IMMUNE RESPONSES AGAINST BOVINE TROPICAL THEILERIOSIS WITH PARTICULAR REFERENCE TO REIMMUNISATION WITH *Theileria annulata* INFECTED CELL LINES.

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ABSTRACT OF THESIS

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This study describes the effect of allogeneic immune responses on immunisation against *Theileria annulata* with a parasite infected cell line. The dynamics of lymphocyte populations during development of the immune response against the parasite after cell line immunisation and sporozoite challenge were investigated in peripheral blood, and lymph efferent from the lymph node draining the site of infection.

T. annulata infected cell lines have been used as vaccines against tropical theileriosis in several countries. Inoculated animals produce a strong response against the allogeneic MHC antigens of the immunising cell line followed by an anti parasite response. There is evidence that immunity to the parasite wanes in the absence of challenge and reimmunisation is often recommended. However, the effect of pre-existing allogeneic responses generated after first immunisation with *T. annulata* infected cell lines upon development of immunity against the parasite at the time of reimmunisation, is not known. To investigate this, an allogeneic response was first generated in the animals followed by immunisation with a *T. annulata* infected cell line of the same BoLA type. A mild allogeneic response generated by inoculation of uninfected leucocytes did not affect the development of immunity during cell line immunisation. However, a strong anti MHC response generated by skin grafting interfered with the development of a parasite specific immune response when the animals were immunised with $1x10^6$ cells. The effect of the allogeneic response was more marked when vaccination was carried out with a lower cell dose of $1x10^4$ cells, where the development of immunity against *T. annulata* was completely blocked. These observations are of immediate importance in endemic areas where *T. annulata* infected cell lines are being used as vaccines to control the disease.

The kinetics of various leucocyte subpopulations in the peripheral blood of animals revealed that leucopenia in response to *T. annulata* infection was caused by decline in the number of circulating neutrophils and lymphocytes. Lymphocytopaenia was caused mainly by reduction in the levels of circulating $CD4^+$ cells and B cells, and to a lesser degree of $CD8^+$ cells and $\gamma\delta$ T cells. Cells of the monocyte/macrophage lineage were least affected. The severity of leucopenia was related to the ensuing parasitological reactions after immunisation with cell line or challenge with sporozoites. The recovery and development of immunity in animals after cell line immunisation and sporozoite challenge was associated with an increase in $CD25^+$ cells in the peripheral blood. A transient increase in the levels of $CD8^+$ cells initially followed by a sustained increase in monocytes was associated with recovery indicating that immunity to *T. annulata* is mainly mediated by mechanisms involving cells of these two types. These cellular changes were not observed in animals undergoing cell line immunisation where the pre-existing allogeneic responses blocked development of parasite specific immune responses.

As initial pathology takes place in the lymph node draining the site of *T. annulata* infection, further studies concentrated on parasite dissemination and T cell activation in the draining efferent lymphatics following sporozoite infection or allogeneic cell line immunisation. During lethal sporozoite infection in the naive animals, the parasite induced strong lymphoproliferation. There was a massive increase in the output of blasting cells in efferent lymph from day 6 with a 5-10 fold increase in the flow rate and cell output. Efferent lymph contained very high levels of interferon-γ between day 5 to 9 after acute sporozoite infection. Parasite infected cells were seen from day 6 onwards. An initial increase in the percentage of $CD2^+$ cells corresponding with an increase in $CD4^+$ cells was observed from day 5. Many $CD4^+$ cells expressed CD25 transiently and became MHC class II^+. This was followed by increased output of blasting $CD8^+$ cells with high MHC class II expression. Blasting $CD4^+$ and $CD8^+$ cells gradually lost CD2 expression with the progression of infection. These cells were MHC class II^+ but had lost CD25 expression suggesting an inappropriate activation of T cells in response to the parasite. Efferent lymph cells were less responsive to Con. A or exogenous IL-2 stimulation *in vitro* during the later stages of infection. The cells did not proliferate *in vitro* in response to autologous parasite infected cells and did not kill autologous parasite infected cells suggesting lymphocyte unresponsiveness.

Animals immunised with $1x10^6$ *T. annulata* infected cells exhibited a 2-4 fold increase in the flow rate and cell output in efferent lymph. Parasite infected cells of the recipient origin were isolated from day 11 onwards, but cells of donor origin were never isolated. Two peaks of blasting cells were observed. The first corresponded to an allogeneic response against MHC antigens of the immunising cell line and the second was associated with the parasite specific response. However, a few $CD4^+$ and $CD8^+$ cells not expressing CD2 were also observed during the second parasite specific phase of the response. At this stage, CD25 and MHC class II expression on T cells increased. The inhibition of parasite development after cell line immunisation as a result of the pre-existing allogeneic response was not associated with any of these changes.

The results indicate that infection with *T. annulata* sporozoites leading to acute theileriosis induces an inappropriate proliferation and nonspecific activation of T cells in the efferent lymph. T cells initially express normal activation markers but lose CD2, a very important adhesion molecule with the progression of the disease. Both specific and some non-specific T cell activation is initiated after immunisation with *T. annulata* infected allogeneic cell lines which induce immunity to sporozoite challenge.

DECLARATION

I hereby declare that the work presented in this thesis is the product of my own efforts, and has not been submitted in any previous application for a degree. The work on which it is based is my own except where stated in the text and in the acknowledgement section.

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ABBREVIATIONS

Abbreviation	Full form
ACD	acid citrate dextrose
APC	antigen presenting cells
BCG	Bacille Calmette and Guerin
BoLA	Bovine leucocyte antigens
BPA	bovine plasma albumin
CD	clusters of differentiation
cDNA	complementary deoxyribonucleic acid
Con. A	concanavalin A
CTL	cytotoxic T lymphocytes
CTLp	cytotoxic T lymphocyte precursors
DLC	differential leucocytic count
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTH	delayed type hypersenstivity
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme linked immunosorbant assay
ELL	efferent lymph lymphocytes
FACS	fluorescent activated cell sorter
Fc	Fragment crystalline
FCS	foetal calf serum
FITC	fluorescein isothiocynate
FL1	fluorescence 1 (green fluorescence)
FL2	fluorescence 2 (red fluorescence)
FSC	forward scatter
GAM	goat anti mouse
GUTS	ground up tick supernates
HBSS	Hank's balanced salt solution
HEV	high endothelial venules
HIV	human immunodeficiency virus
IEF	isoelectric focussing
i.u.	international units
IFAT	indirect fluorescent antibody test
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-2R	interleukin-2 receptor
kd	kilodalton
LCA	leucocyte common antigens
LFA	lymphocyte function antigen
LN	lymph node

log	logarithmic
LPS	lipopolysacchride
mAb	monoclonal antibody
mCi	millicurie
MEM	minimum essential medium
MHC	Major Histocompatibility Complex
MLC	mixed lymphocyte culture
MLR	mixed lymphocyte reaction
mM	millimole
M_r	molecular weight
mRNA	messenger ribonucleic acid
mV	millivolts
NK	natural killer
NMS	normal mouse serum
O.D.	optical density
PBM	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCV	packed cell volume
PE	phycoerythrin
pers. comm.	personal communication
PG	prostaglandin
PMT	photomultiplier detectors
R1	region 1
RAB	rabbit anti bovine
RAM	rabbit anti mouse
RBCs	red blood cells
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
S.G.	specific gravity
SIV	simian immunodeficiency virus
spp.	species
SSC	side scatter
t.e.	tick equivalent
TC	tissue culture
TCGF	tissue culture growth factor
TCR	T cell receptor
TEC	total erythrocytic count
Th	T helper cells
TLC	total leucocyte count
TNF	tumour necrosis factor
VCAM	vascular cell adhesion molecule
WC	workshop clusters

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CHAPTER 1

INTRODUCTION

Rapid population increase in the third world countries necessitates a gigantic growth in food production through improved agriculture and livestock farming. Future growth is likely to occur in the tropical and sub-tropical regions of the world because of the presence of unexploited areas with potential for expanding animal production. Cattle are currently the most important animal resource of food in the form of meat and milk. Productivity of cattle is quite low in the tropics and concentrated attempts for its enhancement are constrained by factors such as: climate, nutrition, management, genetic productivity limits of local breeds and, last but not least, diseases (Pino, 1981).

Infectious blood diseases constitute the greatest single deterrent to the growth of cattle production in the tropics. The most important tick-borne diseases of cattle responsible for the economic losses are theileriosis and babesiosis (Uilenberg *et al.*, 1993). Efforts to improve the productivity of local indigenous cattle in Africa, Asia and the Middle East, by crossbreeding programmes with exotic European cattle of exquisite performance traits, are seriously hampered because of very high susceptibility of both the exotic animals and their crossbreds to tick-borne haemoprotozoan diseases (Gill *et al.*, 1980). Indigenous animals are relatively resistant, probably because they have been living with the parasites for centuries. The initial importation of exotic cattle to the tropical countries sometimes ended up in 100% mortalities particularly because of theileriosis. Crossbreds produced from the imported cattle are also highly susceptible (Oudich *et al.*, 1993).

Bovine tropical theileriosis (Mediterranean Coast fever) and East Coast fever are the two important haemoprotozoan diseases of cattle caused by *Theileria annulata* and *Theileria parva*, respectively. Morbidity and mortality rates of these two diseases in exotic and crossbred animals are very high. Although indigenous animals may be inured to the effects of these diseases, their productivity potential is reduced (Irvin & Morrison, 1987; Brown, 1990). Studies on *Theileria* parasites are also important because of their striking similarities with other apicomplexan parasites, like *Babesia* which infect a number of species, and *Plasmodium* which cause malaria and affect almost 300 million people every year. Malaria infections of man and theileriosis of cattle are thought to be the diseases of greatest significance in the context of third World development (McKeever *et al.*, 1994).

Over 250 million cattle were estimated to be at risk from this disease in 1982 (Robinson, 1982). The disease occurs across a wide zone of tropics in Africa, Europe and Asia which stretches from Morocco to China. The precise economic losses caused by the disease are difficult to calculate as extensive studies on incidence, loss of production and mortality are not available. A recent report from one part of India (Kaira district) where tropical theileriosis is endemic may give an idea of the magnitude of the losses. Out of 40,000 adult crossbred cattle in that district, 3,800 clinical cases of the disease were recorded in the year 1988-89 (Singh, 1991). In addition to direct costs of treatment which are very expensive and unaffordable for many livestock owners in endemic areas, enormous costs are incurred in attempting to control the vector ticks by use of acaricides. Apart from these expenses, losses due to loss of production and mortality are tremendous and difficult to estimate.

T. annulata is transmitted by infected ticks to cattle while feeding. The inoculated sporozoites invade host cells and develop into macroschizonts in the draining lymph node inducing the host cells to become blastoid which divide in synchrony with the macroschizont. As the infection progresses, microschizonts are observed which give rise to merozoites by destruction of the infected cells. Merozoites invade erythrocytes to form piroplasms. Both macroschizonts and piroplasms perhaps contribute to the pathogenesis of the disease leading to severe leucopenia and anaemia (Neitz, 1957; Sharma & Gautam, 1971; Hooshmand-Rad, 1976; Preston *et al.*, 1992a).

The macroschizont stage of the parasite can be cultured *in vitro* leading to attenuation of the parasite (Brown, 1983). Continuously growing allogeneic *T. annulata* infected cell lines have been successfully used as live vaccines in endemic areas (Hashemi-Fesharki & Shad-Del, 1973; Wenshun *et al.*, 1973; Pipano,1981; Singh *et al.*, 1993). Parasite specific cytotoxic T cell activity (Preston *et al.*, 1983; Innes *et al.*, 1989a) and macrophage mediated cytostasis (Preston & Brown, 1988) against parasite infected cells, and humoral responses in the form of antibodies against sporozoites, schizonts and piroplasms (Preston & Brown, 1985; Kachani *et al.*, 1992a) have been observed in the peripheral blood of cell line immunised animals or after drug treatment in sporozoite infected animals. The natural infection with sporozoites in susceptible animals leads to severe disease symptoms and death in 40-60% of the cases without development of protective immune responses. However,

the involvement of various lymphocyte sub-populations and activation of T cells for the development of immunity after infection with *T. annulata* sporozoites or immunisation with parasite infected cell lines is not well understood. Precise knowledge of the mechanisms involved in the induction of protective immunity within the bovine host is an essential pre-requisite for development of effective methods of prophylaxis.

A cell culture vaccine introduces the parasite to the recipient animal in the context of a foreign graft as infected cells express BoLA (Bovine leucocyte antigens) haplotypes identical to those of the original animal from whose lymphocytes they were derived (Spooner & Brown, 1980). Inoculation of *T. annulata* infected cell lines produces a strong allogeneic response against MHC (Major Histocompatibility Complex) antigens of the immunising cell line followed by a parasite specific immune response. Development of protective immunity is associated with the establishment of parasite within the cells of the recipient (Innes *et al.*, 1989a). Duration of immunity after cell line immunisation is poorly defined. There is evidence that immunity against the parasite wanes (Pipano, 1977; Ouhelli *et al.*, 1994) particularly in the absence of the natural tick challenge in the field and there may be need to boost the immunity. Some evidence from both Morocco and Israel suggests that reimmunisation with the same cell line does not boost immunity. This could arise because of the allogeneic responses induced after first cell line immunisation giving rise to a graft rejection response at the time of revaccination. The effect of pre-existing allogeneic responses on the development of immunity against the parasite at the time of second immunisation with the same cell line is not known. Keeping these points in view, the work in this thesis was undertaken with the following objectives:

a. To study the effect of a previously generated allogeneic immune response on immunisation against *T. annulata* with parasite infected cell lines in the context of a need for repeated immunisation against tropical theileriosis.
b. To investigate the dynamics of various lymphocyte populations, in the peripheral blood and efferent lymph from the draining node, during development of an immune response after inoculation with sporozoites or parasite infected cell lines.
c. To characterise and compare the activation of T cells in the lymph efferent from the node draining the site of sporozoite infection or cell line immunisation and their relationship with the development of a protective immune responses.

CHAPTER 2

REVIEW OF LITERATURE

This chapter provides a general background of bovine tropical theileriosis caused by *Theileria annulata*. A brief description of various bovine leucocyte markers relevant to this thesis and general organisation of bovine immune system at the lymph node level is given. This is followed by a review on the current state of knowledge on immunisation and development of immune responses against tropical theileriosis.

The diseases caused by various *Theileria* species (*spp.*) have been recognised since the beginning of this century. Considerable research has been undertaken on these parasites. The main economically important theilerial species for cattle are *T. annulata* and *T. parva* and to a lesser degree *T. sergenti*. This review is mainly concerned with the first two species. More emphasis is laid on *T. annulata* with some aspects of *T. parva* as well for comparison.

2.1 THEILERIOSIS- GENERAL DESCRIPTION

Theilerial parasites are tick transmitted, blood borne protozoa which affect a wide range of mammals mainly ruminants and are particularly important for domestic cattle, sheep and goats in tropical and subtropical countries. Various *Theileria* species have been reviewed from time to time (Sergent *et al.*, 1945; Neitz, 1957; Barnett, 1968; Barnett, 1977; Purnell, 1977; Irvin, 1987; Irvin & Morrison, 1987; Norval *et al.*, 1992).

2.1.1 Historical background and taxonomy

The parasite of the genus *Theileria* was first recognised by Koch in 1897 and were initially assumed to be immature forms of *Babesia bigemina* (Koch, 1903). However these intraerythrocytic organisms were recognised to be distinct from *B. bigemina* when large numbers of cattle suffered from East Coast fever in Rhodesia (Zimbabwe) and Southern Africa. The parasite was then named as *Piroplasma parvum* (Theiler, 1904). The exo-erythrocytic schizont stage inside lymphocytes was discovered by Koch in 1906 and subsequently the parasite was named *Theileria parva* (Bettencourt *et al.*, 1907). Simultaneously, Dschunkowsky and Luhs (1904) discovered a fatal disease in cattle in Southern Russia and called it tropical piroplasmosis caused by *Piroplasma annulatum*, which was also placed later under the new genus *Theileria* and named *T. annulata* (Bettencourt *et al.*, 1907). Further investigations in North Africa, Asia and Europe over the years showed many unidentified types of theilerial parasites widely distributed in these continents (Neitz,

1957). With the passage of time, some of these *Theileria spp.* were defined as new species. The taxonomy of *Theileria* has been a subject of controversy from the very beginning with species being split up, recombined or shifted from one genus to the other with the advancement of knowledge. On the basis of the recent classification of the protozoa by Levine (1988), genus *Theileria* is now classified as follows:

Kingdom	Protista
Subkingdom	Protozoa
Phylum	Apicomplexa
Class	Sporozea
Subclass	Piroplasmia
Order	Piroplasmorida
Family	Theileriidae
Genus	*Theileria*

Taxonomy of the genus *Theileria* has been reviewed by Uilenberg (1981a). Levine (1988) reported 34 species of *Theileria*, but many of these are likely to be synonyms (Irvin, 1987). The main *Theileria* species which affect cattle, as described by Uilenberg *et al.* (1993) and Norval *et al.* (1992), are described in table 2.1.

Table 2.1: *Theileria* species of cattle.

Species	Main known hosts	Main known tick vectors	Disease caused	Reference
T. annulata	Cattle, Asian buffalo	*Hyalomma spp.*	Tropical theileriosis, Mediterranean Coast fever	Dschunkowsky & Luhs, 1904
T. parva	Cattle, African buffalo	*Rhipicephalus spp.*	East Coast fever, Corridor disease, January disease	Theiler, 1904
T. mutans	Cattle, African buffalo	*Amblyomma spp.*	Benign theileriosis	Theiler, 1906
T. sergenti/ buffeli/ orientalis	Cattle, Asian buffalo	*Haemaphysalis spp.*	Oriental theileriosis, Benign cosmopolitan theileriosis	Yakimoff & Dekhteroff, 1930
T. taurotragi	Eland, Cattle, Antelope	*Rhipicephalus spp.*	Benign African Rhipicephaline theileriosis	Martin & Brocklesby, 1960
T. velifera	Cattle, African buffalo	*Amblyomma spp.*	Non-pathogenic	Uilenberg, 1964

The naming of *T. parva* and the diseases it causes has been recently reviewed (Perry & Young, 1993). Some scientists advocate sub-speciation of *T. parva* into *T.p. parva* (East Coast fever) for the parasite causing acute classical disease, *T.p. bovis* (January disease) for the parasite causing mild disease in Zimbabwe and *T.p. lawrencei* (Corridor disease) for the buffalo derived parasite (Lawrence, 1979; Uilenberg, 1981a; Uilenberg *et al.*, 1993). However, this trinomial classification may not be valid as some of the field isolates cross-protected in challenge experiments (Irvin *et al.*, 1989). Further, oligonucleotide probes generated on the basis of the variable region sequence of the subunit ribosomal RNA genes of different *Theileria* spp. were unable to distinguish between various *T. parva* subspecies (Allsopp *et al.*, 1993). There has been confusion over the status of *T. orientalis* as well. Uilenberg (1981a), Uilenberg *et al.* (1985) and Uilenberg *et al.* (1993) considered that *T. sergenti* from Japan, *T. buffeli* from Australia and *T. orientalis* from Europe as different forms of one species and named it as *T. orientalis*. However, Fujisaki *et al.* (1994) have shown that *T. sergenti* is a different species from *T. orientalis* and *T. buffeli* on the basis of 33 kilodalton (kd) piroplasm protein. Recently, a new *Theileria* parasite was isolated from Kenya which could not be transmitted by ticks of the genus *Rhipicephalus* and *Amblyomma* (Ngumi *et al.*, 1994). This species has not yet been fully characterised.

Identification of various species and stocks of *Theileria* was previously based on the morphological characteristics of different parasite stages, geographical distribution, vector specificity, host specificity, cross-immunity and pathological, epidemiological and serological characterisation. More sophisticated techniques are now being used which should help in resolving these controversies. These techniques include the use of monoclonal antibodies (Pinder & Hewett, 1980; Williamson *et al.*, 1989; Musoke & Nene, 1990), characterisation of isoenzyme pattern (Melrose & Brown, 1978; Ben Miled *et al.*, 1994) analysis of protein antigens (Creemers, 1983), use of cDNA probes (Allsopp *et al.*, 1989; Allsopp *et al.*, 1993; de Kok *et al.*, 1993; Fujisaki *et al.*, 1994) and restriction fragment length polymorphism (RFLP) (Morzaria *et al.*, 1990).

2.1.2 Distribution

Bovine tropical theileriosis occurs mainly in tropical and subtropical regions from Portugal, Spain and Morocco in the west, through the Mediterranean coast of

Europe and North Africa, southwards into the Sudan and Eritrea and eastwards into south-east Europe, the Near East and Middle East, southern Russia and Siberia and across the Indian subcontinent through to China and the Far East (Purnell, 1978, Robinson, 1982). *T. parva* is comparatively less widespread than *T. annulata* and is limited to eastern, central and southern Africa (Norval *et al.*, 1992; Uilenberg *et al.*, 1993). Distribution of the two parasites does not overlap except in Sudan where both the parasites cause disease (Norval *et al.*, 1992). Distinct distribution of the two parasites is probably because of the difference in their tick vectors. Figure 2.1 gives an overview of the distribution of these two parasites.

2.1.3 Transmission

Theileria parasites are transmitted trans-stadially by two and three host ticks (from larva to nymph or nymph to adult). Transmission of *T. annulata* has been reviewed by Robinson (1982). *T. annulata* is transmitted exclusively by ticks of the genus *Hyalomma*. The three host tick *H. anatolicum anatolicum* and the two host tick *H. detritum* are the main vectors. *H. a. anatolicum* occurs from northwest Africa eastwards to India. It is replaced by another three host tick *H. lusitanicum* in the southwest mediterranean region and Spain (Barnett, 1977). *H. detritum* ranges from Manchuria through China and India, former southern Soviet Union, south eastern Europe, the Middle East and the Mediterranean littoral of Africa (Norval *et al.*, 1992). Other *Hyalomma* species such as *H. dromedarii, H. a. excavatum, H. marginatum* and *H. scupense* have been shown to transmit the disease in experimental conditions (Barnett, 1977; Uilenberg, 1981a). Ten *Rhipicephalus* and three *Hyalomma* species have been shown experimentally to be possible vectors of *T. parva*. However, the most common vector is *Rhipicephalus appendiculatus* (Norval *et al.*, 1992).

Transmission of the sporozoites from tick to bovine host occurs two to four days after attachment of the tick for feeding on a susceptible host, as the stimulus of a blood meal is required to induce maturation of the parasite in the tick salivary gland (Purnell *et al.*, 1973; Samish & Pipano, 1978; Singh *et al.*, 1979). However, increase in the temperature and relative humidity also leads to maturation of parasites in the salivary glands without any blood meal (Samish, 1977; Young *et al.*, 1979). This suggests that maturation of the parasite in the salivary glands of ticks may occur earlier under natural conditions in hot areas and ticks may transmit the infection as soon as they attach to a susceptible host. Engorgement of an infected tick on a clean

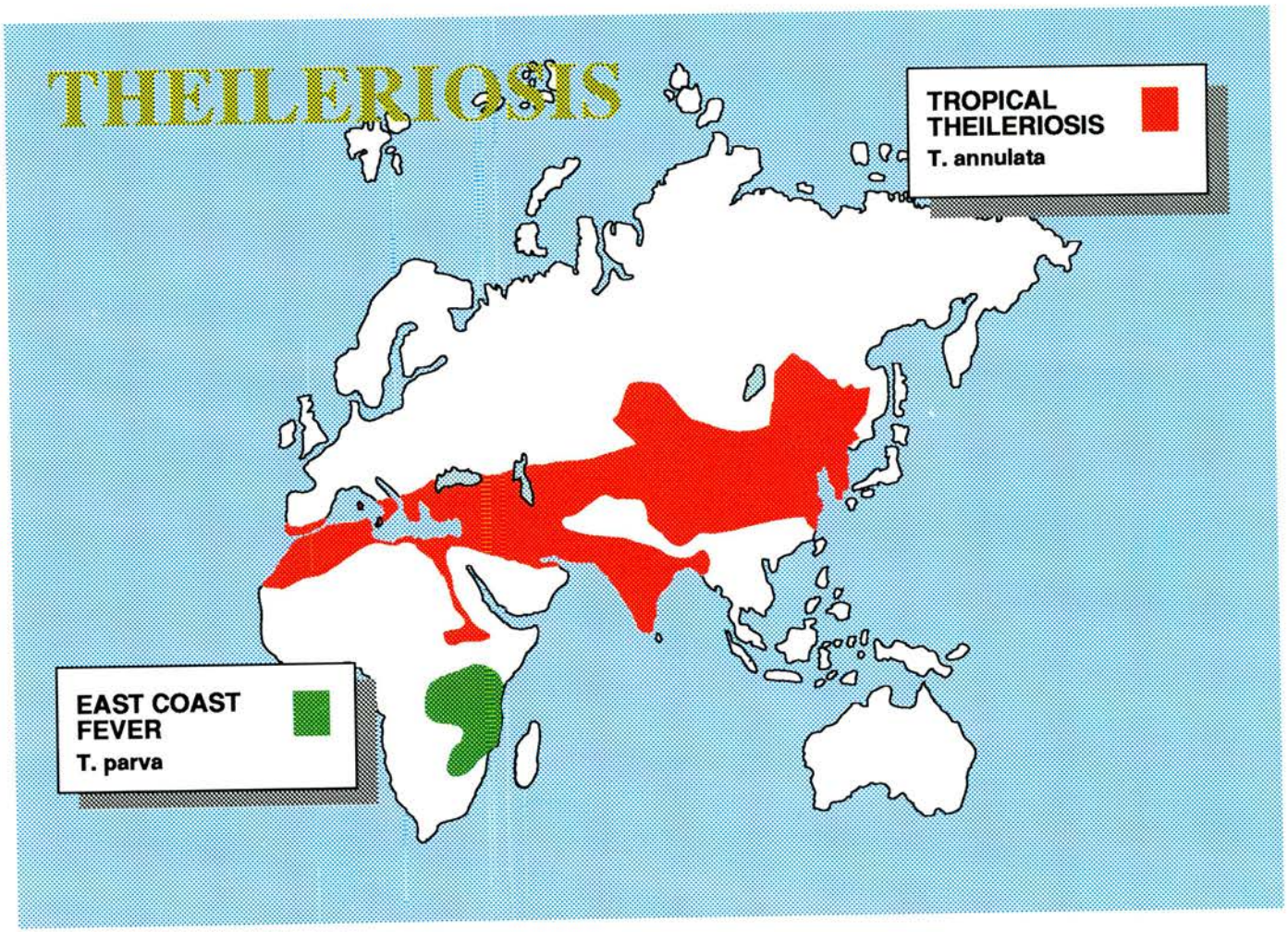


Figure 2.1: Distribution of *Theileria annulata* and *Theileria parva*.

susceptible host makes it clear of the infection in the following instar. However, infection in the tick persists into the following instar if feeding takes place on a non-susceptible host such as rabbits (Bhattacharyulu *et al.*, 1975).

2.1.4 Susceptibility

T. annulata infection mainly causes severe disease in exotic *Bos taurus* breeds of cattle and their crosses, whereas local Zebu and taurine breeds reared in the endemic areas are usually resistant. This has been observed in most of the endemic areas (Singh, 1990; Hashemi-Fesharki, 1991; Flach & Ouhelli, 1992). Young *Bos indicus* calves also suffer from the disease. Adult *Bos indicus* and indigenous taurine breeds are thought to be relatively resistant. Similarly, local breeds were shown to be more resistant than exotic taurine breeds in East Coast fever endemic areas (Guilbride & Opwata, 1963). Moll *et al.* (1986) demonstrated that low numbers of Zebu calves died in endemically stable areas with *T. parva* infection although high numbers of improved taurine cattle would die if introduced to these areas. Recently, Preston *et al.* (1992a) showed that *Bos taurus* x Sahiwal (*Bos indicus*) calves were relatively more resistant to tropical theileriosis than taurine calves.

Hard evidence for genetic control of resistance to tropical theileriosis or East Coast fever is lacking (Spooner & Brown, 1991). In some reports, local cattle reared in disease free conditions for some years suffered more than imported cattle after challenge with *T. annulata* (Sergent *et al.*, 1945). However, recent epidemiological studies in Morocco clearly show that local cattle are more resistant than exotic animals and their crossbreds (Oudich *et al.*, 1993). It is not clear whether the resistance observed in local breeds in endemic areas is because of some genetic factors due to selection over the years of their association with the parasite or because of early exposure of young animals to the parasite making them pre-immune. Valid evidence for genetic resistance can only be obtained from studies within families with the use of highly quantified tick stabilate and clearly definable tests to estimate the level of resistance (Spooner, 1993).

Observations from India suggest that clinical theileriosis in new-born calves up to 8 weeks of age is a serious problem (Grewal *et al.* 1991; Mishra *et al.*, 1994; R.D. Sharma, unpublished observations). In Pakistan, young animals below nine months of age were reported to suffer mostly from clinical theileriosis with the worst affected animals between 2-4 months of age (Hussain *et al.*, 1991). Observations in

Morocco suggest that very young calves are less affected (Flach & Ouhelli, 1992). These differences could be because of different animal management and tick control practices. The second important factor could be the tick vector. In areas where the disease is transmitted by two host ticks, only the adult stage of the tick is capable of transmitting the infection. Adult ticks feed more preferentially on adults than young calves and hence young calves are less affected. In areas where the disease is transmitted by three host ticks, both nymphs and adult ticks can transmit the disease. Immature ticks feed equally well on adult and young animals and, therefore, young animals are infected.

2.1.5 Life cycle

The life cycle of the parasite is illustrated schematically in figure 2.2. Two main stages in the tick: gametogony and sporogony, have been described in detail by Cowdry & Ham (1932) and Mehlhorn & Schein (1984). The life cycle of *Theileria* has also been reviewed by Sergent *et al.* (1945), Neitz (1957), Schein (1975), Uilenberg (1981b) and Mehlhorn & Schein (1993).

The bovine host is infected through inoculation of sporozoites by the saliva of infected ticks during feeding which invade leucocytes probably within 5-60 minutes (min.) as observed during *in vitro* studies on *T. annulata* (Jura *et al.*, 1983) and *T. parva* (Fawcett & Stagg, 1986; Shaw *et al.*, 1991). It is not known where and when the parasite encounters the bovine target cells for invasion *in vivo*. It could be in the mouth parts of the tick, the tick attachment lesion or the pool of cells and fluids that develops below this lesion (Walker & Fletcher, 1986). It could take place in the afferent lymph draining to the node from the site of the tick bite or in the node. Sporozoites probably come in contact with the target cells through a receptor-ligand driven process (Tait & Sacks, 1988) and enter by invaginating the plasma membrane of the target cell in an active temperature dependent process (Jura *et al.*, 1983).

After entry into the host cell, the sporozoite develops into a multi-nucleate trophozoite which after nuclear division develops into a macroschizont transforming the host cell and inducing it to become a large blast cell. Presence of the parasite stimulates the host cell to divide giving rise to a large population of infected cells in the animal. During host cell division, the theilerial particles also divide and are distributed by the host cell spindle apparatus to each of the daughter cells (Hulliger *et al.*, 1964; Hulliger, 1965). After several cycles of clonal expansion, schizonts

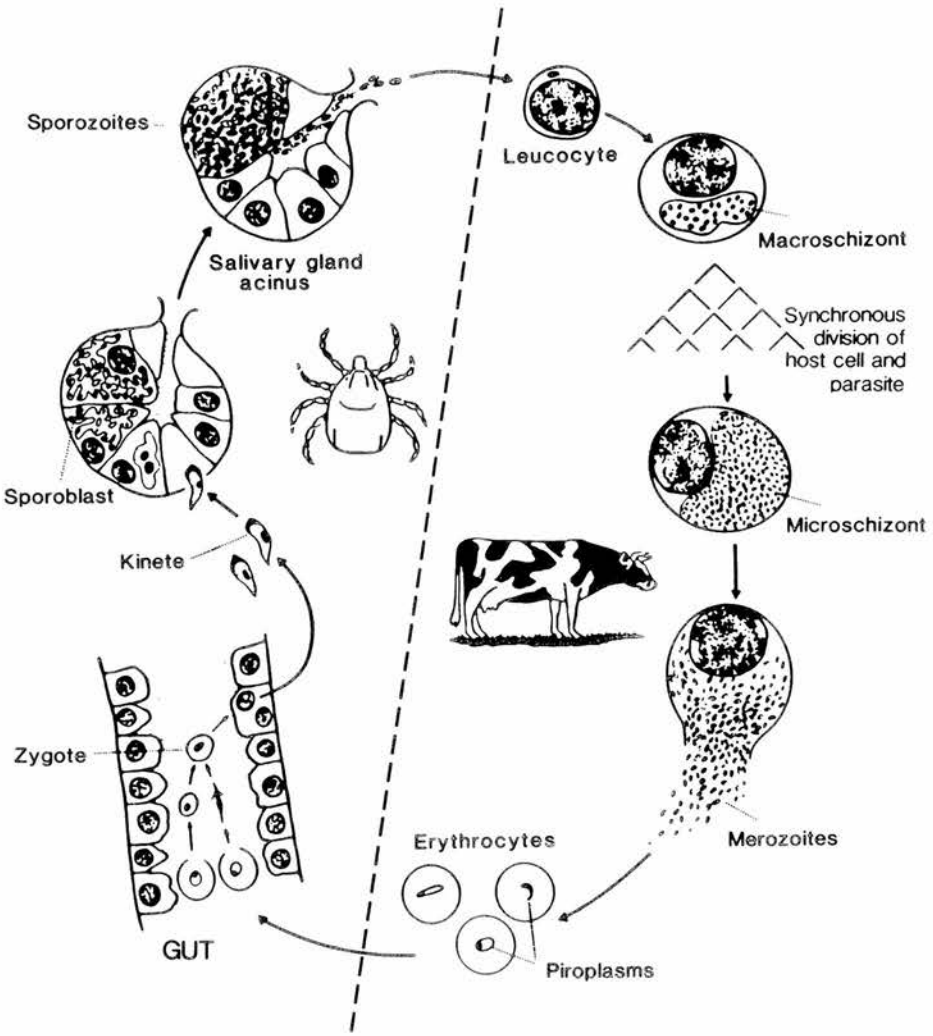


Figure 2.2: Life cycle of *Theileria sp.*

Adapted from "Goddeeris, B.M., Ph.D. thesis, 1986".

differentiate into microschizonts and start producing merozoites *in vivo* in response to some unknown stimuli. One of these stimuli could be fever in the affected animals as merogony can be induced *in vitro* in continuously growing *T. annulata* cell lines by raising the temperature of the culture to 41°C.

Differentiation to the merozoite stage (merogony) takes place within the host cell by increased nuclear division and generation of rhoptry and microneme organelles (Schein *et al.*, 1978; Mehlhorn & Schein, 1984). This process results in significant changes in the antigenic profiles of the parasite, indicating that it is a major point of differentiation in the mammalian phase of the parasite life cycle and involves both positive and negative regulation of gene expression (Glascodine *et al.*, 1990). Efficiency of differentiation from schizont infected cell to merozoite has been shown to be variable in cloned *T. annulata* cell lines. Differentiation to the merozoite stage was found to be a two step process, with a preliminary reversible phase of differentiation leading to a second irreversible phase. This was associated with an increased number of theilerial particles per cell generated by a disruption in the synchrony between parasite growth and host cell division (Shiels *et al.*, 1992). This development leads to destruction of the host cell and release of merozoites into the extracellular environment.

Free merozoites invade erythrocytes and develop into piroplasms which can be detected nine to ten days after experimental infection with ground up tick supernates (GUTS) (Preston *et al.*, 1992a) and slightly later with feeding ticks. The mechanism of penetration of merozoites into erythrocytes is not known and is presumed to be similar to the entry of sporozoites into host cells. The piroplasms undergo division within the erythrocytes by binary fission (Mehlhorn & Schein, 1984) or schizogony (Conrad *et al.*, 1985). Both schizont and piroplasm stages probably contribute to the development of leucopenia and anaemia, which are the main pathogenic features in tropical theileriosis (Sharma & Gautam, 1971; Hooshmand-Rad, 1976; Laiblin, 1978; Preston *et al.*, 1992a). However, the mechanism of development of anaemia and leucopenia is not understood. The erythrocytic parasites persist throughout life in recovered animals (Sergent *et al.*, 1945).

Apart from asexual multiplication of parasite in the bovine host, development of the sexual stages of both *T. annulata* (Sergent *et al.*,1945; Neitz, 1957; Schein, 1975) and *T. parva* (Cowdry & Ham, 1932; Mehlhorn & Schein, 1984) occur within

the tick. Piroplasm infected erythrocytes ingested by the tick during engorgement are lysed within the gut lumen, giving rise to sexual stages (gametes) which combine to form a zygote. The zygote enters gut epithelial cells and develops into a motile club shaped kinete by day 7 after engorgement (Reid & Bell, 1984). There is some synchronisation of kinete formation, which may be controlled by the process of moulting in the tick (Young & Leitch, 1980), as salivary glands are only available for infection by the kinete at the time they re-develop after each moult (Fawcett *et al.*, 1981). Such a mechanism would ensure that the kinete penetrates the salivary gland acini when these would be in a suitable state for development (Reid & Bell, 1984). Kinetes develop in the cells of type II or type III acini (Schein & Friedhoff, 1978). It rounds up forming a sporoblast which can be detected one or two days after the tick completes moulting. When the tick attaches to a new host and commences feeding, nuclear division in the sporoblast (sporogony) produces infective uninuclear sporozoites which are released in the tick saliva and injected into the new host (Fawcett *et al.*, 1985). The number of sporozoites developing in one acinar cell were estimated to be about 50,000. This amounts to an enormous inoculum from one tick, when many acini of the salivary gland are infected (Mehlhorn & Schein, 1993).

2.1.6 Host target cells for invasion by sporozoites

The nature of target cells infected by sporozoites of *T. annulata* and *T. parva*, after inoculation into an animal or *in vitro*, has been a matter of controversy for many years mainly because of lack of suitable reagents to identify populations of potential target cells. Development of monoclonal antibodies (mAbs) which distinguish specific bovine lymphocyte populations (Lalor *et al.*, 1986) has facilitated identification of potential targets for *in vitro* infection by sporozoites. Various cell sub-populations were separated using specific mAbs and infected *in vitro* with sporozoites. *T. annulata* sporozoites infected and transformed cells of monocyte and macrophage lineage and to a lesser degree B cells, but not T cells. On the other hand, *T. parva* sporozoites transformed T cells and B cells, but not monocytes/macrophages (Spooner *et al.*, 1989). Mammary gland macrophages were permissive to infection by *T. annulata* but only to a limited extent by *T. parva*. *T. annulata* preferentially infected MHC class II^+ cells (Glass *et al.*, 1989). Innes *et al.* (1989b) attempted to infect bovine alloreactive cytotoxic T cell lines with sporozoites of both the parasites. The lines were very easily infected by *T. parva* sporozoites, but not by *T. annulata*

sporozoites. Another study with *T. parva* sporozoites showed that the parasite transformed B cells, $CD4^+$ and $CD8^+$ cells and null cells ($\gamma\delta$ T cells), but not monocytes or neutrophils (Baldwin *et al.*, 1988a). Recently, *T. annulata* sporozoites were shown to infect mature monocytes more readily than immature monocytes, although these cells were also easily infectable (Campbell *et al.*, 1994a). It is interesting to note here that Sergent *et al.* (1945) demonstrated that *T. annulata* infected cells of the reticular endothelial system, reticular cells of the node and the spleen, Kupffer cells of the liver and hystiocytes.

Most of the these experiments were done *in vitro*. Further cells infected with either parasite ceased to express a number of surface markers including those for monocytes and B cells (Spooner *et al.*, 1988; Baldwin *et al.*, 1988a). Cells positive for the elastin receptor (a potential ligand for sporozoite entry) or the lipopolysaccharide (LPS) receptor (CD14) lost these expressions after transformation with *T. annulata* sporozoites (Campbell *et al.*, 1994a). Expression of MHC class II antigens has been very high on both *T. annulata* and *T. parva* infected cells (Spooner *et al.*, 1988; Glass *et al.*, 1989). The infected cell lines of both parasites express MHC class I antigens. BoLA specificities of infected cells are unchanged from the original donor lymphocytes (Spooner & Brown, 1980).

Despite extensive studies on finding target cells for these two parasites *in vitro*, little work has been done on identifying the target cells for sporozoite invasion *in vivo*. This is probably due to difficulties in simultaneous monitoring of target cell phenotype and parasitosis, mainly because of changing expression of different cell surface markers after transformation. Presumably, the same cell subpopulations are infectable *in vivo* as well. Some supporting evidence is available in the literature. Baldwin *et al.* (1988a) observed that most of *in vivo* derived *T. parva* cell lines expressed T cell markers. Subsequently, *T. parva* infected cells exiting in the efferent lymph of animals undergoing clinical infection were shown to co-express both CD4 and CD8 T cell markers (Emery *et al.*, 1988). The authors argued that $CD4^+$ cells were initially infected and expressed CD8 upon transformation. These observations have important implications on the pathogenesis and initiation of immunity. The phenotype of the infected cell would determine the migration patterns through various body compartments affecting the severity of lesions in different organs or tissues, secretion and responsiveness to various cytokines and antigen presentation to immuno-

competant cells. Alterations in the expression of surface markers after transformation of the infected cells further complicate the situation.

Theileria parasites must undergo a recognition event for entry into target cells presumably via interaction between ligands on the parasite surface and receptors on the target cells (Tait & Sacks, 1988). Williamson *et al.* (1989) identified an antigen SPAG-1 on the surface of *T. annulata* sporozoites, which could be involved in this process. A mAb against SPAG-1 blocked sporozoite penetration into the target cells. SPAG-1 exhibited a remarkable degree of molecular mimicry to an extracellular matrix protein, elastin (Hall *et al.*, 1992b). It was, therefore, suggested that receptor for elastin which is expressed mainly on monocytes and macrophages might be a ligand for recognition of host cells by the sporozoites. However, *T. annulata* sporozoites infected sorted elastin receptor positive and negative cells with equal efficiency in *in vitro* experiments (Campbell *et al.*, 1994a). The receptor for entry of *T. parva* sporozoites is also not known. It might relate to an epitope on a 67 kd antigen on the sporozoite surface as entry into the target cells can be blocked by antibodies raised against this epitope (Musoke *et al.*, 1984; Dobbelaere *et al.*, 1984). Although, antibodies against MHC class I molecules on the target cells also prevent entry of *T. parva* sporozoites *in vitro* (Shaw *et al.*, 1991), yet MHC class I molecules are unlikely to be the ligands for sporozoite penetration as these are expressed on all nucleated cells and the parasite infects only a small cell subpopulation.

2.1.7 Clinical signs

The incubation period of *T. annulata* or *T. parva* infection following natural tick infestation is around two weeks, ranging between 8-25 days (Sergent *et al.*, 1945; Neitz, 1957). Severity of the disease after *T. annulata* or *T. parva* infection in an animal depends largely upon the dose of initial infection received, the susceptibility of the host and the parasite strain involved (Jarrett *et al.*, 1969; Gill *et al.*, 1977; Samantaray *et al.*, 1980; Dolan *et al.*, 1984c; Preston *et al.*, 1992a). Schizonts are detected in superficial lymph nodes draining the site of tick infection on 5th to 8th day of infection and piroplasms in red blood cells 2-5 days later. Initial symptoms include high fever and swelling of superficial draining lymph nodes followed by symptoms of progressive anaemia (Gill *et al.*, 1980; Samantaray *et al.*, 1980; Uilenberg, 1981b; Preston *et al.*, 1992a). *T. parva* infection is followed by a generalised lymphadenopathy (Morrison *et al.*, 1981a), however, involvement of

other lymph nodes is not seen in *T. annulata* infection. Other clinical symptoms include anorexia, malaise, listlessness, dyspnoea, loss of body weight and digestive disturbances including a localised ulcerative abomasitis (Preston *et al.*, 1992a). Terminal cases show hypothermia, recumbency, rapid breathing and pulse rate. Death can occur between 14-30 days post-infection. Anaemia and jaundice do not normally develop in East Coast fever, but are common terminal features of tropical theileriosis (Hooshmand-Rad, 1976; Dhar & Gautam, 1979). The mortality rate in *T. annulata* infection is 40-60% (Uilenberg, 1981b; Brown, 1990) and in *T. parva* infection is 85-90% (Brocklesby *et al.*, 1961). Animals that survive may undergo a prolonged period of recovery and become piroplasm carriers. This stage may flare up again to acute tropical theileriosis at times of stress or intercurrent disease (Sergent *et al.*, 1945).

A cerebral form of theileriosis has been recorded in some animals infected with *T. parva* usually several months or even years after the initial disease and is known as turning sickness (Mettam & Carmichael, 1936; Giles *et al.*, 1978). Affected animals show ataxia and circling and may die in convulsions. This is associated with the presence of large numbers of infected cells and free schizonts in brain capillaries, with congestion and sometimes necrosis of cerebral brain tissue (Moll *et al.*, 1986). Some animals infected with *T. annulata* also show nervous symptoms in the form of ataxia, convulsions, enlargement and bulging of eye balls followed by blindness, unconsciousness and death (Khanna *et al.*, 1982; Mallick *et al.*, 1986).

2.1.8 Pathogenesis

Pathogenesis of *T. parva* infection has been studied in depth, but literature on other *Theileria spp.* is scanty. Direct extrapolation of findings on *T. parva* for *T. annulata* might not be possible as the two parasites show some striking dissimilarities. The pathology of *T. parva* has been extensively reviewed by Neitz (1957), Wilde (1967), Barnett (1968), Irvin & Morrison (1987) and Norval *et al.* (1992). Accounts on the pathogenesis of *T. annulata* are given in the reviews of Sergent *et al.* (1945), Neitz (1957), Gill *et al.* (1977) and Uilenberg (1981b).

2.1.8.1 Kinetics of parasite dissemination

Following sporozoite inoculation, the parasite has not been followed in great detail till schizonts appear in the draining lymph node. The mode and place of host cell invasion by the parasite are not fully understood. Phenotype of target cells during *in vivo* infection by both the parasites is also not clearly defined. The infection

appears to travel through the lymphatic system as the parasite is first seen in the draining lymph node. Dissemination of the parasite seems to be quite rapid as surgical removal of the draining lymph node, as early as two days after inoculation of *T. annulata* (J.D. Campbell & R.L. Spooner, pers. comm.) or *T. parva* (Emery, 1981b) sporozoites or four days after *T. annulata* infected cell line (P. Goel & R.L. Spooner, pers. comm.), delayed the initial onset of fever but had no effect on subsequent course of infection. These results can be expected if parasite dissemination takes place to more than one lymph node from the site of inoculation. Detection of *T. parva* schizonts in the draining lymph node earlier than non-draining lymph nodes (Morrison *et al.*, 1981a) perhaps reflects a relative enrichment of infection in this location because of its proximity to the site of inoculation, rather than an initial period of establishment of infection in the draining node prior to dissemination. The same might not be true for *T. annulata* infection where schizonts are not usually detected in the non-draining nodes. Dissemination of the parasite might occur through blood circulation to other lymphoid organs.

Schizont parasitosis in *T. parva* infection shows a logarithmic growth phase with a 10 fold growth in approximately three days after initial appearance of schizonts in the lymph node (Jarrett *et al.*, 1969; Radley *et al.*, 1974). There is a dramatic increase in total cellularity and the size of the draining lymph node corresponding to the initial appearance of *T. parva* schizonts (Morrison *et al.*, 1981b). Parasitized cells are detected in efferent lymph at about the same time as in the draining node and are associated with an increase in the total and blasting cell output in efferent lymph (Morrison *et al.*, 1981a; Emery, 1981b). Similar changes are observed in the distant lymph nodes and spleen 2-3 days later except that these are less pronounced and more gradual (Morrison *et al.*, 1981a; Shatry *et al.*, 1981). With the increase in parasite infected cells in the node during later stages of the disease, there is a gradual decrease in the total cellularity of lymphoid tissues, decrease in output of cells in efferent lymph and in the cellularity of thoracic duct lymph (Emery, 1981b; DeMartini & Moulton, 1973b). Enlargement of the draining lymph node is also a common feature in *T. annulata* infection, but similar kinetics of infection and the associated cellular changes in the draining and distant lymph nodes have not been studied. Some of these studies are reported in this thesis.

2.1.8.2 Histological studies

Histological examination of the draining lymph node after inoculation of *T. annulata* sporozoites in a naive animal revealed areas of blasting cells in the medulla by day 4 and throughout the tissue by day 8. By day 10, the node was full of infected cells, normal lymphoid architecture was destroyed with large areas of dead cells, discrete areas of blasting cells and no germinal centres (Campbell *et al.*, in press). During the terminal stages of tropical theileriosis, total cell depletion from the paracortex and medulla with large numbers of infected and blasting cells around follicles were observed (Eisler, 1988). Other internal organs showed signs of congestion and haemorrhage (Sergent *et al.*, 1945). An initial phase of blastogenesis was observed during *T. parva* infection, followed by the appearance of large numbers of infected cells (Barnett, 1968). Uninfected blasting cells were more prominent in the paracortical zone (DeMartini & Moulton, 1973a). Blasting Infected cells were more numerous in T-dependent areas than in B-dependent areas. Germinal centres underwent a rapid involution, although some infected cells were observed within germinal centres (Morrison *et al.*, 1981a). In contrast, inoculation of an allogeneic *T. annulata* infected cell line revealed blasting plasma cells and monocytes initially, followed by appearance of blasting cells in the medullary sisuses. Germinal centre follicles started to appear by day 9. Sections of lymph node collected on day 16 showed large, hyperplastic germinal centres with clear areas of proliferating dark and light zones with no signs of nodal dystrophy (P. Goel & R.L. Spooner, pers. comm.).

2.1.8.3 Leucopenia

Infection with both parasites leads to development of severe leucopenia involving both polymorphonuclear cells and lymphocytes (Wilde, 1967; Sharma & Gautam, 1971; Laiblin, 1978; Morrison *et al.*, 1981b; Preston *et al.*, 1992a). Some previous studies reported leucocytosis with *T. annulata* infection (Sergent *et al.*, 1945; Barnett, 1977), but the animals were infected by mechanical transmission using either infected blood or leucocytes and may not present a true picture. Depletion of lymphocytes is evident from both solid lymphoid tissues and in the recirculating pool which is associated with marked destruction of cells in the later stages of the disease. However, mechanisms involved in the destruction of cells are not clearly known. There is retardation of granulopoeisis in the bone marrow and impairment of lymphopoiesis in bone marrow and thymus after *T. parva* infection (Wilde *et al.*,

1966). Wilde (1967) suggested involvement of an autoantibody dependent mechanism which has not been proved. Polyspecific cytotoxic cells were detected from animals during the terminal phase of East Coast fever which killed a range of different allogeneic infected cells, but not autologous infected cells in *in vitro* assays (Emery *et al.*, 1981a). These cells might be important in causing leucopenia. The parasite might be inducing some kind of stimulation leading to programmed cell death (apoptosis). Some *T. parva* infected cell lines were shown to produce an Interleukin-2 (IL-2) like activity (Brown & Logan, 1986; Dobbelaere *et al.*, 1988). It has been recently confirmed that *T. parva* infected cell lines expressed IL-2 mRNA (Heussler *et al.*, 1992). IL-2 might be responsible for massive proliferation of infected cells and uninfected host cells. Interferon-γ (IFN-γ) is also produced by *T. parva* infected lines, whereas *T. annulata* infected cells probably produce other Interferons (Entrican *et al.*, 1991; DeMartini & Baldwin, 1991; Ahmed *et al.*, 1993). IFN-γ can activate macrophages to produce high levels of tumour necrosis factor-α (TNF-α) which exerts cytostatic and necrotic effects on some target cells (Sugarman *et al.*, 1985). Similar cells might be activated in infection with these two parasites leading to destruction of self lymphocytes.

2.1.8.4 Anaemia

Severe anaemia is a common finding during acute *T. annulata* infection (Sergent *et al.*, 1945; Neitz, 1957; Hooshmand-Rad, 1976; Dhar & Gautam, 1979), but anaemia is usually mild during infection with *T. parva* (Wilde, 1967; Maxie *et al.*, 1982). Anaemia is not aggravated by splenectomy during *T. parva* infection (Barnett, 1968), but parasitaemia flares up even in latent *T. annulata* infection leading to anaemia by splenectomy. Strains of *T. annulata* which had lost the ability to produce microschizonts and, hence, piroplasms could still induce anaemia (Hooshmand-Rad, 1976) suggesting that there may be factors other than intraerythrocytic piroplasms contributing to the cause of anaemia. Erythrophagocytosis has been suggested as a mechanism of erythrocyte destruction rather than a direct effect of parasitaemia on the infected animals (Neitz, 1957; Uilenberg, 1981b). Induction of anaemia by means of autohaemagglutinating antibodies has been suggested, however the events leading to induction of such antibodies remain obscure. Peripheral blood mononuclear cells (PBM) from calves lethally infected with *T. annulata* spontaneously produced TNF-α and IFN-γ (Preston

et al., 1993). A TNF dependent red cell destruction and dyserythropoiesis has been shown in mice infected with *Plasmodium vinkei* (Clark, 1987). It is possible that TNF might be involved in producing anaemia after infection with *T. annulata* by activating macrophages to phagocytose erythrocytes.

2.1.9 Pathological lesions

Pathological lesions are mainly associated with lymphopoietic and vascular systems. Gross post-mortem findings include emaciated and pale carcass, generalised enlargement and oedema of lymph nodes and spleen, massive pulmonary oedema, congestion and consolidation in apical lobes, petechial and ecchymotic haemorrhages of the serosal surfaces (Barnett, 1968; Uilenberg, 1981b). The most striking and frequent lesion is haemorrhagic ulceration of the abomasum commonly called punched necrotic ulcers. The liver is enlarged and haemorrhagic. The gall bladder is often enlarged with dark green viscous bile. There are generalised symptoms of anaemia and jaundice (Sergent *et al.*, 1945; Neitz, 1957). Animals with the cerebral form show congestion of the vessels of the meninges and haemorrhagic necrosis in the central nervous system (Barnett, 1968). Schizonts can be detected from impression smears from most of the internal organs.

2.1.10 Diagnosis

Field diagnosis of tropical theileriosis and East Coast fever still depends upon clinical symptoms, haematological observations, detection of parasite in lymph node biopsies and blood smears and autopsy lesions in dead animals. The most commonly used serum antibody assay for serological surveys has been indirect fluorescent antibody test (IFAT) for *T. annulata* (Burridge & Kimber, 1972) and *T. parva* (Goddeeris *et al.*, 1982). The complement fixation test has also been used in some places (Dhar & Gautam, 1977). Development of an enzyme linked immunosorbant assay (ELISA) (Kachani *et al.*, 1992b; Sunder *et al.*, 1993) and DOT-ELISA (Grewal *et al.*, 1991; Grewal, 1992) has been described for *T. annulata*. Recently, Campbell *et al.* (1994d) developed an immunoperoxidase technique for detection of antibodies against *T. annulata* as a replacement for IFAT. Methyl green pyronin staining has been used to demonstrate infection in the salivary glands of ticks (Walker & McKellar, 1983; Sangwan *et al.*, 1986; Flach *et al.*, 1993). It is hoped that these tests would help in conducting serious epidemiological studies in *T. annulata* endemic areas.

T. parva specific repetitive DNA probes were developed and shown to be useful for the differentiation of the parasite from other *Theileria* species, and for discrimination of stocks within *T. parva* by detection of restriction fragment length polymorphism (Conrad *et al.*, 1987; Allsopp & Allsopp, 1988; Morzaria *et al.*, 1990). Recently, oligonucleotide probes detecting small subunit ribosomal RNA sequences were used to discriminate between six species of *Theileria* (Allsopp *et al.*, 1993). Ben Miled *et al.* (1994) demonstrated considerable diversity amongst various *T. annulata* stocks isolated in Tunisia using two genomic DNA probes. Polymorphism was observed between isolates collected even from the same farm. Similar approaches have also been used for studying polymorphism in *T. sergenti* infection (Matsuba *et al.*, 1993). Currently, these probes only serve as valuable laboratory tools and are not suitable for routine field application mainly because of the use of radiolabelled probes to achieve the desired level of sensitivity. However, use of the polymerase chain reaction (PCR) for *in vitro* amplification of specific DNA sequences would help in making these tests more practicable. This technique has been used to detect *T. annulata* in infected ticks by amplifying a DNA fragment from small ribosomal RNA gene of *T. annulata* using PCR (de Kok *et al.*, 1993). PCR has also been used to amplify p33/34 genes from the piroplasm DNA to distinguish between *T. sergenti*, *T. buffeli* and *T. orientalis* (Kawazu *et al.*, 1992).

2.1.11 *In vitro* cultivation

The methods used for *in vitro* cultivation of *Theileria* parasites and their applications have been extensively reviewed by Brown (1979, 1981, 1983 & 1987). Cell lines can be established from infected lymphoid tissue or peripheral blood from a sick animal or infected lymphoid material from a recently dead animal. Cultures can also be initiated *in vitro* by incubating suspensions of sporozoites, harvested from ticks, with uninfected bovine lymphocytes (Brown, 1987). These transformed parasitised cells can be cultivated *in vitro* for a seemingly indefinite period without addition of exogenous growth factors (Hulliger *et al.*, 1964).

The first reported culture of *Theileria* parasites was achieved by Tsur (1945) with *T. annulata* by culturing liver and spleen explants taken from infected cattle, in plasma clot cultures. This discovery was developed further to cultivate infected cells established either from infected tissues or blood in monolayers (Tsur and Adler, 1962; 1965) or in suspension cultures (Hooshmand-Rad, 1975) and finally led to the

development of a cell culture vaccine against *T. annulata* (Pipano & Tsur, 1966). However, the same technology did not prove very successful for establishment of *T. parva* cell cultures (Brocklesby and Hawking, 1958). The mode of multiplication of theilerial schizonts was conclusively demonstrated in *in vitro* cultures by Hulliger *et al.* (1964). The first successful establishment of a *T. parva* infected cell line *in vitro* was achieved by Malmquist *et al.* (1970), using spleen cells from an infected animal and high concentrations of foetal calf serum. The most important breakthrough came with the work of Brown *et al.* (1973), in which normal lymphoid cells were infected *in vitro* with sporozoites of *T. parva*. This technology was extended to culture other theilerial species (Brown, 1983). This technique has proved to be an invaluable tool for most of the subsequent studies on *T. annulata* and *T. parva*. It created the possibility of generating infected cell lines *in vitro* from naive animals without infecting them. This has been the only stage so far amenable to continuous cultivation in the laboratory. With further developments in *in vitro* culture systems, it is now possible to generate cloned cell lines with one sporozoite infecting one cell (Brown, 1987). The macroschizont infected cell lines of various theilerial species show unlimited growth potential *in vitro* (Brown, 1987) which seems to be induced by the presence of parasite inside the cell. The infected cell stops dividing if the parasite within the cell is killed (Pinder *et al.*, 1981; Ahmed *et al.*, 1992). Bovine fibroblast cell lines can be infected with *T. annulata* or *T. parva* if co-cultivated with parasite infected cell cultures *in vitro* (Brown & Gray, 1981), however the mode of parasite transfer in such conditions remains obscure. Apart from use as a cell culture vaccine in *T. annulata* endemic areas, *Theileria* culture techniques have helped in elucidating the life cycle, taxonomy and chemotherapy and has provided material for immunological, biochemical and molecular studies on these parasites (Brown, 1987).

No other stage of *Theileria* parasites apart from schizonts has been indefinitely grown in culture so far. Infected salivary glands and motile kinete stage of the parasite harvested from backless ticks has been maintained for short periods in culture (Bell, 1980; Bell, 1984). Some workers have produced merozoites *in vitro* (Hulliger *et al.*, 1966; Danskin & Wilde, 1976; Shiels *et al.*, 1992), but no one has been able to infect erythrocytes with these merozoites *in vitro*. Pipano & Fish (1982) could maintain *T. annulata* piroplasms *in vitro* for a few weeks, but were not able to grow these cultures for long periods. Short term cultures of *T. annulata* piroplasms between

10-27 days were maintained by Conrad *et al.* (1985).

2.2 BOVINE LEUCOCYTE MARKERS

With the advent of monoclonal antibody technology, many reagents have been developed which recognise various leucocyte differentiation antigens in man, mouse and other species including cattle (Baldwin *et al.*, 1988c). Monoclonal antibodies generated against human leucocyte differentiation antigens in different laboratories around the world have been tested on a common platform in international workshops. Clusters of differentiation (CD) for classifying antigens and mAbs recognising them have been established on the basis of immunofluorescent cell surface labelling, immunoprecipitation and immunohistology on tissue sections (Bernard *et al.*, 1984). This nomenclature has lead to a practical system for identification of cell surface molecules on the basis of their biochemical structure allowing various cell types to be defined by the expression of "CD antigens".

A similar approach has also been adopted for identification of bovine leucocyte differentiation antigens which have been tested and compared in two international workshops (Howard *et al.*, 1991a; Howard & Naessens, 1993) to date. Clusters of mAbs directed against bovine antigens that were homologous to a human CD antigen were assigned the same number with a prefix "Bo" to facilitate comparison of immunological observations between species. In the case of clusters for which there was no obvious equivalent human CD antigen, a new WC (workshop cluster) number was designated (Naessens, 1993). These two workshops have defined many CD and WC antigens for cattle and other ruminants, some of these relevant to this thesis are described below.

2.2.1 CD2

This antigen is expressed on T cells, is a receptor for sheep red blood cells (RBC) and is also known as E-rosette forming receptor. The antigen appears very early in the ontogeny of T lymphocytes in the thymus and is expressed on both mature and immature lymphocytes with the $\alpha\beta$ T cell receptor (TCR). The natural ligand for this molecule is lymphocyte function antigen (LFA)-3, also known as CD58 (Davis *et al.*, 1987). The molecule in humans is a 45-50 kd transmembrane glycoprotein and is one of the several accessory molecules involved in adhesion and activation of T cells and transduction of activation signals across the cell membrane

(Bierer *et al.*, 1989). The mAbs to bovine CD2 precipitate a 50-60 kd molecule and inhibit rosette formation with sheep RBC (Davis *et al.*, 1988). CD2 is expressed on the majority of $CD4^+$ and $CD8^+$ cells in PBM and a very small proportion of $CD4^-$ /$CD8^-$ cells, but not on $WC1^+$ T cells, B cells and monocytes/macrophages. It is also expressed on some $\gamma\delta$ TCR^+ cells which are not $WC1^+$ (Baldwin *et al.*, 1988b; Davis & Splitter, 1991). The mAbs to bovine CD2 molecule stain the majority of thymocytes in both the cortex and medulla of the thymus except some lymphocytes immediately beneath the thymic capsule, T cell areas in lymph node (LN) paracortex and a few cells in B cell follicular areas (Davis *et al.*, 1988).

2.2.2 CD4

The CD4 molecule in humans is a single chain glycoprotein of 59 kd and is expressed on approximately two third of circulating T cells, including most T cells of helper/inducer type (Terhorst *et al.*, 1980). The mAbs which recognise the bovine analogue precipitate two polypeptides of approximately 52 and 55 kd. It is expressed on about 70% bovine thymocytes, 30% PBM and is not expressed by monocytes or B cells. The expression is restricted to $CD2^+$ and $\alpha\beta$ TCR^+ cells. Cells of this lineage are found in abundance in T dependent areas of lymph node paracortex, but only a few are seen in follicular areas (Baldwin *et al.*, 1986; Bensaid & Hadam, 1991). Cells within this population recognise antigen in an MHC class II restricted manner. CD4 antigen binds to a non-variable part of the β_2 domain of MHC class II molecules on antigen presenting cells (APC) and stabilises APC/T cell interaction. These cells usually do not show cytotoxic activity and proliferate in response to mitogenic and alloantigenic stimulations without addition of exogenous growth factors (Baldwin *et al.*, 1986; Teale *et al.*, 1986) indicating that these cells are capable of secreting IL-2 on activation.

Two subsets of $CD4^+$ cells have been defined in mouse which are stably committed to produce distinct cytokines. "Th1" type CD4 cells produce IL-2, IFN-γ, and TNF, whereas "Th2" type CD4 cells secrete IL-4, IL-5 and IL-6. Th1 and Th2 cells exclusively utilise IL-2 and IL-4, respectively as their growth factor for proliferation in response to an antigen (Coffman *et al.*, 1991). Th1 cells induce delayed type hypersensitivity reactions, activate macrophages and induce B cells to produce IgG_{2a} isotype antibodies. Th2 cells induce B cells to produce IgM and IgG_1 antibodies. Repeated IL-4 stimulations secreted by these cells induce production of

IgE type antibodies by B cells. Th2 cells are usually stimulated in response to persistent antigenic stimulation eg. parasitic infestations (Abbas *et al.*, 1991).

2.2.3 CD8

The CD8 antigen is expressed on a subpopulation of T lymphocytes which show MHC class I restricted cytotoxic effector function (Maddox *et al.*, 1985). It has a molecular weight (M_r) of 34-35 kd and consists of two polypeptide chains α and ß. The antigen binds to a non-variable portion of the α_3 domain of MHC class I molecules on APC and stabilises the interaction between two cells (MacHugh & Sopp, 1991). CD8 is expressed on the surface of approximately 20% PBM, a subpopulation of lymphocytes in T dependent areas of LN and spleen and about 70% thymocytes and is not expressed on B cells, monocytes/macrophages or granulocytes. These cells are rarely seen in the B dependent zone and follicular areas of LN (Ellis *et al.*, 1986). The $CD8^+$ cell population is distinct and non-overlapping from CD4 cells in PBM, but a large population of thymocytes under developmental stages express both the antigens. In the peripheral blood, most bovine $CD8^+$ cells are $CD2^+$ and αß TCR^+, but a few cells also express $\gamma\delta$ TCR^+ (Howard & Naessens, 1993). Most cattle have slightly higher levels of $CD4^+$ cells than $CD8^+$ cells in the peripheral blood.

Most $CD8^+$ cells do not produce growth factors like IL-2. Purified $CD8^+$ cells respond very poorly to mitogens or alloantigens in the absence of exogenous growth factors (Ellis *et al.*, 1986). Cytotoxic effector function resides in the $CD8^+$ population in an allogeneic mixed lymphocyte culture (MLC) or in *vitro* generated cytotoxic T cell lines against cells infected with various microorganisms (Teale *et al.*, 1986; Goddeeris *et al.*, 1986b). Small proportions of both $CD4^+$ or $CD8^+$ cells can express suppressor activities. Some of these cells in man are reported to be $CD11^+$, a marker which is more common on monocytes and granulocytes (Yamada *et al.*, 1985).

2.2.4 CD3

This molecule consists of five different polypeptide chains with M_r ranging from 12-44 kd and is present on all T cells with αß or $\gamma\delta$ TCR, but not on B lymphocytes, monocytes and granulocytes (Davis *et al.*, 1993). The five chains are closely associated with each other and also with TCR which is essential for T cell maturation and function. Incubation of T cells with CD3 antibody induces calcium flux and proliferation. This group of molecules, therefore, may be involved in transmitting signals to the cell interior following binding of antigen to its receptor

(Clevers *et al.*, 1988). Binding of an anti CD3 mAb to bovine $WC1^+$ T cells with natural killer activity augments killing of target cells (Splitter & Choi, 1993), however the mechanism of augmentation is not clearly understood.

2.2.5 $WC1^+$ $\gamma\delta$ T cells

Peripheral blood T cells of man and mouse mainly express differentiation antigens CD4 or CD8 along with CD3 associated αß TCR. There is another minor $CD3^+/CD5^+$ T cell population in peripheral blood and thymus which does not express CD2, CD4 or CD8. Immunoprecipitation studies have shown that these cells do not express αß TCR, but express another form of TCR made up of γ and δ chains (Brenner *et al.*, 1988) and are called $WC1^+$ cells in cattle and sheep (Mackay *et al.*, 1989; Clevers *et al.*, 1990; Morrison & Davis, 1991). Northern blot hybridisation using α, ß, γ and δ probes showed that these cells express γ and δ message (Hein *et al.*, 1989; Clevers *et al.*, 1990).

These cells also originate in the thymus and hence belong to T cell lineage. The peripheral blood of young animals exhibit a substantial population of $WC1^+$ cells where it may be as high as 25% in calves of less than three weeks of age. However, the percentage decreases with age (Clevers *et al.*, 1990). Histological studies show that the cells are mainly concentrated in the thymic medulla, but a few are observed scattered through the thymic cortex. In the LN, cells are 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. In spleen, they are concentrated in marginal zones of the spleen (Clevers *et al.*, 1990). The precise function of $WC1^+$ cells is not properly understood but they may have a role in controlling the proliferation of other lymphocytes. A potential cytolytic activity for these cells has been proposed in cattle, but not clearly established (Howard & Morrison, 1994).

2.2.6 IL-2 receptor

Two surface molecules mediate binding of IL-2 to the cells: a 50 kd glycoprotein CD25 also known as Tac antigen or IL-2Rα which binds IL-2 with low affinity and a 75 kd antigen IL-2Rß which binds IL-2 with slightly higher (intermediate) affinity (Malek *et al.*, 1983; Robb *et al.*, 1987). The heterodimeric complex of both surface molecules forms a functionally effective high affinity molecule which mediates cell proliferation by activating a signal transduction pathway after binding to IL-2. Binding also leads to internalisation of the IL-2/IL-2R complex

leading to downregulation of IL-2R expression on the cell surface. Without continuous stimulation, functional CD25 disappears and the activated cell returns to a resting stage (Cantrell & Smith, 1983).

Unstimulated T and B cells can express IL-2α (CD25) or IL-2ß in very low quantities, but cells express high quantities of IL-2Rα, and to a lesser extent IL-2Rß, after stimulation on the cell surface. So far only IL-2Rα is characterised in cattle (Naessens *et al.*, 1992). The antigen is not detected on resting PBM, but is induced after activation with mitogens. It is also expressed on long-term cultured T cell lines, on $CD2^+CD8^+$ or $CD2^-CD8^+$ T cell clones (Naessens *et al.*, 1992), most *T. parva* infected lines of T or B cell origin and some *T. annulata* infected cell lines of non T cell origin (Dobbelaere *et al.*, 1990; Ahmed *et al.*, 1992). A low level of CD25 expression is seen on resting bovine $WC1^+$ T cells in PBM (Howard & Morrison, 1994). Monocytes and B cells in stimulated bovine PBM cultures also express this antigen at a lower intensity than T cells (Taylor *et al.*, 1992). The mAb to bovine CD25 antigen blocks IL-2 driven proliferation even at high concentrations of IL-2, and is a useful tool for measuring the activation state of particular cells as well as for studying IL-2 dependent cell proliferation (Naessens *et al.*, 1992).

2.2.7 CD45R

This antigen is reported as leucocyte common antigen (LCA) and consists of at least five high molecular weight glycoproteins present on the surface of the majority of leucocytes. Different isoforms arise from a single gene by alternative mRNA splicing of three individual exons potentially generating eight different molecules (Thomas, 1989). The larger intracellular portion is identical in all the isoforms and has tyrosine kinase activity. It can thus potentially interact with intracellular protein kinases and may be involved in triggering cell activation (Clark & Ledbetter, 1989).

CD45R is now divided into three isoforms: CD45RO, CD45RA and CD45RB. The CD45RO isoform is a 180 kd molecule in humans and is expressed on memory T cells or primed T cells. Cells divided from $CD45RO^+$ cells are also born with the same protein and are primed against the same antigen as their progenitor. The CD45RA isoform is a 220 kd molecule expressed on naive or virgin T cells (Akbar *et al.*, 1988). It is believed that a naive T cell which is $CD45RA^+/CD45RO^-$ changes to $CD45RA^+/CD45RO^+$ and finally to $CD45RA^-/CD45RO^+$ after exposure to an

antigen. Memory T cells have been shown to occasionally revert to naive phenotype (Bell & Sparshott, 1990). The third isoform CD45RB consists of four molecules with M_r between 190-220 kd and is expressed on some T cells, and most B cells and monocytes. Naive cells are $CD45RB^+$ (high) and memory cells are $CD45RB^-$ (low) (Streuli *et al.*, 1988).

In cattle, naive $CD4^+$ cells were shown to be $CD45RB^+$ and memory $CD4^+$ cells were $CD45RB^-$, but MHC class I restricted $CD8^+$ cells were heterogeneous with respect to the LCA isoform they express (Howard *et al.*, 1991b). The CD45RO antigen was shown to be expressed on the majority of bovine monocytes, granulocytes and $\gamma\delta$ T cells, variably expressed on $CD2^+$ T cells and absent from B cells. The ability to proliferate in response to recall antigens resided in the $CD45RO^+$ cell population amongst the $CD4^+$ cell population (Bembridge *et al.*, 1993). Sheep B cells, NK cells and naive T cells expressed CD45RA and proliferative ability to recall antigens resided within the $CD45RA^-$ population (memory) amongst the $CD4^+$ cells (Mackay *et al.*, 1990). T cells in afferent lymph expressed a memory phenotype, but most T cells in efferent lymph were naive. The number of naive T cells in the peripheral blood decreased as the animals aged (Mackay, 1993).

2.2.8 B cells

B cells represent about 5-15% of the circulating lymphoid pool and are classically defined by the presence of endogenously produced immunoglobulins (Ig) on the surface. Polyspecific heteroantisera against bovine Ig have been used to identify B cells for many years (Grewal *et al.*, 1978). Isotype specific heteroantisera which react with IgM, IgG_1, IgG_{2a} or IgA produced in goats have also been used to identify B cells (Nielsen *et al.*, 1985). Monoclonal antibodies are now available against most of the bovine Ig isotypes (Naessens *et al.*, 1988; Naessens & Howard, 1991; Mukwedeya *et al.*, 1993). The majority of peripheral blood B cells express mainly IgM antibodies and only a few express other Ig on their surface, although these are present in larger numbers under specific locations like IgA bearing cells in the gut. Subsequently, B cells may change the isotype of antibody produced, but their antigen specificity remains the same (Baldwin *et al.*, 1988c).

Ig on the surface also acts as an antigen receptor for B cells. B cells are initially activated in response to an antigen in T cell areas of the lymph node in association with interdigitating cells and T cell help. On average three activated B

cells colonise a primary follicle and undergo massive clonal expansion to form secondary follicles or germinal centres. Germinal centre B cells may take up antigen held on follicular dendritic cells as an immune complex in unprocessed form, process this antigen and present it to $CD4^+$ cells (MacLennan, 1994). The majority of B cells express MHC class II on their surface, complement and Fc receptor (Kunita *et al.*, 1988). Expression of various antigen markers on maturation stages of B cells viz: Pro-B cell, Pre-B cell, B cell expressing Ig, activated B cell, B cell blast and plasma cell are not clearly defined (Mukwedeya *et al.*, 1993).

2.2.9 Mononuclear phagocytic cells

Cells of this lineage comprise a functionally and morphologically heterogenous population. The two main functions of these cells include phagocytosis of particulate antigens and act as antigen presenting cells to various subpopulations of lymphocytes. Different cells within this population occupy distinct locations within the body tissues or fluids which include blood monocytes/macrophages, Langerhans cells of skin epidermis, Kupffer cells of liver, pulmonary alveolar macrophages, osteoclasts and interdigitating or dendritic cells in solid lymphoid tissues (Splitter & Morrison, 1991). Differentiation of these cells in particular environments may result in the expression of certain antigens and not others. Further, antigens present on cells of this lineage may be expressed on other cell types suggesting a common functional importance of the molecules in myeloid and lymphoid cell differentiation. Monoclonal antibodies against many molecules expressed on the cell surface at various stages of activation and differentiation in cattle have been raised and their functions are being investigated (Baldwin *et al.*, 1988c; Ellis *et al.*, 1988; Splitter & Morrison, 1991; O'Rielly *et al.*, 1991; Hall *et al.*, 1993; Gupta *et al.*, 1993).

2.2.10 Major histocompatibility antigens

The MHC is a chromosomal region that encodes a group of highly polymorphic cell surface glycoproteins essential for recognition of foreign antigens (Klein, 1986). There are two major regions encoding these antigens, namely MHC class I and MHC class II. Bovine MHC (BoLA) class I molecules consist of an MHC encoded heavy chain of M_r 44 kd and a non MHC encoded light chain ß2-microglobulin of M_r 12 kd (Hoang-Xuan *et al.*, 1982). These antigens are expressed on almost every nucleated cell in varying amounts, are involved in the recognition of self and act as classical alloantigens. MHC class I molecules present endogenously

processed peptide antigens to cytotoxic T lymphocytes. Identification of polymorphism of BoLA class I molecules has relied mainly on the use of allo-antisera produced from calf-dam reciprocal immunisations and used in microlymphocytotoxicity assays (Spooner *et al.*, 1979a), and isoelectric focusing (IEF) using mAbs and allo-antisera (Joosten *et al.*, 1988; Al-Murrani *et al.*, 1993). Sera produced in different laboratories have been evaluated in five international workshops and used to identify various BoLA specificities using microlymphocytotoxicity along with IEF using mAbs (Spooner *et al.*, 1979b; Anon. 1982; Bull *et al.*, 1989; Bernoco *et al.*, 1991; Davies *et al.*, 1994b). Some mAbs are available which identify monomorphic determinants (Bensaid *et al.*, 1989; Kemp *et al.*, 1990) or polymorphic determinants (Bensaid *et al.*, 1988) on cell surface associated BoLA class I molecules.

MHC class II gene products or MHC class II antigens are heterodimeric cell surface glycoproteins consisting of a heavy (α) chain of 33-38 kd and a light (ß) chain of 25-28 kd (Kaufman & Strominger, 1979). MHC class II antigens are mainly expressed on B cells, dendritic cells, cells of monocyte/macrophage lineage and on T cells after activation (Hopkins *et al.*, 1993). MHC class II molecules bind to peptides generated by proteolysis of antigen internalised by the cell and present it to $CD4^+$ cells (Germain, 1994). BoLA class II genes are located in two regions referred to as class IIa and class IIb regions. The MHC class IIa region is tightly linked to the class I region and contains bovine DR and DQ genes (Andersson *et al.*, 1986a; 1986b). The class IIb region contains DOB, DYA and DIB genes, and probably also DNA gene (Andersson *et al.*, 1988; Stone & Muggli-Cockett, 1990). MHC class II polymorphism in cattle has been studied by IEF, serology using allo-antisera, PCR-RFLP (Bernoco *et al.*, 1991; Davies *et al.*, 1994a) and single-strand conformation polymorphism (Russell *et al.*, 1994) to characterise class II genes. The mAbs against monomorphic determinants on MHC class II have been widely used for biochemical and functional studies (Baldwin *et al.*, 1988c; DeMartini *et al.*, 1993; Hopkins *et al.*, 1993).

2. 3 BOVINE LYMPHOID SYSTEM

The lymphoid system functions as a single organ despite the fact that it is dispersed all over the body and composed of numerous distinctly structured lymphoid

tissues. Its six major compartments viz: blood, lymph nodes, bone marrow, spleen, gut and thymus, are connected to each other by two vascular networks: the lymphatic system and the blood vascular system. Lymphocytes are the major cellular constituent of this system circulating amongst blood, lymph and interstitial tissue (Trnka & Cahill, 1980). The movement of lymphocytes between these compartments regulates development and maintenance of immunological functions by the system.

The first phase of lymphocytic development occurs in primary lymphoid tissues, namely, bone marrow, thymus and possibly Peyer's patches. Undifferentiated stem cells develop into lymphocytes which mature and proliferate to produce different populations of immunocompetant lymphocytes, each comprising clones of cells capable of recognising and responding to individual antigenic determinants by a process independent of exposure to foreign antigen. The second phase of development occurs primarily in lymph nodes following exposure to foreign antigen which involves proliferation and differentiation of specific immunocompetant lymphocytes to produce antigen specific immune effector cells (MacLennan, 1994).

Events surrounding the initiation of immune responses to antigens in the lymph node can be analyzed by lymphatic cannulation (Hall & Morris, 1962). Examination of cells migrating through afferent or efferent lymph has been extensively studied in sheep, to a lesser extent in cattle, and has provided an understanding of cell kinetics and development of immune responses to various exogenous protein antigens (Hall & Smith, 1971; English *et al.*, 1977; McConnell & Hopkins, 1981; Hopkins *et al.*, 1993) and microorganisms (Hall *et al.*, 1967; Emery, 1981b; Emery *et al.*, 1988; Entrican *et al.*, 1992; Bird *et al.*, 1993; Buxton *et al.*, 1994; McKeever *et al.*, 1994).

2.3.1 Migration of antigen from site of deposition to the lymph node

After gaining access to the tissue spaces in the body, foreign antigens or microorganisms rapidly enter the lymph stream through openings in the walls of adjacent lymphatic capillaries and are transferred via afferent lymphatics to the regional lymph node (Morris, 1972). Studies on migration of cells from the site of antigen deposition to the lymph node are facilitated by the technique of pseudoafferent cannulation where afferent lymphatics are allowed to anastomose with the efferent lymphatic after surgical removal of the lymph node. Cannulation of efferent lymphatic at this stage permits collection of afferent lymph (Emery *et al.*, 1987). Bovine afferent lymph contains around 50% T lymphocytes, 20-25% B lymphocytes and 23%

non-lymphoid cells (Emery *et al.*, 1987). Non-lymphoid cells consist of adherent cells, "veiled" or "frilly" cells known as dendritic cells and Langerhans cells (Bujdoso *et al.*, 1989a). T cells in the normal afferent lymph are MHC class II^+ (Dutia *et al.*, 1993) and express a memory phenotype (Mackay *et al.*, 1992b).

Langerhans cells and dendritic cells are believed to carry antigen and migrate from dermis through afferent lymphatics in the form of veiled cells and enter the lymph node as interdigitating cells (Bujdoso *et al.*, 1989a; McKeever *et al.*, 1991). Interdigitating cells present antigen to the helper T cells in the paracortex of the lymph node (Breel *et al.*, 1988). The origin of interdigitating cells is not clear, but Langerhans cells, veiled cells and interdigitating cells are phenotypically similar and have a common lineage (Kraal *et al.*, 1986), indicating that these cell types presumably represent different migrating stages of the same population (McKeever, 1994). Afferent lymph veiled cells presented soluble antigens to primed T cells *in vitro* very efficiently (Bujdoso *et al.*, 1989a; McKeever *et al.*, 1991). These cells stimulated $CD4^+$ cell responses *in vivo*, when re-inoculated into a syngeneic animal, after priming with antigen *in vitro* (McKeever *et al.*, 1992) indicating that these cells carry antigen from the site of antigen deposition to the lymph node and process it for recognition of T cells.

An increase in the output of lymphocytes was observed in the afferent lymph draining granulomatous and DTH lesions (Smith *et al.*, 1970; Hay *et al.*, 1973). Following primary inoculation of soluble protein antigens, afferent lymph dendritic cells transiently expressed increased levels of CD1 (Hopkins *et al.*, 1989). Secondary antigen challenge in already primed sheep lead to an initial drop in cell output in afferent lymph for 1-3 days, followed by an approximately five fold increase by day five. This was associated with an increased output of MHC class II^+ T cells in afferent lymph, probably because of increased recruitment of activated or memory T cells at the site of antigen challenge. A quantitative increase in expression of MHC class II on dendritic cells and lymphocytes, and CD1 expression on dendritic cells was also observed. These changes in dendritic cells were associated with an increased antigen presenting ability to activated T cells *in vitro* (Hopkins *et al.*, 1989).

2.3.2 Development of immune responses in the draining lymph node

Lymph nodes are encapsulated fixed structures which act as filters for foreign antigen material entering the lymphatic system and provide a favourable environment

for interaction of different cell types in the generation of immune responses. Multiple afferent lymphatics enter the lymph node capsule and empty into the subcapsular sinus (Trnka & Cahill, 1980; Morrison *et al.*, 1986a). Naive T cells enter the lymph node from peripheral blood through specialised endothelial lining of post-capillary venules also called high endothelial venules (HEV) mainly in the paracortex and come in contact with antigen presenting cells entering the paracortex through subcapsular sinuses (Bogen *et al.*, 1991). T cells are stimulated in this perivascular location and start producing IL-2 or IL-4 and form small clusters (Bogen *et al.*, 1993). Activated T cells provide help for the growth and differentiation of antigen specific B cells in the initiation of germinal centre formation in the cortex. With the formation of secondary follicles, responding T cells migrate to the periphery of the follicles where they might help in growth and differentiation of follicular B cells by secreting different cytokines (Bogen *et al.*, 1991; Fuller *et al.*, 1993). Germinal centres mostly comprise B cells with some follicular dendritic cells and T cells. B cells in a single germinal centre are the progeny of very few founder B cells and are responsive to the immunising antigen. T cells within the germinal centre express a memory phenotype, are antigen specific and provide help for B cell proliferation. A physical link between T and B cells in the germinal centre is necessary for B cell proliferation (Fuller *et al.*, 1993). Follicular dendritic cells within the germinal centre contain unprocessed antigen in the form of antigen-antibody complex for very long periods. Retention of antigen in this form maintains circulating antibody levels and regulates production of memory B cells (Klaus *et al.*, 1980).

