CYTOKINE, CELLULAR AND HUMORAL IMMUNE RESPONSES IN CALVES EXPERIMENTALLY-INFECTED WITH *FASCIOLA HEPATICA.*

Thesis submitted in accordance with the requirements
of the University of Liverpool
for the degree of Doctor of Philosophy
by

Atila Akça

October, 1999

ACKNOWLEDGEMENTS

I should like to express my gratitude to the University of Kafkas, Kars and to the Turkish Government for funding this PhD project, and to all those who helped me to complete it.

A special thank you to the staff of the University of Liverpool English Language Unit for their help with my English at the outset, and to all the people who helped me with it subsequently.

I am particularly grateful to Professor Sandy Trees of the Veterinary Parasitology Department for giving me the opportunity to work here for the last five years. Special thanks too to Dr Diana Williams, my supervisor, for her patience, encouragement and constructive criticism from beginning to end of the project. Thanks to their support, not only have I been able to begin to deepen my knowledge and understanding of science, but also to meet and to enjoy exchanging ideas with people from all over the world, and to get to know Liverpool which has made such an impact upon my life, not least as the place where I met my wife, Cathy, and started a family of my own. A special word here for my little boy, Altay, who for the last six months has done everything in his power to distract me from the painful task of writing, without ever quite managing to do so: thank you for making me laugh – now we will have time to play!

I should like to thank Bob Collins, IAH, Compton, for kindly running the IL-4 bioassay; Peter Young, EM Unit, for undertaking the EM sections; Nigel Jones and his co-workers at the animal husbandry farm, Leahurst, for their excellent animal management; Mark Taylor, for providing macrophage mouse cell line, J774; and others too many to mention, for helping in a variety of ways.

Thanks to my colleagues in the Department and to all the many friends who have helped and encouraged me during my time here, especially Sefa, Sabri, Ibrahim, Ibo, Mehmet Ali and above all Luis who was always there to talk or to listen.

Finally, a thank you from the heart to my Mum and Dad, without whose foresight and sacrifice none of this would have been possible.

DEDICATION

It gives me great pleasure to dedicate this work to my wife, Catherine and to my son, Altay.

ABSTRACT OF THESIS

Cytokine, Cellular and Humoral Immune Responses in Calves Experimentally-infected with *Fasciola hepatica*

by Atila Akça

The digenetic trematode, *Fasciola hepatica*, causes disease in a variety of mammalian species, with consequent economic loss. Although fasciolosis may be controlled by eradicating the intermediate host, *Lymnaea* spp., or by chemotherapy, the cost of drugs and drug resistance currently limit its management. A vaccine for *F. hepatica* would make a major contribution to improved control. Cattle are reported to acquire a strong degree of resistance to infection with the parasite, the nature of which, particularly T cell responses and their role in protective immunity, is not fully understood. This study aims to define cellular immune responses in cattle experimentally-infected with *F.hepatica*.

Strong *ex vivo* antigen-specific proliferation of peripheral blood mononuclear cells (PBMC) was detected during the first 4 weeks of a primary infection. From the sixth week of infection, PBMC became unresponsive to fluke antigens. Culture supernatants were tested for antigen-specific interferon-gamma (IFN-γ), interleukin (IL)-2 and IL-4 production, using bioassays or ELISA. The kinetics of IFN-γ and IL-2 production were similar to, and the latter significantly correlated with, those obtained for the proliferation responses of PBMC. Early in the primary infection, relatively high levels of IFN-γ and IL-2 were detected, but levels were undetectable after 6 weeks post-infection (wpi). There was no difference in IL-4 levels in a limited number of culture supernatant tested from infected and control animals. Flow cytometry analysis of fluke E/S and somatic antigen-specific cell lines, generated from PBMC from a hyper-immune cow, showed they were predominantly CD4+ T cells. This indicates that *in vitro* antigen-specific proliferating cells were helper T cells and suggests an immunoregulatory role for this T cell subset in the immunobiology of the infection.

The addition of exogenous recombinant human IL-2 to PBMC cultures restored proliferation during the unresponsive period, indicating that a lack of IL-2 production after 5 to 6 weeks of infection may be responsible for the loss of proliferation. Other immunomodulating factors, nitric oxide (NO) and transforming growth factor-beta$_1$ (TGF-β_1), were also investigated. No increase in nitrite levels was observed in antigen-stimulated culture supernatants, nor did the addition of N^G-Monomethhyl-L-arginine (L-NMMA), an inducible nitric oxide synthase (iNOS) inhibitor, restore proliferation. While there was no difference in TGF-β_1 levels in culture supernatants from infected and control animals, a significant increase in total TGF-β_1 was observed in serum samples from the one infected calf tested.

Changes in peripheral blood leukocytes and lymphocyte sub-populations were investigated by standard haematological techniques and flow cytometry. An increase in eosinophil and B cell numbers was noted soon after the primary and second challenge infections.

Antibody responses of the calves were monitored by ELISA. An early immunoglobulin (Ig) M response was followed by a strong IgG1 and weak IgG2 and IgA responses. This, taken with the elevated number of eosinophils and B cells in circulation, indicates a dominant Th2-like immune response *in vivo*. However, an early peak in IgG2 levels at 2 wpi, returning to lower levels at 4 wpi, suggests the operation of a Th1-like response early in the infection, and supports the cell and cytokine data.

The presence of antigen-specific IFN-γ prompted us to test reactive oxygen and nitrogen intermediates for their ability to kill newly excysted juvenile (NEJ) parasites under cell-free conditions and in the presence of lipopolysaccharide (LPS) and/or IFN-γ activated mouse macrophage cell line (J774). Very high concentrations of hydrogen peroxide (H_2O_2) (0.5-1 mM) were required to kill the parasite. A high concentration of -S-Nitroso-N-acetylpenicillamine (SNAP) killed 50% of the parasites, but low concentrations of each reagent had little or no effect. LPS and/or IFN-γ activated cell line, J774, killed 50 to 100% NEJs and killing was positively correlated with nitrite levels. While the addition of catalase had no effect, addition of L-NMMA reduced killing. Ultrastructural changes in NEJ parasites exposed to NO or H_2O_2 were studied by electron microscopy and NO was shown to cause mitochondrial damage.

Fluke E/S and somatic antigens were partially purified by molecular sieve chromatography using High Pressure Liquid Chromatography (HPLC) and the proliferation and cytokine (IFN-γ) responses of PBMC to each fraction were tested. All fractions induced proliferation, but to a varying degree. The results suggested that fractions containing high molecular weight E/S antigens induce higher proliferation and IFN-γ production. Two fractions of somatic antigen, stimulated high proliferation, but did not induce IFN-γ production, whilst others induced both, indicating that different fluke antigens are capable of inducing different types of immune response.

In conclusion, the results presented here suggest a Th1-like immune response early in *F.hepatica* infection in cattle, which may switch to a Th2-like immune response around patency, and that NO is capable of killing NEJ parasite *in vitro*. This study also highlights the needs for further immunological studies into vaccine candidate parasite antigens.

LIST OF ABBREVIATIONS

µg	microgram
µl	microlitre
µM	micromolar
°C	degree Celsius
bo	bovine as in boIFN-γ (bovine IFN-γ)
BSA	bovine serum albumin
CD	cluster of differentiation
Con-A	concanavalin A
cpm	count per minutes
d	distilled (water)
dd	double-distilled (water)
dl	decilitre
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
E/S	excreted and secreted (antigen)
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immunosorbent assay
EM	electron microscopy
epg	eggs per gram of faeces
et al.	et alia (and others)
FACS	fluorescence activated cell scanner/sorter
FCS	foetal calf serum
Fh12	*Fasciola hepatica* fatty acid-binding protein (12 Kilodalton)
Fig.	figure
FITC	fluorescein isothiocyanate

fl	femtolitre
g	gram
GGT	gamma-glutamyl transferase
GLDH	glutamate dehydrogenase
GPx	glutathione peroxidase
GST	glutathione-S-transferase
h	human as in rhIL-2
HPLC	high performance liquid chromatography
IFN-γ	interferon gamma
Ig	immunoglobulin
IL-	interleukin-
IL-2R	interleukin 2 Receptor
iNOS	inducible nitric oxide syntesase
Krad	Kilorad
l	litre
L-NMMA	N^G-monomethyl-L-arginine
M	molar
mA	milliampere
Mab	monoclonal antibody
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
mg	milligram
ml	millilitre
ml	millilitre
mM	millimolar
n	number of observation/s
NEJ	newly excysted juvenile (parasite)
ng	nanogram
NO	nitric oxide

OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PCV	packed cell volume
pg	picogram
r	recombinant as in rIL-2 (recombinant IL-2)
RAP-1	rhoptry-associated protein-1 (of *Babesia* spp.)
RBC	red blood cell
RNA	ribonucleic acid
RNI	reactive nitrogen intermediates
RNS	reactive nitrogen species
ROI	reactive oxygen intermediates
ROS	reactive oxygen species
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	sodium dodecyl sulphate
SI	stimulation index
SOD	superoxide dismutase
T0	tegumental granule/antigen 0
T1	tegumental granule/antigen 1
T2	tegumental granule/antigen 2
TGF-β	transforming growth factor
Th	helper T cells
TMB	3,3,5,5-tetramethylbenzidine
U	international Unit
UV	ultra violet
V	volt
WBC	white blood cell
wpi	week/s post-infection

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction:

Fasciolosis is a disease caused by digenetic trematodes of the genus *Fasciola*. The disease affects domestic animals, particularly sheep and cattle, and many other mammals including humans. It is distributed worldwide and is prevalent in both developed and developing countries. In temperate and sub-tropical areas *F. hepatica* predominates, whereas *F. gigantica* is the more common parasite in tropical regions.

In domestic animals infection with fasciolosis results in significant economic loss as a consequence of lower milk yields, stunted growth rates, emaciated carcasses, chronic low-grade anaemia, reduced wool production in sheep (Roseby, 1970; Ross, 1970; Harrison *et al.*, 1996) and, above all, livers condemned when animals are slaughtered. It can also cause death. Whilst it may be impossible to calculate precisely the economic impact of fasciolosis, estimates have been made based on the cost of livers condemned in abattoirs. Based on 73,000 cattle slaughtered in Britain in June 1942, an annual loss of over 600 tons of liver valued at about £100,000 (Peters and Clapham, 1942) was estimated. In 1972 the annual loss due to fasciolosis in the UK was estimated at £50 million (Malek, 1980). In a conservative estimate, based only on cattle slaughtered under US federal inspection in 1947 and 1950, Price (1953) suggested a figure of $3 million as the probable annual loss due to fasciolosis at 1953 prices. Undoubtedly that figure would be much higher today given the impact of inflation and the increased value of cattle livers, and taking into account animals slaughtered without formal inspection. In Peru, in 1995, the loss due to fasciolosis amounted to US$ 11 million (WHO, 1995). In 1985 the economic loss worldwide due to fasciolosis was estimated at US$ 2,000 million (Boray, 1985 cited in Spithill and Dalton, 1998).

Currently available control methods include attempts to eradicate the intermediate snail host with molluscicides and by land management, and regular chemotherapy of

mammals with anthelmintics (Roberts and Suhardono, 1996). Each control method has its limitations. The use of molluscicides involves a risk that toxic residues will accumulate and prove damaging to the surrounding fauna in the short or long-term. The use of water supplies treated with molluscicides for higher vertebrate or human consumption would be unacceptable without careful and expensive monitoring and management.

Many drugs are ineffective at the recommended dose against the immature stages of the parasite. Incomplete elimination of the parasite results in subclinical disease and allows continual contamination of pasture. Incomplete removal of immature stages of the parasite may also be fatal due to liver destruction. Triclabendazole, usually given as a single dose effective for all developmental stages of the parasite, is currently the most commonly used method of controlling fasciolosis in developed countries. However, the high cost of the drug limits its use in developing countries such as Peru. Whilst the use of this drug has strengthened optimism about the possibility of control based on chemoprophylaxis, the limitations of this type of control method remain evident. Although there is an overall reduction in the prevalence and intensity of infection soon after treatment (Torgerson and Claxton, 1999), re-infection is often rapid in endemic areas and the transmission rate is high. Mass chemotherapy is expensive, particularly for developing countries where the costs associated with the need to repeat treatments are likely to be prohibitive. Moreover, the possibility must be faced that drug resistant strains of the parasite will ultimately emerge. Resistance to triclabendazole has already been reported in sheep (Mitchell *et al.*, 1998; Overend and Bowen, 1995), suggesting that selection of resistant parasites may ultimately compromise the effectiveness of this drug.

It appears that there is a need to develop alternative, inexpensive and sustainable control methods such as vaccination. Mammalian hosts differ in their ability to acquire resistance to the parasite. Whereas sheep, mice and rabbits are susceptible and show little or no evidence of resistance to *Fasciola* infection, cattle and rats exhibit strong acquired resistance to challenge infections (Haroun and Hillyer, 1986). Cattle have been reported to acquire protective immunity following a primary infection, with self-cure

loss of worm burden occurring around 20 weeks post-infection (Doyle, 1972), and a decrease in the size, number and fecundity of flukes recovered from secondary challenge infection (Doyle, 1973b). Protective immunity can also be elicited in cattle by infection with irradiated metacercariae or drug-abbreviated infections and by immunisation with somatic fluke extract and excreted/secreted fluke antigens (Haroun and Hillyer, 1986). More recently, partial protective immunity was achieved by immunising cattle and sheep with defined fluke antigens: namely the fatty acid-binding protein (Fh12), glutathione-S-transferase (GST), cathepsin L type proteinase and haemoglobin (Spithill and Dalton, 1998). Protection was also transferred to a naïve calf by hepatic lymph node cells from its infected identical twin (Corba *et al.*, 1971).

The evidence cited above and reviewed in Section 1.2.5 indicates that cattle do develop acquired resistance to infection with *F. hepatica*, which suggests that vaccination is a real possibility and that a vaccine could make a major contribution to the control of the disease. However, the nature of that resistance, in particular T cell responses and their role in protective immunity, has not been clearly defined. Both humoral and cell-mediated immune responses appear to be important in resistance to *F. hepatica* infection. However, the inability of immune sera (unless used in very large volumes) to transfer resistance to naïve animals (Armour and Dargie, 1974) and the lack of correlation between antibody titre and protection levels in vaccine trials (Creaney *et al.*, 1995; Dalton *et al.*, 1996; Morrison *et al.*, 1996) suggest that antibodies alone are not sufficient for protective immunity. T cells and cell-mediated immune responses may play a crucial role in resistance to infection with *F. hepatica*. Therefore, this study examines the cytokine and cellular immune responses of cattle experimentally infected with *F. hepatica*.

1.2 Literature Review:

1.2.1 Life Cycle of *Fasciola* spp.:

The parasite has an indirect life cycle in which snail species of the genus *Lymnaea* are the intermediate host and many mammals, including domesticated animals and man, act as final hosts.

The parasite infects the liver of the mammalian host, causing serious damage. It matures two to three months after infection and the adult fluke dwells in the bile ducts where it produces eggs which are carried down into the intestine in the bile. The egg, which is excreted in the faeces, is unembryonated, ovoid, operculate, relatively big, about 63-90 μm X 130-150 μm, and characteristically yellowish-brown in colour. The development of the egg takes place outside the host for about 10 to 15 days under optimum temperature (23 to 25°C) and moisture conditions. The egg is resistant and remains viable for a long time, but is sensitive to, and killed by desiccation or by temperatures below –4°C or above 37°C (Andrews, 1999). The embryonated egg contains a miracidium, which is the infective form of the parasite for the intermediate snail host. Once the eggs are embryonated, the miracidia are ready to hatch with the stimulatory help of sunlight in the natural environment. It has been suggested that light stimulates the miracidium to release a hatching enzyme which digests the cement substance binding down the operculum (Rowan, 1956). The released miracidia, which have a very short lifespan, swim rapidly in the water, searching for a suitable snail host to invade. The development of the parasite in the snail host takes from around 1 to 2.5 months depending upon environmental conditions, particularly temperature. Development halts if temperatures fall below 10°C. The next developmental stage of the parasite, the sporocyst, is found near the site of penetration and develops into rediae which migrate to the distal area of the snail. Within the rediae, the subsequent developmental stage, cercariae - or under certain environmental conditions, a second generation of rediae (daughter rediae) - develop. The cercariae leave the snail and encyst rapidly on the aquatic vegetation, or – much less commonly – encyst freely in the water. The encysted

cercariae, or metacercariae, are resistant and remain viable for up to 6 months at a temperature of 12-14°C (Boray, 1969). The metacercariae are reported to be resistant to freezing up to –10°C, but lose their infectivity at –20°C (Boray and Enigk, 1964).

The mammalian host is infected by consuming aquatic vegetation on which metacercariae encyst or by drinking contaminated water. The metacercariae, soon after being ingested, excyst in the small intestine, penetrate the intestinal wall and migrate through the peritoneal cavity to reach the liver capsule. On arrival, young flukes penetrate the liver capsule and are soon found embedded deep in the parenchyma (Dawes and Hughes, 1964; Boray, 1969). During the migration though the liver parenchyma to the final destination - the bile duct - which takes about 50 to 70 days depending on the host species infected, the young flukes mature. Mature flukes can survive in the bile ducts for several years in some host species, e.g. sheep.

1.2.2 Epidemiology:

The prevalence and incidence of fasciolosis is determined by the interrelationship between the following factors - climate, temperature, humidity, soil composition, water supply and aquatic flora – in a local environment colonised by appropriate snail intermediate hosts. The long lifespan of the fluke in a wide range of mammalian hosts contributes to the maintenance of the infection.

Temperature, relative humidity and an adequate supply of water appear to be the key factors for snail development and for maintenance of the life cycle of the parasite. Although fluke eggs are viable for at least 2 years at 4°C (Boray, 1969), development of egg and parasitic stages within the snail host stops at below 10°C. Above 10°C, the rate of development increases with temperature to an optimum level of around 20°C (Torgerson and Claxton, 1999). A plentiful supply of water is not only vitally necessary for development of snails and for their successful colonisation of the environment, but also for parasite development inside the egg. Rowcliffe and Ollerenshaw (1960) reported three factors critical in the hatching of *F. hepatica* eggs. First, the eggs must become separated from the faeces; on no occasion in their

experiments did any eggs hatch in the presence of faeces. Second, development of the parasite inside the egg is temperature dependent. Third, throughout the entire period from the deposition of the egg in the faeces to the hatching of the miracidium, the egg must be surrounded by a surface film of moisture. Desiccation is lethal for the eggs. It therefore appears that the deposition of faeces in water or heavy rain flushing off the faeces, liberating the eggs and carrying them to pools create the most favourable environment for the development of the parasite within the egg. Furthermore, water is also an absolute requirement if emerging miracidia and cercariae are to swim to snails (Wilson and Taylor, 1978) and aquatic vegetation respectively.

The optimum conditions for development of the parasitic stages outside the mammalian host are also the most suitable for the population dynamics of the snail intermediate hosts and for fluke multiplication within the snail (Kendall, 1953; Over, 1982). With the exception of *Lymnaea columella* in North and South America, the majority of the snail hosts responsible for the transmission of *F. hepatica* are amphibious and primarily mud dwellers preferring slightly acidic and saturated soils. Irrigation ditches, badly drained pasture, springs and water meadows are typical habitats of the snails. The ideal temperature for breeding and development of the snails is 18°C. Below 10°C, snail activity is insignificant (Armour, 1975). In Britain, snail propagation appears only to occur from spring to autumn. In the cold winters, the snail hibernates until the following spring. The number of snail generations per year varies according to the regional climate. Whereas in the west of Scotland one or one and a half generations occur within a year, in southern Britain at least two completed generations per year are reported (Armour, 1975).

Resistance of the metacercariae of both *F. hepatica* and *F.gigantica* to desiccation, on herbage and on hay, has been known for some time in several parts of the world. Detailed information comes from the experimental work of Boray and Enigk (1964) who exposed cysts to different temperatures and relative humidities in controlled climate chambers. The authors found that *F. gigantica* metacercariae survived for longer periods at high temperatures and were more susceptible to desiccation than *F. hepatica* metacercariae. This may indicate why *F. gigantica* is a parasite of tropical

areas with aquatic environments and snails for its transmission. In contrast, *F. hepatica* is a temperate climate parasite surviving all the usual winter conditions in Europe, northern USA and Australia. Metacercariae of both fluke species were resistant to freezing up to –10°C but lost their infectivity at –20°C (Boray and Enigk, 1964).

Ollerenshaw (1959) described two annual cycles of infection in snails in England, based on detailed investigations into the ecology of *L. truncatula*, the seasonal prevalence of fluke infection in livestock, abattoir surveys and analysis of climatic data. The first cycle involves the infection of snails in summer with miracidia from eggs deposited on the pasture in spring by fluke infected animals. This infection in the snail, which is designated as the summer infection takes at least five weeks to mature, and the fresh metacercariae appear on the herbage from mid-August. Ingestion of these metacercariae by the susceptible herbivorous animals takes place any time after mid-August and the clinical fasciolosis occurs from October through to March. If not ingested, a considerable proportion of the metacercariae can survive through the winter and are capable of causing infection in the following spring. In the second cycle, snails are infected with miracidia from eggs excreted in late summer. The infection of snails takes place throughout the autumn. The development of the fluke within the snail stops during the winter hibernation of the snail but resumes in the spring and metacercariae appear on the pasture by mid-summer. This is called the winter infection of the snail and is considered to be of minor importance in most regions of Britain except the southwest (Armour, 1975), probably due to the selective mortality of the infected snails during winter. Nonetheless, it contributes to the maintenance of the life cycle.

With the correlation established between climate and the development both of the parasitic larval stages and of the snail host, the theoretical possibility that the prevalence and incidence of fasciolosis might be forecast based on climate became a real prospect in endemic areas. Ollerenshaw (1966) proposed a method of forecasting the prevalence of fasciolosis in any particular year based on climate, rainfall and mean temperature levels. The broad principals of this forecasting method were originally established using climatic data and records of the prevalence of acute disease in sheep collected over the

ten years between 1947 and 1957 in Anglesey, Britain (Ollerenshaw and Rowlands, 1959).

1.2.3 Control:

The available control methods can broadly be divided into three main categories:

1. control or eradication of the snail intermediate host
2. reduction or eradication of the adult parasite in domesticated animals by the strategic use of flukicides to reduce the contamination of pasture with fluke eggs
3. reduction of the chances of infection by animal husbandry management.

Currently the most effective approach to the control of fasciolosis is by integrating the available control measures.

It is possible to control the snail host to a certain degree with environmental, chemical, or biological methods. The best long-term approach to fluke control via reduction or eradication of the amphibian snail hosts of *F. hepatica* is probably to use habitat management measures, for instance the drainage and filling up of swampy areas. However, the introduction or reconstruction of a farm drainage system is expensive and may take several years to complete (Armour, 1975; Roberts and Suhardono, 1996).

An alternative method of snail control is the use of chemical molluscicides. Among the molluscicides tested against the snail hosts of *Fasciola* spp. are sodium pentachlorophenate (NaPCP), niclosamide (5,2'-dicloro-4'-nitrosalicylanilide), Frescon (N-trityloropholine), and copper sulphate (Torgerson and Claxton, 1999). The susceptibility of individual snail species to a particular chemical compound appears to vary, indicating that surveys may be required in order to determine the most suitable molluscicide for the local snail hosts. Snail re-population after treatment is generally very rapid under optimum climatic conditions (Ollerenshaw, 1971). Therefore, a second respraying is required in summer. However, this may be less effective because the proliferation of herbage at this time of the year impedes contact between the molluscicide and the snail (Roberts and Suhardono, 1996). Moreover, in an

environment where snail development occurs throughout the year, repeated applications of molluscicide may be needed. No molluscicides are licensed for use in the UK.

Although rarely applied, alternative natural snail control measures such as the plantation of plants with molluscicidic effect or the introduction of potential snail competitors such as *Marisa cornuarietis* and predators such as crustaceans, birds, fish, geese and ducks into snail habitats have been proposed (Samson and Wilson, 1973; Roberts and Suhardono, 1996). The leaves of the Eucalyptus species have been reported to have molluscicidal properties and their intermittent fall could effect self delivery if the trees were planted in appropriate places (Hammond *et al.*, 1994).

Reduction or eradication of *Fasciola* in the mammalian host by chemo-therapeutic/prophylactic treatment of infected animals is theoretically an effective method of control by interrupting the life cycle of the parasite, with the added advantage that it improves the general health of the affected domesticated animals. However, the success of this method of control depends primarily on the efficacy of the anthelmintics used and may be limited by their capacity to act against only certain stages of parasite development.

Many of the available flukicides - nitroxynil, rafoxanide, albendazole and hexachlorophene - are effective against either mature or late immature stages of the parasite but not against juvenile parasites (Johns and Dickeson, 1979; Torgerson and Claxton, 1999). The use of these drugs in lactating animals involves the added disadvantage that unacceptable drug residues remain in milk, with a consequent legal requirement that for a specified time after treatment of the animal, its milk must be discharged before the yield is again fit for human consumption. Oxyclozanide, on the other hand, is safe to use in lactating animals but is effective only against the adult parasite. The major disadvantage of using anthelmintics effective only against adult parasites is that surviving juvenile parasites eventually become adults and start excreting eggs to contaminate the pasture. Consequently, a second, and in some cases a third, treatment at two month intervals may be required to eliminate surviving juvenile parasites, escalating the cost of control.

More recently, the introduction onto the market of a new flukicide, triclabendazole, answered some of the questions surrounding the efficacy and safety of treatment based control. However, emerging evidence of a drug resistant fluke population (Mitchell *et al.*, 1998; Overend and Bowen, 1995) and the long withdrawal period required limit its use.

Where possible, one of the most effective control measures against fasciolosis is the reduction of the chances of contracting the infection by animal husbandry. Wet, swampy pasture must be avoided and therefore must be drained where practicable (Armour, 1975). In areas where *F. gigantica* is endemic, acquisition of the infection by animals and the contamination of snail habitats with fluke eggs take place when the animals are watered, rather then when they are grazing on pasture. It has therefore been recommended that avoiding the watering of animals at swampy riverbeds and at waterholes rich in vegetation will considerably reduce the chances of infection (Malek, 1980). However, difficulties are presented in the case of *F. hepatica,* with its amphibious snail intermediate host. Nonetheless, fencing particularly wet areas or avoiding such pasture in autumn and winter is recommended in Britain in years when the Ministry of Agriculture forecasts a high prevalence of fasciolosis (Armour, 1975).

1.2.4 Pathogenesis, Pathology and Clinical Signs:

As the newly excysted parasites penetrate the intestinal wall they cause some damage, but more traumatic and necrotic lesions are produced in the liver parenchyma by migrating immature parasites and later in the bile ducts by the adults. The severity of gross pathology depends primarily on the number of invading parasites and on the host species infected (Boray, 1969).

Probably due to their relatively small size, penetration of newly excysted juvenile (NEJ) parasites through the intestinal wall is not generally associated with clinical signs and gross pathological changes are minimal. More pronounced gross pathological changes are associated with the migrating flukes and are characterised by traumatic hepatitis and fibrosis. The migrating flukes leave tracks, filled with fluke

metabolic waste, blood and necrotic hepatocytes, in the surrounding liver parenchyma, which are then infiltrated by lymphocytes, macrophages, eosinophils and neutrophils. During this stage of infection, due to heavy destruction of parenchymal cells, liver enzymes such as lactate dehydrogenase and glutamate dehydrogenase (GLDH) are released, with a consequent rise in their levels in serum (Anderson *et al.*, 1977; 1981). The latter is widely accepted to be a useful indicator for the diagnosis of the acute phases of the infection. Cattle are generally tolerant of the pathological consequences of the infection. Clinical disease is only seen if the animals are infected with in excess of 1000 metacercariae (Boray, 1969). Typically, haemorrhagic anaemia is seen if the diet of the infected animals is low in protein. Eosinophilia, hypoalbuminaemia and hypergammaglobulinaemia are characteristic in fasciolosis (Anderson *et al.*, 1977, 1981; Behm and Sangster, 1999).

When the parasites enter the bile ducts, the pathology of the disease changes and is characterised by dilated and thickened bile ducts. The size and spiny tegument of the parasite and its blood sucking habit contribute to damage in the bile duct epithelium. However, an increase in proline levels in infected animals' bile has been postulated to be responsible for the hyperplasia of the bile duct (Isseroff *et al.*, 1972; 1977). Due to the destruction of bile duct epithelium, there are increased levels in the serum of gamma-glutamyl transferase (GGT), which is considered to be the enzyme most indicative of the chronic intrabiliary phase of the infection (Anderson *et al.*, 1977).

1.2.5 Resistance to *Fasciola hepatica*:

1.2.5.1 Innate Resistance:

There is a fine distinction between innate resistance to an infectious agent and rapid and effective acquired immunity. The innate immune system includes non-specific effector mechanisms, such as the complement system, macrophages, natural killer cells and probably $\gamma\delta^+$ T cells, and is considered to be the first line of defence against invading organisms. The efficacy of this system is genetically determined, and in many cases, inextricably linked with the development of an adaptive immune response. In

fasciolosis, as in many other parasitic diseases, little is known about the factors which control the host parasite relationship. The susceptibility of different host species to infection with *F. hepatica* varies. While cattle and rats are resistant to the infection, sheep, goats, rabbits and mice are susceptible. It is not known whether this difference is due to the genetically determined innate ability of some species to resist the infection or the inability of others to develop an effective and rapid adaptive immune response. This variation has not yet been thoroughly studied and identification of the mechanism operating the resistance remains a matter of dispute in the immunology of *F. hepatica* infection. In addition, the fact that rats are found to be highly resistant to infection in some laboratories whereas in others resistance is less marked indicates that the strain of rat and level of challenge may be very important. Moreover, susceptibility to a primary infection and the ability to eliminate it varies according to age, sex and the strain of rat (Hughes *et al.*, 1976), although the genetic basis of this resistance has not been examined with reference to *F. hepatica* (Hughes, 1987).

1.2.5.2 Acquired Resistance:

The acquired immune response to *F. hepatica* has been examined in a variety of mammalian hosts. Several species of laboratory animal (Lang, 1967; Hayes *et al.*, 1974a and b) and domesticated animal (Ross, 1967; Doyle, 1971; 1972; Flagstad *et al.*, 1972) have been subjected to an investigation in which the humoral immune response has been measured by examining sera for the presence of parasite-specific antibodies and for the ability of these animals to resist infection, and by monitoring the cell-mediated responses following experimental or natural infection with *F. hepatica*. The experiments have also been extended to define the different body cavities and organs in which the protective immune response operates, by infecting animals with different parasite stages and by different routes. There have also been a number of studies concerned with attempting to immunise animals with attenuated metacercariae and with antigenic material obtained from parasites (Hughes, 1987; Spithill and Dalton, 1998).

1.2.5.2.1 Mice: Although acquired resistance to reinfection with *F. hepatica* in mice following primary infection (Lang, 1967); or immunisation with excreted/secreted products and somatic fluke extract (Lang and Hall, 1977); or the transfer of immune

peritoneal exudate cells from fluke infected mice to naive mice (Lang *et al.*, 1967) has been reported, Chapman and Mitchell (1982) failed to demonstrate this resistance. As a consequence, the mouse is regarded by many authors as a host susceptible to infection with *F. hepatica* which does not develop acquired resistance. Studies to demonstrate resistance in this animal model are probably hampered primarily by the fact that the mouse is extremely susceptible to the pathological consequences of the infection and is killed by 2 to 5 metacercariae. When such small numbers of metacercariae are given, it is difficult to demonstrate a significant immune response by comparing the number of flukes in experimental and control groups.

1.2.5.2.2 Rabbits: Experiments to demonstrate acquired resistance to *F. hepatica* in rabbits following immunisation with X-irradiated metacercariae, drug-abbreviated primary infections or fluke antigens were generally unsuccessful (Hughes, 1987; Haroun and Hillyer, 1986). However, after injecting fluke antigen extract into the hepatic portal vein of rabbits sensitised by a primary infection, Sinclair and Joyner (1974) found a 50% reduction in the subsequent challenge infection. They concluded that an anaphylactic type response in the liver might have been responsible for the reduction.

1.2.5.2.3 Sheep: Sinclair (1967) reported that there was no evidence to suggest that sheep or goats are able to acquire immunity against *F. hepatica.* Durbin (1952) claimed that infection could persist for at least 11 years in experimentally infected sheep. Attempts to produce an acquired resistance to challenge infection in sheep following a primary infection with normal metacercariae (Boray, 1969), X-irradiated attenuated metacercariae or with fluke homogenate antigens (Hughes, 1987) have all been unsuccessful. However, very promising results have recently been obtained by immunising sheep with fluke derived cysteine proteinase (Wijffels *et al.*, 1994) and glutathione-s- transferase (Sexton *et al.*, 1990).

1.2.5.2.4 Rats: The ability of rats to develop acquired resistance against *F. hepatica* infection was demonstrated with a reduction of over 90% of the challenge exposure by Hayes *et al.* (1972). This resistance to challenge infection with *F. hepatica* was

subsequently confirmed following primary sensitising infections with normal (Hayes *et al.*, 1973) or irradiated metacercariae (Corba *et al.*, 1971; Armour and Dargie, 1974). Hayes *et al.* (1974a, b) suggested that 3 to 4 weeks after a primary infection with *F. hepatica* in rats, the protective response to the challenge infection is expressed. However, in their experiment to transfer resistance by immune lymphoid cells, Corba *et al.* (1971) demonstrated that full expression of protective immunity in rats would take more than 4 weeks of exposure to mature. Although transfer of the resistance with lymphoid cells from donor rats 8 to 10 weeks after infection was successful (Corba *et al.*, 1971; Armour and Dargie, 1974), cells taken 4 weeks after infection failed to transfer protection (Corba *et al.*, 1971). The conclusion drawn from these experiments, that the adult fluke is more immunogenic than the juvenile fluke, is doubtful since a high level of protection can be obtained by transferring cells from rats immunised with irradiated metacercariae which do not survive in the host for more then 3 to 4 weeks. The adaptive transfer of lymphoid cells from donors with high parasite burdens (7-8 flukes) also confers significantly more protection to recipients than do cells taken from rats harbouring few flukes (Armour and Dargie, 1974). It therefore appears that the quantity and the duration of the antigenic stimulus are important factors for the full development of immunity.

Further doubt is cast on the conclusion drawn by Armour and Dargie by the work of Hayes *et al.*(1974a and b), who found that rats infected for a short time (2 to 3 weeks) are resistant to challenge by the oral or peritoneal route. This resistance declines over time and after about 6 months the rats are no longer resistant. However, resistance is restored within 2 weeks of reinfection (Hughes *et al.*, 1977). The resistance was also measured by challenging with live adult flukes placed in the body cavity. The resistant rats killed the transplanted flukes within 24 hours (Goose and McGregor, 1973; Bennett *et al.*, 1980). Resistance in the rat does not depend on the presence of the primary infection at the time of challenge, and operates against intraperitoneally-implanted metacercariae, juvenile or adult flukes (Hughes, 1987).

1.2.5.2.5 Cattle: Ross (1965a, b) found that when large numbers (1500-2500) of metacercariae were fed to calves very few flukes reached the bile duct stage; the

majority were trapped and eliminated by tissue reaction during migration through the liver. Cattle are also reported to eliminate an initial infection from the bile ducts and have been found to be resistant to reinfection (Boray, 1969; Doyle, 1971; 1972). This parasite rejection starts at about 20 weeks after infection and occurs when homocytotropic antibodies reach peak levels (Doyle, 1973a) and at about the same time that calcification of the bile ducts develops (Hughes 1987). Acquired resistance in cattle and rats has, therefore, been attributed partly to fibrotic changes induced in the liver and bile ducts during the primary infection, which arrest the migrating juvenile flukes following a challenge infection (Boray, 1969; Ross, 1967; Hughes, 1987). However, there is also evidence to suggest that this resistance is mediated by humoral and cellular immune mechanisms. Eriksen and Flagstad, (1974) reported a 50% reduction of flukes in rats following the surgical transfer of adult flukes to the subcutis when compared to naive rats. Since the liver was intact, no anatomical changes such as fibrosis, which might otherwise have been responsible for the resistance to infection, were observed. It has also been demonstrated that dexamethasone abrogated the protective effect in rats of a previous infection (Hayes and Mitrovic, 1977; Baeza *et al.*, 1994a,b). In addition, protection was transferred to a naive calf by hepatic lymph node cells from its infected identical (monozygotic) twin. The recipient twin showed 79.7% protection to oral challenge with metacercariae (Corba *et al.*, 1971). Although these experiments were carried out with only with one pair of monozygotic twins, they nevertheless support the view that such resistance can be induced without previous liver damage.

Doy and Hughes, (1984b) were able to demonstrate 56% and 94% protection against reinfection 18 or 26 weeks after a primary infection in cattle. In this experiment, only a slight increase in the plasma glutamate dehydrogenase (GLDH) was observed in the resistant calves after challenge, indicating little or no damage in the liver due to migratory flukes from the challenge infection. However, there was no significant difference in the number of flukes recovered from the body cavity of sensitised or naïve control calves at 4 or 14 days after challenge infection. The authors suggested that the liver capsule or parenchyma are the sites at which resistance mechanisms are operating and the invading parasites are killed. Physical barriers may therefore be involved in this resistance (Doy and Hughes, 1984a).

Attempts to immunise cattle against *F. hepatica* using attenuated metacercariae with γ- or x- irradiation produced variable but promising results. Although Boray (1969) was unable to demonstrate any resistance in three calves immunised with 3000 x-irradiated (20 Kilorad-Krad) *F. hepatica* metacercariae on three occasions, significant resistance to homologous challenge infections has been reported in cattle immunised with γ-irradiated metacercariae of either *F. hepatica* (Armour *et al.*, 1974) or *F. gigantica* (Bitakaramire, 1973) [both cited in Haroun and Hillyer, 1986].

Although earlier attempts to immunise cattle with adult fluke somatic extracts were unsuccessful (Ross, 1967b), Hall and Lang, 1978 (cited by Haroun and Hillyer, 1986) reported highly significant resistance (over 90%) to challenge in cattle immunised by a subcutaneous injection of 16 day old somatic fluke homogenate. More recently, partial protective immunity was achieved by immunising cattle with defined fluke antigens: namely the fatty acid-binding protein (Fh12), glutathione-S-transferase (GST), cathepsin L type proteinase and haemoglobin (Spithill and Dalton, 1998).

1.2.5.3 The Mechanisms and Site of Resistance:

The intestinal lumen and the intestinal wall in rats pre-sensitised with a primary infection appear to be the first sites in which acquired resistance against newly excysted juvenile parasites operates. Flukes from the challenge infection were originally reported to be killed during or before their entry into the body cavity (Hayes and Mitrovic, 1977; Rajasekariah & Howell, 1977; Doy *et al.*, 1978;). Burden *et al.* (1983) later reported that the newly excysted juvenile flukes within the gut lumen were coated with antibodies and that between 20 and 30% were unable to penetrate the intestinal wall of resistant rats. Since there was no apparent damage to the gut or tegument of the flukes which failed to penetrate the intestinal wall, the role of antibodies at this level is not fully understood. Mucous production inside the gut lumen may cause mechanical obstruction.

A study on athymic rats (Doy and Hughes, 1982a) revealed that there are two distinct mechanisms of resistance to reinfection with *F. hepatica*: a thymus-independent

resistance which operates in the gut, and a thymus-dependent resistance which operates in the peritoneal cavity. The authors challenged previously sensitised athymic nude rats and their normal heterozygous littermates either orally with metacercariae or directly into the body cavity with newly excysted juvenile flukes. No flukes were recovered from the normal rats, challenged by either route. In contrast, there was no significant reduction in the numbers of fluke recovered from nude rats challenged intraperitoneally, whereas in nude rats challenged orally the fluke burden was effectively reduced (95%). The conclusion drawn here, therefore, is that the mechanism in operation at the gut wall would appear to be thymus-independent and in the body cavity to be T cell-dependent. It is suggested that the T-independent mechanism operating at the level of the gut lumen and/or wall is probably non-specific, because the resistance can be stimulated by the unrelated gut dwelling nematode parasite, *Nippostrongylus brasiliensis* (Doy *et al.*, 1981). However, it is still not clear whether this non-specific resistance is immunological or due to a mechanical barrier and inflammatory changes induced by the infection with *N. brasiliensis*. The occurrence of a non-specific resistance to *F. hepatica* in sheep, after infection with the unrelated parasite *Cysticercus tenuicollis*, has also been speculated upon (Campbell *et al.*, 1977), but Hughes *et al.*, (1978) were unable to confirm this in goats, sheep or cattle.

The role of the intestinal wall in resistance to fluke infection is rather confusing. Hayes and Mitrovic, (1977) and later Hayes, (1978) reported a marked reduction (84-99%) in fluke recovery from the peritoneal cavity of previously immunised rats after 24 or 48 hours of challenge infection. Gross pathological changes in the upper small intestines of the pre-sensitised rats were much more marked than in those rats which only received the challenge infection, indicating that intestinal mucosa is one of the first barriers of resistance to the parasite. Treatment of the rats with the dexamethasone abrogated the protective effect of a previous infection, indicating that a inflammatory response may be responsible for killing the parasite during its migration through the intestinal wall or in the peritoneum. However, a question is raised as to whether the reduced number of challenging flukes collected in the peritoneum was actually due to fact that migrating flukes were immobilised or killed at gut level or whether, as is the case in mice (Harness *et al.*, 1977), there was an accelerated migration of flukes in immune rats so

that by 24 or 48 hours after challenge all the flukes had already crossed the peritoneum and were embedded into the liver. However, when rats with a primary sensitising infection were challenged with newly excysted juvenile parasites given by the peritoneal route they showed little resistance and had considerable damage in their livers, whereas challenge given by the oral route resulted in significant resistance and no liver damage (Rajasekariah & Howell, 1977). Recent studies carried out by van Milligen *et al.* (1998a, b, c) highlighted the importance of intestinal wall as the first and effective immune barrier against *F. hepatica*. Using *ex vivo* gut segments, these authors showed that 77% of the challenging NEJ parasites were unable to cross the intestinal wall of previously infected rats in comparison to naïve rats and that it takes at least two weeks of exposure to primary infection to induce protective immunity at the gut level. They also showed that four weeks after an oral primary infection, frequencies of IgE positive cells, eosinophils and mast cells were significantly increased in the lamina propria. NEJs quickly become coated with IgG1 and IgG2a but not IgE or IgA antibodies and surrounded by eosinophils as they migrated through the submucosa of immune rats (van Milligen *et al.*, 1998c).

Burden *et al.* (1983), on the other hand, using transmission electron microscopy, observed that parasites penetrating the intestinal wall of the previously infected rats were unharmed and, unlike those found in the gut lumen, were free of antibody/antigen complexes (precipitates) on their tegumental surface. Although large numbers of eosinophils and neutrophils were present in the gut wall and around the penetrating flukes, their adherence and degranulation were not evident. The authors suggested two possible explanations for the fact that antibody/antigen precipitates were present on the tegumental surface of the flukes in the gut lumen, but absent from those in the intestinal wall. One is that the precipitate may have been mechanically wiped off the flukes as they forced their way through the tissue of the gut wall. The second is that the flukes shed antibody-bound tegument by the tegumental surface-turnover mechanisms described by Hanna (1980a and b) and Duffus and Franks (1981). However, it is now known that the parasite excretes and secretes proteases in order to penetrate the host tissues and for feeding. Among those proteases, cathepsin L type cysteine proteases cleave immunoglobulins at their hinge regions. However, it is not known whether

flukes migrating through the intestinal wall secrete proteases that proteolytically degrade the antibodies attached to their surface and result in the loss of bound antibody. The attachment and degranulation of rat granulocytes to flukes has been shown to be antibody dependent (Doy *et al.*, 1980). Therefore, the lack of attachment of host granulocytes to flukes seen in the gut wall probably reflects the absence of antibody bound to the tegument.

Those flukes that cross the intestinal wall arrive at the serosal surface of the gut, but soon after are coated with antibodies and host cells including eosinophils, neutrophils, macrophages and mast cells in the peritoneal cavity. The peritoneal cavity therefore appears to serve as the second site of defence against the parasite. The majority of flukes found in the body cavity of sensitised rats were coated with antibodies and host cells including eosinophils which were seen degranulating onto the fluke surface resulting in the erosion of the tegumental syncytium (Burden *et al.*, 1983).

A similar result was obtained in mice in which at 48 hrs after challenge 35% to 59% fewer *F. hepatica* were recovered from the peritoneal cavities of animals immunised with irradiated metacercariae than from unimmunised control mice (Harness *et al.*, 1976).

Bile duct: Hayes *et al.* (1972) noted that in the bile ducts of rats adult flukes from an initial infection were not affected by the development of immunity which destroyed the challenge infection by 90 per cent. Recent studies by Keegan and Trudgett (1992 and Poitou *et al.* (1992) support this early observation and indicate that the bile ducts are an immunologically privileged site for flukes. Hughes *et al.* (1981), were also unable to demonstrate antibodies to T2 antigens (granules) in cattle bile.

1.2.6 Humoral and Cellular Immune Responses:

The role of humoral antibodies in protection to fasciolosis has not been clearly illustrated. Although circulating *F. hepatica* antigen-specific antibodies can be demonstrated in experimentally and naturally infected animals using various serological

tests, the transfer of serum to uninfected animals failed to confer any protection (Corba *et al*, 1971). However, using larger volumes of heterologous (20 ml) and homologous (10 ml) immune sera, Armour and Dargie (1974) have succeeded in the transfer of resistance in rats.

There is a strong antibody response to fluke infection in all host species tested. Antibodies were detectable within 2 weeks following infection in rats, reaching peak levels at around 5 weeks and gradually declining to lower levels after infection reached patency (Oldham, 1985; Keegan and Trudgett, 1992; Poitou *et al.*, 1992). Studies to determine antibody isotype responses revealed that all antibody isotypes (IgM, IgE, IgG1 and IgG2) were produced during the course of *F. hepatica* infection in rats (Pfister *et al.*, 1983; Poitou *et al.*, 1992, 1993b; van Milligen *et al.*, 1998c). Pfister *et al.* (1983) showed that there was a biphasic antigen-specific IgE response in rats: the first peak coincided with the liver migration stages and the second with the flukes becoming established in the bile ducts.

