A STUDY TO IDENTIFY THE PRESENCE OF IL-10 AND IFN-γ IN LEUKOCYTES DURING *THEILERIA PARVA* INFECTION IN CATTLE

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A thesis submitted in partial fulfilment for the degree of Master of Science in Biochemistry of the University of Nairobi.

Declaration

I declare that the work presented in this thesis is my original work and that it has not been presented to any other institution for the purpose of examination.

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Dedication

To my Father and Mother ,

Timothy and Mary Dimbu:

A legacy of your incessant and fervent prayers to God almighty.

Thank you for everything.

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Abbreviations

αβ TcR	Alpha/beta T cell receptor
APTES	Aminopropyl triethoxy silane
BBH-A	Blue Bac His-A plasmid
BBIII	Blue Bac III plasmid
BCIP	5-bromo-4-chloro-3-indoyl phosphate
bp	Nucleotide base pairs
BSA	Bovine serum albumin
BVDV	Bovine viral diarrhoea virus
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CMI	Cell mediated immunity
ConA	Concanavalin A
CPTI	Cooperative programmes, training and information
DAP	Diaminobenzidine
dATP	Deoxy adenine triphosphate
dCTP	Deoxy cytidine triphosphate
DEPC	Diethyl pyrocarbonate
dGTP	Deoxy guanosine triphosphate
Dig	Digoxigenin
Dig-UTP	Digoxigenin conjugated to uridine triphosphate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease

dNTPs	Deoxyribonucleotides
DTH	Delayed type hypersensitivity
DTT	Dithiothreitol
dTTP	Deoxy thiamine triphosphate
EBV	Epstein Barr virus
ECF	East Coast Fever
EDTA	Ethylenediaminetetra acetic acid
FITC	Fluorescin isothiocyanate
GCG	Genetics computer group
γδ TcR	Gamma/delta T cell receptor
HCl	Hydrochloric acid
HRP	Horse radish peroxidase
IAH	Institute for Animal Health (U.K)
IFN-	Interferon
Ig	Immunoglobulin
IL-	Interleukin
ILRI	International Livestock Research Institute (Kenya)
ISH	*In situ* hybridisation
LB	Luria-Bertani (broth)
LGL	Large granular lymphocyte(s)
LN	Lymph node
mAb	Monoclonal antibody
MCS	Multiple cloning site
MDBK	Madin-Darby bovine kidney

MEM	Minimal essential medium
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
MHC	Major histocompatibility complex
Mo/MΦ	Monocyte(s)/macrophage(s)
mRNA	Messenger ribonucleic acid
NaN_3	Sodium azide
NBF	Normal buffered saline
NBT	Nitroblue tetrazolium
NK	Natural killer
nt	Nucleotide bases
NTP	Nucleotide triphosphate
ODA	Overseas Development Agency
Oligo	Oligonucleotide phosphate
PBM	Peripheral blood monocyte(s)
PBS	Phosphate buffered saline
PBSa	Phosphate buffered saline with 0.1% albumin (BSA)
PCR	Polymerase chain reaction
pg	Pico grammes
rbIL-2	Recombinant bovine interleukin 2
R.E	Restriction endonuclease(s)
RNA	Ribonucleic acid
RNase	Ribonuclease
RT-PCR	Reverse transcriptase polymerase chain reaction

RT^{O}C	Room temperature(ambient)
SDS	Sodium dodecyl sulphate
SSC	Sodium saline citrate
T.E	Tris/ethelenediaminetetracacetic acid buffer, pH 8.0
Taq	*Thermus aquaticus*
TBE	Tris/borate/EDTA buffer
TCM	Tissue culture medium
TH 1	T helper 1
TH2	T helper 2
TNF	Tumour necrosis factor
Tris	2-amino-2-(hydroxymethyl) propane-1,3-diol
UV	Ultra violet
WC1	Workshop cluster 1
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

Table of unit measurements

°C	Degree(s) centigrade
hr	Hour(s)
M	Molar ($moleL^{-1}$)
min	Minute(s)
ml	Millilitre(s)
mM	Micromolar
μg	Microgramme(s)
mg	milligramme(s)
μl	Microlitre(s)
μm	Micrometre(s)
ng	Nanogramme(s)
pg	Picogramme(s)
sec	Second(s)

Abstract

The gene expression of two immunoregulatory cytokines, IL-10 and IFN-γ, during infection of bovine leukocytes with *T. parva* was investigated at the single cell level. Initial results obtained from *in vitro* studies with infected cells using RT-PCR showed that both IL-10 and IFN-γ messages were present. The ability of *Theileria*-infected cells to produce IL-10 transcripts was a consistent feature. However, their ability to exhibit IFN-γ messages was less consistent. On application of *in situ* hybridisation technique using bovine IL-10 and IFN-γ-specific riboprobes, it was demonstrated that most infected lymphocytes produced IL-10 and that few of these cells displayed expression of IFN-γ transcripts. Thus, judging from the relative abundance of IL-10 and lack of IFN-γ mRNA, the expression of IL-10, a cytokine known to inhibit IFN-γ production and functions, was shown to be upregulated in leukocytes responding to *T. parva* infection in cattle. These experiments did not demonstrate the production of biologically active IL-10 during the infection. However, the accompanying relative absence of IFN-γ mRNA *in situ* may suggests that biologically active IL-10 was produced and blocked the production of IFN-γ, an important effector TH 1 cytokine. The apparent upregulated expression of IL-10 during infection with *T. parva* is indicative of a regulatory role for this cytokine in the mediation of susceptibility to acute disease. This parasite-instigated induction and upregulation of IL-10 expression may represent an important strategy by which intracellular *T. parva* eludes IFN-γ-dependent cell-mediated immune destruction. Through its induction of tissue-damaging metalloproteinase, IL-10 presence also offers a biochemical explanation to the possible mechanism of propagating the lymphoproliferative, immunopathologic and tissue destructive phenomena observed in ECF.

Chapter 1

Literature Review

Literature Review

1. Introduction

1.1 East coast fever

The apicomplexan parasite *Theileria parva* causes a disease in cattle called theileriosis. This is a tick-borne disease transmitted by the Ixodid tick *Ripicephalus appendiculatus*. Commonly known as East Coast Fever (ECF), theileriosis is an acute lymphoproliferative disease. This disease is endemic to at least eleven countries of eastern, central and southern Africa, where it threatens in excess of 25 million cattle. It thus has a major economic impact on animal production due to mortalities and production losses, as well as, a consequence of the intensive tick-control programmes that are required to prevent the disease. The availability of alternative, less expensive and more environmentally friendly preventive control measures, would substantially relieve this situation.

On inoculation of cattle by the tick-borne vector, *T. parva* infects lymphocytes and induces them to proliferate, allowing the parasite to replicate by clonal expansion and remain in an intra-cellular environment. The resulting disease is characterised by the presence of large numbers of parasitized cells throughout the lymphoid system, lymphocyte destruction and high mortality in susceptible animals.

Presently, the only method for long term protection from a given parasite strain is by immunising cattle using a method of infection and treatment with the drug Tetracycline (Brown *et al.*, 1977). There is strong evidence that cytotoxic T-cell responses are important in mediating this immunity (Emery, 1981a). This method is dependant on the immune status of individual animals (some animals will inevitably come down with disease); the method also poses an inherent danger of possible cross-infection with other

pathogens. It is an expensive and labour intensive method requiring costly drug(s) as well as close management by trained personnel making it inconvenient and of limited widespread applicability in the field.

1.2 Life cycle of *T. parva* in the mammalian host

Knowledge of the life cycle of *T. parva* in the mammalian host is essential to fully appreciate the mechanism of immunity (Fig 1.1). Infection is initiated when sporozoites, deposited by the tick vector during feeding, gain entry into lymphocytes. Various lineages of lymphocytes are susceptible to infection (Baldwin *et al.*, 1988), but T lymphocytes constitute by far the large majority of the parasitised cells in infected cattle (Emery *et al.*, 1988). Within 24 to 48 hours of invading a lymphocyte, the sporozoite develops to a multi-nucleate body named a schizont. This development is in synchrony with the activation of the host cell, which starts to proliferate. At each cell division, the parasite also divides so that both daughter cells get infected (Hullinger *et al.*, 1964). Thus, the infection is established and becomes disseminated by clonal expansion of the small population of lymphocytes initially infected with *T. parva*. Vast numbers of parasitized cells are found throughout the lymphoid system and are responsible for most of the pathology of this disease. The majority of infected animals die within 3 to 4 weeks of infection. In the latter stages of the infection some of the schizonts develop into merozoites which get released and enter erythrocytes to engender piroplasms, which are the tick-infective stage of the parasite.

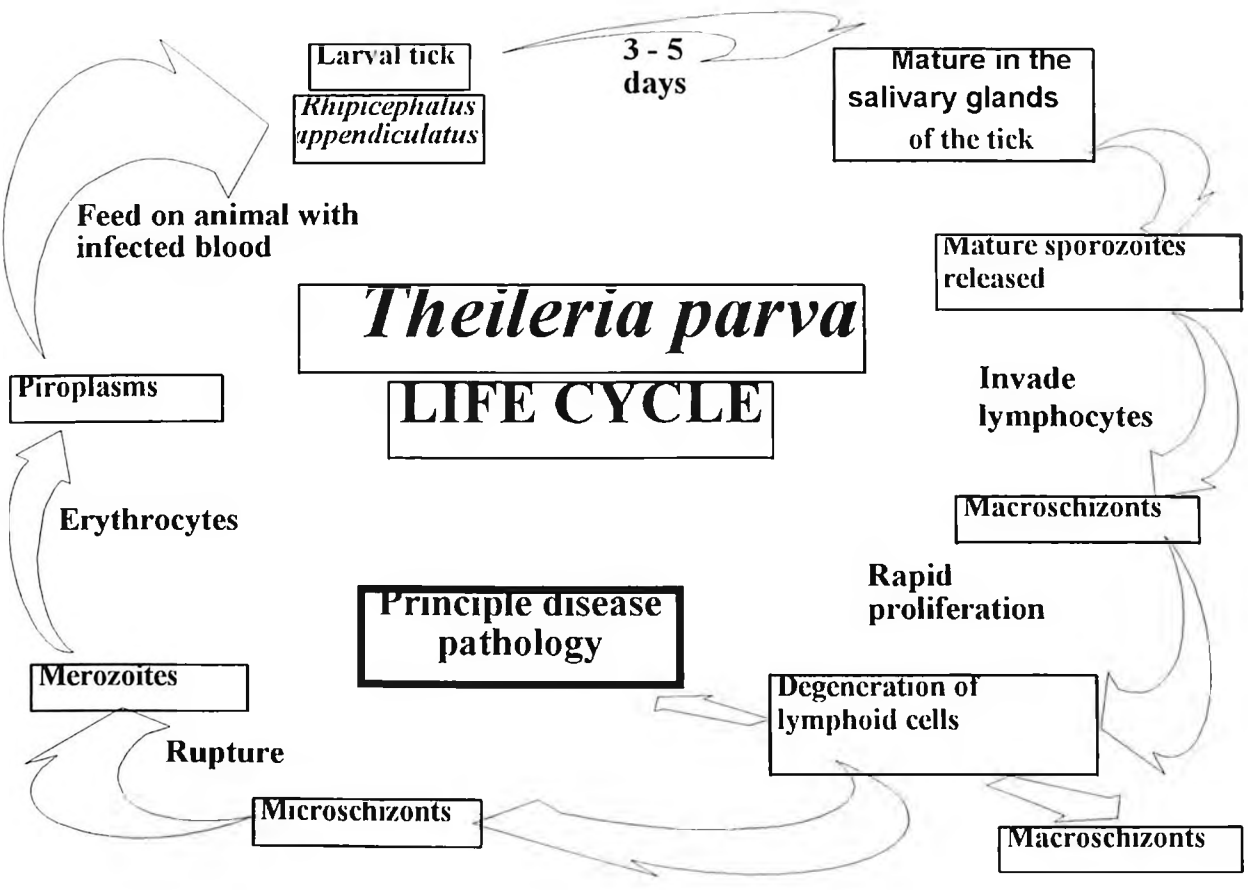

Figure 1.1

Infection is initiated by injection of a sporozoite-laden saliva from an infected tick during feeding. Shortly after invading a lymphocyte, the sporozoite develops to a schizont. This development is synchronously associated with the activation of the host cell, which proliferate. At each cell division, the parasite also divides so that both daughter cells are infected such that, the infection is established and becomes disseminated by clonal expansion of an initial small population of lymphocytes infected with *T. parva*. Infected cells are found throughout the lymphoid system and are responsible for most of the pathology of the disease. Later on in the life cycle, some of the schizonts develop to merozoites get released and enter erythrocytes to give rise to piroplasms, the tick-infective stage of the parasite; the cycle is then repeated.

1.3 Clinical Disease

In the lymphoproliferative stage of infection there is a massive increase in numbers of macroschizont-infected cells, reaching almost lymphosarcomatous proportions (Moulton *et al.*, 1971). This is then followed by a lymphodegenerative stage with lymphoid tissue necrosis. Pathological features at postmortem vary with the severity of infection, but the most striking changes associated with lymphoproliferative theileriosis involve the lymphoid tissue and the lungs (reviewed by Irvin and Morrison, 1987). Typically, there is a generalised enlargement of the lymph nodes and spleen, although in protracted cases these organs may be of a relatively normal size. Histologically, tissue sections of the infected lymph nodes do not display clear demarcation into cortical and medullary regions associated with the normal nodes, and extensive areas of haemorrhage and necrosis are often present. In parallel, the white pulp follicles of the spleen are indistinct and the red pulp of the same is also markedly congested. Usually, the thymus which often contains areas of haemorrhage, is also atrophied. In all ECF postmortem cases, there is consistently, massive pulmonary oedema, depicted as copious oedema fluid in the trachea, bronchi and within the interlobular septae. Focal erythrocyte-filled areas of congestion and consolidation are often present throughout the lung tissue. Petecheal and ecchymotic haemorrhages of the serosal surfaces are common and in certain cases ulcers are observed in the abomasum and/or small intestines.

1.4 Signs and symptoms of the disease

Clinical signs of ECF are for the most first observed 5 to 8 days after an infective tick bite. They include swelling of the drainage lymph node followed by generalized lymphadenopathy and pyrexia. Affected animals may appear listless and, as their condition deteriorates, develop ocular/nasal discharges and dyspnoea, progressing steadily to recumbency and death within 2 to 4 weeks, usually as a result of pulmonary oedema (Irvin and Morrison, 1987).

1.5 Pathogenesis of Disease

The schizont is the pathogenic stage of the *T. parva* parasite; hence consideration of the pathogenesis of the disease has centred around the development and kinetics of multiplication of this stage in the parasite's life cycle. The changes produced in the lymphoid system by infection with a lethal dose of *T. parva* can be considered to progress through three stages: first, an incubation or subclinical period, during which there is no clinical evidence of disease; second, a lymphoproliferative phase, characterised by initial detection and rapid proliferation of parasitized cells throughout the lymphoid system; and third a phase of lymphoid disruption and depletion (Irvin and Morrison, 1987).

1.5.1 Subclinical period

Detailed studies of the phenomena that follow between inoculation of sporozoites by the tick and the initial detection of macroschizonts in the regional lymph are not available due to the difficulty of detecting the organism during this period. Studies *in vitro* on the initial interaction of the sporozoites with the host cells indicate that the organisms are capable of entering cells in a matter of minutes (Fawcet *et al.*, 1982).

Similar events may occur *in vivo* at the site of tick attachment. Maximal release of sporozoites by an infected tick occur 3 to 5 days after the tick has commenced feeding (Nuttall and Hindle, 1913; Purnell and Joyner, 1964), by which time there is a local inflammatory reaction to the tick bite (Cowdry and Danks, 1933). Whether or not this cellular reaction facilitates or hinders establishment of the infection is not known. Infection spreads rapidly from the site of inoculation (Wilde 1966) as shown in the experiment using cattle challenged on the ears by infected ticks. Removal of the ticks and amputation of the relevant part of the ear 5 days after the ticks were applied, did not significantly effect subsequent development of infection.

Infection appears to spread through the lymphatic system, since there is concentration of infected cells in the regional lymph nodes. Because of the finding that surgical removal of the regional node as early as 2 days after challenge of cattle with sporozoites has little effect on the subsequent course of infection, this indicates that there is an early dissemination of infected cells (Emery, 1981b). Whether this occurs entirely via the lymphatics and drainage lymph nodes, or whether sporozoites or infected cells also pass directly into the bloodstream at the site of inoculation has not been established.

Initial detection of infected cells in the drainage node appears to reflect a simple relative enrichment of these infected cells in this location, because of its proximity to the site of inoculation, rather than an initial period of establishment of the infection prior to dissemination to other lymphoid tissues. Judging from observations on the early stages of infection *in vitro* (Stagg *et al.*, 1981; Kurti *et al.*, 1981), the parasite progresses through a period of 2 or 3 days maturation prior to detection of macroschizonts, and induction or proliferation in the host cell.

The time of initial detection of the macroschizonts in the regional lymph node ranges from 4 to 14 days, depending on the number of parasites originally inoculated. Even though cattle exhibiting prolonged prepatent periods have a greater chance of survival, the production of non-lethal infections cannot be achieved reproducibly by titration of infective material (Cunningham *et al*, 1974).

Animals inoculated with infected tick stabilate develop a moderate blast response with the formation of germinal centres in the regional lymph nodes and a concomitant increase in output of cells in efferent lymph 4 to 5 days after inoculation (Emery, 1981b; Morrison *et al*., 1981). However, the response here appears to be directed against tick antigens as similar changes are elicited by uninfected tick stabilate.

1.5.2 Hyperplastic phase

When macroschizonts are first detectable in the regional lymph node, infected animals develop pyrexia which persists as long as the infection lasts. By this time of macroschizont detection, the regional lymph node develops a striking hyperplasia, so that within a 2 to 3 day period the node increases three to sixfold in size (Morrison *et al*., 1981).

This increase in the cell content is maintained for some days and throughout this phase of the infection, there is progressive increase in parasitosis of the lymph node resident cells. Parasitized cells are detected in efferent lymph at about the same time as in the parent lymph node, and the appearance is associated with an increase in cell output and in blast cells in efferent lymph (Morrison *et al*., 1981). The content of blast cells may increase from less than 10% to greater than 50% within 4 to 5 days of initial detection of

parasites, though only a proportion of these blast cells are actually parasitized. The increase in total cellularity of the regional lymph node is also far in excess of that accounted for by an increase in the number in the parasitized cells. The increase in the lymph node cellularity may be due to recruitment of cells into the node; however, the presence of large numbers of infected blast cells suggest that there may be a cellular response to the parasitized cells as well.

The kinetics of cellular changes and levels of parasitosis in the spleen are to a greater degree the same as those observed in non regional lymph nodes (Morrison *et al.*, 1981). However, a notable feature during this phase of the infection is the development of leucopenia, affecting both polymorphonuclear leucocytes and lymphocytes (Wilde, 1966). A drop in the white cell count is already apparent at the time of initial detection of parasitosis.

At the histological level, a progressive increase in the number of blast cells can be seen in the lymphoid tissues during this period (Morrison *et al.*, 1981). Examination of tissue sections by immunoflourescence or immunoperoxidase techniques show macroschizonts within many of these cells. The infected cells do not appear to be restricted to a particular compartment within the lymphoid tissues, although in the lymph node cortices and splenic white pulp there tends to be more in the T dependent areas, than in the B dependent areas. During the later part of this proliferative phase, infected cells can also be detected in the bone marrow, thymus and Peyer's patches and in the lamina propria of the gut and interstitial tissues of the lungs.

1.5.3 Lymphoid disruption and depletion

Approximately 4 to 5 days after initial detection of parasites when the infection reaches levels of 5 to 10% parasitosis in the lymph node and spleen, the architecture of these tissues become peculiarly disorganised in appearance. Despite the continued increase in the percentage of parasitized cells, which may reach levels in excess of 50%, development of a gradual decrease in the total cellularity of the lymphoid tissues is apparent, eventually leading to depletion below resting levels (Morrison *et al.*, 1981). This is parallelled by a decrease in the output of cells from the lymph nodes in efferent lymph and in the cellularity of thoracic duct lymph. In the advanced stages of the disease, the cellular content of the lymph may drop to less than 10% of normal levels (Emery, 1981b). This is also accompanied by a further decrease in blood leucocyte counts which may fall below $1x10^3$ leucocytes per mm^3 (Emery, 1981b).

During this period, there is an increase in the mean number of macroschizonts and piroplasms are initially detected in erythrocytes (Jarrett *et al.*, 1969; Radley *et al.*, 1974). It was demonstrated that in cattle subjected to different levels of challenge with infected ticks, piroplasms first appeared in all animals 15 to 17 days after attachment of the ticks (Jarret *et al.*, 1969). Therefore these events seem to be independent of the level of parasitosis, but are rather time dependent. Based on these results, it was suggested that the differentiation to microschizonts may occur after macroschizonts undergo a finite number of divisions (Jarret *et al.*, 1969).

The gross and histological appearance of the lymphoid tissues early in this phase of the infection resemble that produced by multicentric lymphoid tumours (reviewed by Irvin and Morrison, 1987). Large areas of the lymph nodes and spleen are populated

predominantly by large lymphoid cells. The only compartment which often appears to remain uninvolved is the small lymphocyte component of the B-dependent follicular areas. However, infected lymphoblasts are observed within germinal centres, although germinal centres themselves undergo rapid involution during this phase of the infection. The increase in number of the parasitized cells is associated with an accompanying progressive lymphocytolysis. This is initially observed as small foci of lymphocyte necrosis often associated with sparse infiltrates of neutrophils. These foci eventually expand and coalesce, giving rise to extensive areas of necrosis which involve both parasitized and non-parasitized resident cells. Occasionally there is also vascular damage and thrombosis, giving rise to complete necrosis of the structural elements of the tissue.

Infected cells are abundant in all lymphoid tissues including the lymph nodes, spleen, Peyer's patches, tonsils, thymus, and bone marrow. Involvement of the primary lymphoid organs is of profound significance, because it often results in complete atrophy of normal thymic lymphoid tissue and partial replacement of normal bone marrow haemopoietic tissue by infected lymphoblasts. There is also extensive spreading and infiltration of infected lymphoblasts into a variety of non-lymphoid tissues (Barnett, 1960; Morrison *et al.*, 1981), particularly in the interstitial tissues of the lungs and the lamina propria of the gastrointestinal tract.

1.6 Possible mechanisms of lymphoid distraction

A remarkable feature of the terminal stages of ECF is the heavy depletion of lymphocytes, in the solid lymphoid tissues and in the recirculating pool. This is apparently a result of the destruction of cells within the nodes. However, panleucopenia is also observed and develops before the onset of lymphocytolysis and generalized lymphocyte depletion (Wilde 1966). This study by Wilde provided evidence that the leucopenia involved retardation of granulopoiesis in the bone marrow. There is severe impairment of lymphopoiesis in these tissues during the later stages of the infection. Accordingly, considering chemotherapy of the disease in animals in advanced stages of the disease, some time will be required for the animals to regenerate their haemopoietic tissues and immunocompetence, even if elimination of the parasites is successful.

Polyspecific cytotoxic T-cells have been detected in peripheral blood lymphocytes of cattle during the terminal phase of the lethal disease (Emery *et al.*, 1981). These cells were capable of killing a range of different allogeneic *Theileria* infected cells and uninfected lymphoblasts (Emery *et al.*, 1981), as well as autologous infected cell lines (Taracha, personal communication). Whether or not the cytotoxic cells detected in cattle undergoing lethal infections are themselves infected with the parasite is not known. However, it has been established that infected lymphocytes in some circumstances may exert immunological functions (Emery *et al.*, 1981). Whatever the initial mechanism of lymphocytolysis, it is probable that as increasing numbers of cells are destroyed, material released from the dying cells will have a toxic effect, thus exacerbating the process of cellular destruction.

Evidence for complement system involvement in lymphocytolysis was obtained from studies in cattle, during the advanced stages of a lethal infection with *T. parva* (Shitakha

et al., 1983). Depressed serum levels of haemolytic complement C3 were observed, and with the accompanied absence of detectable immune complexes, it was suggested that activation of complement may be triggered by proteolytic activity of the plasmin on complement components, or by direct activation by free schizonts. In this study, high serum levels of fibrinogen degradation products were also detected and other workers have demonstrated the presence of fibrin monomers in the serum (Maxie *et al.*, 1982). Alternatively, such products may be derived from the extensive deposits of fibrin, which are present at sites of lymphocytosis within the lymph nodes and spleen. In this regard, Shitakha and colleagues suggested that activation of the clotting system may be induced by enzymatic activity released from dying lymphocytes, possibly resulting in the generation of plasmin. Complement products C3a and C5a can directly induce contraction of smooth muscle and increase vascular permeability, as well as releasing vasoactive amines from the mast cells (Hugli, 1978). It has been suggested that the effect of such inflammatory mediators may be accentuated if there is a disturbance in their normal pathways of negative regulation (Chenoweth and Hugli, 1980). A possible example of this, is the terminal depletion of neutrophils, which are involved in inactivation of C5a (Chenoweth and Hugli, 1980). From these observations it would appear that the pathogenesis of pulmonary oedema is closely related to lymphocytolysis of infected cells within the lungs. Thus, the activation of complement may be of particular significance in the development of pulmonary oedema.

An aspect of the cellular immunity that has not been studied in detail in regard to pathogenesis of *T. parva* infection, is the involvement of cytokines, the immune systems cellular messengers. Given their profound influence on many parasitic diseases, it is speculated that cytokines are involved in the mediation of pathogenic effects seen in this disease.

1.7 Biology of cytokines

1.7.1 Introduction

The immune system is regulated by a complex network of cells and soluble factors, the cytokines. Cytokines are protein mediators involved in inflammation, the immune response, cell growth, repair and fibrosis. These proteins are of low molecular weight and many of them are glycosylated and are produced by different types of cells. Generally, many cytokines act in a paracrine fashion, in that they are produced by one cell and act on other(s) adjacent to them, but in some cases they act in an autocrine manner, affecting the same cell(s) that produced them. Because of the diverse, intricate and profound effects they have on the regulatory events in the entire living system, cytokine production is transient and subject to complex and strict control.

1.7.2 Nomenclature

The first time they were studied, a distinction was made between those cytokines that were thought to be produced by lymphocytes, and were named lymphokines, and those produced by monocytes/macrophages named monokines. However, further scrutiny revealed that many cytokines formally designated to be lymphokines or monokines, could also be synthesized by cells of other lineages. This heterogeneity of origin lead to a change in nomenclature such that these terms are no longer applicable in this restrictive sense and are now generally avoided. Additionally, complications became evident when it was found that some activities formally ascribed to different molecules on the basis of diverse biological assays, were in fact mediated by the same cytokine. A new system was therefore adopted, attempting to standardise the nomenclature of cytokines. The term interleukin (IL) was proposed to describe molecularly characterised proteins involved in the intracellular communication between leucocytes (inter + leucocytes), that were soluble, non-immunoglobulin and were numbered sequentially. However this naming system has not been universally applied to the cytokines and some are still known by their original terminology, e.g. interferons (IFN) and tumour necrosis factor (TNF), even though their respective genes have been cloned. Further characterization of various cytokines has shown that even the original definition of an interleukin is no longer tenable. It is clear that many of these cytokines can also be produced by, and act on cells other than leucocytes. In this regard, some interleukins are produced by epithelial cells (for example IL-1), and others can stimulate cells in non-lymphoid organs, such as the brain and liver, and have a multitude of different effects throughout the body. That the same cytokine can have multiple effects on different cells in a variety of tissues underlies the importance of these molecules in regulating and coordinating cellular responses throughout the body, and not just within the immune system.

Since all cytokines are polypeptides and the plasma membrane is impermeable to virtually all such macromolecules, it follows that cytokines cannot directly enter their target cells. Their effects on cellular functions must therefore be exerted via cytokine-specific receptors on the membrane. In general, a cytokine receptor must comprise at least three parts: an extracellular domain to provide the binding site and is specific for each cytokine, a transmembrane region which spans the plasma membrane, and an intracellular domain which either provides enzymatic activity, or binds other molecules to provide a signal inside the cell in response to ligand binding.

1.8 TH1 and TH2 cytokine profiles

Since cytokines control an impressive array of biological functions, it is not surprising that their correct production and action is necessary for good health. *In vivo* different immune responses can be launched in response to pathogens and these are tightly regulated, in part by the production of cytokines by many different cell types. However, the abnormal production of cytokines may lead to the dysregulation of cell growth and cause, or exacerbate the pathology associated with many infections (Parronchi *et al*; 1992; Powrie and Coffman; 1993).

As early as the 1970s, it was suggested that the different types of immune responses may be attributable to heterogeneity within the CD4+ T-cell population. This concept was strengthened during the 1980s by the observations that mouse CD4+ T-cell clones can be divided according to their predominant cytokine secretion profiles (Mosmann *et al.*, 1986). Clones which produced IL-2, IFN-γ and lymphotoxin; and promoted delayed cell mediated immunity (CMI), were designated T helper 1 (TH1) cells (Mosmann *et al.*, 1986; Mosman and Coffman, 1989). While TH2 clones produced cytokines such as IL-4,

IL-5 and IL-10, and were not only able to direct humoral immune responses, but also to promote allergic type responses, with the activation of mast cells and eosinophils (Mosmann *et al.*, 1986; Mosman and Coffman, 1989; Sher and Coffman, 1992). Cross regulation of these subsets has been proposed, where cytokines such as IFN-γ, secreted by TH1 cells, inhibit responses and cytokine production by TH2 cells (Gajewski and Fitch, 1988). Similarly, the production of IL-4 and IL-10 by TH2 cells, in part accounts for much of the down-regulation/suppression of the inflammatory and TH1 or CMI responses (Powrie and Coffman, 1993).