T cell mediated immune responses are also initiated in the paracortical area where naive T cells enter lymph node from high endothelial venules and come into contact with antigen presenting cells. Activated and proliferating T cells travel through the paracortical sinuses into the medullary sinuses through narrow apertures at cortico-medullary junctions (Fahy *et al.*, 1980). Proliferation of cells in response to an antigen leads to expansion of medullary cords (Huang *et al.*, 1990). Medullary sinuses converge at the hilus where they are continuous with one or more efferent lymphatic vessel. Immune responsive cells to a particular antigen are disseminated to the body through efferent lymphatics, entering the circulation through the thoracic duct (Fahy *et al.*, 1980). If the antigen is inoculated only in one lymph node (through a cannulated afferent lymphatic) and all the cells exiting in the efferent lymph are

drained from the challenged node, then the establishment of systemic immunological memory to that particular antigen is prevented and the animal remains naive as far as that antigen is concerned (Hall *et al.*, 1967).

2.3.3 Composition of efferent lymph

Lymphocytes are the predominant cellular constituents of efferent lymph which exit in large numbers from the node. In sheep, efferent lymph contains about 20-30% B cells, 70-80% T cells and no macrophages or monocytes (Cahill *et al.*, 1978; Trnka & Cahill, 1980). Resting bovine efferent lymph contained 58.5% $CD4^+$ cells, 21.5% $CD8^+$ cells, and 17.5% IgM^+ B cells (Emery *et al.*, 1988). A single resting lymph node of approximately 1g weight has an average output of 5 ml lymph and 30-50 million lymphocytes per hr in sheep (Trnka & Cahill, 1980).

2.3.4 Cell shutdown after antigen challenge in the draining node

Antigenic stimulation of a lymph node with many antigens results in retention of lymphocytes in the lymph node transiently. This is evident from decreased cell output in the efferent lymph within first 24 hrs after administration of antigen. The mechanism and significance of this phenomenon termed as "cell shutdown" is not clearly understood. Cell shutdown occurs only on secondary challenge with some antigens. It probably ensures contact of naive T cells with the antigen, as the number of naive T cells able to react to a particular antigen is very low. It can be induced by complement activation leading to retention of B lymphocytes and other cells expressing C3b receptor within the node (McConnell & Hopkins, 1981). LPS, an activator of the classical pathway, has also been shown to induce shutdown (Cahill *et al.*, 1976). Infusion of prostaglandin E_2 (PGE_2) in the afferent lymphatics leads to reduced output of cells in the efferent lymph. Increased secretion of PGE_2 in the node might be due to complement activation or by activated macrophages (Hopkins *et al.*, 1981b). Elevated levels of corticosteroids can also be associated with decreased lymphocyte traffic through the lymph node (Hall, 1986). Intralymphatic infusion of products of primed T cells can also induce cell shutdown (Kelly *et al.*, 1972). IFN-alpha-2a (IFN-α2a) can directly be involved in the regulation of lymphocyte output from the lymph nodes (Hein & Supersaxo, 1988). Cell shutdown has been shown to occur during primary infections with live viruses in sheep (Issekutz, 1985; Smith & Morris, 1970; Bird *et al.*, 1993) and *T. parva* infection in cattle (Emery, 1981b). This phase is specific for the naive population of T cells and the output of memory

T cells from the node is unaffected (Mackay *et al.*, 1992a). Subcapsular and paracortical regions were found to be densely packed with lymphocytes during the period of cell shutdown. Further, there was accumulation of cells around blood vessels in the medulla also suggesting a continued entrance of lymphocytes into the node (Hein & Supersaxo, 1988).

2.3.5 Changes in flow and cellularity of efferent lymph after antigen challenge

Cell shutdown is usually followed by a biphasic increase in the lymphocyte output from the node. The first wave of cells leaving the node is mainly of small lymphocytes which entered the node during cell shutdown (Cahill *et al.*, 1976; Hopkins *et al.*, 1993). The second wave of cells consist of blast cells and antibody producing cells specific for the inoculated antigen generated in the node (Cahill *et al.*, 1976; Hay & Hobbs, 1977). Resting efferent lymph contains only a few blast cells. Blast cells formed in the lymph node in response to an antigen first appear in the efferent lymph during the third day and usually peak between days 4-5 (Trnka & Cahill, 1980). Appearance of blasting cells in the efferent lymph has been observed in response *T. parva* (Emery, 1981b) and *Trypanosoma congolense* (Akol & Murray, 1986) in cattle, and vaccinia virus (Issekutz, 1985), *Toxoplasma gondii* (McColgan *et al.*, 1987), *Chlamydia psittaci* (Huang *et al.*, 1991) and Maedi-visna virus (Bird *et al.*, 1993) in sheep.

There is a marked increase in the blood supply to the node after antigen stimulation leading to increased entry of fluid and lymphocytes to the node (Hay & Hobbs, 1977). Increased entry of cells and reduced exit from the node were supposed to be distinct events occurring independently and possibly mediated by different mechanisms (Cahill *et al.*, 1976). The rate of lymph flow is reported to increase by 2-3 fold around 8-12 days after *T. parva* infection in cattle (Emery, 1981b). About two fold increase in the flow of lymph on days 5-6 was also seen in sheep during *Chlamydia psittaci* infection which declined to pre-infection levels in the following days (Huang *et al.*, 1991). A 2-3 fold increase in the volume of lymph and up to a 10 fold increase in lymphocyte output was observed in the efferent lymph of cattle draining the site of *Trypanosoma congolense* infection (Akol & Murray, 1986). Whether the increased blood flow is mediated by the release of cytokines or vasoactive substances is not clearly known. PGE_2 may be involved in the increased blood flow to the node and enhance vascular permeability to fluid and lymphocytes

(Hopkins *et al.*, 1981b). Other prostaglandins (Trnka & Cahill, 1980), macrophage migration inhibitory factors (Hay, 1973), mitogenic factors (Hay *et al.*, 1973) and cytokines might be responsible for increased output of fluid in the efferent lymph.

2.3.6 Lymphocyte homing and recirculation

Earlier observations that local irradiation of a single lymph node does not result in elimination or reduction of cell output in the efferent lymph suggested that the majority of efferent lymph lymphocytes (ELL) were not generated in the lymph node (Hall & Morris, 1965a). This was further supported by the experiments of Hall & Morris (1965b), when they continuously infused ^{3}H-thymidine into lymph node through a cannulated afferent lymphatic and found only 4% labelled cells in the efferent lymph. It has been shown that about 90% of ELL come from blood into the lymph node by passing between specialised HEV in the paracortex (Hall & Morris, 1965b; Schoefl, 1972), 2-3% arise from proliferation within the node and 5-10% come from afferent lymph in a normal lymph node (Trnka & Cahill, 1980).

Once a primary immune response is initiated in a lymph node to which an antigen has drained, activated lymphocytes of memory phenotype leave the node via efferent lymph and eventually drain into the blood stream through the thoracic duct and are disseminated throughout the body. Memory cells migrate selectively through peripheral tissues, such as skin, where there are more chances to encounter with the antigen, and are drained back to the lymph node via afferent lymph (Mackay *et al.*, 1990). Recent studies have shown that at least a proportion of cells migrate in a tissue selective manner. Washington *et al.* (1988) observed that small $CD4^{+}$ cells were extracted from a normal lymph node more efficiently than other subsets. Small $CD4^{+}$ (possibly naive cells) also showed some specificity to recirculate through the same tissue (Abernethy *et al.*, 1991). Mammary cells exiting the gut lymph nodes preferentially recirculated through gut and migrated poorly through skin (Mackay *et al.*, 1992b). Naive lymphocytes recirculate through lymph nodes via paracortical HEV and leave via efferent lymph. As the frequency of antigen reactive cells within naive cell population is very low, recirculation through lymph node is an effective route for naive cells to make contact with new antigens (Mackay *et al.*,1990).

How naive and memory lymphocytes take different recirculation routes is not properly understood. This presumably depends upon differential homing receptor molecules (Mackay *et al.*, 1992b). A three step model of migration has been

suggested (Mackay, 1993). The first step involves primary interaction between a selectin on the surface of a cell with HEV, followed by some activation signal probably by a cytokine or cell surface interactions leading to changes in ß1 or ß2 integrins. A strong adhesion is formed between the cell and HEV. The final step involves flattening and transendothelial migration of the cell. Expression of ß1 integrin on T cells signifies a differentiation state that correlates either with memory or recent activation (Mackay, *et al.*, 1992b). L-selectin has been shown to be an important adhesion molecule on lymphocytes for LN homing (Howard *et al.*, 1992). $CD4^+$ and $CD8^+$ cells from the gut mucosa were L-selectin negative and only a few were positive in Peyer's patches. Antigen challenged lymph nodes showed an increase in the migration of memory type ß1 $integrin^+$ T cells, and induction of an inflammatory adhesion molecule VCAM-1 on the endothelium (Mackay *et al.*, 1992a). Increase in VCAM-1 on HEV might be one of the molecular mechanisms that accounts for the transient increase of memory type T cell traffic through the node.

2.3.7 Phenotypic changes in efferent lymph following antigen challenge

Various lymphocyte subsets show marked differences in their distribution between blood, afferent and efferent lymph (Mackay *et al.*, 1988). The distribution of CD4, CD8 and $\gamma\delta$ T cells is non-random in these three immunological compartments. Resting efferent lymph had a higher percentage of $CD4^+$ cells and consequently higher CD4/CD8 ratio than peripheral blood (Bujdoso *et al.*, 1989b). Following challenge with human BCG, the percentage of $CD4^+$ cells increased initially, followed by an increase in $CD8^+$ cells (Mackay *et al*, 1992a). Similar changes were also observed in the lymph efferent from the node draining local infection with larvae of blowfly *Lucilia cuprina* in sheep. Output of $CD8^+$ cells was more marked after secondary challenge (Bowles *et al.*, 1994). A marked increase in $CD8^+$ lymphoblasts was observed in the efferent lymph of sheep draining the node infected with Maedi-Visna virus between day six and 11 after infection (Bird *et al.*, 1993). In a recent report, a substantial increase in the proportions of $CD8^+$ cells in efferent lymph was observed between day 8-11 after secondary *T. parva* infection in cattle. The frequency of parasite specific cytotoxic T lymphocyte precursors (CTLp) was as high as 1:32 and confined to the large blasting cell fraction (McKeever *et al.*, 1994).

Activated T cells are lymphoblastoid and express MHC class II and CD25 (Greene *et al.*, 1986; Glimcher & Kara, 1992), whereas resting T cells usually do not express these markers. Primary challenge of the node with ovalbumin was shown to produce little changes in MHC class II expression on T cells. However, secondary challenge produced a two fold increase in the proportion of T cells expressing DR and a 4-5 fold rise in the proportion of DQ^+ T cells which involved all T cell subpopulations (Hopkins *et al.*, 1993). The authors speculated that the increase in MHC class II expression was probably mediated by the release of cytokines.

The reports mentioned above suggest that studies at the local lymph node level are very useful in understanding the initiation and development of immune responses to various antigens. Extensive studies have been done in sheep for understanding basic immunology and development of immunity to various microorganisms using lymphatic cannulation. Surprisingly, similar studies on cattle are scanty in the literature.

2.4 IMMUNITY AGAINST THEILERIOSIS

Immunological studies on *T. annulata* are limited. Most of the studies have been conducted on *T. parva*. Perhaps this is because *in vitro* propagated *T. annulata* infected cell lines were successfully developed as live vaccines in 1966 (Pipano & Tsur, 1966) and have been extensively used in the field. However, attempts to immunise cattle against *T. parva* with infected cell lines have not been very successful. Most of the field immunisation against *T. parva* has relied on the infection and treatment method as discussed below.

2.4.1 Immunisation

Control of theileriosis particularly East Coast fever by immunisation has been reviewed by Brown (1981), Morrison *et al.* (1986b), Dolan & McKeever (1992) and Norval *et al.* (1992). Attempts were initiated to immunise cattle against theileriosis soon after the identification of these parasites in the beginning of this century. Suspensions of lymph nodes and spleen homogenates collected from *T. parva* infected animals were used to immunise susceptible cattle (Theiler, 1911; Spreull, 1914). This approach was soon discontinued because of the erratic availability of immunising material from sick cattle, development of clinical theileriosis following immunisation in many animals and risk of transmission of other diseases. The same approach was

re-investigated several years later by Brocklesby *et al.* (1965), and Pirie *et al.* (1970) without greatly improved results. Sergent *et al.* (1924) demonstrated that unlike *T. parva*, *T. annulata* could be transmitted to susceptible cattle using infected blood, and this was developed as a method of immunisation using strains of low virulence. Continuous mechanical passages of *T. annulata* in cattle resulted in the loss of ability of the parasite to produce infection in ticks but retain immunogenicity (Sergent *et al.*, 1945). This approach was adopted in many countries over the next 40 years, but was also discontinued because of the risk of transmission of other diseases, failure to provide a constant infective material and some mortalities in susceptible animals. With advancements in chemotherapy and *in vitro* cultivation of theilerial parasites, attention was focused on the development of prophylactic methods. In particular using simultaneous infection with sporozoites followed by treatment with a suitable drug and schizont derived cell culture vaccines, which are discussed below.

2.4.1.1 Immunisation by the infection and treatment method

This method of immunisation has been mainly developed and widely used to control *T. parva* infection. It has been known for many years that cattle which recover from theilerial infections become immune to subsequent challenges (du Toit, 1928; Neitz, 1948). Therefore, one of the first approaches to immunisation was to produce a mild self limiting disease as severity of infection was shown to depend on the quantum of initial infection (Barnett, 1957; Wilde, 1967). Further attempts to induce mild non-lethal *T. parva* infection by controlled feeding of infected ticks or titration of GUTS exhibited variable responses in groups of cattle to a given dose of infection (Cunningham *et al.*, 1974; Radley *et al.*, 1974; Dolan *et al.*, 1984c). Similar experiments with graded doses of *T. annulata* infected tick material also showed a dose dependent response (Gill *et al.*, 1977; Samantaray *et al.*, 1980; Preston *et al.*, 1992a). Variable infection rates in different batches of ticks and unpredictable responses in inoculated animals from no reaction to severe disease has made it impossible to use quantum of infection as a means of immunisation.

The observation of Neitz (1953), that cattle were protected against lethal *T. parva* infection by repeated intravenous administration of aureomycin during the incubation period, laid the foundation for development of the so called "Infection and treatment method" of immunisation. He observed that treatment resulted in a decrease and disappearance of schizonts followed by recovery and development of solid

immunity to reinfection. Similar results were reported while treating the animals with oxytetracycline after infection (Neitz, 1957). Another breakthrough in further development of this technique was the production of cryopreserved sporozoite stabilates which allowed infection of cattle with a particular pre-determined dose (Cunningham *et al.*, 1973) along with administration of long acting oxytetracyclines (Radley *et al.*, 1975a; 1975b). Radley (1981) reported further improvements in the technique by using long acting oxytetracycline. This method has now been greatly improved and used commonly for the control of *T. parva* infection. Extensive reviews on this method by Brocklesby & Bailey (1965), Cunningham (1977), Radley (1981) and Brown (1985) have explained development and field application of this technique.

Similar techniques have been developed to immunise cattle against tropical theileriosis using various preparations of tetracyclines with encouraging results (Gill *et al.*, 1976a; Jagdish *et al.*, 1979; Pipano, 1981; Khanna *et al.*, 1983). However, neonatal bovine calves inoculated with one tick equivalent (t.e.) (approximately 10 infected acini) or more freshly prepared GUTS along with long acting oxytetracycline developed clinical theileriosis and died, when immunised with this method. A very low infective dose of 0.035 t.e. GUTS (approximately 5 infected acini) along with the drug engendered protection in 80% animals (Mallick *et al.*, 1987). These experiments suggested that oxytetracycline may not be a drug of choice for immunising very young calves by this method.

With the development of better theilericidal drugs such as parvaquone (Clexon, Wellcome) and buparvaquone (Butalex, Mallincrodt Veterinary Ltd.) (McHardy *et al.*, 1985), successful immunisations have been performed by inoculation of infective *T. parva* stabilate along with treatment using parvaquone (Dolan *et al.*, 1984a) or buparvaquone (Mutugi *et al.*, 1988; Young *et al.*, 1990). Chemoimmunoprophylactic efficacy of buparvaquone against *T. annulata* infection was demonstrated and was found to be better than long acting oxytetracycline (Dhar *et al.*, 1990). Very young calves below four weeks of age were successfully immunised using infective tick material from as little as 2 infected acini (Dhar *et al.*, 1990) to as high as 30 ticks (Kumar *et al.*, 1990) along with buparvaquone treatment. Calves inoculated only with buparvaquone and left in heavily tick infested paddocks also became immune indicating that the drug alone can be used to protect calves born in the disease season for a few days (Kumar *et al.*, 1991). Young calves immunised

with this regime were immune to subsequent potentially lethal challenge immediately after immunisation (Rana & Dhar, 1993), and even if they were already in the incubation period of the disease (Dhar & Rana, 1993). These reports suggest that immunisation by this method can be undertaken even during the disease season and also in very young calves. It is important to note here that immunisation against tropical theileriosis with cell culture vaccine has to be done with caution in very young calves and cannot be done after the onset of the season. Studies on *T. parva* have shown that parvaquone given at early stages of infection could inhibit the development of immunity (Dolan *et al.*, 1988). Ngumi *et al.* (1992) have recently shown that the dose of *T. parva* sporozoites has to be chosen carefully while using buparvaquone for simultaneous treatment, since too high a dose can break through the protection and too low a dose may not produce immunity. However, a wider sporozoite dose range can be used with buparvaquone than with oxytetracycline.

This method of immunisation has some more limitations as well. Production of sporozoite material is very labour-intensive and expensive. There is considerable variation in the infectivity of different batches of ticks. Different vials of the same cryopreserved stock can also produce variable reactions in inoculated animals. Another problem with *T. parva* is heterogenicity amongst various parasite stocks. Some parasite stocks require higher levels of drug in order to control infection than others (Radley, 1981). Immunisation against one stock of *T. parva* did not protect against all stocks of the parasite (Young *et al.*, 1973; Radley *et al.*, 1975b). However, *T. annulata* parasite stocks isolated from different geographical areas show reasonably good cross-protection (Gill *et al.*, 1980). However in most of the studies, the parasite stocks were poorly characterised or quantified, making the situation difficult to interpret. Monoclonal antibodies have been raised against both *T. parva* (Pinder & Hewett, 1980; Minami *et al.*, 1983) and *T. annulata* schizonts (Sheils *et al*, 1986b) for identification of parasite strains. Other methods such as isoenzyme analysis and DNA probes are providing ways of differentiating parasite stocks and characterising their clones, the results cannot yet be related to cross-immunity. Although this method of immunisation is cumbersome, expensive and has many shortcomings, it is the only method available for protection against *T. parva*. The technique has also been developed for *T. annulata*, but only under experimental conditions. It might be useful in immunising very young calves in *T. annulata*

endemic areas which cannot be immunised with the cell culture vaccine.

2.4.1.2 Immunisation with macroschizont infected cells:

Immunisation of susceptible animals with schizont infected cell lines is the most widely used method of immunoprophylaxis against *T. annulata*. Development of cell culture vaccines against tropical theileriosis is mainly attributed to the pioneering work of Israeli workers. Progress and applications of this vaccine have been extensively reviewed by Pipano (1977; 1981; 1984; 1992) and Brown (1981; 1989). Virulence of schizonts was attenuated by prolonged cultivation in cell cultures (Tsur & Adler, 1962) and resulted in milder clinical symptoms and lower parasitaemia on inoculation into susceptible animals (Pipano & Tsur, 1966; Hashemi-Fesharki & Shad-del, 1973; Wenshun *et al.*, 1973; Gill *et al*, 1976b; Stepanova & Zablotsky, 1989; Singh *et al.*, 1993). With loss of virulence in cultures, the parasite lost the capacity to produce piroplasms but retained immunological properties (Pipano & Israel, 1971; Pipano *et al.*, 1973). Cattle vaccinated with completely attenuated schizonts did not provide a source of infection for ticks (Samish *et al.*, 1984). The animals immunised with a cell culture vaccine developed mild parasitological reactions on natural or experimental sporozoite challenge, but were usually protected (Gill *et al.*, 1976b; Pipano, 1981; Ouhelli *et al.*, 1989; Shukla & Sharma, 1991).

These results encouraged immunisation trials against tropical theileriosis under field conditions. Satisfactory protection from the disease was induced in young and adult beef cattle and in female animals up to the age of first pregnancy when inoculated with 1-2x10^6 infected cells. This vaccine could be either used fresh or after cryopreservation, and had four days shelf life at 4°C (Pipano, 1981). Chinese workers were able to preserve the vaccine at 4-6°C for up to 60 days by addition of gelatin (Wenshun *et al.*, 1975). Cell culture propagated schizont vaccine has been used efficiently in many countries where *T. annulata* is endemic, including Israel (Pipano, 1978; 1992), Turkey (Ozkoc & Pipano, 1981), Iran (Hashemi-Fesharki & Shad-Del, 1973; Hashemi-Fesharki, 1988), Iraq (Hooshmand-Rad, 1973), India (Subramanian *et al.*, 1986; Shukla & Sharma, 1991; Singh, 1992), Russia (Stepanova *et al*, 1977), China (Wenshun *et al.*, 1973; Wenshun *et al.*, 1982) and Morocco (Ouhelli, 1991). Attenuated cell lines used as a vaccine in different countries have been developed by continuous passage in culture and tested for immunogenicity by inoculation in

susceptible calves at different passage levels. Sporozoite challenge of animals immunised with such schizont infected cell culture vaccines usually results in mild reactions and protection even against heterologous stocks of the parasite (Pipano, 1981; Brown, 1989; Brown, 1990). Occasionally, highly susceptible adult Friesian cows were inadequately protected and reinforcement with a second immunisation using infected tick material was recommended in such animals (Pipano, 1981).

Cattle immunised with a cell culture vaccine were protected on challenge with sporozoite stocks from remote geographical areas in some experiments (Preston & Brown, 1988; Innes *et al.*, 1989c), but poorly protected in others (Ozkoc & Pipano, 1981; Subramanian *et al.*, 1987). Various *T. annulata* stocks have not been fully characterised and are only known on a geographical basis. Interpretation of cross-challenge studies after cell line immunisation is, therefore, difficult.

Duration of immunity in absence of reinfection after cell line immunisation has not been fully investigated. Pipano (1977) reported that cattle immunised with attenuated schizonts were protected when challenged after 18 months. Animals vaccinated with cell culture vaccine were protected for more than one year (Singh, 1992). In a more promising report, Zablotsky (1991) found that animals challenged 3.5 years after immunisation were immune. Other evidence in the literature suggests that immunity to the parasite declines in the absence of re-challenge (Brocklesby, 1978; Pipano, 1981). Recently, Ouhelli et al. (1994) showed that some of the animals immunised with $1x10^4$ *T. annulata* infected cells were not protected when challenged with sporozoites after seven months.

Little is known about the mechanism by which prolonged culture of cell lines leads to attenuation of the parasite. It might simply be a clonal selection over a period of time, in which case selection pressures will be a priority area of study (Brown, 1990). Studies on glucose phosphate isomerase isoenzyme patterns on *T. annulata* infected cell lines at very early passages revealed multiple banding patterns, but late passages and cloned cell lines only revealed a single triplet (Melrose *et al.*, 1980). Reduction in virulence by prolonged cultivation of *T. annulata* infected cell lines seemed to coincide with a gradual decrease in their reactivity to a schizont specific mAb EU106 and changes in the RFLP patterns leading to selection of a particular genotype (Sutherland *et al.*, 1993). However, any conclusive evidence of association between, disappearance of a particular antigen, or alteration of gene expression in

infected cells with attenuation has not yet been presented.

Immunisation against *T. parva* with allogeneic infected cells has not been very successful as compared to *T. annulata*. Initial studies indicated that at least 10^8 *T. parva* infected fresh or cultured cells were required to induce reliable immunity (Pirie *et al.*, 1970; Brown et al., 1971; Brown *et al.*, 1978a; Emery et al., 1982). However, as few as 10^2 autologous infected cells resulted in sub-patent infection and development of immunity to challenge (Morrison *et al.*, 1981a; Buscher *et al*, 1984). Further, cattle inoculated with 10^3 or 10^5 *T. parva* infected MHC class I matched cells produced immunity, but animals inoculated with similar doses of MHC class I mismatched cells were not protected on subsequent challenge (Teale, 1983 & Dolan *et al.*, 1984b). On the other hand, cell doses of 10^2 (Ouhelli *et al.*, 1989) or even 10^1 (Brown, 1990) allogeneic infected cells would infect and immunise animals against *T. annulata*. Cells infected with both parasites express BoLA types identical to those of the animal from which they were generated (Spooner & Brown, 1980).

During cell line immunisation, the parasite is introduced in the recipient animal within a foreign cell in the form of a graft. Therefore, histoincompatibility between cell line and the recipient may influence successful immunisation on inoculation of a parasite infected cell line. An important feature for the development of immunity after immunisation with allogeneic infected cells is that infection has to transfer and establish into the cells of the recipient animal (Wilde 1967; Pipano *et al.*, 1977; Brown *et al.*, 1978a; Emery *et al.*, 1982; Innes *et al.*, 1989a). However, the mechanism involved in this transfer is unknown. Using karyotypic analysis, it was observed that *T. annulata* would transfer from donor cells and infect cells of the recipient animal within hrs of inoculation {Hulliger et al., 1965 (unpublished) reported in Wilde, 1967}, whereas, parasite transfer was very inefficient with *T. parva* infected cells and could take many days (Brown *et al.*, 1971; Brown, 1981). Innes *et al.* (1989a) elegantly demonstrated the phenomenon of parasite transfer by immunising animals with BoLA mismatched *T. annulata* infected cells and re-isolating parasite infected cells from the recipients. The *in vivo* isolated cell lines expressed the BoLA type of the recipient. It is not clearly known whether transfer of parasite from donor cells to the cells of the recipient takes place before the development of an allogeneic response to MHC antigens of the immunising cell line or thereafter, giving inoculated allogeneic cells some time to proliferate in the recipient. This has

important implications for *T. annulata* cell line immunisation as immunity wanes and reimmunisation is recommended to ensure protection. Histoincompatibility between the donor cell line and the recipient animal might pose similar problems during secondary cell line immunisation against *T. annulata*, as it does during primary immunisation against *T. parva*.

Some attempts have been made to immunise cattle with killed *T. annulata* schizont material. Animals developed high antibody titres after inoculation, but were not immune to sporozoite challenge (Pipano *et al.*, 1977). In a more recent study, two calves inoculated with purified plasma membranes from a *T. annulata* infected cell line were protected on sporozoite challenge with the same parasite stock, but the other two calves immunised with the same material were fully susceptible to challenge with a slightly different stock of the sporozoites (Chaudhri & Subramanian, 1991). In a similar study, Emery *et al.* (1986) showed that 3 out of 4 calves inoculated with purified plasma membranes from autologous *T. parva* infected cell lines were protected on sporozoite challenge, whereas calves inoculated with purified plasma membranes from an allogeneic cell line were not protected. The mechanism of development of immunity against both parasites to such immunising regimes is not understood. It is not clear whether these findings are repeatable as further work in this field is lacking. However, it has been shown that purified plasma membranes can stimulate cytotoxic T lymphocyte responses *in vitro* but only after priming with viable material *in vivo* (Lemonnier *et al.*, 1978; Loh *et al.*, 1979).

2.4.2 Immune responses

Cattle recovered from *T. annulata* or *T. parva* infection are immune to subsequent challenge, indicating that generation of immunity against theileriosis is a possibility (Neitz, 1957). Extensive studies have been conducted over the past few years to understand the development of immunity against these two parasites. Several recent reviews provide a comprehensive cover of present knowledge on immunology of these two parasites (Morrison *et al.*, 1986b; Hall, 1988; Morrison *et al.*, 1989; McKeever & Morrison, 1990; Tait & Hall, 1990; Dolan & McKeever, 1992; Norval *et al.*, 1992; Goddeeris *et al.*, 1994). Studies concentrated on elucidating immune responses to different stages of the parasites during the last few years as discussed below.

2.4.2.1 Immune responses to sporozoites

The current state of recombinant sporozoite vaccines has been briefly reviewed by Musoke & Nene (1990). Although the sporozoite stage of the parasite is exposed to the host immune system for a very short time (Brown *et al.*, 1978b; Jura *et al.*, 1983), the immune serum produced *in vivo* has been able to inhibit entry of sporozoites into uninfected leucocytes *in vitro* (Gray & Brown, 1981; Preston & Brown, 1985; Ahmed *et al.*, 1988). Sera raised in *T. parva* infected animals did not neutralise infectivity of *T. annulata* sporozoites *in vitro* indicating that the immune serum is species specific (Gray & Brown, 1981). Williamson *et al.* (1989) raised mAb against *T. annulata* sporozoites which neutralised sporozoite infectivity *in vitro*. One of these mAb (1A7) recognised a sporozoite surface antigen (SPAG-1) on geographically distinct stocks of the parasite. The gene coding for this protein was expressed only in sporoblasts and sporozoites to a significant degree and not in schizonts or piroplasms. Further characterisation and sequencing of the gene showed regions homologous to repetitive structures present in bovine elastin (Hall *et al.*, 1992a; 1992b; Hall & Baylis, 1993). Neutralising B-cell epitopes recognised by both immune sera and mAb 1A7 were located in the C terminus of this gene (Boulter *et al.*, 1994). The epitope recognised by mAb 1A7 mapped to a 16 amino acid linear sequence and immune bovine sera recognised distinct but adjacent epitopes in the same region (SR1). Immunisation experiments with recombinant protein derived from the cloned gene were carried out to test the level of engendered protection, but the initial results were not very encouraging as all the immunised animals suffered from acute disease on sporozoite challenge (Boulter & Hall, 1994). Meanwhile another *T. annulata* sporozoite surface antigen (SPAG-2) recognised by a mAb 4B11 has also been described. The gene coding for this protein was cloned and expressed as a fusion protein. Hyperimmune serum against this protein raised in rabbits neutralised sporozoite invasion *in vitro* and recognised an antigen on sporozoites and schizont infected cells (Knight *et al.*, 1994).

Antibodies capable of neutralising infectivity of *T. parva* sporozoites were demonstrated in cattle immunised against East Coast fever by the infection and treatment method and subsequently challenged with infective ticks several times (Musoke *et al.*, 1982). Immune sera from such cattle neutralised infectivity of sporozoites of various *T. parva* stocks which were otherwise not cross-protective

(Musoke *et al.*, 1984). A mAb generated against sporozoites of *T. parva* also neutralised invasion of host cells *in vitro* (Dobbelaere *et al.*, 1984; Musoke *et al.*, 1984; Dobbelaere *et al.*, 1985). Immune sera and mAb recognised an antigen (p67) of relative M_r of 67 kd on the surface of sporozoites only (Musoke & Nene, 1990; Iams *et al.*, 1990). The gene coding for p67 protein was cloned and expressed in a plasmid vector (Nene *et al.*, 1992). Partially purified recombinant antigen (NSI-p67) induced sporozoite neutralising antibodies when inoculated into susceptible animals and protected six out of nine cattle on homologous challenge (Musoke *et al.*, 1992). In another experiment, 11 animals immunised with NSI-p67 were challenged with a heterologous Marikebuni stock of *T. parva*, but only six were protected (Musoke *et al.*, 1993). The challenge dose in these experiments was very low (0.1 t.e.), lower than one might get from a single tick. Thus its efficiency in protecting against a field challenge needs testing. Another problem is that an immune response against sporozoites alone might not be adequate to prevent infection of host cells as a few sporozoites might escape neutralisation. Sequence analysis of PCR amplified p67 gene product from six different stocks of the parasite show no variation making it theoretically possible to cross-protect amongst various *T. parva* stocks (Musoke *et al.*, 1993). Use of better adjuvants or live delivery systems might improve the immunising efficacy of these recombinant proteins. This may help in reducing the infective dose to allow the development of a protective immune response to the macroschizont stage or recombinant sporozoite surface antigen could be used as a part of a cocktail vaccine along with antigens from other stages.

As the schizont culture vaccine does not work, there has been a tremendous pressure to develop alternative molecular vaccines against *T. parva*. However, the case for a recombinant vaccine against *T. annulata* is very weak as it has to compete with a highly effective cell culture vaccine.

2.4.2.2 Immune response to macroschizont infected cells

This stage of the parasite seems to be most pathogenic in *T. annulata* as well as *T. parva* infection (Morrison et al., 1981b; Preston et al., 1992a). The parasite lives inside the host cells and divides in synchrony with division of the infected cell. Therefore, cell mediated mechanisms are more likely to be effective than humoral responses.

2.4.2.2a Humoral responses

Animals undergoing immunisation against *T. annulata* or recovering from the infection develop circulating antibodies to all stages of the parasite (Kachani *et al.*, 1992a; 1992b). High antibody titres were detected in cattle after exposure to the parasite (Pipano & Cahana, 1969) and after vaccination with attenuated cell lines (Shukla & Sharma, 1991). Kachani *et al.*, (1992a) demonstrated three antigens between 71 and 73 kd by Western blot analysis which were common to sporozoite, schizont and piroplasm stages of *T. annulata*. Another 32 kd antigen was found to be specific to the piroplasm stage. Several observations suggested that humoral responses may not have any protective role particularly against the schizont stage of the parasite. Immune sera recognised the parasite inside host cells as shown on IFAT slides, but were shown not to recognise parasite associated antigens on the cell surface (Creemers, 1982; Shiels *et al.*, 1989; Glascodine *et al.*, 1990). Passive transfer of immune serum from immunised to susceptible animals did not prevent development of either *T. annulata* (Sergent *et al.*, 1945; Dhar & Gautam, 1978; Samad *et al.*, 1984a) or *T. parva* infection (Muhammed *et al.*, 1975), although sera from immune cattle exhibited antibodies against all stages of the parasite (Burridge & Kimber, 1972). Experiments using lysates or inactivated schizont material to immunise animals have not induced a protective response in either *T. annulata* (Pipano *et al.*, 1977) or *T. parva* (Wilde, 1967; Emery *et al*, 1981b) infection, despite generation of antibodies. Similar attempts to immunise animals against *T. parva* using extracts of piroplasms were unsuccessful (Wagner *et al.*, 1974). Serum from *T. annulata* immune cattle was not opsonic for parasite infected cells and did not lyse these cells in the presence of complement (Ahmed *et al.*, 1988). Similarly, Duffus *et al.* (1978) demonstrated that serum from *T. parva* immune cattle did not lyse infected cells. However, generation of antibodies against schizonts and piroplasms is a useful diagnostic aid for conducting serological surveys and assessing the response of animals after immunisation.

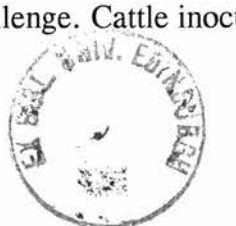
Monoclonal antibodies against *T. annulata* schizonts were raised (Shiels *et al.*, 1986a) which identified three sets of antigens on the surface of infected cells (Shiels *et al.*, 1986b). Two glycoproteins of M_r 100-125 kd and 80 kd were identified on the surface of infected cells. The 100-125 kd molecule was recognised by a mAb 4H5 which was shown to vary in size between various parasite infected lines (Shiels *et al.*,

1989). This mAb induced complement mediated lysis of infected cells (Preston *et al.*, 1986) indicating induction of a cell surface molecule by the parasite which is important immunologically but is not immunodominant for cattle. The role of infection specific antigens in generating immune responses is not very clear. It is not known whether these molecules are parasite gene products or induced by host cells.

2.4.2.2b Cell mediated responses generated *in vivo*

Because of lack of correlation between the presence of antibodies and immunity to the parasite, it was suggested that immunity is mediated by cellular mechanisms. Earlier observations of Singh *et al.* (1977), Ahmed *et al.* (1981) and Samad *et al.* (1983; 1984b) also supported involvement of cell mediated responses in protection. Cattle undergoing lethal *T. parva* infection exhibited increased cellularity and lymphoblasts in lymph efferent from the node draining the site of infection (Emery, 1981b). Protection against concurrent challenge with *T. parva* sporozoites by adoptive transfer of thoracic duct lymphocytes from immune to naive chimeric twin calves (Emery, 1981a) confirmed the role of cellular responses in protection.

A series of further *in vivo* experiments with *T. parva* demonstrated involvement of cytotoxic T lymphocytes in protection. Cattle undergoing immunisation or challenge with *T. parva* transiently expressed cytotoxic cells in efferent lymph and PBM at the time of remission of infection which killed autologous parasite infected cells but did not kill infected cells from unrelated animals (Eugui & Emery, 1981). These effector cells seemed to be T lymphocytes recognising cell surface antigens in the context of MHC molecules. In another experiment, cattle undergoing lethal *T. parva* infection exhibited cytotoxic cells in PBM which killed allogeneic infected cells and xenogeneic murine cells but not autologous infected cells, whereas animals immunised by infection and treatment generated genetically restricted cytotoxic cells which killed autologous infected cells (Emery *et al.*, 1981a). The appearance of cytotoxic cells was transient and coincided with recovery from mild infection. Fractionation and depletion of effector PBM into different cell populations revealed that cytotoxic activity was mediated by T cells (Emery *et al.*, 1981b). Cattle immunised by inoculation of approximately 10^7 allogeneic *T. parva* infected cells generated cytotoxic cells specific for killing the donor cell line and autologous infected cells. Cytotoxicity against autologous infected cells correlated with the subsequent infection and protection on challenge. Cattle inoculated with 10^2-



10^5 autologous *T. parva* infected cells developed cytotoxic responses directed against autologous parasitized cells (Emery *et al.*, 1982). These cytotoxic cells were presumed to be genetically restricted. Cytotoxic cells against autologous *T. parva* infected cells were a feature in immune animals or in animals undergoing immunisation and directly correlated to protection from lethal sporozoite challenge. This mechanism was not stimulated in animals undergoing acute *T. parva* infection.

Studies on *T. annulata* showed that calves recovering from infection produced cytotoxic cells which recognised infected cells in conjunction with self MHC class I molecules (Preston *et al.*, 1983). This was the first evidence of generation of MHC class I restricted cytotoxic cells during *Theileria* infections. Two peaks of cytotoxicity were observed during recovery from *T. annulata* infection. The first peak was BoLA restricted and probably analogous to cytotoxic T lymphocytes, while the second peak was not MHC restricted and probably analogous to natural killer cells. Further evidence of genetically restricted cytotoxic cells came from the experiments of Morrison *et al.* (1987b). They showed that cytotoxic cells generated in East Coast fever immune cattle following sporozoite challenge killed only autologous infected cells or infected cells from other animals expressing matched or half matched BoLA types. The response was found to be biased towards one or the other BoLA type. A mAb specific for bovine CD8 cells partially blocked cytotoxic activity. Cytotoxic activity was also blocked by an MHC class I specific mAb but not by MHC class II mAb indicating that the response was produced by MHC class I restricted cytotoxic T lymphocytes (CTL) of CD8 lineage (Morrison *et al.*, 1987b).

A direct analysis of cell mediated cytotoxic activity against *T. annulata* infected cells was demonstrated by inoculating animals with autologous or allogeneic *T. annulata* infected cell lines by Innes *et al.* (1989a). Animals inoculated with an allogeneic cell line developed a strong cytotoxic response directed against MHC antigens of the immunising cell line followed by development of MHC class I restricted cytotoxic cells against autologous infected cells. Animals inoculated with autologous infected cells developed severe clinical reactions and a low level of cytotoxic activity against parasite infected cells, which was not MHC restricted up to day 20. Sera specific for bovine MHC class I antigens blocked the cytotoxic activity further confirming that the response was MHC class I restricted (Innes, 1988).

A limiting dilution micro-culture system to quantify the frequency of *T. parva* specific CTLp in PBM of immune cattle was standardised (Taracha *et al.*, 1992a). The technique displayed specificity for infected cells, genetic restriction and in some cases strain specificity. Using this technique, a correlation between parasite strain specificity of CTL and *in vivo* cross-protection was confirmed (Taracha *et al.*, 1992b). The most direct evidence that immunity to *T. parva* is mediated by CTL was recently provided by McKeever *et al.* (1994). They showed that frequency of CTLp significantly increased, in lymph efferent from the node draining the site of *T. parva* sporozoite challenge of an immune animal, which mainly resided in the $CD8^+$ cell population. Adoptive transfer of purified $CD8^+$ cells at this stage, from an immune animal to its naive monozygotic twin undergoing lethal *T. parva* infection, protected the recipient calf showing that lethal infection can be controlled by responding $CD8^+$ cells alone. These observations highlight the potential of exploiting parasite antigens recognised by CTL for vaccination (McKeever & Morrison, 1994). However, stimulation of such a mechanism of immunity would require recognition of parasite antigen(s) by the host in the context of self MHC.

Preston (1981) showed a cytostatic effect of adherent cells on *T. annulata* infected cell lines. Adherent cells isolated from peripheral blood of calves immunised using schizont infected cells or sporozoites, exhibited a strong cytostatic effect via a soluble factor on schizont infected autologous as well as allogeneic cell lines (Preston & Brown, 1988). Several studies have reported the presence of a lymphocyte migration inhibition factor in immune animals undergoing challenge with *T. annulata* (Singh *et al.*, 1977; Ahmed *et al.*, 1981; Chaudhri & Subramanian, 1992). Further, certain cytokines have been reported to significantly inhibit *in vitro* development of trophozoite infected cells of *T. annulata* as well as *T. parva*. However, these cytokines had no effect on already established cell lines of both species (Preston *et al.*, 1992b). PBM, pulmonary macrophages and lymph node cells from a calf lethally infected with *T. annulata* spontaneously produced IFN-γ *in vitro* (Preston *et al.*, 1993). Large numbers of $CD25^+$ cells appeared transiently in the draining lymph node after *T. annulata* sporozoite infection. The loss of $CD25^+$ cells from the node coincided with the loss of germinal centres indicating a T cell dysregulation during infection (Campbell *et al.*, 1994b). Immunisation of animals with an allogeneic *T. annulata* infected cell line lead to activation of T cells near HEV and formation of

germinal centres with many blasting cells in the draining lymph node indicating that animals mounted a protective response (P. Goel & R.L. Spooner, pers. comm.). Activation of T cells, secretion of cytokines and their role in immunity and pathogenesis during *T. annulata* and *T. parva* infection has not been extensively studied. Understanding the activation of immunocompetant cells by the parasite and parasite antigens could help in manipulating the immune response in the favour of host during lethal challenge.

2.4.2.2c Generation of T cells *in vitro*

Cellular responses against the parasites were only observed for a short period during *in vivo* studies. In order to expand these responses for identification of antigens recognised by the immune system, it is necessary to generate T cell lines and clones *in vitro*. Early observations demonstrated that irradiated *T. parva* infected cells induced proliferative responses in autologous PBM which killed autologous infected cells as well as uninfected blast cells (Pearson *et al.*, 1979; 1982). In further studies by Eugui & Emery (1981), cytotoxic cells generated by incubation of PBM (collected immediately following challenge) with irradiated autologous *T. parva* infected cells showed low levels of killing of autologous infected cells. Cytotoxic cells generated *in vitro*, by incubation of PBM separated from defibrinated blood of immune or naive cattle with irradiated autologous *T. parva* infected cells, showed no genetic restriction. However, cytotoxic cells generated *in vivo* after challenge of immune animals only lysed autologous infected cells (Emery & Kar, 1983).

The discrepancies in the above results from *in vitro* generated cytotoxic cells related to a number of factors concerning the source of responder and stimulator populations. The infected cell lines used as stimulators were not cloned cell populations. It has been shown that *T. parva* transforms a variety of target cells *in vitro* (Baldwin *et al.*, 1988a). There is a variation in the ability of different cell lines to stimulate proliferation and induce cytotoxic responses in autologous PBM (Goddeeris *et al.*, 1986a). Depletion of monocytes from the responder population tends to give non-specific proliferative responses known as autologous *Theileria* MLR (Goddeeris & Morrison, 1987; Goddeeris *et al.*, 1987). The role of monocytes in generation of a genetically restricted response is not clear. It is possible that initial antigen presentation by monocytes in an MHC class II restricted pathway to $CD4^+$ cells is necessary to stimulate MHC class I restricted $CD8^+$ cells.

Cytotoxic cells specific for autologous *T. parva* infected cells were generated *in vitro* by stimulation of PBM from immune animals with irradiated autologous infected cells but not from PBM of naive animals. Further weekly stimulations of cells from these cultures with irradiated autologous *T. parva* infected cells resulted in enrichment for specific cytotoxic cells. The cultures consisted of mainly $CD4^+$ and $CD8^+$ T cells, but cytotoxic activity was contained in the CD8 fraction only (Goddeeris *et al.*, 1986a). Cytotoxic cells generated *in vitro* seemed to be analogous to *in vivo* generated cytotoxic cells in *T. parva* immune cattle. Cloned T cell populations specific for *T. parva* infected cells were derived from these lines (Goddeeris & Morrison, 1988a). Two types of clones were generated: $CD8^+$ cytotoxic cell clones (Goddeeris *et al.*, 1986b) and $CD4^+$ non-cytotoxic cell clones (Baldwin *et al.*, 1987).

Cytotoxic T cell clones derived from an animal immunised with *T. parva* (Marikebuni) killed infected cells of Marikebuni as well as Muguga stocks. Clones obtained from an animal immunised with *T. parva* (Muguga) only killed parasite infected cells of this stock (Morrison *et al.*, 1987a). This animal was not protected on challenge with sporozoites of Marikebuni stock. On the other hand, two types of cytotoxic T cell clones were generated from another animal immunised with the Muguga stock, which showed a cross-protective response *in vivo*. Some clones were specific for Muguga stock only, whereas others were cross-reactive (Goddeeris & Morrison, 1988b). T cell clones seemed to recognise two antigenic epitopes, one of which was common to both the stocks and the other was specific to Muguga stock only. These studies raised a question as to why some animals immunised with *T. parva* (Muguga) responded to one epitope only, while others responded to both epitopes. Goddeeris *et al.* (1990) showed that selection of the T cell epitope for immune responsiveness was governed by MHC phenotype of the animal. Cytotoxic T cell responses to viral antigens have shown that mice of different MHC phenotypes respond to different epitopes on immunogenic molecules (Vitiello & Sherman, 1983; Townsend & McMichael, 1985). Recently, foot and mouth disease (FMD) peptides recognised by T cells were shown to be influenced by MHC class II types of the recipient animal (Van-Leirop *et al.*, in press).

Further investigations with *T. parva* specific T helper cell ($CD4^+$) clones revealed that these clones identified two sets of antigens on infected cells (Brown *et*

al., 1989b). One antigen was schizont membrane associated and appeared to be expressed on the infected cell surface (Brown *et al.*, 1989c). This antigen could be derived from different parasite stocks and purified schizonts, and was only seen by primed $CD4^+$ cells (Baldwin *et al.*, 1992). The second antigen was a soluble antigenic fraction prepared by high speed centrifugation and seemed to be expressed on the surface of infected cells (Brown *et al.*, 1990). Elimination of schizonts from infected cells by *in vitro* treatment with parvaquone also removed this antigen indicating that the antigen was parasite associated (Brown *et al.*, 1989c). Crude fractionation of this soluble antigen by gel filtration and high performance liquid chromatography demonstrated that the antigen is a 24 kd protein and is recognised by $CD4^+$ cells from immune cattle (Grab *et al.*, 1992).

The main importance of generating $CD4^+$ and $CD8^+$ parasite specific lines and clones is to identify relevant parasite antigens, apart from identification of different parasite stocks. $CD8^+$ cells recognise antigens in association with MHC class I molecules which requires active synthesis and processing. Therefore, the $CD8^+$ cell response to a particular epitope cannot be generated *in vivo* by using killed parasite material or purified antigens. However, it might be possible to introduce candidate antigens into target cells by transfecting parasite gene(s) into a mammalian cell expressing the appropriate bovine MHC molecule(s), or by inserting genes into recombinant virus vectors which could be used to infect bovine cells. Mouse L fibroblast cells have been transfected with a bovine MHC class I gene and expression of MHC class I on the transfected cells has been stable (Toye *et al.*, 1990; Hasima *et al.*, 1992; Sawhney et al., in press). Extensive information on the *T. parva* genome is available (Morzaria & Young, 1993), but initial co-transfection studies of the parasite DNA into bovine MHC class I transfected L cells were not rewarding (Toye *et al.*, 1991).

Another approach of identifying parasite specific epitopes on the cell surface for recognition by $CD8^+$ cells could be by elution of peptides from infected cells and identifying the ones recognised by cytotoxic T lymphocytes. Conze *et al.* (1994) eluted peptides from *T. annulata* infected cells and fractionated them on high performance liquid chromatography. One of these fractions fed to syngeneic cells was recognised by parasite specific cytotoxic cells generated in an immune animal undergoing sporozoite challenge. Further characterisation of this peptide fraction

would help in finding relevant antigenic epitope(s) for $CD8^+$ cells.

There have been fewer studies on *in vitro* T cell responses on *T. annulata* than *T. parva*. Initial observations showed that PBM from immune or naive calves proliferated in the presence of irradiated autologous *T. annulata* infected cells (Preston, 1981), but these findings were never followed. Some success in generating *T. annulata* specific T cell lines from an immune animal which could be maintained for a short period was demonstrated by Innes (1991). The line showed specific cytotoxic activity against autologous and BoLA matched *T. annulata* infected target cells which could be inhibited by addition of immune allo-antisera against MHC class I types but not by anti MHC class II antisera. Cytotoxic effector function resided in the $CD8^+$ cells. Although T cells from immune and naive cattle proliferated in response to irradiated autologous *T. annulata* infected cells *in vitro*, stable T cell lines could not be generated by repeated *in vitro* stimulations of PBM from immune animals with autologous infected cells (Campbell *et al.*, 1994b; A.K. Nichani & R.L. Spooner, unpublished observations). Both $CD4^+$ and $CD8^+$ cells from naive animals proliferated and expressed CD25 and MHC class II when cultured with irradiated autologous *T. annulata* infected cells. Proliferating cells also expressed mRNA for various cytokines viz: IL-2, IL-2R, IL-4 and IFN-γ, suggesting induction of a non-specific polyclonal T cell activation in response to the parasite (Campbell *et al.*, 1994c).

Most lymphocyte effector responses depend on activation of $CD4^+$ cells to secrete cytokines and transmit signals to other cell populations. The fact that animals can be immunised against *T. annulata* emphasize that these responses are generated *in vivo* in immune animals. Inability to generate *T. annulata* specific T cell lines was surprising and unexpected, in contrast to the observations on *T. parva* as discussed above. There could be several reasons for this failure. Both parasites infect and transform different cell subpopulations (Spooner *et al.*, 1989) and, therefore, deliver different signals to T cells. Infected cells of both parasites secrete different cytokines *in vitro*. *T. parva* infected cells produce IL-2 (Dobbelaere *et al.*, 1988; Brown *et al.*, 1989a; Heussler et al., 1992) and IFN-γ (DeMartini & Baldwin, 1991; Entrican *et al.*, 1991; Ahmed *et al.*, 1993) which may at least partially be responsible for stimulating proliferation in culture of PBM with autologous irradiated *T. parva* infected cells. *T. annulata* infected cells produce type 1 IFN (α/β) (Entrican *et al.*,

1991) which might have some suppressive effect on T cells. *T. annulata* infected cell lines generated *in vitro* and *in vivo* have been shown to express different cell surface antigens (Howard *et al.*, 1993). It is possible but unlikely that the parasite infects different cell populations *in vivo* and *in vitro*, and if they do, T cell activation by parasite infected cells *in vivo* and *in vitro* will be different.

2.4.2.3 Immune responses against merozoites and piroplasms

Immune responses against erythrocytic stages are not considered very important since these occur very late in the course of the disease and damage has already been done by that time. Generation of immunity against this stage might be important when sub-unit vaccines containing antigenic determinants from all stages of the parasite are used to block the parasite at all levels. Secondly, it might be important in blocking transmission of parasite to tick vector. Antibody responses to piroplasms have been observed in cattle recovering from infection or undergoing immunisation with either *T. annulata* (Kachani *et al.*, 1992b) or *T. parva* (Burridge & Kimber, 1973). Immune serum from *T. annulata* infected animals has been shown to react with free merozoites produced by lysis of erythrocytes (Ahmed *et al.*, 1988). Monoclonal antibodies generated against *T. annulata* merozoites cross-react with piroplasms (Glascodine *et al.*, 1990).

Extensive research on immunity against theileriosis over the past few years has provided a good understanding on cellular immune effector mechanisms involved in protection against both parasites. Cell culture vaccines have been extensively used to control tropical theileriosis. Reimmunisation is recommended in some endemic areas, but no information on feasibility of reimmunisation is available. Little is known about the cellular interactions involved in activation of immune effector cells leading to the development of immunity. An attempt has been made to address some of these questions in this thesis, particularly elucidating the role of allograft response on cell line immunisation against *T. annulata* and investigating T cell activation responses after immunisation and challenge.

CHAPTER 3

GENERAL MATERIALS AND METHODS

The various materials and techniques used during the persuit of this study are describeded in this chapter. Preparation of common laboratory solutions and media is given in the appendix with a reference in the relevant sections.

3.1 Experimental animals

The animals used in this study were male or female Friesian, Hereford or Charolais calves or their crosses aged between 2-8 months. They were maintained at Dryden Mountmarle farm and fed with a regulated supply of concentrate ration and *ad libitum* hay and water. All the animals were housed together in an animal shed during reimmunisation experiments, however they were housed individually in calf pens during lymphatic cannulation studies where they could easily move around.

3.2 MHC class I (BoLA) typing of animals

MHC class I types of all the animals used in this study were detected by microlymphocytotoxicity as described by Spooner *et al.* (1979a). BoLA types of *T. annulata* infected cell lines isolated from animals after cell line immunisation were also determined by microlymphocytotoxicity as described previously (Spooner & Brown, 1980).

3.2.1 Alloantisera for BoLA Typing

A panel of alloantisera was used to define the BoLA specificities of the animals and *T. annulata* infected cell lines used in this study. These alloantisera were produced in Edinburgh from cattle by reciprocal skin grafting between dam and offspring (Spooner *et al.*, 1979a). These operationally monospecific sera have been tested and compared with sera developed in other laboratories in the five international comparison workshops carried out to date (Spooner *et al.*, 1979b; Anon., 1982; Bull *et al.*, 1989; Bernoco, *et al.*, 1991; Davies *et al.*, 1994b), and have been assigned the workshop specificities. The Edinburgh alloantisera typing panel is listed in appendix (no. 10) and includes sera defining the presently agreed workshop specificities (Davies *et al.*, 1994b).

These typing sera were stored neat in small aliquots at -70°C. Control serum, which was selected for non-reactivity with any of the test cells, was included in the lymphocytoxicity test to estimate baseline test cell viability. Appropriate dilutions of both the control and typing sera were prepared in Hank's balanced salt solution

(HBSS) (appendix, no. 1) and dispersed (1 μl per well) in Terasaki 60 well plastic typing plates (Intermed, Nunc) in a known order under liquid paraffin using a multidispenser Hamilton syringe. Batches of typing plates were also stored at -70°C.

3.2.2 Preparation of test cells for BoLA typing

Preparation of lymphocytes for use in the microlymphocytotoxicity test was carried out essentially as described by Spooner *et al.*, (1979a). Three ml of venous blood, collected in sterile vacutainers containing lithium heparin (Becton Dickinson), was layered onto 2.5 ml Ficoll-hypaque solution of specific gravity (S.G.) 1.077 (Lymphoprep, Nycomed Pharma As, Oslo, Norway) and centrifuged for 15 minutes (min.) at 1500*g*. The lymphocyte layer at the plasma/Ficoll-hypaque interface was removed and washed in HBSS by centrifugation at 400*g* for 5 min. The resultant cell pellet was resuspended in a small volume of HBSS and lysis of any contaminating erythrocytes was achieved by the addition of 2 ml of deionised distilled water to the cell sample with constant mixing, followed rapidly by 2 ml of double strength (2x) HBSS. The cell suspension was then washed twice by centrifugation at 100*g* for 5 min. (to remove platelets) and resuspended in HBSS. Cells were counted in a haemocytometer (Neubauer chamber) using a phase contrast microscope (Diavert, Leitz) and adjusted in HBSS to give a final concentration of $2.5x10^6$ cells per ml.

T. annulata infected cells were washed twice in HBSS by centrifugation at 400*g* for 5 min. The cells were resuspended in HBSS and adjusted to a final concentration of $2x10^6$ cells per ml in HBSS for testing.

3.2.3 Microlymphocytotoxicity test

The microlymphocytotoxicity test was conducted as described by Spooner *et al.* (1979a). The Terasaki typing plates were thawed to room temperature (temp.) before use. All the following incubations were performed at room temp. Details for preparation of complement, eosin dye and fixing solution used for this test are given in appendix (no. 2, 3 and 4). The test cell suspension (1 μl) was added to each well and plates incubated for 30 min. Complement (5 μl) was added to each well of the plate and further incubated for one hour (hr). Following incubation, 2 μl of 5% eosin solution was added to each well and incubated for 5 min. Finally, 5 μl of fixing solution was added. The plates were then read immediately using an inverted phase contrast microscope (Nikon) at 100x magnification or stored at 4°C and read within a week. Test plates were scored as shown in table 3.1 using the system described in

the second international BoLA workshop (Anon., 1982).

Table 3.1: Scoring of Terasaki plates.

Score	% Killed cells
8	80-100
6	60-79
4	30-59
2	10-29
1	0-9

3.3 Generation of allogeneic immune responses

Immune responses against allogeneic MHC antigens in calves were produced either by repeated inoculation of uninfected leucocytes or by repeated skin implantation essentially as described by Pringnitz *et al.* (1982). For preparation of uninfected leucocytes, venous blood was collected in acid citrate dextrose (ACD, appendix, no. 5) and centrifuged at 800*g* for 15 min. Plasma was discarded and RBC from the pellet were lysed by addition of ammonium chloride lysis solution (appendix, no. 6). Cells were pelleted by centrifuging at 300*g* for 10 min. and washed twice with phosphate buffer saline (PBS) pH 7.2 (appendix, no. 7) by centrifugation at 100*g* for 10min. The cells were finally resuspended in PBS at $1x10^8$ per ml and inoculated subcutaneously (s/c) in the neck.

For skin implantation, skin from the donor was removed from the dorsal side of the base of the ear. The site was scrubbed with a disinfectant soap and shaved. About 3 ml of local anaesthetic (Lignavet, C. VET Ltd.) was injected s/c at the site which was then disinfected by spraying with 5% Hibitane (ICI). Skin was lifted with toothed forceps and cut with a scalpel to obtain a piece of tissue approximately one cm in diameter. Pressure was applied to the donor site to stop bleeding. The wound was sprayed with an antibiotic spray (Polybactrin powder spray, The Wellcome Foundation Ltd.) and usually healed without any complications within 2 weeks. The piece of donor skin was immediately transferred into a sterile universal tube containing PBS for transport to the recipient calves.

The recipient calves were implanted with the piece of donor skin in the middle of the neck. The implant site was prepared as described above for collection of skin from the donor. A subcutaneous pocket was created by a single stab with a scalpel. The donor skin was inserted into this pocket and the incision closed with a single suture and sprayed with an antibiotic spray. The incision healed within 2 weeks without complications. This was repeated usually after 4 weeks or later to boost the titre of anti MHC antibodies.

3.4 Generation and maintenance of cell lines and sporozoites

Techniques used for initiation and maintenance of *T. annulata* schizont infected cell lines, uninfected lymphoblastoid lines and *T. annulata* sporozoites are described in this section.

3.4.1 *Theileria annulata* isolates

The species and stocks of *T. annulata* used in this study are detailed in the relevant chapters. Three isolates of *T. annulata*; Gharb (Ouhelli, 1985), Hissar (Gill *et al.*, 1980) and Ankara (Schein, 1975) were used in this work. The parasite material used included cell lines, GUTS and cryopreserved tick stabilates.

3.4.2 Establishment of *T. annulata* cell lines

T. annulata infected cell lines were established *in vitro* as described by Brown (1983). The technique is briefly outlined here.

3.4.2.1 Isolation of peripheral blood mononuclear cells (PBM)

Venous blood was collected in vacutainers containing ACD and centrifuged over Ficoll-hypaque S.G. 1.077 (Lymphoprep) at 1500*g* for 25 min. at room temp. PBM were removed from the interface of plasma and Ficoll-hypaque, and washed in PBS by centrifugation at 300*g* for 10 min. The cells were further washed twice in PBS and once in RPMI-1640 by centrifugation at 100*g* for 10 min. Finally, the cells were resuspended in complete tissue culture (TC) medium (appendix, no. 8), counted and adjusted to the required cell concentration.

3.4.2.2 Ground up tick supernatant (GUTS)

The tick material was prepared and stored by staff at the Centre for Tropical Veterinary Medicine, Edinburgh and kindly provided as and when required. The procedure for obtaining a sterile preparation of GUTS as a source of sporozoite material (Brown, 1983) is briefly described below.

Adult *Hyalomma anatolicum anatolicum* ticks infected with *T. annulata* (Walker *et al.*, 1985) were fed for 3 days on rabbits' ears to stimulate maturation of sporozoites. Partially engorged ticks were removed and washed, once in 1% benzalkonium chloride (Roccal, Winthrop) and a further three times in 70% ethanol. They were then transferred to a sterile container and washed three times in warm Eagle's minimum essential medium (MEM) with Hanks salts, double strength antibiotics i.e. pencillin, 200 i.u. per ml and streptomycin 200 ug per ml (Gibco BRL), and 0.1 mg per ml nystatin (Gibco BRL). They were left for 10 min. in the fourth wash. The medium was discarded, and 2-5 ml of cold MEM with 3.5% bovine plasma albumin (BPA) (Fraction V, Sigma) containing antibiotics as described above was added to the ticks. Ticks were transferred to a sterile mortar and ground with a sterile pestle, with an aliquot of supernatant being removed, and measured quantities of MEM and 3.5% BPA solution (as described above) added. This was repeated until the required concentration of supernatant material (usually 4 t.e. per ml) was reached.

The supernatant was centrifuged for 5 min. at 100*g*. The supernatant (containing the sporozoites) was removed and filtered through a sterile 25mm or 47mm Millipore Swinnex filter with an AP prefilter and 8μ MF filter (Millipore Corporation). The sporozoite strength of the material was expressed as t.e. per ml. The quality of the preparation was assessed using Giemsa (Sigma) stained cytocentrifuge smears prepared to one t.e. per ml.

3.4.2.3 *In vitro* infection of bovine PBM with *T. annulata* sporozoites

This technique is described in detail by Brown (1983) and Brown (1987). PBM at $2x10^7$ cells in one ml TC medium were usually mixed with one ml of the sporozoite suspension (GUTS filtrate) at 1.0 t.e. per ml in a 25 cm^2 tissue culture flask (Intermed, Nunc). The flask was kept in a standing position in a CO_2 incubator at 37°C for two hrs. A further 8 ml of TC medium was added in the flask after two hrs and the flask was placed in the normal horizontal position in the incubator.

For establishing infected cell lines from cryopreserved GUTS, the sporozoite material was rapidly thawed from liquid nitrogen, kept at room temp. to equilibrate for 20 to 30 min. and serially diluted slowly with TC medium containing 20% foetal calf serum (FCS) by three doubling dilutions at 20 min. intervals. Diluted tick material (8 ml) was mixed with 2 ml of PBM at a cell concentration of $1x10^7$ per ml in a 25 cm^2 flask. The flask was kept in an upright position overnight in a CO_2

incubator at 37°C. The next morning, 7 ml of supernate were removed very slowly from the flask and replaced with 7 ml of fresh TC medium. The flask was then placed horizontally in the incubator. Depending upon the amount of tick material available and purpose for which cultures are intended, *T. annulata* cell lines can be generated with varying numbers of PBM and volumes of GUTS in different types of smaller well plates.

The cultures were incubated at 37°C in a 5% CO_2 humidified incubator (Scotlab VSL). The TC medium was subsequently changed at least twice a week according to the metabolic demands of the culture. Aliquots were removed at intervals for cytocentrifuge preparations to monitor infection and transformation rates. Once 30% or more of the cells were infected, the cultures were transferred to a larger culture flask (Intermed, Nunc). After transformation, cells were usually maintained at a concentration between 10^6 and $2x10^6$ per ml.

3.4.2.4 Establishment of parasite infected *in vivo* cell lines

Attempts were made to generate *T. annulata* infected cell lines from animals after cell line immunisation and sporozoite inoculation as described by Brown (1987). For establishing cell lines from lymph node biopsy material, a needle biopsy was collected from the lymph node in RPMI-1640 containing antibiotics (200 i.u./ml penicillin and 200 μg/ml streptomycin) and 10 i.u./ml heparin (Leo laboratories Ltd.). The material was transferred to a universal tube, pipetted vigorously and washed once in TC medium. The cell concentration was adjusted to $2x10^6$ per ml, layered in a 25 cm^2 flask and incubated in a CO_2 incubator at 37°C. For isolating parasite infected lines from blood and/or efferent lymph, PBM (3.4.2.1) and ELL (3.4.5), adjusted to $2x10^6$ cells per ml and seeded at 2 ml per well in 24 well plates. Cultures were monitored for the growth of infected cells as described above for *in vitro* lines.

3.4.3 Establishment of uninfected lymphoblastoid lines

Uninfected lymphoblasts were established by stimulating normal bovine PBM once with 2 μg/ml of concanavalin A (Con. A, Sigma type IV-S) in mixed lymphocyte culture (MLC) medium (appendix, no. 9), as described by Innes *et al.* (1989a). PBM were prepared as described above (3.4.2.1) and incubated with Con. A for 72 hrs in a 5% CO_2 incubator at 37°C. After three days, blast cells were separated by centrifugation over Ficoll-Hypaque solution S.G. 1.077 (Lymphoprep) for 25 min. at 1500*g*. Viable cells were gently pipetted from the interface,

resuspended in RPMI-1640 and centrifuged again at 300g for 10min. followed by another wash for 10 min. at 100*g*. The resultant blast cells were cultured at a concentration of 2.5 x 10^6/ml in MLC medium containing a supplement of 20% T-cell growth factor (TCGF). TCGF was prepared by collecting supernatant from a continuously growing gibbon lymphoid cell line, MLA 144 (Rabin *et al.*,1981; Henderson *et al.*,1983) which releases a factor biologically and chemically similar to human TCGF. The line was grown in 75 cm^2 culture flasks (Intermed, Nunc) using MLC medium. After 48 hrs, when the cells were in exponential growth phase, supernate was collected by centrifuging the cells at 1500*g* for 15 min., filtered through a 0.22μ filter and stored at -20°C in 20 ml aliquots. These cells were used as target cells in the cytotoxicity assays.

3.4.4 Cryopreservation of cell lines and sporozoites

Parasite infected lines, uninfected Con. A blast lines and GUTS were cryopreserved using the methods described by Brown (1983). The infected lines and Con. A uninfected lines were cryopreserved in 10% dimethyl sulphoxide (DMSO) (BDH biochemical). The cells were resuspended in RPMI-1640 supplemented with 50% FCS at a cell concentration of $2x10^7$ per ml and mixed with equal volumes of 20% DMSO in FCS. The final mixture was distributed in 1 ml aliquots and stored at -70°C for 24 hrs and then in liquid nitrogen until required. The GUTS were cryopreserved as a tick stabilate using 7.5% glycerol (BDH biochemicals) as the cryoprotectant in MEM supplemented with 3.5% BPA and stored in liquid nitrogen.

For the resuscitation of infected lines, the method described by Brown (1987) was followed. Vials containing frozen cell lines were thawed rapidly at 37°C and the DMSO was diluted by adding warm (37°C) medium and removed by centrifugation at 200*g* for 10 min. The cells were resuspended in fresh TC medium and incubated at 37°C. Sporozoite stabilates were also thawed rapidly in a 37°C water bath, and left at room temp. to allow equilibration over 30 min. At this stage they were used either undiluted or diluted slowly with MEM containing 3.5% BPA as described above (3.4.2.3) for challenge of animals or generation of *in vitro* parasite infected cell lines.

3.4.5 Preparation of efferent lymph lymphocytes (ELL)

ELL were collected from an indwelling cannula draining the efferent prescapular lymphatic duct. The technique of lymphatic cannulation is described below (3.5). Cells in efferent lymph were usually lymphocytes with less than 1%

contamination of neutrophils and eosinophils. However, at times the lymph contained varying numbers of RBC depending upon damage during surgery and haemorrhages in the lymph node at the later stages of infection with GUTS or *T. annulata* infected cell lines. Freshly collected ELL were washed twice with RPMI-1640 by centrifugation at 300*g* for 10 min. for preparation of cytospin smears. For all other techniques, ELL were purified on ficoll-hypaque as described for PBM above.

3.4.6 Preparation of cytocentrifuge smears

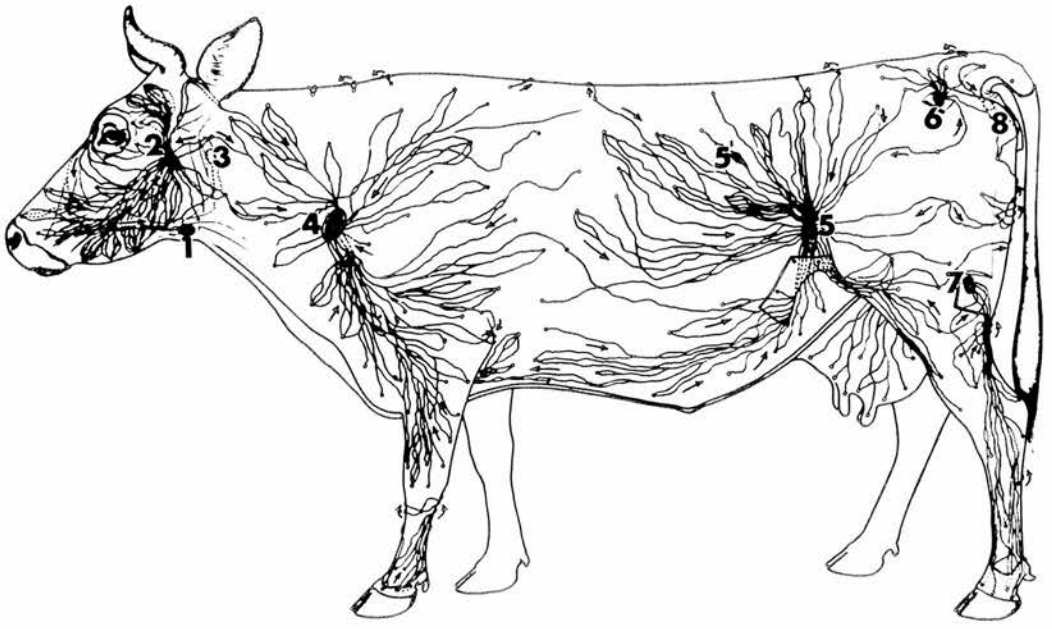
The morphology of ELL, uninfected Con. A blasts, *T. annulata* infected cells and sporozoites in GUTS were examined using Giemsa stained smears of cytocentrifuge preparations. These were prepared by spinning 100 μl aliquots of the material onto a clean glass microscope slides at 110*g* for 7 min. in a cytocentrifuge (Cytospin II, Shandon, Southern Instruments). The slides were first "primed" by 100 μl of RPMI-1640 (Gibco BRL) and centrifugation at 110*g* for 5 min. Cytocentrifuge smears were air dried, fixed for 5 min. in methanol (BDH) and stained for 40 min. with 1:10 dilution of Giemsa stain (Sigma) in Gurr buffer (BDH, one tablet dissolved in 1 litre of distilled water and pH adjusted to 7.2). The stained slides were rinsed in Gurr buffer, air dried and examined under an Ortholux II microscope (Leitz).

3.5 Surgical cannulation of the efferent prescapular lymphatic duct

Efferent lymphatic vessel of the prescapular lymph node (also known as the superficial cervical lymph node) of calves was cannulated to study development of immune responses in the lymph node draining the site of sporozoite infection or cell line immunisation. All the surgical operations were carried out by Dr. Roger Spooner.

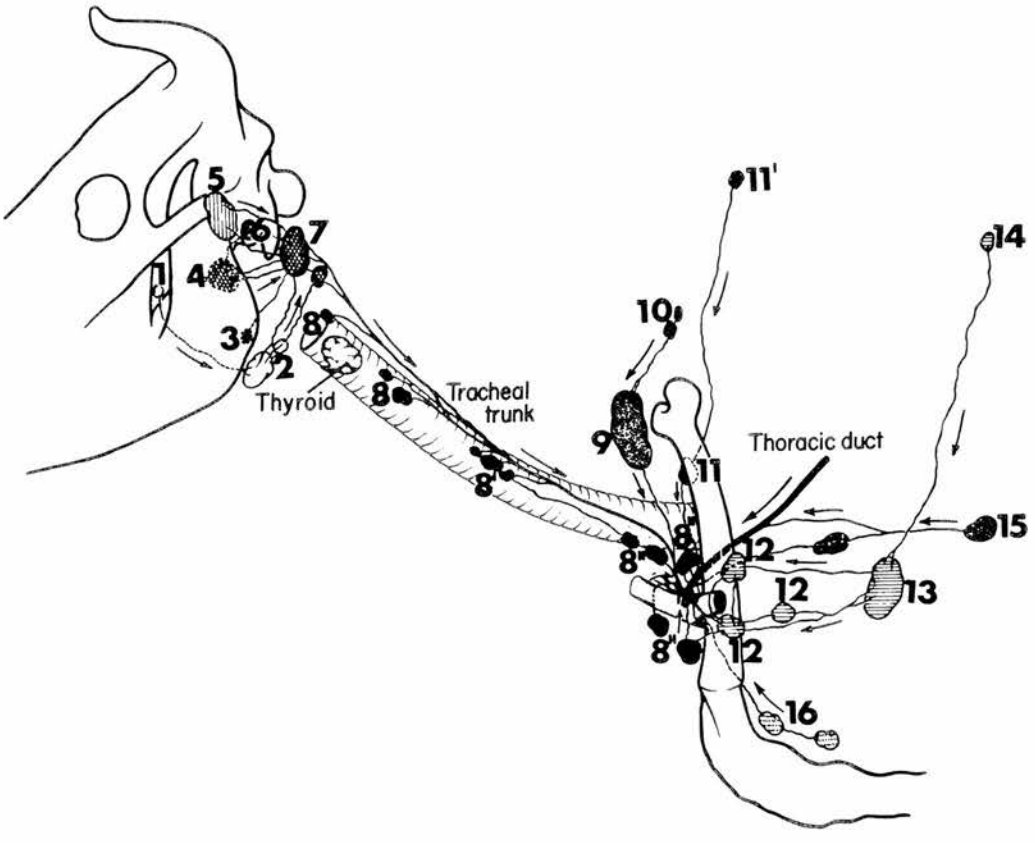
3.5.1 Anatomy of the prescapular lymph node

The anatomical position of the lymph node is shown in fig. 3.1 & 3.2, which have been adapted from, "Sisson and Grossman's the anatomy of the domestic animals" by Getty (1975). The lymph node is located laterally at the cranial border of the supraspinatus muscle on the scalenus muscle. The dorsal two thirds of the lymph node is covered by the omotransversarius and the ventral third by the bracheocephalicus muscle. The efferent lymphatic descends from the node over the scalenus muscle and opens on the right side into the right tracheal trunk. On the left side, it terminates in the thoracic duct or the left tracheal trunk (Saar & Getty, 1975)



1, Mandibular ln.; 2, parotid ln.; 3, lateral retropharyngeal ln.; 4, superficial cervical ln.; 5, subiliac ln.; 5', lnn. of paralumbar fossa; 6, gluteal ln.; 7, popliteal ln.; 8, tuberal ln.

Figure 3.1: Diagram showing the areas drained by afferent lymphatics to various superficial lymph nodes in cattle.
(Figure adapted from "Sisson and Grossman's The Anatomy of domestic Animals" Vol. 1, editor R. Getty, W.B. Saunders Co., 1975).



Lymph flow of the head, neck and shoulder region of the ox.

1, Pterygoid ln.; 2, mandibular ln.; 3, rostral hyoid ln.; 4, medial retropharyngeal ln.; 5, parotid ln.; 6, caudal hyoid ln.; 7, lateral retropharyngeal ln.; 8, cranial deep cervical ln.; 8', middle deep cervical ln.; 8", caudal deep cervical lnn.; 9, superficial cervical ln.; 10, accessory superficial cervical ln.; 11, costocervical ln.; 11', subrhomboid ln.; 12, axillary lnn. of first rib; 13, proper axillary ln.; 14, infraspinous ln.; 15, cranial mediastinal ln.; 16, cranial sternal ln

Figure 3.2: Schematic diagram showing lymph flow in head, neck and shoulder region of cattle.
(Figure adapted from "Sisson and Grossman's The Anatomy of domestic Animals" Vol. 1, editor R. Getty, W.B. Saunders Co., 1975).

as shown in fig. 3.3 & 3.4. The prescapular efferent lymphatic was cannulated with some modifications to the technique described originally by Lascelles & Morris (1961), Hall & Morris (1962) and Hall (1967) for sheep and later by Emery (1981b) and Emery *et al.* (1987) for cattle.

3.5.2 Preparation of cannulae for lymphatic cannulation

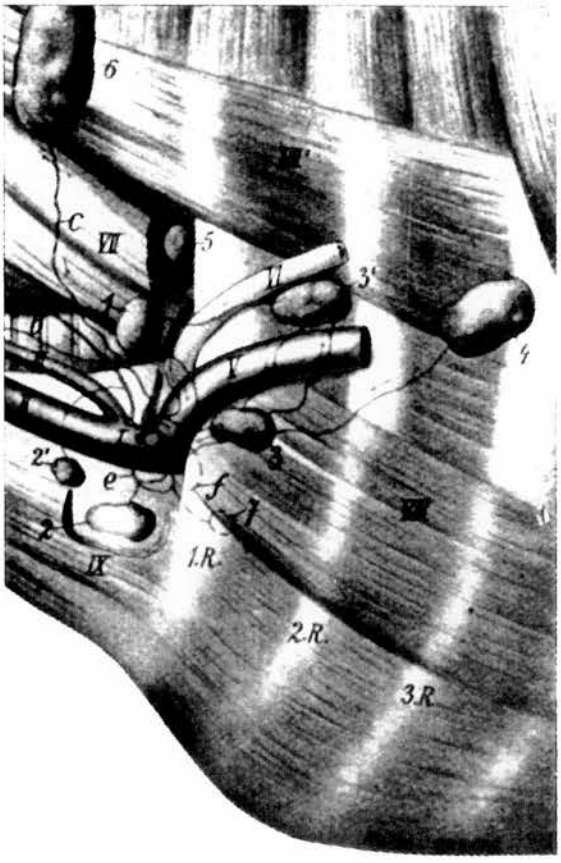
A polvinyl tube (Critchley Electrical Products Pty. Ltd., Australia) of 1.5 mm outer diameter and 1.0 mm internal diameter was used for the efferent cannulation. The cannula was impregnated with heparin before use to reduce clotting and allow better flow of lymph (Hopkins *et al.*, 1993). The tubing was thoroughly rinsed with acetone (BDH), filled with a 2% solution of 3-aminopropyltriethoxy silane (Sigma) in acetone and left for 5 min. It was then rinsed through again with acetone, drained and finally filled with heparin (5,000 i.u. per ml, Leo Laboratories Ltd.). Knots were tied at both ends of the tubing. It was wrapped in aluminium foil and stored at 4°C.

3.5.3 Presurgical procedures and anaesthesia

Animals were starved 24 hrs before surgery. Anaesthesia was induced by intravenous (i/v) injection of 10% thiopentone sodium (RMB Animal Health Ltd) at one ml per 10 Kg. body weight. The animals were intubated immediately using a cuffed endotracheal tube. Anaesthesia was maintained by a mixture of halothane, nitrous oxide and oxygen using a closed circuit anaesthetic machine (BOC Boyle International) fitted with a CO_2 absorber and rebreathing bag. The area around the prescapular lymph node was clipped and scrubbed. Two ml of a blue dye (Bleu patent V, Laboratoire Guerbet, Cedex) was injected s/c at a few different sites in the shoulder area drained by the prescapular lymph node (over the shoulder, above the node and just above and in front of the node) for easy identification of the efferent lymphatic. The operation site was disinfected with 5% Hibitane (ICI).

3.5.4 Surgical procedure

An incision was made parallel to and about 2.5 cm above the jugular vein, directly below the node. The thin layer of the bracheocephalicus muscle was divided by blunt dissection and held open with retractors. Only by digital dissection, the area under the muscle was exposed towards the rear area of fat. The efferent lymphatic follows the anterior surface of fat surrounding the artery and vein which serves the node and skin above it. The duct shows up as blue 10-30 min. after the dye has been injected. The blue dye aids in identification of the duct but is not essential (fig. 3.5).

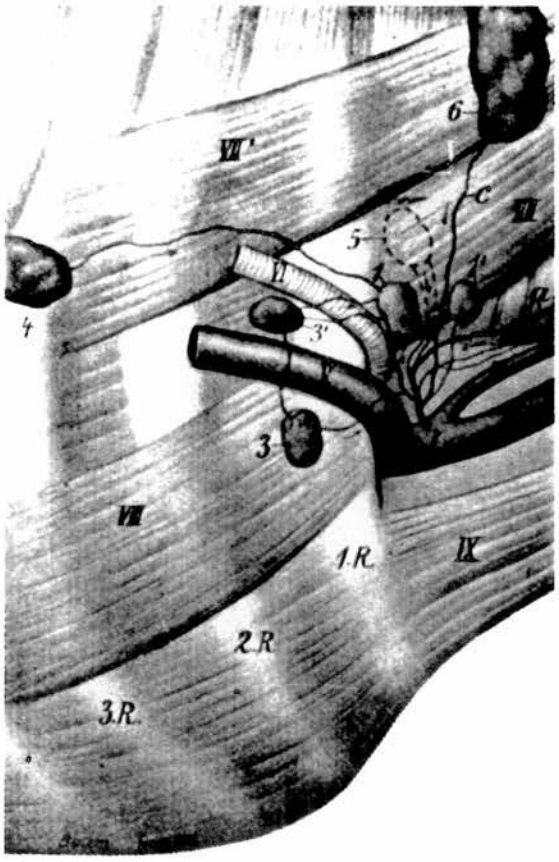


Termination of thoracic duct of ox and left tracheal trunk, left view.

a, Thoracic duct; a', termination of a; b, left tracheal trunk; c, efferents from superficial cervical ln.; e, communicating branch from right tracheal trunk; f, efferents from cranial mediastinal ln.; 1, 2, 2', caudal deep cervical lnn.; 3, 3', axillary lnn. of first rib; 4, proper axillary ln.; 5, costocervical ln.; 6, superficial cervical ln.; 7, cranial sternal ln.; I, common jugular v.; II, left external jugular v.; III, left internal jugular v.; V, left brachial v.; VI, left brachial a.; VII, ventral, and VII', scalenus dorsalis; VIII, rectus thoracis; IX, sternocephalicus; 1.R., 2.R., 3.R. = 1st, 2nd, 3rd ribs.

Figure 3.3: Route of efferent lymphatic from prescapular lymph node of cattle (left side).

(Figures adapted from "Sisson and Grossman's The Anatomy of domestic Animals" Vol. 1, editor R. Getty, W.B. Saunders Co., 1975).



Termination of right tracheal trunk of ox, right view.

a, Right tracheal trunk; c, efferents from superficial cervical ln.; 1, 1', caudal deep cervical lnn.; 3,3', axillary lnn. of first rib; 4, proper axillary ln.; 5, costocervical ln.; 6, superficial cervical ln.; I, common jugular v.; III, right internal jugular v.; IV right external jugular v.; V, right brachial v.; VI, right brachial a.. II, ventral and, VII', scalenus dorsalis; VIII, rectus thoracis; IX, sternocephalicus; 1.R., 2.R., 3.R., = 1st, 2nd, 3rd ribs.

Figure 3.4: Route of efferent lymphatic from prescapular lymph node of cattle on the right side.

(Figures adapted from "Sisson and Grossman's The Anatomy of domestic Animals" Vol. 1, editor R. Getty, W.B. Saunders Co., 1975).

