Bovine Immune Responses to *Fasciola hepatica* During the Early Stages of Infection

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A thesis presented for the degree of Doctor of Philosophy
The University of Edinburgh
2000

Declaration

I declare that the work presented in this thesis is based entirely upon my own research.

Deborah Hoyle

For my parents

ACKNOWLEDGEMENTS

A special thankyou to my supervisor, David Taylor, for his encouragement and for the memorable occasion(s) when he donned a white coat to assist in the lab. I am very grateful to Derek Simmons for technical assistance on busy sample processing days and for his friendship. Thanks also to Karen Fernando, for her friendship and instruction in various laboratory techniques and to John Campbell, for teaching me the basics of tissue culture. I am particularly grateful to Paul Wright and Harry Urquhart, for all their help over the past three years. I very much appreciate the time spent by Nick Ambrose and Darren Shaw in reading my thesis and for their helpful comments regarding the text. I would like to thank all my colleagues and friends within the Faculty, who are not mentioned individually, for their help and support over the past few years.

I am particularly grateful to John Dalton, for inviting me to work for a short period with his research group in Dublin and for his kindness during my stay. I am also grateful to the late Bob Collins, for his time and patience during my week at Compton and to Atila Akca and Diana Williams, for their help in producing one of the protein preparations at Liverpool.

Finally, a heartfelt thankyou to Markus, for all his (almost endless) patience, encouragement and support.

This research was funded by The Wellcome Trust.

ABSTRACT

Fasciola hepatica (liver fluke), is a trematode parasite, particularly of ruminants, that is found throughout the world. Sheep show no resistance to the parasite, cattle may develop partial resistance and rats show complete resistance, which is immune-mediated, to challenge infections. In rats, resistance occurs early during infection, at the level of the gut and/or peritoneum. Little is known regarding the natural immune responses in cattle that occur during this period.

This study was designed to investigate the effect that pre-exposure of cattle to 1-2 and 5-6 day old flukes had upon subsequent challenge infections, when compared to naïve and chronically infected animals. Infection parameters, together with peripheral and local cellular and antibody responses to various *F. hepatica* protein preparations were investigated.

A primary exposure to 5-6 day old flukes, terminated by triclabendazole treatment, was found to reduce the degree of liver damage and eosinophilia experienced after a challenge infection. Lower levels of the enzymes gamma-glutamyl transferase and glutamate dehydrogenase were detected in sera, compared to those in naïve animals ($P < 0.05$). Eosinophilia was also reduced ($P < 0.01$), as was egg output in the faeces during the early patent period ($P < 0.05$). Calves that were pre-exposed to 1-2 day old flukes showed no significant differences in the levels of these parameters, when compared to their naïve counterparts.

Sera taken prior to the secondary challenge from pre-exposed animals and those receiving an unterminated primary infection recognised a variety of proteins in Western blots of whole adult fluke somatic antigen (WFA) and excretory-secretory (ES) preparations. The IgG1 antibody response to protein bands of 96-82, 76-68 and 60-52 kDa predominated. After secondary challenge the response of the chronically infected animals to these protein species was reduced and extremely strong recognition of bands in the region 30-28 kDa was observed. Pre-exposed animals maintained the response to the higher weight bands, showing a similar, but initially stronger recognition pattern, to that of naïve challenged animals. The lower weight protein bands were not detected in these groups until much later.

The IgG1/IgG2 isotype antibody response to purified cathepsin and haem-containing high molecular weight fractions were also examined by ELISA. A

monophasic, IgG1 response was seen to the cathepsin fraction, which occurred late during the infection process and was not seen prior to secondary challenge in any of the four groups. A mixed IgG1/IgG2 antibody response to the haem fraction was seen within 14 days of primary infection. After secondary challenge, this response was boosted in the pre-exposed, but not the chronically infected, animals. No significant difference was noted in antibody titre to either protein fraction between pre-exposed or naïve animals.

Peripheral blood mononuclear cells responded to stimulation with WFA and ES preparations, giving a strong proliferative response as early as 7 days post-infection. This suggests the presence of common antigens between adult and very early fluke stages. Proliferation was decreased after secondary challenge, particularly in the chronically infected group.

In order to examine the local immune response in the 5-6 day pre-exposed group, mesenteric and hepatic lymph nodes were removed 10 days after secondary challenge. Substantial proliferation to WFA was seen with hepatic, but not mesenteric derived lymph node cells, suggesting that the gut response to this antigen preparation was not important. Pre-exposed animals showed a lower mean level of IFN-γ and IL2 cytokines in supernatant fluid. Both groups produced similar levels of B cell stimulating factor. IgG antibody present in hepatic lymph node cell culture supernatant fluid from pre-exposed animals recognised proteins on WFA and ES Western blots of sizes 190-120, 96-82, 72-68 and 60-57.5 kDa. No such response was seen with supernatant fluid from naïve challenged animals.

These results suggest that pre-exposure to flukes of up to 5-6 days old provides a degree of protection against secondary challenge infections in cattle. Peripheral and local antibody is mainly directed against a series of proteins ranging from 96-57.5 kDa in size and to particular bands in metacercarial preparations. Although the main antibody response was IgG1 dominated, the cellular response showed a mixed cytokine pattern, suggesting an unrestricted T helper cell response. Further investigation into the nature of immunoreactive bands identified, together with examination of IgE antibody responses, will aid understanding of the naturally occurring immune response during early infection.

ABBREVIATIONS

$\alpha\beta^+$ T cell	alpha beta T cell
ACD	acid citrate dextrose
kBq	kilobequerel
cDNA	complementary deoxyribonucleic acid
μCi	microcurie
CL1/2	cathepsin-L1/2
Con A	concanavalin A
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
epg	eggs per gram
ES	excretory-secretory products
FACS	fluorescence activated cell sorter
FCS	foetal calf sera
FEC	faecal egg count
FITC	fluorescein isothiocyanate
g	relative centrifugal force
g, mg, μg, ng	gram, milligram, microgram, nanogram
$\gamma\delta^+$ T cell	gamma delta T cell
γ-GT	gamma glutamyl transferase
GLDH	glutamate dehydrogenase
GST	glutathione S-transferase
HMW	high molecular weight fraction
HRPO	horseradish peroxidase
IFN-γ	gamma interferon
Ig	immunoglobulin
IL	interleukin
IU/l	International units per litre
kDa	kilodalton
l, ml, μl	litre, millilitre, microlitre
M, mM	molar, millimolar
mRNA	messenger ribonucleic acid
NEJ	newly excysted juvenile
NK	natural killer cell
NKT	T cell bearing NK cell markers
nm	nanometre
NSS	normal sheep sera
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline/Tween 20
RNA	ribonucleic acid
ROS	reactive oxygen species

RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SDS PAGE	sodium dodecyl polyacrylamide gel electrophoresis
SEM	standard error of the mean
SAC	Scottish Agricultural College
SDS	lauryl sulphate
SI	stimulation index
TBS	tris-buffered saline
TBS-T	tris-buffered saline/Tween 20
TCR	T cell receptor
TEMED	N,N,N',N'-tetramethylethylenediamine
Th0	T helper 0 (unrestricted)
Th1	T helper 1
Th2	T helper 2
v/v	volume per volume
WFA	whole fluke antigen
WHO	World Health Organisation
w/v	weight per volume

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

GENERAL INTRODUCTION & LITERATURE REVIEW

1.1 General introduction

Fasciola hepatica, the common liver fluke, is an important trematode parasite of mammals found in temperate and sub-tropical regions of the world. Distribution of the parasite depends upon the presence or absence of the obligatory intermediate host, snails of the *Lymnaesidae* family. The parasite is seen commonly in Western Europe, particularly in areas with mild, damp climates favoured by the snail host. *F. hepatica* is also widespread across much of Eastern Europe, Russia and associated states, Asia, North and South America and Australasia (Torgerson and Claxton, 1998). Economically, the most important mammalian hosts are domestic ruminants, mainly cattle and sheep. Prevalence rates in cattle in Britain have been estimated at 10% and in Ireland at up to 45% (Torgerson and Claxton, 1998). Fascioliasis outbreaks have increased during the last few years in Scotland, and were particularly bad during 1999 and in the first part of the year 2000 (SAC Veterinary Science Division, 2000).

Infection can result in substantial animal morbidity and mortality, posing a serious financial problem for both subsistence and commercial farmers alike. Combined estimates of the worldwide cost of ruminant infections with *F. hepatica*, *F. gigantica* (tropical liver fluke) and stomach flukes is approximately $3 billion per year (Food and Agricultural Organisation of the United Nations, 1994). Economic losses fall into three main categories: acute loss due to animal mortality; loss at the abattoir, because of liver condemnation and finally, the less visible loss of poor production. Chronic fascioliasis can reduce growth rates, feed conversion ratios (Oakley *et al.*, 1979, Hope Cawdery *et al.*, 1977) and wool production (Hawkins and Morris, 1978).

In developing countries, poor animal productivity has a significant impact on the income and subsequent well being of the farming population. In addition, more direct effects on human health are seen in human cases of fascioliasis. Infection in humans has been seen traditionally as a sporadic and atypical occurrence, however,

the zoonotic potential of *F. hepatica* has recently become more evident (Chen and Mott, 1990; Mas-Coma *et al.*, 1999). Surveys of village populations in the mountainous regions of Bolivia have demonstrated the magnitude of the problem, recording infection prevalences of 23% (Bjorland *et al.*, 1995), 27% (Esteban *et al.*, 1997) and 42% (Hillyer *et al.*, 1992). Many of the infected individuals in these surveys showed signs of acute liver disease. The World Health Organisation has now recognised that *F. hepatica* is a serious zoonosis in certain regions of the world, particularly in South America (WHO, 1995) and a new classification for the epidemiology of fascioliasis in humans has recently been proposed (Mas-Coma *et al.*, 1999). The control of fascioliasis in domestic animals is therefore extremely important from both a public health, as well as an economic, point of view.

Currently, the main method for controlling fascioliasis is by chemotherapy, which eliminates the parasite from the ruminant host. Land management practices, such as restricting grazing to snail free areas, are also employed where adequate resources are available. However, both these methods are expensive and may require repeated attention for effective control to be maintained throughout the year. Consequently, interest in the development of a vaccine for fascioliasis has grown in recent years. Several candidate fluke proteins have been isolated and used in vaccine trials, with variable success (reviewed by Spithill and Dalton, 1998).

The development of effective vaccines against helminth parasites has been an aim of researchers for many years. The discovery of efficacious vaccines protecting against the cystic stages of the tapeworms *Taenia ovis* (Johnson *et al.*, 1989) and *Echinococcus granulosus* (Lightowlers *et al.*, 1996a; 1999) in sheep has at last proven that the design of recombinant helminth vaccines is indeed possible. Much of the research into helminth vaccines has been directed by the need to discover protective vaccines against a variety of parasite diseases of humans, such as schistosomiasis and onchocerciasis. A *Fasciola* vaccine would be of interest not only because the parasite is of great economic importance to the farming community across the world, but also due to its close relationship with the trematode parasite, *Schistosoma sp.*. Antigens that may induce a protective response against *Fasciola*, could have similar effects against *Schistosoma*. Studies with laboratory models have demonstrated that a degree of cross-protection does occur between *F. hepatica* and *Schistosoma mansoni*. Mice infected with one parasite show resistance to challenge

with the other, and vice versa (reviewed by Hillyer, 1984). Vaccination of mice with a purified *F. hepatica* protein, now known as a fatty acid binding protein, induces a degree of resistance to *S. mansoni*, *S. bovis* and *F. hepatica* infection (Hillyer, 1985; Hillyer *et al.*, 1988a; 1988b; López Abán *et al.*, 1999).

Ideally, vaccine design should be based upon a good understanding of the natural immune processes occurring during the various phases of infection. Unfortunately, to date, comparatively few studies have investigated immune responses during infection with *F. hepatica* in the main target hosts, sheep and cattle.

The work described in this thesis investigates immune responses occurring during *F.hepatica* infection in the natural bovine host.

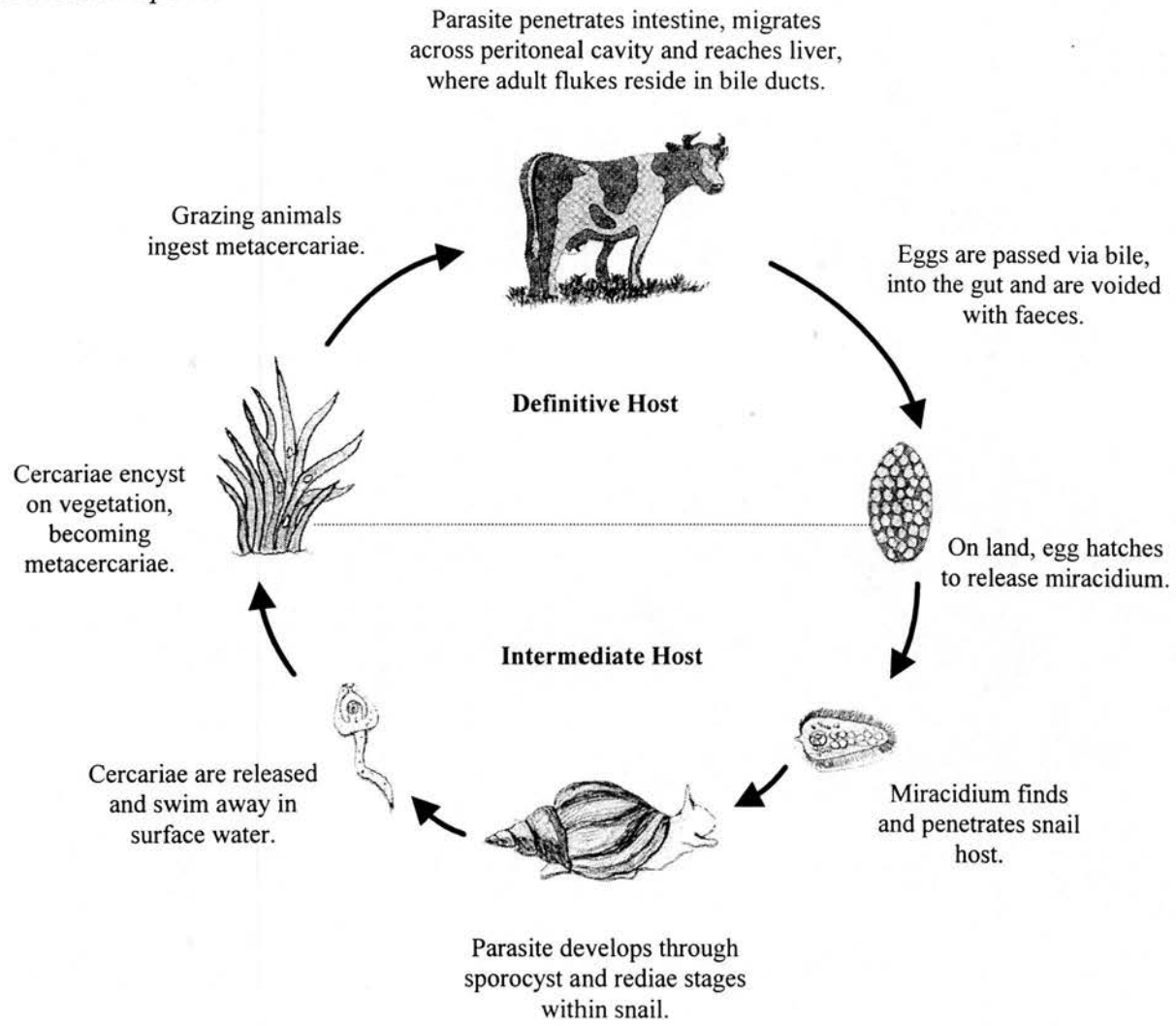
1.2 Literature Review

1.2.1 The lifecycle of *F. hepatica*

The discovery of the lifecycle of *F. hepatica* has formally been attributed to two scientists working towards the end of the nineteenth century, Thomas and Leuckart (reviewed by Andrews, 1988). The parasite has an indirect lifecycle involving a snail intermediate host, within which asexual reproduction occurs and a mammalian definitive host, the site of sexual reproduction (Figure 1.1). Metacercarial cysts released by infected snails are ingested by mammals via contaminated vegetation. Within the mammalian intestine they excyst and as newly excysted juveniles penetrate the intestinal wall and migrate across the peritoneal cavity to the liver, a process that can take as little as 48 hours to complete (Hayes, 1978). The juvenile flukes then migrate through the parenchyma of the liver over a period of weeks, causing gross destruction of parenchymal cells and blood vessels, until they eventually enter the bile ducts, where they reside as adults. The time from ingestion to patency varies between different host species, but on average ranges from eight to ten weeks in sheep and cattle (Boray, 1969). Fluke eggs produced by the adults in the bile ducts are expelled into the gut with bile. They are then voided with the faeces onto pasture-land.

Ambient temperature, moisture and oxygen tension levels all effect the embryonation process within the fluke egg, (Andrews, 1988). When climatic conditions are optimal, i.e. moist and warm (a minimum temperature of 10°C is required for egg development), and in response to appropriate stimuli, particularly light, the eggs hatch to release miracidia. The motile miracidia swim off in surface films of water to find and penetrate the tissues of the snail host. Within the snail the miracidia develop into sporocysts. Asexual multiplication occurs within the sporocyst, with germinal cells forming many rediae. When these are mature the sporocyst bursts, releasing the rediae which become cercariae. The cercariae are subsequently shed from the snail into water and after finding a suitable site on vegetation, they encyst, lose their tails and become metacercariae.

Figure 1.1: Lifecycle of *Fasciola hepatica*



1.2.2 Clinical disease in the ruminant host

The form of disease seen in an animal varies according to several factors, including the host species, ingested metacercarial dose and current health status of the animal (Boray, 1969). A spectrum of disease is seen ranging from a peracute syndrome, with sudden death, to acute or chronic disease (Boray, 1969; Behm and Sangster, 1998). Acute disease is seen more commonly in sheep and young calves, which appear to be more susceptible to the effects of infection, perhaps due to their small size. In the acute form, gross haemorrhage and acute liver failure are seen, caused by the massive structural damage inflicted on blood vessels and the liver parenchyma by migrating juveniles. Unless treated, the effects of circulatory collapse and acute haemorrhagic anaemia often result in death. In comparison, chronic fascioliasis is typified by lethargy, ascites and subcutaneous oedema and a low liveweight gain. The symptoms of chronic disease are a result of impaired liver function, hypoalbuminaemia and a low grade anaemia. In the subclinical form of the disease, whilst no overt clinical signs are evident, reduced production may be noted (section 1.1).

1.2.3 The control of fascioliasis

The efficient control of fascioliasis requires a multifactorial approach, aimed at terminating infections in the ruminant host, reducing snail populations on pasture-land and restricting grazing access of animals to contaminated areas and are discussed below.

1.2.3.1 Elimination from the mammalian host

There are several drugs available which are effective at terminating fluke infections in cattle and sheep. Some, such as nitroxynil and closantel, are only effective against late juvenile and mature fluke stages, whilst others such as albendazole, only kill the adult fluke (McKellar and Kinabo, 1991). However, one drug is effective against very early juvenile and adult stages of the parasite, triclabendazole (Boray *et al.*, 1983; Richards *et al.*, 1990). Triclabendazole is preferred where there is significant risk of acute disease, because it can kill the early migrating juvenile flukes, preventing liver damage. It is also useful in the treatment of herds and flocks where individual animals may harbour differing ages of the

parasite. Drug treatment only removes the current infection and except by possibly reducing transmission levels, does not prevent re-infection. Where infective metacercariae are present on pasture for several months, frequent dosing throughout the grazing season may be required. Chemotherapy is therefore not the ideal means of control, being expensive, manpower intensive and requiring constant access to supplies of the drug. Additionally, the development of *F. hepatica* resistance to triclabendazole has recently been reported in both Australia (Overend and Bowen, 1995) and the United Kingdom (Mitchell *et al.*, 1998; Thomas *et al.*, 2000). Although triclabendazole resistance is not yet a widespread phenomenon, it is a cause for concern and is further evidence for the need to develop alternative methods of fascioliasis control.

1.2.3.2 Snail control

One of the most obvious targets for control is elimination of the snail intermediate host. Chemical treatment of land with molluscides has been tried over the years, but there are many problems associated with this approach (Wilson *et al.*, 1982). Molluscides must be applied on a regular basis for any consistent benefit to be obtained, at least once annually, as snails can repopulate the cleared area rapidly. The chemicals used are toxic and so concern over pollution and hazards to wildlife and humans means this method is not desirable. Another method of control is drainage (Roberts and Suhardono, 1996). The snail species involved in transmission live in wet, mild habitats and are not found in well drained, dry areas. However, drainage may not be beneficial for the local environment, and often acts in opposition to farm irrigation schemes. On a smaller scale, antagonistic trematode schemes have been tried. The snail can play host not only to *F. hepatica*, but also to other trematodes and when infected with one species, it cannot host another (Lie *et al.*, 1966). However, such biological methods of control have not been successful when attempted on a larger scale (Boray, 1969).

1.2.3.3 Grazing management

If the snail population cannot be removed, reducing exposure of stock to snails should be attempted by restricting grazing to safe "snail free" areas. Fencing off wet areas and ditches is simple and cost-effective, but if grazing land is scarce

and much of it is suitable habitat for the snail, then this is not possible.

1.2.3.4 The use of predictive models in control

Various predictive models have been developed to aid in the control of fascioliasis. These are based on climatic conditions, specifically rainfall, but sometimes temperature and provide a measure of the risk of fascioliasis for the following year (Ollerenshaw and Rowlands, 1959; reviewed by Torgerson and Claxton, 1998). More advanced models include data on liver condemnation rates from abattoirs, as well as climatic conditions and are more accurate (Goodall *et al.*, 1993). Sophisticated models, using geographical information systems (GIS), can include data on soil type, hydrology, vegetation and climate (Malone *et al.*, 1998). The forecasting of the risk of fascioliasis, aids farmers in their decisions regarding dosing and grazing strategies. Previously, these models were really only of value to farmers in Western countries, where centralised veterinary support and finances allowed their implementation. However, GIS models have been developed to predict the risk of fascioliasis and in the design of chemotherapy strategies in Ethiopia (Malone *et al.*, 1998). With international support, there is potential for the use of such models in the control of fascioliasis in developing countries.

In summary, the current methods of fascioliasis control are expensive, time consuming and require considerable management. Immunological control in the form of a vaccine on the other hand, could be a more efficient, simple and cost-effective means of reducing disease.

1.2.4 Vaccine development strategies

In order to design an effective vaccine, the type and nature of the immune response in the host to infection must be investigated. Several questions need to be addressed before any significant progress can be made.

- Is there any evidence for the development of immunity during natural infections in the field? Are infected animals able to rid themselves of infection and if so, remain resistant to re-infection? If animals are not able to effectively eliminate parasites and are not resistant to re-infection, how does the parasite evade the hosts immune effector mechanisms? Knowledge of any such evasion tactics may

allow the development of agents which can artificially inhibit or manipulate evasion mechanisms to allow a suitable host-protective response to occur.

- Have cellular and humoral immune responses been investigated longitudinally during the course of primary and secondary infections? This would give a picture of the changing nature of any immune response during the different lifecycle stages of the parasite within the host. The type of immune response occurring is also important and so the contribution of humoral, cellular and accessory cell components needs to be investigated.

- What stage of the parasite is potentially able to stimulate the most protective response and where could this act? From the point of view of the host, killing a small invading fluke is easier than killing a much larger adult. Removing the flukes before they have caused significant structural damage to the liver would also seem most logical. A vaccine that worked by stimulating responses against the adult fluke stages only, would still result in the host being subjected to considerable pathology. Whereas, if juveniles migrating through the gut and peritoneum were targeted, elimination without any damage to the host could be effected.

- The fluke parasite is composed of many thousands of proteins, some involved with metabolism, others having structural roles. Have any of these antigens been identified and how does the host respond to these antigens? Targeting an immune response against proteins that are critical to the survival of the fluke, either for general metabolic processes or for maintaining structural integrity, could result in damage or death to the fluke.

- If any putatively protective responses are identified, how best could these be harnessed to produce a suitable vaccine?

1.2.5 The course of primary and secondary infections in different mammalian hosts

Mammalian species exhibit varying degrees of susceptibility to disease and resistance to re-infection. The majority of current knowledge concerning the nature of fluke infection in the mammal has been derived from examination of the clinical and pathological effects of infection in the two main economically important hosts, cattle and sheep. This has been furthered by the use of experimental systems with laboratory animals, particularly the rat.

1.2.5.1 Infections in sheep and cattle

Sheep and cattle show differing responses to infection with *F. hepatica*. Sheep appear to be particularly susceptible to acute and chronic disease, especially after heavy dose infections (Boray, 1969; Behm and Sangster, 1998). In comparison, sheep that do not succumb to acute disease can remain chronically infected with a primary infection for years (H. Urquhart, personal communication). Sheep show no resistance, in terms of fluke burden, to secondary challenge infections, regardless of dose or the interval between the primary and challenge infections (Sinclair, 1970; 1971; Haroun and Hillyer, 1986). However, flukes recovered from secondary infections in sheep do show evidence of a temporary retardation in development.

Cattle are more resistant to fascioliasis than sheep. Experimentally, adult flukes are lost from the bile ducts of cattle from 20-30 weeks after infection and subsequently cattle show a degree of resistance to re-infection (Boray 1969; Doyle, 1971, Doyle, 1972). The timing of challenge appears critical: challenging animals within 7 weeks of the primary infection does not result in resistance, whereas challenge after 12 weeks does (Doyle, 1973). This suggests that the development of resistance requires exposure of the host to the adult stage in the bile ducts. However, mature, chronically infected cattle have been found in the field situation, suggesting that during natural infections, cattle retain a degree of susceptibility to infection (Clery *et al.*, 1996). This could be due to the ingestion of smaller numbers of metacercariae over a prolonged period.

Sheep and cattle could therefore be considered as representing two distinct types or models of the disease, the susceptible and partially-resistant states, respectively. Identifying the differences in the immune response between these two

species and the reasons for such disparate reactions is important. If resistance in cattle is partially determined by acquired immunological factors, the differences between the two species may highlight the factors responsible for such resistance.

The gross pathology seen in chronically infected sheep and cattle also differs. Cirrhosis and fibrosis of the liver parenchyma and bile ducts occur in both species, but in cattle, unlike sheep, bile duct calcification is also seen (Dow *et al.*, 1967, Sinclair, 1971; Sinclair, 1973). It has been suggested that this calcification and other fibrotic changes of the bile ducts is the reason for the partial resistance seen in cattle, perhaps because it may physically impair fluke feeding (Boray, 1969). Goats, which show similar pathology to sheep, and cattle also respond differently to direct transplantation of adult flukes into the bile ducts (Hughes, 1987). In cattle, if the ducts have been calcified, infections do not "take", whereas where the ducts show less pathology, infection is always successful. However, it is unlikely that calcification or physical impairment are the only factors involved in the partial resistance seen in cattle.

The transfer of lymphoid cells between donor and naïve calves has been found to protect against challenge infection, provided the donor has been infected for a minimum of eight weeks (Corba *et al.*, 1971). Serum from infected cattle is also able to protect against challenge infection in the rat (Haroun *et al.*, 1981). These observations suggest immunological factors are involved in resistance.

1.2.5.2 Infections in the rat

The rat, which has been used widely as an experimental model for fascioliasis, shows a very different response to infection. Rats are susceptible to primary infection but fully resist secondary challenge, although the primary infection flukes remain unharmed (Hayes *et al.*, 1973; Hayes *et al.*, 1974a). Even minimal primary infections prevent super-infection or challenge. No liver calcification is seen and the contribution of physical components appears to be insignificant. Resistance can also be transferred from infected donor rats to those that are naïve, using either serum or lymphoid cell preparations (Hayes *et al.*, 1974b; Hayes *et al.*, 1974c; Corba *et al.*, 1971; Chapman and Mitchell, 1982a; Armour and Dargie, 1974; Rajasekariah and Howell, 1979). Rat strain, dosages and timing of transfers in relation to both donor and recipient primary and challenge infections are important in determining the

outcome of infection.

Resistance in the rat therefore appears to be due, at least in part, to an acquired immunological response. However, the possible incompatibility of the parasite with an unnatural host may also be an important factor. The fact that the rat is only a model must always be borne in mind when extrapolating results from the known situation in the rat to that in the ruminant host.

1.2.6 Evasion of the immune response

Juvenile flukes migrate through the body cavity and liver for several weeks before reaching the bile ducts. During this time they are in constant association with host cells and blood. How is it, therefore, that they survive intact, apparently unharmed by the immune defence mechanisms of the host? Several possible mechanisms by which they may avoid recognition and elimination by the immune system have been proposed.

1.2.6.1 Location of adult flukes

The bile ducts, traditionally considered as being relatively inaccessible to immune effector cells, may act as privileged sites. This would allow long term survival of adult flukes without any risk from host damage. In rats, primary infection flukes present in the bile ducts are not affected by host responses which prevent the establishment of a secondary challenge infection (Hayes *et al.*, 1973; Goose and MacGregor, 1973). However, both cattle and rats eventually lose flukes from the bile ducts (Hughes *et al.*, 1976; Doyle, 1971), although sheep and goats do not. Is this loss a natural physical process, perhaps nutritional, or are the flukes actively removed? No firm evidence indicates which is the case.

1.2.6.2 Juvenile surface coat

Migrating juvenile flukes possess an outer glycocalyx coating (Hanna, 1980a) which stimulates a strong antibody response (Hanna, 1980b; Duffus and Franks, 1981). *In vitro* studies have shown that immunoglobulin is able to adhere to the glycocalyx, but after a period of time both antibody and glycocalyx are sloughed off as a complex from the fluke surface (Hanna, 1980a; Duffus and Franks, 1981; Burden *et al.*, 1982). The sloughed glycocalyx is then replaced with one of similar

antigenicity. Sloughing of this glycocalyx occurs both *in vitro* and *in vivo* and does not require the presence of host antibody (Lammas and Duffus, 1983). Glycocalyx turnover in this manner appears to be a natural process in the juvenile fluke and may therefore act to prevent close association of host effector cells, such as eosinophils or neutrophils, with the fluke surface, by removing opsonising antibody.

1.2.6.3 Cleavage of immunoglobulins

In vitro, flukes produce proteins termed excretory-secretory (ES) products. These contain proteases that are able to cleave the immunoglobulin heavy chain in the hinge region (Chapman and Mitchell, 1982b). Therefore, in addition to sloughing the glycocalyx, flukes can potentially prevent opsonisation by antibodies and the binding of host cells to their surface by cleaving immunoglobulin (Carmona *et al.*, 1993). However, immunoglobulin cleavage has not been demonstrated *in vivo* and whether such activity is a prime function of the proteases within the host is not known.

1.2.6.4 Can host effector cells damage flukes?

Eosinophilia has long been associated with helminth infections, although the role of eosinophils in host protection is not clear. *In vitro*, several studies have demonstrated the ability of eosinophils to damage juvenile and larval helminths in the presence of specific antibody, notably with *S. mansoni* (Veith and Butterworth 1983; Butterworth, 1984) and *Haemonchus contortus* (Rainbird *et al.*, 1998). Electron microscope studies have shown that *in vitro* and in the presence of sera from infected animals, eosinophils are able to bind to juvenile flukes and degranulate, resulting in vacuolation of the fluke tegument (Glauert *et al.*, 1985). After a period of time, these cells and any bound antibody are subsequently shed along with the secreted outer glycocalyx of the fluke, before the fluke can be destroyed by host cell products. Major basic protein, isolated from eosinophils, is able to cause direct damage to the tegument of juvenile flukes, upon co-culture (Duffus *et al.*, 1980).

Demonstrating that host cells can kill or damage flukes, *in vivo*, is not as simple and most evidence is circumstantial. Newly excysted juveniles, injected into the peritoneum of sensitised rats and then recovered after a period of time, were found to be coated in peritoneal cells, mainly eosinophils, but also macrophages and

neutrophils. The eosinophils appeared to adhere, degranulate and destroy the tegument of the fluke, resulting in fluke death. (Davies and Goose, 1981). Adult flukes implanted into the peritoneal cavity also experience similar damage (Bennett *et al.*, 1980). Further evidence for a protective role for eosinophils in *Fasciola* infections in the rat has been obtained using an *ex vivo* gut-loop model, in which protection against re-infection correlated positively with both local gut mucosal and peripheral eosinophil levels (van Milligen *et al.*, 1999).

Epidemiological studies investigating resistance to reinfection with schistosomiasis in humans after chemotherapy, have also demonstrated the importance of eosinophils during infection, with high peripheral eosinophil counts being significantly associated with resistance to reinfection with both *S. haematobium* (Hagan *et al.*, 1987) and *S. mansoni* (Sturrock *et al.*, 1983). However, a further study found no association between eosinophilia and resistance, and it was postulated that the functional activation state of the eosinophil may be more significant that actual peripheral levels (Butterworth *et al.*, 1985).

The importance of IL5-dependent eosinophil responses in protection against infection has also been demonstrated for certain parasites *in vivo* by the ablation of IL5 using anti-IL5 antibodies in mice, for example with *Onchocerca lienalis* (Folkard et al., 1996) and *Strongyloides ratti* (Ovington and Behm, 1997). However, the development of many other parasites, including *S. mansoni*, was unaffected by such treatments (reviewed by Behm and Ovington, 2000). The role of eosinophils in transgenic mice designed to overexpress IL5 has also been studied, again with variable outcome: eosinophilia had no effect on the development of some parasites, but with others burdens were decreased, whilst with *S. mansoni* burdens were increased. *F. hepatica* infections are not affected in mice genetically deficient for IL5 (Ovington and Behm, 1998). The significance of these results with regard to natural host-parasite systems is not clear: mice and humans react differently to infection, for example, with *S. mansoni* infection (Brunet *et al.*, 1998) and it may be that innate immune responses, stimulated by the non-specific inflammation induced by the parasite in the unnatural host, may alter the course and outcome of infection (Meeusen and Balic, 2000).

Host macrophages are also able to damage or kill helminths *in vitro*. Macrophage killing of schistosomula has been demonstrated in the presence of rat

immune sera containing IgE, but not other antibody isotypes (reviewed by Capron *et al.*, 1982). Rat peritoneal cells from *F. hepatica* infected rats kill newly excysted juvenile flukes *in vitro* when incubated with sera from infected animals (Spithill *et al.*, 1997). Killing efficacy correlated with the concentration of nitric oxide measured in the vicinity of the fluke. Macrophage killing of filarial worms by the production of nitric oxide has also been demonstrated with the filarial nematode parasites *Brugia malayi* and *Onchocerca lienalis* (Taylor *et al.*, 1996; Thomas *et al.*, 1997).

From these studies it appears that host effector cells are able to damage trematode parasites *in vitro*, including *F. hepatica*. If such cells are not removed from the fluke surface, for example by glycocalyx shedding, then the toxic products that they release, such as major basic protein and reactive oxygen species, may be involved in parasite killing.

1.2.6.5 Speed of migration

During secondary challenge infections in sheep it has been observed that fluke migration across the body cavity and liver to the bile ducts may be quicker than during the primary infection (Meeusen *et al.*, 1995). Although a good cellular response to the secondary challenge infection was observed in the parenchyma, the flukes left it behind. Empty fluke tracts were surrounded by inflammatory cells, whilst flukes remained relatively clear of host cells. It is possible, therefore, that the speed of migration may be too great for immune effector cells to efficiently bind the flukes. A similar situation has also been seen in mice (Lang, 1967), but other studies in sheep have been contradictory (Sinclair, 1970; 1971; 1973). This theory therefore remains unproven.

1.2.6.6 Modulation of host effector cell function

Fluke products may also be able to modulate the activity of host effector cells by reducing the activity of specifically-stimulated cells, such as lymphocytes, or of general inflammatory cells. *In vitro* studies have shown that *F. hepatica* ES products can modify host immune cell function. ES products can act as chemokines for normal sheep and human neutrophils (Jefferies *et al.*, 1996a) and are able to alter the neutrophil metabolic burst in response to mitogen stimulation (Jefferies *et al.*, 1997). Sheep peripheral blood mononuclear cell responses to mitogens are also affected,

being enhanced at low concentrations, but suppressed at higher concentrations upon co-culture with ES (Jefferies *et al.*, 1996b). ES products also suppress the rat spleen cell response to the mitogen Con A in a dose-dependent manner (Cervi and Masih, 1997), whilst free radical generation and phagocytosis by naïve bovine peripheral blood mononuclear cells have been inhibited by co-culture with ES (Baeza *et al.*, 1993).

1.2.7 Site of resistance to challenge

Where does resistance to secondary challenge infections occur? This is an essential question to address as the answer may indicate which fluke stage induces a protective response and the antigens and immune mechanisms involved. In rats, fluke burdens during early infection can be assessed by collecting flukes from livers and by peritoneal washout techniques. Doy *et al.* (1978), found that after oral challenge with *F. hepatica*, a significant reduction in the number of flukes recovered 48 hours later was seen in sensitised compared to control rats. Resistance occurring so early may therefore have been acting at the level of the gut or peritoneum. This idea was supported by the appearance of gut tissue: the lamina propria of challenged sensitised rats showed an increase in the number of eosinophils when compared to that of control rats. Other studies using similar techniques saw resistance to challenge as early as 24 hours post-challenge and concluded that immunity was effected either in the gut lumen, gut wall or peritoneal wall (Hayes *et al.*, 1977; Hayes, 1978). The small intestinal mucosa of these rats was inflamed and hyperaemic, suggesting that a strong reaction in this site of some sort had occurred. Furthermore, treatment with the anti-inflammatory and immunosuppressive agent dexamethasone, removed the protective effect. Dexamethasone treatment also results in higher fluke burdens in singly infected rats (Baeza and Poitou, 1994).

Intraperitoneal implantation of newly excysted juveniles allows the gut stage to be circumvented. In one such study, resistance was seen only in rats that received oral challenge infections (Rajasekariah *et al.*, 1977), although other evidence does suggest that implanted juveniles can be killed in the peritoneal cavity (Doy *et al.*, 1982). Resistance in rats can be seen at the gut or peritoneal levels. Is this a specific or non-specific effect? Athymic nude sensitised rats are equally resistant to oral challenge as their heterozygous counterparts, but resistance is not seen when they are

challenged intraperitoneally (Doy *et al.*, 1982). Thus in rats, two distinct mechanisms may be responsible for resistance to challenge infections, the first a gut level T cell independent process, possibly eosinophilic and the second, a T cell dependent process acting in the peritoneal cavity or at the liver capsule.

Rats demonstrate more complete resistance to challenge infections than cattle or sheep. Is there any evidence for a similar mechanism of resistance in ruminants? Only one study has been performed in cattle to investigate the site of resistance to re-infection, using a peritoneal wash-out technique (Doy *et al.*, 1984). No reduction in worm burden was found in sensitised calves at either 4 or 14 days after oral challenge when compared to naïve animals, suggesting gut immunity was not significant. Unfortunately, further exploration of this area has not been carried out.

1.2.8 Stage specific antigen expression

Surface expressed antigens alter in nature as the fluke migrates within the host and matures. Cells within the tegument produce vesicles containing proteins that are discharged onto the fluke surface to form the glycocalyx (Hanna, 1980b). The cell types present and the granules they produce change as the fluke matures, with T0 cells and vesicles present in the newly excysted juvenile, T1 during liver migration and T2 vesicles present, but not discharging onto the fluke surface until around the time of entry into the bile ducts (Bennett and Threadgold, 1975; Hanna, 1980b; Burden *et al.*, 1983). Changes in expression of other surface glycoproteins have been noted, particularly during the first 14 days of infection (Lammas and Duffus, 1985; Dalton and Joyce, 1987). Differential expression of somatic and excretory-secretory proteins has also been demonstrated in flukes of different ages (Dalton *et al.*, 1985; Tkalcevic *et al.*, 1996; Oblitas, 1997).

Stage specific expression of antigens does occur and appears to be most marked during the first few days of infection. The most obvious explanations for changing antigen expression include the need for adaptation of the different fluke stages to the different host environments they encounter as they migrate, together with the possibility that antigen patterns change to aid immune evasion.

1.2.9 Host immune responses to infection

1.2.9.1 T helper cells and cytokine production

Three populations of T lymphocyte have been defined in cattle, according to cellular phenotype. $CD4^+$ and $CD8^+$ T cells, which express the αβ T cell receptor, recognise antigen in conjunction with MHC class II or I, respectively (Howard and Morrison, 1994). $CD4^+$ cells generate "help" for the immune response, whilst $CD8^+$ cells have cytotoxic actions on host-infected or neoplastic cells. The third population have a T cell receptor composed of γδ subunits and are thus termed $\gamma\delta^+$ T cells.

$CD4^+$ T cells were originally classified in the murine system into T helper 1 (Th1) and T helper 2 (Th2) subsets by Mosmann *et al.* (1986). This distinction was made on a functional basis according to the nature of cytokine production seen after cell stimulation. The cytokines IFN-γ, IL2 and TNFβ were associated with the Th1 subset and IL4, IL5 and IL6 with the Th2 subset (Cherwinski *et al.*, 1987). Th1 cells can induce a delayed type hypersensitivity response (Cher and Mosmann, 1987) and are associated with cell mediated immunity, whilst Th2 cells provide help for antibody production by B cells. However, Th1 cells are also able to stimulate antibody production, mainly that of the IgG2a isotype (Coffman *et al.*, 1988, Stevens *et al.*, 1988). Both subsets also respond preferentially to different antigen presenting cells, with macrophages stimulating Th1 and B cells Th2 subsets (Gajewski *et al.*, 1991). Antibody production in response to Th2 help is mainly of the IgG1 and IgE isotypes, those typically seen in helminth infections.

Initially, T helper cells were viewed as belonging to one or other of these two exclusive states, which appeared to reciprocally regulate one another. Further study of Th subsets in the murine system and particularly in relation to *Leishmania* infection, reinforced the idea of the polarised response and linked this with infection outcome. With *L. major* infection in humans (Ajdary *et al.*, 2000) and mice (reviewed by Liew and O'Donnell, 1995), the Th1 response is beneficial, resulting in cure and resistance to challenge infections, whilst Th2 driven responses result in pathology and disease exacerbation. Another example of a similar classification was seen with murine *Schistosoma mansoni* infection. Studies suggested the presence of a deleterious Th2 response leading to pathology, whilst an irradiated cercariae vaccine

was found to induce a protective Th1 response (reviewed by Coulson, 1997). More recently, research has suggested that it is the balance of cytokines, rather than a defined Th1/Th2 class of response, that is important in maintaining host resistance to infection. Even in murine schistosomiasis, Th2 cytokines such as IL4 have some beneficial effects and are necessary for host survival (Brunet *et al.*, 1997; 1998).

In human schistosomiasis, field studies suggested that the opposite T helper cell bias to that seen in the mouse was associated with resistance to infection. IgE and IgG1 antibody responses were found to correlate with protection (Hagan *et al.*, 1991; Rihet *et al.*, 1991; Dunne *et al.*, 1992; Demeure *et al.*, 1993) and cellular cytokine production also supported the importance of an IL4/IL5 type response (Roberts *et al.*, 1993; Couissinier and Dessein, 1995). A study investigating resistance to *S. mansoni* in a Brazilian population has also identified the presence of a resistance gene for *S. mansoni* which maps to chromosome 5, in close association with the genes encoding the IL4 and IL5 cytokines (Marquet *et al.*, 1996). These differences between infection in the mouse model and the human highlight the necessity for caution when extrapolating results between species.

Bovine T helper lymphocytes do not appear to segregate strictly into the Th1/Th2 classification system. T cell clones obtained from cattle infected with the parasites *Babesia bovis*, *B. bigemina* or *F. hepatica* showed an unrestricted cytokine profile after stimulation with specific antigen or mitogen, in many cases co-expressing IL4 and IFN-γ (Brown *et al.*, 1993; Brown *et al.*, 1994a). Additionally IL10, which is produced by Th2 cells in the mouse model and acts to downregulate the Th1 response, is produced by bovine T cells expressing IFN-γ and IL4 and is able to regulate proliferation and IFN-γ production in all Th cell types (Brown *et al.*, 1994b; Chitko-McKown *et al.*, 1995). Thus a spectrum of cytokine production, from a Th0 (unrestricted) phenotype, through to a bias towards the Th1/Th2 extremes is seen in cattle. This is similar to the situation seen commonly in humans and with some murine cell clones (Kelso *et al.*, 1991; Kelso, 1995).

Attempts to 'pigeon-hole' immune responses in cattle, particularly those to complex pathogens such as helminth parasites, do not help our understanding of the host immune response to infection.

1.2.9.2 Bovine immunoglobulin isotypes

Genes encoding bovine IgG1, IgG2 (of which two allotypes, A1 and A2 exist), IgG3 (originally termed IgG2b), IgA, IgM and IgE have been cloned (Knight and Becker, 1987; Knight *et al.*, 1988). B cell proliferation and immunoglobulin production is stimulated by IL2 (Collins and Oldham, 1995). IL4 also stimulates B cell activity and preferentially stimulates the production of IgG1 and IgE isotypes, whilst the classical Th1 cytokine, IFN-γ, regulates the production of IgG2 (Estes *et al.*, 1994; 1995; Estes, 1996).

1.2.9.3 Immune responses to **F. hepatica** *in the rat*

Although several fluke antigens have now been isolated and cloned very little is known about the immune response that occurs in the ruminant host during infection and whether it is beneficial, limiting damage, or tolerant, possibly exacerbating infection. More is known regarding immune responses occurring in the rat model, where animals exposed to a primary infection are able to completely eliminate further challenges.