In the human system, these findings remained controversial for some years as polarized populations of human CD4+ T-cell clones producing only TH1 or TH2 cytokines were more difficult to observe (de Vries *at al.*, 1991). In addition, human CD4+ T-cell clones mediating CMI responses were able to produce cytokines of both TH1 and TH2 phenotype (de Vries *at al.*, 1991). Such CD4+ T-cells are described as TH0 cells (Mosman and Coffman, 1989; de Vries *at al.*, 1991;), and can be found in both mouse and human systems. This discrepancy has been answered to a great extent and was probably, initially, due to very different conditions and cell sources used in the murine and human experimental systems. In the mouse, T-cells were often obtained from the spleen and lymph nodes of hyper-immunized animals (Sher and Coffman, 1992); T-cell activation was achieved by stimulation with protein or peptide antigen. In human systems, T-cell clones were often obtained from the peripheral blood of normal individuals, with activation achieved by stimulation with mitogens (Sher and Coffman, 1992). Despite these differences, examples of polarized human TH1- and TH2-type T-cells have now definately been identified, using a source of T-cells obtained from peripheral blood and draining lymph nodes during chronic disease (Romagnani, 1991;

Romagnani *et al.*, 1992).

Development of the appropriate TH subset during infection is important, because certain pathogens are most effectively controlled by either a predominantly cellular (TH1) or a humoral (TH2) type immune response (Scott and Kaufmann, 1991; Sher *et al.*, 1992, Urban *et al.*, 1992). In some chronic diseases, dominance of the improper T helper cell response can exacerbate the disease and lead to an inability to eradicate the invading organism. For example, the strong delayed type hypersensitivity (DTH), a characteristic TH1-type response, that develops in most mice during infection by the parasite *Leishmania major*, results in the eradication of the organism (Scott *et al.*, 1988). In contrast, BALB/c mice mount a mostly humoral (TH2-type) immune response during *L. major* infection and ultimately die of disseminated disease (Heinzel *et al.*, 1989). These two types of responses to *Leishmania* are also seen in the human disease (Carlvalho *et al.*, 1985, Badaro *et al.*, 1986; Sacks *et al.*, 1987). Other human diseases can show similar characteristics. For example, tuberculoid leprosy is accompanied by a strong DTH response that ultimately kills and clears bacilli, though inflicting immunopathology. In contrast, lepromatous leprosy displays a weak CMI combined with the production of TH2 type cytokines, a scenario that results in the organisms multiplying, so that a much more severe disease persists (Yamamura *et al.*, 1991). In other diseases, such as helminth infection (Sher *et al.*, 1992; Urban *et al.*, 1992), human immunodefficiency virus (Clerici *et al.*, 1994), toxoplasma (reviewed by Sher and Coffman 1992) or listeriosis (Magee and Wing, 1988; Bancroft et al., 1989; Tripp et al., 1993) a correlation also exists between the predominant T-cell response phenotype and disease susceptibility. Furthermore, an uncontrolled or chronic T helper cell response can lead to immunopathology (Wierenga *et al.*, 1990; Maggi *et al.*, 1991; Parronchi *et al.*, 1991; Yssel *et al.*, 1991; Alwan *et al.*,

1994). The ability to control the emerging T-helper cell phenotype following exposure to antigens thus offers the potential to induce a response appropriate for a specific pathogen, with minimum pathology. Elucidation of the mechanisms regulating TH phenotype are important to the understanding of immune responses to such pathogens.

Many factors have been shown to influence the generation of TH subsets, including antigen (Mosmann and Coffman, 1989) and antigen presenting cell (Gajewski *et al.*, 1991). However, recently it has become clear that early events in an immune response probably stimulate the production of cytokines by non T-cells that greatly influence differentiation of TH responses (Fig 1.2). In this regard, IFNs (α and γ) have been shown to be important mediators in promoting the differentiation of allergen-specific T-cells into TH0 and TH1 phenotypes, rather than the expected TH2 subset (Parronchi *et al.*, 1992); this function has also been ascribed to IL-12 (Hsieh *et al.*, 1993). Conversely, IL-4 seems to be important in establishing a CD4+ TH2 response, although the initial cell source of IL-4 remains obscure (Bendelac *et al.*, 1992). The ability of IL-4 and IL-10 to inhibit TH1 development and effector functions, as well as the requirement of committed TH1 cell for co-stimulators to induce maximal IFN-γ production, suggests that cell-mediated immunity is under strict control, probably to achieve immunity with minimum immunopathology.

The maintenance and regulation of TH1 and TH2 responses are also tightly controlled by cytokines, which further increases the complexity of the CD4+ T-cell response. Two cytokines which have been shown to play an important regulatory role in the maintenance of a TH1 versus TH2 response are IFN-γ and IL-10.

1.9 Interferon-γ

IFNs were originally identified as factors produced by virally-infected cells, which can protect them against further viral infection (Isaacs and Lindenmann, 1957). It is now clear that IFNs, like other cytokines, can elicit other changes in cell behaviour, including effects on growth, differentiation and regulation of immune responses (reviewed by Bielefeldt *et al.*, 1987). The multiple species of bovine IFNs are classified as type I (α, β, τ and ώ) and type II interferon (γ).

IFN-γ is a pleiotropic cytokine involved in the regulation of nearly all phases of immune and inflammatory responses, including the activation, growth and differentiation of T-cells, B-cells, macrophages, natural killer (NK) cells and other cell types, such as endothelial cells and fibroblasts (review by Bielefeldt *et al.*, 1987).

T lymphocytes and large granular lymphocytes (LGL; a fraction which contains cells with NK activity), are the major producers of the type II, acid labile IFN-γ also termed as immune interferon. Production of IFN-γ can be induced *in vitro* by stimulating T-cells with the activating aspect of specific antigens. Both CD4+ and CD8+ major T-cell subsets have the capacity to produce IFN-γ although the CD4+ helper T-cell is considered the main producer (De Maeyer and De Maeyer-Guignard, 1988). IFN-α induces the production of IFN-γ by NK cells, as well as promoting the differentiation of

allergen-specific T-cells into TH0 or TH1 (Parronchi *et al.*, 1992). This function has also been described for IL-12 (Hsieh *et al.*, 1993), in which IL-12 induces production of IFN-γ by both NK and T-cells (Kobayashi *et al.*, 1989). It has therefore been proposed, that viruses and bacteria stimulate the early production of IFN-γ and IL-12, which induces a TH1 type response (Parronchi *et al.*, 1992). The release of IL-2 by activated T-cells is a main stimulus for further sequential synthesis of IFN-γ and other lymphokines (Kronke *et al.*, 1985). Furthermore, when LGL are treated with IL-2, they release IFN-γ in addition to more IL-2 and this increased production of IFN-γ accounts, in part for the increase in cytotoxicity of these cells (Trincheri *et al.*, 1984; Sandvig *et al.*, 1987).

Te Velde *et al* (1990) made an important observation in which they reported antagonistic activity between IFN-γ and IL-4. It is documented that IFN-γ produced by TH1 cells preferentially inhibits the proliferation of cells with a TH2 phenotype, but not TH1 cells and further that IFN-γ inhibits the release of IL-10 by macrophages (Gajewski and Fitch, 1988). This suggests that the early presence of IFN-γ during an immune response will result in the preferential expansion of TH1 cells and provide the possibility of a negative feedback interaction between the T helper subsets.

IFN-γ has been shown to play an important role in a number of parasitic infections. Intracellular parasite killing is significantly activated when infected macrophages are exposed to IFN-γ, as demonstrated for suspensions of human monocytes infected with *Leishmania donovani* (Hoover *et al.*, 1985). The crucial contribution of endogenous IFN-γ to the resistance of mice to *Trypanosoma cruzi* infection through the use of anti-IFN-γ mAb was demonstrated by Torrico *et al.*, (1991). In this study, both the levels of parasite production and mortality were greatly enhanced in antibody treated mice.

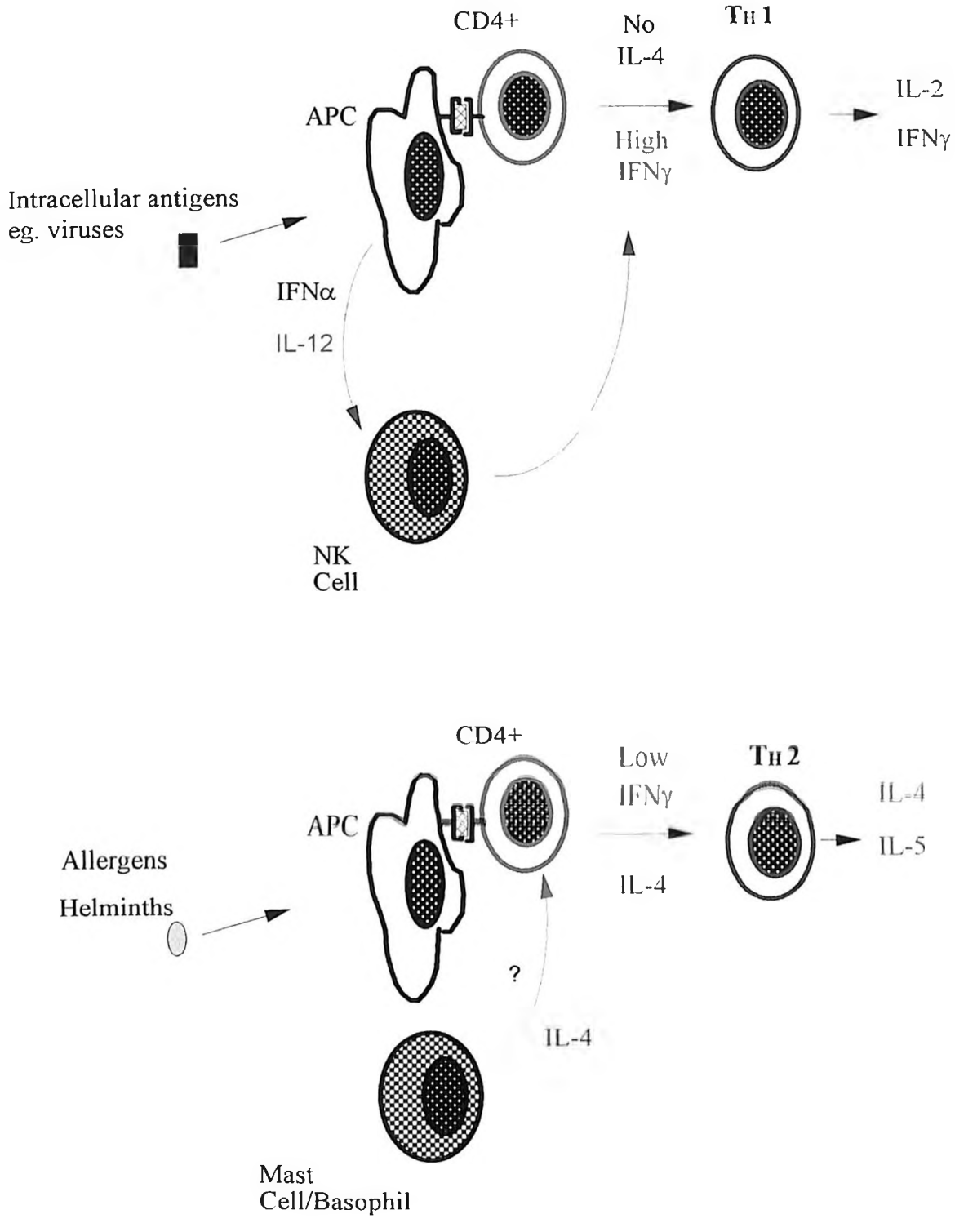

Figure 1.2. The role of cytokines in the generation of T helper (TH) subsets. IFNα and IL-12 generate TH 1 cells due to their ability to induce high levels of IFNγ. In contrast, IL-4 in the presence of low levels of IFNγ generate TH 2 cells.

However, other strains of mice which produce IFN-γ in response to *T. cruzi* have been shown to be susceptible to this parasite (Silva *et al.*, 1992). This susceptibility has been attributed to the co-production of IL-10, which is an inhibitor of IFN-γ, antagonising both its synthesis and biological activity (Silva *et al.*, 1992).

A mechanism involved in the inhibition of intracellular parasites by IFN-γ was identified to be tryptophan starvation. IFN-γ affects the metabolism of tryptophan by parasites, such as *Toxoplasma gondii* (Pfefferkorn, 1984) by inducing an enzyme of tryptophan catabolism, indoleamine 2,3-dioxygenase (Taylor and Feng, 1991). In addition, reactive nitrogen intermediates act as effector molecules of IFN-γ stimulated macrophages (Cenci *et al.*, 1993). These molecules are involved in inhibiting mycobacterial growth in IFN-γ -treated mouse macrophages (Flesch and Kaufmann, 1991). Furthermore, the production of reactive oxygen intermediates and secretion of hydrogen peroxide are correlated with the ability of macrophages to kill intracellular parasites (Murray, 1981). This stimulation of the secretion of reactive oxygen intermediates seems to be an exclusive function of IFN-γ.

1.10 Interleukin 10

First described as cytokine synthesis inhibition factor (Fiorentino *et al.*, 1989), the acid-labile human form of IL-10 (hIL-10) is produced by CD4+ T-cells of both the TH0 and TH2 subsets (Yssel *et al.*, 1992), monocytes/macrophages (de Waal *et al.*, 1991b), and B-cells (Benjamin *et al.*, 1992). Human IL-10 gene was first cloned earlier on in the decade (Vieira *et al.*, 1991) and encodes a 170 amino acid precursor protein, with a single potential N-linked glycosylation site. The mature form of hIL-10 is a protein composed of 160 amino acid residues, which has a 73% identity with mouse IL-10 (Vieira *et al.*, 1991). Recently, the cDNA for bovine IL-10 (bIL-10) has been cloned and encodes a mature protein with 178 amino acids and one potential glycosylation site (Hash *et al.*, 1994). The deduced amino acid sequence of bIL-10 shares an overall 77% and 71% identity with the human and mouse proteins respectively. Unlike the human and murine IL-10, the biological functions of bIL-10 have not to date, been studied in detail.

In models where it has been studied, a key function of IL-10 is the inhibition of the production of cytokines and proliferation of TH1 cells (Macatonia *et al.*, 1993). Studies involving murine TH1 clones and IL-10 showed that the biosynthesis of IFN-γ was consistently suppressed, whereas the synthesis of other cytokines, such as IL-2, was not always inhibitable (Fiorentino *et al.*, 1989). Furthermore, in conjunction with IL-4, IL-10 inhibited many of the functional activities of IFN-γ (Fiorentino *et al.*, 1989). In contrast, IL-10 did not inhibit cytokine expression by murine TH2 clones, including IL-4 and IL-5 leading to the concession that it is an important immunoregulatory cytokine for TH1 specific DTH reactions (Fiorentino *et al.*, 1989).

Studies with human CD4+ TH clones and IL-10, indicated subtle differences in the

biology of this cytokine compared to the mouse system. Unlike in mice, hIL-10 was produced by all CD4+ subsets (TH0, TH1, and TH2) (Yssel *et al.*, 1992a; Del Prete *et al.*, 1993) and inhibited the cytokine production and proliferation of all three subsets (de Waal *et al.*, 1991a). Consistent with the observation in the murine system, the presence of IL-10 in mitogen stimulation assays reduced the production of T-cells, which correlated with the lack of production of IL-2 (Ding and Shevach, 1992; Taga and Tosato, 1992). Exogenous IL-2 could partially restore the proliferative response (Ding and Shevach, 1992), although IL-4 was also required for effective reversing of the suppressive effect of IL-10 on human T-cell responses (Taga and Tosato, 1992).

The mechanism of action of IL-10 on the T-cell response is highly dependent on the type of antigen presenting, or accessory cell. IL-10 only downregulated the cytokine synthesis by TH cells when macrophages and not B-cells were used for the presentation of antigen, superantigen or mitogen (Fiorentino *et al.*, 1989 and 1991; Ding and Shevach, 1992). The inhibition of human, but not murine macrophage function related to the expression of major histocompatibility complex (MHC) class II on macrophages (de Waal *et al.*, 1991a; Ding and Shevach 1992; Ding *et al.*, 1993). However, the diminished accessory cell function of IL-10 treated murine macrophages, was caused by the suppression of the CD80/CD86 costimulatory molecules (Ding *et al.*, 1993). Recently Brown and coworkers (1994), presented evidence to support the concept that bIL-10 primarily affects accessory function in a manner similar to that demonstrated with human accessory cells; bIL-10 does not directly inhibit CD4+ T-cells. It therefore seems reasonable to suppose that *in vivo* activities of bovine IL-10 will resemble those of human IL-10. IL-10 also has a number of other important immunoregulatory properties *in vitro*, including the enhancement of growth and differentiation of B-cells (Defrance *et al.*, 1992), thymocyte

and peripheral T-cells (MacNeil *et al.*, 1990), mast cells (Thompson-Snipe *et al.*, 1991) and cytotoxic T-cells (Chen and Zlotnick, 1991).

1.11 Prospects of IFN-γ and IL-10 involvement in ECF

Considering the polarizing and impressive diverse range of immunoregulatory activities of IFN-γ and IL-10, their correct production and action is necessary for health and resolution from infections. Ironically, *T. parva* infects those cells of the host that regulate this immune response (Emery *et al.*, 1988).

Following infection, the lymphocytes draining the site of parasite inoculation 7 to 8 days post infection contains 20 to 30% lymphoblasts, with only 1% or less of cells in the node infected (Emery, 1981b). These lymphoblasts are made up of both CD4+ and CD8+ T-cells and they do not include parasite-specific cytotoxic T-cells, or their precursors (Morrison, unpublished data). The consequent acute lymphoproliferative response appears unregulated and involves infected and uninfected T lymphocytes. Parasitized cells grown *in vitro* also stimulate potent proliferative responses in autologous T-cells from non-immune cattle (Goddeeris and Morrison, 1987). However this response can be elicited by glutaraldehyde-fixed parasitized cells (Goddeeris and Morrison, 1987), indicating that the secretion of growth factors is not the primary stimulus for proliferation. On the other hand, constitutive expression of IL-2 receptors has been demonstrated on parasitised cells, and at low concentrations IL-2 has been shown to potentiate the growth of infected cells (Coquerelle *et al.*, 1989; Dobbelaere *et al.*, 1990). These observations suggest that primary infection with *T. parva* in cattle may induce an inappropriate cellular immune response, which, rather than controlling the infection, contributes to the pathogenesis of the disease. Although at this point in time the

production of cytokines seem to play a minor role in the lymphoproliferative response, they may potentiate the pathology and growth of parasitised cells.

Apart from the relative absence of IL-2 participation, little is known about the relationship between *T. parva* infection and the cytokines produced in response to this infection. However, certain characteristics of the disease process such as the unregulated expansion of T-cells, early involvement of B-cell growth and differentiation, and loss of lymph node architecture, might indicate that other immunoregulatory cytokines are involved. Two cytokines, IL-10 and IFN-γ, which are intimately involved in the regulation of T and B-cell functions, and whose production is often manipulated by parasites, may play a potential role in the pathology of *T. parva*. This consideration came from preliminary studies showing that IL-10 and IFN-γ are among the major cytokines produced by primary infection of leucocytes by *T. parva* (Collins, unpublished data). Ultimately access to recombinant bovine cytokine genes and their products is crucial to the dissection of these cellular interactions. Recent developments in the cloning of bIL-10 and IFN-γ should enable the development of probes for these cytokines, which will allow the production and establishment of possible role(s) of these molecules.

1.12 Aim and Objectives

The identification and localisation at the single cell level, of cytokines produced in response to *T. parva* infections has not been addressed in the bovine system. Information from such studies would help in broadening the understanding of the host response to general parasitic infection and may lead to the establishment of prophylactic and/or therapeutic regimes. In this study, IL-10 and IFN-γ, two cytokines which most often display reciprocal regulatory activities, are to be investigated to assess their possible contribution to the pathology of *T. parva* infections. The study is designed to detect the production of IL-10 and or IFN-γ transcripts at the single cell level and identify their cellular source(s). To meet this aim the technique of *in situ* hybridisation, together with immunohistochemistry will be applied to identify cells producing IL-10 and IFN-γ during *T. parva* infection.

Chapter 2

Materials and Methods

Materials and Methods

2.1 Tissue culture

2.1.1 Isolation of bovine peripheral blood mononuclear cells

Bovine blood (20ml) was collected into heparin (200 Units (U) ml^{-1}; Leo Laboratories, Risborough, UK) and peripheral blood mononuclear cells (PBM) isolated by ficoll-histopaque density gradient centrifugation. Essentially, 15ml of heparinized blood was added to a Falcon (50ml) tube (Becton Dickinson, Cowley, UK) and mixed gently with an equal volume of phosphate buffered saline (PBS; A22) and underlayed with 15ml of histopaque (Sigma chemicals Co. Ltd, Poole, Dorset, UK; density 1.083). After centrifugation at 120xg for 40min at 20°C, the buffy coat was carefully removed using a sterile pipette. Cells were washed three times with cold PBS by centrifugation at 900xg for 10min at 4°C. PBM were resuspended in tissue culture medium (TCM; A1) or PBS (A22) and their viability determined by the exclusion of 0.1% (w/v) trypan blue.

2.1.2 Culture of 3332 cells

PBM isolated from a *Bos taurus* calf, (2.1.1; animal number 3332, IAH stock), were cultured in presence of 5µg ml^{-1} of concanavalin A (ConA) at 37°C in an atmosphere of 5% carbon dioxide (CO_2), for four days. After this time, 50U ml^{-1} of recombinant (r)bIL-2 was added. After a further four days of culture, 50Units ml^{-1} rbIL-2 and 10% conditioned media (from a 36hr PBM culture stimulated with ConA) were added with fresh medium. Cells were then passaged at 3 to 4 x $10^6 ml^{-1}$ after every 3 to 4 days, by the addition of rbIL-2 (50U ml^{-1}). This formed the 3332 cell line.

2.1.3 Preparation of 3110C cell-line

From a normal *Bos taurus* (animal number 3110C from the IAH stock), PBM were isolated using the Ficoll histopaque density gradient centrifugation (2.1.1). These cells were infected with *T. annulata* sporozoites (provided by Dr. G Brown of the Center for Tropical Veterinary Medicine, Royal (Dick) School of Veterinary Studies, University of Edinburgh) using the following procedures:

2.1.3.1 Thawing of sporozoite stabilate

Sporozoites were supplied as a stabilate at -20°C in 30% (v/v) glycerol. The sporozoites were thawed at 37°C (in a water bath) and then placed at room temperature (RT°C) for approximately 30min. To the thawed sporozoite suspension an equal volume of TCM was added dropwise, with gentle mixing. The sporozoite suspension was left to stand at RT°C for 20min. Then 2ml of sporozoite diluent, comprising Eagles MEM (Life Technologies Ltd (Gibco-BRL), Paisley, UK), containing 3.5% (w/v) bovine serum albumin (BSA; Sigma), was added dropwise with gentle mixing, and allowed to stand for 20min at RT°C. At this time, a further 4ml of the diluent was added. These sporozoites were used to infect bovine PBM cultures. Generally, cultures were infected 30 to 60min after resuscitation of the frozen sporozoites stabilate.

2.1.3.2 Infecting 3110C cells

Serial dilutions of the sporozoite suspension (up to 1/36) was made to give a 100µl volume well^{-1} in a 96 U-well microtitre plate (Sterilin, Hounslow, UK). An equal volume of normal non-*T. annulata*-infected PBM was added at 2 x 10^4 cells well^{-1}. These were cultured at 37°C in 5% CO_2 and fed by the addition of an equal volume of TCM on day 1 and 2 after infection, then subsequently as indicated by culture metabolism.

2.2 Cell surface phenotyping

The phenotype(s) of the cell lines generated (2.1) were determined by looking at the surface expression of selected known leukocyte antigens on these cells. This was achieved using mAbs against these selected antigens and flow cytometry.

2.2.1 Cell staining for phenotype analysis

3110C or 3334 cells (5 to 8 x 10^6 ml^{-1}) stimulated with 10U rbIL-2 were centrifuged at 900xg for 5 min at 4°C. The supernatant was discarded, the cells washed in 10ml of PBS containing 0.1% azide, and spun at 900xg for 3min at 4°C. A panel of mAb were used to stain the cells (Appendix C, Table C1). Essentially, 50µl of mAb (1/10, TCM) was added to the appropriate well and incubated at RT°C for 20min. Cells were then washed three times in PBS/azide by centrifugation at 900xg for 5min. Cells were resuspended in 50µl of goat anti-mouse-fluorescein isothiocyanate (FITC) conjugated antibody (Sera Lab, Crawley, Down, West Sussex, UK) in TCM (A1) containing 0.1% azide at RT°C for 20min, before washing three times in PBS/azide. To permit "live gating" of data, 130µl of propidium iodide solution (2mg ml^{-1}) was added and incubated for 10min at RT°C before washing and resuspending in sheath fluid (filtered PBS). Analysis was then carried out on a Becton Dickinson fluorescent activated cell sorter (FACScan; Becton Dickinson, Mountain View, CA, USA).

2.3 Molecular biology

Many of the protocols used for the manipulation of DNA and RNA were described by Sambrook *et al.*, (1989).

2.3.1 Isolation of total cellular RNA

Total cellular RNA was isolated from both 36hr rbIL-2-stimulated 3332 cells and *T. annulata* infected cell line in log phase of growth. Initially, cell viability was assessed by the exclusion of 0.1% trypan blue. Cells were pelleted at 900xg (4°C) for 5min and lysed in Ultraspec™ RNA reagent (1ml Ultraspec for 0.05 to 1 x 10^7 cells; Biotex Laboratories Inc. USA) by repetitive gentle pipetting. The homogenate was stored at 4°C for 5min to permit complete dissociation of nucleoprotein complexes. Then 0.2ml of chloroform per ml of Ultraspec™ RNA reagent was added and vortexed vigorously for 15sec, before leaving to stand on ice for 5min. The homogenate was then centrifuged at 12000xg (4°C) for 15min. The upper aqueous phase was removed and the RNA precipitated by the addition of an equal volume isopropanol and incubation on ice for 10min, before pelleting at 12000xg (4°C). The supernatant was removed and the pellet washed in 70% ethanol and resuspended in 60µl of diethyl pyrocarbonate (DEPC) treated water. The concentration of the RNA within the sample was calculated based on the assumption that an optical density (OD; 260nm) value of 1.0 corresponded to a concentration of 40µg ml^{-1} of RNA (Sambrook *et al.*, 1989). The ratio of the 260:280 OD was used to give an indication of the quality of the RNA preparation, and a ratio of <1.7 indicated the presence of contaminants, such as protein or phenol in the RNA solution (Sambrook *et al.*, 1989; Appendix AII).

2.3.2 First strand cDNA synthesis

Total RNA (2.2.1; 1 to 10µg) was mixed with 0.5µl of oligo-$(dT)_{12-18}$ (500ng ml^{-1}; Life Technologies) in a total volume of 20µl, incubated at 65°C for 5min and allowed to slowly cool to 25°C. RNasin (0.5µl of 40U μl^{-1}; Life Technologies) and DEPC treated water (12µl) were then added to the reaction mixture. Then 4µl of 5 times first strand buffer (A28) and 2µl of 0.1M dithiothreitol (DTT), followed by 1µl of 10 mM dNTP's (dATP, dGTP, dTTP, and dCTP at pH 7). The contents were mixed gently by tapping the base of the tube. The tube was incubated at 37°C for 2min to equilibrate to this temperature. Finally, 1µl (200 U ml^{-1}) of Superscript™II RNase-H reverse transcriptase (Life Technologies) was added and incubated at 37°C for 1hr. All reaction components including the cocktail of reagents were kept on ice until used.

2.3.3 PCR amplification of cDNA

Amplification by PCR was performed using a programmable thermocycler (Genetic Research Instruments, Dunnow, Essex, U.K), using *Thermus aquaticus (Taq)* DNA polymerase, in reaction buffers supplied by the manufacturers (Gibco-BRL). The reaction components (see below) were assembled on ice in 0.5ml microfuge tubes and overlayed with mineral oil (Sigma). Each set of reactions incorporated a negative control where cDNA was replaced with Tris-ethylenediaminetetraacetic acid (T.E; A8). In addition, the housekeeping gene β-actin was selected as a positive control, as an indicator of the integrity of the isolated cellular RNA. Two further positive controls were also included, which comprised the plasmid DNA carrying the cytokine cDNA(s) of interest (IL-10 and IFN-γ). The oligonucleotide sequences for IL-10, IFN-γ and β-actin are shown in Table 2.1. Oligonucleotides were kindly synthesised by Miss Karen Mawditt of IAH, Compton.

cDNA template	2µl
Reaction buffer (x10; A30)	5µl
$MgCl_2$ (25mM; Gibco)	4µl
5mM dNTP	2µl
5' primer (10 pmol μl^{-1})	2µl
3' primer (10 pmol μl^{-1})	2µl
DEPC water	32µl
Taq DNA polymerase (5 U μl^{-1}; Gibco)	0.5µl
Total volume	50µl

The amplification was carried out using the following program: denaturation (95°C for 1min); annealing (55°C for 1min) and strand extension (72°C for 1min) repeated for 30 cycles, with a final strand extension of 10min (72°C). Samples were analysed immediately (2.3.4) or stored at -20°C.