Antibody responses of experimentally infected calves are detectable as early as two weeks after infection reaching peak levels at around week 9 post-infection (Santiago and Hillyer, 1988; Ortiz-Oblitas, 1997). All antibody subclasses are produced during the course of infection, with IgG1 predominant (Duffus and Franks, 1981; Clery *et al.*, 1996). Despite the presence of antigen-specific circulating antibodies, their role in protective immunity to *F. hepatica* is still the subject of controversy. There is no correlation between antibody titres and the number of flukes recovered following experimental infections (Kendall *et al.*, 1978) and in vaccinated cattle (Spithill and Dalton, 1998). However, antibody-mediated attachment of peritoneal cells (mainly eosinophils) to newly excysted juvenile parasites has been shown (Goose, 1978).

Various attempts have been made to demonstrate the mechanisms of killing. Although the adherence of eosinophils and neutrophils to flukes *in vitro* has been demonstrated (Doy *et al.*, 1980; Duffus and Franks, 1980), neither fluke killing nor tegumental damage were detected. However, Duffus *et al.* (1980) demonstrated the killing of newly excysted juvenile parasites *in vitro*, using major basic proteins from bovine eosinophils.

Davies and Goose (1981) also observed that NEJ parasites were coated by peritoneal cells and rapidly killed when placed in body cavity of previously infected rats.

There have been few attempts to define cellular immune responses in *F. hepatica* infected animals. PBMC collected within 5 weeks of an experimental *F. hepatica* infection in cattle proliferated in response to fluke antigens *in vitro*, but no proliferative response was detectable 5 weeks after a primary infection (Oldham, 1985; Oldham and Williams, 1985). *F. hepatica* somatic homogenate antigen-specific helper T cell clones generated from chronically infected cows expressed either a Th2 or a Th0 cytokine profile (Brown *et al.*, 1994a). In addition, Clery *et al.* (1996) demonstrated a dominant IgG1 isotype response and undetectable levels of IFN-γ in supernatant from fluke antigen stimulated PBMC cultures in naturally exposed cattle following an experimental reinfection with *F. hepatica* metacercariae. In contrast, Clery and Mulcahy (1998) reported IFN-γ production by PBMC from cattle early in primary infection.

1.2.7 Evasion Mechanisms:

1.2.7.1 Host Antigens:

It has been well documented that the blood fluke, *Schistosoma* spp., acquires host antigens to escape or avoid the attack by the host immune system. However, there is no evidence to suggest the acquisition of host antigens in *F. hepatica*. Hughes and Harness (1973a, b) transferred either 18-day old or adult flukes into recipient animals previously immunised against the donor's blood cells. They found that the immunisation had no effect on the parasite. Cattle flukes have been reported to synthesise A, H and Lewis blood group antigens by Ben-Ismail *et al.*, (1982) who suggested that these antigens could be used as surface markers because of their defined nature in immunological and biochemical terms.

1.2.7.2 Replacement and Turnover of Outer Coat or Glycocalyx:

The tegumental surface of the parasite is highly antigenic and is important in the host-parasite relationship. The tegumental surface or glycocalyx is formed by regular and sequential expression of various granules, named T0, T1, T2, and each one appears to be dominant at different developmental stages of the parasite (Bennett and Threadgold, 1973, 1975). The glycocalyx of metacercariae and newly excysted juveniles is derived from T0 type granule producing tegumental cells, which transform into T1 type tegumental cells soon after the parasites penetrate the liver. After this stage, T1 type granules gradually take over from the T0 type and become prominent in the tegument while T2 type tegumental cells are being formed. Prior to entry into bile ducts T2 type granules take over the function of glycocalyx synthesis. This sequential change of the antigenically distinct T1 to T2 granules is reflected in the changes in the antibody responses of the host. The profiles of specific serum antibodies to these sequentially expressed antigens have been described using the indirect fluorescent antibody labelling test (Hanna, 1980a). Antibodies to T0 and T1 antigens were detected first, peaking 3 to 5 weeks after infection after which antibodies to T2 were detected while antibodies to T0 and T1 antigens were declining. In addition, the glycocalyx is continuously shed and replaced by the secretary granules (Hanna, 1980b). Thus, it has been suggested that the ability of the parasite to replace its coat regularly and to turnover its tegumental glycocalyx from T1 to T2, which are antigenically distinct, protects the parasite from the attachment of both antibody and cells, or at least allows attachment for a short time only, therefore preventing any damage to the young fluke (Hanna, 1980b; Duffus and Franks, 1981).

The demonstration of the relationship between antibody response and granule expression has been clarified using infections terminated strategically with anthelmintics (Hanna *et al.*, 1982) and flukes attenuated with irradiation (Hughes, 1987).

The fact that rats which have been sensitised for only 2 to 3 weeks can kill adult flukes placed intraperitoneally would be explained by the fact that adult flukes do express T1

antigens, although to a lesser extent than their predominant T2 antigens. In addition, rats infected with metacercariae attenuated at 4 Krad of γ-irradiation had antibodies to T1 antigens which have been shown not to be expressed by flukes developing from metacercariae attenuated with 4 Krad γ-irradiation (Burden *et al.*, 1983). It was subsequently demonstrated that T0 and T1 contain some similar antigenic determinants using monoclonal antibodies (Hanna and Trudgett, 1983). Although the granule antigens elicit a good humoral and perhaps cellular immune response, and have been considered to be potential candidate antigens for vaccination, their role in host protection needs to be clarified.

1.2.7.3 Sequestered Site:

The fact that whereas adult flukes implanted into the peritoneal cavity of sensitised, but not control, rats are killed, yet adult flukes can persist for a long time in the bile duct in an apparently healthy condition, indicates that the bile duct may serve as an immunologically privileged site for the adult flukes dwelling there.

Although there is also evidence that both cattle and rats eliminate flukes from the bile ducts, no such fluke loss is encountered in sheep and goats. To date, there is no evidence to suggest that the rejection of adult flukes from the bile duct is operated by immunological means. Rather, it has been suggested to be a consequence of nutritional problems for the fluke due to calcified, fibrotic bile ducts in the case of cattle and crowded conditions in the case of rats (Hughes, 1987). Hughes *et al.*, (1981) also reported the lack of antibodies to T2 antigens in cattle bile.

1.2.7.4 Destruction of Host Effector Cells or Antibodies:

It has been demonstrated that excretory and secretary products obtained from fluke caecal contents are toxic to lymphocytes and prevent the attachment of peritoneal cells to newly excysted juvenile parasites in the presence of immune sera *in vitro* (Goose, 1978), and that they suppress the delayed type hypersensitivity to parasite antigens (Cervi *et al.*, 1996). In addition, Chapman and Mitchell (1982) and later Carmona *et al.*, (1993) were able to achieve proteolytic cleavage of immunoglobulins and *in vitro*

prevention of antibody-mediated eosinophil attachment to newly excysted juveniles using the cathepsin B and L-like proteolytic enzymes released by *F. hepatica*.

1.3 Objectives:

1. There is evidence to suggest that acquired resistance to *F. hepatica* infection in cattle is operated by immunological means. However, the mechanisms of resistance and the regulation of the immune response are not clear particularly early in infection and after repeated challenge infections which are likely to occur in the natural environment. The objective of this study was to determine the immunological mechanisms of resistance and in particular the role of T cells.

2. Nothing is known about the nature of T cells and T cell subpopulations involved during primary and subsequent challenge infections with *F. hepatica* in cattle. The aim of this study was to develop and to optimise cytokine assays, mainly IL-2, INF-γ and IL-4, to be able to characterise $CD4^+$ T cell subpopulations stimulated during the infection.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Experimental Animals

2.1.1 Cattle:

Eight three to four month old Belgium Blue Cross Jersey calves were purchased from a farm known to be free of fasciolosis. Faecal samples were examined and serum samples were tested by ELISA to establish that the calves were not infected with *F. hepatica*. The calves were kept on sawdust in an enclosed barn at the Veterinary Faculty's animal husbandry farm, Leahurst, and fed on hay and commercial pelletted concentrate, with water provided *ad libitum*. Two calves were kept as uninfected control animals. The remaining six calves were infected with approximately 1000 viable *F. hepatica* metacercariae; challenged with 700 metacercariae 14 weeks after the initial infection; and treated with 12 mg/kg body weight of triclabendazole, 20 weeks after the initial infection.

Three Belgian Blue cross breed cows, 18 months old, were used in preliminary experiments to determine the optimum assay conditions. One cow (137) was infected orally with 1000 *F. hepatica* metacercariae and challenged twice, at the 25th and 37th week after the first infection, one (136) was used as a control animal throughout the preliminary experiments, and the third cow (BB), which was naturally infected with *Neospora caninum*, was used as a donor animal to obtain PBMC for the IL-2 bioassay (over 20 cows were tested as potential donor animals, see Chapter 3).

2.1.2 Sheep:

Two sheep were infected by oral lavage with *F. hepatica* metacercariae in order to maintain the parasite life cycle.

2.1.3 Rats:

Three-month old Wistar rats were infected with 50 or 100 *F. hepatica* metacercariae by oral lavage and used to harvest the juvenile and adult parasites. The rats were killed by CO_2 suffocation at 4 or 10 weeks after infection for the collection of juvenile and adult flukes respectively.

2.1.4 Procedure for Experimental Infection of the Animals:

Metacercariae, the infective stage of the parasite in mammals, were produced as described in Section 2.3. Metacercariae were encysted on dialysis tubing and then placed in a petri dish. The viable metacercariae, which have characteristic ring-form appearance, were counted under a dissecting microscope. To infect cattle and sheep, a section of dialysis tubing bearing an appropriate number of metacercariae was cut off with a scalpel inserted into a gelatine capsule and administered with a balling gun.

To infect rats, the metacercariae were carefully detached from the dialysing tubes with the back end of a Pasteur pipette. The viable metacercariae were counted and collected with a Pasteur pipette, previously treated with Silicone Repelcote (Hopkin and Williams, Chadwell Health, Essex, UK) and rinsed with distilled water. The appropriate number of metacercariae were transferred into a bijou in 0.5 ml distilled water; and then aspirated into a plastic syringe with a round-ended dosing needle and ejected into the stomach of the rat.

2.2 Collection of Samples:

2.2.1 Blood Sampling:

Blood samples were collected by jugular venepuncture into 10 ml EDTA or heparin coated or non-coated vacutainer (Beckton Dickinson) tubes at one-week intervals. The blood samples in vacutainer tubes with no added anticoagulant, were left at room temperature for 4 hours to clot. They were then refrigerated for 15 minutes and centrifuged at 1000g for 10 minutes in order to separate serum from the clot. The serum samples were aliquoted and stored at -20°C until used.

2.2.2 Faecal Samples:

Faecal samples were collected from the rectum in disposable plastic hand gloves.

2.3 Laboratory Maintenance of *F. hepatica*:

Both the parasite (*F. hepatica*) and the intermediate snail host, *Lymnaea viatrix*, originated from Cajamarca, Peru and were maintained in continuous production in the laboratory at the Department of Veterinary Parasitology in the Liverpool School of Tropical Medicine.

2.3.1 Preparation and Production of Algae:

Continuous production of green algae, *Oscillatoria* spp., was necessary to feed the snails. A clay-rich soil, known to promote rapid growth of the algae was collected from an area known to harbour indigenous snail species near to the River Dee. The soil was crushed using a mortar and pestle and sieved through a 4 mm-mesh sieve to remove stones. The soil, with 0.4g nutrient agar added per kg, was mixed with mineral water (algal fluid) (appendix 1) to form mud which was then sterilised by autoclaving at 120°C for 30 minutes. After cooling, the mud was layered into the bottom of 104x178 mm plastic boxes (Almond Products, Liverpool) giving a depth of about 15 mm. A small amount of algae from a feeder culture was mixed with mineral water, crushed into small pieces in a universal tube and spread over the mud. The box was covered with a lid and the algal plates were left for 5 to 7 days under a fluorescent strip light at room temperature until the mud surface was covered with algae. The culture was inspected daily and watered as necessary to keep the surface moist but not flooded.

Good light and temperature conditions are required for rapid growth of the algae. Although natural sunlight or mercury vapour lamps are reported to be ideal, in our experience, the fluorescent strip light produced an adequate growth rate.

2.3.2 Maintenance and Breeding of the Snail, *Lymnaea viatrix*:

The snails were transferred onto fully confluent algal plates and kept at room temperature. Higher temperatures (24-27°C) were found to speed up the development of the snail and the fluke. Therefore, when there was heavy demand for snails and metacercariae over a short period, the infected or non-infected snails were cultivated in an incubator adjusted to a temperature of 24-27°C.

Snails' egg masses were removed with a fine forceps and transferred to a damp filter paper in a petri dish. The filter paper was kept moist until the eggs hatched in approximately 10 days at 20°C. As soon as the eggs hatched, a small amount of algae from the feeder culture was scraped and transferred on to the filter paper, to feed the young snails. When the young snails were big enough, they were gently transferred to a fresh algal plate with fine forceps or with an artist's brush. Alternatively, parent snails were transferred to a new algal plate, leaving the egg masses on the original plate to hatch, then the young snails were moved to a new algal plate. In optimum conditions (20-24°C temperature with plenty of algae and moisture), young snails grew to a suitable size for infection (3-4 mm) in about 2-3 weeks.

The algal plates and the snails were examined daily, dead snails were removed and any snails on the sides or on the lid of the box were returned to the mud/algal surface. When the algae on the plate had been consumed, the snails were transferred to a fresh algal plate.

2.3.3 Obtaining *F. hepatica* Eggs and Miracidia:

Eggs were separated from faecal samples from a sheep previously infected with the Peruvian strain of *F. hepatica*. The faeces were suspended in tap water in a clean plastic container and passed through a nylon sieve (1 mm) to remove large particles. The suspension was then passed through a sieve of 200 μm aperture into another of 50μm aperture. The eggs passed through the first sieve, but the faecal debris was retained. The eggs trapped on the second sieve were washed under running tap water, transferred into a flat bottomed container and allowed to stand for about 20 minutes,

in which time the eggs settled and supernatant including the fine faecal fibre was decanted. This process was repeated until the eggs were relatively free of debris. The clean egg suspension was transferred into 9 cm plastic disposable petri dishes and incubated for 5 to 7 days at room temperature and then wrapped with aluminium foil and incubated for a further 10 to 12 days at 27°C. When the eggs were embryonated, they were refrigerated. When miracidia were required, the eggs were left on a bench with a light shining on the petri dish. Light and increasing temperature stimulated the miracidia to emerge.

2.3.4 Infection of the Snails with Miracidia:

Two to six miracidia, depending on their activity, were pipetted into each well of a flat bottomed 24-well polystyrene plate (Flow lab., Virginia, USA). The wells were filled with distilled water and a single snail, 3 to 4 mm in size, was placed into each well. The plates were covered with a lid and left for 4-5 hours at room temperature. The plates were regularly checked to ensure that the snails were still in the wells, and that penetration of the snails by miracidia took place. If there were no miracidia swimming in the wells, the snails were considered to be infected. The infected snails were then transferred onto a fresh algal culture and maintained in the way described above. Five weeks after infection, the snails were examined under a dissecting microscope to determine whether they contained *F. hepatica* rediae.

2.3.5 Harvesting and Storage of Metacercariae:

When cercariae were observed inside the snails, six to eight weeks after infection, they were harvested. The snails were gently washed with distilled water to remove algae and mud debris and were placed inside a 4 cm length of dialysis tubing, filled with cold (10°C) distilled water and fastened at each end with clips. The tubes were refrigerated for 10 to 15 minutes in the dark, then placed on a bench at room temperature with a light shining on the snails to induce the release of cercariae. Once the cercariae had stopped emerging from the snails and had all encysted on the dialysis tubing, those snails still alive were removed and placed onto a new algal plate. The tubes containing metacercariae were washed with distilled water, placed into a bijou and kept at 4°C for a maximum period of 6 months.

2.4 Preparation of *F. hepatica* Antigens:

2.4.1 Excretory and Secretary (ES) Antigens:

Rats were infected with 50 to 100 metacercariae and killed 4 or 10 weeks after infection. Live, intact juvenile (4 week old) and adult (10 week old) flukes were collected from the liver tissues and bile ducts respectively. *F. hepatica* infected rats were euthanised by CO_2 suffocation and the carcasses were brought to the laboratory within 30 minutes. Each rat was pinned on a wooden board and its abdominal surface was disinfected with 70% ethanol and dissected under sterile conditions in a hood. The liver was removed intact and placed in a disposable sterile petri dish. In order to collect the adult parasites, an incision was made into the bile duct with fine scissors and forceps, which had been disinfected with 70% ethanol and rinsed with sterile distilled water. The parasites were gently transferred from the bile duct to a universal tube containing sterile, warm PBS. Juvenile parasites were collected from liver parenchymal tissue by cutting the tissue into 1 cm^3 pieces and enabling the parasite to emerge from the tissue into the surrounding medium, after which the parasites were collected with Pasteur pipettes and transferred into a universal tube containing warm, sterile PBS. Having been washed 6 times with sterile PBS and then 6 times with RPMI tissue culture medium (Gibco Ltd., Paisley, Scotland) containing 100 U/ml penicillin and 100 mg/ml streptomycin to remove all traces of blood, host tissue and bile, the flukes were incubated in RPMI medium (1 ml medium per adult and 0.1 ml per juvenile fluke) overnight at 37°C in a humidified atmosphere of 5% CO_2 in air. The following day, the supernatant was collected and centrifuged at 4°C, 10,000g for 30 minutes to remove particulate materials.

2.4.2 Somatic Antigens:

The live flukes, which had been used to obtain ES antigens, were washed with PBS and homogenised using a tissue homogenisor in an ice bath. Alternatively, the flukes were snap frozen in liquid nitrogen and ground in a cold pestle and mortar. The fine powder was suspended in sterile PBS and further sonicated. The homogenated

antigens were then centrifuged at 4°C, 10,000g for 30 minutes and the supernatant was passed through a 0.2 μm filter (Sartorius, Göttingen, Germany). After determination of the protein concentration by Coomassie blue dye based protein assay (see Section 2.5), the ES and somatic antigens were aliquoted in 0.5 and 1 ml volumes in sterile Eppendorf tubes and stored at –80°C.

2.5 Protein Assay:

In order to determine the protein concentration of the antigen preparations and of the antibody solution, a Coomassie blue dye based protein assay (the Bradford assay) was used (Pierce, Rockford, Illinois, USA). The assay was performed according to the manufacturer's instructions using bovine serum albumin (BSA) as an external standard. Briefly, a ready-supplied 2 mg/ml concentration of BSA was diluted in PBS or RPMI medium depending upon the choice of buffer for the sample tested, to give a final protein concentration of 100 μg/ml. Further doubling dilution was performed to produce a range of concentrations from 100 to 6.25 μg/ml. Each concentration range was added in 50 μl volume in duplicate to the wells of a 96 well plate. Samples were also diluted in the same medium to give a range of dilutions from 1:2 to 1:64 and each dilution was added to the plate in 50 μl volume in duplicate. Ready-to-use Coommassie blue dye, 150 μl in volume, was mixed with the standard and test samples in the plate. After 10 minutes incubation, the colour changes were recorded in an automated and computerised spectrophotometer (ELISA reader, Dynex Technologies, Revelation 3.2) at wavelength 570 nm. The machine was blanked using medium alone. The protein concentrations of the samples were extrapolated from a standard curve generated from the optical density reading of each protein concentration of the standard (BSA). Only the sample dilutions that gave optical density readings falling on to the standard curve were used and the final protein concentration was calculated by multiplying the value by the dilution factor.

2.6 ELISA Procedure:

The ELISA was developed to monitor the antibody response of experimentally-infected and control animals. ELISA plates, Immulon-2, (Dynatech Laboratories, Virginia, USA) were coated with 100µl/well of *F. hepatica* E/S antigens at a concentration of 2 µg/ml, in carbonate buffer (0.05 M carbonate, pH 9.6). The plates were covered and left for one hour at room temperature, then overnight at 4°C. The plates were then washed six times (four short washes and two 5 minute washes) with washing buffer (0.01 M phosphate buffer saline, PBS, [pH 7.2] containing 0.05% Tween-20, polyoxyethylene-sorbitan monolaurate [BDH Laboratory Supplies, Poole, England]). The coated wells were incubated with 200 µl blocking buffer (2% skimmed milk [Marvel] (Premier Beverages, Stafford, UK) in washing buffer for 1hr at 37°C to block any non-specific reaction sites. The plates were then washed six times with washing buffer as described above.

One hundred µl of each serum sample diluted in washing buffer (1:100 for total IgG; 1:200 for IgG1, IgG2 and IgA; 1:400 for IgM) were added to each well in duplicate and incubated at 37°C for one hour. The plates were washed as before and incubated with 100 µl per well of optimum dilutions (1:1000) of mouse monoclonal antibodies to bovine IgG1, IgG2, IgA (Serotec Ltd., Oxford UK), IgM (Sigma) or rabbit anti-bovine IgG (1:2000) antibodies conjugated to horseradish peroxidase for 1h at 37°C. After washing, 100 µl of peroxidase-conjugated goat anti-mouse IgG (Sigma) (1:2000) were added to each well and the plates were incubated for 1h at 37°C. After washing as before, 100 µl of freshly prepared substrate (1 mg/ml 2.2',-azino-bis [3 ethylbenz-thiazoline sulfonic acid] peroxidase (ABTS; Sigma) in 1.25mM citrate acid pH4.0 (BDH, Poole, UK) with 0.1% hydrogen peroxide (BDH) (see Appendix 1) were added to the wells. Colour changes were recorded after 20 minutes incubation in the dark, at a wavelength of 405nm on an automatic ELISA reader (Dynex Technologies, Revelation 3.2) against a coated well that had received conjugate alone.

2.7 Separation of Peripheral Blood Mononuclear Cells:

Peripheral blood mononuclear cells (PBMC) were separated using a one step, density gradient centrifugation technique. Heparin or EDTA treated blood was layered over an equal volume of Lymphoprep (Nycomed Pharma, Oslo, Norway) in 15 ml conical centrifuge tubes (17x120mm) (Nunc, Roskilde, Denmark) or in 50 ml centrifuge tubes depending upon the volume of the blood to be processed. After 25 minutes centrifugation at 1200g at room temperature, the red blood cells and polymorphonuclear cells were pelletted at the bottom, the PBMC were retained on the surface of Lymphoprep, and the plasma remained on the top. The cells at the interface between the Lymphoprep and the plasma were transferred into another tube using a sterile Pasteur pipette. The PBMC were then washed three times using PBS + EDTA or RPMI-1640 medium supplemented with 10 u/ml heparin. The first and second washes were performed at 1500g for 10 and 5 minutes, respectively. After the second wash, the supernatant was poured off and the cells at the bottom of the tube were resuspended in 5 ml of complete growth medium (RPMI 1640 cell culture medium supplemented with 10% foetal calf serum, 100U/ml penicillin and 100mg/ml streptomycin, 2 mM L-glutamine). A small amount of the cell solution was mixed with an equal volume of trypan blue, enabling the viability of the cells to be examined, and the cell count was performed using an improved Neubauer haemocytometer counter (Weber Scientific International Ltd., Teddington Neubauer). Finally, the cells were resuspended in complete growth medium giving a final concentration of $2x10^6$ cell/ml.

2.8 Lymphocyte Proliferation Assay:

Lymphocyte stimulation tests were performed at one-week intervals. PBMC were separated as described above and adjusted to a density of $2x10^6$ cells/ml in complete culture medium. One hundred µl of cell suspension were dispensed into each well of a flat-bottomed 96 well plate (Nunclon, Roskilde, Denmark). One hundred µl of

somatic and ES antigens were added to triplicate wells in twofold serial dilutions from 200 to 0.8μg/ml. Each test included cells incubated with Concanavalin A (Con-A) [Sigma] (10μg/ml) or medium alone, and the whole assay was performed under sterile conditions. After incubation for five days in 5% CO_2 at 37°C, each well was pulsed with 1μCi tritium-labelled thymidine (Amersham International, Amersham, UK) and incubated for 5 hours. The cells were harvested onto a filter paper using an automatic harvester. The radioactivity (counts min^{-1}) in each culture was measured by scintillation counting. The proliferative response expressed as a stimulation index (SI) was calculated according to the formula:

SI = Mean counts min^{-1} of stimulated cultures / Mean counts min^{-1} of control cultures

2.9 Generation of antigen-specific cell lines:

PBMC were separated from EDTA-treated blood from *F. hepatica* infected cow 137 as described in Section 2.8 and adjusted to a cell density of $2x10^6$ cell/ml in complete medium. The cells were then stimulated with optimum concentrations of *F. hepatica* E/S (12.5μg/ml) and somatic (100μg/ml) antigens in 24 well plates in a total volume of 2 ml for 5 days at 37°C in 5% CO_2 in air. The proliferating cells were collected and washed with RPMI tissue culture medium supplemented with 2% FCS, resuspended in complete medium, counted and adjusted to a cell density of $1x10^6$ cell/ml and co-cultured for a further 5 days with antigens and Mitomycin C (Sigma) treated autologous PBMC as antigen presenting cells. PBMC from the same animal were separated and adjusted to a density of $5x10^6$ cell/ml and treated with 50 μg/ml final concentration of Mitomycin C and incubated for 30 minutes at room temperature. The cells were then washed three times with 30 ml of RPMI medium each. After the final wash, the cells were adjusted to a density of $1x10^6$ cells/ml and used as antigen presenting cells. The expanding cells were collected from the culture, washed once with RPMI medium, and co-cultured with 10 U/ml of rhIL-2 for 2 days for two cycles. After the final IL-2 incubation, the established cell lines were stained with a panel of bovine lymphocyte sub-population specific monoclonal antibodies

and analysed by flow cytometry to determine their cell composition, as described in Section 2.11. The cell lines were also co-cultured with or without antigens, plus antigen presenting cells and 10 U/ml rhIL-2 for two days, the supernatants were collected and IFN-γ levels measured by ELISA as described in Section 2.10.4.

2.10 Cytokine Assays:

2.10.1 Preparation of supernatants for cytokine analysis:

PBMC were cultured in sterile 24-well plates (Flow lab., Virginia, USA) for 3 days with or without *F. hepatica* ES, somatic antigens and Con-A at final concentrations of 12.5μg/ml, 100μg/ml and 5μg/ml, respectively in a total volume of 2 ml/well. Supernatants were collected and centrifuged on a bench top micro-centrifuge at 10,000g force for 10 minutes to remove cell debris. Supernatants were than aspirated with a sterile pipette and transferred into sterile Eppendorf tubes and stored at -80°C. Supernatants from Con-A stimulated cultures were treated with 0.1 mM of α-methyl-D-mannopyranoside (Sigma) to remove residual Con-A activity.

2.10.2 IL-2 Bioassay:

2.10.2.1 Generation of Short Term IL-2 Dependent Cell Line:

Peripheral blood mononuclear cells from a *Neospora caninum*-infected donor cow were stimulated with Con-A (5μg/ml final concentration) in 24-well sterile plates (Flow lab., Virginia, USA) for 3 days at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells (Con-A blasts) were then collected and washed twice with RPMI tissue culture medium containing 2% FCS to remove dead cells and residual Con-A activity. The cells were counted and resuspended with complete culture medium, giving a final cell concentration of $1x10^6$ cells per ml. The Con-A blast cell suspension was mixed with an equal volume of complete culture medium containing 10 U/ml recombinant human IL-2 (Sigma) and dispensed into a 24-well plate in a total volume of 1.5 ml per well. After 2 days incubation at 37°C, the expanding cells were collected, washed, and further co-cultured with 10 U/ml rhIL-2 for another 2 days and then used in the IL-2 bioassay.

2.10.2.2 IL-2 Bioassay Procedure:

The IL-2 assay was performed essentially as described by Lutje *et al.*, (1995) with some modification. The short-term IL-2 dependent cell line was collected using a 10 ml plastic disposable pipette and transferred into a 50 ml centrifuge tube. After centrifuging at 200g for 10 minutes at room temperature, supernatants were discharged and pelletted cells were resuspended and washed twice in 50 ml washing medium (RPMI-1640 medium supplemented with 2% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin) at 200g for 10 minutes. After the final wash, the cells were suspended in 10 ml complete medium. One hundred μl of cell suspension were mixed with an equal volume of 0.04% trypan blue (Sigma) solution in an Eppendorf tube. Cells were counted and their viability checked. Over 95% viable cells were used in the assay. If the viability fell below this level, the cell suspension was discarded. The cells were resuspended with complete culture medium to give a final cell concentration of $5x10^5$ cells per ml.

The cell suspension was divided into two equal parts in separate 50-ml centrifuge tubes. One half was treated with 30 μg/ml final concentration of monoclonal antibody, IL-A111, which binds to the α-chain of the bovine IL-2 receptor (IL-2R) and blocks IL-2-induced *in vitro* proliferation (Naessens *et al.*, 1992). The other half received an equal volume of PBS only. The cell suspension was immediately distributed into sterile 96-well flat bottom plates in a volume of 50μl per well. The plates were incubated at room temperature for 30 minutes. Meanwhile the culture supernatants were diluted in complete culture medium giving a range of dilution from 1:5 to 1:40. Each dilution of supernatant was added to the cells in a volume of 50μl in duplicate. Recombinant human IL-2, at concentrations ranging from 4 to 0.12 U/ml in duplicate, was run in each assay to produce a standard curve. After 2 days incubation, the plates were pulsed with 1μCi tritium-labelled thymidine (Amersham International, Amersham, UK) and incubated for another 5 hours. The cells were then harvested onto a filter paper using an automatic harvester (Wallac). The radioactivity (counts min^{-1}) in each culture was measured by scintillation counting as described above. IL-2 activity was expressed as the mean cpm of duplicate cultures minus the mean cpm of duplicate cultures which had received the same supernatant but had

been treated with Mab IL-A111. The IL-2 concentration in the supernatants was then estimated from a standard curve plotted using rhIL-2.

2.10.3 IL-4 Bioassay:

IL-4 levels in selected culture supernatants were kindly tested by Dr R.A. Collins, Institute for Animal Health, Compton, UK in a purified bovine B cell proliferation assay (Kuhnle *et al.*, 1996). PBMC from a conventionally-reared *Bos taurus* heifer were isolated by density gradient centrifugation as described before. Cells were adjusted to $3x10^8$ cells/ml in PBS containing 0.5% bovine serum albumin, stained with Mab, IL-A58, specific for the bovine immunoglobulin light chain (Williams *et al.*, 1990). After incubating with anti-mouse immunoglobulins conjugated with superparamagnetic particles (Miltenyi Biotech GmbH, Germany), the labelled cells were isolated on a MiniMacs column (Miltenyi Biotech) following the manufacturer's instructions. The isolated cells were washed and adjusted to $1x10^6$ cells/ml. The viability of the cell preparation was assessed by a trypan blue exclusion test. The purity of the cell population was assessed using a FACScan (Becton Dickinson) flow cytometry which showed that it contained more than 90% B cells.

Five-fold serial dilutions of culture supernatants were tested for B cell growth activity on purified B cells. IL-2 activity in this culture was blocked with Mab, IL-A111 and rbIL-4 was run as a standard. The total culture volume per well was 0.2ml. After 24 hours incubation, the cultures were pulsed with 37kBeq ^{3}H-thymidine (^{3}H-TdR; NEN, Du Pont) and incubated for a further six hours. The radioactivity incorporated into the proliferating cells' DNA was determined by liquid scintillation counting.

2.10.4 IFN-γ ELISA:

The levels of bovine IFN-γ in culture supernatants of PBMC were measured using a commercially available ELISA kit, Bovigam™ (CSL Ltd., Victoria, Australia). The kit was used according to the manufacturer's instructions with recombinant bovine IFN-γ (Ciba Geigy, Switzerland) as a standard.

Anti-bovine IFN-γ coated plates and all the reagents except conjugate were brought to room temperature to equilibrate for approximately 30 minutes. Fifty µl of sample diluent (Green Diluent) were added to the wells, followed by 50µl of the test samples (1:2 dilution) or the rbIFN-γ at a range of dilution from 4750 pg/ml to 147 pg/ml in duplicate. The plates were covered and incubated at room temperature for 60 minutes. After washing six times with wash buffer, 100µl of conjugate reagent (Horseradish peroxidase labelled anti-bovine IFN-γ) were added to each well and incubated for 60 minutes at room temperature. The plates were washed, then 100 µl of freshly prepared enzyme substrate solution (TMB- 3,3',5,5'-tetramethylbenzidine) were added to each well and incubated for 30 minutes at room temperature. The enzyme substrate reaction was stopped with 50 µl/well of the enzyme stopping solution provided (0.5M H_2SO_4) and the absorbance of each well was read using 450nm filter on an automated ELISA reader (Dynex Technologies, Revelation 3.2). The results were expressed as pg/ml based on rbIFN-γ standard. The IFN-γ concentrations of the samples were extrapolated from a standard curve generated from the optical density reading of each concentration of the standard. A typical standard curve is shown in appendix 2.

2.10.5 TGF-β ELISA:

The levels of transforming growth factor-beta 1 (TGF-β1) in the fortnightly culture supernatants of PBMC and serum samples from the calves were measured using a commercially available human TGF-β ELISA kit-TGF-β_1 E_{max}^{TM} ImmunoAssay System (Promega). The kit was used according to the manufacturer's instructions with the supplied recombinant human TGF-β_1 as a standard. We assumed that human TGF-β ELISA kit cross reacts with the bovine TGF-β on the bases of 99% amino acid homology between them. In addition, a human TGF-β detection kit, which, although it was from a different company (R&D Systems) had been used successfully to measure bovine TGF-β in culture supernatants of bovine cells (Nackman *et al.*, 1996).

Flat-bottom 96 well ELISA plates (immulon-4) were coated with TGF-β Coat Mab in carbonate buffer overnight at 4°C. The following day, the plates were removed

from the refrigerator and allowed to warm to room temperature (approximately 15 minutes). Meanwhile, blocking buffer was prepared by mixing 1 part TGF-β Block 5X Buffer with 4 parts deionised water. The contents of the plates were emptied, 270 µl of blocking buffer were added to each well and the plates were incubated at 37°C for 35 minutes. Meanwhile, sample buffer was prepared by mixing 1 part TGF-β Sample 10X Buffer with 9 parts deionised water, and rhTGF-β standard was diluted in the sample buffer to give a range of concentrations from 15.6 to 1000 pg/ml. The plates were washed once by filling the wells with 250 µl of the recommended wash buffer (TBST, 20mM Tris-HCl, 150mM NaCl and 0.05% Tween-20, pH 7.6) using a multi-channel pipettor. The standard and the test samples were added to the plates in duplicate (except for non-simulated culture supernatants, medium alone, which were tested in single wells). The plates were covered and incubated for 90 minutes at 37°C, then washed five times as described above and anti-TGF-β_1-detecting polyclonal antibody (rabbit anti-TGF-β_1 IgG) was added in the recommended dilution of 1:1000. After incubation for 2 hours at 37°C, the plates were washed five times and 100 µl of HRP conjugate (horseradish peroxidase conjugated anti-rabbit IgG, 1:2000 dilution) was added to each well, before a further 2 hours incubation at the same temperature. The plates were washed five times, as above, and 100 µl per well TMB substrate (3,3,5,5-tetramethylbenzidine) solution was added. After fifteen minutes incubation at room temperature the enzyme substrate reaction was stopped with 100µl/well of 1 M phosphoric acid and the absorbance of each well was read using a 450nm filter on an automated ELISA reader (Dynex Technologies). The results were expressed as pg/ml based on rhTGF-β_1 as a standard.

The total TGF-β1 was measured after treatment of the supernatant or serum samples with an acid activation step. The samples were mixed with 1N HCl (10 µl of acid for each 0.5 ml of sample), incubated for 15 minutes at room temperature and neutralised by adding 10 µl of 1N NaOH per 0.5 ml of sample.

2.11 Analysis of Lymphocyte Sub-populations by Flow Cytometry:

Indirect single immunofluorescence staining was carried out using previously described techniques with some modification (Williams *et al.*, 1991). Peripheral blood mononuclear cells were separated from EDTA treated blood samples by density gradient centrifugation as described in section 2.7, except that the washing steps were performed in cold washing buffer at 4°C. All the reagents used in this assay were refrigerated and the whole assay was performed on ice. Depending upon the number of samples to be run, the assay was performed either in 15 ml conical centrifuge tubes or flat-bottomed 96 well micro-plates. RPMI-1640 tissue culture medium supplemented with 2% FCS and 0.1% sodium azide was used as the wash buffer (or FACS buffer). Separated PBMC were adjusted to a density of $5x10^6$ cells/ml in cold FACS buffer and 100µl of this cell suspension were dispensed into the wells of a flat-bottomed 96 well plate. One hundred µl of the optimum dilution of monoclonal antibodies to cell surface markers for bovine lymphocytes were added to the appropriate wells and the plates were incubated for 45 minutes at 4°C. The optimum dilution of each monoclonal antibody used in this assay was determined by chequerboard titration. The lowest possible dilution that gave the highest staining was considered optimum. Table 2.1 summarises the specificity of the Mab and the optimum dilutions used in this assay. The plates were centrifuged at 200g for 5 minutes at 4°C and supernatants were aspirated by a multi-channel pipette without disturbing the layered cells at the bottom of the wells. The cells were washed 3 times with 300µl per well cold wash buffer. When 15 ml centrifuge tubes were used, 10 ml wash buffer was added to the cells and the tubes were centrifuged at 250 g for 5 minutes at 4°C. The supernatants were poured off and the cells were washed once more with 10 ml wash buffer.

Mab	Specific to	Nature	Dilution	Isotype	References
IL-A11	CD4	S/n	1:25	IgG2a	Baldwin *et al.*, 1986 Bensaid and Hadam, 1991
IL-A105	CD8	S/n	1:100	IgG1	Ellis *et al.*, 1986
IL-A42	CD2	S/n	1:100	IgG2a	Davis and Splitter, 1991
IL-A24	Activated macrophages	S/n	1:50	IgG1	Ellis *et al.*, 1988 Splitter & Morrison, 1991
CC15	WC1 (γ/δ T cells)	S/n	1:100	IgG2a	Morrison and Davis, 1991
IL-A59	B cell (Ig light chain)	Ascites	1:500	IgG1	Williams *et al.*, 1990
IL-A111	CD25 (IL-2R α-chain)	S/n or pAb	1:1000 or 30μg/ml	IgG1	Naessens *et al.*, 1992

Table 2.1 Monoclonal antibodies used to analyse circulating lymphocyte sub-populations. S/n; supernatants, pAb; purified antibody.

After the final wash, the supernatants were aspirated and 100μl of FITC conjugated sheep anti-mouse IgG (whole molecule, Sigma) (1:1000 dilution) were added to each well except for the cell control wells which received only medium throughout the procedure described. The plates were incubated for 45 minutes at 4°C in the dark and washed three times as described above. After the final wash, 0.3 ml of 1% paraformaldehyde in filter sterilised PBS were added to each well to fix the cells. The cells were then pipetted into specially designed 12x75mm Falcon test tubes (Becton Dickinson Labware, New Jersey, UK) and the volume was increased to 1 ml by adding more paraformaldehyde solution. The cells were stored in the dark at 4°C and analysed using a FACScan™ (Becton Dickinson, Sunnyvale, California, USA) following day or within 5 days. For each sample, 10 000 cells were counted; forward scatter (FS), side light scatter (SS) and fluorescent intensity (FL1) were recorded. FS

and SS were recorded with linear amplification and FL1 with logarithmic amplification. Analysis was performed on a Hewlett Packard 9153C using Lysis-II™ software. Specific Mab stained cells were expressed as per cent of the total lymphocyte number and then subsequently transformed into absolute number of that sub-population by multiplying the percent value with the total lymphocyte count which was determined by differential blood count.

2.12 Production of Monoclonal Antibodies:

The hybridoma cells that secreted monoclonal antibodies to the surface markers of bovine lymphocyte sup-populations were obtained from EAECC stored in liquid nitrogen.

2.12.1 Retrieval of Hybridoma Cells from Liquid Nitrogen:

Ampoules containing certain antibody producing hybridoma cells were removed from the liquid nitrogen tank and thawed in a 37°C water bath. The outside surfaces of the ampoules were wiped with a tissue soaked in 70% ethanol and were transferred into sterile 15 ml conical centrifuge tubes containing 12 ml pre-warmed complete tissue culture medium (RPMI-1640 tissue culture medium supplemented with 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin [all from GIBCO, Paisley, UK]). The cells were spun at 150g for 5 minutes, supernatants were discharged and pelletted cells were washed once more. The cells were then re-suspended in 10ml pre-warmed complete medium, transferred into two 25 cm^2 culture flasks (Nalge Nunc Int., Denmark) and incubated at 37°C in 5% CO_2 in air.

2.12.2 Maintenance of Hybridomas:

When cells were in exponential growth and had reached confluence (3-4 days), they were sub-cultured. The contents of each flask were diluted 1:6 in pre-warmed complete culture medium and transferred into two 75 cm^2 size or six 25 cm^2 size culture flasks (Nalge Nunc International, Denmark).

To obtain terminal supernatants, hybridoma cells were cultured for a minimum of 10 days. The supernatants were collected and centrifuged at 1000g for 10 minutes to remove cells and debris and aliquoted and stored at –30°C.

2.12.3 Cryopreservation of Hybridomas:

Healthy and exponentially dividing hybridoma cultures were collected and centrifuged at 400g for 5 minutes at 4°C. The supernatant was discarded and the cell pellet was resuspended at a final concentration of $2x10^6$ cells/ml in cold medium containing 20% FCS and 10% dimethyl sulphoxide (DMSO). The cell suspension was immediately dispensed into pre-chilled, sterile freezing vials on ice in one ml of total volume. The vials were then wrapped up in several layers of cotton wool and placed at –80°C overnight for slow and steady freezing. The following day, the vials were moved into liquid nitrogen. One week later one vial was tested for viability.

2.12.4 Preparation of Mouse Thymocytes:

Some hybridomas required mouse thymocytes for optimum growth. The thymus of two-week old BALB/c mice was removed under sterile conditions and sliced with sterile scissors in a petri dish containing 2 ml complete culture medium. The tissue was disrupted by repeated pipetting with a glass Pasteur pipette to obtain a single cell suspension. One million thymocytes were added to each 25 cm^2 tissue culture flask containing hybridoma cells.

2.12.5 Re-Cloning of Anti-Bovine CD25 Antibody Producing Hybridomas, IL-A111:

IL-A111 hybridoma cells were routinely recloned. Thymocytes were prepared as described in Section 2.11.4, adjusted to a density of $5x10^6$ cell/ml and distributed to a 96 well plate in a volume of 100 µl per well. Three dilutions of the hybridoma cells, i.e. 50, 10 and 5 cells/ml respectively, were prepared and added to the plates in a volume of 100 µl per well. Plates were incubated for 10 days, then examined using an inverted microscope and the wells with a single clone were recorded and supernatants tested using 3 day Con-A blast cells by flow cytometry. Two clones

producing the highest antibody level were expanded and used to produce tissue culture supernatants or inoculated into mice to produce ascites as described below.

2.12.6 Ascites Production:

For the IL-2 bioassay, high concentrations of Mab, IL-A111, were required. Since culture supernatants contain relatively low concentrations of antibodies, ascitic fluid containing high amounts of Mab, was produced by injecting hybridoma cells into the peritoneum of BALB/c mice (Harlow and Lane, 1988). Half a millilitre of Pristane (2,6,10,14-tetramethyldecanoic acid) was injected into the peritoneal cavity of adult BALB/c mice and 7-10 days later $2x10^6$ hybridoma cells in 0.5 ml PBS were injected into the peritoneum. When the peritoneal cavities of the mice were visibly enlarged with peritoneal fluid, the mice were killed and ascitic fluid was aspirated with a plastic Pasteur pipette, transferred into 15-ml sterile tube, and centrifuged at 2,000g for 10 minutes. The oil layer at the top of the supernatant was discarded and supernatant was collected without disturbing cell pellet. Ascites was stored at 4°C for up to one week and antibody was purified by protein-G affinity chromatography (Section 2.12.7).

2.12.7 Purification of Mab, IL-A111:

In order to minimise the effect of the possible toxic substances and proteases in culture supernatant and ascites in the IL-2 bioassay, Mab, IL-A111 was purified by affinity chromatography using commercially available, 1ml pre-packed protein-G column kit (Pharmacia) following the manufacturer's instructions. Purified antibody was then desalted using a PD-10 pre-packed Sephadex G25 column (Pharmacia) according to the manufacturer's instructions and eluted in PBS. After sterilising by passage through a 0.2μm filter (Sartorius, Göttingen, Germany), the protein concentration of the antibody solution was determined as described in Section 2.5 and stored at 4°C.

2.13 Separation of Fluke Antigens by Molecular Sieve Chromatography:

In order to partially purify and to define the T cell antigens in fluke E/S and somatic antigens, gel filtration technique was used to separate molecules according to their molecular size.

Fluke E/S and somatic antigens were prepared as described in Section 2.4 and concentrated by ultrafiltration using Centricon™-10 (Amicon Inc., Beverly, USA) following the manufacturer's instructions at 4°C to give a final protein concentration of 5 mg/ml. The concentrated antigens were centrifuged on a benchtop micro-centrifuge at 10,000g for 20 minutes, filtered through a 0.45 μm membrane filter and then applied to high performance liquid chromatography (HPLC) column at room temperature. Pre-packed Superose-12 10/30 column (Pharmacia), attached to an HPLC pump (Pharmacia), was equilibrated with 0.2 μm filter sterilised 0.1M Tris-HCL buffer, pH 7.4. 0.5 ml volume of samples were aspirated into a syringe and applied to the column through its sample application adaptor. The flow rate was 0.5 ml/minute. Fractions were collected after the void volume had passed. The absorbance of the eluates was monitored by a UV detector at 280nm and each peak was collected into a separate tube and stored at –80°C. The relative molecular weight and the purity of the eluted fractions were tested by SDS-PAGE as described in Section 2.13 with a non-reducing sample buffer. The protein concentration of the fractions was estimated by the method described in Section 2.5.