Keegan *et al.* (1992), found that during a primary infection, IgG antibodies to whole fluke antigen appear early, within 7 days of infection and peaked within 3 weeks. No significant correlation was noted between antibody titre and the number of flukes recovered at post-mortem during the primary infection, suggesting that the measured antibody response was non-protective. Rats were resistant to secondary challenge, but whether this was related to the action of antibody was not clear, although the antibody response to challenge was rapid, peaking within 5 days. Antibody was directed against juvenile tegumental and gut cell antigens and adult gut, reproductive and excretory cell antigens. A circulating eosinophilia was seen throughout infection and large numbers of neutrophils, macrophages, lymphocytes and eosinophils were noted in liver sections surrounding flukes and fluke tracts.

Another study confirmed the early appearance of specific antibody to ES proteins (Poitou *et al.*, 1992). IgG1, IgG2a, IgM and IgE antibody isotypes were all detected, but showed differing time-course response profiles throughout the infection period (Poitou *et al.*, 1993). IgM antibody was found throughout infection, not just during the first few days, possibly suggesting the continual sequential release of new antigens throughout fluke development. A total of eight protein fractions ranging in

size from 115 to 22.5 kDa were recognised by antibody (Poitou *et al.*, 1992). Peripheral eosinophil and neutrophil numbers increased during infection and in the spleen the proportion of B cells increased, whilst that of $CD4^+$ and $CD8^+$ T cells decreased. Spleen cell proliferation was seen in response to both mitogen and antigen stimulation, but thymic and peripheral blood mononuclear cells were unresponsive to fluke antigen (Poitou *et al.*, 1993).

Are the events noted in the periphery reflective of the responses occurring at the actual sites of infection? Monoclonal antibodies derived from antibody producing cells of hepatic, splenic and mesenteric lymph nodes of infected rats have been used to examine the local immune response to fluke infection (Meeusen and Brandon, 1994). In chronic primary infections, only hepatic lymph nodes produce parasite specific antibodies, whereas during secondary challenge mesenteric lymph nodes produce antibodies specific to newly excysted juvenile proteins. By comparing responses after oral and intraperitoneal challenge, protection against secondary challenge was found to occur at both the gut and peritoneal levels. Circulating antibody profiles did not always reflect the tissue specific responses seen, suggesting that these may not be a reliable indicator of the immune responses actually occurring at the site of infection. Results from a separate study, using the same monoclonal antibodies to examine stage specific antigen expression and immunity, supported the idea that immature flukes stimulate a more effective protective immune response than adult flukes (Tkalcevic *et al.*, 1996).

Recently, closer examination of the gut response to secondary challenge has shown that resistance to re-infection is significantly correlated with tissue levels of IgE, IgG1 and the presence of eosinophils (van Milligen *et al.*, 1998a). An *ex vivo* gut loop model allowed the gut response to be examined during penetration (van Milligen *et al.*, 1998b). Juvenile flukes that penetrated the gut wall in sensitised rats were covered in IgG1 and IgG2a antibodies and eosinophils. Furthermore, serum titres of IgG1 antibody directed against newly excysted juvenile proteins were highly correlated with protection (van Milligen *et al.*, 1999). Thus, the same sort of response that is seen during a primary infection, i.e. an IgG1, IgE, eosinophilic reaction, which is not able to prevent primary invasion, is also noted during secondary challenge, where it appears to be protective.

1.2.9.4 Immune responses to **F. hepatica** *in cattle and sheep*

In both cattle and sheep, circulating antibodies to ES or somatic fluke preparations are detected within 2 weeks of infection (Santiago and Hillyer, 1988; Chauvin *et al.*, 1995; Clery *et al.*, 1996). IgG1 appears to be the dominant IgG antibody isotype in experimentally and naturally infected cattle (Clery *et al.*, 1996; Bossaert *et al.*, 2000). Immune responses to the tegumental proteins present in the T1 and T2 vesicles have been examined in some detail in cattle. Serum IgG responses to T1 and fluke gut antigens peak at 4-6 weeks post-infection, whilst that to T2 antigens does not appear until week 6 (Hughes *et al.*, 1981). IgG and IgA antibody to T1 and gut antigens, but not to T2 antigens have also been detected in bile. Due to the lack of an effective reagent for detecting bovine IgE, changes in parasite specific levels of this antibody isotype have not been investigated.

Western blot analysis has been used to identify which proteins present in crude fluke preparations are recognised by immune sera. Santiago and Hillyer (1988), found that sheep and cattle recognised different somatic proteins. Cattle sera detected a set of proteins of approximately 56, 64 and 69 kDa that were not present in sheep. Both species recognised bands of 30-38 kDa, to which antibody appeared slightly later than to the higher weight fractions recognised by cattle. Another study found that the appearance of sheep antibody to certain ES proteins was time-dependent, with that to bands of 69 and 51 kDa appearing within 3 weeks, whilst lower weight bands of 20-30 kDa were not detected for a further two weeks (Chauvin *et al.*, 1995). Secondary challenge enhanced the response to the lower weight region only. A similar change in the response pattern to different sized proteins has also been seen in cattle (Itagaki *et al.*, 1995).

The contribution of cell mediated immunity throughout infection is not easy to assess. Peripheral blood lymphocyte responses to specific antigens or mitogens can be investigated by lymphoproliferation and cytokine production assays. However, it is important to realise that circulating lymphocyte reactions are not necessarily reflective of those occurring locally. In cattle, specific lymphocyte proliferation in response to whole fluke antigen is seen within 2-3 weeks of infection, but this effect is transient and by 5-8 weeks cells become unresponsive (Oldham, 1985; Oldham and Williams, 1985; McCole *et al.*, 1999a). T cell sub-set depletion has shown that both $CD4^+$ and $CD8^+$ T cells are responsible for the proliferation seen and $\gamma\delta^+$ T cells

do not appear to be involved (McCole *et al.*, 1999b). Sheep peripheral blood mononuclear cells also show a positive, but irregular proliferative response to fluke proteins (Chauvin *et al.*, 1995)

Cells extracted from the lymph nodes of cattle harbouring chronic primary infections of *Fasciola* have been cloned (Brown *et al.*, 1994a). These cloned cells showed a spectrum of cytokine patterns in response to stimulation with adult fluke extracts and mitogens. The majority of cells expressed IL2, IL4 and IFN-γ cytokines and were classified as Th0 (unrestricted); some produced IL4 only and were designated Th2 types. None were found to conform to the accepted murine Th1 pattern of IL2 and IFN-γ production, with no IL4. It was suggested that this pattern of cytokine production by the cells from infected cattle could indicate that a non-protective Th2 like response dominated during chronic infection. However, as these results were based on clones taken from just two animals at only one time-point during infection, they should be interpreted with caution. IFN-γ production has been noted after stimulation of peripheral blood mononuclear cells from infected cattle with fluke proteins (Clery and Mulcahy, 1998).

Histologically, macrophages, eosinophils, neutrophils and lymphocytes are found in hepatic tissue during primary infections in sheep (Chauvin and Boulard, 1996). During secondary infection, the cellular reaction within the parenchyma changes: the ratio of $CD4^+$ to $CD8^+$ cells increases and eosinophils are more numerous than neutrophils, which predominate during primary infection (Meeusen *et al.*, 1995). B cell infiltrates were also greater in secondary infections.

In contrast to the situation in the rat, where the protective immune response appears to be directed at gut penetrating fluke stages and is manifest by high levels of IgG1, IgE and eosinophilia, immune responses in ruminants are very poorly characterised. Cattle and sheep respond to infection by the early production of antibody and peripheral lymphocytes are reactive to fluke proteins during the first few weeks of infection, mainly throughout the migratory process. However, no association has been noted between antibody levels and fluke burden and there is no clear understanding of which responses may be beneficial to the host, which are merely markers of infection and which may be detrimental, helping to prolong infection.

1.2.9.5 Genetic influence on susceptibility to infection

Studies with *F. gigantica*, the tropical liver fluke, have found that sheep breed affects the outcome of infection. Indonesian Thin Tail sheep are comparatively resistant to infection in comparison to western breeds of sheep (Roberts *et al.*, 1997a) and this is thought to be due to the presence of a major gene which exhibits incomplete dominance (Roberts *et al.*, 1997b). Further studies have shown that whilst both the resistant and susceptible breeds show similar levels of IgG1 and IgE antibody to fluke proteins, levels of IgG2 are significantly lower in the resistant breed (Hansen *et al.*, 1999). It has been suggested that IgG2 acts as a blocking antibody, preventing IgG1 or IgE from promoting a protective response. Thus, an IgG1, IgE, eosinophilic response may be able to induce protection in *Fasciola* infection. Unfortunately, no difference in susceptibility to *F. hepatica* infection was noted between Indonesian Thin Tail sheep and other breeds (Roberts *et al.*, 1997a). As mentioned previously, IgG1 appears to be the dominant antibody present in *F. hepatica* chronically infected cattle. The implications of the discovery of resistance in Indonesian Thin Tail sheep to *F. gigantica* for the understanding of resistance to *F. hepatica* infection therefore remains unclear. Variation in susceptibility to *F. hepatica* infection in sheep has been demonstrated between other exotic and domestic breeds (Boyce et al., 1987), although differences in the immune response were not investigated.

1.2.10 Characterisation of *F. hepatica* antigens

Several antigens have been purified and isolated from crude fluke preparations. The biochemical nature and possible functional role of many of these proteins have been explored. Some have also been tested as potential vaccines with variable success.

1.2.10.1 Glutathione S-transferase (GST)

GST is a member of a multifunctional family involved with the metabolism of xenobiotics and the binding and transport of anionic compounds (Brophy *et al.*, 1990). The presence of GST in flukes was first discovered by biochemical and enzymatic studies (Howell *et al.*, 1988). Multiple isoenzymes were found in adult fluke somatic extract, the two main components having molecular weights of

approximately 25kDa. Immunohistochemistry showed that GST is widespread throughout the tissues of the fluke. Vaccination of rats with purified GST did not protect against challenge, although high antibody titres to GST were seen (Howell *et al.*, 1988).

GST was first used empirically in vaccine trials in sheep following the successful use of GST in protection against *S. japonicum* (Smith *et al.*, 1986) and *S. mansoni* (Balloul *et al.*, 1987) in mice and rats. Vaccination with *F. hepatica* GST reduced fluke burdens in sheep by up to 57% and serum biochemistry showed that establishment of flukes in the bile ducts had also been delayed (Sexton *et al.*, 1990). A degree of protection in cattle has also been demonstrated, although the efficacy of vaccination varied according to the vaccine adjuvant used (Morrison *et al.*, 1996). Protection was also found to be very variable between individual animals and did not correlate with antibody titre. GST isolated from flukes has now been sequenced and cloned (Muro *et al.*, 1993). Some similarity is seen with the GST of *S. mansoni* and *S. japonicum.*

1.2.10.2 Fatty acid binding proteins

Experimentally, a degree of cross-reactivity is seen between the immune response to schistosomiasis and fascioliasis. Mice infected with *F. hepatica* are resistant to challenge with *S. mansoni* and vice versa (Hillyer, 1985). The protein fraction thought to be responsible for this effect was isolated from *F. hepatica* somatic adult extracts and designated FhSMIII(M). Specific antisera raised against this fraction detected common determinants in *S. mansoni* extracts (Hillyer, 1984). Vaccination of mice with purified FhSMIII(M) was found to protect against challenge with both *F. hepatica* and *S. mansoni* (Hillyer, 1985). Cattle have also shown some resistance to challenge with *F. hepatica* after immunisation, with worm burdens reduced by up to 55% (Hillyer *et al.*, 1987).

The active component of FhSMIII(M) is a 12 kDa polypeptide. Vaccination of mice with this purified protein, called nFh12, protects against *S. mansoni* challenge by up to 77% (Hillyer *et al.*, 1988a). During experimental infections with *F. hepatica,* cattle and mice develop anti-Fh12 IgG antibodies within two weeks of infection. This suggests that Fh12 is expressed early during infection of the host by juvenile fluke stages. Mice also develop anti-Fh12 antibodies in *S. mansoni*

infections, albeit at a lower level and not until seven weeks post infection (Hillyer *et al.*, 1988b). Cloning and sequencing of the gene encoding Fh12 has demonstrated that the protein shows similarity to SM14, a schistosome fatty acid binding protein (Rodriguez-Perez *et al.*, 1992). Fatty acid binding proteins are essential for the survival of trematodes as they are unable to synthesise their own long chain fatty acids and must use those of their hosts. Unfortunately immunisation of rabbits with the cloned recombinant protein, rFh15, did not elicit good protection (Muro *et al.*, 1997).

1.2.10.3 Cysteine proteinases

The presence of secreted proteases in the ES products were first noted in experiments examining the action of ES products on immunoglobulins. Mouse, rat, rabbit and sheep immunoglobulin were partially degraded by ES products in a papain like manner (Chapman and Mitchell, 1982b). This proteolysis was enhanced in the presence of cysteine and inhibitor studies suggested thiol or cathepsin like proteases were responsible. The presence of thiol proteases in ES products from juvenile and adult flukes was later confirmed by gelatine gel analysis (Dalton and Heffernan, 1989).

Several groups have now isolated and cloned these cysteine proteinases (McGinty *et al.*, 1993; Heussler and Dobbelaere, 1994; Dowd *et al.*, 1997; Smith *et al.*, 1993; Roche *et al.*, 1997). They appear to exist as a multiple enzyme gene family in the fluke genome, possibly as multiple copies within the genome (Heussler and Dobbelaere, 1994). Five different cathepsin L-like and two different cathepsin B-like proteinases have been cloned and sequenced in one study (Heussler and Dobbelaere, 1994), and another two cathepsin L-like in another, termed CL1 (Roche *et al.*, 1997) and CL2 (Dowd *et al.*, 1997).

What is the function of cysteine proteinases in the fluke? One common method of attacking helminth parasites in mammalian hosts is by antibody-dependent cell mediated cytotoxicity, usually involving eosinophils. Studies have shown that eosinophils are able to bind or associate with flukes, but are often quickly shed. *In vitro* experiments have demonstrated that crude ES and purified cathepsin L-like proteinases from different fluke stages are able to cleave immunoglobulin G and so prevent attachment of eosinophils to newly excysted juveniles (Carmona *et al.*,

1993). This is reversed in the presence of cysteine proteinase inhibitors. Are antibodies directed against cathepsin proteinases able to neutralise this immunoglobulin-cleaving activity? *In vitro* incubation of purified cathepsin L in the presence of polyclonal anti-cathepsin L antisera is able to reverse cleavage and allows eosinophils to bind to newly excysted juveniles (Smith *et al.*, 1994).

Purified CL2 is able to cleave fibrinogen in a non-thrombin like manner, producing fibrin clots (Dowd *et al.*, 1995). This clot formation may act either to prevent excessive host bleeding around feeding adults or to protect the migrating juveniles, acting as a physical barrier against immune attack. Cathepsins are able to digest host tissues. Crude ES and CL1 and CL2 are able to degrade collagen types III and IV, fibronectin and laminin (Berasain *et al.*, 1997). These substances are present in the liver capsule and parenchyma of the mammalian host and so this degradative activity may be important for juvenile migration.

These studies show that the cysteine proteinases of *F. hepatica* appear to have several functions during infection of the mammalian host, including penetration, migration and feeding; possibly immune evasion and clot formation.

The cathepsin proteinases also stimulate some protective effect against fluke infection in the host. Purified CL1, CL2 and a haem-containing protein have been used both separately and combined in a vaccination trial (Dalton *et al.*, 1996): immunisation protected naïve cattle against challenge by reducing parasite burden from between 42.5% (CL1 alone) to 72.4% (CL2 combined with haemoglobin). Fluke egg viability was also substantially reduced, suggesting a possible anti-fecundity effect. No correlation was noted between the circulating IgG antibody titre and the protection seen. In this first vaccination study, Freund's complete adjuvant was used as the adjuvant for the primary inoculative dose. However, a further investigation, using the CL2 and haem fractions as a combined vaccine administered in Freund's incomplete adjuvant, found that no significant protective effect was induced (Mulcahy *et al.*, 1998). Vaccination of sheep with purified cysteine proteinases does not induce significant protection against challenge infection, although a reduction in parasite fecundity does occur (Wijffels *et al.*, 1994).

1.2.10.4 High molecular weight fractions

Two proteins greater than 200 kDa in size have been isolated from *F.hepatica*

ES: one is a haem-containing protein isolated from ES products. In non-reducing SDS PAGE, this fraction migrates as two bands, at a level distinct from bovine haemoglobin, of approximately 200 kDa (McGonigle and Dalton, 1995). Also, specific antisera can be raised by inoculation which does not recognise bovine haemoglobin, indicating that it is indeed a genuine fluke-derived protein. This haem-protein is thought to have a role in oxygen metabolism in the immature fluke, which respires aerobically as it migrates through host tissue (Tielens *et al.*, 1982). As the haem-protein was isolated from adult fluke proteins, and respiration in adult flukes is predominantly anaerobic, it is also thought to be involved in other, oxygen dependent activities in the adult, such as egg production (Tielens *et al.*, 1984). The haem fraction has been used in combination with cathepsin in vaccine trials, where it stimulated a reasonable degree of protection against challenge infection in cattle (Dalton *et al.*, 1996).

The other high molecular weight protein is a dipeptidylpetidase, present in the ES of newly excysted juvenile, liver stage and mature flukes (Carmona *et al.*, 1994). Specific antibody is only detected late during infection, despite the presence of the enzyme in the early juvenile stages. Dipeptidylpetidase is probably involved in the digestion of host molecules, supplying nutrients to the fluke.

1.2.10.5 Leucine aminopeptidase

A membrane bound protein, leucine aminopeptidase, has been partially purified from adult flukes (Acosta *et al.*, 1998). SDS PAGE gel electrophoresis has shown the fraction to be composed of a major 67 kDa product, with minor products migrating in the 30-40 kDa region. Immunohistochemistry has localised the enzyme to the gastrodermis of the fluke; it does not appear to be secreted in ES. Aminopeptidase activity has also been found in *S. mansoni* (Cesari, 1983; Xu and Dresden, 1986). The enzyme is involved in the final stages of protein degradation and is thus thought to play a nutritional role in the fluke. Antibody responses to this fraction during natural infections have not been studied. The results of the only vaccine study conducted to date are so far unconvincing, due to the death of trial stock and extreme differences in fluke burdens between animals within the same group (Piacenza *et al.*, 1999).

1.2.10.6 Antioxidants

Antioxidants have been found in many parasite species and are thought to protect against the potentially damaging effects of reactive oxygen species (ROS). ROS may be produced as a by-product of the parasite's own metabolic processes or by host effector cells attempting to damage and eliminate the parasite. Catalase, one of the more common anti-oxidant enzymes, has not been detected or isolated from the fluke (Pritchard and Schofield, 1971). Alternative mechanisms must therefore be in place to deal with such metabolites. In recent years various molecules showing antioxidant properties have been isolated from *F.hepatica*. Given their essential role in maintaining cellular integrity, antioxidants are an obvious target for vaccine design.

A 21 kDa protein has been identified by screening an expression library with sera from cattle vaccinated with the haem-containing high molecular weight fraction (section 1.2.10.4). The protein is a member of the peroxiredoxin family and is thought to be involved with the breakdown of hydrogen peroxide (McGonigle *et al.*, 1997). A smaller, 12 kDa thioredoxin has been isolated from the tegument of mature flukes. Although humoral responses to thioredoxin have not been studied, T cells from infected cattle are poorly reactive on culture with thioredoxin and so it has not been studied further (Shoda *et al.*, 1999).

Cytochrome c peroxidase activity was detected in crude ES products two decades ago. However, it was only recently that the enzyme was partially purified from ES products and specific activity investigated (Campos *et al.*, 1999). The isolated fraction displayed a major protein of 31 kDa and minor bands which run between 41 and 45 kDa on SDS PAGE gel electrophoresis. Another antioxidant, superoxide dismutase, has been detected in membrane, somatic and ES preparations of juvenile and adult flukes (Piacenza *et al.*, 1998). Enzyme activity is found to be highest in juvenile flukes, reducing as the flukes mature. This reduction in activity could be a reflection of the changes in metabolism that occur as the flukes age, or, if the enzyme is able to protect against host ROS, perhaps indicative of the greater exposure of juvenile flukes to such host products.

Currently, there is a noticeable lack of information regarding the host immune responses to these antioxidants during natural infections. These need to be examined before attempts are made to vaccinate animals experimentally.

1.2.10.7 Other proteins

Screening of adult *F.hepatica* cDNA libraries with specific anti-sera is now being used to identify and characterise further immunogenic fluke proteins. A calcium binding protein of approximately 22 kDa has been identified by this method (Ruiz de Eguina *et al.*, 1999), as well as a protein belonging to the NK lysin family (Reed *et al.*, 2000). The latter is found particularly in immature, but not newly excysted juvenile flukes and runs at 6-8 kDa on SDS PAGE gels. Further work needs to be done to investigate the possible functions and immune responses to these newly identified molecules.

Vaccine trials have been carried out with two other isolated fluke proteins. Paramyosin, a sub-tegumental protein of approximately 94 kDa, has been used to vaccinate sheep and cattle (Spithill *et al.*, 1998). However, it was not protective in sheep and conflicting results were obtained in two cattle trials. Another molecule that was also found to be ineffective as a vaccine is a Kunitz type serine proteinase inhibitor of 6 kDa (Bozas *et al.*, 1995; Spithill *et al.*, 1998). The failure of vaccination using these proteins suggests that the approach of isolating proteins on the basis merely that they are there and can be identified, rather than investigating proteins detected by natural immune responses, may not be helpful.

1.2.10.8 Identifying proteins in newly excysted juveniles

As newly excysted juveniles are so small it is often difficult to obtain adequate material for identifying component molecules. N-terminal sequence analysis requires only a small amount of material and can give an indication of the families to which proteins belong. Seven different proteins present in somatic extracts have been identified by this method (Tkalcevic *et al.*, 1995). Three of these show no similarity to any known proteins, however, two are related to cathepsin B protease, one to cathepsin L protease, another two show similarity with *Schistosoma* haemaglobinases and one is related to a gluconeogenesis enzyme.

1.2.11 Summary

Rats develop immunity to re-infection and this may occur early in infection at either the gut, peritoneal or liver capsule stages. Cattle appear to develop partial resistance to re-infection and experimentally at least, may self-clear primary

infections. It is not known where such immunity is effected in cattle and which components of the immune system are responsible. Additionally, the information about the immune processes occurring in ruminants both experimentally and in the field situation is extremely limited. Those studies which have attempted to address this area have also tended to look mainly at responses during the chronic state, and not at those to the early invasive stages of *F. hepatica*.

Several fluke antigens have been isolated and used in vaccination trials, with mixed success. The majority of these proteins have been isolated from adult fluke protein preparations, often based on the presence of an antibody response found during the later stages of infection. This "isolate and vaccinate" approach is relient upon and dominated by the success of protein isolation methods, rather than being based upon a better understanding of the natural immune responses occurring during infection. Furthermore, although adult and juvenile flukes share many proteins, concentrating on those abundant in the adult may result in a vaccine that is more active in the rejection of more mature parasites. This is of particular significance when considering the clinical implications of infection in the main target hosts. Sheep in particular, are very sensitive to heavy infection, which can be fatal during early fluke migration. Inducing an immune response which targets juvenile flukes, so that they are attacked during early migration could greatly reduce the stress and clinical illness experienced by infected animals. This is also of importance when considering the production capabilities of sub-clinically affected animals.

There is therefore a need for re-examining the approach to fluke vaccine development. Identifying immune responses occurring during the early migratory stages of infection are crucial. Also, further investigation of the immune responses induced by the various trial vaccines should be undertaken. Defining T cell subsets, or rather the cytokines produced during infection, and the antibody isotypes involved, may be necessary for deciding which antigens, adjuvants and inoculation routes should be used for vaccination. Greater knowledge of host reactions to these antigens will also allow optimisation of any protective responses stimulated by vaccination.

1.3 Objectives of the thesis

This study was therefore designed to investigate immune responses occurring in cattle during early experimental infections with *F. hepatica*. In order to limit

exposure to early juvenile stages of the parasite, animals were treated with the drug triclabendazole to terminate infection. The response of such pre-exposed animals to challenge infections was then investigated and compared with that of naïve challenged animals. In schistosomiasis, it has been found that by treating chronically infected people to clear infection and then following the process of re-infection, resistant and susceptible groups of individuals could be identified (Butterworth 1985). Differences in certain immune parameters, particularly levels of IgG1 and IgE antibodies and eosinophils were found between the two groups (Hagan *et al.*, 1987; 1991; Roberts *et al.*, 1993; Demeure *et al.*, 1993). Although the aim of this study was to examine experimental infections terminated during early infection, rather than during a chronic infection state, it was anticipated that similar patterns of response upon re-challenge might emerge.

The results of this study are based on the outcome and analysis of two main experiments:

- In the first experiment, differences in the peripheral antibody and cellular response to challenge infection were examined in naïve, chronically infected and drug-abbreviated pre-exposed animals, together with any alterations in susceptibility to the challenge infection (Chapter 3).

- In the second experiment, local lymph node immune responses occurring shortly after challenge infection were studied in pre-exposed and naïve animals (Chapter 4).

- Antibody and cellular responses to crude fluke protein preparations and to isolated cathepsin protease (Smith *et al.*, 1993) and high molecular weight (McGonigle *et al.*, 1995) fractions were studied in both the experiments (Chapters 5 and 6).

The aims of each experiment are described in more detail in the relevant chapter, together with the rational for the methodology used.

CHAPTER 2

GENERAL MATERIALS & METHODS

Recipes for all solutions and culture media are given in Appendix A.

2.1 Source and preparation of *F. hepatica* protein extracts

2.1.1 Excretory-secretory (ES) and whole adult fluke somatic antigen (WFA) preparations

Live, adult flukes were obtained from the bile ducts of fresh sheep or bovine livers and placed into warm (37°C) PBS, pH 7.4. The flukes were washed once in PBS, then incubated at 37°C for 30 minutes to encourage regurgitation of sheep or bovine blood from the gut into the media (10-15 flukes per 15 ml). Flukes that were clean with little remaining visible gut contents were then selected and washed several times in PBS until they were free from external debris and became pink-coloured. Flukes were placed in 6-well tissue culture plates (Nunc®), with one fluke per 2-3 ml of PBS and cultured for 3-4 hours at 37°C in 5% CO_2 in air. The PBS, containing ES proteins, was aspirated. Live flukes were snap frozen using a mixture of methanol and dry ice and stored at - 70°C until required.

The PBS-ES preparation was centrifuged at 100*g* for 10 minutes to pellet any fluke eggs. The supernatant fluid was decanted, aliquotted into 1.5 ml volumes and centrifuged at 13000*g* for 30 minutes at 4°C. Clarified fluid was then removed from the pelleted cell debris by pipette, pooled, passed through a 0.22 μm syringe filter and aliquotted. Aliquots were stored at -70°C.

WFA was prepared from the frozen flukes collected above. Flukes were rapidly warmed to room temperature (RT) and then placed immediately into a small, home-made "French press" that had been chilled in liquid nitrogen. The flukes were shattered by hitting the rod of the press with a hammer. A 5-10% suspension of fluke powder in ice-cold PBS was prepared and this was incubated on ice, with intermittent vortexing, for 30–60 minutes. Large debris particles were removed by centrifugation at 1200*g* for 10 minutes. The resulting supernatant fluid was then fully clarified by centrifugation at 13000*g* for 30 minutes. Supernatant fluid was aspirated, pooled,

filtered through a 0.22 μm membrane and stored at –70°C until required.

2.1.2 14 day old juvenile fluke somatic preparation

Juvenile flukes were prepared and isolated with the assistance of Dr Diana Williams and Dr Atila Akca at the Liverpool School of Tropical Medicine, University of Liverpool.

Female Wistar rats were dosed with 100 *F. hepatica* metacercariae (Compton Paddock Laboratories, Newbury, Berkshire). On day 14 post-infection, rats were euthanased, the livers removed and juvenile flukes isolated by dissection. The juveniles were washed several times in warm PBS and then frozen at –70°C. Juvenile flukes were defrosted on ice and homogenised with an Ultra-Turrax T8 homogeniser (IKA Laboratories) in ice-cold PBS. The resulting suspension was incubated for 15-30 minutes on ice with occasional vortexing. The mixture was clarified by centrifugation at 13000*g* for 30 minutes at 4°C, however, to prevent unnecessary loss it was not passed through a 0.22 μm filter. The preparation was then aliquotted and stored at –70°C.

2.1.3 Metacercarial crush preparation

F.hepatica metacercarial cysts (Compton Paddock Laboratories) were supplied encysted on cellophane film. Cysts were removed from the film with the blunt edge of a scalpel blade, placed into microfuge tubes and washed 2-3 times with PBS, removing excess fluid by pipette. The cysts were frozen on dry ice and then ground, off ice, using a micropestle (eppendorf). This process was repeated several times. The suspension obtained was made up to a larger volume with PBS and then clarified by centrifugation at 13000*g* for 30 minutes. The supernatant fluid was removed by pipette from pelleted debris, aliquotted and stored at –20°C.

2.1.4 Cathepsin-L protease and haem-containing high molecular weight fraction (HMW)

The cathepsin and high molecular weight fractions used throughout the study were kindly provided by Professor J. Dalton, Dublin City University, Glasnevin 9, Dublin, Ireland. Both fractions were isolated from the excretory-secretory products of

adult flukes (Smith *et al.*, 1993; McGonigle *et al.*, 1995).

It was not clear whether the cathepsin-L pool fraction was supplied in PBS buffer and so buffer exchange was carried out using a PD-10 Sephadex® G-25M column (Pharmacia Biotech) and PBS, pH 7.4. The high molecular weight fraction was used as supplied, in PBS buffer.

2.1.5 Measurement of protein concentration

The protein content of the various preparations used was measured by the bicinchoninic acid method using the BCA Protein Assay Kit (Pierce). The standard microtitre assay was used for samples with expected concentrations above 0.25 μg/ml and the enhanced protocol for those that were found to have concentrations lower than 0.25 μg/ml. The assay was read at 570 nm on a Microplate Autoreader EL311 (BIO-TEK Instruments).

2.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) for separation of proteins

SDS PAGE electrophoresis was performed using the discontinuous method according to Laemmli (1970). Gels were cast and run using both the Mini Protean® II and Protean® II xi gel apparatus (BioRad Laboratories). A 30% stock acrylamide/bis-acrylamide solution (Sigma A-3699) was used to prepare the gel solutions. The separating gel was either a 10 or 12% gel (mini-gels) or 10-15% gradient gel (large gels) in 1.5M Tris pH 8.8 buffer with 0.1% w/v SDS, 0.05% w/v ammonium persulphate and 0.005% v/v TEMED (Sigma T-9281). All stacking gels were 4% acrylamide in 0.5M Tris pH 6.8 buffer with 0.1% w/v SDS, 0.05% w/v ammonium persulphate and 0.005% v/v TEMED. 10-15% gradients were formed using a 385/395 Gradient Former (BioRad Laboratories) and a Micro-Tube Pump MP-3 (Tokyo Rikakika Co., Ltd.).

Samples were mixed with reducing loading buffer and heated to 95°C for 5 minutes prior to loading, or with non-reducing loading buffer (high molecular weight fraction). Gels were run using SDS PAGE running buffer at 200V for 50 minutes (mini-gels) or at 60V overnight (large gels). Broad range markers (BioRad Laboratories) were run with each gel.

2.3 Enzyme-linked immunosorbent assay (ELISA) for detecting IgG1 antibody to excretory-secretory proteins

The wells of a microtitre plate (Immulon-1, Dynex Technologies UK) were coated in 100 µl of ES at 1.5 µg/ml diluted in carbonate-bicarbonate buffer, pH 9.6 (Sigma C-3041), covered in cling-film and incubated overnight at 4°C. The coating solution was then shaken out and the plate was washed with an excess volume of PBS-T buffer, 3 times, using a 3 minute incubation for each wash.

Any unbound sites in the wells were then blocked by adding 200 µl of 4% normal sheep sera (Life Technologies) in PBS-T to each well and incubating for 1 hour at RT. The wash step was repeated.

Test sera was diluted in 1% normal sheep sera in PBS-T and 100 µl added to the respective wells. The plate was shaken for 1 minute on a shaker and incubated for 1 hour at 37°C. The wash step was repeated.

A sheep polyclonal anti-bovine IgG1 horseradish peroxidase conjugated antibody (Bethyl Laboratories Inc., USA) was used to detect bound antibody, as a 1/8000 dilution in 1% normal sheep sera in PBS-T. The plate was shaken to mix and incubated for 1 hour at 37°C. The wash step was repeated.

The reaction was developed using 3, 3', 5, 5'-tetramethylbenzidine, TMB (one-component system, Kirkegaard and Perry Laboratories Inc.). 100 µl of TMB was added to each well and mixed. The reaction was stopped with 0.2 M sulphuric acid. The reaction was read at 450 nm on a Microplate Autoreader EL311 (BIO-TEK Instruments).

This ELISA assay was used to screen stock animals for the presence of antibody to *F. hepatica*, before inclusion in experimental groups. Optimal dilutions of ES, test sera and secondary conjugate antibody were determined by titration in a similar manner to the titrations described in section 5.2. Test, positive and negative control samples were assayed in triplicate. The negative cut-off titre for the reaction was taken as the mean of negative control samples plus 3 standard deviations of the mean. Negative control samples were obtained from 3 uninfected Friesian-Holstein calves and positive control sera from a *F.hepatica* chronically infected animal, calf 95.

2.4 Tissue culture methods

All cell isolation and culture techniques were carried out in a tissue culture facility using Class II vertical laminar flow hoods.

2.4.1 Isolation of bovine peripheral blood mononuclear cells (PBMC)

Blood was taken from the jugular vein with an 18 gauge needle into 10 ml vacutainers® (Beckton Dickinson) containing 2 ml of the anticoagulant acid citrate dextrose (ACD) and processed within 1 hour of collection.

25 ml of the blood-ACD mixture was layered onto 15 ml of warm (37°C) Lymphoprep™ (Nycomed Pharma AS) in a 50 ml centrifuge tube (Corning) and centrifuged in a swing-out rotor at 1500*g* for 25 minutes at RT, without the use of a brake. The interface was carefully collected using a sterile plastic Pasteur pipette and placed into a new tube. The volume was taken to 50 ml with warm PBS and the cells pelleted by centrifugation at 300*g* for 10 minutes. The supernatant fluid was smoothly decanted and the cell pellet resuspended in PBS. The cells were again pelleted by centrifugation at 120*g* for 10 minutes. This last wash and spin process was repeated once more with PBS and once with warm RPMI 1640 media (Sigma R-7388). After this final wash the cells were resuspended in complete media.

2.4.2 Isolation of a single cell suspension from whole lymph node material

The method for isolating cells from bovine lymph nodes was adapted from methods of Dr E. Innes (Moredun Research Institute, Pentlands Science Park, Penicuik, Midlothian) and Gasbarre (1994).

Lymph nodes were taken from animals at post-mortem as soon as possible after death and placed intact into ice-cold lymph node media. The lymph nodes were transferred to a tissue culture facility and processed using aseptic techniques. Lymph nodes were handled with sterile stainless steel forceps.

The lymph node was placed in a petri dish (Bibby Sterilin Ltd.) together with approximately 30 ml of lymph node media and gross debris carefully dissected away. The lymph node was then transferred into a fresh dish containing a similar volume of fluid and chopped into tiny pieces with sterile scissors. The resulting suspension was poured into a sterile bag (Seward Medical), sealed and homogenised for 60 seconds in a Stomacher® 80 machine (Seward Medical). The cell suspension obtained was

then separated from tissue fragments by filtering through a narrow gauge sterile stainless steel sieve (tea-strainer).

The filtrate was transferred into a 50 ml tube and allowed to stand for 5-10 minutes to allow any remaining debris to sediment at the bottom of the tube. The supernatant fluid was then aspirated by pipette, transferred to a fresh tube and pipetted back and forth several times to break up any further cell clumps. The suspension was centrifuged at 120*g* for 10 minutes to pellet cells, supernatant fluid decanted and the pellet resuspended in 2-4 ml of lymph node media.

Red blood cells were lysed at this stage by the addition of 2-4 ml of a 0.83% ammonium chloride solution for a 3 minute incubation period. The solution was then made up to 50 ml with Hank's Buffered Saline Solution (Sigma H-6648). After red cell lysis, the cells were washed twice in lymph node media and once in RPMI 1640 media, pelleting at 100*g* for 10 minutes. Cells were finally resuspended in complete media.

2.4.3 Cell counts using Trypan Blue and a haematocytometer

100 µl of cell suspension was mixed with 400 µl of RPMI 1640 media and 500 µl of Trypan Blue Solution 0.4% (Sigma T-8154) and allowed to stand for 5 minutes. The chamber of an Improved Neubauer haematocytometer (Weber) was filled with the cell suspension and unstained cells counted at 100X magnification. The number of cells per ml was calculated as:

cell count per square (1mm x 1mm) x dilution factor (10) x 10^4

2.4.4 Standard cell proliferation assay

Assays were performed in quadruplicate in 96 well tissue culture plates (Falcon®) at a final cell concentration of 1 x 10^6 cells/ml, using 2 x 10^5 cells per well.

Cells were adjusted to 2 x 10^6 cells/ml in complete media. The protein preparation used to stimulate the cells was prepared at double the required final concentration in complete media and then mixed as a 1:1 ratio with the cell suspension. 200 µl volumes of this mixture were then transferred to the relevant wells. A positive control of cells with the mitogen Concanavalin A (Con A) at a final concentration of 1 µg/ml and a negative control of cells in media alone, were

included for each separate cell sample. Cells were incubated at 37°C in 5%CO_2 in air for 5 days.

On the fifth day, 1 μCi (37 kBq) of ^{3}H-labelled thymidine (Amersham) was added to each well and the cells incubated for a further 6 hours. The cells were harvested onto glass fibre filters (Wallac), using an automatic cell harvester (Harvester 96®, TomTec), and the filters allowed to air dry. Filters were placed in plastic pockets, 4 ml of scintillant fluid, OptiScint "Hisafe" (Wallac) added and the pocket heat-sealed. The incorporation of ^{3}H-thymidine into cellular DNA was then measured using a 1450 Microbeta™ Plus scintillation counter (Wallac).

The arithmetic mean value of quadruplicate replicates was calculated and used in the final analysis.

2.4.5 Cryopreservation of mononuclear cells with dimethyl sulphoxide (DMSO)

Cells in a cell suspension were pelleted by centrifugation at 120*g* for 10 minutes. The pellet was resuspended in a mixture of 90% FCS (Life Technologies) and 10% DMSO (Sigma D-2650) at a concentration of 1 x 10^7 cells/ml and aliquotted into cryogenic vials (Nalgene®). Cells were cooled overnight in a Cryo Freezing Container (Nalgene®) to –80°C and then placed in the vapour phase of a liquid nitrogen cooling facility for long term storage.

2.4.6 Resuscitation of cryopreserved cells

The cryovials were removed from the liquid nitrogen storage facility, immediately placed on dry ice and transported to the tissue culture facility. The cells were rapidly warmed to melting point in a 37°C waterbath and then the contents of one vial (1.5-2 ml) added to 20 ml of complete media (at 37°C). The volume was made up to 50 ml with further media and the cells pelleted by centrifugation at 120*g* for 10 minutes. The cell pellet was resuspended in complete media and the centrifugation step repeated. Care was taken to work rapidly throughout the process, so that the cells could be placed in an incubator as quickly as possible, in order to minimise cell death.

2.5 Fluorescence activated cell sorter (FACS) analysis to characterise cells by phenotype

2.5.1 Staining cells with monoclonal antibodies to cell surface markers

Cells were isolated as described above in section 2.4.1-2, however the final wash step was performed using "cell wash" (FACSflow, Becton Dickinson) and cells were resuspended in "cell wash" at a concentration of 1 x 10^7 cells per ml.

A round bottomed 96-well plate (Falcon®) was used for the staining and washing steps. 5 x 10^5 cells/well were added to the plate and the cells pelleted by centrifuging at 100*g* for 5 minutes. The supernatant fluid was flicked out of the plate and the cell pellet resuspended in 30-50 µl of the primary, anti-cell surface marker, monoclonal antibody at the optimal dilution in cold "cell wash" (Table 2.1). A negative control of mouse serum at a 1/500 dilution in "cell wash" was included for each animal. The plate was incubated on ice for 30 minutes.

The cells were pelleted, as above. The supernatant fluid was flicked out and the cell pellet resuspended in 200 µl of cold "cell wash". The spin step was repeated and the cells washed twice more. After the final spin, the cells were resuspended in 25 µl of the secondary, FITC-labelled antibody conjugate at a 1/500 dilution (anti-mouse polyvalent IgG/A/M, Sigma F1010). The cells were incubated with the conjugate, on ice, in the dark, for another 20 minutes. The washing step was repeated. After washing, the cells were resuspended in 200 µl of "cell wash" and run through the FACScan cytometer within 2 hours.

2.5.2 Sample processing through the FACScan cytometer

Cells were run through a FACScan cytometer (Becton Dickinson). The machine was calibrated using the negative control samples, adjusting forward scatter (FSC) and side scatter (SSC) so that data points fell clearly within a dot-plot. Dead cells and residual erythrocytes were gated out. Fluorescence was measured with the FITC detector (FL1) set at around 620 meV. Settings and gates were optimised for peripheral blood mononuclear cells and lymph node cells and kept constant between samples. The cytometer acquired 10000 cells and results were displayed as frequency histograms.

The proportion of cell staining positive for a particular cell type was

calculated and displayed as a percentage. Results were compared over time or between groups using the Mann-Whitney U test.

Table 2.1: Monoclonal antibodies to bovine cell surface markers used for FACS analysis of cell phenotypes.

Antibody	Specificity	Cell type	Dilution	Reference
CC8[a]	CD4	T helper	1/5	1
SBU-T8[b]	CD8	T cytotoxic	1/1	2
86D[b]	TCR1	$\gamma\delta^+$ T cell	1/1	3
VPM30[b]	Not classified	B cell	1/1	4
VPM65[b]	CD14	Monocyte/ macrophage	1/1	5
Negative control[c]	Mouse sera	N/A	1/500	N/A

Key:

N/A: not applicable

[a] : a gift from Professor C. Howard, Institute of Animal Health, Compton, Berkshire.

[b] : gifts from Professor J. Hopkins, Faculty of Veterinary Medicine, University of Edinburgh.

[c] :Sigma (M-5905)

1: Howard *et al.*, 1989.

2: Maddox *et al.*, 1985.

3: Mackay *et al.*, 1989.

4: Naessens and Howard, 1991.

5: Gupta *et al.*, 1996.

CHAPTER 3

The effect of pre-exposure to restricted stages of juvenile *F. hepatica* on the progression of a subsequent challenge infection in cattle (Experiment A).

3.1 INTRODUCTION

The immune response to the very early fluke stages present during the migratory period from the gut, across the peritoneal cavity and to the liver has not been examined in any detail in the ruminant host. A vaccine that stimulated a protective host response against these early stages would be ideal, eliminating the parasite whilst still small and before serious damage is inflicted upon the liver parenchyma. In a natural infection, examining immune responses to these stages is virtually impossible. No attempts have previously been made to measure specific antibody to fluke proteins in cattle during the first week of infection. Antibody present at later time-points will reflect the changing profile of protein expression as the fluke matures and are necessarily not specific to an individual development stage.

Restricting exposure to early stages would require either physical removal, which would be impossible unless flukes were implanted into the peritoneal cavity within permeable sacks or removal by death of the parasite. Cattle vaccinated with irradiated metacercariae have shown resistance to reinfection (Nansen, 1975) as have rats (Corba *et al.*, 1971). Irradiation is thought to alter parasites in a variety of ways. These include changing the nature of expressed antigens and altering protein synthesis (Wales and Krusel, 1992). Irradiation of *F. hepatica* metacercariae results in the production of newly excysted juveniles with altered carbohydrate and cathepsin-B expression (Creaney *et al.*, 1996). These changes in protein synthesis and antigenic nature may inhibit the natural development of the parasite, perhaps arresting development such that it does not progress beyond a certain stage (Jarrett *et al.*, 1958). However, irradiation is not an exact method of terminating the stage to which a fluke develops. The use of a drug that reliably kills early fluke stages is therefore the best option currently available. In this study, exposure to early restricted stages of the parasite was attempted by drug-abbreviated infections.

The drug triclabendazole is the most effective fasciolicide available for killing immature stages of the parasite. Studies in cattle have shown reductions in fluke burden at 1 week post-infection of 85% (Fuhui *et al.*, 1989), 88% (Richards *et al.*, 1990) and 95% (Boray, 1982) when used at a dose rate of 12 mg/kg and 98% (Boray, 1982) when used at 15 mg/kg. The efficacy of killing earlier stages has only been investigated in sheep, with a 98% reduction in burden found by treating at 1 day post-infection, using a dose rate of 15 mg/kg (Boray, 1983). This and other studies (Dorchies *et al.*, 1983) have shown that killing efficacy is enhanced by increasing triclabendazole dose. In the present study, triclabendazole was used at a high dose rate of 36mg/kg, with the aim of achieving a high kill rate for flukes of less than 1 week of age.