2.3.4 Agarose gel electrophoresis of DNA

DNA samples were routinely analysed by Agarose gel electrophoresis using a mini gel system (Hybaid, Teddington, Middlesex, UK) for the rapid analysis of DNA fragments with an approximate range 0.5 to 1Kb.

Table 2.1. Oligonucleotide primers used in the PCR.

Cytokine	Primers	PCR product size	Restriction endonuclease	Digested product sizes
IL-10	ACAGCTCAGCACTGCTCTGTT CGTTGTCATGTAGGATTCTATG	518	*Bst*EII	295, 223
IFN-γ	GCAAGTAGCCCAGATGTAGC GGTGACAGGTCATTCATCAC	316	*Eco*RV	164, 152
β-actin	CCAGACAGCACTGTGTTGGC GAGAAGCTGTGCTACGTCGC	270	-	-

DNA samples (10µl) were diluted with loading buffer (A18) and examined on 1% (w/v) agarose (Life Technologies) gels prepared in tris-borate buffer (TBE; A11) containing 1µg ml^{-1} ethidium bromide (Sigma). Electrophoresis was carried out at 100 volts (V) for 30min and DNA visualised under low power (long wavelenght) UV light using a transluminator (Phillips Biomedical, London, UK). To allow for the sizing of bands obtained from the various DNA samples, a 100bp DNA ladder (100bp-2000bp; Life Technologies) was run alongside the DNA samples.

2.3.5 Recovery of DNA from agarose gels.

DNA was recovered from agarose gel using the commercial Gene clean™ II kit (Stratech Scientific Ltd, Luton, Bedfordshire, UK) according to the manufacturer's instructions. Essentially, 900µl of sodium iodide (NaI) was added to the excised gel containing DNA of interest followed by 100µl of TBE modifier. The agarose was dissolved at 45 to 55°C and the DNA was adhered to an aliquot of "glass milk" suspension (5µl) by mixing at 4°C for 5 to 10 min and recovered by centrifugation at 9000xg for 3 min. The pellet was washed three times with 300µl of "new wash" solution and the DNA finally eluted from the glassmilk by the addition of 10µl T.E (A8) and incubated at 55°C for 10 min.

2.3.6 Restriction enzyme digestion

DNA products were digested with restriction endonucleases (RE) under the following conditions:

DNA (1 to 2µg)	5µl
T.E (A8)	12.5µl
Reaction buffer (x10)	2µl
RE (8 to 12 Units μl^{-1})	0.5µl
Total volume	20µl

Reaction mixtures were incubated at 37°C for 2hr and then analysed immediately by agarose gel electrophoresis (2.3.4).

2.3.7 Extraction of nucleic acids with phenol/chloroform

DNA was routinely extracted with phenol:chloroform and then ethanol precipitated in order to purify DNA from excess or unwanted buffer salts and other impurities. The DNA sample was equilibrated with an equal volume of phenol/chloroform (1:1; Sigma) by vortexing to form an emulsion. The aqueous and organic phases were subsequently separated by centrifugation at 12000xg for 2min. The aqueous layer containing the DNA was removed, taking care not to disturb the interface, and vortexed with an equal volume of chloroform. The phases were separated as before and the aqueous phase removed and precipitated with 2.5 volumes of "RNA quality" ethanol and 10% (v/v) 2M sodium acetate solution (pH 7.5). The mixture was then centrifuged at 14000xg at 4°C in a bench-top centrifuge for 15min. The pelleted DNA was washed with 70% ethanol (centrifugation at 14000xg at 4°C for 10min), air-dried and resuspended in 100µl of DEPC treated water.

2.4 Vector subcloning

cDNA encoding bovine IFN-γ and IL-10 had previously been cloned in Blue Bac His-A (BBH-A) and Blue BacIII (BBIII) plasmids respectively. For riboprobe synthesis, each cDNA was subcloned into the plasmid, *p*GEM3Z*f*(-) which contained the RNA promoters SP6 and T7.

2.4.1 Preparation of cDNA inserts and *p*GEM3Z*f*(-) for ligation

cDNA encoding bovine IFN-γ and IL-10 were digested out of BBH-A and BBIII respectively, using *Xba*I and *Hind*III in reaction buffer M (A23; Promega) (2.3.6). In another set of reactions 10µg of the *p*GEM3Z*f*(-) was linearized in buffer M (A23; Promega) using *Xba*I and *Hind*III (2.3.6). These products were analysed on a 1% agarose gel (2.3.4, recovered using the Geneclean™ kit (2.3.5) and resuspended in tris-ethylenediaminetetraacetic acid (TE) buffer to give a final concentration of 10 to 100ng μl^{-1}.

2.4.2 Ligation reactions

Ligation of the phosphate groups at the 5' and the hydroxyl groups at the 3' ends of the vector and target DNA sequences were conducted in the following reaction cocktail:

Linearised *p*GEM3Z*f*(-) (2.5.1.2; 100ng μl^{-1})	1µl
Ligase buffer (A33; Promega)	1µl
T4 DNA ligase (1Unit μl^{-1}; Boehringer)	0.5µl
cDNA (10 to 100ng; 2.5.1.1)	7.5µl
Total volume	10µl

For self ligation reactions the cDNA was omitted. The reaction mixtures were incubated

at 16°C overnight and then used immediately to transform competent *Escherichia coli* cells (2.4.3), or stored at -20°C.

2.4.3 Preparation of competent cells

A single colony of *E.coli* (JM109 strain) was inoculated into 10 ml of LB media (A21) and shaken (225 rpm) at 37°C. An aliquot (500µl) of the overnight culture was added to 50 ml LB media and shaken at 37°C. Cells were grown until the culture had reached an OD (600nm) of 0.4 to 0.6. The culture was then incubated on ice for 15 min to inhibit further growth, before the cells were pelleted at 7000 xg at 4°C for 15 min in 50 ml Beckman centrifuge tubes. The supernatant was discarded and the pellet left to stand on ice for 10min. A 2 ml volume of calcium chloride (0.5 ml of 100mM calcium chloride per 10 ml of starting culture was added to the pellet left to stand on ice for 10min, before gently resuspending the cells. The competent cells were left on ice until used in the transformation reaction.

2.4.4 Transformation reactions

To 100µl of competent cells (2.4.3) the ligation reaction components (2.4.2) were added, incubated on ice for 30min, heat shocked at 42°C for 90sec, and placed back on ice for 2 min. LB broth (400µl; A21) was then added to the cells, which were then incubated with shaking at 37°C for 1hr, before an aliquot (50µl, 100µl or 200µl) of the transformed cells were plated onto LB agar plates containing 50µg ml^{-1} ampicillin and 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal; 50µl of 40mg ml^{-1} X-gal stock spread onto the plates). The cell suspension were allowed to soak into the agar and the plates were incubated at 37°C overnight.

2.4.5 Selection of transformants

To allow visual selection of recombinants, disruption of the plasmids *LacZ* gene (containing the MCS) caused the loss of â-galactosidase, producing a white phenotype, whereas non-recombinants produced a blue phenotype in the presence of X-gal.

Single white colonies were inoculated into 5ml of LB broth containing 50µg ml^{-1} ampicillin and grown overnight at 37°C with shaking. 1ml of resultant bacterial suspension was preserved in 1ml glycerol (30% v/v) and stored at -70°C for future use. Small scale plasmid preparations (mini-preps; 2.4.5.1) were performed on each of the remaining cell suspensions, to determine which of the colonies contained an appropriate DNA insert.

2.4.5.1 Purification of DNA from plasmid mini-preparations

Plasmid DNA (20µg) was prepared using Qiagen resin Tips (Tip 20), according to the manufactures instructions, (Qiagen Ltd, Dorking, Surrey, UK). Essentially, a single recombinant colony was used to inoculate 10ml LB broth, containing 50µg ml^{-1} ampicillin and grown at 37°C overnight with constant shaking. Cells were pelleted at 12,000xg for 15 min at 4°C and resuspended in 300µl of buffer P1 (A2). The cells were then lysed by the addition of 300µl of buffer P2 (A3) and left to stand at room temperature for 5 min. Cellular debris and chromosomal DNA was precipitated by the addition of 300µl of chilled buffer P3(A4) and centrifuged at 16000xg for 15 min in a bench top microfuge. The supernatant was passed through a Qiagen resin Tip (Tip 20), which had been equilibrated with 1ml of QBT buffer (A5). The resin was washed four times with 1ml of QC buffer (A6) and DNA was eluted with 800µl of QF buffer (A7). The DNA was precipitated with 0.7volumes of isopropanol (16,000xg for 30 min).

washed with 1ml 70% ethanol, air dried and finally resuspended in 20µl of T.E buffer.

2.4.5.2 Analysis of plasmid preparations

Putative transformed bacterial colonies were taken and their plasmids assessed for the presence of cytokine inserts using restriction enzyme digestion (2.3.6). The identity of the insert fragments was confirmed by R.E digestion using *BstE*II for IL-10 and *Eco*RV for IFN-γ in reaction buffer B (Table 2.1).

2.4.5.3 Establishing the direction of the cloned cDNA

To determine the orientation of the cloned cDNAs in *p*CR™II, restriction digests were performed. For each cytokine a different set of R.E and conditions were used to establish whether the sense strand was under the control of the SP6 or T7 RNA promoters. In this way specific sized fragments would be produced depending on the orientation of the cloned cDNA (Fig. 2.1). The IL-10/*p*CR™II cloned vector was initially digested in the reaction buffer A (A25) with *Eco*RV and then with *Acc*I in reaction buffer B (A9) as described in section 2.3.6. In a separate reaction, the *p*CR™II TA vector carrying the cDNA encoding IFN-γ, was digested (2.3.6) using *Pst*I in buffer H (A10). Finally the digested products were analysed by agarose gel electrophoresis.

2.5 Riboprobe preparation

2.5.1 Digoxigenin labelling of the RNA run-off transcripts

To dig-label RNA transcripts downstream of the SP6 and T7 promoters, the IL10 and IFN-γ plasmids (*p*CR™II) were linearised prior to *in vitro* transcription. For the IL-10 sense riboprobes, *Spe*I (reaction buffer H; A10) was used while *Eco*RV (reaction buffer B; A9) produced the antisense RNA transcript. Similarly *Not*I (reaction buffer H; A10) and *Spe*I (in buffer H) were used for production of the IFN-γ sense and antisense RNA transcripts, respectively. All restriction digests were carried out according to the basic protocol in section 2.3.6.

2.5.2 RNA labelling reaction

The Boehringer Mannheim Biochemica Dig RNA labelling kit (SP6/T7) was used, essentially as described by the manufacturer. The reaction components were assembled on ice as follows:

Control or test DNA (1µg)	2µl
RNA labelling buffer (x10, A32)	2µl
RNasin (20U μl^{-1})	1µl
NTP labelling mixture (A31)	2µl
DEPC water	11µl
T7 or SP6 RNA polymerase (20U μl^{-1})	2µl
Total volume	20µl

Reaction components were incubated at 37°C for 2hrs and then 2µl of RNase-free DNase (10U μl^{-1}) was added and incubated for a further 15min, for the removal of template DNA. The reaction was stopped by the addition of 2µl of 0.2M

ethylenediaminetetraacetic acid (EDTA) solution (pH 8.0) and the labelled RNA precipitated with 2.5µl of 4M lithium chloride (LiCl) and 75µl of prechilled 100% ethanol (-70°C for 30min). The RNA was recovered by centrifugation at 12000xg for 5min and washed in 50µl of 70% cold ethanol. The pellet was air-dried and resuspended in 100µl of DEPC treated water at 37°C for 30min. Finally, 20U (1µl) of RNase-inhibitor was added to inhibit possible contamination by RNases.

2.5.3 Estimating the yield of Dig-labelled riboprobe

To confirm the success of the labelling reaction and estimate the yield of the Dig-labelled probe, ten fold serial dilutions of the labelled control and test RNA preparations were made in DEPC treated water, in a 96U-well plate (Sterilin, Hounslow, UK; table 2.2). The concentration of the control RNA was 100µg ml^{-1} and this was diluted 1:5 to give a starting concentration of 20µg ml^{-1}.

A 100cm^2 nylon membrane (Boehringer) was marked lightly with a pencil to identify the position of each dilution. Serial dilutions of the labelled test RNA were made in DEPC water (table 2.2) and 1µl of each dilution was spotted and covalently linked onto the membrane by either baking at 120°C in an oven for 30 min, or by exposure of the membrane to 100 watts UV light for 3min. The membrane was washed in the buffer 1 (A12) followed by an incubation in buffer 3 for 30min (A13). Meanwhile, sheep anti-Dig conjugated alkaline phosphatase was prepared by diluting 1µl (750Units ml^{-1} antibody) in 10ml Dig-buffer 2 (A29). The membrane was then incubated in the diluted antibody for 30min, washed twice (15min per wash) in buffer 1, followed by an incubation in buffer 3 (A13) for 2min. Buffer 3 was then decanted from the membranes, the substrate solution added and the colour development allowed to occur in the dark

without shaking. The reaction substrate solution had been prepared by mixing 33µl of nitroblue tetrazolium (NBT; 50mg ml^{-1}) and 16.5µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP; 50mg ml^{-1}) solutions in 5ml of buffer 3. When the desired intensity of the dots had developed (typically 30 to 60min),

Table 2.2 Dilution of nucleic acid templates and riboprobes for Dig probing

Labelled or control RNA starting concentration (20µg ml^{-1})	Stepwise dilution of RNA in DEPC water	Final concentration	Total dilution
20 ng μl^{-1}	2µl/38µl	1ng μl^{-1}	1:20
1ng μl^{-1}	5µl/45µl	100pg μl^{-1}	1:200
100pg μl^{-1}	5µl/45µl	10pg μl^{-1}	1:2000
10pg μl^{-1}	5µl/45µl	1pg μl^{-1}	1:20000
1μl^{-1}	5µl/45µl	0.1pg μl^{-1}	1:200000
0.1 pg μl^{-1}	5µl/45µl	0.01pg μl^{-1}	1:2000000

the reaction was stopped by washing the membrane in 50ml of T.E (A8). An estimate of the concentration of the Dig-labelled probes was made by visually comparing the spot intensities of the control and test RNA.

2.5.4 Blotting of nylon membranes

Dot blotting was used as a quick way of qualitative and quantitaitive screening of DNA and RNA. Target containing cDNAs encoding IL-10 and IFN-γ (*p*GEM3Z*f*(-) plasmids) were serially diluted, denatured for 10 min at 95°C and immediately placed on ice.

Similarly, total cellular RNA prepared from 3110C, 3334, and Madin-Darby bovine kidney cells (MDBK), as well as *in vitro* synthesised unlabelled sense and antisense RNA for both IL-10 and IFN-γ were also serially diluted, blotted and fixed onto the nylon membranes (2.5.3).

2.5.5 Hybridisation with Dig RNA probes

The spotted nylon membranes were prehybridised in a sealed plastic bag with 20ml of hybridisation buffer (A17) per 100cm^2 of membrane, by incubation at 68°C for 1hr. The hybridisation solution was occasionally mixed, to prevent membranes from drying between the prehybridisation and hybridisation steps. The hybridisation buffer (A17) was replaced with a solution containing freshly denatured Dig-labelled RNA probe. The optimal concentration of labelled RNA in the mixture depended on the amount of DNA or RNA on the filter to be detected; generally 50 to 200ng of labelled RNA ml^{-1} of hybridisation solution was used. About 2.5ml of the hybridisation solution was required. The membranes were incubated for 6hr (usually overnight) at 68°C with gentle shaking; although blots containing higher concentrations of RNA (up to 200ng ml^{-1}) were only incubated for 2hr. Then the membranes were incubated for 30min with 100ml of buffer 2 (A13), washed twice with 50ml of sodium saline citrate (x2 SSC; A14) containing 0.1% (w/v) sodium dodecyl sulphate (SDS), followed by 2 x 15min washes at 68°C with SSC (x0.1) containing (0.1%) SDS and finally in 50ml of buffer 3 for 5min at RT°C. These membranes were then probed for the detection of RNA transcripts.

2.5.6 Non-isotopic detection of RNA

The membranes from 2.5.5 were then incubated with sheep anti-Dig-alkaline phosphatase mAb (750U ml^{-1}) in Dig-buffer 2 (A29) for 30min. Unbound antibody-conjugate was

removed by washing twice with 100ml of buffer 1 for 5min. The membranes were then equilibrated in 200ml of buffer 3 (A13) for 2min and then incubated in 10ml of freshly prepared colour substrate solution (33µl of NBT solution with 16.5µl of BCIP solution in 5ml of buffer 3), sealed in a plastic bag and placed in the dark. Although the colour precipitate started forming within a few minutes, the reaction was allowed to proceed for 1hr and stopped by washing the membrane in 50ml of T.E (A8) for 5min. Membranes were then dried at RT°C and results documented by photography.

2.6. Preparation of biological and substrate materials

2.6.1 Preparation of lymph node tissue sections

The lymph node tissues collected at post mortem were immediately placed in neutral buffered formalin. The tissues were processed after fixation for 24hr, though the fixation could be extended to up to one week. Once the tissues were paraffin embedded, they were sectioned onto coated slides.

2.6.2 Coating of the glass slides for *in situ* hybridisation

The glass slides were arranged in racks and soaked in 2% solution of Lipsol laboratory detergent (Lip Ltd, West Yorkshire, UK) for more than 1hr. These were then washed in running tap water for 10min followed by rinsing in distilled water and finally a dehydration in 100% ethanol, before drying in a warm oven (37^{0}C). Meanwhile, a 2% solution of 3-aminopropyltriethoxysilane (APTES; Sigma) was prepared in acetone in a fume cupboard and the slides soaked in this solution for 2 to 5min. The slides were then washed quickly in acetone, followed by two changes of distilled water, dried at 37°C and stored in boxes until required.

2.6.3 Alternative glass slide treatment for single cell preparations

The slides were prewashed in hot soapy water (1% Lipsol detergent; Lips), rinsed well in distilled water and air dried. The slides were subbed overnight in a solution composed of 250ml DEPC water, containing 2.5g gelatin (Sigma) and 0.25g of potassium chromate (BDH, Poole, UK). The slides were then washed in distilled water and rinsed in DEPC treated water, air dried at 37°C and stored at -20°C. Cytocentrifuge blocks were also washed with DEPC water and air-dried before use.

2.6.4 Cytospin preparations

Fresh cell suspensions were washed with 500µl of ice cold PBS (A22), containing 0.1% (w/v) BSA (Sigma) and 0.02% (w/v) sodium azide (BDH). The washed cells were resuspended at a density of 1 x 10^5 cells ml^{-1} in PBS/azide and 200µl of this cell suspension was used for subsequent cytofugation. Prior to centrifugation, the coated slides were marked with a diamond pen for their identification. Filter cards (Shandon Inc, Pittsburgh, PA, USA) were carefully aligned onto the slides with the block holders, before placing into the cytofuge block. The filter cards in the cytofuge were prewetted by addition of 200µl of PBS (prepared with DEPC treated water) and centrifuging at 25xg for 2.5min. Then 200µl of the washed cell suspension was loaded into each sample holder and centrifuged at 25xg for 2.5min. Each slide/filter card sandwich was carefully removed from the cytofuge and the cell preparations air-dried before fixing in ice-cold 60% acetone for 40sec. They were then washed in PBS followed by DEPC water and air dried thoroughly. Alternatively, fixation was performed using freshly prepared 4% (w/v) paraformaldehyde (A26).

2.7 *In situ* hybridisation

During the *in situ* hybridisation protocol, the tissue section/cytospin preparations were kept in Terasaki plates (Nunc A/S, Kamstrupvej, Rosklide, Denmark) containing T.E buffer (A8), to prevent the sections from drying out. Prior to unmasking, routinely fixed (with normal formalin buffered saline) and paraffin embedded sections were dewaxed by incubation of sections in xylene (5min) followed by two minute washes each in 100% ethanol, 95% ethanol, 75% ethanol and finally in distilled water.

2.7.1 Unmasking of nucleic acids

Dewaxed tissue sections or fixed cytospins were incubated with proteinase K (100µg ml^{-1}; Gibco BRL) prepared in buffer 1 (A12) at 37°C for 15min. Slides were then washed in DEPC treated water, soaked in 100% ethanol and allowed to air dry.

2.7.2 Denaturation and hybridisation protocol

The Dig-labelled RNA probes were taken from -20°C and thawed at 65°C for 5min. The following probe hybridisation cocktail mixtures were prepared per cytospin/tissue section:

Dig-labelled riboprobe (0.25µg μl^{-1})	0.25µl
Hybridisation mixture (A27)	35µl
Sperm DNA (10mg ml^{-1}; freshly denatured)	2µl
TE Buffer (A8)	12.75µl

Appropriate sense (negative control) and antisense labelled riboprobes were used in the hybridisation cocktail. This probe hybridisation cocktail was applied to the unmasked cytospin and tissue section preparations. The microscopic preparations were then

carefully covered with a cover slip taking care not to introduce air bubbles. The slides were placed back inside the Terasaki plates moistened with SSC (x2; A14) and denatured at 80°C in an oven for 15min. The Teresaki plates were then placed at the hybridising temperature of 55°C for 2hr.

2.7.3 Post-hybridisation washing and blocking of endogenous enzyme activity

The cover slips were carefully removed by gently washing with SSC (x4; A14) and sections/cytospins were then washed several times in SSC (x4). Each washing step lasted for 5min. Slides were then washed twice in SSC (x1) for 5min and then in SSC (x0.1) containing 3% (w/v) BSA/0.05% (v/v) Triton X-100 (Sigma) at 55°C for 15min. The cytospins/tissue sections were then incubated in humidified boxes with 3% (w/v) BSA/0.05% (v/v) Triton X-100 (Sigma) in buffer 1 (A12) for 30min at RT°C. The preparations were then used to detect the hybridised probe (Section 2.7.4).

2.7.4 Detection of hybridised probe

After incubation and removal of the blocking solution, sheep anti-Dig-conjugated alkaline phosphatase (diluted 1:5000) in 3% (w/v) BSA solution was added and the preparations left in humidified boxes for 30min at RT°C. The slides were then washed twice in buffer 1 (A12) for 2 x 5min, equilibrated in buffer 3 (A13) for 2 to 3min and incubated with 200µl of substrate; prepared by mixing 16.5µl of BCIP solution (50mg ml^{-1}) and 33µl of NBT solution (50mg ml^{-1}) in 5ml of buffer 3. The slides were left to develop in humidified boxes in darkness for at least 4hr, after which the reaction was stopped by washing the slides in distilled water. The slides were then counterstained in 1% methyl green, air-dried and mounted in safeclear (Bradley Chemicals Ltd, Standish UK). Microscopic examination and photography was performed on the slides to

document the results.

2.8 Immunoperoxidase staining of tissue sections

Tissues taken from calves at postmortem were snap frozen in liquid nitrogen. Cryostat sections of 5µm thickness were cut, the slides dried and stored at -20°C in the presence of silica gel. Before staining, the slides were warmed to RT°C and fixed in 80% ethyl alcohol containing 0.05% hydrogen peroxide (H_2O_2) for 10min. The sections were stained using a peroxidase mouse IgG Vectastain™ kit (Vector Laboratories, Peterbourgh, Cambrigdeshire, UK). Essentially, test supernatants or mAb tissue culture fluid (1:10 in PBS containing 0.1% azide) were added to the sections and incubated at RT°C for 1hr. The slides were washed in PBS and the sections incubated for 1hr with biotin-conjugated anti-mouse antibody diluted in PBS (0.1% azide). After washing in PBS the sections were incubated for 1hr with Streptavidin-conjugated horse radish peroxidase (HRP), diluted in PBS (0.1% azide) and then washed in PBS. The peroxidase staining was determined by incubating the sections with a diaminobenzidine substrate (Sigma) for 10min, then washed, counterstained in haematoxylin (2.10) and dehydrated through graded alcohols and xylene, before mounting in Safeclear.

2.9 Gordon and Sweet's silver impregnation method for reticulin

Sections were dewaxed in xylene for 5min and washed in 100% ethanol then 95%, 75% through to distilled water for 2min each, followed by incubation in acidified potassium permanganate for 5min, and a rinse in tap water. This was succeeded by bleaching in 1% oxalic acid (5min) and three washes in distilled water. The sections were treated in an iron alum mordant (Sigma; 15min) followed by three washes in distilled water, before treatment with 10% silver nitrate/ammonia solution for 45sec, until the sections became transparent, a step preceding several washes in distilled water. Tissues were reduced in 10% formalin followed by washes in tap water and distilled water. This was followed by toning in a 0.2% gold chloride solution (10 to 15min), followed by a rinse in distilled water. Sections were then fixed in 5% sodium thiosulphate (5min), washed in distilled water and counterstained in neutral red (BDH) for 1min. Sections were quickly dehydrated and mounted in Safeclear.

2.10 Haematoxylin and eosin staining.

Sections were dewaxed in xylene for 5min and washed in 100% ethanol, followed by 2min washes in 95%, and 75% ethanol through to distilled water. Sections were then stained in haematoxylin reagent (Shandon) for 6min, washed and differentiated in running tap water for 3min (until sections became blue) followed by washing in acid-alcohol (0.5% HCl and 99.5% of 70% ethanol) for 5 to 10sec, then by subsequent washes in running tap water (approx. 2min) until they turned blue. A 1% borax solution (BDH) was applied for 1min followed by a wash in tap water. Sections were then counterstained in eosin (Shandon) for 1min. Excess stain was washed off in running tap water followed by a dehydration in xylene and mounting in safe clear.

Chapter 3

Results

Results

3.1 Identification of cell lines synthesising IL-10 and IFN-γ transcripts

To aid the establishment of procedures that would lead to the identification of IL-10 and IFN-γ RNA transcripts at the single cell level, a small panel of leukocyte cell lines (3) were examined to identify whether their total cellular RNA encoded these cytokines. Two of these cell lines, 3110C and 2354, were transformed with *T. annulata* and *T. parva* respectively, while the remaining cell line 3332, was maintained on rbIL-2. The amount of total cellular RNA isolated from one million cells of each cell line was typically 10 to 12μg (Table 3.1).

Table 3.1 Comparison of amounts of total RNA isolated from three cell lines

Cell line	Total RNA per 10^6 cells (μg ml^{-1})
3110C (*T. annulata)*	11
2354 (*T.parva)*	12
3332 (rbIL-2 maintained)	10

Using oligonucleotides specific for β-actin (a ubiquitous eukaryotic house keeping gene), a single 270bp band was obtained with all the three cell lines using the RT-PCR, indicating that the isolation of cellular RNA and the subsequent first strand synthesis reaction was performed successfully (Fig 3.1; lanes 1, 4, and 7). However, differences were observed in the amplification of IL-10 and IFN-γ transcripts between the cell lines.

For the *T. parva* (line 2354) and *T. annulata* (line 3110C) transformed cell lines, PCR products of 300bp and 500bp were detected, which corresponded to the expected sizes for IFN-γ and IL-10 respectively (Fig 3.1). Conversely, only products of the correct size for IFN-γ were detected for the 3332 cell line, while products for IL-10 were never detected using RT-PCR. An interesting observation was made concerning the time-course expression of IFN-γ transcripts in the *T. annulata* cell line. In the earlier passage numbers (up to passage No.10), these cells consistently showed IFN-γ in amplified RT-PCR. However, with higher passage numbers, this reduced progressively till at passage No.25 it was virtually impossible to detect it using RT-PCR.

3.2 Phenotypic analysis of cell lines

The phenotype(s) of the cloned *Theileria*-infected cell line (3110C) and a rbIL-2 maintained cell line (3332) were determined using an indirect immunofluorescent technique. A panel of primary antibodies which recognise various bovine leukocyte surface antigens, including those that differentiate T and B lymphocytes, as well as monocyte/macrophage were used to stain the cells (appendix AIII).

Table 3.2 Phenotypic analysis of 3110C and 3332 cell lines.

Specificity	% Positive cells	
	3110C	3332
control	gated	gated
isotype control	2	0
CD45	6	94
CD2	8	98
CD3	1	99
CD4	2	1
CD8	1	22
CD21	8	0
WC1	1	51
Mo/Mϕ	23	35
MHC I	54	99

Table 3.2 and Figs 3.2a and 3.2b show the typical phenotypic data for 3110C and 3332 cell lines and this data is representative of the population. Surprisingly, 3110C, the cloned *T. annulata*-infected cell line, was shown to be a phenotypically heterogeneous population. This population contained cells that expressed a variety of surface antigens and indicated that subsets were present. The level of expression of all surface antigens tested was low as determined by the lack of intensity of the staining. Characteristically some of these *Theileria*-infected cells expressed the monocytes/macrophage marker recognised by IL-A21. However a proportion of the population also expressed other surface determinants of B cells and T cells, such as CD21 and CD2. In contrast, 3332 cells displayed a phenotype characteristic to T cells as indicated by their expression of CD2, CD3, and CD45 antigens. However, only T cells of the CD8+ subset (about 25% of the total cell population) were found, CD4+ T lymphocytes were absent. This cell population also contained cells which express the WC1 antigen expressed on a subset of gamma/delta T cell receptor positive (γ/δTcR+) T cells. The 3332 cell line had not been cloned and in addition to T cells, contained 35% of total cells expressing the antigen recognised by IL-A21 mAb, most commonly seen on monocytes and macrophages. The majority of the 3332 cells expressed MHC I molecules. The surface phenotype of cells producing IL-10 and or IFN-γ remained obscure. The 2354 (*T. parva*) cell line had previously been characterised (Fiona Houston) and shown to be composed of T cells mainly.

