T CELL ACTIVATION IN *THEILERIA ANNULATA* INFECTION - IMPLICATIONS FOR IMMUNITY & PATHOGENESIS

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Theileria annulata is a protozoan parasite of cattle, causing the lymphoproliferative disease tropical theileriosis. In susceptible animals, disease progresses rapidly, with the host apparently unable to mount an effective immune response. The parasite infects MHC class II$^+$ monocytes and macrophages, during its pathogenic macroschizont stage. This leads to an augmentation of their antigen presenting capability *in vitro* - infected cells (IC) can induce proliferation of autologous resting T cells from naive animals. Preliminary studies showed that such altered APC function was a barrier to isolating *Theileria* specific T cells from immune animals. Infection of APC leading to a change in their function may provide *T.annulata* with a mechanism to evade the host immune response. This thesis sets out to understand interactions between T cells and *T. annulata* infected cells, both *in vitro* and *in vivo* and the consequences for the generation of immunity.

In vitro stimulation of peripheral blood T cells from naive animals by IC caused the cells to proliferate, peaking 5 days post stimulation. Phenotypic analysis showed that CD25 and MHC class II were expressed upon the surface of all T cells (CD4,CD8 and $\gamma\delta$T cells) within 24hrs of stimulation, reaching a peak at 48hrs and remaining stably expressed for up to 7 days post activation. The parasite infected cells could activate both "memory" and "naive" CD4 T cells, with little change in the CD45RB isoforms during activation. Activation of T cells was contact dependant. *T.annulata* infected cells can therefore cause the activation of the majority of T cells from naive animals irrespective of memory status and, presumably, antigen specificity.

The cytokines produced by IC stimulated T cells 1-7 days post stimulation were assessed by reverse transcription polymerase chain reaction (RT-PCR) using primers for IL2, IL2 receptor (IL2R), IL4 and interferon gamma (IFNγ). None of these cytokines were found to be expressed by IC. T cells within PBM expressed mRNA for IL2, IL2R, IL4 and IFNγ 24-48 hours post IC stimulation. IL2 and its receptor were still expressed at day 5 (peak proliferation), and waned by day 7. IFNγ was expressed by all tested animals' cells at all timepoints, while IL4 was intermittently found at day 5 and was always absent at day 7. IL4 was only expressed by CD4 T cells, while IL2/IL2R/IFNγ was expressed by all T cell types. The presence of CD4 cells was required for IL2 and IL2R expression by non CD4 T cells.

In vivo examination was of the draining lymph node (DLN) which is the principal site of parasite development, ideally placing the parasite to interfere with the induction of immunity. DLN exhibited radically altered distributions of T cells expressing activation markers from accepted steps in immune response development. Infected cells were first manifested as proliferating non T non B cells which disseminated throughout the node and which expressed monocyte surface markers. Medullary T cells (predominantly CD4) clustered around IC and expressed CD25 within 2 days of infection. This pattern persisted 4 days post infection, but numbers of CD25 T cells subsequently declined and none were found in the node by 10 days post infection. Altered function of DLN T cells was most obvious in the destruction of DLN germinal centres (GC), particularly the T cell dependent light zones. Although T cell activation marker expression waned, CD3 cells were not depleted from GC light zones or from the node in general. This may suggest that soluble factors play a role in interfering with immunity. Cytokine analysis of DLN cells early in infection demonstrated that production of IL4 within the entire node ceased within 4 days of infection, while in contrast IFNγ production remained and has been shown in other experiments to become elevated. The control of B cell proliferation and differentiation depends partially upon the balance between stimulatory IL4 and inhibitory IFNγ. The dominant production of IFNγ may be leading to the disruption observed in GC by both changing the IL4 production capabilities of GC T cells and directly inhibiting B cell differentiation.

In summary, this thesis has shown that *T.annulata* infected cells posses an innate ability to activate naive T cells. Although all T cell types are activated in PBM, this is dependant upon cytokine release by CD4 cells, subsequently leading to a type 1 response. *In vivo*, a similar mechanism leads to activation of DLN T cells primarily by IC. Such interactions do not lead to the induction of an antigen specific immune response, but to the loss of GC and suppression of further T cell activation.

Declaration

I hereby declare that the work presented in this thesis is my own, except where stated in the text and appendix. The work has not been submitted in any previous application for a degree.

John D.M. Campbell

CONTENTS

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Abbreviations

Abbreviation	Full form
[^{3}H]dThd	tritiated thymidine
ACD	acid citrate dextrose
APC	antigen presenting cell
BCG	Bacille Calmette Guerin
BoLA	bovine lymphocyte antigens
BPA	bovine plasma albumin
CD	cluster of differentiation
cDNA	complimentary DNA
Con A	concanavalin A
CTL	cytotoxic T lymphocytes
DMSO	dimethyl sulfoxide
DLN	draining lymph node
EDTA	ethylene di-amino tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence activated cell scanner
FITC	fluorescence isothiocynate
FSC	forward scatter
GAM	goat anti-mouse
GUTS	ground up tick supernate
HBSS	Hanks balanced salt solution
HEV	high endothelial venule
HIV	Human Immunodeficiency Virus
HPLC	high performance liquid chromatography
IEF	isoelectric focusing
IFAT	indirect fluorescent antibody test
IFN	interferon
Ig	immunoglobulin
IL	interleukin
kD	kilodalton

LN	lymph node
LPS	lipopolysaccharide
mAb	monoclonal antibody
mCi	millicurie
MEM	minimum essential medium
MHC	major histocompatibility complex
MLC	mixed lymphocyte culture
MLR	mixed lymphocyte reaction
mM	millimole
mw.	molecular weight
NK	natural killer cell
NGS	normal goat serum
NRS	normal rabbit serum
PBM	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PCV	packed cell volume
PE	phycoerythrin
PI	propridium iodide
PMT	photomultiplier tube
RAM	rabbit anti-mouse
RBC	red blood cells
RFLP	restriction fragment length polymorphism
spp.	species
SSC	side scatter
t.e.	tick equivalent
TC	tissue culture
Th	T helper
TNF	tumour necrosis factor
WC	workshop culture

CHAPTER 1

Introduction and Review of Literature

INTRODUCTION

Although farming methods have improved greatly in some third world countries eg Asia and South America, others have lagged behind - 80% of farmers in Africa have been estimated to operate on a subsistence level only (Innes *et al*, 1992). One of the most important breeds to these farmers is cattle, but the productivity of local animals in the tropics tends to be low (Pino, 1981). In order to improve productivity, many countries either import or cross breed "exotic" European cattle with local breeds (Ghill *et al*, 1980).

The most serious barrier to this improvement strategy has been identified by Uilenberg *et al* (1993) as the tick borne diseases theileriosis and babesiosis. Theileriosis covers the diseases caused by the protozoan parasites *Theileria annulata* (tropical theileriosis) and *T.parva* (east coast fever). *T.annulata* infection is the subject of this thesis. In 1982, it was estimated that over 250 million cattle were at risk from *T.annulata* infection (Robinson, 1982), with the parasite found in Southern Europe, across the mediterranean littoral, southern former USSR, North Africa, the Near and Middle East, India, China and The Far East (Purnell, 1978; Robinson, 1982). Tropical theileriosis places an enormous burden on the economy of developing countries - in a recent study losses of over $15 million per annum were identified in Morocco alone (R.L. Spooner pers. comm.).

Transmitted by ticks of the *Hyalomma* genus, infective *T.annulata* sporozoites localise in the lymph node draining the site of the tick bite, developing into the macroschizont stage in host leucocytes. These cells are induced to divide rapidly in the node, giving rise to the microschizont stage, followed by host cell rupture to release the merozoite. This stage subsequently invades red blood cells to form the piroplasm. Disease is characterised by high temperature, anaemia and respiratory distress (Barnett, 1977). Infection with *Theileria annulata* leads to morbidity and some mortality in animals native to endemic areas (Brown, 1990). However, the principal problem with associated tropical theileriosis is the extremely high disease susceptibility of European cattle. Mortality rates in these animals range between 40-90%, presenting a severe constraint on cattle improvement in endemic areas

(Robinson, 1982; Uilenberg *et al*, 1993; Hashemi-Fesharki, 1988). Such susceptible cattle seem incapable of mounting an effective immune response, with extreme disease symptoms often leading to death within 2-3 weeks of experimental infection (Samantery *et al*, 1980; Preston *et al*, 1992a). The mechanisms underlying susceptibility of European cattle are not as yet understood.

Parasite control and subsequent immunity can be achieved through the use of "Butalex" which kills the intracellular parasite (McHardy *et al*, 1985; Dhar *et al*, 1990). However, this drug is not in wide use, mainly due to high cost. A degree of control over the disease in some countries has also been obtained through the use of a live attenuated vaccine (Hashemi-Fesharki, 1988; Pipano, 1981). However, there are several problems associated with this vaccine: large cell doses are used in some countries (Adalar *et al*, 1994), and these are laborious and costly to produce; the live vaccine induces carrier status in immunised animals; distribution is problematical; immunity induced by the vaccine appears to wane (Pipano, 1977); revaccination with the same cell line may actively decrease protection (Ouhelli *et al*, 1994; Nichani, 1994). All of these factors have made the design of a vaccine not based on live parasite extremely desirable.

Any novel control methods must combat the acute disease seen in susceptible animals, and there has been a push to find protective parasite antigens over the last few years. However, all of these attempts are largely "shots in the dark" as the mechanisms causing immune response failure and pathogenesis against which they are supposed to protect are unknown. The work presented in this thesis has investigated in detail the causes of immune response failure and pathogenesis during sporozoite infection of susceptible animals, in order to provide fundamental information required for the rational design of improved control methods.

The macroschizont stage of the parasite is the most pathogenic - all the symptoms of tropical theileriosis can be induced by this stage alone (Hooshmand-Rad, 1976). There is strong evidence that the macroschizont is responsible for the immune response failure in primary infections - when animals are drug treated, killing the macroschizont, a protective immunity forms (McHardy *et al*, 1985; Dhar *et al*, 1990) mediated by macroschizont specific cytotoxic T lymphocytes (CTL) (Preston

et al, 1983; Innes *et al*, 1989a). Immune function can therefore be restored by killing the macroschizont. Also, *in vitro* observations suggest that macroschizont infected cells (IC) can influence T cell responses - T cells from naive animals are found to proliferate when cultured with autologous IC (Rintelen *et al*, 1990; Glass and Spooner, 1990a). This apparently innate ability to induce proliferation of T cells can disrupt immune responses *in vitro*. T cells from the blood of immune animals are induced to proliferate "non specifically" blocking the isolation of any parasite specific T cells (appendix 2). If such a phenomenon were to operate *in vivo* it would be a prime candidate for the immune disfunction seen in infected susceptible animals. In this thesis, the role that "non specific" macroschizont infected cell activation of T cells plays in immune disfunction has been investigated in depth. The work is divided into three areas:

1. Detailed examination of proliferation and activation of T cells from naive animals induced by autologous *T.annulata* macroschizont infected cells, in order to define the exact T cell activation capabilities of the parasitised cells.
2. The likely function of *T.annulata* activated T cells, and the role different T cell subsets have in responses, has been assessed by examining cytokine mRNA expression.
3. The role that macroschizont induced T cell activation plays in *in vivo* immune disfunction has been examined in sporozoite infected draining lymph nodes.

The rest of this chapter is devoted to a review of relevant literature, covering the biology of *T.annulata* and the pathogenesis of infection. This is followed by a discussion of bovine T cell effector mechanisms, the induction of primary immune responses in lymph nodes, and immune responses in tropical theileriosis.

REVIEW OF LITERATURE

Tropical theileriosis - *Theileria annulata* infection

In this review of *Theileria annulata* infection, reference to the related parasite *Theileria parva* will also be made. Historically, research has been concentrated on *T.parva* and some parallels can be usefully drawn between the two diseases. However, although basic parasite biology is similar in many areas, care should be taken in drawing parallels between the pathogenesis of and immunity to the different parasites, as there are large differences in key aspects of the two parasites' behaviour.

Taxonomy

The first *Theileria* species to be named was *Theileria parva* by Koch in 1904, followed by the discovery of *T.annulata* by Dschunkowsky and Luhs (1904), although both piroplasm and schizont forms of *T.parva* were almost certainly observed by Koch at the turn of the century (reviewed by Norval *et al*, 1992). Both species were originally named *Piroplasma*, with the change to *Theileria* coming in 1907 (Bettencourt *et al*, 1907). Levine, (1988) lists the names of 39 *Theileria* species, although some of these are almost certainly synonyms (Uilenberg, 1981b). Only *T.annulata* and *T.parva* have been studied in depth, as they are the only highly pathogenic forms of *Theileria*.

The currently accepted classification of *Theileria* is illustrated in the following table (from Irvin, 1987 and Norval *et al*, 1992). *Theileria* diverges from malarial parasites with its classification as Piroplasmia which contains the single order Piroplasmida of which *Theileria* and *Babesia* are the only members (Barnett, 1977). The principal differentiating feature between *Theileria* and the malarial parasites (order Eucoccidia) is the lack of a crystalloid and pigment from the erythrocytic stages in the piroplasmodia.

Kingdom	Protista
Sub Kingdom	Protozoa
Phylum	Apicomplexa
Class	Sporozoea
Sub Class	Piroplasmia
Order	Piroplasmida
Family	Theileriidae
Genus	*Theileria*

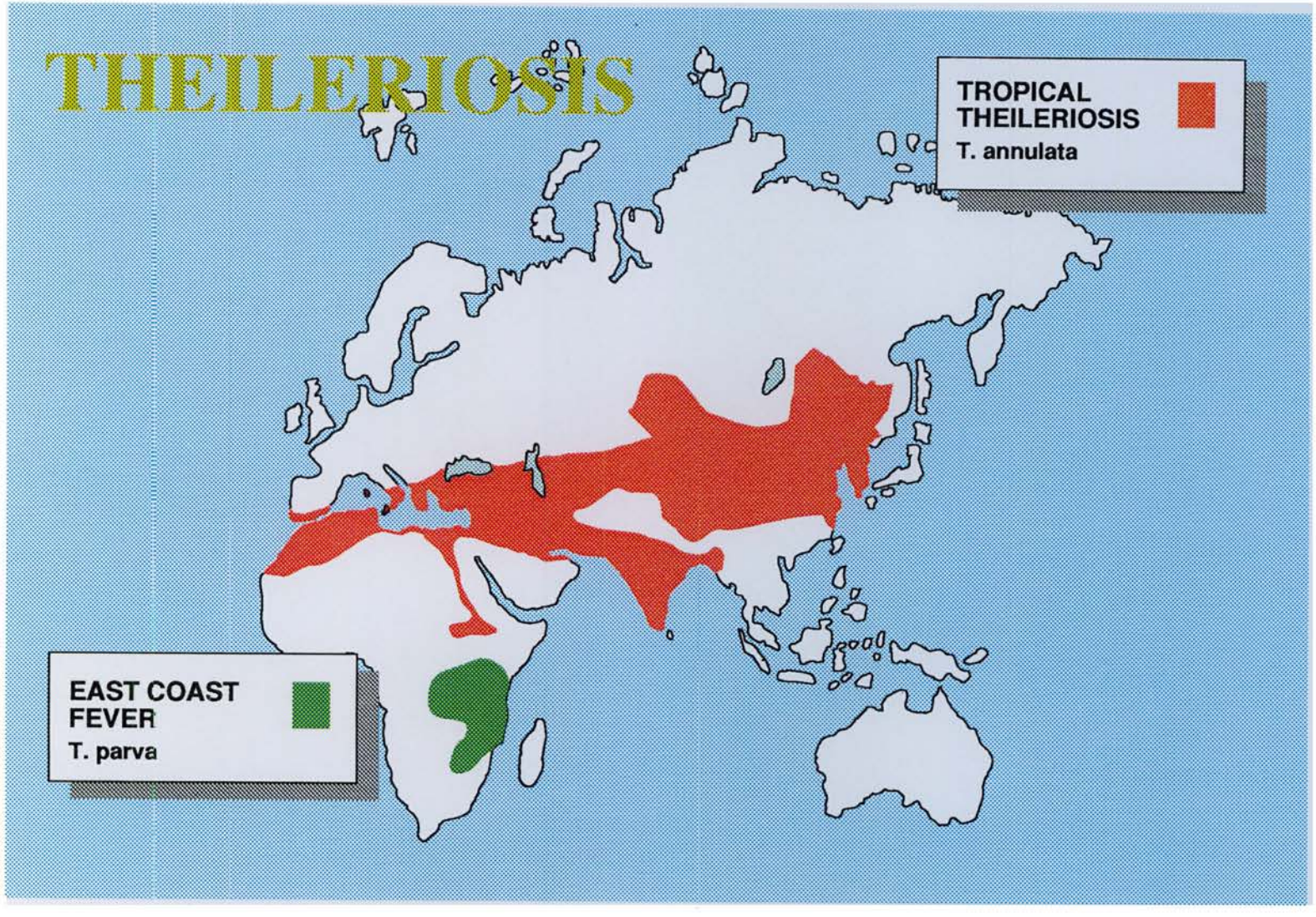
Geographical distribution

T.annulata was originally identified in Southern Russia by Dschunkowsky and Luhs (1904). Today, tropical theileriosis is recognised as an extremely widespread disease, present in Southern Europe, across the mediterranean littoral, southern former USSR, North Africa, the Near and Middle East, India, China and The Far East (Purnell, 1978; Robinson, 1982) (Illustrated overleaf). *T.parva*, in contrast is limited to Africa, particularly the east coast, but also in the central and southern areas (Norval, 1992; Uilenberg *et al*, 1993). The only country where both species have been identified is the Sudan (Norval, 1992).

The geographical localisation of the parasites may be due to the distribution of their vector ticks - *T.annulata* is exclusively transmitted by ticks of the genus *Hyalomma*, while *T.parva* is transmitted by some *Hyalomma* ticks but mostly by the *Rhipicephalus* genus (Purnell, 1978; Norval, 1992). Several species of *Hyalomma* have been implicated in transmitting *T.annulata* - *H. anatolicum anatolicum*, *H. detritum*, *H. a. excavatum, H. lusitanicum*, H. *marginatum* and *H. scupense* were all described by Purnell (1978) as potential vectors in the northern mediterranean littoral, and up to 15 *Hyalomma* species have been demonstrated as capable of disease transmission (Robinson, 1982). *H. anatolicum anatolicum* and *H. detritum* are the two best recognised vectors, with *H. anatolicum anatolicum* present from north west Africa through to India, while *H. detritum* is found in the far East, India and the former USSR. but reaching as far as North Africa. (Norval, 1992).

Host range and susceptibility

Although sporozoites of *T.annulata* have been shown to invade cells from cattle, buffalo, goat and sheep *in vitro* (Steuber *et al*, 1986), field cases are found only in cattle, buffalo, yak, and their cross breeds (Barnett, 1977, Wenshun, 1994). The principal problem presented by tropical theileriosis is the susceptibility of "exotic" european *Bos Taurus* cattle to the disease, with breeds from endemic areas apparently relatively resistant, although Yak crossbreeds with ox are extremely susceptible in China (Wenshun, 1994). Mortality rates of up to 90% in exotic cattle, and of only 5% in indigenous breeds are recorded in the review by Robinson (1982),

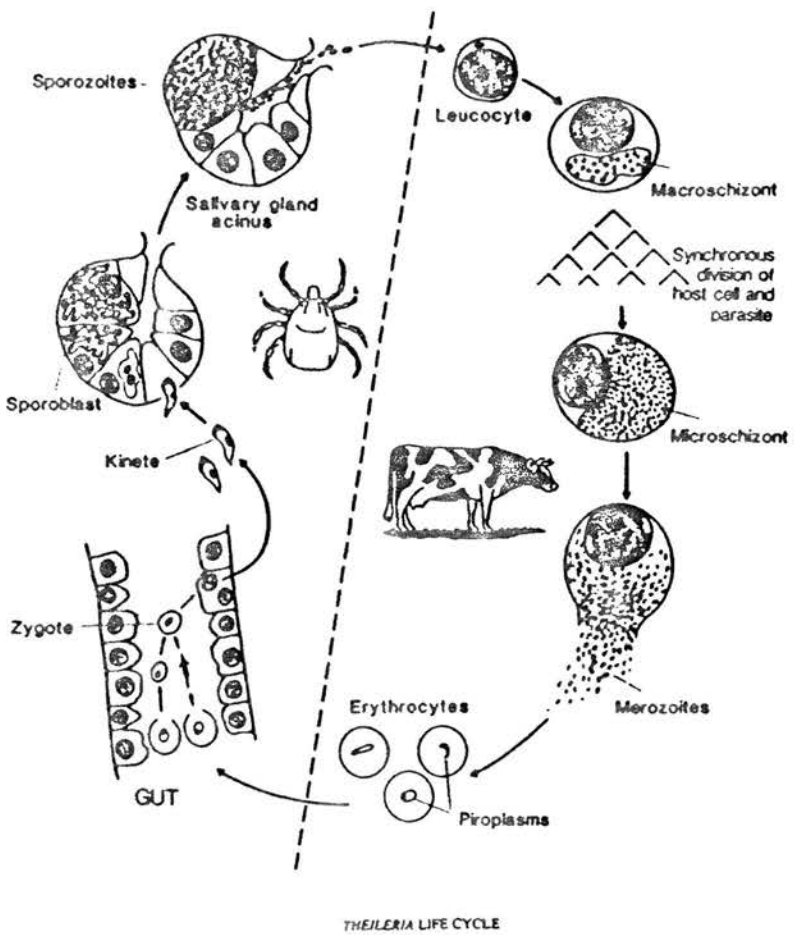

THEILERIOSIS
TROPICAL THEILERIOSIS
T. annulata
EAST COAST FEVER
T. parva

with mortality of 40-70% quoted in other reviews (Uilenberg *et al*, 1993; Brown, 1990). In Iran, mortality of exotic cattle is 40-80%, compared to 10% in indigenous breeds (Hashemi-Fesharki, 1988). In a study of an endemic area of Morocco, local breeds, although largely serologically positive, were much less likely to develop clinical disease than imported Friesians (Oudich *et al*, 1993). There is no hard evidence for genetic resistance in indigenous cattle, although Preston *et al* (1992a) showed that *Bos Taurus* cross *Bos Indicus* calves were marginally more resistant to sporozoite infection than pure *Bos Taurus*. A more likely explanation is that local cattle are exposed to the disease from an early age - 76% of adults were found to be piroplasm carriers in a survey in Morocco (Flach and Ouhelli, 1992). Thus imported adult cattle are compared to a population already exposed to disease. There is also little evidence that cross bred animals born in endemic areas are resistant - in India where the majority of calves are cross breeds, tropical theileriosis is still a major problem (Grewal *et al*, 1991).

Patterns of parasite infection vary between countries - clinical theileriosis affects up to 50% of new-born calves in India, with up to 33% mortality (Grewal *et al*, 1991). In Pakistan, clinical infection is again present in young animals under nine months of age particularly animals between 2-4 months (Hussain *et al*, 1991). In North Africa (Morocco), infection patterns are different, with the percentage of cattle infected progressing with age, until the vast majority of adults have been exposed to the disease. Here, there is little evidence of infection in the new born (Flach & Ouhelli, 1992). The difference in disease may be due to tick feeding patterns, as mature *H.detritum* (the infective stage) in Morocco were found to attach preferentially to older animals (Flach and Ouhelli, 1992). Conversely, the three stage tick *H.a.anatolicum* is the principal vector in India, and spreading of infection by both nymphs and adults may account for the infection in young animals.

Lifecycle

The lifecycle of *T. annulata* is illustrated overleaf. Descriptions of parasite biology are from both *in vivo* and *in vitro* observations. Cells infected with *T.annulata* have been isolated from infected animals and kept in culture over the last fifty years - first



Lifecycle of *Theileria spp.* From Nichani (1994)

reported by Tchernomoretz (latterly known as Tsur) in 1945. A major advance came when Brown *et al* (1973) were able to establish macroschizont cultures by adding sporozoites obtained from ground up *T.parva* infected ticks to uninfected peripheral blood mononuclear cells (PBM). The techniques have been periodically revised and adapted very successfully for *T.annulata* (Brown, 1979;1981;1983;1987). Such culture systems have allowed a great many fundamental questions to be addressed without infection of an animal, or repetitive passaging of the parasite through ticks and cattle.

Development within cattle

Bovine leucocytes are infected by sporozoites produced in the saliva from the bite of an infected tick. There are multiple sites at which the sporozoite can meet its leucocyte target - in the mouth parts of the tick; the bite site; surrounding inflamed tissues; afferent lymphatics; the draining lymph node. Since infection can be satisfactorily induced by experimental subcutaneous injection of sporozoites, the exact site of sporozoite/leucocyte interaction may not be greatly significant. Sporozoites have been shown to quickly enter cells *in vitro* (within 5-60 minutes) in a temperature dependant process of membrane invagination (Jura *et al*, 1983). Although invasion is via the basal end, and capping of sporozoites can be observed, no ligand has yet been identified for sporozoite host cell recognition and invasion. Post cell invasion, the parasite loses its rhoptries and enters the uninucleate trophozoite stage, (Jura *et al*, 1983). 18-24 hours later, following mitosis, the trophozoite then produces independently dividing nuclear particles to form the multinucleate macroschizont. *In vivo*, macroschizont infected cells appear 5-8 days post infection within the draining lymph node. In experimental infections, the time at which macroschizont infected cells are first detected is dependant upon the dose of sporozoites administered (Preston *et al*, 1992a). Macroschizont infected cells leave the draining lymph node soon after their first appearance - a study of efferent lymph from infected nodes detected macroschizont cells leaving the node within 6 days of infection (Nichani, 1994).

Once the macroschizont stage has developed, infected host cells are stimulated into

many division cycles, and as the parasite divides with the host cell parasitaemia within the draining node increases rapidly. Parasite replication occurs in a "window" of the host cycle, with DNA synthesis during the host G_2, and division before the host metaphase (Jura *et al*, 1985). The parasite apparently divides by aligning with the host cell spindle (Hulliger *et al*, 1964), and recent work presented by Shochat (in Hall and Baylis, 1993) suggests that *T.parva* schizonts may associate directly with the host chromatin during division. *In vitro*, macroschizont infected cells divide and grow indefinitely without any exogenous stimuli. Although the mechanism for this apparent transformation is unknown, recent studies have shown that infected cells express the Ki-67 nuclear proliferation antigen (Shayan *et al*, 1994). This antigen, common to many tumours (Gerdes *et al*, 1987), is expressed in cells outwith the G_0 phase of the cell cycle (Gerdes *et al*, 1984), and confirms that infected cells are stimulated to permanently enter the cell cycle.

The microschizont stage, 50-120 small nuclear particles filling the cytoplasm of the cell (Barnett, 1977), forms after several cycles of macroschizont division. The stimulus to form this stage and subsequent merozoites *in vivo* is not known. A potential candidate trigger is fever of the infected animal - macroschizont infected cells, which do not differentiate *in vitro*, can be triggered to do so by heat shock at 41°C (Glascodine *et al*, 1990). Formation of merozoites (merogony) takes place within microschizont infected cells with the formation of rhoptry and microneme organelles (Melhorn & Schein, 1984). New gene expression occurs at this stage, with the suppression of previously expressed genes also present - represented by a loss of schizont surface antigens and expression of novel determinants (Glascodine *et al*, 1990). Formation of the merozoite stage involves breaking the synchrony between division of the host and parasite nucleii (Shiels *et al,* 1992). In the initial stages (lasting 3-4 days), the process is reversible and characterised by an increase in both host and parasite division. Subsequently, host cell division diminishes, while the parasite division continues, at this point the cell is committed to the merozoite stage. Once formed, merozoite release is effected by host cell rupture.

Once released, merozoites invade erythrocytes, again by an unknown mechanism, developing into the piroplasm stage, which can in turn re-infect ticks. In

experimental infections, piroplasms are documented as appearing within 9 days of infection, with the magnitude and speed of onset of parasitaemia again proportional to the dose of sporozoites administered (Preston *et al*, 1992a). Even in recovered animals, piroplasms can usually be detected at low levels throughout life. Even when parasite cannot be visually found in blood smears, or detected by serological tests, polymerase chain reaction (PCR) can detect parasite DNA in the circulating blood (Jongejan *et al*, 1994). Piroplasms can divide asexually within erythrocytes, either by binary fission (Melhorn & Schein, 1984) or schizogony (Conrad *et al*, 1985), although this has not been reproduced *in vitro*. Merozoites produced by intraerythrocytic division may add to persistent parasitaemia in recovered animals.

Development within the tick

Upon ingestion, erythrocytes lyse in the tick gut, initiating the sexual reproductive cycle of the parasite. Gametes can be detected 4-6 hours post ingestion, with zygotes visible within the gut wall by 3 days (Schein, 1975). The next stage to form is the "club-shaped" kinete, which penetrates the gut wall to enter the haemolymph. The time taken for kinete formation and migration to the salivary gland is variable (reported to be 7-20 days post ingestion), and may depend upon the moult status of the tick (Schein and Friedhoff, 1978; Reid and Bell, 1984). Kinetes invade type II or III acinii (Schein and Friedhoff, 1978), and undergo a series of divisions until a large mass of parasite material, visible as membrane bound sub units containing mitochondria and nuclear material, is formed (Reid and Bell, 1984).

Transmission of sporozoites occurs when a previously infected tick feeds on a new susceptible host (Robinson, 1982). Before transmission can take place, the sporozoites require to mature in the salivary gland, stimulated usually by engorgement of the tick. Feeding results in the disaggregation of the "mosaic" of parasite material into discreet particles and the formation of rhoptries (Reid and Bell, 1984). As feeding on a non susceptible host (eg. rabbit) induces maturation but not transmission, this process can be successfully used for obtaining sporozoites for animal or *in vitro* infection. For example, feeding of *H.a.anatolicum* on rabbits produces large numbers of sporozoites peaking after three days feeding (Walker and

McKellar, 1983a). High temperatures can also induce sporozoite maturation (Samish, 1977), so ticks in endemic areas of high temperatures may be potentially infective immediately upon host attachment.

Sporozoite transmission in *T.annulata* is transtadial ie. ticks become infected when feeding and transmit the parasite following the next instar with formation of new mouth parts (Robinson, 1982). For example, the theileriosis season in Morocco correlates well with attachment of adult *H.detritum* to cattle, while the peak larval and nymph feeding times do not induce infection. These immature ticks which have become infected during feeding then overwinter to transmit sporozoites the following summer (Flach and Ouhelli, 1992). In his review, Robinson (1982), considers many reports either supporting or discounting transmission from adults to egg (transovarial transmission). This only has potential relevance in ticks which spend their life on one host eg. *H.scupense*. As Robinson points out, such ticks are not important vectors and thus transovarial transmission, if it occurs, is unlikely to be relevant in the field.

Leucocytes infected by sporozoites

Cells infected by Theileria annulata sporozoites

The preferred target cell for sporozoites of *T.annulata* to infect and subsequently transform into continuously growing cell lines has been a matter for debate for some time, partially due to a lack of reagents to unequivocally identify infected cells. Early attempts at defining infected cells identified "reticular" cells of the lymph node, reticular endothelial system, and the kupffer cells of the liver as infectable with sporozoites (Sergent, 1945). Brown and Gray (1981) infected and maintained cultures of "fibroblastic" cells with *T.annulata* but not *T.parva*. Currently, very little information is available about the cells infected *in vivo*, but a large amount of information has been gathered *in vitro* using monoclonal antibodies (mAb) and cell sorting.

Very clear evidence of the cell types infected by *T.annulata* was obtained by Glass *et al* (1989) and Spooner *et al* (1989), demonstrating that *T.annulata* sporozoites infected MHC class II^+ cells from peripheral blood. The parasite had a marked preference for monocytes and macrophages, infecting B cells to a lesser extent. This

was in marked contrast to *T.parva* which readily infected T cells and B cells, but not macrophages (Spooner *et al*, 1989). The strong preference of *T.annulata* for non T cells was also demonstrated by Innes *et al* (1989b), who found that bovine cytotoxic T cell lines (rapidly infected by *T.parva*) were very poorly transformed by the parasite. In this respect the classification of tropical theileriosis as a "lymphoproliferative" disease is something of a misnomer as the principal cell infected is the monocyte rather than the lymphocyte.

Further sorting experiments have shown that cells expressing CD14 (the LPS receptor) are very good sporozoite targets, and that both mature macrophages and small monocytes within this population are infected (Campbell *et al*, 1994). Although CD14 is expressed upon a small percentage of human peripheral B cells (Ziegler-Heitbrock and Ulevitch, 1993), cell sorting of bovine PBM with anti-CD14 isolates only macrophages (D.J. Brown, unpublished observations). The inability to infect bovine $CD14^-$ cells with sporozoites (Campbell *et al*, 1994) raises questions about the avidity of the parasite for B cells, as the $CD14^-$ fraction of PBM would have contained large numbers of B cells. The situation is further complicated as sporozoites can infect the bovine B cell lymphoma line BL20. However, as this cell line is already transformed and continuously growing, such infected lines may not represent a relevant natural *Theileria* transformation.

One of the most important reasons for identifying target cells is to examine possible ligands for sporozoite attachment and entry to cells. One such candidate ligand was the elastin receptor, as sequences mimicking the binding motifs for this receptor were found within the sporozoite antigen SPAG-1 (Hall *et al*, 1992). Although the elastin receptor is expressed upon macrophages, the principal sporozoite target cell group, *T. annulata* sporozoites infected sorted elastin receptor positive and negative cells equally well in *in vitro* experiments (Campbell *et al*, 1994).

Phenotypes of macroschizont infected cells

Analysis of infected cell lines post sporozoite infection has been extremely useful in consolidating information from sporozoite infection studies. Examining cell lines which have become established from such infection experiments has helped to clarify

the picture. Phenotypic analysis of established infected cell lines has shown that cell lines are MHC class II^+, but do not express CD4, CD8, or surface IgM (Spooner *et al*, 1989). In addition, cell lines are also recognised by the bovine monocyte/macrophage specific mAb IL-A24 (Glass and Spooner, 1990a;Campbell *et al*, 1994). Infected cells have been documented as expressing CD2 (Howard *et al*, 1993), and although this is expressed on T cells this is also found co-expressed with IL-A24 on the surface of ruminant macrophages (Haig *et al,* 1991). Monocytes/macrophages, which expressed the macrophage markers CD14, IL-A109 (thought to be FcγR1, MacHugh *et al*, 1990) and the elastin receptor before infection are found to lose expression of these markers post infection, acquiring the MHC class II^+/IL-$A24^+$ phenotype (Campbell *et al*, 1994). Infection therefore induces a uniform macrophage-like phenotype, irrespective of the initial cell type infected.

Clinical Disease

The time of onset of clinical theileriosis post sporozoite inoculation varies, is dose dependant in experimental infections (Samantery *et al*, 1980; Preston *et al*, 1992a), with natural tick infection taking between 9-25 days (Neitz, 1957). This is also apparently dependant upon the numbers of feeding ticks, but even tick numbers described by Barnett (1977) as "heroic" do not induce symptoms before 5 days. The first sign of a clinical response is usually the appearance of schizonts in and swelling of the draining lymph node within 5-8 days, which usually precedes the onset of fever by 1-4 days depending on dose (Barnett, 1977;Samantery *et al*, 1980; Preston *et al*, 1992a). Piroplasms can be detected in the circulation 2-5 days after lymph node schizonts, and animals become progressively anaemic (these two events are not necessarily connected see below). Other clinical manifestations of disease include cachexia, leucopaenia, inappetance, weight loss, mucous membrane discharge, cessation of rumination, dyspnoea (breathing difficulties) and haemorrhagic diarrhoea (Neitz, 1957;Barnett, 1977;Samantery *et al*, 1980; Preston *et al*, 1992a).

Terminal cases are characterised by high parasitaemia - up to 25% schizonts in the draining lymph node and 65% piroplasms in an experimental study (Samantery *et al*, 1980). Terminal clinical signs include hypothermia, jaundice, recumbency,

respiratory distress, and occasional regression of enlarged lymph nodes (Neitz, 1957; Barnett, 1977). An overall mortality rate of 40-60% has been estimated (Uilenberg, 1981a; Brown, 1990; Hashemi-Fesharki, 1988), with death, probably due to severe anaemia, occurring 8-15 days post clinical manifestation (Neitz, 1957).

Diagnosis

Diagnosing *T.annulata* infection is still largely dependant upon the skill of field workers recognising parasite in biopsies from lymph nodes, piroplasms in the blood and classifying post-mortem lesions. For prevalence studies, great use has also been made of the seroconversion following sporozoite infection of animals (Kachani *et al*, 1992a; 1992b). Antibodies against macroschizont and piroplasm antigens are detected using the indirect fluorescent antibody test (IFAT) (Burridge and Kimber, 1972). In this test, serum from cattle is incubated with macroschizont or piroplasm infected cell antigens, and positive sera are detected by a fluorescent secondary antibody. This test has a number of disadvantages, as preparations cannot be stored for long periods of time and a degree of skill is required in interpreting results. This has prompted the development of enzyme linked immunosorbent assays (ELISA) (Kachani *et al*, 1992b) and DOT-ELISA (Grewal *et al*, 1991; Grewal, 1992), although neither of these assays is as yet commercially available. PCR techniques have also been developed to detect parasite DNA in infected ticks and cattle (de Kok *et al*, 1993; Jongejan *et al*, 1994). These are extremely useful tools in the laboratory, but are not likely to be practical for use in the field in the foreseeable future.

Pathology of infection

Anatomical distribution of the parasite.

Macroschizont infected cells are localised primarily within the draining lymph node, persisting until death of the host, and have been documented as exiting in efferent lymph within 6 days of infection (Nichani, 1994). Macroschizont infected cells leave the node in increasingly large numbers from as early as 6 days post infection (up to 37% of all efferent lymph are infected cells by day 10), with the appearance of infected cells correlating with the onset of fever (Nichani, 1994). Post mortem,

macroschizont infected cells can be detected in most major organs, including the abomasum, adrenal gland and lungs (Forsyth *et al*, 1994). In contrast to *T.parva*, *T.annulata* schizonts are not generally found in other lymph nodes, whereas in *T.parva* up to 30% of all lymph nodes may contain schizonts (Morrison *et al*, 1981). This illustrates different dissemination patterns for the two parasites, which may be related to the cells infected - *T.annulata* primarily infecting macrophages while *T.parva* infects T cells (Spooner *et al*, 1989; Glass *et al*, 1989; Campbell *et al*, 1994). It is possible that infected T cells are able to recirculate and home within the lymphoid system, whereas macrophages cannot do so.

Although not fully understood, the distribution of *T.annulata* schizont infected cells from the lymph node is likely to be "metastatic", with parasite travelling in the blood stream. Recent work by Baylis *et al* (1995) has shown that infected cells produce factors similar to matrix metalloproteases. In *in vitro* assays, macroschizont infected cells were able to digest through and cross protein matrices in a manner dependant upon such metalloproteases. Such a mechanism may be important in *T.annulata* invasion of tissues. Although the main site of schizont infected cell development, removal of the draining node 48 hours post infection does not stop clinical disease, although the onset is delayed (J.D. Campbell & R.L. Spooner unpublished obs.). This may be due to trophozoite infected cells, which are indistinguishable from uninfected cells in conventionally stained blood or lymph preparations, leaving the node immediately upon infection. Alternatively, infection in such cases may be due to formations of infected cells in other areas, eg. inoculation site or dissemination of sporozoites from the efferent lymph to other sites.

Post Mortem Pathological lesions

Pathological lesions are mainly associated with lymphopoietic and vascular systems. Gross post-mortem findings include emaciated and pale carcass, generalised symptoms of anaemia and jaundice; oedema of lymph nodes and spleen but particularly the lungs; haemorrhages of the serosal surfaces (Neitz, 1957; Uilenberg, 1981a). The most common lesions in tropical theileriosis are haemorrhagic ulceration of the abomasum, known as "punched" necrotic ulcers (these may also extend

throughout the entire intestine); enlarged gall bladder with dark green viscous bile; enlarged, discoloured and haemorrhagic kidneys and liver. (Neitz, 1957). It is only recently that detailed histological study of post mortem pathology within differing organs has been carried out. Eisler (1988) found that lymph nodes, particularly the draining lymph node, were oedematous, haemorrhagic with a loss of normal lymphoid architecture. The adrenal gland shows extensive damage to cortex cellular architecture, and the ulcerated abomasum has disruption to villi organisation. In the lungs, alveolar walls are extensively swollen, with disruption of architecture (Forsyth *et al,* 1994).

Mechanisms of pathogenesis

As noted above, principal features of the disease are anaemia, leucopaenia, anorexia and pulmonary oedema. As infection of animals with macroshizont infected cells which have lost the ability to produce piroplasms still results in disease and anaemia (Hooshmand Rad, 1976), piroplasm destruction of erythrocytes may not be the principal mechanism of anaemia. Autohaemagglutinating antibodies can be found in infected animals (Hooshmand Rad, 1976), and phagocytosis of erythrocytes by macrophages via such antibodies has been suggested as a possible mechanism of anaemia (Uilenberg, 1981a), although the mode of action has not been fully elucidated. In a recent study of leucopenia following experimental infection, numbers of circulating leucocytes were found to drop by up to 72%, depending on parasite dose. Losses were found in both the lymphocyte and neutrophil populations (Preston *et al*, 1992a). Again the reasons for this response are unclear, although Eisler (1988) found the bone marrow of acutely infected animals to be lymphoplastic, perhaps indicating a degeneration in lymphopoiesis. Also a feature of *T.parva*, leucopenia has been attributed to retardation of lympho- and granulopoiesis in the bone marrow and also in thymus (reviewed in Wilde, 1967).

The exact causes of the pathogenesis are thus still a matter of debate. A recent finding by Forsyth *et al* (1994) was that lesions within various internal organs were not related to numbers of macroschizont infected cells, and pathology may therefore be due to systemic effects rather than direct action of the parasite within various

sites. A more persuasive model for pathogenesis of tropical theileriosis can perhaps be built around the previous observation and the discovery that macroschizont infected cells express high levels of mRNA for tumour necrosis factor alpha (TNF-α) (Brown *et al*, 1995). TNF-α was also previously detected in PBM from animals lethally infected with sporozoites (Preston *et al*, 1993).

Production of large amounts of TNF-α by macroschizont infected cells and subsequent release to the circulation via the efferent lymph potentially could account for disease pathogenesis in many organs in the absence of infected cells. Anorexia, leucopenia, oedema and fever can be induced directly by experimental infusion of TNF-α in cattle (Bielefeldt Ohmann *et al*, 1989), with lesions, some haemorrhagic, within various organs similar to those seen in tropical theileriosis. In addition, the severity of anaemia in cattle infected with *Trypanosoma vivax* and *T.congolense* can be directly related to production of TNF-α by monocytes (Silegham *et al*, 1994). Production of TNF-α by *T.annulata* infected monocytes therefore has the potential to induce the pathology described in clinical cases. Possible mechanisms aiding such a TNF-α release will be partly examined in this thesis.

Current control measures against tropical theileriosis

Vaccination

Vaccination against tropical theileriosis is carried out in a large number of countries eg. China, Israel, Turkey, Morocco and India (Wenshun *et al*, 1994; Pipano, 1977; Adalar *et al*, 1994; Ouhelli *et al,* 1994; Singh, 1992 respectively). This involves the use of macroschizont infected cell lines which have been attenuated through long term culture *in vitro*. Cell lines do give adequate protection against sporozoite infection, inducing anti-macroschizont cytotoxic T lymphocyte (CTL) responses (Innes *et al*, 1989a) which are discussed in more detail in the "immune responses to tropical theileriosis" section of this review.

Cell line immunisation induces a carrier state in animals, making total eradication of the disease impossible. In addition to this fundamental problem, there are a number of practical difficulties associated with the vaccine which require to be addressed in order to "perfect" vaccination strategies. The number of cells given as

a vaccinating dose is a matter for debate. For example, as few as 10^4 cells give good protection in Morocco while 10^7 are used in Turkey (Ouhelli *et al*, 1994; Adalar *et al*, 1994). As a result, in countries where high doses are used, vaccine production is labour intensive and expensive. There is also no current reliable method for measuring the degree of attenuation of a cultured cell line other than inoculation in an animal. Attenuation of cell lines is measured by the length of time in *in vitro* culture, usually denote by "passage number", The use of this term to denote length of time in culture is somewhat woolly, as there is no agreement on what constitutes a "passage".

Perhaps more alarmingly, immunity following vaccination may wane (Pipano, 1977, Ouhelli *et al*, 1994), and revaccination is questionably effective (Nichani, 1994). The vaccine acts as an allograft in the vast majority of animals, as the major histocompatibility (MHC) types of donor and recipient are mismatched. Recent work by Nichani (1994) has shown that preformed anti-MHC responses can largely block the efficacy of revaccination. As a result, several cell lines of different MHC types may need to be kept as vaccines, which will add to cost and increased administration.

All of the factors discussed above have led to intense activity towards the design of a vaccine not based on live parasite material. Several parasite antigens have been isolated from the sporozoite, macroschizont, merozoite and piroplasm stages (Hall *et al*, 1992; Knight *et al*, 1994; Conze *et al*, 1994; McDonald *et al*, 1994; Glascodine *et al*, 1990). Although all of these stages are recognised by serum from immune animals (Glascodine *et al*, 1990; Kachani *et al*, 1992a; 1992b), their significance for immune responses is unknown. Currently, only a sporozoite antigen is sufficiently well developed to have been used in animal trials, and despite inducing high levels of anti-sporozoite antibody, has not been found to protect against sporozoite challenge (Boulter *et al*, 1995). As mentioned in the introduction, the application of parasite antigens to protective immunity may be a "hit and miss" affair until the mechanisms of immunity leading to parasite induced pathogenesis are fully understood.

Drug treatment

Good control of tropical theileriosis can be achieved through the use of the drug buparvaquone ("Butalex", Mallincrodt Veterinary Ltd.). This napthoquinone drug kills the intracellular parasite (McHardy *et al*, 1985), and confers immunity upon sporozoite infected animals (Dhar *et al*, 1990). Cattle are immune to extremely high doses of sporozoites eg 30 tick equivalents (TE) where an LD50 dose is approximately 1 TE (Kumar *et al*, 1990; Samantery *et al*, 1980; Preston *et al*, 1992a). Infection and treatment of cattle using sporozoites and Butalex could therefore immunise all animals at risk. However, the use of this system for *T.annulata* is not widespread, mainly due to cost. For example, one dose of Butalex costs the equivalent of 1 months wages for a subsistence farmer in India (P. Goel, pers. comm). Also, standardisation of sporozoite doses is somewhat difficult - sporozoites are derived from infected ticks, and the degree of infection varies between batches of ticks.

Both vaccine and drug control methods currently employed therefore have drawbacks which makes an understanding of immune responses, in order to design more rational therapies, a pressing need.

BOVINE T CELLS AND IMMUNE RESPONSES

In this section, the T cell components of the bovine immune system are discussed. As this thesis examines primary immune responses in lymph nodes, the induction of immunity in lymphoid tissue is also examined, with particular emphasis placed upon T cell/APC interactions required to generate immunity.

$CD4^+$ T cells and MHC class II

It is now recognised that the $CD4^+$ T cells form the central component of the vast majority of immune responses, able to influence both antibody and cell mediated immune responses (reviewed Abbas *et al*, 1991). Bovine CD4 expressing T cells were first identified in 1986 (Baldwin *et al*, 1986). Bovine CD4 differs from the human molecule, as it exists in 2 distinct forms (52 and 55kD), while human CD4 is a single molecule of 62 kD (Terhorst *et al*, 1980). Approximately 70% of bovine

thymocytes express CD4, the molecule is found on approximately 30% of PBM and is not detected on monocytes or B cells (Baldwin *et al*, 1986). $CD4^+$ cells also express CD2 and do not express WC1, a marker found on $\gamma\delta$ T cells (Clevers *et al*, 1990). In lymph nodes (LN), $CD4^+$ cells exist primarily in the paracortex, but are also found in the medulla and follicles (Baldwin *et al*, 1986).

Bovine CD4 is found on cells of a "helper" phenotype, defined by Baldwin *et al* (1986) as the ability to produce self growth factors (cytokines) in the absence of other cell types, while not being able to mediate cytotoxicity. While bovine $CD4^+$ cells can act as cytotoxic T lymphocytes (CTL) (Baldwin *et al*, 1992) this is not thought to be their primary function. Interleukin-2 (IL-2) is thought to be the principal cytokine involved in bovine T cell growth (Takamatsu *et al*, 1990). Long term cultured bovine $CD4^+$ and $CD8^+$ T cell lines express receptors for this cytokine, and IL-2 alone can induce DNA synthesis in these cells (Takamatsu *et al*, 1990).

$CD4^+$ T cells recognise antigens (Ag) presented by antigen presenting cells (APC) in the context of Major Histocompatibility Complex (MHC) class II molecules. APC expressing MHC class II have been shown to be essential for isolating antigen specific bovine $CD4^+$ T cells *in vitro* (Glass and Spooner, 1989). The MHC in cattle is named BoLA, analogous to HLA in humans (Spooner *et al*, 1979). At least two BoLA class II loci are transcribed and expressed on the surface of MHC class II^+ cells - *DRA* and *DRB3* and at least one *DQA* and one *DQB* (Joosten *et al*, 1989; Bissumbher *et al*, 1994; Dutia *et al*, 1995). Other BoLA-D antigens have been identified -*DI, DO, DY and DZ* (Andersson *et al*, 1988; Stone and Muggli-Cockett, 1990), but have not as yet been identified as being expressed on the surface of cells.

Recent studies have shown a great deal of similarity between expressed human and bovine DRα and DRβ genes, with a high degree of polymorphism seen in exon 2 of *DRB3*, which represents the antigen binding site (van Eijk *et al*, 1992;Russell *et al*, 1994; Fraser *et al*, 1994). Polymorphism of *DRB3* encoded molecules correlates with restricted presentation of antigen to T cells (Glass *et al*, 1991a). There is also indirect evidence that non *DR* products (presumably *DQ*) play an important role in Ag presentation to bovine $CD4^+$ T cells (Glass *et al*, 1991a). The responses of T

cells to antigens presented through MHC class II molecules has been useful in identifying class II molecule function. This is particularly relevant in vaccine design for an outbred species such as cattle. For example, the response to peptides from foot and mouth disease virus (FMDV) were shown to be variable in cattle but not in in-bred guinea-pigs (Bittle *et al*, 1982; DiMarchi *et al*, 1986). By assessing bovine T cell responses to FMDV peptides it was shown that some peptides were presented to T cells by MHC class II products less efficiently than others (Glass *et al*, 1991b). Use of such knowledge has led to the design of peptides which are efficiently presented by a wide range of MHC class II types, bringing the possibility of effective vaccination of an outbred species closer (Van Lierop *et al*, 1995).

As noted above, the principal function of $CD4^+$ T cells is to produce "help" for immune responses in the form of soluble cytokines. Cytokines produced by $CD4^+$ T cells following activation have been shown to depend on the "memory" status of the cell ie. whether the cell has been previously exposed to antigen. In common with other species, "naive" $CD4^+$ T cells can only produce IL-2, while "memory/effector" cells can produce a wide range of cytokines such as IL-2, IL-4 and interferon-gamma (IFNγ) (Conlon *et al*, 1995; Collins, 1993). Naive $CD4^+$ bovine T cells can be distinguished from memory/effector cells by their expression of different molecular weight (mw.) isoforms of CD45 (Howard *et al*, 1991). As in the mouse and humans (Birkeland *et al*, 1992), responses to recall antigens are mediated by bovine $CD4^+$ T cells expressing the low mw. forms of CD45 (Howard *et al*, 1991). The acquisition of low mw. forms is measured by either losing reactivity with a mAb recognising the high mw. form CD45RB, or by gaining reactivity with a mAb which recognises the truncated form of CD45 - CD45RO (Howard *et al*, 1991; Bembridge *et al*, 1993).

From work originating in mice, $CD4^+$ T cell derived cytokines have been divided into two distinct groups designated T helper (Th) 1 and 2 (Mosmann *et al*, 1986). The Th 1 subset produces cytokines which help cell mediated immune responses eg. IL-2 and IFNγ whereas Th2 cytokines promote humoral responses eg. IL-4 and IL-10 (Mosmann *et al*, 1986; Cher and Mosmann, 1987; Scott *et al*, 1987; Paul and Ohara, 1987; Hsieh *et al*, 1992). For example, immunity to *Leishmania* infections

can be attributed to Th1 cytokines with IFNγ mediated killing of parasite via macrophages (Scott *et al*, 1987; Gaafar *et al*, 1995). In contrast, making a Th2 response in this situation does not control infection. Th2 responses have been shown to be important in malaria, with anti-parasite antibodies vital in stopping disease resurgence (Langhorne *et al*, 1989).

Bovine $CD4^+$ T cell responses can fit into a Th1 or Th2 pattern. *In vitro* recall responses to FMDV peptides from previously immunised cattle are associated with a Th1 response - proliferating cells only make IL-2 and IFNγ (Van Lierop *et al*, 1995). Purely Th2 bovine $CD4^+$ cells (IL-4 producing, non IL-2 dependant) have also been demonstrated (Stevens *et al*, 1992). In mice, a cytokine producing T cell subset which makes both IL-4 and IFNγ as well as IL-2 has been described and designated Th0 (Street *et al*, 1990). It is generally thought that this represents an intermediate stage in the mouse between naive T cells making only IL-2, and a fully differentiated Th1 or Th2 phenotype (Street *et al*, 1990; Swain *et al*, 1990). However, there is good evidence that the Th0 phenotype is not just an intermediate step in bovine T cell cytokine development. $CD45RO^+$ bovine T cells specific for *Babesia bovis* generally secrete IL-4 and IFNγ as well as IL-2 ie. Th0 (Brown *et al*, 1993). Also, $CD4^+$ T cell clones specific for *Fasciola Hepatica* have either Th0 and Th2 profiles (Brown *et al*, 1994). As the responses described here have been generally classified using *in vitro* assays, the *in vivo* significance of such responses remain to be established.

$CD8^+$ T cells and MHC class I

Bovine CD8 is an antigen consisting of an alpha and beta chain mw. 34 and 38 kD respectively and is found on T lymphocytes which mediate cytotoxicity recognising antigens presented in the context of MHC class I molecules (MacHugh and Sopp, 1991). $CD8^+$ cells constitute approximately 20% of PBM, thus forming a smaller proportion than $CD4^+$ cells (Ellis *et al*, 1986). CD8 is expressed by 70% of thymocytes and is not found on B cells, monocytes/macrophages or granulocytes (Ellis *et al*, 1986). $CD8^+$ cells are found in the paracortex of lymph nodes, although at lower levels than $CD4^+$ cells, and at far lower frequencies than $CD4^+$ cells in the

B cell follicles and medulla (Ellis *et al*, 1986). The vast majority of $CD8^+$ cells are $CD2^+$ $\alpha\beta$ TCR^+, but a few cells express $\gamma\delta$ TCR instead of $\alpha\beta$ (Howard and Naessens, 1993).

In common with other species, bovine $CD8^+$ T cells recognise antigen presented by MHC class I molecules (MacHugh and Sopp, 1991). BoLA MHC class I gene products are similar to those of other species, consisting of a heavy chain mwt. 44 kD, comprising three extracelluar domains, a transmembrane section and a cytoplasmic region (Brown *et al*, 1989). A binding groove for Ag is likely to be formed between the non MHC encoded ß2-microglobulin (mwt. 12 kD, Hoang-Xuan *et al*, 1982) and the α3 domain (Bjorkmann *et al*, 1987). Recently, the first report has been made of the motifs necessary for the binding of Ag to bovine MHC class I molecules (Bamford *et al*, 1995).

Historically, identification of BoLA class I molecules has relied on the use of allo-antisera produced by calf-dam reciprocal immunisation which are used in microlymphocytotoxicity assays (Spooner *et al*, 1979). This has been extended by the use of isoelectric focusing (IEF) (Joosten *et al*, 1988; Al-Murrani *et al*, 1993). Until recently only one MHC class I locus, named BoLA-A was recognised (Bernoco *et al*, 1991). A great deal of controversy currently surrounds the number of BoLA class I loci. IEF analysis of bovine MHC class I products reveals several charge variants associated with any particular serotype (Joosten *et al*, 1988; Al-Murrani *et al*, 1993). Joosten *et al* (1988) attributed the complex pattern seen using IEF to post translational modification of a single locus product leading to different charge variants. However, Al-Murrani *et al* (1994) demonstrated that different charge variants could not be accounted for by glycosylation or phosphorylation and suggested that at least 3 loci are present. Strong evidence for more than one BoLA locus has been provided by analysis of mouse cells transfected with bovine MHC class I molecules of one serotype (Sawnhey *et al*, 1995). BoLA serotype A11 is associated with 5 IEF charge variants in PBM, but mouse L cells transfected with a single A11 cDNA show only one IEF band. If IEF bands corresponded to different versions of the same locus product the full pattern should have been seen from the transfected cells. As this is not the case, this provides strong evidence for more than

one locus product being expressed in PBM (Sawnhey *et al*, 1995).

The function of BoLA class I molecules is somewhat less controversial, although functional loci are not yet identified. $CD8^+$ cytotoxic T lymphocytes (CTL) have been shown to mediate anti-MHC responses in MLR reactions in a BoLA class I restricted manner (Teale *et al*, 1985; Spooner *et al,* 1987). Bovine $CD8^+$ CTL have also been shown to mediate cytotoxicity against MHC class I presented antigens from intracellular pathogens (Goddeeris *et al*, 1986b (*T.parva*); Innes *et al*, 1989a (*T.annulata*)). The pathways leading to the activation of $CD8^+$ cells are still unclear. However, it has been shown that $CD8^+$ cells are largely dependant upon IL-2, as they respond poorly to mitogens and alloantigens without the exogenous addition of this growth factor (Ellis *et al*, 1986)

NK cells

A population of $CD2^+$ $CD3^-$ $CD4^-$ $CD8^-$ cells in bovine peripheral blood have been identified and are likely to correspond to NK cells (Cook *et al*, 1989;Evans and Jaso-Friedmann, 1993). Some NK activity may also be found in bovine $CD2^-$ $\gamma\delta$ T cells (see below). *In vitro*, NK-like activity has been described for bovine PBM in the killing of a range of tumour or virus infected cells lines. *In vivo,* clear evidence of non MHC class I restricted cytotoxicity has been shown in killing of bovine herpes virus infected cells (Campos *et al*, 1992; Denis *et al*, 1993), although other roles for NK cells have yet to be demonstrated *in vivo*. The induction of NK activity in cattle is likely to be dependant upon cytokines - bovine NK-like cells acquire killer phenotypes only when incubated with IL-2 or IFNα or γ (Jensen and Schultz, 1990).

$\gamma\delta$ T cells

$CD2^{-/+}$, $CD4^-$ $CD8^-$ $CD3^+$ T cells in humans have been demonstrated to be of thymic origin, and to express a form of TCR made up of γ and δ chains (Brenner *et al.*, 1988). Such cells have been identified in ruminants and northern blot analysis has shown that these cells also express γ and δ message, no α message and only truncated β message (Hein *et al*, 1989; Clevers *et al*, 1990). In cattle, $\gamma\delta$T cells are $CD2^-$ (Clevers *et al*, 1990). In humans, $\gamma\delta$T cells constitute $< 5\%$ of peripheral

blood lymphocytes, but in cattle these cells make up to 25% of the circulating pool in neonates, falling to 15% by 1 year (Clevers *et al*, 1990). Bovine γδ T cells express a surface molecule known as WC1 on their surface (Clevers *et al,* 1990). WC1 exists in two different forms (p215 and p205 both recognised by mAb CC15), is exclusively found on $CD2^-$, $CD4^-$, $CD8^-$ γδ T cells but has not as yet been identified as having any distinct function (Crocker *et al,* 1993). Histological studies show that $WC1^+$ cells are present mainly in the thymic medulla, but a few are scattered through the thymic cortex. In lymph nodes, cells are located in the outer areas of the cortex adjacent to the subcapsular sinuses. A few cells are also present in the sinuses and in the paracortex but not in B cell follicles.

$WC1^+$ cells are also found in the marginal zones of the spleen (Clevers *et al*, 1990). The precise function of, or restriction elements for $WC1^+$ cells remains elusive. These cells can proliferate to mitogens, but do not seem capable of producing their own growth factors, as IL-2 is required to induce proliferation (Clevers *et al*, 1990). This has been confirmed by Collins (1993) who found that $WC1^+$ cells do not make IL-2. NK function has been attributed to $CD2^-$ $CD3^+$ $WC1^+$ cells (Evans and Jaso-Friedmann, 1993), although the mechanisms underlying this activity are unknown. $WC1^+$ cells also produce mRNA for TNFα (Collins, 1993), which may be involved in cytotoxic or cytostatic effector mechanisms involving these cells.

Lymph nodes and the induction of immune responses

Part of the work in this thesis examines the induction of primary immune responses in draining lymph nodes. The lymph node provides the site for the interaction between antigen carrying cells migrating from the areas of inoculation and T cells (Breel *et al*, 1988). The APC:T cell interactions essential in the induction of immunity are discussed here.

LN are specialised structures dedicated to the retention of antigen and the activation of T cells. In common with other species, bovine LN are organised into distinct areas - the follicles where B cell development takes place, and the paracortex which is primarily populated by T cells (Morrison *et al*, 1986). LN are both heavily vascularised and connected to the lymphatic system via afferent and efferent

lymphatics. Cells entering the LN by either the blood or afferent lymphatics leave via the efferent lymph to the thoracic duct and subsequently return to the blood. Systemic immunity is generated in this fashion - collection of efferent lymph via cannulation, thus stopping the return of cells to the blood, blocks the acquisition of immunity (Hall *et al*, 1967).

Afferent lymphatics function principally to transport antigen from the site of inoculation to the LN (Morris, 1972). Bovine afferent lymph is populated with both lymphocytes and antigen presenting cells (APC), which form approximately a quarter of all afferent lymph cells (Emery *et al.*, 1987). APC in afferent lymph consist of both macrophages and "frilly" or "veiled" dendritic cells, and although both subsets can present antigens, the dendritic cell population is far superior to the macrophage (Emery *et al*, 1987; McKeever *et al*, 1991). Bovine afferent lymph veiled cells (ALVC) have been shown to be efficient in mediating both memory T cell responses and in the initiation of primary immune responses. ALVC pulsed with ovalbumin *in vitro* and inoculated into a naive animal induce the formation of anti-ovalbumin T cell responses (McKeever *et al*, 1992). Also, ALVC collected from the sites of antigen inoculation in *T.brucei* immune animals have been shown to efficiently present antigen to T cells *in vitro* via MHC class II, eliciting recall responses (McKeever *et al*, 1992).

Interactions between T cells and cells of dendritic morphology take place in the paracortex of the LN (Breel *et al*, 1988). The APC seen in the paracortex have been described as interdigitating cells (IDC), and these are believed to represent ALVC which have migrated to the LN (Bujdoso *et al*, 1989a; McKeever *et al*, 1991). The paracortex is populated by T cells and is characterised by a network of post capillary venules also known as high endothelial venules (HEV) (Morrison *et al*, 1986). These venules provide an entry point for blood T cells into the LN. The T cells entering from the blood are thought to be primarily naive in phenotype and form the vast majority (approximately 90%) of T cells circulating through LN (Mackay, 1993) Memory T cells, which often express activation markers, enter the node via the afferent lymph (Mackay *et al*, 1992; Dutia *et al*, 1993). The entry of T cells via HEV to the LN is thought to be mediated through adhesion molecules on T cells and

HEV. L-selectin is an important molecule in the association of T cells with HEV. Anti-L-selectin mAB blocks bovine T cell attachment to HEV (Bosworth *et al*, 1993), and HEV express the ligand for L-selectin (Mackay, 1993). T cells in bovine LN have been shown to express L-selectin, and the molecule is expressed on both memory and naive T cells (Howard *et al*, 1992). This may represent a difference between bovine and ovine T cells, in the latter case L-selectin is only expressed on activated or memory cells (Mackay *et al*, 1992) which are thought to enter LN via afferent lymph. Upregulation of adhesion molecules on HEV such as VCAM-1 have been seen during antigen reactions in LN of the sheep (Mackay *et al*, 1992) which may be important in T cell adhesion, but this has not been investigated in cattle.

The activation of T cells in the paracortex has been investigated by immunohistology (Bogen *et al*, 1991;1993). Initial activation of T cells is seen near HEV, where T cells which have newly entered the node are thought to encounter the Ag bearing ALVC, which subsequently assume IDC morphology (Bogen *et al*, 1991; McKeever *et al*, 1991). ALVC (in the form of IDC) activate T cells extremely efficiently, as they can form clusters with the responding T cells (McKeever *et al*, 1991). In experimental models, the appearance of activated T cells is first noted 4 days after inoculation, and the T cell/IDC interactions around HEV appear to peak around day 7 (Bogen *et al*, 1991). The education of T cells with antigen may be facilitated by a phenomenon known as "cell shutdown", where antigen reactive LN decrease cell output in the efferent lymph in the initial stages of a primary immune response (Emery, 1981; Issekutz, 1985; *inter alia*). This decreased cell output does not affect the recruitment of cells via HEV (Hein and Supersaxo, 1988). Thus large numbers of T cells can be retained in the node, increasing the chances of selecting T cells which are specific for the immunising antigen.

Activation of T cells by IDC is a critical step in both the induction of antibody responses and in producing cell mediated immunity. Interaction with APC in the paracortex does not appear to select T cells for the production of one particular response - cells of dendritic morphology can prime T cells to make either predominantly IFNγ or IL-4 (Ronchese *et al*, 1994). Also, both IL-4 and IL-2 producing T cells are seen in the paracortex during primary immune responses

(Bogen *et al,* 1993). In mice, IL-2 and IFNγ are thought to primarily induce cell mediated responses, while IL-4 induces humoral responses (Abbas *et al,* 1991). What influences the development of a B cell or T cell mediated response is not clear, and it seems likely that both often develop, although both may not be protective. For example, in *T.annulata* infection, during the protective immune response seen following Butalex treatment, strong antibody responses can be detected (Kachani *et al*, 1992a; 1992b), but immunity is primarily mediated by $CD8^+$ CTL (Preston *et al*, 1983; Nichani, 1994). It has been suggested that cytokine production at the site of inoculation may influence T cell responses in nodes - Bogen *et al* (1993) detected IFNγ producing NK cells at the site of antigen deposition. Although IFNγ is potentially antagonistic to IL-4 producing cells (Donckier *et al,* 1994), the situation described by Bogen *et al* did not stop IL-4 production. In this particular case, IFNγ production may only help skew a response rather than induce one dominant type.

T cells have been shown to be essential for the formation of B cell germinal centres (GC) (Jacobson *et al*, 1974). Germinal centres are found in B cell follicles after Ag challenge, and are the sites where B cells undergo proliferation and Ab diversity is generated (Maclennan, 1994). B cells require T cell help, both in initial stimulation to proliferate (mediated through interactions between CD40 on B cells and its ligand on T cells (Noelle *et al*, 1992; Armitage *et al*, 1993)), and in subsequent control of proliferation and antibody class switching through cytokine production (Armitage *et al*, 1993; Donckier *et al*, 1994). Following antigen challenge, the T cells which were activated by IDC can be seen to migrate towards B cell follicles and express IL-2 and IL-4 (Bogen *et al*, 1991; 1993). This is followed by the development of B cell responses. This process has not been established in bovine LN. However, as bovine LN contain B cells follicles and GC, and T cells can be detected in GC (Morrison *et al*, 1986), it is likely that a similar response is taking place in cattle. In this study, techniques were developed to examine bovine GC in more detail, and the application of these techniques may be of use in a better understanding of T cell interactions required for bovine B cell immune response development.

The sites for the induction of CTL or other cell mediated responses after the initial T cell/IDC interaction in LN during primary challenges are less well understood. In

the case of CTL, there is good evidence that their antigen "education" takes place in the node. Resting ruminant efferent lymph T cells are predominantly $CD4^+$ (Emery *et al*, 1988; Bujdoso *et al*, 1989b). After Ag challenge $CD8^+$ cells are enriched in efferent lymph, presumably indicating a specific response by these cells (Mackay *et al*, 1992). Cytokine producing $CD4^+$ T cells which have acquired specificity for an Ag in LN primary responses have been recently shown to recirculate to the site of Ag inoculation (Bogen *et al*, 1993). Thus, although cytokines produced by these cells may be "broadcast" systemically in the efferent lymph, initial activation of T cells in LN also appears to induce immune responses at the antigen site through recirculation.

In primary immune responses, T cell/APC interactions in LN can therefore lead to the induction of both humoral and cell mediated immune responses. This thesis has examined the responses in *T.annulata* infected LN in the light of the mechanisms discussed here.

IMMUNE RESPONSES IN TROPICAL THEILERIOSIS

This thesis examines the underlying mechanisms of pathogenesis in tropical theileriosis following sporozoite infection of susceptible animals, which is characterised by an apparent immune response failure. Protective immune responses have been characterised in sporozoite infected animals rendered immune by the drug Butalex, or vaccinated using the live cell line vaccine. The responses generated by such animals are discussed in detail. The limited knowledge regarding primary immune responses to sporozoite infection is discussed in relation to such protective responses.

Immune responses discussed here concentrate on the sporozoite and macroschizont stages, as the pathology of tropical theileriosis is associated largely with macroschizont infected cells (Hooshmand-Rad, 1976), and this stage is formed following sporozoite infection. Antibody responses to piroplasms have been observed in cattle recovering from infection or undergoing immunisation with *T. annulata* (Kachani *et al*, 1992b) and sera from immune animals can opsonise merozoites (Ahmed *et al*, 1988). The antigen seen by such antibodies may be identical on both

parasite stages, as anti-merozoite mAb cross react with piroplasms (Glascodine *et al*, 1990). The significance of these observations for protective immunity is unclear, as these stages are only formed several days after the macroschizont, when the majority of damage already had been done.

Effector mechanisms in T.annulata immune cattle

Cell mediated immunity

Clearance of *T.annulata* infection, both following drug treatment of sporozoite infected cattle and in vaccinated animals is accompanied by a cytotoxic T lymphocyte (CTL) response. In either case, CTL recognise antigens presented via MHC class I on macroschizont infected cells. (Preston *et al*, 1983; Innes *et al*, 1989a). This mechanism of parasite clearance is also seen in immunised animals undergoing secondary sporozoite challenge (Nichani, 1994). Susceptible animals lethally infected with sporozoites do not exhibit any CTL activity in blood or efferent lymph (Nichani, 1994). When such animals are treated with Butalex, killing the intracellular parasite, MHC class I restricted CTL specific for macroschizont infected cells can be found in blood and efferent lymph (Preston *et al*, 1993; Nichani, 1994).

When cell line vaccines are introduced to a naive animal, it is usually in the form of an allograft as the MHC types of donor and recipient are not matched. This is a happy coincidence, as the strong anti-MHC responses are though to aid the anti-parasite response. There are 2 peaks of CTL responses in vaccinated animals, one against the immunising MHC, the second against the parasite (Innes *et al*, 1989a). The anti MHC response is thought to greatly boost anti-parasite responses through initial inactivation of the macroschizont infected cells. Animals inoculated with MHC matched cell lines exhibit severe clinical reactions to the vaccine, and take longer to develop immunity (Innes *et al*, 1989a).

The requirement for the interruption of macroschizont growth either through drug treatment or an anti-MHC response is therefore a prerequisite for the efficient formation of primary CTL responses. Whether CTL are "educated" with parasite antigen by dead macroschizont infected cells, or through uptake of killed schizont antigens by other APC remains unresolved. The latter mechanism has been

demonstrated to be possible, as HPLC fractions from infected cells were presented by uninfected PBM to CTL from recovering animals (Conze *et al*, 1994), resulting in their lysis.

Knowledge about the role of $CD4^+$ T cells in immunity to *T.annulata* is somewhat sketchy. When examined in lymph efferent from draining lymph nodes (LN) in Butalex treated animals, the parasite specific CTL discussed above expressed IL-2R and MHC class II on their surfaces (Nichani, 1994). As the expression of these activation markers is induced by IL-2 (Mauer *et al*, 1984; Smith, 1984;Tomita *et al*, 1991) it seems highly likely that IL-2 is essential in the induction of anti-parasite CTL. IL-2 is not made by *T.annulata* macroschizont infected cells (Brown *et al*, 1995) and bovine $CD4^-$ cells proliferate very poorly without exogenously added growth factors (Baldwin *et al*, 1986). It is therefore reasonable to attribute a role in *T.annulata* immunity to $CD4^+$ T cells, if only in the production of IL-2.

One possible role for $CD4^+$ T cells in immunity could be the activation of macrophages through soluble factors to acquire anti-parasite activity. Such a phenomenon is seen in *Leishmania* infections where killing of intra-macrophage parasites is attributed to Th1 cytokines with IFNγ mediated killing of parasite via macrophages (Scott *et al*, 1987; Gaafar *et al*, 1995). Adherent cells from the peripheral blood of animals infected with *T.annulata* sporozoites or inoculated with cell lines have been shown to exhibit cytostatic effects upon schizont infected autologous and allogeneic cell lines *in vitro* (Preston & Brown, 1988). This is somewhat different from *Leishmania*, as the mechanism appears to operate via uninfected cells rather than on parasitised macrophages. A role for such a mechanism is questionable *in vivo*, as efferent lymph responses in animals recovering from sporozoite infection (Butalex treated) are not characterised by large increases in IFNγ production (Nichani, 1994). If macrophages do have an effector function this may be at localised sites other than the draining lymph node.

Antibody responses

Antibodies recognising all stages of the parasite can be detected in animals recovering from sporozoite infection (Kachani *et al*, 1992a;1992b), and three

antigens have been identified which are common to the sporozoite, schizont, and piroplasm stages (Kachani *et al,* 1992a). Although serum antibody responses are widely used to detect animals which have been exposed to disease, via the IFAT test which clearly depicts stained schizonts and piroplasms within fixed cells, the role of antibodies in protective immunity remains questionable. The effects of antibodies and complement in immunity to *T.annulata* have been studied from several different angles: protection of animals by infusion of immune serum (Dhar and Gautam, 1978;Samad *et al*, 1984); assessing antibody binding and lysis of differing parasite stages by serum from immune cattle and immunised rodents (Preston and Brown, 1985;Ahmed *et al*, 1988); sporozoite neutralisation and macroschizont infected cell lysis by anti theilerial monoclonal antibodies (mAb) (Preston *et al*, 1986; Williamson *et al*, 1989).

At least *in vitro*, a clear role for antibody in blocking sporozoite entry to host leucocytes can be established, as sera from immune animals, immunised mice and anti sporozoite mAb can block transformation (Preston and Brown, 1985; Ahmed *et al,* 1988; Williamson *et al*, 1989). The gene coding for the antigen complex (known as SPAG-1) recognised by neutralising mAb has been cloned and expressed in bacteria (Williamson *et al*, 1989; Hall *et al*, 1992). When fragments of SPAG-1 were fused with a hepatitis core antigen and immunised into calves, strong antibody responses were generated. However, despite these very high antibody titres, protection of animals was not achieved (Boulter *et al*, 1995).