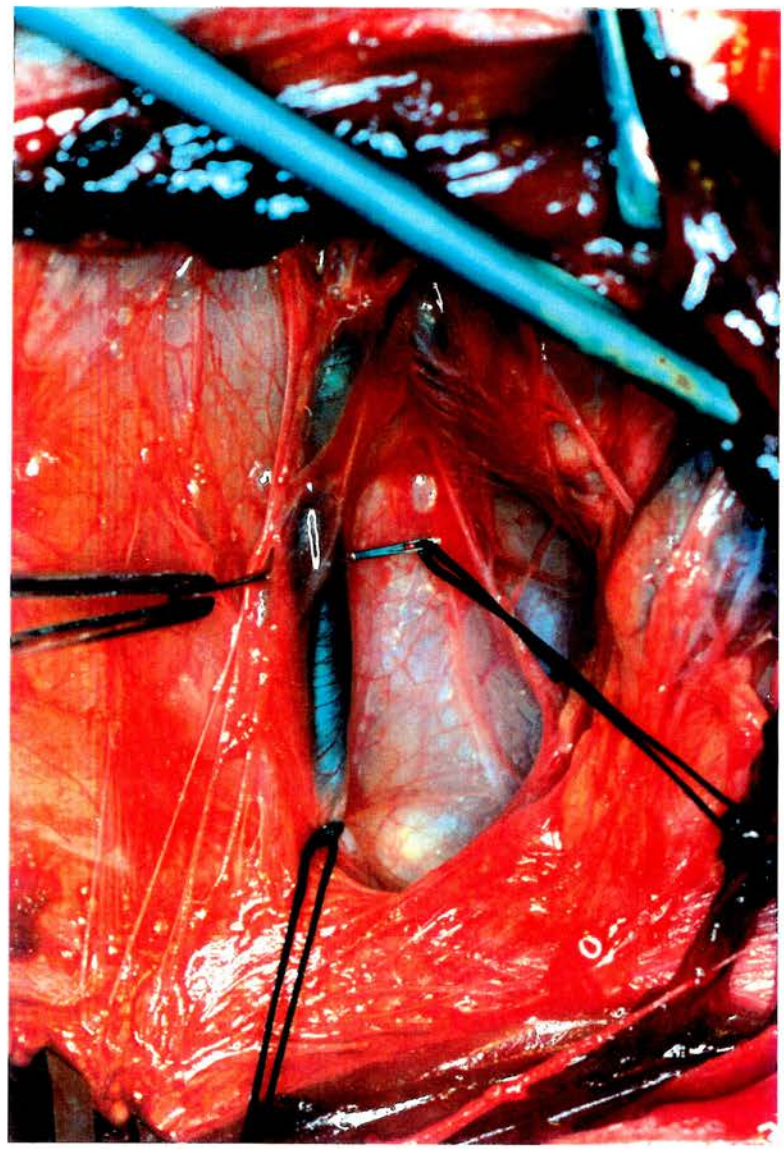


Figure 3.5: Cannulation of efferent lymphatic of prescapular lymph node.

About 2 cm of the duct was exposed but not separated from the fat on which it rests. A braided silk tie was made at the lower end of the exposed vessel, pulled tight and anchored with a haemostat. The vessel then filled up and was easier to see. A further tie was positioned about 1.5 cm nearer the node and the first throw was made but was not pulled tight. The heparin impregnated tubing immersed in alcohol was rinsed with sterile PBS and passed s/c under the skin from the incision site to approximately middle of the neck using a disposable catheter passer (Codman) by making an incision to exteriorise the catheter. The cannula at the lymphatic end was cut at 45° and positioned for introduction into the vessel. The lymphatic duct was then nicked using spring scissors (size 15 cm, Interfocus Ltd.) and a lachrymal probe (Arnolds Vet Products) of appropriate size inserted into the duct towards the node. If valves were encountered, attempts were made to break these down with the probe for providing at least 1 cm of free vessel above the top tie. The cannula was inserted into the vessel gently up to the valve and then withdrawn slightly to keep it clear of the valve and the top knot tied tight. The flow of lymph through the cannula was checked. Fluid level should fall by lifting the free end of the cannula as the fluid presses back against the valve. When the cannula is dropped below the incision site lymph should flow. The cannula was slightly repositioned if it was not flowing well. It was further secured using the original occluding tie. In addition a half hitch of braided silk was tied round the cannula as it emerged from the muscle and attached to the subcutaneous fascia.

The wound was repaired by using continuous sutures (mersilk 2/0 size, W193, Ethicon) for the muscle layer and mattress sutures (Supramid, 4 metric, B. Braun Melsungen AG) for the skin. The point where the cannula exited the skin, was secured with a purse string suture. A small piece of adhesive insulation tape (about 5 cm) was stuck to the cannula at the point where it came out of the skin and stuck back on itself to provide a tag which was sutured to the skin and served as an anchor to the cannula.

3.5.5 Post-operative procedures

An indwelling jugular cannula was inserted and secured for infusion of lymph back into the animal to avoid loss of fluid, electrolytes and proteins, so as not to interfere in the development of immune responses and for simplifying blood sampling during the course of experiments. A plastic collar was placed around the animals neck

onto which a plastic bottle for collection of the lymph was tied. The collar, bottle and the cannulae were covered with an expanding netting (No. 6, Surgifix, manufactured by FRA, Italy; supplied by Vet Drug Co.) anchored distally to a surcingle. The animal was moved to the recovery area and allowed to recover. An injection of long acting penicillin (Duphapen LA, Solvey duphar Veterinary) was given via a deep intramuscular (i/m) route to avoid any secondary infection. Once the animal regained consiousness, a sterile bottle was attached to the collar for collection of lymph as shown in fig 3.6. Heparin (Leo laboratories ltd.) to provide 10 i.u. per ml and antibiotics (Gibco BRL) to provide penicillin 20 i.u. and streptomycin 20 μg per ml based on filled volume were added in the bottle before use.

3.5.6 Monitoring of animals after cannulation

The bottle for lymph collection was changed 2-4 times a day depending upon the rate of flow of lymph. The size of the bottle to be attached was decided based on the rate of lymph flow and size of the animal. The volume of lymph collected was measured with every bottle change and used to calculate the hourly flow rate. A small sample was taken for cell count using trypan blue exclusion and a Neubauer haemocytometer. The lymph was collected for one hr every morning for use in various studies. Remaining lymph was pooled aseptically in 500 ml bottles and infused back slowly into the animal through the jugular cannula. The jugular cannula was flushed at least twice a day with heparinised saline {500 ml pack of sterile 0.9% Sodium Chloride intravenous infusion BP (Baxter Health Care Ltd.) containing 10,000 i.u. of heparin} to prevent clotting. The same cannula was used for collecting blood samples as and when required.

3.6 Clinical observations on infected cattle

Animal experimentation included skin grafting, efferent lymph cannulation, infection by subcutaneous inoculation in the prescapular area with either *T. annulata* macroschizont infected cells or cryopreserved sporozoites. Details of these experiments are given in the appropriate chapters. Observations on clinical, parasitological and haematological parameters were undertaken throughout the experiments.

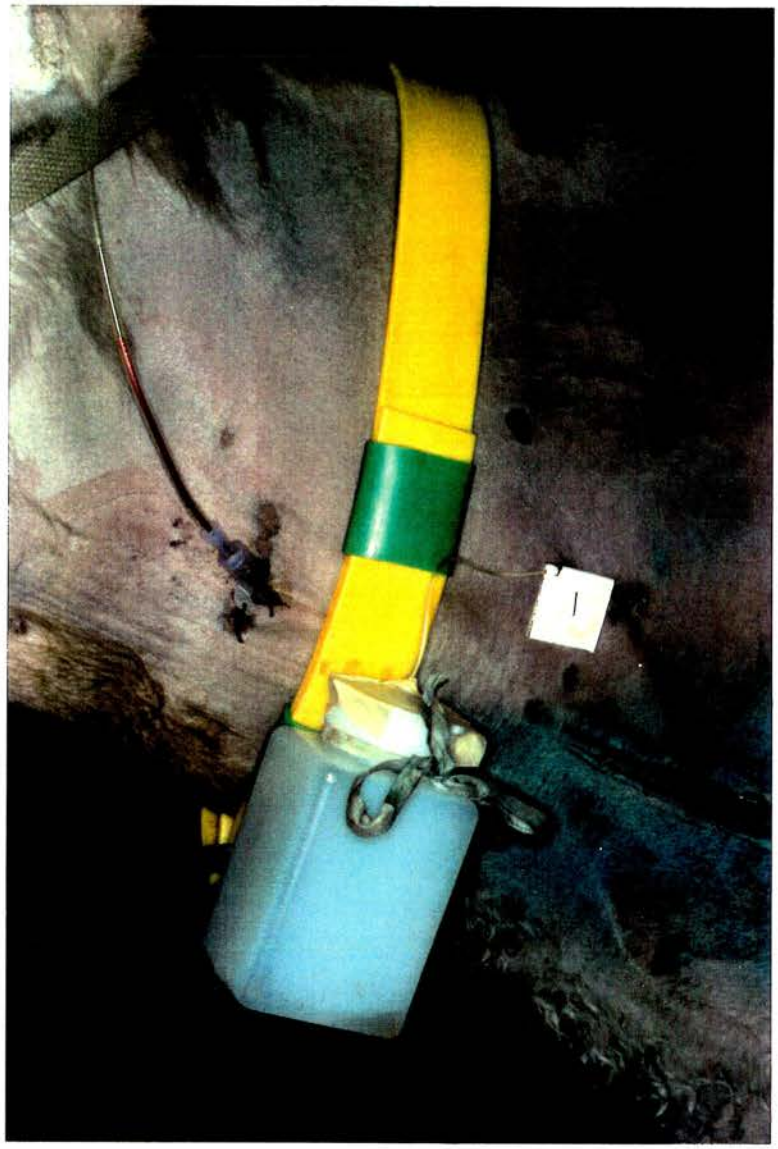


Figure 3.6: Position of cannula and collecting bottle on the animal.

3.6.1 Clinical parameters

Rectal temp. was recorded daily after efferent cannulation and cell line or sporozoite inoculation, and $\geq 39.5^{\circ}C$ was considered a febrile response. The general state of the animals, enlargement of the lymph nodes and colour of mucous membranes were also recorded.

3.6.2 Haematological parameters

Venous blood was collected in 2 ml vacutainers containing disodium EDTA (Becton Dickinson) for haematological examinations. Packed cell volumes (PCV), total erythrocyte counts (TEC) and total leucocyte counts (TLC) were routinely measured usually twice a week after cell line immunisation and sporozoite inoculation. PCV was estimated by a microhaematocrit technique. Blood was drawn in microcapillary tubes, one end was sealed with plasticine and the capillaries were centrifuged at 12,000 rpm for 15 min. in a microhaemotocrit centrifuge (Biofuge, Heraeus Sepatech). PCV was determined using a microhaematocrit reader. TEC and TLC were measured using a coulter counter (Coulter ZBI electronic particle counter, Coulter Electronics Ltd). Blood smears were prepared on glass slides, fixed with methanol and stained with Giemsa stain. These smears were used for differential leucocyte counting (DLC). A total of 200 cells were counted to calculate percent neutrophils, eosinophils, basophils, lymphocytes and monocytes in the blood.

3.6.3 Parasitological parameters

Both blood and lymph node smears were examined for evidence of the parasite in experimentally infected cattle after cell line immunisation and sporozoite inoculation. Thin blood smears were prepared by spreading a small drop of freshly obtained venous blood onto a clear glass slide, using the edge of a second slide, followed by rapid air drying. Lymph node smears were prepared from needle biopsies of infected nodes. This involved insertion of a 20G sterile disposable needle into the lymph nodes where it was rotated several times and withdrawn following closure of the syringe attachment part of the needle with a finger or thumb. The material within the needle was then transferred onto a clean glass slide and was spread and dried as described above for the blood smear. The smears were fixed in methanol for 5 min. and stained with Giemsa stain (1:10 dilution in Gurr buffer) for 40 min. The slides were then rinsed in Gurr buffer and dried prior to microscopic examination.

3.7 Serological observations

Sera samples were collected at weekly intervals from animals after inoculation of uninfected leucocytes, skin grafting, cell line immunisation and sporozoite inoculation and were used to determine humoral antibody responses. Antibody titres against allogeneic MHC antigens after inoculation of uninfected leucocytes, skin grafting and cell line immunisation were measured by microlymphocytotoxicity as described above for BoLA typing (3.2.3) except that serial dilutions of unknown sera were added in Terasaki plates and tested against PBM of known BoLA types (Kachani & Spooner, 1992). Antibody titres against *T. annulata* were measured by an indirect fluorescent antibody test as described below.

3.7.1 The indirect fluorescent antibody test (IFAT)

IFAT was carried out to detect levels of anti-macroschizont or anti-piroplasm antibodies in sera taken from cattle before and after inoculation of *T. annulata* infected cell lines and sporozoite challenge using the method described by Burridge and Kimber (1972). Macroschizont antigens were prepared from a *T.annulata* (Gharb) cell line propagated *in vitro* as described by Burridge and Kimber (1972). Briefly, the cells were washed twice in sterile PBS and once in PBS supplemented with 1% BPA. The cell pellet was resuspended in three times its volume of PBS/BPA. Multiwell glass slides (Flow Laboratories) were coated with this cell suspension by applying 20 μl to each well and then immediately removing it, leaving a thin film of cells on the slide. The slides were quickly dried and fixed in acetone (BDH) for 15 min., air dried and stored at -20°C until used.

Piroplasm antigen was prepared according to the method of Burridge (1971). Briefly, 5 ml venous blood was collected from a calf infected with *T. annulata* (Ankara) showing 20% parasitaemia into a bottle already containing 150-200 ml sterile chilled PBS. It was centrifuged at 400*g* for 10 min. at 4°C. The supernatant and buffy coat were discarded, the erythrocyte pellet was resuspended again in 100 ml PBS and centrifuged as above. The supernate was discarded, pellet was resuspended in 5 ml of 1% BPA in PBS and spun again as above. Finally the pellet was resuspended in 2 volumes of 1% BPA in PBS to 1 volume of erythrocytes. Thin blood smear slides were prepared, air dried and fixed in acetone (BDH). The antigen slides were stored at -20°C until used.

For the test, antigen slides were removed from -20°C and held at 4°C for 30 min. and then at room temp. for a further 30 min. The requisite number of wells were drawn on the piroplasm antigen slides with a diamond pencil. Schizont antigen slides were rinsed three times and piroplasm antigen slides four times in PBS and air dried. Meanwhile four fold dilutions of the sera to be tested were prepared in PBS. The antigen slides were placed in a humidity chamber. Samples (5μl) were pipetted onto designated wells on the antigen slides and incubated for 30 min. at room temp. The slides were washed three times in PBS and dried. A drop of fluorescein isothiocynate (FITC) labelled rabbit anti-bovine (RAB) IgG (whole molecule) conjugate (Nordic) at 1:80 dilution was applied to each well on the slides and incubated for a further 30 min. in the dark. These were washed x3 with PBS and dried. The slides were mounted using a 66% solution of glycerol (BDH) in Tris (BDH) buffer and examined under an ultraviolet epi-illuminated fluorescent microscope (Ortholux II, Leitz) using a 64x oil immersion objective. The highest dilution of the test sera producing a positive fluorescence was recorded.

3.8 Phenotypic analysis of PBM and ELL using flow cytometry

Flow cytometry is an analytical technique which permits accurate quantitation of various parameters of individual cells (size, complexity and fluorescence) in a population of continuously flowing fluorochrome labelled cells. In this work, flow cytometric analysis was performed with a FACScan (Becton Dickinson, Mountain View, CA, U.S.A.). The equipment has a single argon laser which produces a beam of 488 nm wavelength. As the cell passes through the focussed laser beam, it scatters laser light and the fluorescent molecules on its surface are excited to fluoresce. A forward light scatter (FSC) detector collects light at relatively small angles and converts it to an electronic signal which is directly proportional to size of the cell. Signals collected at large angles (90°) gives information on fine internal structure and granularity and termed as side scatter (SSC). Fluorochrome bound to the cell surface absorbs the laser light and emits a light of longer wavelenght. FITC emits light at 530 nm and phycoerythrin (PE) at 580 nm. Fluorescent light is collected by a lens and the different colours produced by different dyes are directed to separate photomultiplier (PMT) detectors. Data on these parameters can be stored and analysed in a computer.

3.8.1 Bovine leucocyte specific monoclonal antibodies

Monoclonal antibodies which react with distinct bovine leucocyte sub-populations were used in this study. The putative specificities of these antibodies, characterised by their cellular distribution and function within the bovine immune system have been tested in two international bovine leucocyte antigen workshops (Howard *et al.*, 1991a; Howard and Naessens, 1993). A summary of the mAbs and the cell surface markers they detect is given in table 3.2.

Table 3.2: List of monoclonal antibodies used in this study.

mAb	Isotype	Specificity	Working dilution
IL-A19	IgG_{2a}	MHC class I	1:1000
IL-A12	IgG_{2a}	CD4	1:1000
IL-A51	IgG_1	CD8	1:500
SBU-T8	IgG_{2a}	CD8	1:1000
IL-A26	IgM	CD2	1:1000
MM1A	IgG_1	CD3	1:100
IL-A29	IgG_1	$\gamma\delta$ T cells	1:1000
CC 15	IgG_1	$\gamma\delta$ T cells	1:1000
IL-A111	IgG_1	CD25	1:1000
CC 76	IgG_1	CD 45RB	1:2000
VPM-30	IgM	Pan B cells	1:1000
IL-A30	IgG_1	Surface IgM	1:1000
IL-A24	IgG_1	Macrophages	1:1000
IL-A109	IgM	Monocytes	1:1500
J11	IgG_1	MHC class II	1:1000
IL-A21	IgG_{2a}	MHC class II	1:1500

The mAb IL-A19 is specific for MHC class I antigens on cell surface (Bensaid *et al.*, 1989), therefore this mAb was used as a positive control in all tests because all viable cells should express MHC I on the cell surface. The mAb IL-A12 identifies a distinct subpopulation of T lymphocytes, bearing the bovine CD4 molecule (Baldwin *et al.*, 1986; Teale *et al.*, 1986). The mabs IL-A51 (Ellis *et al.*, 1986) and SBU-T8 (Maddox *et al.*, 1985) recognise bovine CD8. The mAb IL-A26 is specific for bovine

CD2 (Baldwin *et al.*, 1988b) and mAb MM1A recognises bovine CD3 (Davis *et al.*, 1993). $WC1^+$ $\gamma\delta$ T cells are recognised by mAbs IL-A29 (Morrison *et al.*, 1988) and CC 15 (Clevers *et al.*, 1990). The mAb IL-A111 is a marker for the α chain of the interleukin-2 receptor (IL-2Rα) also known as bovine CD 25 (Tac antigen) on the cell surface (Naessens *et al.*, 1992). The mAb CC 76 binds to the CD45RB molecule on the cell surface (Howard *et al.*, 1991b). Bovine B cells expressing IgM on the surface are recognised by mAb IL-A30 (Naessens *et al.*, 1988). The mAb VPM 30 is specific for B cells (J. Hopkins, pers. comm.; Naessens & Howard, 1991). The mAb IL-A24 recognises a molecule present on bovine monocytes/macrophages associated with antigen presentation (Ellis *et al.*, 1988). The mAb IL-A109 is thought to recognise an antigen analogous to CD64 (FcR1) on monocytes and macrophages (MacHugh *et al.*, 1990). The mAb J11 and IL-A21 recognise a monomorphic determinant on bovine MHC class II molecules (Baldwin *et al.*, 1988c). All mAbs were titrated for optimal working dilutions before use.

3.8.2 Immunoconjugates

A list of various immunoconjugates used as secondary antibodies is given in table 3.3. All conjugates were titrated before use to assess their optimal working dilution.

Table 3.3: Secondary immunoconjugates.

Antibody	Conjugated dye	Specificity	Working dilution
Rabbit anti-mouse Ig (Nordic)	FITC	Polyvalent	1:100
Goat anti-mouse Ig (Sigma)	FITC	IgM	1:100
Goat anti-mouse Ig (Sigma)	PE	IgG	1:100
Goat anti-mouse Ig (Seralab)	FITC	IgG_1	1:200
Goat anti-mouse Ig (Seralab)	PE	IgG_{2a}	1:250

3.8.3 Protocols for Indirect immunofluorescence staining

An indirect immunofluorescence test was used to stain cells for analysis on the FACScan essentially as described by Spooner *et al.* (1988) and Glass & Spooner (1989). For single colour analysis, $5x10^5$ cells in 50 μl of FACS medium {RPMI 1640 with 25 mM Hepes and 5% γ globulin-free horse serum, (Gibco BRL) and 0.1% sodium azide (Sigma)} were mixed with 50 μl of the mAb at a predetermined

optimal dilution and incubated for 30 min. at 4°C on ice in a 96 well round-bottomed culture plate (Nunc). The cells were washed twice in cell wash (Becton Dickinson) by repeated suspension and centrifugation for three min. at 100*g* at 4°C. Supernatant was removed, 25 μl of FITC conjugated rabbit anti-mouse (RAM) Ig (whole molecule, Nordic) at a dilution of 1:100 in FACS medium was added to each well, and the test plate was incubated for 30 min. on ice at 4°C. Plates were covered with tin foil to keep the cells in dark. The cells were washed three times in cell wash as described above and finally resuspended in 100 μl cell wash and either analysed within 3 hrs or stored in the dark at 4°C. The cells were preserved by addition of 100 μl of 1% paraformaldehyde (Sigma) solution in cell wash to each well. The stored cells were analysed within 2 weeks. A negative control sample was included which comprised either cells in medium with no mAb or 50 μl of normal mouse serum (NMS, Seralab) at 1:500 dilution in place of mAb for the first incubation. The second incubation was with the same fluorochrome as used for staining positive cells.

For two colour flow cytometry, cells were dispensed as above and incubated simultaneously with 25 μl each of two mAbs of different isotypes at half of their working dilution. Incubation and washings were done as explained above for single colour staining. This was followed by simultaneous incubation with 25 μl of two appropriate secondary fluorescent reagents, at working dilutions, depending upon the isotypes of primary antibodies. These were two of: 1.Goat anti-mouse (GAM) IgM-FITC (Sigma); 2.GAM IgG-PE (Sigma); 3.GAM IgG_1-FITC (Seralab); 4.GAM IgG_{2a}-PE (Seralab). One conjugate was FITC for green fluorescence and the second was PE for red fluorescence.

3.8.4 Flow cytometry acquisition and analysis

The "lysys II" programme (Becton Dickinson) was used in the acquisition and storage of both the single and two colour flow cytometric parameters of the labelled cells. The "lysys II" and "PC lysys" programmes (Becton Dickinson) were used for analysis of the data.

For single colour parameters, the cytometer configurations were calibrated and set using negative control cells stained with NMS and the appropriate immunoconjugates. FITC fluorescence (530 nm) emission was detected with the PMT voltage set at 600-620 mV (FL1, 1024 channels) as logarithmic (log) amplification scale. FSC and SSC (90°) amplifications were linear and set at EOO (unamplified)

and 400mV, respectively. Using a FSC versus SSC dot plot, lymphocytes were live gated for acquisition and analysis while cell debris, dead cells and RBC were excluded (fig. 3.7a & 3.7b). There were no granulocytes in these cell preparations as the cells were separated on a density gradient (lymphoprep, S.G. 1077) before staining. For acquisition, cell samples were run at approximately 500 cells per second (sec.) and normally data on 5,000 or 10,000 cells were recorded. Data was plotted either as frequency histograms with log fluorescent intensity presented on the x-axis and frequency of cell numbers presented on the y-axis (fig. 3.7c) or as dot plots with log fluorescent intensity presented on the x-axis and FSC on the y-axis (fig. 3.7d).

For two colour parameters, the cytometer configurations were calibrated and set as described above using the negative control cells stained with NMS and appropriate secondary conjugates. The cells were live gated by using a dot plot of FSC verses SSC. The optimum FITC (FL1) and PE (FL2) levels were adjusted to bring the cells to the lower left corner of the box (fig. 3.8a). These optimal PMT voltages were found to be 600-620 mV and 520-540 mV for FL1 and FL2, respectively and both were in the log amplification using 1024 channels.

To compensate for the unwanted spectral overlap of emitted light from the FITC and PE dyes used (fig. 3.8b), the FL1-% FL2 PMT voltage was set between 0.5-1.5% to remove PE signal from FL1 region, and the FL2-% FL1 PMT voltage was set between 30-50% to remove FITC signal from the FL2 region. These settings were done for every test with cells stained with only one primary mAb but both secondary conjugates. Sometimes these settings were slightly different for different mAbs and, therefore, changed accordingly between samples. The data was analysed using dot plots with log FL1 (FITC) intensity on the x-axis and log FL2 (PE) intensity on the y-axis. For statistical analysis, the dot plots were set into 4 calculation regions by setting quadrant markers (fig. 3.8c). Figure 3.8c represents a B cells FITC (FL1) versus CD4 PE (FL2) plot in which quadrant 1 represents CD4 positive cells only, quadrant 2 represents CD4/B cells dual positives, quadrant 3 is the negative population for both primary antibodies while quadrant 4 is the B cells.

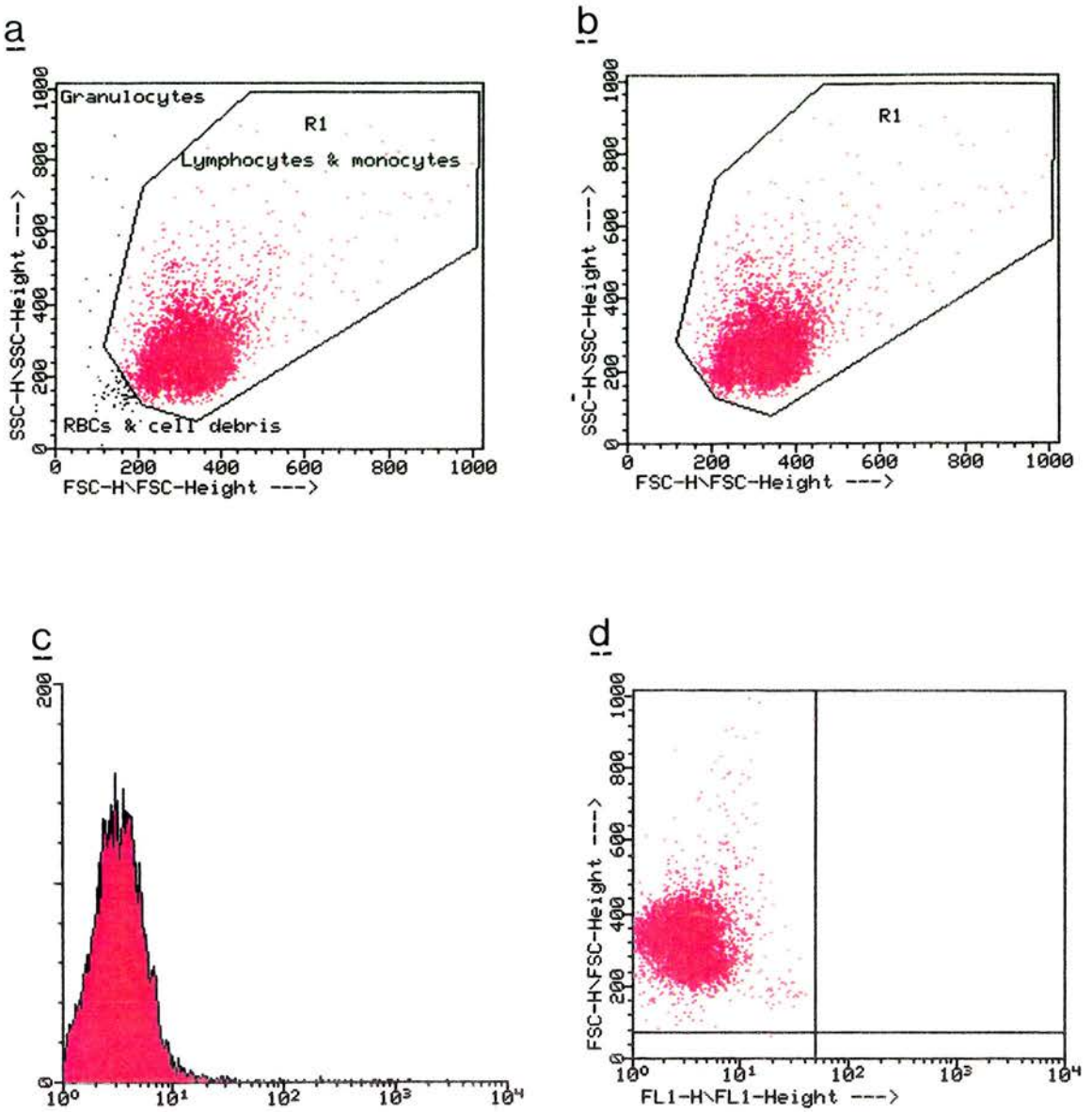


Figure 3.7: Flow cytometry display of cells for live gating during acquisition using forward and side scatter (FSC vs SSC).

a: Dot plot display (ungated) of a negative control sample showing the distribution of various cellular components of blood based on their physical characteristics.

b: Exclusion of dead cells and RBC by live gating during acquisition.

c: Frequency histogram of live gated cells.

d: Dot plot display of live gated cells with log fluorescence on x-axis and FSC on y-axis.

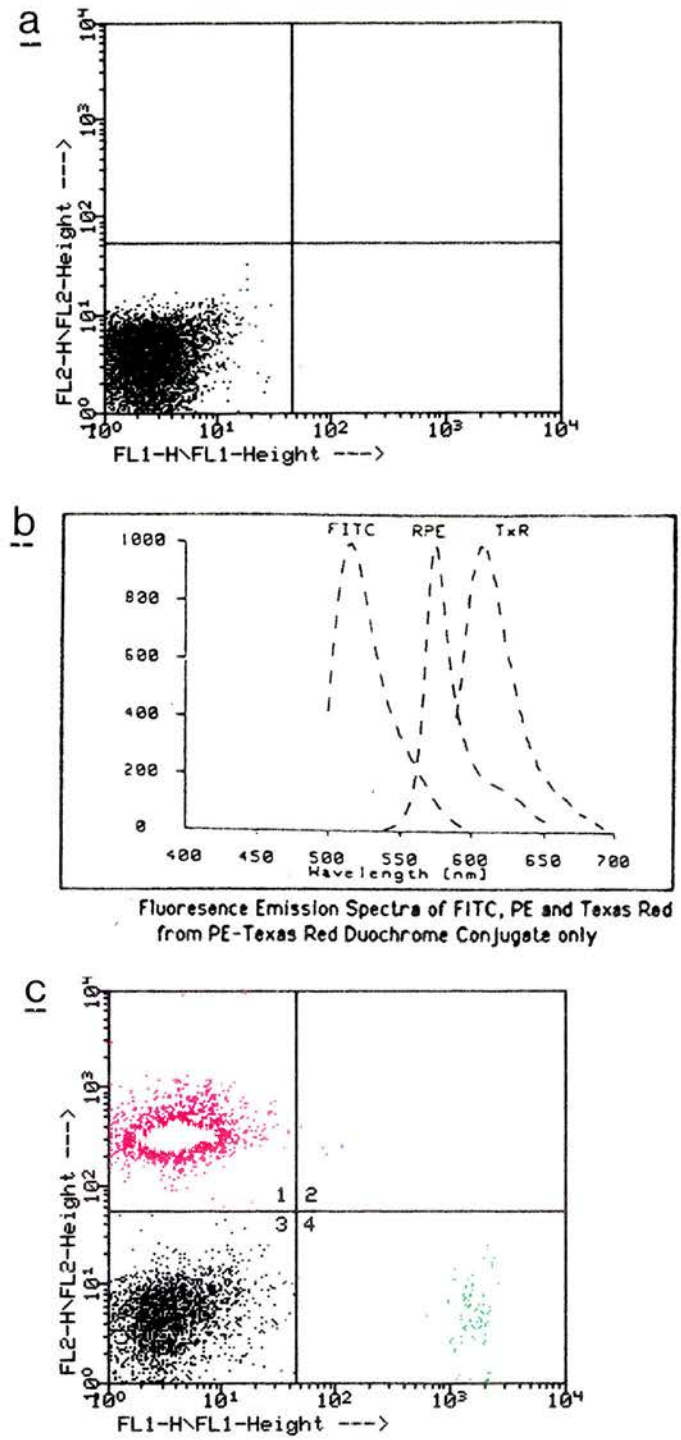


Figure 3.8: Flow cytometry display of cells for two colour analysis.

a: Dot plot display of the negative control sample adjusted for optimal FITC and PE voltage levels

b: Figure showing spectral overlap from emmision of FITC and PE. (Adapted from the working manual of the FACScan, Becton Dickinson).

c: Two colour immunofluorescence dot plot display of cells stained for B cell antibody (FL1) and CD4 antibody (FL2). quadrant 1: CD4+ cells, 2: CD4+ B cells (double positive), 3: double negative (CD4- Bcells), 4: B cells.

3.9 Cytotoxicity Assay:

A standard 4 hr chromium release assay was used to assess cytotoxic activity of effector cells using the technique previously described in detail by Teale *et al.* (1985); Spooner *et al.*, 1987 and Innes *et al.*(1989a).

3.9.1 Effector cells

Cytotoxic activity of ELL and PBM from the animals was assayed at regular intervals after cell line immunisation and sporozoite inoculation. The cells were prepared by centrifugation of blood or efferent lymph on ficoll-hypaque as described above. ELL and PBM stimulated for 5 days with irradiated autologous *T. annulata* infected cells or infected donor immunising cells were also used as effector cells in some experiments. The effector cells were finally resuspended in TC medium. The FCS used in the medium was from a batch that had previously been screened to give low levels of spontaneous ^{51}Cr release from the target cells.

3.9.2 Target cells

Both *T. annulata* infected target cells and uninfected blasts in exponential growth phase were chosen for use as target cells. The cells were washed once in TC medium by centrifugation at 300*g* for 10 min. and resuspended in the same medium at a concentration of $2x10^7$ cells per ml. An equal volume of ^{51}Cr at a concentration of 1 mCi per ml in TC medium (as sodium chromate, Amersham International Ltd.) was added to each target cell suspension. The cells were incubated for 60 min. at 37°C and mixed gently a few times during incubation. Following incubation the labelled cells were washed three times in TC medium (300*g* for 5 min.), after which they were resuspended to give a final cell concentration (based on prelabelling counts) of $1x10^6$ cells per ml. The cytotoxicity assay was performed by adding 25μl (2.5 x 10^4 cells) of the labelled target cell suspension to 100 μl of effector cell suspension in 96-well round-bottomed tissue culture plates (Intermed, Nunc) in duplicate. The concentration of effector cells was varied to give different effector:target ratios i.e 1 $x10^7$ cells per ml for a 40:1 ratio, 5 x 10^6 cells per ml for 20:1 ratio, 2.5 x 10^6 cells per ml for 10:1 ratio and so on. The test plates were incubated for 4 hrs in a 5% CO_2 incubator at 37°C. The contents of each well were individually mixed followed by centrifugation at 170*g* for 5 min. A 25 μl of the supernatant from each well was removed and placed in a disposable 96 well assay plate (Falcon). 100 μl of scintillant (Optiphase safe, LKB Scintillation products) was added to each well. The plates were

sealed with a plate seal (Wallac) and counted in a scintilation counter (Microbeta, Wallac). The percentage cytotoxicity was calculated as:

$$\text{Specific cell lysis (\%)} = \frac{\text{Test release-Spontaneous release}}{\text{Maximum release-Spontaneous release}} \times 100.$$

Spontaneous release of ^{51}Cr from the target cells was measured by incubating 25 μl of target cells with 100 μl of assay medium alone and maximum release was evaluated by mixing 25 μl of target cells with 100 μl of 1% sodium dodecyl sulphate (SDS, Sigma) solution for lysis of all the cells. The spontaneous and maximum release controls were carried out in triplicate wells.

3.10 Proliferation assays

These were performed essentially as described by Glass and Spooner (1990a). Briefly, ELL or PBM (1 x 10^5/well in 50 μl) were used as responders and stimulated by the following (100 μl) in quadriplicate wells: a. Con. A at 4 μg/ml; b. Human recombinant IL-2 (Boehringer Manheim) at 40 i.u./ ml; c. irradiated autologous *T. annulata* infected cells (1x10^4/well); d. irradiated *T. annulata* infected immunising cells (1x10^4/well). Irradiated autologous PBM (1x10^5 /well in 50 μl) were added to provide the antigen presenting cells. PBM were irradiated at 5000 rads, whereas *T. annulata* infected cells were irradiated at 7500 rads. The responder cells with irradiated PBM alone were used as negative control to see baseline proliferation without any stimuli. The test was performed in 96 well flat bottom plates (Inter med, Nunc) in a final volume of 200 μl per well. Cell proliferation was measured after 72 hr of incubation at 37°C in a CO_2 incubator. The cells were labelled with [^{3}H] thymidine (Amersham International, Amersham) for 6 hr and harvested onto a glass fibre filter paper (Wallac) using a cell harvester (Inotech AG). The filters were dried, sealed in plastic bags with 3 ml of scintillant (Optiphase safe, LKB Scintillation products) and counted using a liquid scintillation counter (Microbeta, Wallac).

3.11 Interferon-γ assay

For estimation of IFN-γ in the efferent lymph, an aliquot of fresh lymph collected in the morning was centrifuged at 800*g* for 15 min., cell-free supernate was decanted and stored at -20°C. A solid phase sandwich ELISA using mAbs specific for bovine IFN-γ was used (Wood *et al.*, 1990; Rothel *et al.*, 1990) to measure levels

of IFN-γ in the lymph plasma using a ready made test kit (CSIRO, Australia). The frozen lymph supernates were thawed and brought to room temperature. All the necessary reagents and procedures were provided with the kit. A known positive and negative sample was included in each plate. The plates were read in an ELISA reader (MR 700 Microplate reader, Dynatech) at 450 nm within 20 min. after adding the final solution to stop the enzyme substrate reaction. Serial two fold dilutions of recombinant bovine IFN-γ (Ciba Giegy; a gift from Dr. R.A. Collins, Institute of Animal Health, Compton) were used to plot a standard curve for estimating the biologically active IFN-γ in the lymph samples. Each sample was tested in duplicate and the mean O.D. was used to calculate the ammount of IFN-γ. The samples showing very high levels of IFN-γ were diluted with PBS to get an O.D. within the range of the standard curve.

CHAPTER 4

The effect of anti MHC immune responses on cell line immunisation against *Theileria annulata*

Objectives:

1. To study the feasibility of reimmunisation against *T. annulata* with the cell line used for primary immunisation.

2. To investigate the effect of a previously generated allogeneic immune response on immunisation against *T. annulata* with schizont infected cell lines.

4.1 INTRODUCTION

Theileria annulata or *T. parva* infected cells can be cultured *in vitro* either from the tissues of infected animals (Tsur, 1945; Tsur & Adler, 1962) or by *in vitro* infection of bovine lymphocytes with sporozoites (Brown *et al.*, 1973; Brown, 1983). Continuously growing *T. annulata* infected cell lines have been successfully used as live vaccines in endemic areas of various countries (Hashemi-Fesharki & Shad-Del, 1973; Wenshun *et al.*, 1975; Pipano, 1977; Ozkoc & Pipano, 1981; Stepanova & Zablotsky, 1989; Singh, 1992). A cell line vaccine introduces parasites to the recipient animal in the context of a foreign graft. Therefore, histoincompatibility between the cell line and the recipient may influence successful establishment of the parasite within the recipient, which is important for the development of protective immunity (Brown *et al.*, 1978a; Emery *et al.*, 1982; Innes *et al.*, 1989a). One of the major differences between *T. annulata* and the closely related parasite *T. parva*, is that it is easier to infect and immunise animals with *T. annulata* than with *T. parva* infected cell lines.

Initial attempts to immunise cattle with *T. parva* infected cell lines produced varying results [Brown *et al.*, 1964 (unpublished) reported in Wilde, 1967]. It was later found that 10^8 cells were needed to immunise cattle reproducibly. However, some animals suffered from the disease after cell line immunisation and some others were not protected on sporozoite challenge (Brown *et al.*, 1971; Cunningham, 1977; Wilde, 1977; Brown *et al.*, 1978a). The cell lines infected with both parasites express BoLA phenotypes identical to those of the animal from whose lymphocytes they are derived (Spooner & Brown, 1980). It was proposed that different pathogenic responses observed in animals after inoculation of *T. parva* infected cell lines were caused by a graft rejection phenomenon (Spooner, 1979). This hypothesis was tested by Teale (1983) in Kenya. Cattle inoculated with 10^3 or 10^5 *T. parva* infected MHC class I matched cells were successfully immunised against subsequent challenge. However, animals inoculated with similar doses of MHC class I mismatched *T. parva* infected cells were not protected against challenge (Teale, 1983; Dolan *et al.*, 1984b). About 10^7-10^8 *T. parva* infected cells were needed if they were allogeneic (Brown *et al.*, 1978a; Brown, 1981; Emery *et al.*, 1982).

On the other hand, animals were successfully immunised against *T. annulata* by inoculation of 10^4 to 10^6 allogeneic infected cells (Pipano & Tsur, 1966; Ozkoc

& Pipano, 1981; Hashemi-Fesharki, 1988; Sharma, 1989) and later with as few as 10^2 (Ouhelli *et al.*, 1989) or even 10^1 (Brown, 1990) cells. Extensive field uses of *T. annulata* cell line vaccines in different countries also suggested that graft rejection was not a problem with *T. annulata* and was formally demonstrated by Innes *et al.* (1989c). Inoculation of cattle with an allogeneic *T. annulata* cell line produces a strong cytotoxic T cell response (Innes *et al.*, 1989a) and complement fixing antibodies (Kachani and Spooner, 1992) against the MHC antigens of the immunising cell line, followed by the development of an antiparasite immune response (Innes *et al.*, 1989a; Kachani *et al.*, 1992b). The histocompatibility barrier does not jeopardise infection and primary immunisation against *T. annulata* as it does with *T. parva*.

The duration of immunity after cell line immunisation is poorly defined. There is evidence that immunity wanes (Pipano, 1977; Brocklesby, 1978; Ouhelli *et al.*, 1994), particularly in the absence of natural tick challenge in the field. Thus there may be a need to boost immunity. In Turkey, routine reimmunisation with the same cell line used for the initial immunisation is being done (M. Gunay, pers. comm.). However, there is no information on whether it is necessary or effective. Some evidence from Morocco and Israel suggests that reimmunisation with the same cell line may not be effective (Pipano, 1978; Ouhelli *et al.*, 1994). In an experiment in Morocco, animals inoculated with $1x10^4$ allogeneic *T. annulata* infected cells were immune on challenge one month after the immunisation, but some animals challenged six months after immunisation were not immune. Another group of animals immunised with $1x10^4$ cells, reimmunised with $1x10^6$ cells from the same cell line after seven months and challenged one month later showed mixed reactions from complete susceptibility to mild disease (Ouhelli *et al.*, 1994). This experiment indicated that an anti MHC immune response, generated after first vaccination, might be interfering in the development of immunity at the time of second vaccination with the same cell line. However, another factor influencing this particular experiment was that animals were poorly fed during the trial. It is not known whether a pre-existing allogeneic immune response, produced after first cell line immunisation, would interfere in the parasite transfer and development of immunity against the parasite, if the animal is reimmunised with the same cell line vaccine.

Setting up an experiment where animals are immunised with a cell line vaccine and then left in a *T. annulata* free area for many months or several years until

immunity wanes is clearly very costly, time consuming and logistically difficult. We, therefore, tried to devise a model to address this question over a shorter period of time scale. An allograft response was generated by leucocyte inoculation or skin grafting. It has been shown previously that cell line inoculation produces a similar allogeneic response to that produced by inoculation of uninfected leucocytes or skin implantation (Kachani & Spooner, 1992). We then asked the question whether this interfered with the generation of immunity to *T. annulata*, following primary cell line vaccination, assuming that the first cell line has behaved as a first graft and the cell line gives the second set response.

4.2 MATERIALS AND METHODS

4.2.1 BoLA typing

All animals and *T. annulata* infected cell lines isolated from animals after cell line immunisation were BoLA typed using the microlymphocytotoxicity test described in chapter 3.2 as per the method of Spooner *et al.* (1979a).

4.2.2 Cell lines

Theileria annulata cell lines were established *in vitro* by infection of bovine PBM with sporozoites and maintained in culture as described in chapter 3.4 using the method of Brown (1983). Parasite material used was from the Ankara (Schein, 1975) and the Hissar (Gill *et al.*, 1980) stocks. The cell lines at passage 3 were inoculated s/c above the left prescapular lymph node in one ml volume at varying cell concentrations as shown in table 4.1.

4.2.3 Stabilate challenge

The sporozoites used to provide the lethal challenge were prepared as a stabilate (Cunningham *et al.*, 1973) of *H. a. anatolicum* ticks, infected with the Gharb stock of *T. annulata* (Ouhelli, 1985), as described in chapter 3 (3.4.2.2). Stabilate material (2 t.e. per ml) equivalent to 1 tick (0.5 ml) was inoculated s/c above the right prescapular lymph node.

4.2.4 Animals and experimental design

The animals used in this study were Friesians or Friesian x Brown Swiss or Friesian x Charollais calves aged 3-8 months. The experimental design is shown in table 4.1 and described below.

4.2.4.1 Experiment 1

Five susceptible MHC mismatched calves (group 1) were used in this experiment (Table 4.1a). Two animals (200 & 215) were immunised against MHC antigens by three s/c inoculations of uninfected BoLA mismatched leucocytes from a BoLA A10/A11 animal on day 0, 14 and 28. Seven days after the last priming with uninfected leucocytes, one of the animals was inoculated with a *T. annulata* (Ankara) infected cell line derived from the uninfected leucocyte donor animal. The second animal was inoculated with a *T. annulata* (Ankara) cell line from another animal with a different MHC phenotype (A19/A20). Two other animals not primed with leucocytes were inoculated one with each cell line as immunised controls. The *T. annulata* infected cells were injected s/c at $1x10^6$ per animal. All four animals along with one control were challenged with *T. annulata* (Gharb) sporozoites 26 days later.

4.2.4.2 Experiment 2

Six animals were used in this experiment (group 2) and divided into two subgroups: 2a and 2b (table 4.1b). Animals in group 2a were implanted with skin (x3) from an MHC mismatched animal (BoLA type, A10/A11), as described by Pringnitz *et al.* (1982), to produce an allogeneic immune response. Animals in group 2b were not skin grafted. All animals in group 2 were immunised with $1x10^6$ cells from a *T. annulata* (Hissar) infected cell line derived from the skin donor animal (BoLA type, A10/A11). Animal 838 was implanted with skin on day 0, 28 and 49 and immunised with the cell line 7 days later. Animal 841 was given the skin implants on days 0, 28 and 74 and was immunised 9 days after the last implant. Skin implantations were done on day 0, 28 and 127 in animal 845 which was immunised 11 days after the last implant. All six animals were challenged with 1 t.e. *T. annulata* (Gharb) sporozoites. The period between the day of immunisation to the day of challenge varied from 28 days to 6 months in these animals because they were also used in efferent lymph cannulation experiments described in chapter 7.

4.2.4.3 Experiment 3

Six animals (group 3) were divided into two subgroup: 3a and 3b (table 4.1c). Animals in group 3a were implanted with skin (x3) from the same MHC mismatched animal (BoLA type, A10/A11) as used in experiment 2 on days 0, 27 and 55. Animals in group 3b were not skin grafted. All animals were immunised with $1x10^4$ cells from a *T. annulata* (Hissar) infected cell line derived from the skin donor

Table 4.1: EXPERIMENTAL DESIGN

a. Experiment 1

Animal no.	Priming	Immunisation	Challenge
Group 1			
200	Immunisation with leucocytes from a A10/A11 animal (repeated x3)	TaA (A10/A11), $1x10^6$ cells per animal, (cell line from WBC donor)	Stabilate (TaG) 1.0 t.e./animal
223	-	----do----	----do----
215	Immunisation with leucocytes from a A10/A11 animal (repeated x3)	TaH (A19/A20), $1x10^6$ cells per animal, (cell line from a different animal)	----do----
221	-	----do----	----do----
209	-	-	----do----

b. Experiment 2

Group 2a			
838	Skin grafting from a A10/A11 animal (repeated x3)	TaH (A10/A11), $1x10^6$ cells per animal, (cell line from skin donor)	Stabilate (TaG) 1.0 t.e./animal
841	----do----	----do----	----do----
845	----do----	----do----	----do----
Group 2b			
933	_	----do----	----do----
937	_	----do----	----do----
859		----do----	----do----

c. Experiment 3

Group 3a			
245	Skin grafting from a A10/A11 animal (repeated x3)	TaH (A10/A11), $1x10^4$ cells per animal, (cell line from skin donor)	Stabilate (TaG) 1.0 t.e./animal
246	----do----	----do----	----do----
249	----do----	----do----	----do----
Group 3b			
250	_	----do----	----do----
264		----do----	----do----
273		----do----	----do----

- No action taken ----do---- As above

animal. The immunisation in skin grafted (group 3a) animals was done 5 days after the last implant. All animals were challenged with *T. annulata* (Gharb) sporozoites 32 days after cell line immunisation.

4.2.5 Monitoring of animals

The clinical condition of the animals was assessed by observation of their general condition eg. feeding and by rectal temperature (a febrile response being $\geq 39.5^{o}C$). PCV was measured twice a week by micro-haematocrit. Schizonts in biopsy smears from the draining lymph node and piroplasm in blood smears were assessed, three times a week by Giemsa stained slides, during the period when parasitological reactions were expected. Attempts were made to re-isolate parasite infected cell lines *in vitro* from lymph node biopsies and PBM after cell line immunisation at regular intervals.

4.2.6 Serology

Sera were collected weekly from all the animals after generation of allogeneic responses, cell line immunisation and sporozoite challenge. The level of anti MHC antibodies was measured as described previously by Kachani & Spooner, (1992) using microlymphocytotoxicity. Anti-macroschizont and anti-piroplasm antibodies were determined by IFAT as described by Burridge & Kimber (1972).

Techniques used for clinical monitoring, serology, haematology and re-isolation of parasite infected cell lines from animals after cell line immunisation and sporozoite challenge are described in detail in chapter 3.

4.3 RESULTS

4.3.1 Experiment 1

Two animals inoculated with uninfected leucocytes (200 & 215) developed anti MHC antibodies but exhibited very low titres at the time of cell line immunisation (table 4.2).

Table 4.2: Anti MHC antibody titres (reciprocal) after inoculation of leucocytes in animals of experiment 1.

Animal no.	Day					
	0**	7	14**	21	28**	35
200*	-	-	32	16	8	2
215*	-	-	16	32	32	8

* Titres of sera against PBM of A10/A11 BoLA type.
** Day of inoculation of uninfected leucocytes.

Clinical reactions of calves after cell line immunisation are shown in fig. 4.1 & 4.2. Animal 200 inoculated with a cell line bearing the same MHC antigens against which it had already been preimmunised (A10/A11) showed mild fever for a day on day 7 (fig. 4.1a). Very low levels of schizonts in lymph node biopsies and piroplasms in blood smears could be seen on and after day 17 of cell line immunisation which always remained less than 1% (fig. 4.1c & 4.1d). Its counterpart animal 223 which was inoculated with the cell line only also exhibited similar reactions with the only difference being that fever was seen one day later on day 8 and schizonts were detected in biopsy smears from day 12 and piroplasms in blood smears from day 14 onwards.

Animal 215 was primed against A10/A11 BoLA types followed by immunisation with a *T. annulata* infected cell line of BoLA A19/A20. This animal also showed similar reactions i.e. fever for a day on day 8, a few schizonts (day 14 onwards) in biopsies and very mild parasitaemia in blood smears which was always less than 1%. However animal 221 which was inoculated with the cell line only, suffered from fever for two days initially which reappeared later for three days (fig. 4.1a). The animal exhibited 6% piroplasms in blood and a decrease in haematocrit from 29 to 20% (fig. 4.1b).

The titre of anti MHC antibodies rose sharply in animal 200 after cell line immunisation as this animal already had antibodies to MHC antigens of the immunising cell line. The levels of anti MHC antibodies were similar in all these animals except animal 221 which had a slightly lower titre (table 4.3).

Cultures of *T. annulata* infected cells were re-isolated from all the four animals between day 12-26 after cell line immunisation (table 4.3). BoLA typing of

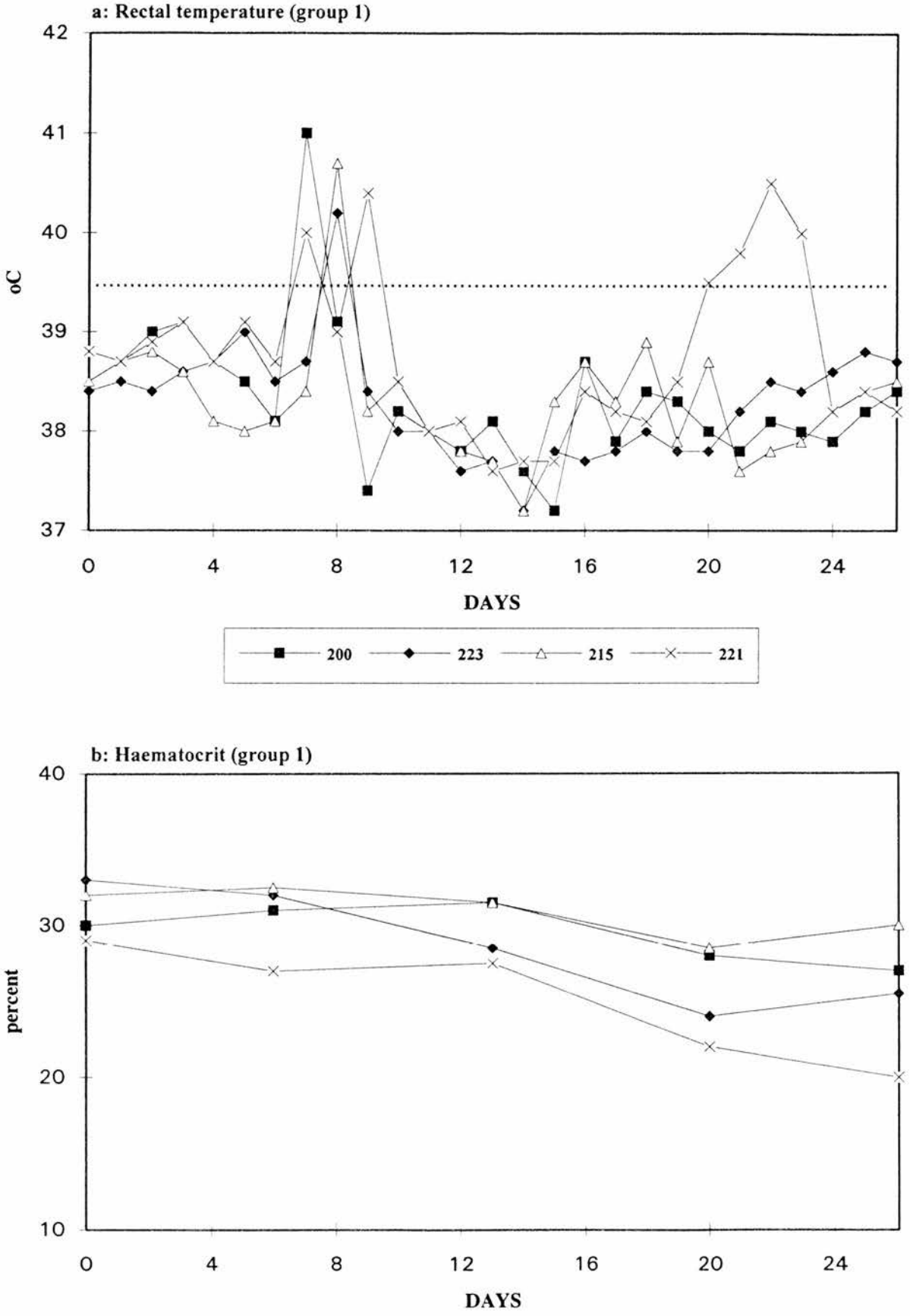
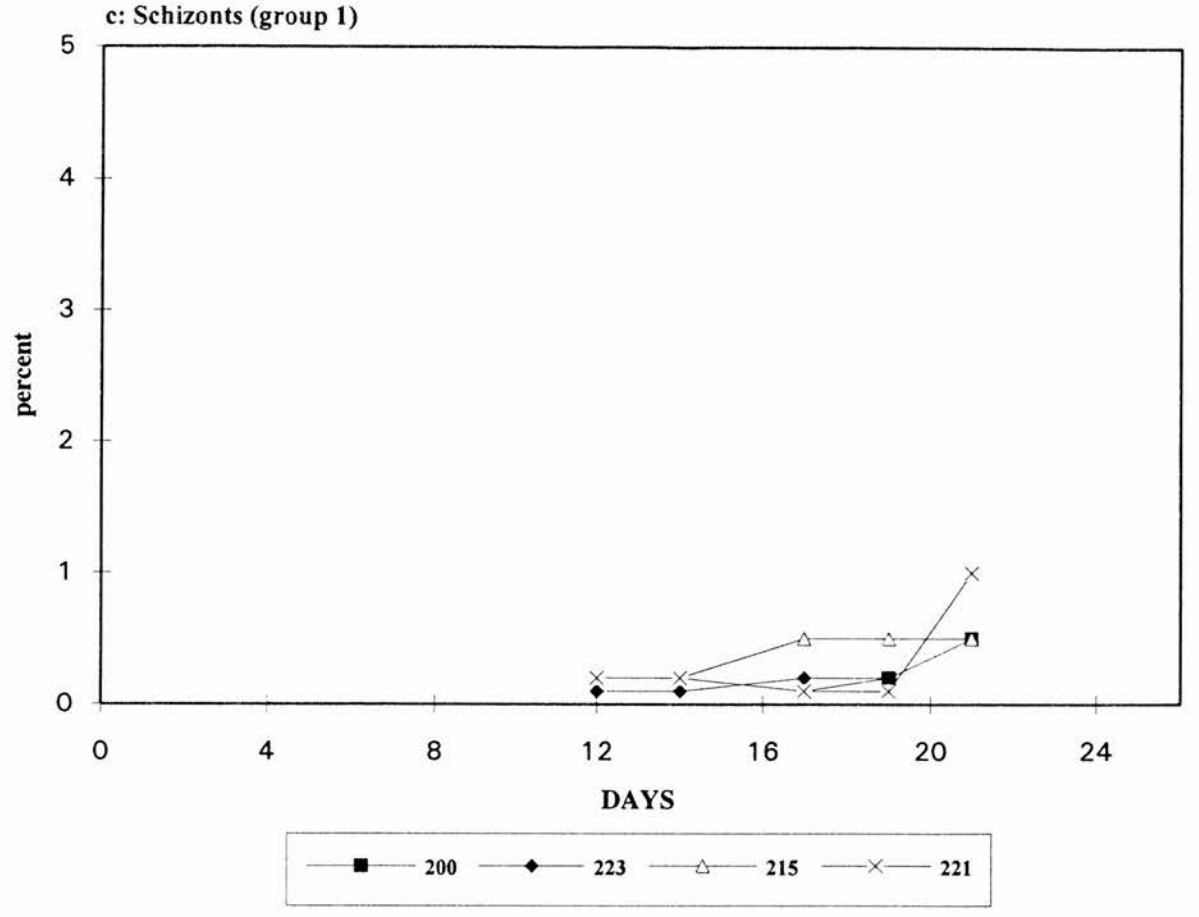


Figure 4.1: Clinical reactions in animals of experiment 1 after immunisation. (a) Rectal temperature. (b) Haematocrit.



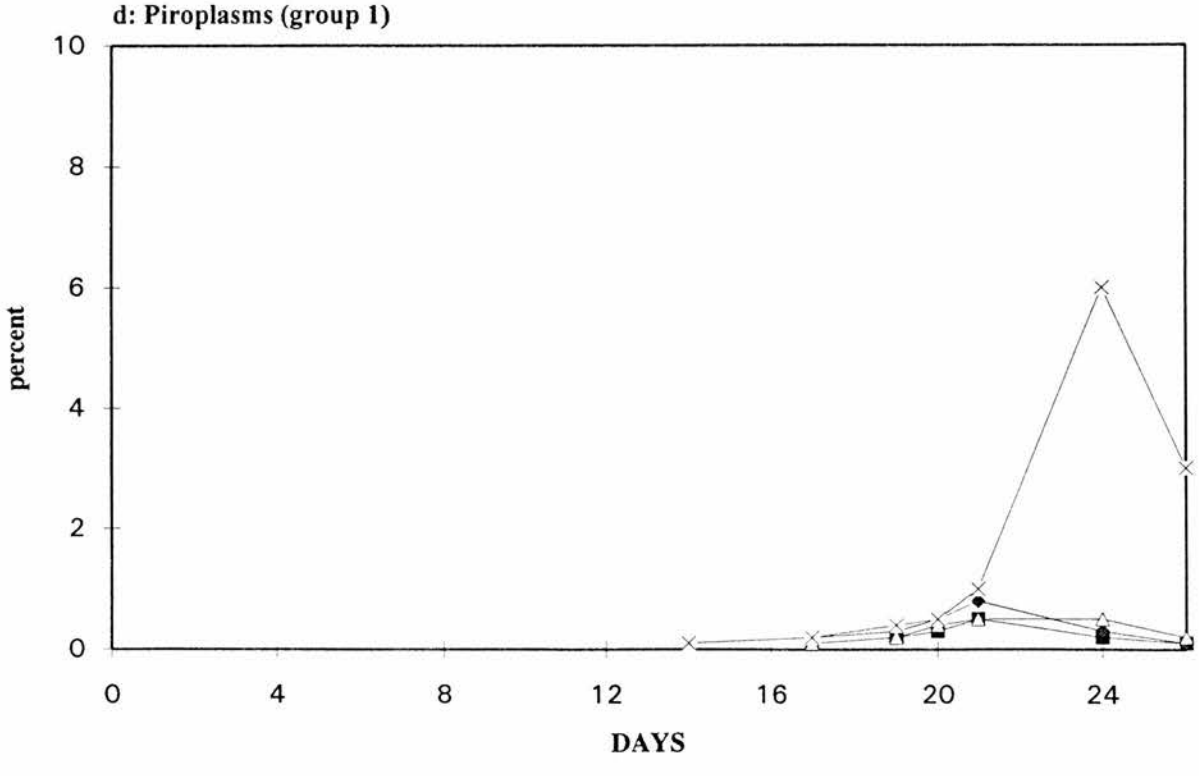


Figure 4.1: Clinical reactions in animals of experiment 1 after immunisation. (c) Schizonts in lymph node biopsy. (d) Piroplasms in blood smears.

these cultures showed that the parasite had transferred from the inoculated donor cell lines to the cells of the recipients. No cell line of donor origin could be isolated either from lymph node biopsies or peripheral blood at any stage after cell line immunisation. The pre-existing anti MHC immune response in animals 200 and 215 seemed to have no effect on parasite transfer which is presumed to be a prerequisite for the development of immunity following cell line immunisation.

Table 4.3: Anti MHC antibody titres (reciprocal) and re-isolation of parasite infected cell lines after immunisation in animals of experiment 1.

Animal no.	Day after cell line immunisation						First day of re-isolation of cell line
	1	7	14	21	28	56	
200*	2	64	128	32	32	8	17
223*	-	-	32	128	64	32	12
215**	-	2	32	128	128	32	14
221**	-	-	2	128	64	32	12
209*	-	-	-	-	-	-	-

* Titre of sera against PBM of an animal having BoLA type A10/A11.
** Titre of sera against PBM of an animal having BoLA type A19/A20.

When these animals were challenged with sporozoites, the reactions were again similar in all the four animals (Fig. 4.2a to 4.2d). The animals reacted mildly and recovered. Maximum piroplasms were always less than 2.5%. However, the challenge control animal (209) suffered from acute clinical theileriosis. This animal developed fever for five days, a sharp drop in haematocrit, 23% schizonts in biopsy smear, 29% piroplasms in blood smear and began to go off its feed by day 12 when it was treated with buparvaquone (Butalex, Mallincrodt Veterinary Ltd.) as described by McHardy *et al.* (1985).

The animals with anti MHC antibodies specific for the vaccine cell line at the time of immunisation had slightly higher anti parasite antibody titres on cell line inoculation, but these were similar in all the animals after challenge (table 4.4).

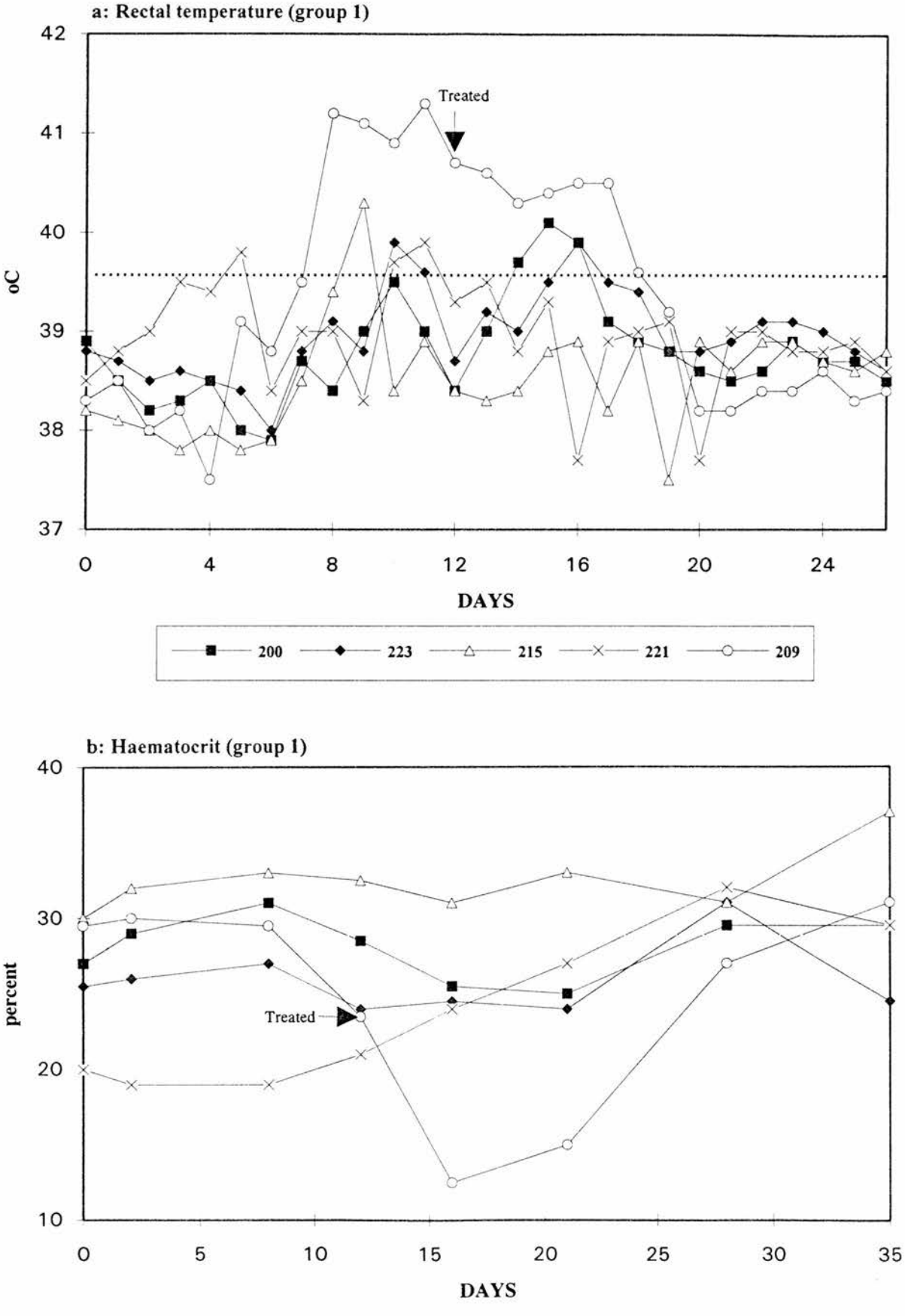


Figure 4.2: Clinical reactions in animals of experiment 1 after challenge.
(a) Rectal temperature. (b) Haematocrit.
(►) Animal no. 209 was treated with buparvaquone on day 12.

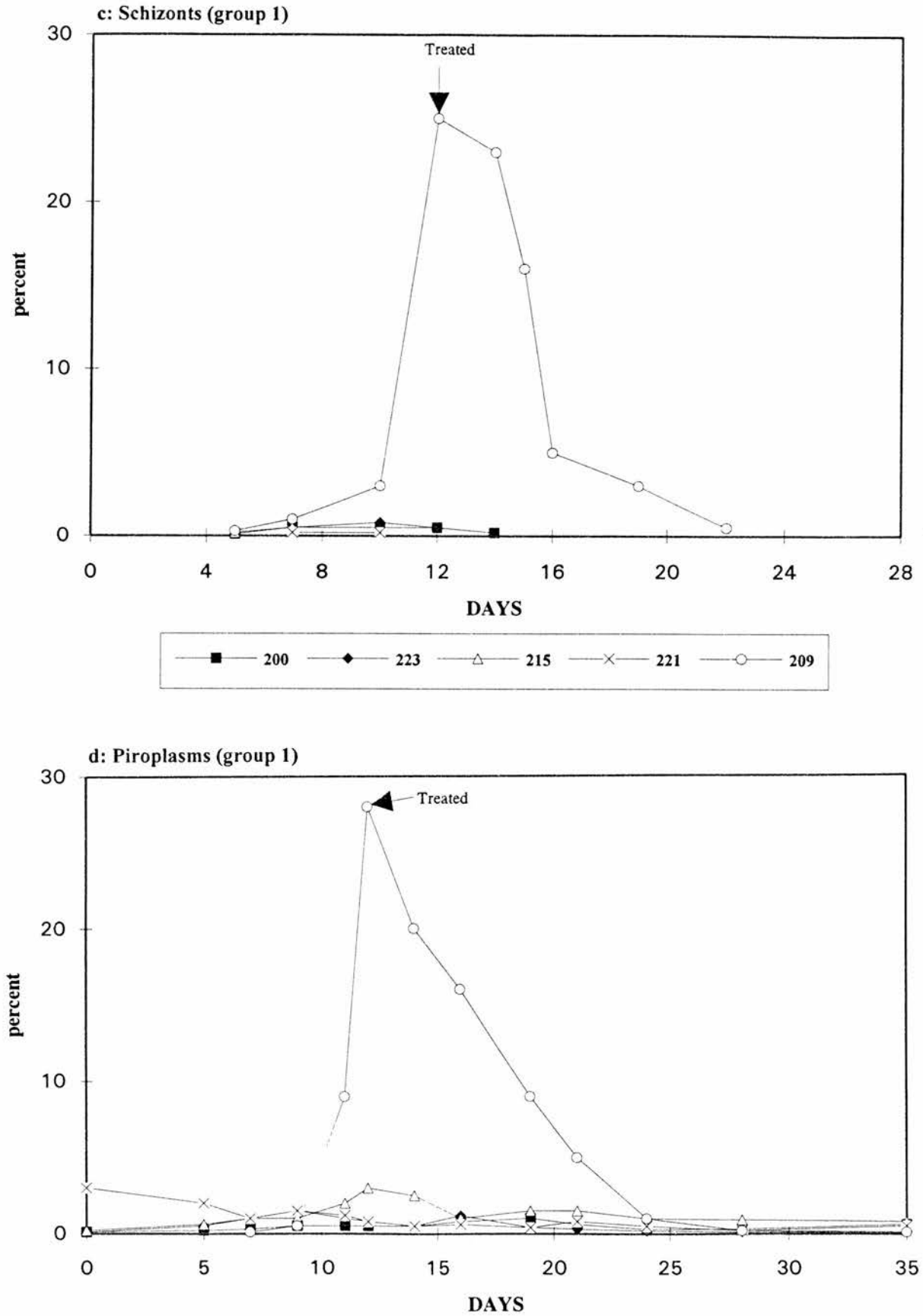


Figure 4.2: Clinical reactions in animals of experiment 1 after challenge. (c) Schizonts in lymph node biopsy. (d) Piroplasms in blood smears.
(►) Animal no. 209 was treated with buparvaquone on day 12.

Table 4.4: Titres (reciprocal) of anti parasite antibodies by IFAT in animals of experiment 1.

a. Schizont antigen

Animal no.	Day					
	1	14	26*	40	47	54
200	10	40	640	2560	2560	2560
223	-	40	160	2560	2560	2560
215	10	10	640	640	2560	2560
221	-	10	160	2560	2560	2560
209**	-	-	-	-	40	2560

b. Piroplasm antigen

Animal no.	Day					
	1	14	26*	40	47	54
200	10	40	640	2560	2560	2560
223	10	40	640	2560	2560	2560
215	-	10	640	2560	2560	2560
221	-	640	2560	2560	2560	2560
209**	-	-	-	-	40	2560

* Day of sporozoite challenge.
** Treated on day 38.

4.3.2 Experiment 2

The animals in group 2a developed a strong allogeneic immune response after skin grafting. The anti MHC antibody titres are shown in table 4.5. The response generated after skin grafting was stronger than the response generated by inoculation of uninfected leucocytes.

Table 4.5: Anti MHC antibody titres (reciprocal) after skin grafting in animals of group 2a.

Animal no.	Day after skin grafting												
	0	7	14	21	28	35	42	49	56	74	83	127	138
838*	-	-	2	32	128	256	512	1024	512				
841*	-	-	16	16	32	128	256	512	256	256	512		
845*	-	-	4	16	32	32	64	128	64	32	16	16	64

* Titres of sera against PBM of A10/A11 BoLA type
The skin grafting was done on day 0, 28 & 49 in 838; 0, 28 & 74 in 841 and 0, 28 & 127 in 845.

After cell line immunisation, the animals in group 2a (skin grafted) exhibited mild fever for a day (fig. 4.3a). Haematocrit values dropped slightly in two animals (fig. 4.3c). Schizonts were seen in one animal (838) and piroplasms in two animals (838 & 845). No parasitological reactions were observed in animal 841 (fig. 4.4a & 4.4c). All three animals in group 2b, which were not skin grafted, exhibited very mild parasitological reactions (fig. 4.4b & 4.4d) after inoculation of $1x10^6$ *T. annulata* infected cells. Haematocrit values dropped in all the three animals. Schizonts and piroplasms were also seen in all the three animals (fig. 4.4b & 4.4d). The skin grafted animals had a strong pre-existing allogeneic response at the time of cell line immunisation. Anti MHC antibody titres increased 2 fold in animal 838, 8 fold in 845, but the titres in 841 decreased (table 4.6). The non skin grafted animals developed allogeneic antibodies after cell line immunisation.

Parasite infected cell lines were re-isolated easily from all the three non skin grafted animals of group 2b between day 12-28 after cell line immunisation. However, cell lines could only be isolated from two of the skin grafted animals (838 & 845) in group 2a between day 17-28. No parasite infected cell line could be isolated from animal 841 (table 4.6) and it did not show any parasitological reactions. All cell lines isolated were of recipient origin.

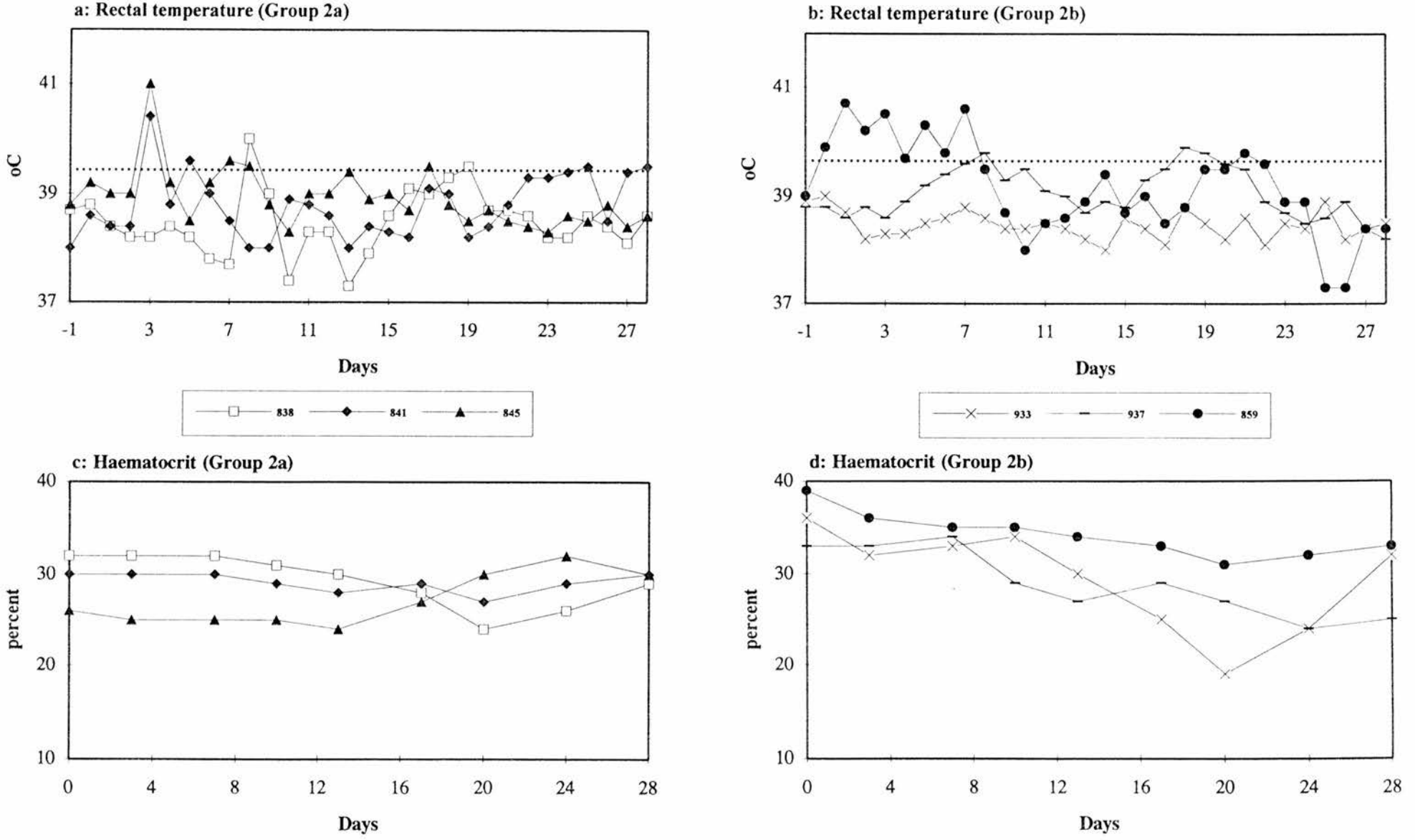


Figure 4.3: Clinical reactions in animals of experiment 2 after immunisation. Group 2a: Skin grafted. Group 2b: not skin grafted. (a & b) Rectal temperature. (c & d) Haematocrit.

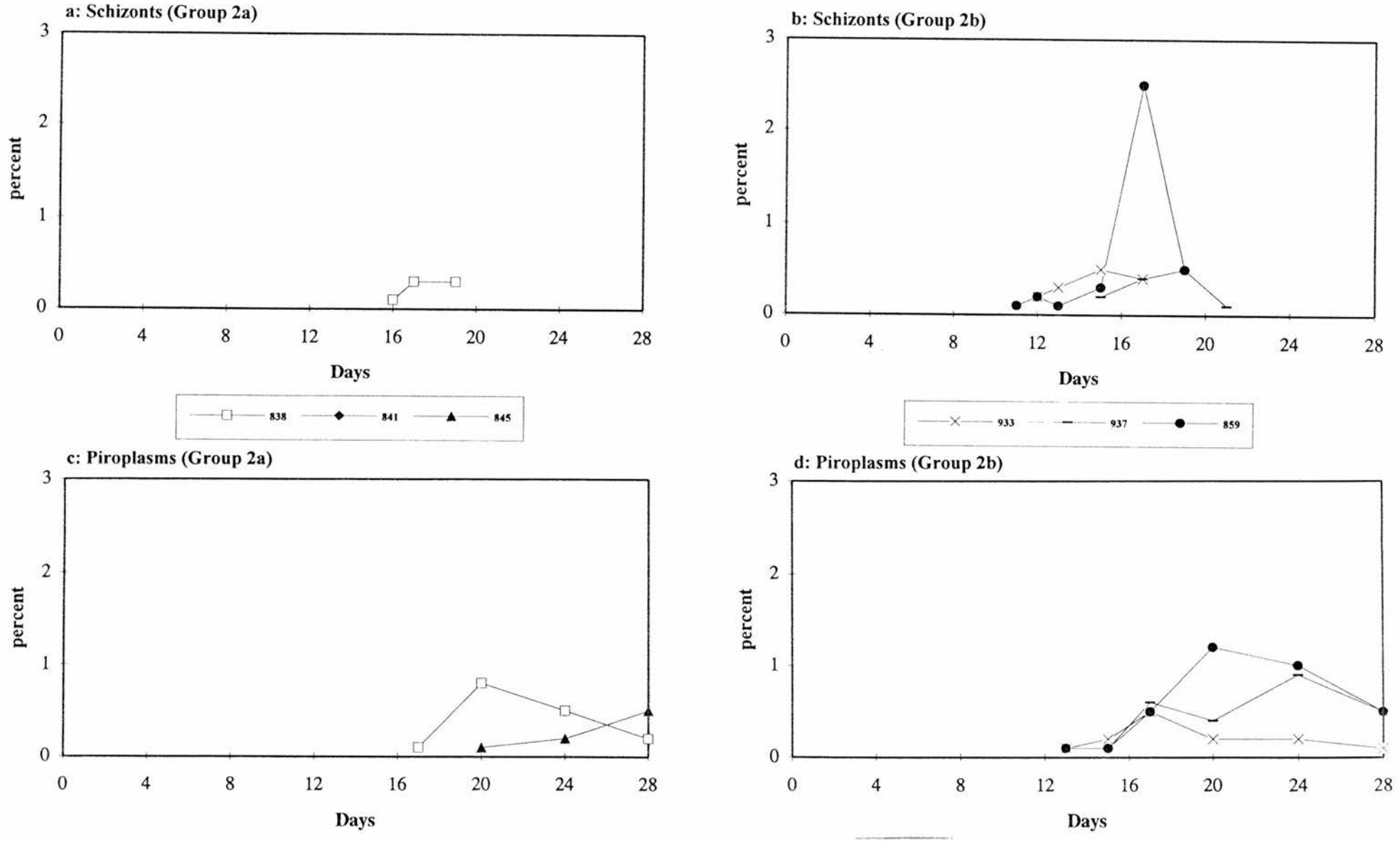


Figure 4.4: Clinical reactions in animals of experiment 2 after immunisation. Group 2a: Skin grafted. Group 2b: not skin grafted. (a & b) Schizonts in lymph node biopsy. (c & d) Piroplasms in blood smears.

Table 4.6: Anti MHC antibody titres (reciprocal) and re-isolation of parasite infected cell lines after immunisation in animals of experiment 2.

Group	Animal no.	Day after cell line immunisation					First day of re-isolation of cell line
		0	7	14	21	28	
2a	838*	256	512	256	128	64	17
	841*	512	256	256	128	128	none
	845*	64	256	512	256	256	21
2b	933*	-	4	32	256	128	12
	937*	-	8	32	128	128	15
	859*	-	4	8	128	256	12

* Titre of sera against PBM of an animal having BoLA type A10/A11.

On challenge with sporozoites, all the animals in group 2a (skin grafted) exhibited fever (fig. 4.5a). Animal 838 suffered from fever for 5 days and 845 for only 2 days, but both recovered spontaneously. Whereas, animal 841 expressed very high fever for 6 days, started to go off its feed and was treated with buparvaquone on day 10. Fever continued for another 7 days after treatment, therefore, a second dose of buparvaquone was given to this animal on day 17. The animal exhibited 42% schizonts in the biopsy smear and 10% piroplasms in the blood smear on day 10 before treatment (fig. 4.6a & 4.6c). The other two animals in this group (838 & 845) only showed very mild parasitaemia and were immune to challenge, which could have been lethal in animal 841 had it not been treated. The animals in group 2b also showed only mild reactions on sporozoite challenge and were immune. Only animal 937 expressed fever for 4 days, but recovered spontaneously (fig. 4.5b). This animal was challenged with sporozoites six months after cell line immunisation. The other two animals in this group (933 & 859) exhibited no rise in rectal temperature. The level of parasitaemia was always less than 1% in these animals (fig. 4.6b & 4.6d). There was a mild drop in haematocrit values in all the animals on challenge except animal 841 where the drop was very severe (fig. 4.5c & 4.5d).

IFAT for anti parasite antibodies indicated that the animal 841 did not develop any antibodies after cell line immunisation, whereas all other animals did. The titres were further boosted after challenge with sporozoites (table 4.7).