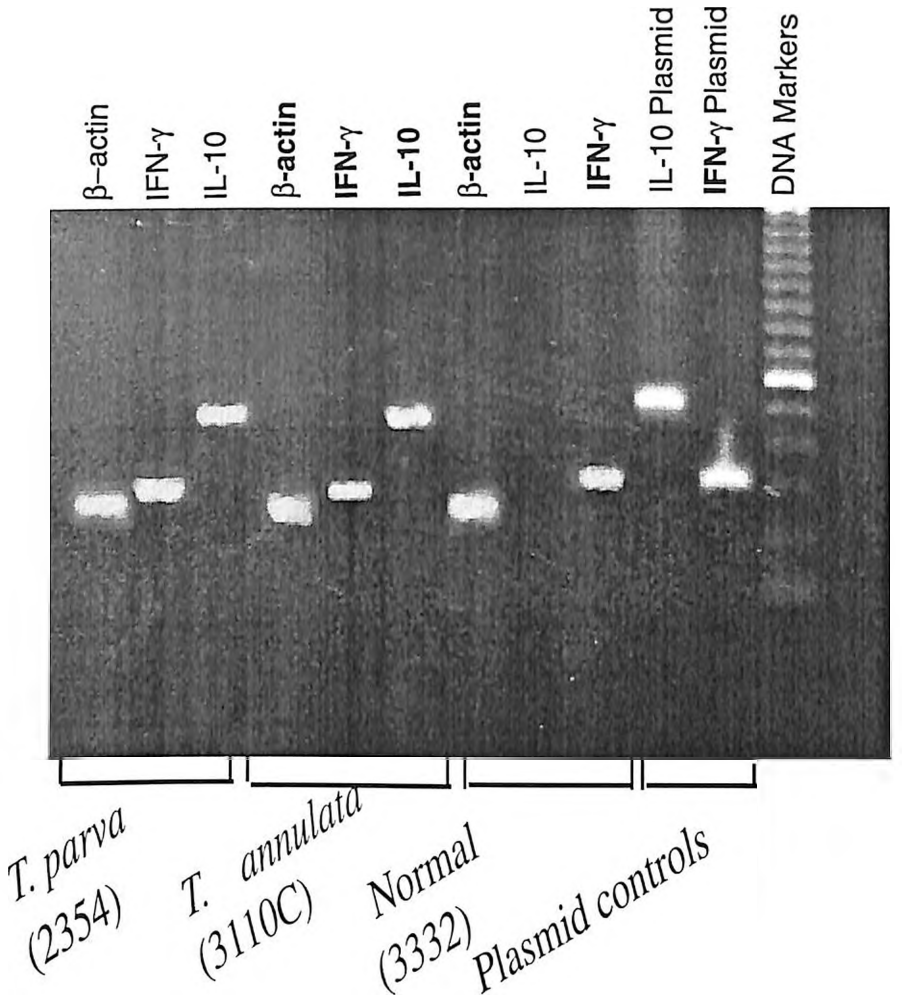

Figure 3.1
Detection of IL10 and IFNγ in T. parva (2354), T. annulata (3110C) and normal cells.

cDNA obtained from cells provided the templates for amplification by PCR, using primers specific for bIL10 and bIFNγ and β-actin. β-actin was used to show the integrity of the mRNA from the various cell lines. The PCR products were loaded onto a 1% agarose gel and electrophoresed to determine their relative sizes by comparison to a marker DNA (lane 12). The marker band sizes differed by 100bp and ranged from 100 to 2000bp with the 600bp band designed to show more intensely than the other bands to ease comparison of band sizes.

3.3 Subcloning of IL-10 and IFN-γ cDNA for production of riboprobes

The cDNAs encoding bIFN-γ and bIL-10 had been cloned into the baculovirus transfer vectors BBH-A and BBIII respectively, and were provided by Dr. R.A Collins. These vectors lacked RNA promoter sequences required for *in vitro* synthesis of RNA. For the production of riboprobes, these cytokine cDNAs were subcloned into the stable non-expressing *p*GEM3Z*f*(-) which contains T7 and SP6 RNA transcriptional promoters that flank the MCS (Appendix AIVa).

Each cytokine cDNA was cut from their respective transfer vectors, using the *Xba*I and *Hind*III R.E. This digestion yielded a heavy, linearised plasmid band of greater than 3000bp for both vectors, but for IFN-γ a further band of approximately 200bp was also produced, and for IL-10 an additional band of 250bp was generated. The excised 250 and 200bp bands for IL-10 and IFN-γ respectively, (Fig3.3) were then ligated into linearised *p*GEM3Z*f(-)* and used to transform JM109 *E. coli* cells and selected with X-gal.

A large number of white clones were obtained (25 and 43 respectively) when the IFN-γ and IL-10 ligation reactions were transformed into competent JM109 and grown in the presence of ampicillin and X-gal (Table 3.3). These white colonies, most likely represented recombinant transformants encoding IFN-γ and IL-10, as the cut vector control produced no white colonies and only one blue putatively non-recombinant colony (Table3.3).

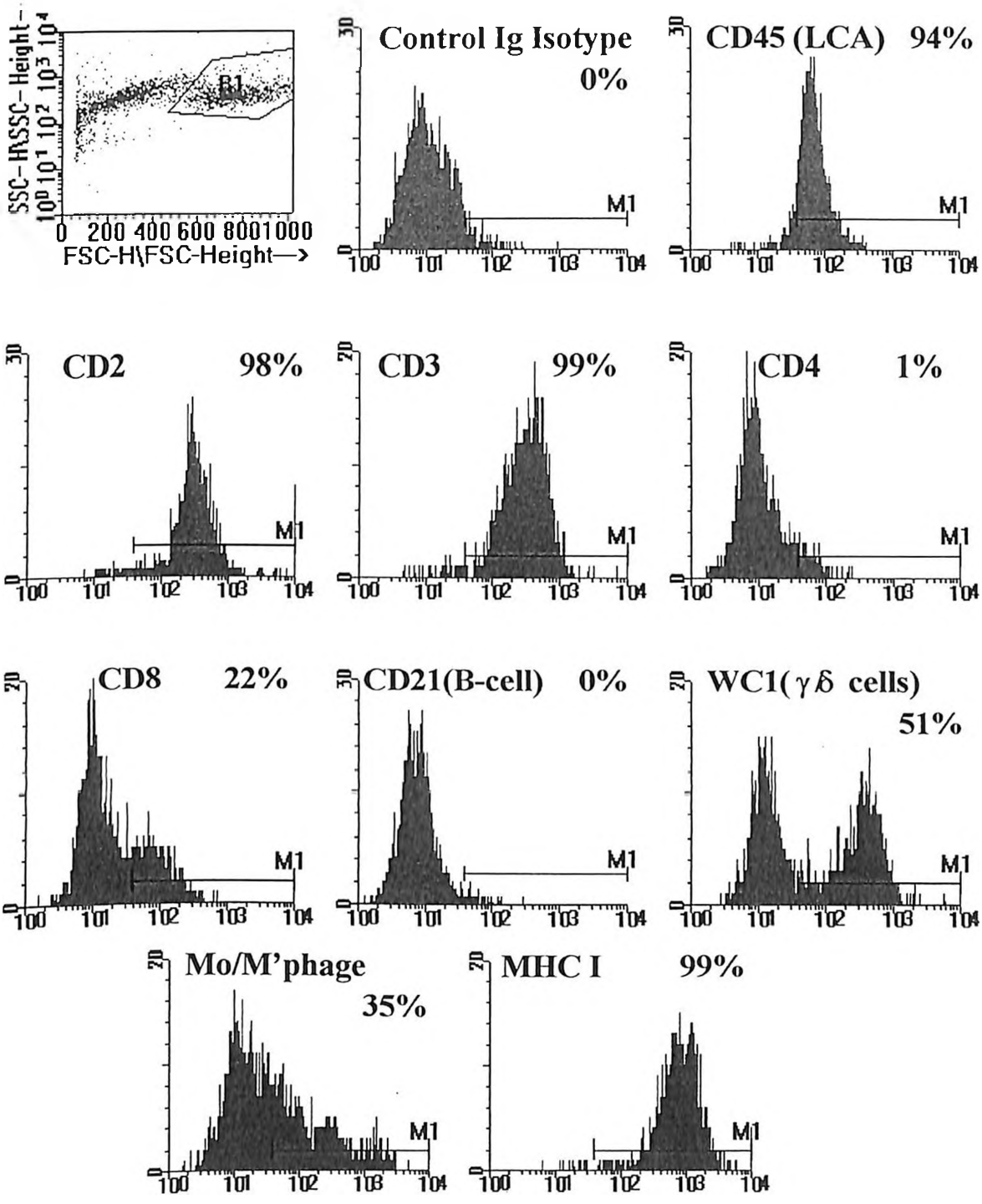

Fig. 3.2a Surface Phenotype(s) of 3332 Cell Line.

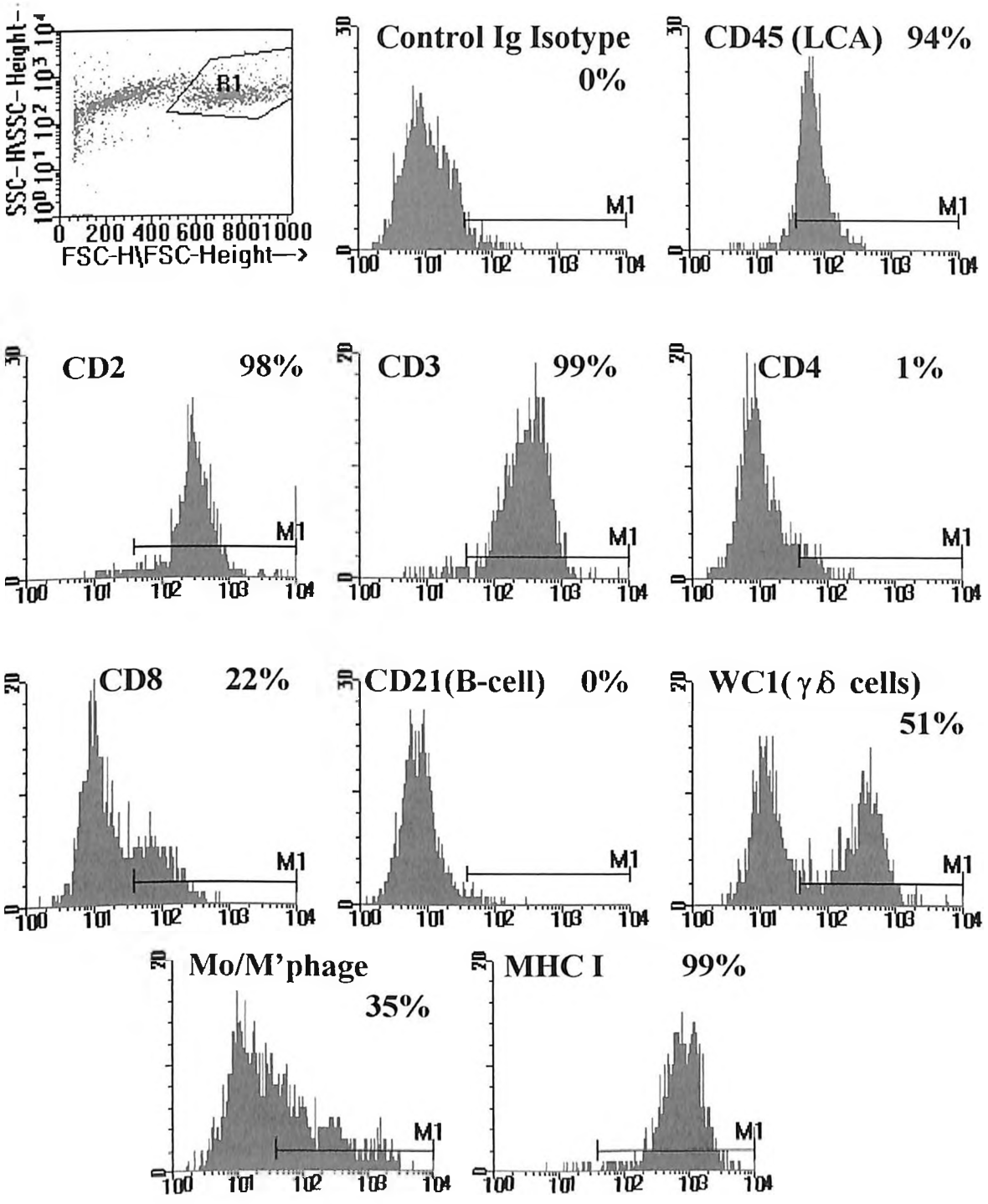

Fig. 3.2a Surface Phenotype(s) of 3332 Cell Line.

Table 3.3 Summary of the transformation

Ligation	Ligation conditions			No. of transformants	
	IL-10	IFN-γ	Ligase	Blue	White
Linearised *p*GEM3Z*f*(-)	-	-	+	1	0
Linearised *p*GEM3Z*f*(-)	-	+	+	1	25
Linearised *p*GEM3Z*f*(-)	+	-	+	2	43
Linearised *p*GEM3Z*f*(-)	-	-	-	0	0

3.4 Identification of bacterial colonies containing IL-10 and IFN-γ recombinant plasmids

To establish whether the IL-10 and IFN-γ cDNA molecules had been successfully subcloned into the *p*GEM3Z*f* plasmid, five white bacterial colonies for each of IL-10 and IFN-γ transformants were selected for further characterisation. Restriction digestion of purified DNA from small scale plasmid preparations, showed that four of the five selected white bacterial colonies for each of the IL-10 and IFN-γ transformants yielded productive plasmid DNA and contained inserts when digested with *Hind*III and *Xba*I (Fig 3.4a and 3.4b). A fragment of about 800bp was obtained with each of the IL-10 *p*GEM3Z*f(-)* transformed cells and compared well with the expected size of ~800bp.

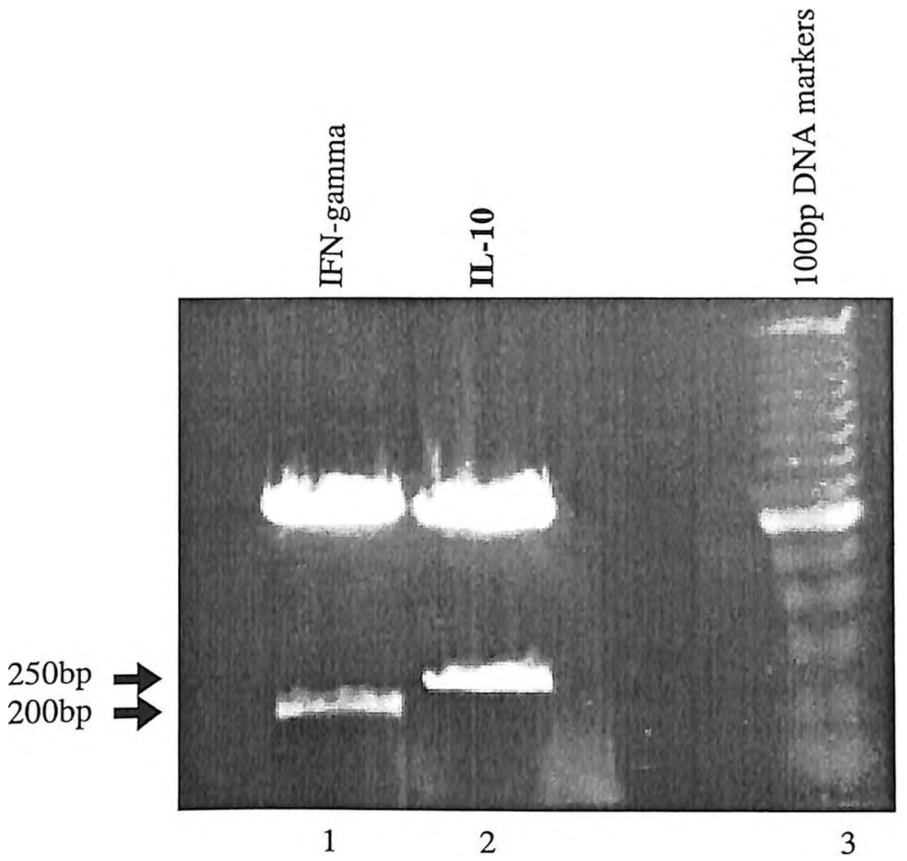

Figure 3.3
Isolation of IL10 and IFNγ gene products from the BBHA and BBIII plasmid. IL10 (lane 1) and IFNγ (lane 2) encoding cDNA were excised from plasmids using XbaI and HindIII and visualised by UV light. Lane 3: DNA markers.

Of this a contribution of ~600bp was from the IL-10 sequence and ~200bp from the host plasmid MCS. Similarly, with IFN-γ the RE digested plasmids produced a product of 700bp, from each of the four colonies which compared well to the expected size of ~700bp for the plasmid and inserted DNA.

3.5 The orientation of IL-10 and IFN-γ sequences in the pCR™II vector Although IL-10 and IFN-γ cDNAs had been successfully subcloned into *p*GEM3Z*f(-)* vectors with T7 and SP6 RNA promoters, a TA vector *p*CR™II, with the same T7 and SP6 promoters and encoding the full length IFN-γ and IL-10 cDNAs, was used for riboprobe synthesis (Appendix AIVb; provided by Dr Bob Collins). The *p*GEM3Z*f*(-) cloned cDNAs lacked the full length sequences; yet the length of the probe is a contributory factor for the enhancement of specificity of the generated riboprobes. Since the target cytokine cDNA sequences had not been directionally cloned into the *p*CR™II vector MCS, their orientation relative to the T7 and SP6 phage RNA promoters was unknown.

To establish the direction (orientation) of the IL-10 cDNA relative to the RNA promoters flanking the MCS, two restriction endonucleases *Acc*I and *Eco*RV were used in a restriction mapping experiment. *Acc*I was selected, because it cut the IL-10 cDNA sequence at a single site at position 469, but failed to cut the host vector (*p*CR™II), while *Eco*RV only digested the vector MCS (with a single cut adjacent to the SP6 RNA promoter) (Fig 3.5a). This double digestion step produced two major products, a fragment of ~549bp represented mostly by the insert and a large fragment, more intense on the gel, of mostly plasmid DNA with greater than 3000bp. Using information from a GCG "Mapsort" package, and in conjunction with this restriction mapping data, it was

verified that the coding strand of IL-10 cDNA sequence had been cloned into the *p*CR™II vector (5' end) adjacent to and therefore under the SP6 promoter (Fig 3.5a). Sense riboprobe synthesis would therefore be under SP6 promoter. In a similar set of experiments the cloned IFN-γ cDNA was digested with a single R.E, *Pst*I. This enzyme cleaved both the vector, at position 335 and 1502, and also the IFN-γ cDNA at position 473 to produce three fragments, ~400bp, ~1900bp and greater than 2000bp (Fig 3.5b). This information indicated that the antisense strand would be under the control of the T7 promoter. Conversely the synthesis of the IFN-γ sense riboprobe transcript would be under the control of the SP6 promoter.

3.6 Templates for riboprobe synthesis

To achieve appropriate lengths of the riboprobes (sense and antisense) for use in the *in situ* hybridisation procedures, plasmids containing the cytokine sequences were linearised with R.E.

For the production of IL-10 and IFN-γ sense templates, *Eco*RV and *Not*I respectively, were used to cut the host vectors downstream the SP6 RNA promoter sites. This resulted in the synthesis of ~500bp sense riboprobes from the SP6 RNA promoter for both IL-10 and IFN-γ (Fig 3.6). Conversely, antisense riboprobes for both cytokines were produced using *Spe*I cleavage and directing the synthesis under the T7 promoter producing ~500bp antisense riboprobes.

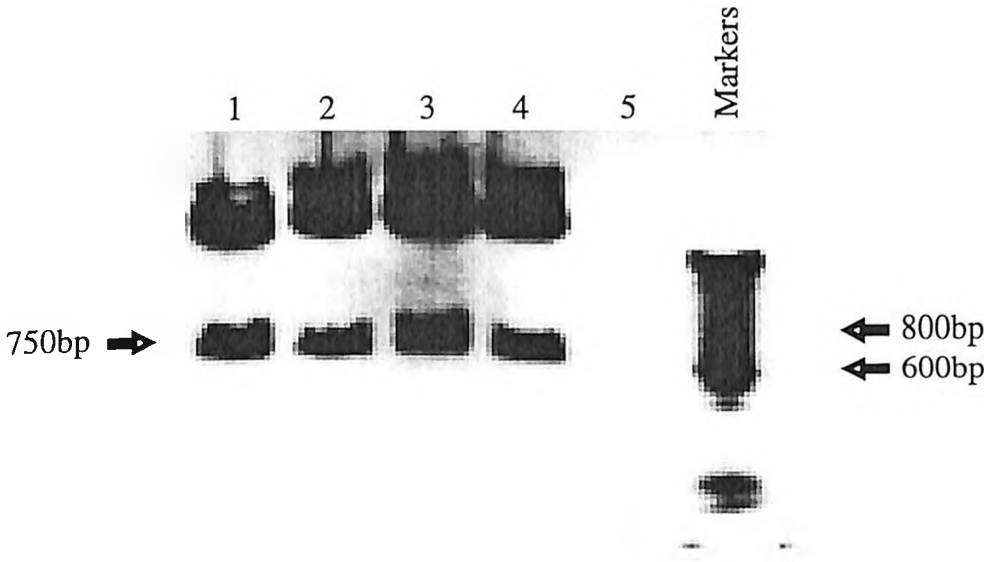

Figure 3.4a

pGEM3Zf(-) cloned IFNγ gene cut from the transformed bacterial colonies using *Hind*IIII and *Xba*I RE. Colony designated as No.5 did not yield a productive recombinant plasmid.

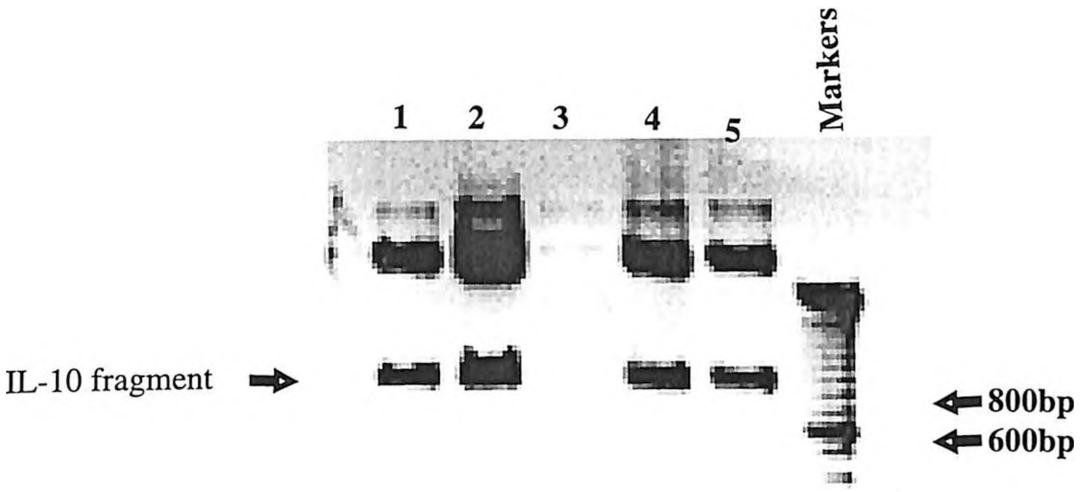

Figure 3.4b
Comparison of digested products of IL10 from the pGEM3Zf() plasmid used to transform E.coli cells. Lanes 1,2,4 and 5 show the presence of plasmid and IL10 DNA, while lane 3 fails to reveal the presence of plasmid DNA or IL10 DNA. The IL10 fragment was released by restriction digestion using HindIII and XbaI. Comparison of the IL10 insert with the markers (lane 6) showed these to represent ~500bp.

3.7 Comparison of yields of IL-10 sense and IFN-γ antisense riboprobes

The IL-10 sense and IFN-γ antisense riboprobes yielded products whose intensities on the gel were similar to those obtained from the SP6 and T7 control templates. However, the IL-10 antisense and sense IFN-γ templates failed to produce bands that were visible on the agarose gel (Fig 3.6). This indicated that these two reaction lacked efficiency or had failed in the production of their probes. However, subsequent spectrophotometric analysis indicated presence of riboprobes in the reaction mixture as shown in Table 3.4.

Table 3.4 Spectrophotometric analysis of riboprobe yields

Template DNA	concentration (μg ml^{-1})	
	antisense riboprobe	sense riboprobe
control	5.76	not measured
IL-10	5.70	7.30
IFN-γ	7.64	5.90

Spectrophotometric comparison of the yields showed that IL-10 sense and IFN-γ antisense reactions produced similar amounts (7.3 to 7.6 μg ml^{-1}) and yielded about 2μg ml^{-1} greater than that seen in the samples from the IL-10 (antisense) and IFN-γ (sense) reactions (Table 3.4). As these methods (3.6 and 3.7) of determining the concentrations of riboprobes appeared to generate variable results, a more sensitive procedure was applied to obtain the accurate yield of Dig-labelled riboprobes. This was done using a nylon membrane dot-blot analysis. Unlike the results from electrophoresis/photography, Dig-labelled products were seen in all labelling reactions (Fig 3.7). Comparing the "end point titres" of the labelled control RNA with probes from the synthesis reactions, all reactions gave same end point as the control except, probe from IFN-γ antisense reaction. Thus the *in vitro* generated probe concentrations were, generally, found to be the same

as the control (Fig 3.7). In this analysis the signal produced by the IFN-γ antisense probe (Fig 3.7 lane 6) was 10 fold less than all other probes. This dot-blot analysis showed that most of the probe preparations contained similar amounts of riboprobe and were considered to be 10μg ml^{-1}, same as the concentration of the control probe. From these considerations, the IFN-γ antisense concentration was therefore considered to be 1μgml^{-1}. Blots derived from the unlabelled RNA (negative control experiment) failed to generate a signal at all dilutions tested as was expected (lane 8).

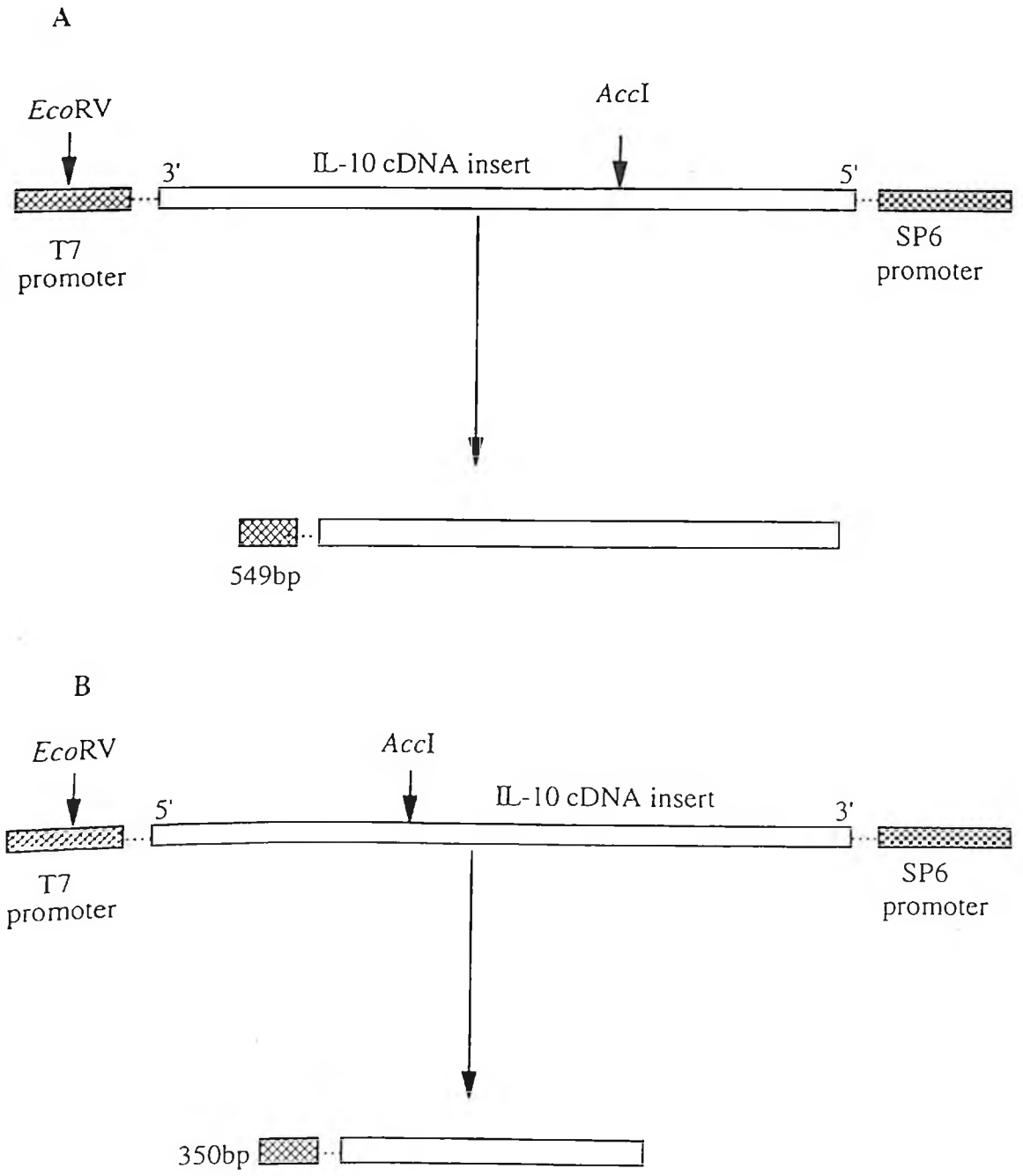

Figure 3.5a Procedure for the determining the orientation of IL-10 inserts.