2.14 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE):

Whole fluke antigens (E/S and somatic) and each fraction collected from the HPLC chromatography were separated on a 10% non-gradient polyacrylamide gel under non-reducing conditions essentially as described by Laemmli (1970).

The preparation of polyacrylamide gels and the buffer solutions are described in Appendix 1. Mini gel apparatus (Bio-Rad) was cleaned with acetone and 70% ethanol and then rinsed with distilled water. After assembling the gel apparatus according to the manufacturer's instructions, 10% resolving gel was poured between the glass plates to 2 cm below the top, and double-distilled water (ddH_2O) was immediately added to the top to level the surface. The gels were allowed to polymerise at room temperature for approximately 1 hour. The distilled water was poured off and 5% stacking gel was poured on top of the polymerised separating gel. A separating comb with ten teeth was placed into the stacking gel and the gel was allowed to polymerise as before. After one hour, the comb was removed, the wells were rinsed with ddH_2O and dried with narrow strips of filter paper. The gel frames were removed from the preparation stand and assembled in the electrode stand, which was then placed into the running tank. The tank was filled with running buffer.

The samples were mixed 1:1 with double stranded sample buffer and were loaded into the wells. Molecular weight markers (Amersham Pharmacia Biotech) were warmed at 40°C for 1 minute and 10 µl loaded into the first well of the gel. The tank was closed and connected to a power supply (Bio-Rad Power Pac 300) and the gels were run at constant 200V (around 20-25mA) for 1h or until the dye (bromophenol blue) reached the end. The gels were gently removed, stained with Coomassie Brilliant Blue R-250 for 1 hour and then destained until the background was clear. Alternatively, the gels were stained with silver stain using Bio-Rad silver staining kit, following the manufacturer's instructions. The relative molecular masses of the protein antigen were estimated by reference to low range molecular weight markers.

2.15 Nitrite Assay:

The Griess reaction (Green *et al.*, 1982) was used to measure the amount of nitrite (as an indicator of NO production) accumulated during the culture. Sixty µl of culture supernatant were added in duplicate to 60 µl of a 1:1 mixture of 1% sulphanilamide in 2.5% phosphoric acid (H_3PO_4) and 0.1% N-(1-Naphthyl) ethylenediamine

dihydrochloride in 2.5% H_3PO_4 in microtitreplates. After 10 minutes of reaction the absorbance was read at 570 nm in an automated ELISA plate reader (Dynatec). The nitrite levels in the culture supernatants were determined with reference to a standard curve generated using sodium nitrite at concentrations ranging from 7.5 to 1000 μM/ml in culture medium.

2.16 Measurement of haematological and biochemical parameters:

Total and differential blood cell counts and levels of serum liver enzymes (GGT and GLDH) were kindly measured by The Department of Veterinary Pathology, The University of Liverpool, Leahurst, Wirral. Blood samples were collected in EDTA-coated vacutainers at weekly intervals and sent to the department within 20 minutes. Red and white blood cell counts were performed on a Coulter Counter (Model ZF, Coulter Electronics Ltd., Bedfordshire, England).

Serum samples were tested for levels of GGT and GLDH using commercially available kits (Randox Laboratories Ltd., Antrim, UK) in an automated enzyme analyser (Kone Instrument Corporation, Espoo, Finland).

CHAPTER THREE

DEVELOPMENT OF A BOVINE IL-2 BIOASSAY AND OPTIMISATION OF ASSAY CONDITIONS

3.1 Introduction:

Laboratory techniques and assays are the essential tools of scientific investigation. The accuracy, sensitivity and specificity of an assay have to be adequate and will generally determine whether an investigation is scientifically acceptable. In comparison to the tools employed in immunological investigations in humans and the mouse model, those available to veterinary science are as yet poor. Studies in fundamental and applied immunology in veterinary science are particularly affected by a lack of reagents essential to the pursuit of high quality research. In particular, investigation into cellular immune responses requires specific reagents.

The discovery of cytokines was a breakthrough for biological science. Cytokines play a multifunctional role and control intercellular communication signals in a variety of cellular responses. Their role is particularly pronounced in immunobiology. During the last two decades, many murine and human cytokines and their receptors have been described, whose biological significance is being studied extensively in laboratories worldwide. Recent advances in molecular biology have contributed enormously to the development of materials and methods in this area. The genes encoding cytokines have been cloned, sequenced and expressed into a variety of systems. Biologically active recombinant forms of many cytokines and antibodies to neutralise their activity or to bind to their respective receptors have been produced. A multitude of immunoassays, bioassays and molecular biological tools have been developed to quantify individual murine and human cytokines, contributing to our understanding of *in vivo* and *in vitro* ongoing immune responses to many pathogens and/or disorders. Many scientific manufacturers are now also able to provide the majority of these reagents, assays, kits etc., making immunology one of the fastest growing scientific disciplines.

However, research in bovine immunology has been hampered by a lack of widely available bovine specific reagents and tools. The reagents urgently required to assist research in bovine immunology are too many to enumerate here. However, those that fall within the scope of the work presented here may be summarised as anti-bovine IgE specific mono- or polyclonal antibodies; recombinant or native bovine cytokines, in particular IL-2, IL-4, IL-5, IL-10; antibodies to bovine cytokines and to their receptors; cytokine detection kits; and competitor cytokine DNA fragments to quantify cDNA amplified by reverse transcriptase-polymerase chain reaction (RT-PCR).

There is a commercially available sandwich ELISA kit for bovine IFN-γ, originally produced for the diagnosis of bovine *Mycobacterium bovis* infection in cattle. To overcome problems such as the low sensitivity (65-72%) and cumbersome manipulations of the tuberculin test and the failure of serological tests to achieve acceptable levels of specificity and sensitivity (Wood and Rothel, 1997), Wood and his co-workers initially developed a test based on the detection of IFN-γ in supernatants of Purified Protein Derivative (PPD) stimulated PBMC by a bioassay (Wood *at al.*, 1990a). To simplify the test, they subsequently produced monoclonal antibodies to recombinant bovine IFN-γ (Wood *et al.*, 1990b) and developed an ELISA (Rothel *et al.*, 1990). This kit is currently used not only for the diagnosis of tuberculosis but also in many research applications to measure production of bovine IFN-γ.

In contrast to humans and mouse, long-term cell lines are not available to detect and quantify bovine IL-2 and IL-4 in body fluids and/or in culture supernatants. However, several attempts have been made to measure those bovine cytokines in lymphocyte culture supernatants using bioassays or to detect cytokine mRNA transcripts by Northern blotting, RT-PCR and quantitative competitive RT-PCR technologies. Initially, Oldham and Williams (1985) attempted to measure bovine T cell growth factors in culture supernatants of PBMC obtained from *F. hepatica* infected cows and stimulated *in vitro* with fluke antigens by a bioassay where 3-day Con-A blast cells were used as responding cells. This test lacked both an external

standard to quantify the amount of cytokine produced and measure the specificity, in that Con-A blast cells respond to cytokines other than IL-2. Attempts to generate long-term bovine IL-2 sensitive T cell lines failed for two reasons: the reproducibility of the test was inconsistent and retrieval of the cells from liquid nitrogen was problematic (R.A. Collins; Compton, UK, personal communication). However, several short-term T cell lines have been generated and successfully used in bioassays to measure bovine IL-2 and IL-2-like activity in culture supernatants (Ayoub and Yang, 1995; Hanby-Flarida *et al.*, 1996; Collins *et al.*, 1994; Brown *et al.*, 1994a). The last two authors used bovine $CD8^+$ T cells in their bioassays, however, Brown *et al.* (1994a) reported that their assay did not discriminate IL-4 from IL-2 activity. The recent availability of a monoclonal antibody (IL-A111) to the IL-2 receptor alpha chain (IL-2R α-chain) that blocks IL-2 dependent proliferation (Naessens *et al.*, 1992) makes a quantitative and specific IL-2 bioassay feasible. Initially, Lutje *et al.*, 1995, using this monoclonal antibody and 3-day Con-A blast cells, developed an IL-2 specific bioassay.

Efforts to develop an IL-4 bioassay received relatively little attention probably due to the species-specific nature of the cytokine and its diverse biological activity on a variety of cell types. Bovine IL-4 has been cloned (Heussler *et al.*, 1992) and expressed in a variety of systems to produce its recombinant form (Estes *et al.*, 1995; Kuhnle *et al.*, 1996; Brown and Estes, 1997). This has made possible the development of a bioassay to measure IL-4 levels in culture supernatants (Kuhnle *et al.*, 1996). In this assay, bovine peripheral blood B cells, separated by magnetic beads, were stimulated with anti-bovine IgM specific monoclonal antibodies (IL-A58 and IL-A59) and co-cultured in the absence or presence of different concentrations of rboIL-4. Subsequently, the IL-4 dependent proliferation of B cells was determined by H-thymidine uptake. Since B cells express IL-2 receptors and respond to it, IL-2 dependent proliferation was blocked by adding IL-A111. More importantly, monoclonal antibodies to bovine IL-2 and IL-4 have recently been produced and sandwich ELISA are being developed (R.A. Collins, personal communication).

The use of molecular techniques such as Northern (RNA) blotting (Brown *et al.*, 1994a; 1996a and b), RT-PCR (Brown *et al.*, 1994a; Ruef *et al.*, 1997) and competitive RT-PCR (Zarlenga *et al.*, 1995; Canals *et al.*, 1997; Ruef *et al.*, 1997) to detect and/or to quantify bovine cytokines has also been widely reported. Although highly sensitive and specific, such techniques are either semi-quantitative or technically difficult to perform, require highly optimised assays and high quality reagents, and are expensive and generally time-consuming. Using such techniques to run the large number of samples required to study the kinetics of cytokines during an infection, is not feasible. These techniques measure only mRNA, but not the expression of protein.

The aim of the work described in this chapter was to develop and optimise assay conditions. In particular, the determination of antigen concentrations, the timing of the collection of supernatants for cytokine testing and the development of a sensitive, specific and quantitative IL-2 bioassay. The optimum assay (ELISA) conditions to monitor the antibody responses of the calves are also reported.

3.2 Results:

3.2.1 Development and optimisation of IL-2 bioassay:

3.2.1.1 Generation of short-term IL-2 sensitive bovine cell lines:

Three-day Con-A stimulated bovine PBMC (Con-A blast cells) were initially used as responder cells in IL-2 bioassays. However, high background readings were obtained even in the absence of exogenous IL-2 (rhIL-2) and a poor IL-2 dose-dependent response was obtained. This made it impossible to use Con-A blast cells as responder cells for an IL-2 bioassay. To eliminate these problems, short-term, IL-2 sensitive bovine cell lines were established. PBMC were activated for 3 days with Con-A and the blast cells were regularly sub-cultured every three days in the presence of 10 U/ml rhIL-2 as described in Section 2.9.2.1. It was possible to keep the cells alive and proliferating for up to two months with this method but, eventually, the cell lines became unresponsive to IL-2. Neither reactivation of unresponsive cell lines with Con-A (even at sub-optimal concentrations-1 to 2 μg/ml) nor the attempts to clone them were successful. Therefore, short-term cell lines generated by three cycles of sub-culturing the blast cells with rhIL-2 for 3 days in each cycle were used in the IL-2 bioassay. This reduced the background proliferation found using Con-A blast cells from over 10,000 cpm to around 1000 cpm.

3.2.1.2 Cell composition of IL-2 specific cell line:

The phenotype of cells in the IL-2 specific cell line was analysed by flow cytometry using monoclonal antibodies specific to the surface cluster of differentiation (CD) antigens on bovine cells (Table 2.1 in Chapter 2). Three day-Con-A blast cells, and cells which had been through one or two cycles of IL-2 stimulation were analysed in order to compare changes in cell population after repeated cultivation in rhIL-2. Con-A blast cells had a mixture of T and B cells, over 90% of which expressed IL-2 receptor. In contrast, less then 4% of the cells in short-term rhIL-2 cultivated cell lines were positive for bovine Ig specific monoclonal antibody (IL-59) and the proportion of $CD4^+$ cells increased from 32% in Con-A blast cells to 54% upon two

cycles of stimulation with rhIL-2. Although a decrease in number of cells expressing IL-2R was observed after the first cycle of IL-2 stimulation, this was restored in the second cycle (Fig.3.1).

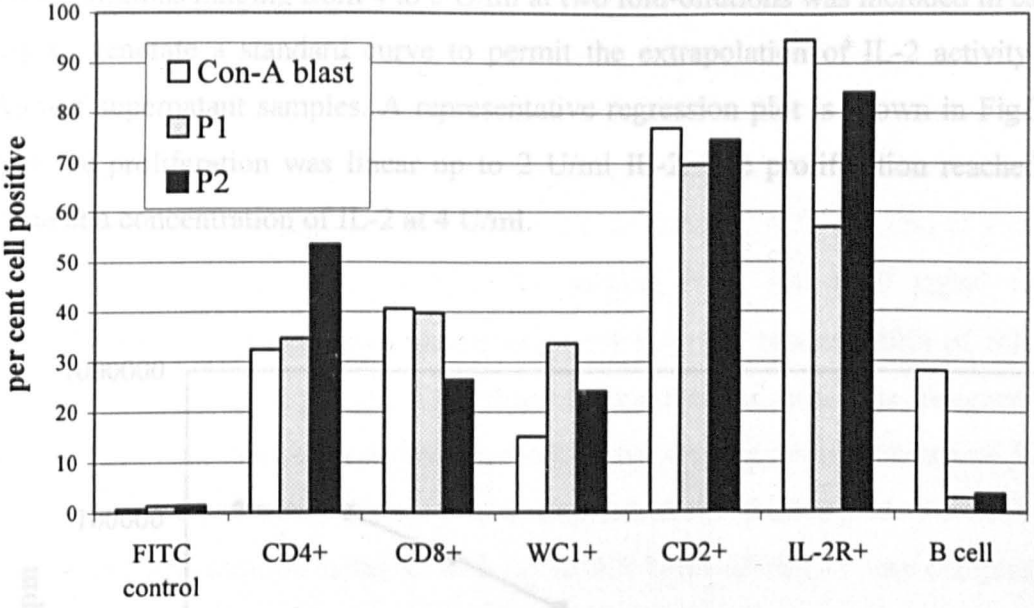


Fig.3.1 Phenotype of cells in Con-A blast cells and one (P1) or two (P2) cycle IL-2 stimulated short-term cell lines. Cells were stained with a panel of bovine cell phenotype specific Mab and analysed by flow cytometry.

3.2.1.3 IL-2 dependence of short-term cell line:

The established short-term cell lines were IL-2 dose-dependent (Fig.3.2). Proliferation, as determined by H^3-thymidine uptake, was significantly correlated with the amount of IL-2 present in the culture ($r=0.98$; $P<0.01$). Recombinant hIL-2 at concentrations ranging from 4 to 0 U/ml at two fold-dilutions was included in each assay to generate a standard curve to permit the extrapolation of IL-2 activity in unknown supernatant samples. A representative regression plot is shown in Fig.3.2 where the proliferation was linear up to 2 U/ml IL-2. The proliferation reached a plateau at a concentration of IL-2 at 4 U/ml.

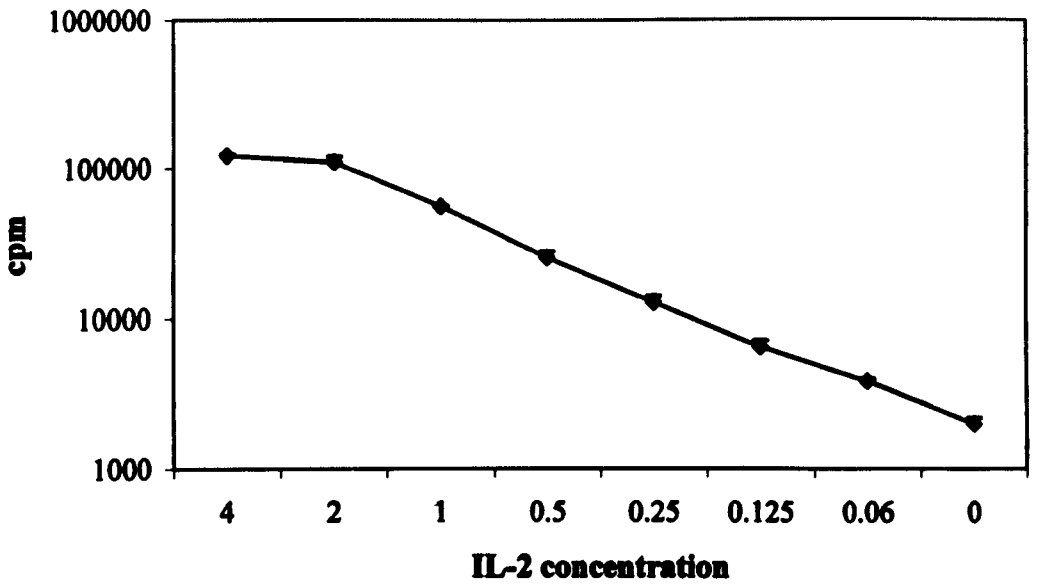


Figure 3.2 IL-2 dependence of short-term cell line. The cell line was stimulated with various concentrations of rhIL-2 and cultured for two days and proliferation determined by H3 thymidine uptake for 5 hours. The results are presented as cpm

3.2.1.4 IL-2 dependent proliferation of cell lines was blocked with a monoclonal antibody (IL-A111) to IL-2R α-chain:

Although the cell lines described here proliferated in response to exogenous rhIL-2, it is likely that these cell lines, as is the case with most activated cell types and those commonly used to measure mouse and human cytokines, respond to multiple cytokines. It is probable that the culture supernatants in which IL-2 levels were to be measured included other cytokines produced by PBMC. Therefore, monoclonal antibody, IL-A111, specific to the alpha chain of the IL-2 receptor, was used to block IL-2 dependent proliferation. To determine the optimum IL-A111 concentration to block IL-2 induced proliferation, cell lines were co-cultured with 10 U/ml of rhIL-2 and two-fold serial dilutions of IL-A111 ranging from 200 to 0 μg/ml final concentration. At concentrations above 25 μg/ml IL-A111 blocked 50% of rhIL-2 induced proliferation (Fig.3.3a). Therefore, the next experiment was designed to determine the reduction in proliferation induced by varying concentrations of IL-2 (from 4 to 0 U/ml), using IL-A111 at a concentration of 30 μg/ml. As seen in Fig.3.3b, the proliferation induced with up to 0.5 U/ml of rhIL-2 was completely blocked with 30 μg/ml of IL-A111.

Supernatants from *F. hepatica* antigen-stimulated cultures were tested at three different dilutions (1:5, 1:10 and 1:20 for supernatants from non-stimulated cultures, and 1:10, 1:20 and 1:40 for supernatants from antigen-stimulated cultures). Levels of IL-2 were subsequently calculated from the proliferation induced by the dilution falling on the linear part of the regression curve where the proliferation induced was lower than that induced by 0.5 U/ml of rhIL-2.

3.2.1.5 Donor animal for PBMC used to establish the cell lines was critical:

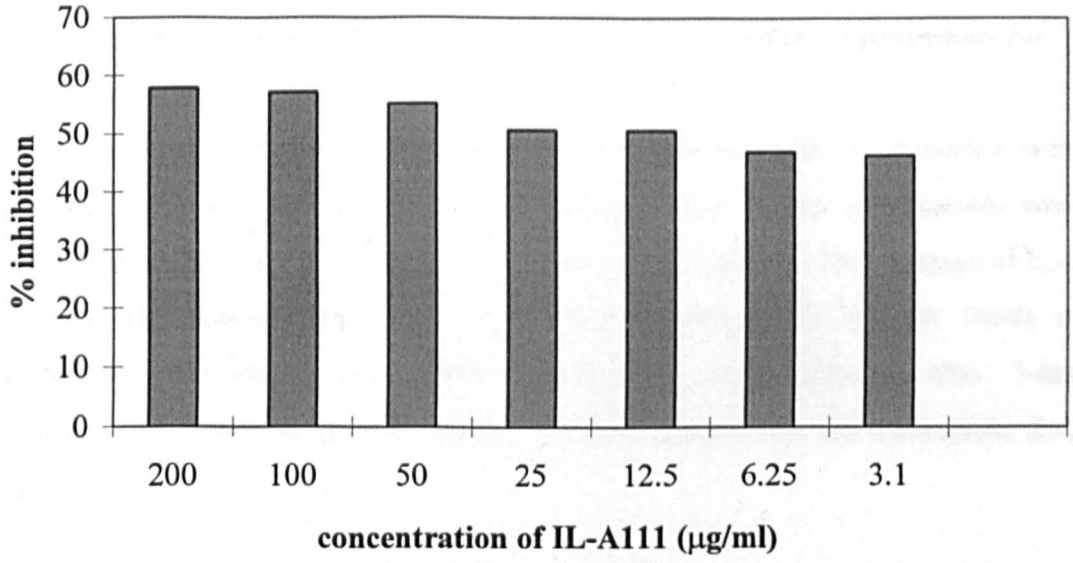
Over 20 cows were tested as PBMC donors. Con-A blast cells generated from the majority of cattle responded poorly in IL-2 assay where the proliferation was either IL-2 independent or showed high background readings and could not be blocked with IL-A111. The proliferation after Con-A activation of PBMC from cows naturally-infected with the intracellular protozoan *Neospora caninum* was generally greater than that obtained from non-infected animals. A single *Neospora caninum*-

infected cow (BB) was used for all IL-2 bioassays used to test IL-2 concentrations in *F. hepatica* stimulated cell cultures.

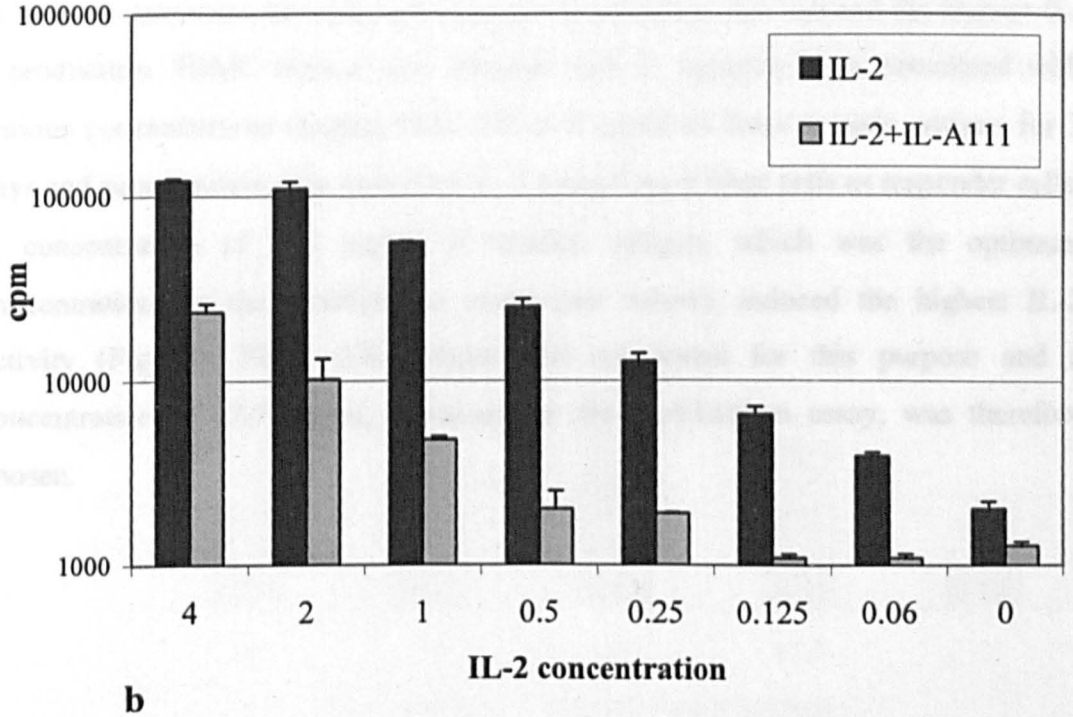
PBMC of the donor animal and the IL-2 sensitive cell lines derived from these PBMC were tested by stimulating *in vitro* with fluke antigens at the concentrations (100 and 12.5 μg/ml for somatic and E/S antigens respectively) used to generate the test supernatants, in order to determine whether they proliferated in response to fluke antigen. Neither PBMC nor the cell lines proliferated in response to fluke antigens. Stimulation indices were always less than 2 to 3 with means of 1.5±0.7 for PBMC and 1.2±0.3 for the cell lines from 8 independent cultures.

3.1.1.6 The effect of fluke antigens on proliferation of IL-2 cell lines:

Since the culture supernatants included fluke antigens and since fluke E/S antigens are toxic to lymphocytes, an experiment was designed to determine whether fluke antigens directly affected the proliferation response of the IL-2 cell line to rhIL-2. The cells were stimulated with varying concentrations of rhIL-2 (from 8 to 0 U/ml) and co-cultured in the presence or absence of *F. hepatica* somatic and E/S antigens at concentrations of 100 and 12.5 μg/ml respectively. The proliferation was measured by H^3-thymidine uptake for the last five hours of the 2-day incubation period. While somatic antigen at the concentration tested had no effect, E/S antigen reduced the IL-2 induced proliferation by 10% with an IL-2 concentration below 1 U/ml.



a



b

Fig.3.3 Inhibition of IL-2 dependent proliferation by monoclonal antibody, IL-A111 to IL-2 receptor α-chain. In (a) the cell line was stimulated with 10 U/ml rhIL-2 and co-cultured with various concentrations of Mab. At this concentration of IL-2 only about 50% inhibition was achieved. In (b) the cell line was stimulated with various concentrations of rhIL-2 and co-cultured with 30 μg/ml IL-A111. Up to 0.5 U/ml rhIL-2 induced proliferation was successfully blocked with this concentration of IL-A111.

3.1.1.7 Determination of optimum time for collection of culture supernatants for IL-2 assay:

The PBMC from a cow (137) experimentally infected with *F. hepatica* were stimulated with 100 μg/ml fluke somatic antigen. The culture supernatants were collected at 12, 24, 48, 72 and 96 hours and were then tested for the presence of IL-2 (Fig.3.4). The supernatants from 3-day cultures induced the highest levels of proliferation. Therefore, the culture supernatants were collected after 3-day stimulation of PBMC with both somatic and E/S antigens for the subsequent IL-2 assay.

3.1.1.8 Determination of the optimum fluke antigen concentration that induced the highest IL-2 production:

In order to determine the optimum antigen concentration that induced the highest IL-2 production, PBMC from a cow infected with *F. hepatica* were stimulated with various concentrations ranging from 100 to 0 μg/ml of fluke somatic antigen for 3 days and supernatants were tested for IL-2 using Con-A blast cells as responder cells. A concentration of 100 μg/ml of somatic antigen, which was the optimum concentration for the proliferation assay (see below), induced the highest IL-2 activity (Fig.3.5). Fluke E/S antigen was not tested for this purpose and a concentration of 12.5 μg/ml, optimum for the proliferation assay, was therefore chosen.

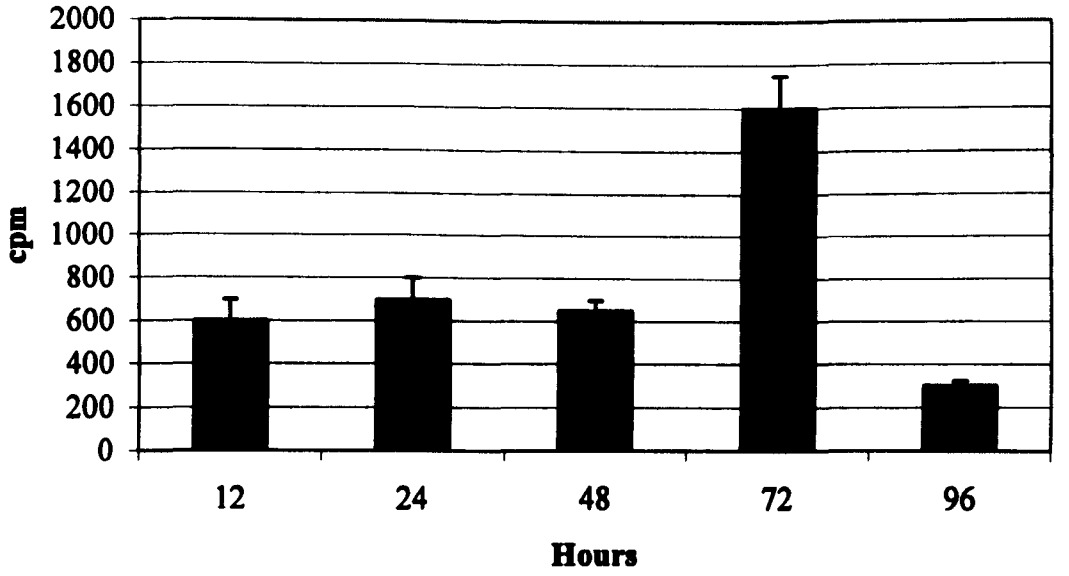


Fig.3.4 Determination of optimum time to collect antigen stimulated culture supernatants for IL-2 bioassay. PBMC from a *F. hepatica* infected cow (137) were stimulated with 100 μg/ml concentration of fluke somatic antigen and the supernatants were collected at various time intervals shown. The IL-2 activity was subsequently tested in IL-2 bioassay using Con-A blast cells as responder cells.

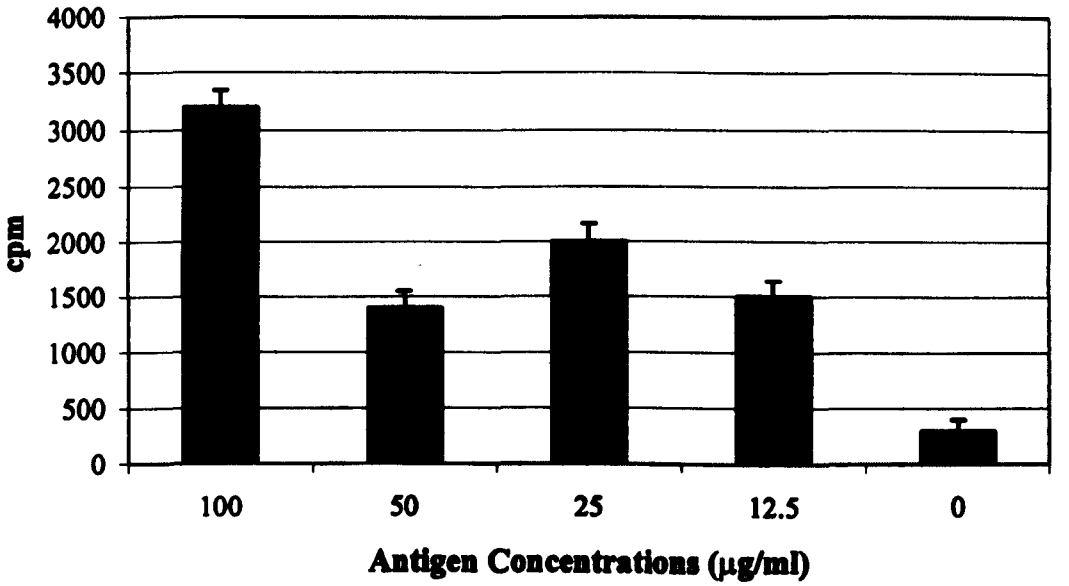


Fig.3.5 Determination of optimum antigen concentration to stimulate the highest IL-2 production. PBMC from an infected cow (137) were stimulated with different concentrations of fluke somatic antigens as indicated for 3 days and culture supernatants were tested for IL-2 activity using Con-A blast cells as responder cells in the IL-2 bioassay.

3.2.2 Optimisation of proliferation assay:

3.2.2.1 Determination of optimum antigen concentration:

In order to determine the optimum antigen concentration that induced maximum proliferation response, PBMC from a cow experimentally infected with *F. hepatica* were stimulated *in vitro* with various dilutions (two-fold dilution) of fluke E/S and somatic antigens for 5 days. The results for each antigen concentration are shown in Fig. 3.6 as stimulation indices (SI). The results shown are representative of five different experiments performed at different times during the infection. Both E/S and somatic antigens stimulated PBMC in a dose-dependent manner. Somatic antigen at 100 μg/ml and E/S antigen at 12.5 μg/ml final concentrations were found to be optimum, inducing maximum proliferation. The highest concentration tested for somatic antigen (100 μg/ml) induced the maximum proliferation with a SI of 160. E/S antigen was tested at concentrations ranging from 100 to 1.56 μg/ml. The highest concentration of antigen induced the lowest proliferation and was found to be toxic to lymphocytes. However, concentrations ranging from 25 to 6.25 μg/ml induced the optimum proliferation, with little or no toxicity to lymphocytes as determined by a trypan blue exclusion test after overnight incubation of the cells in the presence of antigen.

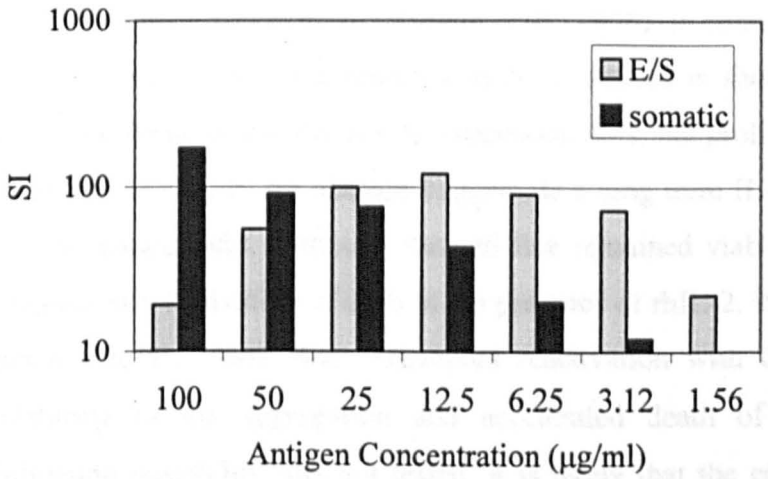


Fig.3.6 Determination of optimum antigen concentration for lymphocyte proliferation assay. PBMC from infected cow (137) were stimulated with different concentrations of fluke E/S and somatic antigens and the proliferation was measured by incorporation of ^{3}H-thymidine for each concentration. The results are expressed as stimulation indices.

3.3 Discussion:

In this chapter, a specific and sensitive IL-2 bioassay to measure biologically active bovine IL-2 in lymphocyte culture supernatants was developed. To date, various attempts have been made to measure bovine IL-2 levels in culture supernatants using bioassays or to detect IL-2 mRNA by RT-PCR. To measure IL-2 bioactivity, many researchers have used Con-A blast cells as responder cells in their bioassay. Initially, we also used three day Con-A blast cells following the method described by Lutje *et al.* (1995). However, high background readings were obtained even in the absence of exogenous IL-2 and a poor IL-2 dose-dependent response was obtained. This made it impossible to use Con-A blast cells as responder cells for an IL-2 bioassay. These problems were probably due to the fact that mitogen-stimulated responder cells themselves kept producing IL-2 together with other cytokines, which in turn increased the background to give a poor dose-dependent response. Difficulty in blocking proliferation with the monoclonal antibody (IL-A111), even at very high concentrations (up to 200 μg/ml), also indicated that blast cells were producing other cytokines and subsequently responding to them. This could also be attributable to the relatively high percentage of B cells in the Con-A blast cells. Flow cytometry analysis of Con-A blast cells revealed that over 25% of the cells in Con-A blasts were Ig^+. Purified bovine peripheral blood B cells have been successfully used to measure IL-4 levels in culture supernatants (Kuhnle *et al.*, 1996). In contrast to Con-A blast cells, two or more cycles in the presence of IL-2 resulted in short-term cell lines which did not proliferate in the absence of exogenous IL-2, but proliferated in a dose-dependent manner to rhIL-2. An attempt to generate a long term IL-2 sensitive bovine cell line was unsuccessful. Although the cell line remained viable for up to two months by regular sub-cultivation of cells in the presence of rhIL-2, it eventually become unresponsive to IL-2 and died. Attempted reactivation with Con-A was unsuccessful, resulting in the aggregation and accelerated death of the cells. Although the following possibility was not tested, it is likely that the cells needed additional signal/s. It is known that T cell activation requires not only the presence of antigens and cytokine signalling but also cell to cell signal transduction. CD40 molecules on the antigen presenting cells and CD40 ligands on the surface of T and

B cells provide an example of this. Additional cytokines, produced by antigen presenting cells such as IL-1 and IL-12, are required to deliver the right signal to keep the cells alive. For example, IL-1 is vital for the maintenance of mouse or human IL-2 sensitive cell lines. Since the short-term IL-2 sensitive cell lines generated were sufficient for our requirements and produced good results, the generation of a long-term bovine IL-2 sensitive cell line, as mentioned above, was not pursued any further. However, our observations here give some indication that the development and maintenance of a long-term IL-2 sensitive bovine cell line with the requisite additional signalling would be worth further investigation.

Although the short-term cell line described here proliferated in response to exogenous rhIL-2 in a dose-dependent manner, it is likely that these cell lines, as is the case with most activated cell types and those commonly used to measure mouse and human cytokines, respond to multiple cytokines. Since up to 0.5 U/ml of the exogenous rhIL-2 induced proliferation was successfully and completely blocked with the addition of 30 μg/ml IL-A111, the levels of cytokine activity measured in culture supernatants are directly attributable to IL-2. In contrast, when cells were cultured in the presence of 10 U/ml rhIL-2, even very high concentrations of IL-A111 (up to 200 μg/ml) were unable to inhibit the proliferation by more than 50%. This unacceptable level of reduction in proliferation was probably due to the excess amount of IL-2 present, which might have competed with antibody for the receptor. We minimised this effect by testing the supernatants with three different dilutions and subsequently determining IL-2 levels in dilutions that corresponded to, at or below the proliferation induced 0.5 U/ml rhIL-2.

The principal advantage of bioassays is that they measure the biologically active and secreted form of the cytokine. However, they possess many disadvantages in comparison to immunoassays and molecular techniques in terms of the accuracy with which the quantity and quality of the cytokine of interest is measured. Many cytokines up- or cross- regulate the function of others. For example, TGF-β and IL-10 down-regulate expression of the IL-2 receptor, therefore the presence of these cytokines in the culture supernatants may adversely affect the measurement of IL-2.

Although our assay was sensitive and specific, it could not exclude this type of possibility. Human IL-15 is also known to bind to the β chain of the IL-2 receptor and induces IL-2 like bioactivity (Ballaun, 1998). It is not known whether the bovine equivalent would share the same specificity to the IL-2 receptor.

CHAPTER FOUR

In Vitro Blastogenesis and Cytokine Responses of Peripheral Blood Mononuclear Cells to Fluke Antigens and Serum Antibody Isotype Responses in Calves Experimentally Infected with *Fasciola hepatica*

4.1 Introduction:

There is considerable evidence to suggest that cattle develop resistance to infection with *F. hepatica* (Haroun and Hillyer, 1986). This resistance has been attributed in part to pathological changes caused by primary infection, as a result of which migrating flukes from the challenge infection are mechanically trapped by fibrotic and calcified parenchymal tissue in the liver. Recent vaccination trials in cattle using defined parasite antigens resulted in variable but significant and promising levels of protection following challenge infections with metacercariae (Spithill and Dalton, 1998). The levels of protection induced in vaccinated animals, unaffected by pathological factors, are directly attributable to an acquired immune response. These results have influenced the balance of opinion in the debate about the mechanisms of resistance away from pathological mechanisms in favour of an acquired immune response.

While there has been much research aimed at demonstrating the resistance or susceptibility of individual host species to *F. hepatica,* and while recent trials have concentrated on the active immunisation of animals with defined parasite antigens, relatively few attempts have been made to define the mechanisms of protective immunity or the immune responses of the host.

Cattle generate antibodies to a wide range of *F. hepatica* antigens during infection (Santiago and Hillyer, 1988; Ortiz-Oblitas, 1997). Antibody responses of experimentally infected calves are detectable as early as two weeks after infection (Santiago and Hillyer, 1988). All antibody subclasses are produced during the course

of infection (Duffus and Franks, 1981). While *F. hepatica* antigen-specific IgG1 is the dominant isotype in experimentally infected cattle, recent findings indicate that both IgG1 and IgG2 antibodies are expressed in naturally exposed animals, with no significant difference between the isotypes (Ortiz-Oblitas, 1997). Despite the presence of antigen-specific circulating antibodies, their role in protective immunity to *F. hepatica* is still the subject of controversy. Antibodies are one of the main mediators of attachment of effector cells, such as eosinophils, macrophages and neutrophils, to the invading organisms. Antibody-dependent cytotoxic cell attachment to the surface of *F. hepatica* has been demonstrated *in vitro* (Duffus and Franks, 1980; Doy *et al.*, 1980; Doy and Hughes, 1982b), without, however, mediating parasite killing. To date, parasite killing *in vitro* has been demonstrated using major basic proteins purified from bovine eosinophils (Duffus *et al.*, 1980). It therefore appears that although antibodies mediate effector cell attachment to the parasite, the killing of parasites requires additional cell activation, probably by the cytokines generated by T cells, such as IL-5 for the activation of eosinophils and IFN-γ for phagocytic cells. Likewise, the requirement of large volumes of antisera for the successful passive transfer of protective immunity (Armour and Dargie, 1974) and the fact that there is no correlation between antibody titres and protection levels achieved in vaccine trials (Creaney *et al.*, 1995; Dalton *et al.*, 1996; Morrison *et al.*, 1996) indicate that antibodies alone are not sufficient for the full expression of protective immunity. On the other hand, significant protective immunity was achieved by adoptive transfer of lymphoid cells (Corba *et al.*, 1971) indicating a role for T cells in protective immunity. However, despite their central role in immunity, T cell function in *F. hepatica* infected individuals has received relatively little attention.

Murine helper T cell clones have been broadly divided into two groups according to their cytokine repertoires: IFN-γ and IL-2 producing Th1 cells and IL-4, IL-5 and IL-10 producing Th2 cells (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989). However, recent studies indicate that there is no such clear-cut differentiation in human beings and cattle based on the cytokine repertoire of cloned T cells. An additional Th0, or unrestricted T helper cell, co-expressing the cytokines IL-4 and

IFN-γ has been demonstrated (Brown and Estes, 1997). Therefore, IFN-γ-dominated responses are designated as Th1 type responses and IL-4-dominated responses as Th2 type (Mosmann and Sad, 1996; Brown and Estes, 1997). In addition, in contrast to the mouse, bovine IL-10 mRNA expression by all three types of helper T cell clones (Th0, Th1 and Th2) specific for either *Babesia bovis* or *F. hepatica* antigens has been reported (Brown *et al.*, 1994b). IL-10 is an immunoregulatory cytokine which plays a crucial role in modulating the nature of the immune response by inhibiting cytokine production of Th1 type cells (Fiorentino *et al.*, 1991a) and of activated macrophages (Fiorentino *et al.*, 1991b). However, bovine IL-10 inhibits the proliferation of all helper T cell subsets, regardless of their cytokine expression, by affecting the antigen presenting cell function (Brown *et al.*, 1994b).

In cattle, although there is as yet no direct *in vivo* evidence, recent *in vitro* studies indicate that, as has been observed in the mouse model, rboIFN-γ stimulates the production of the IgG2 isotype and IgM (Estes *et al.*, 1994), whereas rboIL-4 upregulates IgG1, IgM and IgE antibody production (Estes *et al.*, 1995), by B lymphocytes in the presence of polyclonal B cell activating factors. Interleukin-2 synergises with IFN-γ or IL-4 to potentiate the production of IgM and IgE respectively.

Earlier studies in cattle with experimental *F. hepatica* infection revealed an *in vitro* fluke antigen-specific proliferation of PBMC early in the infection, but no proliferative response was detectable 5 weeks after a primary infection (Oldham, 1985). *F. hepatica* somatic homogenate antigen-specific helper T cell clones generated from chronically infected cows expressed either a Th2 or a Th0 cytokine profile (Brown *et al.*, 1994a). In addition, a recent study by Clery *et al.* (1996) demonstrated a dominant IgG1 isotype response and undetectable levels of IFN-γ in supernatant from fluke antigen stimulated PBMC cultures in naturally exposed cattle followed by an experimental reinfection with *F. hepatica* metacercariae. All these data imply that a Th2 type immune response is associated with chronic *F. hepatica* infection in cattle. In a preliminary experiment using one cow, we observed a strong proliferation response of PBMC to fluke antigens and IFN-γ production early in the

primary infection, however PBMC were unresponsive to fluke antigens by five weeks after infection. A secondary challenge infection did not restore this loss of responsiveness.

The aim of this chapter is to define T cell responses of calves during the course of a primary and secondary experimental infection with *F. hepatica*. *In vitro* fluke antigen-specific proliferative responses and cytokine (IFN-γ, IL-2 and IL-4) production by PBMC were monitored. The antigen-specific serum antibody isotype response was also monitored by solid phase ELISAs. Since the different IgG isotype responses are controlled by different cytokines, particular emphasis was placed on IgG isotype responses to complement the underlying T helper cell response.

4.2 Results:

The following experiments were carried out using seven calves, six of which were infected with 1000 metacercariae and the seventh was left uninfected and used as a control animal throughout. At week 14 after the primary infection the animals were challenged with a further 700 metacercariae and six weeks later treated with triclabendazole.

4.2.1 Lymphocyte Proliferative Response:

4.2.1.1 Kinetics of lymphocyte proliferative response:

The proliferative responses to *F. hepatica* somatic and E/S antigen preparations of PBMC from a group of experimentally infected calves (n=6) and an age-matched uninfected control calf of the same breed, were monitored during the course of a primary and secondary challenge infection by an *in vitro* lymphocyte proliferation assay. Figure 4.1 summarises the dynamics of the proliferation responses of PBMC from infected and control calves to fluke antigens at weekly intervals. Proliferation was detected by week one after infection with mean stimulation indices of 28.6±11.1 (mean±1 standard error of mean = M±SEM) and 119.3±55.8 for E/S and somatic antigens respectively. The response peaked two weeks after infection with mean stimulation indices 467.1±212 for E/S and 379.1±105 for somatic antigen, and gradually decreased until around 5 weeks post infection when it reached pre-infection levels. After the sixth week post-infection, PBMC became unresponsive to both E/S and somatic antigen. A challenge infection administered 14 weeks after the primary infection resulted in a detectable, but weak proliferation response at week 17 post-infection for both E/S and somatic antigen. However, after treatment of animals with a flukicide, triclabendazole, a significant ($P<0.05$) proliferative response of PBMC to fluke antigens was detectable again.