Infusion of immune serum has also not been found to protect animals from sporozoite challenge (Samad *et al*, 1984), and given the strong cell mediated responses detected in infected and immunised animals (Preston *et al*, 1983; Innes *et al*, 1989a; Nichani, 1994) this is perhaps not surprising. However, immune serum in these experiments was not administered until several days post sporozoite infection. As the principal mode of action of antibodies is apparently to block sporozoite entry to host leucocytes, the lack of protection from administering serum long after the sporozoite has entered the host cells is not surprising.

Ahmed *et al* (1988) found that immune serum could not lyse macroschizont infected cells in the presence of complement and postulated that the "neo-antigen" expressed

on the surfaces of infected cells may not be immunogenic. This provides functional evidence for the observations recorded by Shiels *et al* (1989) that immune sera could stain parasite material fixed for IFAT, but not the surface of the infected cell. The failure of serum to lyse macroschizont infected cells is apparently not due to an innate resistance to antibody attack, as a mAb named 4H5 raised against macroschizont infected cells has been shown to cause lysis in the presence of complement (Preston *et al,* 1986).

Ab responses therefore seem likely to play a role primarily against the invasive stages of the parasite and cannot confer immunity alone. Ab responses may be of greater importance in resistance to re-infection in immune animals, as asymptomatic carriers remain serologically positive.

Evidence for Immune disfunction in parasite infection

From the results discussed above, it is clear that cattle can make primary immune responses against *T.annulata*, but the inactivation of the macroschizont infected cells is a prerequisite for doing so. Also, T cell mediated immunity is the principal clearance mechanism. This thesis sets out to understand why T cell responses are not protective in susceptible infected animals. Two clear lines of evidence suggest that T cell mechanisms are "interfered with" by the macroschizont infected cells, rather than simply failing to operate.

In vitro responses

$CD4^+$ T cells have been isolated from the peripheral blood of animals immune to *T.parva* using macroschizont infected cells to stimulate the antigen specific cells (Baldwin *et al*, 1987). However, this has not been possible for *T.annulata* (appendix 2). The inability to isolate parasite specific T cells from the blood of *T.annulata* immune animals is attributed to a phenomenon known as the *"Theileria* autologous mixed lymphocyte reaction (MLR)" (Pearson *et al*, 1979; Goddeeris and Morrison, 1987; appendix 2). This describes the ability of both *T.annulata* and *T.parva* macroschizont infected cells to induce proliferation of autologous PBM from naive animals in the absence of any exogenously added antigen. The net effect of this

phenomenon in *T.parva* using PBM from naive animals is the expansion of T cells which are activated but not parasite specific (Pearson *et al*, 1979). This response does not affect the generation of either $CD4^+$ or $CD8^+$ parasite specific T cells from the blood of immune animals in *T.parva* (Goddeeris *et al*, 1986; Baldwin *et al,* 1987; Goddeeris and Morrison, 1988).

However, the MLR response is sufficiently strong in *T.annulata* to block the isolation of parasite specific T cells, with the expansion of the non functional cells from naive animals described by Pearson *et al* (1979) the only result (appendix 2). These activated cells were IL-2 responsive, but $CD4^+$ cells were relatively short lived in culture, and no cytotoxicity could be detected in $CD8^+$ cells. This provides direct evidence that macroschizont interaction with T cells may affect their ability to mediate immune responses.

In vivo responses

In a study by Nichani (1994) which ran in parallel with the work in this thesis, cells exiting in efferent lymph from sporozoite infected nodes were examined. No evidence was found to suggest that T cells were responding by making a protective immune response to the parasite - no anti macroschizont infected cell CTL responses were present. However, Nichani's study did provide evidence that some form of non protective immune response was occurring. Following sporozoite infection, T cells were initially found to be IL-2 responsive, suggesting that some form of T cell activation was present (Mauer *et al*, 1984; Smith, 1988). IL-2 responsiveness was only short lived in these cells suggesting that a full immune response had not been induced.

Further evidence that some form of T cell activation was present was also indicated by the high levels of IFNγ present in efferent lymph of untreated sporozoite infected animals. As macroschizont infected cells do not make this cytokine (Brown *et al*, 1995) it seems likely that the IFNγ came from some kind of T cell response. Although IFNγ has been shown to have some cytostatic effects on cultured developing macroschizont infected cells (Preston *et al,* 1992b), this does not appear to hold true *in vivo*, as macroschizont infected cells proliferated vigorously in the

draining lymph node during the period of IFNγ production (Nichani, 1994). IFNγ production within the infected lymph node therefore is not likely to represent a protective immune response.

Sporozoite infection of susceptible animals therefore is not pathogenic simply due to a failure to make any immune response. T cells do respond, becoming initially IL-2 responsive and making IFNγ, but this does not appear to hinder parasite infection and is not sustained.

The activated T cells described by Nichani bear more than a passing resemblance to those induced to proliferate non specifically by autologous *T.annulata* infected cells *in vitro* (appendix 2), although cytokine production by these cells was not investigated. Some form of the non functional T cell responses seen from immune animals may therefore be operating during *in vivo* responses to sporozoite infection in naive animals. Such a mechanism may also potentially explain the failure of cattle to form useful primary immune responses without drug or anti-MHC mediated killing of parasitised cells. Investigation of the mechanisms causing this immune response failure form the main body of this thesis.

CHAPTER 2

General materials and methods

GENERAL MATERIALS AND METHODS

All solutions used in this thesis are detailed in appendix 1, which is divided into sections corresponding to each chapter.

Animals

Animals used in this study were castrated Hereford; Friesian; or Ayrshire adult cattle or their crosses.

Bovine Peripheral Blood Mononuclear Cells (PBM)

PBM were isolated as described by Glass and Spooner (1989). Phosphate buffered saline (PBS) was used throughout for washing cells. Whole venous blood was collected, using 20% ACD as an anticoagulant, layered upon Ficoll-Hypaque S.G. 1.077 ("Lymphoprep", Nycomed) and centrifuged at 1500G for 25 minutes. The resulting mononuclear cell layer, was collected and washed in PBS by centrifugation at 300G for 10 minutes. Cells were then washed a further twice in PBS and once in RPMI 1640 medium, centrifuging at 100G for 10 minutes. Cells were then resuspended in TC medium, counted, and adjusted to the required concentration.

THEILERIA ANNULATA PARASITE MATERIAL AND CELL LINES

T.annulata parasite isolates

T.annulata parasites used for the generation of cell lines and infection of animals in this study were derived from three isolates: Hissar (India) (Gill *et al*, 1980); Gharb (Morocco) (Ouhelli, 1985); and Ankara (Turkey) (Schein, 1975). The specific isolates used are detailed in the relevant chapters.

Production of *T.annulata* infected cell lines (Brown 1983)

The infection of PBM with *T.annulata* sporozoites, and production of continuously growing infected cell (IC) lines is described here. Complete tissue culture (TC) medium (Brown 1983) was used for establishing and routine culture of *Theileria annulata* infected cell lines.

Preparation of T.annulata sporozoites

T.annulata sporozoites were provided by the Centre for Tropical Veterinary Medicine, University of Edinburgh. Parasite was prepared as ground up tick supernatant (GUTS) (Brown 1983).

Adult *Hyalomma anatolicum anatolicum* ticks infected with *T.annulata* were fed upon the ears of rabbits for three days to stimulate sporozoite maturation (Walker *et al*, 1985). Partially engorged ticks were removed and first washed in 1% Benzalkonium Chloride (Roccal,Winthrop) (a detergent wash). They were subsequently washed 3 times in 70% ethanol before transfer to a sterile universal tube (Sterilin) where they were washed 3 times with Eagle's minimum essential medium (MEM) (Gibco). The ticks were then allowed to stand for 10 minutes in medium, the supernatant was discarded, and a small amount (2-5 ml depending upon the number of ticks) of cold MEM plus 3.5% bovine plasma albumin (BPA) (Sigma fraction V) was added. The ticks plus medium were then transferred to a sterile mortar and thoroughly ground with a sterile pestle. Measured aliquots of supernate were removed to a sterile universal and replaced with fresh medium.

The freshly prepared GUTS was passed through a sterile 8μm filter (Millipore), and the resulting supernatant's sporozoite content expressed as tick equivalents (TE) per ml ie. the number of ticks used/number of ml supernate obtained. The quality of GUTS produced is routinely assessed at the CTVM by examining Giemsa (Sigma) stained cytocentrifuge preparations (see below) of sporozoites adjusted to 1TE per ml.

GUTS was cryopreserved by adding 7.5% glycerol (BDH) to the sporozoite supernatant in MEM/3.5% BPA, and freezing in liquid nitrogen.

In vitro infection of PBM with sporozoites

T.annulata infected cell lines were produced by infecting PBM using both fresh and cryopreserved GUTS as described by Brown (1983) and (1989).

Fresh GUTS: 1ml of PBM @ 1-2x10^7 cells/ml in TC medium was mixed with 1ml of 1TE/ml GUTS in a 25ml tissue culture flask (Nunclon). The culture flask was

allowed to stand upright for two hours at 37°C in a 5% CO_2 humidified incubator (Scotlab-VSL). 8ml of TC medium was then added, and the flask placed horizontally in the incubator.

Frozen GUTS: When using preserved sporozoites, the GUTS (usually 1ml @ 1TE/ml) was allowed to thaw rapidly and then equilibrate at room temperature for 20 minutes. The parasite material was then serially diluted in doubling quantities of TC medium containing 20% FCS to an approximate volume of 8ml, allowing 20 minutes between dilutions. The sporozoite containing medium was then added to 1-2x10^7 PBM in 2ml in TC. The flask was allowed to stand upright in a 37°C, 5% CO_2 incubator for 24 hrs. 7ml of medium was carefully removed and replaced with 7ml fresh TC, and the flask returned to the horizontal position.

Once IC lines were established, fresh medium was added according to the metabolic demands of the culture (typically every 48-72hrs). Infection could be visualised using cytocentrifuge preparations (Cytospin II, Shandon). Typically 1-2x10^5 cells were centrifuged at 350 RPM for 6 minutes using slide/filter assemblies which had been pre-wet with RPMI 1640 medium. The slides were then air dried, fixed in methanol, and stained for 30-40 minutes in Giemsa stain (Sigma) diluted 1:10 with "Gurr" buffer pH 7.2 (BDH). In well established cultures >95% of viable cells could be identified as containing schizonts. (see Fig. 5.7 for an example of schizont infected cells).

Cryopreservation of cells

All cells were cryopreserved as required using dimethyl sulphoxide (DMSO, Fisons) as the cryoprotectant (Brown 1983). Cells were adjusted to 2x10^7/ml in a cold solution of 50% RPMI1640/50% FCS. An equivalent volume of 20% DMSO in FCS was added dropwise, and the cells were placed in a -70°C freezer for 24hrs before transfer to liquid nitrogen.

Cells were resuscitated by rapidly thawing at 37°C. They were subsequently washed in warm TC medium containing 20% FCS by centrifugation at 200G for 10 min. The cells were then resuspended in fresh TC/20% FCS and incubated at 37°C/5% CO_2.

FLOW CYTOMETRY

Bovine leucocytes were phenotyped by indirect fluorescence using a FACScan flow cytometer (Becton Dickinson). The methods used were based upon those of Spooner *et al* (1988) and Glass and Spooner (1989).

Primary Monoclonal antibodies (mAb)

Details of primary mAb (all of mouse origin) used for FACS analysis are contained in Table 2.1. mAbs were optimally titrated before use. Typically, dilutions between 1:1000 and 1:2000 of ascitic fluid were used. In depth descriptions of mAb and their use are contained within the relevant chapters.

Secondary immunoconjugates

Cells were analysed by both single and double immunofluorescence staining using the secondary immunoconjugates detailed below:

Immunoconjugate	Supplier	Working Dilution
Rabbit anti-mouse (RAM) Ig-FITC	Sigma	1:100
Goat anti-mouse (GAM) IgM-FITC	Sigma	1:100
GAM IgG-PE	Sigma	1:100
GAM IgG_1-FITC	Seralab	1:200
GAM IgG_{2a}-PE	Seralab	1:300

FITC = Fluorescence isothiocyanate

PE = R-Phycoerythrin

Staining of cells for FACS analysis

PBM were isolated as detailed previously, but instead of a final wash in RPMI-1640 medium were washed in cold (4°C) "Cell Wash" (PBS plus NaN_3, pH 7.2) (Becton

Table 2.1 Primary mAb.

mAb (Isotype)	Specificity	Reference
J11 (IgG_1)	MHC Class II (DR)	Dutia *et al*, 1995
IL-A21 (IgG_{2a})	MHC Class II	Davies *et al*, 1994
IL-A26 (IgM)	CD2	Baldwin *et al*, 1988
MM1A (IgG_1)	CD3	Davis *et al*, 1993
IL-A12 (IgG_{2a})	CD4	Baldwin *et al*, 1986
SBU-T8 (IgG_{2a})	CD4	Howard *et al*, 1991
CC63 (IgG_{2a})	CD8	Howard *et al*, 1991
CC76 (IgG_1)	CD45 RB	Howard *et al*, 1991
IL-A30 (IgG_1)	sIgM	Naessens *et al*, 1988
VPM 30 (IgM)	B Cells	Naessens and Howard 1991
CC15 (IgG_{2a})	$\gamma\delta$ T Cells (WC1)	Clevers *et al*, 1990
IL-A111 (IgG_1)	IL2 Receptor	Naessens *et al*, 1992
IL-A24 (IgG_1)	Macrophages	Ellis *et al*, 1988
IL-A109 (IgM)	Monocytes	MacHugh *et al*, 1990

Dickinson). The cells were then resuspended at 1-2x10^7/ml in cold Cell Wash containing 5% γ-globulin free horse serum (Sigma). Tissue culture derived cells were washed twice in cold Cell Wash before resuspension as above.

Single parameter staining

50μl of cell suspension (5x10^5 cells) was mixed with 50μl of optimally diluted mAb in a 96 well round bottomed plate and incubated upon ice for 30 minutes. A negative control was included by incubating cells with mouse serum (diluted 1:1500, Sigma) instead of a mAb. The samples were washed three times with 200μl cold Cell Wash (spinning at 100G, 2 minutes, 4°C), resuspended in 25μl of RAM Ig-FITC and incubated a further 30 minutes upon ice, protected from light. The samples were washed a further three times above before analysis. If not immediately analysed, samples were resuspended in Cell Wash containing 1% w/v paraformaldehyde (Sigma), stored at 4°C, and analysed within 7 days.

Double parameter staining

Cells were treated as above, except 25μl of each monoclonal at half optimal dilution was added in the first step. In addition, 25μl of two appropriate secondary antibodies was added in the second step. eg. in double staining CD4 vs. MHC class II, 25μl of IL-A12 (IgG_{2a}) and J11(IgG_1) diluted 1:500 were added to 50μl of cells. The cells were washed as above and subsequently incubated with 25μl of both GAM IgG_1-FITC and GAM IgG_{2a}-PE for 30 minutes followed by washing.

FACS analysis.

FACScan

The FACScan flow cytometer is equipped with a single argon laser which produces a 488nm wavelength beam. When cells pass through the laser beam changes in the laser radiation are measured using Photomultiplier tubes (PMT) which convert the radiation to electrical impulses. The principal cell parameters measured are size, complexity (granularity), and fluorescence. When a cell passes through the beam, the light is "bent" round the cell. The larger the cell, the larger the amplitude of the

light scatter around it. These changes are detected along the same axis as the incident laser light and are termed forward scatter (FSC), giving a relative measure of a cell's size (Fig. 2.1) Organelles and other intracellular bodies cause the laser light to be refracted at 90° to the incident light axis. The amount of this 90° or side scatter (SSC) is proportional to the amount of cellular complexity, providing a relative measurement. eg. *Theileria* infected macrophages have a much higher SSC than PBM (Fig. 2.1).

Fluorochromes bound to the cell surface absorb the laser light (488nm), and emit light of a longer wavelength eg. FITC emits at 530nm and PE at 580nm. Detectors coupled to optical filters set at appropriate wavelengths measure the amount of emitted light which is proportional to the amount of fluorochrome bound. Although fluorescing at 530nm and 580nm respectively, the emission spectra of FITC (FL1) and PE (FL2) overlap, necessitating the removal (termed compensation) of the spurious signal from each detector (Fig. 2.2). Samples stained positively for either FITC or PE are run separately, and the overlapping signal is set to zero. On the FACScan, the amount of compensation is expressed as the percentage of unwanted wavelength removed from the desired detector.eg FL2-%FL1.

Cytometer calibration (Fig. 2.3)

Before analysing each set of samples, the FACScan was first calibrated using the negative controls. The procedure described is for PBM, but applies to any cell type. FSC vs SSC (size vs complexity) dot plots using a linear scale were used to gate out any dead cells and RBCs ("lymphoprep" removes bovine granulocytes). Fluorescence was measured using a $\log_{10}$ scale with FITC (FL1) and PE (FL2) detectors typically set at 580-620meV and 500-540meV respectively. When double staining, compensation was carried out using samples stained with only one mAb but both secondary antibodies. The FL1 signal was first removed from the FL2 region. FL2-%FL1 was typically set at 25-35% while FL1-%FL2 compensation was usually $<1\%$.

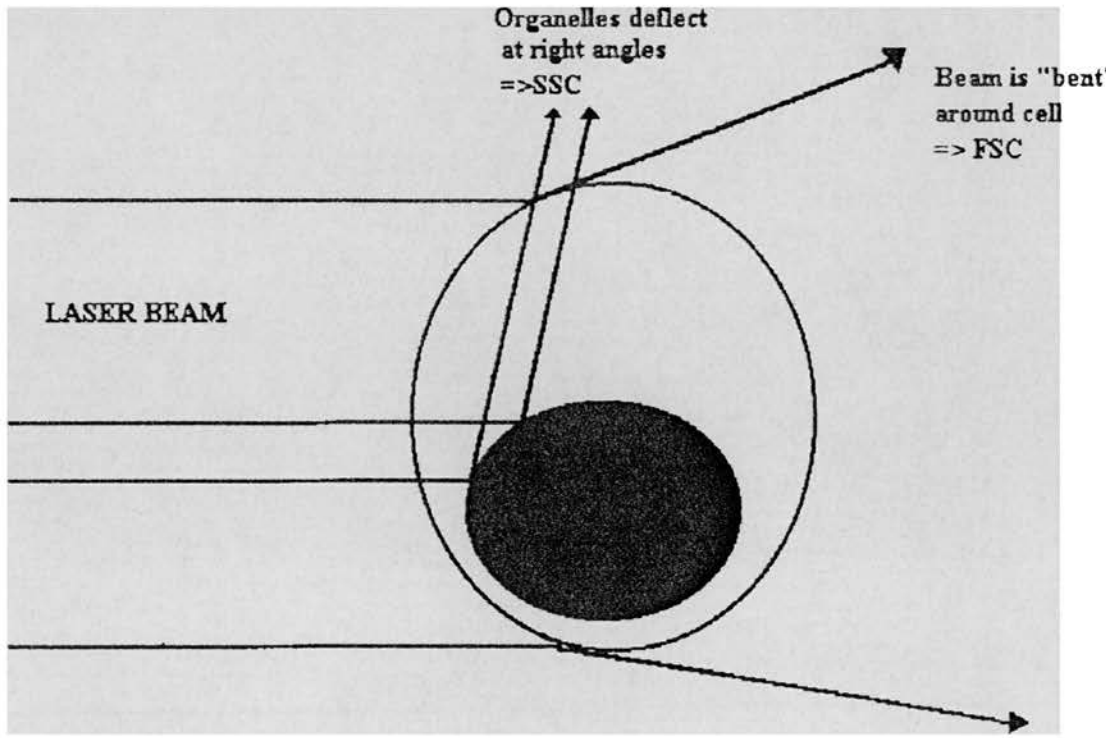


Fig. 2.1 "Side view" of cell in laser beam of a flow cytometer, showing "bending" of the beam to produce the Forward Scatter (FSC) size measurement, and 90° scatter from organelles (Side scatter - SSC)

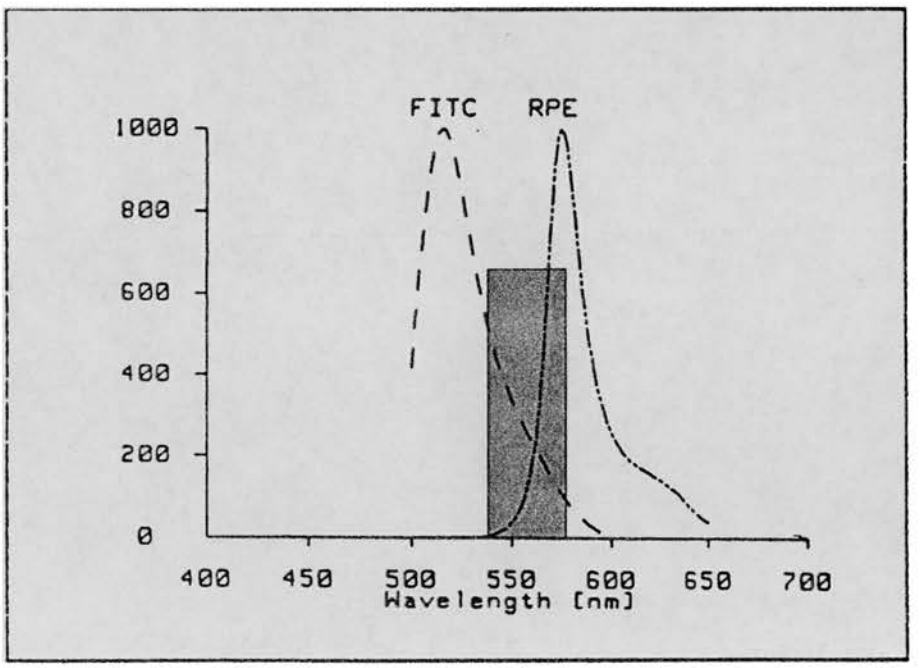


Fig. 2.2 Emission spectra of FITC and PE following excitation at 488nm. Shaded box represents areas of overlap removed during compensation (adapted from "Immunocytometry", Becton Dickinson).

Data acquisition and analysis

"Lysys II" software (Becton Dickinson) was used to acquire and analyse FACS data, "PC Lysys" (Becton Dickinson) was used for analysis of results away from the FACS workstation. Once the cytometer had been calibrated, cell samples were run, acquiring 10000 cells. In the analysis of either single or double stained samples, control fluorescence levels were established using the negative controls. Single stain analysis was plotted as frequency histograms of log fluorescence or as a dot plot of fluorescence vs FSC or SSC. When analysing double colour samples, dot plots of FL1 (x axis) vs FL2 (y axis) were used. Quadrants were set as in Fig. 2.3..

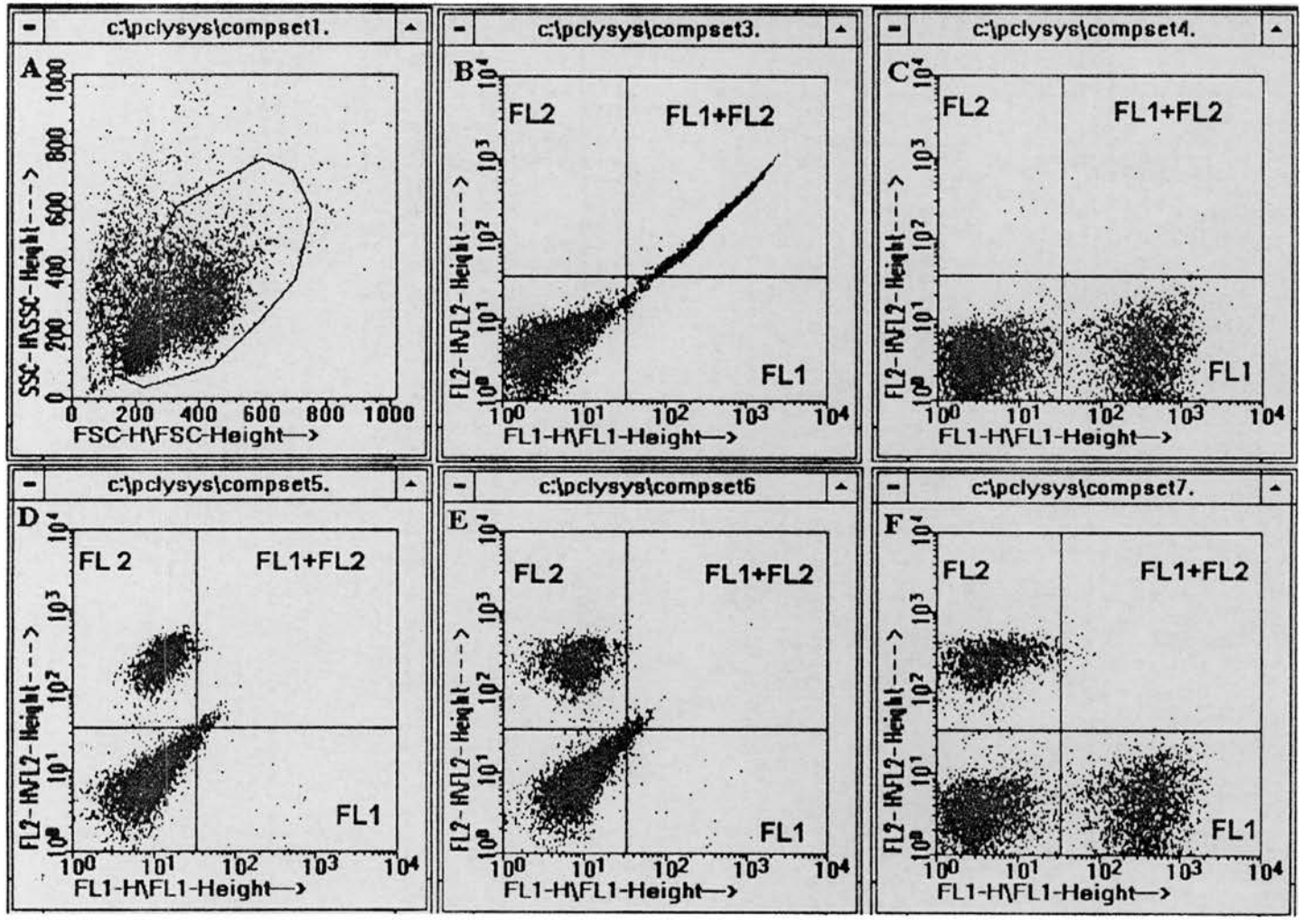


Fig. 2.3 Setting compensation using VPM 30 (anti-B cells) and IL-A12 (anti CD4).

A- PBM are gated to remove debris and RBCs

B+C-Once baseline FL1 and FL2 is set using the -ve control, FL2-%FL1 is set using VPM30 and GAM IgM-FITC, with the FITC signal removed from the FL1+FL2 quadrant

D+E-FL1-%FL2 is set using IL-A12 and GAM IgG_{2a}-PE, with the PE signal placed wholly within the FL2 quadrant.

F- Double stained sample detects both parameters, with no false double positives

CHAPTER 3

In vitro proliferation and activation of T cells induced by *Theileria annulata*

INTRODUCTION

The pathology of *Theileria annulata* infection can be largely attributed to the intraleucocytic macroschizont stage (Pipano and Tsur, 1966; Hooshmand-Rad, 1976), which develops in the draining lymph node after sporozoite inoculation. Unsurprisingly, the macroschizont stage of *T. annulata* has received a great deal of attention in recent years, not least because this form of the parasite can be readily established and maintained in culture (Brown, 1981;1983;1987). The cells infected and "transformed" by *T.annulata* sporozoites into continuously growing lines have been identified as primarily monocytes and macrophages (Spooner *et al*, 1989; Glass *et al*, 1989), and infected lines have a uniform macrophage-like phenotype (Campbell *et al*, 1994). *In vitro* generated lines form a useful model for studying the parasite, which exhibits normal differentiation behaviour *in vitro*:- infected cells established from sporozoite infection remain in the macroschizont stage unless triggered into microschizont formation and merogony by placing them at temperatures of 41°C, corresponding to fever *in vivo* (Glascodine *et al,* 1990; Shiels *et al*, 1992). Therefore, at least in the ability to progress through the parasite lifecycle, *in vitro* generated lines appear to be no different from those *in vivo*.

From an immunological point of view, the macroschizont infected cell is of great interest, as it appears to be the principal target of the host immune response. $CD8^+$ cytotoxic T lymphocytes (CTL) which specifically lyse macroschizont infected cells are in found in the peripheral blood and lymph efferent from draining lymph nodes of "Butalex" treated animals (Preston *et al,* 1983; Nichani, 1994). In addition, animals vaccinated using attenuated macroschizont infected cell lines also exhibit strong anti-parasite CTL responses (Innes *et al,* 1989a; Nichani, 1994). CTL recognise MHC class I restricted antigens on macroschizont infected cells (Preston *et al*, 1983; Innes *et al,* 1989a), and analysis of HPLC fractions from infected cells is now making headway towards identification of these antigens (Conze *et al*, 1994). As the immunogenicity of macroschizont antigens is not in doubt, this makes the inability of susceptible animals, which suffer severe clinical disease, to mount an effective immune response somewhat puzzling. At the same time, the parasite is

presented with a problem, as it is living within a cell which should be easily eliminated by the immune system. As parasite growth is rapid within infected animals, apparently unaffected by any host response, a survival mechanism is evidently in place. It would seem likely that the parasite is inducing changes in the host cells which leads to evasion of immune responses.

As discussed above, *T.annulata* infects macrophages and monocytes, while the related parasite *T.parva* infects T cells and B cells (Spooner *et al*, 1989). Although they infect different cell types, both exhibit a striking phenotypic change upon macroschizont infection with the acquisition of an ability to induce proliferation of autologous PBM. Extensively investigated in *T.parva*, the phenomenon was first reported in 1979 by Pearson *et al*, has more recently been reviewed by Goddeeris and Morrison (1987), and has been dubbed the "*Theileria* mixed lymphocyte reaction". This MLR effect is found in both parasites, and PBM from both uninfected animals and those which have been previously immunised are found to proliferate (Goddeeris and Morrison, 1987; Rintelen *et al*, 1990; appendix 2 this thesis). In *T.annulata*, the continuing presence of the parasite is required for PBM proliferation - "Butalex" treatment of infected cells completely removes any MLR effect (Rintelen *et al*, 1990).

T cells appear to be the principal cell type proliferating in parasite activated PBM (Goddeeris *et al*, 1986; Baldwin *et al,* 1987; Glass and Spooner, 1990a). In naive animals, as proliferation is induced in T cells from animals which cannot possibly have seen *theileria* antigens, macroschizont infected cells presumably express some element which can cross react with other T cell specificities. Such "non specific" activation of T cells is a feature of many diseases, and may be mediated by different antigen types eg. *Trypanosoma cruzi* antigens (Ag) can react with the majority of Vβ genes expressed by T cell receptors (TCR) in human peripheral blood (Piuvezam *et al,* 1993). Pathology in some forms of arthritis has been linked to an over expression of Ag mimicking mycobacterial antigens which cross react with a large range of TCR antigens in the periphery (Iglesias *et al*, 1992). Analogous with the responses to these pathogens, "non specific" activation of T cells by *Theileria* species may present a possible mechanism for subverting the immune response.

There are distinct differences in the PBM responses between *T.parva* and *T.annulata* which suggest that the implications for immune response generation of the MLR differ between the parasites. In *T.parva*, using PBM from immune animals and autologous macroschizont infected cells, it is possible to isolate parasite specific $CD4^+$ and $CD8^+$ T cells and to establish stable lines and clones (Goddeeris *et al*, 1986; Baldwin *et al,* 1987; Goddeeris and Morrison, 1988). Thus the element which induces proliferation of T cells which are not parasite specific apparently does not interfere with genuine immune mechanisms.

In *T.annulata*, information about the effects that macroschizont infected cells have upon naive PBM is somewhat scarce, and only that the phenomenon exists in some form and acts upon T cells is established (Rintelen *et al*, 1990; Glass and Spooner, 1990a). However, a large study of immune PBM failed to isolate parasite specific T cells, with the cells merely proliferating in an autologous MLR manner (appendix 2, this thesis). The MLR effect in *T.annulata* therefore differs from *T.parva* as it appears to be dominant, blocking or masking any genuine anti-parasite response. If such an effect were to operate during primary infections it would have the potential to seriously disrupt the generation of immune responses. Recent evidence would strongly suggest this to be the case:- T cells in lymph efferent from infected draining lymph nodes, although displaying activation markers, are incapable of mediating anti-parasite activity (Nichani, 1994). Treatment with Butalex, which abolishes the MLR inducing potential in macroshizont infected cells (Rintelen *et al*, 1990) is swiftly followed by the production of functional CTL (Nichani, 1994).

Studies throughout this thesis are concerned with understanding the interactions between the immune system and parasite infected cells in an effort to understand why primary immune responses to *T.annulata* are ineffective. In order to assess whether there was a potential role for the autologous MLR in primary immune response failure, studies were designed to examine the interactions between macroschizont infected cells and T cells in the peripheral blood of naive animals. Particular attention was payed to fully classifying the MLR effect in naive animals, the cell types activated, and phenotypes of T cells following "non specific" activation.

Experimental design

Using a group of 6 unrelated animals, *T.annulata* macroschizont infected cell lines were generated *in vitro*. The cell lines produced were used to stimulate autologous PBM in MLR reactions. Proliferation of PBM was monitored daily over a 7 day period, and the effects of differing numbers of stimulating cells assessed. At the same time, in depth phenotypic analysis of responding T cells was carried out, in particular the cell types activated and expression of activation and memory markers.

MATERIALS AND METHODS

T.annulata infected cell lines (IC)

Macroschizont infected cell lines (Ankara or Hisar) from the animals tested were prepared as described in the materials and methods sections. Cell lines were used at low passage number (2-30).

Cell preparation

Peripheral blood mononuclear cells (PBM) were separated as described in materials and methods. Mixed lymphocyte tissue culture medium (MLC) (Glass and Spooner, 1990a) was used throughout the experiments.

Culture of PBM with macroschizont infected cells

Culture of PBM with IC was based upon established protocols for the generation of bovine T cell lines to both intracellular parasites and antigenic peptides (Goddeeris and Morisson, 1987 ; Baldwin *et al*, 1987; and Glass and Spooner, 1990b, respectively). These methods have been previously adapted for antigen presentation assays using IC (Glass and Spooner, 1990a).

PBM were stimulated with autologous irradiated IC in 6x10ml well plates (Nunc, Gibco, Paisley, U.K). IC were irradiated (7500 rads) using a "Gravitom" sealed ^{137}Caesium source. PBM ($8x10^6$/ml):IC ratios were varied from 10:1-40:1, as lowering ratios below 40:1 had been shown to drastically reduce IC induced proliferation (Glass and Spooner, 1990a).

In order to provide a "control" population of stimulated T cells with which to compare IC activated cells, PBM ($2.5x10^6$/ml) were stimulated with 4μg/ml of Con A (Sigma). This amount of Con A has been shown to provide optimal IL2 production, with a peak of proliferation after 48-72 hrs of culture (Seiss *et al,* 1989).

To investigate whether cell-cell contact was essential for the proliferative responses previously described, identical cultures to those described above were set up with PBM separated from IC or from medium containing Con A by a 0.4μm pore membrane (Millicel-CM Insert, Millipore corp., Bedford MA, USA). The total

volume of medium used with inserts was 7ml per well of a 6 well plate. In each case, the PBM (in 4ml of MLC) were first placed in the well of the plate, the insert was then added followed by the stimulator (IC or Con A containing medium). Stimulated PBM were harvested at various times (1-7 days) for FACS analysis.

Proliferation assays

Proliferation assays were performed as previously described (Glass and Spooner, 1990). PBM ($4x10^5$/well) were incubated with irradiated autologous IC (1-$8x10^4$/well) or 4μg/ml Con A for 1-7 days. MLC medium was used throughout, with the 10% FCS replaced by 5% "Serumax" (Sigma), a partially defined serum supplement which has been shown to generate lower background counts. Assays were set up in quadruplicate in 96 well flat bottomed plates (Nunc), and labelled with [^{3}H]dThd (Amersham Int., Amersham, UK) in the last 6 hours of incubation. Assays were harvested using an Inotech 96 well harvester onto a Wallac fibreglass filter mat. Filters were dried at 60°C for 30 minutes, placed in "Optiscint Hi-Safe" scintillant (Wallac) and counted in a Wallac 1450 Microbeta.

As membrane inserts were too large to use in 96 well plates, cells were grown as normal in 6 well plates using inserts and MLC-Serumax. 6 hours before the end of incubation the inserts were removed, the PBM plated out in 96 well plates at the same dilution as normal assays, pulsed, and counted.

FACS Analysis

Cells were stained for dual colour FACS analysis as described in the materials and methods section. Analysis concentrated on the expression of activation markers upon different T cell subsets. MAbs used are listed in table 3.1, followed by a description of the cell surface markers recognised.

T cell markers

MM1A recognises the bovine CD3 complex and strongly stains bovine $\alpha\beta$ T cells and $\gamma\delta$ T cells (Davis *et al*, 1993). CD2, present on $CD4^+$ and $CD8^+$ cells, was identified using IL-A26 (Baldwin *et al*, 1988). CD2 is also expressed upon bovine

Table 3.1

MAb used in analysis of T cell activation

mAb (isotype)	Specificity	Reference
IL-A26 (IgM)	CD2	Baldwin *et al*, 1988
MM1A (IgG_1)	CD3	Davis *et al*, 1993
IL-A12/CC8 (IgG_{2a})	CD4	Baldwin *et al*, 1986/Howard *et al*, 1991
CC63/SBU-T8 (IgG_{2a})	CD8	Howard *et al*, 1991/Meeusen *et al*, 1988
CC15 (IgG_{2a})	$\gamma\delta$ T Cells (WC1)	Clevers *et al*, 1990
J11 (IgG_1)	MHC Class II (DR)	Dutia *et al*, 1995
IL-A21 (IgG_{2a})	MHC Class II	Davies *et al*, 1994
CC76 (IgG_1)	CD45 RB	Howard *et al*, 1991
IL-A150 (IgG)	CD45 R0	Bembridge *et al*, 1993
IL-A111 (IgG_1)	IL2 Receptor	Naessens *et al*, 1992

$CD3^-$ cells, and these are thought to represent bovine NK cells (Evans and Jaso-Friedmann, 1993). The bovine homologues of CD4 and CD8 (recognised by mAbs IL-A12, and CC63/SBU-T8 respectively) have been identified primarily by the functional similarity of the cells recognised by the mAb to their human counterparts (IL-A12 - Baldwin *et al*, 1986; CC8 - Howard *et al,* 1991;SBU-T8 Maddox *et al,* 1985). The production of mAb to bovine T cell receptors (TCR) has proved difficult, and no such mAb were available during this study. Bovine $\gamma\delta$ T cells were stained using mAb CC15 which recognises a surface molecule known as WC1 on the surface of these cells (Clevers *et al,* 1990). WC1 exists in two different forms (p215 and p205 both recognised by CC15), is exclusively found on $CD2^-$, $CD4^-$, $CD8^-$ $\gamma\delta$ T cells but has not as yet been identified as having any distinct function (Crocker *et al,* 1993).

T cell "memory" markers

T cells in humans and mice have been categorised as "naive" or "memory" in phenotype according to their expression of different spliced forms of the cell surface antigen CD45 (Thomas, 1989). The best evidence for similar properties within bovine T cells is the expression of CD45RB recognised by mAb CC76 (Howard *et al*, 1991). $CD4^+$ T cells which stain strongly with CC76 (CD45RB high) have been characterised as naive, while the ability to respond to recall antigens resides in the cells which weakly stain with CC76 (CD45RB low). The previous distinctions have only been established fully for $CD4^+$ T cells (Howard *et al*, 1991). However, memory status can also be assessed in human $CD8^+$ as well as $CD4^+$ T cells by the expression of the CD45RA splice variant. Cells expressing CD45RA are regarded as "naive", while "memory" do not stain with anti CD45RA antibodies but do stain with anti CD45RO antibodies. The mAb IL-A150, which recognises the bovine equivalent of CD45RO (Bembridge *et al*, 1993), was also used in this study.

T cell activation markers

Expression of IL-2 and IL-2 receptor (IL-2R) genes are induced when T cells are activated (Mauer *et al*, 1984; Smith 1988). Bovine T cells rapidly express IL-2R

(Seiss *et al*, 1989), and MHC class II (Glass and Spooner, 1990b) upon activation. IL2R expression was measured using the mAb IL-A111. This mAb recognises the Tac subunit of the IL2R (CD25), does not stain resting T cells, but stains cells post activation (Naessens *et al*, 1992). MHC class II expression was measured using mAbs J11 (Dutia *et al*, 1995) and IL-A21 (Davies *et al*, 1994) both of which recognise bovine MHC class II molecules, but have different isotypes.

FACS analysis

Cells were acquired and analysed using Lysys II software, and gates and compensation were set as described in the materials and methods. Quadrants for analysis of double stained cells were set using negative control samples, with the exception of cells stained with CC76. As staining with this mAb is designated as "high" or "low", gates were set using B cells (mAb VPM30 - Naessens and Howard (1991)), which are stably CD45RB high (Howard *et al*, 1991). Once standard gates had been set, the vertical cross hair was moved to the edge of the B cell population within the CC76 stained cells, while leaving the horizontal crosshair in an identical position. (see Fig. 3.1). Once set, cells in the right hand quadrants were treated as high staining, those in the left as low.

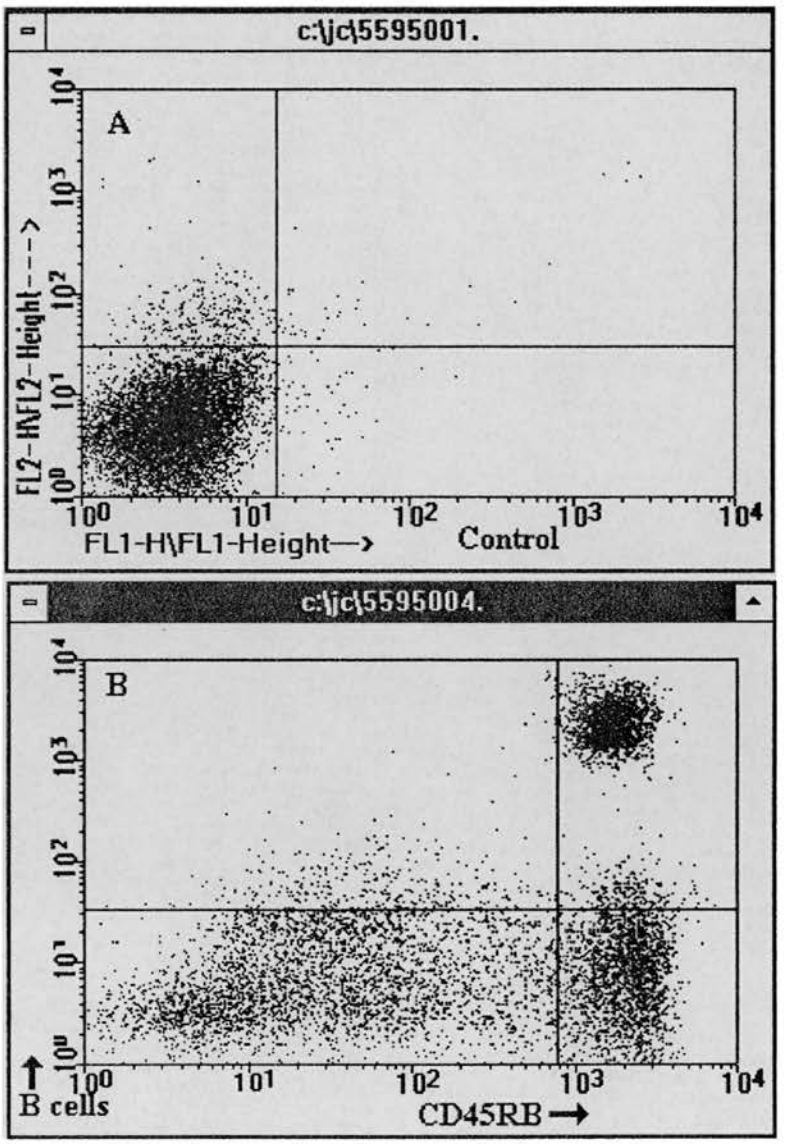


Fig. 3.1 Gating for CD45RB high and low cells using B cells and mAb CC76.
A - Quadrants are set as normal using negative controls.
B- Keeping the horizontal crosshair unchanged, the vertical is moved to the edge of the B cell/CD45RB high population (upper right quadrant). In subsequent samples, cells in the upper right quadrant are designated CD45RB high, and those in the upper left CD45RB low.

RESULTS

PROLIFERATION ASSAYS

In this section, proliferation results are expressed in corrected counts per minute (CCPM) - as the microbeta is a multi detector instrument, results are automatically "corrected" to compensate for different detector efficiencies. Results following stimulation of PBM with Con A or autologous *Theileria annulata* infected cells (IC) are all expressed net of medium control values:

Net Con A induced proliferation = CCPM PBM with Con A - CCPM PBM with medium

Net IC induced proliferation = CCPM PBM with IC - (CCPM PBM with medium + CCPM IC with medium alone)

In all naive animals tested (n=5), incubation of PBM with Con A or autologous IC induced proliferation, although Con A activation always led to greater thymidine uptake than IC activation. The kinetics of proliferation following the two stimuli were dissimilar. Con A induced proliferation was maximal 2-3 days post stimulation, whereas IC stimulated cells showed maximum proliferation at day 5 (n=4) or day 6 (one animal) (Fig. 3.2). Proliferation to IC was first seen at day 3, sometimes plateauing or even falling sharply, before rising again to the peak at day 5-6 (Fig. 3.3).

In cultures examined daily, using IC:PBM ratios of 1:10 and 1:20, the higher number of stimulators generally produced more proliferation (Fig 3.3). Five days post stimulation (peak thymidine uptake), the amount of IC induced proliferation was again dependent upon the numbers of stimulators used, although animals differed in their responses. The highest ratio of IC:PBM - 1:5 - induced maximum proliferation in two animals, with thymidine uptake decreasing with lower concentrations of IC (Fig. 3.4). However, two animals (10795 and 10814) showed markedly lower proliferation at 1:5, with maximum proliferation obtained at ratios of 1:10 and 1:20 respectively (Fig. 3.5). This was not due to the peak day of proliferation being "missed", as daily analysis confirmed that thymidine uptake was maximal at day 5

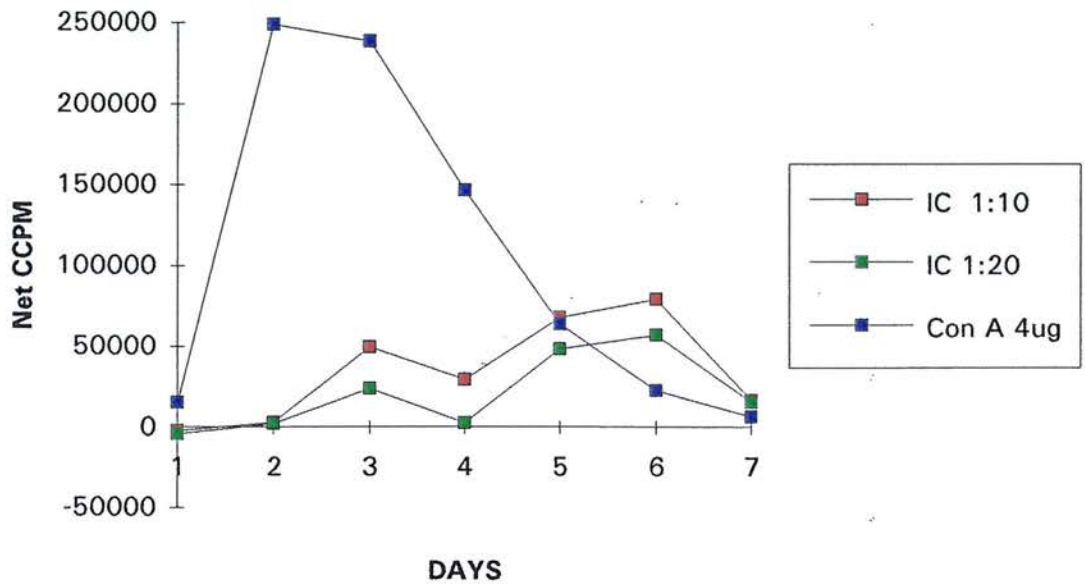
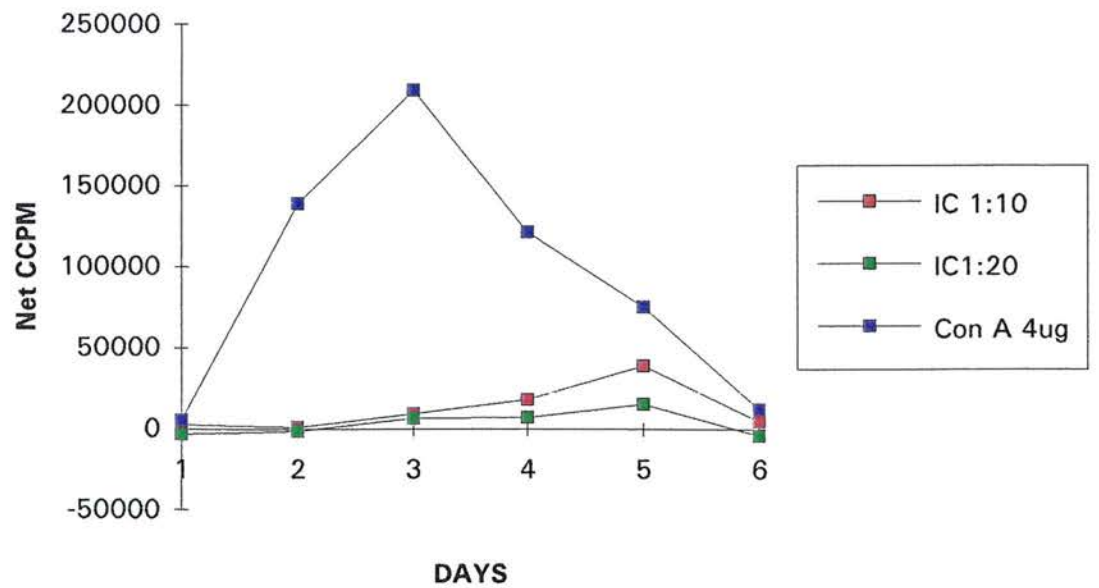


Fig. 3.2 Net proliferation of PBM from 2 animals (13050 above, 10795 below) following Con A and IC stimulation. Con A proliferation peaks at days 2-3, while IC induced proliferation peaks after 5-6 days.



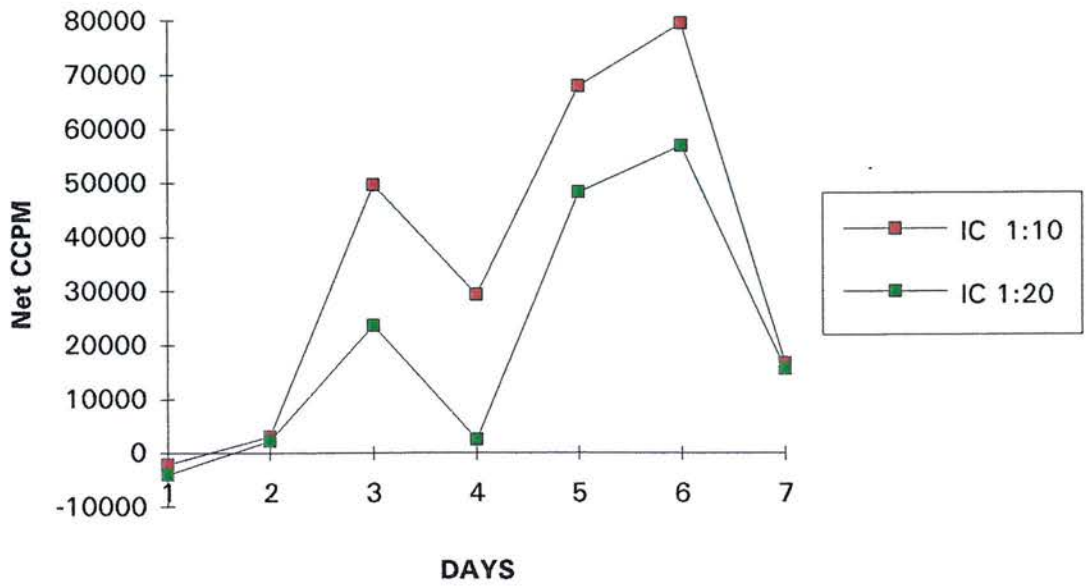
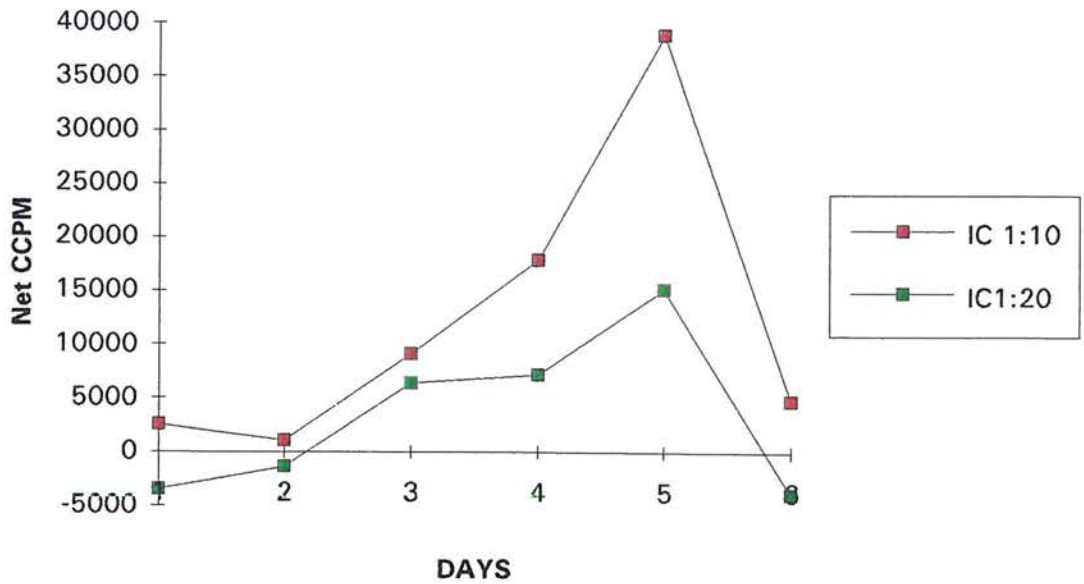


Fig. 3.3 Identical profiles to Fig. 3.2, IC stimulation only. Note distinct double peak of proliferation in 13050 above, while 10795 shows both a "plateau" at day 4 (IC 1:20), and an apparent constant rise in proliferation (IC 1:10).



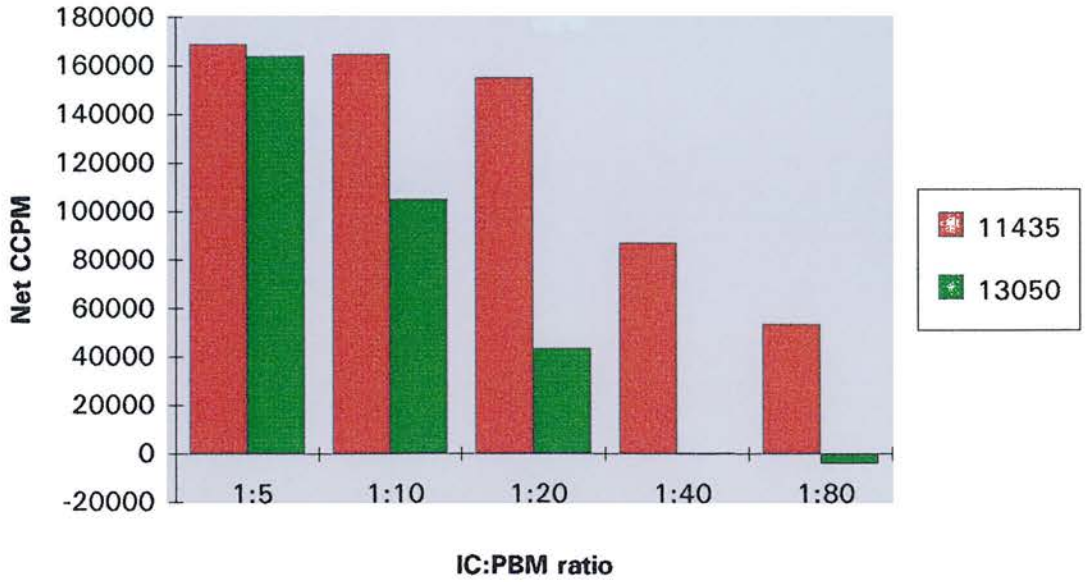


Fig. 3.4 Five days post stimulation, two animals (11435 and 13050), show maximum proliferation at IC:PBM ratios of 1:5.

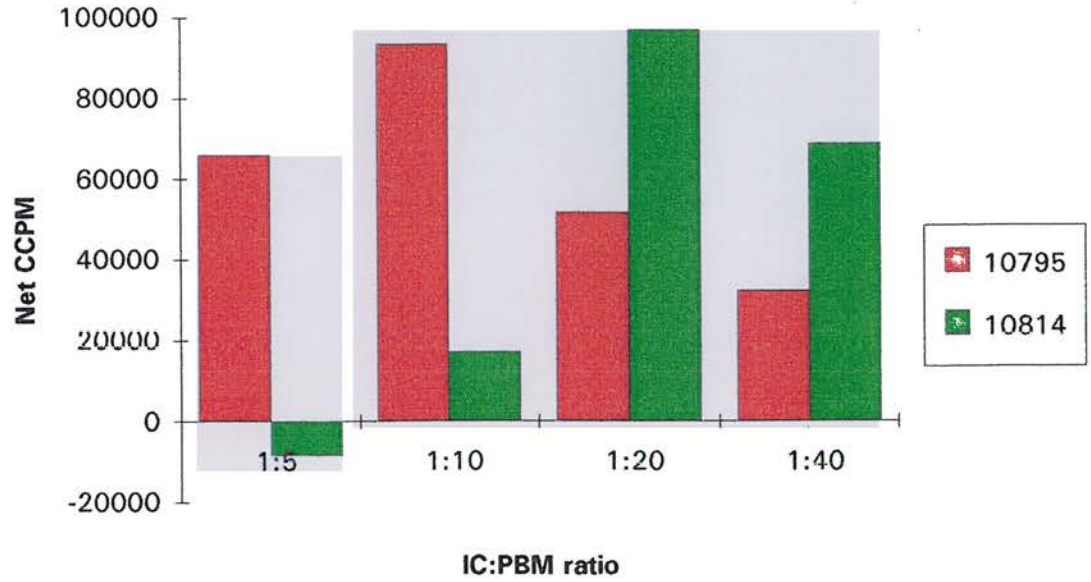


Fig. 3.5 Five days post stimulation, proliferation of PBM from two animals (10795 and 10814) is maximal at IC:PBM ratios of 1:10 and 1:20 respectively.

(10795 illustrated in Fig. 3.3). Maximum net proliferation levels also varied between animals - 170 000 CCPM in 11435 to as low as 50 000 in 10814. Lowering IC:PBM ratios to 1:80 or below resulted in no proliferation in 4 out of 5 animals.
PBM separated from IC by a 0.4μm pore membrane did not proliferate, even at optimal IC:PBM ratios, but separation had no effect upon Con A proliferation (Fig. 3.6).

FACS ANALYSIS OF T CELL ACTIVATION

A total of six animals were used for in depth analysis of T cell activation marker expression. Unfortunately, due to a failure in the data storage system of the FACScan, a large amount of data from two animals was lost. The majority of results are therefore detailed from 4 animals, with numbers used noted within each section. During this study, T cells were principally examined for both their surface expression of activation markers, and for changes in "memory" status. As noted in the materials and methods, cell samples were gated at acquisition to remove dead cells and debris. All quadrant gates used in illustrations were set using the negative controls appropriate to the treatment of the cells and secondary antibodies.

T cells in peripheral blood

T cell populations in freshly isolated PBM

The percentages of different T cell populations within the PBM of 4 healthy animals are detailed in table 3.2. $CD3^+$ cells varied from 55-68% of all PBM, correlating very closely with the total number of cells detected by anti CD4, CD8, and $\gamma\delta$ T cell mAb (maximum 1.8% difference). $CD4^+$ T cells were usually present in larger numbers than $CD8^+$ cells. The numbers of $\gamma\delta$ T cells present was variable and appeared to bear no relation to the numbers of $WC1^-$ cells.
The total numbers of $CD4^+$ and $CD8^+$ T cells could not account for all $CD2^+$ cells observed in PBM. As IL-A26 (anti CD2) does not recognise B cells or monocytes from bovine PBM (Baldwin *et al*, 1988), and bovine $\gamma\delta$ T cells are $CD2^-$ (Crocker *et al*, 1993), these cells are likely to be NK cells (Evans and Jaso-Friedmann, 1993).

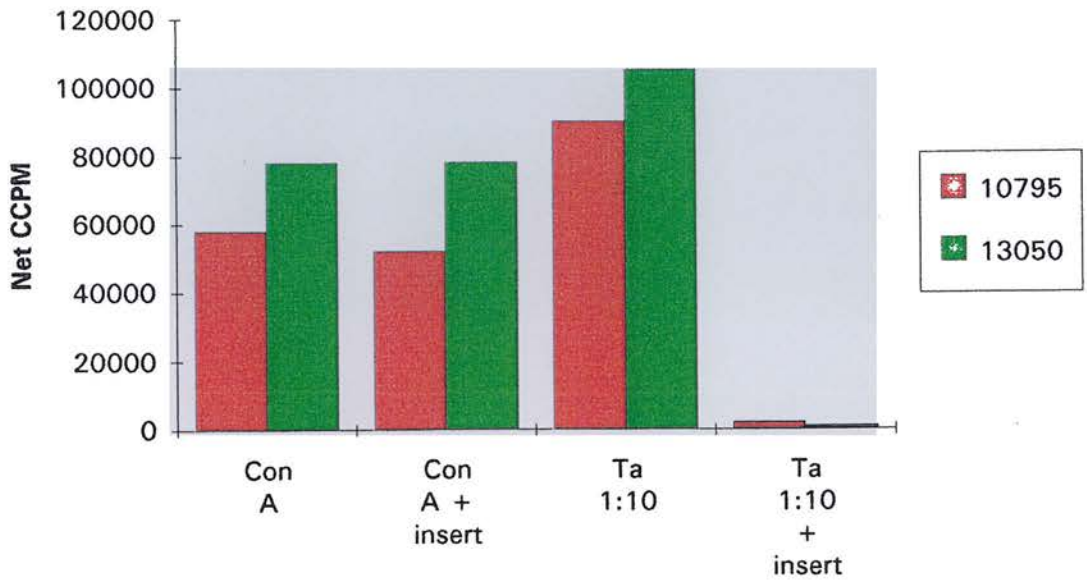


Fig. 3.6 Separation of PBM from stimulatory *T.annulata* IC (Ta) by a 0.4μm pore membrane insert (+I) blocks IC induced proliferation, while Con A stimulation is unaffected. Day 5.

Table 3.2

T cell populations in normal peripheral blood, expressed as percentages of the total population

SEM = Standard error of the mean

Population	10795	10814	11435	13050	Mean ± SEM
CD4	28.2	23.1	36	25.5	28.2 ±2.8
CD8	23.1	24.9	24	18.8	23.6±1.4
CD4 + CD8	51.3	48	60	44.3	50.9±3.4
CD2	61.5	62.2	63.7	52.3	59.9±2.6
γδ T (WC1)	14.4	5.8	6.2	22.1	11.2±3.4
CD3	66.5	55.2	67	68.2	64 ± 3.1
CD4+CD8+ γδT	65.7	53.8	66.2	66.4	63 ± 3.1

Activation and memory markers

The percentage expression of the activation markers IL-2R and MHC class II within $CD4^+$, $CD8^+$ and $\gamma\delta$ T cell populations of freshly isolated PBM are summarised in Fig. 3.7. Approximately 25% of $CD4^+$ cells expressed both activation markers, with very little variation between animals. Similar numbers of $CD8^+$ cells expressed MHC class II but IL-2R expression upon $CD8^+$ cells was consistently lower. $\gamma\delta$ T cells expressed high levels of activation markers ($\geq$40%) in peripheral blood.

Two thirds of $CD4^+$ T cells were present as the CD45RB low effector type (Fig. 3.8), while the majority of $CD8^+$ cells were CD45RB high. Over 85% of all $\gamma\delta$ T cells were CD45RB low. Double staining of CD4/CD8/$\gamma\delta$ T cells with anti-CD45RO was not possible, as this mAb is a mixture of IgG isotypes. However, CD45RO expression on $\alpha\beta$ T cells and NK-like cells was examined by staining with anti-CD2 (IgM-FITC) and anti-CD45RO (IgG-PE). CD45 isoforms expressed by bovine $CD2^+$ NK cells are unknown. Resting human NK cells do not express CD45RO (Shen *et al*, 1995), and the situation would appear similar here, as good correlation was found between the total numbers of $CD4^+$ and $CD8^+$ CD45RB low cells and the percentages of $CD2^+$ $CD45RO^+$ cells (Table 3.3). The behaviour of non T $CD2^+$ cells upon activation is discussed below.

T cell activation by autologous *T.annulata* infected cells

The results of proliferation studies detailed above indicated that although very high IC:PBM ratios of 1:5 and 1:10 induced large amounts of proliferation in PBM in some animals, such ratios could also inhibit proliferation. The vast majority of experiments in this section therefore used IC:PBM ratios of 1:20, which still induced large amounts of proliferation, but had not been shown to be inhibitory in any animals. All data comes from 1:20 ratios unless otherwise stated.

$\gamma\delta$ T CELLS

$\gamma\delta$ T cells are described first due to their extremely characteristic behaviour *in vitro*. In all animals examined (n=6), whether in PBM incubated with medium alone

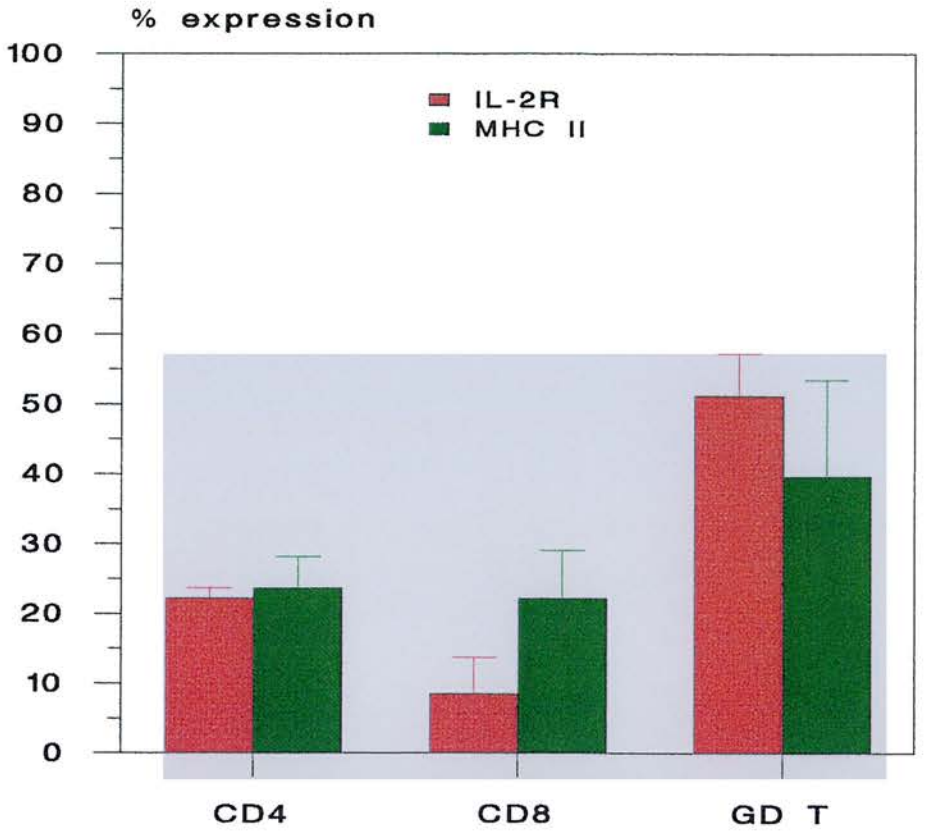


Fig. 3.7 Mean percentage expression of IL-2R and MHC class II on $CD4^+$, $CD8^+$ and $\gamma\delta$ T cells in normal peripheral blood (n=4).

Error bars represent 1 standard deviation.

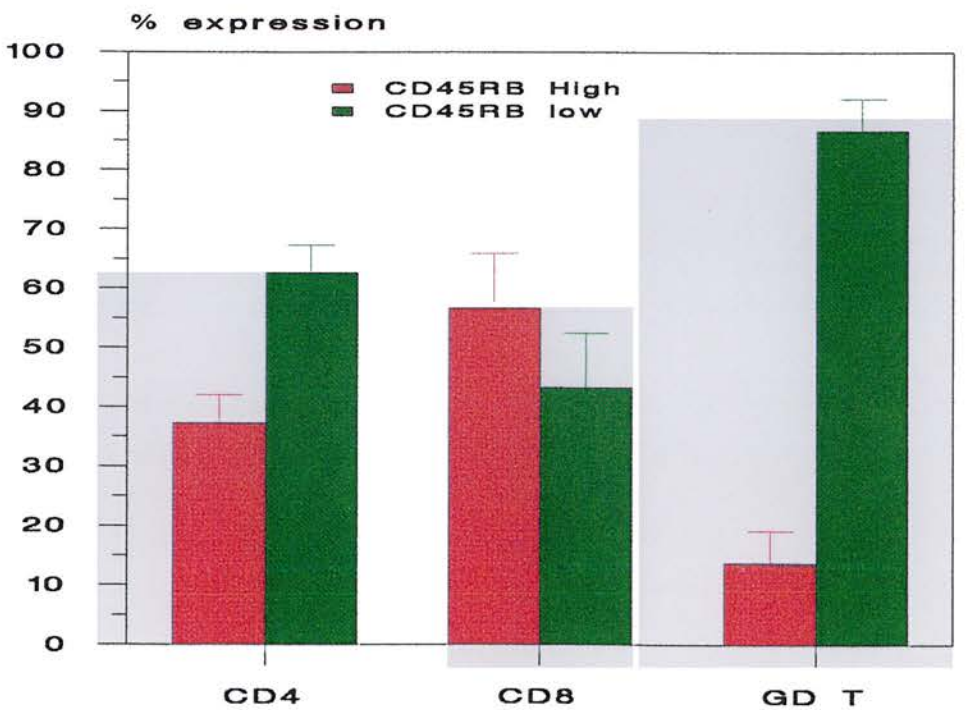


Fig. 3.8 Mean percentage expression of CD45RB low phenotype ("memory/effector") and CD45RB high phenotype ("naive") on $CD4^+$, $CD8^+$ and $\gamma\delta$ T cells in normal peripheral blood (n=4).

Table 3.3
Correlation between CD45RB low and $CD45RO^+$ cells on $CD2^+$ T cells (percentages of total PBM)

SEM = Standard error of the mean

Animal	$CD4^+ + CD8^+$ CD45RB LOW	$CD2^+/CD45RO^+$
10795	28.9%	30.09%
10814	30.14%	28.56%
11435	29.18%	31.9%
13050	25.69%	28.03%
Mean ± SEM	28.48% ±0.96	29.65% ±0.87

(control cultures), IC, or Con A, $\gamma\delta$ T cells expressed extremely high levels of activation markers. A comparison between cells given all 3 treatments for 24 hrs is illustrated in Fig. 3.9. The levels of activation marker expression in the medium only controls is virtually indistinguishable from those given "T cell stimuli". When examined over a 5 day period, 70-80% of $\gamma\delta$ T cells in control cultures continued to express IL-2R, as did those stimulated with IC (Fig. 3.10). The only difference noted was a rise at day 7 in IL-2R expression to 90% in IC cultures, whereas the controls remained constant. Although IC stimulation lead to slightly higher IL-2R expression in the latter stages, MHC class II expression kinetics were identical, peaking at $\geq$95% in both control and IC stimulated cultures, rapidly dropping by day 7 (Fig. 3.10). In all animals, cultured $\gamma\delta$ T cells remained $\geq$90% CD45RB low at all times. Using this system it is therefore impractical to determine any possible activation effects of IC upon $\gamma\delta$ T cells in PBM, as these cells assume an activated phenotype simply by culturing in medium alone. As a result, $\gamma\delta$ T cells are not discussed further.

$CD4^+$ AND $CD8^+$ T CELLS

Early activation events - 24 and 48hrs of culture

After 24 hrs of culture, expression of IL-2R and MHC class II upon $CD4^+$ and $CD8^+$ T cells in control PBM remained essentially the same as in freshly isolated PBM. Con A stimulated cultures exhibited very large increases in activation marker expression - typically 75-85% of both $CD4^+$ and $CD8^+$ cells expressing both IL-2R and MHC class II (examples in Fig. 3.11 and 3.12). *Theileria* stimulated cultures also showed increased activation marker expression, although this was much smaller than Con A and varied tremendously between animals (11435 illustrated in Fig. 3.11 and 3.12). However, the degree of activation was consistent within each animal - increases of only 7% in activation marker expression was found in both $CD4^+$ and $CD8^+$ cells in 10795, whereas increases of approximately 10 and 20% expression of IL-2R and MHC class II respectively were found in both $CD4^+$ and $CD8^+$ cells in 11435.