The restriction enzymes *Acc*I and *Eco*RV were used to cut the cDNA insert encoding IL-10 from the plasmid. 549bp product implied that the sense strand would be under the control of the SP6 promoter and that the antisense would be under the control of the T7 promoter. Conversely, if a 350bp product was produced following the digestion of the clone, the antisense strand would be under the control of the SP6 promoter.

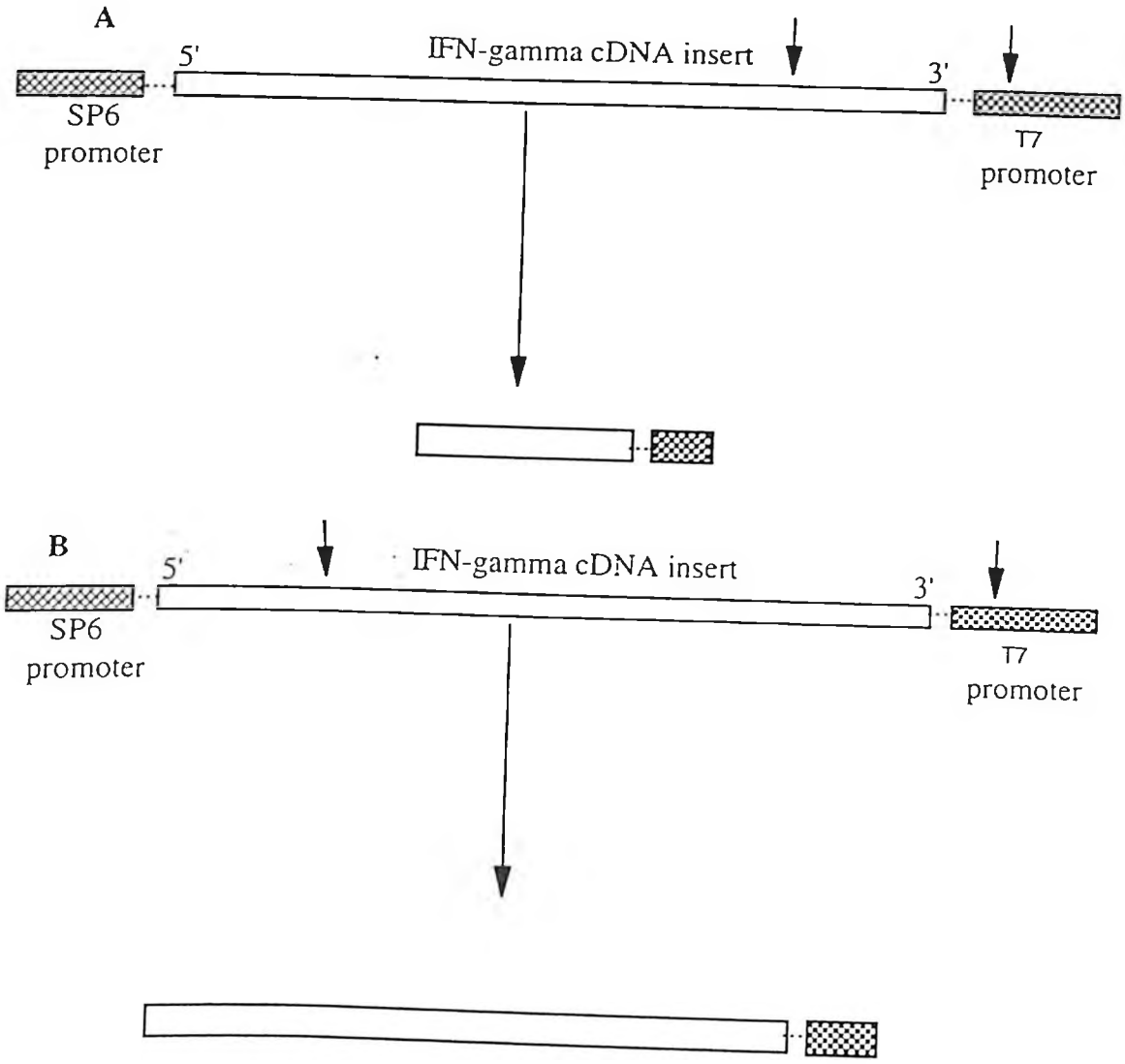

Figure 3.5b. Procedure for determining the orientation of IFN-γ inserts

Restriction of IFN-γ cDNA with *PstI* yielded three fragments. The two larger fragments would be of approximately the same size irrespective of insert orientation. However, the third and smaller fragment size depended on the orientation of the insert in the plasmid. Generaton a fragment of 500bp indicated that the insert was cloned with the sense probe under the SP6 promoter and the antisense probe under the T7 promter.

3.8 RNA dot blots

The specificities of the IFN-γ and IL-10 riboprobes was investigated by probing the *in vitro* generated IL-10 and IFN-γ sense and antisense unlabelled RNA (from *p*CR™II vector), as well as control RNA, RNA from MDBK, 3332, and 3110C cell lines, with antisense labelled probes. This RNA dot blotting was performed, also as a means of rapidly screening the RNA from various sources to determine the presence of target nucleic acid sequences. Labelled antisense IL-10 riboprobe failed to detect complementary sequences in all cell lines except in blots containing the unlabelled sense RNA derived from IL-10 plasmid. Similarly, antisense IFN-γ riboprobe hybridised with only plasmid-derived unlabelled sense RNA and no other sources (Fig 3.8). The results indicated that antisense riboprobes for both IL-10 and IFN-γ were specific to the target sequences. For IL-10, the riboprobe was able to detect complementary sequences to a dilution of one in ten thousands, corresponding to a concentration of 1pg of complementary nucleic acid sequence. For IFN-γ complementary sequence was detected to a dilution of one in a thousand. The results showed that the synthesis of antisense IL-10 riboprobes *in vitro,* was ten times more productive than that for IFN-γ confirming dot blot results of section 3.8. The specificity of IFN-γ riboprobe was not affected despite its low IFN-γ concentration. The failure to detect complementary RNA sequences to the Dig-labelled riboprobes from the various cell lines indicates that either the levels of these cytokine transcripts were too low to be detected by the dot-blot analysis, or that they had been lost upon isolation.

3.9.1 Immunoperoxidase staining of normal (non-infected) LN tissues

Sections of normal lymph node tissue (LN) stained histochemically showed characteristic morphological structures, including T cell and B cell-rich areas (Plate 3.1a and 3.9b).

The underpinning structural framework of the LN as provided by a network of reticulin was clearly distinguishable (Plate 3.2a and 3.10b); few of the reticulin fibres were observed in the germinal centres. Reticulin secreting cells contrasted the more spherical leukocytes in the node and were identified by their elongated shape (Plate 3.2a). The superficial cortex around the follicular areas and adjacent to the subcapsular sinuses contained many more coarse bundles of reticulin, than did the paracortex (Plate 3.2c). Reticulin was seen surrounding blood capillaries and perivascular connective tissue to form a network of fibres (Plate 3.2d). Bundles of reticulin fibres traversing the lumena of the sinuses were also visible. The reticulum cells and reticulin within areas of solid lymphoid tissue were attached to the outer aspect of the sinus walls, hence providing continuity of the network throughout the entire node. The display of the matrix formation was less extensive in the sinuses (both cortical and medullary). The subcapsular sinuses were clearly demarcated and were shown to be mainly devoid of leukocytes. The reticular tissue conferred the node with a conspicuous organised-framework appearance, holding the various cells and node components in their place.

The follicular areas were localized in the superficial cortex and in the deeper cortex adjacent to the sinuses, which extend inwards around the connective tissue trabeculae (Plate 3.3). B-cells were also found diffusely dispersed in the medullary cords and in the cortico-medullary transition areas. A proportion of the follicles were putatively primary follicles (due to their small size) and stained minimally with mAb CC21, whereas others comprised germinal centres surrounded by a mantle of positively staining CC21 cells.

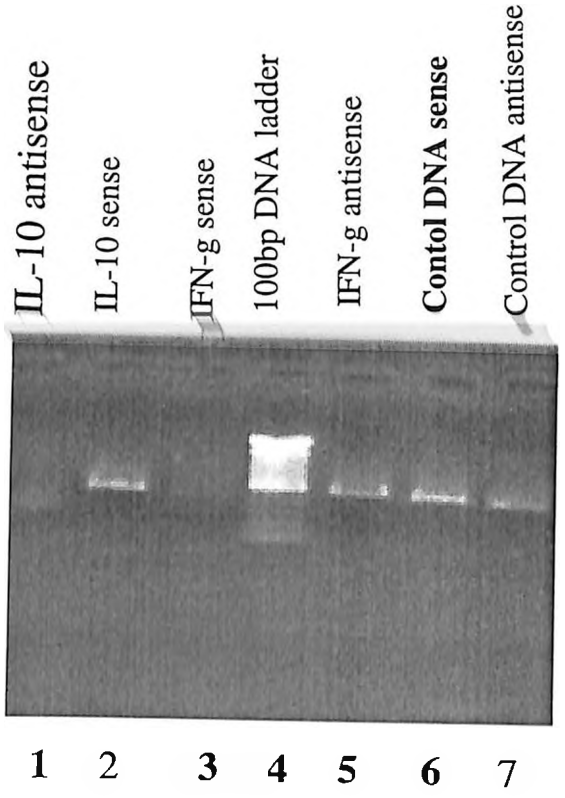

Figure 3.6
In vitro synthesis of riboprobes from bIL10 and bIFN-γ plasmid templates (sense and antisense respectively). Reaction setups designed to generate IFN-γ sense (lane 3) and IL10 antisense (lane 1) riboprobes failed to yield the expected bands.

The active germinal centres were demarcated into two distinct zones, an inner pole distal from the adjacent sinus with many positively staining cells and an outer cap region with fewer positive cells for CC21.

Paracortical areas were shown to comprise mostly CD3+ T cells (Plate 3.4) and a few B cells (CC21), as well as a distinct populations of monocytes/macrophages. T cells were also seen in all zones of the node, but with lower frequencies in the germinal centres and cortical nodules. Both cortical and medullary sinuses were shown to be heavily populated by T cells (Plate 3.1b).

3.9.2 Effect of *T. parva* infection on lymph node tissues

Comparing lymph node tissues from *T. parva* infected calves with those from uninfected calves, a marked reduction in the amount of reticular fibres was observed (Plate 3.5). The reduction in this supporting meshwork gave the tissue an appearance indicative of lymphoid disintegration (Plate 3.5). In comparison to normal LN germinal centres, the immediate surrounding areas mostly lacked reticular fibres. At the periphery of the LN, there was an almost total lack of reticulin fibres associated with the capsule. The overall reduction in reticular fibres in the node indicated a pronounced destruction of the node architectural framework, a consistent feature in *T. parva* infections. Using a *T. parva* schizont-specific mAb, schizont infected cells were seen to be dispersed throughout the lymph node tissue, but were more prevalent in the cortex regions (Plate 3.6). Seemingly, less than 10% of the LN cells were infected and parasitised cells included small, medium and large cells indicating that probably all cell types are infected (Baldwin *et al.*;1988). These parasitised cells displayed intense staining in an area close to the cell membrane periphery (Plate 3.6).

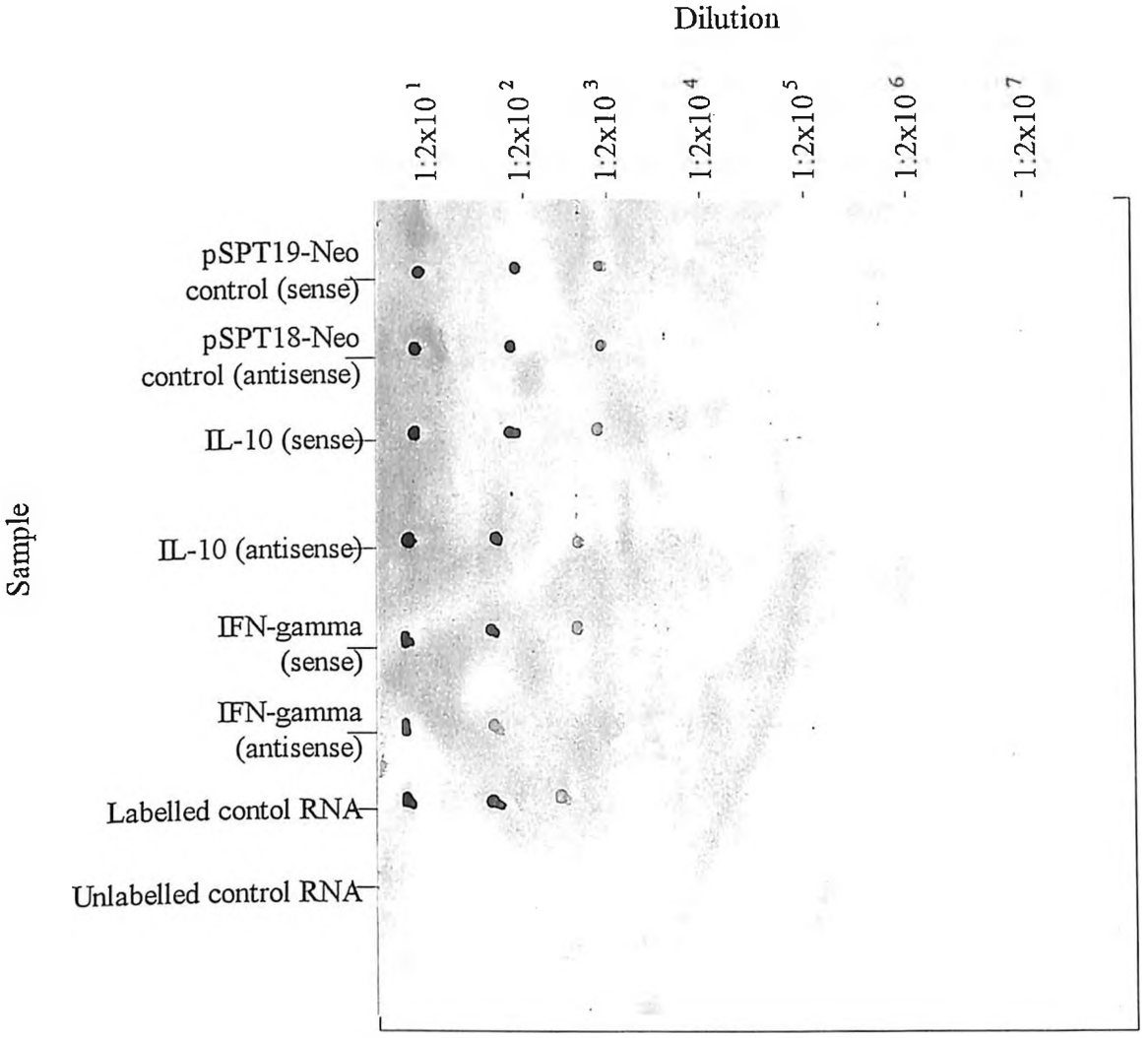

Figure 3.7 Dot blot analysis of the riboprobe yields. Contrasting the photography/electrophoretic observations, RNA was detected in all labelling reactions, although the yield of IFN-gamma was found to be ten fold less than the control concentrations.

Although the framework architecture of the infected lymph node appeared to be degenerating, the characteristic B and T cell-rich (Plate 3.7) zones were still evident although less distinct than those in the uninfected nodes. B cell staining was predominantly localised in germinal centres of the LN (>50% of the staining) with some staining occurring in the cortical nodules lining sinuses adjacent to the trabeculae. In regard to this B cell staining pattern, the staining was more intense at the middle of the LN structures (Plate 3.8). Staining was also markedly intense on cells demarcating the outer confines of the germinal centres (the mantle; Plate 3.8).

With regard the CD4+ T cell subset, these T lymphocytes were restricted to the medullary sinuses, with a few cells showing positive staining in the medullary cord. Within the medullary sinuses, most (more than 50%) of the cells stained positively for CD4 antigen. The germinal centres failed to show any staining which contrasted control tissues, which showed the presence of a few CD4+ cells within their germinal centres. The hilus area was shown to have a few positives for this antigen. The cells in the cortex showing positive staining did not group into clusters but were dispersed randomly within the cortex. Those T cells expressing the γ/δ T cell receptor (γ/δ TcR^+), were mainly located in the subcapsular cortex between the capsule and the cortex. However cells expressing this TcR were also seen in the sinuses, as well as a sporadic staining in the cortex, but with a general lack of staining in the germinal centres. A subset of γ/δ TcR^+ T cells which express the WC1 antigen, were restricted to the paracortex of the medullary cords with a few cells staining in the medullary sinuses. There was generally, a lack of staining in or around the germinal centres. A small proportion of cortical cells stained positive and sporadically, clusters of positive cells could be identified in the cortex. These findings compared well to the staining pattern observed in the controls.

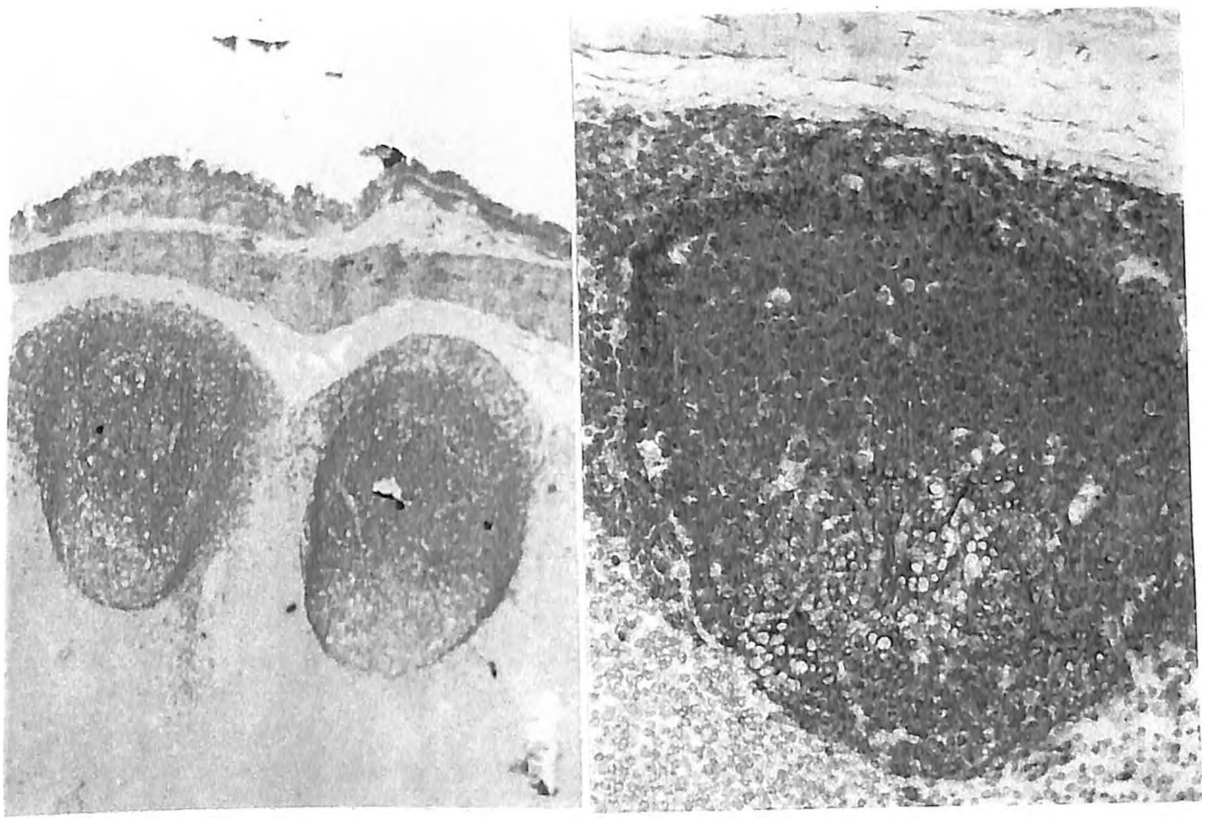

Plate 3.1a B-cell rich areas in a lymph node of a normal non-infected animal

Sections of a portion of a lymph node showing the follicular areas. **Left**: showi the non-specific staining capsule, and subcapsular sinuses with anti-CD21 (x40 **Right**: staining of B-cells expressing CD21 in the germinal centre area (x750).

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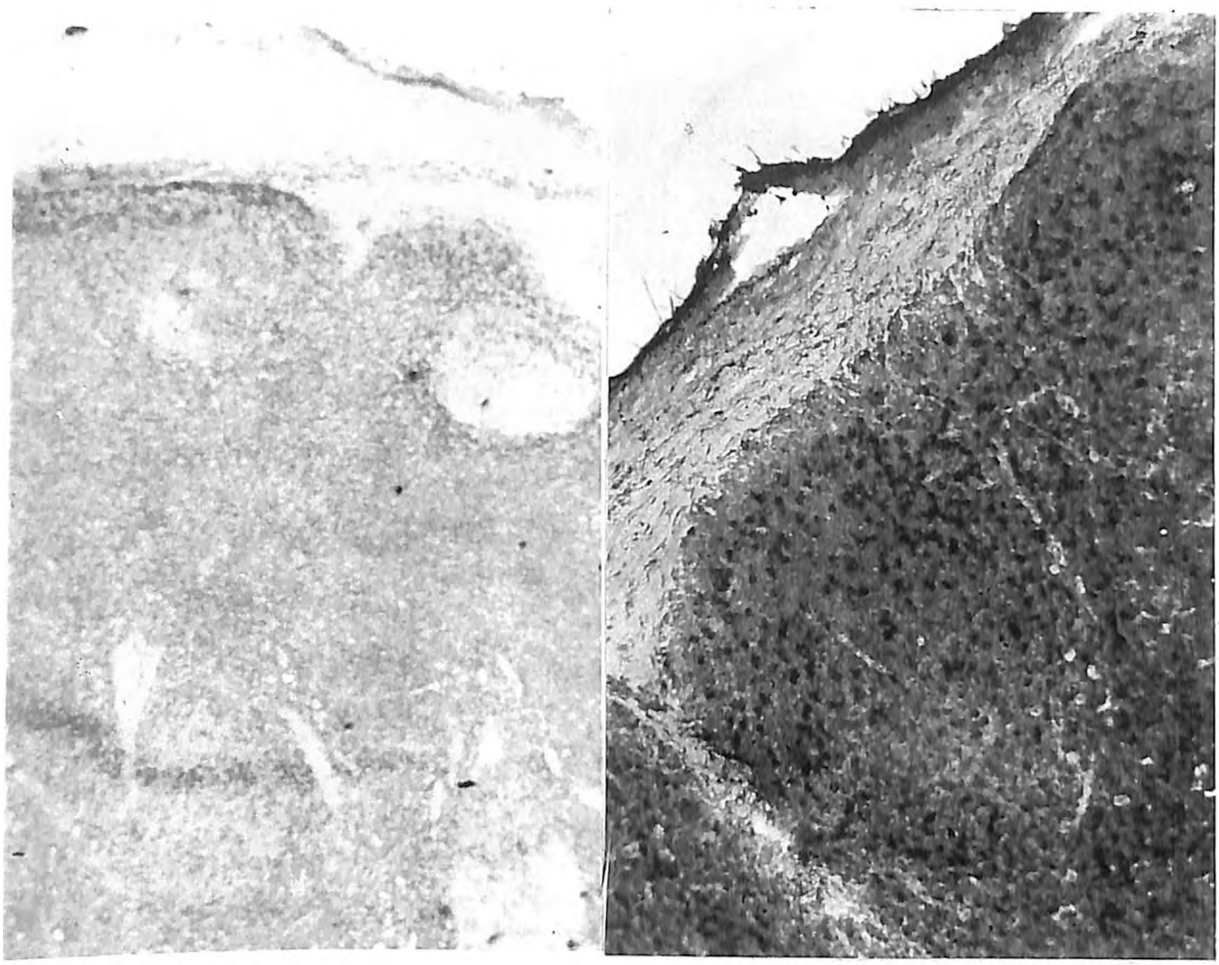


Plate 3.1b T-cell rich areas in a normal non-infected animal's lymph node

Sections of a portion of a prescapular node showing CD2+ T-cells. **Left**: cc (anti-CD2) mAb staining (x500). **Right**: the presence of CD2+ T-cells in germinal centres, shown by the same cc42 mAb (x750).

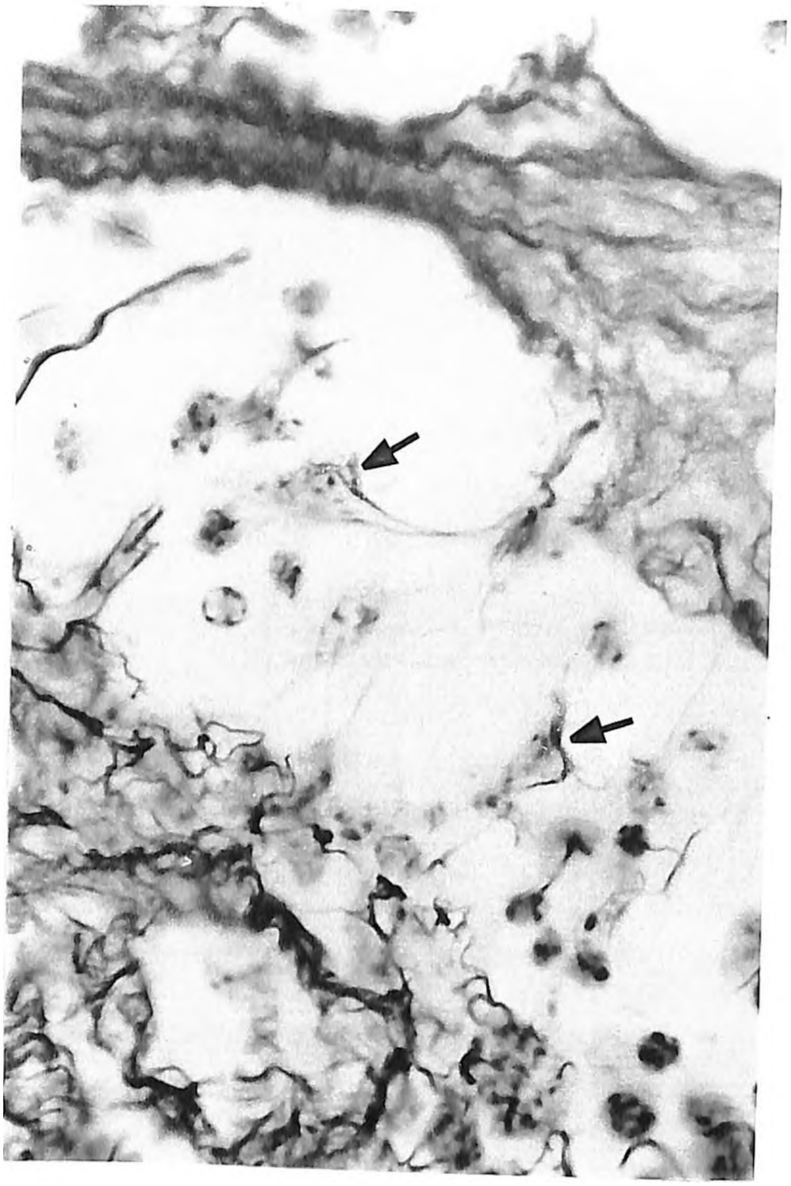


Plate 3.2a Reticulin secreting cells associated with reticulin fibre bundles
Bundles of reticulin fibres with the associated spindle shaped reticulin secreting cells (shown by the arrowheads) as found in the cortices of the non-infected lymph node (Gordon & Sweet staining; x1000).