No antigen-specific proliferation was observed by PBMC obtained from the six calves at three time points before infection (with a mean SI±SD 2.4±3 for E/S and

1.3±0.7 for somatic antigens) and from the control calf throughout the experiment (1.34±1.5 for E/S and 1.8±1.2 for somatic). The T cell mitogen, Con-A, was included in this assay as a positive control. The proliferation induced by Con-A, which was consistently high throughout the experiment, is presented in Fig.4.2

4.2.2 Cytokine Responses of Calves to Fluke Antigens *in vitro*:

4.2.2.1 IFN-γ Levels in Culture Supernatants:

A commercially available sandwich ELISA kit for bovine IFN-γ was used to measure the levels of *F. hepatica* somatic antigen-specific IFN-γ levels in culture supernatants prepared from PBMC of experimentally infected and non-infected calves upon *in vitro* stimulation with fluke somatic antigens for 3 days. PBMC collected from all the calves before infection and from the non-infected control calf throughout the entire experiment produced between 0.6 and 2.2 ng/ml of IFN-γ. IFN-γ was detected in culture supernatants from all six infected calves at two weeks post-infection (Fig.4.3). Levels of IFN-γ increased, reaching a maximum of 8.8 ng/ml at six weeks post-infection and thereafter declined. By ten wpi, levels had decreased to background levels. A second increase in IFN-γ production was observed at week 12 post infection. A slight increase in IFN-γ production, about 1.5 times higher than the levels produced by the non-infected control animal, occurred at 3 weeks after the second challenge infection (week 17). The pattern of IFN-γ levels secreted by PBMC was similar to, but moderately correlated ($r = 0.4$, $t = 1.4$, $p > 0.05$ on a two-tailed regression test) with their proliferation responses to the antigen.

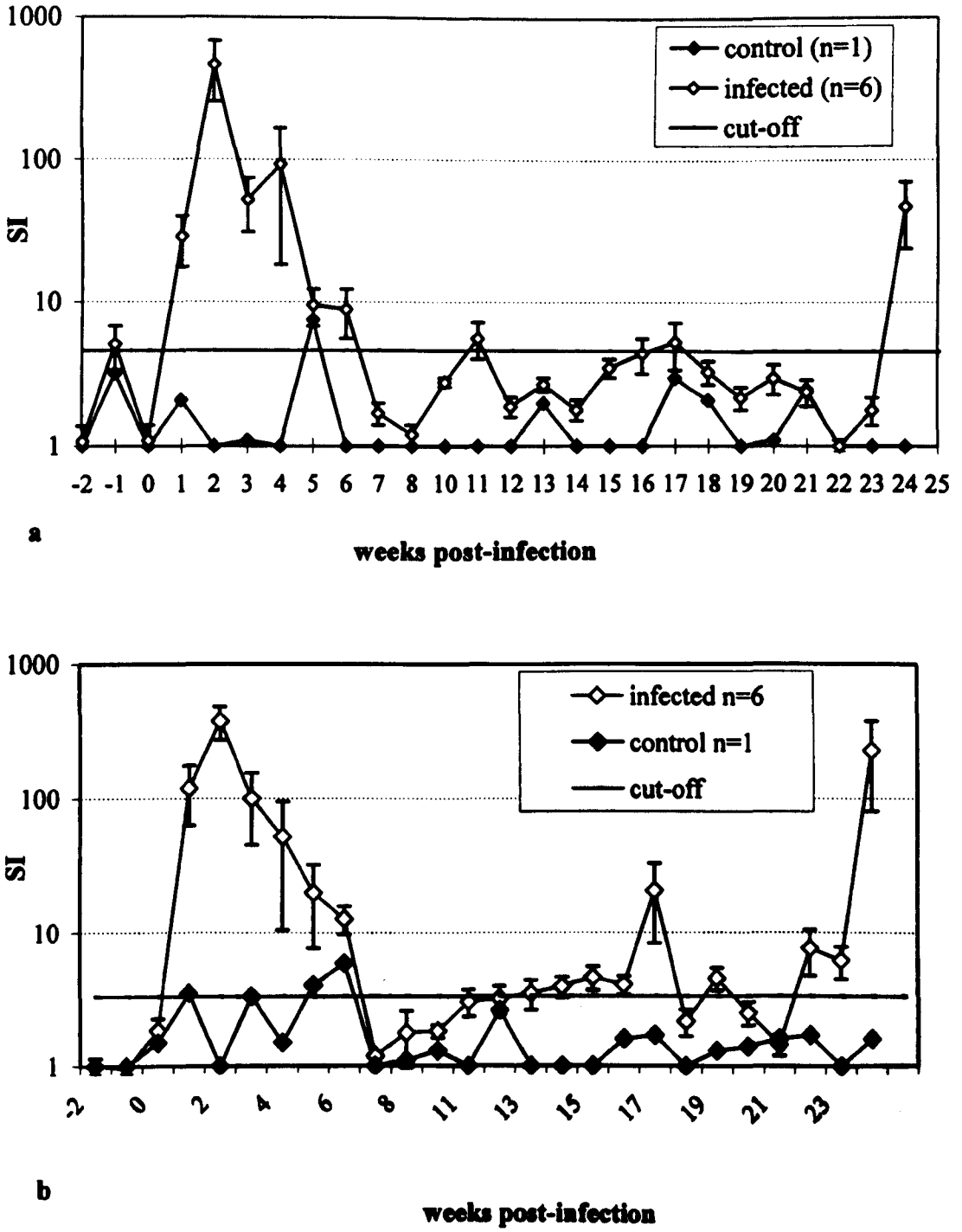


Fig.4.1 Kinetics of *Fasciola hepatica* (a) E/S and (b) somatic antigen-specific proliferation of PBMC *in vitro*. PBMC from infected and control calves at weekly intervals were stimulated *in vitro* with fluke antigens and the proliferation determined by ^{3}H-thymidine uptake. The results are presented as stimulation indices. Cut-off levels were calculated as the mean plus two times the standard deviation of 18 pre-infection SIs and of the SIs of the control animal throughout the experiment.

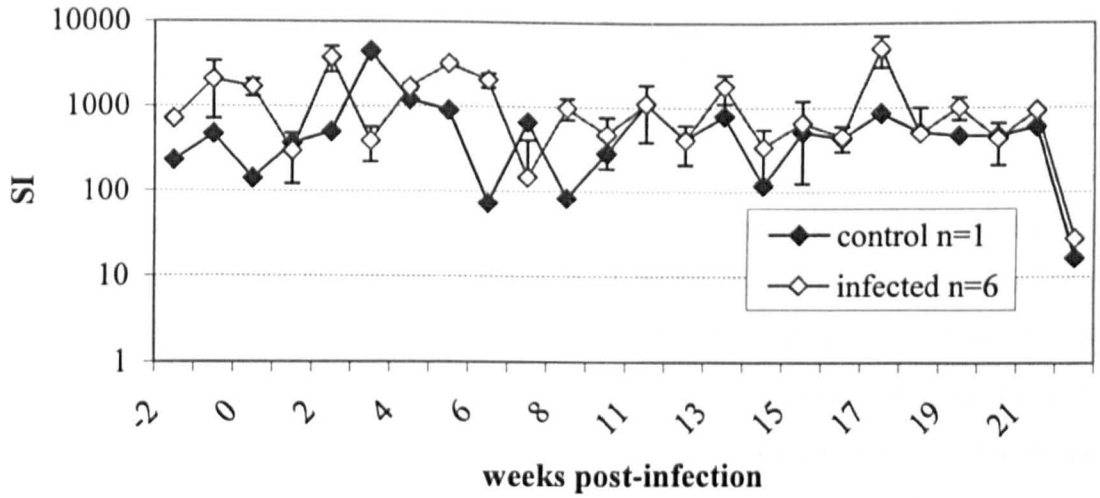


Fig.4.2 Antigen non-specific Con-A induced proliferation of PBMC *in vitro*. PBMC from infected and control calves at weekly intervals were stimulated *in vitro* with Con-A and the proliferation determined by ^{3}H-thymidine uptake. The results are presented as stimulation indices.

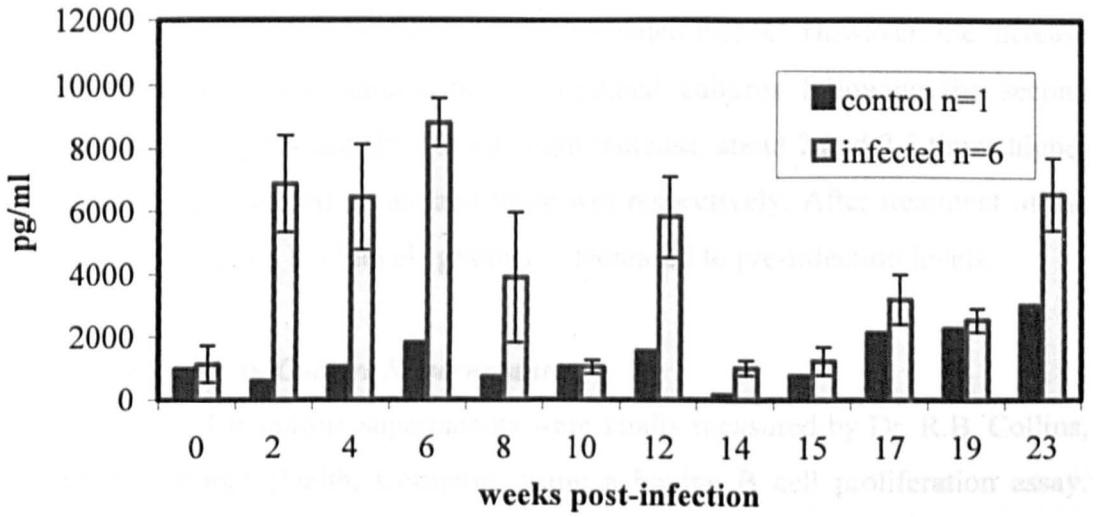


Fig.4.3 *Fasciola hepatica* somatic antigen-specific IFN-γ production of PBMC. PBMC obtained from experimentally infected calves and from the non-infected control calf were stimulated with fluke somatic antigen *in vitro*. IFN-γ levels in culture supernatants were measured at time intervals specified in the figure. The results are expressed as pg/ml after deducting the background levels of IFN-γ measured in the non-stimulated culture supernatants. Error bars represent the standard error of the mean.

4.2.2.2 IL-2 Levels in Culture Supernatants:

Both E/S and somatic antigen-stimulated, together with non-stimulated (medium only) PBMC culture supernatants from all the infected group and from the control calf were tested at weekly intervals for IL-2 production using the T cell proliferation bioassay (Section 2.9.2). IL-2 was undetectable in all the culture supernatants generated from pre-infection samples and from the non-infected control calf. Figure 4.4 shows the kinetics of antigen-induced IL-2 levels in culture supernatants. The profile of IL-2 production was similar to those observed for the proliferative response and IFN-γ production. The background level (0.17 and 0.26 U/ml for E/S and somatic antigens respectively) was set as the cut-off value calculated as the mean of pre-infection and control levels (n=29) plus two times the standard deviation of the mean. IL-2 was detected in culture supernatants from all six infected calves at one week post-infection. Levels of IL-2 increased, reaching a maximum mean of 1.2 U/ml in E/S and 1.95 U/ml in somatic antigen stimulated culture supernatants at three weeks post-infection and thereafter declined. IL-2 decreased to background levels at five wpi in E/S and at seven wpi in somatic antigen-stimulated culture supernatants. After the second challenge infection, there was a modest but consistent increase in IL-2 production by E/S antigen-stimulated PBMC. However, the increase in IL-2 production in somatic antigen-stimulated cultures following the second infection was not significant ($P<0.05$) a slight increase, about 2 and 2.5 times higher than cut-off level occurred at one and three wpi respectively. After treatment of the animals with flukicide, IL-2 levels gradually decreased to pre-infection levels.

4.2.2.3 IL-4 Levels in Culture Supernatants:

The levels of IL-4 in culture supernatants were kindly measured by Dr. R.B. Collins, Institute for Animal Health, Compton, using a bovine B cell proliferation assay. Supernatants of fluke somatic antigen-stimulated cultures from three infected animals (animal no: 123, 125 and 127) and one control animal were tested for IL-4. Samples of supernatants collected pre-infection and at weeks 2,7,12,17 and 22 after infection were tested for IL-4. Low levels of IL-4 were detected, but there was no difference between the infected and non-infected calves at any time point measured (Fig.4.5).

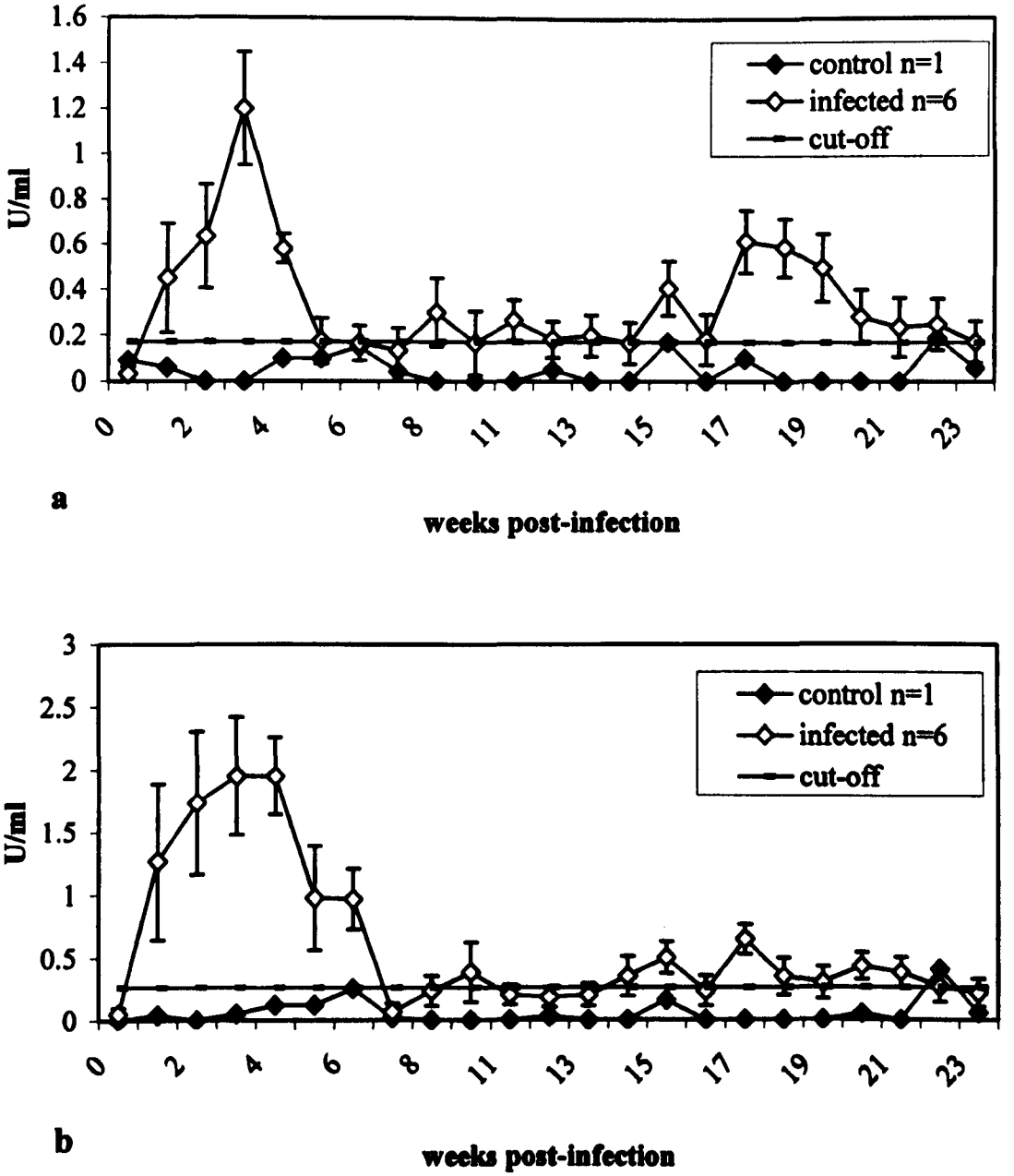


Fig.4.4 Kinetics of *Fasciola hepatica* (a) E/S and (b) somatic antigen-specific IL-2 levels in culture supernatants. PBMC obtained from experimentally infected calves and from the non-infected control calf were stimulated with fluke antigens *in vitro*. Culture supernatants were collected and IL-2 levels were measured at weekly intervals. The results are expressed as U/ml after deducting the background levels of IL-2 measured in the non-stimulated culture supernatants. Cut-off levels were calculated as the mean plus two times the standard deviation of 18 pre-infection levels and of the levels of the control animal throughout the experiment. Error bars represent the standard error of the mean.

4.2.3 Antibody Responses of the Calves:

4.2.3.1 Fluke E/S Antigen Specific IgG Responses:

The kinetics of IgG responses to fluke E/S antigen in calves infected with *F. hepatica* and in the non-infected control calf are presented in Figure 4.6. The cut-off value was calculated as the mean of pre-infection and control levels (n=29) plus two times the standard deviation of the mean. An increase in antigen specific IgG was observed two weeks after infection and reached peak levels at 10 weeks post infection. After the second infection at 14 weeks post primary infection, the antibody levels remained high. The antibody levels in serum samples obtained 11 weeks after the treatment had started to decline.

4.2.3.2 Fluke E/S Antigen Specific IgG Isotype (IgG1 and IgG2) Responses:

A strong IgG1 and a relatively weak IgG2 response of the calves to fluke E/S antigen was observed following infection (Fig.4.7). The kinetics of the IgG1 response were similar to those of total IgG. Antigen-specific IgG1 antibodies were detectable as early as the second week after infection, peaked at around 10 weeks and remained high throughout the experiment. In contrast, although low in comparison with IgG1, IgG2 levels peaked (OD=0.44) as early as the second week after infection, gradually declined to detectable but lower levels (OD=0.34) by 6 weeks post-infection and remained around this level throughout the experiment.

4.2.3.3 Fluke E/S Antigen Specific IgM and IgA Responses:

The cut-off value was set as described above for total IgG. The kinetics of IgM and IgA responses to fluke E/S antigen in calves infected with *F. hepatica* and in the non-infected control calf are presented in Figure 4.8. Both IgM and IgA levels peaked early, by the second week after infection. IgM levels gradually declined to the cut-off level by 6 weeks post-infection and remained low throughout the experiment. No increase in IgM levels occurred after the challenge infection. However, after an initial peak at two weeks post infection, IgA levels reached a plateau, but declined from 20 wpi after treatment.

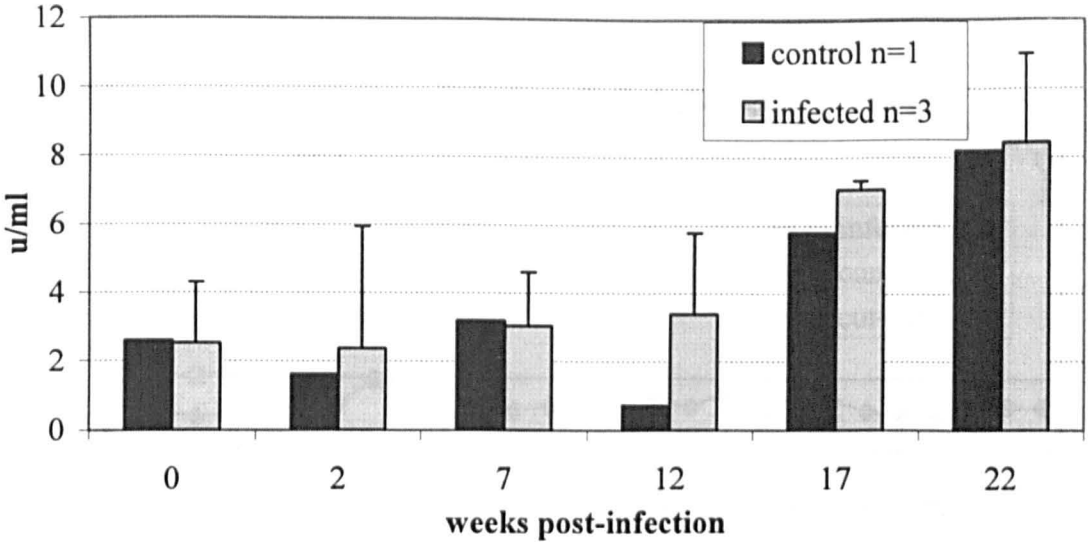


Fig.4.5 IL-4 levels in culture supernatants generated from fluke somatic antigen-stimulated PBMC cultures. IL-4 levels were measured at time intervals specified in the figure. The results are expressed as U/ml. Error bars represent the standard error of the mean.

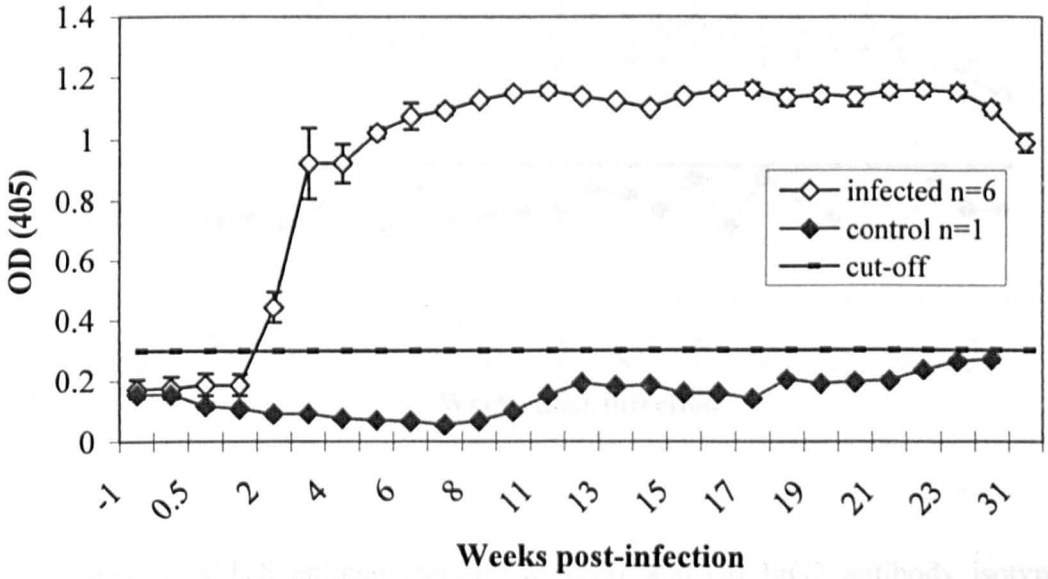


Fig.4.6 Kinetics of E/S antigen-specific total IgG response of calves. Total IgG levels in weekly serum samples were measured by ELISA and the results are expressed as OD values. The cut-off level was calculated as the mean plus two times the standard deviation of 12 pre-infection ODs and of those of the control animal throughout the experiment. Error bars represent the standard error of the mean.

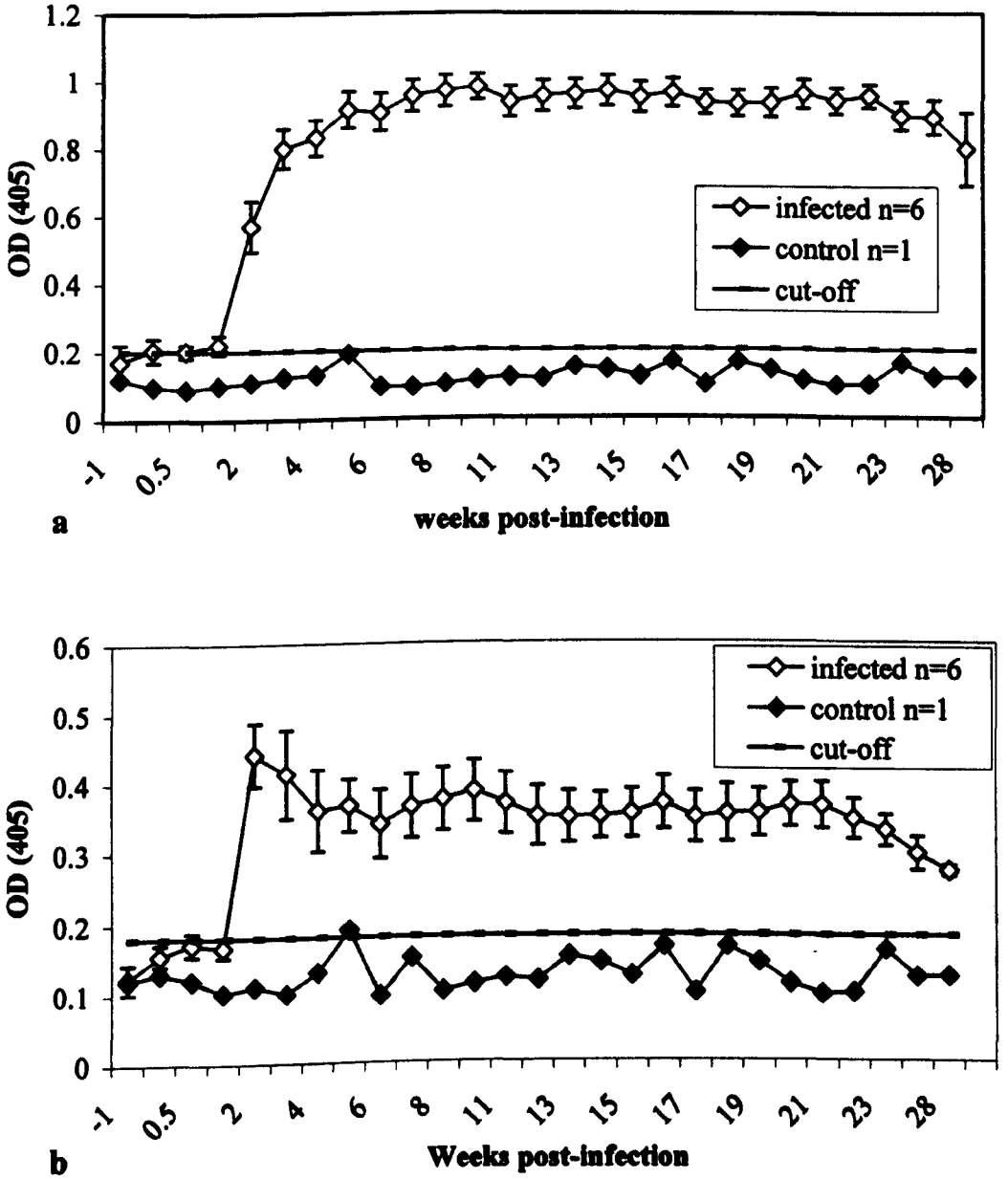
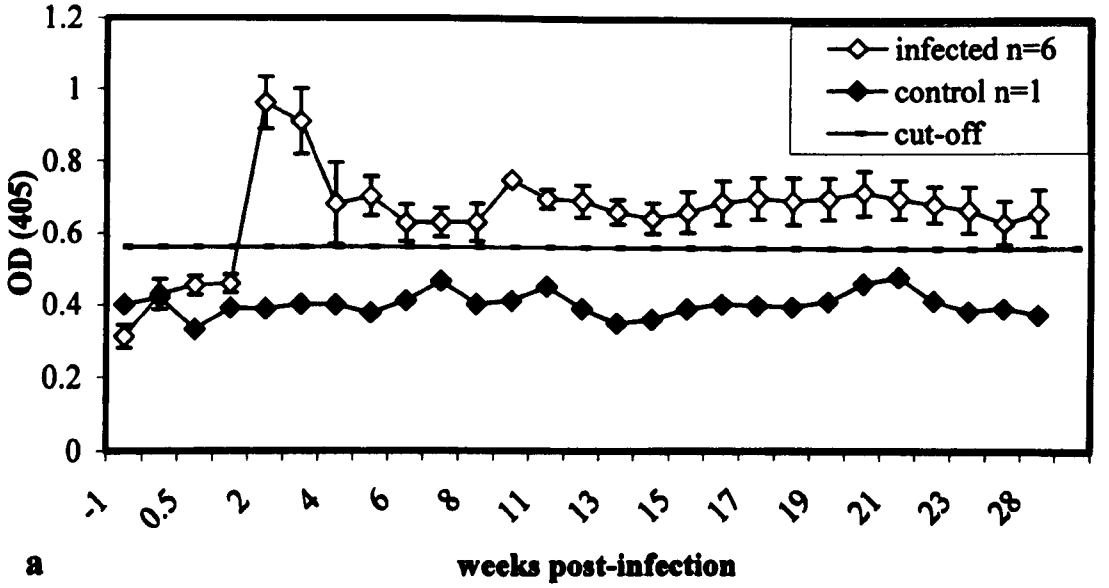
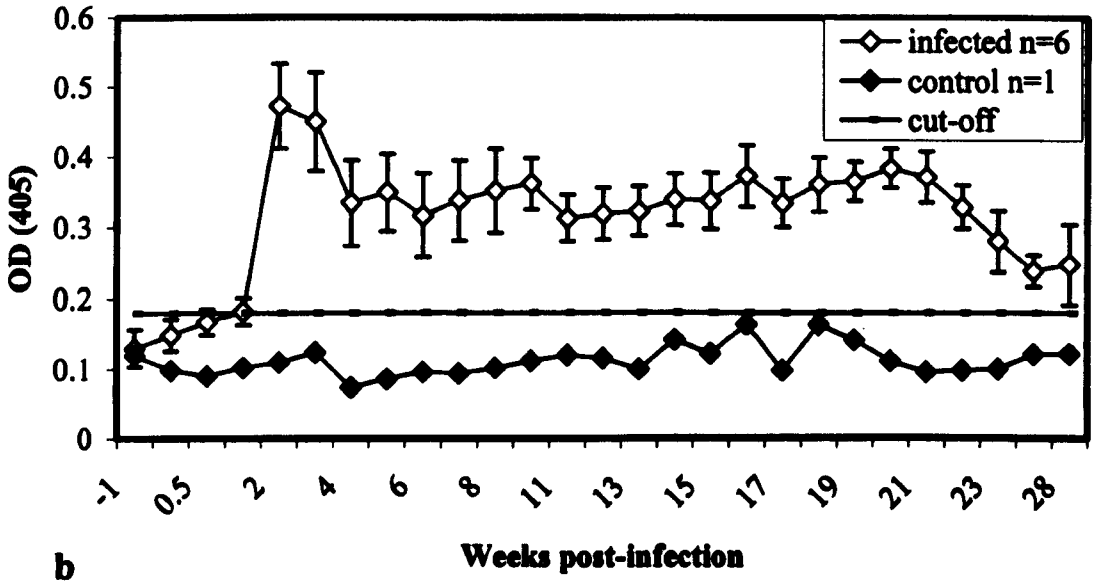


Fig.4.7 Kinetics of E/S antigen-specific (a) IgG1 and (b) IgG2 antibody isotype responses of calves. IgG1 and IgG2 levels in weekly serum samples were measured by ELISA and the results are expressed as OD values. The cut-off level was calculated as the mean plus two times the standard deviation of 12 pre-infection ODs and of those of the control animal throughout the experiment. Error bars represent the standard error of the mean.



a



b

Fig.4.8 Kinetics of E/S antigen-specific (a) IgM and (b) IgA antibody responses of calves. IgM and IgA levels in weekly serum samples were measured by ELISA and the results are expressed as OD values. The cut-off level was calculated as the mean plus two times the standard deviation of 12 pre-infection ODs and of those of the control animal throughout the experiment. Error bars represent the standard error of the mean.

4.3 Discussion:

In this study, we have described the kinetics of proliferation and cytokine production of PBMC from calves experimentally-infected with *F. hepatica* upon *in vitro* stimulation with *F. hepatica* E/S or somatic antigens. In addition, the antibody isotype and sub-isotype responses were examined.

Early in the primary infection, there was a strong proliferation response of PBMC to both fluke E/S and somatic antigens, with mean peak SI levels of 467.1±212 and 379.1±105 respectively. At week 2 post-infection in two individuals, the proliferation induced was as high as that induced by the T cell mitogen Con-A. It might be argued that such an unusually strong response was caused by mitogen-like substances in the antigen preparations. However, since mitogens induce a non-specific response, had they been present in the antigen used in this experiment then proliferation of PBMC obtained before the infection and of PBMC from the control animal would have been expected. While PBMC obtained from the non-infected calf and from the infected group before infection proliferated vigorously upon Con-A activation, they did not proliferate in the presence of fluke antigens *in vitro*, indicating that our antigen preparations did not contain any mitogenic properties and that the proliferation induced after the infection was specific for the antigens. An antigen dose-dependent proliferation response was also observed (Fig.3.6 of Chapter 3), which supports this argument.

The proliferation of PBMC to fluke antigens was detectable as early as the first week after infection, peaked in the second week and gradually decreased to undetectable levels by the sixth week post-infection. An early proliferative response of PBMC to fluke antigen was reported by Oldham, (1985) and Oldham and Williams, (1985) in calves experimentally-infected with *F. hepatica*. These authors showed that PBMC become unresponsive to antigen after the fifth week post-primary infection. Our results support their observation. It is not known why PBMC, while responding vigorously early in the infection, become unresponsive *in vitro* to fluke antigens after 6 wpi. One possibility is that polarisation of a Th1 type response to a Th2 type might

be responsible for the loss of T cell proliferation. All bovine helper T cell subsets have been shown capable of expressing (or producing) IL-10 mRNA transcript (Brown *et al.*, 1994b). Antigen-induced proliferation of bovine T cells (Th0, Th1 and Th2 subsets) was inhibited by 75-94% in the presence of rhIL-10 (Brown *et al.*, 1994b). However, other possible explanations, such as a lack of IL-2 production and elevated levels of TGF-β and NO production by PBMC during the unresponsive period, were investigated and are addressed in Chapter 6.

Transient decreases in T cell reactivity to antigens and/or T cell mitogens have been reported in calves infected with *Ostertagia ostertagi* and *Trichostrongylus axei* (Klesius *et al.*, 1984; Snider *et al.*, 1986; Wiggin and Gibbs, 1990). Recently, Canals *et al.*, (1997) reported a substantial reduction of IL-2 and IFN-γ transcription in calves after an experimental infection with *O.ostertagi*. The authors suggested that this commonly observed unresponsiveness of bovine T cells to *O.ostertagi* antigens and to T cell mitogens might be due either to a general decrease in T cell reactivity or to a down-regulation of Th1 responses. In contrast to the above observations, the responsiveness of PBMC from *F. hepatica* infected calves to the T cell mitogen, Con-A, was consistently high. Therefore, the unresponsiveness to fluke antigens observed in this experiment is unlikely to be the result of a general decrease in T cell reactivity. High levels of IL-2 and IFN-γ production early in the infection, low or undetectable levels later in the infection and a very strong positive correlation between somatic antigen specific proliferation and IL-2 production ($r = 0.7$, $t = 4.45$, $p < 0.01$) (see below) may indicate that down-regulation of IL-2 producing Th1 type cells is responsible for the unresponsiveness.

A challenge infection administered at 14 weeks after the primary infection resulted in a detectable, but weak and non-significant proliferation response at week 17 post-infection to both E/S and somatic antigen. To date there is no conclusive evidence that the low level of response or unresponsiveness to the challenge infection were due to the presence of adult flukes. However, our observation, that after treatment of the animals with a flukicide, triclabendazole, a significant ($P<0.05$) proliferative response of PBMC to fluke antigens was again detectable, suggests that further

research may be warranted to determine whether the immune response is modulated by the presence of adult flukes or of antigen/s released from the late immature or mature flukes. It is well documented that *Schistosoma* egg antigens induce a predominantly Th2 cytokine-mediated immune response (Scott *et al.*, 1989). Detailed time-course studies have revealed that an initial schistosomula-induced Th1 type response is down-regulated following the onset of egg production by egg antigen-induced Th2 type responses (Pearce *et al.*, 1991; Wynn *et al.*, 1993; Capron, 1998;). Moreover, recent studies using cross-regulatory cytokines (IFN-γ, IL-12 vs. IL-4 and IL-10) have documented a complex regulatory network and the feasibility of achieving a novel vaccine strategy in the mouse model of schistosomiasis, based on the administration of rIL-12 with egg antigens (Oswald *et al.*, 1994; Chensue *et al.*, 1994; Wynn *et al.*, 1995; Flores-Villanueva *et al.*, 1996; Boros and Whitfield, 1999). These manipulations have induced a strong inhibition of Th2-type granuloma formation. It is, therefore, very tempting to speculate that *F. hepatica* may be using similar strategies such as the sequential expression of antigen/s, inducing a Th2 type response which, in turn, downregulates the ongoing Th1-like response.

During the early primary infection, fluke antigen-induced IFN-γ and IL-2 were detectable and both were significantly correlated with the proliferation responses of PBMC. There was bi-phasic IFN-γ production during the primary infection. After a strong initial response, between weeks 2 and 6 post-infection, levels returned to the pre-infection levels by week 10 post-infection. A similar result has recently been published by Clery and Mulcahy (1998), who reported IFN-γ production during early primary infection, in contrast to their earlier observation of the failure of IFN-γ production in PBMC obtained from chronically infected cattle (Clery *et al.*, 1996). A second, statistically significant ($p<0.05$) increase in IFN-γ levels occurred at 12 weeks post infection by which time flukes were becoming adult and reaching their final destination in the bile ducts. This was confirmed by the presence of fluke eggs in the faeces from 10 weeks post-infection onwards.

Significant levels of antigen-induced, biologically active IL-2 were detected in supernatants from PBMC early in the primary infection. This is consistent with the

findings reported by Oldham and Williams (1985) who showed significantly elevated levels of IL-2 like T cell growth factor in culture supernatants from cattle one to two weeks after primary infection with *F. hepatica*. Although the authors referred to this T cell growth factor as IL-2, their assay did not discriminate between other factors that induce T cell proliferation. Here we have developed a sensitive and specific bioassay to measure biologically active bovine IL-2. Since proliferation was successfully blocked by a Mab, IL-A111, specific to IL-2 receptor α-chain (Naessens *et al.*, 1992), we were able to measure IL-2 levels accurately in culture supernatants (see Chapter 3).

The antibody response to *F. hepatica* was dominated by an IgG1 response, but an increase in *F. hepatica* E/S antigen-specific IgG2 antibody levels was also observed early in the primary infection. IgG2 and IgM levels peaked at two weeks post-infection and gradually returned to lower but still detectable levels by the fifth week post-infection. Since both IgM and IgG2 secretion by bovine B cells is regulated by a Th1 cytokine, IFN-γ (Estes *et al.*, 1994), this result may indicate that the Th1-like response observed *in vitro* early in the primary infection parallels that observed *in vivo*. However, the presence of an early IgA antibody response and of constantly increasing IgG1 responses that are thought to be regulated *in vitro* by IL-4 (Estes *et al.*, 1995), suggests that the regulation of immune responses *in vivo* is of a complex nature and can not be wholly explained by a Th1/Th2 dichotomy.

In contrast to the primary infection, a super-infection with *F. hepatica* induced neither IFN-γ nor IL-2 production by PBMC *in vitro*. There was also no increase in antigen specific serum IgG2 levels after this super-infection. These results are in agreement with those reported by Clery *et al.* (1996) and Brown *et al.* (1994a). Clery *et al.* reported undetectable levels of IFN-γ in PBMC culture supernatants from chronically-infected cattle. Likewise, *F. hepatica* antigen-specific T cell clones generated from cattle chronically-infected with *F. hepatica* expressed either Th2 or Th0, but no Th1 cytokine profiles (Brown *et al.*, 1994a).

In contrast to IFN-γ and IL-2, no antigen-induced IL-4 was detectable after infection. Two to 4 U/ml of IL-4 were produced during the primary infection and the levels increased to 6 to 8 U/ml after the challenge infection, but there was no difference between the levels produced in infected compared to uninfected calves.

Studies with the closely-related human trematode *Schistosoma* spp. in a mouse model revealed a central role for helper T cell subsets that determines either the protection of the host or the development of fibrotic pathology (Scott *et al.*, 1989; Sher and Coffman, 1992). A Th1 type response is induced during the pre-patent phase of the infection that correlates with the host resistance and leads to protection, whereas, during the onset of egg production by parasites, a Th2 type response is predominant and leads to immunologically-mediated granulamatous pathology (Xu *et al.*, 1991; Pearce *et al.*, 1991). However, recent studies on *Schistosoma* infections in the natural human host suggest a central protective role for antibody-dependent cell cytotoxicity (Capron, 1998).

In conclusion, from the data presented here and the data accumulated to date, it is not possible at this stage to make an assumption that one type of T helper cell response is actually protecting the host, whereas the other type is non-protective and enabling parasite survival as is suggested in *Schistosoma spp.* infections in mice. However, our results suggest that that there was a Th1-like immune response during the early primary infection which probably polarised to a Th2-like response later in the infection.

CHAPTER FIVE

Changes in haematological and biochemical parameters and in lymphocyte sub-populations during the course of *Fasciola hepatica* infection in cattle

5.1 Introduction:

The pathology and pathogenesis of fasciolosis were described in detail during the 1960s and recently reviewed by Behm and Sangster, (1999). Animals vary in their susceptibility to *Fasciola* spp. according to their size, species/breed, the intensity of the infection and the number of infective metacercariae consumed. Cattle, like sheep, are susceptible to the disease, but require over 1000 metacercariae to show clinical fasciolosis (Boray, 1969). The clinical disease in cattle is characterised by anaemia, eosinophilia, weight loss, hypoproteinaemia (loss in serum albumin), elevated liver enzyme levels in the blood and occasionally death (if infected with large doses of metacercariae). However, unlike sheep, cattle are considered to acquire a strong protective immunity (Haroun and Hillyar, 1986) which creates a hostile environment for migrating flukes in the body cavity and liver parenchyma.

Infection with *F. hepatica* causes anaemia, a key contributory factor in morbidity and mortality. The aetiology of anaemia in *F. hepatica* infected animals has long been debated, however it is now widely accepted that it is a haemorrhagic anaemia (Behm and Sangster, 1999). A single fluke has been reported to consume 0.2–1ml of blood per day (Jennings *et al.*, 1956; Dawes and Hughes, 1964 and 1970). However, it has been suggested that the anaemia may be caused not only by the haematophagic behaviour of the flukes but also by blood loss due to extensive haemorrhaging in the liver parenchyma caused by migrating flukes and by ulceration in the bile ducts (Dawes and Hughes, 1970). Moreover, Sewell *et al.*, (1968) considered that the major blood loss was through the bile duct epithelium and that dyshaematopoiesis was not a major factor in the development of anaemia in fasciolosis.

Hypoalbuminaemia and hyperglobulinaemia are common features of fasciolosis. The liver is the only organ that synthesises serum albumin. Liver damage caused by the migrating flukes is thought to jeopardise the liver function and therefore the production of albumin. The decline of plasma albumin in infected animals has been attributed partly to reduced rate of synthesis (Dawes and Hughes, 1964) and partly to the direct loss of blood due to the destructive and blood feeding activities of flukes (Dawes and Hughes, 1970). An increased rate of immunoglobulin synthesis by the lymphocytes during the course of infection may compensate for the loss of albumin and therefore increase the total plasma protein levels.

Eosinophilia, as is the case in many parasitic infections, is a characteristic feature of fasciolosis in all host species. The number of eosinophils in circulation begins to increase 1 or 2 weeks after infection, rising to high levels during the course of infection (Sinclair, 1975; Poitou *et al.*, 1992, 1993). An increase in the numbers of eosinophils together with other leukocytes around the site of infection in the gut epithelium or around the migrating tracks has also been a common feature in *F. hepatica*-infected animals (Doy *et al.*, 1978; Burden, 1983; Meeusen *et al.*, 1995). However, although various attempts have been made to demonstrate the mechanisms of killing using *in vitro* systems, to date only the killing of newly excysted juvenile flukes (NEJ) has been achieved using a major basic protein isolated from bovine eosinophils (Duffus *et al.*, 1980). An antibody dependent adherence of eosinophils and neutrophils to flukes *in vitro* has been demonstrated (Doy *et al.*, 1980; Duffus and Franks, 1980), but without causing lethal damage to the NEJs.

An increase in hepatic enzymes in the blood is commonly seen as a result of damage to liver tissue caused by the flukes. Due to the destruction of liver parenchyma by the migrating flukes, and of bile duct epithelium by the adult fluke, levels of glutamate dehydrogenase (GLDH) and γ-glutamyl transferase (GGT) increase in the plasma. It is known that there is a close relationship between levels of GLDH and GGT in serum and the extent of the damage caused by flukes to liver tissue and bile ducts respectively. These enzymes are considered to be indicators of pathology.

In this chapter, weekly blood samples from the same group of calves used previously (Chapter 4) to evaluate *in vitro* humoral and cellular immune responses were subjected to detailed haematological and biochemical investigation. In an attempt to follow changes in red blood cell numbers and in the quantity of haemoglobin, blood samples were analysed by standard haematological techniques. Changes in leukocyte and/or lymphocyte sub-populations during the course of a primary and a secondary infection and after treatment were also investigated by total and differential cell counts and by flow cytometry. The serum samples taken from the calves at weekly intervals were monitored for the levels and kinetics of the liver enzymes GLDH and GGT. These enzymes are considered to be indicators of pathology. The results of the above work, together with parasitological findings (i.e. egg count) and body weight gain, are discussed in relation to immunological parameters

5.2 Results:

5.2.1 Haematological findings:

5.2.1.1 Red blood cell series and haemoglobin parameters:

The mean red blood cell (RBC) count, packed cell volume (PCV), and mean corpuscular volume (MCV) are shown in Figures 5.1a, b and c respectively. An arbitrary cut off value was estimated for RBC count as the mean minus two standard deviations of the mean of weekly data gathered from the control animal. There was a significant reduction in RBC count after 13 weeks post infection ($P<0.05$, unpaired two-tailed t-test). The reduction was highly significant after the challenge infection administered 14 weeks after the primary infection ($P<0.001$). During the course of the experiment, a decrease in RBC count was also observed in the control animal, although to a lesser extent. Although there was a decrease in the PCVs of the infected group immediately following the primary and secondary infections, the differences were not significant ($P>0.05$) and followed the same trend as the control. There was no difference in MCVs recorded between the mean of the infected animals and of the control animal.

