatible BM cells (Lorenz, 1951). The subsequent work of Till and McCulloch (1961) represented the first quantitative experiments on BM restoration, using limiting numbers of BM cells to rescue lethally irradiated mice. In a series of seminal experiments, it was recognised that the BM contained highly proliferative progenitor cells capable of generating individual macroscopic nodules in the spleen (and BM) of the

irradiated hosts 7 to 14 days post-injection (Till and McCulloch, 1961; Wu *et al*, 1967; Watt *et al*, 1987a). Numerous studies examining this phenomenon demonstrated that these colonies were derived from a single transplanted cell (Becker *et al*, 1963; Wu *et al*, 1967), termed colony forming units of the spleen (or CFU-S) and contained cells of multiple haemopoietic lineages including erythroid, megakaryocytic and myeloid precursors (Till and McCulloch, 1961; Becker *et al*, 1963, Wu *et al*, 1967).

Cells from individual CFU-S were subsequently shown to be capable of initiating further spleen colonies, not only when infused into secondary recipient mice, but also in multiple lethally irradiated mice following serial transfer of the progeny of the incipient CFU-S (Juraskova and Tkadlecek, 1965; Siminovitch *et al*, 1963; reviewed in Dexter and Spooncer, 1987). In conjunction with the observation that the same cell type had the capacity to repopulate the lymphoid system (Wu *et al*, 1968; Lala and Johnson, 1978), it was proposed that the late CFU-S (day 12 to 14) represented a population of radioprotective cells, capable of multilineage differentiation and self-renewal, and thus for many years was equated with the pluri-potential HSC. Subsequent studies however, have demonstrated heterogeneity within the CFU-S compartment with respect to colony size, time of appearance (day 8 or day 12 to 14) and composition (Magli, 1982; Jones, 1990). Furthermore, serial transplantation of spleen colonies led to the formation of smaller secondary spleen colonies in which fewer lineages were represented, and the continued capacity to generate colonies was exhausted within three or four serial transplantations (Morrison *et al*, 1995a; 1995b).

Recent work has demonstrated that, while both long-term and transiently reconstituting multipotent progenitors appear highly enriched for late CFU-S activity, the majority of day 12 CFU-S are transient progenitors (Morrison and Weissman, 1994; Van der Loo *et al*, 1994). The most primitive, long-term reconstituting HSC are perceived to have "pre-CFU-S" activity (Hodgson and Bradley, 1979; Van Zant, 1984; Ploemacher and Brons, 1988; Spangrude and Johnson, 1990) and are therefore too primitive to form a spleen colony within 12 and 14 days, but do give rise to progenitors that can do so.

Various methods which distinguish donor from host cells have confirmed the early work of Wu and collegues (1967), demonstrating the existence of rare stem cells within normal BM, with the capacity for immense proliferation and multilineage differentiation (Fleischman and Mintz, 1984; Spangrude *et al*, 1988; Spangrude, 1989; Lemischka *et al*, 1986). A particularly valuable approach, utilising recombinant retroviruses to "tag" HSC, has made it possible to uniquely mark putative HSC and simultaneously follow multiple progenitor clones based on unambiguous retroviral integration sites (Lemischka *et al*, 1986; Snodgrass & Keller, 1987, Dick *et al*, 1985; Williams *et al*, 1984; Eglitis *et al*, 1985). Utilising this approach, Fraser and collegues have demonstrated that a single stem cell capable of self-renewal *in vitro,* maintained the capacity for reconstitution of the lymphoid and myeloid compartments of lethally irradiated mice (Fraser *et al*, 1990).

Perhaps the most direct evidence for the existence of HSC capable of long term reconstitution of the myeloid and lymphoid compartments has come from studies which involve the physical isolation of candidate stem cells (reviewed in Visser and van Bekkum, 1990). A number of laboratories have reported the isolation of subsets of early murine haemopoietic cells by means of fluorescence activated cell sorting (FACS), based on monoclonal antibody (mAb) staining of specific cell surface molecules and/or specific dye staining (Hoechst 33342 and rhodamine 123) (Visser and de Vries, 1988; McAlister *et al*, 1990; Li and Johnson, 1992; Bertoncello *et al*, 1985; 1991). Spangrude and colleagues (1988) demonstrated that a minor subpopulation of murine BM cells which exhibited a phenotype of Thy-1^{LO}, lineage marker negative and stem cell antigen-1^{+} (Thy-1^{LO} Lin^{-} $Sca1^{+}$) had the capacity to reconstitute the lymphoid and myeloid compartments in lethally irradiated mice (Spangrude *et al*, 1988). Subsequent studies demonstrated that as few as 30 cells, sorted on the basis of this Thy-1^{LO} Lin^{-} $Sca1^{+}$ phenotype in combination with the low retention of the fluorescent supravital dye rhodamine 123 were able to completely repopulate the myeloid and lymphoid compartments of irradiated recipients for many months post-transplant (Li and Johnson, 1992; Szilvassy *et al*, 1989; Wolf *et al*, 1993; Uchida and Weissman, 1992).

1.2.2. Identification Of Candidate Human Haemopoietic Stem Cells.

Since the long term reconstitution of the haemopoietic system of a lethally irradiated recipient represents the only truly valid means of assaying HSC activity, the identification and quantitation of candidate HSC in human haemopoietic tissues has been hampered by the lack of an equivalent assay system. Despite this, there is a general consensus that human HSC reside within a population of BM-derived haemopoietic cells which express on their cell surface, the CD34 antigen (Sutherland and Keating, 1992). The CD34 antigen is a highly glycosylated, integral membrane glycophosphoprotein with an apparent molecular weight ranging from 110 to 120 kD. It is expressed on small-vessel endothelial cells (Fina *et al*, 1990; Beschorner *et al*, 1985), embryonic fibroblasts (Brown *et al*, 1991a) and at low levels by BM stromal precursors (Simmons and Torok-Storb, 1991a; Huang and Terstappen, 1992). Significantly, this molecule is also expressed on approximately 1 to 3% of normal human BM-derived haemopoietic cells (Civin *et al*, 1984; Katz *et al*, 1985; Andrews *et al*, 1986; Watt *et al*, 1987b; Fina *et al*, 1990; Lansdorp *et al*, 1990), whilst less than 0.5% of the peripheral blood cells exhibit labelling with antibodies to this molecule (Krause *et al*, 1996).

Although the $CD34^+$ cell population from normal human BM is enriched in morphologic blast cells (in contrast to the $CD34^-$ cell fraction), it is now apparent that these cells are heterogeneous in composition and functional properties (Civin *et al*, 1984; Katz *et al*, 1985; Andrews *et al*, 1986). Flow cytometric analysis of the $CD34^+$ subfraction has revealed two distinct populations which differ in their relative levels of surface CD34 expression (Andrews *et al*, 1986; Krause *et al*, 1994; Civin *et al*, 1987; Andrews *et al*, 1989). Utilising *in vitro* assay systems (please refer to Section 1.2.4.), the population of cells exhibiting the highest level of CD34 expression ($CD34^{BRIGHT}$), contained the majority of the immature haemopoietic progenitor cells and was enriched for progenitor cells capable of generating colonies in semi-solid medium, including colony-forming units-granulocyte/macrophage (CFU-GM), blast forming units-erythroid (BFU-E), CFU-erythroid (CFU-E), CFU-megakaryocyte (CFU-Meg), and even more highly enriched for the developmentally earlier, multipotential CFU-granulocyte/erythroid/macrophage/

megakaryocyte (CFU-GEMM) (Civin *et al*, 1989; Bernstein *et al*, 1991a), CFU-blast (Leary and Ogawa, 1987) and long-term culture initiating cells (LTC-IC) (Krause *et al*, 1994; Civin *et al*, 1989; Bernstein *et al*, 1991a).

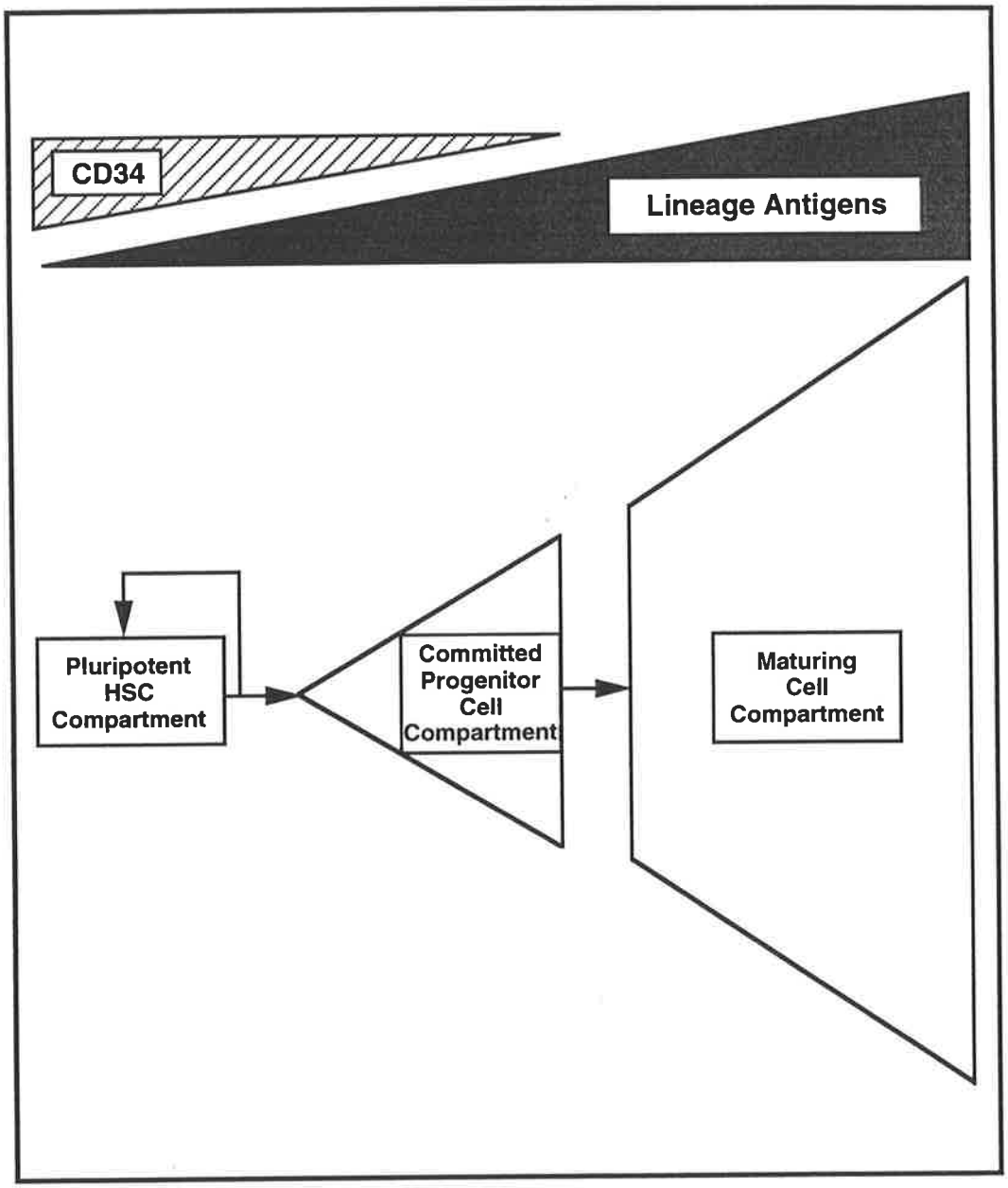
Very early lymphoid progenitors are also $CD34^+$. For example, B-lymphoid-committed progenitors and thymic T-cell precursors marked by the expression of lineage-specific antigens (CD7 and CD19/CD10 for T and B lymphoid precursor, respectively) and terminal deoxynucleotidyl transferase activity (involved in immunoglobulin and T cell receptor gene rearrangement) express the CD34 antigen. In contrast, the developmentally later $CD20^+$ B cells are $CD34^-$ (Pontvert-Delucq *et al*, 1993; Peault *et al*, 1991; Galy *et al*, 1993). Taken together, this implies that the CD34 antigen is expressed at high levels on the earliest haemopoietic cells and decreases to undetectable levels as a function of differentiation (Figure 1.3.).

The most compelling evidence that human HSC reside within the CD34 antigen-positive subfraction of human BM has come from *in vivo* transplantation studies. A number of investigators have demonstrated that $CD34^+$ cells can provide long-term lympho-haemopoietic reconstitution in lethally irradiated baboons and rhesus monkeys. Transplantation with cells that lack the CD34 antigen however, leads to graft failure and transplant-recipient death (Wagermaker *et al*, 1990; Berenson *et al*, 1988). In addition, successful autotransplantation of $CD34^+$ cells after intensive and supralethal cyto-reductive therapy in patients with solid tumours suggests that this population of cells is able to confer long-term reconstitution of haemopoiesis in humans (Berenson *et al*, 1991).

Although these studies imply that human HSC are contained within the $CD34^+$ population, definitive evidence awaits the long-term outcomes of clinical BM transplant trials utilising $CD34^+$ cell allografts (Krause *et al*, 1996). In this setting, if the transplanted allogeneic $CD34^+$ population (as opposed to host stem cells) provide durable, long-term donor multilineage lympho-haemopoiesis in humans, this will imply that these cells are capable both of self-renewal and multilineage differentiation, and thus satisfy the most stringent definition of the stem cell (Krause, 1996).

Figure 1.3. CD34 Antigen Expression Is Lost As A Function Of Differentiation.

CD34 antigen is expressed at high levels on the earliest haemopoietic cells ($CD34^{BRIGHT}$). The level of expression decreases to undetectable levels as a function of differentiation. Concomitantly, maturing cells acquire the expression of activation-(eg. HLA-DR, CD38) and lineage- (eg. CD13, CD33, CD10, CD4, CD8) related antigens.


CD34
Lineage Antigens
Pluripotent
HSC
Compartment
Committed
Progenitor
Cell
Compartment
Maturing
Cell
Compartment

In lieu of a suitable experimental transplantation model (equivalent to the murine transplant model), a number of surrogate *in vivo* and *in vitro* assay systems have been developed to assess the haemopoietic potential of $CD34^+$ cells and various subpopulations thereof.

1.2.3. *In Vivo* Assays For The Assessment Of Candidate Human Stem Cells.

A number of systems reliant on xenogeneic reconstitution have proven useful surrogate *in vivo* models for the study of human haemopoiesis (reviewed in Srour *et al*, 1992). Human chimaeras in immunodeficient mice like the bg/nude/xid (bnx) mouse have been used successfully as recipients of human BM (Srour *et al*, 1992). Although reliant upon the infusion of exogenous human HGF, human macrophage, granulocytic cell, and erythroid cell development has been observed in transplanted mice (Kamel-Reid and Dick, 1988). More recently, a number of investigators (McCune *et al*, 1988; Namikawa *et al*, 1990), have developed the severe combined immunodeficient-human (SCID-hu) mouse model in which human haemopoietic organs were engrafted into the SCID mice (reviewed in Hendrickson, 1993). HLA-mismatched haemopoietic progenitors from human foetal liver, thymus and lymph node were then used to engraft these SCID-hu mice, to examine the transplantation potential of these cells. The HPC were monitored based on the clearly definable donor-type cells over time and the ability to transfer progenitor activity to human foetal bone or thymus in secondary SCID-hu recipients (Peault *et al*, 1991; Chen *et al*, 1994). Using the SCID-hu model, a number of investigators have demonstrated that both myeloid and lymphoid reconstitution potential is restricted to the $CD34^+$ population and subsets thereof (Peault *et al*, 1991; Chen *et al*, 1994; Galy *et al*, 1994).

An alternative, yet equally powerful approach has been developed by Zanjani and collegues. Zanjani *et al* (1991) successfully demonstrated *in utero* transfer of human HSC to pre-immune foetal sheep during early gestation. Sustained chimeric haemopoiesis or long term marrow repopulation (LTMR) was observed in sheep that received either cells from adult human BM (Srour, 1992) or foetal liver (Zanjani, 1992) exhibiting a $CD34^+$ $HLA\text{-}DR^-$ surface phenotype. Moreover, human HSC could be retrieved from sheep BM

more than 3 years following engraftment and used to engraft secondary recipients (Zanjani, 1994). In a like manner, a rare population of cells (refer to Figure 1.4.) derived from adult BM exhibiting a surface phenotype of $CD34^+$ $Thy\text{-}1^+$ Lin^- were also able to give rise to long-term human haemopoiesis when transplanted into foetal sheep (Uchida *et al*, 1994). Another significant finding from the sheep-human model, was the absence of any clinically manifest graft-versus-host disease after transplantation with xenogeneic grafts from immunocompetent donors (Zanjani *et al*, 1992). The use of xenogeneic models of human HSC engraftment provide the ability to study LTMR with subfractions of $CD34^+$ cells, enriched by various cell-surface marker combinations, and complement the various *in vitro* assay systems described below.

1.2.4. *In Vitro* Assays For The Assessment Of Candidate Human Stem Cells.

A variety of *in vitro* assay systems have been developed to quantitate primitive human progenitors and to investigate their stem cell potential. At this juncture, it should be recognised that essentially all of the human *in vitro* assays for HPC are derived from similar methods established using murine cells. These include clonogenic assays performed in semi-solid media, such as the CFU-blast and high proliferative potential colony forming cell (HPP-CFC) assay (Leary and Ogawa, 1987; Bradley and Hodgson, 1979; McNiece *et al*, 1989), the long-term culture initiating cell (LTC-IC) assay (Dexter *et al*, 1977; Moore *et al*, 1979; Gordon *et al*, 1987b) and the pre-CFU-assay (Iscove *et al*, 1989; Smith *et al*, 1991; 1993; Metcalf, 1984). The latter assays represent indirect methods for detecting primitive haemopoietic cells by their ability to generate committed progenitor cells, as measured by the clonogenic assay. The clonogenic assay system provides a means to directly quantitate human progenitor cells *in vitro*. The morphologically-recognisable colonies which include the monopotent BFU-E, CFU-E, CFU-Meg, the bipotent CFU-GM, and the multipotential CFU-GEMM, represent clones of cells produced by a single progenitor cell.

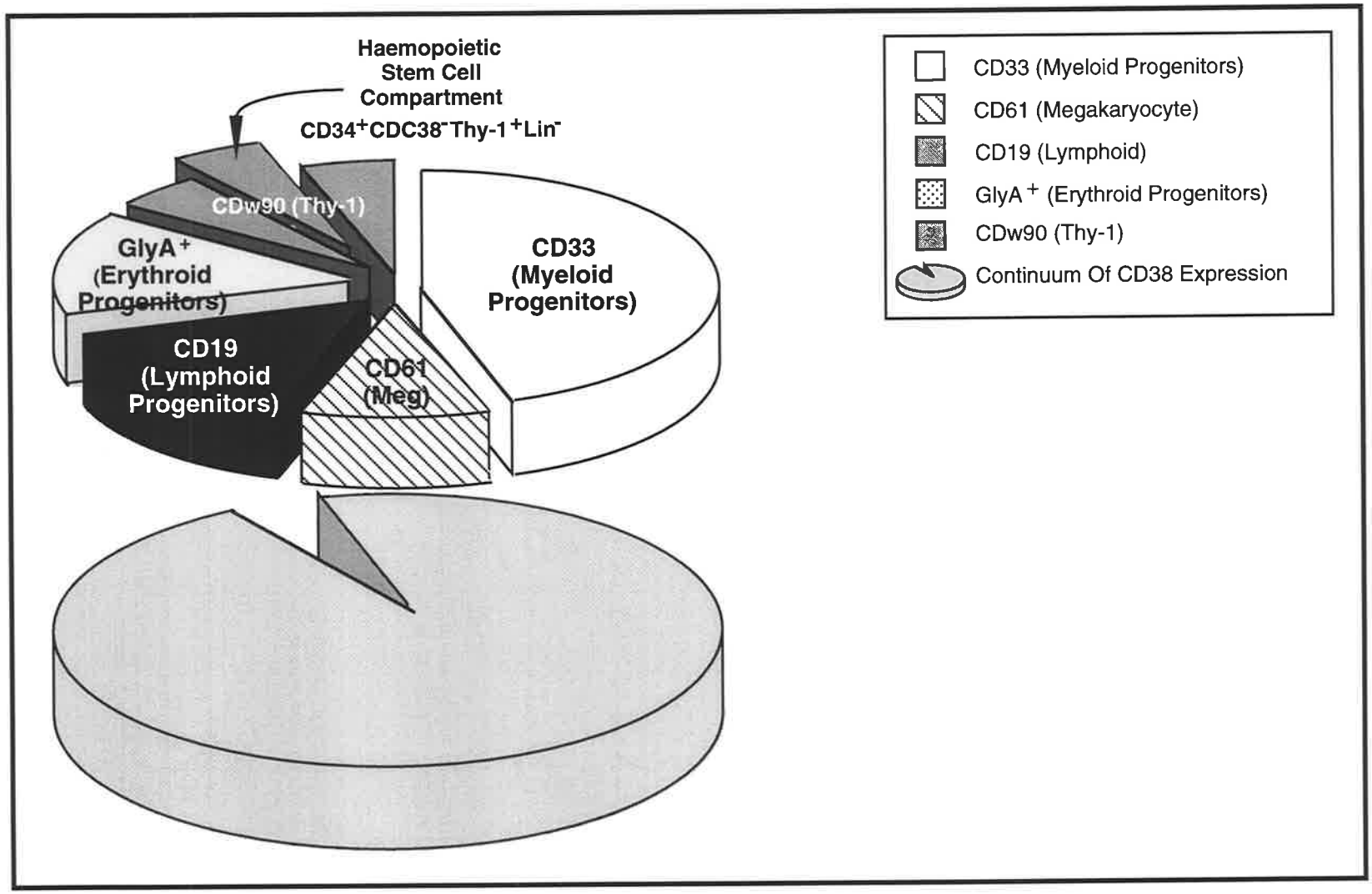
Haemopoietic progenitor cells can be cultured in liquid or semi-solid systems, in the absence of supporting stroma. Various combinations of cytokines (please refer to Section 1.3.) are used in such cultures to promote the proliferation or maintenance of

Figure 1.4. The $CD34^+$ Haemopoietic Stem Cell Compartment Is Heterogeneous In Composition.

Numerous studies have revealed that BM progenitor cells are contained within the subset which is bound by monoclonal antibodies to the CD34 antigen (Civin *et al*, 1984; Ogawa, 1993; Katz *et al*, 1985; Andrews *et al*, 1986). This population however is not uniform, but is comprised of a hierarchy of closely related, yet functionally heterogeneous cell types (Sutherland *et al*, 1989; Smith *et al*, 1991, Brandt *et al*, 1990; Wagermaker, 1990; Berenson, 1988; 1991).

In recent years, a number of promising reagents, including mAbs to the Thy-1 molecule (CDw90; Baum *et al*, 1992; Craig *et al*, 1993; Williams and Gagnon, 1982) have enabled the definition of a subset of $CD34^+$ cells, representing <0.5% of BM mononuclear cells, which are highly enriched for pluripotent stem cells (Craig *et al*, 1993; Baum *et al*, 1992; Simmons *et al*, 1994; reviewed in Ogawa *et al*, 1991).

Furthermore, putative stem cells can be segregated on the basis of lack of expression of the CD38 molecule. The $CD34^+CD38^-$ subpopulation represent approximately 1/5,000 BM mononuclear cells. These cells are quiescent as determined both by flow cytometric analysis using acridine orange (or rhodamine retention, refer to text) and by their resistance to 5-fluorouracil (5-FU) and 4-hydroxyperoxy-cyclophosphomide (4-HC). Preliminary data (Haylock and Simmons, personal communication), indicate that approximately 1/5 to 1/8 $CD34^+CD38^-Thy\text{-}1^+Rh123^{DULL}$ cells is an LTC-IC (refer to text) as assayed at week 5 of the culture, according to the conditions of Sutherland *et al* (1989). Moreover, the $CD34^+CD38^-Thy\text{-}1^+Rh123^{DULL}$ population contains cells with the potential to give rise to any one of a variety of haemopoietic lineages, including erythroid cells, neutrophils, monocytes, megakaryocytes, mast cells and dendritic cells.


Haemopoietic
Stem Cell
Compartment
CD34+CDC38-Thy-1+Lin-
CDw90 (Thy-1)
GlyA+
(Erythroid
Progenitors)
CD19
(Lymphoid
Progenitors)
CD61
(Meg)
CD33
(Myeloid
Progenitors)
CD33 (Myeloid Progenitors)
CD61 (Megakaryocyte)
CD19 (Lymphoid)
GlyA+ (Erythroid Progenitors)
CDw90 (Thy-1)
Continuum Of CD38 Expression

progenitor cells (reviewed in Ogawa, 1993). To date, no combination of cytokines has yet been identified that promotes a significant expansion or long-term maintenance of multipotent progenitors in such cultures. Only myeloid and erythroid differentiation are usually observed, although a means of achieving limited B-lineage differentiation has been reported (Hirayama, 1992).

The blast-colony (CFU-blast) assay devised by Ogawa and collegues identifies a population of late-appearing colonies consisting largely of undifferentiated blast cells that have a high replating ability. Moreover, these cells exhibit the capacity to give rise to both differentiated and undifferentiated colonies in the presence of IL-3, IL-6, IL-1 or a combination of these factors (Leary and Ogawa, 1987; Moreb *et al*, 1989; Moreb, 1990). A modification of this system was used by Terstappen to show that blast colonies derived from $CD34^+CD38^-$ (but not $CD34^+CD38^+$) were capable of extensive generation of new colonies after serial replating of the first-generation colonies (Terstappen *et al*, 1991).

The HPP-CFC assay was originally described in the murine system (Bradley and Hodgson, 1979) and later modified to support the growth of human HPP-CFC by McNiece *et al* (1989). The HPP-CFC assay, like the CFU-Blast assay (refer below), identifies a rare population of cells within normal marrow (~2 per 10^5 mononuclear cells) that are $CD34^+HLA\text{-}DR^-Rh123^{DULL}$, and represents an *in vitro* progenitor phenotype correlated with enrichment of multipotent progenitors (Bradley and Hodgson, 1979; McNiece *et al*, 1989; McNiece *et al*, 1990).

Gordon *et al* (1985), identified a population of early haemopoietic cells (blast-colony forming cells; Bl-CFC) characterised by an ability to specifically adhere to pre-formed marrow stroma within two hours. These adherent cells had stem cell features, including an ability to form undifferentiated blast cell colonies, and generated multipotential and lineage-committed colony forming cells. Further studies revealed that the cell type specifically adhering to the stroma was $CD34^+HLA\text{-}DR^-Lin^-$ and exhibited a capacity for long-term *in vitro* repopulation (Verfaillie *et al*, 1990).

The long-term BM culture (LTBMC), assays cells with the ability to initiate and sustain haemopoiesis in a standard Dexter-type stroma cell-dependent system (please

refer to Section 1.5.3. for detailed discussion). The long-term culture-initiating cells (LTC-IC) responsible for the sustained *in vitro* haemopoiesis observed have been phenotypically defined as cells which exhibit a blast-like morphology (Sutherland *et al*, 1989a), and lack expression of the transferrin receptor (CD71) (Sutherland *et al*, 1989b; Lansdorp and Dragowska, 1992), major histocompatability complex class II, HLA-DR (Sutherland *et al*, 1989a; Moore *et al*, 1980; Keating *et al*, 1984; Brandt *et al*, 1990), various mature lineage markers expressed by both myeloid and lymphoid cells (Baum *et al*, 1992), CD33 (Andrews *et al*, 1989; Bernstein *et al*, 1991a; Simmons and Seed, 1988) and the high molecular weight isoform of the human leukocyte antigen (CD45RA) (Lansdorp *et al*, 1990; Lansdorp and Dragowska, 1992). Further studies have demonstrated that LTC-IC express low levels of the phosphoinositol glycan-linked molecule Thy-1 (Baum *et al*, 1992; Craig *et al*, 1993), and high levels of the proto-oncogene, c-kit (Simmons *et al*, 1994a; Udomsakdi *et al*, 1992; Briddell *et al*, 1992). In addition, LTC-IC have been further defined as quiescent cells as determined by the low retention of the supravital fluorescent dye, Rh123 which accumulates in the mitochondrial membranes, with the subsequent fluorescence activity proportional to the metabolic activity and cell cycle status (Craig *et al*, 1993; Udomsakdi *et al*, 1992). Further affirmation of quiescence has come from studies which show that LTC-IC have limited sensitivity to 4-hydroxyperoxy-cyclophosphomide (4-HC), a cytotoxic compound effecting actively cycling cells (Winton and Colenda, 1989).

Haylock *et al* (1992) have recently developed a stromal cell free, cytokine-dependent suspension culture system for primitive human haemopoietic progenitors (Haylock *et al*, 1992) based conceptually on the studies with murine haemopoietic progenitors originally described by Iscove *et al* (1989). This culture system termed the pre-colony forming unit (pre-CFU) assay, measures the recruitment, proliferation and development of primitive haemopoietic progenitors into lineage restricted haemopoietic progenitors in response to cytokines. The pre-CFU assay was based on the finding that precursors to myeloid progenitors (CFU-GM) that did not grow in semi-solid clonogenic assays in response to defined factors, preferentially differentiated into CFU-GM following

treatment in suspension phase with a combination of IL-1 and IL-3. Assay of CFU-GM present after various periods of time following initiation of pre-CFU suspension culture thus provides a surrogate measure of pre-CFU in the input population of haemopoietic cells. A subfraction of $CD34^+$ cells that lack detectable levels of the surface markers for T-cell, B-cell, natural-killer cells and the myeloid lineage ($CD34^+$ lin^-) were found to yield comparable CFU-GM numbers when compared with 4-HC-resistant $CD34^+$ cells, suggesting that these cells are closely related developmentally to long-term marrow repopulating cells (Haylock *et al*, 1995).

The results from the various *in vitro* assay systems indicate that the primitive HPC (and perhaps HSC), can be discriminated from the lineage-committed progenitors by the expression or absence of a cohort of cell surface molecules. Taken together, these data suggest that a composite phenotype for the human HSC could be; $CD34^+$ $CD38^-$ $Rh123^{DULL}$ $Thy\text{-}1^{LO}$ $CD71^-$ $CD45RA^-$ $c\text{-}kit^+$ $HLA\text{-}DR^-$ (Haylock *et al*, 1995). Conversely, committed progenitor cells would be $CD34^+$, express one or a number of the lineage (eg., CD33, CD10, CD19, CD2, CD7) or activation (eg., HLA-DR, CD38) antigens, and be proliferating cells, as demonstrated by their sensitivity to agents such as 4-HC and 5-fluorouracil (5-FU) and high retention of the supravital dye, Rh123. Furthermore, primitive haemopoietic cells can be discriminated from committed progenitor cells in their requirement for multiple HGF to stimulate proliferation. Committed progenitors in contrast, require only single or a few HGF for maximal stimulation (Moore, MAS., 1991; McNiece *et al*, 1991; Bernstein *et al*, 1991a; 1991b; Lowry *et al*, 1991; Muench *et al*, 1992; Broxmeyer *et al*, 1991). Figure 1.5. summarises both the stage-related phenotypic characteristics associated with stem cell differentiation and the inter-relationship of the various *in vitro* and *in vivo* bioassays discussed above.

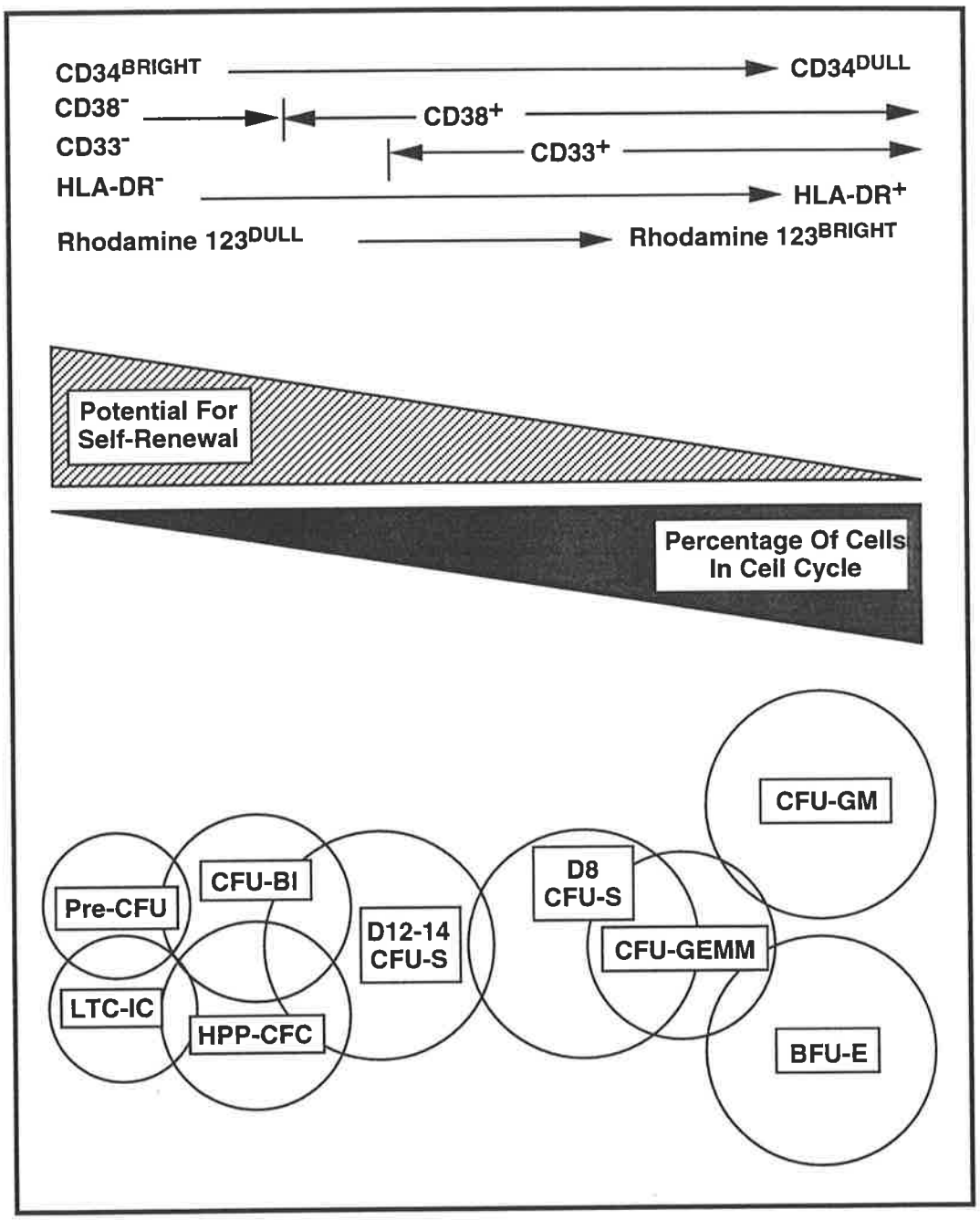
1.3. GROWTH FACTORS IN HAEMOPOIESIS.

1.3.1. Haemopoietic Growth Factors.

Haemopoietic growth factors (HGF) comprise a family of glycosylated polypeptides that were initially described as "unidentified factors" produced by feeder

Figure 1.5. Schematic Diagram Illustrating The Inter-relationship Of The Currently Available *In Vivo* And *In Vitro* Bioassays.

The hierarchical organisation of the stem and progenitor cell compartments are shown in relationship to the proliferative potential and cell-cycle status in normal haemopoiesis. The stage-related phenotypic characteristics which are routinely used for haemopoietic stem cell isolation are also indicated (Adapted from Moore, 1991).


CD34BRIGHT
CD34DULL
CD38-
CD38+
CD33-
CD33+
HLA-DR-
HLA-DR+
Rhodamine 123DULL
Rhodamine 123BRIGHT
Potential For Self-Renewal
Percentage Of Cells In Cell Cycle
CFU-GM
CFU-BI
Pre-CFU
D12-14 CFU-S
D8 CFU-S
CFU-GEMM
LTC-IC
HPP-CFC
BFU-E

cells or present in crude, connective tissue cell-conditioned media that stimulated the proliferation of leucocyte precursor cells (Bradley and Metcalf, 1966; Ichikawa *et al*, 1966; Whetton, 1990).

While self-renewal and differentiation of haemopoietic stem and progenitor cells may be a stochastic process, survival and proliferation is thought to be regulated by HGFs. For example, haemopoietic stem and progenitor cells require HGFs to form and sustain colonies in short-term semi-solid cultures (refer to Section 1.2.4.), and recent studies have demonstrated that cytokines prevent cells from apoptotic cell death (reviewed in Koury, 1992; and Dexter and Spooncer, 1987). Furthermore, myelopoiesis is able to be sustained for several weeks in the absence of an adherent stromal layer if primitive haemopoietic progenitor cells are placed into liquid culture in the continuous presence of multiple HGFs.

To date, 17 interleukins (IL), three colony stimulating factors (CSF), erythropoietin (EPO), stem cell factor (SCF), megakaryocyte growth and development factor (MGDF) and flt 3 ligand (FL), have been molecularly cloned and implicated in the regulation of haemopoiesis and lymphopoiesis (Metcalf and Nicola, 1985; Clark and Kamen, 1987; Zsebo *et al*, 1990; Lyman *et al*, 1993; Hannum *et al*, 1994). Unlike "classical" hormones, HGFs are inducible products of a variety of cell types, including BM stromal elements (refer to Section 1.7.2.), T lymphocytes, endothelial cells, fibroblasts and macrophages (Dexter, 1990; DiPersio, 1990), that exert pleiotropic, additive or synergistic effects on haemopoietic stem and progenitor cells and enhance the functional activities of mature effector cells (Nicola, 1989; Moore, 1990). The cellular specificity and action of the various HGFs is mediated through high affinity receptors expressed on the haemopoietic cell surface, of which many have been characterised at the molecular level (Moore, 1990). While beyond the scope of this review to consider the role of each HGF in detail, Table 1.1. summarises the well-defined HGFs (and their corresponding receptor(s)) which exhibit a role in haemopoiesis. Moreover, a synopsis of the biological activities of each of these glycoproteins is presented in Table 1.2.

Table 1.1. Growth Factors And Their Receptors.

Ligand	MW (kD)	Chromosome location (human)	Source	Receptor	MW (kD)	Expressed By	Subunit Sharing	Other Characteristics
IL-1	14-17	2q	Ubiquitous	IL-1RI	87 - 100	T Lymphocyte Stroma Epithelial Cells	No	-
				IL-1RII	68 - 80	B Lymphocyte Monocytes/Macrophages Stroma	No	-
IL-2	23	4q	T Lymphocytes	p55, p75	55 75	B Lymphocyte T Lymphocyte Natural Killer Cells	No	p55 and p75 dimerise
IL-3	14 - 28	5q	T Lymphocytes Basophils Mast Cells Neuronal Cells BM Stromal Cells?	IL-3-Rα, GM-CSF-Rβ	130 130	Monocytes/Macrophages Lymphocytes Neutrophils Eosinophils Megakaryocytes Basophils	With IL-5-Rα, and GM-CSF-R	α and β chains dimerise
IL-4	18	5q	T Lymphocytes, Mast Cells, Basophils	IL-4R	140	Basophils B Lymphocytes T Lymphocytes Monocytes/Macrophages	No	Receptors are heavily glycosylated
IL-5	40	5q	T Lymphocytes Mast Cells	IL-5-Rα, GM-CSF-Rβ	? 130	B Lymphocytes T Lymphocytes Eosinophils	With IL-3-Rα, and GM-CSF-R	α and β chains dimerise
IL-6	21 - 26	7p	T/B Lymphocytes Monocytes most Mesenchymal Cells	IL-6-Rα, gp 130	80 130	Stromal Cells Megakaryocytes Hepatocytes Endothelial Cells B Lymphocytes T Lymphocytes Monocytes/Macrophages	gp 130 shared with LIF-Rα & IL-11R	LIF-Rα and gp 130 dimerise and bind Oncostatin M as well as LIF
IL-7	17 - 25	8q	Thymus/Spleen Stromal Cells	IL-2R	50 - 75	Megakaryocytes B Lymphocytes T Lymphocytes	?	160 kD receptor maybe a homo-dimer

Table 1.1. Growth Factors And Their Receptors (*continued*).

Ligand	MW (kD)	Chromosome location (human)	Source	Receptor	MW (kD)	Expressed By	Subunit Sharing	Other Characteristics
IL-8	8 - 9	4q	Monocytes Fibroblasts Stromal Cells, Macrophages, T Lymphocytes	IL-8R	58	T Lymphocytes Neutrophils	No	-
IL-10	30 - 35	1q	T/B Lymphocytes, Macrophages Keratinocytes	?	?	?	?	-
IL-11	20 - 23	?	Fibroblasts BM Stromal Cells	IL-11R gp130	130 130	B Lynphocytes Megakaryocytes	gp 130 shared with LIF-Rα & IL-6-Rα	-
IL-12	heterodimeric CK of 70 kD comprised of a 35 & 40 subunits	?	B Lymphocytes Macrophages	IL-12R	110	Activated CD4 and CD8 T cells $CD56^+$ Natural Killer Cells	No	IL-12 shares homology with IL-6 & G-CSF
LIF	58	22q	BM Stromal Cells T Lymphocytes Monocytes/ Macrophages Fibroblasts	LIF-Rα, gp 130	130 130	Megakaryocytes Hepatocytes Endothelial Cells Myocytes Osteosarcoma Monocytes/Macrophages Embryonal Stem Cells	gp 130 shared with IL-6-Rα & IL-11R	LIF-Rα and gp 130 dimerise and bind Oncostatin M as well as LIF
M-CSF (CSF-1)	40 - 90	1p	T Lymphocytes Monocytes/ Macrophages Fibroblasts Neutrophils	*c-fms*	150	Monocytes/Macrophages	No	Cytoplasmic region has tyrosine kinase domains; on chromosome 5q
EPO	18 - 32	7q	Renal Hepatocytes	EPO-R	55	Eosinophils Stromal Cells Megakaryocytes	?	Homodimerisation occurs, receptor on chromosome 19p

Table 1.1. Growth Factors And Their Receptors (*continued*).

Ligand	MW (kD)	Chromosome location (human)	Source	Receptor	MW (kD)	Expressed By	Subunit Sharing	Other Characteristics
SCF	46- membrane bound 30- secreted	12q	Ubiquitous	c-kit	145	BFU-E HPP-CFC Long-Term Culture-Initiating Cells pre-CFU Cells Mast Cells	No	Cytoplasmic region has tyrosine kinase domains; as member of the c-fms, PDGF-R subclass; on chromosome 4
TNF-α	17	6p	Macrophages, B Lymphocytes, NK Cells	TNF R-α TNF R-β	75 55	Ubiquitous Expression Of Both Isoforms	No	Each receptor binds to both TNF-α and TNF-β
MGDF (TPO)	25 - 60 (35)	3p	Adult liver, kidney, spleen and brain.	c-mpl	110-120	PB, BM and CB $CD34^+$ Cells Megakaryocytes Platelets Endothelial Cells	No	N-terminal half of the MGCF protein shares homology with EPO
flt 3 ligand (FL)	40-80	19q13.3	T Lymphocytes BM Stroma?	flt3/flk2 TKR	132 - 158	Restricted to BM $CD34^+$ Cells	No	receptor resembles the c-erbB2, c-kit, c-fms, PDGF and VEGF proteins
G-CSF	18 - 25	17q	BM Stromal Cells Macrophages Endothelial Cells Neutrophils T Lymphocytes	G-CSF-R	150	Neutrophils	None	-
GM-CSF	14 - 35	5q	BM Stromal Cells T Lymphocytes Macrophages Granulocytes Fibroblasts	GM-CSF-Rα, GM-CSF-Rβ	80 - 85 130	Monocytes/Macrophages Neutrophils Eosinophils Erythrocytes	With IL-3-Rα, and IL-5-Rα	α and β chains dimerise

For descriptions of abbreviations, refer to *Abbreviation* section. (Adapted from Bagby, 1994)

Table 1.2. Biological Activities Of Haemopoietic Factors.

Factor	Biological Activity
IL-1	Induces expression of GM-CSF, G-CSF, IL-6 and IL-1 in stromal cells; induces proliferation of pre-activated T cells; promotes the transendothelial passage of neutrophils; synergises with IL-3 in stimulating proliferation in primitive haemopoietic progenitor cells; modulates EGF-R expression; induces IL-2-R number and binding activity and induces maturation of pre-B cells.
IL-2	Stimulates proliferation and activation of T lymphocytes, B lymphocytes, and NK cells; induces expression of IL-1 in monocytes and macrophages; co-induces (with IL-1) expression of interferon-γ in T cells; co-stimulates B cell differentiation and induces non-MHC-restricted cytotoxic T-cell (CTL) activity.
IL-3	Stimulates CFU-GEMM and CFU-S growth; stimulates proliferation of HPP-CFC, BFU-E, B lymphocytes, T lymphocytes (synergistically with IL-2) and growth of myeloid leukaemic cells; induces macrophages to express M-CSF; stimulates growth of mast cells and pre-B cell lines.
IL-4	Induces proliferation of activated B lymphocytes; inhibits IL-2-stimulated proliferation of B cells; co-induces immunoglobulin secretion and isotype switching; co-induces proliferation of T cells and fibroblasts, and co-induces IL-2 receptor expression in T cells; inhibits induction of lymphokine-activated killer cells; inhibits IL-1 synthesis; induces expression of M-CSF and G-CSF genes in monocytes; induces expression of an inhibitor of haemopoiesis by mixed murine marrow stromal cells; enhances murine BFU-E and CFU-E growth (in association with EPO) and enhances CFU-GM growth (with G-CSF and GM-CSF); stimulates expression of MHC Class II antigens on B cells and monocytes.
IL-5	Activates cytotoxic T lymphocytes; co-induces immunoglobulin secretion, stimulates eosinophil production and activation *in vitro* and *in vivo*.
IL-6	Synergistic with IL-3 in stimulating CFU-GEMM colony growth, with M-CSF in macrophage colony growth, and with GM-CSF in inducing granulocyte colony growth; synergistic with IL-4 in inducing T-cell proliferation, haemopoietic colony formation by multipotential progenitor cells; immunoglobulin secretion by B lymphocytes; synergises with IL-2 and IL-1 in inducing T-cell growth; co-induces B-cell growth, and induces terminal differentiation of certain myeloid leukaemic cell lines; co-induces cytotoxic T-cells, stimulates plasmocytoma cell growth; induces acute phase responses *in vivo*; synergistic with IL-3 in stimulating CFU-GEMM and CFU-Meg colony growth; promotes megakaryocyte maturation *in vitro* and stimulates platelet production *in vivo*.

Table 1.2. Biological Activities Of Haemopoietic Factors (*Continued*).

Factor	Biological Activity
IL-7	Co-stimulates T cells (in association with mitogens); enhances growth of allospecific CTLs; induces cytokine secretion by monocytes and macrophages, and induces clonal growth of pre-B cells.
IL-8	Induces neutrophil chemotaxis.
IL-10	Induces proliferation of B lymphocytes and mast cells; inhibits cytokine production by monocytes; inhibits T-lymphocyte proliferation and IL-2 production; inhibits IL-5-mediated antibody secretion; synergises with TGF-β and IL-4 to inhibit macrophage cytotoxicity.
IL-11	Synergises with other cytokines to stimulate haemopoiesis; functions as adipogenesis inhibitory factor.
IL-12	Stimulates the activation of T lymphocytes and natural killer (NK) cells; induces the production of IFN-γ release from TH-1 cells.
LIF	Enhances efficiency of CFU-S infection by retroviral shuttle vectors; induces differentiation of certain murine and human leukaemic cells; inhibits differentiation of embryonal carcinoma cells; stimulates bone resorption; enhances growth of megakaryocytes *in vivo*; augments proliferation of pluripotent human progenitor cells.
M-CSF	Induces monocytes/macrophages growth (murine cells) and differentiation (both murine and human cells); induces phagocytic and secretory function in monocytes and macrophages.
SCF	Markedly enhances clonal growth of GFU-GEMM, BFU-E, and pre-B cells when combined with direct growth factors, IL-3, EPO, or IL-7; enhances growth of mast cells; non-haemopoietic targets include melanocytes, reproductive system, gut and embryonic brain.
EPO	Stimulates clonal growth of CFU-E and a subset of BFU-E; induces globin synthesis in erythroid cells; stimulates murine megakaryocyte colony growth, but has no apparent thrombopoietic activity *in vivo*

Table 1.2. Biological Activities Of Haemopoietic Factors (*Continued*).

Factor	Biological Activity
G-CSF	Stimulates clonal growth of CFU-GM and production of neutrophils *in vivo*; stimulates neutrophil maturation of certain leukaemic cells; activates phagocytic function of mature neutrophils; stimulates quiescent pluripotent progenitor cells to enter G1-S phase; induces migration and proliferation of vascular endothelial cells *in vitro*; stimulates growth of some cancer cells.
GM-CSF	Stimulates growth of CFU-GM and production of monocytes, eosinophils and neutrophils *in vivo*, stimulates growth of CFU-GEMM, BFU-E, and CFU-Meg; primes (pre-activates) phagocytic and chemotactic function of monocytes and granulocytes; stimulates growth of certain leukaemic cells; induces expression of IL-1, TNF-α, and M-CSF by monocytes and IL-1 production by neutrophils; stimulates functional activation of eosinophils; co-stimulates T-cell growth with IL-2; stimulates growth of some cancer cells; induces migration and proliferation of vascular endothelial cells.
flt3 ligand (FL)	FL alone does not support the growth of any type of colony growth, but potentiates myeloid (but not erythroid) colony forming cell proliferation *in vitro*, including high proliferative potential colony forming cells (HPP-CFCs), colony-forming units- blast (CFU-Bl) and long term culture initiating cells (LTC-IC) in the presence of IL-3 and GM-CSF.
MGDF (TPO)	Stimulates both the growth and differentiation of megakaryocytes. Cytokines that interact with MGDF include, SCF which results in an increased numbers of human megakaryocyte colonies *in vitro*; Murine megakaryocyte colony formation is synergistically enhanced with MGDF in combination with EPO, SCF, IL-3 or IL-11. *In vivo* administration of MGDF, results in a 3-4 fold increase in the *de novo* platelet synthesis without effect on red or white cell counts.
TNF-α	Induces expression of GM-CSF, G-CSF, IL-6 and IL-1 in fibroblasts and endothelial cells; enhances mitogen-induced GM-CSF expression in T cells; induces release of GM-CSF and M-CSF *in vivo*, and inhibits the replication of viruses synergistically with interferons; inhibits growth of haemopoietic progenitor cells, lymphocytes, and certain leukaemic cell lines; induces expression of adhesion molecules in myeloid and stromal cells (including endothelial cells) and induces expression of IL-8.

For descriptions of abbreviations, refer to *Abbreviation* section.
(Adapted from Bagby, 1994)

While some factors are primarily stimulatory or inhibitory, most have complex functions in haemopoietic proliferation. This is well exemplified by IL-4, which has been shown to exhibit both stimulatory and inhibitory activities on haemopoietic proliferation (Peschel *et al*, 1987; Rennick *et al*, 1987a). Likewise, from Table 1.2., it is evident that the regulatory pattern attributed to HGFs is one of redundancy and pleiotropy; more than one factor controls cells in one lineage and most factors act on multiple lineages (refer to Figure 1.6.). For example, the generation of granulocytes can be stimulated by IL-3, GM-CSF, G-CSF, IL-6, and even M-CSF in high doses (Metcalf, 1993). Speculative reasons for the redundancy include the necessity for sequential action of various regulators, or the necessity for synergy at the suboptimal concentrations of single HGFs encountered *in vivo*. Although there is minimal amino acid sequence homology between HGFs, it is manifest from X-ray crystallographic analysis of growth hormone (Ultsch *et al*, 1991), GM-CSF (Diederichs *et al*, 1991) and IL-4 (Powers *et al*, 1992) that several may exhibit a similar three-dimensional protein structure, involving four α-helices, two of which are presumed to contribute to receptor binding (Nicola, 1989).

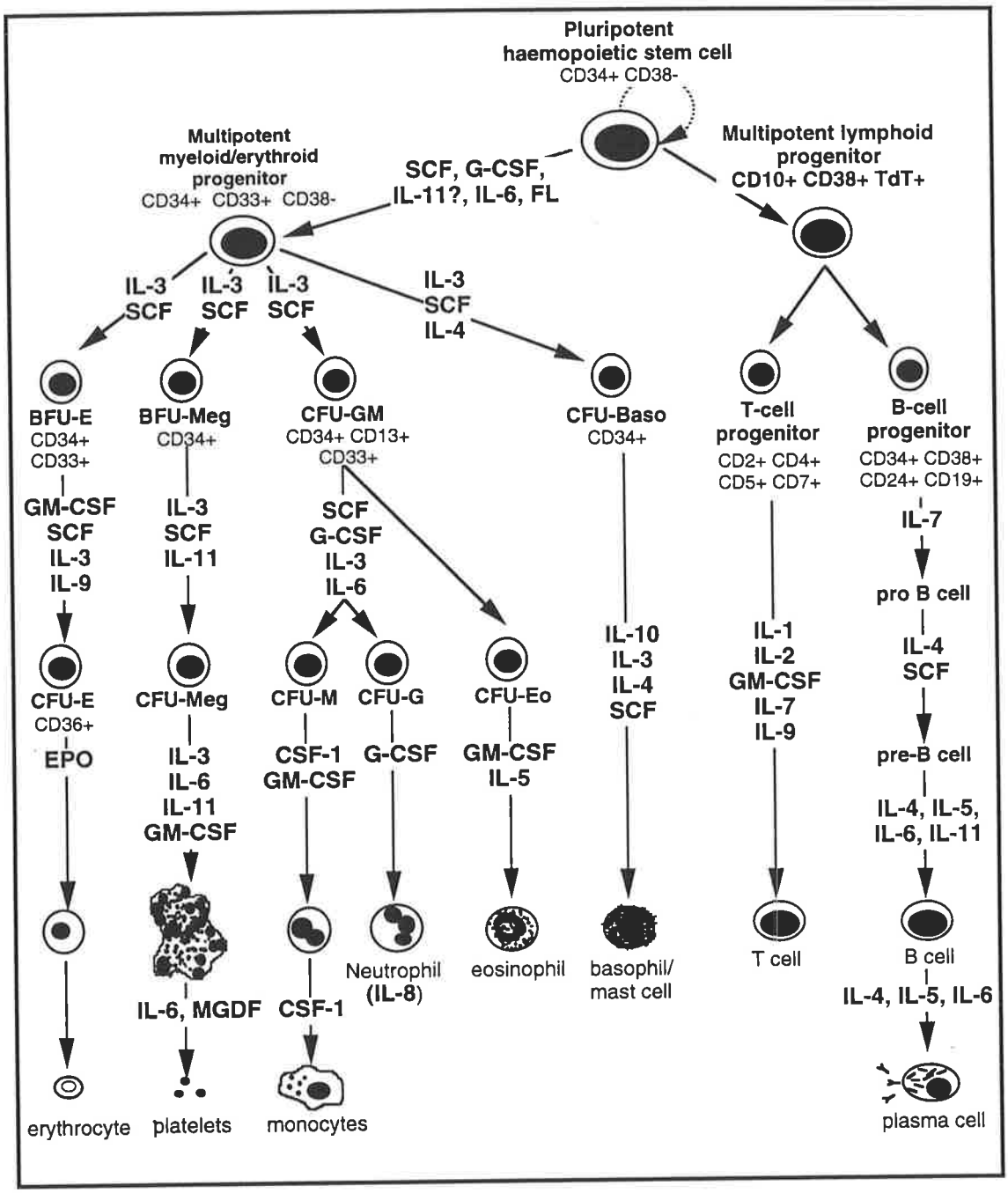
There are a number of modes in which HGFs can be presented to cells expressing the corresponding receptors, and they include; (i) a number of HGFs, including M-CSF (or CSF-1) (Rettenmier *et al*, 1987), FL (Lyman *et al*, 1993; Hannum *et al*, 1994), TGFα (Massagué, 1990) and SCF (Bernstein *et al*, 1968), can be associated with the stromal cell surface via membrane-insertion domains (reviewed in Massagué and Pandiella, 1993); (ii) HGFs may be produced by nearby cells and interact with juxtaposed cells; (iii) HGFs may be associated with BM derived sulphated glycosaminoglycans (GAG). For example, heparan sulphate proteoglycan demonstrates the ability to bind IL-3, GM-CSF and bFGF and present them in a biologically active form to the haemopoietic stem and progenitor cells (Roberts *et al*, 1988a; Gordon *et al*, 1987a; Massagué, 1991); (iv) HGFs including MGDF (reviewed in Hunt, 1995) and EPO (Stephenson *et al*, 1971) may be produced by distant cells which, like traditional hormones, are transported to sites of action via the peripheral circulation. Interestingly, HGFs like M-CSF and SCF may be presented in all these ways.

Figure 1.6. Haemopoietic Growth Factors And Their Effect On Target Cells.

This diagram illustrates the hierachical relationship between pluripotent and multipotent myeloid and lymphoid progenitor cells and haemopoietic growth factors (HGFs). Haemopoietic stem and progenitor cells at various stages of development (as characterised by their expression of lineage-related antigens) are responsive to combinations or individual HGFs.

Growth factors: IL, interleukin-1 to 11; GM-CSF, granulocyte/macrophage colony stimulating factor; G-CSF, granulocyte-colony stimulating factor; M-CSF, macrophage colony stimulating factor; CSF-1 (eqivalent to M-CSF [macrophage-colony stimulating factor]), colony-stimulating factor-1; SCF, stem cell factor; MGDF, megakaryocyte growth and differentiation factor; EPO, erythropoietin; FL, flt3 ligand.

Progenitor cells: BFU-E, blast forming unit-erythroid; CFU-E, colony forming unit-erythroid; BFU-Meg, blast forming unit-megakaryocyte; CFU-Meg, colony forming unit-megakaryocyte; CFU-GM, colony forming unit-granulocyte/macrophages; CFU-G, colony forming unit-granulocyte; CFU-M, colony forming unit-macrophages; CFU-Eo, colony forming unit-eosinophils; CFU-Baso, colony forming unit-basophil/mast; TdT, terminal deoxyribonucleotidyl transferase. (Adapted from Bagby, 1994).


Pluripotent
haemopoietic stem cell
CD34+ CD38-
SCF, G-CSF,
IL-11?, IL-6, FL
Multipotent
myeloid/erythroid
progenitor
CD34+ CD33+ CD38-
Multipotent lymphoid
progenitor
CD10+ CD38+ TdT+
IL-3
SCF
IL-3
SCF
IL-3
SCF
IL-3
SCF
IL-4
BFU-E
CD34+
CD33+
BFU-Meg
CD34+
CFU-GM
CD34+ CD13+
CD33+
CFU-Baso
CD34+
T-cell
progenitor
CD2+ CD4+
CD5+ CD7+
B-cell
progenitor
CD34+ CD38+
CD24+ CD19+
GM-CSF
SCF
IL-3
IL-9
IL-3
SCF
IL-11
SCF
G-CSF
IL-3
IL-6
IL-7
pro B cell
IL-4
SCF
pre-B cell
IL-4, IL-5,
IL-6, IL-11
IL-10
IL-3
IL-4
SCF
IL-1
IL-2
GM-CSF
IL-7
IL-9
CFU-E
CD36+
EPO
CFU-Meg
CFU-M
CFU-G
CFU-Eo
IL-3
IL-6
IL-11
GM-CSF
CSF-1
GM-CSF
G-CSF
GM-CSF
IL-5
IL-6, MGDF
CSF-1
Neutrophil
(IL-8)
eosinophil
basophil/
mast cell
T cell
B cell
IL-4, IL-5, IL-6
erythrocyte
platelets
monocytes
plasma cell

1.4. THE DEVELOPMENT OF THE HAEMOPOIETIC SYSTEM.

1.4.1 The Origin Of Haemopoietic Stem Cells.

The mechanisms underlying the initiation of haemopoiesis during embryogenesis are essentially unknown. Despite this, it is well recognised that vertebrate haemopoietic cells are derived from the splanchnic mesoderm (Rich, 1992; Dieterlen-Lievre, 1975; Dieterlen-Lievre and Martin, 1981; Dieterlen-Lievre *et al*, 1994). In rodents, the mesoderm is formed 7 days postcoitum in the region of the primitive streak and between day 7 and 7.5 of gestation, cells from the mesoderm loosen and begin to migrate along the amniotic wall and visceral endoderm. These migrating cells are responsible for the first visceral blood islands or hemangioblasts, which are later connected by blood vessels forming the primitive circulatory system (Rich *et al*, 1992).

The first morphologically identifiable cells formed are erythropoietin (EPO)-insensitive erythroblasts, although it is speculated that primitive macrophages may also be present in part of the yolk sac blood islands (Rich, 1992). The observation that cells from the extraembryonic environment of the yolk sac migrate to the embryo body gave rise to the suggestion that the yolk sac is the site of *de novo* stem cell formation and responsible for seeding and all other haemopoietic tissues (Moore and Owen, 1967a; Moore and Metcalf,1970). A number of studies have demonstrated that yolk sac progenitors are capable of multilineage progenitor activity *in vivo* (Moore and Owen, 1967b; Moore and Metcalf, 1970) and that cells from 8.5 day old embryos were able to respond to erythropoietin and other haemopoietic factors to produce lineage-restricted colonies in culture (Rich, 1992). To investigate if the observed progenitor activity in the yolk sac was derived from the self-renewing multipotent progenitors or from more committed progenitors, Weissman and colleagues (1978) injected marked, day 9 donor yolk sac cells into the yolk sac cavities of recipient mice and observed the localisation of donor cells to the blood islands. Moreover, myeloid, erythroid and lymphoid progenitors located in the BM of recipient mice were derived from marked-donor yolk sac cells. Similarly, in humans haemopoiesis is initiated in the yolk-sac between days 15 and 18

post-fertilisation, and although it continues for the first six weeks of gestation, it ceases at week 10 of the 38 week gestation period (reviewed in Tavassoli, 1991, and refer to Figure 1.7.).

In addition to the presence of putative HSC in the yolk sac, Godin *et al* (1993) and Medvinsky *et al* (1993) simultaneously reported the progenitor activity in the mouse para-aortic mesoderm. Progenitor cells capable of long-term marrow repopulation (LTMR) of adult recipients were observed in the paraortic mesoderm at 10 days post-coitus (Muller *et al*, 1994). Furthermore, the notion that multipotent progenitors arise simultaneously in the para-aortic mesoderm and in the yolk sac has been further strengthened by the recent work of Godin and colleagues (1995), who established that clones isolated from both the murine yolk sac and para-aortic (splachnopleural) mesoderm at day 8.5 post-coitus are capable of giving rise to B and various myeloid lineages in culture.

1.4.2. The Development Of Foetal And Adult Haemopoiesis.

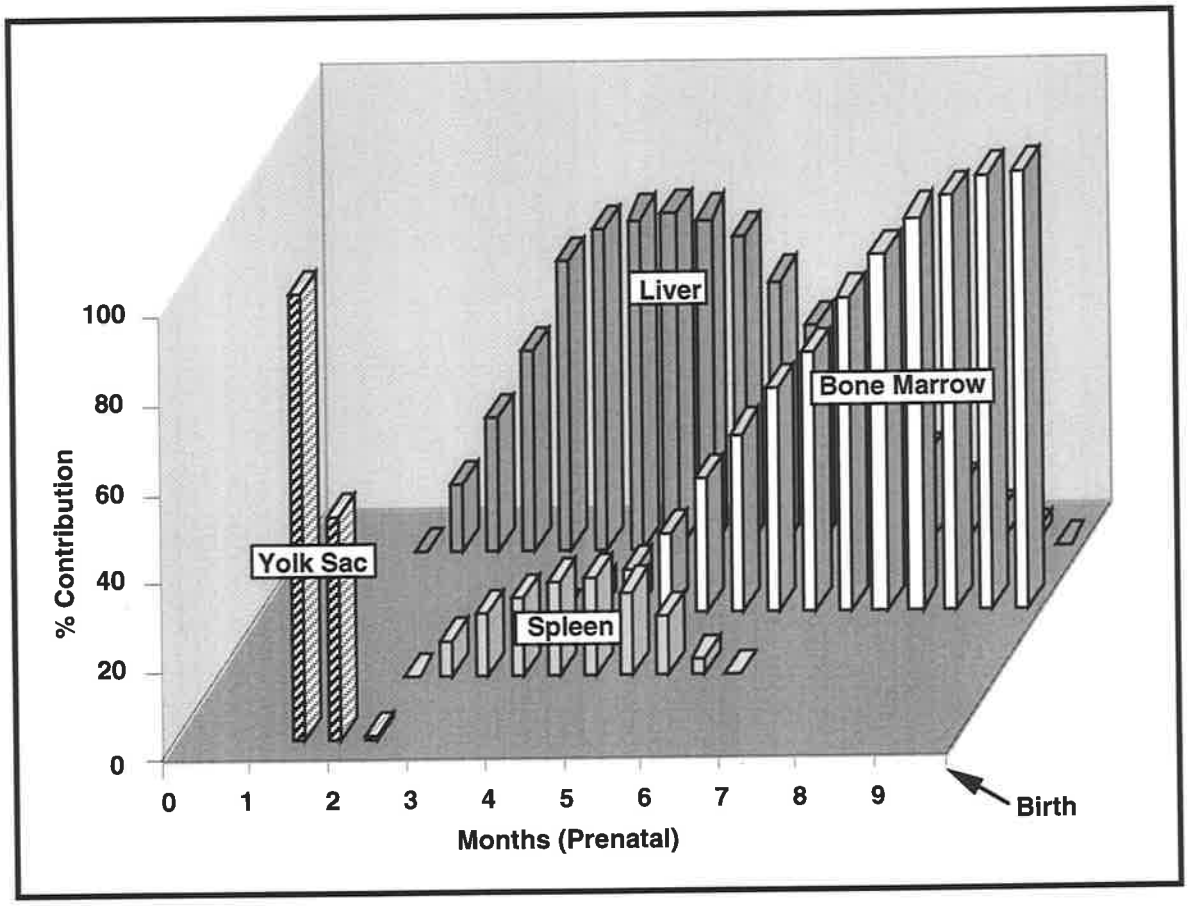
Beyond the yolk-sac, progenitor cells sequentially migrate, via the blood stream, to seed the foetal liver and para-aorta (and rodent spleen), which become transient sites of haemopoiesis. Hepatic haemopoiesis (Petti *et al*, 1985; Houssaint and Hallet, 1988) is detectable in the mouse by day 10 and in humans by week 6 of gestation (Paul *et al*, 1969) and is associated with a gradual decline in yolk sac haemopoiesis. Like the yolk-sac, extravascular erythropoiesis appears to predominate, however some granulopoiesis has been noted.

In the mouse, the spleen is initiated in the task of haemopoiesis 15 days post-coitus, and continues to be active throughout the gestation period. Haemopoietic development is observed in the splenic cords with the last stages of maturation taking place in the splenic sinuses. Although mostly erythropoietic in nature, the rodent spleen retains haemopoietic activity throughout adult life, whilst the human spleen ceases to be haemopoietic at birth (Tavassoli, 1991, refer to Figure 1.7.).

The site of haemopoiesis is finally transferred to the BM, where it assumes the role as the major site of generation of lineage committed and mature cells throughout the life of

Figure 1.7. Dynamic Progression Of Organ-Specific Haemopoiesis During Human Development.

Timetable of succession of haemopoietic organs as a function of gestational age. Early haemopoiesis occurs in the yolk sac, however by 2.5 months of gestation the most active sites are the liver and spleen. The bone marrow, in which a convergence of haemopoietic activity can be found as early as the fourth month, becomes the dominant site of haemopoietic activity by the seventh month and beyond. Despite this, some foci of haemopoiesis in the liver are commonly present at birth.


Liver
Bone Marrow
Yolk Sac
Spleen
100
80
60
40
20
0
% Contribution
0
1
2
3
4
5
6
7
8
9
Months (Prenatal)
Birth

the adult mammal (reviewed in Tavassoli, 1991). By days 17-18 in the mouse and week 20 of gestation in the human, the development of the BM, is associated with the penetration of avascular cartilage by perichondrial mesenchymal cells and their associated blood vessels which leads to calcification of cartilage. The vascular mesenchyme then forms a reticular meshwork (and rudimentary bone) upon which haemopoietic cells can seed and proliferate. The developmental association between the HSC and the non-haemopoietic, fixed tissue mesenchymal elements in the medullary cavity of the BM (collectively referred to as stromal cells (*stroma;* from the Greek-meaning mattress or bed)) are of central importance in the regulation of haemopoiesis, and are dealt with in greater detail below.

1.5. MARROW BIOLOGY AND HAEMOPOIETIC STEM CELLS.

1.5.1. The Bone Marrow Microenvironment.

Haemopoietic precursors have been identified in a number of tissues, however the BM is the only site in normal adult mammals in which myelopoiesis, erythropoiesis and lymphopoiesis proceed simultaneously (Rosse, 1976). Histological examination has revealed that these processes occur within the many cavities of both murine and human bones. The majority of haemopoietic activity is localised to the ossified cancellous tissues at the ends of the human "long" bones and within the shaft cavities of murine "long" bones (Lichtman, 1981; Clark *et al*, 1992). Morphologically recognisable haemopoietic cells are found in the spaces between the marrow draining sinus network of supporting cells and ECM, localising the developing haemopoietic cells into specialised structures or haemopoietic microenvironments (HM).

While the precise mechanisms for control of haemopoietic function remain to be elucidated, there is abundant evidence supporting the existence of the HM (reviewed in Metcalf and Moore, 1971; Trentin, 1975; 1978; Tavassoli, 1975; Price and McCulloch, 1978; Wolf, 1979; Cline and Golde, 1979; Lichtman, 1981; Dexter, 1982; Tavassoli and Friedenstein, 1983). Studies in rodents and humans have demonstrated that for haemopoiesis to become established in recipients of allogeneic and autologous HSC

transplants, HSC were required to 'home' to the BM via the peripheral circulation (Till and McCulloch, 1961; Robertson, 1979). Moreover, when BM from a donor animal was implanted into an ectopic site (such as renal, hepatic and muscular tissue) a stromal organ developed in the recipient animal prior to the establishment of haemopoiesis (Tavassoli, 1968; Tavassoli and Crosby, 1970). The ectopic organs were shown to be of donor stromal cell origin whilst the haemopoietic progenitor cells which seeded the stroma were derived from the circulation of the host animal (Maniatis, 1971; Tavassoli, 1975; Tavassoli and Khademi, 1980). Other studies have demonstrated that in response to mechanical excavation, radiation or cytotoxic agents, the stromal milieu must first regenerate, prior to the resumption of haemopoiesis (Tavassoli, 1974; Knopse *et al*, 1966; Fried, 1976; 1977; Appelbaum, 1980; Ershler, 1980; McManus, 1984; Werts *et al*, 1980; Piersma *et al*, 1983; Perkins and Fleischman, 1988; Patt and Maloney, 1975; Marshall *et al*, 1984).

Although such studies provide evidence of fixed microenvironments within the BM which endow upon it a unique capacity to support haemopoiesis, they do not reveal the role played by the HM. For example, does the HM play a permissive role by merely providing a privileged site for haemopoiesis to occur, or does it fulfil an "instructive" function by determining proliferation and lineage commitment within the haemopoietic system? Two opposing models have been proposed which encompass these notions, namely the "haemopoiesis engendered randomly" or H.E.R. model of Till *et al* (1964) and the "haemopoietic inductive microenvironment" or H.I.M. concept of Trentin and colleagues (Wolf, 1979).

The H.E.R. theory (Till *et al*, 1964), asserts a stochastic model based on chance interactions between pluripotent cells and unspecified regulatory mechanisms that govern the probability of differentiation along a particular pathway. It states that the "decision" to self-renewal or differentiate, is a random event that occurs at the level of the individual haemopoietic stem cell. In contrast, the H.I.M. concept (Curry and Trentin, 1967) proposes the existence within the haemopoietic organs of specialised regions comprised of various mesenchymal stromal elements, assorted ECM glycoproteins, and haemopoietic

growth factors. These H.I.M.'s are thought to selectively "induce one of several possible genetically programmed responses for commitment of a pluripotent stem cell into erythroid, neutrophilic, eosinophilic or megakaryocyte lines of differentiation" (Wolf, 1979). In other words, this model suggests that the stromal cells do not simply supply a supportive framework in which haemopoietic cells lodge and respond to externally derived influences, but have a determinative role in haemopoiesis (Trentin, 1970; Allen and Dexter, 1984, Torok-Storb, 1988).

Aside from the controversies surrounding the mode of action of the HM (H.I.M. 'vs' H.E.R. models) there is little doubt that the HM of the BM influences the pattern of differentiation within the haemopoietic system. Furthermore, in light of recent data (discussed in Section 1.7.1.), protagonists of the H.E.R. model have modified their theory to include the role played by the stromal elements of the BM and soluble/membrane-associated regulatory molecules in determining the probability of certain developmental events (Allen *et al*, 1990). Therefore, the notion of stromal-mediated haemopoiesis is now a widely accepted phenomenon amongst experimental haematologists, although the mechanisms responsible for this dynamic developmental process remain unclear due to the heterogeneity and inherent complexity of the stromal tissue, as discussed below (Allen *et al*, 1990; Wolf, 1968).

1.5.2. The Stromal Elements Of The Bone Marrow Microenvironment: *In Vivo* Studies.

Electron microscopic studies of the structure of the human BM microenvironment have been hampered by the extensive intrusion of trabecular bone and fat cells. As such, the majority of the studies examining the ultrastructure of mammalian BM have been carried out on rodent BM which is largely devoid of trabecular bone. Attempts to classify the various cell populations within the BM on the basis of morphology, association with ECM components, immunochemical studies of antigenic and enzyme components and their association with morphologically recognisable HPC (Westen and Bainton, 1979; Weiss, 1976) have resulted in a cornucopia of descriptive names, as detailed below.

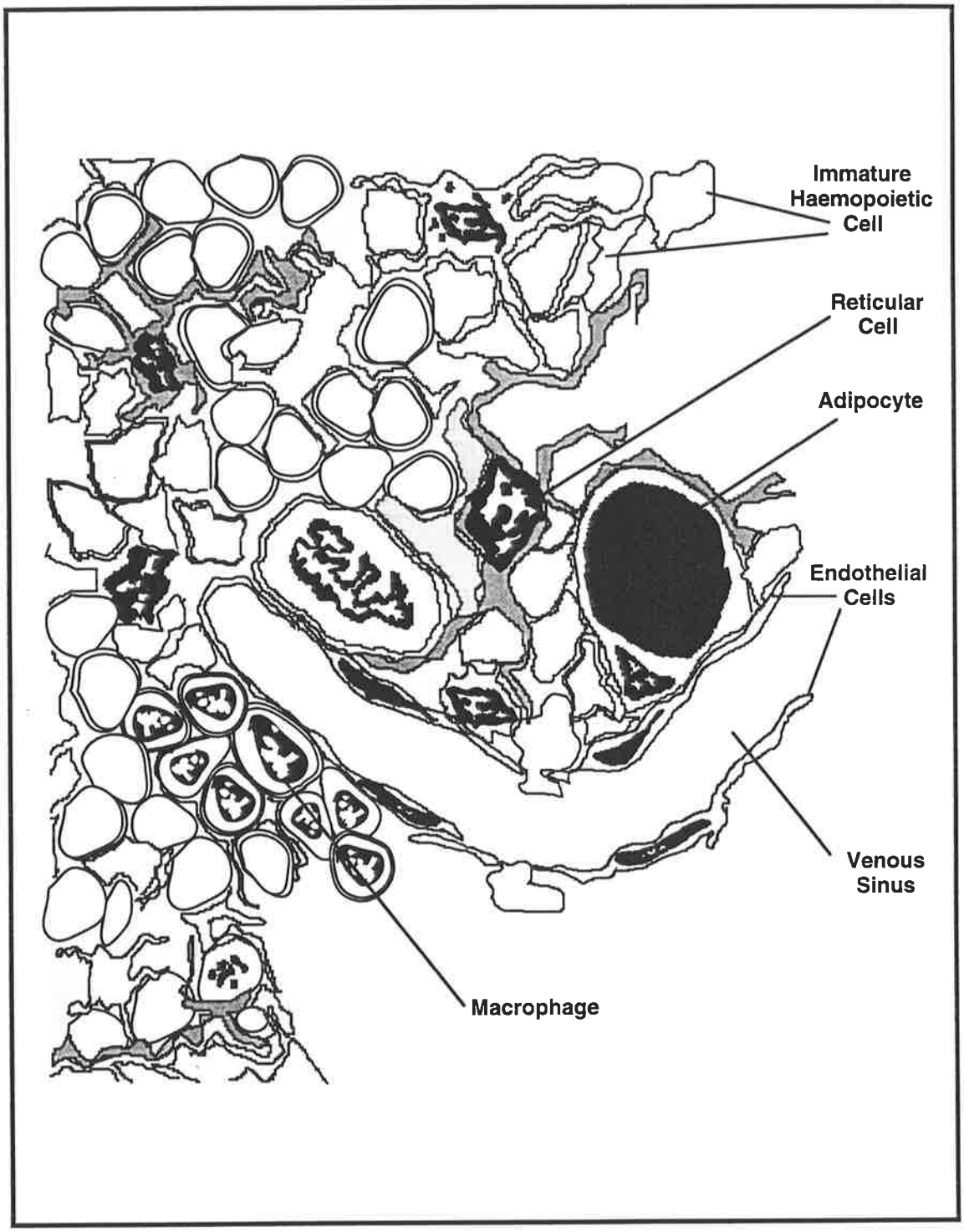
Preparations of murine femur or red marrow from large mammals have revealed a network of loosely woven connective tissue comprised of heterogeneous mesenchymal elements laden with haemopoietic cell clusters and consisting of a large vascular supply made up of thinly walled sinuses (refer to Figure 1.8.). The unicellular layer or endosteum that covers the surface of the bone, including the trabecular regions, is comprised of endosteal cells which have the capacity for differentiation into osteogenic-progenitor cells (Weiss, 1976). This is evidenced by studies which indirectly demonstrate the presence of osteoblasts in BM aspirates, by the formation of bone in diffusion chambers (Owen, 1985) and when implanted into ectopic sites in animals (Friedenstein, 1976; Friedenstein *et al*, 1966; 1987).

Essentially all of the haemopoietic activity takes place in the extravascular spaces between the marrow sinuses where developing blood cells must pass through the sinus wall to enter the circulation (Weiss, 1970; 1976; Lichtman, 1981). Endothelial cells form a complete covering of the inner surface of the sinuses. Morphologically, these cells appear broad and flat with irregular cytoplasmic edges, which can overlap with other endothelial cells forming a barrier between the circulation and the HM (Weiss, 1961; Watanabe, 1966). In addition to lining the large vessels, endothelial cells (in association with smooth muscle cells and pericytes) also line the BM arterioles, and are perceived to play roles in regulating the passage of maturing blood cells into the marrow sinuses and in the 'homing' of transplanted HSC to the HM (Weiss *et al*, 1978; Tavassoli and Hardy, 1990; Issekutz, 1991).

Reticular cells (BM fibroblasts) are the predominant cells of the BM stroma and possess a large irregular morphology, with long cytoplasmic processes innovating the haemopoietic spaces and forming a spongelike network akin to the reticular network of splenic cords. These cells secrete the structural ECM of reticular fibres which support the haemopoietic tissue (Weiss, 1965; Bessis, 1973). The ECM is composed mainly of glycosaminoglycans (GAGs) attached to proteoglycans, fibronectin and collagen types I and III (Bentley, 1981; 1982a; 1982b). Reticular cells which line the abluminal surface of the sinus endothelia (littoral cells), are referred to as adventitial reticular cells (Allen *et al*,

Figure 1.8. Schematic Representation Of The *In Vivo* BM Microenvironment.

Morphologically recognisable haemopoietic cells are found in intimate association with phenotypically heterogeneous, non-haemopoietic mesenchymal elements collectively referred to as BM stromal cells. The cytoplasmic processes of the reticular cells (BM fibroblasts) innovate the extravascular space, forming a scaffolding on which haemopoietic stem and progenitor cells are arranged. Adipocytes (fat cells) represent adventitial reticular cells that have accumulated fat in their cytoplasmic vacuoles. Macrophages occur throughout the marrow, and are generally closely associated with developing haemopoietic cells. The nutrient requirements of the BM are provided by the BM vasculature that permeates the haemopoietic microenvironment. The venous sinuses are lined with endothelial cells which are in turn covered on the adventitial side by an incomplete sheath of reticular cells. Although a direct role for the vasculature in haemopoietic production has not been demonstrated, it may represent the final "gateway" to the entry of newly generated blood cells into the circulation. (Adapted from Allen *et al*, 1993).


Immature
Haemopoietic
Cell
Reticular
Cell
Adipocyte
Endothelial
Cells
Venous
Sinus
Macrophage

1990). The cytoplasmic processes of these cells cover the outer wall of the sinus forming an adventitial sheath (Weiss, 1961; Weiss and Chen, 1975; Allen *et al*, 1990). The reticular cells are found in intimate association with myeloid progenitor cells, implicating a possible role in myeloid development and in particular with granulopoiesis. In addition, it has been proposed that the adventitial reticular cells regulate the migration of blood cells into the circulation by varying their cover over the sinuses (Weiss, 1970).

The lipid-laden adipocytes found in the HM are thought to be derived from lipid-accumulation by adventitial reticular cells (Tavassoli, 1976, Allen *et al*, 1990). Morphologically, these cells are smaller in size and have a different fat content to adipocytes in the nonhaemopoietic areas of the marrow (yellow marrow) (Tavassoli *et al*, 1978). The BM adipocytes appear to increase in numbers in areas of decreasing haemopoietic activity and may provide energy stores for haemopoiesis and/or serve as a mechanism to block the passage of blood cells into the sinuses (Tavassoli *et al*, 1974). Moreover, these cells do not undergo lipolysis in response to acute starvation as do adipocytes in other fat sites (Bathija *et al*, 1988).

The BM macrophages although haemopoietic in origin, are often found in association with haemopoietic clusters and at the sinuses where the adventitial reticular cells reside (Weiss, 1976). Macrophages have a stellate morphology with extended cytoplasmic protrusions and are morphologically distinct from reticular cells due to the presence of large amounts of primary and secondary lysosomes and phagocytized materials. In addition, macrophages stain positive for acid phosphatase and non-specific esterase, whilst reticular cells stain positive for alkaline phosphatase (Westen and Bainton, 1979; Yoffey, 1980; Dexter *et al*, 1984). The macrophages present in the HM are associated with both developing erythrocytes and granulocytes, and are thought to contribute to the maturation of these cells (Weiss, 1976).

Due to the apparent diversity of stromal elements and the complex nature of the BM organ, it has been difficult to ascertain the role played by the individual cellular components of the HM. The development of the long-term BM culture (LTBMC) technique and the generation of numerous stromal cell lines, has provided some insight into the role

of stromal cells in the regulation of haemopoiesis (Dexter *et al*, 1977; Whitlock and Witte, 1982).

1.5.3. The Long-Term Bone Marrow Culture (LTBMC) System.

Sustained *in vitro* haemopoiesis for weeks or months can be attained in culture systems in which primitive HSC and their progeny are maintained in intimate association with an adherent layer of marrow-derived stromal cells. Stromal precursor cells (SPC) from fresh BM aspirates, cultured under appropriate conditions form a complex adherent layer able to support the differentiation and proliferation of HSC for several months.

The LTBMC system was first developed by Dexter and co-workers to support murine progenitor cell (CFU-S) replication (Dexter *et al*, 1977), and subsequently adapted to sustain long term haemopoiesis with marrow from other species, including man (Moore *et al*, 1979; 1980; Gordon *et al*, 1987b; Suda and Dexter, 1981). Moreover, stromal cell cultures have been established that permit either myelopoiesis (Dexter *et al*, 1984) or permit murine long-term *in vitro* lymphopoiesis (Whitlock and Witte, 1982). Stromal cell cultures are capable of supporting the differentiation and sometimes limited self-renewal of haemopoietic progenitors (reviewed in Deryugina and Müller-Sieburg, 1993) and represent the most physiological means of *in vitro* culture of HSC. The LTBMC system enables the assessment of cells with the ability to initiate and sustain haemopoiesis, with the progress of haemopoietic activity monitored by measuring the numbers of clonogenic precursors (CFU-GM) that are released into the culture supernatant (refer to Section 1.2.4.).

A number of studies have implied the necessity of marrow-derived adherent cells for the maintenance of haemopoiesis in long-term cultures. For example, when attachment of cells was inhibited, sustained haemopoiesis was not observed (Dexter *et al*, 1977). Moreover, studies by Reimann and Burger (1979) and Van Den Heuvel (1991) have demonstrated that prolonged haemopoiesis in this culture system appears to be critically dependent on the type of adherent cell population. Adherent stromal layers derived from sources other than bone marrow (eg. spleen, kidney, skin) did not support the

maintenance of haemopoiesis for more than one week (Reimann and Burger, 1979), and adult stroma had a greater capacity to support HSC when compared to stroma derived from neonatal spleen and foetal liver. Furthermore, primitive haemopoietic progenitor cells were shown to adhere only to stroma derived from adult BM and not to stroma derived from foetal BM and foetal liver (Riley and Gordon, 1987).

The stromal cells and matrix components of LTBMC are similar when compared to the structure of the *in vivo* HM, and thus provides an ideal model for studying the interactions between HSC and stromal cells (Wilkins and Jones, 1995). The sections that follow deal with the cellular composition and possible origin of the cells which comprise the adherent layer in LTBMC, and the cellular characteristics and possible functions of stromal cells/cell lines derived from these adherent cells.

1.5.4. Phenotypic Characteristics Of Bone Marrow Stromal Cells: *In Vitro* Studies.

The stromal cells present in the adherent layers of LTBMC are thought to be the *in vitro* equivalent to the stromal cells of the *in vivo* BM HM (Deryugina and Muller-Sieburg, 1993). The adherent layers of human LTBMC consist mainly of BM fibroblasts (60-70%), endothelial cells (10-20%), macrophages (10-20%), adipocytes (5-10%) and an extracellular matrix comprised of cellular proteins including fibronectin, laminin, collagen types I, III and IV and glycosaminoglycans (GAGs) (Strobel, 1986). In addition, smooth muscle cells and osteoblast-like cells have also been detected in the adherent layers of human LTBMC (Charbord *et al*, 1985; Eriksen and Kassem, 1992) and not surprisingly, similar cell types have been noted in murine LTBMCs (Dexter, 1984a).

Fibroblasts represent the most abundant stromal cell type found in LTBMC and are characterised as alkaline phosphatase positive, non-phagocytic cells which secrete various ECM components, including collagen types I and/or III and fibronectin. These large epithelioid-like cells or "blanket cells" are thought to be the *in vitro* equivalent to the adventitial reticular cells. Significantly, a subpopulation of these cells are capable of lipid accumulation and adipocyte differentiation. The appearance of mature fat cells, several

weeks after the establishment of the stromal layer, is associated with haemopoietically active cultures (Allen *et al*, 1990).

Endothelial cells have been shown to secrete ECM components including laminin and collagen type IV (Bentley, 1981). Morphologically, endothelial cells are large cells with thinly spread cytoplasm and appear to overlay haemopoietic cells in a way analogous to the 'blanket cells' (Hasthorpe, 1992). Endothelial cells are detected in the stroma of LTBMC by their expression of the Factor VIII-antigen and by their production of collagen type IV and laminin (Zuckerman and Wicha, 1983; Zuckerman *et al*, 1985). The representation of endothelial cells in LTBMC is difficult to assess over time due to the cessation of Factor VIII-antigen expression in culture (Pedersen, 1980; Liesveld, 1989).

The regulation of haemopoiesis by stromal cells is thought to involve many complex interactions between HSC, stromal cells and the products of stromal cells (cytokines, adhesion molecules, and ECM components; discussed below), however the exact roles of the different stromal cell types remains unclear. It is not known whether a particular stromal cell type, for instance the 'blanket cells', fibroblasts or endothelial cells, control HSC self-renewal, proliferation and differentiation. The development of phenotypically distinct stromal cell lines from primary cultures was expected to resolve the question as to the function of the different stromal cell types, however to date, investigators have been unable to categorically determine which stromal cell type can maintain the HSC pool *in vitro*. At best, some murine stromal cell lines which demonstrate a preadipocyte phenotype have been shown to support both myelopoiesis and lymphopoiesis *in vitro*, whilst the inverse is true for cell lines exhibiting a macrophage-like or fibroblastic stromal cell phenotype (Zipori, 1985; Kodama, 1986; Hunt, 1987). Despite their paucity, these data would suggest that an adventitial reticular-like cell may be responsible for maintaining the primitive stem cell population.

The heterogeneity and turnover of marrow stromal tissue has been attributed to the either (i) the proliferative potential and therefore self-maintaining capacity of each of the stromal populations within the BM or (ii) a minor population of stromal precursor cells (SPC) with the capacity to give rise to each of the recognised stromal elements. As

detailed below, current data would suggest that a multipotent SPC (analogous to the HSC) capable of vast cellular differentiation is responsible for the numerous mesenchymal elements found within the marrow stromal tissue.

1.6. STROMAL PROGENITOR CELLS.

1.6.1. Bone Marrow Stromal Precursor Cells (SPC).

Putative BM SPC have been identified in a number of species, including humans, by their ability to generate colonies of cells morphologically resembling fibroblasts when single cell suspensions of BM are explanted at appropriate densities in liquid culture. The clonogenic stromal progenitor cells responsible for colony growth under these conditions are referred to as fibroblast colony-forming cells (CFU-F) (Friedenstein *et al*, 1970; Castro-Malaspina *et al*, 1980; Wang and Wolf, 1990; Wang *et al*, 1990; Van der Sluijs *et al*; 1990).). This population of SPC were originally identified in rodent BM by Friedenstein and colleagues. Results obtained using a variety of different techniques collectively support the conclusion that each colony is derived from a single cell (Friedenstein *et al*, 1970; Castro-Malaspina *et al*, 1980; Perkins and Fleischman, 1990). In humans, BM-derived CFU-F are alkaline phosphatase positive, synthesise collagen types I and III and are negative for Factor VIII antigen, indicating a reticular cell phenotype.

Characteristic of early progenitor cells (compare with HSC above), CFU-F *in vivo* are almost entirely in a non-cycling state as demonstrated by ^{3}H-thymidine labelling in rodents and by means of the *in vitro* thymidine suicide technique in adult human BM (Keiliss-Borok *et al*, 1971; Castro-Malaspina *et al*, 1980; 1981). CFU-F colonies derived from the BM of virtually all species examined, including humans, are heterogeneous in size and morphology prompting the suggestion that they emanate from clonogenic progenitors at various stages of differentiation (Owen, 1985). A proportion of the fibroblastic colonies formed are large in size and demonstrate extensive replating potential after passaging (Friedenstein, 1976; Simmons and Gronthos, 1991). The high proliferative potential of some clonogenic cells observed in these studies, led Friedenstein to propose that within the CFU-F compartment are SPC with the characteristics of stem cells. Consistent with

this notion, ectopic transplantation of individual fibroblastic clones grown *in vitro* from mouse BM beneath the renal capsule of syngeneic hosts demonstrated that a minor proportion (approximately 15%) produced a BM organ containing the full spectrum of stromal cell types listed above, including bone cells. A further 15% produced bone tissue only while the remainder formed only soft connective tissue or failed to give rise to any tissue (Friedenstein, 1980). Other investigators have shown the induction of adipogenesis in a proportion of BM-derived CFU-F when cultured in the presence of glucocorticoids such as hydrocortisone (Bennett *et al*, 1991, Gronthos and Simmons, personal communication). Approximately 40% of CFU-F clones of both fibroblastic and adipocytic colonies were further induced to form an osteogenic tissue when transplanted *in vivo* using diffusion chambers (Bennett *et al*, 1991).

Based on these studies, Owen and Friedenstein proposed the "stromal stem cell" hypothesis, in which by analogy with the organisation of the haemopoietic system, there exists a hierarchy of cellular differentiation supported at its apex by a small compartment of self-renewing, multipotential stromal stem cells (Owen, 1985; Owen and Friedenstein 1988) (Figure 1.9.). Thus, SPC which yielded BM organs were hypothesised to be stromal stem cells while those CFU-F which gave rise to only bone or soft connective tissue were proposed to be SPC of more restricted developmental potential (committed stromal progenitors). It must be emphasised that while these data provide strong circumstantial evidence for the existence of stromal stem cells, definitive proof of this hypothesis will require more rigorous studies involving, for example, the transplantation of uniquely (retrovirally) marked SPC.

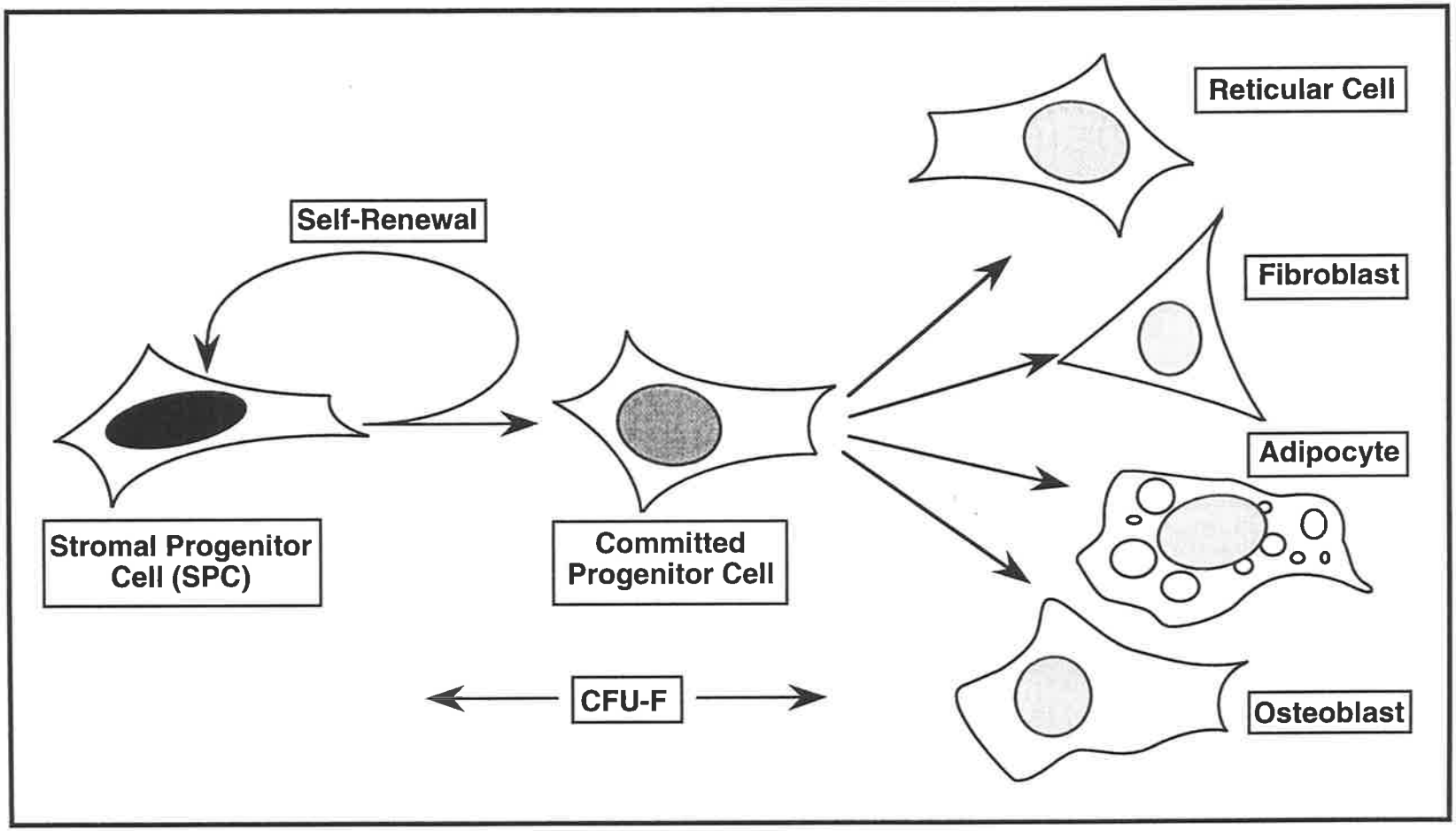
1.6.2 The Ontogeny Of Stromal Progenitor Cells.

Stromal cells, like haemopoietic cells, are derived from the mesenchyme during ontogeny (reviewed in Deryugina and Müller-Sieburg, 1993), however the lineage relationship of stromal and haemopoietic cells in the adult mammal remains a controversial issue. Current data suggest that SPC comprise a lineage distinct from haemopoietic cells, and as such, are frequently defined as non-haemopoietic cells. To

Figure 1.9. Development Of Stromal Cells: A Cellular Hierarchy.

Hypothesised hierarchy of stromal stem cell differentiation. This model proposes the existence within the bone marrow of a developmental hierarchy of stromal cells analogous to that described for the haemopoietic system, in which the differentiated cell types arise from separate progenitors which are in turn derived from multipotent self replicating stromal stem cells.

Putative stromal progenitor cells (SPC) have been identified in the bone marrow of a number of species (including humans) by their ability to form colonies of cells morphologically resembling fibroblasts when single cell suspensions of bone marrow mononuclear cells are explanted at appropriate densities in liquid culture (refer to text). The clonogenic progenitor responsible for colony formation under these conditions is referred to as a fibroblast colony forming unit (CFU-F). (Adapted from Owen, 1985).


Reticular Cell
Self-Renewal
Fibroblast
Adipocyte
Stromal Progenitor Cell (SPC)
Committed Progenitor Cell
CFU-F
Osteoblast

support this notion, a number of studies have demonstrated phenotypic differences between haemopoietic and stromal cells. For example, Van Vlasselaer and colleagues (1994), have recently isolated from the BM of mice 5 days following 5-FU treatment, a population of primitive osteogenic progenitors identified by fluorescence-activated cell sorting (FACS) as $lin^-/Sca\text{-}1^+/WGA^{BRIGHT}$. Although this is a phenotype ostensibly similar to that previously reported for murine HSC, the authors were able to distinguish the two populations based on differences in their light scatter characteristics and by the absence on stromal precursors of haemopoietic surface markers such as Thy1.2 and c-kit (Van Vlasselaer *et al*, 1994).

Similarly, Terstappen and colleagues have conducted an analysis of the cell surface phenotype of SPC in human foetal BM tissue (Huang and Terstappen, 1992). Notably, SPC were shown to express the CD34 antigen at high level but were distinguished from the bulk of haemopoietic progenitors by their lack of CD38 and HLA-DR. Based on FACS studies it was further claimed that within this $CD34^+$ $CD38^-$ $HLA\text{-}DR^-$ subpopulation were so-called "common stem cells" with the potential to develop into both haemopoietic and stromal cell progeny (Huang and Terstappen, 1992). This conclusion was subsequently retracted by the authors based on further analysis of this heterogenous population, which demonstrated that haemopoietic progenitors were contained within a $CD50^+$ (ICAM-3) subset of the $CD34^+$ $CD38^-$ $HLA\text{-}DR^-$ cells while SPC were restricted to a $CD50^-$ subset (Waller *et al*, 1995).

Simmons and Torok-Storb (1991), have recently described a monoclonal antibody, STRO-1 which identifies essentially all assayable CFU-F in aspirates of adult human BM (Simmons and Torok-Storb, 1991a). Although the $STRO\text{-}1^+$ BM mononuclear cell population was heterogeneous in nature, cells selected on the basis of a $STRO\text{-}1^+$ glycophorin A^- phenotype, gave rise to only stromal cells (and not haemopoietic cells) when cultured *in vitro*. In light of these studies, the controversial issue of a common mesenchymal stem cell appears to favour the view that BM stromal cells and haemopoietic cells have a separate ontogeny, although definitive proof of this hypothesis will require more meticulous testing.

1.6.3. Phenotypic Characterisation Of SPC.

Numerous cell surface molecules, identified by either mAbs or lectins, have enabled investigators to isolate candidate human SPC. Consistent with the above cited observations of Terstappen *et al* (1992) in foetal human BM, antibodies to CD34 were recently shown to bind to CFU-F in adult BM (Simmons and Torok-Storb, 1991b). However, unlike the high level of CD34 antigen expression characteristic of foetal BM SPC, adult BM SPC express CD34 at considerably lower levels (at least 10-fold less) than that exhibited by haemopoietic progenitors in the same sample. In addition, not all CFU-F were recovered in the $CD34^+$ fraction, which might reflect either inefficient capture of SPC as a result of low CD34 antigen density, or heterogeneity of CD34 expression within the SPC compartment (Simmons and Torok-Storb, 1991b). As such, the selection of SPC based on the use of a CD34 antibody does not appear to be the method of choice.

In contrast, the novel mAb STRO-1 which identifies essentially all assayable CFU-F in adult human BM aspirates, demonstrates no detectable binding to haemopoietic progenitors (CFU-GM, BFU-E, BFU-Meg, CFU-GEMM) nor to their precursors (pre-CFU) (Simmons and Torok-Storb 1991a; Gronthos and Simmons, unpublished observations) and thus facilitates a clear separation between SPC and haemopoietic progenitors in adult BM. Moreover the antigen(s) identified by STRO-1 is expressed at particularly high copy number by CFU-F (Gronthos and Simmons, 1994) which may in part account for the efficiency of CFU-F isolation with STRO-1.

As the $STRO\text{-}1^+$ cell population is only partially enriched for CFU-F, additional purification steps are required in order to isolate homogeneous populations of SPC. By means of 2- or 3-colour FACS, Simmons and Gronthos (Simmons *et al*, 1994; Gronthos and Simmons, 1995) have demonstrated that a number of cell surface antigens including the endopeptidases CD10 and CD13, and the adhesion molecules Thy-1 (CDw90) and VCAM-1 (CD106), enable the isolation of CFU-F at incidences in the range of 1 per 10-20 cells, which represents up to a 1,000-fold enrichment over starting SPC numbers in unfractionated BM. Moreover, this phenotype is in accord with the recent data of

Terstappen and colleagues regarding the antigenic profile of human foetal BM SPC (Waller *et al*, 1995).

The STRO-1$^+$ cells have also been shown to develop into an adherent stromal layer with an increased capacity to support haemopoiesis when compared to stroma derived from BMMNC (Simmons and Torok-Storb, 1991a; 1991b). Morphologically, the stromal layers formed from STRO-1$^+$ cells consist mainly of fibroblasts, adipocytes and smooth muscle cells but lack macrophages and endothelial cells. As there is a gradual decrease in the expression of the STRO-1 antigen on cultured stromal cells over time, Simmons and Torok-Storb (1991a) have suggested that the STRO-1 antibody identifies a differentiation antigen which decreases in expression as the SPC proliferate and mature into morphologically distinct cells.

Differences between the surface phenotypes of HSC and SPC therefore provide a means to identify and purify human BM SPC with the capacity to develop into an array of phenotypically distinct stromal cell types. The growth and expansion of SPC *in vitro* will enable investigators to explore the mechanisms of stromal-haemopoietic interactions and the role of specific stromal cell types in the regulation of haemopoiesis.

1.7. MECHANISMS OF STROMAL CELL REGULATION OF HAEMOPOIESIS.

1.7.1. Stromal Cell: Haemopoietic Cell Interactions.

As discussed in Section 1.3., the survival of primitive HSC, lineage-restricted progenitor cells and many mature leukocytes, rapidly declines in the absence of exogenous HGFs (Metcalf, 1977). However, the levels of progenitor populations can be maintained or increased *in vitro* when haemopoietic cells are cultured in association with BM-derived stromal cells. This is exemplified in murine LTBMCs, where the survival of the multipotent progenitors (putative HSC) capable of giving rise to both the myeloid and lymphoid pathways are maintained in the presence of an adherent stromal layer (Dexter *et al*, 1977; Dexter, 1982; Johnson and Dorshkind, 1986).

In these LTBMCs, the majority of the stem and progenitor cell activity was found in physical association with the adherent (stromal) layer (Dexter, 1982; Coulombel *et al*,

1983; Dorshkind and Phillips, 1982; Mauch *et al*, 1980), and despite the recent data of Verfaillie (1993), the preponderance of evidence suggests that the survival and differentiation of haemopoietic cells requires intimate interactions between the stromal cells and the developing haemopoietic cells. For example, a number of investigators have observed that separation of the progenitor cells from the stromal cells in a diffusion chamber or a thin agar layer results in reduced myelopoiesis and lymphopoiesis and a decline in the number of primitive cells present in the cultures (Bentley, 1981; Kierney and Dorshkind, 1987). Kodama *et al* (1986) also noted an enhanced CFU-S support capability, when direct contact between MC3TJ-G2/PA6 preadipocytes and haemopoietic cells was permitted (Kodama *et al*, 1986). The early stages of B-cell production were also shown to be dependent upon a close association with stromal cells (Kierney and Dorshkind, 1987).

Although the precise mechanisms which underlie this requirement are largely unknown, a number of suggestions have been made based on recent data. For example, the addition of exogenous HGF to either murine or human LTBMCs results in neither a sustained enhancement nor diminution of haemopoietic progenitor cell numbers (Coutinho *et al*, 1990, Clark *et al*, 1992). Furthermore, conditioned medium harvested from LTBMCs was itself unable to sustain the CFU-S levels in suspension culture, which led Shadduck and colleagues (1983) to suggest that soluble HGFs required for haemopoietic cell maintenance, are not released in appreciable amounts (Shadduck *et al*, 1983). However, an alternative and more plausible explanations for both of these observations are suggested by later experiments which demonstrate that; (i) BM stromal cells constitutively express numerous soluble HGFs which can be sequestered and "compartmentalised" by components of the ECM (Roberts *et al*, 1988a; Gordon *et al*, 1987a), and (ii) a number of HGFs are associated with the stromal cell surface via membrane-insertion domains (Rettenmier *et al*, 1987; Lyman *et al*, 1993; Massagué, 1990; Zsebo *et al*, 1990; Flanagan and Leder, 1990; reviewed in Massagué and Pandiella, 1993).

1.7.2. ECM Proteins And HGF Production By Stromal Cells *In Vitro*.

BM stromal cell-derived HGFs play an important role in regulating the growth and development of haemopoietic stem and progenitor cells. Stromal cells constitutively produce a wealth of HGFs, including granulocyte-macrophage-CSF (GM-CSF), M-CSF, G-CSF, stem cell factor (SCF), leukaemia inhibitory factor (LIF), Interleukin-1β (IL-1β), IL-6, IL-7, IL-8, IL-11 and transforming growth factor beta (TGFβ) (Namen *et al*, 1988; Paul *et al*, 1991; Watson and Mckenna, 1992; Guba *et al*, 1992). Furthermore, stromal cell lines can respond to external stimuli by changing their pattern of HGF production. For example, a variety of agents including IL-1α, tumour necrosis factor alpha (TNFα), bacterial lipopolysaccharides, IL-6, IL-7, and epidermal growth factor (EGF) induce cells to produce elevated levels of IL-6, IL-1β, GM-CSF, and G-CSF (Gimble *et al*, 1989; Sudo *et al*, 1989; Rennick *et al*, 1987b; Abboud and Pinzani, 1991; Slack *et al*, 1990; Yang *et al*, 1988; Wang *et al*, 1991; Umezawa *et al*, 1992; Thalmeier *et al*, 1996). Although most of the studies to date have failed to detect the expression of the pluripotent CSF, IL-3 (known to stimulate a wide range of progenitor cells including primitive HSC (Sonoda, 1988)), one study using the polymerase chain reaction did detect IL-3 mRNA expression in irradiated LTBMCs induced with pokeweed mitogen (Kittler *et al*, 1992).

As alluded to earlier, stromal cell-derived ECM proteins are comprised of a heterogeneous array of molecules including proteoglycans, fibronectin, laminin, and collagen. Many of the molecules in the ECM can interact with each other via glycosaminoglycan (GAG) side chains and GAG binding sites, forming a mesh that embeds stromal and haemopoietic cells (Long, 1992; Coulombel *et al*, 1988). The most abundant ECM component is collagen, and multiple types have been described. It appears that most collagen types are produced in primary cultures, and studies with stromal cell lines have detected various patterns of collagen secretion. Fibronectin and laminin are also produced by the stroma (Long, 1992; Zuckerman and Wicha, 1983). The most complex of the ECM molecules are the proteoglycans. These consist of a protein core to which various GAGs are covalently linked. Of the numerous GAGs identified, several have been reported in the Dexter type cultures (Wight *et al*, 1986). Furthermore, it has been demonstrated that heparan sulphate proteoglycan, a major constituent of BM derived sulphated GAG, has

the ability to bind growth factors IL-3, GM-CSF and bFGF and present them in a biologically active form to the haemopoietic stem and progenitor cells (Roberts *et al*, 1988a; Gordon *et al*, 1987a; Vlodavsky *et al*, 1991; Spooncer *et al*, 1983; refer to Figure 1.10.).

1.7.3. Membrane-Associated HGFs: Stem Cell Factor.

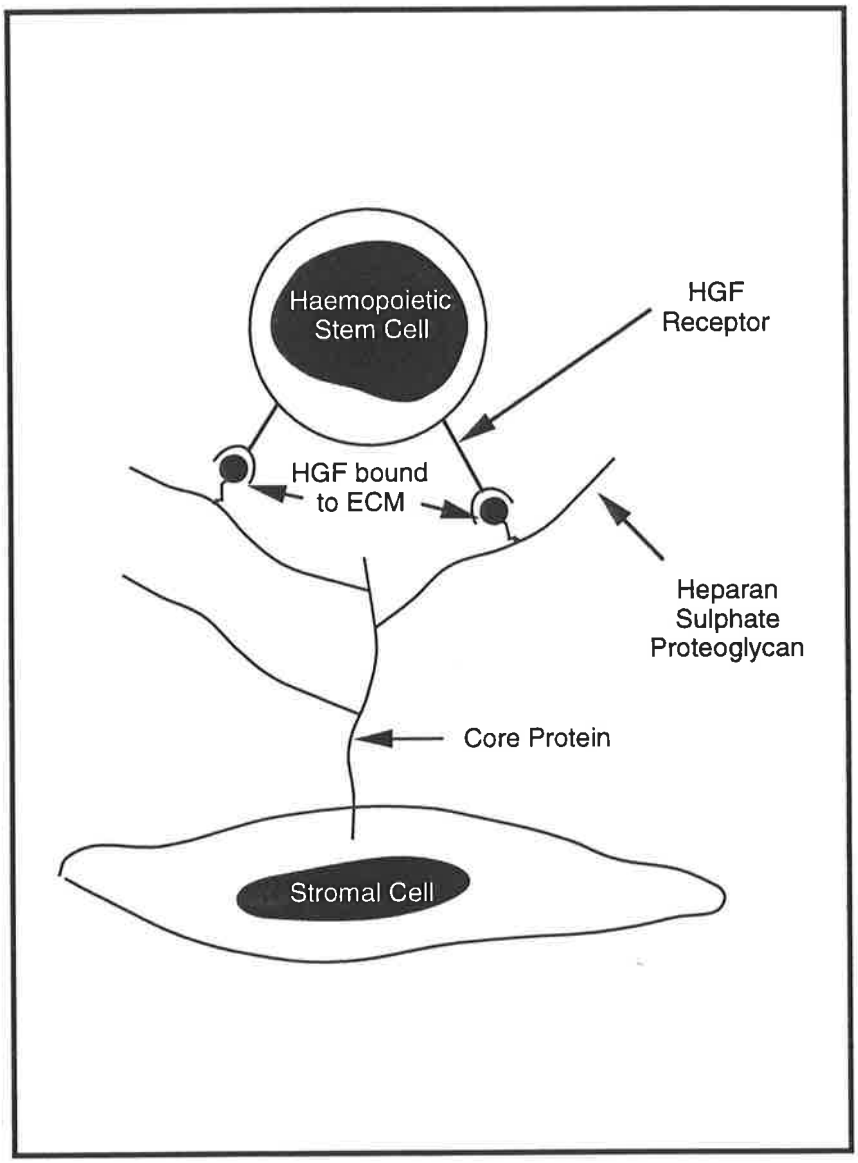
A number of HGFs are associated with the stromal cell surface via membrane-insertion domains, as exemplified by M-CSF (or CSF-1) (Rettenmier *et al*, 1987), FL (Lyman *et al*, 1993; Hannum *et al*, 1994) and the archetypal HGF encoded by the Steel locus (*Sl*) in mice (reviewed in Massagué and Pandiella, 1993).

The molecular cloning of SCF and its receptor c-Kit (CD117), resolved a long standing question regarding the nature of mutations in mice exhibiting defects in haemopoiesis, melanogenesis, and the reproductive system (Godin *et al*, 1991 and reviewed by Galli *et al*, 1994). Mutations at the steel (*Sl*) and dominant white spotting (*W*) loci in mice affect the development of haemopoietic stem cells, primordial germ cells and melanoblasts (Godin *et al*, 1991). The product of the *W* locus is c-*kit* (Qiu *et al*, 1988; Chabot *et al*, 1988; Alexander *et al*, 1991) whilst the *Sl* gene encodes the ligand for this receptor, a peptide growth factor variously referred to as stem cell factor (SCF), mast cell growth factor (MGF) or c-*kit* ligand (KL) (Zsebo *et al*, 1990; Flanagan and Leder, 1990; reviewed in Bernstein *et al*, 1991b). The c-*kit* gene is a proto-oncogene encoding a tyrosine kinase with homology to cell surface growth factor receptors, especially the CSF-1 receptor and the platelet-derived growth factor (PDGF) receptor (Qiu *et al*, 1988; Yarden *et al*, 1987). Recent experiments have demonstrated that its ligand SCF, provides a proliferative and differentiation stimulus for HPC and their progeny, including mast cell and granulocytic colonies (Williams *et al*, 1990; Zsebo *et al*, 1990).

Experimental evidence shows that the nature of the defects from either the *Sl* or the *W* loci are different, although they result in a demonstrably similar phenotype. The *W* mutation results in a defect in the haemopoietic precursor cells, whilst mutations in the *Sl* locus lead to abnormalities in the microenvironment (Dexter and Moore, 1977; Kaneko *et*

Figure 1.10. Model For The Regulation Of Haemopoiesis By Growth Factors And Extracellular Matrix Proteins (ECM).

Schematic diagram of the presumed heparan-sulphate-mediated presentation of haemopoietic growth factors (HGFs) by cellular elements of the BM stroma. In this model, HGFs produced by BM stromal cells are expressed on the stromal cell surface by binding to elements of the ECM (in this case, the heparan-sulphate chains (HS) of transmembrane proteoglycans). Transmembrane-signalling in HSC is initiated following the interaction of integral membrane growth factor receptors on HSC and HS-bound HGFs presented by the stroma. This paracrine mechanism is also thought to provide a mechanism for restricting HGFs to specific microenvironments within the BM. (Adapted from Gallagher and Dexter, 1990; Gordon, 1991).


Haemopoietic
Stem Cell
HGF
Receptor
HGF bound
to ECM
Heparan
Sulphate
Proteoglycan
Core Protein
Stromal Cell

al, 1991). Connected to this finding, a role has been reported for the c-*kit* receptor-ligand complex in providing a homing mechanism during stem cell migration and, later in development, in HSC proliferation, differentiation and survival (Keshet *et al*, 1991). For mast cells, the *c-kit* receptor acts as an adhesion molecule and directs their binding to COS cells transfected with a SCF cDNA (Flanagan *et al*, 1991). Moreover, NIH and BALB/c 3T3 fibroblasts were shown to stimulate the proliferation of co-cultured mast cells from wild type mice (Flanagan and Leder, 1990), whereas fibroblasts established from the embryos of Sl/Sl^d mice failed to support growth of mast cells (Fujita *et al*, 1989).

The amino acid sequence of the SCF from 3T3 cells (and human cells) suggests it is synthesised as an integral transmembrane protein which is further processed by proteolytic cleavage to form a soluble product which is secreted (Huang *et al*, 1990). The Sl^d mutation encodes a truncated c-*kit* ligand, lacking in transmembrane and cytoplasmic domains (Brannan *et al*, 1991). Northern blot analysis shows normal levels of mRNA in Sl^d marrow and studies using recombinant SCF expressed in yeast suggest that the soluble form of SCF of Sl^d mice is as active as the wild type soluble form in a mast cell proliferation assay (Brannan *et al*, 1991). Therefore, to account for the phenotype of the Sl^d, mutation it is now believed that interaction between the membrane form of SCF and the c-*kit* receptor not only provides a proliferative signal (classical HGF action) to HSC, but also serves as an adhesion molecule localising haemopoietic cells to the BM stromal cell surface (Flanagan *et al*, 1991). Thus, molecules which mediate the adhesion of progenitor cells to the BM stroma may not only be instrumental in the restricting HSC and their progenitors to the BM, but may also be critical in regulating the self-renewal and differentiation processes. These cellular adhesion mechanisms are reviewed below.

1.8. CELLULAR ADHESION MECHANISMS INVOLVED IN THE LOCALISATION AND REGULATION OF HAEMOPOIESIS.

1.8.1. Cellular Interactions In Haemopoiesis: A Multiplicity Of Mechanisms.

The cellular interactions that regulate haemopoiesis are regarded to be many and varied. This is to be anticipated when one considers the hierarchical nature of the

haemopoietic system (please refer to Section 1.1.2.), the number of cell lineages generated and the diversity of tissue environments associated with their development and maturation (BM, thymus, secondary lymphoid organs).

In all likelihood the molecules which mediate these interactions may function in a stage and/or lineage-specific manner as occurs, for example in the regulation of T-cell development (Springer, 1990; Dunon and Imhof, 1993). Thus, a variety of molecular species are probably involved in supporting a range of interactions throughout haemopoiesis. Current data support this hypothesis which, in many respects, parallels the diversity of adhesive interactions involved in regulation of the immune system (reviewed in Springer, 1990), particularly those involved between endothelial cells and leukocytes at sites of inflammation (reviewed in Carlos and Harlan, 1994).

In recent years there has been a considerable increase in our understanding of the mechanisms that regulate cell adhesion in multicellular organisms. A broad range of cell surface moieties have been identified that function as cell adhesion molecules (CAMs) for diverse ligands including integral membrane glycoproteins and ECM components (Springer, 1990; Albelda and Buck, 1990; Haynes *et al*, 1989; Bevilacqua and Nelson, 1993). Several distinct superfamilies of CAMs have been identified including the immunoglobulin gene superfamily, integrins, selectins, sialomucins, cadherins and the CD44 family (please refer to Table 1.3.), many of which are expressed by haemopoietic cells. Studies to investigate the function and contribution of particular CAMs to interactions of HPC with the stromal HM are complicated by the diversity of potential ligands for many of these CAMs which are exhibited by marrow stromal tissue (Table 1.4.). This is further compounded by an additional category of interactions which involve HGF-receptor mediated adhesion of HPC to stromal cell-derived HGF presented either as integral membrane proteins (Flanagan *et al*, 1991; Retenmier *et al*, 1987) or bound to ECM molecules (Roberts *et al*, 1988a). This multiplicity of potential adhesive mechanisms presents a major technical and conceptual challenge to the identification of those CAM-ligand pairs which are essential for localisation and regulation of haemopoiesis in the BM.

Table 1.3. Cell Adhesion Molecule Superfamilies.

ICAM, Intercellular Adhesion Molecule; LFA, Leukocyte Funtion Antigen; MHC, Major Histocompatability Antigen; VCAM-1, Vascular Cell Adhesion Molecule-1; PECAM-1, Platelet-Endothelial Cell Adhesion Molecule; N-CAM, Neural-Cell Adhesion Molecule; N-Cadherin, Neural-Cadherin; E-Cadherin, Epithelial-Cadherin; P-Cadherin, Placental-Cadherin; L-selectin, Leukocyte Selectin; E-selectin, Endothelial Selectin; P-selectin, Platelet Selectin; GlyCAM-1, Glycosylated Cell Adhesion Molecule-1; MAdCAM-1, Mucosal Addressin Cell Adhesion Molecule-1; PSGL-1, P-Selectin Glycoprotein Ligand-1.

Table 1.3. Cell Adhesion Molecule Superfamilies.

Immunoglobulin Superfamily Of Adhesion Receptors		
ICAM-1 (CD54)	LFA-2 (CD2)	Thy-1
ICAM-2	LFA-3 (CD58)	MHC Class I & II
ICAM-3	CD3/TCell Receptor	N-CAM
PECAM-1 (CD31)	CD4	c-kit
VCAM-1	CD8	
Cation-Dependent CAMS		
N-Cadherin	E-Cadherin	P-Cadherin
Integrins		
Numerous:	8 β-chains & 15 α-chains in multiple combinations	
Selectins		
L-Selectin (CD62L)	E-Selectin (CD62E)	P-Selectin (CD62P)
Sialomucins		
CD34	GlyCAM-1	PSGL-1
CD43	MAdCAM-1	
Miscellaneous		
CD44	Syndecan	CD36

Table 1.4. ECM, Glycoproteins And CAMs Expressed On Bone Marrow Stromal Cells.

ECM Components	GAGs/Proteoglycans	CAMS
Collagen I	Chondroitin Sulphate	ICAM-1 (CD54)
Collagen lll	Heparan Sulphate	N-CAM (CD56)
Collagen IV	CD44	VCAM-1 (CD106)
Collagen V	Hyaluronate	LFA-3 (CD58)
Collagen Vl	Thy-1	
Fibronectin		
Vitronectin		
Laminin		
Thrombospondin		
Haemonectin		

Based on data from Zuckerman and Wicha (1983), Long and Dixit (1990), Campbell *et al* (1990), Gordon (1988), Gallagher (1989), Simmons *et al* (1992), Kincade *et al* (1989), Teixido *et al* (1992).

Nevertheless, some likely candidates are beginning to emerge and these are reviewed below.

1.9. THE SELECTIN FAMILY.

1.9.1. An Overview.

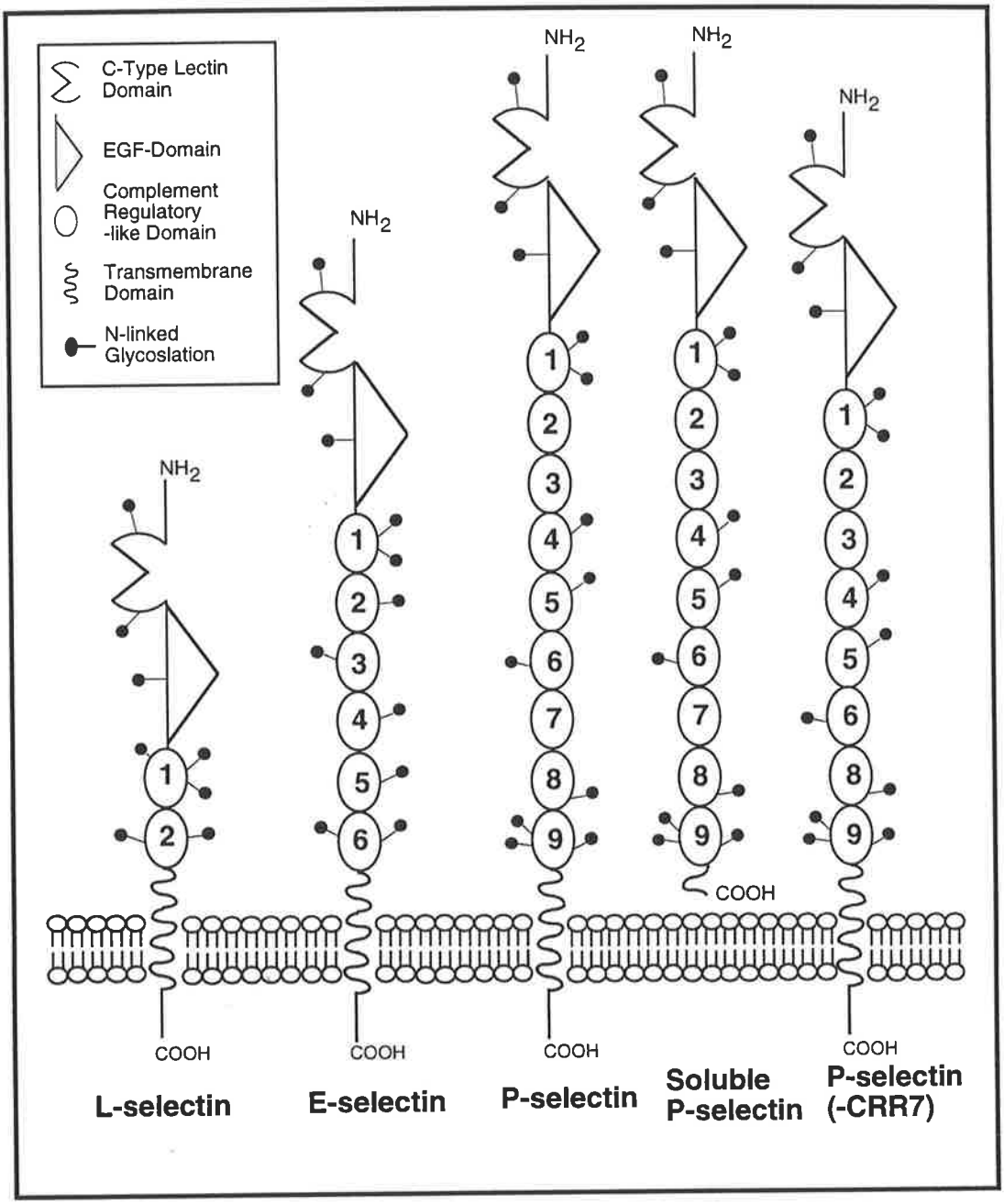
As formerly discussed, mammalian haemopoiesis is restricted to the extravascular compartment of the BM which is separated from the vascular compartment by a single layer of endothelial cells which form specialised vessels termed sinusoids (Weiss, 1976; Lichtman, 1981). Thus in order to enter or egress from the haemopoietic compartment, stem cells must first recognise, or be recognised by, the luminal surface of the endothelium. Molecules which mediate adhesion of haemopoietic stem cells to vascular endothelial cells are therefore likely to play a pivotal role. The selectin family of molecules and specialised carbohydrates displayed by the sialomucin family represent potential receptor ligand pairs which are critically involved in this process.

The selectins are three structurally related glycoproteins designated by the prefixes E (endothelial), P (platelet) and L (leukocyte) (Bevilacqua and Nelson, 1993; Lasky, 1992), that participate in leukocyte adhesion to vascular endothelium and platelets (Siegelman and Weissman, 1989; Bowen *et al*, 1989; Siegelman *et al*, 1989; Lasky *et al*, 1989). E-selectin (CD62E) and P-selectin (CD62P) are expressed by endothelial cells, and L-selectin (CD62L) is expressed on leukocytes. As illustrated in Figure 1.11., the selectins share a number of common structural features including the presence of an amino (NH_2)-terminal Ca^{2+}-dependent (C-type) lectin binding domain, an epidermal growth factor (EGF)-like region, a variable number of short consensus repeats (SCR) homologous to those found in various complement-regulatory proteins, a membrane-anchoring motif, and a short cytoplasmic region (Bevilacqua and Nelson, 1993). The selectins share an overall identity of 40% to 60% at the nucleotide and protein level respectively and moreover, the lectin and EGF domains share 60% to 70% identity (Lasky, 1992). The genes for the selectin family are closely linked (within a 200 kb region) on long arm of the human and murine chromosome 1 (q21-24) (Watson *et al*, 1990; Collins *et al*, 1991) and are located in

Figure 1.11. Conservation Of Domain Structure Of The Selectin Family Of Adhesion Molecules.

The extracellular portion of each selectin contains an amino terminal domain of 117-120 amino acids which are homologous to a variety of Ca^{2+}-dependent animal lectins. This is followed by an adjacent epidermal growth factor-like (EGF) domain of 34-40 amino acids, and a variable number of 62 amino acids, short complement regulatory-like motifs (SRC). L -, E- and P-selectin harbour 2, 6 and 9 SCR domains, respectively (numbered circles). A variant form of P-selectin (-CRR7) which arises by alternative splicing of mRNA, is devoid of complement repeat region 7. This region is followed by a transmembrane sequence and a short cytoplasmic domain at the carboxyl terminus of each selectin molecule. A second variant form of P-selectin which lacks the membrane-anchoring motif (soluble P-selectin) has also been described.

E-selectin is expressed following stimulation of endothelial cells (ECs) with pro-inflammatory cytokines such as IL-1 and TNFα. P-selectin is constitutively expressed by ECs and platelets and is stored in intracellular vesicles (refer to text for details). Following stimulation with agents such as histamine and thrombin, P-selectin is mobilised to the cell surface. L-selectin is expressed on the surface of neutrophils, monocytes, eosinophils and a subset of lymphocytes. Stimulation of lymphocytes leads to the shedding of L-selectin from the cell surface.


C-Type Lectin Domain
EGF-Domain
Complement Regulatory -like Domain
Transmembrane Domain
N-linked Glycoslation
NH2
COOH
L-selectin
E-selectin
P-selectin
Soluble P-selectin
P-selectin (-CRR7)

a region harbouring genes for other complement-binding proteins (C4 binding protein and decay accelerating factor) and coagulation Factor V, suggesting that the selectin family may have arisen by gene duplication (Watson *et al*, 1990).

1.9.2. The Selectin Family: Molecular Cloning And Structural Characterisation.

Human L-selectin (refer to Table 1.5., for alternate nomenclature) represents the homologue of the 90 kD murine peripheral lymph node homing receptor, originally identified by the monoclonal antibody (mAb) MEL-14 (Gallatin *et al*, 1983). Monoclonal Ab MEL-14 was found to block lymphocyte adhesion to high endothelial venules of lymph nodes *in vitro* and blocked lymphocyte homing to lymph nodes *in vivo*. Although initially described as a lymphocyte homing receptor, it was subsequently shown to be constitutively expressed on most other peripheral blood leukocytes including neutrophils and monocytes, and shown to be involved in leukocyte traffic in the systemic microcirculation (Lewisohn *et al*, 1987).

The molecular cloning of human L-selectin unveiled the presence of a number of conserved structural motifs shared by both P- and E-selectins (Tedder *et al*, 1989; Camerini *et al*, 1989; Siegelman and Weissman, 1989; Bowen *et al*, 1989). Translation of the sequence revealed that the L-selectin complementary DNA (cDNA) encoded a core protein of 37 kD which contained two SCR of the complement-regulatory protein domains (Figure 1.11.). Additionally, eight consensus sites for potential N-linked glycosylation were identified. However, no serine- or threonine-rich regions were identified, consistent with the lack of O-liked sugars (Siegelman and Weissman, 1989). The molecular weight of L-selectin differs among lymphocytes (~75 kD), neutrophils (~95 to 105 kD), and monocytes (~110 kD) and this variability is thought to result from cell-specific post-translational glycosylation among these subsets of leukocytes. Sequence analysis also revealed that L-selectin contained 22 cysteine residues, 19 of which are in the SCR and EGF domains, suggesting a significant role for disulfide bond formation in providing appropriate spatial conformation for the lectin region that is the proposed site of ligand binding.

Table 1.5. The Selectin Family: Nomenclature And Expression.

Alternate Nomenclature	Expression Patterns	Surface Expression
L-selectin (CD62L)		
mLHR, Leu8 TQ-1, gp90MEL Lecam-1, Leccam-1 LAM-1	Lymphocytes Monocytes Neutrophils Haemopoietic Progenitor Cells	-Constitutive surface expression. -Conformational changes -Shed following cellular activation.
P-selectin (CD62P)		
PADGEM GMP-140	Platelets Endothelium Bone Marrow Endothelial Cells	-Thrombin, Histamine & others. -From storage granules (minutes). -Cytokine inducible (hours) RNA, protein synthesis
E-selectin (CD62E)		
ELAM-1	Endothelium Bone Marrow Endothelial Cells	-Cytokine inducible (hours) *de novo* RNA, protein synthesis required.

ELAM-1, endothelial-leukocyte adhesion molecule 1; GMP-140, granule membrane protein 140; PADGEM, platelet activation-dependent granule external membrane protein.

(Adapted from Bevilacqua and Nelson, 1993.)

Furthermore, mapping of L-selectin domains by mAbs has established that the NH_2-terminal nine amino acids are critical for ligand binding (Kishimoto *et al*, 1990; Kansas *et al*, 1991). The EGF and SCR units of L-selectin appear to also be important in maintaining the spatial conformation of L-selectin as mAbs that interact with these domains affect binding in the lectin domain (Jutila *et al*, 1992; Siegelman *et al*, 1990; Watson *et al*, 1991).

A differentially spliced form of human L-selectin that lacks the transmembrane anchoring motif and is putatively attached to the cellular membrane by a phosphotidyl-inositol (PI) lipid linkage has been reported by Camerini *et al* (1989). Analysis of exon structure of the murine (Dowbenko *et al*, 1991) and the human (Ord *et al*, 1990) homing receptor genes, and treatment of MEL-14 positive cells with phosphatidylinositol (PI)-phospholipase C however, has led Lasky (Lasky, 1995) to contend that this isoform is due to a cloning artefact. Despite this, there is adequate evidence that L-selectin is shed upon activation of leukocytes, but this is likely to be attributable to proteolytic cleavage near the membrane insertion and not the loss of the reputed PI-lipid linked isoform (Kishimoto *et al*, 1990; Jung and Dailey, 1990). Soluble circulating L-selectin has been measured in the plasma of normal individuals and found to be as high as 1.6 μg/ml (Carlos and Harlan, 1994). Furthermore, plasma-derived soluble L-selectin was able to partially inhibit leucocyte adhesion to cytokine-stimulated endothelium (Schleiffenbaum *et al*, 1992), suggesting that circulating L-selectin retains (at least in part) an active conformation, and may serve an anti-adhesive function in the regulation of leukocyte adhesion to endothelium during inflammation (Lasky, 1992).

P-selectin (refer to Table 1.5., for alternate nomenclature) represents an adhesion protein initially characterised by investigators concerned with the biochemical events associated with platelet activation (Bevilacqua and Nelson, 1993). The production of mAbs against the surface of thrombin-activated platelets resulted in the isolation of a 140 kD transmembrane glycoprotein specifically expressed on the surface of activated but not resting platelets. Immunochemical analysis revealed that this molecule variously termed platelet activation-dependent granule-external membrane protein (PADGEM, Hsu-Lin *et*

al, 1989) or granule membrane protein-140 (GMP-140, McEver and Martin, 1989) was associated with the α-granules in resting platelets (Stenberg *et al*, 1985; Berman *et al*, 1986), and rapidly (within minutes) redistributed to the external plasma membrane upon activation with a variety of agonists including thrombin, histamine and calcium ionophore A23187. Subsequently, P-selectin was shown to be present also in storage granules of endothelial cells known as Weibel-Palade bodies (McEver *et al*, 1989; Bonfanti *et al*, 1989). Following stimulation with thrombin or other inflammatory mediators, the endothelial form of P-selectin is also rapidly translocated to the cell surface following fusion of the storage granules with the plasma membrane (McEver *et al*, 1989; Bonfanti *et al*, 1989).

The molecular cloning and translation of the sequence, engendered P-selectin with a core protein with a predicted molecular weight of 86 kD, nine SCR of the complement-regulatory protein domains and no O-linked sugars in the mature protein (Johnston *et al*, 1989a; 1989b). Twelve potential N-linked glycosylation sites were identified, and if fully exploited render a glycoprotein of 122 kD. The majority of the cysteine residues (54 of 65) are present within the nine SCR of the complement regulatory-like region (six cysteinyl residues per module) and are responsible for the disulfide bridge organisation in P-selectin. Finally, human P-selectin has a cytoplasmic domain of 35-amino acid residues which includes serine and threonine residues which are phosphorylated following platelet activation (Crovello *et al*, 1993; Fujimoto *et al*, 1993).

Two additional, variant isoforms of P-selectin that arise by alternative splicing of mRNA have been described (Johnston *et al*, 1990; please refer to Figure 1.11.). In variant -CRR7, there is deletion of the seventh SCR. Recent work has demonstrated that the number of these complement binding motifs is species specific and as such, suggests a less critical role for the absolute number of these motifs (Lasky, 1995). In the soluble variant, the membrane-anchoring motif is deleted, consistent with a potential secretory form of this adhesion molecule. Concordant with this possibility, soluble P-selectin has been detected in the plasma of normal individuals at levels ranging from 150 to 300 ng/ml, however it is still to be determined whether this represents the secreted form rather than protein shed from platelets or endothelial cells (Carlos and Harlan, 1994; Lasky, 1995).

At this plasma concentration, it has been estimated that 20-40% of the binding sites for P-selectin on neutrophils would be saturated, raising the possibility that soluble plasma P-selectin may inhibit leukocyte adhesion to P-selectin expressed by tissues (Dunlop *et al*, 1992).

Like L- and P-selectin, the discovery of E-selectin (refer to Table 1.5., for alternate nomenclature) involved a mAb-based strategy that identified a 110-115 kD cellular activation antigen that was induced on cultured human umbilical vein endothelium following the stimulation by IL-1 or TNFα (Pober *et al*, 1986) and on endothelial cells at sites of inflammation *in vivo* (Cotran *et al*, 1986). E-selectin was concomitantly shown to mediate adhesion of neutrophils and monocytes to activated endothelium (Bevilacqua *et al*, 1985). Consistent with L- and P-selectin, the molecular cloning of E-selectin revealed the presence of a C-type lectin binding domain, an EGF-like domain, and six complement-regulatory protein motifs (Bevilacqua *et al*, 1989; Hession *et al*, 1990; Polte *et al*, 1990; refer to Figure 1.11.). Furthermore, following sequence analysis, it was apparent that the core protein of E-selectin had a predicted molecular weight of 64 kD with 11 potential N-linked glycosylation sites contributing to almost half of the apparent mass of the molecule. Finally, human E-selectin has a cytoplasmic domain of 32 amino acids which contains a number of tyrosine residues speculated to be involved in the internalisation of E-selectin.

As with P-selectin, the absolute number of complement binding motifs in E-selectin is species-specific and as such is of little consequence to ligand binding capacity (Lasky, 1995). However, an overall sequence homology of approximately 65% within the lectin and EGF-like domains between all three selectins is consistent with their importance in carbohydrate recognition and ligand binding. Mapping of the E-selectin domains with mAbs has demonstrated that the NH_2-terminal nine amino acids of the lectin domain and an epitope within the EGF-like region are critical for ligand binding (Pigott *et al*, 1991; Erbe *et al*, 1992). A study by Erbe *et al* (1992) employing site specific mutagenic analysis and three-dimensional modelling of the lectin domain revealed that three spatially linked, positively charged residues (arginine $(R)^{97}$, lysine $(K)^{111}$ and lysine $(K)^{113}$) are critical for sialylated Lewis X recognition (please refer to Section 1.9.5.) and furthermore K^{111} and

K^{113} are conserved in all three selectins cloned to date. Since all three selectins (Butcher, 1991, Moore *et al*, 1991; Polley *et al*, 1991; True *et al*, 1990; Tyrrell *et al*, 1991) require sialic acid for adhesion, the conservation of these two residues is consistent with a direct role for these residues in sialic acid recognition, putatively via salt-bridge or hydrogen bond formation (Erbe *et al*, 1992).

1.9.3. The Selectin Family: Distribution And Patterns Of Expression.

Although it is abundantly clear that the selectins are closely related in structure and function, their patterns of expression are quite disparate. As alluded to earlier, P-selectin is constitutively synthesised by both platelets and endothelial cells and subsequently targeted to cytoplasmic secretory/storage granules by means of a sorting signal present in the cytoplasmic domain (Koedam *et al*, 1992; Disdier *et al*, 1992). A variety of mediators, including thrombin (Hsu-Lin *et al*, 1984; Stenberg *et al*, 1985), histamine, and other mediators released following mast cell activation (Hattori *et al.*, 1989a, Kubes and Kanwar, 1994, Thorlacius *et al*, 1994), complement C5b-9 complex or C5a fragment (Hattori *et al*, 1989b, Foreman *et al*, 1994), and peroxides (Patel *et al*, 1991) have been shown to elicit a rapid mobilisation of the intracellular pools of P-selectin to the cell surface where it can subsequently mediate adhesion to leucocytes (Bonfanti *et al*, 1989; McEver *et al*, 1989; Hattori *et al*, 1989a; 1989b). The expression of P-selectin at the cell surface is often short-lived, declining considerably within minutes due to shedding of the molecule as described in Section 1.9.2. Furthermore, Handa *et al* (1991) demonstrated that mobilisation of P-selectin to the platelet membrane was inhibited with N,N-dimethyl and N,N-trimethyl derivatives of sphingosine and other selected inhibitors of protein kinase C-dependent phosphorylation (Handa *et al*, 1991), suggesting that phosphorylation of the cytoplasmic serine and threonine was required for translocation of P-selectin to the plasma membrane (Handa *et al*, 1991).

The expression of E-selectin appears to be largely restricted to activated endothelial cells. As alluded to earlier (Section 1.9.2.), stimulation of cultured endothelium with endotoxin (LPS) or inflammatory cytokines, including IL-1 and TNF_{α} results in the

upregulation and expression of E-selectin at the plasma membrane (Bevilacqua *et al*, 1989; Bevilacqua *et al*, 1987). This expression of E-selectin is reliant on *de novo* RNA and protein synthesis, peaks at 4-6 hours post-stimulation and declines to constitutive/basal levels by 24 to 48 hours. Independently, both Doukas, Pober (1990) and Leeuwenberg *et al* (1990) recognised that although unable to induce E-selectin expression, treatment of endothelial cells with Interferon gamma (IFNγ) prolonged its expression following induction with IL-1. Concordant with this observation, examination of the 5' sequence of the regulatory elements of the E-selectin gene revealed the presence of elements necessary for cytokine inducible expression, including sequences consistent with NF-κB and AP-1 binding sites (Collins *et al*, 1991; Becker-Andre *et al*, 1992; Montgomery *et al*, 1991; Whelan *et al*, 1991).

Unlike both P- and E-selectin, only L-selectin expression on the cell surface is constitutive and there is no evidence for accumulation in granules or intracellular vesicles. As previously discussed, membrane associated L-selectin is shed following activation (Jung *et al*, 1988; Kishimoto *et al*, 1989; Berg and James, 1990) and its activity therefore appears to be controlled by regulation of its loss from the cell surface.

1.9.4. Counter Receptors For Selectins.

As detailed in Section 1.9.2., L-selectin was historically characterised as the murine homing receptor that mediated lymphocyte binding to high endothelial venules (HEV) in peripheral lymph nodes (Gallatin *et al*, 1983). In early work, Rosen, Stoolman and collegues examined leukocyte binding to HEVs of frozen sections of lymphoid tissue using the Stamper-Woodruff assay (Stamper and Woodruff, 1986). These investigators demonstrated that the binding of lymphocytes to HEV could be blocked by a number of monomeric, anionically charged sugars, such as mannose-6-phosphate (Stoolman and Rosen, 1983). Later studies demonstrated that more complex carbohydrates, such as a specific subset of polyvalent, anionic sugars such as fucoidin, and yeast cell wall polyphosphomannan ester (PPME) were significantly more effective at abrogating adhesion of lymphocytes to HEV (Rosen *et al*, 1985). Further evidence for a role of

carbohydrate recognition in these adhesive events came from studies of the effects of the enzyme sialidase (neuraminidase) on lymphocyte-HEV binding. Removal of the sialic acid (an acidic/anionic carbohydrate) found on the non-reducing ends of many cell-surface glycoconjugates resulted in a demonstrable reduction in lymphocyte adhesion to HEV of peripheral lymph nodes. These data were thus consistent with the possibility that lymphocytes utilised protein-anionic carbohydrate (carbohydrate moiety) interactions to adhere to the endothelium of peripheral lymph nodes (Lasky, 1995).

This proposal was validated by the molecular cloning of L-selectin that showed a NH_2-terminal C-type carbohydrate binding or lectin domain (Tedder *et al*, 1989; Camerini *et al*, 1989; Siegelman and Weissman, 1989; Bowen *et al*, 1989; Siegelman *et al*, 1989; Lasky *et al*, 1989). The presence of lectin-like domains in both E- and P- selectin suggested that leukocyte binding to these receptors would also involve recognition of carbohydrates. Various antibody studies supported this hypothesis, as mAbs directed against the lectin domain of these molecules abolished cell adhesion (Bowen *et al*, 1990; Erbe *et al*, 1992). In addition, it was established that cell adhesion mediated by these proteins was completely dependent upon calcium, a finding consistent with previous data demonstrating that carbohydrate recognition by the type C lectins was calcium dependent.

As lectin-carbohydrate interactions are characterised by a lower affinity than most well described protein-protein binding interactions (such as antibody-antigen, growth factor-growth factor receptor), it has been essential to characterise lectins according to their binding specificities and to establish their binding affinities (Bevilacqua and Nelson, 1993). To this end, work in recent years from several laboratories has led to an increase in our understanding of the minimal carbohydrate ligands recognised by the three selectin receptors. Additionally, several proteins have been shown to participate in selectin binding and these are reviewed below.

1.9.5. Carbohydrate Ligands For The Selectin Family Of Adhesion Molecules.

During 1990 and 1991, several groups found that antibodies directed against a previously described fucosylated tetrasaccharide antigen, sialyl Lewis X (SLe^X:

NeuNacα2-3Galβ1-4(Fuc1-3)GlcNac; NeuNac: sialic acid, Gal: galactose, Fuc: fucose and GlcNac: N acetyl glucosamine) (CD15s, Figure 1.12. (A)), expressed on neutrophils, monocytes (Walz *et al*, 1990; Munro *et al*, 1992), natural killer (NK) cells (Ohmori *et al*, 1989; Pinola *et al*, 1994) and *ex vivo* activated peripheral blood T and B lymphocytes, were able to block cell adhesion mediated by both E- and P-selectin (Hollenbaugh *et al*, 1993; Bajorath *et al*, 1994; Norgard *et al*, 1993; Graves *et al*, 1994; Drickamer, 1992; Walz *et al*, 1990; Phillips *et al*, 1990; Polley *et al*, 1991). More recently, Tyrell *et al* (1991) demonstrated that both the sialic acid and the fucose linkages were critical for efficient recognition by E-selectin (Tyrell *et al*, 1991; Nelson *et al*, 1993) which was consistent with previous data indicating that sialic acid was an absolute requirement for L- and P-selectin mediated adhesion.

A subset of skin-homing lymphocytes which express the cutaneous lymphocyte antigen (CLA) recognised by the HECA-452 mAb (Berg *et al*, 1991a; 1991b) have also been demonstrated to bind to E-selectin (Picker *et al*, 1991a; 1991b; Berg *et al*, 1991a) and although the CLA-positive cells do not express SLe^X, they do exhibit the Lewis X antigen (Le^X, Figure 1.12. (A)) after treatment with neuraminidase (Berg *et al*, 1991a). Thus, the cutaneous lymphocyte antigen appears to be a sialylated, fucosylated structure closely related to SLe^X.

E-selectin also recognises an isomeric carbohydrate structure related to SLe^X, termed sialyl Lewis A (SLe^a: NeuNacα2-3Galβ1-3(Fuc1-4)GlcNac (Figure 1.12. (B)) (Tyrell *et al*, 1991; Berg *et al*, 1991b). The HECA-452 mAb recognising CLA binds to SLe^a as well as SLe^X (Berg *et al*, 1991b). Consistent with the ability of both oligosaccharides to mediate selectin-carbohydrate recognition (Lasky, 1995), two-dimensional nuclear magnetic resonance (NMR) studies indicate that these closely related structures can adopt related conformations in solution with both the carbohydrate of the sialic acid and the fucose adopting the same directional stoichiometry (Lasky, 1995).

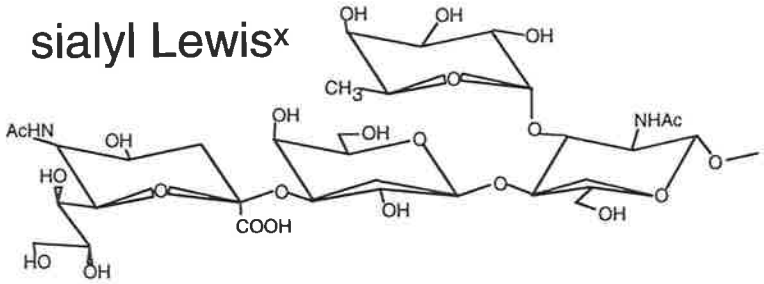
Initial studies demonstrated that P-selectin recognised the Le^X trisaccharide (CD15), Galβ1-4(Fuc1-3)GlcNac (Larsen *et al*, 1990), although the sialylated tetrasaccharide, SLe^X, was subsequently demonstrated to be a higher affinity ligand

Figure 1.12. Oligosaccharide Ligands Of The Selectins.

The structural isomers sialyl Lewis-x (SLe^x, Panel A) and sialyl Lewis-a (SLe^a, Panel B) both contain terminal sialic acid (Neu5Ac) linked α2-3 to galactose (Gal), in turn linked to an N-acetylglucosamine (GlcNAc). Both structures contain a fucose coupled to the GlcNAc. SLe^x and SLe^a differ in the linkages of galactose and fucose (Fuc) to the GlcNAc. The related carbohydrate structures, sulfo $Lewis^x$, sulfo $Lewis^a$, $Lewis^x$ and $Lewis^a$ are also shown. The indicated structures are typically found as terminal residues of larger oligosaccharides on glycoproteins and glycolipids. (Adapted from Bevilacqua and Nelson, 1993; Nelson *et al*, 1995).

A

sialyl Lewis^x



Neu5Ac α2-3 Galβ1-4 GlcNAc——
|
Fucα1-3

sulfo Lewis^x

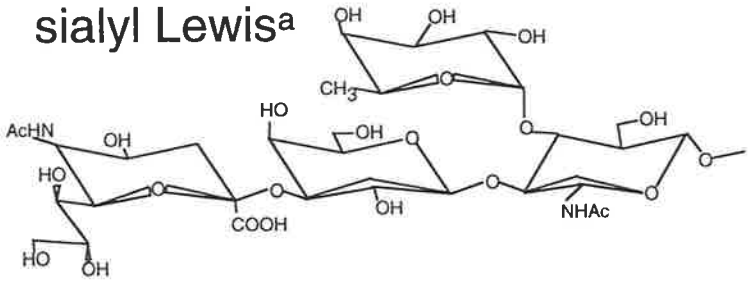
O_3-SO-3Galβ1-4 GlcNAc——
|
Fucα1-3

Lewis^x

Galβ1-4 GlcNAc——
|
Fucα1-3

B

sialyl Lewis^a



Neu5Ac α2-3 Galβ1-3 GlcNAc——
|
Fucα1-4

sulfo Lewis^a

O_3-SO-3Galβ1-3 GlcNAc——
|
Fucα1-4

Lewis^a

Galβ1-3 GlcNAc——
|
Fucα1-4

(Handa *et al*, 1991a; Polley *et al*, 1991; Zhou *et al*, 1991; Foxall *et al*, 1992). Like E-selectin, P-selectin also binds to SLe^a (Handa *et al*, 1991a), however SLe^x and SLe^a fail to compete for binding to activated platelets, suggesting that P-selectin may use other structural modifications (Handa *et al*, 1991a). In this regard, P-selectin was also demonstrated to bind to sulphated glycolipids (Handa *et al*, 1991b, Aruffo *et al*, 1991; Todderud *et al*, 1992; Needham and Schnaar, 1993) and certain sulphated polysaccharides such as an uncharacterised fraction of heparin (Skinner *et al*, 1989).

L-selectin is involved in leukocyte adherence to nonlymphoid microvasculature as well as to peripheral lymph node HEV (Lewisohn *et al*, 1987). Like E- and P-selectin, murine L-selectin has been shown to bind to SLe^x (and SLe^a) (Berg *et al*, 1992; Foxall *et al*, 1992; Green *et al*, 1992). Although mAbs to SLe^x react minimally with nonlymphoid endothelium *in vivo* or *in vitro*, a SLe^x antigen was recently found to be expressed on human HEV, suggesting that it may serve as a ligand for L-selectin (Munro *et al*, 1992; Sawada *et al*, 1993). In the murine system, L-selectin ligands in HEV are sialylated and fucosylated like SLe^x, but also contain sulfate residues which are absolutely required for the high avidity recognition by the lectin domain of L-selectin (Imai *et al*, 1993). Biochemical analysis of the degraded sulfated oligosaccharides, revealed that the two uniquely modified, sulfated forms of the SLe^x carbohydrate, and had the putative structures of NeuNacα2-3(SO_4-6)Galβ1-4(Fuc1-3)GlcNac and NeuNacα2-3Galβ1-4(Fuc1-3)(SO_4-6)GlcNac (refer to Figure 1.12. (A)). L-selectin and P-selectin (but not E-selectin) also recognise sulphatides (Handa *et al*, 1991; Aruffo *et al*, 1991; Todderud *et al*, 1992; Needham and Schnaar, 1993; Skinner *et al*, 1989, Imai *et al*, 1993; 1990) and sulphated-polysaccharides such as fucoidan and heparin (Handa *et al*, 1991; Skinner *et al*, 1989). Recently, heparin-like ligands for L-selectin were identified in cultured nonlymphoid endothelial cells, and these structures are candidates for selectin ligands in the systemic microvasculature (Norgard-Sumnicht *et al*, 1993; Carlos and Harlan, 1994).

The complex nature of the carbohydrate ligands for the selectin family suggests that a diverse group of glycosylating enzymes are required for the generation of these oligosaccharides. As fucose and sialic acid are integral elements of these oligosaccharides,

research has focused on the enzymes that enamour the lactosaminoglycan backbone with these components. To date, three sialyl transferase and a number of candidate fucosyltransferase enzymes have been cloned at the molecular level, however none have been attributed with the function of producing selectin ligands (Kitigawa and Paulson, 1994). More recently however, Sasaki and collegues (1994) have molecularly cloned an enzyme, Fuc-TVII whose characteristics fulfil the requirements of a fucosylating enzyme involved in myeloid SLe^X production (Sasaki *et al*, 1994, Lasky, 1995). These characteristics include: restricted expression in neutrophils; production of a SLe^X carbohydrate that is recognised by a number of anti-SLe^X antibodies; and an ability to confer a number of cell types with the capacity to bind to E-selectin. Despite this, the characteristics of the enzyme(s) involved in the sulfate modification critical for L-selectin binding have yet to be ascertained (Lasky, 1995).

1.9.6. Protein Ligands For The Selectin Family Of Adhesion Molecules: The Emergence Of The Mucin-like Molecule Family.

As alluded to in Section 1.9.5., the lectin-carbohydrate binding critical to selectin mediated adhesion is of low affinity (Bevilacqua and Nelson, 1993), and of insufficient avidity to insure adequate adhesion of leukocytes under the high shear forces encountered in the blood vessel. Coincident with the studies on oligosaccharide ligands of the selectins however, was the realisation that certain integral membrane proteins could also participate in these adhesive interactions by presenting specific carbohydrate moieties to the lectin domains and thus contribute to higher affinity binding of selectins to cellular ligands (Lasky, 1992). The glycoprotein ligands which contribute to selectin binding are discussed below.

The initial breakthrough in elucidating the mechanisms by which selectins increased their avidities for carbohydrate moieties came from the study of endothelial ligands recognised by L-selectin in the murine system. Lasky and colleagues (1992) identified two glycoproteins, a predominant species band of 50 kD and a minor band of 90 kD when ^{35}S-labelled proteins from peripheral lymph node organ culture were precipitated using a

L-selectin-IgG chimeric protein (Lasky *et al*, 1992). Initial studies of the 50 kD sulphated glycoprotein indicated that a majority of the carbohydrate side chains associated with this molecule were O-linked and appeared to be heterogeneous in size (Imai *et al*, 1991; Imai *et al*, 1993). Additionally, studies using organ cultures also revealed that the 50 kD sulphated glycoprotein (sgp50) was rapidly shed from the endothelium (Imai *et al*, 1991; Brustein *et al*, 1992).

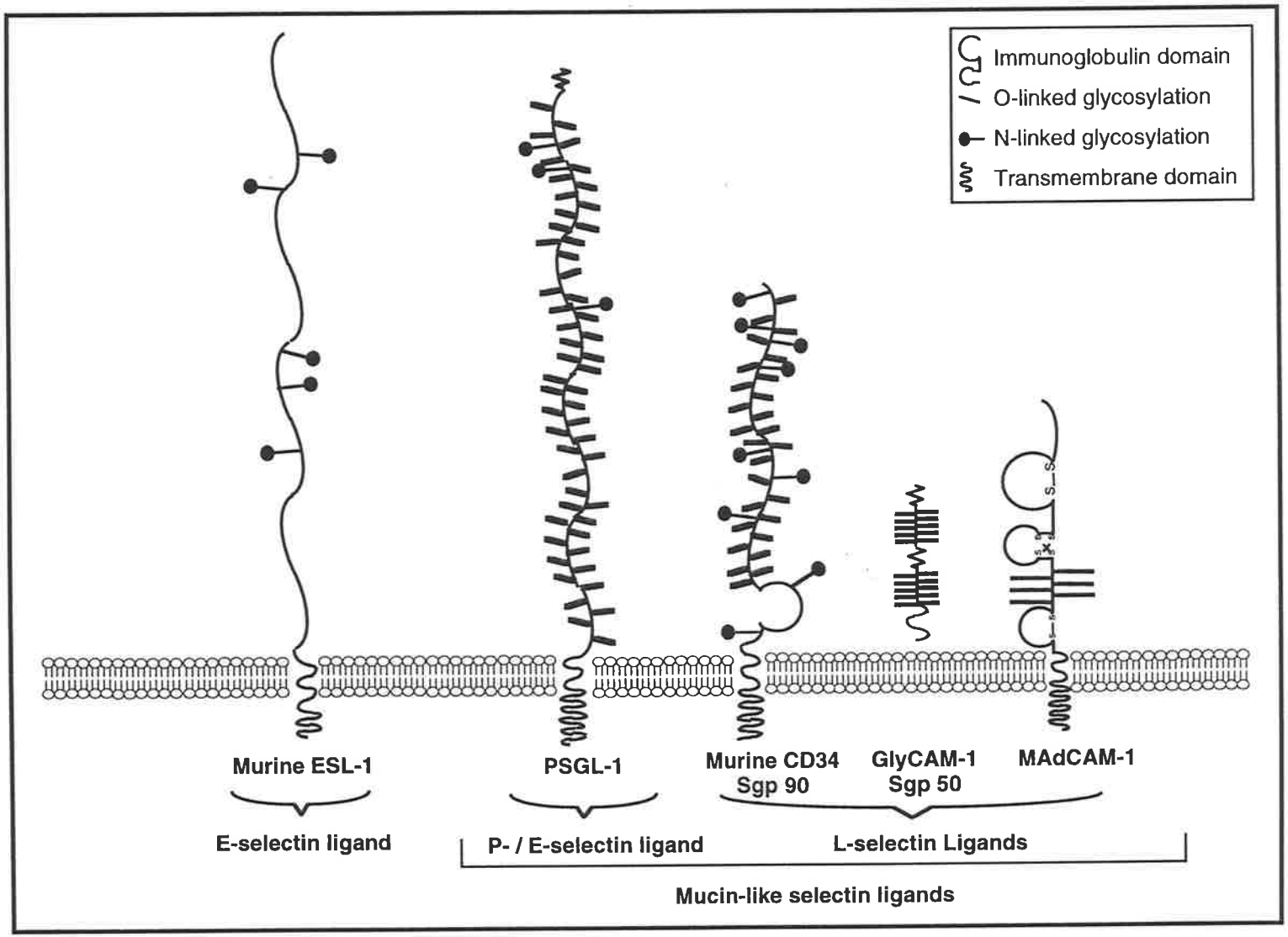
The nature of the 50 kD glycoprotein was subsequently clarified by molecular cloning. Amino acid sequence data enabled the generation of oligonucleotide primers which were used to screen a cDNA library prepared from murine lymph nodes (Lasky *et al*, 1992). Translation of the derived cDNA sequence, predicted a mature protein of 132 amino acids with a core protein molecular weight of approximately 14 kD. Moreover, the protein was comprised of approximately 30% serine and threonine amino acids (putative sites of O-linked glycosylation) clustered into two domains. These distinct regions of O-linked glycosylation are considered to provide a scaffold for the presentation of the polyvalent carbohydrates previously demonstrated to be involved in ligand binding (refer to Section 1.9.5.). The presence of only one consensus region for N-linked glycosylation suggested that 70% of the mass was attributed by O-linked carbohydrates. Because of the apparent dependence on glycosylation, this L-selectin ligand was designated the name glycosylation-dependent cell adhesion molecule-1 (or GlyCAM-1). In addition, the sequence of GlyCAM-1 is similar to other heavily glycosylated proteins such as CD34 and MUC-1 and, like PSGL-1 (see below), represents a new member of an ever-expanding family of sialomucins which have roles in adhesion (Shimizu and Shaw, 1993). Interestingly, analysis of the structure of GlyCAM-1 revealed the absence of a recognisable transmembrane domain, suggesting that GlyCAM-1 may function primarily as a circulating soluble molecule (Lasky, 1995; Brustein *et al*, 1992 and refer to Figure 1.13.). Similarly, a differentially glycosylated, non-L-selectin binding form of this mucin was also produced by mammary epithelial cells during lactation and was isolated from the whey fraction of milk (Dowbenko *et al*, 1993).

Figure 1.13. Glycoprotein Ligands For The Selectin Family: A Prominent Role For The Sialomucin Family Of Adhesion Proteins.

A schematic representation of the five molecules currently identified as ligands for the selectin family. Four of the ligands are mucin-like molecules (sialomucins) and, although exhibiting limited homology at the cDNA level, these molecules all share the common characteristic of being highly glycosylated glycoproteins containing predominantly O-linked carbohydrate side chains linked to serine and threonine residues. The protein backbone is postulated to present clustered arrays of carbohydrates to the lectin domains of the selectins.

Although able to bind L-selectin with high affinity, murine GlyCAM-1 represents a soluble molecule present in both blood and the whey fraction of milk during lactation. Similarly, the vascular isoform of murine CD34 has been shown to bind to lymphocyte L-selectin when this mucin is expressed on the high endothelial venules (HEVs) of peripheral lymph nodes. CD34 is also expressed at all vascular sites in adults and embryos and on all haemopoietic progenitor cells in the BM, although the function of this molecule at these non-lymphoid cells remains to be determined. The recently cloned mucosal addressin, MAdCAM-1, represents a hybrid adhesion protein that contains immunoglobulin-like domains and a mucin-like domain. In addition to functioning as the principle ligand for integrin $\alpha_4\beta_7$, MAdCAM-1 is also able to support the adhesion of leukocytes expressing L-selectin.

PSGL-1 is a mucin-like molecule found on the surface of myeloid cells and activated lymphocytes that present carbohydrates to both P- and E-selectin. Murine ESL-1, unlike the mucin-like molecules described, represents a 150 kD variant of the chicken cysteine-rich fibroblast growth factor receptor which mediates E-selectin-specific cell adhesion of murine polymorphonuclear neutrophils. E-selectin binding to this glycoprotein (which contains 5 sites of N-linked glycosylation) is sensitive to neuraminidase pretreatment and is fucosylation and calcium-dependent. (Adapted from Lasky, 1995; Nelson *et al*, 1995; Tedder *et al*, 1995).


Immunoglobulin domain
O-linked glycosylation
N-linked glycosylation
Transmembrane domain
Murine ESL-1
PSGL-1
Murine CD34
Sgp 90
GlyCAM-1
Sgp 50
MAdCAM-1
E-selectin ligand
P- / E-selectin ligand
L-selectin Ligands
Mucin-like selectin ligands

Additional studies have identified the higher molecular weight sulphated glycoprotein (sgp90) to be identical to murine sialomucin, CD34. The structure of CD34 is similar to GlyCAM-1 in that it contains a large NH_2- terminal serine and threonine -rich O-linked domain that is highly glycosylated (Brown *et al*, 1991a). Unlike GlyCAM-1 however, CD34 contained a definable transmembrane domain and therefore is more likely to function as an endothelial membrane-immobilised L-selectin ligand for leukocytes under conditions of flow (refer to Figure 1.13.). In contrast to GlyCAM-1, whose expression is limited to the HEV of peripheral lymph nodes and to lactating mammary epithelial cells, CD34 is ubiquitously expressed at a variety of endothelial sites (Baumhueter *et al*, 1994). As described in section 1.2.2., CD34 is expressed by vascular endothelium and tissues of haemopoietic origin including haemopoietic progenitor cells and stromal cells, and its glycosylation appears to be tissue-specific (Baumhueter *et al*, 1993). In contrast to CD34 expressed by HEV, haemopoietic cell-associated CD34 does not possess the unique sulphated oligosaccharide recognised by L-selectin (Baumhueter *et al*, 1994) and to date, no studies have documented an equivalent function of human CD34.

Berg and co-workers (1993), have recently demonstrated that a newly cloned ligand for the integrin $\alpha_4\beta_7$, termed mucosal associated addressin (MAdCAM-1, please refer to Figure 1.13.) was able to support the adhesion of leukocytes expressing L-selectin. Although this member of the immunoglobulin (Ig) superfamily contained immunoglobulin-like domains (which mediate integrin recognition), it also harboured a small serine- and threonine-rich region equivalent to those observed in mucins. Moreover, mesenteric lymph node-derived MAdCAM-1 was recently demonstrated to contain sulphated carbohydrate side chains that were recognisable by L-selectin and the anti-peripheral lymph node addressin mAb, MECA 79. MAdCAM-1 therefore, may represent a low-affinity, physiological ligand for L-selectin under reduced flow conditions (Lasky, 1995).