The timing of fluke migration has only been studied in detail in experimental animals such as the rat and mouse. In these species, gut penetration is immediate, with flukes present in the gut mucosa within 2 hours of infection (van Milligen *et al.*, 1998) and present within the peritoneal cavity at 12-24 hours post-infection (Dawes, 1961; Bennett and Threadgold, 1975; Rajasekariah and Howell, 1977). Entry into the liver is seen as early as 2-3 days post-infection in the mouse (Dawes and Hughes; 1964; Tkalcevic *et al.*, 1996) and at 4 days in the rat (Hayes, 1978). There is little data available describing the timing of migration within the ruminant, although flukes have been found at 4 days post-infection in the peritoneal cavity of cattle (Doy and Hughes, 1984). Kendall and Parfitt (1962) state that the time of entry into the liver is approximately 90 hours, although it is not clear whether this relates to the sheep, or another animal. However, given the more complex gut anatomy and larger size of ruminants, it is to be expected that the migration of flukes might take longer than that seen in rats and mice.

This study investigates the host response to two particular stages of fluke migration through the host. The first, the migration of newly excysted juveniles across the gut mucosa and into the peritoneal cavity. The second, the period of migration from the gut, through the peritoneal cavity and into the liver parenchyma. These two migration periods were estimated to last approximately 24 hours and 5 days in cattle, on the basis of the evidence given above. Juvenile flukes were prevented from further migration by the administration of triclabendazole at these two time-points. Animals that were pre-exposed to *F. hepatica* in this manner were

then challenged several weeks later and the progression of infection examined until patency was well established. Serum levels of the two liver enzymes gamma-glutamyl transferase and glutamate dehydrogenase were followed, together with fluke egg levels in the faeces and eosinophilia, in order to give an indication of the degree and the rate of progression of infection. Any differences observed between these parameters in pre-exposed animals, compared to that seen with naïve challenged animals, were investigated.

Experimental Aims

- To investigate the immune responses occurring in cattle to restricted life-cycle stages of *F. hepatica*, specifically those exposed to the first 24 hours or to the first 5 days of infection.

- To compare the immune responses occurring in animals exposed to restricted stages with chronically infected or naïve animals, upon subsequent challenge with the parasite.

- To determine whether exposure to restricted early stages of the parasite confers any benefit to the animal in terms of reduced liver damage or fluke burden, when subsequently challenged.

The immune responses examined included peripheral blood mononuclear cell proliferative responses to crude fluke protein preparations and peripheral antibody responses to crude and specific isolated proteins (antibody responses are examined in Chapters 5 and 6).

3.2 MATERIALS & METHODS

3.2.1 Experimental design

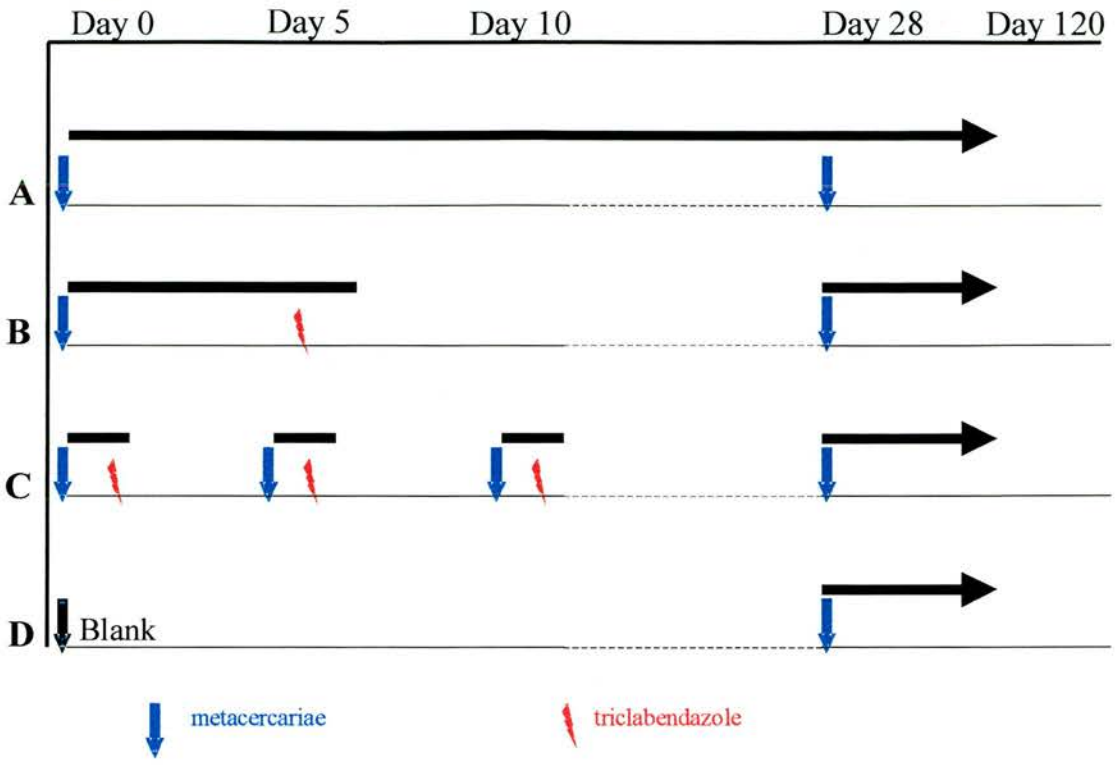
Four groups of ten calves were used in the experiment. Group A, the chronic group, received a primary infection on day 0 and remained infected throughout the experiment, with a superimposed secondary infection given on day 28. Group D, the naïve control group, did not receive a primary infection at day 0, but did receive the day 28 challenge infection. Groups B and C received drug-abbreviated primary infections and were then challenged on day 28. Group B had the primary infection terminated by treatment with triclabendazole on day 5. Group C received three exposures to metacercariae over the first 10 days of the experiment, with infection being terminated by triclabendazole treatment 24 hours following each infection dose. The experimental design is summarised in Figure 3.1 and Table 3.1.

The two control groups A and D were included in the experiment for different reasons:

- Group A was used to compare the early immune response to *F. hepatica* infection in chronically infected animals and in those that had received exposure to restricted juvenile fluke stages during the primary phase of the experiment.

- Group D was used to assess the viability of the metacercariae used in the secondary infection. More importantly, it also allowed a direct comparison of the progression of a chronic infection in naïve and pre-exposed animals, in terms of the liver damage, egg production and immune responses induced.

Figure 3.1: Diagrammatic representation of the experimental design, showing the different treatments that groups A, B, C and D received.



Time is shown along the upper line in days from days 0-120. Each group is illustrated separately on the left hand side. The exposure period to flukes experienced by each group is illustrated by a thick black line; metacercariae administration is shown by vertical arrows and treatment with triclabendazole as jagged lines. Note that the exposure period in groups B and C slightly overhangs the point of triclabendazole treatment. This is to take into account the time needed for the drug, given orally, to reach effective circulating blood levels. The secondary infections in all groups proceed onwards from day 120, shown as arrowheads on the black exposure lines.

Table 3.1: Summary of the primary and secondary infections and triclabendazole doses received by the four groups A, B, C and D.

Group	Primary exposure	Secondary exposure
A	Receive metacercariae on day 0 and remain untreated with triclabendazole.	Receive challenge dose of metacercariae on day 28.
B	Receive metacercariae on day 0 and are treated with triclabendazole on day 5.	Receive challenge dose of metacercariae on day 28.
C	Receive metacercariae on days 0, 5 and 10; and are treated with triclabendazole on days 1, 6 and 11.	Receive challenge dose of metacercariae on day 28.
D	Receive blank filter paper boluses on days 0, 5 and 10; and are treated with triclabendazole on days 1, 6 and 11.	Receive challenge dose of metacercariae on day 28.

3.2.2 Sampling timetable for sera and faeces collection

Routine samples were taken weekly, from day –14 to day 125-156, from all 40 animals in the study, as detailed below.

- Blood samples were taken into EDTA anti-coagulant for a haematological blood screen, including a full differential white cell count.

- Serum from clotted blood was prepared and used for the measurement of levels of the liver enzymes gamma-glutamyl transferase and glutamate dehydrogenase and in studies to investigate antibody responses to fluke proteins (Chapters 5 and 6).

- Per-rectal faecal samples were collected for measurement of faecal egg counts. These were stored at 4°C until processed.

3.2.3 Stock

Castrated male Friesian-Holstein dairy calves, aged between 4-6 months old at the start of the experiment, were used. Prior to the start of the study all animals were screened three times for the presence of *F. hepatica* eggs in the faeces and twice by ELISA, at an interval of three weeks, for IgG1 antibodies to adult fluke excretory-secretory antigens (section 2.3). The ELISA had previously been shown to detect a positive serum response in cattle from as early as two weeks post-infection. One animal appeared positive on the ELISA screen and was not included in the study.

Animals were randomly assigned to the four groups by stratifying all 40 in order of weight and then allocating each to one of the groups in sets of four, using random numbers. This method of allocation ensured that the animals in any particular group were not biased in any way (such as weight or age) when compared to the other groups. Group mean weights and weight ranges at the start of the experiment are shown in Table 3.2.

Calves were penned in the allocated groups in an open-sided barn and were fed a diet of calf rearing nuts with hay and water. One calf in group C failed to thrive for unrelated reasons and was euthanased on humane grounds. All group C results and analyses shown in section 3.3 were performed using the data of the remaining nine animals only.

3.2.4 *F. hepatica* infection

British strain *F. hepatica* metacercariae were supplied encysted on cellulose film by Compton Paddock Laboratories (PO Box 91 Newbury, Berks, RG20 7HB). Metacercariae were counted under a dissecting microscope at 20X magnification and the appropriate number (within +/- 10 cysts) cut from the film. The film, with cysts, was then carefully wrapped within two layers of water-dampened Whatman number 1 filter paper. Cysts were counted within 24 hours of use and stored damp at 4°C. The wrapped dose was placed in a balling gun and administered *per os* to the calves. Animals were kept for several minutes after dosing in a concrete enclosure and fed a small quantity of calf nuts to ensure that the dose had been swallowed.

A metacercarial dose per kg of bodyweight was given to each calf due to the broad range in weight present within the four groups. This was chosen instead of flat dosing, regardless of weight, to prevent the smaller animals being subject to a greater

fluke dose per kg of liver. Flat dosing in this manner could have resulted in much greater stress and interference in liver function in the smaller animals. The dose rate used was 3.6 metacercariae per kg bodyweight, which was a moderate dose. Infection was intended to stimulate an immune response and result in changes in the various infection parameters, but not to produce clinical disease.

The primary dose given to groups A, B and C was calculated using individual mean weights from two readings, taken 7 days apart. The second primary group C dose given on day 5 was identical to the first. The third primary group C dose given on day 10 was based on weights taken at day 7. Challenge secondary doses given on day 28 were again given at a dose rate of 3.6 metacercariae per kg bodyweight, using the mean of two weights taken during the preceding 7 days. The group mean weights and infection doses are shown in Table 3.2.

3.2.5 Termination of parasite infection with the drug triclabendazole

Triclabendazole was used to terminate fluke infections in the two treatment groups B and C (Fasinex^{TM}, Novartis Animal Health UK Ltd, Whittlesford, Cambridge, CB2 4XW). Group B animals were treated *per os* with triclabendazole on day 5 at a dose rate of 36 mg/kg. Group C animals were treated on day 1 and day 11 at a dose rate of 36 mg/kg and on day 6 at a dose rate of 24 mg/kg. The control group, D, was also treated with triclabendazole at the same time points as group C. The drug dose used was higher than the licensed dose rate for the product. It was anticipated that this would improve the efficacy of killing juvenile flukes. Triclabendazole has a very high safety index (Boray, 1983), so no problems were envisaged by using the drug at this dose.

Triclabendazole is oxidised within the blood to two active metabolites, the sulphoxide and sulphone, which do not attain peak concentrations until approximately 18 and 36 hours, respectively, after drug administration. However, detectable levels are present within 5 hours of dosing (Hennessy *et al.*, 1987). Hence, although groups B and C were treated at 5 days and 24 hours post-infection, a window of several hours prior to any fasciolicide action must be taken into account. Primary infection flukes in groups B and C may therefore have reached 6 and 2 days old, respectively.

Table 3.2: Group mean animal live-weights, showing weight ranges and the mean metacercarial doses given, prior to the primary infection (day 0) and secondary challenge infection (day 28).

Group	Primary infection		Challenge infection	
	Weight (range)	Dose (mean)	Weight (range)	Dose (mean)
A	134.3 (162-103)	484	144.5 (173-119)	520
B	137.7 (170-102)	496	149.7 (185-111)	539
C	140.3 (155-81)	484	153.1 (177-144))	551
D	139.0 (171-112)	Not given	155.2 (189-119)	559

3.2.6 Measurement of *F. hepatica* eggs in the faeces

Fluke burden was assessed by the use of faecal egg counts (FEC), measured by a zinc sulphate flotation method. 3g of fresh faecal material were sieved into 45 ml of saturated salt solution until all but insoluble residue remained. The faecal suspension was swirled well to mix and then 13 ml poured into a 15 ml centrifuge tube, which was then centrifuged at 500*g* for 5 minutes. The supernatant was discarded and the pellet resuspended in 13 ml of zinc sulphate solution (specific gravity, 1.3). The tube was then centrifuged again for 3 minutes. The liquid was brought to the brim of each tube without spillage, by adding zinc sulphate solution drop-wise and then a coverslip was placed on the top of the tube. The coverslip was left in place for 4 minutes and then removed, placed onto a glass slide and all fluke eggs present counted under a microscope at 40X magnification. Egg counts per gram were calculated as follows:

Count x 0.33 (scales to 1 g) / 0.28 (proportion of suspension used) x 20/V*

* ,V, volume of centrifuge tube: this terms adjusts for loss during the procedure (M.Sc. Handbook of Helminthological Techniques, Department of Tropical Animal Health, University of Edinburgh 1997).

3.2.7 Preparation of serum samples

Blood was collected into plain glass vacutainers™ (Becton Dickinson) from the jugular vein using a 20 gauge needle. Samples were allowed to clot at room temperature and then chilled for 2 to 4 hours at 4°C, before centrifuging at 1200*g*. Serum was carefully removed from the pelleted clot by pipette and aliquotted into micro-tubes. Serum samples were then stored at –70°C or –20°C for long and short term storage respectively.

3.2.8 Measurement of gamma-glutamyl transferase (γ-GT) and glutamate dehydrogenase (GLDH) levels in serum

Damage to the liver was examined by measuring the serum levels of the two enzymes γ-GT (EC2.3.2.2) and glutamate dehydrogenase GLDH (EC 1.4.1.3). The levels of these enzymes present in serum were measured using Technicon RA® chemistry kits (Miles Inc., Tarrytown, NY, USA) on a Bayer Opera Chemistry System spectrophotometer, by Mr A. Dowell of the Dalghety Laboratory, Faculty of Veterinary Medicine, University of Edinburgh. Levels are expressed as international units per litre, IU/l.

3.2.9 Haematological screens

Blood was collected into vacutainers containing EDTA anticoagulant and differential white cell counts (using blood smears) performed by the Clinical Laboratory, Faculty of Veterinary Medicine, University of Edinburgh. The total number of leucocytes were measured using a coulter counter (Baker); blood films (stained with Modified Wright's stain) were examined microscopically to give the differential cell count.

3.2.10 Peripheral blood mononuclear cell proliferative responses to whole fluke antigen (WFA) and excretory-secretory (ES) preparations

Four animals from each group were selected for analysis of peripheral blood mononuclear cell (PBMC) proliferative responses to the adult WFA and ES protein preparations. The heaviest animals in each group were chosen as this allowed the required volume of blood to be collected within the allowed limit dictated by Home

Office regulations. Those used were as follows: group A, calves 45, 46, 55 and 65; group B, calves 40, 71, 72 and 76; group C, calves 54, 57, 64 and 66 and group D calves 43, 59, 69 and 73. PBMC were prepared as described in section 2.4.1.

It was not possible to perform assays with samples from all sixteen calves on the same day. Eight animals were therefore sampled on one day and the remaining eight on the following day. A negative, baseline assay was carried out on days -14/-13. Those with high or irregular background responses were re-sampled on day –6. Post-primary infection responses were investigated on days 7/8, 14/15 and 26/27. A single secondary post-challenge assay was carried out on days 35/36.

The WFA and ES protein extracts were prepared as described in section 2.1.1 and proliferation assays were carried out according to the procedure outlined in section 2.4.4. The cellular responses to these preparations were titrated for all animals at days -14/-13, -6, 7/8 and 14/15, using WFA and ES concentrations of 1-30 and 1-20 μg/ml respectively. The optimum concentration for each preparation was then selected and used throughout the remainder of the experiment. A positive control of Con A at a concentration of 1 μg/ml and a negative control of culture media alone, were used with all samples at each time-point.

3.2.11 Statistical analysis

The arithmetic group mean values for γ-GT, GLDH, eosinophil and other white cell parameters were used in the analysis. However, the geometric mean was used for representing the group faecal egg output response.

All statistical analyses were performed using the Minitab® 12 software package (Minitab Inc., USA). The non-parametric Mann-Whitney U test was used to investigate group differences for all the parameters studied. The presence of any possible correlation between dose and egg counts or enzyme levels was investigated by the Spearman's rank correlation test.

3.3 RESULTS

3.3.1. Detection of *F. hepatica* eggs in faeces

The pattern of egg production during the latter part of the time-course, from day 105 (week 15) is illustrated in Figure 3.2.

Eggs first appeared in the faeces of animals from group A on day 70. Animals in groups B and C started to produce eggs in the faeces from day 98 onwards, whilst group D animals began to produce eggs slightly earlier, on day 84. Over the sampling period, group A showed a biphasic egg output curve: a sharp peaked rise in egg production between days 114 to 152 was super-imposed upon a lower egg output of approximately 60 eggs per gram. From day 105 onwards, groups B, C and D began to show an appreciable increase in the faecal egg count. The rise was slow and smooth in all three groups, with group D showing the greatest egg output and group B the least. Egg production peaked first in group D at day 138 and then started to drop, whilst that in the two treatment groups, B and C, peaked during the final week of sampling, at day 152. Egg production by the chronically infected group, A, decreased from day 121, so that by the end of the sampling period, all groups showed similar levels of egg production.

The difference in egg output between groups C and D was not significant at any of the time-points examined. However, the clear difference in egg output observed between groups B and D was significant at $P < 0.05$ on days 114-128 (Table 3.3).

Time averaged faecal egg counts

Egg output can be intermittent, so the arithmetic mean faecal egg count for each animal over a period of days was calculated at two points: during the early patent period for groups B, C and D (days 121, 125 and 128) and towards the end of the sampling period (days 138, 152, 154 and 156). Group means (geometric) were then calculated for these two periods. Results are shown in Figure 3.3. The difference between groups B and D for the period 121-128 was significant with $P = 0.02$ and between group A and groups B, C and D for the same period with $P < 0.002$. No significant differences were noted between any of the groups for the latter period examined (days 138 to 156).

Figure 3.2: Longitudinal profile of the geometric mean faecal egg count for groups A, B, C and D from day 105 to day 156. Group mean geometric faecal egg counts (FEC) are shown as eggs per gram (epg). A star marks the points where the difference between groups B and D is significant at $P < 0.05$.

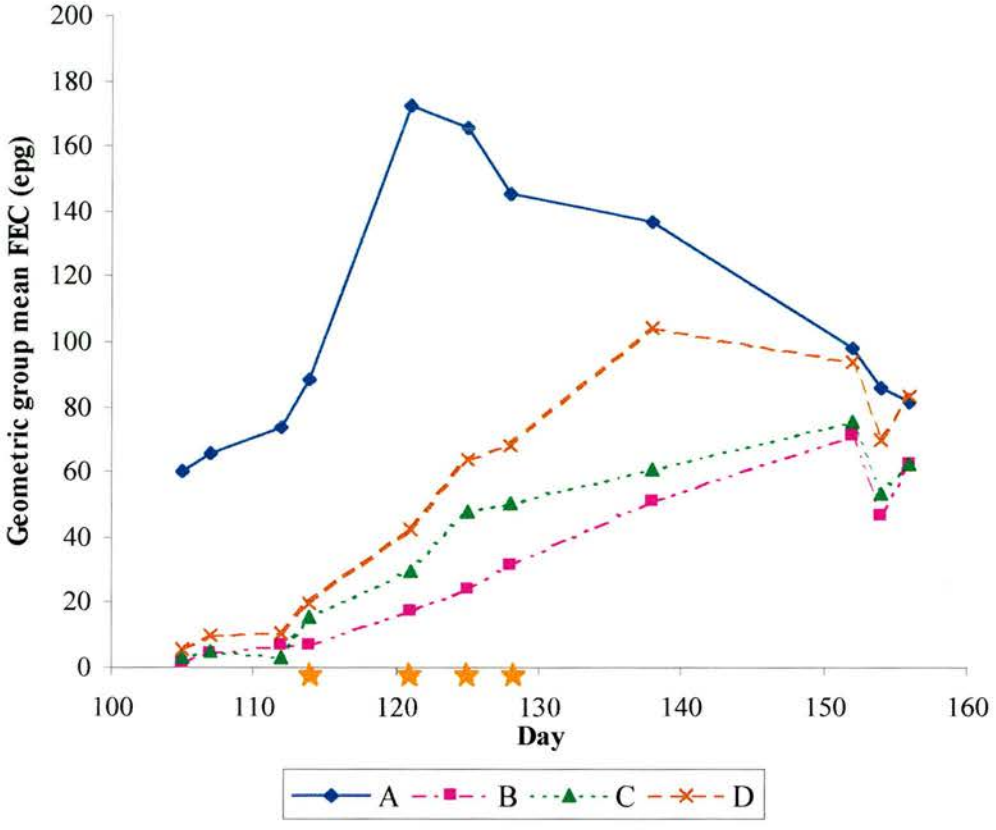


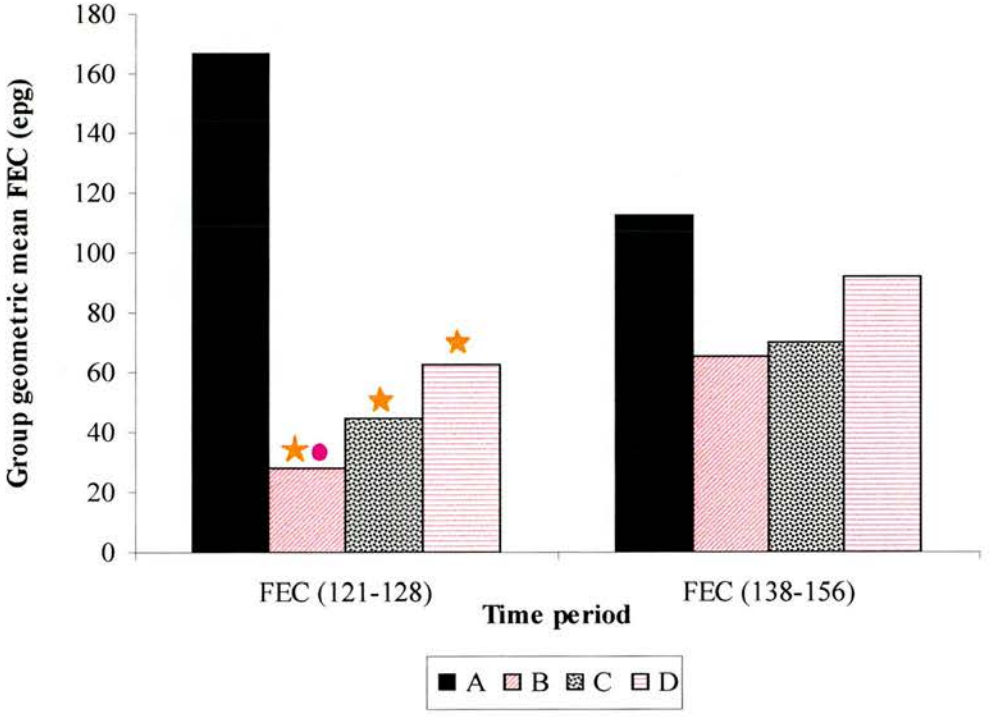
Table 3.3: Results of Mann-Whitney U tests to examine group differences in faecal egg counts, comparing groups B and D (showing *P* values).

Time-point	*P*-value
Day 114	0.049*
Day 121	0.049*
Day 125	0.031*
Day 128	0.038*
Day 138	0.058
Day 152	0.733

Key:

* indicates a result that is statistically significant.

Figure 3.3: Histogram illustrating group geometric mean faecal egg counts for time-averaged counts during the early patent (days 121-128) and late patent (days 138-156) periods. The star indicates that the difference between the group marked and group A is significant at $P < 0.002$ and the circle that the difference between group B and D is significant at $P = 0.02$.



3.3.2 Longitudinal serum profiles of the liver enzymes gamma-glutamyl transferase (γ-GT) and glutamate dehydrogenase (GLDH)

Figure 3.4 shows the profile of serum levels of the enzyme γ-GT throughout the course of the experiment. A sudden and rapid rise in the circulating levels of this enzyme in group A animals was detected from day 49, which peaked at a level of around 500 IU/l at day 77 and remained relatively constant until day 125, when it began to fall. A minor secondary peak was observed during the plateau period, between days 98 and 125, which corresponds to the peak in the challenge control group, D. Levels of γ-GT in the groups B, C and D remained low until day 77, 28 days after the initial rise detected in group A. γ-GT levels rose steeply in both groups C and D from this point onwards, until day 98 in group C and day 119 in group D. Group D reached a similar maximum level to that of group A, whereas levels in group C peaked at around 350 IU/l, before beginning to fall. In contrast, the increase observed in group B occurred about a week later, from day 84 and the rise was slower, reaching a smaller, broader plateau at approximately 200 IU/l. Although there was a striking difference between the enzyme profiles of the four groups, due to within group variation, the differences between groups B and D were only significant with $P < 0.05$ on days 84, 105 and 112 (Table 3.6). No statistically significant differences were noted between the enzyme levels of groups C and D.

The profile of the enzyme GLDH was quite different (Figure 3.5). Resting levels of the enzyme prior to infection fluctuated from week to week with many of the animals. A true rise was detected in group A from day 28 onwards, initially of shallow profile, but rising steeply from day 42 up to a maximum at day 56. A gradual decline in GLDH levels until the end of the sampling period was then observed, although a second minor rise occurred at day 112. Of the other three groups, D started to show an increase in GLDH levels from day 42, with that of B and C delayed by another 4 weeks, until day 70. Enzyme levels in group D rose rapidly up to a peak at day 98, crossing the group A profile, which had started to decline. The changes in GLDH levels in both groups B and C were very similar, although the level in group B was always slightly lower than that of group C. The group C GLDH profile also peaked sharply at day 91, reaching a similar value to that of group D. By day 125, enzyme levels in groups B, C and D had declined significantly, levelling out

to between 100 to 150 IU/l by the end of the sampling period. Although the difference between groups B and D was less marked with this enzyme than with γ-GT, it was significant through a greater part of the time-course (Table 3.6). This was due to a greater consistency in enzyme levels between animals in the same group.

Comparison of peak enzyme levels

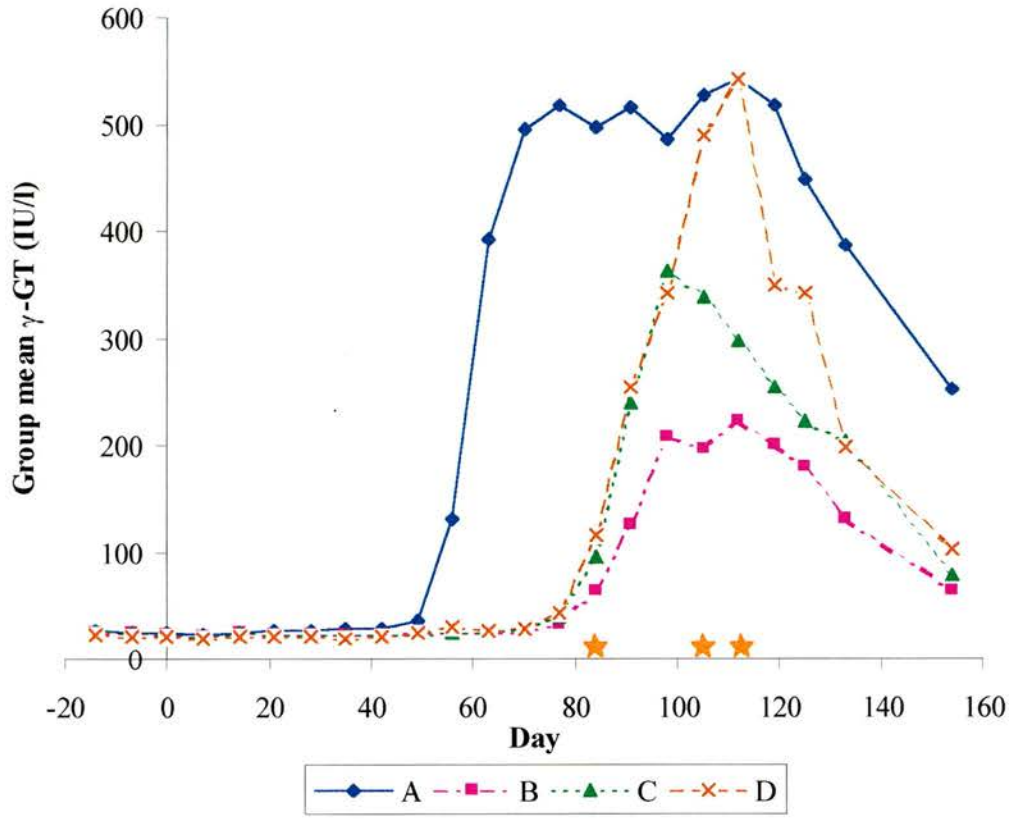
The longitudinal profiles of the liver enzyme response provide a useful indication of the average progression of infection in the four groups. However, not all animals reached peak values for the two enzymes at the same time. Peak values for individual animals were therefore identified and group mean values calculated. Table 3.4 shows the mean peak values for both γ-GT and GLDH enzymes for the four groups. These results support the findings of the longitudinal group analysis, with the difference between peak values in groups B and D remaining significant at $P < 0.05$ for both enzymes studied.

Table 3.4: Group mean peak values for the liver enzymes γ-GT and GLDH, in international units per litre (IU/l).

Parameter	Group			
	A	B	C	D
γ-GT	769	259[a, b]	390[c]	570
GLDH	411	327[a, b]	355[c]	393

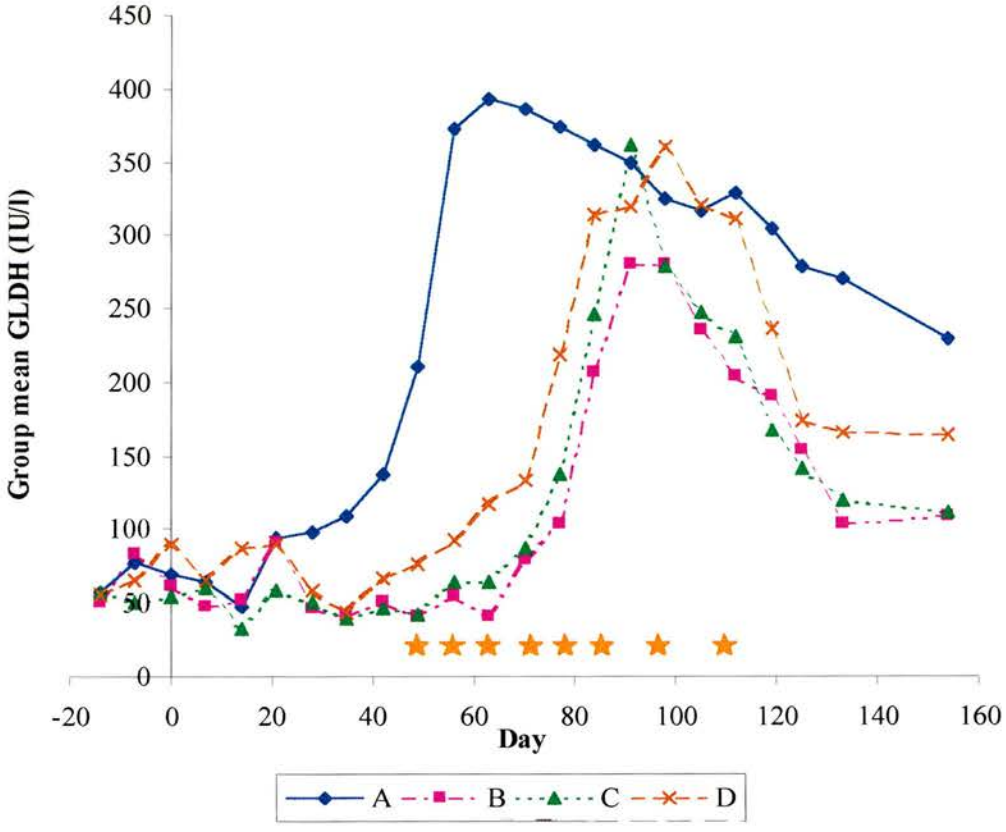
Key:
[a] : the difference between groups B and D is significant with $P < 0.05$
[b] : the difference between groups B and A is significant with $P < 0.01$
[c] : the difference between groups C and A is significant with $P < 0.05$

Figure 3.4: Longitudinal profile of group mean serum levels of the enzyme gamma-glutamyl transferase (γ-GT) over the entire period of study, measured in international units per litre (IU/l). Error bars are not shown to aid clarity, however the points where the difference between groups B and D was significant at $P < 0.05$ are marked with a star.



Note: a copy of this figure, including error bars, is given in Appendix B.1.

Figure 3.5: Longitudinal profile of group mean serum levels of the enzyme glutamate dehydrogenase (GLDH) over the entire period of study, measured in international units per litre (IU/l). Error bars are not shown to aid clarity, however the points where the difference between groups B and D was significant at $P < 0.05$ are marked by a star.



Note: a copy of this figure, including error bars, is given in Appendix B.2.

3.3.3 Peripheral blood eosinophil levels

Animals responded very rapidly to parasite infection by an increase in the proportion of eosinophils in the peripheral circulation (Figure 3.6). Rises were detected in all three groups 7 days after the primary challenge. The increase in both groups B and C was small and transient, lasting only from days 7 to 28. The chronically infected group, A, showed a sustained rise in eosinophil levels from days 7 to 35, reaching a peak of 1.5 x 10^9 cell/l. Eosinophil numbers then fluctuated between 1.5-2.5 x 10^9 cell/l throughout the rest of the sampling period, with a pronounced peak between days 84 to 119 and no apparent tendency towards a decline by the end of the sampling period.

Groups B and C responded within 7 days of the secondary challenge infection (at day 35) with a substantial increase in peripheral eosinophil levels. Animals in group D reacted more slowly to infection, with eosinophil levels rising from day 42, but then increasing in close association with the other two groups. All three groups showed similar peak levels of around 2.0 x 10^9 cell/l at day 49 and then responses in the three groups diverged. Group D maintained a high eosinophilia, not dropping below a level of 1.5 x 10^9 cell/l for the rest of the sampling period and with a peak similar to that observed in group A between days 84 to 119. In contrast, eosinophil counts in groups B and C decreased. Levels in group B dropped rapidly to less than 0.75 x 10^9 cell/l by day 63 and then oscillated around 1.0 x 10^9 cell/l for the remainder of the experiment. The group C response was greater than that of B, but again had dropped substantially by day 125. The difference noted between groups B and D was significant with $P < 0.05$ on days 56, 63 and days 84 through to 112 (Table 3.6), and between groups C and D at days 56 and days 98 through to 125.

Individual animal eosinophil counts were averaged over the periods day 63-125 and day 91-125 and the group means calculated (Table 3.5). For both periods, counts in groups B and C were lower than those of group D and this difference was significant for group B at $P < 0.01$ and for C at $P < 0.05$.

Table 3.5: Time averaged peripheral eosinophil counts, shown as the group mean for the periods days 91-125 and 63-125.

Period	Group mean eosinophil count , x 10^9 cells/l			
	A	B	C	D
Days 91-125	2.12	0.97[a,c]	1.26[b]	2.21
Days 63-125	1.95	0.97[a,c]	1.28[b]	2.02

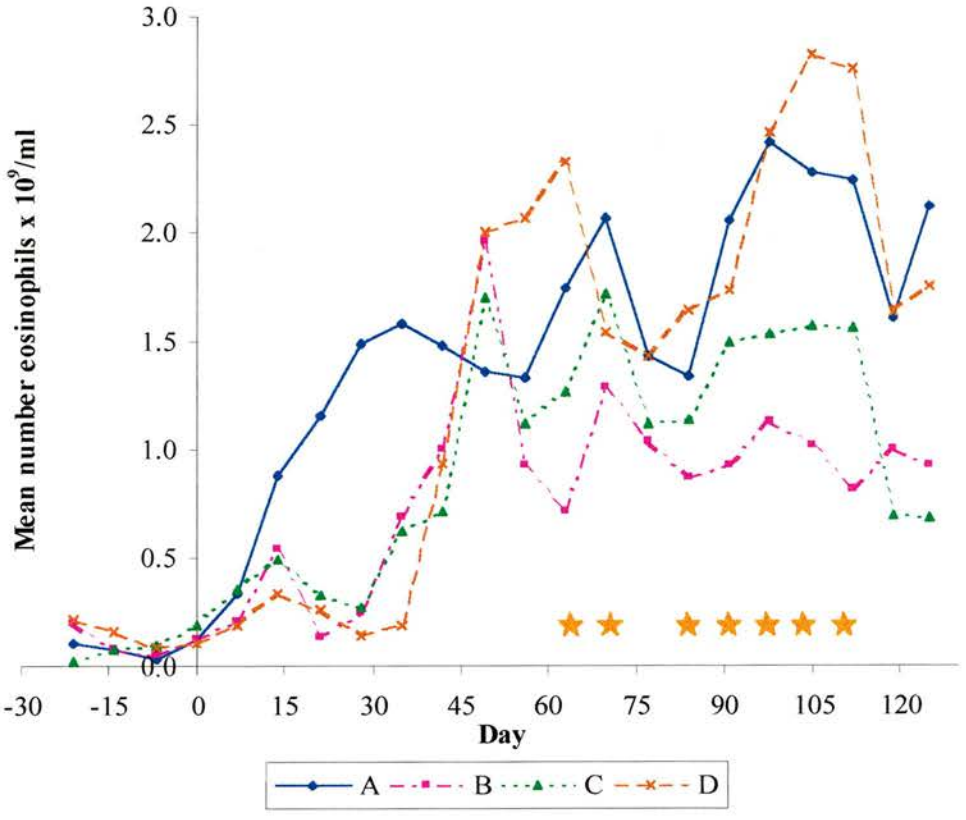
Key:
[a] : the difference between groups B and D is significant with $P < 0.01$
[b] : the difference between groups C and D is significant with $P < 0.05$
[c] : the difference between groups B and A is significant with $P < 0.05$

Table 3.6: Results of Mann-Whitney U tests comparing the difference in liver enzyme and eosinophil levels between groups B and D throughout the later time-course of the experiment (showing *P* values).

Time-point	Calculated *P* values for the given parameters; B versus D		
	γ-GT	GLDH	Eosinophils
Day 49	N/D	0.004**	0.850
Day 56	N/D	0.045*	0.011*
Day 63	N/D	0.0001***	0.006**
Day 70	N/D	0.019*	0.391
Day 77	N/D	0.006**	0.076
Day 84	0.026*	0.014*	0.026*
Day 91	0.104	0.571	0.031*
Day 98	0.130	0.049*	0.001***
Day 105	0.021*	0.055	0.007**
Day 112	0.034*	0.037*	0.001***
Day 119	0.094	0.596	0.111

Key:
*: significant
**: highly significant
***: very highly significant
N/D: not done

Figure 3.6: Longitudinal profile of group mean peripheral eosinophil levels, from day –21 to day 125. Error bars are not shown to aid clarity, however the points where the difference between groups B and D was significant at $P < 0.05$ are marked by a star.



3.3.4 Peripheral blood lymphocyte and neutrophil levels

There was no change in the group mean levels of peripheral lymphocytes and neutrophils throughout the period of study.

3.3.5 Relationship between challenge dose and faecal egg output

Animals were dosed with a fixed number of metacercariae per kg liveweight. As previously mentioned, this was carried out because the weight range within groups was quite wide. In order to investigate the possibility that the differences in faecal egg counts, eosinophil and enzyme levels were related to the initial dose given, scatter plots and correlation analyses were performed.

Figures 3.7 shows scatter plots of the challenge dose of metacercariae against faecal egg counts for individual animals in groups B, C and D, using the time-averaged counts at days 121-128 and 138-156. The results of a Spearman's rank correlation test for association between egg output and the secondary challenge dose are shown in Table 3.7. No association was noted between administered dose and egg output.

Table 3.7: Results of Spearman's rank correlation analysis, comparing the challenge dose and faecal egg output at days 121-128 and 138-156, for groups B, C and D.

Time-point	Spearman's correlation coefficient, showing *P*-value Shown as: coefficient (*P*-value)		
	Group B	Group C	Group D
Days 121-128	-0.285 (0.43)	-0.500 (0.17)	0.261 (0.47)
Day 138-156	-0.103 (0.78)	-0.183 (0.64)	0.321 (0.37)

3.3.6 Relationship between challenge dose and γ-GT and GLDH levels

A similar analysis comparing the liver enzyme response and metacercarial challenge dose was performed using peak γ-GT and GLDH enzyme values. No significant association was detected between peak γ-GT values and metacercarial dose for any of the groups, but there was a significant negative association between peak GLDH levels and dose with group D (correlation coefficient –0.693, $P = 0.03$).

3.3.7 Relationship between GLDH, γ-GT and faecal egg counts

A significant correlation was found between peak γ-GT and GLDH values for groups A (coefficient 0.867, $P = 0.001$) and C (coefficient 0.833, $P = 0.005$), but not for groups B and D. When the data was analysed as a single group, significant correlation was also observed between the two enzyme peak values (coefficient 0.487, $P = 0.002$). Figure 3.8 shows a scatter plot of the data.

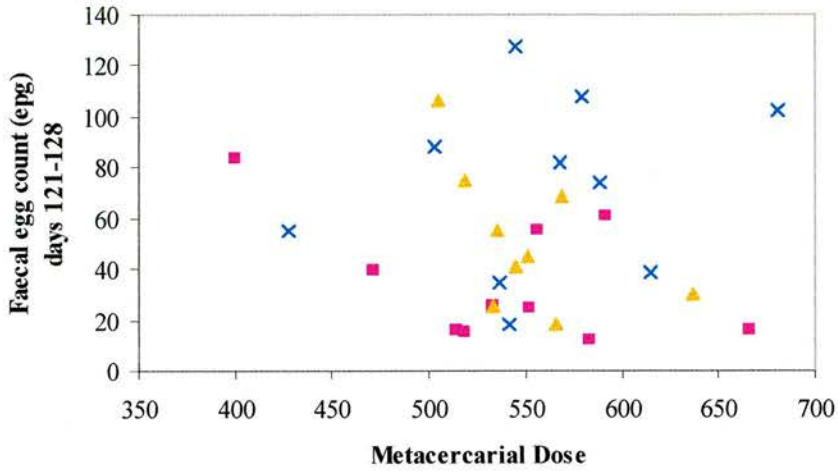
A moderate correlation was detected between both peak γ-GT and GLDH levels and early faecal egg counts (averaged over the period days 121-128) for groups B, C and D analysed collectively (coefficient 0.44-0.45, $P < 0.02$).

3.3.8 Relationship between eosinophilia, challenge dose and faecal egg counts

No significant correlation was detected between the secondary challenge dose and eosinophilia (averaged over the period days 63-125). A significant moderate correlation was found between eosinophilia and early egg output for groups B, C and D analysed as a whole (coefficient 0.582, $P = 0.001$), although when the groups were analysed independently, the correlation was not significant. A scatter plot of eosinophilia against early faecal egg count is shown in Figure 3.9. Analysing groups B, C and D collectively, a moderate association was also detected between eosinophil counts and peak enzyme levels for both γ-GT (coefficient 0.407, $P = 0.03$) and GLDH (coefficient 0.41, $P = 0.03$).

Figures 3.7: Scatter plots of metacercarial challenge dose versus time-averaged faecal egg counts (days 121-128 and 138-156) for individual animals in groups B, C and D. Animals in group B are represented by squares, those in group C by triangles and in D by crosses.

(a) Early patent period (days 121-128)



(b) Late patent period (days 138-156)

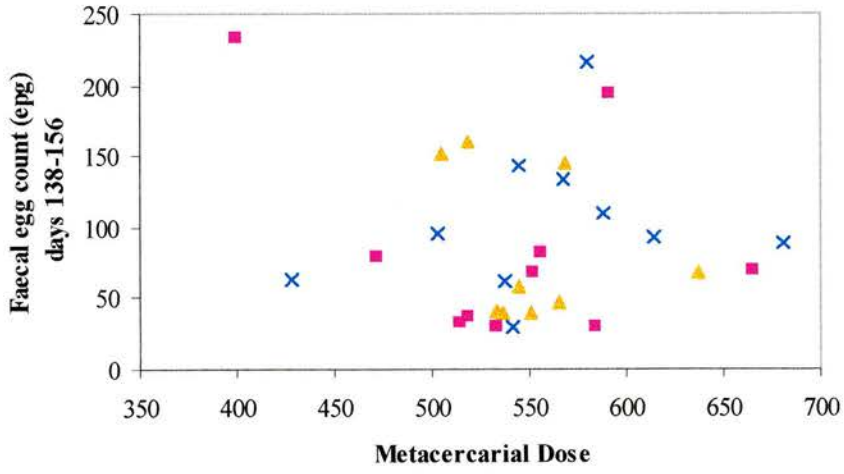


Figure 3.8: Scatter plot of individual animal peak GLDH levels against peak γ-GT levels, for all animals in groups A, B, C and D. Animals in group A are represented by diamonds, those in B by squares, those in group C by triangles and in D by crosses.