There were no differences in haemoglobin (HB, g/dl) concentrations, mean corpuscular haemoglobin concentration (MCHC, g/dl), and mean corpuscular haemoglobin (MCH, pg) between infected and control animals (Fig. 5.2).

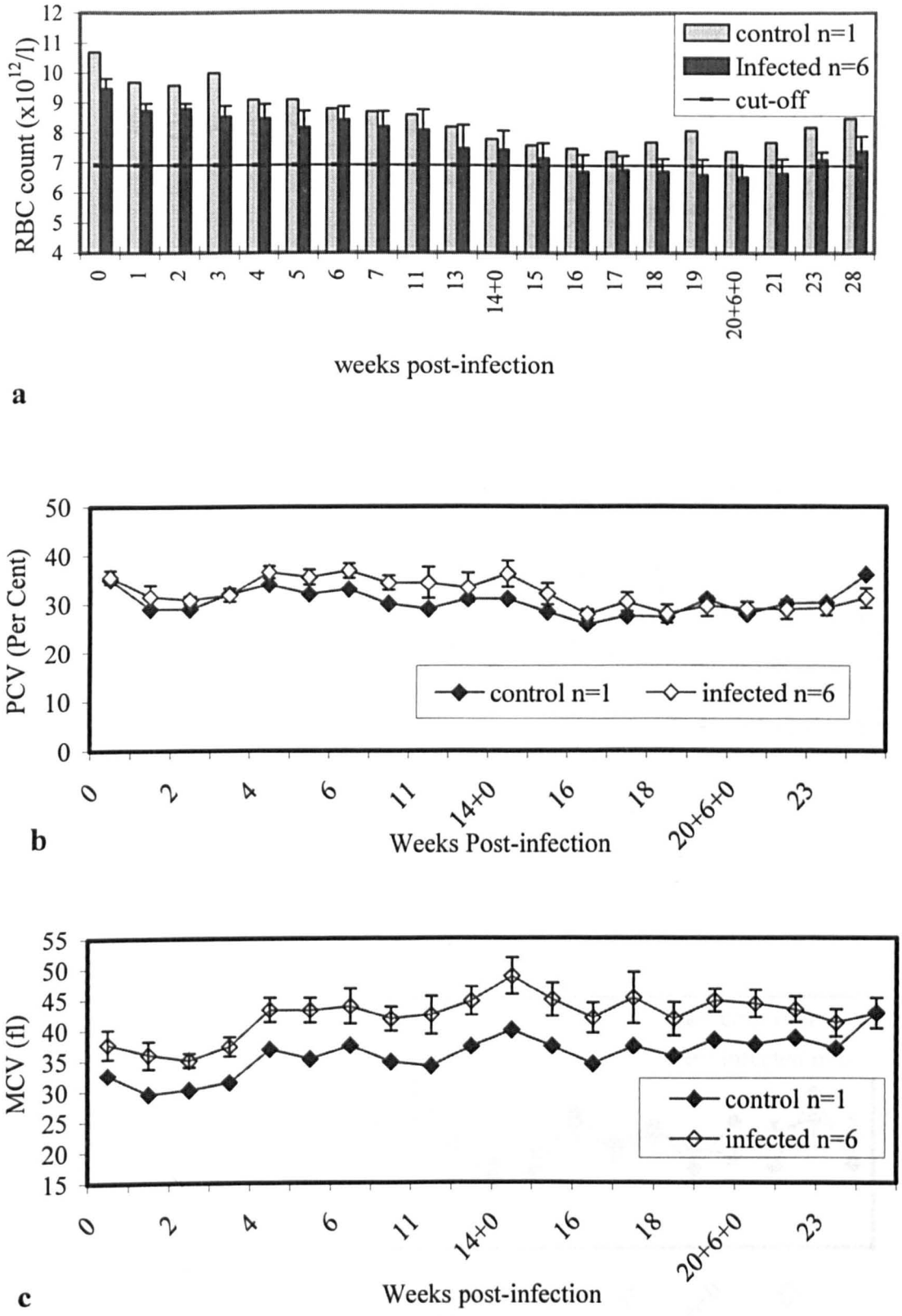


Fig.5.1 Kinetics of (**a**) RBC count, (**b**) PCV and (**c**) MCV values in experimentally infected and control calves. Cut-off value in (a) was calculated as the mean of weekly values of the control animal minus two standard deviations of the mean. $14^{+}0$ and $20^{+}6^{+}0$ indicate the administration of second challenge infection and treatment respectively.

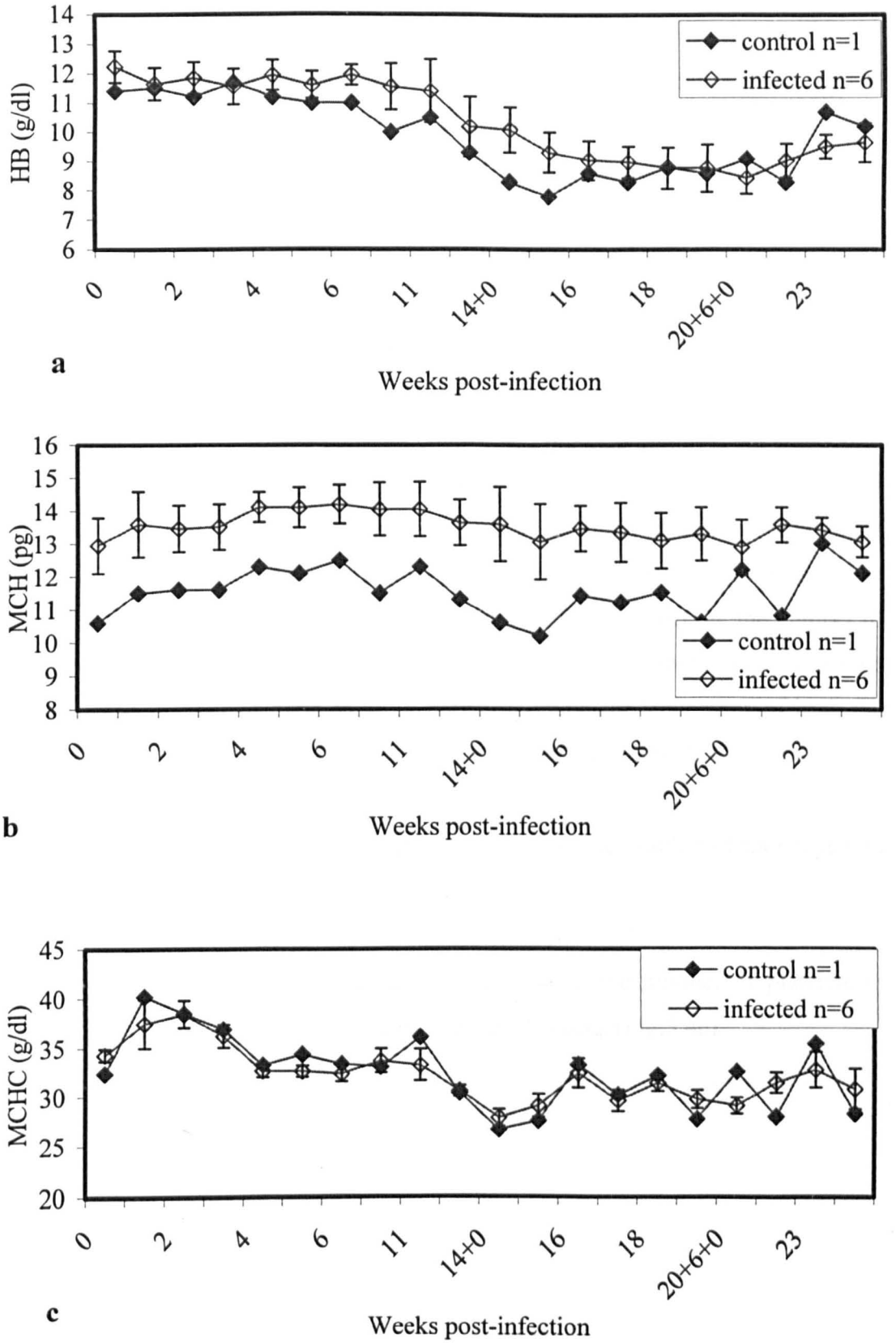


Fig.5.2 Kinetics of (**a**) HB concentration, (**b**) MCH and (**c**) MCHC values in experimentally infected and control calves. $14^{+}0$ and $20^{+}6^{+}0$ indicate the administration of second challenge infection and treatment respectively.

5.2.1.2 White blood cell series:

5.2.1.2.1 Total white blood cell counts: Total white blood cell (WBC) counts increased gradually following infection (Fig.5.3a). Significant increases were observed at 11 and 12 weeks after the primary infection and at 1, 2, 4 and 5 weeks following the challenge infection at week 14 ($P<0.05$, unpaired t-test).

5.2.1.2.2 Polymorphonuclear leukocyte counts: Neutrophils and eosinophils counts are presented in Figure 5.3b and c respectively. There was insufficient data for the number of basophils to allow meaningful analysis (data is not shown). There was no difference in neutrophil counts between control and infected animals, but eosinophil numbers were significantly elevated following infection ($P<0.001$) and remained high until three weeks after treatment. A detectable increase in eosinophil numbers was observed as early as the second week post-infection, peaked at 11 weeks after the infection, thereafter decreasing. Following the challenge infection, the number of eosinophils in circulation again started to increase, peaking 2 weeks post-challenge infection. The number of band form of polymorphonuclear leukocytes of both the control and infected animals followed a similar fluctuating pattern (Fig. 5.4a).

5.2.1.2.3 Monocytes: There was no difference between the number of monocytes in the control and infected animals (Fig.5.4b).

5.2.1.2.4 Platelets: There was a significant increase in the number of platelets in circulation when the infection reached patency at 11 weeks (Fig.5.4c).

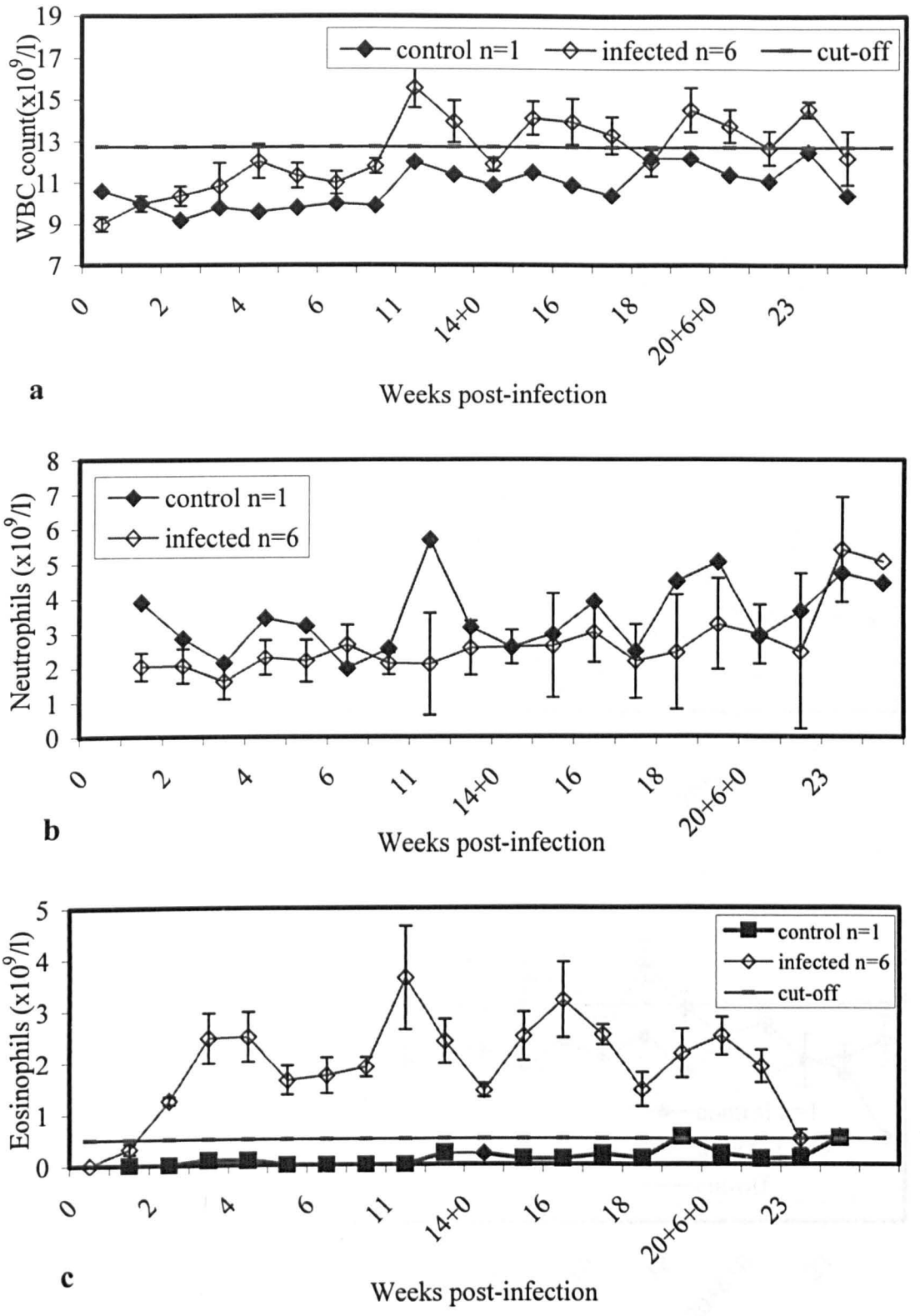


Fig.5.3 Kinetics of total (**a**) WBC, (**b**) neutrophil and (**c**) eosinophil counts in experimentally infected and control calves. Cut-off values in (a and c) were calculated as the mean of the weekly values of the control animal plus two standard deviations of the mean. $14^{+}0$ and $20^{+}6^{+}0$ indicate the administration of second challenge infection and treatment respectively. Error bars represents one standart error of the mean of the infected group.

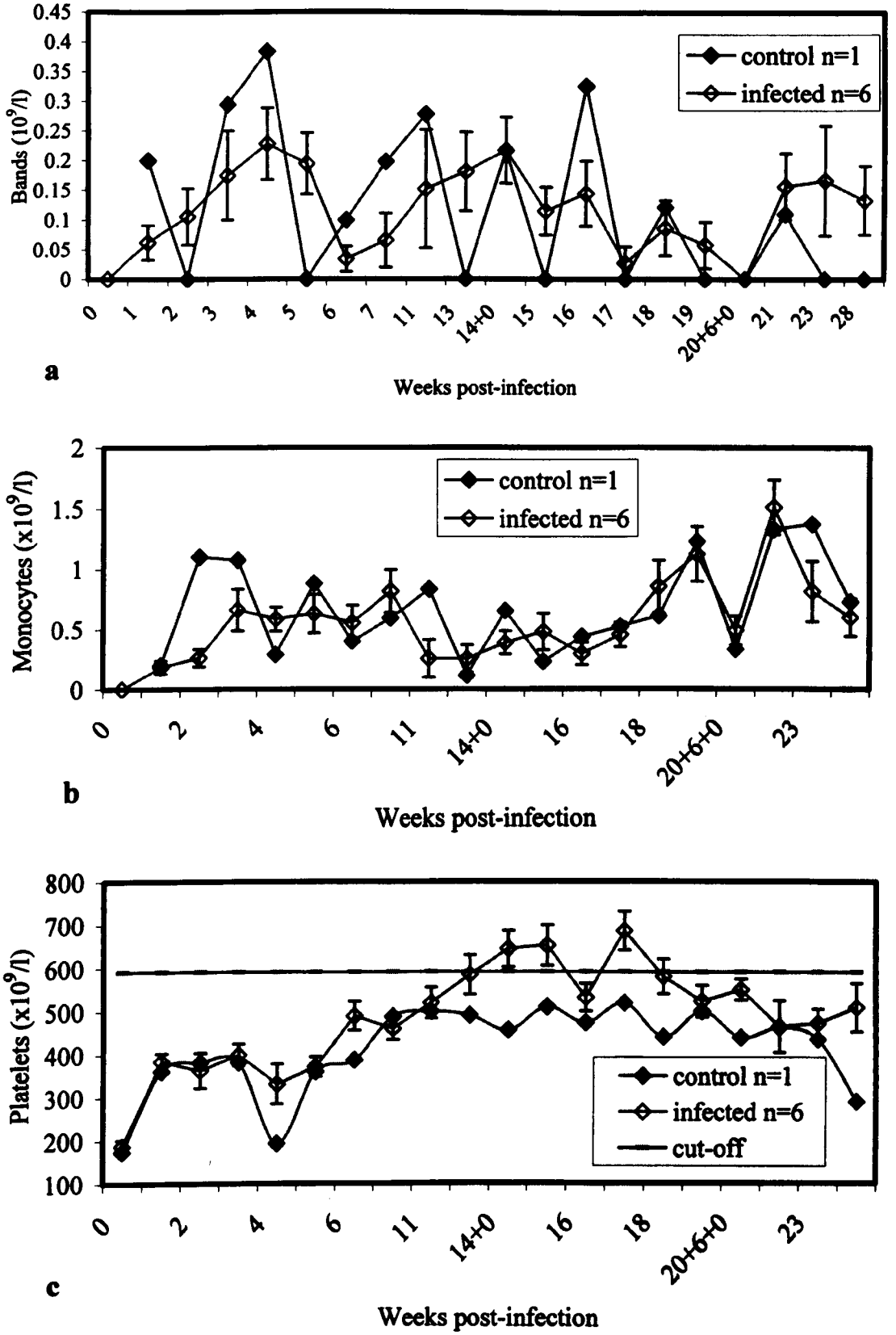


Fig.5.4 Kinetics of (a) bands, (b) monocytes and (c) platelets counts in experimentally infected and control calves. Cut-off value in (c) was calculated as the mean of the weekly values of the control animal plus two standard deviations of the mean. $14^{+}0$ and $20^{+}6^{+}0$ indicate the administration of second challenge infection and treatment respectively. Error bars represent one standard error of the mean in the infected group.

5.2.1.2.4 Lymphocytes: There were no differences in the total number of circulating lymphocyte counts between the infected calves and the control calf (Fig.5.5a). Flow cytometry analysis of PBMC revealed that, with the exception of the B lymphocyte sub-population (Fig.5.5b), there was no difference in the numbers of other circulating lymphocyte sub-populations ($CD4^+$, $CD8^+$, $CD2^+$ and $WC1^+$ T cell populations). Figures 5.5b, 5.6 and 5.7 summarise the results of lymphocyte sub-populations. There were highly significant increases in the number of B cells at 7 and 11 weeks after infection and for four consecutive weeks following the challenge infection ($p<0.001$, unpaired t-test with two tail).

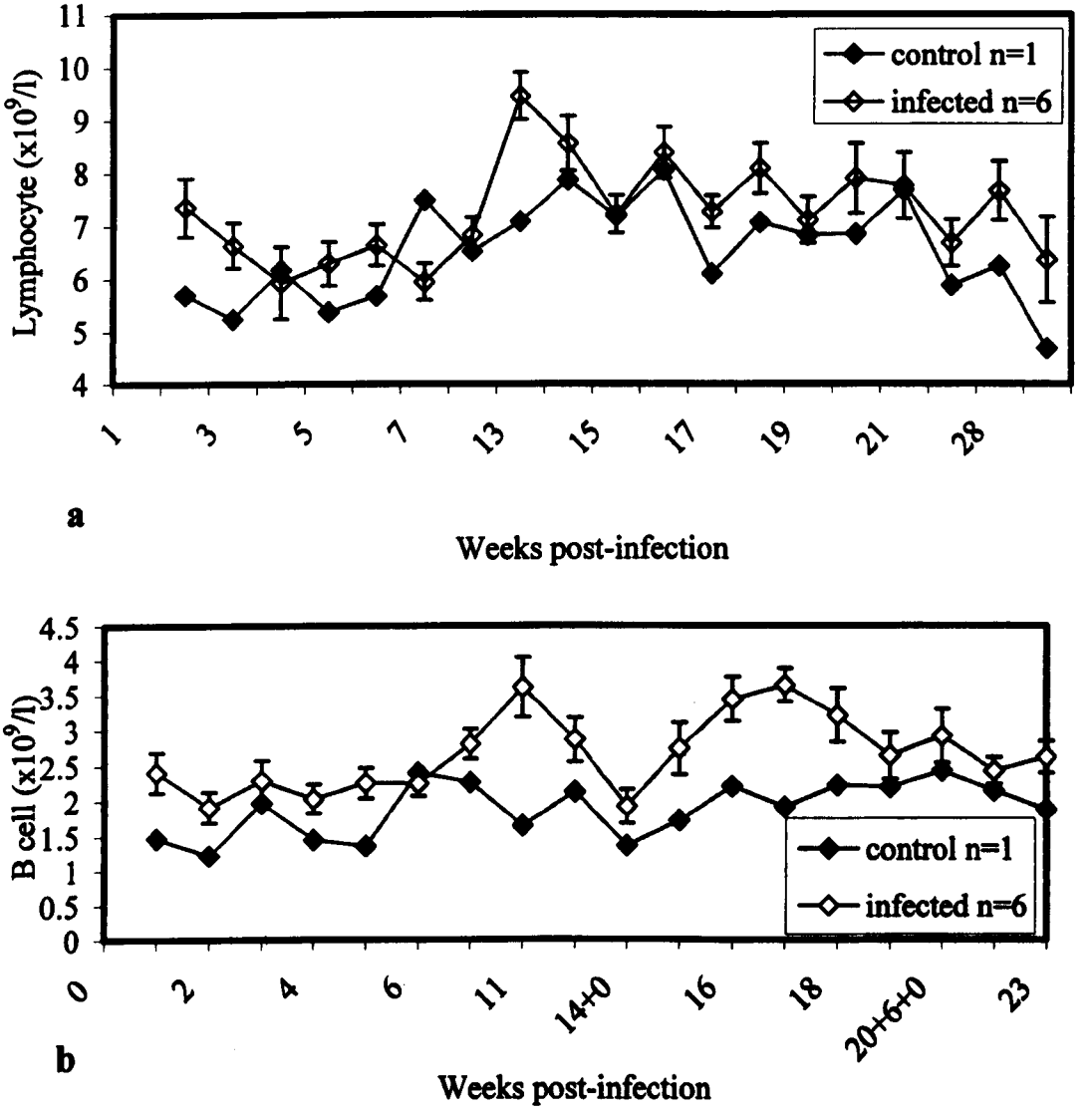
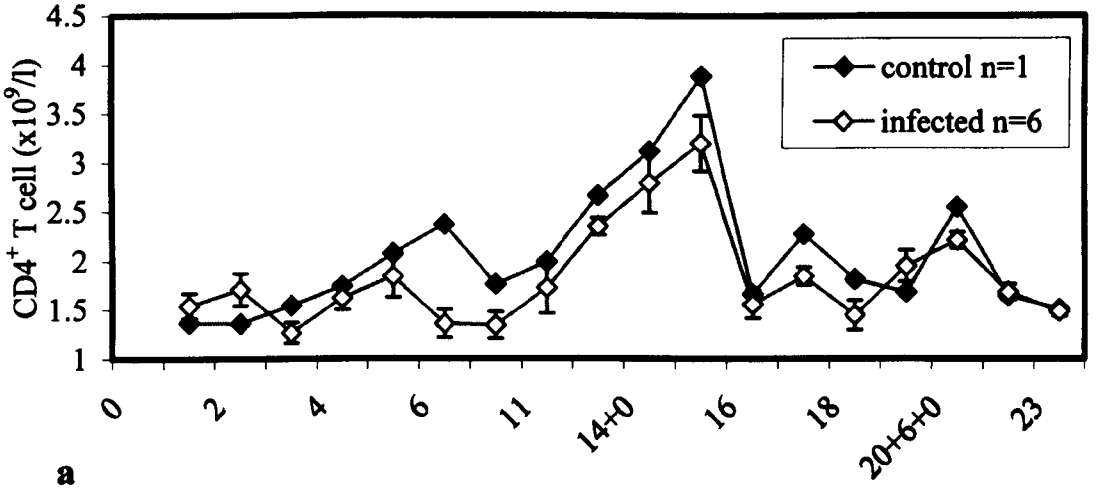
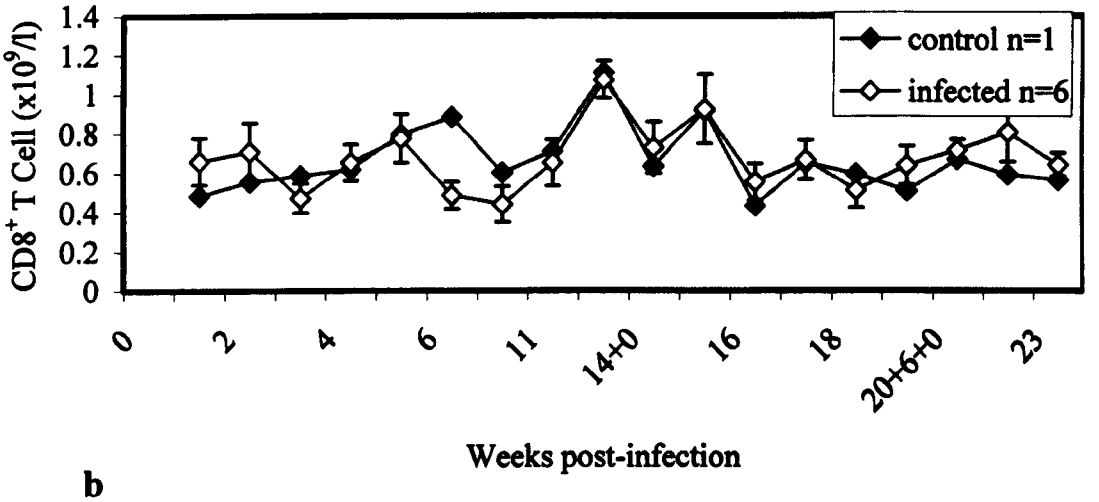


Fig.5.5 Kinetics of (**a**) total lymphocyte and (**b**) B cell counts in experimentally infected and control calves. 14^+0 and 20^+6^+0 indicate the administration of second challenge infection and treatment respectively. Error bars represent one standard error of the mean in infected group.





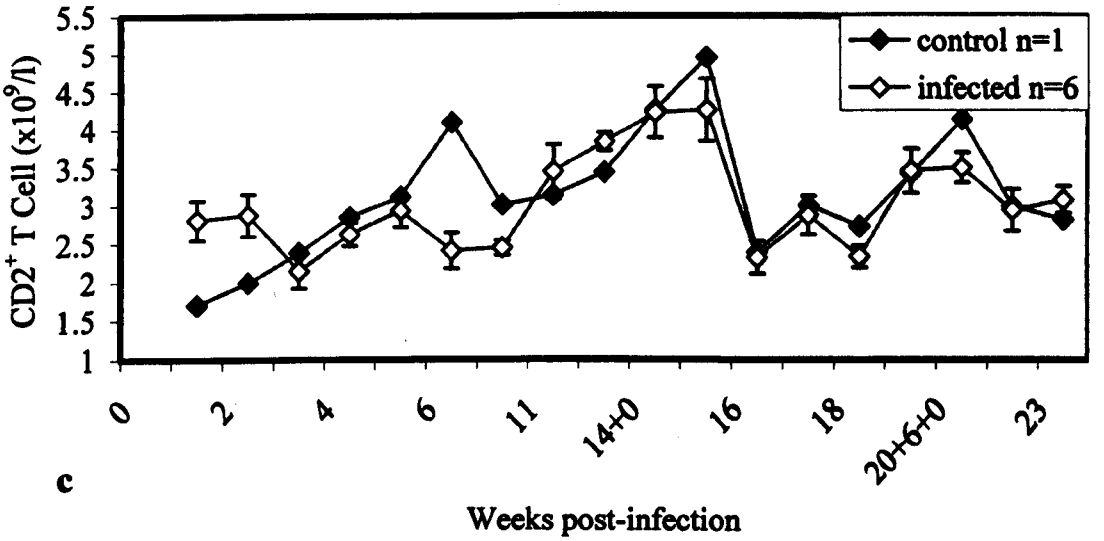


Fig.5.6 Kinetics of (**a**) $CD4^+$, (**b**) $CD8^+$ and (**c**) $CD2^+$ T cell subpopulations in blood circulation of experimentally infected and control calves. 14^+0 and 20^+6^+0 indicate the administration of second challenge infection and treatment respectively. Error bars represent one standard error of the mean in the infected group.

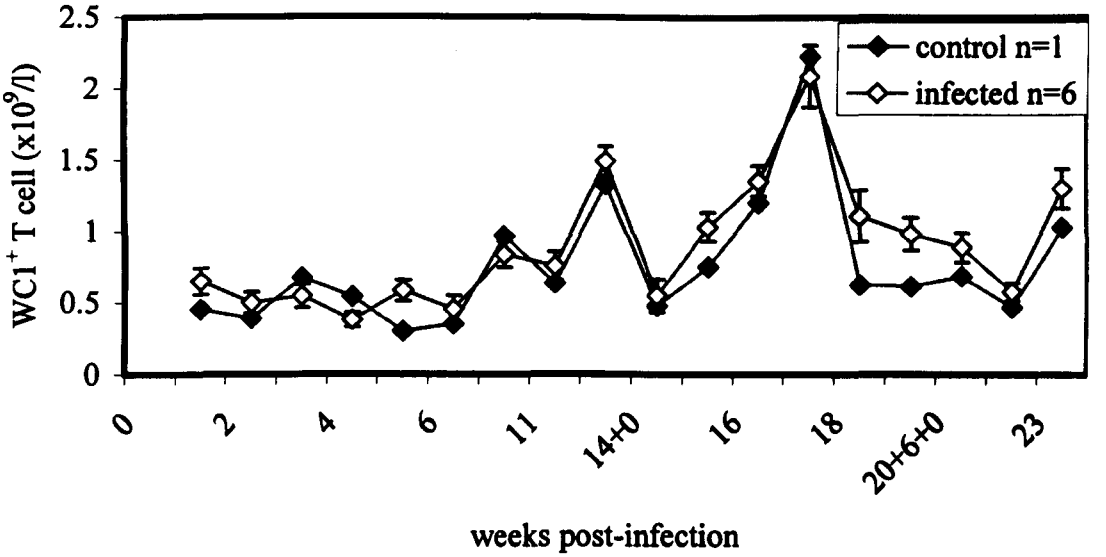


Fig.5.7 Kinetics of $WC1^+$ T cell subpopulation in blood circulation of experimentally infected and control calves. 14^+0 and 20^+6^+0 indicate the administration of second challenge infection and treatment respectively. Error bars represent one standard error of the mean in the infected group.

5.2.2 Biochemistry:

5.2.2.1 The kinetics of GLDH levels in serum samples:

The changes in GLDH levels in serum samples are presented in Figure 5.8a. After the second week post-infection, levels of GLDH in the serum of infected calves started increasing, with a significant increase at 4 weeks post-infection ($P<0.05$). Levels of GLDH in serum gradually increased to highly significant ($P<0.001$) levels at week 7 and reached their highest level at week 11 post-infection. After week 12 of the infection, the levels of GLDH started decreasing. A second increase was observed as soon as one week after the second challenge infection, administered at 14 weeks after the primary infection, after which the levels gradually started decreasing and fell below the cut off level. Following treatment at 20 weeks post-primary-infection, levels of the enzyme gradually decreased to the levels obtained in the non-infected calf. Only one serum sample collected from the control calf at week 6 of the experiment showed a level of GLDH activity elevated above the cut off value.

5.2.2.2 The kinetics of GGT levels in serum samples:

The kinetics of GGT levels in serum samples are shown in Figure 5.8b. Levels of GGT started increasing at week 7 post-infection and peaked at week 13, after which the enzyme levels gradually started decreasing, falling to pre-infection levels after five weeks of treatment. There was no elevated GGT activity in sera collected from the control calf throughout the experiment.

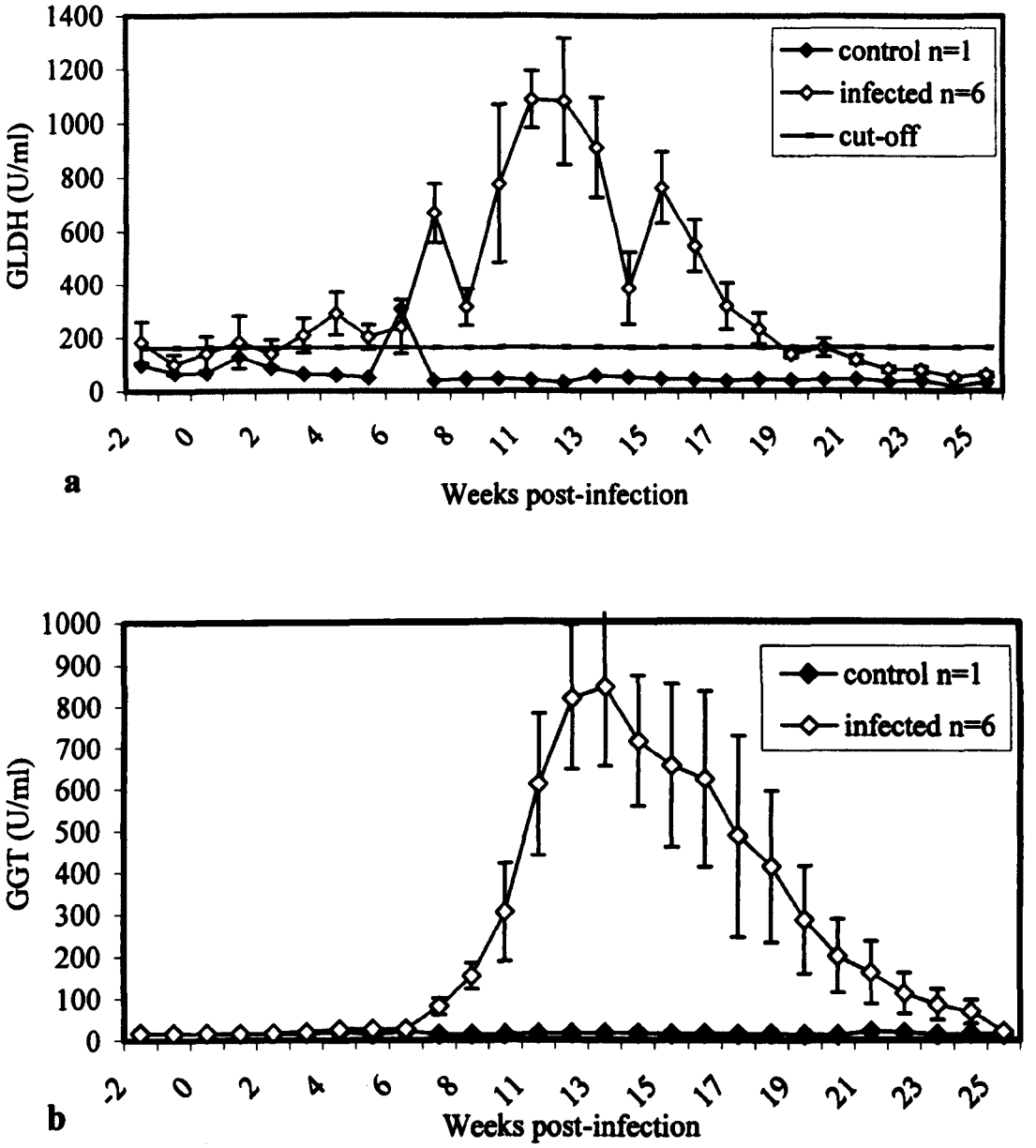


Fig.5.8 Kinetics of (a) GLDH and (b) GGT levels in serum samples collected at weekly intervals from experimentally infected and control calves. Cut-off value in (a) was calculated as the mean weekly enzyme activity recorded in the serum of the control animal plus two standard deviations of the mean. Error bars represent one standard error of the mean in the infected group.

5.2.3 Faecal Egg Counts:

Faecal egg counts are summarised in Figure 5.9. At week 9 post-infection, the faeces of one experimentally-infected calf (127) was positive for fluke eggs. At week 11 all calves were positive, with the number of eggs excreted in the faeces of individual animals variable. Two weeks after treatment, no eggs were found in the faeces of any animal. There was no correlation between the egg counts of individual animals and lymphocyte proliferation to fluke E/S antigens ($r = 0.17$), however there was a positive correlation with the somatic antigen-induced proliferation of PBMC ($r = 0.7$). There was moderate, but negative, correlation ($r = -0.424$) between the egg and eosinophil counts of the individual animals.

5.2.4 Weight gain:

There was no difference in terms of weight gain between infected and control calves (Fig.5.10).

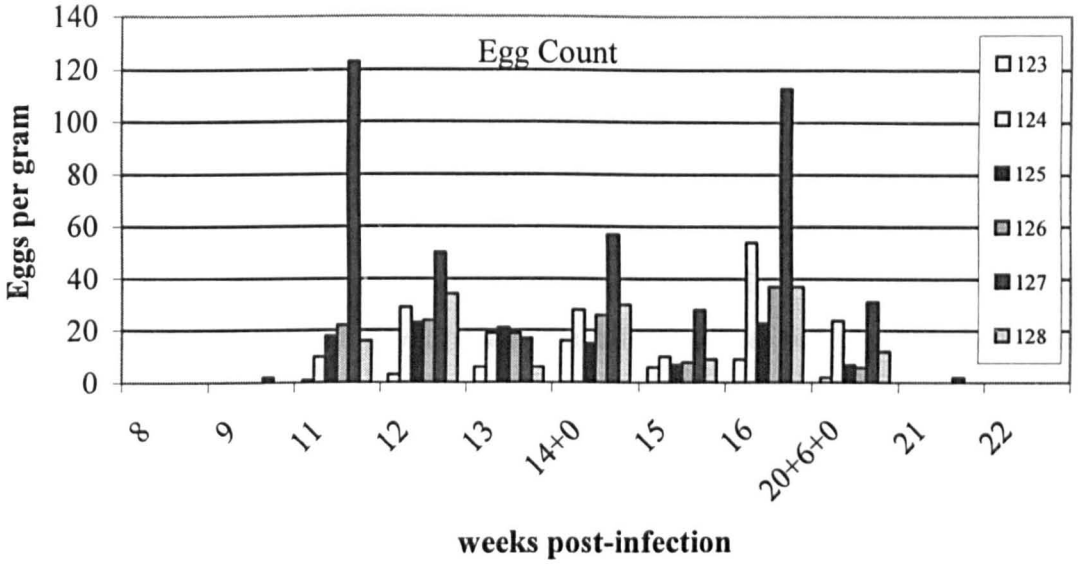


Fig.5.9 Faecal egg count.

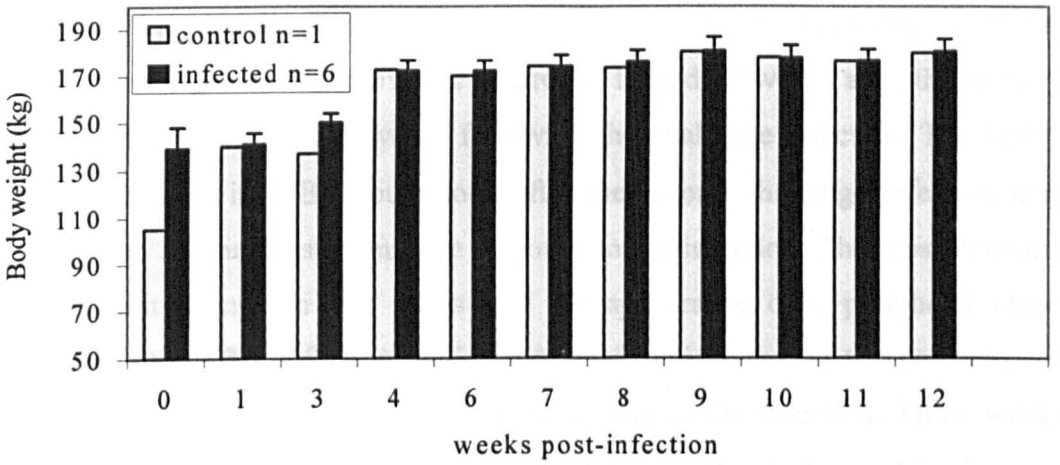


Fig.5.10 Body weight gain of infected and control animals.

5.3 Discussion:

In this study, the only significant reduction in the red blood cell series occurred in the RBC counts of infected animals compared to the total weekly counts of the control calf. Anaemia, observed in the infected calves, was mild and was restricted to a decrease in RBC count, suggesting a haemorrhagic anaemia. A significant reduction in RBC count was only observed when the infection reached patency, becoming more pronounced following the administration of the challenge infection. Anaemia is considered one of the contributors to host mortality and morbidity in fluke infections (Behm and Sangster, 1999) and is caused by the direct blood feeding and destructive activity of flukes to the capillaries. Major hepatic haemorrhages are thought to occur when the late immature or mature flukes pass into the bile ducts. The increase in the number of platelets observed around this time of the infection was probably associated with blood clotting mechanisms and repairing damaged capillaries.

Total white blood cell (WBC) counts gradually increased following infection, reaching levels significantly above the control at 11 and 12 weeks after the primary infection and at 1, 2, 4 and 5 weeks following the challenge infection. The highly significant increase in WBC count soon after the second challenge infection may have indicated an anamnestic immune response to the infection. The most dramatic and significant change in this series was the appearance of a peripheral blood eosinophilia soon after infection, which increased rapidly during the parenchymal stage and persisted at high levels during the course of the infection. Three weeks after treatment, eosinophils returned to similar levels to those observed in the non-infected control animal. Eosinophilia is a common feature of fasciolosis in all host species (Ross *et al.*, 1966; Sinclair, 1973 and 1975; Poitou *et al.*, 1992) and eosinophils are the only effector cells, to date, proven to be able to kill the parasite *in vitro* (Duffus *et al.*, 1980) and *in vivo* (Burden *et al.*, 1983).

In this study, the sedimentation technique was chosen to quantify faecal egg output. This technique is considered to be more sensitive than the flotation technique when a low egg count is suspected (Happich and Boray, 1969a). Nonetheless, variable egg recoveries are reported by different authors, with the sensitivity of the technique

reported to be 25-32% by Happich and Boray, (1969a) and as low as 5% by Claxton, (1996). The recommended sedimentation time of between 5 to 20 minutes was found inadequate to allow all the eggs to settle: our initial observation was that some eggs remained in the supernatants. Therefore, in order to increase the recovery of as many eggs as possible, sedimentation time was increased to one hour. This increased the debris in the sediment and made the recognition of eggs more difficult. Since the sample size in this experiment was relatively low, we adopted the laborious and cumbersome method of diluting the sediment with water and dividing it into several portions in order not to miss eggs and to recover as many as possible.

Faecal egg counts have been reported to correlate with up to 50 flukes recovered at necropsy in sheep. However, due to the effect of crowding on the nutrition of flukes, the egg count reduced dramatically when number of flukes exceeded 100 flukes per liver (Happich and Boray, 1969b). Although the latter finding perhaps brings into doubt the correspondence between the egg counts recorded in our study and the number of flukes present, there was, however, significant correlation ($P<0,05$) between egg output and the levels of liver enzymes (GGT, $r = 0.7$ and GLDH, $r = 0.7$-0.9) in the serum samples of individual animals.

Probably one of the most important findings in this study was the significant positive correlation between the proliferation response of PBMC to fluke antigens *in vitro* and the levels of liver enzymes in serum samples. Similar findings were reported by Clery *et al.* (1996) who demonstrated a strong correlation between antigen-induced proliferation of lymphocytes *in vitro* and fluke burden. Although the authors concluded that the proliferative response was non-protective, given the small amount of experimental data available and the likelihood of interanimal variation in *in vitro* proliferation assays, we have no evidence, except a moderate correlation, to suggest that the cellular immune response, as assessed by *in vitro* proliferation assay, is non-protective in nature. Correlation coefficient and regression analysis, as used in the experiments of Clery *et al.* (1996) to show the association between the *in vitro* proliferation response of PBMC and fluke burden, and in our case between *in vitro* proliferation and hepatic damage, does not necessarily mean that there is a cause-effect relationship.

Levels of GLDH activity in the serum samples of infected calves started to increase three weeks after infection, gradually reaching peak levels at around week 11, before returning to lower levels by week 14, when a second challenge infection was administered. One week post-challenge, GLDH levels peaked, but at a level lower than that caused by the primary infection. Although the reduction in peak level may have been due to immunity or pathology caused by the primary infection, we cannot exclude the possibility that it was due to the administration of lower doses of metacercariae in the second infection (700) as compared to the first (1000). Nonetheless, GLDH activity decreased rapidly after the first week post-challenge, returning to pre-infection levels by week five of the challenge.

Although there was no significant difference in the total number of circulating lymphocytes, flow cytometry analysis revealed a significant increase in B cell population in infected calves at 7 and 11 weeks after infection and for four consecutive weeks following the second challenge infection. The other circulating lymphocyte sub-populations ($CD4^+$, $CD8^+$, $CD2^+$ and $WC1^+$ T cell populations) did not differ significantly from those of control calf. The elevated number of B cells is consistent with the high levels of antibody observed during the course of infection. Antibody levels increased soon after infection, peaked at around 10 to 11 weeks post-infection and remained high throughout the course of infection (see Chapter 4). Hypergammaglobulinamia is seen in fasciolosis, however, its role in protective immunity is still debated.

In conclusion, experimental infection of calves with *F. hepatica* in this study caused a mild, sub-clinical disease, with no sign of anaemia and characterised by eosinophilia and increased B cell levels.