Treatment of the human myeloid cell line HL60 with various proteases resulted in the abrogation of P-selectin-dependent adhesion, suggesting that a glycoprotein scaffold presented SLe^x-related carbohydrate ligands to P-selectin in a unique conformation (Larsen *et al*, 1992). Furthermore, pretreatment of human neutrophils or myeloid cell lines

with a unique protease (O-sialoglycoprotease) that selectively removes highly O-linked glycan (mucin) molecules, resulted in the inhibition of P-selectin binding and was consistent with the ligand belonging to the mucin family (Steininger *et al*, 1992; Norgard *et al*, 1993). Although treatment of myeloid cell lines, such as HL60 cells, with this protease completely abrogated P-selectin binding to these cells, it did not dramatically alter the expression of SLe^x at the cell surface. This was consistent with the notion that most of the SLe^x on the cell surface was not associated with a glycoprotein ligand and is therefore unable to mediate high-affinity binding to P-selectin (Picker *et al*, 1991b). This result therefore implied that the presentation of the carbohydrate in the appropriate conformation by the glycoprotein scaffold is responsible for mediating high-affinity binding of P-selectin (Lasky, 1995), whilst SLe^x not associated with an integral membrane protein is only able to mediate low affinity binding.

Whilst a number of putative ligands for P-selectin have been suggested (Picker *et al*, 1991b; Lenter *et al*, 1994, Norgard *et al*, 1993; Moore *et al*, 1991; Moore *et al*, 1992), insight into the mechanism of high-affinity adhesion was gained following the molecular cloning of a P-selectin ligand, designated P-selectin Glycoprotein Ligand (PSGL-1; refer to Figure 1.13.) (Sako *et al*, 1993). This mucin-like molecule was isolated following the co-transfection of both a specific fucosyltransferase (α(1,3/1,4) fucosyltransferase) and a cDNA library prepared from the human promyelocytic leukaemic cell line HL60. A resultant 220 kD glycoprotein expressed in COS cells was able to confer specific adhesion to both P-selectin (and E-selectin) that was abrogated by EDTA or by functional-blocking selectin-specific mAbs. Analysis of PSGL-1 by SDS-PAGE revealed that following reduction, a single band of 110 kD was obtained, suggesting that PSGL-1 exists as a disulphide-linked dimer.

Sako *et al* (1995) and Pouyani *et al* (1995) concomitantly reported that in addition to the SLe^x presented on O-linked glycans, the determinant that is critical for high affinity binding to P-selectin contains a non-carbohydrate component. Contained within the amino-terminal 19 amino acid residues, the anionic polypeptide segment harboured at least one tyrosine sulfation consensus sequence (Sako *et al*, 1995). Moreover, synthesis of

PSGL-1 in the presence of sulfation inhibitors (sodium chlorate), rendered binding to P-selectin of low affinity. This was confirmed by mutational analysis, where the tyrosine residues were substituted with phenylalanine residues resulting in the complete abrogation of binding to P-selectin (Pouyani and Seed, 1995).

In addition to PSGL-1, an alternate P-selectin-specific leukocyte ligand has been isolated from mouse neutrophils and HL60 cells (Lenter *et al*, 1994). To date, it appears to be a disulphide-linked dimeric molecule (160 kD non-reduced and 80 kD reduced) which exhibits EDTA- and sialidase-sensitive adhesion to P-selectin. Moreover, two additional glycoprotein ligands of 230 and 130 kD have been described, that exhibit promiscuous adhesion to both E- and P-selectin and display sensitivity to O-sialoglycosidase but not N-glycosidase (Lenter *et al*, 1994).

Notwithstanding PSGL-1, glycoprotein ligands for E-selectin remain essentially uncharacterised at the molecular level. Early work by Larsen *et al* (1992), demonstrated that the binding of P-selectin was sensitive to a broad array of proteases, while the binding of E-selectin was protease resistant. Despite this, however, several leucocyte cell surface molecules, modified by SLe^x/Le^x (or related structures) decorations, have been reported to bind to E-selectin, including a member of the selectin family, namely L-selectin (Picker *et al*, 1991; Kishimoto *et al*, 1991). Additionally, the β_2 integrins (Kotovuori *et al*, 1993) and the highly glycosylated phosphoprotein CD66 (belonging to the CEA family of adhesion molecules), known to mediate homotypic adhesion (Watt *et al*, 1991), have been reported to bind to E-selectin (Kuijpers *et al*, 1992). Other studies have shown that the human myeloid ligand for E-selectin is resistant to proteases (Larsen *et al*, 1992) and to O-sialoglycoprotease (Steininger *et al*, 1992), suggesting that the ligand for E-selectin is not a mucin-like glycoprotein.

In contrast, using an E-selectin-IgG chimeric fusion protein as a probe, Levinovitz *et al* (1993) and Lenter and collegues (1994), independently identified a protein ligand with the molecular weight of 150 and 130 kD under reducing and non-reducing conditions respectively, on murine neutrophils and the promyelocytic leukaemic HL60 cell line. E-selectin binding to this glycoprotein was calcium-dependent and sensitive to

neuraminidase pretreatment of the cell lysate, characteristics of E-selectin-dependent leukocyte adhesion (Levinovitz *et al*, 1993). In addition, adhesion to E-selectin also was reduced by pretreatment of leukocytes with N-glycosidase, but not O-sialoglycosidase.

The subsequent molecular cloning of this 150 kD, N-linked glycoprotein, revealed that the murine E-selectin ligand (ESL-1; Figure 1.13.), was a variant of the chicken cysteine-rich fibroblast growth factor receptor (Steegmaier *et al*, 1995). Although dependent upon the co-transfection with the α1->3/4 fucosyltransferase, CHO cells expressing ESL-1 were able to mediate E-selectin-specific cell adhesion. A human homologue of this molecule remains to be defined.

1.9.7. Adhesion Of Haemopoietic Progenitor Cells Mediated By Lectin-Carbohydrate Recognition: A Role For The Selectin Family In Haemopoiesis

Although the selectin family of molecules are clearly of importance in the function of mature leukocytes at the sites of inflammation, their role in human haemopoiesis has yet to be examined in depth. Reports in the literature which have investigated the potential involvement of the selectin family in the interaction of human HPC with the BM microenvironment are few in number, however what is known is discussed below.

In the murine system, Tavassoli and colleagues (Tavassoli and Hardy, 1990; Aizawa and Tavassoli, 1987) have suggested that protein-carbohydrate interactions represent the molecular basis for the homing of stem cells to haemopoietic tissues. Furthermore, Miyake *et al* (1990a) have demonstrated that exposure of murine long term BM cultures (LTBMC) to the anti-murine L-selectin mAb MEL-14 resulted in agglutination of non-adherent cells and slowed rates of mature myeloid cell formation.

L-selectin is expressed on mature myeloid cells (neutrophils, eosinophils and monocytes) and most lymphocytes, mediating the adhesion of leukocytes to endothelium at sites of inflammation and homing of lymphocytes to peripheral lymph nodes (Bevilacqua and Nelson, 1993; Gallatin *et al*, 1983; Tedder *et al*, 1990). Studies by Kansas *et al* (1990), have demonstrated expression of L-selectin on haemopoietic cells which exhibit a primitive morphology. Consistent with this, Griffin and colleagues (1990) have

reported GM-CSF-regulated expression of L-selectin by neutrophils, monocytes and most significantly, their precursors.

Previous studies have documented expression of both P- and E-selectin by a proportion of BM endothelial cells (Beckstead *et al*, 1986; Schweitzer *et al*, 1995; Dercksen *et al*, 1994), although, to date no studies have engendered P- and E-selectin with roles in haemopoiesis. The existence of a soluble form of P-selectin (Gamble *et al*, 1990) (with a possible anti-adhesive function) may however provide a novel mechanism for release of cells, including immature progenitors, from the marrow under both steady-state physiological conditions and during enforced mobilisation as occurs following administration of cytokines or high dose chemotherapy (Sheridan *et al*, 1992; To *et al*, 1990).

1.10. THE INTEGRIN GENE SUPERFAMILY.

1.10.1. An Overview.

Integrins are a large family of CAMs with well documented roles in a variety of cellular functions including cell migration and tissue organisation during embryonic development, cell differentiation, inflammation and metastasis (see reviews by Hynes, 1987; 1992; Hemler, 1990; Ruoslahti, 1991; Arnaout, 1990). Their distribution is universal, and at least one member of the integrin family has been found on every tissue or cell studied (Albelda and Buck, 1990). Integrins mediate both cell-cell and cell-ECM interactions and are so-named because of their ability to integrate the intracellular cytoskeleton with the ECM. Structurally, integrins are heterodimeric transmembrane cell surface proteins consisting of noncovalently associated α and β subunits (Hynes, 1992). Moreover, they represent a phylogenetically conserved family of integral membrane glycoproteins, and studies have revealed that progenitor integrins were present before the divergence of invertebrates and vertebrates (Larson and Springer, 1990). Related $\alpha\beta$ heterodimers have been reported in Drosophila (Wilcox and Leptin, 1985), Xenopus (DeSimone and Hynes, 1988) and chicken (Tamkun *et al*, 1986) and immunologically cross

reacting proteins have been described in nematodes and fungi (Marcantonio and Hynes, 1988).

As transmembrane receptors, the function of integrins was originally thought to be to integrate signals from the external environment with the intracellular skeleton, or "outside-in" signalling (Hynes, 1987; Williams *et al*, 1994). It has been subsequently demonstrated, using T-cell triggering through the CD3 complex, that the affinity of integrins for ligand could be increased, and thus these molecules are also able to participate in "inside-out" signalling (Dustin and Springer, 1989; Ginsberg *et al*, 1992). The mechanisms of regulation of integrin activity are unclear, but investigations have discovered the involvement of RNA splicing, post-translational modifications, phosphorylation and control from the membrane lipid environment (Kirchhofer *et al*, 1991). External influences from growth factors and agonists (ADP, thrombin and phorbol esters) have the ability to up- (or down-) regulate integrin expression and activate their binding capacity (Kirchhofer *et al*, 1991; Levesque *et al*, 1995). Divalent cations are required by all members of the integrin family for ligand binding (Anderson and Springer, 1987) and in their absence, the integrins are not active.

To date, as many as 21 distinct heterodimers formed by non-covalent association between 15 α subunits and 8 different β subunits, each with distinct ligand-binding properties have been characterised (Hynes, 1992; Hemler, 1990; Smyth *et al*, 1993; Dianzani and Malavasi, 1995). Compounding the diversity of adhesive interactions mediated by integrins, most, if not all integrins, can assume multiple functional states (Hynes, 1992). The integrins are subdivided on the basis of β-chain composition (refer to Figure 1.14.), although it is important to note that some α-chains (particularly the α_V chain of the classic vitronectin receptor ($\alpha_V\beta_3$)) can associate with β_1, β_5, β_6, and β_8 subunits with a concomitant change in ligand specificity (Hynes, 1992).

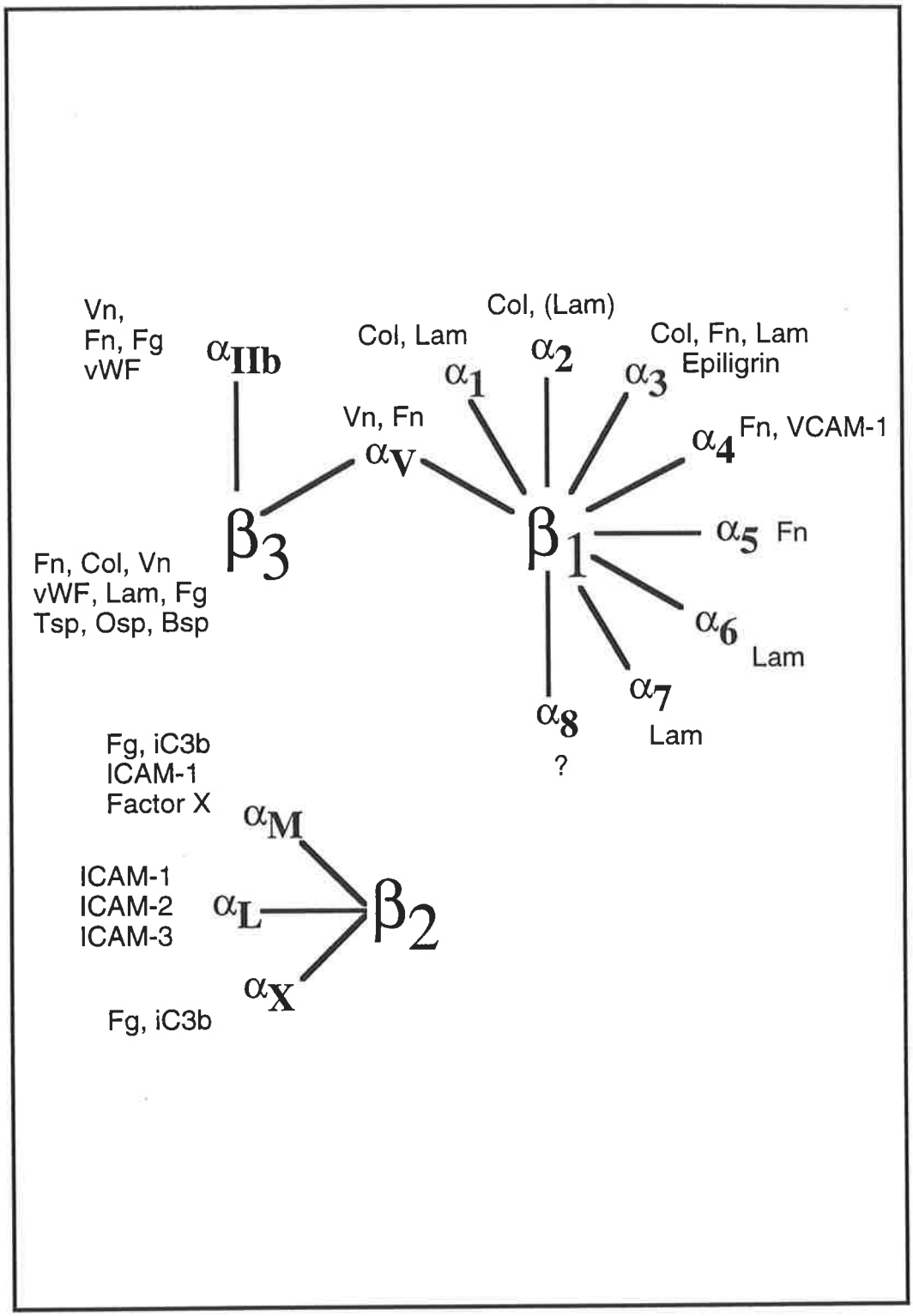
1.10.2. The Integrin Family: Molecular Cloning And Structural Characterisation.

The α subunits and β subunits of the integrins are believed to be synthesised as distinct precursors that are co-translationally glycosylated (Miller and Springer, 1987;

Figure 1.14. Integrin Pairing And Ligand Specificity.

Integrin heterodimers are formed by non-covalent association of unique α chains with one, or more β chains. Composition of subunit pairs determines ligand specificity. The β4, β5, β7 and β8 integrins have not yet been shown to be expressed by haemopoietic cells and have been omitted for simplicity.

Ligands: Col, collagens; Lam, laminin; Fn, fibronectin; VCAM-l, vascular cell adhesion molecule-1; Fg, fibrinogen; vWF, von Willebrand Factor; Tsp, thrombospondin; Osp, osteopontin; Bsp, bone sialoprotein; ICAM-l, intercellular adhesion molecule-1; ICAM-2, intercellular adhesion molecule-2; ICAM-3, intercellular adhesion molecule-3; iC3b, complement factor 3b.


Vn,
Fn, Fg
vWF
αIIb
β3
Fn, Col, Vn
vWF, Lam, Fg
Tsp, Osp, Bsp
Vn, Fn
αV
Col, Lam
α1
Col, (Lam)
α2
α3
Col, Fn, Lam
Epiligrin
α4
Fn, VCAM-1
β1
α5
Fn
α6
Lam
α7
Lam
α8
?
Fg, iC3b
ICAM-1
Factor X
αM
ICAM-1
ICAM-2
ICAM-3
αL
β2
αX
Fg, iC3b

Sastre *et al*, 1986). Association of the α and β subunit precursors occurs in the Golgi apparatus (Ho and Springer, 1983) and the assembled receptors are then transported to the cell surface or to intracellular stores (Todd *et al*, 1984; Miller, 1987). Integrins generally contain a large extracellular domain formed by the α (~1000 residues, 120-180 kD) and β (~750 residues, 90-110 kD) subunits, a transmembrane segment from each subunit, and two short cytoplasmic C-terminal domains, with the exception of β_4, whose cytoplasmic domain exceeds 1000 residues (Hogervorst *et al*, 1990; Suzuki and Naitoh, 1990, Tamura *et al*, 1990). An archetypal integrin is schematically represented in Figure 1.15.

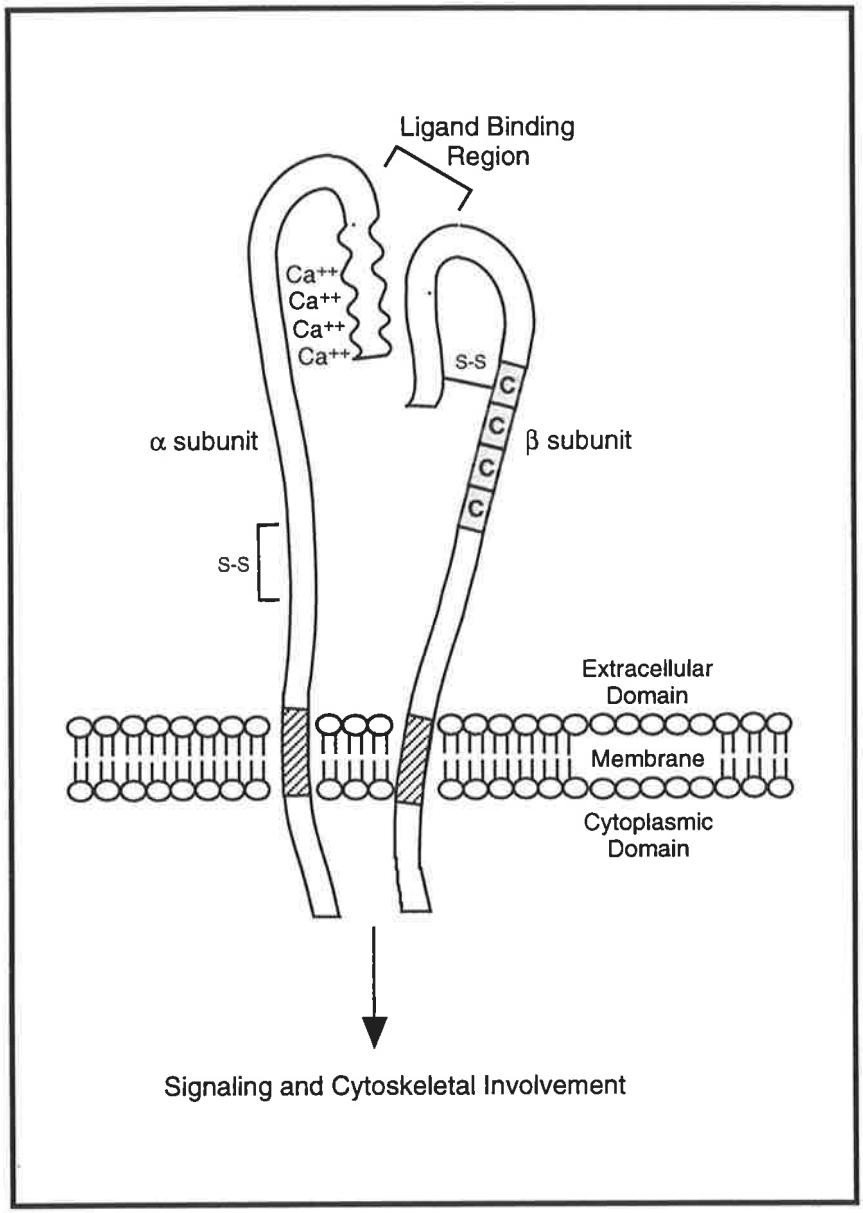
Integrin α chains have several common structural characteristics (Kishimoto and Anderson, 1992; Hynes, 1992; Albelda and Buck, 1990), including up to 7 tandem repeats of approximately 60 amino acids that share homology with EF-hand structures of the calcium-binding proteins of calmodulin and troponin. It is widely held that 3 to 4 of these regions contribute to a divalent cation-binding domain (Albelda and Buck, 1990; Springer, 1990a; 1990b). Divalent cation specificity nevertheless differs among the various α chains with $\alpha_L\beta_2$ CD11a/CD18) and $\alpha_2\beta_1$ (very late activation antigen-2 (VLA-2)) requiring magnesium, whilst $\alpha_5\beta_1$ (VLA-5) requires calcium to function (Hogg, 1991).

A second structural motif of approximately 180 amino acids which shares homology with domains found in von Willebrand factor, cartilage matrix protein and complement factor B is inserted between the divalent cation tandem repeats in several integrins including $\alpha_1\beta_1$ (VLA-1), $\alpha_2\beta_1$ (VLA-2), $\alpha_L\beta_2$ (CD11a or LFA-1), $\alpha_M\beta_2$ (CD11b or Mac-1) and $\alpha_X\beta_2$ (p150,95), and is responsible for conferring ligand specificities for these integrins. For example, the VLA-1 binding site for collagen and laminin (Kern *et al*, 1994), the VLA-2 binding site for collagen (Kamata *et al*, 1994), the Mac-1 binding site for iC3b (Ueda *et al*, 1994), and the LFA-1 binding site for ICAM-1 (Randi and Hogg, 1994) are all located in the I-domain. The α subunits which do not contain the I-domain are generally post-translationally cleaved to render a short transmembrane domain that is disulphide bonded to a large extracellular motif (Dianzani and Malavasi, 1995).

Integrin β chains also exhibit characteristic features (Albelda and Buck, 1990), including the presence of tandem repeats of four cysteine-rich regions conserved among

Figure 1.15. Diagrammatic Representation Of The Structure Of A Typical Integrin.

Integrins are composed of two non-covalently associated subunits designated α and β. Both subunits are integral membrane glycoproteins. The extracellular domains contain the ligand binding region. The α subunit contains areas thought to bind calcium (Ca^{2+}). The β subunit contains four cysteine-rich repeats (C). The large loop in the amino terminus of the extracellular domain of the β subunit is stabilised by intrachain disulphide bonding (S-S). The cytoplasmic domains of both subunits are relatively small and contain regions capable of binding cytoskeletal elements that link the integrins to the cytoskeleton.


Ligand Binding
Region
Ca++
Ca++
Ca++
Ca++
S-S
α subunit
β subunit
C
C
C
C
S-S
Extracellular
Domain
Membrane
Cytoplasmic
Domain
Signaling and Cytoskeletal Involvement

the various β chains and proposed to be essential for tertiary structure (refer to Figure 1.15.). Approximately 100 amino acids from the amino-terminus are additional conserved units that are critical for maintenance of the α/β heterodimer. The introduction of point mutations in either of these regions leads to the absence of expression of the β_2 leukocyte integrins (Arnaout, 1990). The cytoplasmic domain of the β subunit, in concert with the α-subunit, is also necessary for avidity modulation.

An invariant feature of integrin function is the demonstrable interaction of the cytoplasmic tail of both α and β chains with elements of the cytoskeleton. More specifically, the β chain interacts with talin and α-actin, whilst the α chain cytoplasmic domain is thought to be critical in signalling and interactions with unknown elements of the cytoskeleton. For example, truncation of the cytoplasmic domain of both β_1 and β_2 integrins results in the loss of adhesive function (Hayashi *et al*, 1990; Hibbs *et al*, 1991). In contrast, removal of the cytoplasmic domain of β_7 results in the increase in the adhesive function of the $\alpha_4\beta_7$ integrin (Crowe *et al*, 1994), suggesting that receptor avidity is in part conferred by the positive and negative regulatory motifs present within the cytoplasmic domain of the β chain.

1.10.3. Adhesion Of Haemopoietic Progenitor Cells Mediated By Integrin Heterodimers: A Role For The Integrin Family In Haemopoiesis.

To date, the majority of published studies have primarily examined the expression of the β_1, β_2 and β_3 integrins in human BM (Simmons *et al*, 1992; Teixido *et al*, 1992; Soligo *et al*, 1990; Rosemblatt *et al*, 1991; Saeland *et al*, 1992; Liesveld *et al*, 1993; Kerst *et al*, 1993), and therefore the following review will be restricted to these three families.

The β_1 integrins share CD29 as their common β subunit (Hemler, 1990), and this widely distributed family of integrins contains a series of cellular receptors for extracellular matrix proteins including fibronectin, collagen, laminin and vitronectin. The β_1 integrin subfamily consists of the very late acting (VLA) antigens that appear on lymphocytes several days after mitogen stimulation (Hemler *et al*, 1987a) and are reported to have roles in haemopoiesis (Williams *et al*, 1991; Miyake *et al*, 1991a, 1991b; Yanai *et al*,

1994). Investigations into integrin expression in the BM have shown that VLA-2 and VLA-6 are expressed on megakaryocytes and platelets, while human LTBMC show 35-40% expression of both VLA-1 and VLA-3 on adherent cells, including fibroblasts and endothelial cells (Soligo *et al*, 1990). VLA-4 is widely distributed on most haemopoietic cells and binds to both the CS-1 region of fibronectin (Gismondi *et al*, 1991; Rosemblatt *et al*, 1991) and also at a site distinct from that which mediates CS-1 binding to VCAM-1 (Elices *et al*, 1990). It has been shown that murine BM-derived stroma supports haemopoiesis *in vitro*, and that the CFU-S which adhere to the stroma do so via VLA-4/CS-1 interactions (Williams *et al*, 1991) and VLA-4/VCAM-1 interactions (Miyake *et al*, 1991b). Antibodies to VLA-4 inhibit lymphopoiesis and retard myelopoiesis in LTBMC (Miyake *et al*, 1991a, 1991b). Typical localisation for VLA-4 is at sites of intercellular contact, which provides further evidence for its involvement in interactions of haemopoietic stem cells during maturation (Soligo *et al*, 1990). There is also good evidence that $\alpha_4\beta_1$ (CD49d/CD29) is expressed by human LTC-IC (Simmons *et al*, 1992) and this will be dealt with in greater detail below (Section 1.10.4.)

LFA-1, Mac-1 and p150/95 all belong to the β_2 or LEU-CAM subfamily, as they share the β_2 subunit (Larson *et al*, 1989; Albelda and Buck, 1990). These three β_2 integrins are also known as the leukocyte integrins because their expression is restricted to white blood cells. Leukocyte function associated antigen-1 (LFA-1) and Mac-1 both utilise the same ligand, ICAM-1 (Dustin and Springer, 1989; Diamond *et al*, 1991) in addition to other ligands. LFA-1 also binds to ICAM-2 (Seth *et al*, 1991; Nortamo *et al*, 1991; de Fougerolles *et al*, 1991), and Mac-1 has many other ligands including iC3b, coagulation factor X, fibrinogen and endotoxin (Albelda and Buck, 1990). Little is known about the third member of this group, p150/95, other than it too binds iC3b and has some role in the adhesion of monocytes and granulocytes to the endothelium (Stacker and Springer, 1991). The importance of leukocyte integrins is manifested in the congenital leukocyte adhesion deficiency (LAD) type I disorder, in which patients present with recurring infections (often fatal in childhood unless corrected by BM transplantation) as a result of mutations in the common β_2 subunit (Kishimoto *et al*, 1987). Neutrophils from these

patients fail to orient and migrate in response to chemoattractants and are unable to bind to and cross the endothelium at sites of infection (Kishimoto *et al*, 1987).

Of the β_2 (CD18) integrin family, only $\alpha_L\beta_2$ (CD11a/CD18) is detectable on $CD34^+$ cells (approximately 80%) (Tiexido *et al*, 1992; Saeland *et al*, 1992; Liesveld *et al*, 1991). LTCIC however, are restricted to the $CD11a/CD18^-$ subpopulation (Gunji *et al*, 1992). The β_3 subfamily, the cytoadhesins, consists of the platelet glycoprotein IIb/IIIa complex (α_{IIb}/β_3) and the vitronectin receptor (α_v/β_3). Binding of both of these receptors is inhibited by RGD-containing peptides (reviewed in Albelda and Buck, 1990). The platelet glycoprotein IIb/IIIa binds fibrinogen via an RGD recognition sequence. It exists in a non-functional state and requires agonist-activation of platelets for the fibrinogen to bind. Agonists such as RGD peptide bind and cause conformational changes in α_{IIb}/β_3. These changes are associated with activation (the acquisition of fibrinogen binding function) and platelet aggregation (Du *et al*, 1991). On the surface of unstimulated platelets, α_{IIb}/β_3 is randomly dispersed and recognises only immobilised fibrinogen. When the platelets are activated however, α_{IIb}/β_3 becomes a receptor for several soluble adhesion proteins including soluble fibrinogen, von Willebrand factor, fibronectin and vitronectin (Phillips *et al*, 1991).

Other β subunits include the β_P, β_4 and β_5. In mouse the β_P subunit associates with a chain homologous to the human α_4 subunit, to produce the murine homing protein LPAM-1. Like VLA-4, LPAM-1 is expressed on most unstimulated lymphocytes, and the intact heterodimer can be immunoprecipitated with anti-VLA-4 antibodies (Hemler *et al*, 1987b). The presence of Ca^{2+} ions is required for LPAM-1 both to maintain structural integrity and to function in the migration of lymphocytes to lymph nodes and Peyers patches. The alpha subunit α_M is able to associate with both β_P to give the distinct integrin LPAM-1, and β_1 to form the murine equivalent of VLA-4, LPAM-2 (Holzmann and Weissman,1989).

With regard to the primitive HPC compartment, β_3 (CD61) integrin is present on a minor proportion of $CD34^+$ cells (approximately 10%) which are thought to include megakaryocyte precursors (Liesveld *et al*, 1993; Bender *et al*, 1992). The expression of β_3

by more primitive HPC with the capacity to initiate and sustain haemopoiesis in long-term marrow culture (so-called long-term culture initiating cells, LTCIC) (Sutherland *et al*, 1989a; 1989b) however, has not been determined.

1.10.4. Integrin $\alpha_4\beta_1$: Distinct Roles In Cell-Cell And Cell-ECM Adhesion.

Integrin $\alpha_4\beta_1$ is expressed by a wide variety of circulating lymphoid and myeloid cells (Albelda and Buck, 1990; Hemler, 1990) and functions as a receptor for two distinct ligands. The first, vascular cell adhesion molecule-1 (VCAM-1), a member of the immunoglobulin gene superfamily (please refer to Section 1.11.), is an integral membrane glycoprotein which was first identified as an inducible CAM on human endothelial cells by Bevilacqua and colleagues (Rice *et al*, 1990). The second ligand for $\alpha_4\beta_1$ is fibronectin (FN) (Wayner *et al*, 1989), a major component of the ECM synthesised by BM stromal cells *in vivo* and *in vitro* (refer to Section 1.13.). Three distinct binding sites for $\alpha_4\beta_1$ have been identified on the FN molecule (Wayner *et al*, 1989; Mould and Humphries, 1991). The sites on $\alpha_4\beta_1$ involved in interaction with FN are distinct from those which bind to VCAM-I (Elices *et al*, 1990). $\alpha_4\beta_1$ therefore mediates both cell-cell and cell-ECM adhesion. The pioneering work of Miyake, Kincade and colleagues first demonstrated the importance of $\alpha_4\beta_1$ in regulating haemopoiesis *in vitro*. Addition of an anti-α4 subunit monoclonal antibody (mAb) to murine LTBMC was found to completely abrogate lymphopoiesis and to retard myelopoiesis (Miyake *et al*, 1991a; 1991b). It is not clear from these data however, whether inhibition of cellular adhesion is responsible for the observed suppression or whether some antibody-mediated growth modulatory effect in LTBMC may also be involved. Recent data suggest an equally important role for $\alpha_4\beta_1$ integrin in human haemopoietic cell interactions. Between 46-90% of $CD34^+$ BM cells are reported to co-express $\alpha_4\beta_1$ (Simmons *et al*, 1992; Teixido *et al*, 1992; Saeland *et al*, 1992; Liesveld *et al*, 1993; Kerst *et al*, 1993) which include the majority of lineage restricted clonogenic progenitors, CFU-GM and BFU-E (Simmons *et al*, 1992; Kerst *et al*, 1993) and B-cell precursors (Ryan *et al*, 1991). In addition, Simmons *et al* (1992) have shown that LTC-IC express α4β1 and that anti-CD49d antibody partially inhibits their adhesion to

marrow stromal cells *in vitro* (Simmons *et al*, 1992). VCAM-1 is constitutively expressed by human BM stromal cells *in vitro* (Simmons *et al*, 1992; Ryan *et al*, 1991) and *in vivo* (Gronthos and Simmons; unpublished observations) and its expression can be up-regulated by inflammatory mediators such as IL-l, IL-4 and TNFα resulting in concordant increases in $CD34^+$ cell adhesion (Simmons *et al*, 1992). Antibody to VCAM-1 partially inhibits the adhesion of LTC-IC, myeloid and erythroid progenitors, and normal and leukaemic B-lymphoid precursors to cytokine-induced marrow stromal cells (Simmons *et al*, 1992; Ryan *et al*, 1991). Thus, in addition to roles in physiological processes involving migration and localisation of immune cells (Cybulsky and Gimbrone, 1991; Rice *et al*, 1991), the $\alpha_4\beta_1$/VCAM-1 counter receptors also mediate adhesion of primitive HPC to BM stromal cells.

While there is accordance between different groups concerning the role of VCAM-1 in this setting, conflicting observations have been made regarding the role of FN, the alternative ligand for $\alpha_4\beta_1$. A number of groups have reported insignificant binding of HPC to FN (Kerst *et al*, 1993; Liesveld *et al*, 1991), while others have demonstrated adhesion of murine (Williams *et al*, 1991) and human HPC (including LTC-IC) to intact FN or proteolytic fragments thereof (Tiexido *et al*, 1992; Verfaillie *et al*, 1991). Recent work by Hemler and colleagues (Masumoto and Hemler, 1993) may, at least in part, resolve these differing observations. The authors identify 3 activation states of $\alpha_4\beta_1$: the fully active receptor binds both ligands, the inactive form, neither. A third, partially active form of $\alpha_4\beta_1$ binds only to VCAM-1. Thus, in order to bind FN, further activation of $\alpha_4\beta_1$ is required. In accord with this, Kerst *et al.* (1993) and Levesque *et al* (1995), have demonstrated the acquisition of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ dependent adhesion of $CD34^+$ cells to FN following protein kinase C activation, or cytokine (IL-3, GM-CSF or SCF) stimulation, respectively. Therefore, given the high proportion of $CD34^+$ cells which bind to VCAM-1 (Simmons *et al*, 1992), collectively these data suggest that HPC constitutively express the partially active form of $\alpha_4\beta_1$.

Finally, antibodies to $\alpha_4\beta_1$ or VCAM-1 alone or in combination do not completely block the adhesion of HPC to marrow stroma (Simmons *et al*, 1992; Teixido *et al*, 1992;

Liesveld *et al*, 1993) suggesting that more than one CAM interaction is involved in this process and remain the subject of further investigation.

1.11. HAEMOPOIETIC PROGENITOR CELL-GROWTH FACTOR INTERACTIONS.

As detailed in Section 1.3., haemopoiesis is regulated by a wide variety of HGFs. In the context of adhesive interactions between HPC and the stromal HM, HGF are of central importance. Growth factors which act on HPC have been shown to modulate the adhesive interactions of cells by regulating the expression and/or activation state of CAMs (Simmons *et al*, 1992; Bevilacqua *et al*, 1989; Griffin *et al*, 1990; Lasky *et al*, 1992; Nathan and Sporn, 1991; Levesque *et al*, 1995) or by influencing ECM biosynthesis and degradation (Nathan and Sporn, 1991; Nugent, 1989). In addition, cell adhesion has been demonstrated to induce the production of HGF by a variety of cell types (Nathan and Sporn, 1991; Thorens *et al*, 1987). As will be discussed Section 1.13, many HGF are bound in biologically active form by ECM proteins (Roberts *et al*, 1987a; Rathjen *et al*, 1990; and refer to Section 1.3.) and in this form are able to mediate adhesion of HPC (Long *et al*, 1992). In addition, certain HGF can exist in both membrane bound and soluble forms (Flanagan *et al*, 1991; Rettenmier *et al*, 1987; Massague, 1990). The integral membrane isoforms of macrophage colony-stimulating factor (M-CSF) and stem cell factor (SCF) are expressed by marrow stromal cells and have been demonstrated to promote the adhesion of cells bearing the appropriate HGF receptors, c-fms and c-kit, respectively (Flanagan *et al*, 1991; Rettenmier *et al*, 1987). A single receptor-ligand pair is thus able to stimulate HPC proliferation and support HPC-stromal cell adhesion. A number of cell types which express c-kit, including mast cells and megakaryocytes, adhere to marrow fibroblasts via interaction with SCF (Flanagan *et al*, 1991; Avraham *et al*, 1992). c-kit is expressed by 75% of $CD34^+$ cells in the BM, including the majority of CFU-GM, BFU-E and LTCIC (Ashman *et al*, 1991a; Simmons *et al*, 1994a). HPC have been reported to utilise this adhesion mechanism (Sutherland *et al*, 1993) although the adhesion mediated by the c-kit-SCF receptor/ligand pair is of low affinity when compared with *bona fide* adhesion mechanisms.

At this time it is unclear (i) to what extent binding to immobilised HGF is utilised by HPC as an adhesive interaction, (ii) the range of HGF that mediate cell adhesion, and (iii) whether ECM-bound HGF are as effective at promoting adhesion as integral membrane isoforms. This adhesion mechanism clearly warrants further exploration.

1.12. OTHER CANDIDATE CAMs.

Because of the restricted nature of this review, I have of necessity focussed in detail on a limited number of CAM-ligand interactions with roles in supporting adhesion between HPC and the marrow stroma. However, other CAMs are clearly involved in this complex process and as such warrant brief consideration. These include the CD44 family of adhesion molecules (refer to Section 1.12.2.), several CAMs belonging to the Ig superfamily (Williams and Barclay, 1988), and an additional category of interactions which involve HPC adhesion to stromal cell-derived ECM components (refer to Section 1.13.2.).

Ig superfamily members which are expressed by human HPC include PECAM-1 (CD31; Albelda *et al*, 1991), LFA-3 (CD58) (Saeland *et al*, 1992), ICAM-I (CD54) (Saeland *et al*, 1992; Arkin *et al*, 1991), and Thy-1 (Baum *et al*, 1992). ICAM-1 and Thy-l are also present on marrow stromal cells (Teixido *et al*, 1992; Caligaris-Cappio *et al*, 1991), as is N-CAM (CD56) (Kincade *et al*, 1989) but, with the exception of ICAM-1 and PECAM-1 (refer to Section 1.12.1.), have not been shown to participate in adhesive interactions with HPC. CD43, a counter-receptor for ICAM-1 (Rosenstein *et al*, 1991), is expressed at high density by $CD34^+$ cells although its potential role in HPC-stromal cell adhesion remains to be determined.

1.12.1. PECAM-I (CD31): An Amplifier Of Integrin Mediated Adhesion.

Platelet-endothelial cell adhesion molecule-l (PECAM-1 or CD31) is a 130 kD member of the immunoglobulin gene superfamily, that is expressed at high density on endothelium, platelets, granulocytes, monocytes and by a subset of lymphocytes (Albelda *et al*, 1991; Newman *et al*, 1990; Stockinger *et al*, 1990; Torimoto *et al*, 1992; Watt *et al*,

1993; Simmons, DL. *et al* 1990). Although the precise function of CD31 is not known in all cell types that express it, a number of studies clearly document its ability to function as a CAM (Albelda *et al*, 1991; Muller *et al*, 1992; Schimmerti *et al*, 1992).

Studies by Simmons *et al* (1992) demonstrate that in addition to the abovementioned mature leukocyte populations, essentially all $CD34^+$ cells express CD31; BFU-E at low level, CFU-GM at high level and LTC-IC at an intermediate level. Anti-CD31 Fab fragments partially inhibit adhesion of $CD34^+$ cells to marrow stroma (Ashman *et al*, 1991b; Simmons *et al*, 1992). Although the ligand for CD31 is not yet defined, sulphated proteoglycans are a possible candidate (DeLisser *et al*, 1991). The latter are produced in abundant quantities by marrow stromal cells (DeLisser *et al*, 1991) and have been shown to participate in the adhesion of primitive HPC (Siczkowski *et al*, 1992). In addition to its role as a CAM, recent work by a number of investigators (Tanaka *et al*, 1992; Leavesley *et al*, 1994) has demonstrated a role for CD31 in amplifying β_1 and β_2 integrin-mediated adhesion of both T cells (Tanaka *et al*, 1992) and $CD34^+$ HPC (Leavesley *et al*, 1994). Therefore, it would appear that PECAM-1 may be able to control the adhesive interactions of HPC (and their committed progeny) to elements of the BM stroma by regulating the activation states of specific integrins.

1.12.2. Hyaluronate Receptor: CD44.

CD44 (Pgp-1, Ly-24, ECMR III, Hermes Antigen) represents a heterogeneous family of polymorphic integral membrane proteoglycans (PG) expressed on some thymocytes, mature T cells, monocytes, granulocytes, erythrocytes, fibroblasts, keratinocytes, epithelial cells, B cells (Haynes *et al*, 1989; Barclay *et al*, 1993) and on haemopoietic progenitor cells (Lewinsohn *et al*, 1990). It has been identified as being homologous to a receptor for the GAG, hyaluronate (Aruffo *et al*, 1990), a known mediator of cell adhesion, and thus has been attributed a role in cell-cell adhesion, cell migration, embryonic development and pathogenesis (Miyake *et al*, 1990a; 1990b; Culty *et al*, 1990). Moreover, CD44 also binds fibronectin, collagen (Jalkanen and Jalkanen, 1992; Aruffo *et al*, 1990), MAdCAM-1

(Briskin *et al*, 1993) and endothelial proteoglycans (Toyama-Sorimachi and Miyasaka, 1994).

It is apparent from molecular studies that the CD44 locus on the short arm of human chromosome number 11, encodes an mRNA which can be alternatively spliced (Screaton *et al*, 1992). The majority of this heterogeneity is localised to the membrane proximal region which contains at least 12 exons (Screaton *et al*, 1992; Jackson *et al*, 1992). In addition, the CD44 NH_2-terminal domain is homologous with the cartilage proteoglycan core and link proteins. Heterogeneity amongst the CD44 family of molecules, also results from various post-translational modifications including the addition of N- and O-linked glycosylation and GAG side chains (chondroitin sulphate) in the highest molecular weight form. This variability gives rise to a wide pattern of molecules whose molecular weights range from 85 to 250 kD. The isoform expressed is regulated in a tissue-specific and activation dependent manner (Hirano *et al*, 1994; Mackay *et al*, 1994).

Two forms of CD44, one isolated from haemopoietic (80-95 kDa) and the other from epithelial (115-130 kDa) cells have been shown to differ only in their extracellular domains (Stamenkovic *et al*, 1991; Dougherty *et al*, 1991). Biologically, the epithelial form of CD44 differs significantly from the haemopoietic form of CD44. This is evidenced by experiments where B cells transfected with this larger isoform of the protein were unable to bind to endothelial layers, whilst cells transfected with the smaller isoform acquired this capacity (Stamenkovic *et al*, 1991).

Anti-CD44 antibodies identify primitive human HPC (Saeland *et al*, 1992; Lewisohn *et al*, 1990) and marrow stromal cells. Accordingly, the addition of anti-CD44 antibodies to long-term cultures results in the inhibition of both lymphoid and myeloid progenitor development (Miyake *et al*, 1990a). Moreover, binding of B cells to a cloned stromal cell line can be inhibited by treatment of the stroma with hyaluronidase or by pre-incubation of the B cells with certain anti-CD44 antibodies (Miyake *et al*, 1990a), suggesting that HPC and their progeny utilise a CD44-dependent adhesion mechanism.

1.13. ADHESION OF HPC TO EXTRACELLULAR MATRIX (ECM) PROTEINS.

1.13.1. The Adhesion Of HPC To BM Stromal Cell-Derived Extracellular Matrix (ECM) Components *In Vitro*.

Extracellular matrix (ECM) proteins are synthesised in numerous tissues throughout the body (Wicha *et al*, 1982). The composition of the ECM at these sites varies considerably, implying that certain components of the ECM play significant roles in promoting cellular development (Gospodarowicz, 1980; Gospodarowicz *et al*, 1980). A number of ECM components, including fibronectin (FN), heparan sulphate proteoglycans (HSPG), thrombospondin (TSP), various collagens and haemonectin are synthesised by elements of the BM stroma (Table 1.6.). As detailed below, these various ECM components and numerous cytoadhesion molecules (refer to Table 1.7.) are believed to facilitate the binding of HSC and their progeny to the stroma and thus regulate their development (Coulombel, 1983; Ryan, 1992; Verfaillie, 1990; reviewed in Coombe, 1996).

1.13.2. Fibronectin (FN).

The FNs (produced by BM fibroblasts) are disulphide-linked dimeric glycoproteins found in the extracellular matrix of many tissues. They play a crucial role in lymphoid and erythroid precursor homing, and in T cell and phagocyte activation (Lévesque *et al*, 1991). The adhesion of cells to fibronectin has been studied extensively, and several regions of the molecule are known to support cell attachment (Mould *et al*, 1991; Weinstein *et al*, 1988). The binding of mammalian erythroid precursor cells to fibronectin via the Arg-Gly-Asp (RGD) site has been shown using inhibition with monoclonal antibodies directed towards an RGDS-containing peptide, (Patel and Lodish, 1986). Also, as reticulocytes mature into erythrocytes, they lose their capacity for adhesion to fibronectin, and are released into the circulation from the BM (Tsai, 1987; Patel *et al*, 1985). Lymphoid precursor cells adhere to fibronectin at two separate sites. The uncommitted B cell precursors interact with the RGD recognition sequence, but pre-B cells undergoing rearrangement of immunoglobulin genes attach to a different part of the molecule (Bernardi *et al*, 1987). These two receptors on B cells may be expressed during B cell maturation, and may play a role in lymphopoiesis.

Table 1.6. ECM Components Present In The Marrow

Molecule	Possible Source	Marrow Distribution	ECM Interactions	Comments
Collagen I	Fibroblasts	?	Fibronectin, HSPG	-
Collagen III	Fibroblasts	?	Fibronectin, HSPG	-
Collagen IV	Endothelial cells	Vascular basement membrane	Laminin, HSPG	40% LTBMC stroma positive (as determined by immunofluorescence)
Fibronectin	Endothelial cells	Megakaryocytes	Collagens, HSPG	-
Fibronectin	Fibroblasts	Vascular endothelium	Fibrinogen, HSPG	-
Fibronectin	Megakaryocytes	?	?	-
Laminin	Endothelial cells	Vascular basement membrane	?	-
Thrombospondin	Endothelial cells Smooth muscle cells Megakaryocytes	?	?	-
Haemonectin	BM stromal cells	Stromal cell membranes	?	58 kD molecule
Chondroiton Sulphate (CS)	Various	?	?	-
Heparan Sulphate Proteoglycan (HSPG)	Various	?	Fibronectin	Binds HGFs, including IL-3 & GM-CSF
Hyaluronic Acid	Endothelial cells	?	?	70% LTBMC HA in supernatant

(adapted from Clark *et al*, 1992).

Table 1.7. Haemopoietic Cell And Extracellular Interactions.

Matrix Component	Cell Surface Receptor	Cellular Expression
Fibronectin	Fibronectin Receptor Numerous β_1 integrins	Erythroid BFU-E B Cells Lymphoid Cell Lines HL-60 Cells
Fibrinogen	IIb/IIIa	Platelets, Megakaryocytes
Thrombospondin (TSP)	TSP Receptor $\alpha_v\beta_3$	monocytes, platelets, human CFU, CFU-GEMM
Hyaluronic Acid	CD44	T and B cells, Neutrophils, Tumour Cells
Haemonectin	Unknown (58 kD protein)	CFU-GM, BFU-E, Immature Neutrophils
Proteoglycans: Heparan Sulphate	PECAM-1 (CD31)?	BFU-E Bl-CFU
Unfractionated ECM	Unknown	Bl-CFU Bone Marrow Stroma

See *Abbreviations* Section for abbreviations. (adapted from Long, 1992).

The β1 integrin, VLA-4 ($\alpha_4\beta_1$; refer to Section 1.10.4.), has been shown to be involved in the interactions of stem cells with the microenvironment, a process in which VLA-4 binds to the CS-1 peptide of fibronectin (Williams *et al*, 1991). This integrin along with VLA-5 ($\alpha_5\beta_1$), another fibronectin receptor expressed on erythroid precursors (Gismondi *et al*, 1991; Rosemblatt *et al*, 1991), may be responsible for the binding of these cells to the ECM.

1.13.3. Heparan Sulphate Proteoglycans (HSPGs).

Heparan sulphate proteoglycans (HSPGs) are found in cell membranes and enmeshed in the ECM (Gallagher *et al*, 1983; Massagué, 1991; reviewed in Coombe, 1996). Studies of human BM stroma have shown that the HSPGs produced by the stromal cells may have a role in the adsorption and presentation of growth factors in the BM (Morris *et al*, 1991). It has already been shown that GM-CSF and IL-3 bind, in a biologically active form, to heparan sulphate in BM stroma (Gordon *et al*, 1987a; Roberts *et al*, 1988a; and refer to Section 1.7.2.).

One HSPG, isolated from human BM cell membranes and ECM, has been shown to be anchored by glycosyl-phosphatidylinositol linkage (Brunner *et al*, 1991). Soluble (proteolytically cleaved) basic-fibroblast growth factor (bFGF) has been found to associate with this 200 kDa protein to generate a biologically active complex. This suggests the HSPG may exist in 2 forms, one membrane-associated and one free in the ECM. The membrane bound form of the HSPG growth factor complex may be 'shed' from the stromal cell into the surrounding ECM by the action of a cellular phosphatidylinositol-phospholipase C (PI-PLC). Whether this 'shedding' could alter the activity/function of growth factors bound to HSPGs in a manner analogous to that proposed for membrane or soluble SCF (please refer to Section 1.7.3.) is open to further investigation.

1.13.4. Haemonectin.

Haemonectin, a protein of 60 kDa, was originally isolated from bone marrow ECM, and shown to be specific for cells of granulocytic lineage (Campbell *et al*, 1987). It

was discovered by the technique of 'cell blotting', where components of bone marrow ECM were separated using SDS-PAGE, transferred to nitrocellulose, and probed with radiolabelled bone marrow stem cells (Campbell *et al*, 1987). Those cells which bound to the blot were examined by staining and microscopy and shown to be of the granulocytic lineage. Although there is evidence which demonstrates that erythroid progenitors can bind to blotted haemonectin, this result has been variable (Campbell *et al*, 1990).

Haemonectin was shown to be immunologically distinct from the ECM adhesion proteins fibronectin, laminin, collagen type IV or vitronectin (Campbell *et al*, 1987). The organ distribution of this protein is very limited; it can only be detected in bone marrow extracts and not in spleen, mammary gland or kidney (Campbell *et al*, 1987). This implies a specific role in haemopoiesis. Cloned stromal cell lines from *Steel* mutant mice (Sl/Sl^d) show reduced levels of haemonectin when compared to normal littermates, and reduced capacity for stem cell adhesion (Anklesaria *et al*, 1991). The conclusion drawn from this finding, along with the fact that the abnormal haemopoiesis of Sl/Sl^d mice is only seen in the bone marrow and not the spleen (an organ which remains unaffected in this abnormality), is that SCF may contribute in part to the extreme restriction of haemonectin expression. It is suggested that the expression of haemonectin is regulated by specific growth factors, perhaps SCF (produced by the *Sl* gene; see Section 1.7.3.), however further studies are required to verify this postulate (Anklesaria *et al*, 1991).

1.13.5. Thrombospondin (TSP).

Thrombospondin (TSP) is a large trimeric disulphide-linked glycoprotein comprised of 180 kD monomers. A variety of cell types including endothelial cells, smooth muscle cells and megakaryocytes secrete TSP which is subsequently incorporated into the ECM (Lawler, 1986; Frazier, 1987). Multipotent haemopoietic progenitor cells and committed progenitors of the erythroid, granulocytic and megakaryocytic lineages can all bind specifically to TSP while mature granulocytes and erythroid cells loose their ability to attach to TSP as a function of differentiation (Long *et al*, 1992). Purified TSP can interact with SCF *in vitro* to induce a synergistic cytoadhesive response. This results in the

enhanced attachment of primitive haemopoietic progenitors to the protein complex as well as increasing the proliferation of these cells in the absence of a stromal layer (Long and Dixit, 1990).

1.13.6. Collagen.

Collagen types I, III and IV are the main constituents of the BM extracellular matrix (Zuckerman and Wicha, 1983). Early haemopoietic progenitor cells are known to express the adhesion molecule CD44 which recognises collagen (Carter and Wayner, 1988; Kansas, 1990; Lewinsohn, 1990; Brown *et al*, 1991a; 1991b; and refer to Section 1.12.2.). Both myeloid and erythroid progenitor cells have been shown to bind to collagen type I *in vitro* (Koenigsmann, 1992). The inhibition of collagen synthesis in LTBMCs by the addition of cis-4-hydroxyproline interferes with the development of the stromal layer and blocks haemopoiesis (Zuckerman *et al*, 1985). Furthermore, the addition of anti-CD44 antibodies to myeloid and lymphoid LTBMCs inhibits cell proliferation in both types of cultures (Miyake *et al*, 1990a).

Therefore, the binding of HSCs to stromal cells and extracellular matrix components provides a mechanism to control the early progenitor population. The same adhesion molecules and receptors to ECM are also involved with the release of mature blood cells into the circulation and with the 'homing' of HSC to the H.I.M.; lymphocyte tracking and recruitment during an immune response; and the metastasis of malignant tumour cells (Long, 1992; Long *et al*, 1992). The preferential binding of early haemopoietic progenitor cells to the stroma, together with the production of cytokines by stromal cells and their presentation to HSCs, provides strong evidence that the stromal elements and their products are critical in the support and regulation of haemopoiesis.

1.14. CONCLUSIONS AND RESEARCH AIMS.

1.14.1. The Regulation Of Haemopoiesis: A Multiplicity of Mechanisms.

HSC possess the capacity to reconstitute fully the haemopoietic system of compromised animals (Metcalf and Moore, 1971; Metcalf, 1993). As indicated, these stem

cells arise in foetal development and persist throughout the lifetime of the animal. The number of stem cells is maintained at a fairly constant level throughout the lifetime of the animal, indicating that mechanisms exist by which stem cell numbers are constantly maintained despite giving rise to large numbers of differentiated cells of various lineages.

A common feature of primitive HSC, lineage-restricted progenitor cells and even many mature leukocytes (such as T lymphocytes), is that the survival of these cells rapidly declines *ex vivo* in the absence of exogenous stimuli (Metcalf, 1977). However, the levels of progenitor populations can be maintained or increased *in vitro* when haemopoietic cells are cultured in association with BM-derived stromal cells, inferring that in this system, stem cell self-renewal occurs (Dexter *et al*, 1977; Dexter, 1982). Although it is well recognised that supernatants of LTBMC stromal layers contain multiple HGFs required for the proliferation, differentiation and survival (prevention of apoptosis), long term maintenance of HSC populations is not observed in the absence of a stromal layer, suggesting that physical associations with the adherent stromal layer (Dexter, 1982; Coulombel *et al*, 1983) are essential.

This observation can in part be resolved by the finding that a number of the HGFs are expressed as stromal cell membrane- or ECM-associated molecules, implying that haemopoietic cells would need to generate direct cell-cell contact in order to respond to the HGF(s). In addition a multitude of CAMs each with specificity for distinct counter-receptors (ligands) on stromal cells or ECM components of the BM, are also thought to play a critical role in HSC maintenance. Although the nature of the adhesion mechanisms utilised by mature cells is advancing rapidly, much remains to be elucidated regarding the role of CAMs in the retention/regulation of haemopoietic cells within the BM environment. It is likely however, that a similar cohort of adhesive interactions occur between HPC and the BM stroma and play a major role (in association with multiple HGFs) in conferring specialised microenvironmental niches required for the proliferation and differentiation of HSC and their progeny. In addition, it is clear that CAMs are signalling molecules. In the case of the integrins, for example, the signal transduction pathways share a number of features with those of the surface receptor tyrosine kinases for the polypeptide growth

factors EGF, PDGF and insulin-like growth factor (Kornberg *et al*, 1991; Schwartz, 1993; Juliano and Haskill, 1993; Menko and Boettiger, 1987). Thus, the diverse CAM-ligand interactions reviewed here, rather than simply serving to initiate and maintain contact between HPC and stromal cells/ECM components, might also have an additional, more direct role in controlling the growth and development of primitive haemopoietic cells as suggested by the 'niche' model of Schofield (1978).

1.14.2. Aims And Objectives Of Thesis Research.

The aims of the research presented in this thesis, are principally directed towards the molecular characterisation of cell surface molecules (CSMs) that mediate interactions between human HPC and cells of the BM stroma.

In contrast to mature leukocytes, the role of the selectin family in tissues of the haemopoietic system has not been thoroughly investigated. As such, the studies detailed herein were conceived to address the function of E-, P- and L- selectin (and their counter-receptors) in the adhesion and growth regulation of candidate HSCs, using a variety of *in vitro* assay systems.

Moreover, studies presented herein, sought to identify novel structures expressed at the surface of primitive human HPC and cultured BM stromal cells, with possible roles in haemopoietic regulation. This was achieved utilising a newly developed retroviral expression cloning strategy and a panel of mAbs directed against CSMs expressed by tissues of the BM. This approach permitted the molecular characterisation of a CSM expressed by candidate human HSC, which is identified by the mAb HCC-1. Furthermore, it also enabled the molecular isolation of a novel mucin-like transmembrane glycoprotein termed MGC-24v, which functions in mediating stromal cell-stem cell adhesion.

CHAPTER 2

MATERIALS AND METHODS

BUFFER AND MEDIA PREPARATION

2.1. PREPARATION OF BUFFERS

2.1.1. General Considerations

All water used to prepare non-tissue culture solutions was deionised and filtered using the Milli Q RO60 system (Millipore Corporation, USA). Tissue culture solutions were made with Milli-Q water prepared by further purifying the Milli-RO water through two beds of ion exchange resins, a carbon filter and an organic filter using a Milli-QF^{PLUS} system (Millipore). Prepared solutions and buffers were subsequently sterilised by autoclaving or filter sterilisation as indicated. Filter sterilisation was performed using either a sterivex GS 0.22 μm filter system (Millipore Corp., USA, Cat. No. SVGB1010), bottle filter (Corning, USA, Cat. No. 25111), Spin-X filter, (Costar, MA, USA, Cat. No.8160) or by 0.22 μm or 0.45 μm ministart filters (Sartorius, Germany, Cat. Nos. 165 34K and 165 55K, respectively). Following buffer preparation, all solutions were stored at 4°C, -20°C or -80°C as indicated.

2.1.2. Hank's Balanced Salt Solution (HBSS)

Single strength Hank's balanced salt solution (HBSS), pH 7.4 consisted of 0.14 M NaCl (Ajax Chemicals, Australia, Cat. No. 465), 5 mM KCl (Ajax, Cat. No. 383), 0.3 mM $Na_2HPO_4.12H_20$ (BDH, Australia, Cat. No. 10248), 0.4 mM KH_2PO_4 (BDH, Cat. No. 10203), 4.2 mM $NaHCO_3$ (BDH, Cat. No. 10247), 5.5 mM glucose (BDH, Cat. No. 10117), 1% Phenol Red (M&B, UK, Cat. No. P152/18/61, in 0.1 M NaOH) dissolved in Milli-Q water and subsequently autoclaved at 130°C for 20 minutes or filter sterilised as above.

2.1.3. Dulbecco's Phosphate Buffered Saline (PBS)

PBS was comprised of 0.14 M NaCl, 3 mM KCl, 8 mM $Na_2HPO_4.12H_20$, and 1 mM KH_2PO_4 dissolved in Milli-Q water. The pH was adjusted to 7.4 with 1 M HCl prior to sterilisation by autoclaving.

2.1.4. L - Glutamine

A 200 mM (100 X) stock solution of L-glutamine (BDH, Cat. No. 37107) was prepared by dissolving 4 g of L-glutamine in 100 ml of Milli-Q water, followed by incubation at 37°C, to facilitate dissolution. This stock solution was stored as 5 ml aliquots at -20°C.

2.1.5. Sodium Pyruvate

Stock solutions of 100 mM sodium pyruvate (100 X) were prepared by dissolving 1.1 g sodium pyruvate powder (Gibco/BRL, Cat. No. 890-1840) in 100 ml Milli-Q water, filter sterilised and stored at 4°C.

2.1.6. Penicillin - Streptomycin Sulphate For Use in Haemopoietic Stem Cell Assays

20 ml of Milli-Q water was added to a vial of penicillin-streptomycin sulphate (Gibco/BRL, 600-5145AE, 20 X) to give final concentrations of 500 i.u./ml and 500 mg/ml respectively, filter sterilised and stored at -20°C.

2.1.7. 2-Mercaptoethanol (2ME)

A 1 M stock solution of 2ME was prepared by diluting 0.7 ml of 14.7 M β-mercaptoethanol (Sigma, St. Louis, MO, USA, Cat. No. M-3148) into 9.3 ml HBSS and stored at - 20°C. The 1 M stock solution was further diluted 1:10 (in HBSS) to prepare a 0.1 M working solution, filter sterilised and stored as 500 µl aliquots at -20°C.

2.1.8. Folic Acid

To prepare a 10 mg/ml working solution, 100 mg of folic acid (Sigma, USA, Cat. No. F-8758), was dissolved in 10 ml 7.5% bicarbonate solution, filter sterilised and stored at - 20°C.

2.1.9. Myo-inositol

A 1% (w/v) myo-inositol solution was prepared by dissolving 100 mg myo-inositol powder (Sigma, Cat. No. I-5125) in 10 ml Milli-Q water, filter sterilised and stored at -20°C.

2.1.10. Deoxyribonuclease-I Solution (DNase-I)

A DNase-I stock solution was prepared by reconstituting one vial of DNase-I (3.75×10^5 units, Sigma, Type IV, Cat. No. 61362), with 2 ml 0.15 M NaCl, to give a final concentration of 1.875×10^5 units/ml. The DNase-I stock solution was aliquoted into ampoules (67 µl/ampoule; 1.25×10^4 units/ml) and stored at -80°C.

2.1.11. 10% (w/v) Bovine Serum Albumin (BSA)

To prepare 10% (w/v) BSA, 88.4 ml Milli-Q water, 20 g BSA (Sigma, USA, Cohn fraction V, Sigma A-2153) was carefully overlayed on the surface, and allowed to stand at 4°C for 24 hours. Once dissolved, 3 g of Duolite mixed bed resin MB6113 (BDH, Cat. No. 55057) was used to deionise the BSA to ensure the removal of trace elements and contaminants that could potentially inhibit colony growth in the *in vitro* cultures (described below). The beads were removed by filtration through Whatman No. 1 paper, and the procedure repeated 3 times. An equal volume (100 ml) of 2 x IMDM (or 2 x PBS) was added to the BSA and sterilised by filtration through a 0.22 µm bottle filter, prior to storage at - 20°C.

2.1.12. Heat Inactivated Foetal Bovine Serum (ΔFBS)

Heat inactivation of foetal bovine serum (FBS, Gibco/BRL, Cat. No. 200-6140P5, batch No. 660) was achieved by incubating FBS at 56°C for 30 minutes in a water bath. Heat inactivation was performed to ensure inactivation of endogenous bovine complement factors.

2.1.13. Trypsin-EDTA Solution

Trypsinisation solution consisted of 0.054% (w/v) trysin (Difco, USA, Cat. No. 0152-13-1) and 0.54 mM EDTA in PBS. After dissolving the trypsin powder in the EDTA/PBS, the solution was filtered through a low protein binding 0.22 µm filter (Millipore, Cat. No. SLGV025LS), aliquoted and frozen immediately to prevent inactivation of the trysin.

2.2. PREPARATION OF MEDIA

2.2.1. Dulbecco's Modified Eagles Medium (D-MEM)

To prepare D-MEM, one sachet of D-MEM powder (Gibco/BRL, Cat. No. 12800-017), and 3.7 g $NaHCO_3$ (BDH, USA, Cat. No. 10247) were dissolved in 900 mls of Milli-Q water. To this, sterile solutions of N-2-Hydroxyethylpiperazine N'-2-ethaneslphonic acid (HEPES, pH 7.2) (Boehringer-Mannheim, Australia, Cat. No. 737151), penicillin (Sigma, USA, Cat No. P 3032) and streptomycin sulphate (Sigma, USA, Cat. No. S 9137) were added to the final concentration of 15 mM, 100 i.u./ml and 100 µg/ml, respectively. A pH of 7.0 was attained following the addition of 4 ml of 1 M HCl prior to adjustment of the volume to 1 litre with Milli-Q water. The medium was subsequently filter-sterilised using a sterivex GS 0.22 µm filter. Prior to use, the medium was supplemented with 2 mM L-glutamine (BDH, Cat No. 37107) and 10% (v/v) FBS. Medium was replenished with 2 mM L-glutamine at weekly intervals.

2.2.2. Roswell Park Memorial Institute (RPMI-1640) Medium

To prepare RPMI-1640 medium, one sachet of RPMI-1640 powder (Life Technologies, Glen Waverley, Victoria, Australia, Cat. No. 31800-022), 2 g $NaHCO_3$ and 2.5 ml 1 M HCl were dissolved in 1 litre of Milli-Q water. Sterile solutions of HEPES, streptomycin sulphate and penicillin were added to give the final concentrations as above for D-MEM. A pH of 7.4, was attained and the medium was subsequently filter-sterilised using a sterivex GS 0.22 µm filter system. Prior to use, the medium was supplemented with 2mM L-glutamine and 10% (v/v) FBS. Medium was replenished with 2 mM L-glutamine at weekly intervals.

2.2.3. Iscove's Modified Dulbecco's Medium (IMDM)

IMDM was prepared as described for RPMI-1640 with the exception that IMDM powder (Gibco/BRL, Cat. No. 12200-036) was used.

2.2.4. Double - Strength Iscove's Modified Dulbecco's Medium (2 x IMDM)

To prepare 2 x IMDM (Cytosystems, Castle Hill, NSW, Australia, Cat. No. 50-016-PA), one sachet of IMDM powder was dissolved in 390 ml of Milli-Q water, as above. To this, sterile stocks of penicillin, streptomycin, DEAE-Dextran (Pharmacia-LKB Biotechnology AB, Uppsala, Sweden, Cat. No. 170350-01) and L-asparagine were added at concentrations of 200 i.u./ml, 200 µg/ml, 5.2 mg/ml and 0.4 mg/ml, respectively. Filter sterilised medium (as above) was stored as 50 ml aliquots at -20°C or used immediately.

2.2.5. F12 Nutrient Mixture (Ham)

Ham's F12 nutrient medium was prepared by dissolving one sachet of F12 nutrient mixture powder (Life Technologies, Australia, Cat. No. 21700-075), 2 g $NaHCO_3$, 2.5 ml 1 M HCl, 3.57 mg HEPES, 100 mg streptomycin sulphate and 1 x 10^5 IU penicillin were dissolved in 1 litre of Milli-Q water. A pH of 7.4 was attained and the medium subsequently filter-sterilised using a sterivex GS 0.22 µm filter. Prior to use, the medium was supplemented 2 mM L-glutamine and 10% (v/v) FBS. Medium was replenished with 2 mM L-glutamine at weekly intervals.

2.2.6. Alpha Modification Of Eagle's Medium (α-MEM)

To prepare 1 litre of single strength α-MEM, 10 g of α-MEM (with glutamine, without sodium bicarbonate; Flow Laboratories, Australia, Cat. No. 10-311-26) was dissolved in 990 mls of Milli-Q water, supplemented with penicillin (100 i.u./ml), streptomycin sulphate (100 µg/ml) and buffered with 2 g of sodium bicarbonate. The medium was subsequently filter sterilised and stored at 4°C.

2.2.7. Long Term Liquid Culture (LTLC) Medium

To prepare 1 litre of LTLC medium, one sachet of α-MEM (with glutamine, but without sodium bicarbonate; Flow Laboratories, Cat. No. 10-311-26) was dissolved in 990 ml of Milli-Q water. The medium was supplemented with 3.5 ml folic acid (0.01 mg/ml), 4 ml myo-inositol (0.4 mg/ml, Sigma), 0.5 ml (1 mM / litre) hydrocortisone sodium succinate (Upjohn Pty. Ltd., Rydalmere, NSW, Australia), 1 ml (1×10^{-6} M) β-mercaptoethanol, 10 ml (2 mM) L-glutamine, 10 ml (50 i.u./ml and 50 mg/ml) penicillin-streptomycin, 14 ml of 1 M NaCl (to adjust medium to 360 mOsM), 125 ml (12.5%) FBS, and 125 ml (12.5%) horse serum (CSL, Melbourne, Australia).

2.2.8. Methyl Cellulose

Methyl cellulose powder ([8.1 g] A4M premium grade 4000 centipose, Dow Chemicals, Lake Jackson, Texas, USA), was added to a 500 ml Schott bottle, and autoclaved at 15 *psig* (121°C) for 15 minutes. To this, 270 ml sterile 1 x IMDM was added, mixed and left stirring for 48 hour with occasional shaking to assist mixing. When the medium was homogeneously mixed, 180 ml sterile FCS and 60 ml BSA was added and allowed to stir for a further 3 hours. The methyl cellulose mixture was then stored at - 20°C as 25 ml aliquots.

2.2.9. Medium for Pre-Colony Forming Unit (CFU) Assay

a) Serum Replete Pre-CFU Assay Medium

To prepare 300 ml of medium, to 180 ml 1 x IMDM, 90 ml FCS (30% (v/v)), 30 ml BSA (10% v/v), 3 ml L-glutamine (200 mM) and 150 µl 0.1 M 2ME (5×10^{-4} M) were added. The medium was subsequently dispensed into 10 ml aliquots, and stored at -20°C.

(b) Serum Deprived Medium (SDM)

The preparation of SDM for the growth of haemopoietic progenitor cells, was performed essentially as described by Lansdorp and Dragowska (1992). Single strength IMDM was supplemented with 10 mg/ml bovine pancreas derived insulin (Collaborative Research, Bedford, MA, USA, Cat. No. 40205), 2% (w/v) BSA (Sigma, USA, Cat. No. A2153), 40 mg/ml human low density lipoprotein (Sigma, USA, Cat. No. L2139), 200 mg/ml iron

saturated human transferrin (Sigma, USA, Cat. No. T2158), 2 mM L-glutamine, β-mercaptoethanol (5×10^{-5} M) and penicillin/streptomycin at 100 i.u./ml and 50 μg/ml, respectively. The SDM was made fresh as required and was filter sterilised prior to its use.

2.2.10. Medium For Thawing Cryopreserved Cells

To 350 ml of Ca^{2+}, Mg^{2+} -free HBSS, 50 ml acid citrate, 100 ml 10% BSA (in HBSS), and 2 ampoules of DNase-I were added to give a final concentration of 10 mmol/L, 2% and 50 kunitz units/ml respectively. DNase-I was added immediately prior to use.

2.2.11. Immunofluorescence Buffer (IF Buffer)

To prepare 500 ml IF buffer, 25 ml of heat inactivated FBS and 0.4 g sodium azide was added to 475 ml HBSS. Sodium azide was omitted when cells were labelled for cell sorting and culturing.

2.2.12. Blocking Buffer For Flow Cytometric Analysis

Hank's Balanced Salt Solution was supplemented with 10% (w/v) bovine serum albumin (BSA: Cohn fraction V; Sigma, St Louis, MO), 10% (v/v) normal human serum (Red Cross, Adelaide, South Australia) and 5% (v/v) foetal bovine serum (FBS: Gibco/BRL, USA, Cat. No. 200-6140PJ, Batch No. 660). The buffer was filter sterilised through a 0.2 μm bottle filter (Costar) and stored at 4°C.

2.2.13. Flow Cytometry Fixative (FACS Fix)

To 1 litre PBS, 10 ml of formalin (formaldehyde, ACE Chemical Company, Adelaide, Australia), 20 g D-glucose (AJAX Chemical Company), and 0.2 g sodium azide (Sigma, USA, Cat. No. S-2002) were added.

2.2.14. OPI (50 X)

A 50 x OPI stock solution was made by adding 750 ml oxaloacetic acid (Sigma, USA, Cat. No. O-9504), 250 mg sodium pyruvate (Gibco/BRL) and 1000 units of bovine insulin (Sigma, USA, Cat. No. 1-1882; previously prepared by adding 10 ml sterile Milli-Q water and 100 µl glacial acetic acid to 100 mg of insulin powder) to 100 ml DMEM. The medium was filter sterilised and stored at -20°C.

2.2.15. Hybridoma Growth Medium (HGM)

500 ml HGM consisted of 309 ml RPMI-1640, 6 ml HEPES, 0.64 g $NaHCO_3$ 0.8 g glucose, 0.64 ml sodium pyruvate, 5 ml L-glutamine, 50 ml NCTC-109 medium (Life Technologies, Cat. No. 21340-021), 5 ml non-essential amino acids (100 X stock, Multi Cell, Cat. No. 21-145-0100V), 10 ml 50 x OPI (refer to Section 2.2.14), 2.5 mg Ascorbic acid (Sigma, Cat. No. A-4544), 130 mg L-cysteine (Sigma, Cat. No. C-8152), 5 ml penicillin-streptomycin and 100 ml FBS. The medium was filter sterilised and stored at -20°C.

2.3. CYTOKINES AND COLONY STIMULATING FACTORS

2.3.1. Murine Cytokines

Purified recombinant murine (rMu) GM-CSF (4×10^4 units/ml) synthesised in yeast harbouring a GM-CSF expression vector, was kindly provided by Drs. T Wilson and N. Gough (Walter and Eliza Hall Institute, Melbourne, Australia). rMu IL-3 (8.3×10^5 units/ml) synthesised by a recombinant baculovirus vector was a kind gift from Dr. A. Hapel (John Curtin School of Medical Research, Australian National University, Canberra, Australia). Cytokine units were defined, such that 50 units gave 50% of maximal numbers of colonies in soft agar cultures containing 5×10^4 murine bone marrow cells.

2.3.2. Human Cytokines

Recombinant human (rHu), Interleukin-1β (IL-1β), Granulocyte-Colony Stimulating Factor (G-CSF) and Stem Cell Factor (SCF) were generously provided by Amgen Inc, Thousand Oaks, CA, USA.

rHu IL-3, IL-6, Granulocyte/Macrophage-CSF (GM-CSF) were generously provided by Dr. S. Clark, Genetics Institute, Boston, MA, USA.

Erythropoietin was purchased from Eprex; Janssen Cilag, Aukland, New Zealand and rHu tumour necrosis factor alpha (TNFα) was purchased from Pepro Tech Inc., Rocky Hill, NJ, USA.

2.4. CELL LINES AND CELL PREPARATIONS

2.4.1. General Considerations

Prior to use, all cell lines used in these studies were shown to be mycoplasma-free using the Gen-Probe Mycoplasma TC Rapid Detection System (Gen-Probe Inc., San Diego, CA, USA). Cell lines were routinely maintained in sterile plastic flasks (75 cm^2 or 25 cm^2 surface area, Corning, USA, Cat. No. 25110-75 and 25110-25, respectively) in the appropriate medium, supplemented with 10% (w/v) FBS at 37°C in a humidified

atmosphere containing 5% CO_2 in air. Adherent and non-adherent cell lines were subcultured, every 2 to 3 days, to ensure maintenance of a logarithmic phase of growth, between $5x10^4$/ml and $5x10^5$/ml. Cell densities and viabilities were determined using a haemocytometer and trypan blue dye exclusion (0.8% (w/v) in saline), respectively.

All human tissue culture was performed in Class 2 "biohazard" laminar flow hoods (Gelman Sciences). Cell cultures were incubated at 37°C (Forma Scientific air incubator), in an atmosphere containing 5% CO_2 in air to maintain pH 7.0, and a relative humidity of 97%

2.4.2. Murine Haemopoietic Factor-Dependent Cell Lines, FDC-P1 and Baf-3

The murine factor-dependent cell lines, FDC-P1 (Dexter *et al*, 1980) and Baf-3 (Hatakeyama *et al*, 1989), were obtained from Dr. TJ. Gonda (Hanson Centre for Cancer Research, Adelaide) and were cultured in DMEM supplemented with 10% FBS and 80 units/ml murine GM-CSF (FDC-P1 growth medium, FDC-P1 GroM) and 300 units/ml murine IL-3 (Baf-3 growth medium, Baf-3 GroM), respectively. Cultures were initiated with 2×10^5 cells/ml in 25 cm^2 surface area, tissue culture flasks (Corning, USA, Cat. No. 25102-25), and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air, and passaged every 2 to 3 days.

2.4.3. PA317, Ψ_2 And BOSC-23 Retroviral Packaging Cell Lines

The murine fibroblastic, amphotropic virus-packaging cell line, PA317 (Miller and Buttimore, 1986), and two ecotropic virus-packaging cell lines, Ψ_2 (Mann *et al*, 1983) and BOSC-23 (Pear *et al*, 1993), were all obtained from Dr. TJ. Gonda (Hanson Centre for Cancer Research, Adelaide) and maintained in DMEM supplemented with 10% FBS. Cultures were grown as monolayers in 75 cm^2 surface area, tissue culture flasks (Corning, USA, Cat. No. 25110-75), and subcultured every 3 to 5 days. Fibroblast cultures were harvested by trypsin detachment, by washing monolayers twice with HBSS or PBS, followed by the addition of 2 ml trypsin-PBS and incubating the cultures at 37°C for 5 minutes. The trypsin was inactivated by the addition of DMEM supplemented with 10% FBS and each flask subsequently reseeded with 5×10^5 to 1×10^6 cells. Cell densities

were determined using a haemocytometer and the viabilities ascertained using trypan blue exclusion (0.8% w/v in saline). Cultures were incubated at 37°C with 5% CO_2 as described above.

Following infection (refer to Section 2.26), Swiss-3T3, FDC-P1, and Baf-3 cells were selected in the appropriate growth media, containing, 400 µg/ml, 1000 µg/ml and 1,200 µg/ml of Geneticin (G418, Life Technologies), respectively. All cell types were subsequently maintained in growth medium containing 200 µg/ml G418 (maintenance medium, MM).

2.4.4. Murine Stromal Fibroblastic Cell Line, Swiss-3T3

The murine fibroblastic cell line Swiss-3T3 (Rheinwald and Green, 1975a; 1975b), was maintained in DMEM supplemented with 10% FBS. Cultures were grown as monolayers in 75 cm^2 surface area, tissue culture flasks (Corning, USA, Cat. No. 25110-75), and subcultured every 3 to 5 days. Fibroblast cultures were harvested by trypsin detachment, as described above (Section 2.4.3.) and following trypsin-inactivation, flasks were reseeded with 5×10^5 to 1×10^6 cells and subsequently incubated at 37°C with 5% CO_2.

2.4.5. Miscellaneous Cell Lines

(a) CHO Cell Transfectants Expressing The Selectin Family Of Molecules: Chinese Hamster Ovary (CHO) cells expressing human P-, L- and E-selectin were all generously provided by Dr Y. Khew-Goodall, Division of Immunology, Hanson Centre for Cancer Research, Adelaide, Australia. These transfectants were generated by electroporating CHO cells with a linearised full length P-, L- and E-selectin cDNA cloned into the eukaryotic expression vectors pCDM8 (generously supplied by Dr. B. Seed, Massachusetts General Hospital, Boston, MA, USA) or pCDNA1 (Invitrogen Corporation, San Diego, California, USA, Cat. No. V490-20 and Appendix A, Restriction Map #2) together with a 10-fold molar excess of the plasmid pSV2.Neo (Invitrogen Corp., USA) encoding resistance to the neomycin analogue G418. CHO cell clones expressing the various selectin molecules were isolated following 2 weeks selection in

complete Ham's F12 growth medium supplemented with 300 µg/ml G418 (Life Technologies). Selectin expression was assessed following staining of transfectants with a monospecific polyclonal rabbit antiserum, mAb DREG-56 and mAb 5B11, directed against P-, L-, and E- Selectin, respectively (please refer to Table 2.10.1). Clones exhibiting uniform, high levels of Selectin expression were isolated following FACS and single cell deposition (refer to Section 2.10.4) and utilised for all experiments presented herein.

(b) HEL-900: HEL-900 (Martin and Papayannopoulou, 1982), a human erythro-leukaemic cell line was obtained from the American Type Culture Collection (ATCC) and maintained in complete RPMI-1640 medium.

(c) HEL-DR$^+$: HEL-DR$^+$, a human erythroleukaemic subline of the cell line HEL-900 selected on the basis of class II MHC expression, was a kind gift of Dr B. Torok-Storb, Fred Hutchinson Cancer Research Centre, Seattle, Washington. HEL-DR$^+$ cells were maintained in complete RPMI-1640 medium.

(d) K562: K562 (Gahmberg *et al*, 1979), a human erythroleukaemic, glycophorin A positive cell line was a kind gift from Dr H Zola, Flinders Medical Centre, Adelaide, and maintained in complete RPMI-1640 medium.

(e) KG-1: KG-1 (Koeffler *et al*, 1980), a human myeloblastic leukaemic cell line was obtained from the ATCC and maintained in complete RPMI-1640 medium.

(f) KG-1a: KG-1a (Koeffler *et al*, 1980), a more immature subline of the human myeloblastic leukaemic cell line was obtained from the ATCC and maintained in complete RPMI-1640 medium.

(g) HL-60: HL-60 (Collins *et al*, 1977), a human promyelocytic leukaemic cell line was obtained from the ATCC and maintained in complete RPMI-1640 medium.

(h) Human Umbilical Vein Endothelial Cells (HUVECs): HUVECs were generously provided by Dr J. Gamble, Division of Immunology, Hanson Centre for Cancer Research, Adelaide, Australia.

(i) Human Foreskin Fibroblast (HFF-2) : HFF-2 cells were generated by Dr P.J. Simmons and represented a renewable, non-immortalised fibroblastoid cell line. HFF-2 cells were maintained in complete DMEM medium.

(j) Human Foreskin Keratinocytes (HFK) : HFK cells were generated by Dr P. Kaur and represented a renewable, non-immortalised keratinocyte cell line.

2.4.6. Preparation Of Bone Marrow And Peripheral Blood Mononuclear Cells (BMMNC and PBMNC)

Normal bone marrow (BM) was aspirated into preservative-free, sodium heparin-containing tubes (Fisons Pharmaceuticals, Australia, 1,000 units/ml), from the sternum and posterior iliac crest of healthy young volunteers following informed consent. The use of normal BM and PB cells for these studies was approved by the Human Ethics Committee of the Royal Adelaide Hospital. Low density bone marrow mononuclear cells (BMMNC) were collected after centrifugation at 400 x g over Ficoll-Hypaque (Lymphoprep, 1.077 g/dL; Nycomed Pharma AS, Oslo, Norway) for 30 minutes at room temperature. Mononuclear cells were obtained by selecting the interface cells, while granulocytes and erythrocytes were obtained from the pellet. BMMNC were washed thrice by centrifugation at 4°C in "HHF" (Hanks Balanced Salt Solution, [HBSS; Gibco/BRL] supplemented with 20 mM HEPES, pH 7.35 and 5% (v/v) FBS), in preparation for fluorescence activated cell sorting or adhesion assays.

2.4.7. Human Bone Marrow Stromal Cell (HBMSC) Cultures

Stromal cultures were established, essentially as described by Simmons *et al* (1987). BMMNC were prepared by buoyant density gradient centrifugation as described in Section 2.4.6. After washing thrice in HHF, the BMMNCs were resuspended in 10 ml of α-minimal essential medium (refer to Section 2.2.6.) or long term liquid culture medium (refer to Section 2.2.7.) and cultured in 25 cm^2 flask (Corning) at 37°C, 5% CO_2. Upon development of confluent stromal layer, the cells were detached using 0.05% (w/v) trypsin-EDTA in PBS and replated in the same medium at 2 x 10^5 cells per ml in 2 x 75 cm^2 tissue culture flasks, and passaged as required.

2.4.8. Tissue Samples

Human tissue specimens (brain, liver, heart, kidney, lung, spleen, thymus, lymph node, tonsil, small intestine) were obtained from autopsies carried out at the Womens' and Childrens' Hospital, Adelaide and from routine pathological examinations carried out at the Royal Adelaide Hospital. Small specimens approximately 0.5 cm^2 of each tissue type were placed into 25 mm x 20 mm x 5 mm Tissue-Tek II cryomoulds (Miles Laboratories; Naperville, IL) and embedded with O.C.T. compound medium (Miles Laboratories) at -20°C prior to being stored at -70°C. Frozen sections of nerve and muscle tissue were obtained from the Department of Histopathology, Institute of Medical and Veterinary Science (I.M.V.S), Adelaide, Australia. Sections of foreskin were obtained from the Department of Immunology, I.M.V.S.

2.5. HAEMOPOIETIC PROGENITOR CELL ASSAYS

2.5.1. Clonogenic Assays Of Haemopoietic Progenitor Cells

Unfractionated BMMNC, various populations of cells obtained by FACS and cells derived from pre-CFU-assay (refer to Section 2.10.4. and 2.5.4., respectively) were assayed for their content of granulocyte-macrophage colony forming cells (CFU-GM), primitive erythroid progenitors (BFU-E), committed erythroid colony forming cells (CFU-E) and multipotential colony forming cell (CFU-GEMM or CFU-Mix) as previously described (Simmons *et al*, 1990). Cultures were established in triplicate by plating 1 x 10^3 $CD34^+$ cells and 2-5 x 10^4 BMMNC per 35mm dish in 1 ml of IMDM supplemented with 0.9% methylcellulose, 30% FBS, 1% deionised BSA, 3 mmol/L L-glutamine and 5 x 10^{-5} 2-mercaptoethanol. Colony growth was stimulated by the addition of either 5% conditioned medium from the human bladder carcinoma cell line 5637 (5637-CM), 1 ng rHu IL-3 and 4U of rHu-erythropoietin or 10 ng of each of the rHu haemopoietic growth factors; IL-1β, IL-3, IL-6, G-CSF, GM-CSF, SCF and 4U of erythropoietin. CFU-E were scored on day 7 and day 14 as single clusters of haemoglobinised cells. BFU-E were scored on day 14 as multicentric colonies, comprising either large clusters, or a large single colony, of more than 64 haemoglobinised cells. CFU-GM and CFU-GEMM were scored on day 14 of culture with CFU-GM colonies consisting of greater than 40 cells per colony.

CFU-GEMM colonies consisted of a combination of more than 50 erythroid, granulocytic and monocytic cells.

2.5.2. Long Term Bone Marrow Culture (LTBMC)

Populations of cells obtained by FACS and various BMMNC fractions were assayed for their ability to initiate and maintain haemopoiesis in LTBMC as previously described (Simmons and Torok-Storb, 1991a; Simmons and Torok-Storb, 1991b). Established human bone marrow stromal cell (HBMSC) cultures (refer to Section 2.4.7.) were irradiated at 20Gy^{60}Co and each well of a 35 mm 6-well tissue culture clusters (Costar) was seeded with 1-2 x10^5 cells. Cultures were initiated in triplicate, after seeding 1 x 10^4 sorted BMMNC (resuspended in 3 ml LTLC) onto each well of irradiated stroma.

Clonogenic assays (Section 2.5.1.) were performed to provide numbers of input clonogenic cells in each of the haemopoietic cell fractions. Over a period of up to 10 weeks, cultures were maintained by weekly demi-depopulation, whereby 1.5 ml of non-adherent cells were carefully removed from each culture and replaced with an equal volume of pre-warmed LTLC medium. The number of non-adherent cells present in the medium removed at each weekly feed, was determined by counting using a haemocytometer and the number of clonogenic cells (CFU-GM, BFU-E) determined as described above (Section 2.5.1.).

2.5.3. LTBMC: The Effect Of Antibodies In The Initiation And Maintenance Of Haemopoiesis

LTBMC were carried out as described (Section 2.5.2.) with the exception that cultures were supplemented with mAbs to cell surface molecules (CSM) expressed by the stromal or haemopoietic progenitor cells or isotype-matched control antibodies (sterile, azide-free) as indicated in the Results Sections. All antibodies were used at a final concentration of 20 μg/ml. Cultures were established in triplicate and maintained for six weeks with demi-depopulation at weekly intervals and supplementation with fresh

medium containing additional antibody. Cultures were monitored for their production of non-adherent and clonogenic cells as described above (Section 2.5.1.).

2.5.4. Pre-Progenitor Cell (Pre-CFU) Assay

This is a stroma-free, cytokine-dependent suspension culture assay initially described by Iscove *et al* (1989), and modified by Haylock *et al* (1992) which measures the *de novo* generation of CFU-GM as an index of precursors (pre-CFU) of CFU-GM. Immunolabelled BMMNC were sorted into cell fractions using the FACStar^{PLUS} cell sorter and resuspended into pre-CFU medium (IMDM supplemented with 30% FBS, 1% deionised BSA, 3 mM L-glutamine and 5 x 10^{-5} M β-mercaptoethanol) at a concentration of 1 x 10^3 cells/ml. Triplicate 1 ml suspension cultures were established in 24 well plates in pre-CFU medium supplemented with each of the following human haemopoietic growth factors (HGF) at a final concentration of 10 ng/ml: rHu IL-1β, IL-3, IL-6, G-CSF, GM-CSF and SCF. Clonogenic assays were performed in triplicate to determine the number of CFU-GM in the input population of cells used to initiate the pre-CFU cultures. The cultures were incubated at 37°C in 5% CO_2 for 28 days. At days 7, 14, 21 and 28 the contents of each well were removed, washed in IMDM and cell counts performed to determine cell production over the previous week. One tenth of the harvested cells were assayed for their content of CFU-GM (as above), and a further tenth set up in pre-CFU culture with fresh growth medium supplemented with six HGF. The remainder of the cells were used for immunophenotypic analysis or for the preparation of cytospins to assess cell morphology.

2.6. MISCELLANEOUS TECHNIQUES

2.6.1. Cryopreservation of cells

Cells were cryopreserved in the presence of 10% analytical grade dimethyl sulphoxide (DMSO) to prevent the crystallisation and fracturing of the cell membranes. Cells were harvested at log phase and resuspended at 0.5-1x10^7 cells per ml in fresh medium. Immediately prior to freezing, an equal volume of freezing mix (20% DMSO, 30% heat-inactivated FBS, 50% RPMI-1640 or appropriate medium, filter sterilised through a 0.22 μm nitrocellulose filter) was added dropwise with mixing over approximately 2-5

minutes. The cell mixture was aliquoted into cryopreservation ampoules (Nunc, Intermed, Roskilde, Denmark) and control-rate cryopreserved in a control rate freezer (KRYO 10 series) at 1°C/minute from 5°C to -12°C, 4°C/minute from -12°C to -20°C, 0°C for 5 minutes, at 1°C/minute from -20°C to -40°C and 3°C/minute from -40°C to -80°C. The vials were then transferred to liquid nitrogen storage (-196°C).

2.6.2. Thawing Cryopreserved Samples

The appropriate medium was prewarmed to 37°C. Following rapid thawing in a 37°C water bath, the sample was subsequently transferred to a 14 ml polypropylene tube (Corning), and an equal volume of the appropriate medium was added dropwise over a 5 minute period. Following a 10 minute incubation at room temperature, an equal volume of medium was again added. The sample was left to stand at room temperature for an additional 10 minutes, then centrifuged at 200 x g for 5 minutes. The sample was washed twice in medium to remove the residual DMSO prior to culturing.

HYBRIDOMA PRODUCTION AND IMMUNOASSAYS

2.7. PRODUCTION OF MONOCLONAL ANTIBODIES

2.7.1. Immunisation Of Mice

Tertiary passaged, cultured human bone marrow stromal cells (HBMSC) were harvested by trypsinisation as described in Section 2.4.7. and resuspended in 300 µl PBS supplemented with 20 µg muramyl dipeptide (Sigma) as adjuvant. BALB/c mice were immunised intraperitoneally with 5 x 10^6 HBMSC, and subsequently boosted (as above) a further 3 times at three-week intervals to ensure adequate affinity maturation of the immune response. Three days prior to fusion, 5 x 10^6 cells were resuspended in 100 µl PBS and administered via the tail vein. Immediately prior to fusion, mice were sacrificed and their spleens aseptically removed.

2.7.2. Production Of Antibody Secreting Hybridoma Cell Lines

Hybridomas secreting antibodies reactive with cultured HBMSC were generated by fusing the NS1-Ag4-1 murine myeloma cell line and spleen cells derived from BALB/c mice immunised with trypsinised cultured HBMSC (described above). Fusion of splenocytes and myeloma cells was performed essentially as described by Köhler and Milstein (1975) with the following modifications. Following removal of spleens from immunised mice, a single cell suspension of splenocytes was prepared by repeated "flushing" of the spleen with RPMI-1640 medium (supplemented with 2 x penicillin-streptomycin), using a syringe fitted with a 21 gauge needle. To ensure complete removal of the splenocytes, the spleen capsule was gently massaged between the ground glass ends of two sterile microscope slides. Following repeated flushing (as described above), the harvested splenocytes were placed on ice to allow any agglutinated cell debris to settle. The debris-free supernatant (containing the single cell suspension of splenocytes) was removed and transferred to a fresh tube and the splenocytes pelleted by centrifugation (400 x g) at 4 °C. The supernatant was removed by vacuum aspiration, and the cell pellet was resuspended in 10 ml of warm erythrocyte lysing solution (0.75% NH_4Cl in 17 mM Tris-HCl, pH7.4), to ensure complete lysis of contaminating

erythrocytes. Splenocytes were washed twice in serum free-RPMI-1640 and the yield determined by cell counting. The average number of splenocytes obtained ranged from 2 to 3 x 10^8 cells.

The murine myeloma cell line NS1-Ag 4-1 was maintained in RPMI-1640 supplemented with 10% FBS and 2 mM glutamine. Periodically, the cell line was cultured in the presence of 10^{-8} M 8-azaguanine to eliminate revertants which had acquired a functional HPRT gene, however cells were cultured in the absence of 8-azaguanine for at least 7 days prior to performing the fusion. To ensure log-phase growth at the time of the fusion, the NS1-Ag 4-1 cell line was resuspended to 2 x 10^5 cell/ml 24 hours prior. On the day of the fusion, the NS-1 cells were harvested and washed 4 times in serum free RPMI-1640 to ensure removal of residual serum. Splenocytes and NS-1 cells were subsequently mixed at a ratio of 4 to 1 in a 50 ml polypropylene tube (Corning, NY, USA, Cat. No. 25331-50), and pelleted by centrifugation for 10 minutes at 400 x g. The supernatant was removed by vacuum aspiration, leaving a dry cell pellet to which 1 ml of polyethylene glycol 1500 (PEG, Boehringer-Mannheim, Mannheim, Germany), pre-warmed to 37°C, was added over a period of 1 minute. The fusion of splenocytes with the NS-1 myeloma line was facilitated by gentle mixing with the pipette tip. The cell mixture was incubated at 37°C for 1 minute with gentle agitation. The PEG was subsequently diluted by the sequential addition of 1 ml, 1 ml, 3 ml, 5 ml and 10 ml of pre-warmed hybridoma growth medium (HGM; please refer to Section 2.2.15 for preparation) at 1 minute time intervals.

The cells were resuspended in approximately 50 ml with the addition of approximately 25 ml of HGM, and large cellular aggregates were allowed to settle for 5 minutes at room temperature. The supernatant, (containing the fusion bodies) was transferred to a fresh tube, and the cells pelleted by centrifugation at 400 x g for 5 minutes. The supernatant was removed by vacuum aspiration and the cells resuspended in 100 ml of HGM supplemented with 20% conditioned medium derived from the murine macrophage cell line, J774 (J774-CM; negating the requirement for fibroblast feeder layers), and hypoxanthine/thymidine (HT, Boehringer Mannheim) at a final concentration of 0.1 mM and 0.016 mM, respectively. The homogeneously-mixed cell suspension was

dispensed as 100 µl aliquots into 10 flat bottomed, 96 well culture dishes. Cells were cultured for 24 hours at 37°C in a humidified atmosphere containing 5% CO_2 in air, prior to the addition of 100 µl of HGM supplemented with 20% J774-CM, HT and 2 x aminopterin (A; Boehringer Mannheim, Germany; final concentration of 0.8 µM) to each well. Cultures were fed at day 7 by removal of half the medium and replacing with an equal volume of fresh HGM supplemented with 20% J774-CM and HAT. Selection of HAT-resistant hybridomas was allowed to proceed for 10 to 14 days at which time screening was initiated as described below (Section 2.8).

2.8. SCREENING AND SELECTION OF ANTIBODY SECRETING HYBRIDOMA CELL LINES WITH REACTIVITY TO HBMSC

2.8.1. General Considerations

Supernatants from antibody-producing, HAT-resistant hybridomas were screened by means of a multi-tiered screening protocol to select those supernatants which exhibited reactivity with HBMSC and limited reactivity to BMMNC. The selected hybridomas were cloned three times by limiting dilution to ensure monoclonality of the mAb produced (refer to Section 2.8.5) and subsequently isotyped by means of a mouse monoclonal antibody isotyping kit (ISOStrip, Boehringer Mannheim), as recommended by the manufacturer.

2.8.2. Screening Of mAb With Reactivity To HBMSC : "Primary Screen"

The preliminary screen were performed to exclude hybridomas that secreted antibodies that showed extensive reactivity to peripheral blood mononuclear cells (PBMNC) such as the ubiquitous leukocyte antigens HLA-class I and β2 microglobulin. This was performed by means of *in situ* fluorescence assay, where 5 x 10^5 PBMNC (prepared as described in Section 2.4.6.) were added to wells of a flat-bottomed 96 well plate, pre-coated with 50 µl of poly-L-lysine (Sigma, USA). The cells were forced to adhere to the plates by centrifugation of at 300 x g for 5 minutes. Non-adherent cells were removed by vigorous inversion of the plates and the adherent cells were subsequently chilled on ice for 30 minutes following the addition of 100 µl of blocking buffer (Section

2.2.12.) to block potential Fc receptor-mediated binding of the labelling antibody. The blocking buffer was removed by inversion of the plates and 50 µl of hybridoma supernatant was added to the cells and incubated on ice for 60 minutes. Isotype matched, non-binding control mAbs (please refer to Table 2.10.1.) were also included to control for inherent, background fluorescence. The cells were washed thrice with IF buffer (Section 2.2.11.) as described above, and subsequently incubated on ice for a further 45 minutes with 50 µl of a 1/30 dilution (in IF buffer) of Fluorescein isothiocynate (FITC)-conjugated-$F(ab')_2$ sheep anti-mouse Ig (Silenus, Australia, Cat. No. DDAF). The cells were then washed thrice (as above), and resuspended in 100 µl of 1% paraformaldehyde (BDH, Cat. No. 29447) in PBS and incubated at room temperature for 10 minutes. The 1% paraformaldehyde was removed by inversion of the plates (as above), and the individual wells analysed using a fluorescence microscope (Olympus Optical Co. Ltd, Model No. BH2-RFCA, Tokyo, Japan). Wells were scored as positive if more than 5% of the cells in each well were stained (based on visual examination criteria), and eliminated from subsequent analysis.

2.8.3. Screening Of mAb With Reactivity To HBMSC : "Secondary Screen"

Hybridomas producing antibodies "non-reactive" with BMMNCs that were identified in the primary screen were subsequently assessed for their reactivity to HBMSC by means of a peroxidase enzyme-linked immunosorbant assay (ELISA). The appropriate number of wells of a flat-bottomed 96 well plate were seeded with approximately 1×10^4 cultured HBMSC 24 hours prior to the assay, and cultured as previously described (please refer to Section 2.4.7.). After incubation in blocking buffer, 50 µl of hybridoma supernatant (or isotype matched, non-binding control mAb) was added to each well, and incubated on ice for 60 minutes. Wells were washed thrice with 100 µl of IF Buffer, after which 50 µl of a 1/1000 dilution (in IF buffer) of goat anti-mouse IgG conjugated to horse radish peroxide [HRPO] (Caltag, USA, Cat. No. 943-HCG) was added. The cells were washed as above, followed by the addition of peroxidase substrate solution, OPD (O-phenylene diamine, H_2O_2 ; Sigma). The resulting insoluble reaction product was measured using an optical density plate reader at 490 nm. Wells exhibiting an OD reading greater

than that obtained with isotype matched, non-binding control mAbs, were considered positive and retained for subsequent analysis, whilst negative wells were eliminated.

2.8.4. Screening Of mAbs With Reactivity To HBMSC : "Tertiary Screen"

Selected hybridomas fulfilling the criteria of the primary and secondary screens were subsequently used to stain a number of human cell lines and cell preparations, including HUVECs, myeloid cell lines KG1a and HL60, and the erythroleukaemic cell line HEL-DR$^+$ by indirect immunofluorescence as described in Section 2.10.2. Cells exhibiting a unique reactivity profile were isotyped, and subsequently screened for their ability to react with CD34$^+$ cells.

2.8.5. Limiting Dilution Cloning Of Antibody Secreting Hybridomas

Hybridomas exhibiting an appropriate reactivity profile, were subsequently cloned by limiting dilution. Routinely, 500 to 1000 hybridoma cells were initially diluted 2-fold in HGM supplemented with 20% J774-CM and HAT by serial dilution down column one of a 96 well plate. The cells were subjected to a further 2-fold dilution from column 1 through 12, using a multi-channel pipette. Wells were examined for clonal growth (using an inverted microscope) following 10 to 14 days culture. Routinely, 6 to 12 wells exhibiting clonal growth were screened for their maintenance of the reactivity profile (as described above) and positive wells were subject to a further two rounds of limiting dilution cloning. Tertiary clones were then expanded successively into 48 well plates, 24 well plates, 6 well plates and finally 25 cm^2 tissue culture flasks (all from Costar). Stocks of mAb-producing hybridoma cell lines were cryopreserved as described in Section 2.6.1, following the acquisition of sufficient hybridoma supernatant for subsequent characterisation studies or purification of Ig (refer to Section 2.9.).

2.9. PURIFICATION OF MOUSE IgG MONOCLONAL ANTIBODIES (mAb)

2.9.1. Buffers And Reagents Required

(a) 0.1 M phosphate buffer, pH 8.2 : Titrated small amounts of 0.1 M NaH_2PO_4 to a solution of 0.1 M Na_2HPO_4, until a pH of 8.2 was achieved.

(b) 0.1 M citrate buffer, pH 5.5/4.5/5.5 : Titrated 0.1 M citric acid and 0.1 M sodium citrate to achieve the desired pH.

(c) 0.5 M propionic acid : 37 ml propionic acid (Ajax Chemicals, Australia, Cat. No. 693), made up to 1 litre with distilled water.

2.9.2. Protocol For The Purification Of Mouse IgG Monoclonal Antibodies

Monoclonal antibodies were purified essentially as described by Ey *et al*, 1978. A 10 ml plastic-pipette (Linbro, Surgical and Medical Supplies, Cat. No. 612 4407) was packed with 5 ml of Protein A-Sepharose (Pharmacia, North Ryde, NSW, Australia, Cat. No. 17-0780-01) to prepare a re-useable antibody-purification column. The column was subsequently equilibrated with 0.1 M phosphate buffer, pH 8.2 (refer to Section 2.9.1.(a) for preparation) supplemented with 0.1% (w/v) sodium azide and stored in this form at 4°C until required. The column was loaded with the desired mAb in the form of hybridoma tissue culture supernatant or ascitic fluid. Prior to loading on to the column, hybridoma culture supernatant was adjusted to pH 8.2 and filtered through a 0.45 µm filter, whilst ascitic fluid was centrifuged at 100,000 x g (40,000 rpm, Beckman L8.80 ultracentrifuge, Beckman Instruments, Mt. Waverley, Victoria, Australia) for 30 minutes, filtered through a 0.45 µm filter (Sartorius, Germany, Cat. No. 175 98 K) and then diluted 1:1 with 0.1 M Tris-HCl, pH 8.6 prior to addition to the column. Routinely, antibody solution was loaded onto column and allowed to completely discharge by gravity flow. The column was subsequently washed with 0.1 M phosphate, pH 8.2 until the OD_{280} of the effluent was less than 0.05 as measured by spectrophotometry (DU-65 Beckman, Part No. 596791). Monoclonal antibody specifically bound to the Protein A was subsequently eluted with 0.1 M citrate buffer (refer to Section 2.9.1.(b) for preparation) at the appropriate pH (ie IgG_1 were eluted with pH 5.5; IgG_{2a} eluted with pH 4.5; IgG_{2b} and IgG_3 eluted with pH 3.5) and the eluate was collected in 1-2 ml fractions and immediately adjusted to pH 7.0 with the appropriate volume of Tris-HCl pH 8.6 (ie. to change pH 5.5 to pH 7, one volume Tris-HCl was added to 10 volumes eluate; from pH 4.5 to pH 7, 1 volume Tris:4 volumes eluate; from pH 3.5 to pH 7.0, 1 volume Tris:1 volume eluate). Fractions with an OD_{280} of greater than 0.2 were pooled and the purity of the eluate was

checked using the spectrophotometer to scan OD_{220} - OD_{320}. The protein concentration (mg/ml) was determined according to the formula OD_{280} x 0.74 mg/ml. The purified antibody was then dialysed thrice against 500 ml PBS, filter sterilised (as above), aliquoted into appropriate volumes and stored at -80°C until required. Antibody preparations too dilute for further *in vitro* studies, were concentrated using a Centrion gravity concentrator (Amicon, USA, Cat. No. 4304) according to the manufacturers recommendation. Following antibody purification, the column was stripped with 0.5 M propionic acid (refer to Section 2.9.1.(c) for preparation), washed with 0.1 M phosphate, pH 8.2 until neutralised, and finally equilibrated with 0.1 M phosphate buffer supplemented with 0.1% (w/v) sodium azide and stored in this form at 4°C until required.

2.10. IMMUNOLOGICAL STUDIES: IMMUNOFLUORESCENCE LABELLING FOR CELL PHENOTYPING AND CELL SORTING

2.10.1. General Considerations

Flow cytometric analysis was performed using a Coulter Epics-Profile II (Coulter Electronics Corp., Hialeah, Florida). Routinely, 10 to 50,000 events were collected as listmode data and analysed using the Epics-Elite software. Cell sorting was performed on a FACStar^{PLUS} (Becton Dickinson, Sunnyvale, CA) fitted with a 250 mW argon laser that emitted light at a wavelength of 488 nm. Both apparatus were able to simultaneously detect three different fluorochromes including, Fluorescein isothiocyanate (FITC), Phycoerythrin (PE), and energy coupled dye (ECD; a tandem conjugate of PE and Texas Red) detected by 530 nm, 575 nm and 630 nm band pass filters, respectively. Compensation of the three fluorochromes was adjusted using the three directly conjugated mAbs CD4-FITC, CD8-PE and CD3-ECD (all purchased from Coulter, USA).

In addition to assessment of fluorescence, populations of cells were analysed by their light scattering properties. Light scattered in the forward direction, less than 10° from the excitation path (forward scatter), measures the size of the cell, whilst light scattered 90° from the excitation pathway (perpendicular or side light scatter), measures the internal structures such as granules within the cell. Using these two parameters,

different cell populations of lymphocytes, monocytes and granulocytes are discernible (Visser *et al*, 1984). Primitive haemopoietic cells and their immediate progenitors are recognised to lie within the area of lymphocytes and blast cells (Sutherland *et al*, 1989a; 1989b) and as such represented the region selected when progenitor cells were sought.

A list of the antibodies, and a range of target cells including human mononuclear cells derived from bone marrow (BMMNC) and peripheral blood (PBMNC), human bone marrow stromal cells (HBMSC) and a variety of cell lines used in this study are detailed in Tables 2.10.1. and 2.10.2. and Section 2.4, respectively.

2.10.2. Indirect Immunofluorescence Assay

Target cells were thawed (refer to Section 2.6.2.), harvested by trypsin detachment (refer to Section 2.4.3.) or harvested by incubating the cell monolayers at 37°C with 5 mls of 2 mM EDTA in PBS for 10 minutes where possible. The cells were subsequently resuspended in ice-cold IF buffer (Section 2.2.11.), and pelleted by centrifugation at 400 x g in a chilled (4°C) centrifuge. The supernatant was removed by vacuum aspiration and the cells washed twice to remove residual DMSO, trypsin or EDTA accordingly. The cells were resuspended at a concentration of 1×10^7 cells per ml in ice cold Blocking Buffer (Section 2.2.12.) and incubated on ice for 30 minutes in order to block potential Fc receptor mediated binding of the labelling mAb. 50 µl aliquots of this suspension were dispensed into 5 ml polypropylene tubes (Falcon, Becton Dickinson Labware, Bedford, MA, USA, Cat. No. 2058), to which 50 µls of mAb supernatants, appropriately diluted ascites or isotype-matched, non-binding control mAbs were added, mixed by vortexing and incubated on ice for 60 minutes.

When cell viabilities were less than 80%, 10 µl of ethidium monoazide (EMA, Molecular Probes Inc., Portland, Oregon, USA, Cat. No. E-1374) was added, as described by Cantrell and Yielding (1980). Cells treated with EMA were incubated on ice in the absence of light for 30 minutes and then under a fluorescent light box for a further 30 minutes. The cells were then washed 3 times in IF Buffer and the pellets resuspended in either 50 µl of a 1/30 dilution of FITC-labelled $F(ab')_2$ sheep anti-mouse Ig (Silenus, Australia, Cat. No. DDAF) or a 1/20 dilution of PE-conjugated, IgGl-specific $F(ab')_2$

Table 2.10.1. List Of The "Clustered" Monoclonal Antibodies Used In This Study.

Antibody Name	Cluster Designation	CSM Recognition / Cellular Distribution	MWt Of CSM (kD)#	Isotype	Reference/Source
T11	CD2	Leukocyte Functional Antigen (LFA)-2 / T cells, NK cells	45 - 58	IgG_1	Coulter
Leu4	CD3	T Cell Receptor Complex / Thymocytes, PB T cells	45 - 60	IgG_1	Becton/Dickinson
T4	CD4	T Helper / Thymocytes, 2/3 PB T cells	55	IgG_1	Coulter
3A1	CD7	gp40 / Pluripotent T cell precursors	40	IgG_{2B}	Coulter
T8	CD8	T Cytotoxic / Thymocytes/ 1/3 PB T cells	α subunit : 32-34 β subunit : 32-34	IgG_1	Coulter
J5	CD10	Common Acute Lymphoblastic Leukaemia Antigen (CALLA) / B & T lymphoid precursors	100	IgG_{2A}	Coulter
MY7	CD13	Aminopeptidase N / Myelomonocytic lineages	150 - 170	IgG_1	Coulter
LeuM3	CD14	gp55 / Myelomonocytic lineages	monocytes : 53-55 B cells : 64	IgG_{2B}	Becton/Dickinson
LeuM1	CD15	Le^x Antigen (Lacto-N-fucopentaeose III or 3-fucosyl-N-acetyl-lactosamine) / Granulocytes	NA	IgM	Becton/Dickinson
1G10	CD15	Le^x Antigen (Lacto-N-fucopentaeose III or 3-fucosyl-N-acetyl-lactosamine) / Granulocytes	NA	IgG_1	Dr. R. Andrews
WEMG11	CD15	Le^x Antigen (Lacto-N-fucopentaeose III or 3-fucosyl-N-acetyl-lactosamine) / Granulocytes	NA	IgM	Dr. A. Lopez
Leu12	CD19	gp95 / B cell and B cell precursors (expressed prior to CALLA)	95	IgG_1	Becton/Dickinson
B1	CD20	Bp35, B cell specific	33 - 37	IgG_1	Becton/Dickinson
P4C10	CD29	Integrin β_1 subunit / Numerous leucocyte cell types	130	IgG_1	Dr. W. Carter
P5D2	CD29	Integrin β_1 subunit / Numerous leucocyte cell types	130	IgG_1	Dr. E. Wayner
8A2	CD29	Integrin β_1 subunit (mAb shown to activate β_1 integrin function) / Numerous leucocyte cell types	130	IgG_1	Drs. J. Harlan and N. Kovach
61.2C4	CD29	Integrin β_1 subunit / Numerous leucocyte cell types	130	IgG_1	Dr. J. Gamble
5A2.G5	CD31	Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1)/ monocytes, platelets, granulocytes and 50% PBL	130 - 140	IgG_1	Dr. L. Ashman

B2B1	CD31	Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1)/ monocytes, platelets, granulocytes and 50% PBL	130 - 140	IgG_1	Dr. L. Ashman
LeuM9	CD33	gp67 / granulocyte and macrophage precursors- absent on pluripotent stem cells	67	IgG_1	Becton/Dickinson
8G12	CD34	Sialomucin CD34 / immature haemopoietic cells and Endothelial cells	105 - 120	IgG_1	Becton/Dickinson
HPCA-2-PE	CD34	Sialomucin CD34 / immature haemopoietic cells and Endothelial cells	105 - 120	IgG_1	Becton/Dickinson
HPCA-2-FITC	CD34	Sialomucin CD34 / immature haemopoietic cells and Endothelial cells	105 - 120	IgG_1	Becton/Dickinson
12.8	CD34	Sialomucin CD34 / immature haemopoietic cells and Endothelial cells	105 - 120	IgM	Dr. R. Andrews
ICH3	CD34	Sialomucin CD34 / immature haemopoietic cells and Endothelial cells	105 - 120	IgG_{2A}	Becton/Dickinson
Leu17	CD38	gp45 / B and T cell lineages	45	IgG_1	Becton/Dickinson
Plt-1	CD41	gpIIb, IIIa / Platelets and Megakaryocytes	post-translationally cleaved into a 125 and 22 kD subunits	IgM	Coulter
H9H11	CD44	Hyaluronate receptor (Pgp-1) / Most leucocytes	haem. form: 80-95 epithelial form: 130	IgG_1	Dr. P. Simmons
PIG12	CD44	Hyaluronate receptor (Pgp-1) / Most leucocytes	haem. form: 80-95 epithelial form: 130	IgG_1	Telios
KC56	CD45	Leukocyte Common Antigen (L-CA) / on all cells of haemopoietic origin	180 - 240	IgG_1	Coulter
2H4	CD45RA	Leukocyte Common Antigen (L-CA) / B cells, monocytes, $CD4^+$ T cells	180 - 240	IgG_1	Coulter
TS2/7	CDw49a	VLA-α1 subunit	150	IgG_1	Telios
P1E6	CDw49b	VLA-α2 subunit / Platelets, thymocytes	165	IgG_1	Telios
AC11	CDw49b	VLA-α2 subunit / Platelets, thymocytes	165	IgG_1	Telios
P1B5	CDw49c	VLA-α3 subunit / Epithelial cells	150	IgG_1	Telios
P4G9	CDw49d	VLA-α4 subunit / Mononuclear cells	150	IgG_1	Telios
P1D6	CDw49e	VLA-α5 subunit / Platelets, early erythroid	post-translationally cleaved into a 120 and 25 kD subunits	IgG_1	Telios

PHM2	CDw49e	VLA-α5 subunit / Platelets, Early erythroid	post-translationally cleaved into a 120 and 25 kD subunits	IgG_1	Prof. R.A. Aitkins
P3G8	CD51	α_V integrin subunit	post-translationally cleaved into a 125 and 24 kD subunits	IgG_1	Dr. J. Gamble
MEM-43	CD59	Membrane Inhibitor Of Reactive Lysis (MIRL) / Ubiquitous expression on haemopoietic and non-haemopoietic cells	18 - 20	IgG_{2A}	Serotec
2/24	CD59	Membrane Inhibitor Of Reactive Lysis (MIRL) / Ubiquitous expression on haemopoietic and non-haemopoietic cells	18 - 20	IgG_{2B}	Dr A. Fletcher
Leu61	CD61	Integrin β_3 subunit (gpIIIa) / Platelets, Megakaryocyte, Macrophages and non-haemopoietic cells	105	IgG_1	Becton/Dickinson
GMP-140	CD62P	P-selectin (GMP-140, PAGEM) / Megakaryocytes, activated platelets and endothelial cells	140	IgG_1	Dr. M. Berndt
Dreg-56	CD62L	L-selectin (Leukocyte homing receptor) / T and B Lymphocyte subsets, Monocytes and Neutrophils and NK cells	lymphocytes: 75 neutrophils: 95-105 monocytes: 110	IgG_1	Dr. J. Gamble
Leu8	CD62L	L-selectin (Leukocyte homing receptor) / T and B Lymphocyte subsets, Monocytes and Neutrophils and NK cells	lymphocytes: 75 neutrophils: 95-105 monocytes: 110	IgG_1	Becton/Dickinson
1H11	CD62E	E-selectin (ELAM-1) / Activated endothelial cells	110-115 kD	IgG_1	Dr. J. Gamble
5B11	CD62E	E-selectin (ELAM-1) / Activated endothelial cells	110-115 kD	IgG_1	Dr. J. Gamble
T9	CD71	Transferrin Receptor / upregulated on activated leukocytes and proliferating cells	90 - 95	IgG_1	DAKO
5E10	CDw90	Thy-1 / small subpopulations of $CD34^+$ cells, lymphocytes and myeloid cells. Nervous system, connective tissue and various stromal cell lines	18	IgG_1	Pharmingen
1G2	CD105	Endoglin / Endothelium, subset of BM cells, activated macrophages; not on normal B and T cells	95	IgG_3	Dr. H-J. Bühring
YB5.B8	CD117	c-KIT proto-oncogene (SCF receptor) / on all haemopoietic progenitors, but not B-lineage progenitors; on mast cells, melanocytes, spermatogonia and oocytes	145	IgG_1	Dr. L. Ashman
HLA-DR	NA	MHC Class II Antigen (heterodimer of α and β subunits) / B cells, monocytes, macrophages, myeloid & erythroid precursors	α chain : 33 - 35 ß chain : 28 - 30	IgG_{2A}	Coulter
9B3	NA	MHC Class I α subunit (non-covalently associated with β_2 microglobulin / Expressed on most nucleated cells	44	IgG_1	Mr. S. Gronthos

8B4	NA	MHC Class I α subunit (non-covalently associated with β_2 microglobulin / Expressed on most nucleated cells	44	IgM	Dr. L. Ashman
1B4.B12	NA	β_2 microglobulin (associated with MHC Class I α subunit) / Expressed on most nucleated cells	12	IgG_1	Dr. L. Ashman
AA6.C6	NA	Glycophorin A	NA	IgG_3	Dr. L. Ashman
STRO-1	NA	NA	NA	IgM	Dr. P. Simmons
CSLEX-1	CD15s	lacto-N-fucopentaose III (SLe^X) / Expressed on granulocytes, monocytes and both normal (non-myeloid) and tumour cells	NA	IgM	Dr. E. Butcher
HECA-452	CD15s	cutaneous lymphocyte-associated antigen, CLA / skin-associated memory T cells and myeloid cells	NA	rat IgM	Dr. E. Butcher
1B5	NA	Isotype-matched negative control / α-Giardia	NA	IgG_1	Dr. G. Mayrhofer
1A6.11	NA	Isotype-matched negative control / α-Salmonella	NA	IgG_{2B}	Dr. L. Ashman
1A6.12	NA	Isotype-matched negative control / α-Salmonella	NA	IgM	Dr. L. Ashman
1D4.5	NA	Isotype-matched negative control / α-Salmonella	NA	IgG_{2A}	Dr. L. Ashman
HB75	-	Murine MHC Class I ($H\text{-}2^d$ haplotype)	45	IgG_{2A}	Mr E. Bertram

Molecuar Weight ascertained following reduced SDS-PAGE.
• All mAbs are mouse monoclonal antibodies, unless otherwise indicated.
Unless noted otherwise, all antibodies in this study were used as affinity-purified immunoglobulins.
Isotype-matched FITC or PE-conjugated non-binding control antibodies (all from Dako).

Dr. P. J. Simmons, Mr. S. Gronthos, Dr. L. Ashman, Dr. J. Gamble, Dr. A. Lopez, Hanson Centre for Cancer Research, Adelaide, Australia.
Dr. E. Wayner, University of Minnesota Medical School, St. Paul, MN, USA.
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Dr. H-J. Bühring, Eberhard-Karls-Universtat Tubingen, Federal Republic of Germany.
Coulter Corp., Hialeah, FL, USA; Becton Dickinson, Mountain View, CA, USA; Telios Pharmaceuticals Inc. San Diego, CA, USA; DAKO, Dakopatts, A/S, Glostrup, Denmark; American Type Culture Collection, ATCC, Rockville, MD, USA; Pharmingen, San Diego, CA, USA.

Table 2.10.2. List Of The "Unclustered" Monoclonal Antibodies Used In This Study.

Antibody Name	Isotype	MWt Of CSM	Reference/ Source
7H9	IgG_1	45-50	A. Zannettino
11D1.H10	IgG_1	75-80	Dr. L. Ashman
11D3	IgG_1	19	A. Zannettino
12F12	IgG_1	44	A. Zannettino
HCC1	IgM	NA	Ms. B. Swart & Dr. P.J. Simmons
9E10	IgG_3	75-80	A. Zannettino
105.A5	IgM	NA	Dr. H-J. Bühring
CC9	IgG_{2A}	ND	S. Gronthos & A. Zannettino
WM85	IgG_1	100-110	Dr. R. Filshie
CA12	IgG_1	ND	S. Gronthos & A. Zannettino

Molecuar Weight ascertained following reduced SDS-PAGE.
• All mAbs are mouse monoclonal antibodies, unless otherwise indicated.

- ND: Not done.
- NA: No MWt possible.

- Dr. L. K. Ashman, Leukaemic Haemopoiesis Laboratory, Hanson Centre for Cancer Research, Adelaide, Australia.
- Ms. B. Swart, Dr. P. J. Simmons and Mr. S. Gronthos, Matthew Roberts Laboratory, Hanson Centre for Cancer Research, IMVS, Adelaide, Australia.
- Dr R. Filshie, Division of Haematology, Institute of Clinical Pathology and Medical Research, Westmead Hospital, Westmead, NSW, Australia.
- Dr. H-J. Bühring, Eberhard-Karls-Universtat Tubingen, Federal Republic of Germany.

goat anti-mouse Ig (gamma-chain specific, Caltag, Cat. No. M32004, USA) in IF Buffer and incubated at 4°C for a further 45 to 60 minutes. The cells were then washed 3 times as above, and fixed in 300-500 µl of cold 1% paraformaldehyde (in PBS) or FACS Fix (Section 2.2.13). The samples were stored at 4°C or analysed immediately using a Epic's Profile II (Coulter Electronics, USA) with 10-50,000 events collected as listmode data and subsequently analysed using the Epics-Elite software.

2.10.3. Multiple Colour Immunophenotypic Analysis

(a) Phenotypic Analysis Of P-selectin Adherent Population : The phenotype of BMMNC demonstrating specific adhesion to P-selectin together with those BMMNC which failed to bind was assessed by immunofluorescence staining and flow cytometry. The following mAb conjugates were used: Leu4-PE (CD3), LeuM3-PE (CD14), LeuM1-FITC (CD15), Leu12-PE (CD19), HPCA-2-PE (CD34; all from Becton Dickinson, Mountains, View, CA); 3A1-PE (CD7), J5-PE (CD10; Coulter Corp., USA); CD20-PE, glycophorin A-FITC and isotype-matched FITC or PE-conjugated non-binding control antibodies (all from DAKO Corp. Carpinteria, CA, USA). Staining was performed according to manufacturer's recommendations for 45 minutes at 4°C after which cells were washed twice in cold IF buffer and fixed in PBS/1% (w/v) paraformaldehyde. Flow cytometric analysis was performed using a Profile II flow cytometer and 20,000 events were collected per sample as list mode data and analysed using Coulter ELITE software. The cell surface phenotype of $CD34^+$ cells recovered in the P-selectin adherent and non-adherent fractions was assessed in a similar manner. The cells were stained according to manufacturer's recommendations with antibody HPCA2-FITC (CD34) in combination with either LeuM9-PE (CD33), Leu17-PE (CD38), HLA-DR-PE (all from Becton Dickinson), a mixture of J5-PE and Leu12-PE or with appropriate combinations of isotype-matched FITC or PE-conjugated non-binding control antibodies (DAKO). A total of 50,000 events were collected as list mode data for each sample, which was subsequently analysed as above.

(b) P-selectin Ligand Carbohydrate Antigen Expression : Multiple colour immuno-phenotypic analysis was also performed to examine the expression by $CD34^+$ cells of

carbohydrate antigens which have been shown in previous studies to function as P-selectin ligands. BMMNC were stained with HPCA-2-PE in combination with either mAb CSLEX-1 (in the form of tissue culture supernatant) which recognises sialyl Le^x (Fukushima *et al*, 1984) or with an IgM control antibody, 1A6.12 (refer to Table 2.10.1). After a 45 minute incubation at 4°C, cells were washed twice with IF buffer and incubated with a 1:50 dilution of goat anti-IgM-FITC (μ-chain specific: Southern Biotechnology Assoc. Birmingham, AL, USA) for a further 45 minutes. The cells were then washed, fixed and analysed as previously described. To examine the expression of sialyl Le^a antigen on $CD34^+$ cells, BMMNC were incubated simultaneously with the anti-CD34 antibody HPCA-2-PE and either HECA-452 ([rat IgM] Picker *et al*, 1990) or an isotype-matched, non-binding control. After washing, specifically bound antibody was revealed by incubation with a 1:50 dilution of sheep anti-rat-FITC (Silenus) to detect HECA-452 antibody.

Three-colour staining with CD34 antibody HPCA-1 (My10; Becton Dickinson, USA) and HECA-452 in combination with each of the following: LeuM9-PE, Leu17-PE, Leu12-PE or HLA-DR-PE was performed in the following sequence. After sequential incubation with HPCA-1, anti-mouse IgG-biotin (Southern Biotechnology Assoc., USA) and streptavidin-ECD (Coulter) excess sites on the anti-mouse IgG were blocked by 30 minute incubation with a combination of control IgG_1; IgG_{2a} and IgG_{2b} ascites at a 1:100 dilution. This was followed by incubation with HECA-452 together with PE-conjugated antibody or PE-conjugated IgG-control, washing, and a final incubation with sheep anti-rat FITC. A total of 1×10^4 events were collected as list mode data. Immunofluorescence staining with each of the carbohydrate antigen-specific antibodies listed above was also performed before and after neuraminidase treatment of BMMNC.

Three colour staining of BMMNC with CD34 antibody HPCA-2-PE (Becton Dickinson), HECA-452 and CSLEX-1 was performed as follows. After a 45 minute incubation with HPCA-2-PE and HECA-452 at 4°C, the cells were washed twice with IF buffer, and incubated with a 1:50 dilution of sheep anti-rat FITC. Cells were then resuspended in 10% normal rat serum and incubated for 45 minutes at 4°C to block free sites on the sheep anti-rat FITC reagent. The addition of CSLEX-1 or 1A6.12 control

antibody was followed sequentially by monoclonal rat anti-mouse IgM biotin and streptavidin-APC (1:50 dilution; Becton Dickinson). A total of 10^4 $CD34^+$ events were collected as list mode data.

(c) Co-Expression Of Differentiation And Activation Antigens By $CD34^+$$MGC\text{-}24v^+$ Cells: Multiple colour immunophenotypic analysis was performed to examine the expression of activation/differentiation antigens by $CD34^+MGC\text{-}24v^+$ cells. BM-derived 561-Dynabead purified $CD34^+$ cells (refer to Section 2.10.5. (b)) were incubated simultaneously with 50 µl of 10 µg/ml biotinylated 9E10 mAb in combination with FITC-conjugated mAbs to CD33, CD38, CD71, CD117 and HLA-DR. After washing, specifically bound biotinylated 9E10 was revealed by incubation with a 1:50 dilution of streptavidin (SAV)-PE (Coulter) as recommended by the manufacturer. Cells were washed, fixed and analysed as above.

(d) Co-Expression Of Differentiation And Activation Antigens By $CD34^+CD62L^+$ Cells: To examine the expression of activation and differentiation antigens by $CD34^+$ cells which co-expressed L-selectin (CD62L), 3 colour immunophenotypic analysis was performed. BMMNCs were immunolabelled with the CD34 mAb HPCA-1 (My10; Becton Dickinson), and Leu8-PE (anti-L-selectin), in combination with each of the following; Leu-4-FITC (CD3), LeuM3-FITC (CD14), LeuM1-FITC (CD15), Leu12-FITC (CD19), all from Becton Dickinson; 3A1-FITC (CD7), J5-FITC (CD10), MY7-FITC (CD13) and HLA-DR-FITC, or isotype-matched, non-binding, FITC-conjugated control antibodies; all from DAKO. Immunolabelling was performed in the following sequence. After sequential incubation with HPCA-1, anti-mouse-IgG-biotin (Southern Biotechnology Assoc., USA) and SAV-ECD (Coulter), excess sites on the anti-mouse IgG were blocked by 30 minute incubation with a combination of control IgG1; IgG2a and IgG2b ascites at a 1:100 dilution. This was followed by a 30 minute incubation with the directly conjugated FITC and PE mAbs. The cells were subsequently washed and fixed as above. A total of 5×10^4 events were collected as list mode data, and analysed as above.

(e) Detection Of PSGL-1 Protein Expression By BMMNC: 1×10^6 BMMNC were incubated with 50 µl (20 µg/ml) of either an affinity purified rabbit polyclonal antibody against the N-terminus of PSGL-1 or an irrelevant rabbit polyclonal (both kind gifts from

Dr. MC. Berndt, Baker Medical Research Institute, Prahran, Victoria, Australia). After washing, the cells were incubated with a combination of HPCA-2-FITC and biotinylated goat-anti-rabbit (GAR) antibody (Vector Laboratories Inc., Burlingame, CA, USA) as described above. After washing the cells thrice with IF buffer, the biotinylated GAR was detected with SAV-PE (Caltag). Cells were finally washed, fixed and analysed as described above.

2.10.4. Fluorescence Activated Cell Sorting (FACS)

Briefly, cells were resuspended in blocking buffer and incubated for 30 minutes at 4°C. Cells were pelleted in 5 ml polypropylene tubes and resuspended in 50-200 µl of sterile, azide-free primary antibody (or isotype matched control) for one hour at 4°C. Cells were subsequently washed thrice in sterile IF Buffer (without azide), prior to the addition and incubation of the secondary antibody as described above prior to sorting using the FACStar^{PLUS} cell sorter. Antibody binding was measured relative to isotype-matched, irrelevant control antibodies as described above.

2.10.5. Isolation Of Normal Bone Marrow CD34$^+$ Progenitor Cells.

(a) Bone Marrow CD34$^+$ Progenitor Cells: Isolation By FACS

BMMNC obtained as described in Section 2.4.6., were incubated in Blocking Buffer for 30 minutes on ice as described above. Labelling was performed with a variety of directly conjugated anti-CD34 mAbs, including 8G12, ICH3, HPCA-2-PE, HPCA-2-FITC, (all from Becton Dickinson, Mountain View, CA, USA) and 12.8 (a kind gift from Dr. R. Andrews), as recommended by the respective manufacturers. Cell sorting was performed using a FACStar^{PLUS} cell sorter and the threshold for selection of CD34$^+$ cells was based on the level of staining obtained with an isotype-matched control. CD34$^+$ cells within the lymphocyte/blast region (Andrews *et al*, 1989) were sorted into Iscove's modification of DMEM (IMDM) supplemented with 50 Kunitz units/ml DNase I (Sigma, Cat. No. D5025), and 20% FBS. A portion of the sorted cells was analysed immediately to assess purity and viability and was routinely >98%.

(b) Bone Marrow $CD34^+$ Progenitor Cells For Use In Adhesion Assays: Isolation By 561-Dynabeads

$CD34^+$ cells devoid of surface mAb were prepared by magnetic Dynabead-enrichment protocol for use in adhesion assays (refer to Section 2.12.). BMMNC obtained as described in Section 2.4.6., were incubated for 40 minutes at 4°C with a mixture of hybridoma supernatants containing mAbs specific for mature haemopoietic cells (CD2, CD3, CD4, CD5, CD8, CD11b, CD14, and CD19; refer to Table 2.10.1.) and subsequently washed twice with ice-cold IF buffer. Sheep anti-mouse IgG-conjugated magnetic M450 Dynabeads (Dynal, Oslo, Norway) were then added at a 1:1 ratio (beads:cells) and this suspension was incubated at 4°C on a rotary mixer for 60 minutes. Mature cells binding to beads were removed by using the MPC-1 magnetic particle concentrator (Dynal) following the manufactures recommended protocol. Nonadsorbed cells, containing 4 to 10% $CD34^+$ cells, were subsequently incubated for 60 minutes at 4°C with directly conjugated anti-CD34 (561) Dynabeads. $CD34^+$ cells that attached to beads, were captured with the MCP-1 as described above. These cells were subsequently recovered by incubating the cell-bead complexes in the presence of the DETACHaBEAD™ reagent (Dynal), a polyclonal Ab specific for the Fab domain of the anti-CD34 mAb that competitively displaces cells from the beads (Smeland *et al*, 1992). Released cells were eluted into IF buffer, and a portion of the sorted cells were immediately analysed to assess purity and viability, which was routinely between 90 and 95%. Prior to use in adhesion assays, the $CD34^+$ cells were washed twice in IMDM and resuspended at 2×10^5 cells/ml and starved overnight at 37°C in serum-deprived medium (SDM, refer to Section 2.2.9. (b)).

2.10.6. Immunocytoadherence or "Panning"

This procedure represents a modification of a procedure reported previously (Wysoki and Sato, 1978). 25 cm^2 tissue culture flasks were coated with 4 ml of affinity purified goat anti-mouse immunoglobulin (GAM Ig; diluted to 125 µg/ml in PBS, Caltag, USA) and incubated at 4°C for a minimum of 24 hours. Prior to use, the GAM Ig was decanted from the flask and the coated-surface washed 8 to 10 times with PBS, to ensure

complete removal of unbound GAM Ig. The flasks were subsequently blocked for 1 hour at room temperature with Blocking Buffer. During this time, the target cells were harvested, washed 3 times in IF Buffer and resuspended at 2 x 10^7 cells/ml. Aliquots (500 µl) of this cell suspension were dispensed into 10 ml polystyrene tubes and pelleted by centrifugation at 400 x g for 5 minutes at 4°C. To the cell pellets 100 µl of blocking buffer was added and incubated at 4°C for 10 minutes prior to the addition of 100 µl of hybridoma supernatant(s) (a cocktail or singular mAb), or appropriately diluted ascites. The cells were incubated on ice for 60 minutes with intermittent mixing. Following the incubation, the cells were washed twice with the IF Buffer to remove any unbound mAb.

5 x 10^6 cells were added to the flask in a volume of 4 ml. The cells were incubated on the panning surface (on a level table at 4°C) for 90 minutes, and at the midpoint of the incubation the contents were gently swirled to achieve even dispersion of the cells. Following incubation, the non-adherent cells were aspirated with a Pasteur pipette and non-specifically bound cells were removed by washing the panning surface with IF buffer a minimum of 5 times. Strongly immunocytoadherent cells were cultured *in situ*, by adding the appropriate medium and incubating at 37°C, in an atmosphere containing 5% CO_2 (to maintain pH 7.0), and a relative humidity of 97%.

2.10.7. Indirect Immunofluorescent Staining Of Frozen Tissue Sections

Sections of 5 µm were cut from frozen specimens using a 1720 Digital Kryostat (Wild Leitz; Germany) and placed onto glass slides. The slides were then fixed in acetone for 15 minutes at -20°C, washed twice in PBS and then blocked in 5% goat serum (diluted in PBS) for 20 minutes at room temperature. The goat serum was then replaced with optimal concentrations of antibodies directed against human CSM (mouse or rabbit) for one hour at room temperature. Isotype matched, non-binding control mAbs were treated under the same conditions. After washing in PBS the sections were blocked in goat serum for an additional 20 minutes. The secondary antibodies: goat antimouse-µ–specific-FITC (1/30 dilution) plus goat antimouse–γ–specific-Texas Red (TxRd) (1/100 dilution) or goat antimouse–µ–specific-FITC plus goat anti rabbit-biotinylated antibody (1/200) (Vector Laboratories Inc., Burlingame, CA, USA) were diluted in goat serum at optimal

concentrations and incubated with the sections for 30 minutes at room temperature. The slides were then washed three times in PBS. If an anti-rabbit biotinylated second label was used, the slides were then blocked in 1% BSA for 15 minutes after which streptavidin-TxRd (1/200 dilution) (Fisher Scientific, Orangeburg, NY) was added to the slides for a further 30 minutes. After washing in PBS the slides were mounted using 'Gurr' aqueous mountant solution (Uvinert; BDH Ltd., Poole, UK) and analysed with an Olympus BH2-RFCA fluorescent microscope (Olympus Optical Co. Ltd., Tokyo, Japan). Photographs were taken on Ektochrome 400 Kodak colour film.

2.10.8. *In Situ* Staining Of Adherent Cell Populations Using Indirect Immuno-fluorescence

Adherent cell populations were detached with 0.1% trypsin and then re-plated (1 x 10^4 cells/well) into 8 chamber slide culture vessels (Lab-Tek, Nunc Inc.; Naperville, IL) with fresh medium and cultured for one week at 37°C in 5% CO_2. The cells were washed twice with cold RPMI and blocked with 1% BSA in RPMI for 20 minutes at 4°C. Saturating levels of each primary antibody (mouse anti-human IgG monoclonal antibody and the isotype IgG negative control) were added (200 µl/chamber) for 45 minutes at 4°C. After washing three times with cold RPMI the cells were blocked in 1% BSA for a further 20 minutes. The secondary antibody (goat antimouse-γ-specific-TxRd or goat antimouse-γ-specific-FITC) was diluted in cold 1% BSA and added to each chamber for 30 minutes at 4°C. The cells were then washed three times in RPMI and fixed with 2% paraformaldehyde in PBS for 15 minutes at 4°C. After two more washes with PBS, the chambers were detached from rubber gasket and the slides mounted in "Gurr" aqueous mountant (Uvinert; BDH Ltd., UK) and analysed using an Olympus BH2-RFCA fluorescent microscope. Photographs were taken on Ektochrome 400 Kodak colour film and with TMAX 400 Kodak black and white film.

2.11. MISCELLANEOUS PROCEDURES

2.11.1. Soybean Agglutinin (SBA) Enrichment Of BM-Derived Primitive Haemopoietic Progenitor Cells

BMMNC preparations were enriched in immature haemopoietic progenitor cells by differential agglutination using the lectin, Soybean Agglutinin (SBA; Vector Laboratories, Burlingame, CA, USA) as previously described by Reisner *et al* (1980). Non-agglutinated (SBA^-) cells were separated from agglutinated cells by unit gravity separation over 5%BSA, washed in PBS (supplemented with 200 mM D-galactose [Sigma]), to remove residual lectin and then with IF buffer, prior to immunofluorescence staining. The SBA enrichment procedure using SBA routinely resulted in a five to 10-fold enrichment of directly clonogenic cells in the SBA^- fraction with recoveries consistently > 80%.

2.11.2. Rhodamine 123 (Rh123) Staining To Isolate Primitive Haemopoietic Progenitor Cells

Rh123 (Molecular Probes Inc, USA) were stored at 1 mg/ml in PBS at -80°C with a working solution used at 0.1 μg/ml in Hanks Balanced Salt Solution supplemented with 5% FCS (HBSS.5). BM cells were incubated at 10^7/ml for 45 minutes at 37°C, 5% CO_2 and mixed every 15 minutes. Cells were washed twice in HBSS supplemented with 5% FBS, and incubated for a further 15 minutes at 37°C to remove residual unbound Rh123. The cells were washed a further 2 times in HBSS/5% FBS and then stained with antibodies labelled with PE and ECD. The cell samples containing Rh123 were not fixed but analysed or sorted on the day of labelling.

2.11.3. Fluorescein isothiocyanate (FITC)-"Loading" Of Cell Lines

To assess the efficacy of the Dynabead enrichment procedure, FDC-P1 cells (refer to Section 2.4.2.), were fluorescently labelled with FITC. Routinely, 1 $x10^7$ cells were harvested, washed several times with IF buffer, and subsequently resuspended in 100 μl of 10% (w/v) solution of FITC (resuspended in 10% DMSO/serum-free RPMI). Following 30 minutes incubation at 37°C, the cells were washed thrice with IF buffer, and the

resultant uptake of FITC was assessed by either flow cytometry or fluorescence microscopy as described in Sections 2.10.2. and 2.10.8. respectively.

2.12. ADHESION ASSAYS

2.12.1. Source Of P-, E- and L-Selectin

Adhesion assays with BMMNC or $CD34^+$ cells were performed using either Chinese Hamster Ovary (CHO) cells transfected with a human P-, E-, and L-selectin cDNA, or using purified human platelet-derived P-selectin. P-selectin purified from human platelets was prepared as previously described (Skinner *et al*, 1989) and stored at -70°C in 0.02 M Tris buffer, pH 7.4, 0.15 M NaCl, 0.1% Triton X-100 and 0.001 M $CaCl_2$ (storage buffer). Alternatively, adhesion assays were performed with purified recombinant CD62L, CD62P-IgG_1Fc fusion proteins (a kind gift from Dr. L. Lasky, Genentech, San Francisco, USA). To immobilise these constructs, vessel surfaces were coated overnight with an optimised concentration (20 µg/ml) of a monoclonal mouse anti-human IgG_1Fc antibody (hybridoma obtained from ATCC). This served to orient these chimeric molecules such that the lectin domains were presented to opposing cells.

2.12.2. Adhesion Assays With P- And E-Selectin CHO Transfectants

To investigate the ability of $CD34^+$ cells to bind to both P- and E-selectin, $CD34^+$ cells isolated by FACS or 561-Dynabeads, were washed in assay medium (RPMI-1640, 2.5% FCS) and incubated at 10^6/ml in the presence of 50-100 µCi $Na_2{}^{51}CrO_4$ (sodium chromate, New England Nuclear, Boston MA, USA) for 30-60 minutes at 37°C and then washed 3 times in the same medium. Peripheral blood neutrophils were prepared by Ficoll separation of dextran sedimented peripheral blood as previously described (Harlan *et al*, 1985), and subsequently labelled with ^{51}Cr as described above. For the adhesion assays, P- and E-selectin CHO cells (and untransfected CHO cells) were plated in 96-well plates (Nunc) at 3 x 10^4 cells/well 24 hours prior to the assay. ^{51}Cr-labelled cells were added in duplicate at 10^5 ($CD34^+$ cells) or in triplicate at 4 x 10^5/well (neutrophils) and incubated for 30 minutes at 37°C, washed twice to remove unbound cells and then lysed by the addition of 1% Triton X-100. Radioactivity associated with the lysates were quantitated by liquid scintillation counting. In order to demonstrate specificity of

adhesion, assays were performed following the incubation of P- and E-selectin CHO cells with Fab fragments (10 μg/ml) generated from an affinity purified preparation of rabbit anti-human P-selectin antibody (as described by Skinner *et al*, 1991) or function-blocking mAb 5B11 directed against human E-selectin. As a control, cells were incubated with an identical concentration of pre-immune Fab fragments or an isotype-matched, non-binding control mAb.

2.12.3. Adhesion Assays With Purified Platelet-Derived P-Selectin And Recombinant CD62P-IgG$_1$Fc Fusion Protein

CD34$^+$ cells and BMMNC were assayed for their ability to bind purified platelet-derived P-selectin immobilised on plastic. For experiments involving CD34$^+$ cells, 96 or 24 well plates (Nunc) were coated with purified P-selectin diluted in storage buffer to a final concentration of 20 μg/ml, for 4 hours at room temperature. Wells coated in parallel with the storage buffer alone served as a control. Unbound P-selectin was then removed and the wells washed copiously with assay buffer prior to commencing the assay. Alternatively, wells were pre-coated overnight at 4°C with a monoclonal mouse anti-human IgG$_1$Fc antibody (refer to Section 2.12.1.), after which the CD62P-IgG$_1$Fc fusion protein (20 μg/ml in PBS) was allowed to adhere, by incubation at 37°C for 30 minutes or at 4°C for 2 hours.

CD34$^+$ cells were labelled with ^{51}Cr as described above and diluted in assay medium to a final concentration of 5 x 10^4 cells/well and incubated for 30 minutes at 37°C. Wells were then washed twice and assayed for bound radioactivity following addition of Triton X-100 as described above. Alternatively, the assays were performed in 24 well plates by adding un-labelled CD34$^+$ cells (2 x 10^5 cells/ml) at 5 x 10^4/ml per well. Upon completion of the assays, 2% glutaraldehyde (Sigma, USA) in HBSS containing 2 mM Ca^{2+} was added to fix cells and adhesion was quantitated by counting the mean number of cells bound in five randomly selected 100 x fields in each of the 3 replicate wells. Specificity of adhesion to P-selectin was demonstrated by the addition of anti-P-selectin Fab fragments as described above. To assess the role of divalent cations, assays were performed in the presence of 5 mM EGTA or EDTA. Temperature

dependence of adhesion to P-selectin was investigated by performing assays at 4°C and 37°C. The susceptibility of adhesion to shear stress was assessed by performing the assays on an orbital shaker at 60 rpm. Assays involving the myeloid cell line HL60 were performed under identical conditions.

To investigate the adhesion of BMMNC to P-selectin, BMMNC were subjected to a single round of plastic adherence to remove monocytes for 2 hours at 37°C. The plastic non-adherent population was washed twice in assay medium and resuspended to 1 x 10^6/ml and transferred to 90 mm bacteriological grade petri dishes coated with P-selectin as described above. After 30 minutes at 37°C non-adherent cells were carefully removed, the plates washed thrice to remove residual non-bound cells and pooled with the first fraction (P-selectin non-adherent). BMMNC remaining attached to the petri dish (P-selectin adherent fraction) were removed by gentle pipetting in Ca^{2+}/Mg^{2+}-free HBSS supplemented with 0.02% EDTA at 37°C. Both the P-selectin adherent and non-adherent fractions were then washed twice in IF buffer at 4°C prior to immunophenotypic analysis or assay of haemopoietic progenitor cells as described below. An identical procedure was employed in assays performed using $CD34^+$ cells to facilitate phenotypic and functional analysis of P-selectin adherent and non-adherent subpopulations.

2.12.4. Adhesion Of $CD34^+$ Cells To Bone Marrow Stromal Cells

$CD34^+$ cells, prepared with 561-Dynabeads were washed twice and resuspended in 500 µl cell adhesion medium. 50-100 µCi of $Na_2{}^{51}CrO_4$ (New England Nuclear, USA) was added and cells incubated for 1 hour at 37°C. After radio-labelling, cells were washed three times using adhesion assay medium, resuspended to 2-4 x 10^5 cells/ml and chilled on ice for 10 minutes prior to assay. 100 µl of the labelled cell suspension was placed, in triplicate onto 96 wells seeded 24 hours prior with 1-2 x 10^5 human BM stromal cells. The entire procedure was carried out on ice. Plates were centrifuged at 1,000 rpm for 5 minutes at 4°C to sediment cells into direct, uniform contact with treated surfaces. Plates were quickly warmed for 2 minutes to 37°C using a heating block prior to transfer to a humidified (100%) incubator at 37°C for the indicated periods (usually 30-60 minutes). Assay medium was removed by aspiration and wells washed three times by

addition of 150 µl of the adhesion assay medium and flicking off. After the last wash, cell adhesion was examined using an inverted-phase contrast microscope before lysing in 150 µl 1% SDS, 1% NaOH solution. Lysates were counted after 10 minutes using a gamma counter. The percentage of adherent cells was determined by dividing the activity in the adherent fraction by the radioactivity contained in 100 µl of the initial labelled cell suspension.

2.13. ENZYMATIC MODIFICATION OF CSM PROTEINS

2.13. 1. Treatment Of CD34$^+$ Cells With Proteases And Neuraminidase

To investigate the nature of the ligand for P-selectin on CD34$^+$ cells, cell sorter-purified CD34$^+$ cells were washed in serum-free RPMI and then incubated at 10^6/ml for 1 hour at 37°C in the same medium containing the following proteases: bromelain, chymopapain, chymotrypsin, dispase, elastase, papain, pronase, proteinase K, thermolysin and trypsin at a final concentration of 20 µg/ml, with the exception of elastase (20 mg/ml), dispase (4 mg/ml) and proteinase K (1 µg/ml). All enzymes were obtained from Boehringer Mannheim (Mannheim, Germany) with the exception of papain (Sigma, USA), chymopapain (Boots, UK) and elastase (Calbiochem., USA). Following protease treatment, cells were washed twice in assay medium and assayed for their ability to bind to purified P-selectin immobilised on plastic as described above. To determine the role of sialic acid in the binding of CD34$^+$ cells to P-selectin, sorted CD34$^+$ cells or plastic-non-adherent BMMNC were incubated at 10^6/ml for 1 hour at 37°C in PBS/0.1% BSA alone or containing 0.1 units/ml neuraminidase (Arthrobacter ureafaciens; Calbiochem). Digestion was stopped by washing thrice in assay medium. HL60 cells were treated in an identical manner. After enzyme treatment, the cells were either subjected to immunophenotypic analysis as described below or assayed for binding to P-selectin as described above.

2.13.2. Treatment Of BMMNC With Phosphatidylinositol-Specific Phospholipase C (PI-PLC): Cleavage Of GPI-Linked CD59

Loss of antigen expression following treatment with the enzyme PI-PLC, is diagnostic of glycosyl-phosphatidylinositol (GPI)-linkage of membrane proteins. BMMNC, prepared as described in Section 2.4.6., were washed several times in IMDM supplemented with 2% FCS, and resuspended at a final concentration of 5 x 10^6 cells per ml. To this, 50 mU/ml of PI-PLC (Boehringer Mannheim, Germany, Cat No. 114506) was added, and the cells were incubated at 37°C for 2 hours (mock-treated cells were incubated in an equivalent manner in the absence of PI-PLC). Following this, the BMMNC were washed in ice-cold IF buffer in preparation for 2-colour immunophenotypic analysis (please refer to Section 2.10.2).

2.13.3. Treatment Of BMMNC With The Cobra Venom Metalloproteinase, Mocarhagin: Cleavage Of PSGL-1

The binding of ^{51}Cr-labelled $CD34^+$ cells to P-selectin (CHO, platelet and recombinant) was performed as described in Sections 2.12.2. and 2.12.3. To examine the effect of pretreatment of $CD34^+$ cells with mocarhagin on P-selectin binding, cells (0.5-1 x 10^6) were washed in RPMI supplemented with 1% FBS. This was followed by the addition of 10 µg/ml mocarhagin (or an equivalent volume of PBS in "mock"-treated samples), and subsequent incubation at 22°C for 30 minutes. Cells were centrifuged and washed twice with RPMI-1% FBS and their binding to P-selectin assessed as described above.

2.14. MISCELLANEOUS PROCEDURES

2.14.1. Statistics

Data points derived from multiple experiments are reported except where stated, as the mean ± 1 standard error of the mean (SEM). Significance levels were determined by two-sided nonpaired student's t-test analysis or Fridman 2-way analysis (Statview II for MAC, Abacus Concepts Inc., USA).

2.15. CHEMICALS AND REAGENTS

2.15.1. Chemicals

The following chemicals were purchased from Sigma Chemical Co., USA: acrylamide, bisacrylamide (N,N'-methylene-bis-acrylamide), adenosine tri-phosphate (ATP), agarose (Type 1), Ampicillin, bovine serum albumin (BSA), dithiothreitol (DTT), ethidium bromide, ethylenediaminetetra-acetic acid (EDTA), salmon sperm DNA, sodium dodecyl sulphate (SDS), spermidine, Tris base, phenylmethylsulphonylfluoride (PMSF), sodium succinate, N,N,N',N'- tetramethyl-ethenediamine (TEMED) and urea.

Sources of other important reagents were as follows: isopropyl-thiogalactoside (IPTG), 5-bromo-4-chloro-3-indolyl beta-D-galactopyranoside (BCIG); Progen, Darra, Queensland, Australia.; caesium chloride: Boehringer Mannheim, Mannheim, Germany.; ammonium persulphate: Ajax Chemicals, Australia.; chloroform, dimethylsulphoxide (DMSO), polyethylene glycol (PEG) 6000: BDH Chemicals, UK.; phenol: Wacko Pure Chemical Industries, Ltd.; deoxyribonucleotide triphosphates (dNTPs) were purchased from Pharmacia/LKB, Sweden.

All other chemicals and reagents were of analytical grade and maintained as stocks within the Division of Haematology.

2.15.2. Enzymes

All restriction endonucleases used during the course of this work were purchased from either Pharmacia/LKB, Sweden; New England Biolabs, USA; or Amersham, UK; whilst other modification enzymes were obtained from the following sources:

Calf intestinal phosphatase (CIP) and proteinase K, Boehringer Mannheim, Mannheim, Germany.; *E. coli* DNA polymerase I (Klenow fragment) and T4 polynucleotide kinase, Bresatec Ltd., Adelaide, Australia.; lysozyme and ribonuclease A (RNase A); Sigma-[The stock solution of RNase A (10mg/ml) was incubated at 100°C for 10 minutes to inactivate any DNase activity] and T4 DNA ligase, Promega Corporation, Madison, WI, USA., Amplitaq DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA).

2.15.3. Kits

Kits for polymerase chain reaction were obtained from Bresatec or Perkin-Elmer. Long range PCR (XL PCR) kits were a kind gift from Dr. N. Samaras, Perkin-Elmer, Victoria, Australia. Kits for oligo-labelling of DNA and the Super-Base Sequencing Reagent Kit (Bresatec, Cat. No. SBK-2) were purchased from Bresatec. Geneclean II™ and BRESAclean™ kits for the purification of restriction fragments and polymerase chain reaction products were by Bio101 Inc. La Jolla, CA, USA, and Bresatec, respectively. The PRISM™ Ready Reaction Dye Primer Cycle Sequencing Kit and the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit, were purchased from Applied Biosystem, Inc., USA. The QIAquick gel purification system and the QIAGEN-tip 100 Plasmid purification kit were purchased from QIAGEN Inc., Chatsworth, CA, USA. Altered Sites™ *in vitro* Mutagenesis System was purchased from Promega Corporation

2.15.4. Radiochemicals

[α-^{32}P] dATP (3000 Ci/mmol), [α-^{35}S] dATP (1000-1500 Ci/mmol), [α-^{32}P] dCTP (3000 Ci/mmol), and [α-^{32}P] were purchased from Bresatec, Adelaide, Australia.

2.15.5. Miscellaneous

The following items were purchased from the following suppliers:

GF/A glass fibre filter discs and 3MM chromatography paper: Whatman Int. LTD, Maidstone, England.

X-Omat AR diagnostic film: Scientific Imaging Systems, Eastman Kodak Company, , Newhaven, CT, USA.

Fuji RX X-ray film: Fuji Photo Film Co. Ltd, Tokyo, Japan.

Nitrocellulose (BA85) and Nytran 0.45 μm: Schleicher and Schuell Inc. Keene, NH, USA.

Hybond N$^+$ and C extra: Amersham, Poole, England.

Ilford autoradiography cassettes: Ilford, Knutsford, Cheshire, UK.

2.16. GENERAL DNA METHODS

2.16.1. General Considerations

The following methods were performed essentially as described in Sambrook *et al*, 1989: Growth, maintenance and preservation of bacteria; quantitation of DNA and RNA; autoradiography; agarose and polyacrylamide gel electrophoresis; DNA and RNA precipitations; phenol/chloroform extractions; end-filling DNA fragments using the Klenow fragment of *E.coli* DNA polymerase I.

2.16.2. Bacterial Growth Media

Growth media were prepared in double-distilled water and sterilised by autoclaving, antibiotics and other labile chemicals were added after the solution had cooled to 50°C.

(a) Luria-Bertani (LB) broth : 1% (w/v) Bacto-tryptone (Difco, USA, Cat. No. 0123-01), 0.5% (w/v) Bacto-yeast extract (Difco, Cat. No. 0127-01), 1% (w/v) NaCl, adjusted to pH 7.0 with NaOH.

(b) 2 x YT Broth : 1.6% (w/v) Bacto-tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, adjusted to pH 7.5 with NaOH.

(c) TYP Broth : 1.6% (w/v) Bacto-tryptone, 1.6% (w/v) yeast extract, 0.5% (w/v) NaCl, and 0.25% (w/v) K_2HPO_4, with no further adjustment.

(d) Tryptone Broth (TB) : 1% (w/v) Bacto-tryptone, 0.5% (w/v) NaCl.

(e) Super Broth (SB) : 3.2% (w/v) Bacto-tryptone, 2% (w/v) yeast extract, 0.5% (w/v) NaCl, adjusted to pH 7.5 with NaOH.

(f) SOC Medium : 2% (w/v) Bacto-tryptone, 5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl was dissolved in water, the pH adjusted 7.0 and sterilised by autoclaving. Filter sterilised solutions of $MgCl_2$, $MgSO_4$ and D-glucose were added to a final concentration of 10 mM, 10 mM and 20 mM respectively.

(g) Solid Media : Agar plates were prepared by supplementing the above media with 1.5% Bacto-agar (Difco, Cat. No. 0140-01).

In all situations, Ampicillin (50-100 µg/ml) or tetracycline (10 µg/ml) were added where appropriate for growth of transformed bacteria, to maintain selective pressure for

the retention of the recombinant plasmid. MC1061/P3 cells transfected with either pCDNA1 and pCDNA1*neo* were co-selected with 12.5 µg/ml Ampicillin and 7.5 µg/ml Tetracycline.

2.16.3. Buffers

(a) 1 x TNE : 100 mM NaCl, 1 mM EDTA, 10 mM Tris HCl pH 7.5

(b) 1 x SSC : 150 mM NaCl, 15 mM sodium citrate

(c) 1 x TAE : 40 mM Tris-acetate, 20 mM sodium acetate, 1 mM EDTA, pH 8.2

(d) 1 x TBE : 90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, pH 8.3

(e) 1 x TE : 10 mM Tris-HCl pH 7.5, 0.1 mM EDTA

(f) 10 x "One-Phor-All" Restriction Endonuclease Buffer : 100 mM Tris-acetate (pH 7.5), 100 mM magnesium acetate, and 500 mM potassium acetate.

(g) 10 x Annealing buffer : 200 mM Tris-HCl, pH 7.4, 20 mM $MgCl_2$ and 500 mM NaCl.

(h) 10 x Gel Loading buffer : 25% (w/v) Ficoll [type 400 - diluted in water], 0.25% xylene cyanol and 0.25% (w/v) bromo phenol blue.

(i) 10 x Synthesis buffer : 5 mM of each dNTP, 10 mM rATP, 100 mM Tris-HCl, pH 7.4, 50 mM $MgCl_2$ and 20 mM DTT.

(j) Denhardt's solution : 0.1%(w/v) Ficoll, 0.1%(w/v) polyvinylpyrrolidine, 0.1% (w/v) BSA

All buffers were sterilised by autoclaving or where necessary by filtration through a Sartorius™ Minisart NML 0.2µm filter.

2.16.4. Cloning And Expression Vectors

The following vectors were purchased from the indicated supplier or obtained gratis.

(a) pBluescript (Appendix A, Restriction Map #1) series of vectors (approximately 2,961 base pair) were purchased from Pharmacia.

(b) pCDNA1, a 4,188 base pair vector (Appendix A, Restriction Map #2) and pCDNA1.*neo*, a 6,970 base pair vector (containing a neomycin^R cassette between the

MCS and Polyoma ORI of pCDNA1) were purchased from Invitrogen Corporation, San Diego, CA, USA.

(c) pRUF.*neo* (Appendix A, Restriction Map #3) a 5,661 base pair vector was a kind gift from Drs. TJ. Gonda and JR. Rayner, Division of Human Immunology, Hanson Centre for Cancer Research, Adelaide, Australia.

(d) pRUF.(*Not*I)*neo* (Appendix A, Restriction Map #4) a 5,671 base pair vector, represents a modified version of pRUF.*neo*. A *Not*I restriction site was introduced between the *Xho*I and *Bam*HI restriction sites present in the MCS. pRUF.(*Not*I)*neo* was a kind gift from Dr PN. Baird, Matthew Roberts Laboratory, Leukaemia Research Unit, Hanson Centre for Cancer Research, Adelaide, Australia.

(e) pALTER™ a 5,680 base pair vector (Appendix A, Restriction Map #5) was purchased as a component of the Altered Sites™, *in vitro* Mutagenesis System, Promega Corporation, Madison, WI, USA.

(f) pGEM-T a 3,003 base pair vector (Appendix A, Restriction Map #6) was purchased from Promega Corporation, Madison, WI, USA.

2.16.5. Bacterial Strains

The following *E.coli* K12 strains were used as hosts for recombinant plasmids in various recombinant DNA procedures and their respective genotypes are detailed below:

(a) *E.coli* DH5α : *sup*E44, Δ*lac*U169, (p80 *lac*ZΔM15), *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*, *rel*A1, were obtained from the *E. coli* Genetic Stock Centre, Yale University, New Haven, USA.

(b) *E.coli* DH10B : F^-, *ara*D139, (*ara*, *leu*)7697, Δ*lac*X74, *gal*U, *gal*K, *mcr*A, Δ(*mrr- hsd*RMS-*mcr*BC), *rps*L, *dor*, Ø80d*lac*ZΔM15, *end*A1, *nup*G, *rec*A1 were obtained from Life Technologies, Cat. No. 8297SA, Gaithersburg, Maryland, USA.

(c) *E.coli* MC1061/P3 : *ara*D139, D(*ara*, *leu*)7697, *gal*U, *gal*K, D*lac*Y74, hsr^-, *hsrm*+, *str*A, *deo*R+, {p3: amber *amp*R, amber *tet*R, *Km*R}, were obtained from Invitrogen Corporation, San Diego, CA, USA.

(d) *E.coli* JM109 : *end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, λ^-, Δ(*lac-pro*AB), [F', *tra*D36, *pro*A+B+, *lac*I^{q}ΔM15], obtained as a component of the Altered Sites™, *in vitro* Mutagenesis System, Promega Corporation, Madison, WI, USA.

(e) *E.coli* BMH 71-18 mut S : *thi*, *sup*E, λ^-, Δ(*lac-pro*AB), [mutS::Tn10], [F', *tra*D36, *pro*A+B+, *lac*I^{q}ΔM15], obtained as a component of the Altered Sites™, *in vitro* Mutagenesis System, Promega Corporation, Madison, WI, USA.

2.16.6. Preparation Of Electrocompetent Cells

E.coli strains (refer to Section 2.16.5.) were made electrocompetent by the method of Miller *et al*, (1988). An isolated colony was used to inoculate 10 ml of superbroth growth medium (refer to Section 2.16.2. for preparation) and the culture grown overnight at 37°C with continuous shaking. The culture was then subcultured into 1 litre of pre-warmed superbroth growth medium and incubated at 37°C with shaking until an OD_{600} of 0.4 -0.6 was achieved (approximately 2 - 3 hours). The culture was chilled on ice prior to pelleting by centrifugation at 2000 x g for 15 minutes in a pre-cooled JA14 rotor (Beckman J-21C centrifuge). Each pellet was subsequently washed thrice with 250 ml ice-chilled, sterile Milli-Q water, and each pellet resuspended in 5 ml of ice-chilled 10% (v/v) glycerol in water. The cells were pooled into a 35 ml polypropylene Oakridge tube (Nalgene Labware, Nalge, Rochester, NY, USA. Cat. No. 3118-0030) and pelleted by centrifugation at 2000 x g as above. The final pellet was resuspended in 1 ml 10% (v/v) glycerol in water and aliquoted into pre-cooled Eppendorf tubes (Eppendorf, Gerätebau, Federal Republic of Germany), snap frozen on dry ice and stored at -80°C. The transformation efficiency of the cells was determined prior to use, by transforming 10 pg of closed, circular, pBluescript plasmid DNA (refer to Appendix A, Restriction Map # 1), and the efficiency calculated using Formula 2.16.1. below.

Formula 2.16.1:

Transformation Efficiency (CFU/μg) = CFU on control plate x 10^6 pg x dilution factor / # pg pBluescript used.

2.16.7. Transformation Of *E.Coli* With Plasmid Recombinants

Routinely, the ligation reaction products (refer to Section 2.18.7.) to be used for transformation were phenol/chloroform extracted as described in Section 2.18.4. , and resuspended in 10 µl Milli-Q water. In a pre-chilled Eppendorf tube, 2µl of the ligation reaction and 40 µl of thawed electrocompetent cells (refer to Section 2.16.6.) were added and incubated on ice for 1 minute. The mixture was transferred to a pre-chilled electroporation cuvette (Bio-Rad, Hercules, CA, USA, 1 mm electrode distance, Cat. No. 165-2089), and the cells subjected to the following electroporation parameters; 1.6 kV, 200 ohms, 25 µfd, using a Bio-Rad Gene Pulser (Bio-Rad). Time constants between 5.6 and 5.8 were expected and routinely obtained. Immediately following electroporation, 1 ml of SOC medium (refer to Section 2.16.2. for details of preparation) was added to the cuvettes, and the cuvette contents subsequently transferred to a 10 ml polypropylene tube (Disposable products, Adelaide, Australia, Cat. No. 21829), and incubated at 37°C with continuous shaking for 1 hour. The transformed cells were plated onto LB-agar containing 50-100 µg/ml of Ampicillin (or co-selected with 12.5 µg/ml Ampicillin and 7.5 µg/ml Tetracycline, as described above) using a sterile glass spreader. The agar plates were routinely incubated at 37°C overnight. Selection of recombinant colonies was often simplified by the use of the β-galactosidase reporter system in a compatible host and vector. This was achieved by the addition of 20 µl of both 2% BCIG and 2% IPTG prior to the plating of the transformed cells.

2.16.8. Preparation Of Glycerol Stocks

Single colonies of bacteria, obtained by streaking onto agar plates of suitable medium (refer to Section 2.16.2.) were used to inoculate liquid growth medium. Bacterial cultures were grown at 37°C with continuous shaking to provide adequate aeration. The resultant log phase cultures were used to prepare glycerol stock cultures of the bacterial strains (refer to Section 2.16.5.) and recombinant plasmid-transformed bacteria (refer to Section 2.16.4.) by the dilution of an overnight culture with an equal volume of 80% (v/v) glycerol (made in LB broth) and stored at either -20°C, or -80°C for long term storage.

