ASSESSMENT OF CD44 AND K19 AS MARKERS

FOR CIRCULATING BREAST CANCER CELLS

USING IMMUNOBEAD RT-PCR

A Thesis Submitted for the Degree of

Doctor of Medicine

by

Michael Campbell Eaton, MBBS, FRACS

Department of Surgery,

The University of Adelaide.

Submitted for degree in March, 1997.

TABLE OF CONTENTS

Abstract

Declaration

Acknowledgments

Table of Figures

Table of Tables

Table of Abbreviations

ABSTRACT

PURPOSE:

Detection of occult metastases and circulating tumour cells in patients with breast cancer may prove useful in determining prognosis and in planning and monitoring systemic therapies. The identification of carcinoma cells in peripheral blood stem cell (PBSC) harvests may predict relapse after high dose chemotherapy with PBSC transplantation.

This work presents the development of an assay for reverse transcription-polymerase chain reaction (RT-PCR) to be applied to the immunomagnetic isolation of carcinoma cells, as a possible means of detecting small numbers of breast cancer cells in a haemopoietic environment. The messenger RNA expression of two different genes, CD44 and the cytokeratin K19, was assessed for suitability as tumour markers for the Immunobead RT-PCR method, and clinical results using K19 are presented.

METHODS AND RESULTS:

An assay for RT-PCR from very small numbers of cells was developed, using detergent lysis of cells for RNA extraction. Taking fresh tumour cells from a lymph node, it was demonstrated that single metastatic breast cancer cells could express more than one isoform of CD44. CD44 did not prove useful as a tumour marker for Immunobead RT-PCR in a haemopoietic environment, because the CD44 isoform expression of haemopoietic cells could not be distinguished from that of carcinoma cells. The expression of K19 was shown to be specific for epithelial cells, but occasionally K19 transcripts could be amplified from haemopoietic cells of normal individuals. Immunobead RT-PCR using K19 expression as the tumour marker allowed detection of one carcinoma cell amongst one million leukocytes in peripheral blood.

Circulating tumour cells were not detected in any samples of peripheral blood taken at random from patients with advanced breast cancer, nor in any PBSC samples. K19 transcripts were amplified from four samples of peripheral blood taken from patients at the time of PBSC harvest.

CONCLUSIONS:

Immunobead RT-PCR was shown to be a highly sensitive method of detecting breast cancer cells in a haemopoietic environment. Results using K19 as the marker should be interpreted with caution. Results suggested that the mobilisation of stem cells is accompanied by mobilisation of breast cancer cells into peripheral blood, but that circulating tumour cells may be excluded from stem cell harvests.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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

Michael Campbell Eaton

March, 1997.

ACKNOWLEDGMENTS

significance if Dr. Ke-Hui Kui, Ph.D., of the Department of Obstetrics and Gynaecology at The Queen Elizabeth Hospital, had not provided instruction in the use of the micropipette, which he had devised.

Basic laboratory solutions were prepared with the technical assistance of Ms. Jing-Xian Mi. Flow cytometry was performed under the direction of Ms. Belinda Farmer, B.Sc. Bone marrow and stem cell collection samples were kindly prepared by Mr. Mark Shephard, B.Sc.

Ms. Vivian Pascoe, of the Department of Histopathology, is gratefully acknowledged for her work in staining fresh and frozen breast cancer specimens with monoclonal antibodies. Photographs of slides of cytospin and frozen sections were kindly taken by Dr. Bob Dymock, Director of the Department of Histopathology.

Thanks are also due to Professor Guy Maddern of the University Department of Surgery, and Mr. Vlad Humeniuk, Senior Visiting Medical Specialist at The Queen Elizabeth Hospital, for their support.

This thesis presents the results of Immunobead RT-PCR for K19 on 107 separate clinical specimens, and thanks for these samples are largely due to Dr. Dusan Kotasek, MBBS, FRACP. Dr. Kotasek was a central figure in our team researching the detection of circulating cancer cells.

Finally, the assistance of Dr. Alexander Dobrovic must be restated. A project of similar interest and potential significance was not to be found anywhere else in The Queen Elizabeth Hospital in 1993, and now that he has instructed the first student from a surgical background in molecular biology at T.Q.E.H., other surgical students have been able to follow. I thank him for his tireless teaching and direction.

TABLE OF FIGURES

TABLE OF TABLES

TABLE OF ABBREVIATIONS

BC-2	Anti-mucin Monoclonal Antibody
cDNA	Complementary DNA
CD44s	"Standard form" of CD44
CD44e	"Epithelial form" of CD44
CEA	Carcinoembryonic Antigen
CML	Chronic Myeloid Leukaemia
DEPC water	Diethyl Pyrocarbonate-treated Water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EMA	Epithelial Membrane Antigen
FCS	Foetal Calf Serum
G-CSF	Granulocyte-Colony Stimulating Factor
GITC	Guanidinium isothiocyanate
GM-CSF	Granulocyte-macrophage Colony-stimulating Factor
HPW	Hi-Pure Water
MACS	Magnetic Activated Cell Sorter
MCF-7	MCF-7 Breast Cancer Cell Line
MB-453	MDA-MB-453 Breast Cancer Cell Line
MNCs	Mononuclear Cells
mRNA	Messenger RNA
NHL	Non-Hodgkin's Lymphoma
NP-40	Nonidet P-40
PB	Peripheral Blood

PBMNCs	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PBS	Phosphate-buffered Saline
PBSCs	Peripheral Blood Stem Cell Collections
P.E.	Phycoerythrin-conjugated Anti-mouse Immunoglobulin
PMA	Phorbol 12-myristate 13-acetate
PMNs	Polymorphonuclear Cells
PSA	Prostate-Specific Antigen
PSM	Prostate-Specific Membrane
RBC	Red blood Cells
RNA	Ribonucleic Acid
RT	Reverse Transcription
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
T-47D	T-47D Breast Cancer Cell Line
UICC	International Union Against Cancer

CHAPTER ONE:

INTRODUCTION AND REVIEW OF LITERATURE

1.1 BREAST CANCER

1.1.1 Epidemiology

Breast cancer is a major health problem, of great interest to health workers of many disciplines. It is the most common cancer in women in the Western World. In Australia, approximately one in 13 women is likely to develop breast cancer by the age of 74 years, and it has been attributed to the cause of one in 25 of all female deaths. The incidence of the disease is increasing slowly, and in 1990 there were more than 7000 new cases of breast cancer diagnosed in Australia, of which 2000 were in women under the age of 50 years (Coates, 1994; National Health and Medical Research Council, 1995).

1.1.2 Aetiology

The cause of breast cancer is unknown. Prolonged, unopposed oestrogen activity is established as a promoting agent. Associations of lesser strength are the implications of viral and dietary factors as promoting agents (Watt and Spence, 1986). While genetic abnormalities are a feature of malignancy, only 5 to 8% of breast cancers are thought to be hereditary, with a further 10% showing familial clustering without a clear mode of inheritance. The identification of two principal inherited breast cancer-predisposition genes has to date provided little additional information about their possible role in sporadic breast cancer (Hogervorst et al, 1995).

There is a high incidence of loss of heterozygosity at a number of sites in breast cancer, implicating tumour-suppressor genes in the initiation and/or progression of the disease. Breast cancer probably results from a series of molecular genetic events involving the activation of oncogenes and the

1

inactivation of tumour-suppressor genes. The progression from epithelial proliferation through in-situ malignancy to localised invasion and subsequent metastasis probably depends on accumulated genetic alterations, but may also reflect a failure of host defence mechanisms (Harris et al, 1992).

1.1.3   Classification

The major primary division in the classification of carcinomas of the breast is into ductal and lobular carcinomas, with further subdivision into in situ and invasive forms. The ductal and lobular types refer to histologically and cytologically identifiable entities, and not necessarily to the sites of origin. While the vast majority of cancers of "lobular" histological appearance arise in the lobule, some may arise in extralobular ducts. Most ductal carcinomas arise in the "terminal duct-lobular unit", but they may also originate in larger extralobular ductules and ducts (Azzopardi, 1979).

1.1.4   Staging

Staging refers to the classification of a patient's breast cancer according to the anatomical extent of the disease. The system in the most common clinical use currently is the TNM system (description of the primary tumour, the regional lymph nodes and the presence or absence of distant metastases), and has been adopted by the American Joint Committee for Cancer Staging and the International Union Against Cancer (UICC). A summary of this system is provided in Appendix 1 (Beahrs et al, 1988), but a brief working description is as follows:

Stage I   Tumour less than 2cm in size, without evidence of spread.

Stage II   Either: tumour up to 5cm in size, with metastases to movable ipsilateral lymph nodes

Or: tumour more than 5cm in size, without extension beyond the breast or to regional nodes.

2

Stage III   The two major groups within this stage are denoted by fixity of regional lymph nodes and/or local extension of tumour beyond the breast.

Stage IV   Distant metastases.

1.1.5   Treatment

The primary management of carcinoma of the breast can be divided in terms of local and systemic treatment. Local treatment focuses on the breast and, where appropriate, the regional lymph nodes. The main aim of local treatment is the eradication of local disease, with a secondary aim of obtaining information that might dictate adjuvant therapies. In contrast, systemic treatment attempts to eradicate micrometastatic (or occult) disease or palliate metastases.

There are three major factors regarding the management of breast cancer that have revolutionised approaches to clinical treatment and encouraged major interest in basic biological research into breast cancer in recent years.

Firstly, randomised clinical trials comparing breast-conserving treatments with radical surgery have shown no difference in survival between the two groups, demonstrating to clinicians that breast cancer becomes a systemic disease at an early stage in its biological course, and that radical local treatments do not therefore improve the chances of cure (Fisher, 1992).

Secondly, improvements in the technology of radiologic imaging of the breast have led to mass population screening for breast cancer, and the diagnosis and surgical removal of early cancers with a demonstrable improvement in survival (Nystrom et al, 1993).

Thirdly, a major advance has been the development of systemic therapy regimens that can improve disease outcomes. Systemic therapy, given at the

time of primary local treatment of breast cancer in the absence of demonstrable metastases, has been shown to be capable of prolonging disease-free survival and overall survival. In general, chemotherapy has been shown to have a greater effect in premenopausal patients, and hormonal manipulation, chiefly in the form of tamoxifen, has been shown to have a greater effect in post menopausal women (Harris et al, 1992). The demonstrated benefit of adjuvant systemic therapy has stimulated intense clinical and biological research to determine parameters for the prediction of occult metastatic disease so that adjuvant therapies can be prescribed in the most rational manner.

Involvement of regional lymph nodes by tumour is the single greatest predictor of distant disease, and consequently of the likelihood of benefit from adjuvant systemic therapy. For patients whose histological assessment shows no tumour involvement of the regional nodes, the clinician's attempts to predict distant disease are based almost entirely upon characteristics of the primary tumour. More sensitive clinical tools for assessing metastatic disease would be of major clinical interest in rationalising the administration of adjuvant systemic therapies.

The administration of chemotherapy in higher than usual doses with haemopoietic progenitor cell support is becoming accepted treatment in the adjuvant setting in many institutions, despite an absence of randomised clinical trials to demonstrate improved results over standard chemotherapy regimens. An assessment of this form of therapy with respect to the significance of circulating tumour cells forms an important part of this thesis.

Prognosis

Survival from breast cancer is influenced by many factors. In general terms, some Australian data show overall five year survival of 75%, and survival for selected sub-groups of over 90% (National Health and Medical

Research Council, 1995). As a generalisation, approximately 70% of node-negative patients have no further problems after surgical removal of their disease. It is assumed that the remaining 30% harbour metastatic disease that is undetected at the time of initial clinical assessment. Those with poor prognostic indices have an appalling outlook, with a 15 year survival of only around 10% (Galea et al, 1992).

1.2 PREVIOUS METHODS TO DETECT CIRCULATING TUMOUR CELLS

Until approximately 25 years ago, the paradigm underlying the management of breast cancer, and the majority of solid malignancies, was that attributed to William S. Halsted: that metastases occurred by centrifugal spread along anatomical lines, and that regional lymph nodes provided an effective barrier against further spread of tumour cells (Fisher, 1992). The results of clinical management according to the Halstedian paradigm were that surgery was aimed at the removal of all potential local disease to effect a greater chance of cure (Henderson, 1995), and that tumour cells shed into the bloodstream during surgical manipulation of a tumour would increase the likelihood of metastatic disease and hence decrease surgical cure rates (Fisher and Turnbull, 1955). This was associated with much research in the 1950s and 1960s into the question of shedding of tumour cells from primary tumours with and without surgical manipulation (Goldblatt and Nadel, 1965), commencing with the work of Fisher and Turnbull, who in 1955 published their results of cytological assessment of carcinoma cells in mesenteric venous blood of cases of colorectal cancer (Fisher and Turnbull, 1955).

By 1965 more than 5,000 patients had been studied for circulating tumour cells, with reports of 20 different techniques in more than 40 different laboratories (Goldblatt and Nadel, 1965). Taking into account the enormous variation in results from the studies, the large review by Goldblatt and Nadel summarised that the incidence of detection of cancer cells in peripheral venous blood of cancer patients was of the order of 20 to 30 per cent, and that the incidence of detection increased after surgical manipulation of the tumour (Goldblatt and Nadel, 1965).

The techniques to this stage relied on simple light microscopy as the means of detecting cancer cells in blood, with the only difference between the various techniques being in the methods of cancer cell concentration (for example, red cell sedimentation, red cell lysis, floatation, magnetic removal of leukocytes after ingestion of carbonyl iron, filtration, et cetera). The staining methods that were used to assist the cytological diagnosis of cells in blood were essentially non-specific (haematoxylin and eosin, Romanowsky, and Papanicolaou), and the morphologic criteria for identification of cancer cells were confused by the identification of megakaryocytes, endothelial cells, plasma cells, and erythroid, myeloid and lymphoid precursor cells. These cells are large, with high cytoplasmic ratio, dense nuclear chromatin, and multiple nucleoli, complicating their distinction from malignant cells on purely morphological grounds (Goldblatt and Nadel, 1965). The poor specificity for malignant cells in blood based on purely cytological criteria is demonstrated in a recent study which compared conventional cytology with anti-cytokeratin immunocytochemistry for the detection of colorectal cancer cells in venous blood. Circulating tumour cells were identified in only 9% of patients studied by immunocytochemistry compared with 23% of patients in the same study group using conventional cytology (Leather et al, 1993). This may explain the high rate of detection of apparently malignant cells reported in the early studies.

1.3 CURRENT METHODS TO DETECT CIRCULATING TUMOUR CELLS

The modern surgical paradigm governing the management and research of solid tumours has led to a new proliferation of attempts to identify tumour cells in blood. The new paradigm is based on the observation that blood

and lymphatic systems are not independent routes of neoplastic dissemination, and that the formation of metastases is not dictated by anatomical considerations but by factors intrinsic to the tumour cells and the organs to which they gain access (Fisher, 1992).

The acceptance by physicians of the systemic nature of breast cancer is confirmed by the proliferation of adjuvant systemic therapies for disease that appears to be locoregional only, and a dilemma facing clinicians involved in the management of breast and other cancers is which patients should be offered adjuvant systemic therapy (McGuire and Clark, 1992). The limitations of current clinical investigations in detecting small volume metastatic disease demand better techniques, and immunohistochemical and molecular biological techniques to detect minimal volume malignant disease have proliferated.

1.3.1   Immunocytochemistry

Immunohistochemical techniques are based on colour labelling of poly or monoclonal antibodies that have been raised against antigens of human epithelia and epithelial tumours (Edwards, 1985). The principal advantage in applying immunohistochemistry to the diagnosis of circulating tumour cells is that the uncertainty of diagnosing cancer cells purely on the basis of light microscopy morphology is countered by the specificity of a staining technique that can differentiate the target cells from background cells. The accuracy of immunohistochemistry depends on the specificity of the antibody, and in the detection of minimal disease cross-reactivity with any other cells will produce misleading results.

Markers for breast cancer may lack specificity

The tissue-specific antigens thyroglobulin, prostate specific antigen and prostatic acid phosphatase, are examples of markers that have been demonstrated to be reliable detectors of metastatic thyroid or prostate

carcinoma, respectively. The search for a similarly specific antibody for breast tissue or breast neoplasia has been less successful (Almeida and Pestana, 1992).

Early reports of detection of breast cancer micrometastases using immunohistochemical markers failed to adequately define the specificity and sensitivity of the marker by assessing control samples (Thor et al, 1988). A good example of this was the experience using antibodies raised against an antigen in defatted human cream, that was subsequently termed the epithelial membrane antigen (EMA) (Cordell et al, 1985). Studies had suggested that the recognition of breast cancer micrometastases could be significantly increased by using anti-EMA antisera (Sloane et al, 1980; Redding et al, 1983). The likelihood that many of these results may have been false positives has been exposed by subsequent papers that have demonstrated poor specificity of anti-EMA antibodies for detection of cells of mammary origin. Delsol and colleagues used three different monoclonal anti-EMA antibodies to show that EMA was also expressed on plasma cells, some non-Hodgkin's lymphomas, on Reed-Sternberg cells, and could even be induced on normal blood lymphocytes by lymphocyte activators (Delsol et al, 1984).

The same group discussed the applicability of their anti-EMA monoclonal antibody E29 as a marker for diagnosis of epithelial neoplasia, and claimed that haematologic malignancies stained by E29 could be distinguished from epithelial neoplasia on purely morphologic grounds (Cordell et al, 1985). In terms of the application of an anti-EMA antibody to the diagnosis of circulating carcinoma cells however, normal plasma cells could be stained with sufficient frequency to lead to false positive diagnoses of carcinoma cells in blood. When studying bone marrow specimens with and without breast cancer micrometastases using anti-EMA antibody Thor and colleagues were unable to confidently distinguish breast cancer cells in bone marrow from benign

precursor cells, even when using adding the usual criteria for malignancy of large nuclear size, nuclear pleomorphism, and prominent nucleoli (Thor et al, 1988).

This is an important illustration that some normal cells in the haemopoietic compartment may have cytological features of malignancy, and if carcinoma cells are going to be differentiated from haemopoietic cells on the basis of their staining, then the specificity of the antibodies needs to be demonstrated by adequate controls.

Antibody specificity for breast cancer cells

Antibodies reactive to breast cancer can be classified according to the immunogens against which the polyclonal or monoclonal antibody is raised. This separates them into five categories (Thor et al, 1988):

1) membrane-enriched extracts of breast cancers

2) milk fat globule membranes

3) mammary carcinoma cell lines

4) intermediate filaments

5) surface receptors.

The anti-EMA antibody fits into the second category, and is an example of an antibody raised against a mammary extract immunogen whose expression was subsequently demonstrated on non-mammary cells.

Diel's group developed a new monoclonal antibody against the tumour-associated glycoprotein TAG12. They claim that their antibody does not react with mesothelial or mesenchymal tissues, and tested 21 negative controls. The authors concede, however, that they found cross-reactivity with basophilic myelocytes and monocytes in a few cases (Diel et al, 1992).

Anti-mucin monoclonal antibodies (including BC-2, from I.F.C. McKenzie) were used by Hainsworth and colleagues in their study of occult

metastases in lymph nodes (Hainsworth et al, 1993). The mucins are also not exclusive to breast cancer. Negative controls were not addressed by the authors, and they concede that macrophages were "occasionally" stained by their antibodies (Hainsworth et al, 1993).

The most convincing antibodies for detection of breast cancer cells have been directed against the cytokeratin intermediate filaments (Brugger et al, 1994; Cote et al, 1988; Cote et al, 1991; Schlimok et al, 1987; Ross et al, 1993). The specificity is limited to epithelial cells, and the claimed non-reactivity with haemopoietic or mesenchymal cells is supported by the 75 and 50 negative controls respectively of Schlimok and Brugger (Brugger et al, 1994; Schlimok et al, 1987).

Most antibodies show heterogeneous staining of breast cancers

Edwards has stated that the majority of monoclonal antibodies that have been raised to human epithelial tumours bind to only some of the cells in a tumour, showing that tumour cells are very heterogeneous in their expression of antigens (Edwards, 1985). This feature of epithelial cells would predispose to an artificially low rate of detection of epithelial cells in a haemopoietic medium, and, in conjunction with the problems of specificity discussed above, argues for the use of more than one antibody in immunocytochemical studies of breast cancer cells. Edwards (1985) does, however, concede that "there must be some surface molecules present on all the cells of an epithelium that ought to be homogeneously expressed, epithelium-specific antigens".

Of the recent reports of immunohistochemical detection of breast cancer cells in lymph nodes, blood or bone marrow, most groups have used panels of monoclonal antibodies (Mascarel et al, 1992; Cote et al, 1991; Hainsworth et al, 1993; Ross et al, 1993; Brugger et al, 1994). Diel and colleagues used a single

11

antibody directed against an epithelial mucin in their large series of bone marrow biopsies from women with operable breast cancer (Diel et al, 1992).

Most methods concentrate the leukocyte fraction to allow screening of larger volumes

To detect small numbers of carcinoma cells in a haemopoietic medium by simply smearing the medium over microscopy slides would require examination of many slides. One drop of blood would require one slide, and the large numbers of red blood cells could easily obscure the few carcinoma cells that might be present.

Examining larger volumes increases the likelihood of detecting tumour cells in a haemopoietic environment. In their study of tumour cells in bone marrow of primary breast cancer patients, Diel and colleagues took volumes of bone marrow totalling 60ml from each patient (under general anaesthesia). They concentrated the leukocytes by Ficoll-Hypaque density gradient separation, and took their smears from the interface. At least 10 slides were examined for each patient (Diel et al, 1992). Other papers reporting the use of Ficoll-Hypaque separation come from Ross (3-5ml of bone marrow or peripheral blood stem cell harvest), Brugger (peripheral blood, volume not stated), Cote (2-3ml of bone marrow), and Schlimok (6ml of bone marrow) (Diel et al, 1992; Brugger et al, 1994; Ross et al, 1993; Cote et al, 1988; Schlimok et al, 1987). The use of Ficoll-Hypaque density separation relies on the assumption that any carcinoma cells that might be present will separate into the interface layer with the mononuclear cells, and not travel through the Ficoll with the red cells. Cote and colleagues claim that this is the case (Cote et al, 1991).

Sensitivity of detection of tumour cells by immunocytochemistry

To provide a measure of the relative sensitivities of methods for detection of circulating tumour cells, most workers express their results in terms of the number of mononuclear cells required per sample for the detection of one tumour cell. Immunocytochemical methods report sensitivities of the order of one tumour cell in 5×10^4 mononuclear cells (Cote et al, 1988) to 4×10^5 (Brugger et al, 1994) or 5×10^5 (Ross et al, 1993).

The likelihood of detection of the occasional tumour cell in a haemopoietic medium, of course, finally depends upon the volume screened, and the number of slides examined. While Diel and colleagues do not specifically record the sensitivity of their method, they routinely sample approximately 60ml of bone marrow from selected breast cancer patients, under general anaesthesia at the time of surgery (Diel et al, 1992). They state that patients suffer minimal additional morbidity from the bone marrow sampling, and advocate bone marrow biopsy in place of surgical clearance of the axilla in selected cases as the more efficient means of establishing prognosis and planning adjuvant therapies (I.J. Diel, personal communication).

Finally, it must be stated that the overwhelming advantages of immunocytochemical techniques in the identification of tumour cells are that the target cells are visually identified, and may be counted, so that the tumour burden may be quantified.

1.3.2 The Polymerase Chain Reaction (PCR)

The development of the polymerase chain reaction (PCR) and its ability to amplify a specific region of DNA between defined oligonucleotide primers has made an enormous impact upon analysis of nucleic acids. One of the many benefits of PCR technology has been the development of sensitive new assays for minimal residual disease after treatment for haematological malignancies.

13

Several DNA mutations that are consistent and specific have allowed the application of PCR amplification for disease diagnosis in a sensitive and reliable manner.

Cytogenetic abnormalities have allowed application of PCR to haematological malignancies

Examples of these consistent molecular abnormalities are the t(14;18)(q32;q21) chromosomal translocation seen in some lymphomas, and the t(9;22)(q34;q11) in chronic myeloid leukaemia (CML) (Johnson et al, 1995). The t(14;18) translocation is present in 50% of patients with non-Hodgkin's lymphoma, and results in the joining region of the immunoglobulin heavy chain gene being juxtaposed to the *bcl* -2 apoptosis-suppressing gene. This hybrid *bcl* -2/joining region sequence is therefore unique to that clone of lymphoma cells, and since the clusters of break points occur over a short span of base pairs, the translocation is well-suited to PCR amplification from the genomic DNA of lymphoma cells carrying the marker (Schilder, 1995).

The identification of the BCR and ABL genes on either side of the t(9;22) translocation in CML permits reliable detection of cells carrying this translocation by amplification of the specific RNA after reverse transcription (RT-PCR), and has been used for monitoring patients with CML after treatment (Johnson et al, 1995). These molecular techniques allow detection of malignant cells at a level of one cell in one million normal cells (Johnson et al, 1995; Schilder, 1995).

PCR alone is of limited use in detection of cells from solid tumours

Mutations in oncogenes and tumour suppressor genes provide genetic markers that may be studied by PCR and which are specific for neoplasia. These mutations however, occur relatively infrequently within solid tumours, and

14

have different sites of mutation that make localisation for study by PCR difficult.

The K-ras gene has provided one the few useful genetic markers for carcinomas. Mutations in codon 12 of the K-ras gene are seen in up to 90% of cases of adenocarcinoma of the pancreas, and up to 50% of cases of colorectal carcinoma, and can be readily detected by PCR techniques (Tada et al, 1993; Hardingham et al, 1993). In their study of circulating tumour cells in patients with colorectal cancer, Hardingham and colleagues found the K-ras mutation in only 27 primary tumours out of nearly 100 consecutive patients presenting to their institution, which limited the clinical application of their PCR-based technique (Hardingham et al, 1993).

Mutations in other oncogenes or tumour suppressor genes have failed to be of use in PCR screening for solid tumours because they do not occur at such well defined points as in the K-ras gene. For example, sporadic mutations occur in the tumour suppressor gene p53 in approximately one third of breast cancers (Morrison, 1994), but the labour required to target these mutations by PCR would be excessive, since the mutations are found throughout the open reading frame of the p53 gene, and even the "hot spot" regions of mutation extend over four exons (Thompson, 1993; Coles et al, 1992; Johnson et al, 1995).

None of the remaining oncogenes or tumour suppressor genes that have been characterised in sporadic breast cancers (e.g. retinoblastoma gene, nm23, c-myc or ras) have shown sufficient frequency to indicate potential as PCR markers for circulating breast cancer cells (Porter-Jordan and Lippman, 1994).

1.3.3 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR is a sensitive and rapid method for analysis of gene transcripts. The method involves the isolation of RNA from tissues or cells so that it may be

used as a template for reverse transcription to complimentary DNA (cDNA). The cDNA in turn is used as the template for PCR, using primers designed to amplify a selected cDNA region. The amplified cDNA is identified by the size of its PCR product on gel electrophoresis.

Tissue-specific markers for RT-PCR analysis

The absence of common consistent chromosomal abnormalities in solid tumours has led to the search for other targets. There have now been multiple publications of methods for the detection of circulating tumour cells using RT-PCR detection of tissue-specific antigens or enzymes.

In 1991 Smith and colleagues published their method for detection of melanoma cells in blood based on amplification of transcription of the gene for **tyrosinase**, an important enzyme in melanin biosynthesis (Smith et al, 1991). They used reverse transcriptase to prepare complementary DNA (cDNA) from peripheral blood messenger RNA (mRNA) and then targeted the gene for tyrosinase, whose expression should be specific for cells producing melanin. The authors predicted that cells expressing tyrosinase would not normally be present in peripheral blood, so that amplification of the tyrosinase signal should indicate the presence of melanoma cells. Nested PCR was required to reach a level of sensitivity of detection of one tumour cell in 10^5 normal cells in whole blood, and found tyrosinase expression in the peripheral blood of four of seven patients with malignant melanoma, and in none of the eight negative control patients (Smith et al, 1991).

The **neuroendocrine protein gene product 9.5** (PGP 9.5) has been reported to show expression specific to cells of neural crest origin, and was used in an RT-PCR assay to detect neuroblastoma cells in blood at a level of sensitivity of one in 10^7 normal peripheral blood mononuclear cells (MNCs) (Mattano et al, 1992). The authors did concede that analysis by Southern

blotting did detect low levels of transcription of PGP 9.5 in MNCs, but claimed it did not lead to false positive results when applied to peripheral blood (Mattano et al, 1992). Further investigation has discounted the usefulness of PGP 9.5 as a tumour marker due to the expression in haemopoietic cells (Johnson et al, 1995).

Prostate carcinoma cells have been targeted in blood using RT-PCR for the expression of **prostate-specific antigen** (PSA) and **prostate-specific membrane** (PSM) mRNA (Moreno et al, 1992; Deguchi et al, 1993; Israeli et al, 1994). Both markers are claimed to be specific for prostate cells, with the RT-PCR assays providing a sensitivity of detection of one prostate cell in at least one million normal blood cells (Israeli et al, 1994). When nested PCR was applied to the detection of cells by PSM, four out of 40 patient negative controls gave "false positive" results. Two of these "negative controls" were claimed to actually have prostate cancer, leaving two cases without prostatic disease from whose peripheral blood PSM mRNA could be amplified (Israeli et al, 1994).

Carcinoembryonic antigen (CEA) has been investigated as an RT-PCR marker for gastrointestinal and breast cancer, using a nested PCR with a sensitivity of detection of approximately one tumour cell (from a colorectal cancer cell line) in 10^7 normal bone marrow cells (Gerhard et al, 1994). While this study focussed on the gastrointestinal malignancies, four out of six bone marrow aspirates from breast cancer patients were positive for CEA by RT-PCR. The stage of disease and volume of marrow studied were not recorded by the authors. Fifty six control samples from healthy donors and patients with conditions other than carcinomas were negative for CEA expression. This demonstrates specificity superior to other RT-PCR reports, given that the sensitivity of this method was enhanced by both nested PCR and Southern blotting, and that the specificity of the PCR was sufficient to account for the

homology seen in CEA genes expressed in myeloid cells (Gerhard et al, 1994). The relative degrees of expression of CEA by breast cancers compared to colorectal cancers was not addressed by the authors, and is an issue that would require further investigation.

A marker that shows some potential for application to breast cancer cells is MUC1, a core protein of **polymorphic epithelial mucin** (Noguchi et al, 1994). An RT-PCR assay for amplification of MUC1 mRNA has been used to detect breast cancer cells in axillary lymph nodes with a sensitivity of 1 breast cancer cell in 10^6 normal lymph node cells, and an increased rate of detection over immunohistochemical techniques (Noguchi et al, 1994). The expression of MUC1 is not confined to breast tissue however, but none was detected in seven control axillary nodes. It is worth noting that the authors' method differed from most in that neither nested PCR nor Southern blotting and autoradiography were used to enhance sensitivity. The applicability of MUC1 RT-PCR to detection of breast cancer cells in peripheral blood was not discussed (Noguchi et al, 1994).

The DF3 antigen is another member of the family of polymorphic epithelial mucins. Brown and colleagues used RT-PCR amplification of DF3 expression to detect MCF-7 cells in normal blood at a sensitivity of one cell amongst approximately 10^6 normal cells, and found no non-specific expression of the DF3 antigen in normal controls (Brown et al, 1995).

The cytokeratins, particularly cytokeratins 8, 18, 19 and 20 have been studied as RT-PCR markers for epithelial cells (and hence carcinoma cells) in peripheral blood, with very recent controversy regarding the specificity of cytokeratin 19 in RT-PCR studies (Datta et al, 1994; Traweek et al, 1993; Burchill et al, 1995; Krismann et al, 1995). The cytokeratins will be discussed in greater detail later.

Specificity of RT-PCR methods

The results from PCR-based methods for detecting circulating tumour cells appear as bands on gel electrophoresis, and claims of cell detection cannot be supported by a visual image of a cell that results from the immunocytochemical methods. The crucial issue with the PCR-based methods is therefore not the sensitivity of each individual method, but the specificity.

In all the reported RT-PCR assays, total RNA is extracted from an entire sample for reverse transcription to cDNA. The PCR then aims to amplify a rare transcript amongst a host of other transcripts, much like trying to find a needle in a haystack. The power of the PCR allows the "needle" or rare transcript to be detected, but this power also has the potential to amplify cell-specific transcripts that may be expressed at very low levels by non-specific cells. Any RT-PCR signal, however weak, which might arise from non-specific cells, would therefore lead to false-positive results of tumour cell detection. Low-level transcription of tissue-specific genes in non-specific cells has been demonstrated by Chelly and colleagues (Chelly et al, 1989), and is known as "illegitimate" or "ectopic" transcription (Kaplan et al, 1992).

False-positive signals from RT-PCR assays have been amplified from haemopoietic cells of healthy controls using the following markers:

1) Tyrosinase (Smith et al, 1991).

2) PGP 9.5 (Mattano et al, 1992).

3) PSM (Israeli et al, 1994).

4) Cytokeratin 19 (Burchill et al, 1995; Schoenfeld et al, 1994).

Several groups have explained their occasional non-specific results on the basis of illegitimate transcription (Smith et al, 1991; Datta et al, 1994; Brown et al, 1995), and have demonstrated that the incidence of non-specific signals increases when nested PCR is used to enhance assay sensitivity (Smith et al,

1991; Schoenfeld et al, 1994). The means of enhancement of PCR sensitivity are important considerations in determining the reliability of a PCR method, and are summarised in the table below.

Author	Cancer	Marker	PCR method	Autoradiography
Noguchi et al.	breast	MUC1	single round	-
Datta et al.	breast	K19	nested PCR	-
Brown et al.	breast	DF3	single round	+
Burchill et al.	breast/colon	cytokeratins	single round	+
Gerhard et al.	colon/breast	CEA	nested PCR	+
Smith et al.	melanoma	tyrosinase	nested PCR	-
Mattano et al.	neuroblastoma	PGP 9.5	single round	+
Moreno et al.	prostate	PSA	single round	-
Israeli et al.	prostate	PSA/PSM	nested PCR	+
Krismann et al.	lung	cytokeratins	nested PCR	-

Table 1.1:   Summary of markers and the PCR methods. Note that Moreno et al. used PCR product concentration to enhance sensitivity (Moreno et al, 1992).

1.3.4   Flow cytometry

The use of flow cytometry for rare-event detection is limited by the sophistication and cost of the equipment required, and by technical factors such as non-specific staining and autofluorescence. A flow cytometric model has been reported in which cells from a breast cancer cell line were characterised by a cocktail of three different anti-cytokeratin antibodies, each tagged with a colour dye. The non-target haemopoietic cells were tagged with a fourth antibody cocktail which acted as an exclusion colour. The sensitivity of detection in this model was as few as one cancer cell in 10^7 peripheral blood mononuclear cells, but for this level of sensitivity approximately 200ml of blood or 2-20ml of bone marrow was required (Gross et al, 1995).

20

1.3.5 Immunomagnetic cell isolation techniques

Cell lines have been labelled with antibody bound to 50-150nm diameter microbeads and then isolated by a magnetic activated cell separator (MACS). The isolated tumour cells are then identified by RT-PCR or flow cytometry (Mass et al, 1995; Wong et al, 1995). Again, the cost of equipment is high, and technical considerations of the tumour cell identification techniques still apply.

Hardingham and colleagues used antibody-labelled 50μm diameter immunomagnetic beads (Dynal) and simple magnets to isolate cells from the colon cancer cell line SW480 from peripheral blood. Their method of identification of the cancer cells involved a PCR assay to detect the codon 12 mutation in the K-ras gene, and claimed a sensitivity of detection of one SW480 cell amongst 10^5 leukocytes in whole blood (Hardingham et al, 1993). The method has the advantage of being able to screen a relatively large volume of blood, at relatively low cost and minimal technical effort. The disadvantage again lies in the method of tumour cell identification, in that the patient's primary tumour must first test positive for the presence of the codon 12 K-ras mutation before the blood can be screened.

This technique of immunomagnetic cell isolation is the basis of the work in this thesis.

1.3.6 Clonogenic assay

Ross and colleagues developed an assay in which mononuclear cell fractions of fresh or rapidly thawed cryopreserved PBSC and bone marrow specimens from breast cancer patients were plated into culture media and incubated for 14 days. Growth of colonies of breast cancer cells was identified in 21 out of 26 specimens which had been positive for breast cancer cells by

immunocytochemical techniques, thereby confirming that breast cancer cells in fresh and thawed PBSC and bone marrow specimens were viable and capable of clonogenic growth in vitro. The authors suggested that immunocytochemical and clonogenic assay techniques should be considered complementary in the detection of low numbers of tumour cells (Ross et al, 1993).

Technique	Sensitivity	Limitations
Immunocytochemistry	1 in 10^5	Labour-intensive
RT-PCR	1 in 10^6	Marker specificity
MACS	1 in 10^7	Marker specificity; cost
Immunobead-PCR	1 in 10^6	Marker frequecy
Clonogenic culture	-	Technical difficulty

Table 1.2:   Overview of techniques for detection of circulating breast cancer cells.

1.4   CLINICAL RESULTS OF DETECTION OF CIRCULATING TUMOUR CELLS

1.4.1   Bone marrow

The group of breast cancer patients in whom enhanced detection of malignant cells in bone marrow is of clinical interest is that group of patients with primary, operable infiltrating breast carcinoma without clinical evidence of metastatic spread beyond the axilla. Assessment of the "true" incidence of bone marrow micrometastases in these patients should come from work whose controls for the specificity of the results are the most convincing. For the reasons discussed above, most attention here will therefore centre on the results of Cote et al and Diel et al (Cote et al, 1988; Cote et al, 1991; Diel et al, 1992).

The initial study of Cote and colleagues found breast cancer cells in the bone marrow in 18 of 51 patients (35%) with operable breast cancer (no evidence of metastatic disease by conventional staging). Breast cancer cells were found in 6 of 22 (27%) with histologically negative axillary lymph nodes. In

22

none of these samples were breast cancer cells detected by routine histological assessment (Cote et al, 1988).

Follow-up results were consistent with those claimed in 1988 (Cote et al, 1991). After just over 2 years of review, patients who had marrow aspirates that were immunocytochemically positive for breast cancer had a significantly higher rate of relapse (33%) compared to patients with negative bone marrow aspirates (3%).

TNM stage	Number of cases	Number positive
I	13	3 (23%)
II	34	13 (38%)
III	4	2 (50%)

Table 1.3:   Incidence of occult breast cancer cells in bone marrow according to stage (Cote et al, 1988).

The corresponding figures for Diel's group were tabled according to tumour size and nodal status rather than overall clinical stage. Again, none of these bone marrow biopsies had been positive for breast cancer cells by routine cytology. After a minimum follow-up period of 12 months, distant metastases had become clinically apparent in 26 patients, 22 of whom had tumour cells detected in their bone marrow (Diel et al, 1992).

	Number of cases	Number positive
Tumour T1/2	211	79 (38%)
Tumour T3/4	49	36 (73%)
Node Negative	133	40 (30%)
Node Positive	127	74 (58%)

Table 1.4:   Incidence of occult breast cancer in bone marrow according to tumour size and lymph node status (Diel et al, 1992).

There is a paucity of data available of the incidence of breast cancer cells in bone marrow using other methods of detection. Of the RT-PCR publications, only Datta and colleagues have studied bone marrow. Eight samples were tested by RT-PCR for K19, all from stage IV patients, of whom six were positive for K19 transcripts (Datta et al, 1994).

1.4.2 Peripheral Blood

Assessment of an anticipated incidence of breast cancer cells circulating in the peripheral blood is far less reliable than for bone marrow, where the methods of the two investigating teams discussed above appear sound and thorough. There is a dearth of recent data concerning circulating tumour cells despite the plethora of technical publications. Only two groups have reported detection of breast cancer cells in peripheral blood by immunocytochemical techniques, while Datta and colleagues reported the first detection of circulating breast cancer cells using RT-PCR, with a smaller series of results subsequently published by Brown (Brugger et al, 1994; Passos-Coelho et al, 1995; Datta et al, 1994; Brown et al, 1995).

In a study remarkable for its high incidence of detection of breast cancer cells, Brugger and colleagues reported detection of tumour cells in peripheral blood in two of seven stage IV breast cancer patients, and none of two stage II and III patients, in the absence of systemic treatment. Remarkably, after mobilisation of peripheral blood progenitor cells by chemotherapy and G-CSF (granulocyte colony stimulating factor), breast cancer cells were detected in the peripheral blood of all seven stage IV patients, while blood samples from the two patients of lesser stage remained free of tumour cells (Brugger et al, 1994).

Another recent immunocytochemical study showed a lower incidence of detection of breast cancer in peripheral blood: only one out of 23 stage IIIB or

IV breast cancer patients had tumour cells detectable in 5ml of blood (Passos-Coelho et al, 1995).

Datta and colleagues found circulating breast cancer cells stage IV patients only (four out of a total of 19 patients). In a study of blood samples from a series of 12 patients undergoing surgery for primary breast cancer, Brown and colleagues found tumour cells in one patient pre-operatively, four intra-operatively (two grade II, two grade III), and none 24 hours post-operatively (Brown et al, 1995).

With limited data, one is drawn to the conclusion that the likelihood of detection of breast cancer cells in peripheral blood is low, and that detection would correlate with disease of advanced stage. This conclusion is supported by results of studies of other cancers. An initially promising method of detecting circulating melanoma cells found tumour cells in peripheral blood of only three out of 22 patients with metastatic melanoma (Johnson et al, 1995). The incidence of circulating tumour cells in colorectal cancer patients appears higher than for breast cancer patients (Leather et al, 1993), and detection correlates strongly with poor survival (Hardingham et al, 1995).

1.4.3 Peripheral blood stem cell collections

The rationale for high-dose chemotherapy with haemopoietic progenitor cell support

In recent years the treatment of malignant disease with high doses of chemotherapy supported by autologous stem cells has become an established part of oncological practice. This approach is based on the belief that chemotherapy administered at higher and more frequent doses is more likely to destroy the tumour cells that might remain after administration of conventional doses and therefore lead to treatment failure (Barge and Reiffers, 1995).

25

The main factor limiting the treatment doses is haematological toxicity, and haemopoietic support has enabled more rapid recovery of myeloid function, so that the more aggressive therapies can be tolerated by patients. High dose chemotherapy followed by autologous bone marrow transplantation has become the treatment of choice for some patients with acute myeloid leukaemia and non-Hodgkin's lymphoma, but a limitation of the bone marrow transplants is the possibility that clonogenic tumour cells might contaminate the marrow and precipitate relapse when transplanted into the immunocompromised patient (Shpall et al, 1992).

PBSC support has advantages over bone marrow transplantation

An alternative source of haemopoietic stem cells is the peripheral blood. Collections of peripheral blood stem cells (PBSCs) have become increasingly preferred to bone marrow as a means of haemopoietic support, mainly because of the ease of collection combined with good yield of progenitor cells, and, perhaps most importantly, because the incidence of tumour cell contamination of PBSC harvests appears to be less than that of bone marrow harvests in the few solid and haematological malignancies in which this has been studied.

PBSCs are mobilised by chemotherapy and haemopoietic growth factors

In order to harvest enough stem cells from peripheral blood to enable successful transplantation, leukapheresis machines have been devised to remove and concentrate the mononuclear cell fraction (which contains the stem cells) from the peripheral blood, while recycling the remaining blood components (red cells, granulocytes and platelets) back to the patient. In the steady state, a minimum of six leukapheresis procedures is required to harvest enough stem cells to regenerate haemopoiesis comparable to that of bone

marrow (Barge and Reiffers, 1995). The use of chemotherapy (commonly cyclophosphamide) and haemopoietic growth factors (e.g. granulocyte-macrophage colony-stimulating factor, GM-CSF, and G-CSF) to mobilise stem cells from the marrow to the peripheral blood has enhanced the availability of stem cells in the blood to such a degree that the number of leukaphereses required has been drastically reduced, and PBSC engraftments have become more reliable (Barge and Reiffers, 1995).

Do PBSCs contain breast cancer cells, and can tumour cell contamination of PBSCs precipitate relapse?

The presence of contaminating tumour cells in the haemopoietic progenitor cell fractions that are transplanted to the immunosuppressed patient is perhaps the greatest theoretical limitation to high dose therapy with autologous haemopoietic support, and provides an important question to be answered by sensitive methods of tumour cell detection in patients with breast cancer. If it can be established that PBSC collections carry viable tumour cells, then the next question is whether the reinfusion of tumour cells into the patient's bloodstream is a cause of disease relapse.

Some information towards addressing this question comes from studies of residual disease in non-Hodgkin's lymphoma (NHL). There is agreement from studies using sensitive PCR techniques that the presence of small numbers of residual lymphoma cells in bone marrow predicts relapse (Gribben et al, 1993; Hardingham et al, 1995), and data suggest that reinfusion of marrow containing tumour cells is an important cause of therapeutic failure after high-dose therapy and autologous bone marrow transplantation (Shpall and Jones, 1994). The significance of tumour cell contamination in PBSC collections in lymphoma is less clear. Hardingham and co-workers were able to detect tumour cells in the PBSC collections of nearly 50% of their 24 lymphoma

patients receiving high-dose chemotherapy and PBSC transplantation, but were unable to show that this detection conferred any significant effect on outcome (Hardingham et al, 1995). Perhaps detecting tumour cells in the peripheral blood does not carry the same significance in terms of relapse as does detecting cells in bone marrow, because the cells in the periphery may be dead, in the process of dying, or may not have proliferative potential (Schilder, 1995).

Breast cancer cells can contaminate PBSC collections

Despite the growing acceptance of high-dose chemotherapy with PBSC support for the management of patients with poor prognosis breast cancer, the incidence of tumour cell contamination of PBSC collections has not been widely studied.

The work of Ross and colleagues supports the contention that PBSC collections have a lower incidence of tumour involvement in breast cancer patients than do comparable bone marrow harvests (Ross et al, 1993). Using immunocytochemistry with a panel of monoclonal antibodies, they showed that breast cancer cell involvement of PBSC collections occurred significantly less frequently than bone marrow involvement (9 out of 48 patients had tumour cells detected in PBSC collections versus 32 of the same 48 patients having cells detected in their bone marrow). It is noteworthy that all of those nine positive PBSC samples were from patients with known metastatic disease, whereas bone marrow involvement was seen in half of the small number of stage II and III patients. Convincing clonogenic tumour colony growth was demonstrated in a significant number of the PBSC specimens that contained immunocytochemically detectable tumour cells, indicating that breast cancer cells mobilised to the periphery do indeed have proliferative potential (Ross et al, 1993). This suggests that the reinfusion of tumour cells in PBSC transplantation has the potential to precipitate disease relapse.