CHAPTER SIX

Further Investigations of T Cell Responses of Calves to *Fasciola hepatica* Infection:

6.1 INTRODUCTION:

It is well-established that the immune response to *F. hepatica* infection in cattle is characterised by a strong *in vitro* proliferation response by PBMC to fluke antigens early in the infection and a Th1-like cytokine pattern, with both IL-2 and IFN-γ detected (Oldham and Williams, 1985; Clery and Mulcahy, 1998; McCole *et al.*, 1999; see also Chapter 4). This response is down-regulated at around 5 wpi after which PBMC become unresponsive to fluke antigens and cease producing IL-2 and IFN-γ. Moreover, although dominated by IgG1, an E/S antigen-specific IgG2 response was detected in the serum of infected calves at week 2, returning to lower levels by week 4 of the infection (Chapter 4). It has been shown that rboIFN-γ stimulates the production of the IgG2 isotype, whereas rboIL-4 upregulates IgG1 antibody production by B lymphocytes in response to polyclonal B cell activating factors *in vitro* (Estes *et al.*, 1994 and 1995). Furthermore, Brown *et al.* (1999), working on the effect of Th0 helper T cell clones on the enhancement of B cell immunoglobulin production, recently reported that Th0 helper T cell clones specific to *Babesia bigemina* rhoptry-associated protein-1, a vaccine candidate antigen, produced more IFN-γ than IL-4 and enhanced production of both IgG1 and IgG2 isotypes by autologous B cells, whereas *F. hepatica* specific Th0 cells produced more IL-4 than IFN-γ and predominantly enhanced production of the IgG1 isotype. The authors also indicated that the above *in vitro* observation of Th cell activity was representative of the *in vivo* antibody response of RAP-1 immunised cattle, in which both isotype responses were observed, in contrast to the IgG1-biased response observed in *F. hepatica* infected cattle. On the basis of this, our finding of an antigen

specific IgG2 response early in the infection may suggest that an IFN-γ dominated Th1-like immune response might also be present *in vivo* early in the primary infection.

Re-infection of cattle with *F. hepatica* appears to have little or no effect on restoring the responsiveness of PBMC to fluke antigens and/or on the production of Th1-like cytokines (see Chapter 4). Similarly, fluke antigen specific T cell clones prepared from chronically infected cattle showed either a Th0 or Th2 cytokine profile, but no Th1 type clone has been obtained (Brown *et al.*, 1994a). Furthermore, lymphocytes from chronically infected cattle failed to produce IFN-γ in response to fluke antigens in another study (Clery *et al.*, 1996) suggesting a predominantly Th2-like immune response in chronically infected cattle, in contrast to the Th1-like response in the pre-patent phase of the infection.

The unresponsiveness of PBMC to fluke antigens detected after patency may be attributable to the down-regulation of a Th1 type of immune response by cytokines produced by Th2 cells. However, neither this possibility nor other immunomodulating factors such as elevated levels of nitric oxide (NO) and transforming growth factor-beta (TGF-β) have been investigated in fluke-infected cattle. NO is produced mainly by cytokine- (IFN-γ, TNF) and/or LPS-activated macrophages and is thought to be an effector mediator of protective immunity against a wide range of parasites including the trematode parasite *Schistosoma mansoni* (James, 1991; James and Glaven, 1989; James and Hibbs, 1990). In addition to this protective role, it has been postulated that NO and prostaglandins secreted by IFN-γ activated macrophages have a suppressive effect on T cell proliferation in a number of parasitic models (Mills, 1991; Schleifer and Mansfield, 1993; Sternberg and McGuigan, 1992; Rockett *et al.*, 1994). TGF-β, on the other hand, is produced by virtually all cell types but is stored at high levels in the alpha granules of platelets (Ruscetti *et al.*, 1998). The TGF-β is secreted in an inactive (latent) form that requires activation before inducing a biological effect. In mammals the cytokine has three isoforms, TGF-β1, 2 and 3, whose biological properties are almost identical

(Border and Noble, 1994). TGF-β1 has a diverse functional effect on many cell types. In particular, it is involved in the downregulation of IL-2 receptor expression and therefore the inhibition of IL-2 induced proliferation of lymphoid cells (Ruscetti *et al.*, 1998).

As indicated above, PBMC from fluke infected cattle proliferate vigorously *in vitro* early in infection, but it is not known which cell population/s expand in response to fluke antigens. A recent attempt to define T cell subset involvement in the immune response by depleting T cell populations ($CD4^+$, $CD8^+$ or $\gamma\delta^+$) from whole PBMC revealed that while depletion of either $CD4^+$ or $CD8^+$ T cells attenuated proliferation in comparison with responses obtained from whole PBMC, depletion of $\gamma\delta^+$ T cells increased it, although not significantly (McCole *et al.*, 1999). Although this study indicates the importance of $CD4^+$ and $CD8^+$ T cell populations in proliferation response, it does not consider the other cell populations such as B lymphocytes. Probably the most important observation in this whole area to date is that of Brown *et al.* (1994a) who reported a mixture of $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T cells in their fluke somatic antigen-specific T cell lines prepared from two *F. hepatica* infected cows. Overgrowth of $\gamma\delta^+$ T cells was commonly observed in the cell lines and $\gamma\delta^+$ T cell clones generated from these cell lines did not proliferate in response to antigens. In contrast, they did proliferate in response to auto-antigens in an autologous mixed leukocyte reaction (Hanby-Flarida *et al.*, 1996; Okragly *et al.*, 1996). It therefore appears that non-specific proliferation of $\gamma\delta^+$ T cells is problematic for the correct interpretation of *in vitro* proliferation assays.

In recent years, significant progress has been made in the development of a vaccine for *F. hepatica,* based on purified antigens. However, the nature of the protective immune response following a sensitising initial infection or vaccination is still unknown. Very early studies suggested that not antibodies but lymphoid cells effect the transfer of an adoptive immune protection to naïve animals (Corba *et al.*, 1971; Armour and Dargie, 1974). This prompted a few groups to define the cellular immune responses of cattle to *F. hepatica* infection. Despite this, recent successful vaccine trials neglected the immunological investigations which would otherwise

have given some insight into the nature of protective versus non-protective immune responses. Nevertheless, more recently, Mulcahy *et al.* (1999b) re-examined and confirmed the efficacy of fluke derived enzymes (cathepsin L1 and L2) and haemoglobin as vaccines. In their report, PBMC from protected, as compared to non-protected (non-vaccinated), animals showed a higher proliferation response and IFN-γ production in response to fluke antigens *in vitro* following a challenge infection. The authors concluded that IFN-γ producing Th1-like immune responses are important in mediating protection in vaccinated cattle. Support for this hypothesis (unpublished data of O'Neill, Dalton and Mills in mice) was reported by Mulcahy *et al.* (1999a). In this study, the C57/BL mouse strain which developed a Th1-type response was more resistant to infection with *F. hepatica* than the BALB/c and 129SV/EV mouse strains which developed a Th2-type response. Therefore, the identification of fluke antigens with the potential to provoke cell-mediated immune responses, in particular IFN-γ producing Th1-like responses, together with studies to characterise protective and/or non-protective immune responses are vital to the design of rational sub-unit vaccines.

The aim, therefore, of the study described in this chapter was to further characterise T cell responses in cattle and, in particular, to investigate which cell population/s are proliferating in response to fluke E/S and somatic antigen preparations *in vitro*. This chapter also investigates the effect of exogenous IL-2, and of elevated levels of NO and TGF-β on the unresponsiveness of PBMC to fluke antigens observed after patency. We have also attempted to characterise T cell responses to partially fractionated *F. hepatica* E/S and somatic antigens.

6.2 RESULTS:

Experimental Animal: A hyper-immune Belgian Blue cross Jersey cow (137) - which had previously been infected with 1000 *F. hepatica* metacercariae on 10th August, 1995, 1st February, 1996 and 26th April, 1996 respectively for our preliminary experiments - was treated on 30th August 1996 and subsequently re-infected with 1000 *F. hepatica* metacercariae on 30th November, 1998.

6.2.1 Effect of exogenous IL-2 and L-NMMA on the proliferation of PBMC:

The proliferation assay was performed essentially as described in Section 2.8, but with or without the addition of exogenous rhIL-2 and L-NMMA at final concentrations of 10 U/ml and 0.5 mM/ml respectively. PBMC obtained from the cow (137) at 5, 6 and 7 weeks after the final challenge infection were used in this experiment and the results are presented in Fig.6.1a and b. While PBMC did not proliferate in response to fluke E/S antigen in all three weeks, they did proliferate when stimulated with fluke somatic antigens at weeks 5 and 6 (Fig.6.1a) but not at week 7 (Fig.6.1b). The addition of exogenous rhIL-2 to the cultures increased proliferation about 20 fold in E/S stimulated cultures at weeks 5 and 6 but had little or no effect on somatic antigen or Con-A stimulated cultures. At week 7, the proliferation response of PBMC to somatic antigen decreased from an SI of 62 in week 6 to an SI of 3.6. The addition of IL-2 to cultures in this week increased proliferation 13 fold in the E/S cultures and 6 fold in somatic antigen-stimulated cultures. In contrast, the addition of L-NMMA to the cultures had no effect on the proliferation of PBMC in any of the three weeks tested.

6.2.2 Nitric oxide levels in culture supernatants:

Fluke antigen-stimulated and non-stimulated culture supernatants, generated in previous experiments at weekly intervals from one non-infected and six experimentally-infected calves (Chapter 4), were tested for NO levels. The NO was undetectable in the culture supernatants.

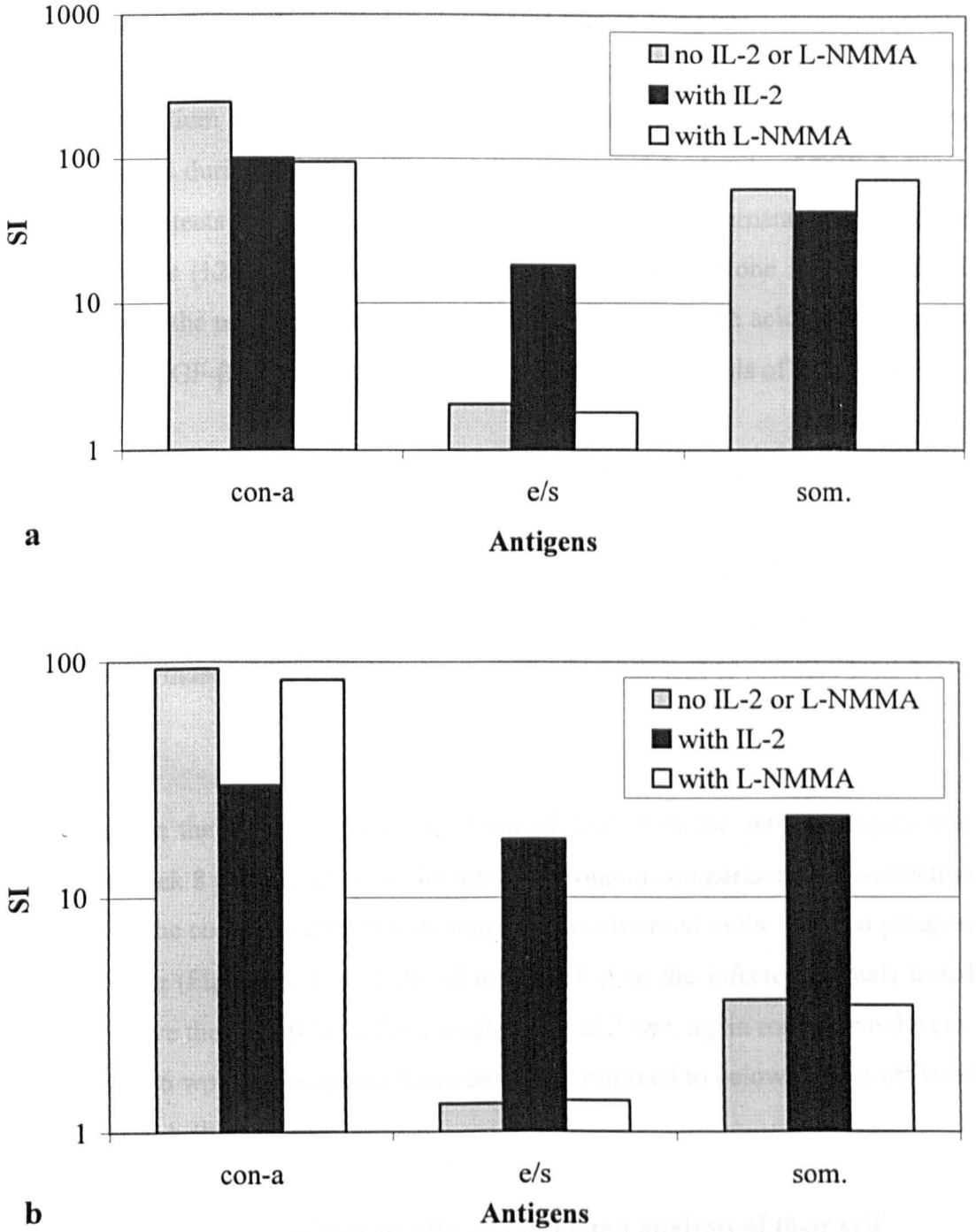


Fig.6.1 Effect of exogeneous rhIL-2 and L-NMMA on proliferation responses of PBMC to *F. hepatica* E/S and somatic antigens during the unresponsive period. PBMC from the infected animal were stimulated with fluke E/S and somatic antigens and co-cultured with or without 10 U/ml rhIL-2 or 0.5 mg/ml L-NMMA at (a) week 6 [week 5 had similar result of week 5] and (b) week 7 post-challenge infection. Som is somatic antigen.

6.2.3 TGF-β levels in culture supernatants and serum samples:

F. hepatica somatic antigen-stimulated culture supernatants together with non-stimulated (medium only) culture supernatants and serum samples obtained at two weekly intervals during infection (Chapter 4) were tested for levels of TGF-β. Since the kit only detects the active form of TGF-β_1, culture supernatants from three infected animals (124, 125 and 127) and serum samples from one infected animal (125) and from the non-infected control animal were treated with acid to activate the latent form of TGF-β and were then tested by ELISA for the levels of total TGF-β.

The levels of active form TGF-β in culture supernatants ranged from about 100 to 385 pg/ml, with no difference between the levels induced in culture supernatants of infected and non-infected animals (Fig.6.2a). However, a twofold increase in the second and fourth week culture supernatants of infected animals was observed in comparison to those of the control animal after acid treatment to measure total TGF-β (Fig.6.2b).

An increase in the levels of the active form of TGF-β in the serum samples was detected at week 8 post-infection in the infected group in comparison to pre-infection levels and to the control animal but no increase was observed in the infected group at any other time (Fig.6.3a). The levels of total TGF-β in the infected animals tested increased above the cut-off level for a single week at 2 wpi, again rose above the cut-off level after 6 wpi, and remained there until they returned to below the cut-off level at 20 wpi (Fig.6.3b).

6.2.4 Establishment of antigen-specific cell lines and analysis of their cell phenotypes:

Three *F. hepatica* somatic and four E/S antigen-specific cell lines were generated from PBMC of a hyper-immune cow (137) following a super-infection with 1000 *F. hepatica* metacercariae. PBMC proliferated vigorously between 1 and 4 weeks post-infection in response to fluke E/S and somatic antigens. Attempts to generate antigen-specific cell lines within these weeks were successful. After 5 days

stimulation with antigen, PBMC transformed into blast cells and proliferated sufficiently to cover the bottom of 24 well plates. The expansion of the cells was observed under an inverted microscope. Antigen priming was essential for the establishment of cell lines. PBMC co-cultured with rhIL-2, but without antigens, did not proliferate and died within 10 days.

Antigen-specific cell lines together with their progenitor PBMC population were analysed by flow cytometry and the results are presented in Fig.6.4 as the mean of 3 somatic and 4 E/S antigen-specific cell lines. While PBMC included a mixture of the cell populations tested, the cell lines were composed of $CD2^+$ and $CD4^+$ T cells. Somatic antigen-induced cell lines had over 95% CD4+ T cells and around 1% $\gamma\delta^+$, $CD8^+$ T cells and B cells. The E/S antigen-specific cell lines showed about 87% $CD4^+$ T cells, 2.5 % $CD8^+$ and 4.4% $\gamma\delta^+$ T cells, but fewer than 1% B cells. About 50% of the cells within the lines expressed IL-2 receptor, as indicated by the expression of CD25.

Cell lines produced low levels of IFN-γ (180 to 535 pg/ml) when cultured alone without stimulation. However when cells were incubated in the presence of antigen and Mitomycin-C treated PBMC from the same animal as antigen presenting cells or with 5 to 10 U/ml rhIL-2, higher levels of IFN-γ were detected (3900-5788 pg/ml somatic-specific cell lines and 1554-6864 pg/ml E/S antigen-specific cell lines).

6.2.5 IFN-γ and proliferative responses of PBMC to partially purified E/S and somatic antigens:

Molecular sieve chromatography using HPLC was used to fractionate *F. hepatica* E/S and somatic antigens and each fraction was run in SDS-PAGE in order to determine its purity and relative molecular mass. After the void volume, a total of 9 fractions were collected for E/S and 6 fractions for the somatic antigen. As seen in Fig.1 in Appendix 2 this attempt to purify fluke proteins was only partially successful. Several proteins were eluted in each fraction and some fractions shared the same molecular weight bands.

The proliferation responses of PBMC from infected cow 137 and from a control animal to each fraction, to whole somatic antigen, to E/S antigens and to Con-A were subsequently tested by an *in vitro* lymphocyte proliferation assay. The proliferation assay was performed before the super-infection and on the two subsequent weeks. The results are shown in Fig.6.5a and b. PBMC from the non-infected control animal did not proliferate in response to whole or fractionated antigen preparations, while proliferating vigorously in response to Con-A. In contrast, PBMC obtained from cow 137 before administration of the challenge infection did proliferate in response to antigens. While the response to whole or fractionated E/S antigens was low, ranging from SI of 1.3 to 11 at this point, proliferation was relatively high to the somatic antigen fractions, ranging from SI of 3 to 22, and to whole somatic antigen the SI was 41. However, following the super-infection, the responses increased. Although PBMC from the infected animal proliferated in response to all the fractions, the response was variable. Fraction 3 of somatic antigen stimulated the highest proliferation, greater than that induced by whole somatic antigen, whereas fraction 1 induced the lowest. In contrast, whole E/S antigen induced the highest proliferation, followed by fraction 2, 1, 3, 4, 6, 7 and 5, while fractions 8 and 9 induced the lowest.

Culture supernatants collected at two weeks post-challenge infection were also tested for the levels of IFN-γ. The levels of IFN-γ in culture supernatants did not correlate with the proliferation ($r = 0.3$ for E/S and $r = 0.2$ for somatic antigen; $P>0.2$ for both). There was a major difference between the fractions in their ability to induce IFN-γ production. Fraction-1 of E/S antigen induced the highest level of IFN-γ, 3.5 times higher than that induced by whole E/S antigen. Moderate levels of IFN-γ were detectable in culture supernatants stimulated by fractions 7, 6, 2, 3 and 4, in descending order respectively. IFN-γ was undetectable in culture supernatants stimulated with E/S antigen fractions-8 and -9. While fraction-3 of somatic antigen induced the highest proliferation, there was no detectable IFN-γ in culture supernatant stimulated with this fraction. Similarly, IFN-γ was undetectable in culture supernatant stimulated with fraction-1, which induced relatively high proliferation. Other fractions and the whole somatic antigens induced high levels of IFN-γ.

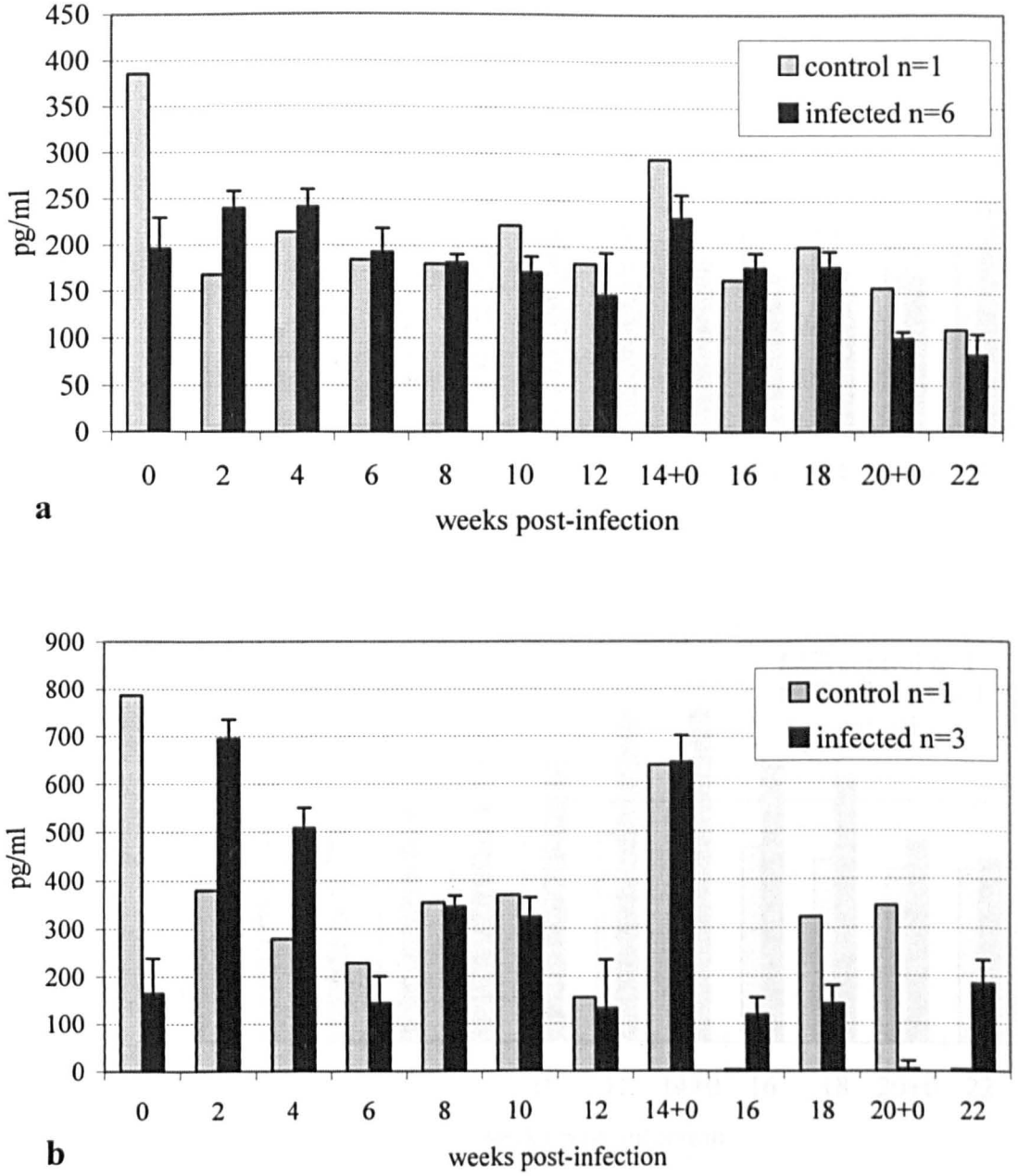


Fig.6.2 *F.hepatica* somatic antigen induced TGF-β levels in culture supernatants. TGF-β levels were measured at two weekly intervals by ELISA (a) before acid treatment to measure the active form of TGF-β and (b) after acid treatment of supernatant to activate the latent form and therefore to measure the total TGF-β. The results are expressed as pg/ml after deducting the corresponding background levels of TGF-β measured in the non-stimulated culture supernatants. 14+0 and 20+0 indicate the second challenge infection and administration of treatment respectively. Error bars represent the standard error of the mean.

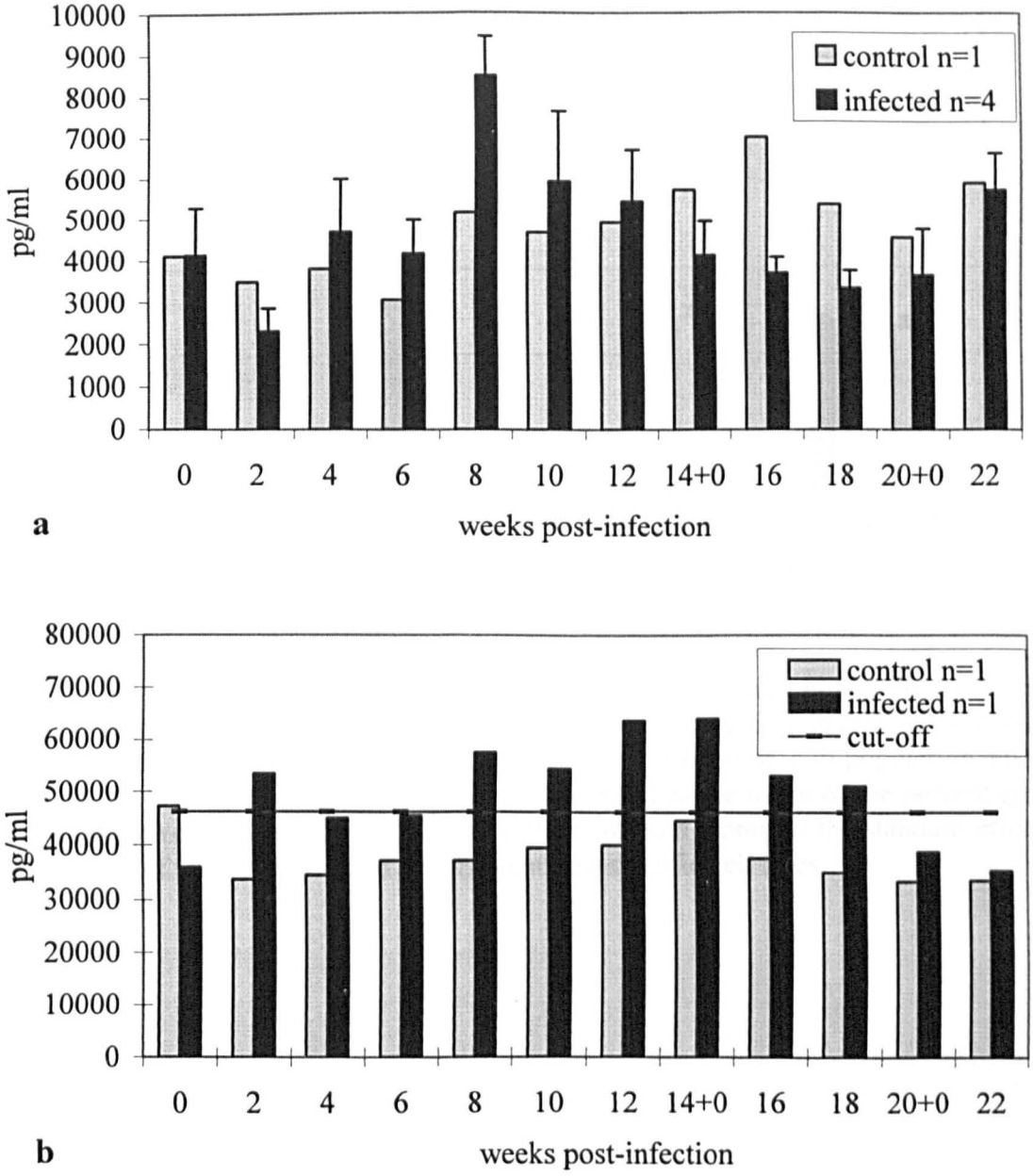


Fig. 6.3 Levels of TGF-β in serum samples from *F.hepatica* infected and non-infected calves. TGF-β levels were measured at two weekly intervals by ELISA (a) before acid treatment to measure the active form of TGF-β and (b) after acid treatment of sera to activate the latent form and therefore to measure the total TGF-β. The results are expressed as pg/ml. Cut-off levels were calculated as the mean of the TGF-β levels obtain in serum samples of the non-infected control calf plus two standard deviations of the mean. 14+0 and 20+0 indicate the second challenge infection and administration of treatment respectively. Error bars represent the standard error of the mean.

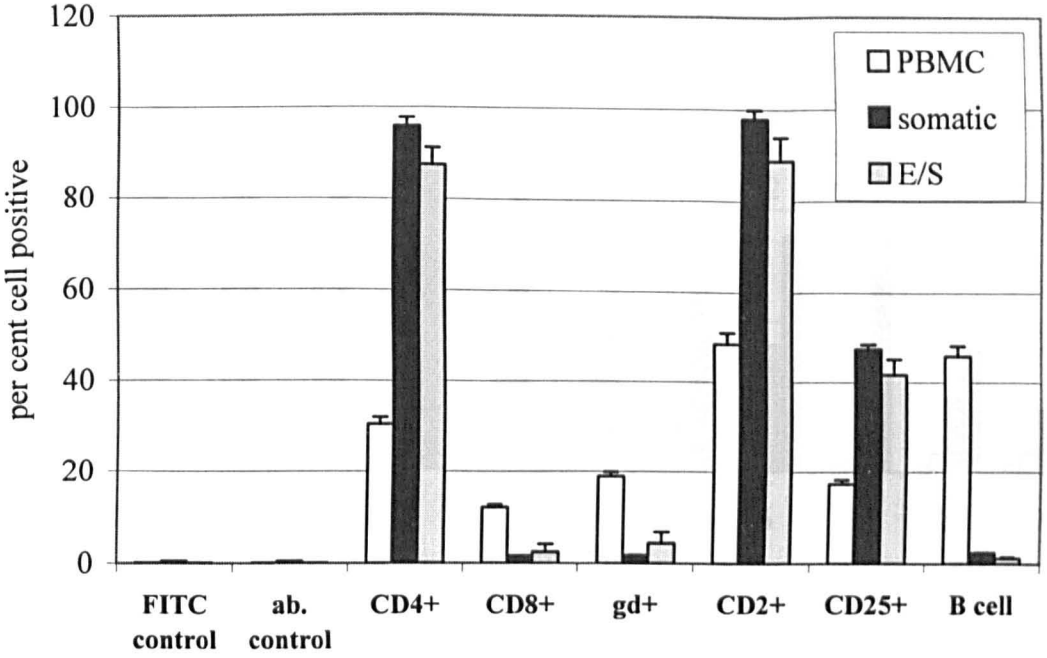
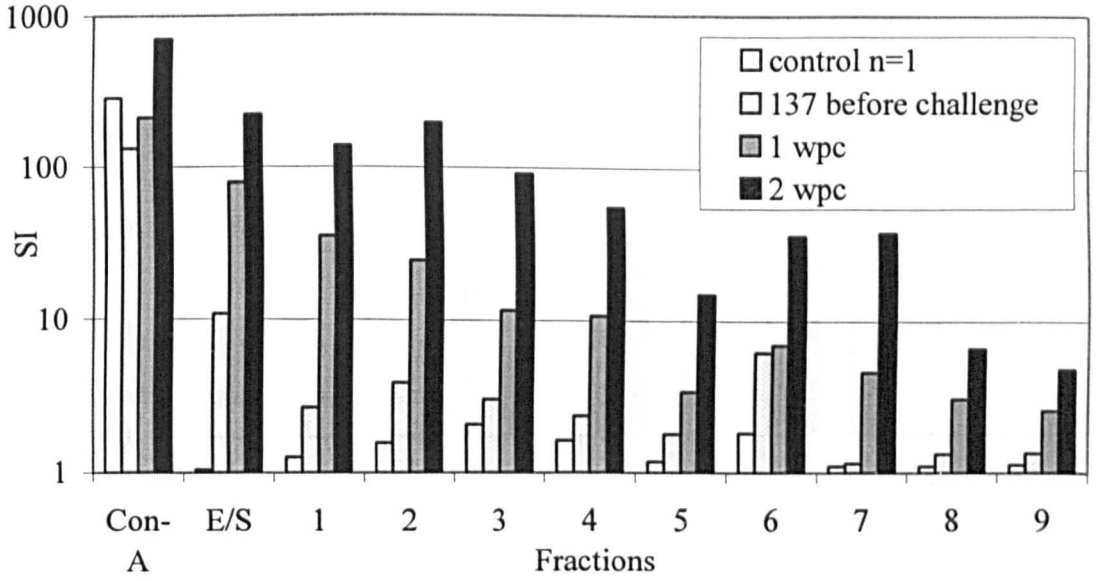
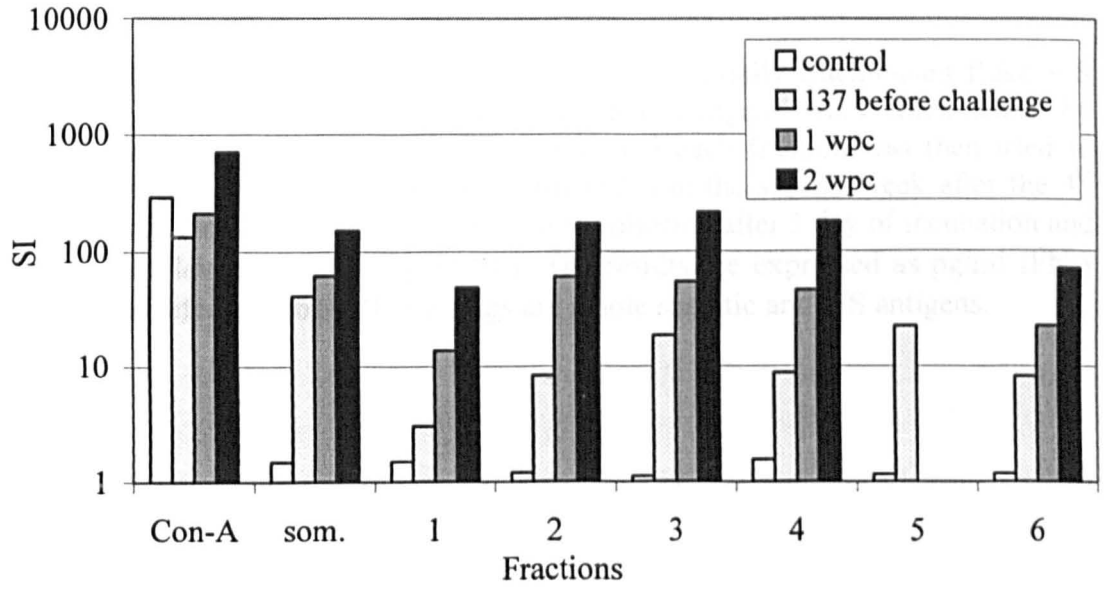


Fig.6.4 Cell phenotypes of *F. hepatica* antigen specific cell lines. Four E/S and three somatic antigen specific cell lines together with their progenitor PBMC population were stained a panel of Mab specific to bovine lymphocyte sub-population and analysed by flow cytometry. The results are presented as the mean of the percentage of cells positive to the specified lymphocytes. Error bars represent the standard error of the mean of three somatic and four E/S antigen-specific cell lines.



a



b

Fig.6.5 Proliferation of PBMC in response to partially fractionated fluke (a) E/S and (b) somatic antigens. *F. hepatica* E/S and somatic antigens were fractionated by molecular sieve chromatography using HPLC and each fraction was then used to stimulate PBMC from a hyper-immune cow (137) following a 4th challenge infection in an *in vitro* lymphocyte proliferation assay. PBMC from a non-infected cow were also included as control. Som is whole somatic antigen and wpc represents weeks after the 4th challenge infection.

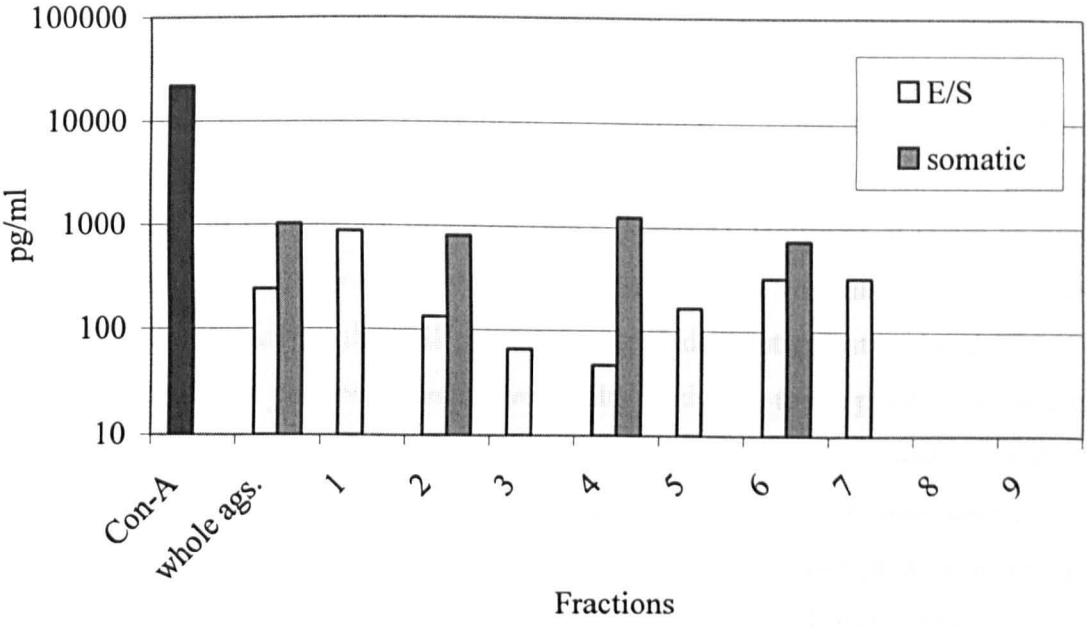


Fig.6.6 IFN-γ production of PBMC in response to partially fractionated fluke E/S somatic antigens. *F. hepatica* E/S and somatic antigens were fractionated by molecular sieve chromatography using HPLC and each fraction was then used to stimulate PBMC from a hyper-immune cow (137) in the second week after the 4th challenge infection. Culture supernatants were collected after 3 day of incubation and tested for the levels of IFN-γ by ELISA. The results are expressed as pg/ml IFN-γ based on the reference to rboIFN-γ. Ags are whole somatic and E/S antigens.

6.3 DISCUSSION:

Peripheral blood mononuclear cells from *F. hepatica* infected cattle proliferated vigorously in response to fluke E/S and somatic antigen preparations *in vitro* during the first 5 weeks of the primary infection (see Chapter 4). However, it is not known which cell sub-population/s were proliferating in response to fluke antigens. Our earlier attempts to analyse the cell phenotypes in 5-day antigen-stimulated PBMC culture cells produced poor or inconclusive results partly due to the presence of many dead cells in the 5 day cultures staining non-specifically with monoclonal antibodies and partly due to the presence of non-proliferating cells in the cultures interfering with the results of flow cytometry analysis. Therefore, we attempted to generate antigen-specific cell lines by culture of antigen-primed cells with fluke antigens plus APC and rhIL-2. The viability of cells in the cell lines was generally over 95% as determined with a trypan blue exclusion test and less then 1% cells were stained with FITC conjugated second antibody or control mouse antibodies in flow cytometry.

Flow cytometry analysis of antigen-specific cell lines revealed that the proliferating cells were $CD4^+$ T helper cells. Other T cell phenotypes ($CD8^+$ and $\gamma\delta^+$) and B cells were recorded as less than 1%, i.e. within background staining. This result indicates an early T helper cell response to infection with *F. hepatica* in cattle. T helper cells are regulatory cells essential for providing help to immune effector mechanisms. While cytokines produced by the Th1 subset enhance the cell-mediated immune responses, the Th2 subset provides help for B cell and humoral-based immune responses. The levels of IFN-γ in culture supernatants of the cell lines were comparable to those produced by antigen-stimulated PBMC reported in Chapter 4. However, the levels of IFN-γ produced by the cell lines were relatively low in comparison to the levels produced by PBMC of cattle infected with the intracellular protozoan parasite, *Neospora caninum* (Williams *et al.*, submitted). Since the levels of other cytokines, in particular IL-4, are not known, it is not certain which subset of $CD4^+$ T cells was responsible for the production of IFN-γ. It is likely that the cell lines were composed of a mixture of all three Th subsets described in cattle. This argument is supported by the fact that while some partially-purified fluke antigens

were capable of inducing a strong proliferation response of PBMC, levels of IFN-γ in culture supernatants stimulated with the same antigenic fractions were undetectable. In contrast, the others induced both strong proliferation and high levels of IFN-γ production.

In this study, we also investigated possible reasons for the unresponsiveness of PBMC to fluke antigens observed in cattle from 5 weeks after infection with *F. hepatica* (Oldham, 1985; Oldham and Williams, 1985; Chapter 4). The cow used in this experiment had been used in our preliminary experiments and had been treated with a flukicide, triclabendazole, then re-infected with *F. hepatica.* The PBMC from this cow after infection proliferated vigorously in response to fluke antigens for the first 4 weeks, but became hyporesponsive subsequently and were unresponsive to both E/S and somatic antigens by week 7 post-infection. This supports our earlier hypothesis that the presence of late immature or adult parasites in the liver or bile ducts might be responsible for the loss of proliferative response. Addition of exogenous IL-2 to the cultures during this hyporesponsive period restored the proliferative response of PBMC to fluke antigens, indicating that a lack of IL-2 production during this period may play a major role in this hyporesponsiveness. Before the PBMC became hyporesponsive and while they were still proliferating in response to fluke somatic antigen preparation at weeks 5 and 6 post-infection, addition of exogenous IL-2 to the cultures did not affect the proliferation. This is probably due to sufficient endogenous IL-2 being produced by PBMC. These results are in agreement with our earlier findings in Chapter 4 where we demonstrated relatively high levels of IL-2 and IFN-γ production during the first five weeks of infection and low levels after 5 weeks post-infection. There was also significant correlation between proliferation and the amount of IL-2 produced. These observations suggest that the hyporesponsiveness results from a lack of IL-2 production by PBMC *in vitro*, but that cells are responsive to exogenous IL-2 and are therefore expressing IL-2 receptors.

Levels of NO in culture supernatants were undetectable. Up to 40 μM/ml nitrite production has been reported in recent publications using bovine cells (Adler *et al.*,

1995; 1996). We measured 2 to 4 μM levels of nitrite, which might be attributable to phenol red in RPMI tissue culture medium. Addition of the iNOS enzyme inhibitor, L-NMMA, did not enhance the proliferative responses of PBMC to fluke antigens during the unresponsive periods indicating that NO is probably not involved in the loss of proliferative response in fluke infected cattle.

This study also examined production of TGF-β_1 in culture supernatants and in serum samples of infected and control animals. TGF-β is produced by many cell types including mitogen-activated non-adherent human PBMC (Derynck *et al.*, 1985), CD4+ and CD8+ human T cells (Kehrl *et al.*, 1986) and has been shown to inhibit IL-2-dependent T cell proliferation by 60-80% by blocking IL-2-induced upregulation of the IL-2 and transferrin receptors (Kehrl *et al.*, 1986). PBMC in our experiment produced moderate levels of TGF-β ranging from 100 to 800 pg/ml. Although this level of TGF-β was found to be sufficient to inhibit 60 to 80 % of IL-2 induced human T cell proliferation, it is unlikely to have caused the unresponsiveness of PBMC to fluke antigens here, since the levels of TGF-β in culture supernatants at weeks 2 and 4 post-infection, when the cells proliferated vigorously, were no lower than in other weeks. Nevertheless, we have no direct evidence to exclude the possibility that this cytokine may be an immunoregulatory/immunomudulatory factor in cattle. Higher levels of TGF-β were observed in the serum samples from the infected calf as compared to the control calf, particularly after week 6 post-infection. However, since this observation was based on a single infected calf, its significance cannot be estimated. Moreover, it is not known whether or not there is any *in vivo* effect on lymphoid cells or on the progress of the infection.

We have attempted to characterise the proliferation responses and IFN-γ production of PBMC to partially fractionated fluke somatic and E/S antigens. The attempt to purify antigens by HPLC was only partially successful but resulted in separation of high, medium and low molecular weight antigens. Although some fractions contained bands of the same molecular weight, most contained one or more unique

protein bands. Each fraction induced different levels of proliferation and IFN-γ production by the PBMC. Fraction 1 of E/S antigen, which included high molecular weight (over 94 kDa) proteins, induced high proliferation and the highest levels of IFN-γ production, four-fold higher than that induced by whole E/S antigen. The successful vaccine candidate component, fluke haemoglobin, eluted from a high molecular weight fraction (>200 kDa) of E/S antigen (McGonigle and Dalton, 1995), together with the fluke derived cysteine proteinase, cathepsin L2, are capable of inducing high levels of protection against experimental *F. hepatica* infection in cattle when used as a vaccine (Dalton *et al.*, 1996). Recently, Mulcahy *et al.* (1999b) reported that cattle vaccinated with these antigens showed a Th1-like immune response with higher lymphocyte proliferation and IFN-γ production following infection. The protection was also associated with the levels of specific serum IgG2 antibody (Mulcahy *et al.*, 1998). It is not known whether fraction 1 of E/S antigen included the fluke haemoglobin in our experiment, however it included high molecular weight E/S antigens and induced the highest levels of IFN-γ production. In contrast to E/S fractions, all of which, except fractions 8 and 9, induced IFN-γ although in varying levels, somatic fractions were more diverse in their capacity to induce proliferation and IFN-γ production. While fraction 3 induced the highest proliferation, there was no detectable IFN-γ production in culture stimulated with this antigenic fraction or with fraction 1. One of the most important findings in this experiment is that since all the fractions induced different levels of proliferation and IFN-γ responses, it is likely that different fluke antigens may be capable of inducing different types of immune response. It therefore appears that further research is warranted into characterising and defining the fluke antigens that induce a protective immune response, which would facilitate the eventual achievement of a subunit vaccine to protect cattle against *F. hepatica* infection.

In conclusion, the data presented here indicate that cells proliferating *in vitro* in response to fluke antigens are CD4+ cells and that a lack of IL-2 production during patency is a likely cause of the unresponsiveness. Although our data suggest that NO and TGF-β may not be involved in the hyporesponsiveness of PBMC to fluke

antigens, more detailed studies are needed on their role in immunomodulation or immunopathology. Since TGF-β is one of the cytokines involved in tissue remodelling and fibrosis (Border and Noble, 1994), our finding of high levels of TGF-β in the serum samples of an infected animal warrants further research into this cytokine. The results of our attempt to characterise the proliferation responses and IFN-γ production of PBMC to partially fractionated fluke somatic and E/S antigens led us to conclude that different fluke antigens may be capable of inducing different types of immune response and to suggest that further research to characterise and define the fluke antigens that induce the requisite immune response, might contribute to the eventual achievement of a subunit vaccine to protect cattle against *F. hepatica* infection.