2.17. ISOLATION AND PREPARATION OF DNA

2.17.1. Isolation And Preparation Of Plasmid DNA: General Considerations

The rapid alkaline hydrolysis procedure, essentially performed as described by Birnboim and Doly (1979), was used for the isolation of plasmid DNA from 2 ml overnight cultures for analytical restriction digests. These methods were also scaled-up for the bulk preparation of recombinant plasmids from 100 ml to 1 litre cultures (as described below) for subsequent use as probes in both Northern and Southern hybridisations. However, a modification of the boiling method described by Sambrook *et al*, 1989 was used to prepare plasmid DNA for sequence analysis (as described in Section 2.17.1. (ii)).

(i) Rapid Small Scale Isolation Of Plasmid DNA ("Mini-Prep." Method)

Isolated colonies were picked from selection plates and cultured overnight in 2 ml of LB broth (refer Section 2.16.2), containing the appropriate antibiotic selection, at 37°C with continuous agitation. Routinely, 1.5 mls of the culture was pelleted by centrifugation for 2 minutes at 10,000 x g at 4°C in a microcentrifuge. The supernatant was removed by vacuum aspiration and the pellet resuspended by vortexing, prior to the addition of 100 µl of Solution 1 (25 mM Tris-HCl pH 8.0, 10 mM EDTA, 15% sucrose and 5 mg/ml chicken egg lysozyme (Sigma, USA), and incubated for 5 minutes at room temperature. Following this, 200 µl of freshly prepared Solution 2 (0.2 M NaOH, 1% SDS) was added and the solution mixed by inverting the tube several times prior to incubating the tube on ice for five minutes. Chromosomal DNA was precipitated by adding 150 µl of ice cold Solution 3 (3 M potassium acetate, pH 4.5) and incubating the mixture on ice for 5 minutes. The mixture was microcentrifuged at 10,000 x g for 10 minutes at 4°C and the supernatant containing the plasmid DNA was carefully removed without disturbing the pellet and transferred to a fresh Eppendorf tube. The plasmid DNA was precipitated by the addition of two volumes of absolute ethanol and then pelleted by centrifugation (10,000 x g) for 10 minutes. Plasmid DNA was subsequently washed once in 70% ethanol, vacuum dried and resuspended in 50 µl of Milli-Q water. Plasmid DNA was subsequently analysed by restriction endonuclease digestion as described in Section

2.18.1. Clones exhibiting the appropriate fragment size(s) after restriction endonuclease digestion analysis were chosen to prepare glycerol stocks as detailed in Section 2.16.8.

(ii) Small Scale Plasmid Preparation Of DNA For Sequence Analysis (TELT Method)

The method described here, represents a modification of the boiling method described by Sambrook *et al*, 1989. A single colony was isolated and used to inoculate 2 ml of LB broth (refer to Section 2.16.2. for preparation) which was cultured overnight at 37°C with shaking. The culture was chilled on ice and the cells subsequently pelleted in a 1.5 ml Eppendorf tube by centrifugation at 10,000 x g for 1 minute at 4°C in a microcentrifuge. The supernatant was aspirated and the pellet resuspended in 250 µl of freshly prepared TELT buffer (50 mM Tris-HCl, pH 7.5; 62.5 mM EDTA, pH 8; 0.4% (v/v) Triton-X100 and 2.5 M lithium chloride). This mixture was vortexed vigorously and left on ice for 5 minutes after which 20 µl of freshly prepared Lysozyme (10 mg/ml) was added. The mixture was vortexed for 3 seconds, boiled for 1 minute and put immediately on ice for approximately 15 minutes. The mixture was then centrifuged for 20 minutes at room temperature and the supernatant transferred to a fresh tube. The plasmid DNA was precipitated by adding 500 µl absolute ethanol to the lysate, mixed by vortexing and centrifuged at 10,000 x g for 15 minutes at 4°C. The DNA pellet was washed in 70% ethanol and vacuum dried. For sequence analysis, the pellet was resuspended in 32 µl of Milli-Q water, of which 2 µl was digested with restriction endonucleases (as described in Section 2.18.1.) in order to determine the yield of plasmid DNA obtained.

(iii) Mid-Scale Plasmid Preparation (QIAGEN™ TIP-100)

The QIAGEN plasmid purification procedure was carried out essentially as recommended by the manufacturer (QIAGEN Inc., Chatsworth, CA, USA, Cat. No. 12143). A single colony was isolated and used to inoculate 2 ml of LB broth supplemented with the appropriate antibiotic and cultured for several hours at 37°C with shaking. This starter or miniculture, was then diluted 1:100 into 200 ml of selective medium and regrown to saturation by overnight incubation at 37°C with shaking. Cells were harvested by centrifugation at 4°C for 15 minutes at 6,000 x g (Beckman JA-10

rotor). The supernatant was removed by inverting the open centrifuge buckets, and the cell pellets were subsequently resuspended in 4 ml of "Buffer P1" [Resuspension buffer, 100 µg/ml RNase A, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0] provided. The cell suspension was then lysed by the addition of 4 ml of "Buffer P2" [Lysis buffer, 200 mM NaOH, 1% SDS], and incubated at ambient temperature for 5 minutes. The lysate was neutralised by the addition of 4 ml of "Buffer P3" [Neutralisation buffer, 3 M potassium acetate, pH 5.5], mixed by inversion, and chilled on ice for 15 minutes. The sample was transferred to a fresh Oakridge tube (Nalgene, Cat. No. 3118-0030), and the insoluble precipitate pelleted by centrifugation at 4°C for 30 minutes at 30,000 x g (Beckman, JA-20 rotor). During this time, a QIAGEN-tip 100 column was equilibrated by the addition of 4 ml of "Buffer QBT" [Equilibration buffer, 750 mM NaCl, 50 mM MOPS, 15% ethanol, pH 7.0, 0.15% Triton X-100], and allowed to discharge from the column by gravity flow. Following centrifugation, the "cleared" supernatant was loaded onto the equilibrated QIAGEN-tip 100 column, and allowed to enter the resin by gravity flow. After the column had emptied, the QIAGEN-tip 100 was washed twice with 10 ml of "Buffer QC" [Wash buffer, 1.0 M NaCl, 50 mM MOPS, 15% ethanol, pH 7.0] to remove contaminants in the DNA preparation. The DNA was eluted into a fresh Oakridge tube following the addition of 5 ml of "Buffer QF" [Elution buffer, 1.25 M NaCl, 50 mM Tris-HCl, 15% ethanol, pH 8.5] to the column. The plasmid DNA was subsequently precipitated with the addition of 0.7 volume (3.5 ml) of isopropanol. The DNA was pelleted by centrifugation at 15,000 x g, at 4°C for 30 minutes (Beckman JA-20 rotor), and thereafter the supernatant was aspirated and the pellet washed with 5 ml of ice cold 70% ethanol. The pellet was air dried for 5 minutes and routinely redissolved in 200 µl of 1 x TE or Milli-Q water. The yield of DNA was determined by UV spectrophotometry (please refer to Section 2.25.2.) and agarose gel electrophoresis (please refer to Section 2.18.2.) and was consistently between 80 - 100 µg per preparation.

(iv) Large Scale Plasmid DNA Preparation

Extraction of plasmid DNA was performed as described by Sambrook *et al*, 1989 and purified on a two-step CsCl gradient by the method of Garger *et al*, 1983. Restriction analysis of the plasmid DNA was performed as described in Section 2.18.1.

(a) Extraction Procedure

Extraction of plasmid DNA was performed from 500 ml Luria-Bertani broth cultures containing Ampicillin (100 µg/ml) and grown for approximately 3-4 hours prior to amplification with 2.5 ml chloramphenicol (34 µg/ml) for 12-15 hours. The culture was centrifuged at 6,000 x g for 15 minutes at 4°C in a Beckman JA-10 rotor. The cells were then resuspended in 16 ml of Solution 1 (25 mM Tris-HCl pH 8.0, 10 mM EDTA, 15% sucrose) freshly supplemented with chicken-egg lysozyme (2.5 mg/ml). The cells were allowed to stand at room temperature for 10 minutes in order to break the cell wall and outer membrane. The resulting spheroplasts were lysed by the addition of 26 ml of fresh Solution 2 (0.2 M NaOH, 1% SDS) and left on ice for 5 minutes. Chromosomal DNA was precipitated by the addition of 14 ml of ice cold Solution 3 (3 M potassium acetate, pH 4.5) and leaving the mixture on ice for 15 minutes. The mixture was centrifuged at 6,000 x g, 20 minutes at 4°C, as described above. The supernatant containing the plasmid DNA, was removed carefully without disturbing the pellet and extracted twice with an equal volume of buffer-saturated phenol/chloroform/isoamylalcohol (25:24:1). The mixture was then centrifuged at 1000 x g (Mistral 3000i, MSE) for 5 minutes to separate the organic and aqueous phases. The plasmid DNA, present in the upper aqueous phase, was transferred to an Oakridge tube and precipitated by the addition of one volume of iso-propyl alcohol (propan-1-ol). The plasmid DNA was then pelleted by centrifugation (10,000 x g, 10 minutes) and subsequently washed once in 70% ethanol. The supernatant was removed and the pellet vacuum dried prior to resuspension in 2.4 ml of Milli-Q water.

(b) Two-Step Caesium Chloride Gradient

Plasmid DNA was further purified on a 2 step caesium chloride (CsCl, BDH, UK, Cat. No. 810709) gradient essentially as described by Garger *et al* (1983). To the DNA

solution, 4.2 g of solid CsCl was added to give a final concentration of 1.8 g/ml. The refractive index of the DNA/CsCl solution was adjusted to 1.4080, prior to the addition of 400 µl of ethidium bromide solution (10 mg/ml). To a Beckman Optiseal tube (Beckman, USA, Cat. No. 361621), 8 ml of CsCl solution (1.470 g/ml, refractive index, 1.3780) was added, and the DNA/CsCl solution was carefully underlaid with a Pasteur pipette. The tubes were completely filled with the less dense CsCl solution (refractive index, 1.3780), balanced by the removal of the lower layer (containing the DNA), heat-sealed, placed in a Beckman TLA-100.4 rotor and subjected to ultracentrifugation in a Beckman Optima TLX ultracentrifuge at 330,000 x g, 5 hours, 20°C. Following centrifugation, the lower plasmid band (supercoiled) was clearly discriminated from the "nicked" - plasmid band, and was removed with a 19 gauge needle and 3 ml syringe. The ethidium bromide was removed by extracting the solution 5 - 6 times with 1 M NaCl-saturated butanol. The extracted-DNA solution was subsequently precipitated with the addition of 2.5 volumes of absolute ethanol and incubated overnight at -20°C. The precipitated DNA was pelleted by centrifugation at 15,000 x g at 4°C, for 20 minutes, washed once with 10 ml of 70% ethanol, vacuum dried and resuspended in 500-1000 µl of Milli-Q water. The yield of DNA was determined by UV spectrophotometry (please refer to Section 2.25.2.) and agarose gel electrophoresis (please refer to Section 2.18.2.) and was consistently between 500-1000 µg per preparation.

2.17.2. Preparation Of High Molecular Weight Genomic DNA

Genomic DNA from eukaryotic cells was isolated by the sodium dodecyl sulphate (SDS)/proteinase K/RNase A protocol, described by Hughes *et al* (1979). Typically 1 x 10^7 cells were harvested and pelleted by low speed centrifugation (400 x g). The cells were subsequently washed thrice by centrifugation at 4°C in PBS and resuspended at 1 x 10^7 per ml in genomic DNA buffer (0.1 M NaCl, 0.05 M Tris-HCl [pH 8.3], 1 mM EDTA). Cells were lysed by incubation in the presence of proteinase K (BDH/Merck, Australia, Cat. No. Cat. No. 24568), SDS and RNase A (Boehringer Mannheim, Germany, Cat. No. 109 169), at final concentrations of 500 µg/ml, 0.5% (w/v) and 100 µg/ml respectively, for 90 minutes at 37°C. After adjusting the concentration of SDS to 1% (v/v), the DNA

was phenol/chloroform extracted as described in Section 2.18.4., and ethanol precipitated by adding 1/10 volume 3M sodium acetate, 2 volumes of 100% ethanol, and incubating at -20°C for 20 minutes. The precipitated DNA was collected by spooling, washed once in 70% (v/v) ethanol and resuspended in sterile Milli-Q water. The genomic DNA was subsequently placed at 4°C for approximately 24 hours to ensure adequate solubilisation. An aliquot was removed and the concentration determined as described in Section 2.25.2. An $OD_{260/280}$ of 1.8 or greater was routinely obtained, and was indicative of a protein-free DNA preparation. Finally, the DNA was resuspended to a final concentration of approximately 1 µg/µl, and stored at -20°C.

2.18. DNA MANIPULATION AND MODIFICATION

2.18.1. Restriction Endonuclease Digestions

In analytical digests, 0.5-1 µg of DNA was incubated with 2-5 units (5 units of enzyme added per µg of DNA) each of the appropriate restriction endonuclease(s) for a minimum of 1 hour in 'One-Phor-All' buffer or the buffer conditions specified by the manufacturer in a total reaction volume of 20-50 µl. Double digests were carried out by digesting the DNA first with the restriction endonuclease (RE) that required the lowest salt concentration for a minimum of 1 hour, and then adjusting the salt concentration appropriately for the second RE. The digests were incubated for a further 1 hour at the requisite temperature prior to terminating the reaction by the addition of 1/10 volume of gel loading buffer (refer to Section 2.16.3. for preparation) or heating to 65°C for 10 minutes. In circumstances where the DNA digest conditions were incompatible, the DNA was phenol/chloroform extracted (refer to Section 2.18.4) prior to the subsequent digestions. If mini-prep DNA (refer to Section 2.17.1.) was contaminated with a substantial amount of RNA, 1 µl of 10 mg/ml RNase A was added in association with the restriction endonuclease.

In preparative digests, 5 to 10 µg of DNA was restricted in a reaction volume of 30 to 50 µl, and the desired DNA fragments were isolated as detailed below.

2.18.2. Analytical And Preparative Separation Of DNA Fragments

Following restriction endonuclease digestion, 1/10 volume of 10 x gel loading buffer (refer to Section 2.16.3. (h), for preparation) was added, and a proportion of this reaction was electrophoresed in 0.8%, 1% or 1.5% (w/v) agarose gels, depending on the expected size of the DNA fragments. Gels were electrophoresed for periods ranging from 30 minutes to 5 hours, at 100 volts in 1 x TAE buffer (refer Section 2.16.3. for preparation details) in a horizontal gel apparatus (Bio-Rad). The gels were stained with ethidium bromide (2 µg/ml in water, Sigma, USA, Cat. No. E-8751) for 5 minutes, and then destained in a large volume of water for 10 minutes with gentle agitation. DNA fragments were visualised by 280 nm short-wave UV light and photographed using Polaroid 667 film. Preparative gels were prepared as described above with the exception that DNA was visualised with 320 nm long-wave UV light to minimise nicking and damage to the fragments required for gel purification and cloning.

2.18.3. Preparation Of DNA Restriction Fragments

Bands representing restriction fragments were visualised under UV light following staining with ethidium bromide, and the appropriate fragment(s) excised from the gel and purified using a number of gel purification protocols (described below).

(a) Geneclean II™ And BRESAclean™ Protocols

These methods were carried out essentially as recommended by the manufacturers' (Geneclean II, Bio101, Cat. No. 3105, and BRESAclean, Bresatec, Adelaide, Australia Cat. No. BC-1) and were used to purify DNA fragments between 0.5 and 7.5 kb from agarose gels. Excised gel slices (containing desired DNA fragment) were placed into 1.5 ml Eppendorf tubes and their mass determined. To this, 2.5-3 volumes of sodium iodide (NaI) solution was added, and the agarose melted at 55°C for 10 minutes. Ten microlitres of resuspended Glassmilk (Geneclean II) or Silica matrix (BRESAclean) was subsequently added, mixed thoroughly, and placed on ice for 5-15 minutes (with intermittent mixing). DNA bound to the glass milk/silica matrix was pelleted by centrifugation (5 seconds, 13,000 rpm), and the NaI supernatant removed by aspiration. The pellet was resuspended, by vortexing and washed 3 times with 800 µl of NEW WASH (Geneclean) or BRESAwash (BRESAclean). Residual wash solution was removed by an additional

centrifugation step and heating the silica matrix/DNA pellet at 55°C for 5 minutes. The DNA was eluted from the silica matrix with the addition of 10-20µl of water or TE, and incubation at 55°C for 10 minutes. The DNA in solution was removed following a brief centrifugation to pellet the silica matrix.

(b) QIAquick Gel Extraction Protocol

This method was carried out essentially as recommended by the manufacturer (QIAGEN Inc. Chatsworth, CA, USA, Cat. No. 28704). Following agarose gel electrophoresis and ethidium bromide staining, separated DNA fragments were visualised by long wave (320 nm) ultraviolet illumination. Gel slices (containing desired DNA fragment) were excised with a clean, sharp scalpel and placed into 1.5 ml Eppendorf tubes. The mass of the gel slice was determined, and 3 volumes of "Buffer QX1" was added prior to incubating the sample at 50°C for 10 minutes. The samples pH was adjusted to less than 7.5 with the addition of 10 µl 3 M sodium acetate, pH 5.0, prior to loading onto a QIAquick spin column (previously inserted into a 2 ml collection tube). The sample was centrifuged at 10,000 x g for 60 seconds in a microcentrifuge, and the flow-through fraction was drained from the collection tube. The QIAquick column was returned to the collection tube, and the bound DNA was washed with the addition of 0.75 ml of "Buffer PE". In general, the DNA fragments were subjected to further enzymatic modification, and as such, the column was allowed to stand for 5 minutes in the presence of the wash buffer, prior to centrifugation as described above. The flow through was drained as above, and the column subjected to centrifugation for an additional 60 minutes to remove residual "Buffer PE". The QIAquick column was transferred to a clean 1.5 ml Eppendorf tube, and the DNA eluted with the addition of 50 µl of Milli-Q water, and centrifuged as above. The concentration of QIAquick-purified DNA fragments were assessed by agarose gel electrophoresis of a proportion of the sample.

2.18.4. Phenol/Chloroform Extraction

This method was carried out essentially as described in Sambrook *et al* (1989), with the following modifications. For limited quantities of DNA, the volume was routinely made up to a volume of 100 µl with Milli-Q water and 1 µl of glycogen (1 mg/ml

stock, Boehringer Mannheim, Mannheim, Germany) was added as carrier. An equal volume of phenol/chloroform was subsequently added, mixed by vortexing and centrifuged at 10,000 x g for 5 minutes to facilitate phase separation. The aqueous phase was then removed and an equal volume of chloroform was added, vortexed and centrifuged as before, to ensure complete removal of residual phenol. The aqueous phase was retained and the DNA precipitated by the addition of 1/10 volume of 3M sodium acetate, pH 4.6 and 2 volumes of ethanol, and incubated on ice for 20 minutes. The DNA was pelleted by centrifugation at 10,000 x g for 20 minutes at 4°C, washed in 70% ethanol and resuspended in water or TE. Phenol/chloroform extraction of genomic DNA was performed essentially as described above, with the omission of the glycogen carrier. Additionally, it was often necessary to extract genomic DNA several times with an equal volume of phenol/chloroform, prior to precipitation as above.

2.18.5. End- Filling Of Restriction Fragments

End-filling of restriction fragments to create blunt-ended DNA molecules was carried out essentially as described in Sambrook *et al* (1989). The DNA to be end filled was ethanol precipitated and resuspended in a reaction volume of 15 µl which contained 50 mM Tris HCl pH 7.6, 10 mM $MgCl_2$, 500 mM DTT, 0.02 mM dNTP's and 2 units of Klenow polymerase. The reaction was incubated at 37°C for 15 minutes, followed by incubation at 75°C for 10 minutes to inactivate the Klenow polymerase. End-filled DNA subsequently used in ligations was not further purified, however if additional enzymatic modification was carried out, DNA was purified by phenol/chloroform extraction (refer to Section 2.18.4.).

2.18.6. Dephosphorylation Of Vector DNA

Plasmids were linearised with the appropriate restriction enzyme(s) as described in Section 2.18.1. To prevent self-ligation of the vector, 5'-terminal phosphate groups were removed by incubation with calf intestinal alkaline phosphatase (CIP, Boehringer Mannheim, Cat. No. 713 023). Routinely, 0.5-1.0 µg of plasmid DNA was incubated with 0.5 units of calf intestinal phosphatase in 1 x CIP buffer comprised of 50 mM Tris-HCl

pH 9.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$. The reaction was carried out in a final volume of 100 µl and subject to two cycles of incubation for 15 minutes at 37°C, followed by 15 minutes at 56°C. The CIP was inactivated by heating at 75°C for 10 minutes in the presence of 5 mM EDTA, pH 8.0 and phenol/chloroform extracted prior to ligation. Alternatively, if further enzymatic modification was required, the vector DNA was electrophoresed on an appropriate percentage agarose gel containing TAE running buffer, excised and purified using a Geneclean II™/BRESAclean™ or QIAquick™ column as described in Section 2.18.3. The amount of vector DNA recovered was estimated following subsequent agarose gel electrophoresis of a proportion of the product.

2.18.7. Ligation Of Restriction Fragments To Vector

A 10µl reaction routinely contained 20-50 ng of vector DNA, the DNA restriction fragment insert, in the presence of 1 x ligation buffer comprised of 50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 1 mM rATP, and 1-2 units of T4 DNA ligase (Promega, Madison, WI, USA, Cat. No. M1801). A 2-3 molar excess of restriction fragment insert to vector DNA was routinely used. The reactions were incubated for either 1 to 4 hours at room temperature, or overnight at 12°C. Control ligations of vector without insert and vector without ligase were usually set up and included in the subsequent transformation to determine background levels of uncut or recircularised vector DNA.

2.18.8. Oligo-Labelling Of DNA

For the oligolabelling of recombinant plasmids and pure fragments, the GIGAprime DNA Labelling Kit (Bresatec, Adelaide, Australia, Cat. No. GPK-1) kit was used, with the following modifications. Routinely, 50-200 ng of DNA in a total of 6 µl water, was denatured by heating at 95°C for 10 minutes and then chilled on ice. To the denatured DNA, 6 µl nucleotide/buffer cocktail, 6 µl decanucleotide solution, 5 µl (50 µCi) of either α^{32}P-dATP or α^{32}P-dCTP (both from Bresatec, Adelaide, Australia, Cat. No. ADA-2 and ADC-2, respectively) and 1 µl (5 units) Klenow polymerase. The labelling reaction was incubated at 37°C for 15-30 minutes before being terminated by the addition of 2 µl of 0.5 M EDTA, pH 8.0. The volume of the reaction was adjusted to 95 µl with Milli-Q

water, and the labelled DNA was precipitated with the addition of 100 µl 7.5 M ammonium acetate, 5 µl of 5 µg/ml salmon sperm DNA, and 400 µl (2 volumes) absolute ethanol. Following incubation on a dry ice/ethanol bath for 15 minutes, the labelled DNA was pelleted by centrifugation for 10 minutes at 4°C in a microcentrifuge at 10,000 x g. The pellets were then washed twice with 70% ethanol to remove excess, unincorporated nucleotides, vacuum dried and resuspended in 100 µl Milli-Q water or TE. Probes were denatured by heating at 100°C for 5 minutes, and placed immediately on ice prior to its addition to the hybridisation solution. This procedure consistently led to efficient incorporation of the radionucleotide(s). The specific activity of probes was generally 1-2 x 10^8 cpm/µg as determined by scintillation counting as detailed below (Section 2.18.9).

2.18.9. Measurement Of Incorporated Radioactivity In Radiolabelled Probes

1-5 µl of each completed reaction sample as well as 1 µl of a 1:500 dilution of the "free" radionucleotide were spotted onto separate Whatman GF/A glass fibre discs, dried under a heat lamp and counted in 3 ml of Optiscint™ (Pharmacia) scintillation fluid in an LKB Wallac 1214 Rackbeta liquid scintillation counter. The percentage of radioactivity incorporated in the probe was subsequently calculated using the counts obtained from each sample and the estimation of the counts from the free radionucleotide initially added to the reaction.

2.18.10. 5'-Phosphorylation Of Oligonucleotides

Phosphorylation of the 5'-terminal nucleotide of oligonucleotides (please refer to Section 2.21. (i) (a & b), was achieved by placing approximately 100 picomoles of the desired oligonucleotide, 2.5 µl of 10 x kinase buffer (500 mM Tris-HCl, pH7.5, 100 mM $MgCl_2$, 50 mM DTT, 1 mM spermidine and 10 mM ATP) and 5 units of T4 polynucleotide kinase into a 1.5 ml microcentrifuge tube. The volume of the reaction was adjusted to 25 µl with the addition of Milli-Q water, and subsequently incubated at 37°C for 30 minutes. The T4 polynucleotide kinase was inactivated by heating the reaction to 70°C for 10 minutes, and used immediately or stored at -20°C until required.

2.18.11. Southern Transfer And Hybridisation

(a) General Considerations

Restricted DNA samples were electrophoresed on 0.8-1.5% agarose gels (depending on the size of the DNA fragments being separated) in 1 x TAE running buffer (refer to Section 2.16.3. for preparation). Following staining with ethidium bromide, the gel was visualised under 320 nm long-wave UV light and photographed. To denature the DNA, the gel was incubated with gentle agitation, in a solution comprised of 0.5 M NaOH and 1.5 M NaCl for 30-60 minutes at room temperature. Gels were subsequently neutralised by incubation in a solution comprised of 1.0 M Tris.HCl, pH8.0, for 30-60 minutes, as described above. The DNA was then capillary-transferred to Hybond-N$^+$ (Amersham, Poole, England) by the method of Southern (1975). Following transfer, the DNA fragments were covalently cross-linked to the membrane by exposure to 0.7 J/cm^2 of UV radiation in a Stratagene UV Stratalinker™ 1800 (Stratagene, La Jolla, CA, USA), according to manufacturer's recommendations. The membrane was prehybridised for 4-8 hours at 42°C in 50% formamide, 5 x SSC, 5 x Denhardt's solution (refer to Section 2.16.3. for preparation), 0.1% SDS, 0.05% sodium pyrophosphate, and 200 µg/ml of sheared salmon sperm DNA. Following addition of 20-50 ng/ml of the appropriate radiolabelled probe, hybridisations were carried out for 10-18 hours under the same conditions. The membrane was washed twice in 2 x SSC, 0.1% SDS for 10 minutes at room temperature, followed by a single wash in 0.5-0.1 x SSC, 0.1% SDS at 65°C for 20-30 minutes. After washing, the membrane was air-dried and exposed to autoradiography film at either room temperature for several hours, or at -80°C for longer exposures as required.

(b) Genomic DNA Digest and Southern Transfer to Determine the Number of Proviral Integrations

The number of the proviral integrations were determined as previously described (Rayner and Gonda, 1994 and Zannettino *et al*, 1996). Briefly, 10 µg of genomic DNA (refer to Section 2.17.2.) was digested with 20-50 units of *Bam*HI, at 37°C for a minimum of 5 hours, in One-Phor-All Plus buffer [10 x buffer: 100 mM Tris-acetate (pH 7.5), 100

mM magnesium acetate and 500 mM potassium acetate, Pharmacia], as specified by the manufacturer and fractionated on a 0.8% agarose gel. The DNA was then transferred to Hybond-N^+ and probed with a 1,090 bp *Bgl*II-*Cla*I fragment corresponding to the Neo^R cassette from pRUF.Neo (refer to Appendix A, Restriction Map # 3), labelled with ^{32}P using random hexanucleotide primers (please refer to Section 2.18.8.).

2.19. DNA SEQUENCE ANALYSIS

2.19.1. Automated DNA Sequence Analysis

(a) PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit

Direct sequencing of double-stranded (ds) PCR products was achieved using the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, California, USA, Cat. No. 401388). Sequencing of PCR products was performed essentially as recommended by the manufacturer. Routinely, 100 ng of gel-purified ds DNA (please refer to Section 2.18.3.) was utilised per 300 bp of PCR product length (i.e. for a PCR product of 1200 bp in length, 400 ng of DNA in a total volume of 9.5 µl of water, was used). To this, 9.5 µl of "terminator premix" [1.58 µM A-DyeDeoxy, 94.74 µM T-DyeDeoxy, 0.42 µM G-DyeDeoxy, 47.37 µM C-DyeDeoxy, 78.95 µM dITP, 15.79 µM dATP, 15.79 µM dCTP, 15.79 µM dTTP, 168.42 mM Tris-HCl (pH 9.0), 4.21 mM $(NH_4)_2SO_4$, 42.10 mM $MgCl_2$, 0.42 units/µl Amplitaq DNA polymerase] and 1 µl of sequencing primer (previously diluted to a concentration of 100 ng/µl) was added to give a final volume of 20 µl. The reaction was overlaid with approximately 40 µl of mineral oil, and the tube was placed in a thermal cycler (Perkin Elmer Cetus, Model No. 480), preheated to 96°C. The thermal cycling parameters used, were as follows: Rapid thermal ramp to 96°C and maintained for 30 seconds, rapid thermal ramp to 50°C and maintained for 15 seconds, rapid thermal ramp to 60°C and maintained for 4 minutes. These cycling "steps" were repeated for 25 cycles, and the resultant products were phenol/chloroform extracted in the following manner. At the end of thermal cycling, 80 µl of Milli-Q water was added to the reaction mix and transferred to a fresh Eppendorf tube. The terminators were extracted following the addition of 100 µl of phenol:water:chloroform (70:20:10) reagent, vortexing and centrifuging the sample at

ambient temperature for 5 minutes at 10,000 x g. The lower organic phase was removed and discarded, and the remaining aqueous phase was re-extracted as described above. The aqueous phase was subsequently removed and transferred to a fresh tube, and the extension products were precipitated by the addition of 10 µl of 3M sodium acetate, pH 5.2, and 300 µl of ice-cold absolute ethanol. The precipitated products were pelleted by centrifugation at 10,000 x g for 15 minutes at 4°C, and the resultant pellets were washed once with 70% ethanol and vacuum dried in preparation for denaturing gel electrophoresis. Prior to electrophoresis, 4 µl of load buffer (deionised formamide, 8.5 mM EDTA) was added to each tube and vortexed. The samples were subsequently denatured by heating the tubes to 90°C for 2 minutes and loaded onto an Applied Biosytems 373A DNA sequencer according to manufacturers instructions. Routinely, 350-450 bp of 5' and 3' sequence data was obtained per clone.

(b) PRISM™ Ready Reaction Dye Primer Cycle Sequencing Kit

Sequencing of double-stranded PCR products which had been cloned into pBluescript/pGEM-T vectors, were routinely performed using the PRISM™ Ready Reaction Dye Primer Cycle Sequencing kit (Applied Biosystems, Inc., USA, Cat. No. 401386). Sequencing of cloned DNA products were performed essentially as recommended by the manufacturer and utilised the M13 RSP and M13 primer recognition sites which flanked the cloned sequence. Routinely, QIAGEN™ tip-100 or TELT-prepared ds DNA (refer to Section 2.17.) diluted to 200-250 ng/µl, provided the highest quality results. For each clone, the following reagents were aliquoted into four 0.5 ml microcentrifuge tubes as described below.

Reagent	A	C	G	T
d/ddNTP mix*	4 µl	4 µl	8 µl	8 µl
DNA Template	1 µl	1 µl	2 µl	2 µl
Total Volume	5 µl	5 µl	10 µl	10 µl

* the composition of the d/ddNTP premix are detailed following protocol description "Kit Reagents".

Each of the reactions were overlaid with approximately 20 µl of mineral oil, and the tubes were placed in a thermal cycler (Perkin Elmer Cetus, Model No. 480), preheated

to 95°C. The thermal cycling parameters used, were as follows: Rapid thermal ramp to 95°C and maintained for 30 seconds, rapid thermal ramped to 55°C and maintained for 30 seconds, rapid thermal ramp to 70°C and maintained for 1 minute. These cycling "steps" were repeated for 15 cycles, and the resultant products were handled as follows: the four extension reactions were transferred and combined in a clean 1.5 ml Eppendorf tube, to which 100 µl of 95% ethanol and 2 µl of 3 M sodium acetate (pH 5.5) were added. The mixture was vortexed thoroughly, and chilled on ice to facilitate the precipitation process. The extension reactions were pelleted by microcentrifugation at 10,000 x g at 4°C for 15 minutes. The supernatant was aspirated and the pellet rinsed with 250 µl of 70% ethanol, centrifuged as above and vacuum dried for 1-3 minutes prior to loading the samples onto an acrylamide gel as described in Section 2.19.1. (a). Approximately, 500-600 bp of 5' and 3' sequence data was routinely obtained per clone.

Kit Reagents

A Mix: 375 µM ddATP, 15.6 µM dATP, 62.5 µM dCTP, 93.8 µM c7dGTP, 62.5 µM dTTP, 0.1 pmol/µl JOE dye primer, 100 mM Tris-HCl (pH 8.9 at room temperature), 25 mM $(NH_4)_2SO_4$, 6.3 mM $MgCl_2$ and 0.18 units/µl Amplitaq DNA polymerase.

C Mix: 187.5 µM ddCTP, 62.5 µM dATP, 15.6 µM dCTP, 93.8 µM c7dGTP, 62.5 µM dTTP, 0.1 pmol/µl FAM dye primer, 100 mM Tris-HCl (pH 8.9 at room temperature), 25 mM $(NH_4)_2SO_4$, 6.3 mM $MgCl_2$ and 0.18 units/µl Amplitaq DNA polymerase.

G Mix: 31.3 µM ddGTP, 62.5 µM dATP, 62.5 µM dCTP, 23.5 µM c7dGTP, 62.5 µM dTTP, 0.1 pmol/µl TAMRA dye primer, 100 mM Tris-HCl (pH 8.9 at room temperature), 25 mM $(NH_4)_2SO_4$, 6.3 mM $MgCl_2$ and 0.18 units/µl Amplitaq DNA polymerase.

T Mix: 312.5 µM ddTTP, 62.5 µM dATP, 62.5 µM dCTP, 93.8 µM c7dGTP, 15.6 µM dTTP, 0.1 pmol/µl ROX dye primer, 100 mM Tris-HCl (pH 8.9 at room temperature), 25 mM $(NH_4)_2SO_4$, 6.3 mM $MgCl_2$ and 0.18 units/µl Amplitaq DNA polymerase.

2.19.2. Manual Sequence Analysis- Dideoxy Sequencing Of DNA

Double stranded sequencing was performed using the Super-Base Sequencing Reagent Kit (Bresatec, Australia, Cat. No. SBK-2) essentially as recommended by the manufacturer. The template DNA was prepared as described in Section 2.17. and denatured by alkaline-treatment as follows:

DNA :	8-12 µl (approximately 2 µg)
2M NaOH :	4 µl
Milli-Q water :	volume up to 20 µl

This was incubated at room temperature for 10 minutes prior to precipitating the DNA by adding the following:

3m NaAc, pH 4.5 :	6 µl
Ethanol :	120 µ1
Water :	14 µl

This was mixed well and incubated at -80°C for 20 minutes. The DNA was pelleted by centrifugation at 10,000 x g for 10 minutes in a microcentrifuge. The pellet was subsequently washed with 70% ethanol and vacuum dried prior to annealing the appropriate sequencing oligonucleotide. The oligonucleotide was annealed to the template by resuspending in:

water :	8 µl
10 x Annealing buffer [1] :	1 µl
Oligonucleotide Primer [2,3] :	1 µl
Total Volume :	10 µl

[1] Please refer to Section 2.16.3., for buffer preparation.

[2] Please refer to Section 2.21.3., for list and location of oligonucleotide primers used.

[3] Primer concentration was adjusted to 100 ng per µl.

This was mixed well and incubated for 30 minutes at 37°C and subsequently incubated at room temperature for 20 minutes. 2.5 µl of the provided A, G, C and T termination mixes were added to individual wells of a microtray (Disposable Products, Australia, Cat. No. 239728) and prewarmed to 37°C on a heating block (Techne block, Techne [Cambridge], Duxford, Cambridge, England). To the annealed primer/template mix the following reagents were added:

Diluted labelling/extension mix [1] :	2 µl
100 mM DTT :	1 µl
water :	1 µl
[α-^{35}S] dATP (10 µCi) [2] :	1 µl
T7 DNA Polymerase (2 units) [3] :	1 µl

[1] The labelling/extension mix was diluted according to the length of the sequence required proceeding the primer binding site. If sequence close to the primer was required i.e. within 5-20 bases, then this mix was diluted 1/15 favouring the generation of short strands of DNA. If a longer extension was required, 1/5 - 1/10 dilution was used.

[2] The [α-^{35}S] dATP was purchased from Bresatec (Australia, Cat. No. SDA-2).

[3] The T7 DNA Polymerase was purchased from Pharmacia (Cat No. 27-0985-04) and diluted to 2 units/µl in the provided enzyme diluent.

Following the addition of the T7 DNA Polymerase enzyme, the reaction was incubated for 5 minutes at 37°C prior to transferring 3.5 µl of each labelling/extension reaction mix to each of the corresponding wells of the microtray plate. The reactions were incubated at 37°C for a further 5 minutes prior to the addition of 4 µl of the stop/loading buffer provided. The reactions were either used immediately or stored at -20°C for up to a week. Samples were denatured at 95°C for a 3 minutes on a heat block and loaded directly onto an acrylamide sequencing gel (please refer to Section 2.19.3.).

2.19.3. Preparation Of Acrylamide Sequencing Gel And Electrophoresis Of Samples

(i) Buffer Preparation

(a) 46% urea solution : Dissolved 460 g of urea (BDH, AnalR, Cat. No. 10290) in Milli-Q water to a final volume of 1 litre. Added 20 - 30 g Analytical Grade resin (AG 501-X8;

Bio-Rad, Cat. No. 142-6424) and stirred for at least 30 minutes. The beads were removed by filtration through Whatman No. 1 paper.

(b) 10 x TBE : Dissolved 121 g of Tris-HCl base, 7.4 g of EDTA and 53.4 g boric acid in Milli-Q water to a final volume of 1 litre. The pH was adjusted to 8.3.

(c) 20% acrylamide solution : Dissolved 193 g of acrylamide (Bio-rad), 6.7 g of N, N' methylene-bisacrylamide (Bio-rad, Cat. No. 161-0144) and 467 g urea in Milli-Q water to a final volume of 1 litre. Added 20-30 g of Analytical Grade resin (AG 501-X8; Bio-Rad, Cat. No. 142-6424) and stirred for at least 60 minutes. This was filtered through Whatman No. 1 paper and stored at 4°C in the dark.

(ii) Gel Preparation And Electrophoresis

To prepare 70 mls of 8% polyacrylamide gel mix containing 7M urea, 35 ml of 46% (w/v) urea, 28 ml of 20% (w/v) acrylamide and 7 ml 10 x TBE were added to a 200 ml glass beaker. To initiate the polymerisation, 350 µl of 10% (w/v) ammonium persulphate and 70 µl TEMED were added, mixed and immediately taken up into the barrel of a 50 ml syringe. This solution was poured in between two previously siliconised (Coatasil, Ajax Chemicals, Cat. No. 2293) glass plates which were securely taped and clamped, and the gel allowed to polymerise for approximately 40 minutes. The comb was subsequently removed and wells were flushed with TBE buffer to remove unpolymerised acrylamide. The gel was assembled onto a BRL vertical electrophoresis apparatus (Bethesda Research Laboratories, BRL, USA) and pre-electrophoresed for 30 minutes at 1700-1800V. The wells were again flushed out prior to loading the denatured samples and electrophoresed for varying time intervals, depending on the length of sequence required. Routinely however, most of the samples were loaded at staggered time intervals and involved loading 2 µl of each denatured sequence reactions (A, G, C, T) and electrophoresed until the xylene cyanol dye front (slow dye front) approached the bottom of the gel. The samples were denatured again and another 2 µl were load and allowed to run until the xylene cyanol dye front was 3/4 of the way down the gel. The third run was allowed to run until the bromophenol blue dye front (fast dye front) approached the bottom of the gel.

Following electrophoresis, the plates were separated and the gel was fixed with 1 litre of a 10% methanol/10% acetic acid solution over a period of 30 minutes. The gel was then transferred to a Whatman 3MM paper and dried under vacuum at 80°C for 45-90 minutes. The dried gels were then autoradiographed for 16-48 hours at room temperature. Sequence data was compiled either manually or using the various DNA analysis software available (see Section 2.25.3.). Analysis of the data was performed using the same programs.

2.20. ISOLATION AND ANALYSIS OF RNA

2.20.1. RNase-Free Buffer Preparation And Special Considerations For RNA Isolation

(a) Preparation of Glassware, Plasticware And Equipment: Sterile, disposable plasticware was considered to be essentially free of RNases, and was used for the preparation and storage of RNA without pretreatment. General laboratory glassware however, was treated by baking at 180°C for a minimum of 8 hours or alternatively filled with a solution of 0.1% (v/v) diethyl pyrocarbonate (DEPC, Sigma, USA, Cat. No. D-5758), a potent inhibitor of RNases. The DEPC-filled glassware was allowed to stand at 37°C for 2 hours, rinsed several times with sterile water and autoclaved for 30 minutes. The autoclave treatment, removes all traces of DEPC that might otherwise modify purine residues in RNA by carboxymethylation. The homogeniser probe (Ultra-turrax homogeniser [Omni-5000, USA]), was treated with a solution comprised of 0.1% (v/v) DEPC and 0.5% (w/v) SDS for a minimum of 5 minutes prior to washing the probe with copious quantities of sterile water to remove traces of DEPC. The poly prep chromatography columns (Bio-Rad, Cat. No. 731-1550) were alkali-treated prior to use by washing the column with a solution of 0.5 M NaOH. To remove residual alkali, the columns were subsequently washed with several column volumes of DEPC-treated water.

(b) Preparation of Solutions : All solutions were prepared in RNase-free glassware, using DEPC-treated and autoclaved water. Alternately, where possible, solutions were treated with 0.1% DEPC for a minimum of 12 hours at 37°C prior to autoclaving as above.

(c) Preparation of Oligo-dT Cellulose : oligo-dT cellulose (Boehringer Mannheim, Germany, Cat. No. 808229) was RNase-treated as follows; the cellulose was resuspended

in 5 ml of 0.5 ml NaOH and subsequently neutralised by washing the cellulose several times with DEPC-treated water and finally resuspended at 0.025 g/ml in Binding Buffer (refer below).

(d) STE Buffer : was comprised of 0.1 M NaCl, 10 mM Tris-HCl and 1 mM EDTA prepared with DEPC-treated water.

(e) Binding Buffer : was comprised of 0.5 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA and 0.1% SDS prepared with DEPC-treated water.

(f) Wash Buffer : was comprised of 0.1 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA and 0.1% SDS prepared with DEPC-treated water.

(g) Elution Buffer : was comprised of 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA and 0.1% SDS prepared with DEPC-treated water.

2.20.2. Preparation Of Polyadenylated (Poly A^+) Messenger RNA

Poly A^+ RNA was prepared essentially as described by Gonda *et al*, (1982). Routinely, 1-5 x 10^7 cells were collected and pelleted in a 50 ml polypropylene tube (Corning, NY, USA, Cat. No. 25331-50), and washed twice in PBS. Pelleted cells were either snap-frozen in liquid nitrogen and subsequently stored at -80°C, or immediately resuspended at 2-5 x 10^6 cells/ml in "STE buffer". Predigested proteinase K (BDH/Merck, Cat. No. 24568) and SDS was added to a final concentration of 200 µg/ml and 0.5% respectively, prior to homogenisation for 30-60 seconds at medium to high speed using an Ultra-turrax homogeniser (Omni-5000, USA). The mixture was subsequently incubated at 37°C for greater than 1 hour. The solution was made up to 0.5 M with respect to NaCl prior to the addition of 2 ml of 50% slurry of oligo-dT cellulose (see Section 2.20.1. for preparation). The oligo-dT cellulose was incubated with the cell lysate for greater than 2 hours with rotation, to facilitate the binding of poly-A^+ mRNA to the oligo-dT cellulose. The oligo-dT cellulose was pelleted by centrifugation at 10,000 x g, for 5 minutes, resuspended in 3 ml of "Binding buffer" and transferred to an alkali-treated poly prep chromatography column (Bio-Rad, Cat. No. 731-1550). Three column volumes (6 ml) of "binding buffer" was added to the column and allowed to slowly pass through under gravity. This was followed by the addition of 2 ml of "wash buffer" which

was allowed to pass through the column prior to the addition of 1.6 ml of "elution buffer". The eluate was collected in a siliconised, RNase-free Corex tube (Corning) and the poly-A^+ mRNA precipitated by the addition of 1/10 volume of 3 M sodium acetate (NaAc, pH 5.6) and 2.5 volumes of absolute ethanol and overnight incubation at -20°C. The mRNA was pelleted by centrifugation at 10,000 x g for 30 minutes (Beckman), at 4°C. The supernatant was aspirated and washed once in 70% ethanol to remove excess salt and subsequently dried under vacuum. The pellet was resuspended in 400 µl TE or Milli-Q water. The concentration was determined as described in Section 2.25.2., the absorbance values at 260 nm and 280 nm of each RNA sample were determined. The A_{260}/A_{280} ratios of the RNA samples were consistently in the range 1.6-2.0. The RNA was used immediately, or stored at -20°C following the addition of 1/10 volume of 3M NaAc and 2.5 volumes absolute ethanol.

2.20.3. Isolation Of Total RNA: "The RNAzol™ B Method"

Total RNA was extracted from tissues and cultured cell lines using the RNAzol™ B Method (Biotecx Laboratories, Inc., Houston, TX, USA, Cat. No. CS-103) which represents a modification of guanidinium isothiocyanate procedure described by Chomczynski and Sacchi (1987). For the isolation of RNA from whole tissues (ie. murine lymph nodes) the organ was first rinsed in PBS and subsequently homogenised in the presence of RNAzol™ B (2 ml per 100 mg of tissue) with numerous strokes in a glass homogeniser. Alternately, 1-5 x 10^7 cultured cells were collected (adherent cells were isolated by tryptic-detachment as described in Section 2.4.3.), pelleted and washed twice in PBS. The sedimented cells were lysed by the addition of 0.2 ml of RNAzol™ B per 10^6 cells and the RNA solubilised by passing the lysate several times through a pipette tip. The RNA was extracted by the addition of 1/10 volume of chloroform, mixed by vortexing and allowed to stand on ice for 5 minutes. The samples were subsequently centrifuged at 12,000 x g for 5 minutes to facilitate phase separation and the aqueous phase removed and transferred to a fresh 1.5 ml Eppendorf tube. The total RNA was then precipitated by the addition of an equal volume of isopropanol, and incubated on ice for 20 minutes. The RNA was pelleted by centrifugation at 12,000 x g for 20 minutes

at 4°C, washed in 75% (v/v) ethanol and dried under vacuum for approximately 10 minutes. The RNA pellet was resuspended in Milli-Q water or TE and the absorbance values at 260 nm and 280 nm of each RNA sample was determined on a Beckman UV spectrophotometer. The A_{260}/A_{280} ratios of the RNA samples were consistently in the range of 1.6-2.0. The relationship of one A_{260} unit equal to 40 µg/ml RNA was used in the calculation of RNA concentrations. The integrity of the RNA was examined by electrophoresis of a sample of the preparation on a 1% agarose/TBE gel. The RNA was used immediately, or stored at -20°C following the addition of 1/10 volume 3M NaAc and 2.5 volumes absolute ethanol.

2.20.4. Northern Hybridisation Analysis Of RNA

(i) Buffer And Reagent Preparation

(a) 10 x Running Buffer : was comprised of 20 mM 3-[N-Morpholino]propane-sulfonic acid (MOPS) (Sigma, USA, Cat. No. M-1254), 1 mM EDTA, 5 mM sodium acetate, pH 5.5. The pH was subsequently adjusted to 7.0 with the addition of NaOH pellets.

(b) Loading Buffer : was comprised of 0.25% bromophenol blue (BDH, UK, Cat. No. 20015), 0.25% xylene cyanol FF (Sigma, NSW, Australia, Cat. No. X 4126) and 20% Ficoll (Type 400, Pharmacia, Sweden, Cat. No. 17-0400-01) in DEPC-treated water.

(c) 1% RNA Agarose Gel : To prepare 100 mls of 1% agarose gels, 1 g of molecular biology grade, RNase-free agarose (IBI, Connecticut, USA, Cat. No. 70040) was dissolved in 72 ml of DEPC-treated water. After cooling to approximately 56°C, 10 ml of 10 x Running buffer (refer above), and 18 ml of 35% formaldehyde solution (final concentration 2.2 M; BDH, AnalR, Victoria, Australia, Cat. No. 10113) was added. Gels were subsequently electrophoresed in 1 x Running buffer containing 2.2 M formaldehyde.

(d) Deionised Formamide : To prepare deionised formamide, 20% (w/v) Analytical Grade mix bed resin AG 501-X8 (Bio-Rad, Cat. No. 142-6424) was added to formamide solution (BDH, AnalR, Victoria, Australia, Cat. No. 103264R) and stirred for several hours at room temperature. Mix bed resin was subsequently removed by filtration through Whatman No. 1 paper, aliquoted and stored at -20°C.

(e) Sample Buffer : 1 ml of RNA sample buffer was prepared by adding together 100 µl 10 x Running buffer, 500 µl deionised formamide, 178 µl 35% formaldehyde solution and 222 µl of DEPC-treated water.

(f) 20 x SSC : To prepare 1 litre of 20 x SSC, dissolved 175.3 g of NaCl and 88.2 g of sodium citrate in DEPC-treated water. The pH was subsequently adjusted to 7.0 and the volume made up to 1 litre.

(g) 100 x Denhardt's Reagent : 100 x Denhardt's reagent was prepared by dissolving 10 g Ficoll Type 400 (Pharmacia, Sweden, Cat. No. 17-0400-01), 5 g polyvinylpyrrolidone (Sigma, NSW, Australia, Cat. No. P 6755) 5 g bovine serum albumin (BSA: Cohn fraction V; Sigma, St Louis, MO) in 500 ml DEPC-treated water.

(h) Prehybridisation Solution : 20 ml of Prehybridisation Solution contained the following: 10 ml deionised formamide; 5 ml of 20 x SSC; 1 ml 100 x Denhardt's reagent; 200 µl 10% SDS; 2 ml of 100 mM HEPES, pH 7.1; 200 µl of 100 mM EDTA, pH 7.5; 200 µl of 200 mM sodium pyrophosphate, pH 7.0; 200 µl of 10 mg/ml, sheared and boiled salmon sperm DNA and 30 µl of 10 mg/ml tRNA (Gibco/BRL, Cat. No. 15401-011). Routinely 5 ml of prehybridisation solution was used per 100 cm^2 membrane in a Hybaid Oven hybridisation bottle.

(ii) Gel Electrophoresis And Northern Hybridisation

Routinely, 2 µg of polyadenylated mRNA or 10 µg of total RNA isolated as described in Sections 2.20.2. and 2.20.3. respectively, was pelleted by centrifugation at 12,000 x g, 4°C for 15 minutes. The RNA was subsequently washed in 70% ethanol, vacuum dried and denatured in the presence of 12 µl of "sample buffer" and 2 µl of 20 µg/ml ethidium bromide. Samples were heated to 60°C for 5 minutes prior to loading on a denaturing 1% agarose gels (refer above for preparation) and electrophoresis in "running" buffer containing 2.2 M formaldehyde. Following electrophoresis, the gels were washed for 30 minutes in water, and ethidium bromide-stained RNA visualised and photographed after ultraviolet illumination. The electrophoresed RNA was subsequently transferred overnight by capillary action in 10 x SSC onto Hybond N^+ (Amersham, Poole, UK, Cat. No. RPN 303B) filters. Following transfer, the filters were washed in 2 x SSC,

air-dried and the RNA covalently cross-linked to the filters by exposure to 0.4 J/cm^2 of UV radiation in a Stratagene UV Stratalinker™ 1800. Filters were prehybridised for 4-16 hours at 42°C in a prehybridisation solution comprised of 50% deionised formamide, 5 x SSC (0.34 M NaCl, 75 mM sodium citrate, pH 7.0), 5 x Denhardt's solution, 0.1% SDS, 10 mM HEPES, 1 mM EDTA, 0.05% (2 mM) sodium pyrophosphate, and 200 µg/ml of sheared salmon sperm DNA. Following the addition of the appropriate denatured, radiolabelled probe (please refer to Section 2.18.8.), hybridisations were carried out for a further 16 hours at 42°C. Filters were washed as described in Section 2.18.11. After washing, filters were air-dried and exposed to Kodak X-Omat autoradiography film, with intensifying screens at -80°C as required.

2.21. SYNTHESIS AND PURIFICATION OF OLIGONUCLEOTIDES

Unless otherwise stated, the following synthetic DNA primers were synthesised "in house" on an Applied Biosystems 391 DNA synthesiser, by A. Mangos and J. Hall, Molecular Pathology Unit, Division of Haematology, Institute of Medical and Veterinary Science, Adelaide, Australia.

2.21.1. De-Protection And Purification Of Oligonucleotides

Oligonucleotides were cleaved from the synthesis columns by inserting a 1 ml syringe at each end of the column. 1 ml of ammonium hydroxide [30% (v/v), BDH/Merck, Cat. No. 1.05428] was subsequently applied to the column, and the plungers of the syringes were moved "to and fro" in order to fill the column with ammonium hydroxide. This was incubated at room temperature for 20 minutes at which time, the ammonium hydroxide was withdrawn and placed into a 2 ml screw capped tube. The above procedure was repeated three times and the oligonucleotides deprotected by the overnight incubation at 56°C. The ammonium hydroxide was removed by vacuum desiccation and the oligonucleotide pellet was dissolved in 200 µl of Milli-Q water and the concentration determined by spectrophotometry as described in Section 2.25.2. and the molarity determined using Formula 2.21.1 below. If full length oligonucleotides were required for site-directed mutagenesis, oligonucleotides were "trityl on" synthesised.

Following cleavage and deprotection, the oligonucleotides were further purified with oligonucleotide purification columns (OPC) as detailed below.

Formula 2.21.1:

Concentration of Oligonucleotide (M) = x μg x 10^{-6} /[MWt of oligonucleotide / 1 x 10^{-3}]

MWt of dNTPs: A=347.2, T=332.2, G=363.2 and C=323.2

2.21.2. OPC Purification Protocol

Prior to the addition of the deprotected oligonucleotide to the Oligonucleotide Purification Column (OPC, Applied Biosystems, Foster City, California, USA, Part No.. A9H053), the cartridge was flushed with 5 ml of HPLC grade acetonitrile, followed by 5 ml of 2 M triethylamine acetate (both reagents from Applied Biosystems, Part No.s 400262 and 400613, respectively). The ammonium hydroxide solution containing the cleaved, deprotected, "trityl-on" crude oligonucleotide was diluted with one-third volume of Milli-Q water, prior to loading approximately 20-40 OD units onto the OPC cartridge with a 5 ml syringe and gently pushed through the cartridge. The eluted fraction was retained and reloaded onto the cartridge and the final eluted fraction was preserved and stored at -20°C. The cartridge was subsequently flushed thrice with 5 ml of diluted ammonium hydroxide (1:10 dilution of concentrated ammonium hydroxide), and then twice with 5 ml sterile Milli-Q water. The OPC-bound oligonucleotide was then detritylated with the addition of 5 ml of 2% (v/v) trifluoroacetic acid solution (Applied Biosystems, Part No. 400137), and the column subsequently washed twice with 5 ml of Milli-Q water. The purified, detritylated oligonucleotide was then eluted by flushing the cartridge with 1 ml of 20% acetonitrile solution. The acetonitrile solution was removed by vacuum desiccation and the oligonucleotide pellet dissolved in 200 μl of Milli-Q water prior to concentration determination by spectrophotometry as described in Section 2.25.2.

2.21.3. Synthetic Oligonucleotides

The oligonucleotides described here, were synthesised and prepared as described in Section 2.21.1.

(i) Oligonucleotides for Mutagenesis:

(a) Ampicillin Repair Oligonucleotide, Phosphorylated (27'mer) :

5'-d(GTT GCC ATT GCT GCA GGC ATC GTG GTG)-3'

(b) MUT 59 Primer, Phosphorylated (36'mer) :

5'-d(ACA GCC GTC AAT TGT GGA TCC GAT TTT GAT GCG TGT)-3'

(containing a *Bam*HI restriction enzyme site, underlined)

(ii) Oligonucleotide Primers For PCR Amplification:

Oligonucleotides directed against pRUF.*neo*

(a) RCF1 (Forward):

5'-d(TTG GGG GAC TCT GCT GAC CAC)-3', which corresponds to the retroviral vector *gag* sequence 98 bp 5' of the MCS

(b) RCR1 (Reverse):

5'- d(CTT GCA AAA CCA CAC ACT GCT CG)-3', which corresponds to the MC1*neo* sequence immediately adjacent to the 3' end of the MCS, approximately 47 bp from the *Xho*I restriction site

(c) RCR2 (Reverse):

5'-d(ATA GCC TCT CCA CCC AAG CG)-3', which corresponds to the MC1*neo* sequence approximately 372 bp 3' from the *Xho*I restriction site.

(iii) Oligonucleotides directed against PSGL-1:

(a) PSGL-1/FP :

5'-d(GCC CTC GAG GAA GCT TTC CCA TGC TCT GCT G)-3'

(b) PSGL-1/RP :

5'-d(AGC GGA TCC GAG GTG GGG TCT TGC CAA AAC AG)-3',

which span the 5' (nucleotides, 15-44) and the 3' (nucleotides, 1282-1304) untranslated regions of the published sequence (Sako *et al*, 1993) respectively and amplify a 1297 bp fragment. *Xho*I and *Bam*HI restriction sites present in PSGL-1/FP and PSGL-1/RP respectively, are underlined.

(iv) Oligonucleotides directed against CD34:

(a) oli 5 (Forward):

5'-d(CTC CAG AGA GAC AAC CTT GAA G)-3' and

(b) oli 6 (Reverse):

5'-d(GGA GTT TGC TGG AAA TTT CTG)-3',

which span exon 3 (654-673) and exon 6 (1040-1051) of the published sequence (He *et al*, 1992), respectively. Oligonucleotides oli 5 and oli 6 were generously provided by Dr. B. Davis, Geraldine Brush Cancer Research Institute, San Francisco, CA, USA, and amplify a 386 bp fragment under the conditions described in Section 2.22.2.

(v) Oligonucleotides directed against GlyCAM-1:

(a) GlyCAM-1/FP :

5'-d(AGC AAG CTT ATG AAA TTC TTC ACT GTC)-3'

(b) GlyCAM-1/RP :

5'-d(AGC GGA TCC TCA TGA CTT CGT GAT ACG)-3',

which span the 5′ (nucleotide 23-40) and 3′ (nucleotide 460-478) of the published sequence (Lasky *et al*, 1992), amplify a 458 bp fragment under the conditions described in Section 2.22.2. *Hind*III and *Bam*HI restriction sites present in GlyCAM-1/FP and GlyCAM-1/RP respectively, are underlined.

(vi) Oligonucleotide Primers For Sequencing:

(a) Universal sequencing primer (USP) :

5'-d(GTA AAA CGA CGG CCA GT)-3'

(b) Reverse sequencing primer (RSP) :

5'-d(CAC ACA GGA AAC AGC TAT GAC CAT G)-3'

(c) M13 RSP :

5'-d(AAC AGC TAT GAC CAT G)-3'

(d) M13 :

5'-d(GTA AAA CGA CGG CCA GT)-3'

USP, RSP, M13 RSP and M13 were utilised for sequencing when vectors contained the complementary binding sites.

(vii) Oligonucleotide Primers For Sequencing From pRUF.*neo*:

(a) Primer 1 (Reverse Primer; anneals to multiple cloning site) :

5'-d(GGA TCA GAT CTA AGC TTG TTA ACG AAT TC)-3'

(b) Primer 2 (Forward Primer; anneals to vector) :

5'-d(ATC GCA GCT TGG ATA CAC)-3'

(c) Primer 1B (Reverse Primer; anneals to multiple cloning site) :

5'-d(GGA TCA GAT CTA AGC TTG TT)-3'

(d) RCF1 (Forward):

5'-d(TTG GGG GAC TCT GCT GAC CAC)-3' , which corresponds to the retroviral vector *gag* sequence 98 bp 5' of the MCS.

(e) RCR1 (Reverse):

5'-d(CTT GCA AAA CCA CAC ACT GCT CG)-3', which corresponds to the MC1*neo* sequence immediately adjacent to the 3' end of the MCS, approximately 47 bp from the *Xho* I restriction site.

(f) RCR2 (Reverse):

5'-d(ATA GCC TCT CCA CCC AAG CG)-3', which corresponds to the MC1*neo* sequence approximately 372 bp from the *Xho* I restriction site.

(viii) Miscellaneous Oligonucleotides

(a) Oligo-dT Reverse Transcription Primer:

5'-d(GAG AGA GAG AGA GAG AGA GAC TCG AGC GGC CGG CTT TTT TTT TTT TTT TTT T)-3' (containing a *Xho* I restriction enzyme site, underlined).

(b) Oligonucleotides For Sequencing CD59 (HCC-1 Clone):

HCC-1/FP1 (Forward):

5'-d(CGC CAG GTT CTG TGG ACA)-3', spans nucleotides 6-23 of the published sequence (Davies *et al*, 1989).

HCC-1/FP2 (Forward):

5'-d(TCC TGT TGG GAA AGA ATA)-3', spans nucleotides 513-530 of the published sequence (Davies *et al*, 1989).

HCC-1/RP1 (Reverse):

5'-d(GTC ACA TGT AGT AGA GTG)-3', spans nucleotides 1175-1192 of the published sequence (Davies *et al*, 1989).

HCC-1/RP2 (Reverse):

5'-d(CTT CCC TGC AAA CAG GAC)-3', spans nucleotides 588-605 of the published sequence (Davies *et al*, 1989).

(c) Oligonucleotides For Sequencing MGC-24v:

MGC-24/FPA (Forward):

5'-d(GCT GAG GAC ACG ATG TCG)-3', spans nucleotides 2-19 of the MGC-24v.

MGC-24/FPB (Forward):

5'-d(ACG ACA GAC TTC TGT TCC)-3', spans nucleotides 326-343 of the MGC-24v.

MGC-24/RPA (Reverse):

5'-d(CAG TGA GTT ACA CAA ATG)-3', spans nucleotides 584-601 of the MGC-24v.

MGC-24/RPB (Reverse):

5'-d(TGA ACA ATA ATC TGT TAT)-3', spans nucleotides 1142-1159 of the MGC-24v.

2.22. REVERSE TRANSCRIPTION (RT) POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION OF DNA

2.22.1. Synthesis Of Complementary DNA (cDNA).

Total cellular RNA was prepared from cell lines or tissues with RNAzol, as described in Section 2.20.3. Routinely, 2 µg of total RNA was reverse transcribed into single-stranded cDNA at 37°C for 120 minutes in a 50 µl reaction containing 40 mM KCl, 50 mM Tris-HCl pH 8.3, 8 mM $MgCl_2$, 0.5 mM dNTPs, 100 ng of poly dT_{12-15} primer (please refer to section 2.21.3., oligonucleotide (viii) (a)), 10 units of RNasin (Promega), and either 100 units of MMLV reverse transcriptase (Life Technologies, Australia) or 30 units of AMV reverse transcriptase (Pharmacia, USA).

2.22.2. Polymerase Chain Reaction (PCR) Amplification Of cDNA

1 to 5 µl of each cDNA synthesis reaction was utilised as template DNA in each PCR. Routinely, the cDNA mixture was added to a 500 µl microcentrifuge tube, (Perkin Elmer), to which 5 µl of 10 x PCR amplification buffer (10 x PCR buffer; 670 mM Tris

HCl pH 8.8, 166 mM $(NH_4)_2SO_4$, 4.5% Triton-X100, 2 mg/ml gelatin, 20 mM $MgCl_2$, 2 mM each dNTP), 100ng of the appropriate "forward" and "reverse" primer sets (please refer to Section 2.21.3.) and sterile Milli-Q water was added to a final volume of 50 µl. This mixture was heated to 96°C for 5 mins, snap chilled, and 2.5 units of Amplitaq DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA) was added. Reaction mixes were overlayed with mineral oil and amplification achieved by incubation in a Perkin-Elmer/Cetus thermal cycler. Typical cycling conditions were 94°C/1-2 min, 55-70°C/2 min, 72°C/2-4 min, for 35-40 cycles, with a final 10 minute extension performed at 72°C. Following amplification, 10 µl of each reaction mixture was analysed by 1.5% agarose gel electrophoresis, and visualised by ethidium bromide staining as described in Section 2.18.2.

2.22.3. PCR Recovery Of Proviral cDNA Inserts From Genomic DNA

(a) Conventional PCR : Typically 1 µg of genomic DNA (prepared as described in Section 2.17.2.) was added to a 500 µl microcentrifuge tube (Perkin-Elmer, USA), to which 5 µl of 10 x PCR amplification buffer (10 x PCR buffer; 670 mM Tris HCl pH 8.8, 166 mM $(NH_4)_2SO_4$, 4.5% Triton X-100, 2 mg/ml gelatin, 20 mM $MgCl_2$, 2 mM each dNTP), 100 ng of the forward oligonucleotide *RCF1* [please refer to Section 2.21.3 (ii)(a)], and 100 ng of either of the two reverse oligonucleotide *RCR1* [please refer to Section 2.16.3 (ii) (b)] or *RCR2* [please refer to Section 2.21.3 (ii) (c)] were added. This reaction mixture was denatured at 96°C for 5 mins, snap chilled, and 2.5 units of Amplitaq DNA Polymerase (Perkin Elmer, USA) was added. Reaction mixes were overlayed with mineral oil and amplification achieved by incubation in a Perkin-Elmer/Cetus thermal cycler. Typical cycling parameters were as follows; denaturation at 94°C for 1 min, annealing at 60°C for 2 min and extension at 72°C for up to 5 minutes for 40 cycles, with a final 10 minute extension at 72°C. Following amplification, 10 µl of each reaction mixture was analysed by 1% agarose gel electrophoresis, and visualised by ethidium bromide staining as described in Section 2.18.2.

(b) Long Range PCR : To amplify long cDNA inserts, extra-long range (XL) PCR was employed in accordance with the manufacturer's recommendations, (reagents kindly provided by Dr. N. Samaras, Perkin Elmer, Scoresby, Victoria, Australia). Briefly, 500 ng of genomic DNA was resuspended in a 20 µl "lower reagent mix", comprised of 1 x XL Buffer, 200 µl dNTPs, 1.1 mM $Mg(OAc)_2$ and 50 ng of each primer, *RCF1* and *RCR1* (see above). Following the addition of one Ampliwax™ PCR Gem 100 (Perkin Elmer) per reaction tube, the wax was melted by heating the tubes at 75°-80°C for 5 minutes and then allowed to set at room temperature. The 30 µl "upper reagent mix" comprising of 1 x XL Buffer and 2 units of the r*Tth* DNA Polymerase was carefully pipetted above the wax layer prior to amplification in a Perkin-Elmer/Cetus thermal cycler. Typical cycling parameters were denaturation at 93°C for 1 min, annealing and extension at 60°C for up to 7 minutes for 40 cycles, with a final 10 minute extension at 72°C. Amplification products were visualised as described above.

2.23. *IN VITRO* MUTAGENESIS OF CD59 cDNA

2.23.1. Altered Sites™ *in vitro* Mutagenesis System : An Overview

To examine the effect of mutagenising the solitary N-linked glycosylation consensus sequence (ser-20 to gly-20) of CD59, the Altered Sites™ *in vitro* Mutagenesis System (Promega Corporation, USA, Cat. No. Q6210) was utilised. This kit, employed a phagemid vector pALTER™ (please refer to Appendix A, Map# 5) which contained two antibiotic resistance genes. The tetracycline resistance gene was always functional, whilst the Ampicillin resistance gene had been inactivated. An Ampicillin repair oligonucleotide (please refer to Section 2.21.3. (i) (a)), when annealed to single stranded DNA (ssDNA), restored Ampicillin resistance to the mutant strand during the mutagenesis reaction. This oligonucleotide, was annealed to the ssDNA at the same time as the mutagenic oligonucleotide (please refer to Section 2.21.3. (i) (b)), and the subsequent synthesis and ligation of the mutant strand linked the two. The DNA was subsequently transformed into a repair minus strain of *E.coli*, BMH 71-18 mut S (please refer to Section 2.16.5. (e)), and the cells grown in Ampicillin, yielding large numbers of colonies. A second round of transformation in the *E.coli* strain JM109 (please refer to Section 2.16.5. (d)), ensured proper segregation of mutant and wild type plasmids and resulted in a moderate proportion of mutants.

2.23.2. Cloning Of CD59 Into The pALTER™ Vector

A 651 base pair (bp) *Sma*I and *Sph*I restriction fragment of the CD59 cDNA (PCR amplification product derived from an FDC-P1 clone specifically expressing the HCC1 antigen, please refer to Section 2.22.3.), was ligated (as described in Section 2.18.7.) into the 5680 bp pALTER™ vector via the unique *Sma* I and *Sph* I sites present in the multiple cloning site. Following ligation overnight, reactions to be transformed were phenol/chloroform extracted as described in Section 2.18.4., and resuspended in 10 µl Milli-Q water. In an electroporation cuvette, 2µl of the ligation reaction and 40 µl of thawed electrocompetent E.coli JM109 cells were added and electroporated as described in Section 2.16.7. The transformed cells were plated onto LB-agar containing 15 µg/ml of

tetracycline with using a sterile glass spreader. The agar plates were incubated at 37°C overnight and selection of recombinant colonies was simplified by the use of the β-galactosidase reporter system, by the addition of 50 µl of each of 50 mg/ml BCIG and 100 µl of 100 mM IPTG prior to plating of the transformed cells.

2.23.3. Preparation Of Phagemid Single-Stranded DNA

An individual tetracycline-resistant colony was inoculated into 2 ml of TYP broth (please refer to Section 2.16.2. (c)) containing 15 µg/ml Tetracycline, and incubated at 37°C overnight with vigorous shaking. 5 ml of TYP broth, containing 15 µg/ml tetracycline, was subsequently inoculated with 100 µl of the overnight culture, and incubated at 37°C for 30 minutes with vigorous shaking prior to the addition of 10 µl of R408 helper phage (multiplicity of infection [m.o.i.] of 10, ie. 10 helper phage particles per bacterial cell). At the time of helper phage addition, K_2HPO_4 was added to a final concentration of 20 mM to improve ssDNA yields, and the culture was incubated overnight at 37°C with shaking. The culture supernatant (containing the phage) was harvested following pelleting of the cells at 12,000 x g for 15 minutes, and transferred to a fresh tube and the supernatant centrifuged again as before. The phage were than precipitated by the addition of 0.25 volumes of phage precipitation solution (3.75 M ammonium acetate, pH 7.5, 20% polyethylene glycol [PEG, MW 8,000]) . The solution was chilled on ice for 30 minutes and subsequently centrifuged for 15 minutes at 12,000 x g and the supernatant thoroughly drained. The pellet was resuspended in 400 µl of 1 x TE buffer, and transferred to a 1.5 ml microcentrifuge tube. To lyse the phage and to remove residual PEG, 0.4 ml of chloroform : isoamylalcohol (24 : 1) was added, vortexed for a 1 minute and microcentrifuged for 5 minutes at 12,000 x g. The upper aqueous phase (containing the phagemid DNA) was transferred to a fresh tube, and phenol:chloroform extracted twice as described in Section 2.18.4. The extracted phagemid DNA was transferred to a fresh tube and precipitated with the addition of 0.5 volumes of 7.5 M ammonium acetate and 2 volumes of absolute ethanol. The sample was incubated at -20°C for 30 minutes, prior to microcentrifugation at 12,000 x g for 5 minutes. The DNA

pellet was washed with 70% ethanol as previously described and resuspended in 20 µl of Milli-Q water.

2.23.4. Mutagenesis Procedure

The mutagenesis reaction, involved the annealing of the Ampicillin repair oligonucleotide (please refer to Section 2.21.3. (i)(a)), and the MUT59 mutagenic oligonucleotide (please refer to Section 2.21.3. (i) b)), followed by synthesis of the mutant strand with T4 DNA polymerase. The heteroduplex DNA was then transformed into the repair minus E.coli strain BMH 71-18 mut S. Mutants were selected by overnight growth in the presence of Ampicillin. Plasmid DNA was then isolated from mutant clones, and used to transform the E.coli strain, JM109.

(a) Annealing Reaction And Mutant Strand Synthesis

To anneal the Ampicillin repair and MUT59 mutagenic oligonucleotides to the recombinant pALTER™ single stranded DNA, the following reagents were placed into a 1.5 ml microcentrifuge tube; 1 µl (0.05 pmol) of the CD59-recombinant pALTER-1 ssDNA, 1 µl (0.25 pmol or 2.2 ng) of the phosphorylated-Ampicillin repair oligonucleotide, 1 µl (1.25 pmol) of the phosphorylated, MUT59-mutagenic oligonucleotide (please refer to Section 2.18.10.), and 2 µl of 10 x "Annealing Buffer" (200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 500 mM NaCl). The volume of the reaction was adjusted to 20 µl, with the addition of Milli-Q water, and heated to 70°C for 5 minutes, prior to allowing the reaction to cool slowly to room temperature (15-20 minutes).

The 20 µl annealing reaction was placed on ice, and the following reagents added; 3 µl of the "Synthesis Buffer" (100 mM Tris-HCl, pH 7.5, 5 mM dNTPs, 10 mM ATP and DTT), 1 µl T4 DNA polymerase (10 units/µl), 1 µl T4 DNA ligase (2 units/µl) and 5 µl of sterile Milli-Q water. The reaction was incubated at 37°C for 90 minutes to allow mutant strand synthesis and ligation.

(b) Transformation Of BMH 71-18 Mut S And pALTER™ Plasmid Miniprep Procedure

The repair minus strain of *E.coli* BMH 71-18 Mut S, was made electrocompetent by the procedure outlined in Section 2.16.6. The annealed and ligated heteroduplex pALTER™ DNA was phenol/chloroform extracted and ethanol precipitated as

described in Section 2.18.4. In an electroporation cuvette, 2µl of the pALTER™ construct and 40 µl of thawed electrocompetent *E.coli* BMH 71-18 Mut S cells (refer to Section 2.16.5. (e)) were added and electroporated as described in Section 2.16.7. Following transformation, the cells were cultured in 4 ml of LB-broth at 37°C for 1 hour with shaking, after which Ampicillin was added to a final concentration of 125 µg/ml and the incubation continued overnight. The cells were pelleted by centrifugation and plasmid DNA prepared as described in Section 2.17.

(c) Transformation Into JM109

A second round of transformation in the *E.coli* strain JM109, was required to ensure proper segregation of mutant and wild type plasmids. In an electroporation cuvette, 2µl of the plasmid DNA and 40 µl of thawed electrocompetent E.coli JM109 cells (refer to Section 2.16.5. (d)) were added and electroporated as described in Section 2.16.7. The transformed cells were spread onto LB-agar plates containing 125 µg/ml of Ampicillin, using a sterile glass spreader. The agar plates were incubated at 37°C overnight and selection of recombinant colonies was simplified by the use of the β-galactosidase reporter system by the addition of 50 µl each of 50 mg/ml BCIG and 100 µl of 100 mM IPTG to the selection plates.

(d) Analysis Of Transformants And Construction of MUT59 And Wild Type (WT) Retroviral Expression Vectors

Mutant CD59 and WT constructs were easily distinguishable due to the incorporation of a *Bam* HI restriction endonuclease site in the sequence of the MUT59 mutagenesis oligonucleotide (please refer to Section 2.21.3. (i)(b)). To confirm this, automated sequence analysis (as detailed in Section 2.19.1.) was performed, utilising the M13 RSP and M13 priming sites present in the pALTER™ vector. To examine protein expression, the WT and mutant isoforms of CD59 were subcloned into the unique *Hpa*I and *Hind*III sites of pRUF.*neo* (please refer to Appendix A, Map# 3) as described in Section 2.18.7. DNA for transfection was prepared as described in Section 2.17.1., and introduced into the transient ecotropic virus-producing cell line BOSC-23 by calcium phosphate transfection as described in Section 2.26.4. (a). The murine factor-dependent cell line, FDC-P1 was infected with recombinant retrovirus as described in Section 2.26.4.

(b), and protein expression was assessed by indirect immunofluorescence and flow cytometry as described in Section 2.10.2.

2.24. LIBRARY CONSTRUCTION

2.24.1. First Strand cDNA Synthesis

Retroviral library construction was performed as described previously (Rayner and Gonda, 1994; Zannettino *et al*, 1996). Poly(A)$^+$ mRNA was isolated as described in Section 2.20.2. from tertiary passaged human bone marrow derived stromal cultures (please refer to Section 2.4.7.).

First-strand cDNA was synthesised in a standard 25 µL reaction (Huse and Hansen, 1988), with 2 µg of poly(A)$^+$ mRNA (pre-heated for 5 minutes at 65°C and snap-cooled on ice to eliminate secondary structure), 1 µg of the reverse transcription oligonucleotide primer [GA]$_{10}$ CTC GAG CGG CCGG CTT (T)$_{16}$ (containing a *Xho*I restriction enzyme site) and incubated for 1 hour at 37°C in a reaction mixture containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 8 mM dithiothreitol (DTT), 4 mM sodium pyrophosphate, 36 units of RNA Guard (Pharmacia, Sweden), 400 µM dATP, 400 µM dGTP, 400 µM dTTP (Pharmacia, Sweden), 200 µM 5-methyl-dCTP (Boehringer-Mannheim, Germany) and 200 units of Superscript Reverse Transcriptase (Life Technologies, Australia). The inclusion of 5-methyl-dCTP ensured methylation of internal *Xho*I restriction sites.

To assess the efficiency of first-strand cDNA synthesis, 5 µl of the reaction mix was immediately removed and placed in a fresh tube. To this, 1 µl of ^{32}P-dATP (3000 Ci/mMole) was added and the reaction was allowed to proceed for 1 hour at 37°C. The cDNA product size (as measured by radionucleotide incorporation) was subsequently analysed by alkaline agarose gel electrophoresis as described by Sambrook *et al* (1989) and autoradiography.

2.24.2. Second Strand cDNA Synthesis

To the first strand cDNA reaction the following components were added: 32 µl of 5 x second-strand cDNA reaction buffer [94 mM Tris-HCl, 453 mM KCl, 23 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$], 4 µl of second-strand deoxynucleoside triphosphates (dNTPs; 10 mM dATP, dGTP, dTTP and 26 mM dCTP) and 6 µl of 100 mM dithiothreitol (DTT). Milli-Q water to a final volume of 160 µl was added prior to the addition of 32 units of

Escherichia coli DNA polymerase I and 0.8 units of *E. coli*-derived RNase H (both from Pharmacia). Second-strand synthesis was allowed to proceed for 2 hours at 16°C at which time the cDNA was ethanol precipitated and resuspended in 43 µl of Milli-Q water in preparation for "flushing" of the ends.

To assess the efficiency of second-strand cDNA synthesis, 5 µl of the reaction mix was immediately removed and 1 µl of ^{32}P-dATP (3000 Ci/mMole) was added. The reaction was allowed to proceed for 2 hours at 16°C, prior to assessing radionucleotide incorporation and ds cDNA synthesis by alkaline agarose gel electrophoresis and autoradiography.

2.24.3. "Blunt Ending" Or "Flushing" Of Double Stranded (ds) cDNA And The Addition Of Cloning Adaptors

To the cDNA, 5 µl of 10 x T4 polymerase buffer [330 mM Tris-acetate, pH 8.5, 660 mM potassium acetate, 100 mM magnesium acetate, 5 mM DTT and 1 mg/ml BSA] and 1 µl of 10 mM dNTP cocktail was added. This was followed by the addition of 8 units of T4 DNA polymerase (Promega Corporation, USA), at which time the reaction was incubated at 37°C for 10 minutes. Polymerase activity was terminated by heating the reaction mixture to 75°C for 30 minutes before adding adaptors to the ends of the ds-cDNA. The reaction was chilled on ice for 5 minutes prior to the addition of 5 µl 10 mM ATP, 5 µl *Bam* HI-*Not* I adaptor (optical density of 0.1 at 260 nm) and 8 units of T4 DNA ligase (all from Pharmacia). The reaction was allowed to proceed for 16 hours at 14°C. In preparation for cloning, the ligase was heat-inactivated as described above and the cDNA phosphorylated by the addition of 2.5 µl of 10 mM ATP, 20 units of T4 polynucleotide kinase and incubation at 37°C for 30 minutes.

2.24.4. Preparation Of ds-cDNA For Cloning

To prepare for cloning, the cDNA was digested with the *Xho* I restriction enzyme. Firstly, the volume of the kinased cDNA was adjusted to 100 µl with the addition of 33 µl of Milli-Q water followed by 2.5 µl of 2 M NaCl, to give a final salt concentration of 150 mM. 20 units of *Xho* I restriction enzyme (Pharmacia) were then added and the

reaction mixture was incubated at 37°C for 3 hours. The cDNA was then phenol extracted and size-selected using a Sephacryl S-400 spin column (Pharmacia) according to the manufacturer's recommendations.

2.24.5. Cloning ds-cDNA Into The Retroviral Vector pRUF.*neo*

The retroviral vector pRUF.*neo* (please refer to Appendix A, Map #3), was digested with *Bam*HI and *Xho*I restriction enzymes to allow for the directional cloning of the cDNA pool into the unique sites present in the multiple cloning site (MCS). Digestion was performed as follows; 5 µg of the supercoiled pRUF.*neo* vector was resuspended in Milli-Q water to a final volume of 76 µl, before the addition of 20 µl 10 x One-Phor-All Plus buffer [100 mM Tris-acetate (pH 7.5), 100 mM magnesium acetate and 500 mM potassium acetate, Pharmacia] to provide an optimal (2 x) salt concentration for both restriction enzymes. Two microlitres (20 units) of both *Bam*HI and *Xho*I restriction enzymes were added and the reaction was allowed to proceed for 2 hours at 37°C. The entire restriction digest was resolved by 0.8% agarose gel electrophoresis and linearised vector excised from the gel and purified using BRESAclean™ (Bresatec, Adelaide, Australia) according to manufacturers' recommendations. The cDNA and vector were quantitated by the "ethidium dot method" as described by Sambrook *et al* (1989) and typically 40 ng of cDNA (average size approximately 2 kb) was ligated into 50 ng of vector in a 10 µl reaction volume comprising 1 x ligation buffer [30 mM Tris-HCl, pH 7.8; 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP] and 3 Weiss units of T4 DNA ligase (Promega). Following overnight incubation at 12°C, the ligation reaction was phenol extracted, ethanol precipitated and resuspended in 10 µl Milli-Q water.

2.24.6. Transformation And Amplification Of Plasmid Library

To amplify the library, multiple 40 µl aliquot's of *E. coli* DH10B electrocompetent cells (Life Technologies) were transformed with 2 µl of the ligation reaction by electroporation, essentially as described by Rayner and Gonda (1994). Following electroporation, cells were immediately dispensed into 1 ml of SOC medium and allowed to recover for 60 minutes at 37°C, with shaking. Each transformation was then "plated

out" onto two 150 mm LB-selection plates (100 μg/ml Ampicillin) and an appropriate dilution was made to assess the number of colonies derived per transformation. The resultant colonies were harvested by gentle scraping (facilitated by the addition of Luria broth), pooled and pelleted by low-speed centrifugation. Plasmid DNA was isolated by standard alkaline lysis and supercoiled DNA for transfection of virus packaging cell line was prepared by CsCl sedimentation as described by Sambrook *et al* (1989).

2.25. MISCELLANEOUS METHODS

2.25.1. Size Determination Of Nucleic Acid Fragments

(a) DNA: The size of restriction fragments were determined by comparing their relative mobilities in agarose gels with those of DNA molecules of known size. The molecular weight markers used routinely were (i) *Hind* III digested bacteriophage lambda (λ) DNA (Boehringer Mannheim, Cat. No. DMW-L1), (ii) *Eco* R1 digested *Bacillus subtillus* phage SPP1 DNA (Bresatec, Adelaide, Australia, Cat. No. DMW-S1) and (iii) 1 kb ladder (Life Technologies, Australia, Cat. No. 5615SB). The estimated sizes of these molecular markers in kilobases are detailed in the Table 2.25.1.

(b) RNA: The size of specific RNA transcripts were made in comparison to 0.24-9.5 kb RNA ladder (Life Technologies, Australia, Cat. No. 15620-016). Please refer to Table 2.25.1., for the detailed sizes of molecular weight markers.

2.25.2. Determination Of DNA, RNA And Synthetic Oligomer Concentration

(a) DNA: The concentration of DNA in solution was determined by measuring the absorption at 260 nm on a Beckman UV spectrophotometer (Beckman Instruments, Mt. Waverley, Victoria, Australia), assuming that an A_{260} of 1.0 represents 50 μg/ml of DNA. Alternatively, the sample was electrophoresed in agarose gels and the intensity of the sample's ethidium bromide-stained bands were compared with the intensities of bands containing known concentrations of DNA standards.

(b) RNA: The concentration of RNA in solution was determined by measuring the absorption at 260 nm on a Beckman UV spectrophotometer as described above, assuming that an A_{260} of 1.0 represents 40 μg/ml of DNA.

Table 2.25.1. Estimation Of Molecular Weight Of DNA And RNA Fragments: Size Determination Markers (sizes in kilobases).

Fragment Number	*Hind* III λ Phage[1]	*Eco* R1 SPP-1 Bacteriophage[2]	1 kb ladder[3]	RNA Markers[4]
1	23.1	8.51	12.216	9.50
2	9.4	7.35	11.198	7.50
3	6.6	6.11	10.180	4.40
4	4.37	4.84	9.162	2.40
5	2.3	3.59	8.144	1.35
6	2.0	2.81	7.126	0.24
7	0.564	1.95	6.108	-
8	0.125	1.86	5.090	-
9	-	1.51	4.072	-
10	-	1.39	3.054	-
11	-	1.16	2.036	-
12	-	0.98	1.636	-
13	-	0.72	1.018	-
14	-	0.48	0.517	-
15	-	0.36	0.506	-
16	-	-	0.396	-
17	-	-	0.344	-
18	-	-	0.298	-
19	-	-	0.220	-
20	-	-	0.201	-
21	-	-	0.154	-
22	-	-	0.134	-
23	-	-	0.075	-

- 1. Boehringer Mannheim, Mannheim, Germany.
- 2. Bresatec, Adelaide, South Australia, Australia.
- 3. Life Technologies, Glen Waverley, Victoria, Australia.
- 4. Life Technologies, Glen Waverley, Victoria, Australia.

(c) Synthetic Oligonucleotides: The concentration of single stranded, synthetic oligonucleotide DNA in solution was determined by measuring the absorption at 260 nm on a Beckman UV spectrophotometer, assuming that an A_{260} of 1.0 represents 33 µg/ml of DNA.

2.25.3. Computer Programs And DNA Analysis Software

Compilation and analysis of DNA sequence data was performed using the following Macintosh molecular biology software: MacVector, version 3.5 (Integrated Sciences), DNA Strider version 1.0, carried out using the HIBIO™ DNASIS-Mac, version 2.0 (Hitachi), DNA and protein sequence analysis system. Database searches were done using the latest release of the HIBIO™ Gene/Protein Sequence Database on compact disc. These discs were distributed quarterly and included the most recent updates of the following databases: GenBank®, EMBL, PIR and SWISS-PROT. Alternatively, sequence data were analysed by accessing the Genbank and European Molecular Biology Laboratory (EMBL) data bases at the National Centre for Biotechnological Information (NCBI).

TRANSFECTION AND RETROVIRAL INFECTION TECHNIQUES

2.26. INTRODUCTION OF cDNA INTO EUKARYOTIC CELLS

2.26.1. Transfection Of Ecotropic Retroviral Packaging Cell Line, Ψ_2 Via The Calcium Phosphate Technique

The calcium phosphate transfection procedure was performed essentially as described by Miller *et al* (1986) and used to introduce various plasmid constructs into the ecotropic retroviral-producing cell line, Ψ_2 (please refer to Section 2.4.3.). 24 hours prior to transfection 60 mm tissue culture dishes (Falcon, USA, Cat. No. 2002) were seeded with $3x10^5$ Ψ_2 fibroblast cells, in a volume of 3 ml of DMEM-10 to ensure 50-60% confluencey on the day of the transfection. Four hours prior to transfection, the medium was aspirated and replaced with 3 ml of fresh DMEM-10 and during this time, the DNA/$CaCl_2$ coprecipitate was formed. The ensuing volumes are representative of the requirements for 1 dish, and are as follows: to a 10 ml conical polypropylene tube (Disposable Products, Australia, Cat. No. 21829) were added; 125 µl 2M $CaCl_2$, 5-10 µg caesium-chloride or Qiagen prepared (please refer to Section 2.17.) circular plasmid DNA constructs in a final volume of 500 µl with sterile Milli-Q water. To prepare 500 µl of 2 x HEBES (HEPES buffered saline), the following reagents were added to a separate 10 ml conical polypropylene tube; 62.5 µl 2M NaCl, 7.5 µl sodium phosphate, pH 7.0, 50 µl 0.5 M HEPES, pH 7.1 and 380 µl sterile Milli-Q water. The DNA/$CaCl_2$ solution was gently mixed and added dropwise, using a plastic transfer pipette, to the 2 x HEBES solution whilst bubbling air through this solution. The 1 ml coprecipitate solution was allowed to stand for 15 minutes at room temperature (after which time a fine precipitate was visible), and was subsequently added dropwise to the monolayer of Ψ_2 cells. Following 24 hours incubation at 37°C with 5% CO_2, the medium/DNA coprecipitate was removed from each dish by vacuum aspiration. The cells were glycerol "shocked" by the addition of 2.5 ml of 15% glycerol in serum-free DMEM for exactly 4 minutes, after which time the glycerol was removed by vacuum aspiration. Following gentle washing with serum free-growth medium, 4 ml of DMEM-10 was added to the adherent cell layer. After 24 hours culture, the cells were harvested by trypsin-detachment, as described in Section 2.4.3. and

subcultured into 15 ml of DMEM-10 supplemented with 400 µg/ml Geneticin (G418, Gibco/BRL, USA, Cat. No. 860-1811) into a sterile plastic flask (75 cm^2 surface area, Corning 25110-75). Cells were fed twice weekly in the presence of selective medium until the mock-transfected cells died. The titre of the retrovirus-producing cell line was assessed by infection of Swiss-3T3 or NIH-3T3 cells, as described in Section 2.26.5.

2.26.2. Library Construction

(a) Transfection Of Amphotropic Retroviral Packaging Cell Line, PA317 Via The Calcium Phosphate Technique

Caesium chloride-purified, supercoiled DNA preparations generated in each of the independent ligations, were pooled in quantities that would ensure proportional representation of each pool in the library. 100 mm dishes (Corning, NY, USA), seeded 24 hours previously with 1 x 10^6 amphotropic-packaging cell line, PA317 cells in 10 ml of medium, were transfected with 40 µg of retroviral plasmid DNA by the calcium phosphate transfection procedure described in Section 2.26.1. Briefly, the calcium phosphate-DNA coprecipitate was incubated with the adherent fibroblasts for 16 hours after which time, the medium was removed and the cells glycerol "shocked". Following gentle washing with serum free-growth medium, 10 ml of DMEM-10 was added to the adherent cell layer and the cells cultured for 48 hours at 37°C with 5% CO_2, prior to harvesting the supernatant containing the retroviral particles. The titre of the retrovirus-producing cell line was assessed by infection of Swiss-3T3 or NIH-3T3 cells, as described in Section 2.26.5.

(b) Retroviral Infection Of The Adherent, Ecotropic Packaging Cell Line Ψ_2, With Amphotropic Retroviral Supernatant-Library Construction

The PA317-derived culture supernatant (containing the amphotropic retroviral particles and constituting the HBMSC library) were harvested from the transiently-transfected PA317 cells, filtered through a 0.45 µm filter (Sartorius, Germany, Cat. No. 165 55K), and stored in 10 ml aliquots at -80°C or used immediately. To facilitate infection of the ecotropic packaging cell line Ψ_2, Hexadimethrine Bromide (Polybrene; Sigma, Cat. No. H-9268) was added at a final concentration of 4 µg/ml to 2 ml aliquots

of the virus-containing supernatant and used to infect 1 x 10^6 Ψ_2 cells seeded and cultured as described in Section 2.4.3. Following 24 hours infection, the cells were trypsinised, harvested, transferred to 225 cm^2 tissue culture flasks and selected with G418 at a final concentration of 400 µg/ml in DMEM-10. The pool of G418 resistant Ψ_2 cells represented the stable library and the size of the library was estimated by plating dilutions of the trypsinised Ψ_2 cell suspensions in G418 containing medium and following 8 days culture, colonies were fixed, stained and enumerated as described in Section 2.26.5.

2.26.3. Retroviral Infection Of Suspension Cells By Co-Cultivation

Cells of the murine factor-dependent lines, FDC-P1 and Baf-3 were infected by co-cultivation with the virus-producing Ψ_2 cells. Briefly, 25 cm^2 tissue culture flasks were seeded with 1 x 10^6 irradiated (30 Gy) Ψ_2 cells and co-cultivated with 4 x 10^5 FDC-P1 or Baf-3 cells in FDC-P1 or Baf-3 GroM (please refer to Sections 2.4.2. and 2.4.3., respectively) for 48 to 72 hours. Following this, the FDC-P1 or Baf-3 cells were harvested by vigorous agitation of the flask and infected cells selected in FDC-P1 or Baf-3 GroM containing 1000 µg/ml and 1,200 µg/ml of G418 respectively. Following 4 to 5 days selection, infected cells were maintained and expanded in FDC-P1 or Baf-3 MM, in preparation for mAb-mediated selection (please refer to Section 2.27.1.).

2.26.4. Transfection Of BOSC-23 Cells And Infection Of FDC-P1 Cells With BOSC-23-Derived Virus

(a) Transfection Of BOSC-23 Cells

The transient ecotropic retrovirus producing cell line BOSC-23 was maintained as described in Section 2.4.3. 60 mm dishes (Corning), were seeded 18 to 24 hours prior to transfection with 1.5 x 10^6 BOSC-23 cells in 4 ml of DMEM-10, to achieve 80% confluencey on the day of the transfection. To increase the viral titre, immediately prior to the transfection, the medium was changed to 4 ml of DMEM-10 supplemented with 25 µM chloroquine. The cells were transfected by the calcium phosphate transfection protocol described in Section 2.26.1., with the following modifications; 5-10 µg of

plasmid construct DNA/$CaCl_2$ in a volume of 500 µl was added dropwise to 500 µl of 2 x HEBES solution by bubbling. The co-precipitate was added immediately (within 1-2 minutes) to the cell monolayers and the cells were subsequently incubated at 37°C in 5% CO_2. Following 7-10 hours incubation, medium containing chloroquine was removed by vacuum aspiration, and replaced with 4 ml of DMEM-10. The cells were cultured for an additional 24 hours prior to retroviral infection of the target cells.

(b) Infection Of FDC-P1 Cells

The murine factor-dependent line FDC-P1, were infected by co-cultivation with the virus-producing BOSC-23 cells. Following the removal of the medium by vacuum aspiration, the BOSC-23 cells were seeded with 3 x 10^5 FDC-P1 resuspended in 3 ml of FDC-P1 GroM (please refer to Section 2.4.2.) supplemented with 4 µg/ml polybrene for 48 hours. Following this, the FDC-P1 cells were harvested by vigorous agitation of the dish and infected cells selected in FDC-P1 GroM containing 1000 µg of G418. Following 4 to 5 days selection, infected FDC-P1 cells were maintained and expanded in FDC-P1 maintenance medium, in preparation for indirect immunofluorescence and flow cytometry (please refer to Section 2.10.2.).

2.26.5. Retroviral Infection Of Swiss-3T3 And NIH-3T3 Fibroblast Cell Lines - Enumeration of Retroviral Titres

The murine fibroblast cell lines, Swiss-3T3 or NIH-3T3 were seeded with 2 x 10^5 in 60 mm tissue culture dishes 24 hours prior to infection, to ensure 10-20% confluencey on the day of the transfection. The medium was removed from the dishes by vacuum aspiration and 1 ml of neat viral supernatant in the presence of 4 µg/ml polybrene was added to the cells. The dishes were incubated for 2 hours at 37°C (with gentle rocking at 30 minute intervals) after which time, 3-4 ml of fresh DMEM-10 was added. 48 hours post-infection, the cells were harvested by trypsin detachment, and "split" 1/20 prior to plating the cells into 3 x 60 mm dishes in 3 ml DMEM-10 containing 400 µg/ml G418. Cells were fed twice weekly until mock infected cells were dead. To determine the titre of the virus, colonies were stained with Diff-Quick (Lab Aids, Australia, LP-64851) and enumerated, taking into account the dilution factors.

2.26.6. Retroviral Infection Of Adherent Swiss-3T3 Fibroblast Cells

The Ψ_2-derived culture supernatant (containing the ecotropic retroviral particles and constituting the HBMSC library) were harvested from the stably-transfected Ψ_2 cells and filtered through a 045 µm filter (Sartorius, Germany). To facilitate infection of the fibroblastic cell line, Swiss-3T3 polybrene was added at a final concentration of 4 µg/ml to 2 ml aliquot's of the virus-containing supernatant and used to infect 1 x 10^6 Swiss-3T3 cells seeded and cultured as described in Section 2.4.4. Following 24-48 hours infection, the cells were trypsinised, harvested (as detailed in Section 2.4.3.), transferred to 75 cm^2 tissue culture flasks and selected with G418 at a final concentration of 400 µg per ml in DMEM-10. The pool of G418-resistant Swiss-3T3 cells expressing HBMSC-derived cDNA clones enabled the selection of antigen expressing cells with antibodies which showed some reactivity to the FDC-P1 cell line.

2.26.7. Recloning Of cDNA Clones Into pRUF.*neo* And Validation Of Surface Antigen Expression

Following PCR recovery of proviral cDNA inserts from genomic DNA, unique restriction sites present in the 5' and 3' flanking regions, were utilised to "reclone" the cDNA into the MCS of the retroviral vector pRUF.*neo*. *E.coli* DH10B cells were transformed as described in Section 2.16.7. and plasmid DNA was isolated using QIAGEN-tip 100 as detailed in Section 2.17.1. (iii). Stable, G418 resistant Ψ_2 virus-producing cell lines were produced by calcium phosphate transfection and used to infect FDC-P1 cells by co-cultivation, as described above. G418 resistant FDC-P1 cells were then analysed for antigen expression by indirect immunofluorescence and flow cytometry as described in Section 2.10.2 and 2.27.2.

2.27. ISOLATION OF INFECTED CELLS WHICH EXHIBIT UNIMODAL ANTIGEN EXPRESSION

2.27.1. Magnetic-Bead/Monoclonal Antibody Capture And Enrichment Of Library Infected FDC-P1, Baf-3 And Swiss-3T3 Cells.

To isolate and enrich infected FDC-P1, Baf-3 or Swiss-3T3 cells expressing antigens of interest, a protocol involving the use of immunomagnetic beads was developed. Prior to immunolabelling, 1 x 10^7-1 x 10^8 infected, G418 resistant cells were incubated in IF Buffer supplemented with 5% (v/v) normal human serum (IF buffer-NHS, "blocking buffer") for 30 minutes on ice to block possible Fc receptor-mediated binding of antibodies. The cells were then incubated with a cocktail composed of mAb supernatants, purified mAb or mAb ascites, each at optimal staining concentration as determined by indirect immunofluorescence and flow cytometry, using HBMSC. After a 60-90 minute incubation on ice (with intermittent mixing), the cells were washed twice in wash buffer and resuspended at a cell concentration, typically in the range of 2-4 x10^7 cells/ml in IF buffer. Sheep anti-mouse (SAM) IgG-conjugated magnetic M450 Dynabeads (Dynal, Oslo, Norway) were then added at a bead to cell ratio of 1:1. The cells were then incubated with the SAM-beads for 2 hours at 4°C on a rotator, after which, cells rosetted by Dynabeads were captured by applying the tube to an MPC-1 (magnetic particle concentrator, Dynal) for 2 minutes. Non-rosetted cells were removed by the addition 3 ml of ice-cold IF Buffer. This was repeated 4 times prior to resuspending the rosetted cells in appropriate medium. The cells were cultured (as described above), for 5 to 10 days to expand the cell number, following which, the selection procedure was repeated. Cells were cultured in the presence of the magnetic beads, with no apparent perturbation in cell growth.

2.27.2. Immunofluorescence Staining And Flow Cytometry

Prior to immunolabelling, cells were incubated in blocking buffer as described above. Aliquots of 5 x 10^5 cells were incubated with 50 µl of the antibody or isotype-matched control (used as culture supernatants or used as at a final concentration of 10 µg/ml for purified mAbs, as detailed in Tables 2.10.1. and 2.10.2.). After a 45 minute incubation at 4°C the cells were washed twice in IF buffer and resuspended in 50 µl IF Buffer containing 1:50 dilution of sheep anti-mouse FITC (Silenus, Australia, DDAF). and incubated for a further 45 minutes at 4°C. Before analysis, cells were washed twice in IF Buffer and resuspended in PBS/1% paraformaldehyde. Flow cytometric analysis was

performed using a Coulter Epics-Profile II flow cytometer (Coulter Corp., Hialeah, FL). Typically, 10,000 events were collected per sample, and stored as list mode data for further analysis using Coulter ELITE software.

2.27.3. Fluorescence Activated Cell Sorting (FACS) And Clonal Isolation Of FDC-P1, Baf-3 And Swiss-3T3 Cells Infectants.

Pooled G418 resistant antigen-expressing FDC-P1, Baf-3 and Swiss-3T3 cells were washed twice by centrifugation at 4°C in IF Buffer and blocked as described above. Surface-antigen expression was detected by labelling 5×10^6 - 1×10^7 infectants with 100 - 200 µl of mAb supernatants or purified mAbs at a final concentration of 10 µg/ml. After a 45 minute incubation at 4°C the cells were washed twice in IF Buffer and resuspended in wash medium containing 1:50 dilution of goat anti-mouse IgG-PE or IgM-PE (Caltag, San Francisco, CA, USA), for a further 45 minutes at 4°C. Prior to sorting, cells were washed twice in IF Buffer and resuspended to 2×10^7 cells per ml. Single cell deposition was performed using the ACDU facility of the FACStar^{PLUS} cell sorter (Becton Dickinson, Mountain View, CA, USA) and the threshold for selection of cells was based on the level of staining obtained with a non-binding isotype-matched control. Single cells were sorted directly into the appropriate medium (with selection) and expanded in culture until sufficient cells were available for immunostaining with mAb, to confirm the unimodal antigen expression by immunofluorescence and flow cytometry.

Alternatively, clones of antigen positive FDC-P1 or Baf-3 cells were isolated by plating populations attained by FACS in semi-solid medium comprising FD GroM supplemented with 0.9% methylcellulose (Dow Chemicals). Following 10 days culture, single colonies were isolated and expanded as described above.

PROTEIN ANALYSIS

2.28. ANALYSIS OF CELL SURFACE ANTIGENS BY IMMUNOPRECIPITATION WITH MONOCLONAL ANTIBODIES

2.28.1. Buffers And Reagents Required

(a) Tris-Saline-EDTA (TSE)

50 mM Tris-HC1, 150 mM sodium chloride, 1 mM EDTA, 0.1% sodium azide, pH 8.0.

(b) 1% NP40-TSE

1% (v/v) Nonidet P40 (NP40, Sigma-Aldrich, Castle Hill, NSW, Australia, Cat. No.. NO896), 50 mM Tris-HC1, 150 mM sodium chloride, 1 mM EDTA, 0.1% sodium azide, in water, pH 8.0.

(c) 0.1% NP40-TSE

0.1% (v/v) Nonidet P40, 50 mM Tris-HC1, 150 mM sodium chloride, 1 mM EDTA, 0.1% sodium azide, in water, pH 8.0.

(d) 100 x Protease Inhibitor Cocktail

To prepare a 100 x protease inhibitor cocktail, 40 mg of each of the following protease inhibitors (all purchased from Sigma Chem. Co., USA) were dissolved in 10 ml of dimethylformamide (DMF); phenyl methyl sulphonyl fluoride (PMSF), L-[tosylamido-2-phenyl] ethyl chloromethylketone (TPCK), 1-chloro-3-tosylamido-7-amino-L-2-heptanone (TLCK), p-nitrophenyl-p'-guanidino-benzoate-HCl (NPGB).

2.28.2. Biotinylation Of Cell Surface Antigens And Preparation Of NP40 Lysates

Biotinylated cell surface lysates were prepared essentially as described by Cole *et al* (1987). Routinely, cell lines were washed thrice in PBS and 0.01% (w/v) sodium azide (PBS + Az), and resuspended to 1 x 10^8 cells per ml. Cell surface antigens were biotinylated with NHSS-Biotin (Bresatec, Adelaide, Australia, Cat. No. BNS-IS), or Sulfo-NHS-Biotin (Pierce Biochemicals, Rockford, IL, USA, Cat. No. 21217) which was added to the cells as a solution of 2.2 mg dissolved in 50 µl PBS per 10^8 cells. The mixture was subsequently chilled on ice for 30 minutes, with occasional gentle mixing. The biotinylated cells were subsequently washed 3 times in PBS + Az and resuspended at a

density of 2 x 10^7 cells per ml. Following biotinylation, the labelled cell suspension was lysed by adding an equal volume of 1% NP40-TSE. To the cell lysate, a 1/100 volume of protease inhibitor cocktail was immediately added. The mixture was subsequently chilled on ice, for 30 minutes and mixed with occasional gentle mixing. The lysates were then centrifuged at 200 x g for 10 minutes at 4°C in a Beckman centrifuge (Beckman Instruments, Mt. Waverley, Victoria, Australia, Model No. J2-21M), to remove nuclei and other large debris. The supernatant was retained, and subsequently centrifuged at 100,000 x g for 30 minutes at 4°C in a Beckman L8-80 ultracentrifuge or alternatively microfuged at 10,000 x g for 15 minutes. Biotinylated lysates were either stored at -20°C or used immediately for immunoprecipitation.

2.28.3. Arming Of Sepharose With mAbs.

Goat anti-mouse Ig-coupled Sepharose (AH-Sepharose 4B, Pharmacia, Cat. No. 17-0470-03; a kind gift from Mr. S. Cole, Leukaemic Haemopoiesis Laboratory, Hanson Centre For Cancer Research, Adelaide, Australia), was washed twice in 1% NP40-TSE by pulse-centrifugation (approximately 10 seconds) in a microcentrifuge. The Sepharose beads were subsequently resuspended in 1% NP40-TSE to prepare a 50% slurry of which 25 µl was added to a 1.5 ml microcentrifuge tube. To each tube, 400 µl of mAb hybridoma supernatant (or purified mAb diluted to 10 µg/ml in PBS supplemented with 0.1% BSA) was added, and the mixture was subsequently incubated at 4°C for a minimum of 6 hours with rotation.

2.28.4. Immunoprecipitation From NP40 Lysates

The resulting "armed" Sepharose was washed twice in 800 µl of 1% NP40-TSE and pelleted by pulse-centrifugation. Excess supernatant was removed by vacuum aspiration to give 12.5 µl of 100% Sepharose. To each tube, a 1 ml aliquot of the appropriate NP40-lysate was added and the samples were subsequently incubated overnight at 4°C on a rotating mixer. The immunoprecipitates were subsequently washed twice in 1% NP40-TSE, once in 0.1% NP40-TSE, and once in TSE, pH 8.0 as described

above. The supernatant was removed and samples stored at -20°C or used immediately for electrophoresis. Each immunoprecipitate represented the material from $1x10^7$ cells.

2.29. GEL ELECTROPHORESIS OF PROTEINS: SODIUM DODECYL SULPHATE - POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS - PAGE)

2.29.1. Buffers And Reagents Required

(a) Sample Buffer

(i) *Reducing Sample Buffer*: 1 ml 0.5 M Tris-HCl, pH 6.8, 800 µl glycerol, 1.6 ml 10% SDS, 400 µl β-mercaptoethanol, 200 µl 0.05% bromophenol blue and 4 ml Milli-Q water.

(ii) *Non-Reducing Sample Buffer*: 1 ml 0.5 M Tris-HCl, pH 6.8, 800 µl glycerol, 1.6 ml 10% SDS, 200 µl 0.05% bromophenol blue and 4.4 ml Milli-Q water.

(b) Electrophoresis "Running" Buffer

0.3% Tris-HCl, 1.44% Glycine, 0.1% SDS, pH 8.3 in water

(c) Semi-Dry Transfer Buffer

0.3% Tris-HCl, 1.44% Glycine, 20% Methanol, pH 8.3 in water

(d) Separating Buffer

1.5 M Tris-HCl, pH 8.8

(e) Stacking Buffer

0.5 M Tris-HCl, pH 6.8

2.29.2. Gel Preparation

SDS-PAGE was performed using the discontinuous buffer system of Laemmli (1970). SDS-PAGE gels of 7.5%, 10%, and 15% were prepared according to the formulations detailed in Table 2.29.1.

TEMED and ammonium persulphate were added to the gel solution immediately before pouring. The gels were poured between two glass plates (dimensions; 10 x 9 cm), separated with 1.0 mm-thick spacers, and immediately after pouring, the gels were overlaid with Milli-Q water or water-saturated butan-1-ol until the polymerisation of the acrylamide was complete. The butan-1-ol was then thoroughly washed away, and a stacking gel added (prepared according to the formulations detailed in Table 2.29.2.).

Table 2.29.1. Separating Gel Preparation Formulations.

	Molecular Weight Separating Range (% Acrylamide Gel)		
	50 - 200 (7.5 %)	**20 - 150 (10 %)**	**10 - 60 (15 %)**
Milli-Q water	12.2 ml	10.1 ml	5.9 ml
1.5 M Tris-HCl pH 8.8	6.25 ml	6.25 ml	6.25 ml
50 % Acrylamide/1.33 % N,N'-methylene bisacrylamide	6.25 ml	8.3 ml	12.5 ml
10% (w/v) SDS	250 µl	250 µl	250 µl
10% Ammonium Persulphate (APS)	100 µl	100 µl	100 µl
TEMED	20 µl	20 µl	20 µl
Total Volume	25 ml	25 ml	25 ml

Table 2.29.2. Stacking Gel Preparation Formulation

Components	Volumes
Milli-Q water	12.2 ml
0.5 M Tris/HCl pH 6.8	5.0 ml
50 % Acrylamide/ 1.33 % N,N'-methylene bisacrylamide	2.6 ml
10 % (w/v) SDS	200 µl
10 % Ammonium Persulphate	100 µl
TEMED	20 µl
Total Volume	20 ml

2.29.3. Sample Preparation And Electrophoresis

Prior to loading, 25 µl reducing or non-reducing sample buffer was added to the biotinylated immunoprecipitate samples. Samples were subsequently boiled for 3 minutes and proteins separated on a SDS-PAGE gel of appropriate acrylamide concentration. 25 to 30 µl of sample buffer was loaded onto empty tracks in order to maintain a level-running buffer front. The size of the immunoprecipitated proteins were determined by comparison with pre-stained molecular weight standards as detailed in Table 2.29.3. The gel was electrophoresed at 20 mA until the bromophenol blue buffer front entered the separating gel, at which time the voltage was raised to 30 mA, until the bromophenol blue buffer front migrated to the end of the gel.

2.29.4. Transfer Of Proteins To Nitrocellulose (Western Blotting) Utilising A Semi-Dry Blotting Apparatus

Following electrophoresis, the gel cartridge was removed from the electrophoresis chamber, and one of the glass plates was removed, exposing one side of the gel. The stacking gel was dissociated from the separating gel and discarded. Prior to blotting, six pieces of Whatman 3MM paper and one piece of nitrocellulose (Hybond-C, Amersham, Poole, UK., RPN. No. 3022) was cut to the exact size of the gel (dimensions; 6 x 8 cm [following the removal of the stacking gel]). Three pieces of 3MM paper were soaked in semi-dry transfer buffer and layed carefully on the wetted anode of the semi-dry blotting apparatus (Hoefer Scientific Instruments, San Francisco, CA, USA) avoiding entrapment of air-bubbles. This was sequentially overlayed with the pre-soaked nitrocellulose (pre-wetted in Milli-Q water for 5 minutes and subsequently soaked in transfer buffer), the gel and three pieces of 3MM paper pre-soaked as described above. The "sandwhich" was covered with the cathode and the proteins were transferred to the nitrocellulose for times ranging from 45 to 90 minutes (depending on the size of the proteins to be transferred) at approximately 40 mA (0.78 mA per cm^2).

Table 2.29.3. Estimation Of Molecular Weight Of Immunoprecipitated And Western Blotted Proteins: Size Determination Markers (sizes in kilodaltons and represent approximations only).

Fragment Number	Seablue Standards[1]	High Range Standards[2]	Low Range Standards[3]
1	250	205	106
2	98	116.5	80
3	64	80	49.5
4	50	49.5	32.5
5	36	-	27.5
6	30	-	18.5
7	16	-	-
8	6	-	-
9	4	-	-

- 1. Novex, French's Forrest, NSW, Australia, Cat. No. LC5625.
- 2. Bio-Rad Laboratories, North Ryde, NSW, Cat. No. 161-0309.
- 3. Bio-Rad Laboratories, North Ryde, NSW, Cat. No. 161-0305.

2.29.5. Locating Immunoprecipitated Antigen

Following Western blotting, the nitrocellulose membrane was blocked by overnight incubation in approximately 100 ml of PBS /3% BSA (blocking buffer) at room temperature. Following the blocking of non-specific protein binding sites the nitrocellulose membrane was washed four times in PBS/0.05% Tween-20 (Sigma, USA), 5 minutes per wash at room temperature and subsequently incubated in 10 ml of 1:1000 dilution of Streptavidin-biotin horseradish peroxidase (HRPO) complex (Amersham, UK, Cat. No.. RPN-1051) in PBS/1% BSA. The filter was washed as described above and immunoreactive proteins were visualised by enhanced chemi-luminescence (ECL, Amersham) as recommended by the manufacturer.

2.29.6. Autoradiography Of Chemiluminescent Proteins

Immunoreactive proteins were visualised by autoradiography. Nitrocellulose membranes were placed in an autoradiography cassette (Ilford; Knutsford, Cheshire, UK), with one sheet of Fuji RX x-ray film at room temperature. The time of exposure was dependent upon the intensity of the signal obtained after an initial exposure for 60 seconds.

2.30. ANALYSIS OF CELL SURFACE ANTIGENS BY WESTERN BLOTTING WITH MONOCLONAL ANTIBODIES

2.30.1. Preparation Non-Biotinylated NP40 Lysates For Western Blotting

(a) Non-Adherent Cell Populations: Non-adherent cell populations were harvested and washed thrice with PBS + Az as described in Section 2.4.3. and resuspended at 2 x 10^8 cells per ml. Lysates were prepared by adding an equal volume of 1% NP40-TSE and 1/100 volume of protease inhibitor cocktail. The mixture was immediately chilled on ice, for 30 minutes with occasional gentle mixing. The lysates were then centrifuged at 200 x g for 10 minutes at 4°C in a Beckman centrifuge (Beckman, Model No. J2-21M), to remove nuclei and other large debris. The supernatant was retained, and subsequently centrifuged at 100,000 x g for 30 minutes at 4°C in a Beckman L8-80 ultracentrifuge or alternatively

microfuged at 10,000 x g for 15 minutes. Non-biotinylated lysates were either stored at -20°C or used immediately for Western blotting.

(b) Adherent Cell Populations: Membrane-lysates of adherent cell populations were prepared by *in situ* lysis of the monolayer of cells. Cells cultured in a 75 cm^2 tissue-culture flask were washed twice with 10 ml of PBS + Az, and lysates were prepared by adding 1 ml of 0.5% NP40-TSE and 10 µl of the 100 x protease inhibitor cocktail. The flask was immediately chilled on ice for 30 minutes, with occasional gentle mixing. The lysed cells were harvested using a rubber policeman and were subsequently transferred to a 10 ml polypropylene tube. To remove nuclei and other large debris, the lysates were centrifuged at 200 x g for 10 minutes at 4°C in a Beckman centrifuge as previously described. The supernatant was retained, and subsequently centrifuged at 100,000 x g for 30 minutes at 4°C in a Beckman L8-80 ultracentrifuge or alternatively microfuged at 10,000 x g for 15 minutes. Non-biotinylated lysates were either stored at -20°C or used immediately for Western blotting.

2.30.2. SDS-PAGE And Western Blotting

To analyse cell surface antigens by Western blotting, protein derived from 1-2 x 10^6 cell equivalents (10-20 µl) were separated by SDS-PAGE as described in Section 2.29. Following gel electrophoresis, the proteins were transferred to nitrocellulose as described in Section 2.29.4., and the filters were subsequently blocked by overnight incubation at 4°C in 5% (w/v) skim milk powder in PBS. The filter was then briefly washed in PBS/0.05% Tween 20 prior to incubating it in 5% (v/v) goat serum/PBS for 1 hour at room temperature with gentle agitation. The filter strips were briefly washed as above and then incubated with 2-5 ml of mAb in the form of hybridoma tissue culture supernatant or purified antibody at a concentration of 10 µg/ml in 1% BSA/PBS. The nitrocellulose filter was washed 3 times in PBS/0.05% Tween-20, 10 minutes per wash at room temperature and subsequently incubated in 2-5 ml of 1:10,000 dilution of goat-anti mouse Ig conjugated to HRPO (Jackson ImmunoResearch Laboratories Inc., USA, Cat. No. 115-036-068) for 1 hour at room temperature. The filter was washed as above and the

immunoreactive proteins were detected by ECL and autoradiography as described in Section 2.29.6.

2.31. MISCELLANEOUS PROTOCOLS

2.31.1. Estimation Of Protein Concentration

The protein concentration of all cell extracts was determined using 2 and 10µl of the extract in the Bio-Rad protein micro-assay. The assay was performed according to the manufacturer's instructions. Bovine serum albumin was used as the protein standard.

CHAPTER 3

STUDIES OF THE ROLE OF SELECTINS IN HUMAN HAEMOPOIESIS

3.1. INTRODUCTION

3.1.1. Cell Adhesion Mediated By Lectin-Carbohydrate Recognition: A Role For The Selectin Family In Haemopoiesis

Haemopoiesis occurs normally in the bone marrow (BM) where the precursors and progeny of multiple myeloid and lymphoid lineages develop in intimate association with cells that comprise the bone marrow microenvironment (Trentin, 1970; Wolf, 1979; Dexter, 1982; Weiss, 1976; Lichtman, 1981; Tavassoli and Friedenstein, 1983; Allen *et al*, 1990; Simmons and Torok-Storb, 1991a; 1991b). Cellular interactions between immature haemopoietic progenitor cells and the stroma are of major importance in the regulation of haemopoiesis, and in all likelihood serve multiple functions ranging from the retention of primitive haemopoietic stem cells within the bone marrow to the regulation of the release of mature haemopoietic cells into the circulation (Trentin, 1970; Dexter, 1982; Dexter, 1979; Torok-Storb, 1988; Kincade *et al*, 1989). In addition, such interactions may be responsible for the 'homing' of haemopoietic stem cells to the marrow following either bone marrow transplantation or endogenous migration (Tavassoli and Hardy, 1990), or to homing to extramedullary sites in disease states which involve new sites of haemopoiesis.

The interactions that occur between primitive haemopoietic progenitors and marrow stromal elements are many and varied, involving both cell-cell and cell-extracellular matrix (ECM) adhesion (Allen *et al*, 1990; Dexter *et al*, 1977; Kincade *et al*, 1989; Tavassoli and Hardy, 1990; Clark *et al*, 1992; Long, 1992; Simmons *et al*, 1994). The cell adhesion molecules (CAMs) which mediate these interactions may function in a stage and/or lineage-specific manner as occurs, for example, in T-cell development (Springer, 1990a; 1990b; Dunon and Imhof, 1993). Current data support the hypothesis that a variety of CAMs are involved in mediating a range of interactions throughout haemopoiesis (Tavassoli and Hardy, 1990; Clark *et al*, 1992; Long, 1992; Simmons *et al*, 1994). Despite intensive research however, the molecular mechanisms responsible for the homing of stem cells to the bone marrow are poorly understood (Tavassoli and Hardy, 1990).

In mammals, haemopoiesis is restricted to the extravascular compartment of the BM which is separated from the vascular compartment by a single layer of endothelial cells which form specialised vessels termed sinusoids (Weiss, 1976; Lichtman, 1981). Thus in order to enter the haemopoietic compartment, stem cells arriving at the marrow must first recognise, or be recognised by, the luminal surface of the endothelium. Molecules which mediate adhesion of haemopoietic stem cells to vascular endothelial cells are therefore likely to play a pivotal role in the phenomenon of stem cell homing but thus far, such molecules have yet to be identified. Nevertheless, it would reasonable to speculate that molecules involved in the adherence of mature leukocytes to the endothelial cell surface, may also play a role in haemopoietic stem cell-endothelial cell interactions.

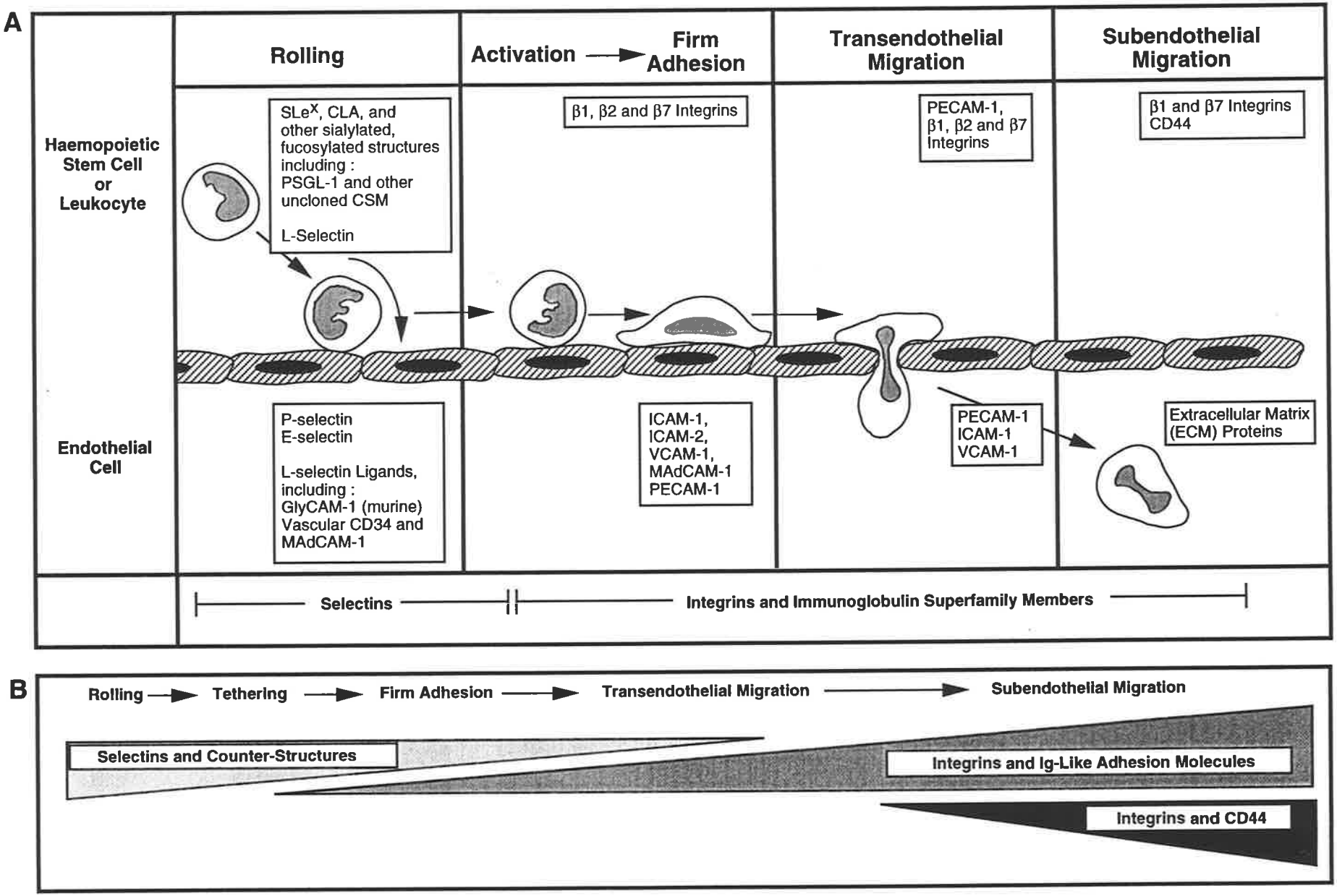
In the past decade, *in vitro* and *in vivo* studies have identified many of the critical structures involved in mature leukocyte adherence to, and migration across endothelium. An operative paradigm of the molecular basis of leukocyte adhesion and extravasation (Figure 3.1.1.) suggests a multi-step phenomenon in which the initial, relatively low-affinity binding event (leukocyte rolling) is mediated by the selectin family of adhesion molecules and carbohydrate-bearing counter-structures (Butcher *et al*, 1991; Lasky *et al*, 1992). Activated leukocytes then induce a higher avidity binding that is mediated by the leukocyte integrins and their cognate endothelial ligands, the immunoglobulin (Ig) superfamily glycoproteins including the intercellular adhesion molecules (ICAMs) and vascular cell adhesion molecules-1 (VCAM-1) (Stoolman, 1989; Springer, 1990; Butcher, 1991; Lasky, 1992) (refer to Figure 3.1.1). Although the adhesion that is mediated by leukocyte integrins is due to protein-dependent binding (Springer, 1990), the selectins bind through the calcium-dependent lectin recognition of carbohydrate ligands on specific endothelial or leukocyte glycoproteins.

The selectins are a family of three structurally related integral membrane glycoproteins that regulate leukocyte adhesion to platelets and/or endothelium during inflammation by means of selective protein-carbohydrate interactions mediated by the lectin-like domain at the N-terminus of each family member (Lasky *et al*, 1989; Johnston *et al*, 1989a; 1989b; Bevilacqua *et al*, 1989; Bevilacqua and Nelson, 1993; Lasky, 1992; Watson *et al*, 1990). Leukocyte(L)-selectin (CD62L) is constitutively expressed by

Figure 3.1.1. Adhesion Molecules Involved In Leukocyte-Endothelial Cell Interactions.

(A) An operative paradigm of the molecular basis of leukocyte adhesion and extravasation proposes a multi-step phenomenon in which the initial, relatively low-affinity binding event (leukocyte "rolling") is mediated by the *selectin family* of adhesion molecules and carbohydrate-bearing counter-structures including *PSGL-1, GlyCAM-1, vascular CD34* and *MAdCAM-1.* Activated leukocytes subsequently induce a higher avidity binding ("firm adhesion") that is mediated by the leukocyte *integrins* (β_1, β_2 and β_7) and their cognate endothelial ligands, the *immunoglobulin (Ig) superfamily glycoproteins* including the *intercellular adhesion molecules* (ICAMs) and *vascular cell adhesion molecules-1* (VCAM-1). Following this, leukocytes enter the subendothelium via a process of transendothelial migration ("diapedesis") which is primarily mediated by members of the integrin and immunoglobulin superfamily of adhesion molecules, including β_1, β_2 and β_7) integrins, PECAM-1 (CD31), ICAM-1 and VCAM-1. Leukocyte migration through the subendothelial environment is subsequently mediated by the integrin family, CD44 and elements of the extracellular matrix (ECM) (Adapted from Carlos and Harlan, 1993).

(B) Schematic representation summarising the relative contributions of adhesion molecules to leukocyte extravasation. The "progression triangles" reflect the changing contributions made by each of the adhesion molecule groups (Adapted from Smith, 1993).


A
Rolling
Activation
Firm Adhesion
Transendothelial Migration
Subendothelial Migration
Haemopoietic Stem Cell or Leukocyte
SLe^X, CLA, and other sialylated, fucosylated structures including : PSGL-1 and other uncloned CSM
L-Selectin
β1, β2 and β7 Integrins
PECAM-1, β1, β2 and β7 Integrins
β1 and β7 Integrins CD44
Endothelial Cell
P-selectin
E-selectin
L-selectin Ligands, including : GlyCAM-1 (murine) Vascular CD34 and MAdCAM-1
ICAM-1, ICAM-2, VCAM-1, MAdCAM-1 PECAM-1
PECAM-1 ICAM-1 VCAM-1
Extracellular Matrix (ECM) Proteins
Selectins
Integrins and Immunoglobulin Superfamily Members
B
Rolling
Tethering
Firm Adhesion
Transendothelial Migration
Subendothelial Migration
Selectins and Counter-Structures
Integrins and Ig-Like Adhesion Molecules
Integrins and CD44

neutrophils, monocytes and lymphocytes (Bevilacqua and Nelson, 1993; Tedder *et al*, 1990) while endothelial cell (E) and platelet (P)-selectins (CD62E and CD62P, respectively) are expressed on activated endothelial cells (Johnston *et al*, 1989a; 1989b; Bevilacqua *et al*, 1989).

P-selectin (PADGEM protein, GMP-140, CD62P) is a 140-kD transmembrane protein expressed by vascular endothelial cells and platelets (Johnston *et al*, 1989a; 1989b; 1989b). The protein is stored pre-formed in the Weibel-Palade bodies of endothelial cells (Bonfanti *et al*, 1989) and in the alpha granules of platelets (Berman *et al*, 1986). Upon stimulation of these cells with a variety of agonists such as thrombin, histamine and calcium ionophore A23187, P-selectin is rapidly (within minutes) translocated to the cell surface (McEver and Martin, 1989; Hsu-Lin *et al*, 1989; Hattori *et al*, 1989a; 1989b) where it mediates leukocyte-platelet (Larsen *et al*, 1989; 1990) and leukocyte-vascular endothelial cell adhesion (Gerig *et al*, 1990; Gamble *et al*, 1990). P-selectin binds to leukocytes by interacting with carbohydrate ligand(s) on opposing cells, (Bevilacqua and Nelson, 1993; Lasky, 1992; Gerig *et al*, 1990) although the precise composition of a number of these reported physiological ligands remain to be determined. The interaction of P-selectin with mature leukocytes is in part responsible for mediating leukocyte rolling on the vascular cell wall (Lawrence and Springer, 1991; Mayadas *et al*, 1993) an early event in leukocyte recruitment at sites of inflammation (Springer, 1990; Atherton and Boon, 1972; Butcher, 1991).

L-selectin (LAM-1, LECAM-1, Leu8, CD62L) was initially described as a lymphocyte homing receptor involved in leukocyte traffic in the systemic microcirculation, however subsequent studies revealed that L-selectin was constitutively expressed on most peripheral blood leukocytes including neutrophils and monocytes (Gallatin *et al*, 1983; Tedder *et al*, 1990; Griffin *et al*, 1990). The molecular weight of L-selectin differs among lymphocytes (~75 kD), neutrophils (~95 to 105 kD), and monocytes (~110 kD) and this variability is thought to result from cell-specific post translational glycosylation among these subsets of leukocytes. Membrane-associated L-selectin is shed following activation (Jung *et al*, 1988; Kishimoto *et al*, 1989; Berg and James, 1990; Bevilacqua and Nelson, 1993) and its activity appears to be controlled by regulation of its appearance and loss

from the cell surface. In the murine system, a number of sulphated endothelial glycoprotein ligands have been described for L-selectin including Sgp50 (GlyCAM-1), Sgp90 (CD34) and MAdCAM-1 (reviewed in Carlos and Harlan, 1994).

E-selectin (ELAM-1, CD62E) is a 110-115 kD cellular activation antigen, whose expression is restricted to the endothelium. Stimulation of cultured endothelium with endotoxin (LPS) or inflammatory cytokines, including interleukin-1 (IL-1) and tumour necrosis factor (TNF), results in the upregulation and expression of E-selectin at the plasma membrane (Bevilacqua *et al*, 1989; Bevilacqua *et al*, 1987). The expression of E-selectin is reliant on *de novo* RNA and protein synthesis and peaks at 4-6 hours post-stimulation then declines to constitutive/basal levels by 24 to 48 hours. *In vitro* studies suggest that CD62E is involved in leukocyte rolling at sites of tissue injury. However unlike CD62L and CD62P, E-selectin is thought to contribute to the later recruitment of leukocytes (Mulligan *et al*, 1991). Although a number of glycoprotein counter-receptors have been suggested (Nelson *et al*, 1995), the best studied ligands for E-selectin are carbohydrate structures including the sialylated form of the Lewis x determinant (CD15), SLe^x, and the closely related structure SLe^a (Nelson *et al*, 1995). These fucosylated lactosamines are found in abundance on circulating monocytes and neutrophils (Fukuda *et al*, 1984; Macher and Klock, 1980; Symington *et al*, 1985; Macher and Beckstead, 1990; Ohmori *et al*, 1989; Munro *et al*, 1992), and a small percentage of blood lymphocytes (Munro *et al*, 1992; Berg *et al*; 1991a; Berg *et al*, 1991b).

In contrast to mature leukocytes, little is known of the role of molecules which facilitate the adhesion of primitive haemopoietic cells to vascular and BM endothelium. This chapter describes various studies which examine the role and expression of members of the selectin family in tissues of the haemopoietic system.

3.2. RESULTS

STUDIES OF THE ROLES OF P-SELECTIN AND E-SELECTIN IN THE ADHESION OF HAEMOPOIETIC PROGENITOR CELLS

3.2.1. $CD34^+$ Cells From The Bone Marrow Adhere To P-Selectin.

$CD34^+$ cells isolated by fluorescence activated cell sorting (FACS) from aspirates of bone marrow from normal, healthy donors were assayed for their ability to bind to CHO cells expressing human P-selectin (Figure 3.2.1.). A mean of 88% (range: 82-100%; n= 5) of marrow $CD34^+$ demonstrated adhesion to CHO-P-selectin transfectants (Figure 3.2.1. (B)) that was specifically inhibited by affinity-purified polyclonal rabbit anti-human P-selectin Fab fragments (Figure 3.2.1. (A1)). A similar proportion of $CD34^+$ cells also adhered to bacteriological dishes coated with purified human platelet-derived P-selectin. Adhesion required the presence of divalent cations, was independent of temperature (ie. occurred equally well at 4°C and 37°C) and occurred under both static and non-static conditions (Figure 3.2.1. (C)). As with assays performed with CHO cell transfectants, adhesion was completely abrogated by anti-P-selectin antibody.

To determine whether adhesion to P-selectin was a specific property of more immature haemopoietic cells, the binding of plastic-adherence depleted, but otherwise unfractionated BMMNC to dishes coated with purified P-selectin was examined. A mean of 25.2% of bone marrow cells specifically adhered to P-selectin, approximately 60% of which were granulocytic cells ($Le^x/CD15^+$) comprising all maturation stages from myeloblasts to segmented neutrophils and an additional 5% expressed the CD14, a marker of the monocyte-macrophage lineage (Table 3.2.1.). A small but reproducible proportion of T-cells bound to P-selectin but the majority were recovered in the non-adherent fraction together with the majority of B-cells and erythroid cells which were correspondingly depleted (approximately 8-fold and 4-fold, respectively) from the P-selectin adherent population (Table 3.2.1.). Significantly, $CD34^+$ cells were 2.5 fold enriched in the P-selectin adherent fraction of the bone marrow and nearly 5-fold depleted in the non-binding population, confirming data obtained using purified $CD34^+$ cells. Of the P-selectin adherent $CD34^+$ cells, a significant proportion co-expressed CD33

Figure 3.2.1. Binding Of $CD34^+$ Cells To P-selectin Is Cation-Dependent, Temperature Independent, Shear-Resistant And Abrogated In The Presence Of α-P-Selectin Antibody.

Adhesion assays were performed using ^{51}Cr-labelled $CD34^+$ cells or neutrophils and CHO-P-selectin cells or untransfected CHO cells. Incubation was carried out for 30 minutes at 37°C after which unbound cells were removed by washing and adhesion quantitated by liquid scintillation counting of Triton X-100 solubilised lysates. Data in **(B)** are presented as the percentage of input radioactive counts and represent the mean ± S.E. of 3 experiments ($CD34^+$ cells, ▨) and 1 experiment (neutrophils, ■), respectively.

The specificity of adhesion of $CD34^+$ cells to the CHO-P-selectin cells is illustrated in **(A1 and A2)**, where adhesion is completely inhibited by addition of rabbit anti-P-selectin Fab fragments (10 μg/ml).

(C) 96- or 24-well plates were coated with purified platelet-derived P-selectin at a final concentration of 20 μg/ml. Purified $CD34^+$ cells were added and incubated as described above, at 4°C or at 4°C on an orbital shaker at 60 rpm to stimulate shear stress. To assess the role of divalent cations, assays were performed in the presence of 5 mM EDTA or EGTA. Upon completion of the assays the cells were fixed and adhesion was quantitated by counting the mean number of cells bound in five randomly selected 100 x fields per well. All assays were established in triplicate. The data in (C) represent the mean ± SE of 3 experiments.

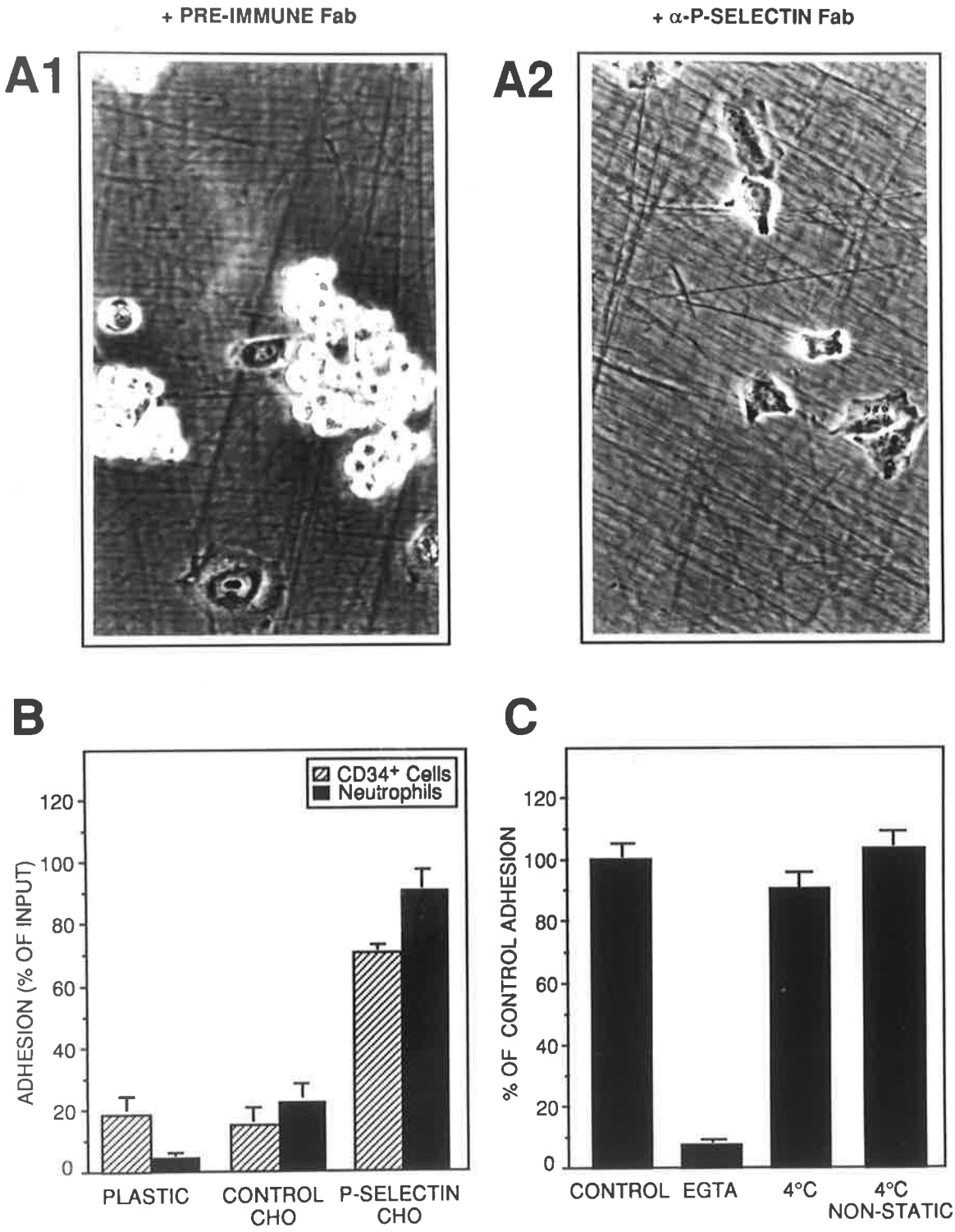

+ PRE-IMMUNE Fab
+ α-P-SELECTIN Fab
A1
A2
B
C
CD34+ Cells
Neutrophils
ADHESION (% OF INPUT)
120
100
80
60
40
20
0
PLASTIC
CONTROL CHO
P-SELECTIN CHO
% OF CONTROL ADHESION
CONTROL
EGTA
4°C
4°C NON-STATIC

Table 3.2.1. Differential Binding Of BM Mononuclear Cells (BMMNC) to P-Selectin.

BMMNC obtained by density separation were subjected to a single round of plastic adherence to remove monocytes. The plastic non-adherent population was transferred to bacteriological grade petri dishes coated with P-selectin. After 30 min. at 37°C, non-adherent cells were carefully removed, the plates washed thrice to remove residual unbound cells and pooled with the first fraction (P-selectin non-adherent). P-selectin adherent cells were removed by the addition of HBSS supplemented with 0.02% EDTA. Both fractions were washed twice in FACS buffer prior to immunofluorescence staining and flow cytometric analysis which was performed using the following antibody conjugates: Leu4-PE (CD3), LeuM3-PE (CD14), LeuM1-FITC (CD15), 3A1-PE (CD7), J5-PE (CD10), CD19-PE, CD20-PE, anti-glycophorin A-FITC, HPCA-2-PE (CD34) and isotype-matched, non-binding FITC and PE-conjugated control antibodies. Cells were analysed using a Profile II flow cytometer. 20,000 events were collected as list mode data per sample and analysed using Coulter ELITE software. Data represent the mean ± SE of 3 experiments.

Table 3.2.1. Differential Binding Of BM Mononuclear Cells (BMMNC) to P-Selectin

	PERCENTAGE POSITIVE CELLS		
CELL POPULATION (Specfic Antigen)	**Unfractionated**	**P-Selectin Adherent**	**P-Selectin Non Adherent**
T-Cells (CD3, CD7)	21.5 ± 6.1	8.6 ± 5.9	32.9 ± 11.0
B-Cells (CD10, CD19, CD20)	7.4 ± 2.6	0.9 ± 0.5	8.9 ± 3.8
Granulocytes (CD15)	27.2 ± 8.8	59.6 ± 15.5	11.0 ± 2.5
Monocytes (CD14)	3.1 ± 1.9	5.7 ± 2.0	0
Erythroid Cells (Glycophorin A)	6.8 ± 3.7	1.8 ± 0.9	16.6 ± 3.1
Progenitor Cells (CD34)	4.2 ± 1.1	10.6 ± 2.3	0.9 ± 0.6

(Andrews *et al*, 1989) and CD38 (Terstappen *et al*, 1991), antigens previously shown to be present on mature myeloid and erythroid progenitor cells (Figure 3.2.2.). Notably, $CD34^+$ cells co-expressing the B-cell restricted antigens CD10 and CD19 were recovered only in the P-selectin non-binding population (Figure 3.2.2.).

3.2.2. Committed Progenitors And Their Precursors Adhere To P-Selectin

To determine whether lineage restricted haemopoietic progenitor cells were present in the population of bone marrow mononuclear cells (BMMNC) which specifically bound to P-selectin, *in vitro* clonogenic assays were performed on adherent and non-adherent fractions. In accord with the above 2-colour phenotypic analyses, virtually all detectable myeloid (CFU-GM) and multipotential colony forming cells (CFU-Mix), together with approximately 80% of erythroid progenitors (BFU-E) were recovered in the population of cells which bound to P-selectin regardless of whether plastic non-adherent BMMNC (Table 3.2.2. (A)) or $CD34^+$ cells (Table 3.2.2. (B)) were employed as the starting population. All three classes of progenitors were correspondingly absent or depleted from the respective P-selectin non-adherent cell fractions and their adhesion to P-selectin was specifically blocked by anti-P-selectin antibody, partially in the case of BMMNC and almost completely in the case of assays performed with purified $CD34^+$ cells (Table 3.2.2. (B)).

In subsequent experiments, hierarchically more primitive haemopoietic precursors (pre-CFU) were examined for their ability to bind P-selectin. Primitive haemopoietic progenitors (pre-CFU) with the capacity to initiate and sustain haemopoiesis *in vitro* represent a minor proportion of the $CD34^+$ population and are characterised, by their lack of expression of CD33 (Andrews *et al*, 1989), CD38 (Terstappen *et al*, 1991) and HLA-DR (Brandt *et al*, 1990). Significantly, $CD34^+CD38^-$ cells were enriched by a mean of 3.5-fold in the P-selectin adherent fraction (range: 2.8-6.4 fold; n=5) and virtually undetectable in the non-adherent population (Figure 3.2.2.). Collectively, these data suggested the likelihood that pre-CFU would exhibit the property of adhesion to P-selectin. Accordingly, BMMNC separated by adherence into P-selectin binding and non-binding populations were assayed for their capacity to initiate haemopoiesis in a

Figure 3.2.2. The Cell Surface Phenotype Of $CD34^+$ Cells Which Bind To P-Selectin.

BMMNC adherent and non-adherent to P-selectin (detailed in Table 3.2.1.) were stained using antibody HPCA-2-FITC (CD34) in combination with either LeuM9-PE (CD33), Leu17-PE (CD38), HLA-DR-PE, a mixture of J5-PE (CD10) and CD19-PE or with appropriate combinations of isotype-matched FITC or PE-conjugated non-binding control antibodies. Cells were washed, fixed and analysed as described (Table 3.2.1). Each 2-parameter histogram was generated from 20,000 events stored as list mode data.

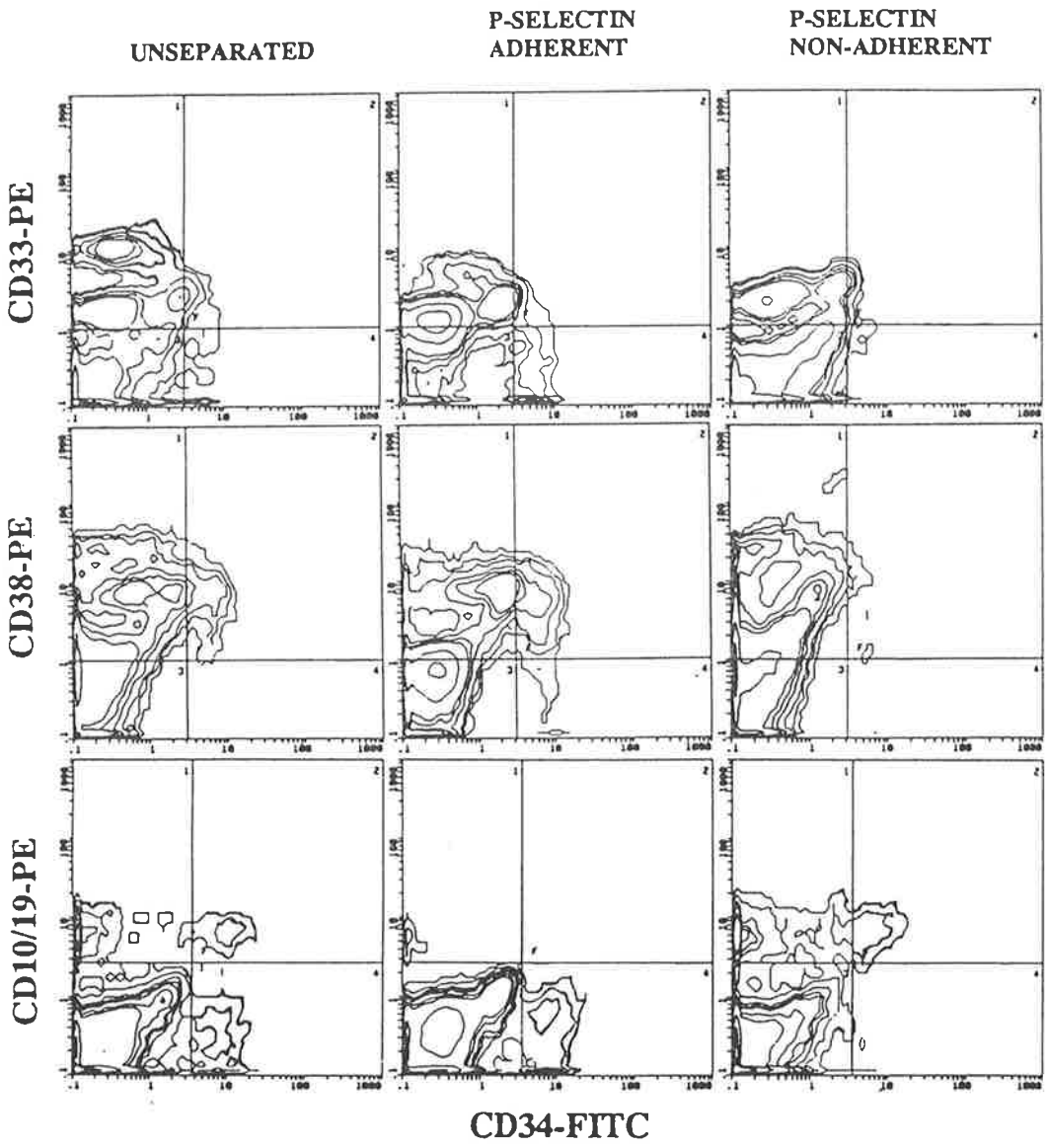

UNSEPARATED
P-SELECTIN ADHERENT
P-SELECTIN NON-ADHERENT
CD33-PE
CD38-PE
CD10/19-PE
CD34-FITC

Table 3.2.2. (A) Committed Haemopoietic Progenitor Cells Adhere To P-Selectin Adsorbed To Plastic.

Haemopoietic progenitor cells in the P-selectin adherent and non-adherent fractions of BMMNC obtained as described in Table 3.2.1. were assayed by plating 2.5 x 10^4 cells in triplicate, in clonogenic assays. CFU-GM, BFU-E and CFU-Mix were scored on day 14 according to standard criteria. Where indicated, affinity-purified rabbit anti-human P-selectin Fab fragments and pre-immune rabbit immunoglobulin Fab fragments were incubated on the P-selectin coated plates at 10 μg/ml for 1 hour prior to the addition of cells. Data represent the mean ± SD; n=2.

Table 3.2.2. (A) Unfractionated BM

Population	P-Selectin Coated	Antibody	% Of Input Cells In Each Fraction	% Clonogenic Cells In Each Fraction	
				CFU-GM	BFU-E
Input *			100	100 ± 51 (140 ± 72)**	100 ± 7 (424 ± 28)
Adherent	-	-	7.6	0 ± 0 (0 ± 0)	0 ± 0 (0 ± 0)
Non-Adherent	-	-	84.8	83 ± 19 (116 ± 26)	96 ± 11 (408 ± 48)
Adherent	+	-	22.7	146 ± 8 (204 ± 11)	139 ± 24 (588 ±100)
Non-Adherent	+	-	81.8	0 ± 0 (0 ± 0)	6 ± 3 (24 ± 11)
Adherent	+	α - P-Selectin	10.6	56 ± 6 (79 ± 8)	26 ± 14 (111 ± 16)
Non-Adherent	+	α - P-Selectin	84.8	64 ± 11 (90 ± 16)	73 ± 19 (308 ± 80)
Adherent	+	Control Ab	19.8	135 ± 16 (189 ± 22)	131 ± 4 (556 ± 19)
Non-Adherent	+	Control Ab	82.9	0 ± 0 (0 ± 0)	4 ± 71 (17 ± 12)

* Ficoll-separated BM Mononuclear cells subjected to one round of plastic adherence (2 hr at 37°C).
** Numbers in parentheses represent actual clonogenic cell numbers.

Table 3.2.2. (B). Committed Haemopoietic Progenitor Cells Adhere To P-Selectin Adsorbed To Plastic.

Purified $CD34^+$ cells obtained as described in Figure 3.2.1. were plated over P-selectin coated plates at 2 x 10^5/ml under conditions identical to those described for BMMNC. Clonogenic assay of committed progenitors in the P-selectin adherent and non-adherent fractions was performed by plating the cells at 10^3/plate as described in Table 3.2.2. (A). Data represent the mean ± SD; n=2.

Table 3.2.2. (B) CD34+ BM

Population	CD62P coated surface	Antibody	% of Input Cells In Each Fraction	% Clonogenic Cells In Each Fraction		
				CFU-GM	BFU-E	CFU-MIX
Input			100	100 ± 5 (81 ± 4)	100 ± 13 (38 ± 5)	100 ± 30 (1 ± 0.3)
Adherent	-	-	2.2	ND	ND	ND
Non-Adherent	-	-	92.5	93 ± 7 (75 ± 6)	97 ± 13 (37 ± 5)	60 ± 30 (0.6 ± 0.3)
Adherent	+	-	84.4	89 ± 8 (72 ± 6)	111 ± 13 (42 ± 5)	100 ± 30 (1 ± 0.3)
Non-Adherent	+	-	13.7	0 ± 0 (0 ± 0)	24 ± 5 (9 ± 2)	0 ± 0 (0 ± 0)
Adherent	+	α- CD62P	1.8	0 ± 0 (0 ± 0)	4 ± 1 11 ± 3	0 ± 0 (0 ± 0)
Non-Adherent	+	α- CD62P	95.3	93 ± 10 (75 ± 8)	105 ± 5 (40 ± 2)	100 ± 30 (1 ± 0.3)

standard stromal cell-dependent LTBMC procedure. As shown in Figure 3.2.3. (A), production of CFU-GM over 5 weeks in culture was only detected in cultures initiated with unfractionated BMMNC or P-selectin adherent BMMNC. The P-selectin non-adherent population demonstrated essentially no potential for generation of clonogenic cells.

An identical trend was observed when $CD34^+$ cells present in unfractionated, P-selectin adherent and P-selectin non-adherent BMMNC were assayed for their ability to generate nascent CFU-GM in a recently described stroma-free, cytokine dependent suspension culture assay (Smith *et al*, 1991, Brandt *et al*, 1990, Haylock *et al*, 1992). (Figure 3.2.3. (B)).

3.2.3. The Nature Of The Ligand For P-Selectin On $CD34^+$ Cells

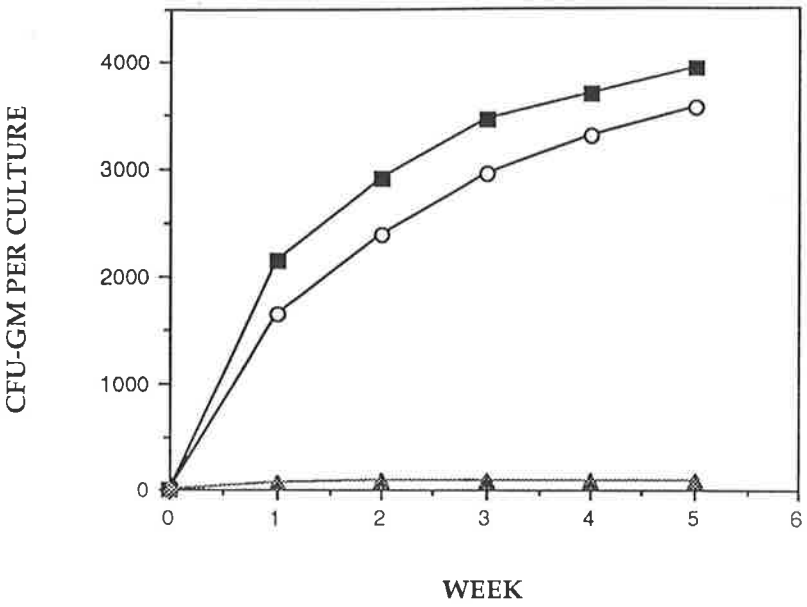
Treatment of $CD34^+$ cells with a variety of proteases (including trypsin, thermolysin, papain, bromelain and elastase) markedly decreased their binding to P-selectin (Figure 3.2.4.(A)) demonstrating that the receptor for P-selectin on $CD34^+$ cells appears to be located on a glycoprotein and not a glycolipid associated with the cell surface. Neuraminidase treatment of $CD34^+$ cells completely abrogated their binding to P-selectin (Figure 3.2.4. (B)) implying a prominent role for sialic acid in the structure and function of the ligand. These data are in accord with the observation that P-selectin is able to bind to the tetrasaccharide sialyl $Lewis^X$ (SLe^X) (Zhou *et al*, 1991; Polley *et al*, 1991; Foxall *et al*, 1992) and a stereoisomer, sialyl $Lewis^a$ (SLe^a). To assess the expression of these carbohydrate antigens on $CD34^+$ cells, monoclonal antibodies (mAb) CSLEX-1 (Fukushima *et al*, 1984) and HECA-452 (Picker *et al*, 1990) were employed. Monoclonal antibody CSLEX-1, specific for the alpha 2-3 sialosylated form of the fucosylated lactosamine, lacto-N-fucopentaose III (SLe^X), is expressed on granulocytes, monocytes and both normal (non-myeloid) and tumour cells (Fukushima *et al*, 1984). Indirect immunofluorescence and flow cytometry revealed that monoclonal antibody CSLEX-1 bound at high levels to a subpopulation (33.5% ± 1.0, n=5) of $CD34^+$ cells (Figure 3.2.5.(A)). Monoclonal antibody HECA-452 which identifies the cutaneous lymphocyte-associated antigen, CLA (Picker *et al*, 1990), reported to function as a ligand for E-selectin

Figure 3.2.3. $CD34^+$ Cells With Long-Term *In Vitro* Repopulating Capacity Bind To P-Selectin.

(A) The P-selectin adherent and non-adherent BMMNC fractions were assayed for their ability to initiate and maintain haemopoiesis in long-term bone marrow culture (LTBMC). LTBMCs were initiated (in triplicate) on confluent, irradiated cultures of allogeneic bone marrow stromal cells with either 1×10^7 unfractionated BMMNC (○) or with the proportion of this number that were P-selectin adherent (■) or non-adherent (▲), respectively. The data are presented as the cumulative production of CFU-GM per culture assayed each week (as described in Table 3.2.2.) and represent the sum of CFU-GM present in the suspension and stromal cell associated phases at each time point. A representative experiment (one of three) is shown.

(B) Assay of the unfractionated $CD34^+$ population (□), $CD34^+$/P-selectin adherent (▨) and $CD34^+$/P-selectin non-adherent (■) populations using a stroma-free, cytokine dependent assay. $CD34^+$ cells were isolated by FACS as described (Figure 3.2.1) from unseparated BMMNC and from the P-selectin adherent and non-adherent BMMNC fractions. Assays were set up in triplicate using 1×10^3 $CD34^+$ cells per group in medium supplemented with 10 ng/ml each of purified recombinant human IL-1β, IL-3, IL-6, G-CSF, GM-CSF and SCF. Additional factors were added at the same concentrations on days 7 and 14. On days 7, 14 and 21 the cells were harvested, washed and assayed for CFU-GM as previously described. The data show CFU-GM production per 1×10^3 $CD34^+$ cells for each group and represent the mean ± S.E. of 3 experimemts.

A


CFU-GM PER CULTURE
4000
3000
2000
1000
0
0
1
2
3
4
5
6
WEEK

B

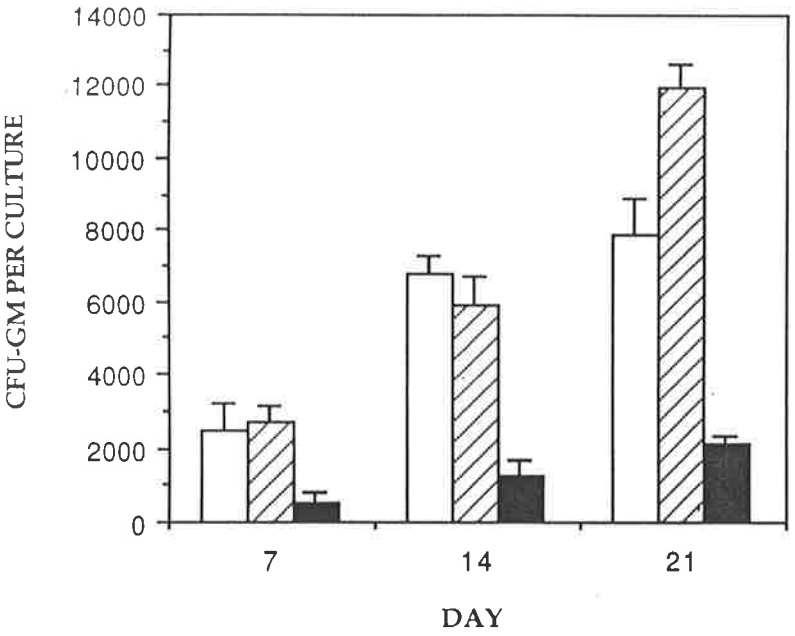

CFU-GM PER CULTURE
14000
12000
10000
8000
6000
4000
2000
0
7
14
21
DAY

Figure 3.2.4.

(A) Effect Of Protease Digestion On The Adhesion Of $CD34^+$ Cells To P-Selectin.

$CD34^+$ cells were incubated at 1 x 10^6/ml for 1 hour at 37°C in RPMI containing proteases at a final concentration of 20 µg/ml with the exception of elastase (20 mg/ml), dispase (4 mg/ml) and proteinase K (1 µg/ml). Following protease treatment, cells were washed and assayed for their ability to bind to P-selectin as described in Figure 3.2.1.(C.).

(B) Neuraminidase Inhibits The Adhesion Of $CD34^+$ Cells To P-Selectin.

Sorted $CD34^+$ cells were incubated at 1 x 10^6/ml for 1 hour at 37°C in PBS/0.1% BSA alone or containing 0.1 unit/ml neuraminidase (*Arthrobacter ureafaciens*). HL60 cells were treated in an identical manner. Digestion was terminated by washing thrice in assay medium, and cells were assayed for adhesion to purified P-selectin as described above. Data represent the mean ± S.E.; n=2. Neuraminidase treated (■); Diluent (▨).

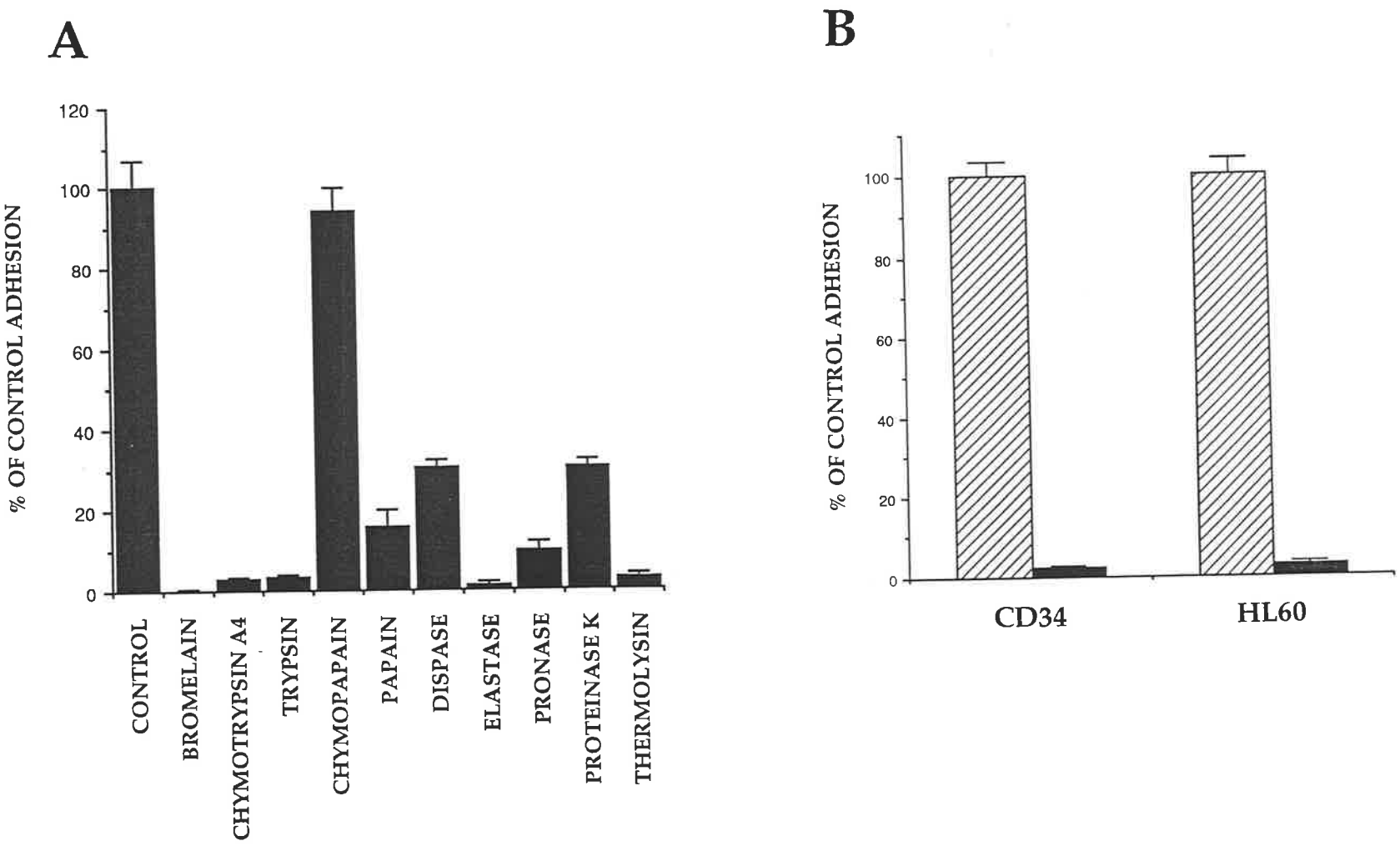
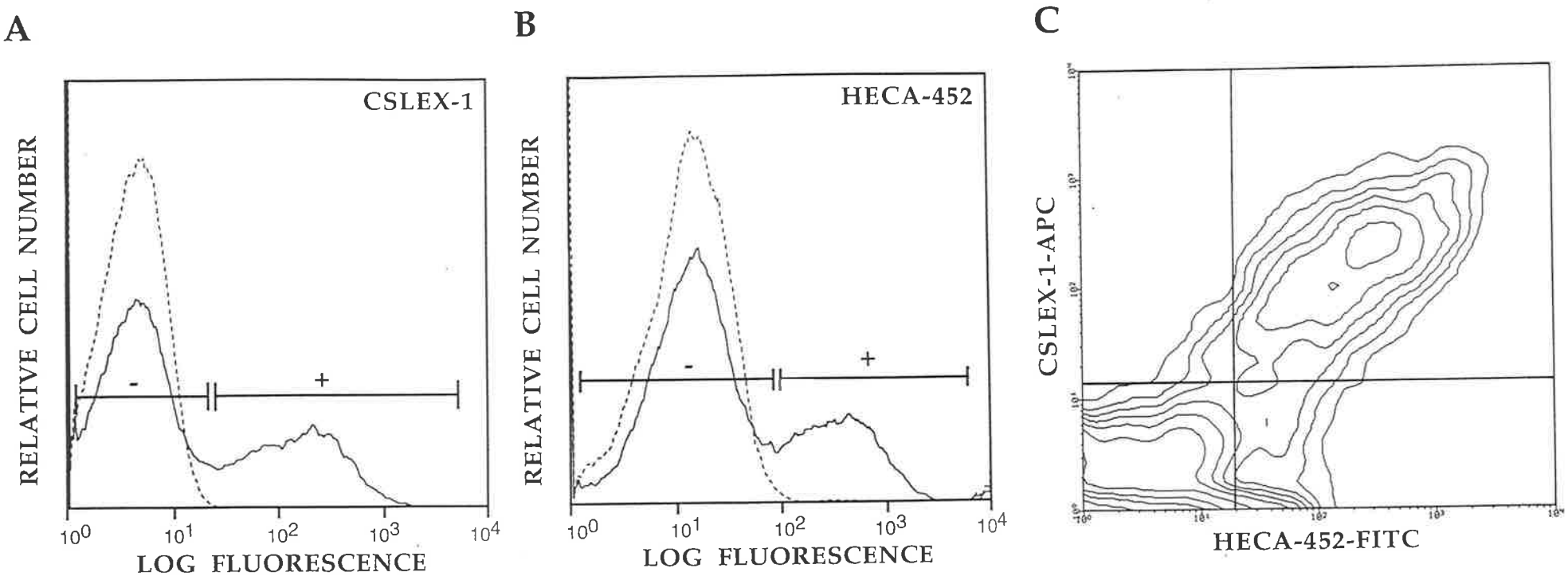

A
% OF CONTROL ADHESION
120
100
80
60
40
20
0
CONTROL
BROMELAIN
CHYMOTRYPSIN A4
TRYPSIN
CHYMOPAPAIN
PAPAIN
DISPASE
ELASTASE
PRONASE
PROTEINASE K
THERMOLYSIN
B
% OF CONTROL ADHESION
100
80
60
40
20
0
CD34
HL60

Figure 3.2.5. $CD34^+$ Cells Display The HECA-452 And CSLEX-1 Antigenic Determinants.

