CYTOKINE GENE EXPRESSION IN A RAT MODEL OF POLYARTHRITIS

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Statement of Originality

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

Ashley Rex Connolly

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Publications

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Abstract

This thesis describes the development of a method used for quantification of cytokine mRNA expression and its application to measuring changes in cytokine expression in the synovial tissue and lymph nodes draining the hind feet of rats with adjuvant arthritis (AA). Competitive reverse transcription polymerase chain reaction assays were developed to quantify mRNA expression of the T-cell cytokines, IFN-γ, IL-2 and IL-4, together with TNF, a pro-inflammatory cytokine and the putative immunomodulatory cytokine TGF-β. IFN-γ and TNF protein levels were measured in AA rats when their respective mRNA levels were found to be elevated above those in control rats.

An investigation into the cytokine mRNA expression of thoracic duct (TD) lymphocytes collected from adjuvant and normal rats was conducted. Cytokine expression in subsets of TD cells expressing multiple activation markers was also examined.

The first section of this thesis describes the application and development of capillary electrophoresis (CE) for the quantitative analysis of PCR products. Aspects of DNA purification and analysis were evaluated which demonstrated that CE was a reproducibly accurate method for the quantification of PCR products.

The second section describes the accuracy and reproducibility of the competitive PCRs. The amplification rate of each competitor used in a PCR was determined and the accuracy of each quantitative PCR was assessed. The competitive PCRs were shown to conform to the basic theoretical requirements predicted by a mathematical model. A discussion is presented on the accuracy of quantitative PCR.

The third section concludes the methodological aspect of the reverse transcription (RT) PCR procedure by assessing the variations associated with extracting mRNA and reverse transcribing

it into cDNA for PCR analysis. Inter-assay and the intra-assay variability of both steps was assessed which indicated that there was considerable inter-assay variability associated with the RT procedure. The most accurate way to measure cytokine mRNA levels required the simultaneous RT of all samples. The development and evaluation of an ELISA used to measure TNF is also described.

Section four describes the application of quantitative PCR for the measurement of cytokine mRNA expression during concanavalin A stimulation of splenocytes. A kinetic study of the quantity of cytokine mRNA accumulating over a period of 3 days is presented. Changes in IFN-γ and TNF gene expression were shown to correlate with changes in the expression of their respective proteins.

The fifth section of this thesis examines the profiles of cytokine mRNA induced in the synovial tissue and the draining lymph nodes of rats during both the development and acute phases of AA. Cytokine protein levels were also measured when the cytokine mRNA levels were elevated. A second experiment was conducted to confirm cytokine mRNA changes during the development of AA. IL-2 and TNF mRNA levels were significantly elevated in the inguinal lymph node 3 days after inoculation and significant increases in TNF and TGF-β mRNA levels were evident in the synovial tissue 12 to 13 days after inoculation.

The sixth section presents the cytokine mRNA levels in TD cells obtained from AA rats at the clinical onset of arthritis. The cytokine profile of CD4$^+$ TD cells displaying cell surface activation markers was determined in addition to the fraction of TD cells that lacked the markers. An increase in the amounts of IFN-γ, TGF-β and TNF were measured in the cells bearing activation markers whereas no significant increase in cytokine mRNA was detected in the population lacking the activation markers.

Abbreviations

AA	Adjuvant arthritis
APC	Antigen presenting cells
BSA	Bovine serum albumin
cDNA	Complementary deoxynucleic acid
CFA	Complete freund's adjuvant
CIA	Collagen induced arthritis
Con A	Concanavalin A
CZE	Capillary zone electrophoresis
DC	Dendritic cell
dNTP	Deoxynucleotide triphosphate
DTH	Delayed type hypersensitivity
FCS	Foetal calf serum
HEV	High endothelial venule
HRP	Horseradish peroxidase
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NK cell	Natural killer cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMNL	Polymorphonuclear leucocytes
RA	Rheumatiod arthritis
RT-PCR	Reverse transcription polymerase chain reaction
SCW	Streptococcal cell wall
Tc-cell	Cytotoxic T-cell
TD	Thoracic duct
TGF-β	Transforming growth factor-beta
Th-cell	T-helper cell
TNF	Tumour necrosis factor
UV	Ultra violet

Chapter 1

Introduction

1.1 Inflammation

1.1.1 Introduction: An Overview of the General Features of Inflammation

Inflammation is a localised protective response elicited by injury or destruction of tissues, which serves to destroy, dilute or wall off both the injurious agent and the injured tissue (Gallin et al. 1988). Acute inflammation is classically characterised by redness, swelling, pain, heat, and loss of function. Vasodilatation of the vessels during the response results in an increase in blood flow to the site which leads to greater perfusion of the tissues producing the characteristic redness associated with inflammation. This increased regional blood flow is often not sufficient to immediately meet the extraordinary metabolic demands of inflamed tissues (James et al. 1990). Increased vascular permeability promotes swelling while the sensitisation of nerve fibres results in increased sensitivity and pain at the site of injury. The inflammatory response is usually accompanied by an increase in tissue temperature. In severe cases inflammation is accompanied by loss of function and temporary or permanent disability.

Inflammation may be classified as either acute or chronic depending on its duration and pathology (Ryan and Majno 1977). Acute or self limiting inflammation is the typical short term response associated with all types of tissue injury. It is generally characterised by the rapid infiltration of neutrophils into the injured tissue which is then followed by a predominant infiltration of phagocytes and lymphocytes. Chronic or self perpetuating inflammation may last for weeks or years due to persistence of a pathogenic antigen as a result of failure of the host to eliminate infection or to discriminate self from non-self. Chronic inflammation is the hallmark of rheumatoid arthritis (RA) and other immunological mediated disorders of inflammation.

An inflammatory response is initiated following tissue injury or infection with a foreign agent. Non-specific destruction of the exogenous material is mediated by neutrophils, eosinophils and mononuclear phagocytes (phagocytic cells) that inhabit, or freely migrate to the site of tissue injury. Polymorphonuclear leucocytes (neutrophils and eosinophils), monocytes or natural killer (NK) cells that are recruited from the blood are responsible for the eradication of foreign material outside the mononuclear phagocyte system.

Specific epitopes on the foreign material can be recognised by B-cell receptors and their derived antibodies or receptors found on T-cells. The recognition of foreign antigen by antibodies or receptors leads to the activation of an amplification system comprising the complement cascade, cytokines, the coagulation cascade, lipid and amine mediators. These mediators increase the flow of blood to the injured tissue, vascular permeability, adherence of circulating leucocytes to vascular endothelium and leucocyte migration into tissue as well as the destruction of foreign material by the stimulated leucocytes.

Immunologically mediated inflammation can be classified into four categories based on histology, physiologic function, clinicopathologic manifestations and the mechanism of induction (Gallin et al. 1988). Each category may partially contribute to a net inflammatory response. The overall inflammatory response may display characteristics of each category.

Allergic inflammation is categorised by the production and secretion of IgE antibodies as a typical response to invasion by extracellular foreign antigens. These antibodies bind to cognate Fc receptors on the surface of mast cells and basophils. Antigen engagement by this cytophilic antibody triggers the release of preformed granules and the synthesis of biologically active inflammatory mediators such as histamine and arachidonic acid. Mononuclear cells are present at the site of inflammation in addition to neutrophils and eosinophils. Asthma and rhinitis are the result of allergic inflammatory reactions.

Inflammation mediated by cytotoxic antibodies is often initiated by the release of complement fixing antibodies such as IgG and IgM following tissue injury. These antibodies can recognise and bind to particular antigens on the surface of circulating cells.

The cell surface bound antibody labels the cell for phagocytic destruction. Binding of complement fixing antibodies to extracellular structures can induce frustrated phagocytosis by neutrophils and other phagocytes which is characterised by exocytosis of degradative lysosomal enzymes and structural damage to the hosts tissues. Many common rheumatologic disorders are characterised by this type of inflammation.

Inflammation can result from the deposition of circulating antibody/antigen complexes in tissues. IgM or IgG complexed with antigen are able to activate the complement cascade and initiate inflammation. The complexes can also be bound by neutrophils and macrophages and thereby initiate the respiratory burst. This involves the generation of toxic oxygen species that cause damage to vessels and tissues. Large amounts of the antigen-antibody complexes that are present in the circulation become localised to small vessels where they initiate inflammation and vasculitis. Phagocytosis of immune complexes initiates the release of cytokines which may either modulate or exacerbate the inflammatory response. Serum sickness is characterised by this type of inflammation.

Chronic inflammation results from interactions between T-cells and antigens. This type of inflammation is often referred to as a delayed type hypersensitivity (DTH) reaction because it is not evident until hours or days after antigen exposure, whereas allergic and immune complex reactions usually occur within minutes to hours after antigen exposure respectively. The antigen is processed then presented by macrophages to T-cells which are activated and secrete cytokines such as IL-2, IFN-γ and TNF. DTH reactions are involved in defence against intracellular bacteria and certain viruses.

1.1.2 Cells Involved in the Inflammatory Response

1.1.2.1 Resident Cells, Endothelial Cells, Platelets, Neutrophils, Eosinophils, Mast Cells, Basophils and Monocytes

An inflammatory response is initiated by resident cells at the site of challenge and subsequently by cells recruited to the site from the circulation. The cellular stage of inflammation is marked by margination and emigration of leucocytes, chemotaxis and phagocytosis of the foreign material or injured tissue.

The accumulation of inflammatory cells is fundamental in inflammation. Cell accumulation is initiated by the production and secretion of chemotactic factors and inflammatory mediators by resident cells at the site of tissue injury. The engagement of these factors by cognate receptors on cells initiates intracellular signalling events that activate the motile machinery of circulating cells. The ensuring motility response can be directionally specific along a concentration gradient towards the site of origin of the chemotactic factor, which is in the direction towards that of the injured tissue.

Endothelial cells that have been activated by these mediators have an increased expression of vascular addressins with specificities for homing receptors on circulating inflammatory leucocytes. Cognate interactions between these molecules are critical for the recruitment of circulating cells to site of inflammation. Endothelial cells also regulate coagulation, fibrinolysis and the exudation of fluid and solutes from the blood into the extravascular space (Rosenberg and Rosenberg 1984).

Platelets contribute to the inflammatory response by releasing a variety of inflammatory mediators. Upon activation by inflammatory stimuli, injury or abnormal blood flow they secrete preformed vasoactive materials from their granules. They release clotting factors, growth factors and other mediators that modulate the inflammatory activities of phagocytes. They activate the complement cascade, and are a source of vasoactive lipids and hydrolases (Packham et al. 1968). Activated platelets are also capable of forming aggregates on plasma membranes resulting in the formation of a primary haemostatic plug that traps leucocytes at the site of inflammation. Through these various mechanisms platelets can contribute in a number of ways to the altered homeostasis of an inflammatory condition.

Leucocytes serve to protect the body against invasion by antigens. They develop from the stem cells located in the bone marrow under the influence of a variety of mediators and growth factors. There are two principal types of leucocytes, the granular cells which include neutrophils, eosinophils and basophils and the non-granular leucocytes which include monocytes, phagocytes and lymphocytes.

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Neutrophils are the first cells to arrive at an inflammatory site. They originate from the bone marrow and move into the circulation where they may remain unactivated for their short life span. Upon activation the neutrophils undergo dramatic changes in their surface characteristics. Receptors for chemoattractants, immunoglobulins and complement proteins are dramatically upregulated. As a result of this "priming" they display increased adherence to endothelia and responsiveness to chemotactic gradients. At sites of inflammation the neutrophils degranulate and secrete degradative enzymes such as elastase and collagenase which contribute to local tissue breakdown. They also secrete chemoattractants and complement activators that amplify the inflammatory response.

Eosinophils are present in large numbers during parasitic and allergic responses in which they engage foreign antigens and exert potent inflammatory effects (Spry et al. 1992). Major basic protein is the principle component of eosinophil granules and is toxic to respiratory epithelium during these responses (Tagari et al. 1992). Eosinophils can also stimulate the release of histamine from mast cells and basophils and are a rich source of arachidonic acid derived lipid mediators (such as LTC_4 and LTB_4) which have multiple effects during an inflammatory response.

Mast cells and basophils develop from a common bone marrow derived haematopoietic precursor cell and have several features in common. Both have cytoplasmic granules containing a variety of chemical mediators of inflammation including histamine and other biogenic amines. In addition both types of cells have high affinity Fc receptors for IgE which upon engagement stimulate the exocytosis of cytoplasmic granules (Segal et al. 1977). Mast cells are commonly found in the tissues and basophils are predominantly found in the circulation. Mast cells and basophils are thought to be involved in allergic responses.

Monocytes are a class of less granular leucocyte which accumulate at an inflammatory site later than granulocytes. Monocytes are phagocytic cells often referred to as macrophages and can engulf larger and greater quantities of material than neutrophils. They release a broad range of inflammatory mediators including free radicals such as superoxide and nitric oxide, a variety of cytokines and macromolecules like fibronectin (Nathan 1987).

Macrophages play a major role in the presentation of antigen to B and T-cells which secrete cytokines that regulate the specific immune response. Due to their abundance in tissues, macrophages can exert important effects on both the immune and inflammatory response.

1.1.3 Specific Immune Response

1.1.3.1 Introduction to B-Cells and T-Cells

The non-specific immune mechanisms such as mucosal barriers, natural killer (NK) cells preformed natural antibodies, complement system and the activity of phagocytic cells provide the first line of action in an inflammatory response. Specific immunity is characterised by antigen specific responses which focus the machinery of non-specific immunity (also known as innate immunity) on antigenic targets. Specific immunity protects against invasive organisms and is often the key to eradication of primary infection and protection against re-infection.

Humoral immunity depends on antibodies secreted by lymphocytes of the B-cell lineage. On their surface B-cells express MHC class II molecules, Fc receptors for immunoglobulins and various complement receptors (Abbas et al. 1991). Following development in the bone marrow, B-cells enter the circulation then migrate to the spleen and various peripheral lymphoid tissues. Immunoglobulins on the surface of B-cells are able to bind cognate foreign antigen resulting in cellular activation. B-cell activation conventionally requires help from T-cells through the secretion of a number of very potent T-cell cytokines that stimulate the division and differentiation of B-cells into immunoglobulin secreting plasma cells. However, there are several T-cell independent mechanisms of B-cell activation which promote plasma cell formation and the secretion of thousands of antibody molecules among which will be antibodies that can engage the foreign antigen usually at low affinities.

Higher affinity antibodies arise through a series of events which include the engagement of antigen by IgM found on the surface of naive B-cells. This is followed by internalisation of the antigen which is directed to the lysosomal compartment where it is partially digested.

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The resulting oligomeric particles are then presented on the cell surface by class II MHC molecules contingent upon the suitability of the polymorphism of the presenting region of the MHC molecule for particular peptides (Unanue et al. 1984). The MHC-peptide complex is then involved in a trimeric interaction with the T-cell receptor (TcR) of a CD4+ T-cell. The specificities for CD4+ T-cells is determined by an interaction between CD4 and a region of class II MHC away from the peptide binding groove. The TcR, like the B-cell receptor and soluble immunoglobulin, has a clonally unique specificity for a peptide which is achieved through rearrangements of multiple germinal components (Meuer et al. 1984). The trimeric interaction between the TcR and the peptide - MHC complex is stabilised by multiple adhesion interactions (ICAM-1, LFA-1, CD-2 and LFA-3) including the interactions of CD40 and CD28 with their respective cognate receptors on antigen presenting cells (a cell type that is especially important in priming the immune response) namely, CD40 ligand and CD80/CD86 (Chambers and Allison 1997; van and Banchereau 1997).

Dendritic cells (DC) are potent antigen presenting cells (APCs) which have the ability to activate naive resting T-cells during primary antigen exposure, leading to a specific immune response (Steinman 1991). DC constantly change their surface phenotype as they mature into potent APCs under the influence of granulocyte macrophage colony stimulating factor (GM-CSF) (Larsen et al. 1994; Symington et al. 1993). They reside in the tissues and upon encounter with an Ag, a complex set of signalling events initiates their egress into the draining lymph nodes where they act as efficient APCs with the capacity to present endogenous and naturally processed self peptides in the context of MHC II molecules. Therefore, DC have been implicated in self tolerance and autoimmune phenomena. DC have been detected during the early stages of autoimmune diseases, clustered together with CD4+ T-cells at the site of immunological activity (Clare-Salzler and Mullen 1992). Furthermore, DC are major producers of the T-cell chemoattractants MIP-1α and MIP-1γ (Heufler et al. 1992; Mohamadzadeh et al. 1996) which are responsible for recruiting T-cells and other DC to an inflammatory site. Several animal models of autoimmune diseases can be transferred to naive recipients with DC and it has

been proposed that they sequester self peptides and may be associated with initiating autoimmune diseases (Knight et al. 1988; Knight et al. 1983).

Mature helper T-cells are also capable of promoting cytotoxic killing carried out by cytotoxic T-cells. CD8 bearing cytotoxic T-cells are able to recognise and lyse host cells that display cognate foreign antigen within the peptide binding groove of class I MHC. This polymorphic molecule has many broad similarities with class II MHC but is presented on most cells. Antigens presented by class II typically arise from within the cytoplasm of cells. The peptides presented are mostly of self origin and elicit no response from T-cells because potentially responsive T-cells have been either deleted in the thymus during development or have been rendered anergic (Antonia et al. 1995; Parker and Eynon 1991). Since viruses need to assume a cytoplasmic location within cells in order to subvert cell machinery for protein synthesis for their own replication, infected cells present viral peptides in association with class I MHC molecules (Braciale et al. 1987). Some infected cells will be lysed by the virus as it disseminates and viral particles will also be taken up by phagocytic cells that will digest viral material in their phagolysosomes and present degraded viral peptides at their surface on class II MHC molecules. This presentation provides the basis of the collaboration of $CD4^+$ T-cells and $CD8^+$ T-cells in a cytotoxic response to viruses infected cells, and immunosurveillance against cancer cells (expressing neo-antigens). The $CD8^+$ cells require the foreign antigen/viral peptide in the context of class I MHC and release lytic agents such as perforin and granzyme at the site of heterotypic cell adhesion (Masson et al. 1986; Young et al. 1986). $CD4^+$ T-cells recognise viral peptide in the context of class II and release co-stimulatory cytokines (for example IL-2, IFN-γ). $CD4^+$ T-cells can also promote the cytotoxic activities of natural killer cells (NK cells) and macrophages through the action of released cytokines.

There is a central role for T-helper cells in generating both the humoral and cell mediated aspects of a specific immune response to invading antigens. T-cell help is most efficiently delivered by cell to cell contact in which the cytokines produced by the T-helper cells are the major factors that determine the outcome of the specific immune response.

1.1.4 Soluble Factors that Regulate Inflammation

1.1.4.1 Introduction to Inflammatory Mediators

Numerous cells contribute to the activation and suppression of the inflammatory response through the secretion of soluble chemical mediators which promote dilation of the capillaries and arterioles in association with increased permeability and blood flow. This results in the exudation of fluids and cells from the blood such as plasma proteins and leucocytes which migrate to the site of immunological activity. The redundancy of these chemicals at mediating the inflammatory response is summarised in Table 1 (Gallin 1989).

Mediators	Function
Histamine, brandykinin, complement, LTC_4, LTD_4, PGE_2, prostacyclins, Hageman factor (activated), high molecular weight kininogen fragments	Increased vascular permeability of small blood vessels
Thromboxane A2, leukotrienes C and D, C5a, LTB_4	Vasoconstriction
C3a, C5a, histamine, LTB_4, LTC_4, LTD_4, thromboxane A2, brandykinin	Smooth muscle contraction
IL-1, TNF, LTB_4	Increased endothelial cell stickiness
CSF's	Influence leucocyte proliferation, differentiation and survival
iC3b, fibronectin, IgG	Adherence, aggregation
C5a, LTB_4, PAF, phagocytic particles, collagen fragments, C-C, C-X-C, C chemokines	Chemotaxis
C5a, PAF, IL-1, TNF, LTB_4	Lysosomal granule release
C5a, TNF, PAF, most chemoattractants,	Oxygen radical release
phagocytic particles IFN-γ enhances C3b, iC3b, IgG (Fc proportion), fibronectin; IFN-γ increases Fc receptor expression IFN-γ, TNF, IL-1	Phagocytosis Granuloma formation
IL-1, TNF, PGE_2	Pyrogens
PGE_2	Pain

Table 1. Soluble factors responsible for mediating an inflammatory response.

1.1.4.2 Histamine and Serotonin

Histamine is one of the first mediators to be produced at the site of tissue injury. It and other vasoactive amines are stored in preformed granules within mast cells, platelets and basophils. The contents of the granules are rapidly secreted upon tissue injury. Histamine contributes to arteriolar dilation and enhanced permeability of the capillaries seen at the site of inflammation (Majno et al. 1969) which are responsible for the erythema of tissue swelling. Histamine can also block the function of cytotoxic T-cells (Falus and Meretey 1992) and can inhibit eosinophil chemotaxis at high concentrations (Clark et al. 1977).

To date the biological activities of serotonin in the inflammatory response remain unclear. However, it has been reported to increase phagocyte responsiveness to chemoattractants, promote fibroblast growth and stimulate collagen formation (Gallin 1989). Aggregated platelets at the site of inflammation liberate serotonin which promotes vasoconstriction and plays a major role in the control of bleeding at the site of tissue injury.

1.1.4.3 Kinins Forming System / Thrombin

The increased permeability and blood flow augmented by histamine at the site of tissue injury allows immunoregulatory constituents from the blood to permeate into the site. Kinins are small peptides which are cleaved from precursors in the blood, and they act to further increase blood flow and vessel permeability. They thereby contribute to the characteristic redness, swelling, warmth (and associated pain) at sites of inflammation. Activation of the kinin system promotes the deposition of a stringy insoluble protein called fibrin. Fibrin accumulation provides the matrix for clot formation, which helps to localise the response within the injured tissue. Coagulation is a fundamental aspect of inflammation. The cascade of proteolytic factors and platelets involved in inflammation also have a key homeostatic role in maintaining the integrity of the intrinsic clotting system.

1.1.4.4 Thromboxanes and Leukotrienes

Phospholipids are a major constituent of cell membranes. These lipids can be degraded by phospholipases which are enzymes found in activated leucocytes and platelets. During inflammation these enzymes are stimulated to degrade phospholipids and thereby release arachidonic acid. Cyclooxygenase and lipoxygenase are enzymes that are capable of metabolising arachidonic acid into prostaglandins and leukotriene derivatives respectively. Different tissues contain various forms of the cyclooxygenase enzyme which gives rise to three classes of mediators called prostaglandins, thromboxanes and prostacyclins. Thromboxanes promote vasoconstriction whereas prostacyclins promote vasodilatation (1997). Prostaglandin derivatives enhance vascular permeability, fever, sensitivity to pain and control the cellular release of inflammatory mediators. Leukotrienes are potent pro-inflammatory mediators that have been implicated in homeostatic immunoregulation. They have been reported to have an effect on the permeability of postcapillary venules, adhesion properties of endothelial cells, cell extravasation and chemotaxis of leucocytes (Parker and Stenson 1989). The metabolism of arachidonic acid to prostaglandins and leukotrienes forms part of a larger biological control system which is capable of regulating the various cellular and homeostatic responses to tissue injury.

1.1.4.5 Complement Cascade and its Implication in Inflammation

The complement system is the primary mediator of the humoral immune response and its products play an important role in amplifying an inflammatory response. The system can be activated through the "classic" mechanism which is initiated by IgG or IgM bound to antigen. Alternatively it can be activated "non-classically" when a constitutively expressed complement protein (C3) binds to complex polysaccharides or enzymes in the circulation (Abbas et al. 1991). Upon engagement, C3 initiates an amplification process leading to the lysis of virally infected cells and bacteria. C3a and C5a are two cleavage products of the complement cascade that can mediate vascular tone and enhance vascular permeability which contributes to the redness and odema associated with inflammation (Hugli 1986). Furthermore, complement products encourage the degranulation of mast cells and

basophils, and the secretion of lysosomal enzymes by neutrophils and mononuclear phagocytes. In addition, C5a is a potent chemoattractant for neutrophils (Elmgreen 1984). Defects in regulation of the complement cascade can lead to immune complex diseases which encompasses a variety of rheumatic diseases such as systemic lupus erythematosus (Walport and Lachmann 1990).

1.1.5 Lymphokines, Chemokines and Growth Factors

1.1.5.1 General Considerations

Cytokines are soluble, short lived, low molecular weight proteins which form a "communication" network for cells of the immune system. The generic word cytokine encompasses a variety of molecules including colony stimulating factors (CSFs), interleukins (ILs) and interferons (IFNs). They bind to specific receptors on the surface of cells and generally act in paracrine or autocrine mechanism to regulate the immune response and direct maturation, migration and the effector functions of inflammatory cells. Cytokines are produced and secreted by many different cells and often have diverse effects on cellular functions. Generally, cytokines function physiologically as colony stimulating factors, mediators of growth and differentiation, immunoregulatory mediators and pro-inflammatory mediators. Many cytokines were originally identified by their secretion from immune cells and their primary effects were exerted on other immune cells (eg. interleukins). Although this is a major role of cytokines, it is now recognised that they are produced by, and influence many cell types.

1.1.5.2 Colony Stimulating Factors

Colony stimulating factors, also called myeloid haematopoietic growth factors, are a group of glycoproteins that primarily regulate the proliferation and differentiation of haematopoietic progenitor cells and the functions of mature blood cells (Lieschke and Burgess 1992). Included in this group of cytokines are granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF) and IL-3. In addition, they are all mediators of inflammation because they exert effects on immunocytes such as lymphocytes,

neutrophils, monocytes and macrophages. CSFs can facilitate chemotaxis of cells towards an inflammatory site and prime granulocytes, mononuclear phagocytes and eosinophils for heightened cytotoxic activity. T-cells, monocytes, macrophages, endothelial cells and fibroblasts are the major cellular sources of haematopoietic growth factors (Groopman et al. 1989).

1.1.5.3 Other Growth Factors

Platelet derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factors (FGFs) and transforming growth factor beta (TGF-β), in addition to some colony stimulating factors, have all been shown to have a primary function associated with growth enhancement of specific cell types (Rowe and Rapoport 1992). They have the potential to mediate the inflammatory response by enhancing or suppressing the proliferation of a variety of different cells at an inflammatory site.

1.1.5.4 T-Cell Cytokines

Interleukin 2, 4, 5, 7, 9, 10, 11, 17 and IFN-γ all play an important role in the inflammatory response because of their regulatory effects on immune cells, particularly macrophages. They are all produced by T-cells during an inflammatory response and can influence the nature and extent of immune responses. These cytokines can enhance cell growth and augment cell function. They have a complex range of activities with both pro and anti-inflammatory effects. For example IL-5 can induce chemotaxis of eosinophils (Warringa et al. 1992) and the production of other inflammatory mediators such as superoxide (Horie et al. 1996). Immunoregulatory cytokines such as IL-10 can have a marked effect on the production of inflammatory cytokines (Pajkrt et al. 1997) while IL-4 and IFN-γ are mutually antagonistic (Gautam et al. 1992).

1.1.5.5 Inflammatory Cytokines Released by Mononuclear Phagocytes

Pro-inflammatory cytokines that have been implicated in the inflammatory responses include TNF, IL-1, IL-6 and IL-8. While the full range of their functions in normal physiology remains to be defined, their role as potent mediators in inflammation and tissue

necrosis is well established. The local production of pro-inflammatory cytokines by activated mononuclear phagocytes triggers the release of additional growth factors which collectively incite the inflammatory response by upregulating the production of other soluble inflammatory mediators (Gallin et al. 1992). TNF and IL-1 are pivotal pro-inflammatory cytokines that are usually produced together during an inflammatory response yet may act separately or synergise in different inflammatory diseases. The recently described cytokines, IL-12 and IL-15 seem to have more focused activities on T-cell function, but are also generally pro-inflammatory (Alleva et al. 1997; Trinchieri 1995).

1.1.6 Control of Cytokine Synthesis and Release

Cytokines all have very potent biological activities and consequently their secretion is usually transient and subject to strict and complex control. They are not generally stored as preformed molecules and their levels are controlled by the translation of short lived cytokine mRNA species. As a result, cytokine secretion is often a brief and highly controlled event. The quantity of a cytokine is further regulated by interactions with specific receptors or antagonists which maybe present on the surface of a cell or secreted into the intercellular space (Balkwill 1991). For example, a specific receptor antagonist of IL-1, known as the IL-1 receptor antagonist (IL-1RA), demonstrates a high level of control of pro-inflammatory cytokine activity (Arend 1993). Cytokines often have a very high affinity for their respective receptors and the receptors themselves are often regulated by soluble mediators which are frequently other cytokines (Abbas et al. 1991).

1.1.7 Cytokine Networks

Activated cells may simultaneously synthesise a variety of different cytokines while an individual cytokine is able to initiate the production and secretion of many others. This results in the formation of an extensive network of cytokines which often has considerable overlapping biological effects. The ability of a single cytokine to enhance or suppress the production of others has important positive and negative regulatory mechanisms for immune and inflammatory responses. The net effect of a cytokine can often be attributed to the action of multiple cytokines which demonstrates the redundant nature of cytokine

networks. Cytokine proteins are rarely released singularly and generally form interacting networks with other regulators such as hormones and neuropeptides (Jorgensen and Sany 1994; Robertson et al. 1992; Rook et al. 1994). As with most biological systems including cytokines, the overall effects will arise from multiple cytokines acting in both synergistic and opposing mechanisms forming a complicated network in which the differentiation state of the target cells will also influence the net biologic response.

1.1.8 T-Cell Cytokines as Determinants of Immune Responses

Cells are able to secrete a variety of cytokines that act as autocrine and/or paracrine regulators of cellular activation, growth and differentiation, immunoregulation and pro-inflammatory factors. Cytokines secreted by circulating and cells resident at sites of inflammation have been implicated in virtually all facets of the inflammatory response. A critical role for cytokines has been established in the development and maintenance of specific immune responses. Such is the pivotal role of T-cell cytokines in determining the nature of immune responses that classification of cellular and humoral immune responses now takes into account the profile of cytokines secreted by helper T-cells in response to a pathogenic stimulus (Mosmann and Coffman 1989).

1.1.9 Cytokines and their Networks as Targets for Intervention

Knowledge of the cytokine network induced during the pathogenesis of a disease has considerable clinical potential in disease intervention. To understand a cytokine network it is necessary to know the basic functions of each cytokine contributing to the network and the environment in which each is produced. A considerable amount of research has been dedicated to studying the immunological properties of individual cytokines as a precursor to elucidating their net effects within the pathogenic network characteristic of a disease. A discussion on the particular cytokines relevant to this thesis will be presented as a basis for elucidating and better understanding the cytokine network responsible for the pathogenesis of adjuvant arthritis (AA). A detailed discussion will be limited to IL-2, IFN-γ, IL-4, TNF and TGF-β which are topical to this thesis.

1.2 Cytokines in Inflammation

1.2.1 Interleukin-2

1.2.1.1 IL-2 Overview

Interleukin-2 (IL-2) is regarded as an immunoregulatory cytokine. IL-2 mRNA is reported to be 704 bps in length, and the deduced amino acid sequence for rat IL-2 is very similar to that of other species (McKnight et al. 1989). IL-2 was originally called T-cell growth factor (TCGF) and is synthesised and secreted primarily by T-helper cells and to a lesser extent by cytotoxic T-cells activated by mitogens or the interaction of the T-cell receptor complex with an antigen in association with MHC (Smith 1984).

1.2.1.2 The Structure of IL-2 and its Receptor

IL-2 is a glycoprotein with an apparent molecular weight of 15-18 kDa and in humans is encoded by a single gene located on chromosome 4 (Seigel et al. 1984). Variable glycosylation of the protein accounts for the range in molecular weights and isoelectric points. Human IL-2 is a 153 amino acid residue polypeptide which contains three cystine residues, two of which form a disulfide bond that is required for biological activity (Tsuji et al. 1987). The crystal structure of IL-2 has shown that it is an α-helical protein with a four fold core and no β-sheet structure (Bazan 1992; Brandhuber et al. 1987). The biological activities of IL-2 are mediated by binding to a multi-molecular cellular receptor which consists of an α-chain (known as Tac antigen, p55 or CD25), a β-chain (also referred to as p70 or p75) and a γ-chain, all of which co-ordinate to form a high affinity receptor capable of transducing the IL-2 signal (Smith 1989). The γ-chain is required for high affinity binding and signalling of IL-2 and in its absence no signal will be transduced (Nakamura et al. 1994; Nelson et al. 1994). The receptor trimer requires IL-2 to be in close proximity with all three components for signal transduction. The respective receptors for IL-4, IL-7, IL-9 and IL-15 are also multimeric and utilise the γ-chain of the IL-2 receptor (Di et al. 1995).

1.2.1.3 Biological Activities of IL-2

IL-2 is responsible for the autocrine clonal expansion of antigen specific T-cells. It also acts as a growth factor for immunocytes and cells outside the immune system. B-lymphocytes and natural killer (NK) cells respond to IL-2 to become plasma cells and lymphokine activated killer cells (LAK cells) respectively. Expression of IL-2 is usually transient and occurs shortly after activation of the immune system which coincides with the transient expression of cell surface IL-2 receptors. Neutrophils and macrophages both demonstrate augmented function in the presence of IL-2 (Djeu et al. 1993; Malkovsky et al. 1987). It stimulates the synthesis of other T-cell derived cytokines such as IFN-γ and lymphotoxin and has been implicated in the development of cell mediated immune responses (Mosmann and Coffman 1989). Failure to secrete IL-2 has been associated with antigen specific T-cell anergy (Johnson and Jenkins 1994; Schwartz 1993). Studies have demonstrated that IL-2 transcription is susceptible to suppression by cyclosporin and glucocorticoids (Daynes and Araneo 1989; Hess et al. 1986; Kelso and Munck 1984). These agents can thereby suppress the secretion of IL-2 and hence cell proliferation.

1.2.2 Interferon-γ

1.2.2.1 IFN-γ Overview

Originally called immune or type II interferon, IFN-γ is a multi-functional protein produced by helper T-cells, cytotoxic T-cells and NK cells. It's transcription is initiated by antigen induced activation and is enhanced by the presence of IL-2.

1.2.2.2 Structure of IFN-γ and its Receptor

It is a homodimeric protein consisting of two 21-24 kDa subunits each consisting of an 18 kDa polypeptide encoded for by a single gene (Rashidbaigi et al. 1986). IFN-γ exists in two different molecular weight species that differ in their degree of glycosylation, which is irrelevant for its biological activity. Human IFN-γ exists in solution as a head to tail dimer with the C-terminus of one monomer aligned with the N-terminus of the other (Ealick et al. 1991; Lunn et al. 1992). The predicted sequence of the IFN-γ receptor displays potential

nitrogen and oxygen linked glycosylation sites and a soluble form has been found in human urine under normal physiological conditions (Novick et al. 1989). Recent evidence suggests that a component of the receptor designated IFN-γRβ, is required for signal transduction following receptor engagement (Pestka 1997; Soh et al. 1994). Engagement of the receptor induces dimerisation and the transmission of an intracellular signal.

1.2.2.3 Biological Activities of IFN-γ

IFN-γ is known now to inhibit viral replication and regulate numerous immunological functions. It regulates the class of antibody secreted by B-cells, upregulates the expression of MHC class I and class II on macrophages and promotes macrophage mediated killing of intracellular parasites (Mogensen and Virelizier 1987). The immunomodulatory effects of IFN-γ are extensive and diverse. It is a potent activator of mononuclear phagocytes and increases IL-1 secretion, the production of platelet activating factor, H_2O_2 and the release of pterin, which are all potent mediators of inflammation (Billiau and Dijkmans 1990; Bulut et al. 1993; De and De 1992; Sen and Lengyel 1992). IFN-γ is produced by helper T-cells and is a key cytokine for promoting cell mediated immune responses. Its production is augmented in the presence of IL-12 and it antagonises IL-4 production. As a result, it is a key cytokine produced during a Th-1 immune response (Mosmann and Coffman 1989). This will be discussed in detail in Section 1.3. IFN-γ protects monocytes against LAK cell lysis, it down regulates IL-8 mRNA expression and induces production of nitric oxide in the presence of lipopolysaccharide (Bulut et al. 1993; Gusella et al. 1993) The synthesis of enzymes that mediate the respiratory burst are induced by IFN-γ and it has been shown to be a monocyte chemoattractant (Issekutz and Issekutz 1993b). IFN-γ can induce its own expression, and its secretion at sites of local inflammation is believed to induce IFN-γ mRNA synthesis by migratory cells (Halloran et al. 1992) and promote lymphocyte adhesion to high endothelial venule cells (Chin et al. 1991).

1.2.3 Interleukin-4

1.2.3.1 IL-4 Overview

Interleukin-4 was first reported as B-cell stimulatory factor (BCSF-1) and B-cell differentiation factor (BCDF). It is produced by helper and cytotoxic T-cells (Mosmann and Moore 1991; Seder et al. 1992), a minor subset of natural killer cells (Yoshimoto et al. 1995), foetal thymocytes (Sideras et al. 1988), mast cells (Bradding et al. 1992) and basophils (MacGlashan et al. 1994).

1.2.3.2 The Structure of IL-4 and its Receptor

Human, mouse and rat RNA code for IL-4 precursor proteins of 153, 140 and 147 amino acids respectively. The signal peptide is cleaved from its precursor to yield a mature protein with multiple glycosylation sites which are believed to be unimportant for biological activity. The IL-4 protein consists of a four helix left-handed antiparallel bundle (Walter et al. 1992). The activity of IL-4 is mediated by high affinity cell surface receptor complexes which consist of a ligand binding subunit (IL-4R) and a common γ-chain (γc) which was originally identified as a component of the IL-2 receptor complex (Kawahara et al. 1994; Russell et al. 1993). Dimerisation of the chains is required in order to transduce the IL-4 signal. A naturally occurring soluble form of the IL-4 receptor has also been identified in mice (Fanslow et al. 1990).

1.2.3.3 Biological Activities of IL-4

IL-4 has stimulatory and suppressive effects *in vitro* that suggest it may play an important role in modulating immune and inflammatory responses. T-helper cells are the major source of IL-4 and it can also be secreted by a subset of NK cells and activated mast cells. It is a pleiotropic cytokine with multiple immune modulating functions and act on a variety of cell types including T and B-lymphocytes, monocytes, neutrophils, macrophages, mast cells, fibroblasts, endothelial cells, osteoblasts, keratinocytes and astrocytes (Paul 1991). IL-4 exhibits anti-inflammatory effects on macrophages and stimulates the production of a humoral immune response in association with activated helper T-cells (Gollob et al. 1997).

It is responsible for decreasing the proliferation of Th-1 cells by antagonising the production of IFN-γ (Dieli et al. 1996; Gautam et al. 1992). As a result, IL-4 is the principal cytokine indicative of a Th-2 response which will be discussed in detail in Section 1.3. It induces antigen specific cytotoxic T-cell development and promotes thymic T-cell differentiation as well as the proliferation of activated T-cells, NK cells, LAK cells and mast cells. IL-4 is also the only known cytokine that acts as a switch factor for IgE production, making it a principle mediator of allergic reactions (Paul 1991). It upregulates the expression of surface MHC class II in association with antigen presentation and the expression of specific adhesion molecules on endothelial cells (Verdegaal et al. 1993). It induces expression of the receptor for IgE (FcεRII) and antagonises the production of inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF. IL-4 inhibits superoxide production (Abramson and Gallin 1990) and the secretion of collagenase by macrophages (Lacraz et al. 1992) yet conversely can stimulate the secretion of collagen by fibroblasts (Sempowski et al. 1994).

1.2.4 Tumour Necrosis Factor

1.2.4.1 TNF Overview

Tumour necrosis factor is also known as cachectin and it has a wide range of biological effects associated with inflammation. TNF is produced by activated T-cells and mainly monocytes and/or macrophages (Vilcek and Lee 1991). TNF knockout experiments have revealed its role in non-specific immunity and toxic shock (Pfeffer et al. 1993; Rothe et al. 1993). It is the principal mediator of both natural and acquired immunity and is an important link between the specific immune response and acute inflammation.

1.2.4.2 Structure of TNF and its Receptors

Rat TNF is a 235 amino acid residue polypeptide with relatively low sequence homology with human TNF (Kwon et al. 1993). It has a precursor sequence containing both hydrophilic and hydrophobic domains and is usually integrated into the cell membrane before it is solubilised by enzymic cleavage of the membrane domain. Currently it is thought that the membrane bound form of TNF exists on the surface of monocytes and

macrophages and serves as a reservoir for the release of the soluble form (Kriegler et al. 1988; Luettig et al. 1989). Biologically active TNF is found as a non-covalently linked trimer. The affinity of TNF for its receptor is relatively low for a cytokine (Loetscher et al. 1991). However, it is usually synthesised in very large quantities and can easily saturate its receptors, which are found on almost all cell types (Tartaglia and Goeddel 1992). The TNF receptor is a heterodimer composed of peptides referred to as TNF-R1 and TNF-RII. TNF-RII (also type A, type-α, 75 kDa or utr antigen) has an apparent molecular mass of 75 kDa while TNF-R1 (also type-B, type-β, 55 kDa, or htr antigen) has an apparent molecular mass of 55 kDa (Tartaglia and Goeddel 1992). The two receptor units are distinct immunologically but their extracellular domains have similarities in amino acid placements. The 55 kDa receptor is responsible for the lytic activity of TNF (Tartaglia et al. 1993b) whereas the 75 kDa unit is believed to be responsible for generating proliferative and regulatory signals in lymphocytes (Tartaglia et al. 1993a; Tartaglia et al. 1991). Shedding of soluble TNF receptors in response to the protein is believed to serve as a mechanism for binding and inhibiting TNF not bound to the surface receptors thus, protecting other cells from the effects of TNF and localising the inflammatory response.

1.2.4.3 Biological Activities of TNF

TNF is a pleiotropic factor, capable of activating multiple signal transduction pathways which have the ability to induce or suppress the expression of a large number of genes including those for growth factors, cytokines, transcription factors, inflammatory mediators and acute phase proteins. TNF is critical for the successful resolution of infectious and metastatic diseases but in an uncontrolled response it can cause damage to the host and is involved in pathological conditions such as cachexia (Oliff 1988), autoimmune disorders such as type 1 diabetes (Pujol-Borrell et al. 1987) and meningococcal septicaemia (Waage et al. 1987). At low concentrations it acts locally as a paracrine and autocrine regulator of leucocytes and causes endothelial cells to become adhesive for neutrophils, monocytes and lymphocytes (Chin et al. 1991; Fan et al. 1993). Many of the biologic actions of TNF are augmented by IFN-γ. TNF can activate inflammatory leucocytes to kill microbes and stimulates mononuclear phagocytes and

other cell types to produce cytokines. It may function as a co-stimulator for T-cell activation and stimulates antibody production by B-cells (Beutler and Cerami 1989). It exerts an interferon-like protective effect against viruses, augments the expression of MHC class I and class II and promotes $CD8^+$ T-cell mediated lysis of virally infected cells. TNF acts on hepatocytes to increase synthesis of certain serum proteins such as serum amyloid A protein (Leist et al. 1995). The spectrum of hepatocyte proteins induced by TNF is identical to that produced by IL-1, and the combination of plasma proteins induced by TNF, IL-1 and other mediators such as IL-6, constitute the acute phase response to inflammatory stimuli which enhances the efficacy of non-specific immunity. Chronic administration of TNF may lead to lymphopenia, immunodeficiency and suppression of T-helper cell functions (Cope et al. 1997). The main impact of TNF on tissue destruction is due to its synergism with IL-1. TNF and IL-1 are pro-inflammatory cytokines that may act independently or synergise during an inflammatory response to induce the production of collagenases and PGE_2 (So et al. 1992). TNF is a potent regulator of inflammation which is capable of inducing a cascade of other inflammatory mediators.

1.2.5 Transforming Growth Factor-β

1.2.5.1 TGF-β Overview

Transforming growth factor beta one (referred to as TGF-β) is a stable, multi-functional polypeptide growth factor that has been found in high concentrations in human platelets and mammalian bone. It is produced by most cells in smaller quantities (Cheifetz et al. 1987; Sporn et al. 1987) and is initially synthesised as part of a larger precursor molecule which contains the mature form of TGF-β at the C-terminus. Biologically active TGF-β is released in an active form from its precursor usually by *in vitro* acidification. Activated endothelial cells (Phan et al. 1992), reactive oxygen species (Barcellos Hoff and Dix 1996) and lysosomal enzymes (Oursler et al. 1993) are believed to control activation of the latent form of TGF-β *in vivo*.

1.2.5.2 Structure of TGF-β and its Receptors

Mature TGF-β is usually found as a disulfide linked dimer of two identical 112 amino acid chains. However, the natural occurrence of heterodimers of some of the TGF-β isotypes have also been reported (Sporn and Roberts 1992; Sporn et al. 1987). The majority of published reports have dealt with the TGF-β1 isotype, however, TGF-β2, TGF-β3, TGF-β4 and TGF-β5 have similar biological functions *in vitro*. The conservation of amino acid sequence between TGF-β1, TGF-β2 and TGF-β3 in different mammalian species is close to 100% although each isotype has a different potency on different cells (Cheifetz et al. 1990). The molecular mass of TGF-β on SDS PAGE is approximately 25 kDa (Hooper 1991). Its effects are mediated by binding to cell surface receptors that are currently incompletely characterised. Most cells have three classes of receptors designated Type I, Type II and Type III (Cheifetz et al. 1986; Massague et al. 1990). Type I and Type II receptors have recently been shown to be involved in the mediation of TGF-β biological responses. The Type III receptor is believed to have no role in signal transduction.

1.2.5.3 Biological Activities of TGF-β

TGF-β has a wide range of biological activities. With only a few exceptions all cells have surface receptors for, and respond to TGF-β. The biological response of cells to TGF-β depends on a variety of conditions including the type of cell, the state of cell differentiation and the presence of other cytokines and growth factors. TGF-β can block the synthesis of plasminogen activator and metalloproteinases in most cells (Matrisian and Hogan 1990) and down regulates the expression of IL-1 receptor which partially explains it's immunosuppressive properties (Dubois et al. 1990). TGF-β has some opposing effects to IFN-γ and TNF which also contribute to its suppressive effects (Wahl 1992). It has been reported to have potent and varied effects on B-cells, T-helper cells, cytotoxic T-cells, chondrocytes, osteoblasts/osteoclasts, monocytes, macrophages and a number of haematopoietic cell types (Roberts and Sporn 1993; Sporn and Roberts 1992; Sporn et al. 1987). The most common effect of TGF-β on these cells is suppressive. However, it has been shown to have stimulatory effects promoting the induction and secretion of growth

factors by macrophages, the synthesis of collagen, fibronectin and some proteoglycans associated with wound repair. It induces monocyte recruitment and activates fibroblasts during acute inflammatory responses (Wahl et al. 1987). It is an important mediator of extracellular matrix formation (Rizzino 1988) and TGF-β has been shown to stimulate osteoblasts and inhibit osteoclasts *in vitro* and is therefore involved in co-ordinating bone remodelling (Oreffo et al. 1989). It is an important modulator of the growth and differentiation of a number of cells involved in both the cellular and humoral immune responses. TGF-β knockout mice die from a diffuse inflammatory syndrome induced by massive infiltration of inflammatory and mononuclear cells (Kulkarni and Karlsson 1993; Shull et al. 1992). Increased expression of TGF-β and other immunoregulatory cytokines such as IL-10, in animal models of oral tolerance is thought to be responsible for generating a subset of T-helper cells called Th-3 cells (Fukaura et al. 1996).

1.2.6 Some Properties of T-Cell Derived Cytokines that Influence their Detection

T-cell derived cytokines are rarely detectable in the circulation whereas those produced at high levels *in vivo*, such as TNF, IL-1 and IL-6 are generally derived from macrophages and cells of the reticulo-endothelial system. A general property of cytokines is their rapid disappearance from the circulation when administered systemically. T-cell cytokines function only as short term, short range mediators and are unlikely to be present in the circulation and are detectable only at a site of immunological activity. Furthermore, the high potency of the T-cell cytokines in biological systems results in strict control of their secretion which is believed to occur intercellularly between the T-cell and the responding cell (Kelso 1993).

1.3 Th-1 and Th-2 Theory

1.3.1 Introduction

A variety of effector mechanisms are induced during an immune response. Regulation of these mechanisms can be critical for host survival and are therefore subject to precise and

complex regulation. The specific arms of an immune response were initially thought to require T-helper cells (Th) to activate both B-cells and cytotoxic T-cells. Therefore, Th-cells were viewed as the essential mediators of antibody production and cytotoxic T-cell killing. The humoral and cell mediated aspects of an immune response are often unequally expressed (Parish 1972) and it was Mosmann and Coffman (Mosmann et al. 1986) who showed that murine Th-cell clones could be divided into Th-1 and Th-2 subsets based on the production of cytokines during an immune response. The often biased nature of a specific immune response was revealed when cytokines produced by Th-cells during a delayed type hypersensitivity response (DTH response) were shown to antagonise the production of T-cell cytokines produced during a humoral immune response. It was hypothesised that the different arms of the specific immune response could be explained by a Th-1 and Th-2 cell dichotomy.

It is generally accepted that help for B-cell growth, differentiation and antibody production *in vitro* and *in vivo* is provided by Th-2 cells in association with a humoral immune response. Th-1 clones can also provide help for antibody production but not as efficiently as Th-2 clones (Coffman and Mosmann 1988). As a result, many of the functions mediated by Th-1 cells are associated with the eradication of intracellular pathogens by cytotoxic T-cells in association with a cell mediated immune response.

Cytokines secreted by Th-cells have autocrine and paracrine effects on cells at the site of immunological activity. Their secretion is not strictly confined to Th-cells which has led to a change in nomenclature from Th-1 and Th-2 to type 1 and type 2 respectively. This more accurately classifies the immune response by the cytokines produced rather than the type of cell from which they are secreted (Clerici and Shearer 1994). Cytokines secreted during type 1 or type 2 responses can influence immunoglobulin isotype class switching in B-cells (Rizzo et al. 1995), the expression of chemokine receptors (Chensue et al. 1996; Schrum et al. 1996) and adhesion molecules (Austrup et al. 1997; Iwamoto and Nakao 1995; Schlaak et al. 1995) which can effect the trafficking and extravasation of leucocytes to the site of immunological activity (Bradley and Watson 1996).

1.3.2 Cytokines and T-helper Cell Responses

The generalisation of the Th-1 and Th-2 classification in murine Th-cell clones was in dispute when human T-cell clones failed to display a similar pattern of cytokine secretion. However, since the original definition murine T-cell clones that do not conform to the Th-1 and Th-2 classification have been isolated (Hatfield et al. 1991 ; Kelso et al. 1995) and there is now concrete evidence from rodents and humans to indicate that Th-cell subsets can be defined *in vivo* and *in vitro* based on the repertoire of cytokines they secrete (Mason and Fowell 1992).

The initial studies on murine Th-1 and Th-2 clones demonstrated that each synthesised either IL-2 and IFN-γ, or IL-4 respectively. Since this original definition Th-cell specialisation has been expanded to incorporate the preferential secretion of a variety of cytokines (Table 2) and several cytokines have been reported to be secreted by both subsets (Maggi et al. 1988; Romagnani 1994; Street et al. 1990). The majority of murine Th-1 clones secrete IL-2, IFN-γ and TNF-β (lymphotoxin) whereas Th-2 clones secrete IL-4, IL-5, IL-6, IL-10 and IL-13. Interleukin-3, GM-CSF and TNF are expressed by both subsets. A similar range of cytokines are produced by human Th-cell clones although the secretion of IL-2, IL-6 and IL-13 by a single Th-cell subset has not been recognised (Mosmann and Sad 1996).

Th-1	Common	Th-2
IFN-γ	IL-3	IL-4
IL-2	GM-CSF	IL-5
TNF-β	TNF	IL-6
	Chemokines	IL-9
		IL-10

Table 2. Cytokines secreted by different subsets of murine T-helper cells.