An important follow-up study using the same techniques was published in 1995 (Passos-Coelho et al, 1995). Patients studied had proven stage IIIB or IV breast cancer, clinically responsive to conventional-dose chemotherapy, and bone marrow biopsies negative for breast cancer by routine cytology. Patients received cyclophosphamide and GM-CSF and underwent a single large-volume leukapheresis for PBSC collection 15 days later.

Only one of 28 patients studied had tumour cells detected in peripheral blood prior to mobilisation, and this patient had tumour cells also detected in the PBSC collection. Only one other PBSC sample from the total of 28 patients had evidence of tumour cell contamination. Only four patients had breast cancer cells detected in bone marrow biopsies by the immunocytochemical method, and only one of these had tumour cell involvement of the PBSC collection. The low frequency of tumour cell contamination of the PBSC collections in these selected patients is of major clinical interest, because their clinical stages reflect those of most patients considered for high-dose chemotherapy (Passos-Coelho et al, 1995).

The other published methods for detection of circulating cells from solid tumours are yet to provide data regarding tumour cell involvement of PBSC collections that is as informative as the results from immunocytochemistry described above. Datta's RT-PCR study of 34 breast cancer patients included PBSC collections from four patients, all with metastatic breast cancer. In none of these could detection of breast cancer cells be claimed (Datta et al, 1994).

1.5   BACKGROUND TO THE DEVELOPMENT OF IMMUNOBEAD RT-PCR

The combination of immunomagnetic isolation of tumour cells with PCR to identify a tumour marker was developed in the laboratory of Alexander Dobrovic, Ph.D., in the Haematology/Oncology Department at the Queen Elizabeth Hospital (Hardingham et al, 1993). This work was aimed at detecting circulating tumour cells from colorectal cancers, using a point mutation in codon 12 of the K-ras gene as the marker for PCR amplification. Immunomagnetic beads were labelled with an epithelium-specific antibody which would bind to epithelial cells, and hence carcinoma cells, in a haemopoietic environment and allow their isolation from the remainder of the haemopoietic compartment when a magnetic field was applied.

1.5.1   Selection of antibodies

The monoclonal antibodies Ber-EP4, anti-CEA (carcino-embryonic antigen) and anti-EMA (epithelial membrane antigen) were tested for their ability to bind to cells from colorectal carcinoma cell lines. Using flow cytometry, Ber-EP4 was shown to be far superior to the other two antibodies in its binding with cell lines. In all of the six colorectal cancer cell lines studied, Ber-EP4 demonstrated better than 99.5% binding to the cells and superior fluorescence, so that Ber-EP4 was chosen as the sole antibody for labelling to the beads, in preference to a "cocktail" of antibodies. These results have been subsequently reproduced by Wong and colleagues, but not acknowledged by them (Wong et al, 1995).

To apply the immunobead cell isolation method to breast cancer, an antibody had to be chosen that would bind to the surface of the vast majority of

30

breast cancer cells. Secondly, a PCR method needs to be chosen that would be applicable to most patients with breast cancer.

Ber-EP4 had to be studied to see if it was as uniform in its binding to breast cancer cells as it was with colon cancer, and it also had to be determined whether any other antibodies might be superior to Ber-EP4 in their binding of breast cancer cells.

Ber-EP4

The monoclonal antibody was developed using MCF-7 cells, and was directed against two 34 and 39 kilodalton surface glycoproteins (Latza et al, 1990). Immunostaining of a panel of different normal tissues with Ber-EP4 showed that all normal epithelial tissues expressed the Ber-EP4 antigen on the cell membrane; only parietal cells in gastric glands and apical cell layers in squamous epithelia were negative. While the staining intensity varied between epithelial cells of different origin, epithelial cells of one type always expressed the antigen homogeneously (Latza et al, 1990). Homogeneity of reactivity of a monoclonal antibody with cells of a single tissue type is said to be unusual (Edwards, 1985), but is obviously highly desirable for the purposes of identifying cells in small numbers.

In Latza's study, all of 37 different carcinoma cell lines stained homogeneously with Ber-EP4, and Ber-EP4 reactivity was seen in 142 of 144 malignant epithelial tumours of various origins. No reaction with Ber-EP4 was seen in cells from 20 cases of leukaemia, two malignant mesotheliomas, or from splenic tissue, peripheral blood or bone marrow (Latza et al, 1990). The Ber-EP4 antigen has, however, been demonstrated on the chronic myeloid leukaemia cell line K562, which interestingly also shows atypical expression of cytokeratins (Zauli et al, 1986). An important feature to note from the viewpoint

of a method for isolating epithelial cells from venesected peripheral blood samples, is that neither epidermis nor endothelia reacted with Ber-EP4 (Latza et al, 1990).

A second group has raised a monoclonal antibody (designated HEA 125) against a 34kD cell surface glycoprotein on the colon cancer cell line HT-29 (Moldenhauer et al, 1987). The authors found that their antibody stained 17 different carcinoma cell lines, including a breast cancer cell line (AlAb), but showed no staining of cell lines from lymphoma, melanoma, sarcoma or neuroblastoma.

Latza and colleagues showed that the Ber-EP4 and HEA125 antibodies precipitated the same antigen, explaining the consistency of results between the two. They noted that the Ber-EP4 antigen was different from all other known epithelial-specific antigens except the HEA125 antigen (Latza et al, 1990).

The specificity of Ber-EP4 has been confirmed in two separate clinical studies, in which all epithelial tumour sections stained with Ber-EP4 (the vast majority homogeneously), and no reactivity was identified in control mesothelial cell samples or sections from lymph nodes (Angelis et al, 1992; Passlick et al, 1994). The staining of the malignant epithelium from carcinomas of the lung by CEA and EMA was seen in only 50% of those cases stained by Ber-EP4 (Angelis et al, 1992).

BC2

The mucins are a family of highly glycosylated, high molecular weight glycoproteins present of the surface of many epithelial cells (Devine et al, 1992). Specifically, they may be found in the apical cell membrane of epithelial cells in human breast, salivary gland, gastrointestinal tract, respiratory tract, kidney bladder and prostate gland (Devine and McKenzie, 1992).

Four distinct gene loci for mucins have been identified in humans, being named MUC1 to MUC4, with MUC1 predominating in mammary epithelium (Devine and McKenzie, 1992). Although many normal tissues express MUC1, the pattern of expression differs and the level of expression is markedly elevated in malignant tumours (Devine and McKenzie, 1992). Increased expression of MUC1 in breast carcinomas makes MUC1 a potential candidate as a tumour marker (Noguchi et al, 1994), and as a potential antigen for binding of immunobeads for isolation of carcinoma cells.

The monoclonal antibody BC2 was raised against human milk fat globule membranes and reacts with the MUC1 core protein (Devine et al, 1992). Studies of reactivity of BC2 demonstrate that MUC1 is expressed as both a membrane-bound form and as a secreted form, and is present on both colon and breast cancer cell lines (Devine et al, 1992)

EMA

Since anti-EMA antibodies were raised against an antigen in defatted human cream (subsequently known as epithelial membrane antigen) it could be anticipated that this antibody might be react specifically with cells of mammary origin (Cordell et al, 1985). As discussed previously, it has been demonstrated that EMA is also expressed on plasma cells, some non-Hodgkin's lymphomas, on Reed-Sternberg cells, and could even be induced on normal blood lymphocytes by lymphocyte activators (Delsol et al, 1984). EMA has been assessed as a potential antibody for use with immunobeads to bind breast cancer cells, but its lack of specificity for epithelial cells virtually precludes its reliable use.

1.5.2 Methods of identification of tumour cells isolated by immunobeads

Once tumour cells have been isolated from a haemopoietic medium by the immunobeads, a method must be devised to demonstrate that cells have in fact been isolated. Light microscopy studies during initial assessment towards immunobead isolation of colon cancer cells showed that at optimum numbers of immunobeads, the beads tended to obscure the tumour cells from view (J.E. Hardingham, Department of Haematology-Oncology, The Queen Elizabeth Hospital, personal communication). Light microscopic methods for identification of tumour cells isolated by the immunobeads were therefore not pursued, and instead detection of K-ras mutations in colon cancer cells by PCR amplification was chosen as the method for demonstration of tumour cell isolation (Hardingham et al, 1993).

The low frequency of mutations in oncogenes and tumour suppressor genes specific for breast cancer makes application of PCR alone to immunobead isolation impractical for clinical studies. The most commonly mutated gene in breast cancers is the p53 gene, and mutations in this gene are seen in no more than one third of cases, and even then they occur throughout the open reading frame of the gene (Morrison, 1994).

The alternative approach of targeting tissue-specific gene transcription to identify the cancer cells was investigated, following the publications available at the time of commencement of this work (Smith et al. 1991, investigating melanoma; Mattano et al. 1992, investigating neuroblastoma; Moreno et al. and Deguchi et al. 1993, investigating prostate cancer; Matsumura and Tarin 1992, investigating alternative splicing of CD44 as a universal tumour marker).

The two criteria fundamental to this approach are:

1) That the RNA marker can differentiate the isolated tumour cells from the background haemopoietic cells.

2) That the RT-PCR can be made sufficiently sensitive to amplify signals from very small numbers of cells.

The expression of messenger RNA from two separate genes was assessed in this work to determine whether either could be coupled to the immunobeads as markers for breast cancer cells in a haemopoietic environment. The first gene evaluated was CD44, followed by cytokeratin 19 after the publication by Datta and colleagues (Datta et al, 1994).

1.5.3   CD44

1.5.3.1 Introduction

In 1992 Matsumura and Tarin published their work on evaluation of the expression of the human CD44 gene, and reported a method by which CD44 expression as measured by RT-PCR could be used to identify the presence of 10 cells from the breast cancer cell line ZR75-1 in a sample of 10^7 leukocytes (Matsumura and Tarin, 1992). The idea for their method arose from their observations that total RNA extractions from malignant tumour tissue displayed a chaotic over-expression of variant isoforms of CD44 when assessed by RT-PCR, whereas normal peripheral blood leukocytes expressed only the standard form of CD44. This work suggested that alternative splicing of CD44 RNA could be used as a generic marker of malignancy, and it therefore appeared that assessment of this phenomenon could be applied to the immunobead technique to demonstrate isolation of carcinoma cells.

1.5.3.2 Definition, structure and function

The term CD44 describes a group of polymorphic cell-surface glycoproteins that are expressed as components of cell membranes and appear to serve a function of adhesion in cell-cell and cell-substrate interactions. CD44 molecules are expressed on B and T lymphocytes, granulocytes, monocytes, erythrocytes and a variety of non-haemopoietic cells, and have apparent molecular weights ranging from 85kDa to more than 200kDa (Jackson et al, 1992). The varying functions that have been attributed to CD44 include: lymphocyte "homing" to lymphoid tissue; adhesion to other cells and to surrounding mesenchymal components including hyaluran, chondroitin sulphate, fibronectin and collagen; T lymphocyte activation; initiation of tumour metastasis formation (Stamenkovic et al, 1991; Tarin and Matsumura, 1993).

The human CD44 gene is composed of at least 19 exons, of which 7 are expressed to form a peptide backbone of about 37kDa which is subsequently glycosylated before being displayed on the cell surface as an 80-90kDa glycoprotein known as the "standard" or "haemopoietic" form (Tarin and Matsumura, 1993).

The region of the CD44 gene that encodes for the extracellular domain spans 25 kb of genomic DNA and contains at least 10 alternatively spliced exons. The exons that encode for the "standard" isoform of CD44 are highly conserved, however the variant region of the gene contains the exons that may be alternatively spliced to produce various cDNA isoforms that differ in a specific region of the extracellular domain, close to the transmembrane segment (Tolg et al, 1993). The variant part of the gene comprises exons 6 to 14, which are designated v2 to v10 according to the nomenclature of Tolg et al (1993).

The "standard" form of CD44 (designated CD44s) is the most commonly expressed, particularly on lymphocytes (Tolg et al, 1993; Mackay et al, 1994). The extracellular domain of the standard form is encoded by exons 1 to 5, and 15 and 16. A larger isoform with an additional extracellular domain has been demonstrated on epithelial cells and has been designated the epithelial form, CD44e (Stamenkovic et al, 1991; Jackson et al, 1992). The "epithelial" isoform of CD44 forms a 180kDa glycoprotein. It is identical to the standard form, but the insertion of transcripts from variant exons 8,9 and 10 leads to an additional 135 amino acids within the extracellular domain of the molecule (Jackson et al , 1992; Tolg et al, 1993).

The various isoforms of CD44, occur by the splicing of transcripts in multiple different combinations into the mRNA strand that corresponds to the standard form (Lazaar and Pure, 1995). The CD44 gene can therefore be potentially expressed as many different alternatively spliced isoforms, often carrying entirely different functions (Screaton et al, 1992; Tarin and Matsumura, 1993).

1.5.3.3 Expression of different CD44 isoforms may confer metastatic potential

While the expression of variant exon epitopes is normally found in a restricted distribution, upregulation of expression of CD44 isoforms has been reported to correlate with increased tumour aggressiveness and metastatic potential (Matsumura and Tarin, 1992; Tanabe et al, 1993; Rudy et al, 1993; Wielenga et al, 1993l; Herrlich et al, 1993; Kaufmann et al, 1995). Gunthert and coworkers identified a new antigenic determinant corresponding to the presence of a CD44 isoform containing an alternatively spliced exon that conferred metastatic potential to a rat adenocarcinoma cell line (Gunthert et al,

Figure 1.1   Schematic representation of the exon map of CD44 (left) and the CD44 protein (right), modified from Koopman et al. (1993).

The exons are numbered on the left column, with the variant exons numbered in red. The CD44 protein spans a cytoplasmic domain, a transmembrane domain and an extracellular domain. The protein encoded by the variant exons is inserted into the CD44 protein in its extracellular domain.

EXTRACELLULAR DOMAIN

5

v1

6   v2

7   v3

8   v4

9   v5

10   v6

11   v7

12   v8

13   v9

14   v10

15

CD44 protein

Protein encoded by variant exons

Insertion site of variant exons

Plasma membrane

CYTOPLASMIC DOMAIN
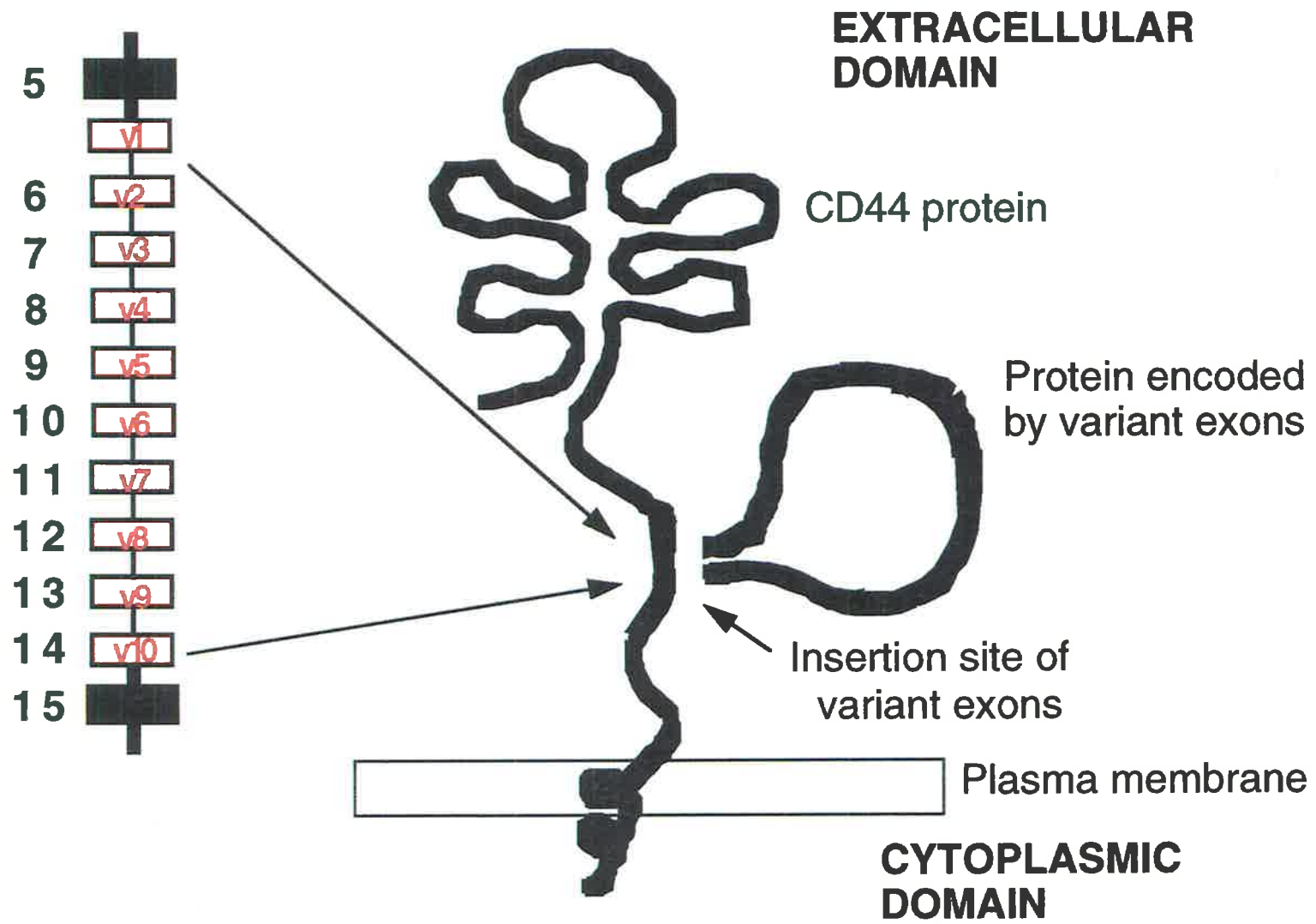
1991). The variant exon v6 has been implicated in initiation of metastasis, and the expression of v6 by tumours is associated with a worse prognosis and is an independent prognostic indicator when studied by multivariate analysis (Kaufmann et al, 1995).

Alternative splicing of mRNA is a well-recognised mechanism of eukaryotic gene expression, especially for mRNA's encoding membrane proteins. Cellular factors regulate the expression of splice variants, and permanent expression on many cases is considered abnormal. However, genetic mutation within a cell may lead to permanent splice variant expression.

In addition to alternative splicing of mRNA, other alterations to the CD44 molecule such as post-translational glycosylation and addition of chondroitin sulphate to the extracellular domain, and phosphorylation of the cytoplasmic domain, have been implicated as possible mechanisms by which the function of CD44 may be modified (Lazaar and Pure, 1995).

1.5.3.4 Distinction of benign from malignant tissues on the basis of CD44 variant expression

Recognising that aberrations of gene function, as seen with the phenomenon of alternative splicing of mRNA, are a common feature of malignancy, Matsumura and Tarin postulated that the over-expression of CD44 splice variants could be used to distinguish malignant from benign tissue (Matsumura and Tarin, 1992).

Using RT-PCR, they examined the CD44 expression from fresh surgical specimens of benign and malignant tumours, predominantly of breast and colon, and compared the results with the patterns of expression in normal tissues. They found that normal PBMNCs, and normal hepatocytes, expressed the standard form of CD44 only, while normal breast, colon and lymph nodes

expressed the standard form and a "trace of a....variant or two" (Matsumura and Tarin, 1992).

In contrast to the normal tissues, Matsumura and Tarin found that all samples of neoplastic tissue showed over-expression of many alternatively-spliced variants of CD44. Furthermore, increased splice variants were seen in those tumours with clinical metastases, moreso than in those local tumours without clinical evidence of metastasis. The authors concluded that the activity of CD44 gene is "severely abnormal in many common human malignant tumours, but not in counterpart tissue from the same patient", and postulated that abnormal expression of CD44 isoforms could become a useful marker for malignancy (Tarin and Matsumura, 1993).

1.5.3.5 A proposed RT-PCR method for detection of circulating tumour cells based on abnormal CD44 expression

Matsumura and Tarin's' method for detection of tumour cells in peripheral blood involved extracting the white blood cell pellets from 20ml of the four volunteers' whole blood (using red cell lysis and centrifugation) and then seeding the pellets with suspensions of ZR75-1 breast cancer cell line cells and then extracting total RNA from the whole for reverse transcription to cDNA. PCR primers were designed to amplify variant exons of CD44 that were known to be expressed by ZR75-1, in the anticipation that the PCR could therefore only amplify CD44 signal from the tumour cells and not the white cells. Using Southern blotting and autoradiography, this method was claimed to be able to detect 10 ZR75-1 breast cancer cells amongst 10^7 leukocytes.

This method depends on two fundamental assertions: firstly, that cells of the haemopoietic compartment express CD44 message only in the standard form; secondly, that the expression of multiple CD44 splice variants by

malignant tissue is seen in single cells as well as multiple cells, and is not just the sum of individual cells expressing different isoforms.

1.5.3.6 CD44 expression in tissues and cells

CD44 expression in tissues versus single cells

All published studies to date using RT-PCR to assess CD44 expression have examined whole tissue samples by GITC extraction of total RNA. This is also relevant to the assessment of CD44 variants as potential prognostic indicators in primary tumours. If a tumour expresses a particular isoform, it is unknown whether all cells of that tumour express that isoform and whether all cells carry the putative poor prognostic indicator, or indeed whether the splice pattern in the malignant cells differs from that of the benign myoepithelial cells that will inevitably contribute to the extracted RNA (Dall et al, 1995). Kaufmann et al. have stated that "we do not yet know whether the epitopes found are part of one and the same CD44 molecule or whether one cancer cell can express several CD44 isoforms" (Kaufmann et al, 1995).

CD44 expression on haemopoietic cells

Central to the claims of Matsumura and Tarin that expression of CD44 variants could be used as a screening test for malignancy is their observation that PBMNCs express only the standard form of CD44 (Tarin and Matsumura, 1993; Tarin and Matsumura, 1993, letter). This is at odds with the results of others.

Salles and colleagues studied CD44 expression on normal, activated, and malignant lymphoid cells by RT-PCR in a semi-quantitative manner. They found that for the wide range of lymphoid cells studied, the predominant PCR product was the standard form of CD44, which of itself contained no additional exons from the variable region (Salles et al, 1993). However, while many

lymphoma samples displayed the CD44s isoform predominantly, activated splenic B cells, more advanced lymphomas epithelial cells and particularly PBMNCs displayed abundant larger CD44 transcripts in addition to CD44s. Amongst the larger transcripts expressed by normal PBMNCs was the epithelial variant of CD44 (Salles et al, 1993).

Mackay and colleagues set out to determine the expression and regulation of CD44 isoforms on leukocytes and epithelia by immunofluorescent staining of isoforms by a panel of monoclonal antibodies, assessed by flow cytometry (Mackay et al, 1994). The antibodies were designed to amplify the variant isoforms CD44-v9 (which would therefore also amplify the "epithelial" variant expressed by variant exons v8,v9 and v10), CD44-v6 (implicated in metastasis) and CD44-v4. Like Salles and colleagues, they found that the predominant isoform expressed on leukocytes was CD44s, but larger isoforms were also expressed, although at low levels. CD44-v9 isoforms (and hence CD44e) were weakly expressed on T and B lymphocytes, monocytes and granulocytes, and only monocytes expressed expressed CD44-v6 and CD44-v4 (Mackay et al, 1994).

It is of considerable interest to this thesis to note that on T lymphocytes, expression of CD44-v9 and CD44-v6 was increased after activation by mitogens, and this upregulation was rapid and transient. This increased expression of CD44 variants after activation of T cells has been demonstrated by others (Arch et al, 1992; Koopman et al, 1993).

CD44 expression on epithelial cells

RT-PCR studies on several carcinoma cell lines by Salles and colleagues showed lower expression of CD44s and higher expression of larger isoforms, relative to the expression seen on lymphocytes (Salles et al, 1993). The

immunofluorescence and flow cytometry study by Mackay et al. concurred, showing that epithelia displayed the most intense and extensive expression of variant isoforms, and the highest levels of variant CD44 exons were seen in the generative epithelial cells. Epithelium from most tissues expressed variant exon 9, but fewer epithelial tissues expressed v6 or v4 (Mackay et al, 1994). It should be noted that the expression of exon v9 is expected to correlate with the expression of v8 and v10 (the "epithelial" variant), and expression of v6 correlates with v7 (the "metastatic variant") (Mackay et al, 1994).

The claim of Matsumura and Tarin that malignant epithelial tissues display a chaotic and permanent over-expression of variant CD44 isoforms (Matsumura and Tarin, 1992; Tarin and Matsumura, 1993; Tarin and Matsumura, 1993, letter) is supported by others (Salles et al, 1993; Mackay et al, 1994; Koopman et al, 1993; Jackson et al, 1992) . Furthermore, the expression of the CD44 v6 isoform has been shown to correlate with tumour progression in colon cancer (Wielenga et al, 1993) and breast cancer (Dall et al, 1995; Kaufmann et al, 1995). However, the specificity of CD44 variant expression as a marker for malignancy is called into question by others who have used immunohistochemistry to demonstrate that many normal epithelia express CD44 variants, including CD44s, CD44-v9 (corresponding to CD44e), and CD44-v6 (Terpe et al, 1994; Fox et al, 1993).

1.5.3.7 Prospects for the investigation of CD44 expression as a potential marker for breast cancer cells using immunobead RT-PCR

Sufficient evidence exists, therefore, to doubt the validity of Matsumura and Tarin's method of using RT-PCR for CD44 variant expression to detect carcinoma cells in peripheral blood. However, the preliminary selection of

tumour cells by the immunobead method may still find CD44 of use as a marker, since the amount of background transcript expression by leukocytes will be greatly reduced. If the limited number of leukocytes remaining after immunobead selection is enough to only show expression of CD44s by RT-PCR, then the amplification of larger CD44 isoforms might indicate the presence of tumour cells bound to the immunobeads.

1.5.4   Cytokeratins

1.5.4.1   Introduction

The cytoplasm of all mammalian cells is structurally supported by various groups of microfilaments and microtubules which together comprise the cytoskeleton. These groups form three filament systems: microfilaments (containing actin), microtubules (containing tubulin), and filaments of intermediate size (7 to 10nm diameter) (Moll et al, 1982; Smack et al, 1994). The intermediate sized filaments display a remarkable specificity of differentiation amongst different cell types, for example, filaments containing keratin-like proteins (cytokeratins) are characteristic of epithelial cells; vimentin filaments occur in mesenchymal cells; desmin filaments are seen in muscle cells; neurofilaments are typical of neuronal cells; and glial filaments are typical of astrocytes (Moll et al, 1982). The conservation of this differentiation specificity amongst intermediate filaments during cell transformation and tumour development has enabled their use in histopathological diagnosis (Moll et al, 1982; Gabbiani et al, 1981).

Approximately 20 cytokeratins have been described, and ordered according to their molecular weights (Traweek et al, 1993). The cytokeratins 8, 18 and 19 have been the most extensively studied because of their occurrence in many different kinds of epithelia, particularly "simple" epithelia, and because

their conservation of expression in carcinomas of lung, breast and gut has led to them being investigated as possible tumour markers (Tsubura et al, 1991; Bader et al, 1988; Moll et al, 1982).

For the purposes of developing a method for detection of tumour cells in peripheral blood using K19 as the marker for the tumour cells, it is important to note that K19 is not expressed in adult skin (Smack et al, 1994). Immunohistochemistry has demonstrated that K8 and K19 are focally expressed in the basal cell layer of foetal skin at around 24 weeks' gestational age, but this expression is lost with increasing maturity. The keratin profile of adult skin shows fewer keratins being expressed, with keratins 5 and 14 predominating in the basal cell layer, and keratins 1 and 10 in the suprabasal compartment (Smack et al, 1994).

1.5.4.2 Expression of cytokeratins in breast epithelium

Immunohistochemical studies have demonstrated that keratin expression in ducts of normal mammary tissue was differentiated according to the cell type. Luminal cells were characterised by expression of the simple epithelial keratins K8, K18 and K19, whereas the basal cells, lying between the luminal cells and the basement membrane, expressed keratins K5 and K14, which are typical of myoepithelial cells in stratified epithelium (Trask et al, 1990). In cell lines from normal and tumour tissues, Trask and colleagues (Trask et al, 1990) showed that the dominant keratins produced in normal cells were K5, K6, K7, K14 and K17, while in tumour cells K8, K18 and K19 were the major intermediate filament proteins. Bartek and colleagues demonstrated immunohistochemical staining of all breast cancers when 116 tumours and 21 metastases were studied with antibodies directed against K19, whereas many cells of the benign tumours studied failed to stain for K19 (Bartek et al, 1985).

K8 and K18 have proved unsuitable as markers for breast cancer cells in blood, since RT-PCR techniques have demonstrated their expression in haemopoietic cells (Traweek et al, 1993; Brown et al, 1995). Assessment of K19 expression haemopoietic cells by RT-PCR methods is the subject of some controversy currently (Burchill et al, 1995).

1.5.4.3   K19 expression in haemopoietic cells

Traweek and colleagues (Traweek et al, 1993) studied K19 expression by RT-PCR in various non-epithelial tissues, peripheral blood and bone marrow, and found that K19 expression was far more limited in these tissues than keratins 8 and 18, and was absent in normal haemopoietic cells. Specifically, reverse transcription followed by 30 cycles of PCR and subsequent Southern blotting displayed strong K19 bands from cultured normal umbilical vein endothelial cells and low intensity signals from cultured normal skin fibroblasts. Samples from 7 normal bone marrows, normal peripheral mononuclear cells, a normal lymph node and the myeloid leukaemia cell line HL-60 were all negative for K19. Of four samples from Non-Hodgkin's lymphomas studied, two cases were faintly positive on autoradiography. The authors suggested that, given the negative results in other haemopoietic cells, these weak positives may have been due to contaminating stromal cells (i.e. fibroblasts and endothelial cells).

Datta and colleagues (Datta et al, 1994) carefully assessed RT-PCR detection of K19 transcripts in bone marrow and peripheral blood samples from either healthy people or patients with conditions other than carcinomas. They were able to exclude amplification of the known K19 pseudogene (Savtchenko et al, 1988) by designing long PCR primers whose 3′ ends differed from the pseudogene, and using stringent PCR conditions. They claimed to consistently

fail to amplify a K19 signal from normal peripheral blood mononuclear cells. Buffy coats from centrifuged samples of peripheral blood or bone marrow were taken and suspended in an ammonium chloride red blood cell lysis buffer, and RNA extracted from the recovered cells for reverse transcription and nested PCR. Using amplification of the ABL gene as controls for reverse transcription, they were unable to amplify K19 message from peripheral blood of 10 control patients, bone marrow samples from 14 healthy donors and 15 patients with CML. A K19 signal was amplified from two separate samples of bone marrow from one CML patient however, and the authors suggested that this may have been the result of low-level "illegitimate transcription" of keratin transcripts in non-specific cells (Datta et al, 1994). This phenomenon of low-level transcription of tissue-specific genes in non-specific cells (Chelly et al, 1989) has been used by other workers in the field of tumour cell detection by RT-PCR as a possible explanation for false-positive results (Datta et al, 1994; Schoenfeld et al, 1994; Smith et al, 1991; Brown et al, 1995). The atypical expression of vimentin and keratin intermediate filaments in the chronic myeloid leukaemia cell line K562 has been discussed above (see section 1.5.1) with respect to the similarly atypical expression of the Ber-EP4 antigen by that cell line (Zauli et al, 1986). The unexpected expression of K19 transcripts in the bone marrow of the CML patient may reflect the same processes occurring in the leukaemic cell line, and may be specific to the malignant cells and not to normal haemopoietic cells.

Schoenfeld and colleagues (Schoenfeld et al, 1994) used RT-PCR for K19 to look for occult metastases in the regional lymph nodes of breast cancer patients. Improved sensitivity of detection over routine histology was demonstrated at 40 cycles of a single stage PCR, and under these conditions none of 11 normal lymph nodes from patients without breast cancer amplified

K19 transcripts, either by ethidium bromide staining or Southern hybridisation. However, when a second stage of PCR was introduced in an attempt to increase the sensitivity of the technique, all 11 nodes displayed K19 product on ethidium-stained gels. The possibility of pseudogene amplification was excluded by digesting the PCR product with *Hae* II (a *Hae* II restriction site was present in the RNA-derived K19 product but not the product from the pseudogene), and demonstrating the two *Hae* II fragments after the second round of PCR. The authors concluded that the K19 expression in normal nodes seen only after two rounds of PCR represented true K19 expression from low-level endogenous RNA template.

1.6 SUMMARY

Breast cancer is the commonest cause of death from cancer in women of western societies. While the management of breast cancer has changed dramatically in recent years, little impact has been made on the mortality of the disease. Clinicians involved in the management of patients with breast cancer need improved methods of assessing or predicting metastatic disease so that patients can be most appropriately selected for systemic therapies. Increased knowledge of the incidence, behaviour and significance of tumour cells in the peripheral circulation is likely to be of major clinical interest.

Initial attempts to quantitate the incidence of circulating tumour cells in patients with solid tumours used light microscopy and conventional cytology, and estimated that tumour cells could be detected in the peripheral blood of 20 to 30% of patients with carcinomas. More sophisticated techniques using immunohistochemistry suggest that these early results overestimated the incidence of circulating tumour cells. Immunohistochemical techniques are more specific, but still have potential problems of cross-reactivity of the antibodies with some haemopoietic cells. A major disadvantage of current immunohistochemical techniques is the labour requirement to screen significant volumes of blood or bone marrow for the presence of tumour cells.

PCR - based methods are limited by the markers that can be used in solid tumours. The use of tissue-specific markers by RNA analysis has led to the development of highly sensitive RT-PCR assays, but more detailed investigation has shown many of these assays to be associated with false-positive results for tumour cell detection.

The potential for clinical application is driving the development of many methods for the detection of tumour cells in the haemopoietic

environment, but the ideal method that combines high sensitivity with specificity has yet to be developed.

The best current methods for detection of breast cancer cells in bone marrow estimate an incidence of micrometastatic disease in the marrow of patients with node-negative breast cancers to be in the order of 30%. The number of recent studies of the incidence of breast cancer cell contamination of peripheral blood and PBSCs is so scant as to make the answer to these questions essentially unknown. Two patterns of breast cancer biology are suggested by the current small studies. Firstly, the incidence of contamination of PBSCs by breast cancer cells does seem to be low, and secondly, some results suggest that administration of chemotherapy is associated with the mobilisation of tumour cells into the periphery. Both of these suggestions require confirmation.

Detection of tumour cells in the peripheral blood probably indicates a large tumour burden and hence poor prognosis. However, the true clinical significance of detection of tumour cells in the haemopoietic compartment requires further investigation.

In terms of the development of a method for the detection of circulating breast cancer cells based on preliminary isolation of tumour cells by immunomagnetic beads, it was necessary to confirm the utility of the monoclonal antibody Ber-EP4. It was also necessary to develop a method for RT-PCR from as few cells as possible, and then applied to apply this method to tumour cells isolated by immunobeads.

Specific questions that had to be addressed with respect to CD44 as a possible marker for immunobead RT-PCR were:

1) the pattern of expression of CD44 isoforms by small numbers of tumour cells and haemopoietic cells, to determine whether the cells

49

could be distinguished from one another on the basis of their CD44 expression

2) whether single or few tumour cells could express more than one CD44 isoform, or whether the phenotype of CD44 expression of a tumour was necessarily the result of the sum of all of its constituent cells.

Specific questions that had to be addressed with respect to K19 as a possible marker for immunobead RT-PCR were:

1) Whether a RT-PCR signal for K19 could be detected from a very small number of cells

2) The incidence of expression of a RT-PCR signal for K19 by tumour cells

3) Whether a RT-PCR signal for K19 could be detected from haemopoietic cells.

CHAPTER TWO:

GENERAL MATERIALS AND METHODS

2.1   REAGENTS AND SOLUTIONS

All reagents were of analytical grade and, unless otherwise stated, were obtained from manufacturers or distributors of Ajax Chemicals (Sydney, Australia), Amersham (Amersham, United Kingdom), BDH Chemicals (Kilsyth, Victoria, Australia), Biorad Laboratories (North Ryde, Australia), Boehringer-Mannheim (North Ryde, Australia), Bresatec (Thebarton, Australia), Commonwealth Serum Laboratories (CSL, Parkville, Australia), Difco (Detroit, USA), GibcoBRL (Life Technologies, Grand Island, New York), Merck (Darmstadt, Germany), Nunc (Naperville, Illinois, USA), Pharmacia LKB, (Uppsala, Sweden), Sigma Chemical Company (St. Louis, USA), Silenus Laboratories (Hawthorn, Victoria, Australia), Progen (Darra, Queensland, Australia), Promega (Madison,WI, USA).

2.1.1   Solutions used for cell culture

Growth Media

The principal growth medium used for the culture of cell lines was Dulbecco's modified Eagle medium (DMEM, Gibco BRL). All media were prepared according to manufacturer's specifications, and filter- sterilised using a 0.22micron disposable cellulose acetate membrane (Corning, New York). DMEM for cell culture was prepared by taking 450ml of medium and adding 1ml penicillin-streptomycin stock (500x, Sigma), 2.5ml L-glutamine (200mM, Sigma), and 50mls FCS (CSL) to make a final concentration of 10% v/v.

Trypsin-versene

In 500ml of Hi-pure water:

Trypsin 1.25g (Difco)

NaCl 4.0g (Ajax)

Na_2HPO_4 30mg (Ajax)

KH_2PO_4 30mg (Ajax)

$NaHCO_3$ 0.25g (Ajax)

Na_2EDTA 0.1g (Sigma)

Glucose 0.5g (BDH)

The solution was filter-sterilised using the 0.22micron filter (Corning).

Penicillin-Streptomycin 500x stock

Benzyl penicillin (Sigma) 766,000units

Streptomycin-sulphate (Sigma) 978,000units

Dissolved in 20ml sterile Hi-pure water and filter-sterilised.

2.1.2   Solutions for molecular biology

Sterile Hi-pure water (HPW)

Sterile, distilled water was filtered through an ion-exchange filter (Milli-Q Plus ultra-pure water system).

Phosphate-buffered Saline

Dulbecco's Phosphate-buffered Saline, without calcium or magnesium (GibcoBRL), was diluted in HPW according to the manufacturer's instructions, then autoclaved.

Diethylpyrocarbonate treated water (DEPC water)

0.2ml DEPC (Sigma) was added to 100ml HPW. The solution was shaken vigorously and then autoclaved.

PBS/FCS/Azide buffer

PBS with 1% foetal calf serum (CSL) and 1% sodium azide (Merck).

Phenol

2-3 grains (0.1%) of 8-hydroxyquinoline (Sigma), were added to 100ml of phenol (Sigma). The phenol was equilibrated by washing it in 100ml of 0.5M

Tris-HCl (pH 8.0). After allowing the mixture to stand, the phenol was drawn from the Tris-HCl, and washed with 100ml of 0.1M Tris-HCl (made with DEPC water, pH 8.0). This step was repeated until the phenol was at pH 7.8. Finally, 0.2% v/v β-mercaptoethanol (Sigma) was added and the phenol stored at 4^0C and used within four weeks.

Solution D

> 4M guanidinium isothiocyanate (Sigma)
>
> 25mM sodium citrate, (BDH) adjusted to pH 7 with citric acid
>
> 0.5% sarcosyl (Sigma)
>
> 0.1M 2-mercaptoethanol (Sigma)

40mM dNTP solution

A 40mM stock solution of dNTPs containing 10mM of each nucleotide was prepared. Fifty mg of each dNTP (2'-deoxy-adenosine-5'-triphosphate disodium salt; 2'-deoxy-cytidine-5'-triphosphate disodium salt; 2'-deoxy-guanosine-5'-triphosphate disodium salt; 2'-deoxy-thymidine-5'-triphosphate disodium salt; Boehringer Mannheim), were pooled and dissolved in 9ml of DEPC water. The pH of the solution was adjusted to pH 7.0 by drop-wise addition of 1M Tris-base, after which the final volume of the solution was adjusted to 10ml. The solution was sterilised through a 0.22micron filter, distributed into sterile Eppendorf tubes and stored at -20^0C.

(6x) Loading buffer

Bromophenol blue 0.25g (BDH) and sucrose 40g (Sigma) was dissolved in 80ml HPW.

5x Tris-Borate (TBE) buffer

To 1600ml distilled water, Trizma base 108g (Sigma), boric acid 55g (Sigma), and 40ml 0.5M EDTA (pH 8.0) was added. The volume was the adjusted to 2 litres with distilled water, as stock solution.

10mg/ml Salmon sperm DNA

100mg salmon sperm DNA (Sigma) was dissolved in 10ml sterile HPW by leaving the mixture in a 37^0C heating block for several hours or overnight. The DNA was sheared using an 18-gauge needle, the mixture boiled for 10 minutes to denature the DNA, and finally stored at 4^0C.

Nasmyth's Solution

In 100ml of HPW, the following reagents were dissolved:

1.1M NaCl (Ajax)

0.3M Na_2HPO_4 (BDH)

0.01M EDTA $Na_2 2H_2 0$ (Sigma).

18.5% dextran sulphate (Pharmacia LKB) was added and allowed to dissolve on a magnetic stirrer. The solution was autoclaved, then 1.85% sodium lauryl sarcosinate (Sigma) was added with constant stirring until dissolved. The volume was made up to 200ml with sterile HPW.

Pre-hybridisation Mix

11ml sterile, distilled water

13.5ml Nasmyth's solution

0.5ml salmon sperm DNA

20xSSC

NaCl 175.3g (Ajax) and sodium citrate 88.2g (BDH) in 800ml HPW. The volume was adjusted to 1 litre with HPW, and then autoclaved.

20% SDS stock

Sodium dodecyl sulphate 40g (Sigma) was dissolved in 180ml HPW, then adjusted to 200ml with HPW.

2.2   CELL CULTURE AND CELL MANIPULATION

2.2.1   Cell Lines and Culture

Cell lines were maintained in culture in DMEM as above, at 37°C, in a humidified atmosphere incubator supplemented with 5% CO_2. When the culture of cells approached confluence, the culture was "split" by treatment with trypsin solution. At this point an aliquot of the mobilised cells would usually be used for laboratory experiments, and the remainder continued in culture in a new flask. All cell lines were taken from laboratory stocks.

The cell lines used in this work were T-47D, MCF-7 and MDA-MB-453 (MB-453). All were cultured as an adherent monolayer, and all had been established from cells obtained from malignant pleural effusions from patients with invasive ductal breast cancers (Hay et al, 1988).

2.2.2   Manipulation of cell lines from culture

Under sterile conditions, culture medium was aspirated and any remaining media washed away with calcium- and magnesium-free sterile PBS. Trypsin-versene solution was added to cover the monolayer of cells, and warmed on a 37°C heating block. Once the adherent layer of cells had been mobilised, the trypsin was washed from the cell suspension by centrifugation at 1700rpm for five minutes. The pellet of cells was resuspended in sterile PBS for manipulation for experiments.

Clumping of cells in suspension made counting of cells and subsequent diluting of cells into small numbers inaccurate. Clumping was minimised by harvesting cells for experiments before their growth reached confluence, and by using a pasteur pipette to mix the cell suspensions as much as possible, both when in suspension in trypsin solution and in PBS.

2.2.3 Isolation of mononuclear cells from whole blood or bone marrow

All manipulations were carried out under sterile conditions, under a laminar flow hood where practicable. Ten millilitres of whole blood or up to two millilitres of bone marrow was centrifuged at 1700rpm for five minutes and the plasma aspirated, leaving the buffy coat behind. The remaining cells were then diluted 1 in 5 with PBS for blood or 1 in 10 for marrow. Seven to ten millilitres of sterile Ficoll-Hypaque (Pharmacia) was layered under the diluted blood or marrow, and centrifuged at 1700rpm for 20 minutes. The mononuclear cell layer was isolated by aspirating the interface layer and transferred to a 10ml tube, and washed in at least a two-fold excess of sterile PBS (centrifuged at 1700rpm for five minutes). Washed cells were resuspended in PBS to 10mls and counted in a haemocytometer (0.1mm deep haemocytometer, American Optical Corporation, Buffalo, N.Y., USA) prior to freezing or manipulations for experiments.

Mononuclear cells from peripheral blood were counted and serially diluted in PBS as described below. An aliquot of 2-3µl of the appropriate mononuclear cell dilution was then transferred to a microfuge tube for reverse transcription.

2.2.4 Freezing cell lines and human samples

Sterile conditions were maintained, and if cell lines were being manipulated, only one cell line was used under the laminar flow hood at a time, to avoid cross-contamination. The suspension of cells was transferred to a sterile 10ml tube and centrifuged at 1700rpm for 5 minutes. The supernatant was removed and the cell pellet resuspended in a cryotube (Nunc) in cold DMEM culture medium with 10% FCS and dimethyl sulphoxide (DMSO, Sigma) added drop-wise with a pasteur pipette to a final concentration of

approximately 20%, in a total volume per cryotube not exceeding 1.5ml. The cryotube containing the cell mixture was placed in a tightly fitting foam box in a -70°C freezer overnight and then transferred to liquid nitrogen for long-term storage.

2.2.5 Recovering cell samples stored in liquid nitrogen

Cryotubes taken from liquid nitrogen storage were rapidly thawed in a beaker of warm water and the contents transferred to a sterile 10ml tube. Cold DMEM growth medium was slowly added to 10ml via a pasteur pipette, keeping the mixture well mixed. The cell suspension was then centrifuged at 1700rpm for 5 minutes to wash out the DMSO. The supernatant was removed and the pellet of cells resuspended in growth medium or PBS as required.

2.2.6 Cell counting and serial dilutions

The method of counting of cells was the same for all cell types, whether cell lines or peripheral mononuclear cells. A small aliquot was taken from the main cell suspension in PBS (after the suspension had been made as uniform as possible) by pipette, placed onto a haemocytometer under a cover slip under 10x light microscopy, and the cell count estimated in terms of millions of cells per millilitre.