Following 48hrs incubation, all animals examined displayed similar patterns of

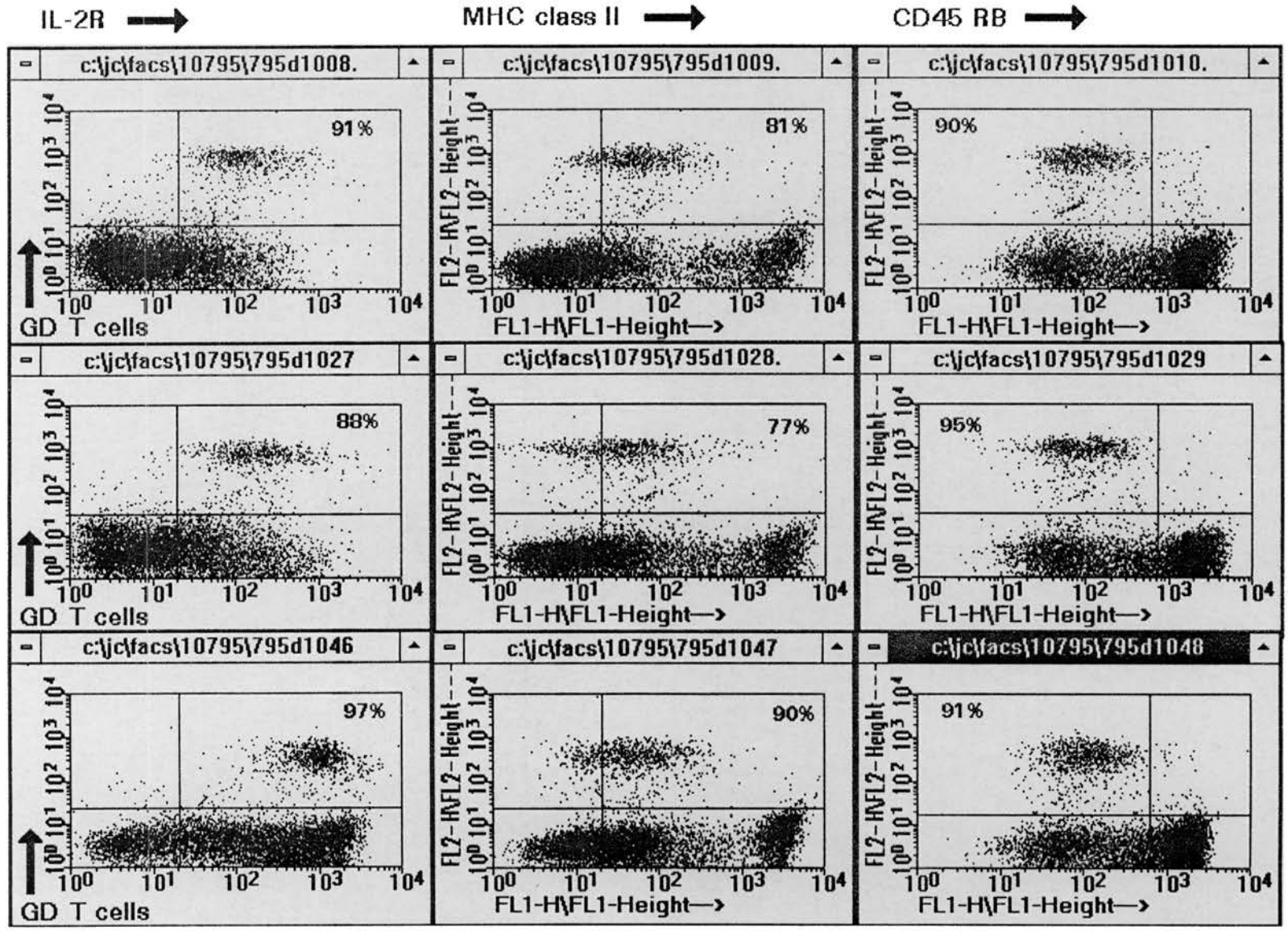


Fig. 3.9 Expression of IL-2R, MHC class II and CD45RB by γδ T cells after 24 hours culture with - medium alone (control), autologous *T.annulata* infected cells (Ta), or Con A 4μg (Con A). Percentages are of total γδ T cells. Animal 10795.

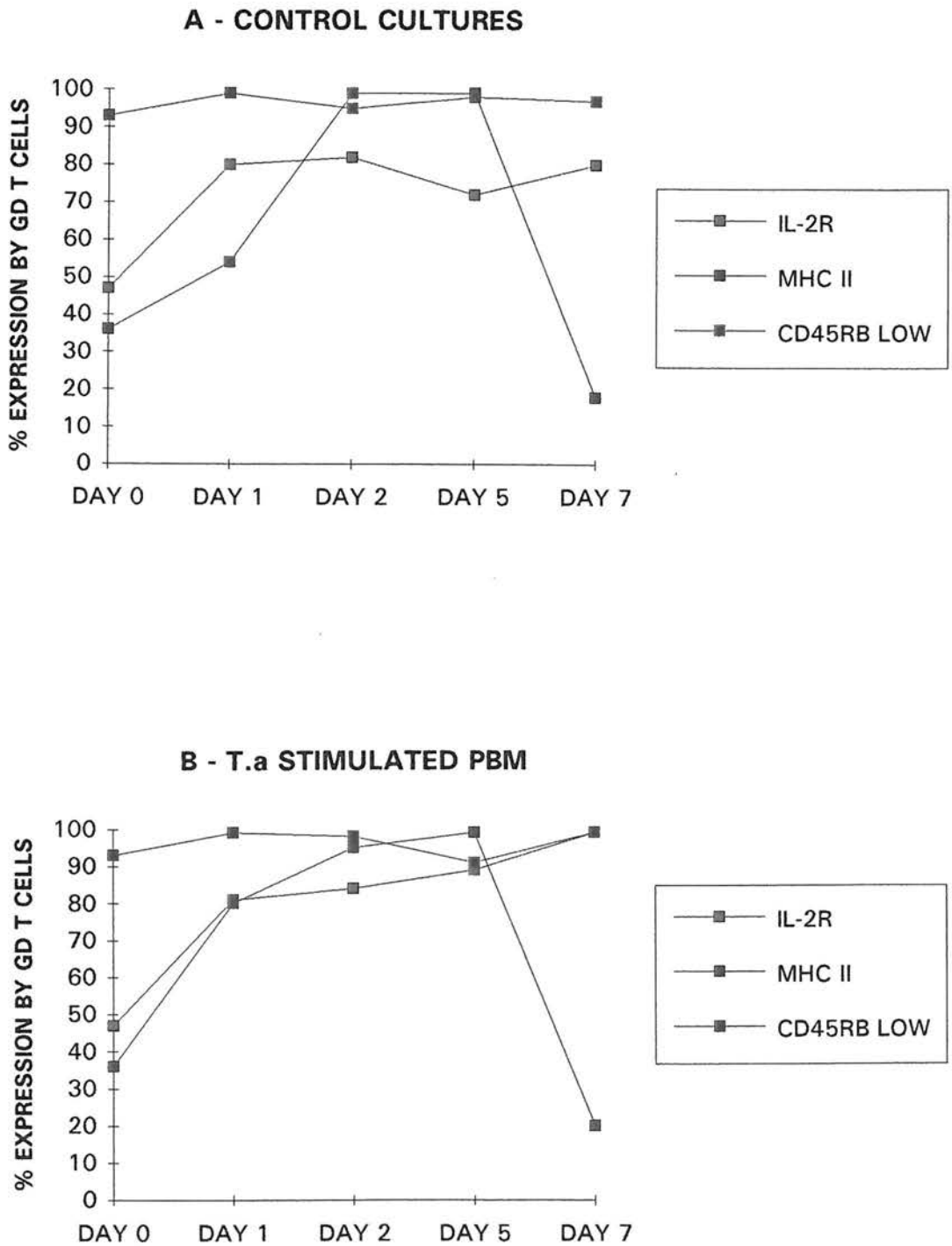


Fig. 3.10 IL-2R and MHC class II expression on $\gamma\delta$ T cells remains extremely high throughout 7 days culture with: A-medium alone or B-*T.annulata* IC. CD45RB low is the predominant phenotype. (Animal 13050).

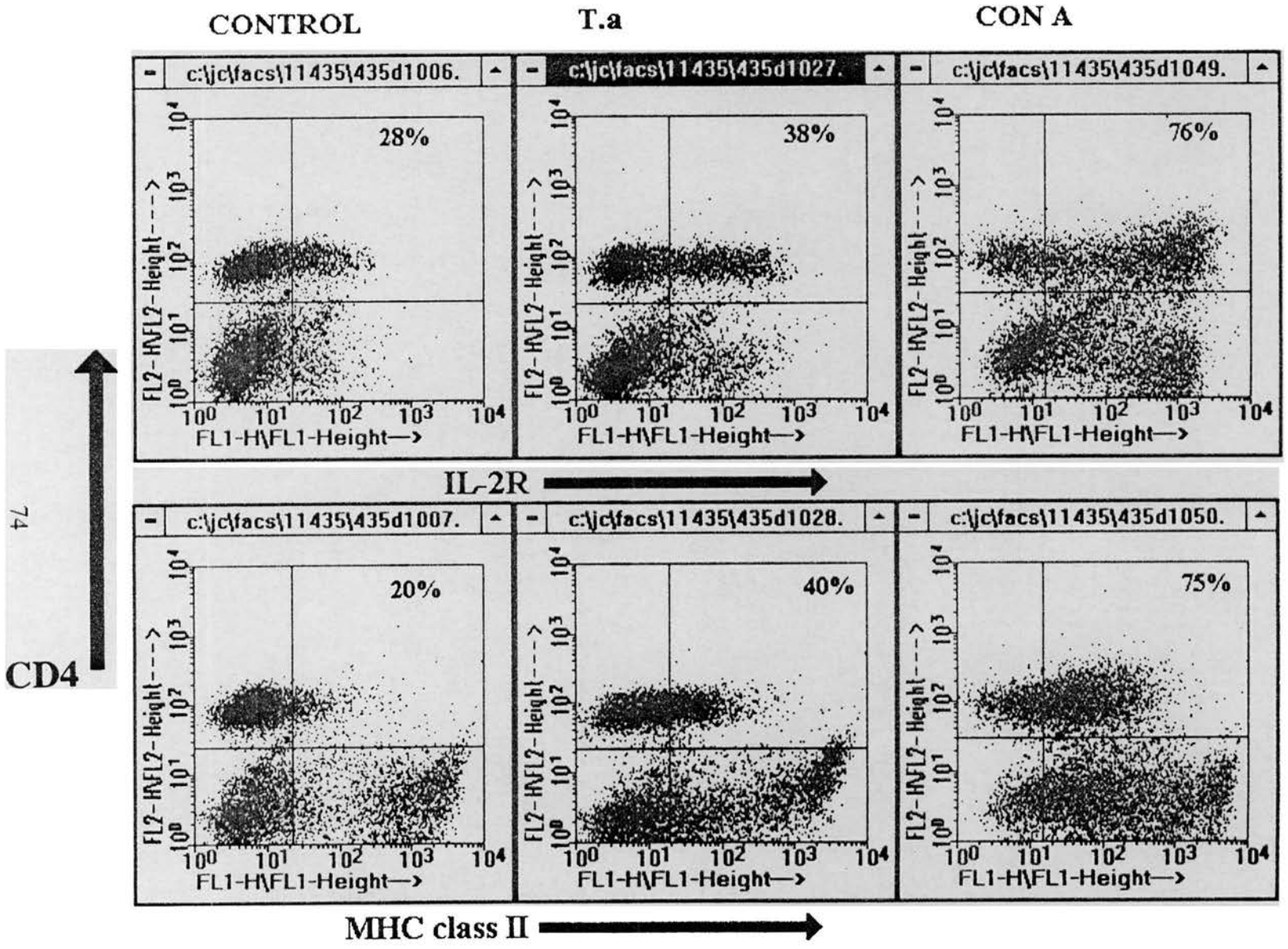


Fig. 3.11 Expression of IL-2R and MHC class II by $CD4^+$ T cells after 24 hours culture with - medium alone (control), autologous *T.annulata* infected cells (Ta), or Con A 4μg (Con A). Percentages are of total $CD4^+$ T cells. Animal 11435

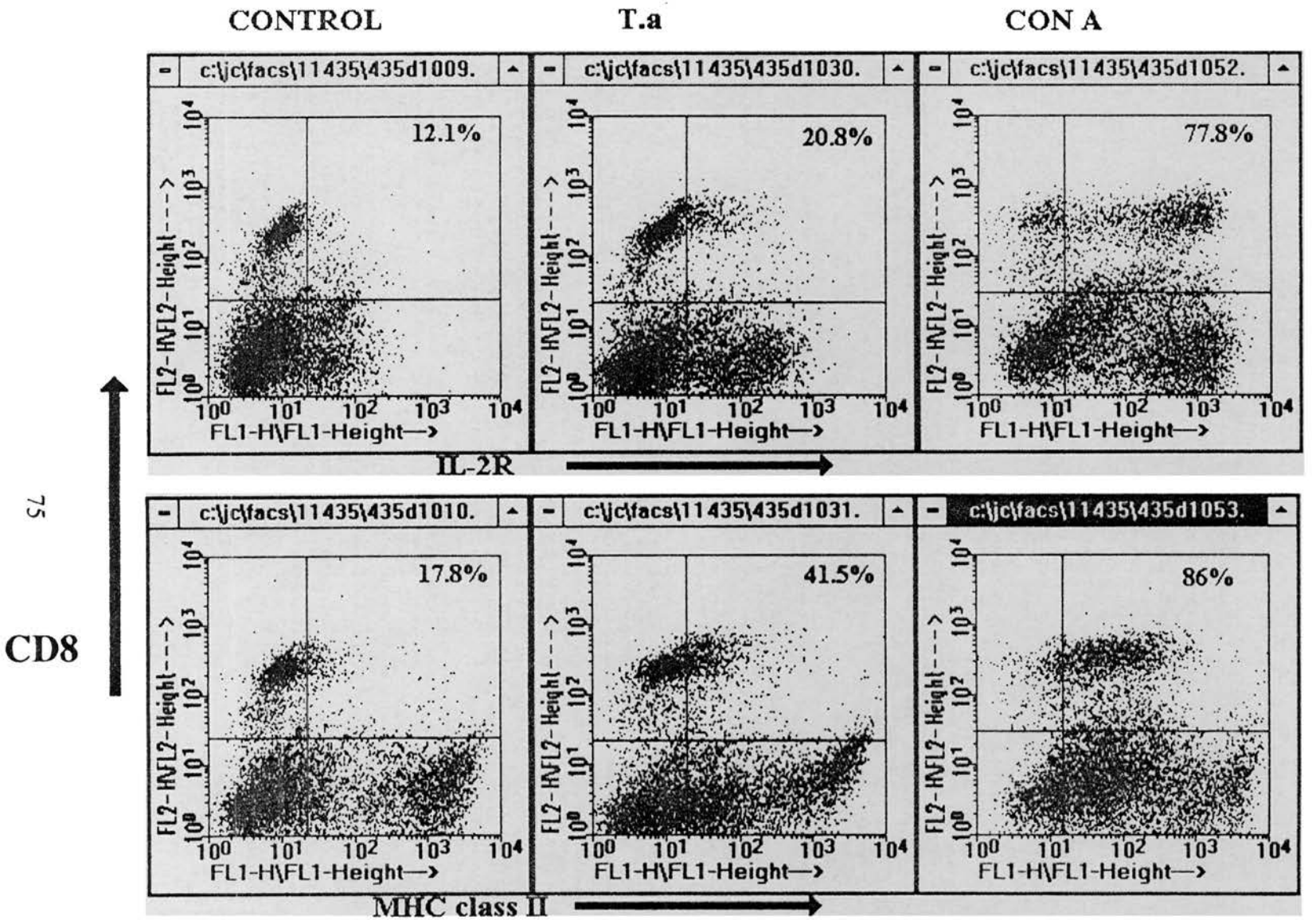


Fig. 3.12 Expression of IL-2R and MHC class II by $CD8^+$ T cells after 24 hours culture with - medium alone (control), autologous *T.annulata* infected cells (Ta), or Con A 4μg (Con A). Percentages are of total $CD8^+$ T cells. Animal 11435

activation marker expression. Data described in this paragraph are summarised in Fig. 3.13 (n=6). The student T-test was used to assess whether the increases in activation marker expression seen were significant (Table 3.4). Control cultures again exhibited similar numbers of activated cells to PBM, although an elevation of up to 10% was seen in some animals. IC induced a highly characteristic pattern of activation in all animals. A mean of 25% more $CD4^+$ cells constantly expressed IL-2R in IC stimulated cultures than in the controls, with MHC class II expression often slightly lower - mean 20% more expression (Fig. 3.14) This situation was reversed in $CD8^+$ cells, with more cells expressing MHC class II (mean 30% above control levels) than IL-2R (mean 18%) (Fig. 3.15). Separation of PBM from IC effectively blocked expression of activation markers upon T cells above control levels, except in IL-2R expression by $CD8^+$ cells. In an animal where IC:PBM ratios of 1:10 had been shown to increase proliferation (11435), such a ratio induced higher levels of activation markers upon T cells - 50% above control values (Fig. 3.16). IL-2R expression upon Con A stimulated cells reached virtually 100% in $CD4^+$ cells, although MHC class II expression was consistently lower - approximately 80%. Both IL-2R and MHC class II were present upon 70-80% of $CD8^+$ cells (Fig. 3.17). Culture of PBM with autologous IC therefore leads to higher expression of activation markers upon both $CD4^+$ and $CD8^+$ cells within 48hrs, although at a lower level than Con A stimulation.

Peak proliferation - day 5 (Fig 3.18)

At day 5, blasting of PBM induced by incubation with IC reached its peak (Fig 3.18A).The student T-test was used to assess whether the increases in activation marker expression seen at day 5 were significant (Table 3.5). IL-2R expression upon $CD4^+$ cells in control cultures remained similar to day 2 - mean 33%. Incubation with IC again had elevated expression of IL-2R to approximately 25% above control values, which again was blocked by separation of IC and PBM by a membrane. MHC class II expression was also elevated upon IC activated $CD4^+$ cells, although not significantly above control values, which also exhibited increased MHC class II expression. However, MHC class II expression following IC stimulation was

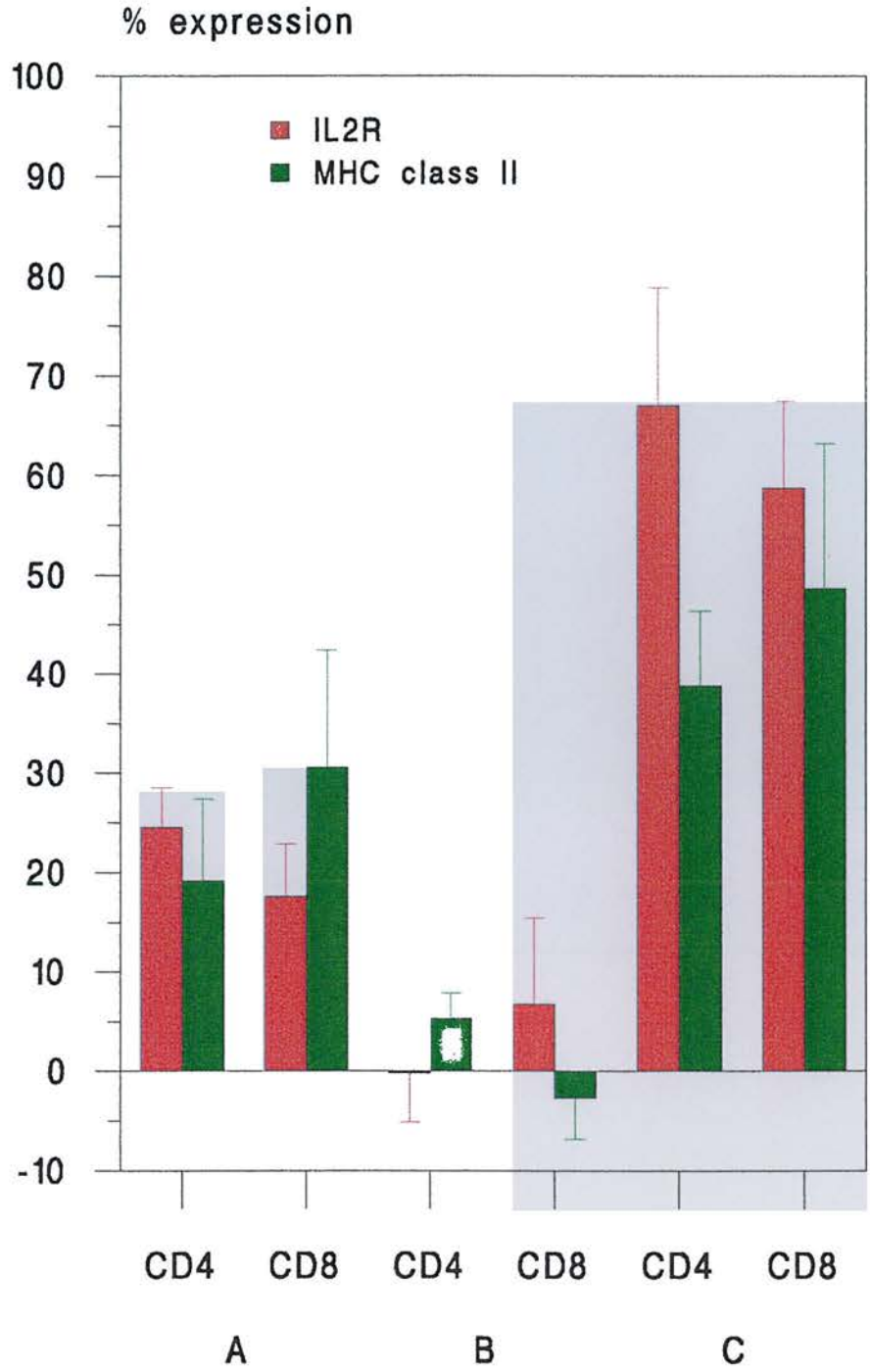


Fig. 3.13 Mean (n=6) IL-2R and MHC class II expression by $CD4^+$ and $CD8^+$ T cells after 48hrs culture with: A - *T.annulata* IC, B - IC separated from the PBM by a 0.4 μm pore membrane, C - Con A 5μg/ml.
Error bars represent 1 standard deviation.

Table 3.4

Student T-test analysis.

Comparison of changes in mean IL-2R and MHC class II expression by $CD4^+$ and $CD8^+$ T cells after 2 days incubation with IC or separated from IC by a membrane. $p \leq 0.05$ is treated as significant.

Cell / Marker	Treatment	Mean % Expression	SD	p value	Significant
CD4/ IL-2R	IC IC + M	24.62 -0.12	3.85 5	$p \leq 0.001$	YES
CD4/ MHC II	IC IC + M	19.2 5.3	8.2 2.6	$p \leq 0.01$	YES
CD8/ IL-2R	IC IC + M	17.5 6.7	5.3 8.77	$p \leq 0.05$	YES
CD8/ MHC II	IC IC + M	30.62 2.75	11.8 4.1	$p \leq 0.001$	YES

IC - Incubated with *T.annulata* IC

IC + M - Incubated with *T.annulata* IC, separated by a membrane

In all cases, incubation of PBM with IC induces significantly higher levels of IL-2R and MHC class II expression on $CD4^+$ and $CD8^+$ cells than, when PBM are separated from IC by a membrane.

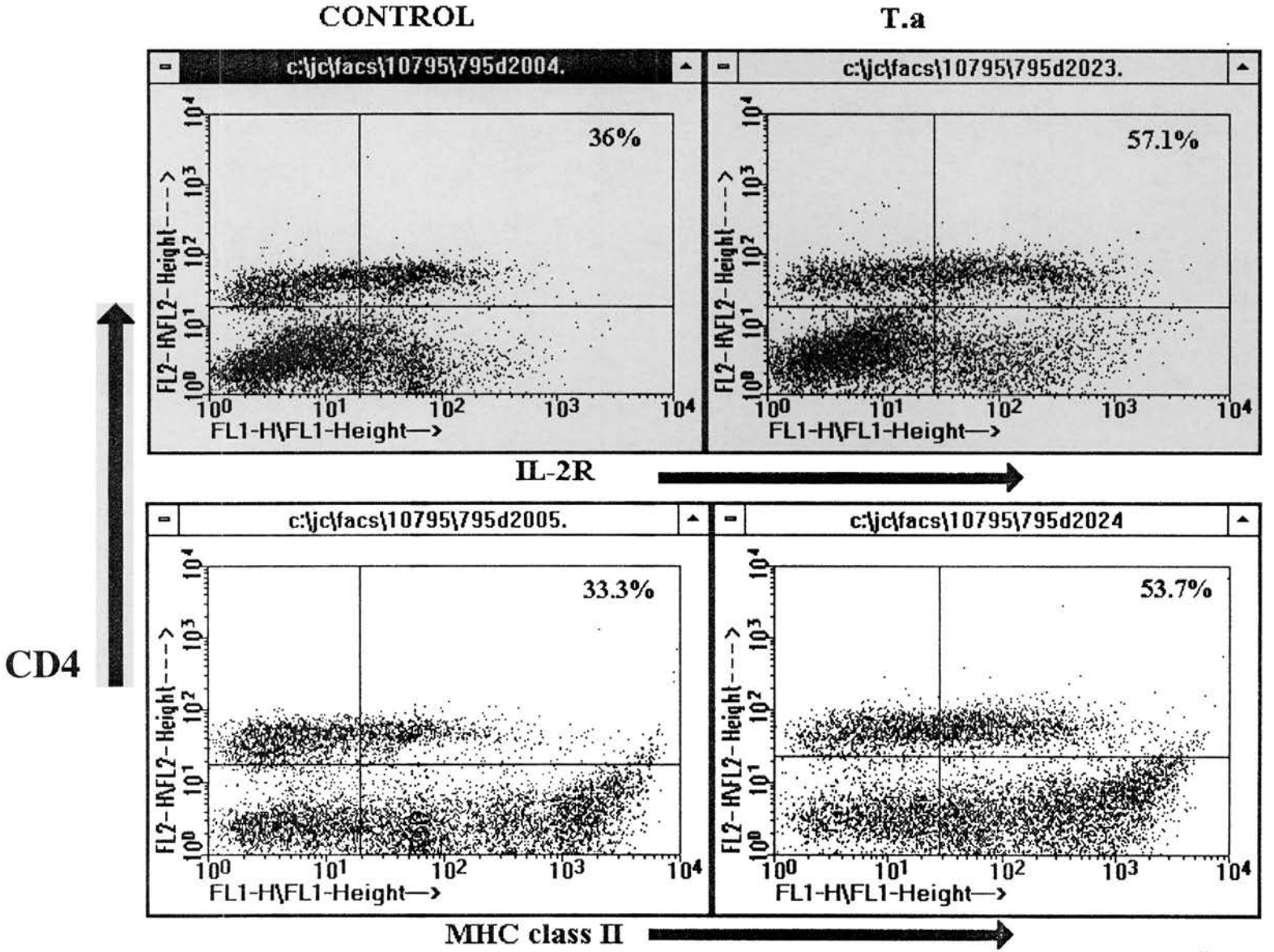


Fig. 3.14 Expression of IL-2R and MHC class II by $CD4^+$ T cells after 48 hours culture with - medium alone (control), or autologous *T.annulata* infected cells (Ta). Percentages are of total $CD4^+$ T cells. Animal 10795

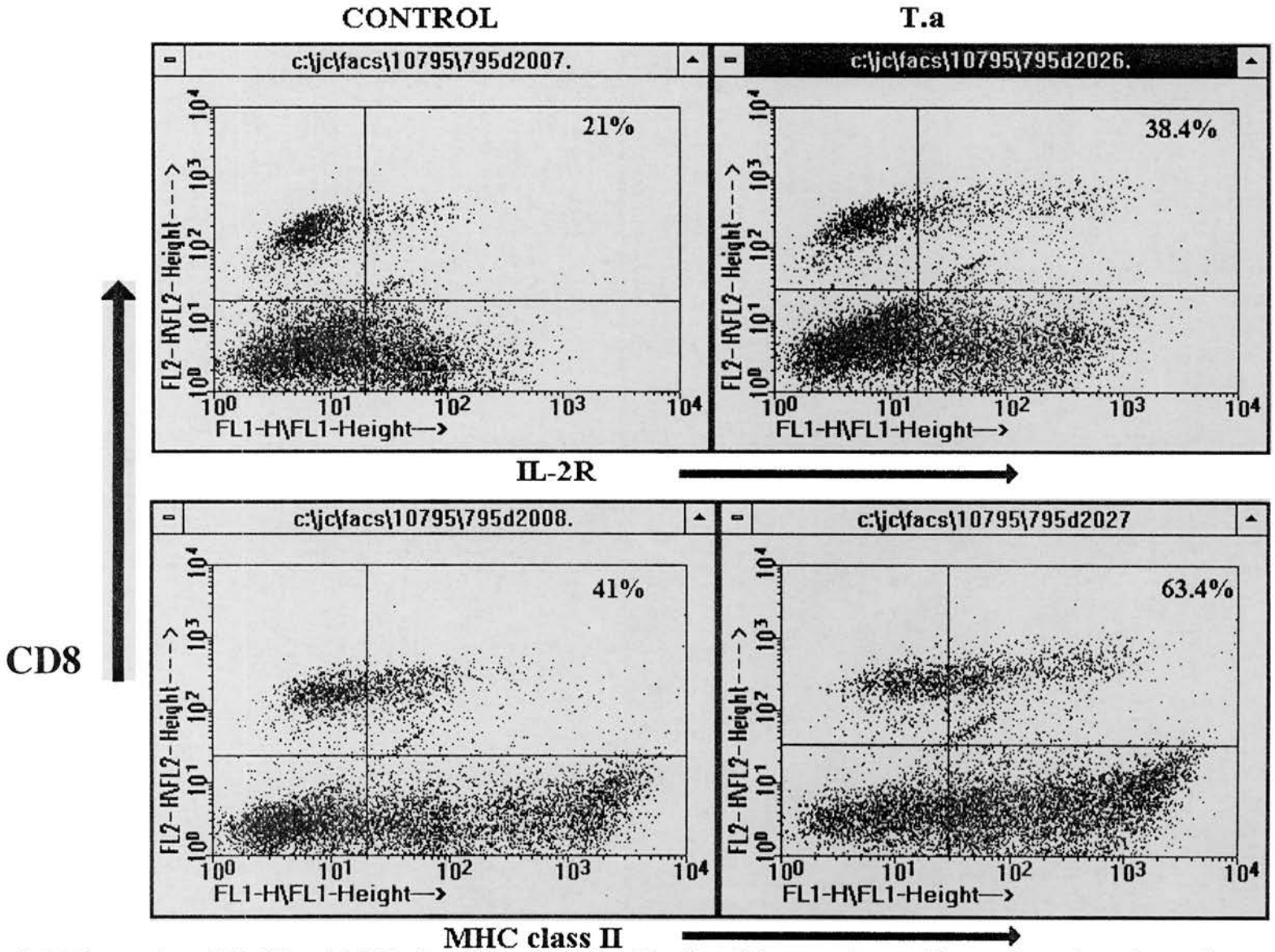


Fig. 3.15 Expression of IL-2R and MHC class II by $CD8^+$ T cells after 48 hours culture with - medium alone (control), or autologous *T.annulata* infected cells (Ta). Percentages are of total $CD8^+$ T cells. Animal 10795

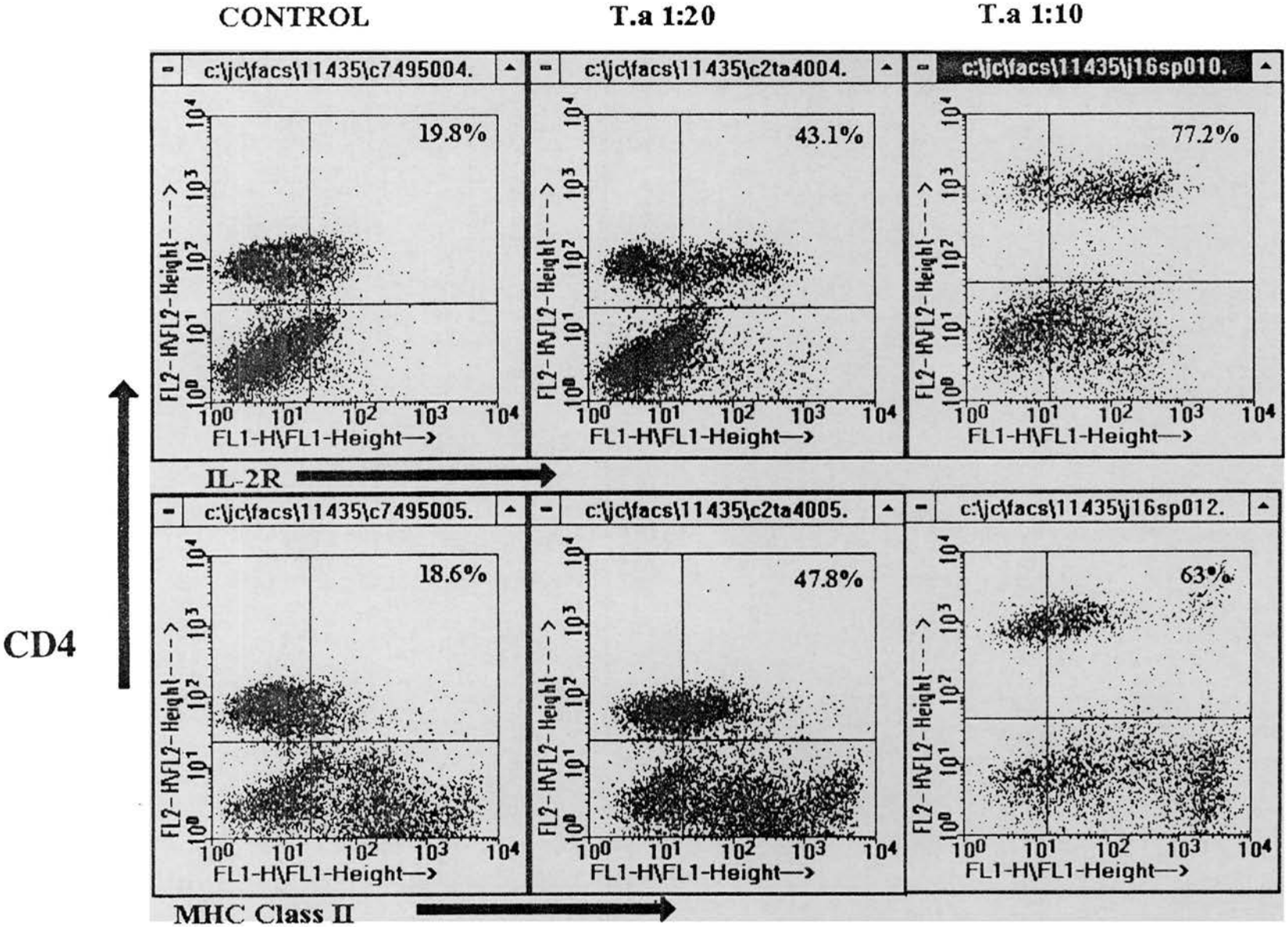


Fig. 3.16 Expression of IL-2R and MHC class II by $CD4^+$ T cells after 48 hours culture with - medium alone (control), or autologous *T.annulata* infected cells (Ta) at ratios 1IC:20 PBM and 1:10. Note large increase in activation at 1:10. Percentages are of total $CD4^+$ T cells. Animal 11435

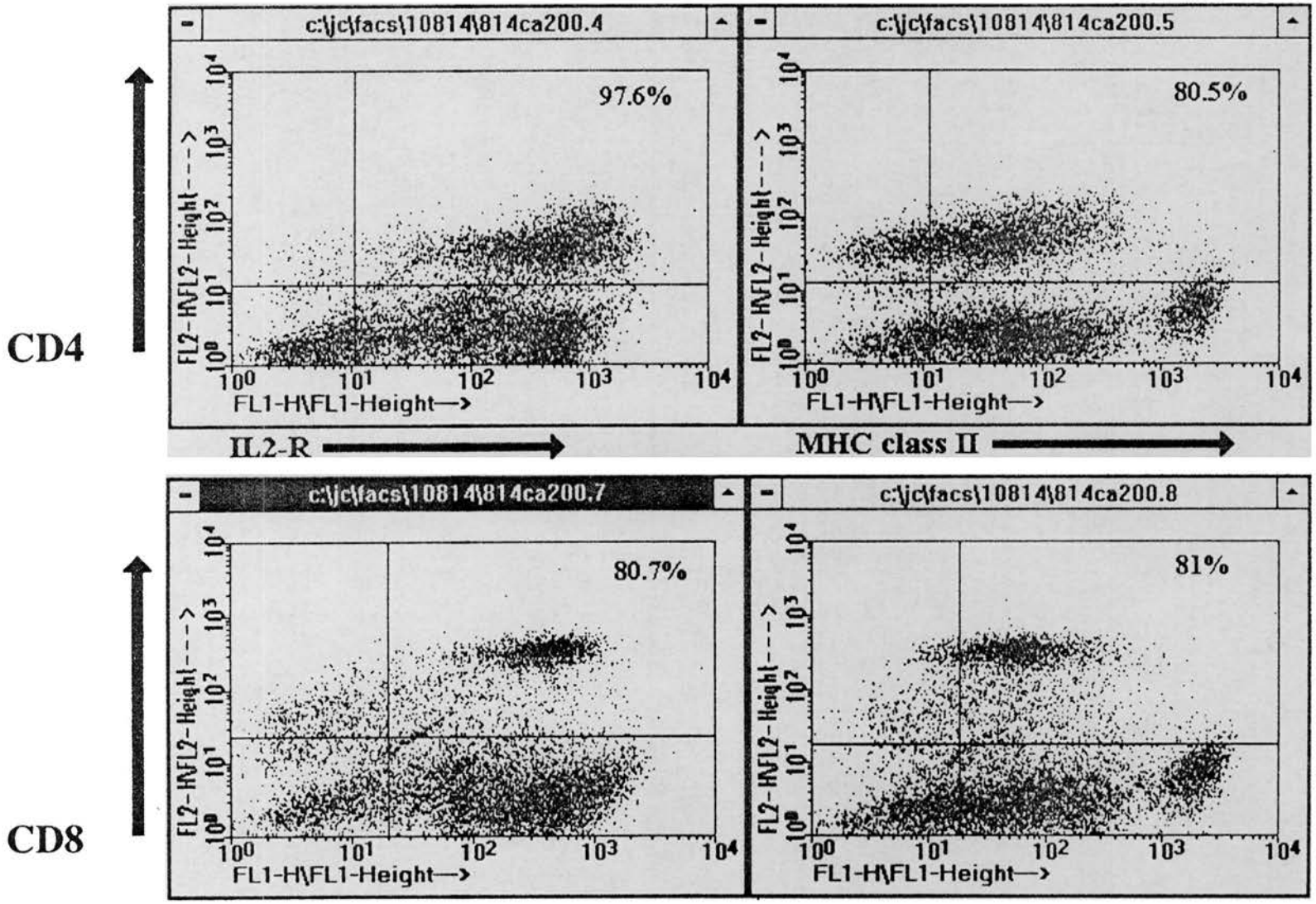
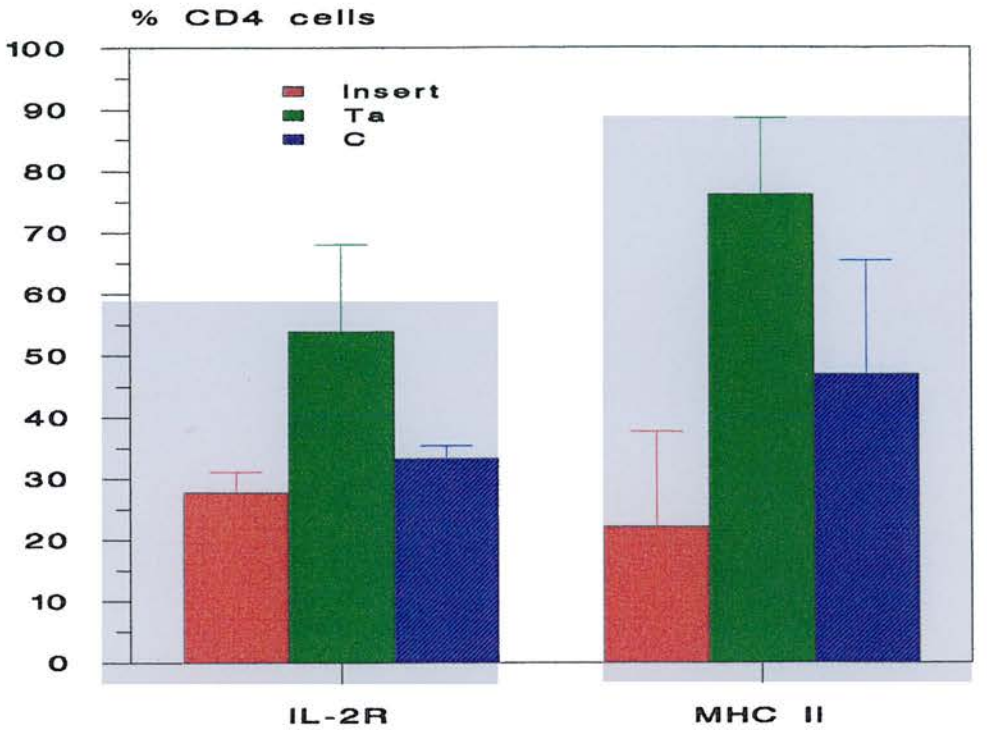


Fig. 3.17 Expression of IL-2R and MHC class II by $CD4^+$ and $CD8^+$ T cells after 48 hours culture with Con A 4μg/ml. Percentages are of total $CD4^+$ or $CD8^+$ T cells. Animal 10184.



Error bars represent 1 standard deviation.

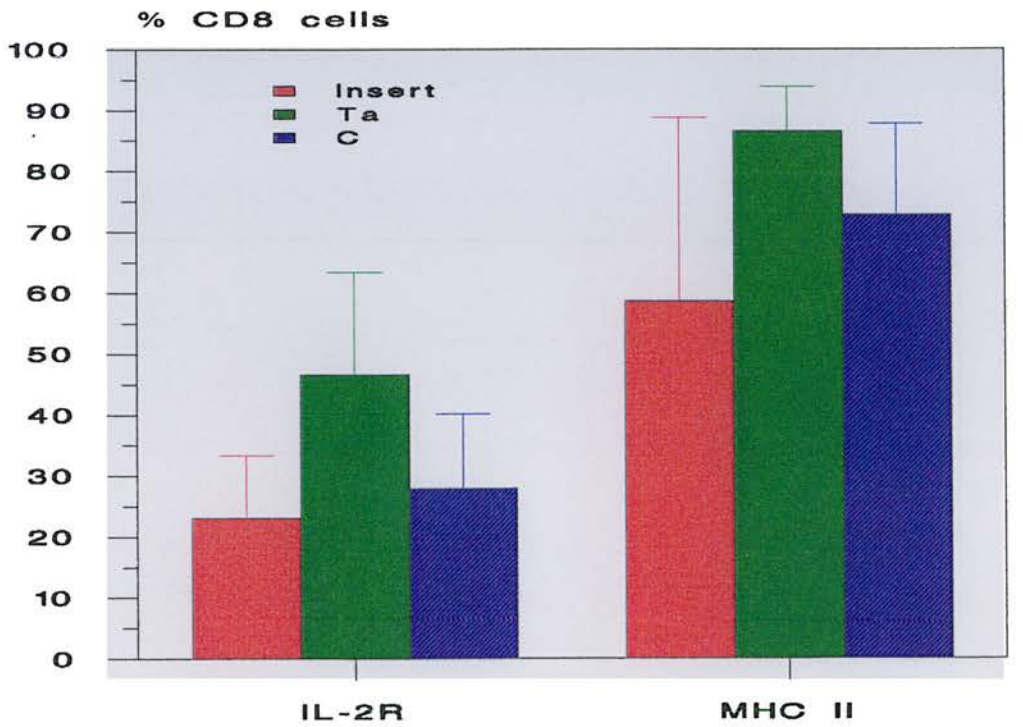


Fig. 3.18 Day 5. Top: Mean (n=4) IL-2R and MHC class II expression by IC stimulated $CD4^+$ cells (Ta) remains significantly higher than with IC separated from the PBM or PBM alone, except in MHC class II expression vs. PBM alone. Although elevated above control levels, $CD8^+$ expression of activation markers is not significantly different (bottom).

Table 3.5

Student T-test analysis.

Changes in IL-2R and MHC class II expression by $CD4^+$ and $CD8^+$ T cells after 5 days incubation.

$p \leq 0.05$ is treated as significant.

A. PBM incubated with IC vs. PBM medium controls

Cell / Marker	Treatment	Mean % Expression	SD	p value	Significant
CD4/ IL-2R	IC Medium	53.98 33.3	14.1 2.08	$p \leq 0.05$	YES
CD4/ MHC II	IC Medium	76.2 47	12.4 18.5	$p \leq 0.1$	NO
CD8/ IL-2R	IC Medium	46.6 27.93	16.87 12.1	$p \leq 0.2$	NO
CD8/ MHC II	IC Medium	86.6 72.83	7.36 14.99	$p \leq 0.3$	NO

IC - Incubated with *T.annulata* IC Medium - Incubated with medium alone

B. PBM incubated with IC vs. PBM/IC cultures separated by a membrane

Cell / Marker	Treatment	Mean % Expression	SD	p value	Significant
CD4/ IL-2R	IC IC + M	53.98 27.8	14.1 3.32	$p \leq 0.02$	YES
CD4/ MHC II	IC IC + M	76.2 22.2	12.4 15.53	$p \leq 0.01$	YES
CD8/ IL-2R	IC IC + M	46.6 23.1	16.87 10.29	$p \leq 0.1$	NO
CD8/ MHC II	IC IC + M	86.6 58.63	7.36 30.1	$p \leq 0.2$	NO

IC - Incubated with *T.annulata* IC

IC + M - Incubated with *T.annulata* IC, separated by a membrane

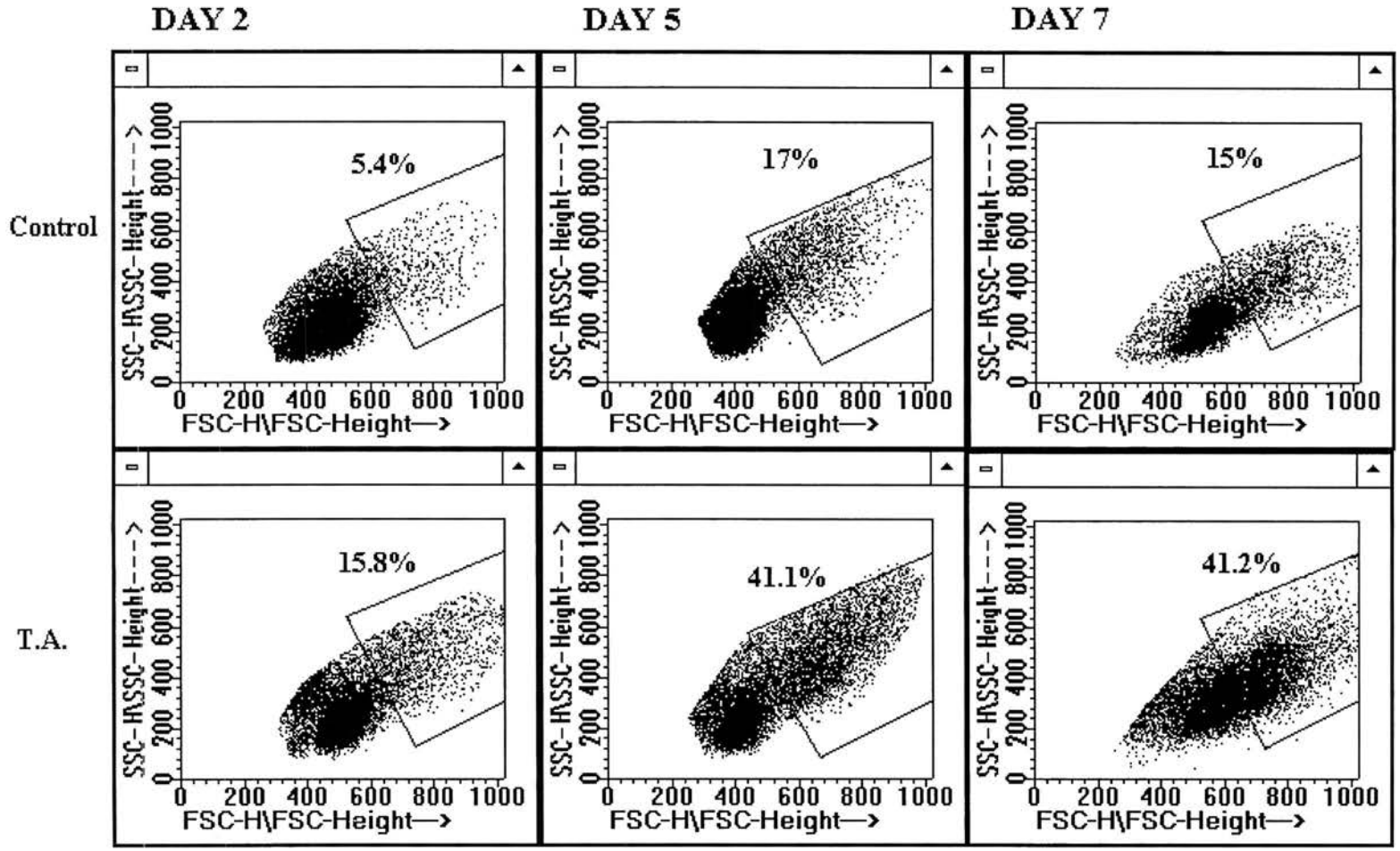


Fig. 3.18A FSC vs. SSC plots showing blasting of PBM induced by incubation with *T.annulata* IC or medium alone. Blasting is maximal in IC cultures after 5 days and remains constant at day 7. Animal 13050.

significantly greater than in IC:PBM cultures with membranes.

Although IL-2R and MHC class II expression on $CD8^+$ cells was greatly elevated in IC stimulated cultures, levels were not significantly higher than in control or membrane separated cultures. In Con A stimulated cultures, expression of IL-2R and MHC class II on both $CD4^+$ and $CD8^+$ remained high ($\geq$ 95%) (Fig 3.19).

Day 7

Unfortunately, sufficient data has been lost from this time point to render analysis of mean activation levels in cultures unreasonable. The principal reason for examining day 7 *T.annulata* activated T cells was to determine whether activated phenotypes were present after proliferation had subsided. Day 5 analysis showed that IL-2R expression upon IC activated $CD4^+$ cells was the only activation marker to be expressed significantly higher than on PBM cultured alone. Data is therefore shown in this section comparing IL-2R expression upon $CD4^+$ cells from control, Con A and IC stimulated cultures at day 7 from 2 animals (Fig. 3.20). IC stimulated $CD4^+$ cells continued to express higher levels of IL-2R than control PBM, although these were still less than Con A activated cells.

CD45RB

In 4 animals examined, $CD4^+$ cells showed a large drop in expression of the CD45RB low phenotype after 5 days incubation with IC (Fig. 3.21), with only a third of all cells expressing this isoform. This essentially reversed the proportions seen in peripheral blood, and was lower than in control PBM and Con A activated cells. However, by day 7, the percentage of $CD4^+$ CD45RB low cells in IC stimulated cultures had returned to the 55-70% levels seen in fresh PBM. This was not observed in positive or negative controls.

$CD8^+$ cells also showed a large change in CD45RB expression in IC stimulated cultures. The percentage of CD45RB high cells fell to 25-35% at day 7 in these cultures, but this was not observed in control or Con A activated PBM (Fig. 3.22). No reproducible pattern within control and Con A cultures were seen, although CD45RB high cells were generally present in greater numbers within controls than

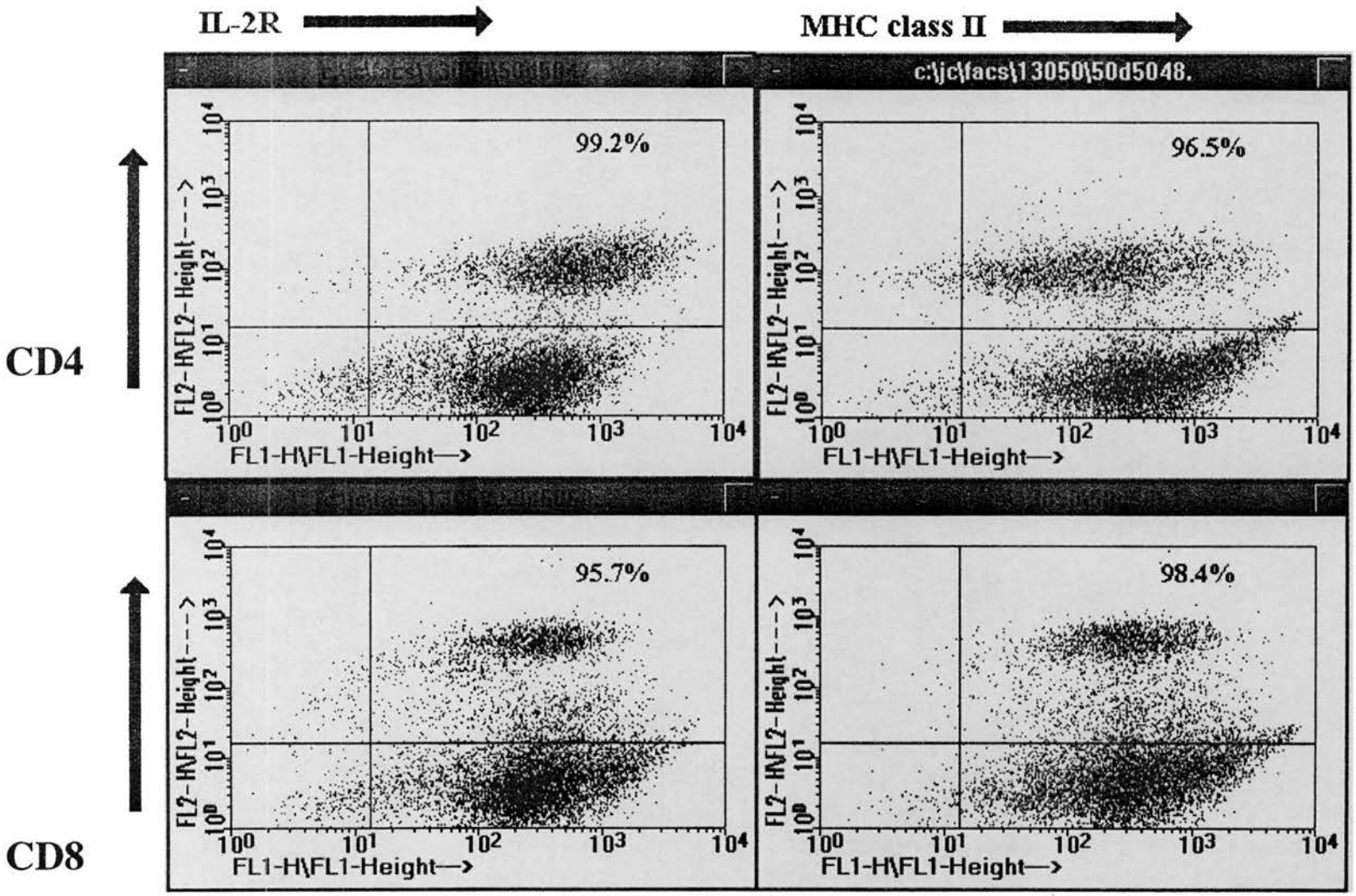


Fig. 3.19 Expression of IL-2R and MHC class II by $CD4^+$ and $CD8^+$ T cells after 5 days culture with Con A 4μg/ml. Percentages are of total $CD4^+$ or $CD8^+$ T cells. Animal 13050.

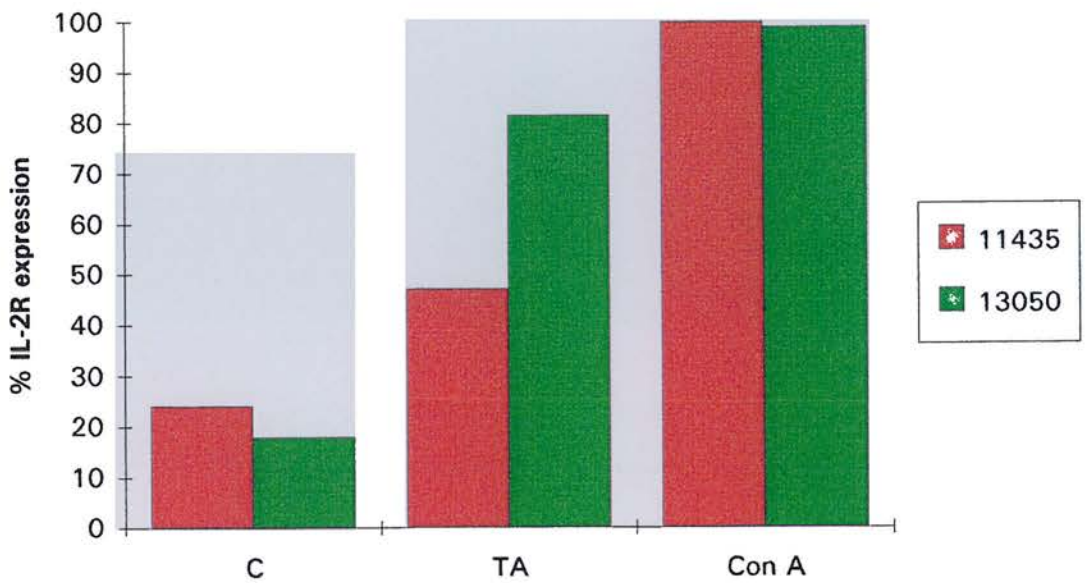
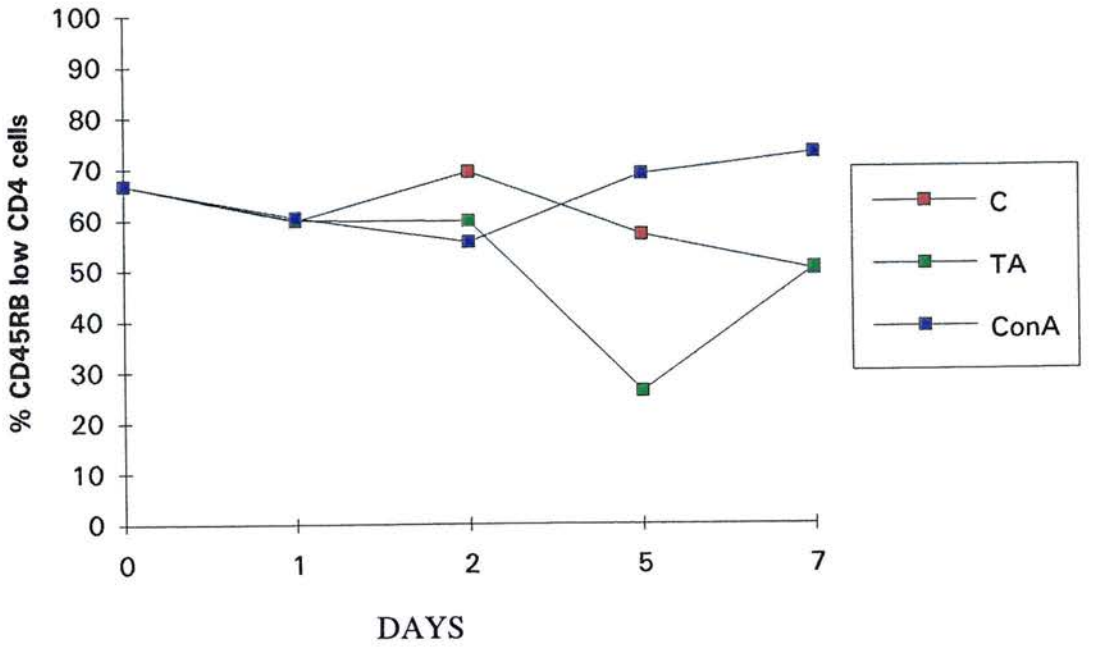


Fig. 3.20 Day 7. IL-2R expression on Con A stimulated $CD4^+$ cells (Con A) remains at virtually 100%. Cells activated with *T.annulata* IC (TA) continue to express higher levels of IL-2R than PBM alone (C).



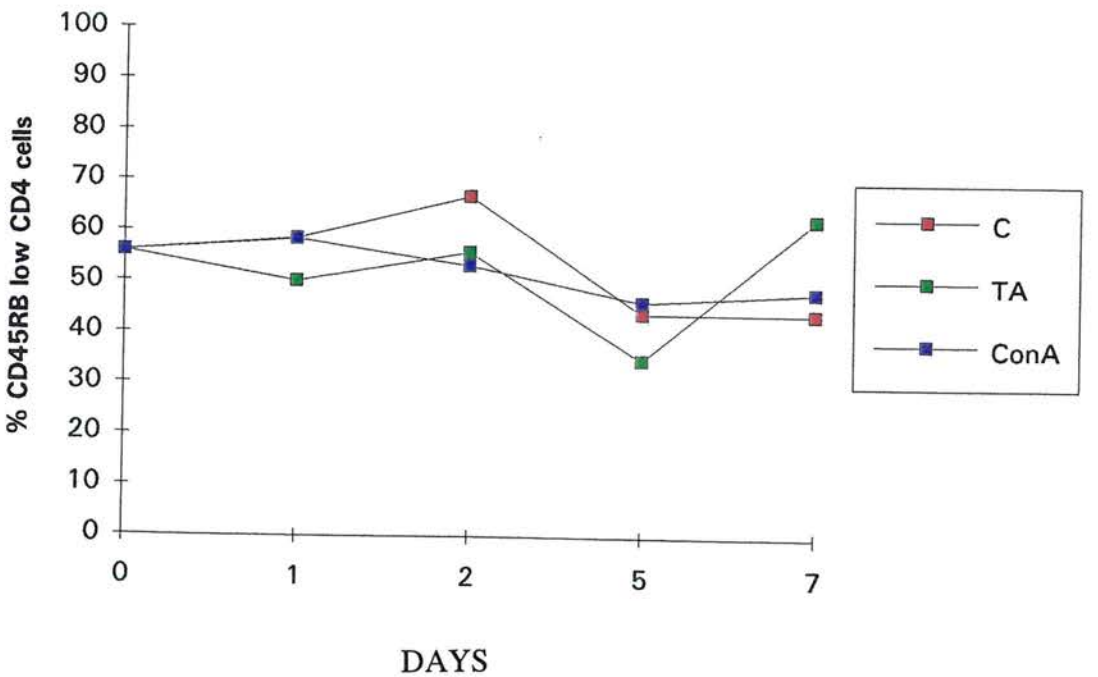
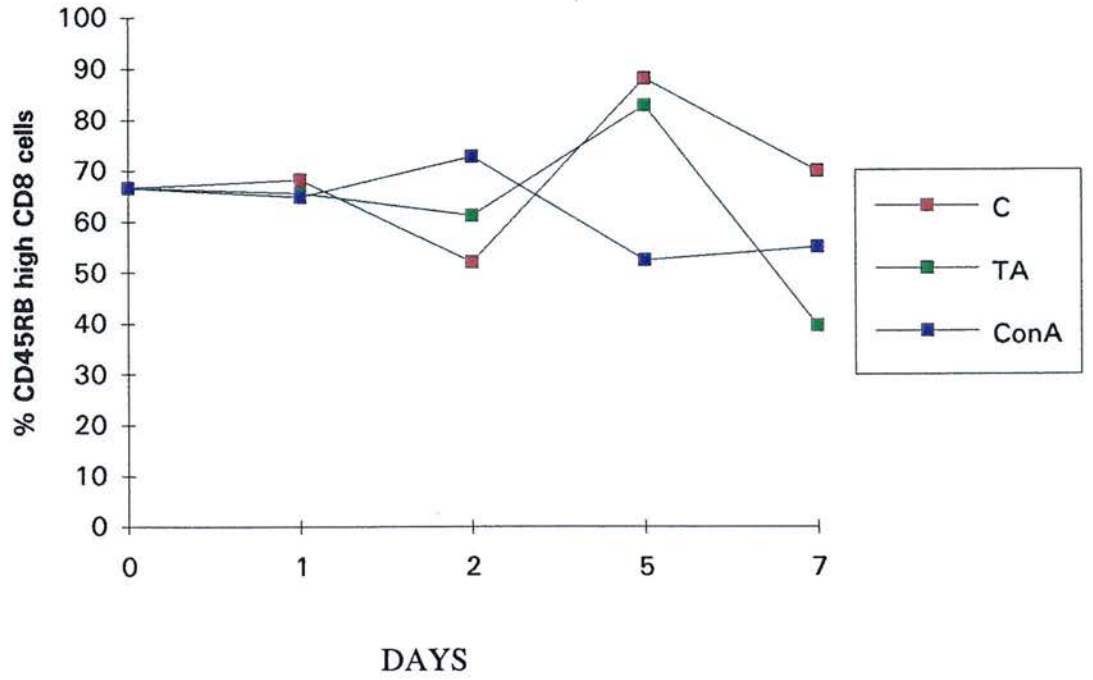


Fig. 3.21 Expression of CD45RB low phenotype upon $CD4^+$ cells drops at day 5, recovering at day 7 when activated with *T.annulata* IC (TA). Animals 13050 (top) and 11435 (bottom).



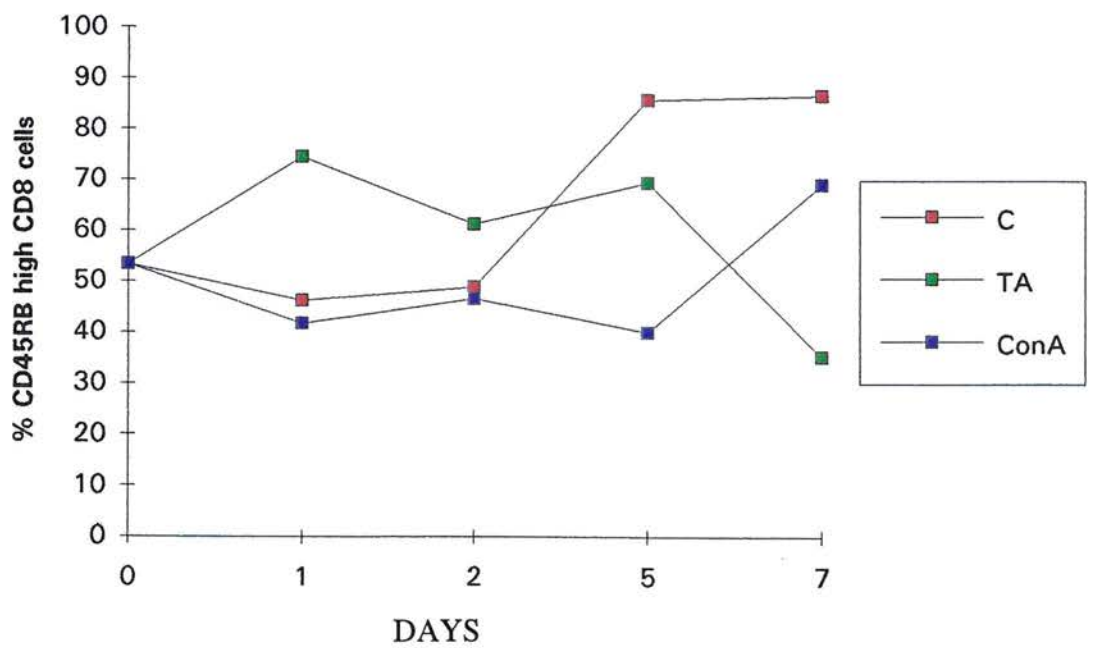


Fig. 3.22 Expression of CD45RB high phenotype upon $CD8^+$ cells drops approx. 40% at day 7 when activated with *T.annulata* IC (TA). Animals 13050 (top) and 11435 (bottom).

in Con A cultures.

CD45RO expression on $CD2^+$ cells

In a preliminary experiment, when the percentages of $CD2^+$ CD45RB low, $CD2^+$ $CD45RO^+$ and the sum of $CD4^+$ and $CD8^+$ CD45RB low cells were compared in PBM cultured alone, the maximum differences between the subsets were found to be within approximately 2.5%, as in normal peripheral blood. However, in both IC and Con A activated cultures, $CD2^+$ $CD45RO^+$ cells were present in larger numbers than $CD2^+$ CD45RB low or (CD4+CD8) CD45RB low cells 5 and 7 days post activation. However the two CD45RB low populations remained essentially identical (Fig. 3.23). IC stimulated cultures were then examined from 3 animals at day 5. The percentage of $CD2^+$ $CD45RO^+$ cells was found to always exceed the numbers of $CD2^+$ or (CD4+8) CD45RB low cells (Fig. 3.24). At the day of peak proliferation, IC stimulation of PBM therefore leads to the expression of CD45RO upon the NK-like $CD2^+$ $CD4^-$ $CD8^-$ cells discussed above.

In summary, incubation of PBM from naive animals with autologous *T.annulata* IC, induces high levels of proliferation in a dose dependant manner. By 48hrs of stimulation, both $CD4^+$ and $CD8^+$ T cells express activation markers at higher levels than control cells, although expression is not as high as Con A activated cells. This elevated level of activation marker expression continues until 7 days post activation (the last time point examined). Separation of IC from PBM blocks activation marker expression. CD45RB expression upon $CD4^+$ and $CD8^+$ cells differs in IC stimulated cultures from PBM incubated alone or with Con A. Incubation with IC also induces the expression of CD45RO upon NK-like $CD3^-CD2^+$ cells.

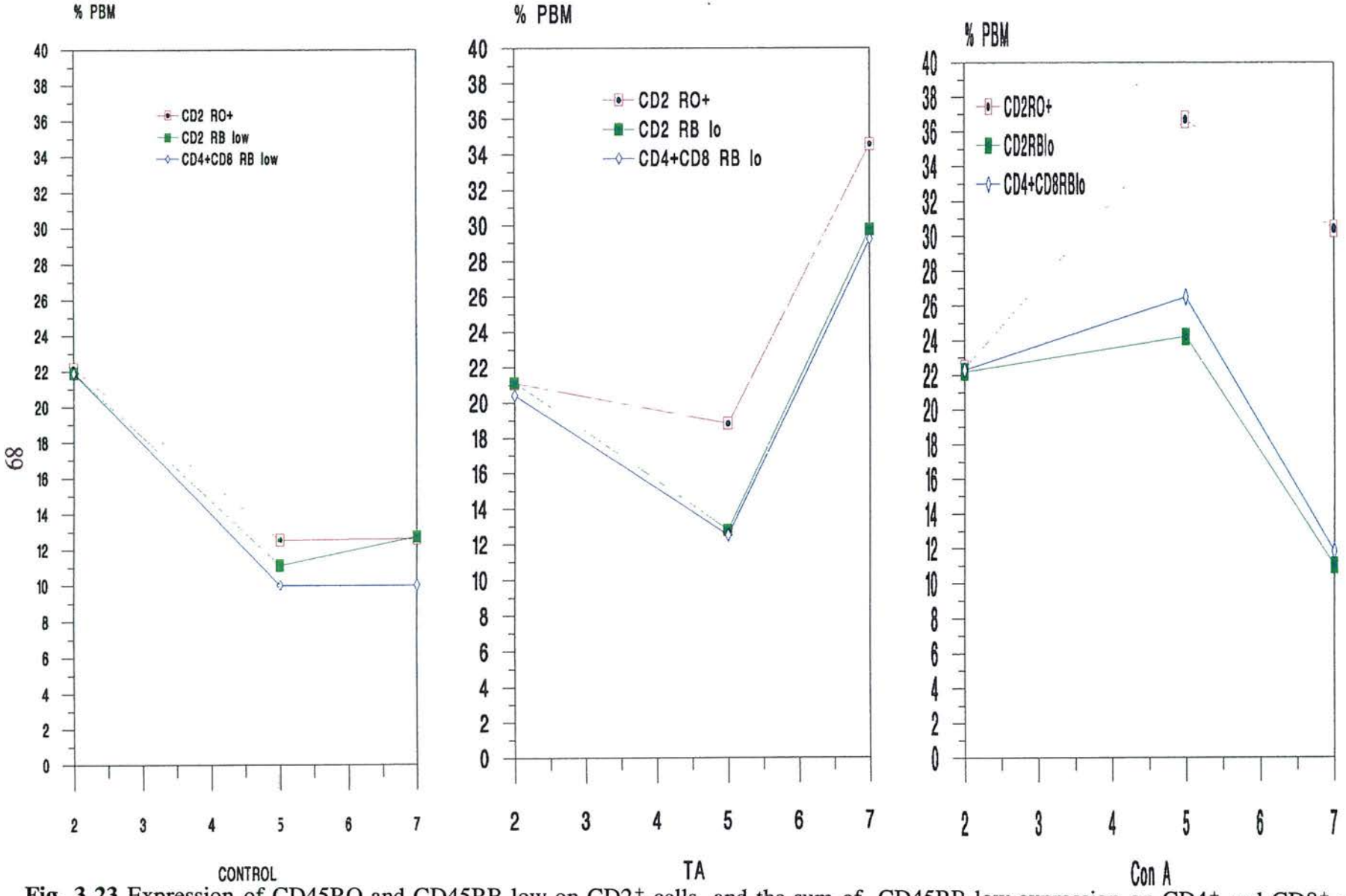


Fig. 3.23 Expression of CD45RO and CD45RB low on $CD2^+$ cells, and the sum of CD45RB low expression on $CD4^+$ and $CD8^+$ cells as a percentage of total PBM. 2, 5, and 7 days post stimulation. Animal 13050.

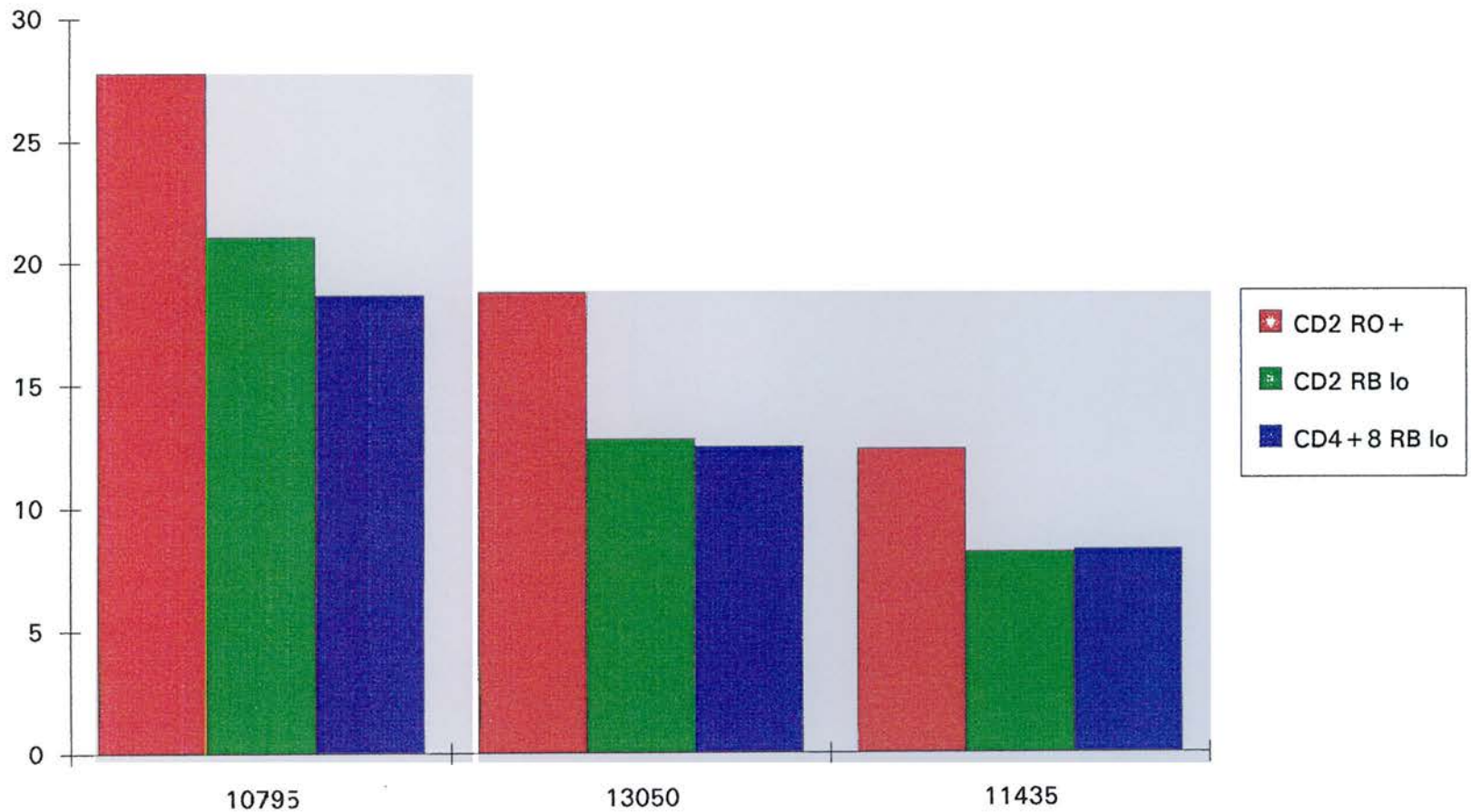


Fig. 3.24 Day 5 post *T.annulata* IC stimulation. Expression of CD45RO on $CD2^+$ cells is elevated above total $CD2^+$ or ($CD4^+ + CD8^+$) CD45RB low cells. Animals 10795, 13050, 11435.