1.3.3 Extension of T-helper Cell Subsets

At least 3 distinct patterns of cytokine secretion exist amongst Th-cells and it has become clear that other patterns can be displayed by long term Th clones. In addition to Th-1 and Th-2, a phenotype called Th-0 has been identified that secretes cytokines representative of both subsets (Firestein et al. 1989). Th-0 cells represent a heterogeneous population of effector cells with the characteristics of both Th-1 and Th-2 subsets (Romagnani 1996). However, it is difficult to reconcile this subset with cytokine function since it simultaneously produces both IFN-γ and IL-4 which are mutually antagonistic cytokines. This subset is believed to represent a population of Th-cells that are at an intermediate stage of differentiation or a population that has been only moderately stimulated (Torbett et al. 1990).

Long term Th-cell clones that predominantly secrete TGF-β and lower amounts of IFN-γ have been considered as fourth helper cell subset classified as Th-3 (Chen et al. 1994). As new cytokines and immunological mediators are discovered, and knowledge about existing cytokines expands, it may prove difficult to classify Th-cells into discrete subsets because of the redundancy of cytokine function. As a result, it has been suggested by Kelso (Kelso 1995) that Th-1 and Th-2 cells are not discrete phenotypes but represent extremes of a spectrum that encompasses cells producing a diverse assortment of cytokines. This is based on the observation that during an immune response individual T-cell clones synthesise a combination of cytokines representative of the Th-1 or Th-2 phenotypes but may not exclusively fit into either classification (Firestein et al. 1989; Maggi et al. 1988; Romagnani 1994; Street et al. 1990). The population of activated Th-cells constituting an immune response can collectively produce a variety of cytokines that may be indicative of a type 1 or type 2 response, but may not exclusively fit into either classification.

1.3.4 Mechanism of Th-1 Responses

Studies of animal diseases have revealed that the cytokine repertoire induced during an immune response can influence the hosts ability to effectively eradicate the invading organism. The cytokine repertoire provokes the effector functions of specific immunity

and therefore is important in determining the hosts resistance or susceptibility to the infectious agent (Sher and Coffman 1992). Th-1 cells secrete IFN-γ and TNF-β which activate macrophages and produce a delayed type hypersensitivity (DTH) response which is best suited to protecting the host against intracellular parasites (Coffman et al. 1991). Extracellular parasites are best combated by Th-0 cells or a combination of cytokines characteristic of the Th-1 and Th-2 phenotype (Daugelat and Kaufmann 1996). Th-2 cells produce IL-4, IL-5, IL-10 and IL-13 which promote IgG1 and IgE production and inhibit macrophage functions. They are best suited to protecting the host against metazoan parasites (Coffman et al. 1991).

Elucidation of some of the mechanisms responsible for biasing Th-cell clones towards either a Th-1 or Th-2 phenotype have been identified by co-culturing Th-cell clones *in vitro* with appropriate cytokines or anti-cytokine antibodies (Maggi et al. 1992 ; Noble et al. 1993; Schmitt et al. 1990). As a result, cytokines present in the local environment of a Th-cell during *in vivo* activation are thought to bias the evolving Th-cell subset (Hsieh et al. 1996). Th-2 clones proliferate strongly in response to both IL-4 and IL-2 and weakly in the presence of IFN-γ whereas Th-1 clones proliferate strongly in response to IL-2 and weakly in the presence of IL-4 (Noble et al. 1993). Furthermore, IL-12 augments IFN-γ production and its secretion promotes the development of a Th-1 phenotype (Scott 1993). IFN-γ, IL-12 and IL-4 are therefore key cytokines that determine the nature of the predominant Th-cell response induced during an immune response.

The early events that lead to Th-1 cell differentiation in mice infected with intracellular pathogens are well understood. The secretion of IL-2 upon stimulation of naive Th-cells promotes a rapid proliferative response that boosts the number of antigen specific effector Th-cells. Restimulation of the expanded population induces secretion of a large number of cytokines. Microbial products induce IL-12 secretion by macrophages which induces natural killer cells (NK cells) to secrete IFN-γ (Biron and Gazzinelli 1995) within 24 hours of infection. Naive Th-cells initiate a specific immune response to the pathogen in an environment containing IFN-γ and IL-12 which assists the induction of a type 1 immune response. IFN-γ regulates Fc receptor expression for IgG2a antibodies (Akiyama et al.

1984) which have been implicated in antibody dependent cell mediated cytotoxicity and cytotoxic killing. Macrophages destroy target cells displaying foreign antigen and IgG2a promotes destruction of the target cells by complement mediated cell lysis.

1.3.5 Typical Th-1 Disease

Resistant C57Bl/6 mice infected with *leishmaniasis major* (*LM*) develop a strong DTH response accompanied by low levels of antibody secretion and no elevation of IgE levels. IFN-γ levels are elevated and the quantity of secreted IL-4 is low. This implies a type 1 response is responsible for the elimination of *LM* in C57Bl/6 mice because the disease is contained and ultimately the mice are cured (Reed and Scott 1993). The involvement of type 2 cells in the pathogenesis of *LM* has been demonstrated by Scott et.al. (Scott et al. 1988) who injected Th-2 specific *LM* clones into *LM* infected mice which resulted in disease exacerbation, whereas Th-1 specific *LM* clones abrogated the infection. A type 1 response is therefore appropriate for elimination of the parasite and resolution of the disease. Susceptible strains of mice respond to *LM* by secreting high levels of IgE antibodies, high quantities of IL-4, low amounts of IFN-γ and display no DTH response. This is characteristic of a type 2 disease and due to the inefficiency of this response the mice ultimately die (Heinzel et al. 1989).

1.3.6 Mechanisms of Th-2 Responses

The early events that lead to Th-2 cell differentiation are not clearly understood. However, it is known that cytokines such as IL-4, IL-5 and IL-6 are able to regulate the growth and differentiation of B-cells during a type 2 response (Matsuda et al. 1989). Antibody isotype switching is mediated by IL-4 which induces IgG1 (Kinashi et al. 1986) and IgE (Coffman and Carty 1986) production. IL-4 increases the expression of Fc receptors for IgE on B-cells and MHC class II expression on macrophages (Galy et al. 1991). IL-3 and IL-4 promote the proliferation of mast cells which themselves are a source of IL-4 (Bradding et al. 1992; Plaut et al. 1989). In the presence of IL-5 eosinophils proliferate and IL-4 promotes antibody isotype switching to IgE which is characteristic of a type 2 response (Paul 1991; Spry et al. 1992). The secretion of IL-10 during an immune response leads to

an inhibition in cytokine synthesis. In particular, it blocks the production of IFN-γ by Th-1 cells and therefore suppresses Th-1 mediated immune functions. This blockade is believed to occur by obstructing the activation of T-cells and macrophages by inhibiting costimulatory signals and antigen presentation respectively (Fiorentino et al. 1989). Therefore, IL-10 acts to suppress the production of Th-1 but not Th-2 cytokines during an immune response.

1.3.7 Typical Th-2 Disease

Helminth parasitic infestations are extracellular pathogens that induce IgE responses, eosinophilia and intestinal mast cell hyperplasia. An example of such a parasite is *Nippostrongylus brasiliensis* (*Nb*) which can be eradicated by the activation of a type 2 like response (Street et al. 1990). Resistant animals produce elevated levels of IL-4 and lower quantities of IFN-γ. The high IgE levels in these animals can be inhibited by administering anti-IL-4 antibody (Finkelman et al. 1988) and the eosinophilia can be blocked with anti-IL-5 antibody (Mosmann and Coffman 1989). Susceptible animals display high levels of IFN-γ and low levels of IL-4 with no significant IgE response. This suggests a type 1 like response is not an effective defence against *Nb* since the parasite persists and susceptible animals ultimately die. A type 2 like response expels worms from the gut and is therefore an effective defence against the parasite (Mosmann et al. 1991).

1.3.8 Factors that Influence Th-1 and Th-2 Bias

It is thought that Th-1 and Th-2 cells differentiate from an uncommitted Th-precursor cell under the influence of cytokines present in the environment of the responding Th-cell. The presence of IL-4 early in the response encourages the development of a type 2 response, whereas IFN-γ and/or IL-12 favours the development of a type 1 response (Seder and Paul 1994). Therefore, factors that induce the production of either IFN-γ and IL-12, or IL-4 during the initiation of an immune response will influence the phenotype of the resulting Th-cell subset.

The adjuvant, antigen, immunological status of the host, genetic background of the host and the status of the hypothalamic-pituitary-adrenal axis all influence the production of

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cytokines and hence, the nature of the Th-cell subset induced during an immune response. Cytokines secreted by the effector cells and the antigen presenting cells also influence cell recruitment to the site of inflammation and the cytokines secreted by these recruited cells.

In a primary immune response IL-12 is secreted by macrophages and cells of the innate immune system (Romagnani 1997). NK cells can secrete IFN-γ which in addition to IL-12, promotes the development of a Th-1 cell phenotype from naive T-cells.

The sources for the early production of IL-4 during an immune response are unclear. Activated mast cells and basophils can produce IL-4 (Plaut et al. 1989). A subset of CD4+ NK1.1+ cells (Yoshimoto and Paul 1994) is able to produce large amounts of IL-4 within a few hours of activation by antigens presented in association with CD1 (Yoshimoto et al. 1995). Activated naive Th-cells are also able to secrete small amounts of IL-4 and indirectly, activated APCs can produce IL-6 which induces the secretion of IL-4 and the proliferation of Th-2 cells (Rincon et al. 1997). Prostaglandin E_2 can inhibit the amplification of Th-1 cells by restricting IL-12 secretion by dendritic cells and IFN-γ secretion by T-cells, which collectively favour Th-2 cell development (Kalinski et al. 1997; Katamura et al. 1995).

Polarised Th-1 and Th-2 cells exhibit preferential expression of activation markers. Selective inhibition of Th-1 or Th-2 cell development with antibodies against B7.1 or B7.2 respectively, has been documented. The contribution of B7.1 and B7.2 to Th-cell development may be dependent on the concentration of the antigen during stimulation (Thompson 1995). CD30 is a member of the TNF receptor family and is preferentially expressed on Th-2 like cells *in vivo* and *in vitro* (D'Elios et al. 1997) whereas lymphocyte activation gene-3 (LAG-3), which is a member of the immunoglobulin superfamily, is preferentially associated with Th-1 like cells (Annunziato et al. 1996). Th-1 but not Th-2 cells are able to bind to P-selectin and E-selectin which indicates that adhesion mechanisms can influence Th-cell trafficking. As a result, Th-1 cells can efficiently enter inflamed sites in Th-1 dominated diseases but are recruited less efficiently in Th2-dominated allergic responses (Austrup et al. 1997).

APCs such as dendritic cells, macrophages and B-cells are capable of inducing Th-1 or Th-2 cell differentiation by selectively providing different accessory signals to the Th-cells (Mosmann and Sad 1996).

Furthermore, a strong adjuvant responses will bias the Th-cell subset. Alum adjuvant provokes an IgE response and a type 2 immune response whereas complete Freund's adjuvant induces IgG2a antibody production and no IgE synthesis which is characteristic of a type 1 response (Lindblad et al. 1997). In the absence of adjuvants other factors that direct the T-cell response come into play such as the strength of the TcR signalling, the route of antigen entry and its dose, which can all influence cytokine production and secretion (Carballido et al. 1997; Constant and Bottomly 1997).

1.3.9 CD8$^+$ T-Cells and Cytokines

Cytokines produced by non-CD4$^+$ T-cells have important roles for *in vivo* responses. Distinctive patterns of cytokine secretion are not confined to CD4$^+$ Th-cells and populations of CD8$^+$ T-cells and $\gamma\delta$ T-cells with type 1 and type 2 profiles have been identified *in vivo* and *in vitro* (Fong and Mosmann 1990). Activation of cytotoxic T-cells (Tc cells) during an immune response often induces a type 1 like cytokine pattern. There is now increasing evidence for the existence of type 2 like CD8$^+$ T-cells (Tc-2 cells) in humans and mice (Coyle et al. 1995; Salgame et al. 1991) Tc-2 cells are believed to have the same cytotoxicity as type 1 cytotoxic T-cells (Tc-1 cells) (Croft et al. 1994), but this is disputable (Sad et al. 1995). Tc-1 or Tc-2 cells promote the development of type 1 or type 2 immune responses respectively.

Tc-cells from mice, humans and rats can secrete high titres of a variety of cytokines in a polarised fashion (Carter and Dutton 1996). To date, little is known about the factors that influence Tc-cell polarisation but they are believed to be similar to those that influence Th-cell polarisation. IL-12 and IFN-γ encourage CD8$^+$ T-cell precursors to differentiate into Tc-1 like cells whereas IL-4 induces the generation of Tc-2 like cells. Commitment of CD8$^+$ T-cells to either the Tc-1 or Tc-2 phenotype occurs at, or shortly after priming, and neither subset can be converted to the reciprocal cytokine secretion pattern thereafter (Sad

et al. 1995). The cross regulatory effects of Th-1, Th-2, Tc-1 and Tc-2 are depicted in Fig. 1.
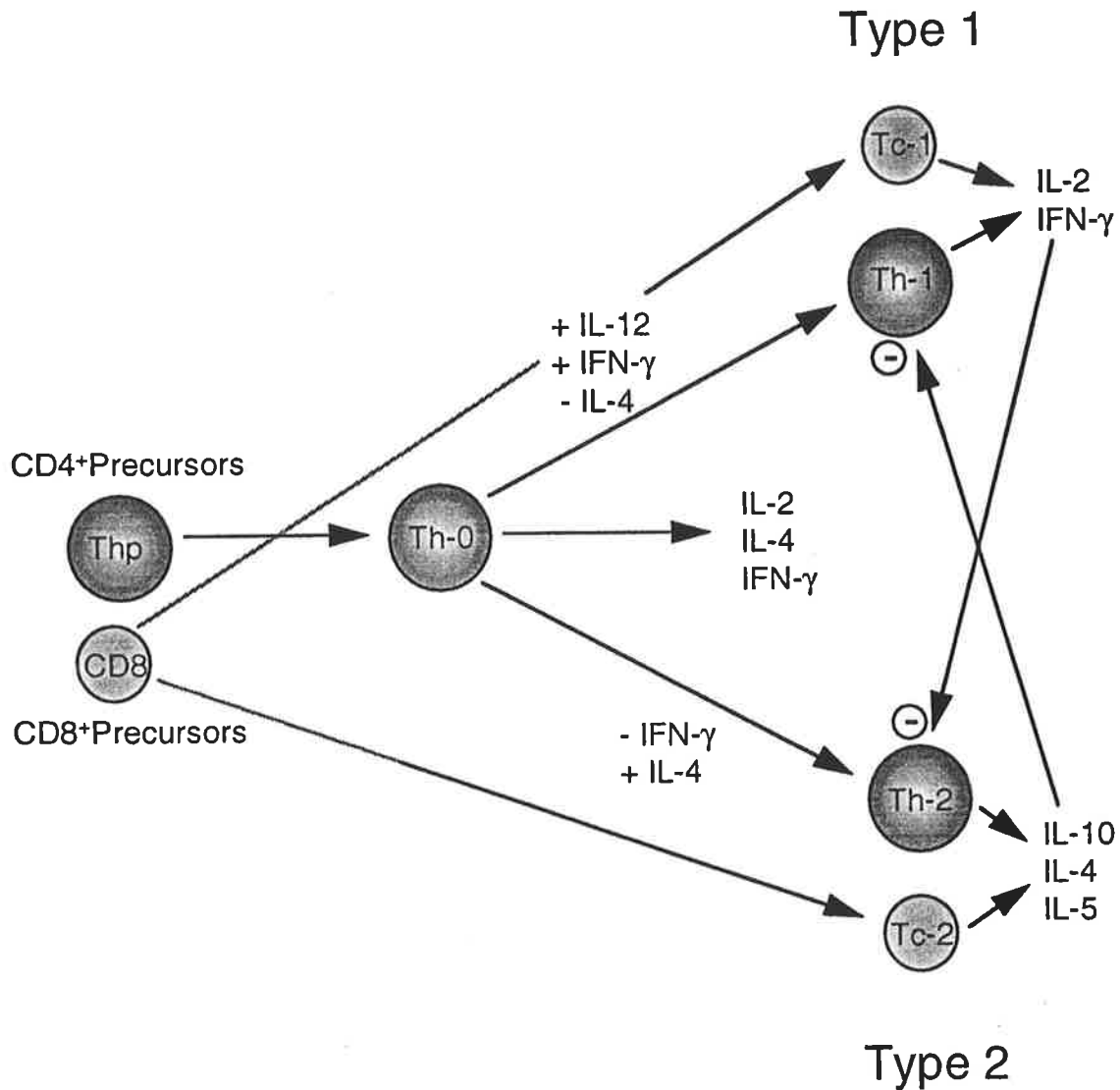
Figure 1. A schematic diagram representing some of the factors that influence CD4^{+} T-cell and CD8^{+} T-cell development and some of the cytokines that they secrete (Mosmann and Sad 1996).

The function of Tc subsets, their role in disease and mutual interactions are still being researched. Polarised Tc cells have been detected during infections in both humans and mice. Tc-1 like cells have been isolated from tuberculoid leprosy patients whereas Tc-2 like cells have been isolated from patients with lepromatous leprosy and patients infected with human immunodeficiency virus, in which there are high IgE levels (Romagnani et al. 1994; Salgame et al. 1991).

It has also been demonstrated that γδ T-cells can secrete type 1 or type 2 cytokines (Ferrick et al. 1995). γδ T-cells are able to produce cytokines rapidly upon first encounter with an antigen and may provide a suitable environment to influence type 1 and type 2 cell production (Hsieh et al. 1996; Subauste et al. 1995).

Many diseases induce a mixture of the two types of specific immune responses especially when the response is not strong or prolonged. Under such conditions IgE production would be limited because of IFN-γ expression. A DTH response may not fully develop because of suppression of a type 1 response by the products of a type 2 response. Both responses can activate B-cells which then become responsive to cytokines produced by either cell subtype. Therefore, antibody responses would be strongly supported by a mixture of type 1 and type 2 responses. The isotype pattern of antibody production depends on the type of immune response, type 1 favouring IgG2a production, and type 2 generating an IgG1 response. This is characteristic of many reported diseases in which variable IgG isotype production is seen without significant IgE or DTH reaction (Stevens et al. 1988). The immune response to an infectious agent may become further complicated if type 1 or type 2 responses occur simultaneously in different anatomical locations or they change at different stages throughout the disease process, as has been implied in experimental allergic encephalomyelitis (EAE) (Issazadeh et al. 1995a) and some animal models of RA (Boissier et al. 1995; Jacob et al. 1989).

1.4 Rheumatoid Arthritis

1.4.1 Introduction

In 1859 the term rheumatoid arthritis (RA) was first introduced by Garrod to describe inflammatory polyarthritis and polyarticular osteoarthritis (Klippel and Dieppe 1994). RA is a common severe inflammatory disorder affecting men and women of all ages. It is of multifactorial origin, encompassing a genetic predisposition characterised by immune driven chronic inflammation. Women contract RA more frequently than men in all populations around the world and in all age groups studied. The mortality associated with

RA has been attributed directly to articular and extra-articular complications and the drugs used in its treatment. The disease is marked by a variable course involving exacerbations and remission of disease activity. Many cases are chronic and progressive and result in severe disability and sometimes premature death.

Diagnosis of RA is dependent on clinical judgement and relies on symptoms and signs that may be transitory or fluctuating since there is no single pathognomonic manifestation or reference test to diagnose the disease. Laboratory abnormalities that accompany systemic RA include elevated erythrocyte sedimentation rate (ESR), rheumatoid factor titre and C-reactive protein levels. These factors are all measures of systemic inflammation and represent early markers of disease activity. Other laboratory abnormalities which include hypergammaglobulinemia, anaemia, occasional hypocomplementemia, thrombocytosis and eosinophilia, occur in patients with more severe RA.

Acute phase reactants are synthesised by the liver in response to inflammation. They include the coagulation protein fibrinogen, complement proteins C3 and C4 and miscellaneous proteins such as transferrin, C-reactive protein, fibronectin and serum amyloid A protein. These proteins are all induced non-specifically in response to inflammation or tissue necrosis.

Most RA patients experience some extra-articular manifestations. The more common systemic features of RA include fatigue, development of rheumatoid nodules, dermal vasculitis, diffuse interstitial pulmonary fibrosis, pericarditis and keratoconjunctivitis sicca (Klippel and Dieppe 1994). Myelopathies, entrapment and ischaemic neuropathies can occur due to postural deformities and compressive soft tissue enlargement. A hypochromic microcytic anaemia develops in active RA. As with other forms of chronic inflammation, impaired iron utilisation in RA results in anaemia which correlates with the activity of the underlying disease (Smith 1977). In addition, gastrointestinal and renal complications can result from drug therapy. RA can be considered a systemic disease that effects the muscles, skin, lungs, heart, eyes, nerves and blood respectively. Thrombocytosis is a frequent finding in active RA that correlates with the number of joints involved with active synovitis, and may be associated with extra-articular features (Hernandez et al. 1975).

Despite systemic involvement, the central feature of RA is a chronic widespread synovitis. No antigens specific to synovial tissue have been defined as primarily arthritogenic despite detailed investigations. No synovium-specific adhesion molecules which might encourage a leucocyte influx have been identified although homing of cells to the synovial tissue results in accumulation of T-cells and macrophages (Kingsley and Panayi 1997).

Rheumatoid factors (Rf) found in the serum of patients suspected of having a clinical diagnosis of RA do not necessarily indicate the presence of RA but may indicate an increased risk to development of RA (del et al. 1988).

Considerable progress has been made in unravelling some of the genetic factors involved in RA, in particular the strong association between certain HLA-DR4 alleles and RA (Wordsworth et al. 1989). While non-genetic factors are believed to provide the initial arthritogenic stimulus, genetic factors may be important in determining the ultimate expression of the disease i.e., self-limiting, chronic, erosive or associated with extra-articular features (Wordsworth 1992).

1.4.2 Rheumatoid Arthritis: Cells and Cytokines

The microscopic anatomy of normal synovial tissue is variable and depends on the nature of the connective tissue on which it lies. The cell lining layer or intima consists of 20%-30% macrophages which have characteristics of tissue macrophages. Their number increases greatly when the synovial tissue is inflamed (Cutolo et al. 1993). Many dendritic cells are present in the synovium of RA patients whereas only few of these cells are present in normal synovial tissue (Thomas and Lipsky 1996). The interdigitating dendritic cells in inflamed synovium display surface MHC class I and are capable of presenting antigen.

The fibroblast-like cells from normal synovium are responsible for glycosoaminoglycan production. In inflamed tissue fibroblasts produce high levels of metalloproteinases at the intimal layer and at the cartilage-pannus junction which results in cartilage destruction (Hauselmann 1997). Polymorphonuclear leucocytes (PMNL) have been implicated in the

pathogenesis of chronic inflammatory diseases such as RA (Brown 1988), inflammatory bowel disease (Kirk et al. 1983), and asthma (Kato et al. 1991).

High endothelial venule formation (HEV) in the synovium of RA patients is associated with enhanced lymphocyte trafficking to the synovium (Cavender et al. 1987) resulting in a lymphocytic infiltration. T-cells are predominate in the chronically inflamed rheumatoid synovial membrane (Van and Paget 1975) and are seen predominantly in the perivascular region of the synovium at an early stage in the disease. Synovial and peripheral blood T-cells from patients with RA are functionally deficient. They display depressed *in vivo* responses to recall antigens (Emery et al. 1984), poor proliferative responses to mitogens (Lockshin et al. 1975; Seitz et al. 1982), poor helper functions (van et al. 1995) and reduced autologous mixed lymphocyte responses (Salmon and Bacon 1988; Smith and DeHoratius 1982). Nevertheless, a substantial improvement in the symptoms of RA may be observed following therapies that reduce the numbers and formation of T-cells. These treatments include thoracic duct drainage, total lymphoid irradiation, Cyclosporin A treatment and treatment with anti-CD4^{+} monoclonal antibodies.

The number of T-helper lymphocytes in RA synovial tissue has been found to consistently to be 4 to 10 fold higher than the suppressor/cytotoxic subset (Duke et al. 1982; Meijer et al. 1982; Nakao et al. 1990) and conflicting findings on the cytokines produced by CD4^{+} T-helper cells in the synovium have been reported. Type 2 cytokines have been found in the synovial fluid of rheumatoid patients and T-cells cloned from RA synovial biopsies (Aljanadi et al. 1996; Cohen et al. 1995; Quayle et al. 1993). However, studies on T-cell cytokine gene expression in the RA synovium have consistently reported the presence of IFN-γ and IL-2 (Dolhain et al. 1996; Miltenburg et al. 1992) suggesting that Th-1 cells are more abundant than Th-2 cells in the joints of RA patients.

Messenger RNA and protein for many pro-inflammatory cytokines such as TNF, IL-1, IL-6, GM-CSF and chemokines such as IL-8 are found in rheumatoid synovial tissue regardless of the patients therapy (Feldmann et al. 1996a). This may be balanced partly by the production of IL-10 and TGF-β which are anti-inflammatory cytokines, IL-1 receptor antagonist and soluble TNF receptor which act as cytokine inhibitors. While there is an

increase in the production of anti-inflammatory cytokines such as IL-10 and TGF-β (Canete et al. 1997; Cohen et al. 1995; Ridderstad et al. 1991), their increased expression in the RA synovium is probably not sufficient to influence regulatory mechanisms since these cytokines and soluble receptors are unable to neutralise the over production of inflammatory cytokines. The major pathology of RA occurs in the synovial tissue whereas the synovial fluid is believed to represent a "sink" containing a mixture of molecules including hyaluronan, other proteoglycans, degradative enzymes and proteases that inhibit the functions of cytokines and promote their degradation. Therefore, cytokine expression in the synovial tissue is more likely to be indicative of RA pathogenesis because it is the principle site of immune and inflammatory activity.

IL-1 and TNF have been detected in the synovial fluid cells and synovial fluid cell culture supernatants from RA patients (Hopkins et al. 1988; Hopkins and Meager 1988) in association with synovial fluid macrophages (Firestein et al. 1990). IL-1 and TNF are also detectable in synovial membrane culture supernatants indicating they are present in quantities large enough to be of biological relevance. Other inflammatory cytokines found in RA synovium or synovial fluid include IL-6 (Hirano et al. 1988), IFN-α (Hopkins and Meager 1988), granulocyte/macrophage colony stimulating factor (GM-CSF) (Williamson et al. 1988), mast cell growth factor (MCGF) (Firestein et al. 1988) and leukemia inhibiting factor (LIF) (Lotz et al. 1992). Platelet derived growth factor, fibroblast derived growth factor-β (Remmers et al. 1991), basic fibroblast growth factor (bFGF) and TGF-β (Goddard et al. 1992) have also been detected. These growth factors may contribute to the hyperplasia of rheumatoid synovial fibroblasts.

1.4.3 Animal Models of Polyarthritis

The early pathogenesis of RA is difficult to study because patients often present with the symptoms of established disease so events which occur before the clinical diagnosis of RA cannot be studied. Inbred laboratory animals typically have well defined genetics and their environment can be closely controlled in terms of diet and exposure to environmental agents. In this regard, they present an advantage for reductionist studies of the pathogenesis of arthritis relative to outbred, free living human subjects. Although there is

no single outstanding animal model of RA there are several commonly used animal models that mimic some of the clinical joint abnormalities, systemic and serological features characteristic of RA. Several micro-organisms are capable of inducing reactive or infectious arthritis in animals. Bacterial cell wall fragments have been reported to induce a chronic polyarthritis (called SCW arthritis) in susceptible strains of rats (Wahl et al. 1991). Inoculation of rats or mice with type II collagen (CII) emulsified in adjuvant results in collagen induced polyarthritis (CIA) (Trentham et al. 1980). CIA in rodents produces a widespread synovitis and may also produce tenosynovitis in the hind foot. Adjuvant arthritis (AA) is another useful animal model for studying the pathogenesis of polyarthritis. AA is a model of acute polyarthritis that can be induced in susceptible rats by the intradermal injection of an emulsion of *Mycobacterium Tuberculosis* (*Mtb*) and Freund's incomplete adjuvant (Pearson 1956). AA is characterised by a destructive arthritis that involves an acute inflammatory phase which to some extent resolves spontaneously. There are several features of AA that have made it a widely studied experimental model of RA.

1.4.4 Adjuvant Arthritis

The systemic nature of AA has been demonstrated to produce some extra-articular manifestations parallel to those observed in RA patients. Rats develop inflammatory nodules e.g., along their tail, not unlike the rheumatoid nodules displayed by some RA patients. C-reactive protein and ESR levels increase prior to clinical signs of inflammation (Liu et al. 1996; Otterness et al. 1991), a moderate anaemia develops after inoculation with the arthritogen (Kweifio-Okai et al. 1994) and defects in the nervous system have been shown to develop in parallel with the polyarthritis (Carleson et al. 1996; Levine et al. 1985).

Despite the similarities AA has with RA, aspects of its induction and pathogenesis are not shared fully with the human disease. In order to achieve a "model" in which polyarthritis can be reliably induced, conditions of adjuvant composition and administration and rat strains have been refined. The killed bacterial component of the adjuvant is not essential since polyarthritis can be induced with Freund's incomplete adjuvant in some rat strains (Lorentzen and Klareskog 1996). Standard conditions of induction of AA yield a disease

which typically has an acute onset followed by a less acute continuing arthritis and irreversible joint damage. AA does not occur spontaneously and needs to be induced with an adjuvant. Like other experimental models of RA, such as CIA and SCW induced arthritis, AA is an acute disease with a chronic progression that displays synovitis, periostitis, pannus formation and erosions. It is an immunologically driven disease in which the aetiological factors are only partially understood, as in RA. The benefit of AA as a model for RA, is that it can be induced under controlled conditions in genetically susceptible rats and immune parameters such as the joint histopathology and blood and liver protein changes resemble those observed in RA. AA has a reliable time course of onset, acute phase and resolution. It can therefore be used to elucidate the mechanisms involved in the induction, treatment and resolution of polyarthritis.

AA is induced in susceptible strains of rats by injection of *Mtb* suspended in an oil adjuvant (complete Freund's adjuvant) intradermally at the base of the tail. The intense cellular reaction at the injection site may be a major factor producing non-specific inflammation and recruitment of mononuclear phagocytes (Audibert and Lise 1993). Studies with complete Freund's adjuvant (CFA) have demonstrated that while intradermal injection of adjuvant was important, the site of injection, volume injected and concentration of *Mtb* in the mixture influenced the frequency with which polyarthritis was induced (Ward and Jones 1962). It has been demonstrated that inflammation at the site of adjuvant deposition is unimportant in AA because removal of the intradermal deposit of CFA by tail amputation prevents disseminated arthritis only if accomplished within 2 hours of inoculation (Ward and Jones 1962). The mechanisms of induction and pathogenesis of AA remain unknown but it has been established that adjuvants spread rapidly to regional lymph nodes where they are disaggregated and phagocytosed (Freund 1951) and the polyarthritis is dependent upon immunological events within regional lymph nodes (Newbould 1964a).

Juvenile rats between 21 and 90 days old are used for studying adjuvant arthritis, because the disease cannot be induced reliably in younger or older rats, nor is it inducible in hamster, mouse, rabbit, gerbil, guinea-pig, dog or man. Injections of CFA into the tail base

of rats produces a non-specific inflammatory swelling at that site. Importantly, an immunologically induced swelling becomes evident in the hind and front limbs of the rats 9-12 days after CFA inoculation. Maximal swelling occurs in the front and hind limbs after approximately 16 days whereas 30-40 days after CFA inoculation the severe inflammation begins to decline as indicated by a return towards normal of plasma proteins such as albumin and fibrinogen (Geiger et al. 1992). Permanent joint deformity and ankylosis in the hind paws occur as a result of bone erosion and periosteal inflammation (Carlson et al. 1985).

Rats with adjuvant disease develop irregularity distributed, focal, infiltrative lesions in certain connective tissues, including effected joints and tendons, the skin (Kuberasampath and Bose 1981) and the ciliary body of the eye (Petty et al. 1989). There is a proliferative connective tissue response, formation of lymphocyte aggregates, bone destruction and periosteal new bone formation. Deposition of fibrinoid material has been reported in vascular walls and extravascular tissue spaces in many lesions, especially those containing polymorphonuclear cells (Burnstein and Waksman 1964).

1.4.5 Lymphatics of the Rat Hind Limbs

The rat demonstrates an extensive network of lymphatic vessels that drain the tail and hind limbs (Tilney 1971). Lymphatic vessels draining the tail region of the rat transverse the lateral scrotal wall and the groin to the inguinal lymph nodes. The inguinal nodes also drain the gluteal regions, thighs and lower abdomen. The caudal node drains the hamstring, gluteal and perineal areas whereas the rostal node drains the anterior thighs and flanks through several small lymph channels. The single popliteal lymph node lies in the lateral aspect of the popliteal space and drains the footpad, foot and hind leg through lymph vessels running with the greater and lesser saphenous veins. The popliteal lymph node serves as a secondary drainage site for the tail.

Inflammation in adjuvant disease is initiated in the synovial and peri-articular connective tissue then spreads quickly to the skeletal muscle and periosteum as reported by Jones and Ward (Jones and Ward 1963). Removal of lymph nodes draining the site of inoculation

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within 5 days of adjuvant administration prevents the development of secondary lesions, however, lymph-adenectomy after the 7th day failed to prevent the disease (Newbould 1964b). This suggests that if adjuvant dissemination was necessary for initiation of the inflammatory process, it occurs early after inoculation.

Lymphocytes are first seen in the synovium in a peri-vascular location and then migrate further into the synovial tissue. Beneath the surface of the synovium large venules are found in association with the arterioles and lymphatics. Capillaries are found in close proximity to synovial intimal cells and may lie within the intima itself. Various adhesion molecules are differentially expressed by the small vessels beneath the intima which may influence the pattern of myeloid and lymphoid cellular infiltrates seen in inflamed tissue (Halloran et al. 1996).

1.4.6 Adjuvant Arthritis: Cells and Cytokines

A common finding in animal and human arthritis is a predominance of mononuclear cells in the synovium and polymorphs in the synovial fluid. Polymorphs appear later than lymphocytes in AA and accumulate within and engorge blood vessels before gaining access to the tissues (Gryfe et al. 1971). Mast cells are the first to appear in abnormal numbers in the inflamed synovial tissues of rats with adjuvant arthritis and they are believed to contribute to the initial part of the phasic inflammatory reaction in adjuvant disease (Gryfe et al. 1971; Sheldon and Bauer 1960).

Healthy rat knees have been reported to have only a limited number of CD8+ cytotoxic cells, B-cells (LCA+) and IL-2 R+ cells (CD25+) and a relatively large number of CD4+ and MHC class II cells (Ia+) which are generally characteristic of a delayed type hypersensitivity reaction. Within 7 days of AA induction there was an increase in the number of blood vessels and an elevated number of cells bearing the IL-2 receptor. 14 days after the induction of AA the number of mononuclear cells appearing in the subintimal area increased (Pelegri et al. 1995a). The phenotype of lymphocytes in the synovial tissue of AA rats is currently in dispute. T-cells, in particular CD4+ T-helper cells, are believed to be the predominate infiltrating lymphocyte into arthritic joints (DeJoy et al. 1990;

Issekutz and Issekutz 1991b; Larsson et al. 1985), however, an increase in CD8$^+$ cells in the synovium of AA rats has also been reported. It has also been speculated that there are elevated numbers of B-cells and CD4$^+$ macrophages in the synovium of AA rats at the peak of inflammation (Pelegri et al. 1995a).

There is strong evidence that adjuvant arthritis is an immunological disease manifest through a type of cellular immune response. This has been demonstrated by the transfer of adjuvant disease to naive recipients with *Mtb* specific cell lines and clones (Holoshitz et al. 1984; Holoshitz et al. 1983; van et al. 1985). Furthermore, transfer of disease from adjuvant donors to syngeneic normal recipients using concanavalin A stimulated lymph node cells or spleen cells has been accomplished. This results in a severe, persistent, erosive synovitis in the syngeneic recipient that clinically, radiographically and histologically resembles adjuvant arthritis (Taurog et al. 1983a; Taurog et al. 1983b). Thoracic duct cells from AA rats are also able to transfer the disease to normal syngeneic recipients without *in vitro* stimulation (Spargo et al. 1996; Whitehouse et al. 1969). In particular, the activated CD4$^+$ TD T-cells are responsible for transferring the disease (Spargo et al. 1998).

AA can be successfully treated with anti-pan T-cell monoclonal antibodies (mAb) (Larsson et al. 1985), anti-CD4 monoclonal antibody (Billingham et al. 1990) and monoclonal antibody to the rat $\alpha\beta$ TcR (Yoshino et al. 1990). These findings emphasise the regulatory role of T-cells, in particular CD4$^+$ T-cells, in the development of AA. Administration of anti-rat CD8 does not mitigate AA (Pelegri et al. 1995b) therefore, CD8$^+$ T-cells are thought not to play a major role in pathogenesis of the disease.

AA can be treated successfully with antibodies that selectively decrease the number of T-cells and is it unable to be induced in congenitally athymic rnu/rnu rats (Kohashi et al. 1982). However, when treated with agents or therapies that non-specifically reduce the number of T-cells, such as neonatal thymectomy, low dose cyclophosphamide or low dose hydrocortisone, a more severe arthritis develops. These therapies presumably reduce the number and formation of suppressor T-cells (Kayashima et al. 1976; Kayashima et al. 1978) or allow the preferential expansion of autoreactive T-cell clones in the absence of

immunomodulatory cells. Cyclosporin A (CsA) is a powerful immunosuppressive drug that therapeutically benefits RA patients (Dougados et al. 1988) by selectively inhibiting the transcription of IL-2 mRNA and other T-cell cytokines (Russell et al. 1992). It has been used successfully in the treatment of AA and other animal models of RA and is now used widely for more severe forms of RA in spite of certain troublesome side effects (del Pozo et al. 1990; Theisen Popp and Muller Peddinghaus 1994).

The aetiology of AA remains unknown and little is known about the lymphoid cells and cytokines involved in the events that regulate its development. The role of cytokines contributing to the pathogenesis of arthritis in animal models has focused principally on their contribution to cartilage and bone destruction, leucocyte chemotaxis and extravasation into the synovial tissue. In various animal models the destructive phase of arthritis can be modulated by neutralising pro-inflammatory cytokines such as IL-1 and TNF and administering immunomodulatory cytokines such as IL-13 and TGF-β (Bessis et al. 1996; Brandes et al. 1991a; Henderson 1995; Staines and Wooley 1994). Knockout mice in which the gene of interest is deleted from the genome, or transgenic animals that have upregulated expression of a cytokine gene or a receptor, have also been used to elucidate the effects that cytokines have on the outcome of experimental models of RA. It has been demonstrated that arthritis develops spontaneously in mice expressing the human TNF transgene (Butler et al. 1997) whereas mice transgenic for the TGF-β receptor display a joint disease similar to that seen in osteoarthritis (Serra et al. 1997).

A basic understanding of the cytokines produced during a disease process may be a prerequisite for modulating the disease. There is strong evidence that AA is initiated and perpetuated by Th-cells although little work has been done to elucidate the type of cellular response involved in the pathogenesis of the disease. There is limited information about cytokine production in the synovium of AA rats since most information is obtained from the lymph nodes draining the limbs. This is in contrast to RA in which the cytokines produced in the synovium are well documented and there is limited information about cytokine production in the lymph nodes draining inflamed joints. The predominant phenotype of the Th-cell subset involved in the pathogenesis of RA is thought to be Th-1

(Dolhain et al. 1996; Miltenburg et al. 1992) although several groups have reported the presence of Th-2 like cytokines (Aljanadi et al. 1996; Quayle et al. 1993; Schlaak et al. 1996).

The aims of this study are to firstly characterise the key components of the cytokine network associated with AA. Particular attention will be paid to the T-cell derived cytokines involved in the early stages and during the development of AA, by analysis of cytokine mRNA in the synovial tissue and lymph nodes draining the hind feet of female DA rats. It is hypothesised that T-cell cytokine mRNA will be present in the synovial compartment and in the inguinal and popliteal lymph nodes of rats with AA. An attempt will be made to define the Th-cell subset responsible for the pathogenesis of AA. An investigation into the patterns of cytokines expressed at different stages during the evolution of polyarthritis should provide important insights into the pathogenesis of inflammatory polyarthritis. Sequential changes in levels of expression of a putative immunoregulatory and a pro-inflammatory cytokine will also be studied as AA develops and progresses.

Messenger RNA for expression of the T-cell cytokines, IFN-γ, IL-2 and IL-4 within the synovial compartment and lymph nodes draining the hind feet of the rats will be assessed. Changes in the gene transcription levels of the immunomodulatory cytokine, TGF-β, and the pro-inflammatory cytokine, TNF, will be monitored in these tissues. IFN-γ and TNF cytokine mRNA levels will be correlated with cytokine protein levels by short term culture of stimulated lymphoid cells.

1.4.7 Lymphocyte Recirculation

1.4.7.1 The Lymphoid System

The lymphoid system is a collection of specialised vessels and organs through which lymphocytes and extravascular fluid travel. The principal components are afferent lymph which moves from the extravascular space in tissues to lymph nodes. Dendritic cells which capture antigen in tissues travel through afferent lymph to the interfollicular areas of lymph nodes. During transit they mature and develop the capacity to process and present

antigen efficiently to lymphocytes. The presentation of antigen by dendritic cells to lymphocytes is a key event in primary immune responses. Lymphocytes with a memory or activated phenotype are also found in afferent lymph (Mackay et al. 1990). The efferent lymphatics originate from the medulla of lymph nodes and join sequentially to form larger vessels with ultimate convergence on the thoracic duct (in the case of lymph from below the diaphragm). Cells from lymph nodes pass into the thoracic duct from which they are delivered into the venous blood. Migrating lymphocytes pass through the thoracic duct into the blood from which they exit into the tissues. The percolation of resting, memory and effector cells through the tissues of the body is a central feature of immunosurveillance against previously encountered invasive organisms.

Thoracic duct lymph contains approximately 85% T-cells as well as cells from the monocyte/macrophage lineage and depending on its origin, red blood cells. Naive T-cells enter lymph nodes directly from the blood through specialised post capillary venules known as high endothelial venules (HEVs). The thymus and spleen are specialised lymphoid organs which lymphocytes and their precursors access directly from the blood. PMNL and mononuclear leucocytes emigrate from the blood stream to sites of injury, infection or immunological reaction. A foreign antigen from the site of infection is transported into lymphoid tissues via the lymph or blood either as cell associated (eg. with dendritic cells) or as non-cell associated material. In the lymph node the antigen is presented in a micro-environment that encourages the proliferation and differentiation of naive antigen specific B and T-cells. In the presence of appropriately presented antigen these lymphocytes undergo transformation to form blasts.

Patterns of lymphocyte recirculation are complex and appear to depend on the tissue of origin and state of activation of lymphocytes. While it has been estimated that the bulk lymphocyte pool may circulate two to three times per day, the rate of recirculation is far from uniform (Freitas et al. 1980; Picker and Butcher 1992; Rowley et al. 1972). Following stimulation in the lymph nodes, blasts enter the thoracic duct lymph before they finally enter the blood and recirculate to inflamed tissues. T-cells generated in the intestine associated lymphoid tissues are preferentially recruited to intestinal mucosal sites while T-

lymphoblasts generated in peripheral lymph nodes are recruited to intestinal and mucosal sites but less to normal intestinal mucosa (Rose et al. 1976a; Rose et al. 1978; Rose et al. 1976b). Small lymphocytes are also present in the thoracic duct lymph but appear to circulate around the body randomly (Freitas et al. 1980).

1.4.7.2 Factors Influencing Recruitment of T-Cells to Inflamed Synovium

Recruitment of blood lymphocytes into inflammatory sites requires a well co-ordinated and dynamic sequence of events involving cell adhesion molecules and chemotactic cytokines. Adhesiveness of the endothelium is under the control of cytokines such as IL-1, TNF and IFN-γ which upregulate surface expression of E-selectin, ICAM-1 and VCAM-1 (Bevilacqua 1993; Hogg and Landis 1993; Springer 1994). T-cells found within the inflamed synovial membrane and the synovial fluid have surface markers characteristic of memory or activated cells including HLA-DR, VLA-1, VLA-4 and CD69 (Burmester et al. 1987; Hemler et al. 1986; Laffon et al. 1991). Adhesion molecules such as ICAM-1, VLA-4, VLA-5, CD44 and LFA-1 are upregulated on T-cells found in synovitis in AA rats (Halloran et al. 1996; Ishikawa et al. 1993; Johnson et al. 1993). These adhesion molecules interact with cognate adhesion molecules expressed on activated endothelium and probably mediate the preferential migration of activated cells into inflamed tissue (Laffon et al. 1991; Postigo et al. 1992). In the inflamed synovial membrane, T-cells may become activated upon interaction with extracellular matrix proteins and APCs (Hynes 1992; Shimizu et al. 1992).

1.4.7.3 Benefits of Thoracic Duct Drainage

Activated T-cells have been implicated in the pathogenesis of RA through the beneficial effects that chronic lymphocyte depletion has on disease severity. T-cell depletion can be achieved by prolonged thoracic duct drainage which depletes cells of the lymphoid system (Edgren et al. 1976; Isaacs et al. 1992; Paulus et al. 1979; Reiter et al. 1991; Wegelius et al. 1970). or by administering anti-T cell antibodies which have also had therapeutic effects in RA patients (Herzog et al. 1989; Perosa et al. 1997). The thoracic duct (TD) is the final common drainage pathway for recirculating lymph that originates from below the

diaphragm. The majority of this lymph drains from the intestine, however, it can be complemented from other sources during injury, infection or immunological activity in the tissues.

During active arthritis there is a rapid and continued recruitment of T-cells and PMNL from the blood into the joint (Issekutz and Issekutz 1991a; Issekutz and Issekutz 1991b). However, the factors involved in the initiation and perpetuation of cell migration are not well understood. TD lymphoblasts in s-phase of the cell cycle are recruited by the synovial tissue of rats with AA to a far greater extent than to the synovial tissue of normal rats (Spargo et al. 1996). Importantly, in contrast to adoptive transfer models of arthritis using cells from solid lymphoid organs (eg. lymph nodes and spleen), with TD cell transfers there is no requirement for deliberate stimulation of the cells with concanavalin A *in vitro*. This has allowed sub-populations of TD cells to be analysed in the search for a population which is responsible for disease transfer. A fraction of TD cells expressing CD4 and cell surface activation markers has been shown to be responsible for the adoptive cell transfer of the disease into naive syngeneic recipients (Spargo et al. 1998).

In an attempt to further elucidate the network of cytokines produced during the initiation AA, the cytokine mRNA pattern of TD cells from arthritic rats was investigated. Furthermore, the pattern of cytokines produced by the fraction of TD cells bearing CD4 and cell surface activation markers was analysed along with the cytokine pattern of those cells lacking the activation markers.

1.5 The Polymerase Chain Reaction and Capillary Electrophoresis

1.5.1 Introduction

Elucidation of the structure of DNA by Watson and Crick (Watson and Crick 1953) was the first step in the era of molecular genetics that has yielded many laboratory techniques for the detailed analysis of nucleic acids. These include restriction endonuclease digestion of DNA, nucleotide sequencing, hybridisation of nucleic acids and the polymerase chain

reaction (PCR). Conventionally, the presence or levels of expression of gene sequences of interest have been analysed by procedures such as Southern and Northern blots, dot blots and in situ hybridisation. However, relatively large amounts of nucleic acids are required for such techniques. PCR can generate millions of DNA copies from a single target sequence contained within a complex mixture of nucleic acids and only a small quantity of nucleic acid is required for analysis.

The sensitivity of the PCR has led to its deployment in a variety of DNA and RNA related techniques. The highly sensitive nature of PCR requires careful laboratory procedures to minimise the risk of PCR contamination (Kwok and Higuchi 1989). Multiple negative controls are therefore necessary to monitor for contaminations which can arise from previous PCR products, exogenous DNA and other cellular materials.

Advances in experimental molecular biology have changed the field of experimental medicine leading to a fresh understanding of the genetic, infectious and neoplastic diseases. PCR has numerous applications in forensic science, pathology, microbiology, haematology and immunology. It is used for the clinical diagnosis of infectious disease, genetic diseases and identifying and testing for cancer. The technique has been widely applied in immunology to define HLA polymorphisms, T-cell receptor and immunoglobulin diversity, pathogen detection, lymphoma and leukemia detection and quantification of gene expression.

Studies of gene expression have been limited because of the lack of sensitivity of standard hybridisation techniques. Several groups have made use of the PCR for the study of gene expression. PCR provides a method for monitoring gene expression through mRNA synthesis. This PCR application has been called RNA-PCR, RT-PCR, RNA phenotyping and message amplification phenotyping (MAPPing). Total RNA is firstly isolated from tissues or cells and used as a template for reverse transcription to complementary DNA (cDNA). RT-PCR has become a popular method for analysing gene transcripts because it is 1000-10,000 fold more sensitive than conventional RNA blotting techniques (Wang et al. 1989).

1.5.2 PCR Theory

RNA isolated from tissues or cells is used as a template for reverse transcription to complementary DNA (cDNA). cDNA serves as the template for PCR amplification using primers that are designed to amplify a specific region of cDNA within a heterogeneous sample of cDNA. The exponential accumulation of PCR products means that picogram amounts of target are sufficient to achieve amplification of a sequence with a specific set of appropriately designed primers. Oligonucleotide primers are therefore an important technical consideration for successful PCR amplification of cDNA. The specificity of the amplification reaction can be controlled by varying the temperature at which the oligonucleotide primers anneal to the native template. Lower binding temperatures will favour non-specific primer binding resulting in undesired products. The concentration of magnesium in the buffers and the concentration of the polymerase enzyme (Saiki et al. 1988) will also influence the interaction of the DNA with the oligonucleotides. There are no rules for the choice of primers except that they be usually 18-25 base pairs long and designed such that sequence motifs which encourage hydrogen bonding at the 3' end of the primers should be avoided in order to limit primer dimer formation. No single PCR protocol is applicable to all situations, consequently each new PCR requires consideration of the cycling parameters, primer design and the magnesium concentration for successful amplification of a specific cDNA.