The technique of serial dilution was designed to provide rapid and reasonably accurate dilutions of a stock cell suspension in 1 in 10 step-wise gradations. Prior to resuspending and counting the cells, several Eppendorf tubes were prepared, each containing 90μl PBS. A 10μl aliquot taken from the stock suspension and mixed by pipette motion into the 90μl volume in the Eppendorf would therefore see a dilution of the original cell suspension of 1 in 10. Ten microlitres taken from the 1 in 10 dilution and mixed into the second 90μl PBS would result in a dilution of 1 in 100 from the original suspension, etc.

The accuracy of this technique was limited by the accuracy of the initial cell estimation under the haemocytometer, and the uniformity of all subsequent diluting cell suspensions. The dilution steps exaggerated errors in the initial cell counts.

2.2.7 Aliquots of cell lines were stored at -80^{0}C for use as positive controls for RT-PCR

In order to provide positive controls for the immunobead RT-PCR method that would mimic as closely as possible the low copy numbers expected of the immunobead RT product, cells from the T47-D or MB-453 breast cancer cell lines were prepared from culture, counted and serially diluted in PBS and stored in microfuge tubes at -80^{0}C, in aliquots of 100 to 250 cells in residual volumes of PBS. When required for use as a positive control, 9.5µl of lysis mix was added to the frozen pellet. In this way the RNasin was immediately active as the cell suspension thawed, thus minimising endonuclease degradation of the RNA. RT-PCR was then completed as per usual protocol.

2.2.8 Micropipette aspiration of cells for RT-PCR

Instruction in the preparation and use of a micropipette for aspiration of single cells was kindly provided by Ke-Hui Cui, Ph.D., of the Reproductive Medical Unit at The Queen Elizabeth Hospital.

A sterile pasteur pipette was heated gently over a low flame from a spirit burner. Both ends of the pipette were grasped, and the pipette rapidly elongated. The heated, stretched tip was then broken at its end with a metal forceps, to leave the tip of the pasteur pipette as a very fine-bore glass pipette. A closed suction tubing was applied to the unchanged end of the pipette, to provide a means by which gentle suction could be applied for the manipulation of cells.

Figure 2.1   The micropipette for selection of single cells from suspension.
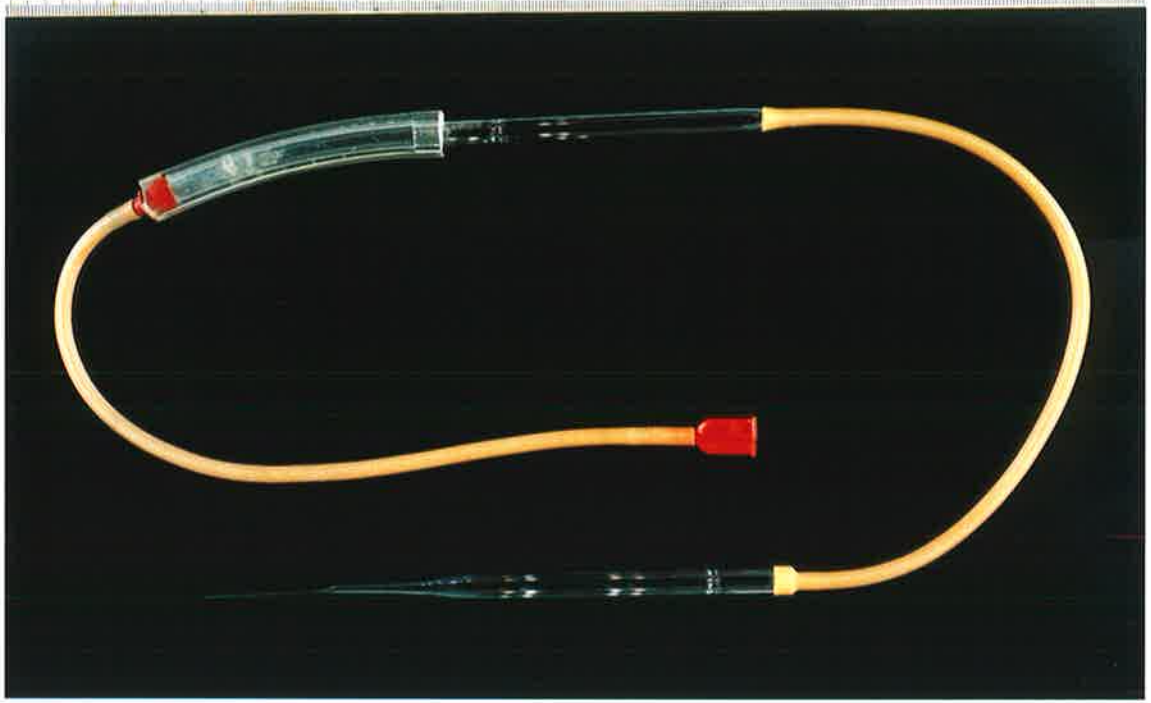
Very small numbers of cells could be manipulated from a petri dish to a lysis buffer in a microfuge tube by keeping cells in a dilute suspension of PBS in the petri dish. Using 10x magnification under an inverse-phase light microscope, individual cells could be easily identified as they were aspirated into the fine tip of the pipette. Dilute suspensions of cells were required for reliable aspiration of single cells. The adherent nature and growth pattern of the carcinoma cells meant that cells adhered to bottom of the dish once contact was made with the plastic. Therefore, aspiration of single cells was also more reliable if the manipulation was performed promptly once the cells had been placed in suspension.

2.3 COLLECTION OF HUMAN SAMPLES FOR DEVELOPMENT OF THE IMMUNOBEAD RT-PCR METHOD

2.3.1 Breast cancer specimens

Specimens of breast cancer tissue from patients with breast cancer at The Queen Elizabeth Hospital were obtained as operative specimens after informed consent and prior arrangement with Histopathologist and Surgeon. Surgical resection specimens were collected fresh in the operating theatres and transported immediately to the Histopathology Department where a specimen for research purposes would be cut from macroscopic tumour by the Histopathologist, and placed immediately in a cryotube and into a portable canister of liquid nitrogen. The cryotube containing the specimen was later transferred to the main liquid nitrogen tank for long-term storage. These specimens were subsequently used for studies of Ber-EP4 staining of primary breast cancers.

2.3.2   Axillary lymph nodes replaced by tumour

Portions of two lymph nodes, from two different patients on separate occasions, and each macroscopically replaced by tumour, were obtained as part of routine surgical clearance of axillary tissue and prepared fresh by macerating and mechanically dissociating the tissue through a 50micron sieve. The cells were sieved into PBS suspension, and centrifuged at 1700rpm for 5 minutes. The cell pellet was resuspended, and, under a haemocytometer, cell viability was confirmed by trypan blue exclusion, and cells were counted. An aliquot of the cell suspension was used for immediate purposes, and the remainder stored in several separate cryotubes, in DMEM culture medium with 10% DMSO in liquid nitrogen.

The lymph node samples were used principally in three separate experiments. The cell suspension from the first lymph node was counted and diluted into PBS suspension such that approximately 500,000 cells were placed into several Eppendorf tubes for incubating with Ber-EP4 and BC-2 monoclonal antibodies for flow cytometric assessment of binding of the antibodies to a suspension of human breast cancer cells (see chapter 3).

Tumour cell suspension from the second lymph node sample was used to provide a dilute suspension for the aspiration of single breast cancer cells by micropipette under light microscopy, for CD44 and K19 RT-PCR (see chapters 5 and 6). A frozen aliquot of this sample was later thawed and used to assess binding of tumour cells by Ber-EP4 and BC-2 using immunohistochemistry (see chapter 3).

2.3.3   Normal lymph node

A third lymph node sample was a normal submandibular lymph node, taken incidentally at the time of surgical resection of a (benign) submandibular salivary gland. This sample was processed through a sieve as described above,

to act as a negative control for the flow cytometry experiments, to establish that neither Ber-EP4 nor BC-2 were binding to lymphoid cells in the nodal suspensions (chapter 3).

2.3.4 Bone marrow and peripheral blood samples

Successful application of an RT-PCR marker to the immunobead-tumour cell isolation technique to differentiate epithelial cells from a large population of haemopoietic cells requires that the marker is not expressed by haemopoietic cells. Samples of haemopoietic tissue that could be readily tested to determine whether the RT-PCR marker could be detected in "normal" samples were bone marrow and peripheral blood.

The bone marrow samples were taken (where excess sample was available), from biopsies taken as part of the routine assessment of patients presenting to the Haematology/Oncology Department of the Queen Elizabeth Hospital with conditions other than carcinoma, and peripheral blood samples taken from a similar patient pool, or from healthy donors. Samples were either used directly in the immunobead RT-PCR technique as described, or processed for total RNA extraction followed by reverse transcription and the polymerase chain reaction. Several cDNA samples from patients with conditions other than carcinoma were generously provided by fellow laboratory workers (D. O'Keefe, Ph.D., P. Laslo, B.Sc., and D. Hussey, B.Sc.).

2.4 MOLECULAR BIOLOGY TECHNIQUES

2.4.1 Preparation of total RNA

Total RNA was extracted from cultures cells or mononuclear cell preparations from whole blood or bone marrow using a modification of the technique of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987).

Five to ten million cells were lysed by vortexing briefly in 500μl Solution D. Extraction was carried out by successively adding 50μl 2M sodium acetate (pH 4.2) 500μl phenol in DEPC water, and 100μl chloroform:isoamylalcohol (49:1, Sigma). The suspension was vortexed after the addition of each solution, and cooled on ice for 15 minutes. Centrifugation at 10,000rpm for five minutes separated the organic and aqueous phases, and the top layer (the aqueous phase) was transferred to a second tube. An equal volume of isopropanol was added to the contents of the second tube, and the RNA precipitated at -80°C for at least one hour (or overnight).

The RNA was sedimented at 14,000rpm for 20 minutes, and the resulting pellet washed in 70% ethanol and vacuum-dried. The RNA pellet was solubilised in 50μl of DEPC water and stored at -80°C. Quantitation was not performed.

2.4.2   The polymerase chain reaction

Design and synthesis of primers

Primers for PCR for CD44 and K19 were designed with the assistance of Amplify 1.0 computer software (Bill Engels, University of Wisconsin, Madison, WI), using published cDNA sequences for CD44 (Dougherty et al, 1991; Stamenkovic et al, 1991) and K19 (Bader et al, 1988; Stasiak et al, 1989).

Oligonucleotides were synthesised in a Pharmacia-LKB Gene Assembler Plus DNA synthesiser according to the manufacturer's instructions, by Mr. Terrence Gooley at The Queen Elizabeth Hospital. Synthesis tubes were initially centrifuged at 4,000rpm for one minute to clear contaminants, then the synthesis cartridge was washed in 28% ammonium hydroxide at 4,000rpm for one minute, to cleave the oligonucleotides from the cartridge support. The cartridge and ammonium hydroxide were incubated at 37°C overnight to complete the cleavage. The ammonium was removed and the oligonucleotide

vacuum-dried. Oligonucleotide pellets were resuspended in 500µl of Tris-EDTA.

Oligonucleotide concentrations were quantitated using spectrophotometry (GeneQuant, Pharmacia) and then diluted in sterile water to give final concentrations approaching 100ng/ml.

PCR amplification conditions

Many of the PCR parameters were changed during the course of this work, but several fundamental conditions were maintained. Reactions were carried out in 50µl volumes in microfuge tubes, using reaction buffer supplied by Boehringer Mannheim, and 200µM of each nucleotide, dATP, dTTP, dGTP and dCTP. The reaction was overlaid with one drop of mineral oil (Sigma) to prevent evaporation of reaction components. The thermal cycler machines were water-cooled, and supplied by ARN Electronics, Belair, South Australia.

2.4.3   Gel electrophoresis

PCR products were run through 1.5% agarose gels (Progen) supported by a Biorad wide minisubmarine apparatus. The electrophoresis buffer was 0.5% TBE to which 100µl of ethidium bromide was added. The gel was visualised under UV light.

2.4.4   Southern blot analysis

When enhanced sensitivity was required (for the majority of this work), PCR products were transferred from the agarose gel to a nylon filter for radioactive labelling by an oligonucleotide specific to the target PCR product.

Southern transfer

A tray was filled with alkali transfer solution (0.4M NaOH, 0.6M NaCl, in HPW), and covered with a sheet of glass, on which two sheets of Whatman 3M filter paper were placed to form a wet wick into the transfer solution. The agarose gel was placed upside down on the wick and surrounded with cling

film. A nylon filter (Hybond-N plus, Amersham) was cut to size and placed in contact with the gel. The filter was overlaid by two more pieces of Whatman 3M paper, cut to size to fit exactly over the nylon filter. All was kept wet by transfer solution, and overlaid by a stack of absorbent paper towels. A glass plate provided light pressure over the paper towels. The Southern transfer was allowed to proceed overnight.

Radio-labelling of oligonucleotide probes

Two oligonucleotides were designed as internal probes, that is, to complement a sequence that is internal to the sequence amplified by the PCR. One was specific for the CD44 system, the other for K19. The details of these oligonucleotides are discussed in Chapters Five and Six.

Oligonucleotides were end-labelled with γ^{32}P-ATP using the Gigaprime DNA labelling kit (Bresatec). 500ng of oligonucleotide DNA were labelled with 100mCi of γ^{32}P-ATP, using polynucleotide kinase, according to the manufacturer's instructions. After end-labelling, the probe was stored in 25ml of prehybridisation mix, at room temperature.

Hybridisation of filters

Prehybridisation and hybridisation steps were carried out in a bottle in a rotary incubation oven. The transfer membrane was taken from the Southern transfer wick and placed in a bottle into which 25ml of prehybridisation mix was poured. For both CD44 and K19 systems the temperature of prehybridisation and hybridisation was 42^{0}C. Prehybridisation was performed for at least one hour. The prehybridisation mixture was then discarded and replaced by the end-labelled oligonucleotide probe, stored in 25ml of similar prehybridisation mix, for incubation at 42^{0}C, usually overnight.

64

Washing the filter

At the completion of hybridisation, the filter was washed in 2xSSC, 0.1% SDS, at 42°C, until the geiger counter measured counts less than 50 per second. Depending on the initial autoradiograph, the filter was washed again, under more stringent conditions (0.1xSSC, 0.1% SDS) if required.

Autoradiography

The filter was wrapped in cling film, excluding all air bubbles, and placed in an x-ray cassette next to x-ray film (Hyperfilm, Amersham). The film was exposed against the radioactive filter at -80°C for a minimum of four hours, and up to several days. The film was developed in an automatic developer (Kodak) in The Queen Elizabeth Hospital's X-Ray Department.

2.5 SUMMARY OF THE IMMUNOBEAD RT-PCR METHOD

The development of the method of application of a sensitive RT-PCR assay to the immunobead-cell isolation technique forms the initial part of the work towards this thesis and is described in Chapters Three, Four and Seven. Since the application of Immunobead RT-PCR to clinical samples formed the bulk of work during the latter half of this thesis, the method is summarised here.

2.5.1 Labelling immunobeads with Ber-EP4

The vial of immunobeads (Dynabeads M-450, Dynal A.S., Oslo, Norway) was taken from storage at 4°C and thoroughly resuspended. Thirty microlitres of bead suspension (12×10^6 beads) were incubated in an Eppendorf tube with 2.5µg of Ber-EP4 antibody, and 200µl of PBS/FCS/Azide buffer. Incubation proceeded at room temperature for two hours. To ensure that the beads were maintained in uniform suspension during the incubation, the tube was applied to a rotating mixer (Dynal).

To wash away any unbound Ber-EP4 antibody, the beads were pelleted by a quick centrifuge spin and the supernatant aspirated. The beads were resuspended in 1ml of PBS/FCS/Azide, and the wash repeated twice. The immunobeads were finally resuspended in 1ml of PBS/FCS/Azide buffer.

2.5.2 The efficacy of labelling of beads

Five microlitres of immunobead-buffer suspension was incubated with 5µl of phycoerythrin-conjugated anti-mouse immunoglobulin (P.E., Silenus) and 200µl PBS/FCS/Azide buffer for at least 15 minutes at room temperature. Any unbound P.E. was then washed away from the beads in the same manner as above, although using 200µl volumes of buffer. The bead pellet was finally resuspended in 200µl of buffer and the indirect binding of P.E. assessed by flow cytometry (Epics Profile II, Coulter corporation). The immunobeads would only be used if the flow cytometry results indicated that the beads were adequately bound by the Ber-EP4.

2.5.3 Incubating samples with the immunobeads

An aliquot of 3µl of the freshly labelled immunobead suspension was diluted 1 in 10 in buffer solution to enable an easier bead count under the haemocytometer. A volume of the freshly prepared stock suspension approximating 2×10^6 beads (usually approximately 150µl) was added by sterile, plugged pipette to the 10ml sterile, polypropylene tube containing the sample of blood or diluted bone marrow or PBSC, and the whole incubated for two to four hours on the rotating mixer (Dynal).

To isolate the immunobeads from the blood, the 10ml polypropylene tube was laid horizontally on a strip of magnets and secured by tape. After 15 minutes the beads were washed in PBS, by aspirating the blood away from the beads with a pasteur pipette, while the beads were held fast by the magnets.

The beads were washed three times, or until the PBS aspirated away from the beads was clear.

The tube was then stood upright on the magnets and the beads washed to the bottom of the tube, again with sterile PBS. With the beads held to the bottom of the tube by the magnets, the supernatant PBS was aspirated, the bead pellet resuspended in residual PBS (approximately 150μl), and transferred by plugged-tipped pipette to a microfuge tube where, against the magnets, the remaining PBS was more accurately aspirated. It was aimed to leave no more than 5μl PBS in association with the beads, to approach the conditions under which the RT-PCR had been optimised as closely as possible.

2.5.4   Application of the RT-PCR assay to the immunobead-cell isolates

Both the lysis and RT mixes were prepared prior to washing the beads, and allowed to stand on ice. All manipulations were performed on ice.

Lysis mix

Immunobead-cell isolates were suspended in a 9.5μl volume lysis mixture containing 0.3% v/v Nonidet P-40 (Sigma), 500ng random hexamers (Pharmacia), 20 units of RNAsin (Promega), 10mM dithiothreitol (DTT, GibcoBRL) and DEPC water.

RT mix

The RT was initiated by adding 9μl of reaction mix to give a final concentration of 50mM Tris-HCl (pH 8.3), 75mM KCl, 3 mM MgCl$_2$, 1mM each of dATP, dGTP, dCTP and dTTP, 15mM DTT and 200units of Superscript II (Gibco BRL), in a final volume of 20μl. The entire mixture was incubated at 37^{0}C for 60 minutes then the reverse transcriptase was inactivated at 70^{0}C for 10 minutes.

PCR

Seven microlitres of the RT product was taken as substrate for PCR in a final volume of 50µl. The other PCR components were added as a master mix: 10mM Tris-HCl (pH 8.3), 1.5 mM MgCl$_2$, 50mM KCl, 200µM each of dATP, dGTP, dCTP and dTTP, 100ng of each primer, sterile distilled water and one unit of Taq polymerase (Boehringer Mannheim). Primers for K19 were:

sense primer 5' GACTACAGCCACTACTACACGACC 3',

antisense primer 5' AGCCGCGACTTGATGTCCATGAGCC 3'.

The PCR conditions were 94^{0}C for 5 minutes, followed by 45 cycles of 94^{0}C for one minute, 68^{0}C for one minute and 72^{0}C for one minute, with a final 7 minute extension at 72^{0}C.

Evaluation of PCR products

A 20µl aliquot of the PCR product was then electrophoresed on a 1.5% agarose gel, visualised by ethidium bromide staining, transferred by Southern blotting to a nylon filter (Hybond-N plus, Amersham) and hybridised with an internal oligonucleotide probe 5' AGATCTGCATCTCCAGGTCGGTTCC 3', which had been end-labelled with γ^{32}P-ATP (Bresatec). Autoradiography was carried out for 4-72 hours.

2.6 COLLECTION OF HUMAN SAMPLES FOR TESTING BY IMMUNOBEAD RT-PCR

2.6.1 Bone marrow biopsies

Because of problems encountered with using archival specimens with the immunobeads (see Chapter Seven), only fresh bone marrow samples were included for study. Biopsy was performed by a Physician from the Haematology/Oncology Department, and was taken under sterile conditions, either under general anaesthesia or local anaesthesia with neurolept

supplementation. Samples were taken by trephine biopsy and by needle aspiration from the patients' iliac crest, and were assessed in the Pathology Department using routine cytology. The remaining bone marrow aspirate available for immunobead RT-PCR was usually of the order of one to two millilitres.

Bone marrow samples were transported in EDTA-containing 10ml polypropylene tubes, and diluted to 10ml in PBS. Approximately 2×10^6 immunobeads labelled with Ber-EP4 were added to the diluted bone marrow sample and incubated as described. The sample was applied to the magnets, and RT-PCR for K19 applied to the washed immunobeads as described.

2.6.2 Peripheral blood samples

Samples of peripheral blood were taken from patients being assessed or reviewed in the Haematology/Oncology Department, and whose disease was therefore in the poor prognosis category. Samples were taken at random, that is, at no specific time with respect to treatment or presentation. Quantity of sample varied from 10 to 20ml. All were transported to the laboratory in 10ml tubes in EDTA. Where two 10ml tubes had been received per patient, beads from both tubes were combined into one microfuge tube for reverse transcription.

2.6.3 Peripheral blood stem cell collections

Patients considered for the high dose chemotherapy program with autologous peripheral blood stem cell support at The Queen Elizabeth Hospital came from two groups:

Stage IV Breast Cancer

Patients included women with hormone-unresponsive metastatic breast cancer, regardless of menopausal status. They may have been previously exposed to chemotherapy or radiotherapy. Patients may have presented with a

diagnosis of metastatic disease or may have been diagnosed as metastatic upon relapse.

Stage II and III Breast Cancer

Patients included pre- and perimenopausal women with stage II breast cancer with oestrogen receptor-positive tumours with more than 10 positive axillary lymph nodes, or oestrogen receptor-negative tumours and more than 5 positive axillary lymph nodes, or stage III breast cancer that was considered to be locally incurable. None of these patients had received adjuvant chemotherapy or radiotherapy prior to entry into the high dose chemotherapy program.

The standard protocol for haemopoietic progenitor cell mobilisation on the Haematology/Oncology ward at The Queen Elizabeth Hospital involved a combination of cyclophosphamide and granulocyte-colony stimulating factor (G-CSF). G-CSF was administered at a dose of 5mg/kg by daily subcutaneous injection, commencing 72 hours after the administration of cyclophosphamide and continuing until the leukocyte count in the peripheral blood exceeded 0.5 x 10^9 per litre. Peripheral blood stem cell collections were begun when the absolute neutrophil count exceeded 0.5 x 10^9 per litre (Kotasek et al, 1994).

Blood progenitor cells were harvested using a COBE "Spectra" Apheresis System, and routine procedure involved concentrating the harvest by centrifugation, and then cryopreserving the cells in liquid nitrogen, without adding dimethyl sulphoxide. Samples of PBSC collection for immunobead RT-PCR testing were taken from the harvest bag in the fresh state, before cryopreservation, in volumes of one to two ml. Samples were quantified according to the approximate number of mononuclear cells per sample tested. The PBSC sample was diluted to 10ml in PBS in a 10ml tube in the same manner as for the bone marrow samples.

2.6.4 Peripheral blood samples taken at the time of PBSC harvest

On the day of PBSC harvest, 10 - 12ml of peripheral blood was taken by cubital fossa venipuncture from the arm contralateral to the side of venous access for the leukapheresis. Blood was transported to the laboratory in EDTA-tubes, for incubation with immunobeads in the established fashion.

The bloods taken for the first three consecutive patients were processed somewhat differently from the remainder, in that each 5ml sample (in EDTA tube) was diluted to 10ml in PBS and incubated with 2×10^6 beads, whereas subsequent samples were combined to 10ml totals and, if the sample exceeded 10ml, the beads from each incubation step were summated to give one bead pellet only for each patient.

CHAPTER THREE:

TUMOUR CELL ISOLATION BY IMMUNOBEADS

3.1   INTRODUCTION

The feasibility of tumour cell isolation from blood using magnetic beads labelled with a monoclonal antibody had already been demonstrated in the Haematology/Oncology Laboratory at The Queen Elizabeth Hospital. Hardingham and colleagues had demonstrated that labelling the immunobeads with the monoclonal antibody Ber-EP4, incubating $2x10^6$ beads with SW480 colon cancer cells in whole blood, and then applying a magnetic field to extract the beads, was a simple and efficient means of isolating tumour cells from suspension (Hardingham et al, 1993).

The antibody Ber-EP4 has now been demonstrated by both Hardingham and colleagues and Wong and colleagues to be superior to a panel of monoclonal antibodies in the efficiency of binding of cells from colon cancer cell lines (Hardingham et al, 1993; Wong et al, 1995), and its binding to colon cancer in the clinical setting has been demonstrated in a clinical study (Hardingham et al, 1995). Since Ber-EP4 is directed against an antigen that is present on both a colon cancer cell line and a breast cancer cell line, it was expected that Ber-EP4 would show similar results with breast tumours as it did with colon tumours.

The flow cytometry experiments comparing the binding of a panel of monoclonal antibodies to colon cancer cells were therefore repeated using cells from breast cancer cell lines. In addition to Ber-EP4 and EMA (Dakopatts, Gestrop, Denmark) the anti-mucin antibody BC2 (supplied by Dr. I. McKenzie of the Austin Hospital, Heidelberg, Victoria) was tested against the breast cancer cell lines. The cross-reactivity of EMA with some haemopoietic cells

virtually precluded its practical use in labelling the immunobeads, but published work regarding BC2 suggested that it might approach the efficacy of Ber-EP4 in recognising epithelial cells (Devine et al, 1992).

3.2 RESULTS OF ANTIBODY BINDING TO BREAST CANCER CELLS

3.2.1 Flow cytometry

Cell lines

Approximately 1 x 10^6 cells from each breast cancer cell line were incubated with the antibodies Ber-EP4, Dako-EMA and BC-2 at concentrations of 0.5µg to 1.25µg per 10^6 cells, indirectly labelled by incubation with 5µl P.E. (Silenus), and assessed by single colour fluorescence using flow cytometry, in the manner described in Chapter 2.5.2.

In all cell lines tested, Ber-EP4 displayed virtually complete binding to the breast cancer cells. The results with the anti-mucin 1 antibody BC-2 were disappointing, with a very poor showing against MB-453. The median fluorescence of flow cytometry was consistently superior with Ber-EP4. The performance of EMA was marginally better than BC-2, but also poor against MB-453. Dako-CEA had been tested against MCF-7 cells, but also performed poorly. Most experiments therefore involved comparisons of Ber-EP4 against BC2 (see figures 3.1 and 3.2).

	ANTIBODY BINDING (median)		
	EMA	BC2	Ber-EP4
MCF-7	87.20%	75.40%	99.90%
T-47D	95.50%	71.00%	99.80%
MB-453	12.00%	nil	100.00%

Table 3.1:   Binding of selected antibodies to breast cancer cell lines tested by flow cytometry.

Fresh breast cancer cells in suspension

Aliquots of 5×10^5 cells from one of the lymph nodes macroscopically replaced by breast cancer metastasis were incubated with the Ber-EP4 and BC2 antibodies, again at concentrations of 0.5μg to 1.25μg per 10^6 cells, and assessed by flow cytometry, as above.

Histograms for Ber-EP4 and BC-2 were similar, with Ber-EP4 showing 91.7% median binding and BC-2 85.0%. Ber-EP4 displayed slightly greater fluorescence (figure 3.2). Many unhealthy cells and other debris (e.g. macerated fibrous tissue) contributed to the "left scatter" on the histograms, so with this background "noise" taken into account, both antibodies showed favourable results against the metastatic human breast cancer cells. The absence of binding of the pan-leukocyte marker CD45 (a pan-leukocyte marker) confirmed the macroscopic and microscopic impressions of total nodal replacement by metastatic tumour.

Normal lymph node

Cell suspension prepared from the normal submandibular node was incubated as above with Ber-EP4 and BC2 antibodies, to act as a negative control, to establish that neither Ber-EP4 nor BC2 were binding to lymphoid cells in the nodal suspensions. In addition, cells were stained with CD45 and CD14 (a monocyte marker) to identify the predominant cell populations.

Fluorescence with CD45 indicated similar leukocyte distributions to other normal nodes (B. Farmer, B.Sc., Haematology/Oncology Department, personal communication), showing 97% binding on histogram analysis. Very few monocytes were present by CD14 staining. Median binding of 1.3% and 2.6% for Ber-EP4 and BC-2 respectively, was consistent with non-specific uptake of P.E. by dead cells and debris (B. Farmer, pers. comm.).

3.2.2 Immunohistochemistry

One fresh-frozen tumour sample and 8 specimens stored in liquid nitrogen were assessed for Ber-EP4 staining by Ms V. Pascoe in the Histopathology Department at The Queen Elizabeth Hospital. Initial comparisons of frozen and paraffin sections from the fresh-frozen tumour sample showed generally better staining of frozen than paraffin-fixed tissue (consistent with the results of Latza et al, 1990). In the frozen tissue all tumour cells were positive for Ber-EP4, whereas paraffin section of the same tumour showed variable staining, with approximately 80% of the tumour cells positive. The intensity of staining of frozen tissue also seemed better: there was a constant, moderate to strong intensity of staining of frozen tissue, and a variable weak to strong intensity with the paraffin-fixed specimen.

Therefore, only freshly frozen tumours were used to assess avidity of Ber-EP4 for tumour cells, and the bank of tumours stored in liquid nitrogen provided the most easily accessible source of specimens. All 8 samples tested showed homogeneous staining of all tumour cells by Ber-EP4 (see figure 3.3).

3.3 DISCUSSION

The ideal antibody for labelling of the immunobeads is one that could be expected to bind reliably to the vast majority of breast cancer cells. Such an antibody would have to satisfy the following criteria:

1) The antibody should recognise an epitope on the surface of the target cell, rather than in the cytoplasm.

2) The antibody should react with the vast majority of target cells.

3) The antibody should recognise its target in a pattern that is homogeneous rather than heterogeneous, such that recognition of the target is as reliable as possible.

Figure 3.1   **Flow cytometry results of antibody binding to cell lines.**
Comparisons of Ber-EP4 and BC-2 are shown for the cell lines
MB-453 and T-47D.

MB-453

BC-2, 2µl
and 5µl

Ber-EP4,
2µl

T47-D

Ber- EP4
2µl

BC-2, 5µl

BC-2, 2µl
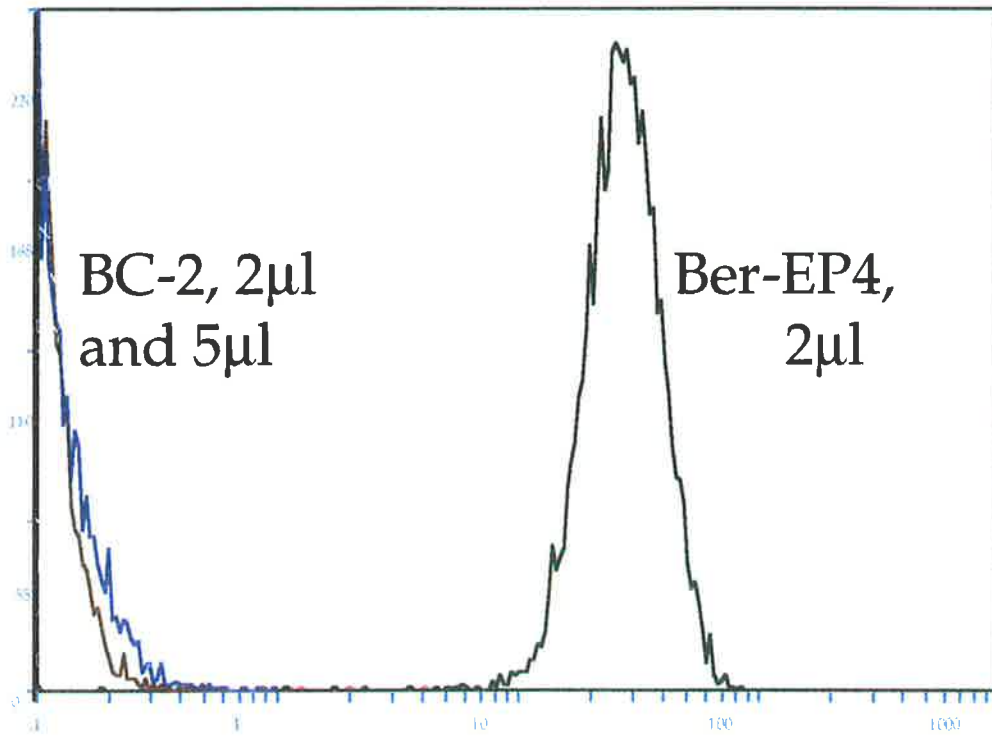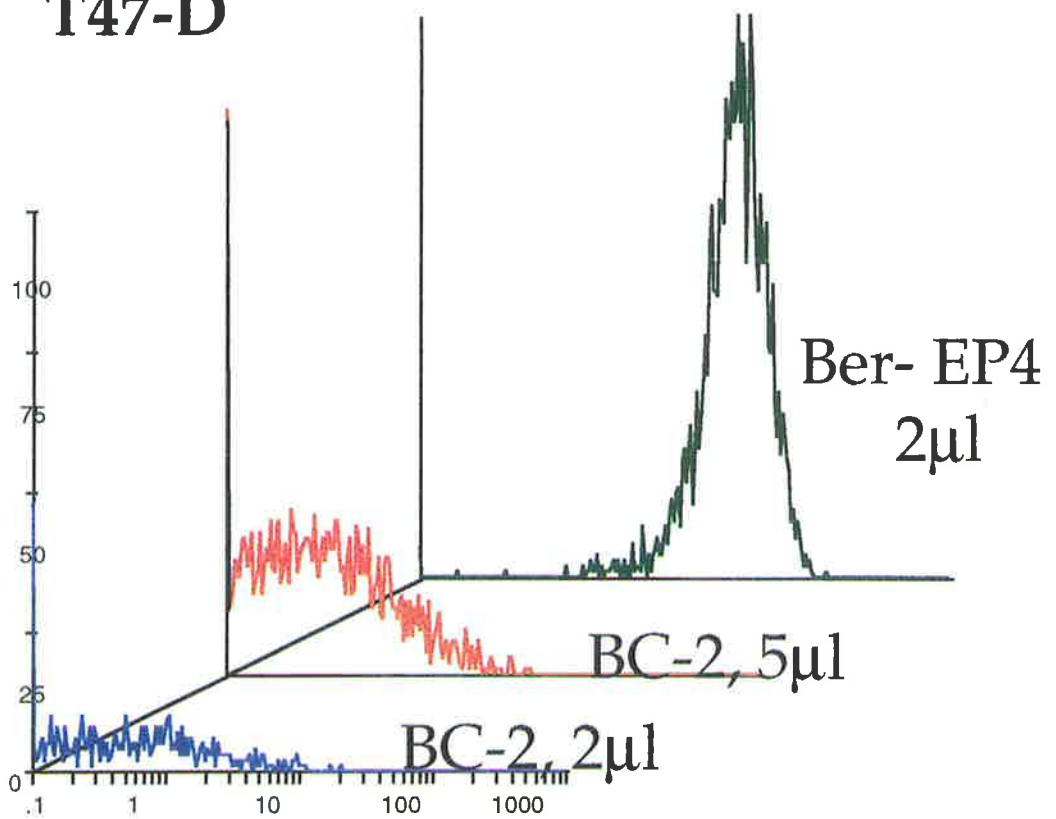
Figure 3.2   Flow cytometry results of antibody binding to the MCF-7 cell line (top) and to metastatic breast cancer cells, taken from a lymph node replaced by cancer (bottom).

MCF-7

130

Count

0

.1   1   10   100   1000

EMA

CEA

Ber-EP4

MALIGNANT LYMPH NODE

200

150

100

50

0

BC-2,5UL

Ber-EP4, 5UL

.1   1   10   100   1000
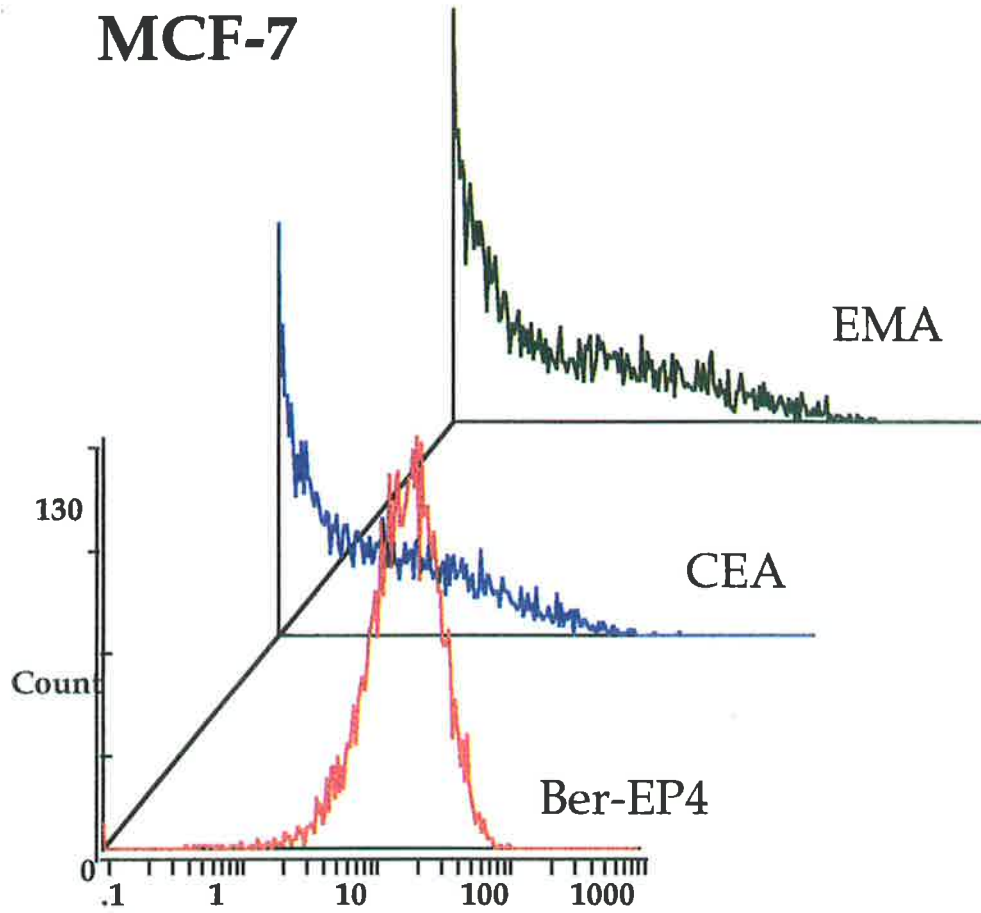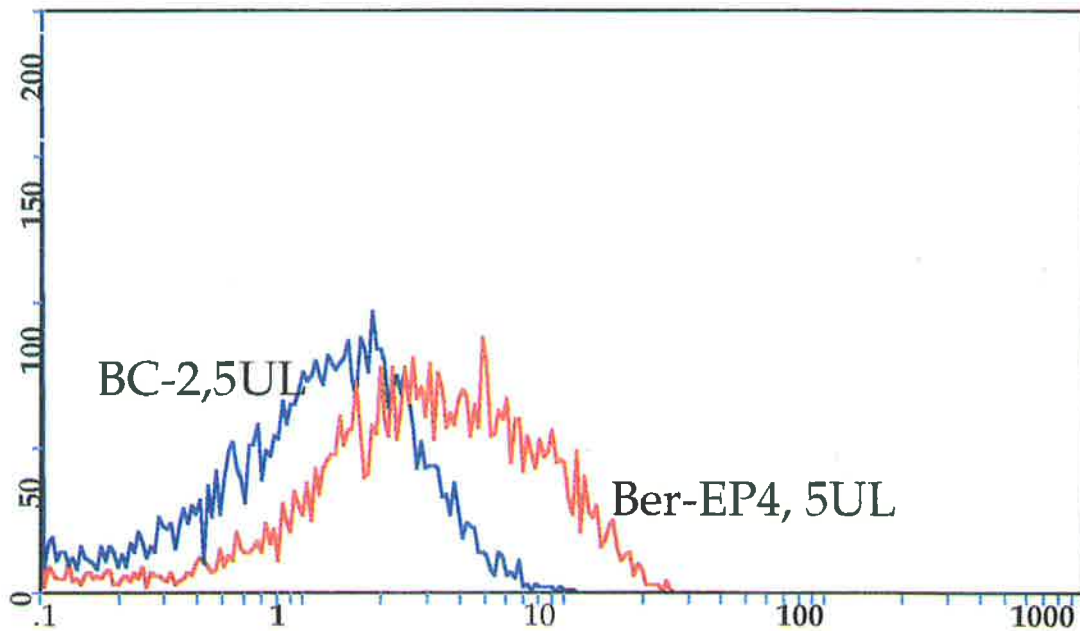
Figure 3.3   **Staining of frozen breast cancer specimen with Ber-EP4.**

A)   Example of frozen breast cancer specimen, without
staining by Ber-EP4.

B)   Section from the same specimen of frozen breast cancer,
showing epithelial cells bound by Ber-EP4, stained red.
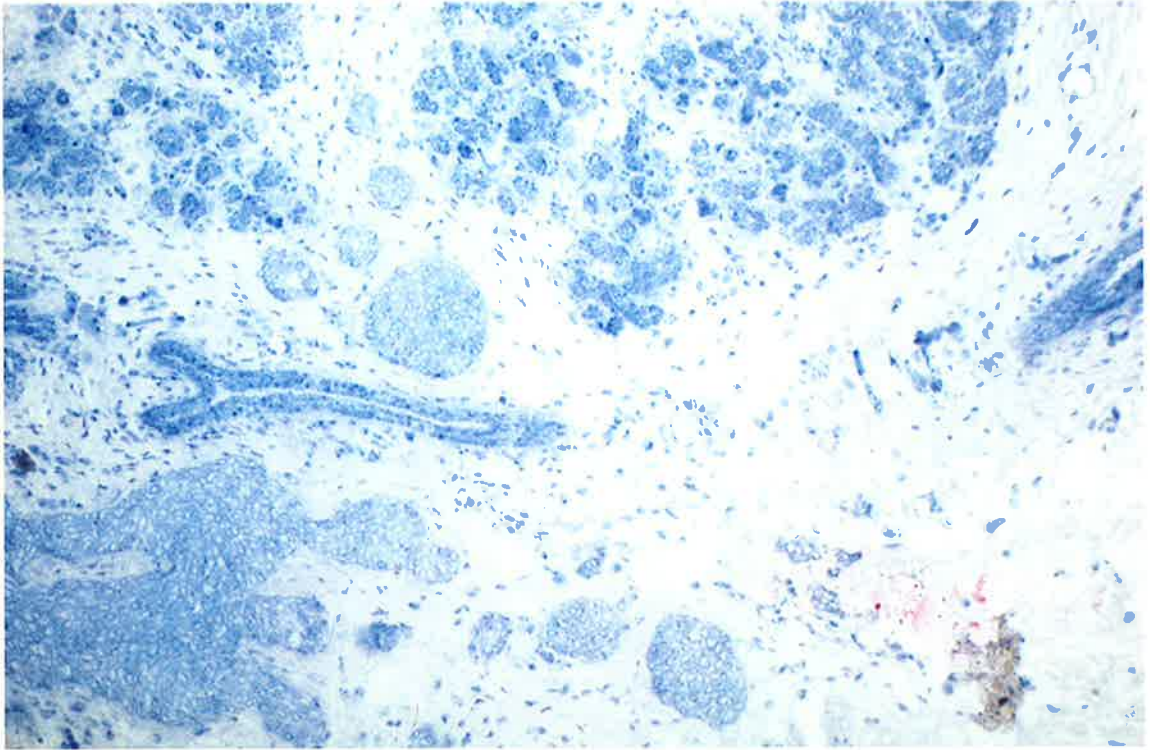
A
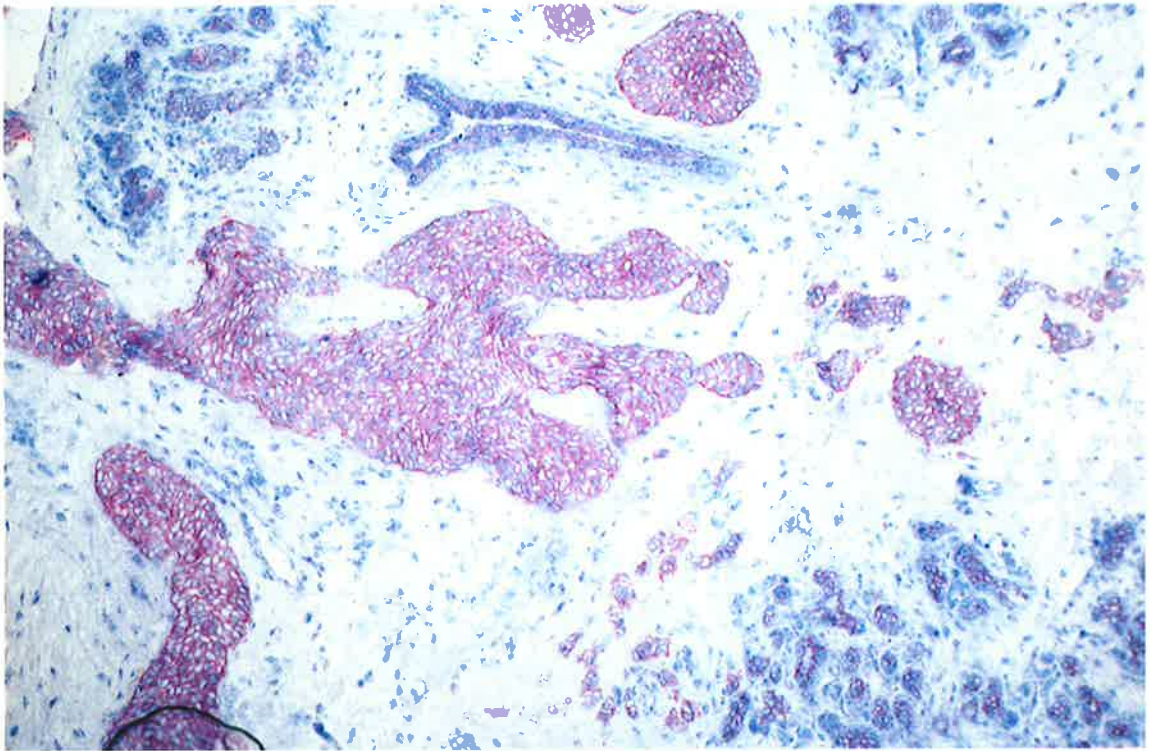
B

4) The antibody should be specific for its target, that is, it should show a minimum of cross-reactivity with non-target cells.