(A) & (B) BMMNC were stained by 2-colour indirect immunofluorescence with CD34 antibody HPCA-2-PE in combination with either HECA-452 or non-binding rat IgM followed by sheep anti-rat Ig-FITC (rat Ig-specific), and CSLEX-1 or IgM control antibody followed by goat anti-IgM-FITC. Data are displayed as single-parameter fluorescence (FITC) histograms of 1×10^4 light scatter gated events collected as list-mode data. Dashed line, mouse IgM and rat IgM control ; dark line, CSLEX-1 and HECA-452.

(C) CSLEX-1 and HECA-452 mAbs recognise a similar glycoprotein antigen on $CD34^+$ cells. BMMNC were stained with $CD34^+$ antibody HPCA-2-PE, HECA-452 and CSLEX-1. CSLEX-1 and HECA-452 were detected with APC- and FITC-conjugated reagents, respectively. A total of 1×10^4 $CD34^+$ events were collected as list mode data.


A
CSLEX-1
RELATIVE CELL NUMBER
-
+
10^0 10^1 10^2 10^3 10^4
LOG FLUORESCENCE
B
HECA-452
RELATIVE CELL NUMBER
-
+
10^0 10^1 10^2 10^3 10^4
LOG FLUORESCENCE
C
CSLEX-1-APC
HECA-452-FITC

on skin-associated memory T-cells (Berg *et al*, 1991a), recognises neuraminidase sensitive structures involving a domain common to both sialyl Le^x and sialyl Le^a (Berg *et al*, 1991b). HECA-452 was found to bind to approximately 35% of $CD34^+$ cells (Figure 3.2.5(B)).

Given the similarity in the carbohydrate epitopes identified by CSLEX-1 and HECA-452, these data suggested the possibility that similar glycoprotein antigens were being identified by the two monoclonal antibodies on $CD34^+$ cells. This was confirmed by subsequent flow cytometric studies (Figure 3.2.5.(C)). No cross-blocking between CSLEX-1 and HECA-452 was observed in these experiments, suggesting that the two antibodies bind to distinct antigenic determinants. Consistent with this observation, three-colour analysis with anti-CD34 antibody, CSLEX-1 and HECA-452 demonstrated co-linear staining of approximately 30% of the $CD34^+$ population (Figure 3.2.5. (C)). Binding of both mAbs was abolished by neuraminidase treatment of $CD34^+$ cells (data not shown). Collectively, these data are consistent with the notion that CSLEX-1 and HECA-452 recognise antigenically distinct epitopes presented on the same glycoprotein structure. Significantly, HECA-452 inhibited the adhesion of both $CD34^+$ cells and the myelomonocytic cell line HL60, to purified P-selectin by 53% and 72% (Figure 3.2.6.) respectively, demonstrating that in addition to its role as a receptor for E-selectin, the HECA-452 antigen also functions as a ligand for P-selectin on haemopoietic progenitor cells. The apparent anomaly between the degree of HECA-452 expression on $CD34^+$ cells (35%) and its ability to block more than 50% of the adhesion to P-Selectin, could be attributed to the IgM isotype of the HECA-452 mAb, as it consistently induced a degree of aggregation of the cells due to cross-linking, leading to an over-estimate of the extent of adhesion-blockade.

Fluorescence activated cell sorting (FACS) of the $CD34^+CSLEX\text{-}1^+$, $CD34^+HECA\text{-}452^+$ and $CD34^+CSLEX\text{-}1^-$, $CD34^+HECA\text{-}452^-$ subpopulations demonstrated clonogenic progenitors in all fractions. Relative to the $CD34^+$ population, BFU-E numbers were consistently diminished in the subpopulations identified by HECA-452 and CSLEX-1, and either unchanged or slightly increased in the corresponding negative subpopulations (Figure 3.2.7. (A)). Pre-CFU activity assayed under stroma-free, cytokine-dependent

Figure 3.2.6. Monoclonal Antibody HECA-452 Partially Blocks The Adhesion Of $CD34^+$ Cells To P-selectin.

Sorted $CD34^+$ cells and HL60 cells were incubated at 5×10^5 cells/ml on plates containing immobilised platelet-derived P-selectin in the presence of control rat IgM antibody (▨) or HECA-452 mAb (■). Adhesion was quantitated as described in Figure 3.2.1. Data represents the mean ± S.E. (n=2)

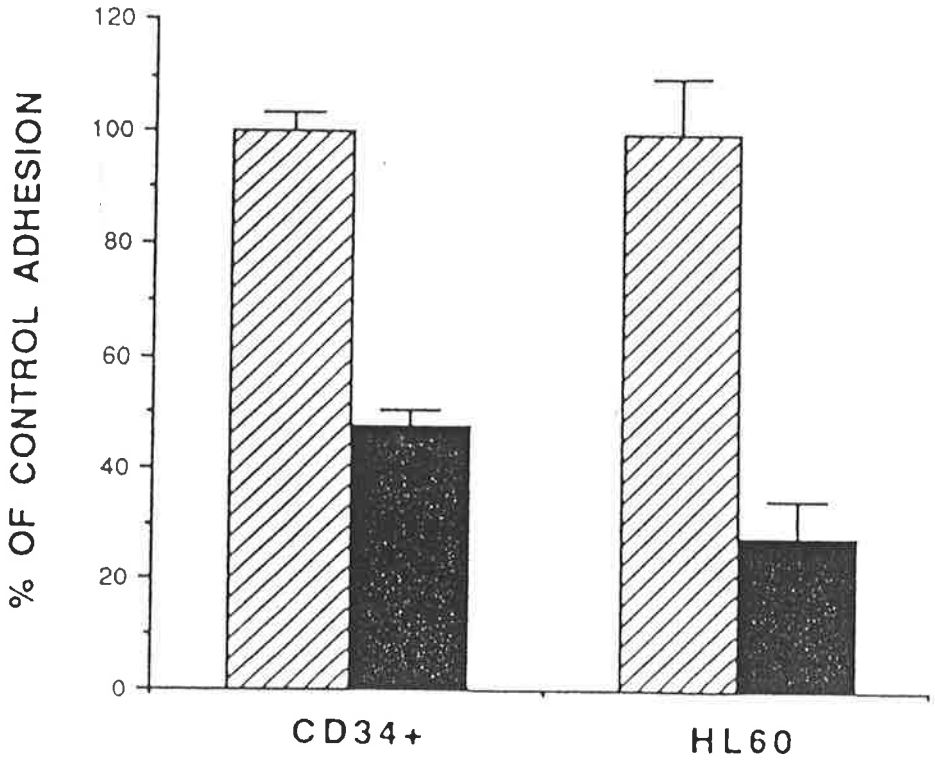
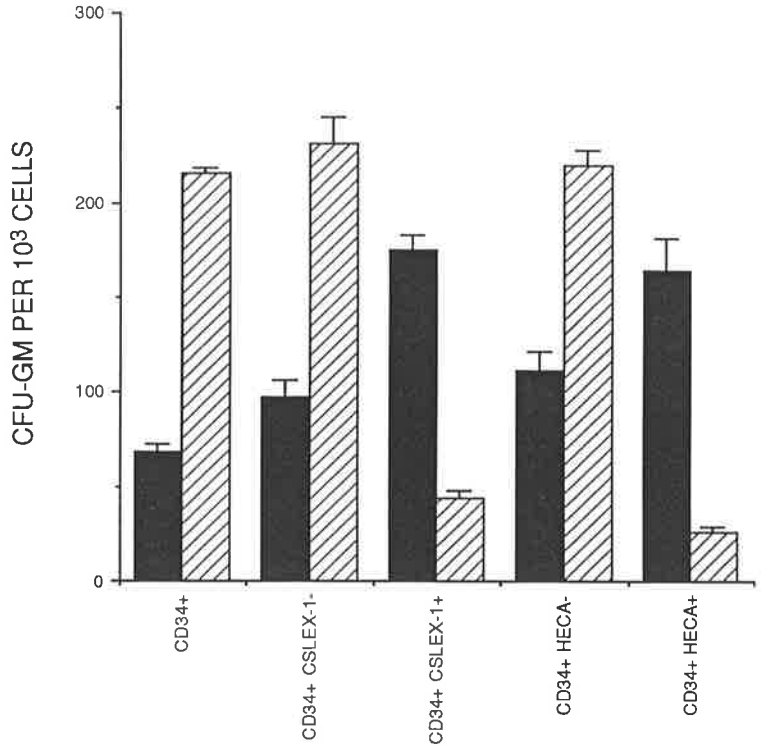

% OF CONTROL ADHESION
120
100
80
60
40
20
0
CD34+
HL60

Figure 3.2.7.

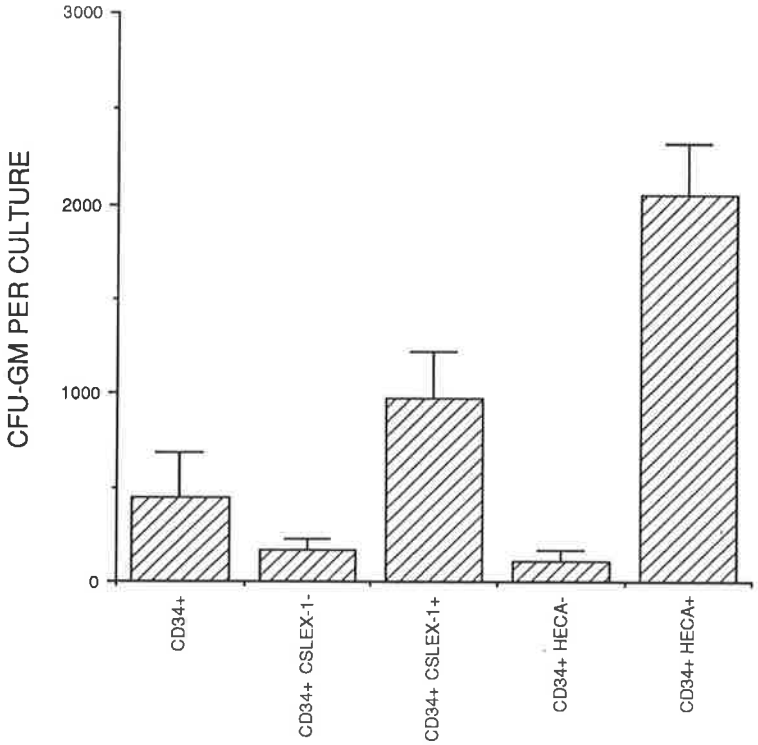
(A) Fluorescence activated cell sorting (FACS) of the $CD34^+CSLEX\text{-}1^+$, $CD34^+HECA\text{-}452^+$ and $CD34^+CSLEX\text{-}1^-$, $CD34^+HECA\text{-}452^-$ subpopulations demonstrates that clonogenic progenitors (CFU-GM, ■) are present in all fractions. Relative to the $CD34^+$ population, erythroid progenitors (BFU-E, ▨) numbers were consistently diminished in the subpopulations identified by HECA-452 and CSLEX-1, and either unchanged or slightly increased in the corresponding negative subpopulations. Results are expressed as the number of CFU-GM at day 14 per 1×10^3 cells plated. Data represent the mean ± S.E. (n=3).

(B) Pre-CFU activity assayed under stroma-free, cytokine-dependent conditions was consistently enhanced in the $CD34^+HECA\text{-}452^+$ and $CD34^+CSLEX\text{-}1^+$ subpopulations and markedly diminished in the respective negative subpopulations. Results are expressed as the mean ± S.E. (n=3) of CFU-GM present in each culture at day 14.

A


CFU-GM PER 10³ CELLS
300
200
100
0
CD34+
CD34+ CSLEX-1-
CD34+ CSLEX-1+
CD34+ HECA-
CD34+ HECA+

B


CFU-GM PER CULTURE
3000
2000
1000
0
CD34+
CD34+ CSLEX-1-
CD34+ CSLEX-1+
CD34+ HECA-
CD34+ HECA+

conditions was consistently enhanced in the $CD34^+HECA\text{-}452^+$ and $CD34^+CSLEX\text{-}1^+$ subpopulations and markedly diminished in the respective negative subpopulations (Figure 3.2.7. (B)). Three colour flow cytometric analyses consistently demonstrated expression of CSLEX-1 antigen by a high proportion (~80%) of the $CD34^+CD38^-$ cells (Figure 3.2.8. (B)). Notably, in all bone marrow samples analysed, the majority (95%) of $CD34^+CD19^+$ B-lymphoid cells lacked detectable expression of CSLEX-1 antigen (Figure 3.2.8. (A)) a phenotype correlating well with their lack of binding to P-selectin as demonstrated above.

During the course of this study, a glycoprotein counter-receptor for P-selectin termed P-selectin Glycoprotein Ligand (PSGL-1) was molecularly cloned from the promyelocytic leukaemia cell line, HL60. This prompted an investigation of the expression of this gene by $CD34^+$ cells or sorted subpopulations. Given the small numbers of cells available, reverse transcription (RT)-PCR amplification was employed. Oligonucleotide primers were designed in accordance with the published sequence to generate a 1297 bp product by RT-PCR amplification. A band of expected size was produced from total $CD34^+$ cells (Figure 3.2.9. (A), lane 5). Subsequent automated sequence analysis revealed 100% sequence homology of this product with the published PSGL-1 sequence (data not shown). In addition, a PCR product of the expected size was observed in both $CD34^+CSLEX\text{-}1^+$ (Figure 3.2.9. (B), lane 1) and $CD34^+CSLEX\text{-}1^-$ (Figure 3.2.9. (B), lane 3) sorted subpopulations.

3.2.4. $CD34^+$ Cells Express The PSGL-1 Protein

To investigate PSGL-1 protein expression by bone marrow-derived $CD34^+$ cells, an affinity purified polyclonal antibody against the N-terminal peptide (Gln^1-Gln^{15}) of the mature, PACE propeptide-cleaved (Rehemtulla and Kaufman, 1992; Sako *et al*, 1993) PSGL-1 protein was utilised (De Luca *et al*, 1995; Phillips and Berndt, 1992). SBA-depleted bone marrow cells, were immunolabelled with mAbs HPCA2-PE (α-CD34) and the α-PSGL-1-peptide polyclonal, and using dual-parameter flow cytometry the level of PSGL-1 expression by $CD34^+$ cells was assessed. Figure 3.2.10. (B), demonstrates that essentially all of the $CD34^+$ cells coexpress the PSGL-1 receptor at low to moderate

Figure 3.2.8.

(A) $CD34^+CD19^+$ B Lymphoid Cells Lack Detectable Expression Of The CSLEX-1 Antigen.

BMMNC were stained by 3-colour immunofluorescence with the directly conjugated mAbs HPCA-2-FITC (α-CD34) and Leu12-PE (α-CD19) in combination with either CSLEX-1 or non-binding IgM control antibody followed by goat anti-IgM-Tricolour (TC). Data are displayed as dual-parameter histograms of 1×10^4 $CD34^+$ events collected as list-mode data.

(B) CSLEX-1 Binds To Approximately 78% Of The $CD34^+CD38^-$ Mononuclear Cells.

BMMNC were stained by 3-colour immunofluorescence with the directly conjugated mAbs HPCA-2-FITC (α-CD34) and Leu17-PE (α-CD38) in combination with either CSLEX-1 or non-binding IgM control antibody followed by goat anti-IgM-Tricolour (TC). Data are displayed as dual-parameter histograms of 1×10^4 $CD34^+$ events collected as list-mode data.

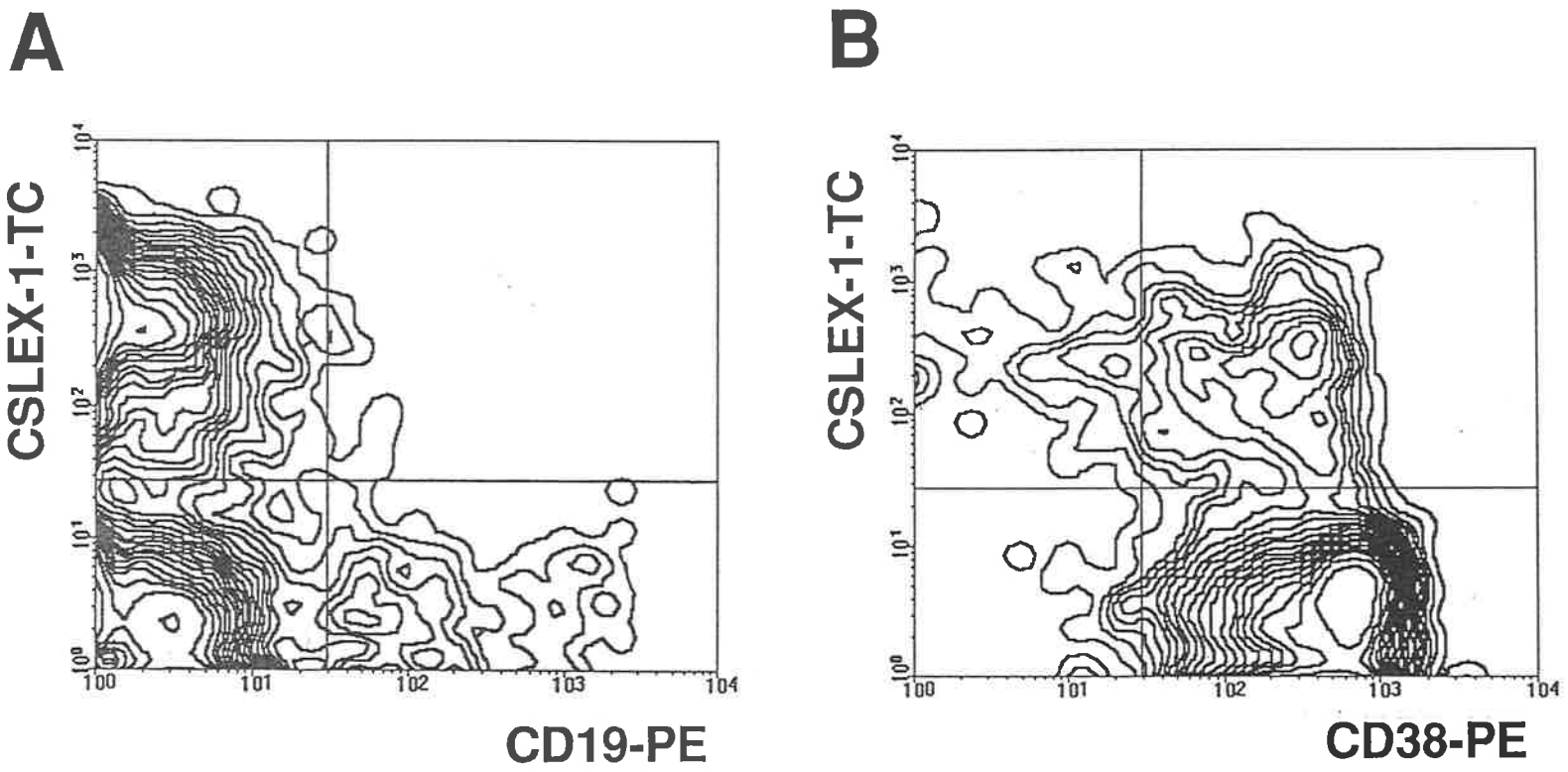

A
CSLEX-1-TC
CD19-PE
B
CSLEX-1-TC
CD38-PE

Figure 3.2.9. $CD34^+$ Cells Express P-selectin Glycoprotein Ligand (PSGL-1) mRNA.

(A) Expression of PSGL-1 was assessed in cell lines HL60, KG1a and BM-derived $CD34^+$ cells by RT-PCR. Expression of CD34 was also assessed in these cell lines and serve as a loading control. Reaction mixes were subjected to electrophoresis on a 1.5 % agarose gel and visualised by ethidium bromide staining. Lane 1, HL60/PSGL-1 PCR; Lane 2, HL60/CD34 PCR; Lane 3, KG1a/PSGL-1 PCR; Lane 4, KG1a/CD34 PCR; Lane 5, $CD34^+$/PSGl-1 PCR; Lane 6, $CD34^+$/CD34 PCR; Lane 7, H_2O control/PSGL-1 PCR; Lane 8, H_2O control/CD34 PCR.

(B) Subdivision of the $CD34^+$ population by means of CSLEX-1 immunoreactivity demonstrates expression of PSGL-1 (by RT-PCR) in both subpopulations. Reaction mixes were electrophoresed and visualised as in (A). Lane 1, $CD34^+$CSLEX-1^+/PSGL-1 PCR; Lane 2, $CD34^+$CSLEX-1^+/CD34 PCR; Lane 3, $CD34^+$CSLEX-1^-/PSGL-1 PCR; Lane 4, $CD34^+$CSLEX-1^-/CD34 PCR; Lane 5, H_2O control/PSGL-1 PCR; Lane 6, H_2O control/CD34 PCR.

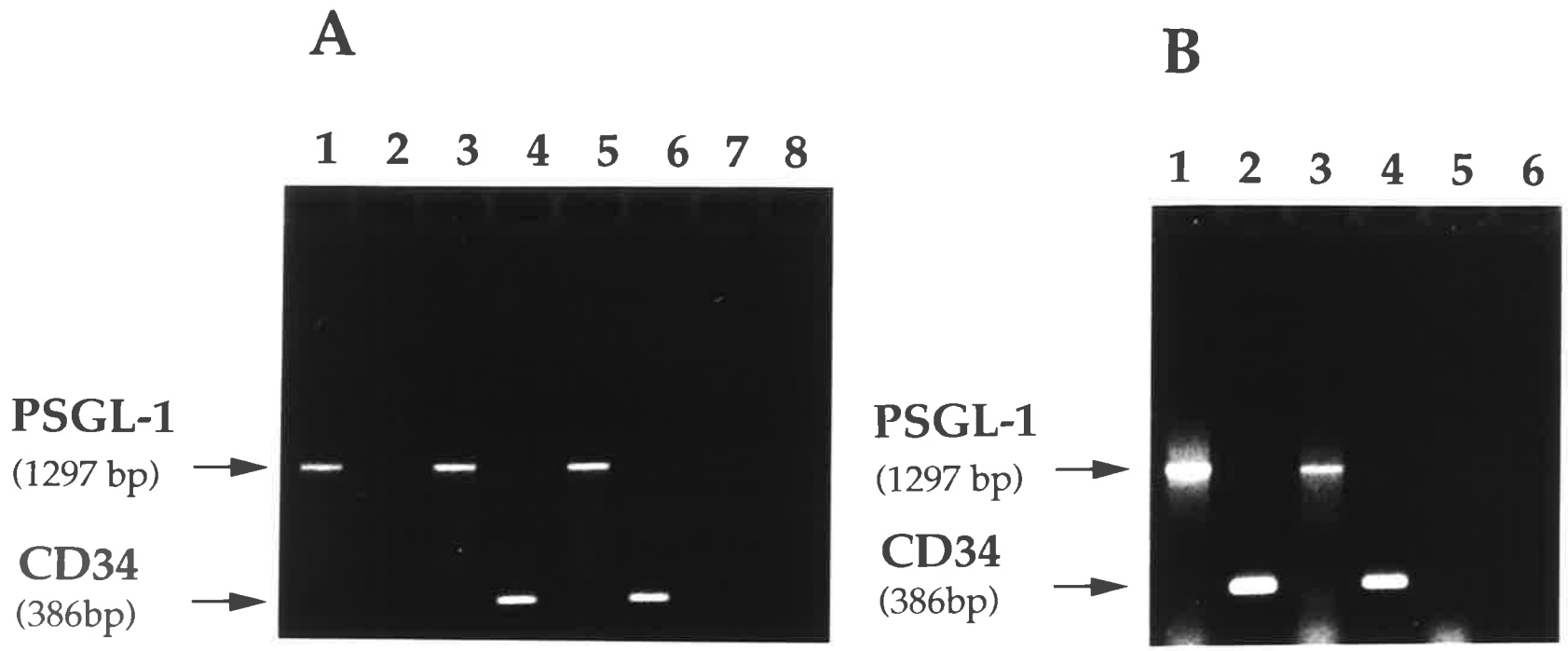
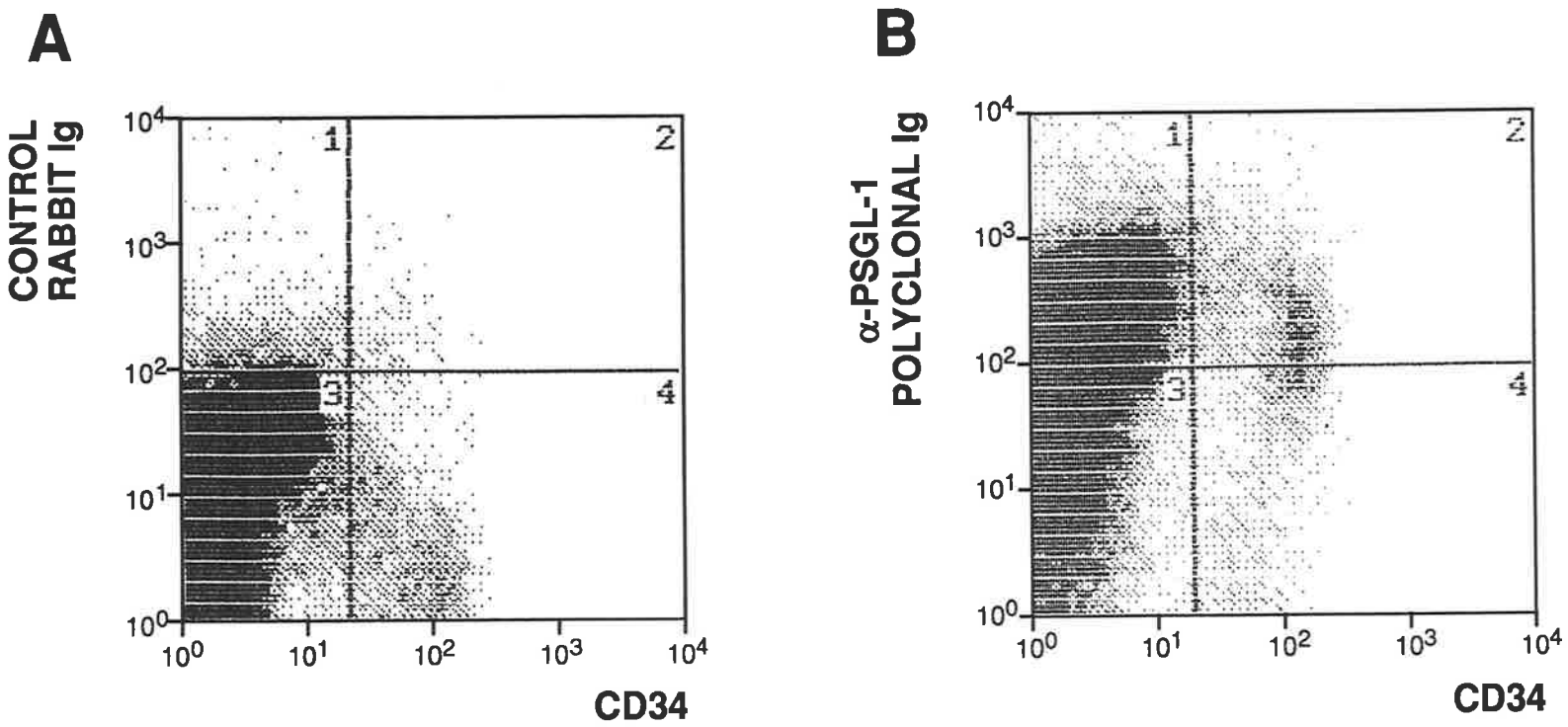

A
1 2 3 4 5 6 7 8
PSGL-1
(1297 bp)
CD34
(386bp)
B
1 2 3 4 5 6
PSGL-1
(1297 bp)
CD34
(386bp)

Figure 3.2.10. $CD34^+$ Cells Express P-Selectin Glycoprotein Ligand (PSGL-1) Protein.

Cell surface-expression of PSGL-1 protein by BM mononuclear cells was assessed by dual-parameter flow cytometry. Soy bean agglutinin (SBA)-depleted bone marrow mononuclear cells, were immunolabelled with mAbs HPCA2-PE (α-CD34) and an affinity purified polyclonal antibody against the N-terminal peptide (Gln^1-Gln^{15}) of the mature, PACE propeptide cleaved PSGL-1 protein (refer to text for details). A total of 1×10^4 $CD34^+$ events were collected as list mode data. Essentially all of the $CD34^+$ cells coexpress the PSGL-1 receptor at low to moderate levels, in accord with the mRNA analysis described above (Figure 3.2.9.)


A
CONTROL RABBIT Ig
10^4
10^3
10^2
10^1
10^0
1
2
3
4
10^0
10^1
10^2
10^3
10^4
CD34
B
α-PSGL-1 POLYCLONAL Ig
10^4
10^3
10^2
10^1
10^0
1
2
3
4
10^0
10^1
10^2
10^3
10^4
CD34

levels, in accord with the mRNA analysis described above. In contrast, PSGL-1 expression by $CD34^+CD19^+$ presumptive B-cell precursors, was undetectable by flow cytometric analysis (data not shown). This was in accordance with the lack of binding of B-cell precursors and their progeny to CD62P.

3.2.5. Contribution Of PSGL-1 In The Adhesion Of $CD34^+$ Cells To P- And E-Selectin: The Effect Of Mocarhagin Treatment On The Adhesion Of $CD34^+$ Cells

A number of reports have demonstrated that in addition to serving as a ligand for P-selectin, PSGL-1 may also bind to E-selectin (Sako *et al*, 1993; Asa *et al*, 1995; De Luca *et al*, 1995; Li *et al*; 1996). In contrast to P-selectin however, the requirements for E-selectin recognition are much less stringent, as E-selectin has been shown to bind a number of sialomucins and glycoprotein structures that co-express sialyl Le^x or the related structure, sialyl Le^a with high affinity (Piker *et al*, 1991; Kishimoto *et al*, 1991; Kuijpers *et al*, 1992; Kotovuori *et al*, 1993; Steegmaier *et al*, 1995).

$CD34^+$ cells isolated by 561-Dynabeads (please refer to *Materials and Methods*, Section 2.10.5. (b)) from aspirates of bone marrow of normal, healthy donors, were assayed for their ability to bind to CHO cells expressing human E or P-selectin. As shown in Figure 3.2.11. a mean of 69% (range: 65-74%; n = 3) of marrow $CD34^+$ demonstrated adhesion to CHO-E-selectin transfectants. Adhesion of $CD34^+$ cells to the CHO-E-selectin transfectants in these assays was, cation-dependent, neuraminidase-sensitive and was specifically inhibited by the function-blocking mAb 5B11. However, unlike binding to the P-selectin expressing CHO cells, adhesion of $CD34^+$ cells to the CHO-E-selectin transfectants was not inhibited by the affinity-purified polyclonal antibody against the N-terminal 15 amino acids of the mature PSGL-1 protein (Figure 3.2.11.).

To further investigate the contribution made by PSGL-1 in the adherence of $CD34^+$ cells to P- and E-selectin, a novel cobra venom metalloproteinase, mocarhagin was utilised (De Luca *et al*, 1995). Mocarhagin (NMM) specifically cleaves between the Tyr^{10} and Asp^{11} residues of the mature, PACE-cleaved PSGL-1 receptor and results in the loss of the N-terminal decapeptide, QATEYEYLDY (Figure 3.2.12. (A)). This region is rich in negatively charged aspartate (D) and glutamate (E) residues, and contains 3 tyrosine (Y)

Figure 3.2.11. $CD34^+$ cell adhesion to P- and E-selectin Expressing CHO Transfectants

BM $CD34^+$ cells isolated with 561-Dynabeads were labelled with ^{51}Cr and assayed for their ability to bind to CHO cells expressing either human E- or P-selectin (compared with untransfected CHO cells). Incubation was carried out for 30 minutes at 37°C after which unbound cells were removed by washing and adhesion quantitated by liquid scintillation counting of Triton X-100 solubilised lysates. Data are presented as the percentage of input radioactive counts and represent the mean ± S.E. of 3 experiments [adhesion of $CD34^+$ cells to P-selectin, (■), E-selectin, (▨) and control CHO cells (□)].

Adhesion of $CD34^+$ cells to both P- and E-selectin was sialic acid-dependent (ie. neuraminidase sensitive) and cation dependent (abrogated in the presence of 5 mM EGTA). Adhesion of $CD34^+$ cells to P- and E-selectin was specifically inhibited with the addition of rabbit anti-CD62P Fab fragments (10 μg/ml) and the function-blocking α-CD62E mAb 5B11, respectively. Unlike binding to P-selectin, adhesion of $CD34^+$ cells to E-selectin was not inhibited by an affinity-purified polyclonal antibody to PSGL-1 protein.

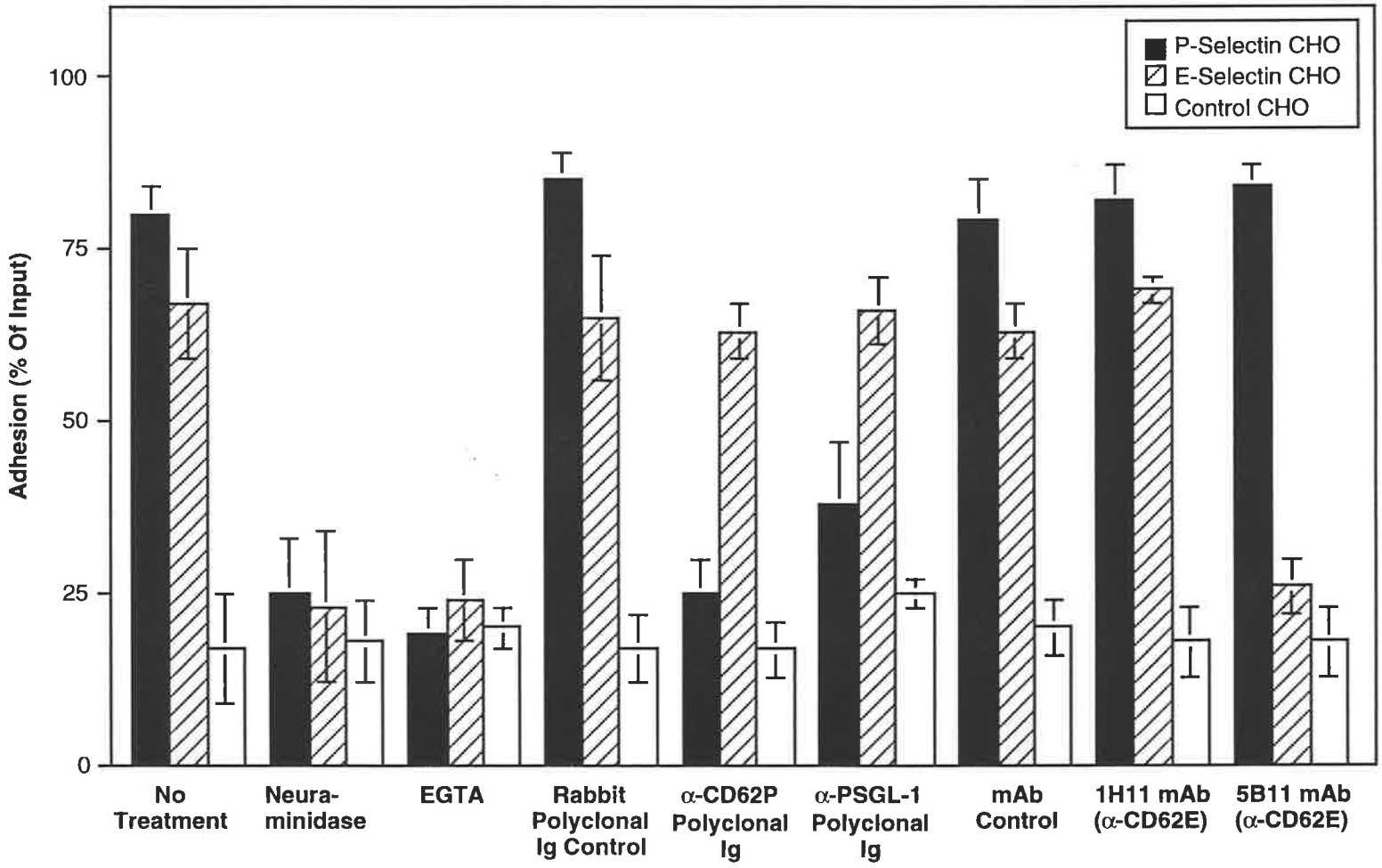

P-Selectin CHO
E-Selectin CHO
Control CHO
Adhesion (% Of Input)
100
75
50
25
0
No Treatment
Neura-minidase
EGTA
Rabbit Polyclonal Ig Control
α-CD62P Polyclonal Ig
α-PSGL-1 Polyclonal Ig
mAb Control
1H11 mAb (α-CD62E)
5B11 mAb (α-CD62E)

Figure 3.2.12. Cobra Venom Metalloproteinase, Mocarhagin Selectively Abrogates The Adhesion Of CD34$^+$ Cells To P-Selectin, But Not E-Selectin.

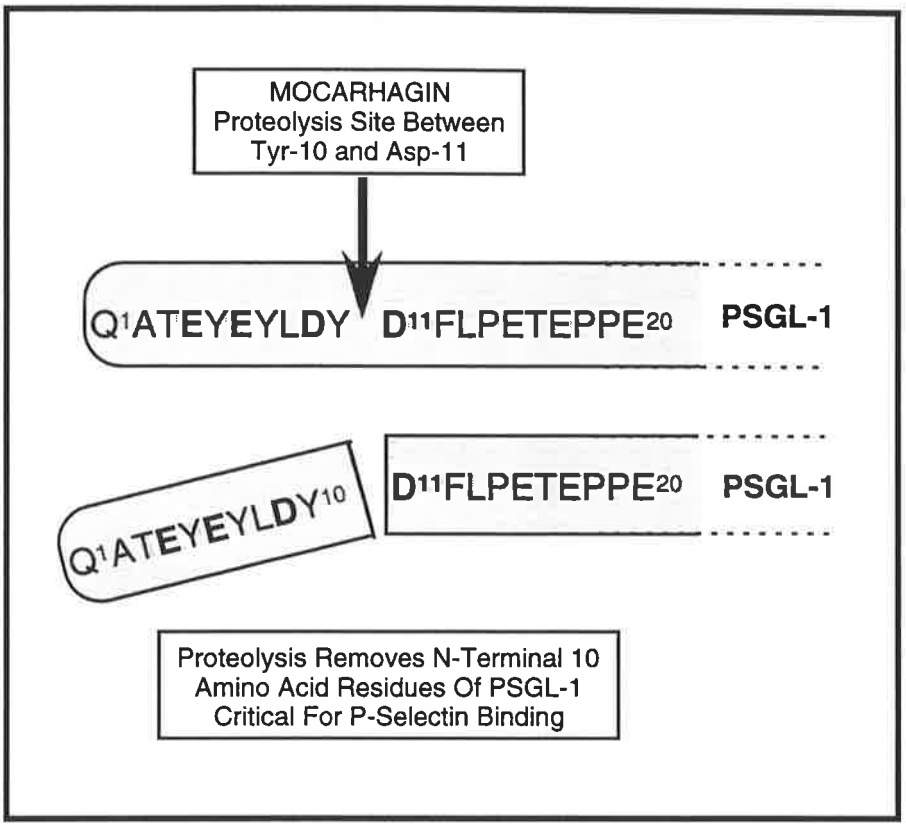
(A) The cobra venom metalloproteinase, mocarhagin (De Luca *et al*, 1995) specifically cleaves between the Tyr^{10} and Asp^{11} residues of the mature, PACE-cleaved PSGL-1 receptor and results in the loss of the N-terminal decapeptide, QATEYEYLDY.

(B) Mocarhagin-treatment inhibits the adhesion of CD34$^+$ cells to P-selectin.

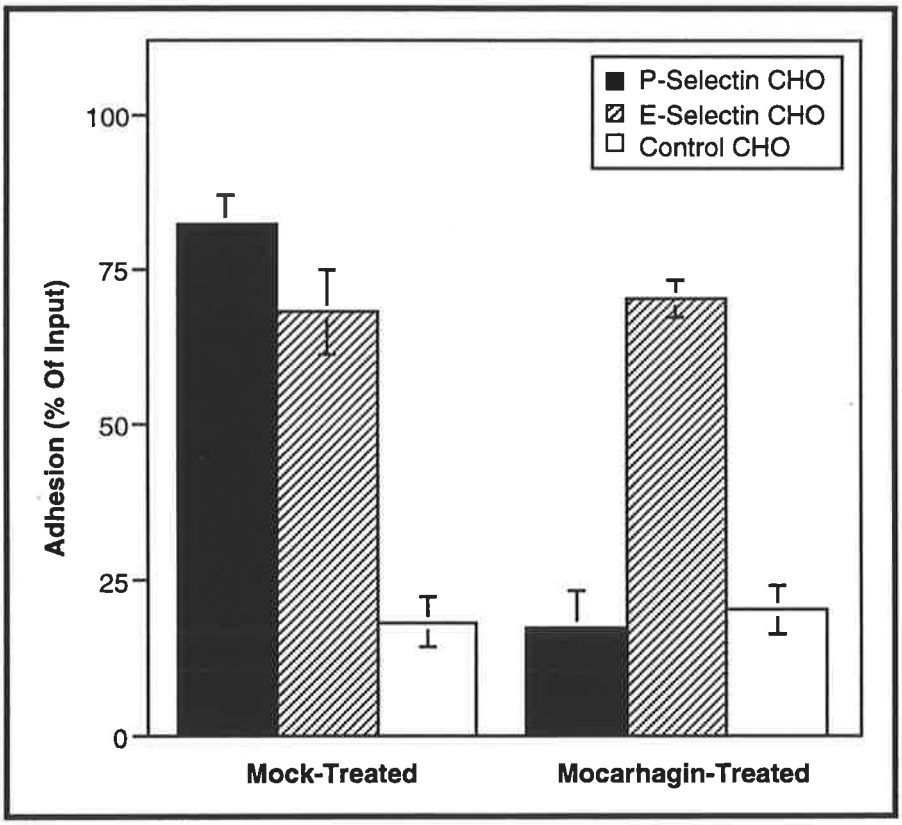
^{51}Cr-labelled, 561-Dynabead-purified CD34$^+$ cells were incubated at 1 x 10^6/ml for 30 minutes at 22°C in RPMI/1% BSA alone or containing 10 μg/ml mocarhagin. Digestion was terminated by washing thrice in assay medium, and cells were assayed for adhesion to P- and E-selectin as described above (Figure 3.2.11.). Data represent the mean ± S.E.; n=3. (adhesion of CD34$^+$ cells to P-selectin, (■), E-selectin, (▨) and control CHO cells (□).

Complete abrogation of binding of CD34$^+$ cells to P-selectin was observed. Significantly, however, adhesion to E-selectin was unaffected. Comparable results were obtained, when the α-PSGL-1 polyclonal was utilised to block CD34$^+$ cell binding to P- and E-selectin (refer to Figure 3.2.11.).

A


MOCARHAGIN
Proteolysis Site Between
Tyr-10 and Asp-11
Q1ATEYEYLDY D11FLPETEPPE20 PSGL-1
Q1ATEYEYLDY10 D11FLPETEPPE20 PSGL-1
Proteolysis Removes N-Terminal 10
Amino Acid Residues Of PSGL-1
Critical For P-Selectin Binding

B


P-Selectin CHO
E-Selectin CHO
Control CHO
Adhesion (% Of Input)
100
75
50
25
0
Mock-Treated
Mocarhagin-Treated

residues that meet the consensus sequence for *O*-sulphation by the Golgi sulfotransferases and combined with the downstream sialylated carbohydrate, is essential for the binding to P-selectin (Pouyani and Seed, 1995 and Sako *et al*, 1995). Treatment of $CD34^+$ cells with NMM resulted in the complete loss of α-PSGL-1-peptide polyclonal-binding activity (data not shown), and moreover, as shown in Figure 3.2.12. (B), resulted in the complete abrogation of binding of $CD34^+$ cells to P-selectin. Significantly, however, adhesion to E-selectin was unaffected.

3.2.6. Committed Progenitors And Their Precursors Adhere To P-Selectin Via PSGL-1

To determine whether lineage restricted haemopoietic progenitor cells were present in the population of bone marrow cells which specifically bound to P-selectin via PSGL-1, clonogenic assays were performed on $CD34^+$ cells treated with mocarhagin. As seen in Figure 3.2.13. the adhesion of committed myeloid (CFU-GM) and erythroid progenitor (BFU-E) to P-selectin, was reduced by approximately 94% and 98%, respectively supporting the hypothesis that PSGL-1 represents the sole high affinity counter-receptor mediating the binding of committed myeloid and erythroid progenitors to P-selectin.

3.2.7. Signals Through PSGL-1 Results In The Negative Regulation Of Haemopoiesis

To investigate if interactions between selectins and their ligands play a physiological role in the regulation of HPC proliferation, soluble P-selectin was utilised to ligate PSGL-1 expressed at the surface of haemopoietic progenitor cells. More specifically, purified $CD34^+$ cells were added to plates coated with purified, human, platelet-derived CD62P, recombinant human-CD62P-IgG_1Fc fusion protein or murine-CD62L-IgG_1Fc (as a control), and subsequently overlaid with agar containing a combination of cytokines shown to optimally stimulate colony formation. In all experiments (n = 3), adhesion to P-selectin was associated with greater than 90% inhibition of both myeloid (CFU-GM) and erythroid colony (BFU-E) formation (Figure 3.2.14.).

3.2.13. Committed Haemopoietic Progenitor Cells Adhere To P-selectin Via PSGL-1.

$CD34^+$ cells were obtained as described in Figure 3.2.12. and incubated at 1 x 10^6/ml for 30 minutes at 22°C in RPMI/1% BSA alone or containing 10 μg/ml mocarhagin. Digestion was terminated by washing thrice in assay medium, and 1 x 10^3 cells were plated over recombinant CD62P-IgG_1Fc fusion protein (or PBS) immobilised on 30 mm tissue culture grade dishes (previously coated with 20 μg/ml of a monoclonal mouse anti-human IgG_1Fc antibody). Adhesion was allowed to proceed for 30 minutes at 37°C, after which unbound cells were removed by gentle washing. Adherent cells were subsequently dislodged by the brisk overlay of 0.9% methylcellulose (in IMDM) supplemented with 10 ng/ml each of purified recombinant human IL-1β, IL-3, IL-6, G-CSF, GM-CSF, SCF and EPO. Relative to the $CD34^+$ population "controls", erythroid (BFU-E, □) and myeloid progenitor (CFU-GM, ■) numbers were consistently diminished following treatment with mocarhagin. These results support the hypothesis that PSGL-1 is the sole high affinity counter-receptor mediating the binding of commited myeloid and erythroid progenitors to P-selectin. Results are expressed as the number of clonogenic cells at day 14 per 1 x 10^3 cells plated. Data represent the mean ± S.E. (n=3).

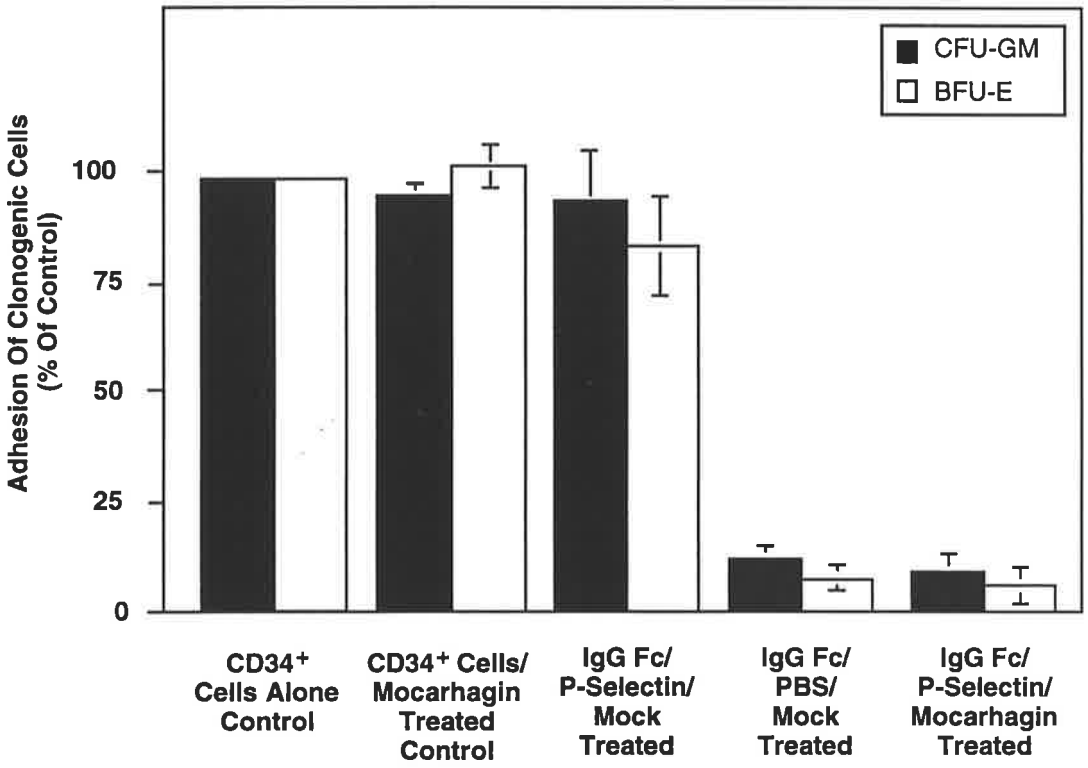
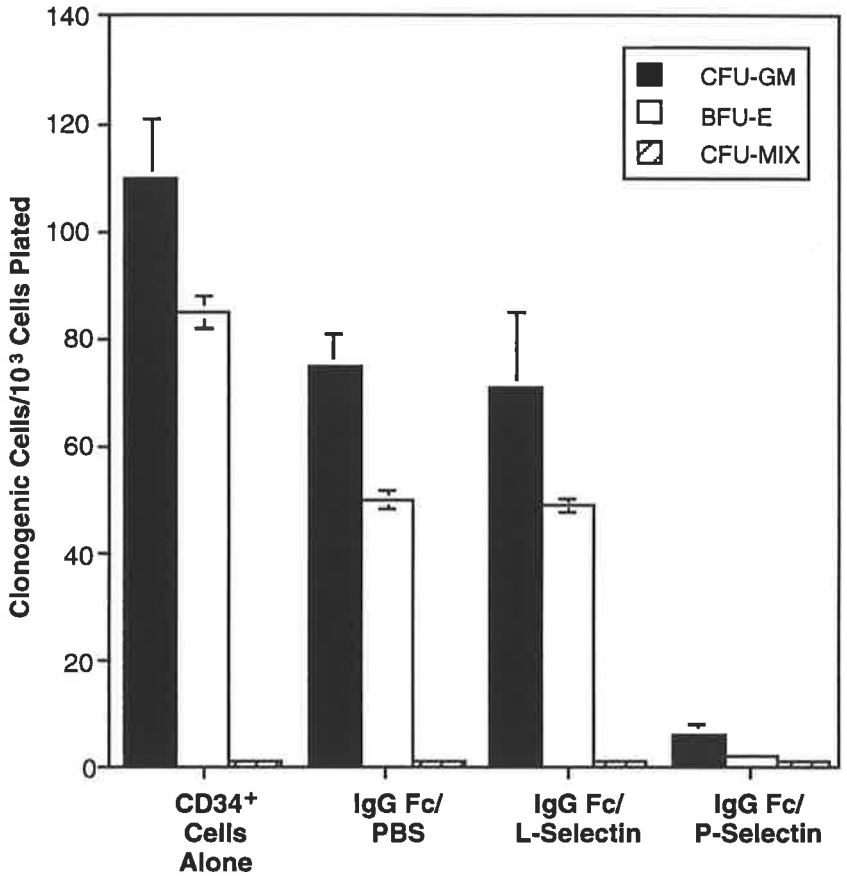

CFU-GM
BFU-E
Adhesion Of Clonogenic Cells (% Of Control)
100
75
50
25
0
CD34+ Cells Alone Control
CD34+ Cells/ Mocarhagin Treated Control
IgG Fc/ P-Selectin/ Mock Treated
IgG Fc/ PBS/ Mock Treated
IgG Fc/ P-Selectin/ Mocarhagin Treated

Figure 3.2.14. Signals Through PSGL-1 Results In The Negative Regulation Of Haemopoiesis.

1 x 10^3 $CD34^+$ cells (obtained as described in Figure 3.2.12.) were plated over recombinant CD62P-IgG_1Fc or CD62L-IgG_1Fc fusion protein (or PBS) immobilised on 30 mm tissue culture grade dishes (previously coated with 20 μg/ml of a monoclonal mouse anti-human IgG_1Fc antibody). Adhesion was allowed to proceed for 30 minutes at 37°C, after which the plates were gently overlayed (ie. not to disrupt the adhesive interaction) with 0.33% agar (in IMDM) supplemented with 10 ng/ml each of purified recombinant human IL-1β, IL-3, IL-6, G-CSF, GM-CSF, SCF and EPO. The number of clonogenic cells (CFU-GM, ■; BFU-E, □ and CFU-Mix, ▨) were assessed at day 14 according to standard criteria, and represent the number of colonies per 1 x 10^3 cells plated [mean ± S.E. (n=3)].


140
120
100
80
60
40
20
0
Clonogenic Cells/10³ Cells Plated
CFU-GM
BFU-E
CFU-MIX
CD34+ Cells Alone
IgG Fc/ PBS
IgG Fc/ L-Selectin
IgG Fc/ P-Selectin

STUDIES OF THE EXPRESSION OF L-SELECTIN (CD62L) BY HAEMOPOIETIC PROGENITOR CELLS

3.2.8. $CD34^+$ Cells From Human Bone Marrow Express L-Selectin

Cell-surface expression of L-selectin (CD62L) by immature haemopoietic progenitors was examined by multiparameter FACS using antibodies Leu8-PE and HPCA-2-PE, which identify L-selectin and the CD34 antigens, respectively (please refer to Table 2.10.1.). To facilitate these studies, sorting was performed using bone marrow samples pre-enriched for the $CD34^+$ cells by differential agglutination using soybean agglutinin (SBA) (Reisner *et al*, 1980; and refer to *Materials and Methods*, Section 2.11.1.). In addition, FACS was carried out using BM samples gated according to their light-scattering properties to include only cells within the lymphoid and blast windows (Figure 3.2.15 (A)), a region previously demonstrated to contain essentially all clonogenic progenitors and the more primitive cells with the capacity to initiate and sustain haemopoiesis in a variety of *in vitro* assays (van den Engh and Visser, 1979; Sutherland *et al*, 1989a; 1989b).

Figure 3.2.15. (B), illustrates a dual parameter contour plot from a representative experiment demonstrating binding of mAbs HPCA-2-FITC and Leu8-PE to light-scatter gated, SBA^- bone marrow cells. A mean of 87.5% (n = 4) of the $CD34^+$ cells were found to coexpress L-selectin (ie. $CD34^+CD62L^+$), whilst 12.5% (n = 4) of the $CD34^+$ cells lacked expression of L-selectin (ie. $CD34^+CD62L^-$).

3.2.9. Immunophenotypic Analysis Of The $CD34^+CD62L^+$ And $CD34^+CD62L^-$ Populations

To further investigate the nature of the differences between the $CD34^+CD62L^+$ and $CD34^+CD62L^-$ populations, BM specimens from four donors prepared as described above were stained by three-colour indirect immunofluorescence using antibodies Leu8 (α-CD62L) and HPCA-2 (α-CD34) in combination with each of a panel of mAbs which identify lineage restricted antigens (myeloid, B cell and T cell) or activation markers. As demonstrated in Figure 3.2.16. and Table 3.2.3, L-selectin expression was consistently found to subdivide, in varying proportions, populations of $CD34^+$ cells exhibiting B cell, T cell, or myeloid restricted antigens. Approximately 19% of $CD34^+$ cells coexpressing the T

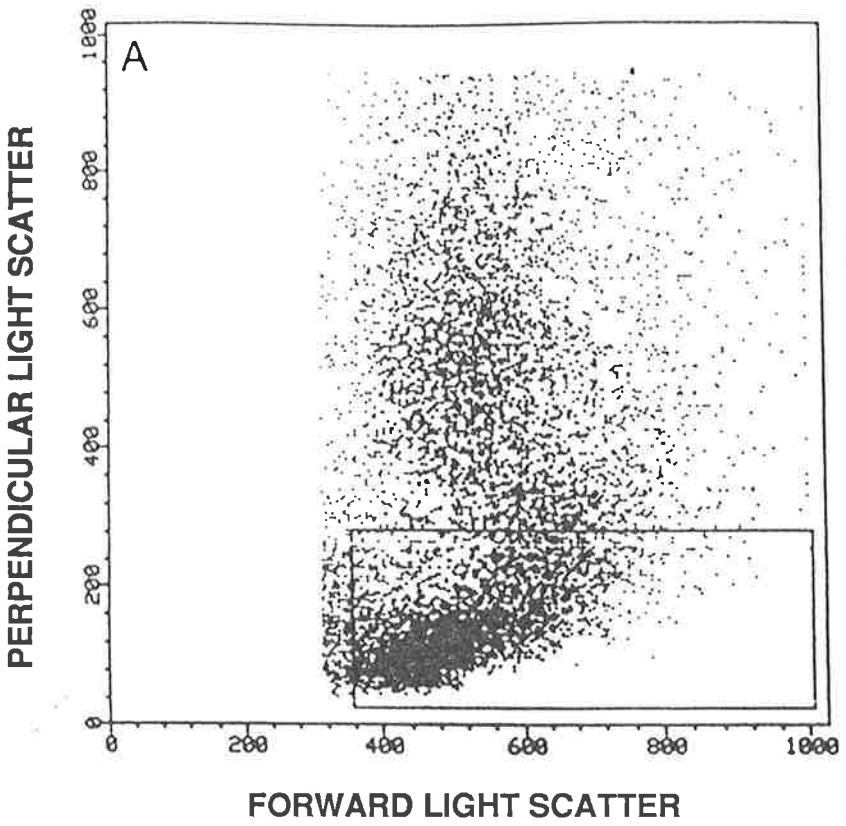
Figure 3.2.15.

(A) Dot plot demonstrating the light-scatter characteristics (forward scatter [FSC] vs. side scatter [SSC]) of soy bean agglutinin (SBA)-depleted BMMNCs. All sorts were performed using the light scatter gates shown, which includes essentially all the lymphoid and blast populations.

(B) Dual-parameter immunofluorescence analysis of the cells shown in the scatter gate in (A), demonstrating the expression of CD34 antigen and L-selectin. SBA^- BMMNC were stained with the directly conjugated mAbs HPCA-2-FITC (α-CD34) and Leu8-PE (α-CD62L). Data are displayed as dual-parameter histograms of 5×10^4 light-scatter-gated events collected as list-mode data.

In addition to subdividing the $CD34^+$ cells into $CD34^+CD62L^+$ and $CD34^+CD62L^-$ subpopulations, the $CD34^+$ cells could be further subdivided into $CD34^+CD62L^{BRIGHT}$ and $CD34^+CD62L^{DIM}$ based on the level of CD62L expression.

A


A
PERPENDICULAR LIGHT SCATTER
0
200
400
600
800
1000
FORWARD LIGHT SCATTER

B

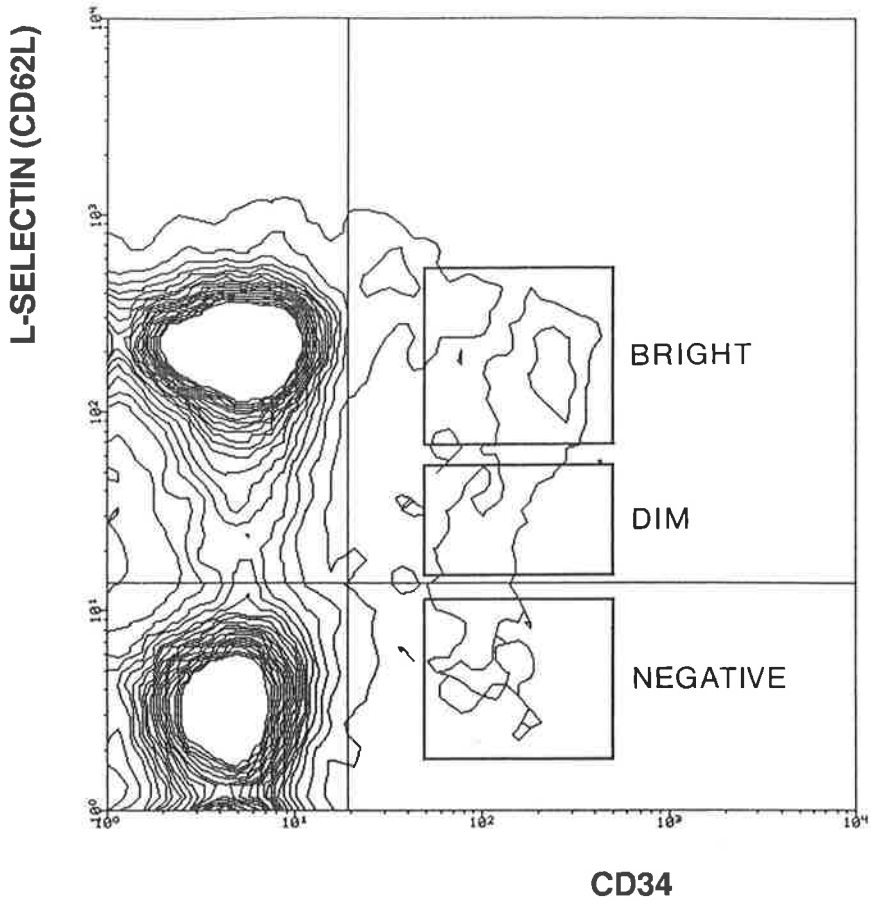

L-SELECTIN (CD62L)
BRIGHT
DIM
NEGATIVE
CD34

Figure 3.2.16. Three-Colour Immunofluorescence Analysis Demonstrating The Co-Expression Of CD62L On Multiple Myeloid And Lymphoid Cell Populations.

BMMNC were stained with Leu8-PE (CD62L) and biotinylated-HPCA-1 (CD34; followed by Streptavidin-ECD) in combination with either LeuM9-FITC (CD33), Leu17-FITC (CD38), HLA-DR-FITC, J5-FITC (CD10), CD19-FITC , 3A1-FITC (CD7) and MY7-FITC (CD13), or with appropriate combinations of isotype-matched FITC or PE-conjugated non-binding control antibodies (refer to *Materials and Methods*, Section 2.10.3. (d)). Cells were washed, fixed and analysed as described previously. Each 2-parameter histogram was generated from 20,000 $CD34^+$ events collected as list mode data using a Profile II flow cytometer and analysed using Coulter ELITE software. Data represent the mean ± SE of 3 experiments.

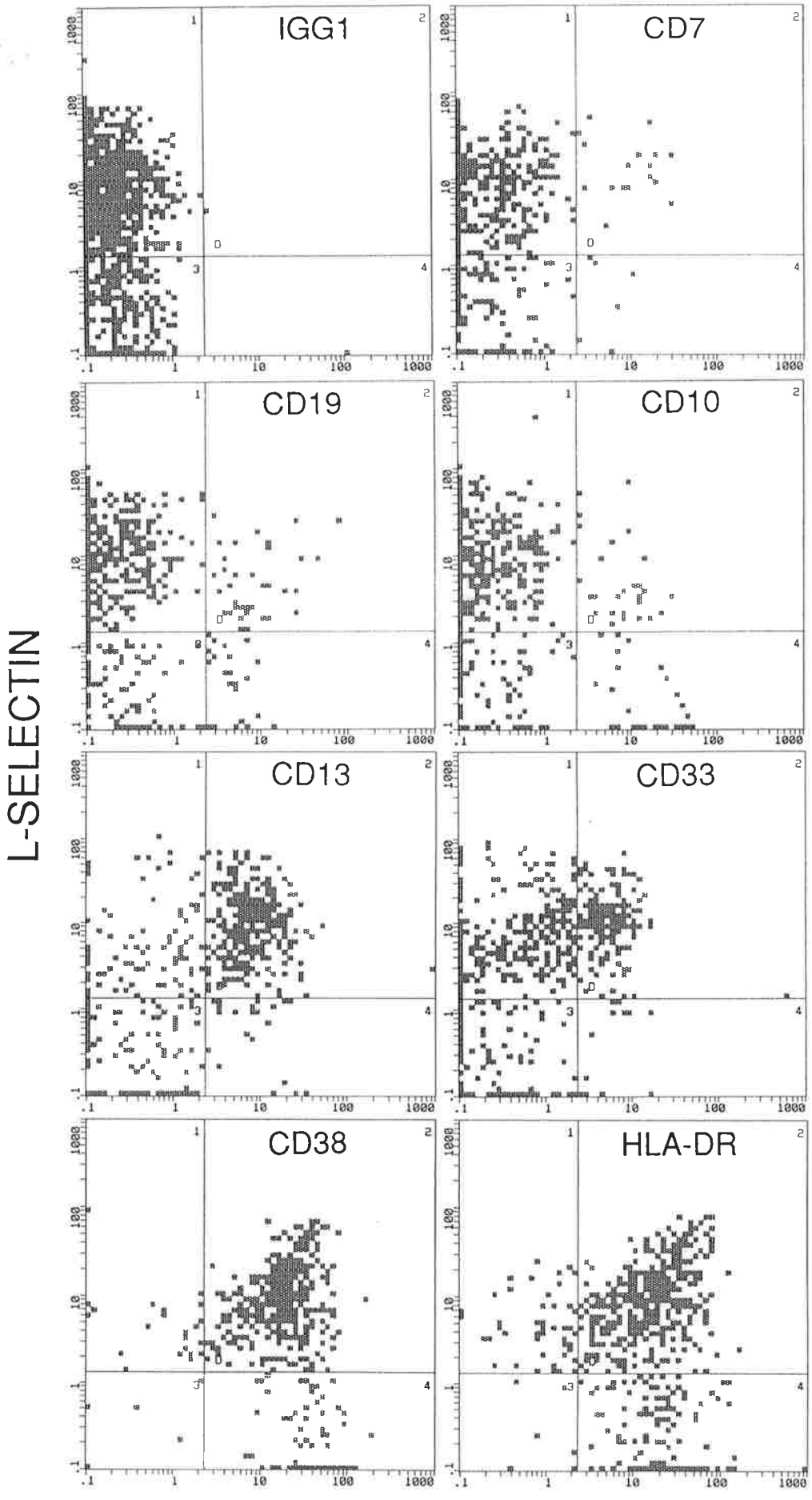

IGG1
CD7
CD19
CD10
CD13
CD33
CD38
HLA-DR
L-SELECTIN
LINEAGE ANTIGEN

Table 3.2.3. L-selectin (CD62L) Expression By $CD34^+$ BM Subsets

Antibody	Expression Of Lineage Antigen By $CD34^+$ $CD62L^+$ BM Mononuclear Cells	Expression Of CD62L By Cells Which Express Both CD34 And Lineage Antigen
Quandrant Number	Region 2 / (1 + 2) - Expressed as Percentage Positive	Region 2 / (2 + 4) - Expressed as Percentage Positive
CD7	18.9	87.4
CD10	13.8	63.6
CD13	81.8	89.0
CD19	15.2	66.8
CD33	60.5	91.9
CD38	48.9	100
HLA-DR	94.0	71.7

Summary of the data obtained from three-colour immunofluorescence and flow cytometric analysis (please also refer to Figure 3.2.16) demonstrating the co-expression of CD62L with early myeloid and lymphoid populations. Information generated from listmode data, and represent 1 x 10^4 $CD34^+$ sorted events. A representative experiments (one of three) is shown.

cell-restricted antigen, CD7 were CD62L$^+$, whilst up to 65% of the early B lymphoid cells, identified by coexpression of CD10 and CD19 also expressed CD62L. Similarly, more than 90% of the CD34$^+$ myeloid precursors, characterised by their expression of either CD38, CD13 or CD38 antigens, also displayed moderate to high levels of L-selectin.

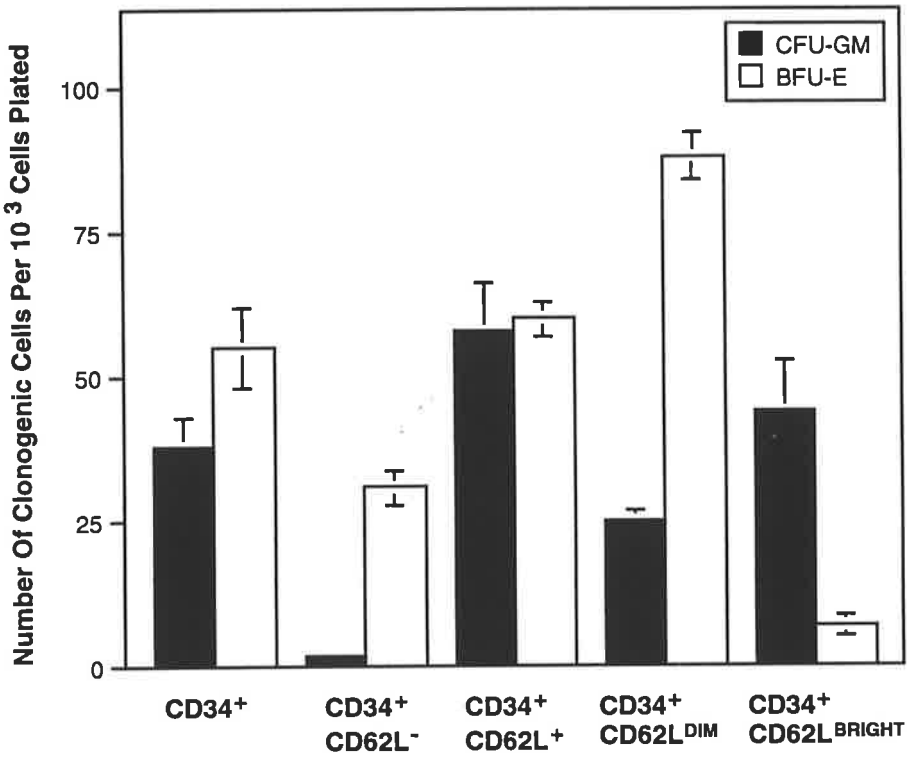
3.2.10. Reactivity Of L-Selectin Antibody Leu8 With Lineage Restricted Clonogenic Progenitors And Their Precursors

In addition to subdividing the CD34$^+$ cells into CD34$^+$CD62L$^+$ and CD34$^+$CD62L$^-$ subpopulations, the CD62L$^+$ population was further subdivided into CD34$^+$CD62L^{BRIGHT} and CD34$^+$CD62L^{DIM} subfractions as shown in Figure 3.2.15. (B)). *In vitro* clonogenic assays were performed to determine which subpopulation harboured the lineage restricted clonogenic progenitor cells. Fluorescence activated cell sorting of BMMNC consistently demonstrated that myeloid cells (CFU-GM) were enriched in the CD34$^+$CD62L$^+$ subpopulation, but were correspondingly absent in the CD34$^+$CD62L$^-$ subpopulation. In contrast, the erythroid progenitors (BFU-E) were present in both fractions, but were consistently diminished in the subpopulation which did not express L-selectin (CD34$^+$CD62L$^-$). Furthermore, myeloid (CFU-GM) progenitors were enriched in the CD34$^+$CD62L^{BRIGHT} when compared with the CD34$^+$CD62L^{DIM} subpopulation, whilst a majority of the BFU-E were present in the CD34$^+$CD62L^{DIM} subpopulation (Figure 3.2.17.). The numbers of CFU-GM in the CD34$^+$CD62L$^+$ and CD34$^+$CD62L^{BRIGHT} were not significantly different (Fridman two-way analysis of variance $p>0.05$)) whilst there was an apparent enrichment in committed myeloid progenitors in the CD34$^+$CD62L^{BRIGHT} fraction when compared to the CD34$^+$CD62L^{DIM} fraction ($p<0.05$). In contrast, there was a statistically significant trend ($p<0.05$) towards high numbers of erythroid progenitors (BFU-E), in the CD34$^+$CD62L^{DIM} subpopulation as compared to their incidence in the CD34$^+$CD62L^{BRIGHT} subpopulation.

3.2.11. Cells Initiating Long-Term Haemopoiesis *In Vitro* Express L-selectin At Low Levels (CD62L^{DIM})

Figure 3.2.17.

Fluorescence activated cell sorting (FACS) of the $CD34^+CD62L^+$, $CD34^+CD62L^-$, $CD34^+CD62L^{BRIGHT}$ and $CD34^+CD62L^{DIM}$ subpopulations (please refer to Figure 3.2.15. (B)) demonstrates that clonogenic myeloid (CFU-GM, ■) cells are enriched in the $CD34^+CD62L^+$ subpopulation and correspondingly absent in the $CD34^+CD62L^-$ subpopulation. In contrast, erythroid progenitors (BFU-E, □) are present in both fractions, but consistently diminished in the subpopulation which did not express L-selectin ($CD34^+CD62L^-$). Myeloid (CFU-GM) progenitors are enriched in the $CD34^+CD62L^{BRIGHT}$ when compared with the $CD34^+CD62L^{DIM}$ subpopulation, whilst the majority of the BFU-E are present in the $CD34^+CD62L^{DIM}$ subpopulation. Results are expressed as the number of CFU-GM at day 14 per 1×10^3 cells plated. Data represent the mean ± S.E. (n=3).


CFU-GM
BFU-E
Number Of Clonogenic Cells Per 10 3 Cells Plated
100
75
50
25
0
CD34+
CD34+ CD62L-
CD34+ CD62L+
CD34+ CD62LDIM
CD34+ CD62LBRIGHT

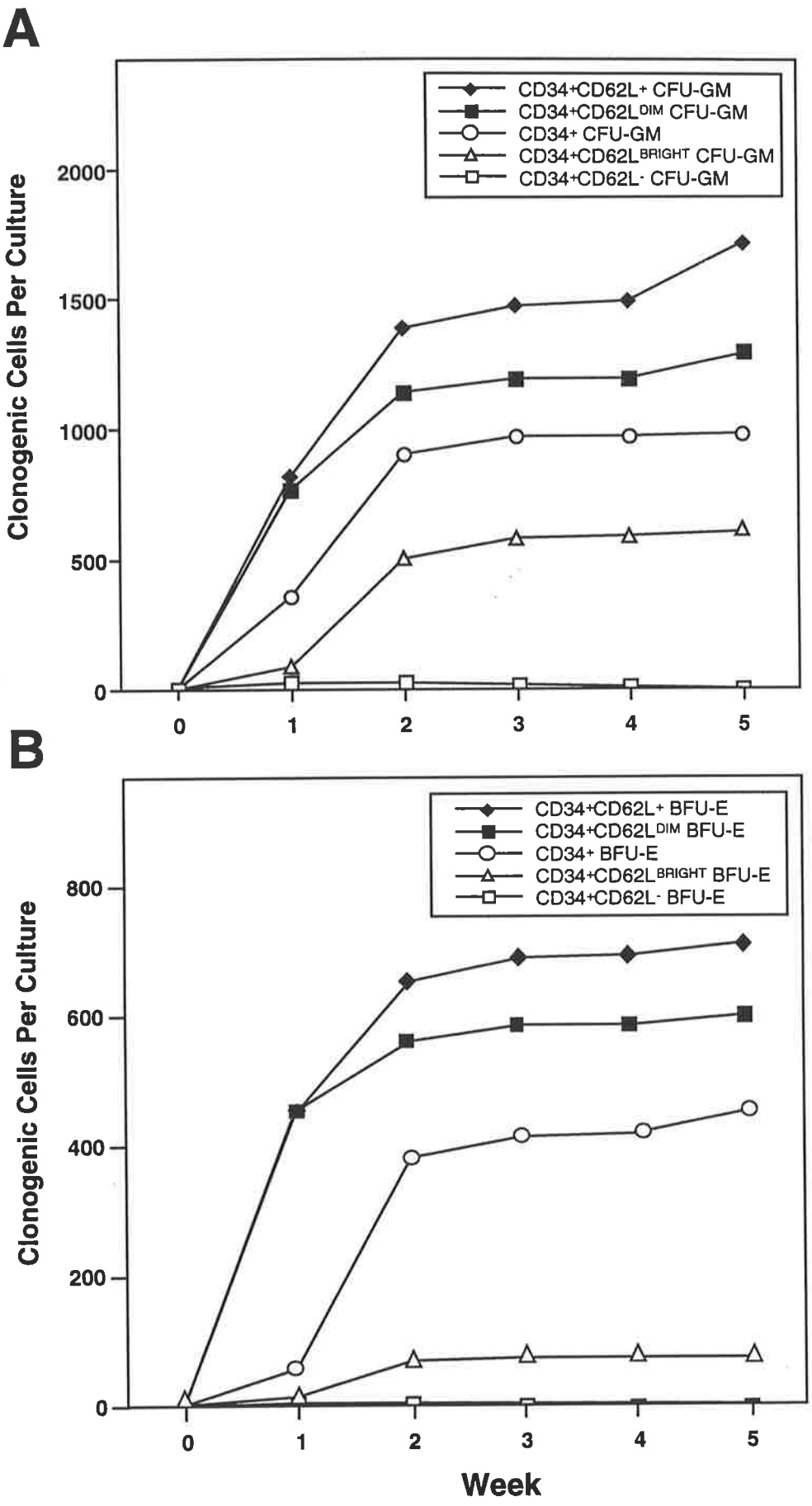
Previous studies have demonstrated that primitive haemopoietic cells have the capacity to initiate and maintain haemopoiesis in standard stromal cell dependent long term marrow culture conditions (long-term culture-initiating cells; LTC-IC (Sutherland *et al*, 1989) or in cytokine driven suspension culture assays (pre-CFU assay, Smith *et al*, 1991, Brandt *et al*, 1990). Furthermore, these small blasts exhibit high levels of the CD34 antigen, but low to undetectable levels of CD33, CD38 or HLA-DR and low retention of the vital fluorescent dye Rhodamine 123 (Andrews *et al*, 1989, Terstappen *et al*, 1991, Brandt *et al*, 1990, Udomsakdi *et al*, 1991; 1992).

To examine whether L-selectin expression was also a characteristic of these primitive haemopoietic cells, $CD34^+CD62L^+$, $CD34^+CD62L^-$, $CD34^+CD62L^{BRIGHT}$ and $CD34^+CD62L^{DIM}$ subpopulations isolated from normal adult BMMNC were assayed for their capacity to initiate and sustain haemopoiesis following co-culture in LTBMC with irradiated allogeneic marrow stromal cells. Figure 3.2.18. illustrates a representative example of the cumulative production of CFU-GM [Figure 3.2.18. (A)] and BFU-E [Figure 3.2.18 (B)] over time in culture from the various sorted populations. Whilst the $CD34^+CD62L^-$ sorted populations demonstrated virtually no ability to generate nascent CFU-GM and BFU-E in LTBMC, myeloid and erythroid production was observed in cultures initiated with total $CD34^+$, $CD34^+CD62L^+$, $CD34^+CD62L^{BRIGHT}$ and $CD34^+CD62L^{DIM}$ subpopulations. Furthermore, and in contrast to the "input" clonogenic assays (described above), the $CD34^+CD62L^{DIM}$ population had a significantly ($p<0.05$) greater capacity to sustain the production of CFU-GM and BFU-E than the corresponding $CD34^+CD62L^{BRIGHT}$ subpopulation at least up to day 35. Notably by the end of week 5 of culture, the $CD34^+CD62L^{DIM}$ population produced more than 2-fold CFU-GM and up to 6-fold more BFU-E than the corresponding $CD34^+CD62L^{BRIGHT}$ subpopulation.

While these data demonstrate that the majority, if not all the LTC-IC are present within the $CD34^+CD62L^+$, and subpopulations thereof, it was conceivable that a proportion of the LTC-IC were present within the $CD34^+CD62L^-$ subpopulation but were not detected in the LTBMC assay, perhaps due their low frequency in the $CD34^+CD62L^-$ population or to their lack of recruitment under these culture conditions. A similar series of experiments were performed to assess the capacity of the various sorted populations

Figure 3.2.18. $CD34^+$ Cells With Long-Term *In Vitro* Repopulating Capacity Express L-selectin At Low Levels ($CD34^+CD62L^{DIM}$).

BMMNC were sorted into $CD34^+$ (○) $CD34^+CD62L^+$ (◆), $CD34^+CD62L^-$ (□), $CD34^+CD62L^{BRIGHT}$ (△) and $CD34^+CD62L^{DIM}$ (■) subpopulations (as described in Figure 3.2.17.) and assayed for their ability to initiate and maintain haemopoiesis in long-term bone marrow culture (LTBMC). LTBMCs were initiated (in triplicate) on confluent, irradiated cultures of allogeneic bone marrow stromal cells with 1×10^3 cells from each sorted population. The data are presented as the cumulative production of clonogenic CFU-GM (A) and BFU-E (B) per culture, assayed each week (as described in the *Materials and Methods*). Data represent the sum of CFU-GM/BFU-E present in the suspension and stromal cell associated phases at each time point. A representative experiment (one of three) is shown.


A
CD34+CD62L+ CFU-GM
CD34+CD62LDIM CFU-GM
CD34+ CFU-GM
CD34+CD62LBRIGHT CFU-GM
CD34+CD62L- CFU-GM
Clonogenic Cells Per Culture
2000
1500
1000
500
0
0
1
2
3
4
5
B
CD34+CD62L+ BFU-E
CD34+CD62LDIM BFU-E
CD34+ BFU-E
CD34+CD62LBRIGHT BFU-E
CD34+CD62L- BFU-E
Clonogenic Cells Per Culture
800
600
400
200
0
0
1
2
3
4
5
Week

to produce nascent CFU-GM in the cytokine driven, stromal cell-free suspension culture (pre-CFU) assay (Smith *et al*, 1991, Brandt *et al*, 1990, Haylock *et al*, 1992). Commensurate with the results obtained in the stromal cell dependent assay, only $CD34^+CD62L^+$ (and not the $CD34^+CD62L^-$) cells sustained the production of CFU-GM in pre-CFU culture conditions (Figure 3.2.19.). Furthermore, subdivision of the $CD34^+CD62L^+$ population into $CD34^+CD62L^{BRIGHT}$ and $CD34^+CD62L^{DIM}$ (refer to Figure 3.2.15 (B)) demonstrated that cells with the capacity for the *de novo* generation of CFU-GM were restricted almost entirely to the $CD34^+CD62L^{DIM}$ subpopulation, with a 4-fold and 25-fold expansion in colony numbers at day 14 and day 21, respectively (Figure 3.2.19).

3.2.12. L-Selectin Interacts With A TNFα-Inducible Bone Marrow Stromal Cell Ligand To Support Haemopoietic Progenitor Cell Adhesion

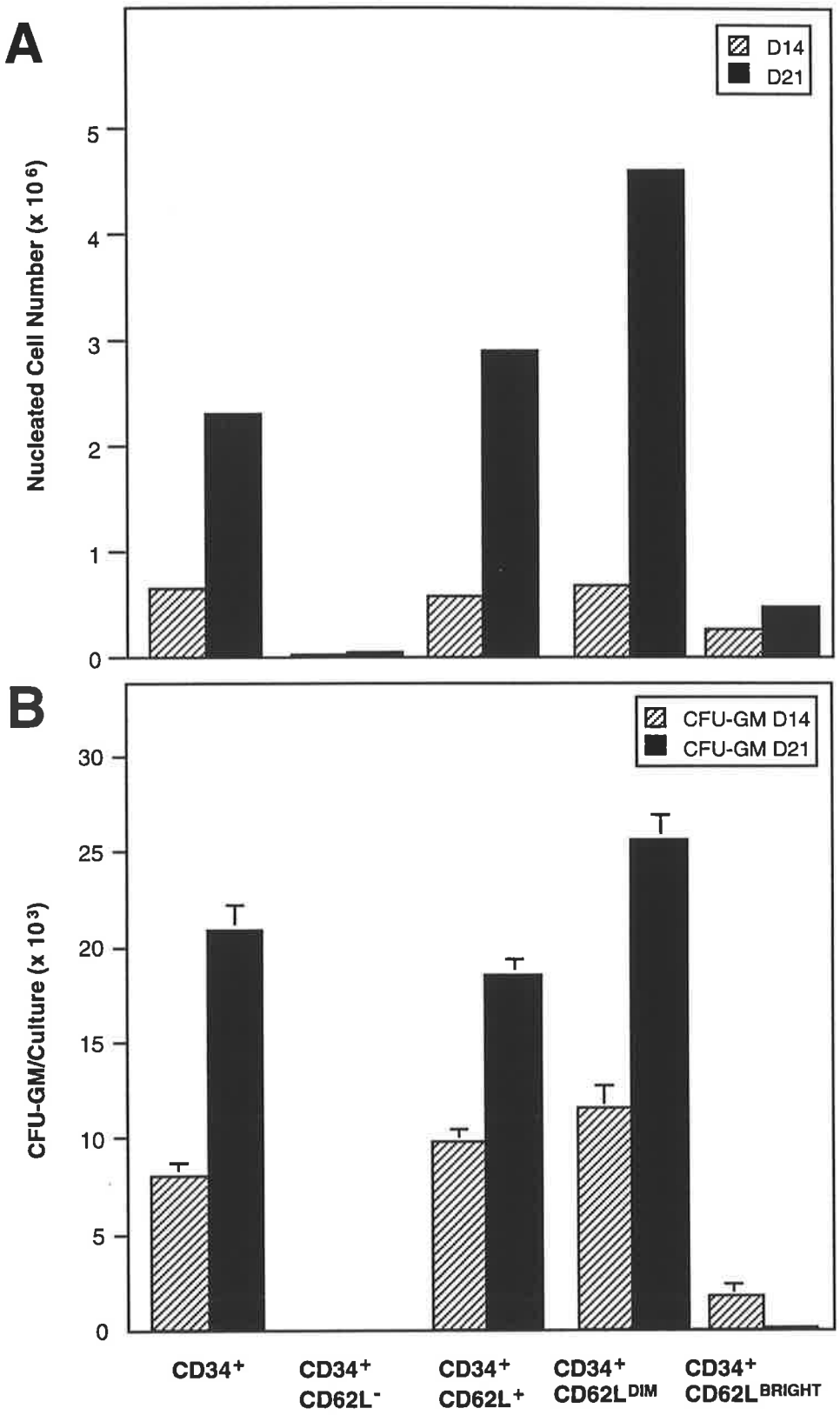
Recently, Spertini *et al* (1991) reported that CD62L was able to mediate the adhesion of both lymphocytes and neutrophils to an inducible, neuraminidase-sensitive ligand on human umbilical vein endothelial cells (HUVECs). Expression of the L-Selectin ligand by endothelium was optimally induced by LPS and the pro-inflammatory cytokines TNFα and IL-1β.

To investigate if the adhesion to a TNFα-inducible ligand was a property of haemopoietic progenitor cells, $CD34^+$ cell adhesion to both unstimulated and cytokine-stimulated HUVECs was examined. FACS-isolated $CD34^+$ cells were preincubated with DREG-56 (a function-blocking mAb to CD62L) or an isotype-matched, non-binding control mAb, prior to their transfer to wells containing cytokine (TNFα) or mock-stimulated HUVECs. While demonstrating no adhesion to unstimulated HUVECs (data not shown), $CD34^+$ progenitor cells exhibited CD62L-specific adhesion (ie. abrogated in the presence of mAb DREG-56) to TNFα-stimulated HUVECs (Figure 3.2.20 (A)).

This observation was replicated when $CD34^+$ cells were transferred to wells containing TNFα-stimulated cultured human BM stromal cells (HBMSC) (Figure 3.2.20. (B) and Figure 3.2.21.), suggesting that a similar (if not identical) L-selectin-specific ligand was also induced in HBMSC.

Figure 3.2.19. $CD34^+$ Cells Initiating Haemopoiesis In The Cytokine-Supplemented (Pre-CFU) Assay Express L-Selectin At Low Levels ($CD34^+CD62L^{DIM}$).

Assay of the same populations as shown in Figure 3.2.18., in a stroma-independent, cytokine supplemented culture. Cultures were established in triplicate using 1 x 10^3 sorted cells per group in medium supplemented with 10 ng/ml each of purified recombinant human IL-1β, IL-3, IL-6, G-CSF, GM-CSF and SCF. Additional factors were added at the same concentrations on days 7. On days 7 and 14 , the cells were harvested, washed and assayed for both nucleated cell number (A) and the number of CFU-GM (B) as previously described. The results are expressed as nucleated cell number and the mean number (±SE) of CFU-GM recoverd at day 14 (▨) and 21 (■) of culture for each group.


A
D14
D21
Nucleated Cell Number (x 10⁶)
0
1
2
3
4
5
B
CFU-GM D14
CFU-GM D21
CFU-GM/Culture (x 10³)
0
5
10
15
20
25
30
CD34+
CD34+ CD62L-
CD34+ CD62L+
CD34+ CD62L DIM
CD34+ CD62L BRIGHT

3.2.20. $CD34^+$ HPC Adhere To Human Umbilical Vein Endothelial Cells (HUVEC) And Human BM Stromal Cells (HBMSC) Via A rhTNFα-Inducible L-Selectin Ligand.

2 x 10^4 primary cultured, HBMSC or HUVECs were transferred to individual wells of an 8 chamber slide 24 hours prior to performing the adhesion assay. HBMSC and HUVEC monolayers were stimulated with 100 U/ml of rhTNFα for 4 hours at 37°C. During this time, FACs-isolated $CD34^+$ cells were incubated on ice at 1 x 10^5/ml in RPMI (supplemented with 2% FCS) containing either 20 μg/ml of the function-blocking mAb to CD62L (DREG-56) or an isotype matched, non-binding control (3D3). 1 x 10^4 cells (in a volume of 100 μl) were subsequently transferred (without washing) to the 8 chamber slide containing HUVECs (A) or HBMSC (B). The cells were incubated at 4°C for 1 hour on an orbital shaker at 60 rpm to stimulate shear stress. At the end of this period, unbound cells were removed by gentle washing, and the cells fixed with the addition of 2% glutaraldehyde in PBS. A representative 200 x field from each sample was photographed with an Olympus BH2-RFCA microscope.

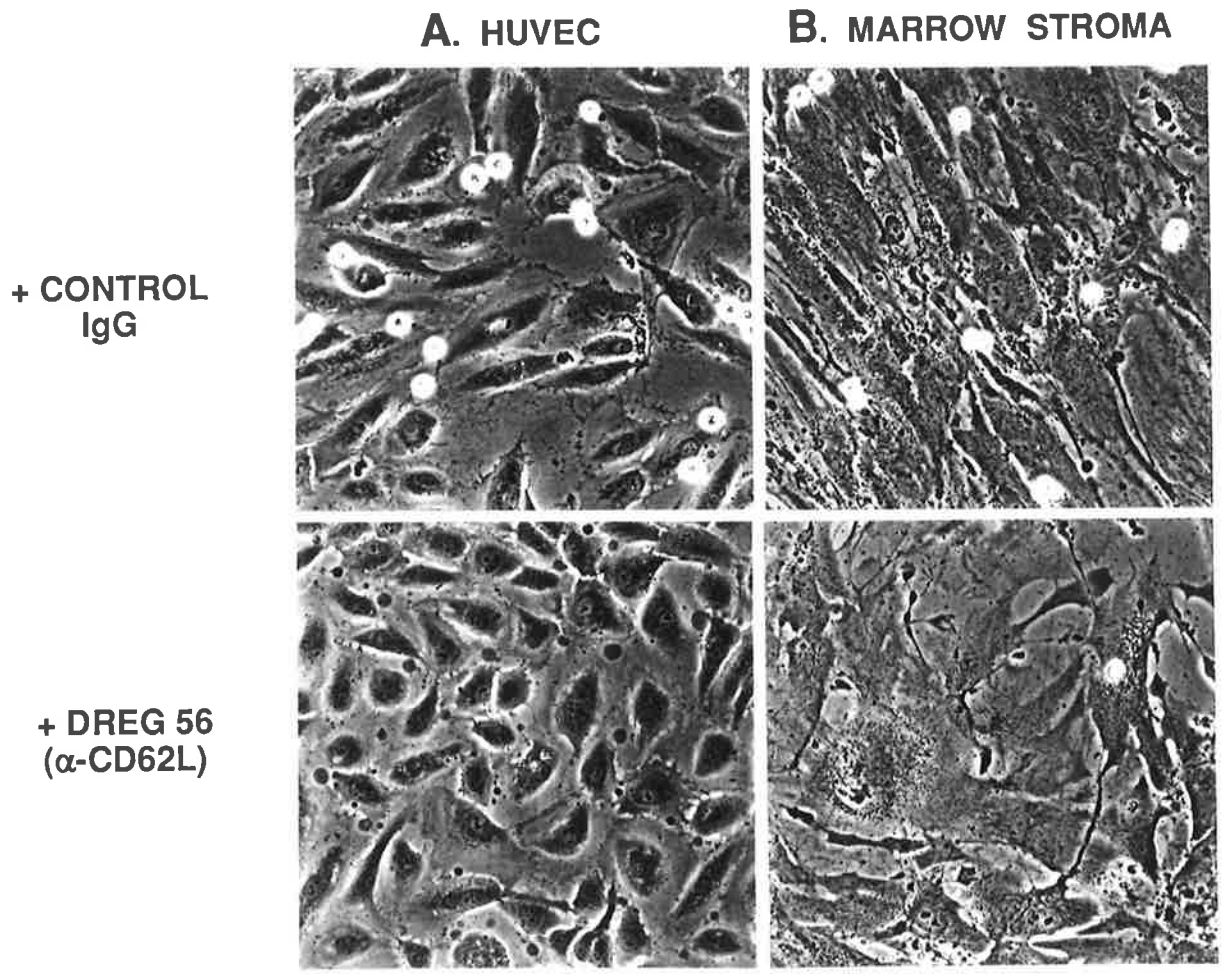
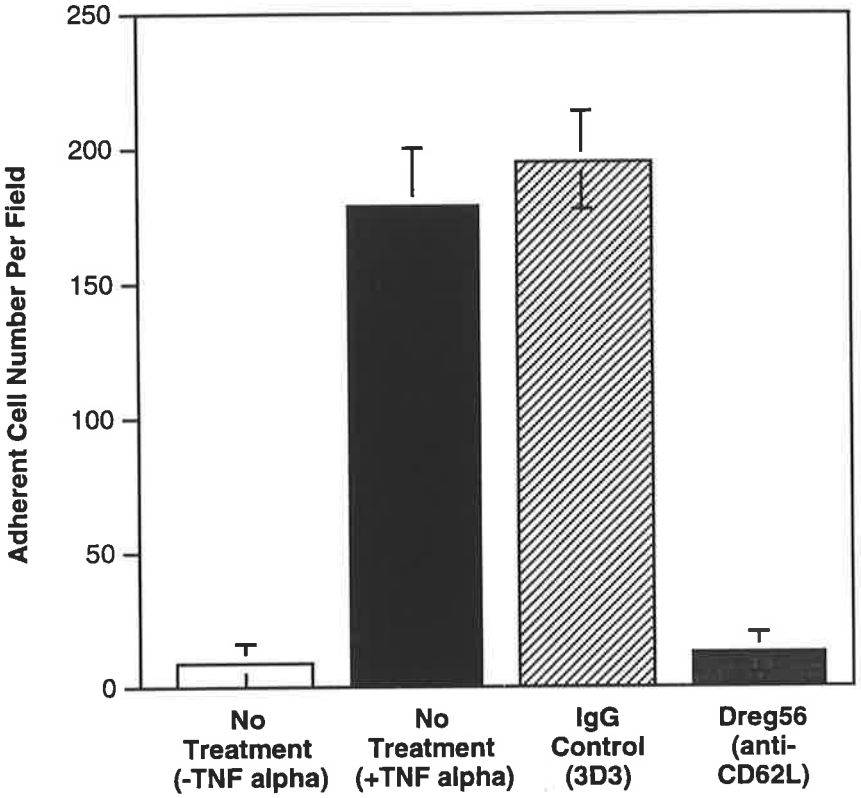

A. HUVEC
B. MARROW STROMA
+ CONTROL IgG
+ DREG 56 (α-CD62L)

Figure 3.2.21. L-selectin Interacts With A rhTNFα-Inducible BM Stromal Cell Ligand To Support Haemopoietic Progenitor Cell Adhesion.

2×10^4 allogeneic BM stromal cells were transferred to each well of an 8 chamber slide 24 hours prior to performing the assay. BM stromal cell monolayers were either left untreated, or stimulated with 100 U/ml of rhTNFα for 4 hours at 37°C. During this time, FACS-isolated $CD34^+$ cells were incubated on ice at 1×10^5/ml in RPMI (supplemented with 2% FCS) containing either 20 μg/ml of the function-blocking mAb to CD62L (DREG-56) or an isotype matched, non-binding control (3D3). 1×10^4 cells (in a volume of 100 μl) were subsequently transferred (without washing) to the 8 chamber slide containing stromal cells. The cells were subjected to conditions of shear (60 rpm on an orbital shaker) for 1 hour at 4°C. Upon completion of the assay, unbound cells were removed by immersing the slide into a bath of 2% glutaraldehyde in PBS. The assay was scored microscopically to assess the number of adherent cells in 5 randomly selected 100 x fields. Data represents the mean number of adherent $CD34^+$ cells (± SE, n=3) per field.


Adherent Cell Number Per Field
250
200
150
100
50
0
No Treatment (-TNF alpha)
No Treatment (+TNF alpha)
IgG Control (3D3)
Dreg56 (anti-CD62L)

3.2.13. The TNFα-Inducible Bone Marrow Stromal Cell Ligand For CD62L Is Unlikely To Be GlyCAM-1

Lasky *et al* (1992), recently identified a murine glycoprotein counter-receptor for CD62L, termed GlyCAM-1 (for Glycosylation-dependent cell adhesion molecule-1) from murine lymph nodes. This prompted the investigation as to whether the human homologue of GlyCAM-1, was (i) equivalent to the TNFα-activated ligand described above and (ii) responsible for mediating the CD62L-specific adhesion of $CD34^+$ cells. Oligonucleotide primers were designed in accordance with the published sequence (Lasky *et al*, 1992) to generate a 458 bp product by RT-PCR amplification. Significantly, a band of expected size was produced from murine lymph node-derived RNA (Figure 3.2.22. (A)). Subsequent automated sequence analysis revealed 100% sequence homology of this product with the published GlyCAM-1 sequence (data not shown).

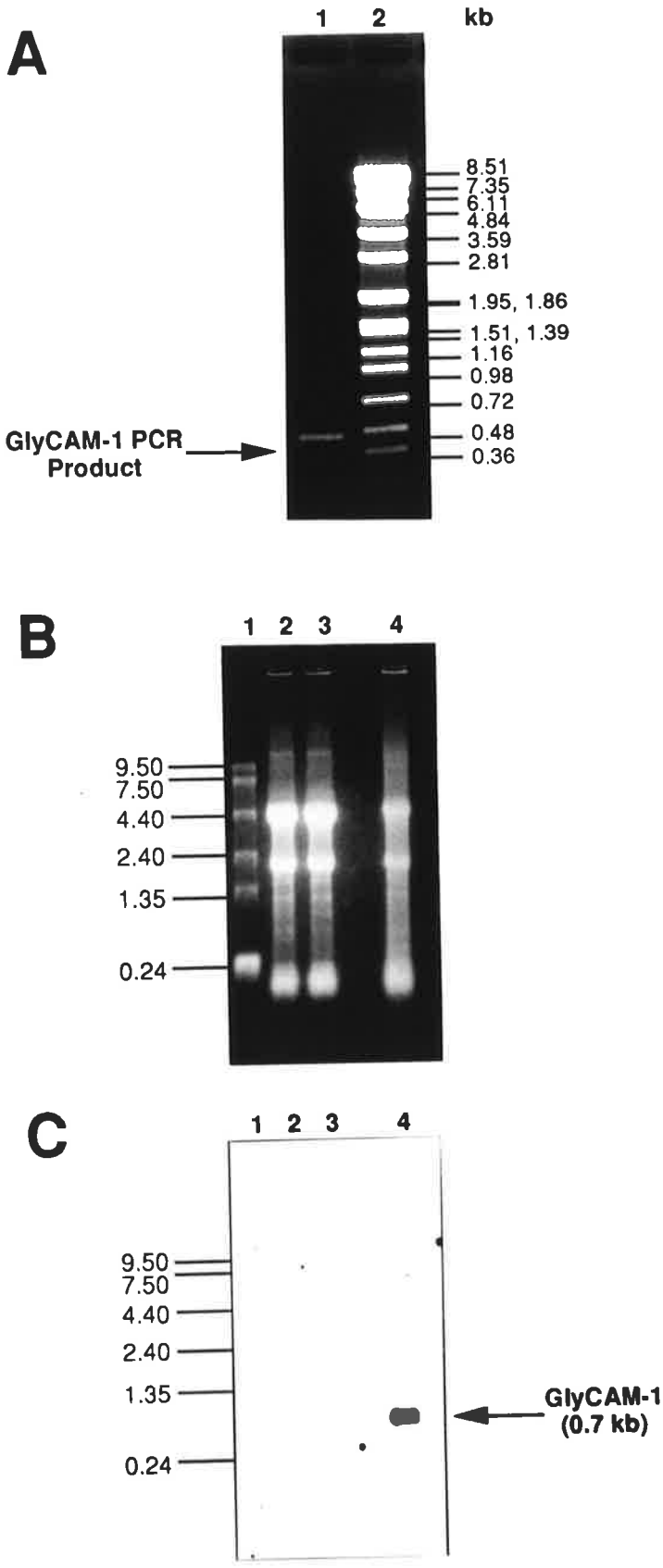
GlyCAM-1 mRNA expression was examined by low-stringency Northern blot analysis of total RNA isolated from TNFα-stimulated human and murine bone marrow stromal cells and unstimulated murine lymph nodes (Figure 3.2.22. (B & C)). Hybridisation of the GlyCAM-1 probe was only observed with the murine lymph node RNA, suggesting that adhesion to cytokine-activated murine and human stromal cells was unlikely to be mediated by the peripheral lymph node mucin-like glycoprotein, GlyCAM-1 or it human homologue.

3.2.22. The rhTNFα-Inducible Bone Marrow Stromal Cell Ligand For CD62L Is Unlikely To Be GlyCAM-1.

(A) Total RNA was isolated from murine peripheral lymph nodes. Oligonucleotide primers (designed in accordance with the published sequence) enabled the generation of a 458 bp product by RT-PCR amplification. Automated sequence analysis revealed 100% sequence homology of this product with the published GlyCAM-1 sequence (data not shown).

(B) 10 μg samples of total RNA derived from rhTNFα-treated murine and human stromal cells, and murine peripheral lymph nodes were subjected to electrophoresis on a 1.0 % formaldehyde-agarose gel and visualised by ethidium bromide staining and photographed. Loading order: Lane 1, RNA Markers (refer to Table 2.25.1.), Lane 2, rhTNFα-stimulated murine stromal RNA; Lane 3, rhTNFα-stimulated human stromal RNA; Lane 4, murine peripheral lymph node RNA.

(C) GlyCAM-1 mRNA expression was examined by low-stringency Northern blot analysis. Following overnight hybridisation at 30°C with the radiolabelled PCR-derived full-length GlyCAM-1 probe, the membrane was washed under low stringency conditions (two times for 15 minutes at 42°C in 2 x SSC/0.1%SDS). Membranes were exposed to Xomat-AR film for 48 hours with intensifying screens. Hybridisation to the 0.7 kb GlyCAM-1 transcript is only observed with the murine lymph node RNA (indicated by arrow).


A
1
2
kb
8.51
7.35
6.11
4.84
3.59
2.81
1.95, 1.86
1.51, 1.39
1.16
0.98
0.72
0.48
0.36
GlyCAM-1 PCR Product
B
1
2
3
4
9.50
7.50
4.40
2.40
1.35
0.24
C
1
2
3
4
9.50
7.50
4.40
2.40
1.35
0.24
GlyCAM-1 (0.7 kb)

3.3. DISCUSSION

3.3.1. Studies Of The Role Of P- And E-Selectin In Human Haemopoiesis: A Summary

Current evidence supports the hypothesis that the restriction of primitive haemopoietic progenitors to the bone marrow involves developmentally regulated adhesive interactions between progenitor cells and various components of the stromal microenvironment (Allen *et al*, 1990; Dexter *et al*, 1977; Torok-Storb, 1988; Kincade *et al*, 1989; Tavassoli and Hardy, 1990; Clark *et al*, 1992; Long, 1992; Simmons *et al*, 1994). A number of reports have documented interactions mediated by integrins, immunoglobulin superfamily members and the CD44 family of CAMs (Lewinsohn *et al*, 1990). In addition, a role for cell-cell adhesion mediated by the specific lectin-like protein-carbohydrate interactions has been demonstrated in murine haemopoietic tissues (Aizawa and Tavassoli, 1987a; 1987b). The selectin family of adhesion molecules function as calcium-dependent lectins (Lasky *et al*, 1989; Johnston *et al*, 1989a; 1989b; Bevilacqua *et al*, 1989; Bevilacqua and Nelson, 1993; Lasky, 1992) but there are few reports which have investigated the potential involvement of selectins in the interaction of human haemopoietic progenitors within the bone marrow microenvironment. This chapter describes various studies which examine the role of P-, E- and L- selectin in tissues of the human haemopoietic system.

Haemopoietic progenitor cells, including lineage restricted clonogenic progenitors and more primitive precursors with the ability to initiate haemopoiesis *in vitro*, exhibit the property of adhesion to P-selectin. Binding of $CD34^+$ haemopoietic progenitors was shown to be cation dependent but independent of temperature and shear stress in accord with characteristics previously described for the adhesion of mature leukocytes to P-selectin (Bevilacqua and Nelson, 1993; Lasky, 1992; Larsen *et al*, 1989; Gerig *et al*, 1990; Gamble *et al*, 1990; Lawrence and Springer, 1991). The majority of erythroid (BFU-E) and essentially all myeloid (CFU-GM) progenitors bound to P-selectin. Erythroid development was accompanied by a loss in ability to bind P-selectin as demonstrated by the recovery of the majority of maturing glycophorin A^+ cells in the P-selectin non-binding fraction of BMMNC. B cells similarly failed to adhere to P-selectin but unlike the erythroid lineage,

presumptive B-cell progenitors identified by their co-expression of CD34 and CD19 antigens (Locken *et al*, 1987) also demonstrated lack of adhesion even at this earliest phenotypically defined stage of B-cell development (please also refer below).

In addition to clonogenic haemopoietic progenitors, P-selectin was also shown to bind more primitive haemopoietic precursors (pre-CFU) as demonstrated by their capacity for the *de novo* generation of clonogenic cells in both standard stroma containing LTBMC and stroma-free, cytokine dependent suspension culture assays. Thus, adhesion to P-selectin is maintained throughout myeloid development from the most primitive to the most mature lineages but is progressively lost as cells develop along the erythroid and B-lymphoid pathways. Collectively, these data demonstrate that committed myeloid progenitors and their precursors, a population closely related hierarchically to haemopoietic stem cells with long term repopulating potential *in vivo*, can be separated from the majority of BMMNC by virtue of their adherence to P-selectin.

At the commencement of this study, the precise nature of the ligand(s) for P-selectin on $CD34^+$ cells remained to be determined. Initial experiments to define the nature of the ligand on $CD34^+$ cells found that, following the incubation of $CD34^+$ cells with a variety of proteases, their binding to P-selectin was markedly decreased, demonstrating that the P-selectin ligand on $CD34^+$ cells was located on a glycoprotein and not on a glycolipid associated with the cell surface. In addition, treatment of $CD34^+$ cells with neuraminidase completely abrogated their binding to P-selectin indicating that sialic acid was essential in the structure and function of the ligand. These data are in accord with previously published observations concerning the characteristics of the P-selectin ligand on the human promyelocytic leukaemia cell line HL60 (Larsen *et al*, 1992; Moore *et al*, 1992) and suggested the possibility that a similar, if not identical sialosylated glycoprotein may comprise a P-selectin ligand on both cell types.

Monoclonal antibodies CSLEX-1 (Fukushima *et al*, 1984) and HECA-452 (Picker *et al*, 1990), recognise neuraminidase sensitive carbohydrate antigens, sialyl Le^x and a epitope common to both sialyl Le^x and sialyl Le^a, respectively. Sialyl Le^x and sialyl Le^a have previously been shown to function as ligands for both E-selectin and P-selectin on other leukocytes (Phillips *et al*, 1990; Berg *et al*, 1991). Both mAbs demonstrated

significant binding to a subpopulation of $CD34^+$ cells from normal marrow, and although consistent with the recent finding of Karakantza *et al* (1994), this is contrary to the previously published findings of Muroi *et al* (1992; 1993a) who reported that normal marrow derived $CD34^+$ cells did not express detectable levels of sialyl Le^x. The reason for this discordant observations are unclear, but may be related to the use of a monoclonal antibody to SLe^x with differing epitope specificity to the CSLEX-1 mAb used by Karakantza and colleagues (1994) and in this study.

Three-colour flow cytometric analysis also demonstrated a precise co-distribution of CSLEX-1 and HECA-452 antigens on $CD34^+$ cells, implying recognition of the same glycoprotein antigen by the two mAbs (Figure 3.2.5. (c)). Importantly, the committed myeloid progenitors (CFU-GM) and more primitive precursors (pre-CFU) were predominantly found in the CSLEX-1^+ and HECA-452^+ sorted subpopulations, consistent with their phenotype of binding to P-selectin. This represents the first report to demonstrate the expression of these carbohydrate structures by primitive human haemopoietic progenitors. In contrast, the number of erythroid progenitors (BFU-E), were consistently diminished in the HECA-452^+ and CSLEX-1^+ subpopulations, somewhat discordant with the observation that the majority of the BFU-E were recovered in the P-selectin adherent fraction of BMMNC or $CD34^+$ cells. This would suggest that unlike myeloid progenitors, BFU-E express alternate carbohydrate ligand(s) not recognised by CSLEX-1 and HECA-452 mAbs. Additionally, as demonstrated herein, approximately 88% of all $CD34^+$ cells bind via P-selectin, yet the antigen(s) recognised by these two mAbs are expressed on only 35% of these cells. This suggests therefore, that the CSLEX-1 and HECA-452 antigens can not account for all the binding observed. Considering these data, these observations suggest that the P-selectin ligand on the remainder of these cells may comprise a form of sialyl Le^x that is additionally sialylated or otherwise modified in such a way as to prevent recognition by sialyl Le^x specific antibodies, CSLEX-1 and HECA-452. An analogous observation was made by Berg *et al* (1991) with regard to the cutaneous lymphocyte-associated antigen, CLA, which represents a ligand for E-selectin on skin-associated memory T-cells.

During the course of this study, a glycoprotein counter-receptor for P-selectin termed P-selectin Glycoprotein Ligand (PSGL-1) was molecularly cloned from the promyelocytic leukaemia cell line HL60 (Sako *et al*, 1993). Analysis by RT-PCR demonstrated expression of PSGL-1 in $CD34^+$ cells. Subdivision of the $CD34^+$ population by means of CSLEX-1 immunoreactivity revealed expression of PSGL-1 in both subpopulations. Based on the RT-PCR and antibody studies, it suggested that sialyl Le^x containing carbohydrate structures decorating PSGL-1 accounted for the P-selectin binding phenotype displayed by $CD34^+$CSLEX-1^+ cells. Moreover, expression of PSGL-1 by the $CD34^+$CSLEX-1^- subpopulation suggested that this protein may also play a role in the adhesion of these cells to P-selectin, presumably through sialic acid containing carbohydrate structures not recognised by CSLEX-1 or HECA-452.

To investigate PSGL-1 protein expression by bone marrow-derived $CD34^+$ cells, an affinity purified polyclonal antibody against N-terminal peptide (Gln1-Gln15) of the mature, PACE propeptide cleaved (Sako *et al*, 1993) PSGL-1 protein was utilised (De Luca *et al*, 1995; Phillips and Berndt, 1992). As shown in Figure 3.2.10., essentially all of the $CD34^+$ cells expressed the PSGL-1 receptor at the protein level, in accord with the mRNA analysis. The level of expression of PSGL-1 by $CD34^+$ cells is consistent with the reported 10-20,000 copies on neutrophils and HL60 cells (Moore *et al*, 1991; Skinner *et al*, 1991). In contrast, PSGL-1 expression by $CD34^+CD19^+$ presumptive B-cell precursors, was undetectable by flow cytometric analysis. This was in accordance with the lack of binding of B-cell precursors and their progeny to CD62P. This observation conforms with the findings of Vachino *et al* (1995), who demonstrated that although mature B cells express PSGL-1, they did not display significant binding to P-selectin. Although the level of expression by these cells is low, the lack of binding to P-selectin was attributed to a deficiency of activation-dependent post-translational events which are essential in the expression of a functional PSGL-1 receptor on lymphocytes (Vachino *et al*, 1995).

A number of reports have demonstrated that in addition to serving as a ligand for P-selectin, PSGL-1 may also bind to E-selectin (Sako *et al*, 1993; Moore *et al*, 1994; Asa *et al*, 1995; De Luca *et al*, 1995). In contrast to P-selectin, however the requirements for E-selectin recognition are much less stringent. E-selectin has been shown to bind a number of

sialomucins and glycoprotein structures that co-express sialyl Le^x or the related structure, sialyl Le^a with high affinity. Consistent with the findings of Dercksen *et al*, (1994), BM-derived $CD34^+$ cells exhibited E-selectin-specific, cation-dependent, neuraminidase-sensitive adhesion to E-selectin CHO cell transfectants (Figure 3.2.11). Unlike the binding to P-selectin, however, adhesion of $CD34^+$ cells to E-selectin was not inhibited when the affinity-purified polyclonal antibody to PSGL-1. Furthermore, treatment of $CD34^+$ cells with novel cobra venom-derived metalloproteinase, mocarhagin, resulted in the complete loss of α-PSGL-1-peptide polyclonal-binding activity, and moreover resulted in the complete abrogation of binding of $CD34^+$ cells to P-selectin. Significantly, however adhesion of $CD34^+$ cells to E-selectin remained unaffected. Thus treatment of cells with mocarhagin, enables the contribution of E- and P-selectin in the adhesion of $CD34^+$ cells to be readily discriminated.

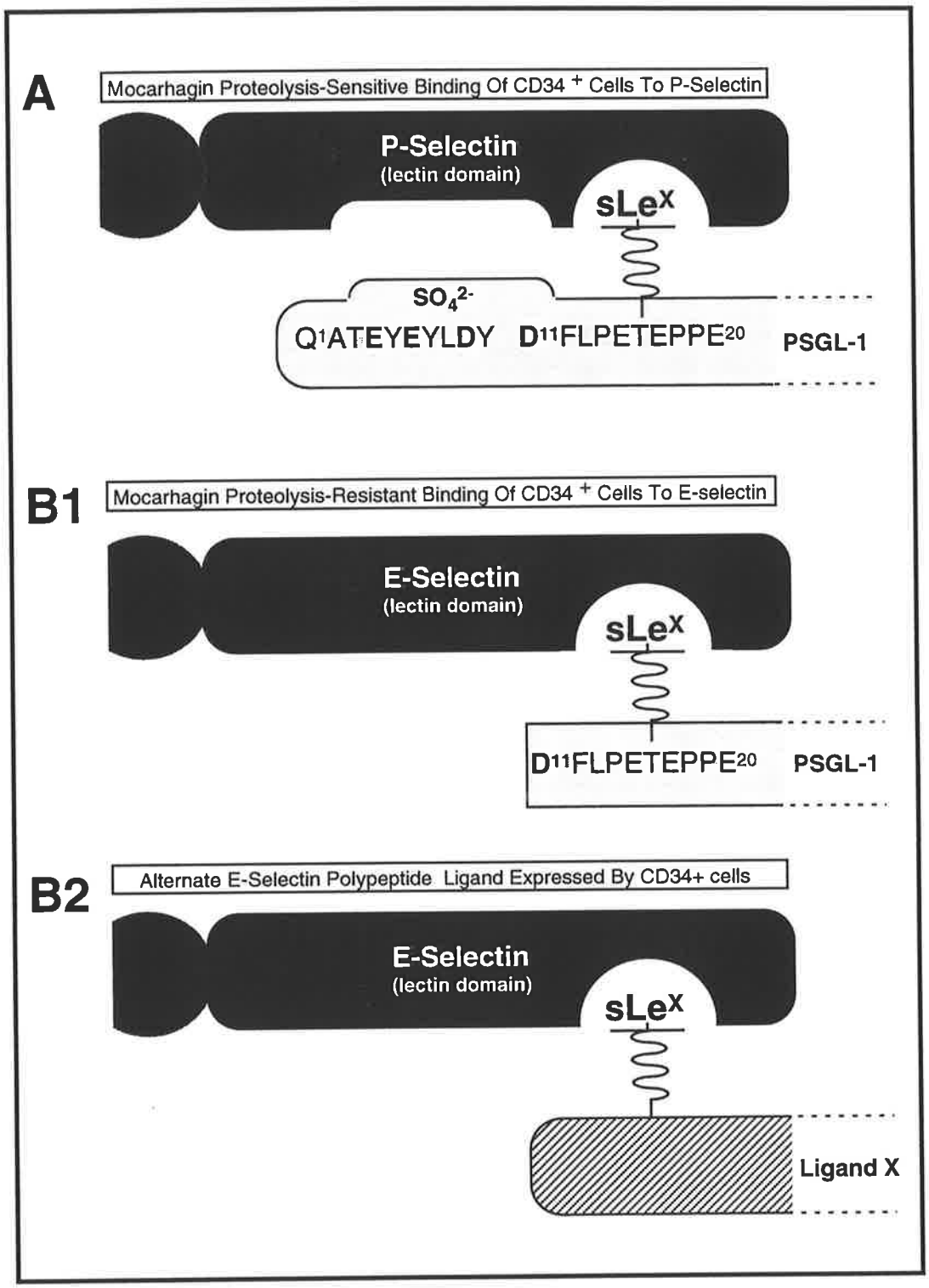
As demonstrated in Figures 3.2.11., 3.2.12. (B) and 3.2.13., P-selectin appears to be highly specific in its recognition of PSGL-1. One explanation, is that P-selectin binding to PSGL-1 is bimodal with P-selectin binding not only involving carbohydrate recognition, but also the negatively charged/sulfated tyrosine cluster (Figure 3.3.1.(A)). The importance of sulfation in P-selectin binding was concomitantly demonstrated by Pouyani and Seed (1995) and Sako *et al* (1995). Although binding of $CD34^+$ cells to E-selectin does not involve the N-terminal region of PSGL-1 (Pouyani and Seed, 1995; Sako *et al*, 1995), the data presented here, do not preclude the possibility that E-selectin interacts with PSGL-1 via the down-stream region bearing clustered sialylated-carbohydrates (Figure 3.3.1.(B)1). Alternatively, the adhesion of $CD34^+$ cells to E-selectin, may involve alternative glycoproteins (or glycolipids) which are capable of presenting sialylated-carbohydrate structures to E-selectin (Figure 3.3.1.(B)2). Consistent with this view, Lenter *et al* (1994) and Levinovitz *et al*, (1993) have independently identified two ligands on HL60 cells which exhibit binding to both P- and E-selectin. The possible existence of such alternative E-selectin ligands on haemopoietic progenitors must remain the subject of future studies. Despite the implication of the existence of additional protein structures on mature myeloid cells that can serve as P-selectin ligands, the data presented here support

Figure 3.3.1. The NH_2-Terminal Decapeptide Of PSGL-1 Is Critical For P-Selectin But Not E-Selectin Binding.

(A) The NH_2-terminal 20 amino acids of the mature, PACE-cleaved (Rehemtulla and Kauffman, 1992; Sako *et al*, 1993) are depicted. A domain spanning amino acid residues 4-11 denotes a region comprising one or more sulphated tyrosines. The structure above the amino acid at position 16, represents an O-linked oligosaccharide modified by sLe^x or an alternate sialylated, fucosylated glycan. Both regions are critical for high affinity binding to P-selectin (Pouyani and Seed, 1995; Sako *et al*, 1995).

(B1) The cobra venom metalloproteinase, mocarhagin (De Luca *et al*, 1995) specifically cleaves between the Tyr^{10} and Asp^{11} residues of the mature, PACE-cleaved PSGL-1 receptor and results in the loss of the N-terminal decapeptide, QATEYEYLDY. As demonstrated in Figure 3.2.12. (B) and Figure 3.2.13., mocarhagin proteolysis of PSGL-1 on $CD34^+$ HPC abrogates their adhesion to P-selectin, *but not to* E-selectin.

(B2) E-selectin may interact with PSGL-1 via the sialylated oligosaccharide on the threonine residue at position 16 (Figure 3.3.1. B1) or undefined, down-stream region bearing clustered sialylated-carbohydrates. Alternatively, the adhesion of $CD34^+$ cells to E-selectin, may involve alternative glycoproteins (or glycolipids) which are capable of presenting sialylated-carbohydrate structures to E-selectin.


A
Mocarhagin Proteolysis-Sensitive Binding Of CD34 + Cells To P-Selectin
P-Selectin
(lectin domain)
sLeX
SO4 2-
Q1ATEYEYLDY D11FLPETEPPE20
PSGL-1
B1
Mocarhagin Proteolysis-Resistant Binding Of CD34 + Cells To E-selectin
E-Selectin
(lectin domain)
sLeX
D11FLPETEPPE20
PSGL-1
B2
Alternate E-Selectin Polypeptide Ligand Expressed By CD34+ cells
E-Selectin
(lectin domain)
sLeX
Ligand X

the hypothesis that PSGL-1 represents the sole P-selectin ligand expressed by primitive human haemopoietic progenitor cells and their committed progeny.

A signalling function for the sialomucin CD34 has recently been postulated by Fackler and collegues (1995). As such, the consequences of adhesion to CD62P on the growth and development of HPC was also examined. In these experiments, adhesion to P-selectin was associated with a >90% inhibition of colony formation (Figure 3.2.13.). Moreover, recent experiments (performed in collaboration with Dr. J-P. Levesque) using the cytokine-stimulated, liquid (pre-CFU) culture system to examine the effects of adhesion to CD62P on more primitive HPC, similarly demonstrated a marked suppression in both nucleated cell number and colony formation.

In accord with these observations, a recent study by Banu *et al* (1995), demonstrated increased numbers of megakaryocytic progenitors in the BM of P-selectin "knockout" mice. Similarly, progenitor cell numbers were also increased in mice deficient in both E-selectin and P-selectin (Frenette *et al*, 1996), suggesting that retention of HPC in a quiescent state within the BM is, in part mediated by the interaction of P-selectin with PSGL-1. These studies indicate a key role for PSGL-1 as a signalling molecule on primitive HPC, and moreover imply that interactions between mucin-like proteins and their counter-receptors negatively regulate HPC proliferation. Despite this, very little is known of the signalling function of PSGL-1 (Weyrich *et al*, 1995). Thus the transduction pathway(s) which are induced in human HPC following PSGL-1 binding to P-selectin and the mechanisms which ultimately contribute to the inhibition of HPC growth remain to be defined.

Questions that arise from these studies include (i) Does the inhibitory effect of CD62P on HPC growth involve the induction of apoptosis or an alternate cytostatic mechanism? This could however be resolved by a number of experimental strategies which include ascertaining whether the apparent stasis is reversible upon removing the cells from the immobilised P-selectin. Alternatively, programmed cell death (apoptosis) could be examined by standard DNA-fragmentation analysis (Herrmann *et al*, 1994), or using one of a number of the commercially available kits. (ii) Does adhesion to CD62P arrest cells at

a particular phase of the cell cycle? This question is amenable to study by flow cytometry using propridium iodide and/or acridine orange.

Although beyond the scope of this thesis, experiments to elucidate the mechanisms underlying these observations will provide valuable information and understanding of the mechanisms responsible for the inhibition of HPC proliferation which occurs as a consequence of PSGL-1-mediated adhesion to CD62P. Moreover, the binding of primitive haemopoietic progenitors to CD62P has several important implications. Firstly, by mediating the initial fast and shear-resistant adhesion and subsequent "rolling" along endothelial cells entering the marrow, CD62P could provide the crucial preliminary contact required for transmigration events mediated by other adhesive mechanisms such as integrin-mediated interactions (Lawrence and Springer, 1991; Butcher, 1991). In accordance with this, a previous report has documented expression of both P- and E-selectin by a proportion of bone marrow endothelial cells (Beckstead *et al*, 1986). Secondly, the existence of a soluble form of P-selectin (Gamble *et al*, 1990) with possible anti-adhesive function might provide a mechanism for the release of cells, including immature progenitors, from the marrow under both steady-state physiological conditions and during enforced mobilisation as occurs following administration of cytokines or high dose chemotherapy (Sheridan *et al*, 1992; To *et al*, 1990).

3.3.2. Studies Of The Role Of L-Selectin In Human Haemopoiesis: A Summary

Leukocytes recirculate continuously between the blood and lymphatic organs and also extravasate into sites of inflammation. The initial step in diapedesis of cells is the binding of specific leukocyte surface molecules to their ligands on vascular endothelial cells (Springer, 1990; Stoolman, 1989; Osborn, 1989; Cotran *et al*, 1986; Butcher, 1991). In recent years, several different receptors involved in leukocyte trafficking have been characterised. Some of them act as adhesion-strengthening molecules in a non-organ-specific manner, while others, like CD62L, mediate tissue-specific binding of leukocytes (Springer, 1990; Stoolman, 1989; Butcher, 1991).

Human L-selectin represents the homologue of the 90 kD murine peripheral lymph node homing receptor, originally identified by the mAb, MEL-14 (Gallatin *et al*, 1983).

Although initially described as a lymphocyte homing receptor, it was subsequently shown to be constitutively expressed on most other peripheral blood leukocytes including neutrophils and monocytes, and shown to be involved in leukocyte traffic in the systemic microcirculation (Tedder *et al*, 1990; Griffin *et al*, 1990).

In this chapter, the expression of CD62L by both committed haemopoietic progenitors and their precursors was examined. The data presented here, confirm and extend previous observations (Lund-Johansen and Terstappen, 1993; Dercksen *et al*, 1995; Spertini *et al*, 1991; Tedder *et al*, 1990; Griffin *et al*, 1990) of the expression of CD62L by $CD34^+$ haemopoietic progenitor cells ($CD34^+$ HPC).

In the current study, multiparameter FACS based on the co-expression of CD34 and CD62L was utilised. The greater sensitivity afforded by the directly-PE conjugated anti-CD62L mAb (Leu8-PE) enabled the clear resolution of the $CD34^+$ cells into $CD62L^+$ and $CD62L^-$ subfractions. Furthermore, the $CD34^+CD62L^+$ was able to be further resolved into $CD34^+CD62L^{BRIGHT}$ and $CD34^+CD62L^{DIM}$ subpopulations.

Studies to examine which input subpopulations harboured the lineage-restricted clonogenic progenitor cells, revealed that the myeloid progenitors (CFU-GM) were predominantly found in the $CD34^+CD62L^+$ fraction, whilst the BFU-E were present in both the $CD34^+CD62L^+$ and $CD34^+CD62L^-$ subpopulations. Similarly, the erythroid progenitors were consistently enriched in the $CD34^+CD62L^{DIM}$ subpopulation, whilst the $CD34^+CD62L^{BRIGHT}$ subpopulation harboured the majority of CFU-GM.

To investigate the role of CD62L in the regulation of hierarchically more primitive progenitors, $CD34^+$ cells sorted on the basis of Leu8 binding (as described above) were assayed for their ability to initiate and sustain myelopoiesis in stroma-dependent LTC. The data presented here, demonstrate that cells with the capacity for *de novo* clonogenic cell production (CFU-GM and BFU-E) were present exclusively in $CD34^+$ cells which demonstrated detectable CD62L expression. The $CD34^+CD62L^-$ subpopulation exhibited virtually no capacity to generate nascent CFU-GM and BFU-E. Moreover, the $CD34^+CD62L^{DIM}$ had a significantly greater capacity to sustain the *de novo* generation of both erythroid (BFU-E) and myeloid (CFU-GM) clonogenic cells when compared with the coincident $CD34^+CD62L^{BRIGHT}$ population.

Although comparable data were obtained when these same sorted populations were assayed in a stroma-free, cytokine-dependent suspension culture system (pre-CFU assay) the cumulative yield of CFU-GM in this system was approximately 20-fold higher (comparison of day 21 clonogenic cells produced by the $CD34^+CD62L^{DIM}$ subpopulation) than the stroma-dependent culture system. Thus it is possible that the suspension culture system allowed more efficient CFU generation, a view supported by the substantially higher yields of CFU-GM. The difference probably reflects, in apart, the production of inhibitory as well as stimulatory factors by the stroma (Eaves *et al*, 1991). Whilst in contrast to the results obtained in pre-CFU/LTBMC assays, the $CD34^+CD62L^{BRIGHT}$ subpopulation harboured the majority of CFU-GM.

Previous studies have shown that human pre-CFU are $CD33^-$ (Andrews *et al*, 1989), $CD38^-$ (Terstappen *et al*, 1991), exhibit undetectable or low expression of HLA-DR (Brandt *et al*, 1990), and lack a variety of lineage-specific antigens (Simmons *et al*, 1994). Using multi-colour flow cytometric analysis, it is evident that of the $CD34^+$ cells with these phenotypes ($CD34^+CD33^-$, $CD34^+CD38^-$, and $CD34^+HLA\text{-}DR^-$), essentially all express CD62L at moderate to high levels (refer to Figure 3.2.16). In addition to the $CD34^+CD62L^+$ population, a significant proportion also expressed the B lymphoid antigens (CD10 and CD19) whilst only a small proportion expressed the T cell antigen (CD7).

Using multiparameter fluorescence with antibodies to well recognised lineage antigens, the study by Lund-Johansen and Terstappen (1993), concluded that CD62L were expressed on $CD34^+$ myeloid progenitor cells and mature granulocytes but down-modulated during the intermediate stages of maturation. In contrast, using *in vitro* assays of committed and primitive haemopoietic cells, the data presented here suggest that all myeloid and erythroid precursors (and their committed progeny) are contained within the $CD34^+CD62L^+$ subpopulation. Moreover, the $CD34^+$ myeloid progenitor cells and their precursors express CD62L at an intermediate level ($CD62L^{DIM}$), whilst the maturation of erythroid precursors was associated with a progressive up-regulation of CD62L expression. Although the functional significance of these data are unknown, they do

indicate that CD62L expression is modulated as a function of haemopoietic cell differentiation.

A recent communication by Spertini *et al* (1991) demonstrated that CD62L was able to mediate the adhesion of both lymphocytes and neutrophils to a cytokine-inducible (TNFα or IL-1β), neuraminidase-sensitive ligand on human umbilical vein endothelial cells (HUVECs). Data presented here demonstrate that a TNFα inducible ligand for CD62L was also expressed by cultured human BM stromal cells. Although the nature of this ligand is unknown, preliminary data suggests that this inducible ligand for L-selectin is unlikely to be the recently cloned glycosylation dependent cell adhesion molecule-1 (GlyCAM-1) (Lasky *et al*, 1992). This is consistent with recent evidence which suggests that the human homologue of the murine GlyCAM-1 will be significantly divergent from the murine molecule (Dr. L. Lasky, personal communication) and as such not detectable by the methods used here. Furthermore, recent data suggests that murine GlyCAM-1 exists as a soluble molecule, and as such, may inhibit (not promote) L-selectin adhesion (Tedder *et al*, 1995), making it an unlikely candidate for the cytokine-inducible ligand observed here.

In conclusion, CD62L is expressed by a subset of immature haemopoietic progenitor cells that are able to initiate myelopoiesis in both stroma-dependent/independent *in vitro* culture systems, the benchmark assays for human haemopoietic stem cells. In addition, data presented here suggests that CD62L may mediate the adhesion of $CD34^+$ HPC to BM stromal cells via a cytokine inducible ligand.

CHAPTER 4

THE DEVELOPMENT OF A NEW TECHNIQUE FOR ISOLATING GENES ENCODING CELL SURFACE MOLECULES USING RETROVIRAL EXPRESSION CLONING

4.1. INTRODUCTION

4.1.1. Cellular Interactions In Haemopoiesis : Molecular Characterisation Of Stem Cell-Stromal Cell Interactions

Cell-cell interactions are of fundamental importance in many cellular systems, both during development and in the adult organism (reviewed in Gilbert, 1991). This requirement is particularly well illustrated by those which regulate the function of the immune system, and (Springer, 1990) in the development of the haemopoietic system (Trentin, 1970; Wolf, 1979; Tavassoli, 1975; Cline and Golde, 1979; Price and McCulloch, 1978; Dexter, 1982; Torok-Storb, 1988). In the adult, haemopoiesis occurs in the extravascular compartment of the BM, where HSC and their progeny develop in intimate association with the stromal tissue of the BM (Dexter, 1982; Weiss, 1976; Lichtman, 1981; Tavassoli and Friedenstein, 1983; Allen *et al*, 1990; Simmons and Torok-Storb, 1991a; 1991b; Dexter *et al*, 1977; Tavassoli and Hardy, 1990). The precise mechanisms underlying the ability of the BM stromal cells (HBMSC) to support HSC development remain to be determined, however current evidence suggests a role for both soluble and membrane-associated haemopoietic growth factors (HGF) and a range of adhesive interactions (Trentin, 1970; Wolf, 1979; Tavassoli, 1975; Dexter *et al*, 1977, Tavassoli and Hardy, 1990; Clark *et al*, 1992; Long, 1992). Studies *in vitro* have demonstrated that although soluble HGF are able to support the proliferation and differentiation of HSC, maintenance only occurs when HSC are co-cultured with marrow stromal cells (Dexter, 1982). These observations, suggest that molecules expressed at the HSC and stromal cell surfaces are of major importance in this process. Although some likely candidates are beginning to emerge (Simmons *et al*, 1992; 1994a; 1994b; Zannettino *et al*, 1995), further research is still required to define additional cell-surface expressed moieties capable of mediating these complex interactions.

Unlike prokaryotic and "lower" eukaryotic systems, molecular genetics and mutational analysis are often difficult to perform in the human system. Of necessity, a number of alternate approaches have been developed to identify and functionally characterise cell surface molecules (CSM) with roles in mediating cellular interactions in

various systems. The primary advance in this area has come from the generation of a large number of monoclonal antibodies (mAbs) directed against molecules expressed at the cell surface (Watt *et al*, 1987a; Andrews *et al*, 1989; Sutherland *et al*, 1989a; 1989b; Terstappen *et al*, 1991; Barclay *et al*, 1993). Monoclonal antibodies provide a means of identifying and characterising the function of many integral membrane glycoproteins, including those which mediate cellular recognition and adhesion (Simmons, DL., 1992). Moreover, a number of expression cloning systems (Kavathas *et al*, 1984; Maddon *et al*, 1985; Young and Davis, 1983) have been developed to enable the isolation of functional cDNA clones corresponding to these CSM. To date, the most successful approach, has been that developed by Aruffo and Seed (Seed and Aruffo, 1987; Seed, 1987; Aruffo and Seed, 1987) and involves the transient expression of cDNA libraries in eukaryotic cells and the specific rescue of cDNA clones by antibody capture and panning (Seed and Aruffo, 1987; Wysoki and Sato, 1978). In the absence of suitable mAbs, specific ligands or cell lines expressing the cognate receptor/ligand have been used to isolate genes encoding growth factor receptors (Gearing *et al*, 1989; Yamasaki *et al*, 1988) and adhesion molecules (Osborn *et al*, 1989; Elices *et al*, 1990).

A number of reports describing the use of retroviral vectors for the construction of stable cDNA expression libraries in eukaryotic cells have now appeared in the literature. The utility of this approach has been demonstrated by the identification of genes with oncogenic potential (Whitehead *et al*, 1995) and by the isolation of cDNAs for factors able to confer autonomous growth on the factor-dependent cell line FDC-P1 (Rayner and Gonda, 1994; Wong *et al*, 1994). Retroviral vectors represent one of the most effective gene transfer systems available, and have proven useful for the transfer of genes into cells (including haemopoietic and primary cells) ordinarily refractory to transduction by other methods (Miller *et al*, 1990; Graham and van der Eb, 1973; Schaffner, 1980; Schaefer-Ridder *et al*, 1982; Zimmerman and Vienken, 1982; Neumann *et al*, 1982; Zenke *et al*, 1990; Stopper *et al*, 1987; Toneguzzo and Keating, 1986). Generally, retroviral vector systems for gene transfer can be divided into two equally important components which include, (i) the retroviral vector, and (ii) the retrovirus-packaging cell line (Miller *et al*, 1993). The retroviral vector is manipulated in its DNA form as part of a bacterial plasmid and,

although it does not encode viral proteins, it serves as a vehicle for the transfer and stable incorporation of genes of up to 10 kb in length into target cells (Miller *et al*, 1993). The retrovirus-packaging cell line (refer to Figure 4.1.1.), provides all of the viral proteins necessary for the encapsidation of the vector RNA into virions and for the subsequent infection, reverse transcription, and integration of the vector into the genomic DNA of target cells. In recent years, numerous modifications in the construction of packaging cell lines has resolved problems of spontaneous helper virus production and poor encapsidation of genomic RNA (Mann *et al*, 1983; Miller, 1990). The host range of the virions produced is dependent upon the choice of the packaging cell lines (Mann *et al*, 1983; Miller and Rosman, 1989; Cone and Mulligan, 1984; and reviewed by Miller *et al*, 1993) which can be broadly divided into two types (i) amphotropic and (ii) ecotropic, with the former able to generate retroviral particles able to infect most mammalian cells of experimental interest, whilst the latter is almost exclusively restricted to rodent cells.

This chapter describes the development of an alternative approach using retroviral expression cloning to isolate genes encoding CSM identified by a panel of mAbs (of unknown specificity) reactive with tissues of haemopoietic origin. More specifically, it deals with (i) the generation of a large, representative human bone marrow stromal cell (HBMSC)-derived cDNA library in the retroviral vector pRUF.*neo* and (ii) the development of a highly efficient selection strategy utilising mAbs and antibody-coated magnetic beads enabling the isolation and enrichment of infected cells exhibiting expression of a desired CSM.

To assess the efficacy of such an approach, a number of mAbs directed against previously defined cell surface antigens were utilised in an attempt to rescue the corresponding cDNA molecules. Following the validation of this approach, the retroviral expression library system was utilised to isolate cDNA clones corresponding to (glyco)proteins expressed at the HBMSC surface and recognised by a panel of newly generated mAbs. The mAbs were selected on the basis of their demonstrable effects in the regulation of haemopoiesis (refer to Chapter 6) or their unique reactivity profile with tissues of the haemopoietic system (refer to Chapter 5 and Chapter 6). The results detailed in this chapter thus confirm previous studies demonstrating the general

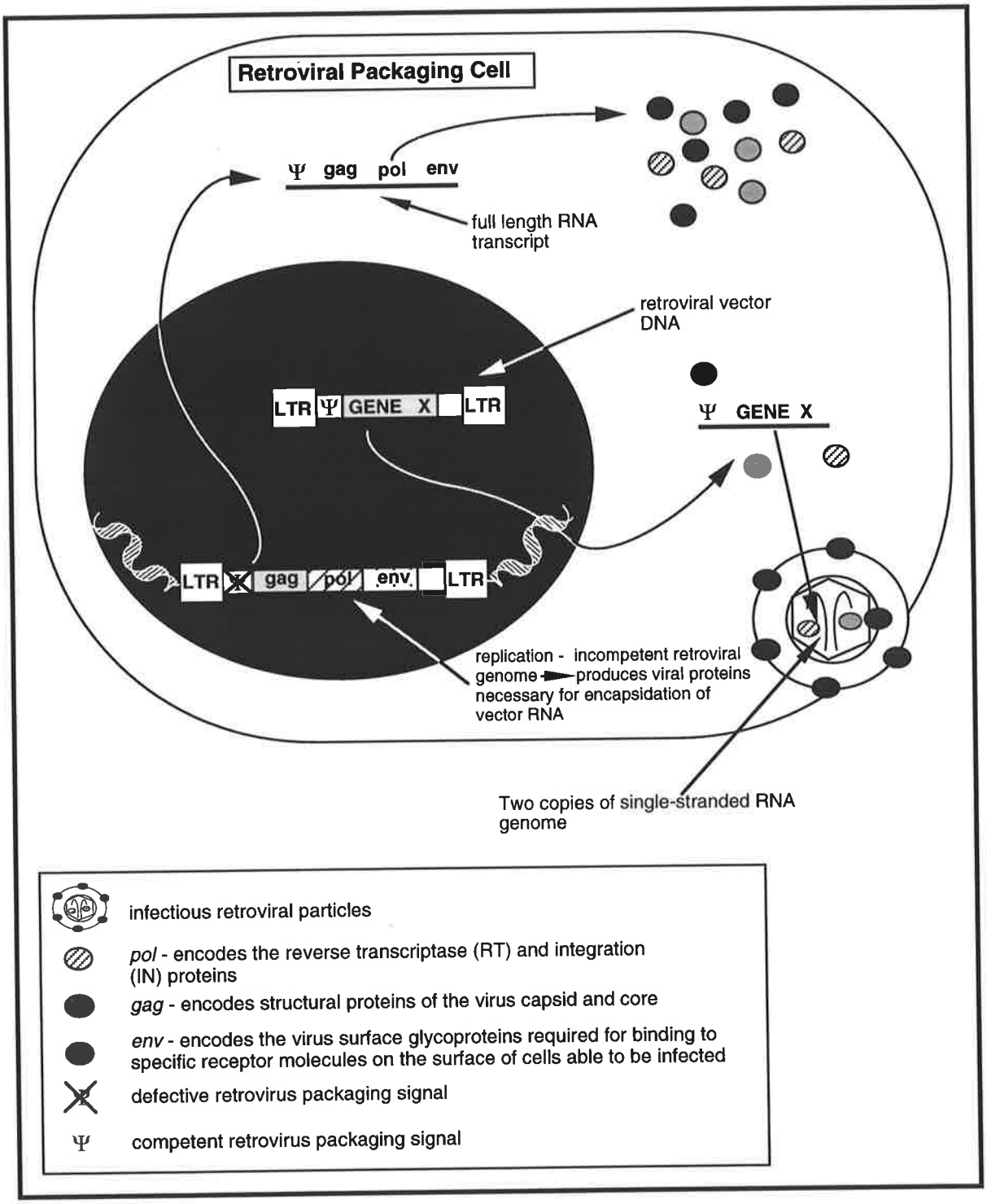
Figure 4.1.1. Retroviral Packaging Cell Line.

A schematic diagram of a retroviral packaging cell line, which harbours a generic helper virus (modelled on the Moloney Murine Leukaemia Virus, MMLV), from which the packaging signal, Ψ (between the 5′ long terminal repeat [LTR] and the *gag* coding region has been deleted.

The defective helper virus produces all the retroviral proteins necessary for viral encapsidation and replication, but is itself incapable of replication (ie. helper virus RNA is poorly encapsidated into virions).

More recent designs of retroviral packaging cells have focused on the separation of the *gag*, *pol* and *env* viral-encoding regions on separate expression plasmids, such that the frequency of recombination events required for spontaneous helper virus production is greatly reduced.

The transfected retroviral plasmid harbouring the gene of interest (GENE X), does not encode viral proteins, but serves as a vehicle for genes to be transferred. The resultant recombinant RNA molecules (containing a competent packaging signal) are subsequently encapsidated by the helper virus-derived proteins, and infectious viral particles produced.


Retroviral Packaging Cell
Ψ gag pol env
full length RNA transcript
retroviral vector DNA
LTR Ψ GENE X LTR
Ψ GENE X
LTR gag pol env LTR
replication - incompetent retroviral genome produces viral proteins necessary for encapsidation of vector RNA
Two copies of single-stranded RNA genome
infectious retroviral particles
pol - encodes the reverse transcriptase (RT) and integration (IN) proteins
gag - encodes structural proteins of the virus capsid and core
env - encodes the virus surface glycoproteins required for binding to specific receptor molecules on the surface of cells able to be infected
defective retrovirus packaging signal
Ψ competent retrovirus packaging signal

application of retroviral cDNA libraries and further extend their utility to the expression-cloning of cDNAs encoding CSMs.

4.2. RESULTS

Outline And Rationale Of Protocol : General Considerations.

As schematically represented in Figure 4.2.1., the protocol used to produce a retroviral expression library commences with the generation of double stranded cDNA from a source that is appropriate for the isolation of gene(s) of interest. The cDNA is directionally cloned into the retroviral vector (refer below), and subsequently amplified in an appropriate *E.coli* bacterial strain. The plasmid DNA thus derived, is used to generate a representative pool of virus producing cells. As illustrated in Figure 4.2.2, this is achieved by first transfecting the plasmid DNA library into the amphotropic packaging cell line (PA317), and the transiently-produced viral pool is then used to infect the ecotropic virus packaging cell line ($\Psi 2$). The infected ecotropic packaging cells are subsequently selected for their expression of the enzyme, aminoglycoside phospho-transferase (encoded by the Neomycin-resistance gene, Neo^R) which confers immunity to the toxic effects of the Neomycin analogue, Geneticin (G418). The resultant Neo^R $\Psi 2$ packaging cells represent the stable retroviral library, and thus serve as a renewable source of virus capable of infecting a target cell population of choice.

The retroviral vector pRUF*neo* (please refer to Figure 4.2.3), possesses a number of attributes which confer upon it a highly efficient gene transfer capability. These salient features include (i) a comprehensive multiple cloning site (MCS), to enable directional cloning of cDNA products; (ii) the MC1*neo* cassette containing the Neo^R gene driven by the f9 polyomavirus enhancer (Thomas and Capecchi, 1987), shown to function effectively in a variety of cell types including, fibroblasts, primary haemopoietic cells and haemopoietic cell lines (Rayner and Gonda, 1994); (iii) the long terminal repeat (LTR) elements derived from the myeloproliferative sarcoma virus (MPSV), known to function well in haemopoietic cells (Bowtell *et al*, 1988; Stocking *et al*, 1985); and (iv) sequences from the rearranged *gag-pol* genes of the M3Neo(myb) provirus integrated in the U22.4 cell line described by Gonda *et al* (1989). This rearrangement resulted in increased expression of the *myb* gene carried by the provirus, and subsequent experiments have indicated that it functions similarly in the pRUF.*neo* vector (data not shown). Expression of *myb* from

Figure 4.2.1. Outline Of The Procedure Employed To Generate A Retroviral cDNA Expression Library.

Please refer to text for details.

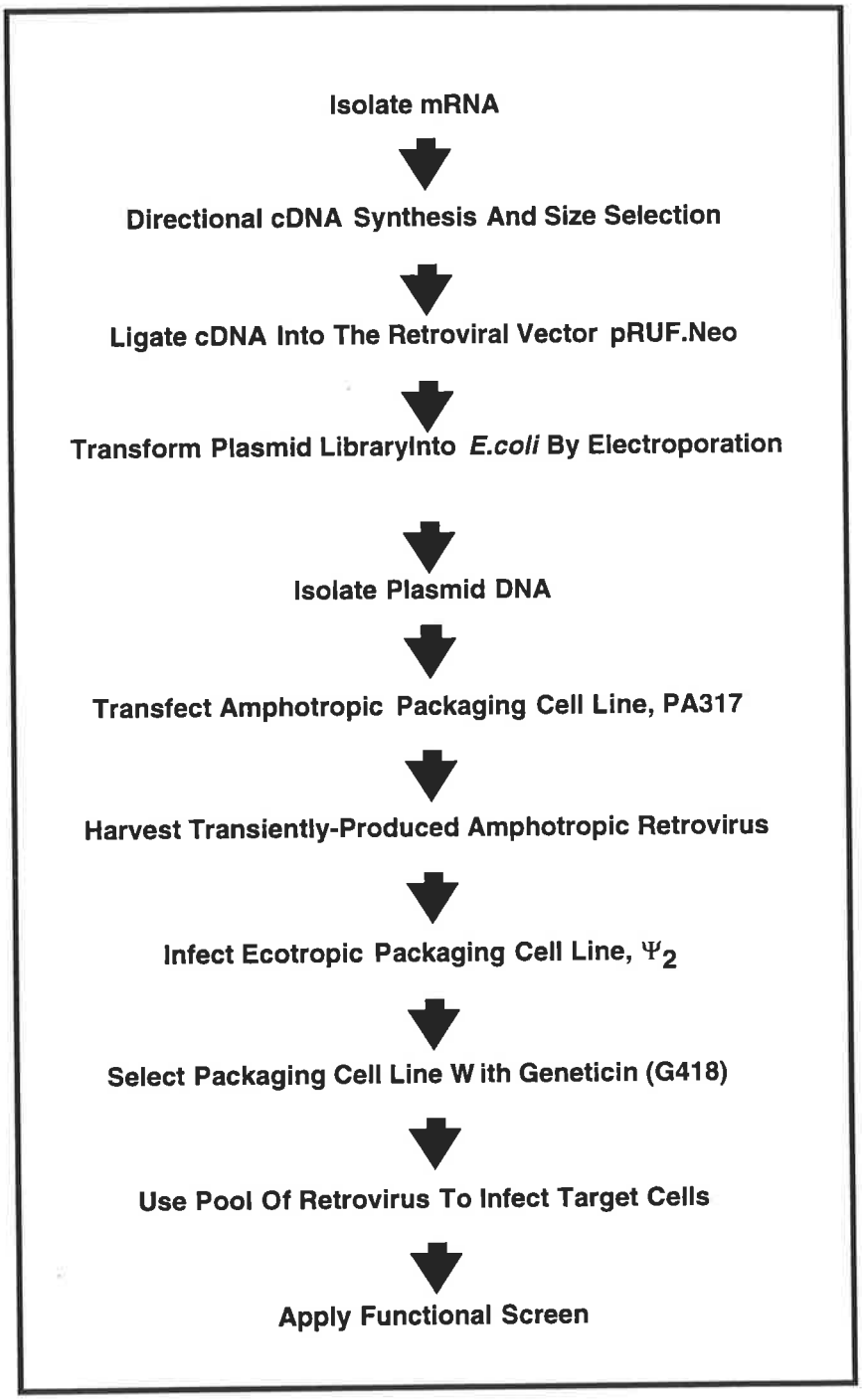

Isolate mRNA
Directional cDNA Synthesis And Size Selection
Ligate cDNA Into The Retroviral Vector pRUF.Neo
Transform Plasmid LibraryInto E.coli By Electroporation
Isolate Plasmid DNA
Transfect Amphotropic Packaging Cell Line, PA317
Harvest Transiently-Produced Amphotropic Retrovirus
Infect Ecotropic Packaging Cell Line, Ψ2
Select Packaging Cell Line W ith Geneticin (G418)
Use Pool Of Retrovirus To Infect Target Cells
Apply Functional Screen

Figure 4.2.2. Production Of Infectious Amphotropic And Ecotropic Viral Particles. Please refer to text for details.

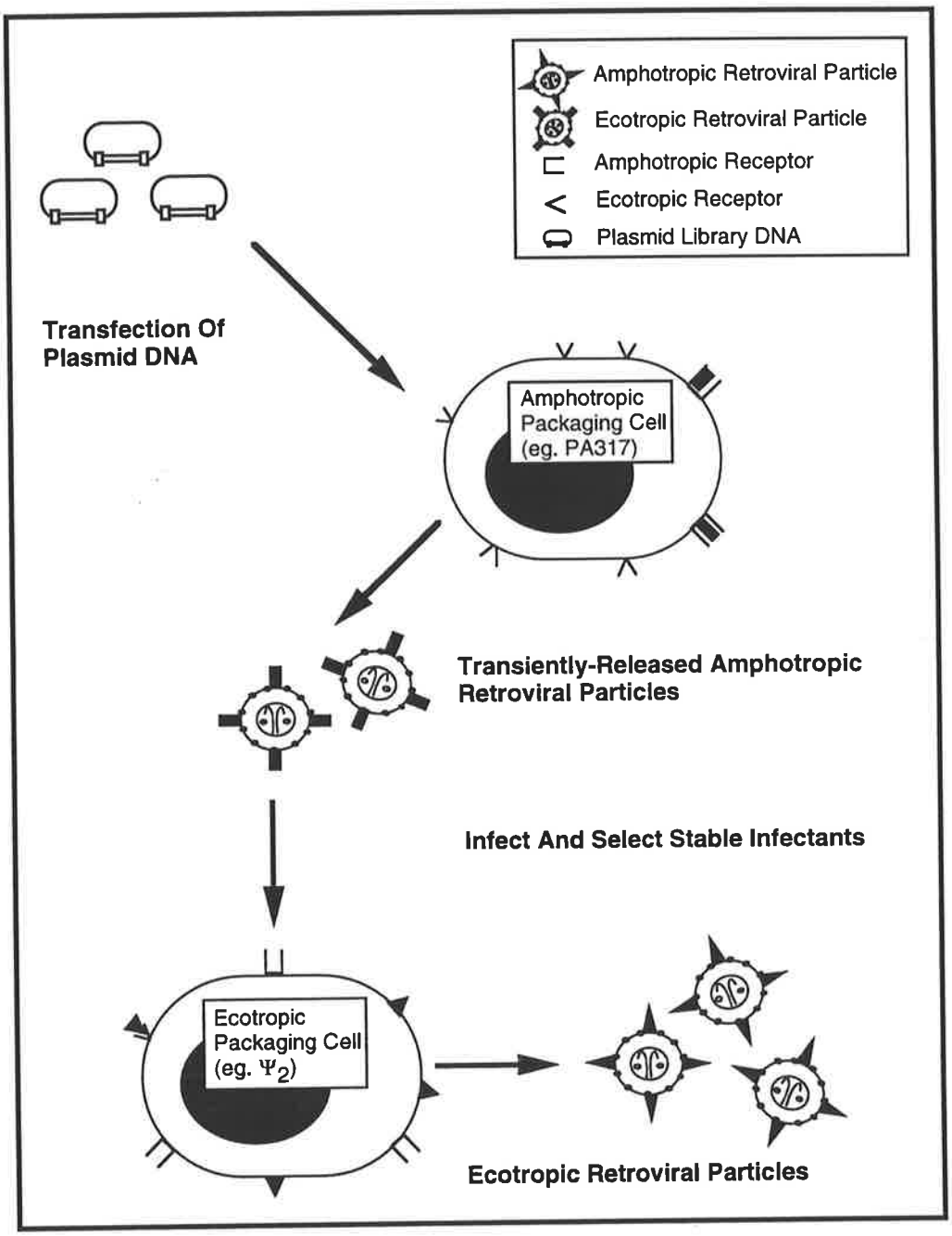

Amphotropic Retroviral Particle
Ecotropic Retroviral Particle
Amphotropic Receptor
Ecotropic Receptor
Plasmid Library DNA
Transfection Of Plasmid DNA
Amphotropic Packaging Cell (eg. PA317)
Transiently-Released Amphotropic Retroviral Particles
Infect And Select Stable Infectants
Ecotropic Packaging Cell (eg. Ψ2)
Ecotropic Retroviral Particles

Figure 4.2.3. Restriction Endonuclease Map Of pRUF.*neo*.

Restriction map of pRUF.*neo* illustrating the salient features, including:

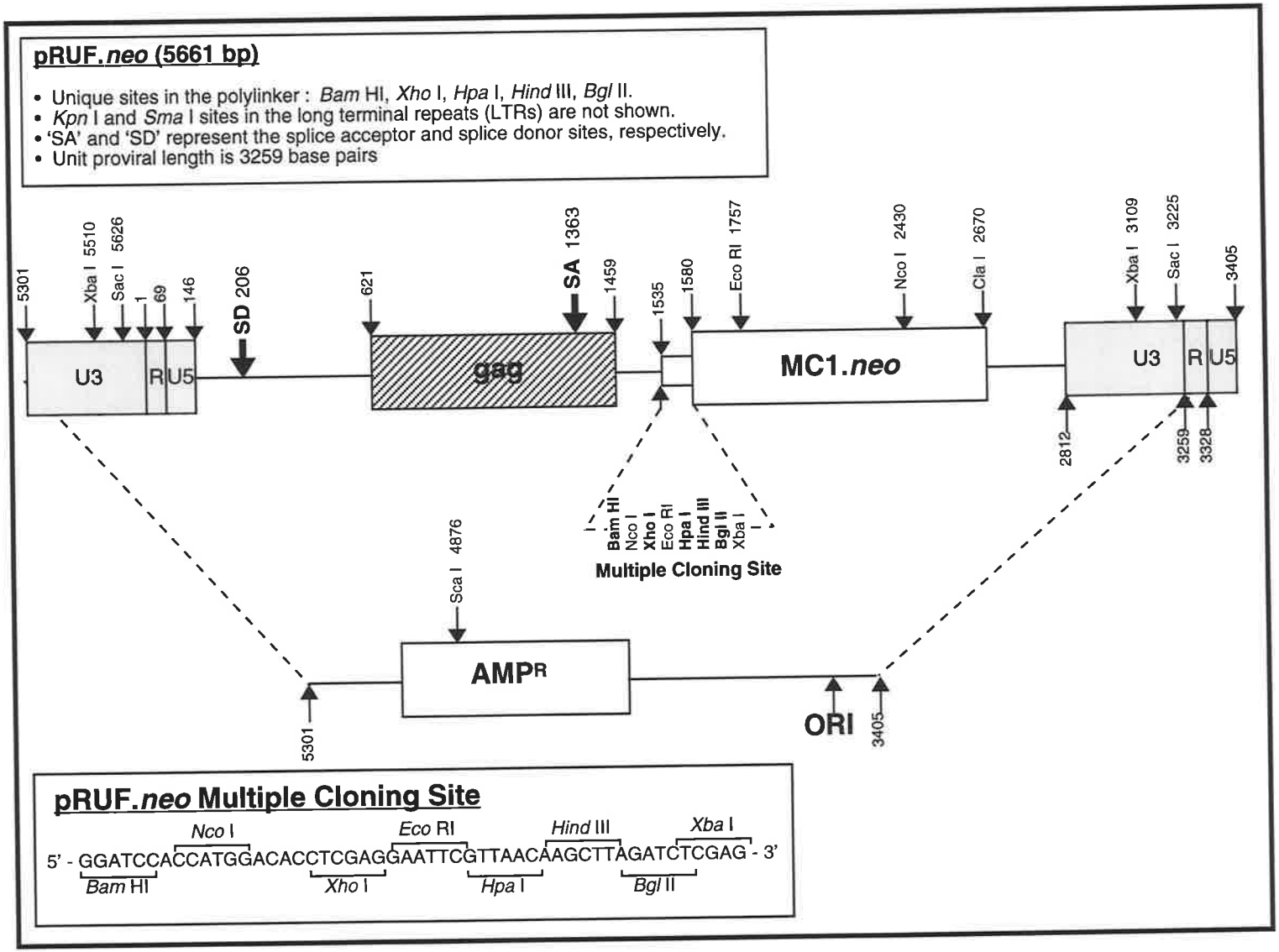
(i) Land mark restriction endonuclease sites

(ii) Restriction cloning sites in the polylinker (MCS), to enable directional cloning of cDNA products

(iii) the MC1.*neo* cassette containing the Neo^R gene driven by the f9 polyomavirus enhancer

(iv) the long terminal repeat (LTR; comprised of U3, R, and U5) elements derived from the myeloproliferative sarcoma virus (MPSV)

(v) the splice donor (SD) and splice acceptor (SA) sites used to generate the subgenomic mRNA (refer to text for details). The nucleotide sequence numbers of the retroviral regions of the plasmid are derived from sequences of the Moloney Murine Leukaemia Virus, MMLV, (please refer to Shinnick *et al*, 1981).


pRUF.neo (5661 bp)
• Unique sites in the polylinker : Bam HI, Xho I, Hpa I, Hind III, Bgl II.
• Kpn I and Sma I sites in the long terminal repeats (LTRs) are not shown.
• 'SA' and 'SD' represent the splice acceptor and splice donor sites, respectively.
• Unit proviral length is 3259 base pairs
5301
Xba I 5510
Sac I 5626
1
69
146
SD 206
621
SA 1363
1459
1535
1580
Eco RI 1757
Nco I 2430
Cla I 2670
Xba I 3109
Sac I 3225
3405
U3
R
U5
gag
MC1.neo
U3
R
U5
2812
3259
3328
Bam HI
Nco I
Xho I
Eco RI
Hpa I
Hind III
Bgl II
Xba I
Multiple Cloning Site
Sca I 4876
AMPR
ORI
5301
3405
pRUF.neo Multiple Cloning Site
Nco I
Eco RI
Hind III
Xba I
5' - GGATCCACCATGGACACCTCGAGGAATTCGTTAACAAGCTTAGATCTCGAG - 3'
Bam HI
Xho I
Hpa I
Bgl II

the U22.4 provirus and of cDNAs inserted into the MCS of the retroviral vector pRUF*neo*, occur via subgenomic mRNA generated by splicing between the normal retroviral splice donor (at nucleotide 206; refer to Figure 4.2.3.) and a cryptic splice acceptor (at nucleotide 1363; refer to Figure 4.2.3.) as previously described (Gonda *et al*, 1987; Gonda *et al*, 1989). In addition, the presence of *gag* sequences has been shown to substantially increase retroviral titres (Armentano *et al*, 1987; Bender *et al*, 1987).

4.2.1. Generation Of A Stable HBMSC cDNA Library: Size And Representation.

Directional cDNA synthesis was performed essentially as described in the *Materials and Methods*, Section 2.24. and as schematically represented in Figure 4.2.4. The size and integrity of the cDNA generated from the reverse transcription of HBMSC-derived mRNA was assessed following alkaline-agarose gel electrophoresis and autoradiography, and found to range from 0.4 to 8.5 kb in size (data not shown). Following the addition of linkers, the cDNA was directionally cloned into the multiple cloning site (MCS) of the retroviral vector, pRUF.*neo* (Figure 4.2.3.). The retroviral vector containing the ligated cDNA was amplified in *E.coli*, and by this method a plasmid library of approximately 1.3×10^6 clones was generated from 130 ng of cDNA. As shown in Figure 4.2.5, restriction analysis of 20 independent clones revealed inserts ranging in size from 0.4 to 8 kb, with the majority (13/20) being greater than 2.5 kb in length.

Central to the construction of any cDNA library, is the requirement for adequate representation of the mRNA species present in the cell type or tissue of origin. Considering previous reports (Bishop *et al*, 1974; Chikaraishi, 1979; Ryfell and McCarthy, 1975), there are between 30,000 to 120,000 different mRNA species present in the cytoplasm of a normal mammalian cell. As such, adequate representation in a cDNA expression library is likely to require at least one order of magnitude more clones than this, as the synthesis of large, full-length cDNAs is a relatively inefficient process. Therefore, the HBMSC plasmid library described here is likely to contain ten to 40-fold more clones, than there are sequences expressed in the mammalian cellular equivalent.