DISCUSSION

The *Theileria* MLR is sufficiently dominant in *T.annulata* to block or mask the generation of parasite specific T cells from the blood of immune animals against antigens on the infected cell surface. In order to assess any potential role for this mechanism in interfering with the generation of primary immune responses, the responses of T cells from naive animals, when co-cultured with autologous *T.annulata* macroschizont infected cells, have been examined in detail.

Kinetics of proliferation

Proliferation within autologous macroschizont infected cell (IC) stimulated PBM was first seen after 2-3 days and peaked at 5-6 days. This was dissimilar to mitogen induced proliferation, which was maximal after 2-3 days culture. The results with mitogen stimulated PBM confirm those of Seiss *et al* (1989) who demonstrated that bovine T cells respond maximally within 72 hours of mitogen treatment. The pattern in *T.annulata* is similar to the kinetics of proliferation in naive PBM induced by *T.parva* (Goddeeris and Morrison, 1987). The element responsible for the induction of proliferation in both parasites thus does not produce proliferation in a similar manner to a mitogen. Proliferation is also not due simply to production of soluble factors by infected cells, as separation of the cells by a membrane which allowed the free passage of Con A in positive controls, totally blocked IC induced proliferation. When bovine T cell responses to recall antigens are assessed *in vitro*, maximum cell proliferation to antigen is usually found at day 3 of culture (Glass and Spooner, 1990b; Howard *et al*, 1991). The first major rise in proliferation to IC is found at this time, and may well involve "memory/effector" cells. However, maximum proliferation was seen at days 5-6, and this would suggest that the antigenic element in *T.annulata* is not stimulating responses to a recall antigen - the cells proliferating are either predominantly naive, or represent memory cells responding to a non recall antigen. This is discussed in further detail in the "CD45" section.

There were distinct differences between animals in the optimal number of stimulatory IC. Virtually complete inhibition of proliferation at high concentrations of IC was

seen in some animals, while in others these concentrations produced maximum proliferation. Inhibition at high stimulator concentrations has been linked to the phenotype of the infected cell in *T.parva* with T cell derived IC inhibiting, while B cell derived IC do not (Goddeeris and Morrison, 1987). Simple surface phenotype examination of *T.annulata* may not be useful in this matter, as surface phenotypes of these cells tend to very similar, irrespective of origin (Spooner *et al*, 1989; Campbell *et al*, 1994). Very recent data has shown that *T.annulata* IC express a wide range of cytokine mRNAs, which could send contradictory signals to T cells eg (IL-10 and IL-12), potentially suppressing responses (Brown *et al*, 1995; D.J. Brown unpub obs.). Also, T cell proliferation correlates with the amount of IL-1α produced by the IC (although cell-cell contact is a primary prerequisite) (Brown *et al*, 1995). Inhibition of proliferation by some cell lines, and induction of high proliferation by others at the same concentration is therefore likely to be related to the levels of potentially stimulatory or suppressive cytokines produced within each cell line.

$\gamma\delta$ T cells

No evidence could be found that $\gamma\delta$ T cells were becoming activated in PBM stimulated with IC, as these cells assumed an IL-2R$^+$, MHC class II$^+$, CD45RB low phenotype, simply upon culture in medium alone. However, these observations do have some bearing upon attempts to determine the restriction element of bovine $\gamma\delta$ T cells. Many more of these T cells express activation markers in the peripheral blood than $\alpha\beta$ T cells, and they acquire a fully activated phenotype within 24 hrs of culture. These cells therefore require only the level of stimuli provided by cytokines and/or accessory molecules in resting PBM to become activated. This must at least question whether $\gamma\delta$ T cells require the strictly regulated antigen presentation pathways of $\alpha\beta$ T cells.

In some ways, the activation behaviour of $\gamma\delta$ T cells is far more reminiscent of NK cells - cells with no known restriction element (Evans and Waldmann, 1993). NK function has been tentatively ascribed to CD2$^-$ CD3$^+$ WC1$^+$ cells (Evans and Waldmann, 1993), as well as "traditional" CD2$^+$ CD3$^-$ NK cells. Bovine NK activity

can be induced by IL-2 and interferons alone (Jensen and Schultz, 1990). If $WC1^+$ cells make up a part of this population this may again point to cytokines as the principal stimulators for this cell type.

Recently, there have been reports that *T.annulata* IC may posses a restriction element for $\gamma\delta$ T cells (Collins *et al*, 1995), as such cells proliferate when cultured with IC. The results seen in this study, at least in the acquisition of an activated cell surface phenotype, would argue that such activation may be achieved by uninfected leucocytes. It is possible that IC possess enough accessory molecules and express sufficient T cell stimulatory cytokines (eg IL-1α, Brown *et al*, 1995) to induce activation of $\gamma\delta$T cells without expressing a specific restriction element.

Activation marker expression by $CD4^+$ and $CD8^+$ T cells

$CD4^+$ cells

In these studies, expression of the activation markers IL-2R and MHC class II (Seiss *et al*, 1989; Glass and Spooner 1990b) on the surface of $CD4^+$ T cells was found to be consistently higher in IC activated cultures than medium controls. This elevated expression of IL-2R was present within 24hrs of culture, and persisted up to 7 days after initial activation. MAb IL-A111, used to detect IL-2R, recognises the p55 sub unit (Tac, CD25) of the bovine IL-2 receptor (Naessens *et al*, 1992). This low affinity receptor has been shown to peak in its expression 48hrs after activation of bovine T cells, remaining at this elevated level for several days thereafter (Seiss *et al*, 1989). This corresponds well with the results for $CD4^+$ cells in Con A and IC cultures described here, which showed little change in the percentage of $IL\text{-}2R^+$ cells between 48hrs and 7 days culture.

The appearance of the p55 subunit of the IL-2R on $CD4^+$ cells, although a reliable marker of activation, may not indicate a large role for IL-2 in proliferation, as p55 is a low affinity receptor. However, at five days, proliferation was maximal shortly after the time when the bovine high affinity IL-2R (a dimer of p55 and the p70 subunit) reaches its maximum surface expression level - 96 hours after T cell activation (Seiss *et al*, 1989). This would suggest that the majority of the proliferating cells at day 5 require IL-2 to proliferate. The levels of MHC class II

expression on $CD4^+$ cells rose at day 5, and the elevation of expression of MHC class II products on T cells is quoted as being IL-2 dependant by Tomita *et al* (1991). This also further implicates IL-2 in proliferation. The expression of IL-2R on $CD4^+$ cells may also explain the vigorous proliferation to IL-2 seen in "non specifically" activated T cells from immune animals (appendix 2). Bovine IL-2R have a high affinity for human IL-2 (Seiss *et al*, 1989) and in these current studies incubation with IC appears to induce an IL-2 responsive state.

Separation of PBM and IC by a membrane blocked expression of activation markers on $CD4^+$ cells. Contact between IC and responding T cell is therefore required to induce both proliferation and an activated cell phenotype. It interesting to note that at the day of peak proliferation, PBM/IC cultures with membranes showed lower activation marker expression than PBM in medium alone. This is further evidence that some of the cytokines produced by *T.annulata* IC may have suppressive effects on T cells, and once the dominant cell contact dependant mechanism is removed, the influence of these factors is amplified. In animals where high IC concentrations did not inhibit, but rather increased proliferation of PBM, $CD4^+$ cells were found to express elevated levels of IL-2R ($\geq$75%) and MHC class II ($\geq$60%) when incubated with IC:PBM at 1:10 rather than at 1:20. When IC cell lines are used that do not inhibit proliferation at high concentrations, it would therefore appear that IC are capable of inducing the activation of the vast majority of $CD4^+$ cells in the periphery.

$CD8^+$ cells

The effects of culture with IC upon activation marker expression by $CD8^+$ cells were somewhat less clear cut. Although clearly activated above control levels at day 2, even at this timepoint, separation from IC by a membrane did not induce a significant drop in IL-2R expression on $CD8^+$ cells. At day 5, IL-2R expression was higher on the surface of IC stimulated cells than controls, although not significantly. MHC class II expression was massively elevated in control, IC, and membrane cultures. IL-2 is thought to increase MHC class II expression on T cells (Tomita *et al*, 1991). It is possible that $CD8^+$ cells require less IL-2 or other soluble factors to

exhibit an activated cell surface phenotype, and the levels within medium controls suffice to induce MHC class II expression. Whatever the cause, the high background control levels of activation marker expression made attributing any *Theileria* specific activation events in $CD8^+$ cells impossible.

Changes in CD45 expression

Macroschizont IC stimulation resulted in distinctive changes in CD45RB phenotypes within both $CD4^+$ and $CD8^+$ T cells.

$CD4^+$ cells

The majority of $CD4^+$ cells expressed the CD45RB low phenotype (memory/effector T cells, Howard *et al*, 1991) in freshly isolated PBM. This had changed by day 5 of IC culture, when $CD4^+$ cells were present primarily as CD45RB high (naive). These naive $CD4^+$ cells subsequently acquired the CD45RB low phenotype by day 7. This pattern was not seen in medium or Con A controls. These observations suggest that incubation with IC is either inducing CD45RB low T cells to lose their effector phenotype, subsequently regaining it, or that a change has occurred within the $CD4^+$ population with naive cells outgrowing memory at peak proliferation. Several factors would suggest that the latter is the more likely.

It is possible for T cells to acquire expression of low molecular weight forms of CD45 (CD45RO/CD45RB low) and then revert to being $CD45RA^+$/CD45RB high, although this has only been established during thymocyte selection (Howard *et al*, 1991; Fuji *et al*, 1992; Wallace *et al*, 1992). The situation in the periphery is somewhat different. Memory/effector $CD4^+$ cells with a CD45RB low phenotype have been shown to be restricted to specific recall antigens (Howard *et al*, 1991; Birkeland *et al*, 1992). The expression of low molecular weight isoforms of CD45 on such cells has been shown to augment signal transduction through the TCR, aiding the decreased antigen response times exhibited in memory/effector cells (Novak *et al*, 1994). For *T.annulata* to effectively de-differentiate memory cells to naive, it would have to abolish or bypass the cell's antigen specificity. Bypassing can be achieved to some extent - addition of IL-2 to CD45RB low cells can restore alloreactivity in cells which were previously not allo responsive (Birkeland *et*

al, 1992), although this was not apparently accompanied by a change in CD45RB expression. As the activated CD4 cells were expressing IL-2R, and IL-2 production is required for expression of its receptor (Mauer *et al*, 1984; Smith, 1988), it may be assumed that IL-2 is being produced within the IC stimulated T cells. The possibility that an IL-2 mediated mechanism of loss of antigen restriction is in operation cannot be totally discounted, although this would not explain a change in CD45 isoform expression.

As discussed above, the first major rise in proliferation to IC is found at 3 days, and may well involve "memory/effector" cells. Such cells have been shown to possess enhanced signal transduction capabilities, probably mediated through low molecular weight isoforms of CD45 (Novak *et al,* 1994). However, the main peak of IC induced proliferation is 5-6 days post activation. This is likely to involve naive cells which have a slower activation response time. For example, biphasic peaks of IL-2 production in mitogen stimulated bovine T cells have been attributed to activation of memory/effector cells followed some time later by naive cells (Weinberg *et al*, 1988). The predominance of CD45RB high $CD4^+$ cells at peak proliferation may therefore reflect the selective proliferation of these naive cells at day 5-6, and the subsequent rise in CD45RB low cells their acquisition of "specificity" for the stimulatory *Theileria* antigen. A similar phenomenon, with very similar peak proliferation times, is seen in *T.cruzi* "non specific" activation of T cells (Piuvezam *et al*, 1993).

The proliferation of IC stimulated PBM peaking at day 5 would suggest that response is not to a recall antigen. The predominance of CD45RB high cells would perhaps confirm that the majority of the cells proliferating at day 5 are naive. Further studies of CD45RB low and high cells in isolation would be required to confirm this.

$CD8^+$ cells

Although the acquisition of an "activated" cell surface phenotype on $CD8^+$ cells was not significantly different between IC and control cultures, large numbers of $CD8^+$ cells did acquire a CD45RB low phenotype after 7 days culture with IC, which was not seen in medium or Con A controls. The acquisition of the CD45RB low

phenotype by $CD8^+$ cells at the same time as the $CD4^+$ cells would suggest that some maturation event is taking place within these cells too. This cannot be linked to the acquisition of an effector CTL phenotype, and antigen specificity, as such cells are distributed evenly within the CD45RB low and high populations (Howard *et al*, 1991). This has also been recorded in mice, with an upregulation in CD44 expression a more reliable indicator for antigen primed $CD8^+$ cells (Pihlgren *et al*, 1995) (anti bovine CD44 mAb were not available during this study).

The percentage of CD45RO expression by $CD2^+$ cells in IC stimulated cultures was found to be at least the same, if not higher, than the percentage of CD45RB low expressing cells at all times (the higher CD45RO expression being due to NK cells, see below). It is therefore reasonable to assume that the majority of CD45RB low $CD8^+$ cells are also $CD45RO^+$. Again, this is not a reliable marker for a CTL effector phenotype (Pihlgren *et al*, 1995), but is associated with a change in the capacity of $CD8^+$ cells to produce cytokines (Conlon *et al*, 1995). Human $CD45RO^+$ $CD8^+$ cells have been shown to produce similar amounts of TNFα to $CD45RO^+$ $CD4^+$ T cells, and levels of IFNγ and GM-CSF significantly higher than $CD45RA^+$ $CD8^+$ cells and even $CD45RO^+$ $CD4^+$ cells (Conlon *et al*, 1995). Stimulation of naive PBM therefore induces a phenotype in the majority of $CD8^+$ cells associated with increased cytokine production, particularly of the Th1 subset.

$CD2^+$ $CD3^-$ cells

As discussed in the results section, the $CD2^+$ $CD3^-$ $CD4^-$ $CD8^-$ $WC1^-$ cells in bovine peripheral blood are likely to correspond to NK cells, although some NK activity is also found in $\gamma\delta$ T cells (Cook *et al*, 1989;Evans and Jaso-Friedmann, 1993). These cells were induced to express CD45RO at days 5 and 7 following IC stimulation of PBM. It is interesting to note that the CD45RB isoform was not detected on $CD2^+$, $CD4^-$ $CD8^-$ cells. As expression of CD45RO is a marker of IL-2 induced human NK cell activation (Shen *et al*, 1995), it is likely that $CD2^+$ NK cells are also becoming activated in IC stimulated PBM. In cattle, NK cells acquire killer phenotypes only when incubated with IL-2 or IFNα or γ (Jensen and Schultz, 1990). As *T.annulata* IC do not normally produce IL-2 or IFNγ (Brown *et al*, 1995), the most likely

source of cytokines to induce NK activation would be the T cells activated by IC. This is further evidence that Th1 cytokines such as IL-2 and IFNγ may be produced following *T.annulata* activation of T cells.

Comparison with other "non specific" T cell stimulatory pathogens

T cell proliferation of PBM from unexposed donors has been demonstrated in response to various pathogens such as *Plasmodium falciparum* and *P.vivax* (Fern and Good, 1992); *Trypanosoma cruzi* (Piuvezam *et al*, 1993); *Borrelia burgdorferi* (Roessner *et al*, 1994); and in rheumatoid arthritis (Iglesias *et al*, 1992). Various types of antigens have been implicated in inducing such proliferation. In arthritis, heat shock proteins which mimic mycobacterial antigens, crossreacting with many memory T cell specificities are thought to induce proliferation (Iglesias *et al*, 1992). This is not likely to be the case in *T.annulata,* as the proliferation kinetics and predominance of CD45RB high cells at peak proliferation would suggest that cells previously unexposed to antigen are reacting. In *T.cruzi*, there is some confusion about the T cell types responding, as proliferation is similar to *T.annulata*, but principally $CD45RO^+$ cells were isolated after proliferation. In *T.annulata,* the CD45RB high cells became CD45RB low after antigen exposure, and this may explain the finding in *T.cruzi*, again suggesting that cross reaction with memory cells is not causing proliferation.

As it seems unlikely that *T.annulata* is solely mimicking a recall antigen, the element inducing proliferation must be able to activate T cells through a mechanism that does not rely on TCR antigen specificity. This phenomenon has been extensively characterised for the members of the "super antigen" family of viral and bacterial peptides which mediate activation through the Vβ sub unit of the TCR (Janeway *et al*, 1989; Marrak and Kappler, 1990). As many TCR types may carry the same Vβ type, T cells with a wide range of TCR specificities can be stimulated. The expression of such an antigen by *T.annulata* could potentially explain the activation of naive T cells, and the apparent bypassing of any parasite specificity seen when T cells from immune animals are activated (appendix 2). As they are expressed in conjunction with MHC class II, superantigens only activate $CD4^+$ T cells (Torres *et*

al, 1993;Janeway *et al*, 1989), and again this would correlate well with the principal cell type activated by *T.annulata* being $CD4^+$.

However, the number of cells activated by *T.annulata* may cast doubt on the infected cells expressing a single "classical" superantigen. At least in some animals, over 75% of all $CD4^+$ cells could be induced to express activation markers using high numbers of stimulators. The numbers of Vβ types in cattle is unknown, but there are 20 in humans and superantigens rarely show specificity for more than 2 Vβ types (summarised by Janeway, 1991). Even common Vβ types have been shown to be expressed upon only up to 10% of peripheral T cells (Marrak *et al*, 1991; Woodland *et al*, 1991). For a superantigen to activate 75% of all $CD4^+$ T cells, it would therefore require to react with a large number, probably more than half, of all expressed Vβ gene products, and this not been described for a single classical superantigen.

Of the six animals tested here, 4 had known MHC class II types, and all were different from each other (E.J. Glass and R.A. Oliver, pers. comm.), yet all induced very similar activation of T cells at IC:PBM ratios of 1:20. Early studies also did not find great differences in responses between unrelated animals (Glass and Spooner, 1990a). This indicates that the *T.annulata* stimulation element is expressed across a range of MHC class II types. This has been implicated as important in *P.vivax, P.falciparum,* and *B. burgdorferi* activation of cells from randomly selected unrelated donors (Fern and Good, 1992; Roessner *et al*, 1994). Although MHC "promiscuity" is not described for *T.cruzi*, this parasite produces a number of antigens which, in combination, interact with the majority of Vβ antigens expressed by human peripheral T cells (Piuvezam *et al*, 1993). From the data in this chapter, if an MHC class II associated antigen is mediating activation, the best estimation of the *T.annulata* stimulation element would be a combination of both the properties described in this paragraph - a group of peptides having an ability to bind to different MHC class II types, with the ability to stimulate many different TCR Vβ segments.

Alternatively, although there is not the body of data available describing a similar phenomenon in other species, a non MHC associated antigen may be in operation.

Although IC always express higher levels of MHC class II on their surface than uninfected macrophages, recent work by Brown *et al* (1995) has shown that the level of PBM proliferation induced by IC does not correlate with numbers of MHC class II molecules on the cell suface. Cells with similar levels of MHC class II can induce widely differing amounts of proliferation, with may suggest that MHC class II is not a major factor in inducing proliferation. The possible action of a non MHC restricted antigen is discussed further in chapter 4 where the roles of differerent T cells in the response to IC is dissected in more detail.

In summary, the data discussed in this chapter defines several facets of *T.annulata* "autologous MLR" activation of T cells. Proliferation responses may involve memory T cells in the first instance, but the principal T cell present at day 5 (peak proliferation) is the CD45RB high naive $CD4^+$ T cell. These cells acquire an effector CD45RB low phenotype after proliferation, generally accepted to indicate the acquisition of antigen specificity. $CD4^+$ cells consistently exhibit elevated levels of activation marker expression, and although these are also elevated on $CD8^+$ cells, this is not significantly different from control levels. The majority of activation that can be detected using the techniques employed in this chapter therefore occurs in $CD4^+$ cells. $CD8^+$ cells acquire an effector CD45RB low phenotype by day 7, as do NK cells, and this has been previously shown to be dependant upon the presence of IL-2 and/or IFNγ leads to the expression of these cytokines by the activated cells. Thus, although direct activation may take place in $CD4^+$ cells, there is evidence that $CD8^+$ and NK cells may aquire effector phenotypes through soluble factors.

T.annulata IC activation of T cells from immune animals does not select parasite specific T cells (appendix 2), and the activation of T from naive animals cells described here, which primarily is focused on naive $CD4^+$ T cells, seems certain to have a similar outcome. If this mechanism were to operate *in vivo*, it could seriously disrupt the generation of immunity. The other chapters of this thesis are concerned with evaluating this hypothesis. The potential that cytokines made by "non specifically" activated T cells have for influencing immune responses is presented

in chapter 4. In chapter 5, *in vivo* immune response development has been examined in depth, paying particular attention to any "MLR" effects and their outcomes.

CHAPTER 4

T cell cytokine production following *Theileria annulata* activation of PBM from naive cattle

INTRODUCTION

In chapter 3 it was shown that *T.annulata* macroschizont infected cells (IC) possessed an ability to activate T cells, particularly $CD4^+$ cells, in PBM from naive animals. After proliferation in response to the macroschizont infected cells, naive $CD4^+$ T cells developed an effector/memory phenotype (CD45RB low), suggesting that they had acquired specificity for the macroschizont antigen causing proliferation. However, as stimulation with this Ag is capable of blocking the recognition of macroschizont antigens by parasite specific T cells (appendix 2), it seems likely that T cells activated in this manner in naive animals are not capable of mediating anti parasite immunity. Although unlikely to play any part in anti-parasite immune responses, the *Theileria* activated T cells have acquired an activated phenotype. One of the principal outcomes of T cell activation, originally attributed to $CD4^+$ cells, but now recognised to be derived from all T cells and a variety of non lymphoid cells, is the production of soluble cytokines.

Originally designated T cell "help", as opposed to cytolytic activity, a large number of cytokines have now been demonstrated to be produced by T cells. Among the bovine cytokine genes currently identified which are relevant to T cell help or development are those for IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IFNγ (Source - EMBL database). In recent years, such cytokines have been divided into two distinct groups designated T helper (Th) 1 and 2 (Mosmann *et al*, 1986). The Th 1 subset contains cytokines which help cell mediated immune responses eg. IL-2, IFNγ and IL-12 whereas Th2 cytokines promote humoral responses eg. IL-4 and IL-10 (Mosmann *et al*, 1986; Cher and Mosmann, 1987; Scott *et al*, 1987; Paul and Ohara, 1987; Hsieh *et al*, 1992; 1993). Th1 and Th2 are used as adjectives for both the cytokine profiles and the cells which produce them. In a recent review (Clerici and Shearer, 1994), it has been suggested that the nomenclature be changed to "type 1 and type 2", as cytokines are not only produced by $CD4^+$ "helper" T cells. For example, IFNγ is produced by activated $CD8^+$ cells and NK cells (Conlon *et al,* 1995; Shen *et al*, 1995), and IL-12 is produced by macrophages (Hsieh *et al,* 1993). Protective immunity, especially to protozoal infections has been extensively

investigated in terms of the Th response required for protection. In some cases the mechanisms are extremely clear cut eg. control of infection in both mouse model and human *Leishmania* infections can be attributed to Th1 cytokines with IFNγ mediated killing of parasite via macrophages (Scott *et al*, 1987; Gaafar *et al*, 1995). This response is so Th1 dependent that inoculation of Th2 producing cells into infected mice actively exacerbates infection. Immunity to malaria is rather more complex, with potentially different mechanisms operating at different disease stages. In *Plasmodium berghei* infections, Th1 responses may be important in disease control, as the neutralisation of IFNγ leads to disease resurgence, while both antibody and cytotoxic T lymphocytes protected against sporozoite challenge (Schofield *et al*, 1987). This differs from other species, as *P.vineki petteri* and *P.chabaudi chabaudi* infections are characterised by cell mediated immunity and IFNγ production in the early stages, followed later by control of infection through Th2 induced antibody production (Langhorne *et al*, 1989; Cavacini *et al*, 1992).

Although the classification into Th1 and Th2 type cytokines has been of great use in defining the cytokine profiles associated with protective immune responses, the division, originally described in mice (Mosmann *et al*, 1986) has now been shown to be less clear cut. Cells which produce both IL-4 and IFNγ as well as IL-2 have been described in mice, and dubbed the Th0 subset (Street *et al*, 1990). This subset is though to arise as a precursor to Th1 and Th2, and may represent an earlier stage in development. This hypothesis is backed up by a better understanding of cytokine secretion by T cells in various stages of development - completely virgin T cells predominantly produce IL-2, acquiring Th1 or Th2 like activity further into development (Swain *et al,* 1990). Thus the development of polarised Th profiles in mice may be a gradual process, progressing from IL-2 production through the Th0 profile to Th1 or Th2 (Street *et al*, 1990; Swain *et al*, 1990).

In cattle, this theory of T cell development only holds true up to a point. $CD45RA^+$ T cells have been shown to produce only IL-2, while $CD45RO^+$ cells can produce a full cytokine repertoire (Collins, 1993), agreeing with the mouse model of progressive acquisition of cytokine producing capacity. However, there is good evidence that the Th0 phenotype is not just an intermediate step in bovine T cell

cytokine development. Fully CD45RO$^+$ antigen specific bovine T cell clones specific for *Babesia bovis* generally secrete IL-4 and IFNγ as well as IL-2 ie. Th0 (Brown *et al*, 1993). Although it is now recognised that IL-2 is not strictly a Th1 cytokine, and is involved in the early development of all Th subsets (Rocken *et al*, 1992), mouse Th2 lines that do not produce or depend on this cytokine for growth have been established (Mosmann *et al*, 1986; Cher and Mosmann, 1987; Fernandez-Botran *et al*, 1986). In cattle this distinction is less clear cut. Although bovine T cell clones have been described which produce IL-4 and are not IL-2 dependant (Stevens *et al*, 1992) corresponding to the situation in mice, CD45RB low T cell lines have been produced in our lab which only make IL-4, but are wholly IL-2 dependant for growth (E.J. Glass and J.D. Campbell, unpub. obs). Caution is therefore advisable in assigning such strict classifications to bovine Th profiles.

The dependence on CTL mediated clearance of parasite (Preston *et al*, 1983; Innes *et al*, 1989a; Nichani, 1994), and the failure of immune serum to protect during primary sporozoite infection (Samad *et al*, 1984) or to lyse macroschizont infected cells (Ahmed *et al*, 1988) would strongly suggest that immunity to *T.annulata* depends primarily on Th1 responses. There is good evidence to suggest that IL-2 rather than IFNγ plays a prominent role in this Th1 response. The appearance of anti-parasite CTL in the efferent lymph of Butalex treated animals is preceded by a period of IL-2 responsiveness in lymph node cells (Nichani, 1994), while IFNγ production shows little change throughout the response. When a susceptible naive animal is infected, and immunity is ineffective, although efferent lymph cells do respond to IL-2 early in infection, this is soon depressed (Nichani, 1994). Also the lymph is found to contain greatly elevated levels of IFNγ, up to 12 times higher than during an effective immune response (Nichani, 1994). Therefore, although an IFNγ dominated form of Th1 response is found naive animals, it is completely different from the IL-2 dominated protective response seen following Butalex treatment. The mechanism for inducing this non protective Th1 immune response is unknown.

In chapter 3, it was shown that when *T.annulata* "non specifically" activates T cells from naive animals, T cells proliferated in a manner which correlated with predicted kinetics of IL-2 production, and that CD8$^+$ cells and NK like cells adopted a

phenotype associated with activation by, or production of IFNγ. To some extent this resembles the situation seen *in vivo*. Non specific activation of T cells may therefore not only disrupt the recognition of parasite antigens, but also induce a cytokine pattern in activated T cells which is completely different to that associated with protection seen *in vivo*.

This chapter sets out to establish whether *T.annulata* activation of T cells from naive animals induces a cytokine profile which is likely to exacerbate or help resolve infection. The work in this chapter included the development of an RT-PCR technique to examine bovine cytokine mRNA expression. Cells were examined for the expression of IL-2, IL-2R (p55 sub unit), IL-4 and IFNγ mRNA at the same timepoints as when they were FACS analysed in chapter 3. As well as establishing the end product cytokine profile and likely function of such activated T cells, several other questions raised in chapter 3 were addressed. The role of IL-2 in proliferation was investigated in more depth by examining its expression patterns throughout T cell activation. By FACS analysis, $CD4^+$ cells appeared to show the most changes after stimulation. Their role in producing cytokines, and the influence they had on other cell types was assessed by sorting $CD4^+$ cells from PBM at different times, and examining ensuing cytokine profiles. In chapter 3, the principal proliferating cell type was seen to be CD45RB high naive $CD4^+$ cells, although the rise in day 3 proliferation may have involved memory cells. As memory and naive bovine $CD4^+$ T cells produce different cytokine patterns (Collins, 1993), the activation of each subset in responding to *T.annulata* could be assessed.

MATERIALS AND METHODS

PBM preparation

Peripheral blood mononuclear cells (PBM) were separated as described in the general materials and methods. Mixed lymphocyte tissue culture medium (MLC) (Glass and Spooner, 1990b) was used throughout the experiments.

Magnetic separation of lymphocyte populations

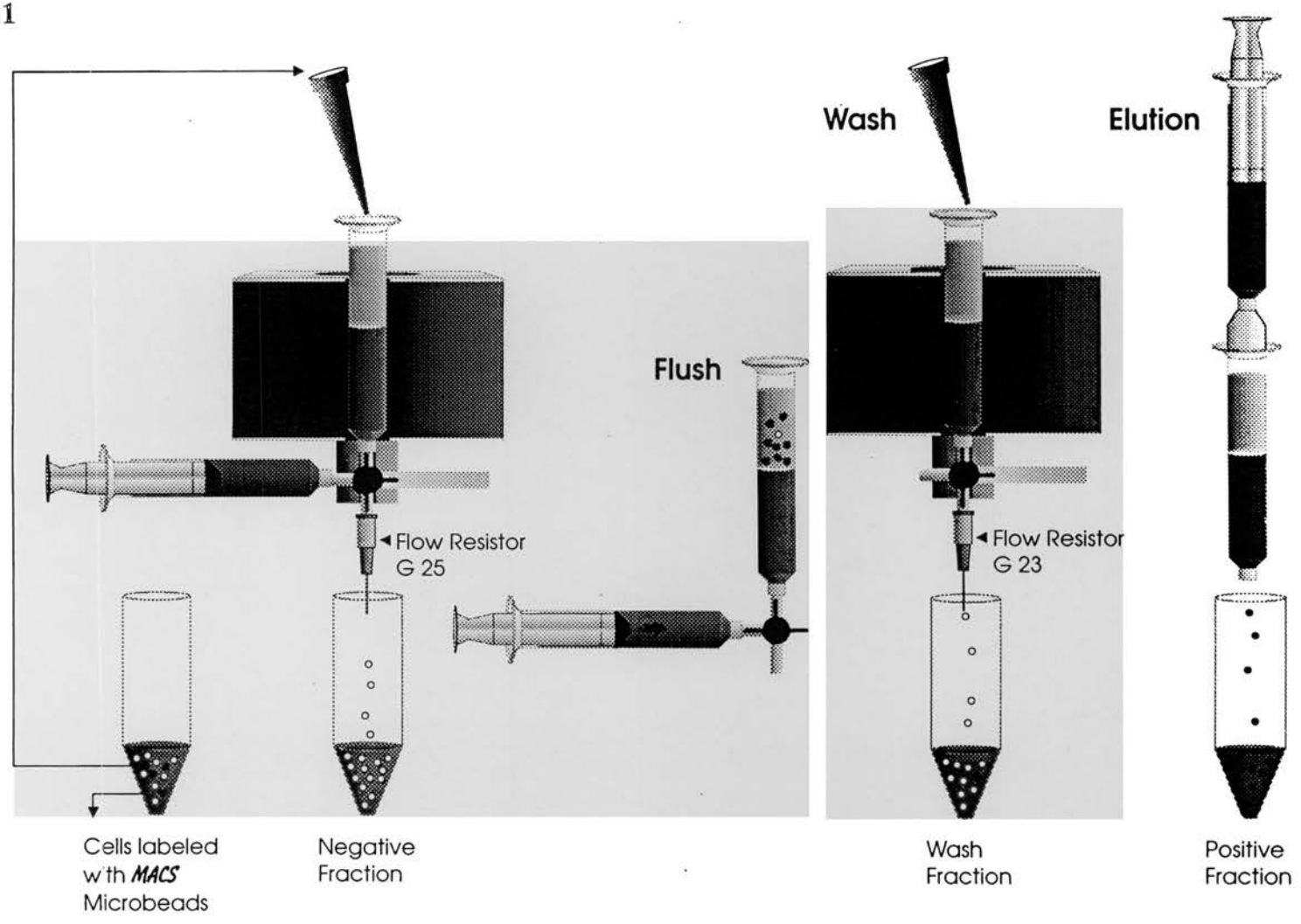
Throughout the experiments in this section, lymphocyte subsets were separated using the MACS magnetic cell sorting system described by Miltenyi *et al* (1990).

Using MACS, cells are first labelled with a monoclonal antibody corresponding to the desired population of cells. The cells are subsequently labelled with magnetic beads conjugated to an appropriate secondary antibody, and then passed through a column containing plastic coated steel wool. The column is placed within a powerful magnet and cell flow rate is controlled using needles of varying diameters. Cells labelled with beads remain trapped within the column, and non magnetic cells are flushed out. The column is then removed from the magnet, allowing magnetic cells to removed (Fig. 4.1). This system differs from other magnetic separation techniques, as the magnetic beads used are extremely small (approximately 50nm diameter). This has several advantages - beads do not have to be detached, membrane perturbation is kept to a minimum, and cells can be FACS analysed immediately post separation.

Preparation of column

Before a separation could take place, the MACS column had to be prepared. An appropriate column for the number of positive cells expected was chosen (see below). The column was attached to a new, sterile stopcock and placed in the MACS magnet as shown in Fig.4.1. The column was then sterilised by filling with 70% ethanol using the syringe at the side. After 10 minutes, several column volumes of PBS/FCS (see below) were passed through from the top, flushing out the alcohol. The column was then allowed to soak in PBS/FCS until just before use, when

Fig. 4.1


Wash
Elution
Flush
Flow Resistor
G 25
Flow Resistor
G 23
Cells labeled
w'th MACS
Microbeads
Negative
Fraction
Wash
Fraction
Positive
Fraction

several volumes of ice cold PBS/FCS were passed through to chill the column. Soaking allows protein to coat the inside of the column, minimising cell damage from contact with the steel wool.

COLUMN	VOLUME	CELL CAPACITY*
A1	2ml	$1x10^7$
A2	2ml	$3x10^7$
B1	5ml	$5x10^7$
B2	5ml	$1x10^8$

* Refers to maximum number of bound positive cells.

Cell labelling

Throughout the procedure, cells were kept on ice and were washed and stained in cold PBS containing 1% FCS (PBS/FCS). PBM were prepared as described in the general materials and methods, receiving a final wash in PBS/FCS rather than RPMI-1640. Tissue culture derived cells were washed twice in PBS/FCS. In this set of experiments, cells were always labelled with anti-CD4 (Howard *et al*, 1991). Cells were adjusted to 10^7 cells/ml and labelled with mAb at a final concentration of 1:200. After 30 minutes incubation on ice, the cells were washed twice in PBS/FCS and resuspended at 10^7 cells per 80μl of PBS/FCS. Appropriate MACS beads eg rat anti mouse IgG_{2a+b} (with azide removed as per the manufacturers instructions) were then added at 20μl per 10^7 cells. The cells were incubated for 20 minutes at 4^0 C in a fridge (ice temperatures inhibit bead movement throughout the solution), washed in PBS/FCS and resuspended at $4x10^7$ cells/ml.

Cell separation

MACS separations were performed to either enrich desired cells or deplete unwanted

cells from PBM. For enrichment, the labelled cells were added to the column and allowed to drip through, followed by 5 column volume washes of PBS/FCS. The column was then removed from the magnet, the cells flushed back using the side mounted syringe, and the column replaced within the magnet. The cells were then washed through as before, using a larger gauge needle to increase the flow speed. Magnetic cells were flushed out after removing the column from the magnet, using a syringe filled with PBS/FCS attached to the side of the column.

To deplete cells, a modified method was used which minimised unwanted cell contamination, although yields were lower than using the standard technique. Initially, cells were added and washed through the column as above. The column was then removed from the magnet, and the positive cells removed by repeated back flushing with the side mounted syringe and decanting the effluate into a tube. The column was then washed 3 times with PBS/FCS before replacing in the magnet. The negative cells were then added to the column again and washed through as before. The resulting negative population typically contained $<1\%$ contaminating positive cells, whereas the positive fraction contained some negative cells which had become non specifically trapped.

Separated cells were washed twice in MLC medium before being used in *in vitro* culture.

T.annulata infected cell lines (IC)

All macroschizont infected cell lines (Ankara or Hissar) from the animals tested were prepared and maintained as previously described in the materials and methods sections. Cell lines were used at low passage number (2-25).

Treatment of IC for stimulation

IC used as antigen presenting cells in this study were treated with Mitomycin C (Sigma) in order to halt their division. IC were washed twice in RPMI medium, and adjusted to 10^7 cells/ml in RPMI containing $50\mu g$/ml Mitomycin C. Cells were then incubated for 30 minutes at 37°C, followed by two washes in RPMI. Cells were finally resuspended at the desired concentration in MLC medium.

Culture of PBM from naive animals with macroschizont infected cells

Culture of whole or separated PBM with IC was carried out in an identical manner to the experiments carried out in chapter 3. Briefly, whole PBM or T cell subsets were stimulated with autologous irradiated IC in 6x10ml well plates (Nunc), with PBM at a concentration of $4x10^6$ cells/ml. As in chapter 3, ratios of PBM:IC were maintained at 20:1 throughout all the assays, - IC were used at $2x10^5$ cells/ml. As a negative control, PBM or T cell subsets ($4x10^6$ cells/ml) were cultured with medium alone. In order to provide a population of activated T cells with which to compare IC activated cells, PBM ($2.5x10^6$/ml) were stimulated with 4μg/ml of Con A (Sigma). Stimulated PBM were harvested at various times (1-7 days) for cytokine analysis. Cells were either used immediately, or frozen in DMSO as described in the general materials and methods section for later analysis.

RT-PCR analysis of cytokine production

In order to analyse the cytokines produced by T cells within PBM following various stimuli, a reverse transcription-polymerase chain reaction (RT-PCR) method was developed. In this technique, total RNA was purified from stimulated cells, followed by synthesis of complementary DNA (cDNA) from the mRNA within the total RNA. The expression of cytokine genes could then be determined by subjecting the cDNA to PCR using specific primers.

Cell preparation

RNA was prepared from either freshly isolated or cryopreserved cells. Cells were washed twice in ice cold PBS, adjusted to 10^7 cells/ml and added at 1ml per tube to autoclaved 1.5 ml tubes (Greiner).

RNA preparation

RNA Isolation.

For RNA work all glassware, tubes, and aqueous solutions were autoclaved. Total RNA was isolated with "RNAzol" solvent (Biogenesis labs, Poole, England), using a modified version of the manufacturer's instructions. Cells were spun for 2 minutes

at 10000RPM in a microfuge and the pellets thoroughly solubilised in 1ml RNAzol. 100μl chloroform supplemented with 4% Iso-Amyl alcohol (Fisons) was added and the samples vortexed for 15 seconds. The tubes were incubated for 10 minutes on ice before spinning for 15 minutes at 14000RPM at 4°C.

The upper aqueous layer (approx. 500μl) was transferred to a fresh sterile 1.5 ml tube, taking care not to transfer any of the protein interface. RNA was then precipitated by adding 800μl ice cold isopropanol (Fisons) and incubating at -20°C for at least one hour. The tubes were then spun at 10000RPM for 15 minutes, 4°C and the resultant pellets washed twice in 800μl ice cold 75%v/v ethanol to remove excess salts. After the second wash, the RNA pellets were dried under vacuum for approximately 10 minutes, before re-suspension in a small volume (10-50μl) of autoclaved dH_2O.

In the later stages of this project, RNA was isolated using the "RNeasy" system (Quiagen). This system, based upon purification columns, is faster than RNAzol, and does not require chloroform extraction. RNA was extracted from cells according to the manufacturer's instructions. Cells were lysed in the supplied lysis buffer @ 10^7 cells per 350μl buffer, and the lysate vigorously passed through a 21G needle several times using a syringe to break up large molecular weight DNA. An equal volume of 70% ethanol was then added to the lysate and gently mixed. 700μl of the mixture was then added to each RNeasy column, and the columns spun for 15 seconds at 14000 RPM. The columns were then washed 3 times in the supplied buffers, and the RNA eluted in 50μl of DEPC treated water.

Measuring RNA yield and purity

The concentration of the RNA obtained was determined by spectrophotometry as described by Sambrook *et al* 1989. An absorbance reading of 1 at 260nm (A_{260}) corresponds to 40 μg/ml RNA (or single stranded DNA). The concentration of RNA (μg/ml) in the sample can therefore be determined by multiplying the A_{260} value obtained by 40. In addition, the purity of RNA can be estimated from the ratio of A_{260} to A_{280} , where A_{280} is the absorbance maximum, for proteins. Ideally, the $A_{260}/A_{280\ ratio}$ will be 1.8-2.

In practice, 1μl of total RNA was diluted in 100μl dH_2O for spectrophotometry.

RNA formaldehyde gel electrophoresis

The integrity of isolated total RNA was also examined by agarose gel electrophoresis under denaturing conditions (Sambrook *et al*, 1989). Gels were run using MOPS buffer. RNA samples, 5-10μg in 12μl of dH_2O were prepared for electrophoresis by adding the following: 5μl 10X MOPS, 8μl paraformaldehyde, 25μl deionised formamide, 1μl of 10mg/ml Ethidium Bromide (EtBr) and heating to 55°C for 15 minutes before running. 3μl of "loading buffer" was added to the samples before overnight electrophoresis at 25 volts. When viewed by UV illumination, ribosomal RNA (rRNA) was clearly visible as 18S and 12S bands. Although not used routinely, the sharpness of the rRNA bands was a useful indicator of the quality of the RNA preparation.

cDNA synthesis

First strand cDNA synthesis was carried out by reverse transcription using the Superscript system (Life Technologies Ltd.), following the manufacturer's instructions. Optimally, 5μg of RNA in 13μl dH_2O was used. The reaction was primed with 1μl of 500μg/ml oligo dT primer, heated to 70°C for 10 minutes using a Techne PHC3 thermal cycler (PCR machine) and then quickly chilled on ice. The resulting mixture, with the oligo dT primer now bound to the polyA tail of the mRNA, was then reverse transcribed to make cDNA. The following was added to the mixture:

2μl 10x synthesis buffer, 2μl 0.1M Dithiothreitol (DTT), 1μl 100mM dNTP mixture and 1μl 200U/μl reverse transcriptase. The reaction was warmed to room temperature for 10 minutes and then incubated at 42°C for 50 minutes. The reaction was terminated by heating to 90°C for 5 minutes and then chilling on ice for 10 minutes. After a brief centrifugation, the cDNA could either be used immediately in a PCR amplification, or stored at -20°C.

PCR amplification of expressed cytokine mRNA

Primer design

Primers for the amplification of cytokine products were designed using PCRPLAN PC/GENE release 6.7 software (IntelliGenetics Inc., Mountain View, California, U.S.A.). This program uses published RNA or DNA sequences to construct oligonucleotide primers. Primers were designed from published sequences of bovine IL-2, IL-2 receptor (p55 sub unit), IL-4 and IFNγ (Reeves *et al*, 1986;Weinberg *et al,* 1988b; Heussler *et al*, 1992; Cerretti *et al*, 1986 respectively). The sequences used were all coding (mRNA) sequences, in order to minimise the possibility of the amplification of genomic DNA. Primer sequences, fragment sizes and predicted melting temperatures (Tm) are listed in table 4.10. The primer for bovine β-Actin was included throughout all experiments as a positive control, as it is constitutively expressed as part of the cytoskeleton. In later experiments, actin was replaced by Glyceraldehyde-3-phosphate dehydrogenase (G3PD), an essential enzyme in glycolysis (Stryer, 1981). G3PD primer sequences were the kind gift of ILRI, Kenya.

PCR amplification

PCR amplification from cDNA was based upon the method of Innis and Gelfand (1990). Although the method used was not quantitative, the same amount of RNA (5μg) was used in each cDNA preparation, and 4μl (equivalent to 1μg RNA) of cDNA reaction mixture was used in each PCR reaction mixture. Control samples were also included which contained primers, but no cDNA, to ensure that detection of products was not due to environmental contamination. As a result, a degree of comparison was possible between assays. PCR was carried out in 50μl reaction volumes, which were comprised as follows:

5μl 10X PCR buffer (Gibco)

2μl 50mM MgCl2 (Gibco)

4μl cDNA

1μl 25μM + primer

1μl 25μM - primer

1μl 10mM dATP (Gibco)

1μl 10mM dCTP (Gibco)

1μl 10mM dGTP (Gibco)

1μl 10mM dTTP (Gibco)

0.4μl (=2U) Taq DNA polymerase (Gibco)

32.6μl dH_2O

Samples were amplified using a Techne PHC3 thermal cycler. Predicted Tm for the primers ranged from 51°C to 61°C. As a result, the primers were first tested using an annealing temperature of 55°C, which was found to be satisfactory for all of the amplifications. The samples were heated to 95°C for 5 minutes ensure that all the RNA/DNA complexes were completely denatured. Amplification consisted of 30 cycles as follows: 95°C 1min (denaturation); 55°C 1min (primer annealing); 72°C 1 min (primer extension). A final incubation at 72°C for 5 min was carried out to ensure complete extension of products. Following amplification, samples were either electrophoresed immediately or stored at -20°C.

Products were analysed by gel electrophoresis at 100V, using TBE as the running buffer in 2% agarose gels. 1μl of running buffer was added per 10μl of sample analysed. As all the fragments produced by the primers were under 600 bp in length, HaeIII size markers (Sigma) were used throughout the experiments. HaeIII markers consist of fragments which form three "blocks" of bands (bp):

587/540/504/458/434

267/234/213/192/184

124/123/104/89/80/64/57/51/21/18/11/8

Bands smaller than the 89 bp fragment were rarely visible.

On a few occasions, when Hae III markers were not available, "1 Kb ladder" markers (Life Technologies) were used. These markers contain of a number of very large fragments (1-12Kb) which were not applicable to the small fragments used in these experiments. However, the lower "block" of fragments (517/506/396/344/298) were suitable for checking that PCR products conformed to the correct sizes.

Table 4.0 PRIMER SEQUENCES

mRNA	STRAND	Tm°C	SIZE bp	5'-SEQUENCE-3'
ACTIN	+	61	288	CTGGCACGACACCTTCAACGAG
	-	61		AGCCAAGTCCAGACGCAGGATG
G3PD	+	57	565	GATGCTGGTGCTGAGTATGTAGTG
	-	57		ATCCACAACAGACACGTTGGGAG
IL-2	+	51	255	AAGTCATTGCTGCTGGATTTAC
	-	52		CCTGTAGTTCCAAAACGATTCTC
IL-2R	+	63	529	GCCAACAAGAGGCTGAAAGGAAACC
	-	63		TGCCCCAGCGTGAAATGGTAGAC
IL-4	+	58	457	GCATTGTTAGCGTCTCCTGGTAAAC
	-	55		CTTCATAATCGTCTTTAGCCTTTCC
IFNγ	+	59	561	GGAGCTACCGATTTCAACTACTCCG
	-	59		GCAGGCAGGAGGACCATTACG

+ coding sequence

- non coding sequence

RESULTS

Throughout this section, lanes on gel photographs are marked with the following abbreviations:

H3 - Hae III markers; 1KB - 1 Kb ladder markers; A - Actin; G3 - G3PD.

In addition, the hyphen is occasionally omitted from IL-2 etc. due to space constraints

T cell cytokine mRNA within *T.annulata* infected cells

T.annulata macroschizont infected cells have a uniform macrophage-like phenotype (Campbell *et al*, 1994). This is reflected in the cytokine mRNAs made by such cells -eg. IL-1α and β, TNFα (Brown *et al*, 1995). Macroschizont infected cells generally do not express T cell cytokine mRNA ie. IL-2, IL-4, IFNγ (Brown *et al*, 1995). This was again found to be the case in this study, as IL-2, IL-2R, IL-4 and IFNγ mRNA were not detected in *T.annulata* IC from animals 11435 and 12929. IC from 13050 were found to express IL-2R mRNA on 2 occasions (Fig. 4.2). The IC which expressed IL-2R were used in an experiment examining PBM activation at day 5, and any bearing this has on the cytokine pattern seen is discussed in the relevant section below.

T cell cytokine mRNA expression in IC activated PBM (Table 4.1)

T cell cytokine mRNA expression in PBM following IC activation was examined in 3 animals (11435, 12929, 13050). As in chapter 3, responses were measured 1,2,5 and 7 days after activation. Medium and Con A controls were also examined.

Cytokine mRNA expression after 24 and 48 hours culture

PBM from all three animals expressed mRNA for IL-2, IL-2R, IL-4 and IFNγ after 24 hours culture with Con A (Fig. 4.3). This pattern was also found in IC activated cultures from 12929 and 11435 (Fig. 4.3), although only IL2 and IFNγ were detected from 13050. Medium controls only expressed IL-2. All four mRNA species could again be detected from IC stimulated cultures (all 3 animals) after 48 hours,

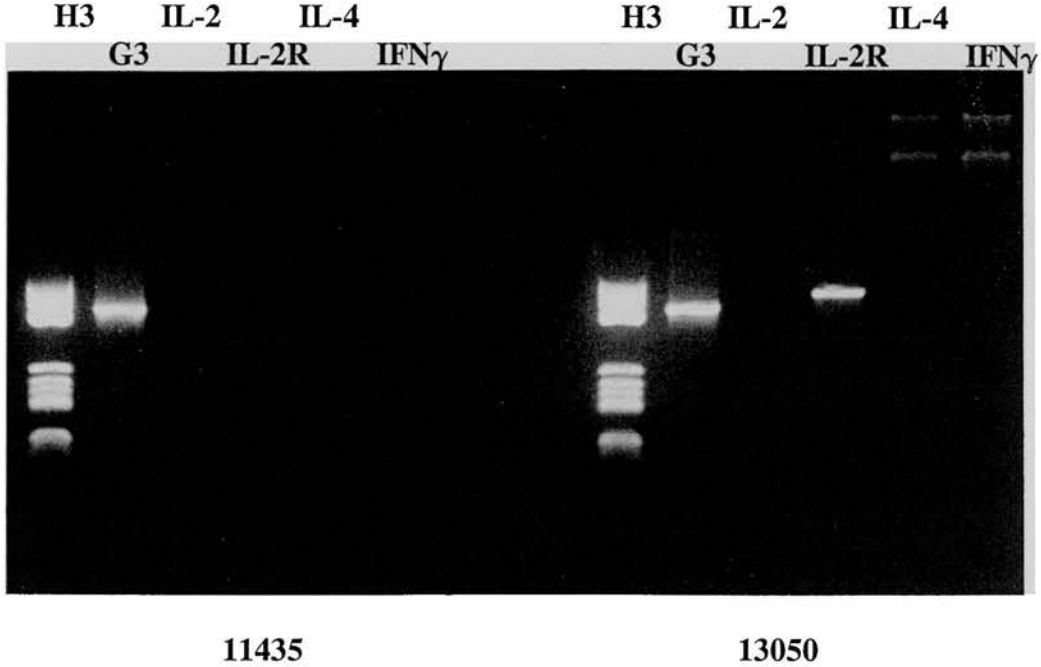


Fig. 4.2 *T.annulata* IC from 11435 (left) do not express mRNA for IL-2, IL-2R, IL-4 or IFNγ. 13050 IC (right) were found to express IL-2R on 2 occasions

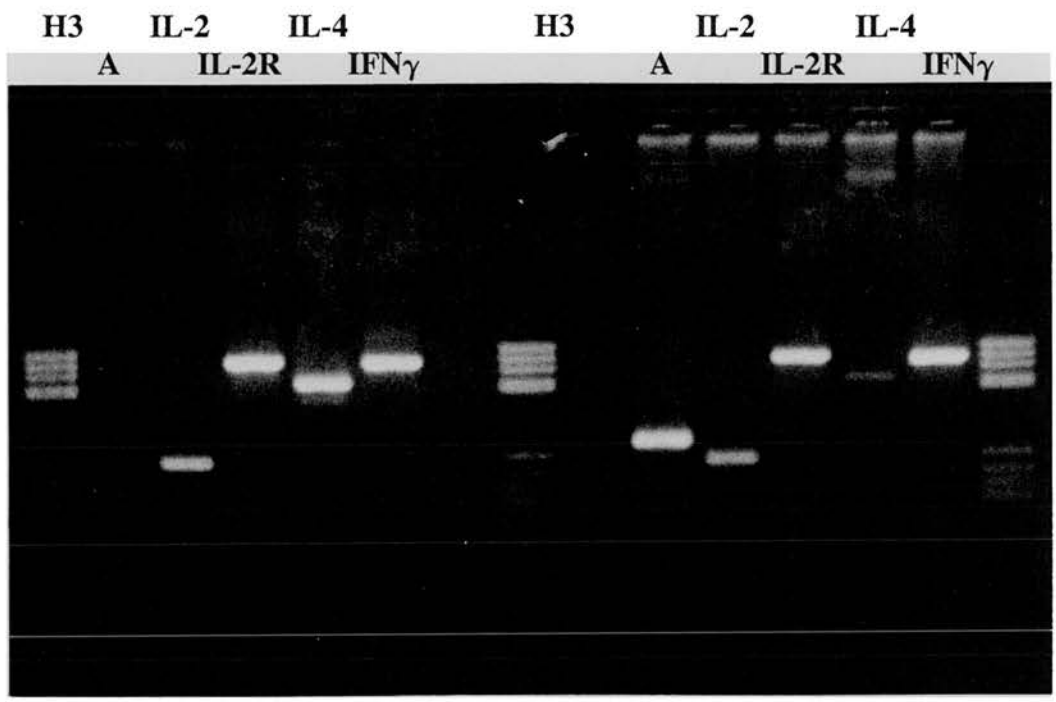


Fig. 4.3 IL-2, IL-2R, IL-4 and IFNγ mRNA are detected from both Con A (left) and *T.annulata* IC activated PBM (right) after 24hrs culture. (Animal 12929). NB actin band was mis-loaded from Con A, susequently re-run and found to be present.

and these were also found in Con A activated PBM (Fig. 4.4). IL-2 could be detected in medium controls, as could IFNγ (Fig. 4.4).

Cytokine mRNA expression 5 and 7 days post activation

After 5 days, when IC induced proliferation of PBM is maximal (see chapter 3), *T.annulata* activated cultures were still found to express mRNA for IL-2, IL-2R and IFNγ. Animals 13050 and 11435 also expressed IL-4 (Fig. 4.5). As noted above, IL-2R expression by 13050 PBM at this time point may have been partially attributable to the IC. As IL-2R was expressed by the other animals' PBM, and 13050 PBM were clearly activated to produce IL-2, IL-4 and IFNγ, it seems unlikely that IL-2R production would be solely limited to IC. IL-4 mRNA was not found from 12929 at day 5 in a total of 4 repeat assays. Con A cultures no longer expressed IL-2 or IL-2R, although IL-4 or IFNγ could be found (Fig. 4.6). No reproducible pattern could be found in control PBM, which ranged from no cytokines detectable to IL-2, IL-2R with or without IFNγ. However, at no time was an identical pattern to IC stimulation seen in control PBM (example Fig. 4.7).

At day 7, cytokine patterns in IC stimulated PBM remained essentially the same as day 5 - all 4 mRNAs were found in 13050 and 11435, while IFNγ message was still present in 12929 although IL-2 had waned, and was only very faintly present (Fig. 4.8). Only IC stimulated PBM were examined at this time point.

Semi-Quantitation of IL-4 and IFNγ mRNA

Very late in this study, a semi-quantitative limited cycle PCR technique was introduced to the department by Mr. D.J. Brown. This involves identical PCR reaction mixtures to those described in the materials and methods being subjected to 20 - 30 cycles of amplification. Comparative measurement of the abundance of mRNA species within assays can be obtained by comparing the number of cycles required for a product to be detected. This was applied to IL-4 and IFNγ production from IC stimulated PBM, 2 and 5 days post activation. At day 2, IFNγ was detected after only 20 cycles, while IL-4 was not detected until 30 cycles. In an animal where IL-4 mRNA was still detected at day 5 (13050), IFNγ continued to be present at 20

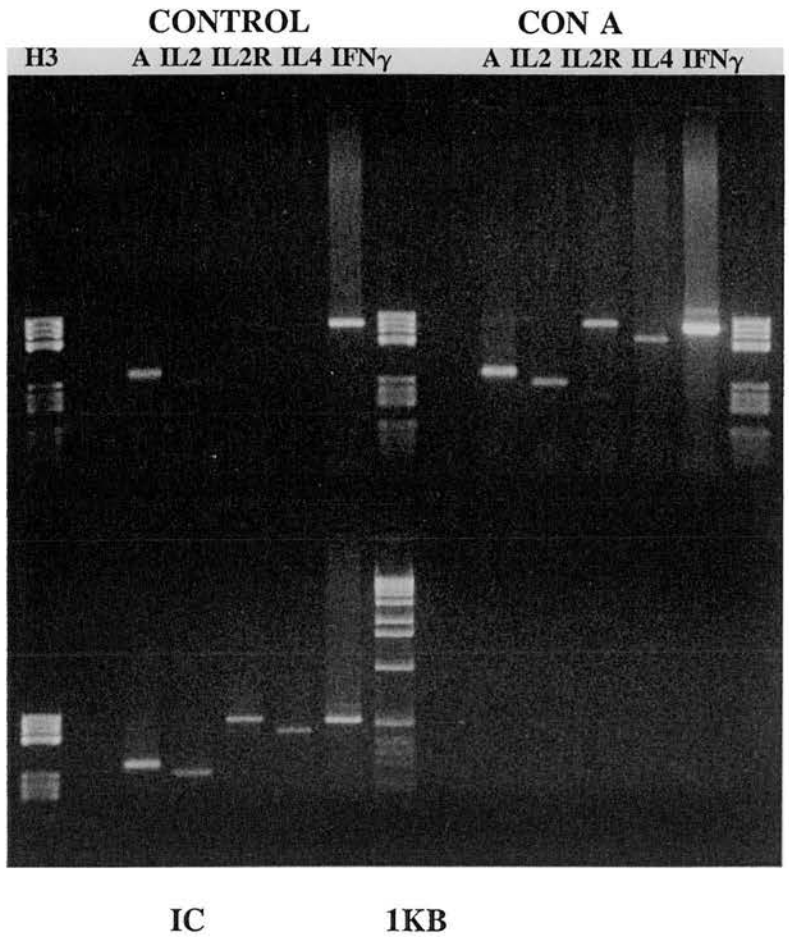


Fig. 4.4 After 48 hours in culture, medium control cultures (top left) express mRNA for only IL-2 and IFNγ. Con A (top right) and *T.annulata* IC (bottom left) activated PBM express mRNA for IL-2, IL-2R, IL-4 and IFNγ. Animal 13050

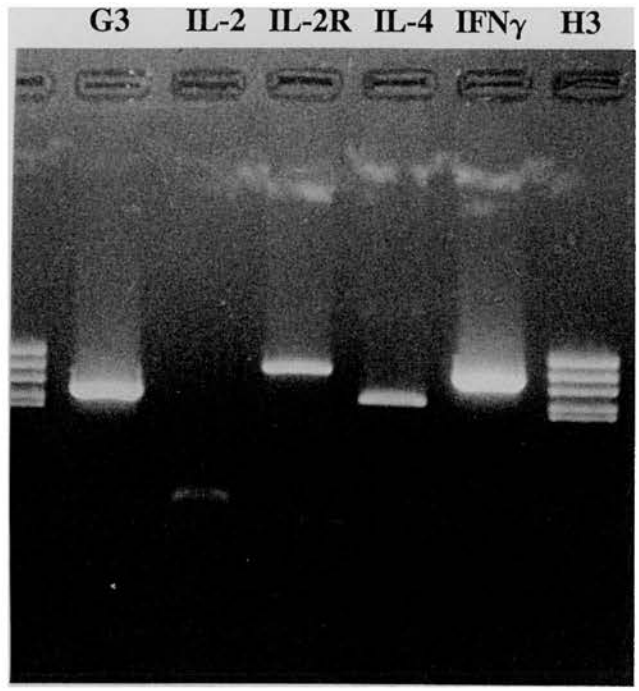


Fig. 4.5 *T.annulata* IC activated PBM from animal 11435 express all cytokine mRNAs at day 5.

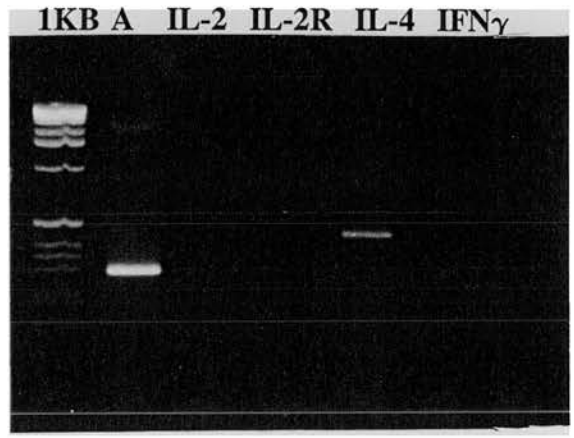


Fig. 4.6 Con A activated PBM no longer produce IL-2 or IL-2R mRNA after 5 days, with only IL-4 remaining in this case. Animal 12929.

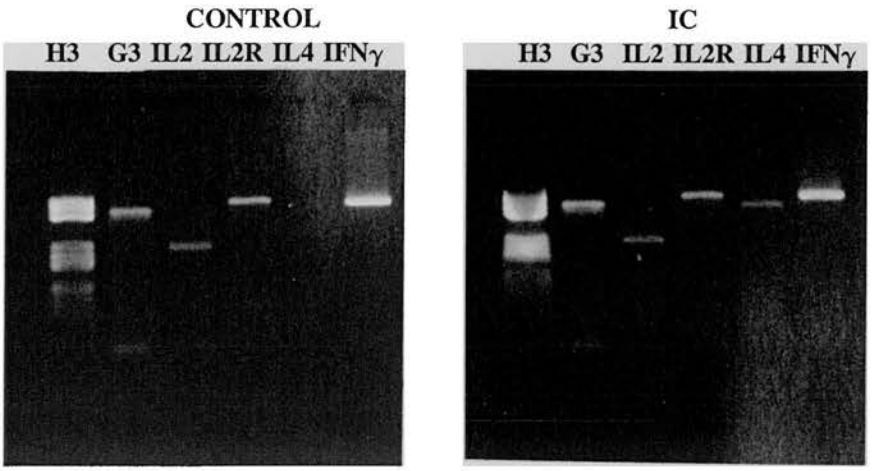


Fig. 4.7 After 5 days culture, medium control cultures (left) express mRNA for only IL-2 IL-2R and IFNγ. *T.annulata* IC activated PBM (right) express mRNA for IL-2, IL-2R, IL-4 and IFNγ. Animal 13050.

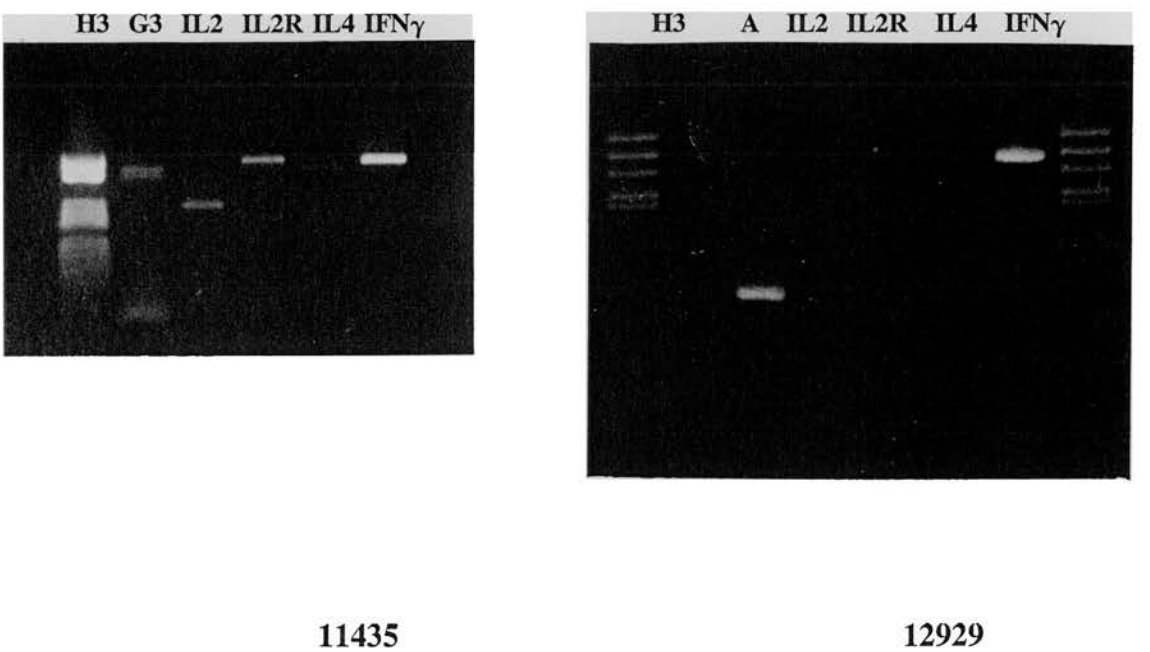


Fig. 4.8 After 7 days culture with IC, PBM from animal 11435 (left) still express all cytokines, although IL-4 is weak. Animal 12929 (right) only expresses IFNγ.

Table 4.1

Cytokine mRNA expression 1,2,5, and 7 days following *T.annulata* IC activation of PBM.

+/- denotes presence or absence of product.

Day	Animal	IL-2	IL-2R	IL-4	IFN gamma
1	12929	+	+	+	+
	11435	+	+	+	+
	13050	+	-	-	+
2	12929	+	+	+	+
	11435	+	+	+	+
	13050	+	+	+	+
5	12929	+	+	-	+
	11435	+	+	+	+
	13050	+	+	+	+
7	12929	+	-	-	+
	11435	+	+	+	+
	13050	+	+	+	+

cycles, while IL-4 was not detected until 28 cycles (Fig. 4.9).

Working at maximum efficiency, PCR amplification is logarithmic, indicating that each molecule of IFNγ cDNA, when detected at 20 cycles, required 2^{18} (262,144) amplifications to detect a product. IL-4 required 28 or 30 cycles - 2^{26} (67,777,216) or 2^{28} (268,435,456) amplifications. IFNγ cDNA is therefore present in far greater amounts than IL-4. Even if the PCR reactions were not functioning at maximum efficiency, the differences between the two cytokines are large enough to signify that IFNγ is produced in far greater amounts than IL-4.

Cytokine mRNA expression within T cell subsets (Table 4.2)

The influence of different T cell subsets upon cytokines produced in PBM after 2 or 5 days culture with IC was examined in two ways:

1. Cytokine expression by $CD4^+$ or $CD4^-$ T cells within whole PBM.
2. Cytokine expression by $CD4^-$ T cells within CD4 depleted PBM.

Cell separations were performed primarily to examine the influence of $CD4^+$ cell derived cytokines upon $CD4^-$ T cells. Accordingly, MACS separations were performed to maximise the removal of $CD4^+$ cells, with "negative" fractions typically $\geq$95% $CD4^+$ free while the $CD4^+$ fraction was typically 85-95% pure (Fig. 4.10).

1. In order to examine the role $CD4^+$ and $CD4^-$ cells in cytokine mRNA expression, whole PBM were incubated with IC as normal, and subsequently sorted into $CD4^+$ and $CD4^-$ fractions after 2 or 5 days culture. After 2 days, IL-2, IL-2R, IL-4 and IFNγ were found in $CD4^+$ cells, but only IL-2 and IFNγ in CD4 depleted cells (Fig. 4.11). This pattern persisted at day 5, although IL-2 message was not present in the $CD4^-$ fraction (Fig. 4.12).

2. Whole PBM were sorted into $CD4^+$ and $CD4^-$ populations before incubation with IC. At day 2, all cytokine mRNAs were again detected in $CD4^+$ cells, but only IFNγ was found in the depleted population. At day 5, $CD4^+$ cells again displayed the same patterns as unseparated PBM, while no cytokine mRNA was found in the CD4 depleted PBM (Fig. 4.13).

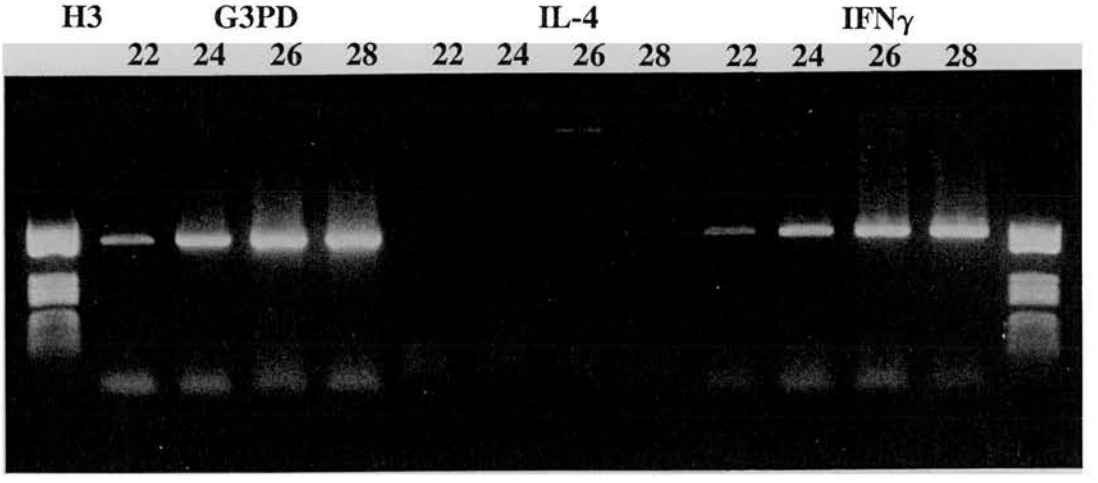


Fig. 4.9 Limiting cycle analysis (cycles 22-28), day 5, IC stimulation. IL-4 is only detected faintly at cycle 28, while G3PD and IFNγ are strongly detected at cycle 22. (G3PD and IFNγ were also found at 20 cycles). Animal 13050.

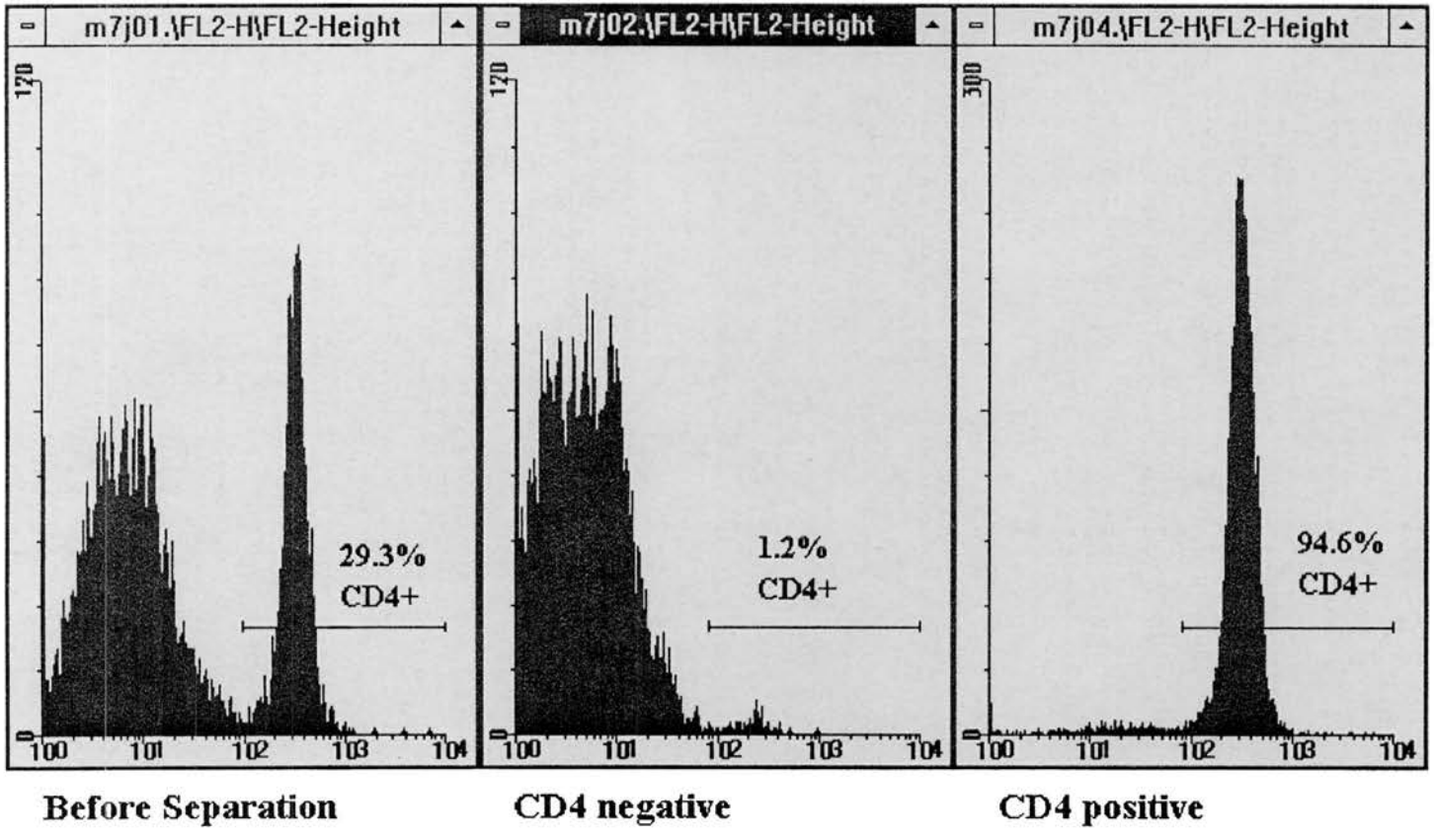


Fig. 4.10 MACS separation of CD4 cells from PBM. A $CD4^+$ population of 30% in normal PBM, is reduced to only 1.2% post depletion, while the $CD4^+$ fraction is enriched to 94.6%.