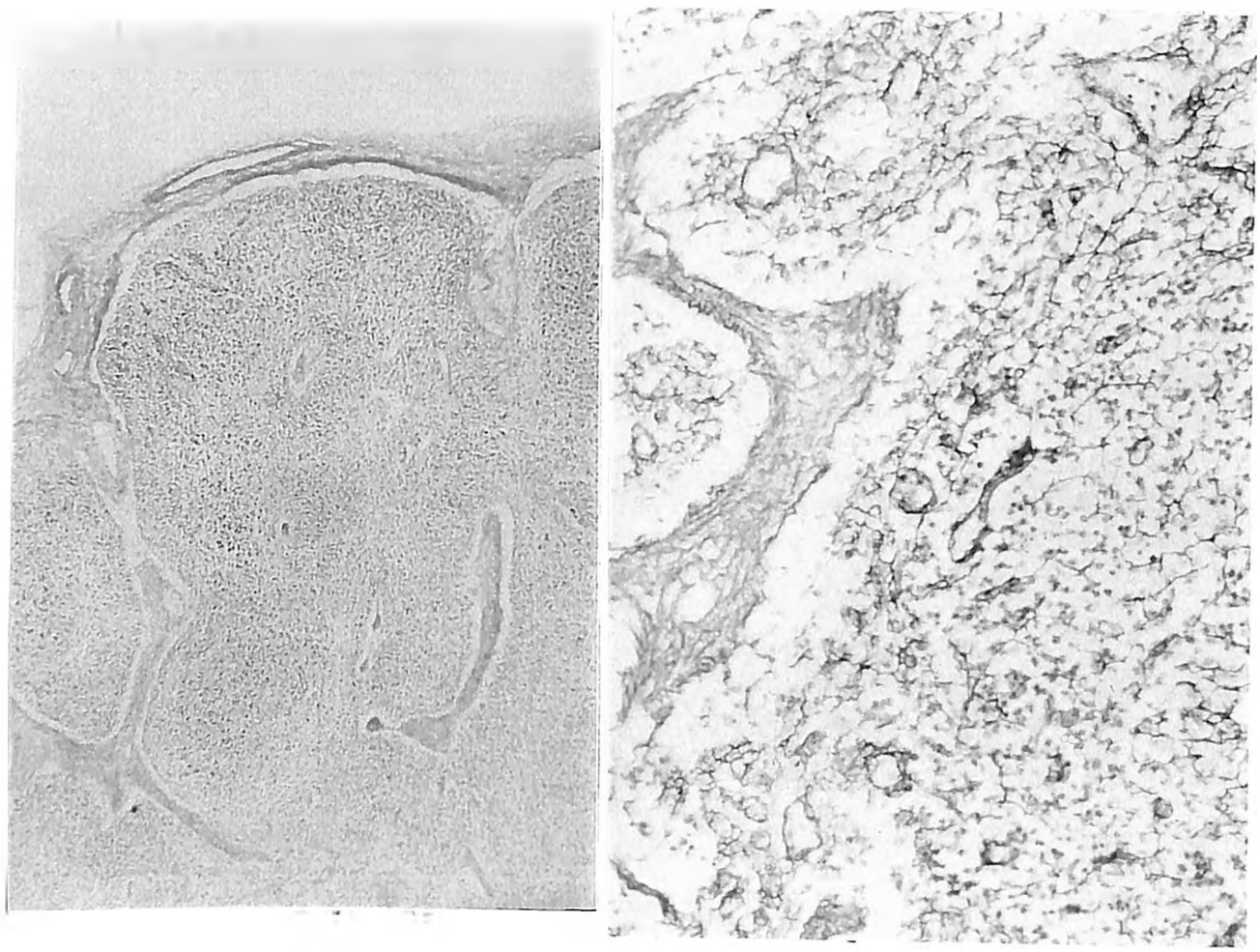


Plate 3.2b Reticulin fibre network formation in lymph node trabeculae

Sections of lymph node tissue showing the network of reticulin fibres along trabeculae. Left: The capsular connective tissue of the node is continuous with a number of trabeculae extending into the substance of the organ (Gordon & Sweet staining; x200). Right: The trabeculae, in turn, connect with the finer meshwork of reticular tissue in the node (Gordon & Sweet staining; x400).

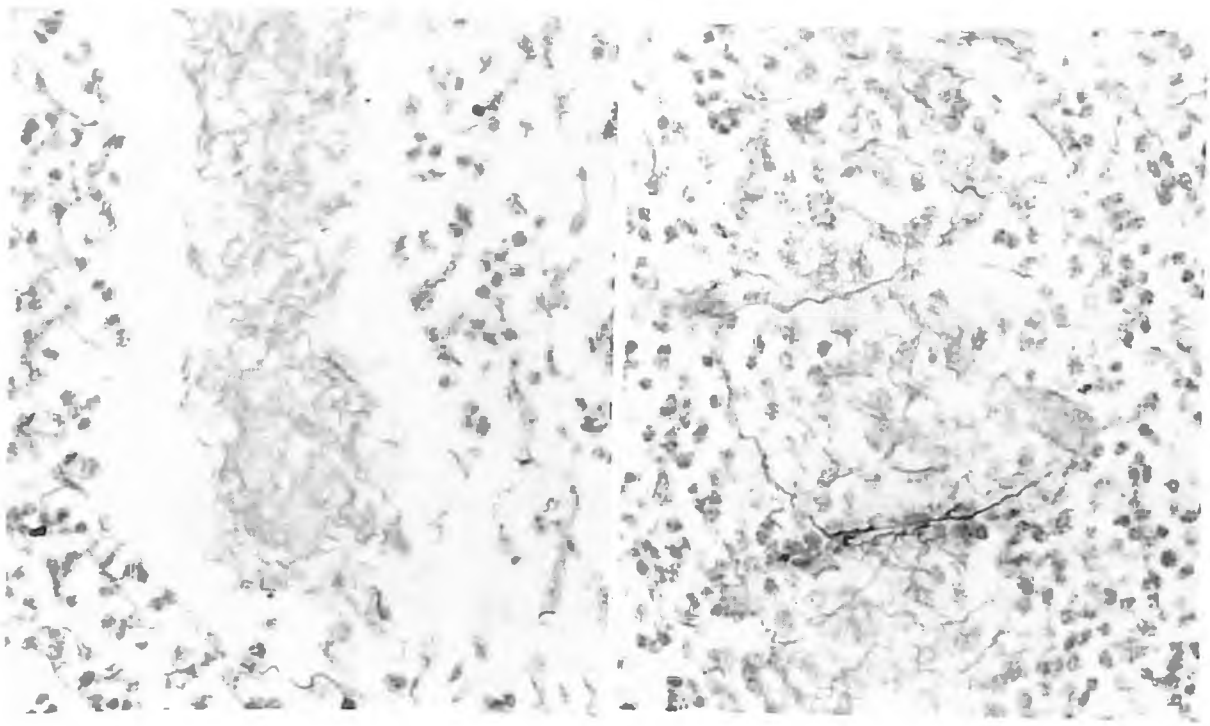


Plate 3.2c Reticulin network in the subcapsular sinuses

The extent of reticulin network observed varied. **Left**: the reticulin networ was composed of coarse reticulin fibre bundles within the trabeculae/subcapsular sinus tissue (G & S staining; x400). **Right**: the diffuse coarse fibre network associated with the paracortex tissue (Gordon Sweet staining; x400).

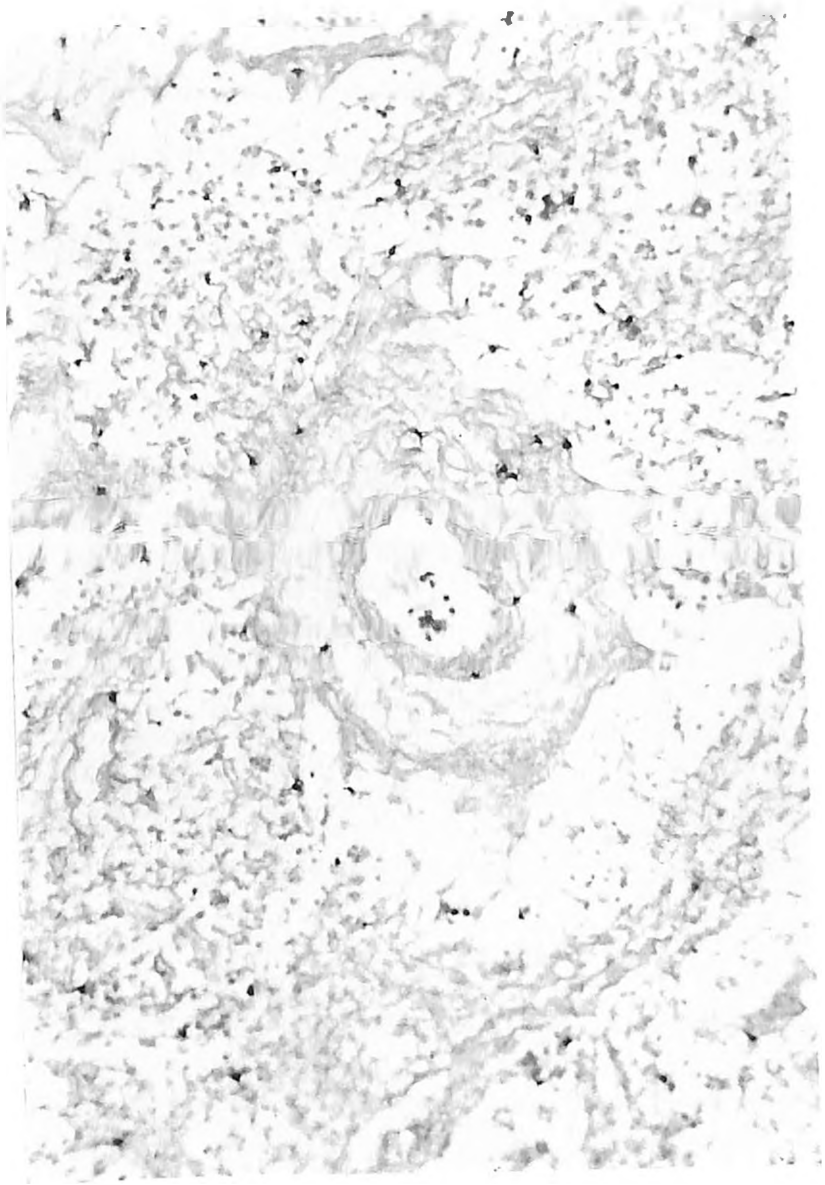


Plate 3.2d Reticulin fibres forming a continuous layer around a blood vessel.

Reticulin fibres form a continuous layer around blood vessels as well as the perivascular tissues of the blood vessels. Erythrocytes (RBCs) can be seen in the lumen of the blood vessel as dark dots. (Gordon & Sweet staining; x400).

The staining of control (uninfected) tissues using a mAb that recognises an antigen on a subset of monocyte/macrophages, staining of these cells was localised to the medullary and cortical sinuses lining the trabeculae and the medullary cords. Likewise, monocyte/macrophages were seen within the cortex (both general cortex and the medullary cord) and the hilum. Very few cells within or around the germinal centre were found to be monocytes/macrophages.

3.10 Identification of IL-10 and IFN-γ at the single cell level

3.10.1 Studies on the substrate for the adherence of cells and tissue sections to glass slides for *in situ* hybridisation

Initial studies were designed to investigate and compare the best medium for the adherence of cells and tissue sections to glass slides for *in situ* hybridisation. Single cell suspensions applied to uncoated slides failed to adhere and were washed away during the unmasking step. Glass slides coated with gelatin provided a good substrate to retain cells onto the slides, however when limited proteolysis with proteinase K was employed, the cells were dislodged from the slides. Coating slides with aminopropyltriethoxy silane (APTES), an organosilane, provided the most effective substrate for retention of single cells and tissue sections without significant cell loss or section lifting during the subsequent proteinase treatment and *in situ* probing.

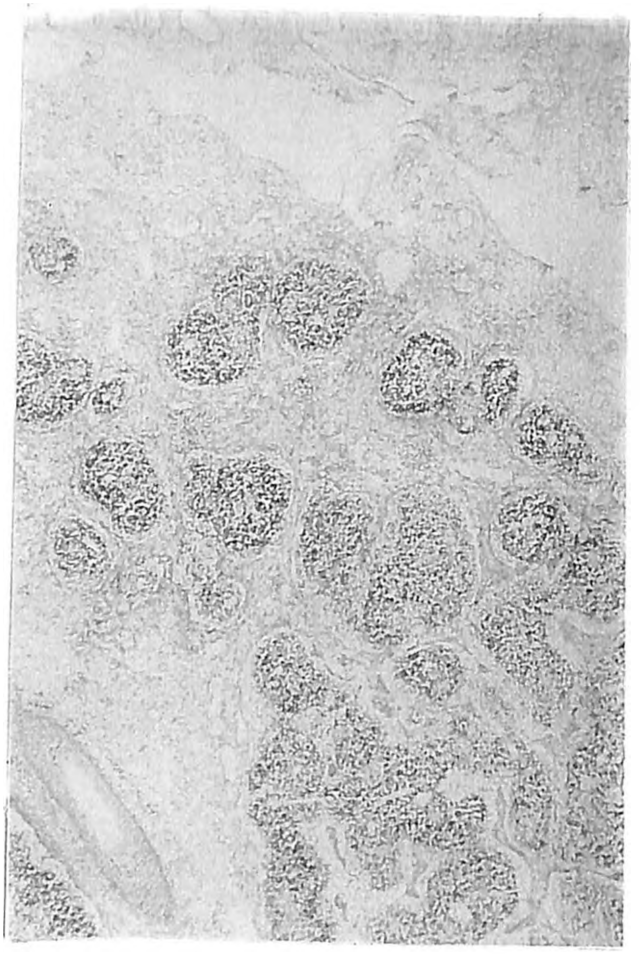


Plate 3.3 Follicular areas (germinal centres) as seen in a normal lymph no

Follicular areas (germinal centres) localised in the superficial areas of the cortex as well as in areas deep in the cortex and adjacent to the medullary sinuses. Some of these follicles are larger in size (putatively germinal centres) and a proportion of them are smaller in size (primary follicles; Haematoxylin & Eosin staining; x200).

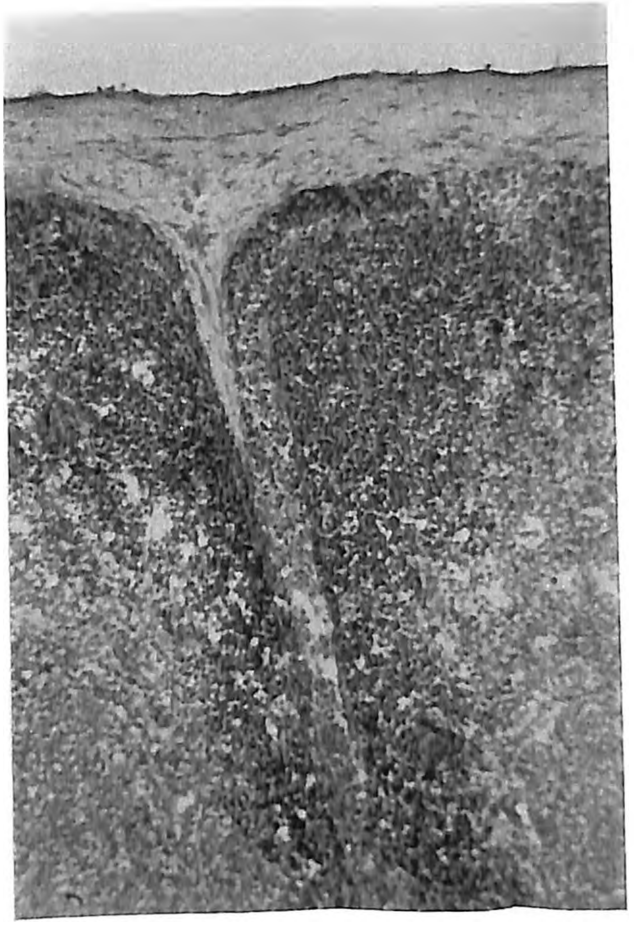


Plate 3.4 CD3+ (T) cells in the paracortex of a non-infected animal.

Section of a lymph node showing the presence of T-cells adjacent the trabecu (in the paracortical region). The section was stained to visualise T-cells with anti-CD3 mAb (counterstained with haematoxylin; x80).

3.10.2 Optimisation of proteinase K treatment for single cell preparations and tissue sections

To establish the optimal conditions for the unmasking of nucleic acids while retaining preservation and morphology of cells, a range of proteinase K concentrations ($5\mu g\ ml^{-1}$ to $20\mu g\ ml^{-1}$) were tested against a range of incubation times (1min to 30min). The optimal concentration was found to be $5\mu g\ ml^{-1}$ for both cell preparations and tissue sections. This concentration did not greatly affect the cell (or tissue) morphology when used with an incubation period of 5min for single cell preparations, and 15min for the formaldehyde fixed tissue sections. Extending the incubation period, or increasing the concentration of the proteinase K resulted in the loss of both cell morphology and lymph node architecture.

3.10.3 Riboprobing for IFN-γ transcripts

Cytospins treated with the IFN-γ antisense probes showed a high level of staining in all cells (Plate 3.9). Staining was not restricted to the cytoplasm but also parts of the nucleus. The reason(s) for this nonspecific staining remained unclear. Similarly, cell preparations treated with the sense IFN-γ probes (negative control) showed staining, albeit lower levels, which included staining of the nuclear compartment. This staining patterns indicated that the signals generated were non-specific (Plate 3.9) and the background staining in IFN-γ probed Cytospins could not be diminished by alterations in the hybridisation conditions.

Plate 3.5 Effect of *T.parva* parasitisation on node architecture.

Lack of reticulin staining in the prescapular node of a *T. parva* infected animal (Gordon & Sweet staining; x200). This lack staining contrasts the abundance of reticulin fibres in the prescapular lymph node of a non-infected animal (cf.: plates 3.2c and 3.2d).

Analysis for the identification of IFN-γ mRNA transcripts in tissue sections of the lymph node isolated from BVDV infected calves (control), showed that the alkaline phosphatase substrate (NBT/BCIP) could stain parts of the tissue non-specifically (Plate 3.10). The intensity of this non-specific staining was related to the incubation time and when the substrate time was increased to twelve hours the background staining was further increased (Plate 3.10). To overcome this problem the incubation period was not extended beyond six hours, an adjustment that resulted in very specific and discrete staining (Plate 3.10). Very few cells indeed showed this positive staining. Using the lymph node tissue sections from a gnotobiotic calf (control), both the sense and antisense IFN-γ riboprobes failed to produce a positive signal. As the failure to detect IFN-γ mRNA in this tissue may simply reflect the status of the lymph node, further experiments were conducted on tissue sections from *T. parva* infected animals. Preliminary experiments showed that both sense and antisense IFN-γ riboprobes failed to produce positive signals on *T. parva* infected lymph node sections (Plates 3.10a and b). Varying the concentration of the IFN-γ riboprobes from 2ng ml^{-1} to 10ng ml^{-1} generated detectable signal in some tissue sections. Similarly, increasing the incubation times from six hours to twelve hours and temperatures ranges (42°C to 80°C) also aided in the generation of specific staining pattern with the IFN-γ riboprobes.

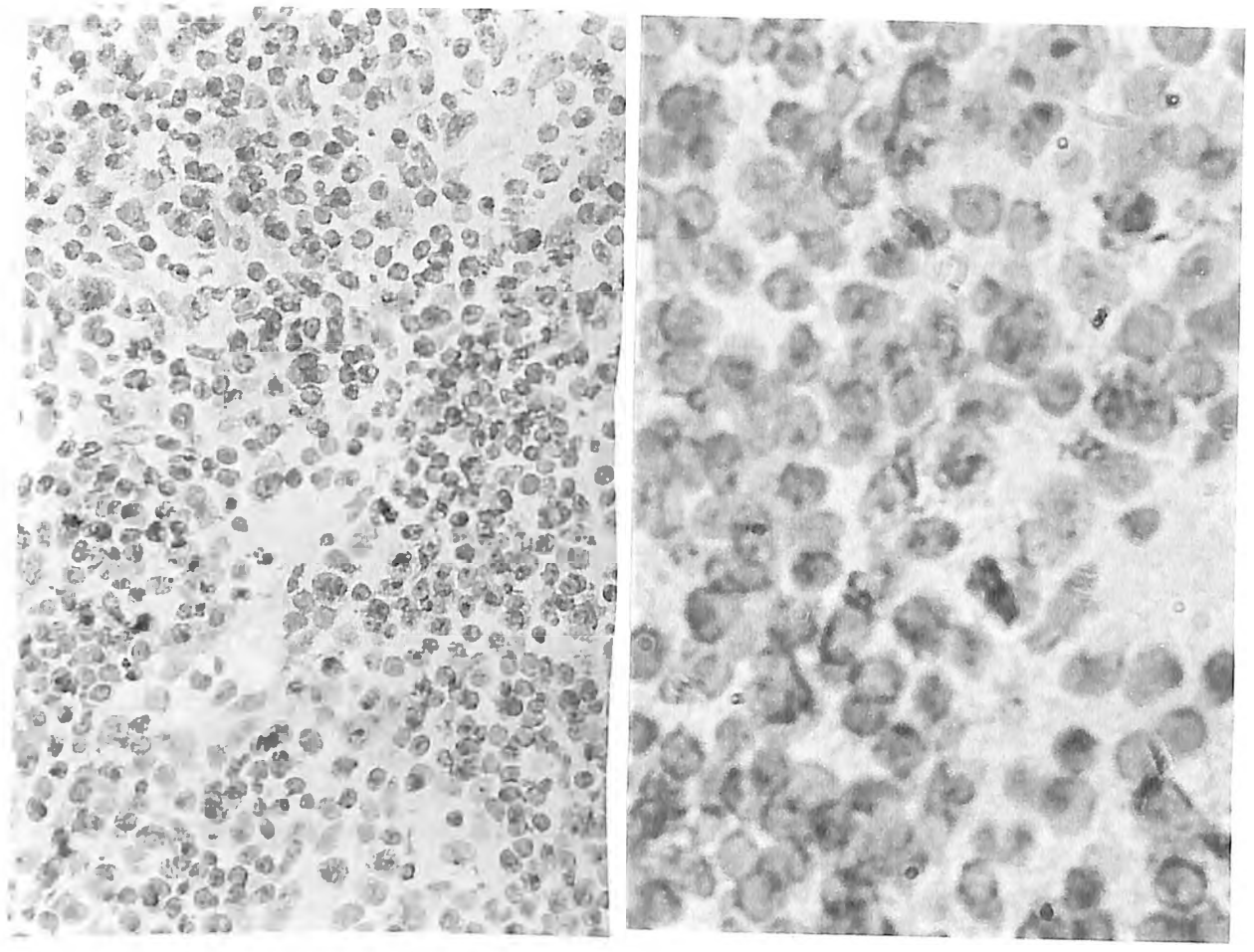


Plate 3.6 Staining of Schizonts with a schizont specific mAb

Sections of a prescapular lymph node tissue from a *T. parva* infected cow. Sections were stained with a mAb directed against the schizont and counterstained with haematoxylin. **Left**: schizonts dispersed throughout the entire lymph node (x200). **Right**: schizonts are localised to the membrane periphery of infected cells in the node (x400).

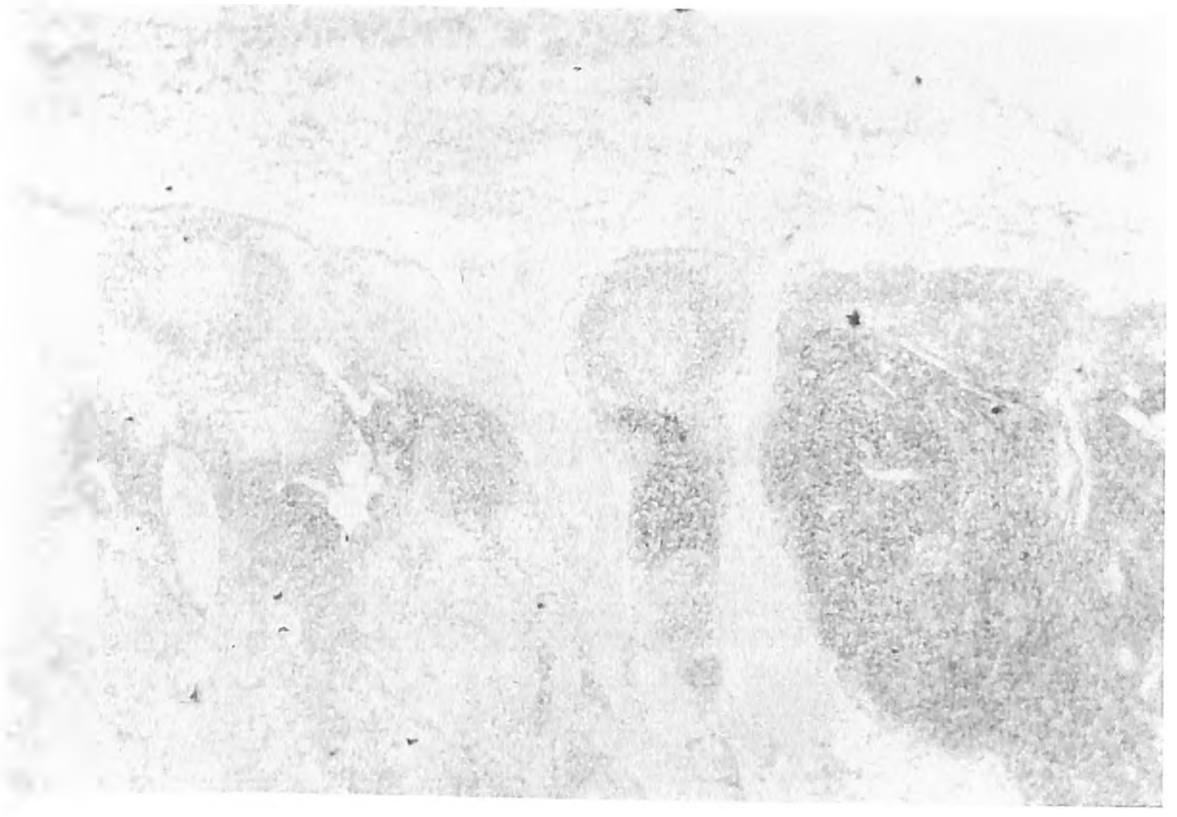


Plate 3.7 T-cells in the *T. parva* infected lymph node

Section through a portion of a lymph node from a *T. parva* infected animal showing the capsule, trabecuale and both the cortex and medulla. T-cells are visualised by staining with anti-CD3 (x20).

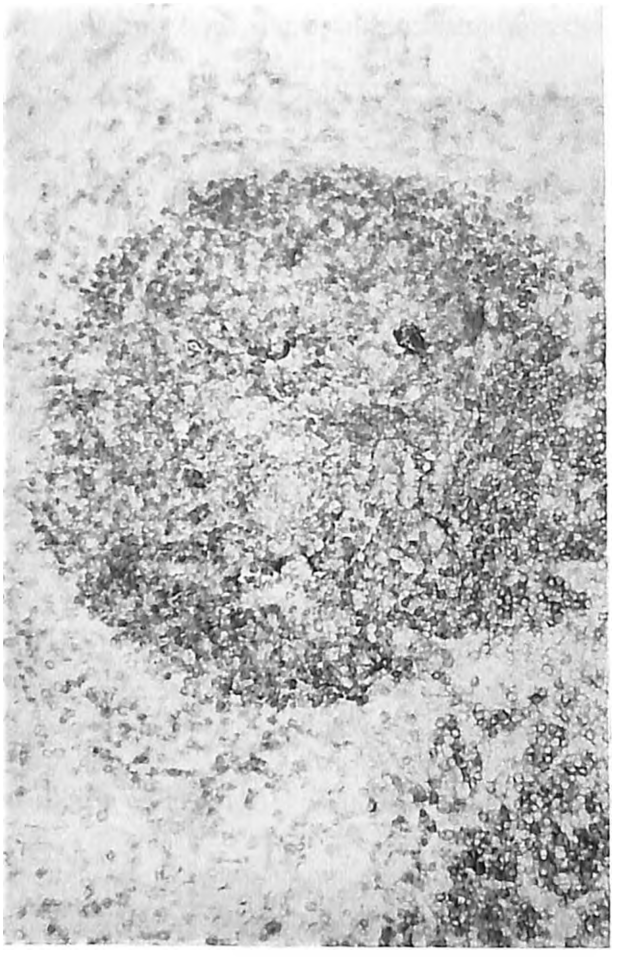


Plate 3.8 Distribution of cc21+ B-cells in the germinal centre

A germinal centre (follicle) stained to show B-cells. Staining is more pronounced in cells in the middle of the follicle and the follicle-surounding cells (mantle)

However, reducing the hybridisation temperature to below 50°C generated moderate levels of nonspecific staining on both sense and antisense probed *T. parva* sections. Tissues from the *T. parva*-infected animal showed positive staining with discrete cells dispersed in the node (Plate 3.12). Compared to the cells shown to produce IL-10, relatively very few cells indeed did produce IFN-γ transcripts. The very low level of IFN-γ expression sharply contrasted with the high level of IL-10 expression observed in these same tissues (section 3.10.4).

3.10.4 Riboprobing for IL-10 Transcripts.

Cytospins of 3110C probed with IL-10 antisense riboprobe produced very intense staining in all cells. This staining was localised mostly to the cytoplasm but did also faintly stain the nuclear region (Plate 3.13). These observations contrasted the lack of staining in 3110C cell preparations probed with the IL-10 sense riboprobe (Plate 3.13). The staining with the sense riboprobe was so low that it was almost impossible to visualise the cells on the glass slides (Plate 3.13).

The optimal hybridisation temperature for signal generation on BVDV/*T. parva* node sections was 55°C. Reducing the hybridisation temperature to below 50°C produced high levels of non-specific staining on these sections (Plate 3.14). Alternatively, increasing the temperature to above 60°C lead to total lack of staining in LN sections (Plate 3.14). When lymph node tissue sections from a gnotobiotic calf were probed no positive signal was detected with IL-10 antisense riboprobes.

However, probing of lymph node tissue sections from BVDV and *T. parva* infected animals, produced intense specific staining (Plates 3.15 and 3.16 respectively). The

intensity of the staining was related to the incubation time, and optimal signal to background ratio was obtained after incubation with the substrate for four hours. In all *T. parva* experiments, LN tissues from three anatomical regions were used. Prescapular LN, which was closest to the point of parasite inoculation, consistently expressed higher levels of staining compared to mesenteric and prefemoral nodes. In some sections isolated from the prefemoral and mesenteric LN, there was total lack of staining with the IL-10 antisense riboprobes but never with the prescapular LN.