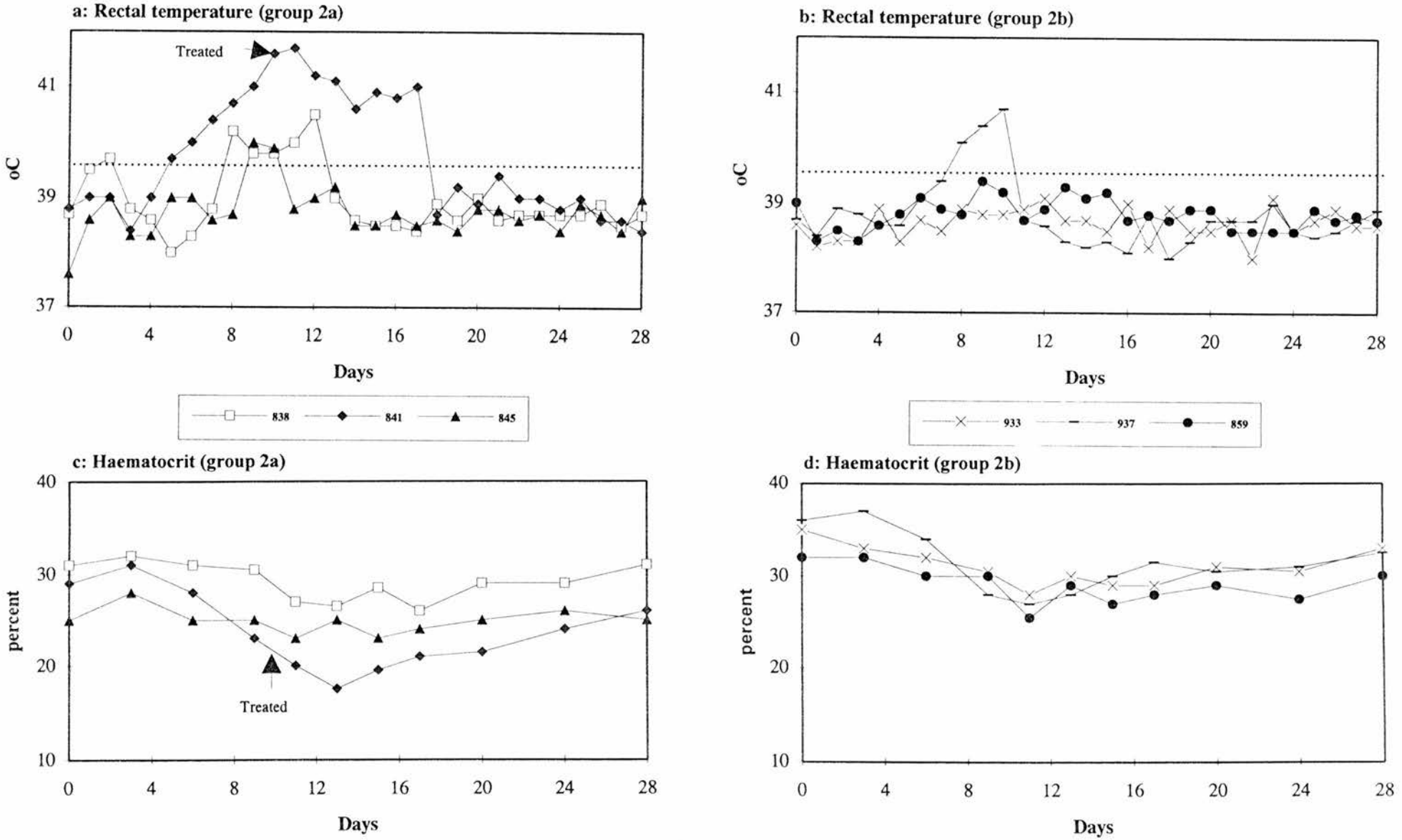


Figure 4.5: Clinical reactions in animals of experiment 2 after challenge. Group 2a: Skin grafted. Group 2b: not skin grafted. (a & b) Rectal temperature. (c & d) Haematocrit. (▸) Animal no. 841 was treated with buparvaquone on day 10 after challenge.

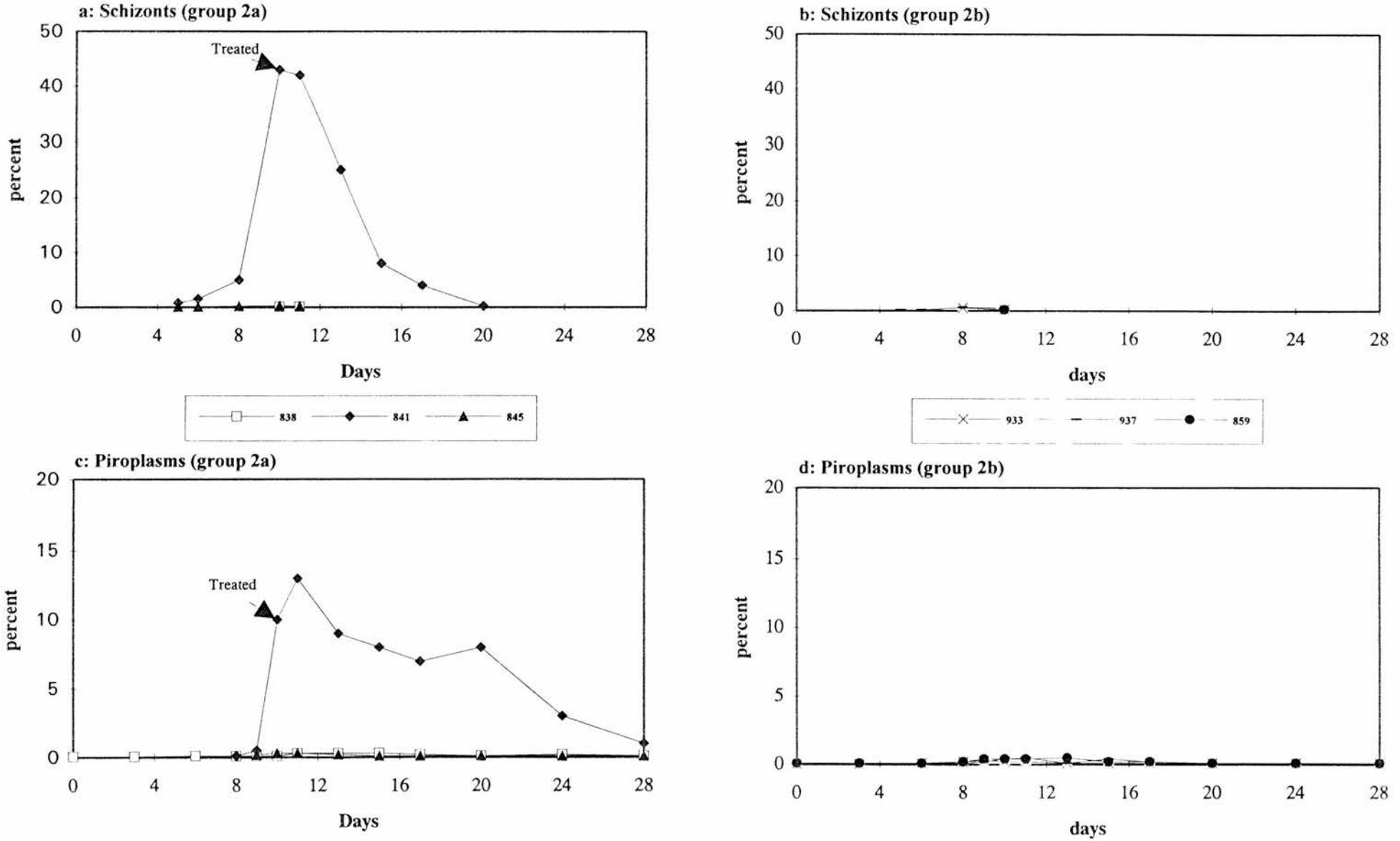


Figure 4.6: Clinical reactions in animals of experiment 2 after challenge. Group 2a: Skin grafted. Group 2b: not skin grafted. (a & b) Schizonts in lymph node biopsy. (c & d) Piroplasms in blood smears.

(►) Animal no. 841 was treated with buparvaquone on day 10 after challenge.

Table 4.7: Titres (reciprocal) of anti parasite antibodies by IFAT in animals of experiment 2.

a. Schizont antigen

Group	Animal no.	Day after immunisation			Day after challenge		
		0	14	28	0	13	28
2a	838	-	10	160	160	640	640
	841*	-	10	10	10	40	2560
	845	-	-	160	160	640	2560
2b	933	-	10	160	40	640	2560
	937	-	10	160	40	160	640
	859	-	40	640	160	640	2560

b. Piroplasm antigen

Group	Animal no.	Day after immunisation			Day after challenge		
		1	14	28	0	13	28
2a	838	-	-	640	640	640	2560
	841*	-	-	10	10	10	2560
	845	-	-	640	40	640	640
2b	933	-	-	160	160	640	640
	937	-	10	640	160	160	640
	859	-	-	2560	640	2560	2560

* Treated on day 10 after challenge.

4.3.2 Experiment 3

Animals in group 3a (skin grafted) developed a strong anti MHC immune response after skin grafting (table 4.8) similar to those developed in the animals of group 2a. The allogeneic antibody titres were very high at the time of cell line immunisation in these animals, whereas there were no antibodies in the animals of group 3b. The anti MHC antibody titres in these animals at the time of and after cell line immunisation are shown in table 4.9.

On inoculation of cell line, the skin grafted animals (group 3a) exhibited mild fever for one day on day 7 or 8 (fig. 4.7a). Haematocrit values dropped very marginally around day 10 (fig. 4.7c). Naive animals in group 3b (not skin grafted) also exhibited fever for a day or two and slight drop in haematocrit (fig. 4.7d).

Schizonts and piroplasms could be detected only in one animal (264). Schizonts were seen in this animal between day 14-18 (max. 0.5%) and piroplasms from day 15 onwards (max. 0.4%). None of the other animals in this experiment (3 skin grafted & 2 non skin grafted) exhibited any parasitological reactions.

Table 4.8: Anti MHC antibody titres (reciprocal) after skin grafting in animals of group 3a.

Animal no.	Day									
	0**	7	14	21	27**	34	41	48	55**	60
245*	-	-	-	16	128	256	128	512	512	512
246*	-	-	2	2	16	32	32	128	64	32
249*	-	-	-	-	32	64	64	64	32	64

* Titres of sera against PBM of A10/A11 BoLA type
** Day of skin grafting.

Attempts were made to re-isolate parasite infected cell lines from lymph node biopsies and blood after cell line immunisation of these animals. It was not possible to isolate any *T. annulata* infected cell lines from the skin grafted animals in group 3a, whereas, parasite infected cell lines were isolated from all three animals in group 3b (table 4.9). Many cell lines were isolated from animal 264 between day 14-28 after cell line immunisation. Two lines were isolated from animal 250 from PBM only, on day 14 and 22 after immunisation. Only one line was isolated from animal 273 also from blood on day 20 after immunisation. BoLA typing of these cell lines revealed that the cell lines originated from the cells of the recipient animal. Titre of anti MHC antibodies started to rise immediately after cell line immunisation in skin grafted animals (group 3a). These antibodies began to appear in animals of group 3b on day 7 and peak titres were seen between day 22-32 (table 4.9).

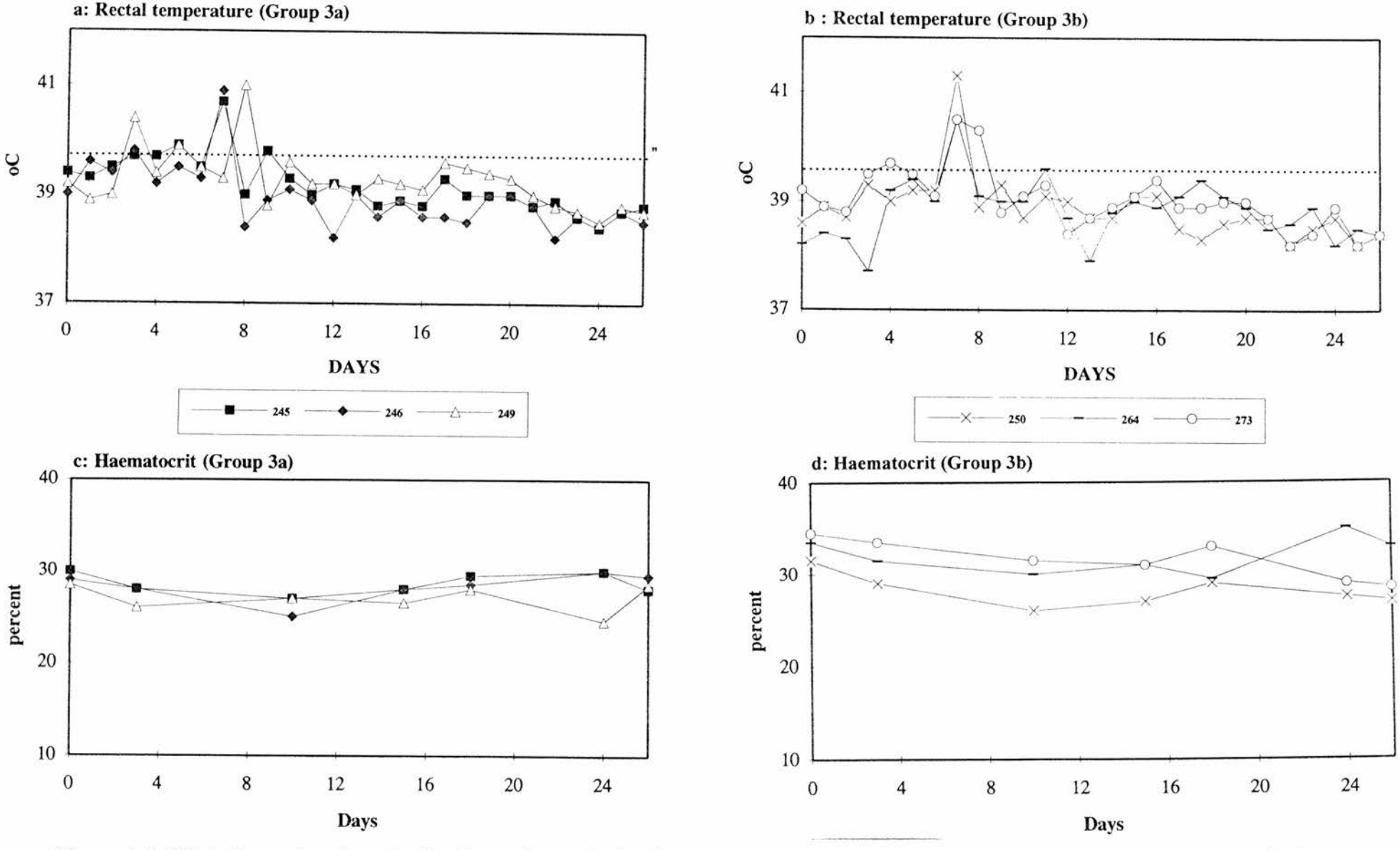


Figure 4.7: Clinical reactions in animals of experiment 3 after immunisation. Group 3a: Skin grafted. Group 3b: not skin grafted. (a & b) Rectal temperature. (c & d) Haematocrit.

Table 4.9: Anti MHC antibody titres (reciprocal) and re-isolation of parasite infected cell lines after immunisation in animals of experiment 3.

Group	Animal no.	Day after cell line immunisation								Day# of re-isolation of cell line
		0	7	14	22	28	32	38	59	
3a	245*	512	256	512	128	32	32	64		none
	246*	32	256	128	512	256	256	128	32	none
	249*	64	128	512	128	256	64	64	16	none
3b	250*	-	8	64	32	64	64	64	4	18 & 22 (two lines)
	264*	-	4	16	64	32	128	64	4	14 (many lines)
	273*	-	4	16	64	64	32	32		20 (one line)

* Titre of sera against PBM of an animal having BoLA type A10/A11.

The IFA titres for anti schizont antibodies were very low in animals of group 3a and one animal in group 3b (maximum 1:40, four weeks after immunisation). The only animal (264) which showed some parasitological reactions after immunisation also showed a titre of 1:640 and the other animal in this group 273 showed a titre of 1:160 four weeks after immunisation. The titres of anti-piroplasm antibodies were very low in all six animals after cell line immunisation (table 4.10).

The three animals in group 3a suffered from acute theileriosis on challenge (fig. 4.8 & 4.9). Rectal temperatures started to rise from day 6 and were more than 41°C on day 10 in all three animals (fig. 4.8a). Schizonts were seen in lymph node biopsies from day 5 onwards and ranged from 33-60% on day 10 (fig. 4.9a). Piroplasms in blood smears were 8-16% on that day (fig. 4.9c). Animal 245 was humanely killed and animal 246 & 249 were treated with buparvaquone (McHardy *et al.*, 1985) on day 10.

The animals in group 3b showed mixed clinical reactions on sporozoite challenge (fig. 4.8 & 4.9). Animal 264 which showed very mild clinical and parasitological reactions on immunisation was immune to sporozoite challenge. It suffered from very mild theileriosis and recovered quickly. Animal 250 exhibited high fever starting from day 6 which continued to rise for 5 days. It then started declining and returned to normal after three more days (fig. 4.8b). Schizonts were detectable from day 5 onwards in lymph node biopsy smears and were 16% on day 12, followed by a decline and were undetectable by day 19 (fig. 4.9b). Similarly, piroplasms rose

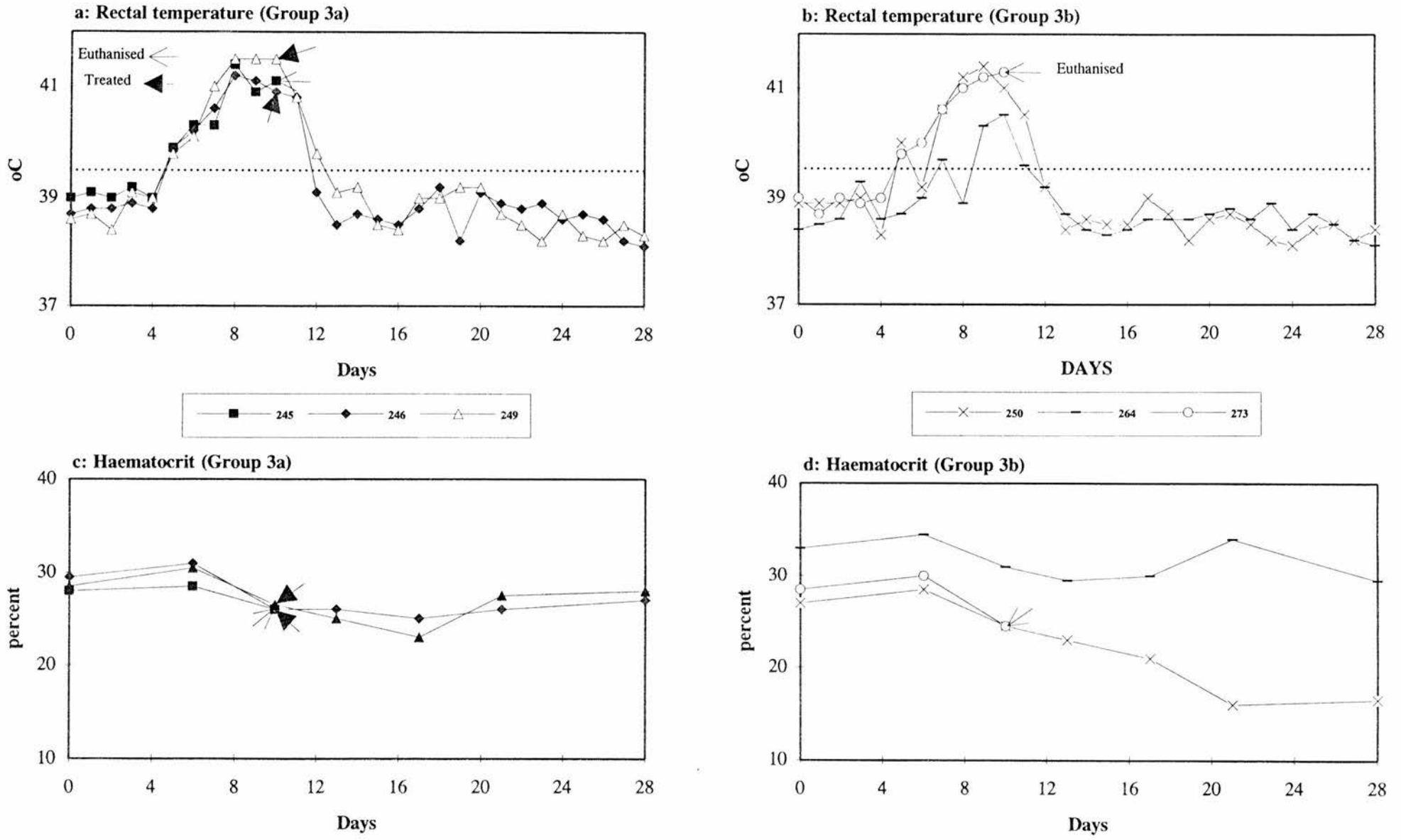


Figure 4.8: Clinical reactions in animals of experiment 3 after challenge. Group 3a: Skin grafted. Group 3b: not skin grafted. (a & b) Rectal temperature. (c & d) Haematocrit. (▸) Animals 246 and 249 were treated with buparvaquone on day 10 after challenge. (→) Animals 245 and 273 were euthanased on day 10 after challenge.

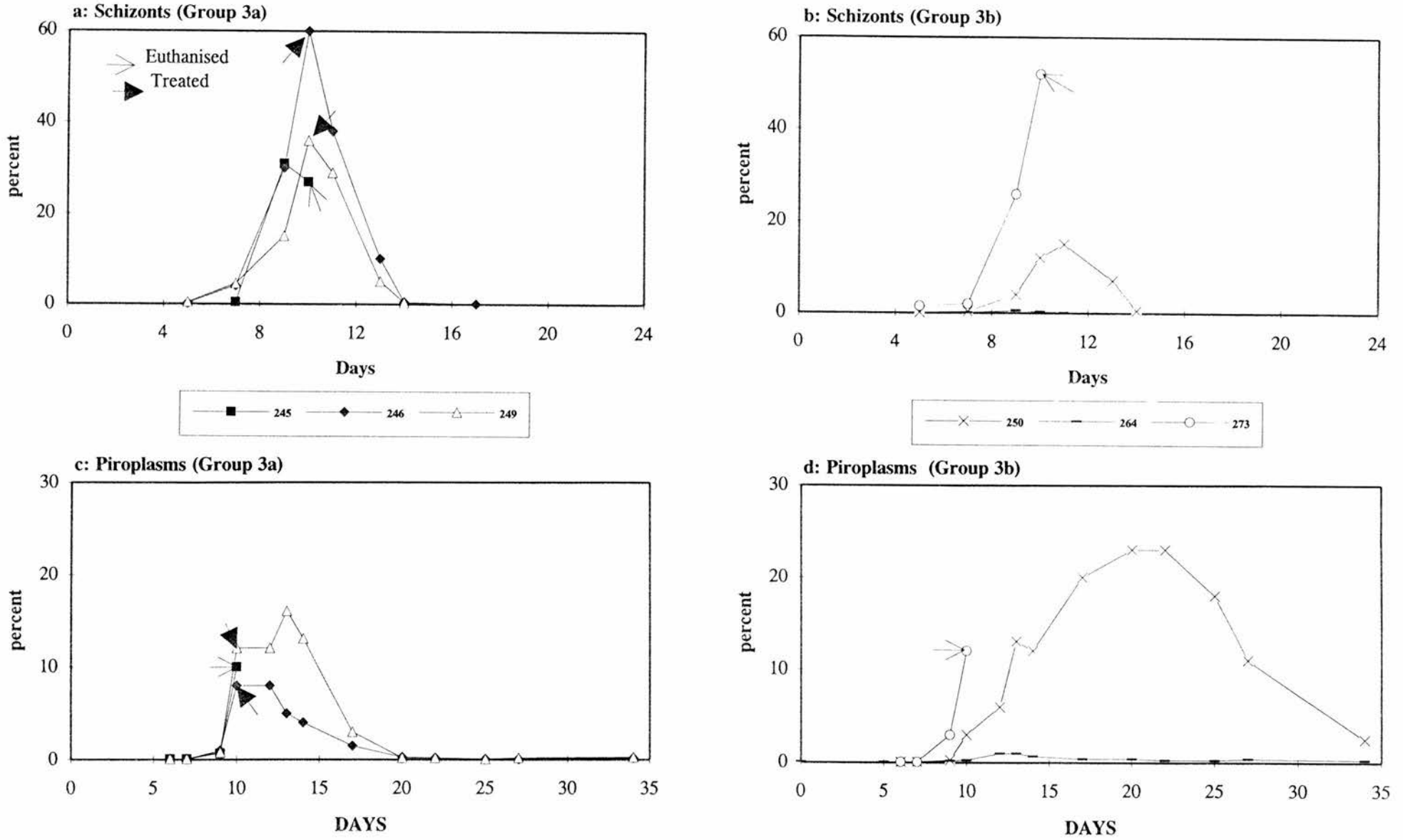


Figure 4.9: Clinical reactions in animals of experiment 3 after challenge. Group 2a: Skin grafted. Group 2b: not skin grafted. (a & b) Schizonts in lymph node biopsy. (c & d) Piroplasms in blood smears. (►) Animals 246 and 249 were treated with buparvaquone on day 10 after challenge. (→) Animals 245 and 273 were euthanased on day 10 after challenge.

up to 23% on day 20 and then started to decline (fig. 4.9d). The haematocrit fell to 14% by day 25 but then started to rise (fig. 4.8d) indicating that the animal was partially immune. The third animal in this group 273 suffered from acute disease. The animal had very high fever which started from day 6. Schizonts were 52% and piroplasms were 12% on day 10 when it was humanely killed. The symptoms of the disease in this animal were similar to those in animals of group 3a (skin grafted).

Table 4.10: Titres (reciprocal) of anti parasite antibodies by IFAT in animals of group 3.

a. Schizont antigen

Group	Animal no.	Day*					
		1	14	28	37	44	58
3a	245**	10	10	10	40		
	246***	-	10	40	160	640	2560
	249***	10	10	40	160	640	2560
3b	250	10	10	40	40	160	2560
	264	-	40	640	640	2560	2560
	273**	10	40	160	160		

b. Piroplasm antigen

Group	Animal no.	Day*					
		1	14	28	37	44	58
3a	245**	10	-	-	10		
	246***	-	-	-	160	160	2560
	249***	-	-	-	10	40	640
3b	250	-	-	10	10	40	2560
	264	-	-	10	10	40	640
	273**	-	-	10	40		

* The animals were challenged on day 32. ** Euthanased on day 10 after challenge.
*** Treated on day 10 after challenge.

4.4 DISCUSSION

These experiments provided a model where questions about reimmunisation with allogeneic *T. annulata* infected cell lines could be addressed in a logistically possible manner within a short time period. Strong allogeneic antibody responses can

be generated by skin grafting between BoLA mismatched animals (Spooner *et al.*, 1978; Spooner *et al.*, 1979a). Inoculation of allogeneic *T. annulata* infected cell lines also produces a strong response to MHC antigens of the immunising cell line comparable to those produced by skin grafting (Kachani & Spooner, 1992). This was further confirmed in these experiments where non skin grafted animals developed strong immune responses against allogeneic antigens of the inoculated cell line after immunisation. Inoculation of *T. parva* infected allogeneic cell lines also gave rise to anti-lymphocyte antibodies in inoculated animals (Cowan *et al.*, 1984). However, precise specificity of these antibodies was not determined. Strong allogeneic immune responses were produced in animals of group 2a and 3a after skin grafting which mimicked the response produced after cell line immunisation. Although, inoculation of uninfected leucocytes only produced mild responses.

No challenge control animals were used in experiments 2 and 3 to test the potency of stabilate used for challenge. However, very high potency of this batch was confirmed by severe disease symptoms in some of the animals (animal 841 in group 2a & all in group 3a), which were not protected and had to be treated or killed. Naive animals in other experiments (chapter 6) inoculated with 0.1 t.e. GUTS (i.e. 1/10th of the sporozoite concentration used in this experiment) also developed acute disease and had to be treated with buparvaquone (McHardy *et al.*, 1985) on day 10 or 11 after infection in order to save their lives. The dose of challenge was 1.0 t.e. in these experiments which was undoubtedly capable of producing potentially lethal infection in naive animals.

In the first experiment, animals were primed against MHC antigens by inoculation of allogeneic uninfected leucocytes. Immunisation of these animals with $1x10^6$ *T. annulata* infected cells, of the same or different MHC phenotype, lead to the development of immunity against potentially lethal sporozoite challenge. The pre-existing anti MHC immune response seemed to have no effect on the development of immunity against the parasite after cell line immunisation, although the day of first isolation of schizonts was delayed. Cell lines were inoculated immediately after priming the animal. In the field situations where animals may be reimmunised with the same cell line vaccine, revaccination would not be carried out for at least one year in the light of the seasonal occurrence of the disease in endemic areas. The level of an allogeneic immune response one year after first cell line vaccination may be low

and comparable to the level of response generated in this experiment.

Keeping in view a weak allogeneic response produced by inoculation of uninfected leucocytes to cell line immunisation, anti MHC responses were produced by repeated skin grafting in animals of group 2a and 3a in further experiments (Spooner *et al.*, 1979a). The animals exhibited a strong response which mimicked the allograft response produced by inoculation of the cell line. Immunisation of animals with $1x10^6$ cells (group 2a), despite the presence of a strong allogeneic response, produced immunity in 2 out of 3 animals but the third animal suffered from acute clinical theileriosis on challenge. This animal appeared fully susceptible to challenge and thus not protected by cell line immunisation. On the other hand, all 3 naive animals (not skin grafted, group 2b) immunised with the same dose of cells were immune to challenge. In the third experiment, using *T. annulata* infected cells at $1x10^4$ per animal, the skin grafted animals (group 3a) were not protected from sporozoite challenge. The animals in group 3b (non-skin grafted) also showed some reactions on challenge. Two of the three animals were immune. Animal 264 withstood challenge very well, 250 suffered moderately and recovered but animal 273 suffered from acute disease. Animal 273 was not protected by immunisation with 10^4 infected cells and was killed on day 10. However, there was a clear difference in the severity of reactions between the two groups. These two experiments clearly indicated that pre-existing allogeneic response inhibited the development of immunity against the parasite.

Sporozoite challenge of immunised animals with *T. annulata* infected cell lines usually results in mild clinical symptoms and recovery (Pipano, 1981). Animals have been successfully immunised with as few as 10^2 infected cells (Ouhelli *et al.*, 1989; Brown, 1990). Development of clinical disease on sporozoite challenge in animal 273 (group 3a; immunised with 10^4 cells) was unusual. Although similar reactions were observed on severe sporozoite challenge in some of the animals inoculated with low cell doses (Shukla & Sharma, 1991). At least with unattenuated cell lines, most other reports in the literature suggest that 10^4 cells engender enough protection against sporozoite challenge (Ouhelli *et al.*, 1989; Innes *et al.*, 1989c; Brown, 1990; Ouhelli *et al.*, 1994).

Critical analysis of parasitological reactions showed that appearance of schizonts and piroplasms was delayed after cell line immunisation in animals with a

pre-existing allogeneic response in all three groups of animals as compared to their non skin grafted immunised counterparts. However, all these animals were immune on sporozoite challenge. In animals where the appearance of schizonts and piroplasms was totally prevented after cell line immunisation (841 in group 2a & all animals in group 3a), severe disease ensued after challenge.

All animals showing parasitological reactions after cell line immunisation irrespective of their group, developed parasite specific antibodies to schizonts. However, animals in which parasitological reactions were totally prevented after cell line immunisation (841 in group 2a & all in 3a), showed no increase in IFA titres to parasite antigens. Parasite specific serological responses after cell line immunisation may not have any protective value, as passive transfer of immune serum (Sergent *et al.*, 1945; Dhar & Gautam, 1978; Samad *et al.*, 1984a) or generation of anti-parasite antibodies by inoculation of killed schizonts (Pipano *et al.*, 1977; Wilde, 1967; Cunningham, 1977), does not protect against sporozoite challenge. However, parasite specific antibodies are a good indicator that the animal is recognising the parasite in the inoculated cell line. Serology has been used to predict whether the animal would be immune on challenge or not (Pipano & Cahana, 1969; Shukla & Sharma, 1989).

Parasite infected cell lines were re-isolated from all animals in groups 1, 2b and 3b and only from 2 animals in group 2a (skin grafted) and these animals exhibited higher anti-macroschizont antibody titres after cell line immunisation. However, isolation of parasite infected cell lines was delayed in animals with a pre-existing allogeneic response as compared to their counterparts. The parasite transfers from donor cells to the cells of the recipient animal during an effective cell line immunisation (Pipano, 1977; Brown *et al.*, 1978a; Innes *et al.*, 1989a). All cell lines isolated were of recipient origin indicating that parasite transferred to the recipient's cells. No cell line of donor origin was isolated at any stage. No cell line was isolated from animal 841 or any animal in group 3a (skin grafted) after immunisation. These animals neither developed anti parasite antibodies nor exhibited any piroplasms in blood smears. The allogeneic response in the animals of group 3a and one animal in 2a prevented the establishment of the parasite in the recipient's cells on cell line immunisation and, as a consequence, the animals were fully susceptible on challenge. The ability to isolate parasite infected cell lines correlated with protection from sporozoite challenge. Even in the non-skin grafted group 3b where one animal was

not protected by 10^4 cells against a very potent sporozoite challenge, only one cell line was isolated with great difficulty, the number and timing of cell line isolation correlated with protection.

There is a strong MHC barrier to immunisation against *T. parva* where animals can only be immunised with $1x10^8$ or more allogeneic schizont infected cells (Pirie *et al.*, 1970; Brown *et al.*, 1971; 1978a). Animals inoculated with $1x10^6$ *T. parva* infected allogeneic cells are infrequently immunised or protected against lethal sporozoite challenge (Brown, 1981). However, 10^3-10^5 *T. parva* infected cells which were MHC class I matched with respect to the recipient did immunise animals against subsequent sporozoite challenge (Teale, 1983; Dolan *et al.*, 1984b). This sort of barrier does not exist against *T. annulata* at the time of primary cell line immunisation, where as little as $1x10^2$ allogeneic infected cells can immunise an animal (Ouhelli *et al.*, 1989). The experiments reported in this chapter indicate that a histoincompatibility barrier boosted artificially, can jeopardise cell line immunisation against *T. annulata* as it does with primary *T. parva* cell line vaccination. Inoculation of higher cell doses during a second immunisation might be able to overcome this situation if anti MHC response is not very strong, as indicated from the experiment 1. Preliminary experiments from Morocco refutes this (Ouhelli *et al.*, 1994). They used 10^6 *T. annulata* infected cells on second inoculation which did not reimmunise. It is possible that cells in the range of 10^7-10^8 would be required for reimmunisation as are needed for primary immunisation against *T. parva*.

The relative ease of immunisation using allogeneic cell lines in the case of *T. annulata* compared to the difficulties with *T. parva* is unlikely to be solely as a result of histoincompatibility, a common element in both cases. It might be because of the ability of the parasite to transfer and infect recipient's cells subsequent to cell line immunisation. It is not known how the parasite transfers from an allogeneic cell line to the recipient's cells. We also do not know the precise mechanism of development of immunity after primary cell line immunisation. An attempt has been made to study the kinetics of cellular changes during development of immunity to *T. annulata* infection in the following chapters. Kinetics of the parasite dissemination in the draining efferent lymph has been studied. Further experiments are needed to explain this. It was demonstrated by karyotypic analysis of infected cells that *T. annulata* would transfer from donor cells to the cells of the recipient within hrs of inoculation

[Hulliger *et al.*, 1965 (unpublished) reported in Wilde, 1967], whereas parasite transfer with *T. parva* was very inefficient and could take many days (Brown *et al.*, 1978a). It is not known whether parasite transfer involves active invasion of host cells by the schizonts or some form of passive transfer (Innes *et al.*, 1992a). The initial immune response induced after the inoculation of an allogeneic infected cell line involves generation of alloreactive cytotoxic T lymphocytes (Innes *et al.*, 1989a) and anti MHC antibodies (Kachani & Spooner, 1992). Innes *et al.* (1992a) proposed that the parasite infected cells are destroyed by these two arms of the immune response and the debris phagocytosed by macrophages. *In vitro* infection studies on various lymphocyte subpopulations suggest that *T. annulata* preferentially infects and transforms macrophages/monocytes and B cells but not T cells, whereas *T. parva* preferentially transforms T cells and not macrophages (Baldwin *et al.*, 1988a; Spooner *et al.*, 1989; Innes *et al.*, 1989b; Glass *et al.*, 1989). However, it is not known which cells are infected *in vivo* during *T. annulata* infection or primary cell line immunisation. Assuming the same cells are infected *in vivo* as *in vitro*, one could postulate that parasite might be phagocytosed by macrophages and infect the recipient's cells by passive transfer. This type of parasite transfer does not take place with *T. parva* as it mainly infects T cells. Therefore, a very high number of allogeneic infected cells is needed to establish infection and immunise against *T. parva*. A precise knowledge of the cells infected after allogeneic cell line immunisation, with both the parasites, could be very useful in designing future immunisation regimes.

In the primary immune response to cell line immunisation, the development of an allogeneic response takes some time and inoculated cells keep on dividing in the recipient's body. The chances of parasite transfer are consequently high. If the animal already has experienced an allogeneic response, the infected cells may be destroyed immediately. The chances of parasite transfer may be reduced because of no time for the multiplication of infected cells. This hypothesis of parasite transfer needs testing. Fibroblasts have been infected *in vitro* by overlaying *T. annulata* infected cells (Brown & Gray, 1981), but purified *T. annulata* or *T. parva* schizont particles would not infect other cells *in vitro* (Innes *et al.*, 1992a) or animals inoculated *in vivo* (Pipano *et al.*, 1977; Wilde *et al.*, 1966). The mechanisms of parasite transfer after cell line immunisation are a topic for future studies.

From these experiments, it seems that an anti MHC immune response can interfere with parasite transfer and the development of an anti parasite immune response following cell line immunisation. If the allogeneic response is strong, it can block the development of immunity. These observations are of immediate importance in endemic areas where cell lines of *T. annulata* schizonts are being used or are planned to be used as vaccines to control the disease. The anti MHC immune response, generated in the animals after first immunisation with a cell line, might interfere with the development of immunity against the parasite if the animals are reimmunised with the same cell line used for first immunisation. Consequently, it is not recommended to use the same cell line for repeated reimmunisation to boost the immunity. Producing a panel of cell lines with different BoLA phenotypes and using one cell line every year might help in overcoming this problem. Although the effect of allogeneic response against one BoLA type against the other needs to be studied in detail before making a firm recommendation.

Similar levels of allograft response prevented parasite transfer and development of immunity in some animals but not in others. The mechanism of this interference cannot be interpreted because of lack of knowledge on the cellular interactions between the parasite and various lymphocyte subpopulations. Little is known about the mechanism of development of immunity after cell line immunisation and sporozoite challenge. Further studies in this thesis are concentrated on understanding the dynamics and activation of various lymphocyte subpopulations and parasite dissemination in the peripheral blood and efferent lymph, both after cell line immunisation and sporozoite challenge, in skin grafted and non-skin grafted animals.

CHAPTER 5

Phenotypic changes in the peripheral blood leucocytes of animals after *T. annulata* cell line immunisation and sporozoite challenge in relation to pre-existing allogeneic responses.

Objectives:

1. To study the effect of cell line immunisation and sporozoite challenge on the kinetics of various lymphocyte subpopulations of peripheral blood in animals with or without a pre-existing allogeneic response.

2. To monitor changes in the peripheral blood leucocytes induced by the parasite leading to leucopenia manifested in the pathogenesis of tropical theileriosis.

5.1 INTRODUCTION

Theileria annulata infection causes a serious lymphoproliferative disease in cattle leading, amongst other things, to severe leucopenia (Sharma & Gautam, 1971; Laiblin, 1978; Preston *et al.*, 1992a). Previous studies have shown the involvement of various subpopulations of leucocytes in the pathogenesis and development of immunity. The parasite preferentially infects and transforms MHC class II^+ monocytes and B cells (Spooner *et al.*, 1989; Glass *et al.*, 1989; Campbell *et al.*, 1994a). Animals, recovering from sporozoite infection or immunised with parasite infected cell lines, generate a strong cytotoxic T lymphocyte response in the peripheral blood which is effective in killing autologous schizont infected cells (Preston *et al.*, 1983; Innes *et al.*, 1989a). Adherent cells isolated from the peripheral blood exhibit a strong cytostatic effect on macroschizont infected cells via a soluble factor (Preston & Brown, 1988). All these observations suggest that considerable alterations of circulating lymphocytes in the peripheral blood are associated with *T. annulata* infection. However, the cause of leucopenia and involvement of various lymphocyte subpopulations are not clearly understood.

Large numbers of lymphocytes continuously enter and exit lymphoid and non-lymphoid tissues using blood as a traffic route with a mean transit time of approximately 30 hrs (Westermann & Pabst, 1990). Therefore, analysis of the cell dynamics and alterations in the various circulating lymphocyte subpopulations could provide useful information on the involvement of various lymphocyte subsets in the development of immunity against the parasite.

In the previous chapter (chapter 4), a pre-existing anti MHC response was shown to prevent development of parasite specific immune responses in some of the animals after immunisation with a *T. annulata* infected cell line. Variable parasitological reactions ranging from acute to subacute and mild forms of the disease were observed in the animals after sporozoite challenge. We decided to investigate phenotypic changes in the peripheral blood lymphocytes of these animals to understand the pathogenesis of *T. annulata* infection and involvement of various lymphocyte subsets in the development of immunity. We hoped to find differences in the response of animals, which became immune after cell line immunisation in the presence of an existing allogeneic response and those which did not respond to immunisation, to further elucidate the effect of allogeneic responses on cell line

immunisation. As *T. annulata* infection leads to severe leucopenia, an attempt was made to define the involvement of various leucocyte subpopulations.

5.2 MATERIALS AND METHODS

5.2.1 Animals and experimental design

Peripheral blood leucocytes were monitored from the animals used in the previous chapter (chapter 4). Two other animals used for lymphatic cannulation studies and inoculated with *T. annulata* sporozoites (details in chapter 6) were also used for these experiments. The different procedures used on these animals are briefly illustrated in table 5.1, with detail in the relevant chapters.

Table 5.1: EXPERIMENTAL DESIGN

Animal no.	Priming	Immunisation	Challenge
Group 1a			
200	Uninfected leucocytes	TaA ($1x10^6$ cells)	Sporozoites
223	-	----do----	----do----
215	Uninfected leucocytes	----do----	----do----
221	-	----do----	----do----
Group 1b			
209	-	-	Sporozoites
903	-	-	----do----
928	-	-	----do----
Group 2a			
838	Skin grafted	TaH ($1x10^6$ cells)	Sporozoites
841	----do----	----do----	----do----
845	----do----	----do----	----do----
Group 2b			
933	-	TaH ($1x10^6$ cells	Sporozoites
937	-	----do----	----do----
859	-	----do----	----do----
Group 3a			
245	Skin grafted	TaH ($1x10^4$ cells)	Sporozoites
246	----do----	----do----	----do----
249	----do----	----do----	----do----
Group 3b			
250	-	----do----	Sporozoites
264	-	----do----	----do----
273	-	----do----	----do----

- No action taken

5.2.2 Monitoring of animals

The clinical, parasitological and haematological reactions of these animals were monitored after primary immunisation with a *T. annulata* infected cell line and after challenge with sporozoites. The detailed clinical, parasitological and haematological observations are illustrated in the relevant chapters.

5.2.3 Leucocyte counts

TLC were monitored in all animals at regular intervals. Blood was collected from the jugular vein in 2 ml vacutainers containing disodium EDTA (Becton Dickinson) for haematological examinations. Leucocytes were counted using a Coulter Counter (Coulter ZBI electronic particle counter, Coulter Electronics Ltd.). Thin blood smears were prepared for differential leucocyte counting on clean glass slides, fixed with methanol and stained with Giemsa stain. A total of 200 leucocytes were counted to calculate percent polymorphonuclear cells (neutrophils, eosinophils and basophils) and mononuclear cells (lymphocytes and monocytes) in the blood. These figures were used to calculate total circulating polymorphonuclear cells and mononuclear cells in the periphery.

5.2.4 Peripheral blood mononuclear cells (PBM)

Collection and separation of PBM has been described in detail in chapter 3 (3.4.2.1). Briefly, PBM were separated by centrifugation of blood collected in ACD on ficoll-hypaque (Lymphoprep). The cells were resuspended in FACS medium for immunostaining and flow cytometry. Cytospin smears were occasionally prepared from these cells to check for polymorphonuclear cell contamination which was usually less than 1% in the purified population.

5.2.5 Monoclonal antibodies

Various mAbs and immunoconjugates have been described in detail in chapter 3 (3.8.1). The following mAbs were used for analysis of various lymphocyte subpopulations in this chapter. The mAb IL-A26 for $CD2^+$ cells, IL-A12 for $CD4^+$ cells, IL-A51 or SBU-T8 for $CD8^+$ cells, IL-A29 for $WC1^+$ $\gamma\delta$ T cells. B cells were identified by mAb VPM 30, monocytes and macrophages by IL-A24. This mAb reacts with monocytes, macrophages and granulocytes. Since granulocytes were removed from peripheral blood by centrifuging on ficoll-hypaque, only monocytes and macrophages were identified with this mAb. MHC class II molecules were identified by J 11 or IL-A21, CD25 by IL-A111 and CD45RB molecules by CC 76.

5.2.6 Immunofluorescent Staining

The technique used for indirect immunofluorescent staining and flow cytometry has been explained in chapter 3 (3.8). This technique was used to analyze phenotypic changes in the PBM following primary cell line immunisation and secondary sporozoite infection.

The results were processed to calculate the percentage of various lymphocyte subpopulations in the peripheral blood. These, along with total mononuclear cell counts, were used to calculate the absolute number of various lymphocyte subpopulations in the peripheral blood.

5.3 RESULTS

5.3.1 Parasitological reactions

The detailed parasitological reactions in the animals used in these experiments have been described in the relevant chapters (chapter 4 & 6). The brief parasitological reactions are shown in table 5.2 for reference. All animals in group 1a exhibited mild parasitological reactions after cell line immunisation and were immune on sporozoite challenge. The naive animals in group 1b were not immunised with a cell line but were inoculated only with sporozoites and acted as challenge controls. All these animals suffered severely from acute theileriosis on sporozoite challenge.

The animals in group 2a were skin grafted to produce an allogeneic response before immunisation with the cell line. Two animals in this group (838 & 845) showed mild parasitological reactions after immunisation and were immune on sporozoite challenge. The third animal (841) did not show any parasitological reactions on immunisation and suffered from an acute disease after challenge with sporozoites. All the animals in group 2b reacted mildly after immunisation and were immune on sporozoite challenge.

The animals in group 3a were also skin grafted. None of these animals showed any parasitological reactions after immunisation with the cell line and suffered from acute clinical theileriosis after challenge with sporozoites. In group 3b, only animal 264 exhibited mild reactions after immunisation. No piroplasms were seen in the smears from the other two animals (250 & 273) in this group after immunisation. However, a parasite infected cell line of recipient origin was isolated from both the animals indicating a very mild response to the cell line. Animal 264 was found to be

immune after sporozoite challenge, animal 250 suffered moderately and recovered but 273 suffered severely. All the animals showing severe disease symptoms were either treated with buparvaquone (Butalex, Mallinkrodt Veterinary Ltd.) or euthanased as shown in table 5.2.

Table 5.2: Summary of parasitological reactions in animals after immunisation and challenge.

Group no.	Animal no.	After immunisation			After challenge		
		Maximum schizonts (%)	Maximum piroplasms (%)	Severity of reaction	Maximum schizonts (%)	Maximum piroplasms (%)	Severity of reaction
1a	200°	0.5	0.5	mild	0.5	1	mild
	223	0	0.8	mild	0.8	1.5	mild
	215°	0.5	0.5	mild	0.5	3	mild
	221	0.5	6	mild	0	2	mild
1b	209*	-	-	-	25	28	severe
	903**	-	-	-	36	10	severe
	928***	-	-	-	10	2.5	severe
2a skin grafted	838	0.3	0.8	mild	0.1	0.3	mild
	841***	0	0	none	43	13	severe
	845	0	0.5	very mild	0.3	0.3	mild
2b	859	2.5	1.2	mild	0.2	0.5	mild
	933	0.2	0.5	mild	0.5	0.3	mild
	937	0.3	0.9	mild	0.6	0.5	mild
3a skin grafted	245¤	0	0	none	31	10	severe
	246***	0	0	none	60	8	severe
	249***	0	0	none	36	12	severe
3b	250	0	0	very mlid	15	23	moderate
	264	0.3	0.2	mild	0.6	1	mild
	273¤	0	0	none	52	12	severe

- No action taken.
° Primed with uninfected leucocytes
¤ Euthanised on day 10

* Treated on day 12.
** Treated on day 11.
*** Treated on day 10.

5.3.2 Total leucocyte counts in the peripheral blood

TLC decreased slightly in most of the animals immediately after cell line immunisation but returned to normal levels by day 10 (appendix, no. 11). The initial mild leucopenia was neither related to the dose of cells used for immunisation nor to the following parasitological reactions in these animals (fig. 5.1). All the skin grafted animals in group 3a which showed no piroplasms or schizonts after immunisation also exhibited a mild drop in TLC which quickly returned to normal levels (fig. 5.1e). However, the drop was less marked and shorter lived in these animals than the ones showing mild parasitological reactions after immunisation (fig. 5.1a, 5.1d and animal 838 & 845 in fig. 5.1c).

Animals showing severe parasitological reactions after sporozoite challenge [all animals in group 1b (fig. 5.1b), animal 841 in group 2a (fig. 5.1c), all animals in group 3a (fig. 5.1e) and 273 in group 3b (fig. 5.1f)] also exhibited severe leucopenia (appendix, no. 12). Whereas, the fall in TLC was less severe in other animals which were immune to sporozoite challenge (group 1a in fig. 5.1a and group 2b in fig. 5.1d). TLC values started to increase in very sick animals after treatment with buparvaquone. Leucopenia would have been very severe had these animals not been treated. The leucopenia corresponded to a decrease in total polymorphonuclear cells (fig. 5.2) as well as total mononuclear cells (fig. 5.3) in the peripheral blood. The drop in total mononuclear cells was due to lymphocytopenia and usually not due to decrease in the number of monocytes.

5.3.3 Changes in the T cell subsets

Initial leucopenia was accompanied by a drop in total $CD2^+$ cells in peripheral blood of most of the animals after cell line immunisation which quickly returned to normal. A slight decrease in total circulating $CD2^+$ cells was also seen in the skin grafted animals which did not show any parasitological reactions after immunisation. The decline in $CD2^+$ cells in animals showing mild parasitological reactions was because of a fall in the total circulating $CD4^+$ cells (fig. 5.4) and $CD8^+$ cells (fig. 5.6). Levels of total $CD4^+$ cells returned to normal by day 10 after immunisation in most of the animals. Animals not showing any parasitological reactions after cell line immunisation also showed a mild transient drop in circulating CD4 cells (fig. 5.4d & animal 841 in fig. 5.4b). An increase in total circulating $CD8^+$ cells was seen between day 8 to 20 in most of the animals showing mild parasitological reactions

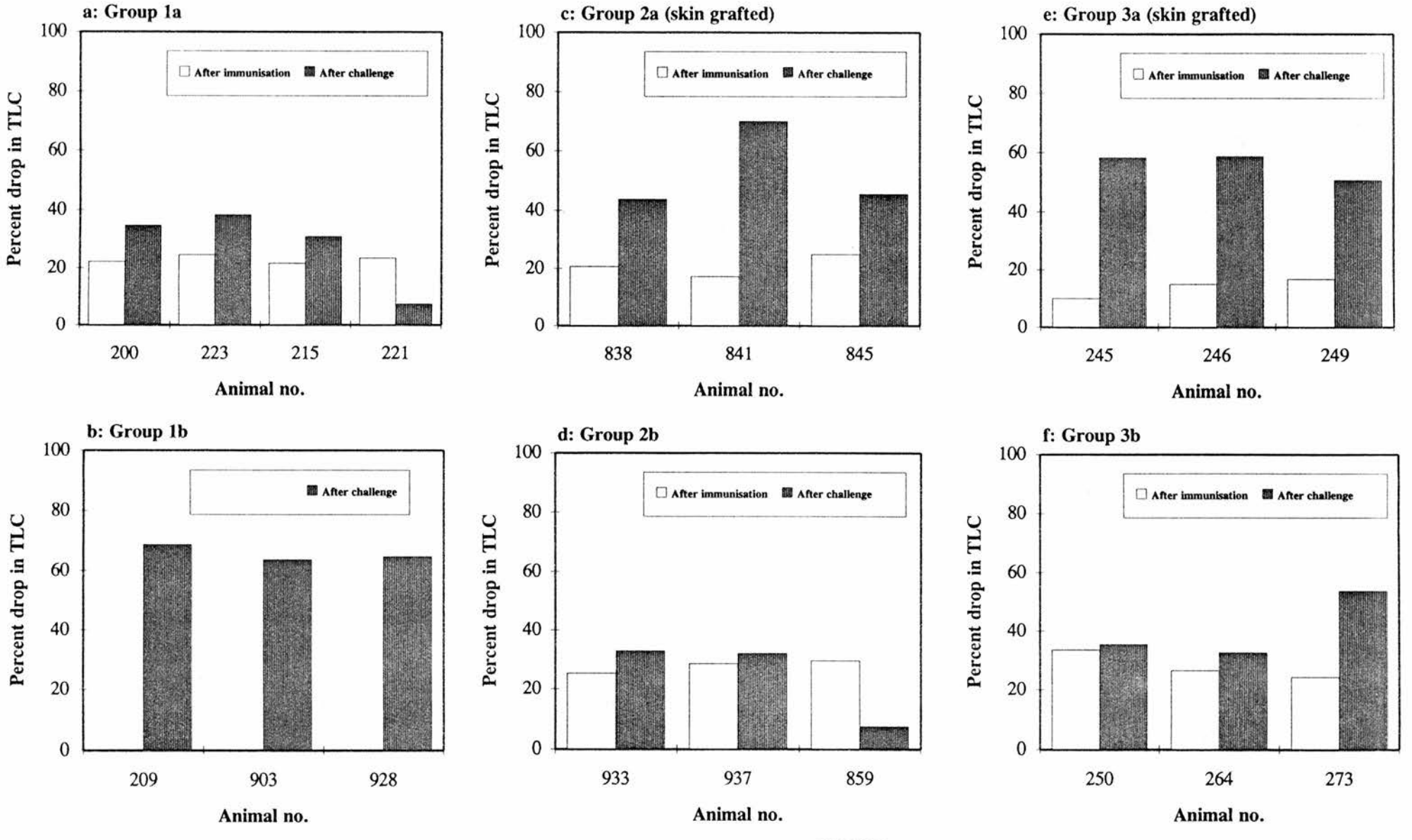


Figure 5.1: Drop in total leucocyte counts in the animals after cell line immunisation and sporozoite challenge.

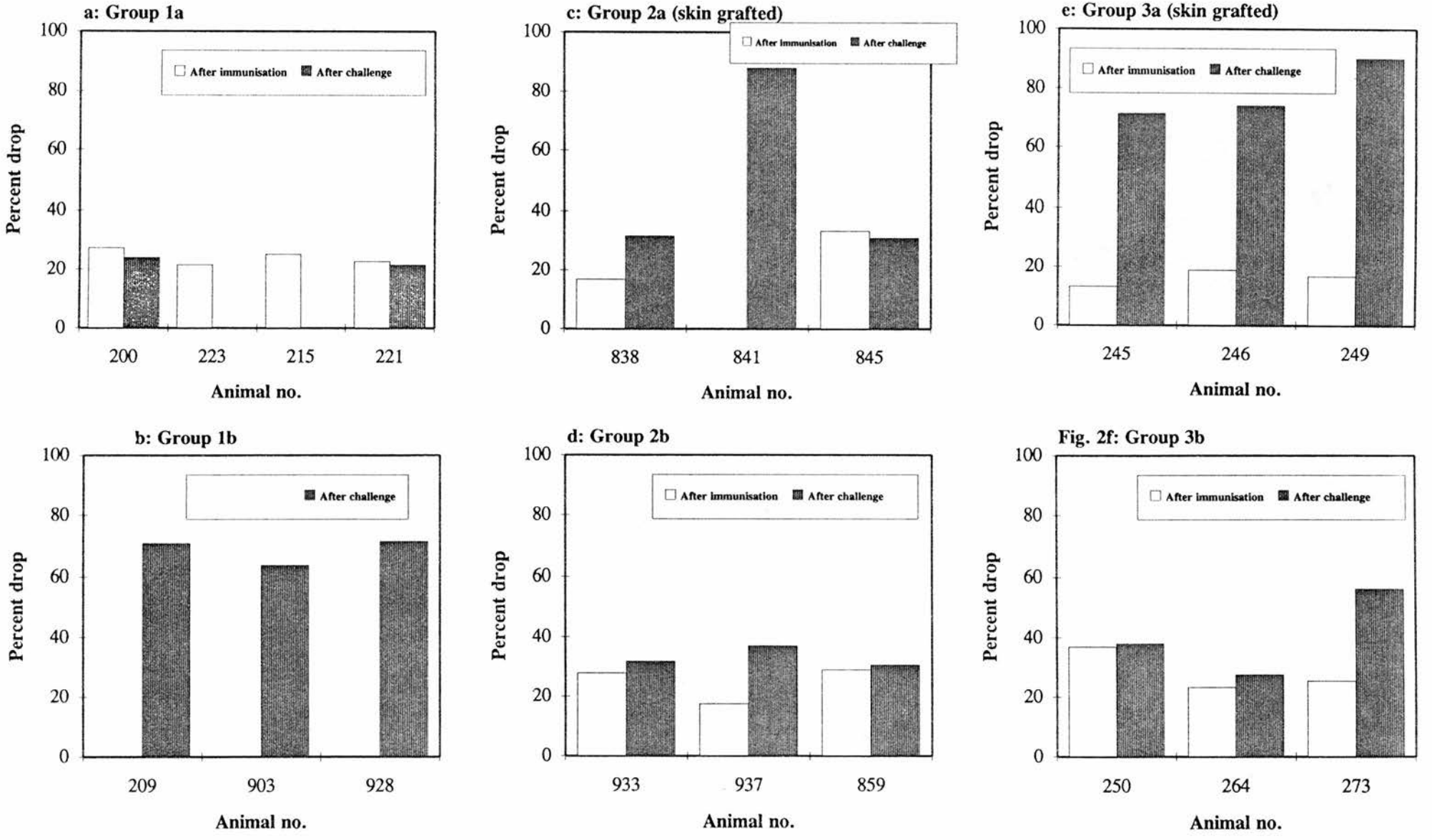


Figure 5.2: Drop in total polymorphonuclear cell counts in the animals after cell line immunisation and sporozoite challenge.

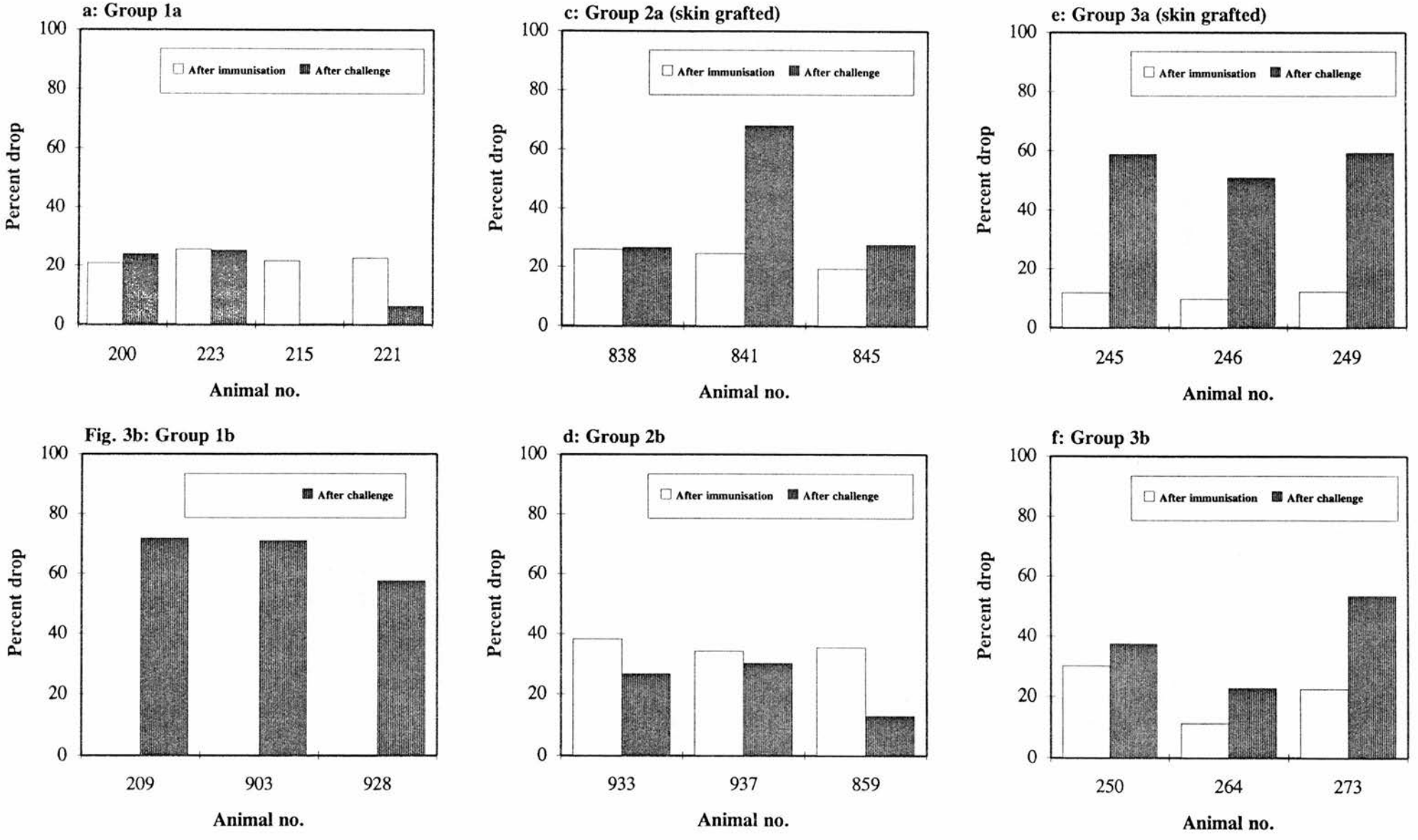


Figure 5.3: Drop in total mononuclear cell counts in the animals after cell line immunisation and sporozoite challenge.

after immunisation (all animals in fig. 5.6a & 5.6c, animals 838 & 845 in fig. 5.6b). The skin grafted animals not showing any parasitological reactions either exhibited no change or a mild early increase in the total $CD8^+$ cells which returned to normal levels soon afterwards (animal 841 in fig. 5.6b and all animals in fig. 5.6d). A slight increase in total circulating $\gamma\delta$ T cells was seen in some animals after immunisation while in others it remained unchanged (data not shown). The levels of circulating $\gamma\delta$ T cells were around $1x10^3$ per cmm in most of the animals.

On challenge with sporozoites, the drop in the circulating $CD2^+$ cells was more pronounced in animals showing severe parasitological reactions than in animals immune to challenge. Levels of total $CD2^+$ cells started to return to normal in animals showing severe disease only after treatment. An initial drop in $CD2^+$ cells after challenge corresponded to a fall in circulating $CD4^+$ and $CD8^+$ cells. It was more severe in animals showing acute disease symptoms {all animals in group 1b (fig. 5.5b) & 3a (fig. 5.5e), animal 841 (fig. 5.5c)}. $CD4^+$ cells returned to normal values very slowly in these animals after treatment. Total circulating $CD4^+$ cells also dropped slightly in immune animals on sporozoite challenge but soon returned to normal or near normal levels (fig. 5.5a, 5.5d & animals 838 & 845 in fig. 5.5c). The number of circulating $CD8^+$ cells initially remained unchanged in immune animals after sporozoite challenge. This was followed by a profound increase between days 8-18 (fig. 5.7a, 5.7d, animals 838 & 845 in fig. 5.7c and animals 250 & 264 in fig. 5.7f). On the other hand, animals showing acute disease symptoms on sporozoite challenge exhibited a gradual reduction in circulating $CD8^+$ cells which became more severe with progression of the disease (fig. 5.7b, animal 841 in fig. 5.7c and animal 273 in fig. 5.7f). These animals later exhibited an increase in total circulating $CD8^+$ cells corresponding to recovery after treatment. There was a slight drop in total circulating $\gamma\delta$ T cells in most of the animals after sporozoite challenge which was more pronounced in the naive animals undergoing acute disease than in the immune animals (data not shown).

5.3.4 CD4:CD8 ratios

The ratio of $CD4^+$ vs $CD8^+$ cells was in favour of $CD4^+$ cells in most of the animals at the time of immunisation with the cell line. It increased in favour of $CD4^+$ cells initially, but fell to unity after day 10 and later below in favour of CD8 cells in most of the animals undergoing mild parasitological reactions after immunisation (fig.

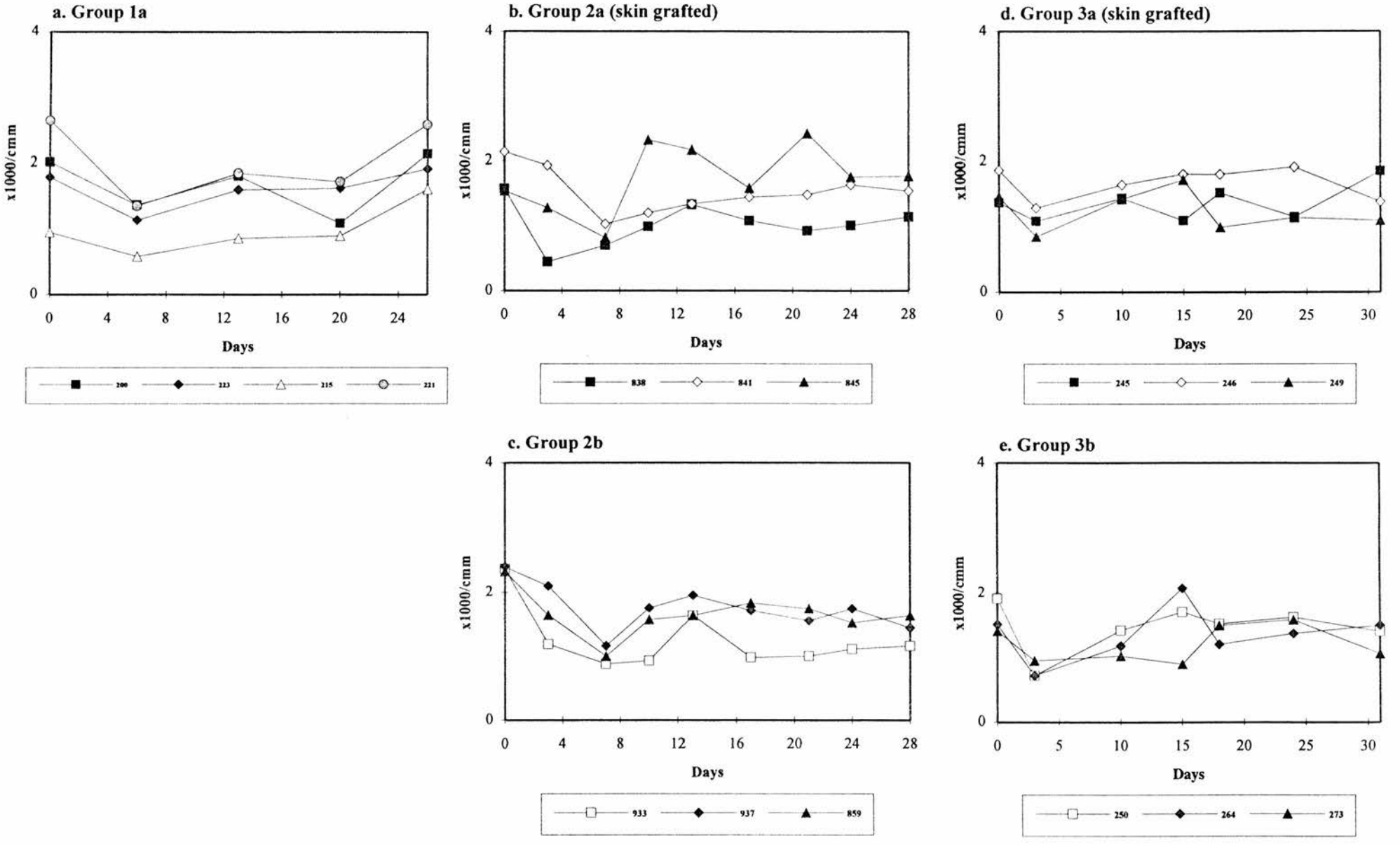


Figure 5.4: Total CD4+ cells in the peripheral blood of animals after cell line immunisation.

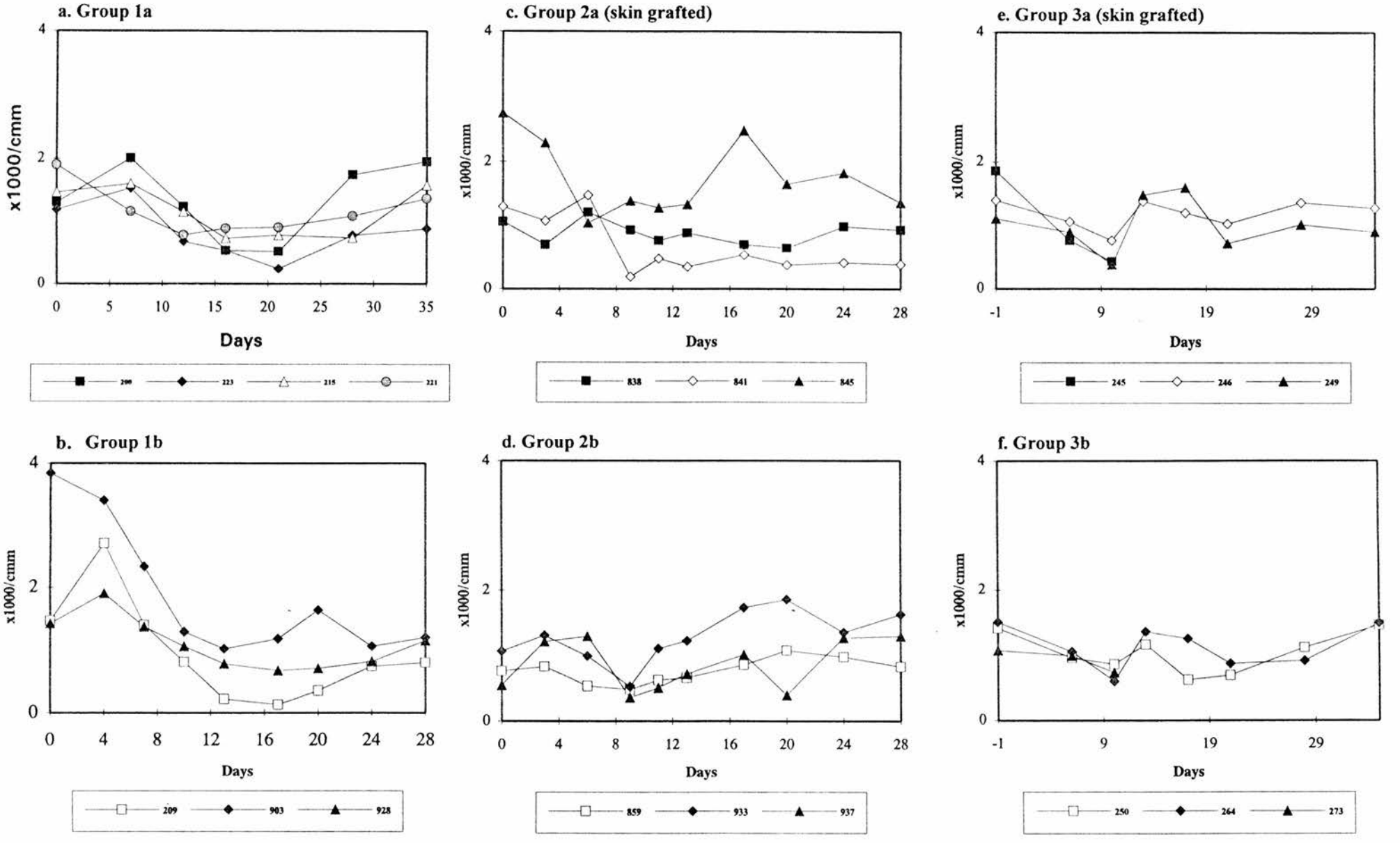


Figure 5.5: Total CD4+ cells in the peripheral blood of animals after sporozoite challenge.

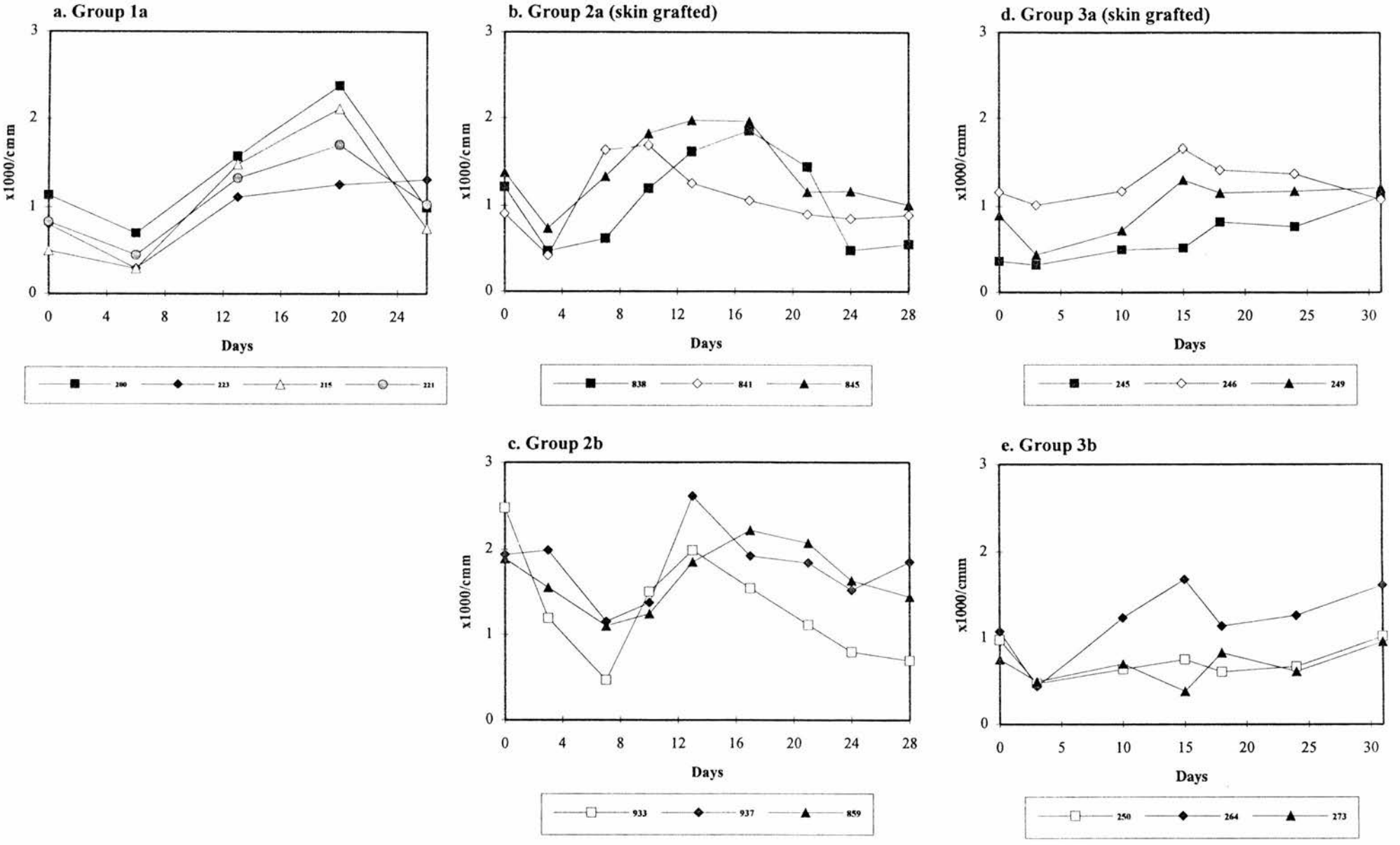


Figure 5.6: Total CD8+ cells in the peripheral blood of animals after cell line immunisation.

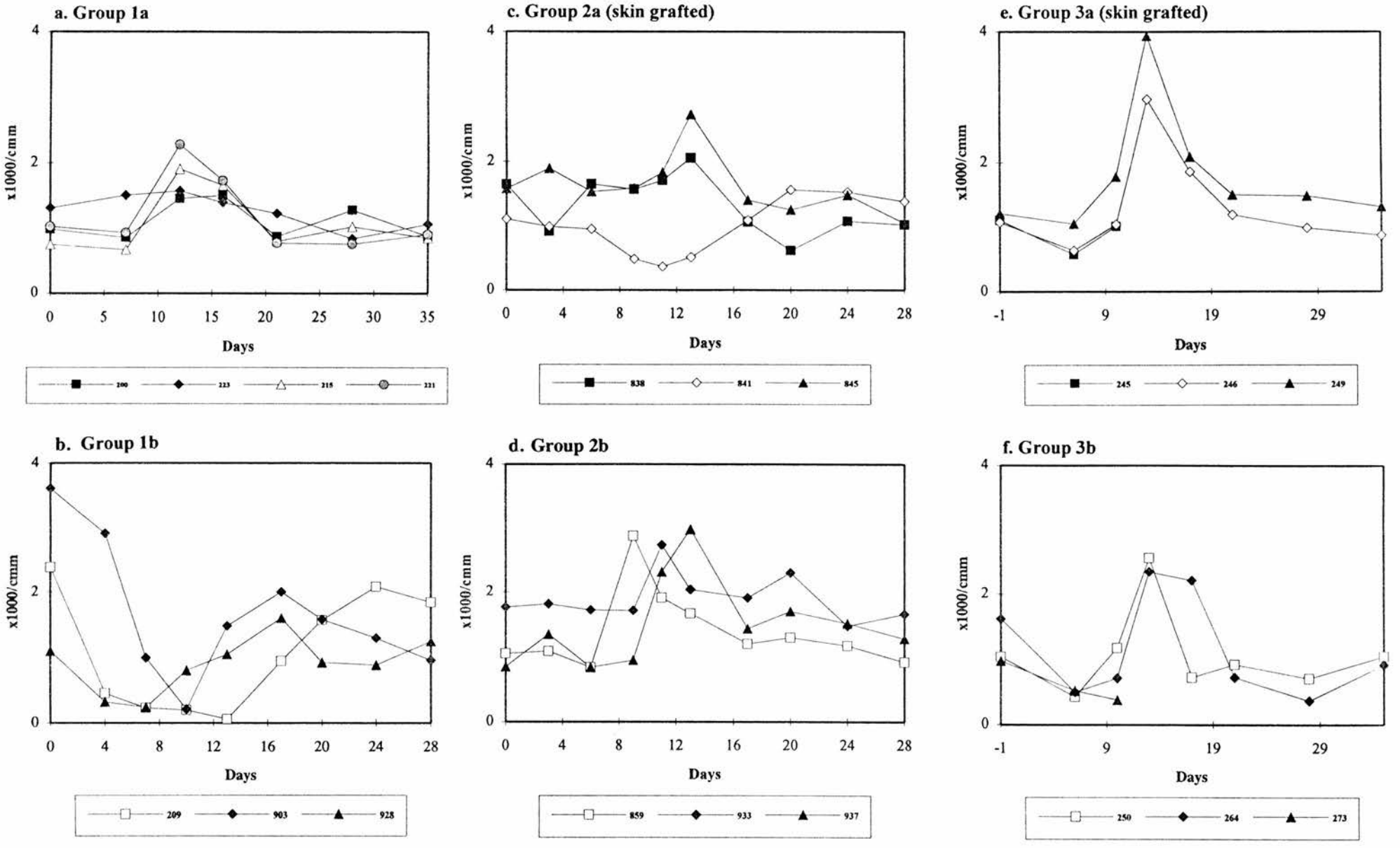


Figure 5.7: Total CD8+ cells in the peripheral blood of animals after sporozoite challenge.

5.8a, fig. 5.8c, animal 838 & 845 in fig. 5.8b and animal 264 in fig. 5.8e). The skin grafted animals not showing any parasitological reactions after cell line immunisation always expressed CD4:CD8 ratios above unity in favour of CD4 cells (fig. 5.8d & animal 841 in fig. 5.8b). After challenge with sporozoites, all immune animals showed a shift in the ratio in favour of CD8 cells after day 9 indicating an increase in circulating CD8 cells as compared to CD4 cells (fig. 5.9a, 5.9d, animal 838 & 845 in fig. 5.9c and animal 250 & 264 in fig. 5.9f). The ratio usually remained higher in favour of $CD4^+$ cells in the animals undergoing acute theileriosis after sporozoite challenge. However, it quickly fell below unity in favour of $CD8^+$ cells in all these animals after treatment (fig. 5.9b, 5.9e, animal 841 in fig. 5.9c).

5.3.5 Changes in B cells

A slight drop in total circulating B cells was observed in most of the animals immediately after immunisation (fig. 5.10). Total B cells began to rise after day 12 in most of the animals reacting mildly after cell line immunisation and becoming carriers (fig. 5.10a, 5.10c, animals 838 & 845 in fig. 5.10b). B cell levels usually remained low and did not show any increase in other skin grafted animals which did not show any parasitological reactions after immunisation (fig. 5.10d & animal 841 in fig. 5.10b). Sporozoite challenge produced a sharp decrease in total B cells corresponding to leucopenia in the naive animals and other skin grafted animals showing clinical disease which stopped falling further in some animals after treatment or started to increase slowly in others (fig. 5.11b, 5.11e & animal 841 in fig. 5.11c). Variable B cell kinetics were seen in the immune animals after challenge. Total circulating B cells either slightly decreased or showed no change in immune animals after sporozoite challenge. This was followed by an increase in their number in some animals usually after day 15 (fig. 5.11a, 5.11d, animals 838 & 845 in fig. 5.11c and animals 250 & 264 in fig. 5.11f).

5.3.6 Monocytes or macrophages

Circulating monocytes/macrophages usually seemed not to be involved in leucopenia. Levels of these cells did not change very much for the first few days after immunisation. Most of the animals reacting mildly to the cell line expressed elevated levels of total circulating monocytes in the peripheral blood usually after day 10 of immunisation (fig. 5.12a, 5.12c, animals 838 & 845 in fig. 5.12b and animal 264 in fig. 5.12e). Levels of circulating monocytes decreased slightly during this period in

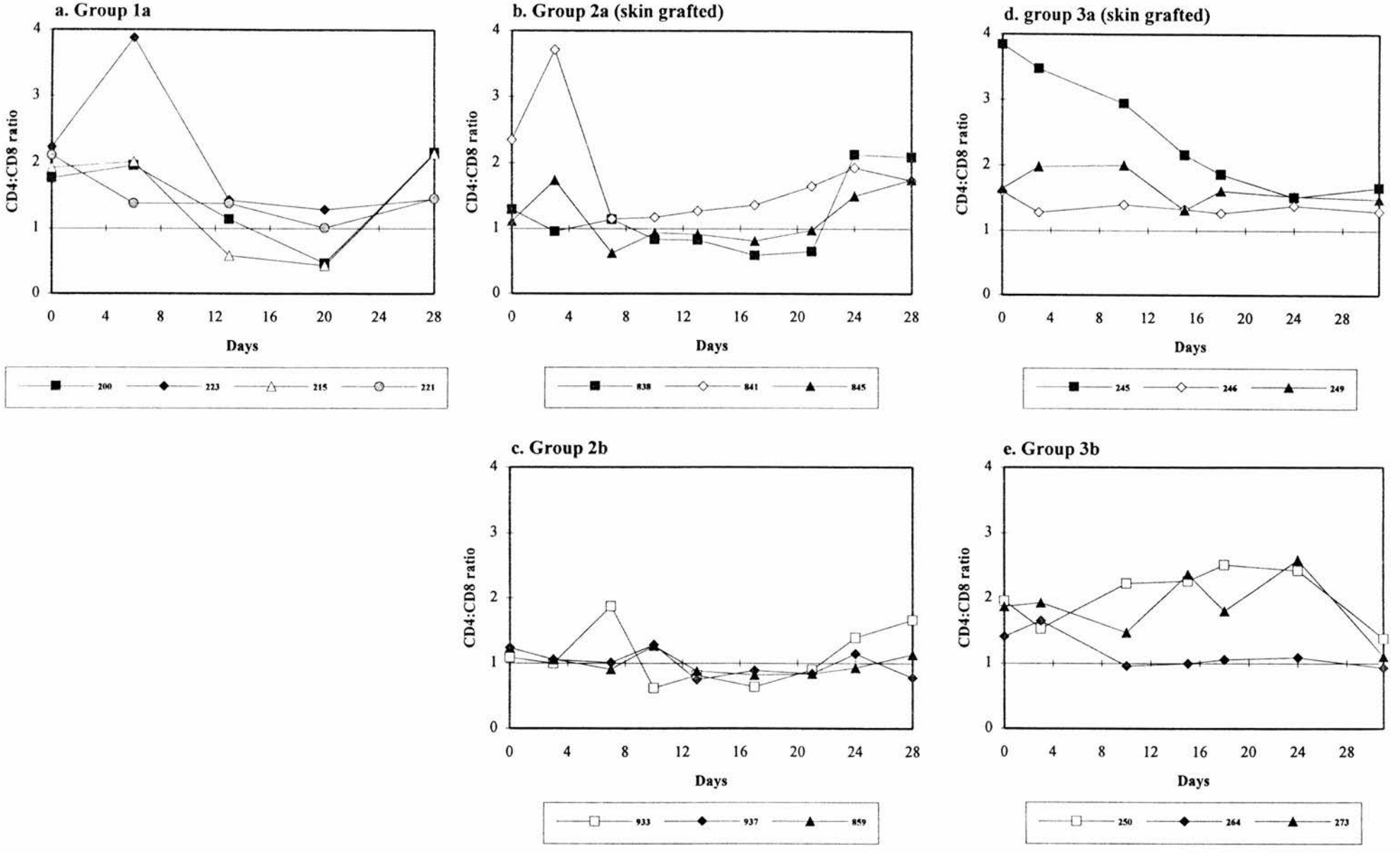


Figure 5.8: Ratio of CD4+ vs CD8+ cells (CD4:CD8) in the peripheral blood of animals after cell line immunisation.