The PCR involves repeated thermal cycling between a denaturation, primer binding and extension temperatures. The later must allow sufficient time for the activity of Taq polymerase to extend DNA sequences complementary to the respective cDNA fragments bound by the oligonucleotide primers. Since the primers each engage a complementary strand of DNA, the extension of one primer amplifies the DNA engaged by the other. These complementary replicated strands synthesised by Taq polymerase serve as further primer binding sites and templates for subsequent primer extension reactions. Repeated cycles of primer extension, denaturisation and annealing sequentially double the amount of DNA product at each cycle resulting in the exponential accumulation of the DNA fragment

whose termini are defined by the 5' ends of each primer. The process of RT-PCR is represented pictorially Fig. 2.
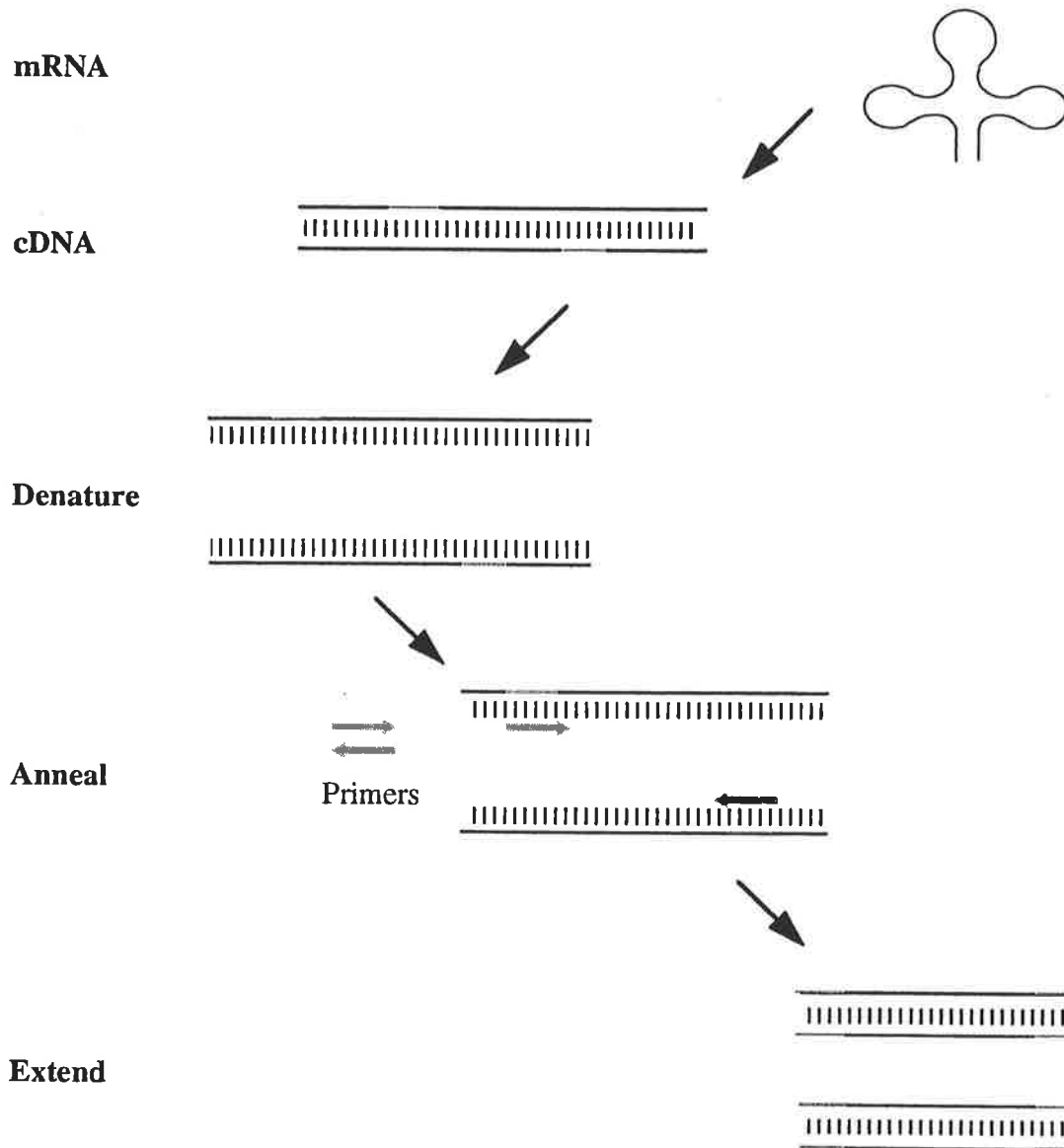
Figure 2. A schematic diagram representing the process of RT-PCR in which the extracted RNA is converted to cDNA which serves as a template for primers to bind and replicate the DNA in the presence of Taq polymerase.

The exponential accumulation of the PCR amplified cDNA depends on the number of amplification cycles and the overall efficiency of the reaction. 20-40 cycles of amplification are performed during a typical PCR. The exponential accumulation of the target DNA over this period can be described mathematically by equation 1.

$$T_j = T_0 (1+E_t)^j \qquad (1)$$

The initial amount of DNA (T_0) is amplified with an efficiency (E_t) over (j) amplification cycles to produce a final amount of product (T_j). In practice the efficiency of the PCR reaction is not constant throughout cycling and the actual amount of product T_j may vary significantly from the theoretical yield. Several parameters influence the reaction efficiency including the quantity of template, the concentration of nucleotides and primers in the reaction, the composition of the buffer, the enzymic activity of the DNA polymerase and the thermocycling parameters. A constant efficiency can be maintained up to an intermediate number of amplification cycles after which the rate of accumulation of the amplified product becomes limited. The levelling of product accumulation in the latter cycles of amplification is referred to as the plateau effect (Pannetier et al. 1993). Several factors influence the onset of the plateau effect, the most important of which is believed to be the activity of the DNA polymerase and the quantity of amplified DNA (Morrison and Gannon 1994).

Although it has been possible to detect and amplify large amounts of rare mRNA transcripts using PCR, it has proven more difficult to quantify small amounts of mRNA extracted from samples. The main constraint in obtaining quantitative data from the PCR is inherent in the amplification process. Initially, amplification is an exponential process and small differences in any of the variables that control the rate of the reaction will dramatically effect the yield of the products. Variables that influence the rate of the reaction include the concentration of polymerase, dNTPs, magnesium, DNA, oligonucleotide primers, annealing, extension and denaturation temperatures, cycle length and cycle number, rate of primer-dimer formation and presence of contaminating cDNA. Even when these parameters are precisely controlled there are often very slight differences between PCR tubes that preclude accurate quantification (Linz 1990). For these reasons PCR has been widely used only as a qualitative technique.

1.5.3 Quantitative PCR

Conventional PCR is not quantitative because the initial amount of the target sequence may not bear a close relationship to the abundance of the target sequence after amplification due to the exponential accumulation of the product. A plateau is reached at higher cycle numbers (Gilliland et al. 1990) and the kinetics of each amplification reaction depend not only on the initial amount of target DNA in each reaction but also the length of the products, the priming of the sequences, potential inhibitors in the sample and variations in the concentrations of the reaction components.

Since the introduction of the PCR, many different techniques have been devised to make it quantifiable. While this has proven difficult due to the exponential nature of the reaction, several novel techniques have been reported. The target cDNA can be quantified relative to a constitutively expressed gene (Noonan and Robinson 1991) or to a known amount of the target cDNA that is amplified in a separate reaction (Ballagi Pordany et al. 1991; Melby et al. 1993). These methods may not compensate for the exponential nature of the amplification process, where tube to tube variations can result in differential accumulation of the PCR products.

A popular quantitative method involves measuring the quantity of the target gene in addition to a constitutively expressed gene. The constitutively expressed gene is usually one associated with the routine functions of a cell and is referred to as a housekeeping gene. Amplification of the target and housekeeping gene are performed in separate reactions. The quantity of the target gene in any sample can then be estimated by normalising it to the amount of the constitutively expressed gene. This method is popular because of its simplicity and ease of implementation. However, there is uncertainty about stability of housekeeping gene expression during the progression and treatment of a disease, and during cell stimulation (Finnegan et al. 1993). Consistent expression of the housekeeping gene is fundamental to the accuracy of this quantitative method. Furthermore, small variations between different PCR tubes can lead to very large differences in the final amount of PCR product. The use of different pairs of primers to

amplify the housekeeping gene and the targeted gene of interest result in each respective reaction proceeding with a different amplification efficiency which discourages accurate quantification. These factors collectively limit the accuracy of this method which is regarded as being suitable only for measuring large changes between different samples.

An improvement on the former technique involves measuring the quantity of the target gene by generating a standard curve. In this method a sample of the amplified target is purified, quantified and serially diluted prior to re-amplification (Robinson and Simon 1991). A standard curve relating the yield of product to the initial concentration of the serially diluted product can be generated from which the quantity of target in an unknown sample can be extrapolated. The accuracy of this method is limited because each reaction is performed in a different tube.

A third quantitative PCR technique involves measuring the quantity of the target gene by co-amplifying a competitor DNA fragment with the target sequence of interest (Gilliland et al. 1990; Wang et al. 1989). This method overcomes several shortcomings of the former methods. The competitor fragment shares the same priming region with the native target which allows them to be co-amplified. The competitor usually differs from the target by having only a small intron, deletion or a mutated internal restriction enzyme site. Target and competitor PCR products can be distinguished because of their size differences using agarose gel electrophoresis, then quantified by measuring the emission of radioactivity, chemiluminescence or UV light of each fragment. The competitor can be titrated in replicate tubes containing a constant amount of target DNA for precise quantification of the target (Connolly et al. 1995). A change in any of the variables that influence PCR should effect the yield of both the competitor and target fragments equally during amplification. The relative amounts of the target and competitor therefore remain conserved throughout the amplification procedure which makes this one of the most accurate techniques for quantifying the PCR.

Several of the PCR assay steps are subject to variability. Variations in extraction of RNA from cells can be addressed by incorporating a competitor RNA template with the target RNA prior to extraction and reverse transcription (Hockett et al. 1995). An improved

competitive PCR technique involves synthesising a RNA competitor with a poly A tail such that the variations in the degradation of RNA during the extraction and reverse transcription procedures will be reflected in the amount of the competitor present at the end of the RT-PCR procedure (Duchmann et al. 1993).

1.5.4 Analysis of PCR Products

cDNA amplified by the PCR is subject to further types of quantitative molecular analysis. The products may be electrophoresed on agarose gels then stained with ethidium bromide to reveal distinct bands of the amplified DNA. PCR products may be probed using Southern transfers or with a dot blots which is essential when non-specifically amplified DNA is generated during the PCR. The PCR products may also be cut with restriction enzymes before being visualised with UV light in agarose gels. All these methods require densitometric quantification of photographs or films. Radioactivity can be counted to quantify the relative abundance of DNA but all of these methods either require subjective crudely quantitative assessments. Capillary electrophoresis (CE) is a rapid and automated technique that can be used to separate and accurately quantify macromolecules and an investigation of its use as a measure of PCR products was undertaken in this thesis.

1.5.5 Capillary Electrophoresis

Capillary electrophoresis (CE) was first employed as a separation technique in 1967, however, published reports describing its routine application did not appear until the early 1980s (Karger et al. 1989). Several modes of CE have developed since its introduction and a large number of examples utilising the various modes have been reported (Baba 1993; Bocek and Chrambach 1991; Guszczynski and Chrambach 1991; Guszczynski et al. 1993; Honda et al. 1992; Nielsen et al. 1991; Olefirowicz and Ewing 1990; Reyes Engel and Dieguez Lucena 1993). Capillary zone electrophoresis has evolved as a method to separate analytes differing in their charge to mass ratio. Micellular electrokinetic electrophoresis utilises variations in the hydrophobicity and the charge to mass ratio of the analytes to achieve separation. Capillary isoelectric focusing is primarily used to separate proteins

with different isoelectric points and capillary isotachophoresis resolves analytes with different mobilities. Capillary gel electrophoresis sieves DNA according to its size.

Electrophoresis in gels (ie. polyacrylamide or agarose) is a powerful tool for separating biological macromolecules such as DNA and for this purpose a variety of different electrophoretic techniques have been developed. DNA separations can be achieved in slab or rod like gels at low electric fields, however, pouring and running the gels can be slow and time consuming and detection and quantification of the products are only possible by using post-separation procedures such as densitometric or image analysis. Since 1981 there has been an increase in biomolecular separations using capillary electrophoresis (Compton and Brownlee 1988; Jorgenson and Lukacs 1983). CE has already established itself as an important analytical tool for the analysis of biomolecules such as proteins, peptides and high molecular weight double stranded PCR products. Capillary gel electrophoresis is an attractive alternative to traditional electrophoretic techniques for the analysis of PCR products. CE has an advantage over conventional slab gel electrophoresis because of its dramatically reduced analysis times, high resolution, reproducibility, quantification and full automation. As with slab gel electrophoresis, DNA separates primarily on a charge to mass ratio in an electric field. However, CE has the advantage of providing accurate quantitative data about the PCR products.

A conventional CE analysis system comprises of dual (+) and (-) power supplies, a fine glass capillary tube, reservoirs of buffers and an online detection system. The fluid filled glass capillary tube provides the electrical contact between two buffer reservoirs (see Fig 3). The analyte can be introduced into the capillary by either pressure injection (positive or negative), hydrostatic injection or electrokinetic injection (Aumatell 1995). Pressure injection requires pressurisation of the loading chamber to force the analyte into the capillary. Hydrostatic injection is achieved by syphoning the sample into the capillary. Electrokinetic injection utilises a voltage to induce the sample to migrate into the capillary. A component of the sample with a with a net charge (z) moves with a velocity (v) when an electric field of strength E is applied according to equation 2.

$$v = Ez/f \qquad (2)$$

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f represents the frictional coefficient of the migrating molecule which varies with its size, charge density and shape in a given solvent (Karger et al. 1989). Electrokinetic injection of charged molecules is an electrophoretic process in which molecules with a low frictional coefficient (f) preferentially migrate into the capillary during the injection process.

Capillary Electrophoresis
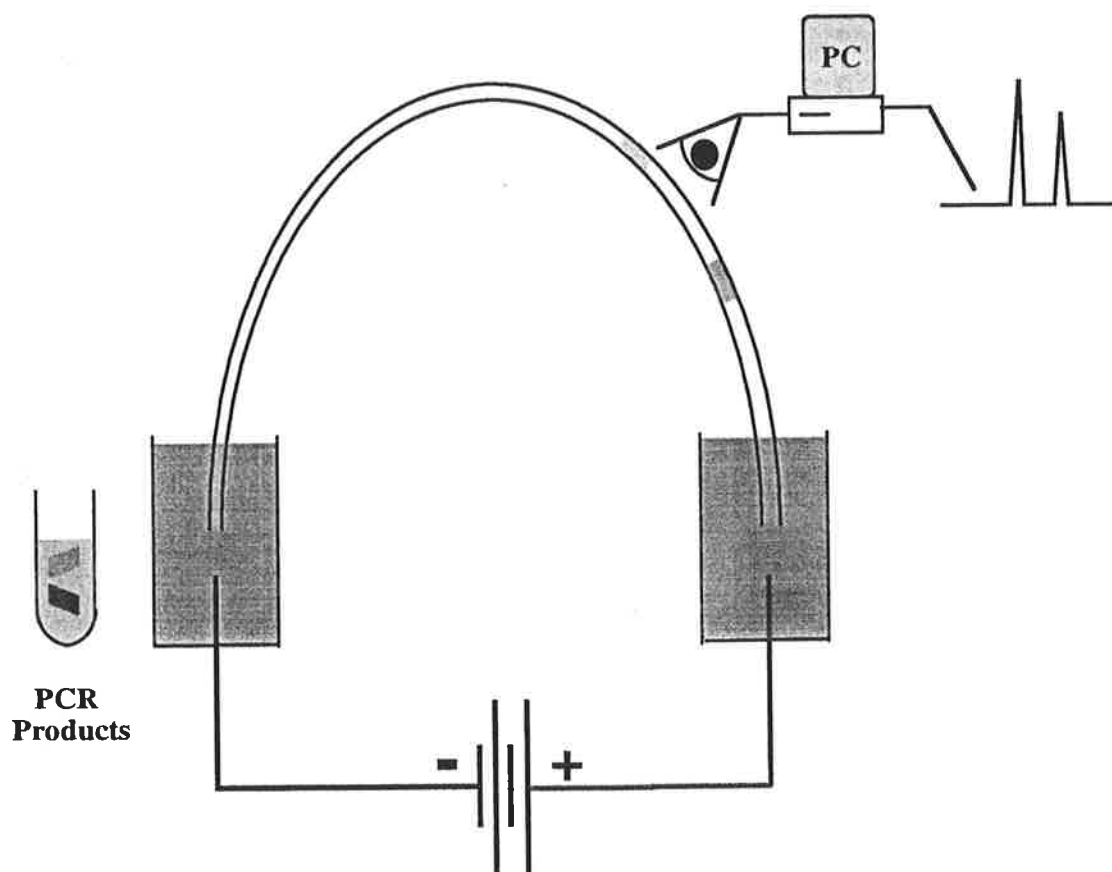
Figure 3. A schematic diagram representing the essential features of a capillary electrophoresis machine. The two buffer reservoirs are maintained at a potential difference by a power supply. The buffer filled capillary sieves DNA based on its charge to mass ratio. An online UV detector monitors the migration of DNA through the capillary and the resultant electropherogram is presented on a personal computer for analysis.

After the sample is introduced into the capillary a voltage is applied across the buffer reservoirs. The applied voltage induces ions in the buffer to migrate. The net migration of ions is called the osmotic flow. Analytes with different mobilities can be separated using

capillary zone electrophoresis (CZE) when the osmotic flow is constant. A detector mounted near the end of the capillary is used to monitor migration of the analytes.

Analytes are monitored as they migrate past the detector. Detectors commonly monitor visible, UV absorption or fluorescence of the analyte (Andrus 1994). The detector output plotted as a function of migration time is represented graphically as an electropherogram.

CZE differs from capillary gel electrophoresis. Nucleic acids co-migrate during conventional CZE because they have a similar charge to mass ratio at a given pH (Heller 1995). Capillaries used in gel electrophoresis are filled with a sieving matrix which is designed to limit the osmotic flow, allowing DNA fragments to be separated according to their length. DNA becomes entangled with the gel matrix as it migrates along the capillary. Larger DNA fragments become more entangled and therefore migrate slower than smaller, less retarded fragments (Barron et al. 1993). Sieving gels such as long chain hydrophilic polymers and porous gels such as polyacrylamide can be used to achieve such a sieving effect (Barron et al. 1993; Quesada 1997). The mechanism of DNA sieving is believed to resemble that of conventional slab gel electrophoresis (Viovy and Duke 1993).

1.5.6 Quantitative PCR and Cytokines

Reverse transcription polymerase chain reaction (RT-PCR) is a very sensitive method for detecting low copy number mRNA transcripts from tissues or cells. Competitive PCR is a technique that has been developed as an accurate quantitative PCR method. In this thesis the quantitative nature of competitive PCR has been exploited to monitor the levels of IL-2, IFN-γ, IL-4, TNF and TGF-β produced in the draining lymph nodes, and synovial tissue during the prodromal and acute phase of AA. The cytokine profile of whole TD cells and the fraction of TD cells bearing activation markers was also quantified using competitive PCR. The sensitivity of the RT-PCR coupled to the accuracy and quantitative features of CE make it an ideal combination for accurate and reproducible quantification of cytokine mRNA.

Chapter 2

Materials and Methods

2.1 RNA Extraction

The method of Chomczynski and Sacchi (Chomczynski and Sacchi 1987) was used to isolate total RNA from tissues and cells. Tissue samples from rat lymph node, spleen or cells from the thoracic duct were placed in a 1.8 mL cryotube (Nunc, Roskilde, Denmark), immersed in liquid nitrogen then crushed into a fine powder with a metal probe. 500 μL of guanidine thiocyanate solution (4.2 M guanidine thiocyanate, 26.4 mM sodium citrate, 0.5% N lauroyl sarcosine and 0.1 M 2-mercaptoethanol), 500 μl of water saturated phenol (Amresco, Solon, U.S.A.), 100 μL of chloroform: isoamylalcohol (49:1 v/v) and 50 μL of 2 M sodium acetate, pH 4) were added to the sample. The mixture was vortexed for 30 sec and stored on ice for 15 min prior to centrifugation at 6500 x g for 30 min at 4°C. The upper aqueous phase was retained and mixed with 500 μL of propan-2-ol before being cooled to -70°C for 30 min. The RNA was pelleted at 4°C for 30 min then the supernatant was decanted and the pellet washed with 200 μL of guanidine thiocyanate solution and 200 μL of propan-2-ol then incubated at -70°C for 30 min. The RNA was pelleted as outlined above then washed twice with 1 mL of 75% ethanol (-20°C). The supernatant was decanted and the RNA pellet was left to dry for 1 hour at room temperature and then redissolved in 52.5 μL of diethyl pyrocarbonate treated water (DEPC water). 2.5 μL of RNA was diluted to 50 μL with DEPC water then quantified by measuring its UV absorbance at 260 nm on a Beckman DU 650 spectrophotometer (Beckman Instruments Inc., California, U.S.A.). Following spectrophotometric quantification, 28 units of RNAsin (Promega Corporation, Madison, U.S.A.) and 1 μL dithiothreitol (DTT) (0.1M) were added to the RNA to limit degradation.

2.2 First Strand cDNA Synthesis

The RNA was incubated for 10 min at 65°C prior to use. 500 ng of RNA was diluted to 10.1 µL with DEPC water then incubated with 4 µL of reverse transcription buffer (250 mM KCl, 100 mM Tris and 12.5 mM MgCl2), 4 µL of dNTP's; 10 µM of each dCTP, dATP, dGTP and dTTP (Promega Corporation, Madison, U.S.A.), 1 µL of 1.5 mg/mL random hexamers (Boehringer Mannheim GmbH, West Germany), 0.004 U of RNAsin (Promega Corporation, Madison, U.S.A.) and 64 U of Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Gibco BRL, Gaithersberg, U.S.A.) at 37°C for 1 hour to generate cDNA. The final product was stored at -20°C.

2.3 cDNA Storage and Dilution

Aliquots of the purified target and competitor cDNA fragments were stored at -70°C then incubated for 10 min at 65°C prior to dilution and subsequent PCR amplification. Storage under these conditions was shown to maintain stable, reproducibly quantifiable amounts of cDNA as assessed by absorbance at 260 nm.

2.4 Polymerase Chain Reaction

Sense and antisense oligonucleotide primers used to amplify β-actin, IL-2, IFN-γ and IL-4 cDNA had been previously published (McKnight et al. 1991). Sense and antisense oligonucleotide primers used to generate TNF and TGF-β PCR products had been previously published (Farges et al. 1995; Mayrhofer). An extensive series of preliminary experiments was undertaken to achieve optimal conditions for amplifying message for the respective cytokines of interest. The product from each optimised PCR was sequenced to confirm its identity.

5 mL of cDNA sample was added to 5 mL PCR buffer (MgCl2 as outlined in Table 1, 500 mM KCl, 100 mM Tris), 34.75 µL Milli-Q water, 0.25 µL (1.25 U) of Taq polymerase (Promega Corporation, Madison, U.S.A.) and 1 µM of each oligonucleotide of the respective primer pairs as indicated in Table 1.

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Cytokine	Primers	Mg++ Conc (mM)	Annealing Temperature	Cycle Number
β-Actin	Sense primer = 5'-ATGCCATCCTGCGTCTGGACCTGGC-3' Antisense = 5'-AGCATTTGCGGTGCACGATGGAGGG-3'	2.0	60°C	27-33
IFN-γ	Sense primer = 5'-ATGAGTGCTACACGCCGCGTCTTGG-3' Antisense = 5'-GAGTTCATTGACAGCTTTGTGCTGG-3'	2.0	60°C	40
IL-2	Sense primer = 5'-CATGTACAGCATGCAGCTCGCATCC-3' Antisense = 5'-CCACCACAGTTGCTGGCTCATCATC-3'	2.0	60°C	40
IL-4	Sense primer = 5'-TGATGGGTCTCAGCCCCCACCTTGC-3' Antisense = 5'-CTTTCAGTGTTGTGAGCGTGGACTC-3'	2.0	60°C	40
TNF	Sense primer = 5'-TACTGAACTTCGGGGTGATCG-3' Antisense primer = 5'-CCTTGTCCCTTGAAGAGAACC-3'	2.0	60°C	35-40
TGF-β	Sense primer = 5'-ATCGACACATGGAGCTGGTGA-3' Antisense primer = 5'-TTCCGTCTCCTTGGTTCAG-3'	0.5	55°C	35-40

Table 1. Oligonucleotide primers, magnesium chloride concentration and cycle number used to amplify the respective PCR products.

Samples were overlaid with mineral oil then denatured at 94°C for 7 min prior to cycling which was carried out in an Hybaid Omni Gene thermal cycler (Hybaid Limited, Middlesex, United Kingdom). It involved a denaturing step at 94°C for 1 min, followed by an annealing step for 1 min at the temperature specified in Table 1, then an extension step at 72°C for 2 min. A final extension step for 7 min at 72°C was carried out after cycling. β-actin primers amplified a 607 bp cDNA fragment and IFN-γ, IL-2, IL-4, TNF and TGF-β primers produced PCR products of 405 bp, 410 bp, 378 bp, 182 bp and 430 bp respectively.

2.5 RNA Denaturing Gel

RNA was electrophoresed through a denaturing formaldehyde gel which was prepared by melting 0.5 g of powdered agarose (Sigma-Aldrich Pty., Ltd., Castle Hill, Australia) in 47.3 mL of Milli-Q water, 2.68 mL of deionised formaldehyde, 50 mg of ethidium bromide and 5 mL of 10 x MOPS buffer (0.2 M 3-[N-morpholino]propane-sulfonic acid, 0.05 M sodium acetate and 0.01 M ethylenediamine-tetraacidic acid). A gel formed as the mixture cooled to room temperature, then 1 μg of RNA dissolved in loading buffer (2.6 μL of 10 X MOPS buffer, 1.8 μL glycerol, 4.2 μL formaldehyde, 13.2 μL formamide (deionised), 0.4 μL bromphenol blue and 2.8 μL of water), loaded onto the gel then electrophoresed at 100 V for 2 hours. The RNA was visualised using a 254 nm UV light.

2.6 Agarose Gel Electrophoresis

25 μL of each PCR product was mixed with 6 μL of loading buffer then 25 μL of this mixture was electrophoresed through a 2% agarose gel at 150 V for 90 min. The gel was stained for 1 hour in 1.3 mM ethidium bromide then photographed with 667 flim (Polaroid, Hertfordshire, United Kingdom) using UV illumination. pUC 19 digested with *Hpa* II (Bresatec, Adelaide, Australia) was used as the molecular weight marker.

2.7 Polyacrylamide Gel Electrophoresis

An 8% polyacrylamide gel was prepared by mixing 3.7 mL of Milli-Q water with 1 mL of 30% acrylamide solution containing 2.7% w/w bis acrylamide, 45 μL of freshly prepared

10% ammonium persulfate and 10 μL of N, N, N', N'-tetramethylenediamine (TEMED) were added to this mixture which was left at room temperature for 15 min to solidify. The DNA was dissolved in 6 μL of loading buffer (40% w/v sucrose in water, 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanole FF) then introduced into the gel and electrophoresed for 1.5 hours at 50 V after which the gel was stained in 1.3 mM ethidium bromide for 15 min then photographed using UV illumination.

2.8 Capillary Gel Electrophoresis of PCR Products

DNA analysis was carried out using a Waters Quanta 4000 capillary electrophoresis system (Waters, Milford, U.S.A.). cDNA was detected using an on column 254 nm UV detector. For DNA separation a 57 cm (μ Sil) DB-17 silica column (Internal diameter (I.D.) = 100 μm) (J &W Scientific, Folsom, U.S.A.) was equilibrated with 0.5% hydroxypropyl-methyl cellulose in TBE buffer (89 mM Tris, 89 mM boric acid and 2 mM EDTA). The PCR product was introduced into the capillary by electrokinetic injection at 3-5 kV for 1 min and separation was achieved at 25°C using a negative power supply operating at approximately 10 kV. Data was processed by on line computational analysis using an Intelligent Machines advanced personal computer (Intelligent Machines Pty. Ltd. Australia) equipped with Millennium 820 V.2.1.5 chromatography software. Quantification of the PCR products was undertaken using CE unless otherwise indicated.

2.9 Polyacrylamide Gel Capillary Electrophoresis of PCR Products

A commercially available kit containing a 65 cm eCAP ds1000 polyacrylamide filled capillary (I.D.= 100 μm) and dsDNA 1000 Gel buffer (Beckman Instruments Inc., Fullerton, U.S.A.) was used to separate PCR product. The product was introduced into the capillary by electrokinetic injection at 3-5 kV for 1 min and separation was achieved at 25°C using a negative power supply operating at 10 kV.

2.10 Preparation of the Target Templates

The product of the PCR using the appropriate sense and antisense primers (listed in Table 1) was electrophoresed through an 8% polyacrylamide gel at 150 V for 4 hours. Gels were

stained for 10 min in 1.3 mM ethidium bromide then cDNA was visualised using 254 nm UV radiation. The correct length bands were excised and soaked in 200 μL of TE buffer (10 mM Tris.Cl pH 7.4 and 1 mM EDTA pH 8.0) at 37°C for 12 hours. The aqueous layer was retained and mixed with 50 μL of Tris saturated phenol (Amresco, Solon, U.S.A.) and 50 μL of chloroform isoamly alcohol (24:1). The mixture was centrifuged for 10 min at 4°C and the upper aqueous layer was retained then washed with 50 μL of chloroform isoamyl alcohol. The upper aqueous layer was retained after a further 10 min spin at 4°C and any residual protein was removed from the DNA product by proteinase K digestion. More specifically, 1.5 mL of 500 mM EDTA (pH 8.0), 15 μL of 0.1 M Tris.Cl (pH 8), 7.5 μL of 10% SDS, 0.75 μL of 10 μg/mL proteinase K (Merck, Darmstadt, Germany) and 25.25 μL of Milli-Q water were added to the aqueous layer and incubated at 37°C for 30 min then transferred to 68°C for a further 10 min incubation. The products were purified by phenol chloroform extraction (Sambrook et al. 1989) then the purified cDNA was precipitated by cooling the solution to -70°C for 10 min after adding 2 μL of sodium acetate (3 M pH=5.5) and 100 μL of ethanol. The cDNA was pelleted by centrifugation at 4°C for 30 min and the pellet was left for one hour to dry at room temperature.

2.11 Selection of PCR Primers

Oligonucleotide PCR primers used to generate competitor cDNA fragments for IL-2, IFN-γ, IL-4, TNF and TGF-β were designed from the respective cDNA sequences that had been submitted to the European Molecular Biology Laboratory (EMBL). The oligonucleotide primers were designed using the computer program DNAIS - Primer Design, and had approximately 50-60 % GC content and a theoretical annealing temperature of 75 - 80°C.

2.12 Preparation of the Competitor Templates

Competitor cDNA templates were made from cDNA according to the method of Celi et al (Celi et al. 1993). Briefly, a nested antisense primer was designed for each cytokine in addition to the two conventional primers. The nested antisense primers are listed in Table 2.

Cytokine	Primers	Mg^{++} Conc (mM)	Annealing Temperature
IFN-γ	5'-CAGGTGCGATTCGATGACAC-3'	2.0	60°C
IL-2	5'-ATCCAACACACGCTGCAGAG-3'	2.0	60°C
IL-4	5'-CCTCAGTTCACCGAGAACCC -3'	2.0	60°C
TNF	5'-CTCCTCTGCTTGGTGGTTT -3'	2.0	55°C
TGF-β	5'-CTGGTAGAGTTCTACGTGT -3'	2.0	55°C

Table 2. Nested antisense oligonucleotide primer sequence, magnesium chloride concentration and annealing temperature used to amplify the respective competitor DNA fragments.

The primers were designed to amplify cDNA fragments of the following sizes: 322 bp (IFN-γ), 299 bp (IL-2), 320 bp (IL-4), 182 bp (TNF) and 430 bp (TGF-β). The nested antisense primer for each cytokine was 5' coupled to the 3' end of the framing antisense primer listed in Table 1. PCR products amplified under standard conditions using the appropriate sense and nested antisense primer were electrophoresed through an 8% polyacrylamide gel at 150 V for 4 hours. Gels were stained for 10 min in 1.3 mM ethidium bromide then cDNA was visualised using UV radiation. The correct length band for each competitor was excised from the gel and purified using phenol, chloroform and proteinase K as outlined in Section 2.10. The purified PCR product, which represents a truncated cDNA fragment of the appropriate cytokine, was stored at -70°C for use in subsequent experiments and from here on is referred to as the competitor cDNA fragment.

2.13 Competitive PCR Using Known Amounts of Target cDNA

The PCR was executed as outlined above in Section 2.4 except the 5 μL of cDNA was comprised of 2.5 μL of a dilution of the competitor and 2.5 μL of the target cDNA. The volume of Milli-Q water was reduced to 32.25 μL to accommodate the addition of 2.5 μL of dNTP's (2 μM of each dCTP, dATP, dGTP and dTTP).

2.14 Competitive PCR

The competitive PCR was executed as outlined above in Section 2.4 except the volume of Milli-Q water was reduced to 32.25 μL to accommodate the addition of 2.5 μL of the appropriate competitor cDNA fragment.

2.15 IFN-γ ELISA

Flexible flat bottom 96 well ELISA plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with monoclonal mouse anti-rat IFN-γ (Biosource International, Camarillo, U.S.A., catalogue #AB-53-010) at a concentration of 1 μg/mL in 50 μL of coating buffer (0.2 M Na_2CO_3, 0.2 M $NaHCO_3$ pH 9.6). Each well was blocked with 50 μL of 2% BSA (Sigma Chemical Co., St. Louis, U.S.A.) in wash buffer (0.05% Tween 20 in PBS) then washed 3 times with 200 μL of wash buffer (0.05% Tween 20 in PBS) and lined with 50 μL of recombinant IFN-γ (Gibco BRL, Gaithersberg, U.S.A., catalogue #3283SB) or the cell culture supernatant diluted in washing buffer containing 1% BSA. After incubation for 24 hours at 4°C the wells were washed 3 times with 200 μL of buffer then coated for 2 hours at 37°C with 50 μL of 2 μg/mL polyclonal rabbit anti-rat interferon-γ (Biosource International, Camarillo, U.S.A., catalogue #AB-54-005). The wells were rinsed 3 times with 50 μL of buffer then incubated at 37°C for 2 hours with 50 μL of 2 μg/mL alkaline phosphatase conjugated sheep anti-rabbit immunoglobulin (Silenus, Victoria, Australia, code#RAP) which was diluted in wash buffer containing 1% BSA and 2% normal mouse serum. The plate was rinsed then developed by adding 50 μL/well of substrate buffer (1.0 M diethanolamine, 0.5 mM $MgCl_2$, 15 mM p-nitrophenyl phosphate, pH 9.8). The optical density at 405 nm was measured on a Bio Rad model 450 ELISA plate reader (Bio Rad, California, U.S.A.) 10-20 min after the addition of the substrate buffer. The detection limit of the assay was approximately 8 ng/mL.

2.16 TNF ELISA

The development of the TNF ELISA is extensively described in Section 5.5. Flexible flat bottom 96 well ELISA plates (Nunc) were coated overnight at 4°C with 50 μL of 4 μg/mL of monoclonal rat anti-mouse TNF (Pharmingen, San Dieago, U.S.A, catalogue #G281-

2626) which was diluted in coating buffer (0.2 M Na2CO3, 0.2 M NaHCO3, pH 9.6). The plate was blocked for 30 min at 37°C with 2% BSA in wash buffer (0.05% Tween 20 in PBS) then each well was rinsed and incubated with 50 µL of recombinant TNF (Serotec Limited, Oxford, United Kingdom, catalogue #PRP16) or the cell culture supernatant for 3 hours at 37°C. The plate was washed then coated with 50 µL of 1 µg/mL polyclonal rabbit anti-rat/mouse TNF (Serotec Limited, Oxford, United Kingdom, catalogue #AAM12) for 1 hour at 37°C. Following washing, the plate was incubated at 37°C for 2 hours with 50 µL of 300 ng/mL donkey anti-rabbit immunoglobulin (Amersham, N.S.W., Australia, catalogue #GE2966) diluted in buffer containing 2% normal donkey serum (Serotec Limited, Oxford, United Kingdom). Each well was washed then incubated for 30 min at 37°C with 50 µL of ABComplex/HRP (Dako, Glostrup, Denmark) diluted 1/50 in PBS according to the manufacturers instructions. The plate was washed and developed by adding 50 µL of substrate buffer (10 mg o-phenylenediamine dihydrochloride, 2 µL of hydrogen peroxide, 0.1 M citric acid, 0.12 M trisodium citrate, pH 4.9) to each well. The colorimetric reaction was stopped after approximately 10 min by adding 50 µL of 2 M sulphuric acid then the optical density at 490 nm was measured. The detection limit of the assay was approximately 5 ng/mL.

2.17 Animals

Female inbred pathogen free Dark Agouti (DA) rats were obtained from either the Gillies Plain Animal Resource Centre (Adelaide, South Australia) or the Animal Resource Centre (Perth, Western Australia). The animals were housed at the Institute of Medical and Veterinary Science and were provided with standard rodent chow and water *ad libitum.* Experiments involved 8 week old aged matched female DA rats housed at 4-5 per cage. Animals were euthanased by asphyxiation in carbon dioxide accordance with ethical guidelines.

2.18 Immunisations

Complete Freund's adjuvant (CFA) was prepared by using a mortar and pestle to crush 100 mg of heat killed *Mycobacterium tuberculosis* H37RA (Difco, Michigan, USA) which was

then suspended in 10 mL of incomplete Freund's adjuvant (Difco, Michigan, USA). Adjuvant arthritis (AA) was induced in 8 week old female DA rats by subcutaneous injection of 100 μL of CFA at the base of the tail. CFA inoculated rats were observed daily, under anaesthesia (halothane or isofluorane and nitrous oxide), for the development of polyarthritis. The severity of polyarthritis was measured by attributing a score for each paw as follows: 0 (no evidence of arthritis), 1 (single focus of redness or swelling), 2 (two or more foci of redness or swelling), 3 (generalised swelling but not global), or 4 (severe global swelling) for each paw. The arthritic index (AI) for each rat is the sum of the scores obtained from the four paws (maximum, 16). Control rats received a subcutaneous injection of 100 μL of saline solution into the base of the tail.

2.19 Tissue Collection For Adjuvant Arthritis Time Trial

Animals were euthanased after inoculation with CFA or 100 μL of saline solution at the base of the tail. Inguinal and popliteal lymph nodes and the ankle synovial tissue were resected, placed in eppendorf tubes and immediately frozen in liquid nitrogen in preparation for PCR analysis.

2.20 Cell Culture

A single cell suspension was prepared by gently extruding lymph node tissue through a 250 micron wire mesh with the plunger of a 20 mL disposable syringe. The cells were then filtered through a fine layer of cotton wool and the erythrocytes were lysed if necessary, by adding ammonium chloride solution (140 mM NH_4Cl, 17 mM Tris, pH 7.6) for 60 sec at 4°C. Leucocytes were cultured in RPMI (Gibco BRL, Gaithersberg, U.S.A.) supplemented with 2 mM L-glutamine, 50 mM 2-mercaptoethanol and 10% (v/v) heat inactivated foetal calf serum (FCS) (Gibco BRL, Gaithersberg, U.S.A.). The cells were cultured in 24 well plates (Nunc) at 37°C in an atmosphere of 5% carbon dioxide at a density of 2×10^6 cells/mL. They were stimulated with concanavalin A type IV (Sigma Chemical Co., St. Louis, U.S.A.) at a concentration of 5 μg/mL, which had been found to provide optimal stimulation in preliminary experiments.

2.21 Cannulation of the Thoracic Duct

Cannulation of the abdominal thoracic duct (TD) was performed as described previously (Bollman et al. 1948; Ford 1978) .TD lymph was collected overnight and the cellular fraction within the lymph was obtained by centrifugation (350 x g for 7 min) at room temperature. The cells were washed twice with RPMI containing 2% FCS and were found to comprise mostly of lymphocytes as assessed by Giemsa staining of cell smears and flow cytometery (Spargo et al.). The cells were then either lysed in 500 μL of guanidine thiocyanate solution and stored at -70°C in preparation for cytokine mRNA analysis, or resuspended for preparation of lymphocyte sub-populations.

2.22 Preparation of TD Lymph Sub-Populations

CD4$^+$ T-cells were prepared from TD lymph by depleting CD8$^+$ T-cells and B-cells with a mixture containing equal volumes of OX8 (CD8), OX33 (CD45 present on B-cells (Woollett et al. 1985)) and Mark-1 (anti κ light chain) (all added in excess). Approximately 8 x 10^8 TD cells were incubated for 30 min at 4°C in a mixture containing 3 mL of each antibody (obtained from cell culture supernatants). The cells were collected by centrifugation and washed three times with 40 mL of RPMI containing 2% FCS and 1% azide to remove the excess antibody. Pre-coated sheep anti-mouse M450 Dynal Beads (Dynal, Oslo, Norway) were incubated with the TD cells (density of 1.5 beads/cell) for 30 min on a rotor at 4°C. The selected cells were collected on a Dynal MPC-6 magnet then washed twice with 40 mL of RPMI/FCS/azide leaving the CD4$^+$ TD cells in solution. The selected cells were collected on a Dynal MPC-6 magnet then washed twice with RPMI/FCS, leaving the CD4$^+$ T-cells in suspension. The selected cells were re-suspended, collected and re-washed to ensure a pure population was collected. The purity of the CD4$^+$ TD T-cells was analysed using a Coulter Epics-Profile II flow cytometer (Coulter Corporation, Florida U.S.A.) with a goat anti-mouse immunoglobulin (IgG) FITC conjugate (Pharmingen, San Diego, U.S.A.).

Following separation of the CD4$^+$ T-cells from the other TD cells, activated CD4$^+$ T-lymphocytes were captured using Dynal Beads to isolate cells bearing the following

surface markers; OX 39 (IL-2 receptor), OX 26 (transferrin receptor), OX 6 (MHC class II) and OX 40 (CD134, expressed on CD4$^+$ T-blasts (Birkeland et al. 1995)). Approximately 3.5 x 10^8 CD4$^+$ TD T-cells were incubated in a mixture containing 3 mL of each antibody listed above for 30 min at 4°C (all obtained from cell culture supernatants). The cells were collected by centrifugation and washed three times with 40 mL of RPMI/FCS/azide to remove excess antibody. The activated sub-population was depleted by incubating CD4$^+$ TD T-cells with pre-coated sheep anti-mouse M450 Dynal Beads at a density of 4.5 beads/cell for 30 min on a rotator at 4°C. The selected cells were collected on a Dynal magnet then washed twice with 40 mL of RPMI/FCS/azide leaving the non-activated CD4$^+$ TD T-cells in solution. The selected cells were resuspended, collected using a magnet and rewashed. The purity of the non-activated CD4$^+$ TD T-cells was analysed using flow cytometery with a goat anti-mouse IgG FITC conjugate (Pharmingen). The activated and non-activated CD4$^+$ TD T-cells were each suspended in 150 μL of RPMI/FCS before being injected into 850 μL of guanidine thiocyanate solution. The samples were stored at -70°C in preparation for cytokine mRNA analysis.

2.23 Flow Cytometric Analysis of CD4$^+$ TD T-Cell Sub-populations

Flow cytometric analysis was used to determine the purity of the CD4$^+$ TD cell population prepared in Section 2.22. 1-2 x 10^6 CD4$^+$ TD cells isolated from TD cells were incubated for 40 min on ice with a mixture containing 100 μL of the following antibodies: OX8, OX33 and Mark-1. The cells were washed twice with 3 mL of buffer (PBS, 0.1 M azide and 2% FCS) then incubated for 40 min on ice with 100 μL of FITC conjugated goat anti-mouse IgG (Pharmingen) in 10% normal rat serum. TD cells were fixed in Facs fix (1 L PBS, 10 mL formalin, 20 g glucose and 0.2 g sodium azide) then analysed using a Coulter Epics-Profile II flow cytometer. As a negative control, the primary antibody was replaced with 200 μL of the monoclonal antibody, 1B5 (anti-Giardia) which was added as neat supernatant from a hybridoma cell line.

A very similar procedure was used to determine the purity of the non-activated CD4$^+$ TD cell population prepared in Section 2.22. Briefly, 1-2 x 10^6 non-activated CD4$^+$ TD T-cells were incubated for 40 min on ice with a mixture containing 100 μL of the following

antibodies: OX39, OX26, OX6 and OX40. The cells were washed twice with 3 mL of buffer then incubated for 40 min on ice with 100 µL of FITC conjugated goat anti-mouse immunoglobulin (Pharmingen) in 10% normal rat serum. Non-activated CD4$^+$ TD cells were fixed in Facs fix then analysed using a flow cytometer.

2.24 Statistical Analysis

The data from quantitative PCRs were analysed using the non-parametric Kruskel-Wallis test and the Mann-Whitney U test. P≤0.05 was regarded as statistically significantly different. In some instances, where doubt remained about the real significance of differences seen amongst multiple analyses, further experiments were undertaken to reassess findings under the conditions that yielded significant differences.

Chapter 3

Capillary Electrophoresis

3.1 Introduction

The separation and analysis of DNA restriction fragments using capillary electrophoresis (CE) was first reported by Strege and Kleemiss (Kleemiss et al. 1993; Strege and Lagu 1991). While recent studies have confirmed the utility of this application, the approach involves certain difficulties depending on the type of column used and other conditions of electrophoresis as well as the source of material under analysis (as discussed below). The present investigations were undertaken to achieve a convenient and reliable method for quantifying cytokine PCR products suitable for routine application.

The major parameters influencing the reproducibility of PCR product analysis using CE are the buffer viscosity and temperature. A single degree variation in the temperature of the running buffer has been reported to cause a 2-3% change in its viscosity which results in a large change in the migration time of the analyte (Guttman and Cooke 1991). Hence, a variation in temperature limits the reproducibility of CE by influencing peak widths and analyte migration time. Variations in the temperature of the running buffer are often attributed to changes in the electric field strength which varies with the length and width of the capillary.

Although the high electric field strengths achievable with CE lead to a high resolution of DNA and reduced acquisition time, the current induced by the electric field heats the buffer within the capillary, promoting thermal diffusion which decreases resolution of the analyte (Slater et al. 1995). Furthermore, heat resulting from the very high electric fields and large currents may vaporise the buffer within the capillary causing a break in the electrical circuit and electrophoretic failure. Heating of the buffer within the capillary has

been shown to be dependent on the internal and external diameters of the capillary (Holzwarth 1996), the molar conductivity and concentration of the buffer (Karger et al. 1989). The use of small diameter capillaries during electrophoresis promotes rapid heat dissipation which minimises thermally induced band broadening. The pH of the DNA solution is also a critical factor in determining the direction of analyte migration during electrophoresis (Khan et al. 1996; Pearce and Watson 1993). The presence of inorganic salts increases the electrical conductivity and temperature of the running buffer and limits resolution of the analyte (Shihabi and Hinsdale 1995). In addition, the concentration of inorganic salts in the analyte influences the method by which the sample can be introduced into the capillary.

Capillary electrophoresis has the advantage that it is a quantitative and automated technique which provides a higher resolution of DNA fragments than that achieved using conventional slab gel electrophoresis. Furthermore, DNA can be detected on line without the need for lengthy post-separation revealing procedures such as staining of DNA followed by densitometery and image analysis, that is required with conventional slab gel electrophoresis. In this Chapter the development of a CE method to quantify PCR products is described.

3.2 Selection of CE System Matrix

3.2.1 Polyacrylamide-Filled Capillaries

The application of CE as a method for DNA separation and analysis initially focused on high resolution and throughput of nucleotide fragments for DNA sequence analysis. High resolution of DNA restriction fragments has been achieved using capillaries filled with solid polyacrylamide (Arakawa et al. 1994; Carson et al. 1993 ; Swerdlow et al. 1992). In order to test the suitability of these gels for the separation and quantification of DNA in our CE system, eCAP polyacrylamide filled capillaries (65 cm in length and 100 μm internal diameter) were used to separate pUC 19 DNA restriction fragments. 500 pg/μL of pUC 19 was electrokinetically injected (at 5 kV for 60 sec) into the capillary and an

electropherogram was acquired with a running voltage of 10 kV over a 30 min period. The procedure was repeated up to 10 times, and in each case the separation of peaks, their height and width were monitored to assess the resolution of pUC 19 in this matrix. This procedure was repeated using three polyacrylamide filled capillaries and in each case the matrix provided very high resolution of the DNA restriction fragments.

Despite the high resolution of DNA, the ends of the capillaries frequently became irreversibly dehydrated when used in the Waters Quanta 4000 CE apparatus and as a result, they became blocked which prevented further loading and migration of the DNA. Careful monitoring of the voltage was also required to avoid excessive heating of the buffer during electrophoresis, failing which, the buffer vaporised and the electrical circuit was broken. This proved very difficult to rectify and because of these difficulties the lifetime of each capillary was limited. The cost of the capillaries and the difficulties associated with their daily use made them unacceptable for routine analysis, which is in agreement with other reports (Barron et al. 1996; Chiari et al. 1992; Kleemiss et al. 1993). A cheaper, more reliable matrix was sought for the analysis of DNA.

3.2.2 Gel-Filled Capillaries

High resolution of DNA fragments has been achieved using capillaries filled with long chain hydrophilic polymers (Baba et al. 1993; Chiari et al. 1992; Heller 1995). These "gel filled capillaries" are reliable and less expensive than polyacrylamide filled capillaries. However, resolution of DNA is generally lower than that obtained using polyacrylamide. The lower resolution is caused by high electro-osmotic flow that is induced when an electric field is applied. Hollow capillaries (eg. DB-17 capillaries) employed in capillary gel electrophoresis have a polymer coating of 50%-phenyl-methylpolysiloxane on the inner wall that acts to retard the osmotic flow of the "gel like" matrix (0.5% hydroxymethylpropyl cellulose (HMPC)) during electrophoresis. The flow is sufficiently restricted to resolve DNA fragments with an efficiency approximating several million theoretical plates (Landers 1993). However, when the capillaries are uncoated and the flow is unrestricted, resolution of DNA fragments rapidly declines.

In order to test the suitability of these gels for the separation and quantification of DNA in our system, gel filled capillaries were used to separate pUC 19 restriction fragments. 500 pg/μL of pUC 19 was electrokinetically injected into a 57 cm DB-17 capillary at 5 kV for 60 sec and an electropherogram was acquired at 10 kV for 30 min. The procedure was repeated up to 10 times on each of three respective gel-filled capillaries and peak separation, heights and widths were monitored to assess the resolution of DNA using this matrix. In each case the DNA restriction fragments were highly resolved.

The lifetime of a gel filled capillary was tested by electrokinetically injecting and separating 500 pg/μL of pUC 19 using the electrophoretic conditions outlined above. The sequence was repeated hundreds of times over a one week period and after each separation resolution of the peaks was monitored to assess the reliability of the matrix in separating DNA. The procedure was repeated approximately 200 times before the DNA bands began to broaden as the resolving power of the capillary diminished. The decrease in resolution was presumably associated with an increase in the electro-osmotic flow that was attributable to the degradation of the inner capillary coating by heat generated during electrophoresis (Martin et al. 1993).

In contrast to studies with this plasmid DNA, the resolution of DNA product within PCR samples decreased rapidly with repeated CE analysis. Since these samples contained traces of mineral oil which is laid over the reaction mix, it seemed possible that the presence of oil may have caused the loss of resolution which was observed after approximately 30 analyses. The oil presumably increased the electro-osmotic flow when it became bound to the polymer coating on the inner wall of the capillary. This interpretation seems valid since after washing the column with 1 mL of dichloromethane and methanol respectively, DNA was able to be separated at high resolution again. The major factor limiting the lifetime of gel filled capillaries thus appeared to be the integrity of the polymer coating. Either degradation or masking of this coating may result in an increased electro-osmotic flow which limits the resolution of DNA.

Since gel filled capillaries gave high resolution of DNA fragments, were durable and easily regenerated when the resolution of DNA deteriorated after multiple analyses of test

samples they were adopted in preference to polyacrylamide filled capillaries for the analysis of PCR products.

3.3 Evaluation of DNA Quantification using Gel Filled Capillaries.

3.3.1 UV Absorbance Spectrum

Quantification of nucleic acids is normally achieved by monitoring the absorption of the constituent nucleotide bases of DNA at 260 nm. A 260 nm ultra violet (UV) lamp was unavailable for the Quanta 4000 CE system therefore, a 254 nm UV lamp was used to monitor the migration of DNA through the capillary. The suitability of this lamp for the detection of DNA was assessed by acquiring a UV absorption spectrum of 2 ng/mL salmon sperm DNA. The spectrum (Fig. 1) depicts a broad absorption peak between 230 nm and 300 nm and shows the absorption of light by DNA at 254 nm to be approximately 97% of the absorption at 260 nm. Hence, the extinction coefficient for DNA at 254 nm (ε_{254}) was approximately 3% lower than the extinction coefficient for DNA at the optimal value of 260 nm (ε_{260}). Therefore, a 254 nm UV lamp was suitable for the detection and quantification of migrating DNA.

3.3.2 Detection Limits

Fluorescence detectors mounted on capillaries enable fentograms of the fluorescently labelled analyte to be detected but requires preparative labelling or staining of the analyte. Furthermore, variations in densities of the label associated with the analyte can lead to variations in peak migration times (Butler et al. 1995) and thus, interfere with peak resolution. Moreover, retention of the label or staining material can complicate subsequent analyses (McCord et al. 1993). UV detectors mounted on capillaries have been reported to be approximately 6 times less sensitive than fluorescence detectors (Milofsky and Yeung 1993) without the inherent problems associated with fluorescent dyes or tags. The detection of DNA using a UV lamp operating at 254 nm has been shown to be suitable for

the detection of DNA, but the slightly lower sensitivity prompted an analysis of the lower limit of DNA detection using CE equipped with a 254 nm UV lamp.