Although it is somewhat elementary to prepare libraries of this complexity as plasmids (refer above), or phages in *E.coli*, the generation of similarly complex libraries in

Figure 4.2.4. Directional cDNA Synthesis And Ligation Into The MCS Of pRUF*neo*.

Schematic representation of directional cDNA synthesis and molecular cloning into the multiple cloning site (MCS) of the retroviral pRUF*neo* vector. Please refer to *Materials and Methods*, Section 2.24., and text for details.

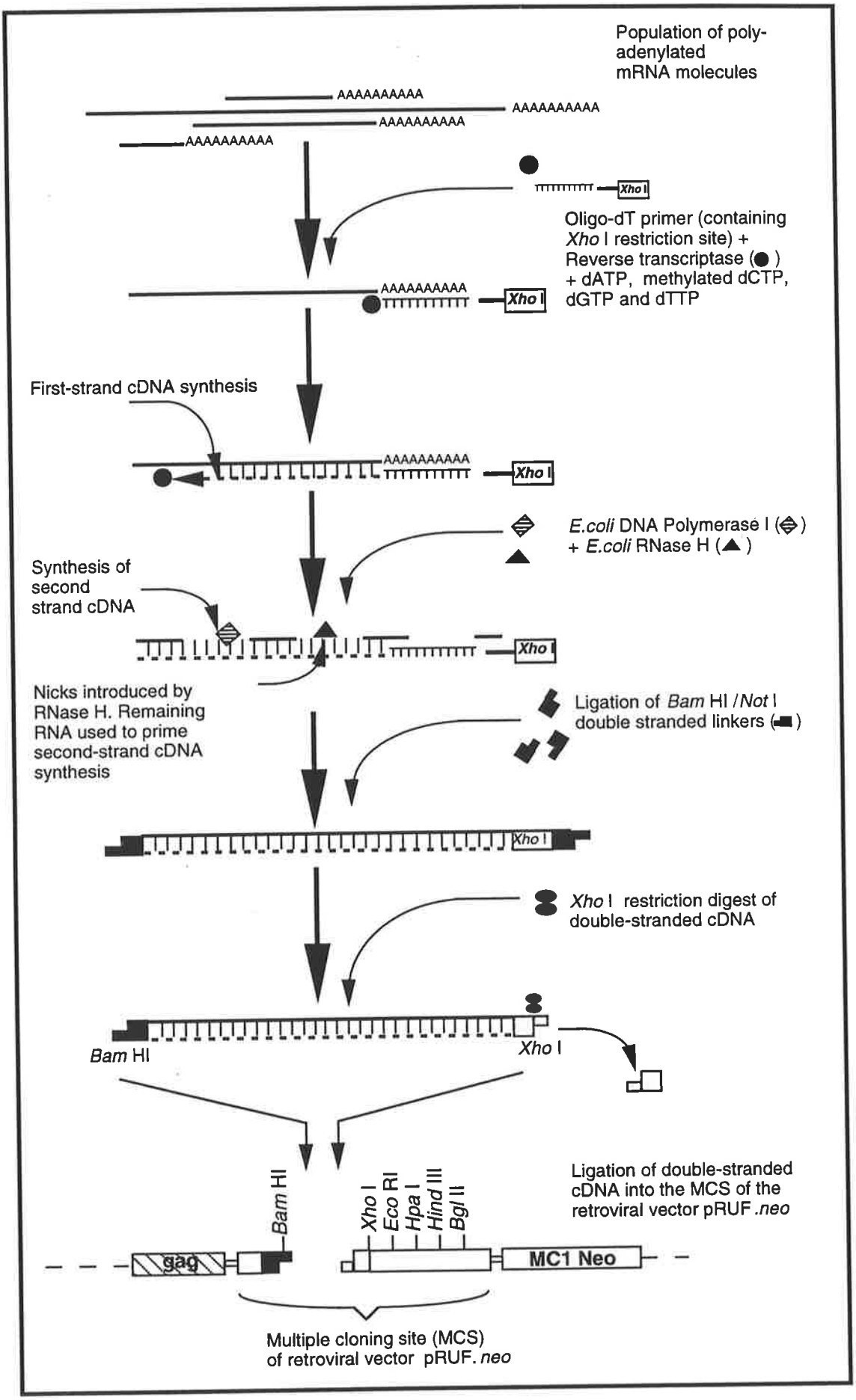
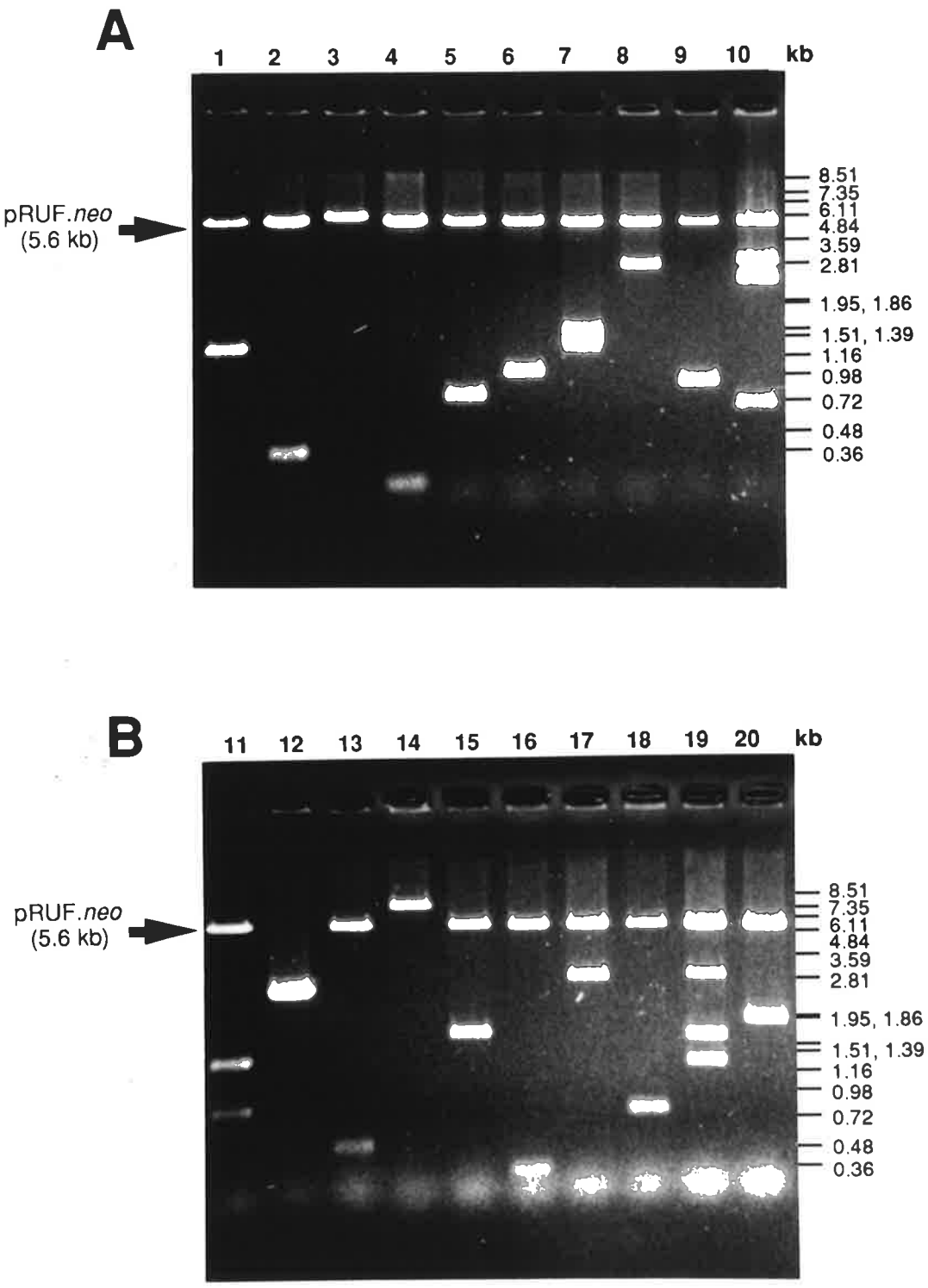

Population of poly-adenylated mRNA molecules
AAAAAAAAAA
Xho I
Oligo-dT primer (containing *Xho* I restriction site) + Reverse transcriptase (●) + dATP, methylated dCTP, dGTP and dTTP
First-strand cDNA synthesis
E.coli DNA Polymerase I (◈) + *E.coli* RNase H (▲)
Synthesis of second strand cDNA
Nicks introduced by RNase H. Remaining RNA used to prime second-strand cDNA synthesis
Ligation of *Bam* HI / *Not* I double stranded linkers (▬)
Xho I restriction digest of double-stranded cDNA
Bam HI
Xho I
Ligation of double-stranded cDNA into the MCS of the retroviral vector pRUF *.neo*
Bam HI
Xho I
Eco RI
Hpa I
Hind III
Bgl II
gag
MC1 Neo
Multiple cloning site (MCS) of retroviral vector pRUF. *neo*

Figure 4.2.5. Restriction Analysis Reveals That cDNA Inserts Range From 0.4-8 kb.

Following transformation of the plasmid library into *E.coli* DH10B, plasmid DNA was isolated from 20 independently isolated bacterial clones. *Bam*HI and *Xho*I restricted samples were subsequently separated on a 1.2% agarose gel, ethidium bromide stained and photographed under ultraviolet illumination.

Due to the presence of internal *Bam*HI and *Xho*I restriction sites, the size of the cDNA inserts represent the sum of the individual fragments (with the exception of the 5.6 kb pRUF.*neo*). The positions of the molecular weight markers (SPP-1 DNA, digested with *Eco*RI) are represented on the right (in kilobase pairs).


A
1 2 3 4 5 6 7 8 9 10 kb
pRUF.neo
(5.6 kb)
8.51
7.35
6.11
4.84
3.59
2.81
1.95, 1.86
1.51, 1.39
1.16
0.98
0.72
0.48
0.36
B
11 12 13 14 15 16 17 18 19 20 kb
pRUF.neo
(5.6 kb)
8.51
7.35
6.11
4.84
3.59
2.81
1.95, 1.86
1.51, 1.39
1.16
0.98
0.72
0.48
0.36

eukaryotic cells is generally more difficult. The protocol described here, has been designed in part to circumvent this problem. As discussed earlier, the initial steps involve (i) transfection of the DNA obtained from the amplification of the plasmid library into the amphotropic packaging cell line, PA317, and (ii) use of the transiently generated retrovirus to infect an ecotropic packaging cell line, Ψ_2.

Using virus generated from the pool of transfected packaging cells to infect an additional packaging cell line provides several advantages. Firstly, infection is a more desirable way to transfer genes into the cells that will constitute the final library of ecotropic virus-producing cells, since it has been shown to yield substantially higher viral titres from these cells (Hwang and Gilboa, 1984; Lynch and Miller, 1991; Miller *et al*, 1986). Secondly, infection generally results in a smaller number of proviral integrations per cell, so that each infected cell in the total pool represents a single (or at most, only few) cDNA species present within the library.

Prior to transfection of the plasmid library into the amphotropic virus-packaging cell line, PA317, plasmid DNA derived from five independent ligations were combined to ensure proportional and adequate representation. Approximately 55 μg of DNA was obtained per 1 x 10^5 bacterial colonies, and a total of 650 μg of CsCl-purified DNA was used to transfect 1.2 x 10^7 PA317 cells. Following transfection, 120 ml of viral supernatant was harvested and the viral titre determined by its ability to confer resistance to G418 following infection of Ψ_2 cells. The titre was estimated to be 3 x 10^4 infectious viral particles per ml of viral supernatant and in view of this, 24 ml of viral supernatant was used to infect 12 dishes of Ψ_2 cells (as described in the *Materials and Methods*), resulting in approximately 7.2 X 10^5 G418 resistant Ψ_2 colonies in four subpools.

4.2.2. Selection Of An Appropriate Target Cell Population For The Functional Screening Of CSM Expression.

Having established a stable HBMSC library of approximately 7.2 X 10^5 independent clones in the ecotropic-virus producing cell line Ψ_2, the murine haemopoietic, factor-dependent cell line FDC-P1 (Dexter *et al*, 1980) was initially chosen as the target cell type for functional screening of surface antigen expression. This choice was made on

the basis of the following criteria; (i) FDC-P1 cells can be infected at high frequency (Rayner and Gonda, 1994) (ii) previous studies (Rayner and Gonda, 1994) revealed that the myeloproliferative sarcoma virus LTR functions well in FDC-P1 cells, (iii) FDC-P1 cells divide rapidly (12 hour doubling time), facilitating the screening procedure (described below) and finally (iv) immunostaining and flow cytometry revealed that there was limited or no cross-reactivity with the "clustered" mAbs directed against human cell surface antigens.

The library was introduced into FDC-P1 cells by co-cultivation with the virus-producing Ψ_2 cells. The efficiency of infection of the FDC-P1 cells was 15-28% (n=3), as determined by plating in agar, in the presence of G418 (Table 4.2.1.). To achieve proportionate transfer of all the cDNAs present in the library into FDC-P1 cells, a total of 6×10^6 FDC-P1 cells were co-cultivated with the stable library in Ψ_2 cells. To prevent the loss cells expressing rare cDNA clones, FDC-P1 cells resistant to G418 (1.26×10^6, as determined by agar plating), were cultured to expand their numbers, such that each independently infected FDC-P1 cell was represented at least 100 times.

4.2.3. Establishment Of A Magnetic-Bead/Monoclonal Antibody Capture And Enrichment Protocol To Screen For CSM Expression.

Initial experiments employing the panning technique established by Wysoki and Sato (1978) and used with great effect by Seed and Aruffo (Seed and Aruffo, 1987; Seed, 1987; Aruffo and Seed, 1987), proved too inefficient for the capture of rare FDC-P1 cells expressing the desired HBMSC antigens. Similarly, screening by preparative fluorescence activated cell sorting (FACs) proved too cumbersome, as large numbers of cells (up to 1×10^8) had to be analysed in order to ensure isolation of CSM-expressing cells present at low incidence in the population (data not shown).

To isolate rare FDC-P1 cells expressing the desired HBMSC CSM, a protocol employing antibody coated magnetic Dynabeads was developed (presented schematically in Figure 4.2.6.). Initial experiments to assess the efficacy of such an approach centred around the ability of the magnetic beads to select and enrich a minor population of "marked" cells. A fixed number of FITC-"loaded" FDC-P1 cells, labelled with mAb

Table 4.2.1. Number Of Clones Derived By Infection Of FDC-P1 Cells With Virus Derived From Ecotropic Packaging (Producing) Ψ_2 Cells.

To determine the infection frequency following co-cultivation with the virus-producing Ψ_2 cell line, FDC-P1 cells were plated in soft agar (as described in *Materials and Methods*) in the presence of G418. Following 10 days culture, colonies were enumerated. Data represents the mean ± SE of three experiments, where 300 cells were plated per dish at day 0.

Table 4.2.1. Number Of Clones Derived By Infection Of FDC-P1 Cells With Virus Derived From Ecotropic Packaging (Producing) Ψ_2 Cells.

	Number of FDC-P1 Colonies Following Co-cultivation With				
Plating Conditions	**Ψ_2 (control)**	**Ψ_2 (Pool A)**	**Ψ_2 (Pool B)**	**Ψ_2 (Pool C)**	**Ψ_2 (Pool D)**
-G418	210 ± 20	230 ± 70	215 ± 15	232 ± 35	190 ± 60
+G418	0 ± 0	34 ± 17	38 ± 9	65 ± 13	48 ± 15
Infection Frequency (%)	0	15	17	28	25

Figure 4.2.6. Schematic Of Magnetic-Bead/Monoclonal Antibody Capture And Enrichment Procedure.

An outline of the magnetic-bead/monoclonal antibody capture and enrichment procedure used to isolate rare cells expressing the desired CSM. (please refer to text for details).

G418 resistant pool of
library-infected target cells

↓

mAb incubation

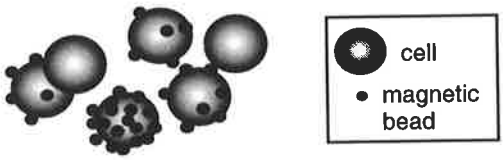
↓

Incubation with sheep anti-mouse (SAM)
IgG-conjugated magnetic M450 Dynabeads

↓

Capture rosetted
cells (bead+) with magnet

↓



↓

Culture to expand cells and
repeat selection

↓

FACS and single cell deposition of bead-
selected target cells

↓

DNA isolation and PCR recovery of
proviral cDNA inserts

directed against murine class I (refer to *Materials and Methods*) were added to an excess of unlabelled FDC-P1 cells at ratios of 1:100 (Figure 4.2.7. (A)) and 1:20 (Figure 4.2.7. (B)). The cell mixture was incubated with antibody-coated magnetic beads and the depletion of labelled cells assessed by flow cytometry. As it can be observed in Figure 4.2.7., the method described here, was effective in removing up to 98% of the labelled cells following *one* round of selection and represented in principal, the technique used to screen the expression library (detailed below).

4.2.4. Isolation Of CSM-Expressing FDC-P1 Cells Infected With The HBMSC Library.

Typically, 1 x 10^8 G418-resistant FDC-P1 cells were incubated (as described in the *Materials and Methods*, Section 2.27.1.) with a "cocktail" of six mAbs as detailed in Table 4.2.2. The mAbs used in this initial study recognise cell surface antigens of which the corresponding cDNA had been previously identified. Their choice was based on a number of parameters including, (i) requisite reactivity with HBMSC and their contrasting lack of reactivity with mock infected FDC-P1 cells (data not shown), (ii) their recognition of antigens with widely varying molecular weights and cDNA sizes and (iii) as demonstrated in Figure 4.2.8. the level of expression of the CSMs recognised by these mAbs differed considerably (ranging from high to low, as determined by flow cytometry) on the HBMSC used to construct the library.

Following the sequential incubation of the mAb cocktail and sheep anti-mouse IgG-coated magnetic Dynabeads, HBMSC antigen-expressing FDC-P1 cells were captured with the use of a magnetic particle concentrator (MPC). Figure 4.2.9. illustrates the sequential enrichment of Ag-expressing FDC-P1 cells following 3 rounds of selection, as assessed by immunofluorescence and flow cytometry and light microscopy, respectively. Magnetic bead selection and enrichment was carried out until homogeneity of antigen expression was achieved, as exemplified in Figure 4.2.9. (C). In some instances, up to five rounds of selection were necessary to achieve homogeneity of Dynabead-rosetting cells, although in the majority of cases, 3 rounds of selection was sufficient.

Having obtained a pool of FDC-P1 cells potentially expressing a variety of HBMSC CSM, the cocktail of mAbs was then divided into its individual components and

Figure 4.2.7. Assessment Of The Efficacy Of The mAb/Magnetic Bead Enrichment Procedure.

FDC-P1 cells were cytoplasmicly-labelled with FITC (please refer to Materials and Methods, Section 2.11.13), and subsequently immunolabelled with either mAb HB75, (directed against Murine MHC Class I [H-2^d haplotype]) or an isotype-matched, non-binding control mAb. 1 x 10^3 and 5 x 10^3 FITC-"loaded"/HB75-labelled FDC-P1 cells were then added to 9.9 x 10^4 (1:100 dilution; panel A) and 9.5 x 10^4 (1:20 dilution; panel B) unlabelled FDC-P1 cells, respectively. To select labelled FDC-P1 cells, 1 x 10^5 sheep-anti-mouse (SAM) IgG-conjugated magnetic beads were added per tube, incubated for 2 hours at 4°C with rotation.

Magnetic bead-"rosetted" (ie. FITC-"loaded/immunolabelled) FDC-P1 cells were captured using a magnetic particle concentrator (MPC). The efficacy of selection was indirectly monitored by flow cytometry (ie. the degree of depletion observed). In both cases (Panel A & B) this procedure was effective in removing up to 98% of the labelled cells following *one* round of selection.

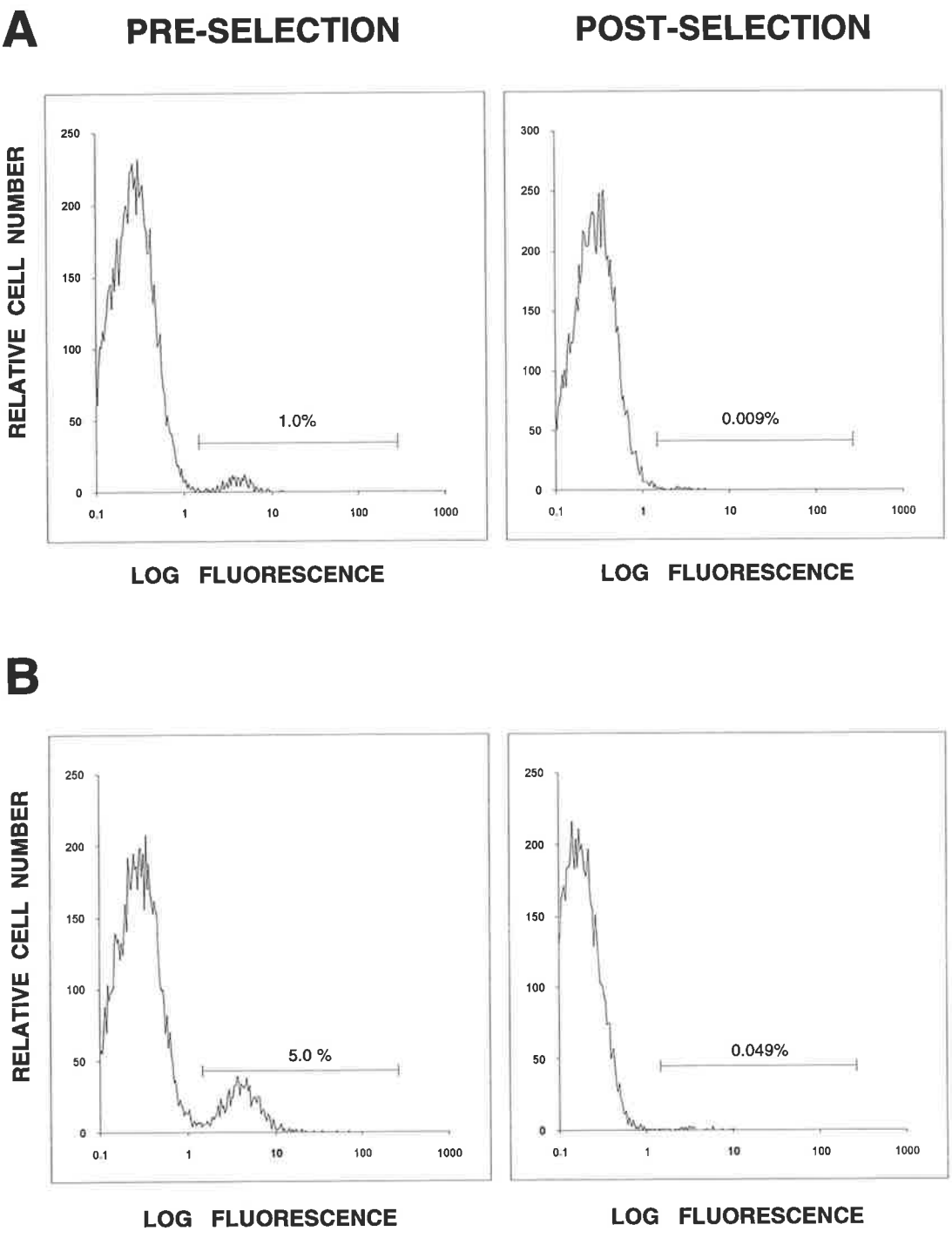

A
PRE-SELECTION
POST-SELECTION
RELATIVE CELL NUMBER
250
200
150
100
50
0
1.0%
0.1
1
10
100
1000
LOG FLUORESCENCE
300
250
200
150
100
50
0
0.009%
0.1
1
10
100
1000
LOG FLUORESCENCE
B
RELATIVE CELL NUMBER
250
200
150
100
50
0
5.0 %
0.1
1
10
100
1000
LOG FLUORESCENCE
250
200
150
100
50
0
0.049%
0.1
1
10
100
1000
LOG FLUORESCENCE

Table 4.2.2. Distribution, Molecular Mass And cDNA Size Of The CSM Identified By The "Clustered" Monoclonal Antibodies Used In This Study.

Antibody Name	CD/Alternate Nomenclature	CSM Recognition / Cellular Distribution	MWt Of CSM(*)	Published Size of cDNA (bp)	Reference/ Source Of mAb
1B4.B12	β_2-microglobulin	β_2-microglobulin (associated with MHC class I subunit) / Ubiquitous expression on haemopoietic and non-haemopoietic cells	12	549 (J00105)	Dr. L. Ashman
5E10	Thy1 (CDw90)	Thy-1 / small subpopulations of $CD34^+$ cells, lymphocytes and myeloid cells. Nervous system, connective tissue and various stromal cell lines	18	523 (H18293)	Pharmingen
MEM-43	MIRL (CD59)	Membrane Inhibitor Of Reactive Lysis (MIRL) / Ubiquitous expression on haemopoietic and non-haemopoietic cells	19	1671 (X16447)	DAKO
9B3	MHC class 1	MHC class I subunit (associated with β_2 microglobulin) / Ubiquitous expression on haemopoietic and non-haemopoietic cells	44	1098 (M32322)	Mr. S. Gronthos
1G2	Endoglin (CD105)	Human TGFβ binding protein / Expressed by endothelial cells and a subset of BMMNC including haemopoietic progenitor cells	95	2620 (J05481)	Dr. H-J. Bühring
P4C10	β1-integrin (CD29)	Integrin β_1 subunit / Numerous leucocyte cell types	130	3614 (X07979)	Dr. W. Carter

The monoclonal antibodies were initially screened by indirect immunofluorescence and flow cytometry to ensure reactivity with HBMSC and lack of binding to FDC-P1 cells, the intended recipient of the retroviral expression library (refer to text).
Numbers in parentheses represent Genbank/EMBL database accession numbers.
* Molecular weights of proteins assessed under reduced conditions.

Figure 4.2.8. HBMSC-Expression Of CSM Identified By The "Clustered" mAbs Used In This Study.

Cultured HBMSCs were immunolabelled with the "clustered" mAbs, detailed in Table 4.2.2. or an isotype matched, non binding control (refer to Table 2.10.1.). Data are displayed as single-parameter fluorescence (FITC) histograms of 1 x 10^4 light-scatter gated events, collected as list mode data.

- - - - -: isotype matched, non-binding control
_____ : labelling mAb

A. mAb 1B4.B12 (β_2-microglobulin).
B. mAb 1G2 (Endoglin, CD105).
C. mAb 5E10 (Thy-1, CDw90).
D. mAb 9B3 (MHC Class I).
E. mAb MEM-43 (MIRL, CD59).
F. mAb P4C10 (β_1 Integrin, CD29).

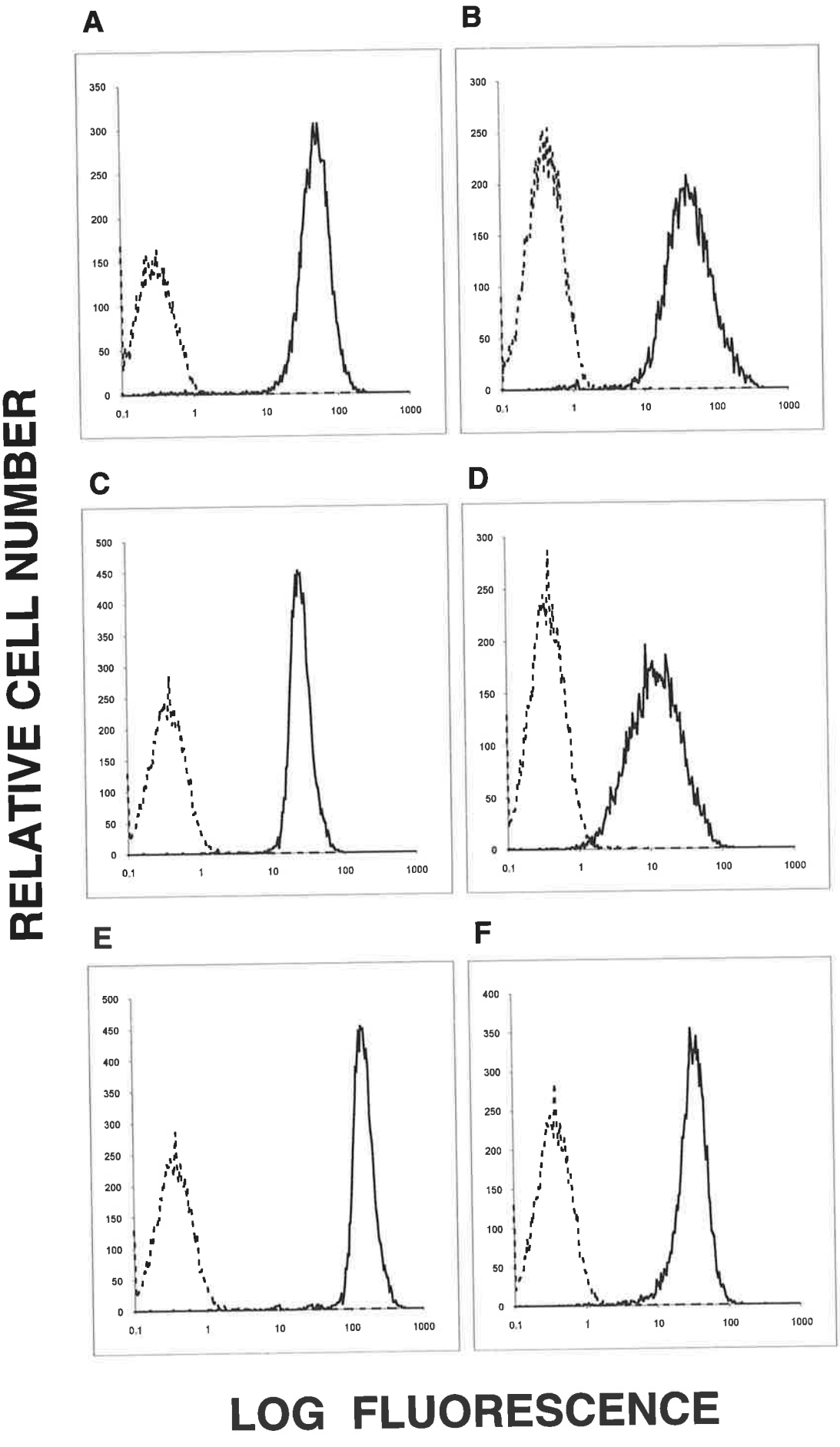
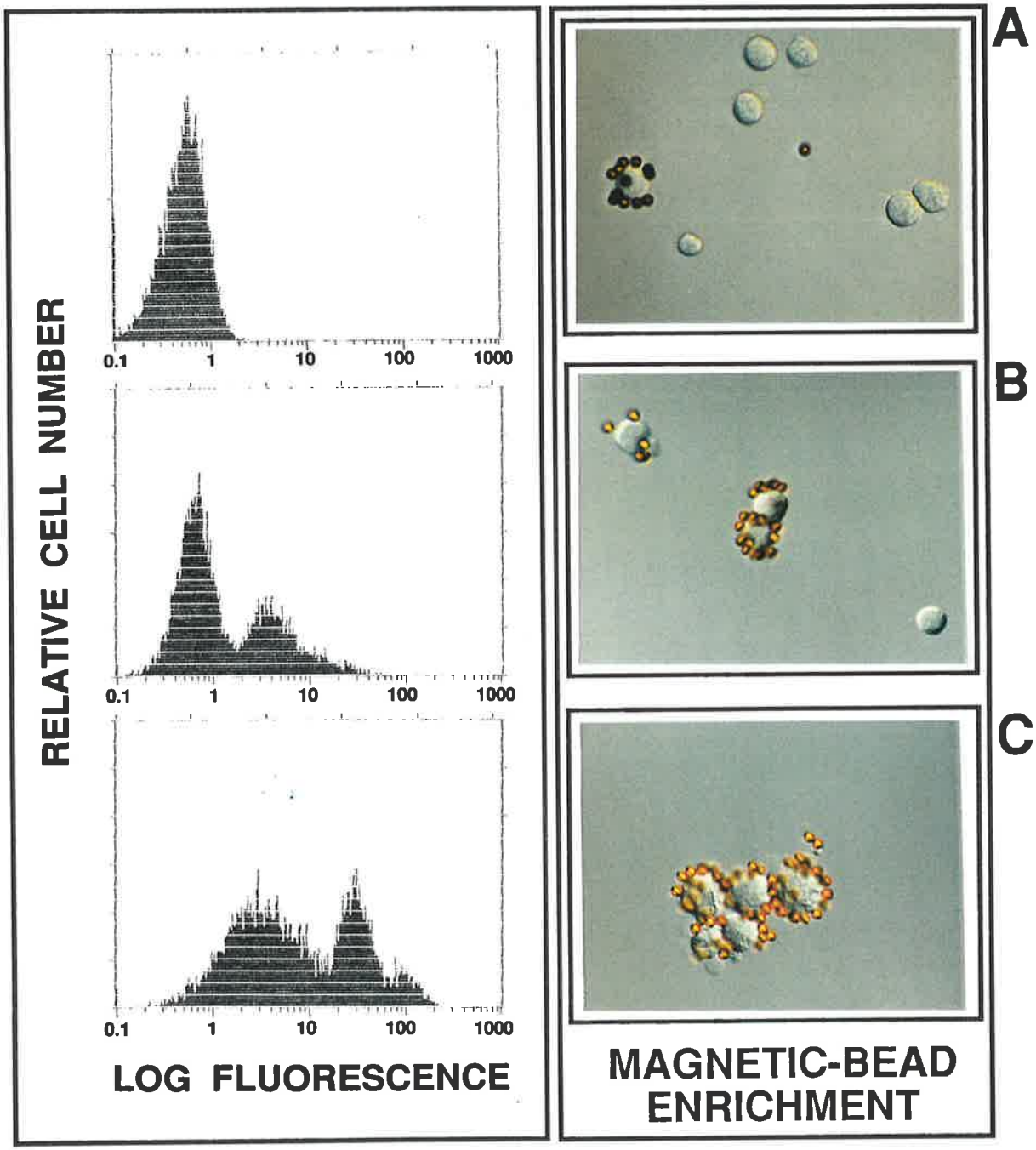

A
B
C
D
E
F
RELATIVE CELL NUMBER
LOG FLUORESCENCE
0.1
1
10
100
1000

Figure 4.2.9. Progressive Enrichment Of FDC-P1 Expressing HBMSC Surface Antigens.

FDC-P1 cells selectively isolated by the magnetic-bead/monoclonal antibody capture and enrichment procedure (please refer to Figure 4.2.6.) were immuno-labelled with the mAb cocktail (Table 4.2.2.) and analysed by flow cytometry and light microscopy (200 x magnification), following (A) one, (B) two and (C) three rounds of selection. Magnetic bead selection and enrichment was carried out until homogeneity of antigen expression was achieved.


RELATIVE CELL NUMBER
0.1
1
10
100
1000
LOG FLUORESCENCE
A
B
C
MAGNETIC-BEAD ENRICHMENT

indirect immunofluorescence and flow cytometry was performed to determine the nature of CSM expressed. Figure 4.2.10. (A) (1, 2 & 3) illustrates the flow cytometric results obtained when the pool of Dynabead-rosetted cells were stained with 1B4.B12 (anti-β_2 microglobulin), 1G2 (anti-Endoglin, CD105) and P4C10 (anti-β_1 integrin, CD29), respectively. Following the determination of HBMSC surface antigen expression, clonal populations of FDC-P1 cells expressing HBMSC CSMs were isolated by fluorescence activated cell sorting (FACS) and single cell deposition, or by a combination of FACS and methylcellulose plating (Figure 4.2.10. (B)) as described in the *Materials and Methods*. Clones were expanded so that surface antigen expression could be assessed by flow cytometry (as described previously and FDC-P1 clones displaying a unimodal distribution of surface-antigen expression were selected and expanded for further analysis (Figure 4.2.10. (C)). Unimodality of CSM expression was used as an indirect measure, to ensure that mixtures of two adjacent clones were not isolated, which could lead to ambiguity in the PCR recovery of proviral cDNA inserts from genomic DNA (discussed below, Section 4.2.6.).

4.2.5. Preliminary Assessment Of The Antigens Expressed By The FDC-P1 Clones And The Analysis Of The Number Of Proviral Integrations.

The molecular weights of the proteins expressed by the FDC-P1 clones were determined following surface biotinylation, immunoprecipitation and SDS-PAGE. The molecular masses of the reduced proteins were in each instance, consistent with the published data for each of the CSM described (Figure 4.2.11. (A) and 4.2.11. (B) and Table 4.2.2).

To ascertain the number of proviral integrations harboured by the various FDC-P1 clones, Southern blot analysis was performed as described in the *Materials and Methods*. The number of proviruses varied between one (MEM-43, P4C10, 1G2 FDC-P1's), two (1B4.B12 FDC-P1's), three (5E10 FDC-P1) and four (9B3 FDC-P1) integrations, confirming the relatively low frequency of infection for each of the FDC-P1 clonal populations (Figure 4.2.12.). Additional Southern blots were performed using other clones

Figure 4.2.10. FACS And Clonal Isolation Of FDC-P1 Cells Expressing HBMSC Surface Antigens.

Following selective magnetic bead enrichment, the pool of Dynabead-rosetted cells (refer to column A) were immunolabelled with the individual components of the mAb cocktail, including (A1) 1B4.B12 [anti-β_2 microglobulin]; (A2) 1G2 [anti-Endoglin, CD105] and (A3) P4C10 [anti-β_1 integrin, CD29]. Furthermore, clonal populations of FDC-P1 were established following FACS and single cell deposition (or by a combination of FACS and methylcellulose plating), by selecting the cells exhibiting the highest expression of the antigen [marker set, R2], (B1->3). Clones were expanded so that surface antigen expression could be assessed by indirect immunofluorescence and flow cytometry. Clones displaying a unimodal distribution of surface-antigen expression were selected and expanded for further analysis (C1->3).

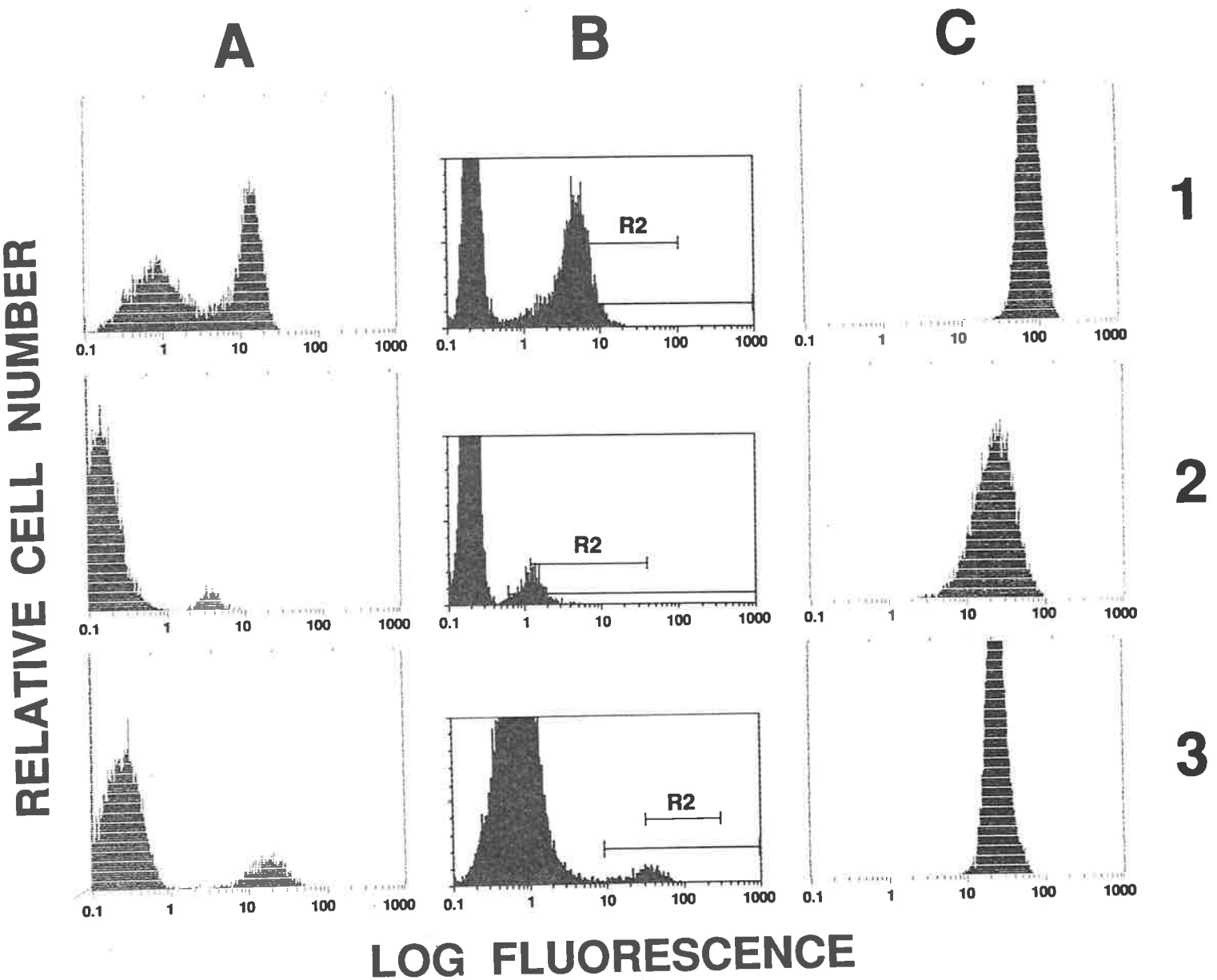

A
B
C
1
2
3
R2
R2
R2
0.1
1
10
100
1000
RELATIVE CELL NUMBER
LOG FLUORESCENCE

Figure 4.2.11. Determination Of The Molecular Mass Of Proteins Expressed By The FDC-P1 Clones Expressing HBMSC Surface Antigens.

Biotinylated membrane preparations of FDC-P1 cells expressing various CSM were co-incubated with the indicated mAb (Table 4.2.2.) for 16 hours at 4°C. Immune complexes were precipitated using goat anti-mouse Sepharose, resuspended in reducing SDS-PAGE buffer, resolved on a (A) 12% or (B) 10% SDS-polyacrylamide gel and visualised with biotin-streptavidin-HRPO complex and enhanced chemiluminescence (ECL).

(A) Lane 1, Thy 1; Lane 2, CD59; Lane 3, β2 microglobulin; Lane 4, MHC class I.
(B) Lane 1, Endoglin; Lane 2, β1 integrin.

Hybrid heterodimers (refer to *Discussion*) between the human and murine polypeptides are observed in (A) lane 3 and 4.

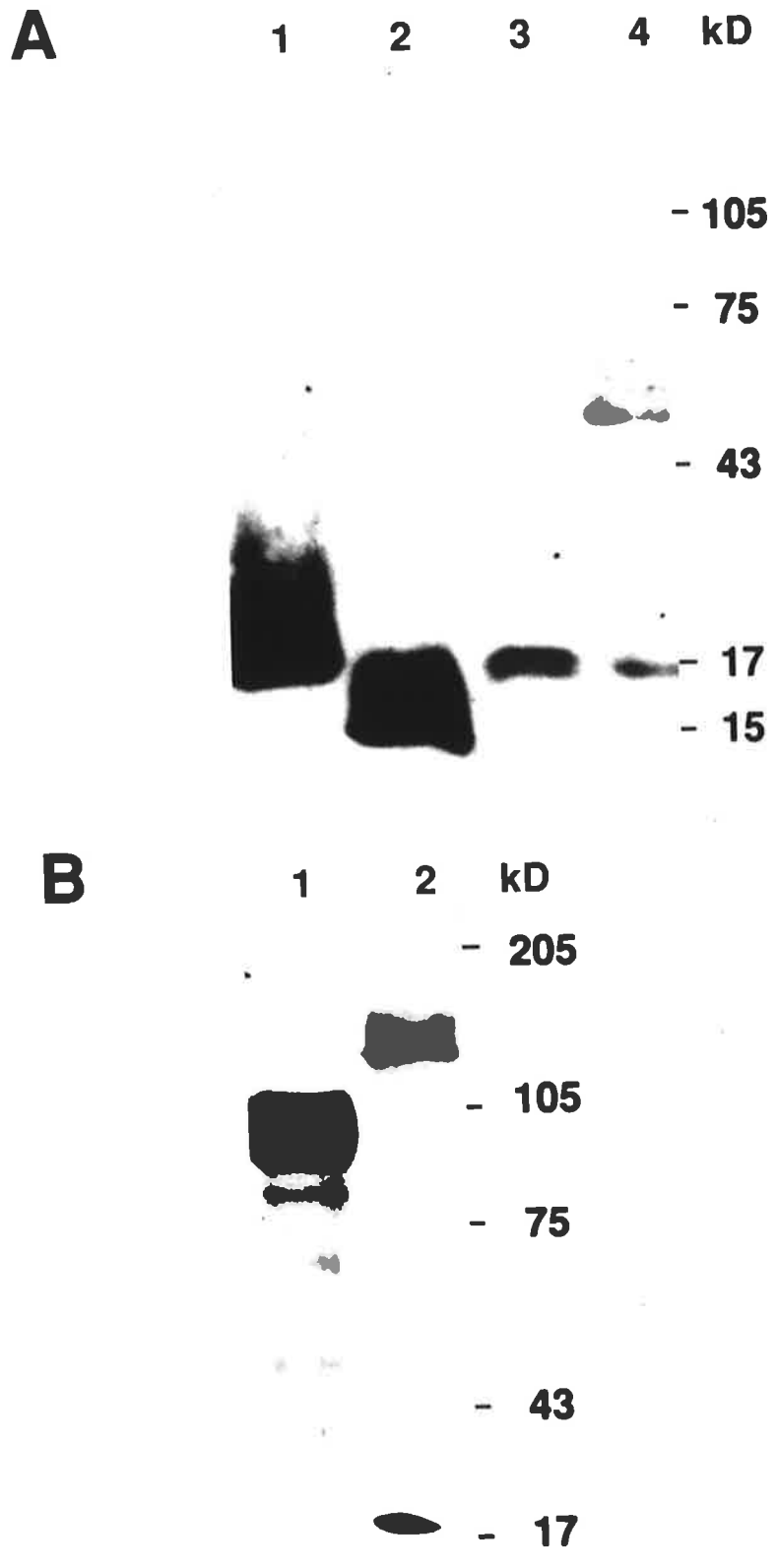
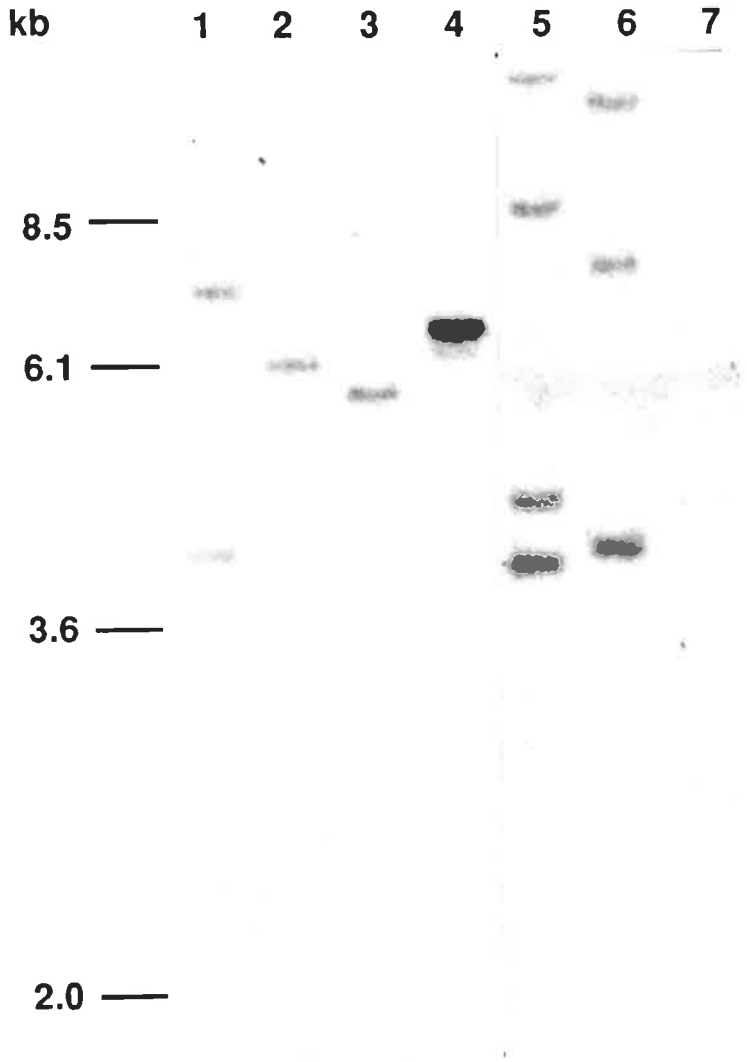

A
1
2
3
4
kD
105
75
43
17
15
B
1
2
kD
205
105
75
43
17

Figure 4.2.12. Southern Blot Analysis Reveals The Number Of Proviral Integrations In Each FDC-P1 Clone.

Southern blot analysis of genomic DNA isolated from HBMSC surface antigen-expressing FDC-P1 clones was performed essentially as described by Rayner and Gonda, 1994. Following restriction digestion with *Bam*H1, genomic DNA was separated on a 0.8% agarose gel, transferred to nitrocellulose, and probed with a ^{32}P radio-labelled Neo^R cassette. The positions of the molecular weight markers (SPP-1 DNA digested with *Eco*R1), are shown on the right (in kilobase pairs). Refer to Table 4.2.2., as nomenclature used in this figure pertains to the mAb used to select the FDC-P1 clonal population. Lane 1, 1B4. B12; Lane 2, MEM-43; Lane 3, P4C10; Lane 4, 1G2; Lane 5, 9B3; Lane 6, 5E10; Lane 7, uninfected FDC-P1 cells. Number of proviral integrations vary from 1 to 4, confirming the relatively low frequency of infection for each of the FDC-P1 clonal populations.


kb
1
2
3
4
5
6
7
8.5
6.1
3.6
2.0

expressing these surface antigens and showed similar copy numbers of virus in these cells (data not shown).

4.2.6. PCR Recovery Of Proviral cDNA Inserts From Genomic DNA.

Following retroviral infection, the proviral DNA is stably integrated in a predictable configuration allowing the rescue of cDNA sequences from the genomic DNA using the polymerase chain reaction (PCR). As illustrated in Figure 4.2.13., primers complementary to sequences adjacent to the multiple cloning site (MCS) in the retroviral vector can thus be used to amplify the cDNA insert responsible for conferring the expression of a particular antigen. The lack of consistent amplification of cDNA inserts greater than 2 kb (eg. Endoglin and β1-integrin) necessitated the use of long range PCR conditions as described in the *Materials and Methods*. In most cases, a single amplified product was obtained for each clone, with sizes ranging from approximately 500 bp to 3800 bp (Figure 4.2.14.). Significantly, in all cases, the size of the amplified cDNA was consistent with the size of the published sequence, when taking into account the additional sequence (approximately 145 base pairs) contributed by the retrovirus.

4.2.7. Partial-Sequence Analysis Of cDNA Inserts.

Gel-purified PCR products were subsequently subcloned into an appropriate sequencing vector (pBluescript or pGEM-T, Appendix A, Restriction Maps # 1 and 6, respectively) to enable partial sequence analysis (refer to *Materials and Methods*, Section 2.19.). The resultant nucleotide sequence was compared with sequences submitted to the Genbank/EMBL databases via standard "FASTA alignment analysis" and selected examples of the results obtained are illustrated in Figure 4.2.15. As demonstrated in Table 4.2.3, sequence analysis of all the FDC-P1 clone-derived PCR products confirmed that the CSM expression as detected by the mAbs was due to the viral transfer of the corresponding cDNA. That is, the sequences obtained conformed entirely with the published sequences for these genes. In all cases, with the exception of the MEM-43 (CD59), the 3' untranslated region of the mRNA, including the poly(A) tract was detectable. This observation however, is in accord with the identification of 4 different

Figure 4.2.13. Schematic Representation Of The Protocol Used To Recover cDNAs From Infected Cells Expressing HBMSC CSMs.

Following infection, the viral DNA is integrated into the host cell genome in a stable and predictable configuration. As such, PCR amplification can be exploited to "rescue" the cDNA cloned into the MCS of the retroviral vector pRUF.*neo*.

Genomic DNA isolated from infected cells is denatured and primers complementary to retroviral sequences which flank the MCS (*RCF1*, *RCR1* and *RCR2*) are therewith annealed. Repeated rounds of denaturation, annealing and extension thus result in the specific amplification of the cloned cDNA. PCR products are separated by agarose gel electrophoresis and subsequently isolated by gel purification.

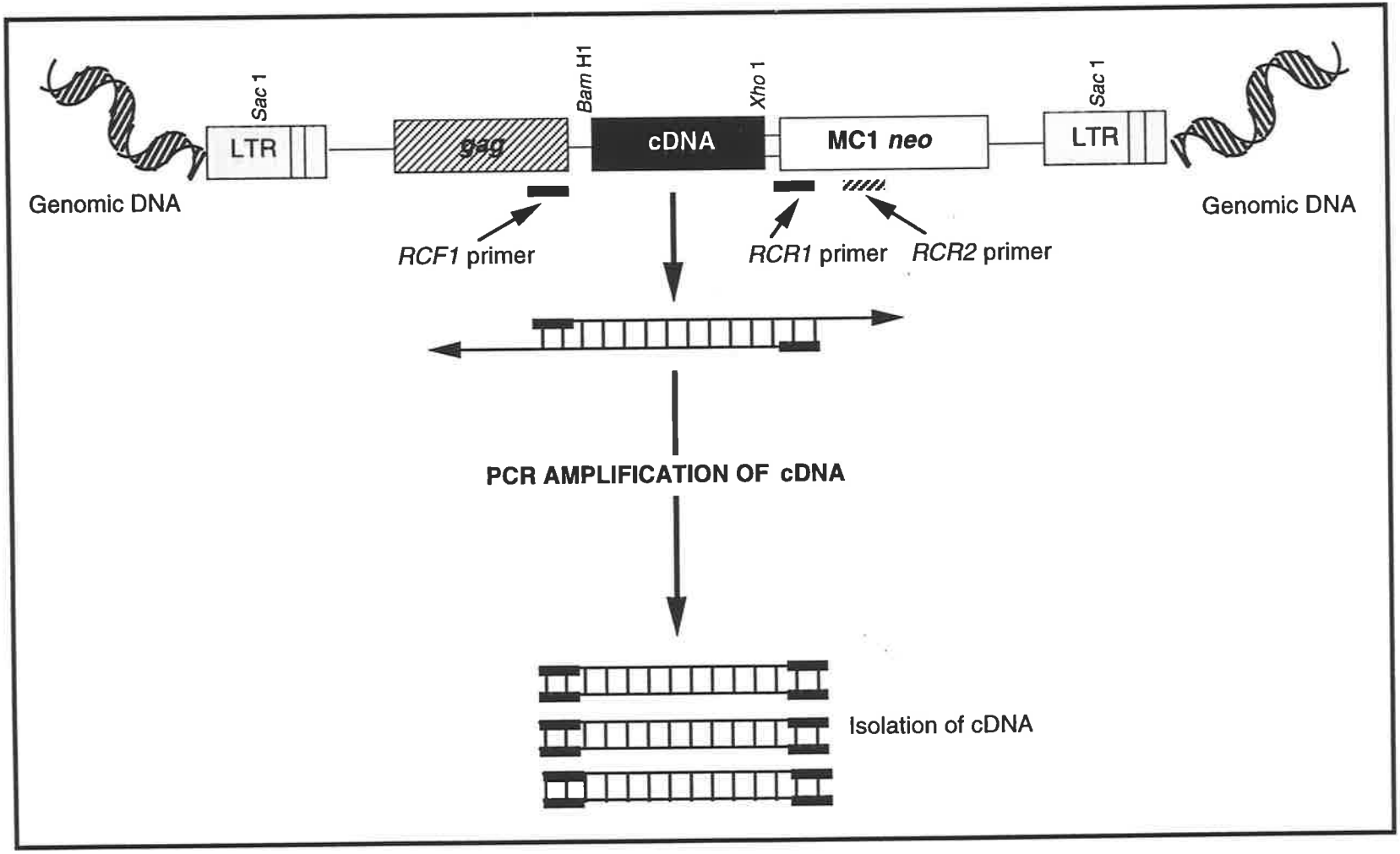

Sac 1
Bam H1
Xho 1
Sac 1
LTR
gag
cDNA
MC1 neo
LTR
Genomic DNA
Genomic DNA
RCF1 primer
RCR1 primer
RCR2 primer
PCR AMPLIFICATION OF cDNA
Isolation of cDNA

Figure 4.2.14. PCR Recovery Of Proviral cDNA Inserts From Genomic DNA Isolated From FDC-P1 Cells Expressing HBMSC Surface Antigens.

Conventional and long range (XL) PCR (refer to *Materials and Methods*, Section 2.22.3. (b)) was used to recover the cDNA inserts from genomic DNA isolated from FDC-P1 cells expressing HBMSC surface antigens. The PCR primers used were complementary to the sequences adjacent to the MCS in the retroviral vector. After amplification the PCR products were separated on a 1.0% agarose gel, visualised by ethidium bromide staining and photographed. Lane 1, 1B4. B12; Lane 2, 5E10; Lane 3, MEM-43; Lane 4, 9B3; Lane 5, 1G2; Lane 6, P4C10; Lane 7, uninfected FDC-P1; Lane 8, water control.

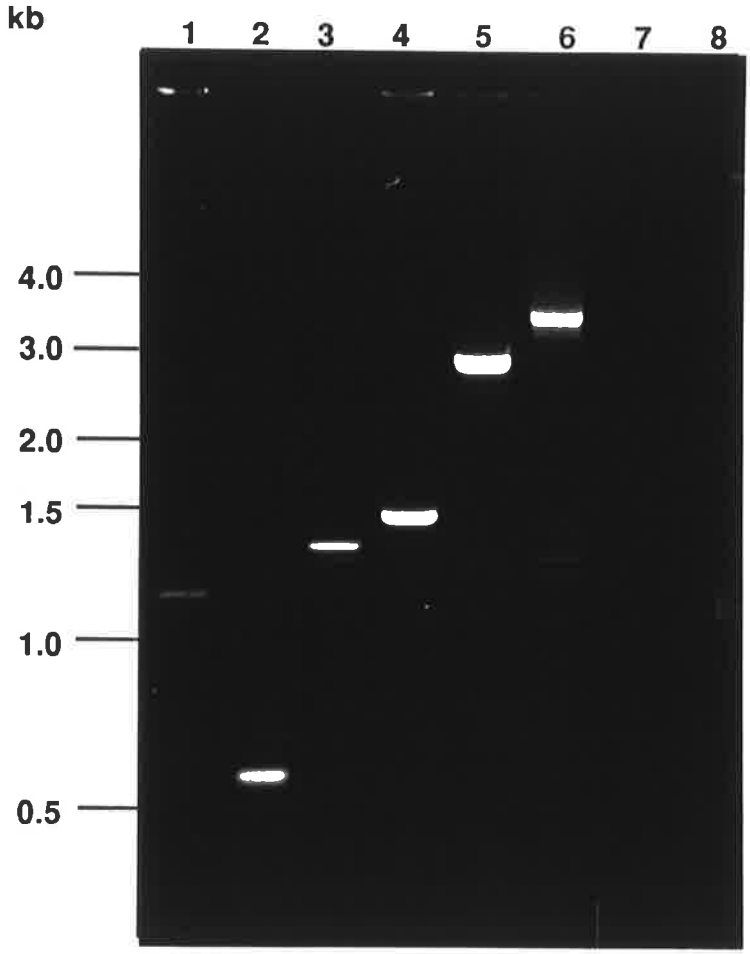

kb
1
2
3
4
5
6
7
8
4.0
3.0
2.0
1.5
1.0
0.5

Figure 4.2.15. FASTA Alignment Analysis Of 1B4.B12 And 9B3 PCR Products.

Following partial sequence analysis, the resultant nucleotide sequence was compared with sequences submitted to the combined Genbank/EMBL database via standard "FASTA alignment analysis".

A. FASTA Alignment Analysis Of 1B4.B12 cDNA

```
gb|S82300|S82300      beta 2-microglobulin C to A point m...  1183  1.1e-93   1
gb|S54761|S54761      beta 2- mu=beta 2-microglobulin [hu...  1169  1.6e-92   1

>gb|S82300|S82300 beta 2-microglobulin C to A point mutation at nucleotide
121 [human, colon cancer cell line HCT, mRNA Mutant, 427 nt], Length = 427
Score = 1183 (326.9 bits), Expect = 1.1e-93, P = 1.1e-93
Identities = 247/260 (95%), Positives = 247/260 (95%), Strand = Plus

Query:   155 GGAATCCGCGGCCGCCAGCATTCGGGCCGAGATGTCTCGCTCCGTGGCCTTAGCTGTGCT 214
             |||  | |  || | ||||||||||||||||||||||||||||||||||||||||||||
Sbjct:     1 GGATCCTGAAGCTGACAGCATTCGGGCCGAGATGTCTCGCTCCGTGGCCTTAGCTGTGCT 60

Query:   215 CGCCCTACTCTCTCTTTCTGGCCTGGAGGCTATCCAGCGTACTCCAAAGATTCAGGTTTA 274
             ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:    61 CGCGCTACTCTCTCTTTCTGGCCTGGAGGCTATCCAGCGTACTCCAAAGATTCAGGTTTA 120

Query:   275 CTCACGTAATCCAGCAGAGAATGGAAAGTCAAATTTCCTGAATTGCTATGTGTCTGGGTT 334
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   121 ATCACGTCATCCAGCAGAGAATGGAAAGTCAAATTTCCTGAATTGCTATGTGTCTGGGTT 180

Query:   335 TAATCCATCCGACATTGAAGTTGGCTTACTGAAGAATGGAGAGAGAATTGAAAAAGTGGA 394
             | ||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct:   181 TCATCCATCCGACATTGAAGTTGACTTACTGAAGAATGGAGAGAGAATTGAAAAAGTGGA 240

Query:   395 GCATTCAGACTTGTCTTTTA 414
             |||||||||||||||||| |
Sbjct:   241 GCATTCAGACTTGTCTTTCA 260

Score = 325 (89.8 bits), Expect = 2.6e-40, Poisson P(2) = 2.6e-40
Identities = 89/119 (74%), Positives = 89/119 (74%), Strand = Plus

Query:   461 CCCCCACTTGTAAAAGAATGGGTATGCCTTCGGTGTGAACCATGTGACTTTTTTAAAGCC 520
             | ||| |    ||||  ||| |||||||| | ||||||||||||||||| | | ||||
Sbjct:   301 CACCCCCACTGAAAAAGATGAGTATGCCTGCCGTGTGAACCATGTGACTTTGTCACAGCC 360

Query:   521 CAGGTTAGTTAAGGGGGGTCGGGGATTTTAAGGGGNATCATGGGGGTTTGAGGTTCCCG 579
             || | |||||||| ||| ||| |   | ||||  | ||||||| ||||||| | | |||
Sbjct:   361 CAAGATAGTTAAGTGGGATCGAGACATGTAAGCAGCATCATGGAGGTTTGAAGATGCCG 419
```

B. FASTA Alignment Analysis Of 9B3 cDNA

```
emb|X13112|HSHLA11K   Human partial mRNA for MHC class I ...   482  4.2e-33   3
dbj|D16842|HUMOKATAK2 Human mRNA for HLA-A11 antigen (A11...   482  8.0e-33   3

>emb|X13112|HSHLA11K Human partial mRNA for MHC class I antigen HLA-A11K, Length =
895. Plus Strand HSPs:
Score = 482 (133.2 bits), Expect = 4.2e-33, Sum P(3) = 4.2e-33
Identities = 115/123 (93%), Positives = 115/123 (93%), Strand = Plus / Plus

Query:   114 GGCCGTCATGGCGCCCCGAACCCTCCTCCTGTTACTCTCGGGGGGCCCTGGCCCTGACCCA 173
             |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct:     3 GGCCGTCATGGCGCCCCGAACCCTCCTCCTGCTACTCTCGGGGGGCCCTGGCCCTGACCCA 62

Query:   174 GACCTGGGCGGGGTCCCACTGCATGAGGTATTTCTACAGCTGGGTGTCCCGGGCCGTCCG 233
             |||||||||||| ||||||| |||||||||||||||| ||  ||||||||| ||| |||
Sbjct:    63 GACCTGGGCGGGCTCCCACTCCATGAGGTATTTCTACACCTCCGTGTCCCGGCCCGGCCG 122

Query:   234 CGG 236
             |||
Sbjct:   123 CGG 125

Score = 90 (24.9 bits), Expect = 4.2e-33, Sum P(3) = 4.2e-33
Identities = 40/45 (89%), Positives = 40/45 (89%), Strand = Plus / Plus

Query:   238 GGAAGCCCCGGTTCATGGGCGTTGGCTACGTGGACGACATGAATT 282
             ||||||||| ||||| |||||||||||||||||||| | | |
Sbjct:   125 GGAAGCCCCGCTTCATCGCCGTGGGCTACGTGGACGACACGCAGT 169
```

Table 4.2.3. Summary Of Results Of Partial-Sequence Analysis Of PCR-Rescued cDNA Clones And Computer Analysis: "Clustered mAbs".

Monoclonal Antibody	Approximate Size Of PCR Product (bp)	cDNA Rescued (bp): Result Of "FASTA" Alignment Analysis
1B4.B12	1200	β2-microglobulin
5E10	560	Thy 1 (CDw90)
MEM-43	1400	CD59
9B3	1600	MHC class 1
1G2	3000	Endoglin (CD105)
P4C10	3800	β1-integrin (CD29)

PCR products were gel purified and subcloned into the pGEM-T vector. Data represents results of partial-sequence analysis (400-700 bp) and DNA homology search (NCBI) for each of the PCR-derived cDNA products.

CD59 mRNA species, differing only in their 3' untranslated region as a result of complex RNA secondary structure around the typical polyadenylation signal, AAUAAA. (Sawada *et al*, 1990; Tone *et al*, 1992).

Application Of The Retroviral Expression Cloning Strategy To Define The CSMs Identified By α-HBMSC mAbs Of "Unknown Cluster" Designation: General Considerations.

Three independent fusions of splenocytes from mice immunised with cultured HBMSC yielded several thousand antibody producing clones. Using the differential screening approach described in *Materials and Methods* (Section 2.7.), 35 clones were identified which exhibited extensive reactivity with surface antigens expressed by cultured HBMSC and limited reactivity with peripheral blood-derived mononuclear cells. The resultant hybridoma clones which met the above criteria were cloned three times to ensure monoclonality of antibody production, and their reactivity with HBMSC re-assessed (please refer to Figure 4.2.16.). The mAb preparations were subsequently isotyped and subjected to a further screening protocol to assess their reactivity with a range of cell lines/cell preparations including HUVECs (Figure 4.2.17.) and from this, six mAbs were selected. Moreover, three additional mAbs of "unknown cluster" were obtained from various sources (detailed in Table 4.2.4.) as part of ongoing collaborative research endeavours. Where possible, the molecular weight of the CSM recognised by the various mAbs were ascertained by immune-precipitation and SDS-PAGE analysis (Table 4.2.4.). To determine the nature of the CSM recognised by these mAbs, the corresponding cDNA was ultimately isolated utilising the above described retroviral expression cloning procedure.

4.2.8. Selection Of An Alternative Target Cell Population For The Functional Screening Of CSM Expression Due To Cross-Reactivity Of mAbs With FDC-P1 Cells.

As detailed in Table 4.2.4., a number of mAbs reactive with HBMSC, including CC9, WM85 and CA12 demonstrated significant cross-reactivity with the murine haemopoietic factor-dependent cell line FDC-P1, necessitating the use of an alternative

Figure 4.2.16. HBMSC-Expression Of CSM identified By The "Unclustered" mAbs Used In This Study.

Cultured HBMSCs were immunolabelled with the "unclustered" mAbs, detailed in Table 4.2.4. or an isotype matched, non binding control (refer to Table 2.10.1.). Data are displayed as single-parameter fluorescence (FITC) histograms of 1 x 10^4 light-scatter gated events, collected as list mode data.

- - - - -: isotype matched, non-binding control
——— : labelling mAb

A. mAb 7H9
B. mAb 11D1.H10
C. mAb 11D3
D. mAb 12F12
E. mAb HCC-1
F. mAb 9E10
G. mAb CC9
H. mAb WM85
I. mAb CA12

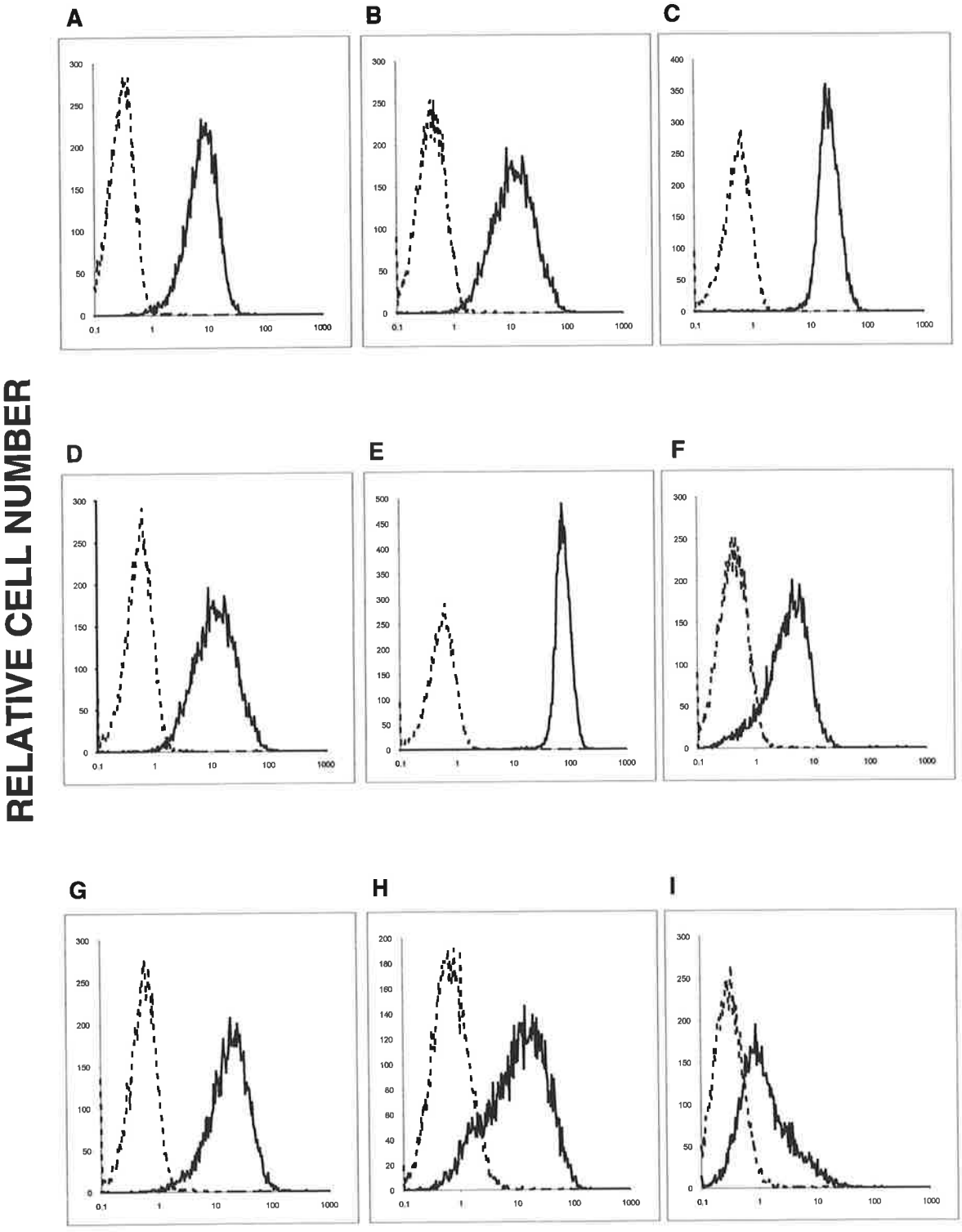

A
B
C
D
E
F
G
H
I
RELATIVE CELL NUMBER
LOG FLUORESCENCE

Figure 4.2.17. ***In Situ*** **Immunofluorescence Staining Of Human Umbilical Vein Endothelial Cells (HUVECs) With "Unclustered" mAbs.**

Primary cultured HUVECs seeded 24 hours prior, were immunolabelled with the "unclustered" mAbs detailed in Table 4.2.4. Bound mAb was detected with FITC-conjugated $F(ab')_2$ sheep anti-mouse Ig, and the samples examined with an Olympus BH2 fluorescence microscope. Cells were viewed at 200 X magnification and photographed.

A. mAb 7H9
B. mAb 11D1.H10
C. mAb 11D3
D. mAb 12F12
E. mAb HCC-1
F. mAb 9E10
G. mAb CC9
H. mAb WM85
I. mAb CA12

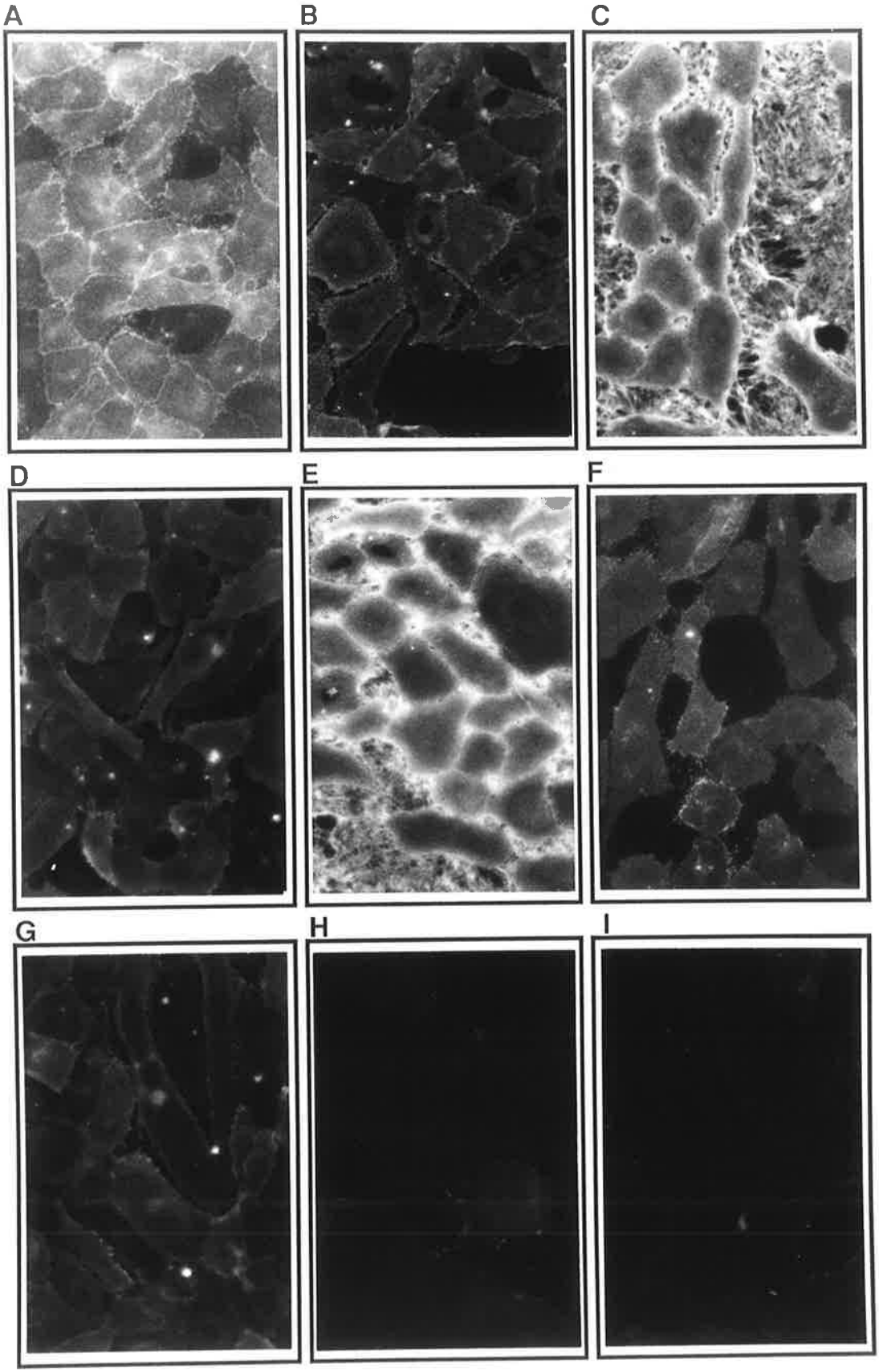

A
B
C
D
E
F
G
H
I

Table 4.2.4. Cellular Distribution And Molecular Weight Of CSMs Identified By "Unclustered" Monoclonal Antibodies.

Data represents a summary of the immunoreactivity profiles obtained when cultured haemopoietic and non-haemopoietic cell lines and cell preparations were examined by single-colour indirect immunofluorescence and flow cytometry.

Immunoreactivity Key : - : negative, ± : limited reactivity, + : low, ++ : medium, +++ : high.

NRP-No result possible, ND-Not done.

(*) Molecular weight deduced following immunoprecipitation of proteins from cultured HBMSC, and reducing-SDS-PAGE.

Table 4.2.4. Cellular Distribution And Molecular Weight Of CSMs Identified By "Unclustered" Monoclonal Antibodies.

Antibody Name	Isotype	MWt Of CSM(*)	Cellular Distribution										Reference / Source
			HBMSC	HUVEC	CHBC	HFF-2	MG63	BMMNC (% + ve) (n=3)	$CD34^+$ Cells	FDC-P1	Baf-3	Swiss-3T3	
7H9	IgG_1	45 - 50	++	+	++	+	+	45.2 ± 4.5	subpopulation	-	ND	ND	A. Zannettino
11D1.H10	IgG_1	75 - 80	++	+	ND	ND	ND	ND	subpopulation	-	ND	ND	Dr. L. Ashman
11D3	IgG_1	19	++	++	+	++	++	85.0 ± 3.2	subpopulation	-	ND	ND	A. Zannettino
12F12	IgG_1	44	++	+	+	+	+	12.5 ± 8.3	subpopulation	-	ND	ND	A. Zannettino
HCC1	IgM	NRP	+++	+++	++	+++	+++	45.9 ± 4.1	subpopulation	-	ND	ND	Ms. B. Swart & Dr. PJ. Simmons
9E10	IgG_3	75 - 80	++	+	+++	+	+	12.3 ± 2.3	subpopulation	-	ND	ND	A. Zannettino
CC9	IgG_{2A}	95 - 110	+++	+	+++	+	-	4.1 ± 2.0	0	+	+	-	S. Gronthos & A. Zannettino
WM85	IgG_1	100	++	±	++	+	-	0	0	+	-	-	Dr. R. Filshie
CA12	IgG_1	ND	+	-	-	-	-	0.9 ± 0.5	0	++	-	-	S. Gronthos & A. Zannettino

HBMSC, cultured human bone marrow stromal cells; HUVEC, human umbilical vein endothelial cells; CHBC, cultured human bone cells; HFF-2, human foreskin fibroblasts; MG63, osteosarcoma; BMMNC, bone marrow mononuclear cells.

target cell. The mAbs in question were thus tested on two substitute cell lines, namely the murine IL-3-dependent cell line, Baf-3 and murine fibroblastic cell line Swiss-3T3, with the resulting reactivity profile summarised in Table 4.2.4. Although the rapid doubling-time manifest by FDC-P1 is not shared by the Baf-3 and Swiss-3T3 cell lines, they do however share a number of the features exhibited by FDC-P1 cells (refer to Section 4.2.2), making them suitable candidate target cells.

The library was introduced into Baf-3 cells by co-cultivation with the four different pools of virus-producing Ψ_2 cells. The efficiency of infection of the Baf-3 cells by the various Ψ_2 pools was 21.5 ± 1.5 % (mean ± standard error of the mean; n=4), as determined by plating in agar in the presence of G418 (Table 4.2.5 (A)). In contrast, virus-containing supernatant was harvested from the various Ψ_2 pools and used to infect the Swiss-3T3 cells. As demonstrated in Table 4.2.5 (B), the average efficiency of infection was determined to be 46 ± 4.6 % (mean ± standard error of the mean; n=4).

To achieve proportionate transfer of all the cDNAs present in the library a total of 6×10^6 Baf-3 and 2×10^6 Swiss-3T3 cells were infected with viral particles produced by the virus-producing Ψ_2 cells. To prevent the loss of cells expressing rare cDNA clones, Baf-3 and Swiss-3T3 cells resistant to G418 (1.19×10^6 and 1.23×10^6, respectively [determined as described above]), were cultured to expand their numbers as described above for the FDC-P1 cells.

4.2.9. Isolation Of CSM-Expressing Target Cells Infected With The HBMSC Library.

To identify the cDNAs corresponding to the CSM identified by the mAbs, 7H9, 11D1.H10, 11D3, 12F12, HCC-1 and 9E10, 1×10^8 G418-resistant FDC-P1 cells were screened as described above. Similarly, to recover cDNAs corresponding to the CSM identified by the mAbs CC9 and WM85, 5×10^7 and 1×10^7 G418-resistant Baf-3 and Swiss-3T3 cells respectively, were incubated sequentially with these "unclustered" mAbs followed by sheep anti-mouse IgG-coated magnetic Dynabeads. The CA12 mAb was included in both the Swiss-3T3 and Baf-3 "screens" in order to maximise the potential for retrieving a cDNA corresponding to this CSM. As described above (Section 4.2.3.) magnetic bead selection and enrichment was carried out until homogeneity of antigen

Table 4.2.5. Number Of Clones Derived By Infection Of (A) Baf-3 and (B) Swiss-3T3 Cells With Virus Derived From Ecotropic Packaging (Producing) Ψ_2 Cells.

To determine the infection frequency following co-cultivation with the virus-producing Ψ_2 cell line, Baf-3 cells were plated in soft agar (as described in *Materials and Methods*) in the presence of G418. Following 10 days culture, colonies were enumerated. Data represent the mean ± SE of three experiments, where 300 cells were plated per dish at day 0.

Alternatively, virus-containing supernatant, harvested from the the various pools were used to infect Swiss-3T3 cells. Following 24 hours infection, cells were harvested by trypsinization (as described in Materials and Methods) and 100 cells plated from each group in the presence of G418. Following 10 to 14 days culture, colonies were enumerated. The data represent the mean ± SE of three experiments.

Table 4.2.5. Number Of Clones Derived By Infection Of (A) Baf-3 and (B) Swiss-3T3 Cells With Virus Derived From Ecotropic Packaging Ψ_2 Cells.

A

	Number Of Baf-3 Colonies Following Co-cultivation With:				
Plating Conditions	**Ψ_2 (control)**	**Ψ_2 (Pool A)**	**Ψ_2 (Pool B)**	**Ψ_2 (Pool C)**	**Ψ_2 (Pool D)**
-G418	173 ± 16	152 ± 6	158 ± 7	132 ± 15	127 ± 4
+G418	0 ± 0	29 ± 2	30 ± 9	31 ± 3	32 ± 2
Infection Frequency (%)	0	19	19	23	25

B

	Number Of Swiss-3T3 Colonies Following Infection With Virus Derived From:				
Plating Conditions	**Ψ_2 (control)**	**Ψ_2 (Pool A)**	**Ψ_2 (Pool B)**	**Ψ_2 (Pool C)**	**Ψ_2 (Pool D)**
-G418	79 ± 1	97 ± 5	86 ± 7	79 ± 7	85 ± 4
+G418	0 ± 0	33 ± 4	42 ± 1	44 ± 3	38 ± 4
Infection Frequency (%)	0	34	49	56	45

expression was achieved. In the case of the "FDC-P1 screen", five rounds of selection were necessary to achieve homogeneity of Dynabead-rosetting cells, whilst the "Baf-3 and Swiss-3T3 screens" required a maximum of 3 rounds of selection. This difference most likely reflects the relatively large number of mAbs included in the screening "FDC-P1 cocktail".

Where applicable, the cocktail of mAbs was divided into its individual components, and the nature of the CSM expressed determined (data not shown). Following this, clonal populations of CSM-expressing cells were isolated by FACS and single cell deposition as described above (Section 4.2.3.). Clones were expanded, surface-antigen expression assessed and genomic DNA isolated in preparation for proviral-integration analysis and PCR-mediated cDNA rescue.

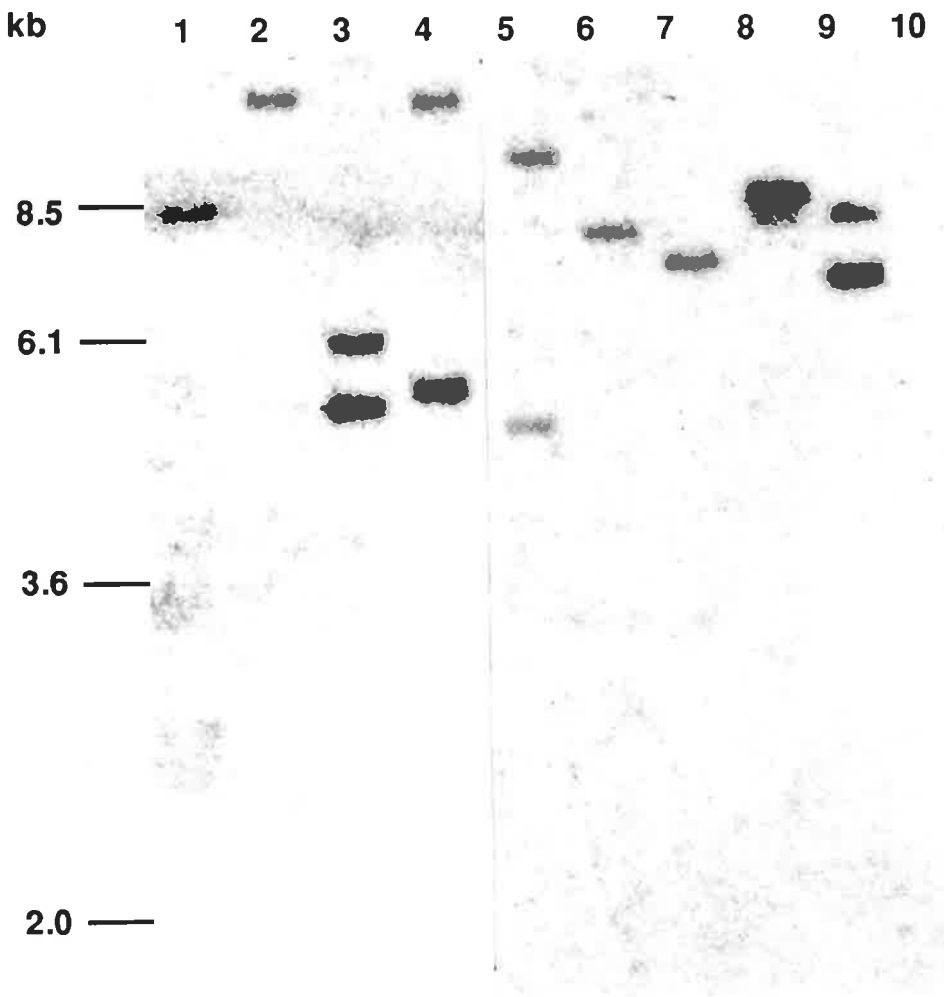
4.2.10. The Analysis Of The Number Of Proviral Integrations And PCR Recovery Of Proviral cDNA Inserts From Genomic DNA.

To ascertain the number of proviral integrations harboured by the various clones, Southern blot analysis was performed as described above. As shown in Figure 4.2.18, the number of proviruses varied between one (7H9-, 12F12-, 9E10-FDC-P1's and CC9-, CA12-Baf-3), two (11D3-,11D1.H10-, HCC-1- FDC-P1's and WM85-Swiss-3T3) integrations, confirming the relatively low frequency of infection for each of the clonal populations as observed above.

As illustrated in Figure 4.2.13., primers complementary to sequences adjacent to the multiple cloning site (MCS) in the retroviral vector were used to amplify the cDNA insert responsible for conferring the expression of a particular antigen. A consistent lack of amplification of cDNA inserts from several of the genomic DNA preparations, prompted the investigation of sequences adjacent to, and including the *RCR1* binding site. As stated in the *Materials and Methods*, the oligonucleotide primer *RCR1* binds to the f9 polyomer enhancer region of the MC1.*neo* cassette adjacent to the MCS (and results in the addition of a 47 bp sequence, 3' to the PCR product, refer to Figure 4.2.13.). Sequence analysis, revealed that this region sustained a high frequency of mutation, with resultant duplications/deletions of short nucleotide sequences (data not shown). This necessitated

Figure 4.2.18. Southern Blot Analysis Reveals The Number Of Proviral Integrations In Each Infected Clone.

Southern blot analysis of genomic DNA isolated from HBMSC surface antigen-expressing clones was performed essentially as described by Rayner and Gonda, 1994. Following restriction digestion with *Bam*H1, genomic DNA was separated on a 0.8% agarose gel, transferred to nitrocellulose, and probed with a ^{32}P radio-labelled Neo^R cassette. The positions of the molecular weight markers (SPP-1 DNA digested with *Eco*R1), are shown on the right (in kilobase pairs). Refer to Table 4.2.4., as nomenclature used in this figure pertains to the mAb used to select the FDC-P1 clonal population. Lane 1, 7H9; Lane 2, 12F12; Lane 3, 11D3; Lane 4, 11D1.H10; Lane 5, HCC-1; Lane 6, 9E10; Lane 7, CC9; Lane 8, CA12; Lane 9, WM85; Lane 10, uninfected FDC-P1 cells. Number of proviral integrations vary from 1 to 2, confirming the relatively low frequency of infection for each of the FDC-P1 clonal populations.


kb
1
2
3
4
5
6
7
8
9
10
8.5
6.1
3.6
2.0

the design of an alternate primer (*RCR2*, please refer to *Materials and Methods*, Section 2.21.3. (ii)(c)) which bound to a conserved region of the neomycin-resistance gene, 3′ to the *RCR1* primer (results in the addition of 372 bp sequence, 3′ to the PCR product, refer to Figure 4.2.13.). PCR amplification using RCF1 and RCR2 resulted in abundant amplified product, as demonstrated in Figure 4.2.19. (A) and (B).

4.2.11. Partial-Sequence Analysis Of cDNA Inserts And The Requirement For Re-expression Analysis.

Gel-purified PCR products were subsequently subcloned into an appropriate sequencing vector (pGEM-T or pBluescript, Appendix A, Restriction Maps # 6 and 1, respectively) or sequenced directly with the use of retroviral sequence-specific oligonucleotide primers (1, 1B, 2, RCF1, RCR1 and RCR2, Section 2.21.3.). The resulting partial-nucleotide sequences were compared with the entries submitted to the Genbank/EMBL databases via standard FASTA alignment analysis as described above. Selected examples of these results are illustrated in Figure 4.2.20. and summarised in Table 4.2.6.

As the number of proviral integrants varied between different clones (Section 4.2.10. and Figure 4.2.18.), the PCR products obtained (Figure 4.2.19. (A) & (B)) were examined for their ability to confer the pertinent CSM expression as demonstrated by appropriate mAb binding. This was ratified in all cases by subcloning the recovered cDNA into the pRUF*neo* vector and introducing these sequences into the either FDC-P1, Baf-3 and Swiss-3T3 cells (where appropriate) by retroviral transduction (refer to *Materials and Methods*, Section 2.26.). The resultant G418-resistant cell populations were tested for their ability to bind the mAb in question by indirect immunofluorescence and flow cytometry. As demonstrated in Figure 4.2.21, the predicted mAb-binding phenotype was observed in each situation.

Figure 4.2.19. (A) And (B). PCR Recovery Of Proviral cDNA Inserts From Genomic DNA Isolated From Infected Cells Expressing HBMSC Surface Antigens Identified With The "Unclustered" mAbs.

Long range (XL) PCR (refer to *Materials and Methods*, Section 2.22.3. (b)) was used to recover the cDNA inserts from genomic DNA isolated from infected cells expressing HBMSC surface antigens detected with the "unclustered" mAbs. The PCR primers used were complementary to the sequences adjacent to the MCS in the retroviral vector pRUF.*neo* (refer to Figure 4.2.13). Amplification was performed as detailed in the *Materials and Methods*, after which the PCR products were separated on a 1.0% agarose gel, visualised by ethidium bromide staining and photographed.

Gel (A)

Lane 1, 11D1.H10; Lane 2, CA12; Lane 3, WM85; Lane 4, HCC-1; Lane 5, CC9.

Gel (B)

Lane 1, 12F12; Lane 2, 11D3; Lane 3, 7H9; Lane 4, 9E10.

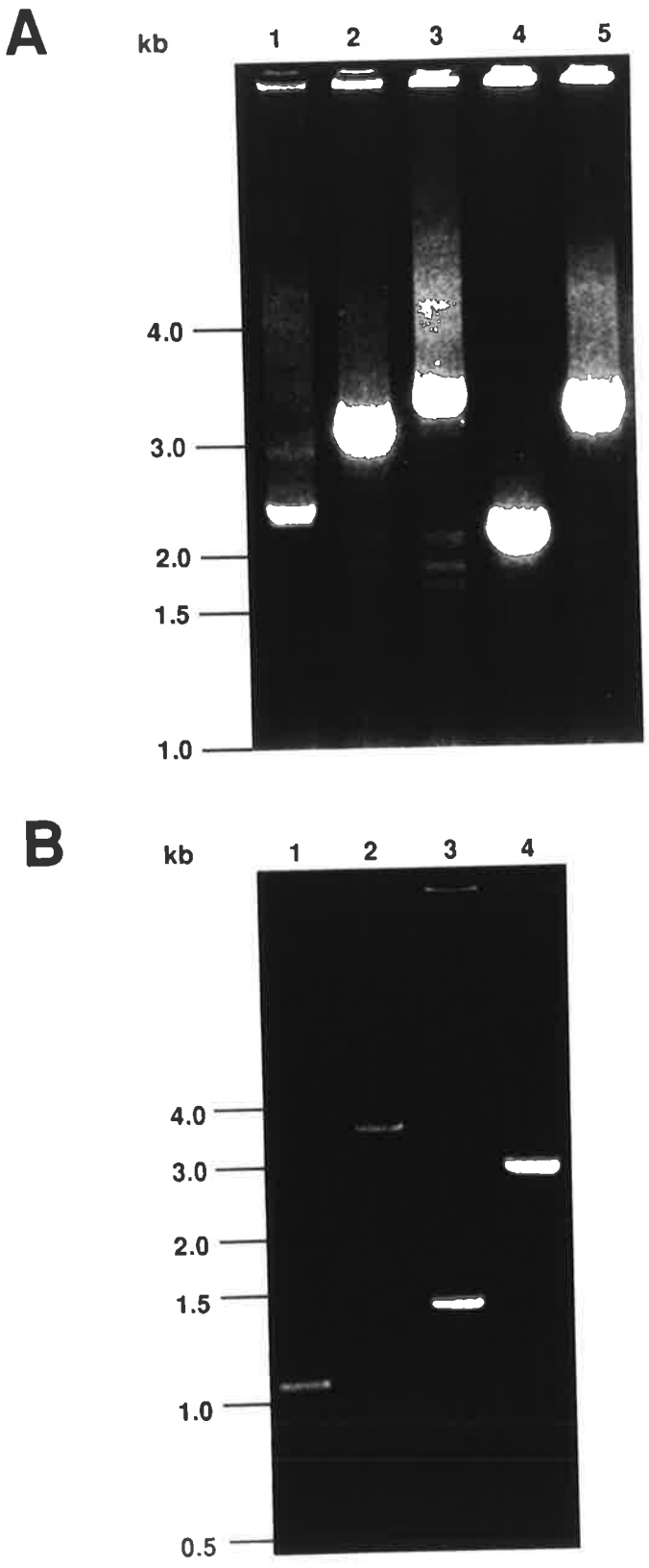

A
kb
1
2
3
4
5
4.0
3.0
2.0
1.5
1.0
B
kb
1
2
3
4
4.0
3.0
2.0
1.5
1.0
0.5

Figure 4.2.20. FASTA Alignment Analysis Of 11D3, WM85, 12F12, CA12 Of PCR Products.

Following partial sequence analysis, the resultant nucleotide sequence was compared with sequences submitted to the combined Genbank/EMBL database via standard "FASTA alignment analysis".

A. FASTA Alignment Analysis Of 11D3 cDNA

```
emb|X59408|HSMCP08   H.sapiens, gene for Membrane cofacto...   621  3.3e-85   4
emb|X59405|HSMCP05   H.sapiens, gene for Membrane cofacto...   621  3.7e-85   4

>emb|X59408|HSMCP08 H.sapiens, gene for Membrane cofactor proteinLength = 3225
Plus Strand HSPs:
Score = 621 (171.6 bits), Expect = 3.3e-85, Sum P(4) = 3.3e-85
Identities = 135/150 (90%), Positives = 135/150 (90%), Strand = Plus / Plus

Query:    83 TATTTTTCCTTTGTAAAGAAAGTGGCTTGAAATCTTTTTTGTTCAAAGATTGATGCCAAC 142
             ||| ||| ||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct:  1619 TATATTTTCTTTGTAAAGAAAGTGGCTTGAAATCTTTTTTGTTCAAAGATTAATGCCAAC 1678

Query:   143 TCTTAGGNTTATTCTTTCACCACCTNTGGAATGAATTTAATATATCGTCCATNGTAAAAG 202
             ||||| | |||||||||||||| || | ||||| |||| ||||||||| ||| ||||||
Sbjct:  1679 TCTTAAGATTATTCTTTCACCAACTATAGAATGTATTTTATATATCGTTCATTGTAAAAA 1738

Query:   203 GCCCTTAAAATTATGTGTATACTACTTTTG 232
             |||||||||| |||||||||||||||||| |
Sbjct:  1739 GCCCTTAAAAATATGTGTATACTACTTTGG 1768

Score = 411 (113.6 bits), Expect = 3.3e-85, Sum P(4) = 3.3e-85
Identities = 109/111 (98%), Positives = 109/111 (98%), Strand = Plus / Plus

Query:   313 TGGGAAAATTCTCTAAAAGTTAATAGGGTAAATTCTCTATTTTTTGTAATGTGTTCGGGT 372
             | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1843 TTGGAAAATTCTCTAAAAGTTAATAGGGTAAATTCTCTATTTTTTGTAATGTGTTCGGTG 1902

Query:   373 ATTTCAGAAAGCTAGAAAGTGTATGTGTGGCATTTGTTTTCACTTTTTAAA 423
             |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1903 ATTTCAGAAAGCTAGAAAGTGTATGTGTGGCATTTGTTTTCACTTTTTAAA 1953
```

B. FASTA Alignment Analysis Of WM85 cDNA

```
gb|M29277|HUMMUC18A/Human isolate JuSo MUC18 glycoprotei. 1341    5.2e-103 2
gb|M28882|HUMMUC18B/Human MUC18 glycoprotein mRNA, compl. 1341    6.2e-103 2

gb|M29277|HUMMUC18A Human isolate JuSo MUC18 glycoprotein mRNA (3' variant),
complete cds. Length = 2943. Plus Strand HSPs:
Score = 1341 (370.5 bits), Expect = 5.2e-103, Sum P(2) = 5.2e-103
Identities = 291/300 (97%), Positives = 291/300 (97%), Strand = Plus / Plus

Query:    59 GGGAAGCATGGGGATTCCCAGGCTGGTCTGCGCCTTCTTGGTCGCCGCCTGTTGGTGCTG 118
             |||||||||||||| |||||||||||||||||||||||| |||||||||| || |||||
Sbjct:     1 GGGAAGCATGGGGCTTCCCAGGCTGGTCTGCGCCTTCTTGCTCGCCGCCTGCTGCTGCTG 60

Query:   119 TCCTCGCGTCGCGGGTGTGCCCGGAGAGGCTGAGCAGCCTGAGCCTGAGTTGGTGGAGGT 178
             |||||||||||||||||||||||||||||||||||||||||| ||||||| ||||||||||
Sbjct:    61 TCCTCGCGTCGCGGGTGTGCCCGGAGAGGCTGAGCAGCCTGCGCCTGAGCTGGTGGAGGT 120

Query:   179 GGAAGTGGGCAGTACAGACCTTCTGAAGTGCGGCCTCTCCCAGTCCCAAGGAAACCTCAG 238
             |||||||||||| |||| |||||||||||||||||||||||||||||| ||||||||
Sbjct:   121 GGAAGTGGGCAGCACAGCCCTTCTGAAGTGCGGCCTCTCCCAGTCCCAAGGCAACCTCAG 180

Query:   239 CCATGTCGACTGGTTTTCTGGCCACAAGGAGAAGCGGACGCTCATCTTCCGTGTGCGCCA 298
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   181 CCATGTCGACTGGTTTTCTGTCCACAAGGAGAAGCGGACGCTCATCTTCCGTGTGCGCCA 240

Query:   299 GGGCCAGGGCCAGAGCGAACCTGGGGAGTACGAGCAGCGGCTCAGCCTCCAGGACAGAGG 358
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   241 GGGCCAGGGCCAGAGCGAACCTGGGGAGTACGAGCAGCGGCTCAGCCTCCAGGACAGAGG 300
```

C. FASTA Alignment Analysis Of 12F12 cDNA

```
gb|M58485|HUMCD63     Human lysosomal membrane glycoprotei...  1243  7.8e-95   1
gb|X07982|HSME491     Human mRNA for melanoma-associated a...  1165  4.0e-88   1

>gb|X07982|HSME491 Human mRNA for melanoma-associated antigen ME491. Length = 826
Plus Strand HSPs:
Score = 815 (225.2 bits), Expect = 2.8e-58, P = 2.8e-58
Identities = 167/172 (97%), Positives = 167/172 (97%), Strand = Plus

Query:   162 GCCGNGGNAAGCCCAGGCCCACCAGCCATGGCGGTGGAAGGAGGAATGAAATGTGTGAAG 221
             |||  || ||||||||||||  ||||||||||||||||||||||||||||||||||||||
Sbjct:     1 GCCTTGGGAAGCCCAGGCCCGGCAGCCATGGCGGTGGAAGGAGGAATGAAATGTGTGAAG 60

Query:   222 TTCTTGCTCTACGTCCTCCTGCTGGCCTTTTGCGCCTGTGCAGTGGGACTGATTGCCGTG 281
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:    61 TTCTTGCTCTACGTCCTCCTGCTGGCCTTTTGCGCCTGTGCAGTGGGACTGATTGCCGTG 120

Query:   282 GGTGTCGGGGCACAGCTTGTCCTGAGTCAGACCATAATCCAGGGGGCTACCC 333
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   121 GGTGTCGGGGCACAGCTTGTCCTGAGTCAGACCATAATCCAGGGGGCTACCC 172


>gb|M58485|HUMCD63 Human lysosomal membrane glycoprotein CD63 mRNA.
Length = 875
Minus Strand HSPs:
Score = 1243 (343.5 bits), Expect = 7.8e-95, P = 7.8e-95
Identities = 255/263 (96%), Positives = 255/263 (96%), Strand = Minus

Query:   421 CCATAAGGAGGNCTGTGTGNANAAGATTGGGGGCTGGCTGAGGAAAAATGTGCTGGTGGT 362
             |||||||||| ||||||| | ||||||||||||||||||||||||||||||||||||||
Sbjct:   613 CCATAAGGAGGGCTGTGTGGAGAAGATTGGGGGCTGGCTGAGGAAAAATGTGCTGGTGGT 672

Query:   361 AGCTGCAGCAGCCCTTGGAATTGCTTTTGTCGAGGTTTTGGGAATTGTCTTTGCCTGCTG 302
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   673 AGCTGCAGCAGCCCTTGGAATTGCTTTTGTCGAGGTTTTGGGAATTGTCTTTGCCTGCTG 732

Query:   301 CCTCGTGAAGAGTATCAGAAGTGGCTACGAGGTGATGTAGGGGTCTGGTCTCCTCAGCCT 242
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   733 CCTCGTGAAGAGTATCAGAAGTGGCTACGAGGTGATGTAGGGGTCTGGTCTCCTCAGCCT 792

Query:   241 CCTCATCTGGGGGAGTGGAATAGTATCCNCCAGGTTTTTCAATTAAACGGATTATTTTTT 182
             |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct:   793 CCTCATCTGGGGGAGTGGAATAGTATCCTCCAGGTTTTTCAATTAAACGGATTATTTTTT 852

Query:   181 CAGAAAAAAAAAAAAAAAAAA 159
             ||||   ||||||||||||||
Sbjct:   853 CAGACCGAAAAAAAAAAAAAAA 875
```

D. FASTA Alignment Analysis Of CA12 cDNA

```
gb|H37944|H37944        yp47b08.r1 Homo sapiens cDNA clone 1... 936     3.e-144 5
emb|X14174|HSAPHOL      Human mRNA for liver-type alkaline p... 1134    1.8e-89 3

gb|H37944|H37944 yp47b08.r1 Homo sapiens cDNA clone 190551 5' similar to
gb:X14174 ALKALINE PHOSPHATASE, TISSUE-NONSPECIFIC ISOZYME, PRECURSOR (HUMAN);.
Length = 484, Plus Strand HSPs:
Score = 936 (258.6 bits), Expect = 3.0e-144, Sum P(5) = 3.0e-144
Identities = 188/189 (99%), Positives = 188/189 (99%), Strand = Plus / Plus

Query:   123 CCGTGCTCCCACGCGCTTGTGCCTGGACGGACCCTCGCCAGTGCTCTGCGCAGGATTGGA 182
             | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:    26 CTGTGCTCCCACGCGCTTGTGCCTGGACGGACCCTCGCCAGTGCTCTGCGCAGGATTGGA 85

Query:   183 ACATCAGTTAACATCTGACCACTGCCAGCCCACCCCCTCCCACCCACGTCGATTGCATCT 242
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:    86 ACATCAGTTAACATCTGACCACTGCCAGCCCACCCCCTCCCACCCACGTCGATTGCATCT 145

Query:   243 CTGGGCTCCAGGGATAAAGCAGGTCTTGGGGTGCACCATGATTTCACCATTCTTAGTACT 302
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   146 CTGGGCTCCAGGGATAAAGCAGGTCTTGGGGTGCACCATGATTTCACCATTCTTAGTACT 205

Query:   303 GGCCATTGG 311
             |||||||||
Sbjct:   206 GGCCATTGG 214

emb|X14174|HSAPHOL Human mRNA for liver-type alkaline phosphatase (EC 3.1.3.1)Length
= 2339Plus Strand HSPs:
Score = 1134 (313.3 bits), Expect = 1.8e-89, Sum P(3) = 1.8e-89
Identities = 228/230 (99%), Positives = 228/230 (99%), Strand = Plus / Plus

Query:   174 AGGATTGGAACATCAGTTAACATCTGACCACTGCCAGCCCACCCCCTCCCACCCACGTCG 233
             |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct:   295 AGGATTGGAACATCAGTTAACATCTGACCACTGCCAGCGCACCCCCTCCCACCCACGTCG 354

Query:   234 ATTGCATCTCTGGGCTCCAGGGATAAAGCAGGTCTTGGGGTGCACCATGATTTCACCATT 293
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   355 ATTGCATCTCTGGGCTCCAGGGATAAAGCAGGTCTTGGGGTGCACCATGATTTCACCATT 414

Query:   294 CTTAGTACTGGCCATTGGCACCTGCCTTACTAACTCCTTAGTGCCAGAGAAAGAGAAAGA 353
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   415 CTTAGTACTGGCCATTGGCACCTGCCTTACTAACTCCTTAGTGCCAGAGAAAGAGAAAGA 474

Query:   354 CCCCAAGTACTGGCGAGACCAAGCGCAAGAGACACTGAAATATGCCCTNG 403
             |||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct:   475 CCCCAAGTACTGGCGAGACCAAGCGCAAGAGACACTGAAATATGCCCTGG 524
```

Table 4.2.6. Summary Of Results Of Partial Sequence Analysis Of PCR-Rescued cDNA Clones And Computer Analysis.

Monoclonal Antibody Name	Approximate Size (bp) Of PCR Product	Results Of "FASTA Alignment Analysis"
7H9	1500	Hyaluronate Receptor, CD44
11D1.H10	2300	4F2 Heavy Chain (CD98)
11D3	3600	Membrane Co-factor Protein (MCP, CD46Q)
12F12	1100	CD63
HCC1*	2000	CD59
9E10#	3000	24 kD Multi-Glycosylated Core protein (MGC-24)
CC9	3150	MUC-18
WM85	3200	MUC-18
CA12	3000	Alkaline Phosphatase

PCR products were gel purified and subcloned into the pGEM-T vector. Data represents the results of partial-sequence analysis (400-700 bp) and DNA homology search (NCBI) for each of the PCR-derived cDNA products.

* : Please refer to Chapter 5.0 for detailed analysis.

: Please refer to Chapter 6.0 for detailed analysis.

Figure 4.2.21. Re-Cloning And Expression Analysis

PCR-derived cDNA products were either cloned into the *Hpa*I (blunt-ended cloning), or the *Bam*HI/*Xho*I (forced-cloning) sites of the retroviral vector pRUF.*neo* (please refer to Figure 4.2.3.). The resultant recombinant plasmids were used to transfect Ψ_2 packaging cells. The virus thus produced was subsequently used to infect the appropriate cell line (as detailed below).

Target cells were selected with G418, and subsequently assessed for CSM expression by indirect immunofluorescence and flow cytometry. Data are displayed as single-parameter fluorescence (FITC) histograms of 1 x 10^4 light-scatter gated events, collected as list mode data.

A. FDC-P1 cells infected with 7H9/pRUF.*neo*-virus and stained with
- - - - - - : isotype matched, non-binding control; or
———: mAb 7H9

B. FDC-P1 cells infected with 11D1.H10/pRUF.*neo*-virus and stained with
- - - - - - : isotype matched, non-binding control; or
———: mAb 11D1.H10

C. FDC-P1 cells infected with 11D3/pRUF.*neo*-virus and stained with
- - - - - - : isotype matched, non-binding control; or
———: mAb 11D3

D. FDC-P1 cells infected with 12F12/pRUF.*neo*-virus and stained with
- - - - - - : isotype matched, non-binding control; or
——— : mAb 12F12

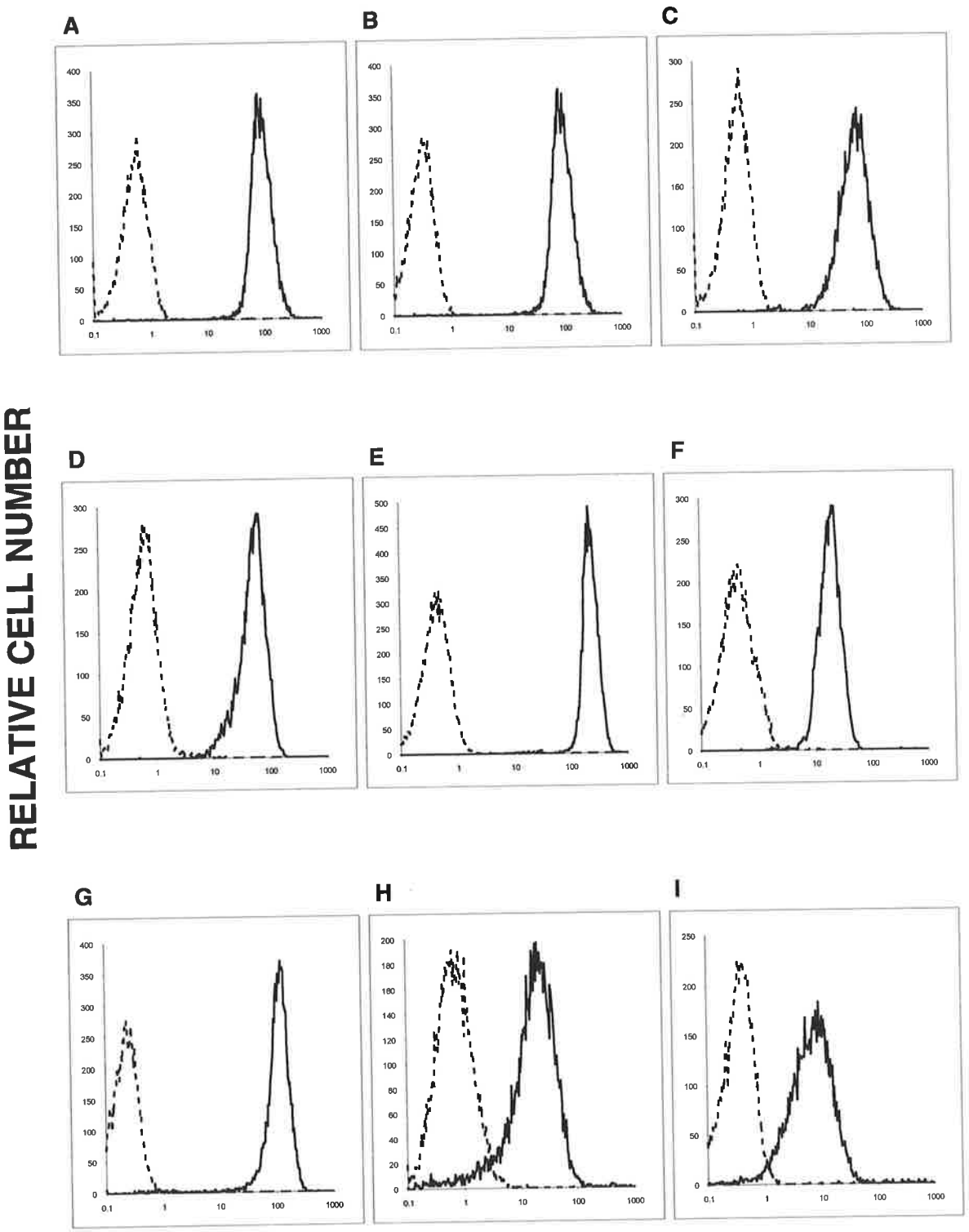
E. FDC-P1 cells infected with HCC-1/pRUF.*neo*-virus and stained with
- - - - - - : isotype matched, non-binding control; or
———: mAb HCC-1

F. FDC-P1 cells infected with 9E10/pRUF.*neo*-virus and stained with
- - - - - - : isotype matched, non-binding control; or
———: mAb 9E10

G. Baf-3 cells infected with CC9/pRUF.*neo*-virus and stained with
- - - - - - : isotype matched, non-binding control; or
———: mAb CC9

H. Swiss-3T3 cells infected with WM85/pRUF.*neo*-virus and stained with
- - - - - - : isotype matched, non-binding control; or
———: mAb WM85

I. Baf-3 cells infected with CA12/pRUF.*neo*-virus and stained with
- - - - - - : isotype matched, non-binding control; or
———: mAb CA12


A
B
C
D
E
F
G
H
I
RELATIVE CELL NUMBER
LOG FLUORESCENCE

4.3. DISCUSSION

4.3.1. Retroviral Expression Cloning Of cDNA Encoding Cell Surface Molecules

In recent years a broad range of cell surface molecules (CSM) have been identified with recognised roles in regulating haemopoiesis and in augmenting the function of the immune system. This increase in our understanding has been facilitated by the advent of a number of cloning strategies that have permitted the molecular characterisation of many novel integral membrane proteins (Kavathas *et al*, 1984; Maddon *et al*, 1985; Young and Davis, 1983; Seed and Aruffo, 1987; Seed, 1987; Aruffo and Seed, 1987; Gearing *et al*, 1989; Yamasaki *et al*, 1988; Osborn *et al*, 1989; Elices *et al*, 1990).

To date, the most successful approach used to clone cDNAs encoding CSM, has been that of Aruffo and Seed (Seed and Aruffo, 1987; Seed, 1987; Aruffo and Seed, 1987). This expression cloning strategy involves the directional cloning of a cDNA pool into a vector such as pCDM8 (Seed, 1987), which is able to replicate in bacteria and to confer expression of cloned genes in a limited number of eukaryotic cell lines, including Chinese Hamster Ovary (CHO) cells and monkey-derived COS cells. Following transient transfection, mammalian cells expressing the desired antigen(s), are selected using antibody capture and panning (Wysoki and Sato, 1978). Episomal plasmid DNA rescued (Hirt, 1967) from the specifically selected cells is amplified in bacteria and the procedure is repeated until clone(s) of cells that render expression of the desired antigen(s) are isolated (Seed and Aruffo, 1987; Seed, 1987; Aruffo and Seed, 1987). Since its development, a large number of CSM have been cloned using mAb (or variations thereof) to screen transiently expressed cDNA libraries (reviewed in Simmons, DL., 1992).

Recently, a number of reports describing the use of retroviral vectors for the construction of cDNA libraries have appeared in the literature. Rayner and Gonda (1994) recently described an efficient procedure for generating stable expression libraries by cDNA cloning in a retroviral vector, pRUF*neo*. They demonstrated the feasibility of this approach by constructing a retroviral library from the activated murine T-cell line, LB3 and then isolating cDNAs for IL-3 and GM-CSF selected on the basis of their ability to confer autonomous growth on the factor-dependent, murine haemopoietic cell line, FDC-

P1. Similarly, Whitehead and collegues (Whitehead *et al*, 1995) illustrated the use of retroviral transfer of cDNA libraries into the murine fibroblastoid cell line NIH-3T3 to isolate cDNAs capable of inducing oncogenic transformation.

In contrast to transfection, the retroviruses can efficiently infect and hence transfer a complex cDNA library to a wide variety of cell types, including cells of haemopoietic origin. This flexibility with respect the target cell is one of the major advantages of this system, particularly when the detection reagent (such as a mAb) demonstrates reactivity with the target cell type. Furthermore, following transduction with a retrovirus, the reverse-transcribed viral DNA stably integrates into the host cell genome in an anticipated configuration, facilitating the recovery of cDNA sequences borne by the provirus (see below). The creation of stably transduced cells also facilitates phenotypic selection of target cells beyond the practical limitations of the current transient expression library systems and conveniently provides cells for further functional analyses.

The results presented in this chapter confirm the utility of retroviral cDNA libraries and extend their use to cloning genes for CSMs. Given that the overall aim of this project was to identify and characterise cell surface molecules (CSM) which are involved in the interaction between the BM stromal cells and developing haemopoietic cells, a retroviral cDNA library from HBMSC was prepared. Using the retroviral vector pRUF.*neo*, a large, stable cDNA library was generated in the ecotropic-packaging cell line, Ψ_2, providing a renewable source of retrovirus capable of infecting target cell types of rodent origin. In the initial studies outlined, the murine haemopoietic cell line FDC-P1 was chosen as the target for selection of CSM expression. As demonstrated above however, alternate murine cell lines (Baf-3 and Swiss-3T3) were required when demonstrable cross-reactivity was observed with FDC-P1 cells and several of the mAbs of "undefined specificity".

In the initial experiments, the representation and integrity of the library was tested by performing an initial screen using mAbs of "known cluster" designation. These mAbs were chosen because of their (i) reactivity with HBMSC, (ii) their recognition of antigens with widely varying molecular weights and cDNA sizes and (iii) the level of expression of the antigens recognised by these mAbs varied considerably. Because initial experiments using the established techniques of panning (Wysoki and Sato, 1978) and FACS proved

ineffective in isolating rare antigen-expressing cells, this necessitated the development of a sensitive, efficient and reproducible technique for selecting such cells from the HBMSC library in FDC-P1 cells. A screening procedure using immunomagnetic Dynabeads was thus developed. In reconstruction experiments, this technique was shown to be both highly sensitive and effective in isolating cells which were present at a frequency of 1 cell in 10^6-10^7 in the original pool FDC-P1 cells (data not shown). Furthermore, this technique was found to be rapid and efficient with an average of only 3 rounds of selection required to obtain a homogeneous pool of antigen-expressing cells. Due to the rapid doubling time of FDC-P1 cells, the Dynabead enrichment procedure was routinely performed every 4 to 5 days, which in practice enabled the isolation of a given cDNA clone within a 3 week period.

The molecular mass of the proteins expressed by the various FDC-P1 clones were determined and in all cases were found to be consistent with the published data (Figure 4.2.11. (A) & (B) and Table 4.2.2.). Significantly, heterodimeric molecules such as β1-integrin, MHC class 1 and β2 microglobulin were adequately expressed in FDC-P1 cells in the absence of their human counterparts. In the case of MHC class 1 and β2 microglobulin it is evident from the immunoprecipitation data (Figure 4.2.11. (A)) that each of these polypeptides (recognised by mAbs 9B3 and 1B4.B12) is able to pair with its murine homologue to form hybrid heterodimeric molecules. In contrast, the human β1-integrin polypeptide, appears to be expressed in the absence of its murine α subunit. (Figure 4.2.11. (B), lane 2). This is contrary to the published findings of Solowska *et al* (1989), who demonstrated that expression of the avian β1 integrin subunit in rodent cells resulted in the expression of functional hybrid heterodimers between the exogenous β1 subunit and endogenous murine α3 and α5 subunits. The functional status of the β1 integrin expressed by the FDC-P1 cells is currently under investigation (Dr. J-P. Levesque, personal communication).

Following clonal isolation of target cells expressing a desired surface antigen (Figure 4.2.10.), the cDNA inserts were rescued from the provirus by PCR amplification. As many of the genes encoding cell surface antigens are large, long range PCR conditions were employed. Interestingly, the average size of the cDNA derived from the infected

clones selected with the "unclustered" mAbs (ie. of unknown cluster designation) was approximately 3,000 bp. This would therefore imply that XL-PCR should be used when target cells are selected using unclustered mAbs to ensure amplification of a cDNA insert of indeterminent length. In most cases a single cDNA product was obtained following PCR amplification from DNA derived from cells harbouring more than one provirus. Recent work would suggest that this is due in part, to the additional inserts being extremely large (6-8 kb) and unable to be amplified under the PCR conditions described (data not shown). Although no evidence exists to suggest rearrangement of the transduced cDNAs (ie. amplified species were derived for all clones tested), there was some indication for rearrangement of sequences in the f9 polyoma enhancer (region to which the *RCR1* oligonucleotide primer bound), necessitating the use of an alternate primer, *RCR2*. Additionally, in circumstances where the infected cell had more than one proviral integration, confirmation that the PCR product was able to confer the appropriate phenotype was essential. This was achieved by re-transfection studies, where the cDNAs were subcloned into the retroviral expression vectors (pRUF.*neo*, pRUF(*Not*I)*neo*) and shown to be capable of transferring antigen expression by immunofluorescence and flow cytometry.

As summarised in Table 4.2.6., following partial sequence analysis and a database alignment analysis, it was apparent that in all cases, the "unclustered" mAbs identified previously identified CSMs. Predicated on cellular reactivity (refer to Table 4.2.4.), the results obtained in almost all cases were somewhat surprising. This observation is well exemplified in the case of mAbs HCC-1 and 9E10 which were found to identify CD59 and the polymorphic epithelial MGC-24. Further studies relating to these mAbs constitute Chapter 5 and 6 of this thesis, respectively.

Monoclonal antibody, 7H9 was not predicted to be an anti-CD44 mAb based on the pattern of reactivity with BMMNC. CD44 represents the well characterised, principle cell surface receptor for hyaluronate (Aruffo *et al*, 1990) and is expressed by cells of lymphoid and myeloid lineages and various cells of mesenchymal origin (reviewed by Stamenkovic *et al*, 1989; 1991). However, since CD44 is highly polymorphic with respect to its exon usage (Jackson *et al*, 1992; Günthert *et al*, 1991), mAb 7H9 may identify the

product of an alternatively spliced exon (or associated carbohydrate decoration) used by HBMSCs. Alternatively, as CD44 exists in an inactive conformation on most cells, mAb 7H9 may recognise an activated form of the molecule. While beyond the scope of this thesis, complete sequence analysis of this cDNA clone is required to address these questions.

To the author's knowledge, this represents the first report demonstrating CD46Q and CD63 expression by HBMSC. Membrane cofactor protein (MCP, CD46Q) is a CSM, expressed by numerous tissues and functions as a complement regulatory factor by binding to C3b or C4b, permitting the plasma serine protease, Factor I, to degrade C3b or C4b (Liszewski *et al*, 1991; Seya *et al*, 1990). It is reasonable, therefore, to speculate that CD46Q may protect HBMSC from autologous complement-mediated lysis. CD63 is a member of an emerging superfamily of tetraspan glycoproteins, which include CD9, CD37, CD53 and the recently cloned PETA-3 (Fitter *et al*, 1995). Moreover, CD63 has been shown to be identical to the melanoma associated antigen, ME491, originally described as an antigen associated with early stages of melanoma tumour progression (Metzelaar *et al*, 1991). CD63 is expressed on activated platelets (Metzelaar *et al*, 1989) and endothelium (Vischer and Wagner, 1993) and is a lysosomal membrane glycoprotein which is translocated to the plasma membrane following activation. CD63 is also expressed on monocytes, macrophages and is weakly expressed on granulocytes, B and T lymphocytes. Recent studies have shown that mAbs to CD63 inhibit adhesion of neutrophils to activated endothelium (Kitani *et al*, 1991) and monocyte adherence to serum-coated plates (Toothill *et al*, 1990), suggesting a role in adhesion. It would therefore be reasonable to speculate that CD63, may play a similar role in the adhesion of BMMNCs to BM stroma, through an as yet undefined ligand. Further studies are required to address this hypothesis.

As detailed in Table 4.2.4., two independently generated mAbs, WM85 and CC9 exhibited limited reactivity with mononuclear cells from BM and PB. Molecular cloning of the corresponding cDNAs revealed that both WM85 and CC9, identified the 110 kD glycoprotein product of the MUC-18 gene. MUC-18 was originally described as a marker

of advanced primary or metastatic melanoma, whose expression was absent on cells from benign naevi (Lehmann et al, 1987).

Recent studies performed in collaboration with Dr. R. Filshie (Filshie, Zannettino, Gronthos, Simmons and Bradstock, manuscript in preparation) revealed that, in contrast to the findings of Lehmann *et al* (1987), all leukaemic T cell lines tested exhibited MUC-18 expression. Moreover, 1/5 B lineage, and 1/5 myeloid leukaemic cell lines exhibited surface MUC-18 expression. These findings were extended by the detection of MUC-18 on cells from patients with acute leukaemia of lymphoid (T or B), or myeloid lineages, as well as a proportion (3/7) with chronic B cell lymphoproliferative disorder. The cells tested were derived from patients of various ages and stages of disease progression and in cases where access to cells from patients in remission were available, a transition from a positive to negative phenotype was observed. Moreover, $CD34^+$ cells derived from PB and BM were also unreactive with WM85 and CC9, suggesting the possibility that MUC-18 expression is correlated with disease progression as has been described for malignant melanoma (Lehmann *et al*, 1987; Lehmann *et al*, 1989).

This also represents the first report describing MUC-18 expression by HBMSC and suggests that this molecule may function in normal haemopoiesis. Being a member of the Immunoglobulin gene superfamily (IgSF) (Lehmann *et al*, 1989; Sers *et al*, 1994), it would be reasonable to speculate that it facilitates the adhesion of haemopoietic progenitor cells (and their committed progeny) to elements of the BM stroma. Evidence for an adhesive role for MUC-18, comes from the work of Shih *et al* (1994), who were able to demonstrate MUC-18-specific adhesion of melanoma cells to immobilised purified MUC-18 protein. Moreover, the existence of an amino acid sequence in the second immunoglobulin loop, very similar to the glycosaminoglycan (GAG) recognition sequence found at the same position in the related IgSF molecules NCAM and PECAM-1, suggests that MUC-18 may bind to elements of the extracellular matrix (ECM) (Lehmann *et al*, 1989). Additional functional studies are required to ascertain the role played by MUC-18 in the haemopoietic system.

Monoclonal antibody CA12 (refer to Table 4.2.4.) was identified on the basis of its binding to the immunising stromal cells, but lack of reactivity with PBMNC, cultures of

skin fibroblasts, keratinocytes and normal human bone cells. CA12 was subsequently found to identify a minor subpopulation of BMMNCs (<1%). FACS was employed to isolate $CA12^+$ and $CA12^-$ subpopulations which were both assayed for their content of clonogenic stromal progenitor cells (also referred to as colony forming units-fibroblast [CFU-F]). The small $CA12^+$ fraction was highly enriched in CFU-F while the corresponding negative population was essentially devoid of these progenitors (Mr. S. Gronthos, personal communication). In accordance with the reactivity of CA12 with CFU-F, 2-colour analysis of BMMNC demonstrated that cells reactive with the CFU-F-specific mAb STRO-1 (Simmons and Torok-Storb, 1991a; Gronthos and Simmons, 1995, Simmons and Gronthos, 1991; Gronthos *et al*, 1994; Simmons *et al*, 1994c) were also reactive with the mAb CA12. Collectively these data demonstrated that in adult human BM, the antigen identified by CA12 is highly specific for stromal elements and their precursors.

Comparisons with sequences in the combined EMBL/Genbank database demonstrated identity with the bone form of the bone/liver/kidney form of alkaline phosphotase ([ALP]; Weiss *et al*, 1986; 1988). In accord with this, the cell line transfected with the cDNA corresponding to the CA12 antigen bound mAb B4-78 (Lawson *et al*, 1985) which recognises an epitope conserved between each of the three isoforms, and also mAb B4-50 (Lawson *et al*, 1985) which has been previously shown to be specific for the bone ALP enzyme. Paradoxically, while B4-50 was reactive with cultured human bone cells, CA12, in accordance with the selection criteria originally employed to identify the antibody, failed to bind to bone cells. Conversely, CA12 was reactive with cultured BM stromal cells but B4-50 was not. A variety of experimental strategies are currently being employed to determine the basis for this novel pattern of reactivity of CA12 for the bone form of ALP (Gronthos, Zannettino and Simmons, manuscript in preparation). These experiments will provide valuable new information regarding the characterisation of the bone cell lineage and the stages involved in the development of bone cells from undifferentiated precursors.

Although submitted to the fourth and fifth International Leukocyte Typing Workshops, mAb 11D1.H10 remained unclustered. Retroviral expression cloning however, revealed that this mAb identified the previously characterised molecule termed 4F2. The

4F2 molecule (recently designated CD98), is a 125 kD disulphide-linked heterodimeric molecule comprised of an 80-90 kD glycosylated heavy chain, and a 40 kD non-glycosylated light chain (Haynes *et al*, 1981). The cDNA encoding the heavy chain of CD98 has been cloned (Teixeira *et al*, 1987, Quackenbush *et al*, 1987), and found to be a Type II integral membrane protein containing 50-81 amino acids in the NH_2-terminal cytoplasmic domain (depending upon which AUG codon is used for the initiation of translation [Quackenbush *et al*, 1987]), a single transmembrane sequence, and a large extracellular domain.

First identified by the mAb 4F2, Haynes *et al* (1981), demonstrated strong reactivity with monocytes and activated leukocytes. Subsequent work however has revealed that CD98 is expressed by all human cell lines studied (Gottesdiener *et al*, 1988). Although the function of CD98 is not yet clear, studies have demonstrated that the mAb 4F2 was able to inhibit Ca^{2+} fluxes, mediated through a Na^{+}/Ca^{+}-exchanger in cardiac and skeletal muscle (Michalak *et al*, 1986). In addition, Haynes *et al* (1981), also showed that mAb 4F2 was able to inhibit lectin-induced mitogenesis of human T cells. To date, no mAbs have been produced that clearly recognise epitopes on the light chain, suggesting that the light chain represents a protein with functional significance that may be conserved across species.

In summary, stably infected FDC-P1 clones have proven to be a valuable resource, not only to screen for additional mAbs, but as tools to investigate the function of a particular CSM. Although this is a particularly powerful application of the retroviral cDNA expression library technique, it is envisaged that many more uses based on this technology will be developed in the near future.

CHAPTER 5

HIGH LEVEL EXPRESSION OF A NOVEL EPITOPE ON CD59 THAT IDENTIFIES A SUBSET OF CD34+ BONE MARROW CELLS HIGHLY ENRICHED FOR PRIMITIVE HUMAN HAEMOPOIETIC PROGENITORS

5.1. INTRODUCTION

5.1.1. Identification Of Cell Surface Molecules (CSM) Expressed By Primitive Human Haemopoietic Cells.

The capacity to purify human haemopoietic stem cells using immunological methods has developed as a result of a large number of studies characterising the cell surface phenotype and the molecules expressed by both primitive cells and cells committed to a specific lineage (Bagby, 1994). To date, more than 150 different cell surface molecules (CSM) have been defined on leukocytes, of which many have been molecularly cloned (Simmons, 1992). An organised nomenclature, called the Cluster of Differentiation or CD system has evolved over the past 15 years to assist in defining and classifying leukocyte surface molecules (Barclay *et al*, 1993).

Moreover, monoclonal antibodies (mAbs) to CSM expressed by subsets of bone marrow (BM) cells have proven useful as markers of haemopoietic cell function. BM cells can be sorted according to the binding of such antibodies and the haemopoietic functions of the resulting populations compared. If the measurable functional properties of the sorted BM cells are reproducible between donors, the binding of the antibody becomes a reliable phenotypic correlate of the functional potential of a BM cell. For example, the finding that all BM progenitor cells are contained within the subset which is bound by mAbs to the CD34 molecule, has greatly facilitated their isolation and analysis (Civin *et al*, 1984; Ogawa, 1993; Katz *et al*, 1985; Andrews *et al*, 1986). Numerous studies however, have demonstrated that this population is not uniform, but is comprised of a hierarchy of closely related, yet functionally heterogeneous cell populations (Sutherland *et al*, 1989a; 1989b; Smith *et al*, 1991, 1991a; Brandt *et al*, 1990; Wagermaker, 1990; Berenson, 1988); Berenson, 1991).

Due to the paucity of mAb reagents specific for subsets of immature cells, initial studies aimed at subdividing the composite $CD34^+$ population were reliant on "negative selection", a process whereby putative stem cells are selected, based on their lack of expression of numerous lineage-specific markers including CD45RA (Lansdorp *et al*, 1990; 1992), CD10, CD19, CD2, CD7, CD38 (Baum *et al*, 1992; Udomsakdi *et al*, 1992),

CD33 (Andrews *et al*, 1989; Bernstein *et al*, 1991), CD71 (Sutherland *et al*, 1989; Lansdorp *et al*, 1992) and HLA-DR (Sutherland *et al*, 1989; Moore *et al*, 1980; Keating *et al*, 1984; Brandt *et al*, 1990).

In recent years however, a number of promising reagents, including mAbs to the Thy-1 molecule (CDw90; Baum *et al*, 1992; Craig *et al*, 1993) and the product of the c-kit proto-oncogene (CD117; Gadd and Ashman, 1985; Ashman, 1987; 1988; 1990; 1991; 1994; Simmons *et al*, 1994; Udomsakdi *et al*, 1992), have been identified, which have enabled the definition of a subset of $CD34^+$ cells, representing <0.5% of the BMMNCs, which are highly enriched for pluripotent stem cells (Craig *et al*, 1993; Baum *et al*, 1992; Simmons *et al*, 1994; reviewed in Ogawa *et al*, 1991). Therefore, mAbs which further subdivide the $CD34^+$ cell population, are of special interest, since they provide a means to describe and isolate functional subsets (a process of positive selection), present within the $CD34^+$ progenitor population at low frequency.

This chapter describes the generation of a murine mAb, HCC-1, which exhibits limited reactivity with both peripheral blood and BM-derived cells and more importantly identifies a subset of the $CD34^+$ cell population which functionally exhibits mulipotentiality in *in vitro* and *in vivo* model systems. The cloning of a cDNA corresponding to the CSM recognised by this mAb was achieved following screening of a human BM stromal cell cDNA library as described in Chapter 4. Surprisingly, HCC-1 was found to bind to a previously undescribed epitope of the CD59 molecule, an 18-20 kD phosphoinositol glycan-linked membrane protein which protects cells against autologous complement attack (Rother *et al*, 1994). Previously published work relating to this molecule has demonstrated uniform expression of CD59 by most nucleated cells of the haemopoietic system, including all the BM-derived $CD34^+$ cells (Terstappen *et al*, 1992; Terstappen *et al*, 1993). In contrast, the HCC-1-defined epitope of CD59 was found to be differentially expressed among $CD34^+$ progenitors with the highest level present on a subset which is highly enriched for pluripotent stem cells. Studies to elucidate the physical basis for this differential expression of the HCC-1 epitope on CD59 molecules support the hypothesis that the binding of the HCC-1 mAb is physically obstructed as a

result of the association of CD59 with an unrelated 80 kD (glyco)protein (gp80) whose expression through haemopoietic cell development appears to be differentiation-related.

5.2. RESULTS

5.2.1. Identification Of Cell Surface Molecules (CSM) Expressed By Primitive Human Haemopoietic Cells: Summary Of Initial Studies

The murine IgM mAb, HCC-1 was generated by Ms. Bernadette Swart in partial fulfilment for the degree of Masters of Applied Science at the University Of South Australia (Swart, 1993). A summary of the features of this mAb are presented in Table 5.2.1. Moreover, utilising the *in vitro* cobble stone area forming cell (CAFC) assay (DiGuisto et al, 1994; Murray et al, 1995) and the SCID-hu bone and thymus mouse model (Chen et al, 1994; Murray et al, 1995), recent studies performed in collaboration with Drs B. Hill and B. Chen of SyStemix Corporation (Palo Alto, CA, USA) (Hill *et al*, 1996), have confirmed and extended these initial studies. As demonstrated in Table 5.2.1. (and associated figures below), mAb HCC-1 identified a subset of BMMNC which exhibit a pluripotent phenotype.

5.2.2. Molecular Characterisation Of The Antigen Identified By mAb HCC-1: Nucleotide And Polypeptide Characterisation.

The finding that HCC-1 was also expressed on various elements of the mesenchyme (Swart, 1993), including cultured human BM stromal cells (Figure 5.2.5.), enabled a cDNA corresponding to this antigen to be isolated (as described in Chapter 4). Subsequent to the isolation of HCC-1 antigen-expressing FDC-P1 clones, genomic DNA was isolated and the corresponding cDNA rescued by PCR (refer to Chapter 4, Section 4.2.10., Figure 4.2.19.). Following partial-sequence analysis, the resultant nucleotide sequence was compared with entries submitted to the Genbank/EMBL databases via standard "FASTA alignment analysis" and found to be 100% homologous to the previously reported cDNA encoding the 18-20 kD glycoprotein variously termed Membrane Inhibitor of Reactive Lysis (MIRL), Homologous restriction factor (HRF20), MEM-43, Protectin, IF-5 and CD59 (Figure 5.2.6.). The CD59 antigen is a structural homologue of the murine Ly-6 antigens (Philbrick *et al*, 1990) and represents a ubiquitously expressed, glycosyl-phosphatidylinositol (GPI)-linked membrane protein

Table 5.2.1. Summary Of The Features Of Monoclonal Antibody HCC-1

Characterisation Of Monoclonal Antibody HCC-1	
Immunogen :	• BM $CD34^+$ cells (intrasplenic).
Isotype :	• IgM.
Characterisation :	• Reacts with approximately 50% BMMNCs ; 10% PBMNCs [refer to Figure 5.2.1. (A) and Figure 5.2.2. (A)].
	• Reacts with approximately 50 and 70 % of the $CD34^+$ population in the PB and BM (steady state or mobilised) [refer to Figure 5.2.1. (B)].
	• Subdivides the CFU-GM and BFU-E populations.
	• Expressed by ≥ 95% of the $CD34^+$ $HLA\text{-}DR^-$ and $CD34^+$ $CD38^-$ cells.
	• Essentially all LTC-IC and pre-CFU are $HCC\text{-}1^+$ in accord with the above phenotype ($CD34^+HLA\text{-}DR^-CD38^-HCC\text{-}1^+$).
	• The $HCC\text{-}1^+$ population in PB and BM is comprised of cells committed to various myeloid and lymphoid lineages [refer to Figure 5.2.3.].
	• Cells with *in vitro* long-term cobblestone area forming cell (CAFC) activity, are highly enriched in the $CD34^+HCC\text{-}1^+$ and $CD34^+HCC\text{-}1^{HI}$ subpopulations derived from BM.
	• The *in vivo* pluripotent SCID-hu bone engrafting activity is restricted exclusively to the $CD34^+HCC\text{-}1^{HI}$ subpopulation.
	• The *in vivo* SCID-hu thymus assay for T cell progenitors, reveals that the majority of the human thymus engrafting activity is detected in the $CD34^+HCC\text{-}1^{HI}$ subpopulation derived from BM.
	• HCC-1 antigen is expressed by committed progenitors and is gradually lost from all cell lineages (with the exception of the erythroid compartment), as they mature into cells found in the peripheral blood and tissues [refer to Figure 5.2.4.].
	• Vascular endothelial cells and BM stromal cells constitutively express the HCC-1 antigen.

Figure 5.2.1. (A) & (B). Summary Of The Expression Of HCC-1 On BM And PB Mononuclear Cells.

(A). The HCC-1 mAb was examined for its reactivity on normal BM and PB mononuclear cells by indirect immunofluorescence and flow cytometry. The pie diagrams represent a summary of the data from 8 normal individuals. HCC-1 antigen was expressed by 45.9 ± 4.1% (mean ± SEM, n=8) and 9.8 ± 1.0% (mean ± SEM, n=8) BMMNCs and PBMNC, respectively.

(B) Dual-parameter immunofluorescence analysis of the cells derived from the 8 normal donors (described above) were immunolabelled simultaneously with mAbs HPCA-2-FITC (α-CD34) and HCC-1 (and subsequently detected with anti-μ-FITC). HCC-1 antigen was expressed by 69.2 ± 5.9% (mean ± SEM, n=8) and 46.4 ± 9.5% (mean ± SEM, n=8) BM and PB derived $CD34^+$ cells, respectively.
(data compiled from Swart, 1993).

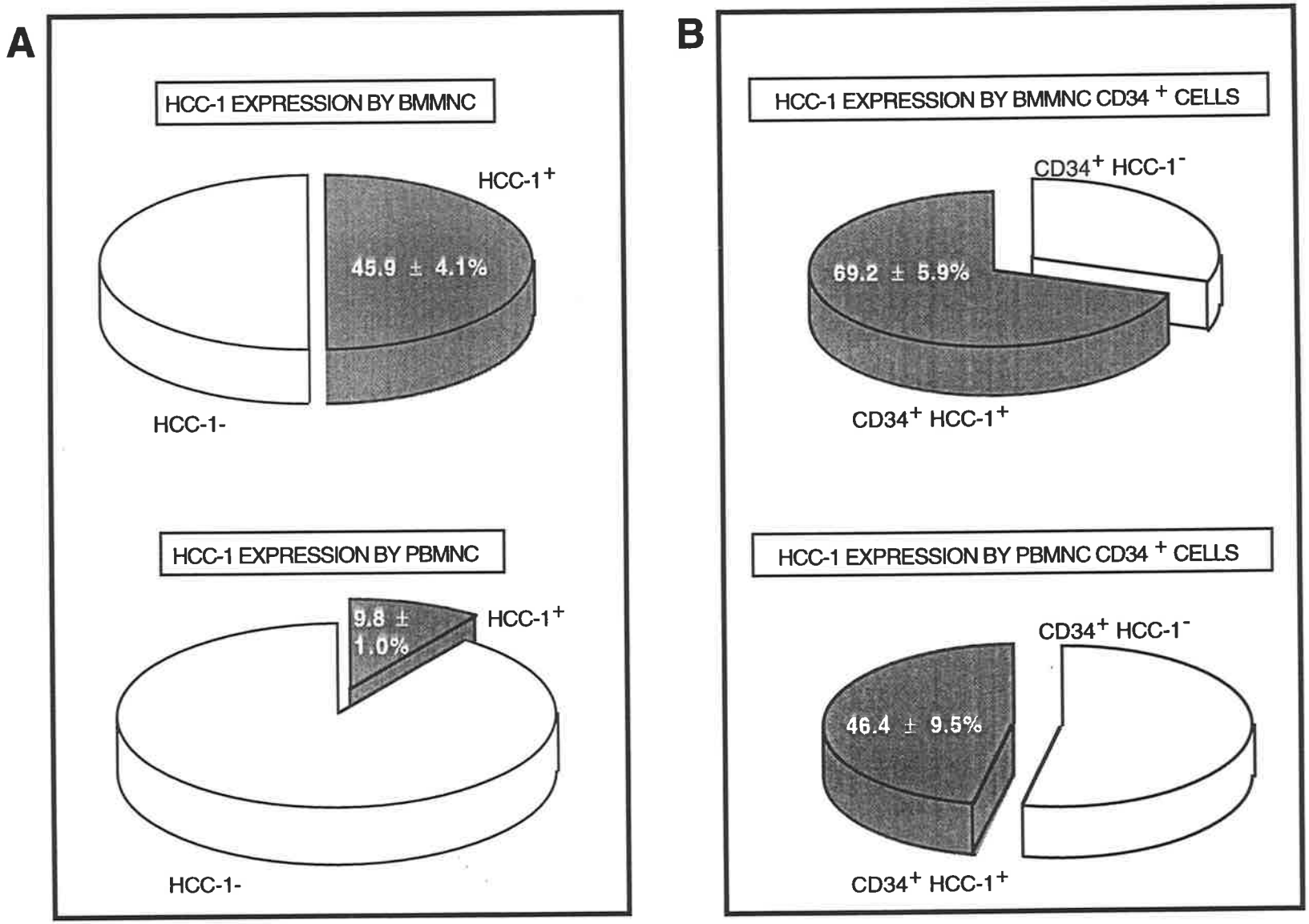

A
HCC-1 EXPRESSION BY BMMNC
HCC-1+
45.9 ± 4.1%
HCC-1-
HCC-1 EXPRESSION BY PBMNC
9.8 ± 1.0%
HCC-1+
HCC-1-
B
HCC-1 EXPRESSION BY BMMNC CD34 + CELLS
CD34+ HCC-1-
69.2 ± 5.9%
CD34+ HCC-1+
HCC-1 EXPRESSION BY PBMNC CD34 + CELLS
CD34+ HCC-1-
46.4 ± 9.5%
CD34+ HCC-1+

Figure 5.2.2. (A) & (B). Two-Colour Immunofluorescence Analysis Demonstrating The Co-Expression Of HCC-1 And CD34 On BMMNCs.

Low density BM mononuclear cells were immunolabelled with HCC-1 and HPCA-2-PE (α-CD34) mAbs. Quandrants were established with isotype controls and used to define the HCC-1^{+} and HCC-1^{-} populations. The HCC-1^{HI} and HCC-$1^{LO/-}$ populations were arbitrarily defined utilising the contour plots.

Dual-parameter histograms was generated from 5×10^4 light-scatter gated events collected as list mode data using a Profile II flow cytometer and analysed using Coulter ELITE software.

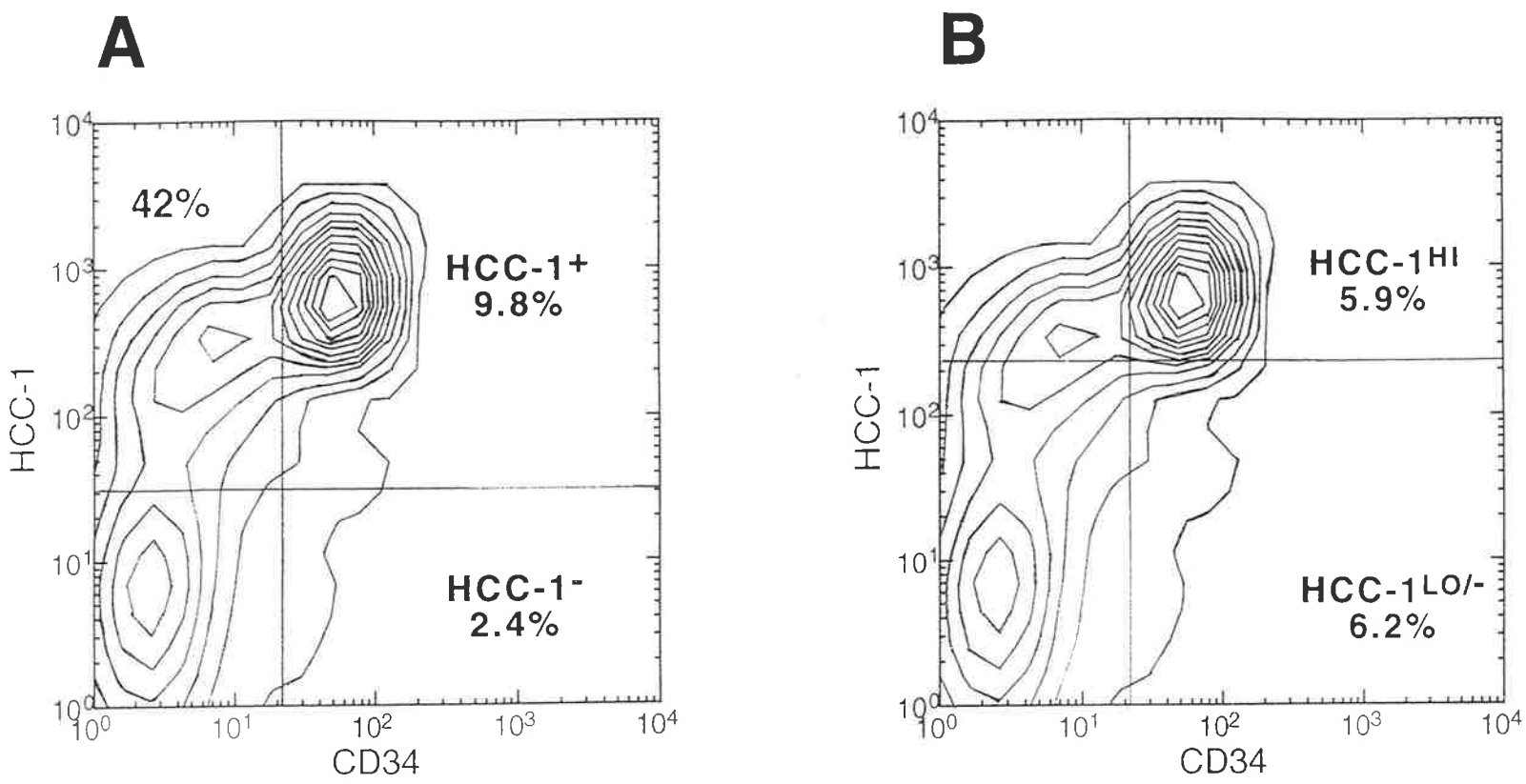

A
42%
HCC-1+
9.8%
HCC-1-
2.4%
HCC-1
CD34
B
HCC-1HI
5.9%
HCC-1LO/-
6.2%
HCC-1
CD34

Figure 5.2.3. Expression Of Lineage Antigens On HCC-1$^+$ BM And PB Mononuclear Cells.

To further characterise HCC-1 expression in the BM and PB, two colour immunofluorescence and flow cytometric analysis was performed using HCC-1 in combination with a panel of mAbs which identify lineage restricted antigens or activation markers. The HCC-1 bound, with varying degrees, to subpopulations of cells co-expressing myeloid (CD33), erythroid (glycophorin A), T lymphoid (CD2, CD7), B lymphoid (CD10, CD19) and the progenitor cell marker CD34. Notably, mature granulocytes and platelets found in the PB did not express the HCC-1 antigen.
(data compiled from Swart, 1993).

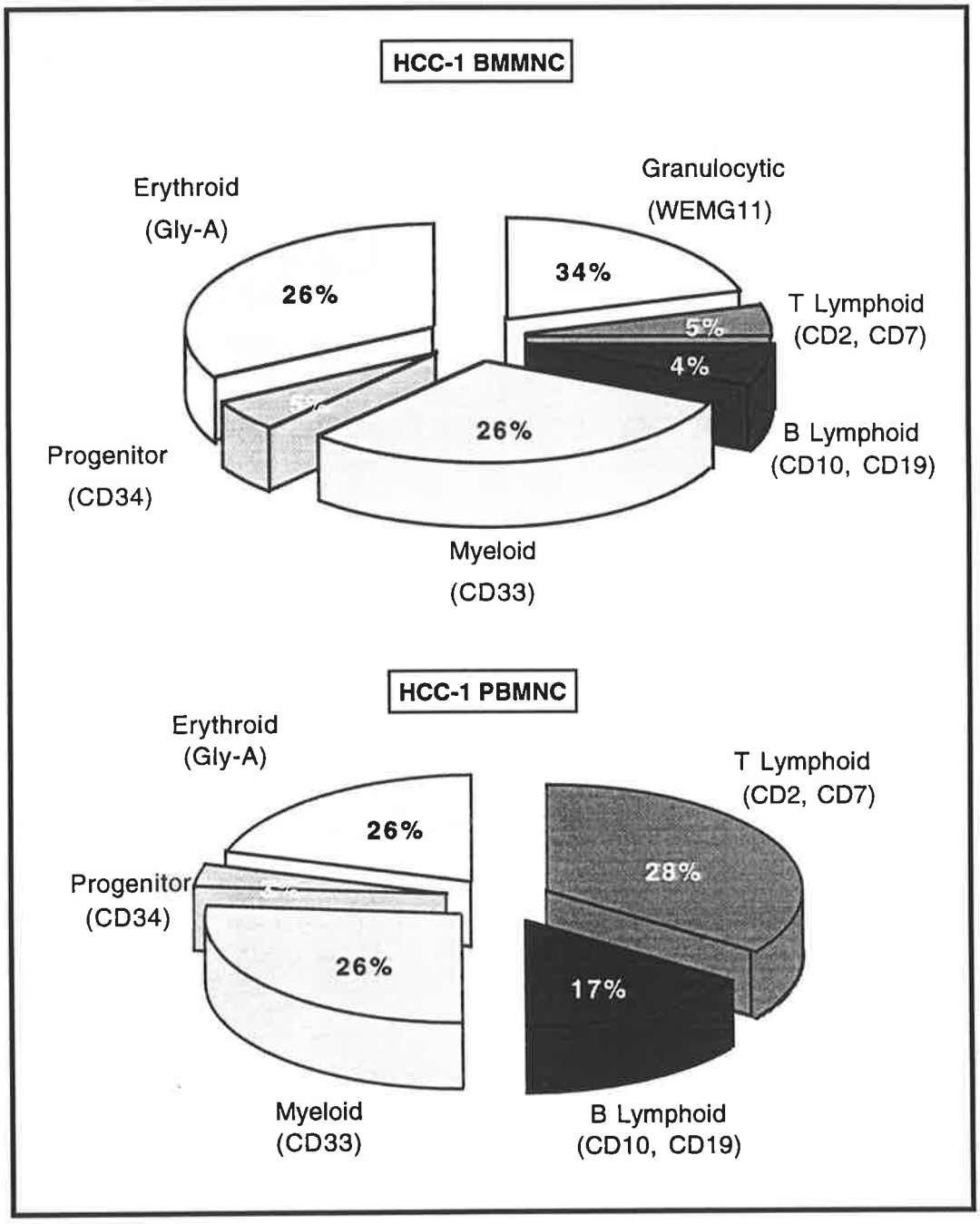

HCC-1 BMMNC
Erythroid
(Gly-A)
26%
Granulocytic
(WEMG11)
34%
T Lymphoid
(CD2, CD7)
5%
4%
B Lymphoid
(CD10, CD19)
Progenitor
(CD34)
26%
Myeloid
(CD33)
HCC-1 PBMNC
Erythroid
(Gly-A)
26%
T Lymphoid
(CD2, CD7)
28%
Progenitor
(CD34)
26%
17%
Myeloid
(CD33)
B Lymphoid
(CD10, CD19)

Figure 5.2.4. (A). Expression Of HCC-1 By Cells Committed To The Lymphoid Lineage.

A schematic diagram, summarising HCC-1 expression throughout lymphoid development. Data compiled from immunophenotypic analyses of tissues from bone marrow, thymus and peripheral blood. (compiled from Swart, 1993)

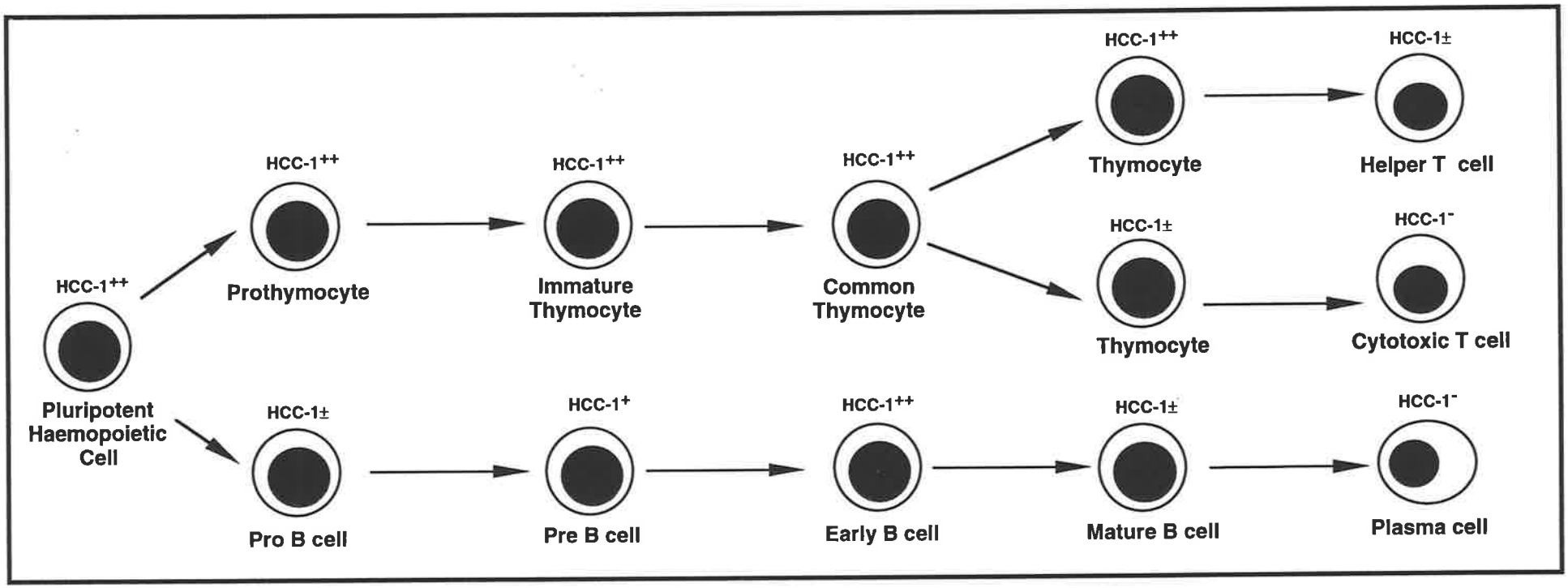

HCC-1++
Pluripotent Haemopoietic Cell
HCC-1++
Prothymocyte
HCC-1++
Immature Thymocyte
HCC-1++
Common Thymocyte
HCC-1++
Thymocyte
HCC-1±
Helper T cell
HCC-1±
Thymocyte
HCC-1-
Cytotoxic T cell
HCC-1±
Pro B cell
HCC-1+
Pre B cell
HCC-1++
Early B cell
HCC-1±
Mature B cell
HCC-1-
Plasma cell

Figure 5.2.4. (B). Expression Of HCC-1 By Cells Committed To The Myeloid Lineage.

A schematic diagram, summarising HCC-1 expression throughout myeloid cell development. Data compiled from immunophenotypic analyses of tissues from bone marrow and peripheral blood. (compiled from Swart, 1993)

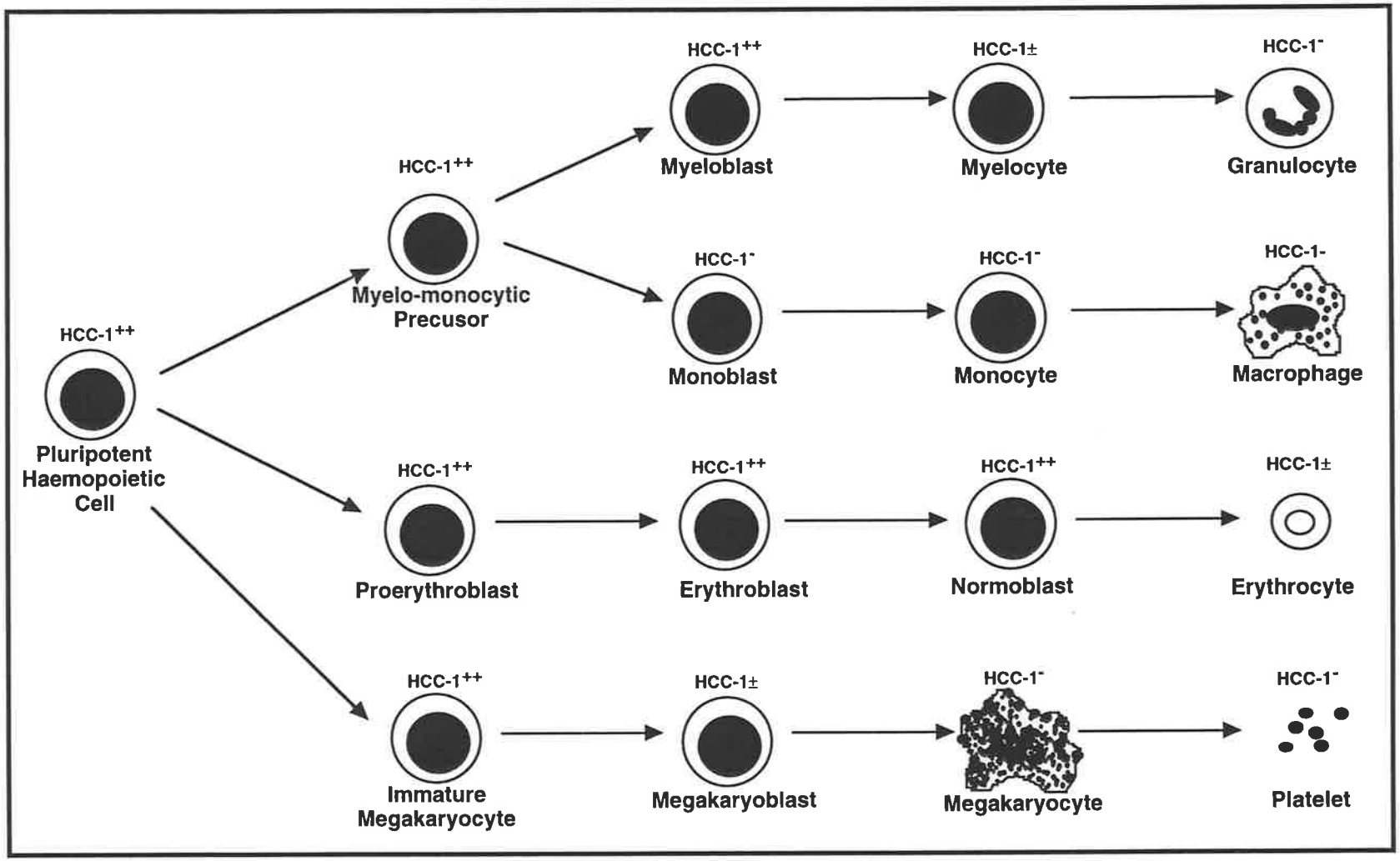
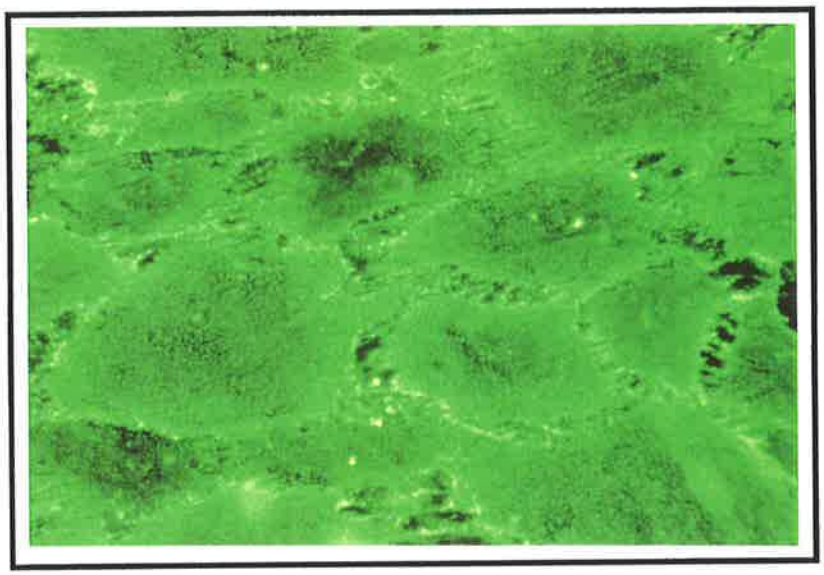

HCC-1++
Pluripotent Haemopoietic Cell
HCC-1++
Myelo-monocytic Precusor
HCC-1++
Myeloblast
HCC-1±
Myelocyte
HCC-1-
Granulocyte
HCC-1-
Monoblast
HCC-1-
Monocyte
HCC-1-
Macrophage
HCC-1++
Proerythroblast
HCC-1++
Erythroblast
HCC-1++
Normoblast
HCC-1±
Erythrocyte
HCC-1++
Immature Megakaryocyte
HCC-1±
Megakaryoblast
HCC-1-
Megakaryocyte
HCC-1-
Platelet

Figure 5.2.5. *In Situ* Immunofluorescence Staining Of Cultured Human BM Stromal Cells.

Primary cultured bone marrow stromal cells, cultivated in 8 chamber culture vessels were immunolabelled with either mAb HCC-1 or STRO-1 as described in the Materials and Methods (Section 2.10.8.). Slides were mounted and analysed using an Olympus BH2-RFCA fluorescent microscope. Cells were photographed at 200 x magnification.