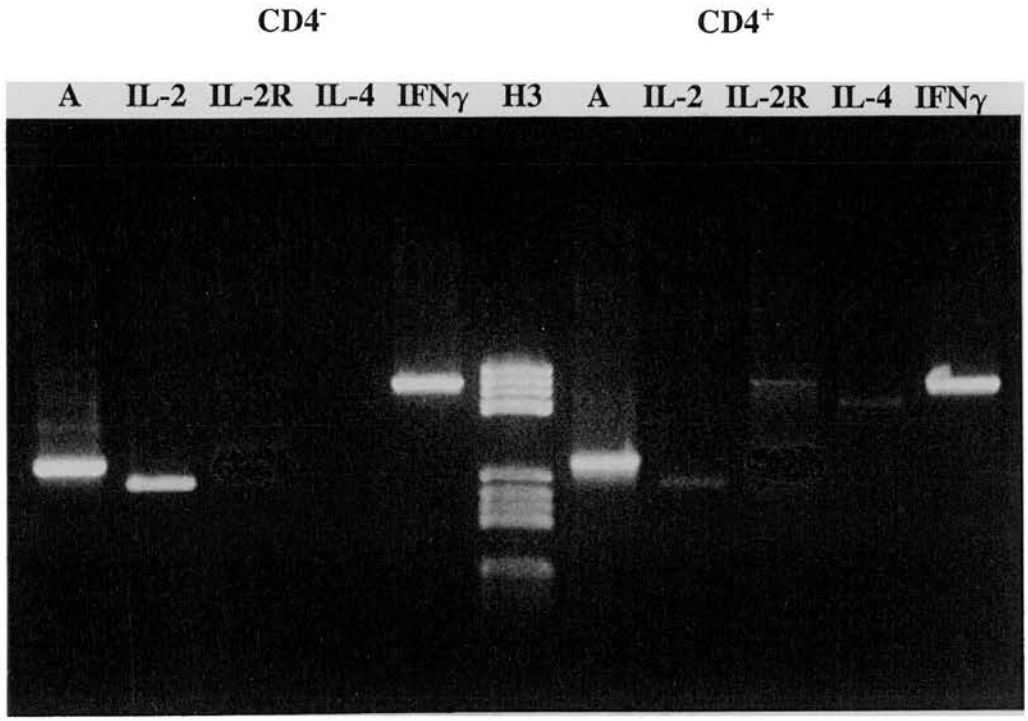


Fig. 4.11 IC stimulated PBM day 2. The $CD4^-$ fraction (left) produces IL-2 and IFNγ mRNA, while the $CD4^+$ fraction (right) produces IL-2, IL-2R, IL-4 and IFNγ. Animal 12929.

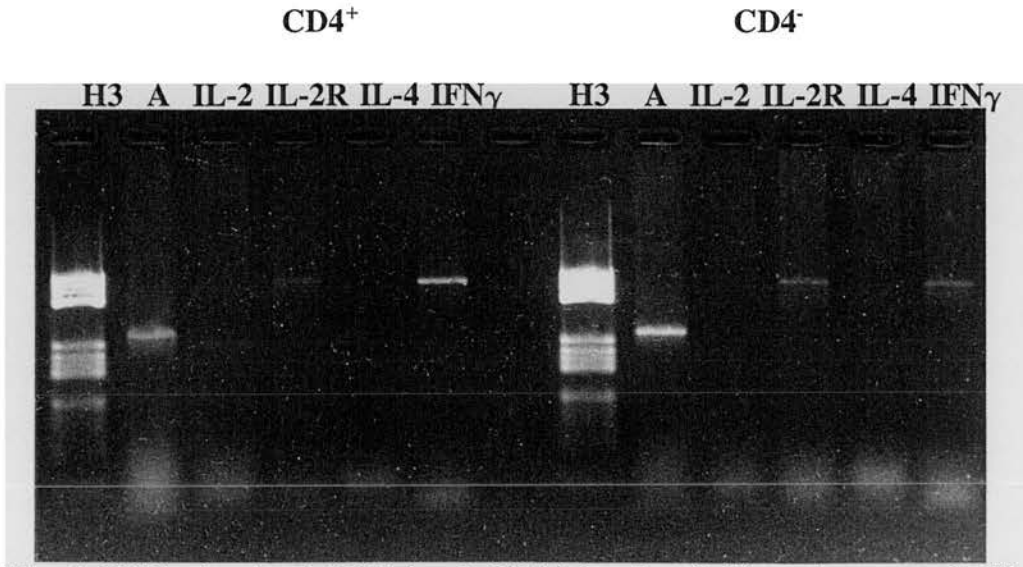


Fig. 4.12 IC stimulated PBM day 5. $CD4^+$ fraction (left) continue to make IL-2, IL-2R and IFNγ (animal 12929 does not make IL-4 at day 5). The $CD4^-$ fraction (right) makes IL-2R and IFNγ mRNA. Animal 12929.

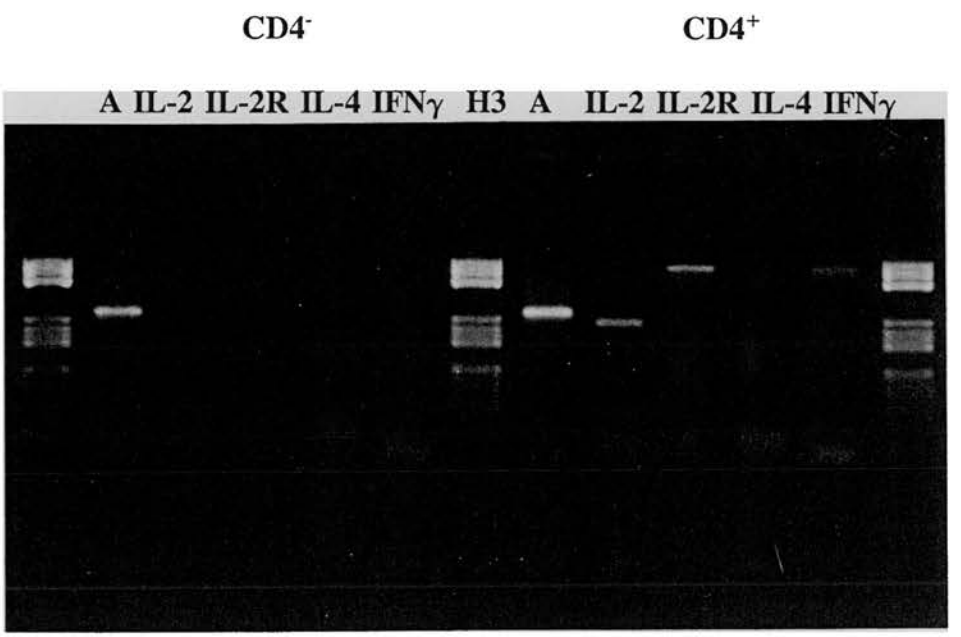


Fig. 4.13 IC stimulated $CD4^+$ T cells or $CD4^-$ PBM day 5. The $CD4^-$ fraction (left) produces none of the cytokine mRNAs in the absence of $CD4^+$ cells, while the $CD4^+$ fraction (right) produces IL-2, IL-2R and IFNγ. Animal 12929 - does not produce IL-4 at day 5.

Table 4.2

T.annulata IC induced cytokine mRNA production by $CD4^+$ and $CD4^-$ cells. Animal 12929 - does not produce IL-4 at day 5.

+/- denotes presence or absence of product.

A. Cytokine mRNA production by $CD4^+$ and $CD4^-$ cells in whole PBM incubated with IC

Day	Subset	IL-2	IL-2R	IL-4	IFNγ
2	$CD4^+$	+	+	+	+
	$CD4^-$	+	-	-	+
5	$CD4^+$	+	+	-	+
	$CD4^-$	-	+	-	+

B. Cytokine mRNA production by PBM sorted into $CD4^+$ and $CD4^-$ fractions before incubation with IC.

Day	Subset	IL-2	IL-2R	IL-4	IFNγ
2	$CD4^+$	+	+	+	+
	$CD4^-$	-	-	-	+
5	$CD4^+$	+	+	-	+
	$CD4^-$	-	-	-	-

In summary, incubation of PBM with IC induces the rapid production of both type 1 and type 2 cytokines. IL-2 and IL-2R persists on the day when proliferation is maximal (day 5), while Con A blasts which reach maximum proliferation after 48-72 hours no longer express these mRNAs at day 5. In one animal, the peak proliferative response is accompanied by the production of a distinct type 1 response, with only IL-2, IL-2R and IFNγ produced. In animals where IL-4 production persists at day 5, this mRNA is produced at much lower levels than IFNγ. In the presence of $CD4^+$ cells, IL-2 and IFNγ production is also found in $CD4^-$ cells. Such mRNA production, at least at day 5, is $CD4^+$ cell dependent - CD4 depleted PBM fail to make any of the cytokine mRNAs when stimulated with IC.

DISCUSSION

Theileria annulata infection in a naive susceptible animal is characterised by a rapid growth of infected cells, and this is accompanied by very large increases in IFNγ in lymph efferent from the node draining the site of infection (Nichani, 1994). This does not correlate with protection, as parasite is not cleared by this response. Also, when a susceptible, sporozoite infected animal is treated with Butalex conferring immunity (Dhar *et al*, 1990) parasite clearance is not accompanied by a large IFNγ response (Nichani, 1994). The source of the IFNγ is unlikely to be macroschizont infected cells, as these cells seldom produce this cytokine (Brown *et al*, 1995).
In chapter 3, it was shown that *T.annulata* macroschizont infected cells (IC) were able to activate the majority of T cells in the peripheral blood of naive animals, possibly acting through $CD4^+$ cells. As naive animals have no anti-parasite memory and the response produced is similar to that which blocks the isolation of parasite specific T cells in immune animals, this activation mechanism is a strong candidate to block the formation of useful immune responses. It is possible that one "side effect" of this T cell activation is the production of an inappropriate cytokine response. This has been investigated in this chapter using RT-PCR to examine cytokine mRNA expression in PBM following IC stimulation. In addition this has clarified several areas of the activation response, such as the memory types of activated cells, and the role played by $CD4^+$ and $CD4^-$ cells in responding to the parasite.

IC activation induces a predominantly Th1 response

This section discusses IL-4 and IFNγ mRNA production. The role of IL-2 in T cell proliferation and differentiation is discussed in a following section. Although both IL-4 and IFNγ mRNA were initially produced following IC induced activation, in one animal's PBM only IFNγ was produced at peak proliferation and thereafter. In animals where IL-4 expression remained, this was shown to be produced at far lower levels than IFNγ, both at day 2 and day 5. Activation of T cells by IC therefore leads to the production of IFNγ alone, or in far greater amounts than IL-4. In either

case, the *in vitro* response is similar to that seen in efferent lymph of naive infected animals characterised by high IFNγ production (Nichani, 1994).

As individual cells were not examined, it was not possible to determine whether the production of IL-4 along with IFNγ was due to a small number of IL-4 producing cells within a predominantly Th1 population, or whether some cells of a Th0 phenotype were produced. The latter phenotype is not thought to be merely an intermediate step in cytokine production by bovine T cells - fully differentiated T cell clones reacting to *Babesia bovis* predominantly produce this cytokine profile (Brown *et al*, 1993). However, the Th0 cells described by Brown *et al* produced IL-4 mRNA in amounts equal to or greater than IFNγ. As IFNγ is detected in far larger quantities than IL-4 in *T.annulata* activated cells, the production of large numbers of Th0 cells is unlikely. The majority of responding cells are therefore of a Th1 phenotype.

The production of an IFNγ dominated response, at least in the initial stages, may not appear to be specifically selected by the parasite antigen, as IL-4 is also produced. The exact factors underlying the selection of a particular Th response still remain rather obscure but are almost certainly not purely antigen driven. For example, in *Leishmania spp.* where a Th1 response induces protection but a Th2 does not (Scott *et al*, 1987; Gafaar *et al*, 1995), identical parasite infections in the same inbred strain of mice can lead to either a Th1 or Th2 response, depending on the cytokines used to influence the response (Wang *et al*, 1994). As IFNγ mRNA was detected at far higher levels than IL-4 even after 48 hours, IFNγ producing cells may be present in larger numbers in the periphery and the "non specific activation" of these cells is reflected in the cytokine response. However, when the cytokine profiles of IC or Con A activated cells are compared at day 5, there is evidence for selection by IC. Mitogen activation of T cells has the potential to induce either Th1 or Th2 cytokine profiles (Rocken *et al*, 1992), and this was seen in the experiments described here, with Con A activated cells expressing either IL-4 or IFNγ at day 5 but never both together. If the "non specific" selection of IFNγ producing cells by *T.annulata* merely reflected the high precursor frequency of these cells in the peripheral blood, a similar response in Con A activated cells would be expected, and is not found.

There is therefore some element of response selection by IC which leads to the activation of IFNγ producing cells.

Although the role of antigen in influencing Th responses is unclear, a distinct role for cytokines, T cell derived or otherwise has been established. *T.annulata* IC express a wide range of cytokine mRNAs - IL-1α, IL-1β, IL-6, IL-10, IL-12 (Brown *et al*, 1995; D.J. Brown unpub. obs.). The only IC cytokine with a currently defined effect on T cells is IL-1α, which is responsible for regulating the magnitude of proliferative responses (Brown *et al*, 1995). This particular cytokine may not particularly influence the Th pattern of responding T cells as bovine T cells which make both IL-4 and IFNγ are induced to proliferate by IL-1 (Stevens *et al*, 1992). Both IL-10 and IL-12 have been shown to modulate T cell cytokine production, with the former inducing Th2 responses, and the latter Th1 (Hseih *et al*, 1992;1993). As a predominantly Th1 response ensues from IC activation of PBM, it seems likely that an IC derived cytokine such as IL-12 is in operation. In chapter 3, it was shown that $CD8^+$ T cells and NK cells acquired a surface phenotype associated with IFNγ production (Conlon *et al*, 1995; Shen *et al*, 1995). IL-12 is capable of inducing such a phenotype in both cell types (D'Andrea *et al*, 1992; Chan *et al*, 1991). Thus an IC derived factor such as IL-12 may aid the development of a Th1 response.

Although IFNγ is the dominant cytokine produced, IL-4 is initially selected persisting at low levels in 2 out of 3 animals, and this may be mediated by IL-10 which is a potent inducer of IL-4 production (Hsieh *et al*, 1992). The extent to which IL-4 persists may be influenced by the relative levels of IL-10 and IL-12 produced by IC from individual animals. "Damping down" of IL-4 production may also be mediated by IFNγ. IFNγ and IL-4 are antagonistic, with the effects of IFNγ apparently dominant, reducing IL-4 production in Th2 cells, or even switching them to IFNγ production (Donkier *et al*, 1994). There may therefore be two stages in the Th1-like cytokine profile seen in IC activated T cells - an initial selection of this phenotype influenced by IL-12 followed by further control thorough IFNγ.

Phenotypes of responding cells

Results in chapter 3 showed that naive CD45RB low cells were the principal cell

type present at peak proliferation. An earlier rise of proliferation suggested that memory cells may also be involved. IL-2, IL-4 and IFNγ mRNA were produced within 24 hours of IC activation of PBM. As naive bovine T cells only produce IL-2 upon initial activation, while CD45RO$^+$ cells produce can produce a wide range of cytokines including IL-2, IL-4 and IFNγ (Collins, 1993), this would confirm that *T.annulata* is also activating memory/effector T cells. Thus although the majority of proliferating cells at day 5 are naive, memory/effector cells are also activated very early following T cell/IC interaction. The element causing T cell activation is thus extremely promiscuous, able to induce the activation of T cells, whether any antigen specificity has been acquired by the cells or not.

Although the cytokine profiles seen in the early stages of activation can be unequivocally assigned to memory/effector cells, the stage at which activated naive T cells contribute to this response is more difficult to ascertain. No current information is available regarding the time post stimulation that naive bovine T cells acquire the ability to produce cytokines other than IL-2. Such experiments in other species have tended to culture cells for 2 weeks or longer, and although a full cytokine phenotype is detected at these times (Rocken *et al*, 1992), this does not pinpoint the change to a full phenotype satisfactorily. The best indicator of cytokine secreting capacity is the expression of a CD45RB low/CD45RO$^+$ phenotype in both CD4$^+$ and CD8$^+$ T cells (Collins, 1993; Conlon *et al*, 1995). As this phenotype was expressed by previously CD45RB high/CD45RA$^+$ CD4$^+$ and CD8$^+$ cells by day 7 of IC culture, it seems likely that these cells are contributing to the cytokine profiles seen at days 5-7.

Cytokine profiles seen 5-7 days post activation are IFNγ dominated, in one animal IL-4 production is not detected at all, and therefore do not differ from those seen in early activation. The factors influencing this response in memory cells were presumably still present 5-7 days post infection, as cytokine mRNA profiles from mitomycin C treated IC have not been found to alter over 7 days of culture (D.J. Brown unpub. obs.). Cytokines from IC may therefore play a part in influencing newly "educated" CD45RB low cells. It is also likely that the IFNγ antagonism for IL-4 discussed above will affect responses. IFNγ is also a potent inducer of its own

production in NK cells and $CD8^+$ T cells, and this mechanism is apparently in operation, as these cells acquire the $CD45RO^+$ phenotype associate with production of this cytokine after 5-7 days culture. As IFNγ is T cell derived is is probable that the activated memory T cells seen early in infection influence the cytokine pattern of subsequently activated naive cells. Therefore, although memory/effector cells may not contribute to the peak of IC proliferation, these cells are activated very early post IC exposure and are likely to play a role in influencing the cytokines expressed by both proliferating naive $CD4^+$ cells and the $CD45RO^+$ $CD8^+$ and NK cells described in chapter 3.

The role of $CD4^+$ T cells and IL-2 in observed responses.

Results in chapter 3 showed that the most prominent changes in activation marker expression were found in the $CD4^+$ subset following incubation with autologous *T.annulata* IC. This was further investigated by sorting $CD4^+$ cells from PBM either before or after IC stimulation and examining cytokine responses. As bovine $\gamma\delta$ T cells do not express IL-2, IL-4 or IFNγ (Collins, 1993), the responses seen in $CD4^-$ cells here are likely to relate only to $CD8^+$ T cells and NK cells. When cells were separated after activation, IL-4 was only ever found in the $CD4^+$ fraction, correlating well with results from human T cells which demonstrate that IL-4 can only be reliably demonstrated within the $CD4^+$ $CD45RO^+$ subset (Conlon *et al*, 1995). IL-2 and IFNγ could be demonstrated within both the $CD4^+$ and $CD4^-$ populations at day 2, although the production of IL-2 had waned by day 5 in the $CD4^-$ cells, with expression of IL-2R remaining. IL-2 from both $CD4^+$ and $CD4^-$ cells may therefore contribute to early T cell proliferation, and as the expression of IL-2 induces its own receptor (expressed in these cells at day 5) (Mauer *et al,* 1984;Smith, 1988) these cells may to some extent be regulating their own proliferation. By the same token, the failure of $CD4^-$ cells to express IL-2 or IL-2R mRNA in the absence of $CD4^+$ cells would suggest that their proliferation would be severely restricted.

As discussed above, IC derived cytokines such as IL-12 may influence IFNγ expression by responding T and NK cells, which acquire a phenotype consistent with

production of this cytokine at days 5-7 (Conlon *et al*, 1995; Shen *et al,* 1995). As IC stimulated $CD4^-$ cells expressed this cytokine at day 2, irrespective of co-culture with $CD4^+$ cells, cytokines such as IL-12 may be able to act directly upon the small numbers of CD45RB low $CD8^+$ cells seen at this time. However, examining PBM depleted of $CD4^+$ cells before IC stimulation demonstrated a distinct role for $CD4^+$ cells in inducing this phenotype at day 5. $CD4^-$ cells did not express IFNγ mRNA at day 5, while this cytokine was expressed by these cells when cultured in the presence of $CD4^+$ cells. IC derived IL-12 alone is therefore insufficient to induce IFNγ expression by $CD8^+$ or NK cells. $CD4^+$ cells were capable of making their own IL-2, and produced IFNγ as normal when incubated alone. It seems likely that the lack of IL-2 production in $CD4^-$ cells resulted in their failure to make IFNγ, as continued IFNγ expression was accompanied by IL-2R expression. A role for IL-2 in IFNγ expression by NK or $CD8^+$ cells seems likely, as all the descriptions of IFNγ production by these cells, whether via mitogen stimulation, cross linking of surface receptors, or in the presence of IL-12 involved systems in which IL-2 was present (Conlon *et al*, 1995; Shen *et al,* 1995; Hseih *et al,* 1993). Similarly, this would question the finding of Jensen and Schultz (1990) that IFNγ alone could induce bovine NK activity, as their experiments were conducted in whole PBM which would have contained a source of IL-2 from $CD4^+$ cells.

The results from this study have shown that $CD4^+$ cells are essential for the induction of full responses in non $CD4^+$ cells. It is therefore extremely unlikely that IC possess a restriction element for cells other than $CD4^+$. Also, cells of memory/effector phenotype are activated, in addition to the naive cells described in chapter 3, so the activating element on IC has a very broad spectrum of activity. As IL-2 and IL-2R mRNA continued to be expressed in IC activated cells at days 5-7, while this had waned in Con A activated cells, this reinforces the conclusion from chapter 3 that the activation is not a simple mitogen mimic.

As discussed in chapter 3, various MHC class II restricted elements have been identified from viruses, bacteria and protozoa which cause the activation of many $CD4^+$ T cells in the periphery (Fern and Good, 1992; Piuvezam *et al*, 1993; Roessner *et al*, 1994; Janeway *et al*, 1989; Marrak and Kappler, 1990). Although

T.annulata may operate through a similar mechanism, the results of Brown *et al* (1995) who found that levels of MHC class II expression on IC do not influence T cell proliferation may cast doubt on *T.annulata* activating cells through a purely MHC class II restricted mechanism.

As activation of PBM is mediated through $CD4^+$ cells, it is possible that part of the IC/T cell interaction is mediated through interactions between CD4 on the T cell and an anti CD4 ligand on the *T.annulata* IC. Such a CD4 recognition molecule has been well characterised in the gp120 envelope protein of HIV (Maddon *et al*, 1986). Although such a molecule could mediate binding of IC to T cells, it is extremely unlikely to result in cell activation by itself - although CD4 is associated with tyrosine kinase activity ($p56^{lck}$, reviewed by Ledbetter *et al*, 1993) engaging CD4 alone has been shown to prime T cells for apoptosis (Banda *et al*, 1992). However, such a CD4 ligand could aid the effect of an MHC bound antigen if it encouraged co-capping of the TCR complex with CD4 (Viellette *et al*, 1989; Zerbib *et al*, 1994). Since cloned IC cell lines with very high levels of MHC class II expression do not induce any more, and sometimes less, proliferation of T cells than those with "normal" levels it is possible that an enhancement mechanism such as the one described in this paragraph is in operation. As the activation of PBM also is partially dependant on IL-1 production by IC, further work will be required to fully characterise the restriction elements responsible for T cell activation.

In summary, work in this chapter has demonstrated that PBM "non specifically" activated by *T.annulata* IC produce a predominantly Th1 response upon activation, with far higher levels of IFNγ mRNA production than IL-4. This response is specifically skewed, almost certainly by both T cell and IC derived cytokines, as genuinely unregulated Con A activated T cells do not develop the same phenotypes. IFNγ and IL-2 is produced by both "helper" $CD4^+$ and "non helper" $CD4^-$ cells, and in this sense is a genuine "type-1" response as defined by Clerici and Shearer (1994). In chapter 5, the type-1 rather than Th1 nomenclature is therefore used. $CD4^+$ cells were demonstrated to play a pivotal role in the activation of other cell types - in the absence of $CD4^+$ cells, $CD8^+$ or NK cells failed to produce IL-2, IL-2R, and at day

5, IFNγ. It is likely that non CD4 cells require $CD4^+$ cell derived IL-2 to proliferate.

CHAPTER 5

Pathology and immune response development within lymph nodes draining the site of *Theileria annulata* infection

INTRODUCTION

Infection with *Theileria annulata* sporozoites in a susceptible (ie European) animal is variously quoted as being fatal in 40-90% of cases (Robinson, 1982; Uilenberg, *et al*, 1993; Hashemi-Fesharki, 1988). Such infected animals seem unable to mount an effective immune response, and large numbers of schizont and piroplasm infected cells develop, apparently unchecked. Post mortem, animals exhibit severe anaemia, pulmonary oedema, haemorrhage in many organs, and ulceration, particularly within the gastro-intestinal tract (Neitz, 1957), with anaemia thought to be the principal cause of death (Barnett, 1977). Although a role in pathology for both macroschizont infected cells (IC) and piroplasm infected RBCs have been proposed (Hooshmand-Rad, 1976; Uilenberg, 1981a), much of the pathology of infection can be attributed to the macroschizont stage of the parasite, as animals are often clinically ill before the appearance of piroplasms. In addition, lethal infections have been induced using strains of parasite which do not produce piroplasms (Pipano and Tsur, 1966; Hooshmand-Rad, 1976).

Treatment of acute infections with the drug "Butalex", which kills the intracellular macroschizont, quickly leads to clearance of parasites and recovery (McHardy *et al,* 1985; Dhar *et al,* 1990). Parasite clearance may be attributable to cell mediated immune mechanisms, as parasite specific $CD8^+$ cytotoxic T lymphocytes (CTL) are detectable in the peripheral blood of treated animals (Preston *et al,* 1983; Nichani, 1994). In addition, animals vaccinated using attenuated macroschizont infected cell lines also exhibit strong anti-parasite CTL responses (Innes *et al,* 1989a; Nichani, 1994). In both cases, CTL recognise MHC class I restricted antigens on macroschizont infected cells (Preston *et al,* 1983; Innes *et al*, 1989a). Cattle are therefore capable of mounting an immune response to macroschizont antigens when the parasite is killed, or attenuated. In this respect the development of immunity is a double edged sword - effective T cell responses require the recognition of macroschizont antigen, but development of this stage is the most pathogenic and often lethal. An understanding of macroschizont development is therefore crucial in any study of the *in vivo* immune response to acute theileriosis.

It has been known for some time that both *T.annulata* (Rintelen *et al,* 1989; Glass

and Spooner, 1990a) and *T.parva* (reviewed Goddeeris and Morrison, 1987) macroschizont infected cells induce proliferation of resting PBM *in vitro*. As discussed in chapter 3, *T.annulata* macroschizont infected cells can induce activation and proliferation in resting autologous T cells from uninfected animals. Although similar properties are found with *T.parva*, such an "autologous MLR" is not a barrier to generating *Theileria parva* specific cytotoxic or helper T cells from immune animals *in vitro* (Goddeeris and Morrison, 1987; Baldwin *et al*, 1987). However, the factors inducing "non specific" T cell activation in *T.annulata* are strong enough to completely block the development of such specific cells from immune animals *in vitro* (appendix 2 this thesis). This inherent overriding ability to stimulate T cells, if exhibited *in vivo*, may provide *T.annulata* with a powerful mechanism to interfere with T cell development.

The macroschizont stages of both *T.annulata* and *T.parva* develop within the local draining lymph node following inoculation of sporozoites. In *T.parva*, detection of macroschizonts within the draining LN (within 4-14 days depending on the dose of sporozoites given), is followed by the appearance of schizonts within other LN (up to 30%of all LN) 2-3 days later (Morrison *et al,* 1981). This is in direct contrast to *T.annulata*, where parasitised cells are very rarely found within non draining LN, although macroschizonts do escape to the periphery and can be detected in many tissues. These differences between the two parasites may be a reflection of their different target cells, with *T.parva* infecting T cells (Spooner *et al,* 1989) and macrophages infected by *T.annulata* (Glass *et al*, 1989; Campbell *et al,* 1994). The appearance of *T.parva* infected cells may be due to homing of infected T cells to LN, a function not normally found in macrophages which are infected by *T.annulata*. If *T.annulata* macroschizont infected cells can interfere with T cell development *in vivo,* the containment of the developing parasite within the draining LN provides an opportunity for the required interactions to take place.

It is possible macroschizont infected cell development is so rapid, that although the host makes an anti-parasite immune response, it is simply overwhelmed by growing parasite. This seems unlikely to be the sole mechanism, as a recent study of efferent lymph from sporozoite infected LN has suggested that T cell function is adversely

affected during sporozoite infection (Nichani, 1994). In particular, T cells gradually become unresponsive to Con A and IL-2 as macroschizonts develop within the node. Similar phenotypes have been associated with T cell responses to other pathogens, notably *Trypanosoma brucei*, where the parasite specifically induces immune suppression (Sileghem *et al*, 1987;1989), and HIV where disease progression is marked by a loss of T cell responses to IL-2 (Reviewed Clerici and Shearer, 1994). It seems likely that *T.annulata* infection is mediating a similar suppression of T cell responses, rather than the parasite simply growing regardless of any immune response.

This chapter is concerned with understanding events during *T.annulata* infection in susceptible animals, in particular, the development of the immune response. In order to test whether the failure of the immune response can be attributed to properties of the macroschizont infected cell, an immunohistochemical study of sporozoite infected LN was initiated, paying particular attention to the parallel development of the parasite and the immune response

Experimental Design

Due to the fire at the CTVM in the winter of 1991, sporozoites were not available at the beginning of this study. Initial work was carried out upon lymph node material kindly provided by Mr. C.G.D. Brown of the CTVM, University of Edinburgh. This consisted of 2 lymph nodes from a healthy animal and a draining lymph node from an animal recovering from severe theileriosis following drug treatment. Immunohistochemical studies were carried out using frozen sections of these lymph nodes in order to determine the usefulness of available antibodies using a well established technique.

Once sporozoites became available again, a series of experiments were carried out in order to examine in detail the events involved in *T.annulata* development, and any effects this had upon the immune response. In this study, the draining lymph nodes were examined during the course of a primary experimental *T.annulata* infection. In susceptible animals, experimental infection usually induces severe disease within 10-

12 days (Samantery *et al*, 1980; Preston *et al*, 1992a). As a result, lymph nodes were examined up to 10 days post infection, in order to obtain a full picture of disease without causing unnecessary suffering.

Three groups of animals were used in this study. The first group was infected with percoll purified sporozoites (Ankara strain) (see below), in order to avoid any adjuvant effect which could be attributed to the residual tick material in GUTS. Draining nodes were removed from the animals at days 4 and 8 post infection. These days were chosen to examine disease development before schizonts are normally first detectable (day 4) and at the peak of lymphoproliferation and lymph node swelling, often associated with the start of temperature elevation, before a heavy parasite burden appears (day 8) (Samantery *et al*, 1980; Preston *et al*, 1992a; C.G.D. Brown pers. comm.)

As the pathology and lymphocyte responses of the draining lymph nodes examined from the first infected group were extremely distinctive, animals in the second two groups were infected with unpurified stabilate. Draining lymph nodes were examined 2 and 4 days post infection in order to clarify early events. Nodes excised 10 days post infection, corresponding to high parasitaemia and morbidity were also examined.

MATERIALS AND METHODS

Animals

Initially examined lymph node material from the CTVM came from one healthy Ayrshire calf, and a Butalex treated calf which was suffering from acute tropical theileriosis following sporozoite infection. For the detailed examination of draining lymph node pathology, three groups of animals were used. Groups 1 and 2 contained 3 animals, group 3 contained 2 animals. All animals used were as described in general materials and methods, but under 8 months old.

Sporozoites

The sporozoites used in this study were prepared as described in the Materials and Methods section.

Animals in group 1 were infected using percoll purified sporozoites (Ankara strain, Schein, 1975) prepared as described by Walker and McKellar (1983a, 1983b). This method yields only approximately half the amount of sporozoites contained in GUTS, but contains only 5% of the contaminating tick material (Walker and McKeller, 1983b). To provide an appropriate control, uninfected ticks were processed thorough percoll in exactly the same way as infected *Hyalomma*. The dose of sporozoites (expressed as tick equivalents (TE)) required to induce acute infection in an animal is somewhat variable, and depends upon the quality of the preparation. Infection with 10 TE is invariably fatal, with 1 TE producing severe clinical disease which is often fatal, although infection with as little as 0.001 TE can lead to death (Samantery *et al*, 1980; Preston *et al*, 1992a; Eisler, 1988). To take account of the loss of parasite in the percoll, it was decided to infect the animals in group 1 with the pre-purification equivalent of 10 ticks each (infected and control).

After the ticks had been ground as described in the general materials and methods, the supernatant from infected and uninfected ticks was centrifuged at 100G for 5 mins. The resulting pellet was resuspended in a known volume of HBSS and passed through an 8μm filter. The supernates were then centrifuged over step-wise percoll (Sigma) gradients: 1.095/1.07/1.05/1.03 g/cm^3 in HBSS for 1 hour, 3000G at 4^{o}C.

The 1.095/1.07 interface was collected, resuspended in PBS, and centrifuged for 30 mins. The resulting pellet was vigorously resuspended in MEM/ 3.5%BPA using a very fine tipped pasteur pipette at 10 TE per ml.

The uninfected control material produced no discernable pellet, but supernate was removed in an identical manner, and resuspension to 10 TE per ml took place in exactly the way as for infected material.

Animals in groups 2 and 3 were infected with 0.5 TE of Gharb (Ouhelli, 1985) stabilate no.63, which had been previously shown to produce high parasitaemia by day 10 of infection (A.K. Nichani pers. comm.)

Immunisations and Lymph node removal

Animals were subcutaneously infected with the previously described sporozoite doses in the left or right shoulder. Draining prescapular lymph nodes were removed on the days specified in table 5.1. Animal 13050 (group 2) was completely untreated, and its lymph node was removed for comparison with the uninfected tick control.

Monitoring of animals

As this study was concerned with events early in *T.annulata* infection, the type of monitoring used for long term or chronic infections eg. changes in PCV, total leucocytic counts, was not applicable, although temperatures and swelling of draining lymph nodes were monitored. Piroplasms were assessed in blood smears prepared by spreading a drop of venous blood on a clean glass slide using a second slide. The development of schizonts within the draining lymph nodes was monitored using either "tissue touch" imprints obtained at lymph node excision or from biopsies. The former were obtained by pressing a freshly cut face of an excised lymph node onto clean glass slides. Needle biopsies were obtained by inserting a 20G needle into the LN, rotating several times, removing the material, placing upon a slide, and smearing as for blood. All preparations were stained with Giemsa as detailed in the general Materials and Methods.

Table 5.1 Immunisations and lymph node removals

GROUP	Animal Number	Immunised	Days post infection LN excised
1	12205	Percoll control	4
	12183	TaA	4
	12170	TaA	8
2	13050	Control	----------
	13058	TaG	2
	13065	TaG	4
3	12245	TaG	10
	12273	TaG	10

TaA - *T.annulata* Ankara strain

TaG - *T.annulata* Gharb strain

Cryostat sections

The method followed was broadly similar to that of Barclay (1981) and Howard *et al* (1988). Lymph nodes were cut into approximately $1cm^2$ pieces and snap frozen in liquid N_2. The samples were then embedded in OCT mounting medium (BDH) and stored at -80°C. Sections were cut at 6μm upon a "Brights" cryostat at -20°C, collected on gelatin coated slides (Fisons, 1% w/v aqueous soln), allowed to air dry, and stored desiccated at -20°C. Before use, slides were fixed in ice cold acetone (BDH) for 10 minutes, followed by three washes in PBS.

Staining of Cryostat sections

Frozen sections were stained in this preliminary study with a limited number of mAbs detailed in Table 5.2.- mAbs J11, IL-A12, and CC63 are described in detail in the Materials and Methods of chapter 3. VPM30 stains all sIg^+ peripheral blood B cells (Naessens and Howard, 1991), although the antigen recognised is unknown. Results in this thesis partly address the specificity of this antibody in lymph nodes. mAbs IL-A24 and IL-A109 (Ellis *et al*, 1988; MacHugh *et al*, 1990) recognise macrophages and monocytes respectively. These were included as cells bearing both these markers are efficiently infected by *T.annulata* sporozoites *in vitro* (Spooner *et al*, 1989; Campbell *et al*, 1994). mAb staining of sections was visualised using fluorescent secondary antibodies detailed in table 5.3. mAbs and secondary conjugates were diluted in PBS pH7.2.

Sections were first incubated with 5% goat serum diluted in PBS for 30 minutes and then washed in 3 changes of PBS. MAbs were used as optimal dilutions of ascitic fluid (typically 1:50 - 1:150) in PBS; tissue culture supernatants were used neat. 10-40 μl of diluted mAb, depending on section size, was incubated for one hour at 4°C and then washed in PBS as above. Double staining of sections was carried out by adding appropriate amounts of both mAbs at twice normal concentration. Secondary conjugates were used diluted 1:100 (1:50 for double staining), and 10-40 μl were incubated on the sections for one hour at 4°C, and then washed with PBS. Sections were then air dried and mounted in UVinert aqueous mounting medium (BDH) before examination. Sections could be stored at 4°C for one week without significant loss of fluorescence.

Table 5.2 mAb used for staining frozen and paraffin lymph node sections

mAb (Isotype)	Specificity	Reference
J11 (IgG_1)	MHC Class II	Dutia *et al*, 1995
IL-A12 (IgG_{2a})	CD4	Baldwin *et al*, 1986
CC63 (IgG_{2a})	CD8	Howard *et al*, 1991
VPM 30 (IgM)	B Cells	Naessens and Howard, 1991
IL-A24 (IgG_1)	Macrophages	Ellis *et al*, 1988
IL-A109 (IgM)	Monocytes	MacHugh *et al*, 1990
IL-A111 (IgG_1)	IL-2R	Naessens *et al*, 1992
MIB 1 (IgG_1)	Ki 67 proliferation antigen	Key *et al*, 1993
1C7 (IgG)	*T.annulata* Schizonts	Shiels *et al*, 1986
Polyclonal Rabbit-anti Human CD3	Human CD3	Dako Ltd. Use in cattle - Ramos-Vara *et al*, 1994

Table 5.3 Secondary immunoconjugates for staining frozen sections.

Immunoconjugate	Supplier	Working Dilution
Goat anti-mouse (GAM) IgM-FITC	Sigma	1:100
GAM IgG_1-FITC	Seralab	1:50
GAM IgG_{2a}-PE	Seralab	1:100

Thin Paraffin sections of Lymph nodes

Paraffin embedding of lymph nodes

For both conventional and immunohistology, lymph nodes were embedded in paraffin wax. Nodes were first cut into approximately $1cm^2$ pieces before fixing in 10% paraformaldehyde (Sigma) in PBS pH7.6 for at least 24 hrs. Nodes were then dehydrated, embedded and blocked in 57^0C melting point paraffin wax, using a Shandon VIP automatic tissue embedder.

2.5-3μm sections were cut from finished blocks upon a Leica microtome. The sections were floated in a 50°C waterbath and collected upon Poly-l-lysine (Sigma) coated slides. Sections were then baked at 56°C for at least 24 hours.

Staining of slides for conventional histology

Lymph node sections were stained using either Haematoxylin and Eosin (H+E) or Toluidine blue for conventional histology. Sections were first dewaxed in 2 changes of xylene, before rehydration through graded alcohols - 100%/95%/70%/50% EtOH/dH_2O v/v. Sections were washed in running tap water before staining in Haematoxylin for 4 minutes. (Harris' Haematoxylin, BDH). After washing in running tap water, sections were placed in "Scott's Tap Water" (Saturated Lithium carbonate soln.) for approximately 1 minute to stain nucleii blue. The slides were then stained in Eosin solution for 30 seconds, washed in water, dehydrated through graded alcohols, cleared in xylene and mounted in DPX mounting medium (BDH). For toluidine blue staining, sections were taken to water, stained in toluidine blue soln. for 4 seconds, washed in water, dehydrated and mounted as above. H+E stains nuclear material dark blue-black with pink cytoplasm. Toluidine blue stains nucleii blue with clear cytoplasm.

Immunohistology

mAb

For immunohistology, optimal concentrations of the mAbs detailed in table 5.2 were determined and used in the range neat-1:10 dilutions of culture supernate or as 1:50-1:100 dilutions of ascites. All but the last 3 mAb in the table have been described elsewhere in the text. MIB 1 recognises the Ki-67 proliferation antigen, which is

expressed in all mammalian cells outwith G_0 stage of the cell cycle (Gerdes *et al*, 1984). MIB 1 is a new mAb recognising this antigen and has been of particular use in paraffin sections (Key *et al*, 1993). Recent studies have shown the mAb to react with *in vitro* cultured bovine cells (Shayan *et al*, 1994). 1C7 recognises *T.annulata* schizonts (Shiels *et al*, 1986) and has been used in the examination of *T.annulata* infected lymph nodes post mortem (Eisler, 1988). Polyclonal rabbit anti-human CD3 has been previously identified as reacting with bovine tissues (Ramos-Vara *et al*, 1994).

mAbs IL-A111 and J11 required the sections to be treated with trypsin digestion solution for 20 minutes at 37°C before staining. Anti-human CD3 required treatment with Pronase digestion solution for 20 minutes at room temperature. MIB 1 required microwave treatment as detailed by Key *et al* (1993). Sections were placed in excess citrate buffer, microwaved on full power for 3x5 minutes and then left to cool for 20 minutes before washing in running tap water.

Staining

MAb staining of formalin fixed sections was performed using standard immunohistochemical techniques. The buffer used throughout was Tris-HCl buffered saline (TBS), as the phosphate in PBS can inhibit alkaline phosphatase. Sections were dewaxed in xylene and rehydrated through alcohols as for conventional histology, washed in running tap water and then in TBS for 5 minutes. If DAB was to be the substrate, endogenous peroxidase activity was blocked by taking sections to 95% ETOH, incubating in 100ml methanol containing 3ml 100 vol hydrogen peroxide for 10 minutes and washing in running tap water. Sections were then placed in a humidified box and blocked in 20% normal rabbit serum (NRS) (SAPU) in TBS for 10 minutes. Excess NRS was then wiped from around each section, and the mAb at the appropriate dilution in 20% NRS added, ensuring that the section was completely covered. The slides were then incubated for 30 minutes. Excess liquid was then tipped off, the sections rinsed briefly in running tap water and washed twice in TBS. The secondary antibody - biotinylated rabbit anti mouse (RAM) Ig (Dako, Glostrup, Denmark) - was diluted 1:400 in 20% NRS and added as above. The sections were incubated for 30 minutes then washed as above. For staining with anti-human CD3,

the normal serum used was goat, and antibody was detected using biotinylated goat anti-rabbit Ig. All dilutions were identical to those for mouse primary antibodies. Staining was demonstrated using the ABC system (Dako) - the avidin/biotinylated enzyme complex was prepared as per the manufacturer's instructions. One drop each of avidin and biotinylated enzyme were added to 5 ml of buffer (50mM Tris-HCl) at least 30 minutes before use. ABComplex was added to the slides, incubated for 30 minutes, then washed as above. Either diaminobenzidine (DAB) or Vector red (Vector labs, Peterborough, U.K.) were used as substrates. DAB (Sigma) was used with ABC-HRP (Horseradish peroxidase), and Vector red (Vector Labs) with ABC-AP (Alkaline phosphatase). DAB was prepared freshly before each use. Vector red has the advantage of being visible by both conventional and ultraviolet/confocal microscopy. This was freshly prepared as per the manufacturer's instructions, with the addition of 1.25mM levamisole (Vector labs) to the substrate. Sections were incubated with the substrate for 20 minutes in the dark and then washed in running tap water. Sections were counterstained in haematoxylin and dehydrated, cleared and mounted as for conventional histology.

The various treatments required by the antibodies meant that double staining was not possible to perform, with the destruction of one or other of the antigens a common problem. However, CD3 and MIB 1 double staining was carried out by first staining with CD3/anti rabbit Ig/vector red followed by MIB 1/anti mouse Ig/DAB. The extremely bright CD3 staining was strong enough to survive microwaving.

Normal/Ultraviolet Microscopy

Sections were examined using a Leitz Ortholux II microscope equipped with both normal and UV light sources, FITC and TRITC filters, and a Wild photoautomat automatic exposure photographic unit. Sections were photographed using 64T ASA film (Kodak) for normal light, and 1600 ASA film for fluorescence photography.

Confocal microscopy

Confocal microscopy was carried out using the Zeiss LSM 10 system in the Dept. of Pathology. Fluorescence was detected using an argon laser wavelength 488nm, and

a TRITC filter. Sections were photographed using Pan F 50 ASA.

DNA Analysis of Paraffin Sections

In order to determine the kinetics of the cell populations within draining lymph nodes, DNA analysis was carried out with formalin fixed, paraffin embedded material by flow cytometry, broadly following the methods of Hedley *et al*, (1983) and Hedley (1989). Cell cycle kinetics can be measured by the binding of DNA specific fluorescent dyes to nuclear DNA. As the amount of dye bound is proportional to the amount DNA, cells in various stages of the cell cycle can be identified by their relative fluorescence.

Isolation of nucleii

Thick (50μm) sections were cut from identical tissue blocks to those used for examination of draining lymph node pathology, floated onto glass slides, and dried for 1 hour at 80°C. Sections were then rehydrated through the following reagents (10 minutes per stage): 2xXylene; 100% EtOH; 99% EtOH; 95% EtOH; 2x dH_2O. The rehydrated sections were then scraped using a clean pasteur pipette into 4ml tubes (Falcon) containing pepsin digestion solution. The tubes were incubated for 1hr at 37°C, vortexing every 10 minutes. The cells were washed twice by adding 1ml PBS and centrifuging at 1800 rpm for 10 minutes, subsequently resuspending in 1ml PBS. The cell suspension was then filtered through cotton wool as follows. A small amount of clean cotton wool was placed within the barrel of a 10ml syringe, and the plunger replaced within the barrel, flattening the cotton wool. The plunger was again removed, and the cell suspension pressed through the syringe. A fresh filter was prepared for each sample.

Propridium iodide staining

Cells were stained by adding 1ml of propridium iodide (PI) solution and incubating for 1 hour at room temperature in the dark, vortexing every 10 minutes. The samples were washed in PBS as before, finally resuspending in PBS. Stained samples could be stored for up to 1 week at 4°C.

Flow cytometry

Flow cytometry for DNA staining was carried out essentially as described for whole

cells, using an EPICS C flow cytometer (Coulter instruments). This flow cytometer, although it has a cell sorting capability, was used for DNA analysis with the laser at 488nm in an identical manner to the FACScan described in the materials and methods. PI fluorescence was detected using a filter at 570nm, equivalent to the FL2 fluorescence on the FACScan. PI stained nucleii produced a typical "DNA histogram", with fluorescence intensity plotted on the x-axis and nuclear number on the y-axis. The large peak of nucleii near the axis origin corresponded to diploid nucleii in the G_0 or G_1 stages of the cell cycle. Mitotic nucleii with double the amount of DNA stain appeared as a small peak with double the x-axis values of the diploid nucleii, with S-phase nucleii occuping the area between the 2 peaks (fig 5.1).

Data Analysis

Cytometer data was analysed in 2 ways - 1. cell cycle analysis and 2. estimation of apoptotic nucleii within the tissue.

1. For cell cycle analysis, DNA fluorescence was measured using a linear scale (rather then the log scale for mAb stained cells), as the amount of PI fluorescence binding to DNA is lower than that of a surface antibody. There are a number of methods for determining the numbers of cells in G_0/G_1, S, and G_2 phases of the cell cycle (reviewed by Baisch *et al*, (1982)). The majority rely upon mathematical models to estimate the areas of the various peaks. Although the assumptions that must be made using these models introduce a degree of error, useful comparisons can be drawn from identically processed samples analysed with the same model. In this case the numbers of nucleii in each peak of the DNA histograms were determined using the method of Baisch *et al* (1975). Essentially, this model identifies the G_0/G_1 and G_2 peaks as normal distributions, and subsequently fits a rectangle between the two peaks to estimate the S phase area. In practice, the model is fitted to the histogram by the computer, first identifying the median values of the G_0/G_1 and G_2 peaks as the centre of the normal distributions (Fig. 5.1) and subsequently adding the S-phase rectangle (Fig 5.2).

2. As nuclear material condenses during apoptosis, its ability to bind PI is diminished. As a result, these nucleii appear as a "sub G_0/G_1 " or "hypodiploid" peak on a DNA

histogram (reviewed by Sherwood and Shimke, 1995). Nucleii were first gated using a logarithmic scale for apoptosis (Fig 5.3). Apoptotic nucleii have less fluorescence but are of a similar size and thus appear "under" the main population of cells when forward scatter (FSC - size) is plotted on the x axis and Fluorescence on the y axis. Debris has a smaller size and therefore appears to the left of the main cell population. The use of this technique is better suited to fresh tissue, which was unfortunately not available, as sheared nucleii from sectioning may lead to a higher than expected "background" stain, although identically treated samples can still yield useful results. Measurement of the hypodiploid peaks was carried out using DNA histograms as obtained using method 1. The hypodiploid region was selected using the samples from normal nodes (Fig.5.3) and remained unchanged throughout all the samples analysed. Apoptosis was assessed in sections from all time points on 2 different occasions ("batch 1" and "batch 2" in the results section).

Analysis of T cell responses early in infection

Although T cells could be identified in paraffin sections on the basis of CD3 and IL2R expression, their exact phenotype could not be determined. As results from group 1 suggested that T cells became activated early in infection, extended analysis of T cell activation was carried out upon group 3 lymph node cells. Cells were isolated from the fresh tissue pre fixation for FACS and cytokine mRNA analysis.

Isolation of lymph node cells

Freshly excised pieces of lymph node were cut into approximately 1cm^2 blocks and placed within a sterile 30ml tube containing 10 ml PBS supplemented with 1% antibiotics (10000 IU/ml penicillin/10 mg/ml streptomycin) (PBS/antibiotics = PBSA). Nodes were washed twice in PBSA to remove surface blood and then placed in a sterile petri dish containing 10ml PBSA. The tissue was then cut into several smaller pieces using a sterile scalpel, and each piece removed to a separate petri dish containing PBSA. Each piece was minced finely using crossed sterile scalpels before further disaggregation of tissue fragments in an autoclaved tissue homogeniser

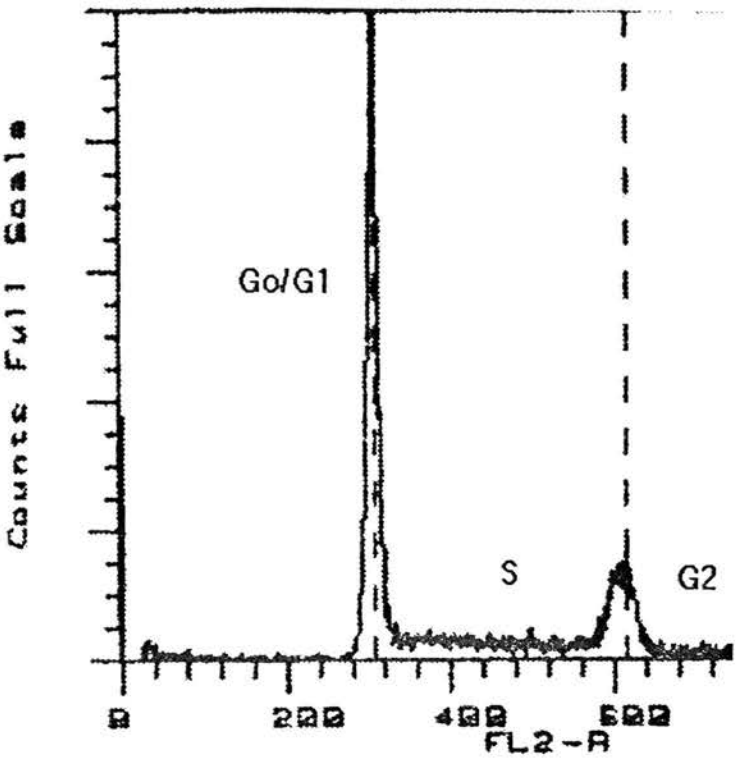


Fig. 5.1 Sample DNA histogram, with G_0/G_1, S, and G_2 peaks marked. Dotted lines represent computer determined median G_0/G_1 and G_2 values.

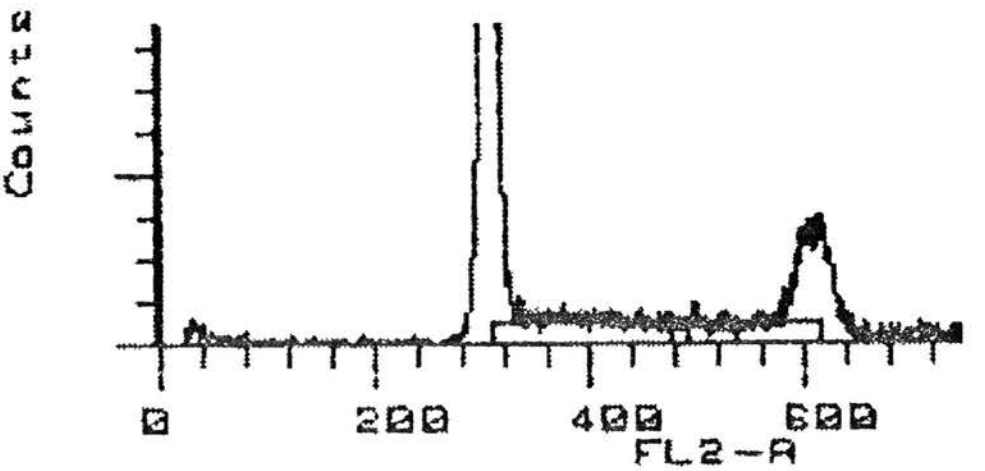


Fig. 5.2 Identical sample to fig. 5.1 enlarged to show rectangle fitted under S-phase

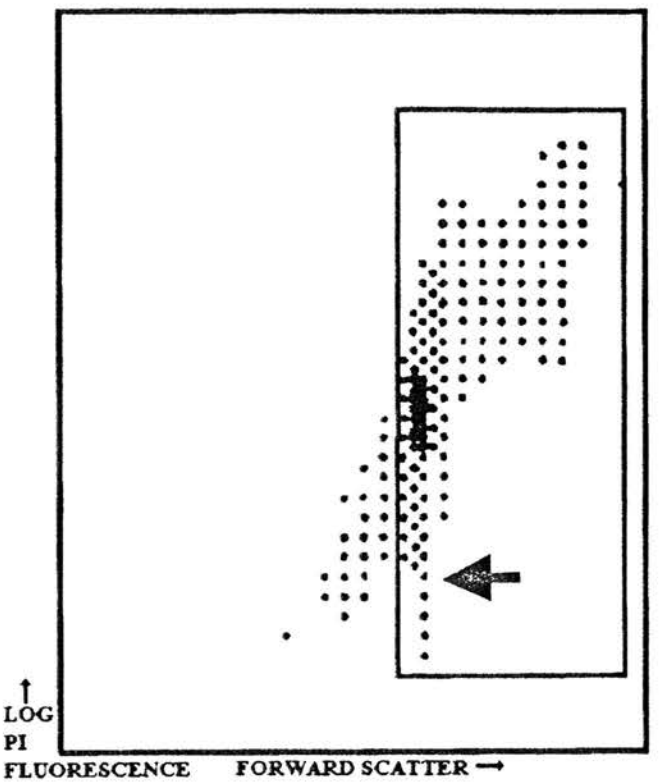


Fig. 5.3 Schematic representation of nucleii gating for apoptosis analysis (above). Apoptotic nucleii (arrow) have identical size to normal nucleii but are less fluorescent. Smaller debris (less forward scatter) is excluded. DNA histogram from normal lymph node sample (below) showing pre-G_0/G_1 (arrows) area corresponding to hypodiploid nucleii

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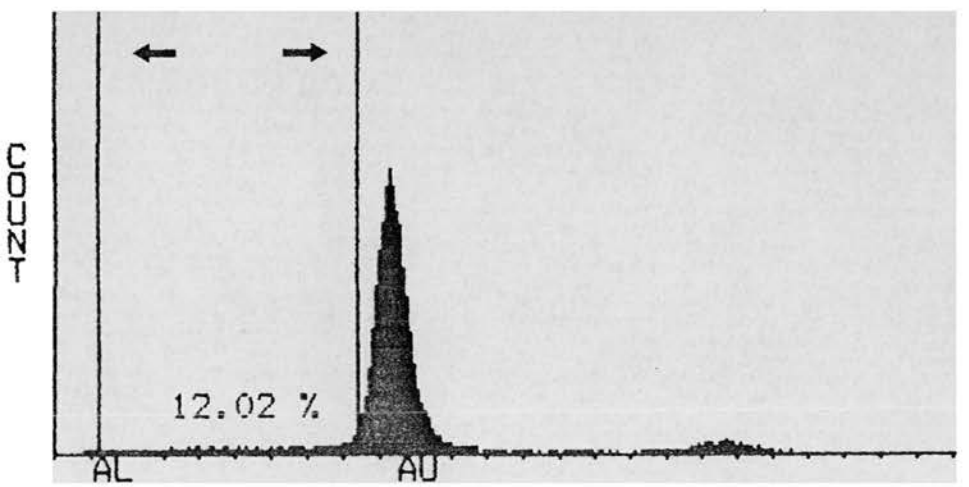


(Jencons Scientific). The cells resulting from each tissue fragment were pooled and washed 3 times in PBS, before FACS staining or cryopreservation in DMSO for future mRNA analysis.

FACS analysis

Lymph node cells (LNC) were stained for FACS analysis as described in the materials and methods. T cell surface markers were stained as described in the materials and methods of chapter 3. Briefly, $CD4^+$, $CD8^+$, and γδ T cells (mAbs IL-A12, SBU-T8, and CC15 respectively) were assessed for their expression of IL-2 receptor and MHC class II (mAbs IL-A111 and J11 respectively).

RT-PCR analysis of cytokine mRNA

The expression of T cell cytokine and cytokine receptor mRNA - IL-2, IL-2 receptor (IL-2R), IL-4 and IFNγ - by LNC was assessed as described in the materials and methods of chapter 4. Briefly, total RNA was isolated using RNAzol B (Biogenesis), followed by chloroform extraction and precipitation in isopropanol. The resulting RNA was washed twice in 75% ethanol followed by drying under vacuum and re-dissolving in sterile water. Five μg of total RNA was reverse transcribed into cDNA using the superscript system (Life Technologies). Four μg of cDNA was then used for 30 cycles of PCR amplification using primers specific for the cytokine mRNAs. Products were analysed by gel electrophoresis, using TBE as the running buffer in 2% Agarose gels.

Culture of LN cells

LN cells were also cultured, primarily in an attempt to isolate infected cells and phenotype them. LN cells were cultured at 10^6 cells per ml in 6 well tissue culture plates using MLC medium.

RESULTS

1. Preliminary studies - Cryostat Sections of Normal and Heavily Infected Draining Nodes. (Fig 5.4-5.6)

Although this part of the study was limited to a normal and a heavily infected node (see introduction), it yielded valuable information about gross changes in immune cell subsets following sporozoite infection. Results obtained here concentrate on immunohistology, as the morphology of frozen sections is inferior to wax, and pathological changes are described in detail in section 2.

Histological changes

Normal bovine lymph nodes (LN) are similar to most mammalian LN (except in size), and can be divided into a cortex, paracortex, and medulla. Conventionally stained sections (Toluidine blue) revealed large differences between normal and infected LN.

In the normal node, the cortex was visible as a homogeneous area of tightly packed small lymphocytes. However, it was not possible to determine germinal centres clearly in the frozen sections. The paracortex was much more sparsely populated, primarily with small lymphocytes, while the medulla was largely empty, characterised by empty sinuses. The main cell type present in the medulla was a larger macrophage-like cell.

In the infected node, small lymphocytes were largely absent, with areas of the node completely devoid of any cells. The predominant population consisted of macrophage-like cells similar to those seen in the medulla of the normal node. In the infected node these cells had disseminated throughout the tissue, encroaching into the severely depleted lymphoid regions.

Immunohistology

Normal LN

The phenotypes of cells within areas defined by conventional histology could be determined using fluorescence microscopy. Unfortunately, mAb CC63 (CD8) and IL-

A24 (Macrophages) did not stain cells in frozen sections. The B cell content of the cortical area was confirmed, as mAb VPM 30 stained the vast majority of cells. The positive areas had very distinct boundaries and contained uniformly stained lymphocyte-like cells (Fig 5.4). The VPM30$^+$ cells stained weakly for MHC class II throughout the cortex, with occasional foci of strongly MHC class II$^+$ cells. They may correspond to germinal centre APC, but section morphology did not allow these areas to be further investigated. VPM 30 also stained occasional cells in the paracortex with lymphocytic morphology. IL-A12 (anti-CD4) stained large areas of lymphocyte-like cells, but positive areas were much less densely packed than VPM 30 positive sites (Fig. 5.5) Double staining (VPM 30-FITC and IL-A12-PE) showed that the CD4$^+$ zones were almost invariably directly adjacent to the B cell areas (Fig. 5.4) corresponding to the paracortex. In addition, the isolated VPM 30$^+$ cells appeared mainly in the CD4$^+$ zones. The large cells in the medulla/paracortex stained strongly with mAb IL-A109 which is thought to recognise FcγR1 (McHugh *et al*, 1990), and this mAb has been shown to particularly recognise cells of monocyte origin (Campbell *et al*, 1994). These cells also stained strongly with anti MHC class II mAb.

Infected LN

Within the heavily infected node, both B and T cell populations were severely depleted. VPM 30 staining was greatly reduced in comparison to normal nodes, confined to small "strips" of positive cells adjacent to the capsule. There was also very little evidence of CD4$^+$ cells, with a few weak positives in the paracortical area. The most striking feature of the infected node was the expansion of the MHC class II$^+$/IL-A109$^+$ cells to fill the majority of the tissue (Fig. 5.6), encroaching into the depleted B cell areas. As the animal concerned had received drug treatment, it was unfortunately not possible to assess the parasite content of these cells.

2. Draining lymph node pathology during the course of sporozoite infection.

Clinical Changes

Control, percoll tick control, and day 2 and 4 infected animals did not show any

elevation of temperature, enlargement of LN, or any parasite in LN or blood smear. The day 8 infected animal had a temperature raised over 39.5°C (fever), a slightly enlarged draining LN containing < 0.1% schizonts, but no piroplasms. The clinical status of animals in group 3 at day 10 post infection is contained in the following table.

Animal No.	Temperature	LN Schizonts	Piroplasms
12245	41.1°C	27%	10%
12273	41.3°C	53%	12%

Schizont infected cells within an impression smear from 12245 are illustrated in Fig 5.7. Both macroschizont and microschizont forms of the parasite were present. Draining lymph nodes were grossly enlarged and haemorrhagic, LN from 12245 and 12273 had grown to 15 cm long, over 4 times the size of LN removed from 13050 and 122205.

The onset of fever and appearance of different parasite stages within the animals were in agreement with previous studies of experimentally induced *T.annulata* infection (Samantery *et al*, 1980; Preston *et al* , 1992a).

Histology

Control lymph nodes

Lymph node sections from animals 12205 (Uninfected tick control) and 13050 (Normal LN) appeared identical. Small lymphocytes predominated in the cortex, but areas of these cells could also be found deeper in the tissue, often closely associated with the nodes' very prominent trabeculae (Fig. 5.8). The area in which these cells were situated, peculiar to bovine lymph nodes, has been referred to as the "cortico-medullary transitional area". This is distinct from the paracortex, as the main cell type is the small sIg^+ B cell (Morrison *et al*, 1986a). Germinal centres (GC) were seen both in the cortico-medullary zones and the cortex (Fig. 5.8). GC were surrounded by small follicular lymphocytes, and contained large cells with prominent nucleoli and

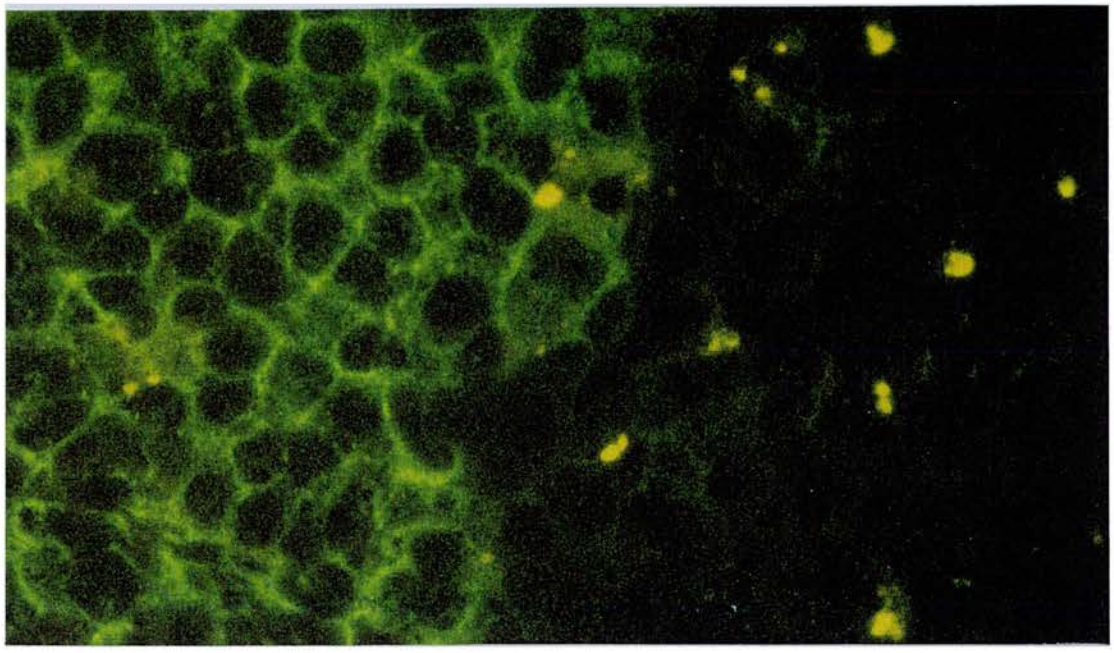


Fig. 5.4 Frozen section, normal lymph node, double stained with VPM 30 and IL-A12 showing a distinct boundary between B cell and T cell zones. VPM 30 (FITC) is stained green, IL-A12 stain (PE) can be seen faintly outwith the B cell zone. x625, U.V. light, FITC filter.

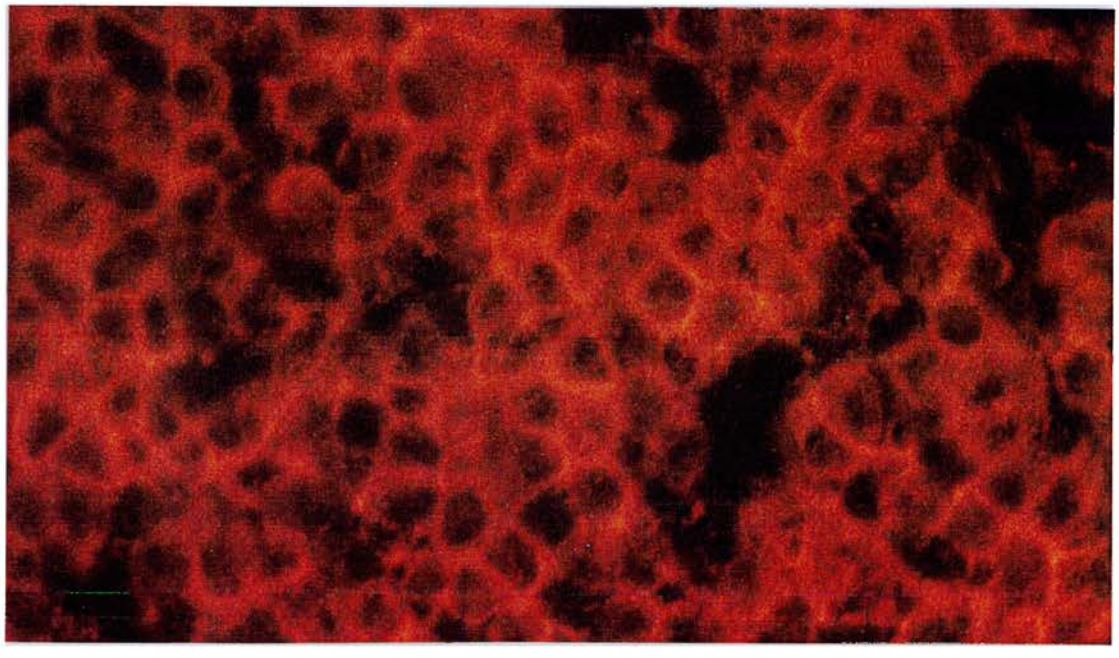


Fig. 5.5 Frozen section, normal node. Paracortex stained with IL-A12 (anti-CD4), T cell zone is more heterogeneous -less tightly packed - than B cell follicles.x625, U.V., TRITC filter

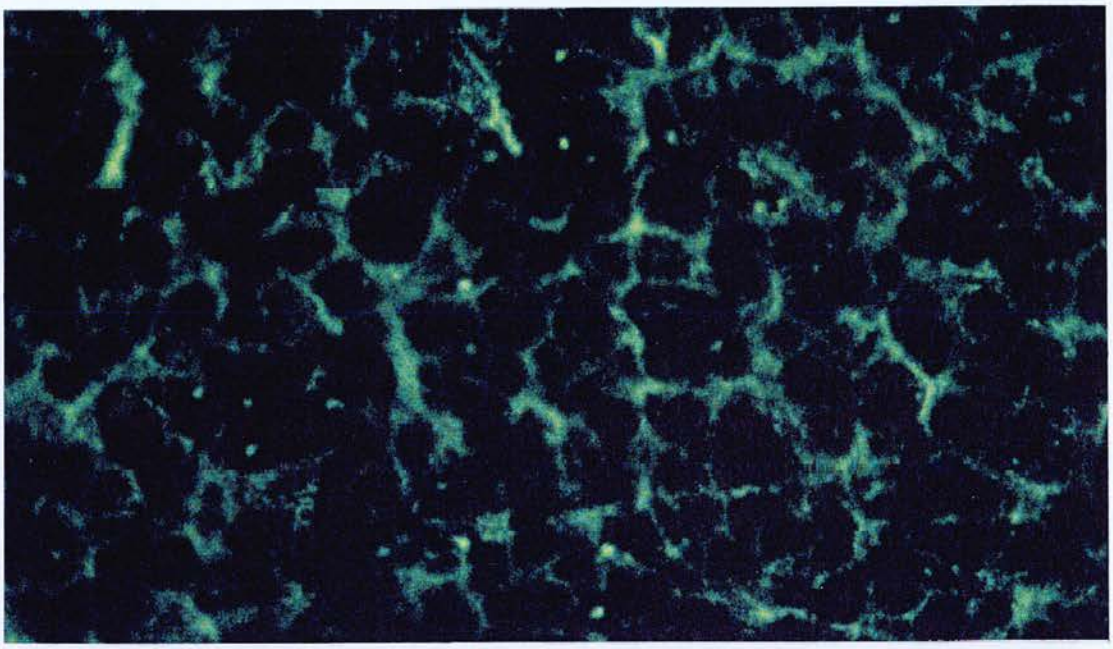


Fig. 5.6 IL-A109 (anti-monocyte) stains the majority of the cells within a heavily infected lymph node. Note large range of sizes and morphology of stained cells. x625, U.V., FITC filter

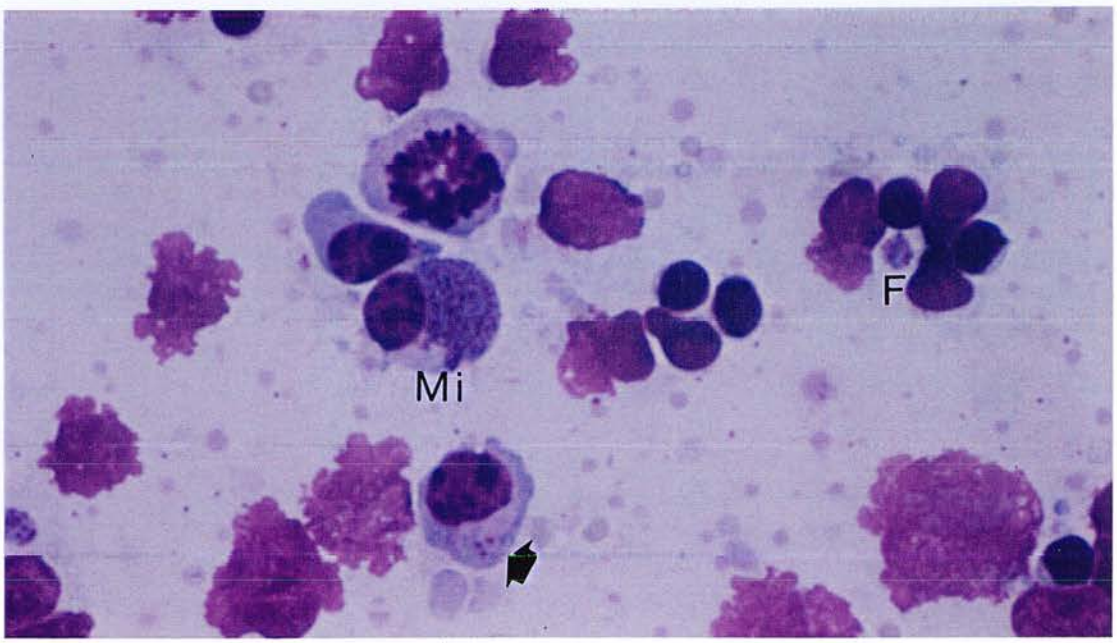


Fig. 5.7 Impression smear from DLN 10 days post infection. Note macroschizont infected cells (arrow), microschizont infected cell (Mi) and free schizont (F).

small lymphocytes, some displaying "condensed" nucleii. In addition, large cells were present which appeared to have endocytosed the condensed nuclear material. These "tingible body macrophages" (TBM) are thought to remove the chromatin of B cells which have died after completing S-phase (Hardie *et al*, 1993). The mantle zone around large GC was well defined around the base and less well defined towards the "apex". See Fig. 5.23 -5.25 for detailed GC anatomy.

The paracortex and medulla of normal lymph nodes contained a diffuse mixture of small lymphocytes and larger non lymphocytic cells. The medullary sinuses of the nodes were largely empty, sparsely populated with small lymphocytes (Fig.5.9).

Infected nodes

Throughout the course of infection, draining lymph nodes became progressively more full of cells, with the open "meshy" appearance of a normal node being replaced by tightly packed cells. The normally prominent trabeculae and sinus architecture were virtually obscured by the mass of cells. By day 10 the tissue was virtually unrecognisable, with evidence of haemorrhage throughout the node.

2 days post infection, draining lymph nodes appeared essentially normal. Germinal centres were present, surrounded by small lymphocytes, and medullary sinuses were empty. The only difference from control nodes was the appearance of small foci of large blast-like cells, visible within the medulla. These cells were 2-3 times bigger than small lymphocytes with abundant cytoplasm and a large granular nucleus.

At day 4, alterations in node composition had become more apparent. The foci of large blast cells had grown considerably larger by day 4, encroaching into the small lymphocytes of the paracortex for the first time (Fig.5.10). The node had become noticeably more cellular and tightly packed with a marked increase in the cell content of the medullary sinuses which were partially filled with small lymphocytes. Germinal centre number and morphology again appeared no different to normal nodes.