The above criteria for antibody selection for use in the immunobead method exclude several antibodies that are in common use in immunohistochemical diagnostic techniques. Anti-cytokeratin monoclonal antibodies are excluded because of the exclusive cytoplasmic location of the cytokeratins (Latza et al, 1990). CEA and EMA have disadvantages because they are not expressed in all epithelia, their expression in a given tumour is often heterogeneous, and they may be expressed in non-epithelial cells (Delsol et al, 1984; Latza et al, 1990).

The results of Ber-EP4 binding to breast cancer cell lines and breast cancer tissues presented above concur with the results of others, as discussed in the introductory chapter. Ber-EP4 has been repeatedly demonstrated to bind to the vast majority of epithelial cells, with minimal non-specific binding. The assertion of Edwards (Edwards, 1985) that "there must be some surface molecules present on all of the cells of an epithelium that ought to be homogeneously expressed, epithelium-specific antigens" is borne out in the case of Ber-EP4. The eight samples of freshly frozen breast cancer tissue all displayed homogeneous staining of their epithelium by Ber-EP4.

The strong binding of Ber-EP4 to the breast cancer cell lines is consistent with the results of Latza and colleagues (Latza et al, 1990), who demonstrated that Ber-EP4 reacted strongly against the five breast cancer cell lines tested, and against both acinar and ductal epithelium of human breast tissue.

Ber-EP4 failed to react with cells from the normal lymph node, tested by flow cytometry. The specificity of Ber-EP4 for epithelial cells is confirmed by the work of Passlick and colleagues, who demonstrated an absence of immunohistochemical staining with Ber-EP4 in lymph nodes from 24 patients

76

with conditions other than carcinomas (Passlick et al, 1994). The specificity of Ber-EP4 for epithelial cells is one its advantages for use with immunobeads, and allows application of the immunobead technique to essentially all carcinomas. The specificity of the immunobead method is therefore determined by the marker chosen for PCR amplification.

While BC2 performed reasonably well against fresh breast cancer cells taken from a lymph node, the result was nevertheless inferior to that seen with Ber-EP4. Ber-EP4 was chosen as the sole antibody to label the immunobeads for breast cancer cell recognition because it satisfied all the criteria listed above and because its binding efficacy to breast cancer samples was demonstrated unequivocally. BC2 was not considered further because of its relatively poor binding to the cell lines.

The cell suspensions taken from the fresh axillary lymph nodes macroscopically replaced by tumour proved an invaluable resource for studying metastatic breast cancer cells. Flow cytometric study of specimens of primary tumour proved unsatisfactory because the fibrous nature of the breast cancers made their dissociation through a sieve difficult, since cells could not be placed into suspension without being destroyed by the mechanical forces required to break up the tumours. Protocols have been described for tumour dissociation by collagenase digestion, but this might decrease the activity of Ber-EP4 since protease treatment is known to decrease Ber-EP4 reactivity (Latza et al, 1990), and the presence of contaminating cells e.g. stromal cells and myoepithelial cells, could artificially decrease the measurements. Lymph node samples have proved easy for flow cytometry assessment of cell surface markers in cases of lymphoma, because the relatively weak fibrous capsule of the lymph node allows easy mechanical dissociation into individual cells. This observation was applied to samples of axillary lymph nodes in breast cancer

patients. Where an axillary node was replaced by metastatic breast cancer, it was easy to dissociate the cancer cells into cell suspension in PBS, with a minimum of contaminating stromal cells or fibrous lymph node capsule or internal architecture, and without the need to add any proteolytic enzymes that might alter cell surface antigens or antibody properties. The only disadvantage of using such lymph nodes is that presentation of a patient with bulky axillary nodal disease is relatively uncommon in modern clinical practice. The two lymph node samples obtained therefore proved to be of considerable value and use in the laboratory.

CHAPTER FOUR:

RT-PCR FOR VERY SMALL NUMBERS OF CELLS

4.1   INTRODUCTION

The application of an RNA-based marker to the immunobead-cell isolation technique required reliable reverse transcription of RNA from as few cells as possible. This chapter describes the development and optimisation of a method for RT-PCR from very low cell numbers. Three major aspects are considered: isolation of RNA from very small cell numbers, reverse transcription of a small amount of RNA, and the polymerase chain reaction.

4.2   RNA EXTRACTION

4.2.1   Introduction

In 1987 Chomczynski and Sacchi published their method of single-step isolation of RNA by guanidinium thiocyanate-phenol-chloroform extraction, and this has since become a widely-used method for rapid RNA extraction from limited quantities of tissue. Guanidinium thiocyanate is a denaturing agent, and a strong inhibitor of ribonucleases, and this is combined with phenol-chloroform extraction of the RNA, followed by precipitation of RNA from the aqueous phase by isopropanol (Chomczynski and Sacchi, 1987). This method requires transfers between tubes and multiple centrifuge steps, all of which introduce potential for losing nucleic acid, and consequently the method is unsuitable for obtaining reverse transcription product from very small cell numbers.

Chomczynski and Sacchi reported successful RNA isolation from one million cells in tissue culture, but the number of cells isolated by immunobeads

in the study of circulating tumour cells was anticipated to be much fewer than this.

4.2.2   Detergent lysis of cells by Nonidet P-40

Introduction

Techniques for RT-PCR from low cell numbers have relied on some form of direct cell lysis to release the nucleic acids The approach used in the development of immunobead RT-PCR was to first establish a means of cell lysis that would allow reverse transcription of the released RNA with minimal degradation or loss of the RNA. It was recognised that the development of an efficient reverse transcription method was fundamental, since the power of PCR to amplify low copy number of target sequence was well established.

Published techniques for cell lysis for RT-PCR vary from detergent lysis (Furukawa et al, 1994) to heat lysis (O'Brien et al, 1994; Kumazaki et al, 1994), hypotonic lysis (Edmands et al, 1994), and freeze-thawing (Eggeling et al, 1995).

The non-ionic detergent Nonidet P-40 (NP-40, Sigma) was chosen for investigation as a means of lysing tumour cells because of its known use in the preparation of nuclei for DNA extraction, and also because it is known to be compatible with reverse transcriptase enzyme, with the ribonuclease inhibitor RNasin (Promega), and with Taq polymerase (Nonidet P-40 is a component of Taq storage buffer).

The concentration of NP-40 required for adequate cell lysis was determined.

Materials and Methods

NP-40 was diluted in DEPC-water to make a final concentration of 1%. Serial dilutions were carried out further in DEPC-water to provide final solutions of 1%, 0.5%, 0.1% and 0.01% NP-40.

Aliquots of 5×10^5 cells from the MB-453 breast cancer cell line in PBS suspension were distributed amongst five Eppendorf tubes. In order to avoid dilution of the detergent solutions by the PBS in the cell suspensions, the Eppendorf tubes were centrifuged at 5,000rpm for five minutes to pellet the cells so that the supernatant PBS could be aspirated and discarded. Each pellet was resuspended in 35μl of one of the four concentrations of NP-40, with the fifth Eppendorf pellet resuspended in DEPC-water as a control. After standing for five minutes an equivalent volume of trypan blue was added to each, and the results assessed by light microscopy.

Results and Discussion

The suspension of MB-453 cells in DEPC-water only showed many intact, unstained cells, with occasional cells that had taken up the trypan-blue stain. At the concentration of 0.01% there were many swollen but otherwise intact cells, with more cells that appeared as pale-blue "spots", with no apparent cell membrane. The appearances suggested inadequate cell lysis at 0.01% NP-40 concentration. At 0.1% and 0.5% NP-40 solutions, there were fewer pale-blue "spots", and the only intact cells that remained were in occasional clumps. There was mostly only debris present, suggesting that the majority of cells had lysed. 1% NP-40 provided the greatest evidence of cell lysis. No intact cells were evident, only very occasional pale-blue "spots".

It was concluded that an NP-40 concentration above 0.1% would provide adequate cell lysis, being considerably better than that provided by DEPC-water alone. These results were reproducible, and were supported by a subsequent experiment in which approximately 10^3 cells from the T47-D cell line were lysed for RT-PCR using primers for CD44 (discussed in Chapter Five), and it was found that 1% and 0.3% NP-40 concentration in the final master mix gave superior results to 0.1% NP-40.

4.2.3   Assessment of methods of cell lysis

4.2.3.1   Introduction

Means of lysing cells for RT-PCR for low copy number have been briefly discussed (Edmands et al, 1994; Kumazaki et al, 1994; O'Brien et al, 1994; Eggeling and Ballhausen, 1995; Furukawa et al, 1994). This method was initiated along the lines of Furukawa and colleagues, using NP-40 detergent lysis with RNasin (Furukawa et al, 1994), but it was necessary to determine whether this was indeed the most efficient mode of cell lysis. NP-40 lysis was therefore compared with heat lysis and with freeze-thawing.

4.2.3.2   Comparing NP-40 with heat lysis

Materials and Methods

Peripheral blood mononuclear cells in PBS suspension were distributed into aliquots of 2μl, by serial dilutions, of cell numbers approximating 700, 70 and 7 MNCs, in two groups. One group had cells lysed in the standard manner (described in section 4.3.2, and in 2.5.4), the other had the NP-40 detergent replaced in the "lysis mix" by DEPC water, and the cells lysed by heating to 90^{0}C for 10 minutes, according to the method of O'Brien (O'Brien et al, 1994).

The PCR was performed using primers for CD44, taking 2μl of reverse transcription product for PCR (see 4.3.2). Southern transfer was not performed.

Results

The lysis method using NP-40 appeared far more efficient than that with heat lysis. On this occasion bands conforming to the standard isoform of CD44 were evident in all NP-40 lanes, being brightest for 700 cells, and even appearing in the lane approximating 7 cells. A weak signal appeared for 700 cells only by the heat lysis method (see figure 4.1).

Figure 4.3   **RNasin and DTT are required for efficient reverse transcription.**

RT-PCR for CD44 on 600, 60 and 6 MNCs, showing best results when RNasin and DTT were included in RT mix.

Lanes 1 and 3:	Positive controls
Lane 2:	Negative control
Lane 4:	600 MNCs, with RNasin and DTT
Lanes 5 and 6:	60 MNCs, with RNasin and DTT
Lane 7:	6 MNCs, with RNasin and DTT
Beyond Lane 7:	No signal from the same cell samples in absence of RNasin and DTT

Figure 4.1   Comparison of NP-40 lysis with heat lysis.

RT-PCR for CD44, using 700, 70 and 7 MNCs.

Lanes 1 and 3:	Positive controls
Lane 2:	Negative control
Lanes 4 to 7:	700, 70, 70 and 7 MNCs respectively, with cell lysis by NP-40
Lanes 8 to 11:	The same cell samples as above, but with cell lysis by heating.

CD44$_E$ —

CD44$_S$ —

1 2 3 4 5 6 7

CD44$_E$ —

CD44$_S$ —

1 2 3 4 5 6 7 8 9 10 11
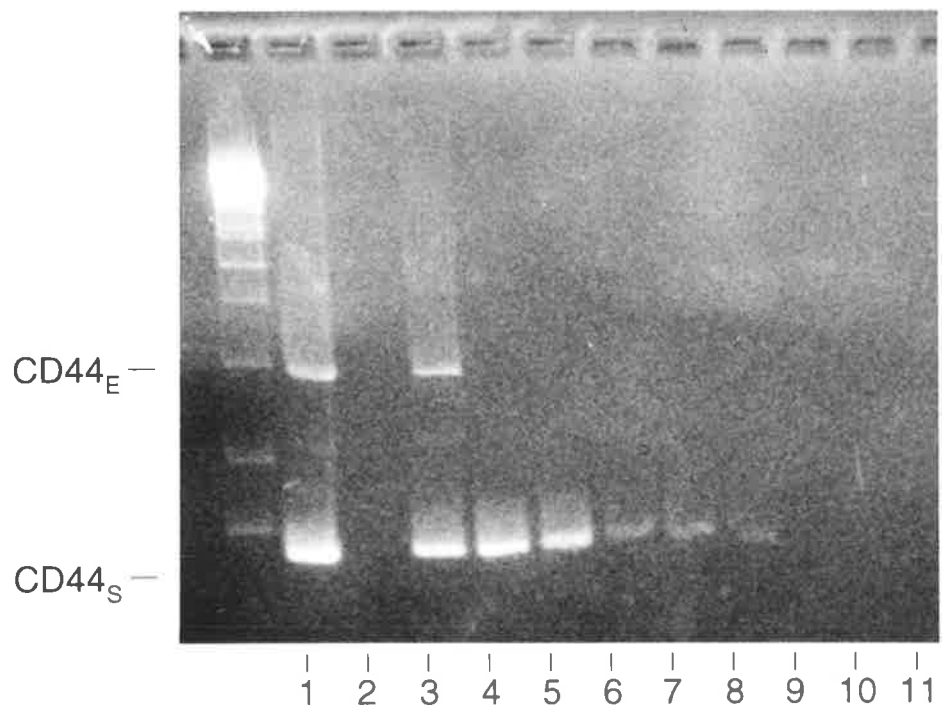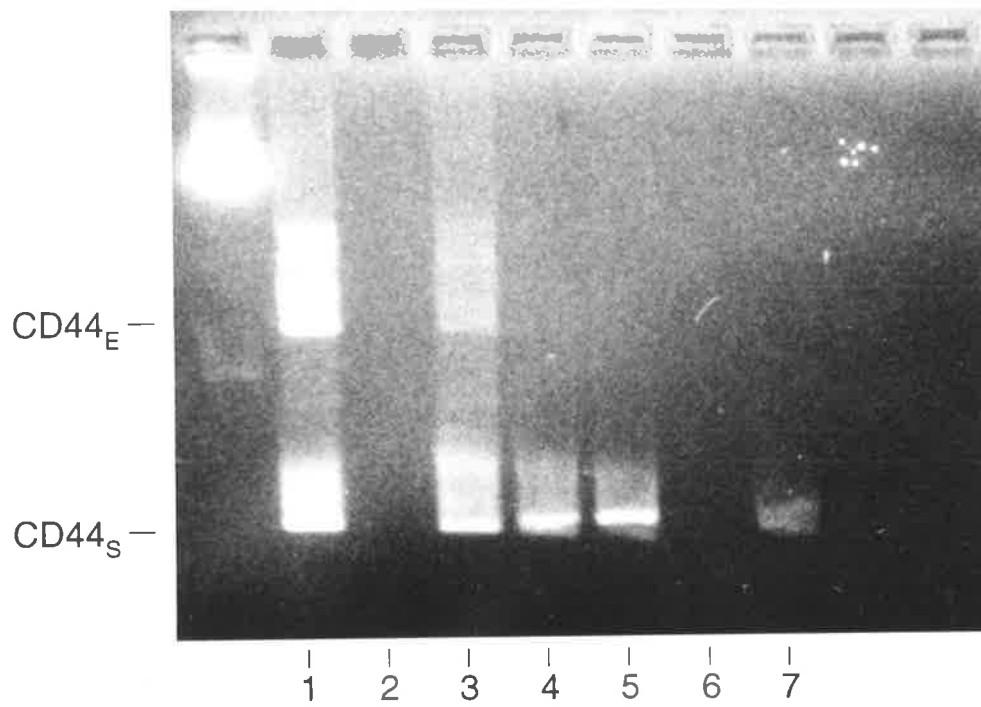
Figure 4.2   **Comparison of NP-40 lysis with freeze-thaw lysis.**

RT-PCR for K19, using approximately 200 T-47D cells per sample.

Lanes 1 and 2:	Positive controls
Lane 3:	Negative control
Lanes 4 to 7:	Two groups of 200 cells, lysed by separate preparations of NP-40
Lanes 8 and 9:	NP-40 cell lysis, at -80^{0}C
Lanes 10 and 11:	Cell lysis at -80^{0}C only
Lanes 12 and 13:	Negative controls
Lane 14:	Positive control

751 bp —

1 2 3 4 5 6 7   8 9 10 11 12 13 14
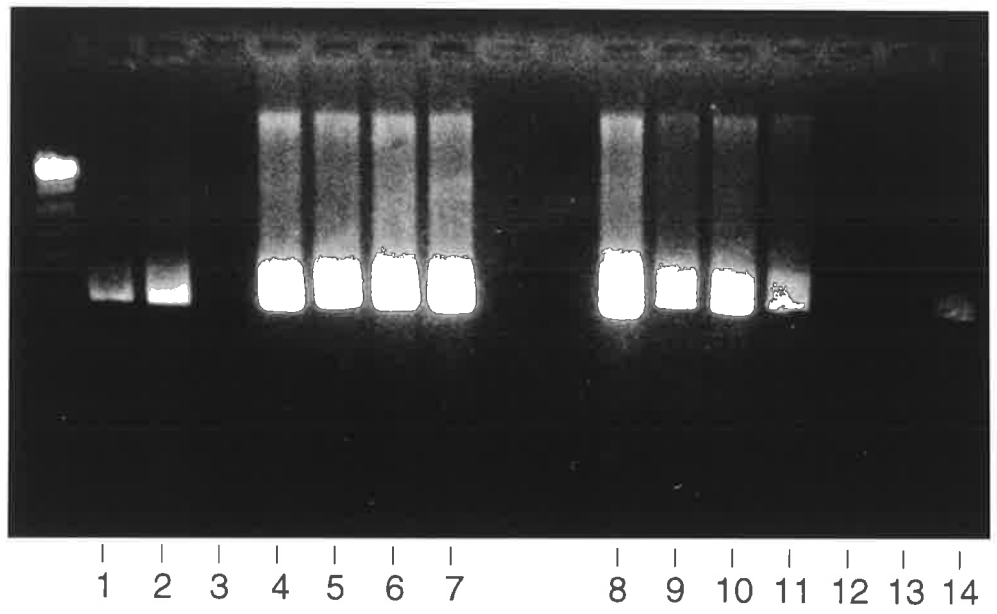
4.2.3.3 Comparing NP-40 with freeze-thawing

Introduction

The following experiment was prompted by the observation that 100 to 200 T47-D cells stored and lysed at $-80^{\circ}C$ in 5 to $10\mu l$ of PBS were proving to be very good controls for reverse transcription from low cell numbers, by displaying strong bands on ethidium bromide staining on RT-PCR for cytokeratin 19 (see Chapter Seven).

Materials and Methods

Two different solutions of 1% NP-40 were compared against 1% NP-40 with freeze-thawing and against freeze-thawing alone.

T-47D cells in PBS suspension were distributed into aliquots of $2\mu l$, each containing approximately 200 cells, in four groups of two. Two groups had cells lysed in the usual manner, at room temperature, using two NP-40 solutions prepared at different times (see "lysis mix", section 4.3.2). The third group had the cells lysed in NP-40, but at a temperature of $-80^{\circ}C$. The fourth group had cells lysed at $-80^{\circ}C$ only, without NP-40. The remainder of the reverse transcription mix was identical for all groups (see 4.3.2).

Two microlitres of the $20\mu l$ reverse transcription product was taken for PCR, on this occasion using primers for K19 (see Chapter Seven). Each well of the agarose gel was loaded with $20\mu l$ of PCR product.

Results

T-47D cells lysed in the NP-40 lysis solutions gave providing K19 bands of maximum intensity. The bands from samples that had been stored at $-80^{\circ}C$ were easily evident on ethidium bromide staining, but generally were not as strong as those from room temperature lysis. While good results were seen with cell lysis from freeze-thawing, they were certainly not superior to NP-40 lysis at room temperature, and adding freeze-thawing to NP-40 lysis did not seem to

improve results, suggesting that the 1% NP-40 lysis mixtures were providing adequate cell lysis (see figure 4.2).

4.3 REVERSE TRANSCRIPTION

4.3.1 Introduction

Routine laboratory protocols for reverse transcription were designed for application to a large RNA template. In preliminary work, reverse transcriptions were performed in 40µl volumes, using 200units of Moloney murine leukaemia virus-reverse transcriptase (MMLV-RT, GibcoBRL), and NP-40 detergent for cell lysis and 40units of RNasin ribonuclease inhibitor (Promega). In these experiments, PCR product, using CD44 primers, could be obtained from only a minimum of 10^3 breast cancer cell line cells . There was little improvement in sensitivity whether the lysed cells were breast cancer cell lines or peripheral blood mononuclear cells. The "best" result using this standard reverse transcription method was CD44 signal on ethidium bromide-stained agarose gel of 200 T-47D cells. This degree of sensitivity was unlikely to prove adequate for an immunobead RT-PCR method.

4.3.2 Reverse transcription from very small numbers of cells

Materials and Methods

A new assay for reverse transcription of low cell numbers was developed, using NP-40 for cell lysis, Superscript II reverse transcriptase (GibcoBRL) and a 20µl volume reverse transcription assay that was a modification of that recommended by the manufacturer (GibcoBRL).

The 20µl reverse transcription reaction volume was divided into two separate "mixes": a "lysis mix" in which the target cells were lysed to release their nucleic acids (in the presence of RNasin and DTT), and a "reaction mix" to add the other components necessary for reverse transcription of the RNA.

Assuming a volume of cells in suspension of approximately 2µl, the 9.5µl "lysis mix" for a single reaction was:

2µl (500ng) random hexamers (Pharmacia)

3µl 1% NP-40 (Sigma)

0.5µl (20units) RNasin (Promega)

1µl 100mM DTT (GibcoBRL)

3µl DEPC-water

Plus cell suspension or immunobead - cell isolate.

The 9µl "reaction mix" for a single reaction was:

4µl 5X first strand buffer (GibcoBRL)

2µl 100mM DTT (GibcoBRL)

2µl 40mM dNTPs

1µl (200units) Superscript II reverse transcriptase (GibcoBRL)

This method is also summarised in Chapter Two.

The 9.5µl "lysis mix" and the 9µl "reaction mix" (per reaction) were prepared in advance in microfuge tubes and stored on ice while the cell suspensions were prepared. The RNasin and reverse transcriptase were the last substrates to be added, to minimise the possibility of enzyme degradation. Cells were counted under a haemocytometer, then serially diluted so that the desired cell number would be contained in no more than 5µl of PBS. The aliquots of cell suspension were then added by pipette to the lysis mixes, mixed by pipette, and the tubes heated to 70^{0}C for 10 minutes to denature the RNA. The tubes were then briefly chilled on ice, briefly centrifuged, then each received the 9µl reaction mix. All reactions were incubated at 37^{0}C for 60 minutes then inactivated at 70^{0}C for 10 minutes. Reverse transcription products were stored at either 4^{0}C or -20^{0}C until used for PCR.

Results

RT-PCR results for low cell numbers using the modified Superscript II protocol for reverse transcription were consistently and dramatically improved. Using serial dilutions, a PCR product could be reliably obtained from less than 20 cells, either PBMNCs or from cultures of T47-D, using PCR primers for CD44. Occasionally CD44 product was seen on ethidium bromide for samples containing what was thought to be (by dilutions) less than 10 PBMNCs. These results were seen during further optimisation experiments, and will be discussed later.

Discussion

1) *Superscript II*

Superscript II reverse transcriptase (GibcoBRL) is a more highly purified version of MMLV-reverse transcriptase (GibcoBRL), with almost total absence of detectable RNase H activity, and is claimed by its manufacturers to give a higher yield of cDNA and a longer cDNA product than MMLV-RT and other reverse transcriptases (GibcoBRL technical literature). It is presented in a storage buffer that includes 1mM DTT and 0.01% (v/v) NP-40 and is packaged with a 5X first strand buffer (250mM Tris-HCl, 375mM KCl and 15mM $MgCl_2$) and 100mM DTT.

2) *The GibcoBRL protocol for Superscript*

The GibcoBRL protocol for first strand cDNA synthesis of minimal RNA target describes a 20µl reaction volume commencing with a 12µl primer mix to which an 8µl RT mix is added. Oligo dT is the recommended RT primer, and the mix is made up in sterile, distilled water. Denaturation of the RNA in the primer mix is recommended, by heating the mixture to 70°C for 10 minutes, followed by a quick chill on ice. The reaction mix containing the first strand buffer, DTT and mixed dNTP stock is then added and the contents gently

86

mixed. Superscript II reverse transcriptase (200units) is then introduced, followed by incubation at 37°C for 60 minutes. Inactivation of the enzyme is recommended by heating to 70°C for 10 minutes.

3) *A modified "reaction mix"*

This protocol was modified to make it more suitable for target RNA obtained by cell lysis. Firstly, the "reaction mix" required 4µl of the 5x first strand buffer in the final 20µl volume to give the appropriate final concentrations of 50mM Tris-HCl (pH 8.3), 75mM KCl and 3mM MgCl$_2$. The Superscript II reverse transcriptase required 2µl 100mM DTT for enzyme stability, making a final 10mM concentration. Two microlitres of the dNTP mix (40mM) made a final concentration of 1mM each of dATP, dGTP, dCTP and dTTP. Adding 1µl of Superscript (200units) gave a total "reaction mix" per reaction of 9µl.

4) *A modified "lysis mix"*

Allowing for a volume of supernatant with the immunobead-cell isolates of approximately 2µl, the remaining volume allowed for the "lysis mix" was approximately 9µl. RNasin (Promega) was believed necessary to inhibit the ribonucleases that would be released by the lysed cells. RNasin has a broad spectrum of activity against ribonucleases, binding the ribonucleases in a 1:1 ratio. RNasin is inactivated at temperatures exceeding 50°C, and requires a minimum of 1mM DTT to maintain activity. The manufacturer's protocol (Promega) recommended that the concentration for use be 1 unit per microlitre, and in an approximately 10µl Lysis Mix this would equate to 10units. RNasin is marketed as a 40units/µl concentration, therefore 0.25µl would be required. For easier manipulation, and to ensure an adequate amount of RNasin in the cell lysate, 0.5µl was included per reaction. The RNasin comes in a buffer that contains 8mM DTT, so dilution in a lysis mix to 11µl would require additional

DTT to maintain a concentration to protect the RNasin. One microlitre of 100mM DTT was therefore added to the "lysis mix".

The random hexamer primers (Pharmacia) were prepared as a 250ng/µl solution, so that 2µl would be added to provide the 500ng required for each reaction, which would be easier to manipulate than a 1µl aliquot. Diluting the 1% NP-40 detergent by one third in the final "lysis mix" would still provide a 0.3% concentration, which previous experiments had demonstrated was adequate for full cell lysis, and which was in the order of the amount used by other investigators (Furukawa et al, 1994).

Oligonucleotides of random sequence ("random hexamers") were chosen in preference to oligo(dT) primers as the means of "priming" the RNA for reverse transcription to cDNA. Oligo(dT) binds to the poly(A) "tail" sequence at the 3' terminus of mRNA and primes the synthesis of the first strand of cDNA from this point. This has the disadvantage of over-representing sequences nearest the 3' end, and potentially may fail to synthesise a desired target sequence that is sited too far from the 3' end. Random hexamers prime cDNA sequences from multiple points on the mRNA template, and are sufficiently diverse to maximise the chances of synthesis from the greatest amount of mRNA. It was felt that random hexamer primers would allow the greatest flexibility in PCR amplification of whatever RNA-based marker was chosen.

5) *Conclusion*

The RT-PCR assay showed a marked improvement in sensitivity using the modification of the GibcoBRL Superscript II protocol. The main factors that had been changed were the reduction in reaction volume from 40 to 20µl, and the change of reverse transcriptase enzyme from MMLV-RT to Superscript II.

4.3.3   RNasin/DTT is necessary for RT by cell lysis

Introduction

In a new system with little information about interactions of many of its components, it was felt necessary to establish the need for the main components. Hence an experiment was designed to investigate whether RNasin and its DTT were required to inhibit ribonuclease degradation of the RNA, or whether RNasin could be omitted from the system.

Materials and Methods

Peripheral blood mononuclear cells in PBS suspension were distributed into aliquots of 2µl, containing two groups, of cell numbers approximating 600, 60 and 6 MNCs (by the technique of haemocytometer counting and serial dilutions). One group had RNasin and DTT included in the "lysis mix" as usual, the other had the RNasin and DTT omitted from the "lysis mix", and the difference made up by DEPC water.

The PCR was performed using primers for CD44, taking 2µl of reverse transcription product for PCR. Southern transfer was not performed.

Results and Discussion

Under ethidium bromide staining bands only appeared in lanes where RNasin and DTT had been used in the "lysis mix", with apparently complete failure of reverse transcription in the absence of RNasin and DTT in the cell lysates. Strong bands conforming to the standard form of CD44 were seen for 600 MNCs and one of the two lanes for 60 cells, with a weak band appearing in the lane for 6 cells, with RNasin and DTT added (see figure 4.3). The absence of a PCR product in the second 60 cell lane may have been due to a sampling error. The positive result for only 6 cells against the negative result for one lane of 60 cells may illustrate the limitations of accuracy of the technique of haemocytometer counting and serial dilutions at low cell numbers.

4.4 CONCLUSIONS REGARDING THE ASSAY FOR REVERSE TRANSCRIPTION

The conclusions drawn from the above experiments were:

1) The non-ionic detergent NP-40 provides cell lysis at room temperature that is not improved upon by combinations of other methods of lysis such as heating or freeze-thawing.

2) A ribonuclease inhibitor is necessary to maintain integrity of RNA for satisfactory reverse transcription to complementary DNA. RNasin (Promega) is a convenient and adequate RNase inhibitor.

3) The reverse transcriptase Superscript II (GibcoBRL) provides potent reverse transcription of low copies of RNA, and works efficiently in a reaction volume of 20µl.

4) The modified Superscript protocol for reverse transcription allows amplification of genetic message by RT-PCR from less than 10 cells.

Heat lysis and lysis by freeze-thawing provide methods for RNA extraction for low cell numbers that are successful (Kumazaki et al, 1994; O'Brien et al, 1994; Eggeling and Ballhausen, 1995) and technically simpler than GITC extraction methods at similar cell numbers (Ziegler et al, 1992). Under the conditions described above, neither has proved as effective as detergent lysis. The inferior results with these methods may be more a result of the experimental design, for example, the extra DEPC-water in each "lysis mix" when NP-40 is not used may be a factor that inhibits the reverse transcription (Eggeling and Ballhausen, 1995), though this is not proven. The poor results with heat lysis may reflect inadequate ribonuclease inhibition. The method of O'Brien and colleagues includes tRNA added to the lysates to act as a substrate for ribonucleases released by the lysed cells (O'Brien et al, 1994); Kumazaki and colleagues do not discuss the issue of ribonucleases (Kumazaki et al, 1994).

NP-40 is combined effectively with RNasin in the "lysis mix" to provide cell lysates with sufficiently intact RNA for reverse transcription. It allows the preparation of samples at room temperature, and does not impede their storage in the frozen state. NP-40 is a component of storage buffers for both Superscript (GibcoBRL) and Taq DNA polymerase, and this, coupled with the indirect evidence from the above experiments, suggests that NP-40 does not inhibit the principal enzymes of RT-PCR.

The important aspect of the reverse transcription assay using cell lysis is not its relative technical simplicity, but its conservation of minimal RNA template.

4.5   THE POLYMERASE CHAIN REACTION

4.5.1   Introduction

Amplification of low number of genetic copies to a level that allows detection by gel electrophoresis is the fundamental utility of the PCR process. However, successful and reliable amplification of low copy number of a genetic sequence depends upon careful choice of PCR reaction conditions. Essentially, conditions that increase the stringency of primer hybridisation favour specificity of amplification, and thus tend to enhance amplification of the specified low copy number sequence. The design of the PCR primers, the relative concentrations of reagents, and the thermal cycling conditions are the parameters that may be modified to optimise the PCR. Throughout this work, the PCR reaction volume remained constant at 50μl. Also remaining constant were PCR cycling conditions of denaturation at 94^{0}C, extension at 72^{0}C for 1 minute, and a total of 45 cycles (the annealing temperature was determined mainly by primer design). Also unchanged were the use of stock 10x amplification buffer (Boehringer-Mannheim) to give final concentrations of

Tris-HCl 10mM, KCl 50mM, and $MgCl_2$ 1.5mM (alterations in $MgCl_2$ concentrations did not increase sensitivity); dNTPs were also unchanged at 200mM each of dATP, dGTP, dCTP and dTTP.

The amount of each primer used per reaction remained at 100ng throughout most of this work; several experiments were performed using 50ng of each primer per reaction with little difference observed. Similarly, little difference was observed between 0.5 and 1unit of Taq polymerase per reaction (whether provided by Boehringer-Mannheim or GibcoBRL), although the larger amount seemed to provide better consistency of results.

4.5.2. The optimum volume of reverse transcription product to add to the PCR mix

Introduction

Conventional RT-PCR methods require the addition of 2 to 5µl of the cDNA solution to a total of 50 to 100µl PCR mix. The published techniques for RT-PCR from low copy number vary in the quantity of RT product made available for PCR, from 10µl (O'Brien et al, 1994) to using the total RT product in single-tube reactions (Furukawa et al, 1994; Ziegler et al, 1992; Eggeling and Ballhausen, 1995; Kumazaki et al, 1994).

RT-PCR product had been obtained for less than 10 cells (by serial dilutions) using 2µl of the RT product (demonstrated in this Chapter). However, it remained to be seen whether increasing the volume of RT product in the PCR would increase the likelihood of successful amplification of low copy number sequences, or whether other factors in the RT mixture would simply inhibit the PCR.

Materials and Methods

Fresh RT product was used to compare PCR results at the single cell level, to determine whether greatest sensitivity was achieved with 2µl of RT

product per 50µl PCR, or 7µl RT product per PCR. Using the micropipette, single T-47D cells were aspirated from PBS suspension and placed into separate "lysis mixes" for reverse transcription. Four separate reactions received only one cell via the micropipette. Reverse transcription from two cells and five cells was performed once only. The RT negative control was the reverse transcription assay components only, without any cells added.

Seven microlitres of each RT reaction were taken for PCR, and in the same PCR, 2µl only from the 2 cell RT, the 5 cell RT and the fourth single cell RT were added to PCR Master Mix in separate PCR tubes, with the 5µl difference made up by sterile, distilled water. The PCR was performed using primers and conditions for K19, and Southern Transfer with hybridisation and autoradiography was required to display the single cell K19 product.

Results

At seven hours exposure of the autoradiograph the bands for the 7µl RT product added to the PCR mix were slightly greater than those for which 2µl of RT product was used, but after 24 hours exposure, the difference was no longer noticeable (see figure 4.4). The results suggested that with fresh cDNA samples PCR results were better with the larger volumes, but confirmed that under optimal conditions the power of PCR was such that very small amounts of target could be amplified.

Discussion

It was hoped that taking a greater proportion of the 20µl RT product for PCR would increase the chances of rare target sequences being included in the PCR substrates, and the above results combined with other observations suggested that volumes larger than 2µl could be added to the PCR without reduced PCR efficiency, and probably with better results.

Indeed, when RT products that had previously been positive for K19 PCR using 2µl of the RT product were repeated comparing 2µl with 5 and 7µl of the same RT product per PCR, most of the previously positive samples now failed to produce a PCR product, and on one occasion that a sample did give a positive result, it had been positive when 5 and 7µl were added for PCR, but not when the previously positive amount of 2µl was used This observation had been made repeatedly throughout this work, namely that with sufficient time, cDNA transcribed from a small amount of substrate will eventually degrade, even when stored at -20°C. It is difficult to explain why an apparently stable cDNA molecule should degrade, especially in the frozen state. Perhaps it is merely a phenomenon of the cDNA adhering to the side of the plastic tube in which it is stored. Regardless of the reasons, the observation was indisputable and had obvious implications for delayed study of immunobead-cell isolates from patient samples.

Experiments repeating K19 PCRs using increased volumes of RT product per PCR showed little difference in sensitivity between 5,7 and 10µl of RT product. If the whole RT product were used, technical failure of the PCR would result in the permanent loss of the entire patient sample. Taking 10µl would leave only enough RT product for one more PCR in the event of wanting to check the cDNA for the presence of other transcripts, and again, failure of a PCR reaction would jeopardise further use of the cDNA. Furthermore, it was observed that the immunobead-cell isolates were often very viscous (reported in Chapter Seven), and pipetting volumes away from the beads often resulted in the beads moving into the pipettes, making it difficult to aspirate small residual volumes of immunobead-RT product. For these reasons 7µl was chosen as a practical volume of RT product to be used as PCR substrate when low copy

94

Figure 4.4   **The effect of the amount of RT product loaded for PCR, assessed by RT-PCR for K19 at the single cell level.**
Agarose gel (top) and corresponding autoradiograph (bottom), showing results for T-47D cells selected by micropipette.

Lanes 1 and 11:	Positive controls
Lanes 2, 3 and 10:	Negative controls
Lanes 4 to 7:	Single T-47D cells (7µl RT product)
Lane 8:	Two cells (7µl RT product)
Lane 9:	Five cells (7µl RT product)
Lane 12:	Two cells (2µl RT product)
Lane 13:	Five cells (2µl RT product)
Lane 14:	One cell (2µl RT product)

751 bp —

1 2 3 4 5 6 7 8 9 10 11 12 13

1 2 3 4 5 6 7 8 9 10 11 12 13 14
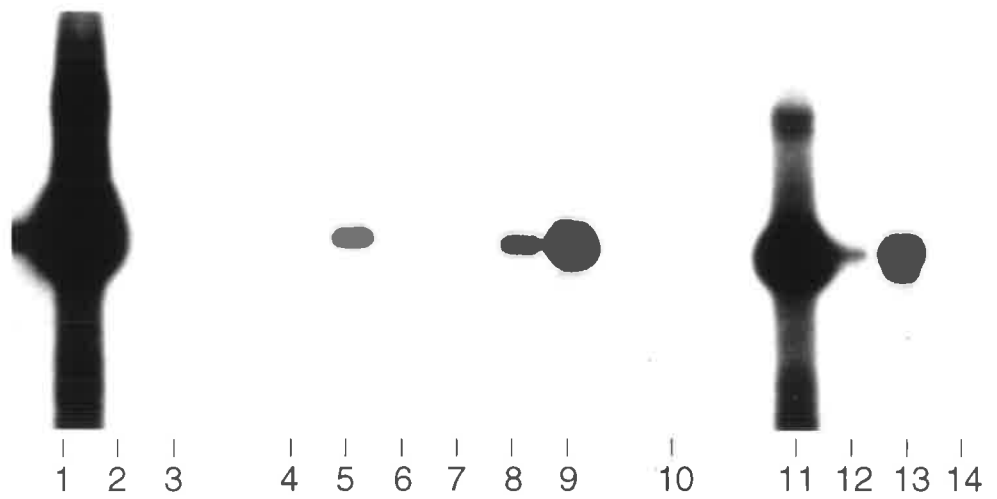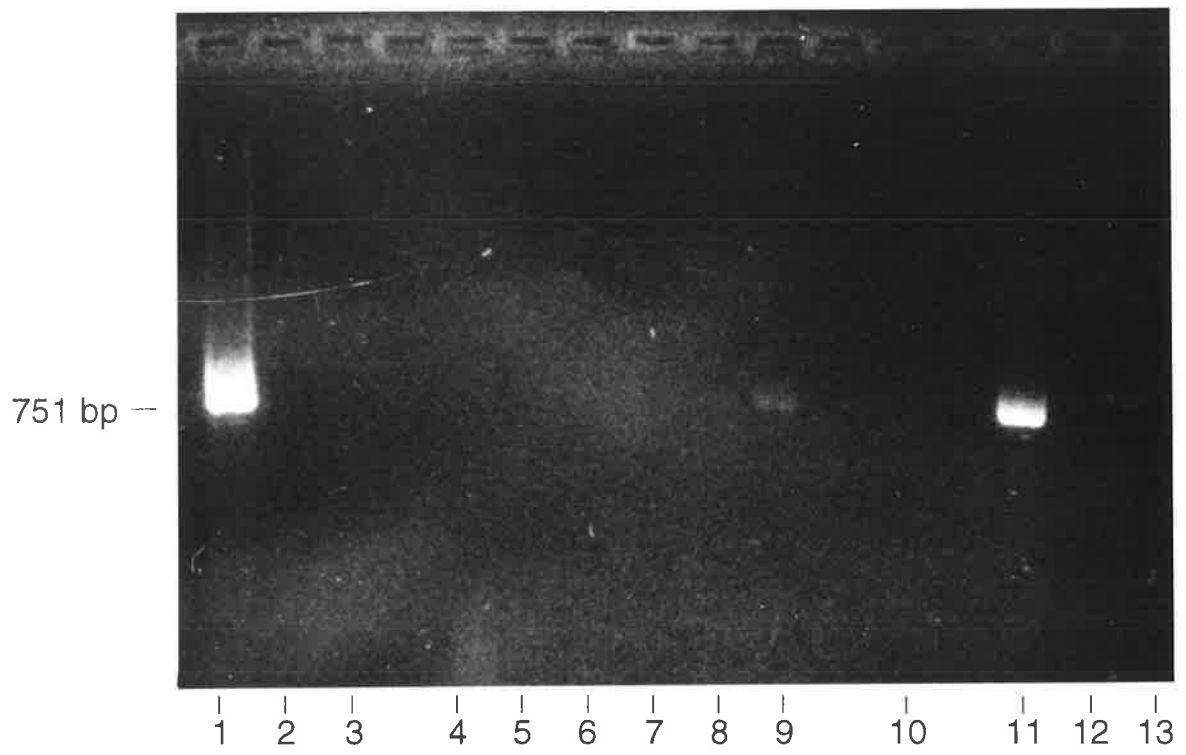
numbers were anticipated, and this was applied to the immunobead RT-PCR method from the time these experiments were performed.

4.5.3. The amount of PCR product loaded for gel electrophoresis

Introduction

Standard laboratory practice is to load 10 to 12μl of the PCR product with loading buffer into each well of an agarose gel for electrophoresis. This volume sits comfortably in the well, with little risk of overflow contamination of the neighbouring well. This results in only approximately one-fifth of the total PCR product being examined at any one time, and one would assume that if a greater proportion the PCR product were loaded into each well, then maybe low levels of amplification would be more easily detected. The following two methods of concentration of the PCR product were tried.

Vacuum-Drying of Product

This method has been described as a means of increasing sensitivity of RT-PCR detection of circulating prostate cancer cells (Moreno et al, 1992), and was applied to K19 RT-PCR, but with poor results. PCR products were aspirated from beneath the overlying mineral oil, transferred to new PCR tubes and vacuum-dried for 60 minutes. In those tubes in which the volume was reduced, it was reconstituted to 20μl with sterile water. Twenty microlitres of PCR product was loaded into each well for agarose gel electrophoresis. Most tubes failed to show any significant reduction in volume of PCR product, possibly because residual mineral oil prohibited evaporation, and the result generally failed to justify the extra effort and risk of loss of PCR product.

Ethanol Precipitation of PCR Product

This technique is mentioned as an accepted method of concentration of DNA for improved results in gel electrophoresis (Sambrook et al, 1989), and the following method was assessed:

1) The 50µl PCR product was aspirated from beneath the overlying mineral oil and transferred to complementary PCR tubes.

2) 5µl of sodium acetate 3M, pH 5.2 was added to each tube (extraction step).

3) 100µl of 100% ethanol was added to each, and all tubes were vortexed.

4) All tubes were precipitated at -800C for 20 minutes.

5) Tubes were centrifuged at 13,000 rpm for 30 minutes, and the supernatant aspirated.

6) The resulting DNA pellets were washed in 50µl of 70% ethanol and then centrifuged at 13,000 rpm for 10 minutes.

7) The supernatant was aspirated and the resulting pellet vacuum - dried.

8) The pellet was resuspended in 10µl of sterile water, then the total resulting volume was run out in an agarose gel.

Some good results were obtained from ethanol precipitation of PCR product, with bands on ethidium bromide staining evident on one occasion for three and ten T47-D cells aspirated by micropipette for RT-PCR for K19. Where bands were strong (for example with positive controls), there was excessive "trailing and smearing" of the large amounts of DNA loaded into the wells (Sambrook et al, 1989).

The problem of applying PCR product precipitation to detection of low transcript number is that in the absence of PCR signal, the only indication that the absence of bands is not due to loss of the product during the many precipitation steps is the presence of primer dimer bands.

Conclusion

The search for PCR signals from immunobead RT-PCR samples on patients will always require enhancement of sensitivity by Southern transfer and autoradiography when results are negative or equivocal, and this is likely to override any advantage that might have been gained by the precipitation steps. The transfer and centrifuge steps create the risk of losing PCR product, which is always a worry when faced with an absence of PCR product on gel electrophoresis.

It was concluded that the extra steps in the immunobead RT-PCR method required for precipitation of PCR product did not justify the marginal improvement in results. As a compromise, the amount of PCR product loaded into the wells of the agarose gels was increased to 20µl (nearly half of the total product).

4.5.4   Other aspects of PCR

Magnesium Chloride (MgCl$_2$)

While each individual system may vary in the concentration of MgCl$_2$ that allows optimum function, it is generally true that excess MgCl$_2$ in the PCR mix leads to accumulation of non-specific amplification products, whereas insufficient MgCl$_2$ tends to decrease the PCR yield. Varying the MgCl$_2$ concentration from the 1.5mM of the supplied 10x PCR buffer (Boehringer) did not offer any improvement in sensitivity of the PCR.

PCR annealing temperature

Higher annealing temperatures should decrease the formation of primer dimers that would tend to occur where there is only a small amount of target sequence, and which would decrease the efficiency of the system (Furukawa et al, 1994). The system for K19 required a relatively stringent annealing temperature of 68^{0}C to avoid amplification of the K19 pseudogene

(see Chapter Six), and there appeared to be equal or even enhanced amplification of PCR controls when the system annealed at 68^{0}C compared with 60^{0}C.