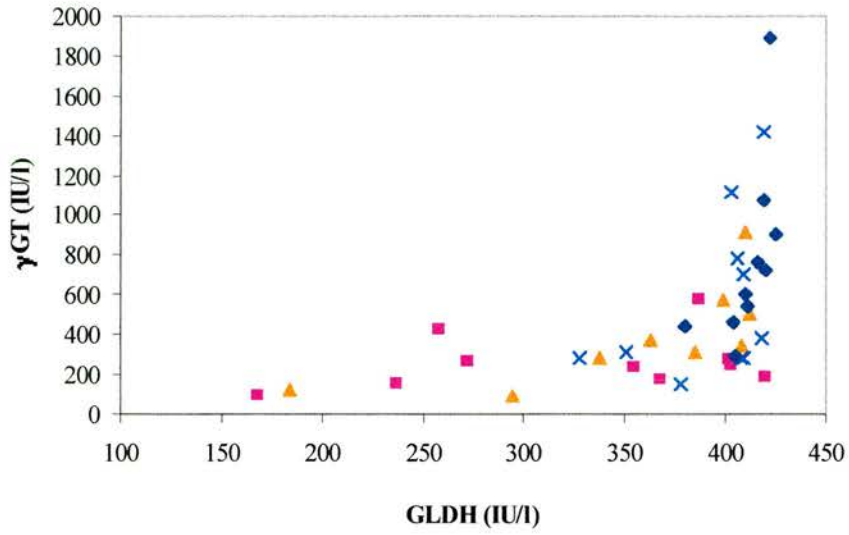
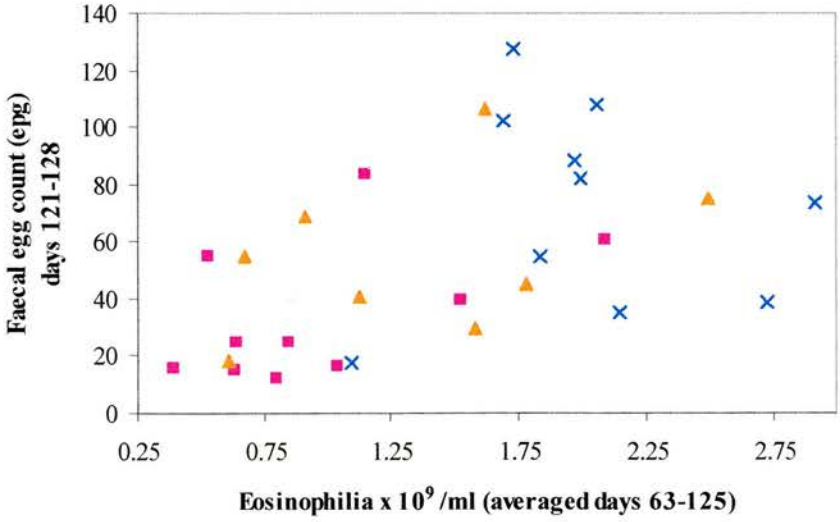


Figure 3.9: Scatter plot of individual animal eosinophil counts (averaged over days 63-125) against early faecal egg output (averaged over days 121-128), for all animals in groups B, C and D. Animals in group B are represented by squares, those in group C by triangles and in D by crosses.



3.3.9 Proliferative responses of peripheral blood mononuclear cells to stimulation with adult fluke protein preparations and Con A

3.3.9.1 Titration of whole adult fluke antigen and excretory-secretory preparations

Proliferative responses to WFA and ES protein preparations were titrated with respect to antigen concentration at days 7 and 14 post-primary infection. The day 14 proliferative responses, shown as group mean gross counts per minute, are displayed in Figures 3.10 and 3.11. The optimum protein concentration that stimulates maximum cell proliferation is likely to vary with each individual when using an outbred population. In this experiment, a choice of optimum protein concentration had to be made for the experiment as a whole. This choice was a compromise between the best overall level of stimulation achieved and availability of the protein preparations. Proliferative responses were vigorous and of similar magnitudes with protein concentrations in the range of 10-30 μg/ml for the WFA preparation and 10-20 μg/ml for the ES preparation. Optimum protein concentrations were therefore taken as 15 μg/ml and 10 μg/ml for WFA and ES preparations, respectively, for the remainder of the experiment.

3.3.9.2 Proliferative responses of unstimulated cells throughout the study

The proliferative activity of negative control, unstimulated cells (background response) altered over the study period (Figure 3.12). Some animals in each of groups A and B showed extremely high background responses shortly after infection at days 7 and 14 (Figure 3.13). There were exceptions in both groups: calves 65 (group A) and 76 (group B), which displayed responses similar to previous, pre-infection levels. However, after secondary challenge at day 35, background responses in groups A and B decreased substantially. Group C animals showed little change in control cell proliferative levels during the experiment and the background response of group D after challenge, at day 35, was also low.

The alteration in proliferative responses of control (unstimulated) cells after infection in some, but not all, animals meant that displaying results of the test (antigen stimulated) response as a ratio of the negative control response, i.e. the stimulation index (SI), was not appropriate. Animals with similar test responses but very different backgrounds responses, in counts per minute, showed widely differing

stimulation indices. For this reason, the net response, calculated as the gross count of test stimulated cells minus that of control (unstimulated) cells was used to display the results. Use of the SI alone would be misleading, suggesting some animals responded poorly to antigenic challenge, when in fact very strong responses were obtained.

3.3.9.3 Proliferative response to whole fluke antigen and excretory-secretory preparations after primary and secondary infections

Figures 3.14 and 3.15 show individual net proliferative responses to WFA and ES preparations at days 14/15, 26/27 and 35/36 for all animals. Considerable variation in cellular responses within the four groups was observed. This is of particular note with group C animals and the response to the WFA preparation: calf 54 showed a strong proliferative reaction at days 14/15 and 26/27, whereas that of calves 57 and 64 was low. In group D, calf 43 also showed a greater response at day 35/36 than the other three animals in the group. In general, responses to the ES preparation were poorer and more variable than those to the WFA preparation.

The group mean net proliferative responses are shown in Figures 3.16 and 3.17. Again, the degree of individual variation is illustrated by the magnitude of the error bars displayed. However, some general comments can be made regarding the overall group response to these proteins. Both groups A and B showed a strong response to stimulation with WFA and ES proteins during the early period after primary challenge at days 7/8 and 14/15. By day 26/27, the group A response was much less prominent, whilst that of group B remained strong. The group C response throughout this period was weaker, although the excessive response of calf 54 raised the group mean value as a whole. After secondary challenge, groups A, B and C showed only a weak proliferative response to stimulation with both WFA and ES preparations, whilst that of group D, previously negative, was moderate. The difference in response between group D and the other groups at day 35/36 was only significant for the group A response to WFA (P = 0.03). Again, group variation, particularly the excessive response of calf 43 in comparison to the other group members, raised the mean value of group D. It should be noted that proliferative responses of the other three individuals in the group were of a similar order of magnitude to that observed with calves 40 and 71 of group B and calf 54 of group C.

Figure 3.10: Proliferation of peripheral blood mononuclear cells at days 14/15 for groups A, B and C; titration of whole fluke antigen (WFA) preparation. Cells were cultured for a 5 day period either unstimulated (control) or with WFA (30–1 μg/ml). Error bars show the standard error of the mean (n=4).

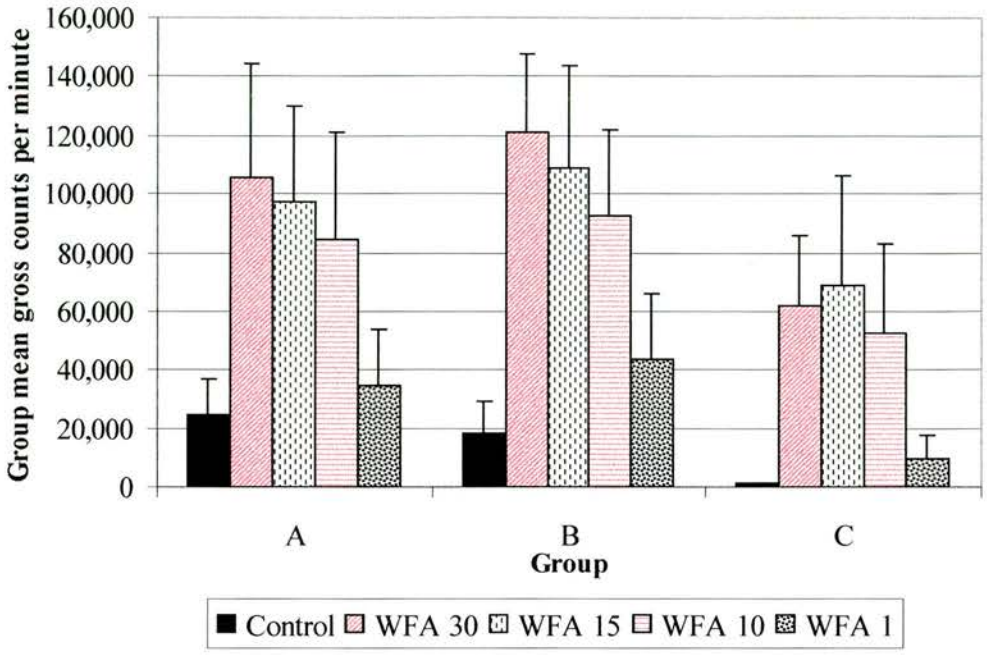


Figure 3.11: Proliferation of peripheral blood mononuclear cells at days 14/15 for groups A, B and C; titration of excretory-secretory (ES) preparation. Cells were cultured for a 5 day period either unstimulated (control) or with ES (20–1 μg/ml). Error bars show the standard error of the mean (n=4).

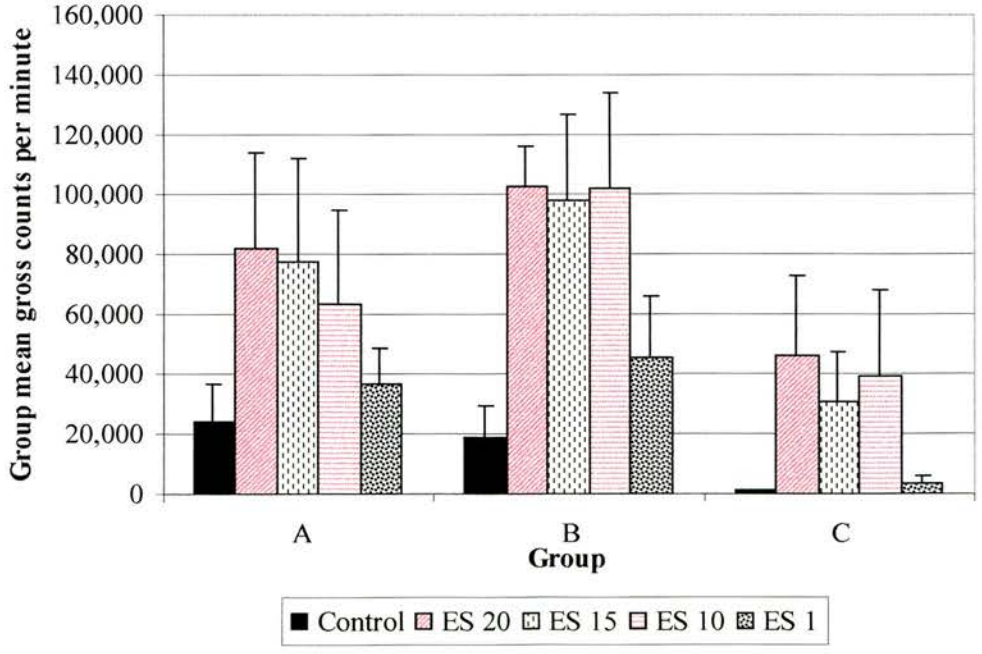


Figure 3.12: Proliferation of unstimulated (control) peripheral blood mononuclear cells after a 5 day culture period, shown as group means, for groups A, B, C and D at all time-points studied. Error bars show the standard error of the mean (n=4).

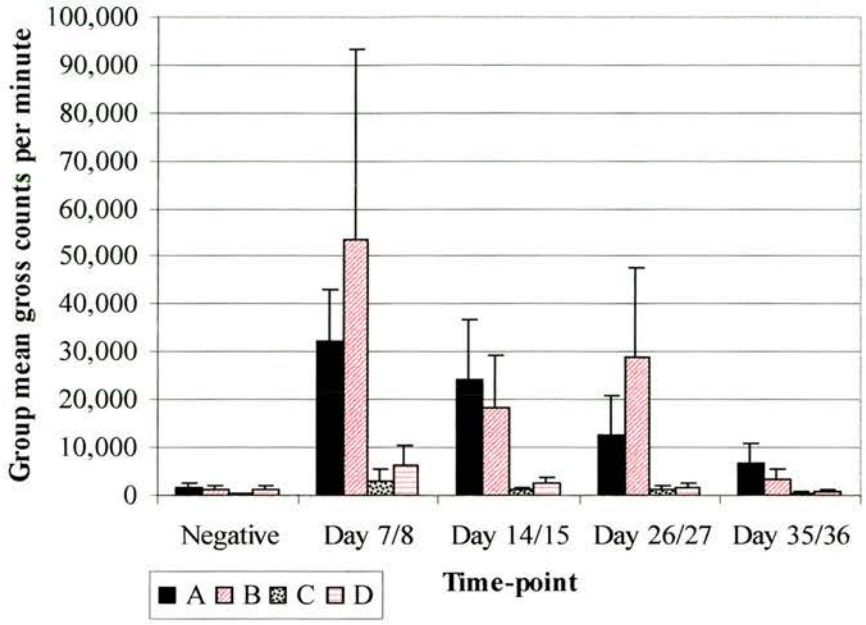


Figure 3.13: Proliferation of unstimulated (control) peripheral blood mononuclear cells for individual animals in groups A and B, for all time-points studied.

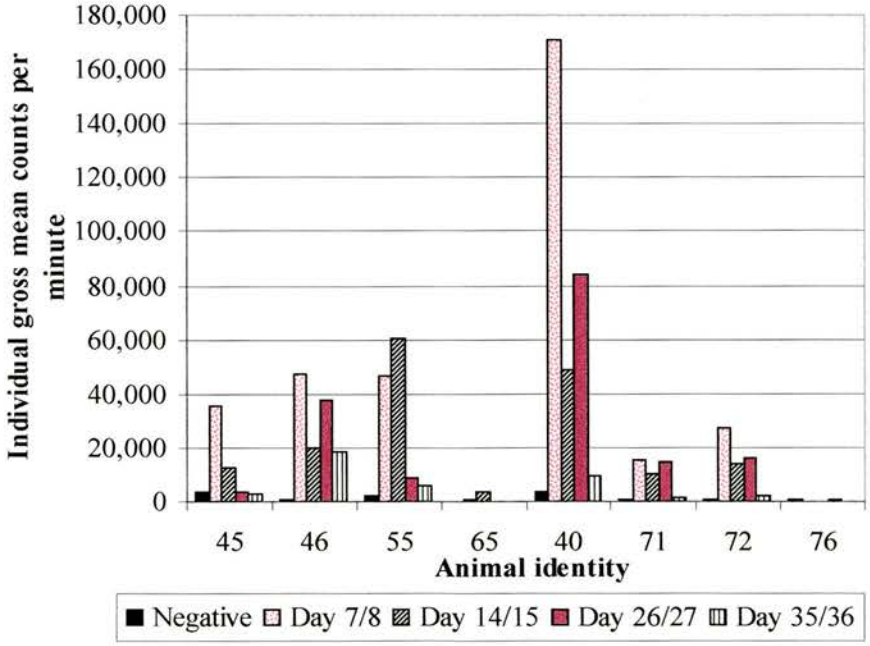
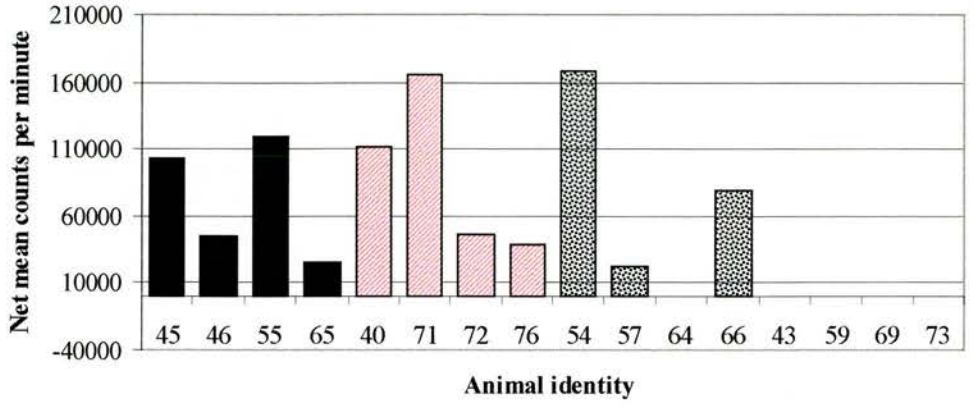
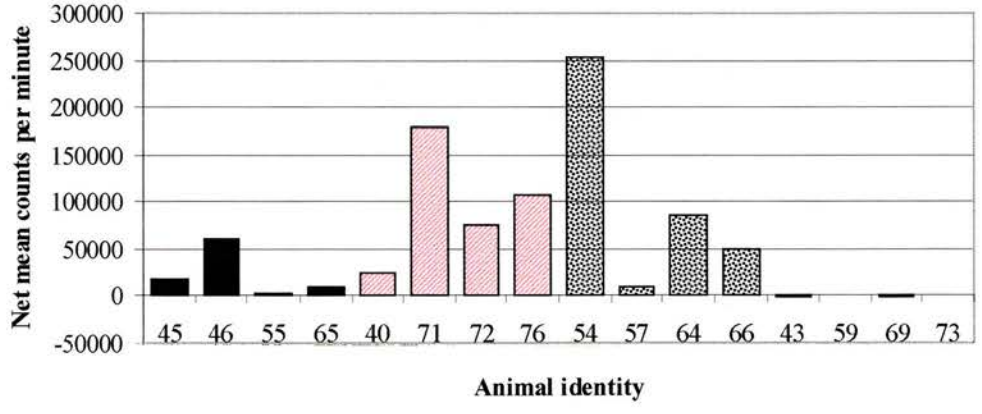


Figure 3.14: Individual peripheral blood mononuclear cell proliferative responses to whole fluke antigen (15 μg/ml) at days 14/15, 26/27 and 35/36 for groups A, B, C and D. Cells were cultured over a 5 day period and results are shown as the net proliferative response (the difference between antigen-stimulated and unstimulated i.e control cultures). Animals in group A are shown as solid bars, in group C as diagonally hashed bars, in group C as speckled bars and in group D as horizontally striped bars.

(a) Day 14/15



(b) Day 26/27



(c) Day 35/36

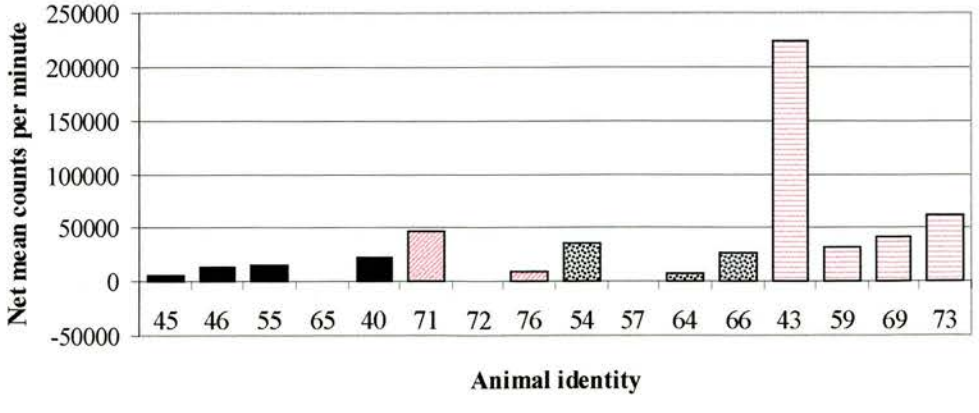
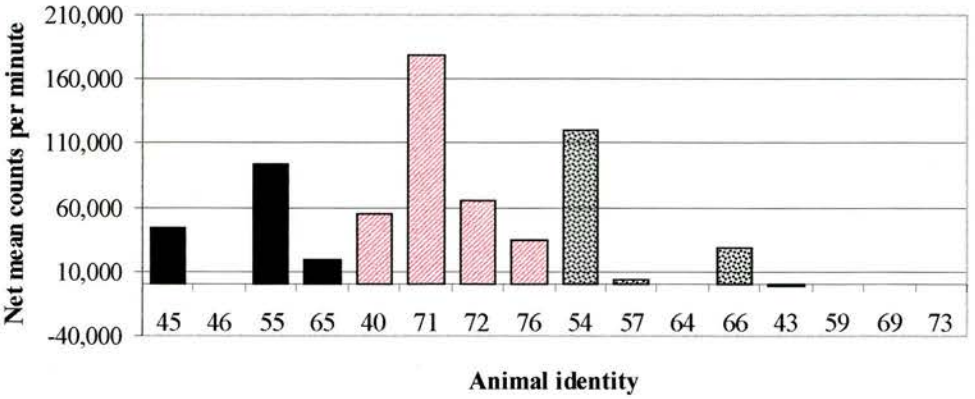
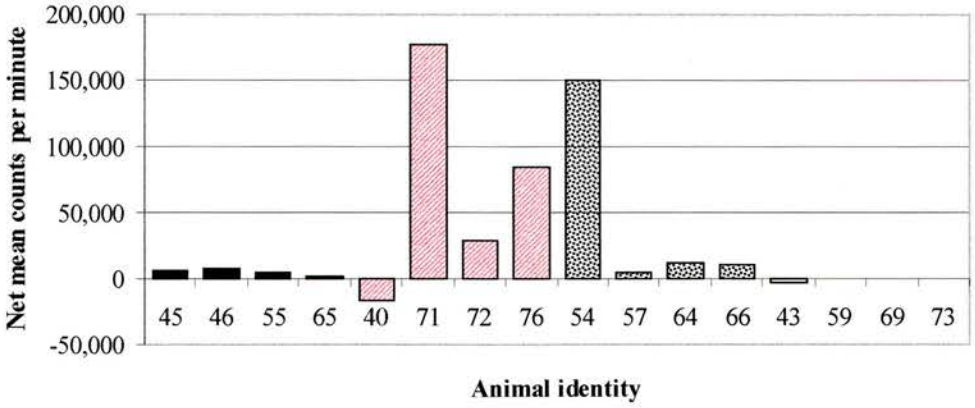


Figure 3.15: Individual peripheral blood mononuclear cell proliferative responses to excretory-secretory preparation (10 μg/ml) at days 14/15, 26/27 and 35/36 for groups A, B, C and D. Cells were cultured over a 5 day period and results are shown as the net proliferative response (difference between antigen-stimulated and unstimulated i.e control cultures). Animals in group A are shown as solid bars, in group C as diagonally hashed bars, in group C as speckled bars and in group D as horizontally striped bars.

(a) Day 14/15



(b) Day 26/27



(c) Day 35/36

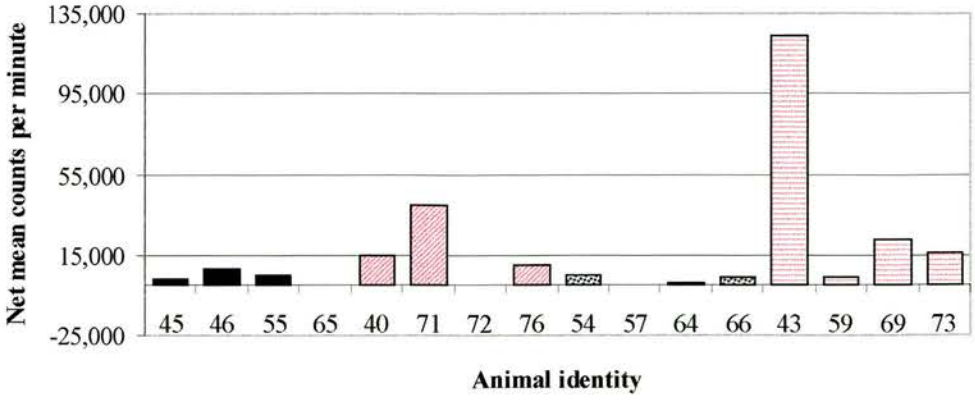


Figure 3.16: Group mean peripheral blood mononuclear cell net proliferative responses to whole fluke antigen (15 μg/ml) for groups A, B, C and D at all time-points studied. Cells were cultured for a 5 day period. Error bars show the standard error of the mean (n=4).

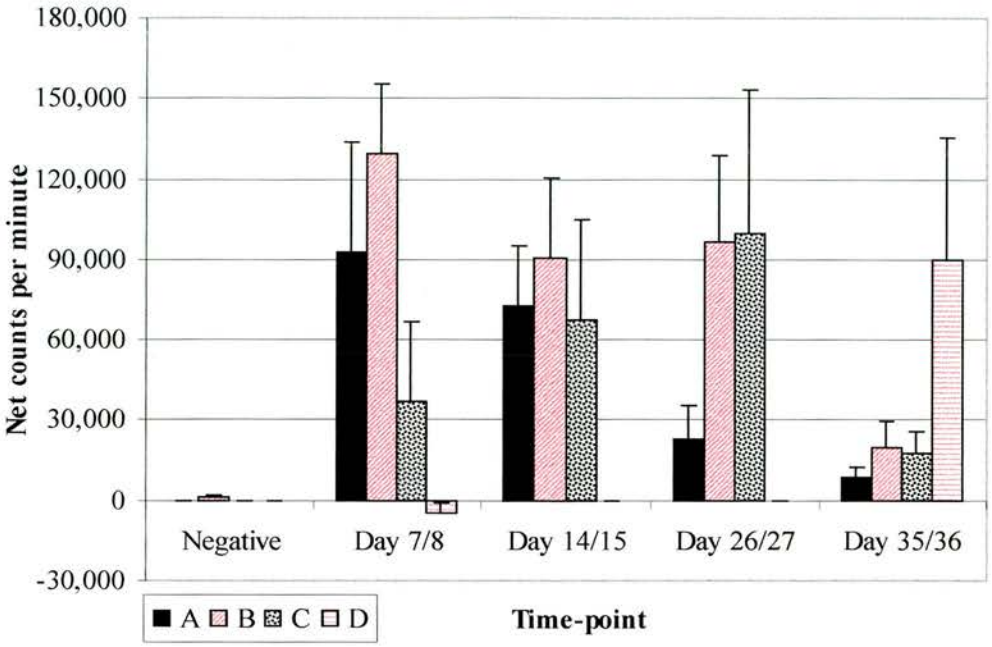
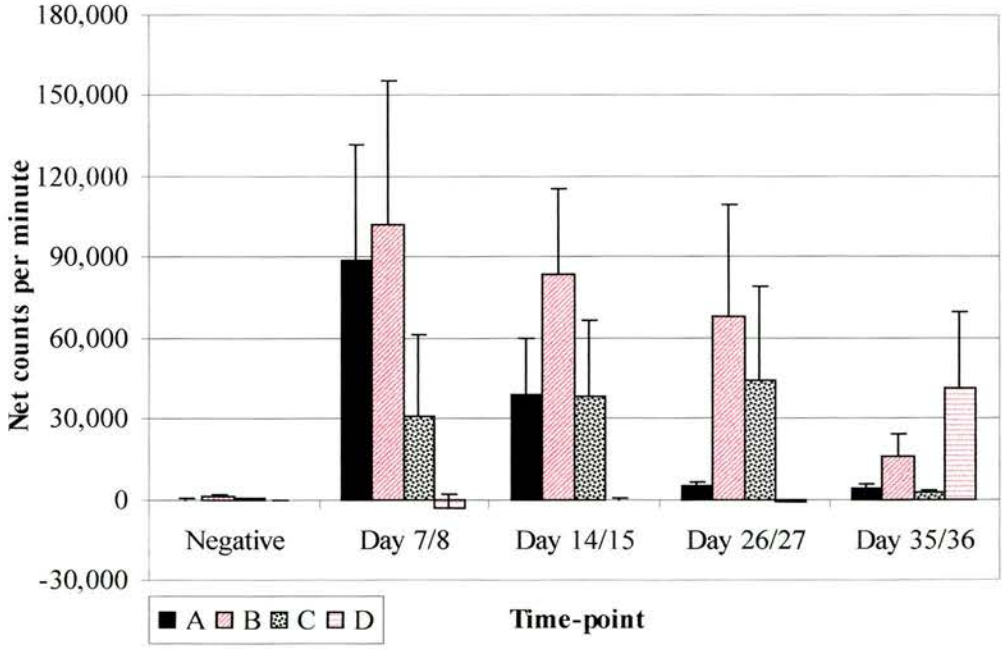


Figure 3.17: Group mean peripheral blood mononuclear cell net proliferative responses to excretory-secretory preparation (10 μg/ml) for groups A, B, C and D at all time-points studied. Cells were cultured for a 5 day period. Error bars show the standard error of the mean (n=4).



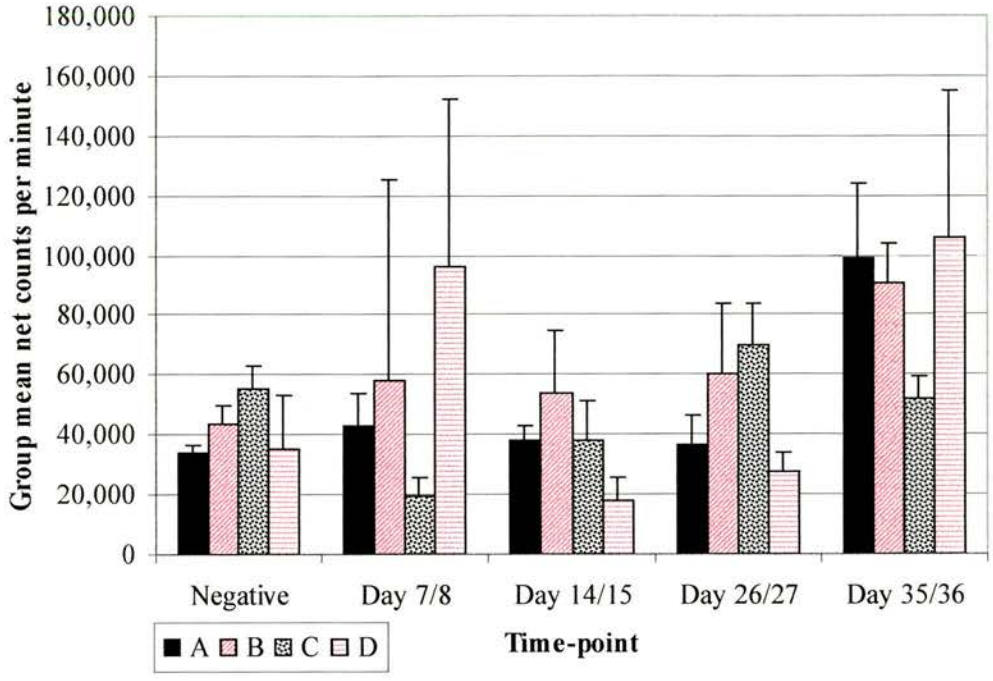
3.3.9.4 Proliferative responses to the mitogen Con A

The mitogen Con A was used as a positive control to check viability of cells. All animals responded well, with strong proliferative responses. Proliferation was relatively uniform within groups at all time-points studied, with the exception of groups B and D on day 7/8 and group D on day 35/36 (Figure 3.18). Inter-group variation was also low when compared to that observed with the WFA and ES preparations. There were no significant changes in the proliferative response to Con A over the study period with any of the groups (comparing pre- and post-infection responses for each group), except for group B at day 35 and group C at day 7 (P = 0.03).

3.3.9.5 Comparison of individual proliferative responses with liver enzyme, eosinophil and faecal egg count levels

Proliferative responses to the WFA and ES preparations varied widely for individual animals within the same group throughout the period of study (Figures 3.14 and 3.15). Individual peak enzyme levels and time-averaged eosinophil and faecal egg counts were examined, in order to determine if extremes in these parameters could account for the observed variation in the proliferative response. Calf 54, of group C, showed a high proliferative response to both ES and WFA preparations, but all infection parameters were of similar values to those of calf 57, which had an extremely low proliferative response. Similarly, calf 43, of group D, had a very high proliferative response to fluke proteins, although the peak GLDH value and eosinophil level were similar to those of calves 69 and 73; calf 43 faecal egg counts also fell in the midrange of the group. In group B, calf 76 had the lowest peak enzyme levels, but the proliferative response was neither the highest nor the lowest. Calf 71 (group B) was an exception to this, showing the greatest proliferation as well as the highest group B GLDH and final egg count levels. These results suggest that the wide range in proliferative responses within the groups cannot be linked to particular trends in the other infection parameters measured.

Figure 3.18: Group mean peripheral blood mononuclear cell net proliferative responses to Con A (1 μg/ml) for groups A, B, C and D at all time-points studied. Cells were cultured for a 5 day period. Error bars show the standard error of the mean (n=4).



3.4 DISCUSSION

3.4.1 Pre-exposure alters the progression of a challenge infection

A clear difference in the blood and faecal infection parameters was observed between the four groups. The chronically infected, group A, animals showed an early increase in circulating levels of the two enzymes, γ-GT and GLDH. *Fasciola* eggs were detected in faeces from day 70 onwards and persistent eosinophilia of between 1.75–2.5 x 10^9 cells/ml was observed throughout the study. In contrast, the two groups B and C, which received drug-abbreviated primary infections, reacted quite differently. Patency was not established in any of the animals from either group until day 98, 70 days after the secondary challenge infection. No change was detected in the liver enzyme profiles during the primary phase of the study. However, both groups B and C did show a small, but transient increase in the circulating eosinophil count during the first three weeks following primary exposure. These observations suggest that the abbreviated primary infections received by these groups had a minimal damaging effect on the liver, did not reach patency within the expected time period for a primary infection, but did stimulate an early circulating eosinophil response.

The presence of the naïve group, D, that received only the secondary infection at day 28, allowed direct comparison between the course of infection in previously unexposed animals and in those that had been exposed to restricted early stages of the parasite. Both the pre-exposed groups B and C had lower mean faecal egg counts from day 112 onwards, when compared to that of group D. However, towards the end of the sampling period egg output in all groups, including the chronic group A, in which egg counts had been very high, converged.

Differences in the degree of liver damage experienced by groups B, C and D were much more striking. The mean γ-GT level in group B was approximately half that of group D during the peak period (days 105-112), with the levels in group C falling midway between the two. Due to the degree of individual variation within groups, this difference between γ-GT levels of groups B and D was only significant at a few time-points. In contrast, GLDH levels in the three groups B, C and D were more similar, although those of groups B and C were still lower than that of group D.

Whilst this difference in group mean GLDH levels between groups B and D was less marked than that for the enzyme γ-GT, it was highly significant over much of the time-course, due to minimal intra-group variation.

The eosinophilic response to the secondary challenge infection in groups B, C and D was complex. All three groups showed an initial sharp rise in circulating eosinophil counts during the first two weeks following secondary infection. However, eosinophilia in group B then decreased rapidly and although elevated above normal levels, remained lower than that of group D. As with all the parameters examined, the group C eosinophilic response fell between that of groups B and D.

These results suggest that group B animals, which had received a 5-6 day drug-abbreviated pre-exposure to juvenile flukes, experienced less liver damage, less eosinophilia and showed a transiently lower faecal egg count than naïve animals upon challenge infection.

3.4.2 Problems associated with estimating fluke burdens

A major problem with both natural and experimental *Fasciola* infections is the measurement of actual fluke burden. Faecal egg counts provide a useful guide to the overall level of infection, but cannot be taken as a definitive measure of burden for a number of reasons. *Fasciola* eggs may be released into the gut from the bile ducts intermittently. Egg counts therefore vary from day to day for a particular animal. The bulk of faecal material within the gut and gut passage time will also affect egg density in the faeces. Although all calves were fed an identical diet, variation in faecal consistency did occur; any transient looseness or diarrhoea will have affected egg count values. The technique used to measure the faecal egg count is another factor affecting the reliability of egg count data. In this experiment, the zinc sulphate flotation technique was used. A sedimentation technique for measuring fluke eggs is more reliable, because all eggs present in the sample are collected. However, the sedimentation method is very time consuming and due to time constraints, was not used in this experiment.

The use of weight dependent metacercarial dosing introduces a further complication into the interpretation of the faecal egg count data, because animals did not receive an identical dose. However, no linear association was detected between

administered dose and egg output within groups. Representing egg counts as a linear function of dose was not, therefore, considered appropriate and gross egg counts were used in the analysis. This does not preclude a more complex relationship between dose and egg output, which perhaps merits further investigation. Furthermore, no association was detected between challenge dose and peak enzyme levels or eosinophilia, excluding the negative correlation between dose and peak GLDH levels in group D. This latter observation was unusual, but does support the view that higher metacercarial doses did not automatically result in higher levels of the various infection parameters.

The rise and subsequent fall in egg output in group A animals between days 112 to 125, eventually leading to a convergence in faecal egg counts between all four groups towards the end of the sampling period, was interesting. This suggested that either incoming secondary flukes began to produce eggs, boosting overall egg output, or that those already resident had a period of increased fecundity. However, this effect was short-lived; why did egg counts drop? The presence of density dependent effects of worm numbers on fecundity has long been postulated in helminth systems (Keymer, 1982, 1987). Evidence supporting the concept of density dependence has been shown for a variety of nematode infections, including *Ostertagia circumcincta* in sheep (Stear *et al.*, 1999), *Heterakis gallinarum* in pheasant (Tompkins *et al.*, 1999) and *Oesophagostomum dentatum* in pigs (Roepstorff *et al.*, 1996). The presence of density dependence in fluke infections has not been investigated, but it is reasonable to expect that within the limiting confines of the bile ducts, available nutrients and space may affect parasite fecundity.

Alternatively, the reduction in group A egg counts could have been due to the death of either the primary or incoming secondary parasites. This may have been a result of natural ageing of the fluke, or indirectly, as a result of excessive host reaction, such as fibrosis around the bile ducts, resulting in an unsuitable environment for fluke survival. Host immune modulation of parasite fecundity is also a possibility. It would have been useful to have continued egg output measurement for a further few weeks in this study, to determine whether or not egg counts stabilised or continued to drop in the chronic group A. Furthermore, any changes seen in egg output in the other groups as infections matured may have provided some indication as to whether the rise between days 112-125 observed in group A was due

to a period of increased fecundity during the primary infection, or as a result of the secondary challenge infection.

3.4.3 Changes in liver enzyme profiles

γ-GT is a membrane bound enzyme; changes in serum levels are associated with damage to the bile ducts, rather than to the liver parenchyma. GLDH, on the other hand, is a cytosolic enzyme found in hepatocytes, which is released passively when cells are damaged (Doxey, 1983). The longitudinal profiles of the two liver enzymes γ-GT and GLDH differed throughout the course of the experiment. GLDH levels rose early, from day 28 onwards in group A and day 49 in group D. This reflects the damage caused to the liver by migration of juvenile flukes during the early stages of infection. Elevation in γ-GT levels did not occur until much later, from day 56 in group A and day 84 in the other groups. This elevation occurred shortly after the expected time of arrival of maturing flukes into the bile ducts (Kendall and Parfitt, 1962; Ross *et al.*, 1966). The rise in γ-GT levels was relatively short-lived in groups B, C and D, but lasted twice as long in group A, probably as a result of the two waves of flukes from both primary and secondary infections reaching the bile ducts. GLDH levels also showed a prolonged elevation in group A, compared to that in the other groups, again indicating that the secondary flukes were actively causing parenchymal damage.

The drop in γ-GT enzyme levels towards the end of the experiment suggests that either the more mature flukes caused less bile duct damage, the bile duct epithelia became unresponsive to damage and enzyme production was reduced or, that flukes were being lost from the ducts. In contrast, GLDH levels were still relatively high at the end of sampling. It is possible that the damage inflicted upon the liver parenchyma by migrating juvenile flukes and the mechanisms involved in healing, such as fibrosis, took longer to resolve than the changes in the bile duct epithelium.

The magnitude of the difference in enzyme levels between groups B and D was much smaller for GLDH than for γ-GT. This difference could be an indication that whilst the damage in the bile ducts in group B, and therefore perhaps the number of flukes, was substantially less than in group D, considerable damage in the

parenchyma still occurred. Host factors, possibly immune-mediated, may have restricted progression of flukes from the parenchyma into the bile ducts. However, the fact that enzyme levels may not be in linear association to the parasite burden must also be considered.

In the present study no correlation was found between peak enzyme levels and faecal egg counts for any of the calves. Fluke burden did not, therefore, appear to be related to the degree of liver damage seen. A moderate positive correlation was noted between peak GLDH and γ-GT levels when the data was examined as a single group, but not with groups B and D when examined individually. Other studies of *F. hepatica* infection in cattle have also demonstrated the lack of correlation between γ-GT levels and fluke burden (Bulgin *et al.*, 1984), although a positive correlation between the number of juvenile flukes recovered from the parenchyma and GLDH levels has been established (Leclipteux *et al.*, 1998).

3.4.4 Eosinophils and *F. hepatica* infections

The early and sudden rise in the number of circulating eosinophils that occurred in group A after primary infection and in groups B, C and D after secondary challenge is typical for *F.hepatica* infections, having been noted previously in rats and sheep (Poitou *et al.*, 1993, Chauvin *et al.*, 1995). The pre-exposed groups B and C also showed a mild, transient eosinophilia shortly after primary infection, in response to exposure to very early fluke stages prior to drug abbreviation. Eosinophil numbers therefore rise rapidly after fluke invasion and this rise may be stimulated by fluke migration across the gut, peritoneal cavity or liver capsule. The high peak level of eosinophilia observed in groups B, C and D after secondary challenge was not maintained in group B, in which a rapid drop to approximately half the peak value occurred. This lower level was maintained until the end of the experiment.

Group B, which experienced the least amount of liver damage and showed transiently lower egg counts, also had a lower final eosinophil count. This could be reflective of a lower overall worm burden. The moderate positive correlation between eosinophilia and early faecal egg counts support this theory. A weak correlation was also detected between eosinophilia and peak enzyme levels.

The reason for the presence of a high peak eosinophilia in group B similar to,

but no greater than that in the other groups shortly after secondary challenge is not clear. The fluke burden during the first days after challenge in groups B, C and D may be no different, thus initial eosinophil counts are similar. The absence of a greater eosinophilia could be taken as an indication of the insignificance of this cell type in protection to infection. However, tissue specific eosinophil counts may be more significant than peripheral eosinophil levels in parasite infections, and these were not evaluated in this study. The functional activation state of the eosinophil may also be more relevant than total cell numbers, in situations where eosinophils are thought to be protective. The importance of the activation state of eosinophils in parasite killing has been shown *in vitro* with schistosomula and human eosinophils (Veith and Butterworth, 1983) and with *Haemonchus contortus* in sheep (Rainbird *et al.,* 1998). The absence of a distinction in peripheral eosinophil levels between groups B, C and D during the early secondary phase of the experiment, therefore, may not accurately reflect possible differences in eosinophil activity between the groups.

3.4.5 The use of chemotherapy to terminate infection

Two disadvantages in the use of drug treatment to terminate infections are firstly confirming that complete clearance of the parasite has occurred and secondly, the possibility that drug treatment *per se* may alter the immune response and thus response to re-infection.

As mentioned in section 3.1, triclabendazole is the most effective fasciolicide available for killing early stages of *F. hepatica*. Confirmation that all primary infections in animals of groups B and C were terminated could only be obtained by liver dissection, which was not carried out. Examination of individual enzyme profiles of the animals in groups B and C gave no indication of an early rise in γ-GT or GLDH levels, although the escape of a small number of flukes may not have resulted in a detectable change in enzyme levels. Sub-optimal doses of triclabendazole in sheep are also able to lengthen the prepatent period (Büscher *et al.*, 1987), so the absence of eggs prior to the time when they first appeared in group D is not conclusive proof that primary infection flukes had not established. However, the evidence given here does support the view that the majority of primary infection

flukes in groups B and C were killed by drug treatment.

In schistosomiasis, praziquantel treatment of chronic infections in humans results in an enhanced humoral and cellular response to specific parasite antigens (Roberts *et al.*, 1993; Grogan *et al.*, 1996a; 1996b; Satti *et al.*, 1996; Mutapi *et al.*, 1998). Drug treatment appears to alter the immune response in a manner not seen in natural infections. Praziquantel acts by both damaging the tegument and paralysing the worm (Harnett, 1988). The former is likely to increase the release of antigens, possibly exposing the host to antigens that may have been previously hidden. The mode of action of triclabendazole on the fluke is not well understood, although the main activity appears to be inhibition of mobility and secretory functions, probably by interference with the microtubule system (Bennett and Köhler, 1987). Integrity of the fluke tegument depends upon continual renewal of surface components, because the glycocalyx is continually shed. Renewal is carried out by the secretory bodies, which carry components from the T0, T1 or T2 cells in the syncitium to the fluke surface. Triclabendazole alters secretory body function, possibly as a result of interference with the microtubule system, leading to loss of the tegument within 24 hours *in vitro* (Stitt and Fairweather, 1993). Tegumental loss, resulting in parasite disintegration, may expose the host to antigens quantitatively or qualitatively different to those seen during live infections. This may have been a factor altering the natural exposure and therefore immune responses to, early infective stages in this experiment.