The large blast cells predominated from the medulla to the capsule of the node by day 8. Outside the medullary sinuses, which were packed with small lymphocytes, the situation was essentially the converse of day 4 with the blast cells a major cellular component (Fig. 5.11 + 5.12). Within the medulla, the vast majority of cells within

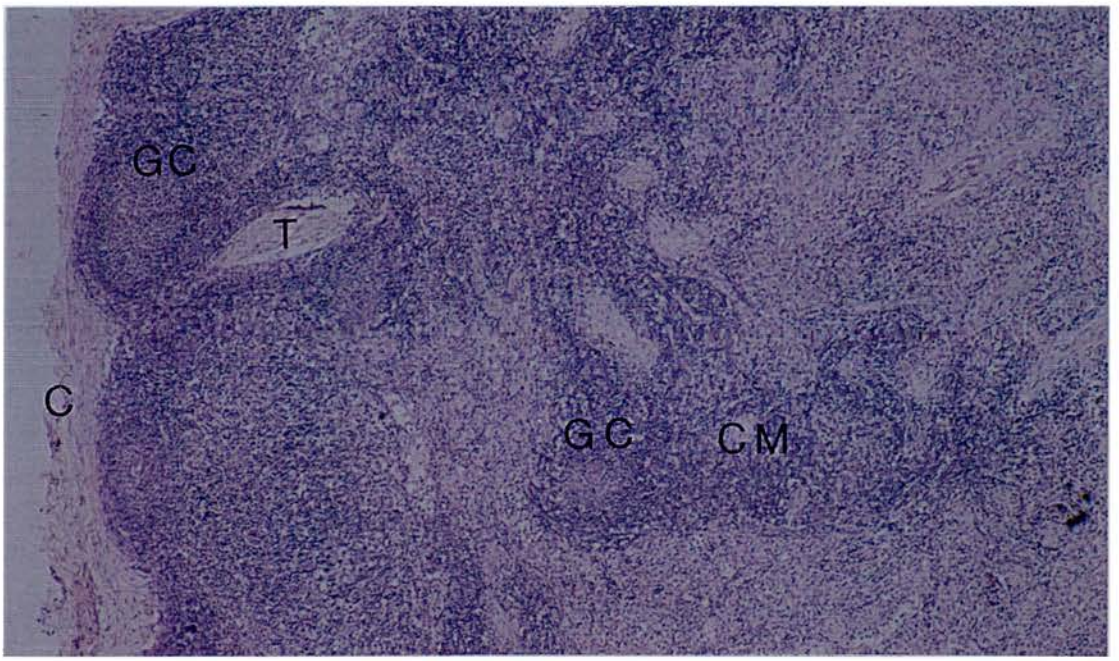


Fig. 5.8 Low power view of a normal bovine lymph node. T - trabeculum. The majority of small lymphocytes are confined near the capsule (C), except in the cortico-medullary (CM) transitional area. Note germinal centres (GC) in both this area and in the cortex.H+E, x50

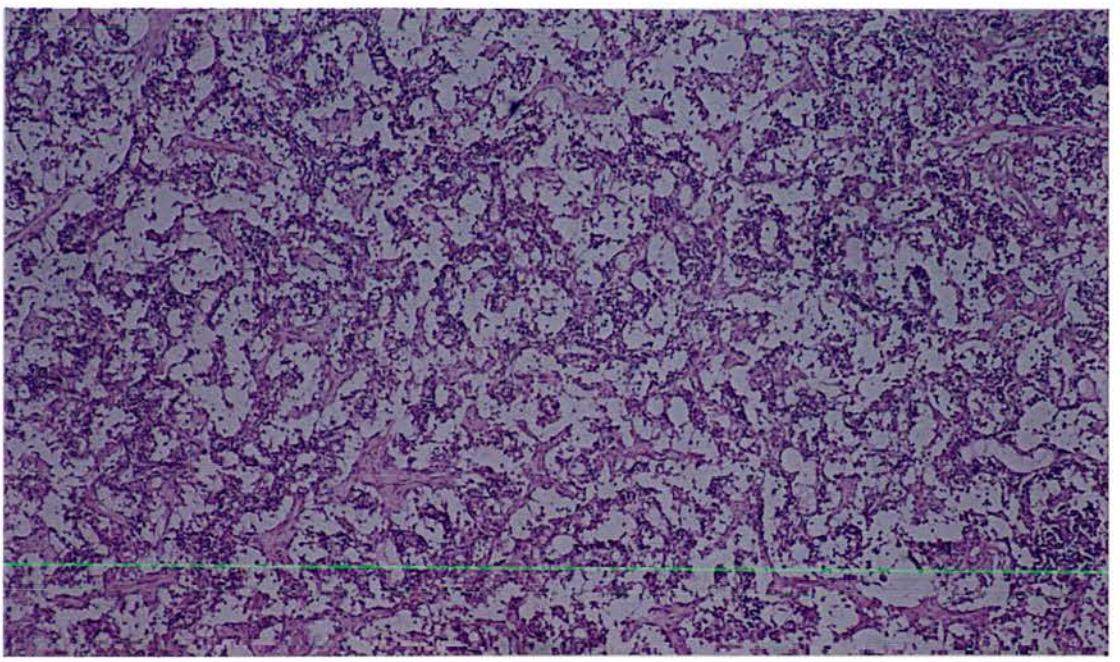


Fig. 5.9 Empty medullary sinuses within a normal lymph node, with scattered small lymphocytes. H+E, x125

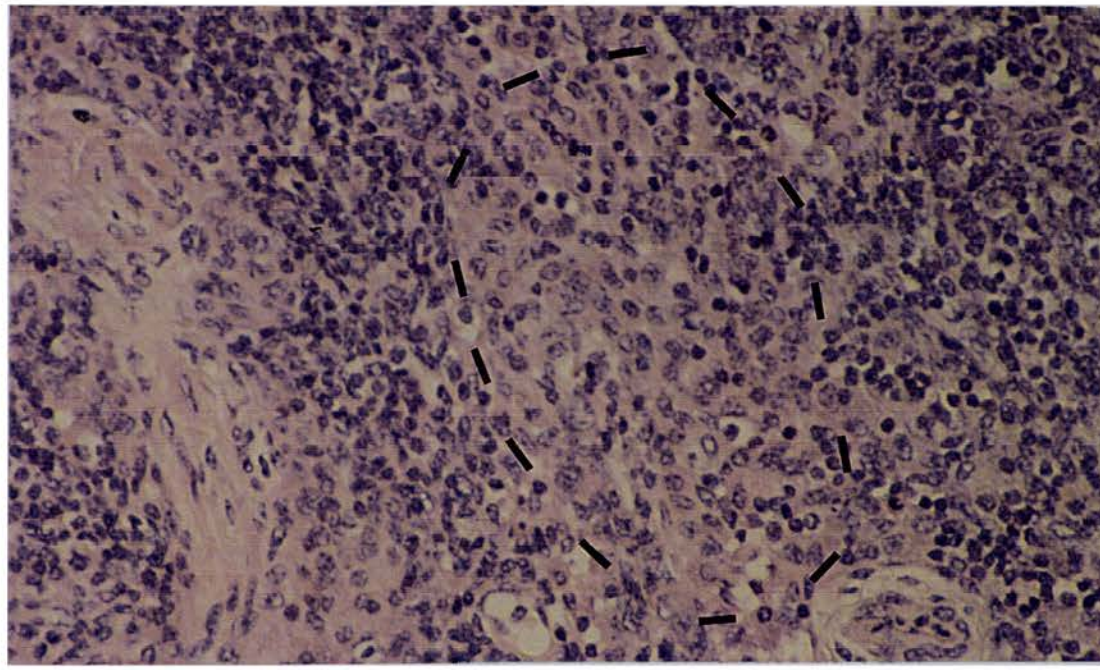


Fig. 5.10 Focus of blast cells (within dotted line) in the medulla of a draining lymph node 4 days post infection. H+E, x400

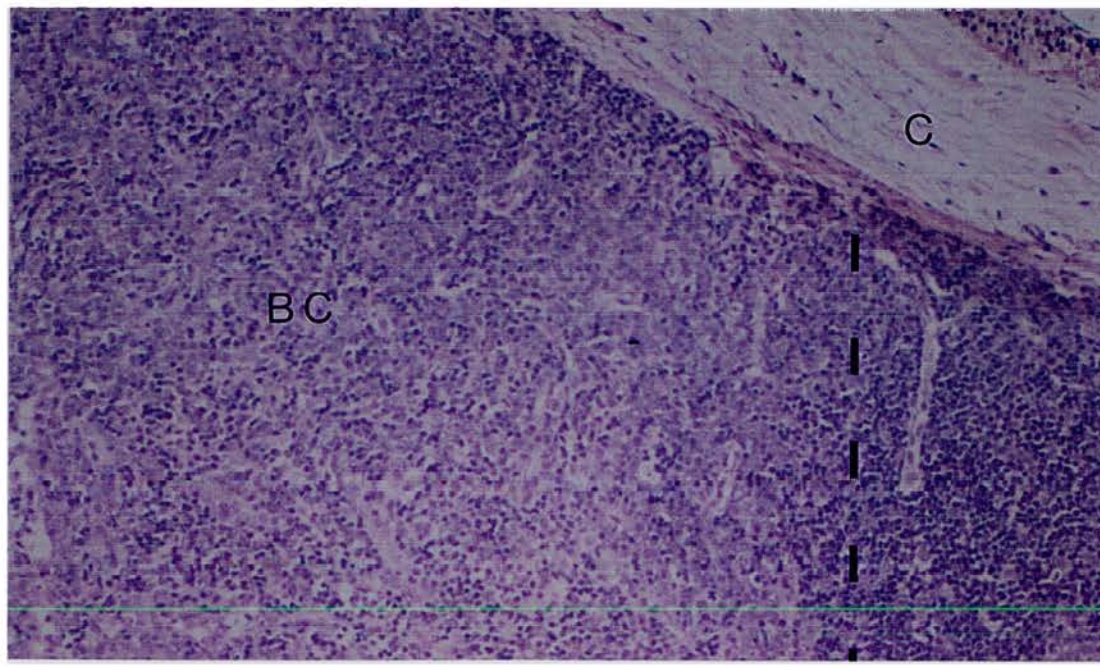


Fig. 5.11 Blasting cells (BC, light staining cytoplasm to left of dotted line) encroaching into the B cell follicular area (small dark lymphocytes) next to the capsule (C). 8 days post infection. H+E. x125.

the sinuses were small lymphocytes, with blast cells numerous outwith the sinuses (see Fig. 5.19 + 5.20). Within the areas of blasting cells, cells with condensed nuclear material were visible (Fig. 5.13). A change was also seen within GC which appeared to be much smaller, both in number and size. Mantle zones appeared intact and proliferating cells were still present within the GC, as were TBM. However, there were no large "polarised" hyperplastic germinal centres present.

At day 10, lymph node architecture was virtually destroyed, with blasting cells as described for day 8 present throughout. Areas of the nodes appeared to be "dead" with only faint blue cytoplasmic stain (see Fig. 5.14). Blasting cells were also interspersed with small patches of lymphocytes, although small lymphocytes were still the main cell type observed in sinuses. Large blasting cells also appeared in the sinuses for the first time. Germinal centre morphology was completely destroyed, no GC could be identified in conventionally stained sections.

Sporozoite infection therefore has a profound effect upon cell subsets and architecture within the draining node. A new population of cells is induced to proliferate and become the dominant cell type within the node. Lymphocyte populations are severely affected with the breakdown of germinal centres and large scale depletion of cortical/paracortical cells.

Immunohistology

mAbs IL-A12 (CD4), CC63 (CD8), IL-A24 and IL-A109 (macrophages and monocytes) did not stain paraffin sections. However, several of the mAb used were extremely effective in identifying cells within the tissue, and a large amount of information was gathered.

Parasite infected cells

Anti-parasite mAb (1C7) did not detect any schizonts within cells at days 2 and 4, and only 2-3 per section by day 8 (corresponding well with the small numbers of schizonts found in impression smears from this time point). Large numbers of cells were stained at day 10, with parasite clearly visible within the cytoplasm (Fig. 5.14). Parasitised cells were MHC class II^+, consisted wholly of the large blast cells, were

MIB 1^+, and were not found in any of the residual lymphocyte areas.

Blast cells

In normal LN, MIB 1^+ cells were largely confined to germinal centres with discrete positive cells scattered throughout the rest of the tissues. MIB 1 stained very strongly in the small foci of blast cells at day 2 (Fig. 5.15). The numbers of MIB 1^+ cells increased steadily throughout the infection with MIB 1 staining residing principally in the large blast cells which grew to dominate the infected nodes. Large blast cells were also MHC class II^+.

It was extremely difficult to determine the phenotype of the blasting cells early in infection. However, when large numbers of cells had developed at day 8, the MIB-1^+ cells could clearly be distinguished as $CD3^-$ (Fig. 5.16). It seems likely that these large non T, non B, MHC class II^+/largely MIB 1^+ cells correspond to the IL-$A109^+$ monocytes found throughout the heavily infected node from the preliminary study.

Lymphocyte interactions with blasting cells

In normal LN, anti CD3 stain revealed that the majority of small lymphocytes were T cells. B cell zones could be identified as non CD3 areas, primarily near the capsule of normal nodes (Fig. 5.17). These were also visible in the cortico-medullary zones surrounding GC deeper in the tissues. Although mAb VPM30 recognises B cells in peripheral blood (Naessens and Howard, 1991), it only stained germinal centres and discrete large cells within the cortex of paraffin sections, with the small mantle zone B cells remaining unstained (see germinal centre section).

Following sporozoite infection, both T and B cells appeared to be steadily displaced by MIB 1^+ blasting cells throughout the course of infection. $CD3^+$ cells surrounded the foci of blasting cells at days 2 and 4. As noted above, MIB 1^+ cells were $CD3^-$, and at day 8 areas of T cells had become isolated by the interstitial blasting cells (Fig. 5.18). The isolated patches of small lymphocytes within day 10 LN were also T cells, while B cells had virtually disappeared. The cells in the sinuses of the LN from day 4 on were completely $CD3^+$, (Fig. 5.19 and 5.20) until day 10, when they were displaced by the large MIB 1^+ cells (Fig.5.21).

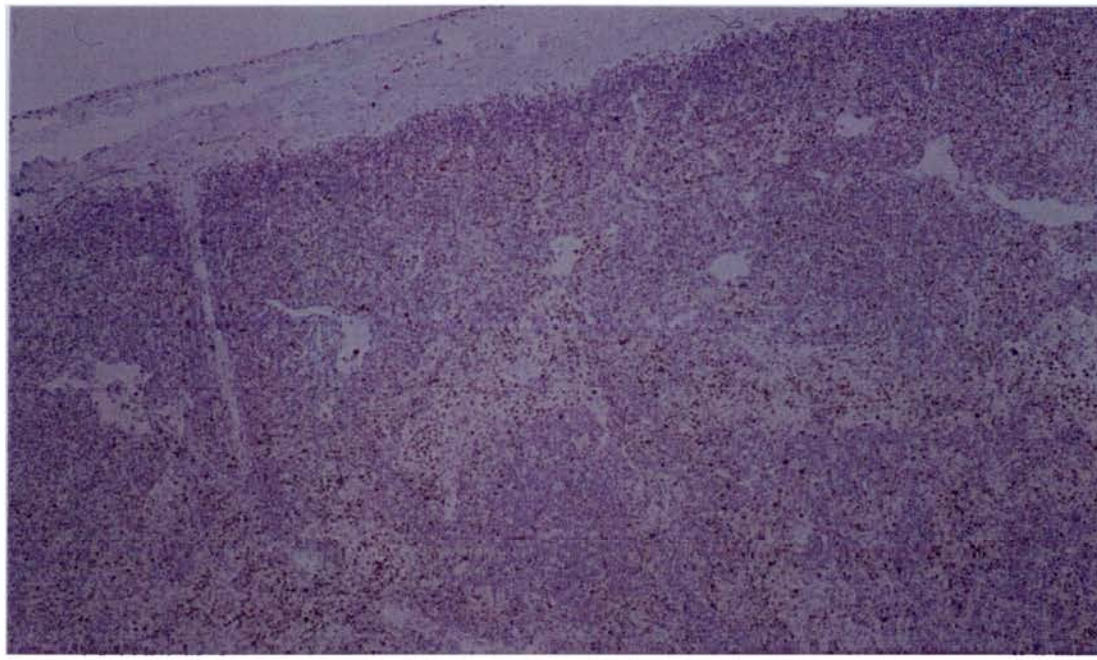


Fig. 5.12 Low power photograph of identical area to section 5.11 stained with MIB-1, showing dark brown stained proliferating cells present within all areas of the tissue except the small blue staining lymphocytes. DAB x50.

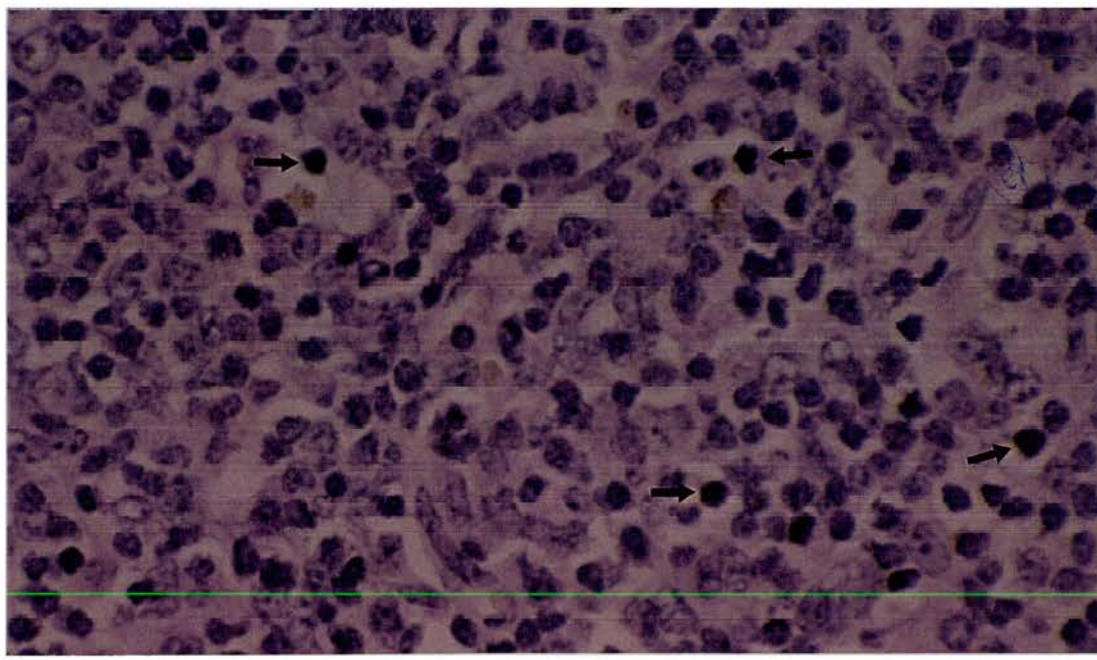


Fig. 5.13 Blasting cells within the paracortex of a day 8 infected node. Note blast cells with large nucleus and pink cytoplasm interspersed with small lymphocytes and cells with condensed nucleii (examples arrowed). H+E, x625

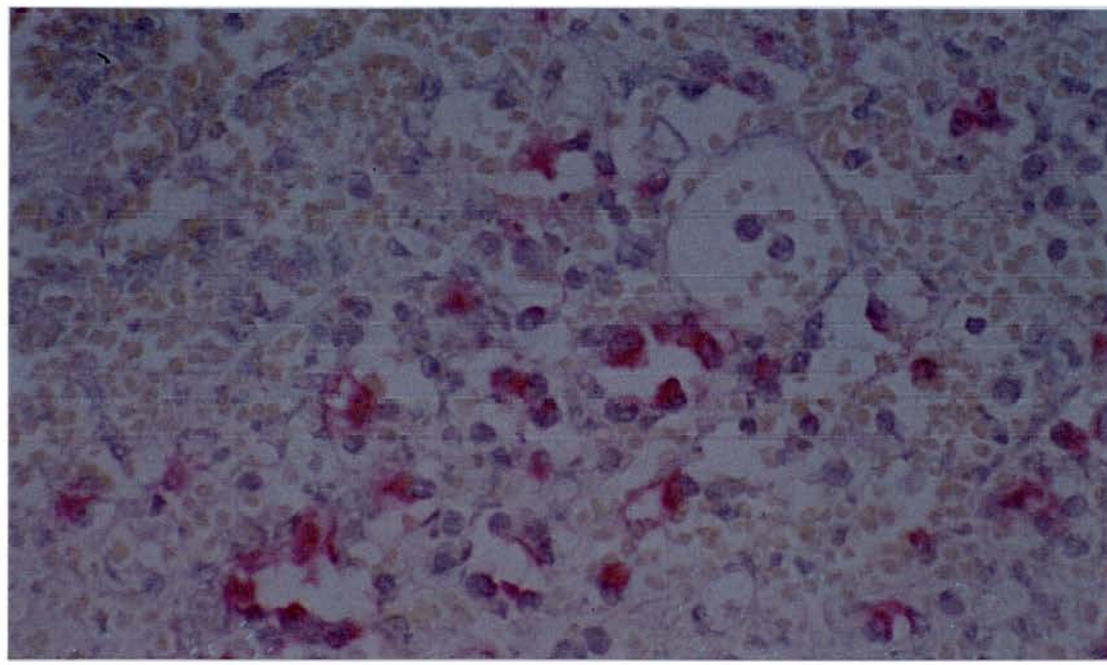


Fig. 5.14 $1C7^{+}$ cells within the paracortex of a day 10 draining node, antibody stained parasite is clearly visible within the cytoplasm of infected cells. Note extensive haemorrhage. Vector red, x500.

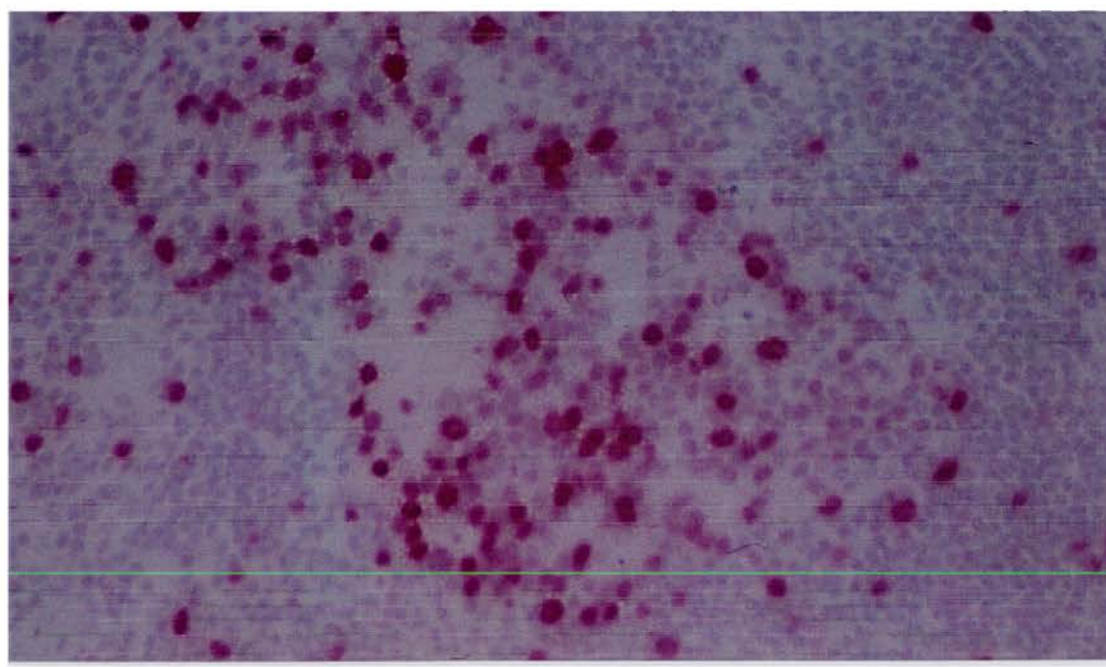


Fig. 5.15 Focus of MIB 1^{+} blast cells (stained red) within the medulla of a draining lymph node 48hrs post infection.Vector red, x500.

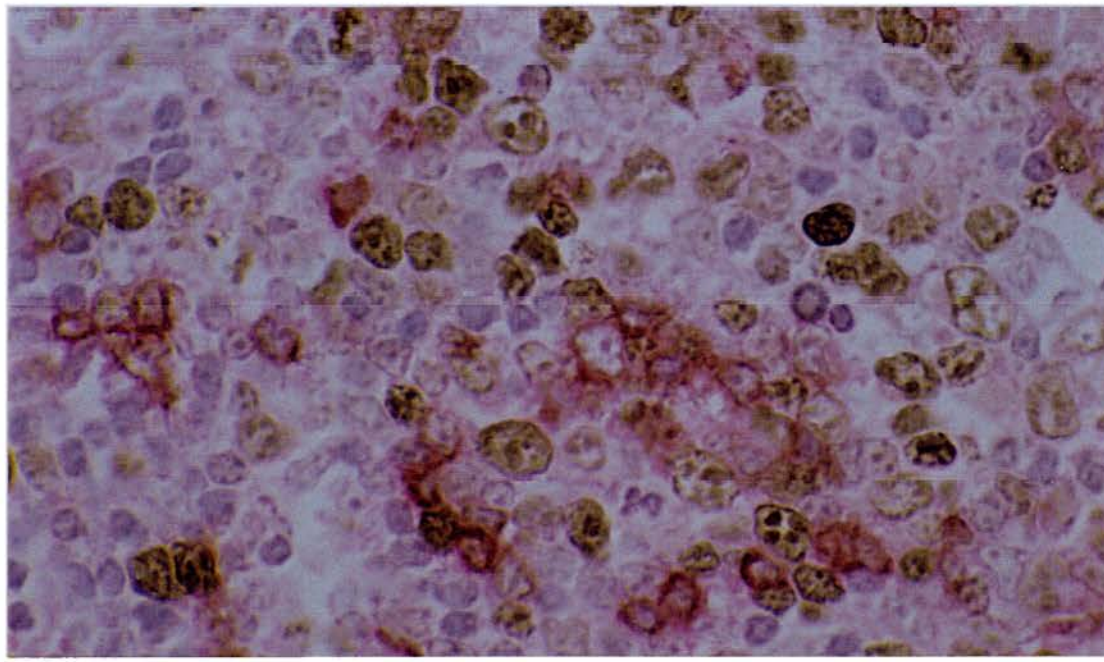


Fig. 5.16 Day 8 infected node double stained with MIB 1 (brown) and anti-CD3 (red). Large brown blasting cells are not stained with red with anti-CD3. DAB and Vector red. x500.

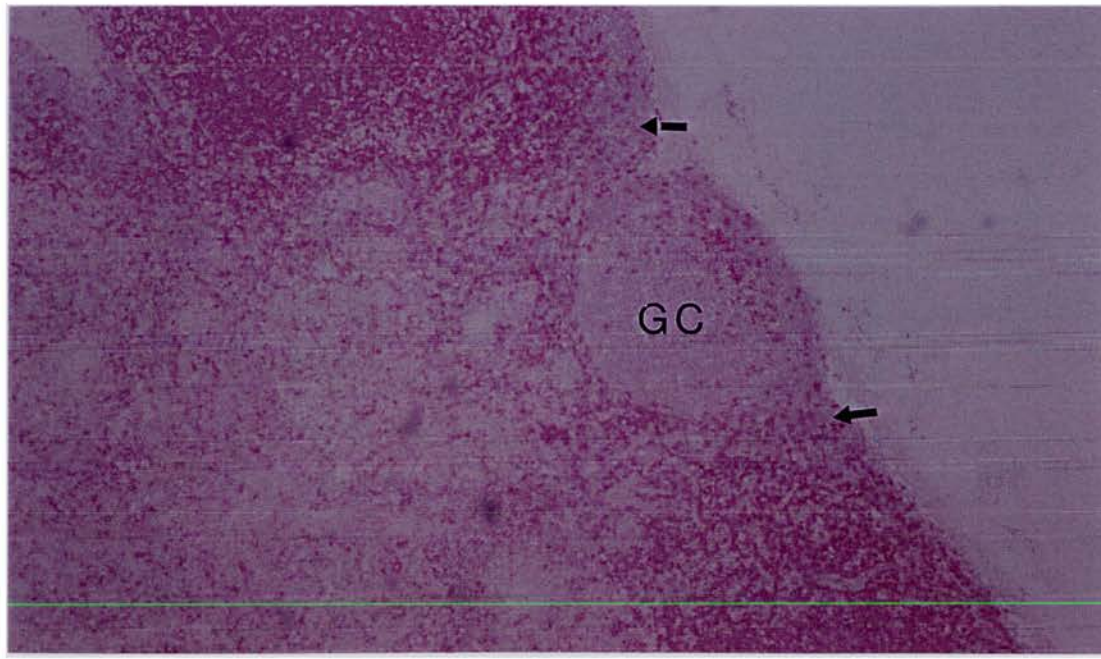


Fig. 5.17 Low power view of a normal bovine node stained with anti-CD3. B cell areas are visible as $CD3^-$ small lymphocytes (examples arrowed) and a germinal centre (GC). Vector red. x50.

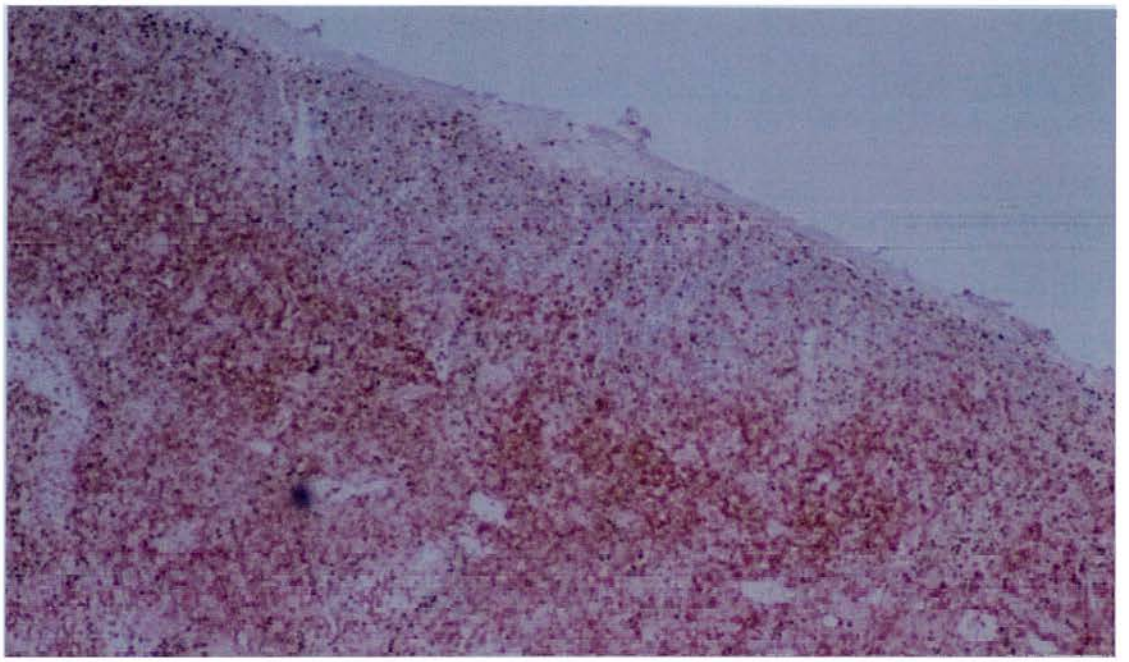


Fig. 5.18 MIB 1^+ cells (brown) present throughout the node, also appearing in between red $CD3^+$ T lymphocytes of a double stained day 8 draining node. DAB and Vector red. x125

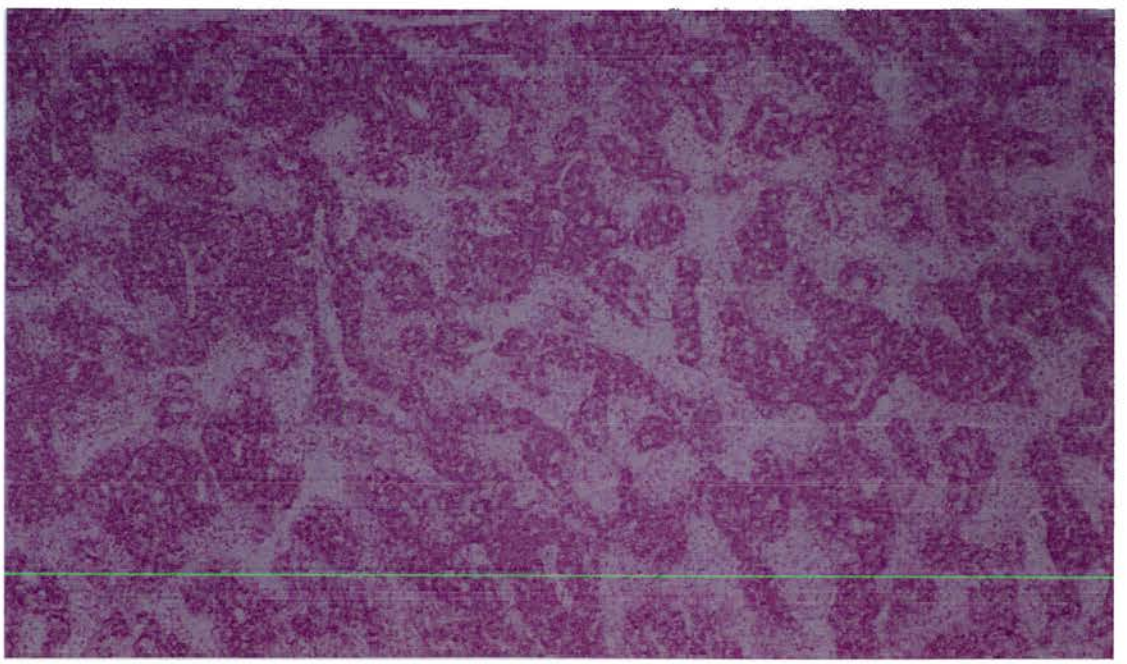


Fig. 5.19 Full medullary sinuses within a day 8 infected node staining strongly for CD3, with few of the interstitial cells positive. Vector red. x50.

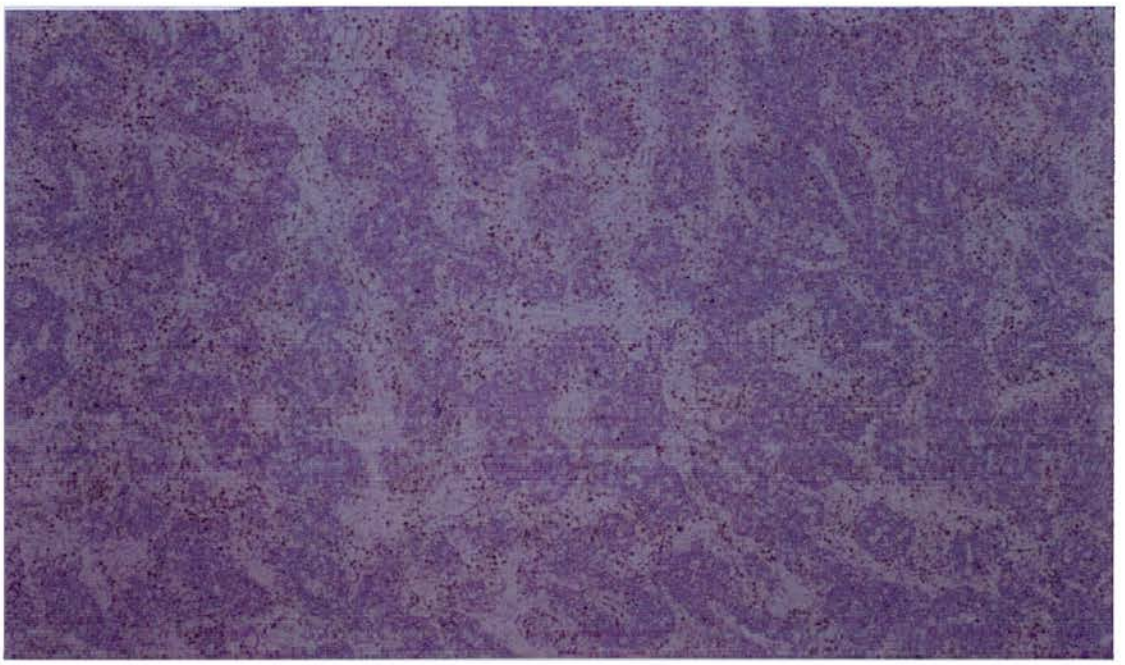


Fig. 5.20 Identical area to Fig. 5.19 showing brown stained MIB-1$^+$ cells largely within the interstitial tissue of the medullary sinuses. DAB, x50.

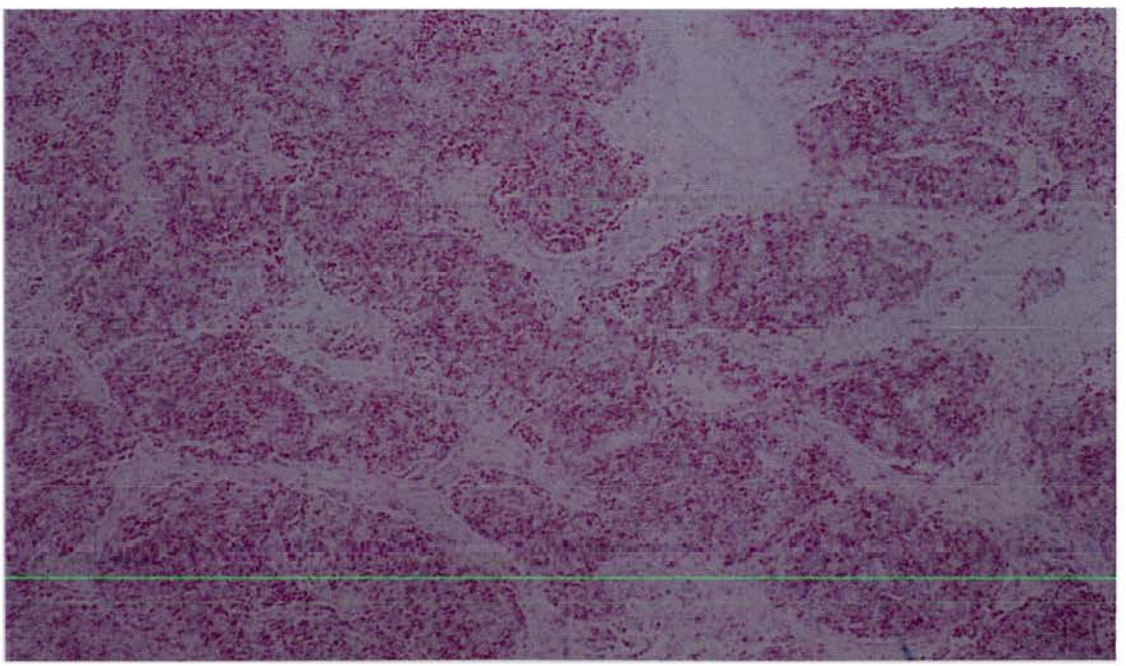


Fig. 5.21 MIB-1$^+$ cells, stained red, are a major component of day 10 infected node medullary sinuses. Vector red, x125.

IL-2 Receptor expression

Within control nodes, mAb IL-A111 (anti bovine IL-2R) stained a few large cells (1-2 per x25 field) mostly in the paracortex, displaying an extremely characteristic cytoplasmic stain. This had changed dramatically, even at day 2, with the appearance of many positive cells within the medulla. This was well established at day 4, with large numbers of blasting cells established around, but not inside, the foci of blasting non-T cells within the medulla (Fig. 5.22). $IL2R^+$ cell numbers within the cortex/paracortex remained unchanged from the control levels. The situation subsequently altered again, as the positive cells within the medulla had completely disappeared at day 8, with very few visible within the rest of the node. $IL2R^+$ cells were virtually absent at day 10.

Germinal centres (GC)

Normal LN

Within the control nodes the constituents of GC could be defined using several mAb. MIB 1 consistently stained normal GC of all sizes. The cells stained by anti-Ki67 mAb such as MIB 1 in GC have been previously defined in humans as proliferating centrocytes within an area known as the "dark zone" (Hardie *et al*, 1993). VPM30 staining was present only in large GC and generally did not stain the MIB 1^+ areas. Small GC were almost exclusively MIB 1^+, while VPM 30^+ cells appeared in larger, well developed GC outwith the MIB 1 areas (Fig. 5.23 and 5.24). In this respect, mAb VPM30 is thought to recognise an area analogous to the human light zone - both in its morphological appearance and in being MIB 1^-. Anti CD3 stain demonstrated that T cells were present within GC, and these were particularly numerous outwith the MIB 1^+ areas (Fig. 5.25). Mantle zone B cells, which did not stain with VPM30, could be identified as small non $CD3^+$ lymphocytes surrounding the GC, especially at the base.

Infected LN

As demonstrated within frozen sections, *T.annulata* infection appears to have a deleterious effect upon B cell development, as cortical areas were found to be

severely depleted within the heavily infected node. In addition, germinal centre morphology became difficult to identify within H&E stained LN sections 8 and 10 days post infection.

At days 2 and 4 post infection, GC appeared essentially identical to control GC, displaying a mantle zone, dark zone, $CD3^+$ cells and a $VPM30^+/CD3^+$ light zone. This changed dramatically at day 8, as GC within the node were present almost exclusively as the small MIB 1^+ type. VPM30 staining was virtually absent from GC, any remaining stain being extremely weak. (Fig. 5.26 and 5.27). The mantle zones were still present although they were often indistinct, and T cells were present in similar numbers to normal GC (fig. 5.28). There was no evidence of large scale cell death, indeed the apoptotic bodies characteristic of normal GC were generally absent, although some were still identifiable in the few remaining large GC (Fig.5.28). At day 10, GC were effectively absent, with only very small clusters of MIB 1^+ cells identifiable. These were often difficult to distinguish from foci of proliferating parasite infected cells, as the mantle zone morphology had been destroyed. Infection therefore severely affected B cell repones, as GC initially lost light zones, followed by total loss of morphology.

DNA analysis of draining lymph nodes

Determination of Mitotic Index

The percentages of the total nucleii (excluding hypodiploid nucleii) within each part of the cell cycle as determined by S-Fit analysis are detailed in the table overleaf. Sporozoite infection led to large numbers of cells entering the cell cycle within 2 days of infection. This correlates well with the appearance of large $MIB1^+$ cells within the medulla of the day 2 node and their subsequent growth throughout the tissue. There appears to be a degree of coordination within the proliferating cells, as there are "peaks" of S phase cells rather than a steady state of division. Daily analysis of cell kinetics would be required to determine whether more than the 2 peaks of S phase cells shown here occur.

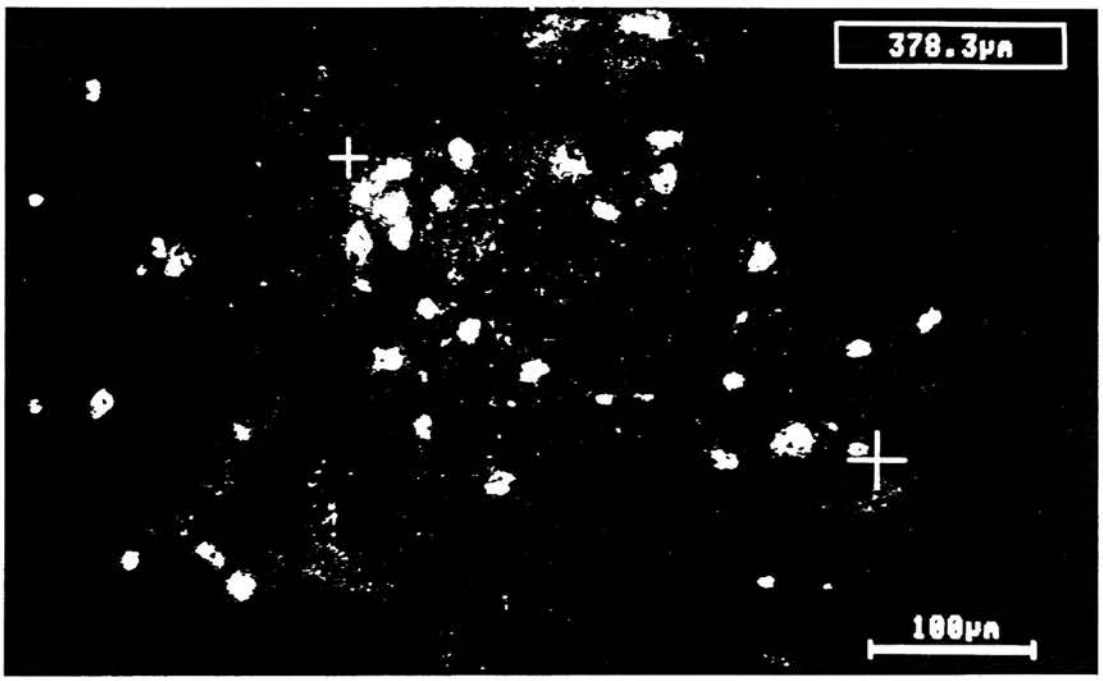


Fig. 5.22 Confocal micrograph of IL-2R$^+$ cells grouped around an area of blasting cells (BC, unstained by this method) within the medulla of a day 4 draining node. Vector red, rhodamine filter. Distance between crosshairs is contined within the box.

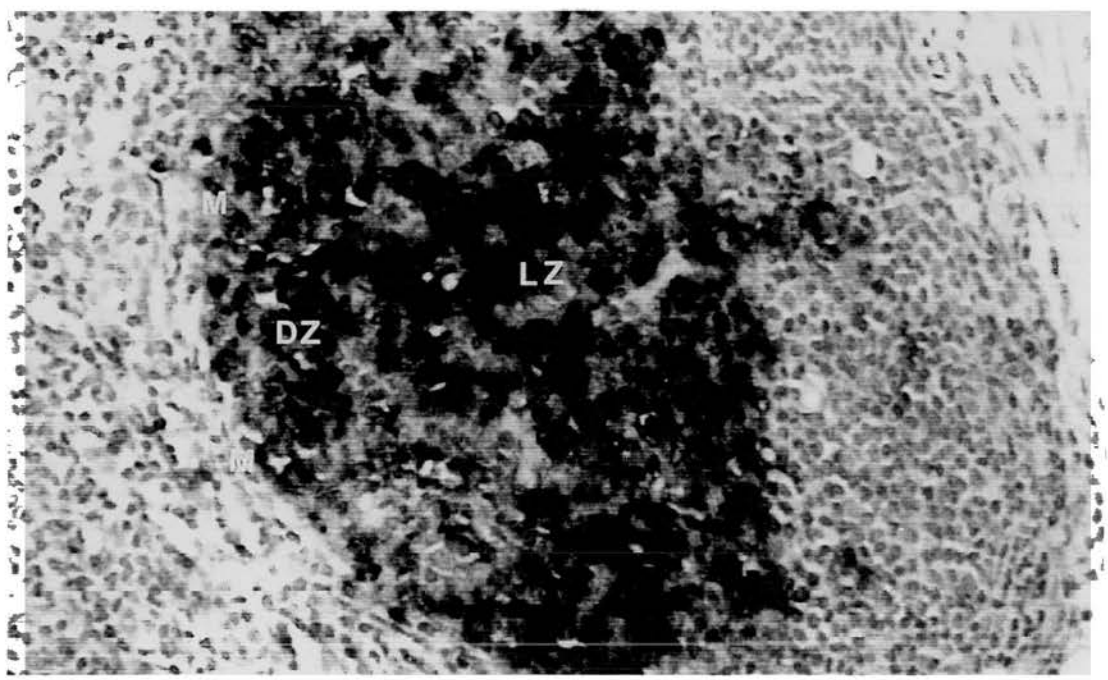


Fig. 5.23 Reverse confocal micrograph (positive cells are black) within a normal, well developed germinal centre stained with VPM30. Stained cells are largely outwith the dark zone (DZ), principally present in the light zone (LZ). Vector red.

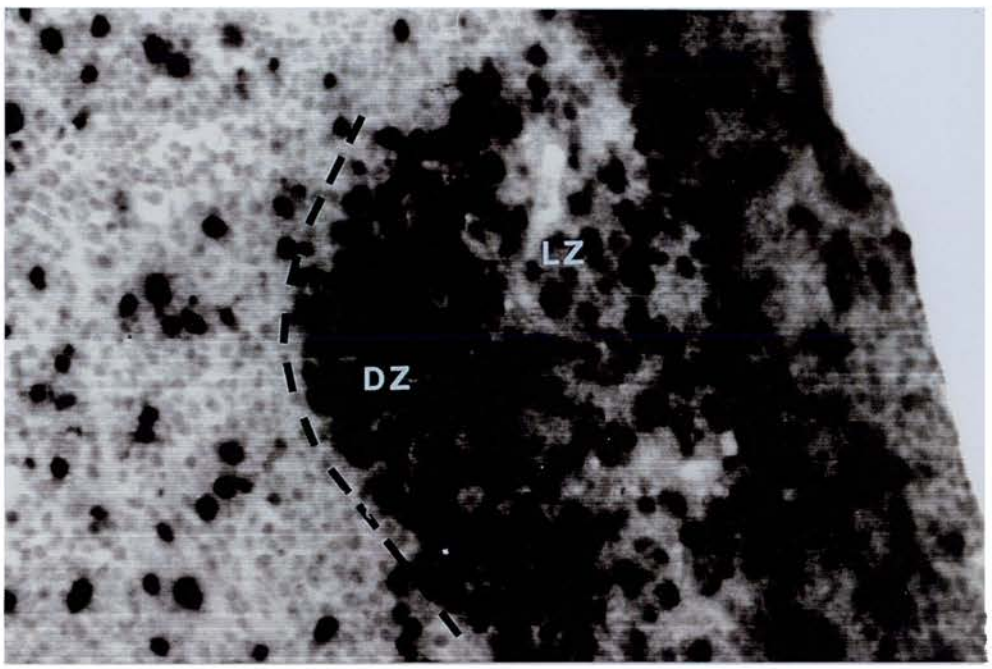


Fig. 5.24 Serial section identical to fig.5.23 stained with MIB 1. Positive cells are largely confined to the "base" of the GC beside the mantle zone - classified as the "dark zone". Vector red.

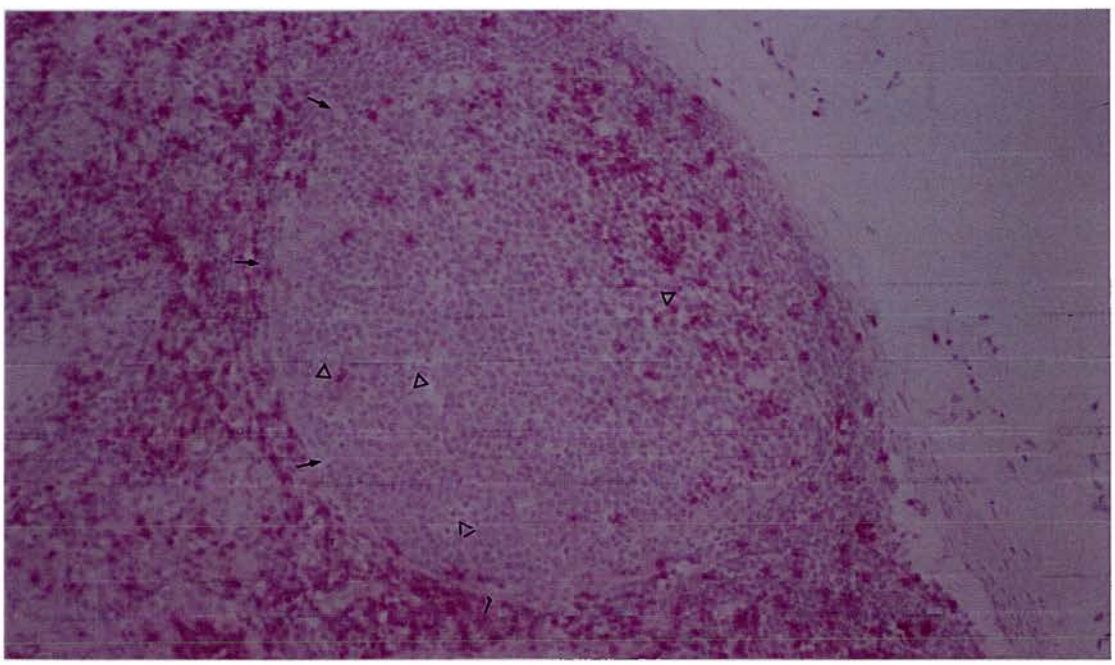


Fig. 5.25 CD3 cells within a well developed germinal centre of a normal node. Note $CD3^-$ small mantle B lymphocytes (black arrow), proliferating dark zone containing "tingible bodies" (white arrow) , and T cells within the light zone. Vector red, x125

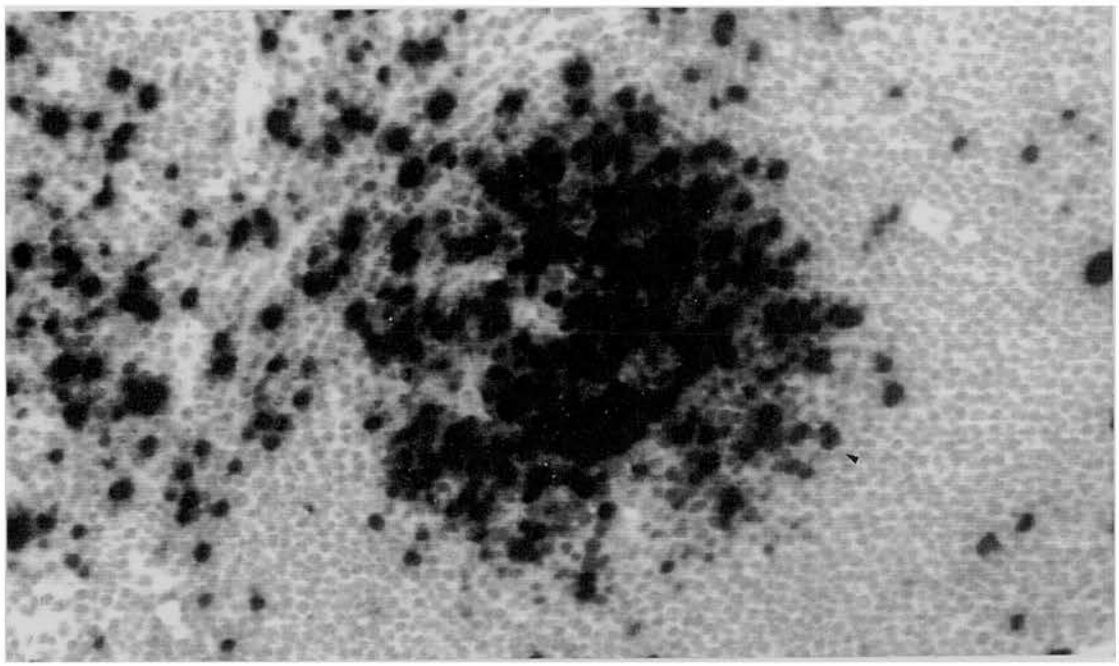


Fig. 5.26 GC within a day 8 draining node, showing MIB 1^+ (black stained cells) dark zone intact. Reverse confocal micrograph, Vector red.

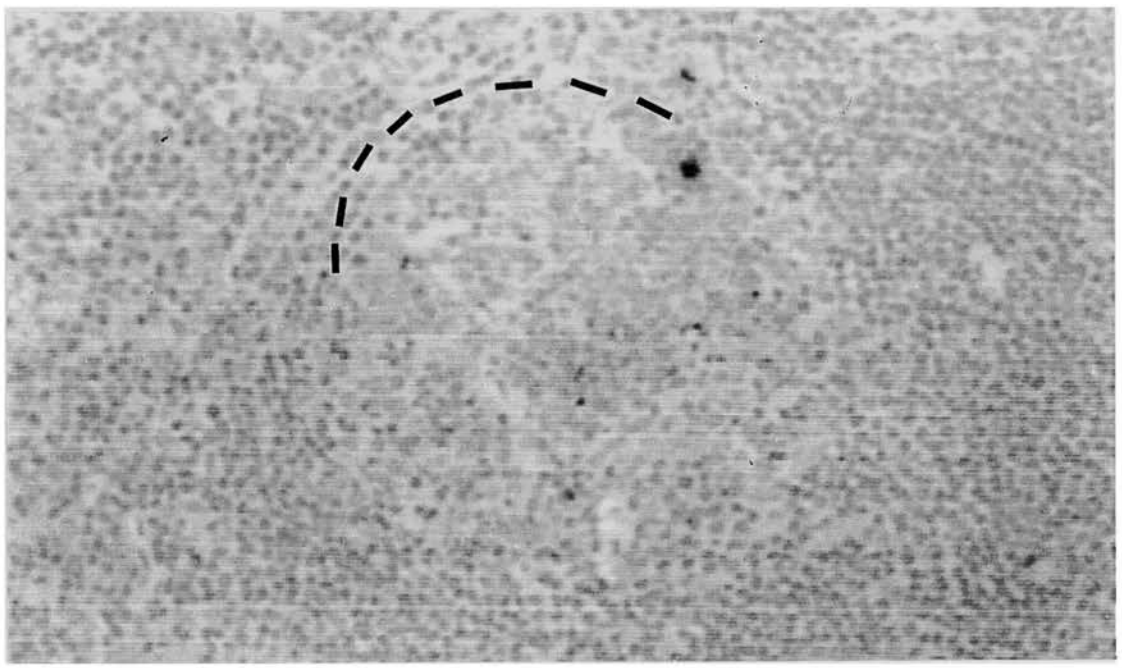


Fig. 5.27 Serial section showing identical GC to fig. 5.26 (mantle zone marked) stained with VPM30. Staining is virtually absent. Reverse confocal micrograph, Vector red.

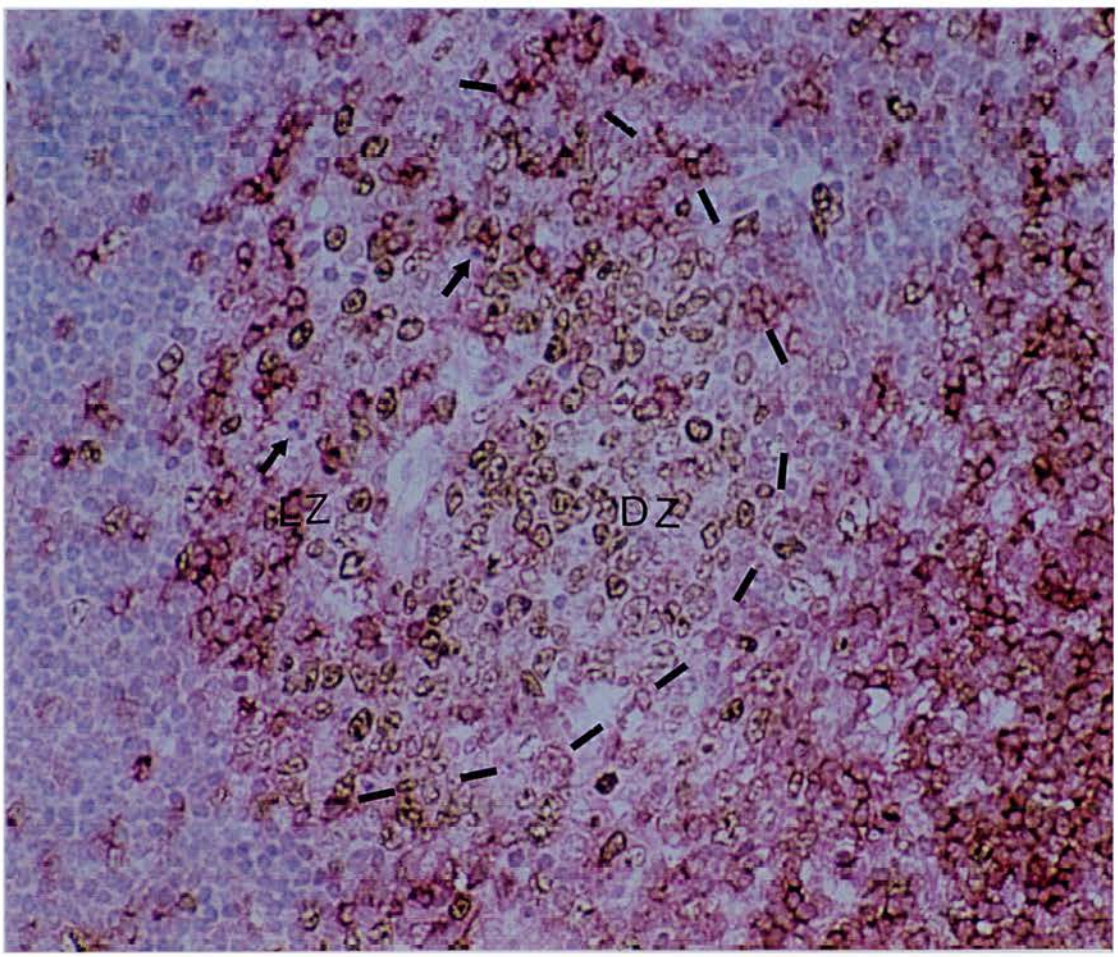


Fig. 5.28 Day 8 draining node GC (VPM 30) stained with MIB 1 and anti-CD3.. $CD3^+$ cells are still present within the light zone (lz) where some apoptotic figures are still present (arrow). MIB 1 still stains proliferating B cells in the dark zone. The morphology of the GC appears to be deteriorating, as the mantle zone is becoming indistinct (compare to fig. 5.25). Vector red, x400.

DAY	G_o/G_1 (%Total)	S	G_2
CONTROL	84.6%	6.7%	8.7%
2	70.2%	19.4%	10.4%
4	78%	7.3%	14.7%
8	70%	18.4%	11.6%
10	78.6%	8.2%	13.2%

Apoptosis

The percentages of cells contained within the sub-G_1 "Hypodiploid" peaks are detailed in the table below. DNA histograms from a normal and day 8 LN (22% apoptosis) are illustrated in Fig 5.29

Day	Control	2	4	8	10
%Hypodip. Batch 1	15	14	17	22	12
%Hypodip. Batch 2	15	14	17	18	13

Apoptotic cells therefore appear soon after blasting MIB 1^+ cells are detected within paraffin sections, with a peak at day 8 when MIB 1^+ have spread throughout the node. At day 10, when MIB 1 only stains fully differentiated parasitised cells, apoptosis subsides to near normal levels.

Cell phenotypes and cytokines in early infection

FACS analysis of T cell activation markers

Expression of T cell activation markers within the control node was at a low level

with <1% of T cells expressing MHC class II, while 8% of $CD4^+$ cells expressed IL-2R (Fig. 5.30A) At day 2, 22% of all LN $CD4^+$ cells expressed IL-2R and MHC class II, but activation markers were expressed upon less than 5% of $CD8^+$ T cells (Fig 5.30B). The percentage of $CD4^+$ and $CD8^+$ cells expressing IL-2R and MHC class II on $CD4^+$ cells remained unchanged by day 4 (Fig 5.30C). IL-2R was also expressed upon γδ T cells both 48hrs and 96hrs post infection, but the very small numbers of these cells (<5%) in the LN made accurate measurement of the level of expression difficult. The majority of cells identified as $IL\text{-}2R^+$ in sections of lymph node taken 2 and 4 days post infection are therefore likely to be $CD4^+$ T cells.

Cytokine expression by LN cells

Cytokine mRNA detected by RT-PCR from control, 2 and 4 days post infection LN are shown in Fig. 5.31. Products for IL-2, IL-2R, IL-4, and IFNγ were all detected within normal lymph node cells. 48 hrs post infection, the IL-4 product had become extremely faint, and was completely absent at day 4. All other products were detected in both lymph nodes, although the IL-2R signal was faint at day 4.

Isolation of parasitised cells from lymph nodes

It was not possible to grow infected cells from either the day 2 or 4 infected lymph nodes.

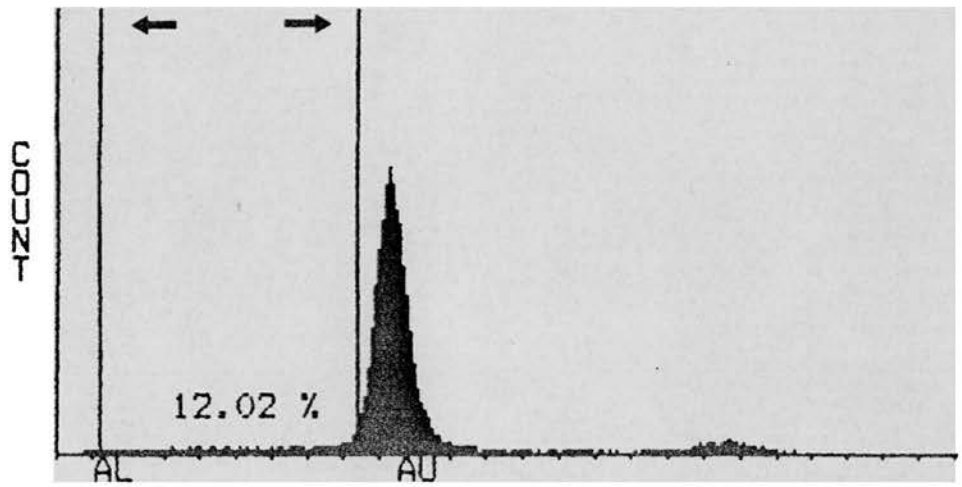
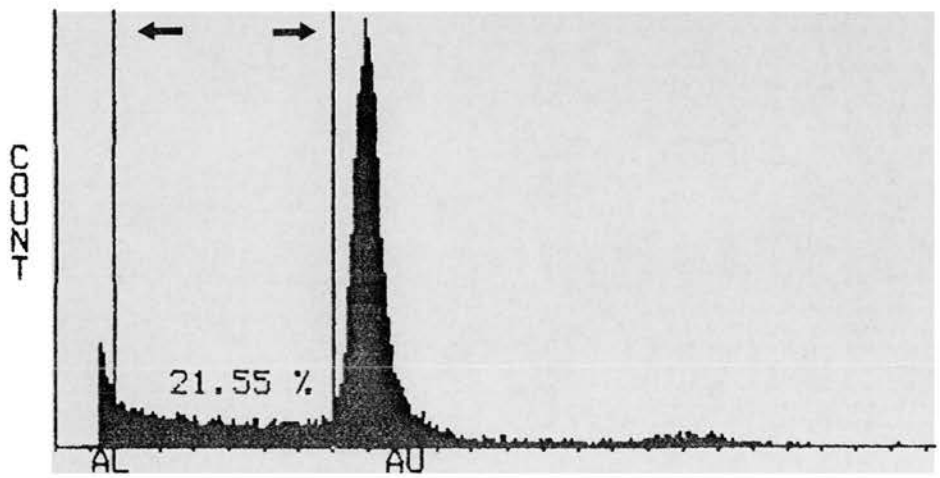


Fig. 5.29 DNA histogram from normal lymph node sample (above) showing pre-G_0/G_1 area corresponding to hypodiploid cells (between arrows). Note increase in this area in heavily parasitised node below.



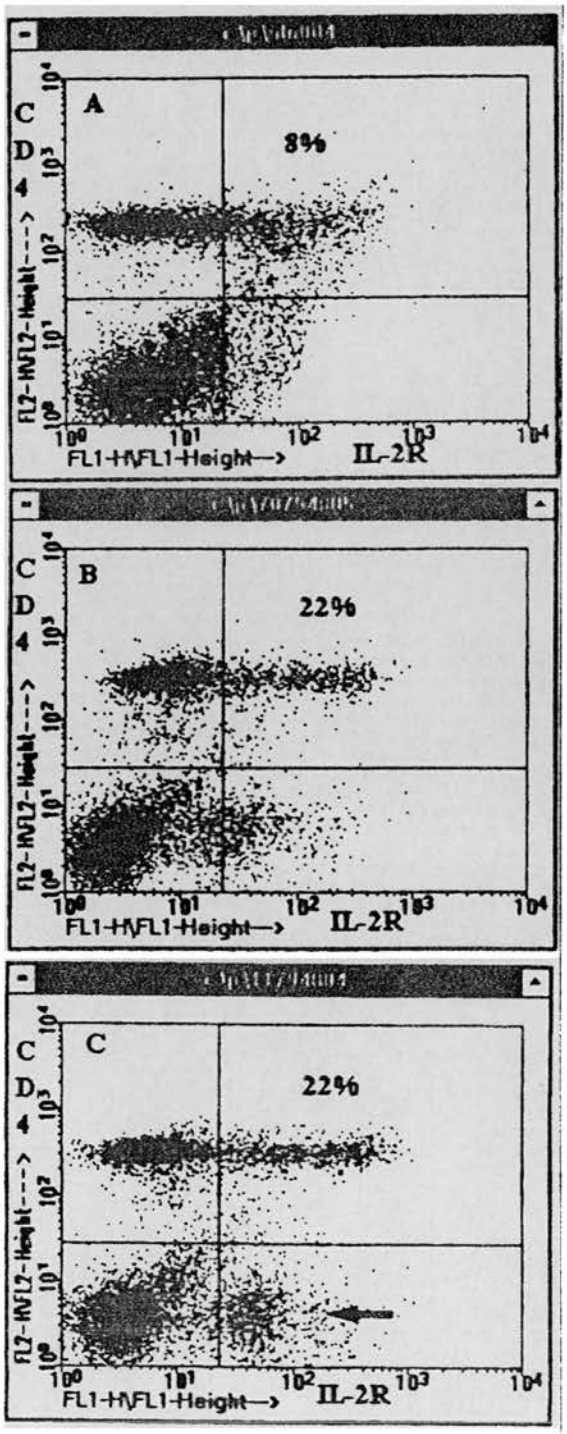


Fig. 5.30 Expression of IL-2R on CD4+ T cells in lymph nodes (LN).
A - Normal LN.
B - 2 days post sporozoite infection.
C - 4 days post sporozoite infection. Note CD4- IL-2R+ population (arrow) corresponding to γδ T cells.

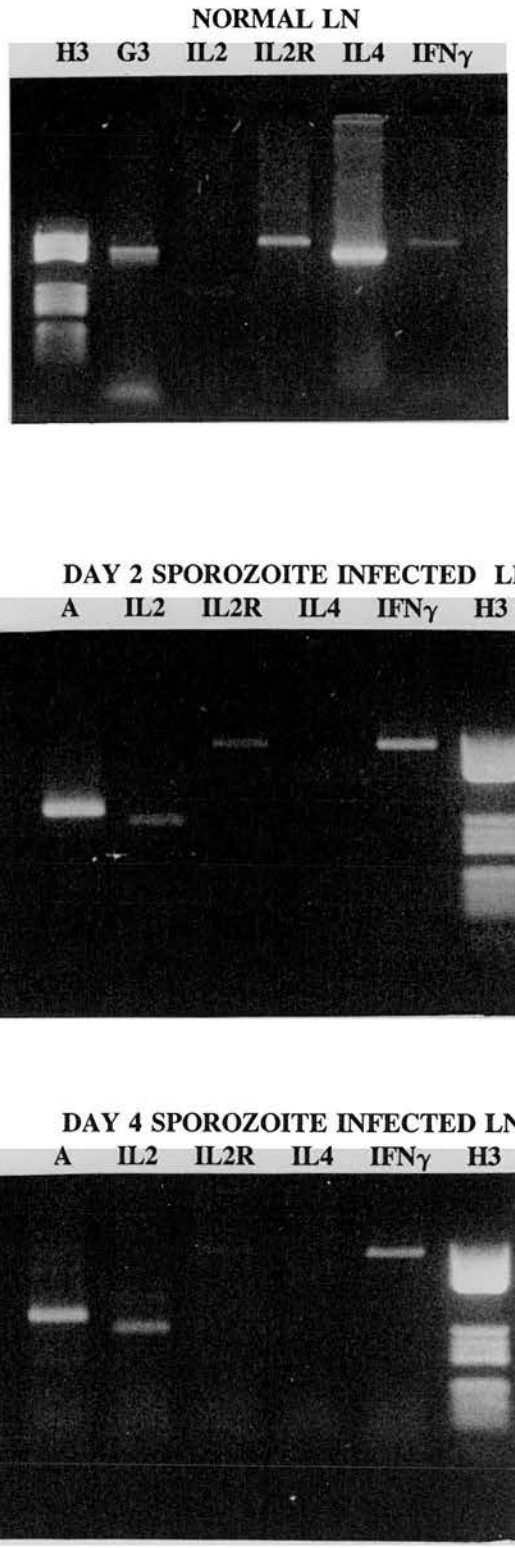


Fig. 5.31 IL-4 is strongly detected in a normal lymph node (LN), but has become extremely faint by day 2 of sporozoite infection and is completely absent by day 4. IL-2R has also become faint by day 4.

DISCUSSION

Parasite development following *T.annulata* sporozoite infection has long been identified as being confined largely to the draining lymph node (DLN). During this period, animals become increasingly ill, with the appearance of piroplasm infected cells a relatively late phenomenon, not necessarily associated with symptoms such as anaemia and pyrexia or even mortality. In this sense, tropical theileriosis presents something of a puzzle - how can the extremely localised development of the parasite cause such widespread pathology? In addition, what role does the immune response play in response to acute infection? As vaccinated or Butalex treated animals make anti - macroschizont infected cell CTL responses, what is the barrier to the immune response in primary infection? By developing immunohistochemical techniques to examine different cell populations the study presented here has been able to examine the interactions of the parasite and the cells within its principal site of development - the draining lymph node.

Parasite infected cell development

Cells infected and events in parasite development

Although the development and dissemination of macroschizont infected cells is well understood from their first appearance approximately 5 days post sporozoite infection, early events in parasite development have remained uncharacterised. In particular, the relationship between the small numbers of macroschizont infected cells and the apparently uninfected "lymphoblastoid" cells which characterise diagnostic biopsies after 5-8 days infection is unresolved. All previous studies, whether of biopsies or efferent lymph have not examined parasite development *in situ*, which has been possible for the first time with the immunohistochemical techniques developed in this study.

Sporozoite infected nodes are characterised by the appearance of "lesions" of large, blasting MIB 1^+ cells exclusively in the monocyte/macrophage rich medulla of the node. These cells steadily grow to become the dominant cell type, and contain schizonts when parasite is detectable. This study has shown that blasting

cells are non T, non B, MHC class II^+ cells, and these cells were identified within heavily infected frozen sections as IL-$A109^+$. This marker, although not fully characterised, is thought to be FcγR1 (MacHugh *et al*, 1990) and is found on bovine cells of monocyte/macrophage lineage, particularly monocytes. Residence of the parasite within these cells corresponds well to *in vitro* data which has identified monocytes and macrophages as the principal cells infected by sporozoites (Spooner *et al*, 1989), with IL-$A109^+$ cells readily transformed (Campbell *et al*, 1994). It is interesting to note that the proliferating cells do not express IL-2 receptors (IL-2R), indeed IL-$2R^+$ cells are markedly lost from the node after the early stages of infection (see below). Some authors have suggested that IL-2/IL-2R signalling may be involved in infected cell growth *in vitro* (Herrmann *et al*, 1989). This is patently not the case *in vivo*. IL-2R are found upon *T.parva* transformed T cells and B cells (Heussler *et al*, 1992b); the lack of such expression upon *in vivo T.annulata* infected cells provides further evidence that these are not the cell types transformed in this case.