While staining with the stromal cell-specific mAb STRO-1 (Simmons and Torok-Storb, 1991b) appears uniform [Panel A], HCC-1 reactivity is largely restricted to sites of cell-cell contact [Panel B].

A



B

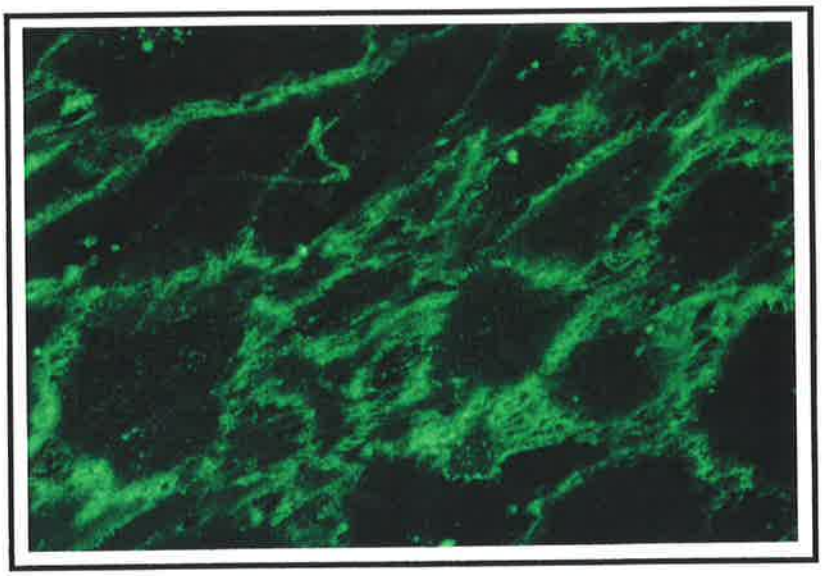


Figure 5.2.6. FASTA Alignment Analysis Of HCC-1 cDNA.

Following partial sequence analysis, the resultant nucleotide sequence was compared with sequences submitted to the combined Genbank/EMBL database via standard "FASTA alignment analysis". The HCC-1 sequence was found to be 100% homologous to the previously reported cDNA encoding CD59 (Sugita *et al*, 1989; Davies *et al*, 1989).

FASTA Alignment Analysis Of HCC-1 cDNA

gb|X16447|HSCD59A Human mRNA for CD59, an LY-6-like pro... 1298 5.1e-101 1

gb|M34671|HUMCD59A Human lymphocytic antigen CD59/MEM43 ... 1193 4.4e-92 1

>gb|X16447|HSCD59A Human mRNA for CD59, an LY-6-like protein regulating complement membrane attack. Length = 1140. Plus Strand HSPs:

Score = 1298 (358.7 bits), Expect = 5.1e-101, P = 5.1e-101

Identities = 265/265 (100%), Positives = 265/265 (100%), Strand = Plus

```
Query:   168 CGAGGCTGGAAGAGGATCCTGGGCGCCGCCAGGTTCTGTGGACAATCACAATGGGAATCC 227
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:    14 CGAGGCTGGAAGAGGATCCTGGGCGCCGCCAGGTTCTGTGGACAATCACAATGGGAATCC 73

Query:   228 AAGGAGGGTCTGTCCTGTTCGGGCTGCTGCTCGTCCTGGCTGTCTTCTGCCATTCAGGTC 287
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:    74 AAGGAGGGTCTGTCCTGTTCGGGCTGCTGCTCGTCCTGGCTGTCTTCTGCCATTCAGGTC 133

Query:   288 ATAGCCTGCAGTGCTACAACTGTCCTAACCCAACTGCTGACTGCAAAACAGCCGTCAATT 347
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   134 ATAGCCTGCAGTGCTACAACTGTCCTAACCCAACTGCTGACTGCAAAACAGCCGTCAATT 193

Query:   348 GTTCATCTGATTTTGATGCGTGTCTCATTACCAAAGCTGGGTTACAAGTGTATAACAAGT 407
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   194 GTTCATCTGATTTTGATGCGTGTCTCATTACCAAAGCTGGGTTACAAGTGTATAACAAGT 253

Query:   408 GTTGGAAGTTTGAGCATTGCAATTT 432
             |||||||||||||||||||||||||
Sbjct:   254 GTTGGAAGTTTGAGCATTGCAATTT 278
```

>gb|M34671|HUMCD59A Human lymphocytic antigen CD59/MEM43 mRNA, complete cds. Length = 1671. Plus Strand HSPs:

Score = 1193 (329.6 bits), Expect = 4.4e-92, P = 4.4e-92

Identities = 242/244 (98%), Positives = 242/244 (98%), Strand = Plus

```
Query:   189 GGCGCCGCCAGGTTCTGTGGACAATCACAATGGGAATCCAAGGAGGGTCTGTCCTGTTCG 248
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:     1 GGCGCCGCCAGGTTCTGTGGACAATCACAATGGGAATCCAAGGAGGGTCTGTCCTGTTCG 60

Query:   249 GGCTGCTGCTCGTCCTGGCTGTCTTCTGCCATTCAGGTCATAGCCTGCAGTGCTACAACT 308
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:    61 GGCTGCTGCTCGTCCTGGCTGTCTTCTGCCATTCAGGTCATAGCCTGCAGTGCTACAACT 120

Query:   309 GTCCTAACCCAACTCCTGACTGCAAAACAGCCGTCAATTGTTCATCTGATTTTAATGCGT 368
             |||||||||||||| ||||||||||||||||||||||||||||||||| ||||||
Sbjct:   121 GTCCTAACCCAACTGCTGACTGCAAAACAGCCGTCAATTGTTCATCTGATTTTGATGCGT 180

Query:   369 GTCTCATTACCAAAGCTGGGTTACAAGTGTATAACAAGTGTTGGAAGTTTGAGCATTGCA 428
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   181 GTCTCATTACCAAAGCTGGGTTACAAGTGTATAACAAGTGTTGGAAGTTTGAGCATTGCA 240

Query:   429 ATTT 432
             ||||
Sbjct:   241 ATTT 244
```

which protects cells against autologous complement attack, by binding to the complement components, C8 and C9, thus inhibiting the formation of the membrane attack complex (MAC) (Meri *et al*, 1990; reviewed in Lachmann, 1991).

As indicated in Figure 5.2.7., complete sequence analysis of the PCR-derived HCC-1 cDNA sequence revealed complete identity (ie. no nucleotide substitutions) with the published CD59 sequence (Genbank accession number: X16447). Computer analysis (MacDNASIS, Version 2.0) of the translated HCC-1 FDC-P1-derived sequence revealed a 128 amino acid polypeptide with a putative molecular weight of 11.5 kD concordant with the molecular mass suggested by Davies *et al* (1989). Following the amino-terminal 25 amino acid putative signal peptide, one potential site of N-linked glycosylation (Asn-Xaa-Ser/Thr) was observed at position Asn^{18} (Figure 5.2.7.). Although a number of potential sites of O-linked carbohydrate (Ser or Thr residues) are present, previous studies suggest that they are not utilised (Davies *et al*, 1989; Ninomiya *et al*, 1992; Nakano *et al*, 1994; Akami *et al*, 1994). Finally, the glycosyl-phosphatidylinositol (GPI) anchor is attached to the carboxyl terminus of the mature peptide at 78^{th} amino acid (Gly^{78}; Williams, 1991; Caras *et al*, 1989; Caras, 1991).

5.2.3. Confirmation That mAb HCC-1 Recognises The Product Of The CD59 cDNA.

As demonstrated in Chapter 4, (Section 4.2.10), the HCC-1 FDC-P1 clone from which the above-described PCR product was derived, harboured 2 retroviral integrations. Therefore to confirm that mAb HCC-1 did indeed identify the product of the CD59 cDNA, a 1.2 kb *Not*I-*Not*I restriction fragment of the CD59 cDNA (harbouring both the entire coding sequence and the 5′ and 3′ non-coding regions) was subcloned from the sequencing vector into the pRUF(*Not*I)*neo* vector (refer to Appendix A, Restriction Map #4) and subsequently introduced into FDC-P1 cells by retroviral transduction (refer to *Materials and Methods*, Section 2.26.). Although the G418-resistant pool of FDC-P1 cells were found to bind mAb HCC-1, the number of $HCC\text{-}1^+$ cells was relatively low (approximately 30%), necessitating HCC-1/magnetic Dynabead enrichment (refer to Chapter 4). Figure 5.2.8. illustrates the fluorescence profile of CD59-expressing FDC-P1 cells immunolabelled with HCC-1 mAb following one round of enrichment. This result

Figure 5.2.7. Nucleotide And Inferred Protein Sequence Of CD59 (HCC-1).

The nucleotide and deduced amino acid sequence of CD59 (HCC-1). The putative signal sequence is underlined. The one potential site of N-linked glycan attachment at Asn^{18} is indicated by asterisks, whilst no sites of O-linked carbohydrate are apparent. Cysteine residues are indicated by boxes. The glycosyl-phosphatidylinositol (GPI) anchor is attached to the carboxyl terminus of the mature peptide at 78^{th} amino acid (Gly^{78}; Williams, 1991). Please note that the nucleotide and amino acid sequence numbering is based on that of Davies *et al* (1989).

				5'-	GTT	CTG	TGG	ACA	ATC	ACA	30
ATG	GGA	ATC	CAA	GGA	GGG	TCT	GTC	CTG	TTC	GGG	62
Met	Gly	Ile	Gln	Gly	Gly	Ser	Val	Leu	Phe	Gly	11
CTG	CTG	CTC	GTC	CTG	GCT	GTC	TTC	TGC	CAT	TCA	95
Leu	Leu	Leu	Val	Leu	Ala	Val	Phe	Cys	His	Ser	22
GGT	CAT	AGC	CTG	CAG	TGC	TAC	AAC	TGT	CCT	AAC	128
Gly	His	Ser	Leu	Gln	Cys	Tyr	Asn	Cys	Pro	Asn	33
CCA	ACT	GCT	GAC	TGC	AAA	ACA	GCC	GTC	AAT	TGT	161
Pro	Thr	Ala	Asp	Cys	Lys	Thr	Ala	Val	Asn	Cys	44
									*		
TCA	TCT	GAT	TTT	GAT	GCG	TGT	CTC	ATT	ACC	AAA	194
Ser	Ser	Asp	Phe	Asp	Ala	Cys	Leu	Ile	Thr	Lys	55
GCT	GGG	TTA	CAA	GTG	TAT	AAC	AAG	TGT	TGG	AAG	227
Ala	Gly	Leu	Gln	Val	Tyr	Asn	Lys	Cys	Trp	Lys	66
TTT	GAG	CAT	TGC	AAT	TTC	AAC	GAC	GTC	ACA	ACC	260
Phe	Glu	His	Cys	Asn	Phe	Asn	Asp	Val	Thr	Thr	77
CGC	TTG	AGG	GAA	AAT	GAG	CTA	ACG	TAC	TAC	TGC	293
Arg	Leu	Arg	Glu	Asn	Glu	Leu	Thr	Tyr	Tyr	Cys	88
TGC	AAG	AAG	GAC	CTG	TGT	AAC	TTT	AAC	GAA	CAG	326
Cys	Lys	Lys	Asp	Leu	Cys	Asn	Phe	Asn	Glu	Gln	99
CTT	GAA	AAT	**GGT**	GGG	ACA	TCC	TTA	TCA	GAG	AAA	359
Leu	Glu	Asn	**Gly**	Gly	Thr	Ser	Leu	Ser	Glu	Lys	110
ACA	GTT	CTT	CTG	CTG	GTG	ACT	CCA	TTT	CTG	GCA	392
Thr	Val	Leu	Leu	Leu	Val	Thr	Pro	Phe	Leu	Ala	121
GCA	GCC	TGG	AGC	CTT	CAT	CCC	TAA	GTC	AAC	ACC	425
Ala	Ala	Trp	Ser	Leu	His	Pro	STOP				128
AGG	AGA	GCT	TCT	CCC	AAA	CTC	CCC	GTT	CCT	GCG	458
TAG	TCC	GCT	TTC	TCT	TGC	TGC	CAC	ATT	CTA	AAG	491
GCT	TGA	TAT	TTT	CCA	AAT	GGA	TCC	TGT	TGG	GAA	524
AGA	ATA	AAA	TTA	GCT	TGA	GCA	ACC	TGG	CTA	AGA	557
TAG	AGG	GGC	TCT	GGG	AGA	CTT	TGA	AGA	CCA	GTC	590
CTG	TTT	GCA	GGG	AAG	CCC	CAC	TTG	AAG	GAA	GAA	623
GTC	TAA	GAG	TGA	AGT	AGG	TGT	GAC	TTG	AAC	TAG	656
ATT	GCA	TGC	TTC	CTC	CTT	TGC	TCT	TGG	GAA	GAC	689
CAG	CTT	TGC	AGT	GAC	AGC	TTG	AGT	GGG	TTC	TCT	722
GCA	GCC	CTC	AGA	TTA	TTT	TTC	CTC	TGG	CTC	CTT	755
GGA	TGT	AGT	CAG	TTA	GCA	TCA	TTA	GTA	CAT	CTT	788
TGG	AGG	GTG	GGG	CAG	GAG	TAT	ATG	AGC	ATC	CTC	821
TCT	CAC	ATG	GAA	CGC	TTT	CAT	AAA	CTT	CAG	GGA	854

TCC CGT GTT GCC ATG GAG GCA TGC CAA ATG TTC 887

CAT ATG TGG GTG TCA GTC AGG GAC AAC AAG ATC 920

CTT AAT GCA GAG CTA GAG GAC TTC TGG CAG GGA 953

AGT GGG GAA GTG TTC CAG ATA GCA GGG CAT GAA 986

AAC TTA GAG AGG TAC AAG TGG CTG AAA ATC GAG 1019

TTT TTC CTC TGT CTT TAA ATT TTA TAT GGG CTT 1052

TGT TAT CTT CCA CTG GAA AAG TGT AAT AGC ATA 1085

CAT CAA TGG TGT GTT AAA GCT ATT TCC TTG CCT 1118

TTT TTT ATT GGA ATG GTA GGA TAT CTT GGC TTT 1151

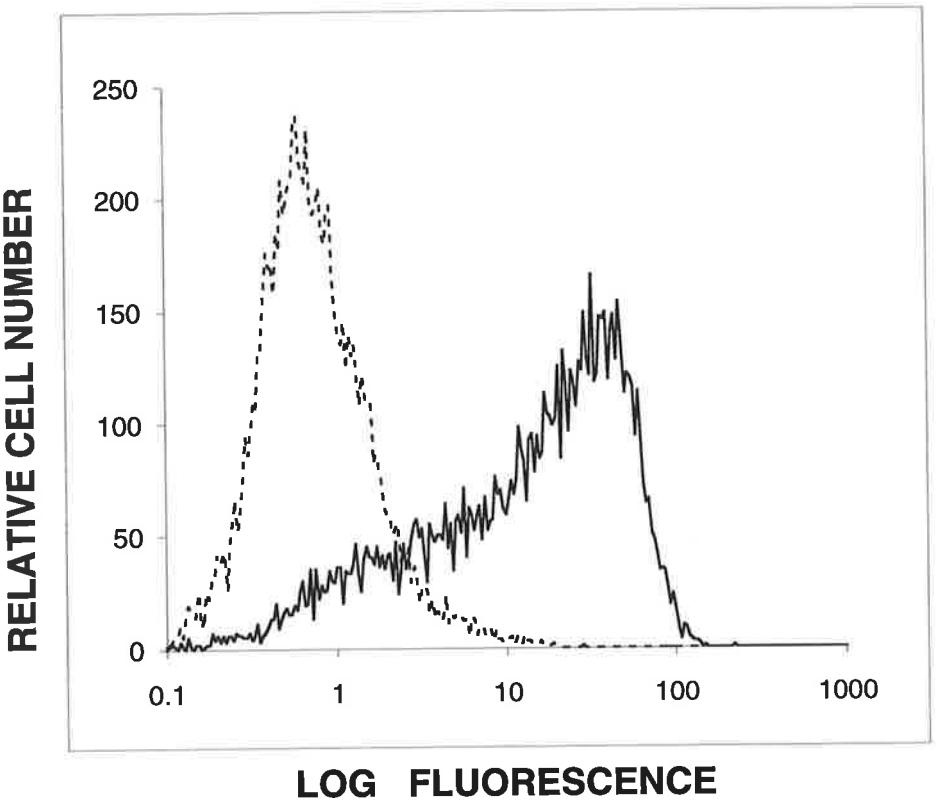
GCC ACA CAC AGT TAC -3' 1166

Figure 5.2.8. Confirmation That mAb HCC-1 Identifies The Product Of The CD59 Gene.

A 2.1 kb *Not*I-*Not*I restriction fragment of the HCC-1 (CD59) cDNA (harbouring both the entire coding sequence and the 5′ and 3′ non-coding regions) was subcloned into the pRUF.*neo* vector and subsequently introduced into FDC-P1 cells by retroviral transduction (refer to *Materials and Methods*, Section 2.26.). The resultant G418-resistant cell population, was subjected to one round of HCC-1/magnetic Dynabead enrichment (refer to text), prior to staining by indirect immunofluorescence and analysis by flow cytometry. Data are displayed as single-parameter fluorescence (FITC) histograms of 1 x 10^4 light-scatter gated events, collected as list mode data.

------ : IgM control mAb

——— : mAb HCC-1


RELATIVE CELL NUMBER
250
200
150
100
50
0
0.1
1
10
100
1000
LOG FLUORESCENCE

thus confirms that mAb HCC-1 identifies a previously undescribed epitope on the CD59 molecule.

5.2.4. Expression Of HCC-1 And 2/24 (α-CD59) Reactive Epitopes By Haemopoietic Cell Lines And Cell Preparations.

Immunofluorescence and flow cytometry was utilised to compare the pattern of expression of CD59 by haemopoietic cell lines and cell preparations as detected by mAb HCC-1 and a mAb previously identified to bind to a peptide-related epitope of CD59, termed 2/24 (Fletcher *et al*, 1992). As demonstrated in Figure 5.2.9., dual colour immunofluorescence of BM cells labelled with both HPCA-2 (α-CD34) and mAb 2/24 (Figure 5.2.9. (A)), reveals that all the $CD34^+$ cells coexpress CD59. In contrast to (and in accord with the findings of Swart, (1993)), only 60% of the $CD34^+$ population coexpress the epitope on CD59 molecules which is detected by mAb HCC-1 (Figure 5.2.9. (B)).

Figure 5.2.10., illustrates the comparative fluorescence histogram profiles obtained when the haemopoietic cell lines K562, HL60, HEL-DR, KG1a and MO7e were immunolabelled with either mAb 2/24 or HCC-1. It is evident from Figure 5.2.10., that the pattern of reactivity which resulted was significantly disparate in all cases, suggesting that the epitope on the CD59 molecule identified by HCC-1 is not present in all cell lines. This is best illustrated with the cell line HL-60, were CD59 expression is apparent (as detected by mAb 2/24), but no HCC-1 binding is evident.

5.2.5. Physical Basis For The Differential Expression Of The HCC-1 Epitope On CD59 Molecules.

In addition to the data presented above, previously published work relating to CD59 (Terstappen *et al*, 1992; 1993) has demonstrated uniform expression of this molecule by most nucleated cells of the haemopoietic system, including all the BM-derived $CD34^+$ cells. As demonstrated above however, the HCC-1-defined epitope of CD59 was found to be differentially expressed among $CD34^+$ progenitors with the highest level present on a subset which is significantly enriched for pluripotent stem cells.

Figure 5.2.9. Expression Of HCC-1 And 2/24 (α-CD59) Reactive Epitopes By Haemopoietic Progenitor Cells.

Dual-parameter immunofluorescence of BM cells labelled with both HPCA-2 (α-CD34) and mAb 2/24 (Panel A), or mAb HCC-1 (Panel B) reveals that all the $CD34^+$ cells coexpress CD59, whilst only 60% coexpress the epitope on CD59 molecules identified by mAb HCC-1. Each 2-colour plot was generated with 2×10^4 light-scatter gated events, collected as list mode data.

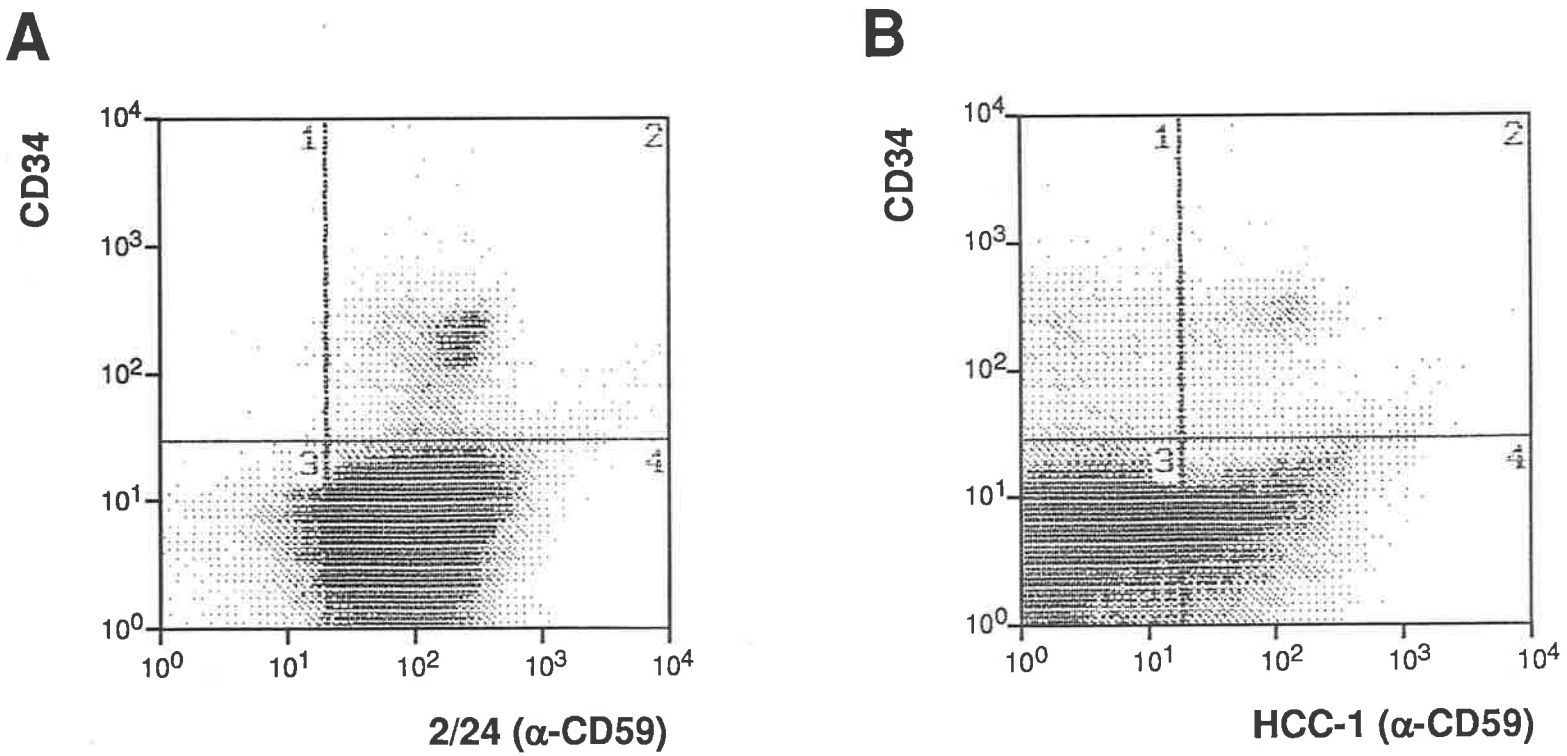

A
CD34
10^4
10^3
10^2
10^1
10^0
10^0 10^1 10^2 10^3 10^4
2/24 (α-CD59)
B
CD34
10^4
10^3
10^2
10^1
10^0
10^0 10^1 10^2 10^3 10^4
HCC-1 (α-CD59)

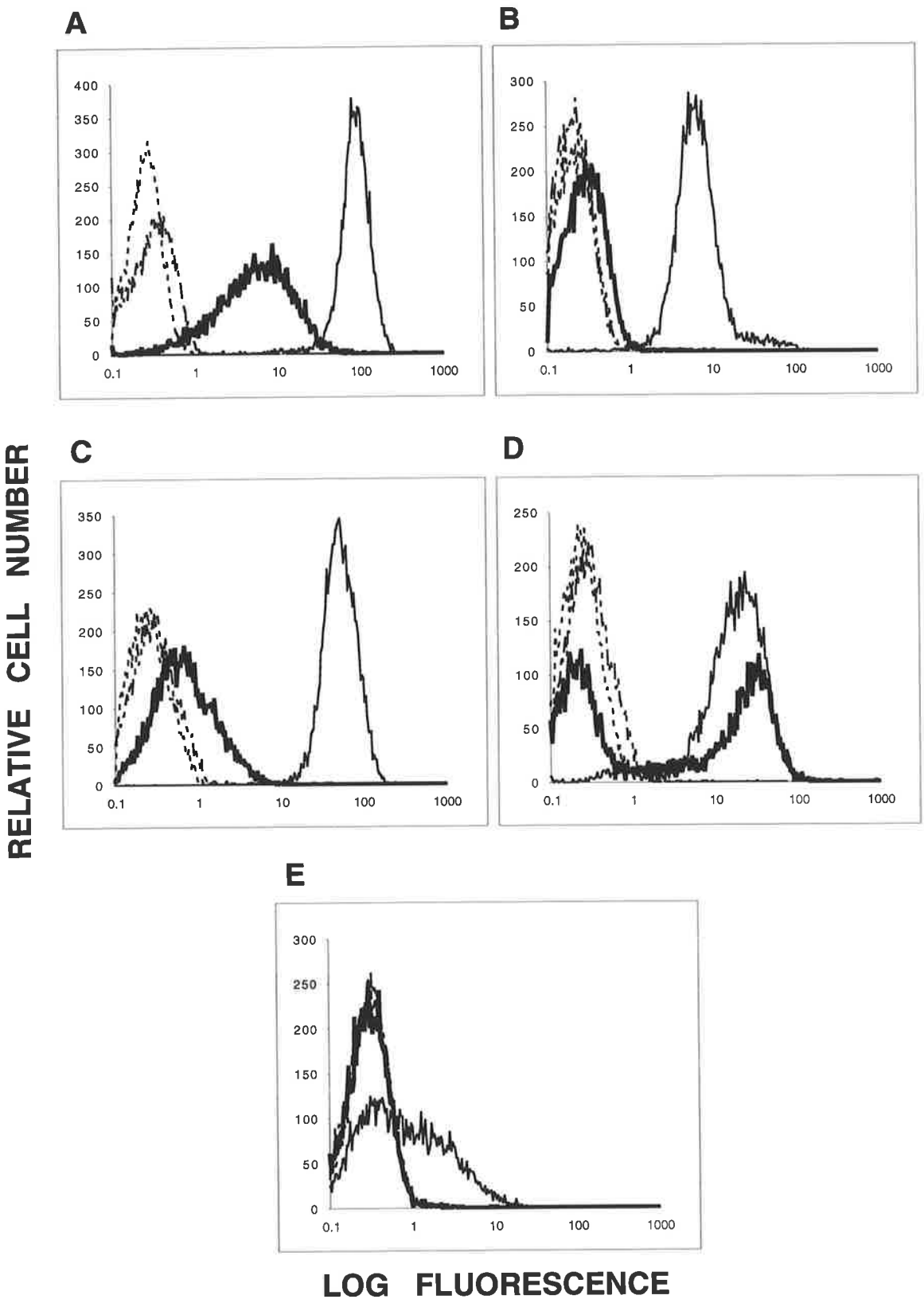
Figure 5.2.10. Expression Of HCC-1 And 2/24 (α-CD59) Reactive Epitopes By Haemopoietic Cell Lines.

Comparative fluorescence histogram profiles obtained when the haemopoietic cell lines K562, HL60, HEL-DR, KG1a and Mo7e were immunolabelled with either mAb 2/24 or HCC-1. Data are displayed as overlays of single-parameter fluorescence (FITC) histograms of 1×10^4 light-scatter gated events, collected as list mode data.

The pattern of reactivity which resulted was significantly disparate in all cases, suggesting that the epitope on the CD59 molecule identified by HCC-1 is not present in all cell lines. This is best illustrated with the cell line HL-60 (panel B), were CD59 expression is apparent (as detected by mAb 2/24), but no HCC-1 binding is evident.

Panel A: K562
Panel B: HL-60
Panel C: Hel-DR
Panel D: KG1a
Panel E: Mo7e

..... : IgG_{2A} control mAb
·–·–·–· : IgG_{2B} control mAb
——— : mAb 2/24
——— : mAb HCC-1


A
B
C
D
E
RELATIVE CELL NUMBER
LOG FLUORESCENCE
0.1
1
10
100
1000

Through the use of mAbs, the study detailed here reveals a unique pattern of CD59 epitope expression not observed in previous examinations of candidate haemopoietic stem cells. The physical basis of the differential expression of the HCC-1 epitope on CD59 is the subject of the following studies.

(i) Do The CD59 Molecules Recognised By mAb HCC-1 Exhibit Differential Sensitivity To Phosphatidylinositol-specific Phospholipase C (PI-PLC)?

The release of alkaline phosphatase and acetylcholinesterase by an enzyme present in the culture medium of several *Bacillus* species led to the identification of phosphatidylinositol-specific phospholipase C (PI-PLC), and henceforth to the discovery of the glycolipid anchor responsible for anchoring CD59 and other GPI-linked proteins to the plasma membrane (Taguchi and Ikezawa, 1978; Low and Finean, 1977; Taguchi *et al*, 1980; Futerman *et al*, 1985; Tse *et al*, 1985; Holder, 1983; Low and Kincade, 1985; Homans *et al*, 1988). Since this phenomenon was first discovered, the specific release of cell surface proteins has become one of the key methods for demonstrating the presence of glycolipid anchors on proteins (Ferguson *et al*, 1988; Stieger *et al*, 1986; Clayton and Mowatt, 1989; Conzelmann *et al*, 1988; Sadeghi *et al*, 1988; Hortsch and Goodman, 1990; Williams *et al*, 1988; and see reviews in Ferguson and Williams, 1988; Low, 1987; 1988; 1989a; 1989b).

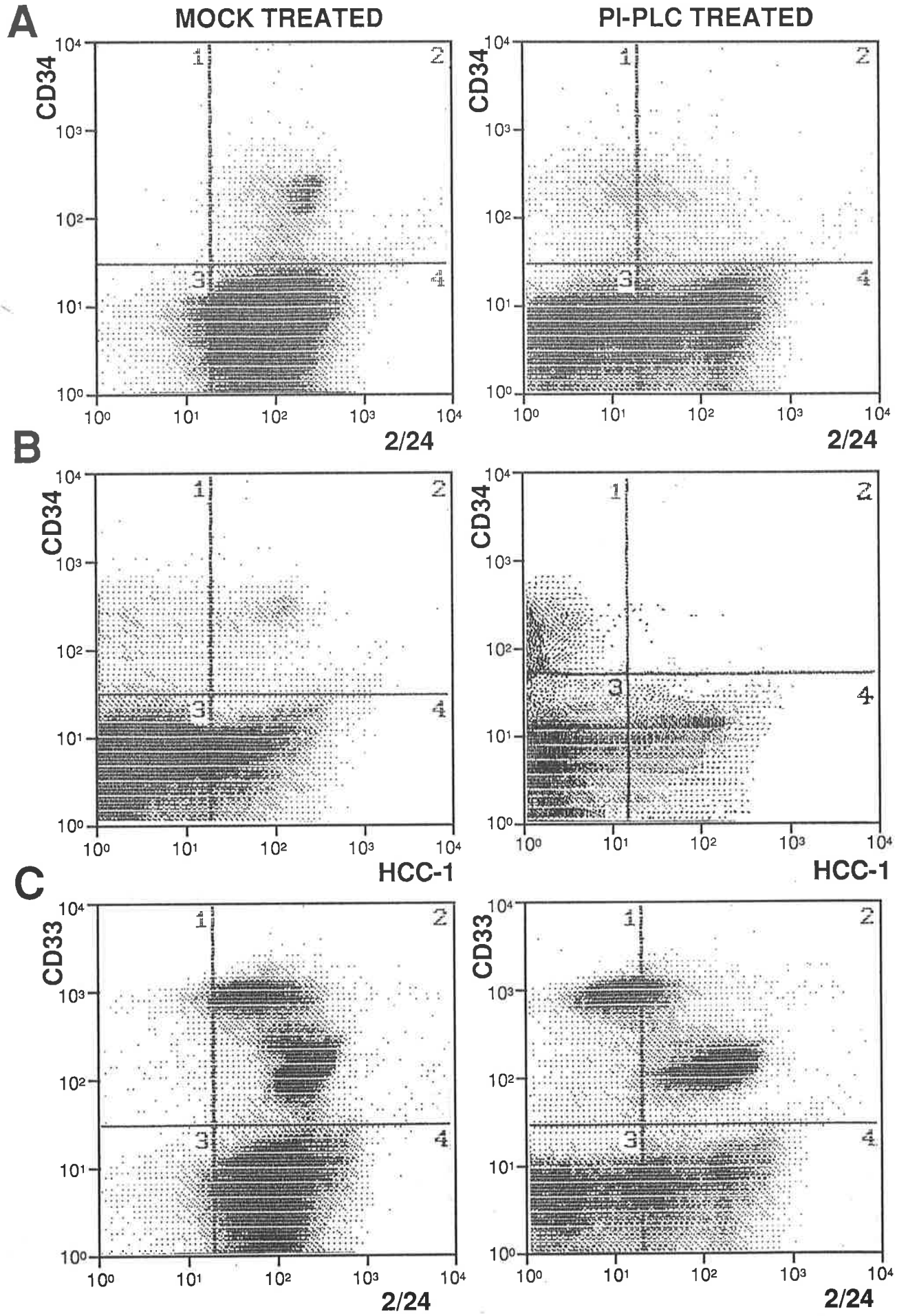
PI-PLC-treatment of BMMNCs revealed that in most instances, HCC-1 binding was lost in accord with the loss of the CD59 molecule from the surface of these cells. In other words, the results obtained with mAb HCC-1 closely paralleled those obtained with the *bona fide* CD59 mAb, 2/24. This included the observation that CD59 (as identified by both 2/24 and HCC-1) was released from BMMNCs, which co-express the mature myeloid antigen CD33 (Figure 5.2.11. (C) and (D)). Moreover, CD59 molecules expressed by human erythrocyte precursors (glycophorin A^+, Figure 5.2.11. (E) and (F)) exhibited resistance to PI-PLC cleavage, which can be attributed to a modification of the inositol ring by a fatty acid chain in these cells, which prevents the release of this molecule (Roberts *et al*, 1987b; Toutant *et al*, 1989; Schmidt, 1978).

Figure 5.2.11. Cleavage Of GPI-linked CD59 On BM Mononuclear Cells With Phosphatidylinositol-Specific Phospholipase C (PI-PLC).

BMMNC were washed thrice with IMDM supplemented with 2% FCS, and resuspended at a final concentration of 5 x 10^6 cells/ml. To this, 50 mU/ml of PI-PLC was added, and the cells incubated at 37°C for 2 hours (mock-treated cells were incubated in an equivalent manner in the absence of PI-PLC). BMMNC were washed in ice-cold wash buffer in preparation for 2-colour immunophenotypic analysis.

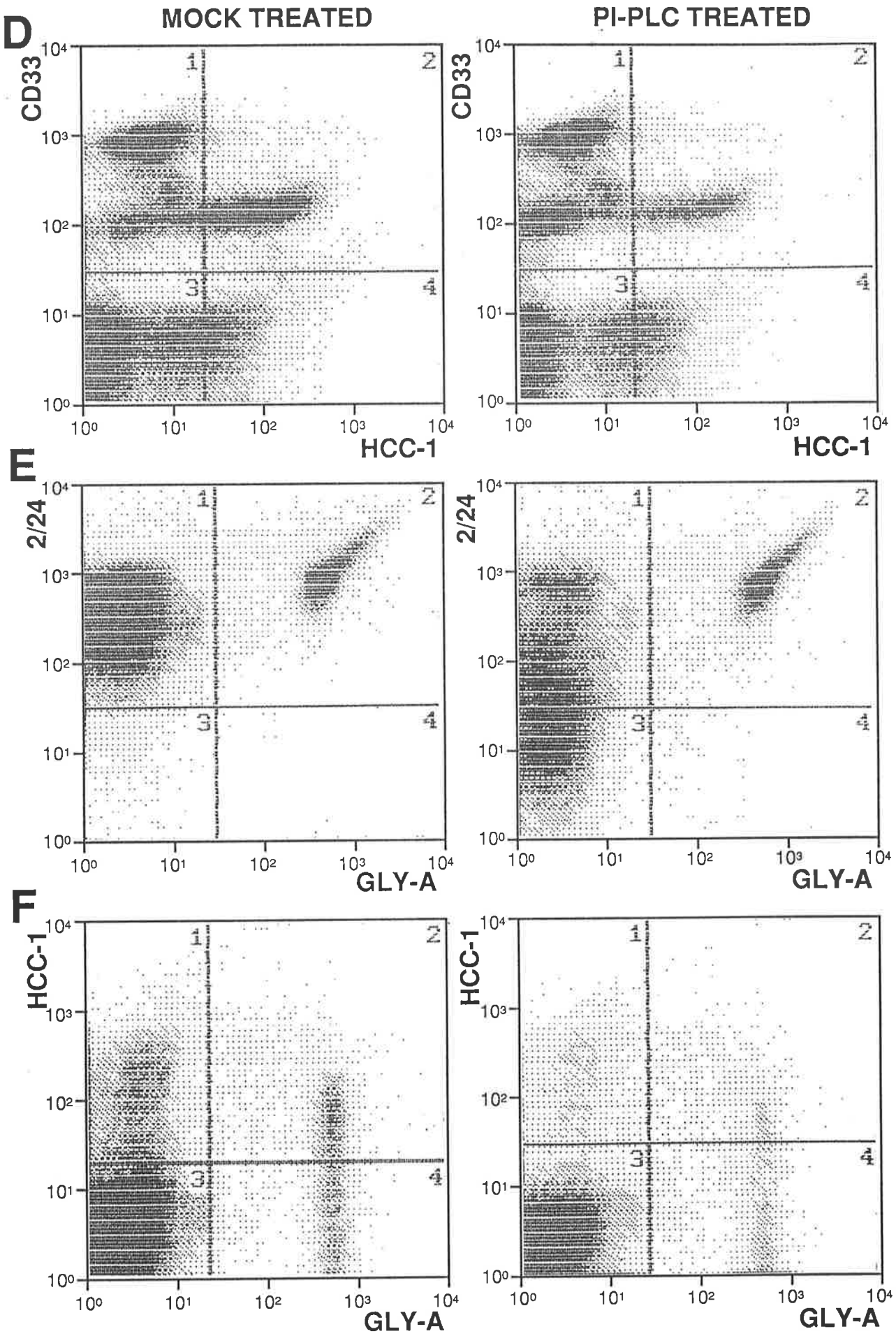
Mock-or PI-PLC-treated BMMNCs were immunolabelled with either HCC-1 or 2/24 in combination with FITC or PE-conjugated mAbs to CD33, CD34 and Glycophorin A (or with appropriate isotype-matched FITC conjugated non-binding control antibodies), as indicated. Each 2-parameter histogram was generated from 2 x 10^4 light scatter gated events collected as list mode data using a Profile II flow cytometer and analysed using Coulter ELITE software. Data represents the mean ± SE of 3 experiments.

(Continued on next page).


A
MOCK TREATED
PI-PLC TREATED
CD34
2/24
B
CD34
HCC-1
C
CD33
2/24
1
2
3
4

(Continued)

Figure 5.2.11. Cleavage Of GPI-linked CD59 On BM Mononuclear Cells With Phosphatidylinositol-Specific Phospholipase C (PI-PLC).


D
MOCK TREATED
PI-PLC TREATED
CD33
HCC-1
E
2/24
GLY-A
F
HCC-1
GLY-A

Despite the observation that the loss of CD59 was paralleled in most instances, one notable exception did exist. As demonstrated in Figure 5.2.11. (A) & (B), following PI-PLC treatment, the progenitor population (as indicated by their expression of the CD34 antigen), sustained a complete loss of HCC-1 expression, whilst 2/24 binding was only lost on a subset of the $CD34^+$ cells (approximately 40%).

(ii) Does mAb HCC-1 Recognise A Carbohydrate Epitope? : *In Vitro* Mutagenesis Of CD59 cDNA To Remove N-Linked Carbohydrates.

Although a number of studies have examined the role of glycosylation with respect to its effect on the complement inhibitory function of CD59 (Nakano *et al*, 1994; Ninomiya *et al*, 1992; Akami *et al*, 1994), no studies have examined whether CD59 is differentially glycosylated as a function of differentiation. Central to this hypothesis is that mAb HCC-1 identifies a variant carbohydrate-epitope presented upon single site glycan attachment at Asn^{18} of the mature polypeptide, whose expression is altered upon haemopoietic cell differentiation. To test whether the binding of the HCC-1 mAb was dependent upon carbohydrate, the solitary N-linked glycosylation consensus site of CD59 was modified to eliminate carbohydrate attachment (as detailed in Figure 5.2.12.) using the Altered Sites™ *in vitro* Mutagenesis System (detailed in *Materials and Methods*, Section 2.23. and schematically illustrated in Figure 5.2.13.).

Briefly, a 651 bp *Sma*I and *Sph*I restriction fragment of the CD59 cDNA was ligated into the 5680 bp pALTER™ phagemid vector via analogous sites present in the multiple cloning site. As detailed in Figure 5.2.13., the mutagenesis reaction involved the annealing of the Ampicillin repair oligonucleotide, and the MUT59 mutagenic oligo-nucleotide to the ssDNA, followed by synthesis of the mutant strand with T4 DNA polymerase I. The heteroduplex DNA was then transformed into the repair minus *E.coli* strain BMH 71-18 mut S and mutants were selected by overnight culture in Ampicillin. A second round of transformation in the *E.coli* strain JM109, ensured appropriate segregation of mutant and wild type (WT) plasmids. Mutant CD59 and WT constructs were easily distinguishable due to the incorporation of a *Bam*HI restriction endonuclease site in the sequence of the MUT59 mutagenesis oligonucleotide (please refer to Section

Figure 5.2.12. Mutagenesis Of N-linked Glycosylation Consensus Sequence.

To test whether the binding of the HCC-1 mAb was dependent upon carbohydrate recognition, the solitary N-linked glycosylation consensus site of CD59 was modified to eliminate carbohydrate attachment. (please refer to text for details).

Mutagenesis of N-linked glycosylation consensus sequence: substitution of amino acid Ser ^{20} with Gly^{20}

N-linked glycosylation
consensus sequence (underlined)

5' - ACA GCC GTC AAT TGT TCA TCT GAT TTT GAT GCG TGT - 3' **Wild Type Strand**
thr^{15} ala^{16} val^{17} asn^{18} cys^{19} ser^{20} ser^{21} asp^{22} phe^{23} asp^{24} ala^{25} cys^{26}

5' - ACA GCC GTC AAT TGT GGA TCC GAT TTT GAT GCG TGT - 3' **Mutagenised Strand**
thr^{15} ala^{16} val^{17} asn^{18} cys^{19} gly^{20} ser^{21} asp^{22} phe^{23} asp^{24} ala^{25} cys^{26}

Introduction of *Bam* HI
restriction endonuclease site
(underlined)

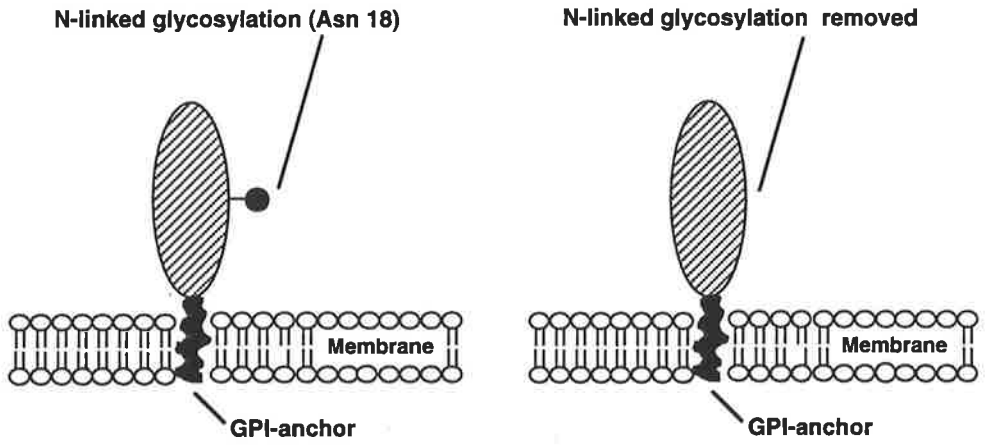
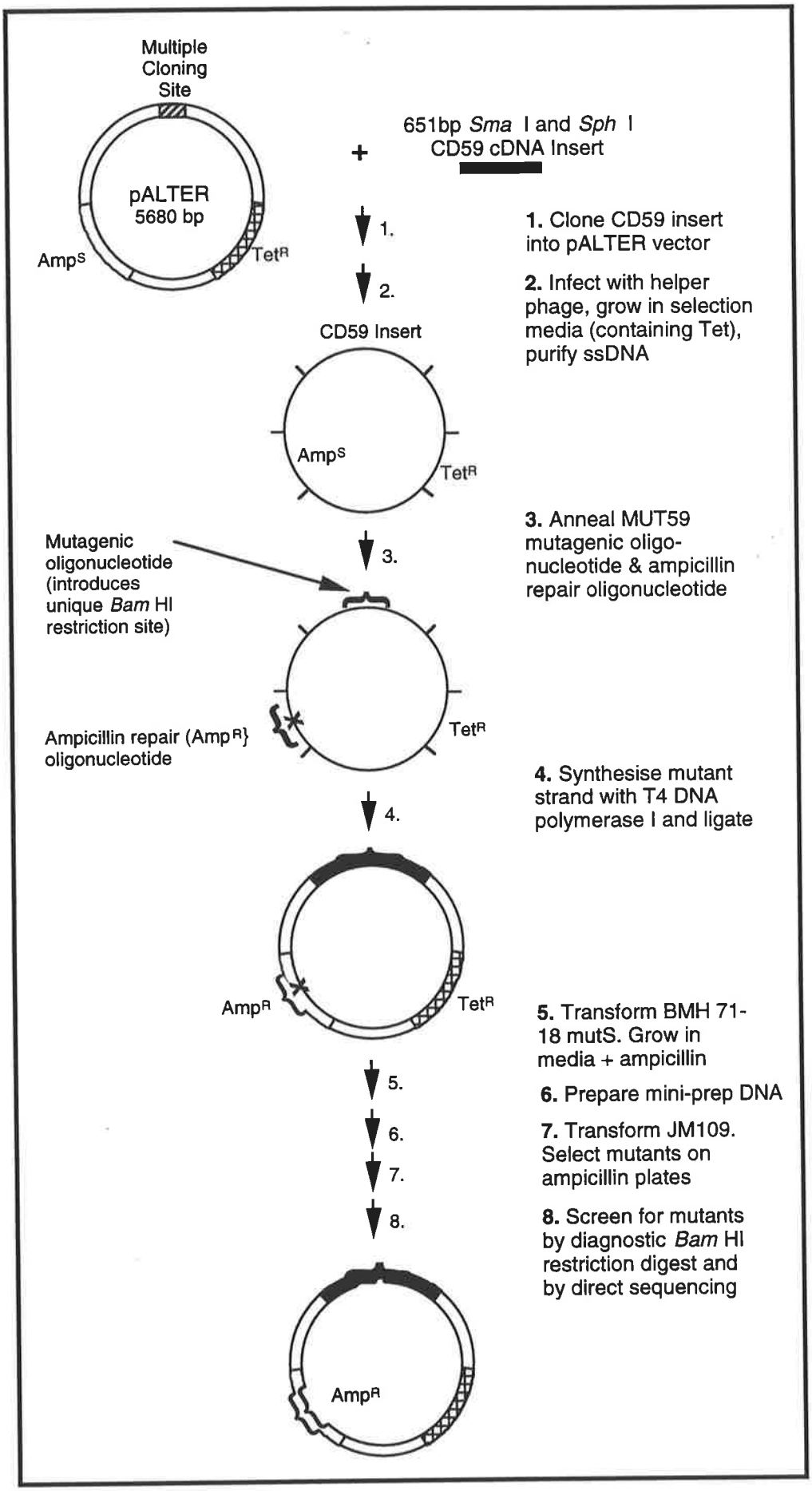


Figure 5.2.13. Schematic Representation Of The Altered Sites™ *In Vitro* Mutagenesis Protocol Used To Alter The N-Linked Glycosylation Site Of CD59

"Please refer to text for details".


Multiple Cloning Site
pALTER
5680 bp
Amp^S
Tet^R
651bp Sma I and Sph I
CD59 cDNA Insert
1.
2.
CD59 Insert
Amp^S
Tet^R
Mutagenic oligonucleotide (introduces unique Bam HI restriction site)
3.
Ampicillin repair (Amp^R) oligonucleotide
Tet^R
4.
Amp^R
Tet^R
5.
6.
7.
8.
Amp^R
1. Clone CD59 insert into pALTER vector
2. Infect with helper phage, grow in selection media (containing Tet), purify ssDNA
3. Anneal MUT59 mutagenic oligo-nucleotide & ampicillin repair oligonucleotide
4. Synthesise mutant strand with T4 DNA polymerase I and ligate
5. Transform BMH 71-18 mutS. Grow in media + ampicillin
6. Prepare mini-prep DNA
7. Transform JM109. Select mutants on ampicillin plates
8. Screen for mutants by diagnostic Bam HI restriction digest and by direct sequencing

2.21.3. (i)(b). and Figure 5.2.14.). Further confirmation of the alteration was achieved following sequence analysis of this region and found to concur with expectations (data not shown). To examine protein expression, the WT and mutant isoforms of CD59 were subcloned into the unique *Hpa*I and *Hind*III sites of pRUF.*neo* (please refer to Appendix A, Map #3). DNA was introduced into the transient ecotropic virus-producing cell line BOSC-23 by calcium phosphate transfection, and the murine factor-dependent cell line FDC-P1, was subsequently infected with recombinant retrovirus produced.

Following selection in G418, the MUT59 and WT59 FDC-P1 cells were immunolabelled with both mAb HCC-1 and mAb 2/24. As demonstrated in Figure 5.2.15. (A) & (B), a comparable binding phenotype was observed between the WT59 and MUT59 FDC-P1 cells, suggesting that HCC-1 binding was not carbohydrate-dependent. A uniform (or "normal") distribution of fluorescence was obtained when both the MUT59 and WT59 cell populations were immunolabelled with mAb 2/24 (Figure 5.2.16. (A) & (B)). In contrast (and somewhat surprisingly), when the cell populations were immunolabelled with mAb HCC-1, a bi-modal pattern of expression was observed. In each instance, only 14% of the MUT59 and WT59 cell population were found to express the HCC-1 epitope at high levels. This observation was consistent with the findings in Section 5.2.6. whereby magnetic bead selection, in conjunction with mAb HCC-1 was required to achieve a homogeneous level of HCC-1 expression. The apparent difference in the pattern of immunolabelling between these FDC-P1 transfectants with mAbs 2/24 and HCC-1 replicates what is observed with the haemopoietic cell line KG1a (please refer above, Section 5.2.8). Thus, the transfected WT59 and MUT59 FDC-P1 cell lines indicate that HCC-1 binding was not dependent upon carbohydrate, and furthermore, provide a model system which replicates the disparate binding pattern observed between HCC-1 and other CD59-defined mAbs on cells of the haemopoietic system.

(iii) Immunoprecipitation Of CD59 From Haemopoietic Cell Lines Reveals The Presence Of An 80 kD (gp80)- "Associated Protein".

Stefanova and Horejsi (1991), recently demonstrated that in addition to their respective antigens, mAbs against human GPI-linked leucocyte surface antigens CD59

Figure 5.2.14. Restriction Analysis Distinguishes The Wild Type (WT) And Mutant (MUT) CD59 Constructs.

The WT and MUT CD59 constructs (cloned into the *Hpa*I and *Hind*III sites of pRUF.*neo* vector) were subjected to *Bam*HI restriction endonuclease digestion, as described in the *Materials and Methods*. Samples were separated on a 1.2% agarose gel, ethidium bromide stained and photographed under ultraviolet illumination.

Mutant and WT CD59 constructs were easily distinguishable due to the incorporation of a *Bam*HI restriction endonuclease site in the sequence of the MUT59 mutagenesis oligonucleotide (please also refer to Figure 5.2.12..).

Lane M: 1 kb DNA Ladder
Lane 1: WT CD59
Lane 2: MUT CD59

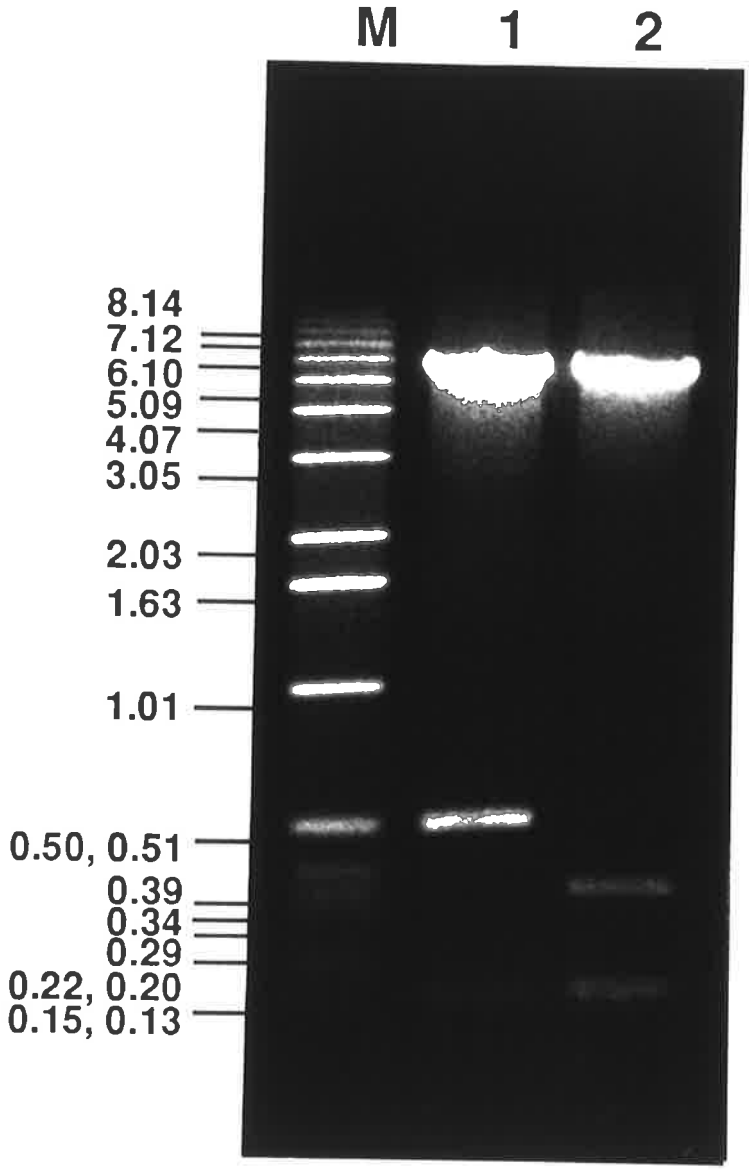

M
1
2
8.14
7.12
6.10
5.09
4.07
3.05
2.03
1.63
1.01
0.50, 0.51
0.39
0.34
0.29
0.22, 0.20
0.15, 0.13

Figure 5.2.15. The Binding Of mAb HCC-1 Is Not Carbohydrate-Dependent.

To examine protein expression, the WT and mutant constructs of CD59 were subcloned into the unique *Hpa*I and *Hind*III sites of pRUF.*neo*. DNA was introduced into the transient ecotropic virus-producing cell line BOSC-23 by calcium phosphate transfection, and the murine factor-dependent cell line FDC-P1, was subsequently infected with recombinant retrovirus produced.

Following selection in G418, the MUT59 and WT59 FDC-P1 cells were immunolabelled with either mAb HCC-1 or mAb 2/24 and protein expression examined by flow cytometry. Data is displayed as single-parameter fluorescence (FITC) histograms of 1 x 10^4 light-scatter gated events, collected as list mode data.

Panel A : WT CD59 transfectant
Panel B : MUT CD59 transfectant

..... : IgG_{2A} control mAb
·–·–·–· : IgG_{2B} control mAb
——— : mAb 2/24
——— : mAb HCC-1

A comparable binding phenotype was observed between the WT59 and MUT59 FDC-P1 cells, suggesting that HCC-1 binding was not carbohydrate-dependent.

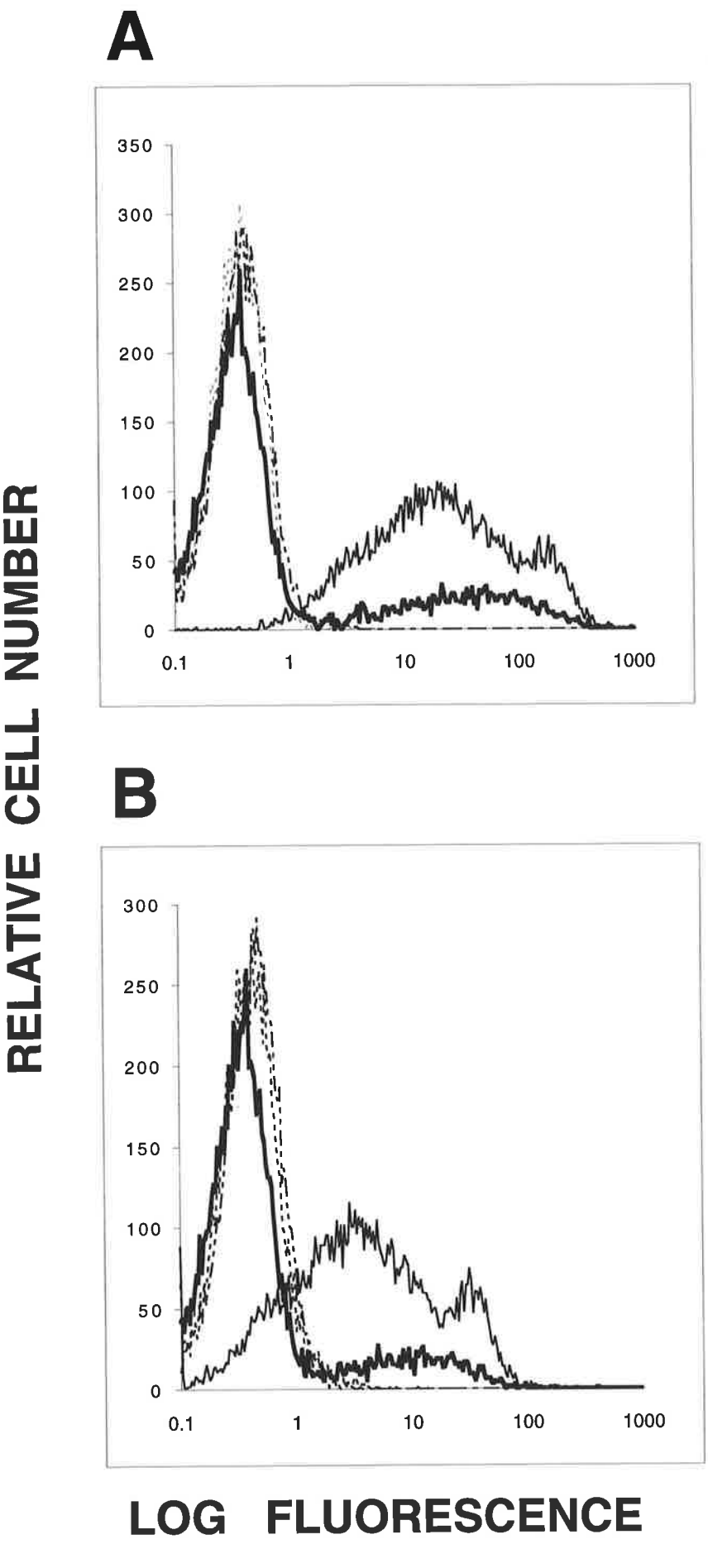
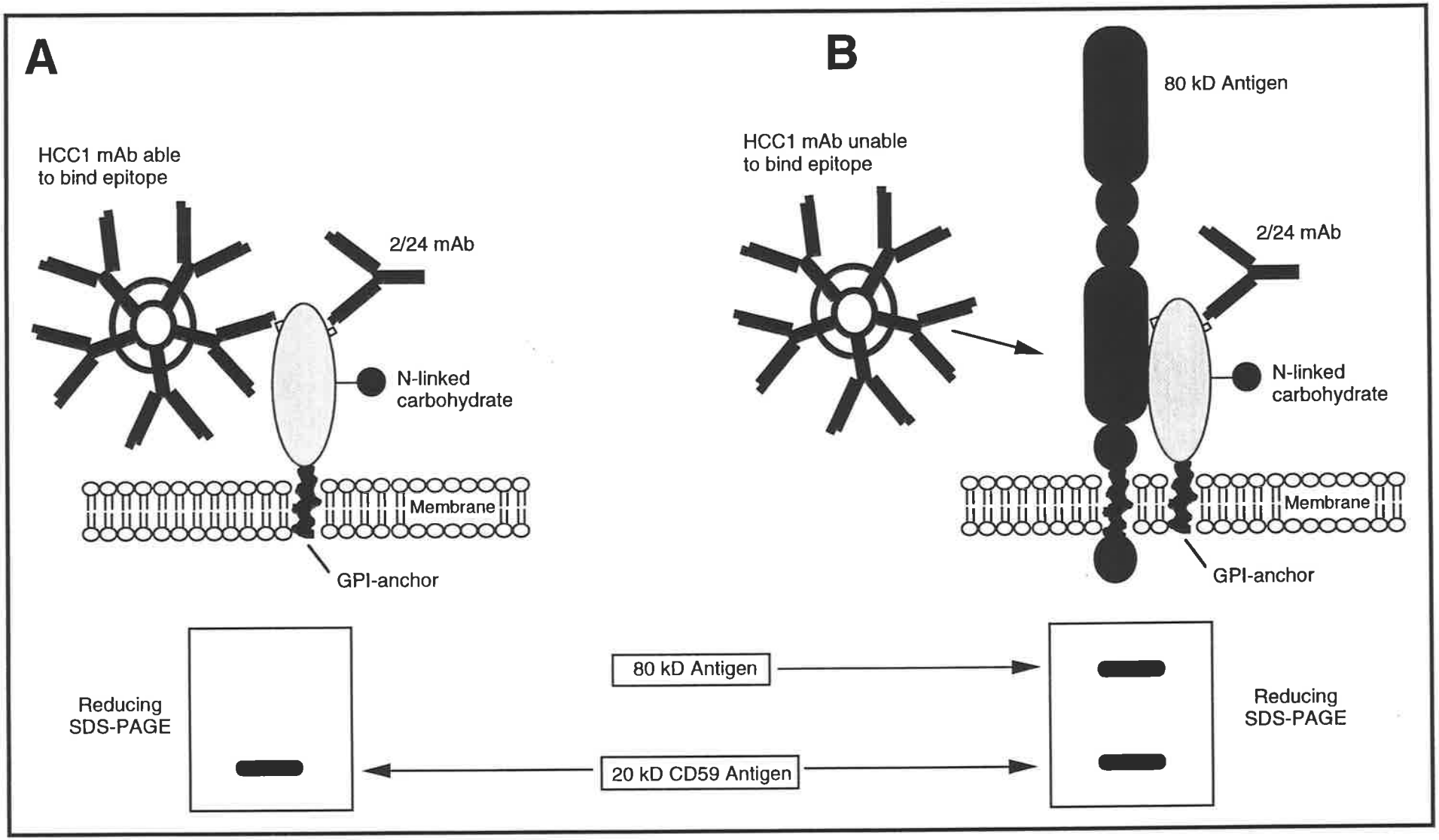

A
350
300
250
200
150
100
50
0
0.1
1
10
100
1000
B
300
250
200
150
100
50
0
0.1
1
10
100
1000
RELATIVE CELL NUMBER
LOG FLUORESCENCE

Figure 5.2.16. Schematic Representation Of The Protocol Used To Determine If Immunoprecipitation Of CD59 With mAb 2/24 Resulted In The Co-Precipitation Of An 80 kd "Associated" Molecule

Immunoprecipitates from cell lines which exhibit no reactivity with mAb HCC-1 will result in the coprecipitation of both CD59 (~20 kD) and a non-covalently-associated 80 kD molecule (gp80) [refer to **(B)**]. In contrast, cell lines which exhibit reactivity with HCC-1 will result in the immune-precipitation of CD59 only [refer to **(A)**].


A
HCC1 mAb able
to bind epitope
2/24 mAb
N-linked
carbohydrate
Membrane
GPI-anchor
B
HCC1 mAb unable
to bind epitope
80 kD Antigen
2/24 mAb
N-linked
carbohydrate
Membrane
GPI-anchor
Reducing
SDS-PAGE
80 kD Antigen
20 kD CD59 Antigen
Reducing
SDS-PAGE

and CD55 consistently immunoprecipitated a common 80 kDa glycoprotein component and (glyco)lipids from detergent lysates of the human T cell line, HPB ALL. These results were attributed to the formation of large, noncovalent complexes resistant to dissociation by mild detergents. The "GPI-complexes" were found to consist of the 80 kD glycoprotein, CD59 and CD55 glycoproteins, relatively tightly bound (glyco)lipids and possibly other so far unidentified components including proteins with tyrosine-kinase activity (Stefanova *et al*, 1991; Morgan *et al*, 1993; Lund-Johansen *et al*, 1993). Subsequent work (Kniep *et al*, 1994; Cinek and Horejsi, 1992), identified the major (glyco)lipid bearing the CD15, CDw17, and CDw65 determinants and co-immunopurifying with CD59 was the glycosphingolipid GM3. Although the nature of the 80 kD surface molecule has not been fully elucidated, studies by Stefanova *et al* (1991), have demonstrated that it is not CD44. Furthermore, recent studies by Horejsi and collegues (Stefanova and Horejsi, 1991; Cinek and Horesji, 1992) have demonstrated that the 80 kD protein may represent the recently described leukocyte antigen, CDw108 (Dr. V. Horejsi, personal communication), however a cDNA corresponding to this molecule has not been identified.

The observations detailed above, and the results obtained in Section 5.2.4., prompted an investigation of whether the existence of closely associated surface molecules could perturb the binding of HCC-1. As the mAb HCC-1 was unable to immunoprecipitate or Western blot the CD59 glycoprotein from detergent lysates (data not shown), this necessitated the design of an alternate strategy in order to test this hypothesis. As schematically represented in Figure 5.2.16., the mAb 2/24 was utilised to immunoprecipitate CD59 (and any associated cell surface molecules) from NP-40 detergent lysates prepared from a number of haemopoietic cell lines which exhibited disparate binding of HCC-1 and 2/24 mAbs. According to this hypothesis, immunoprecipitates from lysates which exhibit no reactivity with mAb HCC-1, should result in the coprecipitation of both CD59 (~20 kD) and gp80.

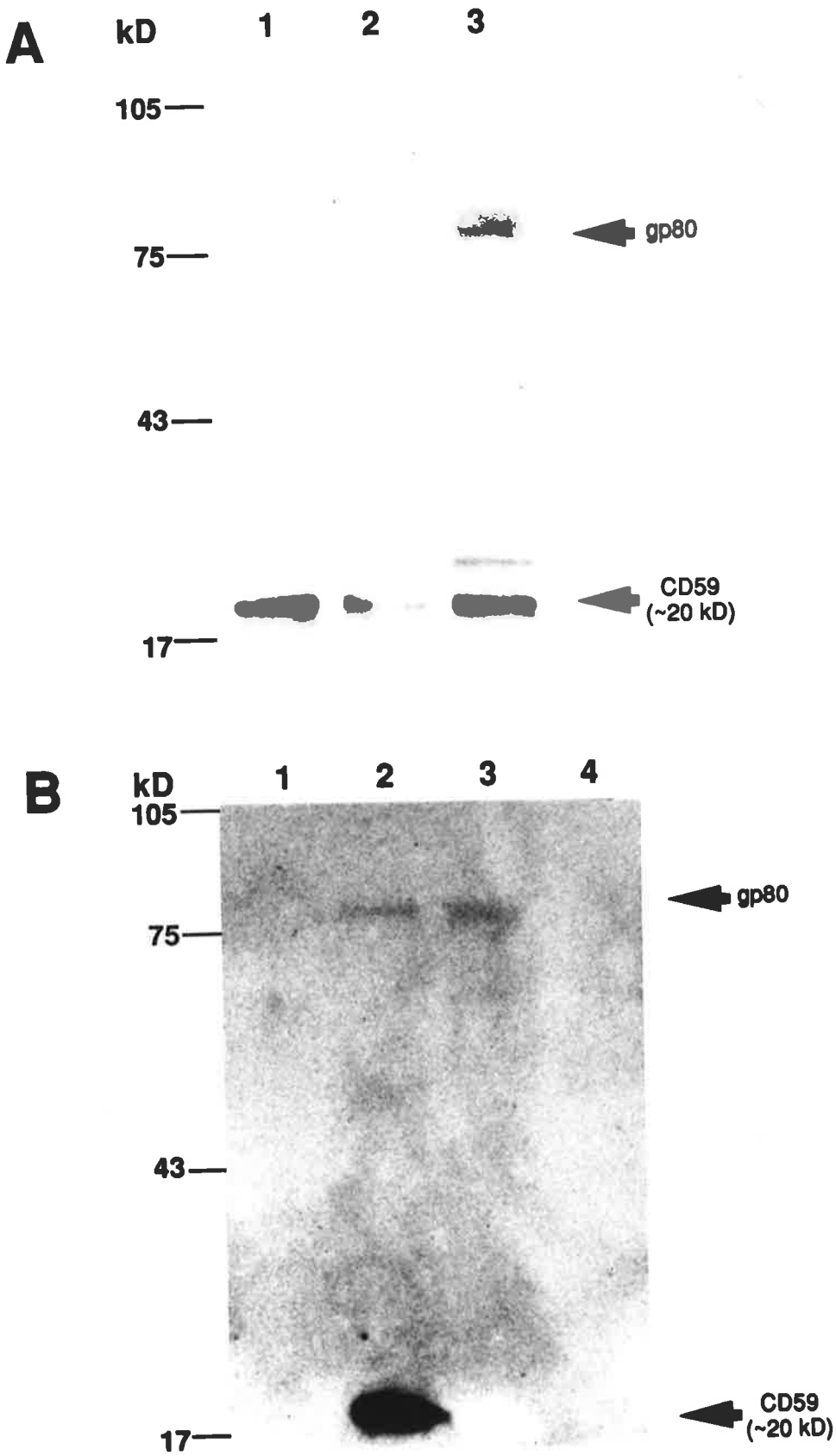
Consistent with this notion (please refer to Figure 5.2.17. (A)), immuno-precipitates from the myeloid cell line HL60 (lane 3), resulted in the co-precipitation of both the CD59 molecule (~20 kD) and a molecule of approximately 80 kD. In addition, immuno-precipitates from the erythroleukaemic cell line K562 (lane 1), resulted in only the

Figure 5.2.17. (A) & (B). An 80 kD "Associated" Molecule May Perturb HCC-1 Binding To The CD59 Molecule.

(A) Biotinylated membrane preparations of Hel-DR$^+$, K562, and HL-60 cells exhibiting disparate binding of HCC-1 and 2/24 were co-incubated with mAb 2/24 for 16 hours at 4°C. Immune complexes were precipitated using goat anti-mouse Sepharose, resuspended in reducing SDS-PAGE buffer, resolved on a 12% SDS-polyacrylamide gel and visualised with biotin-streptavidin-HRPO complex and enhanced chemiluminescence (ECL). (A) Lane 1, Hel-DR$^+$; Lane 2, K562; Lane 3, HL-60.

(B) Biotinylated membrane preparations of WT and MUT CD59 transfectants were co-incubated with mAb 2/24 (or isotype matched, non-binding control) for 16 hours at 4°C. Immune complexes were precipitated using goat anti-mouse Sepharose, resuspended in reducing SDS-PAGE buffer, resolved on a 10% SDS-polyacrylamide gel and visualised with biotin-streptavidin-HRPO complex and enhanced chemiluminescence (ECL).

Lane 1: WT CD59 FDC-P1 transfectants; non-binding control
Lane 2: WT CD59 FDC-P1 transfectants; mAb 2/24
Lane 3: MUT CD59 FDC-P1 transfectants; mAb 2/24
Lane 4: MUT CD59 FDC-P1 transfectants; non-binding control


A
kD
1
2
3
105
75
43
17
gp80
CD59
(~20 kD)
B
kD
1
2
3
4
105
75
43
17
gp80
CD59
(~20 kD)

20 kD CD59 species being resolved, in accord with the proposed model (Figure 5.2.16.) and immunophenotype of these cell lines (Figure 5.2.17. (A)). Moreover, the HEL-DR$^+$ erythroleukaemic cell line revealed an intermediate phenotype, with low but detectable levels of the protein which migrates at approximately 80 kD.

Moreover, biotinylated detergent lysates of the WT and MUT59 FDC-P1 transfectants immunoprecipitated with mAb 2/24, similarly resulted in the co-precipitation of an FDC-P1-derived molecule of approximately 80 kD (Figure 5.2.17. (B)). Although the 17-20 kD CD59 protein is apparent in the lysate from the WT transfectant, it is correspondingly absent in the MUT59 transfectant. This is due to the loss of carbohydrate decoration at Asn^{18}, and accompanied reduction in the molecular weight.

These results provide circumstantial evidence to support the hypothesis that the HCC-1 epitope is obstructed by a closely associated 80 kD glycoprotein (gp80) molecule which is upregulated as a function of differentiation. Although further studies are required to define the nature of this molecule, it is likely the equivalent to the molecule defined by Horejsi (personal communication).

5.3. DISCUSSION

5.3.1. Monoclonal Antibody HCC-1 Identifies An Epitope Expressed By Primitive Haemopoietic Progenitor Cells.

Monoclonal antibodies to haemopoietic cells have been employed to define a particular cell lineage or discrete stages of haemopoietic differentiation and/or activation. The expression of certain antigenic determinants on haemopoietic progenitor cells often correlate with their function and stage in ontogeny and if so, mAbs against these antigens can be used to separate the more primitive haemopoietic cells from their committed progeny within the BM (Andrews *et al*, 1989; Sutherland *et al*, 1989; Terstappen *et al*, 1991; Simmons, 1993; Barclay *et al*, 1993). The finding that all bone marrow progenitor cells were contained within the subset which is bound by mAbs to the CD34 molecule, has greatly facilitated their isolation and study (Civin *et al*, 1984; Ogawa, 1993).

The CD34 antigen is expressed on 1-4 % of the normal adult BM, and includes all the committed progenitors and their precursors (Civin *et al*, 1984; Katz *et al*, 1985; Andrews *et al*, 1986; Sutherland *et al*, 1989a; 1989b; Smith *et al*, 1991, Brandt *et al*, 1990). As haemopoietic cells differentiate, the gradual loss of CD34 expression is accompanied by the acquisition of lineage-restricted antigens (Strauss *et al*, 1986; Knapp *et al*, 1989). Moreover, studies have demonstrated that the $CD34^+$ population is not uniform, but is functionally heterogeneous (Civin *et al*, 1984; Katz *et al*, 1985; Andrews *et al*, 1986). Antibodies which further subset the $CD34^+$ cell population are of special interest as they provide the potential for the isolation of candidate haemopoietic stem cells.

The study detailed in this chapter describes one such mAb, HCC-1 which exhibits reactivity with a subset of the $CD34^+$ cell population (Figure 5.2.2.) which functionally exhibits mulipotentiality in both *in vitro* and *in vivo* assay systems. Initial studies performed by Swart (1993), and summarised herein, demonstrated that the HCC-1 antigen was expressed by cells which exhibited the capacity to initiate and maintain haemopoiesis in standard LTBMC and pre-CFU assay conditions (Table 5.2.1.). Swart (1993), also demonstrated that myeloid (CFU-GM) and erythroid (BFU-E) progenitors were recovered in both the $CD34^+HCC\text{-}1^+$ and $CD34^+HCC\text{-}1^-$ subpopulations, whilst

multipotential clonogenic cells (CFU-GEMM) were recovered only in the $CD34^+HCC\text{-}1^+$ population, suggesting that the HCC-1 antigen is lost upon differentiation (Figure 5.2.4.).

More recently, studies performed in collaboration with Drs B. Hill and B. Chen, (Hill *et al*, 1996) revealed that $CD34^+$ BM HPC exhibiting the highest level of HCC-1 staining were enriched 10-30 fold for cobble stone area forming (CAFC) activity relative to the $CD34^+HCC\text{-}1^{LO/-}$. Even more striking was the observation that the $CD34^+HCC\text{-}1^{LO/-}$ cells were unable to reconstitute BM grafts which had been subcutaneously implanted in immunodeficient (SCID-hu) mice. In contrast, the $CD34^+HCC\text{-}1^{HI}$ cells consistently supported multilineage engraftment potential, a capacity attributed only to the most primitive stem cells (Hill *et al*, 1996).

In contrast to the clear segregation of primitive stem cells by high level binding of the HCC-1 mAb, expression levels of the HCC-1 defined epitope did not completely segregate the thymus repopulating cells, since injection of either $CD34^+HCC\text{-}1^{HI}$ or $CD34^+HCC\text{-}1^{LO/-}$ cells resulted in foetal thymus engraftment. Despite this, the $CD34^+HCC\text{-}1^{LO/-}$ cells demonstrated a lower level and frequency of human thymus engrafting activity relative to $CD34^+HCC\text{-}1^{HI}$ cells (Hill *et al*, 1996). This is most likely due to a lower frequency, rather than a lesser potency, of the thymus engrafting cells within the $HCC\text{-}1^{LO/-}$ population since normal T lymphopoiesis was observed in the $CD34^+HCC\text{-}1^{LO/-}$ reconstituted grafts. As both stem cell and T cell progenitor potential can be measured using the SCID-hu thymus assay (Baum *et al*, 1992; Murray *et al*, 1995; Galy *et al*, 1995), the thymus engrafting activity observed in the case of the $CD34^+HCC\text{-}1^{LO/-}$ population (which is depleted of stem cells), is likely due to small numbers of $CD34^+Lin^-CD10^+$ lymphoid progenitors, a view consistent with the recent observation of Galy and collegues (personal communication). Therefore, the majority of $CD34^+$ progenitors which "read out" in the SCID-hu thymus assay express high levels of the HCC-1 epitope.

5.3.2. Monoclonal Antibody HCC-1 Identifies The Complement Regulatory Protein, CD59.

The finding that the antigen identified by mAb HCC-1 was expressed by BM stromal cells, facilitated the cloning of a cDNA corresponding to this CSM utilising the retroviral expression library, as described in Chapter 4. Significantly, HCC-1 was found to bind to a previously identified, ubiquitously expressed (Lachmann, 1991), 18-20 kD GPI-linked membrane protein most commonly referred to as CD59. This molecule was discovered independently in a number of laboratories (Sugita *et al*, 1989; Davies *et al*, 1989; Holguin *et al*, 1989; Whitlow *et al*, 1990; Taguchi *et al*, 1990; Tomita *et al*, 1991) and maps to human chromosome 11p13 (Heckl-Ostreicher *et al*, 1993). CD59 functions to protect cells against autologous complement attack (Rother *et al*, 1994; Davies and Lachmann, 1993), and has been postulated to prevent the complete unfolding of complement component C9, a process necessary for the subsequent binding of the additional C9 subunits needed to form the pore of the membrane attack complex (MAC) (Meri *et al*, 1990). Although some antibodies to CD59 have been described to neutralise the complement protective function of the CD59 molecule (Jokiranta and Meri, 1993), no deleterious effect of HCC-1 presence on cells in the *in vivo* and *in vitro* assays was observed. In each case, the $CD34^{+}HCC\text{-}1^{HI}$ cell population was at least as potent as the $CD34^{+}$ population and therefore indicates that HCC-1 binding does not block the complement protective function of CD59.

In addition to complement regulation, an adhesive function has also been ascribed to the CD59 molecule. In two independent studies (Deckert *et al*, 1992; Hahn *et al*, 1992), CD59 was identified as a second physiological ligand for the T cell glycoprotein CD2, whose interaction was independent of the adhesion/activation pathway mediated by CD2 and its counter-receptor CD58 (LFA-3). In addition, a recent study by Venneker and Asghar (1992), suggests that CD59 may also be involved in antigen presentation.

Previous studies describing the expression of CD59 by tissues of the haemopoietic system have demonstrated uniform expression by all cells, including BM-derived $CD34^{+}$ cells. In contrast, data presented here clearly demonstrate that the HCC-1-defined epitope of CD59 was found to be differentially expressed among $CD34^{+}$ progenitors with

the highest level present on a subset which is highly enriched for pluripotent stem cells (Figure 5.2.9.). This difference was again observed when a variety of haemopoietic cell lines were examined in a similar manner (Figure 5.2.10.). These data therefore suggest that the population of CD59 molecules expressed by $CD34^+$ cells and numerous cell lines is not homogeneous.

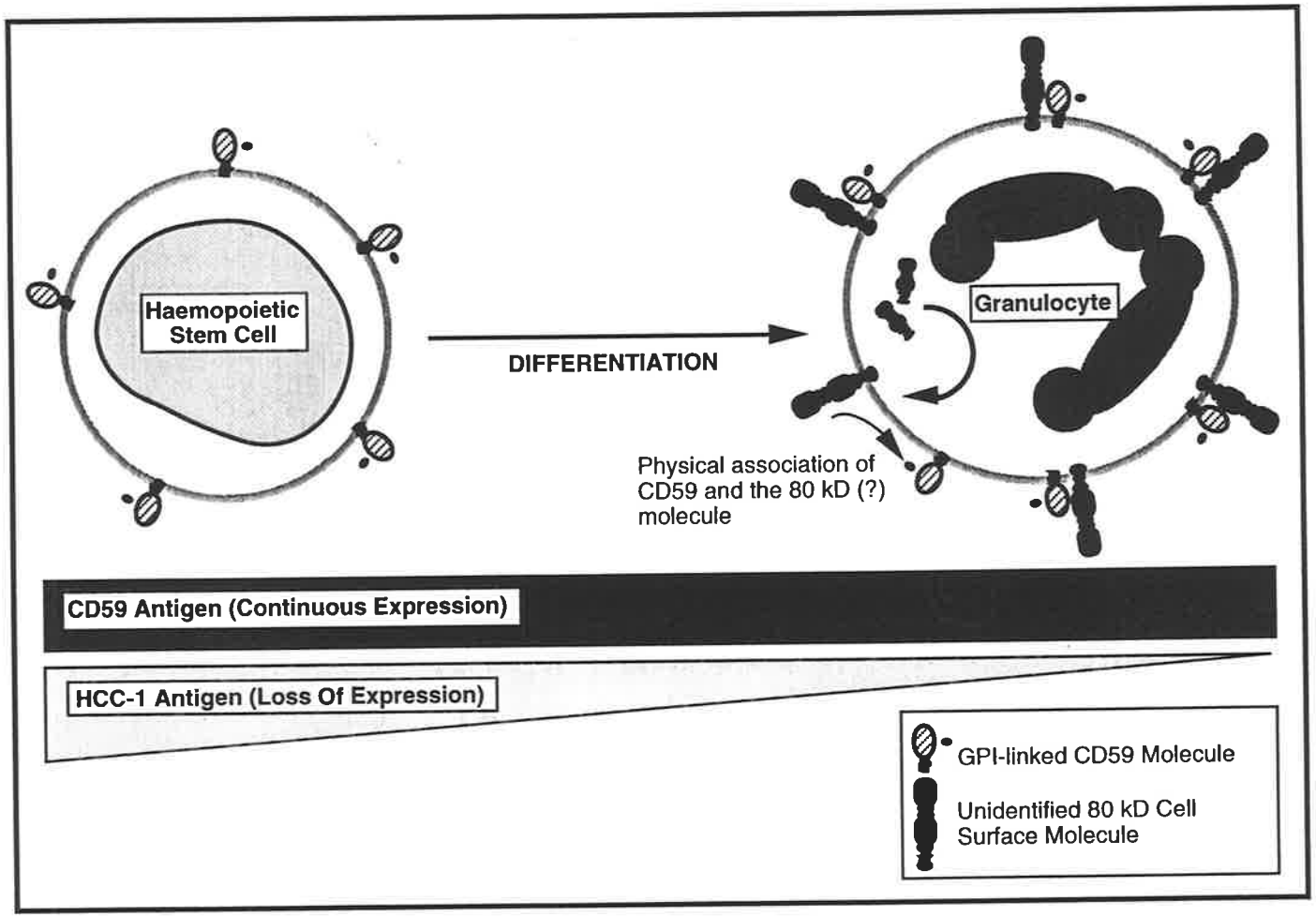
5.3.3. Mechanism For The Differential Expression Of The HCC-1 Epitope By Cells Of Haemopoietic Origin.

Studies designed to address the physical basis of the differential expression of the HCC-1 epitope on CD59 molecules initially contemplated the hypothesis that the mAb HCC-1 identified a variant carbohydrate-epitope whose expression was lost upon differentiation. *In vitro* mutagenesis of this site however, revealed that HCC-1 bound equally well to the mutagenised and wild type CD59 protein, thus indicating that carbohydrate recognition was not responsible for the differential expression of the HCC-1 epitope.

Subsequent analysis of detergent lysates supported the hypothesis that the binding of the HCC-1 mAb was influenced by a closely associated 80 kD glycoprotein molecule that, by inference, is expressed as a function of haemopoietic cell differentiation (Figure 5.3.1.). Previous studies (Stefanova and Horejsi, 1991; Stefanova *et al*, 1991; Cinek and Horejsi, 1992; Morgan *et al*, 1993; Kniep *et al*, 1994) have demonstrated that CD59 can form detergent-resistant complexes in which CD59 is non-covalently associated with other GPI-linked proteins such as CD55 and with an 80 kD glycoprotein component. Although the nature of the 80 kD surface molecule has not been fully elucidated, studies by Horejsi and collegues suggest that it may be equivalent to CDw108 (Dr. V. Horejsi, personal communication). Monoclonal antibody HCC-1 may therefore represent a probe for non-gp80-associated CD59 molecules. Future studies to unequivocally demonstrate that gp80 is responsible for inhibiting HCC-1 recognition of CD59 would require the molecular identification of a cDNA corresponding to this molecule. Furthermore, transfection of this cDNA into an appropriate cell type such as

Figure 5.3.1. The Loss Of HCC-1 Expression By Commited Progenitor Cells: Is It Due To The Acquisition Of A Non-Covalently-Associated 80 kD Molecule ?.

A schematic of the proposed molecular basis for the loss of HCC-1 expression by cells committed to both myeloid and lymphoid development. Based on the studies of Swart (1993) [please refer to Figure 5.2.3.] and those presented herein, HCC-1 expression may be lost due to the acquisition of an 80 kD protein, whose expression is upregulated as a function of differentiation (please refer to text for more details).


Haemopoietic Stem Cell
DIFFERENTIATION
Granulocyte
Physical association of CD59 and the 80 kD (?) molecule
CD59 Antigen (Continuous Expression)
HCC-1 Antigen (Loss Of Expression)
GPI-linked CD59 Molecule
Unidentified 80 kD Cell Surface Molecule

BM stromal cells (which exhibit strong, constitutive expression of the HCC-1 epitope on CD59 molecules), should result in the loss of HCC-1 reactivity.

As alluded to earlier, the carboxyl-terminal portion of the CD59 molecule is covalently linked to an oligosaccharide which, in turn, is glycosydically linked to phosphatidylinositol (PI) anchor. The GPI membrane attachment structure is utilised by a large number of cell surface proteins and occurs in a wide variety of eukaryotes from yeasts to mammals. Furthermore this structure is conserved in protozoa and mammals (Ratnoff *et al*, 1992). Recent data from a number of groups have confirmed that the first step of GPI-anchor biosynthesis is encoded by the PIG-A gene (Miyata *et al*, 1993, Miyata *et al*, 1994; Yamada *et al*, 1995). Mutations in this gene are responsible for the acquired clonal haematological disorder referred to as paroxysmal nocturnal hemoglobinuria (PNH), where there is a deficiency in the synthesis of the GPI molecules that anchor proteins to the cell membrane (Tomiyama *et al*, 1990; Shichishima *et al*, 1991; Schubert *et al*, 1990; reviewed in Rotoli and Luzatto, 1989).

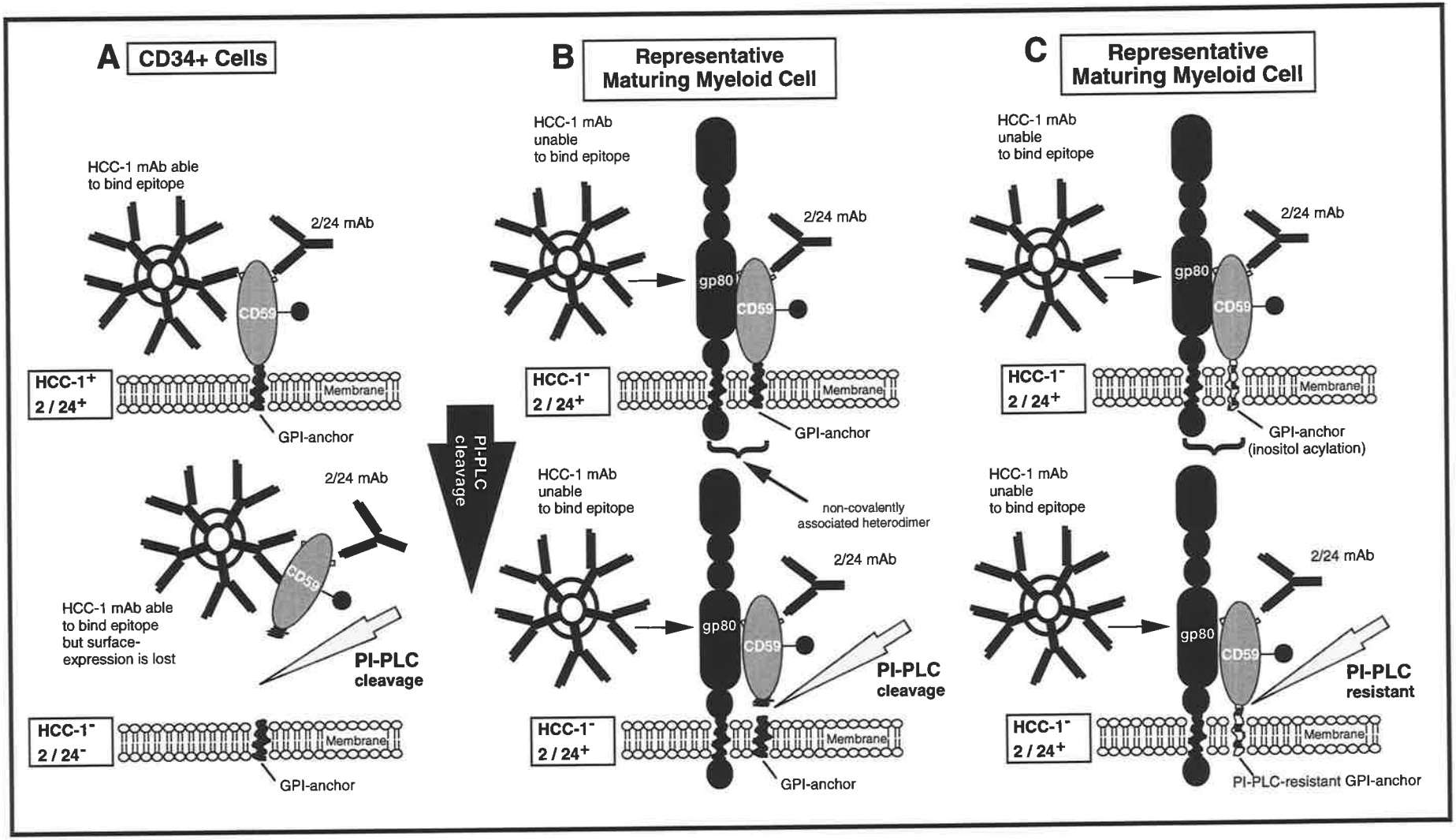
Phosphatidylinositol-specific phospholipase C (PI-PLC) treatment of BMMNCs, revealed that in most instances HCC-1 binding was lost/retained in accord with the loss/retention of the CD59 molecule. This however was not observed within the $CD34^+$ HPC population, where the complete loss of HCC-1, was not associated with a complete loss of CD59 expression. Notwithstanding that this maybe attributed to the phenomenon of inositol acylation (reviewed by Toutant *et al*, 1989), it is more likely due to the retention of CD59 at the cell surface due to its association with gp80 (or other molecules) present within these aforementioned macromolecular complexes (please refer to Figure 5.3.2.) (Stefanova and Horejsi, 1991; Stefanova *et al*, 1991; Cinek and Horejsi, 1992; Morgan *et al*, 1993; Kniep *et al*, 1994).

As this study with HCC-1 illustrates, mAbs provide sensitive probes of structural detail beyond the determination of the presence of a given molecule. Compared to previously described antibodies to CD59, the HCC-1 antibody reveals a subtle difference in the surface molecular detail of stem cells which can be correlated with a primitive functional phenotype. Moreover, this study clearly indicates that the assignment of a

Figure 5.3.2. PI-PLC Resistant CD59 Molecules: Is It Due To The Acquisition Of A Non-Covalently-Associated 80 kD Molecule Or The Use Of An Alternate PI-PLC-Resistant GPI-Anchor?

Proposed molecular basis for the PI-PLC-resistance observed with a proportion of the $CD34^+$ progenitor population. Based on the finding of Swart (1993) [please refer to Figure 5.2.3.] and those presented herein, HCC-1 expression may be lost due to the acquisition of an 80 kD protein, whose expression is upregulated as a function of differentiation (please refer to text for more details).

PI-PLC treatment of BMMNCs revealed that in most instances HCC-1 binding was lost/retained in accord with the loss/retention of the CD59 molecule (refer to Figure 5.2.13.). This however was not observed within the $CD34^+$ HPC population, where the complete loss of HCC-1 (Panel A), was not associated with a complete loss of CD59 expression. Although this maybe attributed to the phenomenon of inositol acylation (Panel C), it is more likely due to the retention of CD59 at the cell surface due to its association with gp80 (Panel B) present within these macromolecular complexes (please refer to text for more details).


A
CD34+ Cells
HCC-1 mAb able
to bind epitope
2/24 mAb
CD59
HCC-1+
2 / 24+
Membrane
GPI-anchor
2/24 mAb
HCC-1 mAb able
to bind epitope
but surface-
expression is lost
CD59
PI-PLC
cleavage
HCC-1-
2 / 24-
Membrane
GPI-anchor
PI-PLC
cleavage
B
Representative
Maturing Myeloid Cell
HCC-1 mAb
unable
to bind epitope
2/24 mAb
gp80
CD59
HCC-1-
2 / 24+
Membrane
GPI-anchor
HCC-1 mAb
unable
to bind epitope
non-covalently
associated heterodimer
2/24 mAb
gp80
CD59
PI-PLC
cleavage
HCC-1-
2 / 24+
Membrane
GPI-anchor
C
Representative
Maturing Myeloid Cell
HCC-1 mAb
unable
to bind epitope
2/24 mAb
gp80
CD59
HCC-1-
2 / 24+
Membrane
GPI-anchor
(inositol acylation)
HCC-1 mAb
unable
to bind epitope
2/24 mAb
gp80
CD59
PI-PLC
resistant
HCC-1-
2 / 24+
Membrane
PI-PLC-resistant GPI-anchor

cluster of differentiation predicated on the cellular immunoreactivity is unreliable, and a molecular cloning approach should be adopted wherever possible.

CHAPTER 6

IDENTIFICATION & FUNCTIONAL CLONING OF MGC-24, A MUCIN-LIKE MOLECULE EXPRESSED BY HAEMOPOIETIC PROGENITORS AND BONE MARROW STROMAL CELLS: A NEGATIVE REGULATOR OF HAEMOPOIESIS

6.1. INTRODUCTION

6.1.1. Mucins: An Emerging Family Of Adhesion Molecules.

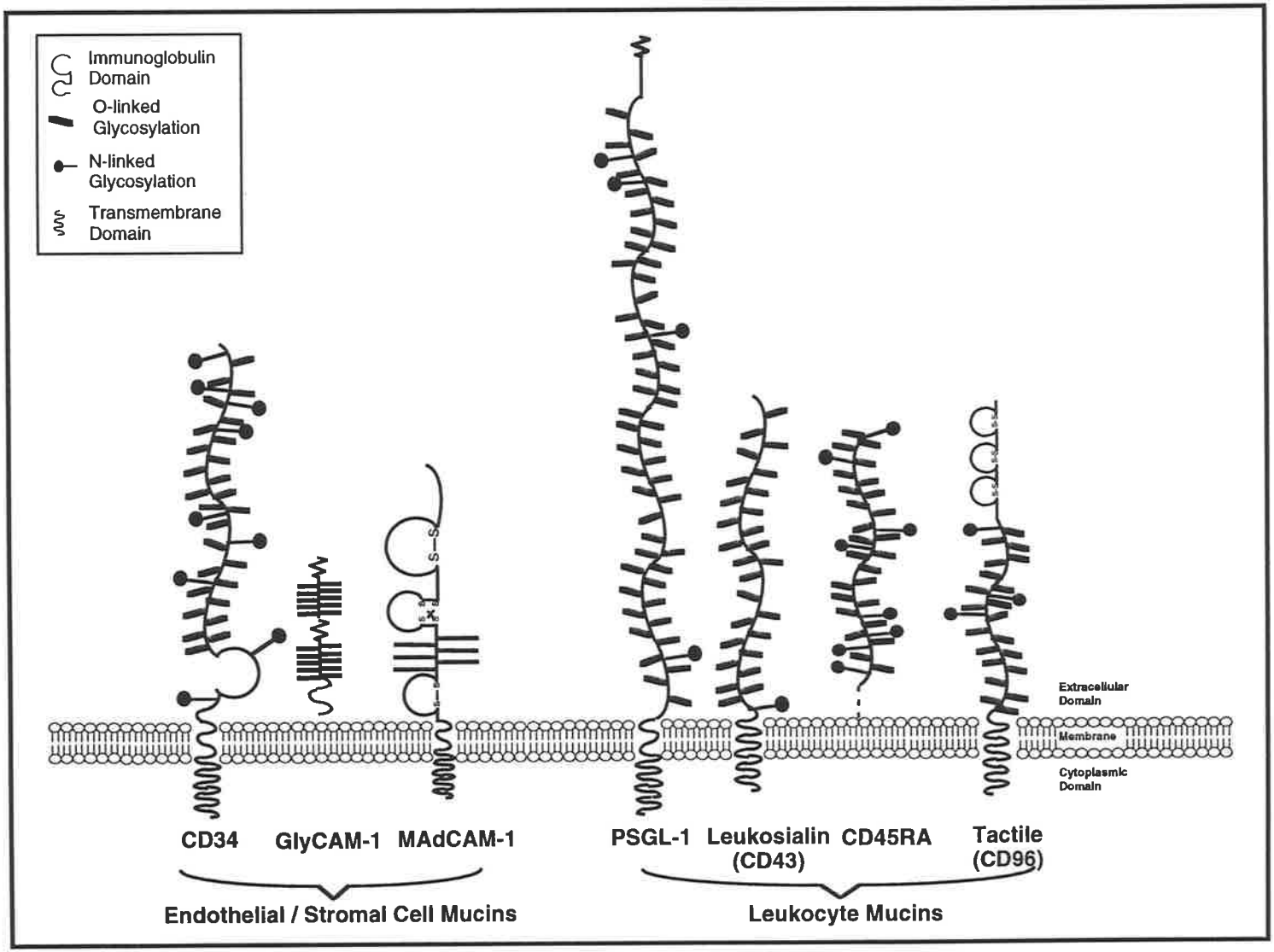
A wide variety of cell surface molecules participate in the regulation of haemopoiesis. Of these, cell adhesion molecules (CAMs) play a major role in mediating interactions between primitive haemopoietic progenitors (HPC) and various components of the bone marrow stroma. Based on domain structure and function, these CAMs can be grouped into 5 main families, including the immunoglobulin superfamily, integrins, cadherins, selectins and mucin-like molecules (Dianzani and Malavasi, 1995; Carlos and Harlan, 1994).

Mucin-like molecules represent an emerging family of glycoprotein molecules of the haemopoietic system, including CD34 (He *et al*, 1992; Imai *et al*, 1993), CD43 (leukosialin, sialophorin) (Shelley *et al*, 1986; Cyster *et al*, 1990; 1991; Manjunath *et al*, 1995), CD45 (Streuli *et al*, 1987; Matthews *et al*, 1989; Dianzani and Malavasi, 1995), CD68 (Holness and Simmons, 1993), CD96 (Tactile) (Wang *et al*, 1992), GlyCAM-1 (Lasky *et al*, 1992), PSGL-1 (Sako *et al*, 1993), and MAdCAM-1 (Briskin *et al*, 1993; Berg *et al*, 1993) (Figure 6.1.1.). Although exhibiting limited homology at the cDNA level, mucin-like molecules all share the common characteristic of being highly glycosylated polypeptides, containing predominantly O-linked carbohydrate side chains linked to serine and threonine residues (Jentoft, 1990; Devine and McKenzie, 1992; Springer, 1990b; Shimizu and Shaw, 1993). Their non-globular, thread-like structure, resembles that of "classical" mucins expressed at the surface of, and in the mucosal secretions of epithelial cells. Carbohydrate chains in mucins are predominantly attached by an α1,3 linkage between N-acetyl galactosamine and the oxygen atom of serine or threonine (O-glycosidic bond), although some oligosaccharides are attached by a linkage between the nitrogen of asparagine and N-acetylgalactosamine (N-glycosidic bond) (Jentoft, 1990; Devine and McKenzie, 1992).

The dense array of O-linked side chains in mucin-like molecules conveys at least

Figure 6.1.1. Endothelial And Leukocyte Mucin-Like Molecules.

A schematic representation of the mucin-like molecules expressed at the surface of endothelial/stromal cells and cells of haemopoietic origin. Although exhibiting limited homology at the cDNA level, mucin-like molecules all share the common characteristic of being highly glycosylated polypeptides containing predominantly O-linked carbohydrate side chains linked to serine and threonine residues. Their non-globular, thread-like structure, enables them to extend well beyond the glycocaylx (a glycoprotein and polysaccharide covering) that surrounds the cell.


Immunoglobulin Domain
O-linked Glycosylation
N-linked Glycosylation
Transmembrane Domain
Extracellular Domain
Membrane
Cytoplasmic Domain
CD34
GlyCAM-1
MAdCAM-1
PSGL-1
Leukosialin (CD43)
CD45RA
Tactile (CD96)
Endothelial / Stromal Cell Mucins
Leukocyte Mucins

two important structural implications which may influence function (Shimizu and Shaw, 1993). The first is the extended structure; the average extension per amino acid is predicted to be 2.5 Å, making many of the mucin-like molecules long enough to extend beyond the 100 Å (10 nm) glycoprotein and polysaccharide covering that surrounds the cell (glycocalyx). The second is the optimal exposure and high multiplicity of the terminal sugars. These two features make mucin-like molecules powerful "two-edge-swords"; they are both pro-adhesive and anti-adhesive (Shimizu and Shaw, 1993). By virtue of their negative charge and extended configuration, mucin-like glycoproteins may act as a repulsive barrier around the cell, however when an opposing cell has specific receptors for the mucin, adhesion surmounts repulsion.

This is well exemplified by the molecular partners for the members of the selectin family including the P-/E- selectin ligand, PSGL-1 (Sako *et al*, 1993) and the L-selectin ligands, GlyCAM-1 (Lasky *et al*, 1992), murine CD34 (Imai *et al*, 1990) and MAdCAM-1 (Briskin *et al*, 1993; Berg *et al*, 1993), which all mediate a rapid pro-adhesive "tethering" under conditions of flow.

The mucin-like molecules CD43, CD45RA and CD68 represent three mucin-like molecules that do not bind to selectin counter-receptors (Barclay *et al*, 1993). The macrophage restricted marker CD68 (Holness and Simmons, 1993; Rabinowitz and Gordon, 1991), is related to the family of acidic, highly glycosylated lysosomal proteins (LGPs) and is thought to protect cells from attack by hydrolases (reviewed in Fukuda, 1991). Although it remains to be confirmed, CD68 is also postulated to bind to tissue and organ-specific lectins which may allow macrophage subsets to home to a particular site (Holness and Simmons, 1993).

CD43 (leukosialin, sialophorin) is a transmembrane molecule expressed by numerous cells of the haemopoietic system, and represents the major sialoglycoprotein of T cells and their precursors (Shelley *et al*, 1986; Bazil *et al*, 1995; 1996). The extracellular domain is highly decorated with O-linked oligosaccharides (Pallant *et al*, 1989) and has been shown to interact with ICAM-1 (CD54) (Rosenstein *et al*, 1991). The cytoplasmic

domain of CD43 is highly conserved across species, implying an important signalling function (Pallant *et al*, 1989). Although constitutively phosphorylated on serine residues, cross-linking of CD43 co-stimulates T-cell proliferation and homotypic aggregation of leukocytes suggesting an ability to trigger "inside-out" signals which modulate cell adhesion (Pallant *et al*, 1989; Piller *et al*, 1991).

The leukocyte common antigen (CD45), as its name implies, is expressed by all nucleated cells of haemopoietic origin (Matthews *et al*, 1989). Although CD45 probably binds several ligands, only the B-cell antigen CD22 (a lectin-like adhesion molecule belonging to the immunoglobulin superfamily) has been identified (Clark, 1993). The intracytoplasmic domain of CD45 has tyrosine phosphatase activity and signals through this molecule are involved in B and T cell activation via the tyrosine kinase pathway (Pingel and Thomas, 1989; Ostergaard *et al*, 1989).

The studies presented in this Chapter, describe the isolation of a cDNA clone that encodes a novel mucin-like glycoprotein termed MGC-24v, expressed by both haemopoietic progenitor cells and elements of the bone marrow stroma. MGC-24v was identified using two novel mAb reagents (9E10 and 105.A5) and the expression cloning strategy described in Chapter 4. Both antibodies detected MGC-24v protein expression by subpopulations of the $CD34^+$ cells, which include the majority of clonogenic myeloid (CFU-GM) and erythroid (BFU-E) progenitors and the hierarchically more primitive precursors (pre-CFU). Biochemical and functional characterisation of MGC-24v, revealed that this protein exists as a homodimeric molecule of 160 kD, that is able to mediate the adhesion of $CD34^+$ cells to BM stroma. Finally, like PSGL-1 (Chapter 3), as yet undefined signals through the MGC-24v resulted in a suppression of *in vitro* haemopoiesis, a feature that appears common to mucin-like molecules.

6.2. RESULTS

6.2.1. Identification Of A Cell Surface Molecule (CSM) Expressed By Primitive Human Haemopoietic Cells And Cultured Human Bone Marrow Stromal Cells.

The murine IgG_3 mAb, 9E10 (generated as described in Chapter 4), was selected primarily on the basis of its immunoreactivity with cultured HBMSC and contrasting lack of immunoreactivity with peripheral blood (PB) mononuclear cells (PBMNC). In addition, 9E10 reacted with a minor population of BMMNC, characterised by low perpendicular light scatter (PLS) and low to moderate levels of forward light scatter (FLS), properties shared with cells which lie within the lymphocyte-blast region (Figure 6.2.1 (A)). Moreover, dual-parameter flow cytometric analysis revealed that a significant proportion of the $9E10^+$ BMMNCs co-expressed the CD34 antigen (Figure 6.2.1. (B)). A mean of 31.43 ± 3.25% (range 29.25%-35.90%; n=4) of the $CD34^+$ cells were found to co-express the 9E10 antigen ($CD34^+9E10^+$).

6.2.2. Reactivity Of 9E10 With Lineage Restricted Clonogenic Progenitors.

To determine whether lineage-restricted haemopoietic progenitor cells were present in the population of bone marrow cells which reacted specifically with 9E10, *in vitro* clonogenic assays were performed on BMMNC-derived, FACS isolated $CD34^+9E10^+$ and $CD34^+9E10^-$ fractions (Figure 6.2.1. (B)). Notably, essentially all the detectable myeloid (CFU-GM), erythroid (BFU-E) and multipotential colony forming cells (CFU-Mix), were recovered in the population of cells which expressed the antigen recognised by the mAb 9E10 (Figure 6.2.2.).

6.2.3. The 9E10 Antigen Is Expressed By Primitive Human Haemopoietic Cells.

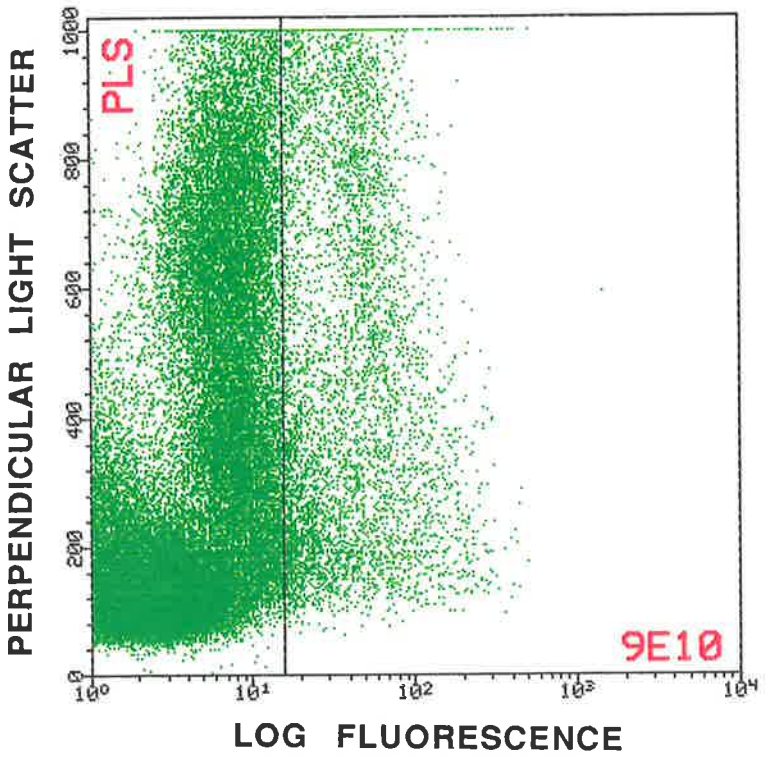
Previous studies have demonstrated that primitive multipotential blast colony-forming cells and cells that initiate long-term haemopoiesis *in vitro* are restricted to a minor proportion of $CD34^+$ cells showing low to undetectable expression of CD33

Figure 6.2.1.

(A) Dot plot demonstrating that 9E10 reacts with a minor population of BMMNC, characterised by low perpendicular light scatter (PLS) and low to moderate levels of forward light scatter (FLS), properties shared with cells which lie within the lymphocyte-blast region.

(B) Dual-parameter immunofluorescence analysis demonstrating the expression of CD34 and 9E10 antigen by BMMNCs. SBA-depleted BMMNC were stained with the directly conjugated mAb HPCA-2-PE (α-CD34) and 9E10 (detected with anti-IgG_3-FITC). Data are displayed as dual-parameter histograms of 5 x 10^4 light-scatter-gated events collected as list-mode data.

A


PERPENDICULAR LIGHT SCATTER
PLS
9E10
LOG FLUORESCENCE

B

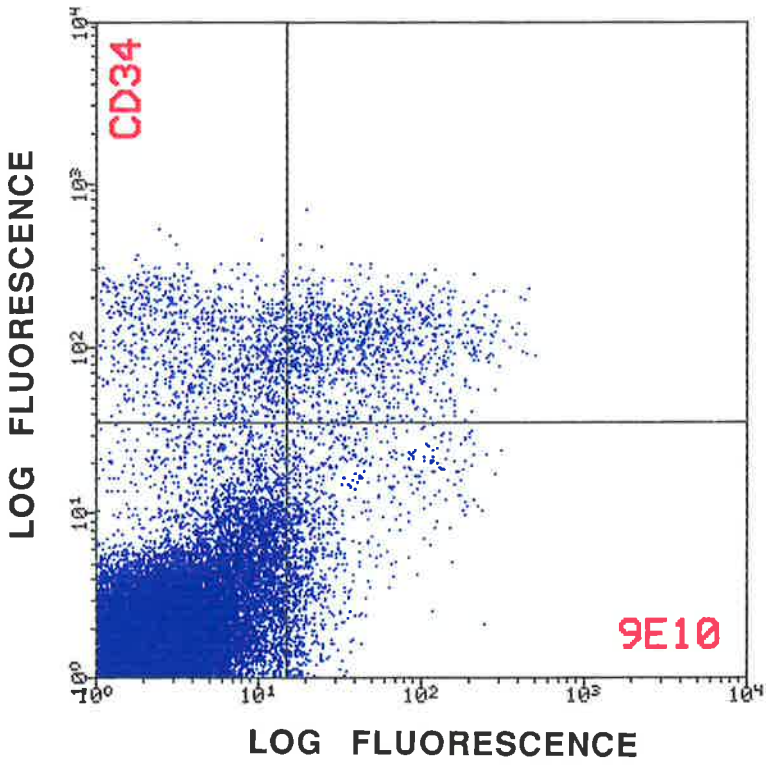
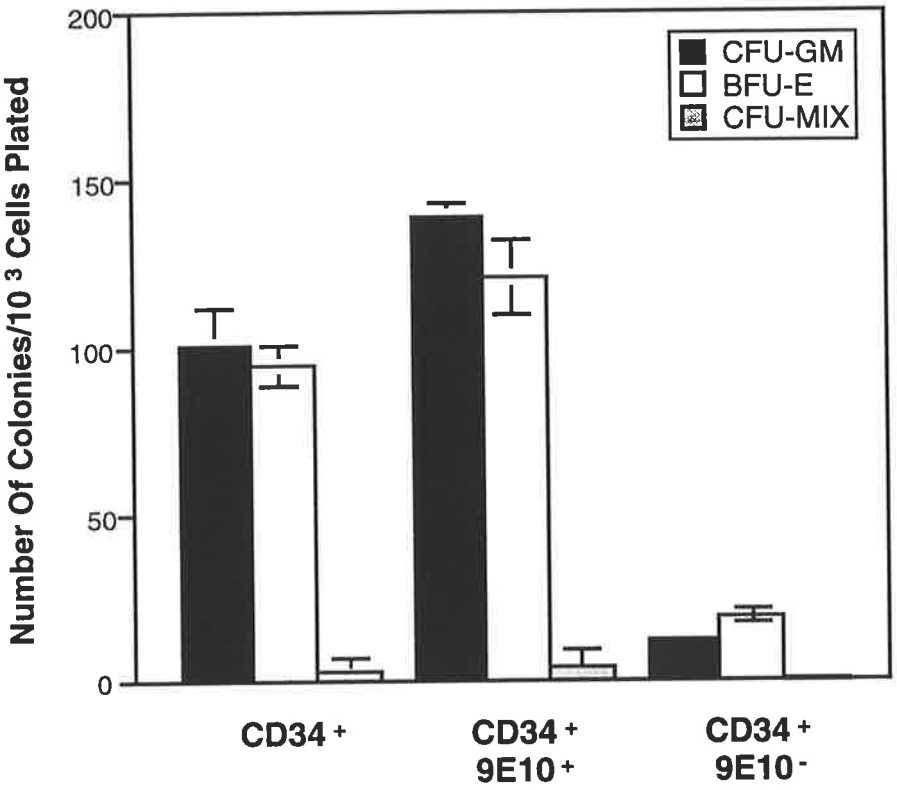

LOG FLUORESCENCE
CD34
9E10
LOG FLUORESCENCE

Figure 6.2.2. Assay Of Clonogenic Progenitors In Populations Sorted On The Basis Of CD34 And 9E10 Antigen Expression.

Fluorescence activated cell sorting (FACS) of the $CD34^+$, $CD34^+9E10^+$, and $CD34^+9E10^-$ subpopulations demonstrates that clonogenic progenitors (CFU-GM, ■, BFU-E, □ and CFU-Mix ▨) are present almost exclusively in the $CD34^+9E10^+$ subpopulation. Results are expressed as the number of CFU-GM at day 14 per 1×10^3 cells plated. Data represents the mean ± S.E. (n=3).


Number Of Colonies/10 3 Cells Plated
200
150
100
50
0
CFU-GM
BFU-E
CFU-MIX
CD34 +
CD34 +
9E10 +
CD34 +
9E10 -

(Andrews *et al*, 1989), CD38 (Terstappen *et al*, 1991), HLA-DR (Brandt *et al*, 1990), or any of a number of lineage-restricted antigens (Baum *et al*, 1992; Udomsakdi *et al*, 1992). Moreover, these cells express low levels of the GPI-linked molecule Thy-1 (Baum *et al*, 1992; Craig *et al*, 1993), and high levels of the proto-oncogene, c-kit (Simmons *et al*, 1994). Therefore, to further characterise the $CD34^+9E10^+$ population, BM $CD34^+$ cells were prepared with 561-Dynabeads (refer to *Materials and Methods*, Section 2.10.5. (b)), and subsequently immunolabelled by two-colour immunofluorescence, using biotinylated 9E10 (followed by streptavidin-PE) in combination with each one of a panel of FITC-conjugated mAbs reactive with lineage-restricted or activation antigens. As shown in Figure 6.2.3 (A) & (B) and Table 6.2.1 (data from a representative experiment), essentially all of the $CD34^+HLA\text{-}DR^{LO/-}$ and $CD34^+CD38^{LO/-}$ [please refer to Figure 6.2.3. (B)] cell fractions express the 9E10 antigen. In addition, CDw90 (Thy-1) expression was more pronounced within the $CD34^+9E10^+$ (compared with the $CD34^+9E10^-$) population, whilst the 9E10 antigen was found to be expressed by essentially all the $CD33^{LO}$ (and $CD33^-$) and the $CD71^{LO}$ (but not $CD71^-$) subpopulations. Finally, a majority of the $CD34^+9E10^+$ cells were found to coexpress CD117 (c-kit), thus confirming that most of the earliest $CD34^+$ haemopoietic progenitor cells identifiable in BM, express the antigen identified by mAb 9E10.

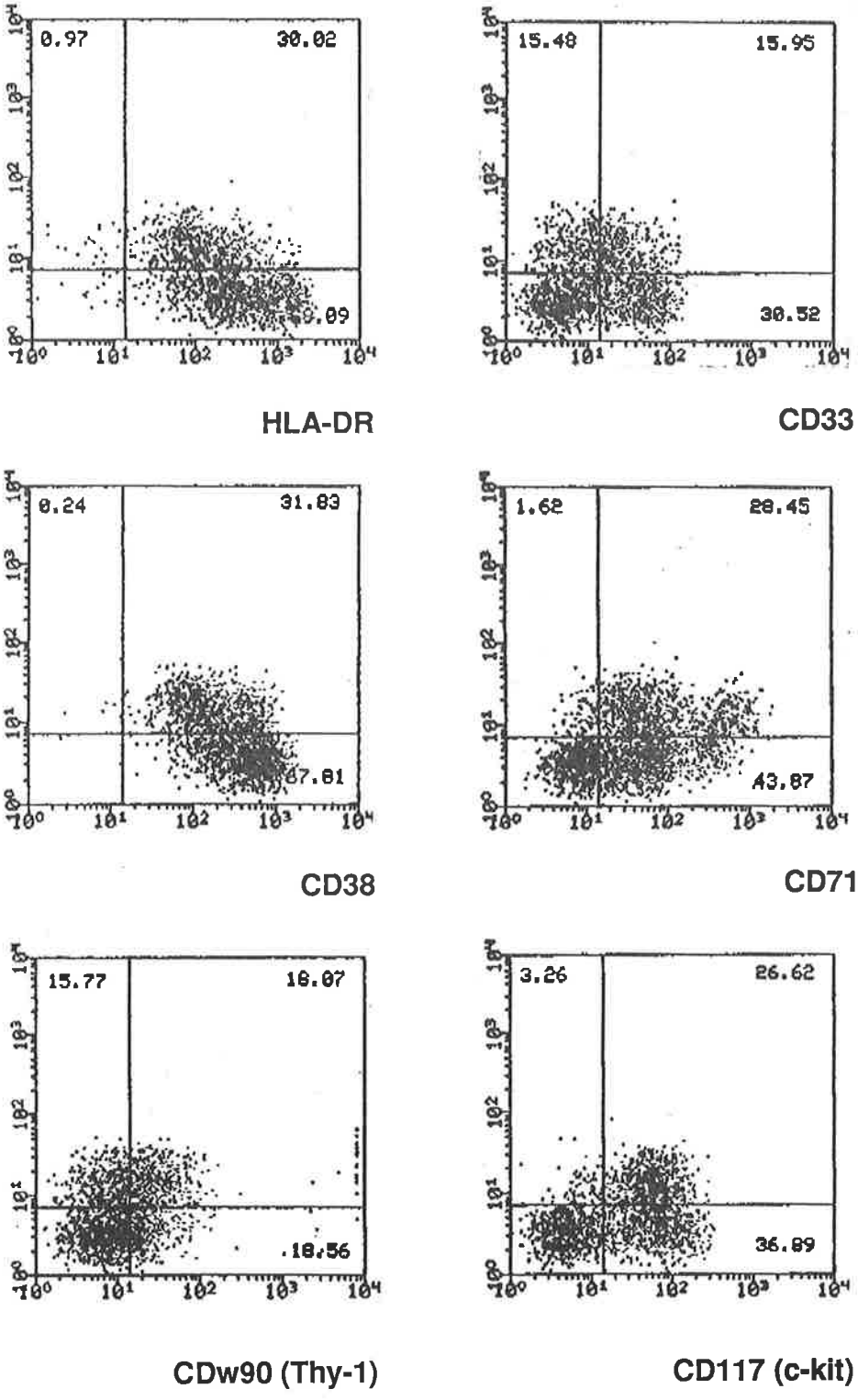
This was subsequently confirmed at a functional level, when $CD34^+9E10^+$ and $CD34^+9E10^-$ fractions were isolated from normal adult BMMNC and assayed for their ability for *de novo* generation of CFU-GM and nucleated cell production in the cytokine driven stromal cell-free suspension culture (pre-CFU) assay. Figure 6.2.4. (A) & (B), illustrate respectively, the production of total haemopoietic cells and CFU-GM over time in culture, from the various sorted populations. The $CD34^+9E10^-$ cells failed to generate CFU-GM in excess of those present in the input population, whilst an 85-fold expansion in the number of clonogenic cells was achieved with the $CD34^+9E10^+$ sorted population, within the 28-day period of the pre-CFU assay (Figure 6.2.4. (B)). Moreover, the $CD34^+9E10^+$ population displayed a two-fold greater capacity to produce nucleated

Figure 6.2.3. (A) Dual-Colour Immunofluorescence Analysis Demonstrating The Co-Expression Of The 9E10 Antigen On Multiple Myeloid And Lymphoid Cell Populations.

(A) 561-Dynabead purified BM-derived $CD34^+$ (refer to *Materials and Methods*, 2.10.5. (b)), were immunolabelled with biotinylated 9E10 mAb (subsequently detected with streptavidin-PE) in combination with FITC-conjugated mAbs to HLA-DR, CD33, CD38, CD71, CDw90 and CD117, or with appropriate isotype-matched FITC conjugated non-binding control antibodies. Each 2-parameter histogram was generated from 2×10^4 $CD34^+$ events collected as list mode data using a Profile II flow cytometer and analysed using Coulter ELITE software. Data represent the mean ± SE of 3 experiments.

(B) (***Please see next page***) Dual-parameter immunofluorescence analysis demonstrating the expression of CD38 and 9E10 antigens by $CD34^+$ cells was performed essentially as described above, with the exception that biotinylated 9E10 mAb was detected with streptavidin-FITC and a PE-conjugated mAb to CD38 (Leu17-PE) was utilised. This afforded greater resolution of CD38 expression by the $CD34^+$ cell population. Data are displayed as dual-parameter histograms of 2×10^4 light-scatter-gated events collected as list-mode data.

A


9E10 ANTIGEN
0.97
30.02
.09
HLA-DR
15.48
15.95
30.52
CD33
0.24
31.83
67.81
CD38
1.62
28.45
43.87
CD71
15.77
18.07
18.56
CDw90 (Thy-1)
3.26
26.62
36.89
CD117 (c-kit)
LINEAGE ANTIGEN

B

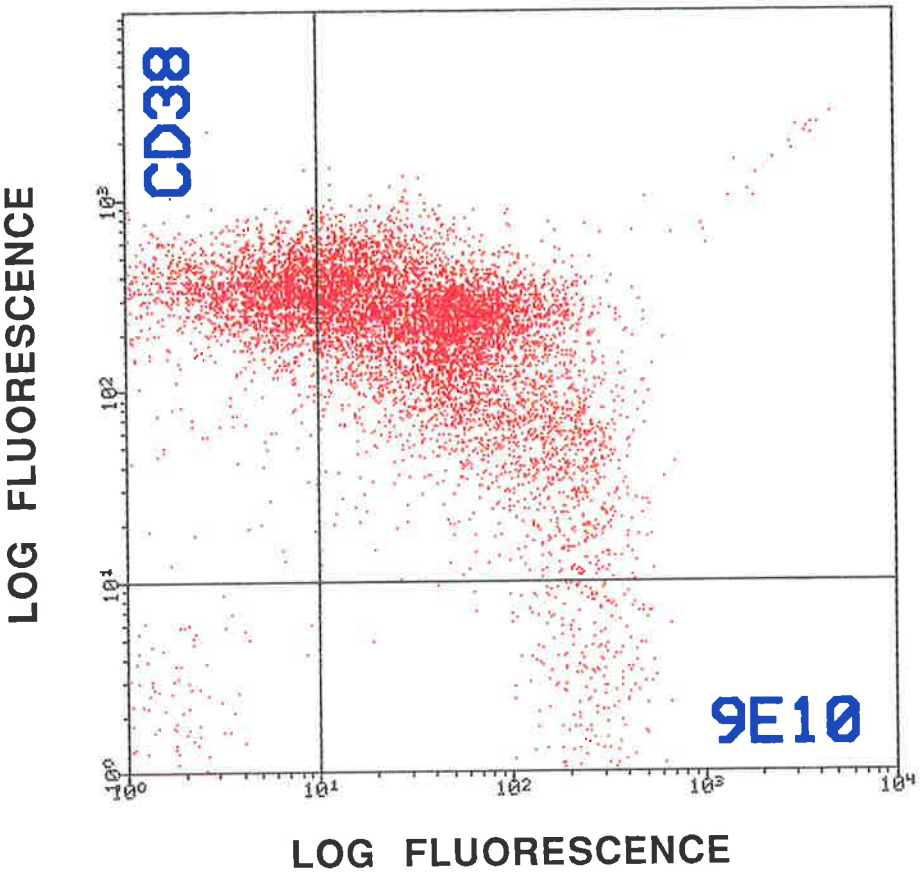

CD38
9E10
LOG FLUORESCENCE
LOG FLUORESCENCE
10⁰
10¹
10²
10³
10⁴

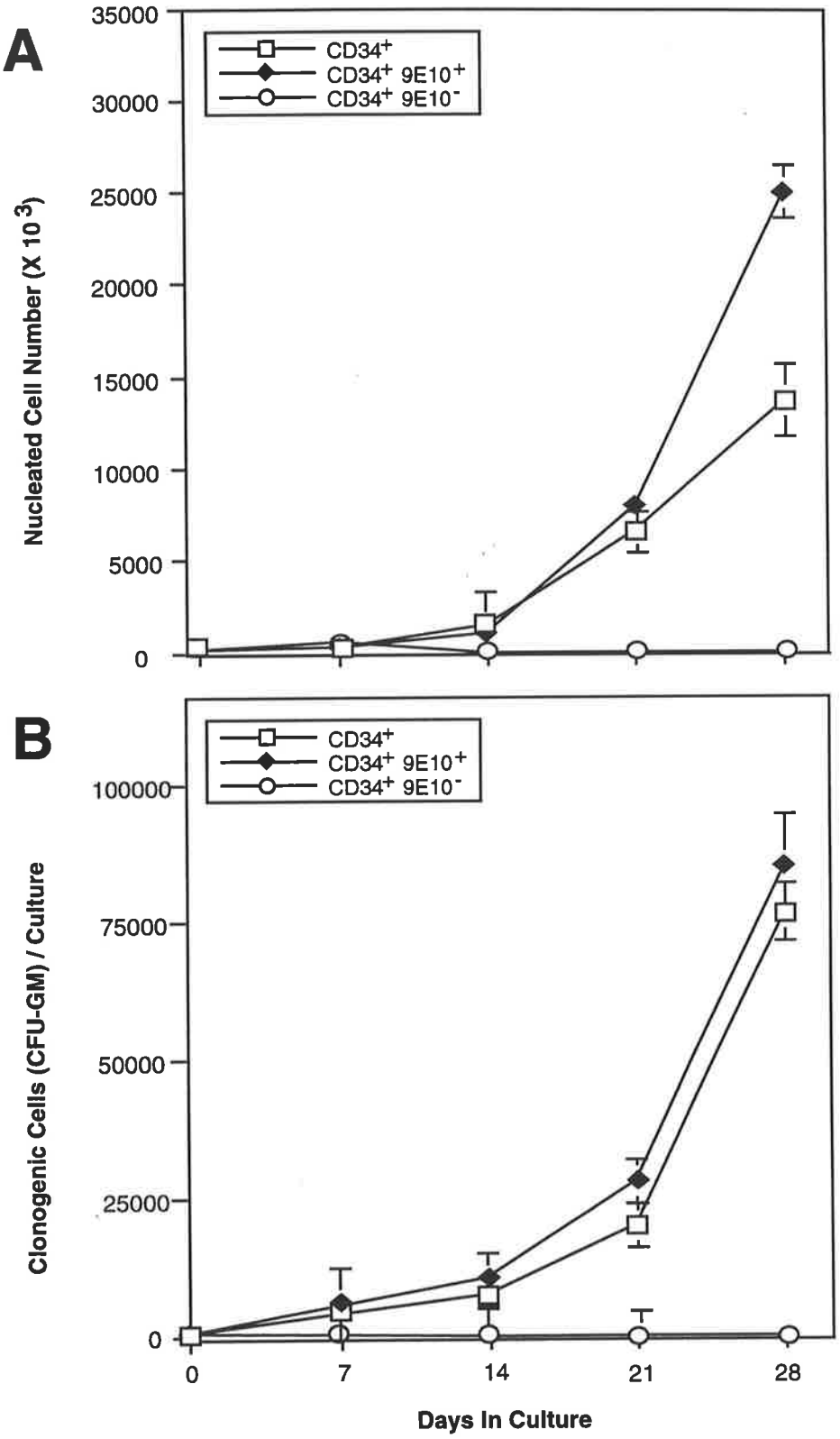
Table 6.2.1. Distribution Of 9E10 Antigen On $CD34^+$ Cell Subsets.

Antibody	Expression Of Lineage Antigen By $CD34^+$ $9E10^+$ BM Mononuclear Cells	Expression Of 9E10 By Cells Which Express Both CD34 And Lineage Antigen
Quandrant Number	Region 2 / (1 + 2) - Expressed as Percentage Positive	Region 2 / (2 + 4) - Expressed as Percentage Positive
CD33	50.7	34.3
CD38	99.2	31.4
CD71	94.6	39.4
CDw90	53.4	49.3
CD117	89.1	41.9
HLA-DR	96.8	30.5

Summary of the data obtained from dual-colour immunofluorescence and flow cytometric analysis (please also refer to Figure 6.2.3.) demonstrating the co-expression of 9E10 with early myeloid and lymphoid populations on $CD34^+$ cells. Information generated from listmode data, and represent 1×10^4 $CD34^+$ sorted events. A representative experiments (one of three) is shown.

Figure 6.2.4. (A) & (B). $CD34^+$ Cells Initiating Haemopoiesis In The Cytokine-Supplemented (pre-CFU) Assay Express The 9E10 Antigen.

BMMNC were sorted into $CD34^+$ (□) $CD34^+9E10^+$ (◆), $CD34^+9E10^-$ (○), subpopulations (as described in Figure 6.2.2.) and assayed for their ability to initiate and maintain haemopoiesis in a stroma-independent, cytokine supplemented culture. Cultures were established in triplicate using 1×10^3 sorted cells per well in medium supplemented with 10 ng/ml each of purified recombinant human IL-1β, IL-3, IL-6, G-CSF, GM-CSF and SCF. Additional factors were added at the same concentrations on days 7, 14 and 21. On days 7, 14 and 21, the cells were harvested, washed and assayed for nucleated cell number and CFU-GM as previously described. The results are expressed as the mean number (± SE) of nucleated cell number **(A)** and CFU-GM **(B)**, recovered at day 7, 14, 21 and 28 for each group. A representative experiment (one of three) is shown.


A
B
CD34+
CD34+ 9E10+
CD34+ 9E10-
Nucleated Cell Number (X 10 3)
Clonogenic Cells (CFU-GM) / Culture
35000
30000
25000
20000
15000
10000
5000
0
100000
75000
50000
25000
0
0
7
14
21
28
Days In Culture

cells than the comparable $CD34^+$ population (Figure 6.2.4. (A)). These data demonstrate that the majority, if not all the pre-CFU are present within the $CD34^+9E10^+$.

6.2.4. The Nature Of The CSM Identified By mAb 9E10.

(i) Biochemical Characterisation Of The 9E10 Antigen.

To ascertain the size of the CSM identified by mAb 9E10, membrane proteins from a variety of haemopoietic and non-haemopoietic cell lines and cell preparations were separated by denaturing SDS-polyacrylamide gel electrophoresis under reducing and non-reducing conditions. Following transfer to nitrocellulose, the filters were incubated with 9E10 mAb supernatant and immunoreactive proteins detected by enhanced chemiluminescence (ECL) as described in the *Materials and Methods* (Section 2.29.5.). As demonstrated in Figure 6.2.5. (A), mAb 9E10 identified two differentially migrating species with an estimated molecular mass of 80 and 160 kD respectively, under non-reducing conditions. Upon reduction however, the intensity of the 160 kD immunoreactive protein was considerably reduced, suggesting that the antigen recognised by the 9E10 mAb may exist as a homodimer of two 80 kD monomers (Figure 6.2.5. (B)). The protein recognised by the 9E10 mAb was expressed in a majority of the cells tested, with the exception of peripheral blood erythrocytes, keratinocytes, Jurkat T cells and PB-derived B cells. Interestingly, the molecular mass of the 9E10 CSM differed marginally amongst the different cells tested, most likely due to cell-specific differential glycosylation.

(ii) Molecular Characterisation

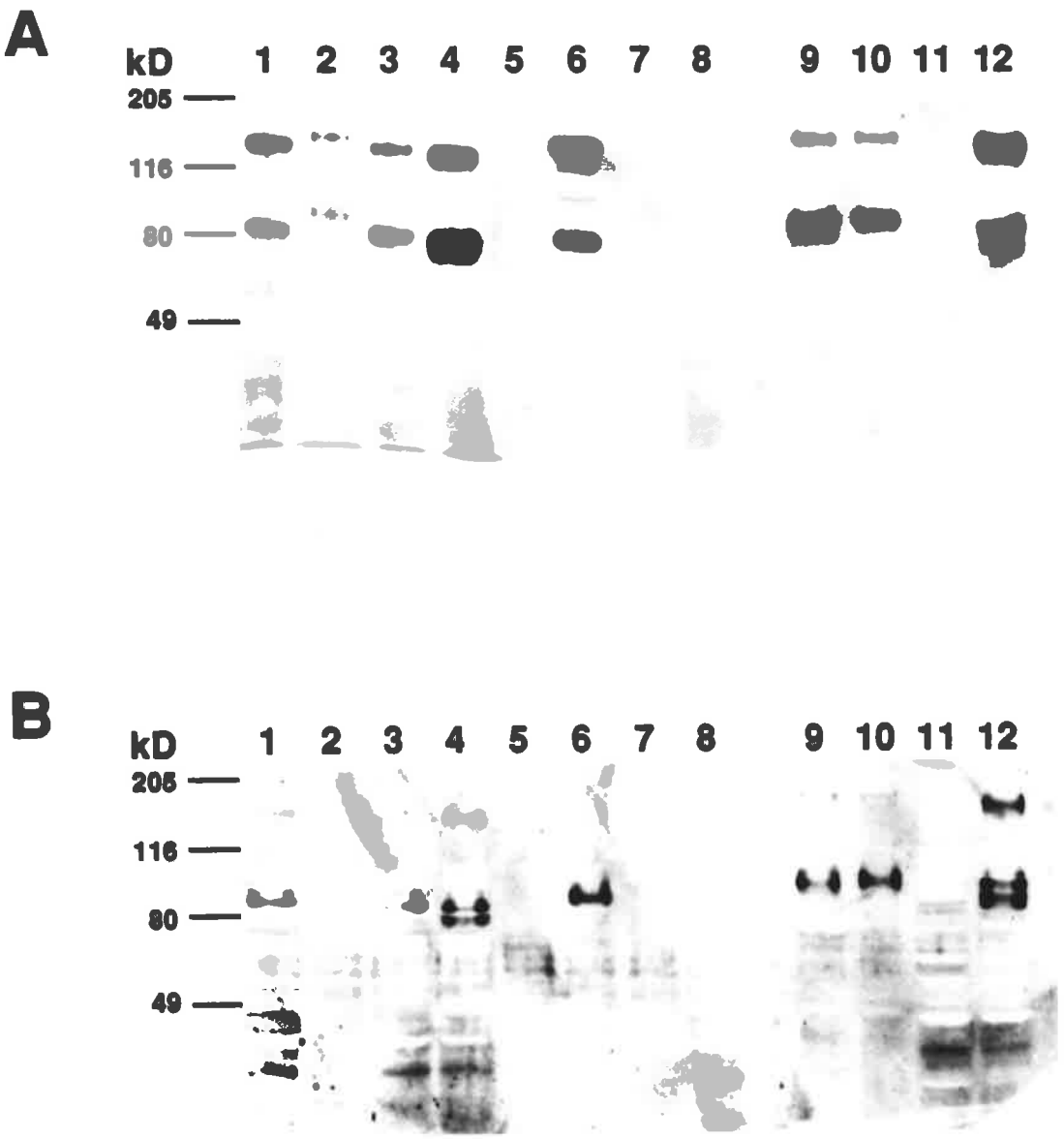
Given its reactivity with human BM stromal cells (refer to Chapter 4, Figure 4.2.16.), mAb 9E10 was used to screen the human BM stromal cell cDNA expression library in the retroviral vector pRUF.*neo* as described in Chapter 4. Subsequent to the specific-isolation of 9E10 antigen-expressing FDC-P1 clones, genomic DNA was isolated and the corresponding cDNA rescued by PCR. Following partial-sequence analysis, the

Figure 6.2.5. (A) & (B). Determination Of The Molecular Mass Of The CSM Identified By mAb 9E10.

Membrane preparations from a variety of haemopoietic and non-haemopoietic cell lines/preparations were separated by 10% SDS-polyacrylamide gel electrophoresis under non-reducing and reducing conditions and transferred to nitrocellulose. The filters were successively incubated with 9E10 mAb supernatant, anti-mouse-HRPO and the immunoreactive proteins detected by enhanced chemiluminescence (ECL) as described in the *Materials and Methods* (Section 2.29.5.). Under non-reducing conditions **(A)**, mAb 9E10 identifies two differentially migrating species with an estimated molecular mass of 80 and 160 kD. Upon reduction **(B)** however, the lower migrating 80 kD band is the major immunoreactive protein.

Panel (A) & (B):

Lane 1, KG1a (progenitor cell line)
Lane 2, HL-60 (promyelocytic cell line)
Lane 3, Hel-DR (erythroleukaemic)
Lane 4, K562 (erythroleukaemic)
Lane 5, red blood cells
Lane 6, Mo7e (megakaryocyte)
Lane 7, Jurkat (T cell line)
Lane 8, $CD19^+$ B cells
Lane 9, human BM stromal cells (HBMSC)
Lane 10, human umbilical vein endothelial cells (HUVECs)
Lane 11, T1 (keratinocytes)
Lane 12, MG63 (osteosarcoma cell line)


A
kD
1 2 3 4 5 6 7 8 9 10 11 12
205
116
80
49
B
kD
1 2 3 4 5 6 7 8 9 10 11 12
205
116
80
49

resultant nucleotide sequence was compared with entries submitted to the Genbank/EMBL databases via standard "FASTA alignment analysis" (Figure 6.2.6) and found to be homologous to a previously reported cDNA isolated from KATO-III human gastric carcinoma cells (Masuzawa *et al*, 1992). The cDNA isolated by Masuzawa and colleagues, encodes a novel polypeptide backbone which is rich in hydroxyl amino acids and cysteine residues, and lacks repeating motifs. This molecule, termed by the authors, MGC-24 (for *M*ulti-Glycosylated Core protein of 24 kD) represents a mucin-like glycoprotein, containing multiple clustered sites of O-and N-linked carbohydrate side chains linked to serine and threonine residues. Using a polyclonal rabbit antiserum, Masuzawa *et al* (1992), demonstrated that the mature MGC-24 glycoprotein derived from tissues of epithelial origin behaved as a high molecular mass (> 200 kD) polymorphic protein. Moreover, treatment of the epithelial-derived MGC-24 glycoprotein with the enzyme endo-α-N-acetyl-galactosaminidase, confirmed the presence of the peanut agglutinin (PNA)-binding site (Galβ1->3GalNAc sequence), a tumour-associated carbohydrate marker, commonly expressed by human carcinomas. Furthermore, the authors found that in addition to some nude mouse-transplanted human colorectal and pancreatic adenocarcinomas, MGC-24 mRNA was also intensely expressed in normal tissues of the colon, small intestine and thyroid.

(iii) Nucleotide And Polypeptide Characterisation

As indicated in Figure 6.2.7., complete sequence analysis of the region encompassing the coding sequence (and the immediate flanking regions), revealed an number of interesting differences to the sequence identified by Masuzawa and colleagues (1992). Although sharing complete identity from nucleotide 1 to 383, the 9E10 cDNA clone exhibited a deletion of 58 nucleotides at this site. This loss is presumably due to the use of alternate 5′ and 3′ splice junction sites present within this region. In accordance to the GT-AG rule described by Mount (1982), analysis of the MGC-24 sequence revealed two splice junction consensus motifs. Seven of the nine-nucleotides of

Figure 6.2.6. FASTA Alignment Analysis Of 9E10 cDNA

Following partial sequence analysis, the resultant 5'-nucleotide sequence was compared with sequences submitted to the combined Genbank/EMBL database via standard "FASTA alignment analysis". The 9E10 sequence was found to be homologous to the previously reported cDNA encoding MGC-24 (for *M*ulti-Glycosylated Core protein of 24 kD, Masuzawa *et al*, 1992).

FASTA Alignment Analysis Of 9E10 cDNA

gb|D14043|HUMMGC24 Human mRNA for MGC-24, complete cds...2427 7.8e-95 1

>gb|D14043|HUMMGC24 Human mRNA for MGC-24, polymorphic epithelial mucin, complete cds. Length = 2427, Plus Strand HSPs: Score = 815 (225.2 bits), Expect = 2.8e-58, P = 2.8e-58. Identities = 403/429 (94%), Strand = Plus

```
                  70        80        90       100       110       120
Sbjct:       CGCTGAGGACACGATGTCGCGGCTCTCCCGCTCACTGCTTTGGGCCGCCACCTGCCTGGG
             X::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Query:       CGCTGAGGACACGATGTCGCGGCTCTCCCGCTCACTGCTTTGGGCCGCCACCTGCCTGGG
                    10        20        30        40        50        60

                 130       140       150       160       170       180
Sbjct:       CGTGCTCTGCGTGCTGTCCGCGGACAAGAACACGACCCAGCACCCGAACGTGACGACTTT
             ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Query:       CGTGCTCTGCGTGCTGTCCGCGGACAAGAACACGACCCAGCACCCGAACGTGACGACTTT
                    70        80        90       100       110       120

                 190       200       210       220       230       240
Sbjct:       AGCGCCCATCTCCAACGTAACCTCGGCGCCGGTGACGTCCCTCCCGCTGGTCACCACTCC
             ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Query:       AGCGCCCATCTCCAACGTAACCTCGGCGCCGGTGACGTCCCTCCCGCTGGTCACCACTCC
                   130       140       150       160       170       180

                 250       260       270       280       290       300
Sbjct:       GGCACCAGAAACCTGTGAAGGTCGAAACAGCTGCGTTTCCTGTTTTAATGTTAGCGTTGT
             ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Query:       GGCACCAGAAACCTGTGAAGGTCGAAACAGCTGCGTTTCCTGTTTTAATGTTAGCGTTGT
                   190       200       210       220       230       240

                 310       320       330       340       350       360
Sbjct:       TAATACTACCTGCTTTTGGATAGAATGTAAAGATGAGAGCTATTGTTCACATAACTCAAC
             ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Query:       TAATACTACCTGCTTTTGGATAGAATGTAAAGATGAGAGCTATTGTTCACATAACTCAAC
                   250       260       270       280       290       300

                 370       380       390       400       410       420
Sbjct:       AGTTAGTGATTGTCAAGTGGGGAACACGACAGACTTCTGTTCCGTTTCCACGGCCACTCC
             ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Query:       AGTTAGTGATTGTCAAGTGGGGAACACGACAGACTTCTGTTCCGTTTCCACGGCCACTCC
                   310       320       330       340       350       360

                 430       440       450       460       470       480
Sbjct:       AGTGCCAACAGCCAATTCTACAGCTAAACCCACAGTTCAG-CCCTCCCCTTCTACAACTT
             ::::::::::::::::::::X   :::  : :   : :   ::::      ::::: :
Query:       AGTGCCAACAGCCAATTCTACAGTACAACAAATAACACTGTGACTCCAACCTCACAACCT
                   370       380       390       400       410       420

               490
Sbjct:       CCAAGACAGTT
                :: ::
Query:       GTGCGAAAG--
                    430
```

Figure 6.2.7. Nucleotide And Inferred Protein Sequence Of MGC-24v.

The nucleotide and deduced amino acid sequence of a variant isoform of *M*ulti-Glycosylated Core protein of 24 kD (MGC-24v). The putative signal sequence is underlined. Potential sites of N-linked glycan attachment are indicated by asterisks (*). Cysteine residues are indicated by boxes (□) Nucleotide sequences which differ from the published sequence (Masuzawa *et al*, 1992) are in bold. A 58 nucleotide deletion between position 383 and 384 is indicated by (▼) and results in a 55 amino acid sequence at the COOH-terminus (italicised) which differs from the published sequence. The putative transmembrane domain (amino acid 140 to 164) is doubly underlined.

File Name: MGC-24v (9E10)
Range (Polypeptide): 14-550
Molecular Weight: 19015.62

5'-													C	GCT	GAG	GAC	ACG	13
ATG	TCG	CGG	CTC	TCC	CGC	TCA	CTG	CTT	TGG	GCC	GCC	ACC	TGC	CTG	GGC	GTG	CTC	67
Met	Ser	Arg	Leu	Ser	Arg	Ser	Leu	Leu	Trp	Ala	Ala	Thr	Cys	Leu	Gly	Val	Leu	18
													□					
TGC	GTG	CTG	TCC	GCG	GAC	AAG	AAC	ACG	ACC	CAG	CAC	CCG	AAC	GTG	ACG	ACT	TTA	121
Cys	Val	Leu	Ser	Ala	Asp	Lys	Asn	Thr	Thr	Gln	His	Pro	Asn	Val	Thr	Thr	Leu	36
□							*						*					
GCG	CCC	ATC	TCC	AAC	GTA	ACC	TCG	GCG	CCG	GTG	ACG	TCC	CTC	CCG	CTG	GTC	ACC	175
Ala	Pro	Ile	Ser	Asn	Val	Thr	Ser	Ala	Pro	Val	Thr	Ser	Leu	Pro	Leu	Val	Thr	54
				*														
ACT	CCG	GCA	CCA	GAA	ACC	TGT	GAA	GGT	CGA	AAC	AGC	TGC	GTT	TCC	TGT	TTT	AAT	229
Thr	Pro	Ala	Pro	Glu	Thr	Cys	Glu	Gly	Arg	Asn	Ser	Cys	Val	Ser	Cys	Phe	Asn	72
						□						□			□		*	
GTT	AGC	GTT	GTT	AAT	ACT	ACC	TGC	TTT	TGG	ATA	GAA	TGT	AAA	GAT	GAG	AGC	TAT	283
Val	Ser	Val	Val	Asn	Thr	Thr	Cys	Phe	Trp	Ile	Glu	Cys	Lys	Asp	Glu	Ser	Tyr	90
				*			□					□						
TGT	TCA	CAT	AAC	TCA	ACA	GTT	AGT	GAT	TGT	CAA	GTG	GGG	AAC	ACG	ACA	GAC	TTC	337
Cys	Ser	His	Asn	Ser	Thr	Val	Ser	Asp	Cys	Gln	Val	Gly	Asn	Thr	Thr	Asp	Phe	108
□			*						□				*					
														58 nucleotide deletion				
TGT	TCC	GTT	TCC	ACG	GCC	ACT	CCA	GTG	CCA	ACA	GCC	AAT	TCT	ACA	G:GT	ACA	ACA	391
Cys	Ser	Val	Ser	Thr	Ala	Thr	Pro	Val	Pro	Thr	Ala	Asn	Ser	Thr	*Gly*	*Thr*	*Thr*	126
□												*						

AAT	AAC	ACT	GTG	ACT	CCA	ACC	TCA	CAA	CCT	GTG	CGA	AAG	TCT	ACC	TTT	GAT	GCA	445
Asn	*Asn*	*Thr*	*Val*	*Thr*	*Pro*	*Thr*	*Ser*	*Gln*	*Pro*	*Val*	*Arg*	*Lys*	*Ser*	*Thr*	*Phe*	*Asp*	*Ala*	144
*																		
GCC	AGT	TTC	ATT	GGA	GGA	ATT	GTC	CTG	GTC	TTG	GGT	GTG	CAG	GCT	GTA	ATT	TTC	499
Ala	*Ser*	*Phe*	*Ile*	*Gly*	*Gly*	*Ile*	*Val*	*Leu*	*Val*	*Leu*	*Gly*	*Val*	*Gln*	*Ala*	*Val*	*Ile*	*Phe*	162
TTT	**CTT**	**TAT**	**AAA**	**TTC**	**TGC**	**AAA**	**TCT**	**AAA**	**GAA**	**CGA**	**AAT**	**TAC**	**CAC**	**ACT**	**CTG**	**TAA**	**ACA**	553
Phe	*Leu*	*Tyr*	*Lys*	*Phe*	*Cys*	*Lys*	*Ser*	*Lys*	*Glu*	*Arg*	*Asn*	*Tyr*	*His*	*Thr*	*Leu*	STOP		178
					□													
GAC	CCA	TTG	AAT	TAA	TAA	GGA	CTG	GTG	ATT	CAT	TTG	TGT	AAC	TCA	CTG	AAG	CCA	607
AAA	TAC	TAT	CTT	TTA	AGA	TGT	CCC	ACA	TGG	AAG	ACG	CTA	TTC	CAG	GAT	CTT	TAA	661
ATT	TCC	ATG	GAT	GCA	TAT	AGG	ATG	TTT	GGG	AGC	ATC	ATC	CGT	GAA	GAA	AAA	ATC	715
AAT	TAA	ATC	ATT	GTG	TTC	AAC	AGG	AAT	ATT	TAA	AAT	ATT	CTG	CAT	GAA	TCC	TGT	769
GGC	TGT	CTT	ATT	TTA	AAT	AGC	TGC	TGC	TGT	GGG	ATT	ATA	TTT	TTT	TTC	CTT	AAC	823
ATG	CCA	AAT	ATA	ACT	TTC	TGA	AAG	TGA	TGG	AAA	ATG	TTG	TCT	TGT	GCA	GAC	AAC	877
ATC	ATG	GCT	CTT	GGC	AGT	TTA	AAT	TTA	GTA	ATT	TTA	ATT	TAG	TGA	ACA	GAA	TTG	931
AGA	AGA	ACG	TGC	CAA	ATG	AGA	ATC	AAT	TAG	GTG	GAT	TTT	TGG	CTG	TCA	TTT	CAA	985
AAG	TGG	AAT	AAA	TTT	ATT	AAT	TTA	GTA	GTA	CTA	AAT	GGT	ATC	CTT	AGA	TTA	AAA	1039
TTT	TGT	GCT	TGA	TAA	CAG	CTG	TTT	TTT	CTA	CAT	TAG	AAA	TAA	GAT	GCC	ACA	CAA	1093
GGA	ACT	ACA	TTC	CAG	ATT	TAA	AGA	AAT	GAA	AGG	ATA	CCA	TTA.....			- 3'		1135

the MGC-24 sequence [**CAG:GTAA**AC] conform with the well recognised 5′ splice junction consensus site [**CAG:GTAAGT**]. Moreover, the short consensus sequence for the 3′ splice junction [CAG:G] is also present, suggesting that the 9E10-derived cDNA represents an alternative-spliced variant of MGC-24. Translation of this new open reading frame (ORF) gives rise to a 178 amino acid polypeptide (from here on termed MGC-24v), divergent from MGC-24 at the COOH-terminus. Examination of sequences 3′ to the MGC-24v ORF, also revealed the presence of 595 bp of sequence unique to the MGC-24v (Figure 6.2.7.-bold script). As this region lies outside the ORF, the functional significance of this additional sequence remains to be defined. Furthermore, consistent with the findings of Masuzawa *et al*, (1992), analysis of MGC-24v revealed a translation start site in the context of a Kozak (Kozak, 1991) consensus sequence (ACACG**ATG**T), with a putative initiator methionine score of 60.

Computer analysis (MacDNASIS, Version 2.0) of the MGC-24v polypeptide, revealed a putative molecular weight of approximately 19.1 kD. This polypeptide sequence was not significantly related to that of any other core protein so far reported (Swiss-PROT search, National Centre for Biological Information, National Institutes of Health, USA). The initiation methionine is followed by a putative 22 amino acid signal sequence, containing a hydrophobic core [amino acids Ala^{23}-Asp^{24}; Figure 6.2.7] (Barclay *et al*, 1993). Following this putative signal peptide, nine potential sites of N-linked glycosylation (consensus sequence: AsnXaaThr/Ser, with the exception of AsnProThr/Ser or AsnXaaThr/Ser-Pro; Barclay *et al*, 1993) are observed on Asn residues at positions 26, 32, 41, 72, 77, 94, 104, 121 and 127 (Figure 6.2.7.). Moreover, the mature 178 amino acid protein is extremely rich in serine and threonine, with 37 (or ~20%) of the encoded amino acids made up of these residues. At least 32 of these residues can serve as attachment sites for O-linked glycans (Devine and McKenzie, 1992), and in combination with N-linked glycans make up approximately 70% of the molecular mass of the mature protein. In addition, the MGC-24v sequence lacks the acceptor site for the addition of a glycosaminoglycan glycan (GAG) chain (Kjellen and

Lindahl, 1991) at position Serine^{142} and Glycine^{143} (SG) present within the MGC-24 polypeptide. Hydropathy analysis (Kyte-Doolittle, GENETYX-MAC/1 3.0.1) of MGC-24v, revealed that the majority of the encoded protein was highly hydrophilic, consistent with the high hydroxyl amino acid content of the protein (Figure 6.2.8.). However, in contrast to the findings of Masuzawa *et al* (1992), examination of the C-terminus of MGC-24v revealed a region (amino acids 140 to 164) of high hydrophobicity that may represent a putative transmembrane-anchoring motif. This putative transmembrane domain is followed by a very short COOH-terminal hydrophilic domain (amino acid residues 165 to 178), consistent with the requirements of a type I transmembrane protein. A schematic diagram demonstrating the salient features of the haemopoietic form of MGC-24v is presented in Figure 6.2.9.

(iv) Expression Of MGC-24v mRNA In Haemopoietic Tissues.

Total RNA isolated from cultured HBMSCs and the candidate myeloid progenitor cell line KG1a (characterised by its high levels of CD34$^+$ surface antigen expression; Koeffler *et al*, 1980) was examined by Northern blot analysis for the presence of MGC-24v transcripts (Figure 6.2.10.). A prominent MGC-24v transcript of approximately 3.0 kb (indicated by the closed arrow) was observed in RNA blots of both cell preparations. Although weak hybridisation with two diffuse species of 4.8 kb and 1.9 kb was also observed in both lanes (Figure 6.2.10., open arrows), this most likely represents cross-hybridisation of the MGC-24v probe with the 28s and 18s ribosomal RNAs, respectively. Therefore, the consistent detection of a single strong hybridising mRNA species, argues against a significant level of alternative RNA splicing, at least in haemopoietic tissues.

6.2.5. Distribution Of MGC-24v On Cell Lines As Detected By mAb 9E10 And An Alternate α-MGC-24v mAb, 105.A5.

Notably, through the retroviral expression-cloning strategy described in Chapter 4, an additional murine IgM mAb 105.A5 (generated independently in the laboratory of Dr.

Figure 6.2.8. Protein Hydropathy Determination Of MGC-24v Amino Acid Sequence.

The hydropathy plot of MGC-24v polypeptide, according to the method of Kyte and Doolittle (1982). Various regions are "bracketed", and identified at the right of the figure. Circled N's represent potential N-linked glycosylation sites.

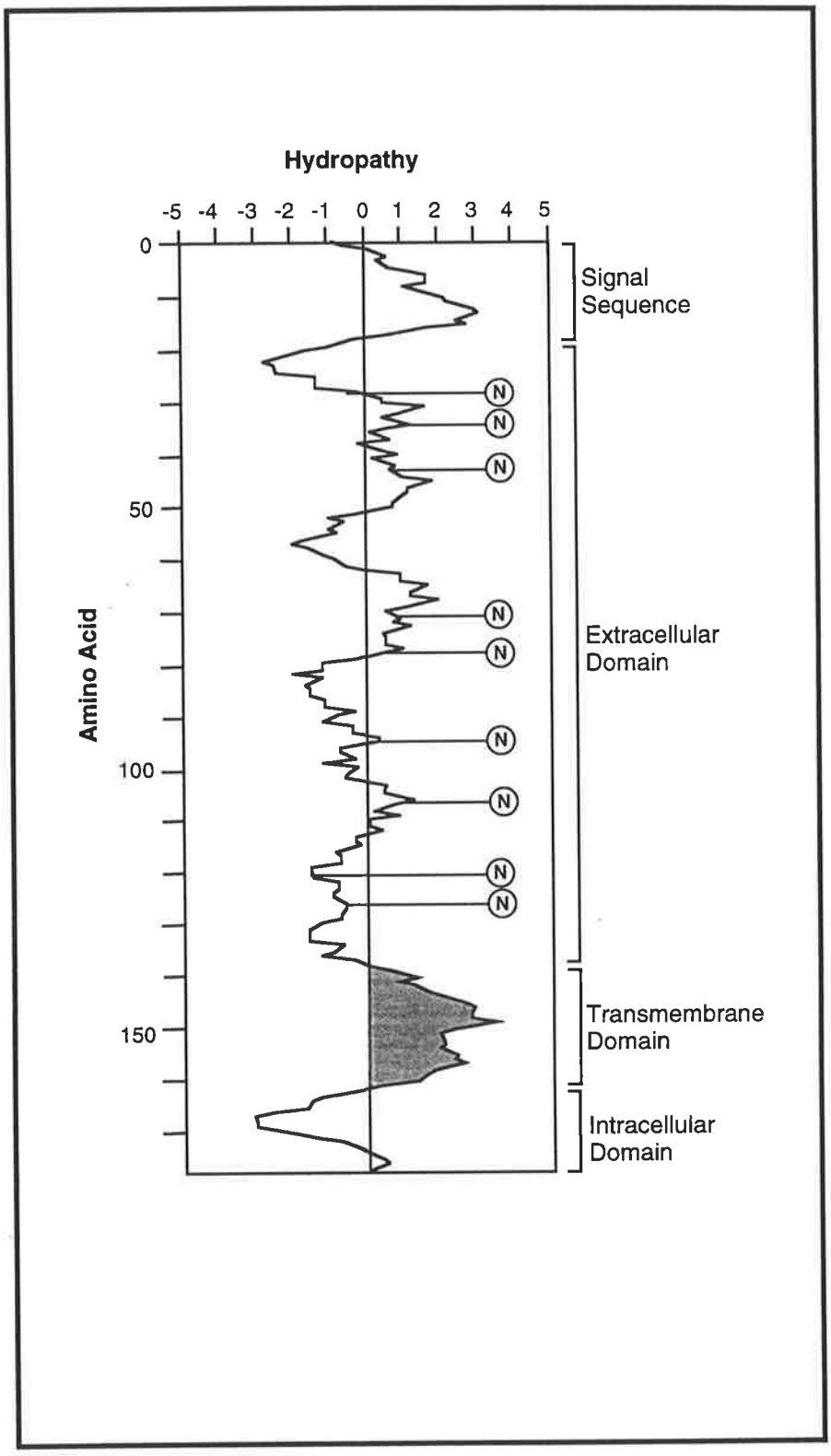

Hydropathy
-5 -4 -3 -2 -1 0 1 2 3 4 5
0
50
100
150
Amino Acid
N
Signal Sequence
Extracellular Domain
Transmembrane Domain
Intracellular Domain

Figure 6.2.9. Schematic Representation Of MGC-24v.

Schematic diagram of MGC-24v, based on biochemical and nucleotide data described in previous figures. The salient features of this molecule include; (i) the numerous (up to 32) potential sites of O-linked glycan attachment, (ii) the 9 possible N-linked glycosylation sites, (iii) the putative transmembrane-spanning domain and (iv) a short cytoplasmic domain.

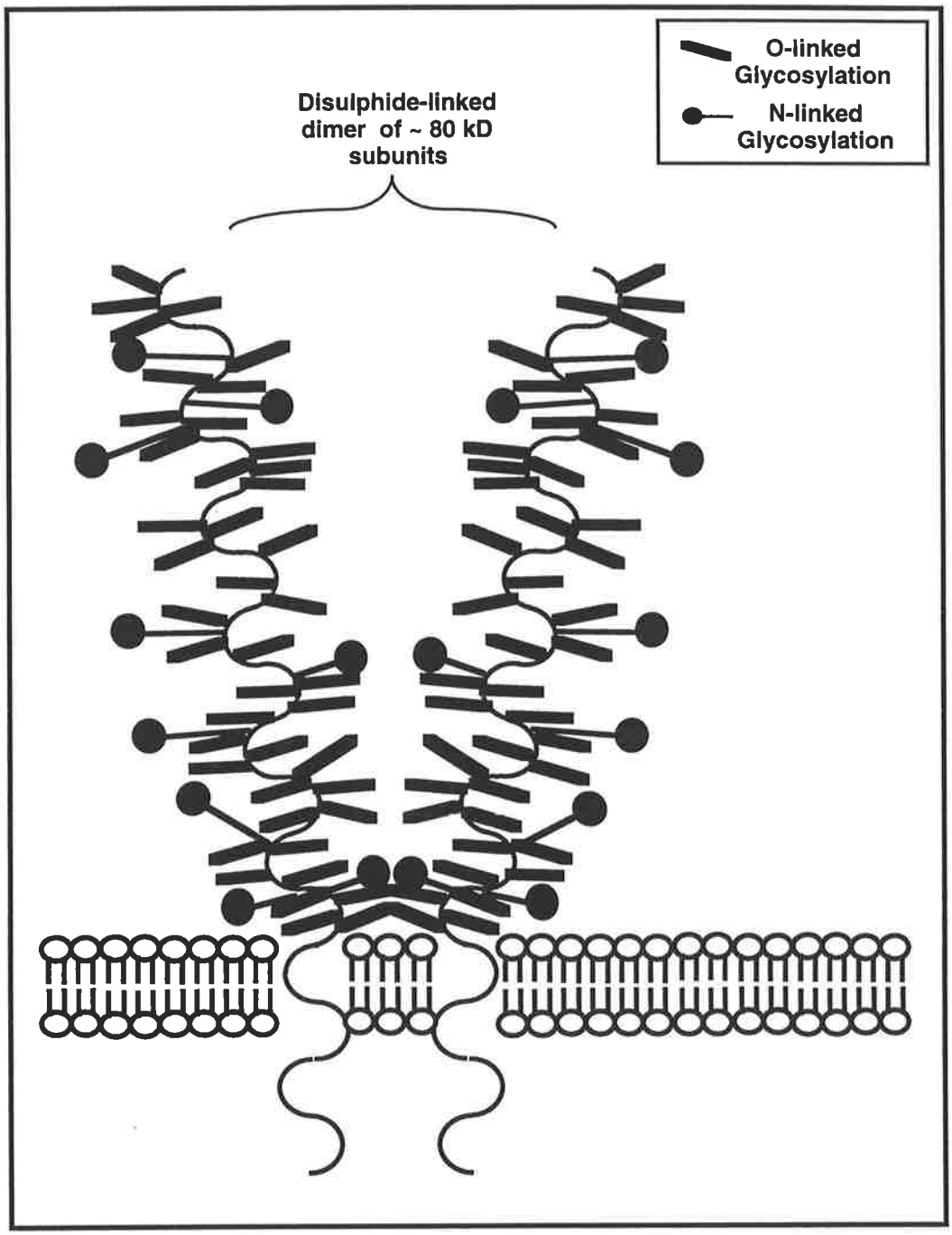
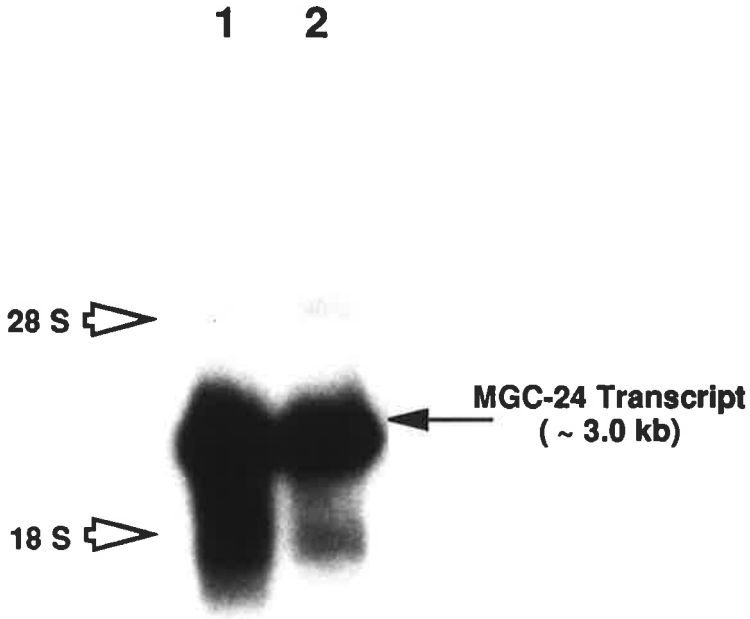

O-linked
Glycosylation
N-linked
Glycosylation
Disulphide-linked
dimer of ~ 80 kD
subunits

Figure 6.2.10. Northern Blot Analysis To Examine MGC-24v Expression In Both Haemopoietic Progenitor And BM Stromal Cells.