3.4.6 Peripheral cellular responses during infection

At day 7 post-infection, all three infected groups, A, B and C showed extremely strong proliferative responses to both the WFA and ES preparations. This indicates that early infection rapidly primed lymphocytes, even when the infection had been terminated by drug treatment. Furthermore, even though all three groups had only experienced invasion by juvenile flukes of up to a week old, cells were able to respond to adult derived proteins. This suggests the presence of common antigens between adult and the very early juvenile fluke stages which are recognised by lymphocytes. As the primary infection progressed, the group A response decreased substantially, whilst that of groups B and C remained relatively high (day 26). A reduction in antigen specific cellular proliferation has been previously documented

with chronic *F. hepatica* infections in cattle (Oldham and Williams, 1985; McCole *et al.*, 1999a); the response of group A was consistent with such findings. Groups B and C may have been able to maintain a good proliferative reaction at this stage due to the absence of maturing flukes. In addition, terminating infection by drug treatment may have enhanced responsiveness by making more antigen available for lymphocyte priming.

However, groups B and C did show a reduction in cellular responsiveness to fluke proteins after the secondary challenge infection. Whilst both groups showed similar levels of response to the WFA preparation, group B had a slightly greater response to the ES preparation. The ability of the secondary infection to stimulate a proliferative response was confirmed by the positive reaction obtained with group D animals. One group D animal, calf 43, showed a much greater response than other animals in the group. If the response of this animal is excluded, it can be seen that both group B and D animals showed similar responsiveness to ES proteins after secondary challenge, at day 35. The secondary challenge infection therefore reduced group A and C proliferative responses to fluke antigen considerably and those of group B to a lesser extent.

3.4.7 Parasites and modulation of host cellular responses

There are several possible reasons for the reduction in peripheral cell reactivity observed during the course of the experiment. As the fluke migrates through the different organs and tissues of the host, it matures through stages, until it reaches the bile ducts. Mature flukes within the duct may be less "visible" to the host immune system, and so provide less antigenic stimulation. Zimmerman *et al.* (1983) suggested that differing stage-specific fluke products might alter cell responses and the presence of a specific "suppressant" factor released by flukes has also been postulated (Oldham and Williams, 1985). However, if the migratory phase in the parenchyma is the main stage stimulating cell proliferation, high responses after secondary challenge should have been observed in group A, when the secondary wave of flukes entered the liver. Thus, the simple movement of primary flukes into the bile ducts, supposedly becoming "less visible", cannot account for the depression in the cellular response of group A. Flukes may release products which are able to alter immune cell function, particularly if present locally in adequate concentrations.

These may affect the interaction between lymphocytes and corresponding antigen presenting cells. Alternatively, the presence of mature flukes may have resulted in an immune response which did not promote a positive cellular response to juvenile migratory stages, perhaps by altering the cytokine environment or activation states of host cells.

ES products have varying effects on host immune cells *in vitro* (section 1.2.6.6). Whether ES products are able to alter immune cell function *in vivo*, and whether this action can persist in cells removed from an infected host and subsequently cultured is not clear. In this experiment the alteration in proliferative activity was noted over time, particularly after challenge infections. It follows that an independent action of the ES or WFA preparations on cell function within the *in vitro* culture system, *per se*, was not responsible. Any change effected by expression of modulating factors by the flukes must therefore have occurred within the host and been maintained after cells were removed for culture. A possible mechanism whereby parasite products could exert such an effect is the down regulation of signalling pathways within the cell.

Parasite-induced modulation of immune cell function has been observed with other helminth parasites. *Echinococcus multilocularis* protoscoleces depress mitogen stimulated proliferation of normal mouse spleen cells in an IL2 dependent fashion (Kizaki *et al.*, 1993). Glycophospholipids isolated from the metacestode may be wholly or partly responsible for this action. Co-culture of healthy peripheral blood mononuclear cells with mitogens and glycophospholipids was found to result in a dose-dependent reduction in cellular proliferation (Persat *et al.*, 1996). This was due to a decrease in IL2 production by the cells, although IL2 receptor expression remained normal. Another cestode, *Taenia multiceps*, produces two factors which have opposing effects *in vitro* on mouse macrophage and T cells (Rakha *et al.*, 1991). One factor enhances stimulation of T cells by macrophages, whilst pre-treatment of macrophages with the second factor results in a reduction in T cell responsiveness after macrophage-T cell interaction. In mice, infection with the filarial parasite, *Brugia malayi*, results in the generation of an IL4-dependent macrophage or eoinophil population present within the peritoneal cavity, which is able to depress lymphocyte proliferative responses to specific antigen (MacDonald *et al.*, 1999). B cells can also be affected by parasite products: the rodent filarial nematode

Acanthocheilonena viteae is thought to depress B cell responses directly by interfering with the protein kinase signalling pathway within the cell (Deehan *et al.*, 1998).

Changes in cell viability did not appear to be responsible for the reduction in proliferative responses detected during this experiment. Cellular proliferation in response to culture with the mitogen Con A remained unchanged throughout the study period. This is in contrast to other ruminant studies: in sheep a reduction in mitogen induced proliferation was noted as infection progressed (Zimmerman *et al.*, 1983), whilst a recent study in cattle observed a transient enhancement of cell responses to Con A during the early infection period (McCole *et al.*, 1999b). In this experiment, *F. hepatica* infection did not modify the host cell response to the mitogen Con A.

The reason for the variable change in background proliferative responses of unstimulated (control) cells in groups A and B after primary infection is not clear, although this phenomenon has been noted previously in *F.hepatica* infection (McCole *et al.*, 1999a). It is possible that early stage fluke infection resulted in non-specific cell activation within the host, and that these cells continued to proliferate once in culture. Nonspecific polyclonal activation of normal lymphocytes has been demonstrated with several helminth parasites, usually through the release of a parasite factor with mitogenic properties. *In vitro* studies examining the effect of antigen stimulation on cell proliferation have found that *Toxocara canis* is able to stimulate macrophages and B cells (Wang *et al.*, 1995), *Heligmosomoides polygyrus* stimulates lymphocytes (Robinson and Gustad, 1997) and *Ascaris* products can act as B cell mitogens (Lee and Xie, 1995). Furthermore, experimental infection of mice with *S. mansoni* can result in B cell activation much greater than would be expected by specific antigen alone (Lopes *et al.*, 1990). In this study a specific mitogenic action of the protein preparations themselves on cells did not appear to be responsible, because group D cellular responses remained at background levels prior to the secondary challenge infection.

An alternative explanation for the enhanced background response could be that activated cells responding to a concurrent, unrelated infection, were included in culture, for example, those responding to a respiratory viral infection. Animals were housed in adjacent pens sharing the same air-space, so all groups would have been

equally affected by a respiratory infection and therefore, it would be expected that cells from all groups would respond in culture in a similar manner. This was not the case, with all group C and D animals showing low background responses, except for calves 66 and 69 at day 7, which had moderately raised background levels.

Examining the nature of the cell phenotypes present in control cultures before and after growth and determining the cytokines released during culture with fluke products, could be useful in the further investigation of the changes observed during this experiment.

3.4.8 Conclusions

Cattle pre-exposed to 5-6 day old fluke infections experienced lower levels of liver damage, eosinophilia and early faecal egg counts after secondary challenge infection, when compared with naïve animals. These results suggest that the actual fluke burden in 5-6 day pre-exposed calves was lower than in naïve animals and that pre-exposure therefore resulted in a degree of resistance to secondary challenge infection.

The results of proliferation studies using peripheral blood mononuclear cells and crude fluke protein preparations indicated that 5-6 day pre-exposed calves were as responsive as chronically infected animals to stimulation with fluke proteins during the primary phase of the experiment. The adult fluke must therefore share antigens which are common to early fluke stages of less than 7 days old. Animals in the 5-6 day pre-exposure group were also able to maintain cellular proliferative response to fluke proteins that was greater than that of chronically infected animals during the 4th and 5th weeks of the study. No significant changes were observed in cellular responses to the mitogen Con A throughout the course of the experiment.

CHAPTER 4

Cellular and cytokine responses of hepatic and mesenteric lymph node cells from *F. hepatica* infected calves (Experiment B).

4.1 INTRODUCTION

Peripheral blood mononuclear cells provide a useful means of examining time-course cellular responses to specific antigens during infection. With localised or compartmentalised infections, however, peripheral cellular responses may be an incomplete reflection of the immune response occurring at the infection site. Furthermore, concurrent unrelated infections, for example subclinical respiratory disease, may influence some of the responses shown by peripheral cells. Respiratory disease is common in young cattle stock housed in a traditional manner and may well have been present in the cattle used in Experiment A (Chapter 3). This problem of concurrent infection becomes more significant when trying to examine the cytokine responses of cells during culture. A low level respiratory viral infection could lead to an enhanced IFN-γ response if virus-activated cells are included in culture. The cytokine response obtained in both unstimulated and antigen stimulated cultures might therefore be distorted.

An alternative, measuring site specific responses, provides a more accurate reflection of the *in vivo* cellular response present during infection. This can be achieved by extracting mononuclear cells from lymph nodes and examining the response to stimulation with specific antigen, as with peripheral cells. There is currently no published working describing local lymph node derived mononuclear cell responses to fluke proteins during the early stages of *F. hepatica* infection in cattle. As juvenile flukes penetrate the gut mucosa prior to migration through the liver parenchyma, the cellular responses at both these sites was investigated in this experiment.

Cytokine production by lymph node cells after stimulation with *F. hepatica* proteins was investigated, as well as cellular proliferative responses. The nature of the cytokines released by lymphocytes indicates the general nature of the effector

immune response (section 1.2.9.1). In the past, much emphasis has been placed on attempting to classify the exact nature of the T cell response, as either Th1 or Th2. This approach detracts from one of the prime reasons for examining the nature of cytokine production, specifically vaccine design. Vaccine adjuvants and antigen dose have a profound influence on the nature of the cytokine response induced to a specific antigen after vaccination (Grun and Maurer, 1989; Constant and Bottomly, 1997). The route of inoculation may also influence the nature of the cytokine response and is illustrated by a recent study in which mice were vaccinated against *F. hepatica* using DNA encoding the glutathione S-transferase protein (Smooker *et al.*, 1999). Intradermal vaccination using a secretory type DNA construct induced an IgG1 and IgE antibody response, whilst intramuscular vaccination with a construct favouring cytoplasmic expression resulted in the production of IgG2a. The recent move towards the use of recombinant cytokines as adjuvants in bovine vaccines also requires knowledge of the type of response to be boosted (Lofthouse *et al.*, 1996). Parasites are complex organisms composed of many antigens able to stimulate differing and possibly opposing immune responses. Recently, attention has turned towards the development of multivalent vaccines, either using various proteins of the same parasite to protect against one infection, or of different parasites to protect against several. Including proteins that are incompatible, and which may stimulate opposing immune responses, could substantially reduce vaccine efficacy.

In the previous experiment (A), it was shown that calves pre-exposed to a 5-6 day period of *F.hepatica* infection showed signs of reduced liver damage and eosinophilia upon subsequent challenge. The peripheral cellular response to fluke proteins in these animals was highly variable. This experiment was therefore designed to examine local, hepatic and mesenteric lymph node responses in equivalent pre-exposed calves and in naïve calves, shortly after a challenge infection. It was anticipated that the study would provide an indication as to which of the two locations, gut or liver, was the main immunoreactive site during challenge infection. A more uniform response between animals in the same group was also anticipated, because fluke infection would be the predominant stimulant of the immune response at both these sites. As hepatic and mesenteric lymph nodes are not easily accessible, this necessitates extraction at post-mortem. The responses at only one time-point could therefore be examined for an individual animal.

Experimental Aims

- To examine proliferative responses of mesenteric and hepatic lymph node cells to a crude *F.hepatica* protein preparation.

- To investigate cytokine production by hepatic lymph node cells after culture with various *F.hepatica* preparations.

- To determine the cell phenotypes present in hepatic lymph nodes after challenge.

- To investigate the antigen recognition profile, if any, of cell culture supernatant from hepatic lymph node cell cultures, by Western blots using various fluke preparations (section 6.3.3).

4.2 MATERIALS & METHODS

4.2.1 Experimental design

Preliminary trial

A preliminary trial was performed prior to the start of the main study, to allow optimisation of the timing and methodology for lymph node extraction. Two female Friesian-Holstein calves, aged 3 months, were given 450 *F. hepatica* metacercariae *per os* and then treated 5 days later with triclabendazole at a dose rate of 36mg/kg. On day 30, both calves were challenged with 500 metacercariae. Hepatic and mesenteric lymph nodes were removed at post-mortem on day 11 (calf 827) and day 14 (calf 813) after the secondary challenge infection.

Cell suspensions were prepared from lymph node material as explained in section 2.4.2. A standard proliferation assay was carried out using whole fluke antigen (WFA) at 15 μg/ml (concentration previously optimised by titration with peripheral blood mononuclear cells taken during the first two weeks of the experiment, data not shown) and Con A at 1 μg/ml. Cells were also cultured unstimulated at a concentration of 2 x 10^6/ml and supernatant fluid collected after a five day period, for later use (section 6.3.3). Proliferation was observed in hepatic node derived cells at both of the time points studied.

Main study

Two groups of four calves were set up: a naïve control group and a 5-6 day exposure group, equivalent to groups D and B of Experiment A, respectively (the groups in this study are therefore also termed D and B). Group B was dosed on day 0 with metacercariae, whilst group D received blank pellets; both groups were then treated on day 5 with triclabendazole at a dose rate of 36 mg/kg (Table 4.1). Both groups B and D were challenged with metacercariae on day 30. On each of days 40 and 41, 10 and 11 days post-secondary challenge, two calves from each group were humanely slaughtered and the liver, hepatic lymph nodes and samples of mesenteric lymph node collected.

Table 4.1: An outline of the experimental design for the main study, showing the timing of the main interventions.

Time-point	Group B (5-day pre-exposure)	Group D (Naïve control)
Day 0	600 metacercariae *per os*	Blank pellet *per os*
Day 5	Triclabendazole at 36mg/kg	Triclabendazole at 36mg/kg
Day 30	375 challenge metacercariae *per os*	375 challenge metacercariae *per os*
Day 40	Collect lymph nodes from two animals (calves 9, 13)	Collect lymph nodes from two animals (calves 16, 17)
Day 41	Collect lymph nodes from two animals (calves 1, 2)	Collect lymph nodes from two animals (calves 6, 12)

4.2.2 Stock

The animals used in the main study were intact, male Friesian-Holstein bull calves aged between 4-6 months at the start of the experiment. Animals were obtained from farms of the University of Edinburgh and had been reared indoors from birth. Prior to the study, all animals were screened by ELISA to test the IgG1 antibody response to fluke excretory-secretory proteins (section 2.3). Animals were screened twice, four weeks apart; all those used were negative.

Animals were allocated into two groups by stratifying for weight and then using random numbers to place weight pairs into either group. The Student's t-test showed no significant difference in the weight distributions between the two groups: means 155 kg and 146 kg, $P = 0.53$. The two groups were then randomly classified as either pre-exposure (B) or control (D).

4.2.3 *F. hepatica* infection

In this experiment a flat dose rate of a given number of metacercarial cysts per animal was used, for two reasons. Firstly, the weight range of the calves was moderate: prior to the start of the experiment, the weight range in group B was 135-170 kg and before secondary challenge the range was 172-210 kg (group B) and 152-

200 kg (group D). Secondly, the main point of interest in this experiment was the immunological response to infection and not the relative levels of the various infection parameters. A primary dose of 600 metacercariae per calf was used, but the secondary challenge dose was reduced to 375 metacercariae per calf, because of limited availability of the parasite. Metacercariae were counted and prepared for dosing as in section 3.2.4.

4.2.4. *F. hepatica* protein preparations

Whole fluke antigen (WFA) and excretory-secretory (ES) protein fractions were prepared as described in section 2.1.1. High molecular weight and cathepsin-L pool fractions (section 2.1.4) were also used in the study.

4.2.5 Sample collection

Sera

Blood was collected weekly from each animal from day –21 until the end of the experiment; sera was prepared (section 3.2.7), aliquotted and stored at –80°C for later analysis of antibody responses (section 6.3.3).

Peripheral blood mononuclear cells (PBMC)

PBMC were isolated from blood as described previously (section 2.4.1) and used in proliferation assays and for fluorescence activated cell sorting (FACS) analysis of cell phenotypes (section 2.5). Proliferation assays were set up at three pre-infection time-points (negative) and on days 7, 14 and 37 post-infection. FACS analysis was carried out once prior to the start of the experiment and on days 7, 20 and 37.

Post-mortem samples

Animals were humanely slaughtered on days 40 and 41. Blood was collected for sera preparation and was aliquotted and stored at -80°C. The livers, including hepatic lymph nodes, and intestines with mesentery were removed from the carcass and samples taken as quickly as possible. Whole mesenteric lymph nodes and half portions of hepatic lymph nodes were placed into ice cold lymph node media and removed to a tissue culture facility, where cells were extracted from the nodes as

described in section 2.4.2. The following assays were carried out with the lymph node derived cells:

- FACS analysis to determine cell phenotype of hepatic lymph node cells (section 2.5).
- Proliferation assays to examine hepatic and mesenteric lymph node cell responses to stimulation with WFA and Con A preparations.
- Cell culture assay to investigate cytokine production by hepatic lymph node cells after stimulation with WFA preparation.
- Unstimulated hepatic lymph node cells were grown over a 4 day period for collection of supernatant fluid to be used in Western blot assays (section 6.3.3).

Excess cells were cryopreserved in 10% dimethyl sulphoxide (DMSO), 90% foetal calf serum (FCS) (section 2.4.5) and stored under vapour phase nitrogen until required.

4.2.6 Cell proliferation assay

PBMC and hepatic and mesenteric lymph node cells were cultured unstimulated and with WFA at 15 μg/ml or Con A at 1 μg/ml in quadruplicate wells, using the standard method (section 2.4.4). The optimum protein concentration for cell stimulation had been chosen from previous titration studies (section 3.3.9.1).

Cryopreserved hepatic lymph node cells that had been in storage for 12 months were defrosted (section 2.4.6) and tested for proliferative responses to high molecular weight, cathepsin-L pool, ES and WFA preparations. As the quantity of high molecular weight and cathepsin preparations was extremely limited, titration for optimisation of cell proliferation could not be done. Proliferation responses to the high molecular weight fraction at 1 μg/ml and 0.2 μg/ml and to the cathepsin-L pool fraction at 10μg/ml were therefore examined (hepatic node cells were found to be unreactive to lower concentrations of the cathepsin fraction in a trial assay). Proliferation assays were also set up using WFA at 15 μg/ml, ES at 10 μg/ml and Con A at 1 μg/ml.

4.2.7. Cytokine production after stimulation of hepatic lymph node cells with fluke proteins

Cytokines produced by fresh hepatic lymph node cells after stimulation with WFA were examined over a 96 hour period. Cells from all animals were cultured in flat-bottomed 6 well plates (Nunc®) at a concentration of 1 x 10^6 cells/ml, either unstimulated (control) or with WFA at 15 μg/ml, under standard conditions. Cells and supernatant fluid from control and antigen stimulated cultures were then harvested 24, 48, 72 and 96 hours later. Cytokine production by cryopreserved cells was also examined in response to culture with the high molecular weight and cathepsin-L pool fractions and the ES and WFA preparations. Cell cultures were set up as for the proliferation assay above. Cell culture supernatant fluid only, was then harvested at 72 hours.

Cells and culture fluid were harvested from the culture plates and the cells pelleted by centrifugation at 120*g* for 10 minutes. The supernatant fluid was smoothly decanted, aliquotted and stored at -20°C. The cell pellets from fresh hepatic node cell WFA-stimulated and control cultures were resuspended and washed in a large volume of cold (4°C) PBS, by repeating the centrifugation step. These cell pellets were then lysed using lysis buffer from either the "Qiagen RNeasy Mini" (Qiagen) or the "SV Total RNA System" (Promega) RNA isolation kits. RNA was extracted from the lysates according to manufacturers' instructions and stored at –20°C. Unfortunately, due to lack of time, it was not possible to investigate the RNA response. Cell culture supernatant fluid was tested by ELISA or bioassay for the presence of the cytokines IFN-γ, IL2 and IL4.

4.2.8 Bovigam™ ELISA assay to measure bovine IFN-γ in cell culture supernatant fluid

The presence of IFN-γ in supernatant fluid was measured using the Bovigam™ ELISA assay system (CSL Limited, Poplar Road, Parkville, Victoria, Australia). Samples were tested in duplicate following the manufacturer's instructions. Recombinant bovine IFN-γ (gift, from the Institute of Animal Health, Compton, Berkshire) was used as the standard for a titration curve, to allow quantification of any IFN-γ present.

4.2.9 Measurement of IL2 by bioassay with an IL2-dependent cell line

The presence of IL2 in supernatant fluid was measured by means of a bioassay, using the bovine IL2-dependent cell line (4325), by Dr R. Collins at the Institute of Animal Health, Compton, Berkshire, according to the method of Kühnle *et al* (1996). Results are presented here as the stimulation index: this was taken as the response ratio between control and antigen stimulated cultures.

4.2.10 Measurement of IL4 by B cell bioassay

The presence of B cell growth factors in hepatic node cell culture fluid was analysed by bioassay using B cells freshly harvested from an uninfected cow. This assay was performed by the author, in conjunction with Dr R. Collins (section 4.2.9), according to the method of Kühnle *et al* (1996). Briefly, B cells were isolated from PBMC obtained from a mature cow, using magnetic bead extraction with the monoclonal antibody ILA58. Purified B cells (1×10^5 per well) were then cultured with 33 µl of supernatant fluid in culture media for 24 hours under standard conditions. Cells were labelled with ^{3}H-labelled thymidine during the last six hours of culture, harvested onto glass fibre filters and the amount of thymidine incorporated into the cells measured by scintillation counter. Results are expressed as the stimulation index. In order to examine whether the observed response is due entirely to IL4, ablation of responses can be attempted using an anti-IL4 antibody. Complete ablation of growth would suggest that the factor within supernatant fluid responsible for growth was indeed IL4. Unfortunately, it was not possible to carry out this assay due to a lack of time and materials.

4.2.11 Production of antibody by unstimulated lymph node cells

Lymph node derived mononuclear cells were incubated at a concentration of 2×10^6 cells/ml in complete media alone for 4-5 days under standard conditions. The supernatant fluid was collected by harvesting the cell suspension and centrifuging at 120*g* for 10 minutes. Supernatant fluid was decanted without disturbing the cell pellet and stored at -20°C. This was used neat as an antibody probe on Western blots of fluke proteins (section 6.3.3).

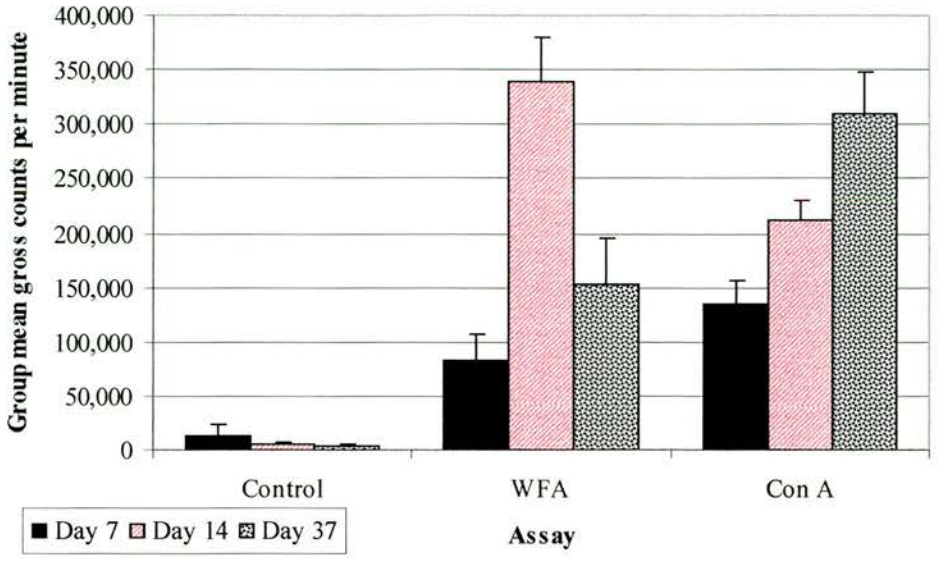
4.3 RESULTS

4.3.1. Peripheral blood mononuclear cell proliferative responses to culture with whole fluke antigen

All group B peripheral blood mononuclear cell samples showed substantial proliferative responses to WFA at both days 7 and 14 (Figure 4.1). The response of individual animals was in many cases greater than or of a similar magnitude to that obtained by culture with the mitogen Con A.

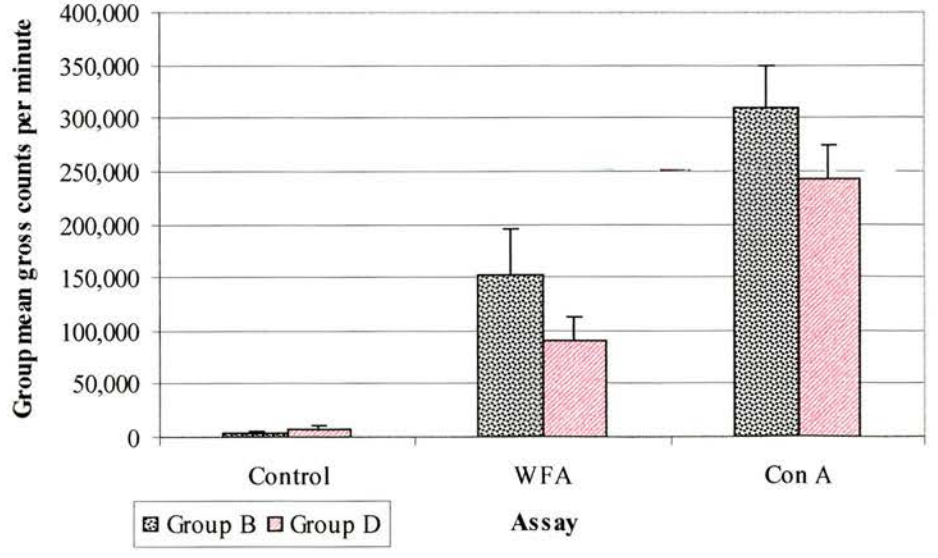
PBMC collected on day 37 from both groups B and D were tested for proliferative responses to WFA after they had been in cryopreservation for several months. Cells were responsive to WFA antigen, with no significant difference in the magnitude of the response between groups B and D (Figure 4.2). This is in contrast to the proliferation results of Experiment A, where the 7 day post-challenge response was lower in the pre-exposed group, B, than in the naïve control group, D (section 3.3.10). It is worth noting that the variability between responses of animals within the same group was reduced in comparison to those obtained in Experiment A. This could be due to the fact that all animals were obtained from a single, closed farm. The response of the cryopreserved cells to the WFA preparation at day 37 was lower than that to the mitogen Con A, unlike the day 14 response obtained with fresh group B cells, where the reverse was seen. Group B cell proliferation to WFA was also reduced after challenge, compared to the day 14 response. This reduction could be due either to altered reactivity or viability of the cells as a result of cryopreservation or as a direct result of the secondary challenge infection.

Figure 4.1: Longitudinal profile of group B peripheral blood mononuclear cell responses to whole fluke antigen (WFA) and Con A at days 7, 14 and 37, shown as group mean gross counts per minute.



Proliferative responses of PBMC after a 5 day culture either unstimulated (control) or with WFA (15 μg/ml) and Con A (1 μg/ml). Error bars indicate the standard error of the mean, where n=4.

Figure 4.2: Group mean proliferative responses of groups B and D peripheral blood mononuclear cells to whole fluke antigen (WFA) and Con A after the secondary challenge infection at day 37, shown as gross counts per minute.



Proliferative responses of PBMC after a 5 day culture either unstimulated (control) or with WFA (15 μg/ml) and Con A (1 μg/ml). Error bars indicate the standard error of the mean, where n=4 for both groups.

4.3.2 Peripheral blood mononuclear and hepatic lymph node cell phenotypes, measured by FACS analysis

Group B longitudinal profile of peripheral blood mononuclear cell phenotypes

There was no change in the proportion of $CD4^+$, $CD8^+$, $CD14^+$ or $\gamma\delta^+$ T cells present in the blood of animals in group B throughout the period of study (Figure 4.3). However, the proportion of B cells was significantly lower at day 37 than at any of the other time-points. Group D cells were also examined on day 37 and no difference was detected in cell phenotype proportions between this group and group B for any cell type. It was expected that the proportion of B cells present in group D at day 37 would have been similar to the level in group B prior to secondary challenge i.e. at day 7. Also, as all other cell types were present at stable levels in group B throughout the study, it is probable that the low percentage of B cells obtained at day 37 was the result of a technical error and was not due to a genuine decrease in the levels of peripheral B cells.

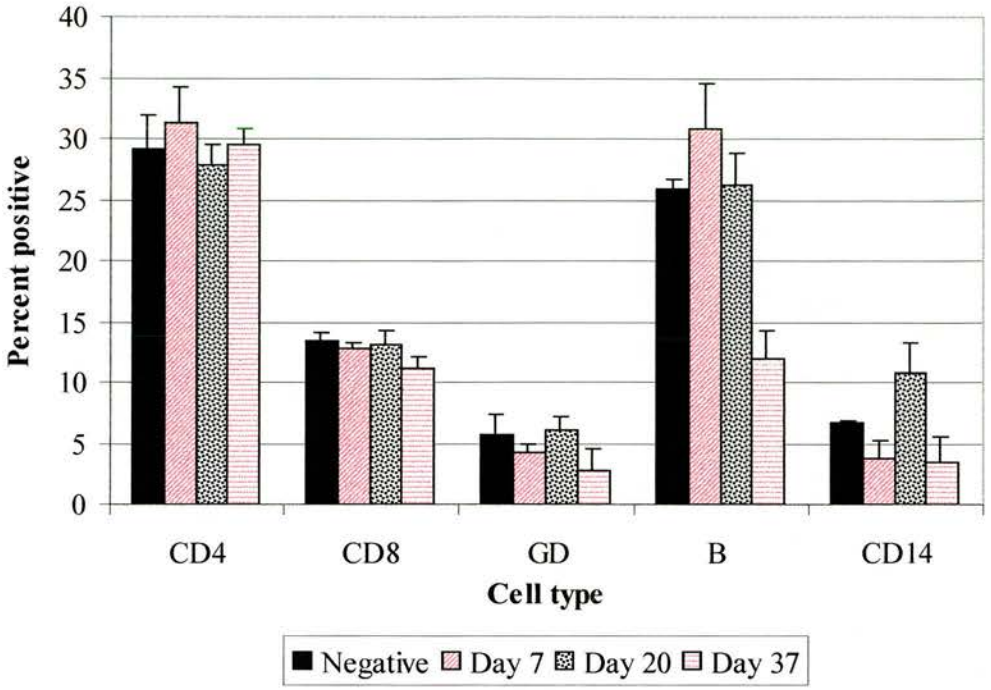
Hepatic lymph node cell phenotypes in groups B and D at day 40/41

The group mean cell phenotypes present in hepatic lymph nodes are shown in Table 4.2. Results of Mann-Whitney U tests showed no significant difference between the two groups for any of the cell types studied. A CD14 positive cell population was not detected in either group. Variability within the two groups was low.

Table 4.2: Proportion of hepatic lymph node cells staining positive for the given cell types, shown as the group mean and giving the standard error of the mean (SEM), where n=4 for both groups.

Cell Type	Monoclonal antibody	Percent positive (SEM)	
		Group B	Group D
$CD4^+$	IAH-CC8	42.4 (4.1)	39.2 (5.6)
$CD8^+$	SBU-T8	12.7 (0.9)	16.4 (1.7)
B cells	VPM30	24.1 (3.7)	20.0 (2.1)
$\gamma\delta^+$ T cells	86D	2.0 (1.0)	0.5 (0.5)

Figure 4.3: Proportion of peripheral blood mononuclear cells staining positive for the given cell populations in group B at days 7, 20 and 37, shown as the group mean.



Cells were taken at a negative time-point (pre-infection) and on days 7, 20 and 37 post-infection. The proportions of cells staining positive for the cell types are shown: CD4, CD8, GD (γδ T cells), B cells and CD14. The group mean is shown and error bars denote the standard error of the mean (n=4).

4.3.3 Lymph node cell proliferative responses to whole fluke antigen and Con A

Preliminary trial

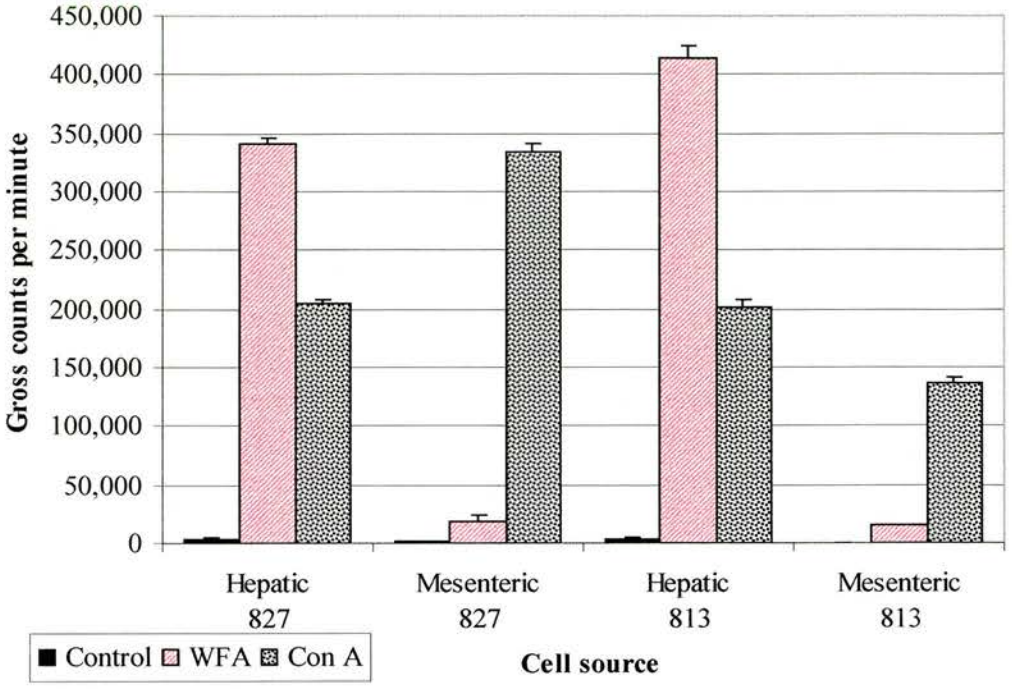
Both trial animals, 813 and 827, showed lymph node proliferative responses of a similar magnitude to one another, despite the three day interval between the two harvests. Hepatic lymph node cell responses to culture with the WFA preparation were much greater than those of mesenteric node derived cells for both animals (Figure 4.4). The response of hepatic node cells to WFA was greater than that to the mitogen Con A, whereas mesenteric node cell responses to WFA were much lower than those stimulated by Con A.

A hepatic lymph node cell response to the adult fluke protein WFA was present 10 days after the secondary challenge infection. A final time-point of days 10 and 11 post-secondary challenge was therefore decided upon for lymph node collection in the main study.

Main Study

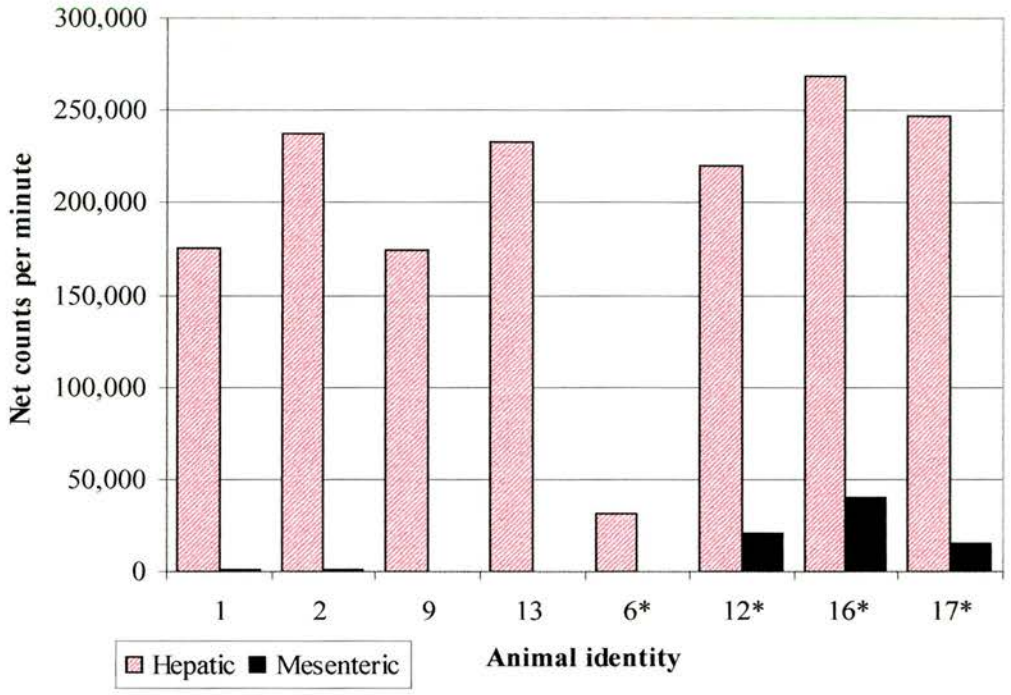
Hepatic lymph node cells from all animals in groups B and D showed a substantial proliferative response to stimulation with the WFA preparation (Figure 4.5). Responses were, in all but one case (calf 6), comparable to or greater than the response obtained by Con A stimulation (Table 4.3). There was no difference in the magnitude of the response between the two groups. In contrast, the mesenteric response to the WFA preparation was low. Mesenteric cells from three of the calves in group D (calves 12, 16 and 17) showed slightly greater proliferation than those from group B animals. Mesenteric cells from calf 9 were contaminated by fungus and the results are not included.

Figure 4.4: Proliferative responses of hepatic and mesenteric lymph node cells of the trial calves 813 and 827 in response to stimulation with whole fluke antigen (WFA) and Con A.



Cells were cultured unstimulated (control) or with WFA (15μg/ml) and Con A (1 μg/ml) preparations over a 5 day period. Mean gross counts per minute of quadruplicate cultures for individual samples are displayed, with error bars showing the standard error of the mean.

Figure 4.5: Hepatic and mesenteric lymph node cell proliferative responses to whole fluke antigen preparation, for individual animals in groups B and D; shown as the net response.



Individual proliferation results for hepatic and mesenteric lymph node cells from group B and D animals after a 5 day culture with WFA (15μg/ml). Results shown are the difference (net counts per minute) between WFA-stimulated and unstimulated (control) cells. Group D (naïve control) animals are marked *. Note that there was no mesenteric sample for calf 9 of group B.

Table 4.3: Hepatic and mesenteric lymph node cell proliferative responses for individual animals, shown as the stimulation index, for whole fluke antigen (WFA, 15 μg/ml) and Con A (1 μg/ml) stimulated cultures.

Animal		Hepatic		Mesenteric	
Group	**Identity**	**WFA**	**Con A**	**WFA**	**Con A**
B	1	152	197	3	629
	2	63	68	3	664
	9	101	102	N/D	N/D
	13	138	79	3	656
D	6	44	383	4	832
	12	94	113	39	322
	16	22	13	176	720
	17	48	39	51	489

Key: N/D = not done

Response to stimulation with Con A

The stimulation indices obtained for mesenteric lymph node cells stimulated with Con A were considerably greater than those of hepatic node cells (Table 4.3). Analysing group B and D responses collectively, the hepatic response to stimulation with Con A was greater than the mesenteric response (215,478 and 176,011 counts per minute, respectively). However, background control counts from unstimulated cell cultures were much lower with mesenteric node cell cultures: a mean of 297 counts per minute was obtained for mesenteric cells, compared to 3692 for hepatic node cells. This difference in the background response accounts for the large discrepancy in Con A stimulation indices between hepatic and mesenteric lymph node preparations.

4.3.4 Cytokine production by fresh hepatic lymph node cells after stimulation with whole fluke antigen

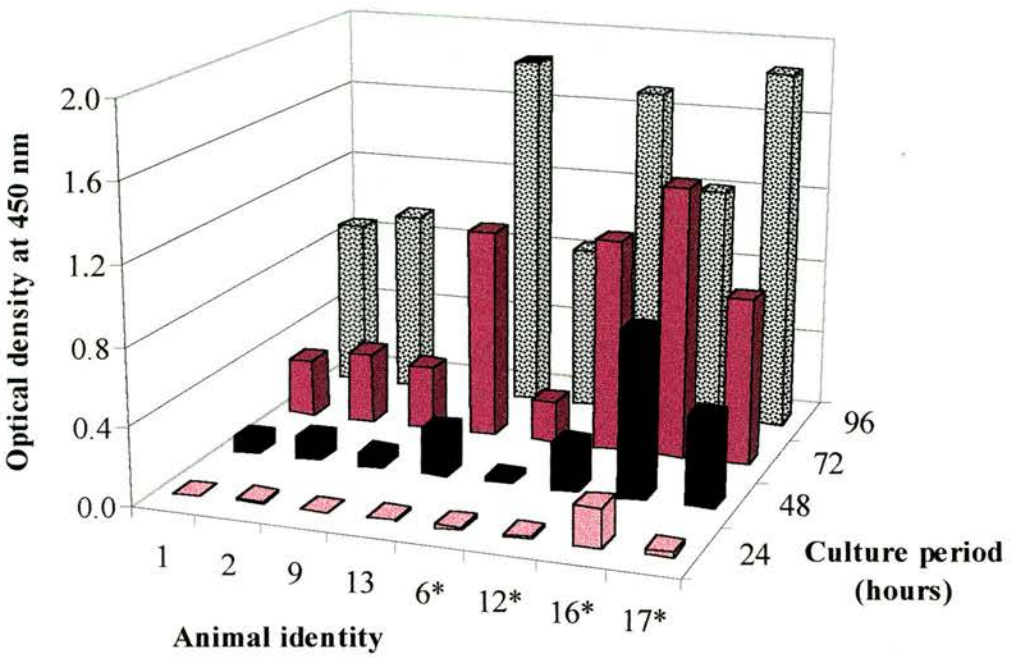
IFN-γ

Optical density values obtained in the Bovigam™ ELISA assay for cell culture supernatant fluid harvested between 24 and 96 hours are shown in Figure 4.6. IFN-γ production by all samples was low at 24 hours; differences between animals became apparent at 48 hours, but by 96 hours all cultures were producing substantial amounts. The amount of IFN-γ present in the day 3 culture supernatant fluid was quantified by titration with recombinant bovine IFN-γ and is shown in Figure 4.7. Overall, three out of the four calves from group B showed low IFN-γ production in WFA stimulated cultures (calves 1, 2 and 9), whereas three out of the four from group D showed high production (calves 12, 16 and 17). Calves 6 and 13 were anomalies in their respective groups. The difference in IFN-γ production between groups B and D was not statistically significant, however, due to the small number of animals involved.

IL2 and B cell growth factor

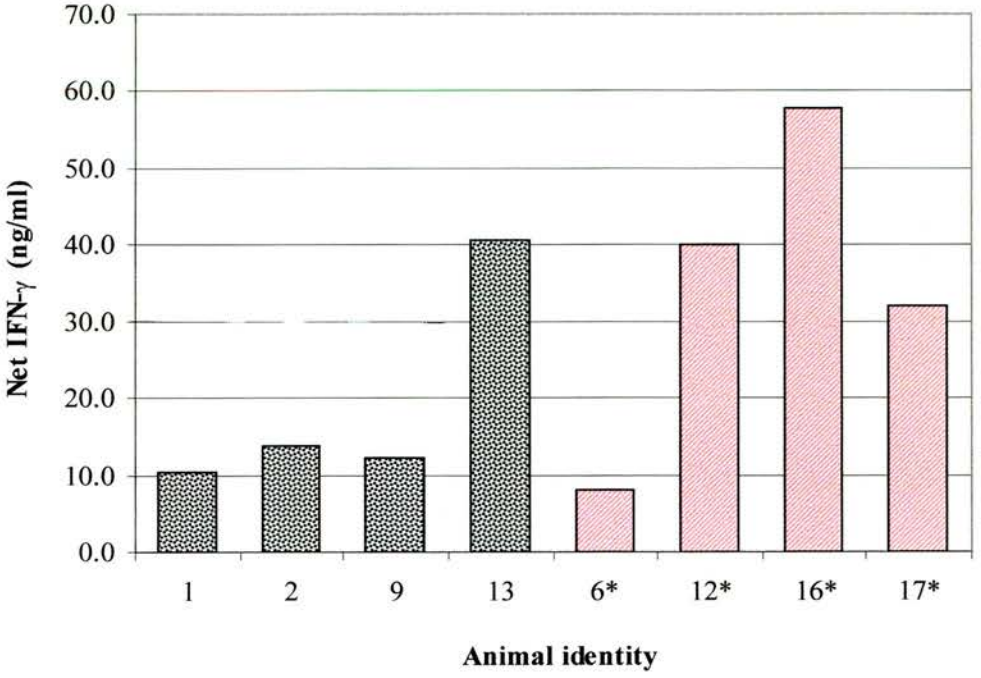
The group mean stimulation indices over the 96 hour period for the IL2 and B cell bioassays after culture with primary cell culture supernatant fluid are shown in Figures 4.8 and 4.9. Stimulation of cell growth by supernatant fluid in both assays was only moderate, although distinct from background responses. Supernatant fluid collected from all WFA-stimulated cultures produced positive responses; there was little difference between the two groups B and D. Cytokine production peaked at 72 hours for both IL2 and B cell growth factor. Supernatant fluid from group D cell cultures resulted in the greatest variation in bioassay responses, particularly with the IL2 bioassay. Samples from calves 16 and 17 showed the earliest production of both cytokines and had the greatest IL2 production. Supernatant fluid collected from lymph node cultures of calf 13 on day 3 and calf 6 on day 4 resulted in the greatest responses in the B cell bioassay.