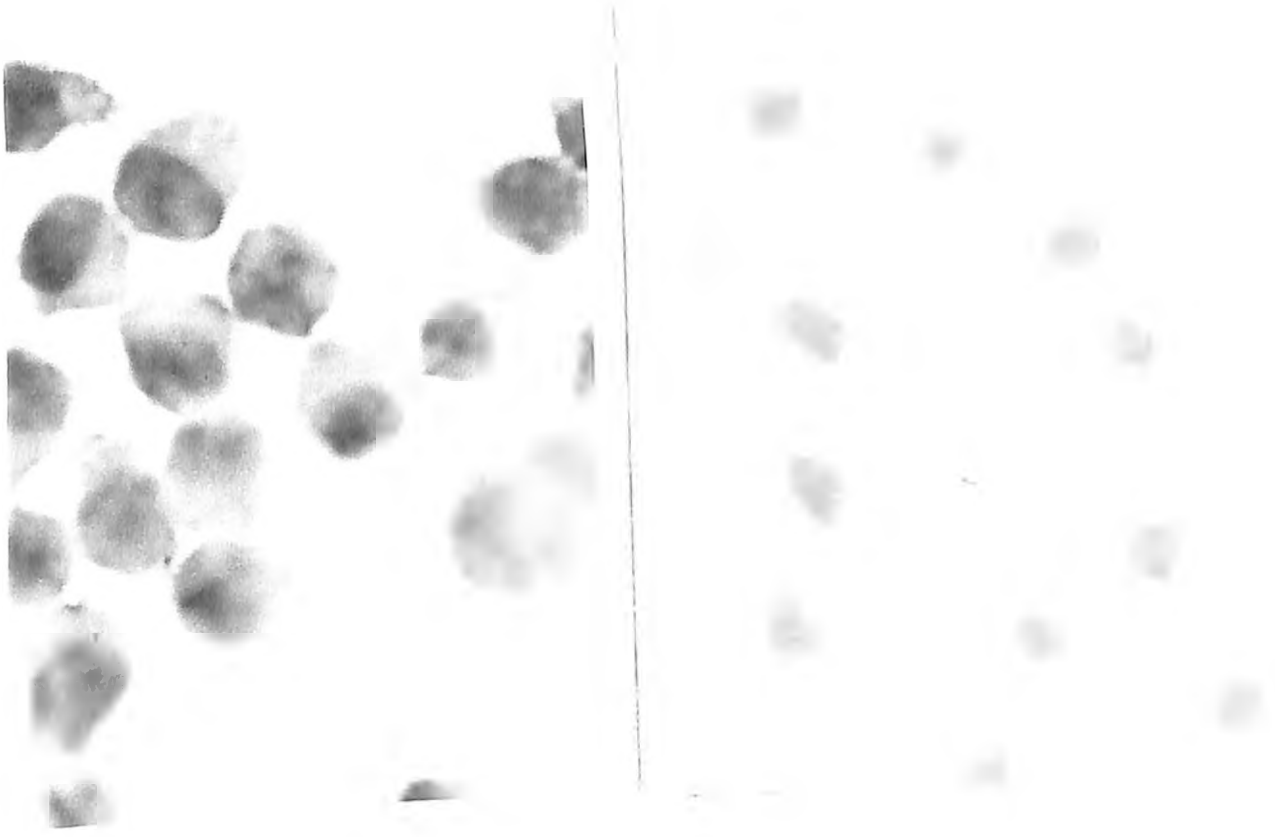


Plate 3.9 *T.annulata* infected cytospin stained with IFN-γ riboprobe

Left: cytospin of *T. annulata* infected cell line, 3110C, stained using an antisense IFN-g-specific riboprobe. Probe staining localised to the cytoplasm of the cells (x100). **Right**: Staining of the same 3110C cells using the sense IFN-γ-specific riboprobe. Background staining occurred in the nucleus implying non-specificity to the target mRNA (x1000).

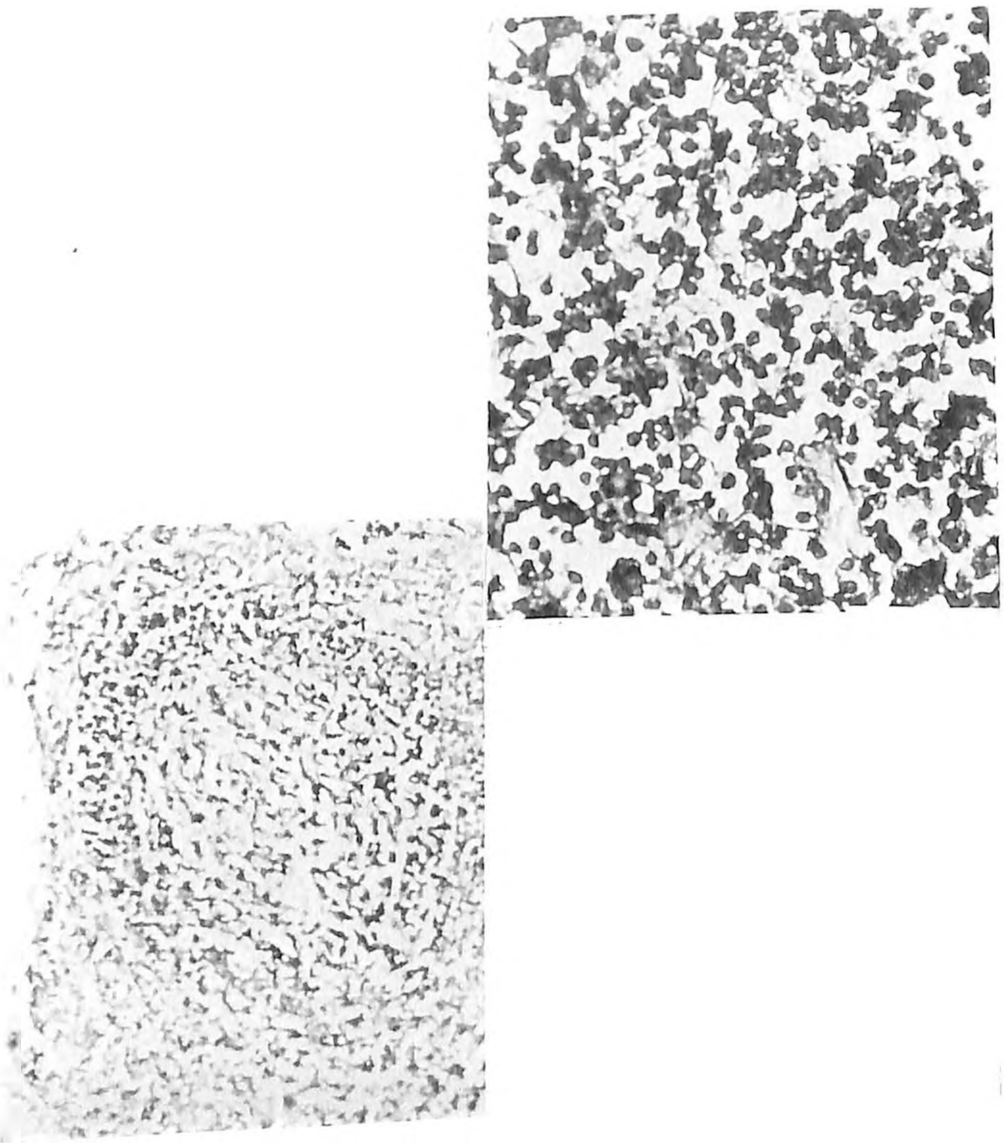


Plate 3.10 BVDV infected cells stained with antisense IFN-γ riboprobe

Left: Non specific staining of BVDV infected nodes using antisense IFN-γ riboprobe (x40). **Top middle:** Increased non-specific staining by the antisense IFN-γ riboprobe due to prolonged incubation times (x80). **Right**: Specific staining in a BVDV infected lymph node obtained by an IFN-γ-specific antisense riboprobe after incubation with the substrate for a four-hour period (x400).

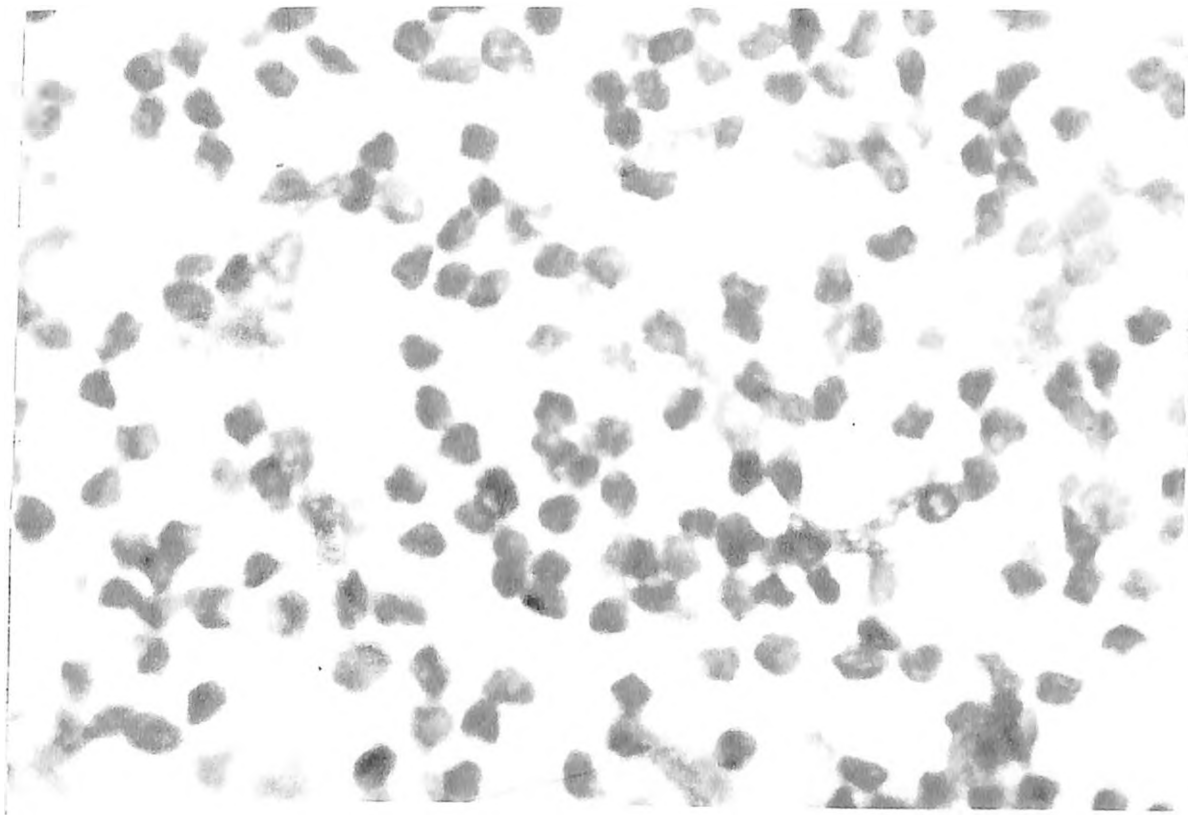


Plate 3.11 Lack of staining in a *T.parva* infected node using antisense IFN-γ probe

Weak or lack of staining in *T. parva* infected lymph node using antisense IFN-γ riboprobe (x400).

Plate 3.12 Positive staining in a *T.parva* infected lymph node using antisense IFN-γ roboprobe

Section of prescapular lymph node (PSLN) tissue from a *T. parva* infected animal showing the presence of IFN-γ transcripts. The section was stained with Dig-labelled IFN-γ antisense riboprobe and counter-stained with methyl green (light green; x400).

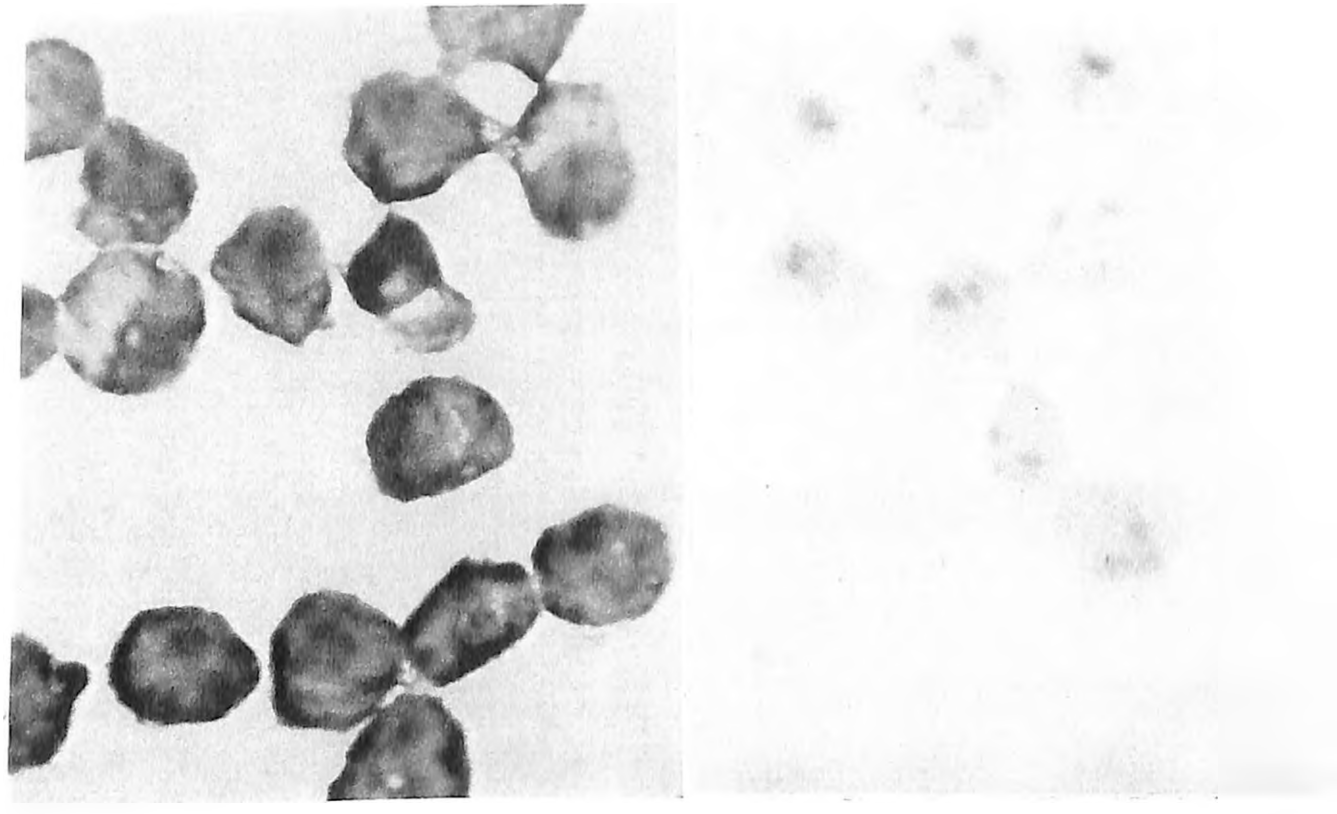


Plate 3.13 Staining of 3110C cytospins using IL-10 riboprobes

Cell suspensions of *T. parva*-transformed cells stained for IL-10 transcrips. Cytospin preparations of 3110C cells were stained with Dig-labelled IL-10 antisense riboprobe (left), or Dig-labelled IL-10 sense riboprobe (right) (x1000).

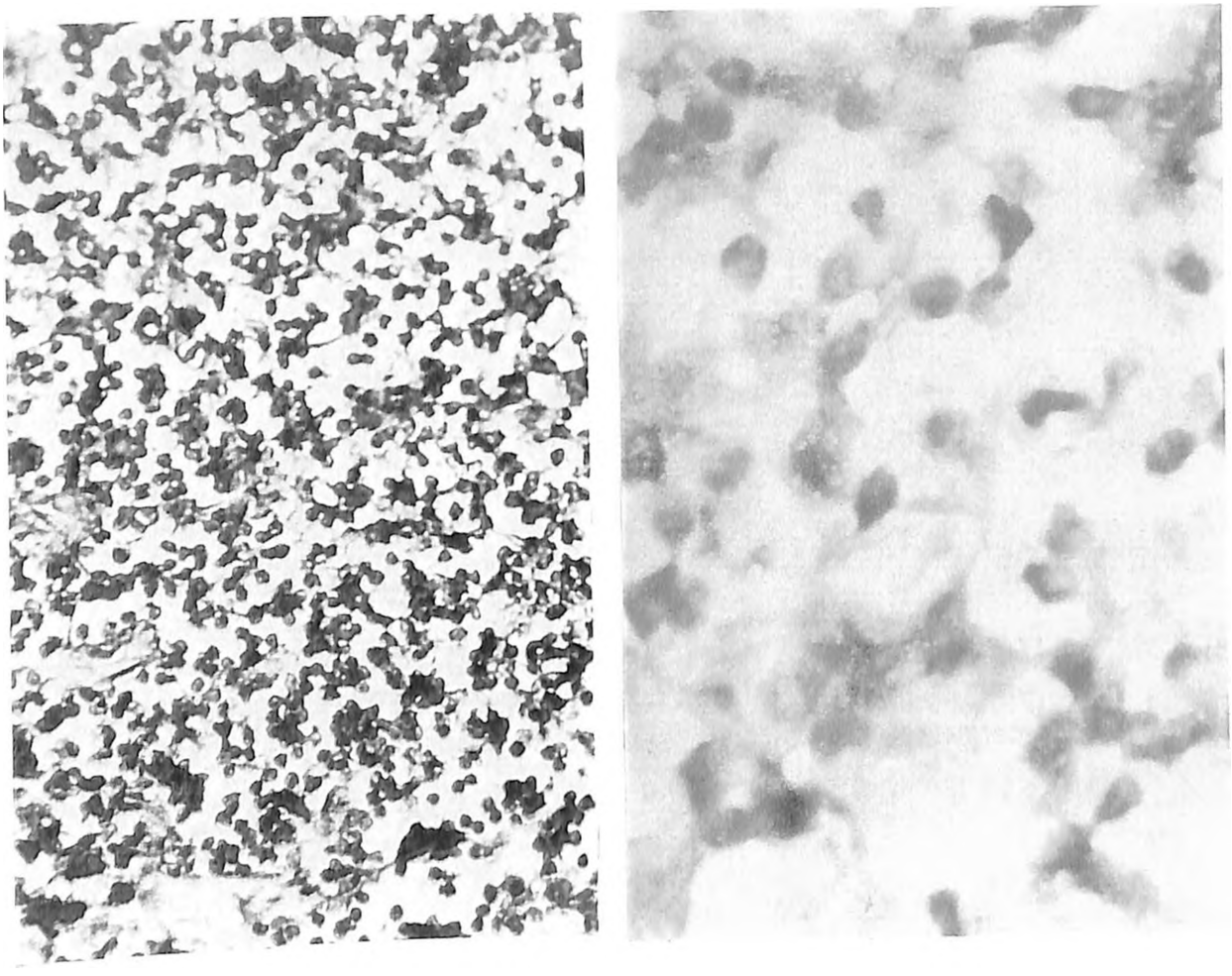


Plate 3.14 Staining of *T.parva* infected lymphnode with IL-10 riboprobes
Sections of PSLN tissue from a *T.parva*-infected animal stained for IL-10 transcripts. **Left**: hybridisation was performed in low stringency conditions (45deg centigrade; x100) or (**right**) high stringency conditions (60 deg centigrade; x300).

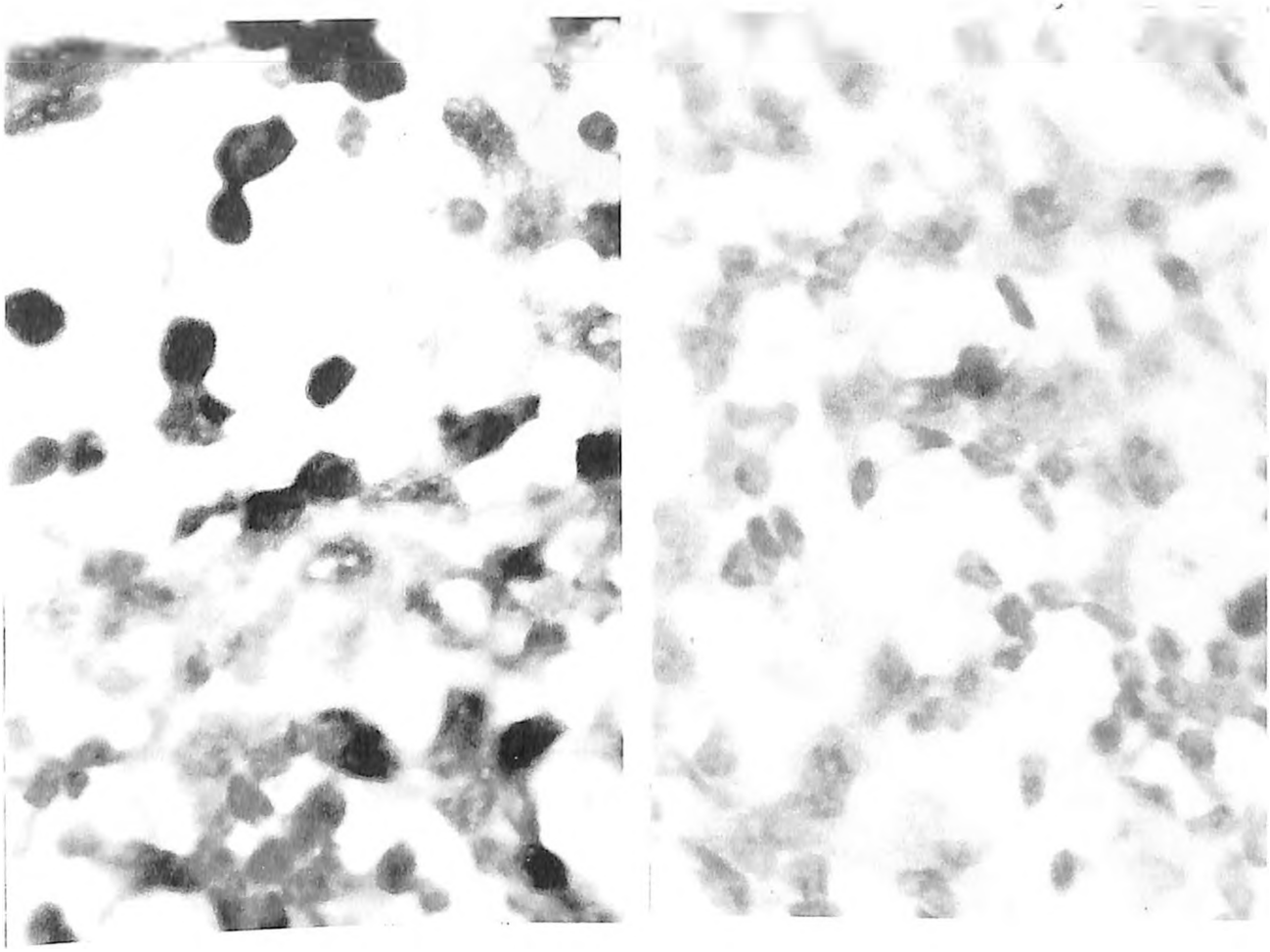


Plate 3.15 Staining of a BVDV infected lymph node with IL-10 riboprobes

(**Left**): Staining of BVDV infected lymph node with antisense IL-10 riboprobe and counter-stained with methyl green. The staining was intense and spread throughout the entire section of the node. However, some of the cells in the node did not stain positively for the expression of IL-10 transcript (x600). (**Right**): node sections probed with sense IL-10 probe there was no staining (x600).

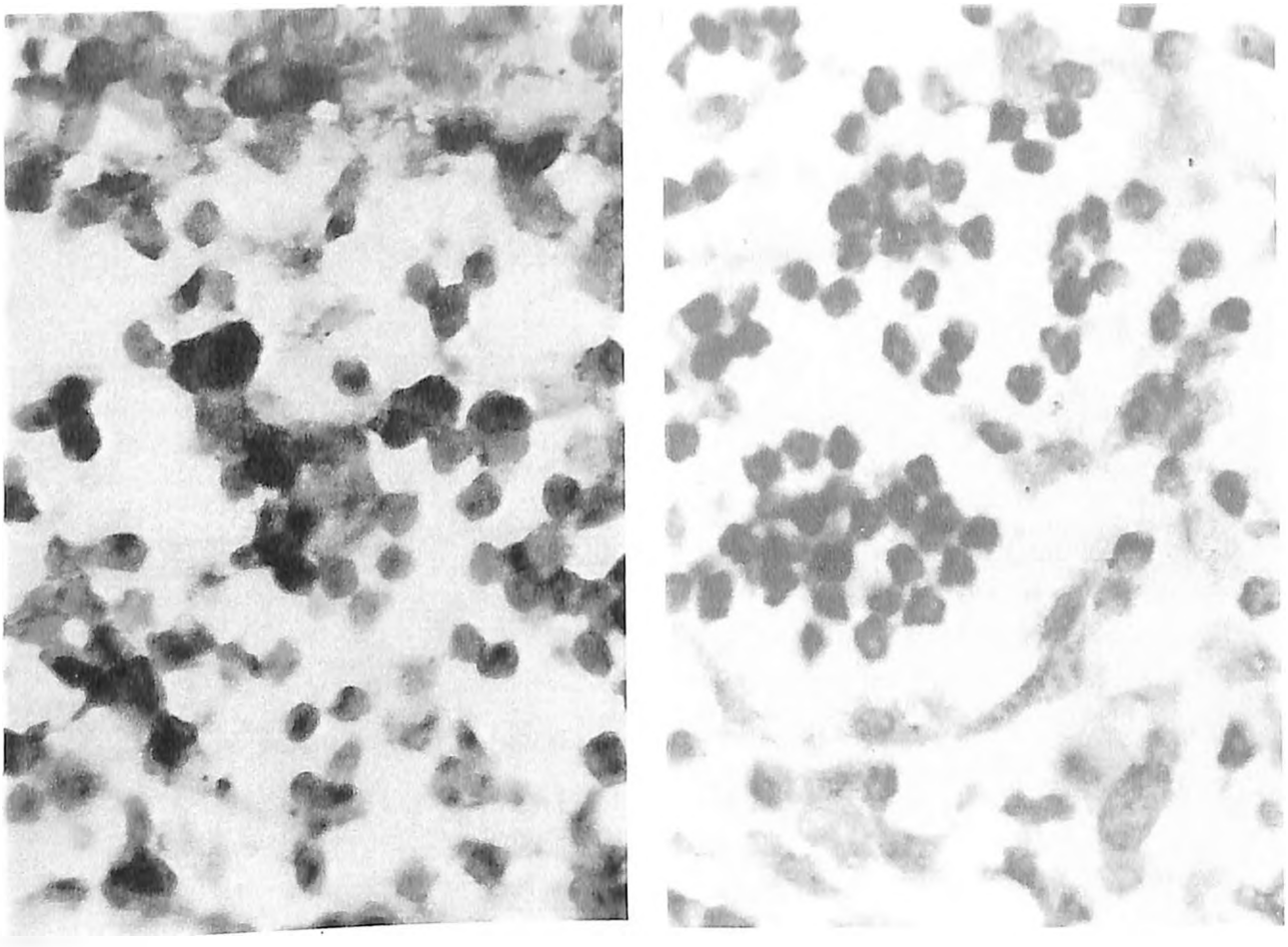


Plate 3.16 *T.parva* infected node probed with IL-10 riboprobes

Left: A section of prescapular lymph node (PSLN) tissue from a *T.parva* infected animal showing the presence of IL-10 transcripts after *in situ* probing. The section was stained with Dig-labelled IL-10 antisense riboprobe and counter-stained with methyl green (light green; x600). **Right**: The same tissue probed with IL-10 sense riboprobes show lack of staining, therefore indicating the specificity of the riboprobes (x600)

Chapter 4

Discussion

Discussion

The polarisation of cytokine profiles instigated by immunoregulatory cytokines has been shown to influence the course of some parasitic infections in rodents (Mosmann and Coffman, 1989; Fiorentino *et al.*, 1989) and man (Romagnani *et al.*, 1992). Such immunoregulatory cytokines are found in the bovine system (Bielefeldt *et al.*, 1987; Hash *et al.*, 1994) suggesting that similar immune responses might be elicited in cattle during parasitic infections. The protozoan *T. parva*, an obligatory intracellular parasite regarding its residence and replicative characteristics, induces the unregulated proliferation of infected and uninfected cells in cattle (reviewed by Irvin and Morrison, 1987). A possible course of generating this proliferative response is through the manipulation of the host's immunoregulatory pathway(s) controlling the generation of specific cytokine profiles. *T. parva* could effect this by initiating in a subtle manner, a state that would lead to cytokine control via one or a combination of any of the following mechanisms: clonal deletion, functional inactivation (anergy), or immunosuppression.

In *T. parva* infections, T cells are the primary targets for the parasite (Emery *et al.*, 1988) and the infected T-cells are presumed to synthesise cytokines as well because *in vitro*, infected cells grow without the need of exogenous growth factors (Dobbelaere et al., 1988). Given that T cells are the leukocytes intimately involved in the immunoregulatory response to protozoan infections, their parasitisation is likely to influence the nature of the immune response mounted by the host. If so, their ability to produce cytokines will modulate part of this response. In many parasitic diseases, the immunoregulatory system is disrupted at the level of cytokine production (for a review, see Baeuerle, 1991). These may include production of ribozymes/ribonucleases that cleave cytokine mRNA (Mahieu *et al.*, 1994), or by direct/indirect induction or inhibition of cytokine transcription factors

(reviewed by Baeuerle, 1991). This influence on the immunoregulatory system at the gene level is crucial to the subsequent immune response, since cytokine synthesis is normally regulated at the level of transcription (Crabtree, 1989; Brorson *et al.*, 1991). Indeed, changes in the cytokine mRNA profiles have been demonstrated in other parasitic diseases (Scott *et al.*, 1988; Heinzel *et al.*, 1989; Yamamura *et al.*, 1991; Scott and Kaufmann 1991; Sher *et al*,. 1992).