This was undertaken using a PCR product of a 404 bp fragment of the IL-2 gene, which was isolated using polyacrylamide gel electrophoresis and purified by organic extraction as outlined in Section 2.10. The concentration of the purified DNA fragment was estimated to be 32 ng/mL by spectrophotometric analysis. The DNA was prepared at concentrations of 2.5 ng/mL, 1.25 ng/mL, 625 pg/mL, 312 pg/mL, and 156 pg/mL then an electropherogram of each dilution was acquired. Each sample was injected using an electrokinetic voltage of 7 kV for 60 sec and an electric field of 210 Vcm^{-1} was applied during acquisition. The lower detection limit of 312 pg/mL was found to be reproducible upon replicate analyses. The signal to noise ratio of the electropherogram at this concentration was approximately 6. An electropherogram representing 312 pg/mL of DNA is displayed in Fig. 2. This limit of detection is comparable to that obtained by others using CE apparatus equipped with UV lamps (Kasper et al. 1988). Although the sensitivity of UV detectors employed in CE analysis can be enhanced by chelating ethidium bromide to the DNA during electrophoresis, this has been reported to increase peak widths and decrease the reproducibility of DNA analysis (Singhal and Xian 1993).

3.3.3 Reproducibility -pUC 19 Analysis Using CE

The reproducibility of DNA migration was investigated by monitoring the migration time of adjacent peaks from electropherograms of pUC 19 plasmid. 500 pg/μL of the plasmid was electrokinetically injected for 60 sec then an electric field of 210 Vcm^{-1} was applied during acquisition. Fig. 3 is a representative electropherogram of the constituent DNA fragments of pUC 19. The reproducibility of CE was determined by monitoring the migration time of the 404 bp and 489 bp fragments on 6 separate days over an 8 day period. The average migration time of peak 1 (404 bp) was 22 min and 15 sec with a standard deviation of $\pm$ 24 sec. The average migration time for peak 2 (489 bp) was 23 min and 33 sec $\pm$ 26 sec. Variation in the migration time of each peak over the 8 day period is represented graphically in Fig. 4.

3.3.4 Quantification of DNA Using CE

CE is an attractive method for the quantification of PCR products because no post-amplification manipulation of the products is required. The reproducibility of CE for the quantification of DNA was assessed by monitoring the area beneath adjacent peaks in an electropherogram of pUC 19. 500 pg/μL of pUC 19 DNA was electrokinetically injected for 60 sec then an electric field strength of 210 Vcm^{-1} was applied to achieve separation. The reproducibility of DNA quantification was determined by monitoring the area beneath the 404 bp (peak 1) and 489 bp (peak 2) peaks acquired from six electropherograms collected on different days over an 8 day period. The ratio of the area beneath the peaks over the 8 day acquisition period is represented in Fig. 4. The percentage ratio of area peak 1:area peak 2 for the acquisition of 6 electropherograms was 81.6% $\pm$ 2.0 %.

Although the molar concentration of each DNA fragment in pUC 19 digested with *Hpa* II is equal, Fig. 3 illustrates that the area beneath each peak of pUC 19 was different. Beers law predicts that the absorption of UV light is directly proportional to the density of DNA (mass/unit volume) therefore, the variation in UV absorption between pUC 19 DNA fragments is largely attributable to the different length fragments having different extinction coefficients (ε_0).

3.3.5 Electrokinetic Loading of Different Sized DNA Fragments

The preferential loading of shorter lengths of DNA (with a low frictional coefficient f) into a capillary using electrokinetic injection has been reported by Kleparnik et. al. (Kleparnik et al. 1995). The products from a competitive PCR differ in length and have different frictional coefficients which may result in preferential loading of one product during electrokinetic injection. The electrokinetic injection of different sized DNA fragments was therefore examined to determine whether preferential loading affected quantification.

The 404 bp IFN-γ target and 321 bp IFN-γ competitor PCR products were isolated using polyacrylamide gel electrophoresis then purified by organic extraction as outlined in Section 2.10. The concentrations of the respective target and competitor DNA fragments were determined spectrophotometrically then eight mixtures were prepared, each of which

contained a different amount of the target and competitor as indicated in Fig. 5. The weight of the target as a fraction of the combined weight of the target and competitor in each mixture was calculated, following which an electropherogram of each was acquired using electrokinetic injection (7 kV for 60 sec) and an electric field strength of 260 Vcm^{-1}. Each electropherogram displayed two distinct peaks with migration times identical to the target and competitor. A representative electropherogram of a single mixture of the target and competitor is displayed in Fig. 6. The respective peaks from each electropherogram were integrated and the area of the target as a fraction of the combined target and competitor areas was calculated. A comparison between the fractional weight and fractional area of the target in each sample is presented in Fig 5.

The fractional weight of the target DNA varies by an average of 3.75% from the fractional area of the target DNA calculated for each sample. This difference was relatively constant over the entire range indicating that there was no preferential electrokinetic injection of the target and competitor DNA fragments despite there being approximately 100 bp difference in length. Hence, the relative area represented by the peak ratio in an electropherogram accurately portrayed the relative weight of DNA in the analyte.

3.4 Preparation of PCR Products for Analysis using CE

Crude PCR products contain residual salts, nucleotides and primers that promote conductivity, peak broadening (Oefner et al. 1992) and decrease the performance of a capillary (Martin et al. 1993). The salts used for the purification of crude PCR products also increase the thermal conductivity during electrophoretic separation which contributes to peak broadening. The electrokinetic injection of analytes provides superior quantification and higher peak resolution than that obtained using hydrostatic injection (Ermakov et al. 1994). However, the successful electrokinetic injection of PCR products requires a desalting step to limit injection of more mobile inorganic salts in preference to the larger, less mobile DNA products. The crude PCR products were therefore desalted and purified prior to analysis using CE.

3.4.1 Desalting of PCR Products

A method to purify and desalt the crude PCR products in preparation for electrokinetic injection and CE analysis was required. Several methods were assessed. IFN-γ target and competitor PCR product fragments (isolated using polyacrylamide gel electrophoresis and purified by organic extraction) were divided into four aliquots.

One sample was desalted using Microcon Spin Columns, in which DNA above 30 kDa. was retained in solution while small inorganic salts were removed. An electropherogram of the purified PCR product was acquired using electrokinetic injection (5 kV for 60 sec) and a running voltage of 210 Vcm^{-1} over a 30 min acquisition period. In the presence of mobile inorganic salts, a small amount of DNA is injected into the capillary and as a result, small peaks representing the DNA are observed in the electropherogram. However, in the absence of inorganic salts, large distinct peaks representative of DNA are observed. Therefore, the suitability of the desalting method was evident in the electropherogram of the desalted PCR products. The separation, height and width of the target and competitor peaks was therefore assessed as an indication of the quantity and purity of DNA. Although Microcon Spin Columns were found to be simple and rapid, and gave rise to high, narrow and well resolved peaks (electropherogram not shown), the columns were too expensive for routine use.

The second sample was desalted using sodium iodide and isopropanol (Wang et al. 1994). 3 μL of a mixture containing 7.6 M sodium iodide and 0.4% SDS was mixed with 10 μL of isopropanol and 20 μL of the crude PCR product. The DNA was pelleted by centrifugation at 4°C for 10 min then washed with 120 μL of 40% isopropanol, inverted and left to dry for one hour at room temperature. The sample was reconstituted with 10 μL of Milli-Q water and an electropherogram was acquired. This desalting method was fast and reproducible. However, the electropherogram consistently displayed a low signal to noise ratio in addition to a large broad peak that had the potential to mask some low molecular weight PCR products and disrupt the reproducibility of CE. This method of desalting the crude PCR products was therefore abandoned.

The third sample was desalted by mixing 40 µL of ethanol with 20 µL of the crude PCR product (Sambrook et al. 1989). The mixture was cooled to -70°C for 30 min following which the DNA was pelleted by centrifugation at 4°C for 15 min. The sample was washed with 120 µL of 70% ethanol, inverted and left to dry for one hour at room temperature and subsequently reconstituted with 10 µL of water. An electropherogram was then acquired. Since the resultant electropherograms consistently displayed no peaks above a flat baseline, this method was also abandoned.

The fourth sample was desalted using sodium acetate and ethanol (Sambrook et al. 1989). 2 µL of 4 M sodium acetate and 60 µL of ethanol were mixed with 20 µL of the crude PCR product. The DNA was cooled to -70°C then pelleted by centrifugation at 25°C for 15 min. The pellet was washed with 120 µL of 70% ethanol, inverted and left to dry for one hour at room temperature. Following reconstitution of the sample with 10 µL of Milli-Q water an electropherogram was acquired. It displayed high, narrow and well resolved peaks with a low level of background noise. The method was reproducible and to the extent that any residual contamination of DNA with salts may have occurred, this did not interfere with the electrokinetic injection procedure. This proved to be the most efficient procedure for desalting and concentrating the crude PCR products for CE analysis and was therefore chosen as the preferred method for preparing PCR products for CE analysis and was used throughout the remaining work in this thesis.

3.4.2 Reproducibility of DNA Precipitation Using Sodium Acetate/Ethanol

The recovery of DNA using sodium acetate/ethanol to desalt the crude PCR products was examined. Triplicate samples containing 325 ng/µL of salmon sperm DNA in 20 µL of Milli-Q water were prepared then mixed with 2 µL of 4 M sodium acetate and 60 µL of ethanol. Each sample was cooled to -70°C for 30 min and the DNA was then pelleted by centrifugation at 25°C for 15 min. The samples were washed with 120 µL of 70% ethanol, inverted and left to dry for one hour at room temperature. All samples were then reconstituted with 50 µL of Milli-Q water then the concentration of DNA was estimated spectrophotometrically and the yield calculated. The mean yield of the three samples was 59% ± 7% (mean ± SD).

3.5 Optimisation of DNA Precipitation Using Sodium Acetate/Ethanol

It was necessary to optimise the conditions by which DNA was precipitated to maximise the yield of the desalted product.

3.5.1 Effect of Temperature on Pelleting DNA

The recovery of DNA using sodium acetate/ethanol in Section 3.4.2 was on average only 59% therefore, the method of desalting the PCR products was further modified in an attempt maximise the recovery of DNA. The temperature at which pelleting of the DNA is carried out has been reported to be a critical factor influencing the yield of product (Sambrook et al. 1989). The recovery of DNA pelleted at different temperatures was therefore examined. 20 ng/mL of salmon sperm DNA was prepared then duplicate mixtures were quantified spectrophotometrically and precipitated by adding sodium acetate/ethanol as detailed in Section 3.4.2. The samples were cooled to -70°C for 30 min then pelleted at either -4°C, 4°C or 25°C prior to being washed with 120 µL of 70% ethanol at the same temperature. The precipitated DNA was spectrophotometrically quantified and the yield was calculated. The relationship between the yield and pelleting temperature is represented in Table 1. The yield as a percentage of the initial amount of DNA fell from approximately 90% at -4°C and 4°C to 65% at 25°C. Thus, in the remaining work of this thesis DNA was desalted with sodium acetate/ethanol and pelleted at 4°C.

3.5.2 Reproducibility of DNA Precipitation Using the Optimised Sodium Acetate/Ethanol Precipitation Method

As shown above (Section 3.5.1), in duplicate samples the mean yield of DNA at 4°C was 90%. The reproducibility of this method was assessed further by precipitating five replicate samples containing 20 ng/mL of salmon sperm DNA using sodium acetate/ethanol. 2 µL of 4 M sodium acetate and 60 µL of ethanol were mixed with 20 µL of each sample. The DNA was cooled to -70°C then pelleted by centrifugation at 4°C for 15 min (at 13 x g). Each sample was washed with 120 µL of 70% ethanol at 4°C, inverted and left to dry for one hour at room temperature. 50 µL of Milli-Q water was used to

reconstitute each sample and the recovered DNA was quantified spectrophotometrically. The average yield was 81.4% ± 10%.

3.5.3 Effect of DNA Concentration on Yield of Precipitation

Precipitation of low quantities of DNA is enhanced when a small amount of heterogeneous DNA carrier is added to the sample and co-precipitated with the DNA of interest (Sambrook et al. 1989). The precipitation of small amounts of DNA was investigated in the absence of carrier DNA. Five different concentrations of a salmon sperm DNA sample between 320 ng/mL and 32 ng/mL were prepared then each sample was spectrophotometrically quantified and precipitated using sodium acetate and ethanol as detailed in Section 3.5.2. Following precipitation, the samples were spectrophotometrically re-quantified and the yield of DNA in each sample was calculated. The percentage yield at each concentration of DNA remained approximately the same over the range tested (approximately 78%) as outlined in Table 2. PCR amplification of DNA is capable of producing up to 5 µg of DNA from 500 ng of starting template (Rodu 1990) suggesting the method is suitable for the precipitation of PCR products in preparation for CE analysis without the addition of carrier DNA.

3.5.4 Sodium Acetate Precipitation Efficiencies at Different Target and Competitor Concentrations

Although it was shown in Section 3.3.5 that there was no preferential loading of different lengths of DNA during elecrokinetic injection into the capillary, selective precipitation of different lengths of DNA could be another factor affecting the quantification of DNA. Differential precipitation of DNA fragments using sodium acetate/ethanol was therefore assessed.

IFN-γ target (404 bp) and competitor (321 bp) were amplified by PCR then isolated using polyacrylamide gel electrophoresis and purified by organic extraction. The concentration of the respective target and competitor DNA fragments were measured spectrophotometrically. Four samples in which the target DNA constituted 10%, 25%, 78% and 95% (by weight) of the total (target + competitor) DNA were prepared. An

electropherogram of each sample was acquired before and after sodium acetate/ethanol precipitation, using a 7 kV loading voltage for 60 sec and an electric field strength of 210 Vcm^{-1}. The area beneath the target and competitor peaks within an electropherogram were measured and the ratio of the respective peak areas was calculated. A comparison of the ratio of peak areas before and after precipitation is presented in Fig. 7. This figure demonstrates that the area of the target as a fraction of the combined target and competitor areas is very similar before and after precipitation. Thus, an electropherogram accurately portrays the relative amount of DNA constituting the analyte when sodium acetate/ethanol is used to precipitate the DNA.

3.5.5 Heteroduplex Formation During PCR Product Analysis

Secondary structure in oligonucleotides has been reported to play an important role in influencing DNA migration during CE analysis (Bianchi et al. 1993; Satow et al. 1993). Similarly, sequence similarities between the target and its respective competitor may promote DNA heteroduplex formation which may limit the accuracy of the competitive PCR and CE procedures. The presence of heteroduplexes in PCR products was investigated using IFN-γ target and competitor which had been purified using polyacrylamide gel electrophoresis and organic extraction. Equal volumes of each purified DNA fragment were mixed and an electropherogram of an aliquot was acquired under standard electrophoretic conditions. The expected two peaks representing the target and competitor DNA fragments were observed in the electropherogram (not shown). An aliquot of the starting mixture was then incubated at 60°C for 5 min and analysed using CE. The presence of several additional high molecular weight bands in the electropherogram (not shown) was interpreted as the formation of heteroduplex which resulted from heating the PCR products. Therefore, it was concluded that heteroduplexes did not form during CE analysis of competitive PCR products unless the products were heated to 60°C. The competitive PCR products are conventionally maintained at or below 26°C and at this temperature there is likely to be insufficient thermal energy available to promote heteroduplex formation. Heteroduplex formation was absent during conventional analysis of PCR products.

3.6 Summary

A study was undertaken to assess the suitability of CE for the quantification of PCR products. Gel filled capillaries were able to sustain more injections and were more reliable than polyacrylamide filled capillaries for the analysis of DNA. A 254 nm UV detector was appropriate for the detection and quantification of DNA and the lower limit of detection using this wavelength was 312 pg/mL. CE was found to be reproducible in that (i) the retention time of peaks in an electropherogram varied by less than 2.2% over an 8 day acquisition period and (ii) electrokinetic injection did not affect the quantification of DNA in the sample.

PCR products needed to be desalted prior to electrokinetic injection and CE analysis. Sodium acetate/ethanol precipitation of the DNA was shown to be compatible with electrokinetic injection and CE analysis. An average 81% yield of DNA was obtained using this procedure and as little as 32 ng/mL of DNA could be reliably precipitated. DNA precipitated using sodium acetate/ethanol was shown to reflect accurately the composition of the test DNA sample since different sized DNA fragments were precipitated equally.

DNA UV Absorbtion Data
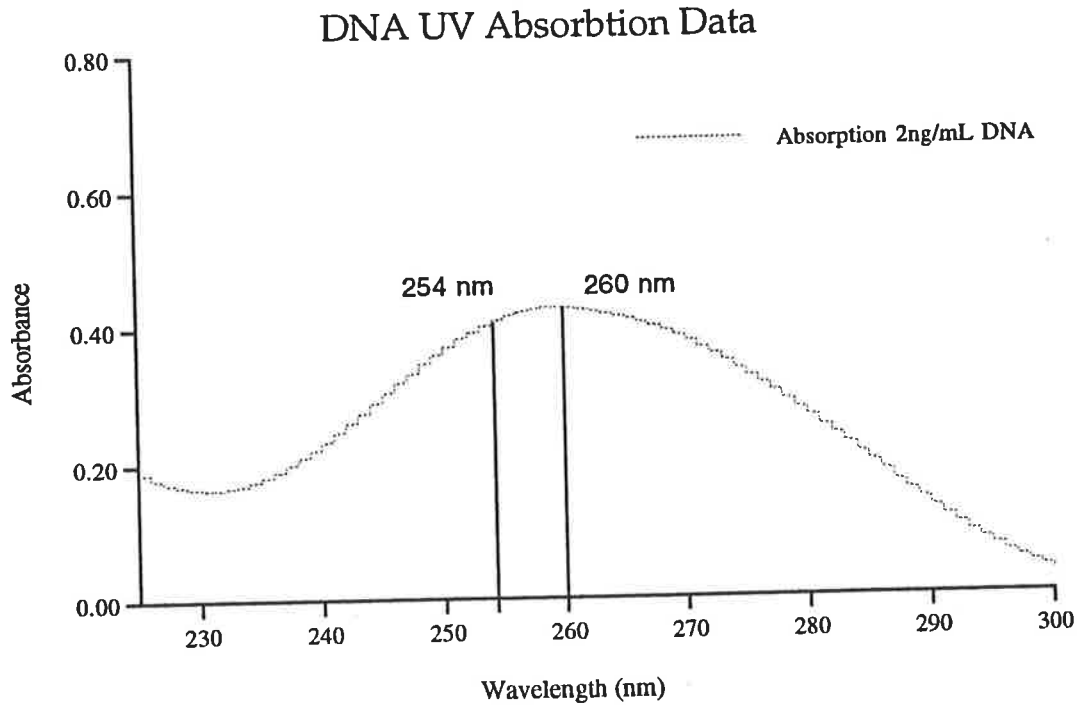
Figure 1. UV absorbance spectrum of 2 ng/mL DNA obtained from salmon sperm.

Capillary Electrophoresis Detection Limit
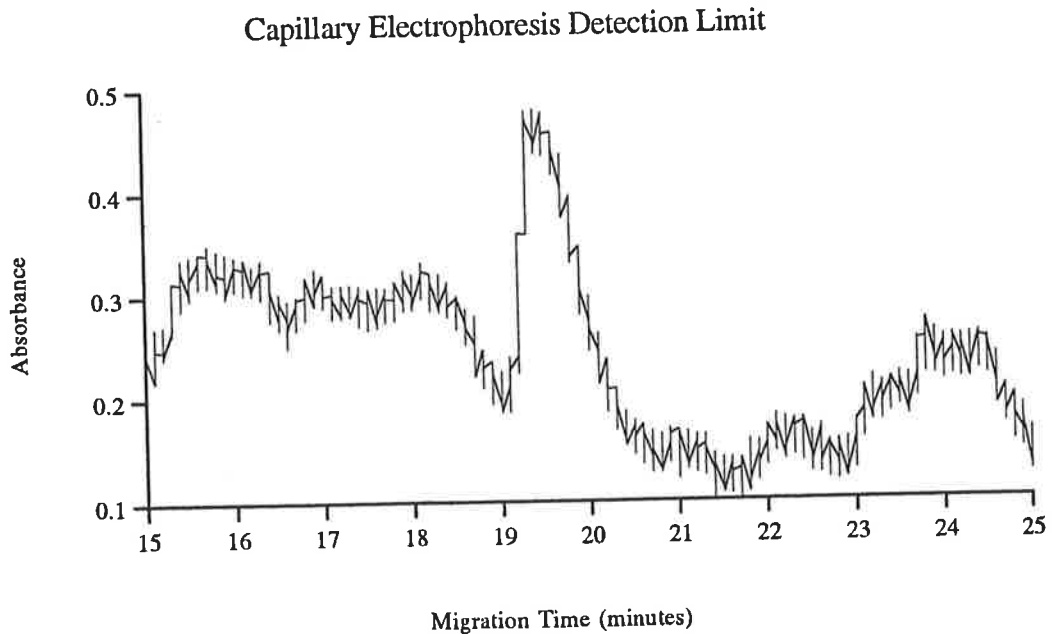
Figure 2. An electropherogram representing 312 pg/mL of IL-2 PCR product (410 bp). A 57 cm (from injection to UV window) DB-17 capillary (i.d. = 100 μm) was filled with 0.5% hydroxymethylpropyl cellulose. DNA was electrokinetically injected at 5 kV for 60 sec and separation was achieved between 25°C and 26°C using an applied voltage of 260 Vcm^{-1}.

pUC 19 Electropherogram
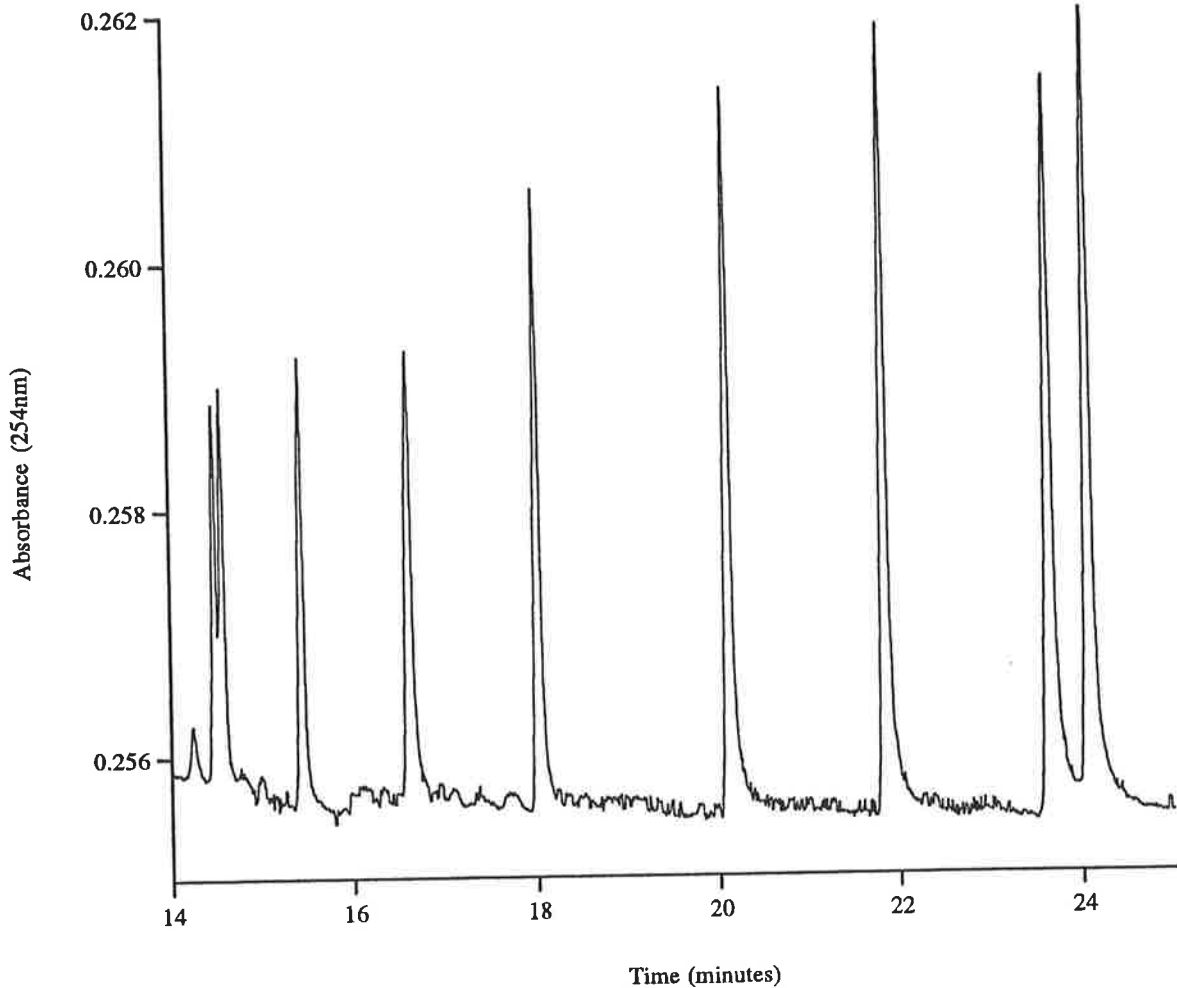
Figure 3. An electropherogram illustrating the resolution of pUC 19 DNA digested with *Hpa* II having fragment lengths of 501 bp (24 min), 489 bp, 404 bp, 331 bp, 242 bp, 190 bp, 147 bp, 111 bp and 110 bp. A 57 cm (from injection to UV window), DB-17 capillary (i.d. = 100 μm)) was filled with 0.5% HMPC. DNA was injected at 5 kV for 1 min and separation was performed at 210 Vcm^{-1}.

Capillary Electrophoresis Peak Reproducibility
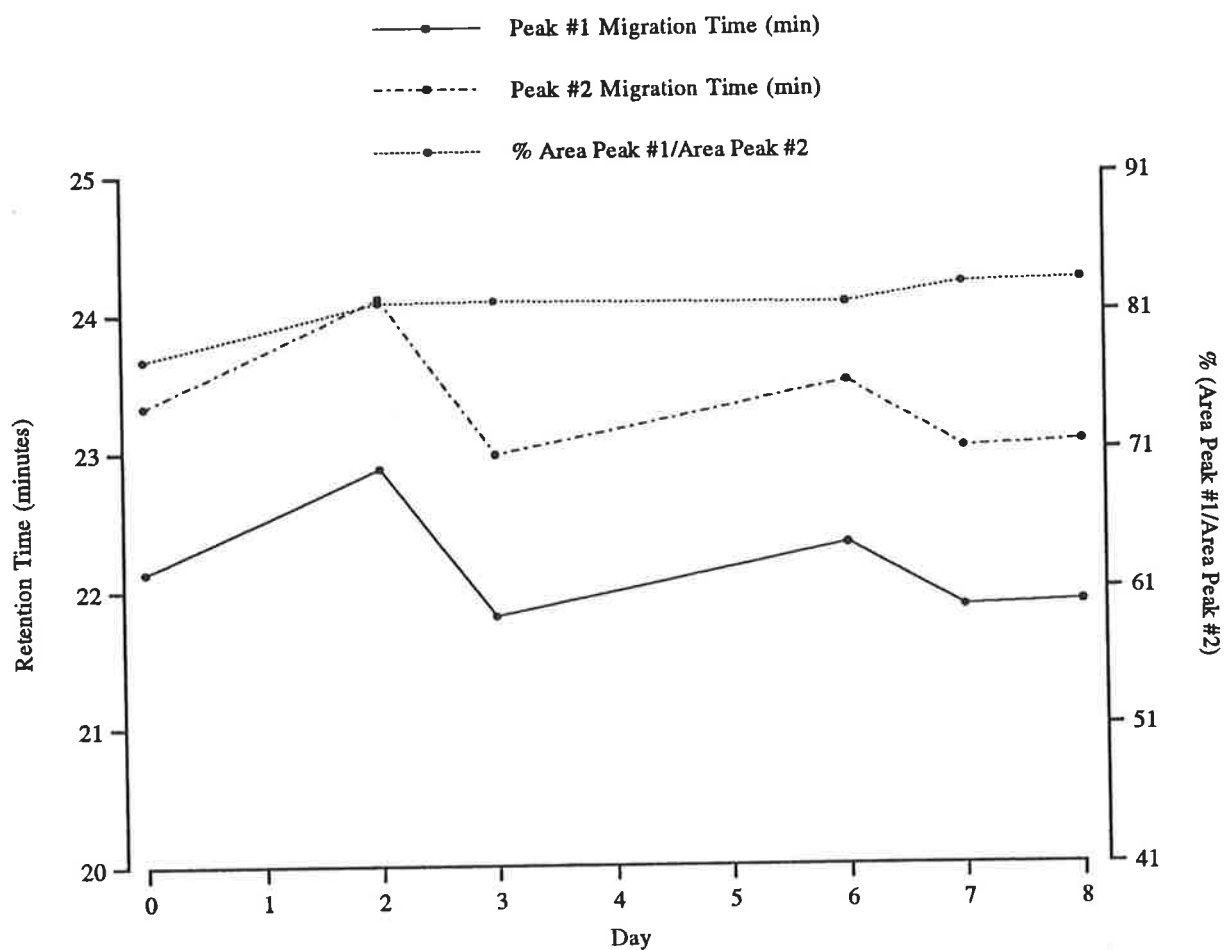
Figure 4. The reproducibility of CE applied to DNA analysis. Retention times of 2 adjacent peaks from a pUC 19 *Hpa* II restricted plasmid were monitored over 8 days. Peak 1 = 404 bp and peak 2 = 489 bp. The retention time of peak 1 varied by 1.08% and the retention time of peak 2 varied by 1.11%. The percentage ratio of peak areas ((area peak 1:area peak 2) x 100%) varied by 2.0% over the 8 day period.

Electrokinetic Loading Efficiency
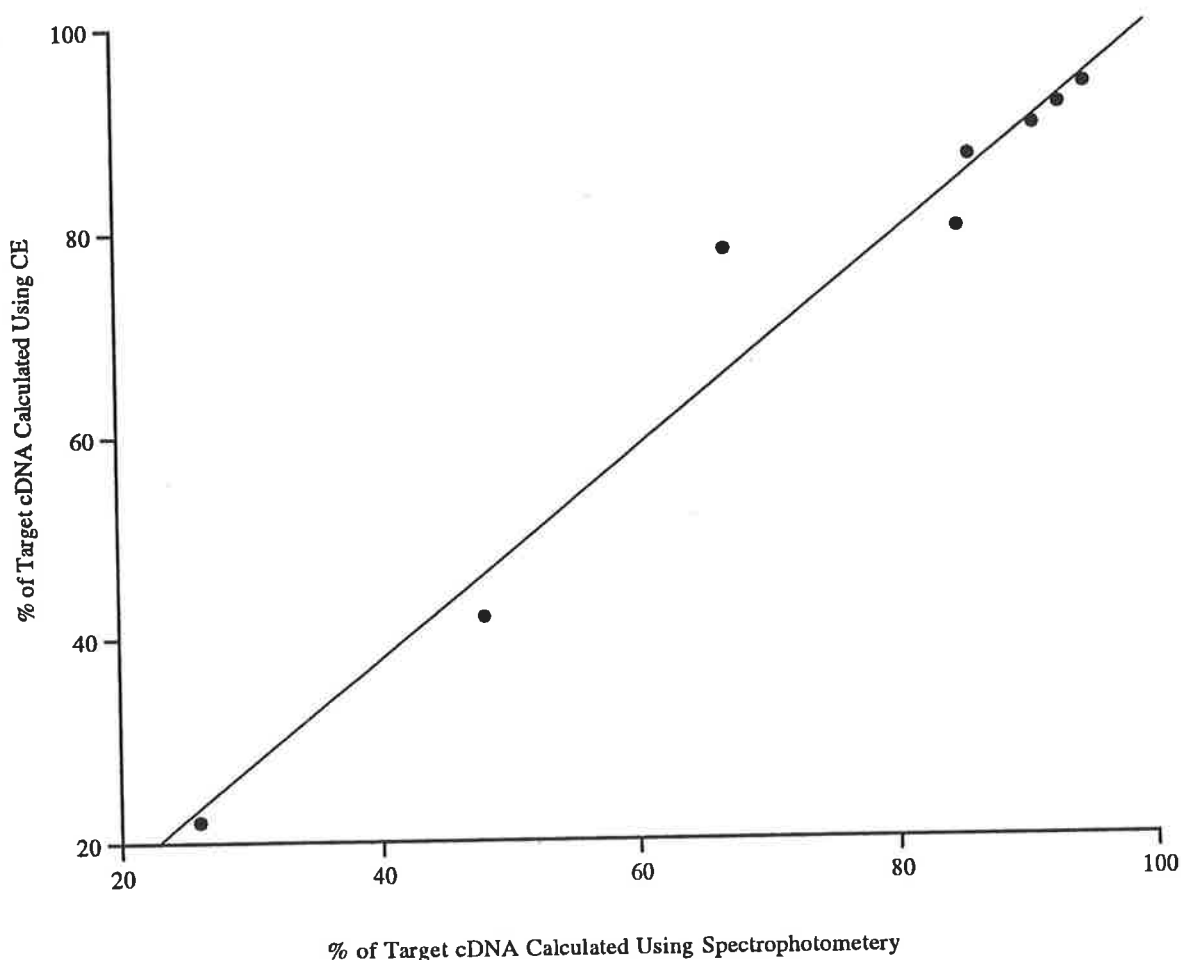
Figure 5. IFN-γ target was quantified spectrophotometrically and added into eight mixtures, each containing a different amount of IFN-γ competitor. An electropherogram of each mixture was acquired and the area beneath the IFN-γ target peak was calculated. The relative amount of target is plotted as a function of the relative area of the target peak calculated from each electropherogram. The line of best fit has the equation $y = 1.039x - 3.618$ $R^2 = 0.916$.

IFN-γ Electropherogram
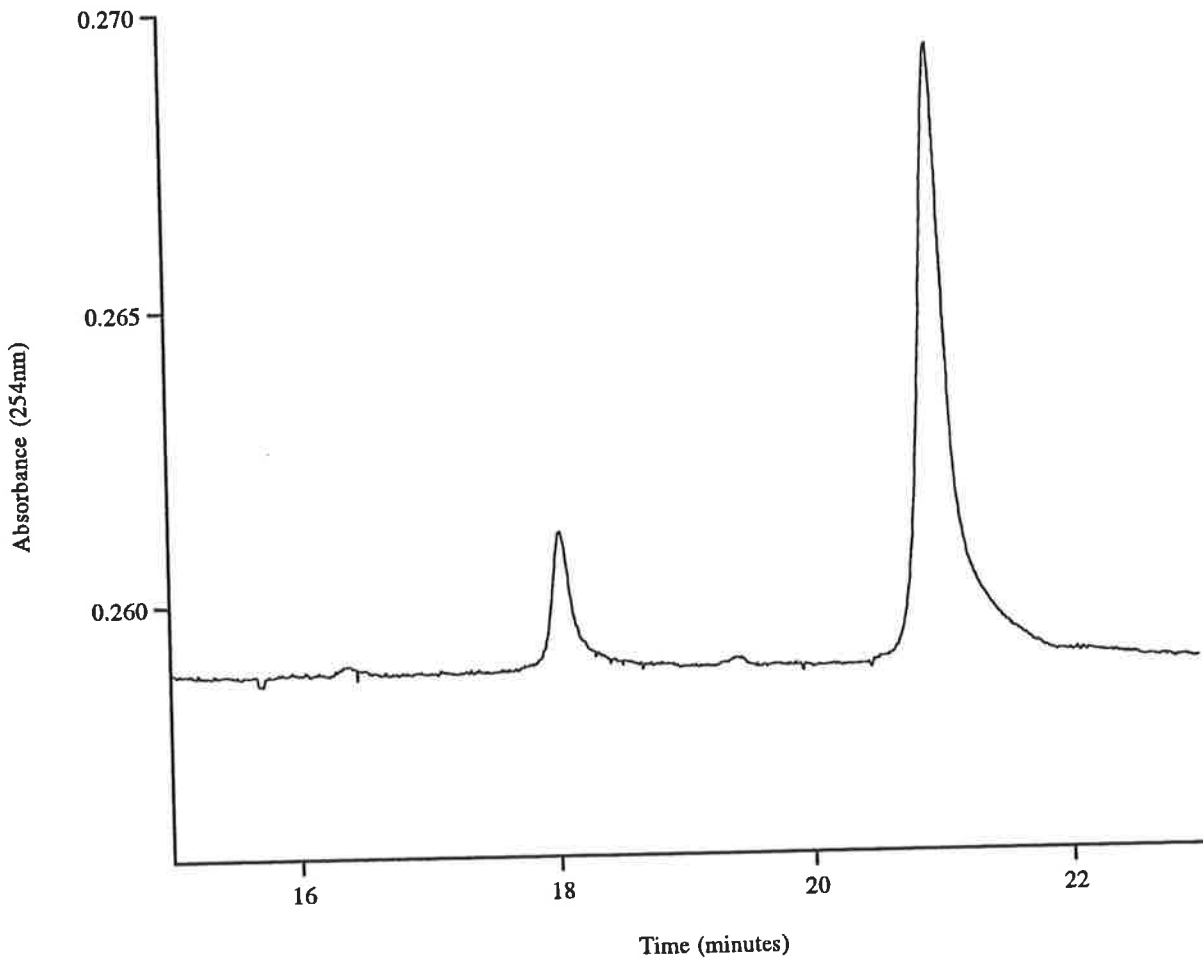
Figure 6. A representative electropherogram showing resolution of purified IFN-γ target (404 bp) and competitor (321 bp) PCR products. Migration times are 21 min for the target and 18 min for the competitor. A 57 cm (from injection to UV window), DB-17 capillary having an internal diameter of 100 μm was filled with 0.5% hydroxymethylpropyl cellulose. DNA was introduced into the capillary using electrokinetic injection at 7 kV for 60 sec and separation was achieved between 25°C and 26°C using an applied voltage of 260 Vcm^{-1}. A 254 nm UV detector measured DNA migration.

DNA Pelleting Temperature (°C)	Percentage Yield of DNA
-4	90.5
+4	93.5
+25	63.0

Table 1. Effect of pelleting temperature on DNA yield. Each point represents the average of 2 precipitation experiments (n=2).

DNA Concentration (ng/mL)	DNA yield (%)
32	75
83	75
151	82
235	84
320	75

Table 2. The percentage yield of DNA obtained by precipitating different concentrations of salmon sperm DNA with sodium acetate/ethanol. The average yield of DNA over the range of DNA concentrations examined was 78%.

DNA Precipitation Efficiencies
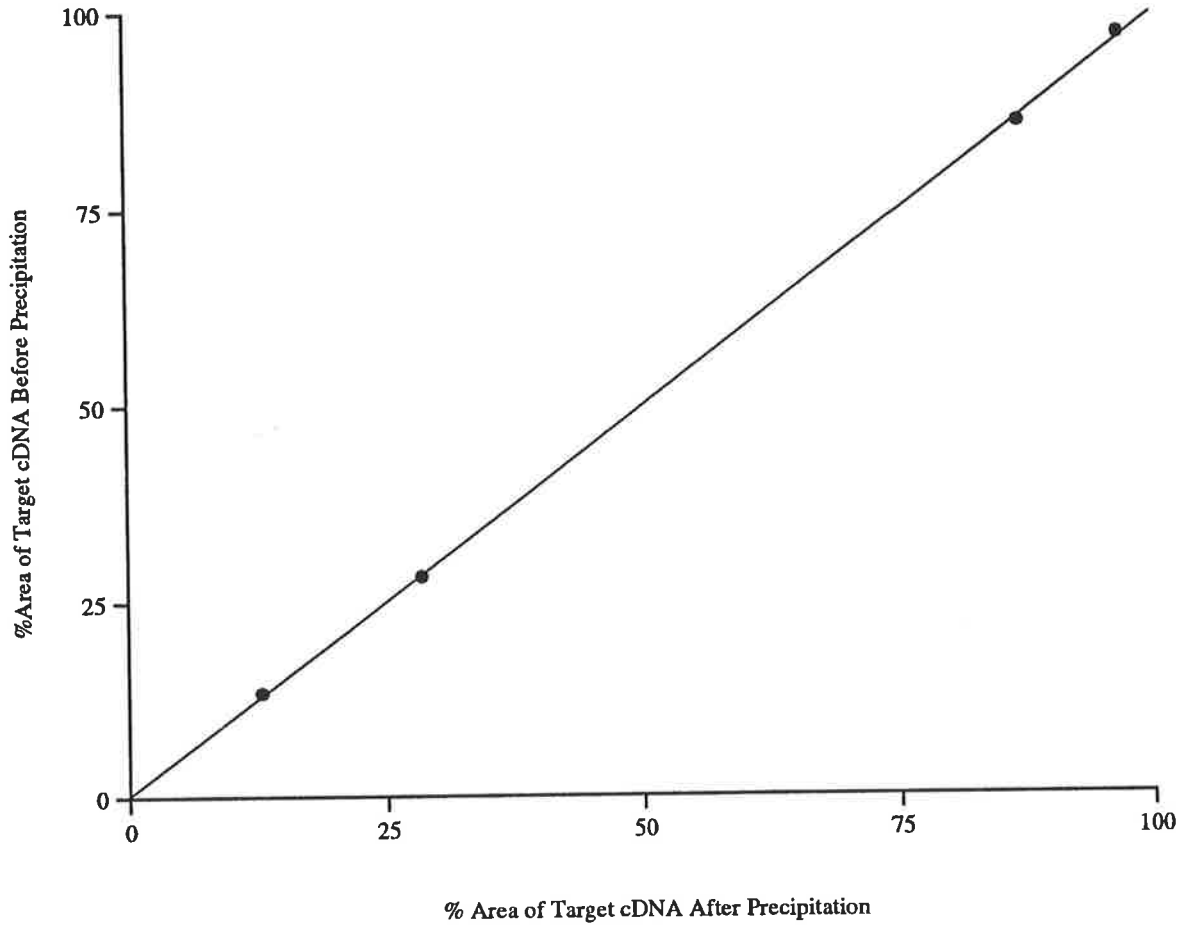
Figure 7. Graphical representation of the percentage area of target cDNA in a mixture before precipitation and the percentage area of the target cDNA after precipitation with sodium acetate/ethanol. A line of best fit has the equation $y = 0.989x + 0.356$ $R^2 = 1.0$

Chapter 4

Competitive PCR

4.1 Introduction

The reverse transcription polymerase chain reaction (PCR) is a sensitive technique that employs oligonucleotide primers to amplify a specific cDNA target from a heterogeneous cDNA sample. Although PCR has been widely used as a qualitative technique, the exponential accumulation of products has the potential to confound quantification. Several methods have been devised to make PCRs more quantifiable. For example, the target cDNA can be quantified relative to a constitutively expressed gene (Noonan and Robinson 1991) or to a known amount of the target cDNA that is amplified in a separate reaction (Ballagi Pordany et al. 1991). These methods may not compensate for the exponential nature of the amplification process, where tube to tube variations can result in differential accumulation of products.

Quantification using a competitive reaction involves the generation of an internal standard curve by co-amplifying serial dilutions of a mutated cDNA competitor with constant amounts of the target cDNA. Competitive PCR has the advantage that it obviates variability in the rate of amplification between different tubes. Numerous competitive PCR procedures have been reported, with a limited number addressing the quantitative nature of the competitive reaction (Santagati et al. 1993). A mathematical model of competitive PCR provided a logical theoretical framework to analyse this key issue more formally (Raeymaekers 1993). PCR assays were used to quantify cDNA for the key T cell rat cytokines IL-2, IFN-γ and IL-4 as well as TNF and TGF-β. The performance of these assays was compared with that predicted by the mathematical model of the competitive

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PCR. The storage and dilution of DNA was established and the lower limit of detection of each PCR assay was assessed. The amplified PCR products were quantified by the capillary electrophoresis (CE) detection system discussed in Chapter 3. The accuracy of the competitive PCRs assays was assessed by comparison with spectrophotometric quantification of purified target cDNA fragments undertaken prior to dilution and subsequent PCR amplification. The mathematical model of competitive PCR was used to formulate a correction strategy which accounted for differences in the amplification efficiencies between the target and competitor DNA fragments. The correction strategy was applied to each competitive PCR and the accuracy of each assay was reassessed.

4.2 Mathematical Modelling of Competitive PCR

4.2.1 Mathematical Model of Competitive PCR

The exponential accumulation of PCR products (before plateau is reached) is well documented and the reaction has been described mathematically as follows (Raeymaekers 1993): The initial amount of target sequence (T_0) amplifies with an efficiency (E_t) over (j) cycles to yield a final amount of PCR product (T_j) according to the equation:

$$T_j = T_0 (1 + E_t)^j$$

Similarly for a competitor (C)

$$C_j = C_0 (1 + E_c)^j$$

From these two equations it can be shown that

$$\log (T_j/C_j) = [\log T_0 - \log C_0] + j \log [(1+E_t)/(1+E_c)] \qquad (1)$$

or $\qquad$ y $\qquad$ = $\qquad$ c $\qquad$ + x $\qquad$ m

Equation 1 predicts a straight line of the form $y = c + xm$ when fixed amounts of the target (T_0) and competitor (C_0) are co-amplified for different cycle numbers (j) to give rise to the

products T_j and C_j. Log $[(1+E_t)/(1+E_c)]$ represents the gradient of the line (m), while log T_0 - log C_0 constitutes the y-intercept (c).

When the amplification efficiency of the target (E_t) is identical to that of the competitor (E_c), log $[(1+E_t)/(1+E_c)]$ equals zero and equation 1 is simplified to equation 2.

$$\log (T_j/C_j) = \log T_0 - \log C_0 \qquad (2)$$

$$y \qquad = \quad c \qquad -1 \quad x$$

Equation 2 predicts a straight line of the form $y = c - x$ when different amounts of the competitor (C_0) are co-amplified with a constant amount of the target (T_0) for a fixed number of cycles. When the final ratio of PCR products, $\log(T_j/C_j)$, is plotted as a function of the initial amount of competitor (C_0) spiked into each tube prior to amplification, a line with a theoretical gradient of -1 is expected. In such a case the amount of target can be quantified by interpolating the x-axis value which will occur when the final amount of target is equal to that of the competitor (therefore $\log (T_j/C_j) = 0$ and $\log T_0 = \log C_0$).

4.2.2 Mathematical Correction of Competitive PCR

There are few published reports analysing the kinetics of competitive PCRs despite the theory being well established (Katz 1994; Lubin et al. 1991). Mathematical modelling of the competitive reaction provides an opportunity to apply a correction factor formulated from observed differences in the amplification efficiency of the target and its competitor.

Accurate quantification of the PCR using the competitive method is based on the assumption that the target and competitor amplify with the same efficiency (ie. $E_t = E_c$). This however, may not always be a correct assumption and if Et $\neq$ Ec then j log $[(1+E_t)/(1+E_c)] \neq 0$. In accordance with equation 2, co-amplification of different amounts of the competitor with a constant amount of the target provides a straight line with a gradient of -1 and a y-intercept (c) such that c deviates by an amount c' which is representative of the difference in the amplification efficiencies (ie. j log $[(1+E_t)/(1+E_c)]$) as outlined in the equation below:

$$\log (T_j/C_j) = \log T_0 - \log C_0 + j \log [\ (1+E_t)/(1+E_c)\ ]$$

$$y \qquad = \quad c \quad -1x \qquad\qquad + c'$$

When the amplification efficiencies differ the y intercept (c) is composed of two contributing factors namely, $\log(T_0)$ and $j \log [\ (1+E_t)/(1+E_c)\ ]$ such that,

$$c = \log(T_0) + j \log [\ (1+E_t)/(1+E_c)\ ]$$

The amount of target (T_0) in an unknown sample can be calculated by including differences in the amplification efficiencies of the target and competitor as outlined in equation 3.

$$\log(T_0) = c - j \log [\ (1+E_t)/(1+E_c)\ ] \qquad\qquad (3)$$

The absolute quantity of the target will be inaccurately estimated if the amplification efficiency of the target differs from that of the competitor ($E_t \neq E_c$). When the final ratio of PCR products, $\log(T_j/C_j)$, is plotted as a function of the initial amount of competitor (C_0) spiked into each tube prior to amplification, a straight line with a theoretical gradient of -1 is expected. Competitive PCR is relatively quantitative if $E_t \neq E_c$ as the straight line is translated along the y-axis by an amount (c') that is indicative of differences in the amplification efficiencies. The preferential amplification of one PCR product therefore induces an error in absolute quantification which can only be determined in experiments in which known amounts of both target and competitor DNA are used. Experiments utilising variable known amounts of target and competitor DNA templates were undertaken in order to establish, using equation 3, an empirically determined correction factor that could be used subsequently to render assays more accurately quantitative.

4.3 Evaluation of the Competitive PCR Using the Mathematical Model

4.3.1 Storage and Dilution of Target and Competitor cDNA Fragments

Storage of the competitor cDNA fragments is fundamental for accurate and reproducible quantitative analysis using competitive PCR. The conditions by which the competitor fragments were stored was therefore assessed in an attempt to maximise the accuracy and reproducibility of the assays. IFN-γ competitor was isolated using PAGE, purified by phenol/chloroform extraction (Section 2.12) and stored at -70°C to limit DNAse activity. However, at -70°C the DNA precipitated, which came to attention after replicate aliquots displayed a 2.5 fold variation in the quantity of DNA estimated using spectrophotometry. This variation in the quantity was eliminated when the stored DNA was incubated at 65°C for 10 min prior to spectrophotometric quantification. This procedure was repeated on four replicate cDNA samples and the quantity of DNA estimated spectrophotometrically was essentially identical upon each analysis. DNA fragments were therefore stored at -70°C then incubated for 10 min at 65°C prior to use. This procedure was shown to maintain stable, reproducibly quantifiable amounts of DNA as assessed by UV absorbance at 260 nm and was adopted as the preferred method of DNA storage and treatment for the remaining work of this thesis.

4.3.2 Detection Limits of PCR Assays

A single copy of DNA can theoretically be amplified up to 10^9 copies in 30 cycles of PCR amplification (Erlich et al. 1991). This capacity for amplification prompted an assessment of the lower limit of detection of each cytokine PCR assay. IFN-γ, IL-2, IL-4, TNF and TGF-β target and competitor PCR products were isolated using PAGE, purified by phenol/chloroform extraction (Section 2.10) and then quantified spectrophotometrically. The detection limit of each cytokine assay was determined by serially diluting each purified DNA fragment from approximately 30 ng/µL to 30×10^{-10} ng/µL. All dilutions were amplified using PCR for 40 cycles as outlined in Section 2.4. The reproducible detection limit of each cytokine PCR assay is listed in Table 1. All PCR assays were able to detect less than 500 copies of DNA and the lowest limit was approximately 30 copies.

The sensitivity of the PCR assays indicates that each is suitable for detecting very low quantities of cytokine cDNA.

4.3.3 Relative Amplification Efficiency of IFN-γ and its Competitor

To assess the relative amplification efficiencies of the IFN-γ target and its competitor cDNA fragments, similar amounts of each were co-amplified for 23, 25, 27, 30, 33 and 35 cycles (Fig. 1). Following amplifications for the specified number of cycles, the target:competitor ratio was calculated using capillary electrophoresis. A plot of this ratio as a function of the cycle number provided a straight line with a very small negative gradient within the range of cycles used. The gradients obtained in separate experiments utilising this strategy are shown in Table 2. The negative gradient of each line indicates that the target amplified slightly faster than the competitor.

In agreement with equation 1, co-amplification of the target with replicate amounts of the competitor for different numbers of cycles yields a straight line with a gradient (m) which is indicative of $\log [(1+E_t)/(1+E_c)]$.

Therefore $\qquad m = \log [(1+E_t)/(1+E_c)]$

or $\qquad 10^m = [(1+E_t)/(1+E_c)]$

When E_c is designated to be 1, a Y% variation in the amplification efficiency of the target is represented by, $\qquad E_t = Y\%$

and $\qquad 10^m = (1 + Y\%)/2$

A Y% variation in the amplification efficiency of the target relative to the competitor is represented by: $\qquad Y\% = (2 \times 10^m) - 1.$ $\qquad$ (4)

The gradients generated by our sets of experimental data (n = 2) indicate the amplification efficiency (E_t) of IFN-γ is between 94.5% and 95% of the competitor efficiency (E_c) (Table 2).