Figure 4.6: IFN-γ levels in whole fluke antigen stimulated cell cultures, during a 24 to 96 hour culture period, shown as optical density values obtained in the Bovigam™ assay.



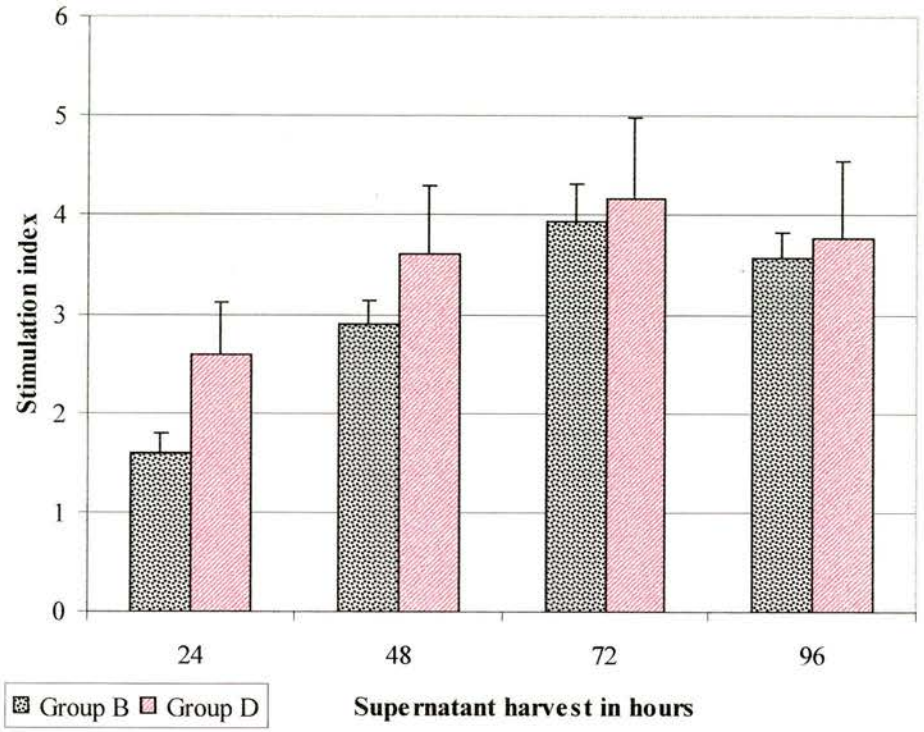
Hepatic lymph node cells were cultured with WFA (15 μg/ml) and cell culture supernatant fluid collected at 24, 48, 72 and 96 hours. This was then tested for the presence of IFN-γ using the Bovigam™ assay. Net optical density values were measured at 450 nm, and results are given as the difference between the value recorded for antigen-stimulated and unstimulated cultures for each animal. Group D animals (naïve control) are marked *. There was no 96 hour sample for calf 9. Bars denote 24 hour (speckled pink), 48 hour (black), 72 hour (dark red) and 96 hour (speckled black) responses.

Figure 4.7: IFN-γ production in ng/ml by whole fluke antigen stimulated hepatic lymph node cell cultures at 72 hours.



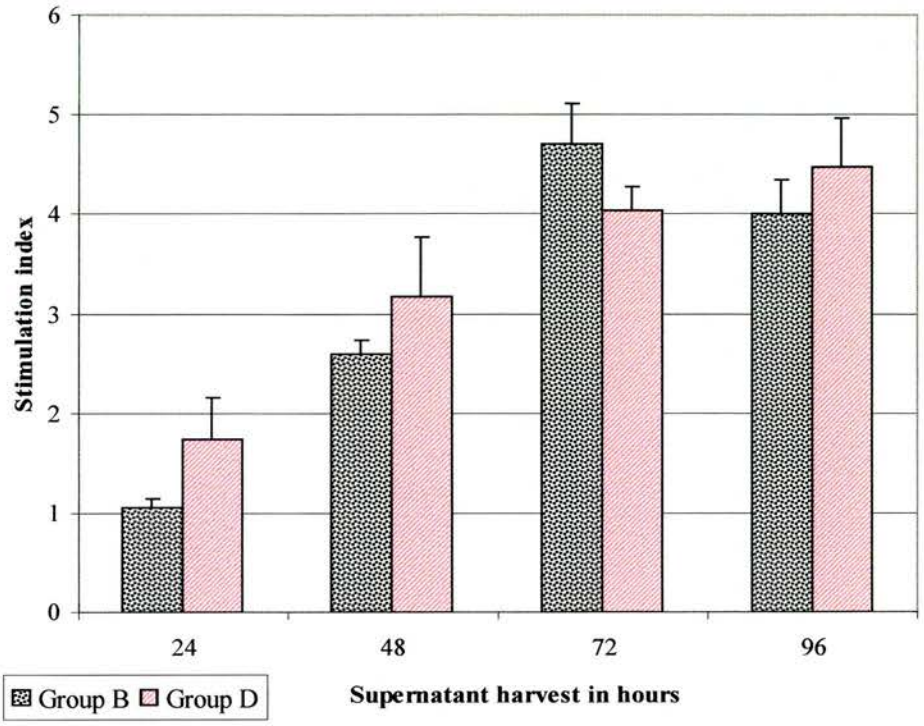
The amount of IFN-γ present in the 72 hour culture supernatant fluid was quantified in the Bovigam™ assay using recombinant bovine IFN-γ as a standard. Net production (difference between antigen-stimulated and unstimulated cultures) is shown in ng/ml. Group B animals are shown as speckled bars and group D as hashed bars, together with an asterisk *.

Figure 4.8: IL2 production by whole fluke antigen stimulated hepatic lymph node cells, during a 24 to 96 hour culture period.



Supernatant fluid harvested from fresh hepatic lymph node cells cultures stimulated with WFA (15µg/ml) was tested for the presence of IL2 using the IL2-dependent cell line 4325. Proliferation of the cell line in response to addition of the primary culture fluid, over a 24 hour culture period, is shown as the stimulation index with n=4 for both groups at 24, 48 and 72 hours and n=3 for group B and n=4 for group D at 96 hours. Error bars show the standard error of the mean.

Figure 4.9: B cell growth factor production by whole fluke antigen stimulated hepatic lymph node cells, during a 24 to 96 hour culture period.



The presence of B cell growth factors present in cell culture fluid between 24 and 96 hours after stimulation with WFA (15 μg/ml). Culture fluid was added to purified bovine B cells and proliferation measured after a 24 hour culture period. Results represent the group mean stimulation index, with n=4 for group D and n=3 for group B (calf 9 not tested). Error bars show the standard error of the mean.

4.3.5 Proliferative responses of cryopreserved hepatic lymph node cells to various *F. hepatic*a protein preparations

Hepatic lymph node cells which had been stored under vapour phase nitrogen were revived after several months of storage and tested for proliferative responses to culture with the following antigen preparations: high molecular weight fraction, cathepsin-L pool fraction, ES and WFA preparations. Cell loss during the defrosting process was approximately 50%. Although the responses of defrosted cells may not accurately reflect that seen with fresh cells, they were useful in indicating any major trends in the cells response to the various fluke preparations. Figure 4.10 shows the group mean net proliferative response to all the preparations studied. Individual responses to the high molecular weight and cathepsin-L pool fractions are shown in more detail in Figure 4.12.

All cells responded well to culture with the WFA and ES preparations. The response to WFA was slightly lower than that obtained with fresh hepatic node cells, but was of a similar magnitude to the Con A response. The response to high molecular weight and cathepsin-L pool fractions varied more widely between animals than those to the WFA and ES preparations.

All animals showed moderate proliferation in response to stimulation with the high molecular weight fraction at 1 μg/ml, with calf 2 showing an excessively high response. The lower concentration of 0.2 μg/ml elicited a much lower response in all calves, except for calf 2, with which the response was similar to that seen with the higher concentration. There was no obvious difference in proliferative responses to the high molecular weight fraction between groups B and D.

In contrast, proliferation in response to the cathepsin fraction was much poorer overall. Cathepsin at a concentration of 1 μg/ml did not elicit a significant response in a trial assay. Due to lack of material, it was not possible to try concentrations above 10 μg/ml. A general trend of slightly lower responses to the cathepsin fraction in group B, compared to group D, animals was evident, but this was not statistically significant. Unlike the response to the high molecular weight fraction, that of calf 2 to the cathepsin fraction was much closer to that of other animals in the group.

4.3.6 Production of IFN-γ by cryopreserved hepatic lymph node cells after culture with various *F. hepatica* protein preparations

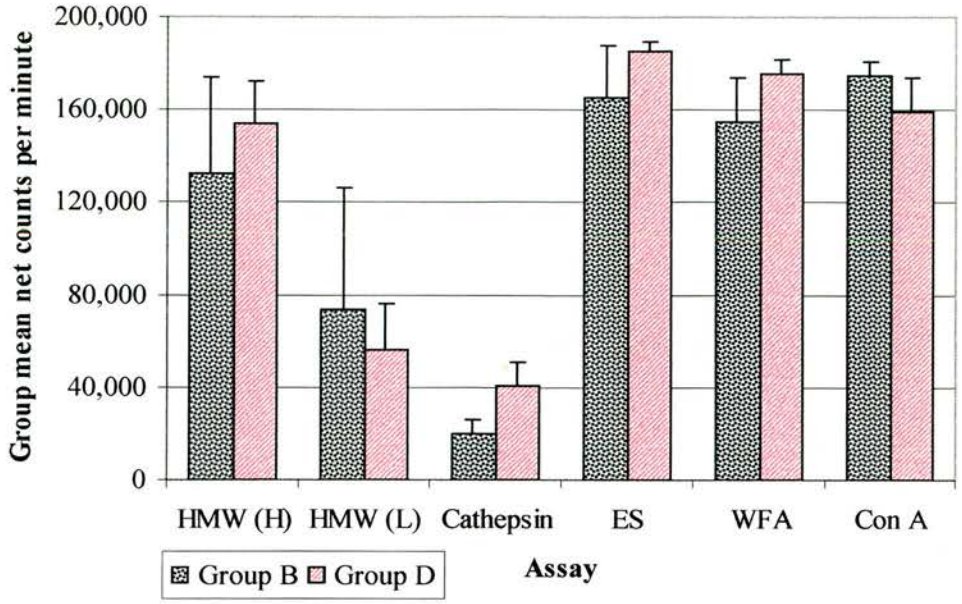
Group mean IFN-γ production at 72 hours for the various fractions tested is shown in Figure 4.11 and individual IFN-γ production to the high molecular weight and cathepsin fractions in Figure 4.13.

The IFN-γ response to the high molecular weight fraction at a concentration of 1 μg/ml was very high for both calves 1 and 2 (group B). The latter animal also had a high proliferative response, whereas that of calf 1 had been average. Stimulation with the high molecular weight fraction at 0.2 μg/ml resulted in a more uniform response with all eight animals, although little IFN-γ was produced by the cells from calf 9 in response to any of the proteins studied.

Cathepsin-stimulated production of IFN-γ by cells from calves 1 and 2 was much lower than that produced in response to the high molecular weight fraction, similar to the pattern seen with the proliferative response. In contrast, all group D samples and calf 13 from group B produced more IFN-γ after stimulation with cathepsin, when compared to that obtained with the high molecular weight fraction. This was despite the fact that in all animals the proliferative responses to the cathepsin fraction were lower than with the high molecular weight fraction. It would therefore appear that although cathepsin did not stimulate a strong proliferative response, cathepsin was a more potent stimulant of IFN-γ production than the high molecular weight fraction.

IFN-γ production by fresh and cryopreserved cells after WFA stimulation was compared and is shown in Table 4.4. Calves 2, 13 and 17 showed very similar responses with both fresh and cryopreserved cells, however cryopreserved cells of calves 1, 6 and 12 produced more, and those of calves 9 and 16 less, IFN-γ than the equivalent fresh cells. Despite these differences in the individual IFN-γ response, the overall pattern of low IFN-γ production by group B animals, versus high IFN-γ production by group D animals was maintained. IFN-γ production in response to stimulation with the ES preparation had not been tested previously with fresh hepatic lymph node cells. However, responses of cryopreserved cells were very similar to those seen with the WFA preparation.

Figure 4.10: Proliferative responses of cryopreserved hepatic lymph node cells to various *F. hepatica* protein preparations.



Group mean net proliferative responses after a 5 day culture period with the following: high molecular weight fraction at 1 μg/ml, HMW (H); high molecular weight fraction at 0.2 μg/ml, HMW (L); cathepsin-L pool at 10 μg/ml; excretory-secretory antigen at 10 μg/ml, ES, and whole fluke antigen at 15 μg/ml, WFA. Con A was also used at 1 μg/ml. Error bars indicate the standard error of the mean, where n=4 for both groups.

Figure 4.11: Production of IFN-γ by cryopreserved hepatic lymph node cells in response to a 72 hour culture period with various fluke protein preparations (as Figure 4.10), shown as the net production (difference between antigen-stimulated and unstimulated cultures).

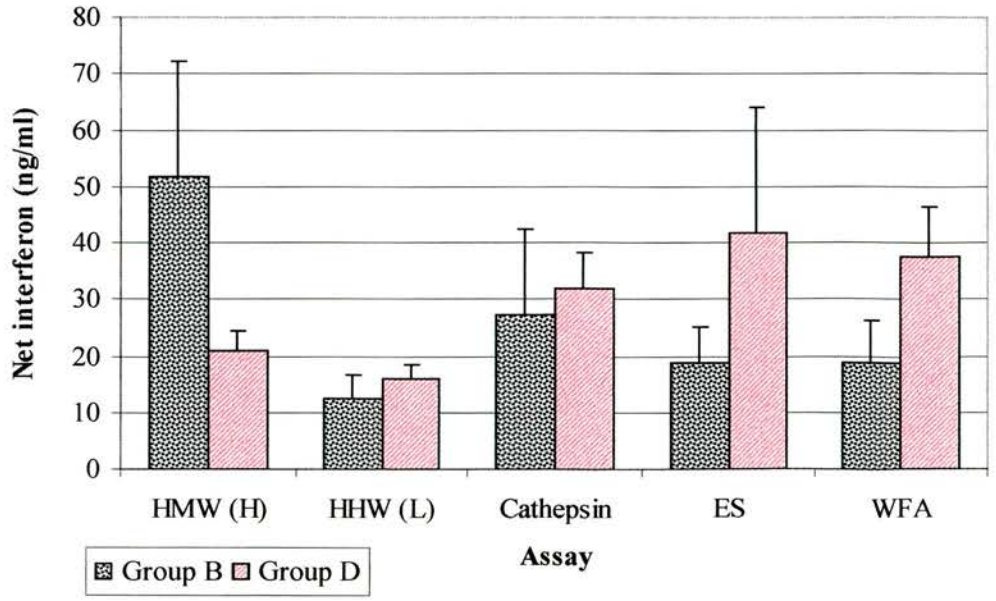
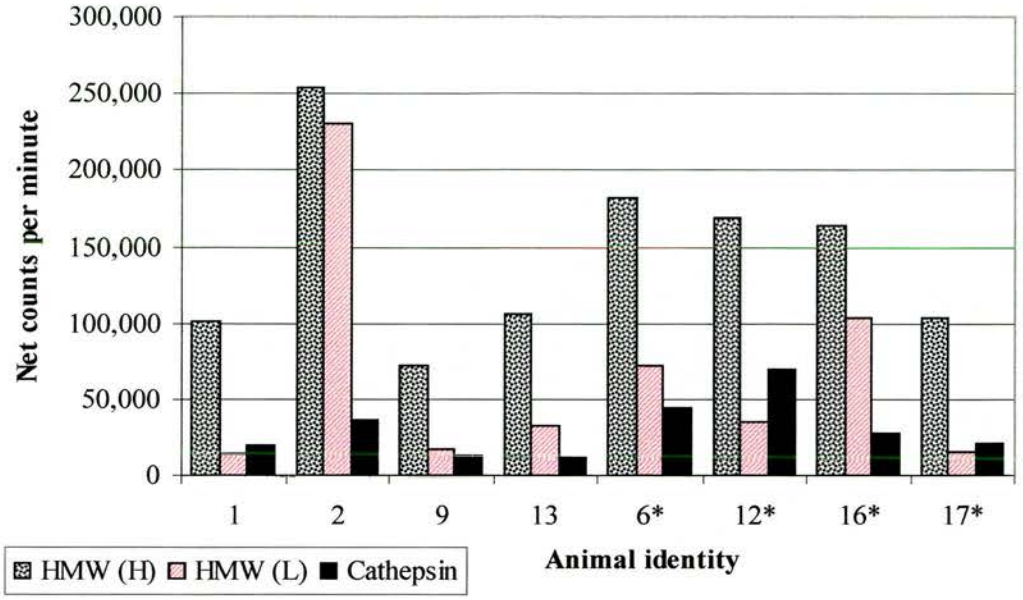


Figure 4.12: Proliferative responses of cryopreserved hepatic lymph node cells to the high molecular weight and cathepsin fractions, for individual animals.



Individual net proliferative responses after a 5 day culture period with the following: high molecular weight fraction at 1 μg/ml, HMW (H); high molecular weight fraction at 0.2 μg/ml, HMW (L); cathepsin-L pool at 10 μg/ml. Group D animals are marked by an asterisk *.

Figure 4.13: Production of IFN-γ by cryopreserved hepatic lymph node cells in response to a 72 hour culture period with high molecular weight and cathepsin fractions (as for Figure 4.12), shown as the net response (difference between antigen-stimulated and unstimulated cultures) for individual animals.

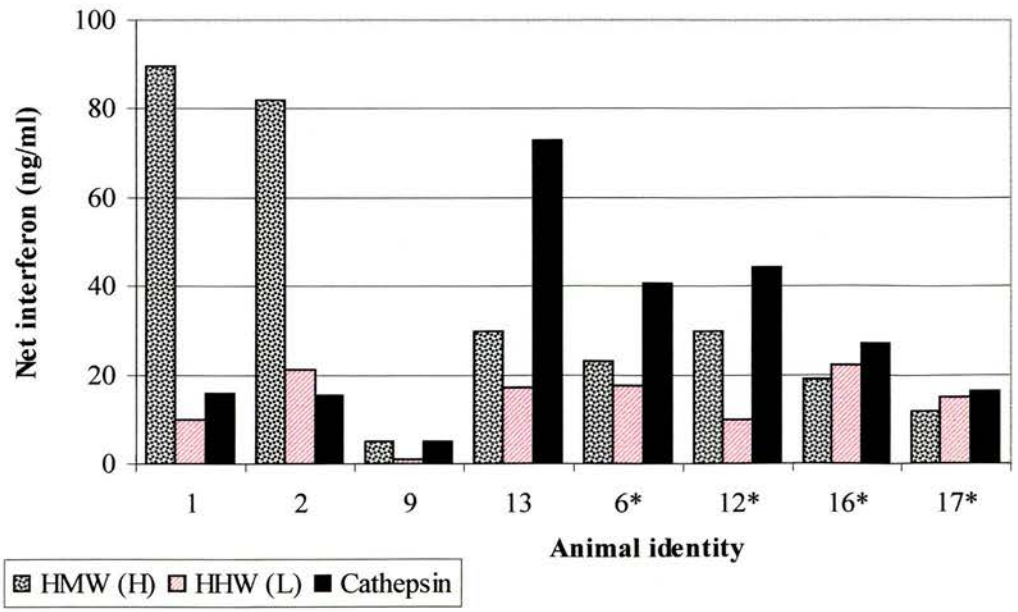


Table 4.4: Comparison of IFN-γ production by fresh and cryopreserved hepatic lymph node cells after a 72 hour culture with whole fluke antigen at 15 μg/ml (group D animals are marked *). Net IFN-γ production (difference between antigen-stimulated and unstimulated cultures) is shown.

Cell source	Animal identity, giving net IFN-γ (ng/ml) in culture fluid							
	1	**2**	**9**	**13**	**6***	**12***	**16***	**17***
Fresh	10.4	13.7	12.3	40.6	8	40	57.7	31.9
Cryo-preserved	18.5	13.9	3.4	38.8	25	63	36.6	26.1

4.4 DISCUSSION

4.4.1 Distinct differences between hepatic and mesenteric lymph node responses

In this study a clear difference was observed between the local lymphocyte response of hepatic and mesenteric lymph node derived cells to stimulation with the WFA preparation. Hepatic node cells from both pre-exposed (group B) and naïve animals (group D) responded vigorously to antigen stimulation, and in many cases this response exceeded or was of similar magnitude to the mitogen induced proliferative response. It can therefore be concluded that within 10 days of infection, juvenile flukes reached the liver and were present for long enough to stimulate an effective lymphocyte response. Proliferative responses were similar in both groups, with the exception of calf 6 (group D), so pre-exposure did not have any modulating effect on the magnitude of the hepatic lymph node cellular response to a secondary infection. This observation is supported by the results of peripheral blood mononuclear cell proliferation assays, in which both groups B and D showed similar proliferative responses to stimulation with WFA at day 37. In contrast, mesenteric node derived cells responded weakly to stimulation with the WFA preparation, although naïve animals showed a slightly greater response than pre-exposed animals. The local response to adult derived *F. hepatica* proteins in this experiment was therefore greatest at the level of the liver and not the gut.

As flukes mature and migrate through the host, their antigenic profiles change, with some proteins being lost and others gained (Lammas and Duffus, 1985; Dalton and Joyce, 1987; Tkalcevic *et al.*, 1996). In this experiment, responses to mature fluke preparations, only, were examined. Ideally, mesenteric cells should have been stimulated with a metacercarial or a newly excysted juvenile preparation, as these are the stages that gut associated mononuclear cells will have been exposed to. Unfortunately, due to cost and the difficulties of supply at the time of the experiment, sufficient parasites were not available to make these preparations. However, distinct hypersensitivity reactions in colonic mucosae from infected rats have been noted *in vitro* in response to specific stimulation with adult-derived antigens (O'Malley *et al.*, 1993). Gut responses in the rat are therefore not entirely specific to early juvenile fluke antigens. The absence of a significant mesenteric response to juvenile antigens in this study would have allowed more definite conclusions to be drawn regarding the

inactivity of mesenteric lymphocytes to fluke proteins after *Fasciola* infection.

4.4.2 Species differences in the gut response

The results of this experiment suggest that the gut response to challenge infection in the pre-exposed group was not significant. The lack of a gut-level response is in agreement with the results of a previous study, in which no difference was found between the number of flukes present in the peritoneal cavity of naïve and sensitised calves after secondary challenge (Doy and Hughes, 1984). However, eosinophilia and an increase in the number of mucosal mast cells have been detected in ileal epithelium after secondary challenge infections in cattle, suggesting the presence of a local cellular reaction in the gut (Wicki *et al.*, 1991). Furthermore, *in vitro* challenge of colonic mucosae, obtained from chronically infected cattle, with *F. hepatica* proteins results in mucosal ion transport, again suggesting specific gut reactivity to adult fluke proteins (McCole *et al.*, 1998). The gut mucosa is therefore reactive during fluke infection; whether a local lymphocyte response is involved and whether the observed mucosal changes are beneficial to the host, is not known.

In contrast, the situation in the rat is quite different. Resistance to challenge infections occurs at both the level of the gut and peritoneum or liver capsule (Doy and Hughes, 1982). Immunohistochemical studies and experiments using *ex vivo* gut loops, have demonstrated that resistance to challenge infection in sensitised rats is associated with an increased number of eosinophils, mast cells and IgE-positive cells in the gut mucosa (van Milligen *et al.*, 1998a; 1999). Mesenteric node cells from mice infected with *F. hepatica* produce cytokines after stimulation with fluke proteins (O'Neill *et al.*, 2000) and mesenteric nodes from infected rats are able to produce fluke-specific antibody in cell culture (Meeusen and Brandon, 1994). These studies show that specific mesenteric node responses to fluke proteins can be elicited during infection in experimental models.

In this experiment, cytokine levels in bovine mesenteric lymph node cell culture fluid were not measured, due partly to the absence of a significant response in the proliferation assay, but also because the assays were very costly. It may be that mice and rats, being inappropriate hosts for *Fasciola*, due to differences in the size ratio between parasite and host, experienced greater gut pathology than would normally be seen in the natural ruminant host. This could account for differences in

the immune responses and specifically in the gut response, between rodents and the natural ruminant host.

4.4.3 Factors affecting the mesenteric lymph node response

One problem with interpreting the mesenteric response is the possibility that responsive cells are not evenly distributed throughout the mesenteric lymph nodes. In this experiment, samples of only one or two nodes were taken. The small intestine is very long and as sampling had to be rapid to preserve the viability of cells and reduce the chances of contamination, node selection was random. Ideally, the intestine should have been unravelled and samples taken from nodes draining equivalent stretches of the intestine. If flukes had penetrated the gut at some distance from the sampling point and reactive cells were few and unevenly distributed, these reactive cells could have been missed by sampling the wrong node.

The location of gut penetration by newly excysted juveniles (NEJs) in the intestine has only been examined in experimental animal models. The results of these studies suggest that the majority of migration occurs through the mucosa of the small intestine, particularly the jejunum (Kawano *et al.*, 1992; van Milligen *et al.,* 1998c). NEJs do reach the colon in challenged rats, but significant penetration here is thought to be unlikely. Gut passage in simple stomached animals with a short intestinal length will be quicker than in ruminant animals. Flukes may therefore be able to penetrate the intestine over a greater part of its length in rats, than in cattle. Whether migration is site restricted and the importance, therefore, of sampling particular regions of mesenteric node tissue in cattle, is not known. It should be noted, however, that in the rat *ex vivo* model, intestinal mucosal responses were not restricted to the site of challenge in the small intestine, but could also be found in large intestinal tissue some distance from the site of NEJ penetration (van Milligen *et al.*, 1998a).

4.4.4 Cytokine production by hepatic lymph node cells

The analysis of cytokine production by bovine cell cultures is severely hampered by the lack of reliable assays for quick, reproducible and accurate quantification. Consequently, full use of the material obtained in this experiment could not be made and only tentative conclusions about cell responses to various

protein fractions could be drawn. The proliferation obtained in the bioassays used to detect the presence of IL2 and B cell growth factor, was only moderate, even with positive control samples. To support the bioassay results, the presence of mRNA in stimulated cells, encoding the various cytokines, should have been examined. Unfortunately, although RNA was prepared, further assays could not be performed due to lack of time.

Hepatic lymph node cells from all animals produced IFN-γ in response to WFA stimulation. Overall, naïve animals (group D) produced more IFN-γ than pre-exposed animals (group B). This pattern was also reflected in the results of the IL2 bioassay: animals producing high levels of IFN-γ, had a high IL2 stimulation index. Little difference was seen in the ability of supernatant fluid from the two groups to stimulate B cell growth. Due to lack of materials, an inhibition study using an anti-bovine IL4 monoclonal antibody was not carried out. If addition of the antibody to bioassay cultures had completely ablated growth of cells, it would be reasonable to assume that the factor in culture supernatant causing B cell growth was indeed IL4.

Only two other studies examining local lymph node cytokine production in responses to *F. hepatica* have been published to date. In a study of sheep, hepatic lymph node cells obtained during early primary infection showed moderate proliferation in response to stimulation with excretory-secretory (ES) preparation, although activity varied considerably between individuals (Moreau *et al.*, 1998). IFN-γ production was detected in only half of the animals sampled at day 10 post-infection, but was not found at any later time-points. *F. hepatica* infected mice respond more consistently, with both hepatic and mesenteric lymph node cell cultures producing cytokines after stimulation with crude adult homogenate at 3 weeks post-infection (O'Neill *et al.*, 2000). Cytokine profiles varied with mouse strain, infective dose and the concentration of antigen used to stimulate cells, however overall, an IL4/IL5 type response appeared to predominate. High dose infections were shown to reduce mitogen-induced production of IFN-γ and IL2 by spleen cells, compared to low dose infections, suggesting some modulation of the lymphocyte response occurred. Whereas in this study, cell responses to stimulation with Con A were strong in both groups of animals.

The results shown here are consistent with the view that during *F. hepatica* infection, bovine T helper cells are able to produce mixed cytokine responses (Brown *et al.*, 1994a): hepatic node cells from groups B and D produced IFN-γ, IL2 and B cell growth factor after stimulation with fluke proteins. This is in contrast to the situation in the mouse, where infection resulted in a predominantly Th2 like response in both hepatic and mesenteric lymph nodes. The slightly lower production of IFN-γ by pre-exposed calves may suggest the presence of a more refined recall response. It must be remembered that the WFA and ES preparations used here for cell stimulation were crude, containing various antigens and therefore probably capable of stimulating a heterogeneous T cell population with opposing effects.

4.4.5 Cellular responses to isolated *F. hepatica* proteins

Cathepsin-L pool and high molecular weight (HMW) fractions became available in limited quantities for testing cellular responses some time after the initial experiment was carried out. Cells used were therefore those that had been cryopreserved in vapour phase nitrogen. Due to lack of antigen material, the assays were not well optimised. However, the results provide a general indication of the local hepatic node responses to these two antigens in infected cattle, which has not been previously examined. Given the interest in these two preparations as potential vaccine candidates (Dalton *et al.*, 1996), understanding lymphocyte responses to these proteins during natural infection is critical.

Cell proliferative responses to the HMW fraction were much greater than to the cathepsin fraction at the concentrations used, with both pre-exposed and naïve animals giving a good proliferative response. With the exception of calf 2, naïve animals (group D) showed a stronger response than those that were pre-exposed (group B) with both protein fractions. IFN-γ production at 72 hours was very variable: high levels were present in culture fluid from calves 1 and 2 (group B) after HMW stimulation, whereas that in all other animals was much lower. Cathepsin induced IFN-γ production was similar to that induced by the HMW fraction, despite the fact that cellular proliferation was lower. It is a possibility that the peak proliferative response to the cathepsin fraction occurred at an earlier time-point than with the HMW fraction and was missed by examining the 5-day response. The timing

of the proliferative response, together with the use of a wider range of cathepsin concentrations, should be further investigated. Individual cathepsin induced IFN-γ production appeared to follow the pattern observed with the WFA and ES preparations, with group D animals producing more IFN-γ than those of group B (excluding calf 13). Unfortunately, it was not possible to measure the levels of IL2 and IL4 in the supernatant fluid by bioassay. Conclusions regarding the overall cytokine profiles induced by these proteins cannot, therefore, be made.

In summary, the high molecular weight fraction induced a moderate to high proliferative response with the majority of samples, whilst stimulation with the cathepsin fraction only resulted in a weak response. In contrast, IFN-γ production was high after stimulation with the cathepsin fraction, but variable in response to the high molecular weight fraction. Both fractions were recognised by host cells during the early stages of *F. hepatica* infection, suggesting that they were either produced by, or cross reactive with proteins of, the early juvenile fluke.

4.4.6 Further limitations in the experimental method

The variability in the response of cryopreserved cells to the isolated fluke proteins between animals of the same group may have been due partly to poor optimisation of the assay, but also to alterations in cell viability after defrosting. Cryopreserved cells did grow well in response to mitogen, WFA and ES stimulation and responses within groups were more consistent with these preparations than with the HMW fraction. However, mitogenic and WFA responses were reduced in comparison to those obtained from cultures set up with equivalent fresh cells. In addition, IFN-γ production by fresh and cryopreserved cells in response to WFA stimulation varied. Survival of cells during resuscitation was approximately 50%. Further losses may have occurred after cell counting, during the plating procedure and during the early hours of culture, which may have introduced further inconsistency into the experiment. Any possible changes in cell phenotype proportions after cryopreservation should have been examined, to determine whether specific cell populations were lost during the preservation process. Ideally, all culture assays should have been performed with fresh cells.

4.4.7 Conclusions

The dominant cellular response at 10 days post-infection to *F. hepatica* whole fluke antigen occurred in hepatic and not mesenteric lymph nodes. There was no difference between the level of the proliferative response of peripheral and hepatic lymph node mononuclear cells from 5-6 day pre-exposed or naïve animals. However, naïve animals did display a weak mesenteric response, which was not detected in the pre-exposed animals. Hepatic lymph node cells stimulated with whole fluke antigen produced IFN-γ, IL2 and B cell growth factor: an unrestricted T helper cell cytokine profile. Cells from naïve animals tended to produce a greater amount of IFN-γ than those from pre-exposed animals.

The phenotypes of peripheral blood mononuclear cells from pre-exposed animals did not change over the period of study. There was also no significant difference in the cell phenotypes present within the hepatic lymph nodes of naïve and pre-exposed animals.

The high molecular weight fraction induced a moderate proliferative response in hepatic node-derived cells, whilst the cathepsin fraction resulted in a poor response. Both fractions stimulated the production of IFN-γ.

CHAPTER 5

IgG1 and IgG2 antibody responses of *F. hepatica* infected calves to purified cathepsin-L1 and L2 pool and high molecular weight fractions.

5.1 INTRODUCTION

Juvenile and adult flukes secrete a variety of proteins *in vitro* culture which are also thought to be released *in vivo*. A number of these proteins have been isolated and characterised, including the cathepsin proteases and a haem-containing high molecular weight fraction.

As discussed previously (section 1.2.10.3), the cathepsin proteases are able to cleave immunoglobulin and prevent eosinophil attachment to newly excysted juveniles *in vitro* (Smith *et al.*, 1993b; Carmona *et al.*, 1993). Although it has not been demonstrated that this is a major activity of the protease within the mammalian host, it is possible that immunoglobulin cleavage is an active immune evasion mechanism. Vaccination of laboratory animals with purified cathepsin proteases results in the production of neutralising antibody (Smith *et al.*, 1994). Cathepsin proteases may therefore be good candidate antigens for use in vaccination against fascioliasis. Antibody to cathepsin stimulated by vaccination could prevent the destruction of antibodies directed against other targets, possibly allowing host-mediated fluke damage and subsequent elimination.

Vaccine trials, using cathepsin protease in conjunction with the haem-containing high molecular weight fraction have had variable results (Dalton *et al.*, 1996; Mulcahy *et al.*, 1998). One characteristic of vaccination was the induction of a strong IgG2 antibody response to fluke products, not an isotype seen typically during natural infections (Clery *et al.*, 1996; Bossaert *et al.*, 2000). This was noted when the vaccine was administered in both Freund's complete and Freund's incomplete adjuvant. However, administration of the vaccine in the latter adjuvant did not elicit significant protection to challenge infections. Furthermore, animals vaccinated with cathepsin/haem fractions in Freund's incomplete adjuvant showed similar correlations between IgG1/IgG2 antibody titres and fluke burden to animals receiving adjuvant alone. Thus vaccination and more specifically the adjuvant used,

appeared to have a strong influence on the isotype pattern observed after challenge infection.

Given the intense interest in these preparations as vaccine antigens, examining antibody responses to these proteins during the early infection period is important. In this experiment, sera from the four groups of calves of Experiment A were tested for antibody responses to purified cathepsin-L1/L2 pool and high molecular weight fractions throughout the course of the study. Both IgG1 and IgG2 isotype responses were examined, in order to determine whether a typical IgG1 response occurred, or an IgG2 response, as for vaccinated animals. Another reason for examining the antibody isotype pattern is that different cytokines preferentially stimulate the IgG1/G2 isotypes (section 1.2.9.2). By determining the IgG isotype response, an indication of the nature of the T helper response can also be obtained.

Antibody responses were measured by indirect enzyme-linked immunosorbent assay (ELISA). A simple analysis, examining optical density values for the two antibody isotypes at selected time-points pre- and post-secondary challenge infection was carried out initially. From these profiles, the time-points where peak reactions occurred were selected and individual sera samples were then fully titrated and group cut-off titre values calculated. A basic analysis was performed, comparing titre values and the various infection parameters measured previously (Chapter 3).

Experimental Aims:

- To investigate the IgG1/G2 antibody isotype responses to cathepsin-L pool and high molecular weight fractions, in the four groups of calves of Experiment A.

- To examine any differences between the responses of pre-exposed and chronically infected animals during the first four weeks of infection and between all groups of animals after secondary challenge.

5.2 MATERIALS & METHODS

5.2.1 Antigen preparations

The high molecular weight and cathepsin-L1/L2 pool fractions, prepared from adult fluke excretory-secretory products, were gifts provided by Professor J. Dalton, Dublin City University, Glasnevin 9, Dublin, Ireland (section 2.1.4).

5.2.2 Sera samples

Sera from all four groups of calves used in Experiment A (Chapter 3) were examined for antibody responses to both fractions. The groups consisted of a chronic infection group, A; a 5 day pre-exposure group, B; a 24 hour pre-exposure group, C and a naïve group, D, which had all then received a secondary challenge infection.

The longitudinal antibody response to the high molecular weight fraction between days 0-98 and to the cathepsin-L1/L2 pool fraction between days 0-112 was investigated. This was performed by examining the group mean optical density values obtained with test sera fixed at an optimised dilution. Individual sera were also titrated out fully for each fraction: at days 28 and 56 with the high molecular weight fraction and at day 105 for the cathepsin-L pool fraction. All samples were assayed in triplicate for the longitudinal screens and in duplicate for the titration series. Known positive and negative control sera was used as the plate standards and for optimising the ELISA systems, which were obtained from a *F.hepatica* chronically infected animal (calf 95).

5.2.3 Secondary and tertiary antibodies

Mouse anti-bovine IgG1 and IgG2 monoclonal antibodies, obtained from the hybridoma clones CVI.BoIgG1.15.8.1a and CVI.BoIgG2.12.5.4c, respectively (ID-DLO, Edelhertweg 15, 8219 PH Lelystad, The Netherlands; Van Zaane and Ijzerman, 1984), were used to detect bovine IgG1 and IgG2 antibody isotypes. A working stock at a concentration of 2 mg/ml was prepared in PBS. Bound monoclonal antibodies were detected using a polyclonal rabbit anti-mouse IgG horseradish peroxidase conjugate (Sigma A-9044).

5.2.4 ELISA optimisation

Optimal concentrations of antigen, test sera and anti-bovine IgG1/G2 monoclonal antibodies were determined by sequential titration, using calf 95 positive and negative sera. The tertiary conjugate antibody was used within the recommended range, at a 1/5000 dilution.

5.2.5 ELISA protocol

Immulon-1 (Dynex Technologies, UK), 96 well microtitre plates were coated with the antigen fraction at the relevant concentration prepared in carbonate buffer, pH 9.6 (Sigma C-3041). The plates were covered with cling-film and left overnight at 4°C. Plates were washed three times with PBS-T (Appendix A), then blocked with 2% normal horse sera in PBS-T for 1 hour at room temperature. Plates were washed twice in PBS-T and then incubated with test sera, at the appropriate dilution in 1% normal horse sera in PBS-T (ELISA diluent) for 1 hour at 37°C. Plates were again washed three times and then incubated with the secondary monoclonal antibody, prepared in ELISA diluent, for 1 hour at 37°C. Plates were washed a further three times with PBS-T. The tertiary polyclonal anti-mouse-Ig horseradish peroxidase conjugate was then used to detect any bound mouse monoclonal; again, a 1 hour, 37°C incubation step was used. After a final three-wash step, bound conjugate was detected with the substrate 3,3',5,5'– tetramethylybenzidine, TMB (two component system, Kirkegaard and Perry Laboratories Inc.). The reaction was stopped with 2M sulphuric acid. All well volumes throughout were 100 µl, except for the blocking step, which was 180 µl per well. Plates were read immediately at 450 nm on a ICN Titertek Multiscan Plus (MK11) for the high molecular weight time-series and on a BIO-TEK Microplate Autoreader (EL311) for all other ELISAs.

5.2.6 Statistical analysis

Statistical analysis was performed using the Minitab 12® software package (Minitab Inc., USA). Group differences for the longitudinal analysis were investigated by the Mann-Whitney U test. Differences in cut-off titre values were examined by the Student's t-test. Any possible association between antibody responses and other infection parameters were analysed by Spearman's rank correlation.

5.3 RESULTS

5.3.1 ELISA optimisation

A summary of the reagent dilutions used for both the high molecular weight and cathepsin-L pool fraction longitudinal ELISAs are shown in Table 5.1. Results of the titration of the high molecular weight fraction are given in the following section to demonstrate the method used for assay optimisation.

Table 5.1: Summary of optimised antigen, sera, monoclonal (secondary) antibody and conjugate (tertiary) antibody dilutions used for the cathepsin and high molecular weight time-series analysis.

Fraction	Antigen concentration	Sera dilution	Monoclonal dilution	Conjugate dilution
High molecular weight	0.5 μg/ml	1/300	1/2000	1/5000
Cathepsin-L1/L2 pool	2.5 μg/ml	1/150	1/2000	1/5000

5.3.2 Titration results for the high molecular weight fraction

Antigen titration

The protein fraction was titrated downwards from a concentration of 4 μg/ml in a doubling dilution series, with sera at 1/200, secondary monoclonal antibodies at 1/2000 and the tertiary conjugate antibody fixed at 1/5000 dilutions. Calf 95 pre-infection and days 20, 48 and 88 post-infection sera were used. The titration curve for the IgG1 isotype is shown in Figure 5.1 and the binding ratios of positive to negative sera at each dilution point for the IgG1 response in Table 5.2. A final concentration of 0.5 μg/ml was chosen for use; this was slightly lower than the optimum determined by binding ratio, but was midway down the linear phase of the titration curve. This offered a good distinction between positive and negative sera whilst conserving the limited amount of material available.

Sera titration

Using a coating antigen concentration of 0.5 μg/ml, calf 95 pre-infection, day 20 and day 68 post-infection sera were titrated from a 1/50 to a 1/3200 dilution in a doubling series. Monoclonal, secondary antibody and tertiary conjugated antibody concentrations were fixed at 1/2000 and 1/5000 dilutions, respectively. Figure 5.2 shows the titration curve obtained for the IgG1 isotype and Table 5.3 shows binding ratios for both IgG1 and IgG2 isotypes. For both isotypes a sera dilution of 1/300 was chosen, as this was within the more linear phase of the titration curve.

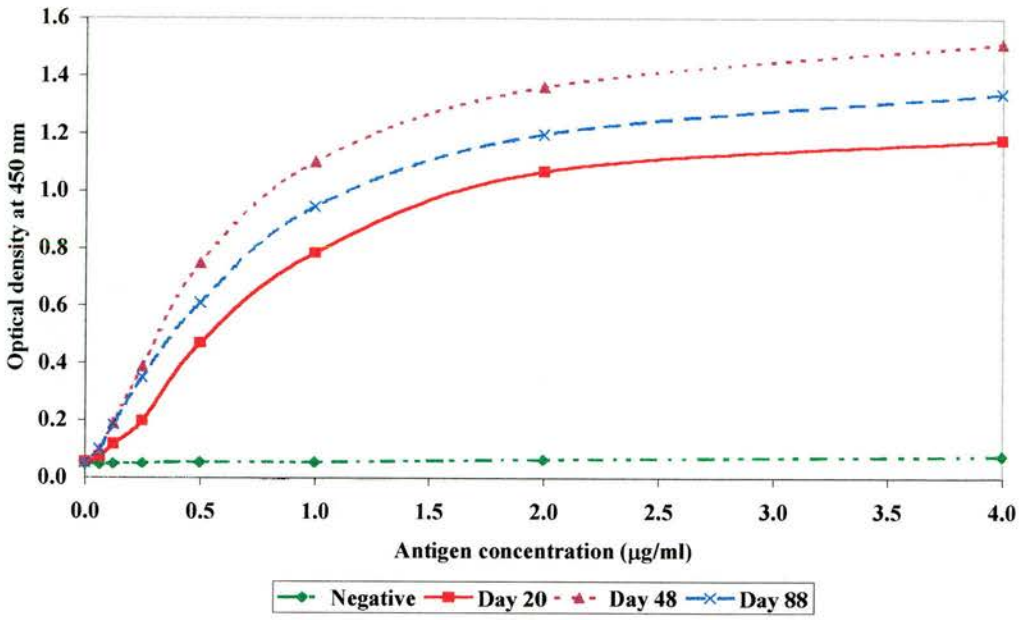
Monoclonal, secondary antibody titration

Anti-bovine IgG1 and IgG2 monoclonal antibodies were titrated in a doubling series from 1/500 to 1/32000 dilutions of the 2 mg/ml working stock, with antigen at 0.5 μg/ml, sera at 1/200 and tertiary conjugate antibody at 1/5000 dilutions. The binding ratios obtained with day 68 post-infection sera are shown in Table 5.4. A dilution of 1/2000 for both monoclonals was decided upon. Again this is slightly lower than optimum, but within the active region of the titration curve.