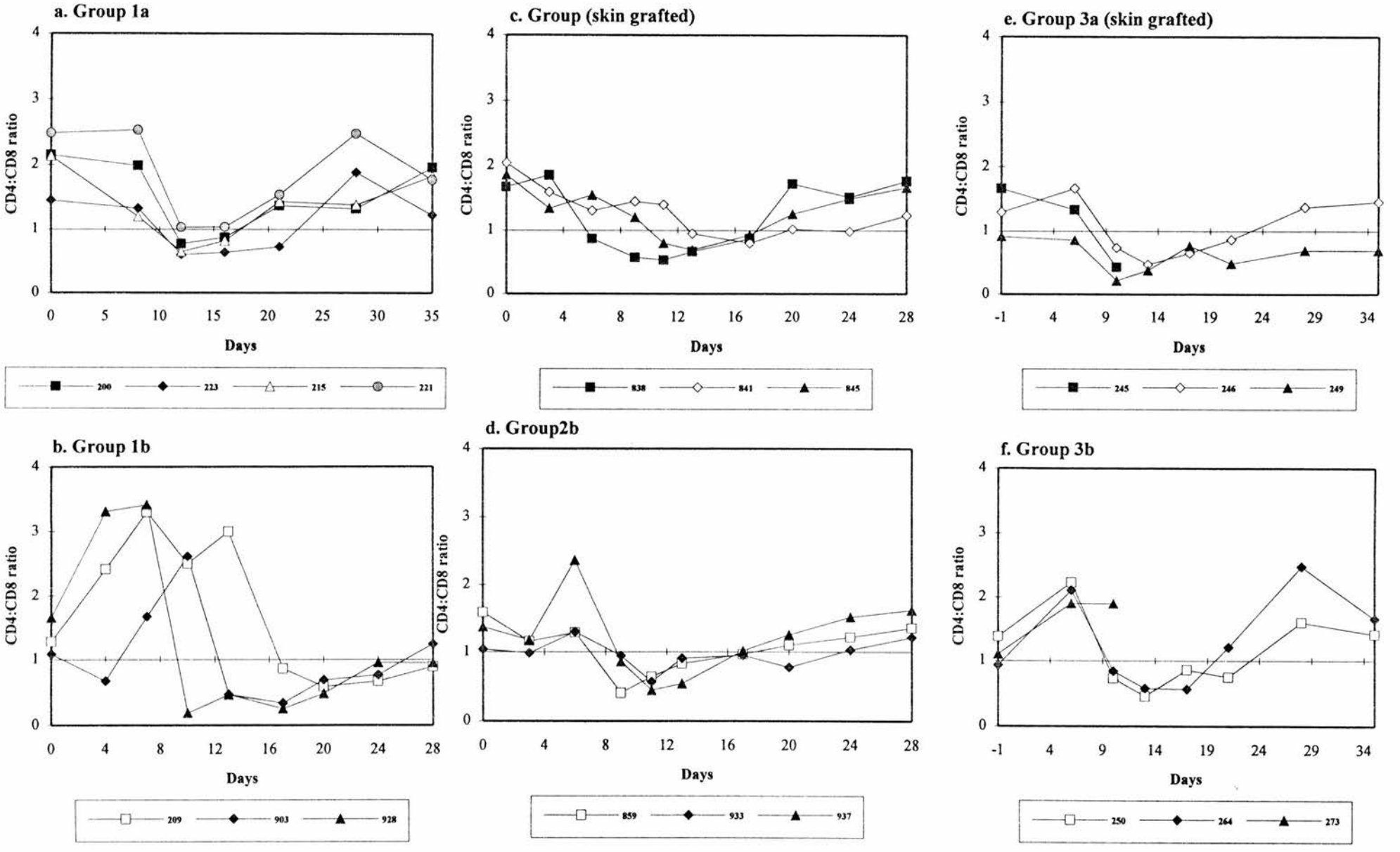


Figure 5.9: Ratio of CD4+ vs CD8+ cells (CD4:CD8) in the peripheral blood of animals after sporozoite challenge.

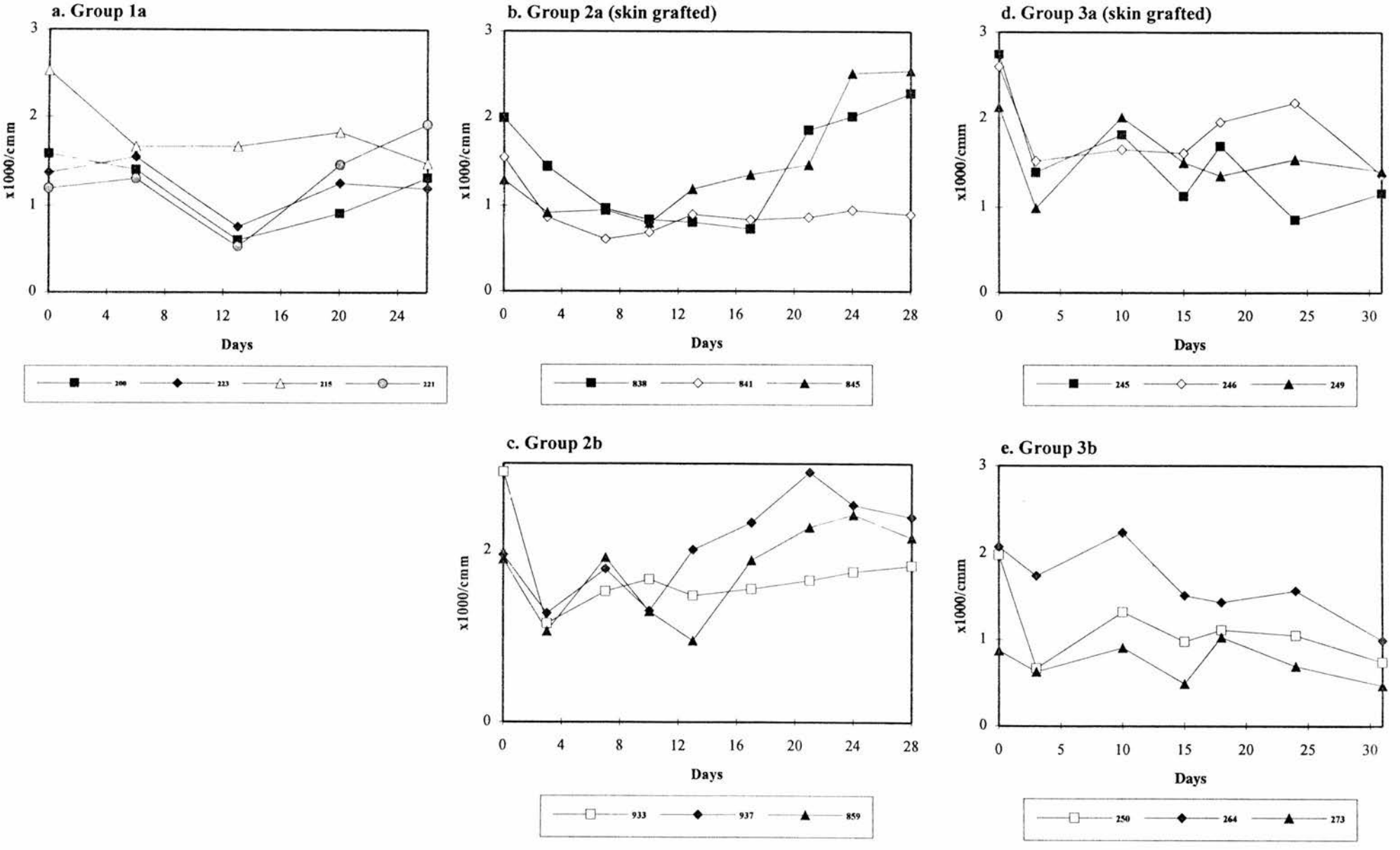


Figure 5.10: Total B cells in the peripheral blood of animals after cell line immunisation.

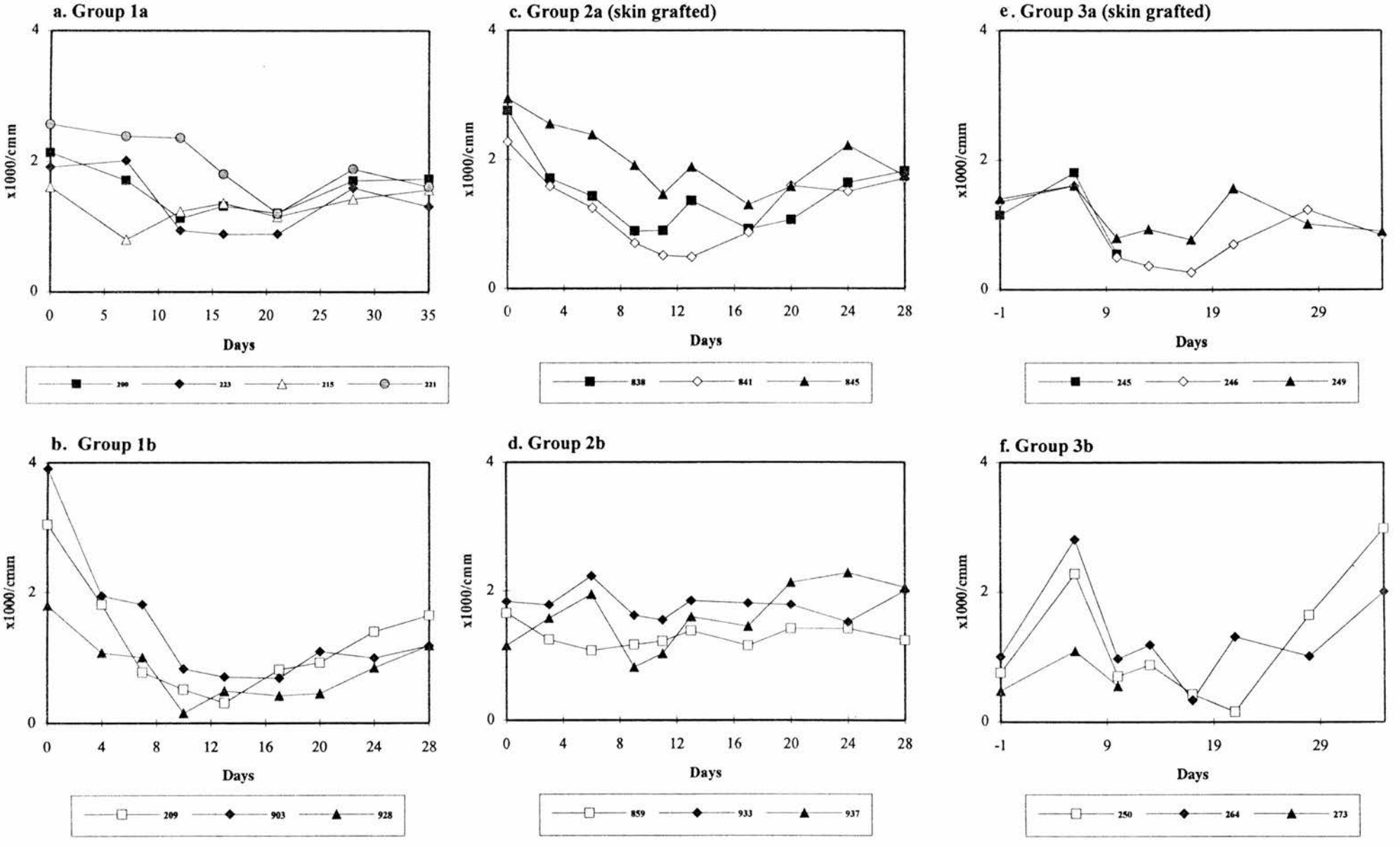


Figure 5.11: Total B cells in the peripheral blood of animals after sporozoite challenge.

skin grafted animals not showing any parasitological reactions after immunisation (fig. 5.12d). The sporozoite challenge revealed an early monocytosis in some of the immune animals (fig. 5.13a, 5.13d, animal 845 in fig. 5.13c & animal 264 in fig. 5.13f) following an increase in total $CD8^+$ cells. Naive animals, undergoing sporozoite challenge, and other skin grafted animals exhibiting acute theileriosis showed no change in the total circulating monocytes initially during acute clinical reactions. However, some animals expressed monocytosis during the recovery phase after treatment (fig. 5.13b & 5.13e).

5.3.7 Expression of IL-2Rα (CD25) on PBM

Only a few cells expressing CD25 were seen in the peripheral blood before cell line immunisation or sporozoite challenge in all the animals. The number of $CD25^+$ cells increased in the periphery between days 7-10 after cell line immunisation. Data from a representative animal (845) is shown in fig. 5.14a. Immune animals also exhibited some $CD25^+$ cells after sporozoite challenge transiently around day 6 (fig. 5.14b shows data from immune animal 859 after sporozoite challenge), although the parasitological reactions were very mild in these animals. Naive animals undergoing an acute infection after sporozoite challenge expressed $CD25^+$ cells in the periphery from day 6 to 13 (fig. 5.14c shows data from animal 841 undergoing acute theileriosis). The skin grafted animals which did not show any parasitological reactions after cell line immunisation exhibited no increase in $CD25^+$ cells in the periphery at any stage.

5.3.8 Expression of CD45RB molecule on PBM

Expression of $CD45RB^+$ (naive phenotype) and $CD45RB^-$ (memory/activated phenotype) cells in the resting peripheral blood is almost equal. The level of $CD45RB^-$ cells initially decreased up to day 10 in the animals undergoing mild parasitological reactions after cell line immunisation. However, the balance turned in the favour of $CD45RB^-$ cells from day 13 onwards indicating the presence of more circulating effector/memory cells which returned to normal levels by day 21 (a representative figure 5.15a from animal 845 after cell line immunisation). Animals which did not show any parasitological reactions after cell line immunisation also did not express any changes in the kinetics of CD45RB positive and negative cells. The immune animals after sporozoite challenge expressed an increase in the $CD45RB^-$ cells from the beginning returning to normal levels by day 17 (a representative fig.

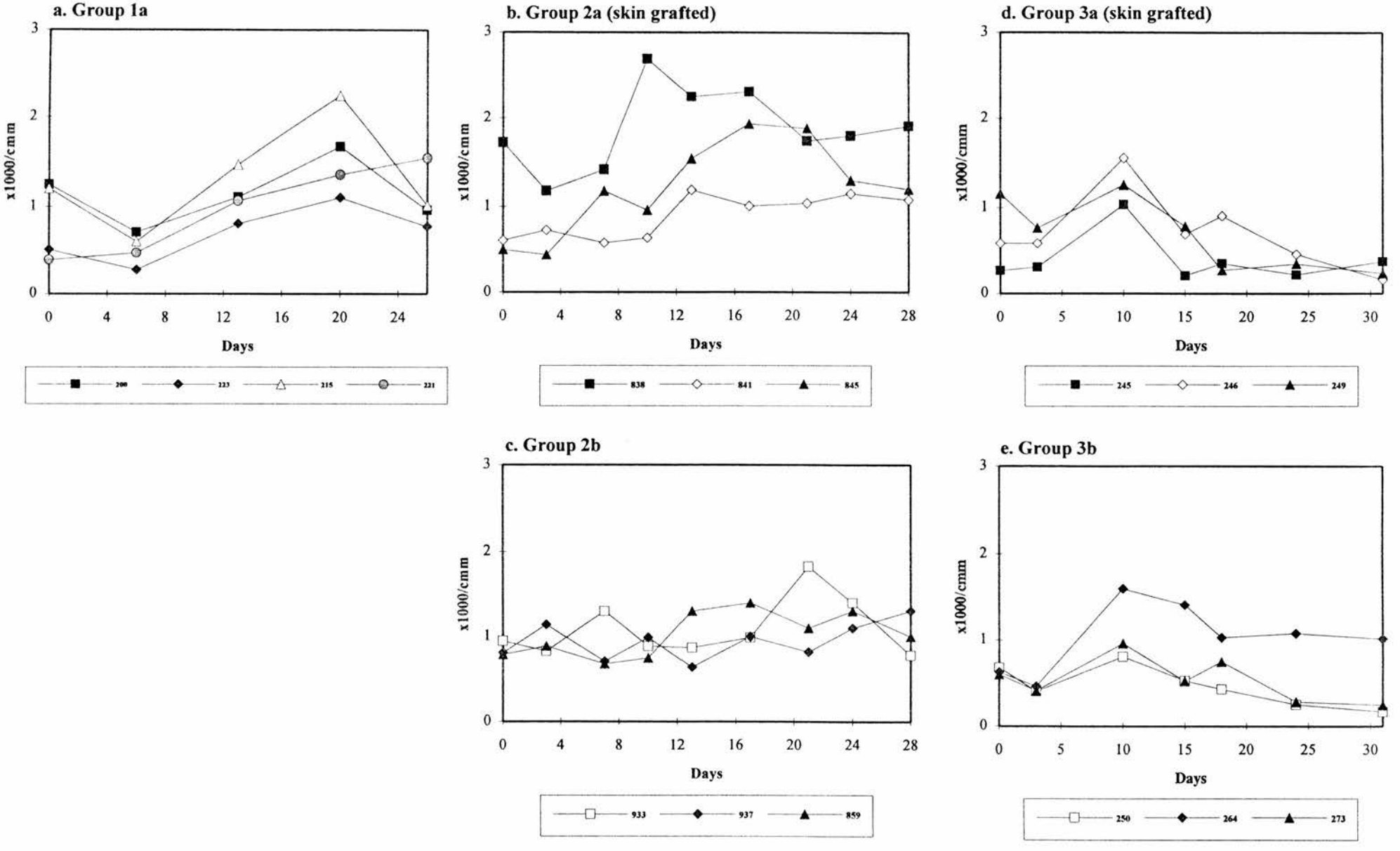


Figure 5.12: Total monocytes/macrophages in the peripheral blood of animals after cell line immunisation.

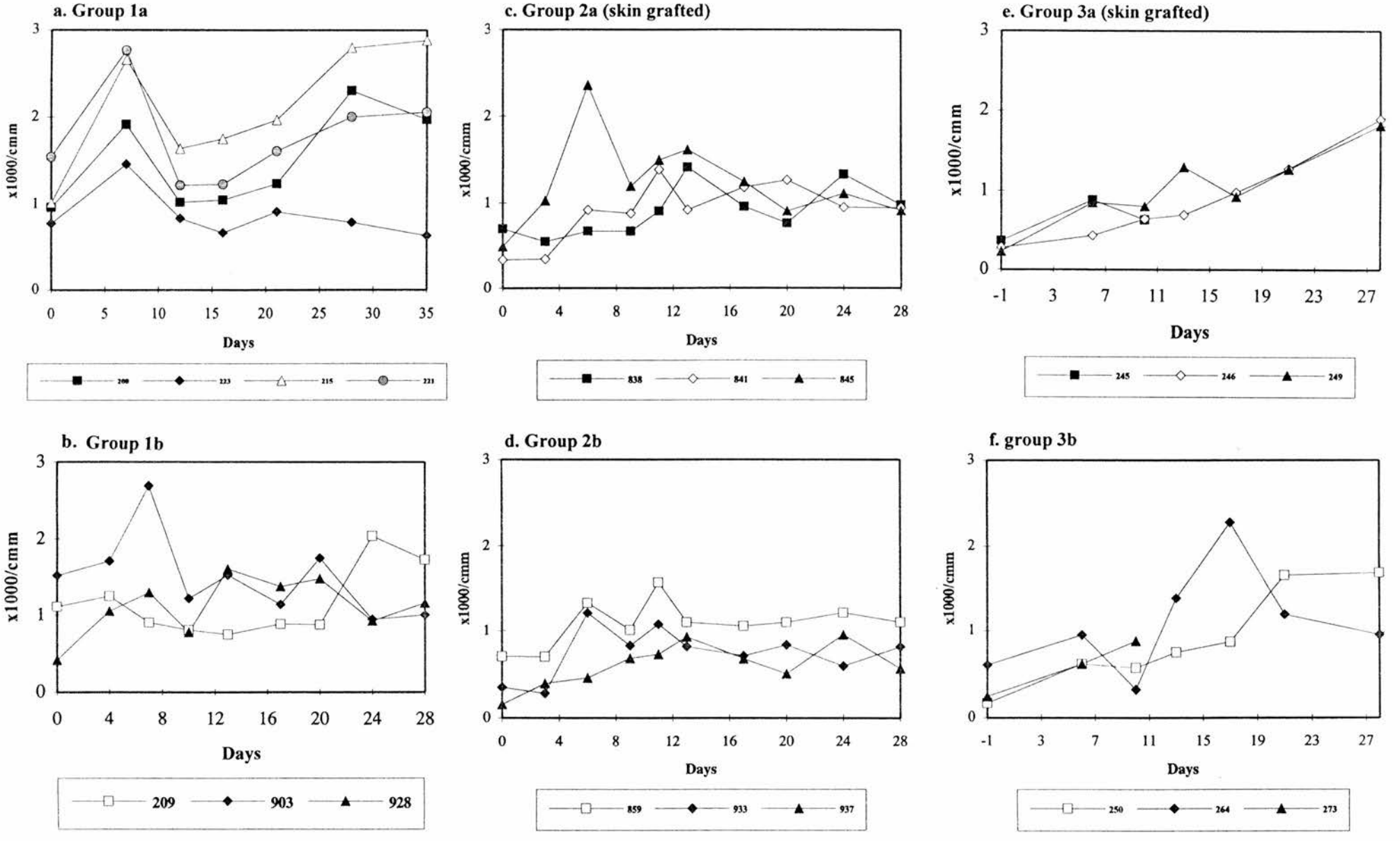
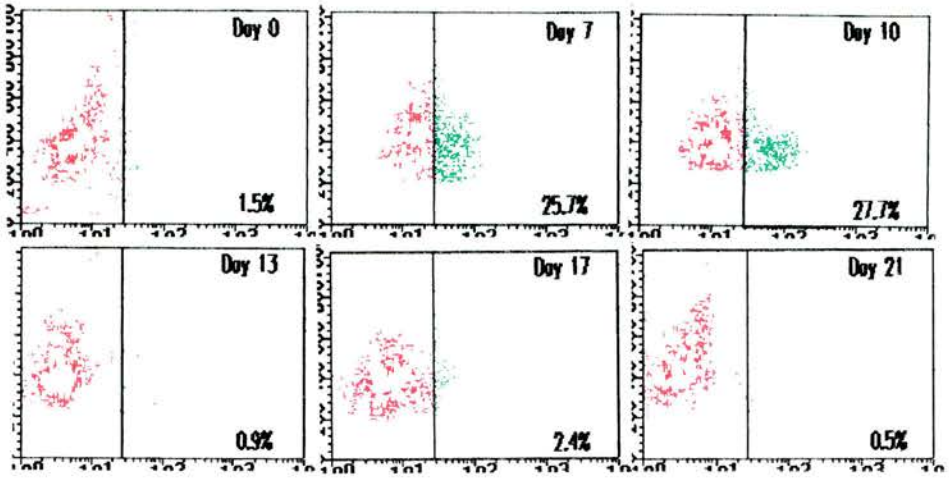
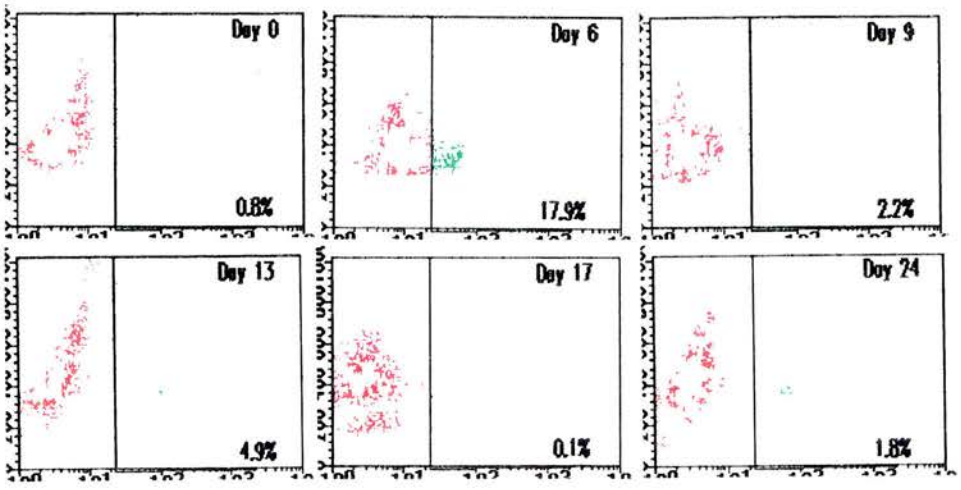


Figure 5.13: Total monocytes/macrophages in the peripheral blood of animals after sporozoite challenge.

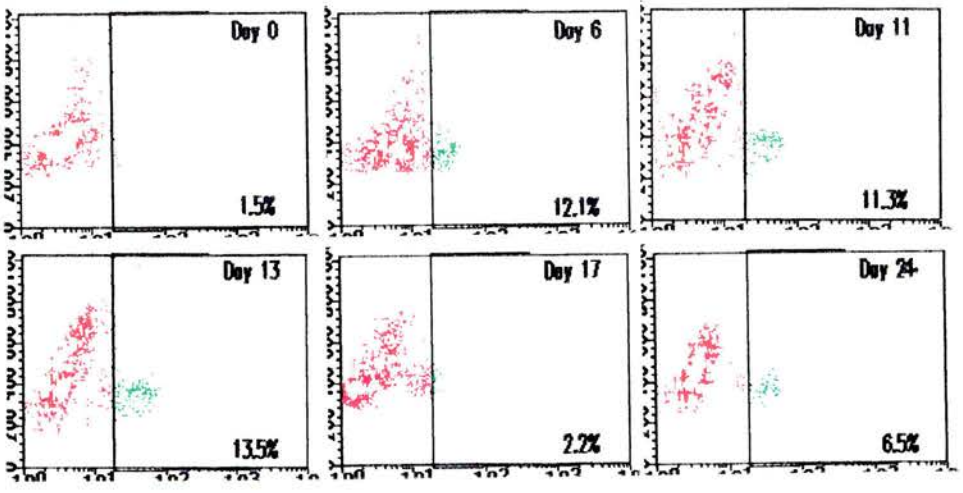
a. Animal no. 845 (mild reactions after cell line immunisation).



b. Animal no. 859 (Immune animal after sporozoite challenge).



c. Animal no. 841 (Acute disease after sporozoite challenge).



Forward scatter (FSC)

CD25+

Figure 5.14: Expression of IL-2 receptor (CD 25) on PBM.

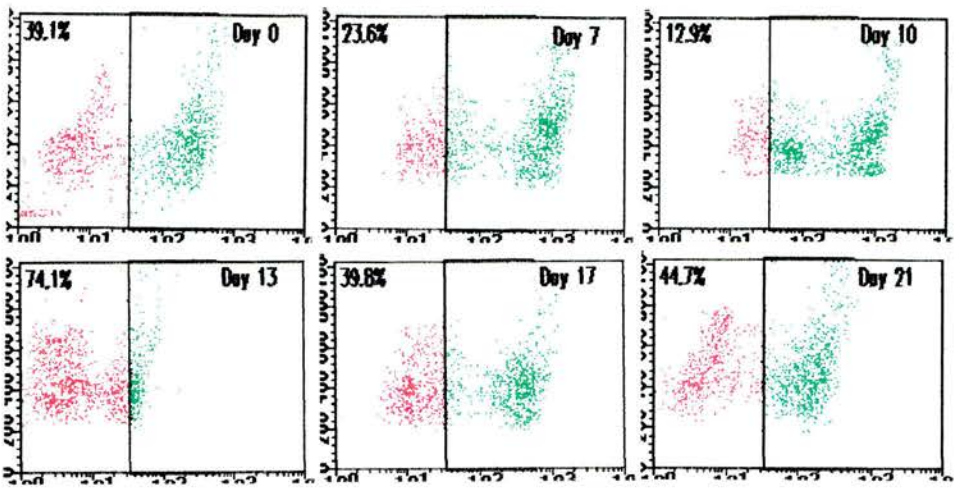
5.15b from animal 859). Increased levels of CD45RB$^-$ cells were seen in the periphery of animals suffering from acute theileriosis only after treatment. The increased numbers of blasting cells in the periphery were associated with CD45RB$^-$ phenotype at this stage (a representative fig. 5.15c from animal 841).

5.4 DISCUSSION

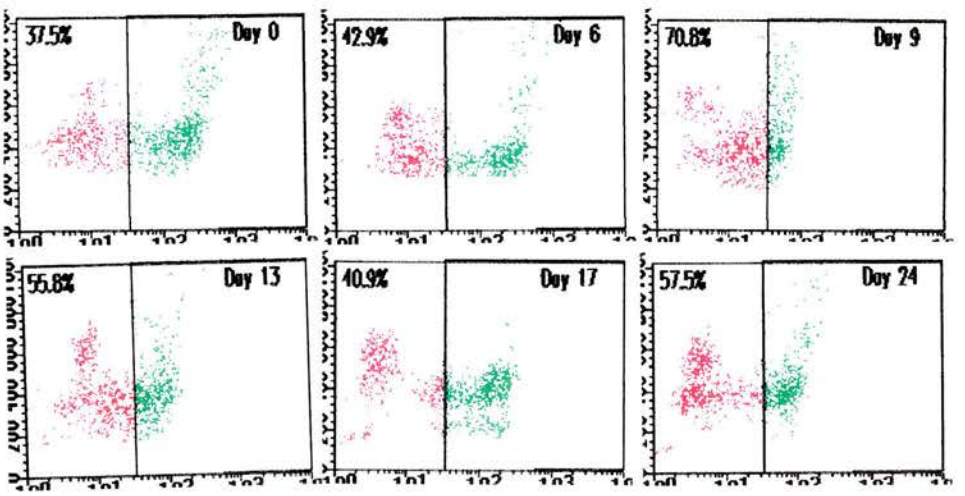
The parasitological reactions within groups were highly variable as described in detail in chapters 4 and 6. Animals having a pre-existing allogeneic response displayed variable parasitological reactions, after primary inoculation of the cell line or secondary sporozoite challenge, even within a group. Differences in the parasitological reactions between animals might have resulted from variation in the kinetics of parasite development and dissemination within and between groups of animals. This could arise either because of varying effects of the pre-existing allogeneic response on the parasite transfer to the recipient's cells, or because of differences in the susceptibility of animals. On the whole, animals either displayed mild parasitological reactions or no reactions after cell line immunisation. Animals could broadly be divided into three groups after sporozoite challenge on the basis of the severity of ensuing clinical reactions. The first being animals which were immune and only exhibited mild reactions and the second group was of animals which showed moderate reactions but recovered. The third group was of animals which suffered from acute theileriosis and were either treated or destroyed. The kinetics of various lymphocyte subpopulations were more related to the severity of ensuing parasitological reactions than to the dose of cell line used for immunisation, level of pre-existing allogeneic response at the time of immunisation or interval between immunisation and challenge. Further, the wide range of pre-infection total and differential cell counts and baseline FACS data impeded proper analysis of the results and demonstration of any statistically significant facts. The results from each animal are, therefore, presented individually. More emphasis is laid on qualitative kinetic trends than actual figures in the results and discussion.

Leucopenia has been reported in the animals undergoing acute *T. annulata* infection after natural or experimental sporozoite challenge (Sharma & Gautam, 1971; Laiblin, 1978; Preston *et al.*, 1992a). However, Hooshmand-Rad (1976) reported little or no change in the TLC and total lymphocytes after infection with infected

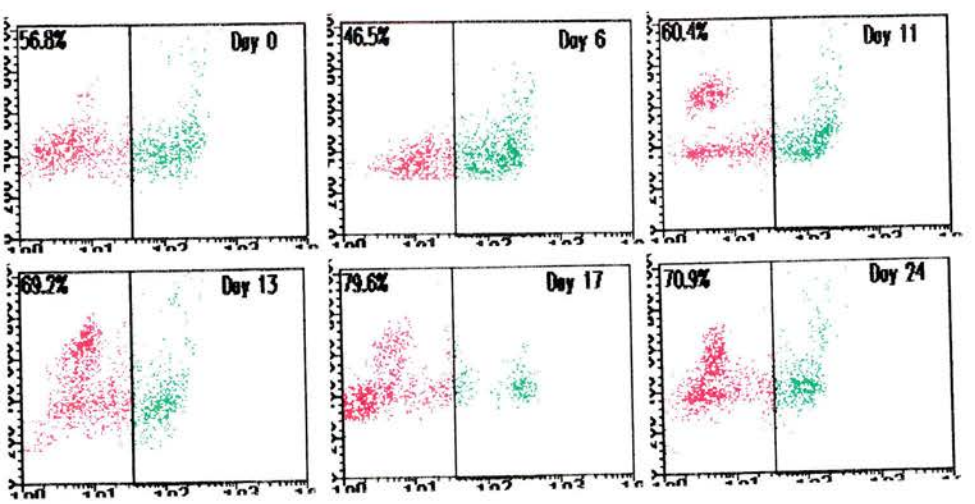
a. Animal no. 845 (mild reactions after cell line immunisation).



b. Animal no. 859 (Immune animal after sporozoite challenge).



c. Animal no. 841 (Acute disease after sporozoite challenge).



Forward scatter (FSC)

CD45RB+

Figure 5.15: Expression of CD45RB on PBM.

blood. The effect of cell line immunisation on circulating leucocytes has not been very well defined. Leucopenia was also a consistent finding in most of the animals in this study after cell line immunisation as well as after sporozoite challenge. Cell line immunisation produced a mild leucopenia of a short duration in the inoculated animals. Very mild leucopenia was seen even in the skin grafted animals which did not show any parasitological reactions. However TLC values returned to normal very quickly and earlier in these animals than those reacting mildly after cell line immunisation. This difference probably could be attributed to the fact that the animals not showing any parasitological reactions after cell line immunisation only produced a mild allogeneic response against the MHC antigens of the inoculated cell line. Whereas, the animals showing mild parasitological reactions first produced an allogeneic response followed by an antiparasite immune response. The fall in peripheral blood leucocytes in these animals was associated with the loss of circulating memory/activated phenotype ($CD45RB^-$) cells from the periphery. It is possible that the cells of memory/activated phenotype have migrated to the lymph node or other body compartments, where the parasite infected cells are present, for immune effector function.

Leucopenia was very severe after sporozoite challenge, especially in the animals showing acute symptoms of the disease. Animals immune to sporozoite challenge also showed mild leucopenia following challenge. All these observations suggest that a drop in TLC is a common finding after primary or secondary *T. annulata* infection, induced either by sporozoites or parasite infected cell lines, affecting both lymphocytes and granulocytes. Monocytes are usually not involved in the loss of white blood cells from the circulation after *T. annulata* infection. The severity of leucopenia was dependent on the ensuing parasitological reactions. Similar findings have been reported earlier in animals infected with varying doses of *T. annulata* sporozoites (Preston *et al.*, 1992a). The development of severe leucopenia is a particular characteristic finding during *T. parva* infection (Wilde, 1967).

The mild transient lymphocytopenia in the animals reacting mildly to the cell line immunisation was caused by the disappearance of $CD2^+$ cells corresponding to $CD4^+$ and $CD8^+$ cells from the periphery. B cells also contributed towards lymphocytopenia to a lesser degree in some animals exhibiting mild reactions after cell line immunisation. The level of $CD4^+$ cells returned to normal values quickly, whilst the

early drop in $CD8^+$ cells was followed by a gradual increase in their numbers in the peripheral blood. This usually exceeded the normal values between day 8-20 to a stage where CD4:CD8 ratios were below unity indicating the development of a CD8 dependent immune response. Animals inoculated with *T. annulata* infected cell lines have been reported to express a strong cytotoxic response in the periphery between day 9 to 23 (Innes *et al.*, 1989a). Initially the cytotoxic response was directed against the allogeneic MHC antigens of the immunising cell line followed by an MHC class I restricted response against autologous *T. annulata* infected cells. The kinetics of the development of cytotoxic responses in the peripheral circulation observed by Innes et. al. (1989a), matches the kinetics of $CD8^+$ cells observed in animals becoming immune after inoculation of a cell line in these experiments. This confirms that $CD8^+$ cells play a major role in the development of immunity after cell line immunisation. Increased levels of circulating $CD8^+$ cells were also observed in immune animals after sporozoite challenge and in seriously sick, sporozoite challenged naive animals but only after treatment. These observations are also compatible with an earlier report that MHC class I restricted parasite specific cytotoxic T lymphocytes appear in the circulation of animals recovering from *T. annulata* infection after sporozoite challenge (Preston *et al.*, 1983).

Lymphocytopenia was severe in animals exhibiting acute symptoms of the disease after sporozoite challenge and usually affected all the lymphocyte subpopulations except monocytes. Animals immune to sporozoite challenge only exhibited transient lymphocytopenia caused by a decline in circulating $CD4^+$ cells, B cells and in some animals by $\gamma\delta$ T cells. Total $CD8^+$ cells and monocytes were usually unaffected initially in the immune animals and exhibited an increase in the later stages. Similar findings have been reported earlier after inoculation of graded doses of *T. annulata* sporozoites in the batches of taurine and taurine x Sahiwal (*Bos indicus*) calves (Preston *et al.*, 1992a). Lethal infections were associated with severe leucopenia because of a continued absence of pan T cells, mature T cells, $CD4^+$ cells, $CD8^+$ cells, B cells and monocytes. In sublethal infections, monocytes, $CD8^+$ cells and B cells generally remained in the blood, increasing gradually in number.

Development of immunity to tropical theileriosis mainly seems to be cell mediated. Antibodies reacting with the macroschizont and piroplasm stages of the parasite have been consistently observed (Preston & Brown, 1985; Ahmed *et al.*,

1988; Kachani *et al.*, 1992b), but inoculation of sera from immune cattle could not protect naive animals from *T. annulata* infection (Dhar & Gautam, 1978; Samad *et al.*, 1984a). However, sera from immune animals can neutralise sporozoite infectivity (Gray & Brown, 1981) and block sporozoite entry into the host cells *in vitro* (Preston & Brown, 1985). Although it does not recognise any determinant on the surface of parasite infected cells (Shiels *et al.*, 1989), indicating that the humoral response might only be partially important in engendering protection. On the other hand, several reports provide evidence for the development of cell mediated immunity against tropical theileriosis. Earlier reports have shown reactions to intradermal inoculation and inhibition of leucocyte migration in response to *T. annulata* piroplasm antigen in the immune animals (Singh *et al.*, 1977). Samad *et al.* (1984b) demonstrated an initial decrease in E-rosette forming cells in the peripheral blood for the first two weeks after immunisation against *T. annulata* with infection and treatment method followed by an increase thereafter reaching a maximum by the fourth week. Development of immunity in cell line immunised animals has also been shown to be associated with an increase in E-rossette forming cells in the periphery (Chaudhri & Subramanian, 1992). Further, a transient appearance of the cytotoxic activity directed against the autologous parasite infected cells has been reported in the immune animals undergoing sporozoite challenge (Innes *et al.*, 1989a; Chaudhri & Subramanian, 1992). Similar patterns of an increase in the circulating $CD8^+$ cells were observed after sporozoite challenge of immune animals in these experiments.

The level of monocytes in the peripheral blood usually increased after cell line immunisation in the animals reacting mildly and after sporozoite challenge in the immune animals. Adherent cells isolated from the peripheral blood of calves immunised using either schizont infected cell lines or sporozoites, have been shown to exhibit a strong cytostatic effect *in vitro* on autologous as well as allogeneic parasite infected cell lines (Preston & Brown, 1988).

The observations from these experiments and *in vitro* bioassays mentioned above (Innes *et al.*, 1989a; Preston & Brown, 1988) suggest that protective immunity against *T. annulata* is mediated by a transient development of $CD8^+$ cytotoxic T lymphocytes and a sustained presence of cytostatic macrophages in the peripheral circulation. However, adoptive transfer experiments have not been undertaken to directly evidence the reliance of immunity on $CD8^+$ cells and/or cells of

monocytes/macrophages lineage during *T. annulata* infection. Recently adoptive transfer of purified responding $CD8^+$ cells from efferent lymph of an immune animal to its naive twin prevented the development of *T. parva* infection in the recipient. These results provide direct evidence for $CD8^+$ cell mediated protective mechanism in *T. parva* infection (McKeever *et al.*, 1994). It may be assumed that $CD8^+$ cells also protect against *T. annulata* infection in a similar fashion.

Development of immunity against *T. annulata* was associated with the appearance of $CD25^+$ cells along with increased levels of MHC class II^+ cells and $CD45RB^-$ cells, in the peripheral blood, after cell line immunisation as well as sporozoite challenge. The expression of MHC class II and CD25 are considered to be markers of activated T cells (Greene *et al.*, 1986; Glimcher & Kara, 1992). However, the appearance of $CD25^+$ cells was seen earlier than the increase in the levels of MHC class II^+ and $CD45RB^-$ cells. Many $CD25^+$ cells were reported to appear in the draining lymph node on day 4 after sporozoite infection in naive animals which disappeared from the node by day 8 (Campbell *et al.*, 1994b). Experiments in this chapter demonstrated the presence of these cells in the peripheral circulation after day 6 of sporozoite infection. Detailed studies on the expression of activation markers on various lymphocyte subpopulations in the peripheral blood was not done which could have provided a better understanding of T cell activation during the course of infection. The immune response after inoculation of parasite infected cell line or sporozoites is initiated in the draining lymph node, therefore, study of efferent lymph draining the site of inoculation provides a better indication of the development of immunity as shown in the next two chapters.

Decline in TLC values and levels of other lymphocyte subpopulations from the periphery observed after cell line immunisation or sporozoite challenge may not represent an actual drop of these cells from the body of an animal. Lymphocytes continuously enter and exit lymphoid and non-lymphoid organs using blood as a traffic route (Pabst & Binns, 1989). In the human body, blood only represents about 2% of the total lymphocytic pool and is assumed to be the same in animals. Small alterations in organs containing many lymphocytes, such as lymph nodes and spleen, and also non-lymphoid organs such as lungs, can cause major alterations in the circulating lymphocytes (Westermann & Pabst, 1990). Initial pathology and parasite development after cell line immunisation or sporozoite challenge takes place in the

draining lymph node leading to its multifold enlargement (Samantaray *et al.*, 1980; Ouhelli *et al.*, 1989; Preston *et al.*, 1992a). The antigen stimulation of a lymph node leads to elevation in the blood flow and increased entry of lymphocytes into the node (Hay & Hobbs, 1977; Mackay *et al.*, 1992b). The mild leucopenia induced after cell line immunisation or sporozoite challenge in immune animals is more likely to be the effect of increased lymphocyte entry into the draining node. This was further supported by the observations that the number of cells with memory/activated phenotype decreased during this period. The mAb used for distinction of naive and memory phenotype (CC 76), only discriminates naive and memory cells of CD4 lineage efficiently and not $CD8^+$ cells (Howard *et al.*, 1991b). Memory T cells recirculate selectively from blood to peripheral tissues to lymph node via afferent lymph (Mackay *et al.*, 1990). Further, a selective non-random migration of $CD4^+$ and $CD8^+$ cells from lymph nodes to blood via efferent lymph has been well documented in response to an antigen (Bujdoso *et al.*, 1989b; Mackay *et al.*, 1992b).

Severe loss of leucocytes during acute *T. annulata* infection cannot be solely attributed to migration of cells, from peripheral blood to lymphoid tissues, because normal levels of circulating leucocytes never return to normal in these animals. Further, the ratio of cells with naive and memory phenotype was not disturbed, until treatment of these animals, indicating a loss of both circulating naive and memory cells. Large numbers of cells with memory phenotype appeared in the circulation after treatment. Campbell *et al.* (1994b) reported a total destruction of the lymph node architecture by day 10 of sporozoite inoculation in naive animals. Many cells undergoing apoptosis were observed in the draining lymph nodes after sporozoite infection in the naive animals. The parasite seems to induce destruction of leucocytes during the course of an acute infection by inducing programmed cell death in the T cells. This might be explained by looking at the activation of T cells in response to the parasite locally in the draining lymph node. Attempts have been made to address some of these questions in the following chapters by examining the cells exiting the draining node via the efferent lymphatics.

Changes in the lymphocyte subpopulations were not accompanied by a decline in circulating monocytes/macrophages, which were shown to be important in the development of immunity to *T. annulata* (Preston & Brown, 1988). An increase in the circulating monocytes observed in these experiments is in accord with the above

findings. An important point to take into consideration is that, unlike lymphocytes, monocytes do not recirculate between lymph, blood and tissues (Springer, 1994). The kinetics of circulating monocytes, therefore, provides a true representation of these cells in the body.

Experiments reported in this chapter showed that reduction in the levels of circulating $CD4^+$ and B cells mainly leads to leucopenia in response to *T. annulata* infection, followed by less involvement of $CD8^+$ cells and $\gamma\delta$ T cells. Cells of the monocyte/macrophage lineage were least affected. Leucopenia is very severe in animals suffering from acute theileriosis after inoculation of sporozoites. Naive animals after cell line immunisation or immune animals after sporozoite challenge only exhibit a mild and transient leucopenia. A transient increase in the levels of circulating $CD8^+$ cells initially followed by a sustained increase in monocytes/ macrophages was associated with recovery, after cell line immunisation and sporozoite challenge, indicating that immunity against *T. annulata* is mediated by $CD8^+$ cells and monocyte dependent mechanisms. During the course of parasite development after cell line immunisation or sporozoite challenge, T cell activation markers appeared transiently on PBM. These changes were not observed in skin grafted animals where no parasitological reactions were seen after cell line immunisation.

Initial pathology and development of immunity takes place in the draining lymph node after inoculation of *T. annulata* sporozoites or cell line. Cells leaving the draining node reflect the immunological events occuring in the node and are responsible for dissemination of immune effector cells to other body compartments. Further experiments, reported in the next two chapters, concentrated on the study of parasite dissemination and development of immunity in the efferent lymph of the node draining the site of *T. annulata* sporozoite challenge or cell line immunisation.

CHAPTER 6

Kinetics of *Theileria annulata* infection and T cell activation in the efferent lymph of cattle

Objectives:

1. To study the kinetics of parasite dissemination in lymph efferent from the node draining the site of lethal *T. annulata* infection with sporozoites.

2. To investigate changes taking place in various lymphocyte subpopulations in efferent lymph from the draining node during *T. annulata* infection.

3. To characterise activation of T cells in response to the parasite in order to understand how immunity develops.

6.1 INTRODUCTION

Following sporozoite infection with *T. annulata* or *T. parva*, multinucleate macroschizonts appear initially in the nearest draining lymph node. However in the case of *T. parva*, two days after the first appearance, almost similar numbers of schizonts can be seen in all the nondraining lymph nodes (Jarret *et al.*, 1969; DeMartini & Moulton, 1973a; Radley *et al.*, 1974; Emery, 1981b; Morrison *et al.*, 1981b). On the other hand, schizonts are seen only in the draining lymph node after *T. annulata* infection and can also be isolated from liver, spleen, kidneys, lungs and abomasum during the later stages of the disease (Neitz, 1957). Only a few infected cells are rarely seen in the nondraining lymph nodes (Sergent *et al.*, 1945; Neitz, 1957; Samantaray *et al.*, 1980; Preston *et al.*, 1992a).

Parasite specific cytotoxic T cell and humoral responses are seen in animals immunised with cell lines or recovering from infection with sporozoites (Preston *et al.*, 1983; Preston & Brown, 1985; Innes *et al.*, 1989a; Kachani *et al.*, 1992a). However, severe infection with sporozoites in naive animals may quickly lead to death without development of protective immunity. Earlier studies in this laboratory had shown great difficulty in generating and maintaining *T. annulata* specific T helper and cytotoxic cells *in vitro* because of apoptosis by T cell lines after 3-4 weeks in culture (Campbell *et al.*, 1994b; A.K. Nichani & R.L. Spooner, unpublished observation). All these *in vitro* and *in vivo* studies were carried out on cells from the peripheral blood of animals which is only a traffic route for circulating lymphocytes (Trnka & Cahill, 1980). Immunological events observed in the peripheral circulation are diluted and may not reflect cellular changes taking place immediately upon introduction of an antigen via the intradermal or subcutaneous port of entry. The nature of early interactions between the pathogen and the host effector cells is very important in determining the outcome of infection. Immunohistochemical studies on lymph node sections following *T. annulata* sporozoite inoculation have revealed that T cell activation was taking place in atypical places and at the wrong times. $CD25^+$ cells for instance observed in the medulla on day 4 had disappeared by day 8 (Campbell *et al.*, in press). In the previous chapter (chapter 5), transient appearance of $CD25^+$ cells was observed in animals after cell line immunisation as well as after sporozoite challenge. To further understand the pathogenesis of *T. annulata* infection and generation of immunity against the parasite, it was decided to study changes

taking place in the draining lymph node *in vivo* using efferent lymphatic cannulation.

There have been extensive studies in sheep for the understanding of *in vivo* immune responses following development of techniques for cannulating efferent lymphatics draining the individual lymph node (Hall & Morris, 1962). This technique enabled extensive studies on lymph node function and development of nodal immune responses to various micro-organisms (Hall *et al.*, 1967; Smith & Morris, 1970; English *et al.*, 1977; Issekutz, 1985; Huang *et al.*, 1991; Bird *et al.*, 1993; Buxton *et al.*, 1994) and exogenous protein antigens (Hall & Smith, 1971; English *et al.*, 1977; Hall *et al.*, 1980; Hopkins *et al.*, 1981a; Bujdoso *et al.*, 1989b; Hopkins *et al*, 1993) in sheep. Surprisingly, reports of similar approach for studies of *in vivo* immunology and development of immune responses in cattle are scarce, apart from a few reports on the pathogenesis and immunology of *T. parva* infection (DeMartini & Moulton, 1973b; Emery 1981a). The kinetics of *T. parva* infection in the lymph efferent from the draining prefemoral node and nondraining lymph nodes were described (Emery, 1981b). *T. parva* infected schizonts were detected in efferent lymph 8 days after challenge, coinciding with a dramatic increase in output of lymphoblasts. Emery & McCullagh (1980a) compared skin graft reactions between chimaeric twins in the draining efferent lymphatics of prefemoral and popliteal lymph node of cattle. Kinetics of the dissemination of *Trypanosoma congolense* and development of humoral responses in the lymph efferent from the node draining the site of local skin lesions induced by the parasite were studied (Akol & Murray, 1986). Responses induced included a 2-3 fold increase in the volume of lymph, up to a 10-fold increase in cell output and appearance of neutralising antibodies. In an earlier report, Brownlie & Scott (1979) established optimal culture conditions for *in vitro* stimulation of bovine ELL with phytohaemagglutinin. Kinetics of MHC class I restricted cytotoxic T lymphocytes in efferent lymph of an immune cattle following *T. parva* sporozoite challenge has recently been described (McKeever *et al.*, 1994). These cells could mediate immunity as shown by adoptive transfer from immune to a naive monozygotic twin undergoing lethal infection. Immunological events occurring in the lymph nodes draining the site of antigen deposition and inflammatory lesions are reflected by cell contents of the efferent lymph draining that node (Trnka & Cahill, 1980; Bujdoso *et al.*, 1989b).

In these experiments, we examined the kinetics of parasite dissemination in efferent lymph draining the site of an acute *T. annulata* infection with sporozoites. Some of the cellular and molecular events occurring within the lymphatic vessel in relation to the progression of the disease were characterised. Cellular traffic from the draining lymph node was monitored to investigate T cell activation and function as a step towards understanding how immunity is generated against the parasite.

6.2 MATERIALS AND METHODS

6.2.1 Cattle

Five Friesian x Brown Swiss or Friesian x Charollais calves aged 3-8 months were used in this study (table 6.1). Efferent lymphatic draining the prescapular node was cannulated. Animals 903 and 928 were infected with *T. annulata* by s/c inoculation of 0.1 t.e. GUTS (Gharb isolate) above the lymph node 24 hrs after surgery to produce clinical theileriosis. These animals were treated with buparvaquone (Butalex, Mallinkrodt Veterinary Ltd.) on day 10 and 11, respectively (McHardy *et al.*, 1985) as soon as the feed intake of the animals started to decline. Animal 264 (solidly immune to sporozoite challenge; chapter 4 for details) was inoculated with 2 t.e. GUTS 24 hrs after surgery and observed for 7 days. The animal was challenged with 2 t.e. GUTS again 6 weeks later on the right side of the neck and the efferent lymphatic of the draining lymph node cannulated on day 5 of challenge and observed further. Observations on this animal after first challenge are mentioned as 264a and after second challenge as 264b in the subsequent text. Animals 249 and 250 were not given any inoculation after cannulation to collect baseline information.

Table 6.1:Details of animals used for lymphatic cannulation and sporozoite inoculation.

Animal no.	Status	Lymphatic cannulation	Sporozoite inoculation	Days of efferent lymph collection
903	Naive	day -1	day 0	day -1 to 13
928	Naive	day -1	day 0	day -1 to 13
264a	Immune	day -1	day 0	day -1 to 6
264b	Immune	day 5	day 0	day 5 to 17
249	Immune	day -1	not done	day -1 to 5
250	Immune	day -1	not done	day -1 to 4

6.2.2 Cell lines

6.2.2.1 *T. annulata* infected cell lines

T. annulata infected cell lines were established *in vitro* by infection of bovine PBM with *T. annulata* (Gharb) sporozoites as described in chapter 3.4.2 (Brown 1983). The resultant continuously growing cell lines were used as targets in cytotoxicity assays and stimulators in proliferation assays.

6.2.2.2 Uninfected lymphoblasts

Uninfected lymphoblasts were established by stimulating PBM once with Con. A and cultured further in MLC medium supplemented with 20% TCGF as described in chapter 3.4.3. These cells were used as targets in cytotoxicity assays.

6.2.3 Cannulation of efferent lymphatic duct

The efferent lymphatic of the prescapular lymph node of animals mentioned above was cannulated as described in detail in chapter 3.5. Lymph was collected in plastic bottles containing 5-10 i.u./ml heparin (Leo Laboratories Ltd.) and antibiotics (Gibco) to provide penicillin 20 i.u. and streptomycin 20 μg per ml when full. The bottle for lymph collection was changed 2-4 times a day depending upon the flow rate. Lymph was collected for one hr every morning for use in various studies. The rest was pooled aseptically and infused slowly into the animal through a jugular cannula in order to minimise the loss of fluid and cells, to maintain fluid, electrolyte and protein balances and not to interfere in the development of systemic immunity.

6.2.4 Infection

The sporozoites used to provide infection were prepared as a stabilate (Cunningham *et al.*, 1973) of *H. a. anatolicum* ticks infected with the Gharb stock of *T. annulata* (Ouhelli, 1985) as described in chapter 3.4.2.2.

6.2.5 Clinical monitoring

The clinical condition of the animals was assessed by daily observation of their general condition and rectal temperature (a febrile response being $\geq$ 39.5°C). Haematocrit and TLC were measured at regular intervals (chapter 3.6 for details). Cytospin smears from efferent lymph were prepared daily and stained with Giemsa for assessment of blasting cells and *T. annulata* infected schizonts. Blasting cells were also assessed by FACS analysis on the basis of FSC vs SSC. The percentage of piroplasm infected erythrocytes was assessed by Giemsa stained thin blood smears. The number of total erythrocytes in the lymph was assessed initially on the basis of

Giemsa stained cytospin smears. But was later measured by a Coulter Counter, when the number of erythrocytes was very high in the lymph.

6.2.6 Cell preparation

ELL collected from indwelling cannulae were counted in a haemocytometer. The viability of cells was always greater than 98% as assessed by Trypan blue dye exclusion. Volume and cellularity of the lymph was measured after every collection to calculate rate of lymph flow and cellular traffic. Cells collected freshly for one hr in the morning were used for flow cytometry, proliferation assays and cytotoxicity assays. An aliquot of lymph was centrifuged at 800*g* for 15 min. and cell-free lymph plasma supernate decanted and stored at -20°C for IFN-γ estimation. ELL and PBM were separated on ficoll-hypaque as described in chapter 3 (3.4.2.1 & 3.4.5). Cell free lymph supernate was collected at short intervals during the first two days after infection. PBM from a different animal (free from *T. annulata* infection) were cultured with this supernate to look for dissemination of sporozoites in the efferent lymph. ELL and PBM were cultured *in vitro* in 24 well plates (Intermed, Nunc) to isolate parasite infected cell lines for studying the kinetics of parasite dissemination.

6.2.7 Monoclonal antibodies

Various mAbs have been described in detail in chapter 3.8.1. Following mAbs were used for analysis of various lymphocyte subpopulations. IL-A26 for $CD2^+$ cells, IL-A12 for $CD4^+$ cells, SBU-T8 for $CD8^+$, IL-A29 for $\gamma\delta$ T cells and VPM 30 for B cells. B cells expressing IgM on the surface were recognised by IL-A30, monocytes and macrophages by IL-A24, MHC class II molecules by J 11, IL-2R α chain (CD25, Tac antigen) by IL-A111 and CD45RB molecules on the cell surface by CC 76.

6.2.8 Immunofluorescent Staining

Various immunoconjugates and techniques used for indirect immunofluorescent staining and flow cytometry have been explained in chapter 3.8. This technique was used to analyze phenotypic changes in various lymphocyte subpopulations in ELL following sporozoite infection. Two colour immunofluorescent staining on ELL was carried out at regular intervals for studying T cell activation following sporozoite infection. Cells were simultaneously incubated with two mAbs of different isotypes. One mAb was used to identify $CD4^+$ or $CD8^+$ cells and the second for the expression of CD25, MHC II, CD2 and CD45RB on the cells. This was followed by incubation of cells simultaneously with two appropriate fluorescent secondary reagents.

Acquisition and analysis on FACScan is described in detail in chapter 3.8.

6.2.9 Proliferation assays

These were essentially as described by Glass and Spooner (1990a) and detailed in chapter 3.10. Briefly, ELL or PBM with irradiated PBM were incubated either alone or with Con. A or Human recombinant IL-2 or irradiated autologous *T. annulata* infected cells. Cell proliferation was measured after 72 hrs by a 6 hr pulse with [^{3}H] thymidine (Amersham) and uptake was assessed by liquid scintillation counting.

T. annulata infected cells were cultured *in vitro* in 96 well flat bottom plates (Intermed, Nunc) at $2x10^4$ cells per well in 100 μl of TC medium with 100 μl of different dilutions of cell free lymph supernates (from day 0 & day 5) or bovine recombinant IFN-γ. Cell proliferation was measured after 48 hrs as described above.

6.2.10 Cytotoxicity Assay

Cytotoxic activity of ELL and PBM from the animals was assayed at regular intervals after infection throughout the experimental period as described in chapter 3.9. Both parasite infected cells and uninfected blasts of known BoLA phenotypes were chosen to provide targets.

6.2.11 Interferon-γ assay

A solid phase sandwich ELISA (enzyme linked immunosorbant assay) using mAbs specific for bovine IFN-γ was used (Wood *et al.*, 1990; Rothel *et al.*, 1990) to measure levels of IFN-γ in efferent lymph using a ready made test kit (CSIRO, Australia) as described in chapter 3.11.

6.3 RESULTS

6.3.1 Clinical reactions

The two control animals which were cannulated but not infected with *T. annulata* (animals 249 & 250) showed no clinical reactions. The immune animal 264 also did not show any clinical reaction after the first or the second sporozoite challenge. Feed and water intake was normal in these animals and they did not exhibit any rise in rectal temp. Two naive animals (903 & 928) which were infected 24 hrs after lymphatic cannulation developed severe clinical theileriosis. Both the animals exhibited fever (rectal temp. $\geq$ 39.5°C) from day 8 onwards (fig. 6.1a). Schizonts were first seen in efferent lymph on day 6 and continued to rise thereafter (fig. 6.1b).

Maximum schizonts were 36% in animal 903 and 10% in animal 928 on day 11. Piroplasms appeared in both the animals on day 9. Maximum parasitaemia was 10% in animal 903 and 2.5% in animal 928 (fig. 6.1c). Haematocrit (fig. 6.1d) and TLC (data shown in chapter 5.3.2) decreased significantly with progression of the disease. Both the animals were seriously ill and were, therefore, treated with buparvaquone (Butalex) as soon as they began to go off feed. Animal 903 was treated on day 11 and 928 on day 10 after sporozoite infection.

6.3.2 Changes in the flow rate and cellularity of efferent lymph

The output of lymph from the prescapular lymph node draining the site of infection is shown in fig. 6.2a. It remained more or less the same for the first four days after sporozoite infection in the naive animals. Following enlargement of the lymph node, lymph flow from the draining node increased significantly during the progression of the disease. Output of fluid from the node was 37 ml and 28.6 ml per hr before infection which increased to 232 ml and 220 ml per hr on day 10 in animals 903 and 928, respectively. It started to decline in one animal (928) after treatment, but kept on increasing (300 ml per hr) in the second (903) even after treatment. There was a marginal increase in the flow rate of lymph in animal 264 after the second sporozoite challenge from day 6 (35.7 ml/hr) which peaked on day 8 (81.2 ml/hr) and declined afterwards.

The output of lymphocytes in efferent lymph increased slowly after infection for the first four days in the naive animals (fig. 6.2b). As enlargement of the lymph node started on day 4, cell output was reduced (cell shutdown) in both the animals up to day 6 ($1.7x10^8$ cells/hr on day 0 & $0.65x10^8$ cells/hr on day 5 in animal 928) followed by a rapid increase. Both animals exhibited a multifold increase in lymphocyte output from the node after day 6 until treatment ($17.8x10^8$ cells/hr on day 11 in animal 928), after which it started to decline. In the later stages, a significant number of RBC appeared in the lymph on day 10 and their number kept on increasing (data not shown). The cell output did not change greatly in the immune animal after first challenge (day -1 to day 6). However the second challenge was accompanied by an immense increase in the cell output peaking on day 7.

The initial increase in lymphocyte traffic in the naive animals was not accompanied by changes in the proportion of blasting cells. However the second peak of lymphocytes, starting from day 6 following cell shutdown between day 4-6, was

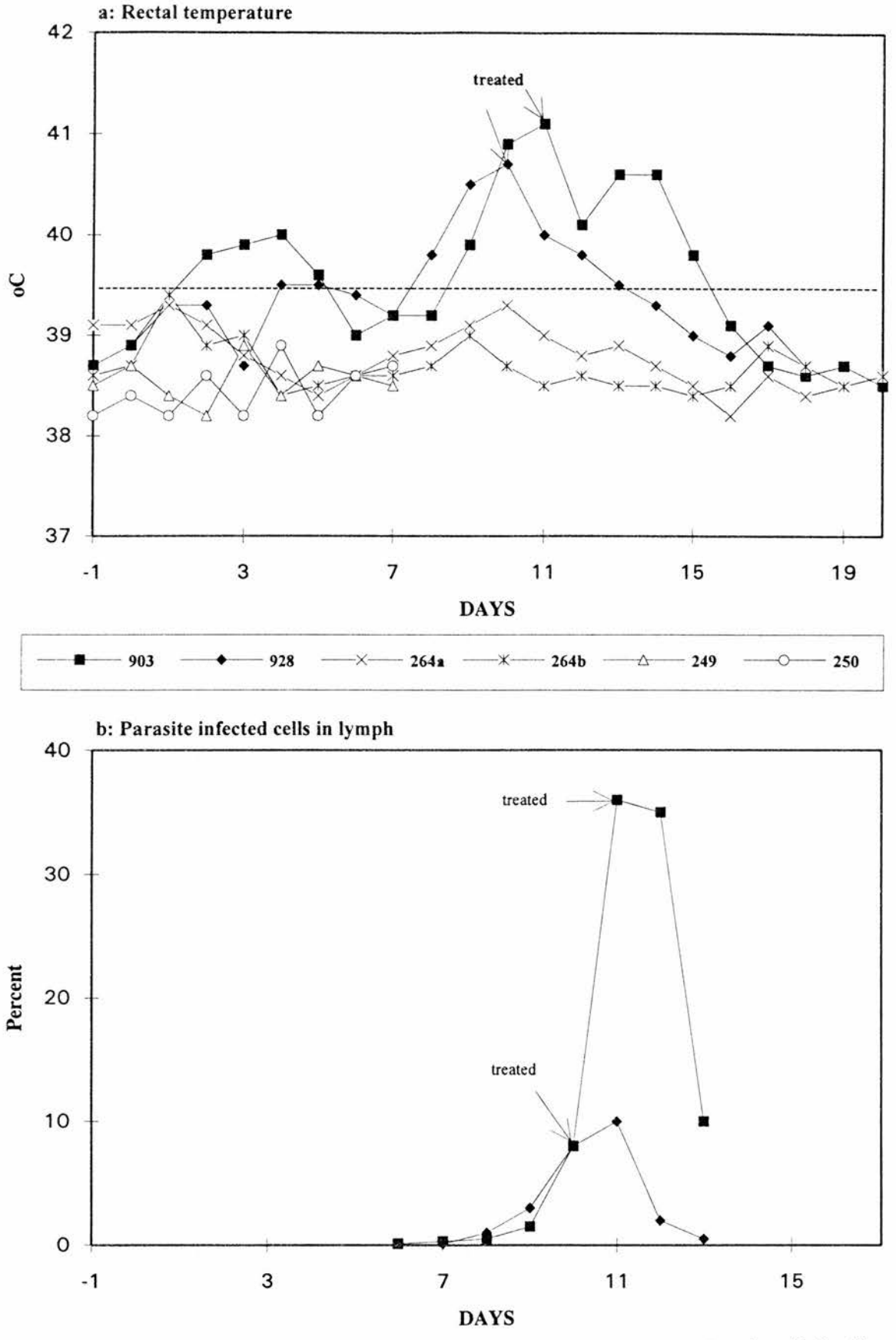


Figure 6.1: Clinical and parasitological reactions in the animals after infection with *T. annulata* sporozoites.

a: Rectal temperature. b: Parasite infected schizonts in the efferent lymph.
(→): day of treatment.

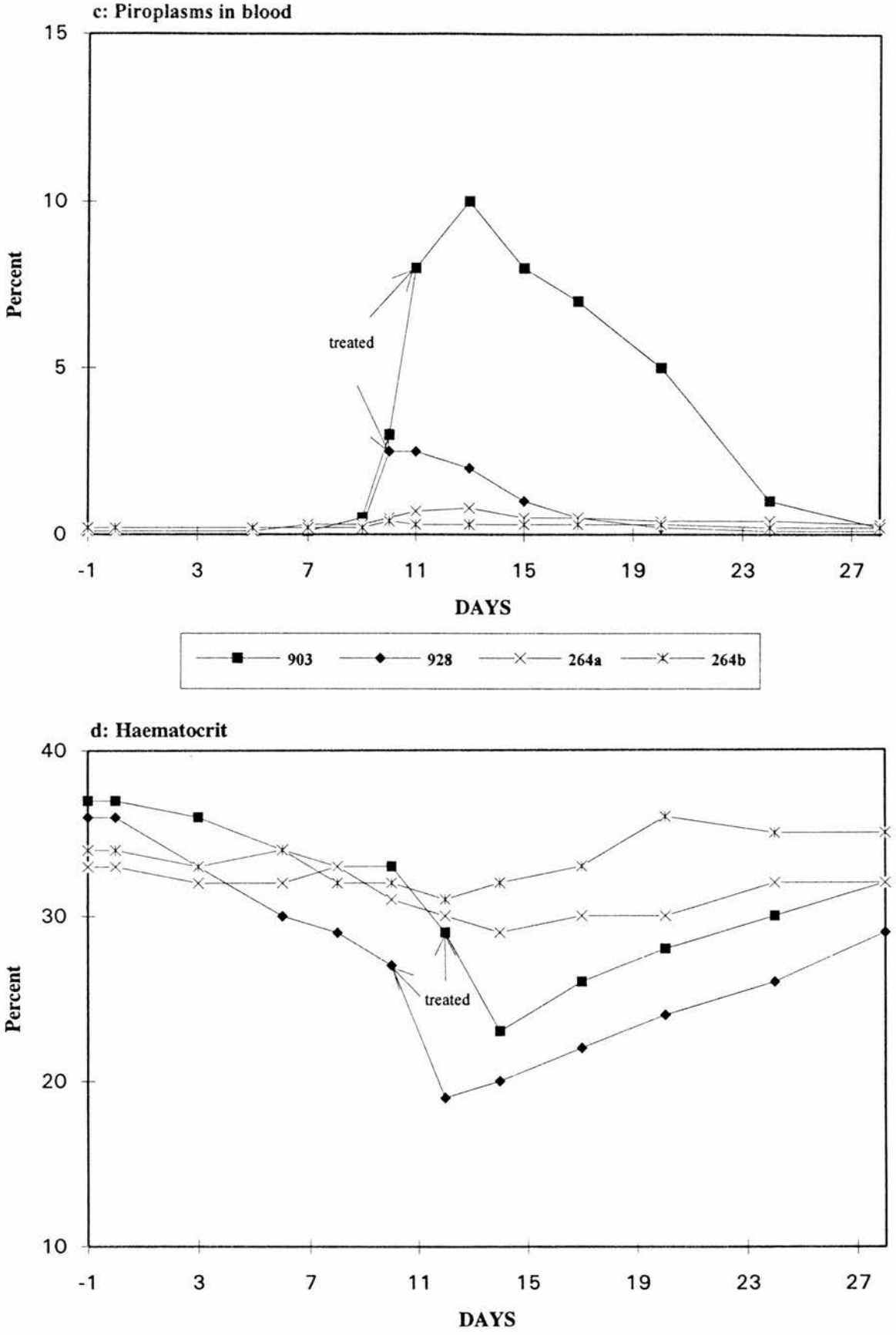


Figure 6.1: Clinical and parasitological reactions in the animals after infection with *T. annulata* sporozoites.

c: Piroplasms in blood smears. d: Haematocrit values. (→): day of treatment.

accompanied by a massive increase in the proportion of blasting cells (fig. 6.2c). Maximum blasting cells were 87% in animal 903 and 91% in animal 928 as counted from Giemsa stained smears. There were no significant changes in the proportions of blasting cells in other animals. FSC vs SSC of the ELL also exhibited an increase in the blasting cells in the naive animals after day 7 as marked by region 1 (R1) in fig. 6.3a., whereas, no increase was observed in blasting cell population of the immune animal (R1 of fig. 6.3b).

6.3.3 Kinetics of parasite dissemination

Culturing of cell free lymph supernates from animals after sporozoite inoculation with PBM from another naive animal did not produce any *T. annulata* infected transformed cell lines. Establishment of cultures in this manner was attempted from naive as well as immune animals, in the period immediately following sporozoite inoculation, to study dissemination of free sporozoites in efferent lymph. Parasite infected cell lines were established *in vitro* from ELL (day 6 onwards) and PBM (day 9 onwards) only from the naive animals. No parasite infected cell line could be isolated from ELL or PBM of the immune animal. Schizonts were first seen from day 6 onwards in Giemsa stained smears and their percentage continued to increase after that. Identification of parasite infected cells was also carried out with mAb 1C7 (Shiels *et al*, 1986a) from the first animal infected with sporozoites. Detection of schizont infected cells was similar to the results obtained from Giemsa stained smears. Therefore, results of only Giemsa stained smears are presented.

6.3.4 Changes in lymphocyte subpopulations in the efferent lymph

The kinetics of various lymphocyte subpopulations in efferent lymph of these animals are shown in fig. 6.4a to 6.4h. Percentage of $CD2^+$ cells in efferent lymph started to increase from day 5 in the naive animals. More than 80% of the cells were $CD2^+$ from day 6-8 (fig. 6.4a). The initial increase of $CD2^+$ cells was accompanied by an increase in $CD4^+$ cells until day 7 (fig. 6.4b) followed by an increase in $CD8^+$ cells to day 10 (fig. 6.4c). Percentage of $CD25^+$ cells increased sharply from day 7 (fig. 6.4f). With the appearance of parasite infected schizonts, the percentage of all these cells decreased from day 10 onwards. But, after treatment with buparvaquone, $CD2^+$ cells started to rise again along with $CD8^+$ cells, whereas, $CD4^+$ cells and $CD25^+$ cells kept on decreasing. The percentage of $\gamma\delta$ T cells decreased slightly during the course of infection (fig. 6.4d). Expression of MHC class II was very low

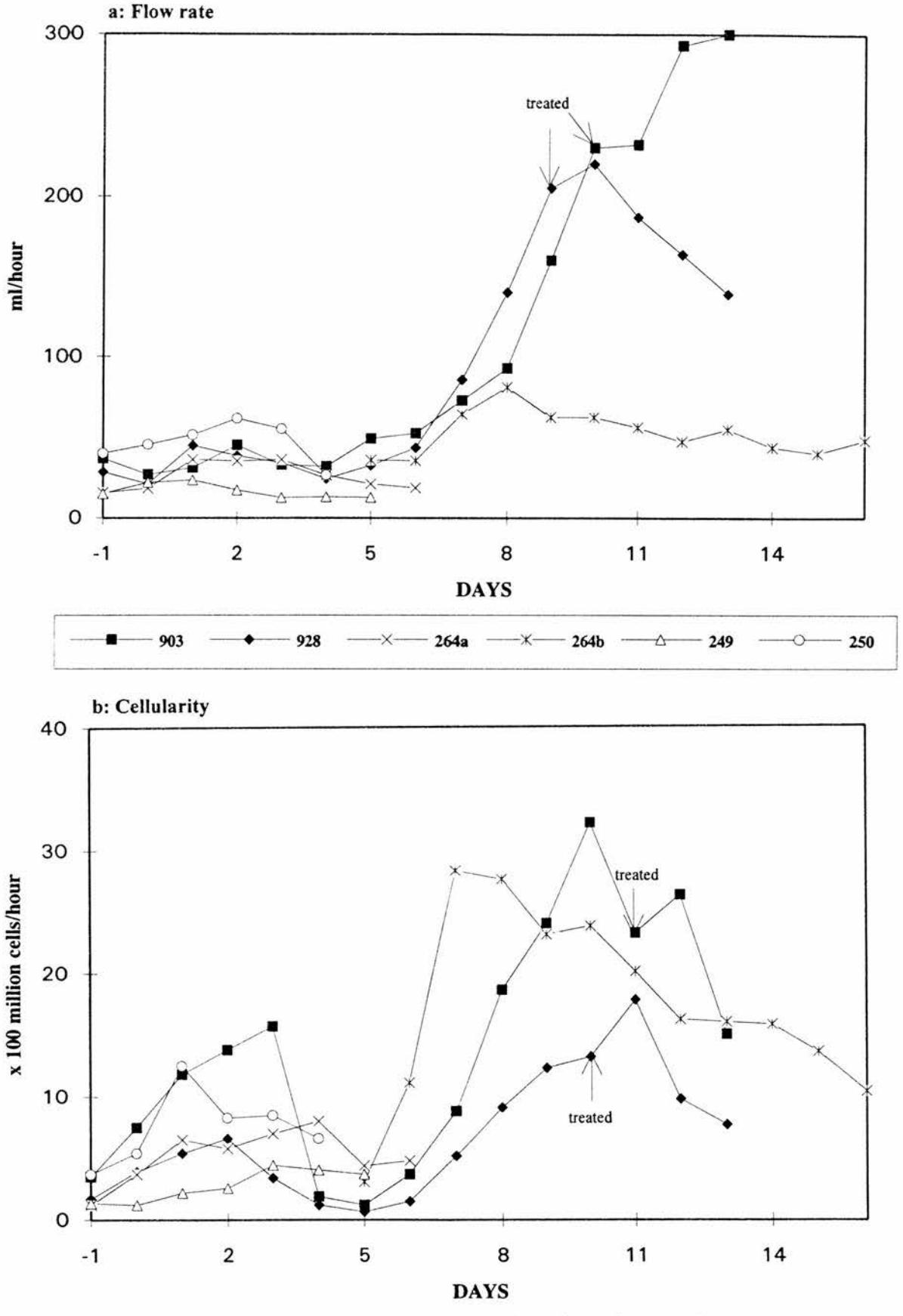


Figure 6.2: Changes in the flow and cellularity of the efferent lymph after infection with *T. annulata* sporozoites .
a: Flow rate. b: Cellularity. (→): day of treatment.

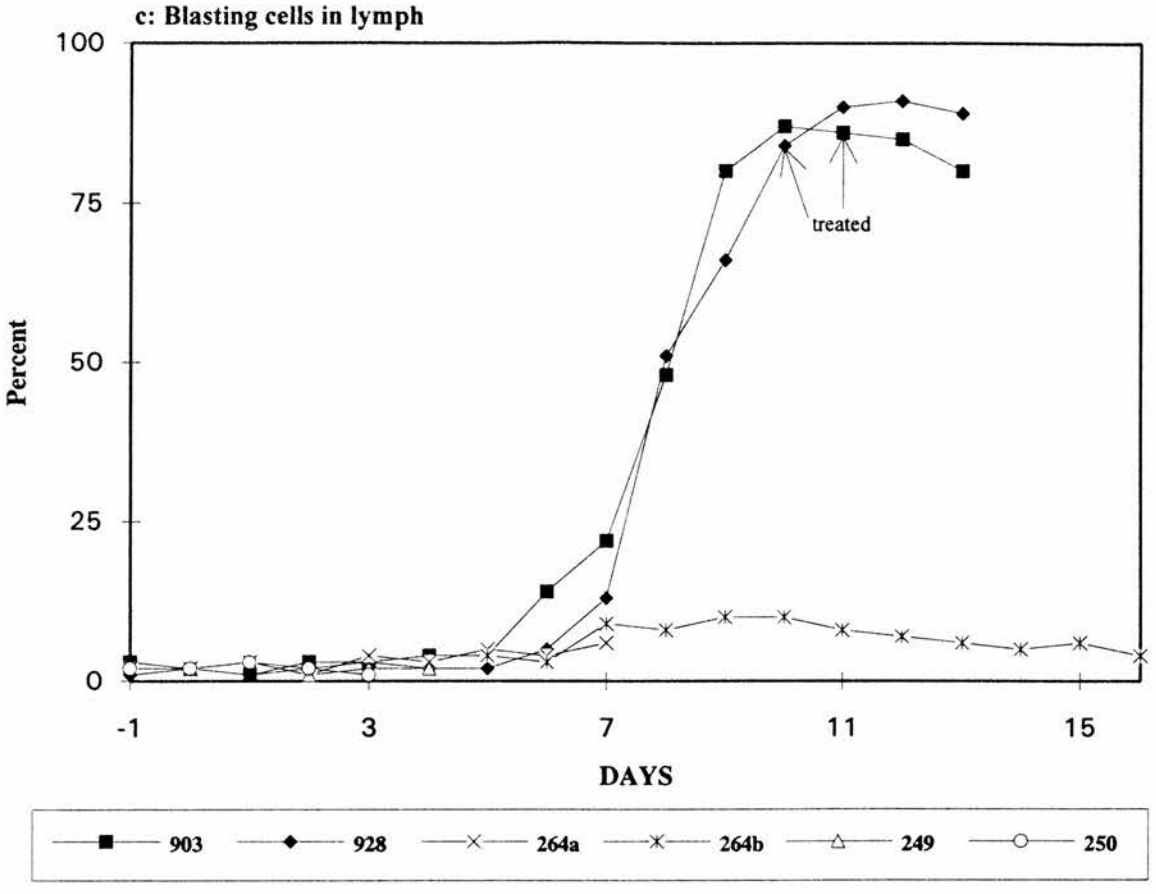


Figure 6.2: Changes in the flow and cellularity of the efferent lymph after infection with *T. annulata* sporozoites.
c: Blasting cells in the efferent lymph. (→): day of treatment.

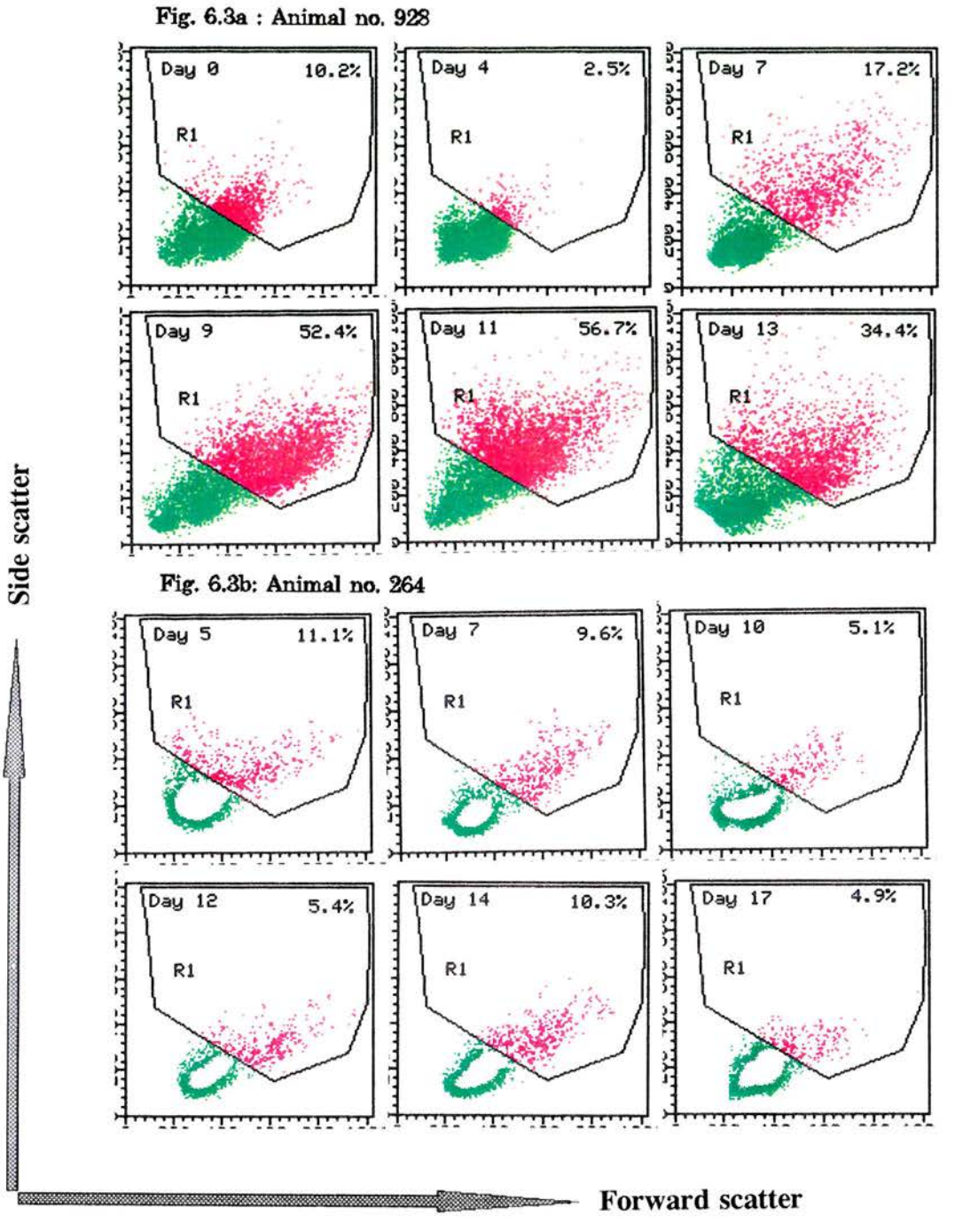


Figure 6.3: Blasting cells in the efferent lymph as assessed by the FACScan. Forward scatter (FSC) of the cells is represented on the X axis and side scatter (SSC) on the Y axis. The blasting cells are highlighted in the region 1 (R1) on the basis of their higher FSC & SSC. a: Animal no. 928 (naive). b: Animal no. 264 (immune).

on resting efferent lymph cells, but rose sharply from day 6 to 10, dropped on day 11 and increased again after treatment (fig. 6.4e). IL-A24$^+$ cells appeared in the efferent lymph from day 9 onwards corresponding to the appearance of infected cells in the lymph (appendix, no. 13). The percentage of total B cells dropped to negligible levels between day 5-10 corresponding to an increase in T cells (fig. 6.4g). IgM positive B cells also dropped to negligible levels from days 5-9 and increased slightly after that (fig. 6.4h). The changes in T cell subsets in the immune animal after both sporozoite challenges were not very pronounced, apart from a slight increase in $\gamma\delta$ T cells from day 11 onwards (fig. 6.4d). The percentage of B cells increased from day 6-11 peaking on day 8 (fig. 6.4g), but the increase was not due to IgM bearing cells (fig. 6.4h).