4.3.4 Relative Amplification Efficiencies of IL-2, IL-4, TNF and TGF-β Targets and Respective Competitors

The amplification efficiency of the IL-2, IL-4, TNF and TGF-β target and respective competitors were examined by co-amplifying each (n = 2) for a different number of cycles. A plot of the target:competitor ratio as a function of the cycle number produced a straight line in each case, with a gradient close to zero within the range of cycles used (Table 2).

The gradients generated by the experimental data were substituted into equation 4 and the amplification efficiency of the target (E_t) relative to their respective competitor (E_c) were calculated (Table 2).

4.3.5 Assessment of Accuracy of Competitive PCR

To assess the accuracy of the IFN-γ competitive PCR at quantifying cDNA, serially diluted samples of a known amount of the competitor were co-amplified with a constant known amount of the IFN-γ target (n = 4). Prior to amplification the IFN-γ target and competitor cDNA fragments were each spectrophotometrically quantified which provided an estimate of the number of copies present. A single dilution of the IFN-γ target was co-amplified with five dilutions of the IFN-γ competitor. In accordance with equation 2, a log plot of the target:competitor ratio as a function of competitor copy number produced a straight line with a gradient approximating -1 (Fig. 2). The number of IFN-γ target copies established by competitive PCR was comparable to the number of copies estimated spectrophotometrically (Table 3).

The accuracy of the IL-2 (n=4), IL-4 (n=3), TNF (n=3) and TGF-β (n=3) competitive PCRs were assessed by co-amplifying serially diluted samples of each competitor with a constant amount of the respective target. The target and competitor cDNA fragments were spectrophotometrically quantified prior to dilution, then subject to PCR amplification. In each case a log plot of the target:competitor ratio as a function of competitor copy number resulted in a straight line with a gradient that approximated -1 (Table 3).

Competitive PCR consistently overestimated the amount of IL-2 target measured spectrophotometrically whereas, the amount of IL-4 and TGF-β target were consistently underestimated. The number of copies of the TNF target determined by competitive PCR was comparable to the number of copies estimated spectrophotometrically (Table 3).

4.3.6 Correction Factors

The number of copies of target cDNA were adjusted by formulating a correction factor to allow for differences in the efficiencies of amplification in the target and competitor DNA templates and thereby achieve more accurate quantification of the target DNA in test samples. The relative amplification efficiency of the target and its respective competitor (Table 2) were substituted into equation 3 with the experimentally determined y-intercept (c) and the number of amplification cycles (j). A corrected value for the amount of target (T_0) was calculated for each competitive PCR assay. The target copy number corrected for differences in amplification efficiency is represented in Table 3.

4.4 Discussion

Differences in the rate of amplification of the target and competitor fragments will limit the quantitative nature of the competitive PCR (Gilliland et al. 1990). The amplification efficiency of the target relative to the competitor cDNA fragment was calculated by comparing our experimental data to equation 1 which mathematically describes competitive PCR. This equation describes a straight line of the form $y = c + mx$ when the final ratio of PCR products $\log(T_j/C_j)$ at different cycles (j) is plotted. The gradient of the line (m) represents $\log[(1+E_t)/(1+E_c)]$ which reflects the difference in the amplification efficiencies of the target (E_t) and competitor (E_c). When both amplify with equal efficiencies, the ratio $[(1+E_t)/(1+E_c)]$ becomes one, and a straight line with a gradient of zero is expected. As E_t and E_c diverge there is preferential accumulation of one PCR product. The larger the difference between E_t and E_c, the further the gradient will deviate from zero and the less accurate will be the competitive PCR.

The decreased length of competitor relative to target cDNA fragments led us to investigate potential differences in their amplification efficiencies. Co-amplifying the target with its respective competitor cDNA fragment for different cycle numbers provided data which when fitted into equation 1 produced a straight line having a gradient which reflected the relative amplification efficiencies (Table 2). The amplification efficiency of the IFN-γ target relative to its competitor was 94.5% to 95% and that of IL-2 and IL-4 were 98.6% to 103.2% and 105.1% to 108.9% respectively. The amplification efficiency of the TNF target relative to its competitor was 101.8% to 102.3% and TGF-β target amplified 100.9% to 101.8% faster than its competitor. A minor difference in the length of the competitor relative to the target cDNA fragment is associated with little difference in the respective amplification efficiencies which is in support of previous findings (Wang et al. 1989). The negative gradient obtained for the IFN-γ PCR indicates the competitor amplified approximately 5% more efficiently than the target whereas the positive gradient obtained for the IL-4, TNF and TGF-β PCRs indicates that the competitor amplified less efficiently than the respective target. The mean values for duplicate experiments suggest that IL-2 target amplified with a very similar efficiency to the IL-2 competitor. However, the correlation coefficient was relatively low in the first experiment and much closer to unity in the second and accordingly, the value obtained in the latter instance may be more accurate. Taq polymerase extends at a rate of 2000 to 4000 bp per min at optimal temperature suggesting that a 2 min extension step during thermal cycling should be adequate for complete extension of both the competitor (approximately 300 bp) and target (approximately 400 bp) cDNA fragments, as indeed appears to be the case.

Under ideal conditions, when the amplification efficiency of the target equals that of the competitor, equation 1 can be simplified to equation 2. The theoretical ideal for equation 2 predicts that a straight line with a gradient of -1 will be obtained when different concentrations of the competitor are co-amplified with a constant amount of the target. To test the quantitative nature of our competitive PCRs using equation 2, known amounts of the target cDNAs were co-amplified with different concentrations of the competitors. The gradient obtained from each competitive experiment approximated the theoretical optimum value of -1. Quantification of the target can be accurately achieved by selecting a range of

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competitor copy numbers C_o such that the target to be assayed T_o falls within the range of C_o values. Interpolation of the x-axis value at a point when the final number of target copies equals that of the competitor ($\log T_j/C_j = 0$ therefore $\log T_o = \log C_o$) provides a more accurate interpolated estimation of T_o than that obtained by extrapolating to the y-intercept (c) since the latter estimate will be more influenced by errors in the slope of the line.

The relative amplification efficiency of the target and respective competitor fragment may have influenced the accuracy of quantification. The IFN-γ target amplified 5% to 6% slower than its respective competitor and accordingly, the competitive PCR overestimated (except in one reaction) the number of target copies predicted spectrophotometrically. The IL-2 target amplified with an efficiency very similar to its competitor and accordingly, the number of target copies predicted by the competitive PCR accurately portrayed that estimated spectrophotometrically. The IL-4 target amplified substantially faster than the competitor, and as a result the PCR consistently underestimated the amount of target, as was the case for TGF-β. The TNF target amplified faster than the competitor yet only in one reaction did the PCR underestimate the amount of target. The quantity of target was otherwise overestimated which may be attributed to limitations of the method used to quantify DNA (Glasel 1995; Wilfinger et al. 1997). Strategies to correct for variations in the amplification efficiencies did not increase the accuracy of the competitive PCRs.

The apparent accuracy of the competitive PCRs will also be influenced by the accuracy of spectrophotometric quantification of the target and competitor cDNA fragments prior to dilution and PCR amplification. This spectrophotometric detection and subsequent dilution will also be subject to random and possibly systematic errors. Our results demonstrate that our competitive PCR systems were sensitive enough to consistently amplify less than 200 copies of each cytokine mRNA transcript and sufficiently accurate to measure between 1.1 and 5.8 fold changes in cDNA levels. This compares favourably to a 120 fold increase in IFN-γ gene expression that we have observed during concanavalin A stimulation of splenocytes (shown in Chapter 6). Non-competitive PCR systems which are based on ten fold serial dilutions of the starting material, allow detection of differences in cytokine

message expression in the order of 10 fold (Dallman et al. 1991). The accuracy of our quantitative PCRs, which is comparable to that of other competitive PCR systems (Pannetier et al. 1993; Piatak et al. 1993), represents a substantial increase in accuracy.

The quantitative nature of competitive PCR has been demonstrated by exploiting the sensitivity, reproducibility and highly quantitative nature of the CE detection system described in Chapter 3. The relative weight of the PCR products in each electropherogram was converted into relative copy number by dividing the area of each peak by the respective molecular weight of the PCR product for accurate quantification. Allowance for differences in the extinction coefficients of the PCR products could increase the accuracy of CE with UV detection, although this refinement would be of marginal value in the present context.

We have calculated small differences in the amplification efficiencies of the targets and competitors and, in accordance with a mathematical model, we have demonstrated that competitive PCR can be used to accurately quantify small changes in cDNA levels relative to those observed in biological systems. Further sources of error of potentially equal or greater importance in practice include variability in extraction of mRNA and the reverse transcription step. In conclusion, the accuracy of competitive PCR is sufficient for reliable quantification of cDNA obtained from biological samples as demonstrated by this favourable comparison of the performance of competitive PCR to a mathematical model of optimal PCR performance.

	IFN-γ	IL-2	IL-4	TNF	TGF-β
Target Detection Limit (Copy Number)	206	110	224	89	131
Competitor Detection Limit (Copy Number)	27	46	127	48	141

Table 1. Purified target and competitor DNA fragments were serially diluted from approximately 30 ng/μL to 30 x 10^{-10} ng/μL in 0.5% Triton. Each dilution was amplified at least in duplicate (n ≥ 2) for 40 cycles. The detection limit of each PCR assay is represented as cytokine copy number per 50 μL PCR reaction.

IFN-γ Amplification Efficiency
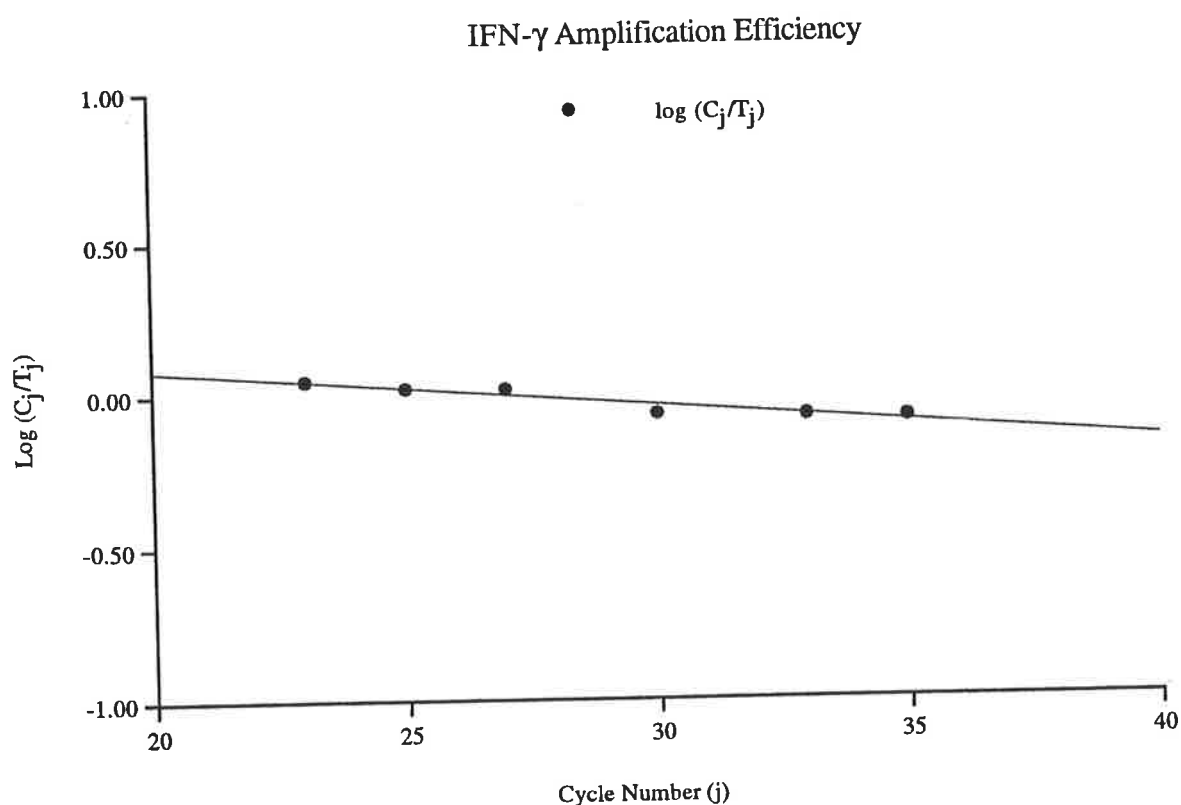
Figure 1. IFN-γ amplification kinetics: Replicate samples containing 96.3 fg of IFN-γ target and 86.5 fg of IFN-γ competitor were co-amplified for 23, 25, 27, 30, 33 and 35 cycles. The PCR products were quantified using capillary electrophoresis then plotted as a function of the cycle number according to equation 1. The line can be described by the equation y = -0.012x + 0.323 R^2 = 0.920

Cytokine	Observed Gradient	R^2	$E_t = Y\% \, E_c$
IFN-γ	-0.012	0.920	94.5%
	-0.011	0.832	95.0%
IL-2	-0.003	0.464	98.6%
	+0.007	0.875	103.2%
IL-4	+0.019	0.970	108.9%
	+0.011	0.658	105.1%
TNF	+0.005	0.889	102.3%
	+0.004	0.885	101.8%
TGF-β	+0.004	0.909	101.8%
	+0.002	0.742	100.9%

Table 2. IFN-γ, IL-2, IL-4, TNF and TGF-β targets were co-amplified with their respective competitors. Amplification reactions were performed on separate occasions for the number of cycles specified in the text. The PCR products were quantified using capillary electrophoresis. The gradient of each line was obtained by plotting log (T_j/C_j) vs cycle number (j) according to equation 1. R^2 is the square of the correlation coefficient obtained for each linear regression analysis. The amplification efficiency of the target relative to the competitor ($E_t = Y\% \, E_c$) was calculated by solving equation 4 having experimentally determined the gradient of each line.
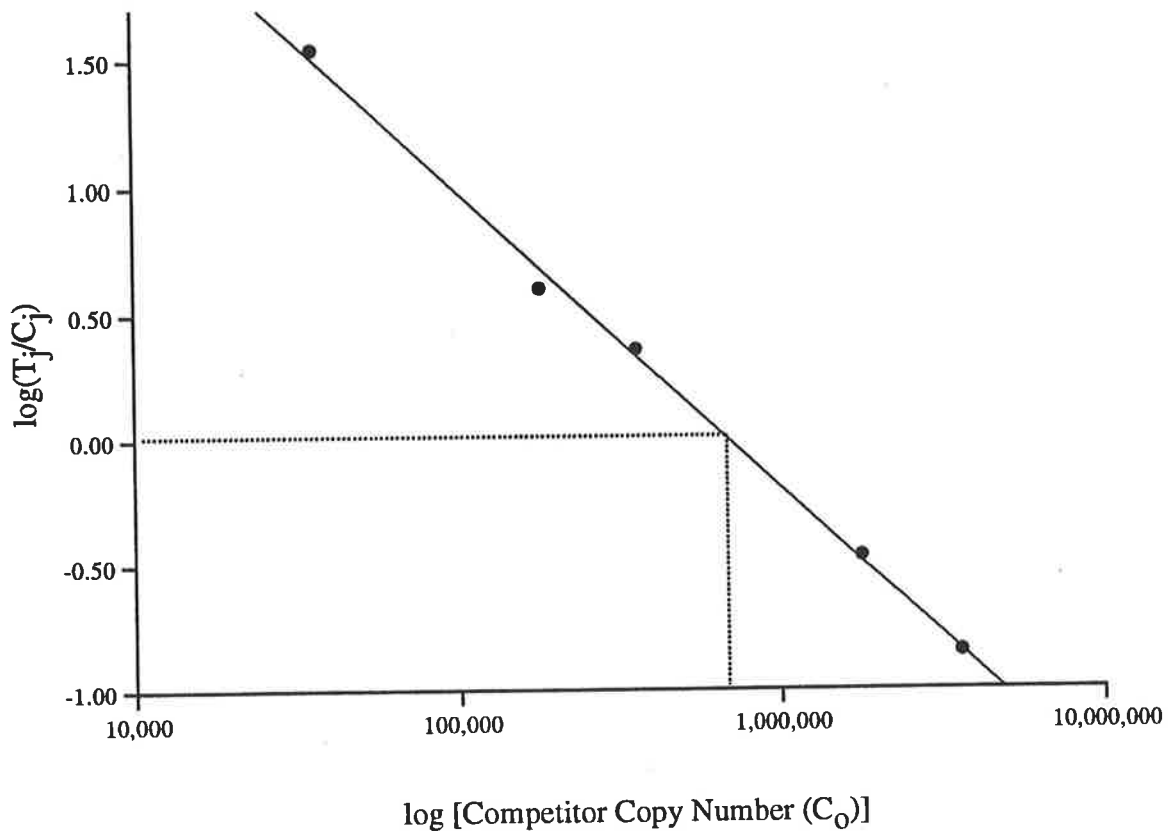
Figure 2. IFN-γ Competitive PCR: Replicate samples containing 52.91 fg (1.3x10^{5} copies) of purified IFN-γ target were each spiked with a different dilution of purified IFN-γ competitor representing respectively 3.57x10^{6} copies, 1.79x10^{6} copies, 3.57x10^{5} copies, 1.79x10^{5} copies and 3.57x10^{4} copies. All samples were co-amplified for 35 cycles. Quantification of the PCR products was undertaken using CE. The line can be described by the equation $y = 6.860 - 1.176 \log x$. The estimated target copy number obtained by interpolation of the x-axis value when $\log (T_j/C_j)$ is zero is 6.8x10^{5} copies which compares with the number of copies (1.3x10^{5} copies) estimated on the basis of starting material which was quantified spectrophotometrically.

Cytokine	Gradient	Initial target copy number estimated by PCR	Initial target copy number assayed by PCR and corrected for differences in amplification efficiency between the target and competitor	Target copy number estimated Spectro-photometrically	Uncorrected fold difference	Corrected fold difference
IFN-γ	-1.176	6.827×10^5	2.985×10^5	1.309×10^5	5.2	2.3
	-1.181	5.462×10^5	2.396×10^5	1.644×10^5	3.3	1.5
	-1.250	6.359×10^5	2.892×10^5	2.326×10^5	2.7	1.2
	-1.067	2.092×10^5	8.410×10^5	2.326×10^5	0.90	3.6
IL-2	-1.324	1.046×10^5	1.284×10^5	7.486×10^4	1.4	1.7
	-1.136	1.294×10^6	2.140×10^6	7.486×10^5	1.7	2.9
	-1.253	2.345×10^5	1.925×10^5	8.751×10^4	2.7	2.2
	-1.218	1.760×10^5	2.123×10^5	8.751×10^4	2.0	2.4
IL-4	-0.849	1.030×10^6	4.588×10^6	4.457×10^6	0.23	1.03
	-0.929	1.947×10^6	6.583×10^6	4.457×10^6	0.44	1.5
	-0.719	1.904×10^7	1.128×10^7	4.457×10^7	0.43	2.5
TNF	-8.00	8.152×10^3	4.620×10^3	8.899×10^3	0.92	0.52
	-0.812	1.390×10^4	1.467×10^3	8.899×10^3	1.6	0.16
	-0.986	1.225×10^4	7.722×10^3	8.899×10^3	1.4	0.87
TGF-β	-0.813	1.757×10^5	1.259×10^5	7.656×10^5	0.23	0.16
	-0.676	2.491×10^5	1.672×10^5	7.656×10^5	0.33	0.22
	-0.907	1.309×10^5	9.700×10^4	7.656×10^5	0.17	0.13

Table 3. Purified PCR products were quantified spectrophotometrically at 260 nm. The initial target copy number estimated by PCR was calculated by interpolating the x-axis value at log $(T_j/C_j) = 0$ when the final ratio of PCR products, log (T_j/C_j), was plotted as a function of the initial amount of competitor C_0 spiked into each tube prior to amplification. The initial target copy number corrected for differences in amplification efficiency between the target and competitor was calculated by substituting the experimental values of E_t and E_c along with the number of amplification cycles (j) into

equation 3. The final ratio of PCR products, log (T_j/C_j), was plotted as a function of the initial amount of competitor C_0 spiked into each tube prior to amplification then interpolation of the x-axis value at log $(T_j/C_j) = 0$ (ie solving equation 1 when $y = 0$) provided a corrected estimate of the initial target copy number estimated by competitive PCR.

Chapter 5

mRNA Extraction, Reverse Transcription Efficiency and Development of a TNF ELISA

5.1 Introduction

Gene expression may be monitored using quantitative RT-PCR which enables a single molecule of RNA to be amplified from a pool of 10,000 to 20,000 different RNAs that are produced by a single cell. Approximately 0.5-1% of total RNA extracted from a cellular source is mRNA, which can be used to monitor gene expression. Total RNA can be extracted from cells and quantified spectrophotometrically after which it is enzymically reverse transcribed into cDNA in preparation for quantitative analysis of the level of gene expression, using competitive PCR.

The method of Chomczynski et. al. (Chomczynski and Sacchi 1987) is used widely to extract RNA from cells or tissue. Contamination of the RNA with phenol and protein are unavoidable using this method (Yamaguchi et al. 1992) and as a result, the accuracy of spectrophotometric quantification of RNA is limited. Spectrophotometry is also used to verify the quality of RNA which is indicated by the UV absorbance ratio at 260 nm and 280 nm (ie., A260/280). Pure RNA has a theoretical A260/280 ratio of 2.0, although a ratio of 1.8 is regarded as a pure preparation in molecular biology manuals (Sambrook et al. 1989). Contamination of RNA with protein and/or phenol decreases the A260/280 ratio whereas contamination of the sample with DNA does not alter the A260/280 ratio but adds to the absorbance at 260 nm resulting in an inaccurate estimation of the amount of RNA (Sambrook et al. 1989). There are numerous reports questioning the reliability of spectrophotometric quantification of RNA as a result of these limitations (Glasel 1995; Huberman 1995; Manchester 1995; Manchester 1996).

The reverse transcription (RT) procedure utilises Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV) to reverse transcribe single stranded RNA into cDNA. RT is optimal when the enzyme is used in conjunction with a specific buffer at 37°C, however, variations in the buffer composition and the reaction temperature affect the rate of the reaction and hence the yield of cDNA. Furthermore, the reverse transcription enzyme and the polymerase enzyme are both inhibited by phenol so any significant contamination of the RNA template with chemicals employed during the extraction procedure are likely to confound quantification.

In this Chapter the reproducibility of RNA extraction, quantification and reverse transcription procedures are assessed and modifications to each procedure are made so that changes in cytokine mRNA expression can be measured accurately. Moreover, changes in cytokine mRNA expression do not always reflect production of the biologically active protein (Tang et al. 1994). Therefore, an ELISA was developed so that secretion of TNF protein could be measured in parallel with changes in the level of cytokine mRNA. The development and evaluation of a TNF ELISA is also described in this Chapter.

5.2 Factors Affecting Spectrophotometric Quantification of RNA

5.2.1 Introduction

Use of the $A_{260/280}$ ratio to evaluate the purity of samples was described by Warburg and Christian who sought to estimate the purity of a protein contaminated with nucleic acids. RNA extracted using the method of Chomczynski et. al. (Chomczynski and Sacchi 1987) is unavoidably contaminated with phenol, protein and DNA. Phenol absorbs UV light at 260 nm and to a lesser extent at 280 nm (Huberman 1995) whereas protein preferentially absorbs UV light at 280 nm and to a lesser extent at 260 nm (Manchester 1996). DNA absorbs UV light at 260 nm and as result, contributes along with phenol and protein to the calculation of the amount of RNA. Therefore, the presence of high purity, intact RNA may only be established by defining the characteristic 18S and 28S ribosomal RNA bands by

gel electrophoresis. However, such a procedure cannot be routinely applied when the quantity of RNA is limited.

In addition, spectrophotometric determination of the quantity and quality of RNA will vary with salt concentration, pH of the solution (Wilfinger et al. 1997) and the calibration of the spectrophotometer when using a published extinction coefficient (Manchester 1995).

5.2.2 Correction for Protein Contamination

Accurate quantification of cytokine gene transcription is dependent on the precision of RNA quantification and the purity of the RNA sample. These aspects have been further analysed by Glasel et. al. (Glasel 1995) who established an association between the quantity of RNA and the purity of the sample (measured by the A260/280 ratio). The purity of nucleic acid having varying degrees of protein contamination was described and a graphical representation of Glasel's data is presented in Fig. 1.

The A260/280 ratio in Fig. 1 may be used to re-estimate the quantity of RNA in a sample. The absorption of UV light by RNA at 260 nm is measured, and the amount of RNA in the sample is calculated using Beers Law (using an extinction coefficient of 40 for RNA at 260 nm) (Sambrook et al. 1989). The RNA concentration is "adjusted" by interpolating the fraction of nucleic acid in a sample using Fig. 1, having determined the A260/280 ratio spectrophotometrically. The adjusted concentration of RNA was calculated by multiplying the interpolated percentage of nucleic acid by the concentration of RNA as outlined below.

Adjusted $Conc^n$ of RNA = $Conc^n$ of RNA x Fraction of Nucleic Acid determined by A260/280

The accuracy of this method in "adjusting" the quantity of RNA due to variations in its purity was evaluated by measuring the amount of IFN-γ mRNA in replicate samples having different A260/280 ratios. Cells collected from the thoracic duct lymph of rats were lysed in guanidine thiocyanate solution then divided into 8 replicate aliquots. The RNA from each aliquot was extracted using the semi quantitative method of Chomczynski et. al. (Chomczynski and Sacchi 1987) and the quantity and quality of each sample was determined spectrophotometrically. This procedure provided samples with an A260/280

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ratio in the range 1.7 to 2.0, indicating the samples were variously contaminated with protein. All the samples were reverse transcribed then an equal amount of IFN-γ competitor was added to 62.5 ng of each cDNA sample and amplified for 40 cycles. The target:competitor ratio was determined using CE and the amount of IFN-γ in each sample was calculated. The results are depicted in Fig. 2 in which, the amount of IFN-γ as a fraction of the highest amount of IFN-γ is plotted as a function of the A260/280 ratio of the respective sample. The solid line represents the fraction of nucleic acid in a sample of known A260/280 ratio determined from the correlation of Glasel et. al.

All aliquots of RNA were of the same origin and all should therefore have an identical amount of IFN-γ. Furthermore, the fraction of IFN-γ mRNA in each sample was expected to decrease as the A260/280 ratio decreased below 2.0, because of an increasing contribution to the absorption of UV light at 260 nm by proteins.

Fig. 2 demonstrates that most of the experimental samples lie above the line defining the percentage of protein at different A260/280 values. This indicates that the percentage of IFN-γ mRNA in these samples was lower than that expected if they were exclusively contaminated with protein. In three cases the experimental samples lay close to but slightly beneath the line, indicating the percentage of IFN-γ in these samples was close to but slightly greater than that predicted from a protein contribution. These small deviations could be contributed to by phenol and DNA absorption of UV light at 260 nm resulting in a deviation in the amount of RNA from the real quantity in the sample of interest.

The observed values for IFN-γ message were clustered around 50% of the maximum value or above except for a single sample showing an absorbance ratio of less than 1.8. This sample displayed an IFN-γ mRNA content of only 15% of the maximum observed. The data supports the suggestion that samples subject to RNA extraction which have an A260/280 ratio greater than 1.8 are suitable for RT-PCR analysis and those that have a lower ratio are not.

5.2.3 β-Actin Housekeeping Gene

Since an activated cell has an increased production of RNA, changes in the amount of mRNA in a cell may be very small or insignificant when quantified relative to total RNA. This limitation is additional to the limits in quantification of RNA due to contamination of the sample with protein, phenol and DNA. Thus, a method for monitoring the extent of mRNA extraction is very desirable. One such method involves monitoring mRNA for a constitutively expressed housekeeping gene such as β-actin. Message for β-actin provides a basis for comparison of cytokine mRNA expression and to monitor the efficiency of mRNA extraction.

β-actin is an essential structural protein of the cytoskeleton and is widely used as a "housekeeping gene" i.e., a gene putatively expressed normally at constituent levels, despite the fact that it may be subject to regulation under certain physiological conditions (Finnegan et al. 1993; Goldsworthy et al. 1993). The integrity of β-actin as a housekeeping gene was examined by assessing the consistency of gene expression in normal and adjuvant arthritic (AA) rats.

The inguinal lymph nodes from four normal and four day 3 AA rats were removed and immediately frozen in liquid nitrogen. The RNA was extracted from a sizeable proportion of each tissue, quantified spectrophotometrically then converted into cDNA. 62.5 ng of cDNA from each sample was subject to PCR analysis using the appropriate oligonucleotide primers for β-actin. The reaction proceeded for 27 cycles, following which, the products were electrophoresed through a 2% agarose gel then stained with ethidium bromide before being photographed using UV light to illuminate the DNA. The intensity of the β-actin bands in the four normal and AA rats is displayed in Fig. 3.

The intensity of each β-actin band was variable and the amount of β-actin in the lymph nodes of the AA rats was generally lower than that in normal lymph nodes, although the conditions by which the RNA was extracted, reverse transcribed and amplified were consistent for each sample. The level of expression of β-actin mRNA in the inguinal lymph nodes appeared to vary between normal and AA rats and thus, β−actin may not be

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suitable for use as a constitutively expressed gene against which to compare cytokine gene expression.

5.2.4 β-Actin Pseudogene

Humans and some rodents (Ng et al. 1985; Raff et al. 1997) have a copy of the β-actin gene that appears to have been incorporated into a retrovirus and re-introduced into the genome without introns. Such a "pseudogene" would militate against the use of β-actin as an internal standard for evaluating cytokine mRNA levels because RNA has an inherent DNA contamination when extracted using the method of Chomczynski et. al. (Chomczynski and Sacchi 1987). A PCR product originating from cDNA would be indistinguishable from one generated from contaminating genomic DNA. Therefore, the presence of a β-actin pseudogene in rat DNA was investigated by attempting to amplify the 607 bp β-actin PCR product from a DNA contaminated RNA sample.

RNA was extracted from the inguinal lymph node of a rat and divided into two samples, of which, one was converted into cDNA. PCR was performed on both samples: 125 ng of RNA and 62.5 ng of the cDNA sample. PCR amplification was performed using oligonucleotide primers designed to amplify a 607 bp fragment of the β-actin cDNA. The products were electrophoresed through a 2% agarose gel and stained using ethidium bromide then photographed using UV illumination. Fig. 4, which depicts the agarose gel of the PCR products, demonstrates that amplification of RNA produced a band equal in length to that obtained upon amplification of cDNA using the same primers. Thus, β-actin is unacceptable for use as a standard for accurate quantification of cytokine gene levels due to contaminating DNA. Although contaminating DNA could be removed by DNAse I treatment at ≥ 4°C for 15 min (Melgar and Goldthwait 1968), such a treatment was regarded as inappropriate for cytokine mRNA due to its short half life at ambient temperatures.

β-actin is therefore unsuitable as a standard for quantifying changes in cytokine mRNA levels because it exists as a pseudogene and constitutive expression of the gene appears to vary. Such variation may be the result of an increase in total RNA production causing a

relative decrease in the quantity of β-actin mRNA. Although it may be more appropriate to quantify changes in cytokine mRNA levels relative to several housekeeping genes (Spanakis 1993), the logistics of such a procedure in the present applications outweigh benefits.

5.3 Extraction and Reverse Transcription Variations

5.3.1 Extraction and Reverse Transcription Variability

Fundamental to the accuracy of the competitive PCR is the reproducibility of RNA extraction and reverse transcription (RT) because full length, intact cDNA is a prerequisite for PCR and is essential if changes in cytokine mRNA levels are to be measured accurately. Accordingly, optimum conditions for both the RNA extraction and RT procedures were determined and the reproducibility of these methods was assessed.

The variability of the extraction and RT procedures were assessed by repeated measurement of a single sample in quadruplicate. Stimulated splenocytes were lysed with guanidine thiocyanate then divided into four equal aliquots. RNA was extracted from each aliquot then a small proportion was converted into cDNA by adding an aliquot of pre-mixed RT reagents (samples A1-4). Four aliquots were withdrawn from the remaining RNA and cDNA was prepared by individually adding each RT reagent to the RNA, in preference to aliquoting pre-mixed reagents (samples B1-4). 62.5 ng of cDNA was withdrawn from each cDNA sample and co-amplified with a known amount of the IFN-γ competitor. The competitive PCR procedure was duplicated and capillary electrophoresis was used to quantify the products, from which the amount of IFN-γ in each sample was calculated. Fig. 5 depicts the variation in the amount of IFN-γ as a fraction of the highest amount of IFN-γ, when identical samples were extracted and RT using a common preparation of the RT reagents (samples A1-4) and individual preparations of the RT reagents (samples B1-4).

All RNA aliquots originated from a common cell source and therefore should have the same amount of IFN-γ mRNA. Fig. 5 demonstrates there was a small variation in the amount of IFN-γ when the extraction and RT procedures were performed sequentially. Samples that were reverse transcribed using the same preparation of the RT reagents had a coefficient of variation (C.V.) of 11%. A C.V. of 28% was calculated when the four RNA samples were reverse transcribed with individual preparations of the RT reagents. The competitive PCR procedure was duplicated, from which it was calculated that the C.V. was 12.5% and 39% for the respective RT reactions.

Minor variations in the concentration of the RT reagents may contribute to the variability in the RT reaction when individual preparations of reagents were employed (average C.V. = 33.5%). Variations in the RT procedure were considerably smaller (average C.V. = 11.7%) when a common preparation of the RT reagents was used. As a result, quantification of cytokine mRNA levels using competitive PCR was reproducible in repeated experiments on the same day using a common preparation of the RT reagents, but was less reproducible on a day to day basis. The precision of the technique can be further increased by generating a synthetic RNA competitor that is firstly RT, then co-amplified with the target cDNA. Such a technique obviates variability in both the RT and PCR procedures and therefore, quantification is more accurate (Souaze et al. 1996; Zhou et al. 1997). Competitive PCR can be made even more accurate if a RNA competitor with a poly-A tail is synthesised so the competitor RNA fragment has an instability comparable to that of the native cytokine target mRNA (Duchmann et al. 1993). Generation of RNA competitors is, however, expensive and labour intensive (Babu et al. 1993; Bouaboula et al. 1992) whereas competitive PCR is suitable for measuring the substantial changes in the levels of cytokine mRNA that can be expected in biological systems.

5.4 Rapid Competitive PCR

5.4.1 Introduction

Quantification of gene transcription levels using competitive PCR typically involving co-amplification of replicate samples of the target (T_0) cDNA with different amounts of the

competitor (Co) is referred to as the "interpolated method". However, if a large number of tissue samples are to be quantified, this procedure becomes labour intensive and impractical.

A modification of the interpolated method has been described (Zachar et al. 1993), in which multiple samples can be analysed by co-amplifying the target (T_0) with a single dilution of the competitor (C_0). Following amplification the target:competitor ratio can be used to calculate the amount of target in the sample. This represents a practical way of quantifying changes in the level of cytokine mRNA in multiple samples. A similar "rapid competitive PCR" method was developed and assessed by Jiang et. al. (Jiang et al. 1996) and shown to be suitable for detecting less than two fold differences in mRNA levels. The accuracy of this rapid competitive PCR procedure was validated prior to its application for the quantification of cytokine mRNA levels, by comparing it to the interpolated method.

5.4.2 Interpolated Competitive PCR Method

Five replicate aliquots containing 62.5 ng of heterogeneous cDNA were added to five different dilutions of the IFN-γ competitor. The mixtures were co-amplified for 40 cycles and the target:competitor ratio was determined using CE. An estimation of the amount of target cDNA was made by interpolating a line defined by the final amounts of PCR product (log (T_j/C_j)) plotted as a function of the initial amount of competitor (log C_0) spiked into each reaction tube as outlined by equation 2 in Section 4.2.1. The data is presented in Fig. 6 demonstrate that 4.80×10^5 copies of IFN-γ cDNA/μg of RNA were quantified by determining the x-intercept of the regression line.

5.4.3 Rapid Competitive PCR Method

The samples used in the interpolated method (in Section 5.4.2) were then analysed using the rapid competitive PCR method to assess its accuracy. The amount of target in each reaction tube from Section 5.4.2 was calculated by solving equation 2 for log (T_0) having experimentally determined the target:competitor ratio (log (T_j/C_j)) for a single dilution of the competitor (log C_0). The number of copies of IFN-γ target in each reaction tube calculated using this method are shown in Table 1.

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The accuracy of the rapid competitive PCR procedure was determined by calculating T_0 having experimentally determined the target:competitor ratio following amplification. The greatest amount of IFN-γ measured using this method was 8.7×10^5 copies/μg and the lowest was 2.8×10^5 copies/μg, which represents a 3.1 fold range. This compares with the value of 4.8×10^5 calculated from Fig. 6 using the interpolated method. The values shown in Table 1 demonstrate that competitive PCR is most accurate when the target is co-amplified with a similar amount of the competitor. When the competitor is in excess the target is underestimated whereas, it is overestimated when the amount of target is in excess.

While the rapid competitive PCR method can be used to accurately quantify relative amounts of target cDNA and is most accurate when the target cDNA is co-amplified with a comparable amount of the competitor, the variation in quantification using this method is likely to be small relative to the variation in biological systems. For example, TNF, IL-10, IL-4 and IFN-γ levels in the spleens of mice injected intraperitoneally with anti-CD3, increased 14, 24, 199 and 851 fold respectively compared to spleens from untreated mice (Zhou et al. 1997). Changes of a similar magnitude have been observed with cytokines in numerous other systems (Ehlers et al. 1992; Prudhomme et al. 1995). A preliminary investigation into cytokine mRNA levels produced by concanavalin A stimulated splenocytes has revealed changes approximating 100 fold. The level of cytokine secretion is, however, dependent on the duration, concentration and the nature of the stimulus (antigen or mitogen) (McHugh et al. 1996). Conversely, changes in cytokine mRNA production can also be very subtle (Baecher Allan and Barth 1993).

5.5 Development and Evaluation of a TNF ELISA

5.5.1 Introduction

Rat TNF is encoded for by a 1805 base pair gene consisting of four exons and three introns. The mature 235 amino acid, unglycosylated protein forms a non-covalently linked biologically active trimer with an apparent molecular mass of 17 kDa (Kwon et al. 1993).

It is normally membrane bound and can be solubilised by enzymic cleavage of the extracellular domain (Perez et al. 1990).

TNF is a key mediator of inflammation and cellular immune responses and is regarded as an inflammatory cytokine because it participates in a cytokine network and stimulates the production and release of factors that are involved in the effector phase of an inflammatory response (Duff 1994). TNF is produced predominantly by activated macrophages and to a lesser extent by lymphocytes, mast cells and polymorphonuclear leucocytes. Most cells have receptors for TNF which suggests that many cell populations can respond to this cytokine. A method was developed to quantify the levels of TNF protein produced during the inflammatory response associated with adjuvant arthritis (AA). The amount of protein was measured to substantiate a correlation between the level of TNF mRNA and protein. This was achieved by developing and evaluating an ELISA for quantifying TNF protein.

5.5.2 Results

5.5.2.1 Components of the TNF ELISA

Although an ELISA kit used to quantify rat TNF was commercially available from Serotec, the kit was only available in the United Kingdom. Therefore, individual commercially available reagents were used to develop a TNF ELISA in house. A schematic representation of the structure of the ELISA is presented in Fig. 7. The bottom of a 96 well microtitre plate was coated with a rat anti-mouse monoclonal capture antibody (Pharmingen) that was specific for rat TNF (see technical data sheet). Serial dilutions of purified recombinant rat TNF (Serotec) were used to generate a standard curve then polyclonal rabbit anti-rat/mouse antibody (Serotec) served as the detecting antibody. The revealing system involved a biotinylated donkey anti-rabbit antibody (Amersham) and an avidin biotin complex (ABComplex), in which the biotin was conjugated to horseradish peroxidase (HRP). After washing to remove biotinylated HRP, o-phenylenediamine dihydrochloride was added as the substrate for HRP and the reaction was terminated by the addition of 4 M sulfuric acid after which the optical density (OD) of each well was measured at 490 nm.

5.5.2.2 Titration of the Revealing Antibody

The concentration of the antibodies used in the ELISA were optimised to ensure assay specificity. Initially a titration of the revealing antibody was performed to establish the concentration at which it non-specifically bound to the protein blocking the microtitre well. This was achieved by incubating different concentrations of the biotinylated donkey anti-rabbit immunoglobulin in a microtitre plate that had been blocked for 30 min with a mixture containing 2% bovine serum albumin (BSA) in 0.5% Tween at 37°C. A broad titration of the revealing antibody was performed initially to establish a starting point from which the ELISA could be optimised. The following dilutions of donkey anti-rabbit immunoglobulin were prepared in PBS containing 0.5% Tween and 1% normal donkey serum; 1/800, 1/1200, 1/1600, 1/2000, 1/3000, 1/5000, and 1/10,000. The plate was incubated with different dilutions of the anti-rabbit immunoglobulin for 2 hours at 37°C. Each well was developed using ABComplex/HRP and o-phenylenediamine dihydrochloride. The OD of each well was measured at 490 nm from which it was concluded that at 1/800 dilution the anti-rabbit immunoglobulin gave rise to an OD value which was marginally above that of the control well which received no anti-rabbit immunoglobulin.

At 1/800 dilution, the OD was due exclusively to donkey anti-rabbit immunoglobulin non-specifically interacting with BSA. OD values of dilutions greater than 1/800 were the same as background levels so therefore, a 1/800 dilution of the donkey anti-rabbit immunoglobulin was employed in the ELISA.

5.5.2.3 Titration of the Detecting Antibody

The concentration of the revealing antibody was established in Section 5.5.2.2 such that other antibodies used in the ELISA could subsequently be optimised. In accordance with the suppliers instructions, the wells of a microtitre plate were coated overnight at 4°C with 2 mg/mL of the monoclonal rat anti-mouse antibody. The plate was then blocked with 2% BSA and a series of standard curves were generated by serially diluting recombinant rat TNF from 20 ng/mL to 156 pg/mL. The plate was incubated at 37°C for 3 hours then the

detecting antibody was introduced into each well. The detecting antibody was incubated at 37°C for 2 hours at the following concentrations; 2 µg/mL, 1 µg/mL, 500 pg/mL, 250 pg/mL, 166 pg/mL and 80 pg/mL. 1/800 dilution of the developing antibody was added to each well then the ELISA was developed using ABComplex/HRP and o-phenylenediamine dihydrochloride. The OD of each well was measured and is depicted in Fig. 8 as a function of the dilution of the detecting antibody.

The concentration of the detecting antibody increased in proportion to the OD. It was concluded from Fig. 8 that 1 µg/mL of the detecting antibody was optimal for detecting TNF protein because 2 µg/mL resulted in only a small increase in the sensitivity but a substantial increase in background noise at low TNF concentrations. At lower concentrations of the primary antibody the sensitivity of the assay decreased, which was evident by the reduced rate of change in OD over a range of TNF concentrations.

5.5.2.4 Accurate Titration of the Revealing Antibody

In section 5.5.2.2 a broad titration of the revealing antibody was performed to provide a starting point from which other antibodies used in the ELISA could be optimised. Subsequently, it was demonstrated in Section 5.5.2.3 that the detecting antibody can used at an optimal concentration of 1 µg/mL and therefore, an accurate titration of the revealing antibody was performed. The wells of a microtitre plate were coated with 2 mg/mL of the monoclonal antibody and blocked with 2% BSA. Serial dilutions of recombinant rat TNF (from 20 ng/mL to 156 pg/mL) were added to the plate, following which it was incubated with 1 µg/mL of detecting antibody for 2 hours at 37°C. The developing antibody was serially diluted from 1/800 to 1/10,000 then incubated with the TNF samples at 37°C for 2 hours. ABComplex and biotinylated HRP were added to the wells. After washing and addition of substrate, the OD was measured and is represented in Fig. 9 as a function of the concentration of TNF.

As the concentration of the revealing antibody decreased, the OD at each TNF concentration decreased and as a result, the detection limit of the ELISA increased. It was concluded from Fig. 9 that 1/1000 dilution of the revealing antibody (which represents 1

μg/mL) was optimal for detecting TNF protein. The lower dilutions generated higher levels of background noise resulting in a decrease in the sensitivity of the assay, whereas dilutions greater than 1/1000 had a slightly lower rate of change in OD as a function of TNF concentration. Furthermore, a lower limit of detection was evident when the TNF standards were incubated with higher dilutions of the revealing antibody.

5.5.2.5 Further Characterisation of the TNF ELISA

Several negative controls were included during the development of the TNF ELISA and the quantification of TNF in experimental samples, to ensure assay specificity for TNF. Negative controls encompassed wells that sequentially excluded the capture antibody, sample, detecting antibody, revealing antibody and ABComplex, and were all consistently negative during analysis. Furthermore, the analysis of a cell culture supernatant that was known to be deficient in TNF protein was consistently negative, whereas the analysis of serial dilutions of a supernatant from concanavalin A stimulated splenocytes demonstrated that the OD decreased proportionally with the titre of diluted supernatant. In addition, substitution of the sample with RPMI containing 10% FCS consistently produced a negative result. Taken together these results demonstrate the suitability of the ELISA assay for measuring TNF in biological fluids.

5.5.2.6 Detection Limit of the TNF ELISA

The detection limit of the TNF ELISA was established using the conditions for optimal detection of the TNF protein. Serial dilutions of recombinant rat TNF between 20 ng/mL and 100 pg/mL were prepared then incubated a microtitre plate that had been pre-coated with 1 mg/mL of the capture antibody. The plate was incubated with 1/1000 dilution of the detecting antibody and 1/1000 dilution of the revealing antibody for 2 hours at 37°C respectively. The ELISA was developed using ABComplex/HRP and o-phenylenediamine dihydrochloride and the OD was measured. The OD is plotted as a function of log TNF concentration in Fig. 10.

The lower limit of detection of the assay was due to a non-specific interaction between the capture and the detecting antibody. This resulted in a low level background noise and as a

result, the detection limit was reproducibly 5 ng/mL. Beyond 1000 ng/mL the standard curve began to plateau which is indicative of the upper limit of detection. The upper and lower detection limits were reproducible and using the conditions established in this Section, TNF protein could be accurately quantified in the range of 5 - 1000 ng/mL.

5.6 Summary

The data presented in this Chapter provide an insight into the accuracy of the multistep quantitative RT-PCR procedure. The study was undertaken to assess the reproducibility of RNA extraction, spectrophotometric quantification, and reverse transcription of RNA into cDNA. Limitations in the accuracy of spectrophotometric quantification of RNA were attributed to the contaminants (such as residual protein) in the RNA after extraction. Attempts to estimate the quantity of RNA more accurately by taking into account its quality were inconclusive.

Changes in mRNA levels are small relative to changes in total RNA production during disease progression as demonstrated in Fig. 3. As a result, β-actin mRNA was investigated as a standard for quantification of cytokine mRNA levels. Changes in cytokine mRNA levels were expected to be relatively larger and more accurately quantified relative to β-actin. However, a β-actin pseudogene was detected in the rat DNA which discounted its use as a standard for mRNA quantification.

The reproducibility of the RT reaction was considerably higher when RT was performed using an aliquot from a common preparation of the RT reagents (C.V.= 11.7%) in preference to individual preparations of the reagents (C.V.= 33.5%).

It was demonstrated that while the "interpolated competitive PCR method" was very accurate for measuring changes in mRNA levels, sufficient quantitative data could be obtained using the "rapid competitive PCR method" because the uncertainty associated with cDNA quantification using this method was considerably smaller than the changes in cytokine mRNA levels expected in biological systems.

Since physiological changes in cytokine mRNA levels are not always supported by changes in secretion of the biologically active protein, a ELISA used to quantify TNF protein was developed and evaluated. The specificity of the ELISA was demonstrated and it was shown to be suitable for detecting TNF protein within the range of 5 - 1000 ng of TNF /mL.

In the remaining Chapters these techniques will be used to monitor changes in cytokine levels during cell stimulation *in vitro* and during the development of adjuvant arthritis (AA) in the rat.

Optical Density ($A_{260/280}$) vs
Percentage of Nucleic Acid in Sample
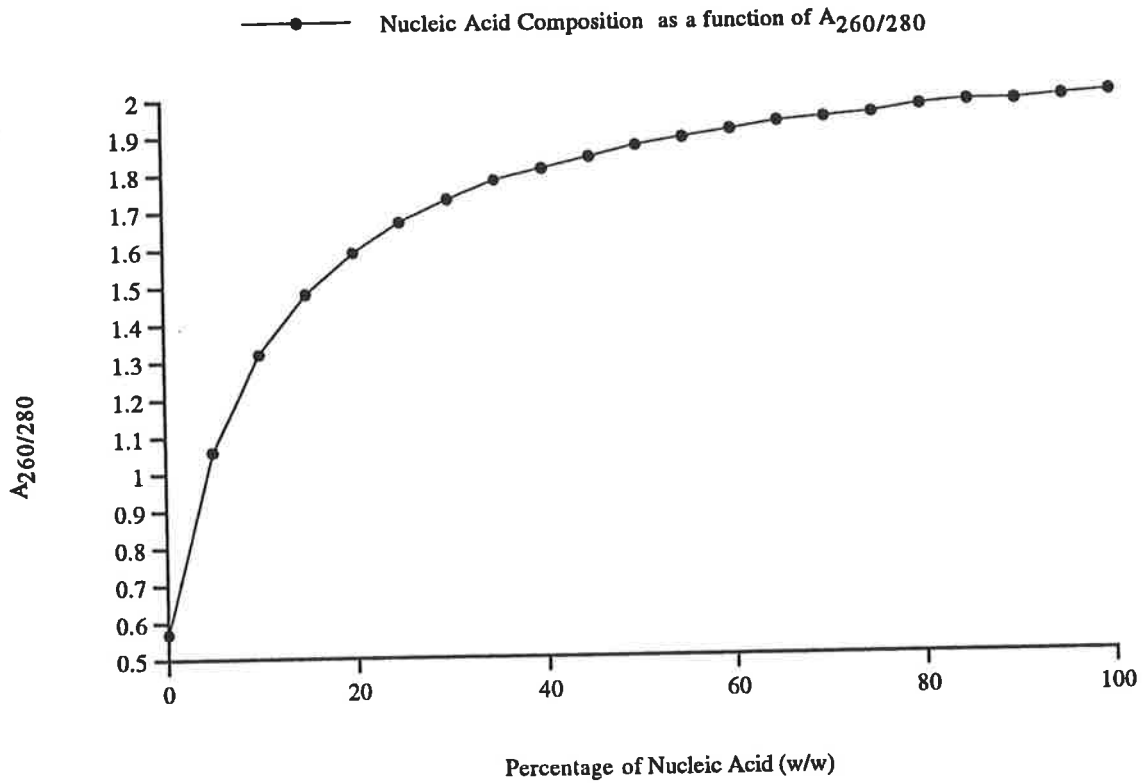
Figure 1. Absorbance ratio A260/280 for specimens containing variable proportions of RNA and protein. The units on the abissa are percentage weight of RNA as a proportion of the total weight of nucleic acid plus protein.