Table 5.2: Antigen titration binding ratios for the high molecular weight fraction and IgG1 antibody isotype, showing the optical density value ratio between calf 95 negative and days 20, 48 and 88 post-infection sera. Sera was used at 1/200, secondary monoclonal antibody at 1/2000 and tertiary conjugate antibody at 1/5000 dilutions.

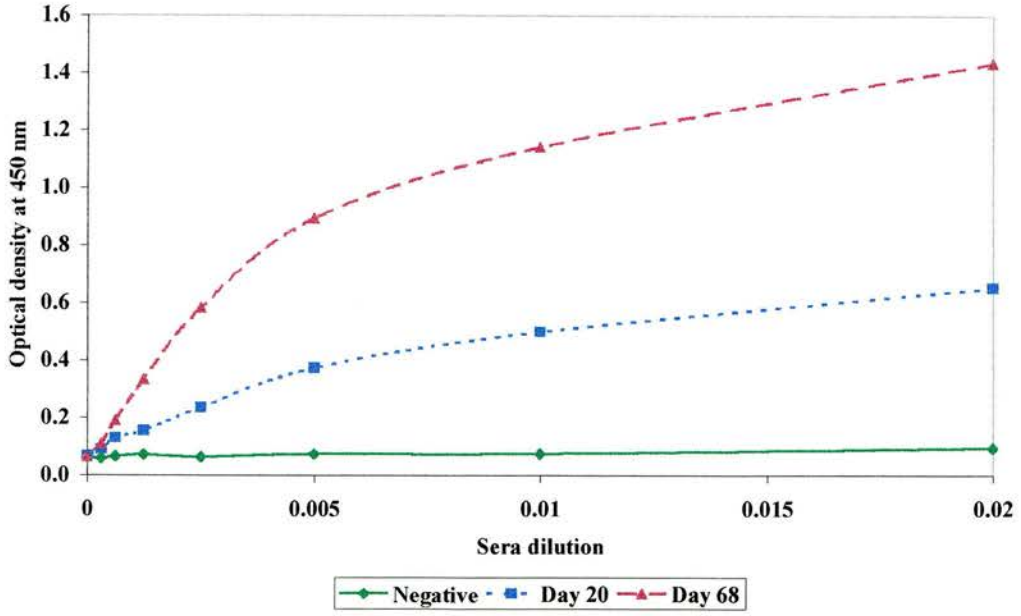
Antigen in μg/ml	Binding ratio		
	Day 20	Day 48	Day 88
4	15.9	20.3	18.0
2	17.0	21.7	19.1
1	14.7	20.6	17.7
0.5	8.8	14.1	11.5
0.25	4.0	7.9	7.1
0.125	2.4	4.0	3.9
0.065	1.5	2.1	2.1
0	1.1	1.1	1.1

Figure 5.1: Antigen titration curve for the high molecular weight fraction and the IgG1 antibody isotype.



Calf 95 pre-infection and days 20, 48 and 88 post-infection sera were used at a 1/200 dilution, with secondary anti-bovine IgG1 at 1/2000 and tertiary antibody conjugate at 1/5000 dilutions.

Figure 5.2: Sera titration curve for the high molecular weight fraction and the IgG1 antibody isotype.



High molecular weight fraction was used at 0.5 µg/ml; secondary anti-bovine IgG1 at 1/2000 and tertiary antibody conjugate at 1/5000 dilutions. Calf 95 pre-infection and days 20 and 68 post-infection sera was titrated from 1/50 to 1/3200 dilutions in a doubling series.

Table 5.3: Sera titration binding ratios for the high molecular weight fraction (0.5 μg/ml) and IgG1/IgG2 antibody isotypes, showing the optical density value ratio between calf 95 negative and days 20 and 68 post-infection sera. The secondary monoclonal antibody was used at a 1/2000 and tertiary conjugate antibody at a 1/5000 dilution.

Sera dilution	Binding ratio			
	IgG1		IgG2	
	Day 20	Day 68	Day 20	Day 68
1/50	7.0	15.4	2.4	2.4
1/100	6.8	15.6	2.0	2.0
1/200	5.2	12.4	1.3	1.3
1/400	3.8	9.4	1.6	1.6
1/800	2.2	4.7	1.3	1.2
1/1600	2.0	3.0	1.0	1.0
1/3200	1.5	1.9	1.1	0.9
0	1.0	1.0	1.2	0.9

Table 5.4: Secondary monoclonal antibody titration for the high molecular weight fraction (0.5 μg/ml) and IgG1/IgG2 antibody isotypes, showing the optical density value ratio between calf 95 negative and day 68 post-infection sera. Sera was used at a 1/200 and tertiary conjugate antibody at a 1/5000 dilution.

Monoclonal dilution	Binding ratio	
	IgG1	IgG2
1/500	20.5	2.2
1/1000	18.7	2.2
1/2000	15.6	2.4
1/4000	12.5	2.6
1/8000	9.4	2.5
1/16000	6.2	2.7
0	1.4	1.9

5.3.3 Longitudinal IgG1 and IgG2 antibody responses to the high molecular weight fraction over the period days 0-98

The group mean optical density values for the IgG1 and IgG2 antibody responses to the high molecular weight fraction over the time-course studied are shown in Figures 5.3 and 5.4. The results of Mann-Whitney U tests to compare differences in optical density values between the four groups are shown in Tables 5.5 and 5.6.

An early rise in both the IgG1 and IgG2 antibody isotype responses was observed with groups A and B. The IgG1 response was highest in group B at day 14, but by day 28 this had altered so that group A showed the higher response. There was a small rise in the group C IgG1 antibody response during this early phase, which reached a similar value to that of group B by day 28. In contrast, both groups A and B showed a consecutive and matched early rise in IgG2 antibody levels during the first 4 weeks of the experiment, whilst the IgG2 response of group C was negligible. The difference in the IgG1 response between groups A and B at day 28 was not statistically significant, whilst that between groups A and C was significant.

After the secondary challenge infection, the IgG1 antibody response was boosted in all groups, although the rise in group A was minimal. Groups B, C and D all showed a similar increase in mean optical density values, rising to a maximum at day 56 and then gradually declining. The group B IgG1 response was higher than that of group D and this difference was significant on days 42 and 56. Although the group C response was similar to that of B, it remained slightly lower and was not significantly different to that of group D at any point. Groups B, C and D all showed significantly higher IgG1 responses than group A at days 56 and 70.

The pattern of the IgG2 response after secondary challenge was similar to that seen with the IgG1 isotype, although actual optical density values were much lower. One notable difference was the fact that with the IgG2 isotype, group D showed a greater response than group B, although this difference was not significant.

Figure 5.3: Longitudinal IgG1 antibody response to the high molecular weight fraction over the period days 0-98, showing the group mean optical density values.

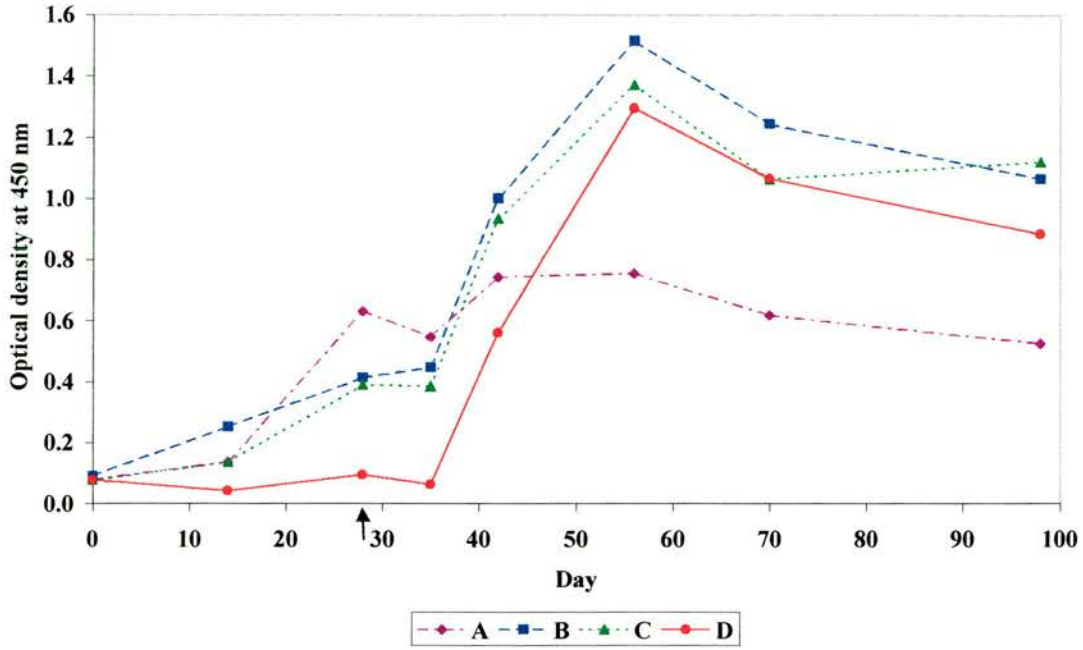
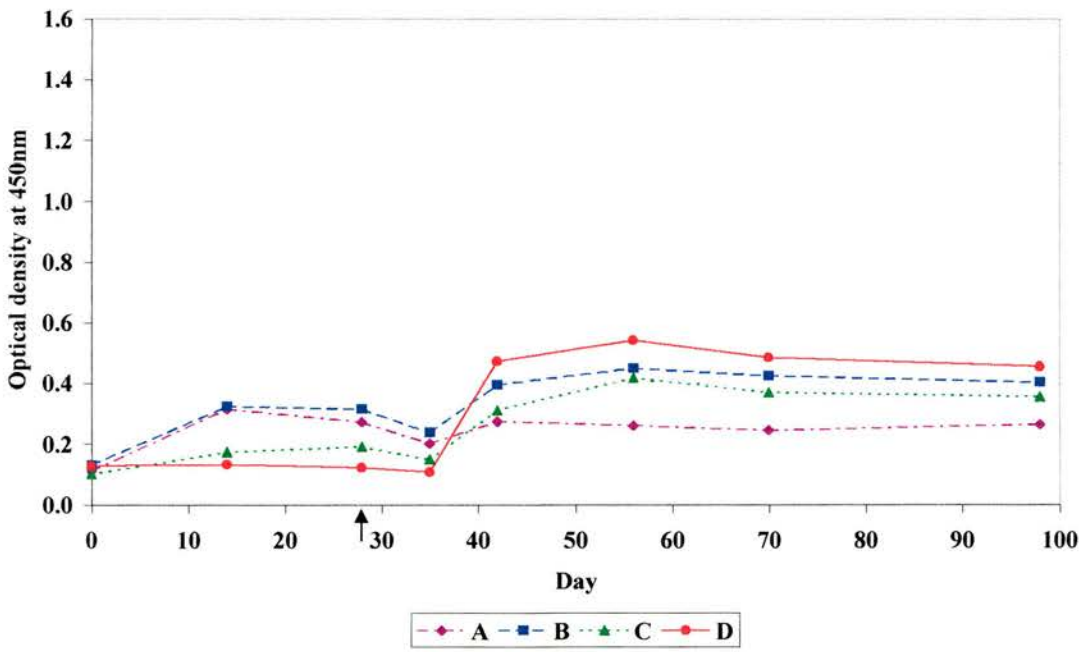


Figure 5.4: Longitudinal IgG2 antibody response to the high molecular weight fraction over the period days 0-98, showing the group mean optical density values.



Figures 5.3 and 5.4: The high molecular weight fraction was used at 0.5 μg/ml, with sera at 1/300, secondary monoclonal antibody at 1/2000 and tertiary conjugate antibody at 1/5000 dilutions. The arrow indicates the time of secondary challenge. Error bars are not shown to aid clarity, however, the results of statistical analysis comparing group differences are shown in Tables 5.5 and 5.6 on the following page.

Note: A copy of Figure 5.3, including error bars, is given in Appendix B.3.

Table 5.5: Results of Mann-Whitney U tests comparing group differences for IgG1 and IgG2 antibody isotype responses to the high molecular weight fraction prior to secondary challenge. The calculated *P* value is shown for the given comparisons.

Time-point	IgG1 response		IgG2 response	
	A: B	A:C	A: B	A:C
Day 14	0.27	0.27	0.41	0.01**
Day 28	0.10	0.05*	0.85	0.09

Key:
A result that is statistically significant is marked * and highly significant **.

Table 5.6: Results of Mann-Whitney U tests comparing group differences for IgG1 and IgG2 antibody isotype responses to the high molecular weight fraction after secondary challenge. The calculated *P* value is shown for the given comparisons.

Isotype	Comparison	Day 42	Day 56	Day 70
IgG1	A:D	0.19	0.0003***	0.001***
	B:D	0.001***	0.05*	0.09
	C:D	0.18	0.39	0.71
IgG2	A:D	0.06	0.001***	0.004**
	B:D	0.82	0.62	0.97
	C:D	0.17	0.24	0.29

Key:
A result that is statistically significant is marked *, highly significant ** and very highly significant ***.

5.3.4 Longitudinal IgG1 and IgG2 antibody responses to the cathepsin L1/L2 pool fraction over the period days 0-112

The longitudinal profile of the IgG1 antibody response to the cathepsin-L pool fraction is shown in Figure 5.5.

IgG1 antibody levels in group A began to rise from day 35, climbing to a sharp peak at day 70, declining to day 84 and then tailing off. Antibody levels in groups B, C and D did not rise until after day 56. The response of group D was the greatest, whilst groups B and C had lower and almost identical responses. The peak response in the latter three groups occurred at day 98. There was no significant difference between the response of group D and groups B or C at any time-point; there was also no significant difference between the peak value of group A and that of groups B, C or D (Figure 5.7).

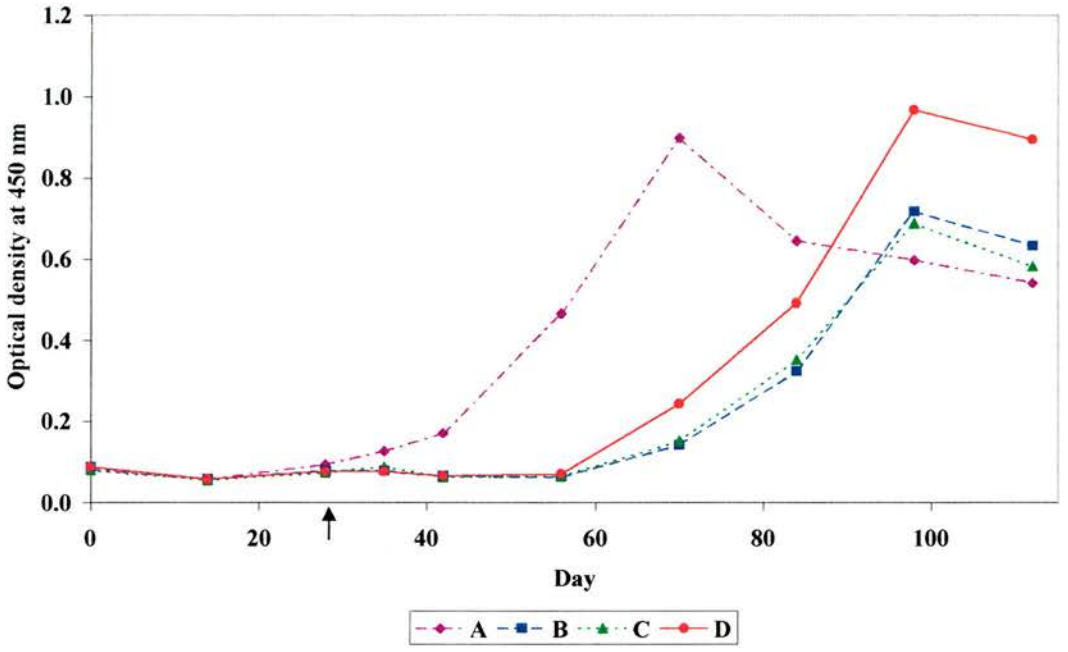
The IgG2 antibody response to the cathepsin fraction was negligible in all four groups and is not shown.

Table 5.7: Results of Mann-Whitney U tests comparing group differences for the IgG1 antibody response to the cathepsin-L pool fraction at the time-points shown. The calculated *P* value is shown for the given comparisons.

Test	A:B	A:C	A:D	B:D	C:D
Day 42	0.001***	0.001***	0.001***		
Day 56	0.0002***	0.0004***	0.0002***		
Day 70			0.001***	0.43	0.96
Day 84			0.35	0.12	0.40
Day 98			0.06	0.27	0.23
Day 112			0.08	0.21	0.10

Key:
Results that are statistically very highly significant are marked ***.

Figure 5.5: Longitudinal IgG1 antibody response to the cathepsin-L pool fraction over the period days 0-112, showing group mean optical density values.



The cathepsin fraction was used at a concentration of 2.5 μg/ml, with sera at 1/150, secondary monoclonal antibody at 1/2000 and tertiary conjugate antibody at 1/5000 dilutions. The arrow indicates the time of secondary challenge. Error bars are not shown to aid clarity, but the results of statistical analysis comparing group responses are shown on the preceding page.

Note: a copy of this figure, including error bars, is given in Appendix B.4.

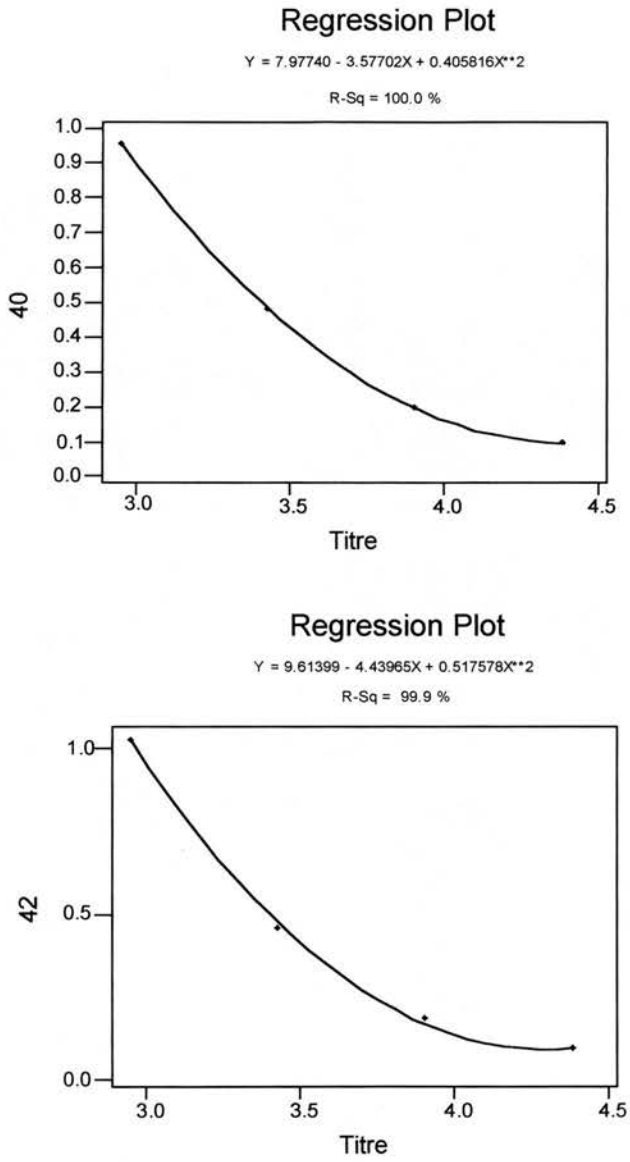
5.3.5. Estimated antibody cut-off titres for the high molecular weight fraction at days 28 and 56

All day 28 and 56 sera were titrated in a three-fold dilution series from a starting dilution of 1/100, for both antibody isotypes. An arbitrary negative cut-off titre for each sera was determined by calculating the dilution giving an optical density value of 0.2 for the IgG1 isotype and 0.15 for IgG2. Negative cut-off titres are often taken as the value given by the negative control standard plus three standard deviations of replicates from the mean value. This convention was not used in this study. Slight variation in the negative control standard value was observed between plates. Although this variation was corrected by plate standardisation, a small difference was still present. The significance of this difference could have been magnified by taking curve intersections at such low optical density values. A higher, arbitrary cut-off value was therefore chosen.

Cut-off titres were calculated by fitting a quadratic polynomial curve through each data set using regression analysis in the Minitab 12® statistical package (Figure 5.6). The data points where the curve tended towards saturation (either high or low) were omitted, because these had the effect of distorting the curve away from the plotted data. The quadratic equation was solved numerically, by standard formula, for x where y = 0.2 (for the IgG1 response) or 0.15 (for the IgG2 response).

Group mean cut-off titre values for IgG1/IgG2 antibody responses to the high molecular weight fraction at days 28 and 56 for the four groups are shown in Tables 5.8 and 5.9 and Figures 5.7 and 5.8. Differences between log titre values were compared using the Student's t-test.

Figure 5.6: Sera titration curves for calves 40 and 42 for the IgG1 antibody isotype response to the high molecular weight fraction on day 56, showing quadratic curves fitted by regression analysis.



Quadratic polynomial curves were fitted through sera titration data points, given as log titre (x axis) and optical density value at 450 nm (y axis) using regression analysis. In this figure, the regression curves for titration series of calves 40 and 42 for the IgG1 response to the high molecular weight fraction at day 56 are shown. The lower two log titre points of log 2.0 and 2.48 (equivalent to a 1/100 or 1/300 dilution) are omitted, because the response had begun to saturate at high optical density values. Cut-off titres were obtained by solving the given quadratic equation for x, where y = 0.2 (for the IgG1 isotype) or where y = 0.15 (for the IgG2 isotype). The r^2 values indicate the goodness of fit for the curve; all r^2 values were greater than 95%.

Cut-off titres for the high molecular weight response

At day 28, group A had the highest IgG1 titre, followed by groups B then C; group D remained effectively negative. The difference in titre between groups A and B, and A and C was statistically significant (Table 5.10). The IgG2 isotype showed a different pattern of response, with similar cut-off titres in groups A and B, whilst groups C and D had low titres. Unlike the IgG1 response, the slight difference between the IgG2 cut-off titres for groups A and B was not significant. These results support the observations from the longitudinal screens, in which group A showed a higher IgG1 response than group B, but a similar IgG2 response during the primary phase of the experiment, just prior to secondary challenge.

After secondary challenge, at day 56, group D had the highest titre for both IgG1 and IgG2 isotypes, followed by B, C and lastly A. This was a slightly different pattern to that observed with the longitudinal analysis, in which group B had a higher optical density value for the IgG1 isotype response than group D. The difference in the IgG1 and IgG2 cut-off titres between groups B and D was not statistically significant, whereas that between C and D for the IgG1 response was. Group A titres were significantly lower than those of the other three groups.

5.3.6 Estimated antibody cut-off titres for the cathepsin-L pool fraction at day 105 (IgG1 isotype only)

Sera were titrated out in a three-fold dilution series from a 1/50 dilution onwards for the IgG1 antibody isotype. An optical density value of 0.2 was again chosen as the negative cut-off point and titres were estimated as explained previously. Results are shown in Table 5.11 and Figure 5.9.

Group D gave the highest antibody titre to the cathepsin fraction, followed by groups B, C and A. Despite the large difference in titre values, no significant difference was detected by Student's t-test between group D and groups B or C (for B versus D, $P = 0.07$ and C versus D, $P = 0.06$). This was due to the extent of titre variation within groups. The titre results are in agreement with the optical density profile obtained in the longitudinal screen (section 5.3.4), in which group D had a higher optical density value than groups B or C.

Table 5.8: Group mean cut-off titre values for IgG1 and IgG2 antibody responses to the high molecular weight fraction at day 28; shown as titre and log titre values.

Group	IgG1		IgG2	
	Log Titre	Titre	Log Titre	Titre
A	3.18	1497	2.72	530
B	2.68	483	2.62	414
C	2.40	251	2.19	157
D	1.35	22	1.90	80

Table 5.9: Group mean cut-off titre values for IgG1 and IgG2 antibody responses to the high molecular weight fraction at day 56; shown as titre and log titre values.

Group	IgG1		IgG2	
	Log titre	Titre	Log titre	Titre
A	3.23	1696	2.51	326
B	3.91	8212	3.25	1799
C	3.69	4937	3.01	1023
D	4.13	13535	3.44	2726

Table 5.10: Results of Students t-tests comparing group mean log cut-off titre values for the high molecular weight fraction at days 28 and 56, showing the *P* value obtained.

Time-point	Comparison	*P* value	
		IgG1	IgG2
Day 28	A vs B	0.02*	0.59
	A vs C	0.004**	0.02*
Day 56	B vs D	0.11	0.33
	C vs D	0.02*	0.08

Key:
Results that are statistically significant are marked * and highly significant ***.

Figure 5.7: Group mean cut-off titres for IgG1 and IgG2 antibody isotype responses to the high molecular weight fraction at day 28.

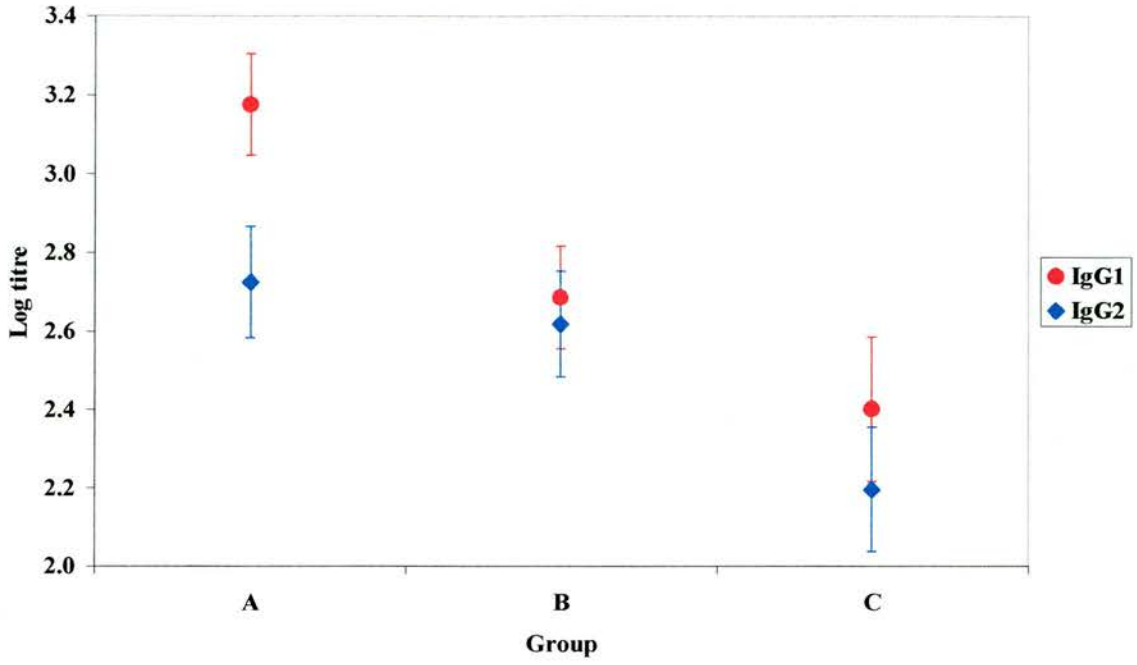
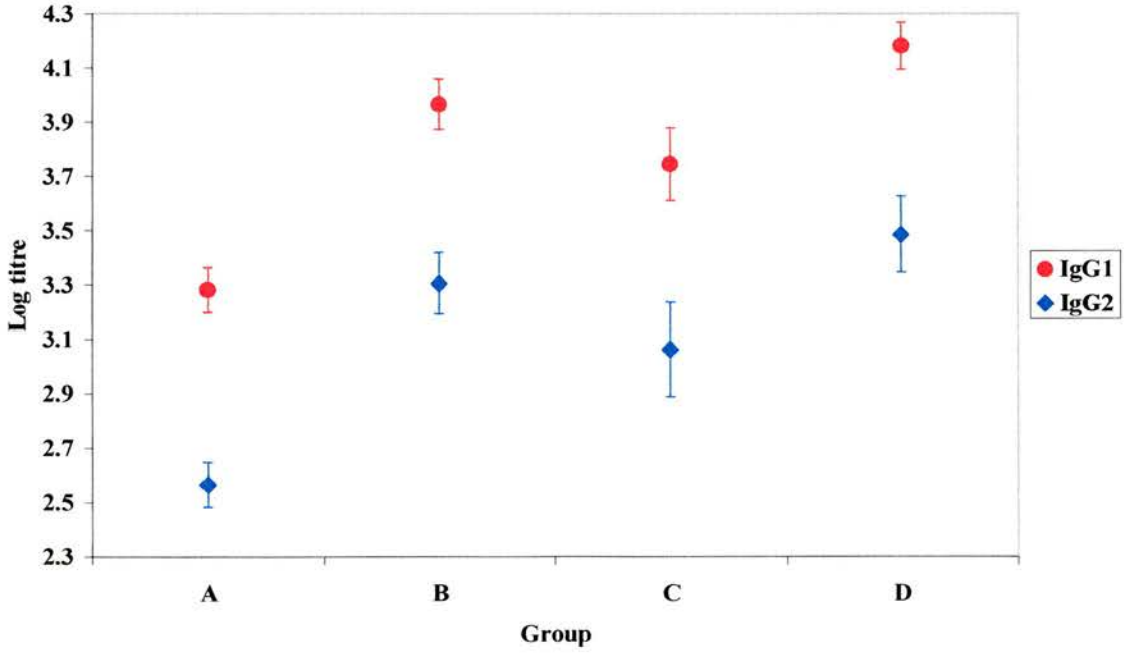


Figure 5.8: Group mean cut-off titres for IgG1 and IgG2 antibody isotype responses to the high molecular weight fraction at day 56.

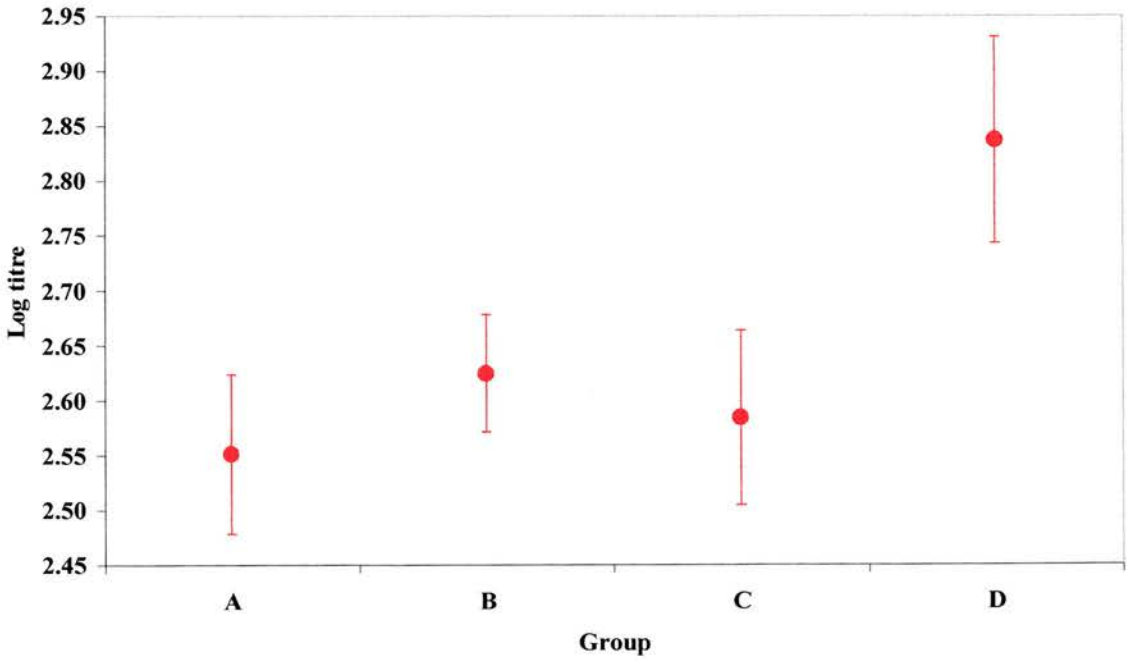


Group mean titre values are shown as either a circle (IgG1) or a diamond (IgG2) for each group, with error bars showing the standard error of the mean.

Table 5.11: Group mean cut-off titre values for the IgG1 antibody response to the cathepsin-L pool fraction at day 105, shown as titre and log titre values.

Group	Log titre	Titre
A	2.55	356
B	2.62	421
C	2.58	384
D	2.84	686

Figure 5.9: Group mean cut-off titre values for the IgG1 isotype antibody response to the cathepsin-L pool fraction at day 105, showing log titre values, with error bars indicating the standard error of the mean.



5.3.7 Associations between antibody cut-off titre values and infection parameters

Associations were investigated using Spearman's rank correlation between log titre values and the following infection parameters: eosinophilia (averaged between days 63 to 125), early faecal egg counts (averaged over the period days 121-128), peak glutamate dehydrogenase (GLDH) and peak gamma-glutamyl transferase (γ-GT) levels. The results are shown in Table 5.12.

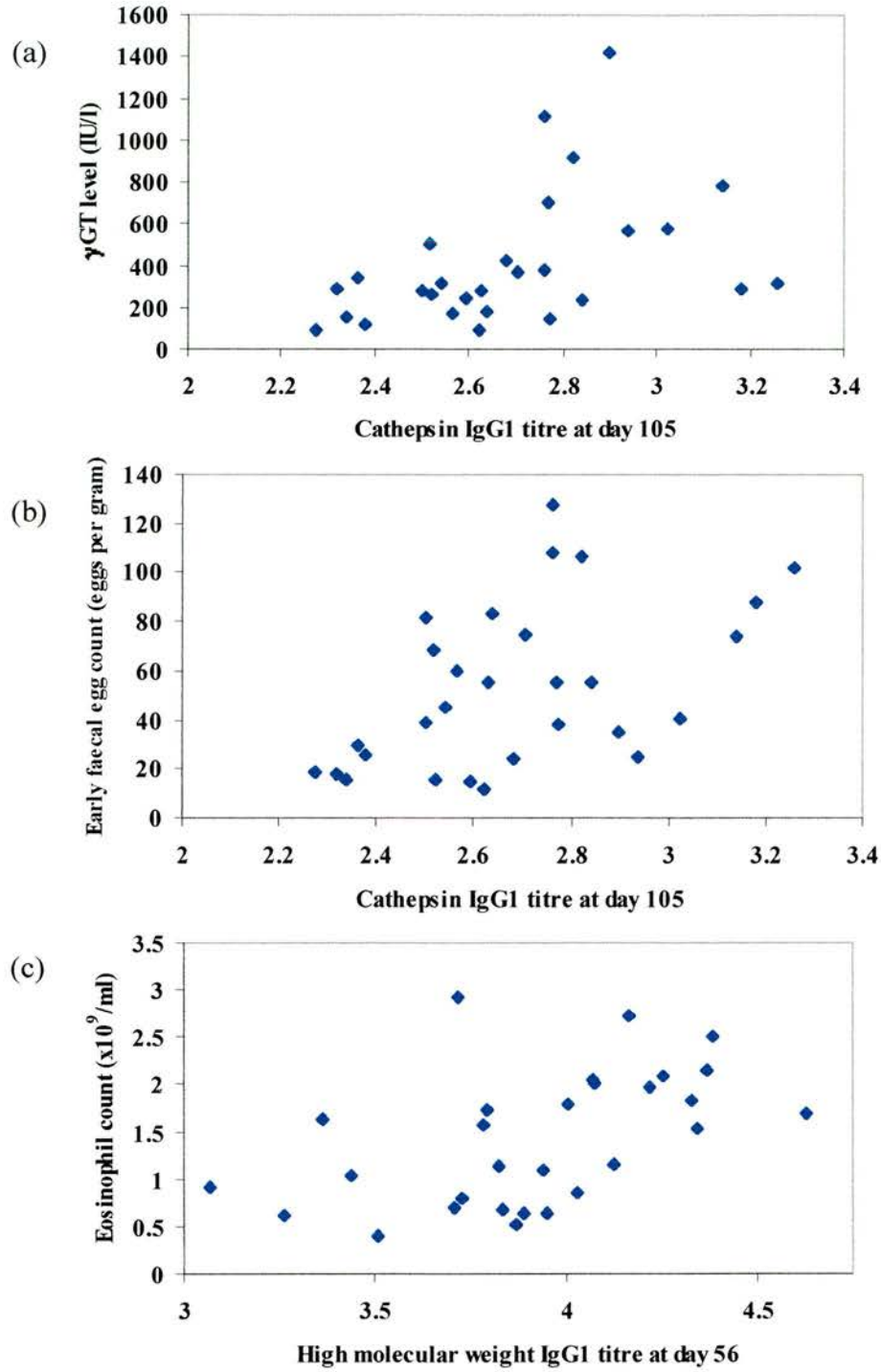
Group B, C and D titres were analysed collectively as one group for the IgG1 responses to the cathepsin fraction at day 105 and for the high molecular weight fraction at day 56. Significant positive correlations were noted between cathepsin titres and early faecal egg out-put, eosinophilia, and peak γ-GT levels. High molecular weight IgG1 titres at day 56 showed a significant positive correlation with eosinophilia and IgG2 titres with peak GLDH levels. Scatter plots of cathepsin titre against early egg output and peak γ-GT values and of high molecular weight titre against eosinophil counts are shown in Figure 5.10. A weak, but significant, positive correlation was detected between cathepsin and IgG1 high molecular weight day 56 titres (coefficient of 0.383, $P = 0.04$).

No significant correlations were detected for high molecular weight day 28 titre values for group B analysed independently, or for groups B and C analysed collectively, and any of the above parameters. However, positive correlations were observed between high molecular weight day 28 IgG1 and day 56 IgG1 titre values (coefficient 0.653, $P = 0.002$) and day 28 IgG2 and day 56 IgG1 values (coefficient 0.467, $P = 0.04$).

Table 5.12: Spearman's rank correlation coefficients showing the relationship between antibody titres and various infection parameters, with the *P* value given in brackets. Results that are statistically significant are highlighted.

Infection parameter	Cathepsin titre day 105, IgG1	HMW titre day 56, IgG1	HMW titre Day 56, IgG2
Eosinophilia	**0.37** (0.05)	**0.55** (0.002)	0.34 (0.07)
Faecal egg counts	**0.48** (0.01)	0.31 (0.11)	0.12 (0.54)
Peak GLDH	0.29 (0.13)	0.18 (0.36)	**0.42** (0.02)
Peak γ-GT	**0.51** (0.005)	0.15 (0.43)	0.22 (0.26)

Figure 5.10: Scatter plots showing the relationship between antibody titres and various infection parameters.

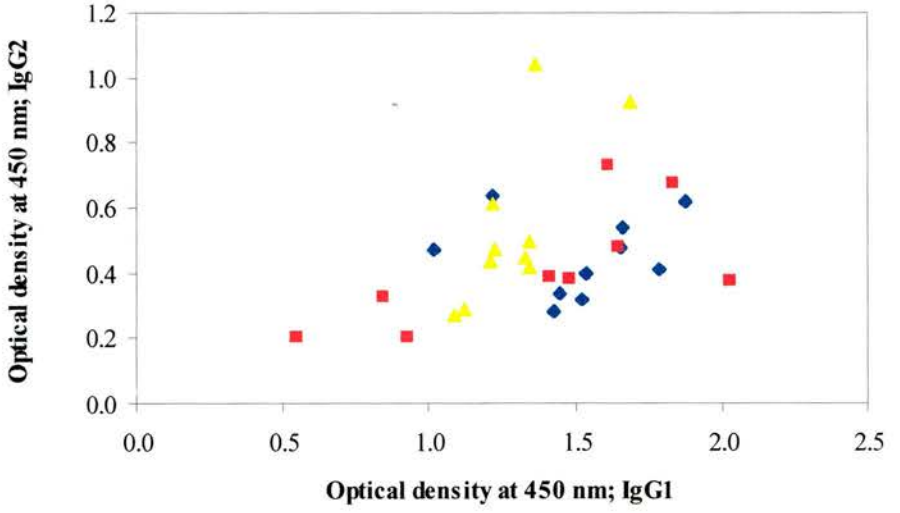


Scatter plots to examine associations between infection parameters and titre values were investigated for groups B, C and D, analysed collectively as a single group. Cathepsin titre against peak γ-GT levels is shown in (a), against faecal egg counts averaged over days 121-128 in (b) and high molecular weight IgG1 day 56 titre against eosinophil counts (averaged from day 63 to 125) in (c).

5.3.8 Association between the IgG1 and IgG2 antibody isotype response

From the longitudinal profiles of the IgG1 and IgG2 antibody response to the high molecular weight fraction (Figures 5.3 and 5.4), it appears that after secondary challenge the IgG2 response mirrored that of the IgG1 response, but at a lower level. Actual optical density values for the two isotype responses cannot be compared directly, due to possible differences in monoclonal antibody affinity for their respective targets, but also because the IgG1 and IgG2 screens were performed independently, on different days. However, a relative comparison between IgG1 and IgG2 responses for an individual animal can be performed by the use of scatter plots, to examine possible correlation. Figure 5.11 shows a scatter plot for the IgG1 response plotted against the IgG2 response for the high molecular weight fraction at day 56. Spearman's rank correlation showed a significant positive association between the IgG1 and IgG2 antibody responses in groups A and D, but not in groups B and C: A, coefficient of 0.77*; B, 0.25; C, 0.65 and D, 0.72* (where * indicates that the result is statistically significant with $P < 0.05$).

Figure 5.11: Scatter plot showing the relationship between IgG1 and IgG2 antibody isotype responses to the high molecular weight fraction at day 56, given as optical density values (450 nm) for individual animals in groups B, C and D. Group B animals are shown as diamonds, group C as squares and group D as triangles.



5.4 DISCUSSION

5.4.1 Antibody responses to the high molecular weight fraction appear early during infection

A biphasic antibody response to the high molecular weight fraction was observed over the time-course in groups A, B and C. In all three groups, an early mixed IgG1/IgG2 isotype response was evident within 14 days of primary infection. After secondary challenge, both pre-exposed groups B and C, together with the naïve group D, showed a rapid increase in antibody levels of both IgG isotypes. The peak group mean optical density value obtained for group B during the second phase of the experiment was greater than that of groups C and D. However, titration of day 56 sera found that the group D response was greater than that of group B. In the latter case, the difference in antibody response between groups B and D was not significant, although the difference between the day 56 IgG1 response of groups C and D was significant. IgG1 antibody levels were always higher than those of the IgG2 isotype. However, as binding of the two monoclonal antibodies for their respective immunoglobulin targets had not been standardised, it was not possible to conclude that the overall response of one isotype was greater than the other, although this does seem likely. The secondary challenge infection in the chronic group A appeared to check the early rise in both IgG1 and IgG2 antibody responses to the high molecular weight fraction.

It can be inferred from these results that the high molecular weight fraction isolated from excretory-secretory products of adult flukes is either present or cross-reactive with a similar protein in the early juvenile fluke. Both group B and C animals were pre-exposed to flukes of less than 6 and 2 days old respectively, and yet they were able to mount an antibody response during the first phase of the experiment. It is possible that the presence of flukes that may not have been killed by drug treatment were responsible for the group B and C response observed during the primary phase. However, it is unlikely that the presence of a small number of escape flukes would result in significant antibody production. Comparing the differences in antibody titres between groups A and B during the primary phase of the experiment, it should be noted that although group B values were significantly lower than those of group A for the IgG1 isotype, they were not for the IgG2 isotype. Early antibody responses to the high molecular weight fraction have been seen previously in

experimentally infected cattle as early as 1 week post-infection (McGonigle and Dalton, 1995). Thus, the responses reported in this experiment with group B and C animals are likely to be a genuine reaction to the short primary exposure that these groups received.

During the primary infection period, group A appeared to develop a rising antibody response to the high molecular weight fraction, which was cut short by the secondary challenge on day 28. Comparing this with the response of group D, a similar early rise of a parallel nature was observed after the secondary challenge infection. However, group D reached a peak optical density value at day 56, 28 days after infection. Why did group A animals not reach a similar peak value during the first 28 days of primary exposure? Two possibilities could account for this observation: either super-infection genuinely checked the rising antibody response to the high molecular weight fraction in group A, or group D animals reacted differently to a primary infection compared to animals of group A.

Considering the latter point, it is possible that the metacercariae of the primary dose differed from those of the secondary dose, for example in terms of viability, despite the fact that they were obtained from the same source. The primary dose may have been less able to stimulate a strong antibody response in group A animals, or metacercariae used for the secondary challenge may have grown more rapidly in group D, releasing more antigen. However, the results of enzyme and eosinophil analyses (section 3.3), show that the timing of events in longitudinal profile for both groups A and D were very similar, and final peak enzyme and eosinophil values were equivalent. This suggests that the course and nature of the primary infections in both groups A and D was similar. Another factor which could be responsible for the difference in the antibody response between the two groups could be the slightly greater age of group D animals at first exposure. The additional one months maturity may have enabled group D animals to mount a swifter immune response to the antigen than group A animals.