6.3.5 Activation of T cells

T cells started to express CD25 from day 7 in the naive animal. CD4$^+$ cells became CD25$^+$ initially, followed by CD8$^+$ cells (fig. 6.5a1 & 6.5b1). Only 5.7% ELL expressed both CD4 and CD25 on day 0. Their percentage increased to 13.4% by day 7. None of the CD8 cells expressed CD25 on day 0, and about 8% ELL expressed both CD8 and CD25 between day 9-13. The Expression of CD25 on CD8 cells was less marked but sustained, and on CD4 cells was transient disappearing by day 11. B cells and only a few CD4$^+$ cells express MHC class II antigens in the resting efferent lymph. Many T cells expressing MHC class II appeared in efferent lymph on and after day 7. CD4$^+$ cells also expressing MHC class II appeared only transiently between day 7-9 and declined by day 11. These cells did not reappear after treatment of the animal (fig. 6.5c1). All CD8$^+$ cells were MHC class II negative in the resting efferent lymph. Some CD8$^+$ MHC class II$^+$ cells started to appear in efferent lymph on day 7 of infection and their number kept on increasing until day 11 (fig. 6.5d1). After treatment on day 11, most of the CD8$^+$ cells expressed MHC class II. $\gamma\delta$ T cells did not express MHC class II at any stage of the disease in efferent lymph (data not shown). In the immune animal (264), expression of CD25 on CD4$^+$ and CD8$^+$ cells changed slightly after sporozoite challenge (fig. 6.5aII & 6.5bII). Expression of MHC class II increased slightly on both cell populations after day 7 (fig. 6.5cII & 6.5dII).

All CD4$^+$ and CD8$^+$ cells normally express CD2 antigen. However, a different picture was seen during *T. annulata* infection. On day 9, a few CD4$^+$ cells

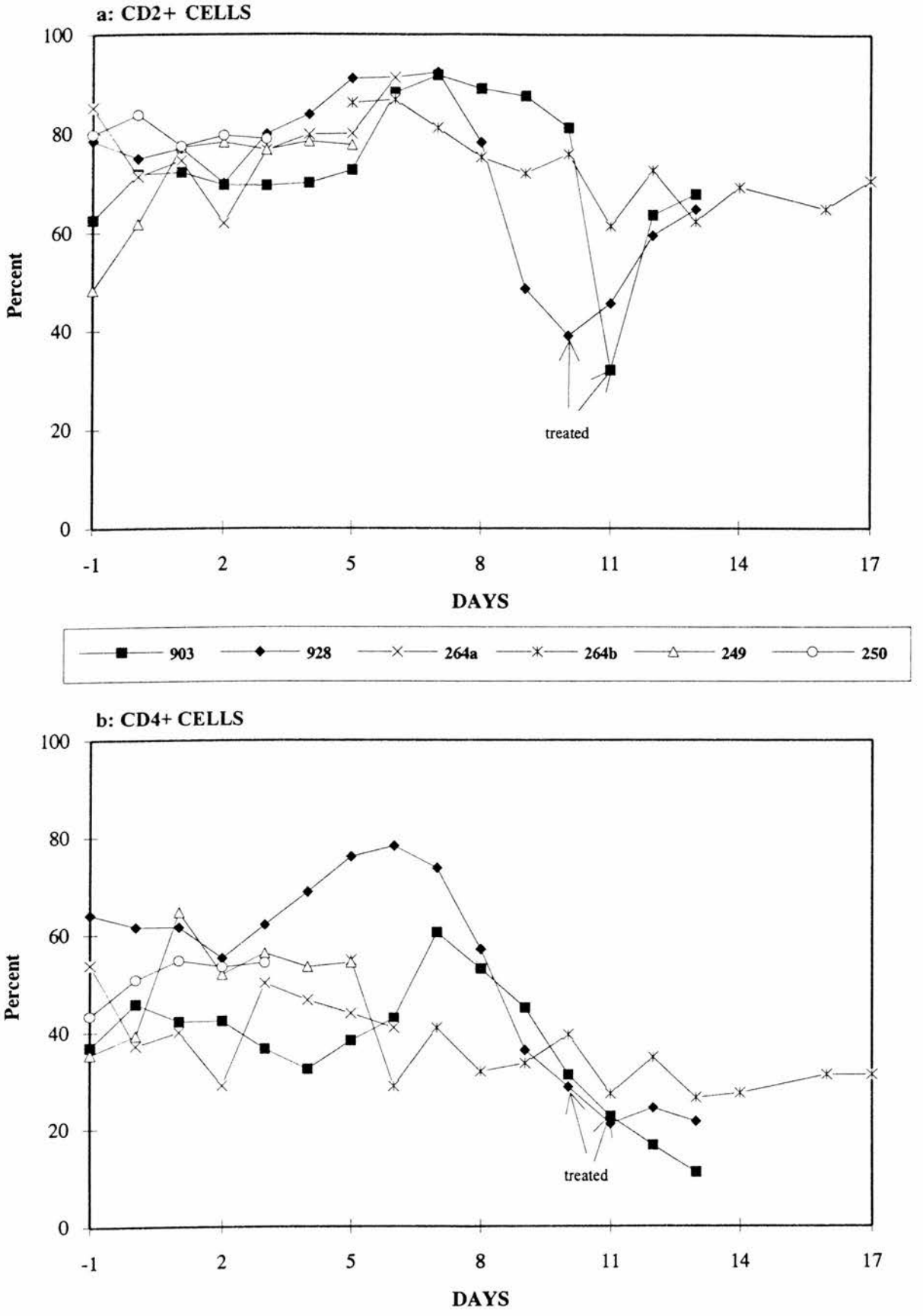


Figure 6.4: Changes in various lymphocyte subpopulations in the efferent lymph after infection with *T. annulata* sporozoites.
a: $CD2^+$ cells. b: $CD4^+$ cells. (→): day of treatment.

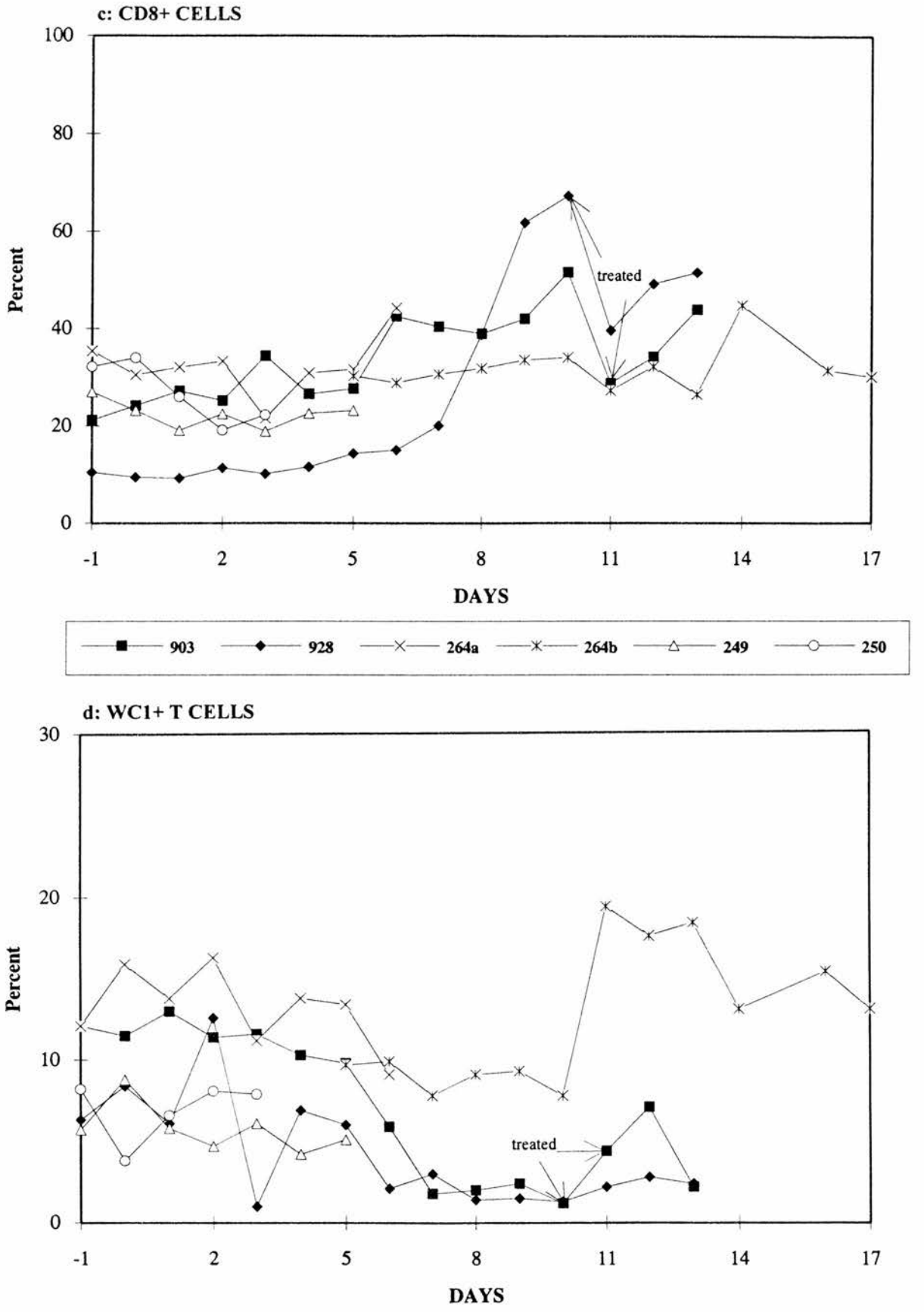


Figure 6.4: Changes in various lymphocyte subpopulations in the efferent lymph after infection with *T. annulata* sporozoites.
c: $CD8^+$ cells. d: $WC1^+$ cells ($\gamma\delta$ T cells). (→): day of treatment.

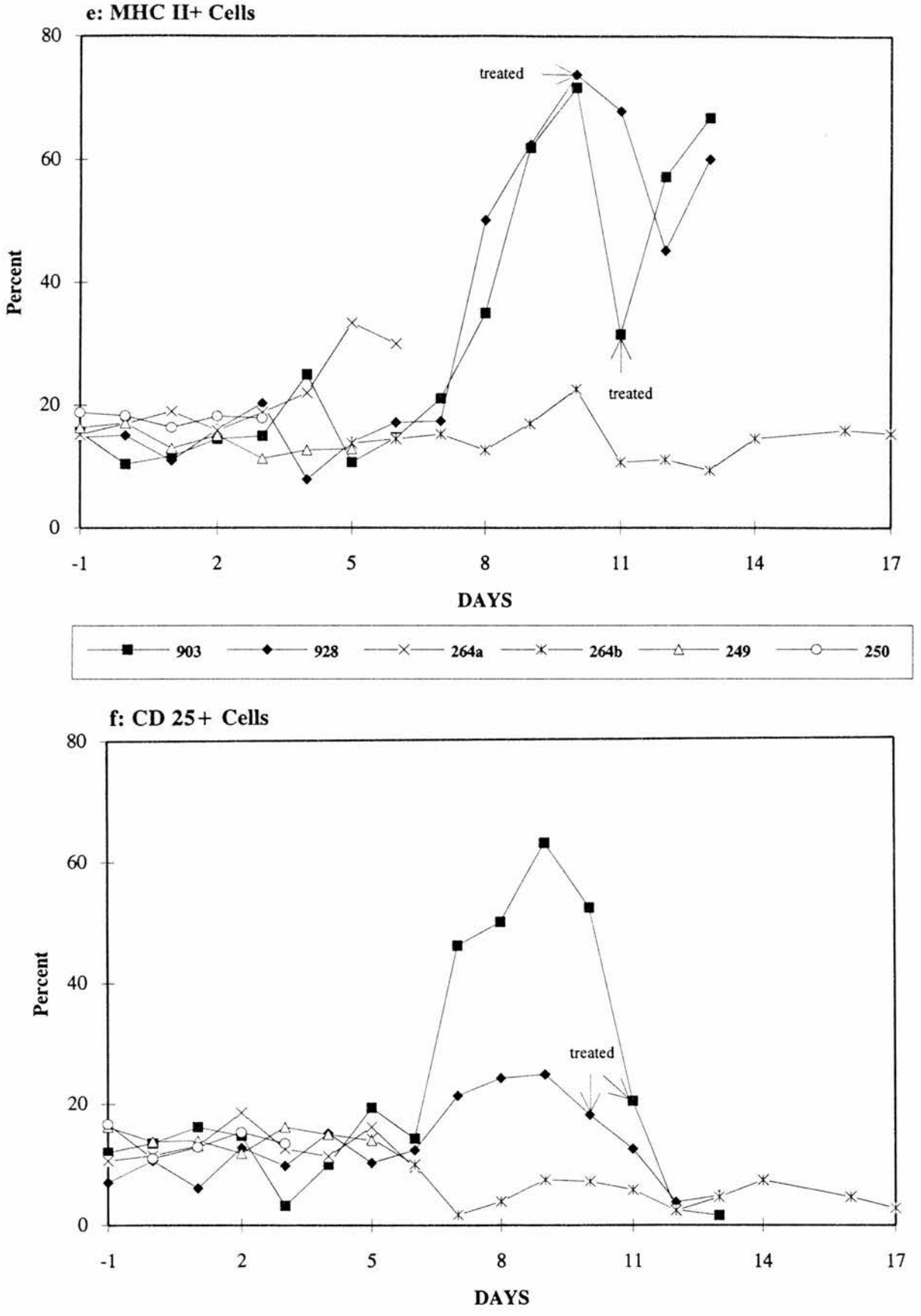


Figure 6.4: Changes in various lymphocyte subpopulations in the efferent lymph after infection with *T. annulata* sporozoites.
e: MHC class II^+ cells. f: IL-$2R^+$ (CD25) cells. (→): day of treatment.

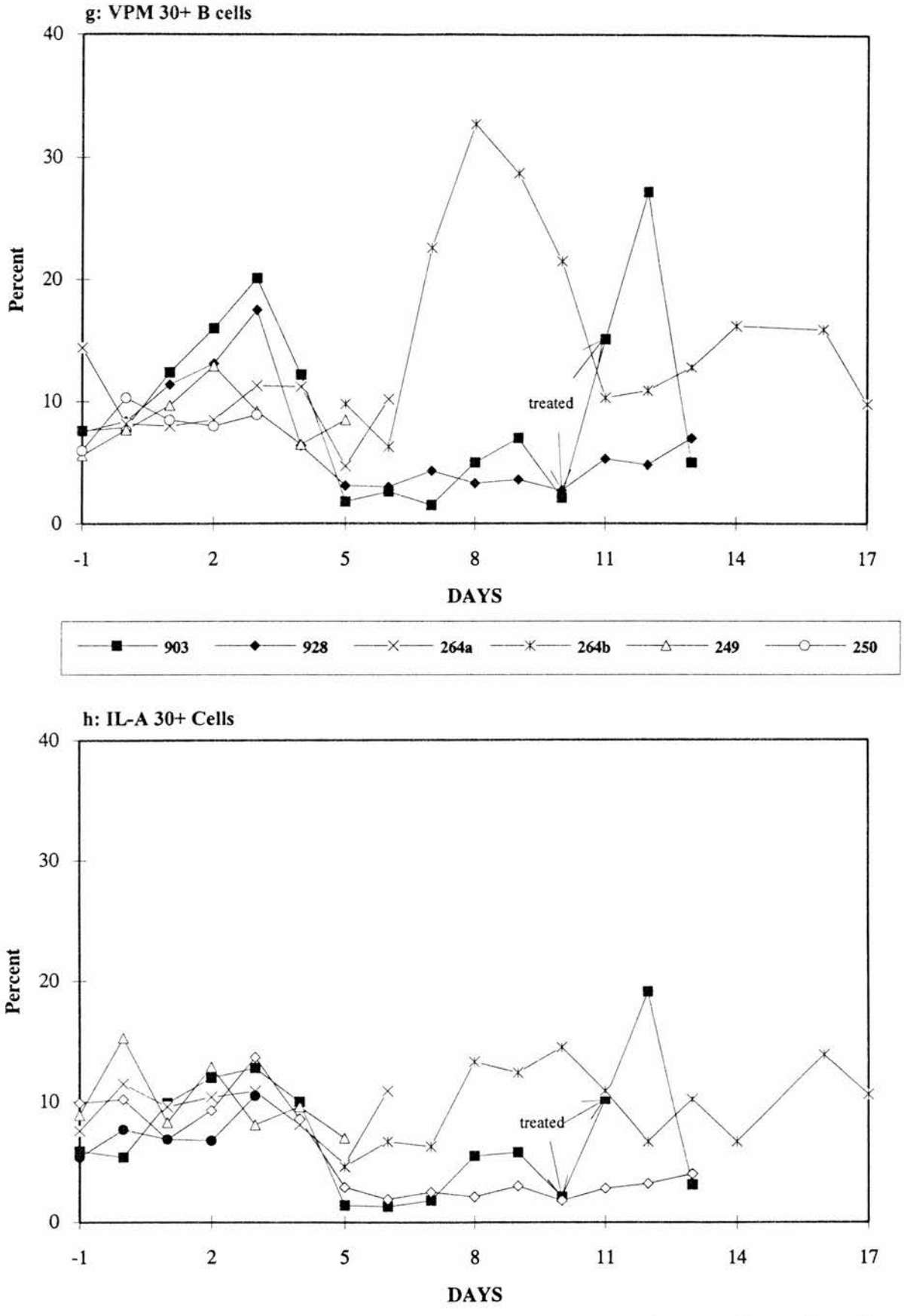


Figure 6.4: Changes in various lymphocyte subpopulations in the efferent lymph after infection with *T. annulata* sporozoites.
g: VPM 30^+ B cells h: IL-A30^+ IgM bearing B cells. (→): day of treatment.

were observed which did not express CD2. The number of $CD4^+$ $CD2^-$ cells slightly increased on day 11. After treatment, all the $CD4^+$ cells again expressed CD2 (fig. 6.5e1). The loss of CD2 expression from CD8 cells was more marked than CD4 cells. A few $CD8^+$ $CD2^-$ cells were observed on day 7 of infection in efferent lymph. The number of $CD8^+$ $CD2^-$ cells increased sharply by day 11 when about 60% of all CD8 cells did not express CD2. As with the $CD4^+$ cells all $CD8^+$ cells expressed CD2 after treatment (fig. 6.5f1). A few $CD4^+$ $CD2^-$ cells were also seen in efferent lymph of this animal on day 12 (fig. 6.5eII). However, $CD8^+$ cells were always $CD2^+$ (fig. 6.5fII).

ELL from the naive animal contained 45.6% CD4 cells on day 0 of infection. Out of which 30.1% expressed naive phenotype (CD45RB high) and 15.6% expressed memory phenotype (CD45RB low) as shown in fig. 6.5g1. The percentage of memory phenotype CD4 cells decreased to 9.3% by day 4, whereas naive CD4 cells increased to 52.5%. Memory CD4 cells exited the node around day 7 which was followed by depletion of both naive and memory CD4 cells from efferent lymph. Output of memory CD4 cells remained more or less constant in the immune animal (fig. 6.5g2). The decrease in CD4 cells corresponded to naive cells only.

6.3.6 Proliferative responses

The functional activation state of ELL was assessed by measuring their *in vitro* proliferative efficiency in response to exogenous stimuli viz: Con. A and recombinant human IL-2 as well as to autologous parasite infected cells. The ability of ELL to proliferate in response to Con. A was greatly reduced during the course of *T. annulata* infection in the naive animal (day 8 & 10 in fig. 6.6a). ELL proliferated in response to exogenous IL-2 on day 8 when the cells had high expression of CD25 and on day 12, when the animal exhibited some specific immune response to the parasite after treatment. However, neither ELL nor PBM proliferated in response to autologous irradiated *T. annulata* infected cells during the course of infection (fig. 6.6a). ELL from the immune animal responded well to Con. A at all stages, however, counts were comparatively lower on day 5 and 7 than days 0, 10, 14 and 17. Cell proliferation in response to IL-2 was higher on day 5 than other days. There was some proliferation in response to autologous *Theileria* infected cells on all the days tested and was slightly higher on days 7 and 10 (fig. 6.6b).

I: Animal no. 928 (naive).

II: Animal no. 264 (immune).

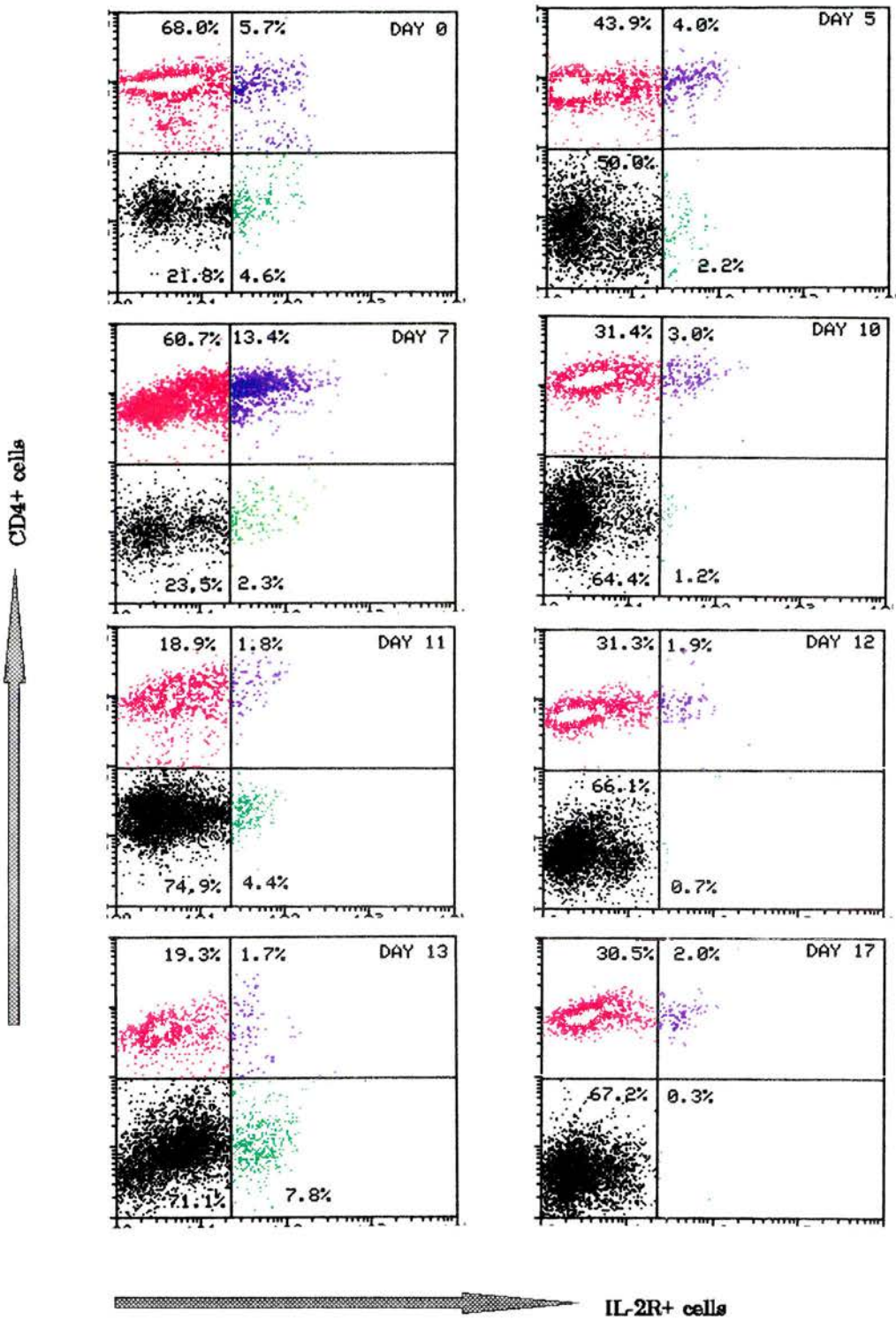


Figure 6.5a: Dot plots of two colour immunofluorescent staining on ELL. $CD4^+$ cells are expressed on the Y axis (FL2). IL-$2R^+$ ($CD25^+$) cells are expressed on the X axis (FL1).

I: Animal no. 928 (naive). II: Animal no. 264 (immune).

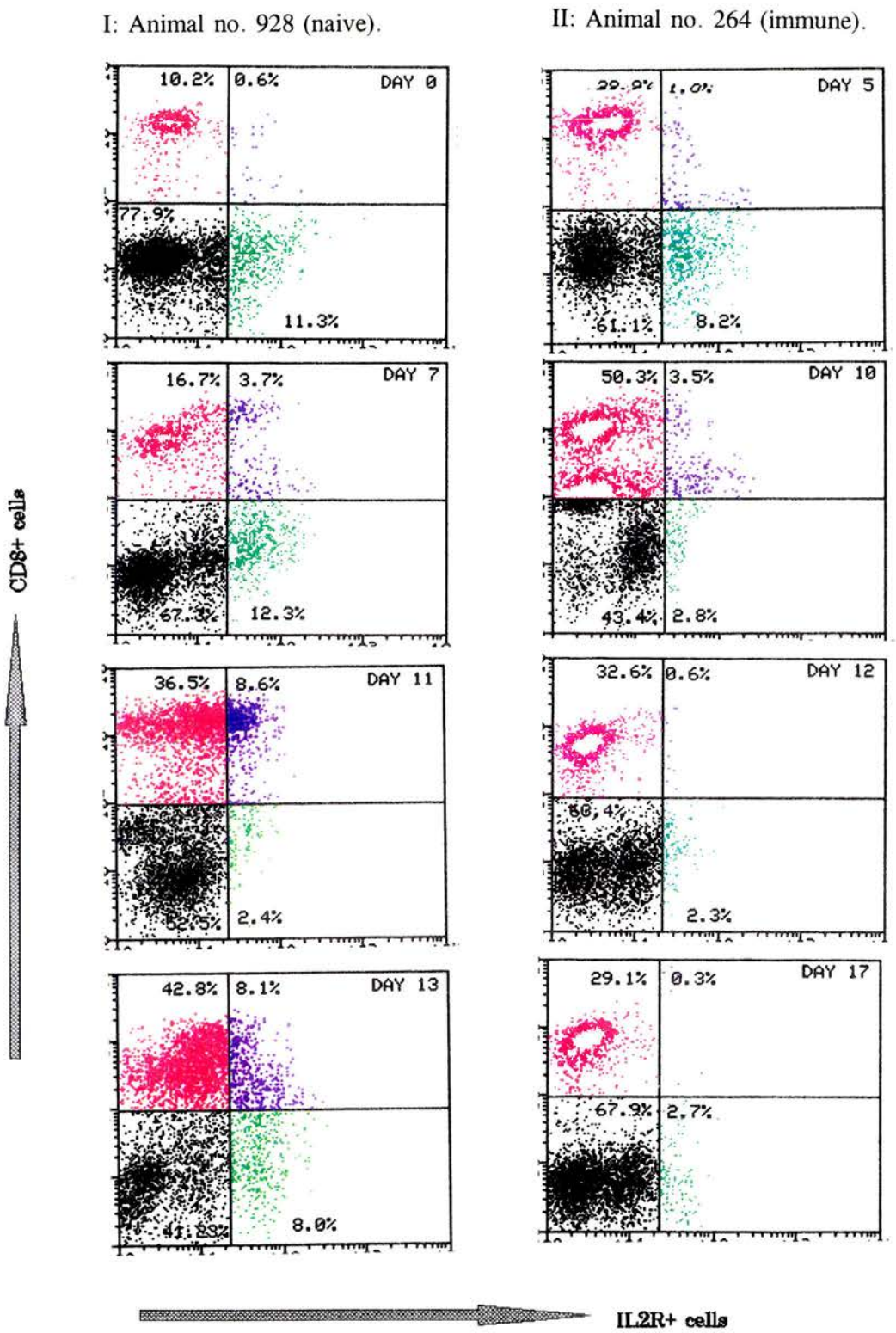


Figure 6.5b: Dot plots of two colour immunofluorescent staining on ELL. $CD8^+$ cells are expressed on the Y axis (FL2). IL-$2R^+$ ($CD25^+$) cells are expressed on the X axis (FL1).
I: Animal no. 928 (naive). II: Animal no. 264 (immune).

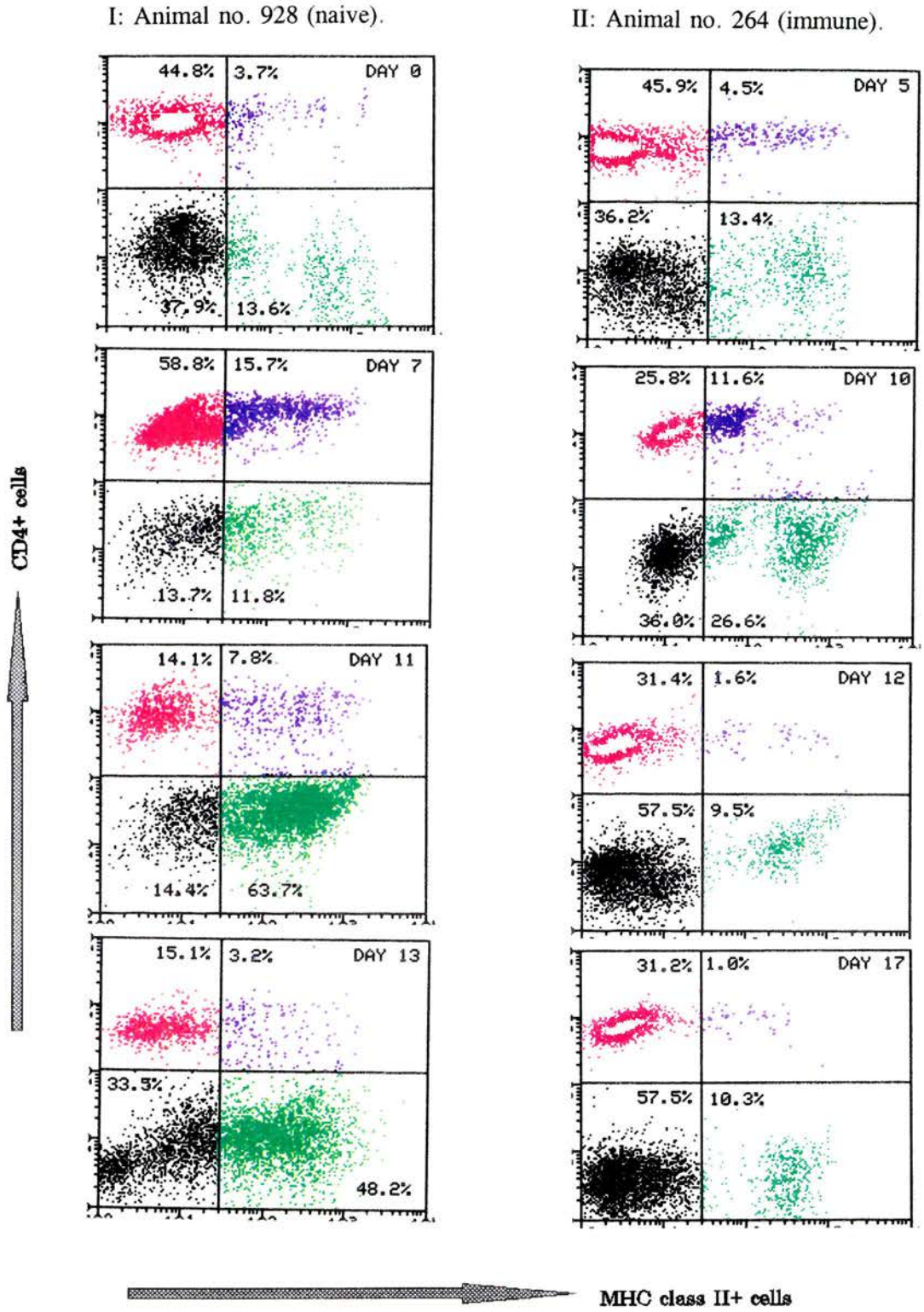


Figure 6.5c: Dot plots of two colour immunofluorescent staining on ELL. $CD4^+$ cells are expressed on the Y axis (FL2). MHC class II^+ cells are expressed on the X axis (FL1).
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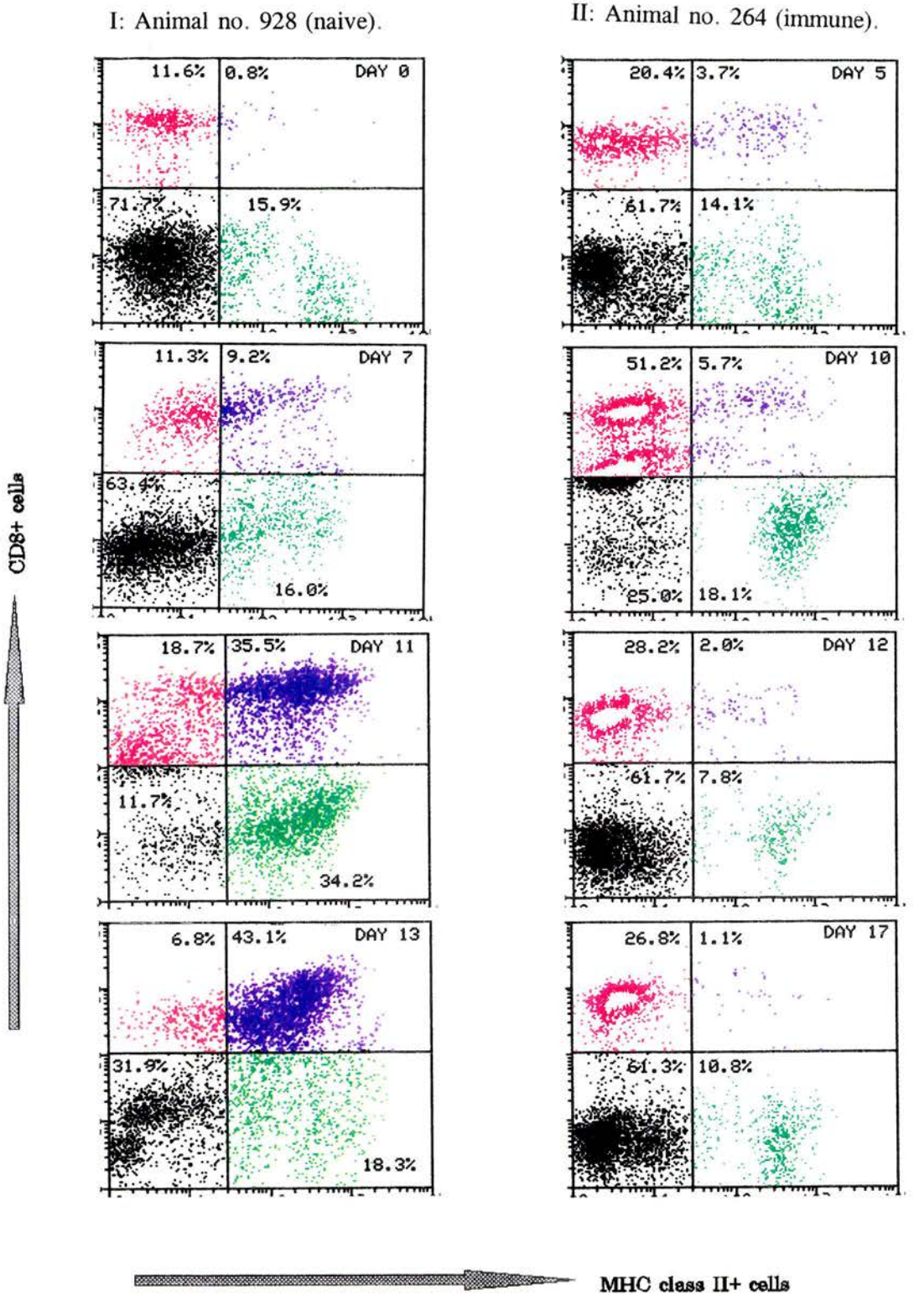

Figure 6.5d: Dot plots of two colour immunofluorescent staining on ELL. $CD8^+$ cells are expressed on the Y axis (FL2). MHC class II^+ cells are expressed on the X axis (FL1).
I: Animal no. 928 (naive). II: Animal no. 264 (immune).

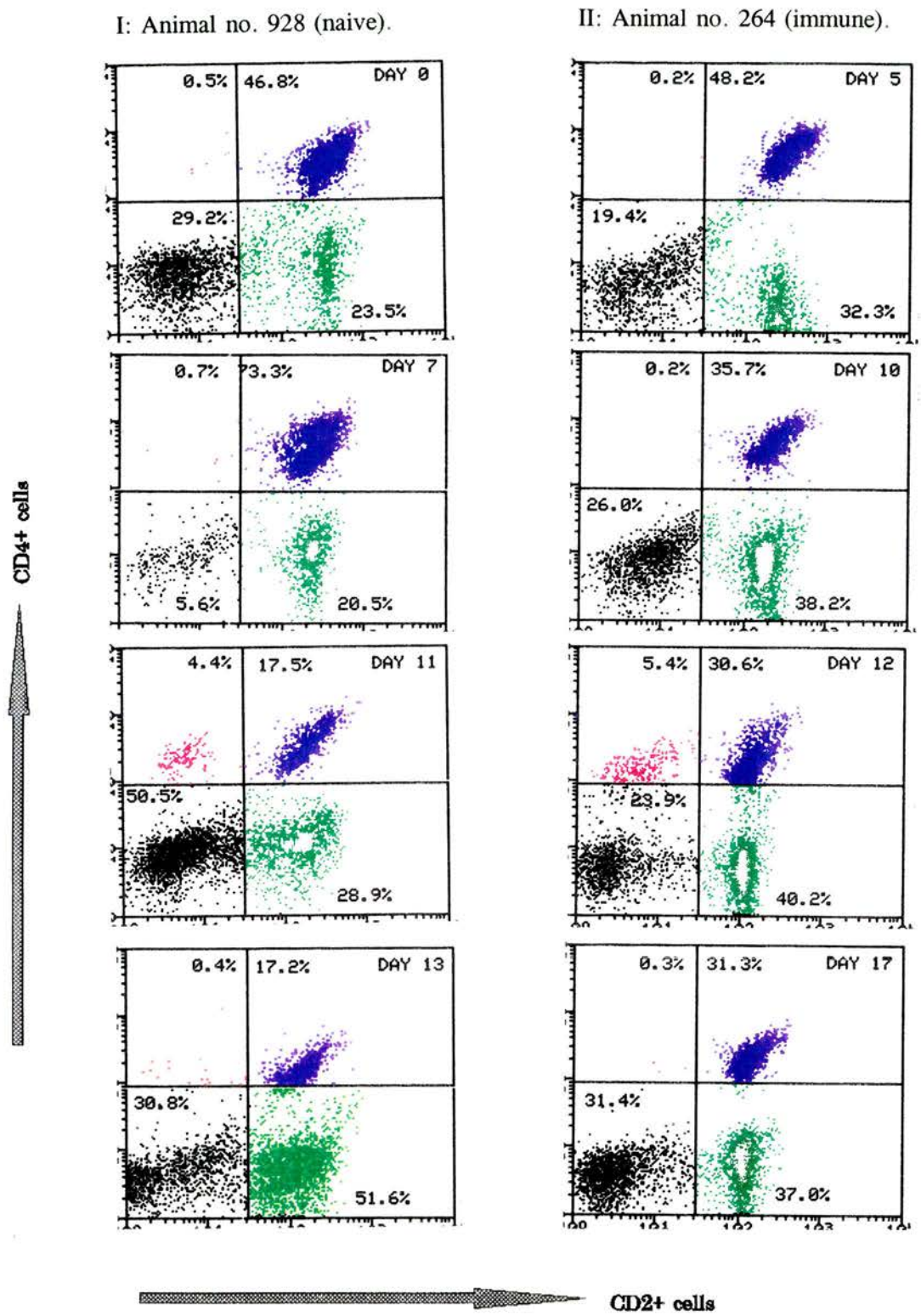

Figure 6.5e: Dot plots of two colour immunofluorescent staining on ELL. $CD4^+$ cells are expressed on the Y axis (FL2). $CD2^+$ cells are expressed on the X axis (FL1).
I: Animal no. 928 (naive). II: Animal no. 264 (immune).

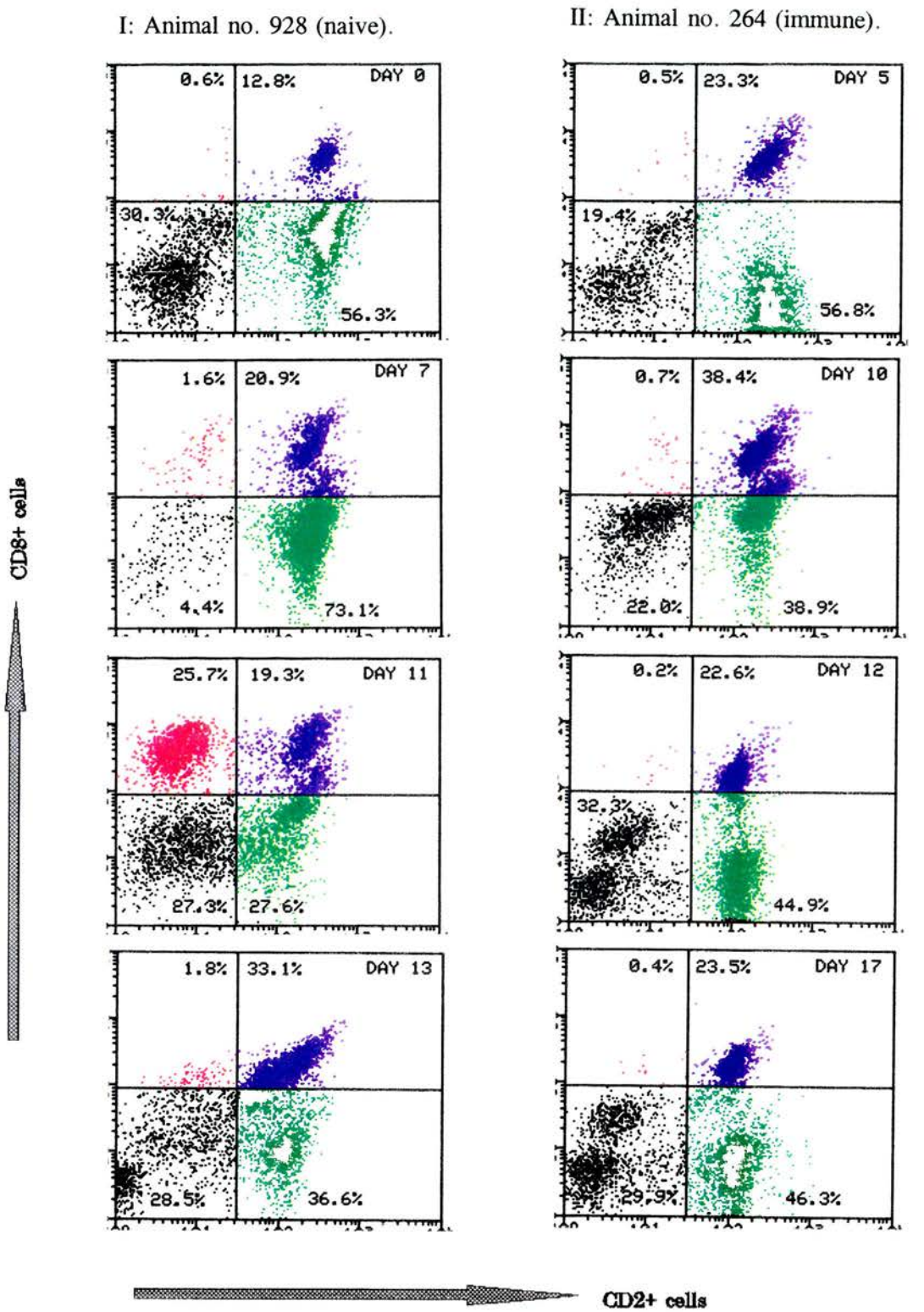

Figure 6.5f: Dot plots of two colour immunofluorescent staining on ELL. $CD8^+$ cells are expressed on the Y axis (FL2). $CD2^+$ cells are expressed on the X axis (FL1).
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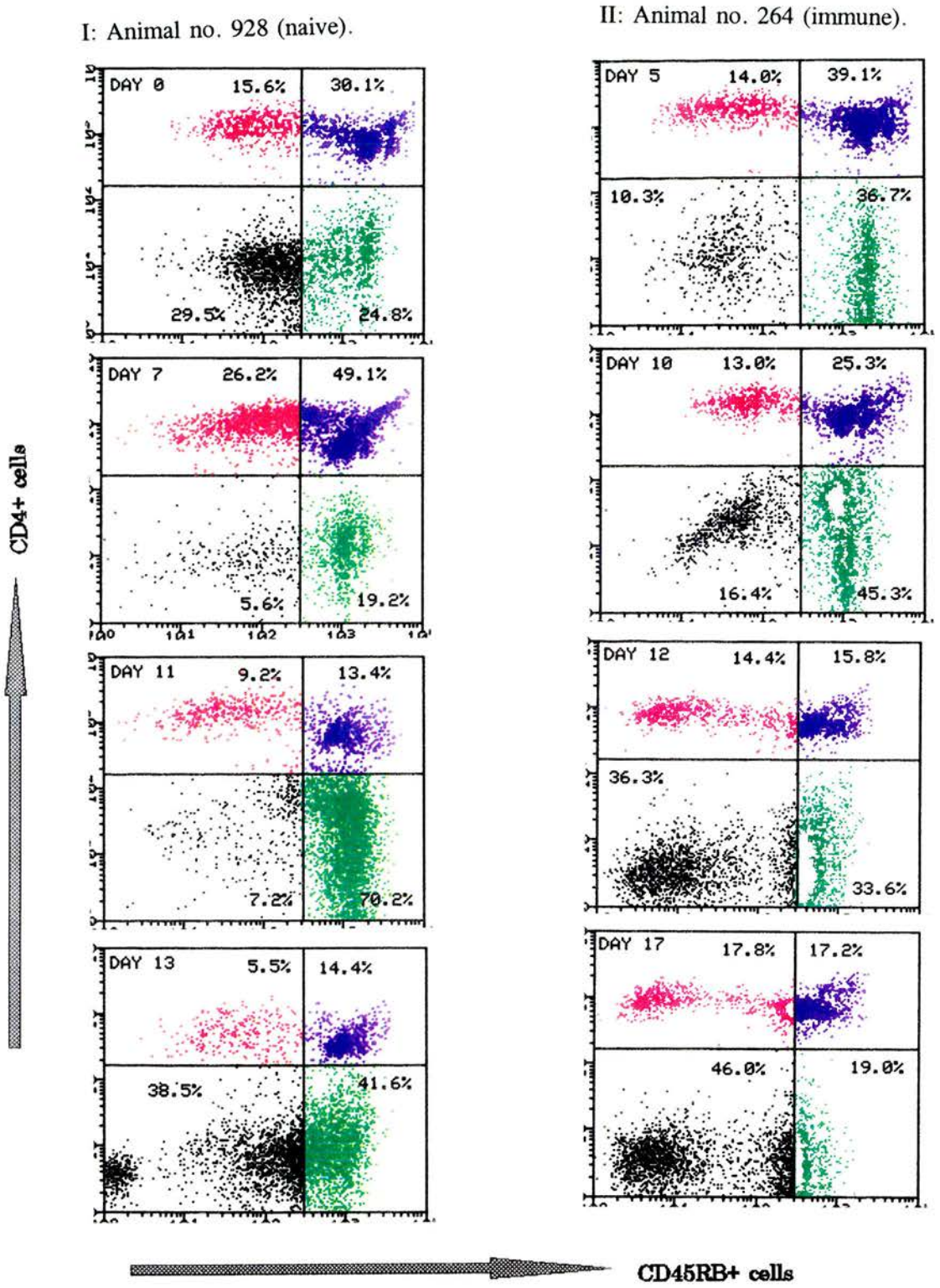


Figure 6.5g: Dot plots of two colour immunofluorescent staining on ELL. $CD4^+$ cells are expressed on the Y axis (FL2). $CD45RB^+$ cells are expressed on the X axis (FL1).
I: Animal no. 928 (naive). II: Animal no. 264 (immune).

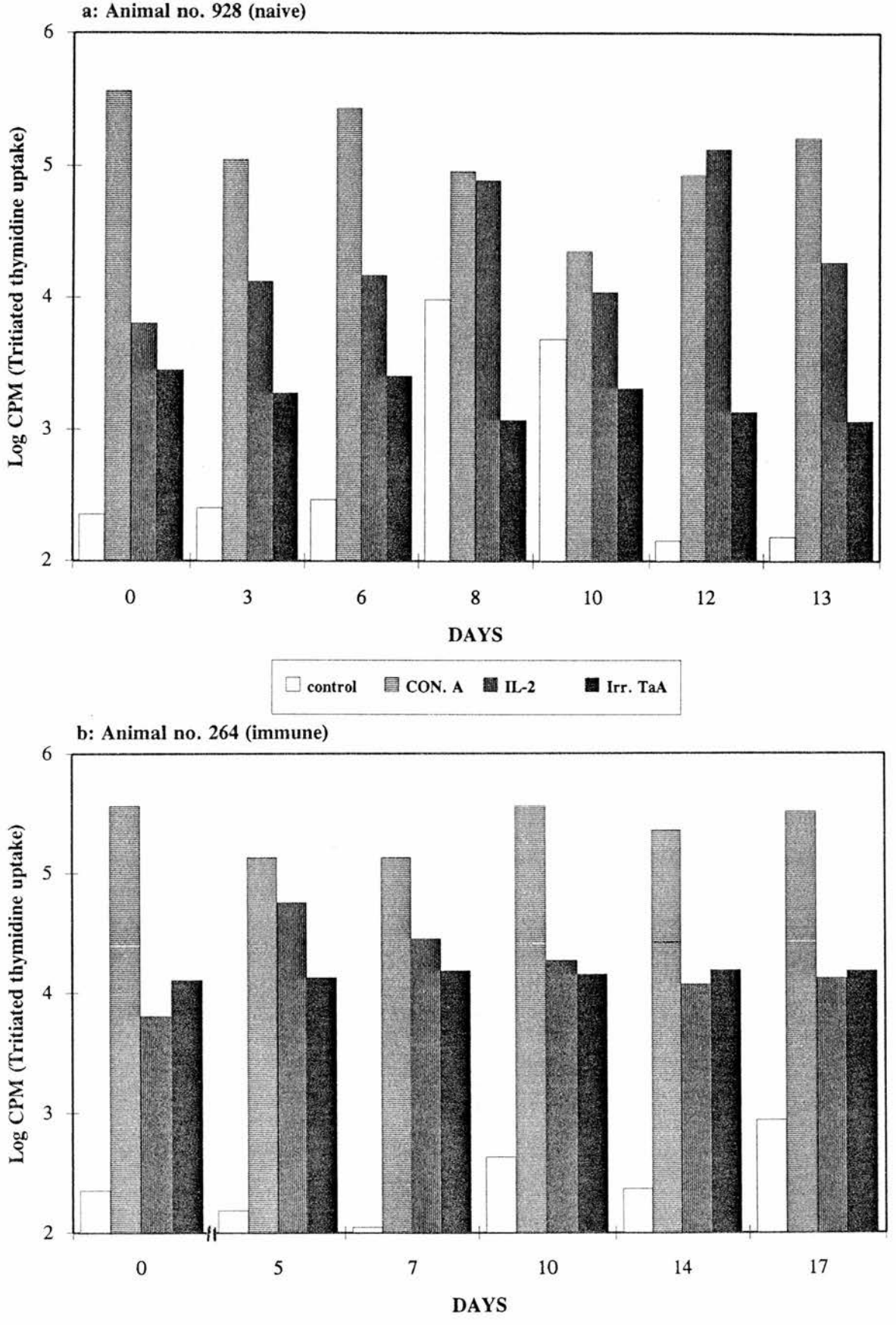


Figure 6.6: Proliferative responses of ELL in response to various antigen stimuli *in vitro*.
Values represent the mean of quadriplicate counts.
a: Animal no. 928 (naive). b: Animal no. 264 (immune).
(Data shown for animal 264 for day 0 for animal 264 was after first cannulation and challenge, and the rest of the data from day 5 to 17 was after second cannulation and challenge).

6.3.7 Cytotoxicity assays

No cytotoxic activity was observed in ELL or PBM of naive animals during the course of *T. annulata* infection in direct cytotoxicity assays against autologous *T. annulata* infected cells (fig. 6.7). MHC class I restricted cytotoxic activity started to appear only after treatment. Cytotoxic activity was 13.6% in ELL on day 12 and 11.6% in PBM on day 12 (40:1 effector:target ratio) against autologous parasite infected cells in animal 928. The parasite specific MHC restricted cytotoxic activity was seen after challenge of the immune animal between day 10 to 14. Peak cytotoxic activity in ELL of immune animal was seen on day 10 (22.5% at 40:1 E:T ratio) and in PBM on day 12 (15.4% at 40:1 E:T ratio) as shown in fig. 6.7.

6.3.8 Interferon-γ assay

High levels of IFN-γ were detected in naive animals undergoing sporozoite challenge from day 4 onwards at the same time as the cell shutdown (fig. 6.8a & 6.8b). The level of IFN-γ started to decrease with the appearance of parasite infected cells in the lymph after day 9. Very low levels of IFN-γ were detected in lymph of the immune animal from day 5-8, but was undetectable during the rest of the period. IFN-γ was undetectable in the lymph of the two uninfected animals. Lymph supernates with high levels of IFN-γ inhibited growth of *T. annulata* infected cell lines *in vitro*. A similar inhibitory effect was also produced by bovine recombinant IFN-γ (fig. 6.9).

6.4 DISCUSSION

6.4.1 Clinical reactions

Two naive animals (903 & 928) developed clinical theileriosis after infection but animal 264 was solidly immune and did not show any reactions after the first or second sporozoite challenges. The naive animals were challenged with only 0.1 t.e. stabilate, whereas the immune animal was challenged with 2 t.e. stabilate on both occasions. A dose of 0.1 t.e. was enough to produce clinical theileriosis. Challenge experiments in chapter 4 were done with 1.0 t.e. stabilate where serious disease was seen in some animals of group 2 and 3. This indicates that tick material used in all these experiments was highly potent in producing clinical theileriosis. Sick animals in chapter 4 exhibited fever from day 5 onwards.

FIG. 7: Killing of autologous T. annulata infected cells in cytotoxicity assays.

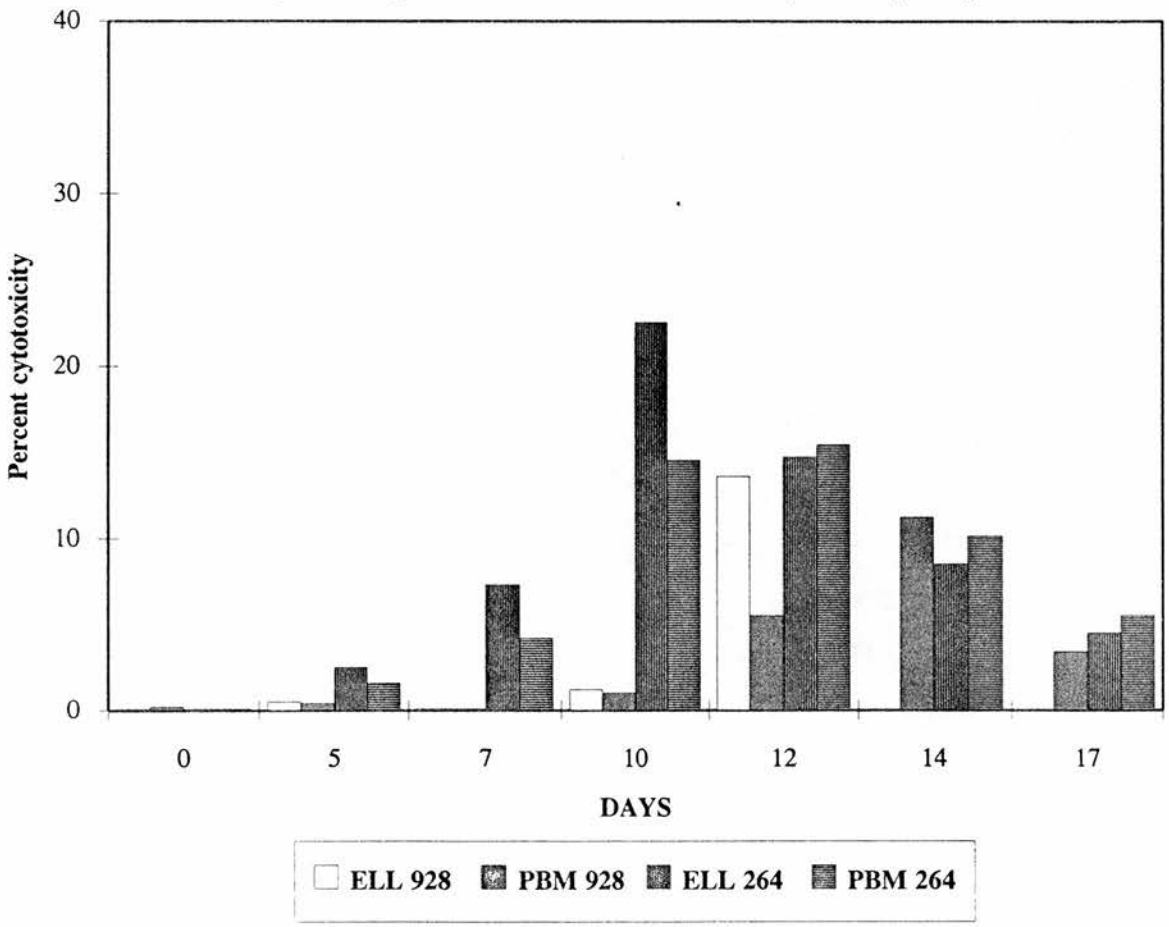
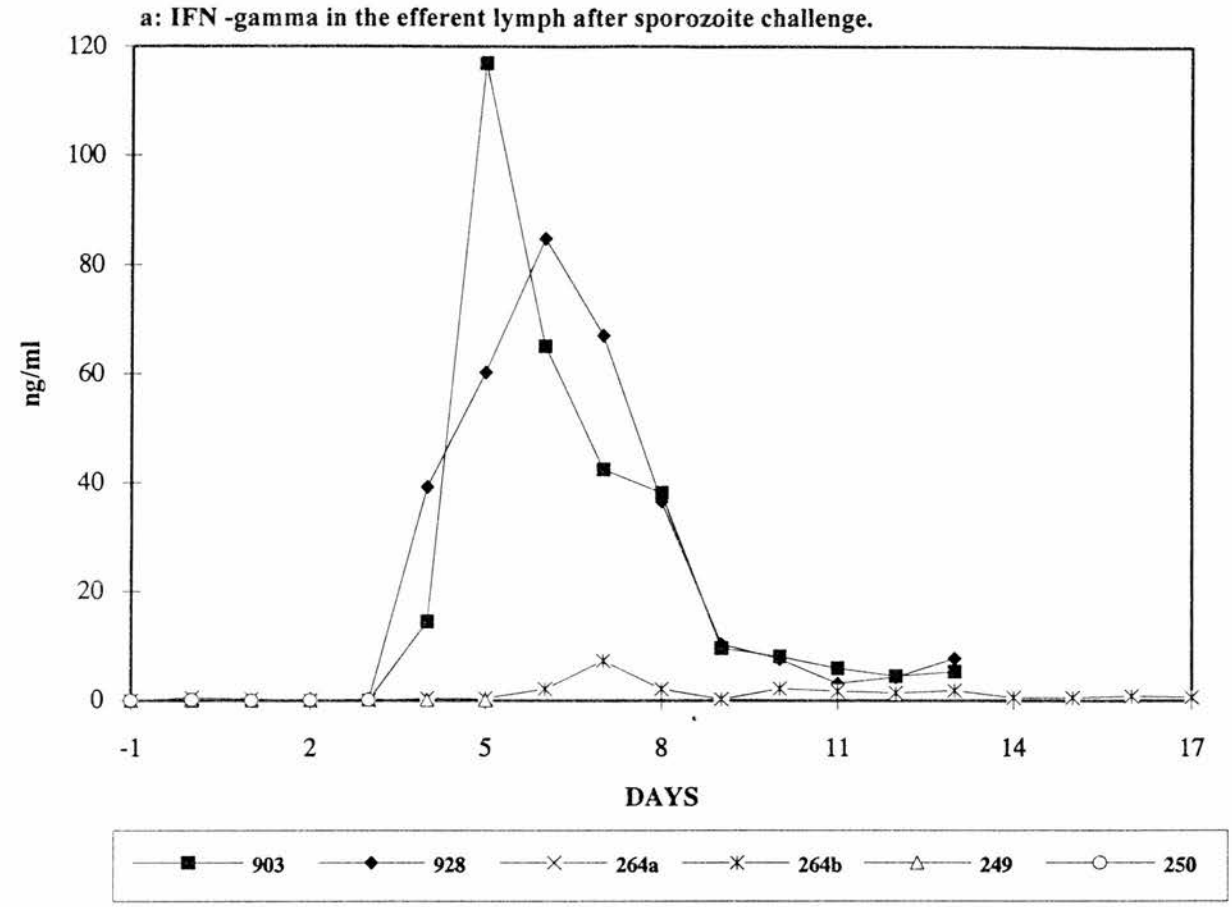


Figure 6.7: Cytotoxic activity of ELL and PBM against autologous *T. annulata* infected cells in cytotoxicity assays after infection with sporozoites.
The figures represent percent cytotoxicity at effector : target ratio 40:1 (Mean of duplicate counts).



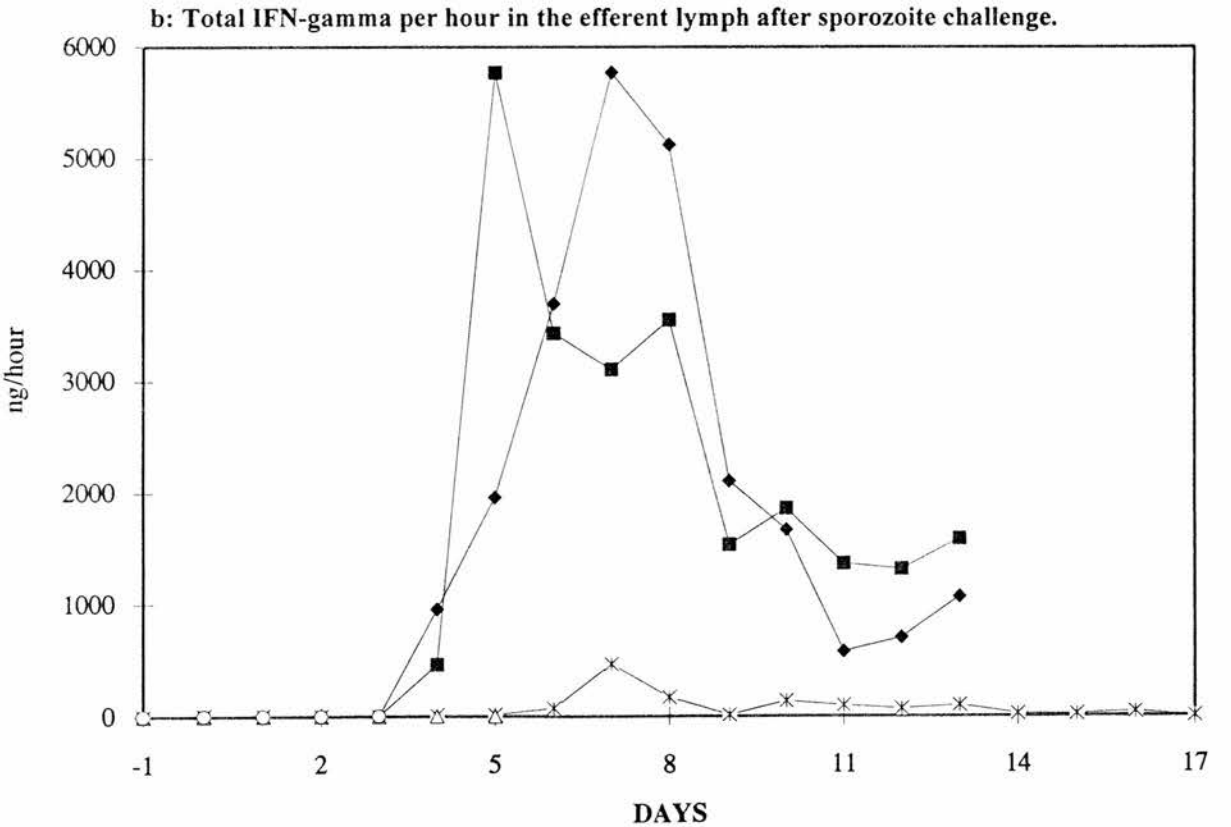


Figure 6.8: Levels of IFN-γ in the draining efferent lymph of animals following infection with *T. annulata* sporozoites.

a:Levels of IFN-γ expressed as ng per ml of lymph. b: Levels of IFN-γ expressed as ng per hour released in the efferent lymph. (→): day of treatment.

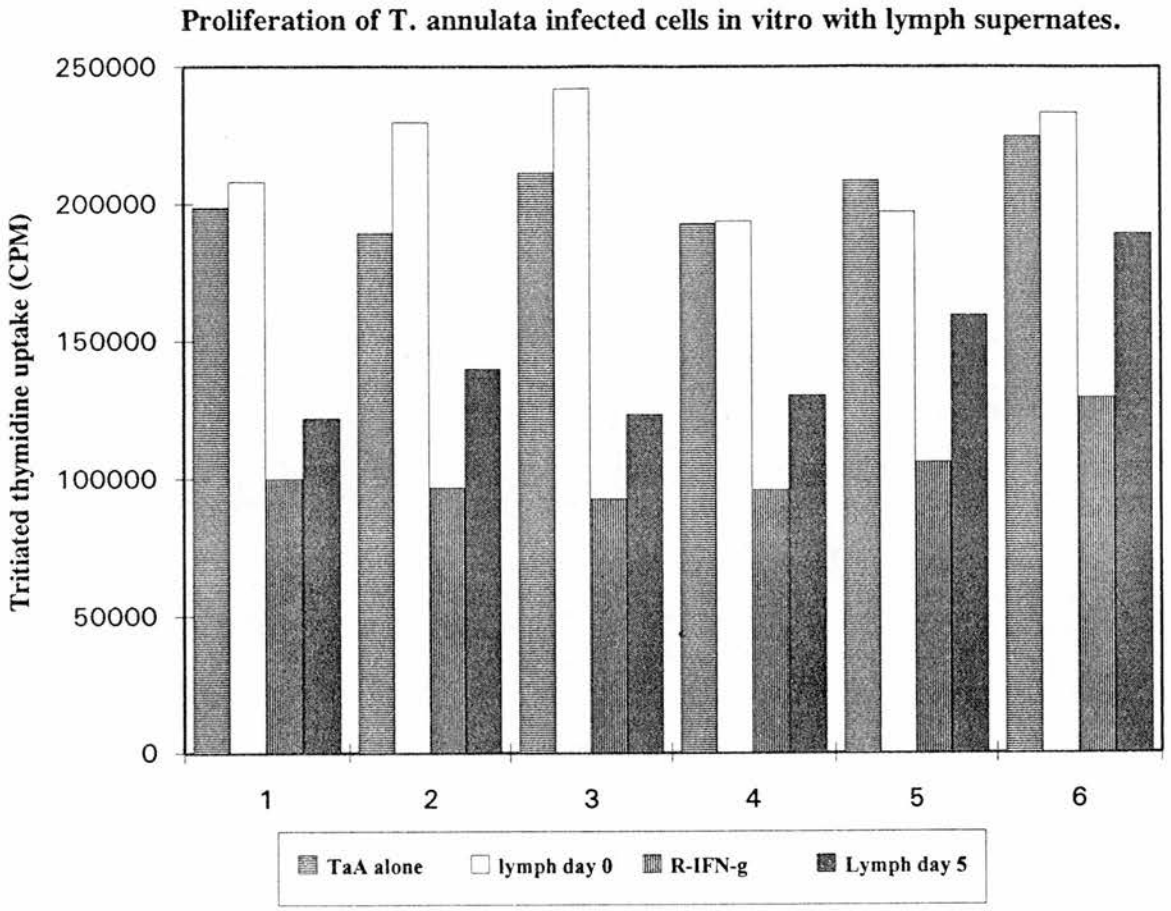


Figure 6.9: Effect of recombinant bovine IFN-γ and lymph supernate with high levels of IFN-γ on proliferation of *T. annulata* cells *in vitro*. (Values represent the mean of quadriplicate counts).

TaA alone: *T. annulata* infected cells in medium only.

Lymph day 0 and lymph day 5: *T. annulata* infected cells with lymph supernate from day 0 (IFN-γ level = negligible) and day 5 (IFN-γ level = 117 ng/ml).
1. 100 μl/well; 2. 50 μl/well; 3. 25 μl/well; 4. 12.5 μl/well; 5. 6.25 μl/well. 6. 3.12 μl/well.

R-IFN-g: *T.annulata* infected cells with serial dilutions of recombinant bovine IFN-γ @ 100 μl per well of: 1. 100ng/ml, 2. 50ng/ml, 3. 25 ng/ml, 4. 12.5 ng/ml, 5. 6.25 ng/ml. 6. 3.12ng/ml.

High levels of IFN-γ were detected in efferent lymph of naive animals from day 4 onwards in the experiments mentioned in this chapter. It is very likely that IFN-γ might be playing a role in pathology of the disease as has also been suggested by Ahmed *et al.* (1993). IFN-γ can stimulate macrophages to secrete high levels of TNF-α. Repeated injections of recombinant bovine TNF-α in cattle produce symptoms of anorexia, depression and reversible debilitating condition (Bielefeldt Ohmann *et al.*, 1989). However, fever appeared from day 8 onwards in these animals. Delay in fever may have occurred for two reasons; IFN-γ leaving in efferent lymph was collected in bottles and infused back later into the animal. The effect produced by immediate infusion may have been different from the slow release of the cytokine in a normal response. The second reason could be the low dose of sporozoites given to these animals.

6.4.2 Kinetics of the parasite dissemination

Examination of efferent lymph draining the site of acute *T. annulata* infection revealed that infected cells entered the circulation via efferent lymph from day 6 onwards. In the sporozoite challenge experiments in chapter 4, schizonts were detected in lymph node biopsies from day 5 onwards. The parasite infected cells seemed to exit in efferent lymph immediately after their appearance in the lymph node. However animals in chapter 4 were challenged with 1 t.e. GUTS as compared to 0.1 t.e. GUTS used for naive animals in this experiment. The number of infected cells in the lymph continued to increase in the following days as was seen in biopsy smears in chapter 4. Parasite infected cells were isolated in culture from efferent lymph from day 6 and in PBM from day 9 onwards, indicating that parasite dissemination from the draining node to other parts of body takes place through the efferent lymph. Similarly, *T. parva* infected cells have been reported to appear in efferent lymph from day 8 of infection (Emery, 1981b) and have been shown to disseminate through central lymph to the rest of the body (DeMartini & Moulton, 1973b).

The striking difference between the two parasites is that systemic spread of *T. parva* infection occurs immediately after sporozoite challenge. Parasite infected cells can be seen in most of distant lymph nodes and other lymphoid organs of the animal, two days after the first appearance of schizonts in the local draining node, leading to a generalised infection (Jarrett *et al.*, 1969; DeMartini & Moulton, 1973a; Radley *et*

al., 1974; Emery, 1981b; Morrison *et al.*, 1981b). Presumably *T. parva* infected schizonts exiting in efferent lymph enter the circulation and spread foci of infection throughout the body of animal. However in the case of *T. annulata*, infected schizonts are infrequently seen in the distant lymph nodes. A few schizonts can be seen in liver, kidney, lungs and peripheral blood during the terminal stages of the disease (Sergent *et al.*, 1945; Neitz, 1957). It was, therefore, anticipated that *T. annulata* infection might be confined to the draining lymph node initially as a localised condition. Immunohistology of the draining lymph node on day 10 of infection showed only abundant parasite infected cells with total destruction of lymphoid architecture (Campbell *et al.*, 1994c). Dissemination of infected schizonts in the efferent lymph raises a question about fate of infected cells exiting the node and their role in the pathogenesis. An important pathological lesion of the disease is "punched up" necrotic ulcers in abomasum and the parasite can be isolated from the liver, spleen, lungs and kidneys during the terminal stages (Sergent *et al.*, 1945; Neitz, 1957; Uilenberg, 1981b). These are probably the site of predilection for infected cells exiting in efferent lymph. *T. annulata* infected cells, when injected s/c in SCID mice, formed tumours at the site of inoculation. Some infected cells were found to infilterate to other tissues mainly to kidneys and lungs (Fell & Preston, 1993), further confirming selective migration of *T. annulata* infected cells to these organs.

The differences in migration between infected cells of *T. annulata* and *T. parva* might be explained by the ability of the two parasites to infect different host cell subpopulations. *In vitro* studies have shown that *T. annulata* preferentially infects cells of macrophages/monocytes lineage (Spooner *et al.*, 1989; Innes *et al.*, 1989b; Glass *et al.*, 1989; Campbell *et al.*, 1994a) and *T. parva* transforms T cells (Baldwin *et al.*, 1988a). Recirculation and homing patterns of T cells and macrophages are different. T cells recirculate either from the blood through tissues into lymph node and back to blood, or from the blood to lymph node through HEV to efferent lymph and back to blood (Mackay *et al.*, 1992b). Monocytes emigrate from the blood stream into the tissues in response to molecular changes on the surface of blood vessels but do not recirculate (Trnka & Cahill, 1980; Springer, 1994). Cells of the macrophage or monocyte lineages do not normally exit in efferent lymph (Cahill *et al.*, 1978; Mackay *et al.*, 1988). Although it is not exactly known whether these two parasites infect the same cell subpopulations *in vivo* as they do *in vitro*. Large numbers of IL-

$A24^+$ cells appeared corresponding to the exit of *T. annulata* infected cells in efferent lymph. Most *T. annulata* infected lines cultured *in vitro* are IL-$A24^+$ (Glass & Spooner, 1990b; Campbell *et al.*, 1994a), which detects a determinant on macrophages (Ellis *et al.*, 1988). Presumably the parasite infected cells exiting the node also expressed the marker. Sporozoite infection and transformation by both parasites induce phenotypic changes on the cell surface (Spooner *et al.*, 1988; Campbell *et al.*, 1994a). The loss or expression of different markers may further alter the migration, recirculation and homing patterns of these cells. Further studies on the phenotype of cells infected *in vivo* and molecular changes induced by the two parasites, on transformation of cells, could be useful in understanding the migration of infected cells and consequently pathogenesis.

The failure to detect or culture *T. annulata* infected cells from efferent lymph of the immune animal, after sporozoite challenge at any stage, indicates that protective immunity against the parasite was effected in the draining lymph node. On the other hand, infected cells from the naive animals could be cultured from day 6 onwards corresponding with appearance of the infected cells in efferent lymph. Parasite infected cells could not be isolated before day 6 from efferent lymph indicating that sporozoite infected lymphocytes perhaps do not exit in efferent lymph until transformed to schizonts. However, infected cells were grown *in vitro* from lymph node cells collected by surgically extirpating the draining lymph nodes on day 2 and 4 after infection (J.D. Campbell, unpublished observations). Culturing of lymph supernates collected at different time intervals immediately after inoculation of sporozoites with PBM from another animal did not produce any parasite infected cell line. Sporozoites are very efficient in infecting and transforming PBM *in vitro* (Brown, 1987). Therefore, it seems unlikely that free sporozoites reach the efferent lymph. These observations suggest that the parasite is localised to the draining lymph node until schizonts are formed. However, other observations in our laboratory do not support this postulate as animals inoculated with sporozoites above the prescapular lymph node developed clinical theileriosis even after removal of the draining lymph node on day 2 or 4 (J.D. Campbell & R.L. Spooner, unpublished observations). Similarly, animals inoculated with *T. annulata* infected cell lines still became carriers of infection even after removal of the draining lymph node on day 4 and 9, (P. Goel & R.L. Spooner, unpublished observations). There could be two possibilities; the first

being that the parasite material inoculated above the prescapular lymph node had drained to some other lymph node, or that a different path other than the efferent lymph exists for dissemination of the parasite.

In the light of these observations, the mode of parasite dissemination needs to be investigated further and could be studied by two approaches. The first could be infusion of parasite material via a cannulated afferent lymphatic and then following it in the lymph node and efferent lymph so that danger of drainage to other lymph nodes is overcome. If an antigen is inoculated via afferent lymphatic and all of the efferent lymph is drained, the response should be confined to the infused node, leaving the rest of the animal immunologically virgin in respect to the antigen used (Trnka & Cahill, 1980). Blood supply in a normal node only provides one way migration of cells from blood to lymph (Hall *et al.*, 1967; Smith *et al.*, 1974). However, cannulating an intact afferent lymphatic would be technically difficult. The second approach could be to label the parasite material with DNA based fluorescent dyes and monitor the migration of cells containing fluorescence in various immunological compartments of the animal body (Horan *et al.*, 1990). A potential problem with this approach could be the dilution of fluorescent material with multiplication of the parasite.

6.4.3 Cell shutdown

A dramatic decrease in lymphocyte output commonly known as the phenomenon of "cell shutdown" frequently but not invariably occurs soon after a lymph node has been challenged with an antigen (Hall & Morris, 1965b; Cahill *et al.*, 1976; Hopkins *et al.*, 1981a; Hein & Supersaxo, 1988; Mackay *et al.*, 1992b). Cell shutdown was observed around day 4-6 of infection in the naive animals as indicated by a very low cell output in efferent lymph, but not in the immune animal. In the case of *T. parva* infection, a slight depression in total cell output was observed in the period immediately after sporozoite challenge followed by increased cell output over the next 6 days (Emery, 1981b). Cell shutdown in the lymph node has been reported to be a normal response to secondary antigenic stimuli, but can also occur shortly after primary challenge before an immune response to the antigen has developed. This can be induced by complement activation (McConnell & Hopkins, 1981) leading to increased secretion of PGE_2 by macrophages in the node (Hopkins *et al.*, 1981b). Elevated levels of corticosteroids can also be associated with decreased lymphocyte

traffic through the lymph nodes (Hall, 1986). IFN-α2a can be involved in regulating lymphocyte output from the nodes (Hein & Supersaxo, 1988). The cell shutdown has also been shown to occur on primary infections with live viruses in sheep (Smith & Morris, 1970; Issekutz, 1985; Bird *et al.*, 1993). However, mechanisms controlling the changes in output of lymphocytes from a lymph node undergoing an immune response are not fully understood.

Cell shutdown observed in the two naive animals undergoing acute theileriosis was not observed immediately after inoculation of sporozoites as reported to occur in the literature (Trnka & Cahill, 1980; Hopkins *et al.*, 1981b), but was observed between day 4-6 corresponding to the initial development of schizonts. During cell shutdown, subcapsular and paracortical regions are densely packed with lymphocytes and there is accumulation of cells around blood vessels in the medulla suggesting continued entrance of lymphocytes into the node during cell shutdown (Hein & Supersaxo, 1988). Accumulation of small T cells and large macrophage like cells in medullary sinuses in the draining lymph node was observed during *T. annulata* infection (Campbell *et al.*, 1994b). Some *T. annulata* infected cell lines have been reported to produce IFN type 1 (α/β) like activity (Entrican *et al.*, 1991). Cell shutdown in these experiments might be IFN mediated as suggested by Hein & Supersaxo (1988) which could be secreted by activated CD4 cells or newly transformed parasite infected cells. However, involvement of other mediators cannot be ruled out as these were not studied in these experiments.

6.4.4 Flow rate and cellularity

Flow rate of lymph in efferent lymphatics draining the site of *T. annulata* infection increased 8-10 fold after day 5 in naive animals and coincided with the enlargement of lymph nodes. The rate of lymph flow has been shown to increase by 2-3 fold around 8-12 days after acute *T. parva* infection in cattle (Emery, 1981b). A two fold increase in flow of lymph has also been reported on days 5-6 in sheep during *Chlamydia psittaci* infection which declined to pre-infection levels in the following days (Huang *et al.*, 1991). There is a marked increase in the blood supply during antigen stimulation resulting in an increase in lymphocyte entry to the node (Hay & Hobbs, 1977). Whether increased blood flow is mediated by release of cytokines or vasoactive substances is not known. However, high levels of IFN-γ were observed between day 5-9 corresponding to the onset of increase in the lymph flow, which

might be a contributing factor. The kinetics of other potential mediators like prostaglandins (Trnka & Cahill, 1980; Hopkins *et al.*, 1981b), macrophage migration inhibitory factors (Hay, 1973), mitogenic factors (Hay *et al.*, 1973) were not studied. Suitable assay techniques for other bovine cytokines secreted by parasite infected cells or activated T cells were not available, which might be responsible for enlargement and later haemorrhages of the lymph node.

There was a multifold increase in total cell output from the node following cell shutdown in naive animals. It has been suggested that cells exiting the node after antigen stimulation comprise lymphocytes which entered the node during cell shutdown and those generated within the node in response to the antigen (Hay & Hobbs, 1977). Total cell output from the node also increased transiently in the immune animal peaking on day 7-8 and started to decline thereafter. An increase in total cell output from the draining lymph node (maximum $8x10^8$ per hr) has also been observed after infection with *T. parva* (Emery, 1981b). Other micro-organisms in sheep like *Chlamydia psittaci* (Huang *et al.*, 1991) and *Toxoplasma gondii* (McColgan *et al.*, 1987; Buxton *et al.*, 1994) have also been shown to cause increased cell output from the node. However, the increase has not been as enormous as observed in these experiments or after *T. parva* infection. This might partially be because of a strong T cell proliferation induced by the parasites (Morrison *et al.*, 1981a) and also shown to occur in response to *T. annulata* (Glass & Spooner, 1990b; Campbell *et al.*, 1994c).

Another important observation was the appearance of large numbers of blasting cells in efferent lymph of naive animals after sporozoite infection, which constituted more than 80% of lymph cells. This also included many parasite infected schizonts during later stages of the infection. A strong lymphocyte proliferation in response to *T. annulata* seemed to contribute in the increased output of total and blasting cells from the node. Appearance of blasting cells of similar levels in efferent lymph (Emery, 1981b) and tracheal and thoracic duct (DeMartini & Moulton, 1973b) has also been reported during *T. parva* infection. A transient appearance of blasting cells in the efferent lymph has been observed in sheep in response to vaccinia virus (Issekutz, 1985), Maedi-visna virus (Bird *et al.*, 1993), *Toxoplasma gondii* infection (McColgan *et al.*, 1987), *Chlamydia psittaci* infection (Huang *et al.*, 1991) in sheep. However blasting cells were reported to appear in the efferent lymph only transiently

in these infections. In contrast, these kept on increasing in the efferent lymph during both *T. annulata* and *T. parva* infection in cattle until the later stages of the disease. Blasting cells observed in these experiments were mainly T cells, further confirming that *T. annulata* induces a strong lymphoproliferation in the lymph node as also reported earlier for both the parasites (Morrison *et al.*, 1981b; Campbell *et al.*, 1994b). Strong proliferation of cells exiting in efferent lymph corresponded histologically to distention of medullary sinuses with accumulation of small and blasting cells. This was followed by disappearance of the existing germinal centres with no sign of *de novo* germinal centre formation in the node and appearance of large areas of dead cells, some small lymphocytes and discrete areas of blasting cells (Campbell *et al.*, 1994c). Increase in T cell output from the draining node is in contrast to leucopenia observed in the peripheral blood during acute *T. annulata* infection (Laiblin, 1978; Preston *et al.*, 1992a; this thesis, chapter 5). The parasite seems to induce a strong proliferation in lymphocytes in the draining lymph node followed by apoptosis, as T cells and particularly CD4 cells are specifically depleted from the peripheral circulation.