Total RNA derived from human BM stromal cells (lane 1) and the primitive myeloid cell line, KG1a (lane 2) were subjected to electrophoresis on a 1.0 % formaldehyde-agarose gel and transferred to a nylon membrane by capillary action. MGC-24v mRNA expression was examined by overnight hybridisation at 42°C with a ^{32}P-radiolabelled full-length MGC-24v probe (9E10-derived PCR product). The membrane was washed as described in *Materials and Methods* (Section 2.20.4.). Membranes were exposed to X-Omat AR film for 24 hours with intensifying screens. Hybridisation to the 3.0 kb MGC-24v transcript is observed in both cell lines (indicated by arrow), whilst possible cross-hybridisation with the 18s and 28s ribosomal RNA is indicated by open arrows.


1
2
28 S
18 S
MGC-24 Transcript
(~ 3.0 kb)

H-J. Bühring, University of Tübingen, Federal Republic Of Germany) was subsequently found to detect the product of the MGC-24v cDNA as illustrated in Figure 6.2.11. This result was somewhat surprising, as mAbs 9E10 and 105.A5 displayed demonstrably different immunoreactivity profiles when a large cohort of cultured haemopoietic and non-haemopoietic cell lines and cell preparations were assessed by single-colour indirect immunofluorescence and flow cytometry (Table 6.2.2). The reason for the disparate pattern of reactivity between the 9E10 and 105.A5 mAbs remains unknown and requires further examination.

6.2.6. Confirmation That mAbs 9E10 And 105.A5 Both Recognise The MGC-24v Glycoprotein.

In light of the disparate reactivity profiles of mAbs 9E10 and 105.A5, a number of approaches were utilised to confirm that both mAbs 9E10 and 105.A5 recognise the MGC-24v glycoprotein. Principally, the 2.7 kb *Bam*HI-*Xho*I restriction fragment of the MGC-24v cDNA (harbouring both the entire coding sequence and the 5′ and 3′ non-coding regions) was subcloned into the pRUF.*neo* vector and subsequently introduced into FDC-P1 cells by retroviral transduction (refer to *Materials and Methods*, Section 2.26.). The resultant G418-resistant cell population was then tested (by indirect immunofluorescence and flow cytometry) for their ability to bind mAbs 9E10 and 105.A5. As demonstrated in Figure 6.2.12., both mAbs demonstrated specific reactivity with the MGC-24v transfectant.

In agreement with this, dual-colour flow cytometric analysis of BMMNC (Figure 6.2.13.) revealed a precise co-distribution following immuno-staining with the 9E10 and 105.A5 mAbs, indicating that the mAbs identify distinct epitopes on the same glycoprotein. As demonstrated above (Section 6.2.1.), the expression of the 9E10 epitope was largely restricted to a subpopulation of BMMNCs which co-expressed the CD34 antigen. In accord with this, dual-parameter flow cytometric analysis revealed that a significant proportion of the 105.A5$^+$ BMMNCs (26.5 ± 4.8%; range 21.5%-32%; n=4)

Figure 6.2.11. FASTA Alignment Analysis Of 105.A5 cDNA.

Following partial sequence analysis, the resultant nucleotide sequence was compared with sequences submitted to the combined Genbank/EMBL database via standard "FASTA alignment analysis". The 105.A5 cDNA sequence was found to be homologous to the previously reported cDNA encoding MGC-24 (for *M*ulti-Glycosylated Core protein of 24 kD, Masuzawa *et al*, 1992).

FASTA Alignment Analysis Of 105.A5 cDNA

gb|D14043|HUMMGC24 Human mRNA for MGC-24, complete cds...2427 7.8e-95 1

>gb|D14043|HUMMGC24 Human mRNA for MGC-24, polymorphic epithelial mucin, complete cds. Length = 2427, Plus Strand HSPs: Score = 815 (225.2 bits), Expect = 2.8e-58, P = 2.8e-58 Identities = 388/390 (99%), Strand = Plus

```
                 70        80        90       100       110       120
Sbjct:   CGCTGAGGACACGATGTCGCGGCTCTCCCGCTCACTGCTTTGGGCCGCCACCTGCCTGGG
         X::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Query:   CGCTGAGGACACGATGTCGCGGCTCTCCCGCTCACTGCTTTGGGCCGCCACCTGCCTGGG
                10        20        30        40        50        60

          130       140       150       160       170       180
Sbjct:   CGTGCTCTGCGTGCTGTCCGCGGACAAGAACACGACCCAGCACCCGAACGTGACGACTTT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Query:   CGTGCTCTGCGTGCTGTCCGCGGACAAGAACACGACCCAGCACCCGAACGTGACGACTTT
                70        80        90       100       110       120

          190       200       210       220       230       240
Sbjct:   AGCGCCCATCTCCAACGTAACCTCGGCGCCGGTGACGTCCCTCCCGCTGGTCACCACTCC
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Query:   AGCGCCCATCTCCAACGTAACCTCGGCGCCGGTGACGTCCCTCCCGCTGGTCACCACTCC
               130       140       150       160       170       180

          250       260       270       280       290       300
Sbjct:   GGCACCAGAAACCTGTGAAGGTCGAAACAGCTGCGTTTCCTGTTTTAATGTTAGCGTTGT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Query:   GGCACCAGAAACCTGTGAAGGTCGAAACAGCTGCGTTTCCTGTTTTAATGTTAGCGTTGT
               190       200       210       220       230       240

          310       320       330       340       350       360
Sbjct:   TAATACTACCTGCTTTTGGATAGAATGTAAAGATGAGAGCTATTGTTCACATAACTCAAC
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Query:   TAATACTACCTGCTTTTGGATAGAATGTAAAGATGAGAGCTATTGTTCACATAACTCAAC
               250       260       270       280       290       300

          370       380       390       400       410       420
Sbjct:   AGTTAGTGATTGTCAAGTGGGGAACACGACAGACTTCTGTTCCGTTTCCACGGCCACTCC
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Query:   AGTTAGTGATTGTCAAGTGGGGAACACGACAGACTTCTGTTCCGTTTCCACGGCCACTCC
               310       320       330       340       350       360

          430       440       450
Sbjct:   AGTGCCAACAGCCAATTCTACAGCTA-AA
         :::::::::::::::::::::::: :: ::
Query:   AGTGCCAACAGCCAATTCTACAGGTACAA
               370       380       390
```

Table 6.2.2. Cellular Distribution Of MGC-24 As Detected By mAbs 9E10 And 105.A5.

Assessment on the distribution of MGC-24 as detected by the mAbs 9E10 and 105.A5, on a large cohort of cultured haemopoietic and non-haemopoietic cell lines and cell preparations by single-colour indirect immunofluorescence and flow cytometry (please also refer to Figure 6.2.3 (A)).

Information generated from listmode data, and represents 1×10^4 light-scatter gated events. A representative experiments (one of three) is shown.

Immunoreactivity Key : - : negative; ± : low reactivity; + : medium reactivity

(Data kindly provided by Dr. H-J. Bühring, University of Tübingen, Germany).

Table 6.2.2. Cellular Distribution of MGC-24 As Detected By mAbs 9E10 And 105.A5.

Cell Line	Characteristics Of Cell Line	Antibody	
		9E10	105.A5
DU.4475	Breast Carcinoma	+	+
NCI-H128	SCLC	+	±
NCI-H69	SCLC	±	±
SK-MES	Lung Carcinoma	+	+
Calu 1	Lung Carcinoma	+	+
Calu 3	Lung Carcinoma	+	±
Calu 6	Lung Carcinoma	±	±
SK-LU1	Lung Carcinoma	+	+
HELA	Cervix Carcinoma	+	+
5637	Urinary Bladder Carcinoma	+	+
IMR-32	Neuroblastoma	±	±
TE-671	Medulloblastoma	+	+
A172	Glioblastoma	+	+
A431	Epidermal Carcinoma	+	+
T-47D	Breast Carcinoma	+	+
HUVEC	Umbilical Vein Endothelial	+	±
UT-7	Eryth./Meg. Leukaemia	+	+
TF-1	Eryth./Meg. Leukaemia	+	+
MO7e	Eryth./Meg. Leukaemia	+	+
MEG-O1	Eryth./Meg. Leukaemia, Ph^+ (CML)	+	+
MOLM-1	Eryth./Meg. Leukaemia, Ph^+ (CML)	+	+
K562	Eryth./Meg. Leukaemia, Ph^+ (CML)	-	±
TMM	B-Lymphoblastic, Ph^- (CML)	-	+
BV-173	B-Lymphoblastic, Ph^+ (CML)	+	+
CML-T1	T-Lymphoblastic, Ph^+ (CML)	-	+
SPI-801	Erythroid (K562-derivative), Ph^+ (CML)	-	±
EM-2	Myeloblastic	-	+
KU.812	Basophilic	+	+

Continued over page

Table 6.2.2. Cellular Distribution of MGC-24 As detected By mAbs 9E10 And 105.A5. (*continued*).

Cell Line	Characteristics Of Cell Line	Antibody	
		9E10	105.A5
HEL	Eryth./Meg. Leukaemia	+	+
KG-1	Myeloblastic Leukaemia	±	+
KG-1a	Immature Subline Of KG-1	-	+
HL60	Myeloblastic Leukaemia	-	+
DU.528	Myeloblastic Leukaemia	-	+
U937	Myeloblastic Leukaemia	-	±
OCI/AML-4	Myeloblastic Leukaemia	+	+
HSB-2	T-Lymphoblastic Leukaemia	-	+
CCRF-CEM	T-Lymphoblastic Leukaemia	-	+
Molt-4	T-Lymphoblastic Leukaemia	-	+
Daudi	B-Lymphoblastic Leukaemia	-	+
Reh	pre B-Lymphoblastic Leukaemia	-	+
U266	B-Lymphoblastic Leukaemia	-	+
Km3	pre B-Lymphoblastic Leukaemia	-	+
Lymphocytes	Derived From Normal PB	-	+
Granulocytes	Derived From Normal PB	-	-
Monocytes	Derived From Normal PB	-	+
Erythrocytes	Derived From Normal PB	-	-
Platelets	Derived From Normal PB	-	±
BMMNCs	Derived From Normal BM	+ (sub. pop.)	+ (sub. pop.)
CD34⁺ BM Cells	Derived From Normal BM	+ (sub. pop.)	+ (sub. pop.)
CML-BC		25/30	ND
AML		32/35	ND
c-ALL		0/20	ND

Figure 6.2.12. Confirmation That mAbs 9E10 And 105.A5 Both Recognise The MGC-24v Glycoprotein.

A 2.7 kb *Bam*HI-*Xho*I restriction fragment of the MGC-24v cDNA (harbouring both the entire coding sequence and the 5′ and 3′ non-coding regions) was subcloned into the pRUF*neo* vector and subsequently introduced into FDC-P1 cells by retroviral transduction (refer to *Materials and Methods*, Section 2.26.). The resultant G418-resistant cell population, was stained by indirect immunofluorescence and analysed by flow cytometry. Data are displayed as single-parameter fluorescence (FITC) histograms of 1 x 10^4 light-scatter gated events, collected as list mode data.

..... : IgG_3 control mAb

–·–·–·–·–·–: IgM control mAb

————: mAb 9E10

————: mAb 105.A5

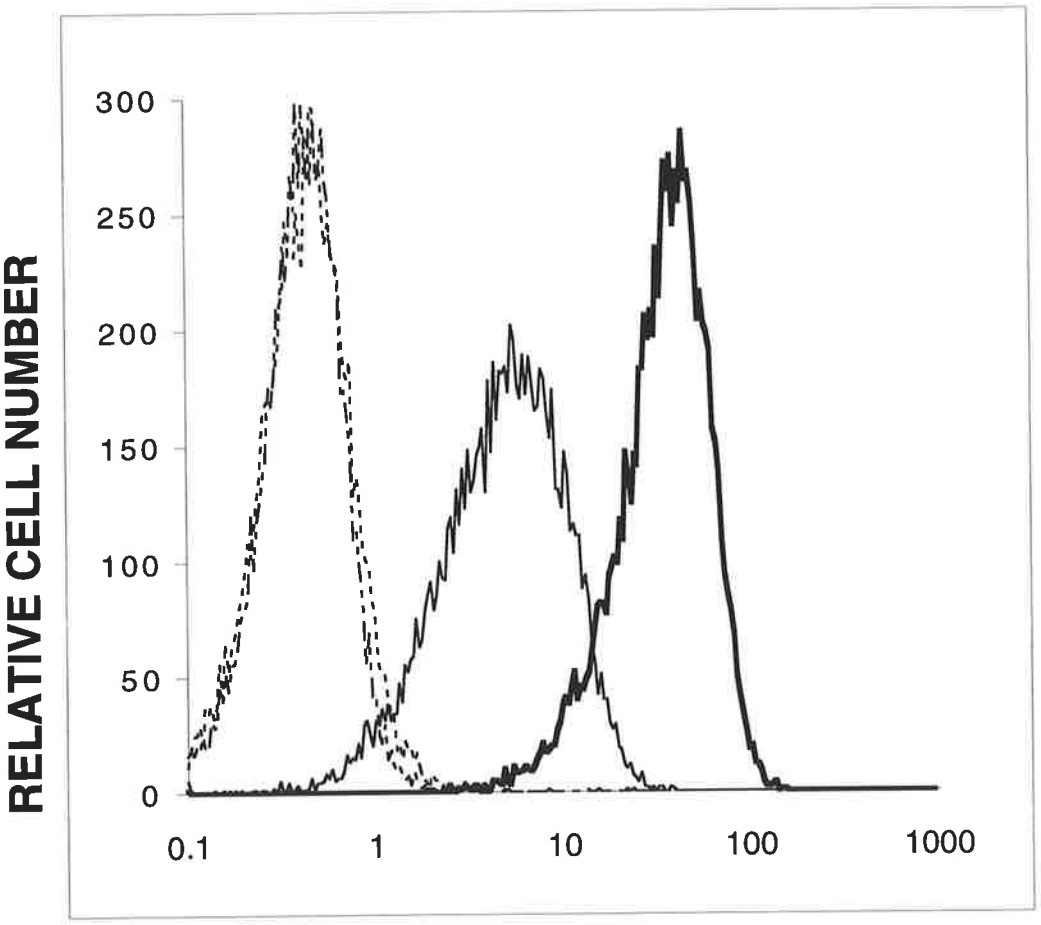
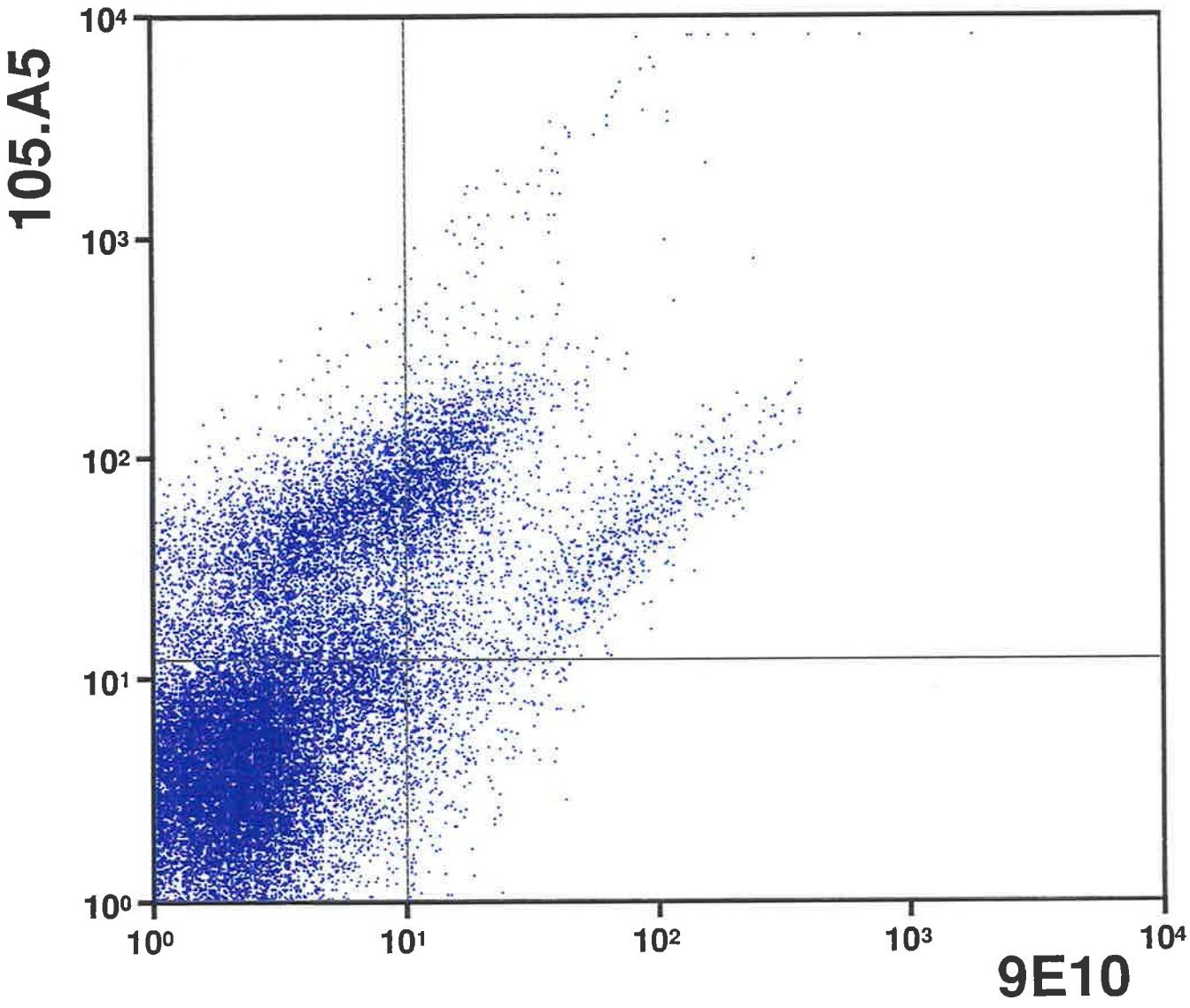

RELATIVE CELL NUMBER
300
250
200
150
100
50
0
0.1
1
10
100
1000
LOG FLUORESCENCE

Figure 6.2.13. 9E10 And 105.A5 mAbs Recognise The Same Glycoprotein Antigen On $CD34^+$ Cells.

BMMNC were stained with 9E10 and 105.A5 and subsequently detected with goat anti-mouse Ig-FITC and goat anti-mouse IgM-PE, respectively. Data are displayed as dual-parameter histograms of 2×10^4 $CD34^+$ cells collected as list mode data.


105.A5
10^4
10^3
10^2
10^1
10^0
10^0
10^1
10^2
10^3
10^4
9E10

also co-expressed the CD34 antigen (Figure 6.2.14. (B)). Moreover, mAb 105.A5 consistently reacted with approximately 11-21% more of the BMMNC fraction which lacked the expression of the CD34 antigen ($CD34^{-}105.A5^{+}$). Subsequent morphological analysis revealed that the $CD34^{-}105.A5^{+}$ fraction harboured cells of all the erythroid stages, including normoblasts. This observation was subsequently confirmed by immunolabelling BMMNCs with both 105.A5 and the erythroid specific antigen, glycophorin A (GLY-A) (data not shown).

To ascertain whether mAb 105.A5, like 9E10, bound to lineage-restricted haemopoietic progenitor cells, *in vitro* clonogenic assays were performed on BMMNC-derived, FACS isolated $CD34^{+}105.A5^{+}$ and $CD34^{+}105.A5^{-}$ fractions (Figure 6.2.14. (B)). In consensus with the results obtained with 9E10 (refer Section 6.2.2.), virtually all detectable myeloid (CFU-GM), erythroid (BFU-E) and multipotential colony forming cells (CFU-Mix), were recovered in the population of cells which expressed the antigen recognised by the mAb 105.A5 (Figure 6.2.15.). Similarly, as demonstrated in Figure 6.2.16. (A) & (B), primitive haemopoietic cells with the capacity to initiate and maintain haemopoiesis in the cytokine driven stromal cell-free suspension culture (pre-CFU) assay (described above) were also restricted to the $CD34^{+}105.A5^{+}$ subpopulation isolated from normal adult BMMNC. In contrast, the $CD34^{+}105.A5^{-}$ cells failed to generate CFU-GM within the 28-day period of the pre-CFU assay. These data demonstrate that the majority, if not all the pre-CFU are present within the $CD34^{+}105.A5^{+}$ subpopulation, commensurate with the results obtained with cells selected with the 9E10 mAb.

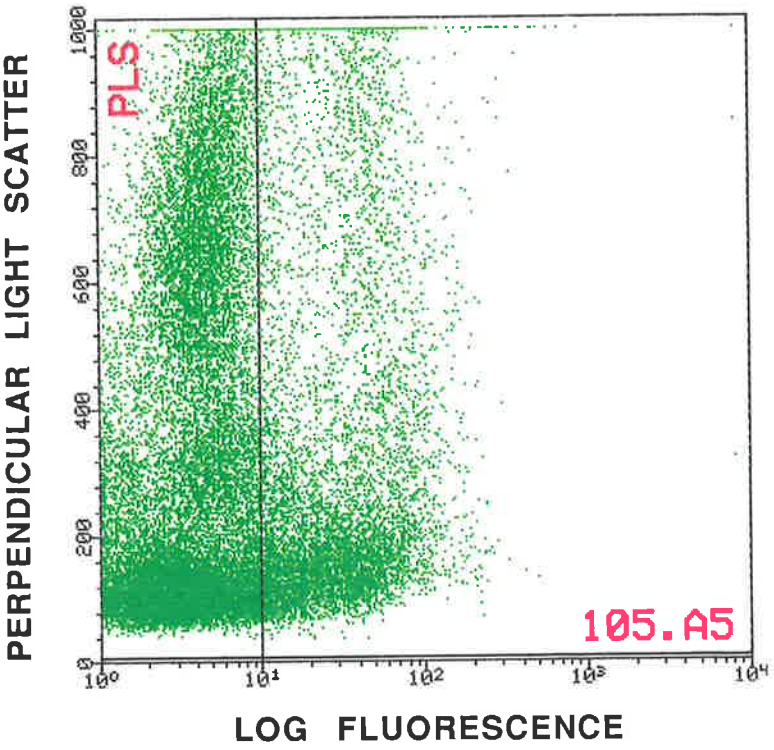
Finally, comparison of the molecular mass of the CSM identified by mAbs 105.A5 and 9E10 by Western blotting revealed that under both reducing and non-reducing conditions, both mAbs identified identical protein species from cultured HBMSCs (Figure 6.2.17.). Furthermore, Western blotting analysis of membrane extracts isolated from FDC-P1 cells selected with the 105.A5 and 9E10 mAbs (105.A5 and 9E10 FDC-P1 clones) revealed that identical recombinant proteins of 80 kD were detected with both mAbs (Figure 6.2.18. (A)). Moreover, immune-precipitation of biotinylated-FDC-P1

Figure 6.2.14.

(A) Dot plot demonstrating that 105.A5 reacts with a minor population of BMMNC, characterised by low perpendicular light scatter (PLS) and low to moderate levels of forward light scatter (FLS), properties shared with cells which lie within the lymphocyte-blast region.

(B) Dual-parameter immunofluorescence analysis demonstrating the expression of CD34 and 105.A5 antigen by BMMNCs. SBA-depleted BMMNC were stained with the directly conjugated mAb HPCA-2-PE (α-CD34) and 105.A5 (detected with anti-IgM-FITC). Data are displayed as dual-parameter histograms of 5 x 10^4 light-scatter-gated events collected as list-mode data.

A


PERPENDICULAR LIGHT SCATTER
PLS
105.A5
LOG FLUORESCENCE

B

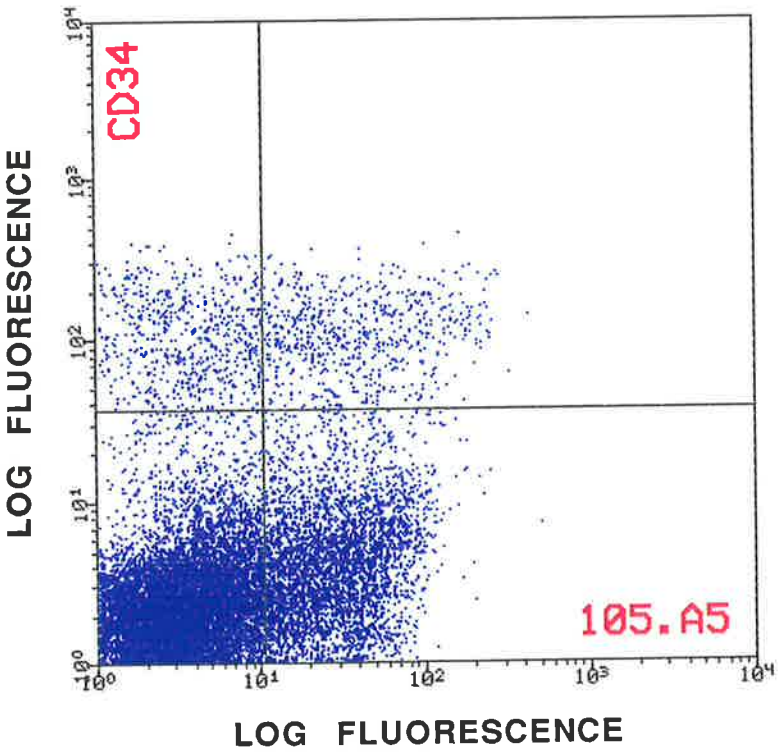

LOG FLUORESCENCE
CD34
105.A5
LOG FLUORESCENCE

Figure 6.2.15. Assay Of Clonogenic Progenitors In Populations Sorted On The Basis Of CD34 And The α-MGC-24v mAb 105.A5

Fluorescence activated cell sorting (FACS) of the $CD34^+$, $CD34^+105.A5^+$, and $CD34^+105.A5^-$ subpopulations demonstrates that clonogenic progenitors (CFU-GM, ■, BFU-E, □ and CFU-Mix ■) are present almost exclusively in the $CD34^+105.A5^+$ subpopulation. Results are expressed as the number of CFU-GM at day 14 per 1×10^3 cells plated. Data represents mean ± S.E. (n=3).

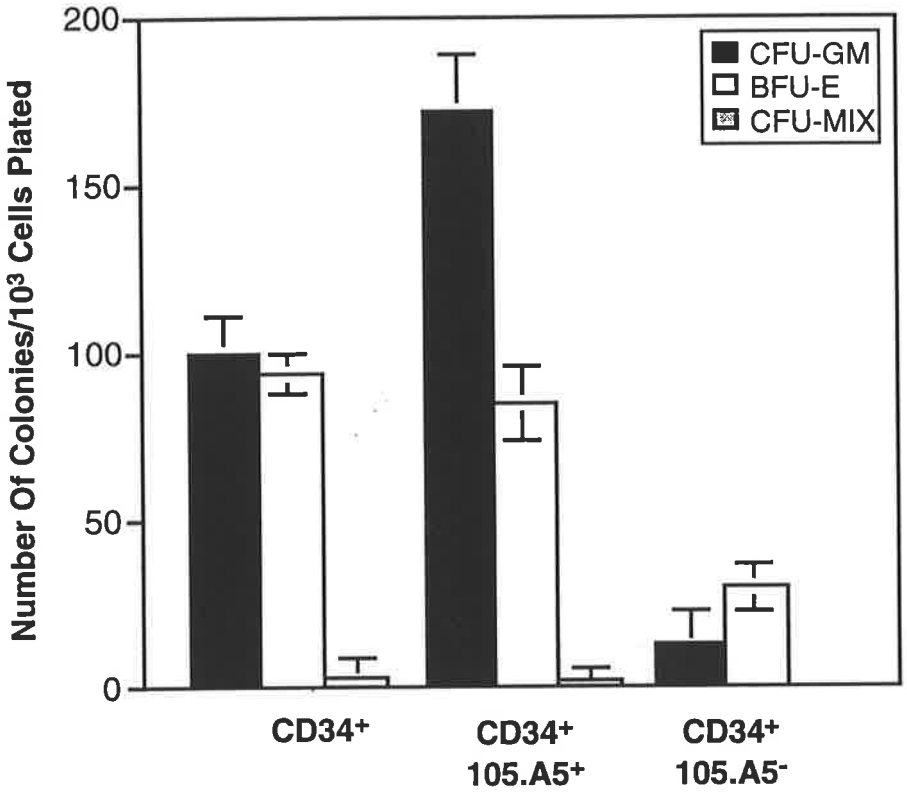

200
150
100
50
0
Number Of Colonies/10³ Cells Plated
CFU-GM
BFU-E
CFU-MIX
CD34+
CD34+ 105.A5+
CD34+ 105.A5-

Figure 6.2.16. (A) & (B). $CD34^+$ Cells Initiating Haemopoiesis In The Cytokine-Supplemented (Pre-CFU) Assay Express MGC-24v As Detected By The 105.A5 mAb.

BMMNC were sorted into $CD34^+$ (□) $CD34^+105.A5^+$ (◆), $CD34^+105.A5^-$ (○), subpopulations (as described in Figure 6.2.14.) and assayed for their ability to initiate and maintain haemopoiesis in a stroma-independent, cytokine supplemented culture. Cultures were established in triplicate using 1×10^3 sorted cells per well in medium supplemented with 10 ng/ml each of purified recombinant human IL-1β, IL-3, IL-6, G-CSF, GM-CSF and SCF. Additional factors were added at the same concentrations on days 7, 14 and 21. On days 7, 14 and 21, the cells were harvested, washed and assayed for nucleated cell number and CFU-GM as previously described. The results are expressed as the mean number (±SE) of nucleated cell number **(A)** and CFU-GM **(B)**, recovered at day 7, 14, 21 and 28 for each group. A representative experiment (one of three) is shown.

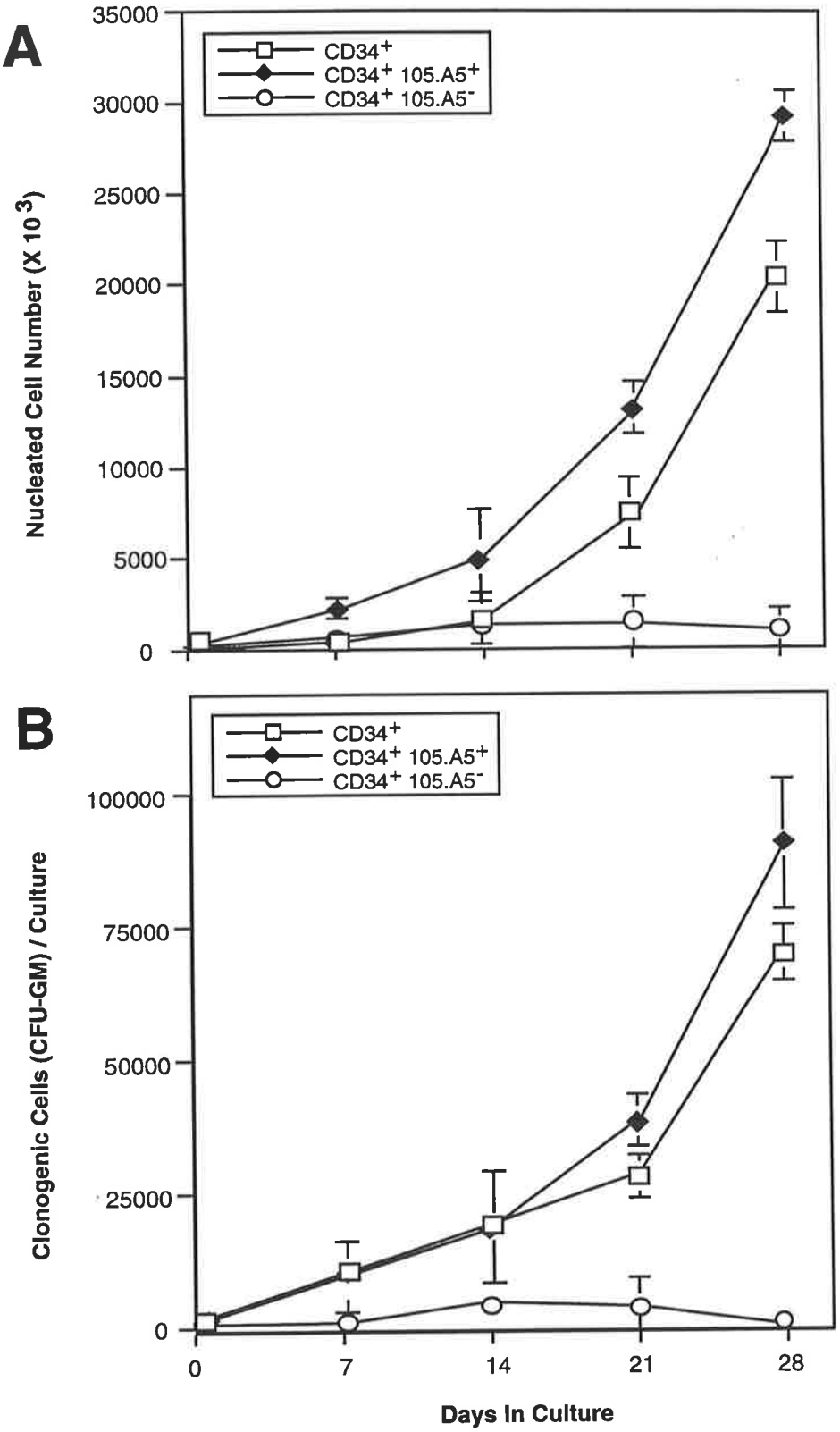

A
B
CD34+
CD34+ 105.A5+
CD34+ 105.A5-
Nucleated Cell Number (X 10 3)
Clonogenic Cells (CFU-GM) / Culture
Days In Culture
35000
30000
25000
20000
15000
10000
5000
0
100000
75000
50000
25000
0
7
14
21
28

Figure 6.2.17. (A) & (B). Western Blot Analysis Cultured Human BM Stromal Cells (HBMSC) With mAb 9E10 And 105.A5.

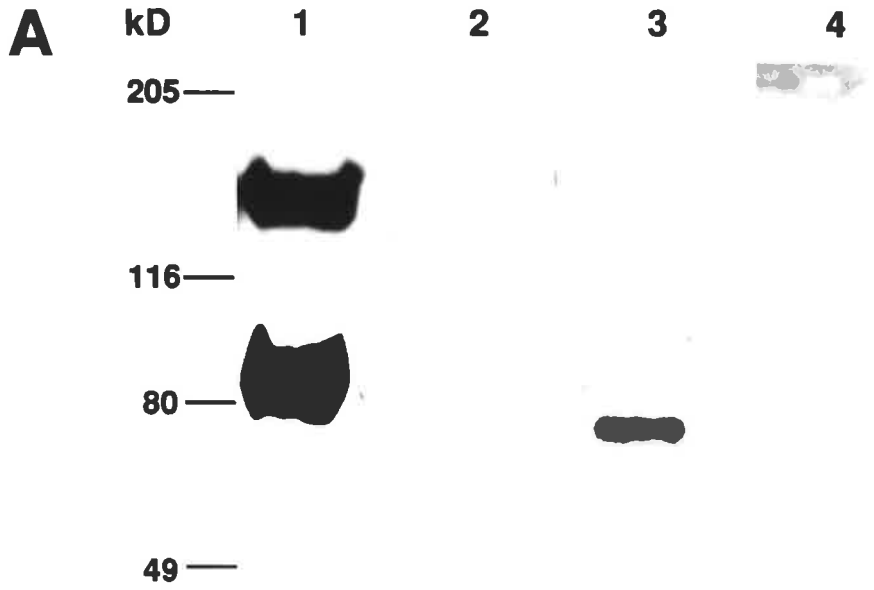
Membrane preparations from cultured HBMSC were separated by 7.5% SDS-polyacrylamide gel electrophoresis under non-reducing and reducing conditions and transferred to nitrocellulose. The filters were successively incubated with either the 9E10 (Panel A) or 105.A5 mAb (Panel B) supernatant (or isotype matched, non-binding controls) and anti-mouse-HRPO. The immunoreactive proteins were detected by enhanced chemiluminescence (ECL) as described in the *Materials and Methods* (Section 2.29.5.). Under non-reducing conditions, mAbs 9E10 and 105.A5 identify two differentially migrating species with an estimated molecular mass of 80 and 160 kD (Panel A & B, lane 1). Upon reduction however, the lower migrating 80 kD band is the major immunoreactive protein (Panel A & B, lane 3).

Panel (A). 9E10 (IgG_3 negative control) immunoblotted HBMSC proteins

Lane 1, non-reduced: 9E10 mAb
Lane 2, non-reduced: IgG_3 negative control
Lane 3, reduced: 9E10 mAb
Lane 4, IgG_3 negative control

Panel (B). 105.A5 (IgM negative control) immunoblotted HBMSC proteins

Lane 1, non-reduced: 105.A5 mAb
Lane 2, non-reduced: IgM negative control
Lane 3, reduced: 105.A5 mAb
Lane 4, IgM negative control


A
kD
1
2
3
4
205
116
80
49

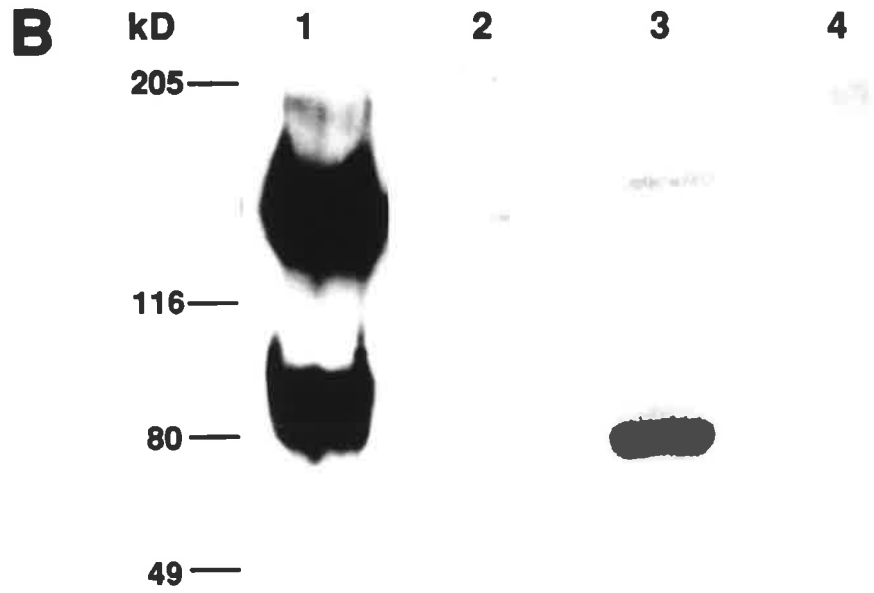

B
kD
1
2
3
4
205
116
80
49

Figure 6.2.18. (A) & (B). Western Blot And Immunoprecipitation Analysis Of Transfectant-Derived Recombinant MGC-24v Protein.

(A) Membrane extracts isolated from FDC-P1 cells selected with the 105.A5 and 9E10 mAbs (105.A5 and 9E10 FDC-P1 clones) were separated by 10% SDS-polyacrylamide gel electrophoresis under reducing conditions and transferred to nitrocellulose. The filters were successively incubated with either the 9E10 (Panel 1) or 105.A5 mAb (Panel 2) supernatant (or isotype matched, non-binding controls), anti-mouse-HRPO and the immunoreactive proteins detected by enhanced chemiluminescence (ECL) as described in the *Materials and Methods* (Section 2.29.5.). Under reducing conditions, mAb 9E10 and 105.A5 identify identical recombinant proteins of 80 kD.

Panel (A) 1.

Lane (a), reduced 9E10 FDC-P1 protein blotted with IgG_3 negative control
Lane (b), reduced 9E10 FDC-P1 protein blotted with mAb 9E10
Lane (c), reduced 9E10 FDC-P1 protein blotted with IgM negative control
Lane (d), reduced 9E10 FDC-P1 protein blotted with mAb 105.A5

Panel (A) 2.

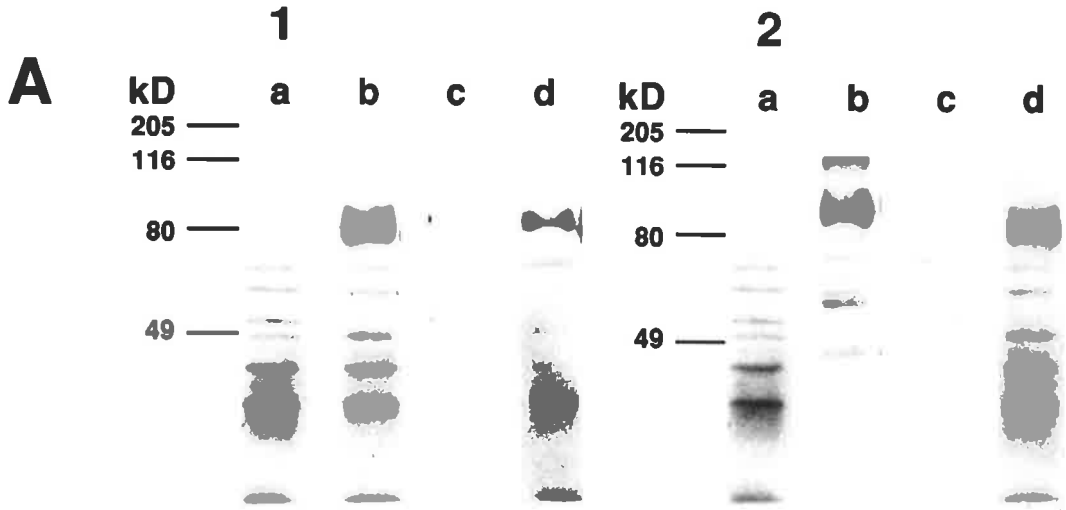
Lane (a), reduced 105.A5 FDC-P1 protein blotted with IgG_3 negative control
Lane (b), reduced 105.A5 FDC-P1 protein blotted with mAb 9E10
Lane (c), reduced 105.A5 FDC-P1 protein blotted with IgM negative control
Lane (d), reduced 105.A5 FDC-P1 protein blotted with mAb 105.A5

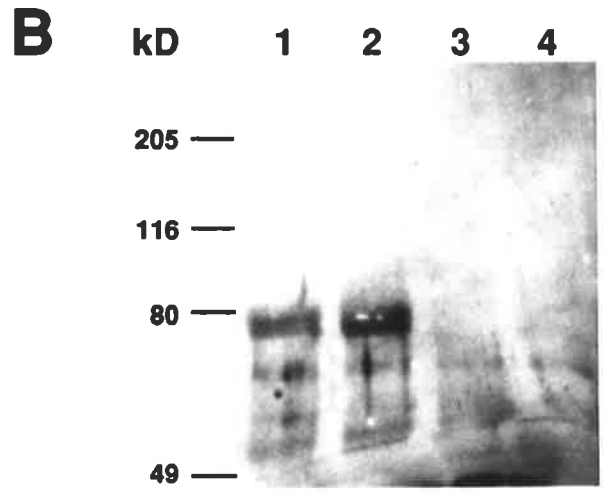
(B) Biotinylated membrane preparations of FDC-P1 cells expressing MGC-24v (ie. 9E10 and 105.A5-selected FDC-P1 cells), were co-incubated with the indicated mAb for 16 hours at 4°C. Immune complexes were precipitated using goat anti-mouse sepharose, resuspended in reducing SDS-PAGE buffer, resolved on a 7.5% SDS-polyacrylamide gel and visualised with biotin-streptavidin-HRPO complex and ECL.

mAb 9E10 immunoprecipitate a protein of the appropriate molecular mass from lysates derived from both 105.A5 and 9E10 FDC-P1 cells.

Panel (B).

Lane 1, 9E10 FDC-P1 lysate immunoprecipitated with mAb 9E10
Lane 2, 105.A5 FDC-P1 lysate immunoprecipitated with mAb 9E10
Lane 3, 9E10 FDC-P1 lysate immunoprecipitated with IgG_3 negative control.
Lane 4, 105.A5 FDC-P1 lysate immunoprecipitated with IgM negative control.


A
1
2
kD
a
b
c
d
205
116
80
49
kD
a
b
c
d
205
116
80
49


B
kD
1
2
3
4
205
116
80
49

lysates revealed that although the IgM mAb 105.A5 was unable to selectively precipitate the MGC-24v protein, mAb 9E10 was able to immune-precipitate a protein of the appropriate molecular mass from lysates derived from both 105.A5 and 9E10 FDC-P1 cells (Figure 6.2.18 (B)).

In summary, both mAbs 105.A5 and 9E10 identify a novel membrane-associated isoform of the recently cloned epithelial, mucin-like protein MGC-24. The studies thus far described, extend its expression to tissues of haemopoietic origin where it exists as a homodimeric cell surface molecule of 160 kD.

6.2.7. Contribution Of MGC-24v In The Adhesion Of CD34$^+$ Cells To Bone Marrow Stromal Cells.

To date, the communication of Masuzawa *et al* (1992) represents the only report describing MGC-24, and although it summarises the molecular isolation of the MGC-24 cDNA, it does not ascribe a function to this mucin-like protein. However, in light of the recent findings that GlyCAM-1, MAdCAM-1 and PSGL-1 (counter-receptors for L- and P-/E-selectin), represent novel mucin-like molecules which participate in adhesive interactions, the possible adhesive function of MGC-24v on CD34$^+$ cell was examined. These assays demonstrated that mAb 9E10 (but not 105.A5) partially blocked (by approximately 30%) the adhesion of chromium-labelled CD34$^+$ cells to allogeneic BM stromal cells *in vitro*, confirming the adhesive function of MGC-24v (Figure 6.2.19.). The lack of adhesion-blockade observed with mAb 105.A5, under the identical conditions probably reflects the low avidity of this IgM mAb, or more conceivably demonstrates that it identifies an epitope not involved in MGC-24v ligand recognition.

6.2.8. Ligation Of MGC-24v On CD34$^+$ Cells Results In The Suppression Of Haemopoiesis : Studies Of Committed Haemopoietic Progenitor Cells And Primitive Haemopoietic Precursors (Pre-CFU).

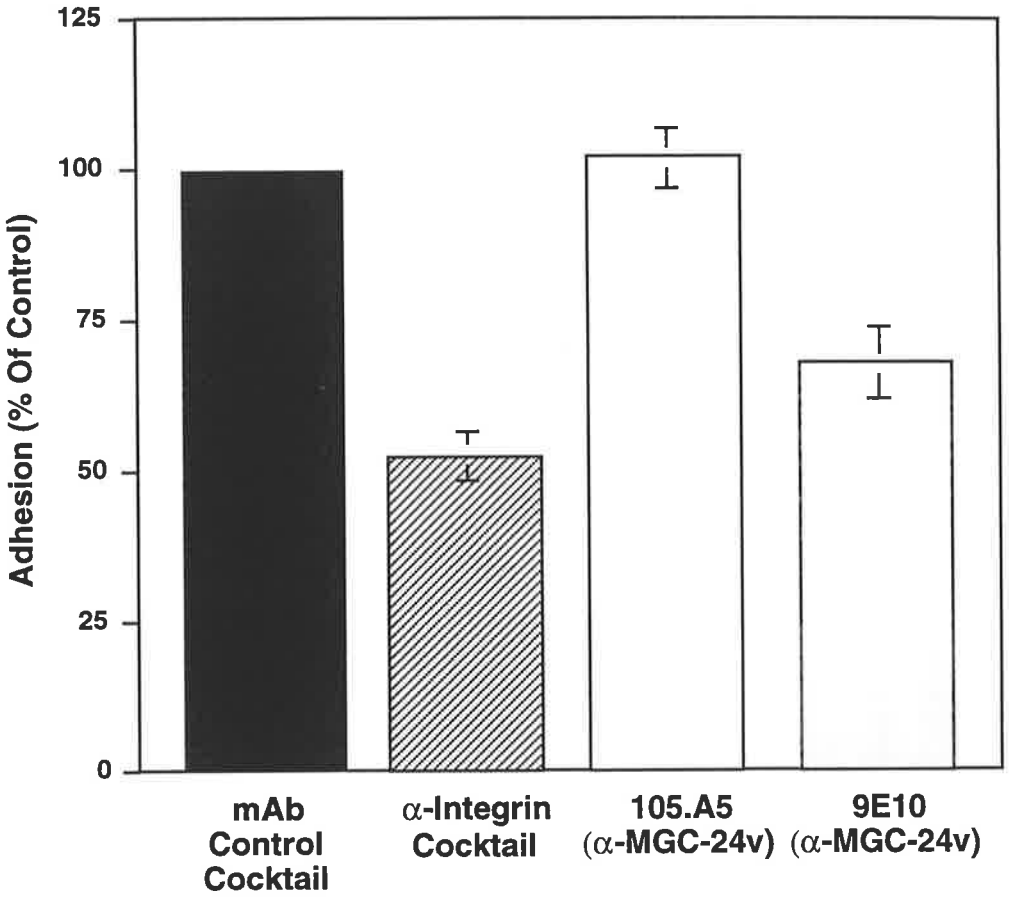
Studies on the ligation of PSGL-1 on human CD34$^+$ cells and the resulting

Figure 6.2.19. The MGC-24v Mucin-Like Glycoprotein Functions As An Adhesion Molecule Expressed By $CD34^+$ And BM Stromal Cells.

1×10^4 allogeneic BM stromal cells were transferred to each well of an 96 well plate 24 hours prior to performing the assay. BM $CD34^+$ cells isolated with 561-Dynabeads were labelled with ^{51}Cr and subsequently incubated on ice at 1×10^5/ml in RPMI (supplemented with 2% FCS) containing either 20 μg/ml of the mAb 105.A5 or 9E10 (or isotype matched, non-binding control mAbs/cocktail of mAbs to β1 integrins). 1×10^4 cells (in a volume of 100 μl) were subsequently transferred (without washing) to each well of the 96 well plate containing stromal cells. Incubation was carried out for 30 minutes at 37°C after which unbound cells were removed by washing. Adhesion was quantitated by liquid scintillation counting of Triton X-100 solubilised lysates.

Data are presented as the percentage of control adhesion obtained in the presence of a cocktail of IgG_3 and IgM non-binding control mAbs and represents the mean ± S.E. of 3 experiments

- ■ cocktail of IgG_3 and IgM non-binding control mAbs.
- ▨ cocktail of mAbs to β1 integrins (refer to Table 2.10.1).
- □ mAb 105.A5
- ▩ mAb 9E10


125
100
75
50
25
0
Adhesion (% Of Control)
mAb Control Cocktail
α-Integrin Cocktail
105.A5 (α-MGC-24v)
9E10 (α-MGC-24v)

suppression of haemopoiesis (please refer Chapter 3, Section 3.2.7.), prompted the investigation of whether ligation of MGC-24v on progenitor cells would have a similar inhibitory effect. Since the natural ligand for MGC-24v has yet to be identified, mAbs 9E10 and 105A5 were used as surrogate ligands.

To examine the effect of the mAbs on the growth and development of committed haemopoietic progenitor cells, purified mAbs were added at incremental concentrations over the range 0.01-30 μg/ml in semi-solid clonogenic assays of committed human haemopoietic progenitors. Following 14 days culture in the presence of 9E10 and 105A5, cultures were scored according to standard criteria for the presence of colonies derived from progenitors of myeloid (CFU-GM) and erythroid (BFU-E) cells. Both 9E10 [Figure 6.2.20.(A)] and 105.A5 [Figure 6.2.20.(B)] resulted in dose dependent inhibition of colony formation of both CFU-GM and BFU-E as compared with isotype matched control antibodies added at the same concentrations. Half-maximal inhibition of both CFU-GM and BFU-E production was obtained when mAb 9E10 was used at 3 μg/ml. Similarly, half-maximal inhibition of both CFU-GM and BFU-E production was obtained when mAb 105.A5 was used at 10 and 3 μg/ml, respectively.

The pre-CFU assay was subsequently performed to examine the effect of engaging MGC-24v on hierarchically more primitive cells. 1×10^3 $CD34^+$ cells per culture (in triplicate) were established in serum-deprived medium supplemented with mAb 9E10 and 105.A5 (or appropriate isotype-matched control mAbs) at a concentration of 10 μg/ml. The production of myeloid (CFU-GM) clonogenic cells was monitored at weekly intervals for 4 weeks as described in the *Materials and Methods* (Section 2.5.4.). As demonstrated in Figure 6.2.21. the addition of either mAb 9E10 or 105.A5 resulted in dose dependent inhibition of haemopoietic cell production, as compared to the non-binding IgG_3 and IgM isotype matched control antibodies added at identical concentrations. More specifically, at day 28 of culture, mAb 105.A5 resulted in > 95% inhibition of cell production when compared with the appropriate control mAb. In comparison, mAb 9E10 resulted in approximately 70% inhibition in cell production at

Figure 6.2.20. (A) & (B). Ligation Of MGC-24v On $CD34^+$ Cells Results In The Suppression Of Haemopoiesis: Studies Of Committed Haemopoietic Progenitor Cells.

To examine the effect of the ligation of MGC-24v on the growth and development of committed haemopoietic progenitor cells, 1 x 10^3 561-Dynabead purified $CD34^+$ cells were incubated with either purified 9E10 (**panel A**) or 105.A5 (**panel B**) [or purified isotype matched, non-binding control mAbs] at incremental concentrations over the range 0.01-30 μg/ml for 1 hour at 4°C. Cells were plated in 0.9% methylcellulose (in IMDM) supplemented with 10 ng/ml each of purified recombinant human IL-1β, IL-3, IL-6, G-CSF, GM-CSF, SCF and EPO. Relative to the negative control mAbs, both erythroid (BFU-E) and myeloid progenitor (CFU-GM) numbers were consistently diminished in a dose dependent manner in the presence of 105.A5 and 9E10. Results are expressed as the number of clonogenic cells at day 14 per 1 x 10^3 cells plated. Data represents mean ± S.E. (n=3).

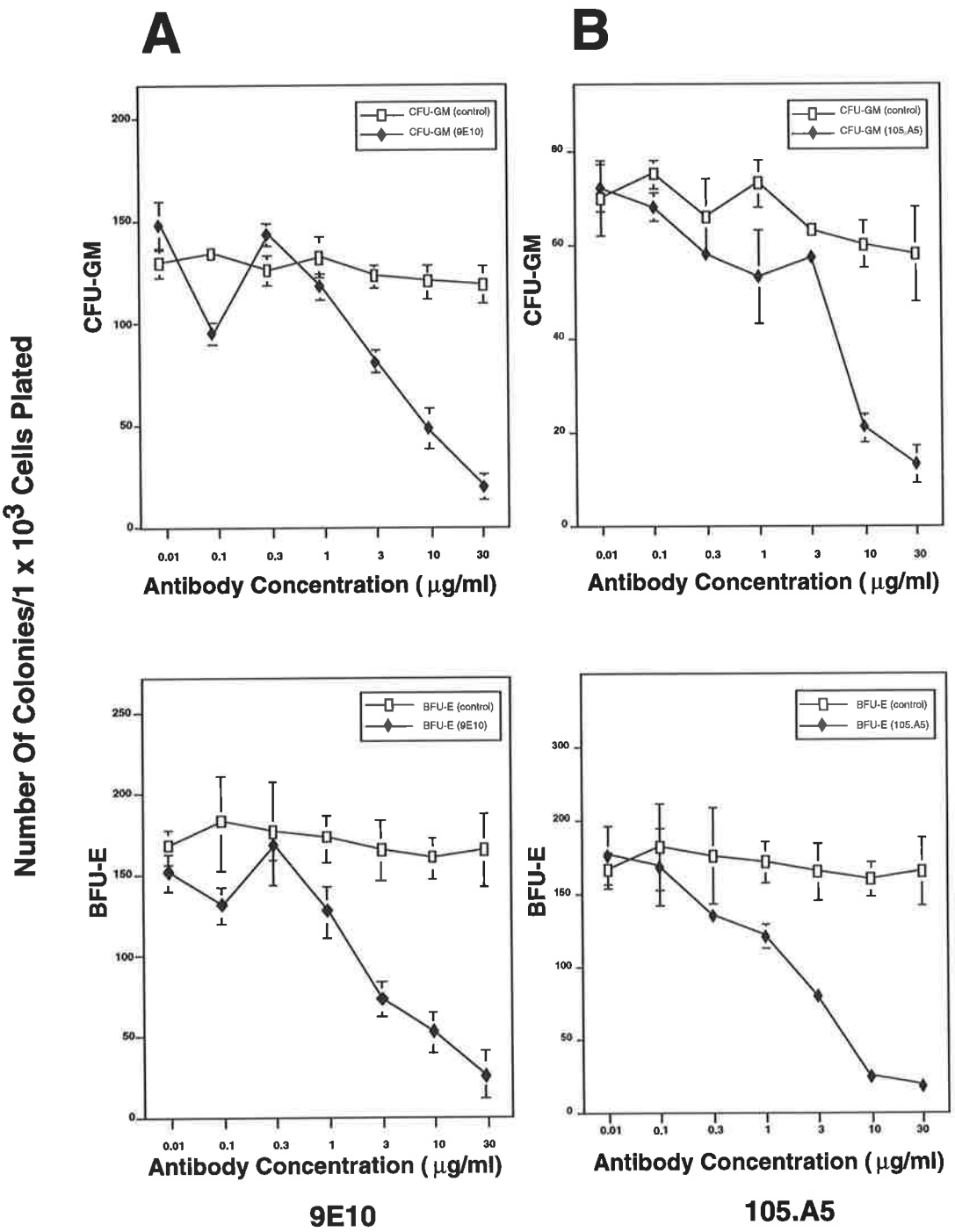
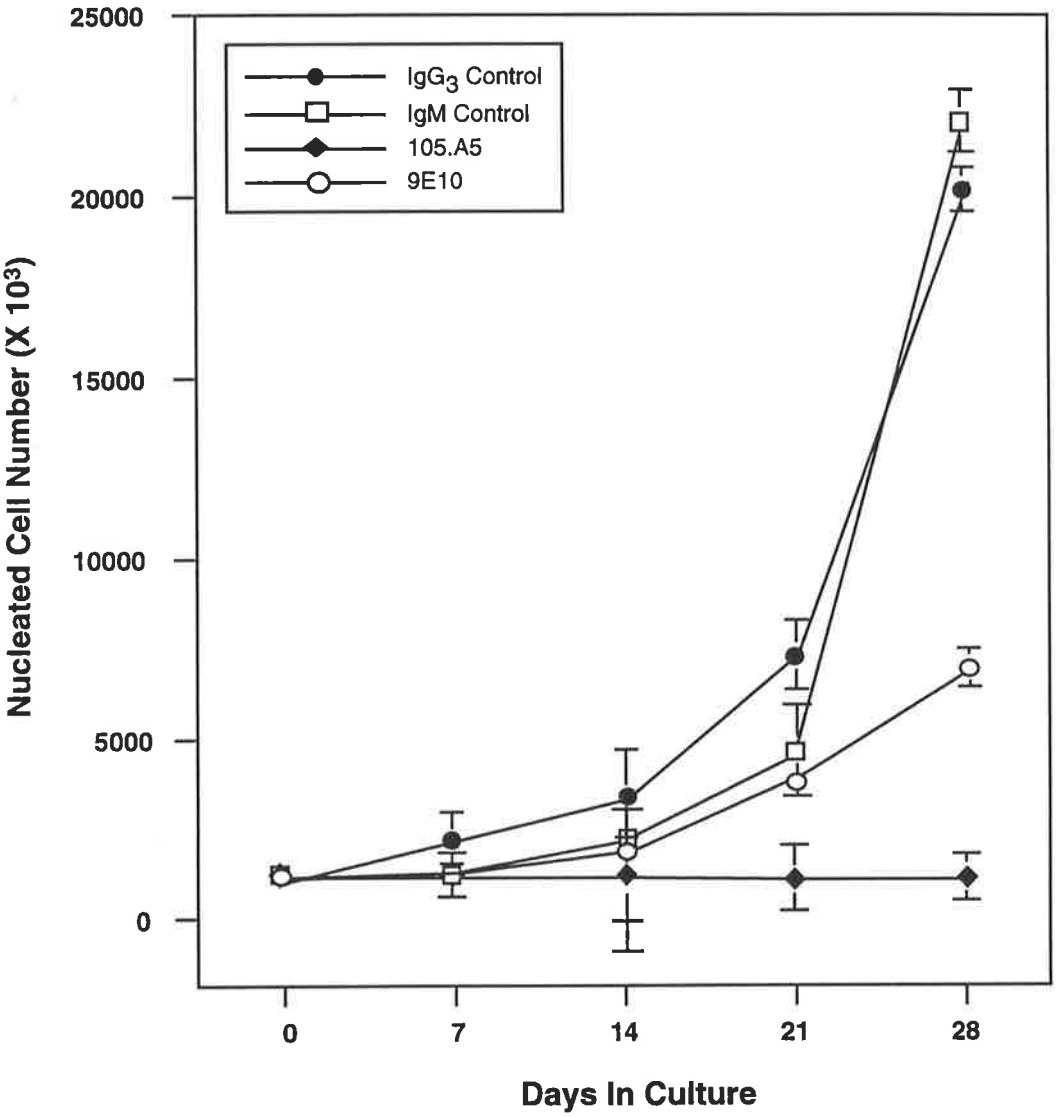

A
B
CFU-GM (control)
CFU-GM (9E10)
CFU-GM (105.A5)
CFU-GM
BFU-E (control)
BFU-E (9E10)
BFU-E (105.A5)
BFU-E
0.01
0.1
0.3
1
3
10
30
Antibody Concentration (μg/ml)
Number Of Colonies/1 x 10^3 Cells Plated
9E10
105.A5

Figure 6.2.21. Ligation Of MGC-24v On CD34$^+$ Cells Results In The Suppression Of Haemopoiesis : Studies Of Primitive Haemopoietic Precursors (Pre-CFU).

1 x 10^3 CD34$^+$ cells per culture (in triplicate) were established in serum-deprived medium containing 10 ng/ml each of purified recombinant human IL-1β, IL-3, IL-6, G-CSF, GM-CSF and SCF. Moreover, this medium was also supplemented with 10 μg/ml of mAbs 9E10 and 105.A5 (or appropriate isotype-matched control mAbs). Additional factors (and mAbs) were added at the same concentrations on days 7, 14 and 21. On days 7, 14 , 21 and 28, the cells were harvested, washed and assayed for nucleated cell number as previously described.(*Materials and Methods* (Section 2.5.4.)).

Monoclonal antibodies 9E10 (○) or 105.A5 (◆) result in dose dependent inhibition of haemopoietic cell production, as compared to the non-binding IgG_3 (●) and IgM (□) isotype matched control antibodies. The results are expressed as the mean number (±SE) of nucleated cells recoverd in each group at the designated time points.


25000
20000
15000
10000
5000
0
Nucleated Cell Number (X 10³)
IgG3 Control
IgM Control
105.A5
9E10
0
7
14
21
28
Days In Culture

day 28. The enhanced ability of mAb 105.A5 (compare with 9E10) to perturb haemopoiesis is probably due to the polyvalency of the IgM antibody and hence greater ability to cross-link cell-surface MGC-24v. The molecular mechanism for this haemopoietic suppression is yet to be defined, however consistent with the ligation of the mucin-like P-selectin counter-receptor PSGL-1 (Chapter 3), it would be reasonable to speculate, that as yet undefined signals transmitted via the MGC-24v cytoplasmic domain result in a marked suppression of cellular proliferation (cytostatic and hence reversible response) or the induction of apoptosis. These questions require further investigation.

6.3 DISCUSSION

6.3.1. Identification Of A Mucin-Like Molecule Expressed By Primitive Human Haemopoietic Cells And Cultured Human Bone Marrow Stromal Cells

Cellular interactions between primitive haemopoietic progenitors and their contiguous stromal microenvironment are of major importance in the regulation of haemopoiesis (Dexter, 1982; Weiss, 1976; Lichtman, 1981; Tavassoli and Friedenstein, 1983; Allen *et al*, 1990; Simmons and Torok-Storb, 1991a; 1991b; Dexter *et al*, 1977; Tavassoli and Hardy, 1990). Accordingly, cell surface antigens which are selectively expressed by either or both components may play an important role in mediating these interactions. The recent cloning of four molecular partners for the selectin family (GlyCAM-1, CD34, MAdCAM-1 and PSGL-1), have led to the identification of a new and emerging family of adhesion molecules variously termed, mucin-like proteins or sialomucins. Mucin-like molecules are characteristically, rod-like, serine- and threonine-rich proteins which are heavily decorated with O-linked glycans.

This present study describes two mAbs, namely 9E10 and 105.A5, which exhibit reactivity to both a minor subpopulation of the $CD34^+$ HPC population and elements of the BM stroma. Upon molecular cloning and partial sequence analysis, mAbs 9E10 and 105.A5 were found to identify a novel transmembrane-containing isoform of a previously identified mucin-like glycoprotein termed MGC-24 (Masuzawa *et al*, 1992). MGC-24 (for Multi-Glycosylated Core protein of 24 kD) was originally cloned from KATO-III human gastric carcinoma cells and found to encode a novel polypeptide backbone, highly decorated with both O-and N-linked carbohydrate side chains.

Complete sequence analysis of the ORFs of both the 9E10 and 105.A5-derived cDNA clones demonstrated that although sharing complete identity with MGC-24 from nucleotide 1 to 383, these cDNA clones exhibited a deletion of 58 nucleotides (a putative alternative-spliced exon) which resulted in a frameshift and hence divergent COOH-terminal domain. In contrast to the epithelial-derived MGC-24 protein, computer

modelling and hydropathy analysis of the 178 amino acid translated region, revealed the presence of both a putative transmembrane-anchoring motif (residues 140-164) and a short cytoplasmic domain (residues 165-178).

Using a polyclonal antiserum to the native deglycosylated core protein, Masuzawa and colleagues (1992) demonstrated that the mature, epithelial-derived MGC-24 glycoprotein behaved as a high molecular mass (>200 kD), principally soluble, polymorphic mucin-like molecule, which contained numerous peanut agglutinin (PNA)-binding sites. In contrast, using mAbs 9E10 and mAb 105.A5 (independently generated by Dr. H.-J. Bühring, University of Tübingen, Germany), Western blot and immunoprecipitation analyses of membrane extracts from cells representing various haemopoietic cell lineages, revealed that MGC-24v exists in its native state, as disulphide linked homodimers of two 80 kD subunits. As this variant isoform lacks the consensus glycosaminoglycan (GAG)-attachment site, it would be fair to speculate that GAG-association or differential glycosylation is responsible for the high molecular mass of the epithelial-derived MGC-24 glycoprotein.

Utilising a variety of *in vitro* haemopoietic cell assays, it was also found that clonogenic myeloid (CFU-GM) and erythroid (BFU-E) progenitors were restricted to the subpopulations of $CD34^+$ cells which expressed MGC-24v. Moreover, the highest level of MGC-24v expression was found on the most primitive progenitors characterised by the $CD34^{BRIGHT}/CD38^-$ phenotype (Figure 6.2.6. (B)), and as demonstrated by the capacity of these cells to initiate and sustain haemopoiesis in the *in vitro* cytokine stimulated pre-CFU assay. Furthermore, in addition to being expressed by haemopoietic precursor cells, Western blot and immunophenotypic analysis using mAb 9E10, revealed that MGC-24v glycoprotein is expressed by $GlyA^+$ erythroid precursors, but is subsequently lost during erythrocyte development. This apparent differentiation-associated loss of MGC-24v membrane expression appears to be a feature shared by lymphoid (B and T) cells, however further studies are required to confirm this observation.

Since MGC-24v was molecularly cloned from a BM stromal cell library, it is

unclear whether an alternate isoform, distinct from both MGC-24 and MGC-24v (but reactive with 9E10 and 105.A5) may be expressed by HPCs and their precursors. Current data do not permit definitive conclusions to be drawn, however several observations suggest that the isoform expressed by haemopoietic cells may be equivalent to that expressed by BM stromal cells. For example, a 3 kb transcript was observed in both BM stromal cells and the candidate myeloid progenitor cell line KG1a by Northern blot analyses (Figure 6.2.10.). In addition, both 9E10 and 105.A5 immunoreactive proteins, derived from various cell preparations (including BM stromal cells and various myeloid cell lines), were of approximately equivalent size (Figure 6.2.5. (A) & (B)). Studies examining the mRNA species present in haemopoietic cells will be required to address this question.

Although mAbs 105.A5 and 9E10 exhibit disparate reactivity profiles (Table 6.2.2), it is clear from the studies presented herein that both mAbs identify the product of the MGC-24v cDNA (Figure 6.2.12.). The mechanism responsible for this phenomenon remains to be determined, however due to the high degree of glycosylation, it is possible that the mAbs 105.A5 and 9E10 identify glycosylation-dependent epitopes and/or heterogeneous glycosylation of MGC-24v molecules. To address this question, future studies could examine their capacity to bind MGC-24v following treatment with a variety of carbohydrate-modifying enzymes to specifically remove sialic acid, O-linked and N-linked glycans. Alternatively, the reactivity of both mAbs with bacterially synthesised, recombinant (non-glycosylated) MGC-24v could be assessed, which will enable the epitope binding-specificity to be determined in the absence of carbohydrate.

The preponderance of data to date, suggests that mucin-like molecules function in a pro-adhesive manner (Sako *et al*, 1993; Lasky *et al*, 1992; Imai *et al*, 1994; Briskin *et al*, 1993; Berg *et al*, 1993). In addition to the aforementioned selectin counter-receptors, studies by May *et al* (1994) suggest that high levels of human $CD34^+$ can mediate the selective adhesion of transfected murine cells to human, but not mouse stroma. However, in contrast, a number of studies have attributed an anti-adhesive function to mucin-like

molecules, by virtue of their negative charge and extended configuration. This was first demonstrated for the neural cell adhesion molecule (NCAM, CD56) (Hoffman and Edelman, 1983; Rutishauser, 1988; Gower *et al*, 1988), and more recently for CD43 (Manjunath *et al*, 1995), episialin (Hilkens *et al*, 1992; Zaretsky *et al*, 1990; Ligtenberg *et al*, 1992), and epiglycanin (Kemperman *et al*, 1993; 1994).

Adhesion assays to examine a possible pro- or anti-adhesive role for MGC-24v were subsequently performed. As demonstrated in Figure 6.2.7., the adhesion of $CD34^+$ cells to allogeneic BM cells *in vitro* was partially inhibited (~30%) in the presence of the mAb 9E10, (but not 105.A5), thus confirming a pro-adhesive function of MGC-24v. The high serine and threonine content of MGC-24v and the existence of nine potential N-linked sites of glycan attachment, indicate that the high degree of glycosylation contributes approximately 70% to the molecular mass of the mature protein expressed by tissues of haemopoietic origin. Moreover, based on the finding with GlyCAM-1 (Lasky,1992) and PSGL-1 (Sako *et al*, 1993), it would be conceivable that the carbohydrate chains of MGC-24v are involved in the adhesive interactions observed between $CD34^+$ and BM stromal cells.

The MGC-24v protein backbone may thus serve as a platform or scaffold for carbohydrate presentation. Such a scaffolding function for a mucin-like glycoprotein is plausible in view of what is currently known about mucin structure. The high carbohydrate content in mucins and mucin-like domains of glycoproteins dominates the physiochemical properties, such that the substituted peptide is highly extended into a rigid rod with all the secondary and tertiary structure precluded (Jentoft, 1990), resulting in a predicted average extension per amino acid of 2.5 Å (Jentoft, 1990; Devine and McKenzie, 1992). In good agreement with this prediction, the leucocyte cell surface molecule, leukosialin (sialophorin, CD43) (Cyster *et al*, 1990; 1991; Fukuda, 1991, Jentoft, 1990; Devine and McKenzie, 1992) contains an extracellular mucin domain of approximately 225 amino acids and has an extracellular extension of 45nm (Cyster *et al*, 1991). Based on this model, the 139 amino acid extracellular domain of MGC-24v would

therefore extend 32 nm above the plasma membrane surface. It is presumed that the role of the mucin domains is to extend the functional domain of these cell surface molecules well beyond the glycocalyx of the cell, and these provide easy access to the opposing counter-receptor or extracellular macromolecules (Devine and McKenzie, 1992).

As MGC-24v appears to facilitate adhesive interactions between haemopoietic and BM stromal cells, it would be tempting to speculate that like PSGL-1, MGC-24v expressed by $CD34^+$ cells may present glycan side chains to E- and/or P-selectin on endothelial cells (vascular or BM). Similarly, BM and stromal cell-expressed MGC-24v may assist in the "tethering" of progenitor cells (and their mature progeny) via interactions with the lectin domain of L-selectin and the clustered carbohydrate side chains presented by MGC-24v. Support for this notion is provided by the recent work of Lenter and colleagues (1994), who have recently identified an alternate P-selectin ligand from the human leukaemic cell line HL60. The molecular weight of this ligand under both non-reducing and reducing conditions, approximates that of MGC-24v and in fact may represent the equivalent cell surface molecule. Whilst an attractive proposition, further studies are required to confirm this possibility.

A number of recent reports have demonstrated that like integrins (Hynes, 1992), mucin-like molecules are capable of integrating external signals into the cytoplasm via their cytoplasmic domains. This was well exemplified by a recent study by Majunith *et al*, (1995), who demonstrated using CD43 "knockout" mice, that this cell-surface sialoglycoprotein was able to negatively regulate T cell activation. Moreover, work by Fackler *et al*, (1995) demonstrated that enforced expression of the full-length form (but not the truncated form) of CD34 inhibited terminal differentiation of murine myeloid M1 cells, strongly suggesting that region(s) in the cytoplasmic domain are responsible for the observed maturation arrest phenotype.

These studies, and those described in Chapter 3 demonstrating that PSGL-1-mediated adhesion to P-selectin results in the suppression of human $CD34^+$ HPC proliferation, prompted the investigation of whether ligation of this MGC-24v on

progenitor cells would have a similar inhibitory effect. Since the physiological counter-receptor(s) for MGC-24v remain to be determined, mAbs 9E10 and 105A5 were used as surrogate ligands. Relative to control mAbs, addition of each of the two anti-MGC-24v antibodies to input clonogenic assays resulted in dose-dependent inhibition of myeloid (CFU-GM) and erythroid (BFU-E) growth, which was almost complete at doses between 10 and 30 μg/ml. Similarly, ligation of MGC-24v on the more primitive culture initiating cells (pre-CFU), resulted in a significant repression of haemopoietic cell proliferation. Whilst the inclusion of mAb 105.A5 resulted in the complete suppression of nucleated cell production, the effect of mAb 9E10 on $CD34^+$ HSC provided only an initial inhibition, allowing the cells to recover from day 7 to day 28 (refer to Figure 6.2.21.). The basis for this difference is unknown and requires further investigation.

These data identify that like PSGL-1, the mucin-like molecule MGC-24v represents a signalling molecules on primitive HPC, and moreover imply that interactions between mucin-like proteins and their counter-receptors negatively regulate HPC proliferation. Despite this, very little is known of the signalling function of MGC-24v, and it remains to be determined whether the inhibitory effect manifested by these molecules, involves the induction of apoptosis, or an alternative cytostatic mechanism (refer to Chapter 3, Section 3.3.1.). Future studies examining the signal transduction mechanisms of MGC-24v should provide an understanding of the mechanisms responsible for the inhibition of HPC proliferation.

Whilst beyond the scope of this thesis, valuable information regarding the function and significance of MGC-24v could be obtained with studies designed to; (i) determine the chromosomal location of MGC-24v, which may identify closely linked molecules of known function (see review by Baird *et al*, 1995); (ii) determine the genomic organisation and exon structure of MGC-24v, which may be predictive of the existence of splice variants; (iii) identify the murine homologue and determine the phenotype exhibited by MGC-24v "knockout" mice, and; (iv) identify the counter-receptor for MGC-24v, which may ultimately indicate function.

CHAPTER 7

GENERAL DISCUSSION

7.1. MOLECULAR DEFINITION OF STEM CELL-STROMAL CELL INTERACTIONS.

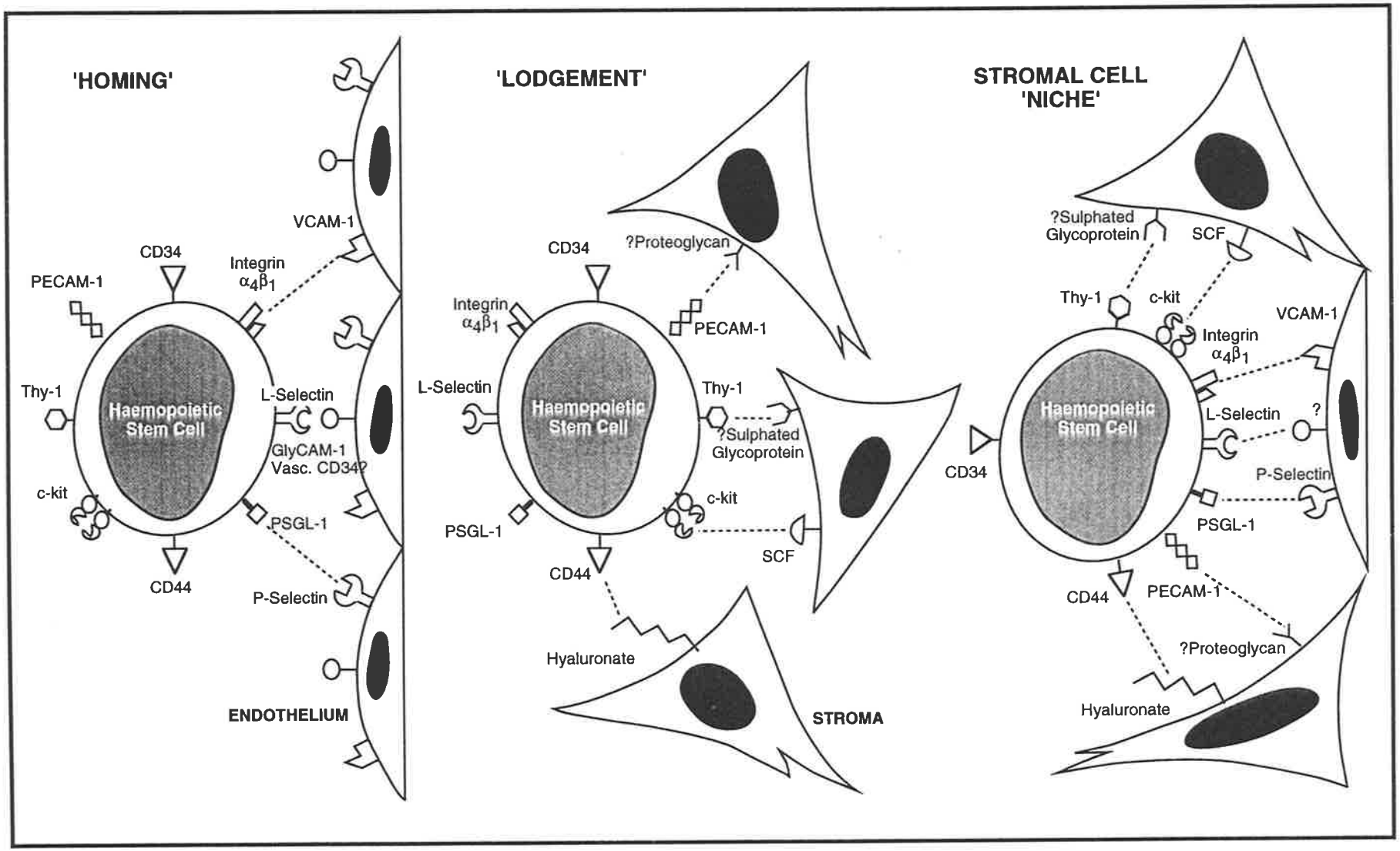
Haemopoiesis occurs in intimate physical association with the stromal elements of the bone marrow (BM) (Trentin, 1970; Tavassoli, 1975; Price and McCulloch, 1978; Wolf, 1979; Cline and Golde, 1979; Dexter, 1979; Dexter, 1982; Torok-Storb, 1988). The various cellular elements of the stroma, together with their associated biosynthetic products constitute the haemopoietic microenvironment (HM) of the BM (Trentin, 1970; Tavassoli, 1975; Wolf, 1979; Dexter, 1982). All haemopoietic cells within the HM are subject to the permissive and most likely directive environment provided by the heterogenous stromal cell population (Tavassoli and Friedenstein, 1983; Allen *et al*, 1990; Dexter *et al*, 1977; Flanagan *et al*, 1991). This level of control is mediated by the cellular interactions between the haemopoietic cells and the stromal cell compartment, either through cell adhesion or interaction with haemopoietic growth factors (HGF) produced and/or presented by the stromal cells (Heinrich *et al*, 1993; Roberts *et al*, 1987a; 1988; Gordon, 1988; Gordon *et al*; 1987a; 1988; 1991; Metcalf, 1984; 1989; 1993; Gabrilove, 1994). In addition, current evidence supports the hypothesis that the restriction of primitive haemopoietic progenitor cells (HPC) to the BM involves developmentally regulated adhesive interactions between HPC and the stromal cell microenvironment (Dexter *et al*, 1977; Tavassoli and Hardy, 1990; Clark *et al*, 1992).

There is now a considerable body of data demonstrating that primitive human HPC exhibit a multitude of cell adhesion molecules (CAMs) each with specificity for distinct counter receptors on marrow stromal cells (Springer, 1990a; 1990b; Albelda and Buck, 1990; Haynes *et al*, 1989; Bevilacqua and Nelson, 1993). Although this may imply redundancy within this repertoire, it most likely suggests that particular CAMs perform separate functions associated with, for example, the homing of HPC to the bone marrow, or their lodgement and retention within the extravascular HM (Figure 7.1.1.). Given the potential complexity of both phenomena, such an array of CAMs on HPC might be anticipated.

Support for this notion comes from studies of the mechanism of entry of mature leukocytes into tissues at sites of inflammation. From such studies has come the realisation that this multi-step process is controlled by a dynamic interaction between an

Figure 7.1.1. Hypothetical Scheme Illustrating The Potential Role Of CAMs And Their Stromal Ligands In The Homing And Lodgement Of HPC To The Bone Marrow Microenvironment.

Selectins and Integrins are envisaged to participate in the homing of HPC to the marrow endothelium while other CAM-ligand pairs may contribute to their subsequent lodgement in the extravascular haemopoietic compartment. Alternatively, multiple CAM ligand pairs may be required to promote adhesion of HPC to a specialised stromal cell niche.


'HOMING'
VCAM-1
CD34
Integrin
$\alpha_4\beta_1$
PECAM-1
Thy-1
Haemopoietic Stem Cell
L-Selectin
GlyCAM-1
Vasc. CD34?
c-kit
PSGL-1
CD44
P-Selectin
ENDOTHELIUM
'LODGEMENT'
?Proteoglycan
CD34
Integrin
$\alpha_4\beta_1$
PECAM-1
L-Selectin
Haemopoietic Stem Cell
Thy-1
?Sulphated Glycoprotein
c-kit
PSGL-1
SCF
CD44
Hyaluronate
STROMA
STROMAL CELL 'NICHE'
?Sulphated Glycoprotein
SCF
Thy-1
c-kit
VCAM-1
Integrin
$\alpha_4\beta_1$
CD34
Haemopoietic Stem Cell
L-Selectin
?
P-Selectin
PSGL-1
CD44
PECAM-1
?Proteoglycan
Hyaluronate

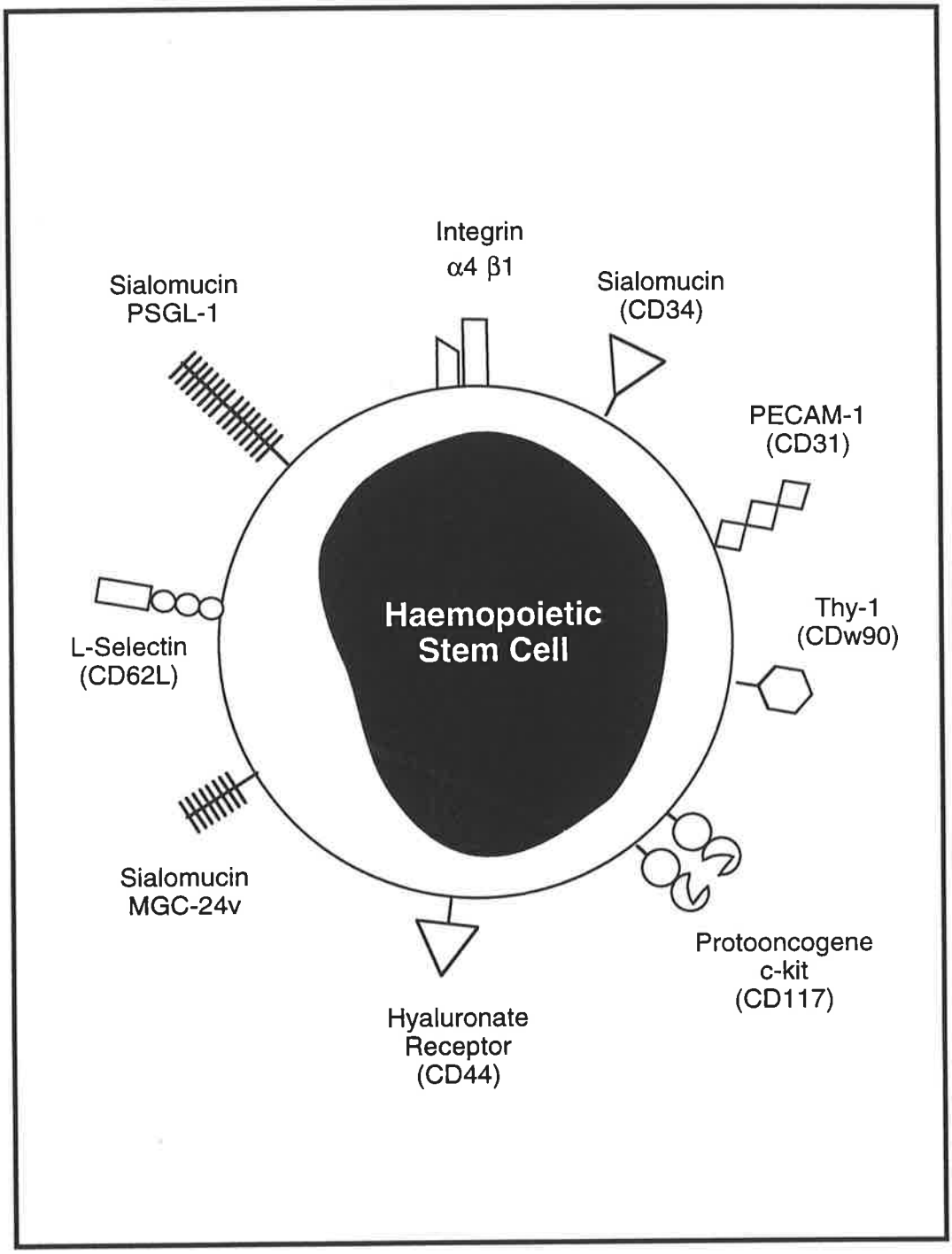
ever increasing number of CAM families expressed by both leukocytes and endothelial cells (Lawrence and Springer, 1991; Mackay and Imhof, 1993; Hogg, 1991; 1992). As illustrated in Figure 3.1.1. the initial attachment and "rolling" of leukocytes along the endothelium is mediated by members of the *selectin* family binding to their carbohydrate-bearing, *mucin-like* ligands. The next stage involves firm attachment which is accomplished by a triggering event that causes activation of *integrins* thereby facilitating binding to their counter-receptors, the various members of the *immunoglobulin gene superfamily*. The final stage, extravasation, involves a change in the shape of the leukocyte and transmigration through the endothelium. Thus, rather than being dependent on a single binding event, the specificity and diversity of leukocyte-endothelial cell interactions relies on sequential and combinatorial adhesive interactions (Butcher, 1991). Given that HPC express a similar cohort of CAMs to those found on circulating lymphocytes (Figure 7.1.2.) (Springer, 1990; Albelda and Buck, 1990; Bevilacqua and Nelson, 1993; Hemler, 1987a; 1987b; 1990; Torimoto *et al*, 1992) a similar scenario of events may be involved in the "homing" of HPC to the bone marrow (Figure 7.1.1.).

In order to enter the haemopoietic compartment, stem cells arriving at the marrow must first recognise, or be recognised by, the luminal surface of the endothelium. Once in the haemopoietic compartment, HPC interact with stromal elements that regulate their subsequent growth and development. It remains to be determined if the CAMs responsible for guiding HPC to the marrow endothelium are the same as those involved in binding to marrow stromal cells or whether distinct CAMs regulate the latter process of "lodgement" and retention within the "stromal cell niche" (Figure 7.1.1.).

The data presented in this thesis principally dealt with the molecular characterisation of CSMs which are expressed by haemopoietic progenitor cells (and their precursors) and cells of the bone marrow stroma, which may function in (i) augmenting haemopoietic cell development, (ii) homing and retention of HPC within the extravascular haemopoietic compartment of the BM, and (iii) their egress into the peripheral circulation.

Figure 7.1.2. Primitive Human HPC Exhibit An Extensive Array Of CAMs.

Primitive human HPC exhibit an extensive array of CAMs, each of which interacts with specific ligands expressed by cells of the stromal microenvironment. CAMs expressed by HPC include members of the integrins, the selectins, the sialomucins and the immunoglobulin gene superfamily (*refer to text for details*).


Integrin
α4 β1
Sialomucin
PSGL-1
Sialomucin
(CD34)
PECAM-1
(CD31)
Haemopoietic
Stem Cell
Thy-1
(CDw90)
L-Selectin
(CD62L)
Sialomucin
MGC-24v
Protooncogene
c-kit
(CD117)
Hyaluronate
Receptor
(CD44)

7.2. SUMMARY OF DATA.

7.2.1. The Role Of P- And E- Selectin.

The selectins are a family of three structurally related integral membrane glycoproteins that regulate leukocyte adhesion to vascular endothelium during inflammation, by means of selective protein-carbohydrate interactions mediated by the C-type lectin domain at the N-terminus of each family member (Lasky *et al*, 1989; Johnston *et al*, 1989a; 1989b; Bevilacqua *et al*, 1989; Bevilacqua and Nelson, 1993; Lasky *et al*, 1992; Watson *et al*, 1990).

P-selectin (CD62P) is a 140 kD transmembrane protein which is stored pre-formed in the Weibel-Palade bodies of endothelial cells, including BM endothelium (Bonfanti *et al*, 1989; Johnston *et al*, 1989a; 1989b; Beckstead *et al*, 1986; Schweitzer *et al*, 1995, Rafii *et al*, 1994) and in the alpha-granules of platelets (Berman *et al*, 1986). Following stimulation by a variety of agonists, P-selectin is rapidly translocated to the cell surface where it supports the shear-resistant adhesion ("rolling") of mature leukocytes to sites of inflammation (McEver and Martin, 1989; Hsu-Lin *et al*, 1989; Hattori *et al*, 1989a; 1989b; Gerig *et al*, 1990; Gamble *et al*, 1990; Lawrence and Springer, 1991, Mayadas *et al*, 1993; Springer, 1990a; 1990b; Atherton and Boon, 1972; Butcher, 1991). Data presented in Chapter 3, demonstrate that in addition to its well documented role as CAM for mature leukocytes, P-selectin also supports the adhesion of candidate human haemopoietic progenitor cells ($CD34^+$ HPC) and of their lineage-restricted clonogenic cell progeny (CFU-GM, BFU-E)). This represents the first report establishing a role for P-selectin as an adhesion molecule for human $CD34^+$ HPC.

Studies in a number of laboratories have demonstrated that P-selectin binds to a single class of receptor on neutrophils and HL60 cells (Moore *et al*, 1992; Sako *et al*, 1993), that through expression cloning was subsequently identified as a 220 kD, disulphide linked homodimeric sialomucin, termed by the authors P-selectin glycoprotein ligand-1 (PSGL-1) (Sako *et al*, 1993). Data presented herein provides several lines of evidence to demonstrate that PSGL-1 is expressed by $CD34^+$ HPC, and represents the sole ligand for P-selectin on these cells: (i) RT-PCR and flow cytometric analyses demonstrated that marrow-derived $CD34^+$ cells express both PSGL-1 mRNA and protein, (ii) adhesion of

$CD34^+$ cells to P-selectin is completely abolished by treatment with anti-PSGL-1 antibody and the highly specific cobra venom metalloproteinase, mocarhagin (De Luca, 1995).

A number of reports (Sako *et al*, 1993; Moore *et al*, 1994; Asa *et al*, 1995; De Luca *et al*, 1995; Li *et al*; 1996) have demonstrated that in addition to serving as a ligand for P-selectin, PSGL-1 may also bind to E-selectin. E-selectin (CD62E) represents a 110-115 kD, cytokine-inducible transmembrane protein whose expression is restricted to cells of endothelial origin (Bevilacqua *et al*, 1989; Bevilacqua and Nelson, 1993; Dercksen *et al*, 1994; Schweitzer *et al*, 1995). As E-selectin expression at the plasma membrane is reliant on *de novo* RNA and protein synthesis (reviewed in Carlos and Harlan, 1994), it is postulated to be involved in the delayed recruitment of leukocytes to sites of inflammation (Mulligan *et al*, 1991).

The requirements for E-selectin recognition are much less stringent than that for P-selectin, as CD62E has been shown to bind (with high affinity) to a number of sialomucins and glycoprotein structures that co-express sialyl Le^x or the related structure, sialyl Le^a (Picker *et al*, 1990; 1991a; Kishimoto *et al*, 1991; Kuijpers *et al*, 1992; Kotovuori *et al*, 1993; Steegmaier *et al*, 1995). Data presented in Chapter 3, demonstrate that BM-derived $CD34^+$ cells exhibit cation-dependent, neuraminidase-sensitive adhesion to E-selectin. Adhesion of $CD34^+$ cells to E-selectin however, was unaffected by treatment of the $CD34^+$ cells with mocarhagin, thus enabling the contribution of E- and P-selectin in the adhesion of $CD34^+$ cells to be readily discriminated.

Although data presented here and elsewhere (Pouyani and Seed, 1995; Sako *et al*, 1995) demonstrate that binding of $CD34^+$ cells to E-selectin does not involve the N-terminal region of PSGL-1, they do not preclude the possibility that E-selectin interacts with PSGL-1 via a more C-terminal region bearing clustered sialylated-carbohydrates (refer to Figure 3.3.1.). Alternatively, $CD34^+$ cell binding to E-selectin may involve alternative glycoproteins (or glycolipids) which are capable of presenting sialylated-glycan structures to E-selectin. This view is consistent with the recent findings from two independent groups (Lenter *et al*, 1994; Levinovitz *et al*, 1993), who described the isolation of a 150 kD glycoprotein from both murine and human myeloid cells which exhibited E-selectin-specific adhesion.

7.2.2. The Role Of L-Selectin.

Human L-selectin represents the homologue of the 90 kD murine peripheral lymph node homing receptor, originally identified by the mAb, MEL-14 (Gallatin *et al*, 1983). Although initially described as a lymphocyte homing receptor, it was subsequently shown to be constitutively expressed on most other peripheral blood leukocytes including neutrophils and monocytes, and shown to be involved in leukocyte traffic in the systemic microcirculation (Tedder *et al*, 1990; Griffin *et al*, 1990).

Although a number of reports examining the expression of L-selectin by $CD34^+$ HPC (Lund-Johansen and Terstappen, 1993; Turner *et al*, 1995; Dercksen *et al*, 1995), have been published, the data presented in Chapter 3, represent the first studies to investigate the *in vitro* potential of $CD34^+$ progenitor cells selected on the basis of CD62L expression.

In addition to subdividing the $CD34^+$ cells into $CD62L^+$ and $CD62L^-$ subfractions, stringent dual colour FACS enabled the separation of the $CD62L^+$ population into $CD34^+CD62L^{BRIGHT}$ and $CD34^+CD62L^{DIM}$ subpopulations. *In vitro* assays to measure the capacity to initiate and sustain haemopoiesis (LTBMC and pre-CFU assays), consistently demonstrated that the $CD34^+CD62L^{DIM}$ population had a significantly greater capacity to sustain the *de novo* generation of both erythroid (BFU-E) and myeloid (CFU-GM) clonogenic cells, when compared with the coincident $CD34^+CD62L^{BRIGHT}$ population. Moreover, the $CD34^+CD62L^-$ subpopulation exhibited virtually no capacity to generate nascent CFU-GM and BFU-E.

Studies to examine which subpopulations harboured the lineage-restricted clonogenic progenitor cells revealed that the myeloid progenitors were enriched in the $CD34^+CD62L^+$ fraction, whilst the erythroid progenitors were present in both the $CD34^+CD62L^+$ and $CD34^+CD62L^-$ subpopulations. Similarly, the erythroid progenitors were consistently enriched in the $CD34^+CD62L^{DIM}$ subpopulation, whilst in contrast to the results obtained in pre-CFU/LTBMC assays, the $CD34^+CD62L^{BRIGHT}$ subpopulation harboured the majority of CFU-GM.

A corollary of these data suggest that all myeloid and erythroid precursors (and

their committed progeny) are contained within the $CD34^+CD62L^+$ subpopulation. Moreover, the $CD34^+$ myeloid progenitor cells and their precursors express L-selectin at an intermediate level ($CD62L^{DIM}$), whilst the maturation of erythroid precursors was associated with a progressive up-regulation of L-selectin expression. Although the functional significance of these data are unknown, they do indicate that L-selectin expression is modulated as a function of haemopoietic cell differentiation.

A recent communication by Spertini *et al* (1991) demonstrated that L-Selectin was able to mediate the adhesion of both lymphocytes and neutrophils to a cytokine-inducible (TNFα or IL-1β), neuraminidase-sensitive ligand on HUVECs. Data presented in Chapter 3 similarly demonstrate that a TNFα inducible ligand for L-selectin was expressed by cultured human BM stromal cells. Although the nature of this ligand is unknown, preliminary data suggests that this inducible ligand for L-selectin is unlikely to be the recently cloned glycosylation dependent cell adhesion molecule-1 (GlyCAM-1) (Lasky *et al*, 1992). Further studies are required to identify and characterise the nature of this CSM.

7.2.3. Retroviral Expression Cloning; An Alternate Approach To Isolate cDNAs Corresponding To Novel CSMs.

The relative intractability of mammals to genetic analysis has precluded traditional genetic approaches to haemopoiesis. Despite these formidable challenges, remarkable progress has been made with the use of a number of alternate approaches. The advent of hybridoma technologies (Kohler and Milstein, 1975), provided a means of generating a wide variety of mAb probes, specific for molecules expressed at the surface of various tissue and cell preparations (Knapp *et al*, 1989). Furthermore, mAbs have enabled the identification and characterisation of the function of many integral membrane glycoproteins, including those which mediate cellular recognition, adhesion or haemopoietic cell regulation (reviewed by Simmons, 1992).

The concomitant and unprecedentedly swift developments in expression cloning and recombinant DNA technologies (Young and Davis, 1983; Kavathas *et al*, 1984; Maddon *et al*, 1985) have enabled the isolation of functional cDNA clones corresponding to many of these CSM, and has opened up a new era in biology and medicine. To date,

the most successful approach has been that developed by Aruffo and Seed (Seed and Aruffo, 1987; Seed, 1987; Aruffo and Seed, 1987; Gearing *et al*, 1989), however in recent years, a number of reports describing the use of retroviral vectors for the construction of stable cDNA expression libraries in eukaryotic cells have appeared in the literature (Rayner and Gonda, 1994; Wong *et al*, 1994; Whitehead, 1995). These retroviral-vector based systems appear to overcome a number of the inherent shortcomings of conventional expression cloning techniques. In contrast to transfection, retroviruses represent one of the most effective gene transfer systems available, and have proven useful for the transfer of genes into cells (including haemopoietic and primary cells) ordinarily refractory to transduction by other methods (reviewed by Miller *et al*, 1993).

Chapter 4 described studies which were principally designed to identify additional molecules that participate in the regulation of haemopoiesis. A panel of mAbs reactive with tissues of haemopoietic origin were generated and selected based on their demonstrable effects in augmenting haemopoietic cell proliferation/differentiation *in vitro*, or their ability to subset the primitive haemopoietic stem cell compartment. To determine the nature of the molecules identified by these mAbs, a molecular cloning approach was employed to functionally clone their corresponding cDNAs. A novel method for rapidly isolating genes encoding CSMs from a human BM stromal cell cDNA library was constructed in the retroviral vector, pRUF.*neo*. This was further facilitated by the development of a highly efficient selection strategy, utilising mAbs and antibody-coated magnetic beads. Following critical testing, this technique was employed to isolate cDNA clones corresponding to surface (glyco)proteins expressed at the HBMSC and HSC surface, recognised by mAbs of undefined specificity. The results detailed in Chapter 4 confirm previous studies demonstrating the general application of retroviral cDNA libraries and further extend their utility to the expression-cloning of cDNAs encoding CSMs. Moreover, these studies provided numerous observations that precipitated the subsequent investigations detailed in Chapters 5 and 6.

7.2.4. Molecular Characterisation Of HCC-1: A CSM Expressed By A Primitive Subset Of The Haemopoietic Stem Cell Compartment

In recent years, mAbs to haemopoietic cells have been employed to (i) define

particular cell lineages, (ii) discrete stages of haemopoietic differentiation/activation and (iii) to detect the presence of CSMs with roles in mediating cell adhesion (Bagby, 1994; Simmons, 1992; Barclay *et al*, 1993). Moreover, it is well recognised that the expression of certain antigenic determinants on haemopoietic progenitor cells often correlate with their function and stage in ontogeny and as such, can be used to separate the more primitive haemopoietic cells from their committed progeny within the BM (Andrews *et al*, 1989; Sutherland *et al*, 1989a; 1989b; Terstappen *et al*, 1991 Watt *et al*, 1987a). This is well exemplified by the finding that all BM progenitor cells are contained within the subset which was bound by mAbs to the CD34 molecule (Civin *et al*, 1984; Ogawa, 1993).

The subject matter detailed in Chapter 5 represents an extension of the work initiated by Swart (1993) using the mAb HCC-1. Swart (1993) demonstrated that this mAb delineated a subpopulation of $CD34^+$ HPC from PB and BM, that are able to initiate haemopoiesis *in vitro*. In accord with these original observations, recent *in vivo* studies (Hill *et al*, 1996), have demonstrated that $CD34^+$ HPCs expressing the CSM identified by mAb HCC-1, also exhibit multilineage engraftment potential in the SCID-hu mouse model.

The finding that the HCC-1 antigen was expressed by BM stromal cells, enabled the cloning of a cDNA corresponding to this CSM, utilising the retroviral expression library described above. From this, HCC-1 was found to bind to the CD59 molecule, a previously identified 18-20 kD phosphoinositol glycan-linked membrane protein with reported roles in autologous complement regulation, adhesion and antigen presentation (Rother *et al*, 1994; Deckert *et al*, 1992; Hahn *et al*, 1992; Venneker *et al*, 1992). Previous studies have demonstrated uniform expression of CD59 by most nucleated cells of the haemopoietic system, including all the BM-derived $CD34^+$ cells (Terstappen *et al*, 1992; Terstappen *et al*, 1993). In contrast however, the HCC-1-defined epitope of CD59 was found to be differentially expressed amongst $CD34^+$ progenitors, with the highest level present on a subset which is highly enriched for pluripotent stem cells.

Studies to determine the physical basis of the differential expression of the HCC-1 epitope on the CD59 molecule, investigated the hypothesis that the mAb HCC-1 identified a variant carbohydrate-epitope presented upon a single site of glycan attachment at Asn^{18}, whose expression was lost upon differentiation. *In vitro*

mutagenesis of this site however, revealed that HCC-1 bound equally well to the mutagenised and wild type CD59 protein, thus indicating that carbohydrate recognition was not responsible for the differential expression of the HCC-1 epitope.

Stefanova and Horejsi (1991) recently demonstrated that in addition to their respective antigens, mAbs against human GPI-linked leucocyte surface antigens CD59 and CD55 consistently immunoprecipitated additional glycoprotein and (glyco)lipid components from detergent lysates of the human T cell line, HPB ALL. Studies to investigate the hypothesis that molecules associated with CD59 were responsible for perturbing HCC-1 binding, resulted in the identification of an 80 kD (glyco)protein. Although gp80 expression through haemopoietic cell development appears to be differentiation-related, further studies are required to define the nature of this molecule.

PI-PLC-treatment of BMMNCs, revealed that in most instances HCC-1 binding was lost/retained in accord with the loss/retention of the CD59 molecule. This however was not observed within the $CD34^+$ HPC population, where the complete loss of HCC-1 was not associated with a complete loss of CD59 expression. Notwithstanding that this maybe attributed to the phenomenon of inositol acylation (reviewed by Toutant *et al*, 1989), it is more likely due to the retention of CD59 at the cell surface due to its association with gp80 (or other molecules) present within these aforementioned macromolecular complexes (please refer to Figure 5.3.2.) (Stefanova and Horejsi, 1991; Stefanova *et al*, 1991; Cinek and Horejsi, 1992; Morgan *et al*, 1993; Kniep *et al*, 1994).

Thus, based on the observations detailed in this Chapter, assignment of a cluster of differentiation (CD) predicated on the cellular immunoreactivity of a particular mAb reagent is fraught with pitfalls. As illustrated here, only through the expression cloning strategy was it possible to assign a corresponding cDNA and hence protein to which mAb HCC-1 bound.

7.2.5. The Identification And Molecular Characterisation Of MGC-24v: A CSM Expressed By Primitive Haemopoietic Progenitors With A Role In Mediating Stromal Cell-Stem Cell Adhesion.

Accumulating data suggests that numerous CSMs participate in the regulation of

haemopoiesis (Springer, 1990a; 1990b; Albelda and Buck, 1990; Bevilacqua and Nelson, 1993; Hemler, 1990; Torimoto *et al*, 1992). Of these, cell adhesion molecules (CAMs) play a major role in mediating interactions between primitive HPC and various components of the marrow stroma. Based on domain structure and function, these CAMs can be grouped into 5 main families of adhesion molecules including the immunoglobulin superfamily, integrins, cadherins, selectins and mucin-like molecules (Dianzani and Malavasi, 1995).

Mucin-like molecules represent an emerging family of glycoprotein molecules expressed by tissues of the haemopoietic system. An adhesive function for many of the mucin-like proteins can be inferred from the recent molecular cloning of glycoprotein counter-receptors for members of the selectin family. Studies presented in Chapter 6 described two mAbs, namely 9E10 and 105.A5, with reactivity to both a minor subpopulation of the $CD34^+$ HPC population and elements of the BM stroma. Upon molecular cloning and partial sequence analysis, mAbs 9E10 and 105.A5 were found to identify a novel transmembrane-containing isoform of a previously identified mucin-like glycoprotein termed MGC-24 (Masuzawa et al, 1992).

MGC-24 (for Multi-Glycosylated Core protein of 24 kD) was originally cloned from KATO-III human gastric carcinoma cells and found to encode a novel polypeptide backbone, highly decorated with both O-and N-linked carbohydrate side chains. Using a polyclonal antiserum to the native deglycosylated core protein, Masuzawa and colleagues (1992) demonstrated that the mature, epithelial-derived MGC-24 glycoprotein behaved as a high molecular mass (>200 kD), principally soluble, polymorphic mucin-like molecule, which contained numerous peanut agglutinin (PNA)-binding sites. In contrast, immunoprecipitation and Western blotting analyses (using the mAbs 9E10 and 105A5) revealed that in tissues of haemopoietic origin, this glycoprotein behaved as a membrane-associated, disulphide-linked 160 kD homodimer, comprised of two 80 kD monomers. This apparent disparity in molecular weight was resolved following complete sequence analysis of the ORFs of both the 9E10 and 105.A5-derived cDNA clones. Although sharing complete identity from nucleotide 1 to 383, the 9E10 and 105.A5-derived cDNA clones exhibited a deletion of 58 nucleotides (a putative alternative-

spliced exon) which resulted in a frameshift and hence divergent COOH-terminal domain. In contrast to the epithelial-derived MGC-24 protein, computer modelling and hydropathy analysis of the 178 amino acid translated region, revealed the presence of a putative transmembrane-anchoring motif (residues 140-164), which was followed by a short cytoplasmic domain (residues 165-178). Moreover, this variant isoform, termed MGC-24v, lacked the consensus glycosaminoglycan (GAG)-attachment site, in accord with the notion that GAG-association was responsible for the high molecular mass of the epithelial-derived MGC-24 glycoprotein.

Immunophenotypic studies utilising mAbs 9E10 and 105.A5 revealed that MGC-24v was expressed by a subpopulation of the $CD34^+$ cells, which included the majority of clonogenic myeloid (CFU-GM) and erythroid (BFU-E) progenitors and their hierarchically more primitive precursors (pre-CFU). Since MGC-24v was molecularly cloned from a BM stromal cell library however, it is unclear whether an alternate isoform, distinct from both MGC-24 and MGC-24v (but reactive with 9E10 and 105.A5) may be expressed by HPCs and their precursors. Current data do not permit definitive conclusions to be drawn, however several observations suggest that the isoform expressed by haemopoietic cells may be equivalent to that expressed by BM stromal cells. For example, a 3 kb transcript was observed in both BM stromal cells and the candidate myeloid progenitor cell line KG1a by Northern blot analyses (Figure 6.2.10.). In addition, both 9E10 and 105.A5 immunoreactive proteins, derived from various cell preparations (including BM stromal cells and various myeloid cell lines), were of approximately equivalent size (Figure 6.2.5. (A) & (B)). Studies examining the mRNA species present in haemopoietic cells will be required to address this question.

To date, the communication of Masuzawa *et al* (1992) represents the sole report describing the MGC-24 glycoprotein, and although it summarised the molecular isolation of the MGC-24 cDNA, it did not ascribe a function to this mucin-like protein. In light of the recent findings that both GlyCAM-1 and PSGL-1 are also mucin-like molecules which participate in adhesive interactions (counter-receptors for L- and P-selectin respectively), the possible adhesive function of the membrane-associated MGC-24v glycoprotein was examined. The data presented in Chapter 6 demonstrated that 9E10 (but not 105.A5)

was able to partially block (~30%) the adhesion of $CD34^+$ cells to allogeneic BM stromal cells *in vitro*, thus confirming the adhesive properties of MGC-24v.

The high serine and threonine content (sites of O-linked glycosylation), and the existence of 9 potential sites of N-linked glycosylation in the extracellular domain of MGC-24v, may indicate that the protein backbone serves only as a platform or scaffold for carbohydrate presentation. Such a scaffolding function for a mucin-like glycoprotein is conceivable in view of what is currently known about mucin structure (Jentoft, 1990). Comparing the structures of PSGL-1, GlyCAM-1 and CD34 with MGC-24v, it would be plausible to speculate that MGC-24v may present sialic acid-containing glycan chains to members of the selectin family. These hypotheses await further examination.

7.2.6. Mucin-Like Proteins: Negative Regulators Of Haemopoiesis.

As detailed in Chapters 3, the consequences of adhesion to CD62P on the growth and development of HPC was also examined. In all the experiments performed, adhesion to P-selectin was associated with an inhibition of colony formation. Similarly, (as demonstrated in Chapter 6), ligation of MGC-24v on $CD34^+$ HPC with either mAb 9E10 or 105A5, resulted in a dose-dependent inhibition of CFU-GM and BFU-E growth in both clonogenic and pre-CFU assay systems.

In accord with these observations, a recent study by Banu *et al* (1995), demonstrated increased numbers of megakaryocytic progenitors in the BM of P-selectin "knockout" mice. Similarly, progenitor cell numbers were also increased in mice deficient in both E-selectin and P-selectin (Frenette *et al*, 1996), suggesting that retention of HPC in a quiescent state within the BM is in part, mediated by the interaction of P-selectin with PSGL-1. These studies indicate a key role for PSGL-1 (and by inference, MGC-24v) as signalling molecules on primitive HPC, and moreover imply that interactions between mucin-like proteins and their counter-receptors negatively regulate HPC proliferation. Despite this, very little is known of the signalling function of either PSGL-1 or MGC-24v, and it remains to be determined whether the inhibitory effect manifested by these molecules involves the induction of apoptosis, or an alternative cytostatic mechanism. Some insight into this question may be gained from the recent studies of Bazil and

colleagues (Bazil *et al*, 1995; 1996), who demonstrated that cross-linking of the mucin-like molecule CD43 (leukosialin) on human HPC (using antibody MEM-59, which identifies a sialic acid dependent epitope on human CD43) resulted in the induction of apoptosis of the more committed HPC. Although expressing CD43 at high levels, the HSC were spared the inhibitory effect mediated by signals transmitted through this mucin-like molecule. It is likely that the growth inhibitory properties of CD43 on HPC are related to the adhesive properties of this sialomucin. In previous studies, CD43 was shown to bind ICAM-1 (CD54) (Rosenstein et al, 1991), however subsequent studies have failed to confirm these observations (Bazil *et al*, 1995; 1996).

Moreover, a signalling function for the sialomucin CD34 has been recently postulated by Fackler and colleagues (1995). Enforced expression of the full-length (but not the truncated) isoform of the human CD34 protein in murine M1 myeloid leukaemia cells, (which can be terminally differentiated to macrophages by treatment with IL-6 or LIF), resulted in the retardation of terminal differentiation. Significantly, while the extracellular domain of CD34 shows little conservation between species, the cytoplasmic region exhibits a high degree of sequence similarity with 90% amino acid identity and 92% nucleotide identity between human, murine and canine CD34 (Krause *et al*, 1996). Accordingly, Fackler *et al* (1995), suggest that the cytoplasmic region of CD34 is therefore responsible for the observed maturation arrest phenotype. Insight into the mechanism was gained following examination of the cytoplasmic tail of CD34, where several potential binding sites for candidate transducing proteins like protein kinase C (PKC) were found. PKC, is well recognised to be involved in haemopoietic cell proliferation and differentiation (Abrahm and Smiley, 1981; Heyworth *et al*, 1993; Resnitzky *et al*, 1992).

Similar examination of the short cytoplasmic domain of PSGL-1 revealed the presence of a PKC and a casein kinase II (CaKII) phosphorylation site at Ser 336 and 348. This indicates that these enzymes (or related enzymes) may play a pivotal role in the transduction of PSGL-1-mediated growth inhibition. Although MGC-24v did not harbour these recognised sites, alternate (and as yet undefined) enzymes/binding sites may function in a similar manner. Whilst beyond the scope of this thesis, future studies examining the signal transduction mechanisms of both PSGL-1 and MGC-24v should

provide valuable new information and understanding of the mechanisms responsible for the inhibition of HPC proliferation.

7.3. CONCLUSIONS AND FUTURE DIRECTIONS.

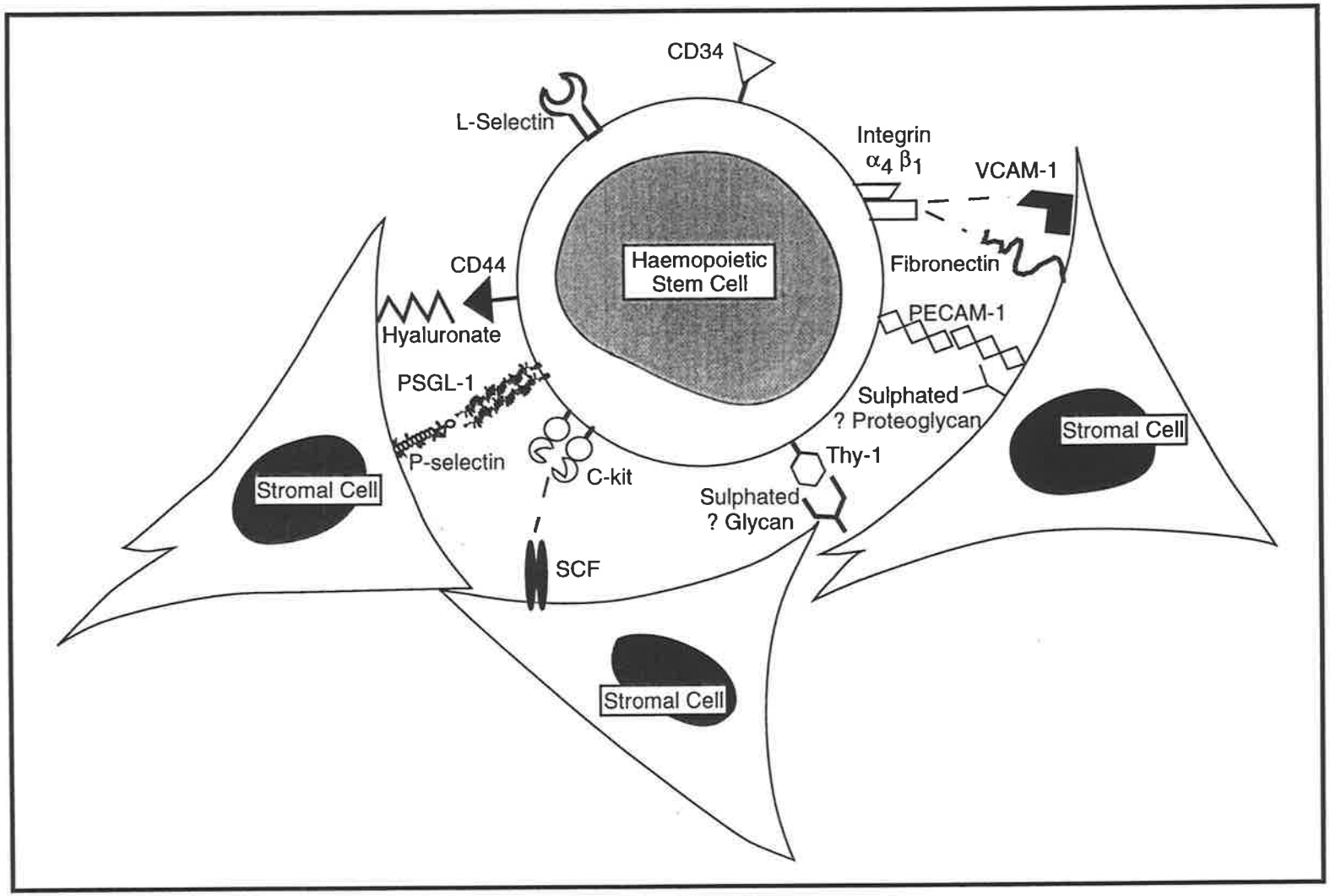
Primitive human HPC exhibit an extensive array of CAMs (Figure 7.1.2) , each of which interacts with specific ligands expressed by cells of the stromal microenvironment (Figure 7.3.1). CAMs expressed by HPC include members of the integrins, the selectins, the sialomucins and the immunoglobulin gene superfamily. In documenting the expression and function of these CAMs, one can begin to appreciate the true level of complexity of adhesive interactions that are involved in the regulation of normal haemopoiesis.

Moreover, aberrant interaction between haemopoietic cells and stromal cells may contribute to the evolution and pathophysiology of a number of haemopoietic disorders, including myeloid leukaemias and lymphoid malignancies (Soligo *et al*, 1990; Gordon *et al*, 1987c; Emerson *et al*, 1989; Denkers *et al*, 1992; Inghirami *et al*, 1988). While beyond the scope of this thesis, it is likely that the abnormal interactions observed in these diseases will be mediated through the modulation of one or more of the specific adhesive interactions described here. A major objective for the future will be to understand the mechanisms that regulate CAM expression and function in HPC and BM stromal cells.

It is now clear from numerous studies that CAMs, including members of the integrin family, are signalling molecules (Kornberg *et al*, 1991; Schwartz, 1993; Juliano and Haskill, 1993). Like integrins, data presented here demonstrates that the mucin-like molecules (sialomucins), PSGL-1 and the newly described glycoprotein, MGC-24v, also exhibit signalling function. Thus the diverse CAM-ligand interactions presented here, rather than simply serving to initiate and maintain contact between HPC and stromal cells, might also have an additional, more direct role in controlling the growth and development of primitive haemopoietic cells as suggested by the 'niche' model depicted in Figure 7.1.1.(based on that of Schofield, 1978). Such a model has obvious implications for the regulation of haemopoiesis, both normal and abnormal, and remains a provocative line of investigation for the future.

Figure 7.3.1. Hypothetical Scheme Illustrating The Potential Role Of CAMs And Their Stromal Ligands In The Growth And Development Of Haemopoietic Progenitor And Stem Cells.

Haemopoiesis occurs in intimate physical association with the stromal elements of the bone marrow (BM). The various cellular elements of the stroma and their associated biosynthetic products (including haemopoietic growth factors and extracellular matrix components) constitute the haemopoietic microenvironment (HM) of the BM. Current evidence supports the hypothesis that the retention of HPC within the HM is reliant on a multitude of cell adhesion molecules (CAMs) each with specificity for distinct counter receptors on marrow stromal cells. In addition to mediating cell-cell adhesion, this model proposes that signals transduced following receptor-ligand binding may contribute directly to the regulation of HPC proliferation and development.


CD34
L-Selectin
Integrin
α_4 β_1
VCAM-1
Haemopoietic
Stem Cell
CD44
Fibronectin
PECAM-1
Hyaluronate
PSGL-1
Sulphated
? Proteoglycan
Stromal Cell
P-selectin
C-kit
Thy-1
Stromal Cell
Sulphated
? Glycan
SCF
Stromal Cell

BIBLIOGRAPHY

Abboud, SL., Pinzani, M. (1991). Peptide growth factors stimulate macrophage colony-stimulating factor in murine stromal cells. Blood. 78: 103.

Abrahm, JL., Smiley, R. (1981). Modification of normal human myelopoiesis by 12-O-tetradecanoylphorbol-13-acetate (TPA). Blood. 58: 1119.

Aizawa, S., Tavassoli, M. (1987a). In vitro homing of hemopoietic stem cells is mediated by a recognition system with galactosyl and mannosyl specificities. Proc. Natl. Acad. Sci. (USA) 84: 4485.

Aizawa, S., Tavassoli, M. (1987b). Interaction of murine granulocyte-macrophage progenitors and supporting stroma involves a recognition mechanism with galactosyl and manosyl specificities. J. Clin. Invest. 80: 1698.

Akami, T., Arakawa, K., Okamoto, M., Akioka, K., Fujiwara, I., Nakai, I., Mitsuo, M., Sawada, R., Naruto, M., Oka, TAD. (1994). Enhancement of the complement regulatory function of CD59 by site-directed mutagenesis at the N-glycosylation site. Transplant. Proc. 26: 1256.

Albelda, SM., Buck, CA. (1990). Integrins and other cell adhesion molecules. FASEB J. 4: 2868.

Albelda, SM., Muller, WA., Buck, CA., Newman, PJ. (1991). Molecular and cellular properties of PECAM-1 (endoCAM/CD31): a novel vascular cell-cell adhesion molecule. J. Cell Biol. 114: 1059.

Alexander, WS., Lyman, SD. and Wagner, EF. (1991) Expression of functional c-kit receptors rescues the genetic defect of W mutant mast cells. EMBO. J. 10: 3683.

Allen, TD., Dexter, TM. (1984). The essential cells of the haemopoietic microenviroment. Exp. Haematol. 12: 517. (editorial).

Allen, TD., Dexter, TM., Simmons, PJ. (1990). Marrow Biology and Stem Cells. In: Dexter TM, Garland JM, Testa NG (eds): Colony Stimulating Factors: Molecular and Cellular Biology. Marcel Dekker. New York. pp. 1.

Anderson, DC., Springer, TA. (1987). Leukocyte adhesion deficiency: An inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. Annu. Rev. Med. 38:175.

Anderson, DM., Lyman, SD., Baird, A., Wignall, JM., Eisenman, J., Rauch, C., March, CJ., Boswell, HS., Gimpel, SP., Cosman, D., Williams, DE. (1990). Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms. Cell. 63: 235.

Andrews, RG., Singer, JW., Bernstein, ID. (1986). Monoclonal antibody 12.8 recognises a 115 kd molecule present on both unipotent and multipotent hematopoietic colony-forming cells and their precursors. Blood. 67: 842.

Andrews, RG., Singer, JW., Bernstein, ID. (1989). Precursors of colony-forming cells in humans can be distinguished from colony-forming cells by expression of the CD33 and CD34 antigens and light scattering properties. J. Exp. Med. 169: 1721.

Anklesaria, P., Greenberger, JS., Fitzgerald, TJ., Sullenbarger, B., Wicha, M., Campbell, A. (1991). Hemonectin mediates adhesion of engrafted murine progenitors to a clonal bone marrow stromal cell line from Sl/Sl^d mice. Blood. 77: 1691.

Appelbaum, FR., Fefer, A., Cheever, MA., Sanders, JE., Singer, JW., Adamson, JW., Mickelson, EM., Hansen, JA., Greenberg, PD., Thomas, ED. (1980). Treatment of aplastic anamia by bone marrow transplantation in identical twins. Blood. 55: 1033.

Arkin, S., Naprstek, B., Guarini, L., Ferrone, S., Lipton, JM. (1991). Expression of intercellular adhesion molecule-1 (CD54) on hematopoietic progenitors. Blood 77: 948.

Armentano, D., Yu, S-F., Kantoff, PW., von Ruden, T. Anderson, WF., Gilboa, E. (1987). Effect of internal viral sequences on the utility of retroviral vectors. J. Virol. 61: 1647.

Arnaout, AM. (1990). Structure and function of the leukocyte adhesion molecules CD11/CD18. Blood. 75: 1037.

Aruffo, A., Kolanus, W., Walz, G., Freedman, P., Seed, B. (1991). P-selectin recognition of myeloid and tumor sulfatides. Cell. 67: 35.

Aruffo, A., Seed, B. (1987). Molecular cloning of a CD28 cDNA by a high efficiency COS cell expression system. EMBO. J. 6: 3313.

Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, CB., Seed, B. (1990). CD44 is the principal cell surface receptor for hyaluronate. Cell 61: 1303.

Asa, D., Raycroft, L., Ma, L., Aeed, PA., Kaytes, PS., Elhammer, AP., Geng, JG. (1995). The P-selectin glycoprotein ligand functions as a common human leukocyte ligand for P- and E-selectins. J. Biol. Chem. 270: 11662.

Ashman, LK., Buhring, H-J., Aylett, GW., Broudy, VC., Muller, C. (1994). Epitope mapping and functional studies with three monoclonal antibodies to the C-Kit receptor tyrosine kinase, YB5.B8, 17F11 and SR-1. J. Cell. Physiol. 158: 545.

Ashman, LK., Cambareri, AC., Eglinton, JM. (1990). A MoAb that inhibits the action of GM-CSF on normal but not leukaemic progenitors. Leuk. Res. 7:637.

Ashman, LK., Cambareri, AC., To, LB., Levinsky, RJ., Juttner., CA. (1991a). Expression of the YB5.B8 antigen (c-kit proto-oncogene product) in human normal bone marrow. Blood. 78: 30.

Ashman, LK., Gadd, SJ., Mayrohofer, G., Spargo, LDJ., Cole, SR. (1987). A murine monoclonal antibody to an acute myeloid leukaemia-associated antigen identifies tissue mast cells. Myeloid Workshops, M5.10: 726.

Ashman, LK., Roberts, MM., Gadd, SJ., Juttner, CA. (1988). Expression of a 150kD cell surface antigen indentified by MoAb YB5.B8 is associated with poor prognosis in acute non-lymphoblastic leukaemia. Leuk. Res. 12:923.

Atherton, A., Boon., GVR. (1972). Quantitative investigation of the adhesiveness of circulating polymorphonuclear leukocytes to blood vessel walls. J. Cell. Physiol. 222: 447.

Avraham, H., Scadden, DT., Chi, S., Broudy, VS., Zsebo, KM., Groopman, JE. (1992). Interaction of human bone marrow fibroblasts with megakaryocytes: Role of the c-kit ligand. Blood. 80: 1679.

Bagby, G. (1994). Molecular basis of blood diseases. Stamatoyannopoulos, G. et al. (eds). W.B. Saunders, Philadelphia, second edition.

Baird, PN., D'Andrea, RJ., Goodall, GJ. (1995). Cytokine receptor genes: structure, chromosomal location, and involvement in human disease. Leuk. Lymph. 18: 373.

Bajorath, J., Hollenbaugh, D., King, G., Harte Jr, W., Eustice, DC., Darveau, RP., Aruffo, A. (1994). CD62/P-selectin binding sites for myeloid cells and sulfatides are overlapping. Biochemistry. 33 (6): 1332.

Banu, N. (1995). Role of P- and E-selectin in megakaryocytopoiesis. Blood. 80: 284 (abstract).

Barclay, AN., Birckeland, ML., Brown, MH., Beyers, AD., Davis, SJ., Somoza, C., Williams, AF. (1993). The leukocyte antigen facts book. Academic Press.

Bathija, A., Davis, S., Trubowitz, S. (1988). Bone marrow adipose tissue: Response to starvation. Am. J. Hematology. 6: 191.

Baum, CM., Weissman, L., Tsukamoto, AS., Buckle, AM., Peault, B. (1992). Isolation of a candidate human haemopoietic stem-cell population. Proc. Natl. Acad. Sci., (USA). 89: 2804.

Baumhueter, S., Kyle, C., Mebius, R., Dybdal, N., Lasky, LA. (1994). Global vascular expression of murine CD34, a sialomucin-like endothelial ligand for L-selectin. Blood. 84: 2554.

Baumhueter, S., Singer, MS., Henzel, W., Hemmerich, S., Renz, M., Rosen, SD., Lasky, LA. (1993). Binding of L-selectin to the vascular sialomucin, CD34. Science. 262: 436.

Bazil, V., Baudys, M., Hilgert, I., Stefanová, I., Low, MG., Zbrozek, J., Horejsí, V. (1989). Structural relationship between the soluble and membrane-bound forms of human monocyte surface glycoprotein CD14. Mol. Immunol. 26: 657.

Bazil, V., Strominger, JL. (1991). Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes. J. Immunol. 147: 1567.

Becker, AJ., McCulloch, EA., Till, JE. (1963). Cytological demonsration of the clonal nature of spleen colonies derived from mouse marrow cells; Nature; 197: 452.

Becker-Andre, M., Van Huijsduijnen, RH., Losberger, C., Whelan, J., DeLamarter, JF. (1992) Murine endothelail leukocyte-adhesion molecule-1 is a close structural and functional homologue of the human protein. Eur. J. Biochem. 206: 401.

Beckstead, JH., Stenberg, PE., McEver, RP., Shuman, MA., Bainton, DF. (1986). Immunohistochemical localization of membrane and α-granule protein in human megakaryocytes: Application to plastic-embedded bone marrow biopsy specimens. Blood. 67: 285.

Bender, JG., Williams, SF., Myers, S., Nottleman, D., Lee, WJ., Unverzagt, KL., Walker, D., To, LB., and van Epps, DE. (1992). Characterisation of chemotherapy-mobilized peripheral blood progenitor cells for use in autologous stem cell transplantation. Bone Marrow Transplant. 10: 281.

Bender, MA., Palmer, TD., Gelinas, RE., Miller, AD. (1987). Evidence that the packaging signal of Moloney murine leukemia virus extends into the *gag* region. J. Virol. 61: 1639.

Bennett, JH., Joyner, CJ., Triffitt, JT., Owen, ME. (1991). Adipocyte cells cultured from marrow have osteogenic potential. Journal of Cell Science. 99: 131.

Bentley, SA. (1981). Close range cell: cell interactions are required for stem cell maintenance in continuous bone marrow culture. Exp. Hematol. 9: 308.

Bentley, SA. (1982a). Bone marrow connective tissue and the haemopoietic microenvironment. Br. J. Haematol. 50: 1.

Bentley, SA., Alabaster, O., Foidart, JM.. (1981). Collagen hetergeneity in normal human bone marrow; Br. J. Haematol. 48: 287.

Bentley, SA., Knutsen, T., Whang-Peng, J. (1982b). The origin of the hematopoietic microenvironment in continous bone marrow culture. Exp. Hematol. 10: 367.

Berenson, RJ., Andrews, RG., Bensinger, WI., Kalamasz, DF., Knitter, G., Buckner, CD., Bernstein, ID. (1988). Antigen $CD34^+$ marrow cells engraft lethally irradiated baboons. J. Clin. Invest. 81: 951.

Berenson, RJ., Bensinger, WI., Hill, RS., Andrews, RG., Garcia-Lopez, J., Kalamasz, DF., Still, BJ., Spitzer, G., Buckner, CD., Bernstein, ID., Thomas, ED. (1991). Engraftment after infusion of $CD34^+$ marrow cells in patients with breast cancer or neuroblastoma. Blood. 77: 1717.

Berg, EL., James, SP. (1990). Human neutrophils release the Leu-8 lymph node homing receptor during cell activation. Blood. 76: 2381.

Berg, EL., Magnani, J., Warnock, RA., Robinson, MK., Butcher, EC. (1992). Comparison of L-selectin and E-selectin ligand specificities: The L-selectin can bind the E-selectin ligands sialyl Le^x and sialyl Le^a. Biochem. Biophys. Res. Commun. 184: 1048.

Berg, EL., McEvoy, LM., Berlin, C., Bargatze, RF., Butcher, EC. (1993). L-selectin-mediated lymphocyte rolling on MAdCAM-1. Nature. 366: 695.

Berg, EL., Robinson, MK., Mansson, O., Butcher, EC., Magnani, JL. (1991b). A carbohydrate domain common to both sialyl Le^a and sialyl Le^x is recognised by the endothelial cell leukocyte adhesion molecule, ELAM-1. J. Biol. Chem. 266: 14869.

Berg, EL., Yoshino, T., Rott, LS., Robinson, MK., Warnock, A., Kishimoto, TK., Picker, LJ., Butcher, EC. (1991a). The cutaneous lymphocyte antigen is a skin lymphocyte homing receptor for the vascular lectin endothelial cell-leukocyte adhesion molecule 1. J. Exp. Med. 174: 1461.

Berman, CL., Yeo, EL., Wencel-Drake, JD., Furie, BC., Ginsberg, MH., Furie, B. (1986). A platelet alpha granule membrane protein that is associated with the plasma membrane after activation. J. Clin. Invest. 78: 130.

Bernardi, P., Patel, VP., Lodish, HF. (1987). Lymphoid precursor cells adhere to two different sites on fibronectin. J. Cell Biol. 105: 489.

Bernstein, A., Forrester, L., Reith, AD., Dubreuil, P., Rottapel, R. (1991b) The murine W/c-kit and steel loci and the control of hematopoiesis. Semin. Hematol. 28: 138.

Bernstein, I., Leary, A., Andrews, R., Ogawa, M. (1991a). Blast colony-forming cells and precursors of colony-forming cells detectable in long-term marrow culture express the same phenotype ($CD33^-CD34^+$). Exp. Hematol. 19: 680.

Bernstein, ID., Andrews, RG., Zsebo, KM. (1991). Recombinant human stem cell factor enhances the formation of colonies by $CD34^+$ and $CD34^+lin^-$ cells and the generation of colony-forming cell progeny from $CD34^+lin^-$ cells cultured in interleukin-3, granulocyte colony stimulating factor, or granulocyte-macrophage colony stimulating factor. Blood. 77: 2316.

Bertoncello, I., Bradley, TR., Watt, SM. (1991). An improved negative immunomagnetic strategy for the purification of primitive hemopoietic cells from normal bone marrow. Exp. Hematol. 19: 95.

Bertoncello, I., Hodgson, GS., Bradley, TR. (1985). Multiparameter analysis of transplantable hemopoietic stem cells: The separation and enrichment of stem cells homing to marrow and spleen on the basis of Rhodamine-123 fluorescence; Exp Hematol; 13: 999.

Beschorner, WE., Civin, CI., Strauss, LC. (1985). Localization of hematopoietic progenitor cells in tissue with the anti-My-10 monoclonal antibody. Am. J. Pathol. 119: 1.

Besmer, P., Murphy, JE., George, PC., Qui, F., Bergold, PJ., Lederman, L., Snyder, HW., Brodeur, D., Zuckerman, EE. and Hardy, WD. (1986). A new acute transforming feline retrovirus and relationship of its oncogene v-kit with the protein kinase gene family. Nature. 320: 415.

Bessis, M. (1973). Living Blood Cells and Their Ultrastructure; Springer-Verlag, New York; p 477.

Bevilacqua, MP., Butcher, EC., Furie, B., Gallatin, M., Gimbrone, MA., Harlan, J., Kishimoto, K., Lasky, L., McEver, R., Paulson, J. (1991). Selectins: a family of adhesion receptors. Cell 67: 233.

Bevilacqua, MP., Nelson, RM. (1993). Selectins. J. Clin. Invest. 91: 379.

Bevilacqua, MP., Pober, JS., Mendrick, DL., Cotran, RS., Gimbrone, MA. (1987). Identification of an inducible endothelial-leukocyte adhesion molecule. Proc. Natl. Acad. Sci. (USA). 84: 9238.

Bevilacqua, MP., Pober, JS., Wheeler, ME., Cotran, RS., Gimbrone, MA. (1985). Interleukin 1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes, and related leukocyte cell lines. J. Clin. Invest. 76: 2003.

Bevilacqua, MP., Stengelin, S., Gimbrone, MA. Seed, B. (1989). Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. Science. 243: 1160.

Birnboim, HC., Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7: 1513.

Bishop, JO., Morton, JG., Rosbash, M., Richardson, M. (1974). Three abundance classes in HeLa cell messenger RNA. Nature. 250: 199.

Blackburn, MJ., Patt, HM. (1977). Increased survival of haemopoietic pluripotent stem cells in vitro induced by a marrow fibroblast factor. Br. J. Haemtol. 37: 337.

Bonfanti, R., Furie, BC., Furie, B., Wagner, DD. (1989). PADGEM (GMP 140) is a component of Weibel-Palade bodies of human endothelial cells. Blood. 73: 1109.

Bowen, BR., Fennie, C., Lasky, LA. (1990). The Mel 14 antibody binds to the lectin domain of the murine peripheral lymph node homing receptor. J. Cell Biol. 110 (1): 147.

Bowen, BR., Nguyen, T., Lasky LA., (1989). Characterisation of a human homologue of the murine peripheral lymph node homing receptor. J. Cell. Biol. 109: 421.

Bowtell, DDL., Cory, S., Johnson, GR., Gonda, TJ. (1988). Comparison of expression in hematopoietic cells by retroviral vectors carrying two genes. J. Virol. 62: 2464.

Bradley, TR., Hodgson, GS. (1979). Detection of primitive macrophage progenitor cells in mouse bone marrow. Blood. 54: 1446.

Bradley, TR., Metcalf, D. (1966) The growth of mouse bone marrow cells in vitro. Aust. J. Exp. Biol. and Med. Sci. 44: 287.

Bradley, TR., Pilkington, G., Garson, M., Hodgson, GS., Kraft, M. (1982). Cell lines derived from a myelomonocytic leukaemia. Br. J. Haematol. 51: 595.

Brandley, BK., Swiedler, SJ., Robbins, PW. (1990). Carbohydrate ligands of the LEC cell adhesion molecules. Cell 63: 861.

Brandt, J., Srour, EF., Besien, KV., Briddell, RA., Hoffman, R. (1990). Cytokine-dependent long-term culture of highly enriched precursurs of hematopoietic progenitor cells from human bone marrow. J. Clin. Invest. 86: 932.

Brandt, JE., Srour, EF., van Besien, K., Briddell, RA., Hoffman, R. (1990). Cytokine-dependent long-term culture of highly enriched precursors of hematopoietic progenitor cells from human bone marrow. J. Clin. Invest. 86: 932.

Brannan, CI., Lyman, SD., Williams, DE., Eisenman, J., Anderson, DM., Cosman, D., Bedell, MA., Jenkins, NA., Copeland, NG. (1991). Steel-Dickie mutation encodes a c-Kit ligand lacking transmembrane and cytoplasmic domains. Proc. Natl. Acad. Sci. (USA). 88: 4671.

Breems, DA., Blokland, EA., Neben, S., Ploemacher, RE. (1994). Frequency analysis of human primitive hematopoietic cell subsets using a cobble stone area forming cell assay. Leukaemia. 8: 1095.

Briddell, RA., Broudy, VC., Bruno, E., Brandt, JE., Srour, EF., Hoffman, R. (1992). Further phenotypic characterization and isolation of human hemopoietic progenitor cells using a monoclonal antibody to the c-kit receptor. Blood. 79: 3159.

Briskin, MJ., McEvoy, LM., Butcher, EC. (1993). MAdCAM-1 has homology to immunoglobulin and mucin-like adhesion receptors and to IgA1. Nature. 363: 461.

Brown, J., Greaves, MF., Molgaard, HV. (1991a). The gene encoding the stem cell antigen, CD34, is conserved in mouse and expressed in haemopoietic progenitor cell lines, brain, and embryonic fibroblasts. Int. Immunol. 3: 175.

Brown, TA., Bouchard, T., St-John, T., Wayner, E., Carter, WG. (1991b). Human keratinocytes express a new CD44 core protein (CD44E) as a heparan-sulfate intrinsic membrane proteoglycan with additional exons. J. Cell. Biol. 113: 207.

Broxmeyer, HE., Cooper, S., Lu, L.. (1991). Effect of murine mast cell growth factor (c-kit proto-oncogene ligand) on colony formation by human hematopoietic progenitors. Blood. 77: 2142.

Brunner, G., Gabrilove, J., Rifkin, DB., Wilson, EL. (1991). Phospholipase C release of basic fibroblast growth factor from human bone marrow cultures as a biologically active complex with a phosphatidylinositol-anchored heparan sulfate proteoglycan. J. Cell Biol. 114: 1275.

Brustein, M., Kraal, G., Mebius, RE., Watson, SR. (1992). Identification of a soluble form of the lymphocyte homing receptor. J. Exp. Med. 176: 1415.

Burg, DL., Feldbush, TL. (1989). Late events in B cell activation. Expression of membrane alkaline phosphatase activity. J. Immunol. 142: 381.

Butcher, EC. (1991). Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. Cell. 67: 1033.

Caceres-Cortes.J., Rajotte, D., Dumouchel, J., Haddad, P., Hoang, T. (1994). Product of the steel locus suppresses apoptosis in hemopoietic cells. Comparison with pathways activated by granulocyte macrophage colony-stimulating factor. J. Biol. Chem. 269(16): 12084.

Caligaris-Cappio, F., Berguli, L., Gregoretti, MG. (1991). Role of bone marrow stromal cells in the growth of human multiple myeloma. Blood. 77: 2688.

Camerini, D., James, SP., Stamenkovic, I., Seed, B. (1989). Leu-8/TQ1 is the human equivalent of the Mel-14 lymph node homing receptor. Nature 342: 78.

Campbell, A., Wicha, MS. and Long, M., (1985). Extracellular matrix promotes the growth and differentiation of murine hematopoietic cells in vitro. J. Clin. Invest. 75: 2085.

Campbell, AD., Long, MW., Wicha, MS. (1987). Haemonectin, a bone marrow adhesion protein specific for cells of the granulocytic lineage. Nature. 329: 744.

Campbell, AD., Long, MW., Wicha, MS. (1990). Developmental regulation of granulocytic cell binding to haemonectin. Blood. 75: 1758.

Campbell, DG., Gagnon, J., Reid, KBM., Williams, AF. (1981). Rat brain Thy-1 glycoprotein. The amino acid sequence, disulphide bonds and an unusual hydrophobic region. Biochem. J. 195: 15.

Cantrell, CE., Yielding, KL. (1980). Binding of ethidium monoazide to the chromatin in human lymphocytes. Biochem. Biophys. Acta. 609: 173.

Caras, IW. (1991). An internally positioned signal can direct attachment of a glycophospholipid membrane anchor. J. Cell Biol. 113: 77.

Caras, IW., Weddell, GN., Williams, SR. (1989). Analysis of the signal for attachment of a glycophospholipid membrane anchor. J. Cell Biol. 108:1387.

Carlos, TM., Harlan, JM. (1994). Leukocyte-endothelial adhesion molecules. Blood. 84: 2068.

Carter, WG., Wayner, EA. (1988). Characterization of the class III collagen receptor, a phosphorylated, transmembrane glycoprotein expressed in nucleated human cells. J. Biol. Chem. 263: 4193.

Castro-Malaspina, H., Day, RE., Resnick, G., Kapoor, N., Meyers, P., Chiarieri, D., McKenzie, S., Broxmeyer, HE., Moore, MAS. (1980). Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. Blood. 56: 289.

Cepko, LC., Roberts, BE., Mulligan, RC. (1984). Construction and applications of a highly transmissable murine retrovirus shuttle vector. Cell 37:1053.

Chabot, B., Stephenson, DA., Chapman, VM., Besmer, P., Bernstein, A. (1988). The proto-oncogene c-kit encoding a transmembrane tyrosine kinase receptor maps to the mouse W locus. Nature. 335: 88.

Charbord, P., Gown, AM., Keating, A., Singer, JW. (1985). CGA-7 and HHF, two monoclonal antibodies that recognise muscle actin and react with adherent cells in human long-term bone marrow cultures. Blood. 66: 1138.

Chen, BP., Galy, A., Kyoizumi, S., Namikawa, R., Scarborough, J., Webb, S., Ford, B., Cen, D-Z., Chen, S. (1994). Engraftment of human hematopoietic precursor cells with secondary transfer potential in SCID-hu mice. Blood. 84: 2497.

Chikaraishi, DM. (1979). Complexity of cytoplasmic polyadenylated and non-polyadenylated rat brain ribonucleic acids. Biochemistry. 18: 3249.

Chomczynski, P., Sacchi, N. (1987). Single-step method of RNA isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform extraction. Anal. Biochem. 162: 156.

Cinek, T., Horejsi, VAD. (1992). The nature of large noncovalent complexes containing glycosyl-phosphatidylinositol-anchored membrane glycoproteins and protein tyrosine kinases. J. Immunol. 149(7): 2262.

Civin, C., Strauss, LC., Brovall, C., Frackler, MJ., Schwartz, JF., Shaper, JH. (1984). Antigenic analysis of hematopoiesis III: A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG1a cells. J. Immunol. 135: 157.

Civin, CI., Banquerigo, ML., Strauss, LC., Loken, MR. (1987). Antigenic analysis of hematopoiesis VI: Flow cytometric characterization of My-10 positive progenitor cells in normal human bone marrow. Exp. Hematol. 15: 10.

Civin, CI., Trischmann, T., Fackler, M., Bernstein, I., Brunning, J., Campos, L., Greaves, M., Kamoun, M., Katz, D., Lansdorp, P., Look, A., Seed, B., Sutherland, D., Tindle, R., Uchanska-Ziegler, B. Report on the CD34 cluster workshop, in Knapp, W., Dorken, B., Gilk, W., Rieber, E.,

Stein, H., Schmidt, R., von den Borne, A. (eds): Leukocyte Typing IV. Oxford, UK., Oxford University Press, 1989. p. 818.

Clark, BR., Gallagher, JT., Dexter TM. (1992). Cell adhesion in the stromal regulation of haemopoiesis. Bailiere's Clin. Hematol. 5: 21.

Clark, EA. (1993). CD22, a B-cell-specific receptor, mediates adhesion and signal transduction. J. Immunol. 150:4715.

Clark, SC., Kamen, R. (1987). The human hematopoietic colony stimulating factors. Science. 236: 1229.

Clayton, CE., Mowatt, MR. (1989). The procyclic acidic repetitive proteins of Trypanosoma brucei. Purification and post-translational modification. J. Biol. Chem. 264: 15088.

Cline, MJ., Golde, DW. (1979). Cellular interactions in haematopoiesis. Nature 277: 177.

Cole, SR., Ashman, LK., Ey, PL. (1987). Biotinylation: An alternative to radioiodination for the identification of cell surface antigens in immunoprecipitates. Mol. Immunol. 24: 699.

Collins, SJ., Gallo, RC., Gallagher, RE. (1977). Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. Nature. 270: 347.

Collins, T., Williams, A., Johnston, GI., Kim, J., Eddy, R., Shows, T., Grimbone, MA., Bevilacqua, MP. (1991). Structure and chromosomal location of the gene for endothelial-leukocyte adhesion molecule 1. J. Biol. Chem. 266: 2466.

Cone, RD., Mulligan, RC. (1984). High-efficiency gene transfer into mammalian cells: generation of helper-free recombinant retrovirus with broad mammalian host range. Proc. Natl. Acad. Sci. (USA). 81: 6349.

Conforti, G., Dominguez-Jimenez, C., Zanetti, A., Gimbrone, MA., Cremona, O., Marchisio, PC., Dejana, E. (1992). Human endothelial cells express integrin receptors on the luminal aspect of their membrane. Blood. 80: 437.

Coombe, DR. (1996). The role of stromal cell heparan sulphate in regulating haemopoiesis. Leukemia and Lymphoma. 21: 399.

Cotran, RS., Gimbrone Jr, M., Bevilacqua, MP., Mendrick, DL., Pober, JS. (1986). Induction and detection of a human endothelial activation in vivo. J. Exp. Med. 164: 661.

Coulombel, L., Eaves, AC., Eaves, CJ. (1983). Enzymatic treatment of long-term human marrow cultures reveals the preferential location of primitive hemopoietic progenitors in the adherent layer. Blood. 62: 291.

Coulombel, L., Vuillet, MH., Tchernia, G. (1988). Lineage and stage specific adhesion of human hematopoietic progenitor cells to extracellular matrices from marrow fibroblasts. Blood. 71: 329.

Coutinho, LH., Will, A., Radford, J., Schiro, R., Testa, NG., Dexter, TM. (1990). Effects of recombinant human granulocyte stimulating factor (CSF), human granulocyte-macrophage-CSF and gibbon interleukin 3 on hematopoiesis in human long term bone marrow culture. Blood. 75: 2118.

Craig, W., Kay, R., Cutler, RL., Lansdorp, PM. (1993). Expression of Thy-1 on human hemopoietic progenitor cells. J. Exp. Med. 177: 1331.

Crovello, CS., Furie, BC., Furie, B. (1993). Rapid phosphorylation and selective dephosphorylation of P-selectin accompanies platelet activation. J. Biol. Chem. 268: 14590.

Crowe, DT., Chiu, H., Fong, S., Weissman, IL. (1994). Regulation of the avidity of integrin alpha 4 beta 7 by the beta 7 cytoplasmic domain. J. Biol. Chem. 269: 14411.

Culty, M., Miyake, K., Kincade, PW., Silorski, E., Butcher, EC., Underhill, C. (1990). The hyaluronate receptor is a member of the CD44 (H-CAM) family of cell surface glycoproteins. J. Cell Biol. 111: 2765.

Curry, JL., Trentin, JJ. (1967). Hemopoietic spleen colony studies. 1. Growth and differentiation. Devl. Biol. 15: 395.

Cyster, J., Shotton, DM., Williams, AF. (1991). The dimensions of the T lymphocyte glycoprotein leukosialin and identification of linear protein epitopes that can be modified by glycosylation. EMBO. J. 10: 893.

Cyster, J., Somoza, C., Killeen, N., Williams, AF. (1990). Protein sequence and gene structure for mouse leukosialin (CD43), a T lymphocyte mucin without introns in the coding sequence. Europ. J. Immunol. 20: 875.

Dancey, JT., Deubelbeiss, KA., Harker, LA., Finch, CA. (1976). Neutrophil kinetics in man. J. Clin. Invest. 58: 705.

Davies, A., Lachmann, PJAD. (1993). Membrane defence against complement lysis: the structure and biological properties of CD59. Immunol. Res. 12: 258.

Davies, A., Simmons, DL., Hale, G., Harrison, RA., Tighe, H., Lachmann, PJ., Waldmann, H. (1989). CD59, an LY-6-like protein expressed in human lymphoid cells, regulates the action of the complement membrane attack complex on homologous cells. J. Exp. Med. 170: 637.

De Luca, M., Dunlop, LC., Andrews, RK., Flannery, JV., Ettling, R., Cummings, DA., Veldman, GM., Berndt, MC. (1995). A novel cobra venom metalloproteinase, Mocarhagin, cleaves a 10-amino acid peptide from the mature N-terminus of P-selectin glycoprotein ligand receptor, PSGL-1 and abolishes P-selectin binding. J. Biol. Chem. 270: 26734.

de Fougerolles, AR., Stacker, SA., Schwarting, R., Springer, TA. (1991). Characterization of ICAM-2 and evidence for a third counter-receptor for LFA-1. J. Exp. Med. 174: 253.

de Sauvage, FJ., Hass, PE., Spencer, S., Malloy, BE., Gurney, AL., Spencer, SA., Darbonne, WC., Henzel, WJ. (1994). Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand. Nature. 369: 533.

Deckert, M., Kubar, J., Zoccola, D., Bernard, Pomier, G., Angelisova, P., Horejsi, V., Bernard, AAD. (1992). CD59 molecule: a second ligand for CD2 in T cell adhesion. Eur. J. Immunol. 22(11): 2943.

DeLisser, HM., Muller, WA., Newman, PJ., Albelda, SM. (1991). PECAM-1 (CD31) mediates heterophilic cell-cell adhesion. J. Cell Biol. 115: 2317.

DeLisser, HM., Yan, HC., Newman, PJ., Muller, WA., Buck, CA., Albelda, SM. (1993). Platelet/endothelial adhesion molecule-1 (CD31)-mediated cellular aggregation involves cell surface glycosaminoglycans. J. Biol. Chem. 268: 16037.

Denkers, IA., de Jong-de Boer, JJ., Beelen, RH., Ossenkoppele, GJ., Langenhuijsen, MM. (1992). VLA molecule expression may be involved in the release of acute myeloid leukaemia cells from the bone marrow. Leuk. Res.16: 469.

Dercksen, MW., Gerritsen, WR., Rodenhuis, S., Dirkson, MK., Slaper-Cortenbach, IC., Schaasberg, WP., Pinedo, HM., von-dem-Borne, AE., van-der-Schoot, CEAD. (1995). Expression of adhesion molecules on CD34+ cells: CD34+ L-selectin+ cells predict a rapid platelet recovery after peripheral blood stem cell transplantation. Blood. 85: 3313.

Dercksen, WM., Cazemier, MWH, van der Linden, PJE., Kerst, JM., Pinedo, HM., Von dem Borne, AEGK, Gerritsen, WR., van der Schoot, CE. (1994). Binding of hematopoietic progenitor cells to endothelium is mediated by E-selectin and VLA-4. Blood. 84: 236a (abstract).

Deryugina, EI., Müller-Sieburg, CE. (1993). Stromal cells in long-term cultures: Keys to the elucidation of hematopoietic development? Crit. Rev. Immun. 13: 115.

DeSimone, DW., Hynes,RO. (1988). Xenopus laevis integrins. Structural conservation and evolutionary divergence of integrin β subunits. J. Biol. Chem. 263: 5333.

Devine, PL., McKenzie, IFC. (1992). Mucins: structure, function, and association with malignancy. Bioessays. 14: 619.

Dexter, TM. (1979). Cell Interaction in vitro. Clinics in Hematol. 8: 453.

Dexter, TM. (1982). Stromal cell-associate haemopoiesis. J. Cell. Physiol. Suppl 1: 87.

Dexter, TM. (1987a). Growth factors involved in haemopoiesis. J. Cell Sci. 88: 1.

Dexter, TM. (1987b). Stem cells in normal growth and disease. Br. Med. J. 295: 1192.

Dexter, TM. (1990). Introduction to the haemopoietic system. pp 1-5. Colony-stimulating factors: molecular and cellular biology. Immunology Series, vol 49, Marcel Dekker.

Dexter, TM., Allen, TD., Lajtha, LG. (1977). Conditions controlling the proliferation of haemopoietic stem cells in vitro. J. Cell. Physiol. 91: 335.

Dexter, TM., Garland, J., Scott, D., Scolnick, E., Metcalf, D. (1980). Growth of factor-dependent hemopoietic precursor cell lines. EMBO. J. 4: 2575.

Dexter, TM., Moore, MAS. (1977). In vitro duplication and 'cure' of haemopoietic defects in genetically anaemic mice. Nature. 269: 412.

Dexter, TM., Simmons, P., Purnell, RA., Spooncer, E., Schofield, RA. (1984). The regulation of haemopoietic cell development by the stromal cell environment and diffusable regulatory molecules. In: Aplastic Anaemia: Stem Cell Biology and Advances in Treatment, NS Young, AS Levine, RK Humphries, eds. New York: Alan R. Liss. pp 13.

Dexter, TM., Spooncer, E. (1987). Growth and differentiation in the hemopoietic system. Ann. Rev. Cell Biol. 3: 423.

Dexter, TM., Spooncer, E., Simmons, P., Allen, TD. (1984a). Long term bone marrow culture: an overview of techniques and experience. In: Long term Bone Marrow Culture, DG Wright, JS Greenberger, eds. New York: Alan R. Liss: 57-96. Kroc Foundation Series Vol 18; Alan R. Liss, Inc., New York; p. 57-96, 1984.

Dexter, TM., Spooner, E., Scofield, R., Lord, BI., Simmons, PJ. (1984b). Haemopoietic stem cells and the problem of self-renewal. Blood Cells. 10: 315.

Diamond, MS., Staunton, DE., Marlin, SD., Springer, TA. (1991). Binding of the integrin Mac-1 (CD11b/CD18) to the third immunoglobulin-like domain of ICAM-1 (CD54) and its regulation by glycosylation. Cell. 65: 961.

Dianzani, U., Malavasi, F. (1995). Lymphocyte adhesion to endothelium. Crit. Rev. Immunol. 15 (2): 167.

Dick, JE., Magli, MC., Huszar, D., Phillips, RA., Bernstein, A. (1985). Introduction of a selectable gene into primitive stem cell capable of the long term reconstitution of the hematopoietic system of W/Wv mice. Cell. 42: 71.

Diederichs, K., Jacques S., Boone, T., Karplus, PA. (1991). Low-resolution structure of recombinant human granulocyte-macrophage colony stimulating factor. J. Mol. Biol. 221: 55.

Dieterlen-Lievre, F. (1975). The origin of haemopoietic stem cells in the avian embryo: an experimental approach. J. Embryol. Exp. Morphol. 33: 607.

Dieterlen-Lievre, F., Godin, IE., Garcia-Porrero, JA., Marcos, MAR. (1994). Initiation of hemopoiesis in the mouse embryo. Ann. NY. Acad. 718: 140.

Dieterlen-Lievre, F., Martin, C. (1981). Diffuse intraembryonic hemopoiesis in normal and chimeric avian development. Dev. Biol. 88: 180.

DiGuisto, D., Chen, S., Combs, J., Webb, S., Namikawa, R., Tsukamoto, A., Chen, BP., Galy, AMH. (1994). Human fetal bone marrow early progenitors for T, B, and myeloid cells are found exclusively in the population expressing high levels of CD34. Blood. 84: 421.

DiPersio, J.F. (1990). Colony-stimulating factors: enhancement of effector cell function, pp81. Colony-stimulating factors: molecular and cellular biology. Immunology Series, vol 49, Marcel Dekker.

Disdier, M., Morrissey, JH., Fugate, RD., Bainton, DF., McEver, RP. (1992). Cytoplasmic domain of P-selectin (CD62P) contains the signal for sorting into the regulated secretory pathway. Mol Biol. Cell 3: 309.

Dorshkind, K. (1990). Regulation of hemopoiesis by bone marrow stromal cells and their products. Ann. Rev. Imunol. 8: 111.

Dorshkind, K., Phillips, RA. (1982). Maturational state of lymphoid cells in long-term bone marrow cultures. J. Immunol. 129: 2444.

Dougherty, GJ., Lansdorp, PM., Cooper, DL., Humphries, RK. (1991). Molecular cloning of CD44R1 and CD44R2, two novel isoforms of the human CD44 lymphocyte "homing" receptor expressed by hemopoietic cells. J. Exp. Med. 174: 1.

Doukas, J., Pober, JS. (1990). IFN gamma enhances endothelial activation induced by tumor necrosis factor, but not IL-1. J. Immunol. 145: 1727.

Dowbenko, DJ., Diep, A., Taylor, B A., Lusis, AJ., Lasky, LA. (1991). Characterisation of the murine homing receptor gene reveals correspondence between protein domains and coding exons. Genomics. 9: 270.

Dowbenko, DJ., Watson, SR., Lasky, LA. (1993). Cloning of a rat homologue of mouse GlyCAM-1 reveals conservation of structural domains. J. Biol. Chem. 268: 14399.

Drickamer, K. (1992). Engineering galactose-binding activity into a C-type mannose-binding protein. Nature. 360: 183.

Du, X., Plow, EF., Frelinger, AL., O'Toole, TE., Loftus, JC., Ginsberg, D. (1991). Ligands "activate" integrin $\alpha_{IIb}\beta_{III}$ (platelet GPIIb-IIIa). Cell 65: 409.

Dunlop, LC, Skinner, MP., Bendall, LJ., Favaloro, EJ., Castaldi, PA., Gormann, JJ., Vadas, MA., Berndt, MC. (1992). Characterization of GMP-140 (P-selectin) as a circulating plasma protein. J. Exp. Med. 175: 1147.

Dunon, D., Imhof, BA. (1993). Mechanisms of thymus homing. Blood. 81: 1.

Dustin, ML., Springer, TA. (1989). T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. Nature. 341: 619.

Eaves, CJ., Cashman, JD., Kay, RJ., Dougherty, GJ., Otsuka, T., Gaboury, LA., Hogge, DE., Lansdorp, PM., Eaves, AC., Humphries, RK. (1991). Mechanisms that regulate the cell cycle status of very primitive hemopoietic cells in long-term human marrow cultures: analysis of positive and negative regulators produced by stromal cells within the adherent layer. Blood. 78: 110.

Ebell, W., Castro-Malaspina, H., Moore, MAS., O'Reilly, RJ. (1985). Depletion of stromal cell elements in human marrow grafts separated by soybean agglutinin. Blood. 65: 1105.

Eglitis, MA., Kantoff, P., Gilboa, E., Anderson, WF. (1985). Gene expression in mice after high efficiency retroviral mediated gene transfer. Science. 230: 1395.

Elices, MJ., Oxborn, L., Takada, Y., Crouse, C., Luhowskyj, S., Hemler, ME., Lobb, RR. (1990). VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. Cell. 60: 577.

Emerson, SG., Guba, SC., Upadhyaya, GH., Deisseroth, AB., Thomas, S. (1989). Chronic myelogenous leukaemia progenitor cells are deficient in cell surface LFA-3 and are not recognised by autoregulatory T lymphocytes. Clin. Res. 37: 901A.

Erbe, DV., Wolitzky, BA., Presta, LG., Norton, CR., Ramos, RJ., Burns, DK., Rumberger, JM., Narasinga-Rao, BN., Foxall, C., Brandley, BK., Lasky, LA. (1992). Identification of an E-selectin region critical for carbohydrate recognition and cell adhesion. J. Cell Biol. 119: 215.

Eriksen, EF., Kassem, M. (1992). Fluoride stimulates proliferation and differentiation of human marrow stroma. Bone and Mineral. 17 (suppl 1): 196 (abstract).

Ershler, WB., Ross, J., Finlay, JL., Shahidi, NT. (1980). Bone marrow microenvironment defect in congenital hypoplastic anemia. The New England Journal of Medicine. 302: 1321.

Erslev, AJ. (1983). Production of erythrocytes. In: Williams, WJ., Beutler, E., Erslev, AJ., Lichtman, MA. New York, New York, McGraw-Hill. pp. 365.

Ey, PL., Prowse, SJ., Jenkin, CR. (1978). Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein A sepharose. Immunochem. 15: 429.

Fackler, M., Krause, D., Smith, O., Civin, C., May, W. (1995). Full length but not truncated CD34 inhibits hematopoietic cell differentiation of M1 cells. Blood. 85: 3040.

Favaloro, EJ., Moraitis, N., Bradstock, K., Koutts, J. (1990). Co-expression of haemopoietic antigens on vascular endothelial cells: a detailed phenotypic analysis. Br. J. Haematol. 74: 385.

Ferguson, MA., Homans, SW., Dwek, RA., Rademacher, TW. (1988). Glycosyl-phosphatidylinositol moiety that anchors Trypanosoma brucei variant surface glycoprotein to the membrane. Science. 239: 753.

Ferguson, MAJ., Williams, AF. (1988). Cell-surface anchoring of proteins via glycosyl-phosphatidylinositol structures. Ann. Rev. Biochem. 57: 285.

Fina, L., Molgaard, HV., Robertson, D., Bradley, NJ., Monaghan, P., Delia, D., Sutherland, DR., Baker, MA., Greaves, MF. (1990). Expression of the CD34 gene in vascular endothelial cells. Blood. 75: 2417.

Fitter, S., Tetaz, TJ., Berndt, MC., Ashman, LK. (1995). Molecular cloning of cDNA encoding a novel platelet-endothelial cell tetraspan antigen, PETA-3. Blood. 86: 1348.