The very early appearance of blasting cells and their large expansion before schizonts are detectable can lead to two different conclusions - either the parasite is capable of causing division of host cells before formation of visible macroschizonts, or the blasting cells contain a small number of infected cells inducing proliferation of uninfected cells. The latter explanation is favoured in the case of *T.parva* as the non parasitised blast cells do not appear until schizont infected cells are well established (Morisson *et al*, 1981). Uninfected blasting cells in *T.parva* are principally T cells, and this is postulated to be a possible mechanism for the parasite to expand its target cell population (Mckeever and Morrison, 1994). Although T cells do become activated during infection (see below), the vast majority of blasting cells in sporozoite infected nodes were $CD3^-$. *T.annulata* infected cells are something of a natural oddity as they are proliferating macrophages, ie residing outwith G_0 of the cell cycle (Spooner *et al*, 1989;Glass *et al*, 1989; Campbell *et al* 1994; Shayan *et al*, 1994). Monocyte/macrophages are described by Cotter *et al* (1994) as "end stage G_0 cells" ie. cells which do not normally divide once they have reached maturation,

with cytokines leading to changed phenotype rather than division. It is therefore extremely unlikely that any event other than infection of the cell could lead to the induction of cell cycling seen in non T non B cells in the DLN.

In infected nodes, proliferating cells are detected early in infection with increasing numbers of cycling cells seen before schizonts are visible. The first detection of schizonts by light microscopy is almost simultaneous in all infected cells with the sudden appearance of massive schizont parasitaemia (27 and 53% of all LN cells in the 2 animals examined at day 10, with only $<1\%$ of cells displaying schizonts at day 8). When the conditions required for parasite division are considered this coordination of parasite appearance would provide strong evidence that the majority of dividing cells are infected.

The trophozoite stage of the parasite undergoes binary fission within 18-24 hours of entry to the host cell, leading to formation of the schizont (Jura *et al*, 1983). Although not identifiable using light microscopy, schizonts can unequivocally be demonstrated within cells 24-48 hours post infection using electron microscopy (Jura *et al*, 1983). Thereafter, the replication of the schizont is intimately associated with division of the host cell, taking place once the cell reaches G_2 in the cell cycle (Jura *et al*, 1985). The parasite must therefore induce the division of the host cell in order to survive. It seems probable that the host cell will have to undergo several divisions before sufficient parasite material is present to be visible by light microscopy. This is exactly the position in infected nodes, with an increase in the number of cells in S-phase, G_2/M of the cycle throughout infection. In addition, cycling (MIB 1^+) cells appear in an area enriched for known sporozoite targets, expanding rapidly, leading to a simultaneous appearance of schizonts in many cells late in infection. It is therefore likely that the apparently uninfected blast cells seen in LN biopsies and this study are mostly infected cells which have been induced to divide.

T.annulata infected DLN are characterised by a steadily rising number of apoptotic cells, which appear once large numbers of cells have entered the cell cycle. As discussed below, B cell areas do not exhibit any large increase in apoptotic cells, if anything GC apoptosis decreases. Apoptosis seems to be

associated with the dividing ($CD3^-$) parasitised cells - the majority of apoptotic cells were present within proliferating lesions and reached a peak in numbers at the same time as MIB 1^+ cells were most prevalent. Once large numbers of cells with well developed schizonts were present, numbers of both cycling and apoptotic cells dropped. The decrease in cycling cells may well represent further differentiation to the microschizont stage with the loss of parasite control of the host cell cycle (Shiels *et al*, 1992) - microschizont infected cells were visible within day 10 DLN. If infected cells were destroying lymphocytes by the release of soluble factors, this would be expected to continue when large numbers of schizonts were visible, but this is not the case. Possible mechanisms for apoptosis or rescue of infected cells are discussed in a following section.

Sites for parasite invasion of host cells

Although the principal area in which *T.annulata* macroschizont infected cells develop is undisputed, the site in which the sporozoite infects its targets *in vivo* remains unknown. As particles eg. dyes pass swiftly from injection site to the draining node (within 30 minutes - R.L. Spooner pers. comm.) it is possible that sporozoites may drain directly into the node and infect cells *in situ*. Alternatively, as sporozoites can attach to cells within 5 to 60 minutes *in vitro* (Jura *et al*, 1983), it is possible that cells may enter the LN from the tissues already infected with parasite. Within the draining LN examined here, blasting cells appear within 48hrs in the medulla - an area shown to be populated primarily with IL-$A109^+$ monocytes/macrophages in frozen sections. Such cells are readily sporozoite infectable *in vitro* (Campbell *et al*, 1994). Medullary monocytes/macrophages are a relatively static population of cells, and are thought to be derived from precursors within surrounding tissues entering via the afferent lymphatics and sub capsular sinus (Mebius *et al,* 1991a). However, the passage of a cell from the surrounding tissue via the subcapsular sinus to the medulla is relatively slow - in murine studies where afferent lymphatics were severed, macrophage passage to the node was not markedly affected for 4-7 days (Mebius *et al*, 1991b). If cells were entering from the tissues already infected, they should be first detected in

the sub capsular sinus. As concentrations of MIB 1^+ cells are only present in the medulla and not the subcapsular sinus 48-96 hrs post infection, this would suggest that infection is confined to cells already *in situ*.

Bovine afferent lymph contains a mixture of phagocytic cells expressing various combinations of macrophage and dendritic cell markers (CD1; IL-A109 antigen; the antigen recognised by IL-A24 known as p110/75; and CD14 among others, McKeever *et al,* (1991); A.K. Nichani and P. Goel pers. comm.). Sporozoites entering the afferent lymph are therefore in contact with potential targets. $CD14^+$ cells are highly infectable with sporozoites *in vitro* (Campbell *et al*, 1994). The $CD14^+$ macrophage/monocyte fraction is thought to home to the medulla as discussed above, and it is therefore possible that some of the infected cells may have originated from afferent lymph macrophages. The $CD1^+$, primarily $CD14^-$ interdigitating afferent lymph cell (IDC) forms up to 20% of afferent lymph antigen presenting cells (Mckeever *et al,*1992; A.K. Nichani and P. Goel pers. comm). This cell type has been previously shown to home specifically to the paracortex (McKeever *et al* (1992); Bogen *et al*, (1991)(1993)). However, proliferative lesions are not seen in the paracortex in parallel with those in the medulla, suggesting that such cells are not infected to any great degree. This last observation may also point to a higher degree of specificity in target selection within DLN than *in vitro* - macrophages which express the antigen p110/75 (mAb IL-A24) are readily infectable *in vitro* (Spooner *et al*, 1989), but are localised in the paracortex *in vivo* (McKeever *et al*, 1991) ie. outwith the principal sites of early parasite development. Thus sporozoites may be selecting monocytes in the medulla in preference to paracortical cells of myeloid origin.

DLN immune responses and implications for parasite development

T cell responses

One of the most important questions addressed in this study was whether macroschizont infection simply overwhelms the primary immune response, or whether the parasite specifically interferes with developing immunity. A recent study of lymph efferent from infected DLN suggested that the latter was the case.

Although T cells bearing activation markers (IL-2R and MHC class II) were found to be leaving the node, they were apparently not functioning normally, as they did not proliferate to IL-2 or recognise autologous infected cells (Nichani, 1994). By examining the developing immune response *in situ*, this study has been able to determine key events in T cell reactions to *T.annulata*.

In normal and control DLN, IL-2R$^+$ cells were confined largely to the CD3$^+$ paracortex and the cortex, in agreement with previous studies using IL-A111 (Naessens *et al*, 1992). Infection was characterised by the rapid appearance of CD4$^+$/IL-2R$^+$ cells, particularly surrounding the "lesions" of developing infected cells. The numbers of IL-2R$^+$ cells then dropped back to that of normal nodes by day 8, and positive cells were virtually absent by day 10. It seems likely that the CD4$^+$/IL-2R$^+$ cells found to be exiting in DLN efferent lymph (Nichani, 1994) correspond to the cells of this type developing in the medulla early in infection. Sporozoite infection therefore leads to a rapid activation of T cells, these cells then leave the node and T cells bearing activation markers are subsequently largely absent from the node.

This study has used the expression of IL-2R to identify activated T cells, and this marker was shown to be expressed largely upon CD4$^+$ cells in DLN. As IL-2 and IL-2R expression is simultaneous when T cells are activated (Mauer *et al*, 1984;Smith, 1988), useful comparisons can be made to other studies of T cell activation in DLN which examined IL-2 producing T cells (Bogen *et al*, 1991;1993). A possible criticism of this method is that activated IL-4 producing type-2 cells may be missed - some bovine *in vitro* derived, IL-4 producing clones have been characterised as IL-2 independent (Stevens *et al,* 1992). The principal cytokine response detected from T cells early in infection was type-1 - IL-2/IL-2R/IFNγ with IL-4 expression rapidly disappearing. It is therefore unlikely that IL4 producing cells are playing a major part in the observed immune response. In addition, results from chapters 3 and 4 showed that $\geq$ 95% of ConA activated CD4$^+$ T cells expressed IL-2R, and IL-4 was produced within this population. Thus IL-2R is a suitable for detecting the vast majority of activated bovine T cells, irrespective of cytokines produced by the cells.

Examination of patterns of T cell activation in draining nodes during primary immune responses to soluble antigens have shown a radically different pattern to those seen in this study. Using the production of IL-2 and IL-4 to identify activated T cells. Bogen *et al*, (1991;1993) determined that such cells appear 3-4 days post injection and localise exclusively in the parafollicular area near the paracortex/cortex junction. Such cells subsequently increase in numbers and migrate to B cell follicles, with maximal numbers (approximately 10% of all LN cells) being reached 11 days post inoculation. This is in direct contrast to the situation in *T.annulata* infected nodes, with the maximal appearance of activated T cells at days 2-4, confined almost exclusively to the medulla. At day 10, when large numbers of activated T cells should be present in the B cell zones, they are virtually absent. T cell activation in infected nodes therefore takes place in non conventional sites, far earlier than expected, and is absent at a time when activation should be maximal.

The localisation of T cells within the paracortex/cortex junction during normal immune responses is largely due to the heavy vascularisation within this area. The vast majority (approximately 90%) of T cells circulating through lymph nodes are "naive" in phenotype and enter nodes through interactions between high endothelial venules (HEV) which express the ligand for L-selectin, expressed on naive T cells (Mackay, 1993; Mackay *et al*, 1992; Howard *et al*, 1992). T cells are thought to localise here in order to interact with Ag carrying antigen presenting cells (Bogen *et al*, 1991). In particular, ruminant afferent lymph "veiled" cells are very potent T cell stimulators (Bujdoso *et al*, 1989a; McKeever *et al*, 1991), antigen primed bovine veiled cells have been shown to induce primary T cell immune responses *in vivo* (McKeever *et al*, 1992). Activated T cells in *T.annulata* DLN do not appear in the recognised sites for interaction with these cells and do not migrate to the B cell zones. Thus, in infected LN, the majority of T cell activation is not mediated in recognised sites by "normal" APC with a subsequent lack of normal post activation T cell reactivity patterns.

Results in chapters 3 and 4 of this thesis have demonstrated conclusively that macroschizont infected cells can activate T cells *in vitro* upon contact,

irrespective of memory status or commitment to a particular pattern of cytokine expression. Such activation is primarily mediated through $CD4^+$ cells. The appearance of elevated numbers of activated $CD4^+$ cells, early in infection and associated virtually exclusively with developing parasitised cells would strongly suggest that *T.annulata* infected cells are functioning in a similar manner *in vivo*. *In vitro*, such activation does not produce *Theileria* specific T cell responses, even in immune animals (appendix 2). There is little evidence to suggest that the activation of T cells seen *in vivo* represents a genuine immune response against the parasite either. The cytokine responses are very similar to those seen *in vitro* - cytokine production within the node skewed rapidly with a loss of IL-4. In addition, germinal centre morphology was first altered and eventually virtually eliminated and parasitised cells grew unchecked.

Implications for immunity and parasite development of altered T cell responses

How can indiscriminate T helper cell activation within the medulla lead to the apparent breakdown in immune responses? In the first instance, infected cells apparently randomly activate T cells around them in the tissue. Activation of $CD4^+$ and $CD8^+$ T cells "non specifically" by *T.annulata in vitro* does not confer any anti parasite activity to these cells (appendix 2), and this mechanism also seems to apply *in vivo*, with activated T cells in efferent lymph unable to kill or proliferate to autologous parasite infected cells (Nichani, 1994). The creation of a population of T cells unable to mediate anti parasite responses will presumably aid parasite survival. Secondly, and almost certainly with more serious consequences, infection in the node produces a type-1 like cytokine response - IL-4 production is lost from the node, and IFNγ production elevates and remain high throughout infection (Nichani, 1994). IFNγ is most likely to come from the activated T cells - infected cells rarely if ever make this cytokine *in vitro* (Brown *et al*, 1995) and *in vitro* parasite stimulated T cells produce IFNγ mRNA at far higher levels than IL-4. Elevated IFNγ production early in infection can potentially influence the three major aspects of the anti parasite response - B cell germinal centre development (discussed in depth in the next section), the

formation of "normal" T cell responses, the development of the parasitised cells and subsequent pathology.

It has been suggested that early IFNγ production during lymph node responses may influence subsequent T cell activation (Bogen *et al*, 1993). IFNγ is potently antagonistic to IL-4 producing cells, and may even switch such cells to IFNγ production (Donckier *et al,* 1994). A similar mechanism is apparently in operation early in infection, as IL-4 production is quickly lost within the DLN and efferent lymph subsequently contains greatly elevated levels of IFNγ 4-10 days post infection (Nichani, 1994). Thus, even if T cells are encountering APC in relevant sites later in infection, their subsequent cytokine profiles are unlikely to develop freely into an optimised response to eject the parasite. IFNγ is however unlikely to affect migration of lymphocytes or APC into the node, as both cell types show increased adhesion to HEV in the presence of IFNγ (Kraal *et al*, 1994; Pabst and Westermann, 1994).

Could there be any role for *Theileria* activated T cell derived cytokines in the development of infected cells? Evidence of a role for soluble factors in macroschizont infected cell growth *in vitro* is somewhat contradictory. Macroschizont infected cell lines grow vigorously in culture without the addition of any exogenous growth factors and both Nichani (1994) and Preston *et al* (1992b) have shown that the addition of IFNγ may decrease proliferation of *in vitro* derived lines. This is completely opposite to the case *in vivo*, where maximal infected cell proliferation takes place at the same time as peak IFNγ production (Nichani, 1994). Although *in vitro* limiting dilution analysis has shown that sporozoites and macrophages seeded as low as one cell per well can result in the establishment of infected lines, this is extremely inefficient and cell lines are established much more efficiently from mixed cultures of cells. (Spooner *et al*, 1989; R.L. Spooner pers comm.; J.Campbell unpub. obs). Infected cell lines can be derived from peripheral blood macroshizont infected cells once an animal is heavily infected, but cells isolated from infected DLN in this study early in infection failed to grow into infected lines. Taken together this evidence suggests that fully differentiated infected cells have little need for other growth factors,

while the early stages are more dependant upon their microenvironment.

The early stages of parasite development in the DLN are characterised by rapid cell division. A major potential hazard for the parasite once host cell division has been initiated is apoptosis of the cell. Monocytes and macrophages are inherently non dividing cells, movement into the cell cycle greatly increases the chance of inducing apoptosis in these cells (Cotter *et al*, 1994) and apoptotic cells are a feature of *in vivo* parasite development. Apoptosis of macrophages *in vitro* can be averted via the action of tumour necrosis factor alpha (TNFα) and IL-1β (Mangan and Wahl, 1991). TNFα has been shown to be produced by PBM from infected animals (Preston *et al*, 1993), and when macroschizont infected cells, which constitutively express mRNA for TNFα and IL-1β *in vitro* (Brown *et al*, 1995) are fully developed, apoptosis in the node is decreased. TNFα production is therefore present during infection and may play a role in stabilising the otherwise potentially apoptotic infected cells. IFNγ is a potent inducer of TNFα production by macrophages (Philip and Epstein, 1986), and the action of IFNγ alone can mediate macrophage rescue from apoptosis to a large extent (Mangan and Wahl, 1991). The increased production of IFNγ in *T.annulata* infection therefore has the potential to induce TNFα secretion by both normal and infected macrophages. Thus activation of T cells by the parasite to produce IFNγ may lead to better survival of infected cells via the induction of TNFα. As apoptotic cells are seen in DLN, this rescue system may be far from perfect, but even in optimised *in vitro* systems 10-20% of cells fail to be rescued by TNFα or IFNγ (Mangan and Wahl, 1991).

Although of potential significance for parasite survival, the induction of TNFα secretion via IFNγ activation of macrophages almost certainly is of great importance in inducing disease pathology. The symptoms of tropical theileriosis are extremely similar to those attributed to the action of TNFα in cattle - anorexia, anaemia, leucopenia and pyrexia (Bielefeldt Ohmann *et al*, 1989; Silegham *et al*, 1994). Although infected cells are largely localised in the DLN, activated T cell derived IFNγ and macrophage/infected cell TNFα will be disseminated throughout the body via afferent lymph. These cytokines are strong

candidates to be the causative agents of the widespread pathogenesis caused by disease, particularly in the absence of any correlation between pathological lesion in an organ and the presence of infected cells (Forsyth *et al*, 1994).

Alterations in germinal centre responses

In this study, identification of the functional compartments of bovine GC has been possible using several different mAb. Previous histological descriptions have concentrated upon identifying cell types using PNA, polyclonal antisera against Ig to identify B cells, and anti T cell mAb (which are not available in this country) (Morrison *et al*, 1986; Galeotti *et al*, 1993). Using MIB 1 we have been able to compare bovine and human GC, with the dark zone of proliferating centroblasts (Hardie *et al*, 1993) clearly visible. T cell zones were identified using anti CD3 Ab. The light zone was also distinguishable as a more sparsely populated area, MIB 1^- and containing $CD3^+$ cells. mAb VPM 30 (expressed on all peripheral blood and efferent lymph B cells, Naessens and Howard, 1991; Nichani, 1994) stained B cells only in the light zone of large, well developed GC, and stain was absent from the mantle zone or dark zone. In this respect, VPM 30 appears to recognise a differentiation antigen acquired by B cells upon passage to the light zone. As such movement is dependant upon cognate interactions with T cells to rescue somatically mutated centroblasts (MacLennen, 1994), the expression of the antigen recognised by VPM30 on B cells is likely to be dependant upon T cell help.

A most striking feature of sporozoite infected nodes was the alteration seen in GC light zones. The light zone $VPM30^+$ cells seen in large GC early in infection are not likely to represent a *theileria* specific response, as even the most immunogenic of soluble antigens do not produce visible light zones until at least 4 days post inoculation (reviewed by Nieuwenhuis *et al*, 1992). At day 8 most B cell follicles were small, only displaying mantles and dark zones. These may represent *de novo* follicles which are unable to form into GC with light zones. In the very few larger GC with morphologically distinguishable light zones, VPM 30 staining was completely absent although T cells remained. This suggests that B

cells are not developing properly by this stage in infection. By day 10, even dark zones were virtually eliminated. Thus parasite infection causes a gradual loss of B cell differentiation and, presumably, function, initially stopping normal development of B cells in light zones followed by a general inhibition of proliferation.

For the successful formation of GC, B cell development is dependant upon 2 types of accessory cells - T cells and antigen presenting cells known as follicular dendritic cells (FDC). Briefly, antigen is presented to B cells by FDC who in turn present to T cells. T cell "help" then causes the B cells to proliferate rapidly and undergo antibody diversification. Finally, B cells are selected for their ability to recognise antigen upon FDC and to present Ag to T cells. This final selection takes place in the light zone, where B cells are either selected and become functional, or fail and die by apoptosis (Liu *et al*, 1989; Maclennan, 1994). If B cells were reaching this stage in *T.annulata* infection, only for the selection mechanisms to be absent, mass apoptosis of cells would be predicted (Liu *et al*, 1989). As neither mature ($VPM30^+$) B cells or mass apoptosis were visible in GC light zones, this suggests that the majority of B cells are being blocked from entering this stage of development.

In vivo, GC are completely dependant upon T cell help (Jacobson *et al,* 1974) and T cell interactions are essential in GC development before the selection step discussed above - in inducing B cell proliferation when GC are first formed (Kosco and Gray, 1992, Noelle *et al*, 1992) subsequently influencing class switching and enhancing Ig secretion (Armitage *et al*, 1993 *inter alia*). In considering what mechanisms may be acting upon T cells and B cells the requisite interactions will be discussed in the order they appear to break down - failure of B cells to progress to selection in the light zone, followed by a diminution of dark zone proliferation.

Infected nodes show a loss of IL-4 mRNA expression, while IFNγ expression continues unabated and is detected at elevated levels in DLN efferent lymph throughout infection (Nichani, 1994). As discussed in chapter 4, *T.annulata* infected cell *in vitro* stimulated T cells continue to produce far greater amounts of

IFNγ mRNA than IL-4. The control of B cell proliferation *in vivo*, is achieved through a fine balance of B cell stimulatory IL-4 (reviewed Paul and Ohara, 1987) and B cell inhibitory IFNγ (Reynolds *et al*, 1987). Disease models characterised by hyper Ig production (Donckier *et al*, 1994), and altered B cell growth in HIV infection (Clerici and Schearer, 1993) have been attributed to over production of IL-4 by $CD4^+$ T cells causing unregulated B cell growth. Such conditions can be experimentally treated by adding exogenous IFNγ (Field *et al*, 1992; Donckier *et al*, 1994). In *T.annulata* infection the situation is completely reversed - elevated IFNγ plus decreased or absent IL-4 - and this has the potential to seriously depress B cell responses. It is interesting to note that in *T.parva* infection, where infection of T cells does not change the cytokine profile of the infected cells and both IL-4 and IFNγ continue to be produced (Brown *et al*, 1995), that GC remain apparently normal during infection (Morrison *et al*, 1986) despite the presence of large numbers of infected cells.

There are 2 mechanisms proposed for the IFNγ/IL-4 regulation of B cell development - either IFNγ is antagonistic to IL-4 producing $CD4^+$ T cells (Donckier *et al,* 1994), or has direct inhibitory effects on B cells (Abed *et al*, 1994a;1994b). Significantly, both mechanisms affect proliferating B cells, ie. after initial stimulation but before selection in light zones. The predominantly type 1 T helper responses induced in T cells by *T.annulata* both in infected nodes and *in vitro* may lead to the inhibition of IL-4 production by germinal centre T cells. Such a response has even been shown to switch the phenotype of IL-4 producing T cells to IFNγ production (Donckier *et al*, 1994). A lack of IL-4 during this stage may decrease B cell proliferation, but more importantly, inhibit the production of Ig (Armitage *et al*, 1993; Donckier *et al*, 1994). IFNγ can also directly affect B cell development. B cells only express receptors for IFNγ once they are proliferating, and then only shortly before the production of Ig (Abed *et al*, 1994a). *In vitro*, exposure to IFNγ at this stage leads to a profound suppression of Ig production, even in the presence of T cells and/or exogenous IL-4 (Abed *et al*, 1994b). GC in infected nodes are "stuck" at the dark zone stage, as B cells are not passing into the light zone. B cells enter this zone after

an intense period of Ig hypermutation (Maclennen, 1994) and such maturation does not occur until after the start of Ig production (Berek *et al*, 1985). During parasite infection, blocking of initial Ig secretion by IFNγ in the absence of IL-4 may block maturation of the Ab response and subsequent passage of cells to the light zone.

The initialisation of dark zone proliferation appears to be the stage of B cell development affected to a lesser extent during infection. The induction of B cell proliferation and thus initiation of GC before Ig production was elegantly demonstrated by Hodgkin *et al* (1991) to be cytokine independent - B cell proliferation was initiated using membranes from both activated Th1 and Th2 T cells. It was only after such proliferation that Th2 cytokines - the ratio of IL-4 to IFNγ - became essential for Ig secretion. This is backed by the finding of Kosco and Gray (1992) that anti-IL-4 mAb did not stop initial clustering of B cells/APC/ T cells in an *in vitro* model. The cytokine independent induction of proliferation has been demonstrated to be initiated via CD40 expressed upon the surface of B cells (reviewed by Noelle *et al*, 1992). The ligand for CD40 is expressed by activated T cells and has been shown to be directly mitogenic for B cells (Armitage *et al*, 1993). The persistence of small GC with proliferating B cells in infected nodes suggests that this mechanism is intact, at least 8 days post infection.

The reduction of GC to virtually indistinguishable clusters of MIB 1^+ cells by day 10 of infection may be due to IFNγ mediated inhibition after the initial cytokine independent initiation of proliferation. In addition, the development of new GC is usually accompanied by the migration of IL-2^+ T cells, into primary follicles in order to provide "help" (Bogen *et al*, 1991; 1993). Such activated T cells are found in the medulla of infected lymph nodes, not the B cells zones, and have virtually disappeared at the time when T cells are normally seen in large numbers in follicles (day 11 post inoculation Bogen *et al*, 1991). The failure to generate new GC may therefore also be due to the lack of new local T cell help within the B cell zones.

In summary, studies of DLN have demonstrated that *in vivo* schizont infected cell development takes place broadly in two stages: rapid proliferation of infected cells accompanied by apoptosis, reaching a peak approximately 8 days post infection. This is followed by a decrease in cell cycling and apoptosis when large numbers of schizont infected cells become visible. This may represent further differentiation to microschizonts, an event associated with the loss of parasite induced host cell division. The rapid appearance of dividing cells within the medulla strongly suggests that the majority of cells infected are resident within the DLN rather than entering pre infected via other routes. The initial confinement to the medulla of infected cells is likely to represent a higher degree of target specificity than *in vitro* - cells with a surface phenotype which are readily infected in culture reside principally outwith the areas where infection is initiated.

T.annulata infection also leads to a profound alteration of T cell reactivity within DLN, with LN resident T cells being activated by infected cells. Such activation may aid evasion of the immune response, as activated efferent lymph T cells have previously been shown to be incapable of mediating anti parasite responses. Activated T cells generate of a Th1 type cytokine response which may subsequently affect "genuine" T cell activation events. The Th1 cytokines produced following "non specific" T cell activation may play a large role in pathology by aiding infected cells growth, interfering with germinal centre development, and causing pathology of the disease.

CHAPTER 6

Summary and Conclusions

SUMMARY AND CONCLUSIONS

The principal problem associated with tropical theileriosis is the extremely high mortality seen following infection of European cattle (40-90% mortality Robinson, 1982; Uilenberg *et al*, 1993; Hashemi-Fesharki, 1988). Such susceptible cattle seem incapable of mounting an effective immune response, with experimental infection often leading to death within 2-3 weeks (Samantery *et al*, 1980; Preston *et al*, 1992a). As drug treatment is expensive, and there are a number of problems associated with the live attenuated vaccine, current research into disease control is directed towards the production of a vaccine which does not involve live parasite material. One of the principal stumbling blocks in the development of such vaccines is the lack of knowledge about the mechanisms underlying the failure of the immune response to sporozoites in susceptible animals. Working towards a vaccine to prevent the induction of an unknown pathogenetic mechanism is somewhat difficult. The work in this thesis directed at understanding the interactions between the parasite and the host in naive susceptible cattle, with the aim of identifying the mechanisms of parasite survival and any effects these had on the developing immune response.

Most of the pathology of tropical theileriosis can be attributed to the macroschizont stage (Hooshmand-Rad, 1976), which develops in the draining lymph node. When infected animals are treated with Butalex, killing the macroshizont, the animal recovers (McHardy *et al*, 1985), is subsequently immune (Dhar *et al*, 1990) and treated animals generate anti macroschizont infected cell cytotoxic T lymphocytes (CTL) (Preston *et al*, 1983; Innes *et al*, 1989a Nichani, 1994). This restoration of immune function suggests that although the host is capable of generating an immune response, the parasite infection interferes with normal immune function. *In vitro* observations suggested that macroschizont infected cells (IC) may have an inherent capability to interfere with T cell development - IC induce proliferation of autologous resting T cells from both naive (Rintelen *et al*, 1990; Glass and Spooner, 1990a) and immune cattle and this phenomenon blocks the isolation of parasite specific T cells from the immune animals (appendix 2). It was postulated that inappropriate activation of T cells by parasitised cells may explain the inability of

the host to mount an effective immune response. This thesis has characterised in depth the activation effects of IC upon naive cells *in vitro*. Whether or not this phenomenon affected immunity *in vivo*, was examined in draining lymph nodes during sporozoite infection.

Examination of lymph node (LN) T cell populations showed that $\gamma\delta$ T cells formed an extremely small percentage (approx 4%) of LN cells (this study), and no expansion or phenotypic change in this population has been noted in studies of efferent lymph responses to *T.annulata* in naive or immune animals (Nichani, 1994). *In vitro* observations showed that the $\gamma\delta$ T cell subset of bovine PBM expressed extremely high levels of activation markers and an effector CD45RB low phenotype (Howard *et al*, 1991), simply upon culture with medium alone. It was therefore not possible to examine any IC induced activation effects upon these cells using phenotypic analysis. Due to these factors, discussion in this chapter concentrates on $CD4^+$, $CD8^+$ and NK cells, and the term "T cells" refers to $CD4^+$ and $CD8^+$ T cells only, unless otherwise indicated.

Interaction with T.annulata infected cells leads to T cell activation in vitro and in vivo

Incubation with autologous IC induced proliferation of PBM from naive animals, in agreement with Rintelen *et al* (1990) and Glass and Spooner (1990a). The latter authors had previously shown that T cells were the responding population. It was shown that both naive and memory/effector cells T cells were responding. Proliferation was first detected after 2-3 days, the time at which bovine memory T cells respond to recall antigens (Glass and Spooner, 1990b), and analysis of T cell subsets showed that CD45RB high $CD4^+$ cells, were the dominant $CD4^+$ T cell present at the day 5-6 peak of proliferation. The involvement of memory effector cells was confirmed by analysis of cytokine profiles from responding T cells - IL-2, IL-4 and IFNγ were produced by T cells within 24 hours of incubation with IC - naive bovine T cells are incapable of making such a cytokine response (Collins, 1993). As both naive and memory/effector T cells were induced to proliferate by IC

it seems likely that *T.annulata* macroschizont infected cells posses an ability to activate T cells irrespective of antigen specificity.

IC induced activation was primarily mediated through $CD4^+$ cells. The activation markers IL-2R and MHC class II on $CD4^+$ cells were expressed at consistently higher levels than medium controls, while this effect was not seen to a significant extent on $CD8^+$ T cells. This was confirmed when the cytokine profiles of $CD4^-$ PBM were examined after culture with IC in the absence of $CD4^+$ cells. While $CD4^+$ cells produced all the cytokine mRNAs found in whole PBM, $CD4^-$ cells only produced IFNγ at day 2 and none of the cytokine mRNAs at day 5. As IC cannot induce the IL-2/IL-2R autocrine cycle associated with T cell proliferation (Mauer *et al*, 1984; Smith, 1988) in $CD4^-$ cells it would seem unlikely that they possess an antigen capable of stimulating these cells. $CD4^-$ cells did however, contribute to the responses seen in whole PBM as they produced IL-2 and IL-2R mRNA when they were co-cultured with $CD4^+$ cells. It seems likely that they are dependent upon $CD4^+$ cells for an initial IL-2 stimulus.

Although the expression of IL-1 by IC is thought to be important in the induction of T cell activation (Brown *et al*, 1995) the proliferation of PBM and expression of activation markers on $CD4^+$ cells was blocked by separating the IC from the PBM by a porous membrane which did not block Con A activation. Activation of T cells therefore is not mediated by soluble factors alone. Indeed, as activation marker expression in membrane cultures was lower than in medium only controls, suppressive factors may be produced by the IC although their effect is only noticeable when the contact mediated activation is withdrawn.

Activation of PBM from naive animals by IC therefore leads to direct activation of $CD4^+$ T cells *in vitro* and, through IL-2 production, these cells induce activation of other cell types. As activated PBM from unexposed animals cannot contain parasite specific T cells, such activated cells although IL-2 responsive, are extremely unlikely to be of any use to the host in mounting an immune response. This resembles closely the responses found in PBM from immune animals (appendix 2), where activated IL-2 responsive T cells were also not specific for relevant parasite antigens.

T.annulata IC also activated $CD4^+$ T cells *in vivo*, with a distinctive clustering of

IL-2R$^+$ cells, shown by FACS analysis to be primarily CD4$^+$, around developing parasitised cells in the medulla of sporozoite infected LN. This was seen within 48 hrs of sporozoite infection. This is extremely unlikely to represent a genuine immune response. The induction of primary bovine lymph node CD4$^+$ T cell responses has been shown to be dependant upon interaction with Ag primed interdigitating cells (IDC) (McKeever *et al*, 1992). In other species T cells have been shown to interact with such APC in the paracortex 4-7 days post inoculation at the earliest (Bogen *et al*, 1991;1993). Given the incorrect anatomical location, and the extremely quick induction of large numbers of activated T cells (approx. 25% of all LN CD4$^+$ cells) the reactions seen in infected lymph nodes are likely to correspond to the "non specific" T cell activation seen *in vitro*.

A recent report described an elevation in IL-2 responsive IL-2R$^+$ CD4$^+$ T cells in lymph efferent from sporozoite infected nodes. However these cells did not appear to have any parasite specificity (Nichani, 1994). It seems likely that the IL-2 responsive T cells represent the parasite activated T cells, and this being the case, such cells correspond exactly to the T cells isolated using IC from the blood of immune animals (appendix 2) - IL-2 responsive but not parasite specific. This study set out to ascertain whether any role existed for non specific T cell activation in primary immune response failure to *T.annulata* sporozoite infection. As activation by IC leads to the production of IL-2 responsive but non parasite specific T cells both *in vitro* and *in vivo,* a role in immune response failure may be assigned to this phenomenon.

Consequences for immunity and pathology of parasite induced T cell activation

The early studies in this thesis showed evidence of a complete breakdown of immune responses in sporozoite infected LN which was characterised by a dominant population of infected cells, and a virtually complete loss of B cell germinal centres (GC). The lack of control over growth of macroschizont infected cells is likely to be attributable to the activation of a non parasite specific population of T cells - could this in some way affect GC? GC are formed from a complex interaction

between B cells, T cells and APC, with T cell cytokines playing an important role in development (reviewed by Kosco and Gray, 1992). In order to assess any role that IC activated T cell cytokines could have on GC, the cytokines produced by these T cells were examined along with a detailed study of GC morphology throughout infection.

The phenotypes of IC stimulated PBM suggested that IFNγ may have been playing a role in influencing activated cell phenotypes - both $CD8^+$ cells and NK cells acquired a $CD45RO^+$ phenotype which has been associated with activation in these cell types, and the acquisition of an IFNγ producing phenotype (Conlon *et al*, 1995; Sher *et al*, 1995). Cytokine analysis confirmed that both $CD4^+$ and $CD4^-$ cells produced IFNγ in IC activated PBM. IFNγ mRNA was produced in far higher quantities than IL-4. IC activated cells thus induce a type-1 cytokine response in PBM. This was dramatically demonstrated to also take place *in vivo*, as IL-4 mRNA production was completely lost from sporozoite infected nodes by the fourth day of infection, while IFNγ mRNA production was unchanged. Also, IFNγ production in lymph efferent from sporozoite infected nodes has been shown to increase during infection (Nichani, 1994).

In the light of this IFNγ dominated response from parasite activated T cells, the following mechanism of *T.annulata* pathology may be proposed (summarised in Fig 6.1), resulting in a loss of GC and aiding parasite growth:

Elevated IFNγ production, especially in a total absence of IL-4 is likely to account for the destruction of GC. GC were found to be arrested at the dark zone stage, with no B cells entering the light zone. Entering the light zone phase of the GC is dependant upon the expression of Ag specific Ig (Maclennan, 1994). B cells are particularly sensitive to IFNγ after initial proliferation, before Ig is expressed (Abed *et al*, 1994a), a lack of IL-4 also inhibits Ig production (Armitage *et al*, 1993; Donckier *et al*, 1994). Parasite activation of T cells to produce an IFNγ dominated cytokine response is therefore a strong candidate mechanism for failure of B cell responses during sporozoite infection.

IFNγ production by T cells may also contribute directly to the survival of the parasite. *T.annulata* infection induces the division of the host macrophage, a cell

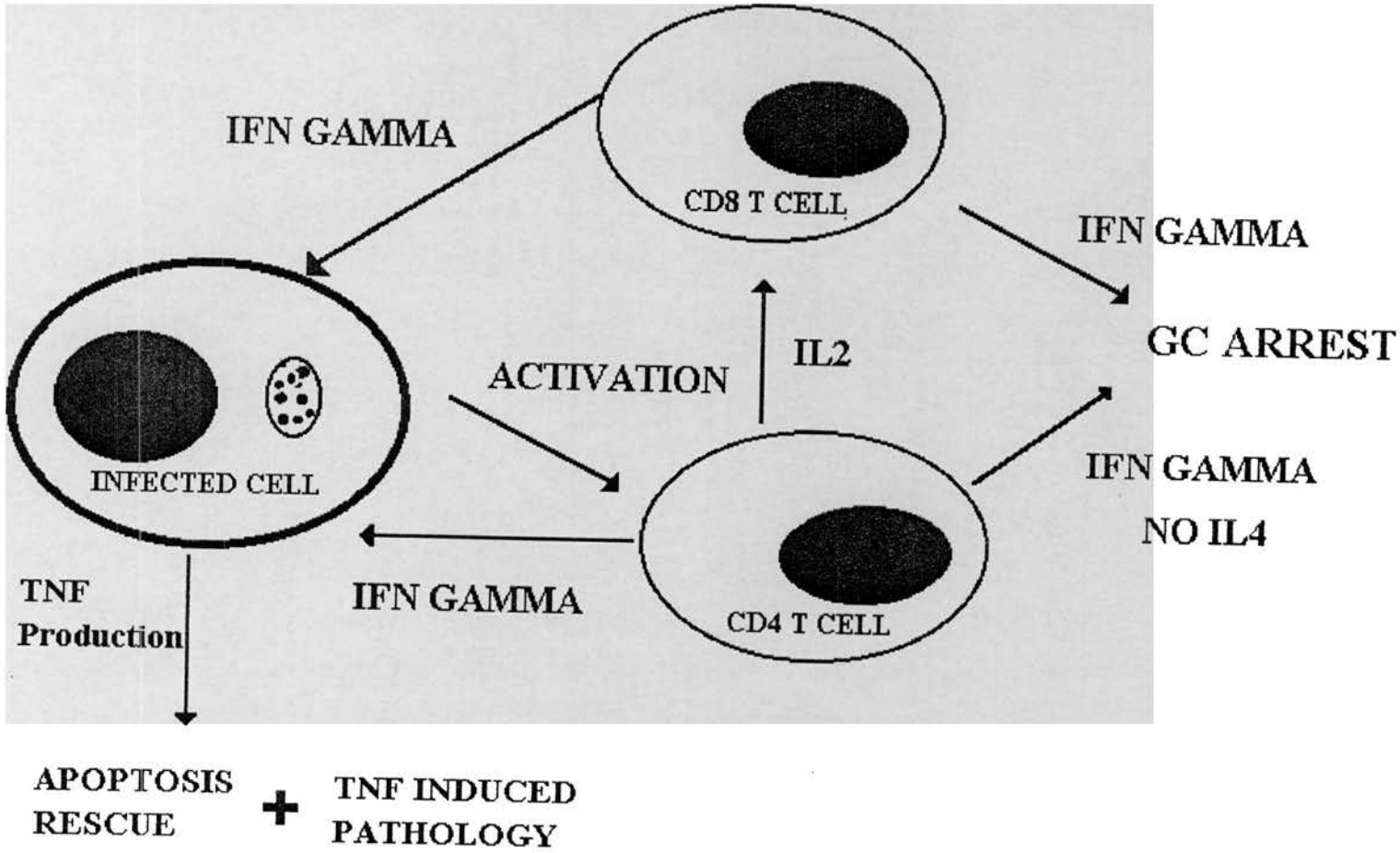


Fig. 6.1 Proposed mechanism of T cell activation induced pathology.

type which readily apoptoses when induced to divide (Cotter *et al*, 1994). A large rise in apoptotic cells was seen in infected nodes. Macrophage rescue from apoptosis can be mediated by IFNγ (Mangan and Wahl, 1991), thus parasite activated T cells are induced to express a cytokine which should aid parasite survival. Rescue of macrophages by IFNγ induces the production of TNFα (Mangan and Wahl, 1991) and fully differentiated *T.annulata* infected macrophages express mRNA for this cytokine (Brown *et al*, 1995). The symptoms of *T.annulata* infection correlate very well with those induced by TNFα (Bielefeldt Ohmann *et al.*, 1989; Silegham *et al*, 1994). It is therefore also extremely likely that activation of T cells to produce IFNγ at least boosts the TNFα producing capacity of macrophages, infected or otherwise, contributing to disease pathology.

This thesis set out to evaluate any possible role for interactions between *T.annulata* infected cells and T cells in immune response failure during sporozoite infection. The results have shown that macroschizont infected cells can activate $CD4^+$ T cells irrespective of T cell memory status, and through these $CD4^+$ cells also activate $CD8^+$ and NK cells. Activated PBM produce a cytokine profile which is predominantly type-1, characterised by IFNγ mRNA production. T cell activation in sporozoite infected LN is extremely similar to that seen *in vitro*, with infected cell induced T cell activation dominant in the early stages of infection. Such activated cells do not represent the induction of a genuine immune response. A strong type-1 cytokine response ensues with a complete loss of IL-4 mRNA production in the node. This IFNγ response is a prime candidate mechanism for the destruction of GC seen in infected nodes, and is also likely to contribute to infected cell growth and TNFα mediated pathology. Thus, non specific T cell activation by *T.annulata* not only blocks immune responses by interfering with the induction of genuinely antigen specific T cells, but the cytokines from aberrantly activated cells are likely to exacerbate disease pathology.

The findings in this thesis have serious implications for the future design of vaccines. If any antigen is to be used to induce a protective immune response, it would be highly unlikely to succeed if the T cells responding to it produced large amounts of IFNγ. Also, the use of a peptide solely to induce CTL effector mechanisms may not

be effective, as their response would be developing in line with the aberrant $CD4^+$ T cell activation. The efficacy of any vaccine based on merozoite antigens would be highly questionable, as this work establishes that pathogenetic mechanisms are already operating well before merozoite formation. Sporozoite antigens which can induce neutralising antibodies have not been successful in protecting against sporozoites (Boulter *et al*, 1995). Pursuing these Ag as vaccines would seem questionable - only a small minority of the inoculated sporozoites would need to evade antibody, infect macrophages, and start pathogenesis mechanisms. Also, if used in field conditions, sporozoite infection before antibody responses have formed could actively suppress a protective response through IFNγ interference with GC formation.

To make full use of the findings discussed here, vaccine design should perhaps concentrate on identification of the trigger responsible for non specific activation. Characterisation of this element is essential if strategies are ever to be developed to bypass its effects. The most realistic goal in this area would be to identify the Ag and its presentation pathway in order to try and block its effects. Most readily applied *in vitro*, blocking the activation effect could lead to isolation of genuinely antigen specific T cells. A combination of protective antigens recognised by $CD4^+$ and $CD8^+$ T cells has the potential to effective. This is presumably the combination used in immune animals, although blocking of the "non specific" element may also be required.

The association of pathology with T cell activation has also some potentially immediately beneficial outcomes. A major problem in assessing the suitability of an infected cell line for vaccine use is that there is currently no method available to establish the degree to which it has been attenuated. Although direct production of TNFα may partially cause a cell line to be pathogenic, T cell activating capability is also likely to be a factor in pathogenesis. There is also a current desire to investigate mechanisms underlying natural resistance in species native to endemic areas. The relationship established between T cell activation and pathogenesis established in this thesis is also likely to be of use here. For example, T cells of resistant cattle may be refractory to *T.annulata* non specific activation or produce

different, helpful cytokines upon activation. The *in vitro* techniques developed in this thesis which largely mimic the response seen *in vivo* would be of use in testing such hypotheses without the use of full scale animal trials.

Chapter 7

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APPENDIX

Chapter 2. GENERAL MATERIALS AND METHODS

PBS 0.9% NaCl, 0.107% Na_2HPO_4 and 0.051% NaH_2PO_4 (all Fisons) w/v aqueous soln. pH 7.2.

Complete TC medium RPMI-1640 medium containing 25mM Hepes, supplemented with 2mM glutamine, 100 IU/ml penicillin,100 μg/ml streptomycin (all Gibco BRL), and 10% foetal calf serum (Meridien or Globepharm labs)

ACD 2.5% D-Glucose and 2.05% Di-Sodium Hydrogen Citrate (Both Fisons) w/v aqueous soln.

MEM Eagle's minnimum essential medium supplemented with Hanks' Bakanced Salts, 200IU/ml penicillin, 200μg/ml streptomycin, and 100μg/ml nystatin (all Gibco BRL).

Chapters 3 and 4. *IN VITRO* STUDIES/PCR

MLC RPMI 1640/25mM HEPES supplemented with 10% FCS, 2mM L-Glutamine, 100μg/ml Gentamicin (Gibco) and $5x10^{-5}$M β-Mercaptoethanol (Sigma)

RNA gel 2g agarose (Sigma), 140ml dH_2O, 40ml paraformaldehyde (Sigma), and 20ml 10X MOPS buffer.

10X MOPS 0.2M MOPS (3-(*N*-morpholoino)propansulphonic acid), 50 mM sodium acetate, 10mM EDTA (all Sigma) pH7, autoclaved and stored in the dark.

Deionised formamide (Fisons) prepared by adding 1g/100ml Amberlite MB-1 resin (BDH), mixing for one hour, and filtering twice through Watman no. 1 filter paper to remove the resin. Deionised formamide was stored at -20°C.

Loading buffer 50% v/v glycerol (Fisons), 1mM EDTA (Sigma), 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF.

PCR buffer (Gibco) 200mM Tris-HCl ph. 8.4, 500mM KCl

dNTPs (Pharmacia). 100mM stock diluted to 10mM in TE buffer.

TE 10mm Tris-HCl pH 7.6/1mM EDTA

Agarose Sigma type 1-A

TBE 10x stock solution: 108g Tris, 55g boric acid, 40ml 0.5M EDTA (pH8) in 1 litre dH_20, Eqivalent to 0.045 Tris borate, 0.1mM EDTA when diluted to 1x.

Paraffin oil Fisons liquid paraffin 0.860-0.890

HaeIII size markers (Sigma) HaeIII digest of PBR322 plasmid.

Chapter 5. LYMPH NODE STUDIES

Eosin Soln. aqueous solution of 1% w/v Eosin and 1% w/v Phloxine (BDH)

Toluidine Blue Soln. 1% w/v Toluidine blue in 1% w/v aqueous soln. Borax, (both BDH)

TBS 0.9% NaCl buffered with 50mM Trisma base (Sigma), adjusted to pH 7.6 with 1N HCl.

Trypsin Digestion Soln. 0.1%w/v Trypsin 1:250 (DIFCO, Detroit, USA) and 0.1% $CaCl_2$ (BDH, Poole, UK) in distilled H_2O.

Pronase Digestion Soln. 75mg per 100ml Pronase (Sigma) in TBS. Equivalent to 4.1 U/ml.

Citric Acid Soln. 1.05g citric acid (BDH) in 500ml dH_2O, pH 6

DAB substrate Buffer 50mM Tris-HCL pH 7.6 plus 0.01M Imadizol (Sigma). Add 5mg DAB and 1 drop 30 vol H_2O_2 to 5ml buffer just before use.

Pepsin Digestion Soln. 0.5% pepsin (Sigma) in 0.9% NaCl solution pH 6.

Propridium Iodide Soln. 100μg/ml aqueous soln. propridium iodide (Sigma) with 0.04% RNAse. (Sigma RNAse A from bovine pancreas).

2 - Attempts to generate *T.annulata* specific $CD4^+$ T cell lines *in vitro*

This appendix describes research carried out by the author in conjunction with Dr. K.A. Odling and Dr. E.J. Glass from 1990-1992.

Introduction

By 1990, it was well established that *Theileria parva* macroschizont infected cells induced proliferation of PBM from autologous naive animals (first examined by Pearson *et al,* 1979, discussed in detail by Goddeeris and Morrison, 1987). This ability to activate PBM is referred to as the "*theileria* autolgous MLR reaction". However, this MLR effect had not proved to be a barrier to the generation of MHC restricted *T.parva* specific $CD8^+$ or $CD4^+$ T cell lines and clones from the blood of immune animals undergoing sporozoite challenge (Goddeeris *et al*, 1986; Baldwin *et al,* 1987; Goddeeris and Morrison, 1988). Thus the autologous MLR effect did not prevent specific T cells from recognising parasite antigens via MHC class I and MHC class II on macroschizont infected cells.

Although a similar "MLR" effect had been noted with *T.annulata* infected cells (Rintelen *et al*, 1990; Glass and Spooner, 1990), this did not block or inhibit APC function in autologous *T.annulata* infected macrophages which were able to present soluble antigens to antigen specific T cell lines (Glass and Spooner, 1990). If any effect was present, the MLR stimulatory element appeared to increase the antigen specific response (Glass and Spooner, 1990). As the *T.annulata* autologous MLR did not interfere with presentation of third party soluble antigens via MHC class II, it was reasonably assumed that this phenomenon should not affect the presentation of parasite antigens through this pathway. Attempts were therefore made to generate *T.annulata* specific $CD4^+$ T cell lines from the blood of immune animals undergoing sporozoite challenge using macroschizont infected cells.

Similar studies were carried out by A.K. Nichani and R.L. Spooner to generate $CD8^+$ cytotoxic T cell lines from immune animals. These studies used similar techniques to those described below, and their findings are included in the discussion.

Experimental protocol

Based on protocols used for generation of *T.parva* specific lines and clones (Baldwin *et al*, 1987 (CD4); and Goddeeris and Morrison, 1988 (CD8)) and antigen specific T cells using *T.annulata* infected cells (IC) (Glass and Spooner, 1990), PBM were isolated from 3 previously sporozoite immunised and butalex treated animals undergoing further sporozoite challenge. PBM were incubated with autologous irradiated IC at IC:PBM ratios of 1:10 or 1:20 for 7 days and viable cells isolated using ficoll-hypaque. Isolated cells were then alternately incubated weekly with 20IU/ml rhuIL-2 and autologous irradiated PBM or autologous IC. Attempts were made to clone T cells by limiting dilution and incubation with rhuIL-2, IC and irradiated PBM 2, 4, 6,and 8 weeks post initial stimulation.

Summary of results

Lines were generated from 3 immune animals, and as the results obtained from each line were similar, the data from only one animal is presented in detail. The animal described here, 11434, was immunised with sporozoites 4 times, and attempts to generate lines were made from the second immunisation on. After initial stimulation with IC, large numbers of blasting T cells were recovered, which expanded vigorously in rhuIL-2. Tritiated thymidine incorporation assays using IC as stimulators demonstrated that the freshly isolated PBM proliferated vigorously. However, when compared to autologous MLR proliferation from a naive animal with an identical MHC type (animal 11435, homozygous twin of 11434), proliferation was no higher in the immune animal (Fig. 1).

In initial experiments, culture with IC produced T cell lines containing both $CD4^+$ and $CD8^+$ cells, and the ratio of approximately 2 CD4:1 CD8 cells found in PBM was maintained (Fig.2a & b). No expansion of gamma delta T cells from the percentages found in PBM was observed (results not shown). It was therefore decided to deplete $CD8^+$ cells using mAb CC63 and complement in order to enrich $CD4^+$ T cells. Depletions were carried out either from fresh PBM, or after 2 weeks of culture. When CD8 depleted PBM were used, lines $\geq 85\%$ $CD4^+$ were produced. However, it was not possible to produce lines which were stable in culture and the

mean survival time of lines made from whole PBM or CD8 depleted PBM was the same (PBM=4.9 weeks (n=7);$CD8^-$=5 weeks (n=7) ie. 3 stimulations with IC). Death of cells was synchronous eg. a culture containing approximately $2x10^8$ blasts after IL-2 expansion was found to contain $\leq 10^6$ viable cells 48hrs after culturing with IC. Death of cultures usually occurred during the 3rd stimulation with IC. In total, over 30 lines were established from 3 animals, and all died within 2 months. Clones were only ever established from cells cloned 2 weeks after first stimulation. On three occasions it was possible to establish clones from $CD4^+$ lines, and two of these clones survived their establishing lines by 4 weeks, became $\geq 95\%$ $CD4^+$ (Fig. 2c), but died before sufficient numbers could be produced to test their specificity.

Conclusions

Using standard T cell culture methods, it did not prove possible to isolate stable lines or clones of $CD4^+$, *T.annulata* specific T cells. Similarly, no $CD8^+$ cells which lysed macroschizont infected cells could be generated by Nichani and Spooner. Thus, although T cells from immune animals were induced to proliferate by autologous IC, and became IL-2 responsive, this stimulation did not produce either $CD4^+$ or $CD8^+$ effector cells. It seems unlikely that the few clones which survived for up to 4 weeks after their parent line represent genuinely specific cells - *T.parva* specific T helper clones were stable throughout repeated restimulations, with up to 3 weeks steady growth before fresh stimulators were required (Baldwin *et al*, 1987).

T.annulata immune animals can clear parasite via T cell mediated immune mechanisms, with macroschizont specific CTL present in the blood and efferent lymph (Innes *et al*, 1989; Nichani, 1994). It therefore seems extremely unlikely that the failure to expand such cells *in vitro* was due to a lack of suitable precursors. The most likely explanation for the observed proliferation in *T.annulata* activated cells, is that the autologous MLR stimulating element is sufficiently strong to interfere with the generation of parasite antigen restricted T cells. To induce proliferation without the generation of "cytolytic lymphocytes" ie genuine effector cells, mirrors exactly the results of Pearson *et al* (1979) when describing the effects of the *T.parva* autologous MLR in naive animals and is apparently the result using PBM from

immune cattle in *T.annulata*. Although some genuine activation may have occurred, the "non specific" activation of irrelevant T cells, and their subsequent IL-2 responsiveness appears to have led to the selection of these cells rather than establishing parasite antigen specific T cells

It is interesting to note that the autologous MLR did not override the recognition of soluble antigen using 100% antigen specific T cell lines (Glass and Spooner, 1990a). However, these lines were generated using standard PBM APC, and were only restimulated once using IC, when they were established antigen specific lines, specifically for APC assays. This may further support the view that some parasite specific T cells are generated in naive PBM, but are overwhelmed by non specifically activated cells - the "non specific" activation could not operate when interacting with a homogeneous population of T cells actively responding to a single antigen.

It can therefore be concluded that the ability of *T.annulata* macroschizont infected cells to activate T cells "non specifically" is sufficiently powerful to override genuine recognition of parasite antigen by specific T cells. In this respect, *T.annulata* is distinctly different from *T.parva*, as specific T cells can be selected using macroschizont infected cells of the latter parasite.

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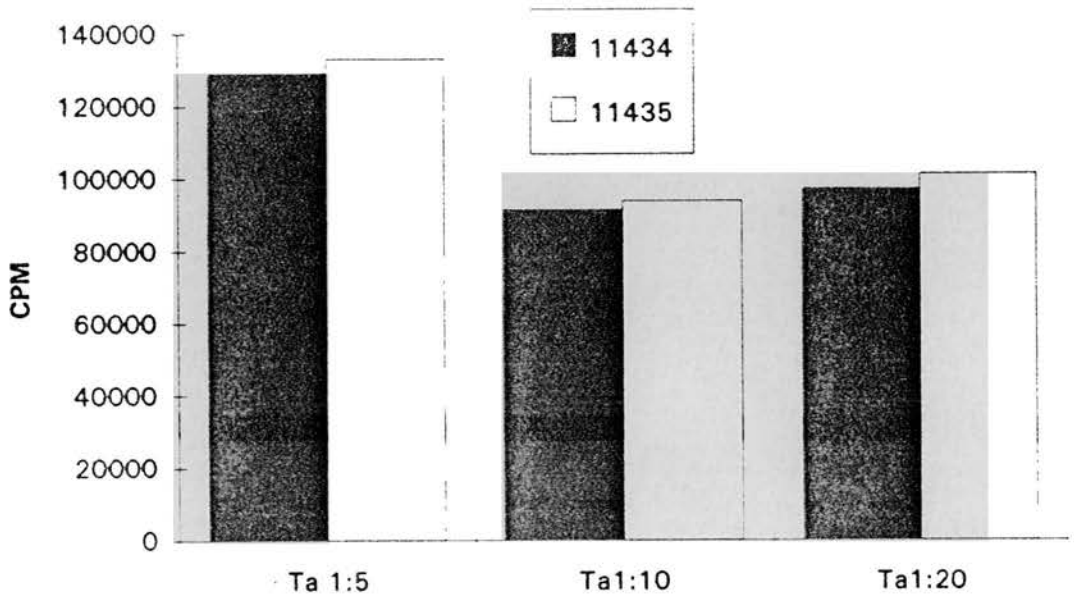

Fig. 1 Proliferation in freshly isolated PBM to graded numbers of autologous *T.annulata* macroschizont infected cells after 5 days incubation is no higher in an immune animal (11434) than in its naive twin (11435). Tritiated thymidine uptake, expressed as counts per minute (CPM).

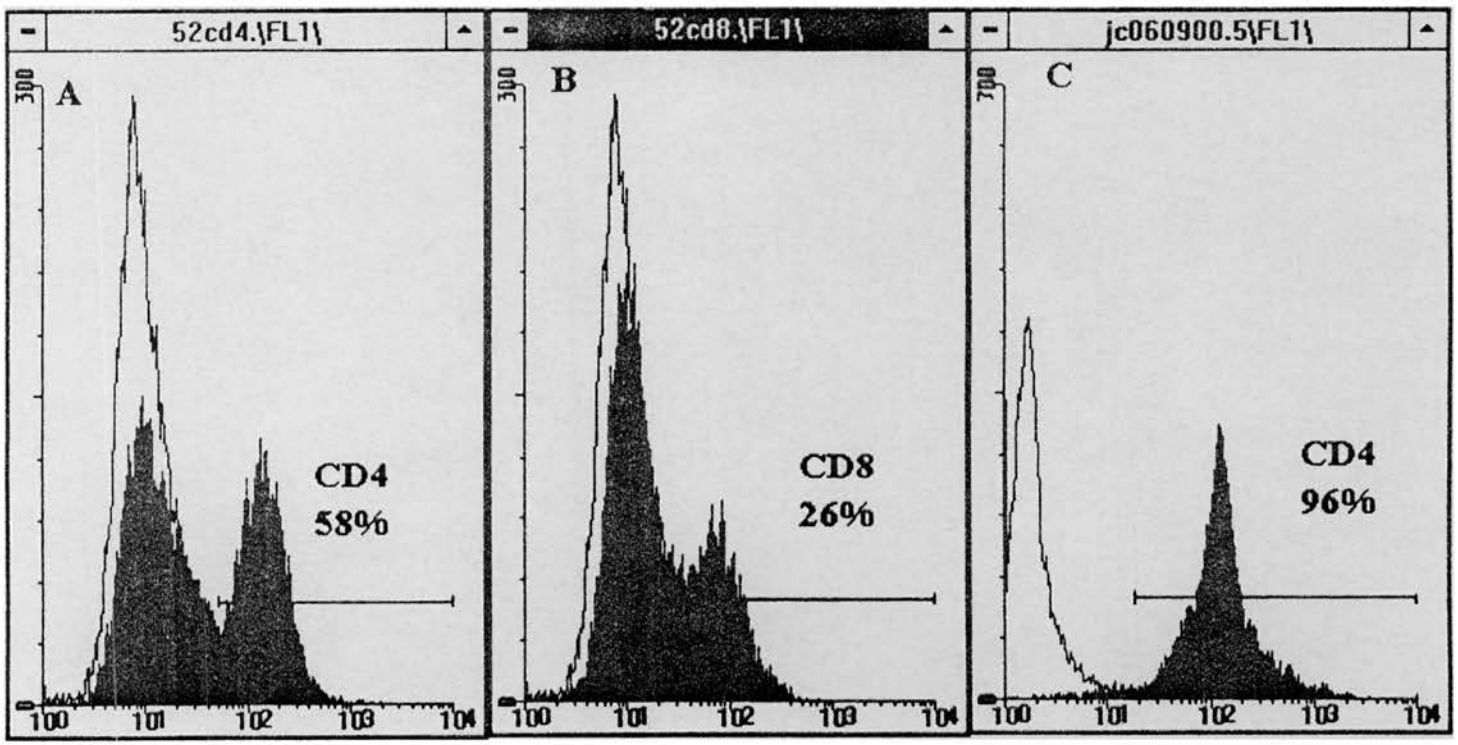

Fig. 2. A+B. After 4 weeks *in vitro* stimulation, a T cell line consists of 58% CD4 T cells and 26% CD8, maintaining the approximate 2:1 ratio found in peripheral blood.
C. After depletion of CD8 cells using mAb and complement, a T cell clone consisting of ≥ 95% CD4 cells was derived. (but it still died!).

Publications

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Brief communication

Theileria annulata sporozoite targets

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SUMMARY

Bovine peripheral blood mononuclear cells (PBM) infected in vitro *with* Theileria annulata *sporozoites have previously been characterized as MHC class II$^+$ mature macrophages. The ability of* T. annulata *sporozoites to infect different subpopulations of MHC class II$^+$ bovine monocytes was investigated. Cells were labelled with monocyte specific monoclonal antibodies (MoAb) and isolated using magnetic cell sorting (MACS). Sporozoites infected both immature and mature monocytes, but more readily infected the mature population. A potential ligand for sporozoite entry is the elastin receptor which is expressed mainly on the immature population of monocytes and not on B cells or T cells.* T. annulata *sporozoites infected elastin receptor positive and negative cell populations equally well. Infected immature cells lost the expression of elastin receptors and the immature marker, subsequently expressing the mature marker. All monocytes lost the expression of CD14 (the LPS receptor) upon infection with sporozoites. The infection of specific populations and subsequent alterations in phenotype may alter the function of these cells and play an important role in disease pathogenesis.*

Keywords T. annulata *monocytes, FACS analysis, MACS separation, limiting dilution*

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INTRODUCTION

Theileria annulata is a tick transmitted protozoan parasite which affects cattle in Southern Europe, North Africa, India, the Middle East and Southern Russia, causing tropical theileriosis, a life threatening disease. We have shown *in vitro* that sporozoites invade major histocompatibility complex (MHC) class II$^+$ cells, particularly cells of the macrophage/monocyte lineage and also, though much less efficiently, B cells (Spooner *et al.* 1989, Glass *et al.* 1989). Whatever the origin of the initial infected cell, all cell lines apparently had a similar phenotype as judged by the available markers. Infected cells were nonspecific esterase$^+$ plus MHC class II$^+$ and surface IgM$^-$ and T cell marker$^-$ (Spooner *et al.* 1988, 1989, Glass & Spooner 1990). The monoclonal antibody (MoAb) IL-A24 detects a determinant on macrophages associated with antigen presentation (Ellis *et al.* 1987). This ligand is also expressed upon sporozoite infected cells, which can present antigen via MHC class II (Glass & Spooner 1990).

Distinct ligands upon the surface of the target cells may be required for sporozoite attachment and invasion. Identification of these ligands and further characterization of the primary cells which sporozoites infect could be of prime importance in understanding the pathogenesis of this disease and the variation seen in responses to currently used cell line vaccines (Ouhelli *et al.* 1989).

This study extends our previous findings and more fully characterises the phenotype of cells infected by *T. annulata*, using three recently available MoAbs to potentially relevant surface antigens. The MoAbs are IL-A109, VPM65 and BCZ.

IL-A109 recognizes monocytes and macrophages and is thought to recognize an antigen analogous to CD64 (FcR1) (MacHugh, McKeever & Goddeeris 1990).

VPM65 recognizes ovine CD14 (VK Gupta, personal communication) and crossreacts with bovine macro-

phages and B cells, although cell sorting using this MoAb isolates only monocytes/macrophages (see below) (D.J.Brown, unpublished observations). In humans, CD14 is a 55 kDa glycoprotein expressed as a cell surface molecule on macrophages and a small population of B cells, and acts as a receptor for LPS (reviewed in Zeigler-Heitbrock & Ulevitch 1993).

BCZ recognizes the elastin receptor (Mecham *et al.* 1989). Elastin binds to its receptor via a repeating hexapeptide: Val-Gly-Val-Ala-Pro-Gly (VGVAPG) (Mecham *et al.* 1989). Recently, the gene coding for a *T. annulata* sporozoite surface antigen (SPAG-1) involved in invasion of cells has been isolated, expressed in *E. coli* (Williamson *et al.* 1989), and found to contain VGVAPG (Hall *et al.* 1992). Thus the elastin receptor is a candidate ligand for sporozoite binding.

Using these novel MoAbs, bovine monocyte/macrophage cells from PBM were characterized, their susceptibility to sporozoite infection assessed, and the subsequent phenotype of the infected cells examined.

Characteristics of peripheral blood monocyte populations

Peripheral blood mononuclear cells (PBM) from Fresian cows were obtained by Ficoll-Hypaque separation (Spooner *et al.* 1989). *T. annulata* does not infect T cells (Spooner *et al.* 1989, Glass *et al.* 1989), and preliminary studies showed that $CD4^+$ and $CD8^+$ T cells are not stained by BCZ, VPM65, IL-A109 or IL-A24 (results not shown). In order to enrich cells of interest in PBM, T cells were removed by lysis with MoAbs CC8 (CD4) and CC63 (CD8) (Howard *et al.* 1991), and rabbit complement (Seralab) according to the method of Teale *et al.* (1986). The resulting T $cell^-$ population was enriched for B cells and monocytes.

T cell-PBM were stained by indirect immunofluorescence and analysed with a FACScan essentially as described previously (Glass & Spooner 1990). Double staining was performed by first incubating with both MoAbs (of different isotypes) followed by appropriate fluorescent secondary reagents: Goat anti mouse (GAM) IgM-FITC; GAM IgG-PE (Sigma, Poole, UK); GAM IgG_1-FITC; GAM IgG_{2a}-PE (Seralab).

The size of the populations stained by the MoAbs was determined by the cells' forward scatter (FSC) upon the FACScan. The majority of IL-$A109^+$ monocytes were small monocytes (mean FSC = 33; Figure 1a) whereas IL-$A24^+$ monocytes were large (mean FSC = 77; Figure 1b). VPM65 recognized both IL-$A109^+$ and IL-$A24^+$ cells. MACS sorted $VPM65^+$ cells (see below) contained three distinct populations in terms of size—small, intermediate and large monocytes respectively (see Figure 1d). Phenotyping of these cells confirmed that three distinct phenotypes were present IL-$A109^+$/IL-$A24^-$ (small); IL-$A109^+$/IL-$A24^+$ (intermediate); IL-$A109^-$/IL-$A24^+$ (large). It is likely that the small IL-$A109^+$ monocytes are immature, becoming larger and IL-$A24^+$ upon activation and maturation.

BCZ stained approximately 16% of T cell depleted PBM, and the majority of these cells (82%) had a distinctive low FSC (mean FSC = 31; Figure 1c) similar to IL-$A109^+$ cells. Double staining with BCZ and macrophage markers revealed that this population was identical to the IL-$A109^+$/IL-$A24^-$ cells described above. The remainder of the BCZ^+ cells were contained in both IL-$A24^+$ populations. B cells were negative for BCZ (results not shown). Thus the elastin receptor is mainly expressed on a discrete population of monocytes and not T or B cells in peripheral blood.

Purification and infection of monocytes

The populations identified by BCZ, VPM65 and IL-A109 were purified using magnetic cell sorting (MACS sorting: see Miltenyi *et al.* 1990 for a review of this method), of T $cell^-$ PBM. The cells were labelled using either VPM65 or BCZ at 1/100 dilution of ascitic fluid, followed by the appropriate MACS bead conjugate (e.g. rat anti-mouse IgG1). The magnetic cells were run through the column twice, and the purity of the separated cells was always > 90%, and usually > 95%. Insufficient IL-A109 was available for MACS sorting. Therefore IL-$A109^+$ monocytes were produced by negative selection in the following way: B cells and T cells were removed by MACS sorting using IL-A30, CC15, CC8 and CC63 (anti sIgM, WC1, CD4 and CD8 respectively. Howard *et al.* 1991). The resulting pure monocytes were subsequently MACS sorted using IL-A24. The IL-$A24^-$ population was 100% IL-$A109^+$ and is referred to in the text as IL-$A109^+$.

The positive and negative cell populations obtained were infected with sporozoites of *T. annulata* (Gharb & Ankara strain) (Brown 1987) in $\log_3$ dilutions, and infection was assessed as previously described (Spooner *et al.* 1989). Frequencies of cells which were transformed were calculated according to Lefkovits & Waldmann (1979) and Brett, Kingston & Colston (1987). Statistically significant differences between cell populations were calculated using analysis of variance. Transformation frequencies of the sorted cells are detailed in Table 1.

Although T cell depleted PBM (T $cell^-$) were not significantly more infectable than PBM ($P < 0{\cdot}1$),

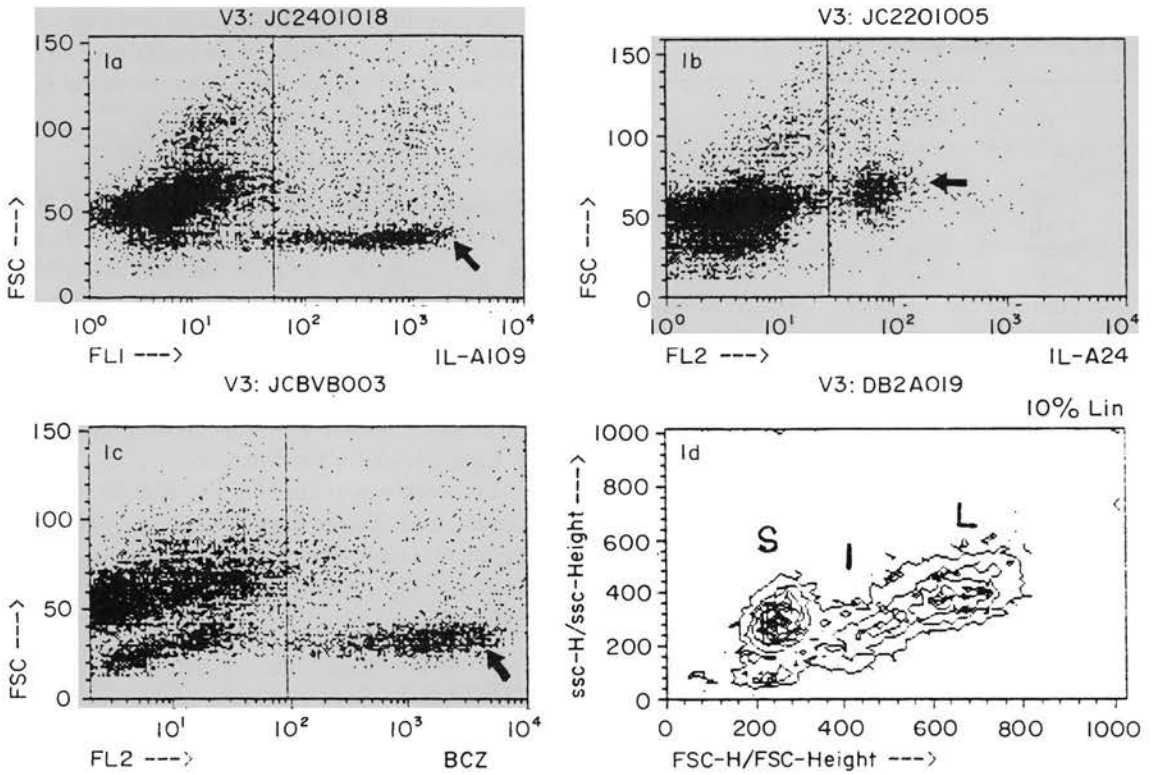

Figure 1 FACS analysis of T cell depleted PBM and CD14$^+$ MACS sorted cells. a–c: Dot plots of T cell depleted PBM stained with a—IL-A109. The majority of cells (arrow) have a low forward scatter (FSC). Mean FSC = 33. b—IL-A24. Stained cells (arrow) have a much larger mean FSC (77) than IL-A109$^+$ cells. c—BCZ. The majority of BCZ$^+$ cells (arrow) have a similar mean FSC (31) to IL-A109. Positive/negative regions are set upon negative controls. d—FSC *v.* SSC contour plot of VPM65$^+$ MACS sorted cells showing small (S), intermediate (I) and large (L) cells.

removing T cells did substantially increase the precursor frequency of infectable cells. Both BCZ$^+$ and BCZ$^-$ cells were highly infectable with *T. annulata*. Thus although the elastin receptor may be a ligand for sporozoite attachment it certainly is not the only ligand. Sporozoites efficiently infected CD14$^+$ cells, IL-A24$^+$ monocytes and IL-A24$^-$ monocytes but not CD14$^-$ cells (primarily B cells). Taken together, these results confirm the parasite's marked preference for bovine monocytes. We have previously shown that IL-A24$^+$ cells are major targets for *T. annulata* with a very high infection frequency (1 : 9 within an individual experiment) (Glass *et al.* 1989). The results in this paper show that *T. annulata* preferentially infects these mature monocytes, although it also efficiently infects the more immature IL-A109$^+$ monocytes. Many monocytes within bovine lymph nodes are IL-A109$^+$ (J.Campbell, S.Howie, E.Glass, manuscript in preparation). As the draining lymph node is a major site of parasite development *in vivo* the demonstration of cells within lymph nodes which are potentially infectable with sporozoites is an important finding.

FACS analysis of sporozoite infected cell lines

Cell lines generated from the above cell subpopulations were phenotyped once sufficient infected cells had grown—usually within two weeks. All cells lines were from individual 200 μl wells but not necessarily clonal.

All cell lines produced from *in vitro* infections had the phenotype 100% IL-A24$^+$ and MHC class II$^+$. These results are similar to those reported previously (Spooner *et al.* 1989, Glass *et al.* 1989). Thus even the cell lines derived from the IL-A109$^+$ population had become IL-A24$^+$. Only one of three IL-A109$^+$ cell lines examined contained any IL-A109$^+$/IL-A24$^+$ cells (approximately 25%). Within ten days of first being analysed, this line became IL-A109$^-$. All infected cells were found to be completely negative for CD14 despite this marker being expressed on all monocytes and the CD14$^+$ cells being $>$ 95% VPM65$^+$ when infected. In addition, four *T. annulata* infected cell lines derived previously from *in vitro* infection of PBM had similar phenotypes (results not shown). None of the lines expressed CD14 as

Table 1 Frequency of cells transformed by *T. annulata* as determined by limiting dilution analysis

Experiment	Cell population	Frequency of transformed cells	*P* value	Parasite
1	PBM	1:1055	a	Gharb
	$T\ cell^-$	1:297	a	
	BCZ^-	1:38	b	
	BCZ^+	1:20	ND	
2	PBM	1:771	a	Ankara
	$CD14^+$	1:116	b	
	$CD14^-$	1:6897	a	
3	PBM	1:330	a	Gharb
	$IL\text{-}A24^+$ monocytes	1:16	b	
	$IL\text{-}A109^+$ monocytes	1:52	a	

[1]Within each experiment the same letter denotes no significant difference between frequencies; different letters denote significant differences between frequencies ($P < 0{\cdot}05$).

detected by VPM65. Low numbers of $IL\text{-}A109^+$ and BCZ^+ cells were detected in two of the cell lines (11% and 18%) and this expression was only seen at low passage. Thus it appears that whatever the initial phenotype of the monocytes infected, infected cells become $IL\text{-}A24^+$, $IL\text{-}A109^-$, BCZ^- and $VPM65^-$.