Optical Purity vs IFN-γ Copy Number
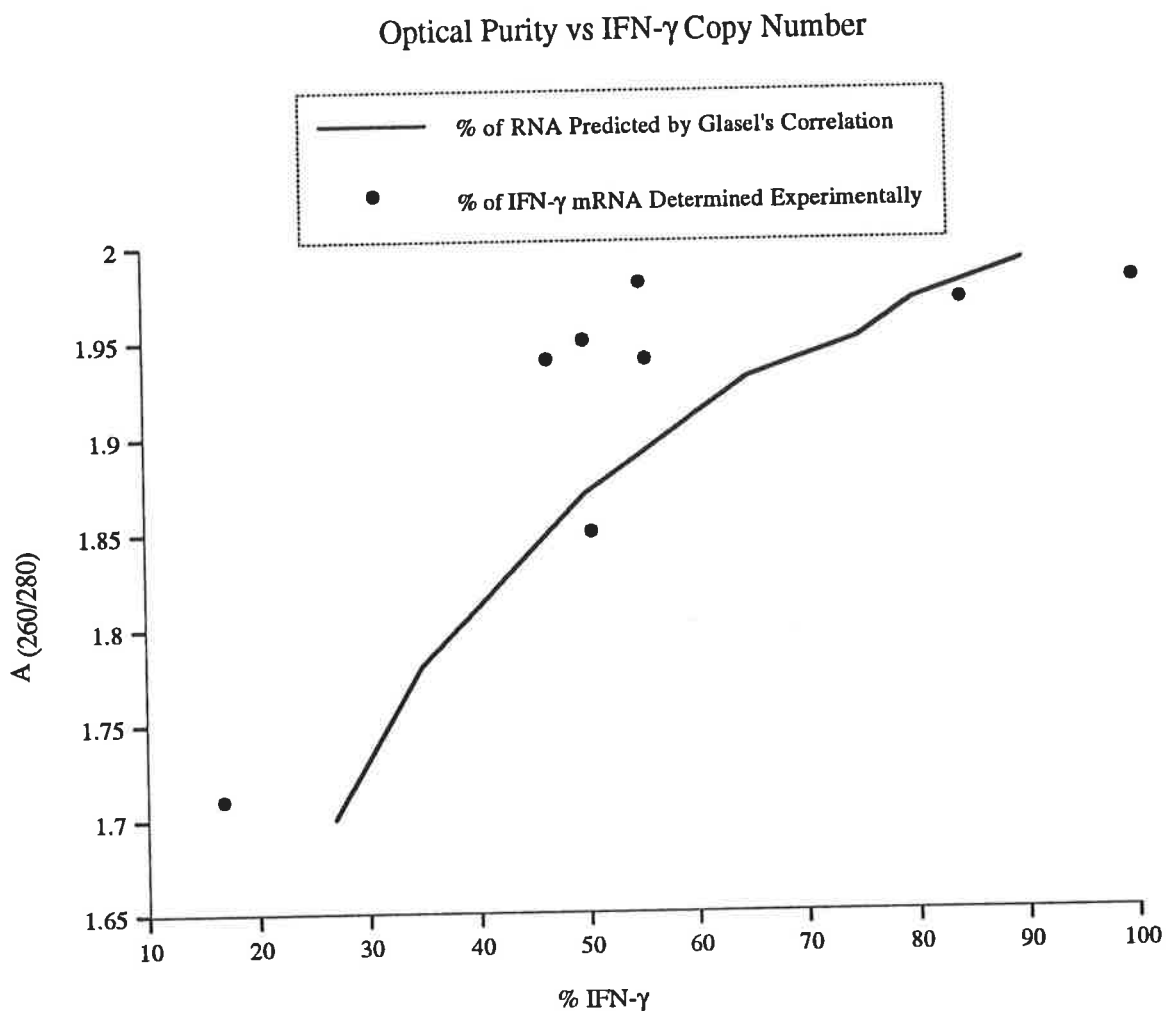
Figure 2. RNA was extracted from eight replicate cell aliquots, reverse transcribed then the amount of IFN-γ in each sample was measured using competitive PCR. The quantity of IFN-γ is expressed as a fraction of the maximum amount of IFN-γ measured (ie. measured amount/maximum measured amount x 100%) and plotted as a function of the A260/280 ratio. The line is a representation of Glasel's correlation which describes the percentage of nucleic acid in a sample as a function of A260/280.
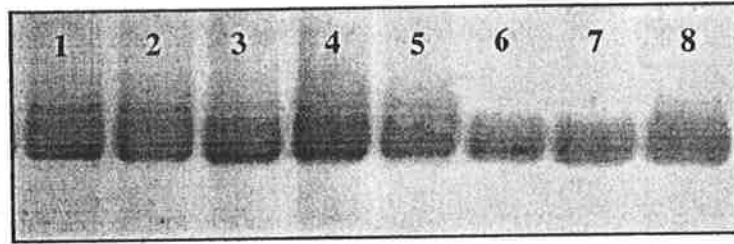
Figure 3. β-actin band intensity from the inguinal lymph node of four normal rats (lanes 1-4) and four day 3 AA rats (lanes 5-8).
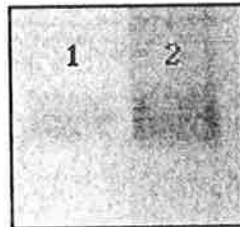
Figure 4. PCR amplification of 62.5 ng of cDNA (lane 1) and 125 ng of RNA (lane 2). The PCR was performed with β-actin primers using standard conditions for 27 cycles.

Extraction and Reverse Transcription Reproducibility
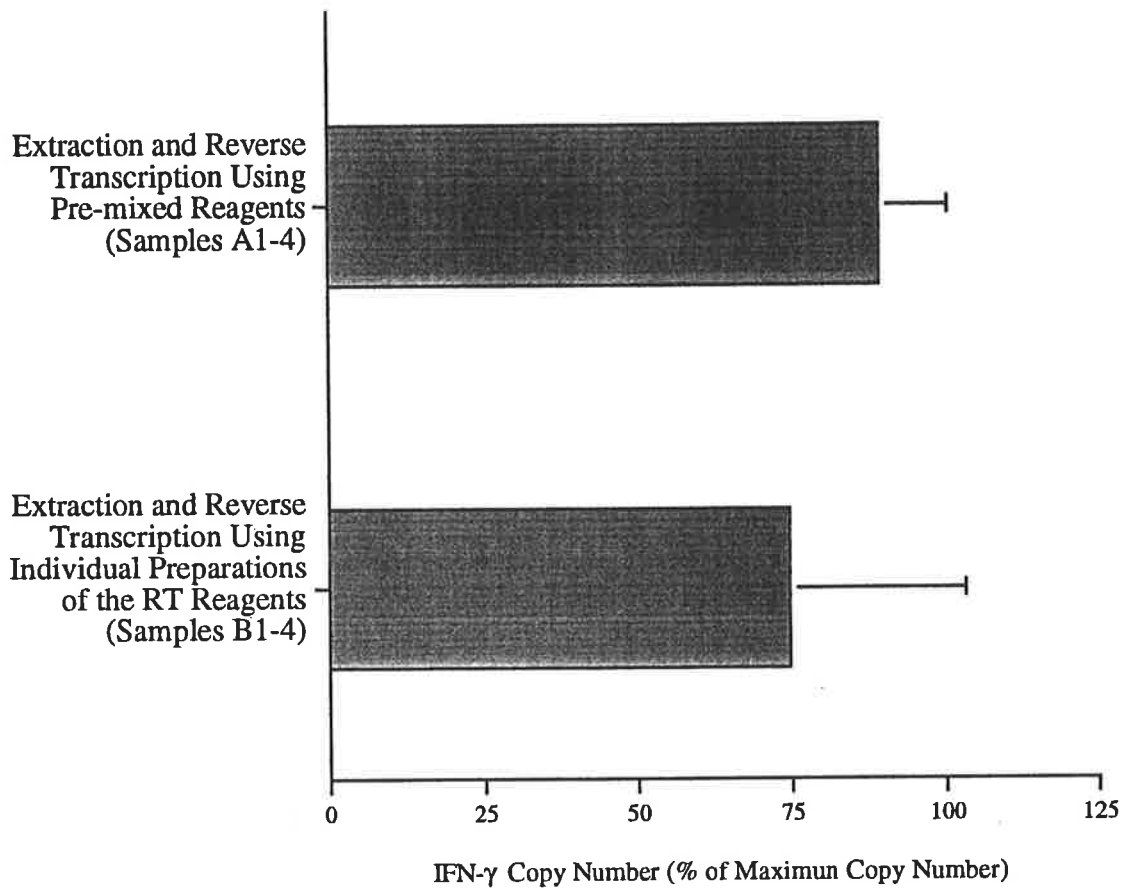
Figure 5. Cell lysates were divided into 4 replicate samples. RNA was extracted from each sample and reverse transcribed by adding an aliquot of the pooled RT reagents (samples A1-4). The RT procedure was then repeated on the RNA, using individual preparations of the RT reagents (samples B1-4). The level of IFN-γ in each sample was measured using competitive RT-PCR and expressed as a percentage of the maximum amount of IFN-γ measured in each procedure. Each row represents the mean ± % C.V. of four samples.

IFN-γ Competitive PCR

log (T_j/C_j)
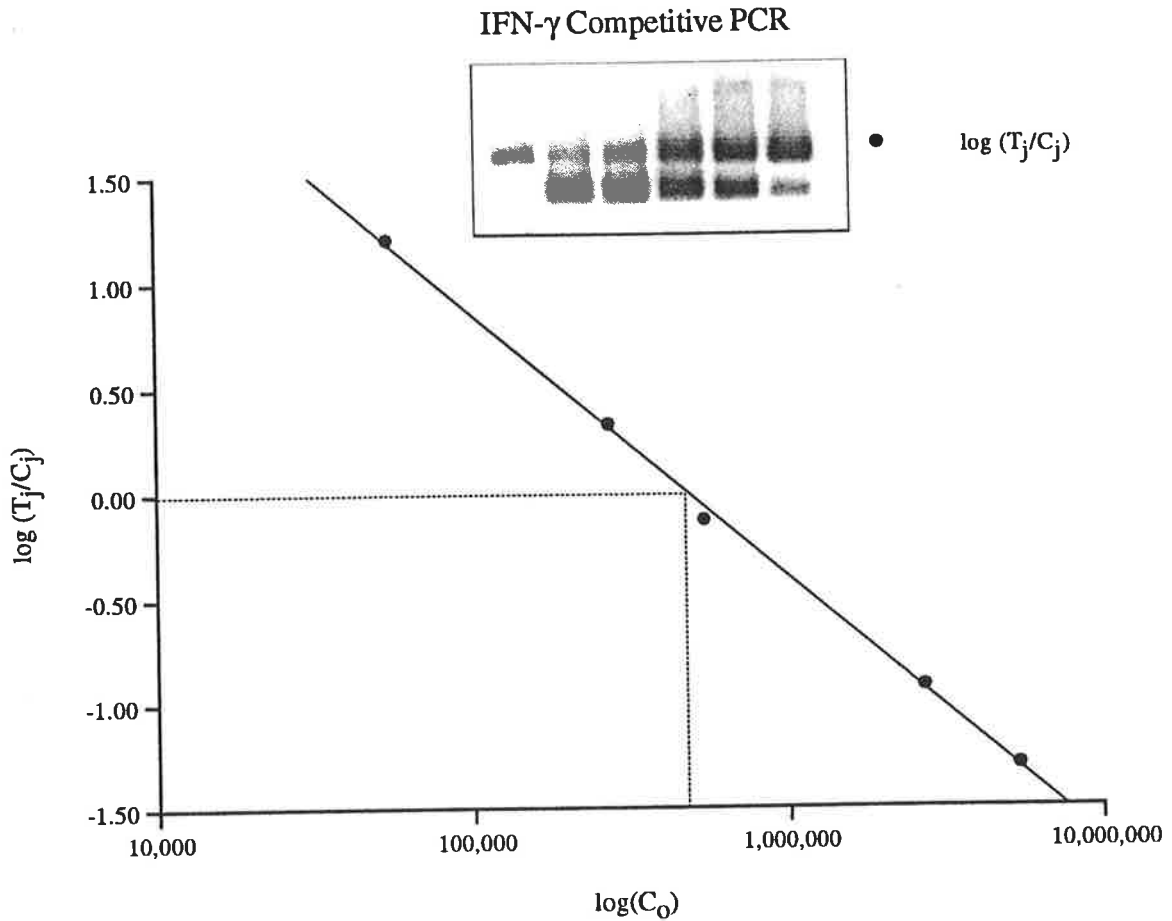
Figure 6. Five replicate samples of cDNA prepared from con A stimulated splenocytes were co-amplified with the IFN-γ competitor. CE was used to determine the target : competitor ratio in each sample and the amount of IFN-γ was determined by solving the equation y = -1.250 log(x) + 7.102 for x when y = 0. The inset depicts agarose gel analysis of the competitive PCR products with the far left hand lane representing IFN-γ target positive control.

Sample	Log (T_i/C_i)	Copy Number (C_o)	Copy Number (T_o)
1	-1.289	54×10^5	2.8×10^5
2	-0.920	27×10^5	3.3×10^5
3	-0.126	5.4×10^5	4.0×10^5
4	0.329	2.7×10^5	5.8×10^5
5	1.207	0.54×10^5	8.7×10^5

Table 1. Replicate samples of the target were co-amplified with different concentrations of the competitor. The target:competitor ratio in each replicate was measured using CE and the quantity of IFN-γ was calculated by solving equation 2 for T_o.
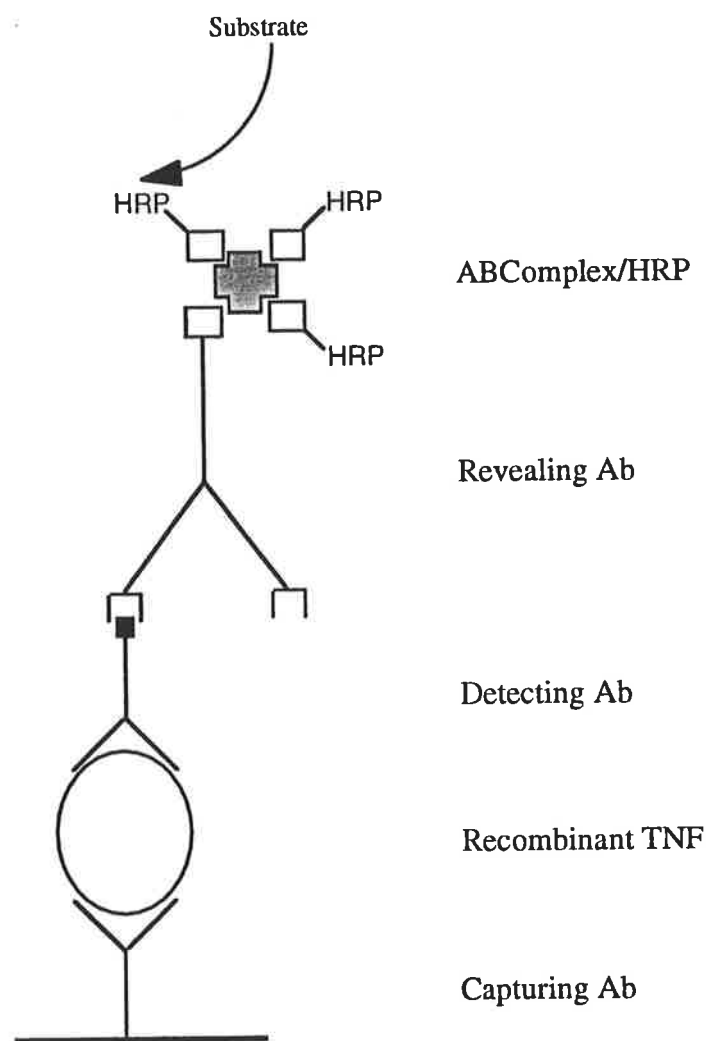
Figure 7. Schematic representation of the ELISA used to quantify rat TNF.
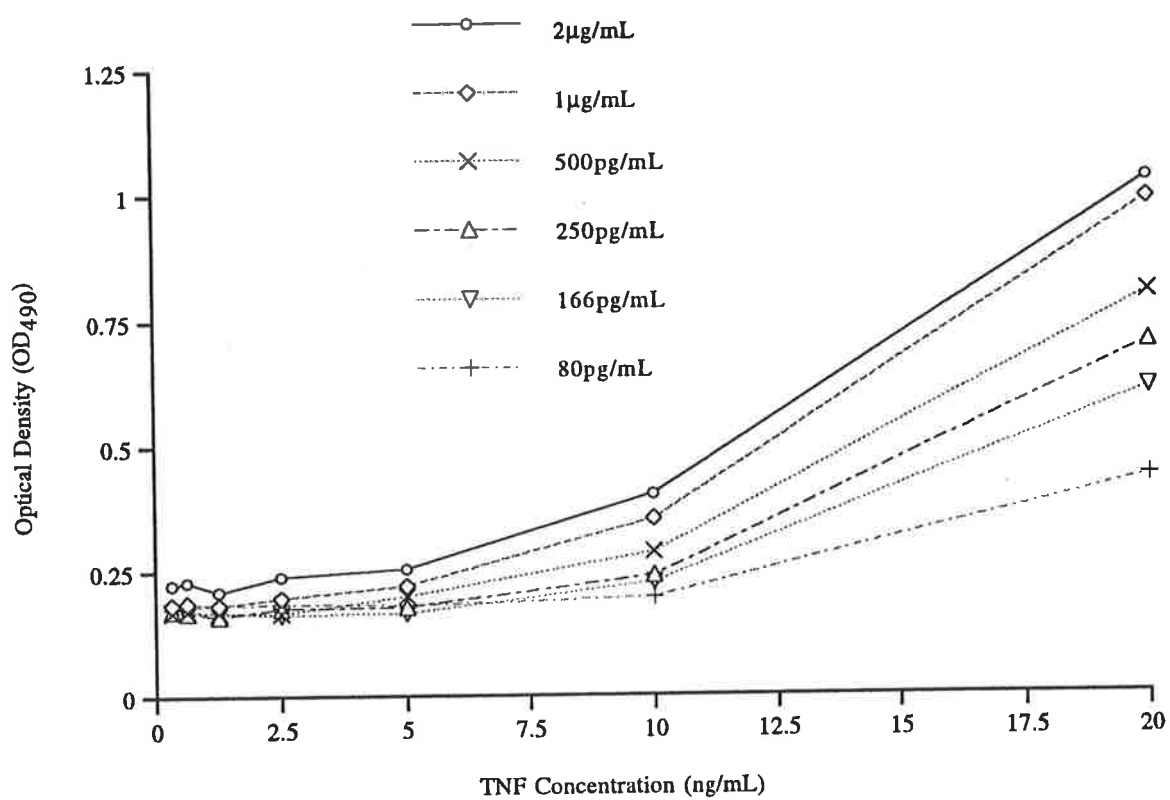
Figure 8. Standard curves generated by titrating the detecting antibody.

Titration of the Revealing Antibody
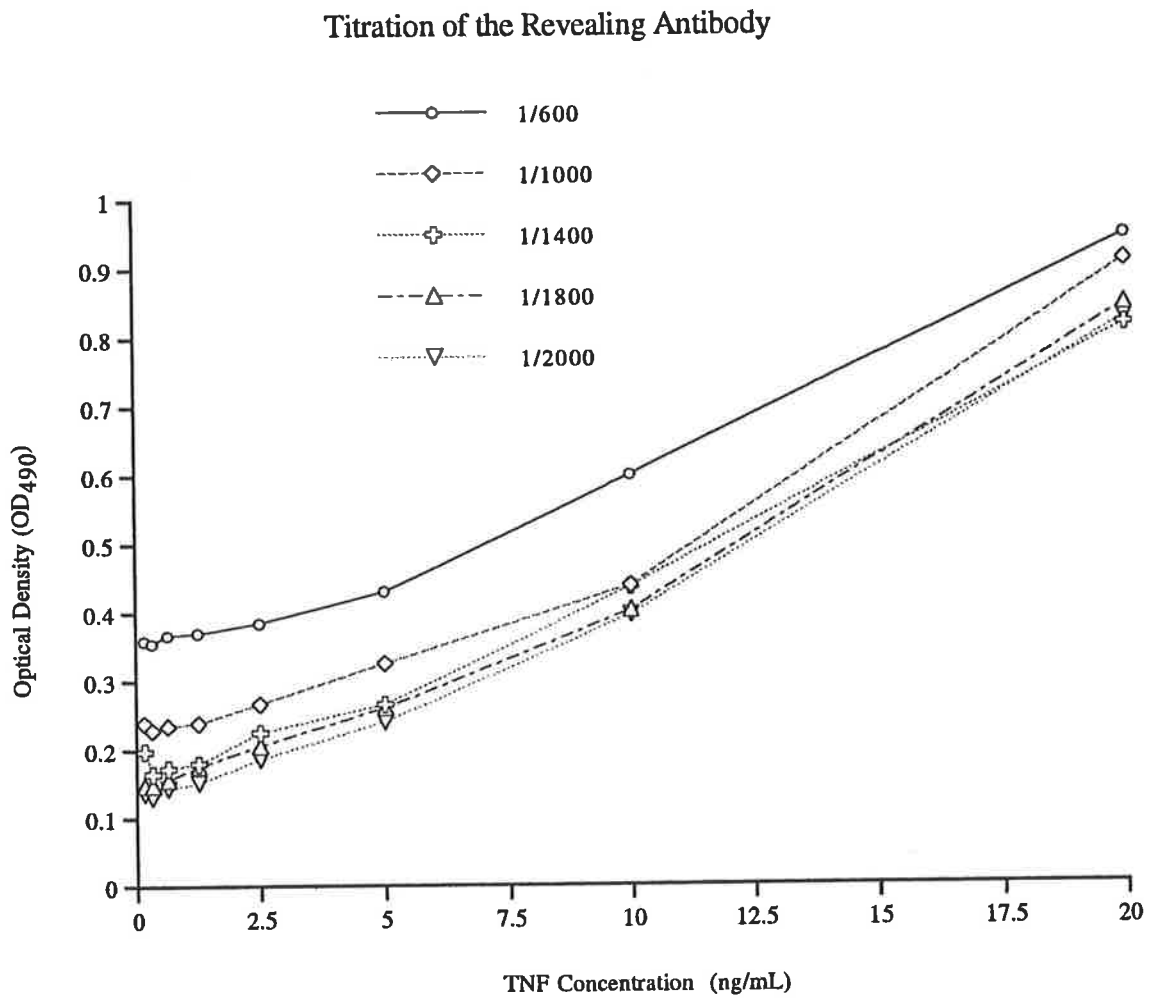
Figure 9. Standard curves generated using different dilutions of the revealing antibody (not all dilutions are shown).

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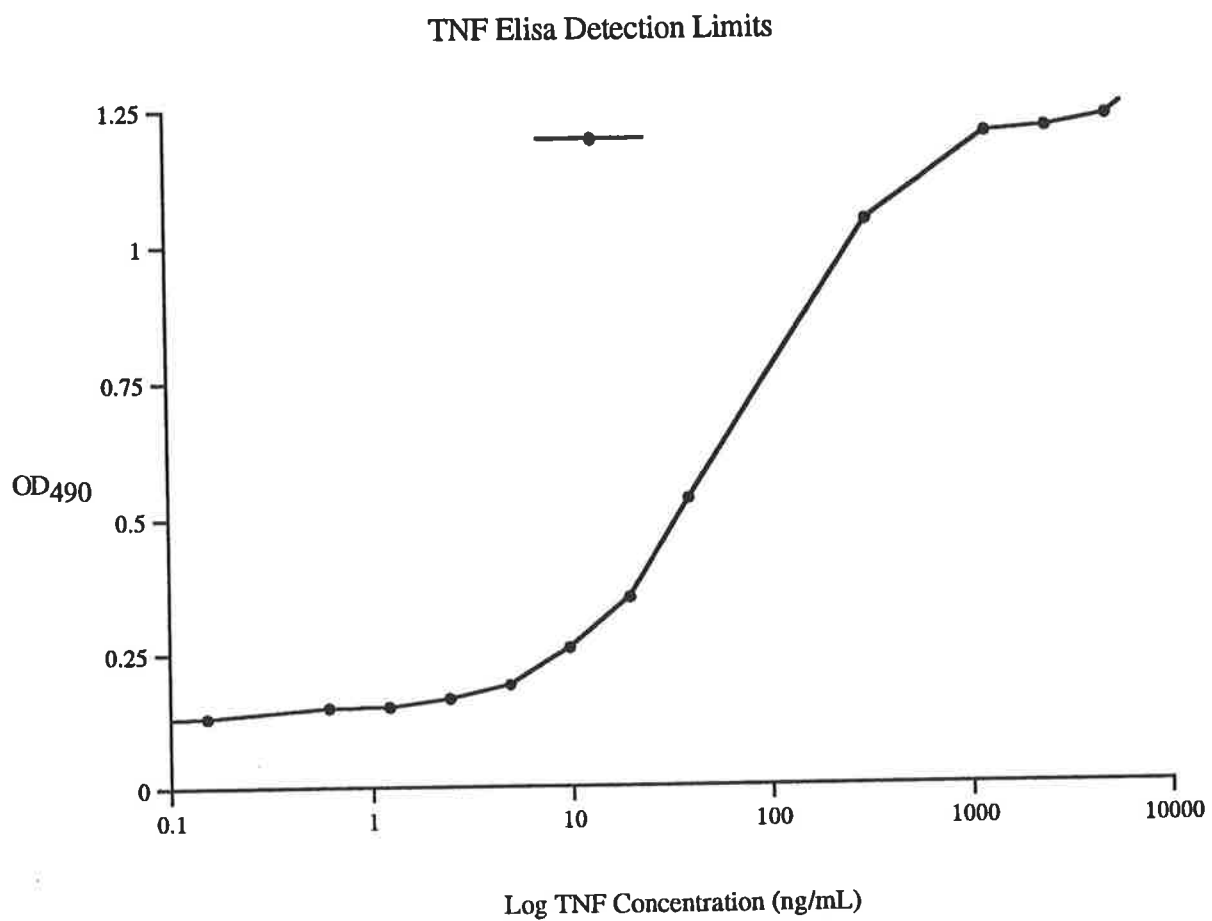
Figure 10. The upper and lower limits of detection of TNF protein by ELISA. The lower and upper limits of detection are 5 ng/mL and 1000 ng/mL respectively.

Chapter 6

Kinetics of Cytokine mRNA and Protein Secretion During Concanavalin A Stimulation of Rat Splenocytes

6.1 Cytokine mRNA Expression in Con A Stimulated Rat Splenocytes

6.1.1 Introduction

Cytokines are potent mediators of an immune response that are usually produced in small quantities by a variety of different cell types. A single cytokine is able to stimulate the production of many others which results in the formation of a complex, dynamic cytokine network. Sequential measurement of changes in cytokine gene expression provides important information about the networks that control an immune response and the factors that may initiate and perpetuate the response. The amount of cytokine mRNA produced during cell activation does not always reflect production of the biologically active protein since cytokine gene expression may be controlled at the transcriptional and post-transcriptional levels (Stoeckle 1991). Furthermore, message for most cytokines possess several repeats of the AUUUA pentamer in the 3' untranslated region which confers instability of the mRNA. This contributes to the short half life of many cytokine mRNA transcripts (Akashi et al. 1994) which is in the order of approximately 20 minutes to one hour (Bethea et al. 1992; Bollon et al. 1988; Shaw and Kamen 1986). As a result of their potent biological activity, cytokines are usually secreted at very low concentrations (Balkwill 1991; Kaplan 1996). Furthermore, T-cells are usually in a resting or semi-activated state, during which they secrete only very small amounts of a cytokine whereas upon activation, they become primed and secrete cytokines in short bursts (Kaplan 1996; Kelso 1993). This is in contrast to macrophage and monocyte derived cytokines, such as TNF and TGF-β, which are produced in relative high amounts and are often secreted non-directionally into the extracellular environment where they exert their effects.

Minor differences in cellular physiology caused by genetic background (Szeliga et al. 1996), age, (Hobbs et al. 1993), tissue origin, the timing and nature of exogenous stimulation, as well as subtle variations in environmental conditions (Karp et al. 1994) can all effect the level of cytokine gene expression in an immune response. It is therefore valuable to have an assay that can reliably and accurately detect low quantities of cytokine mRNA. The quantitative RT-PCR procedure described in Chapters 4 and 5 has a detection limit approximating 100 copies of cytokine cDNA. Furthermore, the reproducibility of the assay using the conditions outlined in Sections 5.3 and 5.4 make the technique suitable for monitoring changes in the levels of low abundance cytokine genes.

This Chapter describes changes in the steady state levels of mRNA for the T-cell cytokines; IFN-γ, IL-2 and IL-4, the putative immunoregulatory cytokine, TNF, and the immunomodulatory cytokine, TGF-β, at various times during concanavalin A (con A) stimulation of splenocytes. Although the mechanisms underlying the activation of cells by con A remain undefined, it is known to cross link a number of glycosylated cell surface receptors (Burger and Noonan 1970), including the T-cell antigen receptor (Weiss et al. 1987). Con A activates T-cells which leads to the production of T-cell cytokines and subsequently, macrophage-derived cytokines. Quantitative RT-PCR was used to measure changes in the levels of steady state cytokine mRNA. Changes in the levels of cytokine mRNA and protein were measurable over 72 hours of stimulation of spleen cells with con A. This was a convenient system in which to measure changes in cytokine expression by cells. Since changes in cytokine gene transcription do not always reflect production of the biologically active protein (Tang et al. 1994), the levels of IFN-γ and TNF proteins were measured along with changes in the levels of the respective cytokine mRNA. IFN-γ protein was measured using an established ELISA assay and the levels of TNF were measured using an ELISA assay developed and evaluated as described in Section 5.5.

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6.1.2 Results.

6.1.2.1 Profiles of Cytokine mRNA Production Following Con A Stimulation of Rat Splenocytes

Changes in the levels of cytokine mRNA were monitored during *in vitro* stimulation of splenocytes with con A. A single cell suspension of splenocytes from a DA rat was prepared (Section 2.20) and the contaminating erythrocytes were lysed using ammonium chloride solution. The resultant splenocytes were cultured at a density of 2×10^6 cells/mL in a 24 well plate then 5 μg/mL of con A was added to selected wells. Both stimulated and unstimulated splenocytes were harvested after 0h, 3h, 6h, 9h, 12h, 18h, 24h, 30h, 36h, 48h and 72h of culture. At the designated time, the cells were collected and lysed in 500 μL of guanidine thiocyanate solution and stored at -70°C in preparation for quantification of cytokine gene levels using quantitative RT-PCR. Similarly, aliquots of the supernatants at each time point were taken, passed through a 0.45 μm filter then stored at -70°C in preparation for cytokine protein analysis (see Section 6.2).

RNA was extracted from cell lysates (Section 2.1) and cDNA prepared (Section 2.2) and the amount of each cytokine mRNA was quantified using competitive PCR (Section 2.14). The levels of IFN-γ, IL-2 and IL-4 were determined by amplifying multiple dilutions of the appropriate competitor with replicate samples of the target cDNA, which is referred to as the "interpolated competitive PCR method" in Section 5.4.2. Due to the large number of samples to be analysed, this method became labour intensive and impractical. Therefore, the amount of TNF and TGF-β cDNA present at each time point was determined by co-amplifying a single dilution of the appropriate competitor with a single aliquot of the target which is referred to in Section 5.4.3 as the "rapid competitive PCR method". CE was used to determine the target:competitor ratio from each competitive PCR following which, the amount of cytokine cDNA at each time point was calculated. Fig. 1 illustrates changes in the level of cytokine cDNA during the time course of con A stimulation.

The abundance of mRNA for each cytokine during the first phase of stimulation varied. TGF-β was the most abundant followed by IFN-γ, TNF, IL-2 then IL-4. TGF-β mRNA

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expression peaked at 12 hours and 36 hours after con A stimulation after which it declined to background levels at 48 hours. The kinetics of TNF mRNA production were similar to TGF-β, however, the overall amount of TNF was considerably lower. Following a peak in production at 12 hours, the level of IFN-γ mRNA reached a plateau, then after 39 hours it slowly declined to baseline levels. The amount of IL-2 peaked after 12 hours of stimulation then slowly decreased to background levels as the stimulation time extended towards 72 hours. IL-4 mRNA levels remained very low throughout the stimulation time course.

Cytokine gene expression was similarly quantified in a duplicate con A stimulation experiment using the time points, 0h, 12h, 24h, 48h and 72h. Although the absolute quantities of cytokine gene expression differed somewhat from the first trial (data not shown), the relative changes were similar to those outlined in Fig 1.

The amount of cytokine mRNA produced by unstimulated cells was examined using quantitative RT-PCR. Although the levels of IFN-γ, IL-2 and IL-4 mRNA in unstimulated cells were below the detection limit of the PCR assay, the levels of TNF and TGF-β mRNA were quite high (approx. 100,000 and 250,000 copies/μg respectively). The amounts of TNF and TGF-β mRNA produced by stimulated and unstimulated splenocytes during the first experiment are illustrated in Fig. 2 and Fig. 3 respectively.

6.1.3 Discussion

The kinetics of IFN-γ, IL-2, IL-4, TNF and TGF-β mRNA expression by rat splenocytes in response to con A simulation is illustrated in Fig. 1. A similar hierarchal production of IFN-γ, IL-2 and IL-4 mRNA in murine splenocytes stimulated with con A has been reported (Monteyne et al. 1992). IFN-γ protein produced by mice splenocytes peaked approximately 35 hours after stimulation with con A whereas IL-4 protein produced by the splenocytes underwent phasic production, peaking firstly at 25 hours then again 50 hours after stimulation (Sander et al. 1989).

It is evident from Fig. 1 that the production of T-cell derived cytokines was generally lower than that of TNF and TGF-β. This may in part be due to the relative abundance of

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cells producing the latter cytokines. The spleen is a rich source of phagocytic cells including macrophages and dendritic mononuclear phagocytes that can secrete TNF and TGF-β upon activation whereas, it contains fewer T-cells and thus, T-cell derived cytokines (Junqueira et al. 1989). Furthermore, "T-cell derived" cytokines are secreted only by a few types of cells, whereas TNF and TGF-β are produced by numerous types of cells. For example IL-2 is produced only by activated T-cells. IFN-γ is secreted by natural killer cells as well as activated T-cells (Rager-Zisman and Bloom 1985). IL-4 is secreted by activated T-cells and by mast cells (Bradding et al. 1992). In contrast, neutrophils, macrophages, lymphocytes, NK cells and endothelial cells are all capable of secreting TNF (Vassalli 1992). Platelets are a major source of TGF-β (Massague 1987) and they secrete it readily upon stimulation. TGF-β is secreted as a pro-cytokine that requires enzymic cleavage to achieve biological activity. The latent form of TGF-β is secreted by almost all types of cells (Rifkin et al. 1993) and as a result, it is difficult to assign a biological function to TGF-β without monitoring secretion of the biologically active protein (Sporn et al. 1987). The abundance of different types of cells in the spleen may in part be reflected in the relative amounts of various cytokines produced.

It is conceivable that production of T-cell cytokines is subject to strict regulation and therefore, these cytokines may be produced in smaller amounts. The quantity of cytokine mRNA produced may be indicative of its biological activity with locally acting cytokines produced in lower amounts. This is consistent with lower production of T-cell derived cytokines and the abundance of TNF and TGF-β following stimulation. The production of significant amounts of TNF and TGF-β mRNA by unstimulated splenocytes, as depicted in Figs. 2 and 3 may represent a preformed pool of mRNA, which can be rapidly translated into protein if required. This could also be explained by the cells being partially stimulated during preparation for culture.

IFN-γ and IL-4 are well known to be mutually antagonistic cytokines (Fernandez-Botran et al. 1988; Greenbaum et al. 1988; Maggi et al. 1992). This was evident in Fig. 1 which depicted low levels of IL-4 mRNA and high levels of IFN-γ mRNA throughout the time course of stimulation.

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This Chapter describes the application of quantitative RT-PCR to measuring changes in cytokine mRNA levels following *in vitro* stimulation of splenocytes. The data indicated the magnitude of change that can be expected when measuring changes in cytokine mRNA in association with *in vitro* mitogen stimulated biological system. A change as large as 150 fold was observed for IFN-γ whereas, very little change in mRNA production was observed for IL-4. Thus, the precision of the quantitative RT-PCR procedure which was established in Chapter 4 has been demonstrated to be suitable for quantifying changes in the levels of cytokine mRNA in a biological system.

6.2 Cytokine Protein Secretion During Con A Stimulation of Rat Splenocytes

6.2.1 Introduction

Physiological changes in the level of cytokine mRNA are often reported without relevant data supporting secretion of cytokine protein. Changes in cytokine mRNA levels during stimulation of splenocytes with con A were analysed using quantitative RT-PCR in Section 6.1.2.1. In this Section, changes in the level of IFN-γ and TNF protein were measured during the stimulation of splenocytes with con A to ascertain a relationship between cytokine mRNA production and secretion of the respective proteins. IFN-γ protein was measured in the supernatants of cultured cells using an established ELISA whereas, the amount of TNF protein was measured in the same samples using the ELISA developed in Section 5.5.

6.2.2 Quantification of IFN-γ and TNF Protein Levels During Con A Stimulation of Splenocytes

Changes in the levels of cytokine mRNA were monitored during stimulation of splenocytes with con A *in vitro* as previously outlined. Briefly, a single cell suspension of splenocytes from DA rats was prepared which were stimulated with 5 µg/mL of con A as described in the experiment outlined in Section 6.1.2.1. Cell culture supernatants were

harvested after 0h, 3h, 6h, 9h, 12h, 18h, 24h, 30h, 36h, 48h and 72h of stimulation. The amount of cytokine protein at each time point was measured in triplicate using an ELISA. The kinetics of IFN-γ protein and mRNA accumulation is plotted in Fig. 4, and the kinetics of TNF protein and mRNA accumulation is plotted in Fig. 5.

IFN-γ protein remained undetectable until 12 hours after con A stimulation. Thereafter, the amount detected steadily increased until the measurements were terminated after 72 hours. The IFN-γ protein measured most likely represents its accumulation over the 72 hour period which is evident during the later time points in which steady state mRNA levels decline to baseline levels whereas, the quantity of secreted protein continues to increase. Since protein synthesis cannot be maintained without new message being transcribed, once mRNA production falls to zero there is no further increase in protein levels as seen in Figs. 4 and 5. Protein levels began to rise several hours after the peak of mRNA production and gradually rose thereafter, reaching maximal levels at 72 hours.

In contrast, TNF protein remained undetectable in the superatants of con A stimulated cells until only 4 hours post stimulation. Thereafter the amount rapidly increased up to 18 hours after which, the absolute quantity of TNF varied slightly but remained elevated until the measurements were terminated after 72 hours. There was an initial burst of mRNA production after approximately 12 hours of stimulation which may have been a precursor to the rapid increase in the quantity of TNF protein that was observed between 4 and 12 hours of stimulation (as with IFN-γ protein levels). The kinetics of TNF protein production depict in Fig. 5 most likely represents the accumulation of TNF over the 72 hour period. There was approximately a 4 hour time delay between upregulation of mRNA and detection of the protein.

IL-4 is key cytokine that is relevant to predicting the predominating T-helper cell subset induced during an immune response. However, IL-4 mRNA levels during con A stimulation were very low and therefore no attempt was made to measure the protein levels. Attempts to quantify TGF-β using Immunohistochemistry and Western blots failed, mainly due to the lack of specificity of the polyclonal antibodies.

6.2.3 Conclusions

Con A interacts with the T-cell receptor and other surface molecules resulting in the rapid induction of T-cell cytokine expression without the requirement for antigen processing and presentation. The peak level of cytokine protein production is the result of a balance between production, removal and degradation. For example IL-2 production is known to upregulate IL-2 receptor expression on T-cells, which results in increased binding and removal of IL-2 from the extra cellular environment (Minami et al. 1993). Furthermore, cytokines may be cross regulatory, for example binding of IFN-γ to its specific receptor is known to down regulate IL-4 production (Maggi et al. 1992). Thus, the production of cytokines seen in this *in vitro* system may be influenced by homeostatic cross-regulatory mechanisms thereby explaining the low levels of IL-4 production seen in association with substantial IFN-γ production.

There is good correlation between IFN-γ and TNF mRNA production, and secretion of the respective proteins *in vitro*. This indicates that mRNA expression should be a good indicator of protein synthesis *in vivo*. However, this may not be applicable for all mRNA and proteins, particularly those that are regulated principally at the translational or post-translational level. TNF protein production is controlled at these levels as well as at the transcriptional level (Sung et al. 1992) and in this *in vitro* system TNF mRNA and protein levels do correspond well.

6.3 Summary

A study was undertaken to measure changes in cytokine mRNA levels using quantitative RT-PCR following stimulation of splenocytes *in vitro*. The data demonstrated the magnitude of change associated with cytokine mRNA production which are expected within a mitogen stimulated cellular system. A change as large as 150 fold was observed for IFN-γ whereas very little change in mRNA production was observed for IL-4. Thus, the precision and accuracy of the quantitative RT-PCR procedure that was developed in

Chapter 4 was suitable for quantification of changes in the levels of cytokine mRNA in a biological system.

Since physiological changes in cytokine mRNA levels are not always supported by changes in secretion of the biologically active protein, the levels of IFN-γ and TNF protein were measured during stimulation of splenocytes with con A. This was accomplished by developing and evaluating an ELISA to measure TNF protein levels while IFN-γ protein levels were measured by an ELISA that was already established in our laboratory. There was a good correlation between IFN-γ and TNF mRNA production and secretion of the respective proteins *in vitro*. This verified that mRNA expression was a good indicator of protein secretion *in vitro*, at least in relation to these cytokines.

Capillary electrophoresis was shown to be suitable for quantification of PCR products as demonstrated in Chapter 3 whereas, the accuracy and reproducibility of the competitive RT-PCR procedure was established in Chapter 4. Furthermore, an insight into the accuracy of quantifying RNA and the reproducibility of the extraction and reverse transcription steps were presented in Chapter 5. In this Chapter the competitive RT-PCR procedure was shown to be suitable for monitoring changes in the levels of cytokine mRNA by cells *in vitro* and that increased message was associated, after a period of delay, with increased protein synthesis. In summary, the competitive RT-PCR procedure has been thoroughly evaluated and shown to be suitable for monitoring changes in cytokine mRNA levels by cells *in vitro*.

IFN-γ, IL-2, IL-4, TNF, TGF-β mRNA Profile During Con A Stimulation of Spleen Cells
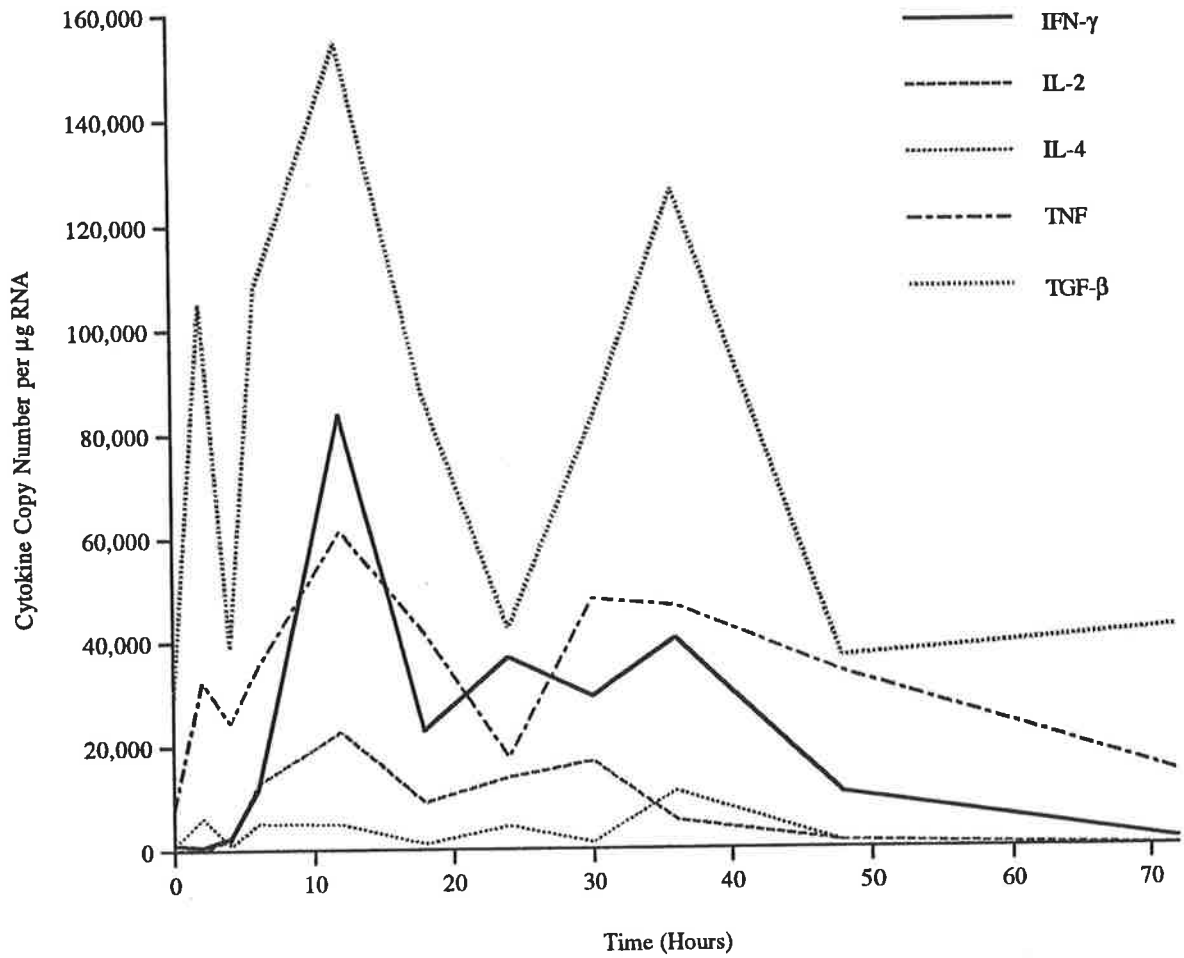
Figure 1. A time course of IFN-γ, IL-2, IL-4, TNF and TGF-β mRNA expression during stimulation of splenocytes (2×10^6 cells/mL) with 5 μg/mL of con A.

TNF Stimulation Profile
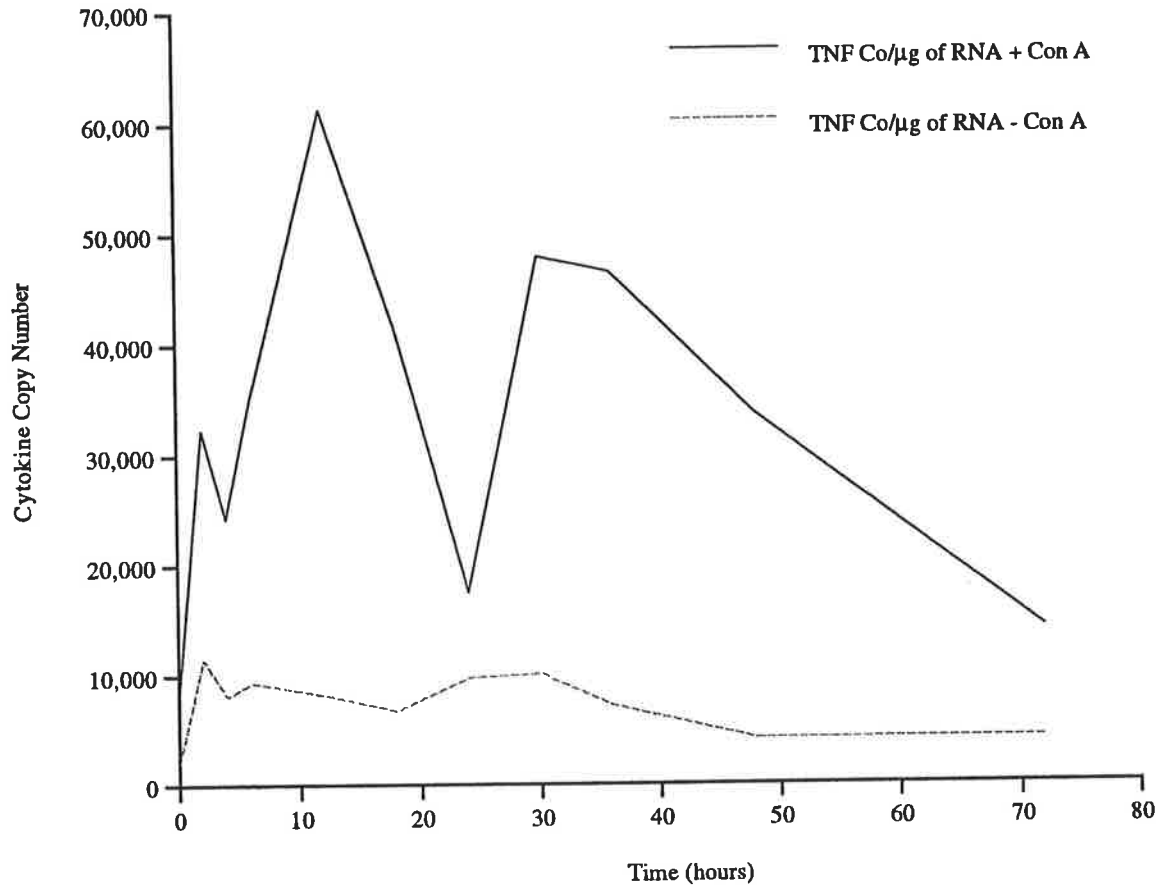
Figure 2. A time course of TNF mRNA expression in unstimulated splenocytes and splenocytes stimulated with 5 μg/mL of con A.
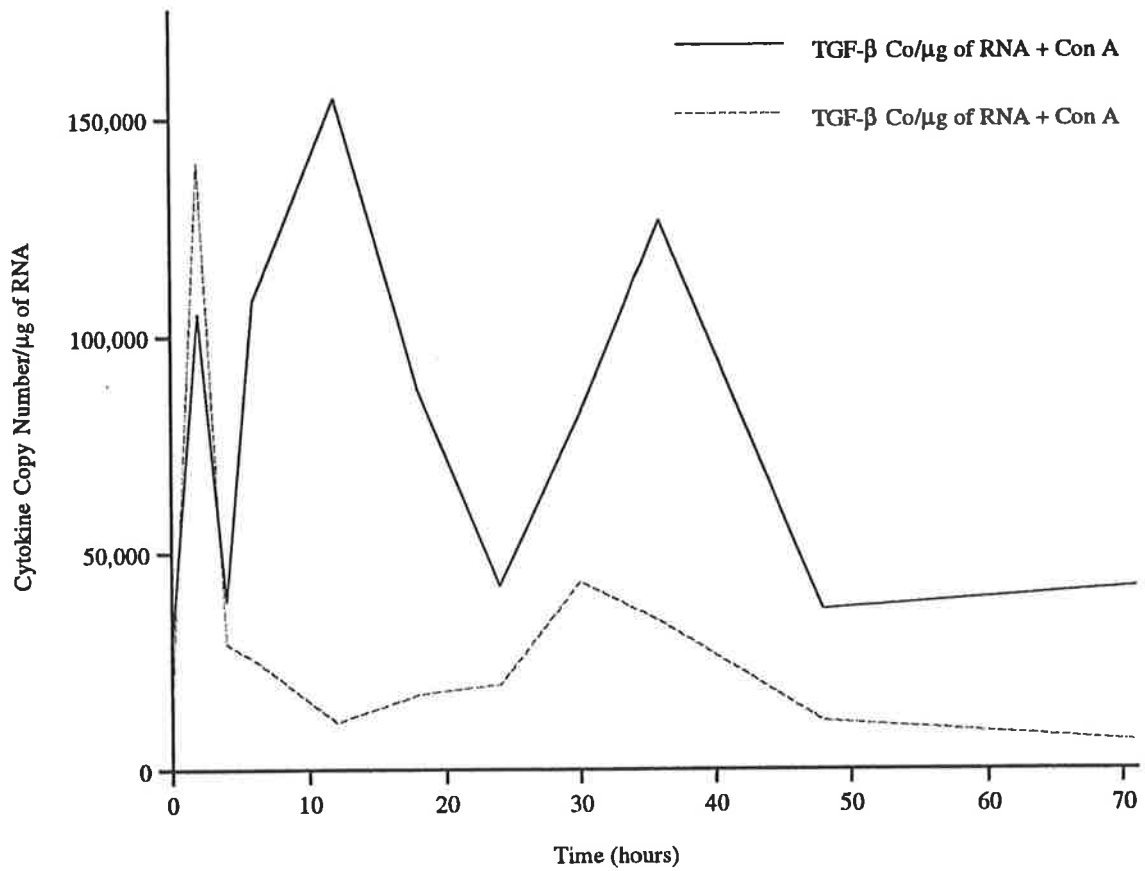
Figure 3. A time course of TGF-β mRNA expression in unstimulated splenocytes and splenocytes stimulated with 5 μg/mL of con A.