Hot Start PCR

A true "hot start" PCR involves withholding a critical reaction component until the reaction has attained a stringent primer annealing temperature. This minimises non-specific primer extension during the denaturation period and in theory improves efficiency. "Hot start" can be performed by manually adding the last reagent when the reactions reach approximately 80^{0}C, or by employing a wax layer that separates two reaction phases until the wax melting temperature is reached. The method used in work towards this thesis was to keep the PCR reactions on ice until the thermal cycler reached a temperature of 80^{0}C, before placing the PCR tubes in the thermal cycler. The value of this method was not assessed, but it was thought that the temperature of the ice would at least minimise non-specific primer extension prior to heating of the thermal block.

Radioactive PCR

The advantage of radioactive PCR was that the time-consuming hybridisation steps and steps to wash background radioactivity from the transfer filters could be avoided. Radioactive PCR was found to be sensitive to the single-cell level. However, the technique increased the risk of radioactivity exposure, particularly to other workers in the laboratory, and since the method was not more sensitive than labelled oligonucleotide probes, it was not pursued.

PCR product contamination

Contamination of a PCR mixture by products of previous PCR reactions is a well-recognised problem of the technique (Erlich et al, 1991), and was a

recurring problem during the establishment of this method. The extreme sensitivity of Southern transfer and autoradiography highlighted even the smallest amounts of contamination, such that not all negative controls would carry the false-positive messages. The problem of low-level contamination was only mastered when published strict laboratory procedures were followed (Erlich et al, 1991): RT and PCR mixes were prepared in a room separate from the areas in the laboratory where DNA was handled, with pipettes specifically dedicated to that purpose; "plugged tips" (disposable pipette with cotton wool barriers preventing contamination of the pipette barrel) were used whenever DNA was being manipulated in the main laboratory, and gloves were worn and changed regularly, especially when moving between areas in the laboratory.

Sub-aliquotting reagents

Another recognised laboratory technique to demonstrate its purpose during the course of this work was the method of sub-dividing regularly used reagents or samples into smaller aliquots. The aim is to work with small volumes to minimise the number of times that a frozen sample might be thawed, and to minimise the extent of solution contamination should contamination occur.

The enzyme stabilising agent DTT is an example of a solution best stored frozen that may degrade if thawed too often. At one point during this work the RT lysis method was failing completely. All critical variables were tested, and it was only when the old DTT sample was replaced with a fresh one that the function of the RT method returned. This event emphasised the importance of RNasin and its dependence upon DTT, and also the importance of maintaining fresh reagents by the rapid turnover of small volumes.

4.6   SUMMARY

The application of an RNA-based marker to the immunobead-cell isolation technique required reliable reverse transcription of RNA from as few cells as possible. A method for RT-PCR from very low cell numbers was developed, based on release of cellular nucleic acid by detergent lysis of cells.

The reverse transcription method was efficient enough to allow PCR amplification of genetic signal from a single cell when detergent lysis was combined with ribonuclease inactivation and a powerful reverse transcriptase, in a 20µl reaction volume.

The PCR reaction was not inhibited by the components of the RT product, up to a volume of 10µl of RT product in a final PCR reaction volume of 50µl. For practical reasons 7µl was chosen as the volume of RT product to add to the PCR reaction.

Methods of concentration of PCR product were not found to be of practical benefit for the immunobead RT-PCR method, since Southern transfer of PCR product and autoradiography would virtually always be performed.

The sensitivity of this cell lysis technique for RT-PCR to the single cell level will be demonstrated in the succeeding chapters.

CHAPTER FIVE:

ASSESSMENT OF CD44 FOR IMMUNOBEAD RT-PCR

5.1   INTRODUCTION

With the observation that malignant tissues display a chaotic and permanent over-expression of variant isoforms of CD44, it was hoped that assessment of CD44 spiced variant expression could be used as a universal marker for malignancy (Tarin and Matsumura, 1994; Matsumura et al, 1994), and that this could then be applied to Immunobead RT-PCR for detection of circulating carcinoma cells.

The method of Matsumura and Tarin (Matsumura and Tarin, 1992) for detection of breast cancer cells in whole blood has been described in the opening chapter. Their method of differentiating benign from malignant tissue by CD44 expression depends on the non-malignant cells expressing exclusively the standard isoform of CD44. Other work reviewed in the opening chapter, reporting variant isoform expression by normal peripheral blood MNCs, makes it unlikely that their method is reliable. Their method has not been reproduced in the literature since the initial publication. Furthermore, the expression of CD44 variants by benign tissues has been noted by Fox and colleagues to render the PCR assessment of CD44 variants "unlikely to be of practical value for tumour diagnosis or cancer screening" (Fox et al, 1993).

The approach of preliminary tumour cell isolation or "enrichment" by immunobead selection of cells encouraged further investigation of CD44 as a potential tumour marker for the isolated cells, because the immunobead isolation step provides epithelial cell specificity, and could therefore theoretically compensate for a less specific PCR tumour marker. Using the analogy of finding a needle in a haystack, the immunobeads would draw the

101

needle from the haystack, so that the PCR marker would still have to identify the "needle", but would not have to deal with the background noise of the "haystack".

Investigation of the applicability of CD44 as a tumour marker for the immunobead RT-PCR method could therefore be defined to two major issues. Firstly, the expression of CD44 by haemopoietic cells trapped with the beads after the blood had been washed away, would have to be of the standard isoform of CD44 only. This could be possible if only a small number of leukocytes remained with the beads, and if so few leukocytes remained that the complicating presence of the "activated" lymphocyte minority would be unlikely.

Secondly, it remained to be demonstrated whether the few tumour cells that would be isolated by the immunobeads would show PCR expression of variant CD44 isoforms, so that tumour cell expression could be differentiated from the predominantly CD44s expression of the associated leukocytes. To date, all tumour expression of CD44 by PCR has been assessed at the tissue level, so that it remains unknown whether the phenotype of multiple isoform expression by malignant tumours is the result of over-expression by single cells, or is merely the result of the sum of total cellular expression.

5.2   MATERIALS AND METHODS

5.2.1   PCR Primers for CD44

The nucleotide sequence for the CD44 gene was obtained (Dougherty et al, 1991; Stamenkovic et al, 1991). The aim of primer design was to enable amplification of both the standard form and any isoforms that might arise from alternative splicing of the variant exons. Primers were therefore designed to flank the variant region of the CD44 gene (exons 6 to 14), from constant region

"upstream" (exon 5) to the constant region "downstream" (exon 15) (Henk et al, 1993; Screaton et al, 1992). An oligonucleotide that was internal to these primers, and complementary to the constant region, was used for radioactive probing and autoradiography to highlight the standard CD44 isoform and all CD44 variants.

The primers designed to amplify across the variant part of the CD44 mRNA were modifications of those used by Henk (Henk et al, 1993): the sense primer recognising a sequence in constant exon 5 (5′ AAGACATCTACCCCAGCAACCCT 3′) and the antisense primer recognising constant exon 16 (5′ GATGCCAAGATGATCAGCCATTCT 3′). The sense primer contained an extra three bases added to the "C13" primer of Henk and colleagues, to produce a 23 - base oligonucleotide with an approximate melting temperature of 70^0C. The antisense exon 16 primer of Henk ("C2A") was closely related to the constant exon 16 primers of others (Salles et al, 1993; Matsumura and Tarin, 1992; Jackson et al, 1992) and was modified to produce a 24-base oligonucleotide with an approximate melting temperature of 70^0C.

The oligonucleotide used as an internal end-labelled probe for autoradiography was designed to be antisense to exon 15, the first exon in the constant region downstream from the insertion site for the variant exons. This antisense exon 15 oligonucleotide was located 37 bases downstream from the insertion site, was 24 bases long, with an approximate melting temperature of 68^0C, and its sequence was 5′ ATCTGATTCAGATCCATGAGTGGT 3′.

5.2.2 PCR Conditions for CD44

The cell lysis assay described in Chapter Two was used to reverse transcribe mRNA from low cell numbers to cDNA, and the PCR for CD44 was also as described, with cycling conditions being: 94^0C for 5 minutes, then 45

```
   1   CCAGCCTCTGCCAGGTTCGGTCCGCCATCCTCGTCCCGTCCTCCGCCGGCCCCTGCCCCG
  61   CGCCCAGGGATCCTCCAGCTCCTTTCGCCCGCGCCCTCCGTTCGCTCCGGACACCATGGA
 121   CAAGTTTTGGTGGCACGCAGCCTGGGGACTCTGCCTCGTGCCGCTGAGCCTGGCGCAGAT
 181   CGATTTGAATATAACCTGCCGCTTTGCAGGTGTATTCCACGTGGAGAAAAATGGTCGCTA
 241   CAGCATCTCTCGGACGGAGGCCGCTGACCTCTGCAAGGCTTTCAATAGCACCTTGCCCAC
 301   AATGGCCCAGATGGAGAAAGCTCTGAGCATCGGATTTGAGACCTGCAGGTATGGGTTCAT
 361   AGAAGGGCACGTGGTGATTCCCCGGATCCACCCCAACTCCATCGTGCAGCAAACAACAC
 421   AGGGGTGTACATCCTCACATCCAACACCTCCCAGTATGACACATATTGCTTCAATGCTTC
 481   AGCTCCACCTGAAGAAGATTGTACATCAGTCACAGACCTGCCCAATGCCTTTGATGGACC
 541   AATTACCATAACTATTGTTAACCGTGATGGCACCCGCTATGTCCAGAAAGGAGAATACAG
```
1 601   AACGAATCCTG**AAGACATCTACCCCAGCAACCCT**ACTGATGATGACGTGAGCAGCGGCTC
```
 661   CTCCAGTGAAAGGAGCAGCACTTCAGGAGGTTACATCTTTTACACCTTTTCTACTGTACA
 721   CCCCATCCCAGACGAAGACAGTCCCTGGATCACCGACAGCACAGACAGAATCCCTGCTAC
 781   CA*ATATGGACTCCAGTCATAGTACAACGCTTCAGCCTACTGCAAATCCAAACACAGGTTT
 841   GGTGGAAGATTTGGACAGGACAGGACCTCTTTCAATGACAACGCAGCAGAGTAATTCTCA
 901   GAGCTTCTCTACATCACATGAAGGCTTGGAAGAAGATAAAGACCATCCAACAACTTCTAC
 961   TCTGACATCAAGCAATAGGAATGATGTCACAGGTGGAAGAAGAGACCCAAATCATTCTGA
1021   AGGCTCAACTACTTTACTGGAAGGTTATACCTCTCATTACCCACACACGAAGGAAAGCAG
1081   GACCTTCATCCCAGTGACCTCAGCTAAGACTGGGTCCTTTGGAGTTACTGCAGTTACTGT
1141   TGGAGATTCCAACTCTAATGTCAATCGTTCCTTATCAGGAGACCA**AGACACATTCCACCC
```
3 1201   CAGTGGGGGGGTCCCAT**ACCACTCATGGATCTGAATCAGAT**GGACACTCACATGGGAGTCA
```
1261   AGAAGGTGGAGCAAACACAACCTCTGGTCCTATAAGGACACCCCAAATTCC**AGAATGGCT**
```
2 1321   **GATCATCTTGGCATC**CCTCTTGGCCTTGGCTTTGATTCTTGCAGTTTGCATTGCAGTCAA
```
1381   CAGTCGAAGAAGGTGTGGGCAGAAGAAAAAGCTAGTGATCAACAGTGGCAATGGAGCTGT
1441   GGAGGACAGAAAGCCAAGTGGACTCAACGGAGAGGCCAGCAAGTCTCAGGAAATGGTGCA
1501   TTTGGTGAACAAGGAGTCGTCAGAAACTCCAGACCAGTTTATGACAGCTGATGAGACAAG
1561   GAACCTGCAGAATGTGGACATGAAGATTGGGGTGTAACACCTACACCATTATCTTGGAAA
1621   GAAACAACCGTTGGAAACATAACCATTACAGGGAGCTGGGACACTTAACAGATGCAATGT
1681   GCTACTGATTGTTTCATTGCGAATCTTTTTTAGCATAAAATTTTCTACTCTTTTTAA
```

Figure 5.1   cDNA sequence of "epithelial form" of CD44.

PCR primers are shown underlined.

1   Denotes sense primer (recognizes a sequence in exon 5)

2   Antisense primer (recognizes a sequence in exon 16)

3   Internal oligonucleotide (exon 15)

*   Denotes end of exon 5

**   Denotes beginning of exon 15

Figure 5.2   Autoradiograph of RT-PCR for CD44 on 300, 600 and 1500 MNCs.

Lanes 1 to 3:	Positive controls (from T-47D cells)
Lane 4:	Negative control
Lanes 5 and 6:	300 MNCs
Lanes 7 and 8:	600 MNCs
Lanes 9 and 10:	1500 MNCs

CD44$_E$ —

CD44$_S$ —

1 2 3   4 5 6 7 8 9 10
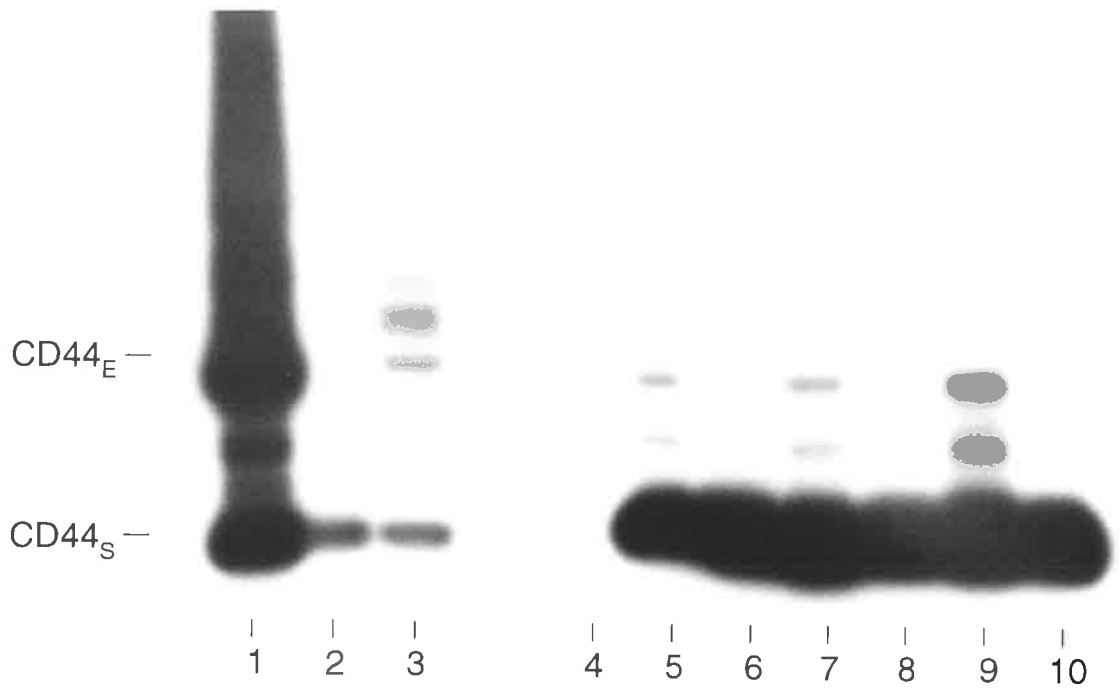
Figure 5.3   **Autoradiograph of CD44 expression from 600, 60 and 6 MNCs (the corresponding agarose gel photograph is seen in figure 4.3).**

RT-PCR product from the "standard" and "epithelial" forms of CD44 are designated CD44s and CD44e, respectively.

Lanes 1 and 3:	Positive controls
Lane 2:	Negative control
Lane 4:	600 MNCs
Lanes 5 and 6:	60 MNCs
Lane 7:	6 MNCs

CD44$_E$ —

CD44$_S$ —

1	2	3	4	5	6	7

cycles of 94°C for one minute, 55°C for one minute and 72°C for one minute, with a 7 minute extension at 72°C.

5.3   RESULTS

5.3.1   CD44 expression by haemopoietic cells

With large numbers of haemopoietic cells, the RT-PCR product for CD44 was predominantly the standard or haemopoietic form, conforming to a 330 base-pair band on gel electrophoresis. Larger isoforms were expressed irregularly, so that for apparently identical mononuclear cell numbers the PCR products could be quite different at the levels of sensitivity afforded by autoradiography. Figure 5.2 demonstrates autoradiograph results from PCR for CD44 on cell lysates of approximately 300, 600 and 1500 peripheral blood mononuclear cells. The standard isoform predominates, but in some lanes larger isoforms are expressed. In these cases the appearances differ from the results seen with cancer cell lines only in the relative amounts of standard form expressed - the expression of the standard form is relatively greater from mononuclear cells than epithelial cells.

This apparent irregularity of CD44 expression by haemopoietic cells suggests that a small population of cells may be responsible for the expression of isoforms larger than the standard, so that when these occasional cells are included in the sample the "phenotype" of expression is that of the standard isoform with additional splice variants. In terms of combining these results with immunobeads, the addition of any splice variants makes differentiation from bound epithelial cells very difficult, since this relies entirely on the demonstration of expression of larger CD44 isoforms.

At lower cell numbers than above, the CD44 expression from mononuclear cells was essentially the standard form only. Figure 5.3 shows

104

results on autoradiography from 600, 60 and approximately 6 mononuclear cells prepared by serial dilution in PBS. The expression of the standard form only is in contrast to the T47-D positive controls, which display two principal bands, conforming to the standard form and the 741 base-pair band that would be expected from the "epithelial" form. In these cases of low numbers of mononuclear cells, the expression of CD44 isoforms on autoradiography was not merely a function of duration of exposure of the autoradiograph. Even if the lane for less than 10 cells was excised from the nylon filter and exposed alone for many days, the standard isoform remained the only isoform detected on the autoradiograph.

The limit of sensitivity for CD44 expression from mononuclear cells was 5 to 10 cells selected by micropipette. In these examples the only the standard isoform was demonstrated.

5.3.2   CD44 expression by breast cancer cell lines

5.3.2.1   CD44 expression from low cell numbers prepared by serial dilution

Large numbers of cells from both the T47-D and MCF-7 cell lines, after RNA extraction by the GITC method, consistently displayed two major bands of approximately equal intensity, which conformed to the expected sizes of the standard and epithelial isoforms of CD44.

The CD44 isoforms expressed by lesser numbers of T47-D cells showed some variation between samples, but generally at cell numbers greater than 100 or so both the standard and epithelial isoforms were expressed, again approximately in equal proportions. Figure 5.4 shows CD44 expression demonstrated by RT-PCR from 140 and 14 T47-D cells. The three lanes for 140 cells show the two major bands that predominate for larger cell numbers, whereas at the low number of approximately only 14 cells the standard or

haemopoietic form is consistently absent. The epithelial isoform is the smallest expressed in this example, with the third 14 cell lane displaying a larger band.

If these results could be consistently reproduced, CD44 would show some potential as a marker for epithelial (and hence carcinoma) cells using the immunobead technique. Isolated tumour cells could be differentiated from the background leukocytes by their predominant expression of the epithelial isoform, in contrast to the expression of the standard form by up to several hundred leukocytes.

Expression of CD44 isoforms from cell lines by the RT-PCR method was not constant, however, suggesting that CD44 variant isoform expression would not be a reliable indicator for tumour cells isolated by immunobeads. Lanes carrying RT-PCR product from supposedly the same number of cells could show apparently conflicting results. Lanes 2 and 3 in figure 5.2 show CD44 isoforms expression from two separate T-47D cell suspensions, but both thought to represent approximately 400 T-47D cells. Lane 2 shows only the standard isoform, whereas in lane 3 the standard form is seen with an array of larger isoforms, including the epithelial form. The expression of only the standard, or haemopoietic, isoform by 400 T47-D cells in lane 2, seemed incongruous, given that it was expected that the epithelial form should predominate in epithelial cells. These results could have been influenced by sampling error or by PCR artefact.

Perhaps the technique of estimating the cell number and then arriving at a designated low number of cells by serial dilutions results in such an inaccurate and unequal distribution of cells for lysis that comparisons by this method are meaningless. If cells are allowed to grow to confluence before harvesting, they tend to clump together and become more difficult to count and distribute amongst different tubes, thereby accentuating the inaccuracies

106

Figure 5.4   **Agarose gel (top) and autoradiograph (bottom) of CD44 expression from 140 and 14 T-47D cells.**

Lanes 1 and 3:   Positive controls

Lane 2:   Negative control

Lanes 4 to 6:   140 cells

Lanes 7 to 9:   14 cells

CD44_E —

CD44_S —

1 2 3 4 5 6 7 8 9

CD44_E —

CD44_S —

1 2 3 4 5 6 7 8 9
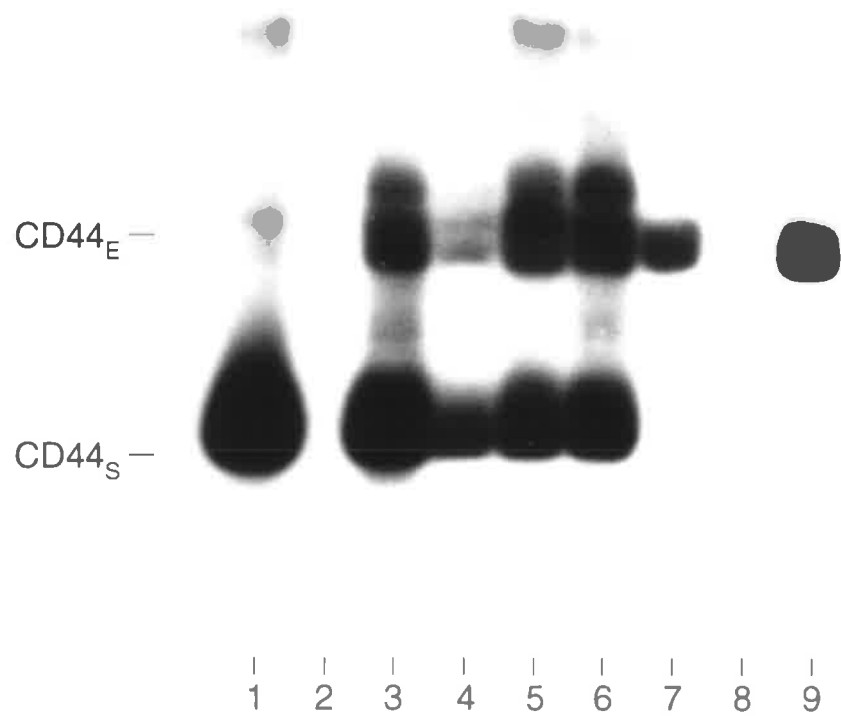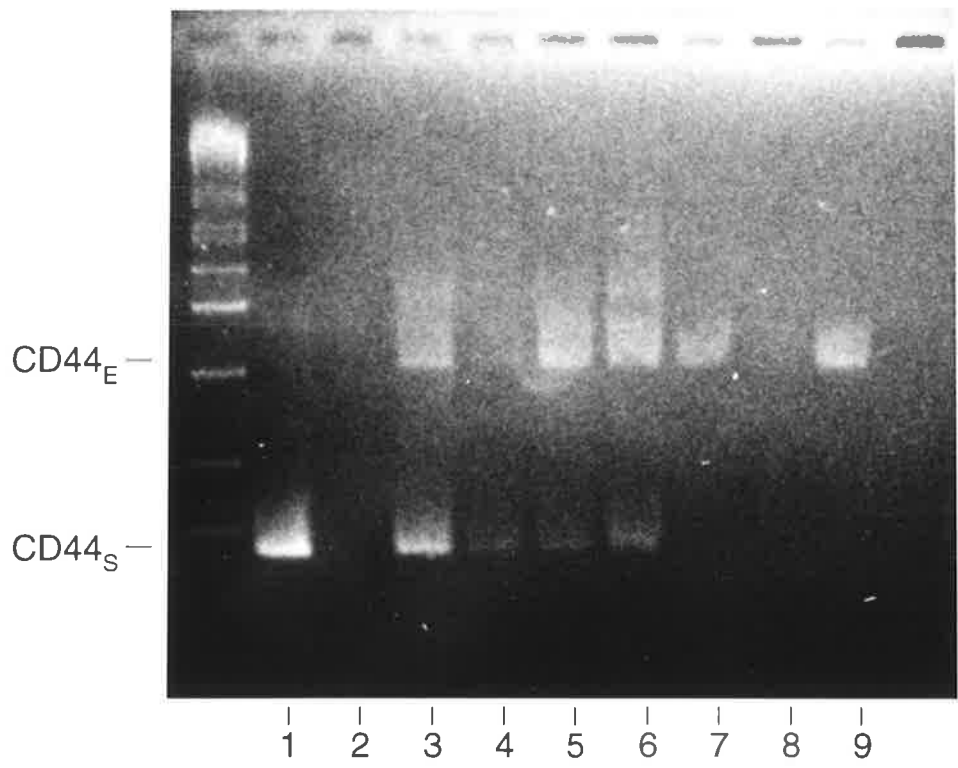
inherent in the count estimation and serial dilutions. The cell contact by clumping may alter expression of the cell surface molecules such that CD44 expression is altered in this case to produce irregular results. Perhaps the differences in CD44 expression seen between epithelial cells, and especially between epithelial cells and haemopoietic cells, are merely an artefact of the PCR. If the standard form of CD44 is ubiquitously expressed and larger isoforms are expressed in addition on some non-haemopoietic cells, then at low copy number of CD44 message the PCR might amplify only that isoform which anneals to primers in the initial cycles. Whichever isoform was favoured early would then increase its copy number in the exponential manner of PCR, and all remaining low copy number sequences would be swamped by the exponentially increasing isoform, and none would be amplified.

The above results have the shortcoming that the cell distribution is unreliable and that the true sensitivity of the RT-PCR assay for CD44 is unknown.

5.3.2.2 CD44 expression from very small numbers of cells selected by micropipette

Figure 5.5 shows the CD44 expression by RT-PCR of four separate single cells and five cells from the T-47D cell line. Two lanes showed single bands, corresponding to the 741-bp bands expected of the epithelial isoform, and consistent with the results for 14 cells shown above. However, another single cell RT-PCR showed a strong standard band in addition to the band of the epithelial variant. The lane of the fourth single cell harvested showed one isoform only, of size intermediate between the standard and epithelial forms. The RT-PCR product from 5 cells displayed a strong standard band but a weaker epithelial band. The lane for 2 cells failed to display a product for CD44. RT products from each of the cells aspirated by pipette amplified signals for

K19 by PCR, including the lane for two aspirated cells which failed to produce a signal for CD44 (results not shown).

Results with cells selected by micropipette confirm the sensitivity of the RT-PCR assay by cell lysis at the single cell level.

This study of CD44 expression of T47-D cells by RT-PCR suggests that single carcinoma cells display a heterogeneity of expression of CD44, even when studied from the same cell line (in which cells should arise from the same clone and therefore have the same genetic phenotype). The standard and epithelial forms are the predominant forms expressed, but individual cells may express other variants exclusively. Again it is curious that some single cells may express the standard isoform so strongly, while the 14 cells taken by dilution (figure 5.4) expressed the epithelial variant exclusively. This result was reproducible - on two occasions groups of 10 to 20 cells aspirated by the micropipette expressed only the epithelial isoform of CD44.

The apparent irregularity of CD44 expression seen in the dilution experiments described above is therefore confirmed, and the possible explanations for the anomalies are the same, namely that either this represents a true result, or that it represents a an artefact of PCR at low copy number. Irregularity of CD44 expression by cell lines has been described also by Tarin and Matsumura, who have claimed that cell lines which have been serially propagated in vitro do not give reliable results compared to fresh tissues (Tarin and Matsumura 1993).

5.3.3   CD44 expression by metastatic human breast cancer cells

After mechanical dissociation of the malignant lymph node surgically removed from the axilla of a patient with breast cancer, inspection of the cell suspension under light microscopy showed a dominant cell population of large cells, sometimes single but mostly in large grape-like clusters. These cells

rapidly adhered to the plastic of the petri dish (a characteristic of epithelial cells) and could only be mobilised for aspiration into the pipette by a jet of PBS from a second pipette. Some smaller cells and occasional spindle-shaped cells were seen. The cells selected for RT-PCR assessment were from the population of large cells, when in single cell suspension.

The single metastatic breast cancer cells displayed a consistency of CD44 expression that was not seen in the cell line samples (figure 5.6). The PCR band conforming to the haemopoietic or standard isoform was dominant in all PCR products. Bands conforming to the expected size of the epithelial isoform were seen in PCR products from five cells, but an additional isoform larger than that expected for the epithelial variant was seen in most lanes, along with the standard form.

The following results confirm that the large, single cells taken by the micropipette were truly tumour cells and not just, for example, activated lymphocytes:

1) *Histopathology*

Histopathology of the surgically resected lymph node confirmed the macroscopic impression of complete replacement of the node by metastatic breast cancer.

2) *Cytopathology*

A cytospin preparation of an aliquot of the cells in suspension, stained with May-Grunwald-Giemsa stain, was examined by a cytopathologist from the Pathology Department at The Queen Elizabeth Hospital. All cells seen in the cytospin preparations were considered to have cytological features of malignancy, that is, they were large cells with large, pleomorphic nuclei (see figure 5.7).

3) RT-PCR for K19

PCR using primers for K19 (see Chapter Six) was performed on residual reverse transcription products from the micropipetted cell samples. All cell samples produced PCR signals for K19 except the fourth single cell aspirated. The K19 PCR is specific for epithelial cells, and does not amplify a K19 signal from lymphocytes under normal conditions (see Chapter Six).

All available evidence therefore indicates that the CD44 expression demonstrated above has come from individual metastatic breast cancer cells.

Metastatic breast cancer cells can express multiple CD44 mRNA isoforms

Increased expression of variant isoforms of CD44 in malignant tissues has been consistently demonstrated by others, but to date CD44 expression by individual tumour cells has not been addressed, and it has not yet been demonstrated whether one cancer cell can express several CD44 isoforms (Kaufmann et al, 1995). Cells from this particular group demonstrate consistent expression of two major mRNA isoforms: the ubiquitous standard form and a larger isoform whose exon composition has not been characterised. Additional isoforms are seen as the amount of target nucleic acid is increased, in the five cell lanes. It remains uncertain whether these extra isoforms enhance the metastatic capability of the tumour cells, or whether they simply represent loss of regulation of mRNA expression in the malignant state. The CD44 expression from this group of breast cancer cells, as determined by the cell lysis RT-PCR method, is consistent with the finding of Salles et al., in that the relative expression of CD44s is lower, and the expression of larger isoforms is higher, relative to that seen with MNCs (Salles et al, 1993).

Figure 5.5   **Single cell RT-PCR for CD44 using T-47D cells aspirated from suspension by micropipette.**

Lanes 1 and 4:	Positive controls
Lanes 2 and 3:	Negative controls
Lanes 5 to 8:	Single T-47D cells
Lane 9:	Two cells
Lane 10:	Five cells

Figure 5.6   **CD44 isoform expression by single metastatic human breast cancer cells, assessed by RT-PCR.**

Lanes 1 and 2:	Positive controls
Lane 3:	Negative control
Lanes 4 and 5:	One cell
Lanes 6 and 7:	Two cells
Lane 8:	Three cells
Lane 9:	One cell
Lane 10:	One cell
Lanes 11 to 14:	Groups of approximately five cells

741 bp —

330 bp —

1   2   3   4   5   6   7   8   9   10

330 bp —

1   2   3   4   5   6   7   8   9   10   11 12 13 14
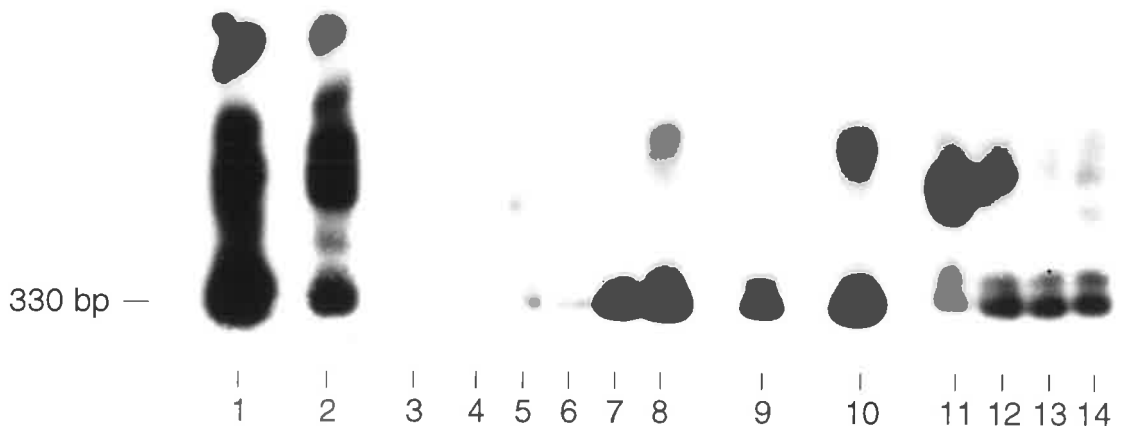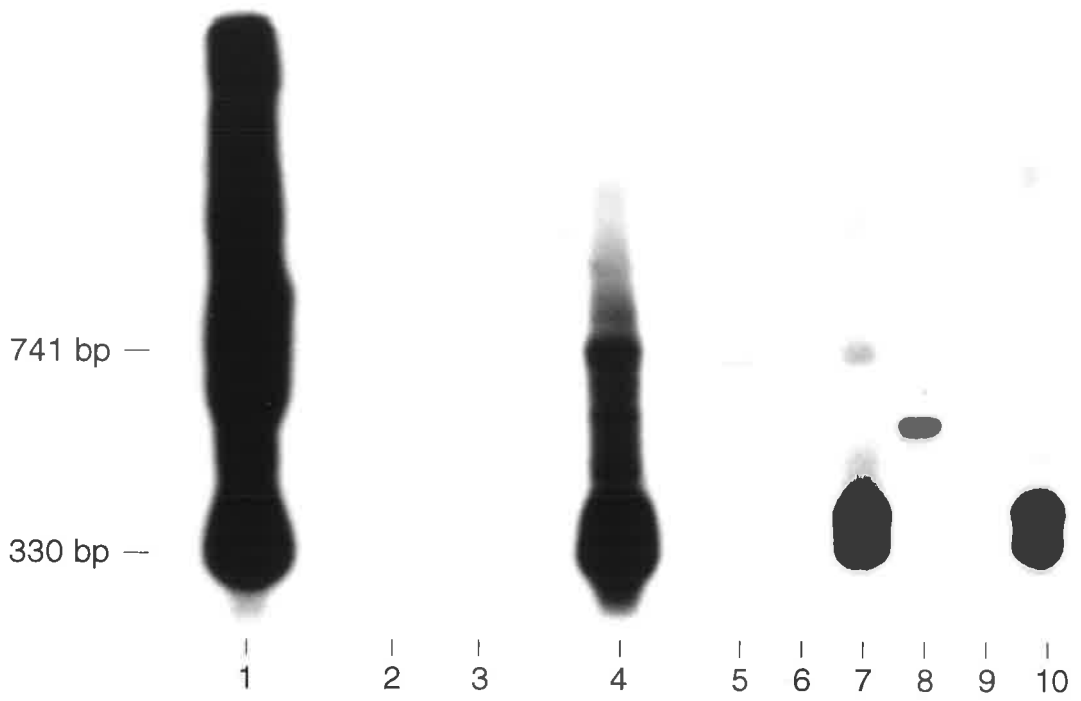
Figure 5.7   Photomicrograph of a cytopsin preparation of metastatic human breast cancer cells, taken from an axillary lymph node, used for assessment of CD44 expression by RT-PCR.
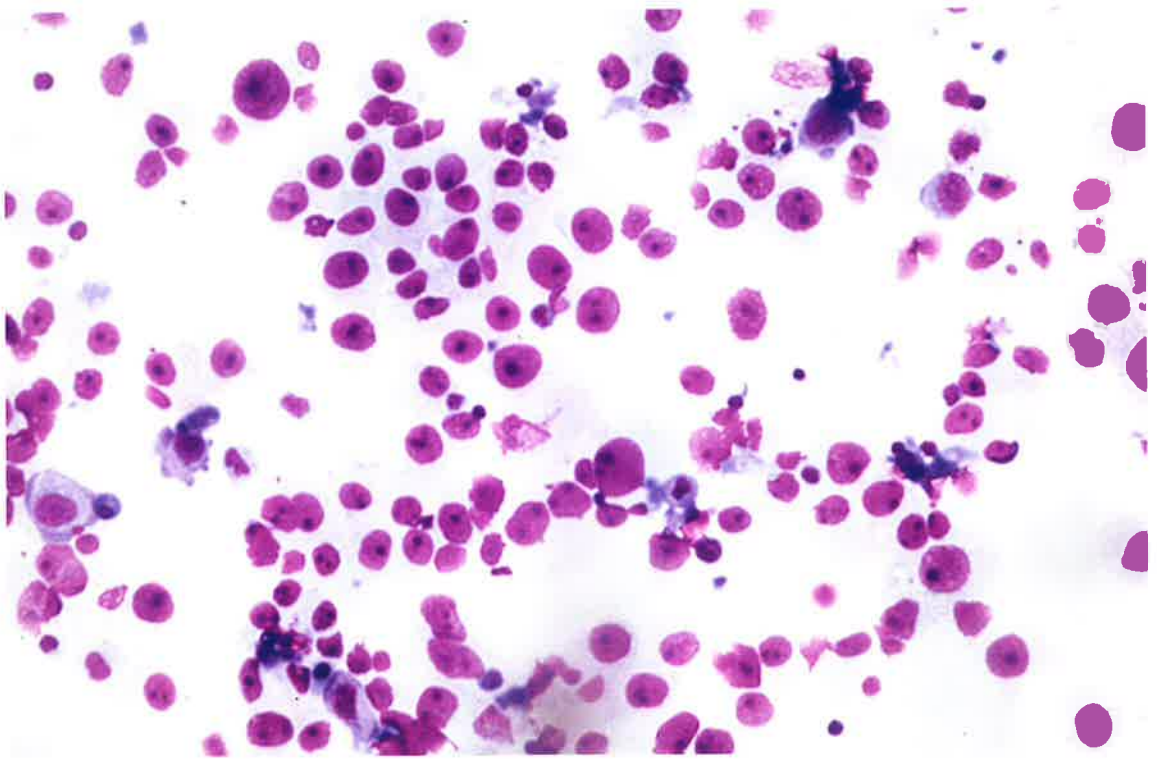
CD44 PCR is unlikely to be able to differentiate carcinoma cells from normal MNCs, even when selected by immunobeads

If these breast cancer cells were bound by immunobeads, their differentiation from the background of white blood cells on the basis of their CD44 signals would be difficult. The predominant isoform, the standard form, would be indistinguishable from the strong standard isoform signal that would be expected from the leukocytes, and the recognition of a bound malignant cell would depend entirely upon the amplification of the generally weaker larger isoform. It could be expected that the PCR would preferentially amplify the signal that would be in gross excess, that is, the standard CD44 signal, and in this case the low copy number of the larger isoforms might not be recognised by the PCR primers.

Even if larger isoforms were identified in the PCR product, it would be difficult to be certain that they were not merely the result of expression by activated lymphocytes (Arch et al, 1992; Mackay et al, 1994; Salles et al, 1993).

5.3.4   Immunobead RT-PCR for CD44 using whole blood only

Results of the immunobead RT-PCR technique for CD44, used to test peripheral blood samples taken from healthy volunteers, or patients being investigated for conditions other than carcinoma, are shown on the autoradiograph in figure 5.8. Of the nine immunobead RT-PCR reactions shown, seven displayed bands after Southern transfer and autoradiography that conformed to the expected size of the standard CD44 isoform. It is important to note that in three of these samples, bands conforming to larger CD44 isoforms are also seen, in lanes 5 (patient with NHL), 9 (healthy donor) and 11 (patient with disseminated non-seminomatous germ cell tumour of the testis). If CD44 had been used as a marker for circulating carcinoma cells, then these three people would have been wrongly diagnosed as having carcinoma

cells in their peripheral blood, on the basis of increased expression of CD44 splice variants, in their immunobead-cell isolates. It is possible that the increased expression of splice variants seen in these three cases is due to the presence of activated lymphocytes trapped with the immunobeads. The results are certainly not due to isolated carcinoma cells, however, which excludes CD44 as an immunobead RT-PCR marker for carcinoma cells in blood.

Figure 5.8   Immunobead RT-PCR for CD44, using peripheral blood samples from patients without carcinoma, or healthy controls.

Lane 1:   Positive control

Lane 2:   Negative control

Lanes 3 to 11:   Samples from control subjects

330 bp —
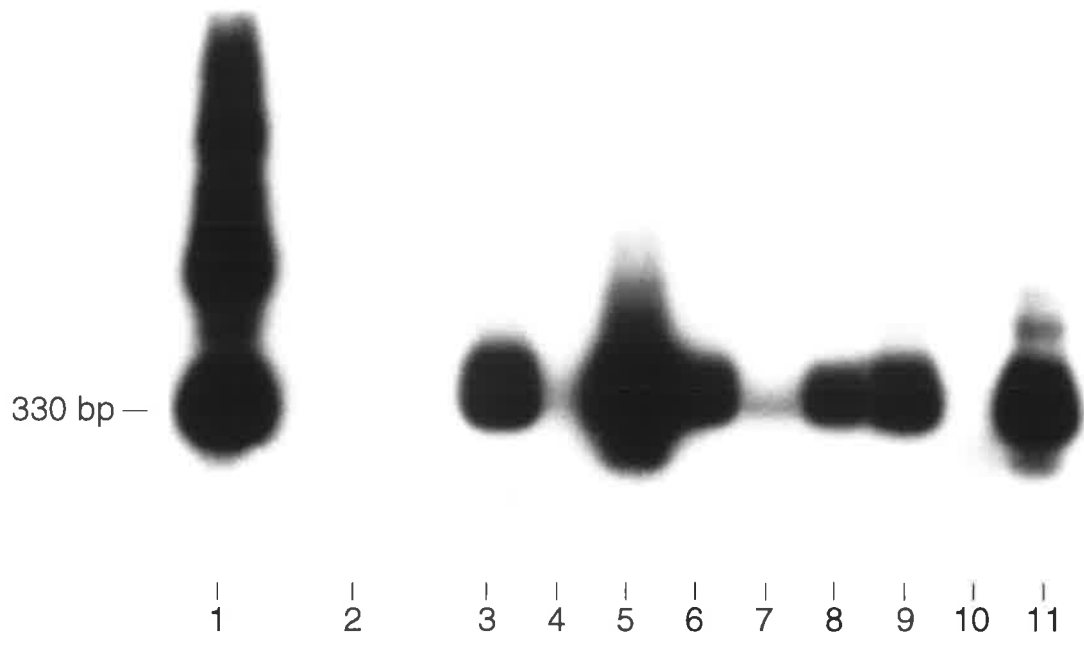
CHAPTER SIX:

RT-PCR FOR K19

6.1   INTRODUCTION

The immunobead PCR technique for detection of colorectal cancer cells in peripheral blood using PCR amplification of mutations in codon 12 of the KRAS gene as the marker for the malignant cells was developed in the Haematology-Oncology Laboratory at The Queen Elizabeth Hospital (Hardingham et al, 1993; Hardingham et al, 1995). This approach has the disadvantage that circulating tumour cells can only be detected in patients whose primary tumour contains the K-ras codon 12 mutation. In the absence of a sufficiently frequent gene mutation in breast cancers, attention was directed towards mRNA-based markers that have the advantage of application to virtually all carcinomas. During work towards the development of the immunobead RT-PCR technique, published work suggested that cytokeratin 19 could be an appropriate marker for cells from the majority of carcinomas (Traweek et al, 1993; Datta et al, 1994; Schoenfeld et al, 1994).

The human cytokeratin 19 gene comprises 4667 nucleotides, with five introns and a total length of mRNA and corresponding cDNA of 1394 nucleotides (Bader et al, 1988. There is considerable variation in the sizes of the introns, with intron one having 2571 nucleotides, intron two 196 nucleotides, intron three 305, intron four 113 and intron five 145 nucleotides.

When K19 cDNA is amplified by PCR, amplification of contaminating genomic DNA can be avoided by using PCR primers that specify a region of the K19 that involves two separate exons, since amplification of genomic DNA will be prohibited by the intervening exon. However, if a pseudogene sequence corresponds to the cDNA sequence, it may be amplified. The cell lysis technique

113

for reverse transcription of cellular RNA exposes total cellular nucleic acid to the reverse transcriptase, and does not exclude DNA. This provides potential for PCR amplification of pseudogenes, since pseudogenes have no introns.

A processed pseudogene is a DNA segment that arises when a reverse transcript of RNA is integrated into the genome. Processed pseudogenes do not generally code for functional proteins and lack the introns that are seen in functional genes. The sequence of a pseudogene commences at the 5′ end of the mRNA and contains a poly(A) tail at the 3′ end. The incorporation of a reverse transcript into the genome is believed to be a phylogenically ancient event and occurs only in those genes that are expressed in cells of the germ-line (Savtchenko et al, 1988). Consequently, the genes that have accompanying pseudogenes are commonly those that encode for "housekeeping" proteins, which include tubulin, actin, ribosomal proteins and cytokeratins 8, 18 and 19, which are the intermediate filament proteins that show the earliest appearance in embryogenesis (Bader et al, 1988; Savtchenko et al, 1988).

The sequence of the K19 pseudogene was published by Savtchenko and colleagues in 1988, and shows 82% homology with the human K19 gene (Savtchenko et al, 1988). Burchill and colleagues have suggested that more than one K19 pseudogene may exist (Burchill et al, 1995), but the principle references on the subject do not confirm this (Bader et al, 1988; Savtchenko et al, 1988). Therefore, it should be possible to design a PCR that exploits differences in nucleotide sequence between the functional gene and its corresponding pseudogene, to exclude the pseudogene from amplification.

Assessment of K19 as a possible tumour cell marker using immunobead RT-PCR is described below. Results of assessment of the K19 RT-PCR assay for exclusion of pseudogene amplification, for sensitivity, and for specificity in a haemopoietic environment are presented.

114

6.2   CONDITIONS   FOR   EXCLUSION   OF   PSEUDOGENE AMPLIFICATION

It was necessary to establish that the K19 PCR was specific for K19 message, and would only amplify a product from cDNA, not genomic DNA. Two PCR annealing temperatures were compared to assess which would provide the greatest specificity for PCR on samples of genomic DNA.