The finding that certain cytokines such as IL-10 are produced by all *T. parva* infected cell lines might suggest a role for this protein in the pathology of the disease. However, the RT-PCR technology also provided evidence that IFN-γ an IL-10 antagonist, was expressed by the same cell lines. These result demonstrate a simultaneous production of IL-10 and IFN-γ at the transcriptional level in *T. parva* infected leukocytes, though it was not possible by this PCR procedure to obtain a quantitative measure of the two cytokines. Similarly, another member of *Apicomplexa*, *T. annulata*, a parasite infecting bovine monocyte/macrophages mainly, also produced transcripts for IL-10 but less consistently those encoding IFN-γ. The expression of similar cytokines by *T. parva* and *T. annulata* infected cells suggest that *T. annulata* and *T. parva* may influence the host immune response in a similar manner.

As evidenced in this work, *Theileria*-infected cells produce IL-10 transcripts and presumably the active protein. IL-10 is a cytokine with important regulatory functions on lymphoid and myeloid cells, which include potent suppression of many effector activities of other cytokines (Fiorentino *et al.*, 1989; Macatonia *et al.*, 1993;). Of these, IL-10 downregulates expression and effector functions of IFN-γ (Silva *et al.*, 1992), a cytokine whose function among others, is to upregulate the expression of both major

histocompatibility complex class I (MHC I) and MHC II antigens (Lindahl *et al.*, 1976; de Waal *et al.*, 1991). Indeed, the observed low level expression of MHC I antigens in the *T. annulata* infected cells (3110C) may be attributable to the downregulation of IFN-γ effector functions by the apparent presence of IL-10 in the cells. Reduced expression of MHC antigens results in a consequential fall in the antigen presenting potential of antigen presenting cells implying an ensuing immunosuppression.

The manipulation of expression of modulating immunoregulatory cytokines is one strategy that has been adopted by some parasites in search of circumvention of the host's immune response. Most notable are parasites that have salvaged or captured immunoregulatory cytokine gene(s) or gene product(s), which they express during parasitisation, such that parasite infestation is favoured. An example is elaborated by Epstein Barr virus (EBV), a virus that encodes a molecule (vIL-10) which is structurally and functionally similar to human IL-10 (Moore 1990; Niiro *et al* 1992; Swaminathan *et al* 1993). One may also speculate on use of other alternative strategies which would include the production of proteases that cleave specific cytokines or their receptors to regulate responses, as well as the documented evidence of production of soluble cytokine receptors to specific cytokines such as IFNs (reviewed by Smith; 1993). These last two mechanisms if present, would effectively nullify the action of secreted cytokines, leading to the dysregulation of the immune functions. Whether *T. parva* uses any or a combination of such strategies for immune subversion is presently unknown.

A limitation of the RT-PCR is the lack, in mixed cell preparations, of information on the cell source of the target RNA. To obtain this information, another procedure, *in situ* hybridisation, which detects complementary nucleic acid sequences in cells with a lower

limit of 10 to 20 nucleic acid copies per cell was used. When *in situ* hybridisation is performed in conjunction with immunohistochemical staining, the phenotype of the cytokine producing cell can be determined. Unlike RT-PCR, which theoretically requires only one copy of the target RNA or DNA to be amplified, the procedure of *in situ* hybridisation is a direct detection method, requiring an RNA/DNA probe labelled with a reporter molecule. For the detection of low copy numbers, such as mRNA, non-radioactive probes are considered more sensitive and suitable because of their stability and unlimited shelf life. In the present study, riboprobes were labelled with digoxigenin, a steroid isolated from the foxglove plant (*Digitalis lanata* or *Digitalis purpurea*; Holtke and Kessler, 1990) permitting their detection with antibodies raised against this hapten.

For the production of riboprobes an optimal nucleotide length of about 500 has been suggested (Holtke *et al.*, 1990). Probes of greater than 1000nt are considered too long to allow them to readily enter into permeabilised cells, while those of less than 300nt length are often not retained, or can bind non-specifically leading to difficulties in the interpretation of results. For these reasons the present study produced riboprobes for IL-10 and IFN-γ of between 550 to 600nt length. However, although the *in vitro* transcription produced riboprobes of comparable length for both IL-10 and IFN-γ the yield for antisense IFN-γ probe was consistently ten fold less. The reason(s) for the lower efficiency of transcription in this reaction remained unclear.

Although initial experiments on cytokine plasmid cDNAs confirmed the specificity of the Dig-labelled IL-10 and IFN-γ riboprobes, the ability of these probes to detect cytokine mRNA from total cellular RNA extracts of MDBK, 3332, and 3110C cells failed. The results obtained with 3332 and 3110C RNA extracts were unexpected, since

the RT-PCR had shown that transcripts for these cytokines were present. Given that the blotting procedure is capable of detecting 1pg of complementary sequence equivalent to approximately 20 nucleic acid copies per cell (Sambrook *et al.*; 1989), the problem was unlikely to be due to the lack of sensitivity. A likely reason may have been the loss of the RNA integrity due to digestion by RNases during the procedural manipulations involved in isolation of the cellular RNA. This reasoning seems plausible, because IL-10 transcripts were detected in later studies using 3110C single cell preparations.

Evidence accrued from cytospins prepared from *T. annulata* infected cells indicated presence of IL-10 with high specificity. Contrastingly, both sense and antisense IFN-γ *in situ* probing resulted in cytospin staining. Seeking to overcome this non-specificity displayed in IFN-γ probing, combinations of various parameters and conditions for *in situ* hybridisation were tried for optimisation. These included changes in probe concentration, adjustments in hybridisation temperatures and alterations in the stringency of washings. However, even with all these variables considered the background staining could not be reduced to reasonably acceptable low levels. Given that the riboprobe was synthesised from full length cDNA, the possibility of errors arising from non specificity of the probe were considered unlikely. The reason(s) for this non specific staining remained unclear and deductions about the *in situ* localisation of IFN-γ transcripts in these cytospins could not reliably be made from the results/observations.

The success of the *in situ* hybridisation procedure relies much on accessing/unmasking the target nucleic acid by limited proteolysis. In performing this function, proteinase K has since been used successfully in many *in situ* hybridisation studies (Angerer *et al.*, 1987). This is due to the properties of this fungal serine protease, which includes its

ability to inactivate endogenous nucleases such as RNases and DNases (Weigers and Hilz 1971; Weigers and Hilz 1972). However, when used at relatively high concentrations, proteinase K destroyed the morphology of the cells. Thus a concession was reached in the amount of proteinase K used, which would not destroy cell morphology, and at the same time unmask RNA for probing. Although the concentrations of proteinase K for the unmasking of target RNAs can vary, the amount required for IL-10 and IFN-γ on single cell preparations was considered to be the same, judging from cell morphology after proteinase treatment. However, initial problems in detecting IFN-γ but not IL-10 transcripts in tissue sections may suggest that the unmasking conditions are different for these cytokines with fixed tissues.

Studies using normal LN tissues showed that, transcripts encoding both IL-10 and IFN-γ could only be detected in a few cells at low levels, and were most often seen in both the cortex and paracortex zones of the lymph nodes, indicating that these cytokines are not normally expressed at high levels by cells in the node. The occurrence of IL-10 transcripts in the paracortex and the cortex zones in normal uninfected tissues, might also indicate that IL-10 plays little or no role in B cell lymphopoiesis, owing to its absence in the germinal centres.

The finding that most cells in the prescapular lymph node tissue from a *T. parva* infected animal, with approximately 10% of the cell population presenting as parasitised, stained positively for the presence of IL-10 transcripts contrasted the normal tissues. The loss in reticulin fibres and pronounced destruction of the underlying node architecture observed in *T. parva* infected LN is documented (Irvin and Morrison, 1987). However, the coincidental IL-10 expression with significant changes in stromal morphology may indicate a mechanism involving IL-10 by which the presence of the parasite induces this tissue disruptive/immunopathologic saga. A model which links cytokine dysregulation to the pathology of ECF is invoked in this study. One possibility pertinent to this study is that the initial hyperplasty, followed in advanced stages of the disease by reduction in cellularity of the infected node, is through the uncontrolled upregulated expression of IL-10, via IL-10's haemopoetic and tissue disruptive potential. The appearance of IL-10 transcripts in cells throughout these infected tissues certainly gives evidence for this protein's upregulated expression. Indeed the amount of IL-10 staining seen in tissue sections paralleled parasite inoculation point and hence the degree of parasitisation, with lymph nodes distant from the point of parasite inoculation displaying little evidence for the presence of schizonts and showing only a few cells as expressing IL-10.

The expression and presence of this TH2 immunoregulatory cytokine, IL-10, inevitably has a biasing consequence towards the pathway taken by the responding leukocytes to the infection and hence the pathological repercussion displayed. IL-10 along with IL-4 drive the immune response towards a TH2 profile (Powrie and Coffman, 1993), a state favouring the domination of the immune system by the parasite due to the lowered IFN-γ-mediated cytolytic potential. Secondly, the initial upregulation and presence of IL-10 may induce enhancement of growth and proliferation of B-cells (Defrance *et al.*, 1992), thymocyte and T-cells (MacNeil *et al.*, 1990), mast cells (Thompson-Snipe *et al.*, 1991) and cytotoxic T-cells (Chen and Zlotnick, 1991) leading to the increase in cellularity of LN observed during the hyperplastic phase of *T. parva* infection. However, as the infection progresses and more IL-10 is produced by these increased numbers of cells, the IL-10 may then begin to manifest its effects on T cell apoptosis (Clerici *et al.*, 1994). Induction and maintenance of this apoptotic state by the IL-10 may lead to selective depletion of cells resulting in the reduction of cellularity evident in the lymphoid destructive phase of ECF disease.

Given that a relatively small cellular fraction of the node comprised parasitised cells, and that IL-10 transcripts were present in most of the cells in the prescapular lymph node, it is evident that both parasitised and non-parasitised cells produce this cytokine, including both B and T-cell components as well as monocytes and macrophages. A variety of mechanisms may exist for the pan-production of IL-10 in parasitised lymph nodes. Suggestions include their actions via both paracrine and autocrine systems for non-parasitised and parasitised cells.

Another possible association of IL-10 overexpression and pathology is that the presence

of IL-10 may evoke destruction of the LN tissue structure through induction of mechanisms that produce matrix-digesting metalloproteinases. Matrix metalloproteases (MMP) constitute a family of proteolytic enzymes capable of degrading every component of the extracellular matrix (reviewed by Conca, 1993). For example, tissue destruction observed in inflammatory joint disease is largely accounted for by the action of these enzymes (Conca, 1993). MMP activity is inhibited by tissue inhibitors of metalloproteases (TIMP). Previous studies have demonstrated that various cytokines can modulate MMP and TIMP gene expression (Conca, 1993), among them IL-10.

IL-10 has a diverse influence on the modulation of many immune functions and controls the signal transduction pathway leading to the production of MMPs by leukocytes. Reitamo *et al* (1994) demonstrated that recombinant human IL-10 affects the expression of several genes involved in extracellular matrix synthesis and remodelling in human dermal fibroblast cultures, a scenario in which type I collagen gene expression was downregulated, while collagenase and stromelysin gene expression were markedly enhanced by IL-10. These results demonstrate the potential role of IL-10 in the induction and maintenance of destruction of tissue structural components. Taken together with demonstrated presence of IL-10 in *Theileria*-infected cells/tissues, it may be said that the presence of IL-10 in ECF provokes the loss of tissue integrity by induction of matrix metalloproteinases.

Conversely, Shapiro *et al* (1990) demonstrated that IFN-γ specifically and selectively suppresses the lipopolysaccharide-induced production of the same two metalloproteinases, collagenase and stromelysin. These *in vitro* studies may represent events occurring *in vivo*. The downregulatory effects of IL-10 upon IFN-γ demonstrated

in *T. parva*-infected tissues would abrogate IFN-γ effects, leading to unregulated expression of these metallo enzymes with pathological consequence. This possibly is the case in ECF, leading to the observed loss of reticulin and lymphoid destruction.

Indeed, *T. annulata*-infected cells have been shown to produce abundant metalloproteinases *in vitro* (Baylis *et al.*, 1992). Thus the paradigm associating the presence of IL-10 to matrix metalloprotease-induced pathology seems plausible although this has not been studied in the *T. parva* context. In this respect, studies with cytokine inhibitors as well as TIMP on *T. parva* infected tissues/cells might serve to indicate inhibition or postponement of the crippling consequences of metalloproteases, if indeed they are involved int ECF pathogenesis. This is a point requiring investigation in the *T. parva* pathology.

The downregulation of IFN-γ expression leading to the relative absence of the effector functions of this cytokine would result in the inhibition of TH1 responses (Silva *et al.*, 1992) with an ensuing lack in cytolytic potential. Susceptibility to protozoan, helminth and retroviral infections in animal experimental models have been associated with distinct, mutually exclusive profiles of cytokine production (Sher *et al.*, 1992; Cenci *et al.*, 1993; Clerici *et al.*, 1994) and such a phenomenon is anticipated in ECF. For example, genetically susceptible mice have relatively high levels of IL-4 (TH2 associated) and low levels of IFN-γ (TH1 associated) in their lymphoid tissue during infection with *Leishmania major* (Carlvalho *et al.*, 1985), whereas the opposite pattern is observed with genetically resistant mice, which recover from infections with this parasite (Carlvalho *et al.*, 1985). This dichotomy of response dictates that protective immunity will depend on the activation of appropriate T helper subsets. Furthermore,

cytokines produced by these polarised CD4+ T helper cell subsets can downregulate the activity and function of the other subsets (Seder and Paul, 1994). This study has demonstrated that in *T. parva* infections, there is an upregulation of the production of IL-10 and a depressed IFN-γ expression implying a TH2 type of response. Through the demonstrated low level expression of IFN-γ its downregulated expression is attributed to inhibitory activity upon it by IL-10, such that as with parasitic diseases in other species, the TH1/TH2 paradigm seems to operate in *Theileria* infections. The downregulation of, and hence the relative absence of IFN-γ leading to the inhibition of cytotoxic TH1 responses will lower the cytolytic capabilities of the immune system allowing the parasite to prosper and the consequent uncontrolled parasite proliferation as observed in the hyperplastic phase in the disease, with consequent increased pathology. The presence *perse,* of IL-10 transcripts in the infected tissues, may or may not represent the proposed mechanism(s) of immunopathology and whether this presence of IL-10 in responding tissues is beneficial to the host or to the parasite, it is not clear. Yet, judging by concordant IL-10 presence and disruption of the architecture seen in *T. parva* infected nodes, IL-10's upregulated expression is probably detrimental to the host.

The natural host of *T. parva* is the African buffalo, *Syncerus caffer*. *T. parva* infects *S. caffer*, without provoking the overt clinical signs seen in cattle (Young *et al.*, 1973). This may be attributed to a long-term parasitic relationship between *S. caffer* and *T. parva*. Conversely, the parasitic relationship between *T. parva* and *Bos indicus* may be considered to be a recent venture while that between *T. parva* and *Bos taurus* as a more recent one. These considerations arise from the high mortality rates observed in untreated cases of ECF, situations in which there are strain specific resistance: Boran cattle (*B. indicus*) showing higher natural resistance as compared to Fresian (*B. taurus*)

cattle. An interesting study in this respect would be to investigate the expression of IL-10 and IFN-γ in *S. caffer* following infection with *T. parva* and comparing findings to observation made in ECF with *B. indicus* and *B. taurus*. Results from such investigations will provide confirmation on cytokine-profiles in *T. parva* infection as well as providing solid clues to cytokine influence in "immune" versus "diseased" states, with a view to indicating favourable and injurious cytokine depictions.

Conclusion

Using RT-PCR, IFN-γ and IL-10 transcripts have been shown to be produced *in vitro* by both *T. parva* and *T. annulata* infected cells. *In situ* hybridisation protocols specific to the identification of both IL-10 and IFN-γ at single cell level in bovine single cells and lymph node tissues have been developed. These protocols could be appropriated to the study of other cytokines on the same tissues as well as same/other cytokines in other tissues. Employing this technique, very low level expression of IFN-γ and high level expression of IL-10 transcripts were demonstrated in *T. parva* infected nodes. The upregulation of IL-10 expression induced in ECF disease indicates a TH2 type of response to *T. parva* infection, a situation which seems to favour parasite persistence and proliferation due to lack of IFN-γ a major player in the IFN-γ-dependent cell-mediated immune destruction. This reduction in cytolytic potential towards infected cells would lead to increase in pathology. According to a proposed model relating cytokine expression to pathology, the presence of IL-10 appears to partly account for the unregulated proliferation of cells resulting in the observed hyperplasty in initial phases of ECF. In this model, chronic/persistent IL-10 overexpression should also lead to the reduction in cellularity in advanced stages of the disease as a result of IL-10 induced T-cell apoptosis. The documented display of occasional vascular damage and thrombosis, as well as the node architecture disturbances that give rise to complete necrosis of the structural elements of the tissues in ECF can all be attributed to IL-10 induced tissue-destructive matrix metalloproteinases.

Appendixes

Appendix A. Media and Buffers

A1 Tissue culture medium (TCM)

1.RPMI 1640 + Glutamax (Gibco,UK)	90ml
2.FBS (10%)	10ml
3.Penicillin and streptomycin	0.1ml
4.2-Mercaptoethanol	0.1ml

This medium was filtered through a 0.2µm pore filter (Schleicher & Schuell, Germany) and stored at 4°C until required.

Composition of Buffers

A2 Buffer P1 (Resuspension buffer)	100µg/ml Rnase A, 50mM Tris/HCl, 10mM EDTA, pH8.0
A3 Buffer P2 (Lysis buffer)	200mM NaOH, 1% SDS
A4 Buffer P3 (Neutralisation buffer)	3.0M K acetate at, pH5.5
A5 Buffer QBT (Equilibration buffer)	750mM NaCl, 50mM MOPS, 15% Ethanol, pH7.0, 0.15%Triton X-100

A6 Buffer QC (Wash buffer)	1.0M NaCl, 50mM MOPS, 15% Ethanol, pH 7.0
A7 Buffer QF Buffer)	1.25M NaCl, 50mM Tris/HCl, 15% (Elution Ethanol, pH 8.5
A8 TE	10mM Tris/HCl, 1mM EDTA, pH 8.0
A9 Buffer B	10mM Tris/HCl, 5mMMgCl_2, 100mM NaCl, 1mM 2-Mecarptoethanol, pH8.0
A10 Buffer H	50mM Tris/HCl, 10mM $MgCl_2$, 100mM NaCl, 1mM Dithioerythritol, pH7.5
A11 0.5X TBE buffer	44.5mM Tris-base, 44.5mM boric acid, 0.1mM EDTA, pH 8.0
A12 DIG Buffer I	100mM Maleic acid, 150mM NaCl, pH 7.5

A13 Buffer 3	100mM Tris-HCl, pH 9.5, 100mM NaCl, 50mM $MgCl_2$
A14 20X SSC	3M NaCl, 300mM Sodium citrate, pH 7.0
A15 Standard Hybridisation Buffer	5X SSC, 0.1% Sodium lauroylsarcosine, 0.02% SDS, 1% blocking reagent from stock.
A16 10X MOPS	200mM Morpholinopropansulfonic acid, 50mM Sodium acetate, 10mM EDTA, pH 7.0,autoclaved.
A17 Modified hybridisation buffer (+ 50% formamide)	5XSSC, 50% formamide(deionised), 0.1% sodium lauroylsarcosine, 0.02% SDS, 2% blocking reagent.
A18 6X Loading buffer	0.26% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water
A21 LB Medium	10g bacto-trypton, 5g bacto-yeast extract, 10g NaCl, dissolved in 1L distilled water, pH 7, then autoclaved.
A22 phosphate buffered saline (PBS)	0.17M NaCl, 3.4mM KCl, 9.2mM Na_2HPO_4, 1.8mM KH_2PO_4, pH6.8

A23 Buffer M	10mM Tris-HCl, 10mM $MgCl_2$, 50mM NaCl, 1mM dithioerythritol, pH 7.5
A24 LB agar	LB (A20), in 1% agarose
A25 Buffer A	33mM Tris Hcl, 10mM Mg acetate, 66mM K acetate, 0.5mM dithiotreitol, pH 7.9
A28 5 x reaction buffer	250mM Tris-HCl (pH8.3), 0.375M KCl, 15mM $MgCl_2$
A29 Dig buffer 2	1% (w/v) Blocking reagent in buffer 1(A12)
A30 10X *Taq* reaction Buffer	200mM Tris-HCl (pH 8.4), 500mMKCl
A31 10X NTP labelling mixture	ATP, 10mM; CTP, 10mM; GTP, 10mM; UTP, 6.5mM; Dig-UTP, 3.5mM; pH7
A32 10x RNAtranscription buffer 100mM DTT, 100mM NaCl, 20mM	400mM Tris-HCl, pH8.0, 60mM $MgCl_2$, spermidine, RNase inhibitor 1U μl^{-1}.
A33 10x T4 DNAligase buffer 100mM DTT, 5mM ATP.	300mM Tris-HCl, pH 7.8; 100mM $MgCl_2$,

A26 Preparation of 4% paraformaldehyde

The paraformaldehyde was made by dissolving 0.4 g of the paraformaldehyde ash in 10 ml of PBS in a 65°C water bath. 5µl of 10M NaOH was added to assist dissolution. The solution was then filtered through a 0.2µm filter (Schleicher & Schuell, Germany). DEPC (20µl) was added to 100ml of the fixative immediately before fixation of cytopreps in the coplin jars.

A27 Composition of the hybridisation mixture

Formamide (Molecular Biology Grade) (Life-Technologies UK)	5ml
50% Dextran Sulphate, Mw 500000 (in DEPC water) (Sigma, UK)	1ml
20x SSC (DEPC treated)(Sigma, UK)	1ml

This hybridisation mixture was stored at 4°C until needed.

Appendix AII: Formulae

1. [RNA] = OD_{260} x 40 x dilution factor = $\mu g ml^{-1}$

eg. Concentration ($\mu g/ml$) = 0.349x40x60 = 837.6$\mu g ml^{-1}$

2. The number of cells per ml = the number of cells from cytometer reading x 10^4 x dilution factor.

eg. Cells ml^{-1} =48x10^4x2 = 9.6x10^5

3.Purity of total RNA =ratio of OD_{260}/OD_{280} should lie between 1.8 and 2.

eg. OD_{260}= 0.349

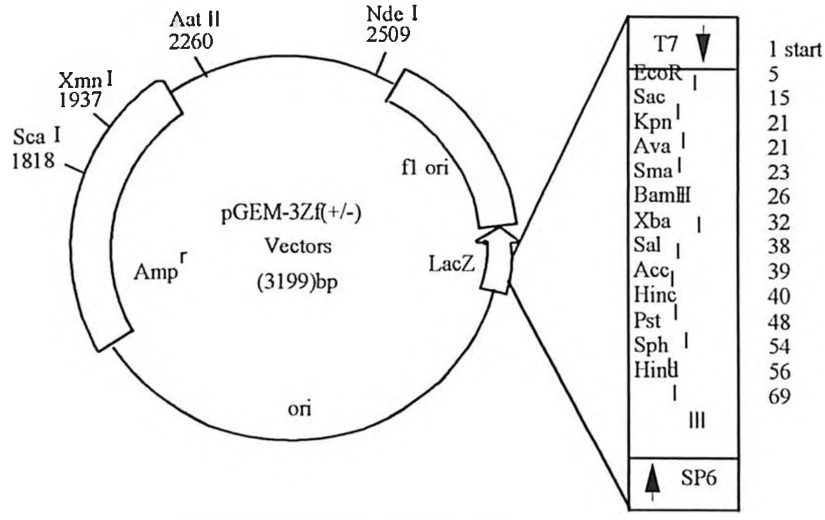
OD_{280}= 0.194

Ratio of OD_{260}/OD_{280} = 1.826 =the RNA sample extracted pure

Appendix AIII: Immunofluorescent/immunoperoxidase staining

Table C1. Monoclonal antibodies and their specificities.

Monoclonal antibody	Cluster of differentiation	Source of mAb	Specificity
CC1	CD45	Bembridge *et al.*, 1993	Pan leucocytes
CC42	CD2	Davis and Splitter. (1991)	T-cells, NK-cells
MM1A	CD3	Davis *et al.*, 1993	Thymocytes / mature T-cells
CC8	CD4	Bensaid and Hadam, 1991	T-helper cells, macrophages, monocytes
CC63	CD8	MacHugh-ND & Sopp-P (1991)	T-cytotoxic / suppressor cells
CC21	CD21	Sopp, 1996	Mature B-cells, follicular dendritic cells
CC15	WC1	Clevers *et al.*, 1991	γ/δ T cell subset
IL-A88	MHCI	Dr J Naessens, ILRI, personal communication	All nucleated cells
IL-A21	Mo/Mϕ	Taylor *et al.*, 1993	Monocytes / macrophages



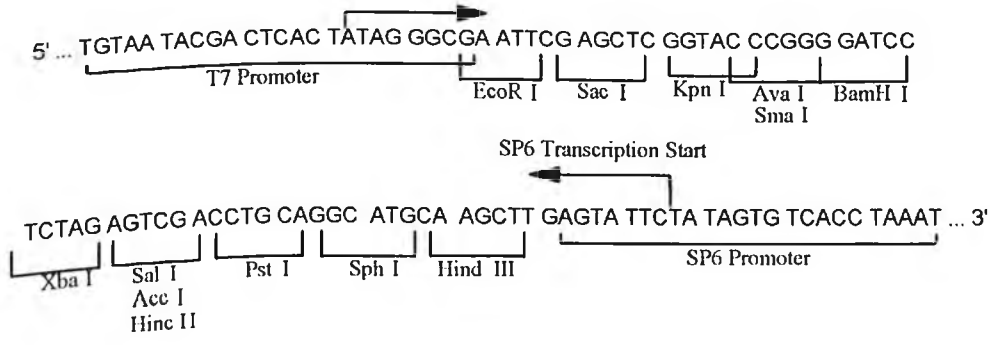
Appendix IVa pGEM-3Zf(+/-) Vectors promoter and multiple cloning site sequence.

Vector Map Notes:

1. Sequence reference points:

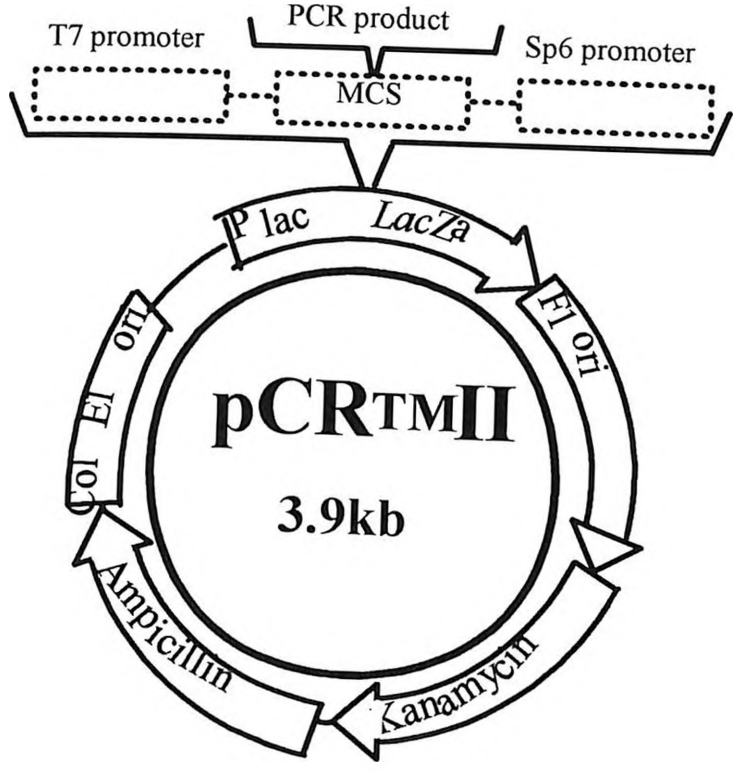
a.	T7 RNA Polymerase transcription initiation site	1
b.	SP6 RNA Polymerase transcription initation site	69
c.	T7 RNA Polymerase promoter	3183-6
d.	SP6 RNA Polymerase promoter	64-86
e.	multiple cloning sites	5-61
f.	lac Z start codon	108
g.	lac operon sequences	3020-3180; 94-323
h.	lac operator	128-144
i.	β-lactamase (Amp^r) coding region	1265-2125
j.	phage f1 region	2564-3019
k.	binding site of pUC/M13 Forward Sequencing Primer	3140-3150
l.	binding site of pUC/M13 Reverse Sequencing Primer	104-120

2. Use the SP6 or pUC/M13 Reverse Primer to sequence ssDNA produced by the pGEM-3Zf(-) Vector.
3. Use the T7 or pUC/M13 Forward Primer to sequence ssDNA produced by pGEM-3Zf(+) Vector.



The sequence shown corresponds to RNA synthesised by T7 RNA Polymerase and is complementary to RNA synthesised by SP6 RNA polymerase. The strand shown is complementary to the ssDNA produced by the pGEM-3Zf(+) Vector and the same as the ssDNA strand produced by the pGEM-3Zf(-) Vector.

Appendix AIVb



Lac Z gene: bases 1-571

Sp6 promoter: bases 239-255

Multiple Clonong Site: bases 269-381

T7 promoter: bases 388-407

F1 origin: bases 572-986

Kanamycin resistance: bases 987-2114

Ampicillin resistance: bases 2133-2992

ColE1 origin: bases 3182-3765

This represents the pCR™II vector sequence with a PCR product inserted by TA cloning.

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