6.4.5 Activation of T cells

Phenotypic changes in various lymphocyte subsets of efferent lymph after *T. annulata* infection in the naive animals revealed an initial increase in $CD2^+$ cells corresponding with an increase in $CD4^+$ cells after day 5. A large number of these cells were $CD25^+$ and MHC class II^+. This was followed by an increased output of $CD8^+$ cells with high expression of MHC class II and CD25. Similarly, blasting $CD4^+$ cells were reported to appear in efferent lymph from day 6 followed by a switch to $CD8^+$ cells around day 9 or 10 in sheep after infection with a live incomplete strain of *Toxoplasma gondii* (Innes *et al.*, 1992b). A non-random migration of T lymphocytes in efferent lymph has been suggested following ovalbumin or BCG challenge to the node, with initial increase in $CD4^+$ cells followed by $CD8^+$ cells (Bujdoso, *et al.*, 1989b; Mackay *et al.*, 1992b). However, $CD8^+$ cells exiting the node from naive animals, despite showing activation markers, did not kill autologous infected cells in cytotoxicity assays. MHC class I restricted parasite specific cytotoxic cells were seen only transiently in the efferent lymph after treatment of these animals. Activated T cells have been reported to be lymphoblastoid and expressing MHC class II and CD25 (Greene *et al.*, 1986; Glimcher & Kara, 1992),

whereas resting T cells usually do not express MHC II and CD25. Many $CD25^+$ cells were observed in sections of the draining node on day 4 but not on day 8 and 10 following *T. annulata* infection. $CD25^+$ T cells were seen in the medulla with loss of germinal centres from the node suggesting that activated $CD4^+$ cells were not capable of providing help to B cell follicles for formation of the germinal centres (Campbell *et al.*, 1994b). However, high levels of IFN-γ were being secreted in the node at this stage. In the event of a normal protective immune response against the parasite, activated $CD4^+$ cells should interact with interdigitating cells in the paracortex (McKeever *et al.*, 1992) or B cell follicles to initiate formation of germinal centres (Bogen *et al.*, 1993). However in these experiments, T cells seemed to be nonspecifically stimulated by the parasite in the medulla, which exited in the efferent lymph allowing proliferation of parasite infected cells in the node. Functional assays on these cells have further shown that these cells were not specific for parasite antigens.

6.4.6 Role of IFN-γ in the lymphocyte activation and pathogenesis

High levels of IFN-γ were observed corresponding to an initial increase in $CD4^+$ cells with increased MHC class II and CD25 expression. Low levels of IFN-γ were also observed in efferent lymph of immune animal after challenge. An earlier report also showed that IFN-γ is secreted in efferent lymph from the node during primary responses to protein and polysaccharide antigens (Emery *et al.*, 1990). Increased levels of IFN-γ were also detected transiently after secondary challenge of the immune animal. IFN-γ has been reported to induce MHC class II expression on T cells and regulate production and secretion of IL-2 (Schreiber *et al.*, 1989). IFN-γ also induces MHC class II expression on macrophages (Benoist & Mathis, 1990; Dalton *et al.*, 1993) and activates them to produce TNF-α, which can exert cytostatic and necrotic effects on some target cells (Sugarman *et al.*, 1985; Aiyer *et al.*, 1986). A similar protective mechanism appears to be operative in tropical theileriosis as sustained presence of cytostatic macrophages in the circulation of calves recovering from the disease has been reported (Preston & Brown, 1988; this thesis, chapter 5). There is evidence for activation of adherent cells and PBM by IFN-γ to secrete TNF-α (Preston *et al.*, 1992b).

Interestingly, levels of IFN-γ in efferent lymph decreased with appearance of the parasite, which suggests that it is not produced by infected cells. Most of *T.*

annulata infected cell lines maintained *in vitro* also do not produce IFN-γ, whereas most of *T. parva* cell lines do (Entrican *et al.*, 1991; DeMartini & Baldwin, 1991; Ahmed *et al.*, 1993). Blasting CD4 cells perhaps produce this cytokine, as appearance of blasting CD4 cells correlates very well with the peak of IFN-γ. Further cell sorting studies are needed to establish the phenotype of cells secreting this and other cytokines and their effect on T cell activation and parasite control. Anti-viral activity of IFN-γ is well known. It has recently been shown to inhibit growth of *Toxoplasma gondii* as well *in vitro* (Oura *et al.*, 1993; Johnson *et al.*, 1993). IFN-γ seems to inhibit growth of *T. annulata* infected cells *in vivo*. As soon as parasite infected cells are transformed in the node, IFN-γ some how shuts down probably because activated $CD4^+$ cells exit the node and are replaced by $CD8^+$ cells. The inhibitory effect of IFN-γ on infected schizonts diminishes and further proliferation of schizonts continues. This hypothesis is supported by the inhibitory effect of recombinant bovine IFN-γ and IFN-γ rich lymph supernate on *T. annulata* transformed cell lines *in vitro* observed in these experiments. Bovine recombinant IFN-γ and bovine recombinant TNF-α are reported to inhibit establishment of trophozoite infected cell cultures *in vitro* (Preston *et al.*, 1992b).

6.4.7 Loss of CD2 adhesion molecule

It seems that animals infected with *T. annulata* mount a T cell activation response initially as evident from the appearance of activated CD4 cells followed by CD8 cells. However, increasing multiplication of the parasite in the lymph node seems to induce an inappropriate proliferation and not activation of T cells as many blasting CD4 and CD8 cells were observed to lose CD2 expression with the progression of the infection. These cells expressed MHC class II but had lost CD25 expression. Further, ELL lost responsiveness to Con. A and exogenous IL-2 stimulation *in vitro* during the latter stages of the infection. This was not because the cells were already committed to parasite specific antigens as they did not proliferate *in vitro* in response to autologous parasite infected cells. These observations suggest a state of T lymphocyte unresponsiveness as previously reported to be induced in some viral infections either because of anergy or suppressor lymphocyte activity (Reinherz *et al.*, 1980; Carney *et al.*, 1981; McChesney & Oldstone, 1989; Gruters & Miedema, 1992). Although efferent lymph from the naive animal contained abundant $CD8^+$ cells, these were $CD2^-$ and did not kill autologous parasite infected

cells in chromium release assays until treatment. Similar $CD8^+$ $CD2^-$ cells have also been observed during *T. parva* infection, but their function was not established (E.A. Innes, N.D. MacHugh & W.I. Morrison, unpublished observations). Induction of $CD8^+$ T cell activation in a defective maturation state has also been reported in HIV infection in man (Pantaleo *et al.*, 1990; Salazar-Gonzalez *et al.*, 1985) and SIV infection in rhesus monkeys (Reimann *et al.*, 1991). The circulating $CD8^+$ MHC class II DR^+ cells in HIV-infected patients did not express CD25. These cells could not be stimulated *in vitro* to proliferate in response to mitogens and other stimuli, even in the presence of additional IL-2 or IL-4 (Pantaleo *et al.*, 1990). Cattle seem to mount a similar defective activation in response to *T. annulata* infection where $CD8^+$ cells become MHC class II^+ but are CD25 negative and lose expression of CD2, an important adhesion molecule for their function. The CD2 molecule binds to LFA-3 to mediate CTL-target cell adhesion and conjugate formation, leading to activation of the cell for its effector function, which is independent of antigen recognition by the TCR (Bierer *et al.*, 1989). The number of cells bearing this defective phenotype increased with dissemination of the parasite in the efferent lymph. ELL were also unresponsive to other exogenous stimuli at this stage, suggesting this to be a parasite induced phenomenon which encourages its dissemination. On the other hand, only a few $CD4^+$ $CD2^-$ cells were observed in the immune animal on day 12, and $CD8^+$ cells were always $CD2^+$. MHC class II and CD25 expression was induced on a few $CD8^+$ cells during day 7 to 12 of the challenge. Functional assays on the ELL revealed parasite specificity indicating stimulation of a normal secondary immune response. Unresponsive lymphocytes have been recorded in the acute response to Epstein-Barr virus and cytomegalovirus (Reinherz *et al.*, 1980; Carney *et al.*, 1981) because of activation of TH_2^+ type T cells (suppressor T cells). TH_1 and TH_2 subsets are not well characterised in cattle. High levels of IFN-γ in the lymph of naive animals suggests a TH_1 like activation of T cells initially leading to anergy in these experiments.

In summary, experiments reported in this chapter suggest that *T. annulata* sporozoites induce a strong lymphoproliferation in the draining lymph node of the naive animals leading to an enormous increase in output of fluid and cells from the node. Cell output from the node increased transiently in the immune animal as well, but output of fluid was unaffected after challenge with sporozoites. A period of cell

shutdown was observed in the naive animals, corresponding to the initial transformation of schizonts in the node. This was followed by a massive output of blasting cells from the node along with exit of parasite infected cells. Blasting cells were not observed in the immune animal. Schizonts were seen in efferent lymph of the naive animals as soon as they were expected to appear in the lymph node. Parasite isolation in culture was possible from efferent lymph earlier than from peripheral blood, indicating that the parasite is disseminated to the rest of the body through efferent lymph. Large amounts of IFN-γ were observed in efferent lymph which might be responsible for some of the pathology of the parasite.

Phenotypic analysis of ELL showed an increase in T cell output which initially comprised $CD4^+$ cells followed by a huge output of $CD8^+$ cells. Naive animals initially seemed to mount a normal T cell activation response after sporozoite challenge in the form of transient CD25 and MHC class II expression on CD4 cells, followed by a strong proliferation of $CD8^+$ cells with high expression of MHC class II but not CD25. However, these cells were not parasite specific as evident from the functional assays. Parasite specific T cells were observed in the immune animal after day 7, but only after treatment in the naive animals. The parasite induced an inappropriate proliferation and nonspecific activation of T cells in the lymph node as the cells lost CD2 expression, an important adhesion molecule for normal T cell effector function.

These experients provided an understanding of nonspecific T cell activation induced by the parasite during acute infection, when a parasite specific immune response is not generated. Inoculation of a *T. annulata* infected cell line in an animal produces a parasite specific immune response. In the final chapter of this thesis, development of local immune response in the efferent lymph of the node draining the site of cell line immunisation was studied in comparison to the responses generated in the presence of allograft responses, in order to understand the generation of immunity against the parasite.

CHAPTER 7

Kinetics of parasite dissemination and T cell activation in the efferent lymph during immunisation with a *T. annulata* infected cell line in naive and skin grafted animals.

Objectives:

1. To investigate the kinetics of parasite development and dissemination in the lymph efferent from the node draining the site of immunisation with an allogeneic *T. annulata* infected cell line.

2. To study the dynamics of various lymphocyte subpopulations in the lymph efferent from the node draining the site of cell line immunisation in relation to the development of immunity.

3. To characterise T cell activation in response to immunisation with an allogeneic *T. annulata* infected cell line.

4. To study the effect of a pre-existing allogeneic response on parasite and cell dynamics in the efferent lymph after cell line immunisation.

7.1 INTRODUCTION

Theileria annulata infected cell lines have extensively been used as vaccines in cattle to control tropical theileriosis in endemic areas. Inoculation of an allogeneic cell line initially produces a response against MHC antigens of the immunising cell line followed by a parasite specific immune response in the recipient animals (Innes *et al.*, 1989a). Transfer of parasite from the donor cell line to cells of the recipient is an important pre-requisite for effective cell line immunisation (Pipano *et al.*, 1977; Brown *et al.*, 1978a; Innes *et al.*, 1989a; this thesis, chapter 4). Little is known about the mode of the parasite transfer and dissemination of the parasite infected cells. The initial cellular interactions and activation of various lymphocyte subpopulations leading to the development of immunity after cell line immunisation are not very well understood.

Previous studies have shown a transient appearance of MHC class I restricted cytotoxic T lymphocytes (Innes *et al.*, 1989a) and a more sustained macrophage mediated cytotoxic activity against the parasite infected cells (Preston & Brown, 1988) after immunisation with *T. annulata* infected cell lines. A transient increase in circulating $CD8^+$ cells and sustained presence of monocytes/macrophages in the peripheral blood were observed after cell line immunisation during these investigations (chapter 5). In chapter 6, progression of acute theileriosis was found to be associated with a non-specific activation of T cells leading to loss of CD2, an important adhesion molecule, on the cell surface. The cells activated during acute theileriosis resulting from inoculation of sporozoites displayed no evidence of parasite specificity in the functional assays.

Cells leaving in efferent lymph from an antigen primed node reflect immunological events occurring in the lymph node and are responsible for systemic dissemination and amplification of the local immune responses (Trnka & Cahill, 1980; Bujdoso *et al.*, 1989b; Mackay *et al.*, 1992b). The experiments in this chapter were, therefore, undertaken to investigate kinetics of parasite dissemination, changes in cell dynamics and T cell activation in the lymph efferent from the node draining the site of immunisation with a *T. annulata* infected cell line in naive animals.

Critical analysis of the effect of a pre-existing allogeneic response on cell line immunisation against *T. annulata* in chapter 4 revealed that development of parasite specific immunity after cell line immunisation was blocked in some animals. Cell

dynamics in the peripheral blood were mostly unaltered in the animals which did not develop immunity to the parasite after immunisation with a cell line. Experiments were therefore further extended to investigate the kinetics of cellular changes in the efferent lymph after cell line immunisation in animals already exhibiting a strong allogeneic response.

7.2 MATERIAL AND METHODS

7.2.1 Animals and experimental design

Four calves (1 Friesian, 1 Hereford & 2 Friesian x Charollais) aged 3-7 months were used in these experiments. BoLA types of the animals were determined by microlymphocytotoxicity (Spooner *et al.*, 1979a) as described in chapter 3.2 to ensure that BoLA types of the recipient animals were different from the animal chosen as the skin donor and to produce the immunising cell line (animal 10769; BoLA type A10/A11). Allogeneic immune responses were generated in animals 838 and 841 by implantation of skin collected from animal 10769 (details in chapter 3.3). Animals 933 and 859 were not skin grafted. The efferent lymphatic draining the prescapular lymph node of all the four animals was cannulated as described in chapter 3.5. The animals were inoculated s/c with $1x10^6$ cells of *T. annulata* (Hissar) infected immunising cell line 24 hours after surgery above the prescapular lymph node.

7.2.2 Cell lines

T. annulata infected cell lines were established *in vitro* by infection of PBM with sporozoites as described previously (Brown, 1983) and detailed in chapter 3.4. The cell line from the skin donor animal was used to immunise animals after cannulation of the efferent lymphatic. The cells of this line were also used as stimulators in proliferation assays and MLC, and as targets in cytotoxicity assays to measure the allogeneic response. Autologous *T. annulata* infected cells were used as targets in the cytotoxicity assays and as stimulators in the proliferation assays and MLC to evaluate the parasite specific immune responses.

Uninfected lymphoblastoid cell lines were generated from all these animals by stimulating PBM once with Con. A (Sigma) and cultured further in MLC medium containing a supplement of 20% TCGF as described in detail in chapter 3.4.3. These cells were used as target cells in the cytotoxicity assays.

7.2.3 Cannulation of efferent lymphatic duct

The efferent lymphatic of the prescapular lymph node was cannulated as described in detail in chapter 3.5. Lymph was collected into plastic bottles containing 5-10 i.u./ml heparin (Leo Laboratories Ltd.) and antibiotics (Gibco) to provide penicillin 20 i.u. and streptomycin 20 μg per ml when full. The bottle for lymph collection was changed 2-3 times a day depending on the flow rate. Lymph was collected for one hr every morning for use in various studies. The rest of the lymph was pooled aseptically and infused slowly into the animal through a jugular cannula to minimise the loss of fluid and cells, to maintain fluid, electrolyte and protein balance and not to interfere in development of the immune response.

7.2.4 Clinical monitoring

The clinical condition of the animals was assessed by observations on their general condition, rectal temperature (a febrile response being $\geq$ 39.5°C) daily, haematocrit and TLC at regular intervals (chapter 3.6). Cytospin smears from efferent lymph were prepared daily and stained with Giemsa for assessment of blasting cells and *T. annulata* infected schizonts. Blasting cells were also assessed by FACS analysis using FSC vs SSC to identify large cells. Percentage of piroplasm infected erythrocytes was assessed by Giemsa stained thin blood smears. Details of the clinical reactions are presented in chapter 4 and are discussed very briefly in this chapter.

7.2.5 Cell preparation

ELL collected from indwelling cannulae were counted in a haemocytometer. The viability of the cells was always greater than 98% as assessed by Trypan blue dye exclusion. Volume and cellularity of the lymph was measured after every collection to calculate rate of lymph flow and cellular traffic. Freshly collected ELL for one hr in the morning and PBM were purified on ficoll-hypaque (Lymphoprep) as described in chapter 3.4.2.1 and 3.4.5. These cells were used for flow cytometry, proliferation assays and cytotoxicity assays and as responders in MLC. An aliquot of lymph was centrifuged at 800*g* for 15 min. and cell-free lymph plasma supernate was decanted and stored at -20°C for IFN-γ estimation. ELL and PBM were cultured *in vitro* in 24 well plates (Intermed, Nunc) to isolate parasite infected cell lines for studying the kinetics of dissemination of parasite infected cells. Cultures were established daily from ELL and every 2-3 days from PBM.

7.2.6 Monoclonal antibodies

Various mAbs have been described in detail in chapter 3.8.1. The following mAbs were used for analysis of various lymphocyte subpopulations. IL-A26 for $CD2^+$ cells, IL-A12 for $CD4^+$ cells, SBU-T8 for $CD8^+$ cells, IL-A29 or CC 15 for $\gamma\delta$ T cells, VPM 30 for B cells. B cells expressing IgM on the surface were recognised by mAb IL-A30. IL-A24 for monocytes and macrophages, J 11 or IL-A21 for surface MHC class II molecules, IL-A111 for CD25 (IL-2 receptor α chain, Tac antigen) and CC 76 for CD45RB molecules on the cell surface.

7.2.7 Immunofluorescent Staining

Various immunoconjugates and techniques used for indirect immunofluorescent staining and flow cytometry have been explained in chapter 3.8. This technique was used to analyze phenotypic changes in the ELL following cell line immunisation. Two colour immunofluorescent staining was done at regular intervals to investigate T cell activation in ELL. One mAb was used to identify $CD4^+$ or $CD8^+$ cells and the second mAb of a different isotype for staining CD25, MHC class II, CD2 or CD45RB molecules on the cell surface. This was followed by simultaneous incubation of cells with two appropriate fluorescent secondary reagents as described previously.

7.2.8 Mixed lymphocyte culture (MLC)

ELL and PBM were cultured *in vitro* with irradiated autologous *T. annulata* infected cells or cells used for immunisation, in 6x10 ml well plates (Intermed, Nunc) for five days, in MLC medium at regular intervals after cell line immunisation. Each well contained 5 ml of ELL or PBM at $4x10^6$ cells per ml, 2.5 ml of irradiated (5000 rads) autologous PBM at $4x10^6$ cells per ml and 2.5 ml of irradiated (7500 rads) parasite infected autologous or immunising cells at $4x10^5$ cells per ml to give a responder vs stimulator ratio of 20:1. The stimulated cells were used, after five days, as effector cells in the cytotoxicity assays to measure allogeneic and parasite specific cytotoxic activity.

7.2.9 Proliferation assays

These were essentially as described by Glass and Spooner (1990a) and detailed in chapter 3.10. Briefly, ELL or PBM with irradiated PBM were incubated either alone or with Con. A or Human recombinant IL-2 or irradiated autologous *T. annulata* infected cells or irradiated cells used for immunisation in quadruplicate wells of 96 well flat bottom plates. Cell proliferation was measured after 72 hours of

incubation at 37°C in a 5% CO_2 incubator by a 6 hr pulse with [^{3}H] thymidine (Amersham) and the uptake was assessed by liquid scintillation counting.

7.2.10 Cytotoxicity Assays

The cytotoxic activity of freshly prepared ELL and PBM from the immunised animals and after stimulation with irradiated cells for five days (7.2.8) was assayed at regular intervals after immunisation throughout the experimental period. Autologous parasite infected cells, autologous uninfected blasts, parasite infected immunising cells, uninfected lymphoblasts from the animal used to create the immunising cell line and BoLA mismatched parasite infected cells were chosen as targets. The details of the technique are described in chapter 3.9.

7.2.11 Interferon-γ assay

A solid phase sandwich ELISA (enzyme linked immunosorbant assay) using mAbs specific for bovine IFN-γ was used (Wood *et al.*, 1990; Rothel *et al.*, 1990) to measure levels of IFN-γ in the efferent lymph using a ready made test kit (CSIRO, Australia) as described in chapter 3.11.

7.3 RESULTS

Efferent lymph cells were monitored as long as the cannulae kept flowing. Once the cannula stopped, some of the observations were continued on PBM and lymph node cells collected by a needle biopsy up to three weeks after cell line immunisation. The cannula remained patent for 12 days in animal 933, 18 days in 859, 21 days in 838 and 9 days in 841.

7.3.1 Clinical reactions

The detailed clinical, parasitological and haematological reactions in these animals after cell line immunisation are described in chapter 4. Briefly, animals 933 and 859 expressed mild parasitological reactions in the form of schizonts and piroplasms after cell line immunisation. Out of the two animals expressing a pre-existing allogeneic immune response, one animal (838) exhibited very low levels of schizonts in efferent lymph and piroplasms in blood smears similar to the other two animals mentioned above. The second animal (841) did not show any parasitological reactions. The size of the draining lymph node enlarged in animals 933, 859 and 838, but no enlargement was observed in animal 841. On challenge with potentially lethal *T. annulata* sporozoites, animals 933, 859 and 838 were immune but animal 841

suffered from acute theileriosis (details in chapter 4).

7.3.2 Changes in the flow rate and cellularity of efferent lymph

The output of lymph from the prescapular node draining the site of cell line immunisation is shown in fig. 7.1a. The flow rate increased slightly in all the animals immediately after cell line immunisation and dropped back to normal by day 8 in the three animals showing mild parasitological reactions after cell line immunisation. The flow rate dropped back to normal on day 5 in animal 841 which showed no parasitological reactions. The magnitude of increase was only 1.5-2 fold in all the animals. A second peak of increase in the flow of lymph was observed in the animals exhibiting parasitological reactions after day 11. The output of total lymphocytes in the efferent lymph increased three fold in one animal (933), whereas, it was marginal in animal 859 and 838 and only seen between day 6 to 9. The cell output from animal 841 which did not show any parasitological reactions decreased slightly after day 4 (fig. 7.1b).

Blasting cells (fig. 7.2a) were seen in cytospin smears prepared from the efferent lymph from day 5 onwards in all the animals. Only a slight increase was seen in animal 841. The other three animals which showed parasitological reactions after immunisation exhibited two peaks of blasting cells. The first increase was observed between day 6-11 and was associated with the development of an allogeneic response against MHC antigens of the immunising cell line. The second increase was more pronounced, started from day 13 and was associated with the parasite specific immune response and dissemination of the parasite in the efferent lymph. A similar pattern of blasting cells was seen when FSC vs SSC of ELL was examined on FACScan (R1 in fig. 7.3a for animal 859 and 7.3b for 841).

7.3.3 Kinetics of the parasite dissemination

T. annulata infected cells appeared in the efferent lymph of animals 933, 859 and 838. No infected cells were seen in the animal 841 at any stage. Infected cells were seen in animals 933 and 859 from day 11 and in 838 from day 16 onwards. Maximum schizonts were always less than 1% of ELL in animals 933 and 838, and 2.5% in 859 (day 17) as shown in fig. 7.2b. Parasite infected cell lines could be cultured from ELL on day 11-12 in animal 933, day 11-18 in animal 859 and day 16-18 in animal 838. Parasite infected cells were isolated from PBM of animal 933 from day 14 onwards, animal 859 from day 13 onwards and animal 838 from 18 onwards.

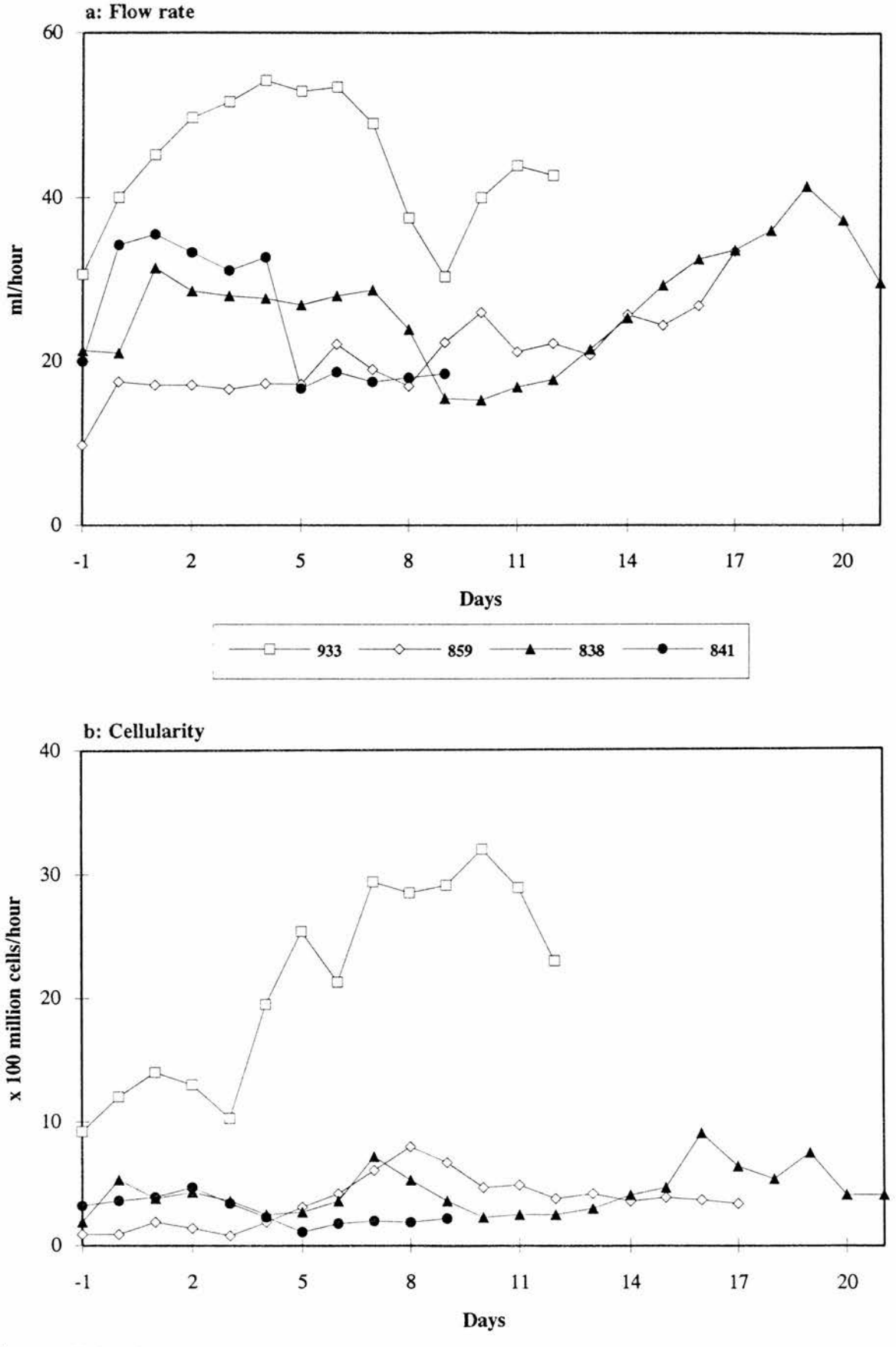


Figure 7.1: Changes in the flow and cellularity of the efferent lymph after immunisation with *T. annulata* infected cell line.
a: Flow rate. b: Cellularity.

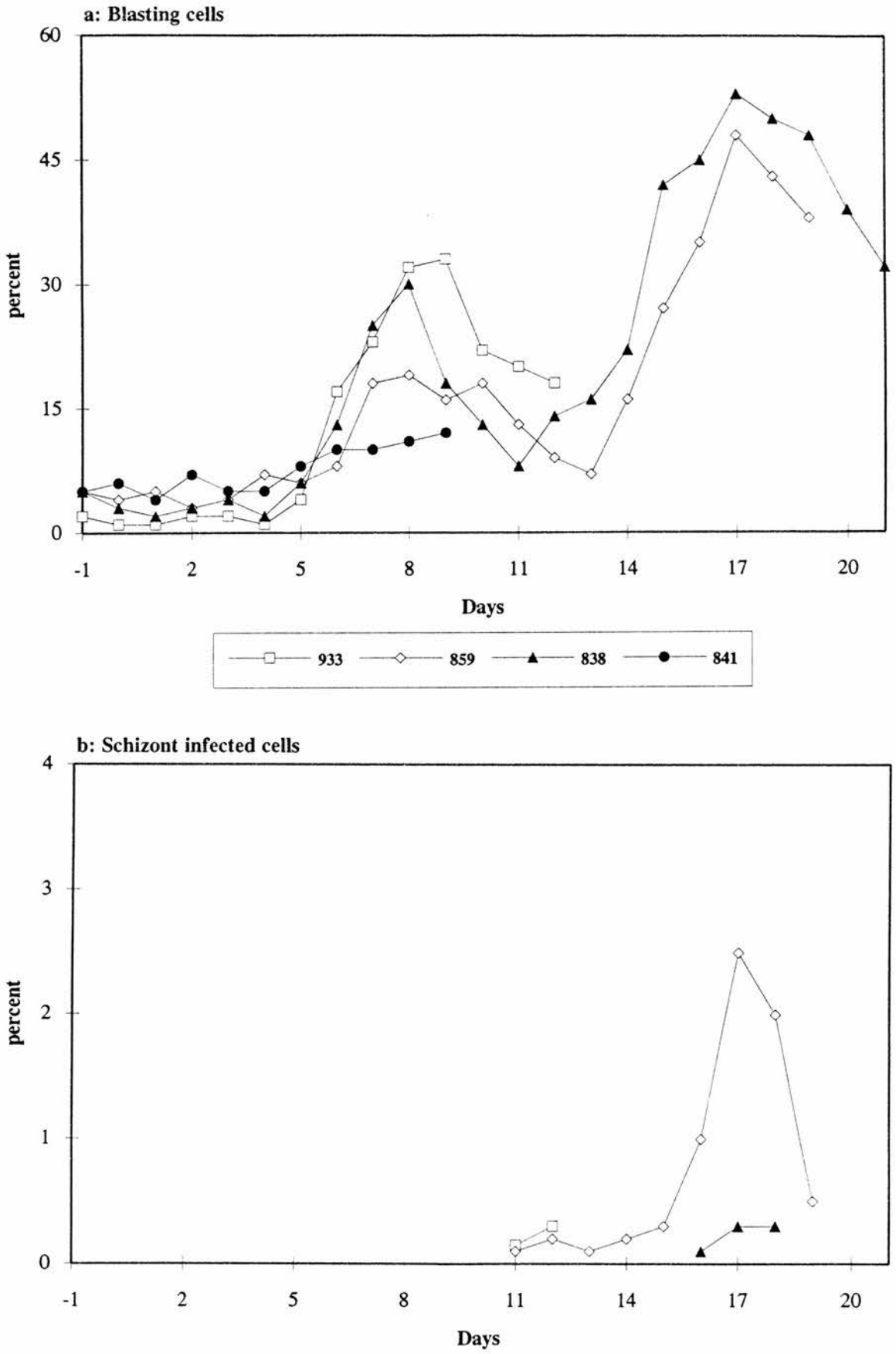


Figure 7.2: Blasting and parasite infected cells in the efferent lymph after immunisation with *T. annulata* infected cell line.
a: Blasting cells. b: parasite infected cells.

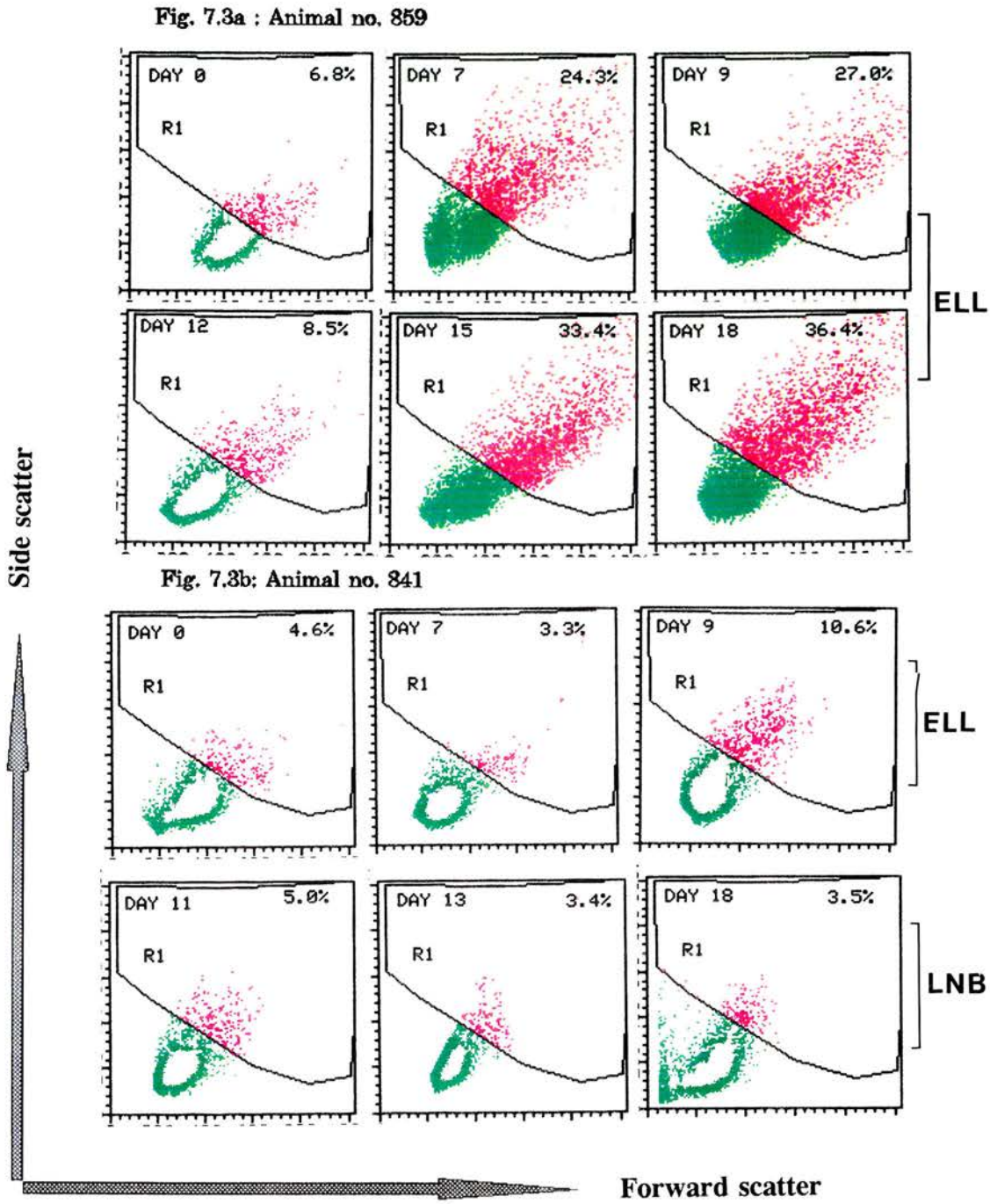


Figure 7.3: Blasting cells in the efferent lymph after immunisation with *T. annulata* infected cell line as assessed by the FACScan. FSC of cells is represented on the X axis and SSC on the Y axis. Blasting cells are highlighted in the region 1 (R1) on the basis of higher FSC & SSC. ELL: (Efferent lymph lymphocytes). LNB: (Lymph node biopsy cells).
a: Animal no. 859 (naive). b: Animal no. 841 (skin grafted).
(The day 11, 13 & 18 figures for animal no. 841 are from lymph node biopsy cells).

All the cell lines isolated from these animals were found to be of the recipient's origin (animal's own infected cells) on BoLA typing. No cell line of the donor origin could be isolated from ELL or PBM at any stage, despite very intensive sampling especially for the first two days after immunisation.

7.3.4 Changes in lymphocyte subpopulations in the efferent lymph

The kinetics of various lymphocyte subpopulations in efferent lymph of animals after immunisation is shown in fig. 7.4a to 7.4h. The percentage of total $CD2^+$ cells ranged between 61.9-82% in the resting efferent lymph of these animals. It started to increase after day 4 of cell line immunisation peaking between day 6-8 and returned to resting levels by day 9 or 10. The increase in animal 841 which did not show any parasitological reactions was only marginal from 80% to 87.5%. The other three animals exhibited a second peak in the percentage of $CD2^+$ cells starting from day 11 or 12 to day 18 (fig. 7.4a). The first peak of $CD2^+$ cells was associated with an increase in the percentage of both $CD4^+$ cells (fig. 7.4b) and $CD8^+$ cells (fig. 7.4c), but the second peak was mainly associated with an increase in $CD8^+$ cells. Resting levels of $CD8^+$ cells were 18.3% and 21.3% in animals 859 and 838, respectively. A maximum of 55.4% and 59.9% $CD8^+$ cells were observed in animal 859 on day 17 and 838 on day 18, respectively. The output of $\gamma\delta$ T cells slightly declined in the efferent lymph after immunisation in all the animals and began to rise from day 15 only in animal 838 (fig. 7.4d). There was a mild increase in MHC class II^+ cells immediately after cell line immunisation which declined by day 4, followed by an increase from day 7 onwards which was prominent during the parasite specific response (fig. 7.4e). Expression of CD25 on ELL increased in two animals immediately after immunisation, whereas it was unaltered in the other two (fig. 7.4f). A small increase was seen in the percentage of B cells in efferent lymph immediately after immunisation between day 0-3 followed by a mild second increase between day 8-14 (fig. 7.4g & 7.4h).

7.3.5 Activation of T cells

The expression of CD25 first increased on $CD4^+$ cells from day 9 followed by $CD8^+$ cells from day 15 after cell line immunisation (fig. 7.5a & 7.5b). Expression of MHC class II on $CD4^+$ cells slightly increased from day 13. However, MHC class II expression increased significantly on $CD8^+$ cells from day 13 after immunisation (fig. 7.5c & 7.5d). Only 1.1% $CD8^+$ MHC class II^+ cells were

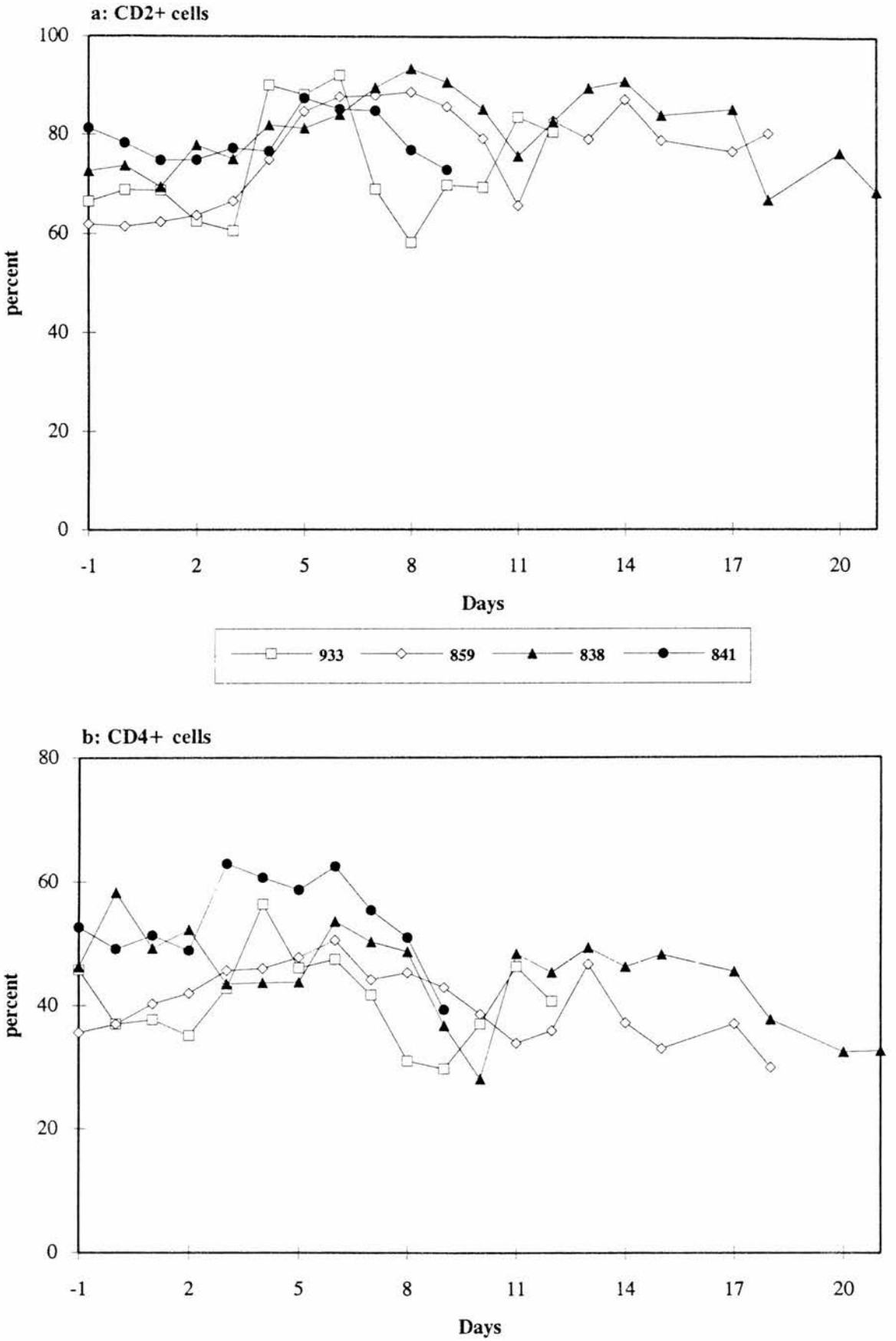


Figure 7.4: Changes in various lymphocyte subpopulations in the efferent lymph after immunisation with *T. annulata* infected cell line.
a: $CD2^+$ cells. b: $CD4^+$ cells.

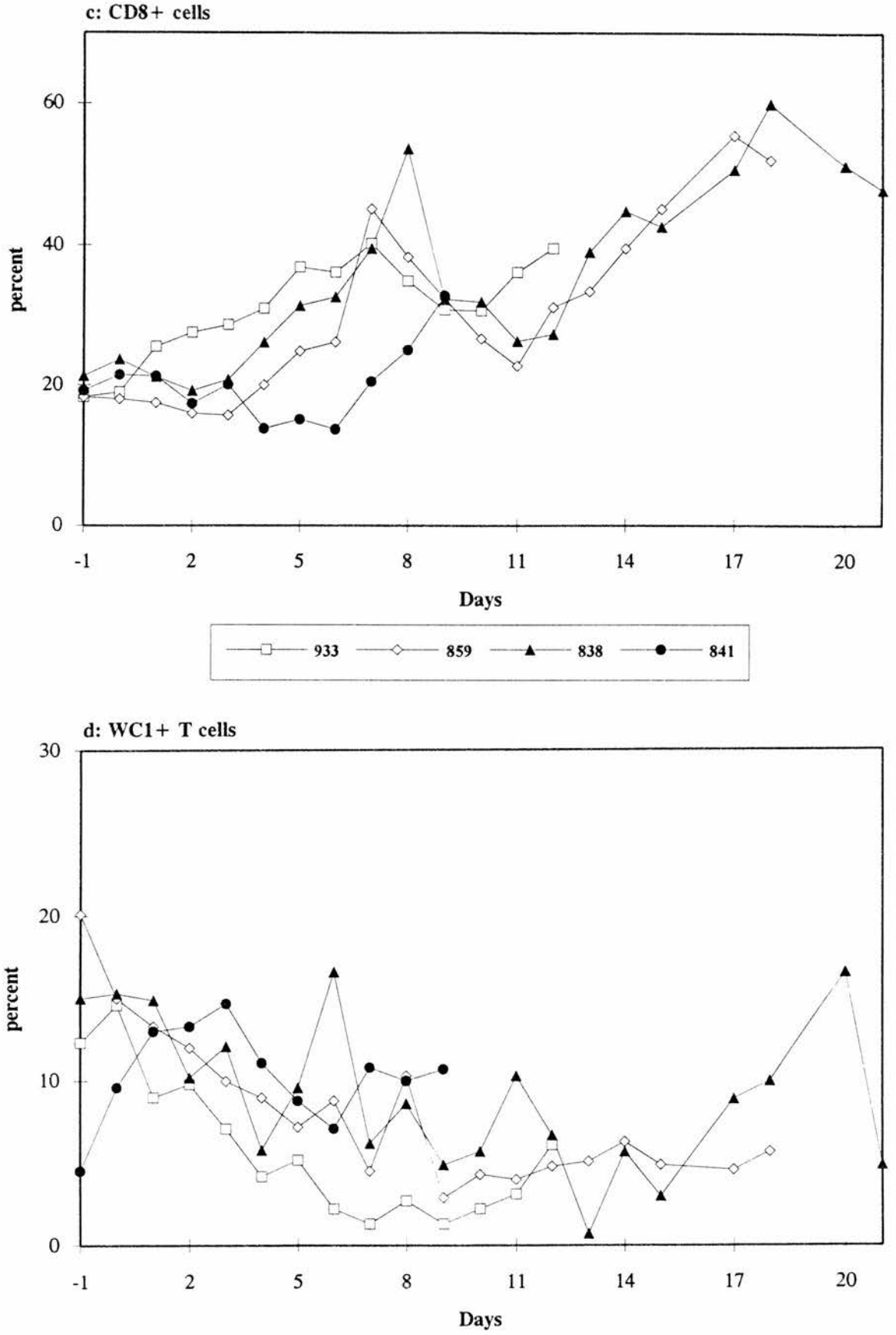
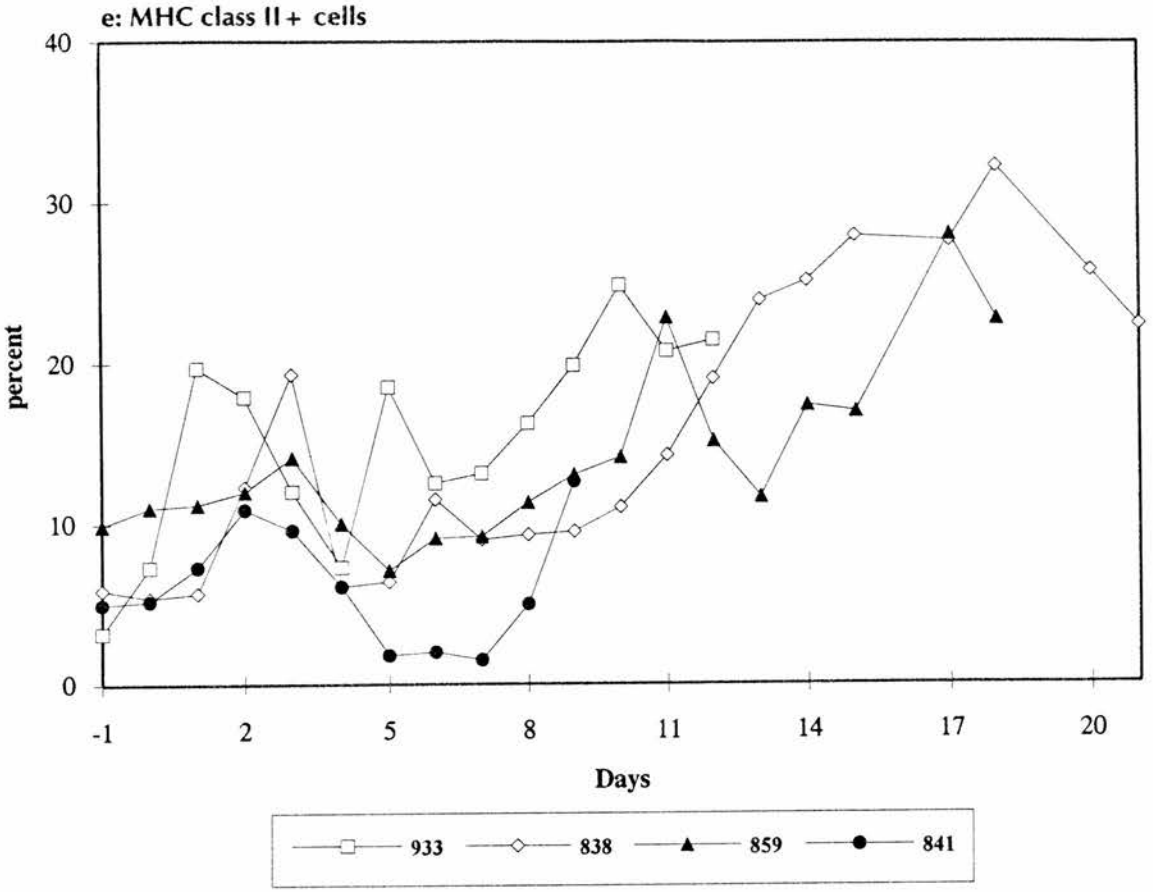


Figure 7.4: Changes in various lymphocyte subpopulations in the efferent lymph after immunisation with *T. annulata* infected cell line.
c: $CD8^+$ cells d: $WC1^+$ cells ($\gamma\delta$ T cells).



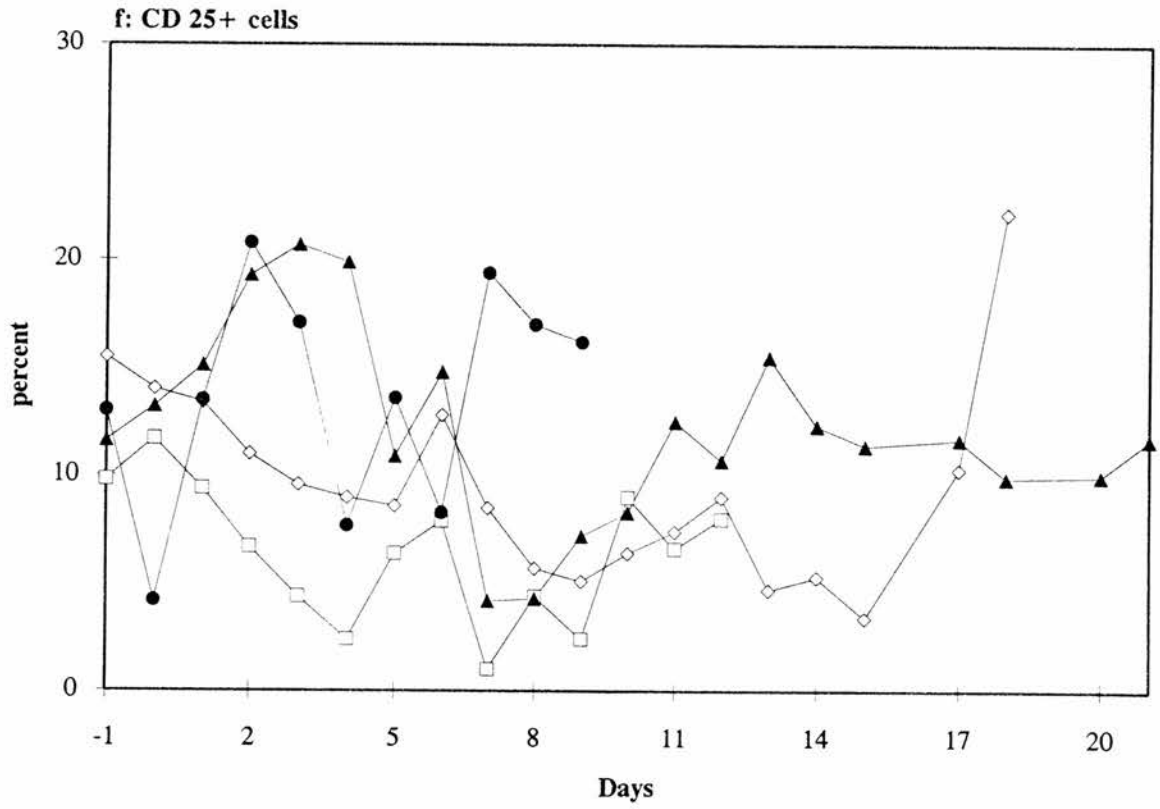
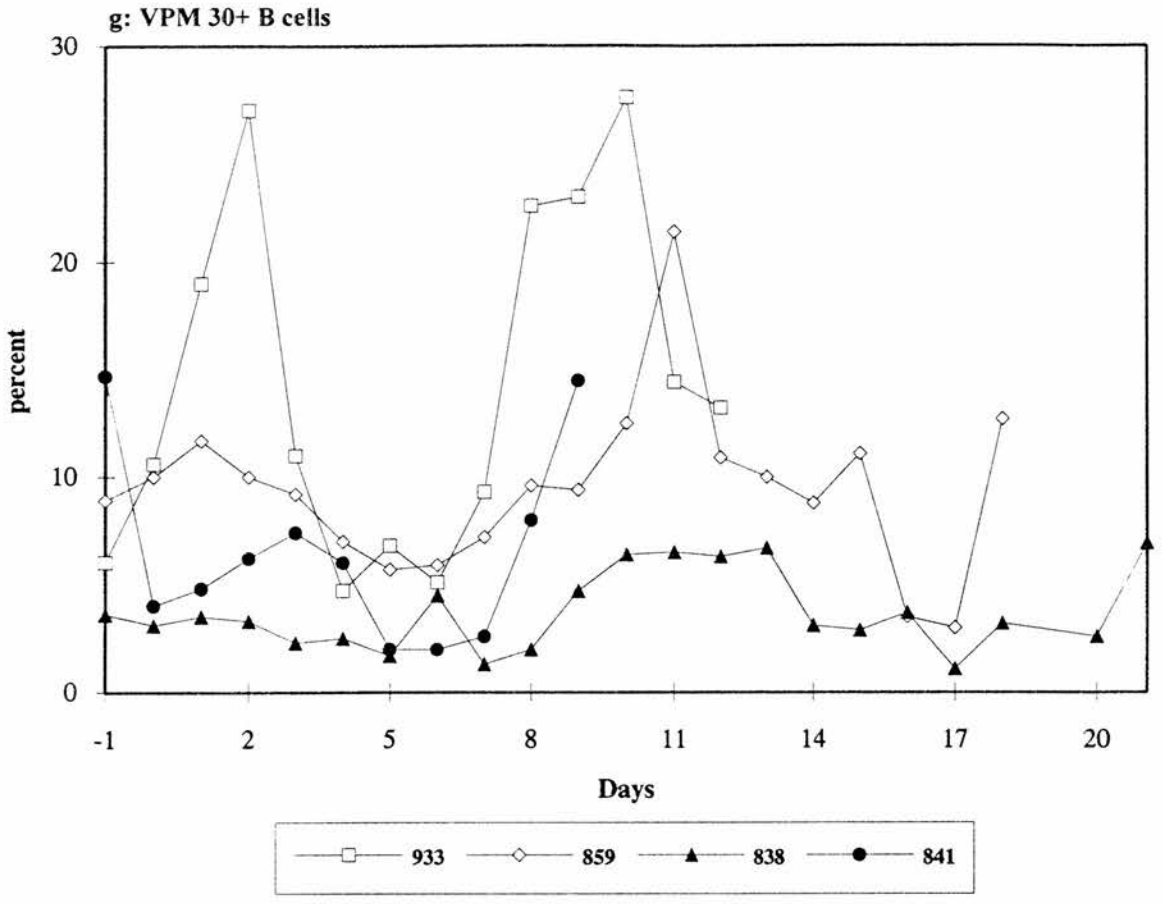


Figure 7.4: Changes in various lymphocyte subpopulations in the efferent lymph after immunisation with *T. annulata* infected cell line.
e: MHC class II^+ cells f: CD 25^+ (IL-2R, α chain) cells.



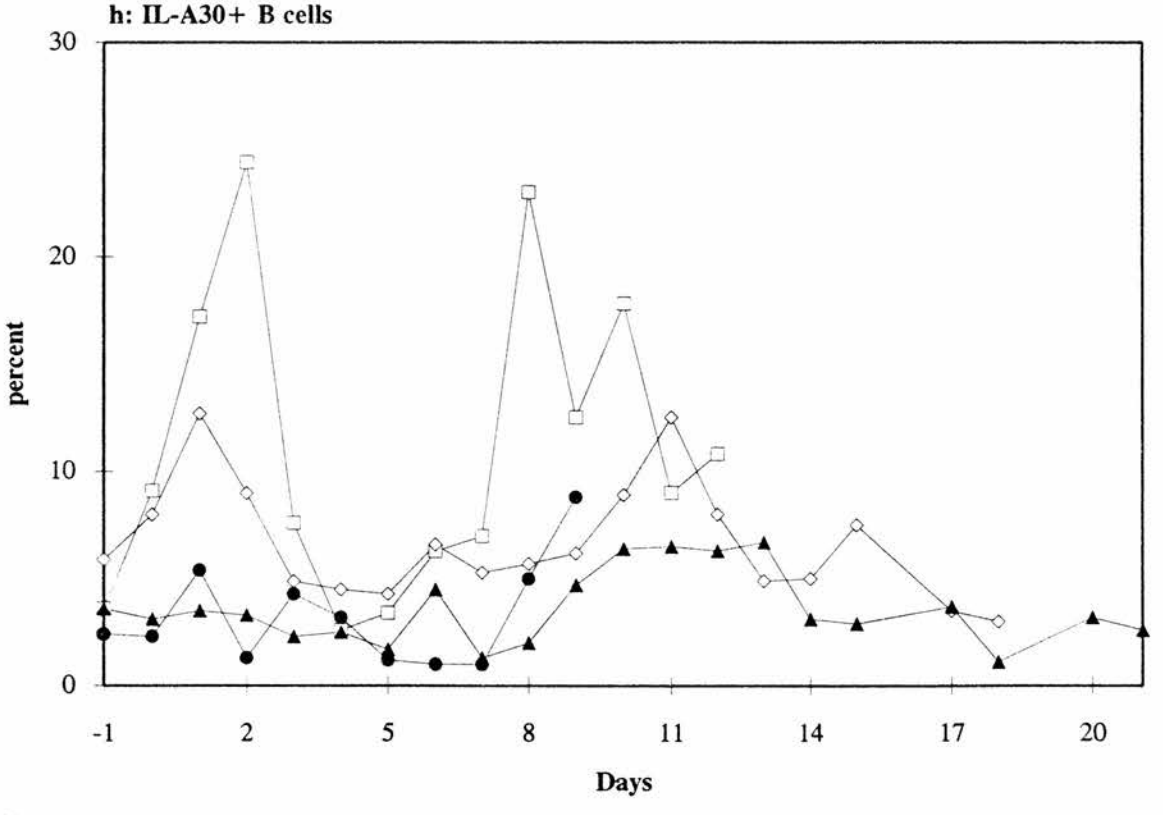


Figure 7.4: Changes in various lymphocyte subpopulations in the efferent lymph after immunisation with *T. annulata* infected cell line.
g: VPM 30^+ B cells h: IL-A30^+ IgM bearing B cells.

observed on day 0 as compared to 25.7% $CD8^+$ MHC class II^+ cells on day 18. Increased expression of CD25 and MHC class II on T cells was mainly associated with the parasite specific immune response and was less marked during the allogeneic response against MHC antigens of the immunising cell line. Expression of these activation markers on T cells was not observed in animal 841 which did not develop any parasitological reactions after immunisation.

Resting $CD4^+$ and $CD8^+$ cells express CD2. A few $CD4^+$ and $CD8^+$ cells lost CD2 expression during the second parasite specific phase of the immune response (fig. 7.5e & 7.5f). Loss of CD2 expression from T cells after cell line immunisation was less marked than observed after sporozoite inoculation in the naive animals as reported in the chapter 6. Only 4.5% $CD4^+$ $CD2^-$ and 9.9% $CD8^+$ $CD2^-$ cells were observed on day 18 in animal 859. Loss of CD2 expression on T cells was not observed in ELL or lymph node biopsy cells of animal 841 which did not show any parasitological reactions after immunisation.

An initial increase in the output of $CD4^+$ cells was observed immediately after immunisation. The increase was associated with the appearance of more $CD45RB^+$ (naive) $CD4^+$ cells. The percentage of $CD45RB^-$ (memory) $CD4^+$ cells initially declined to almost half in all the animals after immunisation up to day 9 during the period of allogeneic response. The parasite specific response was associated with the increase in $CD45RB^-$ $CD4^+$ (memory) cells and a gradual loss of $CD4^+$ $CD45RB^+$ (naive) cells (fig. 7.5g) indicating the development of a parasite specific response.

7.3.6 Proliferative responses

The functional activation state of ELL (fig. 7.6) and PBM (fig. 7.7) was assessed by measuring their proliferative efficiency in response to Con. A, IL-2, irradiated autologous *T. annulata* infected cells (Ta self) and irradiated parasite infected immunising cells (TaH 769). Proliferative ability of ELL and PBM in response to Con. A was greatly reduced after cell line immunisation. This effect was more pronounced during the second parasite specific phase of the response. The ability of these cells to proliferate in response to exogenous IL-2 increased gradually after day 7 of immunisation. Increased proliferative responses were observed in response to immunising cell line after day 7 and in response to autologous infected cells after day 12. These changes in the proliferative responses to various stimuli were associated only with animals exhibiting mild parasitological reactions after

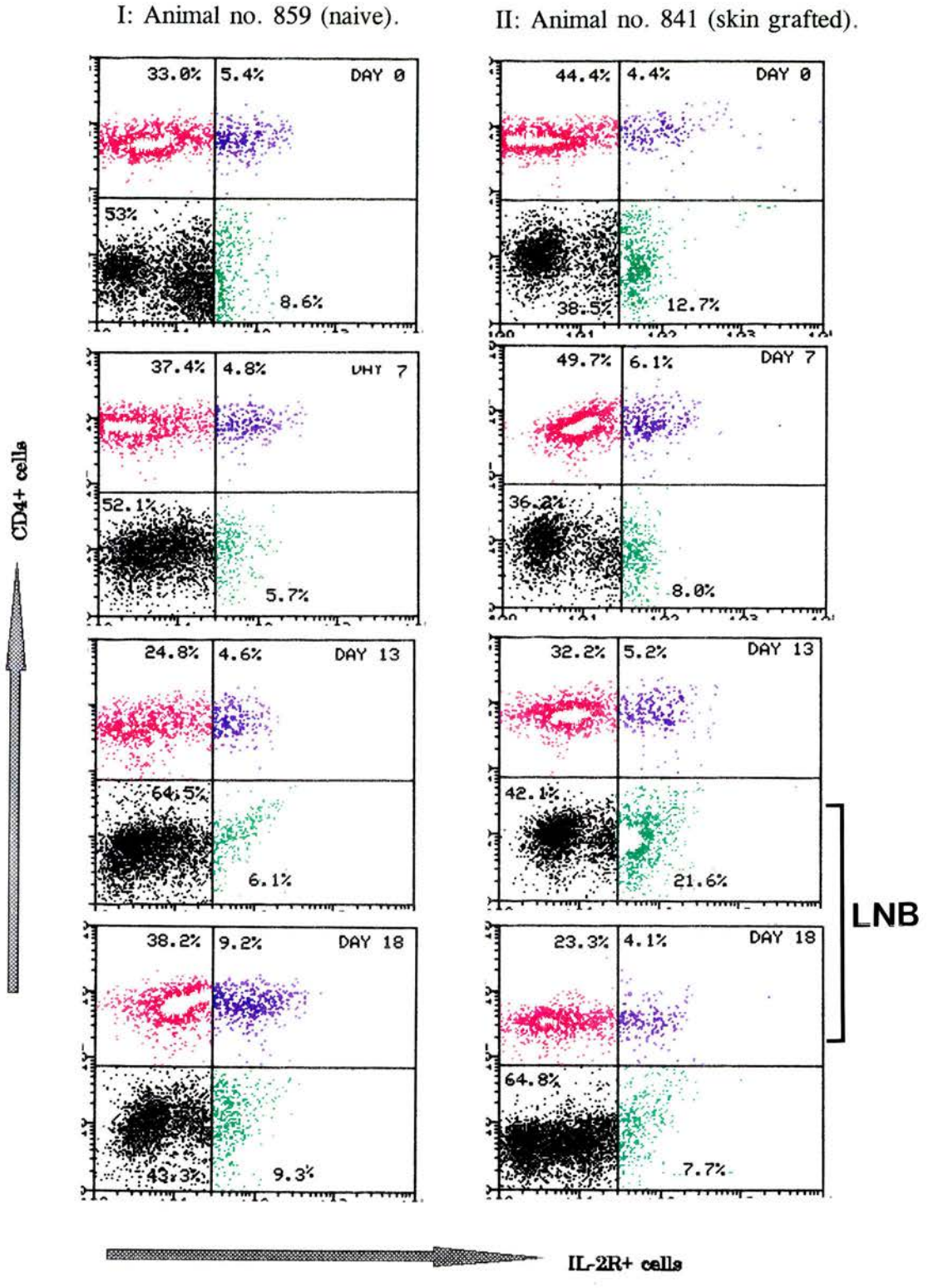


Figure 7.5a: Dot plots of two colour immunofluorescent staining on ELL. $CD4^+$ cells are expressed on the Y axis (FL2). IL-$2R^+$ (CD25) cells are expressed on the X axis (FL1).
I: Animal no. 859 (naive). II: Animal no. 841 (skin grafted).
(LNB: The day 13 & 18 figures for animal no. 841 are from lymph node biopsy cells).

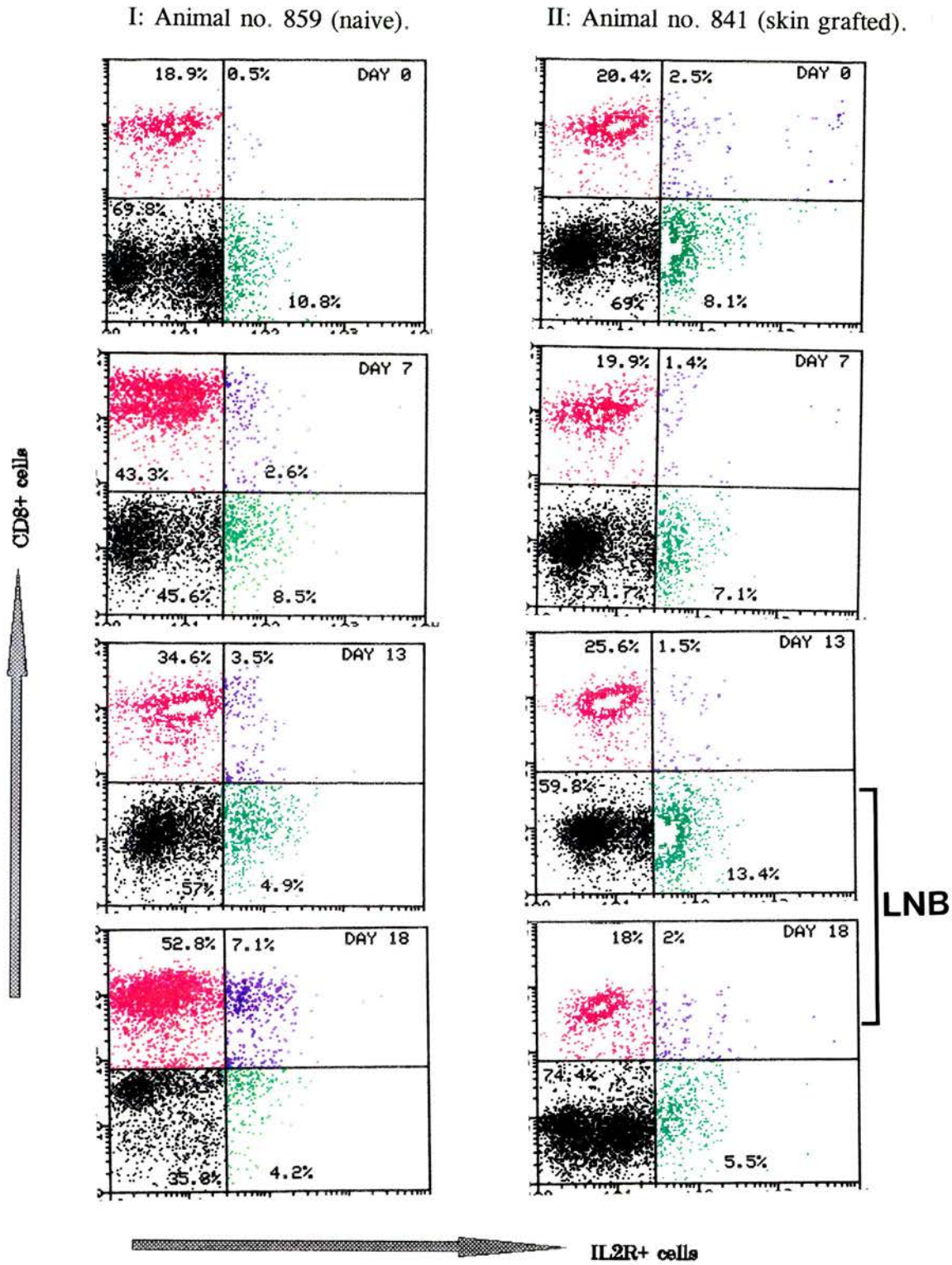


Figure 7.5b: Dot plots of two colour immunofluorescent staining on ELL. $CD8^+$ cells are expressed on the Y axis (FL2). IL-$2R^+$ (CD25) cells are expressed on the X axis (FL1).
I: Animal no. 859 (naive). II: Animal no. 841 (skin grafted).
(LNB: The day 13 & 18 figures for animal no. 841 are from lymph node biopsy cells).

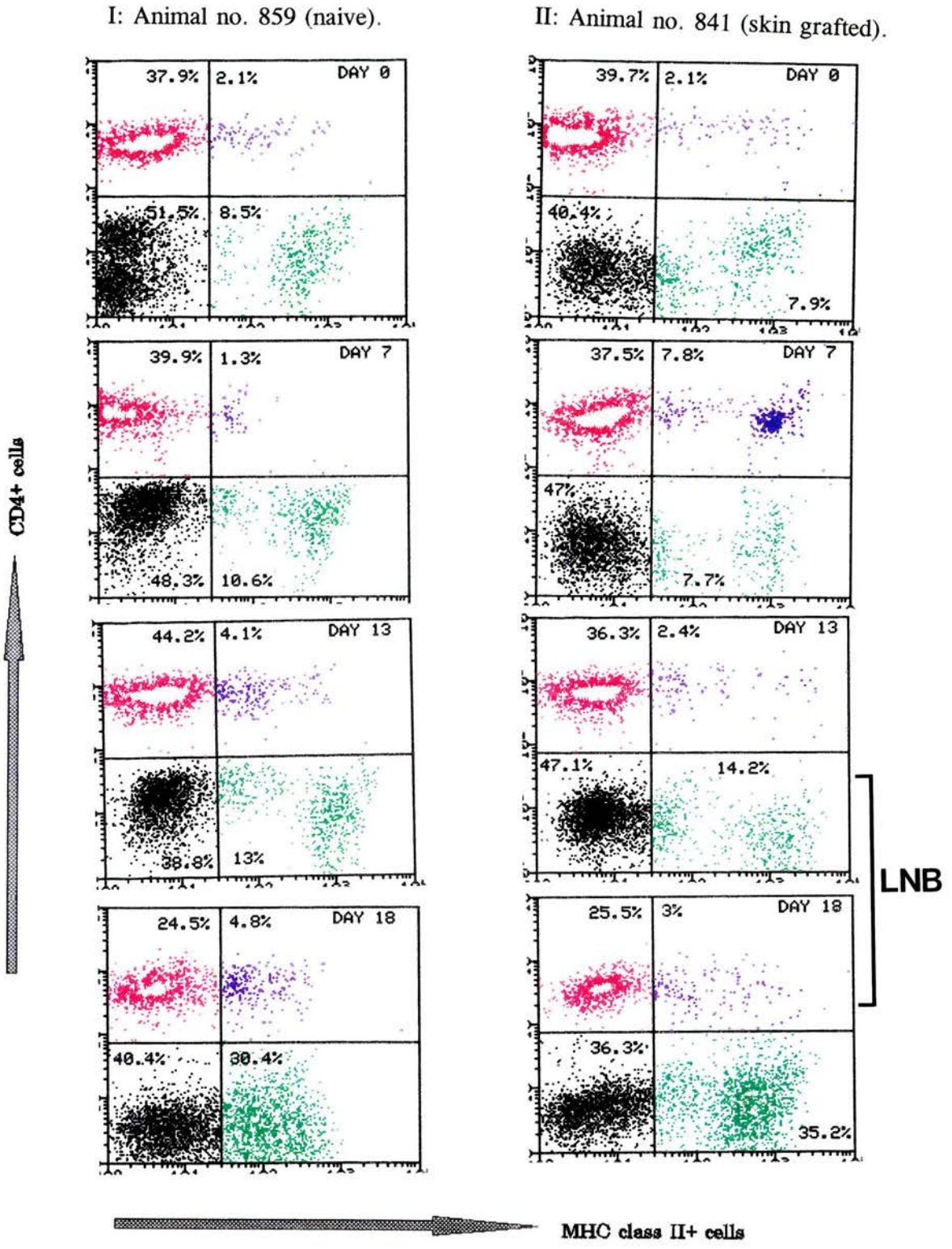


Figure 7.5c: Dot plots of two colour immunofluorescent staining on ELL. $CD4^+$ cells are expressed on the Y axis (FL2). MHC class II^+ cells are expressed on the X axis (FL1).
I: Animal no. 859 (naive). II: Animal no. 841 (skin grafted).
(LNB: The day 13 & 18 figures for animal no. 841 are from lymph node biopsy cells).

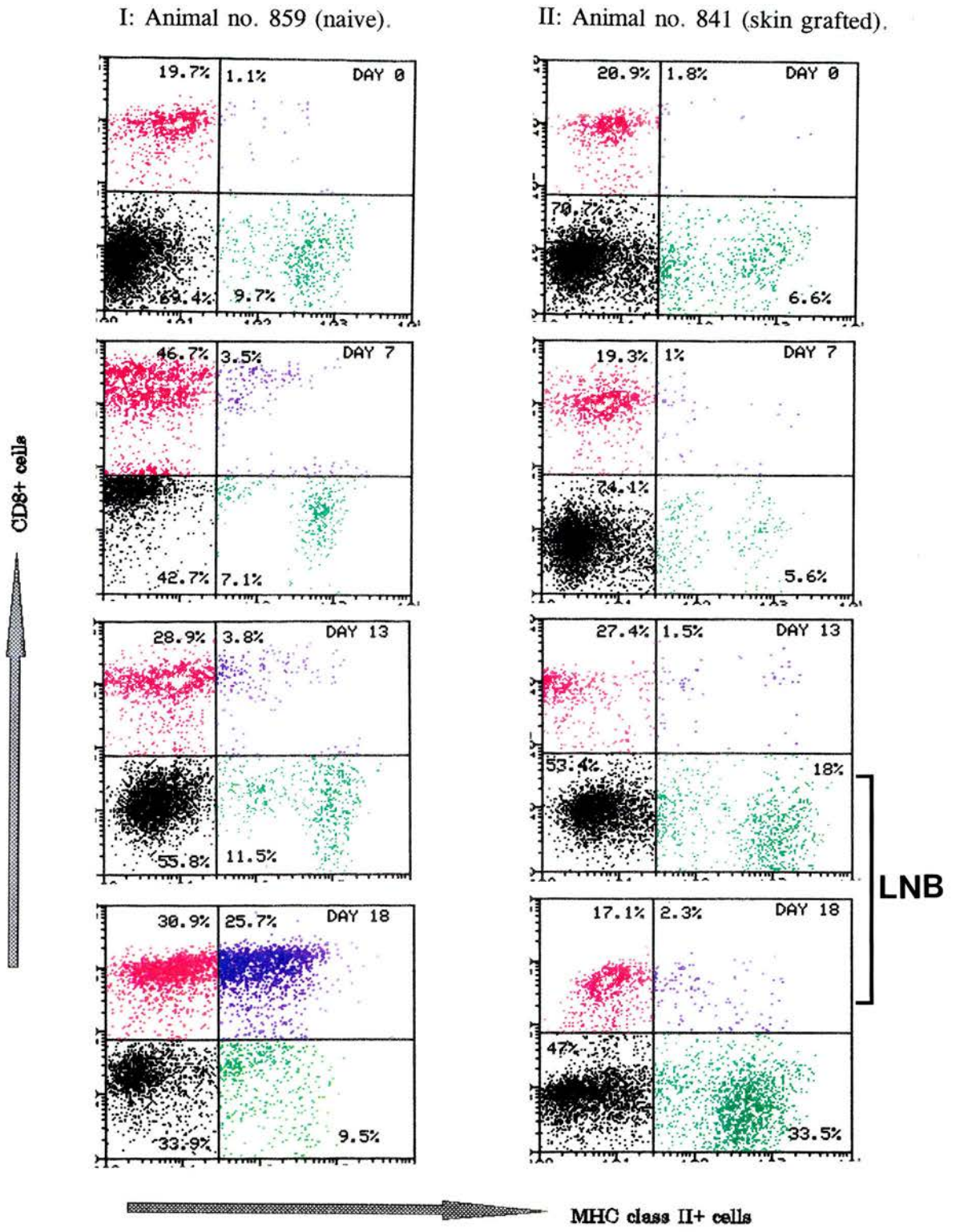


Figure 7.5d: Dot plots of two colour immunofluorescent staining on ELL. $CD8^+$ cells are expressed on the Y axis (FL2). MHC class II^+ cells are expressed on the X axis (FL1).
I: Animal no. 859 (naive). II: Animal no. 841 (skin grafted).
(LNB: The day 13 & 18 figures for animal no. 841 are from lymph node biopsy cells).

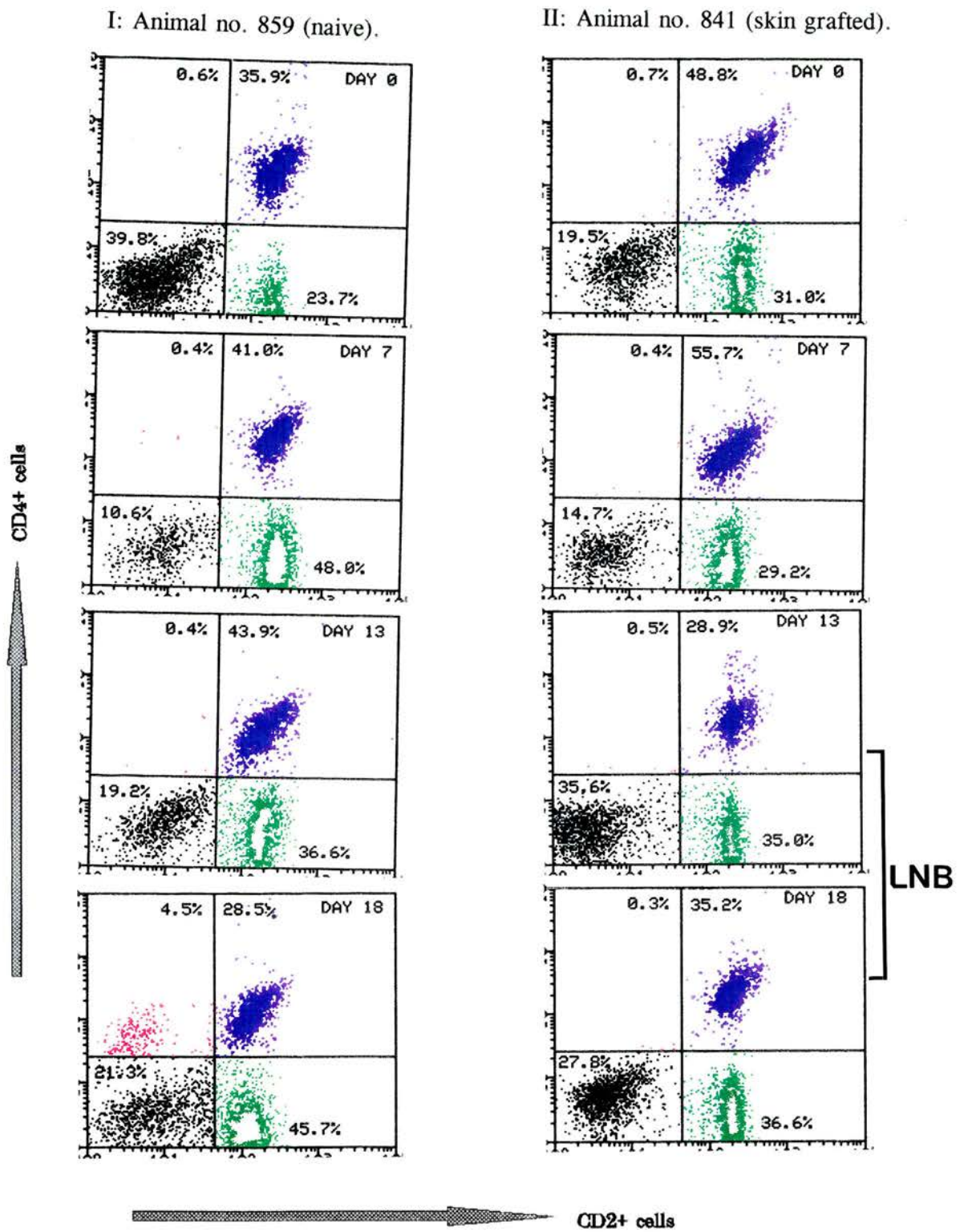


Figure 7.5e: Dot plots of two colour immunofluorescent staining on ELL. $CD4^+$ cells are expressed on the Y axis (FL2). $CD2^+$ cells are expressed on the X axis (FL1).
I: Animal no. 859 (naive). II: Animal no. 841 (skin grafted).
(LNB: The day 13 & 18 figures for animal no. 841 are from lymph node biopsy cells).

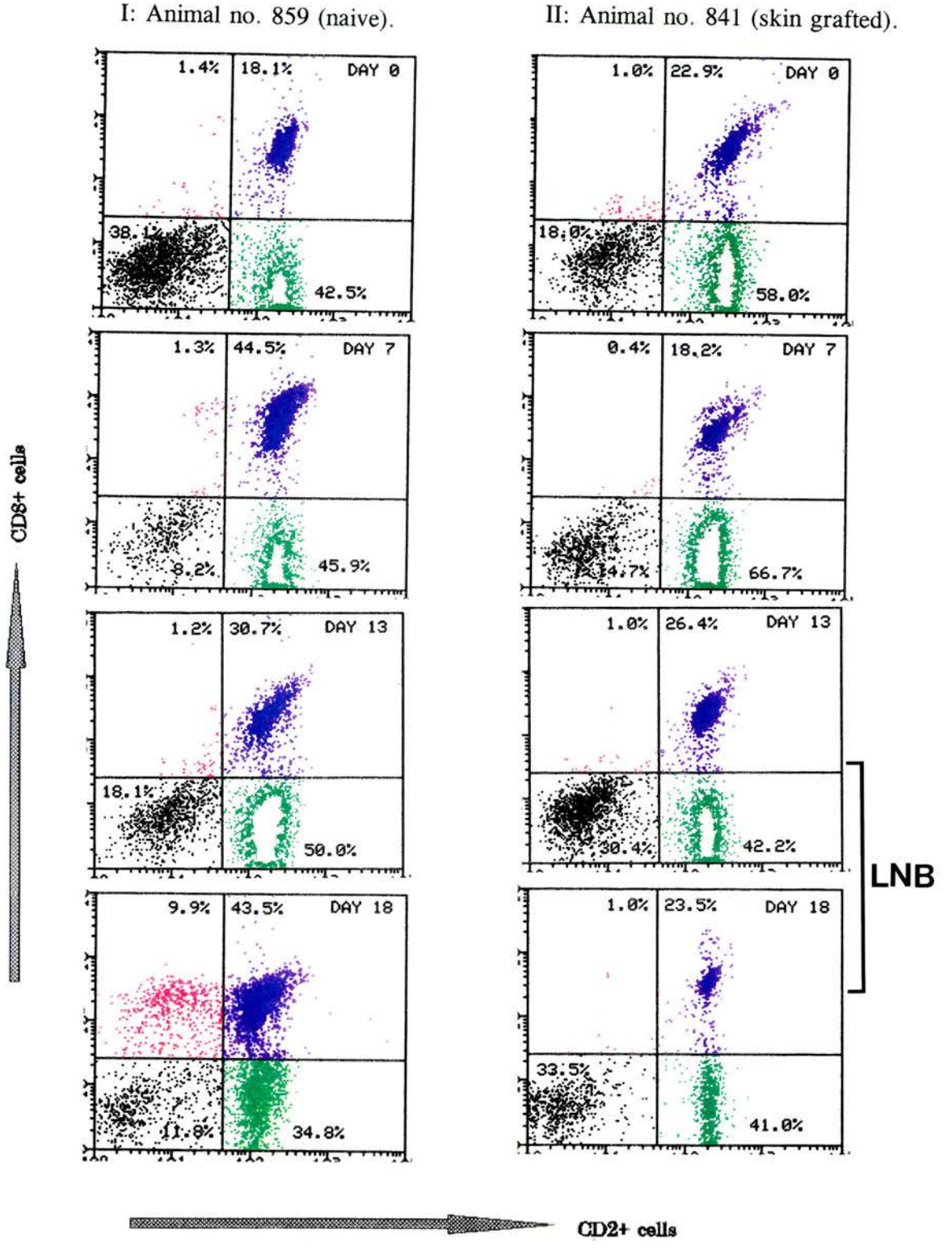


Figure 7.5f: Dot plots of two colour immunofluorescent staining on ELL. $CD8^+$ cells are expressed on the Y axis (FL2). $CD2^+$ cells are expressed on the X axis (FL1).
I: Animal no. 859 (naive). II: Animal no. 841 (skin grafted).
(LNB: The day 13 & 18 figures for animal no. 841 are from lymph node biopsy cells).