Flanagan, J.G., Chan, D.C. and Leder, P. (1991). Transmembrane form of the kit ligand growth factor is determined by alternative splicing and is missing in the Sl^d mutant. Cell. 64: 1025-1035.

Flanagan, JG., Leder, P. (1990). The kit ligand: A cell surface molecule altered in Steel mutant fibroblasts. Cell. 63: 185.

Fleischman, RA., Mintz, B. (1984). Development of adult bone marrow stem cells in H-2-compatible and-incompatible mouse fetuses. J. Exp. Med. 159: 731.

Fletcher, A., Bryant, JA., Gardner, B., Judson, PA., Spring, FA., Parsons, SF., Mallinson, G., Anstee, DJAD. (1992). New monoclonal antibodies in CD59: use for the analysis of peripheral blood cells from paroxysmal nocturnal haemoglobinuria (PNH) patients and for the quantitation of CD59 on normal and decay accelerating factor (DAF)-deficient erythrocytes. Immunology. 75(3): 507.

Foreman, KE., Vaporciyan, AA., Bonish, BK., Jones, ML., Johnson, KJ., Glovsky, MM., Eddy, SM., Ward, PA. (1994). C5a-induced expression of P-selectin in endothelial cells. J. Clin. Invest. 94: 1147.

Fowler, JH., Wu, AM., Till, JE., McCulloch, EA., Siminovitch, L. (1967). The cellular composition of haemopoietic spleen colonies; J. Cell. Physiol. 69: 65.

Foxall, C., Watson, SR., Dowbenko, D., Fennie, C., Lasky, LA., Kiso, M., Hasegawa, A., Asa, D., Brandley, BK. (1992). The three members of the selectin receptor family recognize a common carbohydrate epitope, the sialyl Lewis-x oligosaccharide. J. Cell. Biol. 117: 895.

Frazier, WA. (1987). Thrombospondin: a modular adhesive glycoprotein of platelets and nucleated cells. J. Cell Biol. 105: 625.

Frenette, PS., Mayadas, TN., Rayburn, H., Hynes, RO., Wagner, DD. (1996). Susceptibility to infection and altered hematopoiesis in mice deficient in both P- and E-selectins. Cell. 84: 563.

Fried, W., Chamberlin, W., Kedo, A., Barone, J. (1976). Effects of radiation on hemopoietic stroma. Exp. Hematol. 4: 310.

Fried, W., Kedo, A., Barone, J. (1977). Effects of cyclophosphamide and of bulsulfan on spleen colony-forming units and on haemopoietic stroma; Cancer Res. 37: 1205.

Friedenstein, AJ. (1976). Precursor cells of mechanocytes. Int. Rev. Cytology. 47: 327.

Friedenstein, AJ. (1980). Stromal mechanisms of bone marrow: cloning in vitro and retransplantation in vivo. Immunology of Bone Marrow Transplantation. (ed). Thienfelder, S. Springer-Verlag Berlin. p.19.

Friedenstein, AJ., Chailakhyan, RK., Gerasimov, UF. (1987). Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. Cell Tissue Kinetics. 20: 263.

Fujimoto, T., McEver, RP. (1993). The cytoplasmic domain of P-selectin is phosphorylated on serine and threonine residues. Blood. 82: 1758.

Fujita, J., Onoue, H., Ebi, Y., Nakayama, H., Kanakura, Y.(1989). In vitro duplication and in vivo cure of mast-cell deficiency of Sl/Sl^d mutant mice by cloned 3T3 fibroblasts. Proc. Natl. Acad. Sci. (USA). 86: 2888.

Fukuda, M. (1991). Leukosialin, a major O-glycan-containing sialoglycoprotein defining leukocyte differentiation and malignancy. Glycobiology. 1: 347.

Fukuda, M., Spooncer, E., Oates, E., Dell, A., Klock, JC. (1984). Structure of sialylated fucosyl lactosaminoglycan isolated from human granulocytes. J. Biolchem. 259: 10925

Fukushima, K., Hirota, M., Terasaki, PI., Wakisaka, A., Togashi, H., Chia, D., Suyama, N., Fukushi, Y., Nudelman, E., Hakamori, S. (1984). Characterisation of sialosylated Lewis^x as a new tumor-associated antigen. Cancer Res. 44: 5279.

Futerman, AH., MG. Low, KE. Ackermann, WR. Sherman, and I. Silman. (1985). Identification of covalently bound inositol in the hydrophobic membrane-anchoring domain of Torpedo acetylcholinesterase. Biochem. Biophys. Res. Commun. 129: 312.

Gabrilove, JL., White, K., Rahman, Z., Wilson, L. (1994). Stem cell factor and basic fibroblast growth factor are synergistic in augmenting committed myeloid progenitor cell growth. Blood. 83: 907.

Gadd, SJ., Ashman, LK. (1985). A murine monoclonal antibody specific for a cell-surface antigen expressed by a sub-group of human myeloid leukaemias. Leuk. Res. 9: 1329.

Gallagher, JG., Dexter, TM. (1990). Binding of growth factors to heparan sulphate: implications for the regulation of haemopoiesis by bone marrow stromal cells. Haematology Today.

Gallagher, JT., Spooncer, E., Dexter, TM. (1983). Role of the cellular matrix in haemopoiesis. I. Synthesis of glycosaminoglycans by mouse bone marrow cell cultures. J. Cell Sci. 63: 155.

Gallatin, WM., Weissman, IL, Butcher, EC. (1983). A cell-surface molecule involved in organ-specific homing of lymphocytes. Nature. 304: 30.

Galy, AHM., Cen, D., Travis, M., Chen, S., Chen, BP. (1995). Delineation of T progenitor cell activity within the CD34$^+$ compartment of adult bone marrow. Generation of T cells from cytokine-mobilised peripheral blood and adult bone marrow CD34$^+$ cells. Blood. 85: 2770.

Galy, AHM., Verma, S., Barcena, A., Spits, H. (1993). Precursors of CD3$^+$CD4$^+$CD8$^+$ cells in the thymus are defined by expression of CD34: Deliniation of early events in human thymic development. J. Exp. Med. 178: 391.

Galy, AHM., Webb, S., Cen, D., Murray, LJ., Condino, J., Negrin, RS., Chen, B. (1994) Generation of T cells from cytokine-mobilised peripheral blood and adult bone marrow CD34$^+$ cells. Blood. 84: 104.

Gamble, JR., Skinner, MP., Berndt, MC., Vadas, MA. (1990). Presentation of activated neutrophil adhesion to endothelium by soluble adhesion protein GMP-140. Science. 249: 414.

Garger, SJ., Griffith, OM., Grill, LK. (1983). Rapid purification of plasmid DNA by a single centrifugation in a two-step caesium chloride-ethidium bromide gradient. Biochem. Biophys. Res. Comm. 117: 835.

Gartner, S., Kaplan, HS. (1980). Long-term culture of human bone marrow cells. Proc. Natl. Acad. Sci. (USA). 78: 4756.

Gearing, DP., King, JA. Gough, NM., Nicola, NA. (1989). Expression cloning of a receptor for human granulocyte-macropohage colony stimulating factor. EMBO. J. 8: 3667.

Gerig, JG., Bevilacqua, MP., Moore, KL., McIntyre, TM., Prescott, SM., Kim, JM. Bliss, GA., Zimmerman, GA., McEver, RP. (1990). Rapid neutrophil adhesion to activated endothelium mediated by GMP-140. Nature. 343: 757.

Gilbert, SF. (1991). Developmental Biology. Fourth edition, Sinauer Associates, Inc. Sunderland, Massachusetts, USA.

Gimble, JM., Pietrangeli, C., Henley, A., Dorheim, MA., Silver, J., Namen, A., Takeichi, M., Goridis, C., Kincade, PW. (1989). Characterization of murine bone marrow and spleen-derived stromal cells: analysis of leukocyte marker and growth factor mRNA transcript levels. Blood. 74: 303.

Ginsberg, MH., Du, X., Plow, EF. (1992). Inside-out integrin signalling. Curr. Opin. Cell Biol. 4: 766.

Gismondi, A., Morrone, S., Humphries, MJ., Piccolo, M., Frati, L., Santoni., A. (1991). Human natural killer cells express VLA-4 and VLA-5, which mediate their adhesion to fibronectin. J. Immunol. 146: 384.

Godin, I., Deed, R., Cooke, J., Zsebo, K., Dexter, M., Wylie, CC. (1991). Effects of the steel gene product on mouse primordial germ cells in culture. Nature. 352: 807.

Godin, IE., Dieterlan-Lievre, F., Cumano, A. (1995). Emergence of multipotent hemopoietic cells in the yolk sac and para-aortic splanchnopleura in mouse embryos beginning at 8.5 dpc. Proc. Natl. Acad. Sci. (USA). 92: 773.

Godin, IE., Garcia-Porrero, JA., Coutinho, A., Dieterlan-Lievre, F., Marcos, MAR. (1993). Para-aortic splanchnopleura from early mouse embryos contains B1a cell progenitors. Nature. 364: 67-70.

Gonda, TJ., Cory, S., Sobieszczuk, P., Holtzman, D., Adams, JM. (1987). Generation of altered transcripts by retroviral insertion within the c-*myb* gene in two murine monocytic leukemias. J. Virol. 61: 2754.

Gonda, TJ., Ramsay, RG., Johnson, GR. (1989). Murine myeloid cell lines derived by in vitro infection with recombinant c-*myb* retroviruses express *myb* from rearranged vector proviruses. EMBO. J. 8: 1767.

Gonda, TJ., Sheiness, DK. Bishop, JM. (1982). Transcripts from the cellular homologs of retroviral oncogenes: distribution among chicken tissues. Mol. Cell. Biol. 2: 617.

Gordon, M. (1988). Extracellular matrix of the marrow microenvironment. J. Haematol. 70: 1.

Gordon, MY. (1991). Hemopoietic growth factors and receptors: Bound and free. Cancer. Cells. 3: 127.

Gordon, MY., Atkinson, J., Clarke, D., Dowding, CR., Goldman, JM., Grimsley, PG., Siczkowski, M., Greaves, MF. (1991). Deficiency of a phosphatidylinositol-anchored cell adhesion molecule influences haemopoietic progenitor cell binding to marrow stroma in chronic myeloid leukemia. Leukemia. 5: 693.

Gordon, MY., Dowding, CR., Riley, GP., Clark, D., Goldman, JM., Greaves, MF. (1987c). Altered adhesive interactions with marrow stroma of hematopoietic progenitor cells in chronic myeloid leukaemia. Nature. 328: 342.

Gordon, MY., Hibbin, JA., Dowding, C., Gordon-Smith, EC., Goldman, JM. (1985). Separation of human blast progenitors from granulocytic, erythroid, megakaryocytic and mixed colony-forming cells by "panning" on cultured marrow-derived stromal layers. Exp. Hematol. 13: 937.

Gordon, MY., Riley, GP., Clarke, D. (1988). Heparan sulfate is necessary for adhesive interactions between human early hemopoietic progenitor cells and the extracellular matrix of the marrow microenvironment. Leukemia. 2: 804.

Gordon, MY., Riley, GP., Greaves, DR. (1987b). Plastic adherent progenitor cells in human bone marrow. Exp. Hematol. 15: 772.

Gordon, MY., Riley, GP., Watt, SM., Greaves, MF. (1987a). Comparmentalization of a haemopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. Nature. 326: 403.

Gospodarowicz, D. (1980). Extracellular matrix and control of proliferation of vascular endothelial cells. J. Clin. Invest. 65: 1351.

Gospodarowicz, D., Delegado, D., Vlodavsky, I. (1980). Permissive effect of the extracellular matrix on cell proliferation in vitro. Proc. Natl. Acad. Sci. (USA). 77: 4094.

Gottesdiener, KM., Karpinski, BA., Lindsten, T., Strominger, JL., Jones, NH., Thompson, CB., Leiden, JM. (1988). Isolation and structural characterization of the human 4F2 heavy-chain gene, an inducible gene involved in T-lymphocyte activation. Mol. Cell Biol. 8: 3809.

Gower, HJ., Barton, CH., Elsom, VL., Thompson, J., Moore, SE., Dickson, G., Walsh, FS. (1988). Alternative Splicing Generates a Secreted Form of N-CAM in Muscle and Brain. Cell. 55: 955.

Graham, FL., Van der Eb, AJ. (1973). A New technique for the assay of infectivity of human adenovirus 5' DNA. Virology. 52: 456.

Graves, BJ., Crowther, RL., Chandran, C., Rumberger, JM., Li, S., Huang, KS., Presky, DH., Familletti, PC, Wolitzsky, BA., Burns, DK. (1994). Insight into E-selectin/ligand interaction from the crystal structure and mutagenesis of the lec/EGF domains. Nature. 367: 532.

Green, PJ., Tamatani, T., Watanabe, T., Miyasaka, M., Hasegawa, A., Kiso, M., Yuen, CT., Stoll, MS., Feizi, T. (1992). High affinity binding of the leukocyte adhesion molecule L-selectin to 3'-sulphated-Le^a and -Le^x oligosaccharides and the predominance of sulphate in this interaction demonstrated by binding studies with a series of lipid-linked oligosaccharides. Biochem. Biophys. Res. Commun. 188: 224.

Griffin, JD., Spertini, O., Emst, TJ., Belvin, ME., Levine, HB., Kanakura, Y., Tedder, TF. (1990). Granulocyte-macrophage colony-stimulating factor and other cytokines regulate surface expression of the leukocyte adhesion molecule-1 on human neutrophils, monocytes and their precursors. J. Immunol. 145: 576.

Gronthos, S., Ohta, S., Graves, S., Simmons, PJ. (1994). The STRO-1^+ fraction of adult human bone marrow contains the osteogenic progenitors. Blood. 4164.

Gronthos, S., Simmons, PJ. (1995). The growth requirements of STRO-1-positive human bone marrow stromal precursors under serum-deprived conditions in vitro. Blood. 929.

Guba, SC., Sartor, CI., Gottschalk, LR., Ye-Hu, J., Mulligan, T., Emerson, SG. (1992). Bone marrow stromal fibroblasts secrete interleukin-6 and granulocyte-macrophage colony-stimulating factor in the absence of inflammatory stimulation. Blood. 80: 1190.

Gunji,Y., Nakamura, M., Hagiwara, T., Hayakawa, K., Matsushita, H., Osawa, H., Nagayoshi, K., Nakauchi, H., Yanagisawa, M., Miura, Y., Suda, T. (1992). Expression and function of adhesion molecules on human hematopoietic stem cells: $CD34^+LFA\text{-}1^-$ cells are more primitive than $CD34^+LFA\text{-}1^+$ cells. Blood. 80: 429.

Günthert, U., Hofmann, M., Rudy, W., Reber, S., Zöller, M., Haußmann, I., Matzku, S., Wenzel, A., Ponta, H., Herrlich, P. (1991). A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. Cell. 65: 13.

Hahn, WC., Menu, E., Bothwell, AL., Sims, PJ., Bierer, BEAD. (1992). Overlapping but nonidentical binding sites on CD2 for CD58 and a second ligand CD59. Science. 256: 1805.

Handa, K., Igarashi, Y., Nisar, M., Hakomori, SI. (1991b). Downregulation of GMP-140 (CD62P or PADGEM) expression on platelets by N, N-dimethyl and N, N, N-trimethyl derivatives of sphingosine. Biochemistry. 30: 11682.

Handa, K., Neudelman, ED., Stroud, MR., Shiozawa, T., Hakomori, S. (1991a). Selectins GMP-140 (CD62; PADGEM) binds to sialosyl-Le^x and sialosyl-Le^a, and sulfated glycans modulate this binding. Biochem. Biophys. Res. Commun. 181: 1223.

Hannum, C., Culpepper, J., Campbell, D., McClanahan, T., Zurawski, S., Bazan, JF., Kastelein, R., Hudak, S., Wagner, J., Mattson, J. (1994). Ligand for FLT3/FLK2 receptor tyrosine kinase regulates growth of haematopoietic stem cells and is encoded by variant RNAs. Nature, 368(6472): 643.

Harrison, DE., Astle, CM., Lerner, C. (1988). Number and continuous proliferative pattern of transplanted primitive immunohematopoietic stem cells. Proc. Natl. Acad. Sci. (USA). 85: 822.

Hasthorpe, S., Bogdanovski, M., Rogerson, J., Radley, JM. (1992). Characterization of endothelial cells in murine long-term marrow culture. Implication of hemopoietic regulation. Exp Hematol. 20: 476.

Hatakeyama, M., Mori, H., Doi, T., Taniguchi, T. (1989). A restricted cytoplasmic region of IL-2 receptor β chain is essential for growth signal transduction but not for ligand binding and internalization. Cell. 59: 837.

Hattori, R., Hamilton, KK., Fugate, RD., McEver, RP., Sims, PJ. (1989a). Stimulated secretion of endothelial von Willebrand factor is accompanied by rapid redistribution to the cell surface of intracellular granule membrane protein, GMP-140. J. Biol. Chem. 264: 7768.

Hattori, R., Hamilton, KK., McEver, RP., Sims, PJ. (1989b). Complement proteins C5b-9 induces secretion of a high molecular weight multimers of endothelial von Willibrand factor and translocation of granule membrane protein GMP-140 to the cell surface. J. Biol. Chem. 264: 9053.

Hayashi, Y., Haimovich, B., Reszka, A., Boettiger, D., Horwitz, A. (1990). Expression and function of chicken integrin beta 1 subunit and its cytoplasmic domain mutants in mouse NIH 3T3 cells. J. Cell Biol. 110: 175.

Haylock, DN., To, LB., Dowse, T.L., Juttner, CA., Simmons, PJ. (1992). Ex vivo expansion and maturation of peripheral blood $CD34^+$ cells into the myeloid lineage. Blood. 80: 1405.

Haylock, DN., To, LB., Makino, S., Dowse, TL., Juttner, CA., Simmons, PJ. (1995) Ex vivo expansion of human hemopoietic progenitors with cytokines. In Hematopoietic Stem Cells: Biology and Therapeutic Applications. Levitt, D., Mertelsmann, R. (eds)., Marcel Dekker, New York, pp. 491.

Haynes, BF., Hemler, ME., Mann, DL., Eisenbarth, GS., Shelhamer, J., Mostowski, HS., Thomas, CA., Strominger, JL. Fauci, AS. (1981). J. Immunol. 126: 1409.

Haynes, BF., Telen, MJ., Hale, LP., Denning, SM., (1989). CD44-A molecule involved in leukocyte adherence and T-cell activation. Immunol. Today. 10: 423.

He, X-Y., Antao, VP., Basila, D., Marx, JC., Davis, BR. (1992). Isolation and molecular characterization of the human CD34 gene. Blood. 79: 2296.

Heckl-Ostreicher, B., Ragg, S., Drechsler, M., Scherthan, H., Royer-Pokora, BAD. (1993). Localization of the human CD59 gene by fluorescence in situ hybridization and pulsed-field gel electrophoresis. Cytogenet. Cell. Genet. 63: 144.

Heinrich, MC., Dooley, DC., Freed, AC., Band,L., Hoatlin, ME., Keeble, WW., Peters, ST., Silvey, KV., Ey, FS., Kabat, D. (1993). Constitutive expression of steel factor gene by human stromal cells. Blood. 82(3): 771.

Hemler, ME. (1990). VLA proteins in the integrin family: structures, functions and their roles on leukocytes. Ann. Rev. Immunol. 8: 365.

Hemler, ME., Huang, C., Schwarz, L. (1987a). The VLA protein family. Characterization of five distinct surface heterodimers each with a common 130,000 molecular weight β subunit. J. Biol. Chem. 262: 3300.

Hemler, ME., Huang, C., Takada, Y., Schwarz, L., Strominger, JL., Clabby, ML. (1987b). Characterization of the cell surface heterodimer VLA-4 and related peptides. J. Biol. Chem. 262: 11478.

Hendrickson, EA. (1993). The SCID mouse: Relevance as an animal model system for studying human disease. American. J. Pathol. 143: 1511.

Herrmann, M., Lorenz, H-M., Voll, R., Grunke, M., Woith, W., Kalden, JR. (1994). A rapid and simple method for the isolation of apoptotic DNA fragments. Nuc. Acids. Res. 22: 5506.

Hession, C., Osborn, L., Goff, D., Chi-Rosso, G., Vassallo, C., Pasek, M., Pittack, C., Tizard, R., Goelz, S., McCarthy, K., Hopple, S., Lobb, R. (1990). Endothelial leukocyte adhesion molecule 1: Direct expression cloning and functional interactions. Proc. Natl. Acad. Sci. (USA). 87: 1673.

Heyworth, CM., Dexter, TM., Nicholls, SE., Whetton, AD. (1993). Protein kinase C activators can interact synergistically with granulocyte colony-stimulating factor or interleukin 6 to stimulate colony formation from enriched granulocyte-macrophage colony-forming cells. Blood. 81: 894.

Hibbs, ML., Xu, H., Stacker, SA., Springer, TA. (1991). Regulation of adhesion of ICAM-1 by the cytoplasmic domain of LFA-1 integrin beta subunit. Science. 251: 1611.

Hilkens, J., Ligtenberg, MJ., Vos, HL., Litvoniv, SV. (1992). Cell membrane-associated mucins and their adhesion-modulating property. Trends Biochem. Sci. 17: 359.

Hill, B., Rozler, E., Travis, M., Chen, S., Simmons, PJ., Zannettino, ACW., Galy, A., Chen, B., Hoffman, R. (1995) High Level Expression of a Novel Epitope of CD59 Identifies a Subset of $CD34^+$ Bone Marrow Cells Highly Enriched For Pluripotent Stem Cells. Exp. Haematol. 24: 936.

Hirano, H., Screaton, GR., Bell, MV., Jackson, DG., Bell, JI., Hodes, RJ. (1994). CD44 isoform expression mediated by alternative splicing: tissue-specific regulation in mice. Int. Immunol. 6: 49.

Hirayama, F., Shih, JP., Awgulewitsch, A., Warr, GW., Clark, SC., Ogawa, M. (1992). Clonal proliferation of murine lymphohemopoietic progenitors in culture. Proc. Natl. Acad. Sci. (USA). 89: 5907.

Hirt, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. 26: 365.

Ho, M-K., Springer, TA. (1983). Biosynthesis and assembly of the alpha and beta subunits of Mac-1, a macrophage glycoprotein associated with complement receptor function. J. Biol. Chem. 258: 2766.

Hodgson, GS., Bradley, TR. (1979). Properties of haemopoietic stem cells surviving 5-fluorouracil treatment: evidence of a pre-CFU-S cell. Nature. 281: 381.

Hoegervorste, F., Kuikman, I., vondem Borne, AEGK., Sonnenberg, A. (1990). Cloning and sequence analysis of beta 4 integrin cDNA: an integrin subunit that contains a unique 118 kD cytoplasmic domain. EMBO. J. 9: 765.

Hoffman, S., Edelman, GM. (1983). Kinetics of homophilic binding by embryonic and adult forms of the neural cell adhesion molecule. Proc. Natl. Acad. Sci. (USA). 80: 5762.

Hogg, N. (1991). An integrin overview. Chem. Immunol. 50: 1.

Hogg, N. (1992). Roll, roll, your leukocyte gently down the vein. Immunol. Today. 13: 113.

Holder, AA. (1983). Carbohydrate is linked through ethanolamine to the C-terminal amino acid of Trypanosoma brucei variant surface glycoprotein. Biochem. J. 209: 261.

Holguin, MH., Fredrick, LR., Bernshaw, NJ., Wilcox, LA., Parker, CJ. (1989). Isolation and characterization of a membrane protein from normal human erythrocytes that inhibits reactive lysis of the erythrocytes of paroxysmal nocturnal hemoglobinuria. J. Clin. Invest. 84: 7.

Hollenbaugh, D., Bajorath, J., Stenkamp, R., Aruffo, A. (1993). Interaction of P-selectin (CD62) and its cellular ligand: analysis of critical residues. . Biochemistry. 32(12): 2960.

Holness, CL., Simmons, DL. (1993). Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. Blood. 81: 1607.

Holzmann, B., Weissman, IL. (1989). Integrin molecules involved in lymphocyte homing to Peyer's patches. Immunol. Rev. 108: 45.

Homans, SW., Ferguson, MAJ., Dwek, RA., Rademacher, TW., Anand, R., Williams, AF. (1988). Complete structure of the glycosyl phosphatidylinositol membrane anchor of rat brain Thy-1 glycoprotein. Nature. 333: 269.

Houssaint, E., Hallet, MM. (1988). Inability of adult circulating stem cells to sustain hemopoiesis in mouse fetal liver microenvironment. Immunology. 64: 463.

Hsu-Lin, SC., Berman, CL., Furie, BC., August, D., Furie, B. (1989). A platelet membrane protein expressed during platelet activation. J. Biol. Chem. 259: 9121.

Huang, E., Nocka, K., Beier, DR., Chu, T-Y, Buck, J., Lahm, HW., Wellner, D., Leder, P., Besmer, P. (1990). The hematopoietic growth factor KL is encoded at the Sl locus and is the ligand of the c-kit receptor, the gene product of the W locus. Cell. 63: 225.

Huang, S., Terstappen, LWMM. (1992). Formation of hematopoietic microenvironment and hematopoietic stem cells from single human bone marrow stem cells. Nature. 360: 745.

Hughes, SH., Payvar, F., Spector, D., Schimke, RT., Robinson, HL., Payne, GS., Bishop, JM., Varmus, HE. (1979). Heterogeneity of genetic loci in chickens: analysis of endogenous viral and normal genes by cleavage of DNA with restriction endonucleases. Cell. 18: 347.

Humphries, RK., Eaves, AC., Eaves, CJ. (1981). Self-renewal of hemopoietic stem cells during mixed colony formation in vitro. Proc. Natl. Acad. Sci. (USA). 78: 3629.

Hunt, P. (1995). The physiological role and therapeutic potential of the Mpl-ligand in thrombopoiesis. Stem Cells. 13: 579.

Hunt, P., Robertson, D., Weiss, D., Rennick, D., Lee, F., Witte, ON. (1987). A single bone marrow derived stromal cell type supports the in vitro growth of early lymphoid and myeloid cells. Cell.48: 997.

Huse, WD., Hansen, C. (1988). cDNA cloning redefined. A rapid, efficient, directional method. Strategies Mol. Biol. 1:1.

Hwang, WD., Gilboa, E. (1984). Expression of genes introduced into cells by retroviral infection is more efficient than that of genes introduced into cells by DNA transfection. J. Virol. 50: 417.

Hynes, RO. (1987). Integrins: A Family of cell surface receptors. Cell 48: 549.

Hynes, RO. (1992). Integrins: Versatility, modulation and signalling in cell adhesion. Cell 69: 11.

Ichikawa, Y., Pluznick, DH., Sachs, L. (1966) In vitro control of the development of macrophage and granulocyte colonies. Proc. Natl. Acad. Sci. (USA). 989: 111.

Imai, Y., Lasky, LA., Rosen, SD. (1993). Sulphation requirement for GlyCAM-1, an endothelial ligand for L-selectin. Nature. 361: 555.

Imai, Y., Singer, MS., Fennic, C., Lasky, LA., Rosen, SD. (1991). Identification of a carbohydrate based endothelial ligand for a lymphocyte homing receptor. J. Cell Biol. 113: 1213.

Imai, Y., True, DD., Singer, MS., Rosen, SD. (1990). Direct demonstration of the lectin activity of gp^{90mel}, a lymphocyte homing receptor. J. Cell Biol. 11: 1225.

Inghirami, G., Wieczorek, R., Zhu, B-Y., Silber, R., Dalla-Favera, R., Knowles, DM. (1988). Differential expression of LFA-1 molecules in non-Hodgkin's lymphoma and lymphoid leukaemia. Blood. 72: 1431.

Iscove, NN., Shaw, AR., Keller, G. (1989). Net increase of pluripotential haemopoietic precursors in suspension culture in response to IL-1 and IL-3. J. Immunol. 142: 2332.

Issekutz, TB. (1991). Inhibition of in vivo lymphocyte migration to inflamation and homing to lymphoid tissues by the TA-2 monoclonal antibody: A likely role for VLA-4 in vivo. The Journal of Immunology. 147: 4178.

Jackson, DG., Buckley, J., Bell, J. (1992). Multiple variants of the human lymphocyte homing receptor CD44 generated by insertions at a single site in the extracellular domain. J. Biol. Chem. 267: 4732.

Jalkanen, S., Jalkanen, M. (1992). Lymphocyte CD44 binds the COOH-terminal heparin-binding domain of fibronectin. J. Cell Biol. 116: 917.

Jentoft, N. (1990). Why are proteins O-glycosylated?. TIBS. 15: 291.

Johnson, A., Dorshkind, K. (1986). Stromal cells in myeloid and lymphoid long-term bone marrow cultures can support multiple hemopoietic lineages and modulate their production of hemopoietic growth factors. Blood. 68: 1348.

Johnson, GR., Li, CL. (1992) Rhodamine-123 reveals heterogeneity within murine lineage negative Sca-1 positive haemopoietic cells. J. Exp. Med. 175: 1443.

Johnson, GR., Metcalf, D. (1977). Pure and mixed erythroid colony formation in vitro stimulated by spleen conditioned medium with no detectable erythropoietin. Proc. Natl. Acad. Sci. (USA). 74: 3879.

Johnston, GI., Bliss, GA., Newman, PJ., McEver, RP. (1990). Structure of the human gene encoding granule membrane protein-140, a member of the selectin family of adhesion receptors for leukocytes. J. Biol. Chem. 265: 21381.

Johnston, GI., Cook, RG., McEver, RP. (1989a). Cloning of GMP-140, a granule membrane protein of platelets and endothelium: sequence similarity to proteins involved in cell adhesion and inflammation. Cell 56: 1033.

Johnston, GI., Kurosky, A., McEver, RP. (1989b). Structural and biosynthetic studies of the granule membrane protein, GMP-140 from human platelets and endothelial cells. J. Biol. Chem. 264: 1816.

Jones, RJ., Wagner, JE., Celano, P., Zicha, MS., Sharkis, SJ. (1990). Separation of pluripotent haemopoietic stem cells from spleen colony-forming cells. Nature. 347: 188.

Juliano, RL., Haskill, S. (1993). Signal transduction from the extracellular matrix. J. Cell Biol. 120: 577.

Jung, TM., Dailey, MO. (1990). Rapid modulation of homing receptors ($gp90^{MEL-14}$) induced by activators of protein kinase C: Receptor shedding due to accelerated proteolytic cleavage at the cell surface. J. Immunol. 144: 3130.

Jung, TM., Gallatin, WM., Weissman, IL., Dailey, MO. (1988). Down-regulation of homing receptors after T cell activation. J. Immunol. 141: 4110.

Juriskova, V., Tkadlecek, L. (1965). Charater of primary and secondary colonies of hematopoiesis in the spleen of irradiated mice. Nature. 206: 951.

Jutila, MA., Watts, G., Walcheck, B., Kansas, GS. (1992). Characterization of a functionally important and evolutionary well-conserved epitope mapped to the short consensus repeats of E-selectin and L-selectin. J. Biol. Chem. 175: 1565.

Kamata, T. Puzon, W., Takada, Y. (1994). Identification of a putative ligand binding sites within I domain of integrin alpha 2 beta 1 (VLA-2, CD49b/CD29). J. Biol. Chem. 269: 9659.

Kamel-Reid, S., Dick, D. (1988). Engraftment of immuno-deficient mice with human hematopoietic stem cells. Science. 242: 1706.

Kaneko Y., Takenawa J., Yoshida O., Fujita K., Sugimoto K., Nakayama H., Fujita J. (1991). Adhesion of mouse mast cells to fibroblasts: Adverse effects of the steel (Sl) mutation. J. Cell Physiol. 147: 224.

Kansas, GS. Spertini, O., Stoolman, LM., Tedder, TF. (1991). Molecular mapping of functional domains of the leukocyte receptor for endothelium, LAM-1. J. Cell Biol. 114: 351.

Kansas, GS., Muihead, MJ., Daily, MO. (1990). Expression of the CD11/CD18, leukocyte adhesion molecule 1, and CD44 adhesion molecules during normal myeloid and erythroid differentiation in humans. Blood. 76: 2483.

Karakantza, M., Gibson, FM., Cavenagh, JD., Ball, SE., Gordon, MY., Gordon-Smith, EC. (1994). SLE^{X} expression of normal CD34 positive bone marrow haemopoietic progenitor cells. Brit. J. Haem. 86: 883.

Katz, F., Tindle, RW., Sutherland, DR., Greaves, MF. (1985). Identification of a membrane glycoprotein associated with hemopoietic progenitor cells. Leuk. Res. 9: 191.

Kavathas, P., Sukhatme, VP., Herzenberg, LA., Parnes, JR. (1984). Isolation of the gene encoding the human T-lymphocyte differentiation antigen Leu-2 (T8) by gene transfer and cDNA subtraction. Proc. Natl. Acad. Sci. (USA). 81: 7688.

Keating, A., Powell, J., Takahashi, M., Singer, JW. (1984). The generation of human long-term marrow cultures from marrow depleted of Ia (HLA-DR) positive cells. Blood. 64: 1159.

Keating, A., Singer, JW., Killen, PD., Striker, GE., Salo, AC., Sanders, J., Donnall, Thomas, D., Thorning, D., Failkoow, P.J. (1982). Donor origin of the in vitro haematopoietic microenvironment after marrow transplantation in man. Nature. 298: 280.

Keiliss-Borok, IV., Latzinik, NV., Epichina, SY., Friedenstein, AJ. (1971). Dynamics of the formation of fibroblast colonies in monolayer cultures of bone marrow according to ^{3}H thymidine incorporation experiments. Cytologia. 13: 1402.

Kemperman, H., Wijnands, D., de Rijk, D., Roos, E. (1993). The integrin alpha 6 beta 4 on TA3/Ha mammary carcinoma cells is involved in adhesion to hepatocytes. Cancer Res. 53: 3611.

Kemperman, H., Wijnands, D., Meijne, AML., Roos, E. (1994). TA3/St, but not TA3/Ha, mammary carcinoma cell adhesion to hepatocytes is mediated by alpha 5 beta 1 interacting with surface-associated fibronectin. Cell Adhesion Commun. 2: 45.

Kern, A., Briesewitz, R., Bank, I., Marcantonio, EE. (1994). The role of the I domain in ligand binding of the human integrin alpha 1 beta1. J. Biol. Chem. 269: 22811.

Kerst, BJM., Sanders, JB., Slaper-Cortenbach, ICM., Doorakkers, MC., Hooibrink, B., van Ders, RHJ., von dem, Borne, AEGK., van der Schoot, CE. (1993). $\alpha 4\beta 1$ and $\alpha 5\beta 1$ are differentially expressed during myelopoiesis and mediate the adherence of human CD34 cells to fibronectin in an activation-dependent way. Blood. 81: 344.

Keshet, E., Lyman, SD., Williams, DE., Anderson, DM., Jenkins, NA., Copeland, NG., Parada, LF. (1991). Embryonic RNA expression patterns of the c-kit receptor and its cognate ligand suggest multiple functional roles in mouse development. EMBO.J. 10: 2425.

Kierney, PC., Dorshkind, K. (1987). B lymphocyte precursors and myeloid progenitors survive in diffusion chamber cultures, but B cell differentiation requires close association with stromal cells. Blood. 70: 1418.

Kinashi, T., Springer, TA. (1994). Steel factor and c-kit regulate cell-matrix adhesion. Blood. 83(4): 1033.

Kincade, PW., Lee, G., Pietrangeli, CE., Hayashi, SI., Gimble, JM. (1989). Cells and molecules that regulate B-lymphopoiesis in bone marrow. Ann. Rev. Immunol. 7: 111.

Kirchhofer, D., Grzesiak, J., Pierschbacher, MD. (1991). Calcium as a potential physiological regulator of integrin-mediated cell adhesion. J. Biol. Chem. 266: 4471.

Kishimoto, TK., Anderson, DC. (1992). The role of integrins in inflammation. In. Inflammation: Basic principles and clinical correlates. Gallin, JI., Goldstein, IM., Snyderman, R. (eds). New York, NY, Raven Press, pp. 353.

Kishimoto, TK., Hollander, N., Roberts, TM., Anderson, DC., Springer, TA. (1987). Heterogeneous mutations in the β subunit common to the LFA-1, Mac-1, and p150,95 glycoproteins causes leukocyte adhesion deficiency. Cell. 50: 193.

Kishimoto, TK., Jutila, MA., Berg, EL., Butcher, EC. (1989). Neutrophil Mac-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors. Science (Wash. DC). 245: 1238.

Kishimoto, TK., Jutila, MA., Butcher, EC. (1990). Identification of a human peripheral lymph node homing receptor: A rapid down-regulated adhesion molecule. Proc. Natl. Acad Sci. (USA). 87: 2244.

Kishimoto, TK., Warnock, RA., Jutila, MA., Butcher, EC., Lane, C., Anderson, DC., Smith, CW. (1991). Antibodies against human neutrophil LECAM-1 (LAM-1/Leu-8/Dreg-56 antigen) and endothelial ELAM-1 inhibit a common CD18-independent adhesion pathway in vitro. Blood. 78: 805.

Kitani, S., Berenstein, E., Mergenhagen, S., Tempest, P., Siraganian, RP. (1991). A cell surface glycoprotein of rat basophilic leukaemic cells close to the high affinity IgE receptor (Fc epsilon R1). Similarity to human melanoma differentiation antigen ME491. J. Biol. Chem. 266: 1903.

Kitigawa, H., Paulson, JC. (1994). Differential expression of five sialyltransferase genes in human tissues. J. Biol. Chem. 269: 17467.

Kittler, ELW., Mcgrath, H., Temeles, D., Crittenden, RB., Kister, VK., Quesenberry, PJ. (1992). Biological significance of constitutive and subliminal growth factor production by bone marrow stroma. Blood. 79: 3168.

Kjellen, L., Lindahl, U. (1991). Proteoglcans: structures and interactions. Annu. Rev. Biochem. 60: 443.

Klein, J. (1990). Blood cells and their origin. In Immunology. pp. 8.

Knapp, W., Dorken, B., Gilks, WR., Rieber, EP., Schmidt, RE., Stein, H., Von dem Borne, AEGKR. (1989). Leukocyte typing IV. White cell differentiation antigens. Fourth International workshop and conference on human leukocyte differentiation antigens. Oxford University Press.

Kniep, B., Cinek, T., Angelisova, P., Horejsi, VAD. (1994). Association of the GPI-anchored leucocyte surface glycoproteins with ganglioside GM3. Biochem. Biophys. Res. Commun. 203(2): 1069.

Knopse, WH., Blom, J., Crosby, WH. (1966). Regeneration of locally irradiated bone marrow. Blood. 28: 398.

Knospe, WH., Gregory, SA., Hussain, SG., Fried, W, Trobaugh, FE. (1972). Origin and recovery of colony-forming units in locally curettedbone marrow of mice. Blood. 39: 331.

Kodama, H., Hagiwara, H., Sudo, H. (1986). MC3T3-G2 PA6 preadipocytes support in vitro proliferation of haemopoietic stem cells through a mechanism different to that of interleukin-3. J. Cell Physiol. 129: 11.

Koedam, JA., Cramer, EM., Briend, E., Furie, B., Furie, BC., Wagner, DD. (1992). P-selectin, a granule membrane protein of platelets and endothelial cells, follows the regulated secretory pathway in AtT-20 cells. J. Cell Biol. 116: 617.

Koeffler, HP., Billing, R., Lusis, AJ., Sparkes, R., Golde, DW. (1980). An undifferentiated variant derived from the human acute myelogenous leukaemia cell line (KG-1). Blood. 56: 265.

Koenigsmann, M., Griffin, JD., DiCarlo, J., Cannistra, SA. (1992). Myeloid and erythroid progenitor cells from normal bone marrow adhere to collagen type I. Blood. 79: 657.

Köhler, G., Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. 256: 495.

Kornberg, L., Earp, HS., Turner, C., Prokop, C., Juliano, RL. (1991). Signal transduction by integrins: increased protein tyrosine phosphorylation caused by clustering of beta 1 integrins. Proc. Natl. Acad. Sci. (USA). 88: 8392.

Kotovuori, P., Tontti, E., Pigott, R., Sheperd, M., Kiso, M., Hasegawa, A., Renkonen, R., Nortamo, P., Altieri, DC., Gahmberg, CG. (1993). The vascular E-selectin binds to the leukocyte integrins CD11/CD18. Glycobiology. 3: 131.

Koury, MJ. (1992). Programmed cell death (apoptosis) in hematopoiesis. Exp. Hematol. 20: 391.

Kovach, NL., Carlos, TM., Yee, E., Harlan, JM. (1992). A monoclonal antibody to β_1 integrin (CD29) stimulates VLA-4-dependent adherence of leukocytes to vascular cell adhesion molecule-1 (VCAM-1). J. Cell Biol. 116: 499.

Kozak, M. (1991). An analysis of vertebrate mRNA sequences: intimations of translation controls. J. Cell. Biol. 115: 887.

Krause, DS., Fackler, MJ., Civin, CI., May, WS. (1996). CD34: Structure, biology, and clinical utility. Blood. 87: 1.

Krause, DS., Ito, T., Fackler, MJ., Collector, M., Sharkis, S., May, WS. (1994). Characterization of murine CD34, a marker for hematopoietic progenitor and stem cells. Blood. 84: 691.

Kubes, P., Kanwar, S. (1994). Histamine induces leukocyte rolling in post-capillary venules: a P-selectin-mediated event. J. Immunol. 152: 3570.

Kuijpers, TW., Hoogerwerf, M., van der Laan, LJW., Nagel, G., van der Schoot, CE., Grunert, F., Roos, D. (1992). CD66 non-specific cross reacting antigens are involved in neutrophil adherence to cytokine-activated endothelial cells. J. Cell. Biol. 118: 457.

Kuzu, I., Bicknell, R., Fletcher, CDM., Gatter, KC. (1993). Expression of adhesion molecules on the endothelium of normal tissue vessels and vascular tumors. Lab. Invest. 69: 322.

Kyte, J., Doolittle., RF. (1982). A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157: 105.

Lachmann, PJ. (1991). The control of homologous lysis. Immunol. Today. 12: 312.

Laemmli, UK. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680.

Lajtha, LG. (1979). Stem cell concepts. Differentiation 14: 23.

Lala, PK., Johnson, GR. (1978). Monoclonal origin of B lymphocyte colony forming cells in spleen colonies formed by multipotential haemopoietic stem cells. J. Exp. Med. 148: 1468.

Lansdorp, PM., Dragowska, W. (1992). Long-term erythropoiesis from constant numbers of $CD34^+$ cells in serum-free cultures initiated with highly purified progenitor cells from human bone marrow. J. Exp. Med. 175: 1501.

Lansdorp, PM., Sutherland, HJ., Eaves, CJ. (1990). Selective expression of the CD45 isoforms on functional subpopulations of $CD34^+$ hemopoietic cells from human bone marrow. J. Exp. Med. 172: 363.

Larsen, E., Celi, A., Gilbert, GE., Furie, BC., Erban, JK., Bonfanti, R., Wagner, DD., Furie, B. (1989). PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. Cell. 59: 305.

Larsen, E., Palabrica, T., Sajer, S., Gilbert, GE., Wagner, DD., Furie, BC., Furie, B. (1990). PADGEM-dependent adhesion of platelets to monocytes and neutrophils is mediated by a lineage-specific carbohydrate, LNF III (CD15). Cell. 63: 467.

Larsen, GR. Sako, D., Ahern, TJ., Shaffer, M., Erban, J., Sajer, SA., Gibson, RM., Wagner, DD., Furie, BC., Furie, B. (1992). P-selectin and E-selectin: distinct but overlapping ligand specificities. J. Biol. Chem. 276: 11104.

Larson, RS., Corbi, AL., Berman, L., Springer, TA. (1989). Primary structure of the LFA-1 alpha subunit: An integrin with an embedded domain defining a protein superfamily. J. Cell. Biol. 108: 703.

Larson, RS., Springer, TA. (1990). Structure and function of leukocyte integrins. Immunol. Rev. 114: 181.

Lasky, LA. (1992). Selectins: interpreters of cell-specific carbohydrate information during inflammation. Science. 258: 964.

Lasky, LA. (1995). Selectin-carbohydrate interactions and the initiation of the inflammatory response. Ann. Rev. Biochem. 64: 113.

Lasky, LA., Singer, MS., Dowbenko, D., Imai, Y., Henzel, WJ., Grimley, C., Fennic, C., Gillet, N., Watson, SR., Rosen SD. (1992). An endothelial ligand for L-selectin is a novel mucin-like molecule. Cell. 69: 927.

Lasky, LA., Singer, MS., Yednock, TA., Dowbenko, D., Fennie, H., Rodriguez, T., Nguyen, S. Stachel, S., Rosen, SD. (1989). Cloning of a lymphocyte homing receptor reveals a lectin domain. Cell. 56: 1045.

Lawler, J. (1986). The structural and functional properties of thrombospondin. Blood. 67: 1197.

Lawrence, MB., Springer, TA. (1991). Leukocytes roll on selectin at physiologic flow rates: distinction from the prerequisite for adhesion through integrins. Cell. 65: 859.

Lawson, GM., Katzmann, JA., Kimlinger, TK., O'Brien, JF. (1985). Isolation and preliminry characterization of a monoclonal antibody that interacts preferentially with the liver isoenzyme of human alkaline phosphatase. Clin. Chem. 31: 381.

Leary, A., Ogawa, M. (1987). Blast colony assay for umbilical cord blood and adult bone marrow progenitors. Blood. 69: 953.

Leavesley, DI., Oliver, JM., Swart, BW., Berndt, MC., Haylock, DN., Simmons, PJ. (1994). Signals from platelet/endothelial cell adhesion molecule-1 (CD31) enhance the adhesive activity of the very late antigen-4 integrin of human $CD34^+$ hemopoietic progenitor cells. J. Immunol. 153: 4673.

Leeuwenberg , JFM., von Asmuth, EJU., Jeunhomme, TMAA., Buurman, WA. (1990). IFN gamma regulates the expression of the adhesion molecule ELAM-1 and IL-6 production by human endothelial cells in vitro. J. Immunol. 145: 2110.

Lehmann, JM., Holzmann, B., Breitbart, EW., Schiegelow, P., Riethmuller, G., Johnson, JP. (1987). Discrimination between benign and malignant cells of melanocytic lineage by two novel antigens, a glycoprotein with a molecular weight of 113,000 and a protein with a molecular weight of 76,000. Cancer Res. 47: 841.

Lehmann, JM., Riethmuller, G., Johnson, JP. (1989). MUC18, a marker of tumor progression in human melanoma, shows sequence similarity to the neural cell adhesion molecules of the immunoglobulin superfamily. Proc. Natl. Acad. Sci. (USA) 86: 9891.

Lemischka, IR., Raulet, DH., Mulligan, RC. (1986). Developmental potential and dynamic behavior of hematopoietic stem cells. Cell. 45: 917.

Lenter, M., Levinovitz, A., Isenmann, S., Vestweber, D. (1994). Monospecific and common glycoprotein ligands fo E- and P-selectin on myeloid cells. J. Cell. Biol. 125 (2): 471.

Levesque, J-P., Hatzfeld, J., Hatzfeld, A. (1991). Mitogenic properties of major extracellular proteins. Immunol. Today. 12: 258.

Levesque, J-P., Leavesley, D., Niutta, S., Vadas, M., Simmons, PJ. (1995). Cytokines increase human haemopoietic cell adhesiveness by activation of VLA-4 and VLA-5 integrins. J. Exp. Med. 181: 1805.

Levinovitz, A., Muhlhoff, J., Isenmann, S., Vestweber, D. (1993). Identification of a glycoprotein ligand for E-selectin on mouse myeloid cells. J. Cell. Biol. 121: 449.

Lewinsohn, DM., Nagler, A., Ginzton, N., Greenberg, P., Butcher, EC. (1990). Hematopoietic progenitor cell expression of the H-CAM (CD44) homing-associated adhesion molecule. Blood. 75: 589.

Lewis, JP., Trobaugh, FE. (1964). Haematopoietic stem cells. Nature. 204: 589.

Lewisohn, DM., Bargatze, RF., Butcher, EC. (1987). Leokocyte-endothelial cell recognition: Evidence of a common molecular mechanism shared by neutrophils, lymphocytes, and other leukocytes. J. Immunol. 138: 4313.

Li, CL., Johnson, GR. (1985). Stimulation of multipotential, erythroid and other murine haematopoietic progenitor cell lines by adherent cell lines in the absence of detectable multi-CSF (IL-3). Nature. 316: 633.

Li, CL., Johnson, GR. (1992). Rhodamine 123 reveals heterogeneity within the lin^-, $sca\text{-}1^+$ hemopoetic cells. J. Exp. Med. 175: 1443.

Li, F., Wilkins, PP., Crawley, S., Weinstein, J., Cummings, RD., McEver, RP. (1996). Post-translational modifications of recombinant P-selectin glycoprotein ligand-1 required for binding to P- and E-selectin. J. Biol. Chem. 271: 3255.

Lichtman, MA. (1981). The ultrastructure of the hemopoietic environment of the marrow: A Review. Exp. Hematol. 9: 391.

Liesveld, JL., Abboud, CN., Duerst, RE., Ryan, DH., Brennan, JK., Lichtman, MA. (1989). Characterization of human marrow stromal cells: role in progenitor cell binding and granulopoiesis. Blood. 73: 1794.

Liesveld, JL., Winslow, JM., Frediani, KE., Ryan, DH., Abboud, CN. (1993). Expression of integrins and examination of their adhesive function in normal and leukaemic hematopoietic cells. Blood. 81: 112.

Liesveld, JL., Winslow, JM., Kempski, MC., Ryan, DH., Brennan, JK., Abboud, CN. (1991). Adhesive interactions of normal and leukaemic human $CD34^+$ myeloid progenitors: role of marrow stromal, fibroblast and cytomatrix components. Exp. Hematol. 19: 63.

Ligtenberg, MJL., Buijs, F., Vos, HL., Hilkens, J. (1992). Suppression of cellular aggregation by high levels of episialin. Cancer Res. 52: 2318.

Lim, B., Izaguirre, CA., Aye, MT., Huebsch, L., Drouin, J., Richardson, C., Minden, MD., Messner, HA. (1986). Characterization of reticulofibroblastoid colonies (CFU-RF) derived from bone marrow and long-term marrow culture monolayers. J. Cell Physiol. 127: 45.

Liszewski, MK., Post, TW., Atkinson, JP. (1991). Membrane cofactor proteins (MCP or CD46): newest member of the regulators of complement activation gene cluster. Annu. Rev. Immunol. 9: 431.

Locken, MR., Virendra, OS., Dattilio, KL., Sivin, CI. (1987). Flow cytometric analysis of human bone marrow: normal B lymphocyte development. Blood. 70: 1316.

Lok, S., Kaushansky, K., Holly, RD., Kuijper, JL., Lofton-Day, CE., Oort, PJ., Grant, F., Heipel, MD., Burkhead, SK., Kramer, JM. (1994). Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo. Nature. 369: 565.

Long, MW. (1992). Blood cell cytoadhesion molecules. Exp. Hematol. 20: 288.

Long, MW., Briddell, R., Walter, AW., Bruno, E., Hoffman, R. (1992). Human hematopoietic stem cell adherence to cytokines and matrix molecules. J. Clin. Invest. 90: 251.

Long, MW., Dixit, VM. (1990). Thrombospondin functions as a cytoadhesion molecule for human hematopoietic progenitor cells. Blood. 75: 2311.

Lorenz, E., Uphoff, D., Reid, TR., Chelton, E. (1951). Modification of irradiation injury in mice and guinea pigs by bone marrow injections. J. Natl. Cancer. Inst. 12: 197.

Low, MG. (1987). Biochemistry of the glycosyl-phosphatidylinositol membrane protein anchors. Biochem. J. 244: 1.

Low, MG. (1988). Glycosylphosphatidylinositol: an anchor for eukaryotic membrane proteins. Biochem. Soc. Trans. 16: 271.

Low, MG. (1989a). Glycosyl-phosphatidylinositol: a versatile anchor for cell surface proteins. FASEB. J. 3: 1600.

Low, MG. (1989b). The glycosyl-phosphatidylinositol anchor of membrane proteins. Biochim. Biophys. Acta 988: 427.

Low, MG., Finean, JB. (1977). Release of alkaline phosphatase from membranes by a phosphatidylinositol-specific phospholipase C. Biochem. J. 167: 281.

Low, MG., Kincade, PW. (1985). Phosphatidylinositol is the membrane-anchoring domain of the Thy-1 glycoprotein. Nature. 318: 62.

Lowry, PA., Zsebo, KM., Deacon, DH. (1991). Effects of rrSCF on multiple cytokine responsive HPP-CFC generated from Sca-1$^+$Lin$^-$ murine hematopoietic progenitors. Exp. Hematol. 19: 994.

Lund-Johansen, F., Olweus, J., Symington, FW., Arli, A., Thompson, JS., Vilella, R., Skubitz, K., Horejsi, VAD. (1993). Activation of human monocytes and granulocytes by monoclonal antibodies to glycosylphosphatidylinositol-anchored antigens. Eur. J. Immunol. 23(11): 2782.

Lund-Johansen, F., Terstappen, LWAD. (1993). Differential surface expression of cell adhesion molecules during granulocyte maturation. J. Leukoc. Biol. 54: 47.

Lyman, SD., James, L., Bos, TV., de Vries, P., Brasel, K., Gliniak, B., Hollingsworth, LT., Picha, K. (1993). Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells. Cell. 75: 1157.

Lynch, CM., Miller, AD. (1991). Production of high-titre helper virus-free retroviral vectors by cocultivation of packaging cells with different host ranges. J. Virol. 65: 3887.

Macher, BA., Beckstead, JJ. (1990). Distribution of VIM-2 and SSEA-1 glycoconjugate epitopes among human leukocytes and leukaemia cells. Leuk. Res. 14: 119.

Macher, BA., Klock, JC. (1980). The isolation and chemical characterization of neutral glycosphingolipids of human neutrophils. J. Biol. Chem. 255: 2092.

Mackay, CR., Imhof, B. (1993). Cell adhesion in the immune system. Immunol. Today. 14: 99.

Mackay, CR., Terpe, HJ., Stauder, R., Marston, WL., Stark, H., Gunthert, U. (1994). Expression and modulation of CD44 variant isoforms in humans. J. Cell Biol. 124: 71.

Maddon, PJ., Littman, DR., Godfrey, M., Maddon, DE., Chess, L., Axel, R. (1985). The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4: A new member of the immunoglobulin gene family. Cell. 42: 93.

Magli, MC., Iscove, NN., Odartchenko, N. (1982). Transient nature of early haemopoietic spleen colonies. Nature. 295: 527.

Maniatis, A., Tavassoli, M., Crosby, WH. (1971). Origin of osteogenic precursor cells in extramedullary marrow implants. Blood. 38: 569.

Manjunath, N., Correa, M., Ardman, M., Ardman, B. (1995). Negative regulation of T-cell adhesion and activation by CD43. Nature. 377: 535.

Mann, R., Mulligan, RC., Baltimore, D. (1983). Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell. 33: 153.

Marcantonio, EE., Hynes, RO. (1988). Antibodies to the conserved cytoplasmic domain of the integrin β_1 subunit react with proteins in vertebrates, invertebrates, and fungi. J. Cell Biol. 106: 1765.

Marshall, MJ., Nisbet, NW., Evans, S. (1984). Donor origin of the in vitro hemopoietic microenvironment after marrow transplantation in mice. Experimentia. 40: 385.

Martin, P., Papayannopoulou, T. (1982). HEL cells: A new human erythroleukaemic cell line with spontaneous and induced globin expression. Science. 216: 12333.

Massagué, J. (1990). Transforming growth factor alpha: a model for membrane-anchored growth factors. J. Biol. Chem. 265: 21393.

Massagué, J. (1991). A helping hand from proteoglycans. Cell-surface proteoglycans help present some polypeptide growth factors to their receptors and may act as a reservoir for others. Curr.Biol. 1: 117.

Massagué, J., Pandiella, A. (1993). Membrane-anchored growth factors. Ann. Rev. Biochem. 62: 515.

Masumoto, A, Hemler, ME. (1993). Multiple activation states of VLA-4. Mechanistic differences between adhesion to CS-1/fibronectin and to vascular cell adhesion molecule-l. J. Biol. Chem. 268: 228.

Masuzawa, Y., Miyauchi, T., Hamanoue, M., Ando, S., Yoshida, J., Takao, S., Shimazu, H., Adachi, M., Muramatsu, T.A.D., (1992). A novel core protein as well as polymorphic epithelial mucin carry peanut agglutinin binding sites in human gastric carcinoma cells: sequence analysis and examination of gene expression. J. Biochem. Tokyo. 112(5): 609.

Matthews, RJ., Pingel, JT., Meyer, CM., Thomas, ML. (1989). Studies on the leukocyte-common antigen: structure, function, and evolutionary conservation. Cold Spring Harb. Symp. Quant. Biol. 54 (Pt 2): 675.

Mauch, P., Greenberger, JS., Botnick, L., Hannon, E., Hellman, S. (1980). Evidence for structural variation in self-renewal capacity within long-term bone marrow culture. Proc. Natl. Acad. Sci. (USA). 77: 2927.

May, G., Healy, L., Gale, K., Greaves, M. (1994). Functional analysis of the stem cell antigen CD34 in transgenic mice. Blood. 84: 871.

Mayadas, TN., Johnson, RC., Rayburn, H., Hynes, RO., Wagner, DD. (1993). Leukocyte rolling and extravasation are severely compromised in P-selectin-deficient mice. Cell. 74: 541.

McAlister, I., Wolf, NS., Pietrzyk, ME. (1990). Transplantation of hematopoietic stem cells obtained by a combined dye method fractionation of murine bone marrow. Blood. 75: 1240.

McCune, JM., Namikawa, R., Kaneshima, H., Shultz, LD., Liberman, M., Weissman, IL. (1988). The SCID-hu mouse: murine model for the analysis of human hematolymphoid differentiation and function. Science. 241: 1632.

McEver, RP., Beckstead, JH., Moore, KL., Marshall-Carlson, L., Bainton, DF. (1989). GMP-140, a platelet α-granule membrane protein, is also synthesized by vascular endothelial and is localized in Weibel-Palade bodies. J. Clin. Invest. 84: 92.

McEver, RP., Martin, MN. (1989). A monoclonal antibody to a membrane glycoprotein binds only to activated platelets. J. Biol. Chem. 259: 9799.

McManus, PM., Weiss, L. (1984). Bulsulfan-induced chronic bone marrow failure: Changes in cortical bone, marrow stromal cells and adherent cell colonies. Blood. 64: 1036.

McNiece, IK., Bertoncello, I., Kriegler, AB., Quesenberry, PJ. (1990). Colony forming cells with high proliferative potential (HPP-CFC). Int. J. Cell Cloning. 8: 146.

McNiece, IK., Langley, KE., Zsebo, KM. (1991). Recombinant human stem cell factor synergises with granulocyte- and granulocyte-macrophage-colony stimulating factor, interleukin-3 and erythropoietin to stimulate human progenitor cells of myeloid and erythroid lineages. Exp Hematol. 19: 226.

McNiece, IK., Stewart, DM., Deacon, DS., Temeles, DS., Zsebo, KM., Clark, SC., Quesenberry, PJ. (1989). Detection of human CFC with high proliferative potential. Blood: 74: 609.

Medvinsky, AL., Samoylina, NL., Muller, AM., Dzierzak, EA. (1993). An early pre-liver source of CFU-S in the developing mouse. Nature. 364: 64.

Menko, AS., Boettiger, D. (1987). Occupation of the extracellular matrix receptor, integrin, is a control point for myogenic differentiation. Cell. 51: 51.

Meri, S., Morgan, BP., Davies, A., Daniels, RH., Olavesen, MG., Waldmann, H., Lachmann, PJ. (1990). Human protectin (CD59), an 18,000-20,000 MW complement lysis restricting factor, inhibits C5b-8 catalysed insertion of C9 into lipid bilayers. Immunology. 71: 1.

Meri, S., Väkevä, A., Laari, T., Lachmann, PJ. (1991). Soluble forms of CD59-antigen: Distribution in body fluids and functional activity. Complement. Inflamm. 8: 193.(abstract)

Metcalf, D. (1977). Haemopoietic colonies. pp 27. Berlin: Springer Verlag.

Metcalf, D. (1984). The hemopoietic colony stimulating factors. Amsterdam: Elsevier.

Metcalf, D. (1988). Molecular control of blood cells. London. Harvard University Press.

Metcalf, D. (1989). The molecular control of cell division, differentiation, commitment and maturation in haemopoietic cells. Nature. 339: 27.

Metcalf, D. (1993). Hematopoietic regulators: redundancy or subtlety?. Blood. 82: 3515.

Metcalf, D., Moore, MAS. (1971). Haemopoietic cells. Frontiers of Biology series: North-Holland Publishing Company Amsterdam. pp 71.

Metcalf, D., Nicola, NA. (1985). Synthesis by mouse peritoneal cells of G-CSF, the differentiation inducer for myeloid leukemia cells: Stimulation by endotoxin, M-CSF and Multi-CSF. Leuk. Res. 9: 35.

Metzelaar, J. M., Wijngaard, PLJ., Peters, PJ., Sixma, JJ., Nieuwenhuis, HK., Clevers, H. (1991). CD63 Antigen, a novel lysosomal membrane glycoprotein, cloned by screening procedure for intracellular antigens in eukaryotic cells. J. Biol. Chem. 266: 3239.

Metzelaar, MJ., Sixma, JJ., Nieuwenhuis, HK. (1989). In Leucocyte Typing IV: White cell differentiation antigens. W. Knapp, B. Dorken, W. R. Gilks, P. Rieber, R. E. Schmidt. H. Stein, and A. E. G. K. von dem Borne, eds. Oxford University Press, Oxford. pp. 1039

Michalack, M., Quackenbush, E., Letarte, M. (1986). J. Biol. Chem. 261: 92.

Michl, J., Qiu, Q-Y., Kuerer, HM. (1991). Homing receptors and addressins. Curr. Opin. Immunol. 3: 373.

Migliaccio, G., Migliaccio, AR., Petti, S., Mavillio, F., Russo, G., Lazzaro, D., Testa, U., Marinucci, M., Peschle, C. (1986). Human embryonic hemopoiesis. J. of Clin. Invest. 78: 51.

Miller, AD., Buttimore, C. (1986). Redesign of a retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol. Cell. Biol. 6: 2895.

Miller, AD., Miller, DG., Garcia, JV., Lynch, CM. (1993). Use of retroviral vectors for gene transfer and expression. Methods in Enzymology. 271: 581.

Miller, AD., Rosman, GJ. (1989). Improved retroviral vectors for gene transfer and expression. Biotechniques. 7: 980.

Miller, AD., Trauber, DR., Buttimore, C. (1986). Factors involved in production helper virus-free retrovirus vectors. Somatic Cell Mol. Genet. 12: 175.

Miller, JF., Dower, WJ., Tompkins, LS. (1988). High-voltage electroporation of bacteria: genetic transformation of Campylobacter jejuni with plasmid DNA. Proc. Natl. Acad. Sci. (USA). 85: 856.

Miller, L., Albert, G., Vann-Blank, N., Ludowyke, R., Metzger, H. (1990). Transmembrane signalling in P815 mastocytoma cells by transfected IgE receptors. J. Biol. Chem. 21: 12444.

Miller, LJ., Springer, TA. (1987). Biosynthesis and glycosylation of p150,95 and related leukocyte adhesion proteins. J. Immunol. 139: 842.

Miltenyi, S., Muller, W., Weichel, W., Radbruch, A. (1990). High Gradient Magnetic Cell Separation With MACS. Cytometry. 11: 231.

Miyake, K., Kincade, PW. (1990). A new cell adhesion mechanism involving hyaluronate and CD44. Curr. Top. Microbiol. Immunol. 166: 87.

Miyake, K., Medina, KL., Hayashi, SI., Ono, S., Hamaoka, T., Kincaide, PW. (1990a). Monoclonal antibodies to Pgp-1/CD44 block Lympho-hemopoiesis in long-term bone marrow cultures. J. Exp. Med. 171: 477.

Miyake, K., Medina, KL., Ishihara, K., Kimoto, M., Auerbach, R., Kincade, PW. (1991a). A VCAM-like adhesion molecule on murine bone marrow stromal cells mediates binding of lymphocytic precursors in culture. J. Cell Biol. 114: 557.

Miyake, K., Underhill, CB., Lesley, J., Kincade, PW. (1990b). Hyaluronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. J. Exp. Med. 172: 69.

Miyake, K., Weissman, K., Greenberger, JS., Kincade, PW. (1991b). Evidence for a role of the integrin VLA-4 in lympho-hemopoiesis. J. Exp. Med. 173: 599.

Miyata, T., Takeda, J., Iida, Y., Yamada, N., Inoue, N., Takahashi, M., Maeda, K., Kitani, T., Kinoshita, TAD. (1993). The cloning of PIG-A, a component in the early step of GPI-anchor biosynthesis. Science. 259: 1318.

Miyata, T., Yamada, N., Iida, Y., Nishimura, J., Takeda, J., Kitani, T., Kinoshita, TAD. (1994). Abnormalities of PIG-A transcripts in granulocytes from patients with paroxysmal nocturnal hemoglobinuria. N Engl J Med. 330:283.

Montgomery, KF., Osborn, L., Hession, C., Tizard, R., Goff, D., Vassallo, C., Tarr, PI., Bomsztky, K., Lobb, R., Harlan, JM. (1991). Activation of endothelial-leukocyte adhesion molecule-1 (ELAM-1) gene transcription. Proc. Natl. Acad. Sci. (USA). 88: 6523.

Moore, KL., Eaton, SF., Lyons, DE., Lichenstein, HS., Cummings, RD., McEver, RPAD. (1994). The P-selectin glycoprotein ligand from human neutrophils displays sialylated, fucosylated, O-linked poly-N-acetyllactosamine. J. Biol. Chem. 269: 23318.

Moore, KL., Stults, NL., Diaz, S., Smith, DF., Cummings, RD., Varki, A., McEver, RP. (1992). Identification of a specific glycoprotein ligand for P- selectin (CD62) on myeloid cells. J. Cell Biol. 118: 445.

Moore, KL., Varki, A., McEver, RP. (1991). GMP-140 binds to a glycoprotein receptor on human neutrophils: evidence for a lectin-like interaction. J. Clin. Invest. 112: 491.

Moore, MAS. (1990) Haemopoietic growth factor interaction: in vitro and in vivo preclinical evaluation, Colony-stimulating factors: molecular and cellular biology. Immunology Series, vol 49, Marcel Dekker.

Moore, MAS. (1991). Clinical implications of the positive and negative hematopoietic stem cell regulators. Blood. 78: 1.

Moore, MAS. (1993). Ex vivo expansion and gene therapy using cord blood $CD34^+$ cells. J. of Hematotherapy. 2: 221.

Moore, MAS., Broxmeyer, HE., Sheridan, APC., Meyers, PA., Jacobson, N., Winchester, RJ. (1980). Continuous human bone marrow culture: Ia antigen characterization of probable pluripotential stem cells. Blood. 55: 682.

Moore, MAS., Metcalf, D. (1970). Ontogeny of the haempoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo. Br. J. Haematol. 18: 279.

Moore, MAS., Owen, JJT. (1967a). Stem cell migration in developing myeloid and lymphoid systems. Lancet. (ii): 658.

Moore, MAS., Owen, JJT. (1967b). Chromosome marker studies in the irradiated chick embryo. Nature. 215: 1081.

Moore, MAS., Sheridan, APC., Allen, TD., Dexter, TM. (1979). Prolonged hematopoiesis in a primate bone marrow culture system: Characteristics of stem cell production and the hematopoietic stem cells in vitro. Blood. 54: 775.

Moreb, J., Zucali, JR., Rueth, S. (1990). The effects of tumor necrosis factor-alpha on early human hematopoietic progenitor cells treated with 4-hydroxyperoxycyclophosphamide. Blood. 76: 681.

Moreb, J., Zucali, JR., Weiner, RS. (1989). The role of interleukin 3 and interleukin 6 in the protection from 4-hydroxyperoxycyclophosphamide and the proliferation of early human hematopoietic progenitor cells. Exp. Hematol. 17: 1022.

Morgan, BP., van den Berg, CW., Davies, EV., Hallett, MB., Horejsi, VAD. (1993). Cross-linking of CD59 and of other glycosyl phosphatidylinositol-anchored molecules on neutrophils triggers cell activation via tyrosine kinase. Eur. J. Immunol. 23(11): 2841.

Morris, AJ., Turnbull, JE., Riley, GP., Gordon, MY., Gallagher, JT. (1991). Production of heparan sulphate proteoglycans by human bone marrow stromal cells. J. Cell Sci. 99: 149.

Morrison, SJ., Hemmati, HD., Wandycz, AM., Weissman, IL. (1995a). The purification and characterization of fetal liver hematopoietic stem cells. Proc. Natl. Acad. Sci. (USA). 92: 10302.

Morrison, SJ., Uchida, M., Weissman, IL. (1995b). The biology of hematopoietic stem cells. Ann. Rev. Cell Dev Biol. 11: 35.

Morrison, SJ., Weissman, IL. (1994). The long term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. Immunity. 1: 661.

Mould, AP. Humphries, MJ. (1991). Identification of a novel recognition sequence for the integrin $\alpha4\beta1$ in the COOH-terminal heparin-binding domain of fibronectin. EMBO J. 10: 4039.

Mould, AP., Komoriya, A., Yamada, KM., Humphries, MJ. (1991). The CS5 peptide is a second site in the IIICS region of fibronectin recognized by the integrin $\alpha_4\,\beta_1$. Inhibition of $\alpha_4\beta_1$ function by RGD peptide homologues. J. Biol. Chem. 266: 3579.

Mount, SM. (1982). A catologue of splice junction sequences. Nucleic Acids Re. 10: 459.

Muench, MO., Cupp, J., Polakoff, J., Roncarolo, MG. (1994). Expression of CD33, CD38 and HLA-DR on $CD34^+$ human fetal liver progenitors with high proliferative potential. Blood. 83: 3170.

Muench, MO., Schneider, JG., Moore, MAS. (1992). Interactions among colony-stimulating factors IL-1, IL-6 and kit-ligand in the regulation of primitive murine hematopoietic cells. Exp. Hematol. 20: 339.

Muller, AM., Medvinsky, AL., Strouboulis, J., Grosveld, F., Dzierzak, EA. (1994). Development of hematopoietic stem cell activity in the mouse embryo. Immunity. 1: 291.

Muller, WA., Berman, ME., Newman, PJ., DeLisser, HM., Albelda, SM. (1992). A heterophilic adhesion mechanism for the platelet/endothelial cell adhesion molecule-1 (CD31). J. Exp. Med. 175: 1401.

Mulligan, MS., Varani, J., Dame, MK., Lane, CL., Smith, CW., Anderson, BC., Ward, PA. (1991). Role of endothelial/leukocyte adhesion molecule-1 (ELAM-1) in neutrophil-mediated lung injury in rats. J. Clin. Invest. 88: 1396.

Munro, JM., Lo, SK., Corless, C., Robertson, MJ., Lee, NC., Barnhill, RL., Weinberg, DS., Bevilacqua, MP. (1992). Expression of sialyl-$Lewis^x$, an E-selectin ligand, in inflammation immune processes, and lymphoid tissues. Am J. Pathol. 141: 1397.

Muori, K., Suda, T., Nojiri, H., Amemiya, Y., Miura, Y., Singhal, H., Hakomori, S. (1993a). Expression of sialyl-$Lewis^x$ in CD34-positive leukemic blasts. Leukemia. 7: 336.

Muori, K., Suda, T., Nojiri, H., Ema, H., Amemiya, Y. Miura, Y., Nakauchi, H., Singhal, H., Hakomori, S. (1992). Reactivity profiles of leukemic myeloblasts with monoclonal antibodies directed to sialyl-Le^x and other lacto-series type 2 chain antigens: absence of reactivity with normal hemopoietic progenitor cells. Blood. 79: 713.

Murray, L., Chen, B., Galy, A, Chen, S., Tushinski, R., Uchida, N., Negrin, R., Tricot, G., Jagannath, S., Vesole, D., Barlogi, B., Hoffman, R., Tusukimoto, A. (1995). Enrichment of human

hematopoietic stem cell activity in the $CD34^+Thy\text{-}1^+Lin^-$ subpopulation from mobilized peripheral blood. Blood. 85: 368.

Nakahata, T., Ogawa, M. (1982). Identification in culture of a class of hemopoietic colony-forming units with extensive capability to self-renew and generate multipotential hemopoietic colonies. Proc. Natl. Acad. Sci. (USA). 79: 3843.

Nakano, Y., Noda, K., Endo, T., Kobata, A., Tomita, MAD. (1994). Structural study on the glycosyl-phosphatidylinositol anchor and the asparagine-linked sugar chain of a soluble form of CD59 in human urine. Arch. Biochem. Biophys. 311(1): 117.

Namen, AE., Lupton, S., Hjerrild, K., Wignall, J., Mochizuki, DY., Schmierer, A., Mosley, B., March, C.J., Urdahl, D., Gillis, S., Cosman, D., Goodwin, RG. (1988). Stimulation of B-cell progenitors by cloned murine interleukin-7. Nature. 333: 571.

Namikawa, R., Weilbaecher, KN., Kaneshima, H., Yee, EJ., McCune, JM. (1990). Long-term human hematopoiesis in the SCID-hu mouse. J. Exp. Med. 172: 1055.

Nathan, C., Sporn, M. (1991). Cytokines in context. J. Cell Biol. 113: 981.

Neben, S., Donaldson, D., Sieff, C., Mauch, P., Bodine, D., Ferrara, J., Yetz-Aldape, J., Turner, K. (1994). Synergistic effects of interleukin-11 with other growth factors on the expansion of murine hematopoietic progenitors and maintenance of stem cells in liquid culture. Exp. Hematol. 22: 353.

Needham, LK., Schnaar, RL. (1993) The HNK-1 reactive sulfoglucoronyl glycolipids are ligands for L-selectin and P-selectin but not E-selectin. Proc. Natl. Acad. Sci. (USA). 90: 1359.

Nelson, RM., Dloich, S., Aruffo, A., Cecconi, O., Bevilacqua, MP. (1993). Higher affinity oligosaccharide ligands for E-selectin. J. Clin. Invest. 91: 1157.

Neumann, E., Schaefer-Ridder, M., Wung, Y., Hofschrekler, P.H. (1982). Gene transfer into mouse myeloma cells by electroporation in high electric fields. EMBO J. 1:841.

Newman, PJ., Berndt, MC., Gorski, J., White, C., Lyman, S., Paddock, C., Muller, WA. (1990). PECAM-1 (CD31) cloning and relationship to adhesion molecules of the immunoglobulin gene superfamily. Science. 247: 1219.

Nicola, NA. (1989). Haemopoietic cell growth factors and their receptors. Annu. Rev. Biochem. 58: 45.

Nicola, NA., Johnson, GR. (1982). The production of committed haemopoietic colony forming cells from multipotential precursors in vitro. Blood. 60: 1019.

Ninomiya, H., Stewart, BH., Rollins, SA., Zhao, J., Bothwell, AL., Sims, PJAD. (1992). Contribution of the N-linked carbohydrate of erythrocyte antigen CD59 to its complement-inhibitory activity. J. Biol. Chem. 267(12): 8404.

Norgard, KE., Moore, KL., Diaz, S., Stults, NL., Ushiyama, S., McEver, RP., Cummings, RD., Varki, A. (1993). Characterization of a specific ligand for P-selectin on myeloid cells: A minor glycoprotein with sialylated O-linked oligosaccharides. J. Biol. Chem. 268: 12764.

Norgard-Sumnicht, KE., Varki, NM., Varki, A. (1993). Calcium-dependent heparin-like ligands for L-selectin in non-lymphoid endothelial cells. Science. 261: 480.

Nortamo, P., Salcedo, R., Timonen, T., Patarroyo, M., Gahmberg, CG. (1991). A monoclonal antibody to the human leukocyte adhesion molecule intercellular adhesion molecule-2. Cellular distribution and molecular characterization of the antigen. J. Immunol. 146: 2530.

Nottenburg, C., Rees, G., St John., T. (1989). Isolation of mouse CD44 cDNA: Structural features are distinct from the primate cDNA. Proc. Natl. Acad. Sci. (USA). 86: 58521.

Ogata, S., Hayashi, Y., Takami, N., Ikehara, Y. (1988). Chemical characterization of the membrane-anchoring domain of human placental alkaline phosphatase. J. Biol. Chem. 263: 10489.

Ogawa, M. (1993). Differentiation and proliferation of hematopoietic stem cells. Blood. 81: 2844.

Ogawa, M., Matsuzaki, Y., Nishikawa, S., Hayashi, S-I., Kunisada, T. Sudo, T., Kina, T., Nakauchi, H., Nishikawa, S-I. (1991). Expression and function of c-kit in hemopoietic progenitor cells. J. Exp. Med. 174: 63.

Ohmori, K., Yoneda, T., Ishihara, G., Shigeta, K., Hirashima, K., Kanai, M., Itai, S., Sasaoki, T., Aril, S., Arita, H., Kannagi, R. (1989). Sialyl SSEA-1 antigen as acarbohydrate marker on human natural killer cells and immature lymphoid cells. Blood. 74: 255.

Ord, DC., Ernst, TJ., Zhou, LJ., Rambaldi, A., Spertini, O., Griffin, J., Tedder, TF. (1990). J. Biol. Chem. 265: 7760.

Osborn, L., Hession, C., Tizard, R., Vassallo, C., Luhowskyj, S., Chi-Rosso, G., Lobb, R. (1989). Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell. 59: 1203.

Ostergaard, HL., Shackelford, DA., Hurley, TR., Johnson, P., Hyman, R., Sefton, BM., Trowbridge, IS. (1989). Expression of CD45 alters phosphorylation of the lck-encoded tyrosine protein kinase in murine lymphoma T-cell lines. Proc. Natl. Acad. Sci. (USA). 86: 8959.

Owen, ME. (1985). Lineage of osteogenic cells and their relationship to the stromal system. Bone and Mineral Research. 3: 1.

Owen, ME., Friedenstein, AJ. (1988). Stromal stem cells: marrow derived osteogenic precursors. CIBA Foundation Symposium. 136: 42.

Pallant, A., Eskenazi, A., Mattei, MG., Fournier, RE., Carlsson, SR., Fukuda, M., Frelinger JG. (1989). Characterization of cDNAs encoding human leukosialin and localization of the leukosialin gene to chromosome 16. Proc Natl Acad Sci U S A 86: 1328.

Patel, KD., Zimmerman, GA., Prescott, SM., McEver, RP., and McIntyre, TM. (1991). Oxygen radicals induce human endothelial cells to express GMP-140 and bind neutrophils. J. Cell Biol. 112: 749.

Patel, VP., Ciechanover, A., Platt, O., Lodish, HF. (1985). Mammalian reticulocytes lose adhesion to fibronectin during maturation to erythrocytes. Proc. Natl. Acad. Sci. (USA). 82: 440.

Patel, VP., Lodish, HF. (1986). The fibronectin receptor on mammalian erythroid precursor cells: Characterisation and developmental regulation. J. Cell Biol. 102: 449.

Patt, HM., Maloney, MA. (1975). Bone marrow regeneration after local injury: A review. Exp Hematol. 3: 135.

Paul, J., Conkie, D., Freshney, RI. (1969). Erythropoietic cell population changes during the hepatic phase of erythropoiesis in the foetal mouse. Cell Tissue Kinet. 2: 283.

Paul, SR., Yang, YC., Donahue, RE., Goldring, S., Williams, DA. (1991). Stromal cell associated hematopoiesis: immortalization and characterization of a primitive derived stromal cell line. Blood. 77: 1723.

Pear, WS., Nolan, GP., Scott, ML., Baltimore, D. (1993). Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl. Acad. Sci. (USA). 90: 8392.

Peault, B., Weissman, IL., Baum, C., McCune, JM., Tsukamoto, A. (1991). Lymphoid reconstitution of the human fetal thymus in SCID mice with CD34+ precursor cells. J. Exp. Med. 174: 1283.

Pedersen, DC., Fass, DN. (1980). Failure to detect Willebrand factor in cultured porcine endothelial cells. Circulation. 62 (suppl 3): 169.

Perkins, S., Fleischman, RA. (1988). Hematopoietic microenvironment. Origin, lineage and transplantability of the stromal cells in long-term bone marrow cultures from chimeric mice. J. Clin. Invest. 81: 1072.

Perkins, S., Fleischman, RA. (1990). Stromal cell progeny of murine bone marrow fibroblast colony-forming units are clonal endothelial-like cells that express collagen IV and laminin. Blood. 75: 620.

Peschel, C., Paul, WE., Ohara, J., Green, I. (1987). Effects on B cell stimulatory factor-1/interleukin-4 on hematopoietic progenitor cells. Blood. 70: 254.

Petti, S., Testa, U., Migliaccio, AR., Mavillio, F., Marinucci, M., Lazzaro, D., Russo, G., Mastroberardino, G., Peschle, C. (1985). Embryonic hemopoiesis in the human liver. Prog. Clin. Biol. Res. 193: 57.

Philbrick, WM., Palfree, RG., Maher, SE., Bridgett, MM., Sirlin, S., Bothwell, ALAD. (1990). The CD59 antigen is a structural homologue of murine Ly-6 antigens but lacks interferon inducibility. 20: 87.

Phillips, DR., Berndt, MC. (1992). Platelet membrane glycoprotein V purification. Methods Enzymol 215: 176.

Phillips, DR., Charo, IF., Scarborough, RM. (1991). GPIIb-IIIa: The responsive integrin. Cell 65: 359.

Phillips, ML., Nudelman, E., Gaeta, A., Perez, M., Singhal, AK., Hakamori, S., Paulson, JC. (1990). ELAM-1 mediates cell adhesion by recognition of a carbohydrate ligand, sialyl-Le^x. Science. 250: 1130.

Picker, LJ., Kishimoto, TK., Smith, CW., Warnock, RA., Butcher, EC. (1991a). ELAM-1 is an adhesion molecule for skin-homing T cells. Nature 349: 796.

Picker, LJ., Michie, SA., Rott, LS., Butcher, EC. (1990). A unique phenotype of skin-associated lymphocytes in humans. Ass. J. Pathol. 136: 1053.

Picker, LJ., Warnock, RA., Burns, AR., Doershuk, CM., Berg, EL., Butcher, EC. (1991b). The neutrophil selectin LECAM-1 presents carbohydrate ligands to the vascular selectins ELAM-1 and GMP-140. Cell. 66: 921.

Piersma, AH., Ploemacher, RE., Brockbank, KGM. (1983). Transplantation of bone marrow fibroblastoid stromal cells in mice via the intravenous route. Br. J. Haematol. 54: 285.

Pigott, R., Needham, LA., Edwards, RM., Walker, C., Power, C. (1991). Structural and functional studies of the endothelial activation antigen endothelial leukocyte adhesion molecule-1 using a panel of monoclonal antibodies. J. Immunol. 147: 130.

Pike, BL., Robinson, WA. (1970). Human bone marrow colony growth in agar gel. J. Cell Physiol. 76: 77.

Piller, F., Le Deist, F., Weinberg, KI., Parkman, R., Fukuda, M. (1991). Altered O-glycan synthesis in lymphocytes from patients with Wiskott-Aldrich syndrome. J Exp Med 173: 1501.

Pingel, JT., Thomas, ML. (1989). Evidence that the leukocyte-common antigen is required for antigen-induced T lymphocyte proliferation. Cell 58: 1055.

Pinola, M., Renkonen, R., Majuri, M-L., Tiisala, S., Saksela, E. (1994). Characterization of the E-selectin ligand on NK cells. J. Immunol. 152: 3586.

Ploemacher, RE., Brons, NHC. (1988). Cells associated with marrow and spleen repopulating ability and cells forming spleen colonies on day 16,12 and 8 are sequentially ordered on the basis of increasing Rhodamine-123 retention. J. Cell Physiol. 136: 531.

Ploemacher, RE., Van der Sluijs, JP., Van Beurden, CA., Baert, MR., Chan, PL. (1991). Use of limiting dilution type long-term marrow cultures in frequency analysis of marrow repopulating and spleen colony forming hematopoietic stem cells in the mouse. Blood. 78: 2527.

Ploemacher, RE., Van der Sluijs, JP., Voerman, JSA., Brons, NHC. (1989). An in vitro limiting-dilution assay of long-term repopulating hematopoietic stem cells in the mouse. Blood. 74: 2755.

Ploemacher, RE., van Soest, PL., Voorwinden, H., Boudewijn, A. (1993). Interlukin-12 synergises with interleukin-3 and steel factor to enhance recovery of murine hemopoietic stem cells in liquid culture. Leukaemia. 7: 1381.

Pober, JS., Bevilacqua, MP., Mendrick, DL., Lapierre, LA., Fiers, W., Gimbrone, MA. (1986). Two distinct monokines, interleukin 1 and tumour necrosis factor, each independently induce biosynthesisand transient expressionof the same antigen on the surface of cultured human vascular endothelial cells. J. Immunol. 136: 1680.

Polley, MJ., Phillips, ML., Wayner, E., Nudelman, E., Singhal, AK., Hakomori, SI., Paulson, JC. (1991). CD62 and endothelial cell-leukocyte adhesion molecule-1 (ELAM-1) recognize the same carbohydrate ligand, sialyl-lewis x. Proc. Natl. Acad. Sci. (USA). 88: 6224.

Polte, T., Newman, W., Gopal, TV. (1990). cDNA for endothelial leukocyte adhesion molecule 1 (ELAM-1): Sequence differences. Nucleic Acids Res. 18: 1083.

Pontvert-Delucq, S., Breton-Gorius, J., Schmitt, C., Hibner, U., Vilmer, E., Baillou, C., Rohrlich, P., Heymann, D., Najman, A., Guigon, M., Lemoine, FM. (1993). Characterization and functional analysis of adult human bone marrow cell subsets in relation to B-lymphoid development. Blood. 82: 417.

Pouyani, T., Seed, B. (1995). PSGL-1 recognition of P-selectin is controlled by tyrosine sulfation consensus at the PSGL-1 amino terminus. Cell. 83: 333.

Powers, R., Garrett, DS., March, CJ., Frieden, EA., Gronenborn, AM., Clore, GM. (1992). 1H, 15N, 13C, and 13CO assignments of human interleukin-4 using three-dimensional double- and triple-resonance heteronuclear magnetic resonance spectroscopy. Biochemistry. 31: 4334.

Prchal, JT., Throckmorton, DW., Carroll, AJ., Fusion, EW., Gams, RA., Prchal, JF. (1978). A common progenitor for human myeloid and lympoid cells. Nature. 274: 590.

Price, GB., McCulloch, EA. (1978). Cell surfaces and the regulation of hemopoiesis. Semin. Hematol. 15: 283.

Quackenbush, E., Clabby, M., Gottesdiener, KM., Barbosa, J., Jones, NH., Strominger, JL., Speck, S., Leiden, JM. (1987). Proc. Natl. Acad. Sci. (USA). 84: 6526.

Quesenberry, PJ., Lowry, PA. (1992). The colony stimulating factors: an overview. Cancer. 70: 909.

Qui, F., Ray, P., Brown, K., Barker, PE., Jhanwar, S., Ruddle, FH., Besmer, P. (1988). Primary structure of c-kit: Relationship with the CSF-1/PDGF receptor kinase family-Oncogene activation of v-kit involves deletion of the extracellular domain and C-terminus. EMBO. J. 7: 1003.

Rabinowitz, SS., Gordon, S. (1991). Macrosialin, a macrophage-restricted membrane sialoprotein differentially glycosylated in response to inflammatory stimuli. J. Exp. Med. 174: 827.

Rafii, S., Shapiro, F., Rimarachin, J., Nachman, RL., Ferris, B., Weksler, B., Moore, MAS., Asch, AS. Isolation and characterization of human bone marrow microvascular endothelial cells: hematopoietic progenitor cell adhesion. Blood. 84: 10.

Randi, AM., Hogg, N. (1994). I domain of beta 2 integrin lymphocyte function-associated antigen-1 contains a binding site for ligand intercellular adhesion molecule-1. J. Biol. Chem. 269: 12395.

Rathjen, PD., Toth, S., Willis, A., Heath, JK., Smith, AG. (1990). Differentiation-inhibiting activity is produced in matrix-associated and diffusible forms that are generated by alternate promotor usage. Cell. 62: 1105.

Ratnoff, WD., Knez, JJ., Prince, GM., Okada, H., Lachmann, PJ., Medof, MEAD. (1992). Structural properties of the glycoplasmanylinositol anchor phospholipid of the complement membrane attack complex inhibitor CD59. Clin. Exp. Immunol. 87: 415.

Rayner, JR., Gonda, TJ. (1994). A simple and efficient procedure for generating stable expression libraries by cDNA cloning in a retroviral vector. Mol. Cell. Biol. 14: 880.

Rehemtulla, A., Kaufman, RJ. (1992). Protein processing within the secretory pathway. Current Opinions in Biotechnology. 3: 560.

Reimann, J., Burger, H. (1979). In vitro proliferation of haemopoietic cells in the presence of adherent cell layers. II. Differential effect of adherent cell layers derived from various organs. Exp. Hematol. 7: 52.

Reisner, Y., O'Reilly, RJ., Kapoor, N., Good, RA. (1980). Allogeneic bone marrow transplantation using stem cells fractionated by lectins: In vitro analysis of human and monkey bone marrow cells fractionated by sheep red blood cells and soy bean agglutinin. Lancet. 2: 1320.

Rennick, D., Hunte, B., Dang, W., Thompson-Snipes, L., Hudak, S. (1994). Interleukin-10 promotes the growth of megakaryocyte, mast cell, and multilineage colonies: analysis with committed progenitors and Thy-1^{lo}Sca1^{+}stem cells. Exp. Hematol. 22: 136.

Rennick, D., Yang, G., Gemmell, L., Lee, F. (1987b). Control of haemopoiesis by bone marrow stromal cell clone: lipopolysaccharide-and interleukin-1-inducible production of colony-stimulating factors. Blood. 69: 682.

Rennick, D., Yang, G., Muller-Sieberg, C., Smith, C., Aria, N., Takabe, Y., Gemmell, L. (1987a). Interleukin-4 (B cell stimulatory factor-1) can enhance or antagonize the factor-dependent growth of hemopoietic progenitor cells. Proc. Natl. Acad. Sci. (USA). 84: 6889.

Resnitzky, D., Tiefenbrun, N., Berissi, H., Kimchi, A. (1992). Interferons and interleukin 6 suppress phosphorylation of the retinoblastoma protein in growth-sensitive hematopoietic cells. Proc. Natl. Acad. Sci. (USA). 89: 402.

Rettenmier, CW., Roussel, MF., Ashmun, RA., Ralph, P., Price, K., Sherr, CJ. (1987). Synthesis of membrane bound colony-stimulating factor-1 (CSF-1) and down-modulation of CSF-I receptors in NIH-3T3 cells transformed by co-transfection of the human CSF-I and c-fms (CSF-I receptor) genes. Mol. Cell. Biol. 7: 2378.

Rheinwald, JG., Green, H. (1975a). Formation of a keratinizating epithelium in culture by a cloned cell line derived from a teratoma. Cell. 6: 317.

Rheinwald, JG., Green, H. (1975b). Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell. 6: 331.

Rice, GE., Munro, JM., Bevilacqua, MP. (1990). Inducible cell adhesion molecule 110 (INCAM-110) is an endothelial receptor for lymphocytes. J. Exp. Med. 171: 1369.

Rice, GE., Munro, JM., Corles,s C., Bevilacqua, MP. (1991). Vascular and non-vascular expression of INCAM-110. A target for mononuclear leukocyte adhesion in normal and inflarned tissues. Am. J. Pathol. 138: 385.

Rich, IN. (1992). The developmental biology of hemopoiesis: Effect of growth factors on colony formation by embryonic cells. Exp. Hematol. 20: 368.

Richman, CM., Weiner, RS., Yankee, RA. (1976). Increase in circulating stem cells following chemotherapy in man. Blood. 47: 1031.

Riley, GP., Gordon, MY. (1987). Characterization of cultured stromal layers derived from fetal and adult haemopoietic tissues. Exp. Hematol. 15: 78.

Roberts, R., Gallagher, J., Spooncer, E., Allen, TD., Bloomfield, F., Dexter, TM. (1988a). Heparan sulphate bound growth factors: a mechanism of stromal mediated haemopoiesis. Nature. 332: 376.

Roberts, RA., Spooncer, E., Parkinson, EK., Lord, BI., Allen, TD., Dexter, TM. (1987a). Metabolically inactive 3T3 cells can substitute for marrow stromal cells to promote the proliferation and development of multipotent haemopoietic stem cells. J. Cell. Physiol. 132: 203.

Roberts, WL., Kim, BH., Rosenberry, TL. (1987b). Differences in the glycolipid membrane anchors of bovine and human erythrocyte acetylcholinesterases. Proc. Natl. Acad. Sci. (USA) 84: 7817.

Roberts, WL., Myher, JJKuksis, A., Low, MG., Rosenberry, TL. (1988b). Lipid analysis of the glycoinositol phospholipid membrane anchor of human erythrocyte acetylcholinesterase. Palmitoylation of inositol results in resistance to phosphatidylinositol-specific phospholipase C. J. Biol. Chem. 263:18766.

Robertson, M. (1979). Bone-marrow transplantation: Man and mouse. Nature. 280: 720.

Roodman, GD., Bird, A., Hutzler, D., Montgomery, W. (1987). Tumor necrosis factor-alpha and hematopoietic progenitors: Effects of tumor necrosis factor on growth of erythroid progenitors CFU-E and BFU-E and the hematopoietic cell lines K562, HL60 and HEL cells. Exp. Hematol. 15: 928.

Rosemblatt, M., Vuillet-Gaugler, MH., Leroy, C., Coulombel, L. (1991). Co-expression of two fibronectin receptors, VLA-4 and VLA-5 by immature erythroblastic precursor cells. J. Clin. Invest. 87: 6.

Rosen, SD., Singer, MS., Yednock, TA., Stoolman, LM. (1985). Science. 228: 1005.

Rosenstein, Y., Park, JK., Hahn, WC., Rosen, FS., Bierer, BE., Burakoff, SJ. (1991). CD43, a molecule defective in Wiscott-Aldrich syndrome, binds ICAM-1. Nature. 354: 233.

Rosse, C. (1976). Small lymphocyte and transitional populations of the bone marrow; their role in the mediation of immune and progenitor cell functions. Int. Rev. Cyt. 45: 155.

Rother, RP., Rollins, SA.,Mennone, J., Choder, A., Fidell, SA., Bessler, M., Hillmen, P.,Squinto, SP. (1994). Expression of recombinant transmembrane CD59 in paroxysmal nocturnal hemoglobinuria B cells confers resistance to human complement. Blood 84: 2604.

Rothstein, L., Pierce, JH., Klasser, V., Greenberger, JS. (1985). Amphotrophic retrovirus vector transfer of the V-ras oncogene to human hematopoietic and stromal cells in continuous bone marrow culture. Blood. 65: 744.

Rotoli, B., Luzzatto, L. (1989). Paroxysmal Nocturnal Haemoglobinuria. Bailliere's Clin. Haematol. 2: 113.

Ruoslahti, E. (1991). Integrins. J. Clin. Invest. 87: 1.

Rutishauser, UA., Acheson, A., Hall, AK., Mann, DM., Sunshine, J. (1988). The neural cell adhesion molecule (NCAM) as a regulator of cell-cell interactions. Science (Wash. DC.)240: 53.

Ryan, DH., Nuccie, BL., Abboud, CN., Liesveld, JL. (1992). Maturation-dependent adhesion of human B-progenitor cells to marrow stromal cells involves heparan sulphate. Blood. 80: 912.

Ryan, DH., Nuccie, BL., Abboud, CN., Winslow, JM. (1991). Vascular cell adhesion molecule-1 and the integrin VLA-4 mediate adhesion of human B cell precursors to cultured bone marrow adherent cells. J. Clin. Invest. 88: 995.

Ryfell, GU., McCarthy, BJ. (1975). Complexity of cytoplasmic RNA in different mouse tissues measured by hybridization of polyadenylated RNA to complementary DNA. Biochemistry. 14:1379.

Saeland, S., Duvert V., Caux, C., Pandrau, D., Favre, C., Valle, A., Durand, I., Charboard, P., de Vries, J., Banchereau, J. (1992). Distribution of surface membrane molecules on bone marrow and cord blood $CD34^+$ hematopoietic cells. Exp. Hematol. 20: 24.

Saiki, RK. (1990). Amplification of genomic DNA, p. 13-20. In PCR protocols: a guide to methods and applications. Innis, MA., Gelfand, DH., Sninsky, JJ., White, TJ. (eds). Academic Press, Inc., San Diego, Calif.

Sako, D., Chang, XJ., Barone, KM., Vachino, G., White, HM., Shaw, G., Veldman, GM., Bean, KM., Ahern, TJ., Furie, B., Cumming, DA., Larsen, GR. (1993). P-selectin glycoprotein ligand-1 (PSGL-1) is the high affinity counter-receptor for P-selectin on myeloid cells. Cell. 75: 1179.

Sako, D., Comess, KM., Barone, KM., Camphausen, RT., Cumming, DA., Shaw, GD. (1995). A sulfated peptide segment at the amino terminus of PSGL-1 is critical for P-selectin binding. Cell. 83: 323.

Sambrook, J., Fritsch, EF., Maniatis, T. (1989). In Molecular cloning: a laboratory manual., 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sasaki, K., Kurarta, K., Funayama, K., Nagata, M., Watanabe, E. Ohta S., Hanai, N., Nishi, T. (1994). Expression cloning of a novel alpha 1,3-fucosyltransferase that is involved in biosynthesis of the sialyl Lewis x carbohydrate determinants in leukocytes. J. Biol. Chem. 269: 14730.

Sastre, L., Kishimoto, TK., Gee, C., Roberts, T., Springer, TA. (1986). The mouse adhesion proteins Mac-1 and LFA-1: Studies on mRNA translation and protein glycosylation with emphasis on Mac-1. J. Immunol. 137: 1060.

Sawada, M., Takada, A., Ohwaki, I., Takahasi, N., Tateno, H., Sakamoto, J., Kannagi, R. (1993). Specific adhesion of a complex sialyl Lewis X antigen on high endothelial venules of human lymph nodes: Possible candidate for L-selectin ligand. Biochem. Biophys. Res. Commun. 193: 337.

Sawada, R., Ohashi, K., Anaguchi, H., Okazaki, H., Hattori, M., Minato, N., Naruto, MAD. (1990). Isolation and expression of the full-length cDNA encoding CD59 antigen of human lymphocytes. DNA-Cell-Biol. 9: 213.

Schaefer-Ridder, M., Wang, Y., Hofschneider, PH. (1982). Liposomes as gene carriers: Efficient transformation of mouse L-cells by the Thymidine Kinase gene. Science. 8: 166.

Schaffner, W. (1980). Direct transfer of cloned genes from bacteria to mammalian cells. Proc. Natl. Acad. Sci. (USA). 77: 2163.

Schimmerti, LA., Yan, H-C., Madri, JA., Albelda, SM. (1992). Platelet endothelial cell adhesion molecule PECAM-1, modulates cell migration. J. Cell Physiol. 153: 417.

Schleiffenbaum, B., Spertini, O., Tedder, TF. (1992). Soluble L-selectin is present in human plasma at high levels and retains functional activity. J. Cell. Biol. 119: 229.

Schmidt, MFG. (1989). Fatty acylation of proteins. Biochim. Biophys. Acta 988: 411.

Schofield, R. (1978). The relationship between the spleen colony-forrning cell and the haemopoietic stem cell. Blood Cells. 25: 195.

Schubert, J., Uciechowski, P., Delany, P., Tischler, H-J., Kolanus, W., Schmidt, RE. (1990). The PIG-anchoring defect in NK lymphocytes of PNH patients. Blood. 76: 1181.

Schwartz, GN., Hudgins, WR., Perdue, JF. (1993). Glycosylated Insulin-like growth factor II promoted expansion of granulocyte-macrophage colony-forming cells in serum-deprived liquid cultures of human peripheral blood cells. Exp. Hematol. 21: 1447.

Schwartz, MA. (1993). Transmembrane signalling by integrins. Trends Cell Biol. 2: 304.

Schweitzer, CM., Dräger, AM., van der Valk, P., Thijsen, SFT., Zevenbergen, A., Theijsmeijer, AP., van der Schoot, CE., Langenhuijsen, MMAC. (1995). Constitutive expression of E-selectin and VCAM-1 on endothelial cells of haemopoietic tissues. Am. J. Pathol. 148: 165.

Screaton, GR., Bell, MV., Jackson, DG., Cornelis, FB., Gerth, U., Bell, JI. (1992). Genomic structure of CD44 reveals at least 12 altematively spliced exons. Proc. Natl. Acad. Sci. (USA) 89: 12160.

Seed, B. (1987). An LFA-3 cDNA encodes a phospho-linked membrane protein homologous to its receptor CD2. Nature. 329: 840.

Seed, B., Aruffo, A. (1987), Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. Proc. Natl. Acad. Sci. (USA). 89: 3363.

Sers, C., Riethmuller, G., Johnson, JP. (1994). MUC18, a melanoma-progression molecule, and its potential role in tumor vascularization and hematogenous spread. Cancer Res. 54: 5689.

Seth, A., Gote, L., Nagarkatti, M., Nagarkatti, PS. (1991). T-cell-receptor-independent activation of cytolytic activity of cytotoxic T lymphocytes mediated through CD44 and $gp90^{MEL-14}$. Proc. Natl. Acad. Sci.(USA). 88: 7877.

Seya, T., Hara, T., Matsumoto, M., Akedo, H. (1990). Quantitative analysis of membrane cofactor protein (MCP) of complement. High expression of MCP on human leukemia cell lines, which is down-regulated during cell differentiation. J. Immunol. 145: 238.

Shadduck, RK., Waheed, A. Greenberger, JS., Dexter, TM. (1983). Production of colony stimulating factors in long-term bone marrow culture. J. Cell Physiol. 102: 287.

Shaw, LM., Mercurio, AM. (1993). regulation of the $\alpha_6\beta_1$ integrin laminin receptor fuction by the cytoplasmic domain of the α_6 subunit. J. Cell Biol. 123: 1017.

Shaw, LM., Messier, JM., Mercurio, AM. (1990). The activation dependent adhesion of macrophages to laminin involves cytoskeletal anchoring and phosphorylation of the $\alpha_6\beta_1$ integrin. J. Cell Biol. 110: 2167.

Shelley, CS., Remold-O'Donnell, E., Davis, AE III., Bruns, GA., Rosen, FS., Carroll, MC., Whitehead, AS. (1986). Molecular characterization of sialophorin (CD43), the lymphocyte surface sialoglycoprotein defective in Wiskott-Aldrich syndrome. Proc. Natl. Acad. Sci. (USA). 86: 2819.

Sheridan, WP., Begley, CG., Juttner, CA., Szer, J., To, LB., Maher, D., McGrath, KM., Morstyn, G., Fox, RM. (1992). Effect of peripheral blood progenitor cells mobilized by filgrastim (G-CSF) on platelet recovery after high dose chemotherapy. Lancet. 339: 640.

Shichishima, T., Terasawa, T., Hashimoto, C., Ohto, H., Uchida, T., Maruyama, Y. (1991). Heterogenous expression of decay accelerating factor and CD59/membrane attack complex inhibition factor on paroxysmal nocturnal haemoglobinuria (PNH) erythrocytes. Br. J. Haematol. 78: 545.

Shih, I-M., Elder, DE., Speicher, D., Johnson, JP., Herlyn, M. (1994). Isolation and functional characterisation of the A32 melanoma-associated antigen. Cancer Res. 54: 2514.

Shimizu, Y., Shaw, S. (1993). Mucins in the mainstream. Nature. 366: 630.

Shimizu, Y., van Seventer, DA., Ennis, E., Newman, W., Horgan, KJ., Shaw, S. (1992). Cross-linking of the T cell-specific accessory molecules CD7 and CD28 modulates T cell adhesion. J. Exp. Med. 175: 577.

Shinnick, TM., Lerner, RA., Sutcliffe, JG. (1981). Nucleotide sequence of Moloney murine leukaemic virus. Nature (London). 293: 543.

Siczkowski, M., Clark, D., Gordon, MY. (1992). Binding of primitive haemopoietic progenitor cells to marrow stromal cells involves heparan sulphate. Blood. 80: 912.

Siegelman, MH., Cheng, IC., Weissman, IL., Wakeland, EK., (1990). The mouse lymph node homing receptor is identical with the lymphocyte cell surface marker Ly-22: Role of the EGF domain in endothelial binding. Cell. 61: 611.

Siegelman, MH., van de Rijn, M., Weissman, IL., (1989). Mouse lymph node homing receptor cDNA clone encodes a glycoprotein revealing tandem interaction domains. Science. 243: 1165.

Siegelman, MH., Weissman, IL. (1989). Human homologue of mouse lymph node homing receptor: Evolutionary conservation at tandem cell interaction domains. Proc. Natl. Acad. Sci. (USA). 86: 5562.

Siegers, MP., Feinendegen, LE., Lahiri, SK., Cronkite, EP. (1979). Relative number and proliferation kinetics of hemopoietic stem cells in the mouse. Blood Cells. 5: 211.

Siminovitch, L., McCulloch, EA., Till, JE. (1963). The distribution of colony-forming cells among spleen colonies., Journal of Cellular and Comparative Physiology. 62: 327.

Simmons, DL., (1992). Cloning cell surface molecules by transient expression in mammalian cells. In Cellular Interactions in development: A practical approach. (ed) Hartley, DA., IRL Press.

Simmons, DL., Seed, B. (1988). Isolation of a cDNA encoding CD33, a differentiation antigen of myeloid progenitor cells. J. Immunol. 141: 2797.

Simmons, DL., Walker, C., Power, C., Pigott, R. (1990). Molecular cloning of CD31, a putative intercellular adhesion molecule closely related to carcinoembryonic antigen. J. Exp. Med. 171: 2147.

Simmons, PJ., Aylett, GW., Niutta, S., To, LB., Juttner, CA., Ashman, LK. (1994a). c-kit is expressed by primitive human hematopoietic cells that give rise to colony-forming cells in stroma-dependent or cytokine -supplemented culture. Exp. Hematol. 22(2): 157.

Simmons, PJ., Bartelmez, S., Torok-Storb, B. (1990). Human long-tern culture repopulating cells are $CD34^{+}/SBA^{-}/Rh\text{-}123^{dull}$. Exp. Hematol. 18: 550 (abstract).

Simmons, PJ., Bartelmez, S., Torok-Storb, B. (1990). Re-establishment of haemopoiesis in human long-term cultures with soybean agglutinin-negative (SBA)/$CD34^{+}$/Rhodamine 123^{DULL} pre-CFC. Exp. Hematol. 18: 550.

Simmons, PJ., Gronthos, S. (1991). Isolation and characterization of human bone marrow stromal cell precursors. Int. J. Cell Cloning. 9(4): 408 (abstract).

Simmons, PJ., Gronthos, S., Zannettino, A., Ohta, S., Graves, SE. (1994c). Isolation, characterization and functional activity of human marrow stromal progenitors in hemopoiesis. Advances in Bone Marrow Purging and Processing. Fourth International Symposium. Progress in Clinical Biological Research. 389: 271.

Simmons, PJ., Leavesley, DI., Levesque, J-P., Swart, BW., Haylock, DN., To, LB., Ashman, LK., Juttner, CA. (1994 d). The mobilization of primitive hemopoietic progenitors into the peripheral blood. Stem Cell. 12 (suppl.1): 187.

Simmons, PJ., Masinovsky, B., Longenecker, BM., Berenson, R., Torok-Storb, B., Gallatin, WM. (1992). Vascular cell adhesion molecule-1 expressed by bone marrow stromal cells mediates the binding of hematopoietic progenitor cells. Blood. 80: 388.

Simmons, PJ., Niutta., S., Ashman, LK., Berndt, MC., Juttner, CA. (1992). Primitive human bone marrow cells express L-selectin and PECAM-l. Exp. Hematol. 20: 329. (abstract).

Simmons, PJ., Przepiorka, D., Thomas, ED., Torok-Storb, B. (1987). Host origin of marrow stromal cells following allogeneic bone-marrow transplntation. Nature. 328: 429.

Simmons, PJ., Torok-Storb, B. (1991a). Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. Blood. 78: 55.

Simmons, PJ., Torok-Storb, B. (1991b). CD34 expression by stromal precursors in normal human adult bone marrow. Blood. 78: 2848.

Simmons, PJ., Zannettino, A., Gronthos, S., Leavesley, D. (1994b). Potential adhesion mechanisms for localisation of haematopoietic progenitors to bone marrow stroma. Leuk. Lymph. 12: 353.

Singer, JW., Charboard, P., Keating, A., Nemunaitis, J., Raugi, G., Wight, TN., Lopez, JA., Roth, GJ., Dow, LW., Fialkow, PJ. (1987). Simian virus 40-transformed adherent cells from human long-term marrow cultures: Cloned cell lines produce cells with stromal and hematopoietic characteristics. Blood. 70: 464.

Singer, SJ., Nicolson, GL. (1972). The fluid mosaic model of the structure of cell membranes. Science 175: 720.

Skinner, MP., Fournier, DJ., Andrews, RK., Gorman, JG., Chesterman, CN., Berndt, MC. (1989). Characterization of human platelet GMP-140 as a heparin-binding protein. Biochem. Biophys. Res. Commun. 164: 1373.

Skinner, MP., Lucas, CM., Burns, GF., Chesterman, CN., Berndt, MC. (1991). GMP-140 binding to neutrophils is inhibited by sulphated glycans. J. Biol. Chem. 266: 5371.

Slack, JL. Nemunaitis, J., Andrews, DF., Singer, JW. (1990). Regulation of cytokine and growth factor gene expression in human bone marrow stromal cells transformed with simian virus 40. Blood. 75: 2319.

Small, D., Levenstein, M., Kim, E., Carow, C., Amin, S., Rockwell, P., Witte, L., Burrow, C., Ratajczak, M., Gewitz, A., Civin, C. (1994). STK-1, the human homolog of Flk-2/Flt-3, is selectively expressed in $CD34^+$ human bone marrow cells and is involved in the proliferation of early progenitor/stem cells. Proc. Natl. Acad. Sci. (USA). 91: 459.

Smeland, EB., Funderud, S., Kvalheim, G., Gaudernack, G., Rasmussen, AM., Rusten, L., Wang, MY., Tindle, RW., Blomhoff, HK., Egeland, T. (1992). Isolation and characterization of human hematopoietic progenitor cells: an effective method for positive selection of $CD34^+$ cells. Leukemia 6: 845.

Smith, C., Gasparetto, C., Collins, N., Gillio, A., Muench, MO., O'Reilly, RJ., Moore, MAS. (1991). Purification and partial characterisation of a human hematopoietic precursor population. Blood. 77: 2122.

Smith, LG., Weissman, IL., Heimfeld, S. (1991a). Clonal analysis of hematopoietic stem-cell differentiation in vivo. Proc. Natl. Acad. Sci. (USA). 88: 2788.

Smith, SL., Bender, JG., Maples, PB., Unverzagt, K., Schilling, M., Lum, L., Williams, S., Van Epps, DE. (1993). Expansion of neutrophil precursors and progenitors in suspension culture of $CD34^+$ cells enriched from human bone marrow. Exp. Hematol. 21: 870.

Smyth, SS., Joneckis, CC., Parise, LV. (1993). Regulation of vascular integrins. Blood. 81: 2827.

Snodgrass, R., Keller, G. (1987). Clonal fluctuation within the haematopoietic system of mice reconstituted with retrovirus-infected stem cells. EMBO. J. 6: 3955.

Soligo, D., Schiro, R., Luksch, R., Manara, G., Quirici, N., Parravine, C., Deliliers, GL. (1990). Expression of integrins in human bone marrow. Br. J. Haematol. 76: 323.

Solowska, J., Guan, J-L., Marcantonio, EE., Trevithick, JE., Buck, CA., Hynes, RO, (1989). Expression of normal and mutant avian integrin subunits in rodent cells. J. Cell. Biol. 109: 853.

Sonoda, Y., Yang, Y-C., Wong, GG., Clark, SC., Ogawa, M. (1988). Analysis in serum-free culture of the targets of recombinant human hematopoietic growth factors: IL-3 and GM-CSF are specific for early developmental stages. Proc. Natl. Acad. Sci. (USA). 5: 4360.

Southern, EM. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503.

Spangrude, GJ. (1989). Enrichment of murine haemopoietic stem cells: Diverging roads. Immunol.Today. 10: 344.

Spangrude, GJ., Heimfeld, S., Weissman, IL. (1988). Purification and characterization of mouse haemopoietic stem cells. Science. 241: 58.

Spangrude, JG., Johnson, GR. (1990). Resting and activated subsets of mouse multipotent hematopoietic stem cells. Proc. Natl. Acad. Sci. (USA). 87: 7433.

Spertini, O., Luscinskas, FW., Kansas, GS., Munro, JM., Griffin, JD., Gimbrone, MA. Jr., Tedder, TFAD. (1991). Leukocyte adhesion molecule-1 (LAM-1, L-selectin) interacts with an inducible endothelial cell ligand to support leukocyte adhesion. J. Immunol. 147(8): 2565.

Spooncer, E., Gallagher, JT., Krizsa, F., Dexter, TM. (1983). Regulation of hematopoiesis in long-term bone marrow cultures IV. Glycosaminoglycan synthesis and the stimulation of haematopoiesis by beta-D-xylosides. J. Cell Biol. 96: 510.

Springer, TA. (1990a). Adhesion receptors of the immune system. Nature. 346: 425.

Springer, TA. (1990b). The sensation and regulation of interactions with the extracellular environment: The cell biology of lymphocyte adhesion receptors. Annu. Rev. Cell. Biol. 6: 359.

Srour, E., Brandt, JE., Briddell, RA., Grigsby, S., Leemhuis, T., Hoffman, R. (1993). Long-term generation and expansion of human primitive hematopoietic progenitor cells in vitro. Blood. 81: 661.

Srour, EF., Hoffman, R., Zanjani, ED. (1992). Animal models for human hematopoiesis. J. Hematother. 1: 143.

St John, T., Meyer, J., Idzerda, R., Gallatin, WM. (1990). Expression of CD44 confers a new adhesive phenotype on transfected cells. Cell. 60: 45.

Stacker, SA., Springer, TA. (1991). Leukocyte integrin P150,95 (CD11c/CD18) functions as an adhesion molecule binding to a counter-receptor on stimulated endothelium. J. Immunol. 146: 648.

Stamenkovic, I., Amiot, M., Pesando, JM., Seed, B. (1989). A lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family. Cell. 56: 1057.

Stamenkovic, I., Aruffo, A., Amiot, M., Seed, B. (1991). The hematopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronate-bearing cells. EMBO.J. 10: 343.

Stamper Jr., HB., Woodruff, JJ. (1986). Lymphocyte homing into lymph nodes: In vitro demonstration of the selective affinity of recirculating lymphocytes for high endothelial venules. J. Exp. Med. 144: 828.

Steegmaier, M., Levinovitz, A., Isenmann, S., Borges, E., Lenter, M., Kocher, HP., Kleuser, B., Vestweber, D. (1995). The E-selectin-ligand ESL-1 is a variant of a receptor for fibroblast growth factor. Nature. 373: 615.

Stefanova, I., Horejsi, VAD. (1991). Association of the CD59 and CD55 cell surface glycoproteins with other membrane molecules. J. Immunol. 147(5): 1587.

Stefanova, I., Horejsi,V., Ansotegui, IJ., Knapp, W., Stockinger, HAD. (1991). GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. Science. 254: 1016.

Steininger, CN., Eddy, CA., Leimgruber, RM., Mellors, A., Welply, J. (1992). The glycoprotease of Pasturella haemolytica A1 eliminates binding of myeloid cells to P-selectin but not E-selectin. Biochem. Biophys. Res. Commun. 188: 760.

Stenberg, PE., McEver, RP., Shuman, MA., Jacques, YV., Bainton, DF. (1985). A platelet α-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. J. Cell. Biol. 101: 880.

Stephenson, JR., Axelrad, AA., Mcleod, DL., Shreeve, MM. (1971). Induction of colonies of hemoglobin-synthesizing cells by erythropoietin in vitro. Proc. Natl. Acad. Sci. (USA). 68: 1542.

Stieger, A., Cardoso de Almeida, ML., Blatter, M-C., Brodbeck, U., Bordier, C. (1986). The membrane-anchoring systems of vertebrate acetylcholinesterase and variant surface glycoproteins of African trypanosomes share a common antigenic determinant. FEBS Lett. 199: 182.

Stocking, C., Kollek, R., Bergholz, U., Ostertag, W. (1985). Long terminal repeat sequences impart hematopoietic transformation properties to the myeloproliferative sarcoma virus. Proc. Natl. Acad. Sci. (USA). 82: 5746.

Stockinger, H., Gadd, SJ., Eher, R., Majdic, O., Schreiber, W., Kasinrerk, W., Strass, B., Schanbl, E., Knapp, W. (1990). Molecular characterisation and functional analysis of the leukocyte surface protein CD31. J. Immunol. 14: 3889.

Stoolman, LM. (1989). Adhesion molecules controlling lymphocyte migration. Cell. 56: 907.

Stoolman, LM., Rosen, SD. (1983). Possible role for cell surface carbohydrate-binding molecules in lymphocyte recirculation. J. Cell Biol. 96: 722.

Stopper, H., Jones, H., Zimmerman, U. (1987) Large scale transfection of mouse L-cells by electropermealization. BBA. 900: 3844.

Strauss, LC., Rowley, SD., LaRussa, VF., Sharkis, SJ., Stuart, RK., Civin. CI. (1986). Antigenic analysis of hematopoieisis V. Characterization of My-10 antigen expression by normal lymphohematopoietic progenitor cells. Exp. Hematol. 14: 878.

Streuli, M., Hall, LR., Saga, Y., Schlossman, SF., Saito, H. (1987). Differential usage of three exons generates at least five different mRNAs encoding human leukocyte common antigens. J Exp Med 166: 1548.

Strobel, ES., Gay, RE., Greenberg, PL. (1986). Characterization of the in vitro stromal microenvironment of human bone marrow., Int. J. Cell Clon. 4: 341.

Suda, T., Dexter, TM. (1981). Effects of hydrocortisone on long-term human marrow cells. Br. J. Haematol. 48: 661.

Sudo, TM., Ito, M., Ogawa, Y., Iizuka, M., Kodama, M., Kunisada, T., Hayashi, M., Ogawa, M., Sakai, K., Nishikawa, S., Nishikawa, S-I. (1989). Interleukin-7 production and function of stromal cell-dependent B cell development. J. Exp. Med. 170: 333.

Sugita, Y., Tobe, T., Oda, E., Tomita, M., Yasukawa, K., Yamaji, N., Takemoto, T., Furuichi, K., Takayama, M., Yano, S. (1989). Molecular cloning and characterization of MACIF, an inhibitor of membrane channel formation of complement. J. Biochem. 106: 555.

Sutherland, DR., Keating, A. (1992). The CD34 antigen: structure, biology and potential clinical applications. J. Hematother. 1: 115.

Sutherland, HJ., Eaves, CJ., Eaves, AC., Dragowska, W., Lansdorp, PM. (1989a). Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro. Blood. 74: 1563.

Sutherland, HJ., Eaves, CJ., Eaves, AC., Lansdorp, PM. (1989b). Differential expression of antigens on cells that initiate haemopoiesis in long-term human marrow culture. In Leucocyte Typing IV: White cell differentiation antigens. W. Knapp, B. Dorken, W. R. Gilks, P. Rieber, R. E. Schmidt. H. Stein, and A. E. G. K. von dem Borne, eds. Oxford University Press, Oxford. pp. 910.

Sutherland, HJ., Hogge, DE., Cook, D. and Eaves, CJ. (1993) Alternative mechanisms with and without Steel Factor support primitive human hematopoiesis. Blood 81: 1465.

Suzuki, S., Naitoh, Y. (1990). Amino acid sequence of a novel integrin beta subunit and primary expression of the mRNA in epithelial cells. EMBO. J. 9: 757.

Swart, B., (1993). Development and Characterisation of monoclonal antibodies to primitive haemopoietic cells. Master of Applied Science Thesis. University Of South Australia. Adelaide. Australia.

Symington, FW., Hedges, DL., Hakomori, S. (1985). Glcolipid antigens of human polymorphonuclear neutrophils and the inducible HL60 myeloid leukaemia line. J. Immunol. 134: 2498.

Szilvassy, SJ., Lansdorp, PM., Humphries, RK., Eaves, AC., Eaves, CJ. (1989). Isolation in a single step of a highly enriched hemopoietic stem cell population with competitive long-term repopulating ability. Blood. 74: 930.

Taguchi, R., Asahi, Y., Ikezawa, H. (1980). Purification and properties of phosphatidylinositol-specific phospholipase C of Bacillus thuringiensis. Biochim. Biophys. Acta. 619: 48.

Taguchi, R., Funahashi, Y., Ikezawa, H., Nakashima, I. (1990). Analysis of PI (phosphatidylinositol)-anchoring antigens in a patient of paroxysmal nocturnal hemoglobinuria (PNH) reveals deficiency of 1F5 antigen (CD59), a new complement-regulatory factor. FEBS Lett. 261: 142.

Taguchi, R., Ikezawa, H. (1978). Phosphatidyl inositol-specific phospholipase C from Clostridium novyi type A. Arch. Biochem. Biophys. 186: 196.

Takada, Y., Puzon, W. (1993). Identification of a regulatory region of integrin β_1 subunit using activating and inhibiting antibodies. J. Biol. Chem. 268: 17597.

Tamkun, JW., DeSimone, DW., Fonda, D., Patel, RS., Buck, C., Horwitz, AF., Hynes, RO. (1986). Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. Cell. 46: 271.

Tamura, R. Rozzo, C., Starr, L., Chambers, J., Reichardt, L. (1990). Epithelial integrin $\alpha 6\beta 4$ complete primary structure of $\alpha 6$ and variant forms of $\beta 4$. J. Cell. Biol. 111: 1593.

Tanaka, Y., Albelda, SM., Horgan, KJ., van Seventer, GA., Shimizu, Y., Newman, W., Hallam, J., Newman, PJ., Buck, CA., Shaw, S. (1992). CD31 expressed on distinctive T cell subsets is a preferential amplifier of β_1 integrin-mediated adhesion. J. Exp. Med. 176: 245.

Tavassoli, M. (1975). Studies in hemopoietic microenvironments. Exp. Hematol. 3: 213.

Tavassoli, M. (1976). Ultrastructure development of bone marrow adipose cell. Acta Anat. 94: 65.

Tavassoli, M. (1991). Embryonic and fetal hemopoiesis: An overview. Blood Cells. 1: 269.

Tavassoli, M., Crosby, WH. (1968). Transplantation of marrow to extramedullary sites. Science. 161: 54.

Tavassoli, M., Crosby, WH. (1970). Bone marrow histogenesis: A comparison of fatty and red marrow. Science. 169: 291.

Tavassoli, M., Friedenstein, AJ. (1983). Hemopoietic stromal microenvironment. Ann. J. Hematol. 15: 195.

Tavassoli, M., Hardy, CL. (1990). Molecular basis of homing of intravenously transplanted stem cells. Blood. 76: 1059.

Tavassoli, M., Houchin, DN., Jacobs, P. (1978). Fatty acid composition of adipose cells in red and yellow marrow. Scand. J. Haematol. 18: 47.

Tavassoli, M., Khademi, R. (1980). The origin of hemopoietic cells in ectopic implants of spleen and marrow. Experientia. 36: 1126.

Tavassoli, M., Maniatis, A., Crosby, WH. (1974). Induction of sustained haemopoiesis in fatty marrow. Blood. 43: 33.

Tavassoli, M., Sacks, PV., Crosby, WH. (1975). The effect of in vitro irradiation on the subsequent growth of ectopic splenic autoimplants. Proc. Soc. Exp. Biol. Med. 148: 780.

Tavassoli, M.., Takahashi, K. (1982). Morphological studies on long-term cultutre of marrow cells: characterisation of the adherent stromal cells. Am. J. Anat. 164: 91.

Tedder, TF., Isaacs, CM., Ernst, TJ., Demetri, GD., Adler, DA., Disteche, CM. (1989). Isolation and chromosomal localization of cDNAs encoding a novel human lymphocyte cell surface molecule, LAM-1. Homology with the mouse lymphocyte homing receptor and other human adhesion proteins. J. Exp. Med. 170: 123.

Tedder, TF., Penta, AC., Levine, HB., Friedman, AS. (1990). Expression of the human leukocyte adhesion molecule, LAM1: Identity with the TQ1 and Leu8 differentiation antigens. J. Immunol. 144: 532.

Tedder, TF., Steeber, DA., Chen, A., Engel, P. (1995). The selectins: vascular adhesion molecules. FASEB. J. 9: 886.

Teixeira, S., Di Grandi, S., Kuhn, LC. (1987). J. Biol. Chem. 262: 9574.

Teixido, J., Hemler, ME., Greenberger, JS., Anklesaria, P. (1992). Role of β1 and β2 integrins in the adhesion of human $CD34^{HI}$ cells to bone marrow stroma. J. Clin. Invest. 90: 358.

Terstappen, LWMM., Huang, S., Stafford, M., Lansdorp, PM., Loken, MR. (1991). Sequential generations of hematopoietic colonies derived from single nonlineage-committed $CD34^{+}CD38^{-}$ progenitor cells. Blood. 77: 1218.

Terstappen, LWMM., Nguyen, M., Huang, S., Lazarus, HM., Medof, EM. (1993). Defective and normal haematopoietic stem cells in paroxysmal nocturnal hemoglobinuria. Br J Haematol. 84: 504.

Terstappen, LWMM., Nguyen, M., Lazarus, HM., Medof, EM. (1992). Expression of the DAF (CD55) and CD59 antigens during normal haematopoietic cell differentiation. J Leuk Biol. 52: 652.

Thalmeier, K., Meißner, P., Reisbach, G., Hültner, L., Mortensen, BT., Brechtel, A., Oostendorp, RAJ., Dörmer, P. (1996). Constitutive and modulated cytokine expression in two permanent human bone marrow stromal cell lines. Exp. Hematol 24: 1.

Thomas, PS., Pietrangeli, CE., Hayashi, S-I., Schachner, M., Goridis, C., Low, MG., Kincade, PW. (1989). Demonstration of neural cell adhesion molecules on stromal cells which support lymphopoiesis. Leukemia. 2: 171.

Thorens, B., Mermod, J-J., Vassalli, P. (1987). Phagocytosis and inflammatory stimuli induce GM-CSF mRNA in macrophages through post-transcriptional regulation. Cell. 48: 671.

Thorlacius, H., Raud, J., Rosengren-Beezley, S., Forrest, MJ., Hedqvist, P., Lindbom, L. (1994). Mast cell activation induces P-selectin-dependent lekocyte rolling and adhesion in postcapillary venules in vivo. Bichem. Biophys. Rex. Commun. 203: 1043.

Till, JE., McCulloch, EA. (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells.Radiation Reasearch. 14: 213.

Till, JE., McCulloch, EA., Siminovitch, L. (1964). A stochastic model of stem cell proliferation, based on the growth of spleen colony-forming cells. Proc. Natl. Acad. Sci. (USA). 51:29.

To, LB., Sheppard, KM., Haylock, DN., Dyson, PG., Charles, P., Thorp, DL., Dale, BM., Dart, GW., Roberts, MM., Sage, RE., Juttner, CA. (1990). Single High Doses of Cyclophosphamide Enable the Collection of High Numbers of Haemopoietic Stem Cells from the Peripheral Blood. Exp. Hematol. 18: 442.

Todd, RF., Arnaout, MA., Rosin, RE. (1984). Subcellular localisation of the large subunit of Mo 1, (Mo 1 alpha, formerly gp110), a surface glycoprotein associated with neutrophil adhesion. J. Clin. Invest. 74: 1280.

Todderud, G., Alford, J., Millsap, KA., Aruffo, A., Tramposch, KM. (1992). PMN binding to P-selectin is inhibited by sulfatide. J. Lekoc. Biol. 52: 85.

Tomita, M., Tobe, T., Choi-Miura, N., Nakano, Y., Kusano, M., Oda, E. (1991). Structural analysis of (MACIF) CD59. Complement. Inflamm. 8: 233. (abstract)

Tomiyama, J., Ninomiya, H., Abe, T. (1990). Enhanced complement-susceptibility and dysfunction of lymphocytes in paroxysmal nocturnal haemoglobinuria (PNH). Br. J. Haematol. 76: 540.

Tone, M., Walsh, LA., Waldmann, HAD. (1992). Gene structure of human CD59 and demonstration that discrete mRNAs are generated by alternative polyadenylation. J. Mol. Biol. 227: 971.

Toneguzzo, F., Hayday, A., Keating, A. (1986) electric field-mediated DNA transfer: Transient and stable gene expression in human and mouse lymphoid cells. Mol. Cell Biol. 2: 703.

Toothill, VJ., Van Mourik, JA., Niewenhuis, HK., Metzelaar, MJ., Pearson, JD. (1990). Characterization of the enhanced adhesion of neutrophil leukocytes to thrombin-stimulated endothelial cells. J. Immunol. 145: 283.

Torimoto, Y., Rothstein, DM., Dang, NH., Schlossman, SF., Morimoto, C. (1992). CD31, a novel cell surface marker for CD4 cells of suppressor lineage, unaltered by state of activation. J. Immunol. 148: 388.

Torok-Storb, B. (1988). Cellular Interactions. Blood. 72: 373.

Toutant, J-P., Roberts, WL., Murray, NR., Rosenberry, TL. (1989). Conversion of human erythrocyte acetylcholinesterase from an amphiphilic to a hydrophilic form by phosphatidylinositol-specific phospholipase C and serum phospholipase D. Eur. J. Biochem. 180: 503.

Toyama-Sorimachi, N., Miyasaka, M. (1994). A novel ligand for CD44 is sulfated proteoglycan. Int. Immunol. 6: 655.

Trentin, JJ. (1970). Influence of hematopoietic organ stroma (Hematopoietic Inductive Microenvironments) on stem cell differentiation. In: Regulation of Hematopoiesis. Vol. 1., Gordon, S., (ed). Appleton-Century-Crofts, New York. pp. 161.

Trentin, JJ. (1976). Hemopoietic microenvironments. Transplant Proc. 10: 77.

Trowbridge, IS., Hyman, R., Mazauskas, C. (1978). The synthesis and properties of T25 glycoprotein in Thy-1-negative mutant lymphoma cells. Cell. 14: 21.

True, DD., Singer, MS., Lasky, LA., Rosen, SD. (1990). Requirement for sialic acid on the endothelial ligand of a lymphocyte homing receptor. J. Cell Biol. 111: 2757.

Tsai, S., Patel, V., Beaumont, E., Lodish, HF., Nathan, DG., Sieff, CA. (1987). Differential binding of erythroid and myeloid progenitors to fibroblasts and fibronectin. Blood. 69: 1587.

Tse, AGD., Barclay, AN., Watts, A., Williams, AF. (1985). A glycophospholipid tail at the carboxyl terminus of the Thy- 1 glycoprotein of neurons and thymocytes. Science. 230: 1003.

Turner, ML., McIlwaine, K., Anthony, RS., Parker, ACAD. (1995). Differential expression of cell adhesion molecules by human hematopoietic progenitor cells from bone marrow and mobilized adult peripheral blood. Stem. Cells. Dayt. 13: 311.

Tyrrell, D., James, P., Rao, N., Foxall, C., Abbas, S., Dasgupta, F., Nashed, M., Hasegawa, A., Kiso, M., Asa, D., Kidd, J., Brandley, BK. (1991). Structural requirements for the carbohydrate ligand of E-selectin. Proc. Natl. Acad. Sci. (USA) 88: 10372.

Uchida, N., Combs, J., Conti, A., Chen, S., Gianni, AM. (1994). The in vivo hematopoietic population of rhodamine 123 low population of human marrow cells. Exp. Hematol. 22: 755 (abstract).

Uchida, N., Weissmann IL. (1992). Searching for hematopoietic stem cells: evidence that Thy-1.1^{lo}Lin^{-}Sca-1^{+} cells are the only stem cells in C57BL/Ka-Thy-1.1 bone marrow. J. Exp. Med. 175: 175.

Udomsakdi, C., Eaves, CJ., Sutherland, HJ., Lansdorp, PM. (1991). Separation of functionally distinct subpopulations of primitive human hematopoietic cells using Rhodamine-123. Exp. Hematol. 19: 338.

Udomsakdi, C., Lansdorp, PM., Hogge, DE., Reid, DS., Eaves, AC., Eaves, CJ. (1992). Characterization of primitive hematopoietic cells in normal human peripheral blood. Blood. 80: 2513.

Ueda, T., Rieu, P., Brayer, J., Arnaout, MA. (1994). Identification of the complement iC3b binding site in the β2 integrin CR3 (CD11b/CD18). Proc. Natl. Acad. Sci. (USA). 91: 10680.

Ultsch, M., de Vos, AM., Kossiakoff, AA. (1991). Crystals of the complex between human growth hormone and the extracellular domain of its receptor. J. Mol. Biol. 222: 865.

Umezawa, A., Maruyama, T., Segawa, K., Shadduck, RK., Waheed, A., Hata, JI. (1992). Multiple marrow stromal cell line is able to induce hematopoiesis in vivo. J. Cell Physiol. 151: 197.

Vachino, G., Chang, XJ., Veldman, GM., Kumar, R., Sako, D., Fouser, LA., Berndt, MC., Cumming, DAAD. (1995). P-selectin glycoprotein ligand-1 is the major counter-receptor for P-selectin on stimulated T cells and is widely distributed in non-functional form on many lymphocytic cells. J. Biol. Chem. 270: 21966.

Van Den Heuvel, R., Schoeters, G., Leppens, H., Vanderborght, O. (1991). Stromal cells in long-term cultures of liver, spleen and bone marrow at different developmental ages have different capacities to maintain GM-CFC proliferation. Exp. Hematol. 19: 115.

Van der Loo, JCM., van den Bos, C., Baert, MRM., Wagemaker, G., Ploemacher, RE. (1994). Stable multilineage hematopoietic chimerism in alpha thalassemic mice induced by a bone marrow subpopulation that excludes the majority of day 12 spleen colony forming units. Blood. 83:1769.

Van der Sluijs, JP., de Jong, JP., Brons, NHC., Ploemacher, RE. (1990). Marrow repopulating cells, but not CFU-S, establish long-term in vitro hemopoiesis on a marrow-derived stromal layer. Exp. Hematol. 18: 893.

Van Vlasselaer, P., Falla, N., Snoeck, H., Mathieu, E. (1994). Characterization and Purification of Osteogenic Cells from Murine Bone Marrow by Two-Color Cell Sorting Using Anti-Sca-1 Monoclonal Antibody and Wheat Germ Agglutinin. Blood. 84(5): 753.

Van Zant, G. (1984). Studies of hematopoietic stem cells spared by 5-fluorouracil. J. Exp. Med. 159: 679.

VanZant, G., Goldwasser, E. (1979). Competition between erythropoietin and colony stimulating factor for target cells in mouse marrow. Blood. 53: 946.

Venneker, GT., Asghar, SSAD. (1992). CD59: a molecule involved in antigen presentation as well as downregulation of membrane attack complex. Exp. Clin. Immunogenet. 9: 33.

Verfaillie, C., Blakolmer, K., McGlave, P. (1990). Purified primitive human hematopoietic progenitor cells with long-term in vitro repopulating capacity adhere selectively to irradiated bone marrow. J. Exp. Med. 172: 509.

Verfaillie, CM. (1990). Direct contact between human primitive in vitro repopulating capacity adhere selectively to irradiated bone marrow stroma. J. Exp. Med. 172: 509.

Verfaillie, CM. (1993). Soluble factors(s) produced by human bone marrow stroma increase cytokine-induced proliferation and maturation of primitive hematopoietic progenitors while preventing their terminal differentiation. Blood. 82: 2045.

Verfaillie, CM., McCarthy, JB., McGlave, PB. (1991). Differentiation of primitive human multipotent hematopoietic progenitors into single lineage clonogenic progenitors is accompanied by alterations in their interaction with fibronectin. J. Exp. Med. 174: 693.

Vischer, UM., Wagner, DD. (1993). CD63 is a component of Weibel-Palade bodies of human endothelial cells. Blood. 82: 1184.

Visser, JWM., Bauman, JGJ., Mulder, AH., Eliason, JF., de Leew, AM. (1984). Isolation of murine pluripotent hemopoietic stem cells. J. Exp. Med. 159: 1576.

Visser, JWM., de Vries, P. (1988). Isolation of spleen colony-forming cells (CFU-S) using wgeat germ agglutinin and rhodamine 123 labelling. Blood Cells. 14: 369.

Visser, JWM., van Bekkum, DW. (1990). Purification of pluripotent hemopoietic stem cells: past and present. Exp. Hematol. 18: 248.

Vlodavsky, I., Bar-Shavit, R., Ishai-Michaeli, R., Bashkin, P., Fuks, Z. (1991). Extracellular sequestration and release of fibroblast growth factor: a regulatory mechanism?. Trends Biochem. Sci. 16: 268.

Vogel,H., Niewisch, H., Matioli, G. (1968). The self-renewal probability of haemopoietic stem cells. J. Cell Physiol. 72: 221.

Wagermaker, G., van Gils, FCJM., Bart-Baumeister, JAK., Wielenga, JJ., Levinsky, RJ. (1990). Sustained engraftment of allogeneic CD34 positive hemopoietic stem cells in rhesus monkeys. Exp. Hematol. 18: 704.

Waller, EK., Olweus, J., Lund-Johansen, F., Huang, S., Nguyen, M., Guo, G-R., Terstappen, L. (1995). The "Common Stem Cell" Hypothesis Reevaluated: Human Fetal Bone Marrow Contains Seperate Populations of Hematopoietic and Stromal Progenitors. Blood. 85(9): 2422.

Walz, G., Aruffo, A., Kolanus, W., Bevilacqua, M., Seed, B. (1990). Recognition by ELAM-1 of the sialyl-Le^x determinant on myeloid and tumor cells. Science. 250: 1132.

Wang, PL., O'Farrell, S., Clayberger, C., Krensky, AM. (1992). Identification and molecular cloning of tactile: A novel human T cell activation antigen that is a member of the Ig gene superfamily. J. Immunol. 148: 2600.

Wang, QR., Wolf, NS. (1990). Dissecting the hematopoietic microenvironment. VIII. Clonal isolation and identification of cell types in murine CFU-F colonies by limiting dilution. Exp. Hematol. 18: 355.

Wang, QR., Yan, ZJ., Wolf, NS. (1990). Dissecting the hematopoietic microenvironment. VI. The effects of several growth factors on the in vitro growth of murine bone marrow CFU-F. Exp. Hematol. 18: 341.

Wang, SY., Su, CY., Hsu, ML., Chen, LY., Tzeng, CH., Ho, CK. (1991). Effect of lipo-polysaccharides on the production of colony-stimulating factors by the stromal cells in long-term bone marrow cultures. Exp. Hematol. 19: 122.

Watanabe, Y. (1966). An electron microscopic study on the reticuloendothelial system in bone marrow. Tohoku J. Exp. Med. 89: 167.

Watson, JD., Mckenna, HJ. (1992). Novel factors from stromal cells: bone marrow and thymus microenvironments. Int. J. Cell Cloning. 10: 144.

Watson, JV. (1980). Enzyme kinetic studies in cell populations using fluorogenic substrates and flow cytometric techniques. Cytometry. 1:143.

Watson, JV. (1981). Dual laser beam focussing for flow cytometry through a single crossed cylindrical lens pair. Cytometry. 2:14.

Watson, ML., Kingsmore, SF., Johnston, GI., Siegelman, MH., LeBenu, MM., Lemons, RS., Bora, NS., Howard, TA., Weissman, K., McEver, RP., Seldin, MF. (1990). Genomic organisation of the selectin family of leukocyte adhesion molecules on human and mouse chromosome 1. J. Exp. Med. 172: 263.

Watson, SR., Imai, Y., Fennie, C., Geoffrey, J., Singer, M., Rosen, SD., Lasky, LA. (1991). The Complement binding-like domains of the murine homing receptor facilitate lectin activity. J. Cell Biol. 115: 235.

Watt, SM., Gilmore, DJ., Davis, JM., Clark, MR., Waldmann, H. (1987a). Cell-surface markers on haemopoietic precursors. Reagents for the isolation and analysis of progenitor cell subpopulations. Molecular and Cellular Probes. 1: 297.

Watt, SM., Karhi, K., Gatter, K., Furley, AJW., Katz, FE., Healy, LE., Atlass, LJ., Bradley, NJ., Sutherland, DR., Levinsky, R., Greaves, MF. (1987b). Distribution and epitope analysis of the cell membrane glycoprotein (HPCA-1) associated with the human hemopoietic progenitor cells. Leukemia. 1: 417.

Watt, SM., Sala-Newby, G., Hoang, T., Gilmore, DJ., Grunert, F., Nagel, G., Murdoch, SJ., Tchilian, E., Lennox, ES., Waldmann, H. (1991). CD66 identifies a neutrophil-specific epitope within the hematopoietic system that is expressed by members of the carcinoembryonic antigen family of adhesion molecules. Blood. 78: 63.

Watt, SM., Williamson, J., Genevier, H., Fawcett, J., Simmons, DL., Hatzfeld, A., Nesbitt, SA., Coombe, DR. (1993). The heparin binding PECAM-1 adhesion molecule is expressed by $CD34^+$ hematopoietic progenitor cells with early myeloid and B-lymphoid cell phenotypes. Blood. 82: 2649.

Wayner, E A., Garcia-Pardo, A., Humphries, MJ., McDonald JA., Carter, WG. (1989). Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. J. Cell Biol. 109: 1321.

Weinstein, R., Riordan, MA., Wenc, K., Kreczko, S., Zhou, M., Dainiak, N. (1988). Dual role of fibronectin in hematopoietic differentiation. Blood. 73: 111.

Weiss, L. (1961). An electron microscopic study of vascular sinuses of the bone marrow of the rabbit. Bull Johns Hopkins Hosp. 108: 171.

Weiss, L. (1965). The structure of bone marrow. Functional interrelationships of vascular and hemopoietic compartments in experimental haemolytic anaemia: an electron microscopic study. J. Morph. 117: 467.

Weiss, L. (1970). Transmural cellular passage in vascular sinuses of rat bone marrow. Blood. 36: 189.

Weiss, L. (1976). The hematopoietic microenvironment of the bone marrow: an ultrastructural study of the stroma in rats. Anat. Rec 186: 161.

Weiss, L., Che, LT. (1975). The organisation of hemopoietic cords and vascular sinuses in bone marrow. Blood Cells. 1: 617.

Weiss, MJ., Henthorn, PS., Lafferty, MA., Slaughter, C., Raducha, M., Harris, H. (1986). Isolation and characterization of a cDNA encoding a human liver/bone/kidney-type alkaline phosphotase. Proc. Natl. Acad. Sci. (USA). 83: 7182.

Weiss, MJ., Ray, K., Henthorn, PS., Lamb, B., Kadesch, T., Harris, H. (1988). Structure of the human liver/bone/kidney alkaline phosphotase. J. Biol. Chem. 263: 12002.

Weissman, IL., Papaioannou, V., Gardner, R. (1978). Fetal hematopoietic origins of the adult hematolymphoid system. In: Cold Spring Harbor Conference on Cell Proliferation. Vol. 5: Differentiation of Normal and Neoplastic Hematopoietic Cells, ed. Mark, PA., Till, JE. New York: Cold Spring Harbor Lab. pp. 33

Wendling, F., Maraskovsky, E., Debili, N., Florindo, C., Teepe, M., Titeux, M., Methia, N., Breton-Gorius, J., Cosman, D., Vainchenker, W. (1994). c-Mpl ligand is a humoral regulator of megakaryocytopoiesis. Nature. 369: 571.

Werts, ED., Gibson, DP., Knapp, SA., DeGowin, RL. (1980). Stromal cell migration precedes hemopoietic repopulation of the bone marrow after irradiation. Radiat. Res. 81: 20.

Westen, H., Bainton, DF. (1979). Association of alkaline phosphatase-positive reticulum cells in bone marrow with granulocytic precursors. J. Exp. Med. 150: 919.

Weyrich, AS., McIntyre, TM., McEver, RP., Prescott, SM., Zimmerman, GA. (1995). Monocyte tethering by P-selectin regulates monocyte chemotactic protein-1 and tumor necrosis factor-a secretion. J. Clin. Invest. 95: 2297.

Whelan, J., Ghersa, P., Van Huijsduijnen, RH., Gray, J., Chandra, G., Talabot, F., DeLamarter, JF. (1991). An NF kappa B-like factor is essential but not sufficient for cytokine induction of endothelial leukocyte adhesion molecule (ELAM-1) gene transcription. Nucleic Acids Res. 19: 2645.

Whetton, AD. (1990). The biology and clinical potential of growth factors that regulate myeloid cell production. Trends Pharm. Sci. 11: 285.

Whitehead, I., Kirk, H., Kay, R. (1995). Expression cloning of oncogenes by retroviral transfer of cDNA libraries. Mol. Cell. Biol. 15: 704.

Whitlock, CA., Witte, ON. (1982). Long-term culture of B lymphocytes and their precursors from murine bone marrow. Proc. Natl. Acad. Sci. (USA). 79: 3608.

Whitlow, MB., Iida, K., Stefanová, I., Bernard, A., Nussenzweig, V. (1990). H19, a surface membrane molecule involved in T-cell activation, inhibits channel formation by human complement. Cell. Immunol. 126: 176.

Wicha, MS., Lowrie, G., Kohn, E., Bagavandoss, P., Mahn, T. (1982). Extracellular matrix promotes mammary epithelial growth and differentiation in vitro. Proc. Natl. Acad. Sci. (USA). 79: 3213.

Wight, TN., Kinsella, MG., Keating, A., Singer, JW. (1986). Proteoglycans in human long-term bone marrow culture: Biochemical and ultrastructural analysis. Blood. 67: 1333.

Wilcox, M., Leptin, M. (1985). Tissue-specific modulation of a set of related cell surface antigens in Drosophila. Nature. 316: 351.

Wilkins, BS., Jones, DB. (1995). Immunohistochemical characterization of intact stromal layers in long-term cultures of human bone marrow. Br. J. Haematol 90: 757.

Williams, AF., Barclay, AN. (1988). The immunoglobulin superfamily. Annu. Rev. Immunol 6: 381.

Williams, AF., Tse AG, D., Gagnon, J. (1988). Squid glycoproteins with structural homology to Thy-1 and Ly-6 antigens. Immunogenetics. 27: 265.

Williams, D., Nathan, DG. (1991). Introduction: The molecular biology of hematopoiesis. Semin. Hematol. 28: 114.

Williams, DA., Lemischka, IR., Nathan, DG., Mulligan, RC. (1984). Introduction of new genetic material into pluripotent hematopoietic stem cells of the mouse. Nature. 310: 476.

Williams, DA., Rios, M., Stephens, C., Patel, VP. (1991). Fibronectin and VLA-4 in haematopoietic stem cell-microenvironment interactions. Nature. 352: 438.

Williams, DE., Eisenman, J., Baird, A., Rauch, C., Van Nebs, K., March, JC., Park, LS., Martin, U., Mochizuchi, DY., Bosnell, HS., Burgess, GS., Cosman, D., Lyman, SD. (1990). Identification of a ligand for the c-kit proto-oncogene. Cell. 63: 167.

Williams, M.J., Hugues, PE., O'Toole, TE., Ginsberg, MH. (1994) The inner world of cell adhesion function: integrin cytoplasmic domain. Trends Cell Biol. 4: 109.

Winton, EF., Colenda, KW. (1989). Use of long-term human marrow cultures to demonstrate progenitor cell precursors in marrow treated with 4-hydroxyperoxycyclophosphamide. Exp. Hematol. 15: 710.

Wolf, NS. (1979). The hemopoietic microenvironment. Clinics in Hematol. 8: 469.

Wolf, NS., Kone, A., Priestly, GV., Bartelmez, SH. (1993). In vivo and in vitro characterisation of long-term repopulating primitive hematopoietic cells isolated by sequential Hoechst 33342-rhodamine 123 FACS selection. Exp. Hematol. 21: 614.

Wolf, NS., Trentin JJ. (1968). Hemopoietic colony studies. Effects of haemopoietic organ stroma on differentiation of pluripotent stem cells., J. Exp. Med. 127: 205.

Wong, BY., Chen, H., Chung, S-W., Wong, PMC. (1994). High-efficiency identification of genes by functional analysis from a retroviral cDNA expression library. 1994. J. Virol. 68: 5523.

Wu, AM., Till, JE., Siminovitch, L., McCulloch, EA. (1967). A cytological study of the capacity for differentiation of normal hemopoietic colony-forming cells. J. Cell. Physiol. 69: 177.

Wu, AM., Till, JE., Siminovitch, L., McCulloch, EA. (1968). Cytological evidence for a relationship between normal hematopoietic colony-forming cells and cells of the lymphoid system. J. Exp. Med. 127: 455.

Wysoki, LJ., Sato, VL. (1978). Panning for lymphocytes: A method for cell selection. Proc. Natl. Acad. Sci. (USA). 75: 2844.

Yamada, N., Miyata, T., Maeda, K., Kitani, T., Takeda, J., Kinoshita, TAD. (1995). Somatic mutations of the PIG-A gene found in Japanese patients with paroxysmal nocturnal hemoglobinuria. Blood. 85: 885.

Yamasaki, K., Taga, T., Hinta, Y., Yawata, H., Kawanishi, Y., Seed, B., Taniguchi, T. Hirano, T., Kishimoto, T. (1988). Cloning and expression of the human interleukin-6 (BSF 2/IFN β-2) receptor. Science. 241: 825.

Yanai, N., Sekine, C., Yagita, H., Obinata, M. (1994). Roles for very late activation antigen-4 stroma-dependent erythropoiesis. Blood. 83: 2844.

Yang, YC., Tsai, S., Wong, GG., Clark, SC. (1988). Interleukin-1 regulation of hematopoietic growth factor production by human stromal fibroblasts. J. Cell Physiol. 134: 292.

Yarden, Y., Kuary, W., Kuang, W-J., Yarg-Feng, T., Coussens, L., Munemitsu, S., Dull, T.J., Chen, E., Schlessinger, J., Franke, U., Ullrich, A. (1987) Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an identified ligand. EMBO. J. 11: 3341.

Yayon, A., Klagsbrun, M., Esko, JD., Leder, P., Ornitz, DM. (1991). Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell 64: 841.

Yoffey, JM., Yaffe, P. (1980). The phagocytic central reticular cell of the erythroblastic island in rat bone marrow. J. Recticuloendothel. Soc. 28: 37.

Yong, K., Khwaja, A. (1990). Leucocyte cellular adhesion molecules. Blood Rev. 4: 211.

Young, RA., Davis, RW. (1983). Efficient expression of influenza virus NS1 nonstructural proteins in Eschericia coli. Proc. Natl. Acad. Sci. (USA). 80: 6105.

Zanjani, ED., Flake, AW., Rice, H., Hedrick, M., Tavassoli, M. (1994). Long-term repopulating ability of xenogeneic transplanted human fetal liver hematopoietic stem cells in sheep. J. Clin. Invest. 93: 1051.

Zanjani, ED., Mackintosh, FR., Harrison, MR. (1991). Hematopoietic chimerism in sheep and nonhuman primates by in utero transplantation of fetal hematopoietic stem cells. Blood Cells. 17: 349.

Zanjani, ED., Pallavincini, MG., Ascensao, JL., Flake, AW., Langlois, RG. (1992). Engraftment and long-term expression of human fetal hematopoietic stem cells in sheep following transplantation in utero. J. Clin. Invest. 89: 1178.

Zannettino, ACW., Berndt, MC., Butcher, C., Butcher, EC., Vadas, MA., Simmons, PJ. (1995). Primitive human haemopoietic progenitors adhere to P-selectin (CD62P). Blood. 85: 3466.

Zannettino, ACW., Rayner, JR., Ashman, LK., Gonda, TJ., Simmons, PJ. (1996). A powerful new technique for isolating genes encoding cell surface antigens using retroviral expression cloning. J. Immunol. 156: 611.

Zaretsky, JZ., Weiss, M., Tsarfaty, I., Hareuveni, M., Wreschner, DH., Keydar, I. (1990). Expression of genes coding for pS2, c-erbB2, estrogen receptor and the H23 breast tumor-associated antigen. A comparative analysis in breast cancer. FEBS. Lett. 265: 46.

Zenke, M., Steinlein, P., Wagner, E., Cotten, M., Beung, H., Birnsteil, M.L. (1990). Receptor-mediated endocytosis of transferrin-polycatination conjugates: An efficient way to introduce DNA into haemopoietic cells. Proc. Natl. Acad. Sci. (USA). 87: 3655.

Zhou, DFH., Ding, JF., Picker, LJ., Bargatze, RF., Butcher, EC., Goeddel, DV. (1989). Molecular cloning and expression of Pgp-1. The mouse homolog of the human H-CAM (Hermes) lymphocyte homing receptor. J. Immunol. 143: 3309.

Zhou, Q., Moore, K., Smith, DF., Varki, A., McEver, RP., Cummings, RD. (1991). The selectin GMP-140 binds to sialyated, fucosylated lactosaminoglycans on both myeloid and nonmyeloid cells. J. Cell Biol. 115 (2): 557.

Zimmerman, GA., McIntyre, TM., Mehra, M., Prescott, SM. (1990). Endothelial cell-associated Platelet-activating factor: A novel mechanism for signaling intercellular adhesion. J. Cell Biol. 110: 529.

Zimmerman, U., Vienken, J. (1982). Electric field-induced cell-to-cell fusion. J. Memb. Biol. 67: 165.

Zipori, D., Toledo, J., von der Mark, K. (1985). Phenotypic heterogeneity among stromal cell lines from mouse bone marrow disclosed in their extracellular matrix composition and interactions with normal and leukemica cells. Blood. 66: 447.

Zsebo, KM., Williams, DA., Geissler, EN., Broudy, VC., Martin, FH., Atkins, HL., Hsu, RY., Birket, NC., Okino, KH., Murdock, DC., Jacobsen, FW., Langly, KE., Smith, KA., Takeishi, T., Cattancah, BM., Galli, SJ., Sugg,s SV. (1990). Stem cell factor is encoded at the SL locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. Cell. 63: 213.

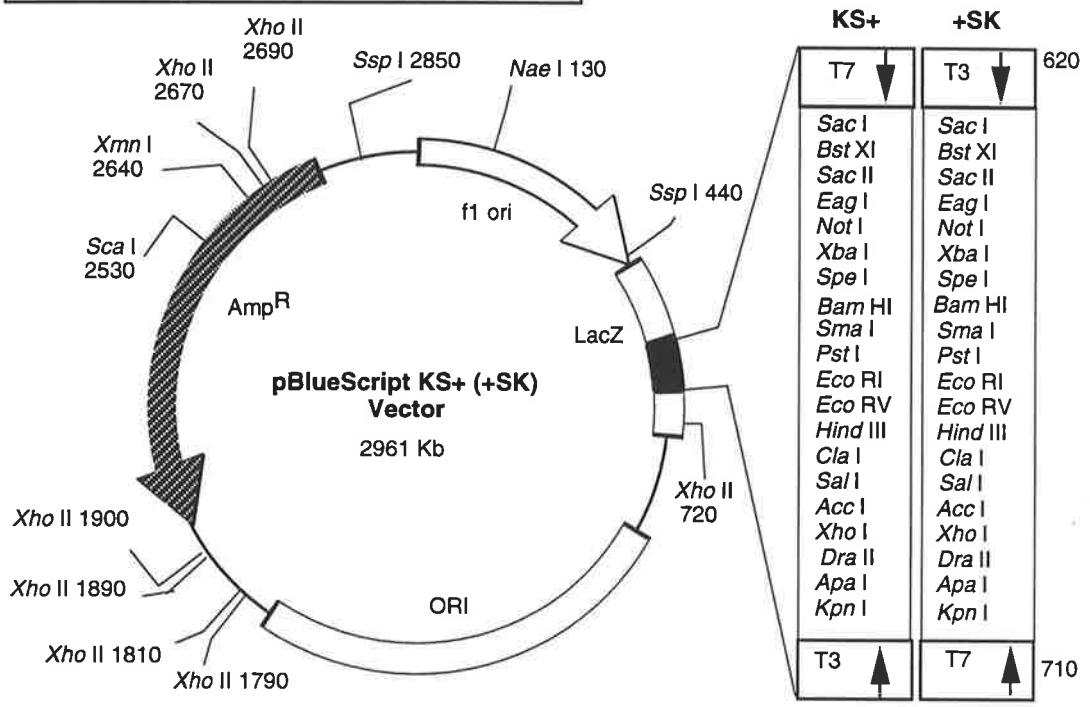
Zuckerman, KS., Rhodes, RK., Goodrum, DD., Patel, VR., Sparks, B., Wells, J., Wicha, MS., Mayo, LA. (1985). Inhibition of collagen deposition in the extracellular matrix prevents the establishment of a stroma supportive of hematopoiesis in long-term murine bone marrow cultures. J. Clin. Invest. 75: 970.

Zuckerman, KS., Wicha, MS. (1983). Extracellular matrix production by the adherent cells in long-term murine bone marrow cultures. Blood. 61: 540.

APPENDIX A

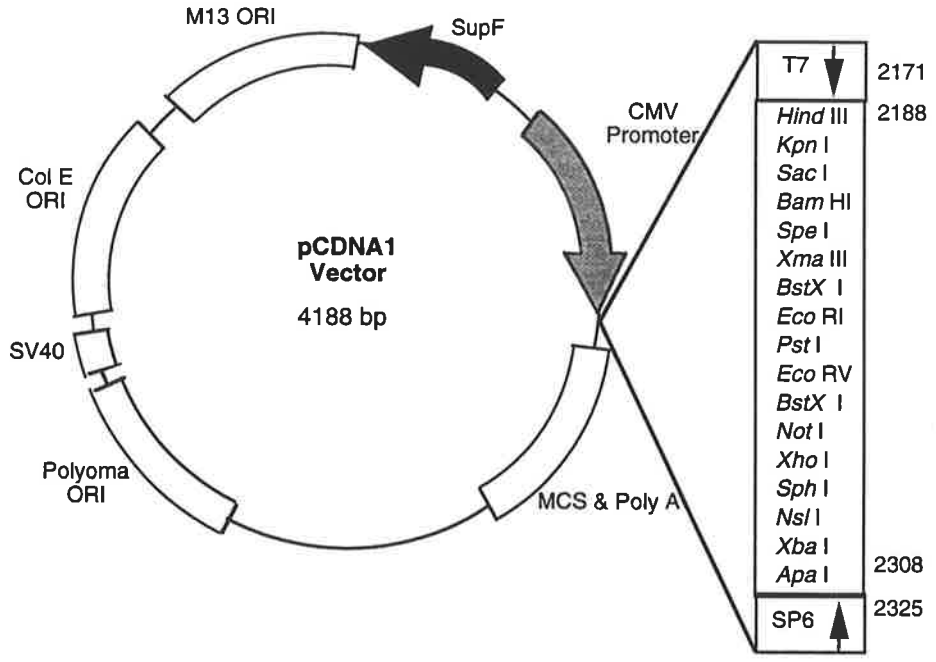
Plasmid name: pBlueScript KS+ and +SK
Plasmid size: 2961 bp

1



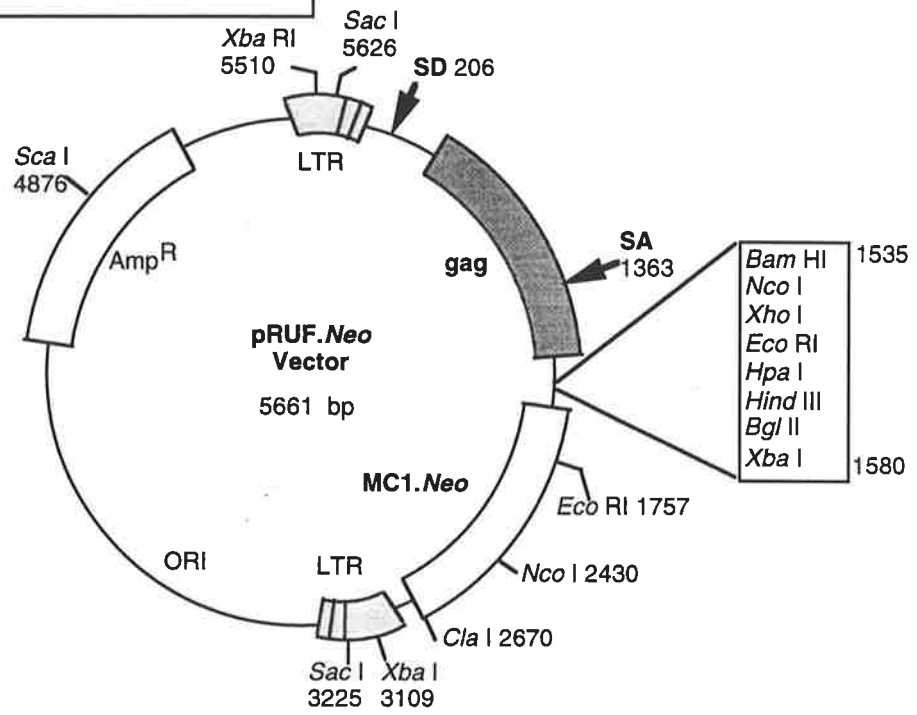
Plasmid name: pCDNA1 Vector
Plasmid size: 4188 bp

2



Plasmid name: pRUF.*Neo* Vector
Plasmid size: 5661 bp

3



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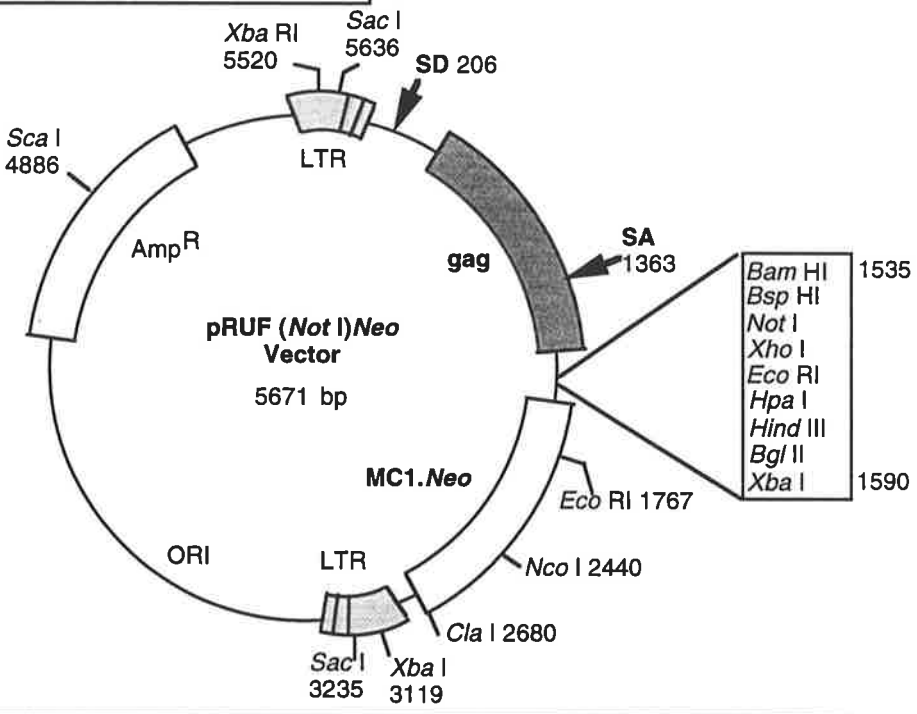
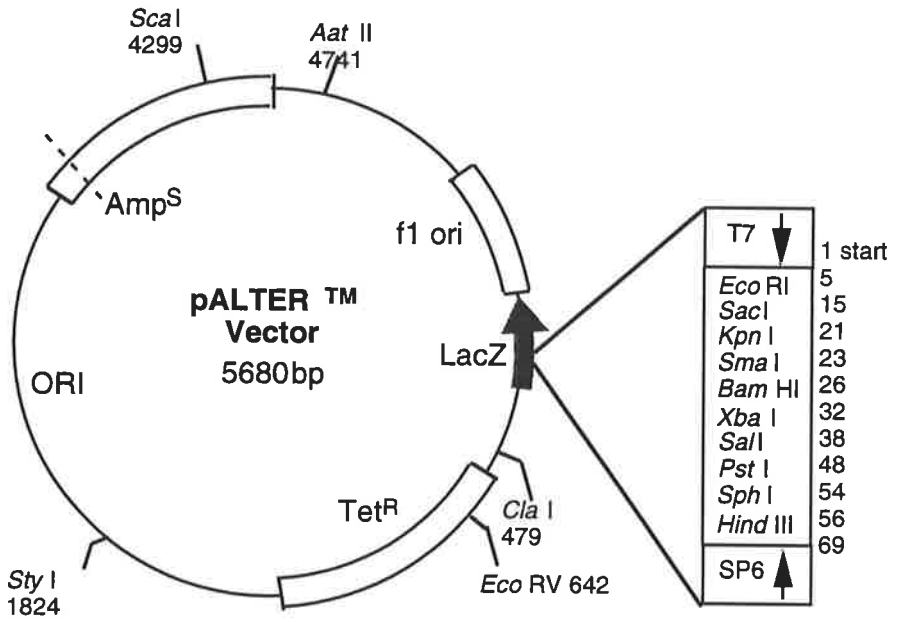
Plasmid size: 5671 bp

4



Plasmid name: pALTER ™ Vector
Plasmid size: 5680bp

5



Plasmid name: pGEM-T Vector
Plasmid size: 3003bp

6