Since expression of the determinant recognised by IL-A109 and elastin receptors appears to be restricted to immature monocytes, this evidence suggests that sporozoite infection and subsequent transformation may induce differentiation to a specific phenotype similar to mature monocytes with concomitant loss of 'immature' markers. Alternatively, since the infected cells were not clonal, $IL\text{-}A24^+$ cells may be the only cells transformed, subsequently out growing all others. We favour the former, as the loss of CD14 expression shows that sporozoite infection does induce phenotypic change. As $IL\text{-}A109^+$ cells were produced by removing $IL\text{-}A24^+$ cells, infection cannot be attributed to $IL\text{-}A109^+/IL\text{-}A24^+$ cells. In addition, the 12% of $IL\text{-}A24^+$ cells in PBM (see Figure 1b) gave a precursor frequency of 1:330, the precursor frequency of 1:52 observed in the $IL\text{-}A109^+$ cells would require an extremely high number of contaminating $IL\text{-}A24^+$ cells.

The loss of particular markers may alter the function of parasitized monocytes. For example the loss of FcR may affect the cell's ability to take up antigen and the loss of CD14 may alter the ability of parasitized cells to respond to activation through the LPS receptor pathway. Indeed, we have previously shown that parasitism by *T. annulata* alters antigen presenting function (Glass & Spooner 1990). We are currently investigating some of these phenotypic changes as they may play an important role in the pathogenesis and outcome of the disease.

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The influence of MHC polymorphism on the selection of T-cell determinants of FMDV in cattle

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SUMMARY

There is a quest for the development of a new generation of vaccines consisting of well-defined subunit antigens. For a number of practical reasons it is attractive to develop vaccines on the basis of synthetic peptides. However, their efficacy may be limited by genetic restrictions imposed on T-cell recognition via major histocompatibility complex (MHC) polymorphism, as shown by many studies using inbred animal species. To study the effect of MHC polymorphism in an outbred species, we selected four cattle homozygous for different A-DR-DQ haplotypes, and another four cattle which shared one haplotype in combination with a haplotype of one of the MHC homozygous animals. We analysed responses to synthetic peptides comprising defined T-cell epitopes of foot-and-mouth disease virus (FMDV) in this selected group of FMDV-vaccinated cattle. This analysis shows that even in outbred animals, MHC polymorphism influences the responses to synthetic peptides. Interestingly, one of the peptides, VP4[20–34], was recognized in association with at least four different MHC haplotypes. Fine specificity analysis of this peptide revealed subtle shifts in the core epitope recognized. All peptides that induced lymphocyte proliferation *in vitro* were found to induce a T-helper type-1 (Th1) type of response, irrespective of the MHC haplotype involved. Together, these data support the notion that individuals carrying distinct MHC types can be vaccinated successfully by vaccines that include only a limited number of peptides. In the design of a peptide vaccine against FMDV we suggest inclusion of the highly conserved VP4 sequence 20–34.

INTRODUCTION

One of the novel approaches in vaccine research is the development of vaccines consisting of well-defined subunits of pathogen-derived antigens. Most attractive is the development of vaccines based on synthetic peptides comprising one or more B-cell epitopes and one or more T-cell epitopes of the pathogen. The advantages of such peptide vaccines include reduced costs and improved safety and quality in comparison to vaccines comprising the whole pathogen. They also comply with the growing requirement for marker vaccines that can discriminate between vaccinated and infected individuals. Moreover, by peptide vaccination it is possible to manipulate the immune system in such a manner that the production of protective neutralizing antibodies is induced,[1] or that a non-responder to native antigen is converted into a responder.[2]

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However, especially in the development of such peptide vaccines, three aspects need special attention. First of all, polymorphism in pathogen-derived proteins often occurs in those parts of the proteins which are involved in immune recognition. In particular, RNA viruses, including foot-and-mouth disease virus (FMDV), contain regions with extremely high sequence variability.[3] Consequently, T cells primed and antibodies elicited by a vaccine composed of sequences selected from such highly variable regions of the virus might not cross-react with virus occurring in the field.

A second major aspect to consider is the variability of the host immune response. A T-helper cell only recognizes an antigenic peptide in association with one of the major histocompatibility complex (MHC) class II products, expressed by antigen-presenting cells (APC).[4] Each individual expresses its own set of MHC products coded by genes with a large allelic variation (i.e. MHC polymorphism). Hence, the MHC-restricted T-cell response frequently implies an individual-specific T-cell response towards peptides. This has been clearly demonstrated in many studies in which inbred mice strains were used.[5,6]

nally, the type of T-cell help induced by a peptide vaccine as to be considered. Different cytokine profiles produced helper cells lead to different effector functions.[7] Again, s in mice suggest that the type of effector function might e controlled by MHC class II. In these studies a single de was able to elicit different types of T-cell help in ent strains of mice.[8]

ere we studied the influence of MHC polymorphism on responses to peptides after vaccination with the whole gen in an outbred species, i.e. cattle. It has already been n that T-cell responses are under control of MHC class II tle.[9–12] Extensive studies on the MHC typing of cattle revealed a high polymorphism comparable to that in ns.[13] Upon detailed MHC typing of a large cattle herd, ated with whole inactivated FMDV, we were able to four animals homozygous for A-DR-DQ haplotypes and elated heterozygous animals. In this way proliferative and ne responses to three previously defined FMDV T-cell es[14] in association with six different MHC class II (DH) types could be examined.

MATERIALS AND METHODS

antigen was prepared as described previously.[14] Briefly, V strain A_{10}Holland was grown on a baby hamster cell line (BHK21). Collected supernatant was treated ·04 M binary ethyleneimine to inactivate the virus, and ns were precipitated twice with 9% and 6% (w/v) hyleneglycol (6000 MW), respectively. Intact virus was d on a $CsCl_2$ equilibrium gradient. Purity was confirmed S–urea–PAGE, as described by Meloen & Briaire.[15] preparation was dialysed against phosphate buffer 5), precipitated with 2 volumes of acetone at −20° and ended at a concentration of 1 mg/ml for *in vitro* ulation of bovine peripheral blood mononuclear cells oliferation assays.

es

5–53] (IMDRFVKINSLSPTHVIDL), VP2[74–88] HLTKLELPTDHH) and VP4[20–34] (SIINNYYMQ-SM) were synthesized by standard solid-phase Fmoc try.[16] These peptides were purified by reversed-phase erformance liquid chromatography (HPLC) and the ce was checked by amino acid composition analysis. Ten ping 15-mers, two 10-mers and one 7-mer covering the mino acid sequence 17–40 (indicated in Table 5) were ed by automated simultaneous multiple peptide syn- as described previously.[14,17] All peptides were synthe- ith an amide group at the C-terminus.

s

Holstein–Friesian cows were used. These animals were l from the Wageningen Agricultural University (WAU) he Netherlands. Selection was based on MHC type and relationship. Four animals were homozygous for both class I and class II, the other four animals were MHC ygous. This latter group included the four MHC class II pes of the homozygous group, plus two additional ones. of the heterozygous animals had inherited the same l haplotypes as three animals of the homozygous group

Table 1. Interrelationship and MHC class II haplotypes of the selected cattle

Animal no.*	Sire-code father†	Sire-code via dam‡	Paternal haplotype	Maternal haplotype
A1983	026B	021A	DH7A§	DH7A
A2399	060A	046A	DH8A	DH8A
A2346	060B	035B	DH22B	DH22B
A2390	051A	045A	DH24A	DH24A
A2396	060A	18*4	DH8A	DH18A
A2420	060B	011A	DH22B	DH7A
A2351	057B	159A	DH22H	DH18A
A2362	051A	159A	DH24A	DH18A

* Animals A1983, A2399, A2346 and A2390 were homozygous for MHC class I and MHC class II haplotypes. Animals A2396, A2420, A2351 and A2362 were heterozygous. Two extra DH haplotypes (22H and 18A) were included in this latter group.

† Code number of the fathers; note that three fathers of the homozygous animals were shared by three animals from the heterozygous group.

‡ Code number of the sire of which the haplotype was inherited via the mother.

§ Class II haplotype (DH), as shown in Table 2.

(Table 1). The age of the animals varied from 3 to 5 years. They all had been vaccinated at least once with the generally used trivalent FMDV vaccine, which is a mixture of strains A_{10}Holland, O_1BFS1860 and C_1Detmold, and were last revaccinated with monovalent vaccine type A, 6 months before the start of the experiments.

MHC typing

Class II polymorphism was established by serological, biochemical and DNA typing techniques. Serological typing was performed by the two-colour fluorescence technique (TCT), as originally described by van Rood *et al.*,[18] with minor modifications as described by Nilsson *et al.*[19] Biochemical typing [immunoprecipitation and one-dimensional isoelectric focusing (1D-IEF)] was performed as described by Joosten *et al.*[20] and Davies *et al.*[21] The MHC class II antigens were precipitated with 3 μl of the monoclonal antibody IL-A21 (a gift of Dr A. J. Teale, ILRAD, Nairobi, Kenya). Molecular typing was performed by two methods: detection of DRB3 polymorphism by polymerase chain reaction–restriction fragment length polymorphism (DRB3-PCR-RFLP) and detection of polymorphism of DQA and DQB by RFLP analysis. DRB3-PCR-RFLP typing was carried out essentially as described by van Eijk *et al.*[22] DQ-RFLP was performed as described by Siguardardottir *et al.*[23] For DQA–DQB typing, first and second domain (exon 2 and exon 3) DQA and DQB probes were used (clone DQA: W1, clone DQB: Y1). The sizes of the molecular weight fragments were adjusted on the basis of the BRL/Lifetechnologies (Baltimore, MD) 1 kb ladder. The nomenclature used for serotyping (Dw, Dc), 1D-IEF (DRBF) and DQA–DQB-RFLP was based on the nomenclature proposed at the Fifth International BoLA Workshop reported by Davies *et al.*[24] The nomenclature used for DRB3-PCR-RFLP was based on the one proposed by van Eijk *et al.*[22] Based on the combination of results of these four typing methods,

Table 2. Defined MHC class II haplotypes (DH)

H*	Ds (serology†)	DRBF (IEF†)	DRB3 (PCR-RFLP†)	DQA (RFLP†)	DQB (RFLP†)
	Dw2	02	7	2	2
	Dw1; Dc12	06	8	12	12
A	Dw1.4; Dc7	04	18	5	5
B	Dw1; Dc9	07	22	9B	9B
H‡	Dw1, Dc9	07	22	9B	9A
A	Dw3	03	24	1A	1

* Class II haplotype (DH) reference numbers, according to the Fifth ernational Bovine Lymphocyte Antigen (BoLA) workshop.[24]
† The method of typing is shown between parentheses.
‡ This haplotype was not officially defined during the Fifth Inter- ional BoLA workshop. The nomenclature used is as suggested by s workshop.[24]

ss II haplotypes were defined. For each class II haplotype a haplotype (DH) code comparable to the one introduced in class II report of the Fifth International BoLA Workshop[24] s used (Table 2). As MHC class I and class II are strongly ked, additional class I typing of the eight animals used in the dy and class I and class II typing of related animals further nfirmed the defined MHC class II haplotypes.

oliferation assay

oliferation assays were performed as described previously.[14] efly, peripheral blood mononuclear cells (PBMC) from the ht FMDV-vaccinated cattle were restimulated by *in vitro* ubation with 0·25 μg/ml viral antigen for 7 days. After an pansion period of another 7 days in medium containing IU/ml recombinant human interleukin-2 (IL-2) (Sanofit, ulouse, France), cells were tested in a proliferation assay: ls were incubated for 5 days in 96-well flat-bottomed tissue ture plates (Costar, Cambridge, MA) at a concentration of × 10^5 cells/ml in the presence of freshly isolated irradiated ologous PBMC (1 : 1) and different concentrations of viral igen or peptides. All cultures were performed in triplicate. ring the last 18 hr of culture 0·4 μCi [^{3}H]thymidine (specific ivity 1·0 Ci/mmol; Amersham International, Amersham, UK) s added to each well. Incorporation of label was assessed by uid scintillation counting in a Betaplate-counter (Pharmacia, psala, Sweden). Results were obtained as mean c.p.m.

okine assays

measurement of interferon-γ (IFN-γ), IL-2 mRNA and 4 mRNA, cells were pretreated as described above for the liferation assay. Incubations with peptides were also formed in 96-well flat-bottomed plates (Costar) at an al concentration. However, to minimize contamination of RNA samples with RNA from APC, only adherent cells m 10^5 irradiated PBMC/well) were used as APC. After 24, and 72 hr of incubation with peptides, non-adherent cells e harvested and washed with cold phosphate-buffered saline S) for RNA isolation, and supernatants were collected for ermination of IFN-γ. Different dilutions of the supernatants e assayed in duplicate for IFN-γ using a commercial bovine N-γ EIA kit (Commonwealth Serum Laboratories, Parkville, Victoria, Australia) according to the manufacturer's instructions. The use of this kit was described for the first time by Rothel *et al.*[25]

Table 3. Cytokine primer sequences

mRNA	Strand	Sequence
Actin	+	5′ CTGGCACGACACCTTCAACGAG 3′
	−	5′ AGCCAAGTCCAGACGCAGGATG 3′
IL-2	+	5′ AAGTCATTGCTGCTGGATTTAC 3′
	−	5′ CCTGTAGTTCCAAAACGATTCTC 3′
IL-4	+	5′ GCATTGTTAGCGTCTCCTGGTAAAC 3′
	−	5′ CTTCATAATCGTCTTTAGCCTTTCC 3′

+ reads 5′–3′.
− reads 3′–5′.

Total RNA was isolated from 10^7 cells by the use of RNAzol™ B (Campro Scientific, Veenendaal, the Netherlands) according to the manufacturer's instructions. The amount of RNA was determined by OD_{260} and, if available, 10 μg of each sample was checked by electrophoresis in 2% agarose/20% formaldehyde/20 mM 3-morpholino propane sulphonic acid (MOPS)/5 mM sodium acetate/1 mM EDTA gel. Three samples of 5 μg RNA were used for reverse transcriptase-mediated synthesis of cDNA using the SuperScript™ pre-amplification system (Gibco BRL, Paisley, UK) with oligo d(T) as a primer, according to the manufacturer's instructions. From each reverse transcriptase reaction mixture (volume 20 μl) 5-μl samples were subjected to PCR. Cytokine primers were designed from published cDNA sequences for bovine IL-2[26] and IL-4,[27] using PC/Gene release 6.7 software (IntelliGenetics Inc., Mountain View, CA), and were synthesized by OSWEL DNA services (University of Edinburgh, Edinburgh, UK). Sequences are shown in Table 3. Primers did not amplify products from genomic DNA. Actin primers were included as a control for quality of the cDNA product and PCR. PCR was performed at a final concentration of 10 mM Tris–HCl pH 8·3, 50 mM KCl, 0·1 mg/ml gelatin, 8 mM $MgCl_2$, 0·2 mM dNTP, 0·5 μM of the 5′ and 3′ primer, 2·5 U Thermus aquaticus DNA polymerase (Taq DNA polymerase) (Gibco BRL) in a total volume of 50 μl. The mixture was overlaid with paraffin oil (Sigma, St Louis, MO) and then amplified using a Techne PHC-3 thermal cycler. The amplification consisted of 30 cycles of the following profile: denaturation at 95° for 1 min, primer annealing at 55° for 1 min, and extension at 72° for 1 min. Twenty-five microlitres of each PCR reaction mixture was electrophoresed in 2% agarose gels in Tris borate/EDTA buffer. Gels were stained with ethidium bromide and photographed. Samples were indicated positive for the relevant product when bands of the expected size were visible.

RESULTS

In this study the influence of six bovine MHC class II haplotypes on the proliferation and cytokine production of T cells in response to three T-cell sites of FMDV (VP1[35–53], VP2[74–88] and VP4[20–34]), was examined. The six MHC class II haplotypes were present in eight cattle (Tables 1 and 2). PBMC from these cattle were restimulated once *in vitro* with

Table 4. Proliferation of PBMC from selected cattle to intact inactivated FMDV and to peptides, representing three T-cell sites of FMDV

Animal no.		A1983	A2399	A2346	A2390	A2420	A2396	A2351	A2362
DH*		7A/7A	8A/8A	22B/22B	24A/24A	22B/7A	8A/18A	22H/18A	24A/18A
BG†		4547	660	1285	6438	355	8603	123	4890
FMDV-antigen‡	Conc. 2·5 μg/ml	**42**	**68**	**17**	**21**	**122**	**23**	**57**	**32**
VP1[35–53]§	10^{-5} M	**6·9**	1·2	1·1	0·3	**5·7**	**12**	**86**	**28**
	10^{-6} M	**6·6**	1·1	1·5	0·5	**3·0**	**20**	**62**	**23**
	10^{-7} M	**3·6**	1·6	0·8	0·4	1·0	**20**	**48**	**18**
VP2[74–88]	10^{-5} M	1·8	2·0	**15**	0·5	**303**	1·3	**204**	2·4
	10^{-6} M	1·5	2·3	**8·3**	0·7	**282**	1·0	**198**	1·8
	10^{-7} M	1·3	1·8	**4·7**	0·9	**75**	1·8	**101**	1·7
VP4[20–34]	10^{-5} M	**31**	**66**	**7·1**	0·4	**114**	**8·7**	**151**	**26**
	10^{-6} M	**24**	**44**	**3·0**	1·1	**13**	**5·3**	**74**	**16**
	10^{-7} M	**17**	**11**	2·7	1·0	**4·2**	**3·1**	**27**	**4·3**

* Class II haplotypes of the animals as shown in Table 1 and Table 2.

† BG, background proliferation of PBMC presented as c.p.m. The value obtained is the mean of 12 cultures.

‡ Proliferation to purified FMDV strain A_{10}Holland presented as stimulation index (SI = proliferation to antigen/background proliferation).

§ Proliferation to three molar concentrations of the peptide indicated, also presented as SI.

Bold numbers indicate SI values of 3·0 or higher. The standard deviation of the mean was 20% or less.

purified FMDV. Thereafter, cells were incubated with each peptide, representing one of the three T-cell sites, during which both proliferation and cytokine production were measured. Proliferation assays were performed using cells that were cultured for 5 days in the presence of different concentrations of each peptide or intact inactivated FMDV. Results are shown in Table 4. All cattle responded very well to intact FMDV: in all cases the stimulation index was greater than 15. Seven out of the eight animals also responded to one or more of the peptides. Peptide VP1[35–53] was recognized by PBMC from animals carrying the DH7A or DH18A haplotypes. VP2[74–88] was recognized by PBMC from animals carrying the DH22B or DH22H haplotypes. Notably, peptide VP4[20–34] was recognized by PBMC from most animals, indicating a promiscuous recognition in association with haplotypes DH7A, DH8A, DH22B and DH18A. The animal homozygous for the DH24A haplotype did not recognize any of the peptides.

Apart from a clear selectivity in the responses to the peptides, a difference in the magnitude of the responses was also observed. Animals carrying the DH7A haplotype responded more strongly to the VP4 than to the VP1 peptide, whereas the opposite proved to be the case for two out of three animals carrying the DH18A haplotype. In animals carrying the DH22B or DH22H haplotypes, the response towards the VP2 peptide was most dominant, exceeding the response to the VP4 peptide. Similarly, the heterozygous animal A2420, typed as DH22B/DH7A, responded to the three peptides, VP2[74–88], VP4[20–34] and VP1[35–53], with decreasing magnitude. Heterozygous animal A2351, typed as DH22H/DH18A, responded strongest to VP2[74–88] due to the presence of the DH22H haplotype. The responses to the VP1 and VP4 peptides were more or less equally high, due to the presence of the haplotypes DH18A and DH22H, respectively.

For refinement of the recognition of the VP4 T-cell site by the different animals, a panel of 10 overlapping peptides, each 15 amino acids long, covering residues 17–40 of VP4, were tested using PBMC from animals A1983 (DH7A), A2399 (DH8A) and A2362 (DH18A/DH24A). Unfortunately, we were not able to use PBMC from animal A2346, which excluded analysis in the context of DH22B. The results are presented in Table 5. PBMC from animal A1983 responded to six of the peptides, covering amino acid sequence 17–36. Peptides covering amino acid sequence 20–39 elicited the strongest proliferation of PBMC from animal A2399. High responses to all peptides, with the exception of VP4[26–40], were found with PBMC from animal A2362. Since it was shown that none of these peptide-specific responses was likely to occur via the DH24A haplotype, DH18A must have been the haplotype responsible for this effect. Deduced from these results, three shorter peptides were synthesized and tested. As expected, VP4[22–31] was recognized in association with haplotypes DH7A and DH18A, and VP4[25–34] was recognized in association with haplotypes DH8A and DH18A. However, the 7-mer VP4[25–31] was not recognized.

Cytokine production by the PBMC following peptide-specific stimulation was measured after 24, 48 and 72 hr incubation with no peptide or one concentration of each peptide. The cytokine assays were performed using PBMC from the three homozygous cattle (A1983, A2399 and A2346), which proliferated to one or two of the three peptides (Table 4). Results of the IFN-γ assay are shown in Table 6. IFN-γ was found in all supernatants. However, a significantly higher concentration of IFN-γ was present in 24-, 48- and 72-hr supernatants of cells that specifically proliferated to peptide, as shown by enhanced [^{3}H]thymidine incorporation after 5 days of incubation. The presence of transcripts for IL-2 and IL-4 was tested by PCR using specific primers. In all 24- and 72-hr samples, IL-2 mRNA could be detected easily (data not shown). IL-4 mRNA was not detected in any sample of cells specifically proliferating to peptide. IL-4 mRNA was only

Table 5. Proliferation of PBMC to a panel of overlapping VP4 peptides

Animal no.	A1983	A2399	A2362
DH	7A/7A	8A/8A	24A/18A
BG*	565	588	589
FMDV-antigen†	+ + +	+ +	+ + +
VP4 peptides			
17–31‡	+ + +§	–	+ + +
18–32	+ + +	–	+ + +
19–33	+ + +	+	+ + +
20–34	+ + +	+ +	+ + +
21–35	+ + +	+ + +	+ + +
22–36	+ +	+ + +	+ + +
23–37	–	+ + +	+ + +
24–38	–	+ + +	+ + +
25–39	–	+ +	+ +
26–40	–	+	–
22–31	+ +	–	+ + +
25–34	–	+ +	+ +
25–31	–	–	–

* BG, background proliferation of PBMC presented as c.p.m. The value obtained is the mean of 12 cultures.

† Proliferation to 2·5 μg/ml purified FMDV strain A_{10}Holland.

‡ Proliferation to 5×10^{-5} M of the VP4 peptides. Peptide sequence is indicated by first and last amino acid of the VP4 amino acid sequence.

§ + + + = SI > 100; + + = 10 < SI < 100; + = 3·0 < SI < 10; – = SI < 3·0. (SI = stimulation index). The standard deviation of mean was 20% or less.

Table 6. Proliferation, IFN-γ secretion and detection of IL-4 mRNA by or in PBMC incubated with no peptide or with each peptide, representing one of the three FMDV T-cell sites

		SI*	Relative IFN-γ conc.†			IL-4 mRNA
Incubation time (hr)		114	24	48	72	48/72
A1983‡	No peptide	1·0	1·0	1·0	1·0	+
(DH7A/DH7A)	VP1[35–53]§	**3·1**	**1·8**	**1·4**	**1·4**	–
	VP2[74–88]	1·0	1·0	1·0	1·0	+
	VP4[20–34]	**19·3**	**3·4**	**4·7**	**3·1**	–
A2399	No peptide	1·0	1·0	1·0	1·0	+
(DH8A/DH8A)	VP1[35–53]	1·0	1·0	0·9	1·0	+
	VP2[74–88]	1·0	1·0	1·1	1·0	+
	VP4[20–34]	**7·8**	**2·4**	**3·9**	**3·5**	–
A2346	No peptide	1·0	1·0	1·0	1·0	+
(DH22B/DH22B)	VP1[35–53]	1·3	1·2	1·2	1·0	+
	VP2[74–88]	**22·2**	**2·7**	**3·5**	**3·1**	–
	VP4[20–34]	**3·4**	**2·3**	**2·6**	**3·2**	–

* Proliferation of cells was measured by a 5-day proliferation assay. SI (stimulation indices) are shown.

† Eight times diluted supernatants of cells were tested in an IFN-γ ELISA kit. Relative IFN-γ concentration was calculated as the ratio of the OD_{450} value obtained from the supernatant and the OD_{450} value obtained from the supernatant of the same cells incubated without peptide.

‡ Animal from which PBMC were tested with, between parentheses, the MHC class II haplotype (DH) as shown in Table 2.

§ PBMC were incubated with 10^{-6} M of the peptides indicated.

Bold numbers indicate SI > 3·0, OD_{450} ratios > 1·3 and samples in which no IL-4 mRNA was detected.

clearly detectable in 48- and 72-hr samples derived from cells proliferating non-specifically (Table 6). The enhanced IFN-γ secretion and no or decreased IL-4 transcription by cells specifically proliferating to the peptides indicated a shift towards a T-helper type-1 (Th1) type of response.

DISCUSSION

An attractive approach in vaccine development is the generation of vaccines based on synthetic peptides. However, since it is known that T cells recognize antigenic peptides only in association with products of the polymorphic MHC,[4] such a peptide vaccine may be effective only in a selected group of individuals. Here we studied the effect of bovine MHC class II polymorphism on the proliferative and cytokine responses to three T-cell sites of FMDV.

To refer to the complete MHC class II haplotype, we used the D haplotype (DH), as defined by several typing techniques for both the DR and DQ regions (= Ds type + DRB3 PCR-RFLP type + DRBF-type + DQA-RFLP type + DQB-RFLP type). A clear correlation between the MHC class II haplotype and the response to a particular peptide was found, which is summarized in Table 7. Although Holstein–Friesians were used throughout this study, D haplotypes 7A, 8A, 18A and 22B have also been found in other breeds of cattle.[24] So the three peptides are likely to be effective in other breeds of cattle as well. It is not certain whether the recognition of VP4[20–34] is possible in the context of the DH22H haplotype. This haplotype was only present in a heterozygous animal (A2351) that also carried the DH18A haplotype, which itself is responsible for a response towards this peptide. However, VP4[20–34] is recognized in association with the DH22B haplotype (animal A2346) which is, with respect to DR type, identical to the DH22H haplotype. Furthermore, the magnitude of the response to VP4[20–34] by PBMC from animal A2351 was clearly enhanced compared to the response to this peptide in the context of the DH18A haplotype only (as shown by animal A2362). Based on both observations, we suggest that VP4[20–34] might also be recognized in the context of the DH22H haplotype.

The recognition of this VP4 T-cell site in the context of the DH7A, DH8A and DH18A haplotypes was studied in more detail. A difference in the 'core' sequences was observed for

Table 7. Association between the T-cell sites recognized by PBMC from FMDV-vaccinated cattle and their MHC class II haplotype (DH) or associated DRB3-PCR-RFLP type

DH	DRB3-PCR-RFLP	VP1[35–53]	VP2[74–88]	VP4[20–34]
7A	7	+	–	+
8A	8	–	–	+
18A	18	+	–	+
22B	22	+	+	+
22H	22	+	+	+
24A	24	–	–	–

recognition in the context of these different haplotypes: amino acid 22 (to 31 or less) of VP4 was essential for recognition in the context of the DH7A haplotype: for the DH8A haplotype, amino acid 33 (from 26 or more) was required, although responses increased dramatically as soon as amino acid 34 was included; and finally, in the context of the DH18A haplotype, the critical sequence seemed to include amino acid 25 (to 31 or less). However, the 7-mer VP4[25–31] was not recognized in the context of the DH18A haplotype. Apparently this relatively short peptide is much less effective in MHC binding and/or T-cell activation, as has been found in many other studies on T-cell responses to truncated peptides.[28,29] Another possibility is that in the context of the DH18A haplotype more than one 'core' sequence within the VP4 sequence 17–39 is present, one within region 22–31 and the other within region 25–34, each binding to the same or to different MHC class II molecules (e.g. DR and DQ). These kinds of shifts in 'core' sequences within one promiscuous T-cell site of an antigen have been described before.[30] Analysis of such a T-cell site indicated that the peptide assumed different conformations upon interaction with different MHC molecules.[31]

We did not observe a significant shift in cytokine profiles of PBMC that were derived from animals carrying different MHC class II haplotypes, but that proliferated to the same peptide. Similarly, no differences in cytokine profiles of PBMC that were derived from a single animal but that specifically proliferated to different peptides could be found. PBMC that specifically proliferated to peptide were all found to secrete more IFN-γ and less IL-4 than non-specifically proliferating cells, indicating a shift towards T-helper cells of the Th1 subtype upon peptide-specific stimulation.[7]

We do not know whether the responses to the FMDV peptides, reported here, are restricted via DR, DQ or maybe even other products expressed from the bovine MHC class II region.[32,33] Evidence of DR and/or DQ restriction might be obtained by testing other MHC-typed cattle matched either for DR or DQ. However, selection of such animals will not be easy, since DQ and DR are in strong linkage disequilibrium.[34,35] Alternatively, the use of blocking DR- and DQ-specific monoclonal antibodies (mAb) or direct MHC–peptide binding assays could be informative in this matter. However, well-defined mAb are currently unavailable for these studies.

Knowledge of the precise restriction element is not necessarily essential for the prediction of the effectiveness of a peptide in a MHC-typed animal. Cross-overs between the different MHC class II loci appear to be limited, leading to a strong linkage between DR, DQ and probably also other products.[34,35] Thus, in general, typing for only one MHC class II product might be sufficient to predict the outcome of the response towards a peptide. Although about 600 animals of the WAU herd are typed for DRB3 by PCR-RFLP, only a few animals are typed for the complete MHC class II haplotype. If we consider our findings for this population of 600 animals, the following predictions can be made with regard to the successful implementation of peptide vaccines (for this purpose the DRB3-PCR-RFLP types, associated with the DH haplotypes studied so far, are included in Table 7). Complete non-responsiveness to the three FMDV peptides was observed for the DH24A haplotype. Seventeen animals in the typed WAU herd were found to be homozygous for DRB3*24, a DRB3 type which is found to occur exclusively in the DH24A haplotype. Thus, only 3% of non-responders from the total herd of 600 animals would be expected, on the basis of the frequency of the DH24A haplotype. To date, 32 DRB3-PCR-RFLP types have been defined. So far, four of these have been shown to be associated with a response to VP4[20–34]. Yet, 60% of the WAU herd was positive for one or two of these four responder DRB3-PCR-RFLP types. If the distribution of class II haplotypes is representative for the Holstein–Friesian breed in general, VP4[20–34] would be a good candidate for inclusion in a peptide vaccine, especially since this site is derived from a highly conserved region of the virus as well. The amino acid sequence of VP4[20–34] is 100% identical between serotypes A, O and C of FMDV. An effective synthetic vaccine might be generated for example by coupling the VP4 T-cell site in combination with one or more B-cell epitopes to the lipotripeptide (P_3CSS), as studied by Wiesmuller *et al.*[36] or by inclusion of the peptides into liposomes.[37]

In conclusion, these findings show for the first time that a strong MHC-restricted selection of T-cell epitopes also occurs in an outbred population like cattle. This will have major implications for research on vaccine development, and for the design of synthetic vaccines in particular. The influence of MHC polymorphism may be less apparent in the response to complete viral proteins. Still, the effect of MHC polymorphism must not be under-estimated when testing new subunit vaccines in randomly chosen groups of animals. In order to establish the efficacy of the vaccine in different MHC backgrounds, the animals used should be typed in advance. Then groups of animals can be selected in which the most frequent MHC class II types are represented and equally distributed. For the development of a peptide vaccine, MHC polymorphism will surely have an influence, as we have shown here. In order to design a peptide vaccine which is effective in all animals, the response to different T-cell epitopes should be tested for all possible MHC haplotypes. For this kind of study homozygous animals are indispensable. Finally, a combination of a limited number of peptides, covering a T-cell response in association with all haplotypes should be included in the vaccine. The present study shows that for this purpose conserved and promiscuous epitopes of a complex pathogen like FMDV can be found that largely circumvent the MHC-related restriction even in an outbred population.

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Theileria annulata induces abberrant T cell activation *in vitro* and *in vivo*

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SUMMARY

The protozoan parasite of cattle, *Theileria annulata*, causes a severe lymphoproliferative disease, developing initially in the draining lymph node, which is often fatal in naive animals. Infection of macrophages with *T. annulata* leads to an augmentation of their antigen-presenting capability *in vitro* and infected cells can induce proliferation of autologous resting T cells from naive animals. This inappropriate activation of T cells may play an important role in the failure of the host to mount an effective immune response *in vivo*. To investigate this hypothesis we characterized further the response of T cells from naive cattle to infected cells *in vitro*, and also examined the development of the immune response in lymph nodes draining the sites of *T. annulata* infection. Both $CD4^+$ and $CD8^+$ T cells from naive peripheral blood mononuclear cells (PBMC) were induced to proliferate and express the activation markers IL-2R and MHC class II when cultured with infected cells. This effect was seen in both 'naive' and 'memory' T cells, and was dependent upon contact with infected cells. *In vitro*, infected cells are therefore capable of activating T cells irrespective of their antigen specificity or memory status. In draining lymph nodes, although large numbers of IL-$2R^+$ cells developed following infection, these activated cells were only associated with areas of parasite-induced proliferating cells, and subsequently disappeared from the node. Cells expressing IL-2R were not present in recognized sites for T cell development. Germinal centres were severely affected, losing T cell-dependent zones followed by a total destruction of morphology. T cell function is therefore severely disrupted within draining nodes. This study has shown that parasitized cells supply sufficient signals *in vitro* to activate T cells irrespective of specificity. T cells also are not stimulated in a conventional manner *in vivo*, and this may play an important role in preventing an effective immune response from being generated.

Keywords *Theileria annulata* T cells antigen presentation activation markers

INTRODUCTION

leria annulata is a tick-transmitted protozoan parasite, ting cattle in Southern Europe, North Africa, India, the dle East and Southern Russia, causing tropical theileriosis. e is known about the primary immune response to *nnulata* in naive animals, where infection quickly leads to n. Parasite development takes place within the lymph node ing the site of initial infection, and is therefore in a position terfere with immune response development. When infected als are treated with the drug 'butalex', which kills the cellular parasite, the animal recovers [1] and is subse-tly immune. Animals recovering from infection generate parasite infected cell $CD8^+$ cytotoxic T lymphocytes (CTL) This restoration of immune function suggests that although nost is capable of generating an immune response, the site infection overwhelms normal immunity.

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In vitro it has been previously shown that *T. annulata* preferentially infects cells of monocyte/macrophage lineage [4,5], and transforms them into continuously growing cell lines. These macroschizont infected cells can act as antigen-presenting cells (APC) and present third party peptides via MHC class II to antigen-specific $CD4^+$T cells *in vitro* [6]. Infected cells exhibit augmented APC function, inducing greater proliferation to peptide than normal APC. In addition, infected cells also induce 'non-specific' proliferation of autologous resting T cells from naive cattle in the absence of any exogenously added antigen [6,7]. These observations suggest that infected cells may have an inherent capability to activate T cells. We postulate that inappropriate activation of T cells by parasitized cells may explain the inability of the host to mount an effective immune response. Here we have characterized the activation effects of infected cells upon naive cells *in vitro*. We have also investigated whether this phenomenon affects immunity *in vivo*, by examining the development of the primary immune response to *T. annulata* in draining lymph nodes.

MATERIALS AND METHODS

Animals

Hereford calves aged approximately 4 months were used for the examination of draining lymph nodes. All other animals used in this study were normal Friesian female or castrated male cattle aged 6 months or older.

Immunizations

Theileria annulata sporozoites were prepared from infected ticks, prepared as previously described [8]. Lymph nodes were obtained from two calves infected with percoll-purified sporozoites of *T. annulata* [9] (Ankara strain). A third control calf was immunized with the appropriate percoll fraction from uninfected *Hyalomma* ticks. In addition, the draining lymph nodes from two calves infected with unpurified Gharb strain sporozoites were also examined 10 days post-infection.

Theileria annulata-*infected cell lines*

Macroschizont infected cell lines (Hissar or Gharb strain) from the animals tested were prepared as previously described [6]. Cell lines were used at low passage number (2–20).

Cell preparation

Peripheral blood mononuclear cells (PBMC) were separated using Ficoll–Hypaque ('Lymphoprep', Nycomed, Oslo, Norway) as previously described [10]. Complete tissue culture medium was used throughout the experiments [6].

Culture of PBMC

PBMC were cultured with *T. annulata*-infected cells essentially as described previously [6]. Briefly, PBMC were cultured with autologous irradiated (75 Gy) infected cells in 6×10 ml well plates (Nunc, Gibco, Paisley, UK). PBMC (8×10^6/ml): infected cell ratios varied from 10:1 to 40:1. In addition, PBMC ($2{\cdot}5 \times 10^6$/ml) were stimulated with 5 μg/ml concanavalin A (Con A) to provide a 'control' population of stimulated T cells. Identical cultures with PBMC separated from infected cells or from medium containing Con A by a 0·4-μm pore membrane (Millicel-CM Insert, Millipore Corp., Bedford, MA) were also established in six-well plates. Stimulated PBMC were harvested at various times (1–7 days) for FACS analysis.

Proliferation assays

Proliferation assays were performed as previously described [6]. Briefly, PBMC (4×10^5/well) were incubated with irradiated autologous infected cells (2×10^4/well) or 5 μg/ml Con A for 1–9 days. Proliferation was measured using liquid scintillation following a 6-h pulse of ^{3}H-dThd (Amersham, Aylesbury, UK). As the membrane inserts were too large to use in 96-well plates, cells grown in the presence of inserts in six-well plates for the desired length of time were plated out in 96-well plates at the same dilution as normal assays, pulsed, and counted.

MoAbs

MoAbs used in these experiments are detailed in Table 1. MoAbs were used at optimal concentrations (1:1000 dilution of ascitic fluid or 1:20 dilutions of culture supernatant) for FACS analysis. The use of MoAb for staining sections is detailed under immunohistology.

Table 1.

MoAb	Specificity
IL-A12	Bovine CD4 [11]
J11	Bovine MHC class II [12]
IL-A111	Bovine IL-2 receptor [13]
CC63	Bovine CD8 [14]
1C7	*T. annulata* macroschizonts [15]
VPM 30	Pan B cells in peripheral blood, germinal centres [
CC76	Bovine CD45RB [14]
MIB-1	Ki-67 proliferation antigen [17]

FACS analysis

Cells were analysed using the MoAbs described above o Becton Dickinson FACScan essentially as previously descril [10]. Double staining was performed by first incubating w both MoAbs (of different isotypes) followed by appropri fluorescent secondary reagents: goat anti-mouse (GA IgM–FITC; GAM IgG–PE (Sigma, Poole, UK); GA IgG1–FITC; GAM IgG2a–PE (Seralab).

Lymph nodes

The right prescapular lymph nodes (draining the site infection) were taken from the control and one infec animal at day 4 post-infection. Nodes were taken from other percoll-purified sporozoite-infected animal at day 8, a from the two Gharb-infected animals at day 10. Pieces of n approx. 0·5–1 cm^2 were fixed in 10% formal buffered saline 7·4 and subsequently embedded in paraffin wax. Secti (2·5–3 μm) were cut from paraffin wax-embedded lyn nodes. Sections were stained with haematoxylin and ec (H&E) or Giemsa for conventional histological examinati Infection with macroschizonts was assessed in Giemsa-stai needle biopsies from the nodes.

Immunohistology

Sections were stained with the MoAbs detailed above. Opti concentrations of MoAbs were determined and used in range neat–1:10 dilutions of culture supernate or 1:50–1:100 dilutions of ascites.

MoAbs staining of formalin-fixed sections was perfor using standard immunohistochemical techniques. Secti were dewaxed in xylene and rehydrated through alcol MoAbs IL-A111, J11, and IL-A12 required the sections tc treated with trypsin digestion solution for 20 min at 3 before staining. (Digestion solution: 0·1% w/v Trypsin 1: (Difco, Detroit, MI) and 0·1% $CaCl_2$ (BDH, Poole, UK distilled H_2O.) Staining was visualized using the ABC- system (Dako, Glostrup, Denmark) with Vector red (Vec Labs, Peterborough, UK) as the substrate. Vector red the advantage of being visible by both conventional ultraviolet/confocal microscopy.

RESULTS

Activation of T cells from naive animals

Previous studies have shown that naive T cells proliferate infected cells, and that the magnitude of proliferation proportional to the ratio infected cells:PBMC or T cells [

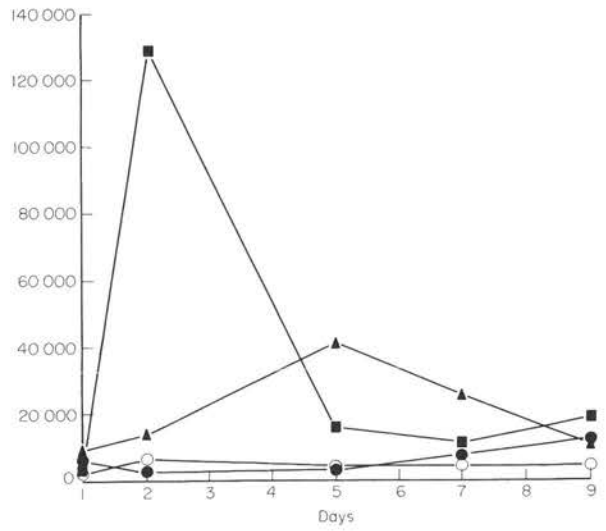
order to analyse this response and phenotype the responding lls, PBMC from naive animals were cultured with irradiated tologous infected cells. PBMC were also incubated with Con as a positive activation control. Increasing the numbers of fected cells:PBMC in these experiments again led to creased proliferation (results not shown). The results esented here used infected cells:PBMC ratios of 1:20, cept where indicated.

Both infected cells and Con A induced proliferation, hough the time courses were dissimilar (Fig. 1). With fected cells, proliferation peaked after 5 days as described eviously [6], whereas with Con A proliferation peaked much rlier at day 2. In addition the amount of proliferation induced infected cells was always substantially less than with on A.

Incubation with infected cells increased the numbers $CD4^+$ and $CD8^+$ T cells expressing IL-2R and MHC ss II molecules on their surface by approximately 25% ig. 2). These determinants were also expressed upon the rface of Con A-activated T cells (Fig. 2). IL-2R d MHC class II expression by T cells was detected after h of culture, plateaued by 48 h, and was stable for up to days (Fig. 3). Increasing the ratio of infected cells:PBMC 1:10 led to increased expression of IL-2R and MHC ss II upon $CD4^+$ and $CD8^+$ cells (48 h timepoint shown Fig. 3). IL-2R and MHC class II were expressed upon th CC76 (CD45RB) 'high' and 'low' staining cells (results t shown). Separating PBMC from the infected cells by a 4-μm membrane completely blocked the expression of any of e activation associated markers on T cells (Fig. 2), but had no ect upon Con A stimulation (results not shown).

aining lymph nodes

eileria annulata macroschizonts usually appear in the aining lymph node 5 days post-infection, reaching a peak



. 1. Proliferative responses of peripheral blood mononuclear cells BMC) from a naive animal to infected cells or concanavalin A on A). cct/min, Corrected counts per minute. Values are means of idruplicate samples, ○, PBMC; ●, *Theileria annulata*; ▲, PBMC + *annulata*; ■, Con A 5 μg.

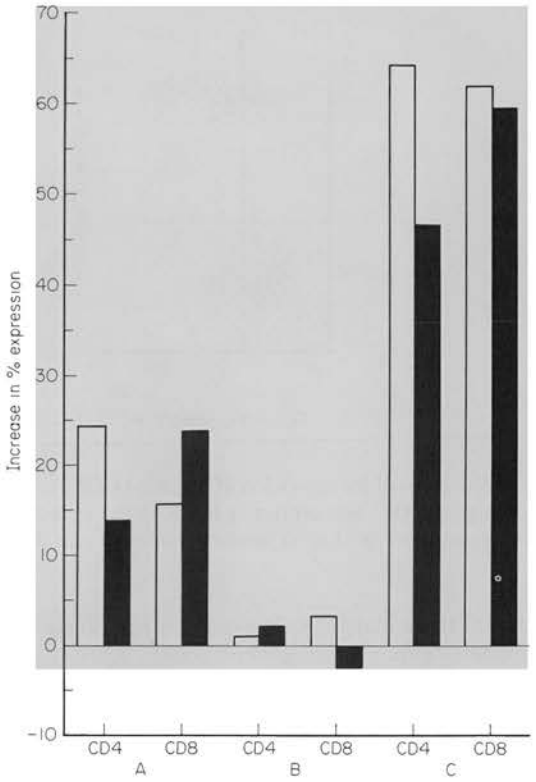


Fig. 2. Changes from control levels of expression of IL-2 receptors (□) and MHC class II (■) by bovine T cells in peripheral blood mononuclear cells (PBMC) after 48 h culture with: A, infected cells (1:20); B, infected cells (1:20) separated from the PBMC by a 0·4-μm membrane; C, concanavalin A (Con A) 5 μg/ml. Results are means from four different animals.

at days 8–9, with European cattle rarely surviving beyond day 10 or 11 (C.G.D. Brown, personal communication). Here we examined draining lymph nodes at days 4, 8 and 10 in order to study the immune response before macroschizonts are detectable, and during the period when parasite growth is at its peak. The description of the pathology of *T. annulata* infection within draining lymph nodes is limited to specific points concerned with the induction of the immune response.

Macroschizonts were not present in control or day 4 Giemsa-stained biopsies, and day 8 biopsies contained <1% macroschizonts. Lymph nodes removed from the two animals at day 10 contained 27% and 53% macroschizonts, respectively, within the biopsies, and most cells within sections of these nodes were $1C7^+$.

The percoll control draining node showed no signs of reaction, with normal germinal centre morphology, small lymphocytes within the cortex, and empty medullary sinuses. The most striking feature of the *T. annulata* infection was the appearance of foci of large blasting cells in the medulla of the day 4 draining node, spreading throughout the tissue by day 8 (Fig. 4a). On day 10, these were the predominant cell type present, all normal morphology having disappeared from the nodes. The blasting cells stained strongly with MoAb MIB-1 (anti-proliferation antigen) and anti-MHC class II MoAb. Studies of frozen sections from infected animals have shown

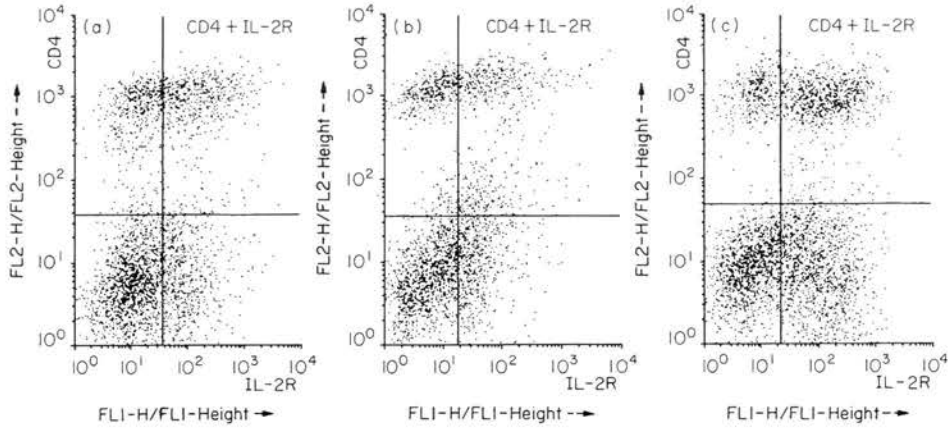


Fig. 3. FACS analysis of the expression of IL-2R on $CD4^+$ cells from a naive animal after: (a) 48 h and (b) 7 days culture with infected cells (1 : 20 The percentage of $CD4^+$ cells expressing IL-2R (50%) is unchanged between 48 h and 7 days. (c) IL-2R expression upon CD4 cells from the sam animal is increased (to 70%) upon incubating infected cells: PBMC 1 : 10 for 48 h.

that many of these blasting cells express macrophage markers (J. Campbell, unpublished observations). At day 10, the blasting cells were $1C7^+$.

IL-A111 (anti-IL-2R) stained the cytoplasm of very few cells in sections from control nodes. However, within the day 4 draining node there were many $IL\text{-}2R^+$ cells, grouped around, but not inside, the areas of blasting cells in the medulla (Fig. 4b). By day 8, the number of $IL\text{-}2R^+$ cells had returned to control levels, disappearing completely by day 10.

Normal germinal centres (GC) had a mantle zone, characterized by small lymphocytes. $MIB\text{-}1^+$ (Ki67) stained proliferating cells in an area at the 'base' of the GC analogous to the human GC dark zone [18]. VPM 30 staining was present only in well developed GC and generally did not stain the $MIB\text{-}1^+$ areas in these GC, recognizing an area analogous to the human light zone [18]—both in its morphological appearance (Fig. 5a) and in being $MIB\text{-}1^-$. Smaller GC with primarily 'dark zone' $MIB\text{-}1^+$ cells did not stain with VPM30. By day 8, germinal centres could only be distinguished as having a mantle and dark zone. All VPM 30 staining was lost from GC, and only blasting $MIB\text{-}1^+$ area cells persisted (Fig. 5b,c). No germinal centres were distinguishable by day 10.

DISCUSSION

Although *T. annulata* infections in naive cattle are usually fatal, parasite-specific CTL and serum antibodies have been detected in animals recovered from *T. annulata* infection [2,3]. Removing live parasite therefore removes any constraints upon the ability to generate immunity. In a previous report it was shown that infection of macrophages with *T. annulata* altered their antigen-presenting function *in vitro*, as infected cells induced proliferation of resting autologous T cells from naive animals [6,7]. Infected cells may therefore have an innate ability to activate T cells. In this study we have characterized activation of T cells by infected cells *in vitro* and examined the effects such activation may have upon immune response development *in vivo*. Here we show that infected cells can induce activation markers upon resting T cells *in vitro*, irrespective of their memory status. *In vivo*, T cells are not activated in conventional sites. In addition, profound changes are seen in th development of germinal centres.

Expression of IL-2 and IL-2R genes is induced when T cel are activated [19,20]. Bovine T cells rapidly express IL-2R [21 and MHC class II [22] upon activation. In this study, bot IL-2R and MHC class II were expressed on the surface c T cells when activated by the mitogen Con A. We show her that *T. annulata*-infected cells induce the expression of IL- receptors and MHC class II molecules on the surface of CD4 and $CD8^+$ T cells from animals which have not been infecte with the parasite, and these naive PBMC are induced t proliferate by the infected cells. *Theileria annulata*-infecte cells are therefore capable of producing enough stimuli t activate T cells in the absence of specific antigen.

When PBMC were separated from infected cells, T ce activation was no higher than that seen in PBMC incubate alone. Infected cells therefore require contact to activate T cell and although soluble factors may be important they are no sufficient alone. The numbers of cells activated increased b adding more infected cells, and cells of both memory an 'naive' phenotypes were seen to be activated. It therefor seems likely that infected cells are capable of activating restin $CD4^+$ or $CD8^+$ T cells irrespective of phenotype, and the mai limiting factor is infected cell: T cell contact.

The peak of proliferation induced by infected cells wa similar to that induced by MHC class II associated antigen [10 whereas Con A-activated T cells showed maximum prolifera tion at day 2 (Fig. 2). This suggests that the 'non specifi activation of T cells by infected cells is not due to a parasit associated mitogenic factor. Members of the 'superantige family of viral and bacterial peptides, and heat shock protei which mimic mycobacterial antigens, have been associated wit 'non-specific' activation of $CD4^+$ and $CD8^+$ T cells. The latt is also linked to an increase in expression of MHC class II c aberrant APC [23–25]. Antigens from *Trypanosoma cruzii* hav been shown to induce strong proliferation of naive PBMC the antigen can react with the majority of $V\beta$ genes expresse by T cell receptors [26]. Given the kinetics of infected c activation of T cells, an antigen presented in this manner is more likely candidate than a mitogen or a superantige

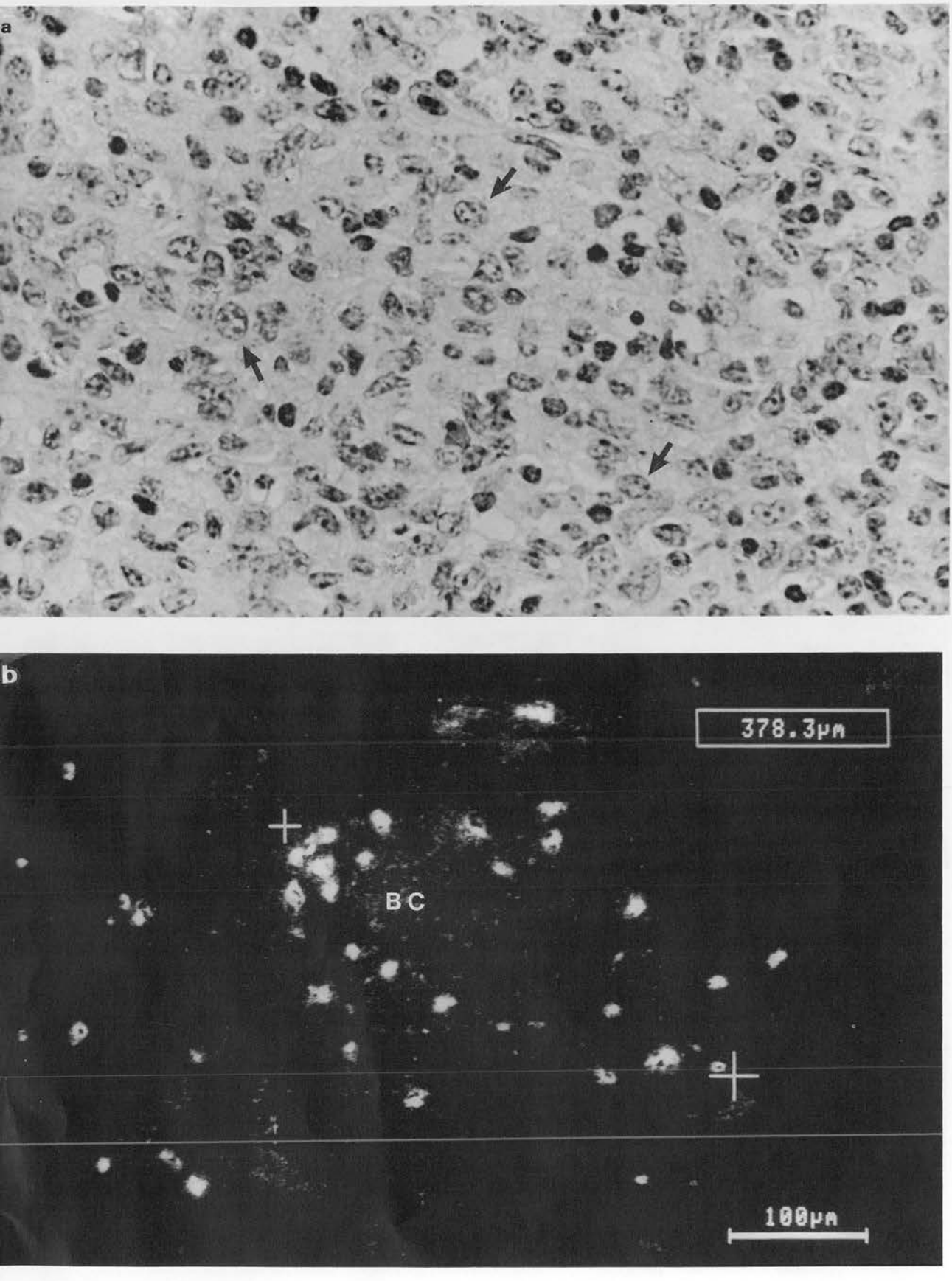
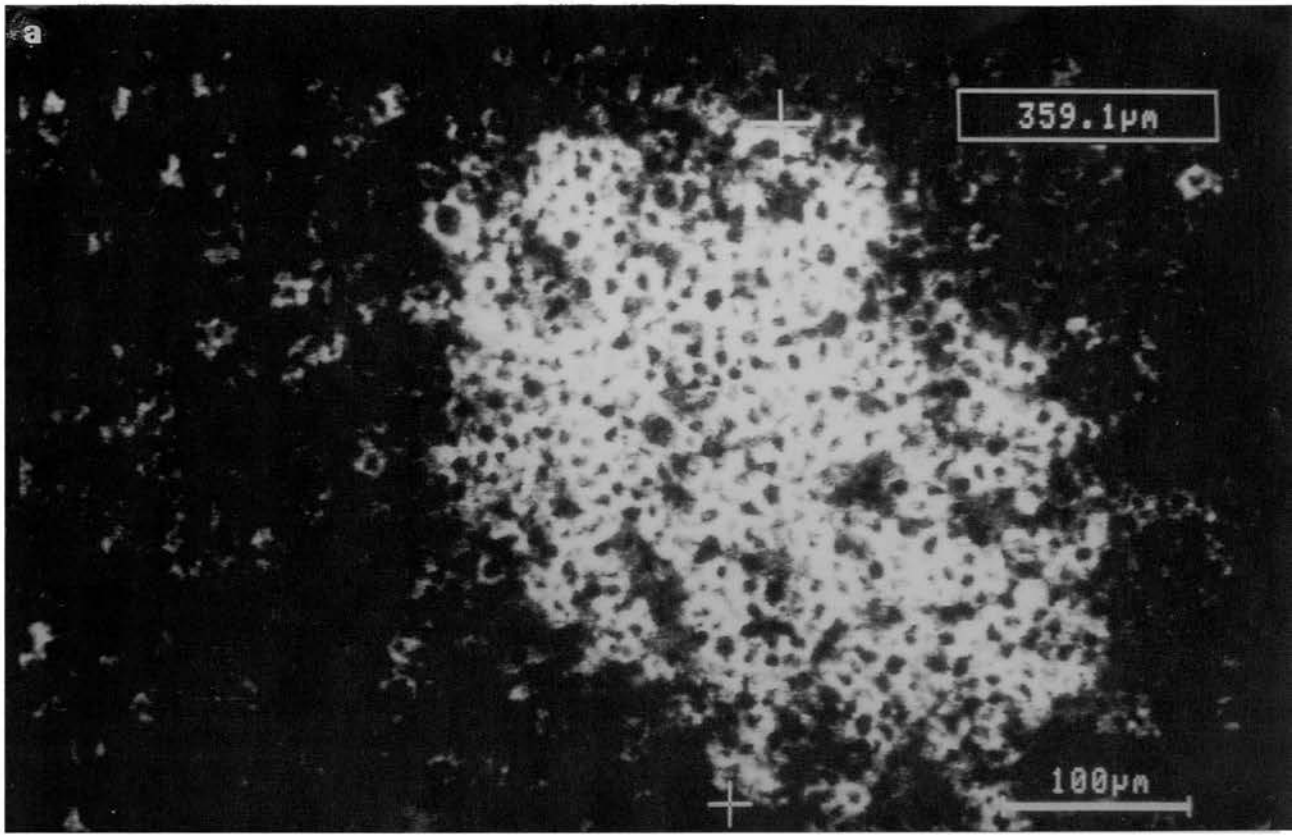


Fig. 4. Paraffin sections of bovine lymph node. (a) Large blasting cells which predominate throughout the draining lymph node 8 days post *Theileria annulata* infection. Examples are arrowed. (Haematoxylin and eosin, ×500.) (b) Confocal micrograph using Vector red substrate and rhodamine filter. Distance between crosshairs is contained in box. IL-2R$^+$ cells grouped around an area of blasting cells (BC; unstained by this method) in the medulla of a day 4 *T. annulata*-infected lymph node.



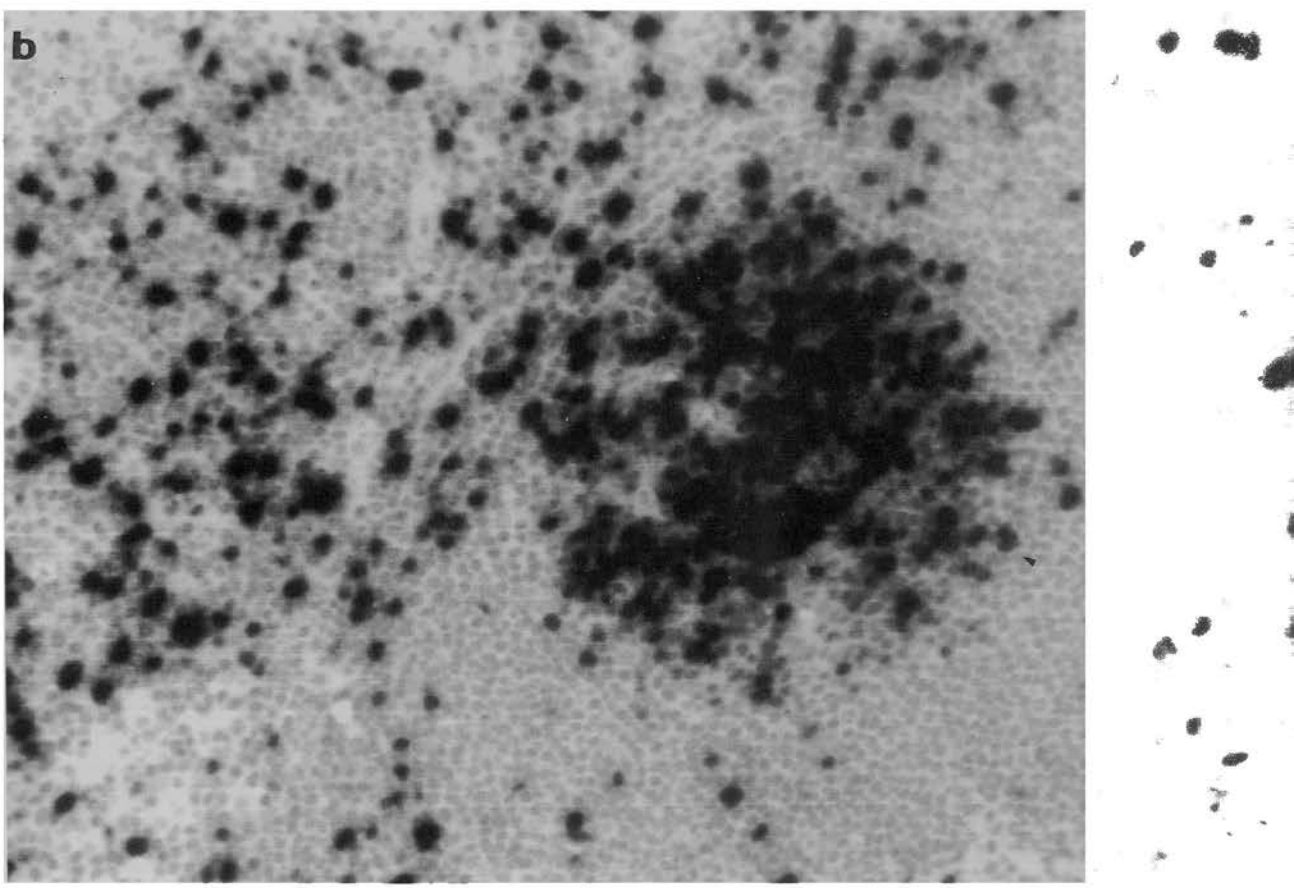


Fig. 5. Confocal micrographs using Vector red substrate and rhodamine filter. Distance between crosshairs is contained in box. (a) Normal bovine germinal centre (GC) stained with MoAb VPM30. The MoAb predominantly stains an area analogous to the human 'light zone', (b,c) Serial sections from a day 8 *Theileria annulata*-infected lymph node. Stained with (b) MIB-1 and (c) VPM30. Although MIB-1 'dark zone' staining persists, all VPM30 'light zone' staining is absent.

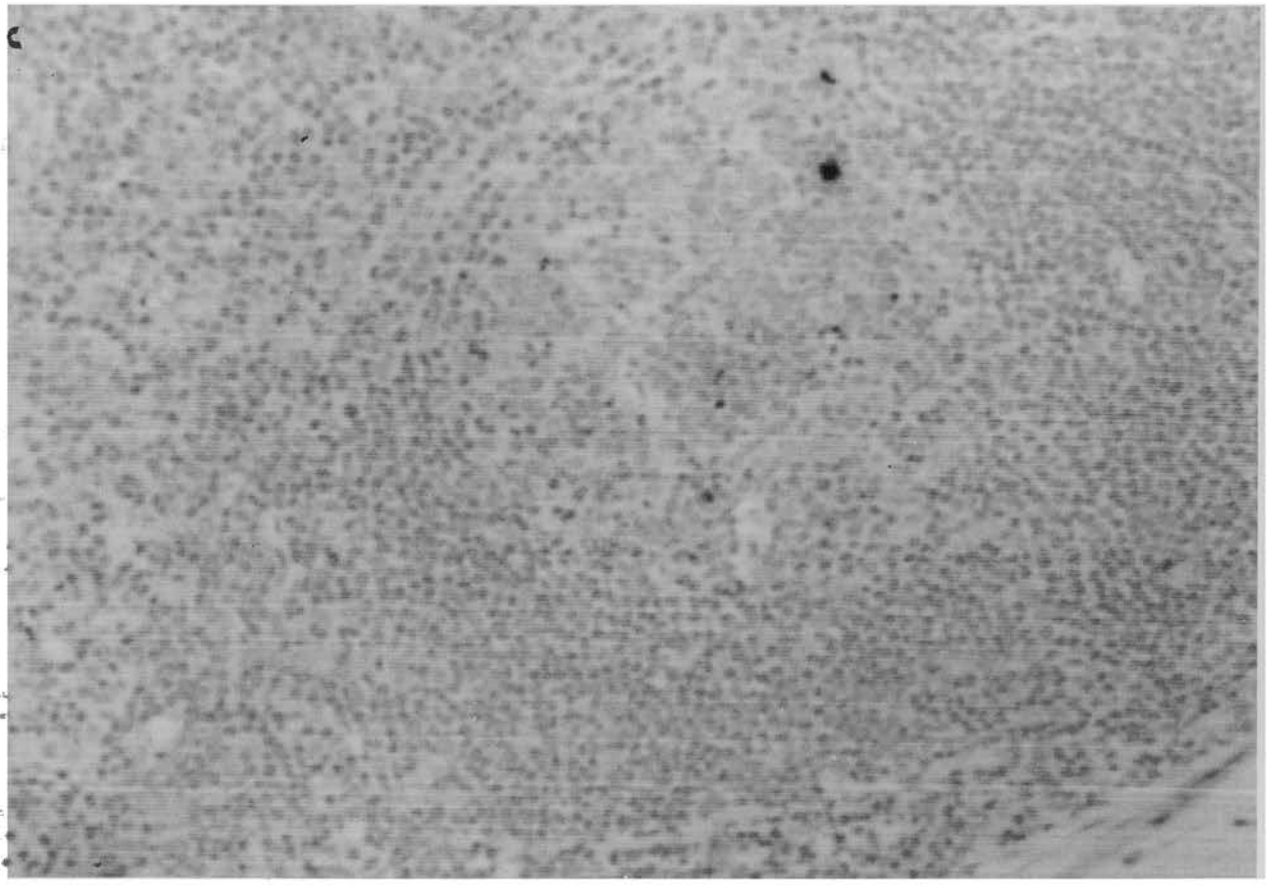


Fig. 5. Continued.

her analysis of the activation of $CD4^+$ and $CD8^+$ subsets, the antigens presented by MHC class II on infected cells, be required in order to characterize further the antigenic ent.

n the lymph nodes draining the sites of *T. annulata* infection, expressing IL-2R associated only with areas of parasite-ced proliferating cells in the medulla. Many of the latter cells MHC class II^+, express macrophage markers, and are found e parasitized when schizonts are detectable. It seems likely the *in vivo* appearance of IL-$2R^+$ cells in the medulla reflects non-specific' activation of T cells by parasitized cells *in vitro*. gen-primed interdigitating cells (IDC) have been shown to a major part in primary bovine $CD4^+$ T cell responses [27]. tudies of the induction of primary immune responses to ide antigens, it has been shown that T cells initially interact these cells in the paracortex 4–7 days post-inoculation, in vascular foci [28]. The appearance of IL-$2R^+$ cells exclusively e medulla suggests that *T. annulata* infection interferes with l activation in two ways: not only is T cell activation induced ne parasite-associated blasting cells, but also T cells are not g primed in normal anatomical sites. *Theileria annulata* tion therefore interferes with the normal pathways of T APC interaction.

Germinal centres were severely affected by the parasite tion, initially losing the 'light zone', followed by total of morphology. This is indicative of aberrant T cell function, as germinal centres are T cell-dependent [29]. The initial loss of the 'light zone' at a time when the proliferating dark zone is relatively unaffected also suggests that T cells are affected, as T cells are an essential component of the GC light zone [18,30], involved in cognate interactions to rescue somatically mutated (dark zone derived) centroblasts [31].

In this study we have shown that *T. annulata*-infected APC have the potential to interfere with immune response development as they can 'non-specifically' activate T cells irrespective of their antigen specificity *in vitro*. More importantly, a similar phenomenon is seen *in vivo* with the rapid appearance of activated T cells within the draining lymph node. Subsequent T cell priming in recognized sites of the lymph node does not occur. The alteration of T cell function is most dramatically manifested by the loss of the T cell-dependent compartments of germinal centres, followed by complete germinal centre breakdown.

Our findings also have important implications for vaccine design, as any vaccine would have to enhance the specific immune response without promoting non-specific proliferation. It seems unlikely that directing T helper cells towards direct interactions with infected cells would be effective, and indeed may be deleterious. We are currently studying the interactions of CD4 T cells from immune animals with infected cells and APC from within lymph nodes in order to determine which determinants are recognized by the 'specific' immune response.

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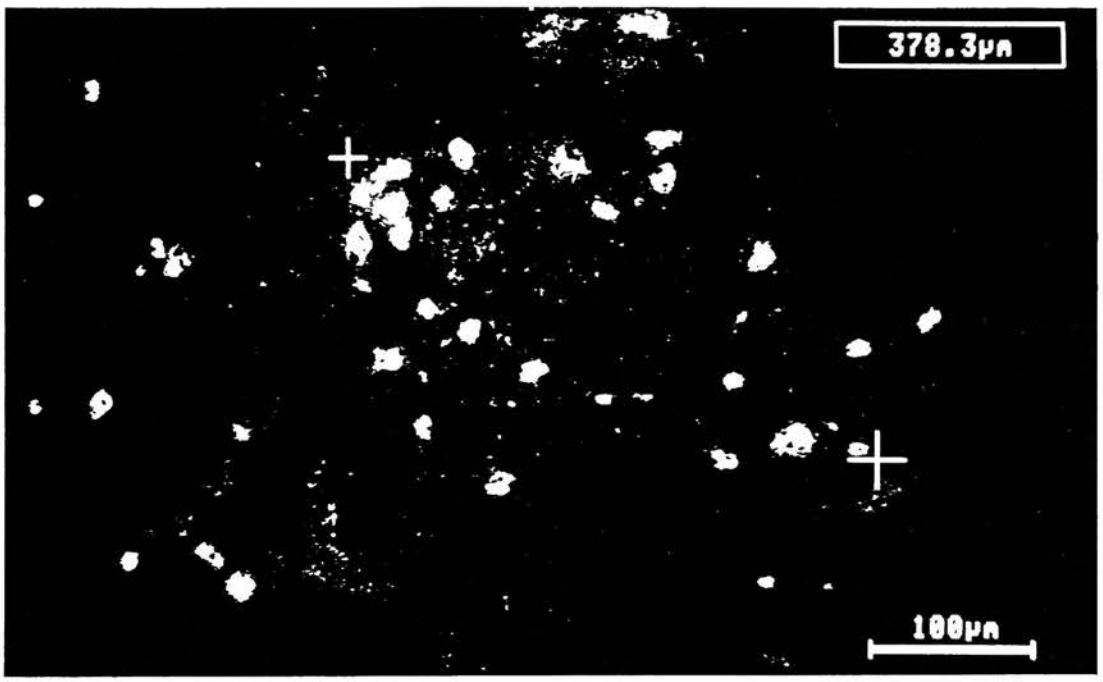

Fig. 5.22 Confocal micrograph of IL-2R$^+$ cells grouped around an area of blasting cells (BC, unstained by this method) within the medulla of a day 4 draining node. Vector red, rhodamine filter. Distance between crosshairs is contined within the box.

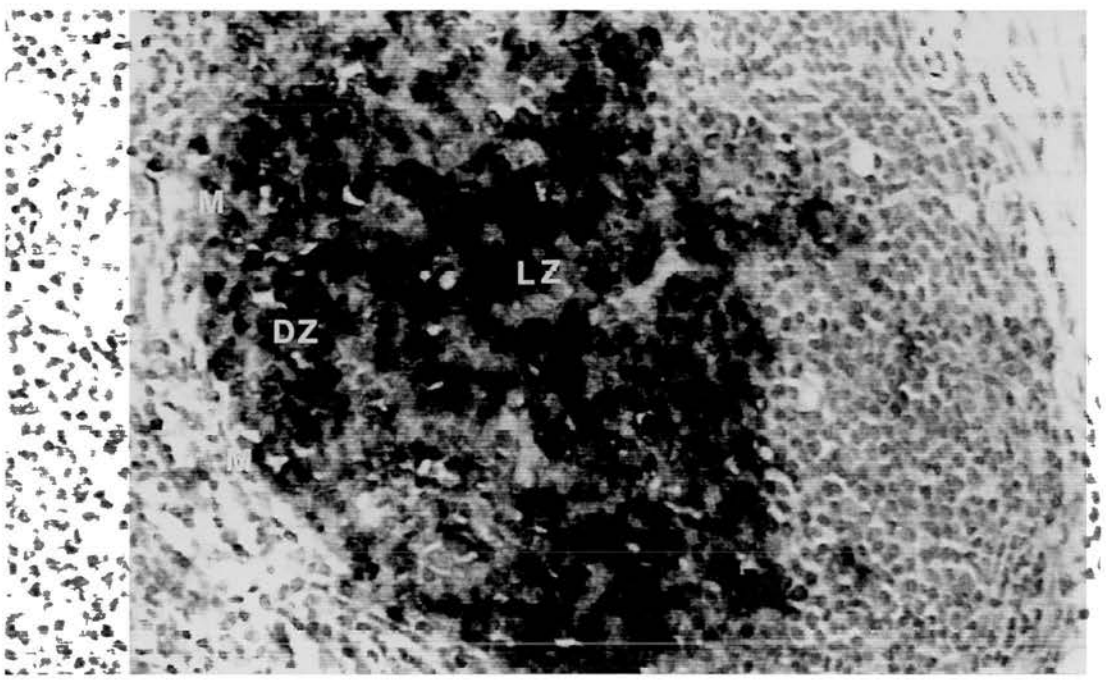

Fig. 5.23 Reverse confocal micrograph (positive cells are black) within a normal, well developed germinal centre stained with VPM30. Stained cells are largely outwith the dark zone (DZ), principally present in the light zone (LZ). Vector red.