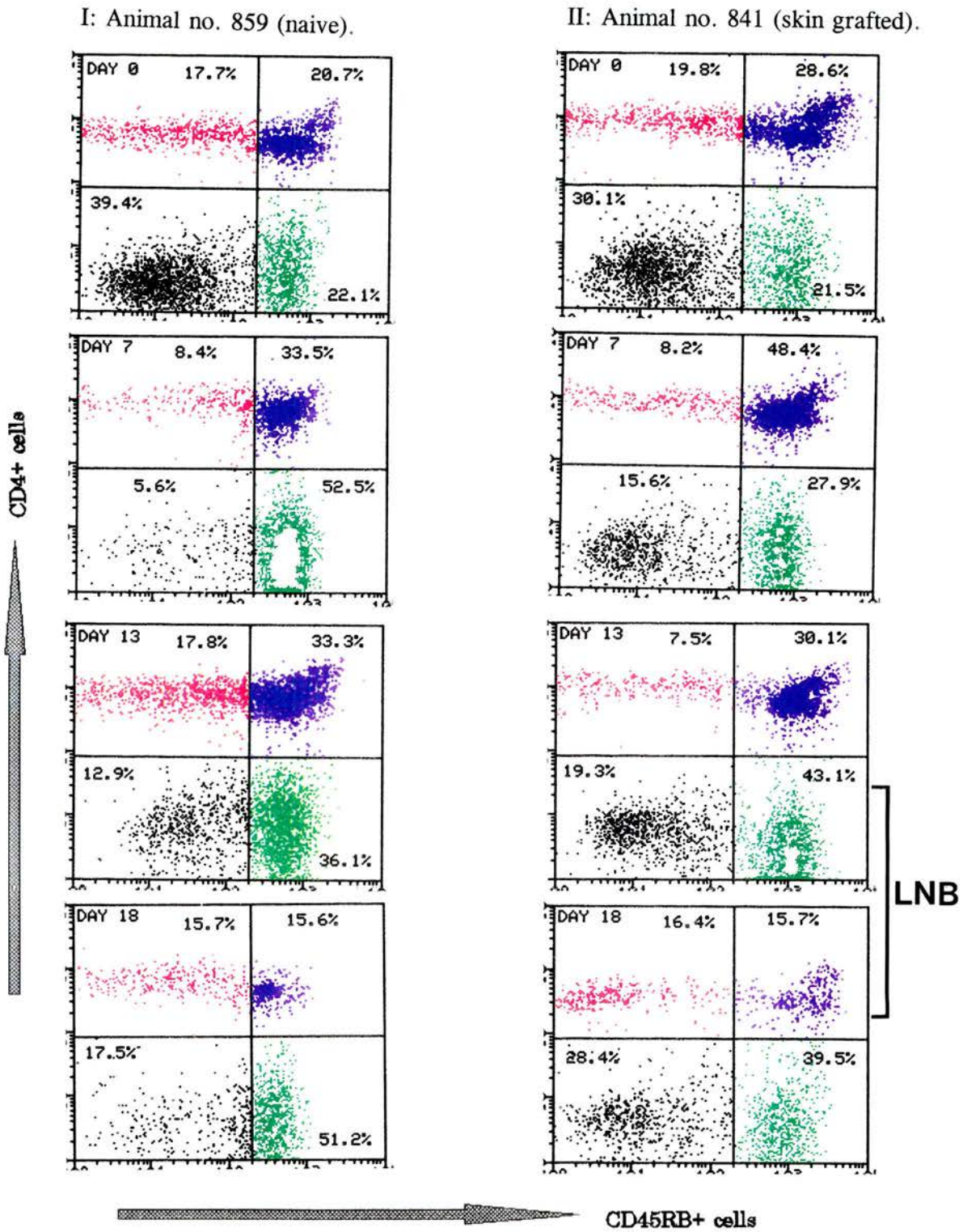


Figure 7.5g: Dot plots of two colour immunofluorescent staining on ELL. $CD4^+$ cells are expressed on the Y axis (FL2). $CD45RB^+$ cells are expressed on the X axis (FL1).
I: Animal no. 859 (naive). II: Animal no. 841 (skin grafted).
(LNB: The day 13 & 18 figures for animal no. 841 are from lymph node biopsy cells).

immunisation (fig. 7.6a & 7.7a for animal 933; fig. 7.6b & 7.7b for animal 838). ELL and PBM from animal 841 which did not show any parasitological reactions exhibited no variation in the proliferative ability to various stimuli after immunisation (fig. 7.6c & 7.7c).

7.3.7 Cytotoxicity responses

Cytotoxic activity of ELL and PBM was measured against several target cells in chromium release assays. Direct assays were done using freshly harvested ELL and PBM as effector cells. The data presented in fig. 7.8 shows percent killing at effector vs target ratio of 40:1. ELL and PBM were stimulated with irradiated autologous *T. annulata* infected cells or irradiated parasite infected cells of the immunising cell line for five days to enrich for cytotoxic effector cells. The stimulated cells were used as effectors in chromium release assays against various targets. Fig. 7.9 shows percent killing of target cells at 20:1 effector vs target ratio by using stimulated ELL or stimulated PBM as effectors.

7.3.7.1 Direct assays

Cytotoxic activity directed against parasite infected cells used to immunise animals was observed in freshly harvested ELL from day 5-6 and in PBM from day 7-8 onwards (fig. 7.8a & 7.8b). A similar degree of lysis was seen against uninfected lymphoblasts derived from the animal which was used to generate the immunising cell line and donate skin for implantation (data not shown). This indicated that cytotoxic response was directed against MHC antigens of the immunising cell line. Peak cytotoxic activity against the allogeneic antigens of the immunising cell line appeared between day 7-10 in ELL and 11-13 in PBM. The response began to wane by day 15 in the efferent lymph and by day 18 in the peripheral blood.

A moderate cytotoxic response started to develop against autologous *T. annulata* infected cells from day 11-12 in the efferent lymph and day 12-13 in the PBM (fig. 7.8c & 7.8d). Autologous uninfected blasts were not killed by ELL or PBM at any stage indicating that the response was not directed against the blasting cells (data not shown). Furthermore, a *T. annulata* infected cell line of a mismatched BoLA type different from the BoLA type of the immunising cell line and recipient animal was also not killed (data not shown) indicating it to be an MHC class I restricted parasite specific response. The peak of the parasite specific response was observed between day 15-18 in ELL and PBM. Animal 841 which did not show any

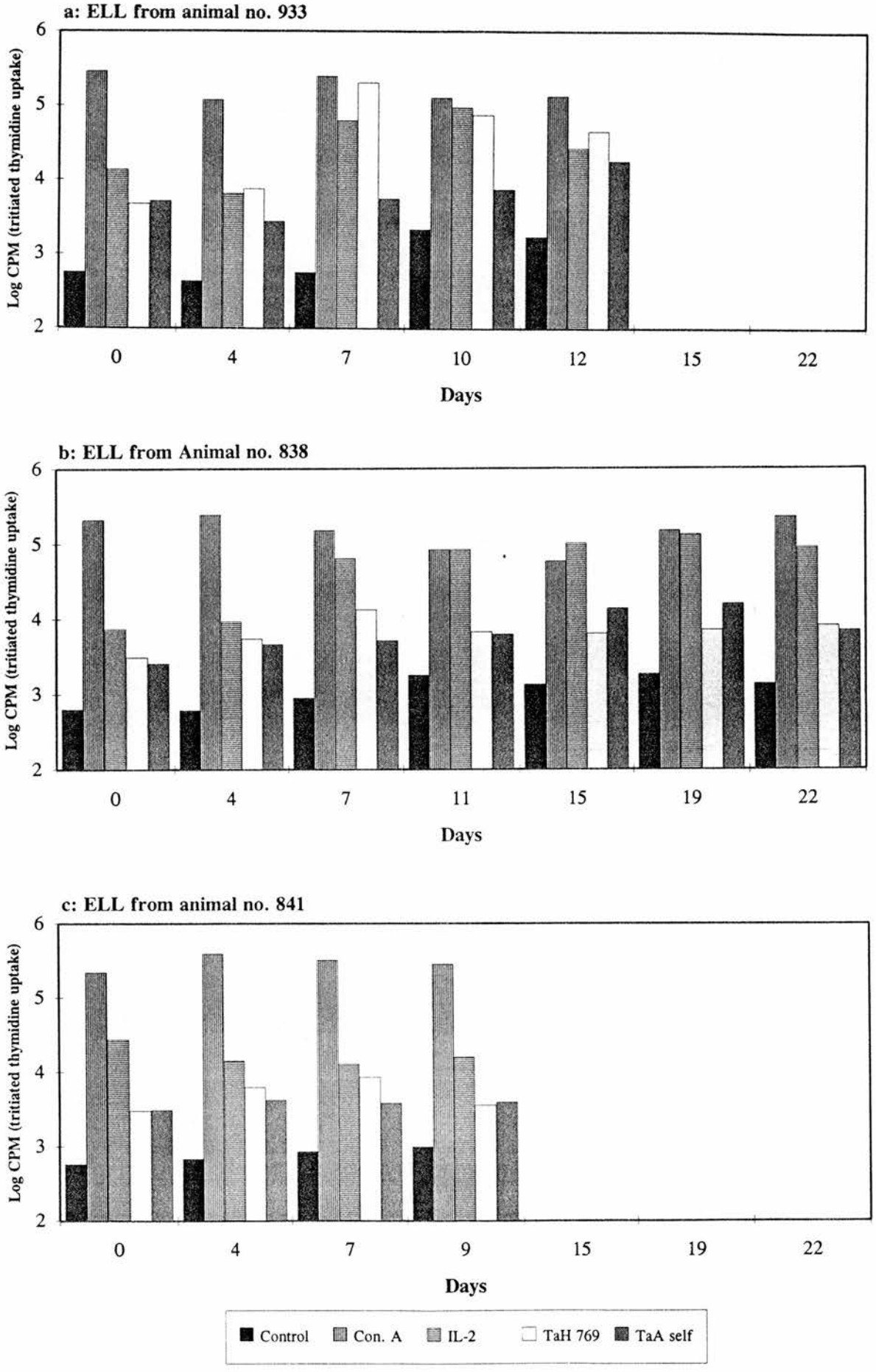


Figure 7.6: Proliferative responses of ELL in response to various antigen stimuli *in vitro* after immunisation with *T. annulata* infected cell line.
Values represent the mean of quadriplicate counts.
a: Animal no. 933. b: Animal no. 838. c: Animal no. 841.

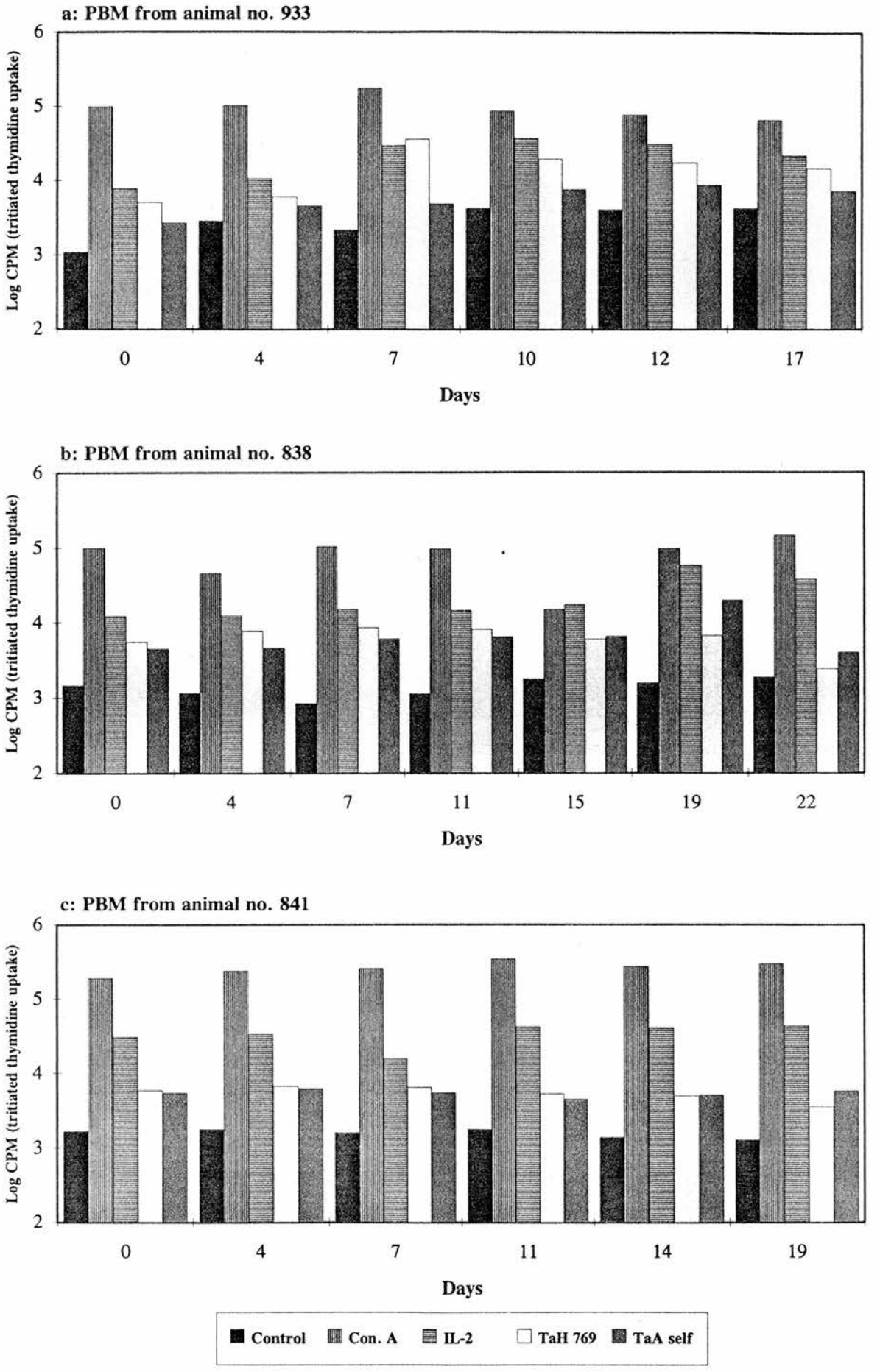


Figure 7.7: Proliferative responses of PBM in response to various antigen stimuli *in vitro* after immunisation with *T. annulata* cell line.
Values represent the mean of quadriplicate counts.
a: Animal no. 933. b: Animal no. 838. c: Animal no. 841.

parasitological reactions after immunisation, exhibited only allogeneic cytotoxic activity and no MHC class I restricted parasite specific killing.

7.3.7.2 Cytotoxic activity of stimulated cells

Stimulation of ELL or PBM with irradiated immunising cells or irradiated autologous *T. annulata* infected cells helped in observing a magnified cytotoxic response against allogeneic antigens of the immunising cell line and the parasite, respectively (fig. 7.9). Stimulation of ELL or PBM with irradiated immunising cells on day 0 of immunisation produced some allogeneic response in animals 838 and 841 as these animals had already been primed against MHC antigens by skin implantation before immunisation (fig. 7.9a & 7.9b). The allogeneic response was not stimulated from cells collected pre-immunisation from animals 933 and 859. The stimulated allogeneic cytotoxic response gradually became stronger after immunisation in all the four animals. ELL and PBM stimulated with irradiated autologous *T. annulata* infected cells produced a very mild parasite specific cytotoxic response up to 7 days after immunisation. Afterwards, the response gradually became stronger in all the animals except animal 841, which never showed an amplified parasite specific response (fig. 7.9c & 7.9d).

7.3.8 Interferon-γ assay

Levels of IFN-γ in the efferent lymph of animals after cell line immunisation are shown in figure 7.10. IFN-γ was detected in efferent lymph of animal 933 and 859 from day 2 to 7 after immunisation. The levels were very high in animal 933 as compared to 859. Levels of IFN-γ were undetectable in animals 838 and 841 at this stage. Low levels of IFN-γ were detected again in the efferent lymph of animals 859 and 838 from day 12 onwards during the parasite specific phase of the response. IFN-γ was not detectable in the efferent lymph of animal 841 throughout the experiment.

7.4 DISCUSSION

Two naive animals (933 & 859) exhibited mild parasitological reactions after cell line immunisation. It has been well documented in the literature that animals inoculated with allogeneic *T. annulata* infected cell lines develop mild parasitological reactions and immunity to subsequent sporozoite challenge (Pipano, 1981; Ouhelli *et al.*, 1989; Brown, 1990). These two animals were subsequently immune to potentially lethal sporozoite challenge as shown in chapter 4. A strong allogeneic response was

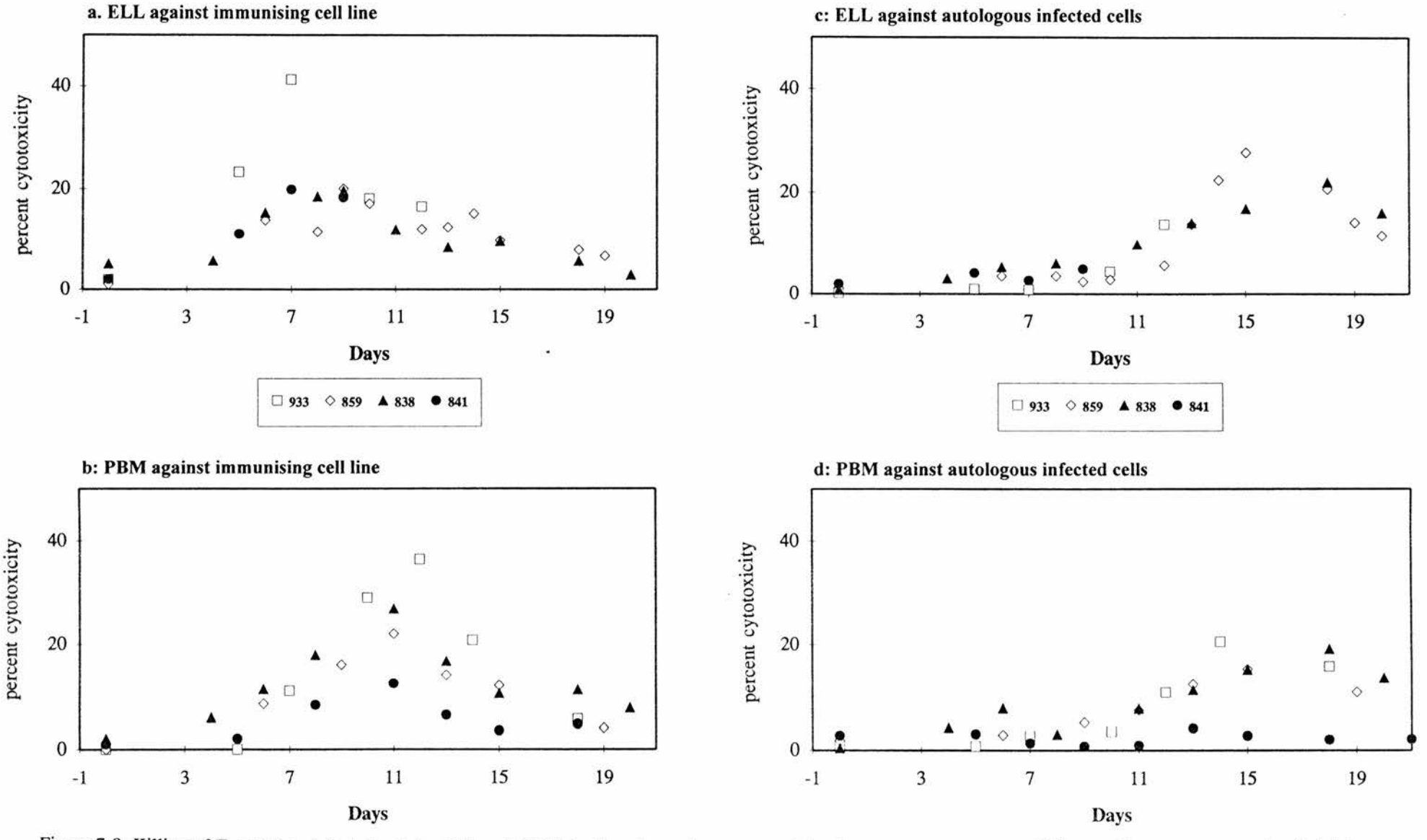


Figure 7.8: Killing of *T. annulata* infected cells by ELL and PBMs in chromium release assays. The figures represent percent killing at effector vs target ratio 40:1 (Mean of duplicate counts). **8a:** Cytotoxic activity of ELL against immunising cells; **8b:** Cytotoxic activity of PBMs against immunising cells; **8c:** Cytotoxic activity of ELL against autologous parasite infected cells; **8d:** Cytotoxic activity of PBMs against autologous parasite infected cells.

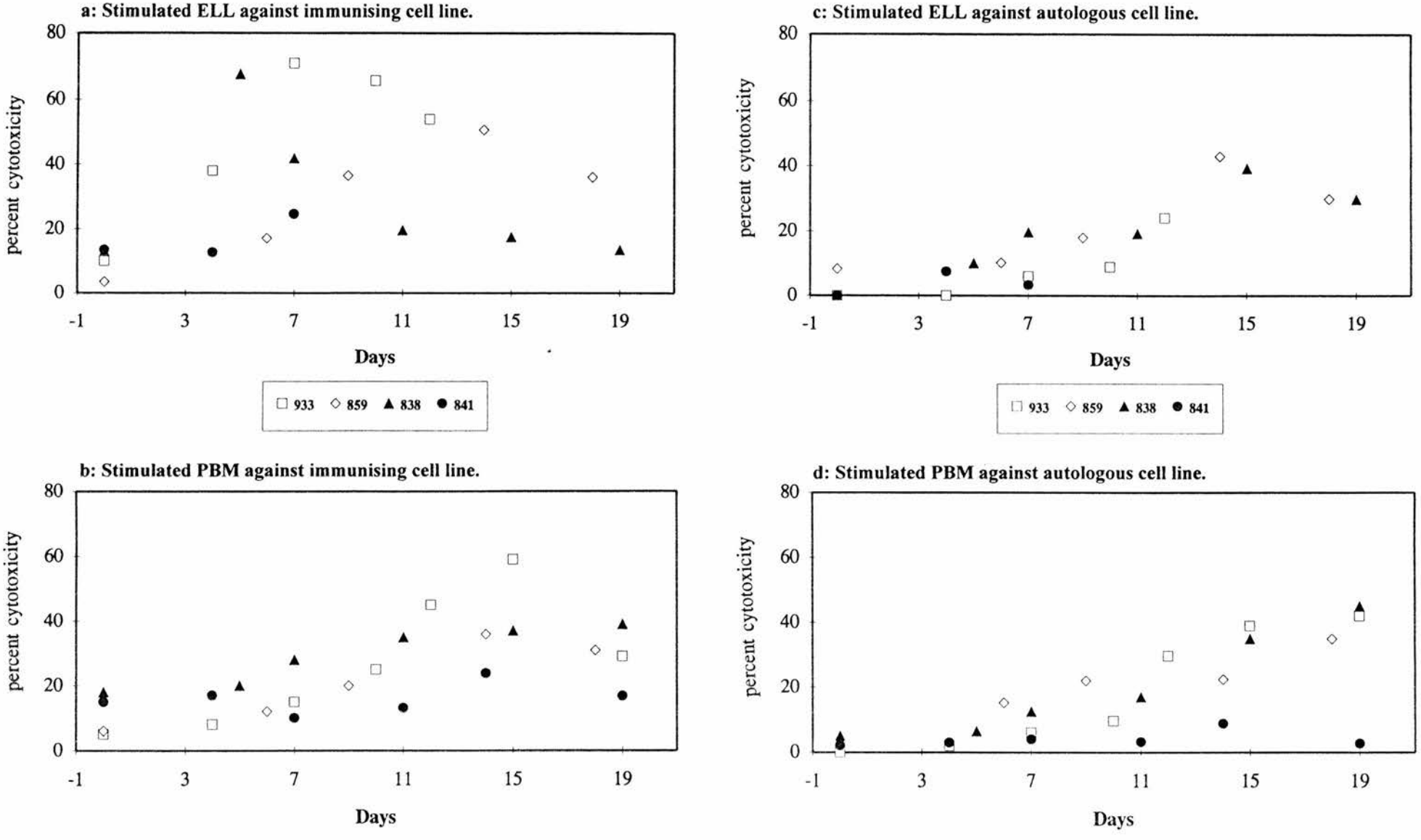


Figure 7.9: Killing of *T. annulata* infected cells by stimulated ELL and PBMs (stimulated with irradiated immunising cells or autologous infected cells) in chromium release assays. The figures represent percent killing at effector : target ratio 20:1 (Mean of duplicate counts). **9a:** Stimulated ELL against immunising cells; **9b:** Stimulated PBMs against immunising cells; **9c:** Stimulated ELL against autologous infected cells; **9d:** Stimulated PBMs against autologous infected cells.

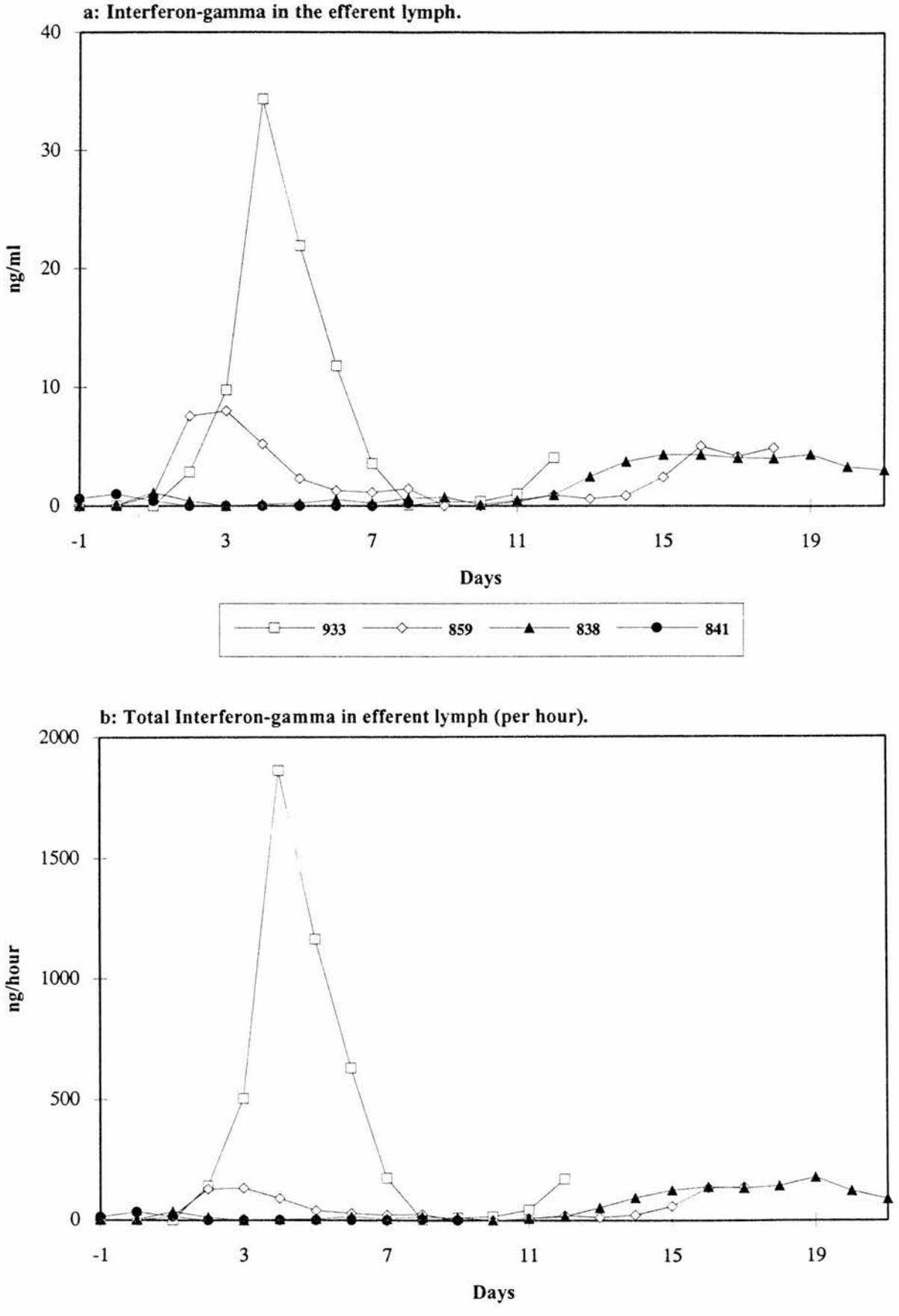


Figure 7.10: Levels of IFN-γ in the draining efferent lymph of animals after cell line immunisation.

a: IFN-γ expressed as ng per ml in the efferent lymph.

b: Levels of IFN-γ expressed as ng per hour released in the efferent lymph.

induced by skin implantation in the other two animals (838 & 841) before inoculation of the parasite infected cell line derived from the skin donor animal. One of the animals exhibited mild parasitological reactions after immunisation, but no parasites were seen in the second animal, although similar titres of anti MHC antibodies were evident in both the animals at the time of immunisation (chapter 4).

The three animals reacting mildly to the immunising cell line (2 naive & 1 skin grafted) displayed a biphasic immune response in the efferent lymph and peripheral blood, the first directed against MHC antigens of the immunising cell line, followed by a response directed against autologous parasite infected cells. Such a biphasic response has earlier been reported in the peripheral blood after cell line immunisation by Innes *et al.* (1989a). The animal 841 only showed an allogeneic response after immunisation and no parasite specific response, indicating that the pre-existing allogeneic response blocked the generation of parasite specific immune response. Innes (1988) and Preston & Brown (1988) also observed no parasite development or boost in the parasite specific immune response when the animals were reimmunised with the same allogeneic cell line used for first immunisation. Although experiments in the above two reports were undertaken with different objectives, indirect evidence that reimmunisation with the same cell line may not be useful in boosting the parasite specific immune response was obtained. Observations on animal 841 and the experiments in chapter 4 clearly showed that development of the parasite specific immune responses could be inhibited partially or completely by a pre-existing allogeneic response.

Studies on the draining efferent lymph also revealed that the pre-existing allogeneic response delayed or prevented the development of parasite after cell line immunisation. *T. annulata* infected cells were isolated from efferent lymph of the two naive animals from day 11 onwards after cell line immunisation. Infected cells were isolated from only one of the other two animals (838) exhibiting a pre-existing allogeneic response, only from day 16. No cell line was isolated from animal 841, showing complete prevention of parasite development. Furthermore, schizonts were observed in the efferent lymph only for 3 days (day 16-18) in animal 838 (skin grafted) as compared to 9 days (day 11-19) in animal 859 showing that the parasite development was delayed in the former. It is possible that most of the infected cells were destroyed immediately after immunisation by the pre-existing allogeneic

response diluting parasite load in the animal body. If so, the parasite took longer to attain detectable levels. This allowed enough time for the development of a parasite specific immune response, and in turn cleared the parasite soon after its appearance. Parasite infected cell lines were isolated from efferent lymph a few days before these could be isolated from PBM. This observation was consistent with the lymphatic system being a route for transfer of the parasite from the draining lymph node to other body organs, as observed in chapter 6 after sporozoite inoculation and reported to occur for *T. parva* (Emery, 1981b).

T. annulata infected cell lines isolated from the efferent lymph expressed the BoLA type of the recipient animals, indicating that the infected cells exited in the efferent lymph only after parasite transfer. An important pre-requisite for the development of parasite specific immune response after cell line immunisation is that the parasite must transfer from donor cells to the cells of the recipient (Pipano *et al.*, 1977; Brown, 1981; Innes *et al.*, 1989c; this thesis, chapter 4). Cell lines of donor origin were never isolated from efferent lymph or peripheral blood at any stage. The place and mode of parasite transfer and phenotype of recipient's cells infected by the parasite is not known. This is an important topic for future research to further understand the cellular interactions leading to the development of a biphasic immune response after cell line immunisation. The precise role of the allogeneic responses on development of immunity against the parasite at the time of first or second immunisation could only be elucidated after finding an answer to these questions. These findings would also be important in understanding the development of immunity after cell line immunisation against *T. parva*. Immunisation with allogeneic *T. parva* infected cells is only possible by inoculation of as high as 10^7 to 10^9 cells (Pirie *et al.*, 1970; Brown *et al.*, 1971; Brown *et al.*, 1978a; Brown, 1981; Morrison *et al.*, 1981a), whereas, immunisation against *T. annulata* could be effective with as low as 10^2 (Ouhelli *et al.*, 1989) or 10^1 (Brown, 1990) allogeneic infected cells.

The present experiments indicate that parasite transfer takes place somewhere between the site of inoculation of the cell line and the draining node as only infected cells of the recipient origin were isolated in the efferent lymph. A few parasite infected cells were observed in the lymph node biopsies after inoculation of $1x10^8$ allogeneic *T. parva* infected cells for the first four days and then around day 10 (C.G.D. Brown, unpublished observations). It seems likely that schizonts observed

for the first four days were of donor origin followed by the appearance of recipient infected cells later. These observations and the results in this chapter suggest that the parasite is confined to the draining lymph node until development of the infected cells of recipient origin. This is in contrast to the finding that parasite development took place even after surgical removal of the draining lymph nodes after day 4 and 9 following immunisation with allogeneic *T. annulata* infected cell line before appearance of schizonts of the recipient origin (P.K. Goel & R.L. Spooner, pers. comm.). If infected cells are confined to the draining lymph node, removal of the node should have stopped further parasite development and spread. The cell line was inoculated subcutaneously in that experiment. In that instance, as with these studies, there is a possibility that some of the inoculated cells were drained to some other lymph node. Further studies by inoculation of labelled parasite infected cells through intact afferent lymphatics and following their dissemination might help in understanding the development of the parasite in the recipient and the mode of parasite transfer.

Interestingly, the cell shutdown in the draining lymph node, as reported to occur frequently after antigen challenge (Hall & Morris, 1965b; Hopkins *et al.*, 1981b) and also observed after inoculation of sporozoites in the naive animals (chapter 6), was not seen after immunisation with the parasite infected cell line in any of the animals. Total lymphocyte output was reported to remain relatively constant for the first 150 hrs, followed by a marked elevation afterwards, in the efferent lymph from the prefemoral node draining skin allografts (Emery & McCullagh, 1980a). Inoculation of the allogeneic parasite infected cell line is directly comparable to the response generated against a skin allograft as both instances lead to generation of a graft rejection response in the lymph node. Migration of cells from the lymph node was selective in these experiments, as evidenced by a decline in $CD4^+$ cells of the memory phenotype ($CD45RB^-$) soon after immunisation, followed by a gradual increase in memory CD4 cells and decrease in naive CD4 cells after day 7. It has been shown earlier that initiation of primary immune responses to a protein antigen in the lymph node was followed by exit of $CD4^+$ memory cells via efferent lymph into the blood (Mackay *et al.*, 1990).

Increase in the flow of lymph in the efferent lymphatics was observed in two naive animals after immunisation during the early allogeneic phase of the response.

This was not observed in the other two animals which had been presensitized to the MHC of the immunising cell line. Increase in the flow rate, which was marked in one of the two naive animals, was associated with about a three fold increase in the cell output and high IFN-γ levels during the allogeneic phase of the response. The smaller increase in the flow rate of the second animal was associated with about a two fold increase in the cellularity and marginal increase in the levels of IFN-γ in the efferent lymph at this time. Perhaps the severity of these reactions was related to level of the ensuing allogeneic reactions, as cytotoxicity assays revealed a very strong allogeneic response in the former and only a mild response in the second naive animal. An increase in the flow rate and output of total and blasting cells in the efferent lymph was also reported after inoculation of allogeneic lymphocytes in the draining area or afferent lymphatics of the node (Emery & McCullagh, 1980b).

The second parasite specific phase of the immune response was associated with almost a two fold increase in the flow of lymph in the animals while they underwent mild reactions to immunisation, but the total cell output was almost unchanged. A transient increase in the output of lymph has also been reported in sheep after infection with *Chlamydia psittaci* (Huang *et al.*, 1991) and a strain of *Toxoplasma gondii* which does not establish persistent infection (Buxton *et al.*, 1994). The increased flow rate was probably attributable to the enlargement of the lymph node and increase in blood supply to the node as suggested by Hay & Hobbs (1977) and also discussed in chapter 6.

A biphasic output of blasting cells was observed in the efferent lymph of animals exhibiting mild parasitological reactions after cell line immunisation. The first peak of blasting cells corresponded to the allogeneic response against MHC antigens of the immunising cell line. The second peak was stronger and coincided with the appearance of parasite specific cytotoxic response. The percentage of blasting cells increased above 50% along with very low levels of IFN-γ in the efferent lymph. Blasting cells have been reported to appear in the draining efferent lymph in response to various micro-organisms (Emery, 1981b; Issekutz,1985; McColgan *et al.*, 1987; Huang *et al.*, 1991; Bird *et al.*, 1993; Buxton *et al.*, 1994). A massive output of blasting cells was observed in response to inoculation of *T. annulata* sporozoites leading to acute clinical theileriosis (chapter 6). These blasting cells were not involved in controlling the infection as cytotoxicity and proliferation assays on ELL did not

reveal parasite specificity as discussed in chapter 6.

Allogeneic responses were shown to be induced in peripheral blood around day 9 after inoculation of animals with a *T. annulata* infected BoLA mismatched cell line. The specificity of the cytotoxic response changed, becoming directed against autologous parasite infected cells around day 13 (Innes *et al.*, 1989a). Similar kinetics of the cytotoxic response in the peripheral blood were observed after immunisation in animals reacting mildly to the cell line. The cytotoxic response in the efferent lymph appeared earlier, was stronger and more short lived than that detected in the peripheral blood, indicating that the initiation of immune response takes place in the draining lymph node. The biphasic cytotoxic response associated with two peaks of blasting cells and $CD8^+$ cells in the efferent lymph reflected a change in the antigens being presented to the immune system. The first response reflected specificity to the allogeneic antigens of the immunising cell line. After parasite transfer and infection of the recipient's cells, the immune response received a second priming by parasite specific antigens in the context of self MHC. A similar biphasic cytotoxic response was observed in peripheral blood of the animals after inoculation of 10^7 or more *T. parva* infected allogeneic cells (Emery *et al.*, 1982).

Phenotyping of ELL revealed a change in the initial dominance from $CD4^+$ cells to $CD8^+$ cells during the course of the immune response. Similar findings in the efferent lymph have been reported after immunisation with an incomplete strain of *Toxoplasma gondii* tachyzoites (Innes *et al.*, 1992b) and infection with *Chlamydia psittaci* (Huang *et al.*, 1991) in sheep. Increased output of $CD8^+$ cells in efferent lymph is in accord with increased levels of circulating $CD8^+$ cells in the peripheral blood as shown in chapter 5 and also reported earlier in animals recovering from *T. annulata* sporozoite infection (Preston *et al.*, 1992a). It has been shown earlier that cells exiting in the efferent lymph are responsible for systemic dissemination of the immune response (Fahy *et al.*, 1980; Trnka & Cahill, 1980). Transfer of syngeneic thoracic duct leucocytes from a calf immune against *T. parva* to its naive chimaeric co-twin has been shown to provide resistance to lethal sporozoite challenge (Emery, 1981a). In a recent report, increase in the precursor frequency for *T. parva* specific MHC class I restricted cytotoxic T lymphocytes was observed in the efferent lymph of an immune animal undergoing sporozoite challenge. Adoptive transfer of purified $CD8^+$ cells from ELL of the immune animal at this stage prevented the development

of disease in its naive monozygotic twin undergoing a potentially lethal infection (McKeever *et al.*, 1994). Similar experiments have not been done with *T. annulata* specific $CD8^+$ cells, but the kinetics of the cytotoxic responses observed here suggests that the same would also be true for *T. annulata*.

Expression of activation markers in the form of MHC class II and CD25 on T cells (Greene *et al.*, 1986; Glimcher & Kara, 1992) slightly increased during the phase of allogeneic response. The increase in the expression of activation markers was more marked during the parasite specific phase of the response. Many $CD4^+$ and $CD8^+$ cells expressed MHC class II after activation but CD25 appeared only on a few cells. Expression of MHC class II but not CD25 on T cells has been shown to be a defective maturation state appearing in response to various lentivirus infections (Panteleo *et al.*, 1990; Reimann *et al.*, 1991; Bird *et al.*, 1993). However, responsiveness of ELL and PBM to *in vitro* stimulation with IL-2 gradually increased and specific cytotoxic activity appeared after cell line immunisation indicating the activated state of the cells. ELL and PBM were less responsive to Con. A after immunisation, but their responsiveness to irradiated immunising cells and autologous infected cells gradually increased. This indicates that cells exiting in the efferent lymph were already committed either to allogeneic antigens of the immunising cell line or the parasite antigens and were, therefore, less responsive to Con. A. This is in contrast to the observations in chapter 6 where both ELL and PBM were less responsive to all exogenous stimuli during the period of acute clinical theileriosis after inoculation of sporozoites, which seemed to be either because of lymphocyte anergy or suppression.

All the above observations on ELL and PBM after immunisation with allogeneic *T. annulata* infected cell lines demonstrate the development of a normal immune response which is dominated by a cytotoxic T cell response. However during the parasite specific phase of the response, a few $CD4^+$ and $CD8^+$ cells not expressing CD2 were also observed. The percentage of $CD4^+$ or $CD8^+$ cells not expressing the CD2 molecule was very low in contrast to the appearance of large numbers of $CD4^+$ and $CD8^+$ cells with loss of CD2 expression observed during acute *T. annulata* infection with the sporozoites (chapter 6). Loss of CD2 expression on T cells was also observed during *T. parva* infection (E.A. Innes & G. Lamb, unpublished observations, reported in Innes *et al.*, 1992a). As discussed in chapter

6, loss of CD2 expression was associated with a non-specific activation of T cells by *T. annulata*, leading to some kind of suppression where T cells neither responded *in vitro* to irradiated parasite infected cells nor to other exogenous stimuli like Con. A and IL-2. The parasite was also able to cause non-specific stimulation at least in some T cells after cell line immunisation, although the amount of parasite load was very low in these animals. Nevertheless, a parasite specific cytotoxic T cell response also developed.

The two animals implanted with skin developed similar levels of allogeneic response, but parasite transfer to the recipient's cells took place only in one animal which was immune to subsequent sporozoite challenge. The pre-existing allogeneic response did not allow parasite development in the second animal which was fully susceptible to the sporozoite challenge. Why these two animals showed different responses after cell line immunisation despite similar pre-existing allogeneic response is not clear and could not be explained from these experiments. It has been previously shown that animals inoculated with killed *T. annulata* infected cells developed antibody titres to a schizont antigen, but were not immune on sporozoite challenge (Pipano *et al.*, 1977). More studies are required to understand how parasite transfer takes place and what is the precise role of allogeneic response on the parasite infected cells used for immunising the animals. Further, it is important to know which cells are infected by the parasite during the parasite transfer. There is a possibility that a mild allogeneic response is beneficial for the parasite transfer as it would attract immunologically active cells to the site of infection or the draining lymph node. Alloreactive cytotoxic cells generated against MHC antigens of the immunising cell line perhaps only destroy the infected cell but not the parasite within it, which is able to infect other cells. If that is the case, reimmunisation with an increased dose of allogeneic *T. annulata* infected cells might enable some parasites to transfer into the recipient's cells. However, it was recently shown that reimmunisation of calves with $1x10^6$ allogeneic *T. annulata* infected cells seven months after the first immunisation with $1x10^4$ cells did not boost immunity (Ouhelli *et al.*, 1994). Possibly cell numbers in the range of 10^7-10^9 would be required for effective cell transfer at the time of reimmunisation as is needed for the first immunisation against *T. parva* (Pirie *et al.*, 1970; Brown *et al.*, 1971; Brown *et al.*, 1978a; Brown, 1981; Emery *et al.*, 1982).

The results presented in this chapter showed that animals immunised with

$1x10^6$ *T. annulata* infected cells exhibited a mild increase in the output of lymph and total cells from the node. The output of blasting cells from the node showed a massive elevation showing two peaks of the blasting cells. The first corresponded to an allogeneic response against MHC antigens of the immunising cell line and the second was associated with the parasite specific response. Parasite infected cells of the recipient origin appeared in efferent lymph from day 11 onwards, but cells of donor origin were never isolated and established in culture. Isolation of parasite infected cells from the efferent lymph was accomplished earlier than peripheral blood, further confirming that parasite dissemination after cell line immunisation also takes place through efferent lymph. A biphasic increase in the output of $CD8^+$ cells in efferent lymph was observed after cell line immunisation. The increase was more pronounced during the second parasite specific phase than the first allogeneic phase. A few T cells expressed CD25 as compared to many T cells expressing MHC class II. ELL and PBM exhibited increased proliferation in response to *in vitro* stimulations with human recombinant IL-2, irradiated autologous *T. annulata* infected cells and irradiated immunising cells. Cytotoxic responses against MHC antigens of the immunising cell line followed by MHC class I restricted parasite specific cytotoxicity, were observed in both efferent lymph and peripheral blood. However, a few $CD4^+$ and $CD8^+$ cells not expressing CD2 were also observed during the second parasite specific phase of the response. At this stage, CD25 and MHC class II expression on T cells increased. The inhibition of parasite development after cell line immunisation as a result of the pre-existing allogeneic response was not associated with T cell activation in response to the parasite and only allogeneic responses were observed.

CHAPTER 8

SUMMARY AND CONCLUSIONS

Theileria annulata infected macroschizont cell lines have been widely used as vaccines in endemic areas to control tropical theileriosis (Hashemi-Fesharki & Shad-Del, 1973; Wenshun et al., 1973; Stepanova et al., 1977; Pipano, 1977; Singh, 1992). However, the duration of immunity engendered by these vaccines is poorly defined. Immunity has been shown to decline after vaccination (Pipano, 1977; Ouhelli et al., 1994) and reimmunisation is recommended in some countries (Pipano, 1978). It would be particularly relevant in areas where the disease season is very short and endemic stability does not exist. All the animals do not encounter ticks to get a boost in immunity during a particular season. This situation has been reported in Morocco where only 15.6% animals were infected by ticks in one season, as shown by seroconversion (Flach & Ouhelli, 1992). The cell lines of *T. annulata* used as vaccines for control of the disease in endemic areas are invariably allogeneic to the recipient animals. Some evidence in the literature suggests that reimmunisation with the same cell line may not be effective (Pipano, 1978; Ouhelli et al., 1994). It could arise because of the allograft responses induced against MHC antigens of the immunising cell line after first vaccination. Experiments in this thesis were initiated to address this question i.e. to elucidate the effect of a previously generated allogeneic response on immunisation with a *T. annulata* infected cell line.

It has been shown earlier that allograft reactions produced by *T. annulata* infected cell lines are similar to the responses produced by skin grafting (Kachani & Spooner, 1992). These findings were exploited further and developed as a model to study reimmunisation in these experiments. The allograft responses generated after first cell line immunisation were imitated either by inoculation of uninfected leucocytes or by skin grafting. This was followed by inoculation with a *T. annulata* infected cell line which acted as the second immunisation. The development of immunity was assessed by challenging the animals with a potentially lethal dose of sporozoites.

Allogeneic responses generated by the inoculation of uninfected leucocytes were very mild as compared to the strong responses after skin grafting. The animal showing a mild allograft response, induced by the inoculation of uninfected cells, was immune to lethal sporozoite challenge following immunisation with $1x10^6$ *T. annulata* infected cells. In contrast, animals exhibiting a strong anti MHC response induced by skin grafting exhibited variable reactions on cell line immunisation. Of the skin

grafted animals, one out of three inoculated with $1x10^6$ infected cells and all three inoculated with $1x10^4$ infected cells were not immunised. The parasite did not transfer into cells of the recipient from the immunising cells and the animals suffered from an acute disease on sporozoite challenge. The pre-existing allogeneic response prevented the appearance of parasitological reactions and development of immunity after cell line immunisation. The two other animals which became immune despite an allogeneic response, exhibited delayed parasitological reactions and presumably development of immunity.

The ability to isolate parasite infected cell lines from the peripheral blood after cell line immunisation was also delayed or prevented in the presence of a pre-existing allograft response. This ability correlated with protection on sporozoite challenge. Animals were fully susceptible to sporozoite challenge if no parasite infected cell line could be isolated after immunisation. There is an indirect evidence in the literature that transfer of the parasite from inoculated cells to the cells of the recipient is important for the development of immunity against *T. annulata* as well as *T. parva* (Brown et al., 1978b; Emery et al., 1982; Innes et al., 1989a). This was conclusively demonstrated by the experiments in this thesis. Where no parasite infected cell line was isolated, as after immunisation from some of the skin grafted animals, such animals were fully susceptible on sporozoite challenge.

Although the importance of allograft responses in the development of immunity during immunisation with *T. annulata* infected cell lines was highlighted in these experiments, similar levels of allograft responses were found to prevent parasite transfer and the development of immunity in some animals but not in others. The mechanism of this interference is not understood because of lack of knowledge of the cellular interactions between the parasite and various lymphocyte subpopulations. We know little about the cellular events leading to the development of immunity after cell line immunisation and subsequent sporozoite challenge. Further studies in this thesis thus concentrated on attempts to understand the dynamics of parasite dissemination and activation of various lymphocyte subpopulations. To do this, changes occurring in peripheral blood and lymph efferent from the node draining the site of *T. annulata* cell line immunisation and sporozoite challenge were monitored, in skin grafted and non-skin grafted (naive) animals.

Peripheral blood acts as a traffic route for circulating leucocytes and, therefore, can provide useful information on the involvement of various cell subpopulations in the development of immunity (Westermann & Pabst, 1990). The dynamics of various leucocyte subpopulations in peripheral blood revealed that mild and transient leucopenia was a common finding, after immunisation with *T. annulata* infected cell lines or challenge of immune animals. In contrast, leucopenia was severe and progressive in susceptible animals developing acute disease after sporozoite challenge. A very small and transient fall in circulating leucocytes was also observed in the skin grafted animals which showed no sign of parasite development after cell line immunisation. Leucopenia was caused by a fall in the number of circulating granulocytes and lymphocytes. In response to *T. annulata* infection, a fall in the levels of circulating $CD4^+$ cells and B cells is directly responsible for this lymphocytopaenia, followed by lesser involvement of $CD8^+$ cells and $\gamma\delta$ T cells. Cells of the monocyte/macrophage lineage were least affected.

In the convalescent animals, there was a transient increase in the total circulating $CD8^+$ cells initially, followed by a sustained increase in monocytes/macrophages. These changes were associated with recovery in cell line immunised animals and also after treatment in animals showing clinical theileriosis. These observations further confirmed that immunity against *T. annulata* is mediated by $CD8^+$ cells and monocyte dependent mechanisms.

During the course of parasite development after cell line immunisation or sporozoite challenge, T cell activation markers in the form of CD25 (IL-2R, α chain) appeared transiently on PBM and the percentage of cells bearing a memory phenotype ($CD45RB^-$) increased. These changes were not observed in skin grafted animals where no parasitological response was seen after cell line immunisation.

Initial pathology and development of immunity takes place in the draining lymph node after sporozoite challenge or cell line immunisation. Cells leaving in the efferent lymph of an antigen primed node reflect the immunological events occurring in the node and disseminate immune effector cells to rest of the body (Trnka & Cahill, 1980). Further experiments concentrated on understanding parasite dissemination and T cell activation in the lymph efferent from the node draining the site of sporozoite challenge or cell line immunisation.

T. annulata sporozoites induced a strong lymphoproliferation in the draining

lymph node of naive animals, leading to a 8-10 fold increase in the output of fluid and cells from the node. Cell output from the node also increased transiently in the immune animal challenged with sporozoites, but output of fluid was unaffected. Animals immunised with $1x10^6$ *T. annulata* infected cells only exhibited a mild and transient increase (1.5 to 2 fold) in the output of lymph and total cells from the node.

Infection with sporozoites in naive animals induced a period of cell shutdown corresponding to the initial appearance of schizonts in the node. This was followed by a massive output of blasting cells (more than 80% of total ELL on day 10) accompanied by the exit of parasite infected cells in the efferent lymph. Only a few blasting cells were observed in the immune animal. In the cell line immunised animals, output of blasting cells from the node increased gradually, showing two peaks. The first corresponded to an allogeneic response against MHC antigens of the immunising cell line and the second was associated with a parasite specific response. The maximum proportion of blasting cells in efferent lymph was about 50% during the second phase. A slight increase in the output of lymph and blasting cells was also observed in the skin grafted animals which showed no parasitological reactions after cell line immunisation.

Schizonts appeared in the efferent lymph of naive animals after sporozoite challenge from day 6 onwards, very shortly after they would be expected to appear in the lymph node. Infected cells were not observed in the immune animal. As judged by isolation of parasite *in vitro*, the cell line immunised animals showed infected cells of recipient origin in efferent lymph from day 11 onwards. Infected cells of donor origin were never isolated. Appearance of schizonts was delayed to day 16 in one of the skin grafted animals after cell line immunisation and completely prevented in the other. Isolation of parasite infected cells in the efferent lymph was accomplished earlier than from peripheral blood, indicating that the parasite dissemination takes place through efferent lymph to the rest of the body.

Large amounts of IFN-γ were observed in efferent lymph, coinciding with the cell shutdown in naive animals undergoing acute disease after sporozoite challenge. This was followed by comparatively lower levels of IFN-γ detected during the course of infection, which might be responsible for some of the initial pathology. Two low level peaks of IFN-γ were observed in naive animals after cell line immunisation. The first transient and relatively stronger peak was observed between day 2-7, followed

by very low levels of IFN-γ after day 12 which corresponded to the appearance of the parasite. IFN-γ was not detected in the skin grafted animal not responding to the cell line immunisation.

Phenotypic analysis of ELL showed an increase in T cell output, which initially comprised $CD4^+$ cells, followed by a massive output of $CD8^+$ cells after sporozoite challenge or cell line immunisation of naive animals. A biphasic increase in the output of $CD8^+$ cells was observed after cell line immunisation. The increase was more pronounced during the second parasite specific phase than the first allogeneic phase.

Naive animals initially seemed to mount a parasite specific T cell activation response after sporozoite challenge in the form of transient CD25 and MHC class II expression on $CD4^+$ cells, followed by a strong proliferation of $CD8^+$ cells with high expression of MHC class II but not CD25. In cell line immunised animals, few $CD4^+$ and $CD8^+$ cells expressed CD25. In contrast, many T cells which were mainly $CD8^+$ cells expressed MHC class II, mainly during the parasite specific phase of the response.

Functional assays on ELL and PBM from calves undergoing acute theileriosis after sporozoite challenge revealed that the response of these cells was not parasite specific. The cells showed no parasite specific cytotoxic activity and were less responsive to exogenous Con. A, IL-2 or irradiated autologous infected cells *in vitro*, as if in a state of suppression. Parasite specific T cells were observed in the immune animal and after treatment in naive animals. On the other hand, ELL and PBM from cell line immunised animals exhibited increased proliferation *in vitro* in response to stimulation with IL-2, irradiated autologous *T. annulata* infected cells or irradiated immunising cells. Cytotoxic responses were observed against MHC antigens of the immunising cell line followed by MHC class I restricted parasite specific cytotoxicity.

The parasite induced an inappropriate proliferation and abnormal activation of T cells in the lymph node after sporozoite challenge as T cells gradually lost CD2 expression with the progression of the disease. CD2 is an important adhesion molecule for normal T cell effector function and is required for initial binding with LFA-3 on target cells (Bierer et al., 1989). Loss of CD2 expression was more pronounced on $CD8^+$ than $CD4^+$ cells. In cell line immunised animals, a few $CD4^+$ and $CD8^+$ cells not expressing CD2 were also observed during the second parasite

specific phase of the response along with appearance of normal immune effector cells. At this stage, CD25 and MHC class II expression on T cells increased. Inhibition of the parasite development after cell line immunisation, as a result of the pre-existing allogeneic response, was not associated with T cell activation in response to the parasite and only allogeneic responses were observed.

These experiments show that pre-existing allograft immune responses, generated in animals after first cell line immunisation, may block parasite transfer and further development or enhancement of immunity against *T. annulata* on second immunisation with the same cell line. The situation at the time of reimmunisation with the same cell line against *T. annulata* looks similar to primary cell line immunisation against *T. parva* where a large number of cells are required for parasite transfer. These observations are of immediate importance in endemic areas where *T. annulata* infected cell culture vaccines are being used, or their application is planned for the future. They are even more relevant in countries where animals are regularly moved between theileriosis free and endemic areas. It may not be advisable to reimmunise the animals with the same cell line as that used for first vaccination. The problem might be overcome by using cell lines with different MHC phenotypes. However, further experiments will be needed to confirm the effectiveness of using different cell lines before recommending it for field conditions. Alternatively, parasite infected cell lines lacking MHC expression might be useful as these would not induce an allograft response. Some *T. parva* infected MHC class I and class II negative cell lines have been generated (A.J. Teale, pers. comm.), but similar *T. annulata* infected lines are not available.

Leucopenia, corresponding to the severity of parasitological reactions, as a result of loss of both circulating polymorphonuclear cells and mononuclear cells, was observed after sporozoite challenge and after cell line immunisation of naive animals. However, studies on the efferent lymph exhibited an increase in the total cell output from the lymph node draining the site of infection, as a result of T cell proliferation in that node. The parasite seemed to induce a non-specific proliferation and inappropriate activation of T cells as it was not associated with normal T cell activation markers. Appearance of CD25 was transient, T cells lost CD2 expression and activated $CD4^+$ cells did not express the memory phenotype during the course of acute disease. Some non-specific T cell activation was also observed in cell line

immunised animals corresponding to the small parasite load. The non-specifically activated T cells are possibly destroyed by apoptosis leading to a decrease in the circulating leucocytes.

This study provided more understanding of parasite dissemination and some of the cellular and molecular interactions leading to development of immunity against *T. annulata*, in the efferent lymph and peripheral blood, both after sporozoite challenge and cell line immunisation. The findings are relevant for *T. parva* and other apicomplexan parasites of man and animals like *Plasmodium* where these type of studies may not be possible. Similar experiments in animals exhibiting a pre-existing allograft response only showed that parasite transfer after cell line immunisation can be delayed or prevented. This observation cautions against repeated use of the same allogeneic cell line for boosting immunity under field conditions. The precise mechanism, which prevents the development of parasite specific immune response because of the presence of an allograft response, was not determined. An understanding of the mechanism of parasite transfer from donor cells to the cells of the recipient would be a high priority topic for future studies. This would help in improving control measures for *T. annulata* and also help in defining and developing a suitable cell culture vaccine for the closely related parasite *T. parva*.

CHAPTER 9

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APPENDIX

1. Hanks balanced salt solution

HBBS supplied in powder form (Gibco BRL, Life technologies) containing phenol red was reconstituted with deionised distilled water. Sodium bicarbonate (Fisons) was added at the rate of 0.35g per litre and the pH of the HBBS solution was adjusted to 6.8 with a pH meter (Russell pH limited) using 1N HCL or 1N NaOH. The final volume was made up to 100 ml to make 10x (10 times concentrated) HBSS solution. It was sterilised by filtration (0.22μ, Millipore Corporation) and stored at 4°C until required. The solution was made up to 1x before use by addition of sterilised distilled water.

2. Complement

Pooled rabbit sera collected from a commercial slaughterer was used as a source of complement. Batches of complement were screened for lack of inherent cytotoxicity and for potency in the microlymphocytotoxicity test using test cells and antisera of known reactivity. Suitable batches of complement were then stored at -70°C until required in the test.

3. Eosin dye

A 5% w/v stock solution of Eosin powder (Koch Light Laboratories Ltd) in HBSS was prepared and stored at -20°C in 1 ml aliquots. The solution was thawed just before use.

4. Fixing solution

Fixing solution was prepared by adding 5 ml of 0.15M disodium hydrogen phosphate (Fisons) in deionised distilled water to 95 ml of formalin (40% formaldehyde in 0.9% saline) and was stored at room temperature.

5. Acid citrate dextrose

ACD consisted of 2.5% D-Glucose (Fisons) and 2.05% di-sodium hydrogen citrate (Fisons) in distilled water. The solution was autoclaved and added to 20 ml vacutainers or 500 ml bottles for collection of blood at 1 ml for 4 ml of blood (4 ml ACD & 16 ml blood in 20 ml vacutainer; 100 ml ACD & 400 ml blood in the bottle).

6. Ammonium chloride lysis solution

It consisted of two solutions: Solution A, 0.16M Ammonium Chloride (Fisons); Solution B 0.17M Tris (Fisons). Both the solutions were prepared separately in distilled water, autoclaved and stored at room temperature. Solution A and B were warmed to 37°C and mixed in a ratio of 9:1 just before use. Blood was centrifuged at 1500g for 20 min., supernate plasma was discarded. To 15 ml cell pellet, 35 ml of mixture of sol. A and B was added. The tubes were incubated for a few min. in 37°C water bath until colour of the solution became black. It was centrifuged at 300g for 10 min. in a refrigerated centrifuge. The cell pellet was washed twice with PBS by centrifugation at 100g for 10 min.

7. Phosphate buffer saline

PBS consisted of 0.9% Sodium Chloride, 0.107% di-Sodium Hydrogen Phosphate and 0.051% Sodium di-Hydrogen Orthophosphate (all Fisons) w/v aqueous solution in distilled water. The pH of the solution was adjusted to 7.2 using 1N HCl or 1N NaOH, autoclaved and stored at room temperature.

8. Complete tissue culture medium

Theileria annulata infected cell lines were established and maintained in sterile TC medium comprising RPMI-1640 with 25mM Hepes (Gibco BRL), 2mM L-glutamine (Gibco BRL) and supplemented with 10% FCS (Meriden), 100 i.u. per ml penicillin and 100 μg per ml streptomycin (Gibco) as described by Brown (1983). Aliquot of TC medium were tested for sterility before use in cultures.

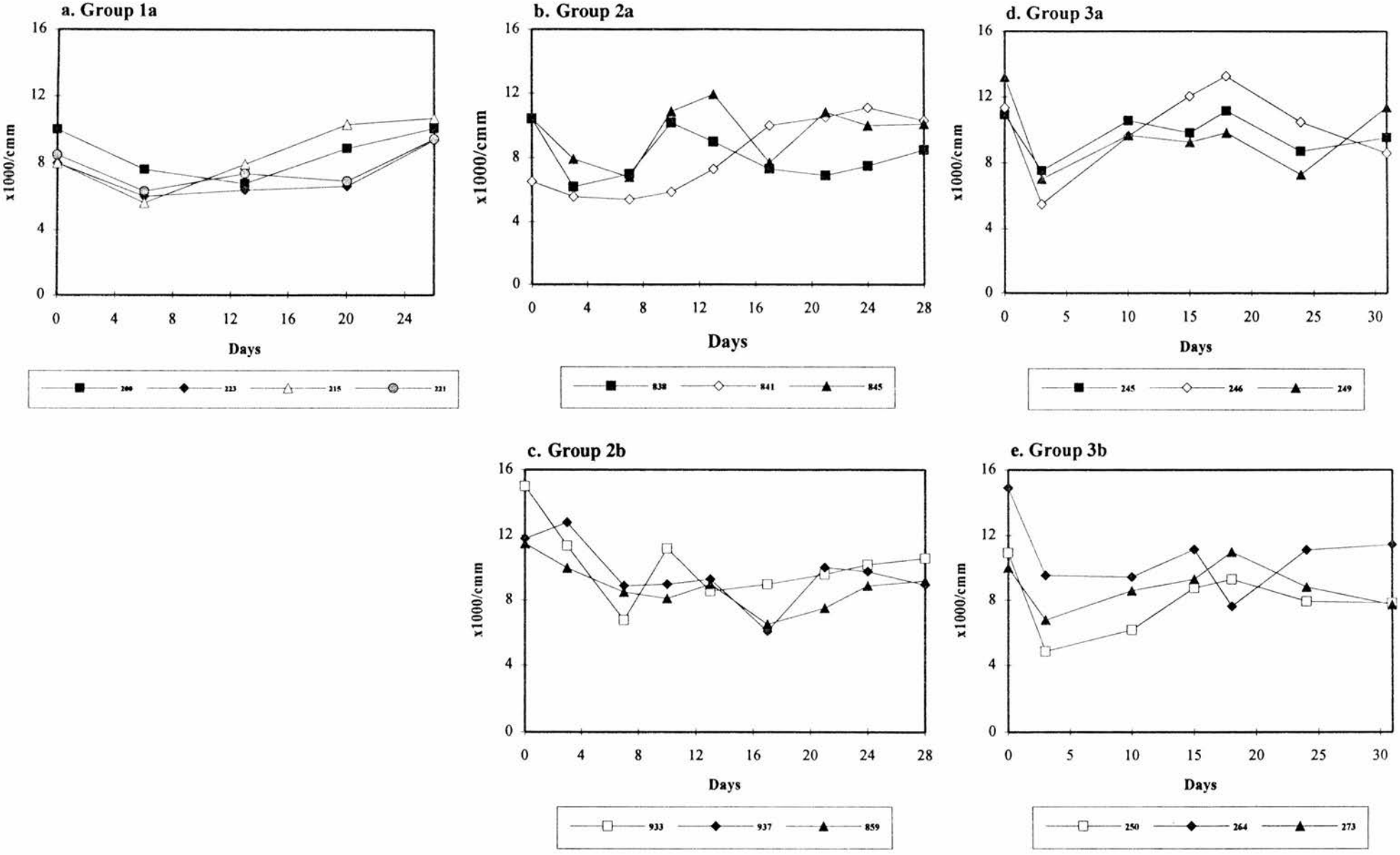
9. Mixed lymphocyte culture medium

MLC medium was used for mixed lymphocyte culture of PBM or ELL with irradiated self *T. annulata* cell cultures, initiation and maintenance of uninfected lymphoblastoid cell lines and proliferation assays. The medium contained RPMI 1640 (Gibco BRL) supplemented with 25mM Hepes, 2mM L-glutamine (Gibco BRL), $5x10^{-5}$ M 2-mercaptoethanol (BDH) and supplemented with 10% foetal calf serum (Imperial) and 50 μg/ml gentamycin (Gibco BRL) as described by Glass and Spooner (1990). Aliquots of MLC medium were tested for sterility before use.

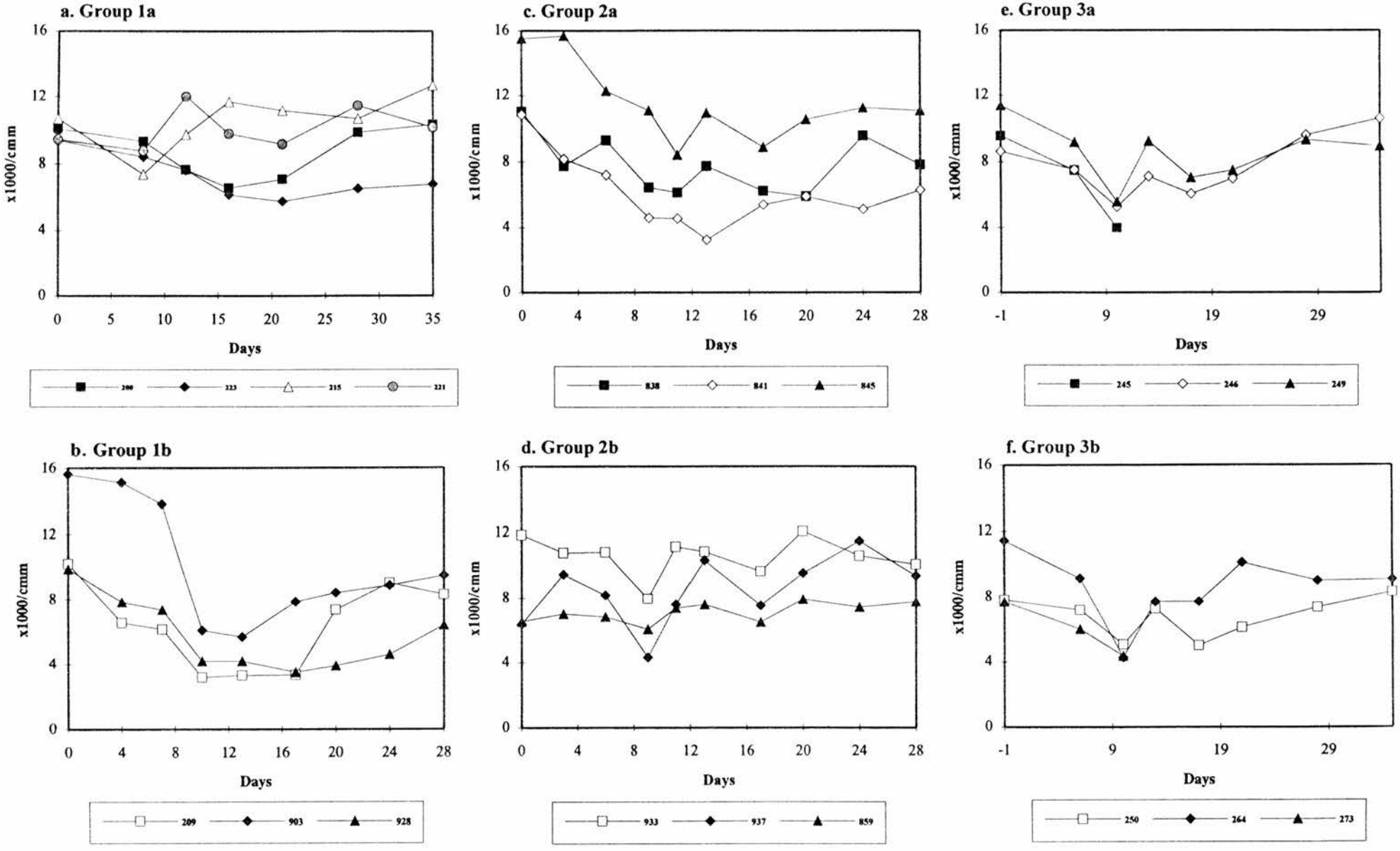
10. BoLA specificities based on 5th international workshop and defining sera used.

Antigen specificity	Previous designation*	Sera	Antigen specificity	Previous designation*	Sera
W1		1,8,83,112,151	A15	A8.2	105,152
A2		2,62,64,65,115	A16		38,43,131,114
A3		39	W17	W6.1	12,92,93
W4		82,117	A18	W6.2	81,86,113
A5		7,90	A19	W6.4	100
A6		9,10,66,75,97	A20		78,121
A7		11,67,68,132	A21		107,123
A8		13	W25		111,132
A9		14,140	A31		99,127
A10		69,71	A32		40,124,126
A11		73,76,110,102	W36		121
A12		47,133	W50		85
A13		5,136,109	BoLy R'		94
A14	A8.1	80,138			

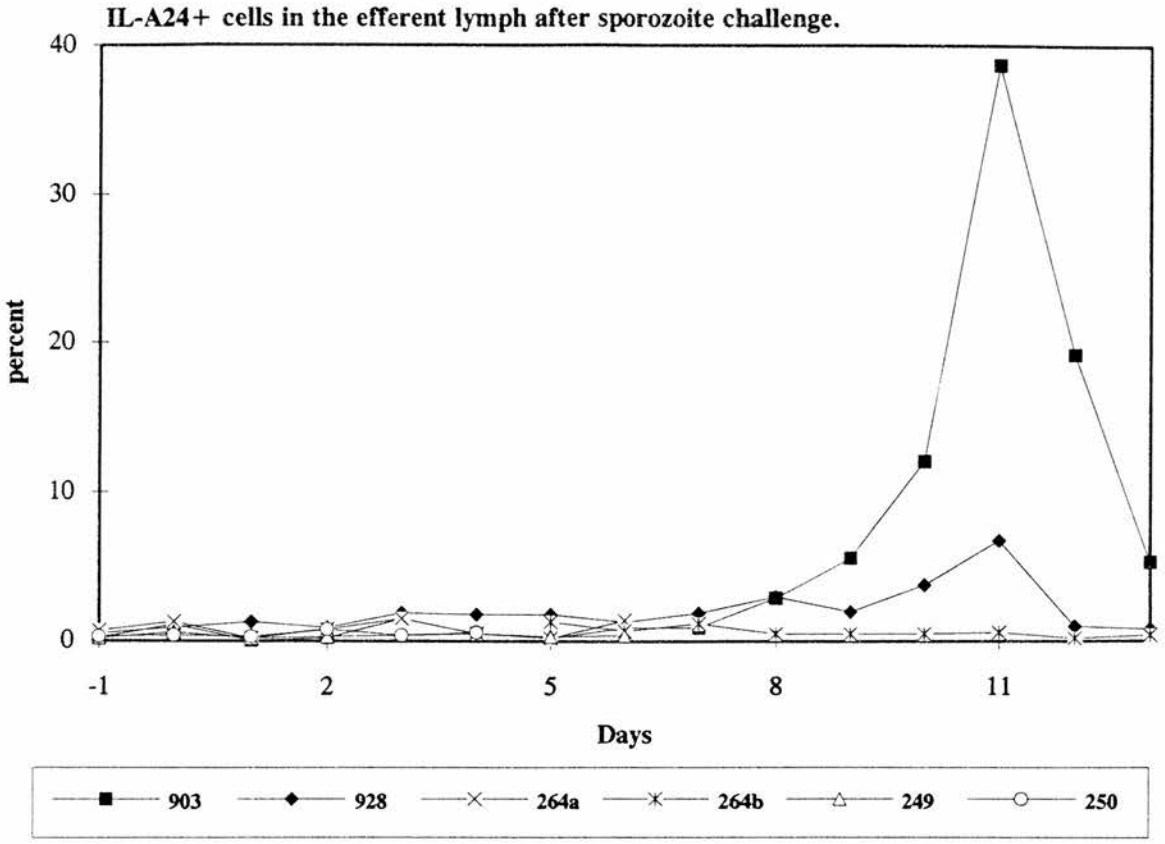
* Previous designation if any.

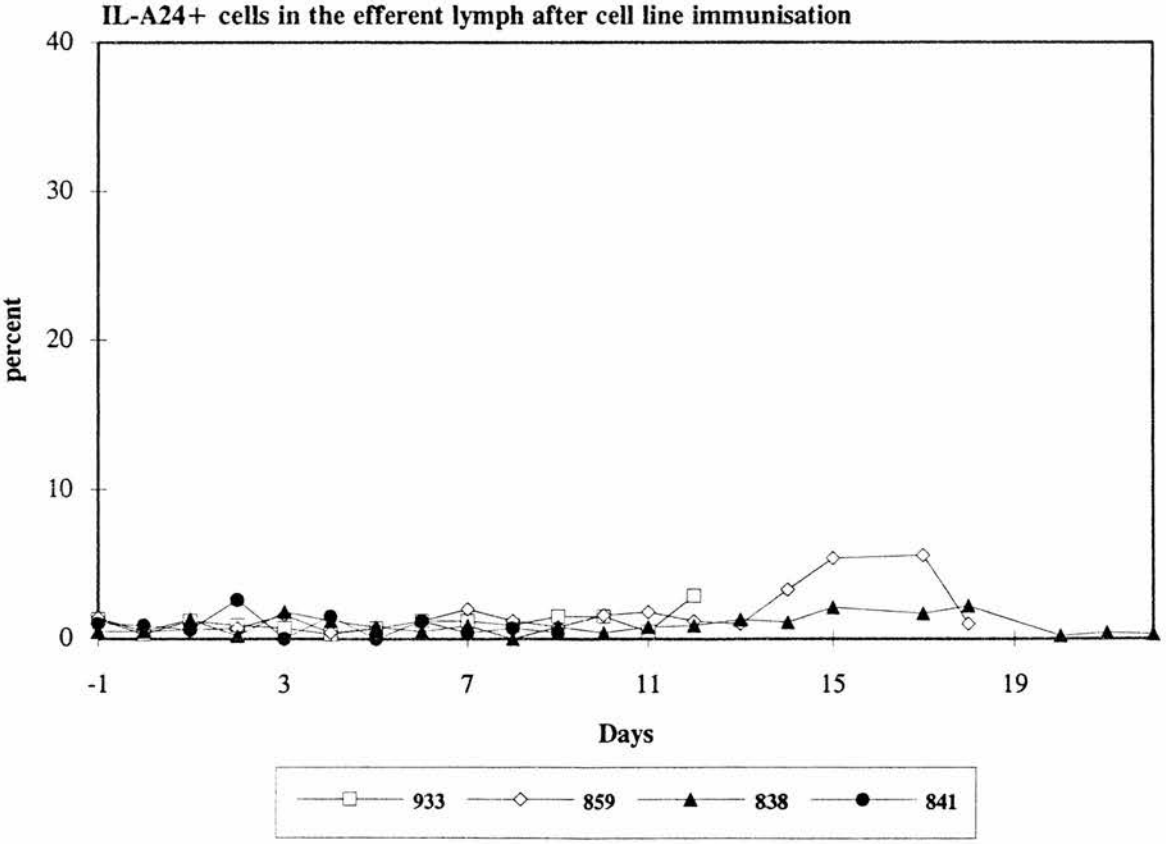


11: Total leucocyte counts in the animals after cell line immunisation.



12: Total leucocyte counts in the animals after sporozoite challenge.





13: appearance of IL-A24$^+$ cells in the efferent lymph after infection with *T. annulata* sporozoites or parasite infected cell line.

LIST OF PUBLICATIONS FROM THE THESIS

1. **Nichani AK.** (1993) The effect of anti MHC response on cell line immunisation against *Theileria annulata*. In: Proceedings of a meeting "*Tropical Theileriosis research: current status*", held at University of York, 29-30 March, 1993. p. 12.

2. **Nichani AK & Spooner RL.** (1994) Changes in T cell subsets in efferent lymph of cattle during *Theileria annulata* infection. *Journal of Cellular Biochemistry*. **S18D**: 422.

3. **Nichani AK, Millar P, Maxwell MH, Robertson GW, Brown, CGD & Spooner RL.** (1994) Allogeneic immune responses can block the development of immunity following cell line immunisation against *Theileria annulata*. In: *European Union third coordination meeting on Tropical Theileriosis*, held at Antalya, Turkey, 4-9 October, 1994.

4. **Nichani AK, Craigmile SC, Thorp BH, Ritchie M, Brown CGD & Spooner RL.** (1994) Response to *Theileria annulata* infection in efferent lymph leaving the node draining the site of infection. In: *European Union third coordination meeting on Tropical Theileriosis*, held at Antalya, Turkey, 4-9 October, 1994.

The effect of anti MHC response on cell line immunisation against *Theileria annulata*

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Theileria annulata infected lymphoblastoid cells have been successfully used as a live vaccine which produces a strong cytotoxic response and complement fixing antibodies against the allogeneic MHC antigens along with an anti parasite immune response. However, the histocompatibility barrier does not jeopardise infection and primary immunisation against *T. annulata*. The duration of immunity after cell line immunisation is not clearly known and reimmunisation is sometimes recommended. However, it is not clear whether the anti MHC response interferes in the parasite transfer and development of immune response at the time of reimmunisation. This experiment was undertaken to see the effect of preformed anti MHC response on cell line immunisation by immunising animals against MHC antigens and later inoculating *T. annulata* infected cell lines and challenging with stabilate of *T. annulata* sporozoites. The role of preformed anti MHC response on cell line immunisation against *T. annulata* will be discussed.

Proceedings of a meeting "*Tropical Theileriosis research: current status*", held at University of York, 29-30 March, 1993. p. 12.

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V 649 CHANGES IN T CELL SUBSETS IN EFFERENT LYMPH OF CATTLE DURING *Theileria annulata* INFECTION

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Theileria annulata is a protozoan parasite which causes a serious lymphoproliferative disease of cattle: Tropical theileriosis in tropical and subtropical countries. The parasite is transmitted by ticks of the genus *Hyalomma* and preferentially invades monocytes/macrophages and B cells. Much of the pathology and lymphoproliferation takes place in the lymph node draining the site of infection. We are studying immune responses to the parasite in the efferent lymph of the draining lymph node in a potentially lethal infection as a model for understanding immunity to intracellular parasites. The parasite induces strong lymphoproliferation and the blasting cells start appearing in the efferent lymph after day 6 of infection. Initially, the blasting cells are CD4+ followed by a strong proliferation of CD8 cells. Most of the blasting cells are IL-2R+ as well at this stage. The resting T cells in the efferent lymph are MHC class II negative, but majority of blasting CD4 and CD8 cells have high MHC class II expression as well. However, the CD8 cells do not kill autologous parasite infected cells. The cells proliferate strongly in response to con A and exogenous IL-2, but don't proliferate in response to irradiated autologous *Theileria* infected cells. These findings are discussed in relation to the progression of the infection. Further studies on the function of the various activated cell populations are in progress.

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THIRD EU COORDINATION MEETING ON

TROPICAL THEILERIOSIS

Antalya, Turkey, 4-9th October 1994

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ALLOGENEIC IMMUNE RESPONSES CAN BLOCK THE DEVELOPMENT OF IMMUNITY FOLLOWING CELL LINE IMMUNISATION AGAINST *THEILERIA ANNULATA*

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T. annulata infected cell lines have been used as vaccines against tropical theileriosis in several countries. The inoculated animals produce a strong cytotoxic T cell and humoral response against the allogeneic MHC antigens of the immunising cell line followed by a parasite specific immune response. The duration of immunity after cell line immunisation is not known. There is evidence that immunity to the parasite wanes and reimmunisation is often recommended. However, it is not clear whether an allogeneic response generated after first immunisation would interfere with development of immunity against the parasite at the time of reimmunisation with the same cell line. These experiments were undertaken to study the effect of pre-existing allogeneic response on cell line immunisation. The animals were immunised against MHC antigens by inoculation of uninfected leucocytes or skin grafting and later immunised with a *T. annulata* infected cell line of the same BoLA type. A mild allogeneic response generated by the leucocyte inoculation didn't affect the development of immunity following cell line immunisation. But a strong allogeneic response generated by skin grafting, which mimics the response produced by inoculation of the cell lines, interfered with the parasite transfer and development of a parasite specific immune response when the animals were immunised with $1x10^6$ cells. The allogeneic response generated in this manner totally blocked parasite transfer at a lower immunising cell dose of $1x10^4$ cells where animals suffered from acute disease on sporozoite challenge. These observations are of immediate importance in endemic areas where cell lines of *T. annulata* schizonts are being used as vaccines to control the disease. The allogeneic immune response generated in the animals after first immunisation with a cell line might block development of immunity against the parasite if the animals are reimmunised with the same cell line used for first immunisation.

European Union third coordination meeting on Tropical Theileriosis,
held at Antalya, Turkey, 4-9 October, 1994.

THIRD EU COORDINATION MEETING ON

TROPICAL THEILERIOSIS

Antalya, Turkey, 4-9th October 1994

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RESPONSE TO *THEILERIA ANNULATA* INFECTION IN EFFERENT LYMPH LEAVING THE NODE DRAINING THE SITE OF INFECTION

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Following *T. annulata* infection, the initial pathology takes place in the nearest draining lymph node. We are studying the pathogenesis and T cell activation in the draining efferent lymphatics following sporozoite challenge or allogeneic cell line immunisation to understand the development of immunity. During a potentially lethal sporozoite challenge, the parasite induced strong lymphoproliferation with a massive increase in output of blasting cells in the efferent lymph from day 6 with a 5-10 fold increase in the flow rate and cell output. Parasite infected lymphoblasts were seen from day 6 onwards. An initial increase in the percentage of $CD2^+$ cells corresponding with an increase in $CD4^+$ cells was observed from day 5. Many $CD4^+$ cells expressed IL-2R transiently and became MHC II^+. This was followed by an increased output of blasting $CD8^+$ cells with high MHC II expression. Blasting $CD4^+$ and $CD8^+$ cells gradually lost CD2 expression with progression of the infection. These cells were MHC class II^+ but had lost IL-2R expression suggesting an inappropriate activation of T cells in response to the parasite. Efferent lymph cells were less responsive to Con. A or exogenous IL-2 stimulation *in vitro* during the later stages of infection. The cells didn't proliferate *in vitro* in response to autologous parasite infected cells and didn't kill autologous parasite infected cells suggesting lymphocyte unresponsiveness either due to anergy or suppression. The animals inoculated with a *T. annulata* infected cell line exhibited a 2-4 fold increase in the flow rate and cell output in the efferent lymph. Parasite infected cells of recipient origin were seen from day 11 onwards, however cells of donor origin were never isolated. Two peaks of blasting cells were observed, the first corresponded to an allogeneic response against MHC antigens of the immunising cell line and the second was parasite associated. A few $CD4^+$ and $CD8^+$ cells not expressing CD2 were also seen during the second parasite specific phase of the response. At this stage, IL-2R and MHC II expression on T cells increased. The results indicate that *T. annulata* induces an inappropriate proliferation and not activation of T cells as T cells express normal activation markers but lose CD2 which is a very important adhesion molecule, however both specific and nonspecific T cell activation is seen during immunisation with cell lines of infected schizonts which induces immunity to sporozoite challenge.

European Union third coordination meeting on Tropical Theileriosis,
held at Antalya, Turkey, 4-9 October, 1994.