CHAPTER SEVEN

Susceptibility of Newly Excysted Juvenile *Fasciola hepatica* to Nitric Oxide and Hydrogen Peroxide in cell free culture and in IFN-γ/LPS activated macrophage culture

7.1 Introduction:

It has been demonstrated that reactive nitrogen and oxygen intermediates play a significant role in the defence of the host against many pathogenic bacteria (Turco *et al.*, 1998; Rhoades and Orme, 1997; Chan *et al.*, 1992; Pacelli *et al.*, 1995; Way and Goldberg, 1998), protozoa (Gale *et al.*, 1998; Green *et al.*, 1990; Ghadirian and Denis, 1992; Hughes, 1988) and multicellular helminths (Taylor *et al.*, 1996; James and Glaven, 1989; Rajan *et al.*, 1996).

Reactive nitrogen and oxygen intermediates are produced and released mainly by appropriately activated phagocytic cells as part of the host defence. Two synergistic signals are required for the activation of these cells, in particular macrophages: one, a macrophage activation factor such as the cytokines IFN-γ, TNF-α, granulocyte-macrophage colony-stimulating factor and macrophage colony-stimulating factor; the other, receptor binding components such as lipopolysaccharides (LPS) (James and Hibbs, 1990). These activation signals prompt the cells to generate and release NO and other reactive species (O_2^{-}*, H_2O_2, *OH, HOCl), some of which are toxic for intracellular and extracellular targets (Nathan *et al.*, 1980; Hughes, 1988). NO is generated in the body through the action of an enzyme, nitric oxide synthase (NOS) which utilises the amino acid L-arginine as a substrate and liberates NO and L-citrulline. There are at least two isoforms of NOS: the constitutive enzyme (cNOS) is responsible for basal NO synthesis in both endothelial cells and the nervous system and is calcium (Ca^{2+})-dependent, whereas the cytokine inducible enzyme (iNOS) is Ca^{2+}-independent (Liew and Cox, 1991; James and Hibbs, 1990). It has been postulated that NO reacts with Fe-S groups of mitochondrial enzymes, resulting in

the formation of iron-nitrosyl complexes, therefore causing the inactivation and degradation of Fe-S containing enzymes in the mitochondrial electron transport chain (James and Hibbs, 1990; Liew and Cox, 1991; Hibbs, 1992). Alternatively, NO can react with the oxygen anion radical $O_2^{-}\bullet$ to form peroxynitrite anion ($ONOO^{-}$) which decays rapidly once protonated to form the reactive hydroxyl radical HO and the stable free radical nitrogen dioxide NO_2 (Hibbs, 1992).

Macrophages activated *in vitro* with IFN-γ and/or LPS have been shown to have a powerful cytotoxic effect on many of the parasites studied, including schistosomula of *Schistosoma mansoni* (James and Hibbs, 1990) and microfilariae of filarial nematodes (Taylor *et al.*, 1996). In many cases, killing was positively correlated with the amount of NO produced and completely or partially inhibited by N^{G}-monomethyl-L-arginine (L-NMMA), a structural analogue of L-arginine which is a competitive inhibitor of iNOS (James and Glaven, 1989; James, 1991). Recent studies indicate that NO may have similar defensive role *in vivo*. Administration of the iNOS inhibitor, aminoguanidine, abrogated the resistance to filarial nematodes in nonpermissive mice (Rajan *et al.*, 1996) and in another study significantly reduced the parasitaemia in *Babesia bovis* infected cattle (Gale *et al.*, 1998). Moreover, treatment of permissive, immunodeficient mice with a chemical compound that releases NO conferred resistance to the filarial nematode, *Brugia malayi* (Rajan *et al.*, 1996).

The susceptibility of parasite species and the stages of a particular parasite to reactive molecules has been reported to be variable (James and Hibbs, 1990; Hughes, 1988). This was partly attributed to a parasite's ability to induce different levels of anti-oxidative metabolisms.

In *F. hepatica* infection, an accumulation of inflammatory cells around the site of infection has been demonstrated (Meeusen *et al.*, 1995). An increase in peritoneal cells, the majority of which were macrophages and neutrophils, has been reported in parasite infected rats (Keegan and Trudgett, 1992). An accumulation of macrophages, together with lymphocytes, around the migration tracks in the liver

parenchyma of *F. hepatica* infected sheep has also been demonstrated (Meeusen *et al.*, 1995). However, the role of macrophages in the defence of the host and in the killing of flukes has not been studied. Although there is clear evidence to suggest that IFN-γ activated macrophages generating NO are responsible, at least in part, for killing the closely related human parasite *Schistosoma mansoni* (James and Glaven, 1989; James, 1991), there has been no research published on macrophages as possible effector cells for controlling *Fasciola* spp. Rather, there is an assumption that there may be a Th2 type immune response to infection with *F. hepatica* has focused attention away from an analysis of pro-inflammatory responses in fluke-infected animals. Our finding that there is strong antigen-specific proliferation of PBMC and IFN-γ production in cattle early in infection with *F. hepatica*, prompted us to investigate the susceptibility of the parasite to the reactive oxygen and nitrogen intermediates. This is the first report indicating that reactive nitrogen and oxygen intermediates might be capable, at least in part, for killing NEJ parasites *in vitro*.

7.2 Materials and Methods:

7.2.1 Excystment of Metacercariae *in vitro*:

Metacercariae were produced as described in Section 2.3 of Chapter 2 and were carefully detached from the dialysis tubing with the back end of a Pasteur's pipette. The viable metacercariae were collected with a Pasteur pipette, previously treated with Silicone Repelcote (Hopkin and Williams, Chawell Health, Essex, England), rinsed with distilled water, and transferred to a sterile petri dish. The excystment of metacercariae *in vitro* was performed as described elsewhere (Hanna, 1980) with some modification. The metacercariae were incubated in 0.2% (w/v) sodium hypochlorite (Milton; Procter and Gamble Ltd., Weybridge, Surrey, UK.) for up to 30 minutes at room temperature to remove the outer cyst wall. All the procedures were then performed under sterile conditions.

Metacercariae with the outer cyst wall removed were collected with a sterile, silicone treated Pasteur pipette, transferred to a sterile universal tube and washed thoroughly (10 times, each with 20 ml) with sterile distilled water to remove residues of sodium hypochlorite. The metacercariae were then placed into 25 cm^2 tissue culture flasks and incubated in the excystment medium (mixing equal volumes of 0.05 M HCl with a solution of 1% sodium bicarbonate, 0.8% sodium chloride, 0.2% sodium taurocholate and 0.8% L-cysteine) at 37°C for 4 hours or until 50% excystment was achieved.

The newly excysted juvenile parasites (NEJ) were collected from the excystment medium with a Pasteur pipette and rinsed with several changes of warm, complete tissue culture medium (CM) (RPMI-1640 medium supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin). Unexcysted metacercariae were returned to the incubator for further excystment.

7.2.2 *In vitro* Culture Conditions:

7.2.2.1 Cell Free Culture:

The susceptibility of NEJ parasites to H_2O_2 and NO was tested in cell free culture conditions. The NEJ parasites were incubated in the presence or absence of different concentrations of H_2O_2 (Sigma) and of NO generated from S-nitroso-N-acetylpenicillamine (SNAP) (Calbiochem, Novabiochem, La Jolla, USA). Twenty NEJs were placed into the wells of 24-well tissue culture plates in 1 ml CM and incubated at 37°C for at least 30 minutes prior to the start of the experiments after which all the parasites were examined under an inverted microscope at 50x and 100x magnification to ensure that all the parasites were motile, healthy and undamaged. Meanwhile the reagents, H_2O_2 and SNAP, were freshly diluted in CM to give the desired concentrations (2 mM for H_2O_2 and 10 mM for SNAP). Further serial dilutions of the reagents in CM were performed. Each dilution was added in 1 ml to the wells in duplicate. The final concentrations of the reagents in the wells were therefore half the initial concentrations. One set of wells received medium only and served as an untreated control. Combinations of H_2O_2 and SNAP were tested in similar fashion. The plates were incubated at 37°C in 5% CO_2 in air. The motility of the parasites was assessed microscopically at various time intervals during the culture (Section 7.2.3). At the termination of the experiment after 72 hours of incubation, parasite viability was assessed using a trypan blue exclusion test (Section 7.2.4).

7.2.2.2 Activated Macrophage Culture:

Mouse macrophage cell line, J774, recombinant mouse IFN-γ and lipopolysaccharide (LPS) were kindly provided by Dr. Mark Taylor (Liverpool School of Tropical Medicine). The cell line was routinely cultured in CM in tissue culture flasks (25 and 80 cm^2) at 37°C in 5% CO_2 in air. When proliferating macrophages completely covered the surface of the flasks, they were scraped with a sterile cell scraper, and the detached cell suspension was centrifuged and washed once with RPMI-1640 tissue culture medium. Pelletted cells from one flask were then resuspended in CM and divided between three new flasks. When sufficient macrophages were obtained, they were counted and resuspended with CM to give $2x10^5$ cells/ml. The viability of

the cells was determined by a trypan blue exclusion test and cell suspension containing over 95% viable cells was used.

J774 cells were dispensed into the wells of 24-well tissue culture plates, 0.5 ml or $1x10^5$ cells in each well, and activated with 100 U/ml final concentration of recombinant murine IFN-γ (Pharmingen), 1 μg/ml final concentration of LPS (Sigma) or a combination of both, in a volume of 0.5 ml. Reagents used to modulate activation, catalase (C-10 from bovine liver, Sigma) and N^G-monomethyl-L-arginine, monoacetate salt (L-NMMA) (Calbiochem®-Novabiochem Corporation, La Jolla, USA) were added simultaneously with IFN-γ and/or LPS in some cultures. Catalase was used at 10 μg/ml final concentration which has been shown to neutralise H_2O_2 activity (Taylor *et al.*, 1996). L-NMMA was used at 0.5mM/ml final concentration to block NO production (Hibbs *et al.*, 1987). Twenty parasites per well (5000 cells per parasite) were added directly after stimulation in 0.5 ml of CM, giving a final well volume of 1.5 ml. Control cultures received either parasites only in 1.5 ml CM or parasites co-cultured with same amount of non-activated, -modulated macrophages. The reagents used to activate (IFN-γ and LPS) or modulate the activation (catalase and L-NMMA) were also tested to determine whether they were in themselves toxic to the parasite. Parasites were cultured with medium containing the same amount of reagent used to activate macrophages, but without macrophages. The plates were then incubated at 37°C in 5% CO_2 in air. The motility of the parasites was assessed microscopically at various time-intervals during the culture (Section 7.2.3). At the termination of the experiment after 90 hours of incubation, supernatants were taken in order to measure nitrite levels (Section 7.2.5) and parasite viability was assessed using a trypan blue exclusion test (Section 7.2.4).

7.2.3 Visual Assessment of Parasite Motility:

Under the *in vitro* culture conditions used in this experiment, NEJ parasites were highly motile. However, lapses in their motility and short resting periods between the very active and vigorous movements were noted in a preliminary assessment experiment. Therefore, rather than scoring the motility of the parasites collectively,

the motility of each individual parasite in a culture condition was observed for an extended time and scored individually. The overall motility of parasites for given culture conditions was subsequently determined as the mean of the individual motility scores for all the parasites in that culture.

The scoring system used was:
5: very active and vigorous movement;
4: active movement;
3: no active movement, but movement on suckers noticeable;
2: movement in the gut;
1: on a higher magnification, some movement of muscles;
0: no movement at all.

7.2.4 Assessment of Parasite Viability:

Assessment of parasite viability was made using a trypan blue exclusion test. One hundred µl of 0.4% trypan blue (Sigma) was added to each well giving a final trypan blue concentration of 0.04% in the well. After 30 minutes, the trypan blue stained medium was aspirated using a Pasteur pipette and the wells were washed with PBS until the background was clear. The plates were examined under a dissecting microscope, dead parasites were stained with blue colour and non-stained live parasites were counted. Killing was expressed as the percentage of dead parasites over the total number of parasites in each culture condition.

7.2.5 Nitrite Assay:

The Griess reaction (Green *et al.*, 1982) was used to measure the amounts of nitrite (as an indicator of NO production) accumulated during the culture. Sixty µl of culture supernatants in duplicate were added to 60 µl of a 1:1 mixture of 1% sulphanilamide in 2.5% phosphoric acid (H_3PO_4) and 0.1% N-(1-Naphthyl) ethylenediamine dihydrochloride in 2.5% H_3PO_4 in microplates. After 10 minutes of reaction the absorbance was read at 570 nm in an automated ELISA plate reader

(Dynex Technologies). Nitrite was determined with reference to a standard curve generated using sodium nitrite at concentrations ranging from 7.5 to 1000 μM/ml in culture medium.

7.2.6 Ultrastructural Observations of NEJ Parasites Exposed to SNAP and H_2O_2 by Electron Microscopy:

NEJ parasites (20 parasites each) were exposed to 2.5 mM SNAP and 0.1 mM H_2O_2 for 6 hours. Both groups and the control group, which was incubated in medium only, were processed for electron microscopy by Mr Peter Young (E.M. Unit, Liverpool School of Tropical Medicine, Liverpool). The parasites were fixed in 3% glutaraldehyde in 0.1M cacodylate buffer for 1 hour. The samples were washed twice and placed in 1% osmium tetroxide for 1 hour, then dehydrated in ethanol. Following polymerisation for 24 hours at 70°C, the samples were fixed in resin and stained with uranyl acetate (20 minutes) and lead citrate (5 minutes). Sections were then examined on a Philips CM10 electron microscope at 80 kv.

7.3 Results:

7.3.1 Cell Free Culture:

7.3.1.1 Susceptibility of NEJ Parasites to H_2O_2:

The susceptibility of NEJ parasites to various concentrations (1, 0.5, 0.1, 0.01 and 0.001 mM/ml) of H_2O_2 was studied. The parasites showed dose dependent susceptibility to H_2O_2 (Fig.7.1a). H_2O_2 rapidly reduced the motility of the parasites in a dose- and incubation time-dependent fashion. While 1 and 0.5 mM H_2O_2 immobilised the parasites within 20 minutes incubation time, 10 and 1 μM concentrations had little or no effect over the time (72 hours) studied (Fig.7.2a). A trypan blue viability exclusion test showed comparable results to those of the motility assessment. Whereas 1 and 0.5 mM H_2O_2 killed 100 and 98% parasites respectively, 100, 10 and 1 μM concentrations killed only 16, 7 and 5% parasites respectively. The parasites were also incubated with 1 and 0.5 mM concentrations of H_2O_2 in a separate experiment, and each culture was sequentially terminated at various time intervals (10, 20, 40 minutes and 6 hours) and viability assessed by trypan blue exclusion test to determine the time required to kill the parasites. 1 mM H_2O_2 killed all the parasites after 20 minutes incubation whereas 6 hours incubation time was required for the 0.5 mM concentration of H_2O_2 to kill 90% of the parasites (Table 7.1). Photographs 7.1a and b show a representative example of the success of the trypan blue exclusion test.

7.3.1.2 Susceptibility of NEJ Parasites to NO generated from SNAP:

A SNAP dose- dependent reduction in the motility of NEJ parasites was observed over 72 hours of incubation time. A reduction in motility of the parasites co-cultured with 5 and 2.5 mM SNAP was noted after 10 to 20 minutes incubation and was maintained throughout the experiment. However, although lower concentrations of SNAP (1, 0.1 and 0.01 mM), reduced motility early in the incubation period (20 minutes), the motility of the parasites was restored after 40 minutes incubation (Fig.7.2.b). At the termination of the experiment at 72 hours, a trypan blue viability

test revealed SNAP-mediated killing which was dose dependent (Fig.7.1.a), but the results were variable between the duplicate cultures. Therefore, although there was substantial killing (between 18 and 28%) with 1 and 2.5 mM concentrations of SNAP, only the 5 mM SNAP reduced viability significantly ($P<0.05$).

In a separate experiment, 2.5 and 5 mM concentrations of SNAP were tested to determine the minimum time required to produce a lethal effect on NEJ parasites. Twelve to 20 parasites were incubated with SNAP and cultures were sequentially terminated at various time intervals (10, 20, 40 minutes and 6 hours). Viability was assessed by a trypan blue exclusion test to determine the time required to kill the parasites. Killing was first detected at 40 minutes incubation with 5 mM SNAP, when 20% of parasites were dead. After 6 hours of incubation with 2.5mM SNAP, only one parasite out of 12 was dead (Table 7.1)

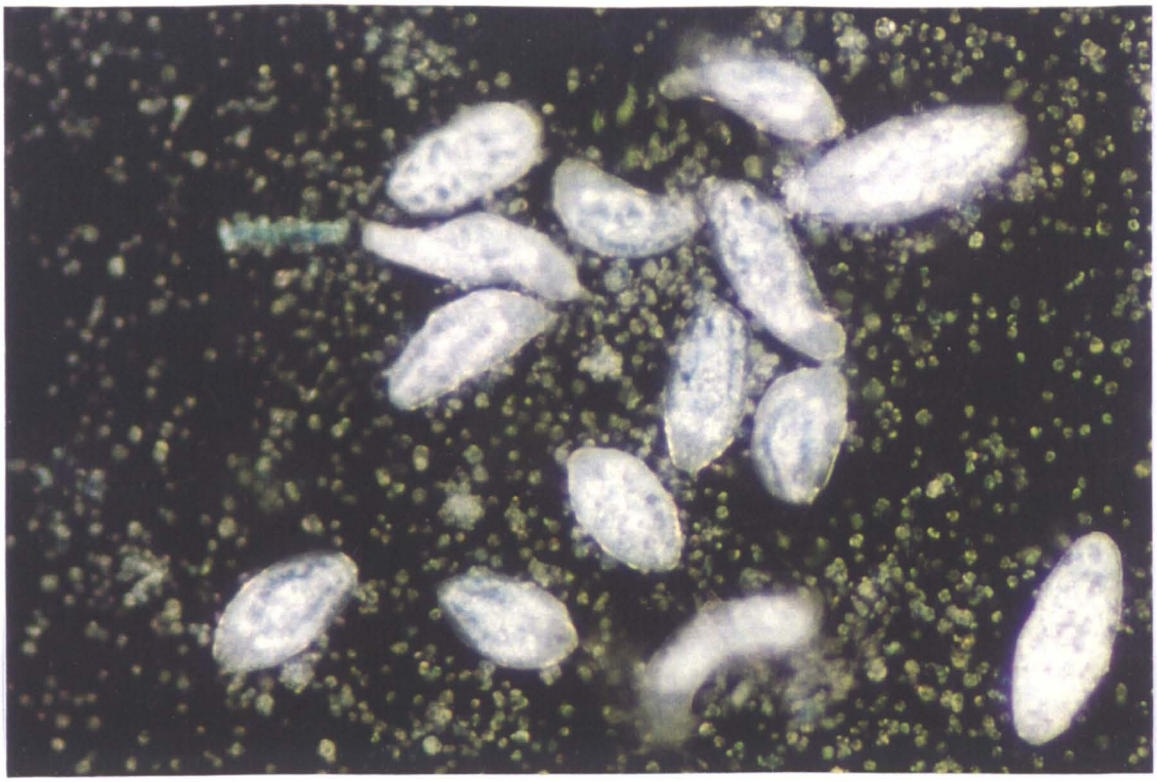
7.3.1.3 Susceptibility of NEJ Parasites to Combined Doses of H_2O_2 and NO:

When the SNAP in various concentrations was used in combination with various concentrations of H_2O_2, the reduction in the viability of the parasites was slightly, although not significantly ($P>0.05$), greater than that observed when the individual chemicals were used separately at the same concentrations (Fig.7.3).

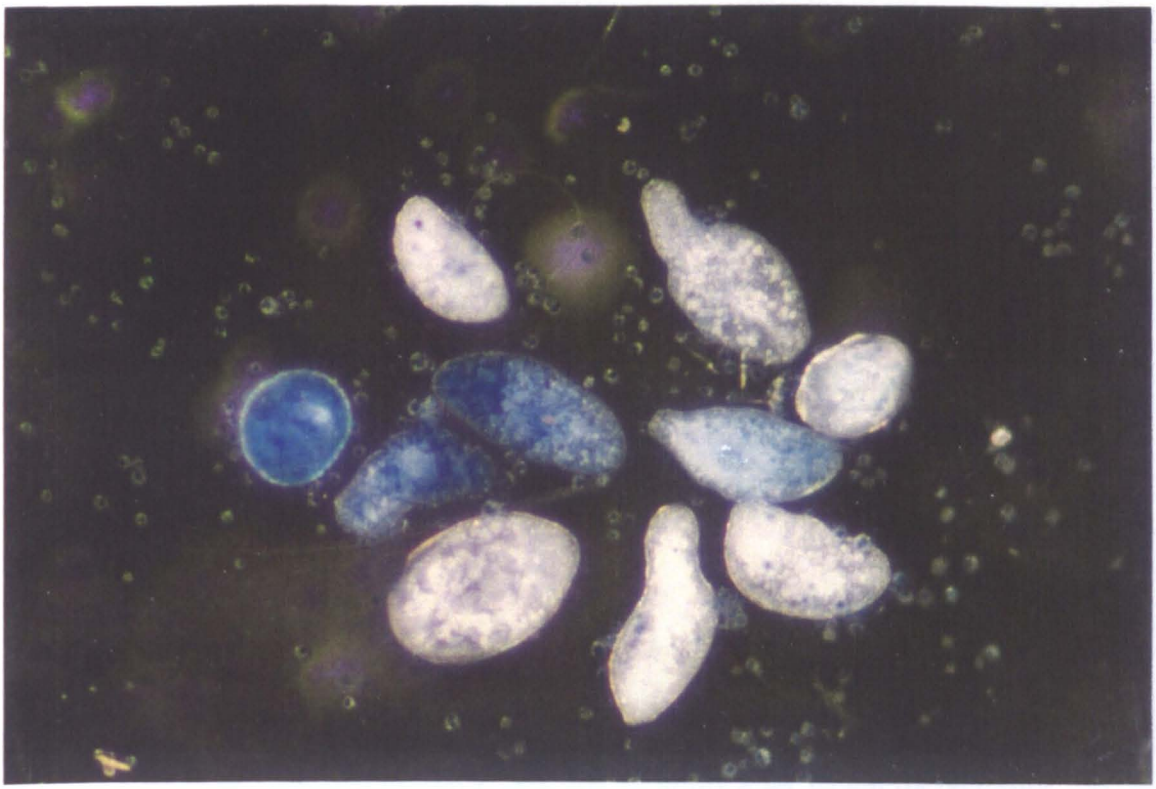
Time	1 mM H_2O_2	0.5 mM H_2O_2	5 mM SNAP	2.5 mM SNAP
10 minutes	0/12	0/19	0/15	0/16
20 minutes	13/13	0/13	0/12	0/12
40 minutes	15/15	1/14	2/10	0/14
6 hours	20/21	9/10	2/12	1/12

Table 7.1 Determination of time required for H_2O_2 and SNAP at specified concentrations to kill NEJ *F. hepatica*. NEJ parasites were exposed to two different concentrations of H_2O_2 and SNAP for the various times specified and the viability of the parasites was determined by trypan blue exclusion. On the right is the number of parasites exposed to the chemicals and on the left is the number of parasites killed at the specified times.

a)



b)



Photograph 7.1 Success of the trypan blue exclusion test. (a) shows live parasites and (b) shows live and dead parasites, with dead parasites stained blue.

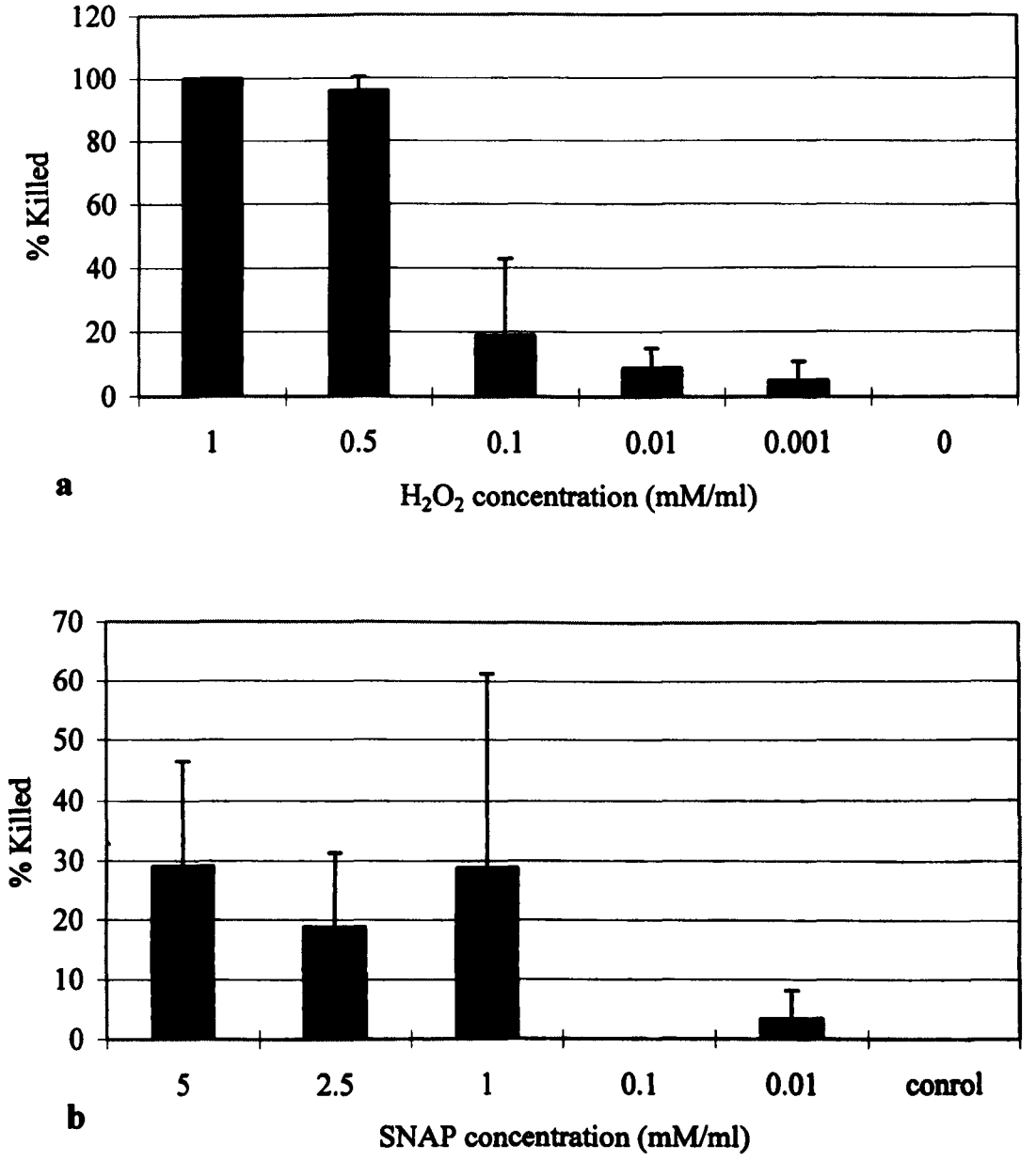


Fig.7.1 Susceptibility of NEJ *F. hepatica* to (a) hydrogen peroxide and (b) nitric oxide generated from SNAP. NEJ parasites were exposed to different concentrations of H_2O_2 and SNAP, as indicated on the x axis, for 72 hours after which the viability of the parasites was determined by trypan blue exclusion. The results are expressed as mean of the four experiments. Each experiment was performed with 20 parasites per concentration Error bars represent standard deviation of the mean.

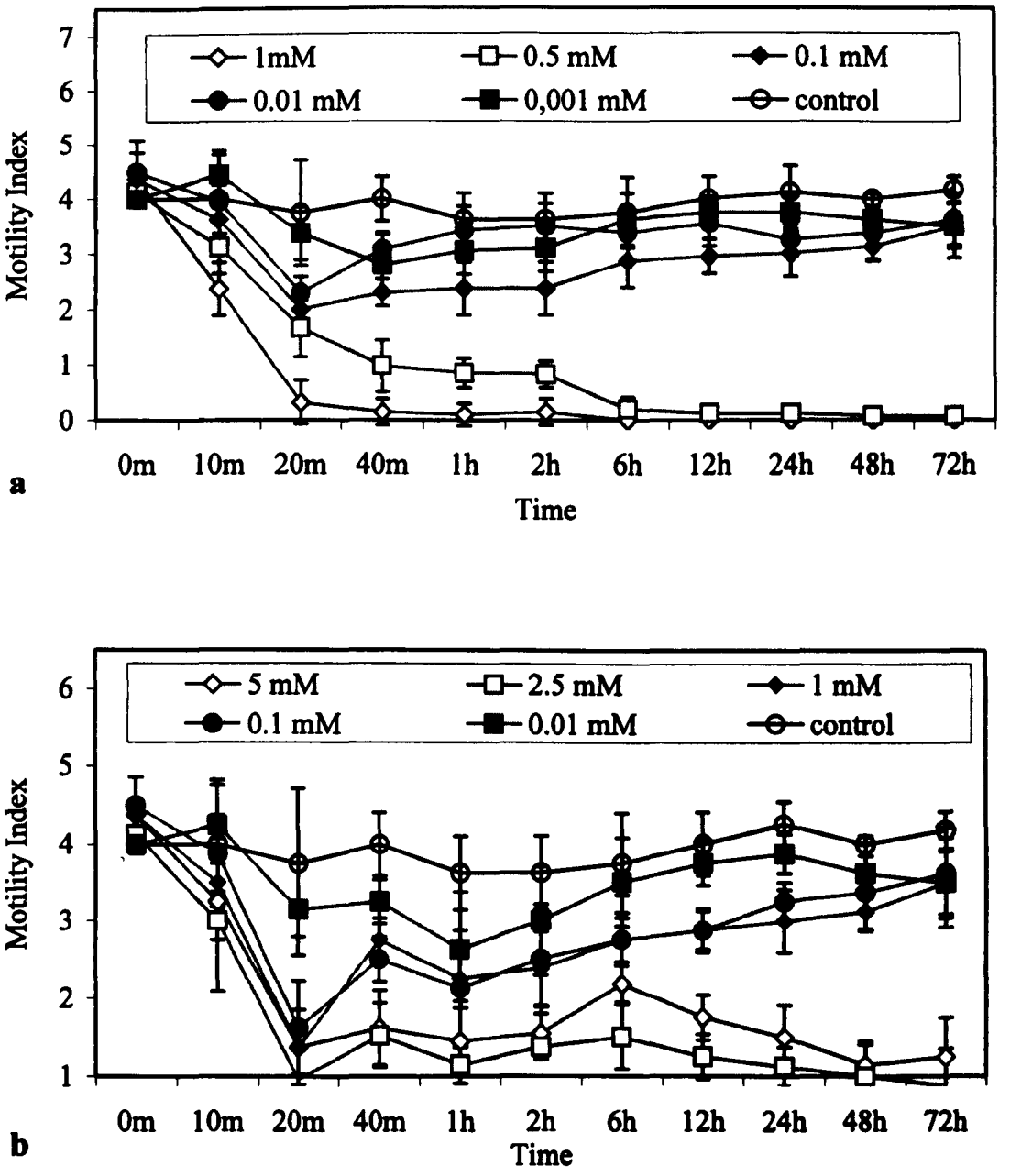


Fig.7.2 Effect of various concentrations of (a) hydrogen peroxide and of (b) SNAP on the motility of NEJ *F. hepatica*. NEJ parasites were exposed to different concentrations of H_2O_2 and SNAP, as indicated on the figure legends, and the motility of the parasites was recorded at various times as indicated on the x axis. The results are expressed as the mean of the motility indexes of the four experiments. Each experiment was performed with 20 parasites per concentration Error bars represent standard deviation of the mean. m is minute and h is hours.

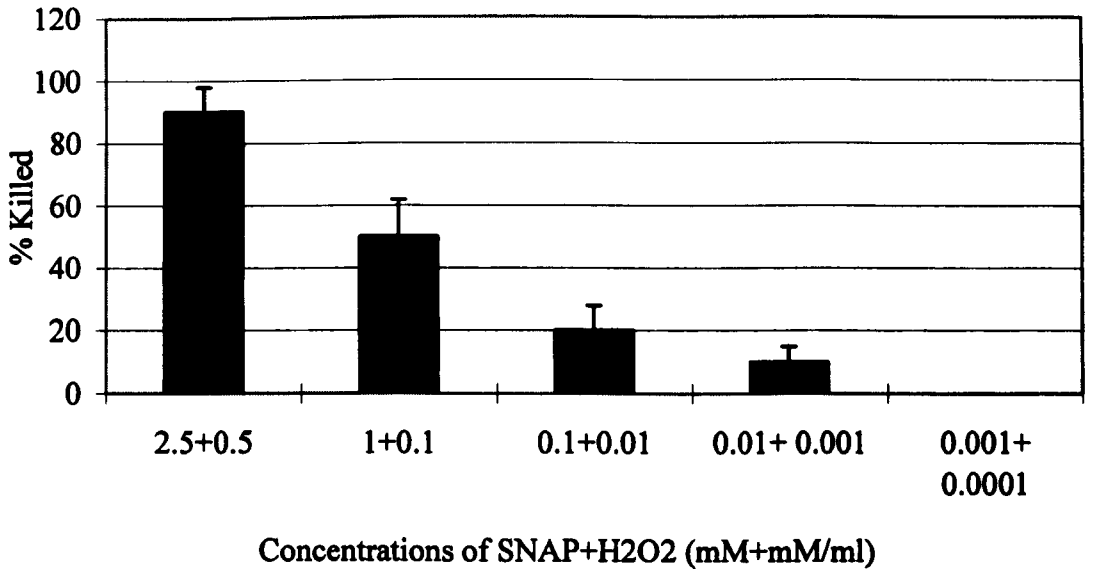


Fig.7.3 Susceptibility of NEJ *F. hepatica* to combined doses of hydrogen peroxide and nitric oxide generated from SNAP. NEJ parasites were exposed to combinations of different concentrations of H_2O_2 and SNAP, as indicated on x axis, for 72 hours after which the viability of the parasites was determined by trypan blue exclusion. The results are expressed as the mean of the four experiments. Each experiment was performed with 20 parasites per concentration Error bars represent standard deviation of the mean.

7.3.2 Activated Macrophage Cultures:

7.3.2.1 Susceptibility of NEJ Parasites to Killing by IFN-γ and/or LPS Activated Macrophages:

The amount of nitrite produced by activated macrophages and parasite viability are shown in Figure 7.4 and changes in parasite motility are summarised in Table 2. Macrophages activated with IFN-γ produced only 28 μM/ml nitrite, whereas LPS and the combination of LPS and IFN-γ induced 109 and 125 μM /ml nitrite respectively. Parasite killing was significantly correlated ($r=0.98$; $P<0.01$) with the amount of nitrite produced. While all the parasites were alive in culture activated with IFN-γ alone, 50% and 80% of the parasites were killed in cultures activated with LPS and the combination of LPS and IFN-γ respectively. In control cultures, where the parasites were either incubated without macrophages or with non-activated macrophages, there was no detectable nitrite and all the parasites survived. By 12 hours, a reduction in the motility of the parasites was observed in cultures activated with IFN-γ and/or LPS.

Killing of the parasites was not associated with cell adherence. In non-activated cultures, the parasites were coated with macrophages, but this had no effect on the motility or the viability of the parasites. In the activated cultures there was less macrophage adherence to the surface of the parasites compared to cultures with non-activated cells.

7.3.2.2 Inhibition of H_2O_2 and NO with Catalase and L-NMMA from IFN-γ and LPS Activated Macrophages:

The macrophage cell line was activated with a combination of IFN-γ and LPS, and the specific activity of H_2O_2 and the production of NO were inhibited by catalase and L-NMMA in some cultures (Fig.7.4). Although the addition of catalase (10 μg/ml) to the culture prevented the reduction in motility before 12 hours, it did not prevent a reduction in motility after 12 hours of incubation nor the killing of the parasites (Table 7.2). All the parasites were killed in these cultures. However, when NO

production was inhibited by the addition of the specific inhibitor, L-NMMA, the reduction in motility was partially prevented. Although L-NMMA significantly reduced the amount of nitrite, it was unable to eliminate NO production completely and 3 out of 20 parasites were dead. However, the addition of L-NMMA together with catalase, completely prevented the reduction in motility and the killing. In this culture nitrite levels were low, about 12 μM/ml.

The reagents used to activate or to modulate the activation had no adverse effect on the motility or the viability of the parasites. The parasites cultured with the same amount of reagent used to activate macrophages were all alive at the termination of the experiment at 90 hours (Fig.7.4).

7.3.3 Ultrastructural Observations on NEJ Parasites Exposed to SNAP and H_2O_2:

The NEJ parasites were exposed to 2.5 mM SNAP and 0.1 mM H_2O_2 for 6 hours and examined under by electron microscopy to determine whether NO or H_2O_2 could mediate damage to the parasites at an ultrastructural level. In control cultures where the parasites were incubated in medium, all had an intact ultrastracture (Fig.7.5). In contrast, there was evidence of extensive damage in mitochondria in the parasites exposed to SNAP (Fig.7.6 and Fig.7.7). There was also some evidence of detachment of the tegumental glycocalyx in some parasites exposed to H_2O_2 (Fig.7.8)

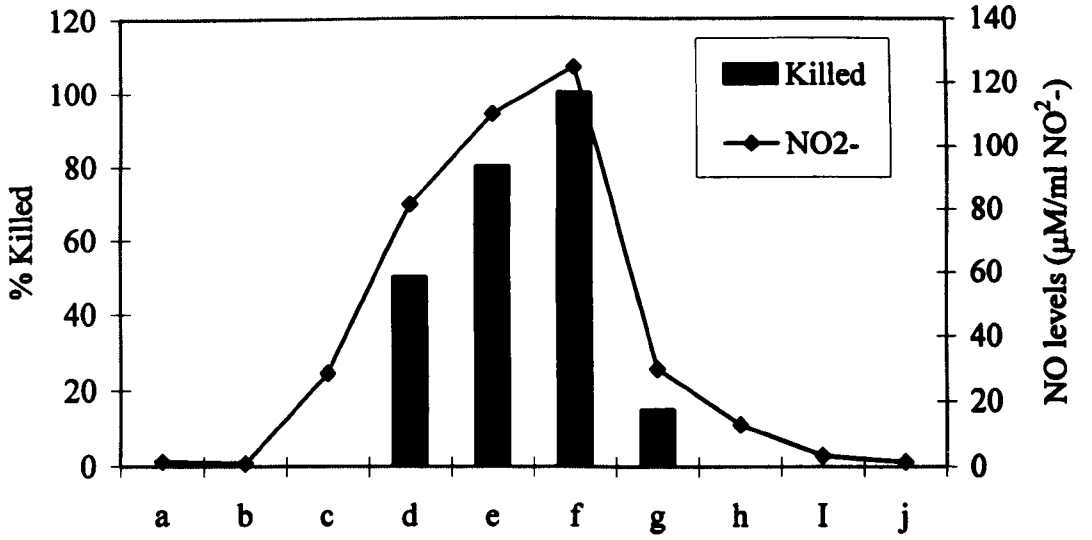


Fig.7.4 Susceptibility of NEJ *F. hepatica* to killing by IFN-γ and/or LPS activated macrophages. Mouse macrophage cell line, J774, was activated with mouse rIFN-γ and/or LPS and 20 NEJ parasites were co-cultured for 90 hours. Some of the cultures were modulated with catalase and L-NMMA to block H_2O_2 activity and NO production respectively. At the termination of the cultures at 90 hours, culture supernatants were tested for nitrite levels and parasite viability assessed by trypan blue exclusion. The results are expressed as the percentage of parasites killed in specified culture conditions (see below for details) and NO levels were expressed as μM nitrite per ml of culture supernatant.

a) control: parasites in culture medium, no cells
b) control: parasites were co-cultured with non-activated cells
c) parasites were co-cultured with 100 U/ml IFN-γ activated cells
d) parasites were co-cultured with 1μg/ml LPS activated cells
e) parasites were co-cultured with 100 U/ml IFN-γ and 1 μg/ml LPS activated cells
f) as in (e) but activation modulated with 10 μg/ml catalase to block H_2O_2 activity
g) as in (e) but activation modulated with 0.5 mM L-NMMA to inhibit NO production
h) as in (e) but activation modulated with a combination of 10 U/ml catalase and 0.5 mM L-NMMA.
i) control: parasites cultured with medium including 100 U/ml IFN-γ and 1 μg/ml LPS to ensure that these reagents had no effect on the viability of the parasites.
j) control: parasites cultured with medium including 10 μg/ml catalase and 0.5 mM L-NMMA to ensure that these reagents had no effect on the viability of the parasites.

Culture conditions*/time	0 h	1 h	6 h	12 h	24 h	48 h	90 h
a	4	4.2	4.1	4.2	4.1	4	4.1
b	4	4.1	4	3.8	4	4	4.2
c	4.1	4.5	4	3	2.6	3	2.6
d	4.2	4	3.4	2.5	2.1	2	1
e	4.1	3.8	3	2.1	1.6	1.2	0.2
f	4	3.8	3	3	1	0.5	0
g	4.3	4	3.5	3.6	2.8	2	2.5
h	4.2	3.8	4	4	4.2	4	4.1
i	3.6	4	4.1	4.2	4.2	4	4.2
j	4.3	4.2	4.3	4.4	4.2	4	4.2

Table 7.2 Effect of activated mouse macrophage cell line on the motility of NEJ *F. hepatica*. NEJ parasites were co-cultured with macrophages and their motility assessed at various times as indicated on the table. * See Fig.7.4 legend for the culture conditions

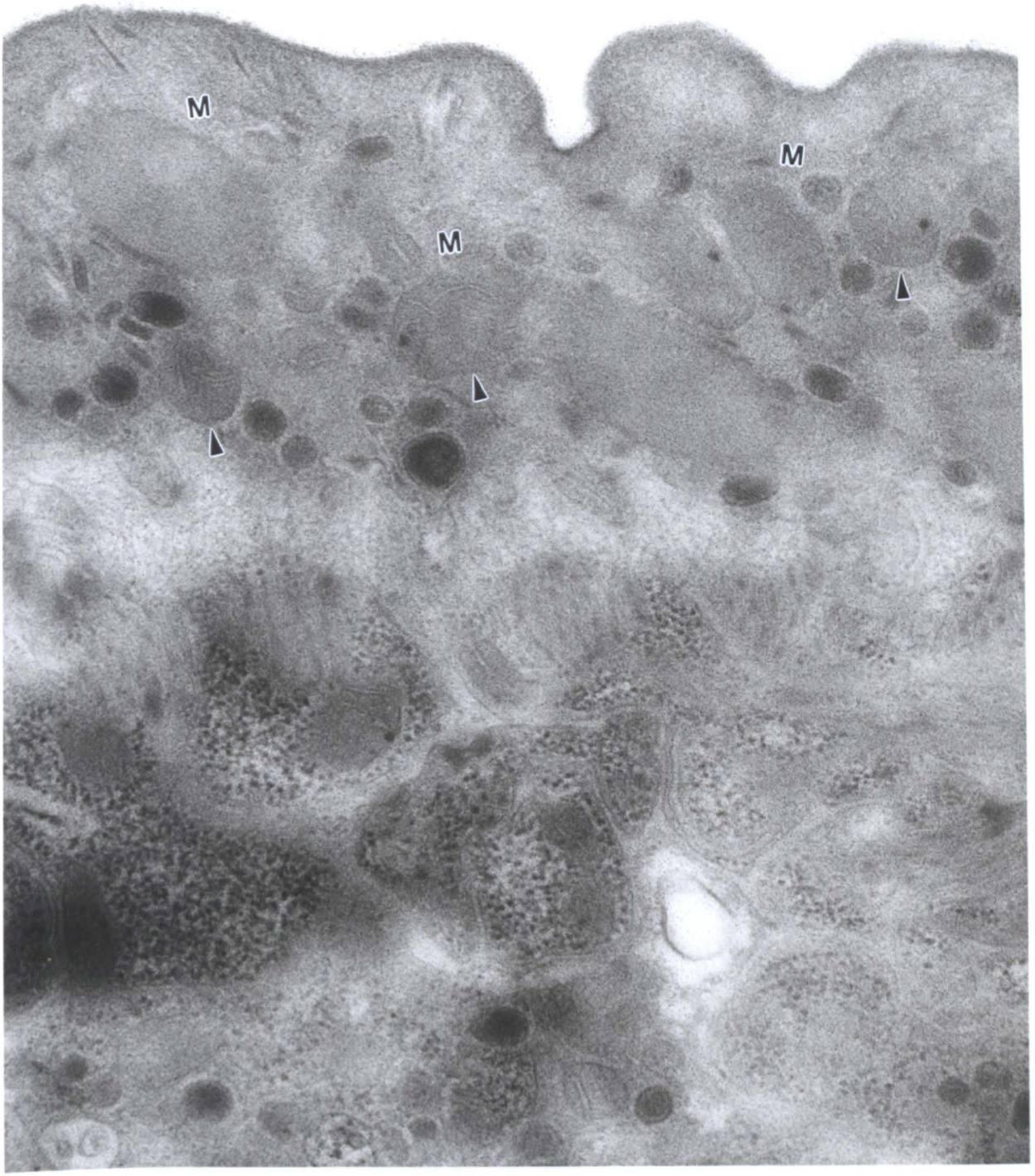


Fig.7.5 NEJ parasites were incubated for 6 hours with medium only. Normal structure of the parasite is shown. No tegumental or mitochondrial damage. M mitochondria, D tegumental granule 0 (T0). Magnification x 52,000.