Results from six samples of genomic DNA preparations, applied to two separate K19 PCRs, one with annealing temperature of 60°C, the other 68°C, are shown in figure 6.1. Three samples were from peripheral blood mononuclear cells of normal donors, one was prepared from cells of the breast cancer cell line T-47D, one from peripheral blood mononuclear cells of a patient with non-Hodgkin's lymphoma (NHL), and the last was taken from a preparation of cells from a normal lymph node.

On ethidium bromide-stained agarose gels, no PCR product was evident from genomic DNA samples in the PCR annealed at 68°C, whereas bands conforming to the expected size for K19 were seen for two of the three DNA preparations from normal peripheral blood mononuclear cells and for the NHL patient's DNA.

After autoradiograph exposure of 24 hours, K19 signals were seen from all genomic DNA samples annealed at 60°C, with the exception of the sample from the normal lymph node. There was no evidence of K19 PCR product from any of the DNA samples annealed at 68°C, even with prolonged exposure time of autoradiography.

There was little difference in band intensity for the three positive controls whether the PCR annealing temperatures were 60°C or 68°C. The first control was cDNA from the MCF-7 cell line. The second and third controls were cDNA preparations from limited numbers of cells, specifically 250 and 200 cells

115

respectively from the breast cancer cell line MB-453, frozen at -80°C in 2-5μl of PBS.

Reverse transcriptase-negative controls were also negative for K19 RT-PCR at a PCR annealing temperature of 68°C. Aliquots of approximately two hundred cells from the MB-453 cell line, stored at -80°C in 5μl of PBS, were thawed and each divided into two separate lysis mixes - one with Superscript II reverse transcriptase, the other without reverse transcriptase. After autoradiography a PCR product conforming to the expected size for K19 was evident where the reverse transcriptase had been included, but not where reverse transcription had not occurred. This demonstrated that the amplification of the K19 signal was truly occurring from cDNA reverse transcription product, and not from "contaminating" genomic DNA.

6.3   SENSITIVITY OF K19 RT-PCR

Cell lines by GITC RNA extraction

Strong signals for K19 PCR product were seen on ethidium bromide staining of agarose gels for all test samples from epithelial cell lines or tissues. These cDNA samples were prepared by the GITC method of RNA extraction from the breast cancer cell lines T-47D, MB-453 and MCF-7, the colon cancer cell line LIM 1899, and from a specimen of human colon cancer (generously donated by Denise O'Keefe, Ph.D.). The amount of RNA template for reverse transcription had not been quantified.

Cell lines by serial dilution

Cells from breast cancer cell lines, diluted in PBS and subjected to the cell lysis RT-PCR assay, provided PCR products that could be easily visualised on agarose gel electrophoresis at levels of 80 to 100 cells. Figure 6.2 shows an example of the sensitivity of the K19 RT-PCR assay when serially diluting a cell

116

sample to small numbers of cells. Aliquots of approximately 140 and 14 T-47D cells were taken for reverse transcription (the same reverse transcription product was used for CD44 assessment, see Chapter Five) and K19 PCR applied. PCR products for K19 were easily visible on ethidium bromide staining for 140 T-47D cells, but were faint for 14 cells. After 24 hours exposure of the autoradiograph, strong K19 expression was seen for all lanes, suggesting that cells from the breast cancer cell line T-47D would express K19 transcripts in sufficient quantity to permit detection of K19 product from a single cell using cell lysis RT-PCR.

Cell lines by micropipette selection

Cells from the breast cancer cell line T-47D were prepared as a dilute suspension in PBS for aspiration by micropipette under light microscopy, and selected for reverse transcription. PCR reliably displayed K19 bands from single T-47D cells selected by the micropipette when enhanced by Southern transfer and autoradiography. In the example shown in figure 6.3, three out of four single cells aspirated from suspension by micropipette displayed PCR product for K19 on autoradiography, while two and five cells aspirated by pipette gave relatively strong signals.

Cell lines frozen as positive controls

Aliquots of 100 to 250 cells from cell lines in residual volumes of PBS, stored in microfuge tubes at $-80^{0}C$, could be thawed in "Lysis mix" to produce an RT-PCR product for K19 that could be reliably identified on an ethidium bromide stained agarose gel. Multiple samples were therefore stored in microfuge tubes at $-80^{0}C$ for up to six weeks for use as reverse transcription positive controls, since these more closely represented the level of sensitivity required of the RT-PCR assay than did larger amounts of RNA obtained by GITC extraction of RNA from large cell numbers.

Figure 6.1   **Autoradiograph showing K19 PCR on samples of genomic DNA, comparing PCR annealing temperatures of 68^0C and 60^0C.**

No K19 bands are seen with the annealing temperature at 68^0C, with strong positive controls at 68^0C.

Lanes 1 to 3:	Positive controls
Lanes 4 and 5:	Negative controls (no DNA)
Lanes 6, 7 and 8	PBMNCs from 3 normal donors
Lane 9	T-47D cell line
Lane 10	Patient with NHL
Lane 11	Normal lymph node.
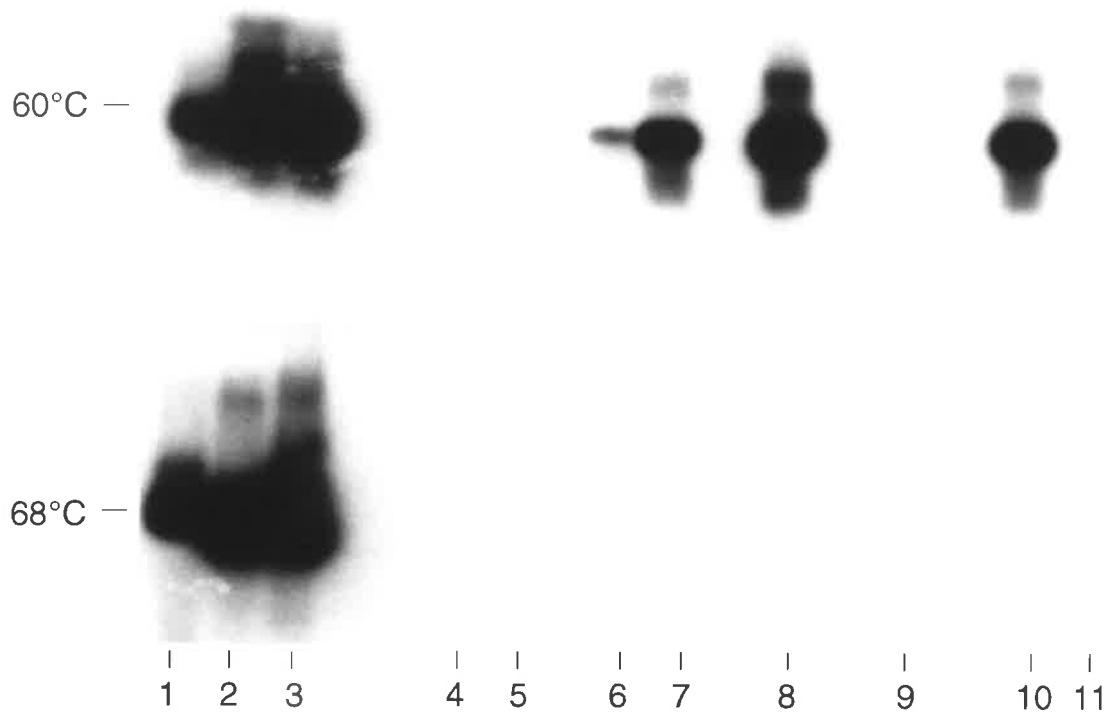
Figure 6.2   Agarose gel (top) and autoradiograph (bottom) showing K19 RT-PCR results for 140 and 14 T-47D cells, prepared by serial dilution.

Lanes 1 and 3:	Positive controls
Lane 2:	PCR negative control
Lanes 4,5 and 6:	140 cells
Lanes 7, 8 and 9:	14 cells

751 bp —

1   2   3   4   5   6   7   8   9

751 bp —

1   2   3   4   5   6   7   8   9
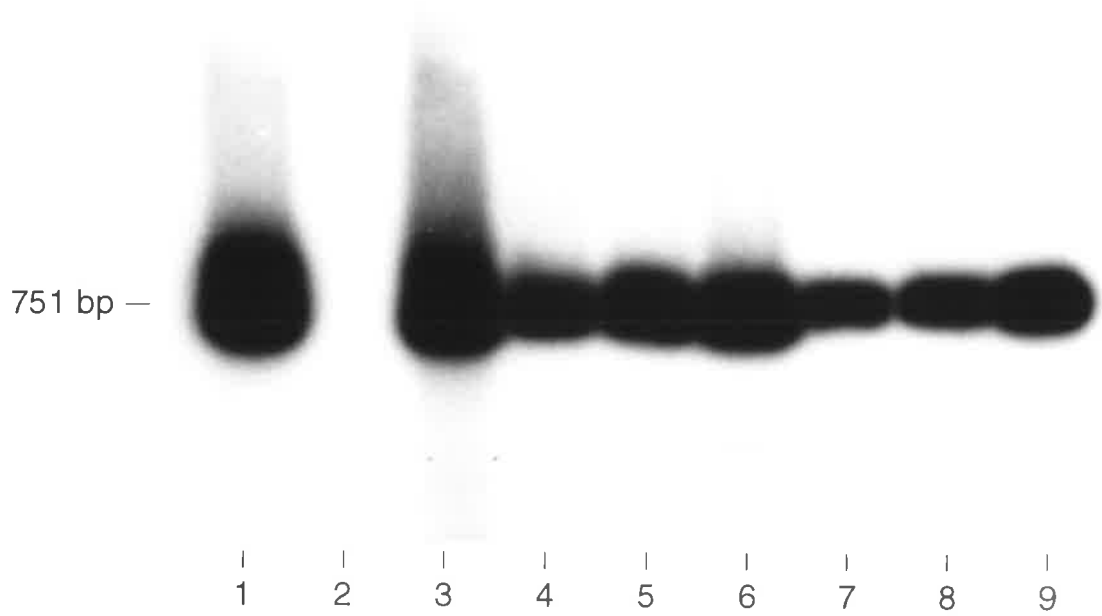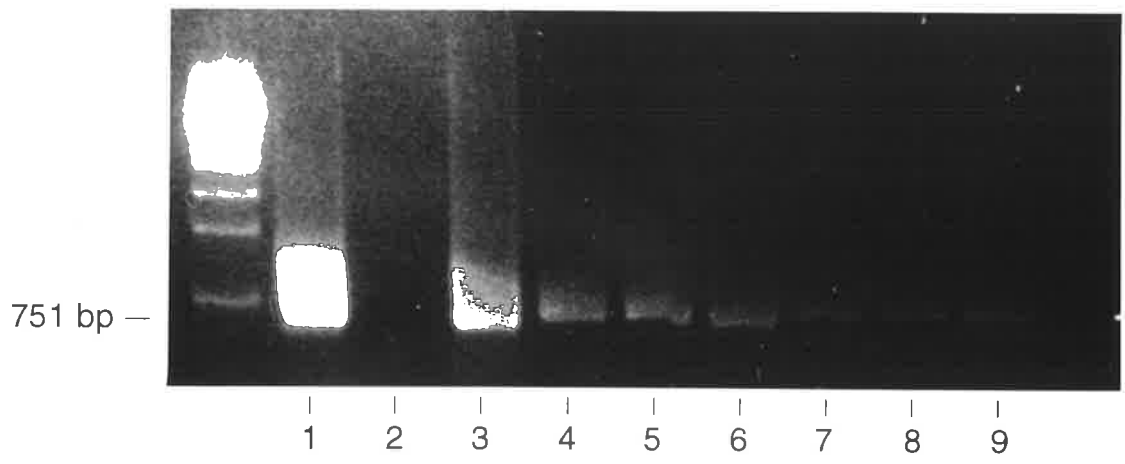
Figure 6.3   **Single cell RT-PCR for K19 using T-47D breast cancer cells aspirated from suspension by micropipette.**

Lanes 1 and 10:	Positive controls
Lanes 2 and 9:	Negative controls
Lanes 3 to 6:	Single T-47D cells
Lane 7:	Two cells
Lane 8:	Five cells

1　2　3 4 5 6 7 8　9　10
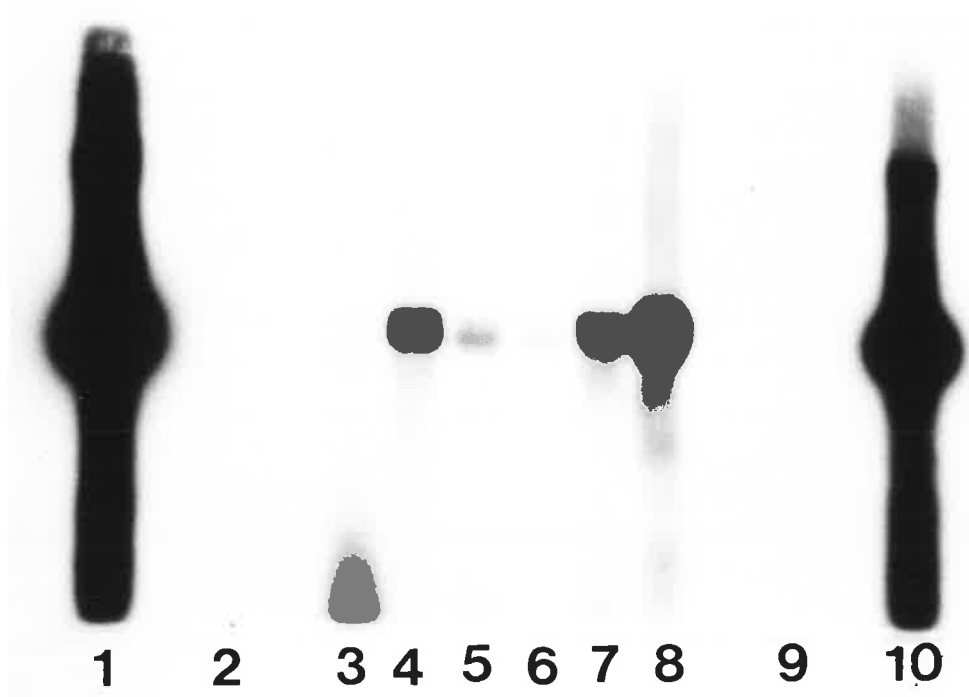
Human breast cancer cells

As stated in Chapter 5.4, RT-PCR product for K19 was seen at autoradiography on all samples of breast cancer cells aspirated by micropipette from a suspension of cells from a lymph node macroscopically replaced by breast cancer, except the fourth lane of single cells aspirated (data not shown). This confirmed the sensitivity of the K19 RT-PCR for single human tumour cells, and, as discussed below, proved useful in confirming that the CD44 PCR signals obtained from these aspirated cells did indeed come from cells of epithelial origin (see Chapter Five).

6.4 EXPRESSION OF K19 BY HAEMOPOIETIC CELLS

Expression of K19 in haemopoietic cells by GITC extraction of RNA from large numbers of cells

To determine whether K19 transcripts could be detected by RT-PCR in "normal" haemopoietic tissues, a total of 15 haemopoietic samples had cDNA prepared by GITC RNA extraction and reverse transcription.

Six bone marrow samples were taken from patients with pathologies varying from acute lymphocytic leukaemia to chronic myeloid leukaemia and NHL. The nine peripheral blood samples were taken from five healthy donors, two leukaemics, and two were samples of neonatal umbilical cord blood (supplied courtesy of the Department of Obstetrics and Gynaecology, The Queen Elizabeth Hospital).

The relative amounts of RNA extracted from the above samples were not quantified, and the methods of reverse transcription may have differed for the samples donated by other laboratory workers. The important aspect of assessment however, is that intact cDNA should be present in each sample. This was assessed by PCR for CD44, since the standard (or haemopoietic) form is

ubiquitously expressed on haemopoietic cells, and the variable coding region of the CD44 gene ensures that PCR product for CD44 is derived entirely from cDNA.

PCR for CD44 demonstrated strong expression of the standard isoform in all samples, both for bone marrow and peripheral blood. Southern transfer and autoradiography enhancement of sensitivity was not performed in this case because of the unequivocal results on ethidium bromide staining alone.

None of the total of 15 haemopoietic specimens expressed K19 by RT-PCR analysis, even after Southern transfer and autoradiography enhancement.

Expression of K19 in haemopoietic cells by immunobead RT-PCR for K19

The immunobead RT-PCR method was applied to 15 bone marrow samples taken from patients with conditions other than carcinoma, and 13 peripheral blood samples from healthy volunteers or patients with conditions other than carcinoma. All 15 bone marrow samples were negative for K19 transcripts after autoradiography, but one of the 13 peripheral blood samples was positive for K19 by immunobead RT-PCR. This sample had been taken from a "healthy volunteer", towards the conclusion of work towards this thesis, after many of the patient results had been collected.

	RT-PCR by GITC	Immunobead RT-PCR
Bone marrow	6/6	15/15
Peripheral blood	9/9	12/13

Table 6.1   Numbers of clinical haemopoietic samples which were negative for K19 expression.

6.5   DISCUSSION

6.5.1   Design of primers for K19 PCR

The primers designed for K19 immunobead RT-PCR were modifications of those designed by Datta and colleagues, which were specifically designed to avoid amplification of the K19 pseudogene (Datta et al, 1994). The primers of Datta were designed to generate a PCR product that spanned all five introns of the K19 gene and produce an mRNA-specific product. To avoid amplification of the known processed pseudogene, the primers were further designed to incorporate differences between the gene and the pseudogene at the 3′ ends of the sequence targeted by the primers (Datta et al, 1994). Oligonucleotide primers hybridise specifically to their complementary sequences at the 3′ ends of each strand of the target sequence, so that increasing the mismatches between gene and pseudogene in these regions decreases the likelihood of primer extension from the pseudogene sequences (Watson et al, 1992).

Having chosen the best 3′ sites, they then made their primers unusually long (up to 30 bases), leading to high primer melting temperatures which would tolerate high PCR annealing temperatures for maximum specificity.

The K19 primers designed for immunobead RT-PCR were modifications of the two internal primers (designated "C" and "D") of Datta's nested PCR (Datta et al, 1994). The "sense" primer (modified from "C") was located in exon one, totalled 24 bases and had an approximate melting temperature of 74^{0}C (5′ GACTACAGCCACTACTACACGACC 3′).

The "antisense" primer (modified from "D") was located in exon six, totalled 25 bases and had an approximate melting temperature of 80^{0}C: (5′ AGCCGCGACTTGATGTCCATGAGCC 3′). The K19 sequence, with primers

120

```
   1   CGGGGGTTGCTCCGTCCGTGCTCCGCCTCGCCATGACTTCCTACAGCTATCGCCAGTCGT
  61   CGGCCACGTCGTCCTTCGGAGGCCTGGGCGGCGGCTCCGTGCGTTTTGGGCCGGGGGTCG
 121   CTTTTCGCGCGCCCAGCATTCACGGGGGCTCCGGCGGCCGCGGCGTATCCGTGTCCTCCG
 181   CCCGCTTTGTGTCCTCGTCCTCCTCGGGGGGGCTACGGCGGCGGCTACGGCGGCGTCCTGA
 241   CCGCGTCCGACGGGCTGCTGGCGGGCAACGAGAAGCTAACCATGCAGAACCTCAACGACC
 301   GCCTGGCCTCCTACCTGGACAAGGTGCGCGCCCTGGAGGCGGCCAACGGCGAGCTAGAGG
 361   TGAAGATCCGCGACTGGTACCAGAAGCAGGGGCCTGGGCCCTCCCGC**GACTACAGCCACT**
```
     D   **A**   **T**
```
1  421   **ACTACACGACC**ATCCAGGACCTGCGGGACAAG*ATTCTTGGTGCCACCATTGAGAACTCCA
2  481   GGATTGTCCTGCAGATCGACAACGCCCGTCTGGCTGCAGATGACTTCCGAACCAA*GTTTG
   541   AGACGGAACAGGCTCTGCGCATGAGCGTGGAGGCCGACATCAACGGCCTGCGCAGGGTGC
   601   TGGATGAGCTGACCCTGGCCAGGACCGACCTGGAGATGCAGATCGAAGGCCTGAAGGAAG
3  661   AGCTGGCCTACCTGAAGAAGAACCATGAGGAG*GAAATCAGTACGCTGAGGGGCCAAGTGG
   721   GAGGCCAGGTCAGTGTGGAGGTGGATTCCGCTCCGGGCACCGATCTCGCCAAGATCCTGA
   781   GTGACATGCGAAGCCAATATGAGGTCATGGCCGAGCAGAACCGGAAGGATGCTGAAGCCT
4  841   GGTTCACCAGCCGG*ACTGAAGAATTGAACCGGGAGGTCGCTGGCCACACGGAGCAGCTCC
   901   AGATGAGCAGGTCCGAGGTTACTGACCTGCGGCGCACCCTTCAGGGTCTTGAGATTGAGC
5  961   TGCAGTCACAGCTGAGCATG*AAAGCTGCCTTGGAAGACACACTGGCAGAAACGGAGGCGC
  1021   GCTTTGGAGCCCAGCTGGCGCATATCCAGGCGCTGATCAGCGGTATTGAAGCCCAGCTGG
```
                           A T
```
  1081   CGGATGTGCGAGCTGATAGTGAGCGGCAGAATCAGGAGTACCAGC**GGCTCATGGACATCA**
  1141   **AGTCGCGGCT**GGAGCAGGAGATTGCCACCTACCGCAGCCTGCTCGAGGGACAGGAAGATC
```

Figure 6.4   The K19 sequence, showing PCR primers and primer

mismatches with the processed pseudogene.

Primers are marked in bold underlined print.

The pseudogene sequence mismatches are designated

immediately above the corresponding base.

D   Denotes a deletion in the pseudogene sequence, which

therefore causes mismatches with the pseudogene upstream of

the deletion.

*   Denotes intron-exon boundary, with the number in the

left margin designating the intron.

and mismatches with the processed pseudogene, is shown in Figure 6.4. These primers gave an expected PCR product of 751 base pairs, in comparison to the 745 base pair product of Datta's equivalent primers, and were modified specifically to try to minimise complementarity between primers.

The oligonucleotide used as a labelled internal probe for Southern transfer and autoradiography comprised 25 bases and had an approximate melting temperature of 78^0C (5′ AGATCTGCATCTCCAGGTCGGTTCC 3′).

6.5.2 Choice of PCR annealing temperature for exclusion of pseudogene amplification

The experiment performed comparing the annealing temperatures of 60^0C and 68^0C demonstrated that the primers would amplify pseudogene sequences in the PCR at an annealing temperature of 60^0C, but at the higher annealing temperature pseudogene amplification was abolished. At lower annealing temperatures, primers can anneal to the DNA template at sites where sequences differ slightly from the target sequences, even with 3′ mismatches. Amplification can occur when mismatching primers are close together on opposite strands of the DNA. Because correct complementary sequences for the primers are incorporated into the synthesised fragments, an unwanted sequence is produced with ends that precisely match the primers. Therefore, an unwanted sequence becomes efficiently amplified, leading to false positive results, and amplification of the desired sequence is less efficient. At the higher PCR annealing temperature, annealing of primers to sites other than the desired ones is minimised, reducing the likelihood of amplification of mismatched sequences and improving the efficiency of amplification of target sequence (Watson et al, 1992).

The positive controls suggest that there has not been any compromise of sensitivity at the higher annealing temperature of 68^0C, in fact there may

have been a marginal improvement (Figure 6.1). Several factors may be responsible for this: apart from the increased efficiency, and reduced primer dimer formation (Furukawa et al, 1994), the Taq DNA polymerase enzyme has a reported optimal working temperature of around 75^0C (Boehringer Mannheim product information), and its efficiency may be reduced by as much as a factor of two at 60^0C and by a factor of ten at 37^0C (Sambrook et al, 1989).

A total of ten different genomic DNA specimens have been tested for K19 amplification by PCR at an annealing temperature of 68^0C. None of these have amplified a pseudogene. If the assertion by Burchill and colleagues that more than one K19 pseudogene exists is correct (Burchill et al, 1995), then one would have expected other pseudogenes to have been revealed.

An alternative method of demonstrating absence of "false positive" RT-PCR product (i.e. pseudogene amplification) from genomic DNA is to use a "reverse transcriptase-negative control". In this case, a positive signal by RT-PCR should be absent from the same material if the reverse transcriptase enzyme has not been added to the system, that is, reverse transcription of the RNA to cDNA has not occurred. The reverse transcriptase-negative controls demonstrated that the PCR product obtained by the cell lysis RT-PCR technique from low numbers of MB-453 cell line cells taken from -80^0C storage was not due to amplification of the K19 pseudogene.

It was therefore concluded that the PCR design adequately excluded amplification of the K19 pseudogene.

6.5.3 Sensitivity of K19 RT-PCR

Since the number of tumour cells isolated from 10 to 20ml of blood by the immunobeads would be very small, the RT-PCR assay to detect the bound cells must be as sensitive as possible, and sensitivity to the level of a single cell would be desirable.

The development of the RT-PCR assay was carried out using cells from cell lines in culture, as described in Chapter Four. Results presented here demonstrate that a K19 signal can be amplified from a single cultured cell, and that the cell lysis method for reverse transcription of cellular RNA provides the potential for an RNA-based marker to be used with the immunobeads for detection of carcinoma cells in a haemopoietic environment.

The technique of aspiration of single cells from dilute suspension by the micropipette allowed the sensitivity of the RT-PCR to be demonstrated with greater certainty than would be allowed by the method of serial dilutions of counted cells.

Abundant cellular expression of K19 transcripts is not unique to cell lines in culture. Amplification of K19 transcripts from single human metastatic tumour cells suggests that these cells express K19 transcripts at similar levels to cell lines in culture, and further demonstrates the potential of K19 as a marker for immunobead RT-PCR in the clinical setting.

6.5.4   Specificity of K19 RT-PCR

The use of K19 to differentiate epithelial cells from a large population of haemopoietic cells requires that K19 is not expressed by haemopoietic cells. Most workers studying the expression of K19 by RT-PCR to date have published evidence suggesting that this is the case (Datta et al, 1994; Schoenfeld et al, 1994; Traweek et al, 1993), but there is some controversy about this in the recent literature (Johnson et al, 1995; Krismann et al, 1995). It was therefore important to attempt to replicate the work of others, particularly Datta and colleagues, to determine whether expression of K19 mRNA by haemopoietic cells can be detected by RT-PCR.

Initial results using cDNA prepared by GITC extraction from large cell numbers, and from immunobead-reverse transcription samples from bone

123

marrow and peripheral blood suggested that amplification of K19 transcripts was not seen in haemopoietic cells. Many clinical samples had been tested using K19 immunobead RT-PCR before the positive K19 signal from the blood of the healthy volunteer was found. The proportion of haemopoietic samples amplifying K19 transcripts in this series of one in 43 is coincidentally similar to the one in 39 of Datta and colleagues (Datta et al, 1994). An important difference however, is that the K19-positive sample of Datta and colleagues was a bone marrow biopsy from a patient with CML, in contrast to the K19-positive blood sample from a healthy volunteer in this series.

The amplification of K19 message from peripheral blood of a healthy volunteer by RT-PCR stimulated review of the specificity of K19 expression when assessed by RT-PCR. Since this came towards the end of collection and assessment of clinical samples, and since it led to new work, the results of the review of K19 are examined later, in Chapter Nine.

CHAPTER 7:

TECHNICAL ASPECTS OF IMMUNOBEAD RT-PCR

7.1 INTRODUCTION

The results of in vitro studies to assess combination of the K19 RT-PCR assay with tumour cell isolation by the immunomagnetic beads are discussed.

7.2 THE OPTIMUM NUMBER OF IMMUNOBEADS PER SAMPLE

7.2.1 Introduction

Most of the technical aspects of working with immunobeads had been assessed in the Haematology/Oncology Laboratory at the Queen Elizabeth Hospital (Hardingham et al, 1993), prior to the commencement of work towards this thesis. Indeed, the efficiency of the immunobead isolation of tumour cells in whole blood had been indirectly confirmed by the excellent clinical results seen in its application to colon cancer patients (Hardingham et al, 1995).

7.2.2 Results

The optimum number of immunobeads per sample in a 10ml tube had been determined by reconstruction experiments to be 2×10^6 (Hardingham et al, 1993). This was confirmed in a reconstruction experiment using cells from the T-47D cell line, in which 50 and 500 cells (prepared by serial dilutions) were incubated in separate samples of 10ml whole blood against 1×10^6, 2×10^6 and 4×10^6 immunobeads. After RT-PCR, strong bands conforming to the expected 751 base pair product were seen on agarose gel electrophoresis in those samples incubated with 1×10^6 and 2×10^6 immunobeads. Bands were only faint for those samples incubated with 4×10^6 immunobeads. Two million beads was the number chosen to mix with the samples.

7.3   SENSITIVITY OF IMMUNOBEAD RT-PCR

7.3.1   Introduction

Previous work of immunobead-PCR for colon cancer cells had published a sensitivity of detection of one cell from the colon cancer cell line SW480 per 10^5 leukocytes in whole blood (Hardingham et al, 1993). Subsequent work demonstrated the sensitivity to be more in the order of one SW480 cell amongst 10^6 leukocytes in whole blood (J.E. Hardingham, personal communication).

Experiments were repeated using cells from either the T-47D or MB-453 breast cancer cell lines, in 10ml of either whole blood or PBS, and tested by the immunobead RT-PCR method for K19.

7.3.2   Results and Discussion

An example of the results of sensitivity testing using T47-D cells is shown in figure 7.1, showing the K19 signals on autoradiography for 500, 50 and 25 cells from the T-47D cell line, each in 10ml whole blood. The general decrease in strength of the K19 signal from 500 cells down to 25 cells is evident. This particular example proved to be around the limit of sensitivity of immunobead RT-PCR using K19, being of the order of 20 to 25 cells per 10ml of whole blood, with 50 cells being reliably detected. This approximates detection of one tumour cell amongst 2×10^6 leukocytes.

When samples were performed in duplicate, there was variability of signal intensity amongst two tubes carrying supposedly the same number of tumour cells. Such variability might be expected however, given the possible variables involved in: 1) beads binding to the cells, 2) recovery of beads after washing and transfer from the 10ml tube to the microfuge tube, 3) efficiency of lysis of a relatively small number of cells in the presence of so many beads, 4)

Figure 7.1 **The sensitivity of immunobead RT-PCR, using T-47D cells in 10ml whole blood.**

The 751-bp product for K19 is displayed.

Lane 1:	Positive control
Lane 2:	Negative control
Lane 3:	500 T-47D cells
Lane 4:	50 cells
Lane 5:	25 cells

1   2   3   4   5
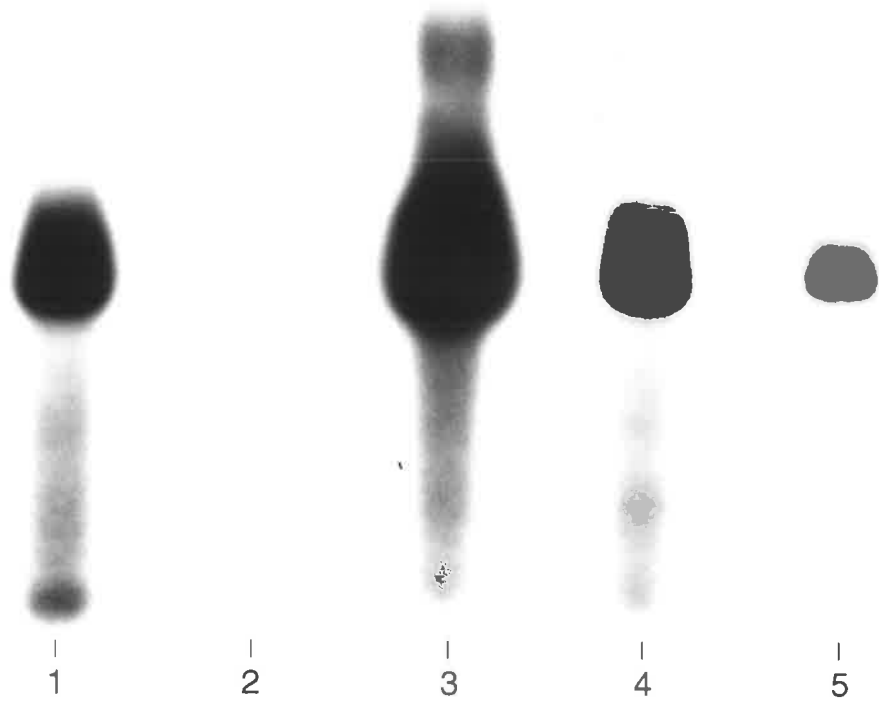
efficiency of reverse transcription with low numbers of cells, and 5) variables within the PCR reactions in different tubes, for low copy number of target.

The calculated sensitivity of detection of immunobead RT-PCR using K19 of one tumour cell amongst 2×10^6 leukocytes compares favourably with other techniques. The RT-PCR methods that have been published to date use PCR for a tissue-specific transcript on reverse transcription product from RNA extracted from an entire sample, and, as discussed in Section 1.3.3, demonstrate detection sensitivities of between 1 in 10^4 to 1 in 10^7 normal leukocytes.

The application of the RT-PCR assay to the immunobeads provides sensitivity of tumour cell detection that is at least the equal of the immunobead-PCR technique (Hardingham et al, 1993), but may be less reliable. Reconstruction experiments were not always as successful as the example shown. Of six experiments performed at different times, using either the T-47D or MB-453 cell lines, two failed to give results of expected sensitivity. No conclusion was reached to explain the discrepancy in the results in these two experiments. At the time of these two experiments, cells were often allowed to grow to confluence in culture before harvesting for counting and dilution. Inaccuracies in counting and subsequent distribution are more likely if cells are grown to confluence in culture, because they are more likely to clump together, making the already approximate nature of counting even less precise. While the application of trypsin was effective in mobilising the monolayer of culture for cell harvesting, repetitive mixing of the cells could not break the cell clusters that occurred in confluent cultures.

During the course of this work it became apparent that the timing of cell harvest from culture was important if the optimal results were to be obtained, so that the explanation for failure of the experiments could simply be

127

that clumping of confluent cells created such inaccuracies in counting that no cells were distributed to the blood samples for incubation with the beads.

On several occasions of selecting cells under light microscopy by micropipette for RT-PCR, it was observed that a "clump" of cells (approximately 10 to 20) aspirated by the pipette would give an RT-PCR product of no greater intensity than that from single cells, or of unexpectedly low intensity, given some of the excellent results seen at the single cell level. An explanation for this was not found. It is possible that the NP-40 concentration was not sufficient to lyse cells within a cluster - this seems unlikely considering that some of the strong PCR bands that have been demonstrated from 500 or so cells suggest that the lysis mix has no trouble dealing with a relatively large number of cells. Another explanation is that expression of K19 mRNA is down-regulated when cells are in such contact with each other that they are "clumped" or cultured to confluence. Since keratins are fundamental to the cytoskeleton of epithelial cells, one could postulate that their production is up-regulated at times of cell growth, and down-regulated at times of growth arrest; and that cells are in a stage of growth arrest when cultured to confluence. The implications of this hypothesis for the application of immunobead RT-PCR using K19 are unknown.

The sensitivity of the immunobead RT-PCR technique derives principally from the preliminary isolation of tumour cells by the immunobeads. The prior tumour cell enrichment by the immunobead isolation provides a total amount of RNA that is much smaller than that of other RT-PCR methods that work from RNA extracted from the entire samples being screened for tumour cells. The reverse transcription product is derived from the putative tumour cells and the few white blood cells trapped by the beads. The exclusion of non-target cells from the RT-PCR should decrease the likelihood of amplification of

low-level transcription of tissue-specific genes from non-specific cells, which has been used as an explanation for false positive results by investigators who use total RNA extraction for RT-PCR (Smith et al, 1991; Datta et al, 1994; Brown et al, 1995). This is discussed in Chapter One.

7.4   TECHNICAL DIFFICULTIES OF WORKING WITH ARCHIVAL SPECIMENS

7.4.1   Introduction

Early in the course of this work bone marrow biopsies from patients with breast cancer being assessed by the Haematology/Oncology Unit at The Queen Elizabeth Hospital were stored in 10% DMSO and DMEM culture medium in liquid nitrogen. It was hoped that these specimens could be thawed at a later date for testing when the tumour marker for use with the immunobeads had been developed. In addition, aliquots of all stem cell harvests prepared at the hospital had also been stored in 10% DMSO in liquid nitrogen. It was anticipated therefore that an archive of material, with immediate information on patient outcome, was waiting for study.

It was found, however, that the cells lysed rapidly when thawed, centrifuged to wash out the DMSO (which is toxic to cells at room temperature), and resuspended into 10ml PBS for incubation with the immunobeads. The sticky DNA and proteins released by the lysed cells caused the immunobeads to clump together, prohibiting their binding to target cells and prohibiting their attraction by the magnets. Many of the immunobeads were therefore washed away with the remainder of the PBS, so that target cell binding and immunobead retrieval were very poor. Lysis of cells in peripheral blood stem cell collections is a recognised problem of thawing of the patients' PBSC samples, and granulocytes in the impure PBSCs are thought to be the quickest

cells to disintegrate. The cell lysis is exacerbated by a centrifuge step to wash out the DMSO. Stem cell harvests are therefore infused directly into the recipient's peripheral bloodstream, without washing out the DMSO (M. Shepherd B.Sc., T.Q.E.H. Department of Haematology-Oncology, personal communication).

The incubation period required to allow rosetting of immunobeads around target tumour cells was ample time for cell lysis, even without washing out the DMSO, and even with more "physiological" media like 10% foetal calf serum in culture media.

Two samples of archival PBSCs stored in liquid nitrogen were thawed under the gentlest possible conditions to try to minimise loss of cells by lysis.

7.4.2   Materials and Methods

An aliquot of archival PBSC from one patient with NHL and the other with chronic myeloid leukaemia (CML), stored in 10% DMSO in a cryotube in liquid nitrogen were thawed rapidly at 37^0C, suspended in culture medium (DMEM with 10% FCS and 1% glutamine), and washed by gentle centrifuge at 900rpm at 4^0C for 10 minutes (Beckman). The resulting pellets were gently resuspended in culture medium (DMEM/FCS/glutamine).

The stem cell suspensions were filtered through a 40µm nylon mesh and the residue examined by light microscopy.

7.4.3   Results and Discussion

Clumps of cell debris were present in suspension after the wash. These clumps were removed by the filtration step, but light microscopy showed that many intact cells were trapped in the amorphous debris. Further cell lysis and clumping occurred as the cells stood in suspension.

The progression of cell lysis throughout the time of incubation with the beads would lead to progressive loss of cells and of beads caught in the debris,

and would obviate any advantage of removing the initial clumps by filtration. The loss of cells through lysis would also greatly decrease the chances of detection of contaminating tumour cells.

If the putative contaminating tumour cells lyse as readily as the polymorphonuclear and mononuclear cells, they are unlikely to be sufficiently viable to survive reinfusion and to establish new metastatic colonies in the recipient of the stem cell transplant. The problem with the immunobead technique for archival specimens is not the loss of tumour cells as such, but the lysis of leukocytes which leads to loss of the immunobeads and hence failure of the immunobead-tumour cell isolation step.

Assessment of the thawed samples requires a method that deals with the cells as soon as they are thawed. Therefore, RT-PCR based on total RNA extraction, or immediate fixation of cells for immunocytochemistry assessment, are probably the most practical means of assessing archival samples for the presence of contaminating tumour cells.

7.5 DENATURATION OF RNA CAN BE AVOIDED FOR IMMUNOBEAD RT-PCR

7.5.1 Introduction

Many protocols for reverse transcription recommend a heating step prior to initiation of reverse transcription to denature the RNA (Kumazaki et al, 1994; O'Brien et al, 1994). The protocol supplied with Superscript II reverse transcriptase by GibcoBRL recommends heating the primer mix of RNA, primers and sterile distilled water (equivalent to the "lysis mix" in this modification) to $70^{0}C$ for 10 minutes, then quickly chilling the mix on ice. It was observed on several occasions that immunobeads that had been incubated with blood or bone marrow became sticky and clumped after the denaturation step

131

(perhaps by denaturation of cytoplasmic proteins). Clumping of the beads makes them more difficult to work with, and may theoretically restrict access of components of the reverse transcription mix to RNA template that might be trapped between the "sticky" beads. An experiment was designed to try to determine whether the denaturation step was necessary for efficient reverse transcription at limited cell numbers.

7.5.2   Materials and Methods

T-47D cells were distributed amongst microfuge tubes giving counts of approximately 50 cells and 100 cells, in 2µl PBS. Lysis mix was added to two sets of duplicates of 50 and 100 cells. One set of duplicates was heated to 70°C for 10 minutes and then quickly chilled on ice before the reverse transcription mixes were added. The second set of duplicates was not heated, but immediately received the reverse transcription mixes. Both sets of duplicates were incubated at 37°C for 60 minutes, then inactivated at 70°C for 10 minutes. PCR for K19 was performed, and the products electrophoresed on an agarose gel and visualised by ethidium bromide staining.

7.5.3   Results

The bands for K19 were visible on ethidium bromide for the 50 and 100 cell tubes, with very little difference between those which had been denatured at 70°C and those which had not. The denaturation step of heating the cell lysate to 70°C for 10 minutes was not necessary for strong expression of RT-PCR product for K19. It was a technical advantage to omit the denaturation step for immunobead RT-PCR.

7.6 POSITIVE CONTROLS FOR IMMUNOBEAD RT-PCR

7.6.1 Introduction

An appropriate positive control for reverse transcription is necessary to ensure that the components of a reaction have been added properly and that components are functionally intact. RNA stored at -80^{0}C is an appropriate control for most reverse transcription reactions, since most are working from total RNA extractions. GITC extraction of total RNA from breast cancer cell lines was initially used for positive controls, but this was an inadequate control for RNA released by cell lysis. Reverse transcription reactions on RNA preparations do not require functioning NP-40, RNasin or DTT, and do not replicate the relatively low amount of target seen in immunobead-cell isolates.

7.6.2 Results and Discussion

A K19 RT-PCR product could be reliably seen on an agarose gel after ethidium bromide staining for 100 cells stored at -80^{0}C. Cell preparations stored in this way provided convenient reverse transcription controls, but were still limited in that they only demonstrated that the components of the reverse transcription were intact (separate controls for PCR components were used). The best form of control for intact cDNA from the immunobead - cell isolates became apparent towards the end of this work, and was PCR for CD44 (see Chapter Nine). The standard (or haemopoietic) form of CD44 was that which was predominantly expressed by the majority of immunobead samples from patients after PCR, and these were easily evident on ethidium bromide only.

Most RT-PCR methods use PCR directed against a ubiquitously expressed mRNA as a marker to demonstrate an intact reverse transcription product, for example, β-actin (Deguchi et al, 1993; Moreno et al, 1992; Noguchi et al, 1994), ABL (Datta et al, 1994; Mattano et al, 1992), β-globin (Smith et al, 1991), and glyceraldehyde phosphate dehydrogenase (Burchill et al, 1995;

Schoenfeld et al, 1994). CD44 primers could be easily applied to the immunobead-cell lysate reverse transcription product because the PCR sensitivity had already been demonstrated, the standard form had been shown to be ubiquitously expressed, and the absence of a CD44 pseudogene excluded the possibility of false positive results from contaminating genomic DNA.

7.7 SUMMARY

The optimum number of immunobeads for incubation with 10ml of whole blood had been previously determined to be approximately $2x10^6$ (Hardingham et al, 1993), and experiments with application of the K19 RT-PCR assay to the immunobeads also found that this number of beads gave optimum results.

Sensitivity of the immunobead RT-PCR method for detection of tumour cells equated to detection of 25 cells in 10ml of whole blood, or approximately one tumour cell amongst $2x10^6$ leukocytes. This sensitivity compares favourably with other published methods, and has a theoretical advantage in specificity because the immunobeads select the target cells at the exclusion of non-target cells, thereby theoretically minimising chances of amplification of low-level transcription of tissue-specific genes in non-specific cells.

Archival specimens could not be thawed for practical use with the immunobeads, because DNA and proteins released from lysed cells caused clumping of the beads and subsequent loss of the beads during the washing step. Cell lysis progressed with duration of incubation time with the beads. Clumping of beads was also more noticeable after the denaturation step at 70^0C, recommended in reverse transcription protocols. It was demonstrated that K19 RT-PCR maintained its sensitivity when the 70^0C denaturation step was omitted.

CHAPTER EIGHT:

PATIENT RESULTS

8.1 BONE MARROW BIOPSIES

8.1.1 Introduction

Patients with breast cancer are referred to the Haematology/Oncology Department at The Queen Elizabeth Hospital for consideration of systemic treatment if the clinical and/or pathological stage of their disease places them in a poor prognosis category. Patients in this category include those with metastatic breast cancer and pre- and perimenopausal women with tumour involvement of axillary lymph nodes or poor prognostic features of the primary tumour. For patients in this category, chemotherapy administration in the form of a combination of cyclophosphamide, methotrexate and fluorouracil is established (Coates, 1994; Harris et al, 1992). In recent years the Haematology/Oncology Department at The Queen Elizabeth Hospital has joined others in embarking on high dose chemotherapy regimens for women with metastatic or poor prognosis primary breast cancer, in the form of a clinical trial (Kotasek et al, 1994). Full clinical staging of these patients was required for consideration of this dose intensive therapy, and bone marrow biopsy was part of routine assessment for these patients.

8.1.2 Results

Bone marrow samples from eleven patients were tested by immunobead RT-PCR. The patients' ages ranged from 34 to 67 years, with a median of 46 years. Four patients had stage IV disease (metastatic disease), two stage III (advanced locoregional disease), four stage IIB, and one stage IIA, according to the UICC staging system (Beahrs et al, 1988). A review of the TNM and UICC staging system is provided in Appendix I.

Patient	Stage	Cytology	K19 RT-PCR
1	IIA	-	-
2	IIB	-	-
3	IIB	-	-
4	IIB	-	-
5	IIB	-	-
6	III	-	-
7	III	-	+
8	IV	+	+
9	IV	+	+
10	IV	-	+
11	IV	-	-

Table 8.1: Detection of tumour cells in bone marrow of breast cancer patients using immunobead RT-PCR.