IFN-γ mRNA / Protein Secretion Following Con A Stimulation of Splenocytes

Figure 4. The kinetics of IFN-γ mRNA and protein accumulation during con A stimulation of splenocytes.
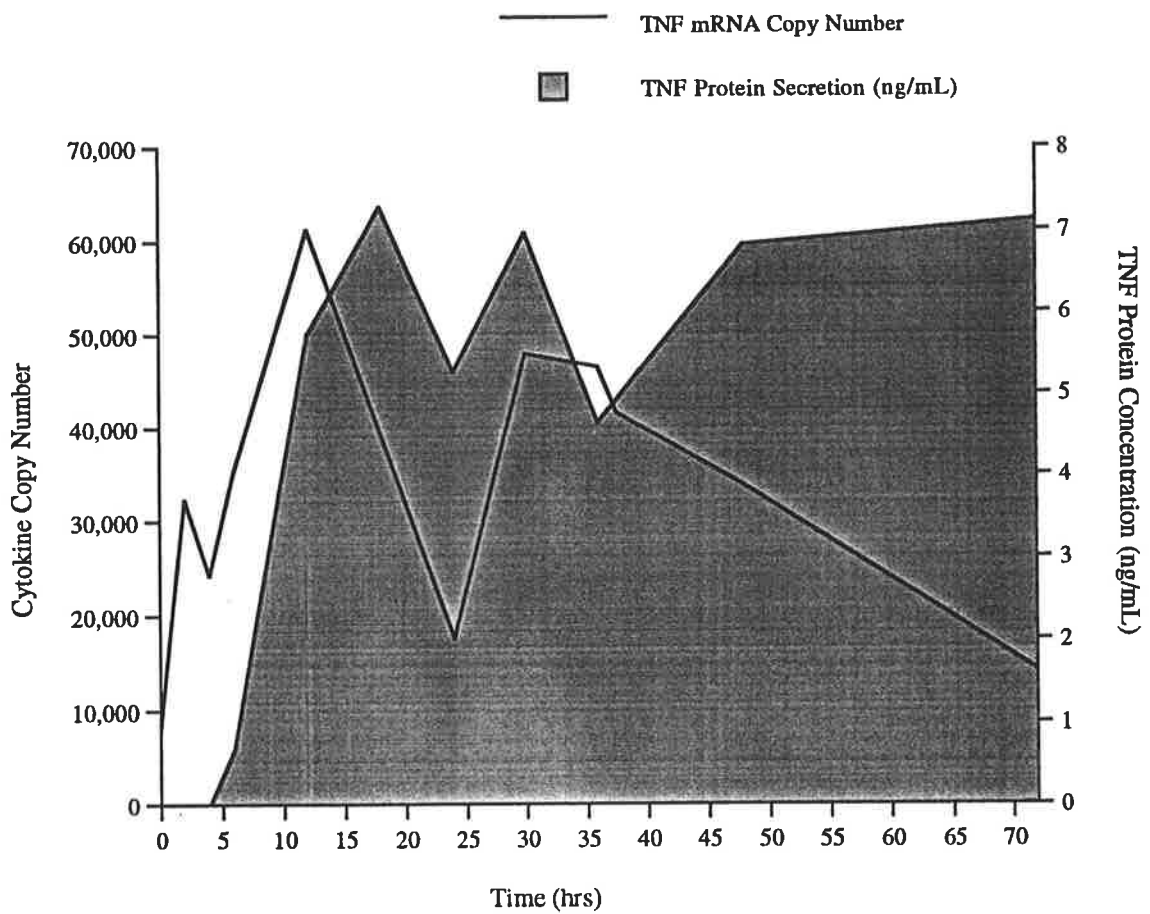
Figure 5. The kinetics of TNF mRNA and protein accumulation during con A stimulation of splenocytes.

Chapter 7

Kinetics of Cytokine Expression During the Induction of Adjuvant Arthritis in Rats

7.1 Adjuvant Arthritis Time Trial

7.1.1 Introduction

Animal models of rheumatoid arthritis (RA) provide an opportunity to study the aetiology, pathophysiology and treatment of the disease under controlled conditions using protocols often not feasible or ethically acceptable in humans. Information gained from studies using animal models of RA can be particularly informative regarding induction of the disease considering early changes in RA are difficult to study because onset of the disease often proceeds clinical diagnosis. Adjuvant arthritis (AA) provides a model to study the induction of polyarthritis that, like RA, leads to joint destruction. Insights into immuno-physiological events in AA should provide guidance for less accessible and more difficult investigations in RA.

Adoptive transfer experiments and various T-cell directed therapies have demonstrated that CD4$^+$ T-cells play a critical role in the induction and pathogenesis of both RA and AA. The T-cell dependence associated with the induction and pathogenesis of AA is well established (Billingham et al. 1990; Pelegri et al. 1995b; Yoshino et al. 1990). However, the presence of T-cells in synovial tissue during the later stages of AA in rats is controversial (Pelegri et al. 1995a) whereas detection of T-cells in synovial tissue of RA patients is well established (Van and Paget 1975).

The presence of T-cells in the synovial tissue of rats with AA was reported by Larsson and De Joy (DeJoy et al. 1990; Larsson et al. 1985). Furthermore, T-cell accumulation in AA synovia was quantified by Issekutz et. al. (Issekutz and Issekutz 1991a). However, Pelgri

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et.al. used immunohistochemical techniques to demonstrate there were few or no lymphocytes in the synovial tissue of rats with AA on days of maximum inflammation (Pelegri et al. 1995a).

Initially, a functional bias in cytokines secreted by CD4$^+$ T-cells was evident in animal models of human diseases in which the immune system was chronically stimulated. It has been demonstrated that human CD4$^+$ T-cells can become polarised into Th-1 or Th-2 types (Romagnani 1991). Th-1 cells promote cell mediated or delayed type hypersensitivity responses and the secretion of IFN-γ and IL-2, whereas Th-2 cells promote humoral or allergic responses and the secretion of IL-4, IL-10 and IL-13.

Inflammatory arthritis such as RA, is characterised by phagocyte and lymphocyte cell infiltration into the synovial tissue and the subsequent production of multiple cytokines. There is evidence suggesting Th-1 cells are responsible for the pathogenesis of RA (Miltenburg et al. 1992) as well as counter evidence implicating Th-2 responses with progression of the disease (Quayle et al. 1993). In this Chapter, the production of cytokines during the evolution of AA was assessed in an attempt to establish the type of T-cell and immunoregulatory cytokines involved in the induction and expression of AA.

7.2 Results

7.2.1 Incidence and Severity of AA in CFA Inoculated Rats

Arthritis was induced in female DA rats by injection of complete Freunds adjuvant (CFA) into the tail base as outlined in Section 2.18. Arthritis developed in all rats within two weeks of CFA inoculation. The pathogenesis of AA was monitored by assessing weight loss and the arthritic index (AI) of individual rats. The AI scale provided a measure (from 0 to 4) of the severity of inflammation and oedema. 0 represented a normal paw and 4 represented a severely inflamed paw. The maximum achievable joint score was 16 following which, the animal was euthanased in accordance with ethical guidelines. The development of AA as indicated by the AI and weight loss is depicted in Fig. 1. Animals

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with severe clinical synovitis (determined by the AI) were euthanased prior to the ethically accepted disease duration of 14 days. As a result, 3 rats with AA were euthanased on day 12 and 13. On these days all rats had severe arthritis and their data was combined and represented as day 12/13 in Fig. 2, 3 and 4.

Eight rats inoculated with saline solution served as the controls. Four were euthanased on day 0 and day 15. Since findings in these control rats were similar, they were combined and are represented as day 0 in Figs. 1, 2, 3 and 4. These rats had no evidence of joint swelling or oedema and experienced an average increase in body weight of 14 grams.

7.2.2 The Kinetics of IL-2, IFN-γ, IL-4, TNF and TGF-β mRNA Expression in the Inguinal Lymph Nodes of CFA Inoculated Rats (Experiment 1)

Changes in cytokine mRNA levels in the inguinal lymph node, popliteal lymph node and synovium were monitored during the development of AA in experiment 1. Changes in cytokine mRNA levels in the inguinal lymph node were evident 3 days after CFA inoculation whereas changes in cytokine mRNA levels in the popliteal lymph node were elevated approximately 6 days after CFA inoculation. Therefore, experiment 2 involved monitoring changes in cytokine mRNA levels in the inguinal lymph node and popliteal lymph node 3 and 6 days after CFA inoculation respectively.

In experiment 1, cytokine mRNA levels were assessed during the development of AA by inoculating each of twenty rats with 100 μL of 10 mg/mL CFA. One day after inoculation, three rats were euthanased and the inguinal lymph nodes, popliteal lymph nodes and synovial tissue were removed and immediately frozen in liquid nitrogen in preparation for quantification of cytokine mRNA using competitive PCR. This procedure was repeated on days 3, 6, 9 and 12/13 after CFA inoculation (experiment 1).

RNA was extracted from each tissue sample and reverse transcribed in preparation for PCR analysis. Competitive PCR was performed by adding a constant amount of competitor fragment to 62.5 ng of cDNA. PCR amplification was performed using the conditions outlined in Section 2.14 with oligonucleotide primers designed to amplify IL-2,

IFN-γ, IL-4, TNF and TGF-β. The PCR products were desalted then quantified using CE from which the amount of cytokine cDNA in each sample was calculated.

Intradermal injection of CFA into the tail base of female DA rats induced IFN-γ mRNA expression in the inguinal lymph node 3 days after inoculation (Fig. 2). The levels of IFN-γ mRNA rose significantly from control values on day 3 and remained elevated until day 12/13 when all rats with AA were euthanased. IL-2 mRNA levels rose sharply on day 1 then decreased but remained significantly different from the control levels until day 6. TNF mRNA levels were significantly elevated from those in the control rats 1 day after CFA inoculation, peaking at day 3 and remaining elevated until day 9. TGF-β mRNA levels were significantly elevated from those in the control rats 1 day after inoculation and remained elevated until day 6, after which they fell to control levels. IL-4 mRNA levels were also monitored throughout the time course of AA development yet at no stage were they significantly different from the levels measured in control rats.

7.2.3 The Kinetics of IL-2, IFN-γ, IL-4, TNF and TGF-β mRNA Expression in the Popliteal Lymph Nodes of CFA Inoculated Rats (Experiment 1)

Changes in cytokine mRNA levels in the popliteal lymph node were assessed during the development and pathogenesis of AA as described in experiment 1, Section 7.2.2.

Intradermal injection of CFA into the tail base of female DA rats induced an upregulation of IL-2 mRNA in the popliteal lymph node 6 days after immunisation whereafter, the levels were similar to those in the control rats (Fig. 3). A tentative increase in IFN-γ mRNA level was observed on days 3 and 6 otherwise they were similar to the control level. The amounts of TNF mRNA from AA rats did not differ significantly from those in the control rats, with low levels of message being found in all but a single rat on day 1. TGF-β mRNA levels rose significantly from control values on days 9 and 12/13 and were otherwise similar to the control values. IL-4 mRNA levels were also monitored throughout the time course of AA development yet, as with the levels of IL-4 in the inguinal lymph node, at no stage were they significantly different from the levels measured in the control rats.

7.2.4 The Kinetics of IL-2, IFN-γ, IL-4, TNF and TGF-β mRNA Expression in the Synovial Tissue of CFA Inoculated Rats (Experiment 1)

Changes in cytokine mRNA levels in the synovial tissue were assessed during the pathogenesis of AA as outlined in Section 7.2.2. Intradermal injection of CFA into the tail base of female DA rats failed to induce IL-2 mRNA expression in the synovial tissue for the duration of the experiment despite the high sensitivity of the PCR assay (Fig. 4). IFN-γ mRNA levels in both AA and saline inoculated rats did not differ significantly throughout the course of AA. IL-4 mRNA levels were monitored in the synovial tissue throughout the development of AA yet at no stage were they significantly different from control values. TNF mRNA was undetectable in the synovial tissue of AA rats until day 9, but was increased significantly on day 12/13. TGF-β mRNA levels in AA rats decreased below control values on days 1 and 3, then rose and were significantly elevated from the control values on day 12/13.

7.2.5 Cytokine mRNA Expression in the Inguinal Lymph Nodes 3 Days After CFA Inoculation (Experiment 2)

The data from experiment 1 indicated that the levels of IFN-γ, IL-2, TNF and TGF-β mRNA in the inguinal lymph node were elevated 3 days after CFA inoculation. There were no direct time control rats for days 1, 3, 6 or 9 because of the excessive number required for the experiment. Furthermore, multiple comparisons can yield misleading differences on statistical analysis. Therefore, experiment 2 was conducted in which changes in cytokine mRNA levels were re-analysed on day 3 in the inguinal lymph nodes and day 6 in the popliteal lymph nodes.

In experiment 2, four rats were inoculated with 100 µL of 10 mg/mL CFA then 3 days later they were euthanased and the inguinal lymph nodes were removed and immediately frozen in liquid nitrogen in preparation for cytokine mRNA quantification using competitive PCR. Likewise, four control rats were inoculated with 100 µL of saline solution and the inguinal lymph nodes were removed 3 days after immunisation as outlined above. Competitive PCR was performed as outlined in Section 7.2.2. The reproducibility

and accuracy of the quantitative PCR has been analysed in Sections 4.3.5 and 5.3.1 from which it was shown to be relatively quantitative when all samples were RT using a common preparation of the RT reagents. Hence, while the proportion of cytokine mRNA, which is reverse transcribed to cDNA, can vary between experiments, within an experiment the conversion should be consistent and indicative of changes in mRNA expression.

The levels of IL-2 and TNF mRNA expression were significantly elevated in the inguinal lymph nodes of AA rats (Table 1). Mean IL-2 mRNA levels rose to 9,618 copies/μg of RNA in AA rats which was 2.0 fold higher than those in control rats. An average 6,866 copies of IFN-γ mRNA/μg of RNA was measured 3 days after induction of AA which represents on average a 4.7 fold rise above the levels measured in the controls. A mean of 956,275 copies of TNF mRNA/μg of RNA was obtained in AA rats which represents a 2.0 fold increase above the control levels. Mean TGF-β mRNA levels rose to 54,532,657 copies/μg of RNA 3 days post-immunisation which represented a 1.6 fold rise but this did not differ statistically to the levels in the control rats (Mann-Whitney U test).

7.2.6 Cytokine mRNA Expression in the Popliteal Lymph Nodes 6 Days After CFA Inoculation (Experiment 2)

The data from Fig. 3 indicated that IFN-γ, and IL-2 mRNA levels in the popliteal lymph node were elevated 6 days after CFA inoculation. However, no controls were sacrificed on this day. Therefore, four CFA and four saline inoculated rats were euthanased 6 days after immunisation and IFN-γ, and IL-2 cytokine mRNA levels in the popliteal lymph node were measured as outlined in Section 7.2.2.

On average 137,881 copies of IL-2 mRNA/μg of RNA were quantified in the popliteal lymph node of AA rats which represents a 1.8 fold increase above the levels in the control rats (Table 2). The levels of IFN-γ mRNA averaged 3,435 copies/μg of RNA in AA rats which was 6.5 fold higher than the control values. Despite the elevation in IL-2 and IFN-γ mRNA levels in AA rats, they were similar statistically to the control values.

7.2.7 Cytokine mRNA Expression in the Synovium 12/13 Days After CFA Inoculation

The data from Fig. 4 indicated that TNF and TGF-β mRNA levels in the synovial tissue were elevated significantly 12/13 days after CFA inoculation. However, no controls were sacrificed on this day. Therefore, changes in TNF and TGF-β mRNA levels in the synovial tissue were evaluated with the four control rats euthanased on day 15. The data are presented in Table 3.

An average 2,856 copies of TNF mRNA/μg of RNA was measured 12/13 days after the induction of AA whereas TNF was undetectable in all but one control rat. TGF-β mRNA rose to 261,609 copies/μg of RNA in AA rats which was 6.5 fold higher than the control values.

7.2.8 IFN-γ Protein Secretion in the Inguinal Lymph Nodes 3 Days After CFA Inoculation

IFN-γ mRNA expression in the inguinal lymph node appeared to increase 3 days after CFA inoculation (Fig. 2). However, such an increase was not evident in experiment 2, in which the levels of IFN-γ from AA rats were similar to those in control rats (Table 1). To verify the mRNA data, changes in the level of IFN-γ protein were analysed in the inguinal lymph node 3 days after CFA inoculation as outlined in experiment 2 (Section 7.2.5). Inguinal lymph nodes were removed from the four CFA and four saline inoculated rats 3 days post immunisation. Mononuclear cells (MNC) from the nodes of individual rats were cultured with concanavalin A (con A) and the amount IFN-γ protein accumulation in the supernatants was measured over a 3 day period using an ELISA assay.

The quantity of IFN-γ produced by the inguinal lymph node cells of AA and control rats is displayed in Fig. 5. The amount of IFN-γ protein was significantly elevated in supernatants acquired from AA rats after 1 day of con A stimulation and was undetectable in the control rats. IFN-γ protein levels in AA and control rats were not different statistically after 2 and 3 days of con A stimulation, but the levels in AA rats were generally greater than those in the control rats.

7.2.9 TNF Protein Secretion in the Inguinal Lymph Nodes 3 Days After CFA Inoculation

The level of TNF mRNA in the inguinal lymph node of AA rats rose significantly from control values on day 3 (Fig. 2 and Table 1). To verify changes in the levels of TNF mRNA, the amounts of TNF protein in the inguinal lymph node were analysed on day 3. Inguinal lymph nodes were removed from AA and saline inoculated rats 3 days post immunisation and MNC were cultured with con A as described in Section 7.2.7. The amounts TNF protein accumulating in the supernatants were measured over a three day period using an ELISA developed in Section 5.5.

The amount of TNF protein in the inguinal lymph node of AA and control rats is displayed in Fig. 6. Stimulation of inguinal lymph node cells for 1 and 2 days with con A lead to the secretion of TNF at a level similar to the lower detection limit of the assay. After 1 day of con A stimulation, TNF levels in AA rats were on average 3.9 ng/mL which represented a 1.7 fold rise above those in the control rats. 2 days after con A stimulation TNF levels were on average 4.8 ng/mL in AA rats, a 1.8 fold increase above the control levels. Moreover, the levels of TNF in rats with AA were well above the lower detection limit of the assay after 3 days of stimulation and were found to be statistically different from the control values. AA rats secreted on average 14.6 ng/mL of TNF after 3 days of stimulation which represented a 1.9 fold increase above the levels in measured in the control rats.

7.3 Discussion

Mechanisms responsible for induction and perpetuation of arthritis were examined by studying IFN-γ, IL-2, IL-4, TNF and TGF-β gene expression in the inguinal and popliteal lymph nodes, and the synovial tissue of CFA immunised rats using a reproducible and accurate quantitative RT-PCR technique. IFN-γ and TNF protein levels in the inguinal lymph nodes of AA and normal rats were evaluated which substantiated changes in the respective cytokine mRNA levels. Different cytokines were produced in different tissues

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during the progression of AA and the levels of T-cell derived cytokines were generally considerably lower than those of TNF and TGF-β.

In some instances, the amount of cytokine mRNA varied considerably between different rats. This may be due to substantial biological variability between rats resulting in a slightly different time course of cytokine mRNA production during the pathogenesis of AA. Furthermore, the variability in cytokine mRNA levels were substantiated by variability in cytokine protein production. Differences in cytokine message levels may also be the result of inaccurate quantification. Necrotic regions found in the lymph nodes of adjuvant rats may also account for differences in cytokine levels since cytokine mRNA is very susceptible to degradation. However, distinct rats with low levels of a given cytokine did not necessarily have low levels of all cytokines suggesting the mRNA was intact. Therefore, it is likely that biological differences account for most of the variations in the level of cytokine production. Limitations in the quantitative RT-PCR procedure may also contribute.

The data described in this Chapter is unique because it analyses progressive changes in the levels of T-cell derived and immunomodulatory cytokines during both the prodromal and established stages of AA. Changes in the levels of cytokine expression were monitored in the inguinal lymph nodes which principally drain the tail base and the popliteal lymph nodes which drain the hind paws, an important site of synovial inflammation in AA. Progressive changes in T-cell, pro-inflammatory and immunomodulatory cytokine mRNA levels in the synovial tissue of rats with AA were also reported for the first time.

Identification of type 1 and type 2 responses has come to dominate perspectives on immune responses to infection, in which preferential expansion of one subset can determine recovery during the pathogenesis of a disease (Kroemer et al. 1996; Romagnani 1995). The type of response generated by products of a pathogen used to *in vitro* stimulate cells may differ from the *in vivo* response due to the cell culturing conditions and the particular component used as the stimulus to upregulate cytokine production (Akiyama et al. 1983; Monteyne et al. 1992; Sander et al. 1989). Cytokines secreted by T-cells cloned from diseased tissues have also been used to estimate the frequency of the different types

of clones. Such a procedure also requires *in vitro* stimulation of cells which may bias the production of cytokines, bearing in mind a clone can change its pattern of cytokine production (Rocken et al. 1992). Cytokines produced by diseased tissue *in vivo*, most accurately reflect cytokine production *in situ* and hence, provide a more accurate representation of the immunological mechanisms associated with the pathogenesis of a disease. Tissues were removed from CFA inoculated rats during the development of adjuvant disease and cytokine mRNA was quantified without any requirement for *in vitro* stimulation.

There was a statistically significant increase in the quantity of IL-2, IFN-γ and TNF mRNA in the inguinal lymph node 3 days after the induction of AA in two separate experiments. The increases in IFN-γ and TNF mRNA in AA rats were supported by increases in secretion of the respective proteins (Figs. 5 and 6). By contrast, cytokine mRNA expression in the popliteal lymph node of AA rats did not differ significantly from the levels measured in control rats. The levels of TNF and TGF-β mRNA expression in the synovial tissue were significantly elevated during the established phase of adjuvant disease (day 12/13 in Fig. 4).

The inguinal nodes drain the site of adjuvant deposition and the hind limbs, and appear to be involved in the pathogenesis of AA (Kleinau et al. 1995; Koga et al. 1976; Newbould 1964a; Spargo et al. 1996). The production of IFN-γ, IL-2 and TNF in the inguinal nodes increased early in the course of adjuvant disease, well before the emergence of hind limb swelling. The rise in IL-2 production was most likely induced by inflammation at the site of immunisation at the tail base, which became visible within two days of CFA inoculation. The burst of IL-2 production in the inguinal node 3 days after immunisation is characteristic of a primary immune response in which IL-2 functions as a T-cell growth and activating factor resulting in the proliferation of antigen activated T-cells and bystander cells (Smith 1984). Upregulation of IL-2 in the inguinal lymph node has been reported in collagen induced arthritis (CIA) (Mussener et al. 1995; Stasiuk et al. 1996) and has been indirectly implicated in the amelioration of AA by treatment with cyclosporin A (which inhibits IL-2 expression) (Haynes et al. 1996). Furthermore, the suppression of

experimental arthritis by prophylactic regimes of treatment with monoclonal antibodies that deplete T-cells is associated with a decreased production of IL-2 during the early phase of disease (Jacobs et al. 1994; Yoshino 1995). This provides additional evidence that IL-2 plays a role in the induction of experimental arthritis.

An increase in IFN-γ mRNA expression in the inguinal lymph node was measured 3 days after CFA inoculation (Fig. 2). IFN-γ increases the phagocytic activity of mononuclear phagocytes and increases the expression of MHC class II on their surface. IFN-γ also amplifies the cognitive phase of an immune response by activating MHC class II and CD4$^+$ T-cells which enhances the cellular immune response to foreign antigens (Ijzermans and Marquet 1989). IFN-γ is also a defining cytokine of type 1 immune responses. The early upregulation of IFN-γ in the inguinal lymph node parallels that of IL-2 production, suggesting a type 1 response prevails in the inguinal lymph node of CFA inoculated rats. The upregulation of IL-2 and IFN-γ is consistent with an early role for the inguinal lymph nodes in the induction phase of AA. A phasic effect of IFN-γ on the course of both AA and CIA has been suggested previously (Boissier et al. 1995; Jacob et al. 1989) with arthritis being initiated by putative type 1 cells and perpetuated by type 2 cells. This is consistent with the present findings in as much that there was a burst of type 1 cytokines (IL-2 and IFN-γ) in the inguinal lymph nodes that drain the CFA inoculation site 3 days after inoculation. However, upregulation of a characteristic type 2 cytokine, namely IL-4, during the established phase of the disease was absent (Fig. 2). The kinetics of pro-inflammatory cytokine production in CIA and experimental allergic encephalomyelitis (EAE) were examined and in both studies, the production of pro-inflammatory cytokines (ie. TNF and IL-1β) was paralleled by T-cell activation events such as upregulation of IFN-γ, which stimulates macrophage function (Issazadeh et al. 1995b; Marinovamutafchieva et al. 1997). A lack of IFN-γ during the late phase of CIA was reported by Mussener et. al. (Mussener et al. 1997b) which suggested there was a difference in the cytokine secretion pattern at various stages of the disease, with T-cell derived cytokines being present during the initiation phase and monokines being present at all stages of the disease. This appears to be more consistent with the current findings in that the early increase in T-cell cytokine production was accompanied by a rise in TNF

production. Cytokines produced in the inguinal lymph node after injection of CFA into the tail base suggest a type 1 response in these nodes is integral to, or creates the environment for the development of pathogenic T-cells which migrate from the nodes through the efferent lymph and disseminate through the blood to cause a polyarticular synovitis.

TNF is a pro-inflammatory cytokine that can activate lymphocytes, synovial cells and induce fibroblast proliferation. In addition, it has been implicated as a major factor in the pathogenesis of experimental models of polyarthritis (Brennan 1994). Its expression in the inguinal lymph node was upregulated during the early phase of adjuvant disease and declined before the onset of clinically evident synovitis, in parallel with resolution of inflammation at the tail base (Fig. 2). A rapid accumulation of TNF mRNA expression has also been reported in the inguinal lymph node of rats with CIA and the levels remained elevated throughout the time course of the disease (Mussener et al. 1995). The increased production of TNF three days after CFA inoculation is presumably triggered by primary inflammation in response to CFA inoculation or the elevated levels of IFN-γ which were evident in the inguinal lymph node one day after CFA inoculation. Native collagen may also stimulate the production of TNF, not only through antigen processing by antigen presenting cells and conventional presentation to T-cells, but by binding to several types of receptors, such as those within the integrin family, which are known to be expressed on lymphoid cells (Hynes 1992; Springer 1990). It has been reported that binding of collagen type IV to collagen receptor integrins VLA-1 and VLA-2, which are expressed on activated T-cells, stimulates TNF production (Miyake et al. 1994). TNF expression can also be induced in T-cells and macrophages by binding to extracellular matrix components such as fibronectin and laminin (Hershkoviz et al. 1993). The production of TNF in the inguinal lymph nodes may therefore be contributed to by a number of tissue components that could arrive in lymph nodes via the afferent lymphatics following injury to the tissues at the base of the tail.

The popliteal lymph nodes are of special interest since they drain the hind limbs of arthritic rats and become enlarged during the evolution of polyarthritis (Kleinau et al. 1995; Newbould 1964). Furthermore, immunosuppressive drugs have been shown to influence

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the cellular composition of the popliteal lymph nodes (Haynes et al. 1996) in association with the amelioration of AA (Kinne et al. 1995). In the present studies, no constituent change in cytokine gene expression in the popliteal lymph node was found during the course of AA (Fig. 3 and Table 2) and there was a large amount of scatter of the data in the popliteal lymph node, with some rats having very high amounts of cytokine mRNA (for example TNF in Fig 3). This may in part be due to the biological variability that is associated with the onset of adjuvant disease in which the severity of inflammation in individual hind limbs can vary considerably.

The synovial tissue is the usual index site of inflammatory disease in RA. Pannus formation and joint destruction are important features which are seen in both RA and AA. Despite the abundance of T-cells in RA synovial tissue, T-cell cytokines have proved difficult to detect. Likewise, T-cell derived cytokines were also difficult to detect in the synovium of AA rats. IL-2 mRNA was undetectable throughout the course of adjuvant disease (Fig. 4), despite the high sensitivity of the PCR assay. IL-4 and IFN-γ mRNA were detectable at low levels in the synovial tissue of AA rats, yet at no stage were their levels measurably different from those in the control rats. There was an increase in the level of TNF and TGF-β gene expression in the synovial tissue of rats with adjuvant disease which correlated with visible signs of hind limb inflammation.

A quantitative increase in TNF expression has also been demonstrated in the synovial tissue of AA rats (Smith Oliver et al. 1993) and the joint fluid of rats inoculated with zymosan (Pettipher and Salter 1996). Similarly, the production TNF is pivotal for the induction and pathogenesis of CIA (Brahn et al. 1992; Feldmann et al. 1996b). TNF is a pro-inflammatory cytokine which has been the target for immunological intervention in experimental arthritides as well as RA. Anti-TNF therapy has been reported to inhibit the development of experimental arthritis in animals (Issekutz et al. 1994; Joosten et al. 1996; Piguet et al. 1992) and RA in humans (Feldmann et al. 1996b). Although the mechanism of action has not been fully elucidated, TNF has been shown to activate endothelial cells to express adhesion molecules that facilitate transendothelial migration of leucocytes (Lindsley et al. 1992). TNF is a potent chemotactic agent for macrophages which may

explain the large infiltration of macrophages into the synovial tissue of rats with AA (Pelegri et al. 1995a). TNF secretion by T-cells and macrophages can be induced directly *in vitro* i.e. in the absence of presentation of antigen in the context of MHC class II, by engagement of immobilised components of the extra cellular matrix by integrins (Hershkoviz et al. 1993). This mode of action may contribute to TNF production and hence inflammation in adjuvant disease. Intra-articular injection of TNF has been shown to accelerate the progression of CIA (Santambrogio et al. 1993) and has been implicated in cartilage degradation presumably by increasing the synthesis of collagenase by fibroblasts and synovial cells (Beutler and Cerami 1989). Extracellular matrix damage can also be attributed to the inhibition of proteoglycan synthesis by TNF (Saklatvala 1986) and in collaboration with IL-1, TNF may induce bone resorption which is evident in AA (Gowen et al. 1983). Intra-articular injection of TNF during the late prodromal phase of CIA increases the severity of arthritis (Santambrogio et al. 1993). Rats administered TNF continually, using osmotic infusion pumps, develop more severe CIA than rats receiving no TNF treatment (Brahn et al. 1992). Conversely, repeated administration of TNF has been reported to delay the onset of AA (Ando and Sun 1990). This delay may be associated with a decreased expression of the p75 component of the soluble TNF receptor which has a suppressive effect on T-cell proliferative responses (Cope et al. 1995). A pro-inflammatory role of TNF can be inferred from its presence in the rheumatoid synovium, synovial effusions and the success of TNF blockade therapies with monoclonal antibodies and soluble TNF receptor constructs (Breedveld and van der Lubbe 1995; Brennan and Feldmann 1996; Brennan et al. 1992).

In addition to TNF, there was an increase in the level of TGF-β gene expression in the synovial tissue of rats with adjuvant disease, which was heightened with the development of inflammation in the hind limbs. Pro and anti-inflammatory effects attributable to exogenous TGF-β have been reported, the outcome of which are influenced by the concentration, timing or route of TGF-β administration (Wahl 1994). It is shown in Fig. 4 that TGF-β mRNA was most elevated in the synovial tissue at the time of clinically evident synovitis. It has been found in the synovium of rats with CIA where its expression was shown to increase following the emergence of synovitis (Mussener et al. 1997a),

perhaps suggesting a pro-inflammatory effect of this cytokine. Intra-articular injection of TGF-β into the ankle space of rats with CIA accelerated and intensified the arthritis (Cooper et al. 1992). By contrast, its administration during the late prodrome phase of CIA protected against disease onset which illustrates the anti-inflammatory effect of TGF-β (Santambrogio et al. 1993). Furthermore, injection of TGF-β at the time of induction of CIA prevents disease onset (Kuruvilla et al. 1991) and treatment with TGF-β has also been shown to reduce inflammatory cell infiltration, pannus formation and joint erosion in streptococcal cell wall (SCW) induced arthritis (Brandes et al. 1991a).

TGF-β protein is expressed chronically inflamed tissues (Csernok et al. 1996; Mussener et al. 1997a; Wahl et al. 1988) including the synovium of RA patients (Lafyatis et al. 1989) and rodents with experimentally induced arthritis (Allen et al. 1990; Lafyatis et al. 1989) and TGF-β mRNA expression in the inflamed synovium of AA rats has been described in this Chapter. Repeated intra-articular injections of TGF-β have been shown to stimulate the synthesis of articular cartilage proteoglycan, inflammatory cell influx, fibrosis and osteophyte formation (van Beuningen et al. 1993). It increases the production of chemotactic agents such as IL-1 and IL-8 and acts as a strong chemoattractant for neutrophils, monocytes and T-cells (Adams et al. 1991; Brandes et al. 1991b; Wahl et al. 1987) and induces the production of matrix degrading enzymes (Wahl 1992). TGF-β may stimulate immature T-cells whereas after activation, T-cells increase their expression of TGF-β receptors and are growth arrested once they are engaged (Kehrl et al. 1986). The levels of TGF-β mRNA are elevated in the synovium during the established phase of AA (Table 3). However, it is uncertain whether TGF-β is behaving predominantly as a pro-inflammatory cytokine encouraging the migration of inflammatory cells to the synovium or an immunomodulatory cytokine acting to dampen the inflammatory response.

Early expression of the IFN-γ, IL-2 and TNF genes in the inguinal lymph node of AA rats reflects the nature of immunological events within lymph nodes that drain the disease inducing adjuvant injection site and suggests a type 1 bias immune response. Subsequent increased expression of TNF and TGF-β in clinically inflamed synovium presumably

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reflects a role for these cytokines in the effector phase of the immunological response that mediates disease expression (Imrich et al. 1994; Ohmori et al. 1992).

In this chapter, the profile of expression of cytokines that are upregulated during different phases in the progression of AA was measured. This provided an insight into the immunological mechanisms responsible for the development and expression of adjuvant disease.
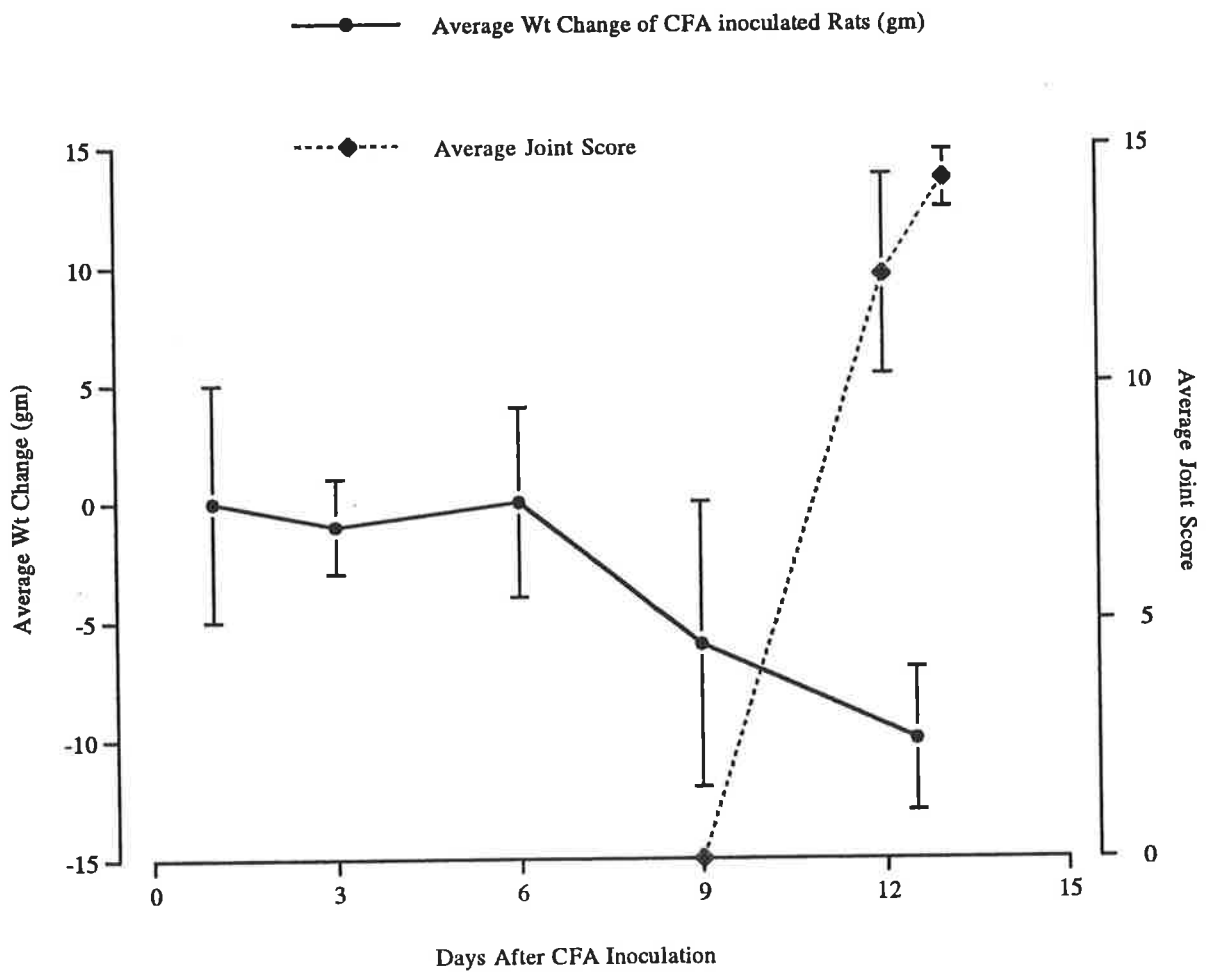
Figure 1. The progression of AA indicated by the loss of weight and AI of CFA inoculated rats. Each point represents the average of three individual rats ± standard deviation.

Inguinal Lymph Node Cytokine Profile During the Development of AA

IL-2

IFN-γ

IL-4

TNF

TGF-β

Cytokine Copy Number /μg of RNA

Days After CFA Inoculation
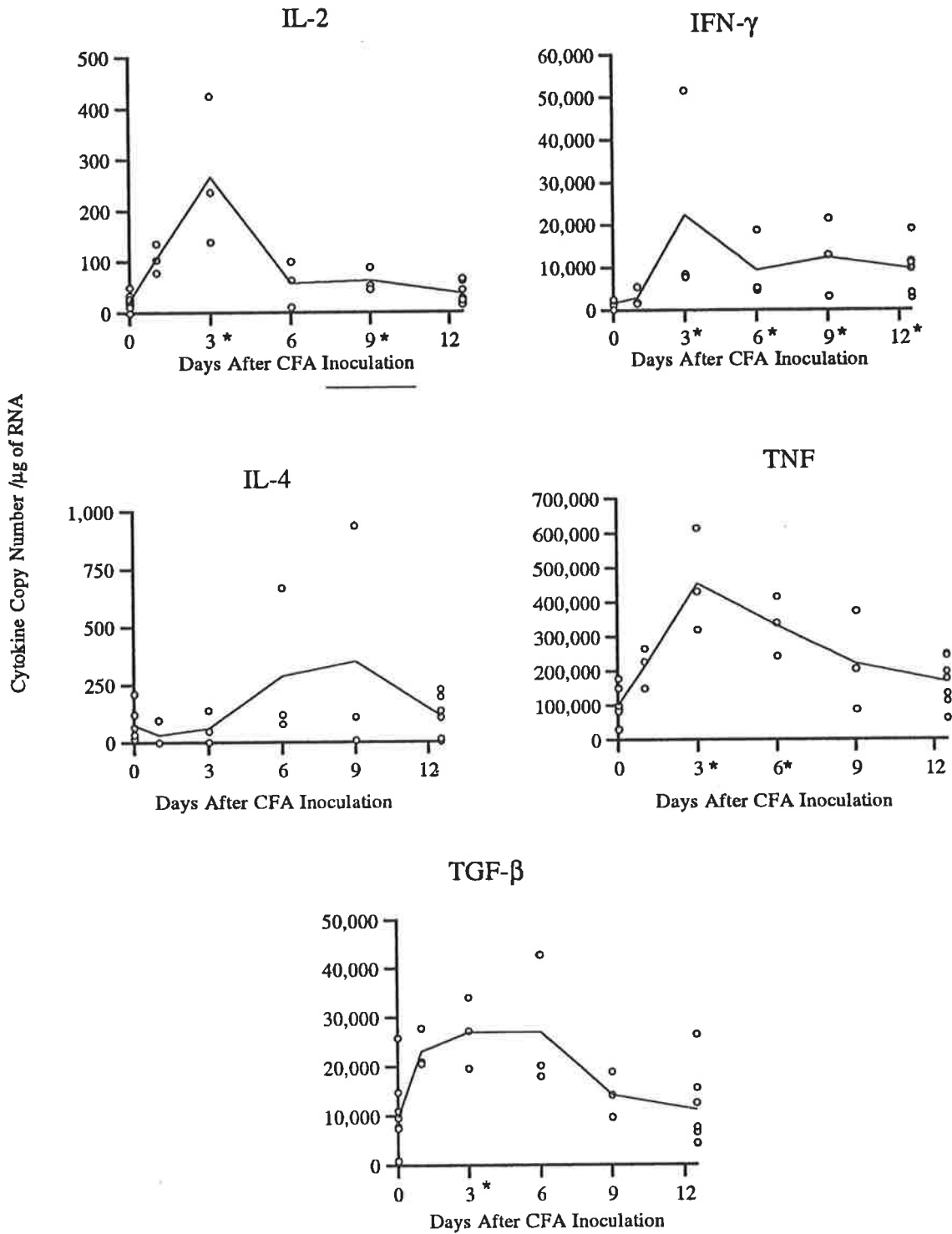
Figure 2. The kinetics of cytokine mRNA levels in the inguinal lymph node of CFA inoculated rats. Day 0 values represent the eight control rats (combined day 0 and 15) which were inoculated with saline solution. *P ≤ 0.05 (Mann Whitney U test).

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Popliteal Lymph Node Cytokine Profile During the Development of AA
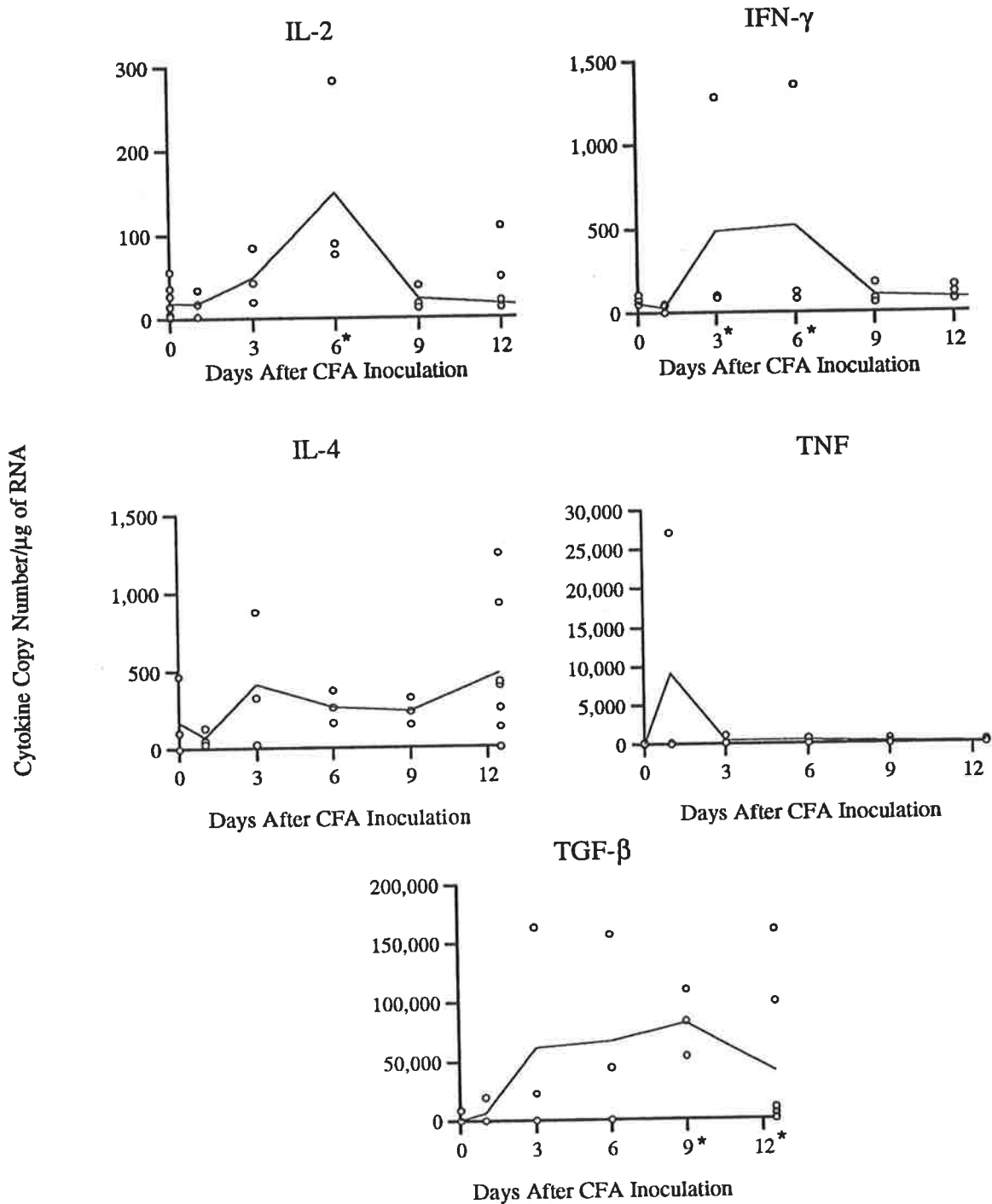
Figure 3. The kinetics of cytokine mRNA accumulation in the popliteal lymph node of CFA inoculated rats. Day 0 values represent the eight control rats (combined day 0 and 15) which were inoculated with saline solution. The triangles represent the number of cytokine transcripts determined in individual rats. The mean level of cytokine production at each time point is represented by the solid line. *P<0.05

Synovial Tissue Cytokine Profile
During the Development of AA

IFN-γ

IL-4

TNF

TGF-β
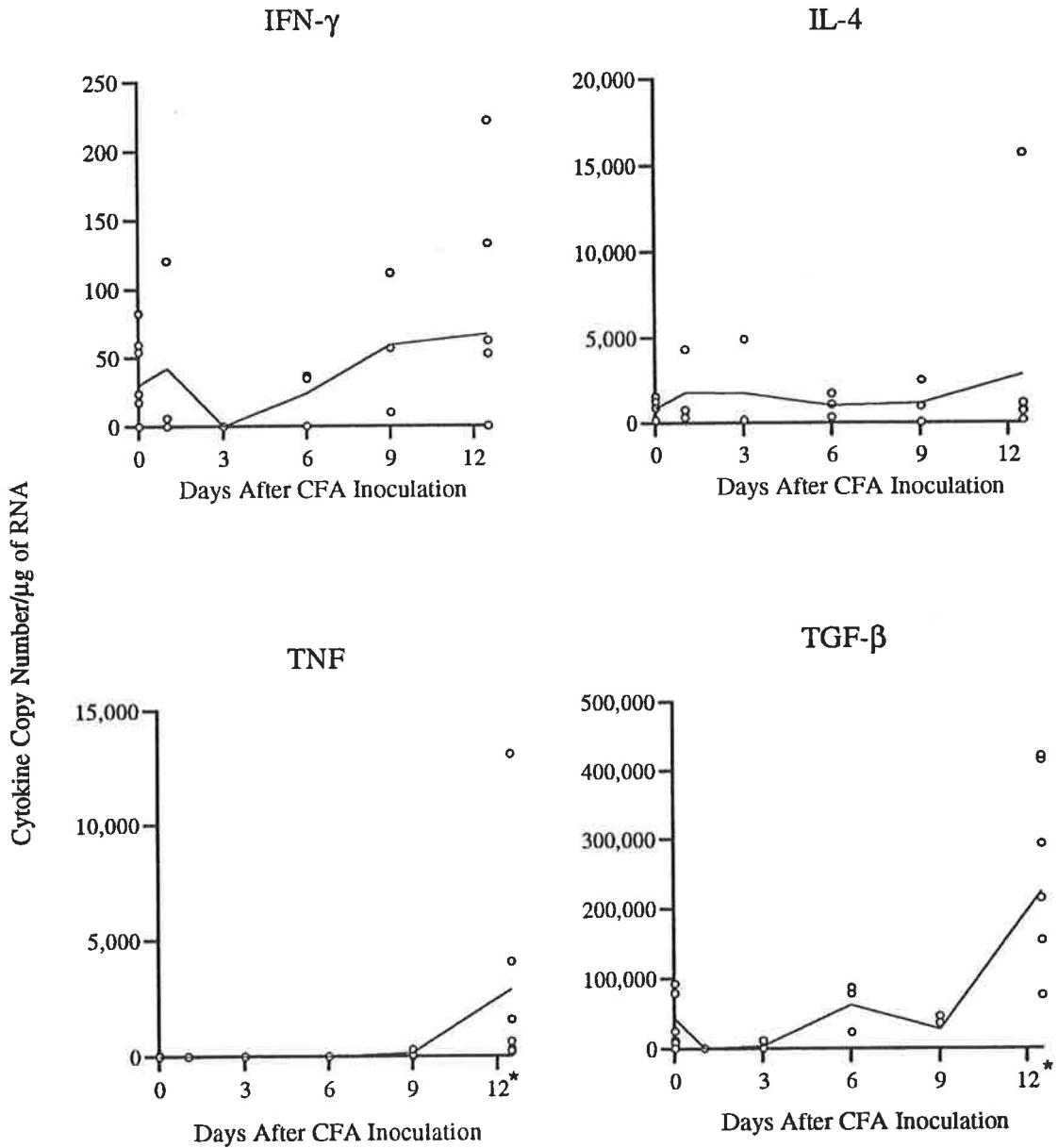
Figure 4. The kinetics of cytokine mRNA accumulation in the synovial tissue of CFA inoculated rats. Day 0 values represent the eight control rats (combined day 0 and 15) which were inoculated with saline solution. The triangles represent the number of cytokine transcripts determined in individual rats. The mean level of cytokine production at each time point is represented by the solid line. *P<0.05

Cytokine Copy Number/μg of RNA	Normal Rats	CFA Inoculated Rats (day 3)
IL-2	5,209	15,055
	6,179	8,010
	5,544	9,052
	2,175	6,355
Mean	**4,777**	**9,618***
IFN-γ	555	1,044
	685	3,253
	3,561	10,105
	1,088	13,064
Mean	**1,472**	**6,866**
TNF	636,754	931,890
	483,195	949,544
	430,398	1,123,947
	414,253	819,717
Mean	**491,150**	**956,275***
TGF-β	20,874,565	47,113,842
	83,453,128	51,790,647
	28,647,504	84,749,160
	4,612,602	34,476,980
Mean	**34,396,949**	**54,532,657**

Table 1. Quantification of cytokine mRNA in the inguinal lymph nodes of CFA and saline inoculated rats 3 days after immunisation. The plain text represents the number of cytokine transcripts determined in individual rats. The bold text represents the mean number of cytokine transcripts/μg of RNA. *$P<0.05$ (Mann Whitney U test).

Cytokine Copy Number/μg of RNA	Normal Rats	CFA Inoculated Rats (day 6)
IL-2	11,012	136,741
	100,785	249,958
	24,634	66,068
	175,810	98,756
Mean	**78,060**	**137,881**
IFN-γ	245	621
	1,291	6,416
	231	410
	340	6,294
Mean	**527**	**3,435**

Table 2. Quantification of cytokine mRNA accumulation in the popliteal lymph nodes of CFA and saline inoculated rats 6 days after immunisation. The plain text represents the number of cytokine transcripts determined in individual rats. The bold text represents the mean number of cytokine transcripts/μg of RNA. *P<0.05

Cytokine Copy Number/μg of RNA	Normal Rats	CFA Inoculated Rats (day 12/13)
TNF		295
		182
	55	13,066
	0	4,038
	0	220
	0	1,569
		626
Mean	**14**	**2,856***
TGF-β		291,909
	11,890	75,063
	8,126	213,985
	24,789	414,070
	91,936	153,864
		420,761
Mean	**34,185**	**261,609***

Table 3. Quantification of cytokine mRNA accumulation in the synovial tissue of CFA and saline inoculated rats 12/13 and 15 days after immunisation respectively. The plain text represents the number of cytokine transcripts determined in individual rats. The bold text represents the mean number of cytokine transcripts/μg of RNA. *P<0.05

IFN-γ Protein Expression in the Inguinal Lymph Nodes of AA and Normal Rats.