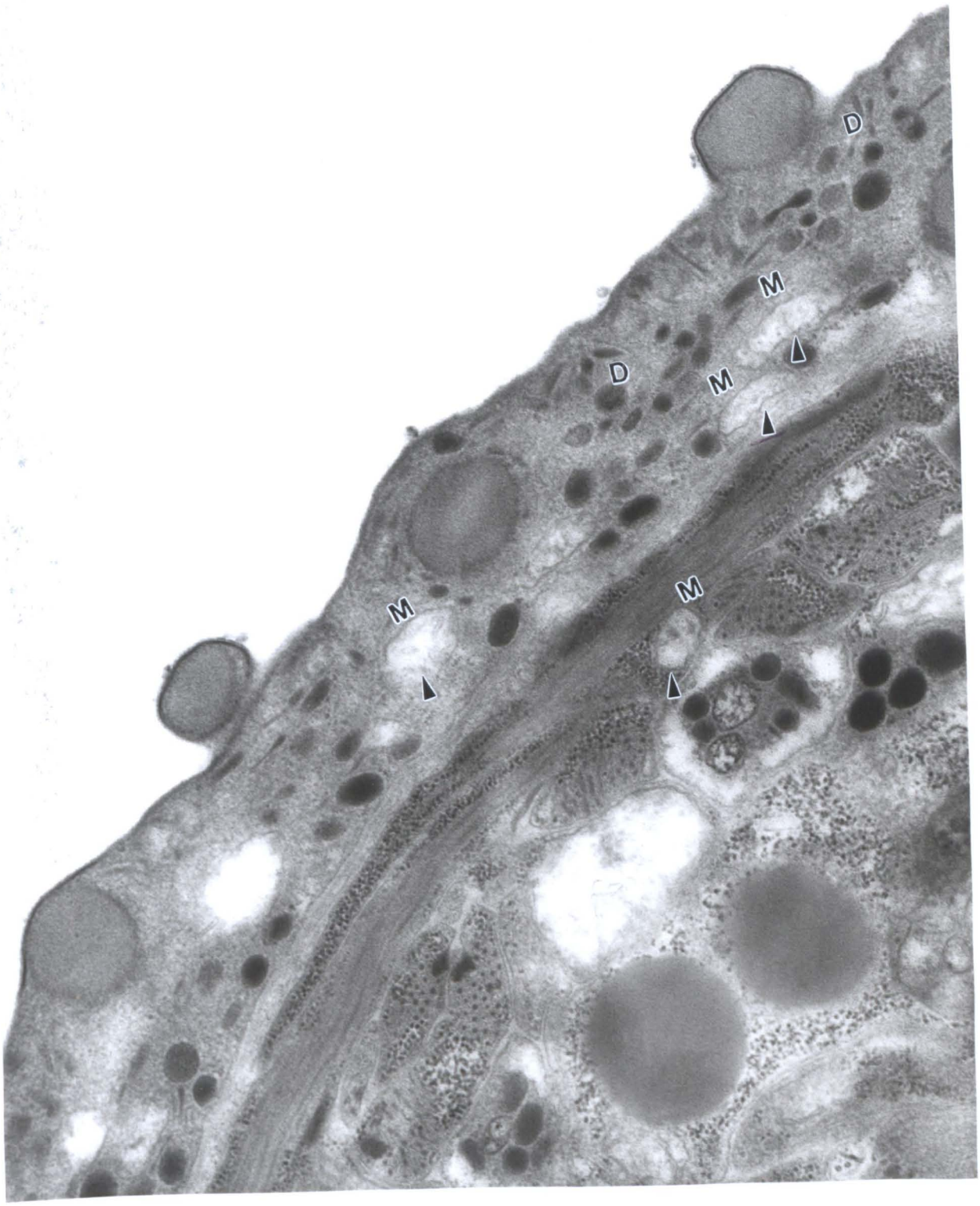


Fig.7.6 NEJ parasites were incubated for 6 hours with 2.5 mM SNAP. Note damage to the mitochondria (M). D is T0. Magnification x 38,000.

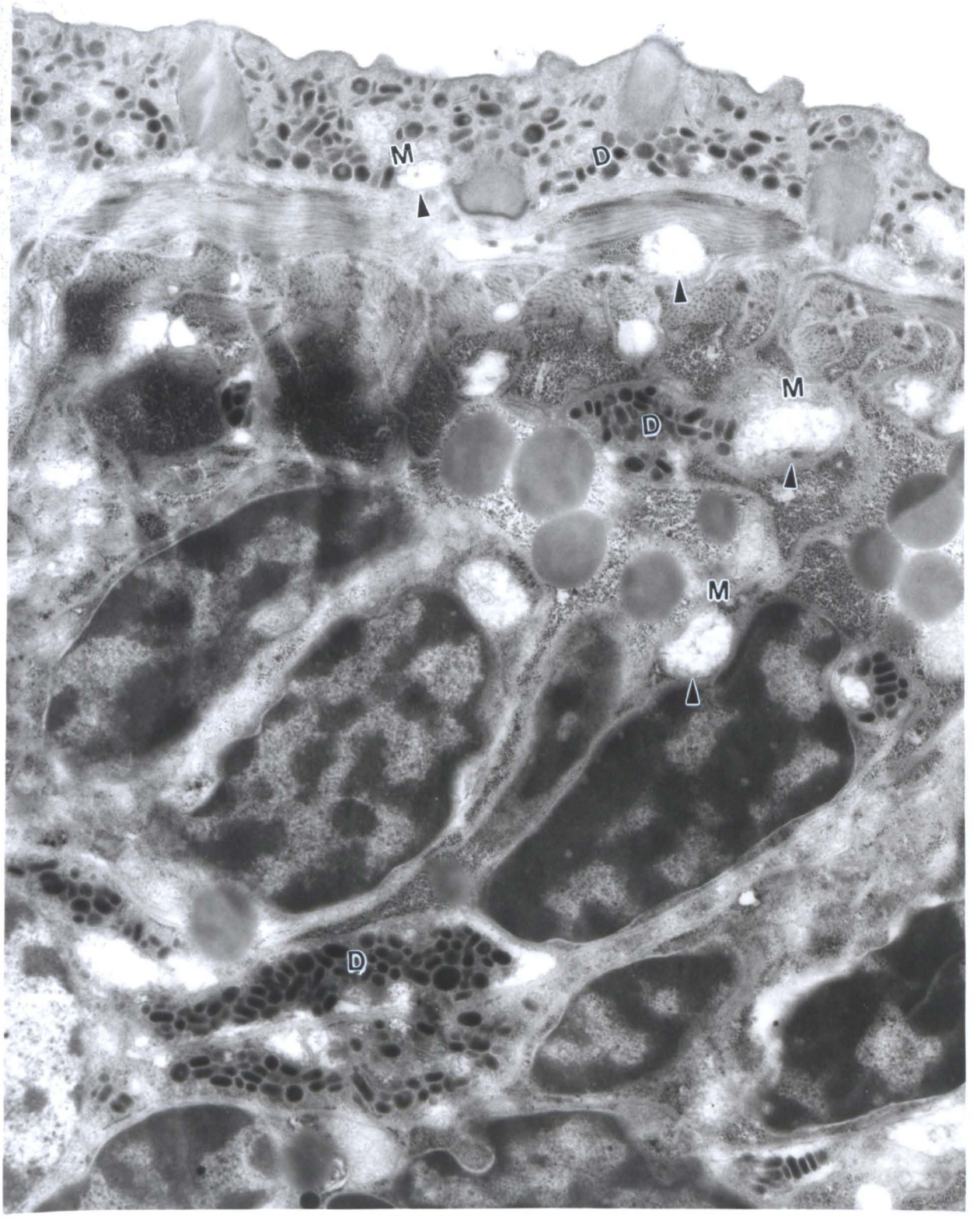


Fig.7.7 NEJ parasites were incubated for 6 hours with 2.5 mM SNAP. Note damage to the mitochondria (M), D is T0. Magnification x 18,500.

7.4 Discussion:

The results of this study show that NEJ of *F. hepatica* are susceptible to NO and H_2O_2 in a cell-free environment and to the substances generated by an activated mouse macrophage cell line, J774, *in vitro*. Dose-dependent susceptibility of the parasites to SNAP and the highly significant correlation between the viability of the parasites and the nitrite produced in experiments with activated macrophages indicate that this may due to the generation of NO.

In a cell free system, NEJs were killed at high concentrations of NO and H_2O_2. Nitric oxide generated from 5 and 2.5 mM SNAP significantly reduced motility of the parasites and 18 to 30 % parasites were killed after 72 hours exposure. The parasites were immobilised within 20 minutes of exposure to SNAP at concentrations ranging from 0.1 to 5 mM. However, the motility of NEJs exposed to 0.1 and 1 mM concentrations of SNAP was gradually restored over time and by the end of the experiment was the as same as the motility of those in control cultures. This result may indicate that the spontaneous release of NO from SNAP might last for about 20 minutes, after which parasites receiving lower doses of NO recover while those exposed to higher doses are severely damaged and eventually die. Since neither the duration of NO release from SNAP nor its quantity are known, this hypothesis is based on the fact that NEJs lost motility at around 20 minutes of exposure regardless of SNAP concentration. Only a proportion of parasites in each well were killed. There are two possible explanations for this discrepancy: first, the genetic diversity of individual parasites may mean that some are resistant to the lethal effects of NO; second, that all parasites exposed to the same concentration of NO had damage to critical organelles but that death took longer than the 90 hours of this assay. The second possibility is the more likely one, since all the parasites exposed to SNAP for 6 hours showed evidence of mitochondrial damage, but were still alive before collection for processing for EM.

The ultrastructural study to identify the damage caused by NO revealed a disruption of mitochondria within the muscle and tegumental cells. This result is consistent with

the proposed reactivity of NO with iron and therefore with targeting the Fe-S containing enzymes in the mitochondrial electron transport chain (Ding *et al.*, 1988; Hibbs, 1992). Similar ultrastructural changes have also been demonstrated in larvae of the closely related trematode parasite *Schistosoma mansoni* following *in vitro* incubation with cytokine-activated macrophages (McLaren and James, 1985). There was also some evidence of detachment of the tegument in NEJs exposed to H_2O_2. Excretory granules around the gut epithelium were less prominent in this group.

Killing increased with increased incubation time. High concentrations of H_2O_2 were very potent mediator but lower concentrations had little or no effect. With a similar experiment, Taylor *et al.* (1996) observed that microfilariae of *Onchocerca lienalis* were highly susceptible to H_2O_2 with concentrations as low as 0.001 mM inducing rapid reduction in motility and viability of the microfilariae. In our experiment, 100 x higher concentration of H_2O_2 only killed about 18% of the NEJs. This discrepancy could be due to the methods used to measure the viability. In this experiment, we measured killing directly by trypan blue exclusion, whereas Taylor *et al.* (1996) measured killing by an MTT reduction assay. Alternatively, NEJs may be less susceptible to H_2O_2 than microfilariae of *O. lienalis*. The inability of catalase to block the killing by activated macrophages, and, in cell-free conditions, the requirement of high concentrations to kill the parasites, support this argument and indicate that H_2O_2 is an unlikely candidate for involvement in immune mediated oxidative damage.

This resistance to H_2O_2 concentrations at or below 100 μm may be a strategy of NEJ parasites to evade immune damage. Reactive oxygen species (ROS) are the product of the normal oxidative metabolism of living organisms in an aerobic environment. High concentrations of ROS generated by the parasitic organism itself or by the immune cells of the host can cause serious damage to membrane lipids, nucleic acids and proteins. Organisms, therefore, neutralise these potentially harmful metabolites by producing antioxidants and enzymes which repair oxidatively damaged molecules (McGonigle *et al.*, 1998). It has also been postulated that the production of appropriate detoxification enzymes is one of the mechanisms by which parasites

evade immune mediated oxidative damage (Callahan *et al.*, 1988; McGonigle *et al.*, 1998). Antioxidant enzymes, such as catalase and superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase and the recently described antioxidant family, peroxidoxins, have the ability to neutralise or to decompose ROS-alkyl hydroperoxides and H_2O_2. The presence of SOD (main O_2^{-*} scavenger) activity has recently been reported in E/S and somatic extract of immature and mature *F. hepatica* (Barrett, 1980; Piacenza *et al.*, 1998). SOD catalyses the spontaneous dismutation of O_2^{-*} to H_2O_2 and molecular oxygen (O_2). It is possible that these fluke-derived molecules protect against the cytotoxic effects of H_2O_2.

In conclusion, NO does appear to damage and kill NEJs of *F. hepatica*. Whether this mechanism operates *in vivo* and contributes to the host protective mechanisms against *F. hepatica* awaits further investigation.

CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSIONS

The study described in this thesis investigated the cell-mediated immune responses, in particular T cell and IL-2, IL-4 and IFN-γ responses, of cattle to experimental infection with *F. hepatica*. T cell responses were investigated by *in vitro* proliferation and cytokine (IFN-γ, IL-2 and IL-4) production of PBMC stimulated with fluke antigens. Fluke E/S and somatic antigen-specific cell lines were also generated from PBMC of a hyper-immune cow collected within 5 weeks of a super-infection and the composition of the cell populations was analysed by flow cytometry to determine the antigen-responding cell population/s.

PBMC from the infected calves proliferated vigorously *in vitro* in response to fluke E/S and somatic antigens early in the primary infection but were unresponsive after 6 wpi (Chapter 4). This finding confirms those made previously (Oldham and Williams, 1985; Clery and Mulcahy, 1998; McCole *et al.*, 1999) and verifies the fact that the immune response to *F. hepatica* infection in cattle is characterised by a transient proliferation of PBMC to fluke antigens *in vitro*. However, it is not known which cell population/s are proliferating or expanding in response to fluke antigens *in vitro* early in infection. Bovine PBMC include a relatively high number of $\gamma\delta^+$ T cells and overgrowth of $\gamma\delta^+$ T cells has been commonly observed in cell lines (Brown *et al.*, 1994a). While cloned $\gamma\delta^+$ T cells failed to proliferate in response to antigens, they did proliferate in response to auto-antigens in an autologous mixed leukocyte reaction (Hanby-Flarida *et al.*, 1996; Okragly *et al.*, 1996). It follows that non-specific proliferation of $\gamma\delta^+$ T cells may impede the correct interpretation of *in vitro* proliferation assays. It is not certain whether the proliferation response observed in our study and elsewhere is directly attributable to antigen responding $\alpha\beta^+$ T cells or to outgrowth of antigen non-specific $\gamma\delta^+$ T cells. A recent attempt to characterise T cell subset involvement in cattle infected with *F. hepatica* by sequential depletion of T cell subsets ($CD4^+$, $CD8^+$ and $\gamma\delta^+$) from whole PBMC

revealed that depletion of both $CD4^+$ and $CD8^+$ T cell subsets ablated the proliferation to fluke antigens *in vitro* of the remaining cells (McCole *et al.*, 1999). Although that study indicates a role for $CD4^+$ and $CD8^+$ T cells in proliferation response to fluke antigens *in vitro*, it does not consider other cell populations such as B lymphocytes. In this study, we sought to deliver a direct answer to the question of which cells are proliferating in response to fluke antigens early in the infection by establishing *F. hepatica* antigen-specific cell lines, which were then labelled with a panel of bovine lymphocyte subset-specific monoclonal antibodies and analysed by flow cytometry. Flow cytometry analysis of three somatic and four E/S antigen-specific cell lines revealed that the proliferating cells were predominantly $CD4^+$ T cells (Chapter 6). While less than 1% of the cells stained with monoclonal antibodies specific to bovine CD8, γδ T cells and B cells, over 95% in somatic and 87% in E/S antigen-specific cell lines stained positively with Mabs to CD4 and the pan-T cell marker, CD2. This result contradicts, in part, those reported by McCole *et al.* (1999) who also suggested a role for $CD8^+$ T cells in the proliferation response. The difference may be attributable to the varying methods used in these studies. On the other hand, using a similar approach to ours, Brown *et al.* (1994a) reported a mixture of $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T cells in their fluke somatic antigen-specific cell lines prepared from two cows chronically-infected with *F. hepatica*. In contrast to our results, overgrowth of $\gamma\delta^+$ T cells was commonly observed in their cell lines, but the authors stated that cell lines containing less than 10% $CD8^+$ T cells or depleted of $\gamma\delta^+$ T cells proliferated vigorously in response to fluke antigen. This result, together with the lack of $CD8^+$ and $\gamma\delta^+$ T cell populations in our study, suggests that these cell populations are not the predominant responding cell population *in vitro*, and emphasises the involvement of $CD4^+$ T helper cells in the immune responses of cattle to infection with *F. hepatica*. Nevertheless, the recently described $CD8^+$ T cell phenotype, which can switch development to a $CD8^-CD4^-$ Th2-like phenotype when activated in the presence of IL-4 (Erard and Gros, 1994), should not be forgotten, nor the proposed role of $CD8^+$ T cells in fluke infection (McCole *et al.*, 1999).

$CD4^+$ helper T lymphocytes are immunoregulatory cells which initiate and control on-going immune responses by secreting cross-regulatory cytokines. This cell

population was initially divided into two groups according to the cytokine repertoires of cloned murine cells: IFN-γ and IL-2 producing cells were named as Th1; IL-4, IL-5 and IL-10 producing cells as Th2 (Mosmann and Coffman, 1989). While IFN-γ dominated Th1 type immune responses are involved in cell-mediated inflammatory reactions, IL-4 dominated Th2 type cells assist antibody production and antibody-mediated immune mechanisms. Although recent evidence accumulated from humans and bovine $CD4^+$ T cell clones suggests the presence of Th0 subsets with rather unrestricted cytokine profiles (Mosmann and Sad, 1996; Brown and Estes, 1997; Brown *et al.*, 1998), the Th1/Th2 dichotomy, or IL-4/IFN-γ dominated responses with diverse regulatory effects, remains an important functional division in the immune system (Mosmann and Sad, 1996). It was therefore anticipated that the measurement of cytokine profiles during the course of infection with *F. hepatica* might provide some insight into T cell function during the immune response to *F. hepatica*. Levels of cytokines, IL-2, IL-4 and IFN-γ in culture supernatants were measured by bioassays or ELISA. During the early primary infection, fluke antigen-induced IFN-γ and IL-2 were detectable in culture supernatants. However, when the infection reached patency, the levels of these cytokines in culture supernatants were either undetectable or similar to the control animal's. The kinetics and pattern of IL-2 and IFN-γ levels were similar to, and in the case of IL-2 significantly correlated ($P<0.05$) with, the proliferation responses of PBMC. In contrast, no antigen-induced IL-4 was detectable after infection. These results, together with those of Clery and Mulcahy (1998) and Brown *et al.* (1994a), would suggest that a Th1-like immune response is elicited early in the infection, which may polarise to a Th2 type response after 6 wpi.

Antibody isotype responses to *F. hepatica* were measured in order to define to some extent the underlying T helper cell functions. The antibody response to *F. hepatica* was dominated by an IgG1 response, but an increase in fluke E/S antigen-specific IgG2 antibody levels was also observed early in the primary infection. IgG2, IgM and IgA levels peaked at two weeks post-infection and then returned to lower but still detectable levels by the fifth week post-infection (Chapter 4). In cattle, although there is as yet no direct *in vivo* evidence, recent *in vitro* studies indicate that rboIFN-γ

stimulates the production of IgG2 and IgM (Estes *et al.*, 1994), whereas rboIL-4 upregulates IgG1, IgA and IgE antibody production (Estes *et al.*, 1995), by B lymphocytes co-stimulated with polyclonal B cell activating factors. Dominant IgG2 antibody responses are commonly observed with infections that induce strong IFN-γ production, whereas helminth infections which induce an IgG1 antibody response produce elevated levels of IL-4. These observations suggest that the *in vitro* cytokine-regulated immunoglobulin isotype switch might be relevant *in vivo*. It is, therefore, tempting to postulate, on the basis of the presence of IgG2 antibody response early in the infection, the existence of a Th1-like response *in vivo*, supported by the *in vitro* observations. However, the presence of an early IgA and of constantly increasing IgG1 responses suggests that regulation of antibody responses *in vivo* may be of a complex nature, which cannot be explained completely by a Th1/Th2 dichotomy.

The unresponsiveness of PBMC to fluke antigens is detected in cattle after 6 wpi. Our earlier observations suggested that down-regulation of an IL-2 producing Th1-like immune response might be responsible for this (Chapter 4). There was a significant positive correlation ($P<0.05$) between antigen-specific proliferation and IL-2 production. High levels of IL-2 and IFN-γ produced early in the infection, during which strong proliferation responses of PBMC were observed, and low or undetectable levels of these cytokines later in the infection coincided with the loss of proliferation responses of PBMC to fluke antigens. Addition of exogenous rhIL-2 restored the proliferation responses of PBMC to fluke antigens. Other immunomodulating factors such as NO and TGF-β were also investigated, but although a non-specific increase in nitrite levels in culture supernatants was observed, addition of L-NMMA, an iNOS enzyme inhibitor, did not enhance the proliferation responses of PBMC to fluke antigens during the hyporesponsive periods, indicating that NO is probably not involved in the loss of proliferation response in *F. hepatica*-infected cattle. There was also no difference in TGF-β_1 levels in culture supernatants collected during responsive and hyporesponsive periods (up to 6 wpi and after 6 wpi respectively) or from infected and non-infected control animals. This result indicates that TGF-β is an unlikely candidate for the

unresponsiveness observed. On the other hand, when compared with the control calf, elevated levels of total TGF-β_1 were observed in the serum samples of an infected calf (although only a single infected calf was tested). Since it is speculated that TGF-β is involved in tissue fibrosis (Border and Noble, 1994), this finding may be relevant to the formation of liver fibrosis in fasciolosis in cattle. In a recent review, Meeusen (1999) classified the immunopathology of helminth infections in three categories, including fibrosis associated with IL-4 and TGF-β producing novel Th3 type cells in the category of delayed type hypersensitivity III.

A challenge infection administered 14 weeks after the primary infection resulted in a detectable, but weak and non-significant, proliferation response of PBMC to both E/S and somatic antigens. Similarly, antigen-induced IL-2 and IFN-γ production was detectable but the levels were significantly lower ($P<0.05$) than those induced early in the primary infection. These results are comparable to earlier reports, in which strong antigen-induced proliferation and/or IFN-γ production were observed early in primary infections (McCole *et al.*, 1999; Oldham and Williams, 1985), in contrast to weak proliferation and undetectable/no IFN-γ production following experimental re-infection of cattle harbouring a natural fluke infection (Clery *et al.*, 1996). These results, together with persistently high numbers of eosinophils, an increase in the number of circulating B cells and no increase in serum fluke antigen-specific IgG2 levels following a second infection, indicate a Th2-like immune response in chronically-infected cattle.

However, after treatment of the animals with the flukicide, triclabendazole, strong and significant ($P<0.05$) antigen-specific proliferation and IFN-γ production were again detectable. This data, together with our observation that PBMC from a hyper-immune but treated cow, previously infected on 3 occasions, proliferated vigorously and produced IFN-γ in response to fluke antigens during the early stages of the re-infection, similar to that seen early in a primary infection, suggests that the immune response might be modulated by the presence of adult flukes or of antigen/s released from late immature or mature flukes. This observation appears to indicate strongly that further research may be warranted in this area. In this context, it is well-

documented that an initial schistosomula-induced Th1 type response is down-regulated following the onset of egg production by egg antigen-induced Th2 type responses (Pearce *et al.*, 1991; Wynn *et al.*, 1993; Capron, 1998). It is, therefore, tempting to speculate that *F. hepatica* may be using similar strategies, such as the sequential expression of antigen/s, inducing a Th2 type response which, in turn, downregulates the ongoing Th1-like response. Our attempt to define the cellular responses of cattle to partially-purified fluke antigens showed that, while stimulating high proliferation, some antigenic fractions did not induce IFN-γ production. In contrast, others induced both proliferation and IFN-γ production to varying degrees of magnitude, indicating that different fluke antigens may be capable of inducing different types of immune response (Chapter 6). This latter observation suggests that further research into this area would potentially be of great value for the achievement of a subunit vaccine based on defined antigens with known capacity to induce the desired immune response. However, the nature of protective immune response to *F. hepatica* infection in cattle is still largely unknown. Chronically-infected cattle with strong evidence of Th2-like immune response can be successfully re-infected with *F. hepatica* (Clery *et al.*, 1996). Moreover, there is no conclusive evidence that cattle develop a protective immunity under constant natural exposure in endemic areas (Ortiz-Oblitas, 1997). The Th2-like immune response to fluke infection may not be protective against re-infection. However, the following evidence suggests that IFN-γ producing Th1-like immune responses might correlate with protection. First, Mulcahy *et al.* (1999b) reported partial protection, with a 55% reduction in fluke burdens in calves vaccinated with fluke-derived enzymes (cathepsin L1 and L2) and haemoglobin. PBMC from vaccinated, as compared to non-protected/non-vaccinated calves, showed higher proliferation and IFN-γ production in response to fluke antigens *in vitro* following a challenge infection. In this experiment, serum samples from vaccinated calves also contained high levels of IgG2 antibody to specific fluke antigens. Second, in another study (unpublished data of O'Neill, Dalton and Mills reported by Mulcahy *et al.*, 1999a), it was reported that the C57/BL mouse strain which developed a Th1 type response was more resistant to infection with *F. hepatica* than the BALB/c and 129SV/EV mouse strains which developed a Th2 type immune response. Third, our results demonstrated that NEJ parasites are susceptible

and killed *in vitro* by NO generated from chemical sources (SNAP) or generated by IFN-γ and/or LPS activated mouse macrophage cell line (J774). This result urgently requires confirmation by similar studies using bovine macrophages and *in vivo* experiments in gene knockout mice or mice with enhanced Th1 type immune response.

In conclusion, data presented in this thesis suggest the expression of a Th1-like immune response early in primary *F. hepatica* infection in cattle, which is probably regulated to a Th2-like response later in the infection. Our *in vitro* studies indicate that IFN-γ producing Th1-like response is capable of killing NEJ parasites through the release of NO by macrophages. However, it is difficult to say, at this stage, whether this mechanism would kill the parasite *in vivo*. To clarify this, further *in vivo* work is needed. Our preliminary observations of the existence of a Th2-like immune response coincident with the presence of late-immature and mature parasites indicate that the polarisation of an existing Th1 response to a Th2 response might originate from parasite. Finally, the fact that while some fractionated parasite antigens are capable of inducing IFN-γ others are not indicates the need for further research to pinpoint the antigenic structure capable of producing protective immune responses, which will eventually contribute to the development of a sub-unit vaccine.

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APPENDICES

Appendix 1: Preparation of Buffers

Preparation of Algal Fluid

Solution A

KNO_3	8.0 g/l
KH_2PO_4	10.32 g/l
NaCl	0.16 g/l
Na_2CO_3	6.0 g/l
$Na_3C_6H_5O_7\ 2H_2O$	0.08 g/l

Solution B

	mg/ 100 ml
NH_4VO_3	2
H_3BO_3	286
$CO(No_3)_2\ 6H_2O$	5
$CuSO_4\ 5H_2O$	8
$MgSO_4\ 7H_2O$	25 g
$MnCl_2\ 4H_2O$	180
$Na_2MO\ O_4\ 2H_2O$	20
$ZnSO_4\ 7H_2O$	2

Solution C

$CaCl_2$	5.5 g/ 100 ml

25 ml solution A to ~ 900 ml H_2O then add 1 ml solution B and 1 ml solution C. Make up to 1.0 litre.

Reagents for Enzyme-Linked Immunosorbent Assay (ELISA)

Carbonate Coating Buffer

Dissolve 1.59 g sodium carbonate and 2.93 g sodium bicarbonate in distilled water to a total volume of 1L. Adjust the pH to 9.6 and sterilise by filtration.

ELISA Substrate Solution

Dissolve 10 mg ABTS in 670 µl distilled water. Add 200 µl of the ABTS solution to 10 ml citric acid solution (105 mg citric acid in 10 ml distilled water, adjusted to pH4 with sodium hydroxide solution). Just before use, add 10 µl hydrogen peroxide.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1.5M Tris Buffer pH8.8

Dissolve 18.5 g Tris(hydroxymethyl)methylamine in 25 ml distilled water, adjust to pH8.8 with 50% hydrochloric acid and make up to 100 ml with distilled water.

0.5M Tris Buffer pH6.8

Dissolve 3 g Tris(hydroxymethyl)methylamine in 25 ml distilled water, adjust to pH6.8 with 1 N hydrochloric acid and make up to 50 ml with distilled water

Separating Gel (10%) for SDS-PAGE Minigel

Mix 7.5 ml 40% acrylamide solution, 11.2 ml 1.5M Tris buffer pH8.8 and 11.2 ml double distilled water.
Just before use add 150 µl 10% sodium dodecyl sulphate (SDS), 100 µl 10% ammonium persulphate solution and 20 µl TEMED.

Stacking Gel (5%) for SDS-PAGE Minigel

Mix 1.25 ml 40% acrylamide solution, 1.25 ml 0.5M Tris buffer pH6.8 and 7.44 ml double distilled water.
Just before use add 100 µl 10% SDS, 50 µl 10% ammonium persulphate solution and 10 µl TEMED.

Sample Buffer

Mix 1.88 ml 0.5M Tris buffer pH6.8, 6 ml 10% SDS, 3 ml glycerol and 2.12 ml double distilled water.

Running Buffer pH8.3

Dissolve 6.06 g Tris(hydroxymethyl)methylamine, 28.84 g glycerine and 1 g SDS in 1L distilled water, adjust to 2L with distilled water and check pH.

Excystment Medium

0.05M HCl
Add 250 µl of HCl in 50 ml distilled water

Excystment solution

Sodium bicarbonate	1.0 g
Sodium chloride	0.8 g
Sodium taurocholate	0.2 g
L-cysteine HCl	0.8 g
	ddH_2O to 100 mls

Mix equal volumes of 0.05M HCl with excystment solution.

Appendix 2:

Y = +0.1189 +0.0006 X

R-Squared = 0.9951

Curve fit Q.C. : 0.9951 >= 0.0000
: 0.0000 <= 0.1189 <= 1.0000
: 0.0000 <= 0.0006 <= 1.0000

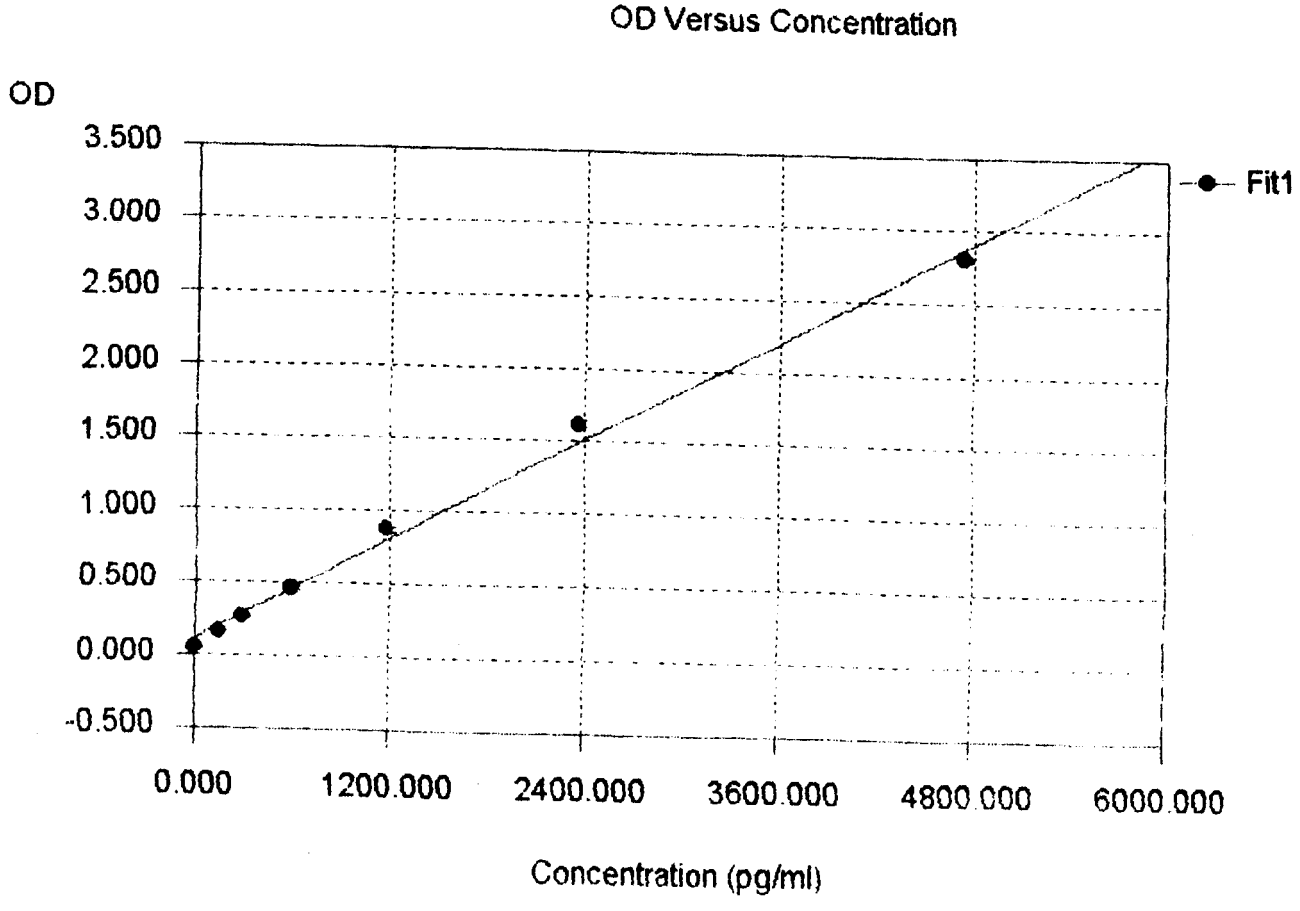


Fig.A1. A typical standard curve for IFN-γ. The IFN-γ concentrations of the samples were extrapolated from standard curves generated from the optical density reading of each concentration of the standard (BSA). The above is a representative standard curve plotted using software incorporated into the ELISA reader (Revelation 3.2, Dynex Technologies).

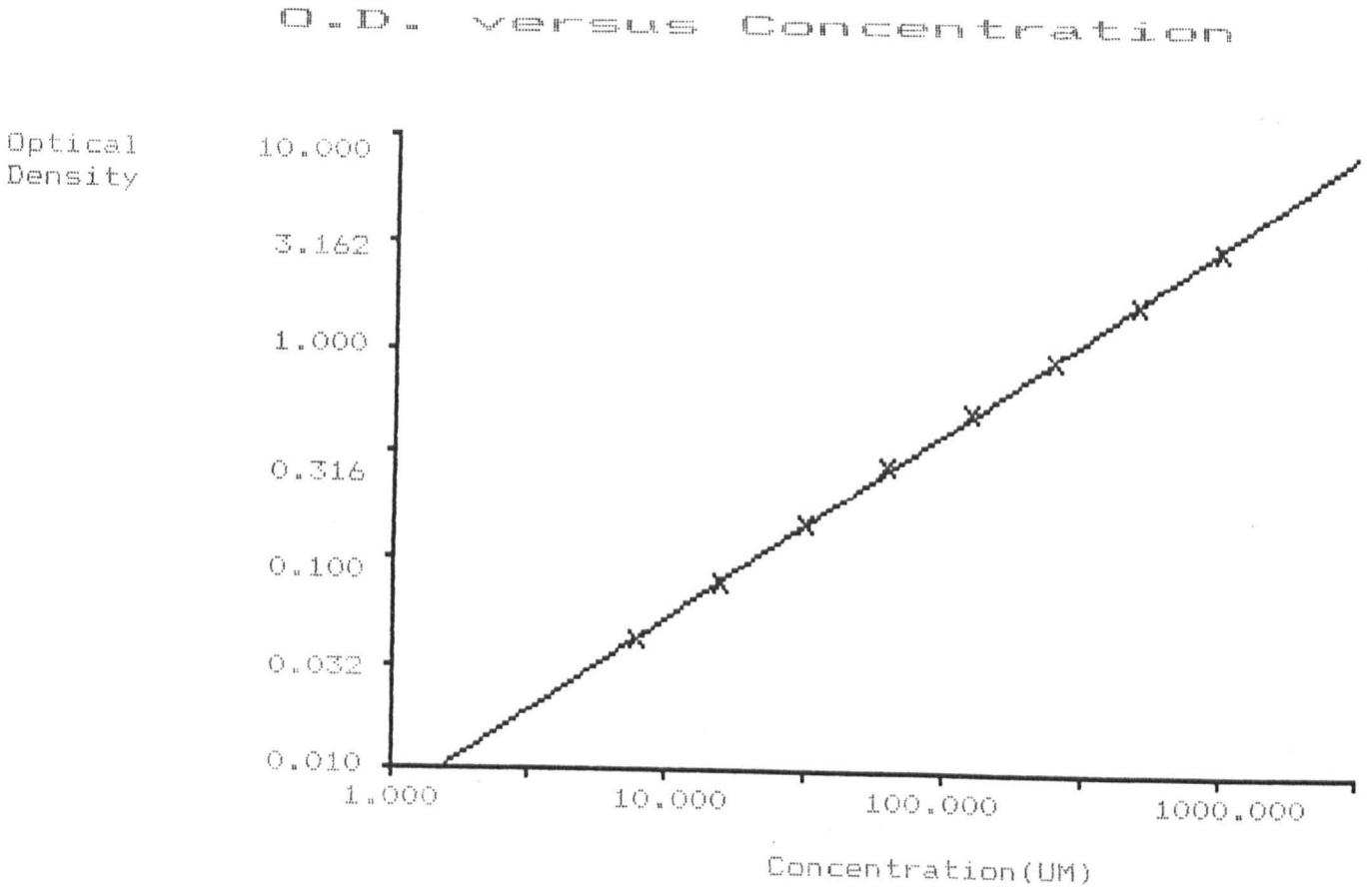


Fig.A2. A typical standard curve for the nitrite assay. Nitrite levels in culture supernatants were extrapolated from standard curves generated from optical density readings of each concentration of the standard (sodium nitrite). The above is a representative standard curve plotted using software incorporated into the ELISA reader (Revelation 3.2, Dynex Technologies).

Y = +0.1834 +0.0014 X

R-Squared = 0.9995

Curve fit Q.C. : 0.9995 >= 0.0000
: 0.0000 <= 0.1834 <= 1.0000
: 0.0000 <= 0.0014 <= 1.0000

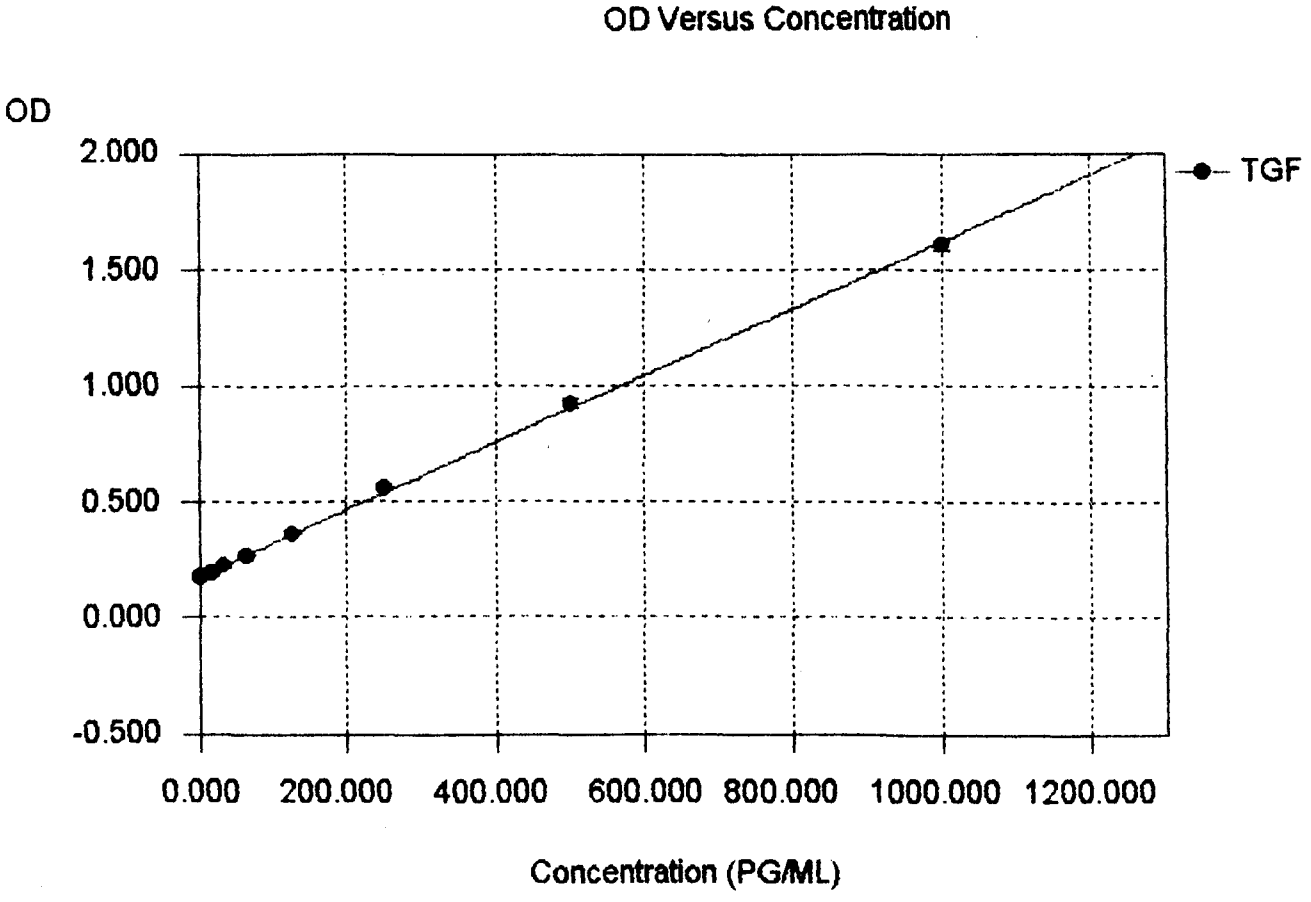


Fig.A3. A typical standard curve for the TGF-β assay. TGF-β in culture supernatants and serum samples was extrapolated from standard curves generated from optical density readings of each concentration of the standard (recombinant human TGF-β1). The above is a representative standard curve plotted using software incorporated into the ELISA reader (Revelation 3.2, Dynex Technologies).

Fig.A4. SDS-PAGE analysis of *F. hepatica* E/S and somatic antigenic fractions. Whole fluke E/S and somatic antigens and each fraction collected from the HPLC chromatography were run on a 10% non-gradient polyacrylamide gel under non-reducing conditions and the gels were stained with Coomassie blue and/or silver stain.

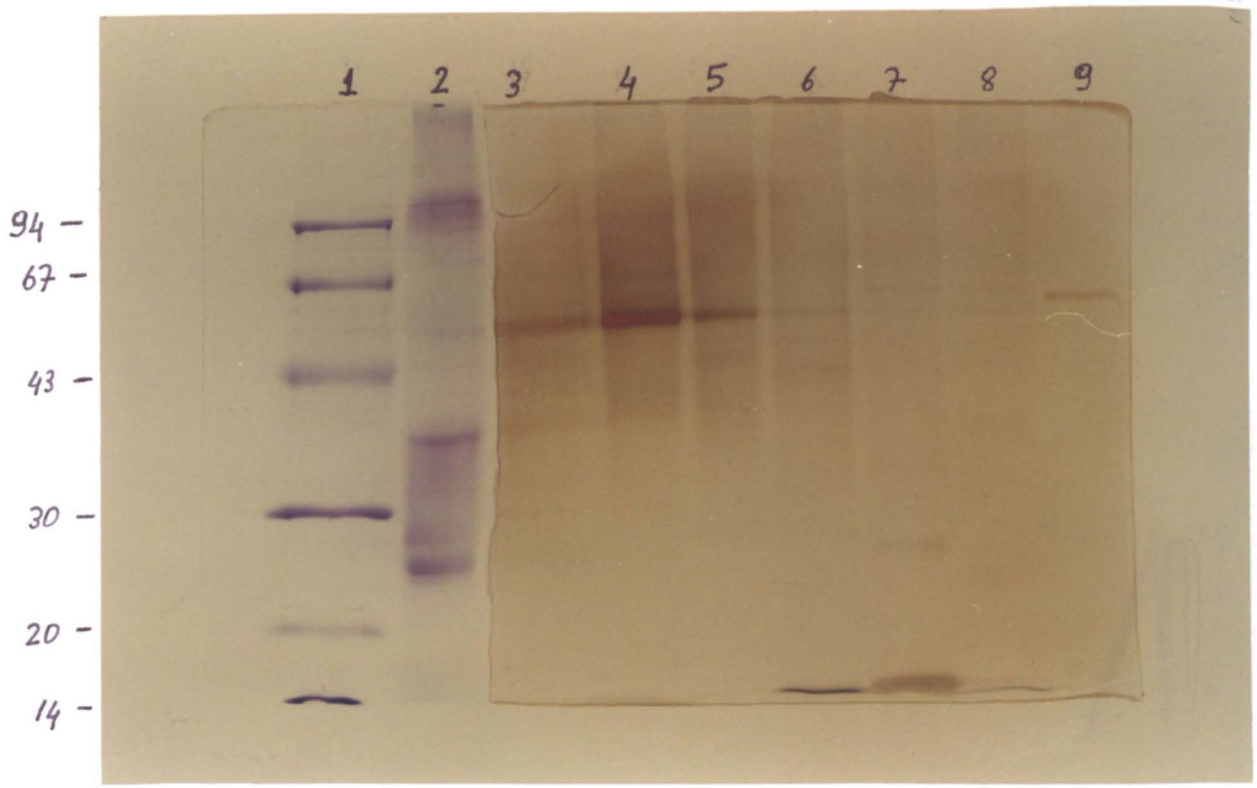
Gel-1 Somatic antigen

Lane-1. Molecular marker (stained with Coomassie blue)
Lane-2. Whole somatic antigen (stained with Coomassie blue)
Lane-3. Fraction-1 (stained with silver stain)
Lane-4. Fraction-2 (stained with silver stain)
Lane-5. Fraction-3 (stained with silver stain)
Lane-6. Fraction-4 (stained with silver stain)
Lane-7. Fraction-5 (stained with silver stain)
Lane-8. Fraction-6 (stained with silver stain)
Lane-9. E/S antigen Fraction-9 (stained with silver stain)

Gel-2 E/S antigen (stained with silver stain)

Lane-1. Molecular marker
Lane-2. Whole E/S antigen
Lane-3. Fraction-1
Lane-4. Fraction-2
Lane-5. Fraction-3
Lane-6. Fraction-4
Lane-7. Fraction-5
Lane-8. Fraction-6
Lane-9. Fraction-7
Lane-10. Fraction-8

Gel-1



Gel-2