Of the eleven, four were positive for K19 transcripts by immunobead RT-PCR - three of the four stage IV patients, and one of the three stage III patients. Only two patients had bone marrow biopsies that were positive for breast cancer on routine cytology, one on bone marrow trephine, the other on aspirate. Each had bulky primary disease (T4 tumours), but the only other apparent distant disease was an involved supraclavicular node in one. Both of these bone marrow biopsies returned K19 RT-PCR signals that were strong enough to be easily evident on ethidium bromide staining alone. Therefore, the K19 RT-PCR results are borne out by correlation with clinical data (although this is limited to two cases). Both were in remission eight months after high dose chemotherapy with autologous PBSC support.

The third stage IV patient with a positive bone marrow biopsy by K19 immunobead RT-PCR was a 67 year old woman whose node-negative 12mm primary tumour had been resected 18 months earlier. Her initial clinical stage was therefore T1N0M0, but her disease relapsed approximately six months after surgery and she received combination chemotherapy. A bone marrow

136

biopsy to complete her restaging prior to chemotherapy was positive on routine cytology. A clinical response to chemotherapy was seen, and this bone marrow biopsy performed eight months after chemotherapy was now negative for breast cancer by routine cytology, but positive by immunobead RT-PCR. She suffered a local recurrence two months later, then relapsed, predominantly in the central nervous system, and died two years and five months after her initial diagnosis.

The positive stage III patient had a primary tumour measuring 8 cm and had associated extensive intraduct disease, but the lymph nodes cleared from the axilla were free of disease by routine histological assessment (T3N0M0). She developed a local recurrence two years after initial diagnosis and underwent high dose chemotherapy with autologous PBSC support. The bone marrow biopsy was performed at this point to assess the patient for treatment, and was negative for breast cancer by routine cytology but positive by immunobead RT-PCR. She was in clinical remission at review three years after diagnosis.

The stage IV patient whose bone marrow biopsy was negative for K19 PCR product had a heavy overall tumour burden, reflected in the TNM stage of T4N2M1. Her site of clinical metastasis was predominantly extensive supraclavicular and axillary nodal involvement. While this patient's bone marrow sample was negative for breast cancer on both routine cytology and immunobead RT-PCR, it is of interest that K19 transcripts were detected in her peripheral blood on the day of stem cell harvest (see PBSC results).

The remaining six bone marrow samples that were negative for breast cancer both on routine cytology and immunobead RT-PCR came from one patient with stage IIA disease (T2N0M0), four with stage IIB disease (all T2N1M0) and one with stage III disease (T4N1M0). One of the stage IIB

patients, a 55 year old woman with a 3.3cm poorly differentiated primary tumour with heavy involvement of axillary lymph nodes, has relapsed with multiple bony metastases (demonstrated by whole body bone scan) 10 months after diagnosis, and six months after PBSC transplant. The other five were in clinical remission at between four and fourteen months follow-up. All except the patient with stage IIA disease have received high dose chemotherapy with PBSC support. Again, it is of interest that the stage III patient, while being apparently negative on bone marrow assessment, had K19 transcripts detected in a sample of peripheral blood taken on the day of autologous stem cell harvest.

8.1.3 Discussion

While the results of tumour cell detection in bone marrow samples seem consistent with clinical stage, results and outcome, the incidence of tumour cell detection by immunobead RT-PCR in this small series falls short of results using immunocytochemistry (Cote et al, 1991; Diel et al, 1992). The results of Cote and Diel taken together suggest that approximately one third of stage II breast cancer cases and between 50 and 70% of locally advanced cases should have micrometastatic disease detectable in bone marrow. In this series, K19 transcripts were amplified only from patients with stage III or IV disease. The series is however comparable to the only published data that use RT-PCR technology - Datta and colleagues detected K19 transcripts by RT-PCR in six out of eight bone marrow biopsies, all from stage IV breast cancer patients (Datta et al, 1994).

A significant difference between the immunobead RT-PCR data and those of Diel and colleagues is that the latter group screened 60ml volumes of marrow from each patient, compared with the 1 to 2ml used in the immunobead results. As a small series, the immunobead RT-PCR results

resemble the results of Ross and colleagues, obtained using a cocktail of antibodies, including cytokeratins, in immunocytochemical study of 3 to 5ml of bone marrow taken from each patient (Ross et al, 1993). They found breast cancer cells in biopsies from one of two stage II patients, three out of six stage III patients, and 28 out of 40 patients with stage IV disease.

While the immunobead method has the advantages of being relatively cheap and simple to perform, and potentially able to screen large sample volumes, some technical limitations of the technique were demonstrated. The presence of fat in the biopsy specimen interfered with immunobead recovery in the same way as lysed cells in thawed specimens, and probably also interfered with immunobead binding of any tumour cells.

No account is made in these results of the diluting effect of a "bloody tap", which may lead to a marrow sample being mostly blood rather than marrow. This can be especially significant when the volumes being incubated with the beads are only small. There were no controls for quality of the samples.

The most practical use for detection of occult breast cancer in bone marrow in current medical practice would be to aid existing prognostic indicators in determining which stage I or II patients might benefit from adjuvant systemic therapy, or even which stage II patients could be recommended for high dose chemotherapy. The results of Diel and colleagues (1992) suggest that their immunocytochemical method may have greater potential for clinical application than the method of immunobead RT-PCR as it is applied to bone marrow biopsies in this series.

8.2   PERIPHERAL BLOOD STEM CELL HARVESTS

8.2.1   Results

Peripheral blood stem cell collections from eight consecutive patients were studied by immunobead RT-PCR. Four of these had metastatic (stage IV) disease, while four had locally advanced disease (stage III). The patients' ages ranged from 37 to 51 years, with a median of 43 years.

Samples from four consecutive days of PBSC harvest were studied in two patients, while lesser numbers of samples were taken from the others either because of technical reasons or because of fewer harvest days being required. The number of mononuclear cells per sample tested varied from 25 x 10^6 to 140 x 10^6 cells in total, reflecting the daily variations between harvests.

None of the nineteen PBSC samples tested from the eight patients were positive for K19 transcripts by immunobead RT-PCR (see Table 8.2).

8.2.2   Discussion

The results of immunobead RT-PCR for K19 were not what was anticipated. It was expected that if circulating tumour cells were to be detected in any media other than bone marrow, it would have been in the PBSC samples, since a larger number of haemopoietic cells is generally sampled in these PBSC collections than in the samples of whole blood, and since tumour cell contamination of PBSC collections is a major theoretical limitation of high dose chemotherapy with PBSC support (Shpall and Jones, 1994). The marrow involvement (albeit microscopic) in patients 2 and 3 would make these most likely in theory to show tumour cell contamination of their PBSCs, or even their peripheral bloods, after stem cell mobilisation by the cyclophosphamide and G-CSF. However, all of the samples from these two patients were negative for K19 transcripts.

8.3   PERIPHERAL BLOOD SAMPLES

Results and Discussion

Random peripheral blood samples were tested by immunobead RT-PCR on a total of 22 occasions on 20 different patients. Seven patients were stage II, three were stage III, and ten stage IV. In none of these patients taken at random were K19 transcripts detected by immunobead RT-PCR. This contrasts with the results using immunobead-PCR to detect circulating tumour cells in patients with colon cancer. Circulating K-ras mutant cells were detected in perioperative blood samples in 9 out of 27 consecutive patients with K-ras mutations in their primary tumour, and detection of tumour cells was associated with a dismal prognosis. Interestingly, in six of these nine, tumour cells were detected in pre-operative samples, suggesting that colon cancers are continually shedding tumour cells into the bloodstream (Hardingham et al, 1995).

If primary breast cancers constantly shed tumour cells into the circulation, then immunobead RT-PCR using K19 has been unable to detect them. Three of the specimens came from patients whose tumours would have provided ample opportunity for release of tumour cells into the peripheral circulation, having bulky locoregional disease, and having not yet received any treatment (clinical stages T4N1, T4N2 and T3N1). Blood samples from two patients, hospitalised in the terminal stages of metastatic breast cancer, also failed to produce evidence of K19 transcripts by immunobead RT-PCR.

The stage IV patient who had random peripheral blood samples taken on three occasions did have K19 transcripts detected in one sample of her peripheral blood taken on day of stem cell harvest (patient number 8, Table 8.2, in Section 8.4.1). The three peripheral blood samples were taken at times unrelated to stem cell harvest: one before PBSC mobilisation, and two taken

141

two and five weeks after harvest respectively. No evidence for circulating tumour cells was detected in any of these samples.

Similarly, one of the three stage III patients whose random peripheral blood sample was negative for K19 transcripts by immunobead RT-PCR had K19 transcripts amplified from peripheral blood taken on day of peripheral blood stem cell harvest (patient number 7, Table 8.2).

Was the absence of detection of tumour cells in peripheral blood due to poor binding of tumour cells by Ber-EP4 and hence a failure of the immunobeads to isolate the cells? Two of the 12 peripheral blood samples from stage IV patients included samples from patients whose bone marrow samples were positive for K19 transcripts - one of these had been positive also by routine cytology. It would be reasonable to assume that the cells from these tumours, demonstrated to be positive for Ber-EP4, would continue to be positive if shed into the periphery, and would therefore be bound by the immunobeads if present in the blood sample. One therefore assumes that, at least in these two cases, absence of detection of tumour cells in peripheral blood was not due to failure of the immunobeads to isolate the cells.

8.4   PERIPHERAL BLOOD SAMPLES TAKEN AT THE TIME OF PBSC HARVEST

8.4.1   Results

Results are shown in Table 8.2. On four occasions K19 transcripts were amplified from peripheral blood samples taken on the day of stem cell harvest. These positive blood samples came from three patients (patients 6, 7 and 8, see Table 8.2), two stage IV and one stage III, and all from samples in which the aliquots of blood in the 5ml tubes were pooled to give a single 10ml sample for incubation with the beads.

142

Follow-up periods for these patients at the time of writing varies from six to twelve months, with a median of ten months. The first five patients listed in Table 8.2 were all in clinical remission at the time of writing. All of these five were clear of clinical disease at the time of high dose chemotherapy and PBSC support. Of the two stage IV patients (2 and 3, see Table), patient 2 had metastatic disease demonstrated only in her bone marrow biopsy (by both routine cytology and immunobead RT-PCR), while patient 3 had an involved left supraclavicular lymph node surgically excised, and also had involvement of her bone marrow biopsy by both routine cytology and immunobead RT-PCR.

Patient 6 (stage IV at time of PBSC harvest) died from cerebral metastases within six months of transplant. Patient 8 (stage IV) remains alive at twelve months follow-up, but has untreatable recurrent disease involving her ipsilateral axilla and chest wall. In contrast, patient 7 (stage III) has remained in clinical remission eleven months after transplant.

Patient	Stage	PBSC	PB	Outcome
1	III	-	N	CR
2	IV	---	---	CR
3	IV	----	----	CR
4	III	--	--	CR
5	III	--	--	CR
6	IV	----	N++-	Deceased
7	III	--	-+	CR
8	IV	N-N	-+-	PD

Table 8.2:   Results of K19 immunobead RT-PCR for consecutive patients undergoing PBSC mobilisation and harvest, showing disease stage and PBSC samples and peripheral blood samples taken on the day of harvest, and outcome after median follow-up of 10 months.   CR = Clinical Remission, PD = Progressive Disease, N = sample not taken.

8.4.2   Discussion

It was indeed an unexpected result to claim detection of circulating tumour cells in peripheral blood samples taken at the time of PBSC harvest, particularly when one considers that the bulk of tumour had been surgically eradicated in these patients. This is emphasised in the case of patient 7 (see Table 8.2), who had only stage III disease, with all clinically detectable tumour removed, yet the K19 results still claim detection of tumour cells circulating in the periphery. This same patient had three other blood samples that were taken, before and well after administration of cyclophosphamide and G-CSF, and all were negative for K19 transcripts by immunobead RT-PCR (see Peripheral Blood Samples Results). Indeed, other samples taken at random from two stage III patients failed to detect circulating tumour cells.

Patients 6 and 8 were both stage IV (their disease had been debulked by removal of their primary tumours). Patient 8 had three different blood samples tested by immunobead RT-PCR, one prior to mobilisation and two well after mobilisation. All three were negative for K19, therefore acting as a form of control for the samples taken during harvest. In addition, as recorded above, in no blood samples taken at random from patients with metastatic breast cancer could detection of circulating tumour cells be claimed, even in those patients who had not yet received any form of treatment for their bulky locoregional disease and metastases.

Despite some uncertainty regarding the reproducibility of the immunobead RT-PCR method (see Chapter Seven), the above results suggest that either very few tumour cells are released into the circulation by breast cancers, or the level of circulating tumour cells is below the limits of detection by the immunobead RT-PCR method. Therefore, if circulating tumour cells are only detected at the time of greatest mobilisation of stem cells into the

periphery, one must conclude that the mobilisation of haemopoietic progenitor cells is associated with the mobilisation into the periphery of large numbers of breast cancer cells.

The suggestion made by these results, namely that chemotherapy and/or growth factors could mobilise tumour cells in addition to the stem cells, has some support in the literature, although not until 1994 (Shpall and Jones, 1994). Brugger and colleagues used immunohistochemistry to show that after mobilisation with VP16, ifosfamide and cisplatin, supplemented by G-CSF, there was a substantial recruitment of tumour cells (from solid tumours, including breast) into the peripheral circulation, and that it was more pronounced in the presence of documented bone marrow infiltration (particularly in the case of breast cancer) (Brugger et al, 1994). Furthermore, recent work using an RT-PCR assay for "maspin" mRNA has claimed to detect breast cancer cells in the peripheral blood of 3 out of 9 patients (33%) with stage IV breast cancer receiving systemic therapy, but only 1 of 11 patients (9%) with stage IV cancer not receiving systemic therapy (Luppi et al, 1995).

Again, one did not expect that circulating breast cancer cells would be detected in peripheral blood but not in PBSC harvests, especially considering that the PBSCs had been taken from the same medium, and at basically the same time as those samples that gave positive results. A simple explanation for this observation is that the "continuous flow cell separator" effect of the COBE "Spectra" machine for leukapheresis separates breast cancer cells from the blood stem cells, so that the collection of stem cells excludes the tumour cells, which are then returned to the patient with the remaining components of the blood.

8.5 ARE CIRCULATING CARCINOMA CELLS SEPARATED FROM MONONUCLEAR AND STEM CELLS DURING LEUKAPHERESIS?

8.5.1 Introduction

Technical information provided with the COBE "Spectra" Apheresis System (COBE, Denver, Colorado, USA) describes how the peripheral venous blood is taken from a patient and centrifuged in the COBE leukapheresis machine. The centrifugal force separates the components of the blood according to specific gravity, so that the red blood cells lie most peripherally in the centrifuge chamber as it spins, with larger cells also lying in this region and the neutrophils and then the mononuclear cells and smaller stem cells lying most centrally. Figure 8.1 is a diagrammatic representation of this, showing an "interface" separating the layer of small stem cells from the remaining larger components of the blood.

The COBE "Spectra" is designed so that the operator of the machine determines the contents of the collection by altering the "plasma pump" (see Figure 8.1). Increasing the flow through the pump lowers the level of the interface relative to the collection flange, having the effect of decreasing the collection of the neutrophil layer and making the collection of stem cells more exclusive. Correspondingly, the colour of the effluent in the "collect tube" (see Figure 8.1) becomes paler, since red cells are more likely to be excluded.

Conversely, decreasing the flow of the "plasma pump" raises the level of the interface, so that more neutrophils are collected with the stem cells, and the presence of increasing numbers of red blood cells darkens the colour of the effluent in the "collect tube".

The established practice in the Haematology/Oncology Unit at The Queen Elizabeth Hospital is to maintain a pale effluent in the "collect tube" to obtain a collection that is as pure as possible for stem cells. This purity of

146

RBC AND PLASMA OUT

COLLECT
TUBE

PLASMA OUT

BLOOD IN
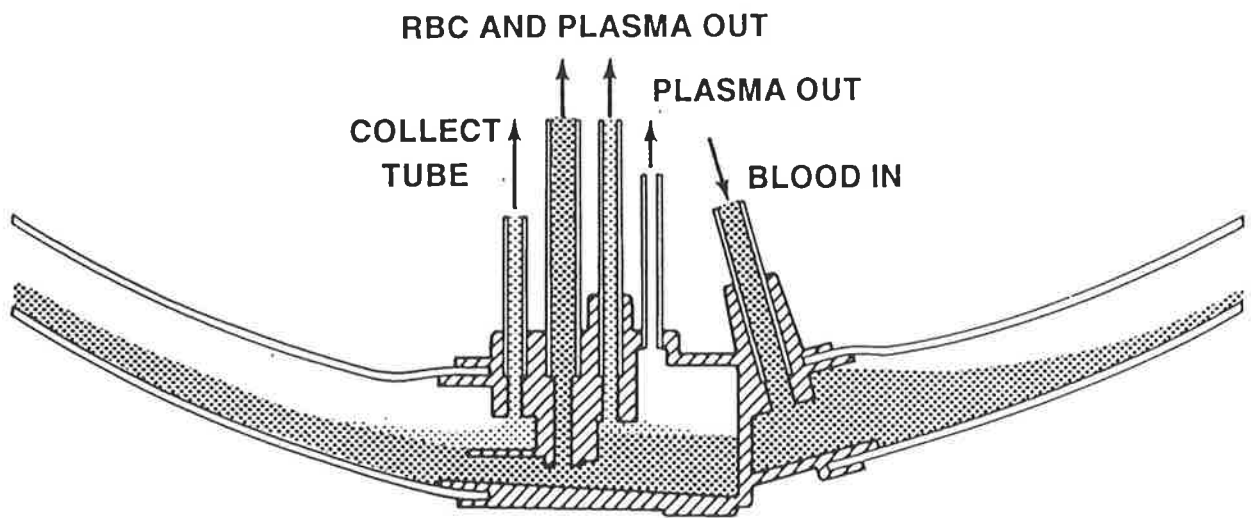
Figure 8.1   Diagrammatic representation of cell separation by centrifugation in the Cobe Spectra apheresis system. The dark stippling represents the major blood components, while the lighter stippled area represents plasma and smaller cells (e.g. mononuclear cells and stem cells). The position of the light/dark interface relative to the collection flange determines the purity of collection of the small stem cells and mononuclear cells.

collection is confirmed by flow cytometry studies of the PBSCs showing that most cells collected are CD34-positive (B. Farmer, B.Sc., Dept. of Haematology-Oncology, T.Q.E.H., personal communication).

Contamination of PBSCs from patients with NHL undergoing high dose chemotherapy with PBSC support has been demonstrated by others (Gribben et al, 1993) and importantly at The Queen Elizabeth Hospital by Hardingham et al. (Hardingham et al, 1995) using the same COBE leukapheresis machine. Lymphoma cells, like stem cells, are small, and therefore may not separate from the stem cell layer during leukapheresis, leading to a risk of tumour cell contamination in PBSCs taken from patients with lymphoma.

It has not yet been established where cells from solid tumours like breast or colon will lie during centrifugation. A simple way of explaining why tumour cells have been claimed to be detected in peripheral blood but not in PBSCs is that breast cancer cells are large cells, so they are therefore centrifuged away from the interface and are not collected with the stem cells, but remain with the remainder of the blood components which are returned to the patient.

A means of enhancing this theory was to try to assess the blood components being returned from the "Spectra" during leukapheresis to the patient, for the presence of tumour cells. K19 transcripts were therefore sought by immunobead RT-PCR in these fluids in the succeeding breast cancer patients undergoing stem cell harvest.

8.5.2 Methods and Patients

Three patients who underwent PBSC harvest for poor prognosis breast cancer were studied. High dose chemotherapy with PBSC support was planned for these patients in the adjuvant setting for stage IIB breast cancer (all had large primary tumours with heavy nodal involvement). Clinical screening tests

147

had failed to show evidence of metastatic disease, and bone marrow biopsies were negative by routine cytology. Only one of these biopsies was tested by immunobead RT-PCR, and was negative for K19 transcripts (the other two were not tested for technical reasons).

Peripheral blood samples were tested by immunobead RT-PCR prior to the administration of cyclophosphamide and G-CSF in patients 1 and 2 (see Table 8.3). Aliquots of the fresh PBSC were tested by immunobead RT-PCR in the usual manner, and 10ml of peripheral blood taken on the day of harvest were also tested. Single harvests only were required for patients 2 and 3, because of excellent retrieval of stem cells. Two harvest days were required for patient 1, but for the following technical reasons only one was included for results.

In the initial attempt to collect the blood components being returned to the patient, the neutrophil and red blood cell layers were harvested separately, and incubated with immunobeads in separate 10ml tubes. Profound lysis of neutrophils occurred within the two hours of incubation, causing much clumping and subsequent loss of beads, invalidating the results. Subsequent samples were processed by mixing the red cell and neutrophil collections 50/50 into 10ml tubes with EDTA before incubation with immunobeads.

8.5.3   Results

The results are summarised in Table 8.3. In keeping with previous results, all three PBSC samples were negative for K19 transcripts. The peripheral blood samples taken prior to mobilisation with cyclophosphamide and G-CSF were negative for K19, as were the three samples taken on day of PBSC harvest.

148

Patient	Stage	PBSC	PB	rbc/pmn
1	IIB	-	-	-
2	IIB	-	-	-
3	IIB	-	-	+

Table 8.3: K19 Immunobead RT-PCR results for three consecutive patients, testing PBSC samples, peripheral blood taken on the day of the PBSC harvest, and mixtures of blood components being returned to the patient (rbc/pmn - predominantly red blood cells and neutrophils).

The volumes of the red blood cell/neutrophil mix that would have been returned to the patient but were studied by immunobead RT-PCR were 30 and 20ml respectively for patients 1 and 2, and two separate aliquots of 10 and 20ml for patient 3. Mixing the harvested neutrophils into a solution more in keeping with whole blood was associated with much less cell lysis, and allowed more efficient use of the immunobeads. Both samples were negative for patients 1 and 2, but the 10ml aliquot of red blood cell/neutrophil mix from patient 3 gave a signal for K19 RT-PCR that was not detectable on ethidium bromide staining, but was evident on autoradiography after 7 hours exposure. The 20ml sample from patient 3 was negative for K19.

Discussion

The positive signal for K19 by immunobead RT-PCR for patient 3 indicates that breast cancer cells were present in the blood being returned to the patient after the stem cells had been harvested. This, plus the continued absence of breast cancer cells from the PBSCs, suggests that the breast cancer cells migrate by the centrifugal force in the COBE "Spectra" cell separator to the peripheral layers with the red cells and the neutrophils and are therefore not

included in the stem cell harvest, but are returned to the patient along with the remaining blood components.

CHAPTER NINE:

CAVEATS

9.1   INTRODUCTION

The RT-PCR marker chosen for application to the immunobead-tumour cell isolates must satisfy two criteria to enable reasonable conclusions to be drawn from the clinical results: 1) it must be specific for the target cells, and not be amplified by the background cells, and 2) it must be sufficiently sensitive to allow detection of small cell numbers. As has been discussed in Chapter Six, towards the end of work towards this thesis, K19 transcripts were amplified by RT-PCR from the peripheral blood of a "healthy volunteer". It was also around this time that the first publication questioning the specificity of K19 as an RT-PCR marker for epithelial cells appeared (Burchill et al, 1995). The implications of these events and the work that followed are discussed in this chapter.

A false positive for K19

All nine peripheral blood samples from healthy controls or patients without carcinoma were negative for K19 RT-PCR from RNA extracted by the GITC method. All six samples of GITC-extracted RNA from bone marrow biopsies from patients with conditions other than carcinoma were negative for K19 by RT-PCR. All fifteen bone marrow samples from patients with conditions other than carcinoma were negative for K19 by immunobead RT-PCR, but of thirteen peripheral blood samples from healthy volunteers or patients with conditions other than carcinoma, one was positive for K19 by immunobead RT-PCR.

Repeated testing showed that amplification of K19 transcripts after immunobead RT-PCR on peripheral blood from this healthy person was intermittent, that is, not all cDNA preparations from this person's immunobead

samples gave a K19 product by PCR. To investigate this further, RT-PCR for K19 on total RNA extractions from mononuclear cell preparations from this person's whole blood were performed. Results were inconsistent, in that an RNA preparation from one peripheral blood mononuclear cell collection would display K19 expression by RT-PCR, whereas an RNA preparation from another cell collection would not. During these experiments PCR contamination by K19 product was confidently excluded, and CD44 transcripts were amplified from all samples, demonstrating intact cDNA.

One out of 44 haemopoietic samples from individuals acting as negative controls for K19 therefore demonstrated non-specific amplification of K19 transcripts. This proportion was similar to that of Datta and colleagues, who tested blood and bone marrow samples from patients with conditions other than carcinomas by K19 RT-PCR and found that 10 blood samples were negative for K19 but bone marrow from one patient out of 30 was consistently positive for K19 (Datta et al, 1994). This differed slightly from results in this thesis in that the false positive in Datta's series was from a patient with CML, whose atypical expression of the cytokeratin might be explained on the basis of ectopic expression of K19 by a haematological malignancy.

Why should peripheral blood from a healthy volunteer have unusual expression of K19? This expression, when present, was strong enough to be detected on ethidium bromide staining, making it stronger than most of the clinical specimens in which detection of circulating breast cancer cells was observed. One unusual feature of this otherwise healthy laboratory worker that differentiated him from other healthy controls was the fact that he was undergoing repeated venesections for his own research. Could haemopoietic proliferation be a possible common factor between this person and the CML positive of Datta and colleagues, and the two out of four NHL samples that

were found to be weakly positive on autoradiography after K19 RT-PCR by Traweek and colleagues (Traweek et al, 1993)? Had the healthy researcher's repeated venesections produced a haemopoietic aberration (for example, activation of peripheral blood lymphocytes) that led to non-specific expression of K19 by a population of his haemopoietic cells? If this were the case, could any breast cancer patient with activated lymphocytes (for example) give false positive results by immunobead RT-PCR using K19?

Contradictions regarding K19 in other published works

The work of Burchill and colleagues was the first published work to cast doubt on the validity of using RT-PCR for K19 as a means of detecting carcinoma cells in blood, when they reported their results that K19 product was detected by RT-PCR in blood samples from 6 out of 15 normal volunteers (Burchill et al, 1995). While they and others (Brown et al, 1995) have demonstrated expression of other cytokeratins in haemopoietic cells, other work, in addition to this thesis, has suggested that mRNA expression of K19 shows greater specificity than other keratins (Datta et al, 1994; Schoenfeld et al, 1994; Traweek et al, 1993).

The RT-PCR method of Burchill and colleagues is summarised below.

A commercially produced kit was used to extract "total cellular RNA" from blood. This "total" extraction is similar to the techniques of Traweek and Datta (Datta et al, 1994; Traweek et al, 1993), but differs from the "total" RNA extraction in work towards this thesis, in which a predominantly mononuclear cell preparation was used as the source for RNA.

Analysis was made on blotted RT-PCR products hybridised with an internal probe. The RNA samples were DNase treated and reverse transcriptase-negative controls were included for all RT-PCR reactions, so theoretically controls against pseudogene amplification should have been

adequate. However, the controls were not shown and PCR conditions were not described, and there was considerable primer sequence homology with the known K19 pseudogene, which may have allowed amplification if any residual DNA were present. The primers gave a 214 base pair band on gel electrophoresis.

While much relevant technical detail was missing from this publication, the authors have considerable experience in this field (Smith et al, 1991; Burchill et al, 1995; Burchill et al, 1994), and the controls described appear sound.

The dilemma therefore is how to explain the contradictions between the published reports of K19 amplification from haemopoietic cells. Burchill and colleagues claim that more than one K19 pseudogene exists, and that these other pseudogenes would therefore not differ from the designed PCR primers at the 3' ends, so that pseudogene amplification would still occur. The references they supply for this statement (Bader et al, 1988; Savtchenko et al, 1988) have been studied by this author, who is unable to find reference in either paper to the identification of more than one pseudogene for keratin 19.

If Burchill's data are not the result of pseudogene amplification, then perhaps there is another factor, like the nature of the sample population, that causes their different results. Could their "normal" controls have a factor in common with the "normal" laboratory worker in this series whose blood was intermittently positive by RT-PCR for K19? Can haemopoietic cells, or a population of cells within the haemopoietic compartment, be activated to non-specifically express keratin 19 mRNA?

9.2 SOME POPULATIONS OF HAEMOPOIETIC CELLS CAN EXPRESS K19 mRNA

9.2.1 Materials and Methods

cDNA samples for the following PCR experiments were obtained from Mr. P. Laslo, B.Sc., of the Transplantation Immunology Laboratory, T.Q.E.H. cDNA samples used were prepared by Mr. Laslo, from reverse transcription of RNA extractions from the cell lines K562 (raised from CML), HUT 78 (T lymphocyte) and THP.1 (monocyte), and from peripheral blood mononuclear cells taken from healthy donors, some stimulated by PMA, others not stimulated. Briefly, peripheral blood mononuclear cells had been prepared via a Ficoll-Hypaque gradient, washed and resuspended in culture medium, at a concentration of 10^6 MNCs per millilitre. Five millilitres were incubated in a culture flask to which PMA (Phorbol 12-myristate 13-acetate, Sigma) was added in a final concentration of 10ng per millilitre. A further 5ml had been incubated in a second culture flask, without the addition of PMA, to act as an unstimulated control. After a designated incubation time, the total cell population was harvested by trypsinization and centrifugation, and pelleted for RNA preparation by the GITC method, and reverse transcribed.

PCR reactions were performed for K19 according to usual protocol, using 5µl of RT product per 50µl reaction, and annealing at 68^0C. Genomic DNA was used as a control for pseudogene amplification. The PCR products were run on an agarose gel, Southern transferred, hybridised and autoradiographed.

Integrity of the cDNA samples was checked by PCR for CD44.

9.2.2 Results

The K19 signals from the K562 cell line and MNCs exposed to PMA were strong enough to be easily evident on ethidium bromide staining alone. cDNAs from THP.1 and HUT 78 cell lines, and the unstimulated MNCs, failed

to amplify K19 signals, even after exposure of the autoradiograph for three days. No K19 signal was seen from genomic DNA. CD44 signals from the cDNA samples were visible on ethidium bromide staining.

9.2.3   Discussion

The absence of K19 mRNA expression as measured by RT-PCR from normal human mononuclear cells and the leukocyte cell lines HUT 78 and THP.1 was consistent with conclusions from the literature that expression of K19 is specifically not found in haemopoietic cells, either by immunohistochemistry (Bader et al, 1988; Moll et al, 1982) or by RT-PCR (Datta et al, 1994; Traweek et al, 1993). Non-specific expression of cytokeratins has been demonstrated in the leukaemic cells from the K562 cell line, which also expresses the Ber-EP4 antigen (Latza et al, 1990).

The aberrant K19 results of K562 and the MNCs treated by PMA demonstrate that certain abnormal conditions can activate the expression of some mRNAs which would not normally be expressed by that particular cell type.

9.3   PMA ACTIVATES NORMAL MNCs TO EXPRESS K19

9.3.1   Introduction

The activation of MNCs by PMA demonstrated in the above experiment was repeated using MNC preparations from other normal controls to see whether the results were reproducible.

9.3.2   Materials and Methods

Mononuclear cell preparations from samples of whole blood from three different healthy controls were individually resuspended in culture medium. Each was divided into two equal aliquots and incubated in a culture flask, one aliquot treated with PMA, the other acting as an untreated control. After two

hours incubation, total cell populations were harvested and total RNA extractions performed. Complementary DNA was prepared from each by reverse transcription (cDNA samples were obtained from Mr. P. Laslo, B.Sc.).

PCR was performed under the usual conditions, taking cDNA from each of the three donors and comparing stimulated MNC cDNA with unstimulated (control) cDNA. A genomic DNA control against K19 pseudogene amplification was included. Analysis was performed by agarose gel electrophoresis, Southern transfer, hybridisation and autoradiography

9.3.3 Results

Results are shown in Figure 9.1. The PCR and genomic DNA controls demonstrated absence of PCR product contamination and pseudogene amplification. Unstimulated MNCs from all three donors failed to show K19 expression by RT-PCR, even after exposure of the autoradiograph for three days. In contrast, stimulation by PMA activated the expression of K19 in all three, evident on ethidium bromide alone. PCR products for CD44 confirmed the integrity of cDNA samples.

Other established means of cell activation were compared (e.g Ionomycin, Forskolin, Con A, and Mixed Lymphocyte Culture, prepared by Mr. P. Laslo) but while activation of MNCs to express K19 could be demonstrated on occasions with these stimuli also, it could not be consistently reproduced. PMA was the only agent that was able to consistently demonstrate activation of K19 expression by otherwise normal MNCs. The activation of MNCs by other agents, as measured by K19 RT-PCR, was irregular and not reproducible on each occasion.

9.3.4 Discussion

Lymphocytes become activated, and may subsequently replicate or differentiate, in order to develop cellular and humoral immune responses . The

157

Figure 9.1   Autoradiograph showing activation of K19 RT-PCR expression in MNCs of normal donors after treatment by PMA.

|	
Lane 1:	Positive control
Lane 2:	Negative control (i.e. no cDNA)
Lane 3:	Genomic DNA (i.e. no amplification of pseudogene)
Lane 4:	Subject 1, unstimulated
Lane 5:	Subject 1, stimulated by PMA
Lane 6:	Subject 2, unstimulated
Lane 7:	Subject 2, stimulated by PMA
Lane 8:	Subject 3, unstimulated
Lane 9:	Subject 3, stimulated by PMA

1   2   3   4   5   6   7   8   9
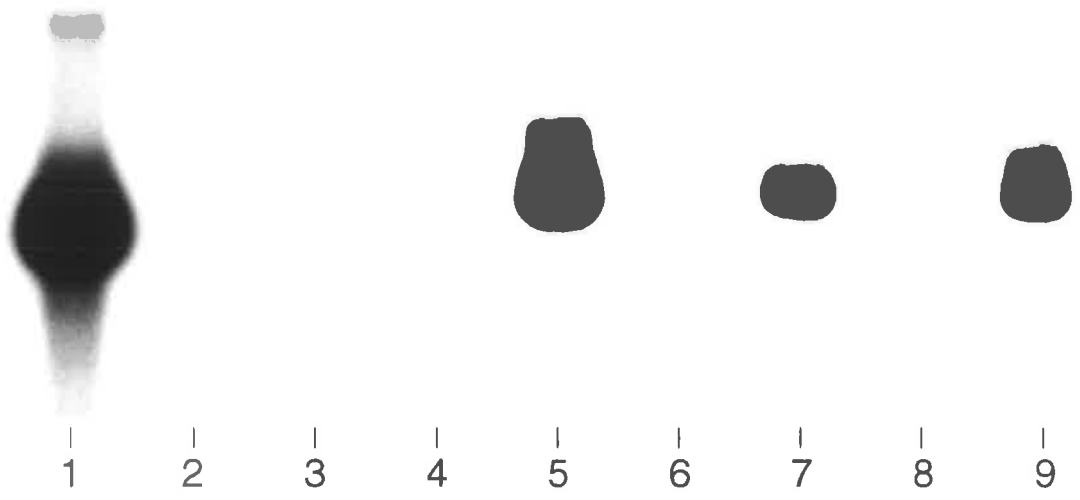
processes of lymphocyte activation are complex, with the end result being DNA synthesis and mitosis, which may be manifested as increased expression of cell receptors or release of growth factors or cytokines . Lymphocyte activation is therefore associated with upregulation of transcription of certain mRNAs, and perhaps even the expression of genes that might normally lie dormant in particular cell types (Williams et al, 1990).

Transduction of an activation signal from the cell surface to the nucleus involves the binding of a signalling ligand to a cell-surface receptor protein. By a cascade pathway, the changes in the receptor brought about by the ligand binding results in the production of two compounds, inositol triphosphate and diacylglycerol, that then initiate separate cascade pathways.

Inositol triphosphate activates pathways by which calcium channels across the cell membrane are opened, increasing intracellular calcium which can then activate the calcium binding regulatory protein calmodulin. Calmodulin can then bind and regulate many different cell proteins, including kinases, to modify cell function.

Diacylglycerol activates protein kinase C, which acts as a cofactor for many cellular responses. Diacylglycerol is indirectly activated by a variety of extracellular stimuli, making it a potent messenger in the activation process.

The end results of activation are increased DNA and mitosis. In particular, increases in the transcription of the proto-oncogenes c-myc, c-fos, and c-myb, and of receptors for interleukins have been demonstrated (Williams et al, 1990).

Cellular mitogens have been shown to produce the same biochemical effects on cells that are seen in during cell activation. Phorbol 12-myristate 13-acetate (PMA) is one of a group of mitogens known as phorbol esters, whose activation properties have been well documented. Phorbol esters are

compounds extracted from croton oil, and are well known to act as tumour promoters. They are structurally very similar to diacylglycerol, and their binding and activation of protein kinase C in the same way as diacylglycerol explains many of their effects on cells.

PMA therefore acts at a pivotal point in the activation cascade. Its activity as a mitogen and as an upregulator of mRNA expression is frequently used in experiments demonstrating factors regulating expression of various mRNAs. (Sato et al, 1995; Chotani et al, 1995). The demonstration of K19 expression in activated lymphocytes is not of itself the point of interest here, but its importance lies in the implications for K19 as a marker for carcinoma (or indeed any RNA-based markers for cancer). While it has not been demonstrated whether mitogens as strong as PMA are active in the clinical setting, the potential weakness of K19 remains. Positive clinical results using RNA-based markers should be supported by other evidence to avoid false positive results.

The contradiction between the results of Burchill and colleagues (Burchill et al, 1995) and those of other workers (Datta et al, 1994; Schoenfeld et al, 1994; Traweek et al, 1993) could be explained either on the basis of false positive results by Burchill (pseudogene amplification), or perhaps because they were sampling a different population of "normal" controls, that is, healthy people with some activated lymphocytes.

9.4   CD44 PCR TO TEST PATIENT NEGATIVES AND SAMPLES

9.4.1   Introduction

The above results indicate that genes which are "switched off" in some cells in the normal state can be "switched on" under the influence of certain stimuli to become non-specifically expressed. While the expression of the keratin 19 gene is normally limited to epithelial cells, haemopoietic cells also

159

have the capacity, under certain conditions, to express keratin 19 mRNA. The specificity of K19 expression as a marker for carcinoma cells in a haemopoietic environment is now under serious question. While the majority of haemopoietic samples fail to show evidence of non-specific K19 expression, it is virtually impossible to tell whether expression in sporadic haemopoietic samples is due to the presence of a few carcinoma cells, or to a population of cells that is normally present in that environment, but just behaving abnormally.

The number of negative controls from clinical samples that have failed to demonstrate K19 expression suggests that the true incidence of activation of leukocytes in vivo to express K19 is low, but the above results raise the possibility that the small number of immunobead samples that happened to be positive for K19 were so either because the reverse transcription happened to work in those cases, and the trapped leukocytes therefore provided K19 signals, or because more leukocytes were trapped with the beads, subsequently providing more opportunity for abnormal K19 expression by a leukocyte.

The positive controls that had been used to this point for immunobead RT-PCR have been discussed already in Chapter 7 (section 7.6), and were previously prepared aliquots of small numbers (80 to 200 cells from T-47D or MB-453 breast cancer cell lines). These controls were chosen over total RNA preparations from cell lines because they provided more appropriate assessment of the efficiency of the RT-PCR system, having greater similarity to the substrate being tested. However, these controls only confirmed that the components of the reverse transcription reaction were functioning and had not degraded; they were unable to confirm whether each individual reverse transcription had been successful or not. Was it possible that a K19 signal only arose from a patient's immunobead-cell sample when leukocytes were trapped with the beads and the reverse transcription happened to work?

The use of ubiquitously expressed mRNA from "housekeeping genes" as controls for intact cDNA reverse transcription product has been discussed in Section 7.6. CD44 primers had been assessed already in this work, and could be conveniently applied to the immunobead-cell lysate reverse transcription product because the PCR sensitivity had already been demonstrated, the standard form had been shown to be ubiquitously expressed, and the absence of a CD44 pseudogene meant that cDNA could be distinguished from genomic DNA.

CD44 had been used successfully to demonstrate intact cDNA in reverse transcription products from RNA extractions taken from patient and volunteer samples tested as negative controls. PCR for CD44 was now applied to immunobead-cell isolates to assess firstly the frequency of leukocyte entrapment by the immunobeads, and secondly the expression of CD44 variant isoforms by those leukocytes that remained with the beads.

9.4.2 Materials and Methods

Reverse transcription products from immunobead - cell isolates were taken from storage at -20^0C, thawed at room temperature and briefly centrifuged to ensure recollection of all cDNA sample with the immunobead pellet. Where aliquots had been taken from immunobead - reverse transcription products only once for PCR testing, at least 10µl of product remained for further PCR, and in these cases a 5µl aliquot could easily be taken for CD44 PCR. For some other samples, 5µl was difficult to take, or less than 5µl was available. In these cases the maximal remaining amount was taken for PCR. PCR conditions in all other aspects complied with the usual protocol. PCR products were electrophoresed through an agarose gel, transferred to a nylon membrane, hybridised and autoradiographed as described.

161

cDNA products from all bone marrow and peripheral blood samples from negative controls were tested for CD44 expression by PCR. Other immunobead samples tested by PCR for CD44 were: 1) from the more recent PBSCs (patients 3, 5, 6, 7 and 8, see Table 8.2), 2) peripheral bloods taken at the time of PBSC harvest from patients 6, 7 and 8 (see Table 8.2) and 3) immunobead samples from the COBE "Spectra" leukaphereses being returned to the patient after the stem cell collection.

9.4.3   Results

Negative Controls

The majority of immunobead samples arising from incubation with whole blood or bone marrow biopsies taken from healthy volunteers, or patients being investigated for conditions other than carcinoma, gave strong signals for the standard isoform of CD44 that could be visualised on ethidium bromide staining of the agarose gel.

Of the peripheral bloods from negative controls, 13 samples had been negative for K19, and 10 remained available for further PCRs. Six displayed bands on ethidium bromide staining that conformed to the expected size of the standard CD44 isoform, and the added sensitivity of Southern transfer and autoradiography allowed the appearance of a CD44 band in one extra case. The autoradiograph for nine of these samples is shown in Figure 5.8. Included in this autoradiograph in lane 5 is the result from immunobead RT-PCR for CD44 from a bone marrow biopsy from a patient with NHL. Splice variants of CD44 are demonstrated in the larger isoform bands from the samples in lanes 5 (patient with NHL), 9 (healthy donor) and 11 (patient with disseminated non-seminomatous germ cell tumour of the testis).

Of the bone marrow negative controls, a total of 15 had been negative for K19, and 11 remained available for CD44 PCR. Nine displayed bands that

conformed to the standard isoform for CD44, and which were evident on ethidium bromide staining alone.

PBSCs for CD44

Twelve immunobead samples from PBSCs were evaluated by PCR for CD44. As recorded in Chapter 8, none of these PBSCs were positive for K19. Eight of the 12 produced CD44 signals conforming to the standard isoform on gel electrophoresis, with ethidium bromide staining alone. The extra sensitivity of autoradiography did not add to the number of samples that displayed CD44 isoforms, but it did display expression of larger isoforms in six of the eight. Examples are shown in Figure 9.2.

Peripheral bloods at time of PBSC harvest

Ten of the peripheral venous blood samples taken at the time of PBSC harvest were tested for CD44 expression. Nine gave bands conforming to the standard isoform that could be visualised on gel electrophoresis. Again, Southern transfer and autoradiography did not increase the numbers expressing CD44, but demonstrated expression of larger CD44 isoforms in many. Four of these samples had given signals for K19 by RT-PCR, and had therefore been interpreted as demonstrating detection of tumour cells. There was no correlation between the samples displaying CD44 splice variants and those displaying expression of K19 (see Figure 9.3).

Blood products returned to the patient from the COBE "Spectra"

PCR for CD44 was performed on two of the patient samples tested for the presence of circulating tumour cells in the leukapheresis products from the COBE "Spectra" that were being returned to the patient after stem cell harvest (patients 1 and 3, see Table 8.3). These provided bands for the standard isoform of CD44, with autoradiography showing larger CD44 isoforms in three samples.

Figure 9.2   **Immunobead samples from PBSCs, evaluated by PCR for CD44.**

Ten of the 12 immunobead samples from PBSCs evaluated by PCR for CD44 are shown (lanes 1 to 10). None of these PBSCs were positive for K19, but intact cDNA is demonstrated in six of the 10 samples, including additional CD44 isoforms in four (lanes 1, 3, 4 and 6).

CD44$_S$ —

1   2   3   4   5   6   7   8   9   10
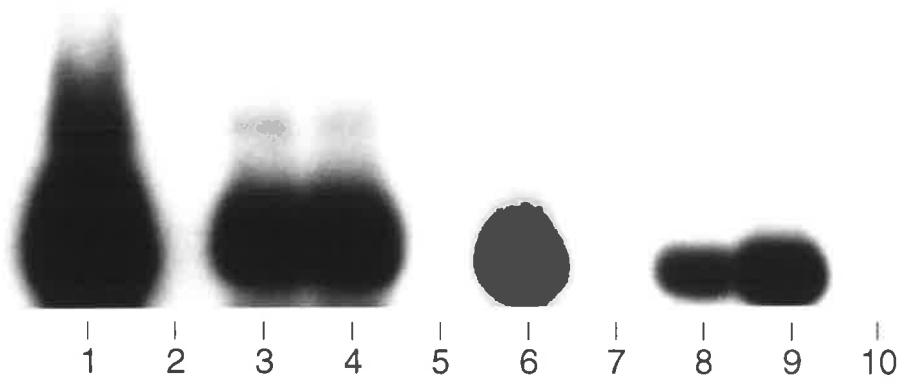
Figure 9.3   **Evaluating peripheral venous blood samples taken at the time of PBSC harvest for CD44 expression.**

Samples in lanes 3, 4, 7 and 9 had been positive for K19. Note that there is no correlation between the samples displaying CD44 splice variants and those displaying expression of K19.

Lane 1:	Positive control
Lane 2:	Negative control
Lanes 3 to 10:	Examples of CD44 expression from peripheral blood samples taken at the time of PBSC harvest.

Figure 9.4   **Evaluating leukapheresis products returned to the patients after PBSC harvest for CD44 expression.**

The sample that was positive for K19 (lane 4) does not show increased expression of CD44 isoforms. The samples studied are from patients 1 and 3, Table 8.3.