Figure 5. IFN-γ protein production measured in triplicate by ELISA. The inguinal lymph nodes were removed from four rats 3 days after inoculation with either saline solution or CFA. MNCs were cultured with con A for 1, 2 and 3 days. Values represent the average of triplicate measurements ± standard deviation.*P<0.05, UD = not detected.

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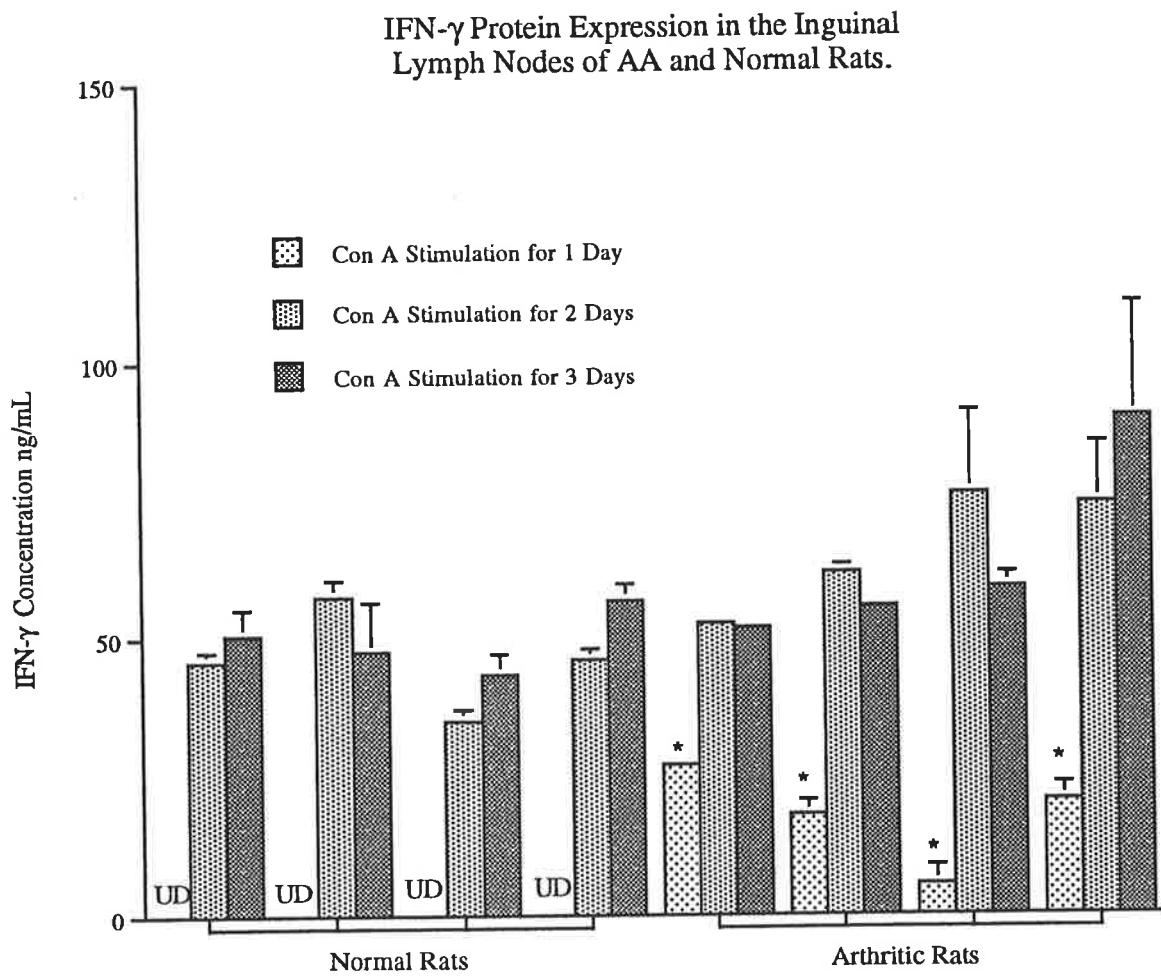
TNF Protein Expression in the Inguinal Lymph Nodes of AA and Normal Rats.
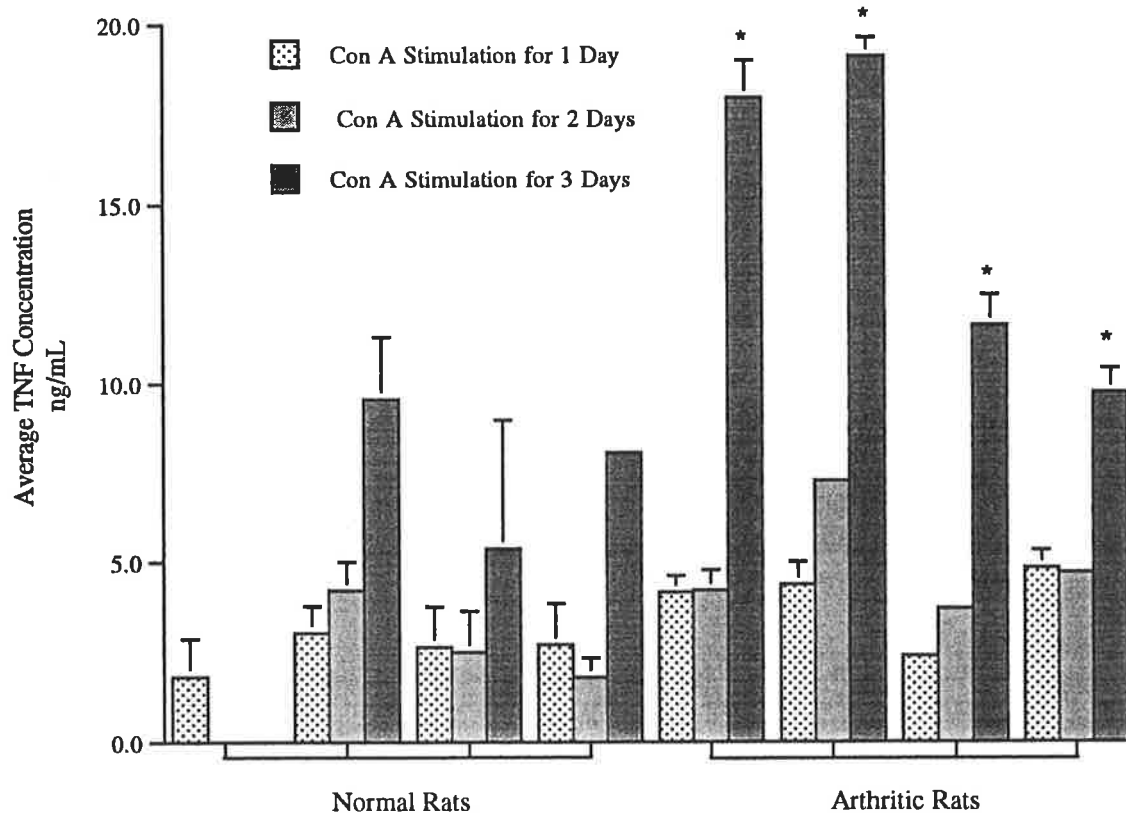
Figure 6. TNF protein production measured in triplicate by ELISA. The inguinal lymph nodes were removed from four rats 3 days after inoculation with either saline solution or CFA. MNCs were cultured with con A for 1, 2 and 3 days. Values represent the average of triplicate measurements ± standard deviation. *P<0.05, UD = not detected.

Chapter 8

Cytokine Profiles Expressed by Arthritogenic T-Cells in Thoracic Duct Lymph

8.1 Introduction

Leucocyte extravasation from the blood entails a series of well co-ordinated events involving cognate interactions between adhesion molecules expressed by leucocytes and endothelial cells, that are upregulated in response to cytokines such as IL-1 and TNF. The response involves leucocyte rolling, tethering, firm adhesion of leucocytes to endothelial cells and the transmigration of leucocytes through the vessel wall (Shimizu et al. 1992). This step involves the release by leucocytes of degradative enzymes that digest the vascular basement membrane. Chemokines influence vascular adhesion molecule expression and determine the directional migration of leucocytes in the extra-vascular space, in a cell type specific manner (Miller and Krangel 1992). Within the extra-vascular space the leucocyte adhesion molecules interact with matrix proteins which can presumably influence their migration through, and retention at the sites of inflammation (Hogg and Landis 1993; Jutila 1992). These events are complex, highly regulated and involve some redundancy in the actions of both adhesion molecules and soluble mediators.

During adjuvant arthritis there is recruitment of T-cells, monocytes and polymorphonuclear leucocytes from the blood into the joint (Issekutz and Issekutz 1991a; Issekutz and Issekutz 1991b). The expression of adhesion molecules such as VLA-4 on vascular endothelium, mediates T-cell migration (Issekutz et al. 1996). LFA-1 / Mac-1 (CD11a / CD18), have been shown to be involved in mononuclear cell migration into arthritic joints (Issekutz and Issekutz 1993a). Furthermore, the expression of specific

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adhesion molecules has been associated with the preferential recruitment of Th-cell subsets to the site of inflammation (Austrup et al. 1997).

The fate of circulating leucocytes which are recruited into the tissues will depend on the cell type and the presence and nature of the inflammatory response within the tissues. Phagocytic cells (monocytes and neutrophils) recruited to sites of inflammation execute effector functions (release degradative enzymes, cytokines, eicosanoids, and reactive oxygen species) and most perish in the tissues or in the case of synovium, the synovial fluid. The fate of lymphocytes in inflamed tissue such as synovium will depend on multiple influences including whether or not they encounter cognate antigen, the manner in which it is presented and the presence or absence of costimulatory molecules and cytokines. Infiltrating lymphocytes may undergo apoptosis or pass through the tissues and exit via the afferent lymphatics through which they travel to draining lymph nodes.

Most lymphocytes entering afferent lymph bear memory and activation cell markers. Naive lymphocytes enter lymph nodes directly from the blood, presumably through HEVs. They must be activated in lymph nodes through interactions with dendritic cells (DC). DCs are thought to comprise distinct lineages; a lymphocyte derived lineage which is found in the follicular areas of lymph nodes and a non-lineage which includes Langerhans cells and interstitial dendritic cells (Steinman 1991). The latter arrive in peripheral tissues as precursors and undergo maturation under the influence of GM-CSF to acquire antigen capture functions (Heufler et al. 1988). They mature further under the influence of TNF and then exit the tissues via the efferent lymphatics and travel to the lymph nodes during which they mature in transit and display antigen processing and presentation capabilities upon arrival. DCs seem capable of stimulating naive T-cells and inducing primary immune responses (Inaba et al. 1990). The potential antigen presenting capabilities of dendritic cells is the basis of the autologous mixed lymphocyte reaction (Flechner et al. 1988). DCs are specialised in the capture of antigen in tissues and presentation of the antigen in lymph nodes. They are retained in abnormal numbers in the synovium where they may be able to stimulate lymphocytes in a manner not generally seen in peripheral tissue (Wilkinson et al. 1990).

Thoracic duct (TD) cells comprise mostly small lymphocytes, many of which have the phenotype of naive cells (Westermann et al. 1994). A minority of TD cells are large lymphocytes which display activation markers (ie. TD lymphoblasts) and are believed to home rapidly to specific tissues (Jalkanen et al. 1989; Pietschmann et al. 1992), whereas small TD lymphocytes circulate randomly (Freitas et al. 1980).

The site of lymphocyte activation and regional differences in the properties of vascular endothelium affect the recruitment of activated T-cells (Parrott and Wilkinson 1981). T-cells generated in the mucosal-associated lymphoid tissues appear to be recruited preferentially to intestinal mucosal sites whereas T-lymphoblasts generated in peripheral lymph nodes are recruited to inflammatory sites in addition to the intestinal mucosa (Rose et al. 1976a; Rose et al. 1978; Rose et al. 1976b).

TD drainage results in cellular depletion of the lymphoid system and leads to immunosuppression (Paulus et al. 1979; Wegelius et al. 1970). TD drainage has been reported to ameliorate clinical disease in a significant number of RA patients (Paulus et al. 1977; Ueo et al. 1979) and delay the development of AA in rats (Spargo et al.). Moreover, adjuvant disease is transferable to syngeneic recipients with TD cells (Quagliata and Phillips Quagliata 1972; Whitehouse et al. 1969) and TD lymphoblasts from rats with AA are recruited to both normal and inflamed synovium (Spargo et al. 1996). In studies described in this Chapter, the cytokine profile of TD cells and CD4$^+$ TD lymphoblasts obtained from AA rats was examined to ascertain the profile of cytokines produced by cells capable of transferring adjuvant disease between arthritogenic and naive rats by adoptive cell transfer. The markers of cell activation used to isolate the TD lymphoblasts included OX 39 which binds CD25, the IL-2 receptor, OX 26 which binds CD72, the transferrin receptor, OX 6 which binds MHC class II and OX 40 which binds CD134, a member of the TNF receptor superfamily which is expressed on rat CD4$^+$ T-blasts (Birkeland et al. 1995).

8.2 Results

8.2.1 Cytokine mRNA Expression in Unfractionated TD Cells

The steady state levels of IFN-γ, IL-2, IL-4, TNF and TGF-β mRNA expressed by TD cells from AA and normal rats were assessed using quantitative RT-PCR. TD lymph was collected from four AA rats following cannulation of the thoracic duct 9-10 days after CFA inoculation. The TD lymph from four saline injected rats was also collected using this procedure. All TD lymph samples were collected over a period of approximately 12 hours following which, the cells were pelleted and lysed in guanidine thiocyanate solution then stored at -70°C in preparation for cytokine mRNA analysis using quantitative RT-PCR.

Cytokine gene expression was measured in each TD cell sample using competitive PCR. This was performed by adding a constant amount of the appropriate competitor fragment to 62.5 ng of cDNA (see Section 5.4.3). PCR was performed using the conditions outlined in Section 2.14 with oligonucleotide primers designed to amplify IL-2, IFN-γ, IL-4, TNF and TGF-β. The PCR products were desalted then quantified using CE from which the amount of the respective cytokine cDNA in each sample was calculated.

A low level of IL-2 mRNA was detected in a single AA and control rat. An average 2,570 copies/μg of IFN-γ mRNA were measured in the TD lymphocytes from AA rats which represents an average, a 10 fold increase above the levels found in the controls. A mean 1,026 copies of IL-4 mRNA was quantified in the TD cells from AA rats, which was a 1.5 fold rise above the control levels. TNF mRNA expression averaged 164,782 copies/μg RNA in TD cells from AA rats which was approximately the same as that in the controls. TGF-β mRNA expression in TDL from AA rats averaged 407,360 copies/μg RNA representing a 2.1 fold rise above the control levels. These data are shown in Table 1.

8.3 Cytokine Profiles in CD4$^+$ T-Cells in Thoracic Duct Lymph

8.3.1 Introduction

Although the majority of thoracic duct lymphocytes are small cells, a limited number of blasts also appear in the thoracic duct lymph. The blasts can adoptively transfer adjuvant disease which is not achievable with small non-blast TD lymphocytes (Spargo et al. 1998). TD cells were collected from CFA and saline inoculated rats then CD4$^+$ TD T-cells were isolated and the CD4$^+$ TD T-cell blasts were separated from the non-blast cells (Section 2.22). The expression of cytokine mRNA by blast and non-blast cells from the TD lymph of both CFA and saline inoculated rats was analysed to determine whether the cells that adoptively transfer AA display a distinctive pattern of cytokine mRNA expression.

9-10 days after CFA inoculation the TD cells from three AA rats were collected and pooled. CD8$^+$ T-cells and B-cells were depleted using the monoclonal antibodies OX 8 (CD8), Mark-1 (anti κ light chain) and OX 33 (CD45 found exclusively on B-cells (Woollett et al. 1985)). Flow cytometric analysis on the residual population showed greater than 96% staining for CD4$^+$ T-cells while less than 4% were CD8$^+$ T-cells and B-cells (Table 2). Of this highly purified CD4$^+$ TD T-cell population, approximately 30% stained positive for cells bearing activation markers recognised by the monoclonal antibodies; OX26, OX40, OX39 and OX6. Cells bearing the activation markers were captured using Dynal Beads and designated "activated". The residual cells of the CD4$^+$ TD T-cell population constituted the "non-activated" fraction and flow cytometric analysis revealed greater than 98% staining for CD4$^+$ T-cells and less than 2% stained with the cocktail of activation markers (Table 3). Both fractions were lysed in guanidine thiocyanate solution and stored at -70°C in preparation for cytokine mRNA analysis. A similar procedure was performed on a sample of cells collected from the thoracic duct of three normal rats. The levels of cytokine mRNA in the activated and non-activated fractions of CD4$^+$ T-cells from the TD of both AA and non-inoculated rats was measured as outlined in Section 8.2.1.

8.3.2 Cytokine Profiles of CD4+ T-Cells From the TD Lymph of CFA Inoculated Rats

IFN-γ, TNF and TGF-β mRNA expression in activated CD4+ T-cells from the TD lymph of AA rats was significantly higher than the levels in the non-activated fraction. The levels of IL-2 and IL-4 mRNA were not statistically different between the two cell fractions. A graphical representation of the data is depicted in Table 4.

A mean 1,057 copies/μg of IL-2 mRNA was found in the activated CD4+ T-cells from AA rats which represented a 0.74 fold difference from the levels measured in the non-activated fraction. IFN-γ levels reached on average, 200,507 copies/μg of RNA in the activated fraction of CD4+ T-cells from TD lymph of AA rats whereas it was undetectable in the non-activated fraction. 103 copies of IL-4 mRNA were measured in activated fraction, a 0.36 fold difference from that measured in the non-activated fraction. TNF mRNA expression in activated CD4+ T-cells reached 260,774 copies/μg of RNA, a 13.5 fold rise above the level found in the non-activated fraction. 5,202,992 copies of TGF-β/μg of RNA were present in the activated cells which represents a 73 fold increase above the level measured in the non-activated fraction.

8.3.3 Cytokine Profiles of CD4+ T-Cells From the TD Lymph of Normal Rats

The levels of IFN-γ, IL-2, IL-4, TNF and TGF-β mRNA in activated and non-activated fractions of CD4+ T-cells within lymph collected from normal rats were not statistically different. The data is presented in Table 5.

8.3.4 Comparison of the Cytokine mRNA Expression Profiles in the Non-Activated Fractions of CD4+ T-Cells From TD Lymph of AA and Normal Rats

The activated CD4+ T-cells from AA rats displayed elevated levels of IFN-γ, TNF and TGF-β mRNA in comparison to their non-activated counterparts (Section 8.3.2) whereas, cytokine mRNA levels were not significantly different in both CD4+ T-cell fractions from the TD lymph from normal rats (Section 8.3.3). In the following Sections, cytokine levels in the non-activated CD4+ T-cell fractions of TD lymph from AA and normal rats were compared. Likewise, cytokine levels in the activated CD4+ T-cell fractions of TD lymph

from AA and normal rats were compared to ascertain whether the activated CD4$^+$ T-cells that adoptively transfer AA display a characteristic cytokine mRNA expression profile.

There was no statistical difference in the levels of cytokines measured in the non-activated fraction of CD4$^+$ TD T-cells from CFA inoculated and normal rats (Table 6).

8.3.5 Comparison of Cytokine mRNA Expression Profiles in the Activated Fractions of CD4$^+$ T-Cells From the TD Lymph of AA and Normal Rats

The activated CD4$^+$ T-cells from the TD lymph of AA rats displayed significantly elevated levels of IFN-γ, TNF and TGF-β mRNA compared to their counterparts in normal rats (Table. 7). IL-2 and IL-4 mRNA levels varied but were statistically similar in the two samples.

The amount of IFN-γ mRNA measured in the activated cell fraction of CD4$^+$ T-cells from the TD lymph of AA rats was on average 200,507 copies/μg of RNA which was approximately 1,000 fold higher than the average amount measured in activated CD4$^+$ T-cells from TD lymph of normal rats. TNF mRNA expression in the activated CD4$^+$ T-cells from the TD lymph of CFA inoculated rats was on average 260,774 copies/μg of RNA which represents a 6 fold increase above levels quantified in the activated fraction from normal rats. TGF-β levels averaged 5,202,992 copies/μg of RNA in the activated fraction of CD4$^+$ T-cells from TD lymph of AA rats. This level was 79 fold higher than that measured in the activated CD4$^+$ T-cells from TD lymph of normal rats.

8.4 Discussion.

The transfer of arthritis using lymphocytes or T-cell lines has been well documented in animals (DeJoy et al. 1989; Holoshitz et al. 1984; Nakajima et al. 1993; Taurog et al. 1983b; Whitehouse et al. 1969). Likewise, the migration and homing of lymphocytes from arthritic donors, to the synovial tissue of syngeneic recipients has been implicated with the development of arthritis (Mikecz and Glant 1994). TD lymphoblasts from rats with AA can be found in the paws of recipient rats after adoptive transfer to naive rats and their

recruitment is increased when the recipient has established arthritis (Spargo et al. 1996). Furthermore, activated CD4+ T-cells from the TD lymph of rats in the late prodrome for AA can also adoptively transfer arthritis to naive recipients (Spargo et al. 1998).

Cytokine mRNA expression in CD4+ T-cells obtained from the TD lymph of AA and normal rats was assessed to elucidate the cytokine mRNA expression profile of these cells. Cytokine mRNA produced by both the activated and non-activated fractions of CD4+ T-cells were measured because activated CD4+ T-cells from TD lymph of AA rats are able to adoptively transfer disease, whereas non-activated CD4+ T-cells are unable to transfer the disease (Spargo et al. 1998). The level of IFN-γ, IL-2, IL-4, TNF and TGF-β mRNA expression in unfractionated TD cells and the activated and non-activated sub-populations of CD4+ T-cells from TD lymph of both AA and normal rats was measured.

The data presented in Table 1 indicates that TD cells from rats with AA have an elevated expression of IFN-γ. Furthermore, in Table 4 the increased expression was shown to occur in the activated sub-population of CD4+ T-cells which also express more TNF and TGF-β mRNA than the non-activated CD4+ T-cell population in the TDL from AA rats. The rise in TNF and TGF-β mRNA levels in the activated sub-population was presumably not significant enough to influence the analysis of whole TD cells since the cells were diluted in whole TD lymph by the presence of other cells including B-cells and CD8+ T-cells. The amounts of IFN-γ, TNF and TGF-β mRNA in activated CD4+ T-cells from TD lymph of AA rats were significantly greater than the levels from their normal counterparts (Table. 7). The expression of cytokine message by the CD4+ T-cells without activation markers from the TD lymph of AA and control rats was similar (Table 6). Likewise, the expression of cytokine message by the activated and non-activated CD4+ T-cells from TD lymph of non-inoculated rats were also similar (Table 5).

IFN-γ mRNA levels in the unfractionated TD cells from AA rats was 10 fold higher than levels in the normal rats (Table 1) and IFN-γ expression was shown to occur in CD4+ T-cells bearing activation markers (Table 4). It has been shown that this sub-population can adequately transfer adjuvant disease (Spargo et al. 1998). IFN-γ has been implicated in the pathogenesis of various animal models of arthritis (Cooper et al. 1988; Jacob et al. 1989;

Mauritz et al. 1988; Stasiuk et al. 1996; Wiesenberg et al. 1989) but can also down-regulate inflammatory responses (Nakajima et al. 1990; Nicoletti et al. 1993; Wahl et al. 1991). IFN-γ has been reported to have a phasic effect on the course of collagen induced arthritis (CIA) in which the disease severity was dependent on the dose and the route of administration (Boissier et al. 1995). Administration of recombinant IFN-γ shortly before the induction of AA (-1 day) increased the severity of limb inflammation whereas neutralisation of IFN-γ 1 to 2 days before induction of the disease decreased the severity of arthritis (Jacob et al. 1989). IFN-γ has therefore been implicated in the early phase of AA which is consistent with the data presented in Chapter 7. The effects of IFN-γ on the pathogenesis of AA are complicated since an arthritogenic T-cell clone was found to secrete low amounts of IFN-γ whereas, a clone that inhibited the development of AA secreted high amounts of IFN-γ and proliferated in the presence of exogenous IFN-γ (Jacob et al. 1989). The data presented in Sections 7.2.2 and 7.2.8 indicates there is a rise in the level IFN-γ mRNA in the inguinal lymph node of AA rats early (3 days) after the induction of the disease, which suggests that IFN-γ mRNA expressing cells may migrate from the inguinal lymph node via the TD to sites where they encounter antigen and exert their immunological effects.

Since IFN-γ has been implicated in the regulation of lymphocyte migration (McMurray 1996) a rise in the level of IFN-γ mRNA expression within TD lymphoblasts (as depicted in Table 4) could be associated with homing of the blasts to inflammatory sites during adjuvant disease. Furthermore, IFN-γ is in part responsible for HEV formation (Hendriks et al. 1989; Manolios et al. 1988) and therefore increases the adhesion of lymphocytes to endothelium (Chin et al. 1991; Yu et al. 1985) enhancing the transmigration of cells (May and Ager 1992): more specifically, it promotes the migration of T-cells from the blood and retards their entry back into the lymphatics which results in cells becoming effectively trapped in the tissue and whereby perhaps increasing their chance of encountering a targeted antigen (Westermann et al. 1993). The priming of CD4^{+} T-cells for the production of IFN-γ within TD lymph may be important for the accumulation of T-cells with arthritogenic potential within synovial tissue.

The gut-associated lymphoid tissue is unique in that it favours the induction of Th-2 cells and cells that secrete TGF-β (Weiner 1997). Migrating cells from the lymphoid tissues that serve the gut, reach the circulation via the TD. It is therefore not surprising that measurable levels of IL-4 mRNA, a typical Th-2 cytokine, were found in TD cells from both AA and normal rats (Table 1). There was generally a very low level of IL-2 mRNA expression in TD cells (Table 1) which may reflect the low level of cytokine production by T-cells (Kelso 1993) or more likely, degradation of IL-2 mRNA at room temperature (25°C) over the 10 hour period during which the TD cells was collected. IL-2 is a potent cytokine and may be subject to strict control such that its production may be down regulated until it is confined to a suitable anatomical location, such as a lymph node, where it can effectively exert its biological actions.

TGF-β has diverse effects on the immune system and the regulatory effects that it exerts on the production T-cell derived cytokines are controversial. It has been reported to augment delayed type hypersensitivity responses (DTH responses) by promoting the secretion of IFN-γ and IL-2 by CD4^{+} T-cells (Mosmann and Sad 1996). Under the appropriate conditions it can exert the opposite effect on the expression of these cytokines and promote the expression of IL-4, IL-5 and IL-10 which are associated with humoral immune responses (Maeda and Shiraishi 1996). As demonstrated in Table 7, the levels of TGF-β mRNA in the CD4^{+} lymphoblasts from the TD lymph of AA rats were 79.2 fold higher than their counterparts from non inoculated rats. CD4^{+} T-cells from the TD of arthritic donor rats have been shown to accumulate in the synovial tissue of arthritogenic recipient rats (Spargo et al. 1996). In the previous Chapter (Section 7.2.6) it was shown that elevated levels of TGF-β mRNA occur in the synovium of rats with actively induced AA. The circulating TD lymphoblasts may provide the source of TGF-β mRNA found in the synovium. There was however, an absence of IFN-γ mRNA expression in the synovial tissue of AA rats despite its abundance in TD cells. This may be due to the antagonistic effect of TGF-β on IFN-γ production (Kulkarni et al. 1993; Wahl 1992).

TGF-β is also capable of orchestrating the recruitment and activation of leucocytes by up regulating adhesion molecule expression and acting as a chemotactic agent inducing

cytokine secretion, which initiates the formation of a cytokine network (Wahl et al. 1993b). Local secretion of TGF-β can promote an inflammatory response (Cooper et al. 1992; Wahl et al. 1993a) whereas systemic administration of TGF-β generally inhibits inflammation (Brandes et al. 1991a; Kuruvilla et al. 1991; Wahl et al. 1991). When high levels of TGF-β are achieved in the circulation recurrent infections can develop (Lowrance et al. 1994) suggesting excess systemic therapeutic immunomodulation with this cytokine can comprise protective immunity. Overall, it seems likely that local production of TGF-β within the synovium promotes synovitis and that CD4+ T-cells which are recruited to the synovium may contribute to its production.

Table 7 also demonstrates that TNF was produced by activated CD4+ T-cells from TD lymph of AA rats, in which the levels were 5.7 fold higher than those of their counterparts in normal rats. Up regulation of TNF expression has been implicated in the inflammatory response (Brennan et al. 1995) and has been shown to regulate T-cell proliferation during the later stages of an immune response (Scheurich et al. 1987). The central role of TNF in the arthritic process has been demonstrated by intra-peritoneal treatment of AA rats with TNF antiserum which caused a significant decrease in the severity of limb inflammation (Issekutz et al. 1994). The treatment was shown to be specific for inflammation of the joint, in preference to other sites, which demonstrated the susceptibility of the synovial tissue to the inflammatory effects of TNF. Likewise, TNF blockade during the preclinical phase of CIA can inhibit the onset of the disease (Piguet et al. 1992; Thorbecke et al. 1992) and blocking the pro-inflammatory effects of TNF using anti-TNF monoclonal antibody therapy also ameliorates RA, although the effect is transient, repeated treatments can be given with benefit (Elliott et al. 1993).

The propensity of lymphocyte homing to the limbs of rats with AA is unknown since the arthritogenic antigen remains elusive. However, specific homing receptors on endothelial cells have been reported to be upregulated in the synovial tissue of rats with AA (Halloran et al. 1996). Selectins on the circulating cells are partly responsible for mediating the recruitment of cells to sites of inflammation in particular, the trafficking of T-helper cell subsets (Austrup et al. 1997). More specifically, Th-1 cells are able to bind to P- and E-

selectin and are efficiently recruited during a type 1 immune response. Cutaneous lymphocyte antigen (CLA) is involved in the selective migration of T-cells to sites of DTH expressing E-selectin (Berg et al. 1991) and the induction of CLA is mediated in part by TGF-β (Picker et al. 1993) and IL-12 (Leung et al. 1995), the former being a characteristic Th-1 cytokine (Manetti et al. 1993). In this Chapter it has been shown that activated CD4^{+} T-cells in TD lymph from AA rats has a type 1 like cytokine profile as indicated by the elevated expression of IFN-γ mRNA. The expression of P-selectin is upregulated by TNF at an inflammatory site (Weller et al. 1992). Optimal migration of leucocytes into arthritic joints is P-selectin dependent (Walter and Issekutz 1997). Therefore, it is hypothesised that type 1 CD4^{+} TD lymphoblasts may be recruited to the synovial tissue of rats with AA due to cytokine induced expression of T-cell adhesion molecules, such as CLA on T-cells, and P-selectin and E-selectin on endothelial cells. The array of cytokine induced cell adhesion molecules expressed on TD lymphoblasts and their respective ligands on endothelial cells may therefore promote the migration of T-cells into the synovium where they may initiate or perpetuate the inflammatory response.

Cytokine Copy Number/µg of RNA	Normal TD Cells	AA TD Cells
IL-2	0	9
	31	0
	0	0
	0	0
Mean	**8**	**2**
IFN-γ	0	1,366
	413	2,512
	324	4,614
	267	1,538
Mean	**251**	**2,570***
IL-4	1,128	827
	527	824
	287	980
	800	1,474
Mean	**685**	**1,026**
TNF	129,261	173,989
	138,296	100,258
	209,525	102,705
	195,330	282,175
Mean	**168,103**	**164,782**
TGF-β	37,153	318,360
	143,397	838,333
	30,695	168,975
	556,624	303,770
Mean	**191,967**	**407,360**

Table 1. Changes in the amounts of cytokine mRNA from TD cells obtained from AA and normal rats. The plain text represent the number of copies of cytokine mRNA in individual rats. The bold text represents the mean cytokine copy number. $*P<0.05$ (Mann Whitney U test).

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	Normal	AA
CD4 αβ-TcR	≥96	≥96
CD8 κ-Light Chain CD45 (B-cell specific)	≤3.2	≤3.9

Table 2. The purity (percentage staining) of the CD4^{+} T-cells from TD lymph of normal and adjuvant rats following selection with OX8, Mark-1 and OX33.

	Normal	AA
CD4 αβ-TcR	≥98	≥98
MHC class II CD134 IL-2 R Transferrin Receptor	≤0.5	≤1.8

Table 3. The purity (percentage staining) of unactivated CD4^{+} T-cells from TD lymph of normal and adjuvant rats following selection with OX39, OX 26, OX26 and OX40.

Cytokine Copy Number/μg of RNA	Non-Activated CD4$^+$ TD Cells From AA Rats	Activated CD4$^+$ TD Cells From AA Rats
IL-2	3,828	0
	909	3,239
	988	0
	0	990
Mean	**1,431**	**1,057**
IFN-γ	0	215,055
	0	435,867
	0	87,397
	0	63,710
Mean	**0**	**200,507***
IL-4	253	127
	333	223
	478	54
	95	8
Mean	**290**	**103**
TNF	31,453	309,235
	16,841	409,555
	16,338	208,272
	12,526	116,035
Mean	**19,289**	**260,774***
TGF-β	74,063	1,429,311
	133,702	5,321,089
	67,512	3,643,311
	10,804	10,418,256
Mean	**71,520**	**5,202,992***

Table 4. Changes in the amounts of cytokine mRNA in the activated and the non-activated fraction of CD4$^+$ TD cells from AA rats. The plain text represent the number of copies of cytokine mRNA in individual rats. The bold text represents the mean cytokine copy number. *P<0.05

Cytokine Copy Number/μg of RNA	Non-Activated CD4$^+$ TD Cells From Normal Rats	Activated CD4$^+$ TD Cells From Normal Rats
IL-2	0	2,535
	435	689
	807	0
	5	321
Mean	**311**	**886**
IFN-γ	162	0
	0	669
	0	0
	0	135
Mean	**41**	**201**
IL-4	237	679
	333	207
	235	121
	476	140
Mean	**321**	**287**
TNF	0	38,120
	12,618	118,367
	44,313	14,296
	18,696	12,216
Mean	**18,907**	**45,750**
TGF-β	0	0
	0	262,542
	79,531	0
	0	0
Mean	**19,883**	**65,635**

Table 5. Changes in the amounts of cytokine mRNA in activated and the non-activated fraction of CD4$^+$ TD T-cells from non-inoculated rats. The plain text represent the number of copies of cytokine mRNA in individual rats. The bold text represents the mean cytokine copy number. *P<0.05

Cytokine Copy Number/μg of RNA	Non-Activated CD4+ TD Cells From Normal Rats	Non-Activated CD4+ TD Cells From AA Rats
IL-2	0	3,828
	435	909
	807	988
	5	0
Mean	**311**	**1,431**
IFN-γ	162	0
	0	0
	0	0
	0	0
Mean	**41**	**0**
IL-4	237	253
	333	333
	235	478
	476	95
Mean	**321**	**290**
TNF	0	31,453
	12,618	16,841
	44,313	16,338
	18,696	12,526
Mean	**18,907**	**19,289**
TGF-β	0	74,063
	0	133,702
	79,531	67,512
	0	10,804
Mean	**19,883**	**71,520**

Table 6. Changes in the amounts of cytokine mRNA in the non-activated fraction of CD4+ TD T-cells obtained form normal and AA rats. The plain text represent the number of copies of cytokine mRNA in individual rats. The bold text represents the mean cytokine copy number. *P<0.05

Cytokine Copy Number/μg of RNA	Activated CD4$^+$ TD Cells From Normal Rats	Activated CD4$^+$ TD Cells From AA Rats
IL-2	2,535	0
	689	3,239
	0	0
	321	990
Mean	**886**	**1,057**
IFN-γ	0	215,055
	669	435,867
	0	87,397
	135	63,710
Mean	**201**	**200,507***
IL-4	679	127
	207	223
	121	54
	140	8
Mean	**287**	**103**
TNF	38,120	309,235
	118,367	409,555
	14,296	208,272
	12,216	116,035
Mean	**45,750**	**260,774***
TGF-β	0	1,429,311
	262,542	5,321,089
	0	3,643,311
	0	10,418,256
Mean	**65,635**	**5,202,992***

Table 7. Changes in the amounts of cytokine mRNA in the activated fraction of CD4$^+$ TD T-cells obtained form normal and AA rats. The plain text represent the number of copies of cytokine mRNA in individual rats. The bold text represents the mean cytokine copy number. *$P<0.05$

Summary and Future Directions

9.1 Methodology

The application of CE for separation and quantification of PCR products was demonstrated. Gel filled capillaries were shown to be very reliable and ideally suited to the resolution of DNA peaks. Their durability allowed more than 200 injections with highly reproducible migration of DNA through the capillaries. The PCR products required desalting prior to electrokinetic injection and CE analysis, which was achieved by precipitating the DNA with sodium acetate/ethanol. This method was also shown to reflect accurately the composition of the DNA sample. Furthermore, electrokinetic injection of DNA into the capillary did not affect quantification of the PCR products. The lower limit of detection of DNA using CE was 312 pg/mL which made the technique very applicable for the analysis of competitive PCR products.

The accuracy and reproducibility of competitive PCR was thoroughly assessed in accordance with a mathematical model of competitive PCR. At most there was a only a small difference in the amplification efficiency of the target and its respective competitor DNA fragment. It was demonstrated that competitive PCR can be used to accurately quantify small changes in cDNA levels although errors associated with diluting the competitor cDNA fragment limited the accuracy of quantification. Competitive PCR was shown to be sufficiently accurate for the reliable quantification of cDNA as demonstrated by the favourable comparison of its performance to a mathematical model of the reaction.

Further sources of error in the quantitative PCR procedure, of potentially equal or greater importance include variability in the extraction and reverse transcription (RT) of RNA. An insight was gained into the variability of these procedures by assessing their day to day

reproducibility. The variability of the RT reaction was considerably lower when performed using an aliquot from a common preparation of RT reagents in preference to individual preparations of the reagents. Furthermore, RT was reproducible in replicates analysed on the same day and less reproducible on a day to day basis. The daily variability of the reverse transcription procedure was a major factor limiting the accuracy of quantitative PCR.

The accuracy of the quantitative PCR procedure was also limited by the precision with which RNA was quantified. The amount of RNA in a sample was determined by measuring its optical absorbance at 260 nm. This was limited in precision because residual protein, DNA and reagents used during RNA extraction contaminated the RNA extract which confounded accurate quantification. Attempts to estimate the quantity of RNA more accurately by taking into account the quality of each sample were inconclusive.

Precise quantification of the amount of RNA added to a competitive PCR is fundamental for accurate quantification of cytokine mRNA expression. There are limitations to measuring the quantity of RNA spectrophotometrically therefore, an alternative method for standardising the quantity of RNA added to each competitive PCR was investigated. β-actin mRNA was assessed as a standard to which changes in cytokine mRNA levels could be measured. β-actin message levels were expected to be modest thereby allowing cytokine mRNA expression to be substantial in relation to this constitutively expressed housekeeping gene rather than total RNA. However, a β-actin pseudogene was detected in genomic DNA which negated its use as a valid standard for mRNA quantification in the rat. The levels of cytokine mRNA were therefore standardised to the quantity of RNA added to each PCR.

Quantitative RT-PCR was applied to measuring changes in cytokine mRNA levels following *in vitro* stimulation of spleen cells. This indicated the magnitude of change in cytokine mRNA levels following mitogen stimulation *in vitro*. A change as large as 150 fold was observed for IFN-γ whereas very little change in the level of mRNA production was observed for IL-4. Thus, the precision and accuracy of the quantitative RT-PCR

procedure was suitable for measuring large changes in the amount of cytokine mRNA seen in a biological system.

A poor signal to noise ratio was observed when quantitative RT-PCR was used to measure changes in the lymph nodes draining the hind limbs and the synovial tissue of rats during the development of AA. A comparison of the variability of cytokine mRNA levels with data obtained using cell culture supernatants suggests it was not attributable fully to variations during extraction, quantification, reverse transcription of RNA and competitive PCR. Differences in cytokine mRNA levels may be attributable to errors in sampling tissue from the lymph node. Distinct regions in a node may have different levels of cytokine mRNA due to various states of cellular activation in the node. Furthermore, necrotic regions in a node may promote mRNA degradation hence, variability in the levels of cytokine mRNA in individual rats. An influx of immunocytes into the lymph nodes and synovial tissue of rats with AA may result in an increase in RNA levels and since cytokine mRNA constitutes only a small fraction of total RNA, the increase in RNA may outweigh the rise in cytokine mRNA levels, making the effective changes small or even insignificant. Other physiological and biological differences between individual rats may also contribute to variation in cytokine message levels which were also evident at the protein level.

Changes in cytokine mRNA levels were evident on analysis of unseparated TD cells between AA and normal rats. Analysis of a subset of cells expressing CD4 and certain activation markers yielded a much larger difference (1000 fold for IFN-γ) between arthritic and normal rats. These findings suggest that a sub-population of TD cells express IFN-γ very strongly and the signal from this population had been diluted by the much larger number of cells not expressing IFN-γ among unseparated TD cells. The results suggest cytokine mRNA analysis can be much more meaningful when performed on a sub-population of cells isolated from the tissue of interest. Furthermore, this strategy allowed increased cytokine expression to be observed without deliberate stimulation *in vitro* which differs from other studies seeking evidence for cytokine expression *in vivo* in which stimulation with con A has been necessary to increase differences in cytokine mRNA to

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considerable levels. This strategy of deliberate stimulation raises questions regarding the extent to which observed findings mirror *in vivo* conditions.

A sub-population of cells can be selected from a cell suspension using magnetic beads coated with antibodies which bind cell surface markers of interest. The magnetic beads form rosettes around cells engaged by antibodies against surface molecules. These cells can then be isolated in a magnetic field. This technique allows cells bearing single or multiple cell surface markers to be isolated and is limited only by the availability of antibodies. The benefit of this method was evident during the analysis of CD4$^+$ TD sub-populations in which, significant changes in cytokine mRNA levels were measured in activated CD4$^+$ TD cells which were not evident during analysis of unseparated TD cells.

Several methods can be used to quantify cytokine production at either the mRNA or the protein level. *In situ* hybridisation and *in situ* PCR can be used in preference to quantitative PCR to monitor cytokine mRNA levels. Both procedures provide information on the type of cells producing a given cytokine but *insitu* hybridisation lacks sensitivity and both methods have a limited quantitative capacity. Intracellular cytokine protein levels can be detected using flow cytometry. This technique has the advantage that different fluorochrome labelled monoclonal antibodies can be used to label simultaneously, extracellular surface antigens and intercellular cytokines in permeablised cells. Two colour flow cytometry may therefore be used to elucidate the production of a cytokine by cells of a particular phenotype (eg. the proportion of IFN-γ secreting T-cells). The intensity of fluorescence provides a measure of intensity of cytokine expression, however, a period of stimulation *in vitro* coupled with blockade of cytokine secretion is required to achieve accumulation of cytokine that can be measured by this method. This requirement for *in vitro* stimulation casts some doubt on the relevance of findings to actual levels of expression *in vivo*.

Magnetic beads can be used to isolate a sub-population of cells bearing specific cell surface markers. Cytokine production in the isolated cell population can then be analysed using either flow cytometry or quantitative RT-PCR. More specifically, a single cell suspension prepared by collagenase digestion of the synovial tissue from AA rats may be

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subdivided into populations based on cell phenotype (eg. T-cells) or activation status. It is conceivable that a sub-population of cells from the synovium of AA rats could be isolated using magnetic beads after which, quantitative RT-PCR could be used to elucidate the profile of cytokines secreted by the isolated cell population. The limitations of this method are the cell numbers hence, the quantity of RNA extracted and a knowledge of the nucleotide sequence of the cytokine cDNA which is required to design oligonucleotide primers.

Furthermore, it is possible to enzymically detach magnetic beads from a rosetted cell after subdivision of the cell population. This is of substantial benefit since cells rosetted with magnetic beads are not suitable for flow cytometric analysis. Magnetic beads may be used to isolate a specific population of cells (eg. T-cells). Cytokine expression by the cells may be analysed by RT-PCR and 2 colour flow cytometry. As discussed above, measurement of cytokine production using flow cytometry usually requires *in vitro* stimulation of the cells with a mitogen or antigen which may not always accurately reflect cytokine production *in vivo*. Therefore, the measurement of cytokine protein levels using flow cytometry would ideally complement the measurement of cytokine mRNA levels using quantitative RT-PCR. Collectively, these procedures should prove suitable to achieve a substantial improvement in quantitative analysis of cytokine mRNA and protein levels and should be beneficial for measuring the production of key cytokines such as IL-12, IL-15, IL-10 and the recently described cytokine IL-17, during the development and pathogenesis of AA. However, it remains to be demonstrated whether collagenase digestion of the synovium strips the cells of their cell surface markers, which would compromise cell isolation using magnetic beads.

To date, all techniques used to measure cytokine production have limitations. Imperfections in measuring the level of cytokine gene expression using quantitative RT-PCR were evident in the variability of cytokine copy number. This may in part be due to biological variability between different rats as well as variability in the extraction, reverse transcription and PCR procedures. A considerable amount of time is required to optimise each competitive PCR and each reaction requires occasional "fine tuning" to maintain

specificity for the desired cDNA sequence. Despite these drawbacks, quantitative PCR is very sensitive and was successfully applied to measuring changes in cytokine mRNA levels without the need for *in vitro* stimulation. It was very applicable for measuring changes in cytokine message levels in a sub-population of TD cells although information on cytokine protein levels is required to substantiate changes in cytokine message levels.

9.2 A Model for the Pathogenesis of AA Based on Cytokine mRNA Expression

The time course of cytokine expression during the development of adjuvant arthritis has been established. Mechanisms responsible for induction and perpetuation of arthritis were examined by studying IL-2, IFN-γ, IL-4, TNF and TGF-β gene expression in the inguinal lymph nodes which principally drain the tail base, the popliteal lymph nodes which drain the hind paws, and the synovial tissue, an important site of inflammation in AA. The data presented is unique because it analyses progressive changes in the levels of T-cell derived cytokines and immunomodulatory cytokines in different tissues during both the prodromal and established stages of AA.

It is demonstrated that TD cells from rats with AA have an elevated expression of IFN-γ which was expressed in high copy numbers by a sub-population activated of CD4$^+$ T-cells. These cells also expressed elevated levels of TNF and TGF-β mRNA relative to their non-activated counterparts.

Some of the events leading to the induction and pathogenesis of AA can be postulated based on the profile of cytokines detected in the secondary and tertiary lymphoid tissues during the developmental and acute phases of adjuvant disease.

Intradermal inoculation of CFA into the base of the tail of DA rats initiates an immune response. Dendritic cells (DC) residing in the tissues most likely encounter the antigen and initiate a complex set of events culminating in the recruitment of T-cells and other DC to the inflammatory site. DCs with an endogenous antigenic peptide/MHC class II complex on their surface travel via the afferent lymphatics to the draining (eg. inguinal) lymph nodes where they interact with naive T-cells. This interaction leads to T-cell activation and

subsequently, the production of T-cell derived cytokines. In this thesis it was demonstrated that there was an up regulation of IFN-γ, IL-2 and TNF cytokine gene expression in the inguinal lymph nodes three days after inoculation with CFA. Cytokine up regulation was most likely the result of a primary immune response to CFA. IFN-γ and IL-2 are representative of a type 1 immune response whereas up regulation of TNF is consistent with an inflammatory response in the inguinal lymph nodes.

Endothelial cells in the inguinal lymph nodes are transformed in the presence of TNF, into HEVs which recruit circulating cells to the lymph node. Naive T-cells are amongst the recruited cells and upon exposure to appropriately presented antigen they develop into blast cells. T-lymphoblasts are believed to home selectively to specific tissues. It is hypothesised that the homing of T-lymphoblasts is determined by the adhesion molecules they express which is influenced by cytokines secreted by these cells and/or the cytokines produced in the inguinal lymph nodes during T-cell activation. In AA there is a type 1 like cytokine profile in the inguinal lymph nodes which may favour the recruitment of cells to the synovium.

Blast cells traffic via the efferent lymphatics which ultimately converge on the thoracic duct (TD). TD cells comprise mostly naive cells that circulate randomly (Freitas et al. 1980). However, a significant number of TD cells are lymphoblasts which are believed to home rapidly to specific tissues (Jalkanen et al. 1989; Pietschmann et al. 1992). In this thesis it was demonstrated that TD cells from AA rats displayed elevated levels of IFN-γ. Furthermore, message for IFN-γ was expressed at high copy numbers by activated CD4^{+} T-cells in TD lymph. These cells also displayed elevated expression of TNF and TGF-β mRNA than their counterparts from normal rats. The elevated levels of IFN-γ may promote the retention of circulating cells in tissues (Westermann et al. 1993; Westermann et al. 1994) and HEV formation at the inflammatory site. The production of TNF and TGF-β by circulating CD4^{+} T-cells may be associated with up regulation of adhesion molecules which are involved in cognate interactions between T-cells and microvascular endothelial cells.

At the site of inflammation, the expression of vascular adhesion molecules is upregulated by chemokines which also guide the migration of leucocytes into the extra-vascular space in a cell type specific manner (Proost et al. 1996). TNF and TGF-β can influence cell migration by up regulating vascular adhesion molecule expression (Vassalli 1992; Wahl et al. 1993b). It was demonstrated that these cytokines were up regulated in the synovial tissue of rats with AA. TNF can promote joint erosion whereas the predominant effect of TGF-β, amongst its multiple functions, may be determined by the presence of other cytokines produced in the synovial tissue. The detection of biologically active TGF-β protein in the synovium of AA rats is required to complement the mRNA data and it remains to be determined whether TNF is secreted by the activated T-cells in the synovium.

It is conceivable that TD lymphoblasts home to the synovium because of cytokine induced expression of adhesion molecules on their surface and the presence of cognate ligands on endothelial cells. The expression of specific adhesion molecules has recently been associated with preferential recruitment of Th-cell subsets to an inflammatory site (Austrup et al. 1997). Th-1 cells are able to bind to P- and E-selectin and were more efficiently recruited to sites dominated by a type 1 response. P-selectin is upregulated by TNF at inflammatory sites and E-selectin can be upregulated by TGF-β and IL-12. Therefore, type 1 cells from the inguinal lymph node may be preferentially recruited to inflamed synovial tissue due to the cytokine induced expression of P and E-selectin on vascular endothelium in the synovial tissue.

However, the mechanism by which inflammation in the synovial tissue is initiated remains elusive. It is possible that non-specific inflammatory insults could prepare the synovium to receive potentially arthritogenic cells by amplification of a subset of autoreactive T-cell clones or clones that cross react with an unidentified antigen in the synovial tissue. It has been demonstrated that TD lymphoblasts obtained from AA rats can adoptively transfer adjuvant disease to normal recipient rats and therefore, they play a role in initiating synovial tissue inflammation (Spargo et al. 1996). These observations suggest that migratory T-cells are involved in the dissemination of polyarthritis and that early events in

the development of arthritogenic T-cells occur in lymph nodes. The studies in this thesis suggest the cells that can adoptively transfer or disseminate arthritis may have a type 1 pattern of cytokine production (IFN-γ dominant) and they arise from the inguinal lymph nodes.

A fundamental understanding of cytokine costimulation or the networks responsible for up regulation of adhesion molecule expression in the synovium and on the circulating arthritogenic T-cells is required to elucidate the events leading to synovial tissue inflammation. Such a network is destined to develop new features as novel cytokines and adhesion molecules are characterised. It is fundamental to establish an understanding of the cytokine network involved in the development and perpetuation of AA because targeting key cytokines in an animal model of arthritis may lead to therapeutic interventions in the development and/or progress of RA. The findings presented in this thesis provide fundamental technical and immunological insights into the population of cells and the cytokines that are responsible for the development and pathogenesis of AA as a model for RA.

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