Lane 1:	Patient 3, PBSC sample
Lane 2:	Patient 3, peripheral blood sample on day of harvest
Lanes 3 and 4:	Patient 3, leukapheresis products returned to the patient
Lanes 5 and 6:	Patient 1, leukapheresis products returned to the patient
Lane 7:	Patient 1, PBSC sample
Lane 8:	Patient 1, peripheral blood on day of harvest.
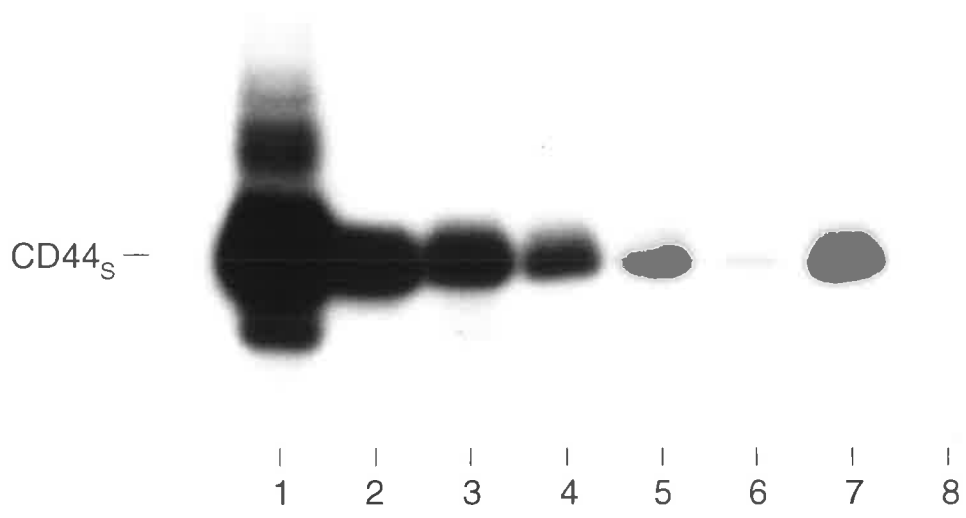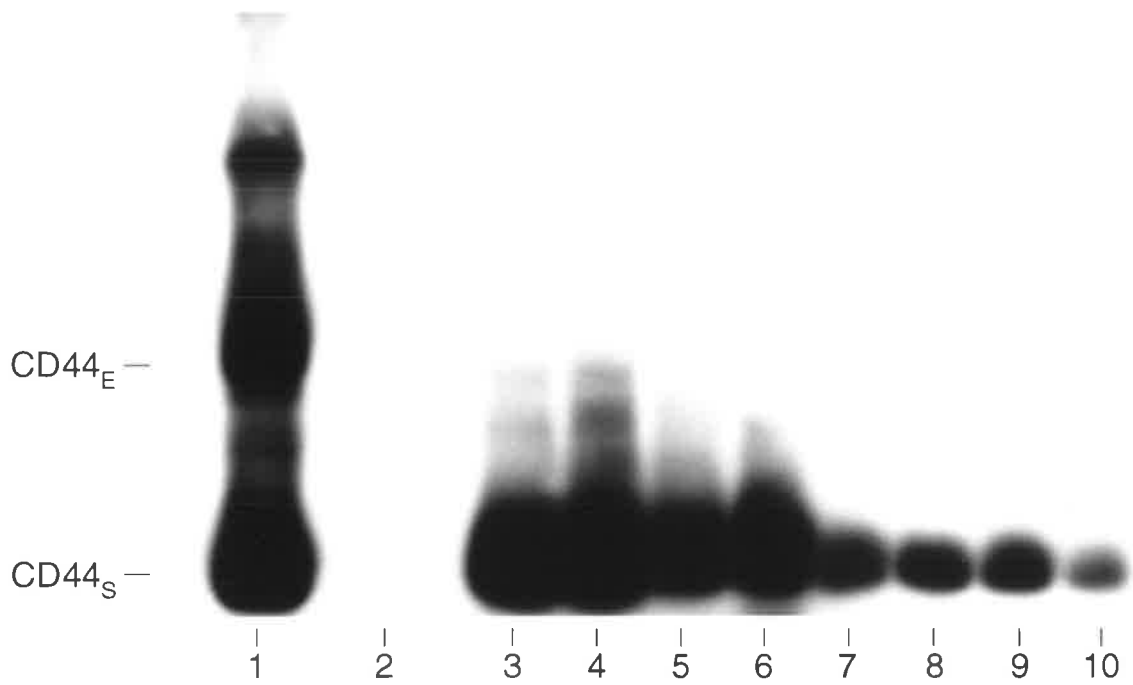
The specimen that was positive for K19 transcripts did not display expression of CD44 splice variants (figure 9.4).

9.4.4   Discussion

Immunobeads incubated with clinical samples and subjected to the RT-PCR technique in most cases provide PCR products for CD44. The bands are usually strong enough to be seen on an ethidium bromide-stained agarose gel, and the brighter the band, the more likely is the autoradiograph to display larger isoforms in addition to the standard (or haemopoietic) isoforms seen on ethidium bromide staining.

Given that the signals seen by RT-PCR on very small numbers of peripheral blood mononuclear cells generally require autoradiography to be identified, one could guess that the number of white blood cells that end up in association with most immunobead samples without being bound by the Ber-EP4 antibody, and despite the washing steps, is of the order of at least 100 cells, or thereabouts. It would be reasonable to conclude that the expression of CD44 splice variants by RT-PCR using the cell lysis method is related to the number of leukocytes that stay in association with the beads after the haemopoietic medium is washed away. This is supported by the early results of RT-PCR on peripheral blood mononuclear cells for CD44 using cell lysis (see Chapter 5).

From the viewpoint of the immunobead RT-PCR results for K19 presented in this thesis, it is reassuring to note that the expression of K19 does not correlate with the intensity of the CD44 signal, or the amount of expression of CD44 splice variants. The RT-PCR does not appear to be positive just because the number of leukocytes that have been trapped by the beads is larger than usual. It is also unlikely that a K19 signal is amplified just because an activated lymphocyte gets caught up in the beads, because this event would be more likely to occur when more leukocytes are trapped, and an activated lymphocyte

itself might be more likely to express CD44 variants (Koopman et al, 1993; Mackay et al, 1994; Salles et al, 1993). The immunobead RT-PCR expression of CD44 variant isoforms does not correlate with K19 positivity by immunobead RT-PCR.

The in vitro activation of human peripheral blood lymphocytes by various mitogens to increase expression of CD44 splice variants is well documented (Koopman et al, 1993; Mackay et al, 1994). It is of interest to this work that Koopman and colleagues used PMA, amongst other stimuli, to activate lymphocytes to express variant CD44 glycoproteins. The results here demonstrate that PMA can activate lymphocytes to abnormally express K19 mRNA. If the positive results for immunobead RT-PCR for K19 were due not to circulating tumour cells but to activated lymphocytes, then surely that degree of activation would produce expression of splice variants by CD44 PCR.

One of the important conclusions from these results is that the theories of Matsumura and Tarin regarding tumour cell identification by CD44 splice variant expression (Matsumura and Tarin, 1992; Tarin and Matsumura, 1993) cannot be applied to the isolation of limited numbers of cells by immunomagnetic beads. These results support the conclusions made earlier in this work regarding the use of CD44 as a tumour marker for immunobead RT-PCR.

CHAPTER TEN:

SUMMARY, CONCLUSIONS AND FURTHER RESEARCH

10.1   INTRODUCTION

This study is the result of two years of laboratory-based research aimed at the issues regarding circulating tumour cells in patients with breast cancer. These issues are of particular relevance in breast cancer because metastases tend to occur relatively early in the course of the disease (in comparison with other solid tumours), because systemic therapies have been shown to confer improvements in patient outcomes, and because breast cancer is the most common malignancy affecting women in developed countries.

A major question in the clinical management of patients with breast cancer is which women should be offered systemic therapy in the adjuvant setting, and a method for detecting occult metastatic disease could be of great clinical use in making these decisions in breast cancer patients whose axillary lymph nodes are uninvolved by tumour.

High dose chemotherapy with PBSC support is becoming established in the treatment of selected patients with poor prognosis breast cancer, but a major theoretical limitation to its use is the possibility of tumour cell contamination of the PBSC samples being reinfused into the immunosuppressed recipient. Very little data from which to make a judgement on the incidence or significance of tumour cell contamination in PBSCs exists. A method for detection of breast cancer cells in PBSC harvests could provide information of major interest to oncologists treating patients with breast cancer patients.

166

The primary aim of this work therefore was to try to develop a method for the detection of breast cancer cells in haemopoietic media, with the intention of seeking answers to the above questions.

10.2 IMMUNOMAGNETIC CELL ISOLATION CAN BE APPLIED TO BREAST CANCER CELLS

The technique of immunomagnetic cell isolation coupled with PCR to identify the presence of isolated tumour cells had already been developed in laboratories of the Haematology-Oncology Department at The Queen Elizabeth Hospital, using PCR amplification of mutation in codon 12 of the K-ras gene as a marker for colon carcinoma cells (Hardingham et al, 1993). Since a similar single gene mutation does not exist in sufficient frequency in breast cancers to enable PCR alone to be used to diagnose the presence of breast cancer cells isolated by the immunobeads, it firstly had to be determined whether an RNA-based marker would be appropriate for carcinoma cells, and secondly whether an RT-PCR assay could be developed that could amplify mRNA transcripts from very small numbers of cells. Work presented in this thesis demonstrates that the principle of tumour cell isolation from blood by tiny magnetic beads labelled with an antibody that recognises epithelial cells can potentially be applied to the detection of breast cancer cells, and indeed potentially any carcinoma cells, by applying a sensitive RT-PCR assay. The sensitivity of detection of carcinoma cells in blood using this technique is of the order of detection of one carcinoma cell for every two million leukocytes.

The results of Hardingham and colleagues (1993), subsequently replicated by Wong et al. (1995), with respect to the binding of the antibody Ber-EP4 to colon cancer cells, have been confirmed for breast cancer cells, using both cell lines and fresh and archival human tissue.

10.3 CD44 EXPRESSION BY RT-PCR CANNOT DIFFERENTIATE CARCINOMA CELLS FROM HAEMOPOIETIC CELLS

The expression of the CD44 gene was studied by RT-PCR, and used to develop an RT-PCR assay based on detergent lysis of cells to release RNA for reverse transcription. Of the several published methods for RT-PCR from very small cell numbers (Furukawa et al, 1994; Kumazaki et al, 1994; Edmands et al, 1994; Eggeling et al, 1995; O'Brien et al, 1994; Ziegler et al, 1992), only the report of Furukawa and colleagues describes the use of cell lysis by detergent, and only three of these reports confirmed the sensitivity of their RT-PCR assay on single cells obtained by micropipette aspiration (Furukawa et al, 1994; Kumazaki et al, 1994; Ziegler et al, 1992). A technique of collection of cells by micropipette was learned during the course of this work, and used to demonstrated the sensitivity of the cell lysis RT-PCR assay to the single cell level.

By obtaining a surgically excised specimen of axillary lymph node replaced by metastatic breast cancer and macerating the tissue into cell suspension, the expression of CD44 by individual metastatic breast cancer cells was studied. Increased expression of CD44 variant isoforms has been demonstrated in malignant tissues, and has been reported to correlate with increased tumour aggressiveness and metastatic potential (Kaufmann et al, 1995; Matsumura and Tarin, 1992; Tanabe et al, 1993; Rudy et al, 1993; Wielenga et al, 1993). These studies have only addressed the CD44 expression of whole tissue samples however, and if CD44 variant expression is truly associated with increased metastatic potential of cancer cells, an outstanding question has been whether or not individual metastatic carcinoma cells show increased expression of variant isoforms (Kaufmann et al, 1995). This thesis reports that single metastatic breast cancer cells can express more than one CD44 isoform, when

studied by RT-PCR, with the standard or haemopoietic isoform of CD44 being most commonly expressed.

The work of Matsumura and Tarin encouraged the study of CD44 expression as a possible tumour cell marker for RT-PCR analysis (Matsumura and Tarin, 1992; Tarin and Matsumura, 1993), claiming that carcinoma cells could be differentiated from white blood cells on the basis of their "chaotic and permanent over-expression of variant CD44 isoforms" relative to white cells (Matsumura and Tarin, 1992). Results presented in this thesis dispute the findings of Matsumura and Tarin, and are in agreement with others, that expression of CD44 variant isoforms is not exclusive to malignant cells, and indeed may be expressed by normal leukocytes (Salles et al, 1993; Mackay et al, 1994; Arch et al, 1992; Koopman et al, 1993). It has been demonstrated that even the preliminary selection of tumour cells by the immunobead method does not allow the use of CD44 expression as a marker for carcinoma cells, since larger CD44 isoforms can still be amplified from the leukocytes remaining in association with the immunobeads.

10.4 RESULTS OF CYTOKERATIN 19 AS A MARKER FOR CARCINOMA CELLS USING IMMUNOBEAD RT-PCR

Initial reports of cytokeratin 19 mRNA expression suggested that K19 would become a useful RT-PCR marker to differentiate carcinoma cells from haemopoietic cells. After excluding pseudogene amplification by PCR design, K19 was successfully used as a marker for the immunobead RT-PCR method, detecting cells from a breast cancer cell line in whole blood with a sensitivity that compared favourably with other published methods of tumour cell detection in peripheral blood. The preliminary selection of the carcinoma cells theoretically improves the specificity of the immunobead RT-PCR technique

over other RT-PCR methods, since minimising the non-target cells should decrease the likelihood of amplification of low-level "illegitimate" transcripts.

Using K19 as the tumour cell marker for immunobead RT-PCR, detection of tumour cells in bone marrow samples from stage III and IV breast cancer patients was consistent with their clinical stage. Tumour cells were not detected in stage II patients, which has been claimed in methods using immunocytochemistry (Cote et al, 1991; Diel et al, 1992). In no patients were circulating breast cancer cells detected in random peripheral blood samples, even in patients prior to treatment, and carrying a large tumour burden. This method for detecting tumour cells in a haemopoietic environment has therefore failed in one of the major aims - additional prognostic information to assist in clinical treatment decisions has not been forthcoming. Even allowing for occasional failure of the immunobead RT-PCR technique, the absence of amplification of K19 signals from the peripheral blood of cancer patients suggests, in contrast to the results in colon cancer using immunobead-PCR (Hardingham et al, 1995), that the number of breast cancer cells circulating in peripheral blood at any one time is low.

The striking result from the immunobead RT-PCR studies has been that while no circulating tumour cells were detected in the peripheral blood taken at random from breast cancer patients, and while no tumour cells were detected in PBSC harvests, on four occasions K19 transcripts were amplified from peripheral blood samples taken on the day of stem cell harvest.

It was concluded from this unexpected result that the mobilisation of haemopoietic progenitor cells into the peripheral circulation is associated with mobilisation of larger numbers of breast cancer cells into the periphery. This conclusion is supported by the work of two other groups (Brugger et al, 1994; Luppi et al, 1995), and led to the hypothesis that the leukapheresis process

170

separates circulating carcinoma cells from the smaller mononuclear and stem cells, harvesting the smaller cells and excluding the mobilised tumour cells from the PBSC harvest.

To attempt to prove this hypothesis, leukapheresis products from the next three consecutive PBSC harvest breast cancer patients were tested for K19 transcripts by immunobead RT-PCR, in addition to the peripheral blood samples and PBSC harvests. These were all stage IIB patients, and none demonstrated amplification of K19 transcripts from peripheral blood or PBSC sample. On one out of the three occasions a K19 signal was amplified from leukapheresis product being returned to the patient.

If these results are valid, one can suggest that carcinoma cells are selectively excluded from PBSC harvests. If this is the case, a major theoretical limitation to the use of high dose chemotherapy will have been discounted.

10.5   LIMITATIONS OF THE CLINICAL RESULTS USING K19

Initial studies of K19 expression by RT-PCR declared great promise for K19 as a specific marker for epithelial cells (Datta et al, 1994; Traweek et al, 1993), and the initial results in this thesis concurred. This specificity is now under question, both by work presented in this thesis, and elsewhere (Burchill et al, 1995). However, further work with K19 as a marker for carcinoma cells has recently been published, with the authors prepared to accept a low "false positive" rate of K19 amplification (Fields et al, 1996). At best, it can be said that the results presented in Chapter Nine provide indirect evidence for the validity of the K19 immunobead RT-PCR results. This study of K19 expression has demonstrated that normal peripheral blood mononuclear cells can be "activated" to express mRNA transcripts that have hitherto been considered "specific" for cells from other tissues. The "false positive" clinical results

171

reported in the RT-PCR studies for detection of circulating tumour cells may not be simply due to low-level "illegitimate transcription" (Brown et al, 1995; Datta et al, 1994; Schoenfeld et al, 1994; Smith et al, 1991), but also to expression of tissue-specific transcripts by non-specific cells that have been "activated".

It could be argued that the positive immunobead RT-PCR results in the peripheral blood samples taken at the time of stem cell harvest were in fact due to K19 expression by leukocytes that had been "activated" by either the cyclophosphamide or the G-CSF that had been administered to mobilise the stem cells.

Were breast cancer cells detected in the peripheral blood of the patients undergoing PBSC harvest? Given the limitations of K19 as an RT-PCR marker for carcinoma cells, these clinical results cannot be proven. The limitations of specificity of RT-PCR signals for K19 must stand as a warning for all methods of tumour cell detection that are base on RT-PCR. Problems with false positive detection of tumour cells by RT-PCR methods have been discussed in the introductory chapter. The problem with all of these published methods is that one cannot actually identify the cell from which the target mRNA transcript was amplified, to prove that the signal came from a circulating tumour cell and not from a haemopoietic cell. One can only demonstrate the statistical likelihood of the signal arising from a tumour cell, in relation to the number of clinical negative controls (similar haemopoietic samples taken from persons not diagnosed with a carcinoma). Any RT-PCR method is therefore only as good as its negative controls, and the results must be interpreted with caution.

10.6   FURTHER RESEARCH

It is plausible that mobilisation of stem cells for PBSC harvest is associated with mobilisation of breast cancer cells into peripheral blood, and

that the mobilised breast cancer cells are separated from the PBSCs during leukapheresis, and are therefore excluded from PBSC collections. This is suggested but not proven beyond doubt by the results presented in this thesis.

This hypothesis demands further work, since its implications are of major clinical interest. The conclusions of this thesis take the emphasis of tumour cell detection away from screening for occult metastatic disease and towards answering specific questions of individual clinical significance. In this aspect, the advantages of the Immunobead RT-PCR approach over immunocytochemistry, namely lower cost and lesser technical demand, are of lesser importance. It is suggested above that proof of the hypothesis cannot be obtained by a method that relies on RT-PCR. While immunocytochemical techniques are also suffer inaccuracies, they have the major advantage over RT-PCR methods of visually presenting the target tumour cell. Immunocytochemistry subjects the putative tumour cell to study of its identity by both immunologic and cytologic methods, which remain visually demonstrated for independent assessment by others. The labour required for further study of the hypothesis regarding carcinoma cell separation during leukapheresis would be justified by the clinical importance of the question.

APPENDIX

The following TNM definitions and stage groupings for carcinoma of the breast are taken from the UICC/TNM classification, as recorded by Beahrs and colleagues, in the third edition of the Manual for Staging of Cancer (Beahrs et al, 1988).

TNM CLASSIFICATION

Primary Tumour (T)

TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma *in situ*
T1	Tumour 2 cm or less in greatest dimension
T2	Tumour more than 2 cm but not more than 5 cm in greatest dimension
T3	Tumour more than 5 cm in greatest dimension
T4	Tumour of any size with direct extension to chest wall or skin, including inflammatory carcinoma

Regional Lymph Nodes (N)

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis to movable ipsilateral axillary lymph nodes
N2	Metastasis to ipsilateral axillary node(s) fixed to one another or to other structures
N3	Metastasis to ipsilateral internal mammary lymph node(s)

Distant Metastasis (M)

MX   Presence of distant metastasis cannot be assessed

M0   No distant metastasis

M1   Distant metastasis (includes metastasis to ipsilateral supraclavicular lymph nodes)

STAGE GROUPING

Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1, N2	M0
Stage IIIB	T4	Any N	M0
	Any T	N3	M0
Stage IV	Any T	Any N	M1

BIBLIOGRAPHY

Almeida PCCD, Pestana CB (1992). Immunohistochemical markers in the identification of metastatic breast cancer. Breast Cancer Research and Treatment 21:201-210.

Angelis MD, Buley ID, Heryet A, Gray W (1992). Immunocytochemical staining of serous effusions with the monoclonal antibody Ber-EP4. Cytopathology 3:111-117.

Arch R, Wirth K, Hofmann M, Ponta H, Matzku S, Herrlich P, Zoller M (1992). Participation in normal immune responses of metastasis-inducing splice variant of CD44. Science 257:682-685.

Azzopardi JG (1979). Problems in Breast Pathology. W.B. Saunders, London, pp 240-256.

Bader BL, Jahn L, Franke WW (1988). Low level expression of cytokeratins 8, 18 and 19 in vascular smooth muscle cells of human umbilical cord and in cultured cells derived therefrom, with an analysis of the chromosomal locus containing the cytokeratin 19 gene. European Journal of Cell Biology 47:300-319.

Barge A, Reiffers J (1995). The role of haematopoietic growth factors Consultant Series: Peripheral blood progenitor cell rescue, vol. 12. Gardiner-Caldwell Communications Ltd., pp 5.

Bartek J, Taylor-Papadimitriou J, Miller N, Millis R (1985). Patterns of expression of keratin 19 as detected with monoclonal antibodies in human breast tissues and tumours. International Journal of Cancer 36:299-306.

Beahrs O, Henson D, Hutter R, Myers M (1988). Manual for Staging of Cancer, Third ed. J.B. Lippincott, Philadelphia, pp 145-148.

Brown DC, Purushotham AD, Birnie GD, George WD (1995). Detection of intraoperative tumor cell dissemination in patients with breast cancer by use of reverse transcription and polymerase chain reaction. Surgery 117:96-101.

Brugger W, Bross KJ, Glatt M, Weber F, Mertelsmann R, Kanz L (1994). Mobilization of tumour cells and hematopoietic progenitor cells into peripheral blood of patients with solid tumours. Blood 83:636-640.

Burchill SA, Bradbury MF, Smith B, Lewis IJ, and Selby P (1994). Neuroblastoma cell detection by reverse transcriptase polymerase chain reaction (RT-PCR) for tyrosine hydroxylase messenger RNA. International Journal of Cancer 57:671-675.

Burchill SA, Bradbury MF, Pittman K, Southgate J, Smith B, Selby P (1995). Detection of epithelial cancer cells in peripheral blood by reverse transcriptase-polymerase chain reaction. British Journal of Cancer 71:278-281.

Chelly J, Concordet J-P, Kaplan J-C, Kahn A (1989). Illegitimate transcription: transcription of any gene in any cell type. Procedings of the National Academy of Science USA 86:2617.

Chomczynski P, Sacchi N (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry 162:156-159.

Chotani MA, Payson RA, Winkles JA, Chiu I-M (1995). Human fibroblast growth factor 1 gene expression in vascular smooth muscle cells is modulated via an alternate promoter in response to serum and phorbol ester. Nucleic Acids Research 23:434-441.

Coates AS (1994). Management of newly diagnosed early breast cancer. A national approach to breast cancer control. In: Coates A (ed.) National Breast Cancer Consensus Conference, November 1994. The Medical Journal of Australia 161(Supplement), 6-7.

Coles C, Condie A, Chetty U, Steel CM, Evans HJ, Prosser J (1992). p53 mutations in breast cancer. Cancer Research 52:5291-5298.

Cordell J, Richardson TC, Pulford KAF, Gosh AK, Gatter KC, Heyderman E, Mason DY (1985). Production of monoclonal antibodies against human epithelial membrane antigen for use in diagnostic immunocytochemisty. British Journal of Cancer 52:347-354.

Cote RJ, Rosen PP, Hakes TB, Sedira M, Bazinet M, Kinne D, Old LJ, Osborne MP (1988). Monclonal antibodies detect occult breast carcinoma metastases in the bone marrow of patients with early stage disease. The American Journal of Surgical Pathology 12:333-340.

Cote RJ, Rosen PP, Lesser ML, Old LJ, Osborne MP (1991). Prediction of early relapse in patients with operable breast cancer by detection of occult bone marrow micrometastases. Journal of Cinical Oncology 9:1749-1756.

Dall P, Heider K-H, Sinn H-P, Skroch-Angel P, Adoli G, Kaufmann M, Herrlich P, Ponta H (1995). Comparison of immunohistochemistry and RT-PCR for detection of CD44v-expression, a new prognostic factor in human breast cancer. International Journal of Cancer 60:471-477.

Datta YH, Adams PT, Drobyski WR, Ethier SP, Terry VH, Roth MS (1994). Sensitive detection of occult breast cancer by the reverse-transcriptase polymerase chain reaction. Journal of Clinical Oncology 12:475-482.

Deguchi T, Doi T, Ehara H, Ito S-I, Takahashi Y, Nishino Y, Fujihiro S, Kawamura T, Komeda H, Horie M, Kaji H, Shimokawa K, Tanaka T, Kawada Y (1993). Detection of micrometastatic prostate cancer cells in lymph nodes by reverse transcriptase-polymerase chain reaction. Cancer Research 53:5350-5354.

Delsol G, Stein H, Pulford KAF, Gatter KC, Erber WN, Zinne K, Mason DY (1984). Human lymphoid cells express epithelial membrane antigen. The Lancet 2:1124-1129.

Devine PL, Birrell GW, Whitehead RH, Harada H, Xing P-X, McKenzie IFC (1992). Expression of MUC1 and MUC2 mucins by human tumor cell lines. Tumor Biology 13:268-277.

Devine PL, McKenzie IFC (1992). Mucins: structure, function, and associations with malignancy. BioEssays 14(9):619-624.

Diel IJ, Kaufmann R, Goerner R, Costa SD, Kaul S, Bastert G (1992). Detection of tumour cells in bone marrow of patients with primary breast cancer: a prognostic factor for distant metastasis. Journal of Cinical Oncology 10:1534-1539.

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. Journal of Experimental Medicine 174:1-5

Edmands S, Kirk J, Lee A, Radich J (1994). Rapid RT-PCR amplification from limited cell numbers. PCR Methods and Applications 3:317-319.

Edwards PAW (1985). Heterogenous expression of cell-surface antigens in normal epithelia and their tumours, revealed by monoclonal antibodies. British Journal of Cancer 51:149-160.

Eggeling FV and Ballhausen W (1995). Freezing of isolated cells provides free mRNA for RT-PCR amplification. Biotechniques 18:408-410.

Erlich HA, Gelfand D, Sninsky JJ (1991). Recent advances in the polymerase chain reaction. Science 252:1643-1651.

Fields KK, Elfenbein GJ, Trudeau WL, Perkins JB, Janssen WE, Moscinski LC (1996). Clinical significance of bone marrow metastases as detected using the polymerase chain reaction in patients with breast cancer undergoing high-dose chemotherapy and autologous bone marrow transplantation. Journal of Clinical Oncology 14:1868-1876.

Fisher E, Turnbull RJ (1955). The cytologic demonstration and significance of tumor cells in the mesenteric venous blood in patients with colorectal carcinoma. Surgery, Gynaecology and Obstetrics 100:102-108.

Fisher B (1992). The evolution of paradigms for the management of breast cancer: A personal perspective. Cancer Research 52:2371-2383.

Fox SB, Gatter KC, Jackson DG, Screaton GR, Bell MV, Bell JI, Harris AL, Simmons D, Fawcett J (1993). CD44 and cancer screening (letter). The Lancet 342:548-549.

Furukawa T, Zitnik G, Leppig K, Papayannopoulou T, Stamatoyannopoulos G (1994). Coexpression of and · globin mRNA in cells containing a single human globin locus: results from studies using single-cell reverse transcription polymerase chain reaction. Blood 83:1412-1419.

Gabbiani G, Kapanci Y, Barazzone P, Franke W (1981). Immunochemical identification of intermediate-sized filaments in human neoplastic cells. American Journal of Pathology 104:206-216.

Galea MH, Blamey RW, Elston CE, Ellis IO (1992). The Nottingham Prognostic Index in primary breast cancer. Breast Cancer Research and Treatment 22:207-219.

Gerhard M, Juhl H, Kalthoff H, Schreiber HW, Wagener C, Neumaier M (1994). Specific detection of carcinoembryonic antigen-expressing tumor cells in bone marrow aspirates by polymerase chain reaction. Journal of Cinical Oncology 12:725-729.

Goldblatt SA, Nadel EM (1965). Cancer cells in the circulating blood: A critical review. Acta Cytologica 9:6-20.

Gribben JG, Neuberg D, Freedman AS, Gimmi CD, Pesek KW, Barber M, Saporito L, Woo SD, Coral F, Spector N, Rabinowe SN, Grossbard ML, Ritz J, Nadler LM (1993). Detection by polymerase chain reaction of residual cells with the bcl-2 translocation is associated with increased risk of relapse after autologous bone marrow transplantation for B-cell lymphoma. Blood 81:3449-3457.

Gross H-J, Verwer B, Houck D, Hoffman RA, Recktenwald D (1995). Model study detecting breast cancer cells in peripheral blood mononuclear cells at frequencies as low as 10^7. Procedings of the National Academy of Science USA 92:537-541.

Gunthert U, Hoffmann M, Rudy W, Reber S, Zoller M, Haubmann I, Matzku S, Wenzel A, Ponta H, Herrlich P (1991). A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. Cell 65:13-24.

Hainsworth PJ, Tjandra JJ, Stillwell RG, Machet D, Henderson MA, Rennie GC, McKenzie IFC, Bennett RC (1993). Detection and significance of occult metastases in node-negative breast cancer. British Journal of Surgery 80:459-463.

Hardingham JE, Kotasek D, Farmer B, Butler RN, Mi J-X, Sage RE, Dobrovic A (1993). Immunobead-PCR: A technique for the detection of circulating tumor cells using immunomagnetic beads and the polymerase chain reaction. Cancer Research 53:3455-3458.

Hardingham JE, Kotasek D, Sage RE, Eaton MC, Pascoe VH, Dobrovic A (1995). Detection of circulating tumor cells in colorectal cancer by immunobead-PCR is a sensitive prognostic marker for relapse of disease. Molecular Medicine 1:789-794.

Hardingham JE, Kotasek D, Sage RE, Gooley LT, Mi J-X, Dobrovic A, Norman JE, Bolton AE, Dale BM (1995). Significance of molecular marker-positive cells after autologous peripheral-blood stem-cell transplantation for non-Hodgkin's lymphoma. Journal of Cinical Oncology 13(5):1073-1079.

Harris JR, Lippman ME, Veronesi U, Willett W (1992). Breast Cancer (review). New England Journal of Medicine 327(7):473-480.

Hay R, Macy M, Chen TR, McClintock P, Reid Y (1988). Catalogue of cell lines and hybridomas, Sixth ed. American Type Culture Collection.

Henderson IC (1995). Paradigmatic shifts in the management of breast cancer. New England Journal of Medicine 332: 951.

Henk D, Weering JV, Baas PD, Bos JL (1993). A PCR-based method for the analysis of human CD44 splice products. PCR Methods and Applications 3:100-106.

Herrlich P, Zoller M, Pals S, Ponta H (1993). CD44 splice variants: metastases meet lymphocytes. Immunology Today 14:395-399.

Hogervorst FBL, Cornelis RS, Bout M, Vliet MV, Oosterwijk JC, Olmer R, Bakker B, Klijn JGM, Vasen HFA, Meijers-Heiboer H, Menko FH, Cornelisse CJ, Dunnen JTD, Devilee P, Ommen G-JBV (1995). Rapid detection of BRCA1 mutations by the protein truncation test. Nature Genetics 10:208-212.

Israeli RS, Miller WH, Jr., Su SL, Powell CT, Fair WR, Samadi DS, Huryk RF, DeBlasio A, Edwards ET, Wise GJ, Heston WDW (1994). Sensitive nested reverse transcription polymerase chain reaction detection of circulating prostatic tumor cells: comparison of prostate-specific membrane antigen and prostate-specific antigen-based assays. Cancer Research 54:6306- 6310.

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. The Journal of Biological Chemistry 267:4732-4739.

Johnson PWM, Burchill SA, Selby PJ (1995). The molecular detection of circulating tumour cells. British Journal of Cancer 72:268-276.

Kaplan J-C, Kahn A, Chelly J (1992). Illegitimate transcription: its use in the study of inherited disease. Human Mutation 1:357-360.

Kaufmann M, Heider K-H, Sinn H-P, Minckwitz GV, Ponta H, Herrlich P (1995). CD44 variant exon epitopes in primary breast cancer and length of survival. The Lancet 345:615-619.

Koopman G, Heider K-H, Horst E, Adolf G, van den Berg F, Ponta H, Herrlich P, Pals ST (1993). Activated human lymphocytes and aggressive non-Hodgkin's lymphomas express a homologue of the rat metastasis- associated variant of CD44. Journal of Experimental Medicine 177:897-904.

Kotasek D, Sage RE, Dale BM, Norman JE, Bolton A (1994). Dose intensive therapy with autologous blood stem cell transplantation in breast cancer. Australian and New Zealand Journal of Medicine 24:288-295.

Krismann M, Todt B, Schroder J, Gareis D, Muller K-M, Seeber S, Schutte J (1995). Low specificity of cytokeratin 19 reverse transcriptase-polymerase chain reaction analyses for detection of hematogenous lung cancer dissemination. Journal of Cinical Oncology 13:2769-2775.

Kumazaki T, Hamada K, Mitsui Y (1994). Detection of mRNA expression in a single cell by direct RT-PCR. Biotechniques 16:1017-1018.

Latza U, Niedobitek G, Schwarting R, Nakarda H, Stein H (1990). Ber-EP4: new monoclonal antibody which distinguishes epithelia from mesothelia. Journal of Clinical Pathology 43:213-219.

Lazaar AL, Pure E (1995). CD44: a model for regulated adhesion function. The Immunologist 3:19-25.

Leather AJM, Gallegos NC, Kocjan G, Savage F, Smales CS, Hu W, Boulos PB, Northover JMA, Phillips RKS (1993). Detection and enumeration of circulating tumour cells in colorectal cancer. British Journal of Surgery 80:777-780.

Luppi M, Morselli M, Bandieri E, Sabbatini R, Marasca R, Frassoldati A, Barozzi P, Torelli G, Federico M (1995). Detection of tumor cells into bone marrow and peripheral blood of patients with breast cancer by RT-PCR (abstract). Breast Cancer Research and Treatment vol. 37 (Supplement). From: 18th Annual San Antonio Breast Cancer Symposium, December, 1995.

Mackay CR, Terpe H-J, Stauder R, Marston WL, Stark H (1994). Expression & modulation of CD44 variant isoforms in humans. The Journal of Cell Biology 124:71-82.

Mascarel ID, Bonichon F, Coindre JM, Trojani M (1992). Prognostic significance of breast cancer axillary lymph node micrometastases assessed by two special techniques: reevaluation with longer follow-up. British Journal of Cancer 66:523-527.

Mass RA, Bruning PF, Breedijk AJ, Top B, Peterse HL (1995). Immunomagnetic purification of human breast carcinoma cells allows tumor-specific detection of multidrug resistance gene 1-mRNA by reverse transcriptase polymerase chain reaction in fine-needle aspirates. Laboratory Investigation 72:760-764.

Matsumura Y, Tarin D (1992). Significance of CD44 gene products for cancer diagnosis and disease evaluation. The Lancet 340:1053-1058.

Matsumura Y, Hanbury D, Smith J, Tarin D (1994). Non-invasive detection of malignancy by identification of unusual CD44 gene activity in exfoliated cells. British Medical Journal 308:619-624.

Mattano LA, Moss TJ, Emerson SG (1992). Sensitive detection of rare circulating neuroblastoma cells by the reverse transcriptase-polymerase chain reaction. Cancer Research 52:4701-4705.

McGuire WL, Clark GM (1992). Prognostic factors and treatment decisions in axillary node-negative breast cancer. New England Journal of Medicine 326:1756.

Moldenhauer G, Momburg F, Moller P, Schwartz R, Hammerling GJ (1987). Epithelium-specific surface glycoprotein of Mr 34,000 is a widely distributed human carcinoma marker. British Journal of Cancer 56:714-721.

Moll R, Franke WW, Schiller DL (1982). The catalogue of human cytokeratins: Patterns of expression in normal epithelia, tumors and cultured cells. Cell 31:11-24.

Moreno JG, Croce CM, Fischer R, Monne M, Vikho P, Mulholland SG, Gomella LG (1992). Detection of hematogenous micrometastasis in patients with prostate cancer. Cancer Research 52:6110-6112.

Morrison BW (1994). The genetics of breast cancer. In: Shapiro CL, Henderson IC (Eds.) Hematology/Oncology Clinics of North America, vol. 8, pp 20-22.

National Health and Medical Research Council (1995). Clinical practice guidelines for the management of early breast cancer. Australian Government Publishing Service.

Noguchi S, Aihara T, Nakamori S, Motomura K, Inaji H, Imaoka S, Koyama H (1994). The detection of breast carcinoma micrometastases in axillary lymph nodes by means of reverse transcriptase-polymerase chain reaction. Cancer 74:1595-1600.

Nystrom L, Rutqvist LE, Wall S, Lindgren A, Lindqvist M, Ryden S, Andersson I, Bjurstam N, Fagerberg G, Frisell J, Tabar L, Larsson L-G (1993). Breast cancer screening with mammography: overview of Swedish randomised trials. The Lancet 341:973-978.

O'Brien DP, Billadeau D, Ness BV (1994). RT-PCR assay for detection of transcripts from very few cells using whole cell lysates. Biotechniques 16:586-588.

Passlick B, Izbicki JR, Kubuschok B, Nathrath W, Thetter O, Pichlmeier U, Schweiberer L, Riethmuller G, Pantel K (1994). Immunohistochemical assessment of individual tumor cells in lymph nodes of patients with non-small-cell lung cancer. Journal of Cinical Oncology 12(9):1827-1832.

Passos-Coelho J, Ross AA, Moss TJ, Davis JM, Huelskamp A-M, Noga SJ, Davidson NE, Kennedy MJ (1995). Absence of breast cancer cells in a single-day peripheral blood progenitor cell collection after priming with cyclophosphamide and granulocyte-macrophage colony-stimulating factor. Blood 85:1138-1143.

Porter-Jordan K, Lippman ME (1994). Overview of the biologic markers of breast cancer. Hematology/Oncology Clinics of North America, vol. 8, pp 73-100.

Redding WH, Monaghan P, Imrie SF, Omerod MG, Gazet J-C, Coombes RC, Clink HM, Dearnaley DP, Sloane JP, Powles TJ, Neville AM (1983). Detection of micrometastases in patients with primary breast cancer. The Lancet 2:1271-1273.

Ross AA, Cooper BW, Lazarus HM, Mackay W, Moss TJ, Ciobanu N, Tallman MS, Kennedy MJ, Davidson NE, Sweet D, Winter C, Akard L, Jansen J, Copelan E, Meagher RC, Herzig RH, Klumpp TR, Kahn DG, Warner NE (1993). Detection and viability of tumour cells in peripheral blood stem cell collections using immunocytochemical and clonogenic assay techniques. Blood 82:2605-2610.

Rudy W, Hofmann M, Schwartz-Albiez R, Zoller M, Heider K-H, Ponta H, Herrlich P (1993). The two major CD44 proteins expressed on a metastatic rat tumour cell line are derived from different splice variants: each one individually suffices to confer metastatic behaviour. Cancer Research 53:1262-1268.

Salles G, Zain M, Jiang W-m, Boussiotis V, Shipp M (1993). Alternatively spliced CD44 transcripts in diffuse large-cell lymphomas : Characterisation and comparison with normal activated B cells and epithelial malignancies. Blood 82:3539-3547.

Sambrook J, Fritsch EF, Maniatis T (1989). In: Molecular cloning: a laboratory manual, Second Ed., vol. 2. Cold Spring Harbor Laboratory Press.

Sato K, Yamazaki K, Shizume K, Kanaji Y, Obara T, Ohsumi K, Demura H, Yamaguchi S, Shibuya M (1995). Stimulation by thyroid-stimulating hormone and Graves' immunoglobulin G of vascular endothelial growth factor mRNA expression in human thyroid follicles in vitro and flt mRNA expression in the rat thyroid in vivo. Journal of Clinical Investigation 96:1295-1302.

Savtchenko ES, Schiff TA, Jiang C-K, Freedburg IM, Blumenburg M (1988). Embryonic expression of the human 40-kD keratin: evidence from a processed pseudogene sequence. American Journal of Human Genetics 43:630-637.

Schilder R (1995). Molecular markers and stem-cell transplants: are they made for each other? Journal of Cinical Oncology 13:1052-1054.

Schlimok G, Funke I, Holzmann B, Gottlinger G, Schmidt G, Hauser H, Swierkot S, Warnecke HH, Schneider B, Koprowski H, Reithmuller G (1987). Micrometastatic cancer cells in bone marrow: in vitro detection with anti-cytokeratin and in vivo labeling with anti-17-1A monoclonal antibodies. Procedures of the National Academy of Science USA 84:8672-8676.

Schoenfeld A, Luqmani Y, Smith D, O'Reilly S, Shousha S, Sinnett HD, Coombes RC (1994). Detection of breast cancer micrometastases in axillary lymph nodes by using polymerase chain reaction. Cancer Research 54:2986-2990.

Screaton GR, Bell MV, Jackson DG, Cornelis FB, Gerth U, Bell JI (1992). Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. Procedures of the National Academy of Science USA 89:12160-12164.

Shpall EJ, Stemmer SM, Johnston CF, Hami L, Bearman SI, Berenson R, Jones RB (1992). Purging of autologous bone marrow for transplantation: the protection and selection of the hematopoietic progenitor cell. Journal of Hematotherapy 1:45-54.

Shpall EJ and Jones RB (1994). Release of Tumour cells from bone marrow. Blood 83:623-625.

Sloane JP, Ormerod MG, Imrie S, Coombes RC (1980). The use of antisera to epithelial membrane antigen in detecting micrometastases in histologic sections. British Journal of Cancer 42:392-398.

Smack DP, Korge BP, James WD (1994). Keratin and keratinization. Journal of the American Academy of Dermatology 30:85-102.

Smith B, Selby P, Southgate J, Pittman K, Bradley C, Blair G (1991). Detection of melanoma cells in peripheral blood by means of reverse transcription and polymerase chain reaction. Lancet 338:1227-1229.

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. The EMBO Journal 10(2):343-348.

Stasiak PC, Purkis PE, Leigh IM, Lane EB (1989). Keratin 19: predicted amino acid sequence and broad tissue distribution suggest it evolved from keratinocyte keratins. Journal of Investigative Dermatology 92:707-716.

Tada M, Omata M, Kawai S, Saisho H, Saiki RK, Sninsky JJ (1993). Detection of ras gene mutations in pancreatic juice and peripheral blood of patients with pancreatic adenocarcinoma. Cancer Research 53:2472-2474.

Tanabe KK, Ellis LM, Saya H (1993). Expression of CD44R1 adhesion molecule in colon carcinomas and metastases. The Lancet 341:725-726.

Tarin D, Matsumura Y (1993). Deranged CD44 gene activity in malignancy (Editorial). Journal of Pathology 171:249-250.

Tarin D, Matsumura Y (1993). CD44 and Cancer (letter). The Lancet 341:252-253.

Tarin D, Matsumura Y (1994). Recent advances in the study of tumour invasion and metastasis. Journal of Clinical Pathology 47:385-390

Terpe H-J, Stark H, Prehm P, Gunthert U (1994). CD44 variant isoforms are preferentially expressed in basal epithelia of non-malignant human fetal and adult tissues. Histochemistry 101:79-89.

Thompson AM (1993). p53 and breast cancer. The Breast 2:8-10.

Thor A, Viglione MJ, Ohuchi N, Simpson J, Steis R, Cousar J, Lippman M, Kufe DW, Schlom J (1988). Comparison of monoclonal antibodies for the detection of occult breast carcinoma metastases in bone marrow. Breast Cancer Research and Treatment 11:133-145.

Tolg C, Hofmann M, Herrlich P, Ponta H (1993). Splicing choice from ten variant exons establishes CD44 variability. Nucleic Acids Research 21:1225-1229.

Trask DK, Band V, Zajchowski DA, Yaswen P, Suh T, Sager R (1990). Keratins as markers that distinguish normal and tumor-derived mammary epithelial cells. Procedures of the National Academy of Science USA 87:2319-2323.

Traweek ST, Liu J, Battifora, H (1993). Keratin gene expression in non-epithelial tissues. American Journal of Pathology 142:1111-1118.

Tsubura A, Okada H, Senzaki H, Hatano T, Morii S (1991). Keratin expression in the normal breast and in breast carcinoma. Histopathology 18:517-522.

Watson JD, Gilman M, Witkowski J, Zoller M (1992). Recombinant DNA, Second Ed. Scientific American Books.

Watt PCH, Spence RAJ (1986). Pathology for Surgeons. I.O.P. Publishing Ltd., pp 305-6.

Wielenga VJM, Heider K-H, Offerhaus GJA, Adolf GR, van den Berg FM, Ponta H, Herrlich P, Pals ST (1993). Expression of CD44 variant proteins in human colorectal cancer is related to tumor progression. Cancer Research 53:4754-4756.

Williams WJ, Beutler E, Erslev AJ, Lichtman MA (1990). Hematology, Fourth ed. McGraw-Hill, Inc., p. 130.

Wong LS, Bateman WJ, Morris AG, Fraser IA (1995). Detection of circulating tumour cells with the magnetic activated cell sorter. British Journal of Surgery 82:1333-1337.

Zauli D, Gobbi M, Crespi C, Tazzari PL, Miserocchi F, Magnani M, Testoni N (1986). Vimentin and keratin intermediate filaments expression by K562 leukemic cell line. Leukemia Research 10:29-33.

Ziegler BL, Lamping C, Thoma S (1992). Single-cell cDNA-PCR: removal of contaminating genomic DNA from total RNA using immobilized Dnase1. Biotechniques 13:726-729.