Even if optical density and titre values at day 28 were indeed the group A maximum for a single primary infection, the fact still remains that the secondary challenge infection did not appear to boost the levels of antibody to the high molecular weight fraction in group A animals. If the response was predominantly stimulated by early juvenile expression of the protein and not by the presence of more mature flukes, a further rise in response to the second wave of juveniles might

be expected. One possible explanation for the dampened response could be that the primary infection flukes were able to reduce the antibody response in some way to the secondary challenge. Modulation of the observed antibody response could have been brought about by diversion of antibody production to alternative, perhaps more abundant antigenic targets; by direct regulation of B cell function (Deehan *et al.*, 1998); or by switching the antibody response to an isotype that was not measured in this experiment. The presence of another antibody isotype, sharing the same antigenic epitope, but present in greater quantities, could block binding of the isotype under investigation in the ELISA reaction.

Alternatively, such a quantity of antigen may have been released by the parasites into the peripheral circulation of the host that all available antibody was bound in antigen-antibody complexes, a Gell and Coombs type III hypersensitivity reaction (Meeusen, 1999). The quantity of free antibody in sera would be reduced, giving the impression that the antibody response had decreased.

5.4.2 Antibody responses to the cathepsin-L pool fraction are not significant during early infection

In contrast, antibody recognition of the cathepsin fraction was monophasic and appeared later during the infection process. The response was dominated by the IgG1 isotype, with negligible changes in the IgG2 antibody response throughout infection. An initial low rise in optical density values was observed in group A from day 28 onwards, with the main increase occurring from day 42 and peaking at day 70. There was no detectable antibody response in groups B and C during the primary infection period. IgG1 levels subsequently rose in groups B and C and in group D after the secondary challenge, peaking in all three groups at day 98, 28 days after the peak observed with group A. Although both optical density and titre values suggested that the response in groups B and C was lower than that in D, this difference was not significant.

The appearance of antibody to cathepsin relatively late during the infection process is consistent with the results from other studies. Control calves in cathepsin protease vaccine trials showed only a minimal rise in antibody levels by ELISA at 3 weeks post-infection, but distinct recognition of doublet bands in Western blots of excretory-secretory products, migrating at the cathepsin level, was noted from week 6 (Dalton *et al.*, 1996). Recently, a comparison of diagnostic assays found that

antibody responses to crude excretory-secretory products and cathepsin-L1 peptide fragments (peptides predicted by sequence analysis) were first detected from 4 weeks post-infection after a heavy metacercarial challenge, whereas antibody to purified whole cathepsin-L1 showed a lag of a further week (Cornelissen *et al.*, 1999). The presence of cathepsin-L proteases, as characterised by substrate analysis, has been demonstrated in excretory-secretory products of newly excysted juvenile and three-week-old flukes (Carmona *et al.*, 1993). However, isolation and cloning of the only major gelatin gel reactive protease in the 29 kDa region, present in newly excysted juvenile ES, found that the protease was of the cathepsin-B class (Wilson *et al.*, 1998). N-terminal sequence analysis of newly excysted juvenile proteins has demonstrated the presence of amino acids with homology to both cathepsin-B and human cathepsin-L proteases (Tkalcevic *et al.*, 1995). From these studies it is apparent that early juvenile flukes do secrete a major protease which appears to be of the cathepsin-B class, although the possibility of cathepsin-L protease secretion cannot be ruled out. Absent or low expression of cathepsin-L by young flukes would explain the later reactivity recorded in this study, in comparison to that observed with the high molecular weight fraction.

It is interesting, given the low IgG response to the high molecular weight fraction observed in group A after secondary challenge, that peak values for cathepsin-specific antibody in groups A and D were so similar. Super-infection appeared to have no modulating effect on the response to cathepsin, but additionally, secondary challenge did not appear to boost the response either. Perhaps a secondary infection has the action of prolonging the antibody response? If the antibody profile to the cathepsin fraction had been followed for a longer period, differences in the duration of response could have been examined. Alternatively, secondary flukes may not have been able to mature in calves already carrying a primary infection. Certainly, liver enzyme profiles were extended in group A animals compared to those of group D (section 3.3.2), suggesting that the second fluke infection had a damaging action on the liver and did reach the bile ducts. Directly testing the fate of primary and secondary infections is difficult, although tagging metacercariae with a marker resistant to shedding is a possibility. ^{75}Selenomethionine has been used to label *Schistosoma mansoni* cercariae, allowing the parasites to be localised within host tissue after infection (Dean *et al.*, 1984; Wilson *et al.*, 1986).

5.4.3 Associations between antibody titre and infection parameters

Moderate positive correlations were observed between cathepsin titres and early faecal egg output, eosinophilia and peak gamma-glutamyl transferase levels, suggesting that the cathepsin antibody levels acted in this study partly as markers of infection. Associations between the response to the high molecular weight fraction and infection parameters were less obvious, although moderate correlations were noted with eosinophilia and peak glutamate dehydrogenase levels. A strong positive association was also observed between pre- and post-challenge titres to the high molecular weight fraction, indicating that animals that responded well during the primary phase, were also those responding well after challenge. The analysis gave no indication that higher antibody titres were associated with lower levels of infection. However, Spearman's rank correlation is not a particularly powerful method for measuring associations between parameters. A more detailed analysis, with the use of regression models, should be attempted.

5.4.4 Specificity of reagents

To distinguish effectively between IgG1 and IgG2 isotype antibody responses, the specificity of the detecting reagent is crucial. In situations where very strong IgG1 or IgG2 responses are expected, a small degree of cross-reactivity is not critical. However, where the response of an isotype may be negligible or very low, specificity is important. Several large research establishments produce their own in-house monoclonal antibodies to bovine IgG1 and IgG2 isotypes; for those working independently, sources are more restricted. The monoclonals used in this study were chosen because they were available in reasonable lot sizes as purified material (in some cases, monoclonal antibodies are supplied in the form of unpurified cell culture supernatant fluid) and could be used at a high dilution, reducing the cost of screening a large number of samples.

IgG1 and IgG2 antibody responses to the high molecular weight fraction did show a positive association. However, the presence of significant correlation does not imply any causative effect and the fact that samples were positive for both IgG1 and IgG2 may be due to an independent factor. The scatter plot showed that in many cases, there were groups of points with a close range in IgG1 values, but a wide range in IgG2 values. This would suggest that the IgG2 value obtained was not associated with that of the IgG1 value. However, it is also true to say that those that

had a low IgG1 response, also had a low IgG2 response i.e. poorly responsive individuals were low to negative with both isotypes. This latter point may have affected the correlation observed.

In summary, some degree of cross-reaction between the isotypes may have occurred and requires investigation using purified bovine immunoglobulins. However, the group B response during the primary phase of the experiment, during which period the IgG1 and IgG2 responses showed similar optical density and titre results, suggests that an independent IgG2 response was also present.

5.4.5 Conclusions

An early mixed IgG1/G2 antibody response to the high molecular weight fraction was detected in the animals receiving a 5-6 day drug-abbreviated primary infection, indicating that this protein was expressed by very young flukes. IgG1 antibody titres at day 28 were highest in chronically infected animals, whilst IgG2 antibody titres were similar in chronically infected and 5-6 day pre-exposed animals. After secondary challenge, 5-6 day and 24-48 hour pre-exposed animals developed a predominantly IgG1 antibody response, of similar magnitude to that seen in naïve animals. Pre-exposure did not, therefore, alter the progression of the antibody response to this fraction. In contrast, the antibody response after secondary challenge in chronically infected animals was minimal and did not increase substantially above the primary infection levels, suggesting that the antibody response was "inhibited" by the presence of maturing flukes.

The antibody response to the cathepsin-L pool fraction appeared later during the infection process, was dominated by the IgG1 isotype and did not appear to be involved in the dynamics of the early immune response. Moderate positive correlations between cathepsin titre and some infection parameters suggest that antibody responses to the cathepsin fraction may act as markers of infection.

CHAPTER 6

Peripheral and local IgG antibody responses to various *F. hepatica* protein preparations in infected calves.

6.1 INTRODUCTION

In the previous chapter, IgG1 and IgG2 antibody responses to two isolated fluke proteins, cathepsin-L protease and a haem-containing high molecular weight fraction, were examined. Although various other proteins have been isolated from whole fluke (WFA) and excretory-secretory (ES) preparations (section 1.2.10), these were not available for use in the current study. In order to examine antibody responses to other fluke proteins during the early infection period in the various groups of both Experiments A and B and to identify any differences between groups, Western blot analysis was used. Of main interest was comparison of the pre-secondary challenge antibody responses between chronically infected (group A) and 5-6 day pre-exposed (group B) animals and post-secondary challenge responses between the latter and naïve animals (group D). As well as investigating antibody responses to adult-derived proteins, those to metacercarial and 14 day juvenile preparations were examined.

Antibody produced locally, at the site of infection, may differ to that seen peripherally, in sera. In *F. hepatica* infected rats, antibody produced by mesenteric, hepatic and splenic lymph nodes recognise different proteins in homogenates prepared from different ages of fluke (Meeusen and Brandon, 1994). The predominant antibody isotype was also found to differ between the three nodes: IgA was associated with the mesenteric node and IgE with the hepatic node. Although the current study is not directly comparable, given the differences in protection against challenge between sensitised cattle and rats (section 1.2.5), the methodology is appropriate. Hepatic node cell culture fluid collected during Experiment B (section 4.2.11) was used to probe Western blots of fluke preparations, to examine the local antibody response and to compare it with that seen in sera.

6.2 MATERIALS & METHODS

6.2.1. Protein preparations

The following protein preparations were used: adult whole fluke antigen, adult excretory-secretory protein, juvenile (14 day) fluke homogenate, metacercarial crush preparation, high molecular weight fraction and cathepsin-L pool fraction (section 2.1). WFA, ES, juvenile and metacercarial crush extracts were run under reducing conditions on a large 10-15% SDS PAGE gradient gel (section 2.2) to investigate the relationship between the various protein bands in the four preparations.

6.2.2 Sera samples

Sera samples from calves of Experiment A (section 3.2.1) were tested on blots of WFA and ES preparations. 7-8 animals from each group were used to examine responses to WFA and 5 animals from each group for responses to the ES preparation. Sera was tested at various time-points from the negative (pre-infection) period to day 70 post-infection. Antibody responses to the cathepsin-L pool fraction and juvenile and metacercarial crush preparations were tested on a limited number of samples.

Sera samples from the 8 animals of Experiment B (section 4.2.1) were tested for antibodies to WFA, ES, juvenile and metacercarial crush preparations. Samples taken during the negative (pre-infection) period and on days 26 and 40/41 were tested.

Hepatic lymph nodes from Experiment B animals were cultured unstimulated in complete media as described in section 4.2.11. Cell culture supernatant fluid was harvested after a 4 day incubation period and used neat as the primary antibody probe on Western blots of WFA, ES, cathepsin-L pool and high molecular weight preparations.

6.2.3 Secondary and tertiary antibody reagents

Polyclonal horseradish peroxidase conjugated anti-bovine IgG (Sigma A-5295) and anti-bovine IgG1 (Bethyl Laboratories Inc., USA), or monoclonal anti-bovine IgG1/G2 antibodies (working stock 2 mg/ml; section 5.2.3), together with a

polyclonal horseradish peroxidase conjugated anti-mouse IgG (Sigma A-9044) were used to detect bound test antibody.

6.2.4 Polyacrylamide gel electrophoresis and Western blotting

Protein preparations were separated under reducing conditions on SDS PAGE mini-gels of an appropriate acrylamide percentage, or on large 10-15% gradient gels (section 2.2). The high molecular weight fraction was run under non-reducing conditions. The separated proteins were then transferred onto 0.45 μm nitrocellulose membrane (Amersham) using a semi-dry blotting system (TransBlot® SD, BioRad Laboratories). Nitrocellulose membrane, Whatman 3M filter paper and the acrylamide gel were incubated in transfer buffer for 30 minutes at room temperature (RT). The gel sandwich was assembled and proteins transferred at 18-20V for 40 minutes. After transfer, the membrane was washed briefly in Tris-buffered saline/Tween 20 (TBS-T, Appendix A).

The lanes containing the protein standards and some of the separated sample proteins were cut off and stained with colloidal gold solution (BioRad Laboratories) to show the position of the markers and sample proteins. The main blot was placed in blocking buffer and incubated at RT with agitation for approximately 1 hour. The blocking buffers varied according to the antibody conjugate that was used. Table 6.1 shows the type of gel, blocking buffer and antibody conjugates used with the various protein preparations.

The blocked membrane was cut into 3-4mm strips (mini blots) or 5mm strips (large blots) and rinsed briefly in TBS-T. Test sera was prepared at the required concentration in blocking buffer and incubated with the membrane strips for 1 hour at RT, with agitation. The strips were then washed four times in TBS-T. Strips were incubated with either a directly conjugated horseradish peroxidase-labelled polyclonal anti-bovine IgG or IgG1 reagent, or with unconjugated monoclonal anti-bovine -IgG1 or -IgG2 antibodies, diluted in the appropriate blocking buffer, for 1 hour. The strips were again washed four times in TBS-T. Where a monoclonal antisera had been used for the secondary antibody step, a further incubation was carried out using a rabbit polyclonal anti-mouse IgG horseradish peroxidase-labelled conjugate, for 1 hour at RT. After washing, any bound horseradish peroxidase

conjugate was detected using 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Vector Laboratories, Inc.). The reaction was stopped with water and strips air dried in the dark. Strips were mounted and scored visually.

The conditions used for each protein preparation, including gel type, sera dilution and the nature of the secondary antibody reagent, are shown in Table 6.2.

Table 6.1: Secondary and tertiary antibody reagents used for Western blot analysis, showing the antibody target, nature and source together with the dilution and blocking agent used. The optimum dilution of the reagents was determined by titration.

Target	Type	Source	Conjugate	Dilution	Block
Bovine IgG1	Polyclonal	Sheep	HRPO	1/2000	2% NSS
Bovine IgG	Polyclonal	Rabbit	HRPO	1/2000	2*-3% soya milk
Bovine IgG1	Monoclonal	Mouse	None	1/1000	3% soya milk
Bovine IgG2	Monoclonal	Mouse	None	1/1000	3% soya milk
Murine IgG	Polyclonal	Rabbit	HRPO	1/4000 or 1/5000	3% soya milk

Key: NSS = normal sheep sera

HRPO = horseradish peroxidase conjugate

*: 2% soya milk was used for Western blots performed with cell culture supernatant fluid and 3% soya milk for all other blots.

Table 6.2: Conditions used for Western blots of the various protein preparations. Optimal sera dilutions were determined by titration.

Protein preparation (μg)	Gel type, with acrylamide %	Sera dilution	Secondary and tertiary reagents
WFA (120 μg)	Large 10-15%	1/400	Polyclonal anti-bovine IgG1
WFA (120 μg)	Large 10-15%	1/400	Monoclonal anti-bovine IgG1/G2
ES (50 μg)	Mini 10 or 12%	1/200	Polyclonal anti-bovine IgG
ES (50 μg)	Mini 10 or 12%	1/200	Monoclonal anti-bovine IgG1/G2
Juvenile (30 μg)	Mini 12%	1/200	Polyclonal anti-bovine IgG
Metacercarial (9 μg)	Mini 12%	1/200	Polyclonal anti-bovine IgG
Cathepsin-L pool (15 μg)	Mini 14%	1/200	Polyclonal anti-bovine IgG
High molecular weight (10 μg)	Mini 7.5%	1/200	Polyclonal anti-bovine IgG

Key: WFA: whole fluke antigen preparation
ES: excretory-secretory preparation

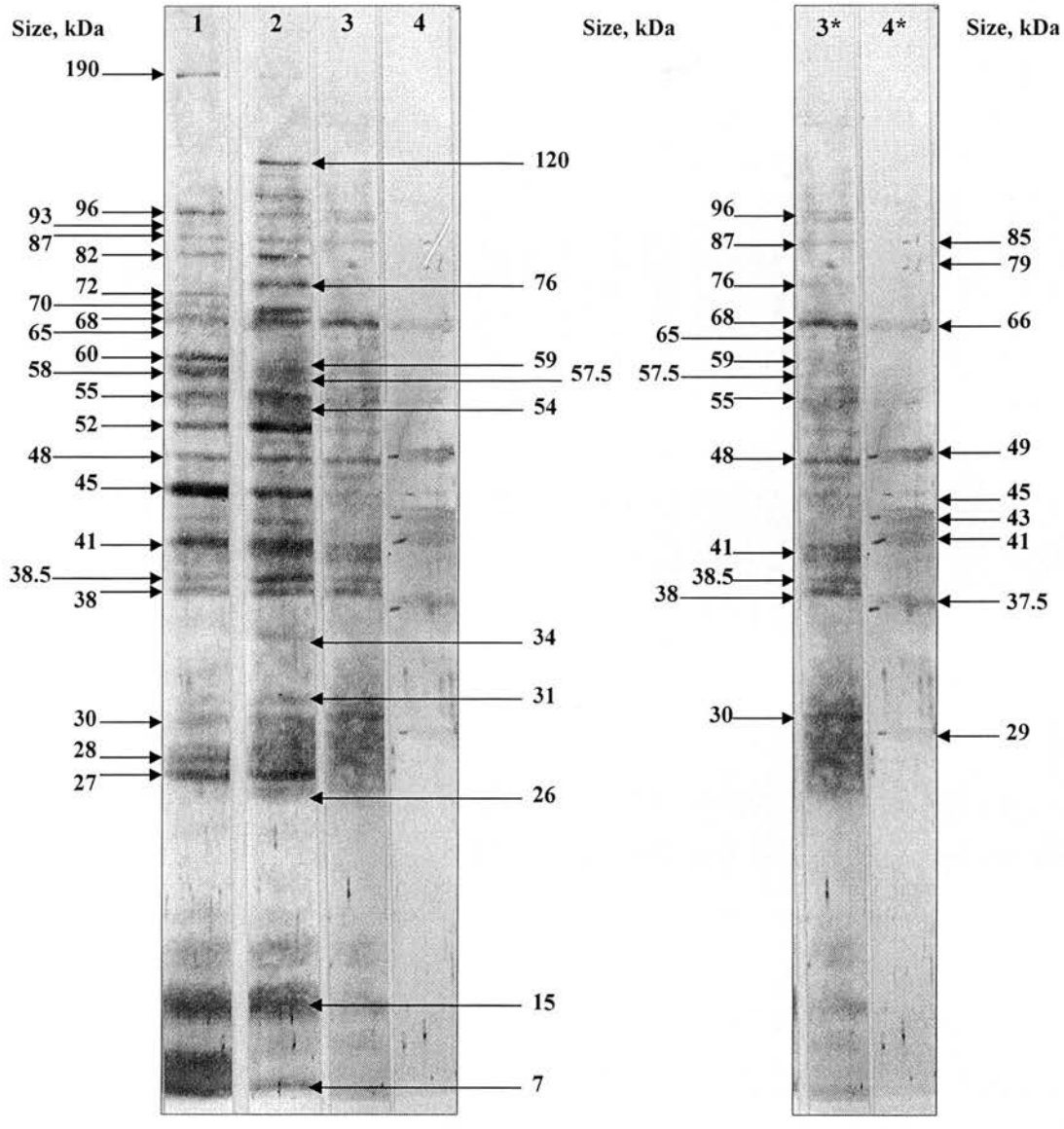
6.3 RESULTS

6.3.1 Comparison of protein bands present in whole fluke antigen, excretory-secretory, 14 day juvenile and metacercarial preparations

Figure 6.1 demonstrates the relationship between bands present in the four preparations. Many of the proteins bands present in the ES and WFA preparations were common to both, although a few protein bands were distinct in each of the two preparations. Approximate band sizes in kDa were calculated from relative migration distances, using a standard curve of known protein markers (Broad range markers, BioRad Laboratories). Table 6.3 shows the size of immunoreactive bands in WFA and ES preparations.

The juvenile preparation contained fewer protein bands than were present in the WFA and ES extracts. The majority of reactive bands in the juvenile preparation were common to those in either WFA or ES preparations. Nine reactive bands were noted in the metacercarial crush preparation. Only one, of 45 kDa, appeared to be common to bands present in the other preparations, although in some cases band sizes were very close. Table 6.4 shows the size of immunoreactive protein bands present in the juvenile homogenate and metacercarial crush preparations.

Figure 6.1: Colloidal gold stain of blotted whole fluke antigen (WFA), excretory-secretory (ES), juvenile and metacercarial preparations to show band relationships.



ES, 4 μg (lane 1), WFA, 4 μg (lane 2), juvenile, 4 μg (lane 3, 3*) and metacercarial crush, 1 μg (lane 4, 4*) preparations were separated on a 10-15% gradient SDS PAGE reducing gel and blotted onto nitrocellulose membrane. The positions of the main immunoreactive bands are marked by arrows, with their size given in kDa.

Table 6.3: Comparison of band relationships between whole fluke antigen (WFA) and excretory-secretory (ES) preparations, showing sera reactive bands and their estimated size in kDa.

Band size in kDa	WFA	ES	Band size in kDa	WFA	ES
190	✓	✓	54	✓	✗
160	✓	✓	52	✓	✓
140	✓	✗	48	✓	✓
120	✓	✗	45	✓	✓
96	✓	✓	43	✓	✓
93	✓	✓	41	✓	✓
87	✓	✓	38.5	✓	✓
82	✓	✓	38	✓	✓
76	✓	✗	34	✓	✗
72	✗	✓	33	✓	✗
70	✓	✓	31	✓	✓
68	✓	✓	30	✓	✓
65	✗	✓	28	✓	✓
60	✗	✓	27	✓	✓
59	✓	✗	26	✓	✗
58	✗	✓	15	✓	✓
57.5	✓	✗	7	✓	✓
55	✓	✓			

Where ✓ indicates the band is present and ✗ that the band is absent.

Table 6.4: Protein bands present in *F. hepatica* 14 day juvenile and metacercarial preparations, showing the band size in kDa.

Juvenile Homogenate	Metacercarial Crush
96	85
87	79
76	66
70	49
65	45*
59	43
57.5	41
55	37.5
48	29
45	21
41	
38.5	
38	
30	
28	

All protein bands in the metacercarial preparation, except for band 45 marked *, had differing relative migration (RF) values to the bands in the other fluke preparations, even though calculated band sizes, to the nearest kDa, were the same (see Figure 6.1).

6.3.2 Results of Western blots performed with samples from Experiment A

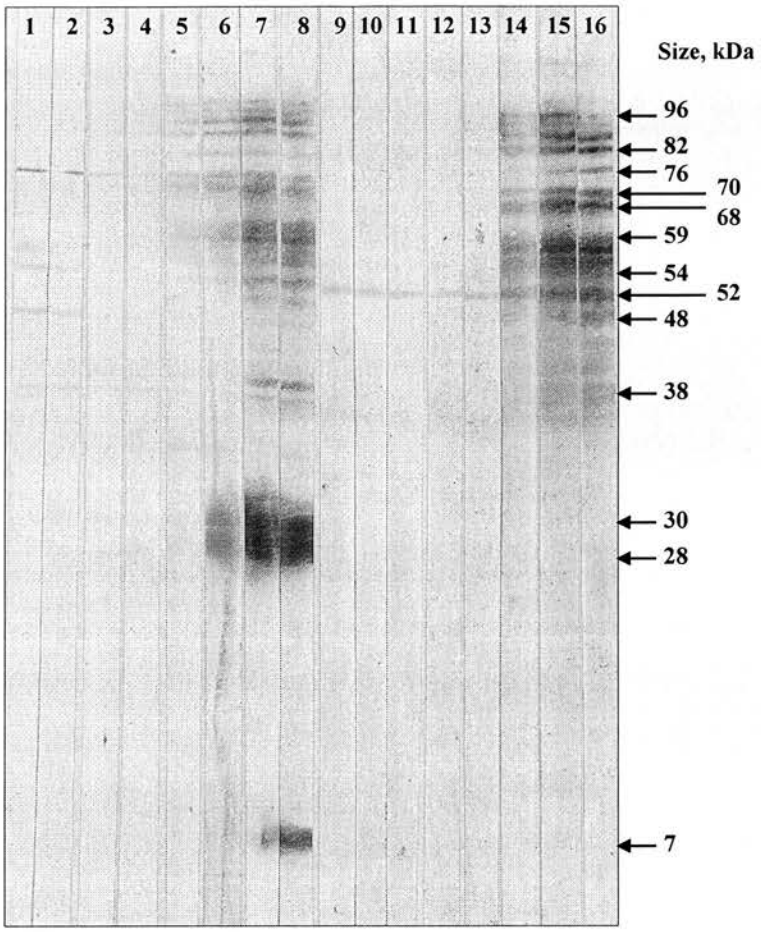
6.3.2.1 Longitudinal IgG1 antibody responses to whole fluke antigen

A longitudinal analysis of sera IgG1 antibody responses to WFA at days 0 to 49 was carried out with the sera from one animal in each of the groups A (calf 46), B (calf 40), C (calf 66) and D (calf 69). The results are shown in Figures 6.2 and 6.3.

The group A animal, calf 46, showed little response to infection until day 28, at which time there was weak reactivity to bands of size 96-82, 70 and 68, 59 and 57.5-45 kDa. After secondary challenge, at days 35 to 49, the response to these bands was much stronger; in addition, there was a strong reaction to two bands of size 30 and 28 kDa. During the first 28 days of infection, sera from the group B animal (calf 40) only detected one band of 52 kDa in the WFA preparation. However, after secondary challenge, calf 40 responded strongly to the 96-82, 70 and 68 kDa bands and particularly to bands of 59 and 57.5-54 kDa. The pattern of response with calf 66, a group C animal, was very similar to that of calf 40, although reactivity to bands 70-68 and 59-57.5 kDa appeared slightly earlier, at day 21. Sera from the group D animal (calf 69) was negative until after secondary challenge, after which point 96-82, 70 and 68, 59 and 57.5-45 kDa bands were recognised, as for groups B and C. There was no reaction to the 30 and 28 kDa bands at any point with sera from the three animals of groups B, C and D.

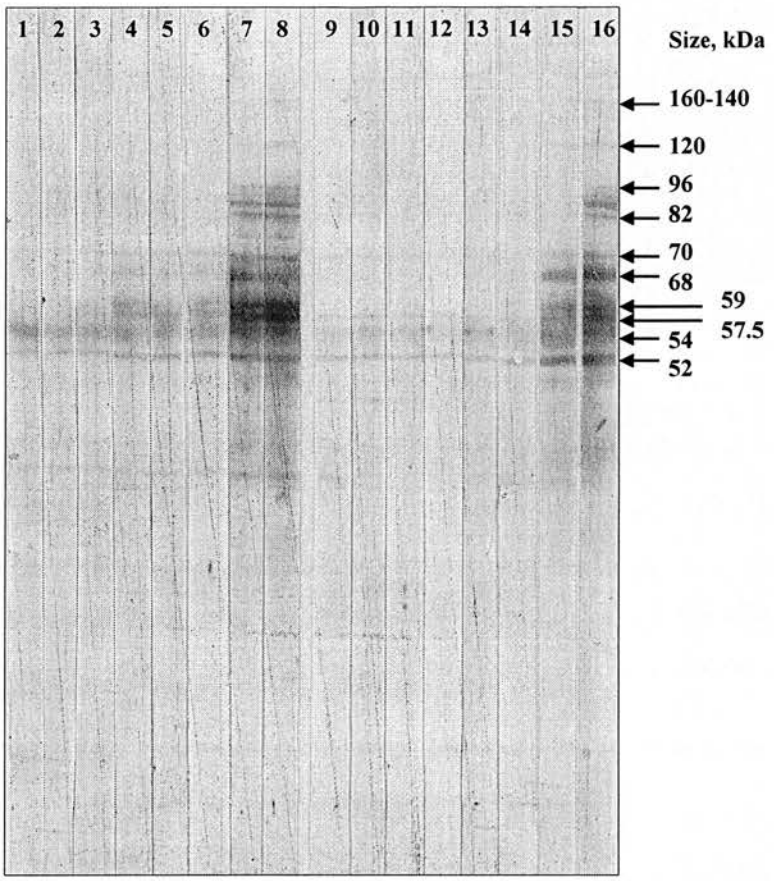
The longitudinal antibody response to WFA was also examined with sera from 8 animals in each of the four groups at days 0, 28 and 42 (mini time-series). Separate blots were used for the different groups, however, conditions were standardised, to allow comparison between groups. The response of group A is shown in Figure 6.4. These blots confirmed the trends observed in the longitudinal weekly series analysis, although the reactivity of group B animals at day 28 to bands of 96-82, 76-68 and 59-52 kDa was greater than observed with calf 40, above (Table 6.5).

Figure 6.2: Experiment A; longitudinal IgG1 antibody responses to whole fluke antigen for calf 46 (group A) and calf 40 (group B), from days 0 to 49.



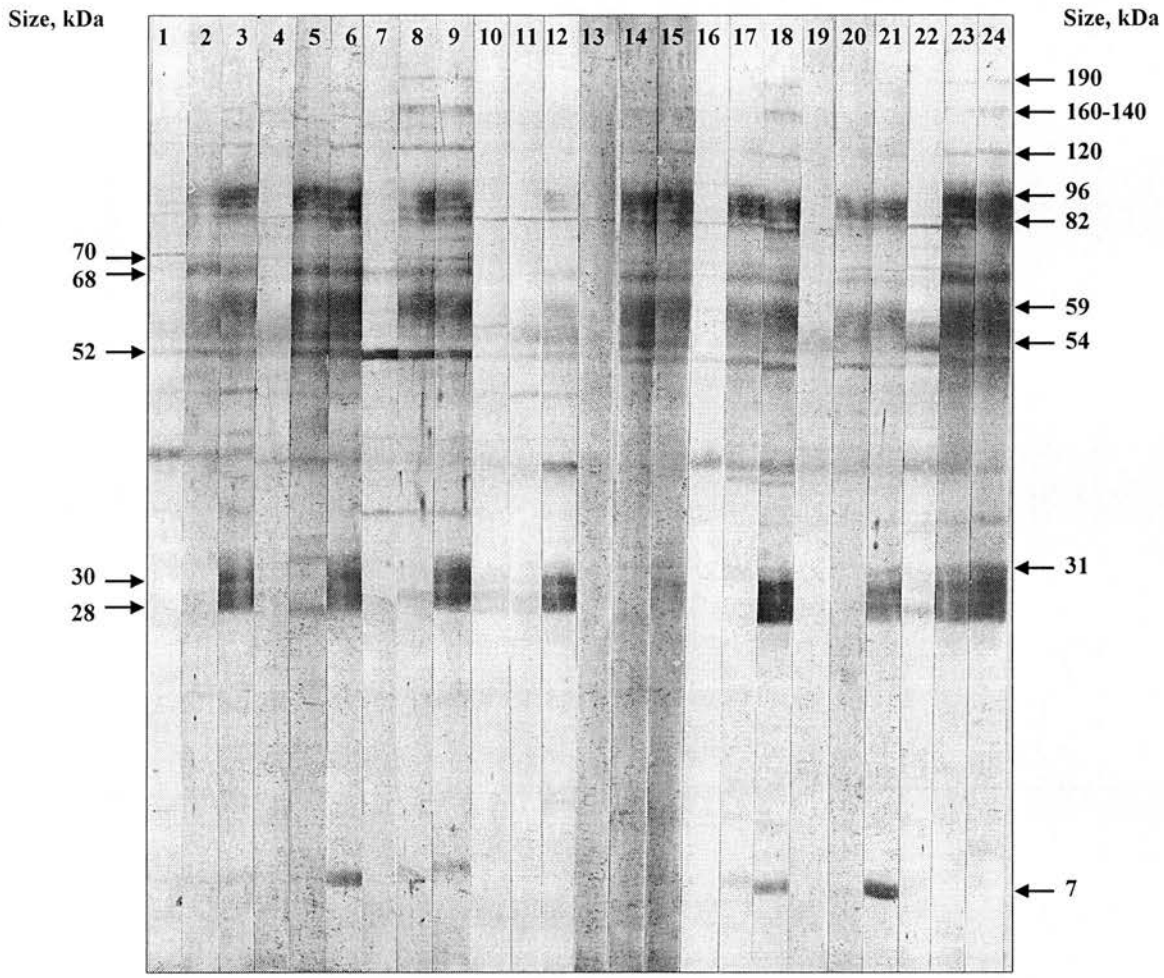
Lanes 1-8 (calf 46, group A) and 9-16 (calf 40, group B) were incubated with sera taken at days 0, 7, 14, 21, 28, 35, 42 and 49 post-primary infection, respectively. The positions of the main reactive bands are marked by arrows, with the band size shown in kDa. WFA was separated on a 10-15% SDS PAGE gradient gel under reducing conditions.

Figure 6.3: Experiment A; longitudinal IgG1 antibody responses to whole fluke antigen for calf 66 (group C) and calf 69 (group D), from days 0 to 49.



Lanes 1-8 (calf 66) and 9-16 (calf 69) were incubated with sera taken at days 0, 7, 14, 21, 28, 35, 42 and 49 post-primary infection. Major reactive bands are shown by arrows, with band sizes marked in kDa. WFA was separated on a 10-15% SDS PAGE gradient gel under reducing conditions.

Figure 6.4: Experiment A; longitudinal IgG1 antibody responses to whole fluke antigen for eight group A animals, using sera from days 0, 28 and 42.



Lanes 1-3, 4-6, 7-9, 10-12, 13-15, 16-18, 19-21 and 22-24 show the IgG1 antibody responses of 8 individual group A animals at days 0, 28 and 42 post-primary infection, respectively. The positions of the major reactive bands are shown by arrows, and band sizes marked in kDa. WFA was separated on a 10-15% SDS PAGE gradient gel under reducing conditions.

6.3.2.2 Fixed time-point group screens for IgG1 antibody responses to whole fluke antigen

The results of the longitudinal analysis indicated that the most important period in terms of group differences in band reactivity lay between days 28 and 49. By day 49, group A animals recognised 19 different protein bands. Sera antibody responses of all four groups to WFA were therefore examined at days 28, 42, 49, 56 and 70. The same blot and identical conditions were used for all samples at a given time-point, except for day 28 samples. Figures 6.5 and 6.6 show blots tested with day 42 and day 70 sera, respectively. The day 28 response (pre-secondary challenge) was investigated using the mini time-series blots described in section 6.3.2.1. The day 28 response was compared between groups by scoring the response of every animal to each band in the preparation (as either present or absent) and then collating the total for the group. The number of animals in each group positive for a particular band at day 28 is shown in Table 6.5.

(a) Early, pre-secondary challenge response

The group A response to bands 96-82, 70, 68 and 59-52 kDa at day 28 was strong, with 6/8 to 8/8 animals recognising each band. The response to the lower region bands of 30 to 27 kDa was faint and seen in only 1/8 to 2/8 animals. Sera from group B also reacted to the 96-82, 70, 68, 59, 57.5 and 52 kDa bands, although the response was not as great as for group A; no response to the 30-28 kDa bands was detected for any group B animal. The group C response was similar in pattern to that of group B, although it was weaker and bands were positive less frequently.

(b) Post-secondary challenge response

The band response pattern changed after the secondary challenge infection. At day 42, 14 days post-challenge, group A animals still recognised the bands detected at day 28, but additionally, the two bands of 30 and 28 kDa were strongly recognised by all animals. In contrast, the group B response to the mid region bands of 96-82, 70, 68 and 59-54 kDa was enhanced after challenge, whilst no antibody response to the 30 and 28 kDa bands was detected. Groups C and D also recognised the mid region bands, but with less intensity than group B, with the group D response being the weakest. By day 49, the differences between group A and the other three groups

were quite pronounced: the antibody response to the lower region 30 and 28 kDa bands was outstanding in group A and virtually absent in the other groups. In contrast, recognition of bands 96-82, 70, 68, 59 and 57.5-54 kDa was very strong in group B, but very weak in group A, and weak to moderate in group D. By day 70, this difference in the band recognition pattern of lower weight versus mid weight proteins, between group A and the other three groups, was further accentuated. Although groups B, C and D did show some reactivity to the 30 and 28 kDa bands at day 70, this was markedly less than that seen with group A.

Bands of 190, 160, 140 and 120 kDa were weakly recognised by sera from various animals in all groups inconsistently after secondary challenge. The response to the 120 kDa band was the most regular.

(c) IgG1/IgG2 antibody isotype responses at day 56

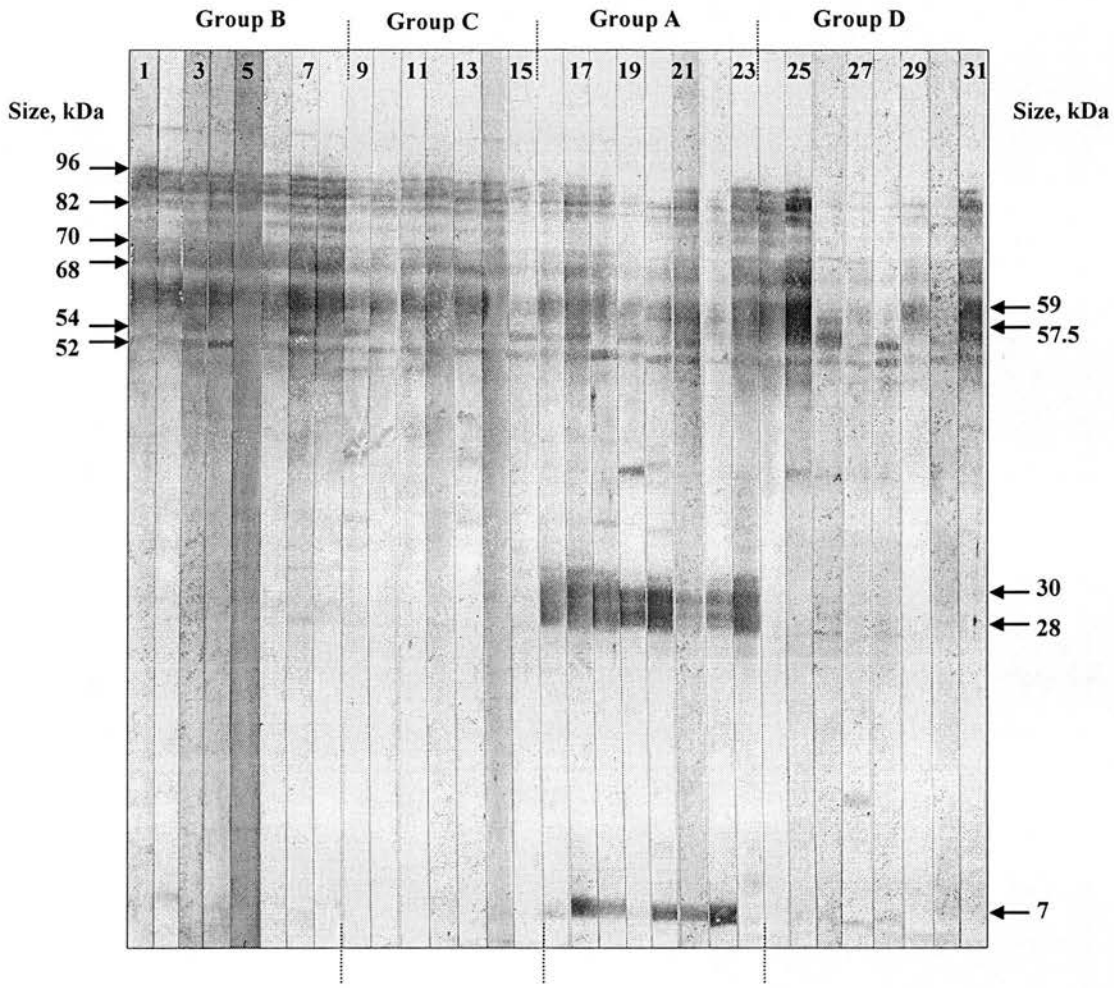
The IgG1 antibody response pattern to the WFA protein described above, was examined using a polyclonal antisera. Monoclonal antisera to both IgG1 and IgG2 antibody isotypes became available later in the study. A further blot was performed using day 56 sera from four animals in each of groups A, B and D, to examine the IgG1/IgG2 isotype response. The IgG1 response pattern was similar to that observed at days 42 and 49 using the polyclonal anti-IgG1 reagent for all three groups. The IgG2 response at day 56 was minimal: weak reactivity to some of the bands recognised by the IgG1 isotype was detected. However, no moderate or strong IgG2 band reactivity was observed with any of the sera tested, suggesting the absence of a specific IgG2 response at day 56.

Table 6.5: IgG1 antibody responses to whole fluke antigen at day 28 in groups A, B and C, shown as the group total scores for individual bands.

Band kDa	Group A	Group B	Group C	Band kDa	Group A	Group B	Group C
190	2/8	0	0	**54**	**8/8**	4/8	5/8
160	2/8	0	0	**52**	**7/8**	5/8	**6/8**
140	0	0	0	**48**	3/8	2/8	0
120	2/8	0	0	**45**	2/8	1/8	2/8
96	**7/8**	5/8	2/8	**43**	0	1/8	3/8
93	**7/8**	5/8	2/8	**41**	3/8	1/8	2/8
87	**7/8**	**6/8**	2/8	**39**	5/8	2/8	3/8
82	**7/8**	**6/8**	2/8	**38**	1/8	0	0
76	1/8	4/8	2/8	**34**	2/8	0	2/8
70	**6/8**	4/8	3/8	**33**	1/8	0	2/8
68	**7/8**	5/8	3/8	**31**	2/8	0	1/8
59	**7/8**	**6/8**	4/8	**30**	1/8	0	1/8
57.5	**7/8**	**6/8**	5/8	**28**	2/8	0	1/8
55	**8/8**	4/8	0	**27**	1/8	0	1/8

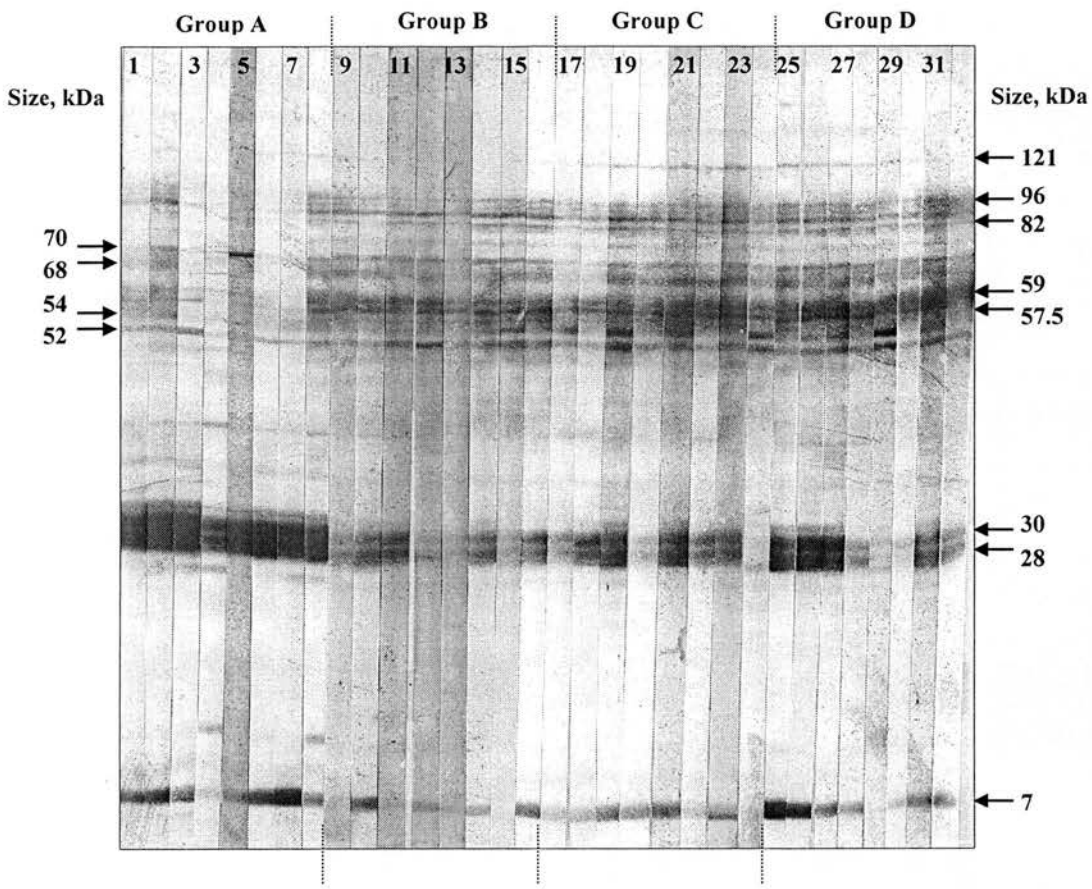
Key:
The score is highlighted in bold where more than three-quarters of animals in a group recognised a particular band.

Figure 6.5: Experiment A; IgG1 antibody responses to whole fluke antigen at day 42 for groups A, B, C and D.



Day 42 IgG1 antibody responses to WFA preparation for individual animals in groups B (lanes 1-8), C (lanes 9-15), A (lanes 16-23) and D (lanes 24-31). Alternate lanes are numbered. The positions of major reactive bands are shown by arrows, with band sizes marked in kDa. WFA was separated on a 10-15% SDS PAGE gradient gel under reducing conditions.

Figure 6.6: Experiment A; IgG1 antibody responses to whole fluke antigen at day 70 for groups A, B, C and D.



Day 70 IgG1 antibody responses to WFA preparation for individual animals in groups A (lanes 1-8), B (lanes 9-16), C (lanes 17-24) and D (lanes 25-32). Alternate lanes are numbered. The positions of major reactive bands are shown by arrows, with band sizes marked in kDa. WFA was separated on a 10-15% SDS PAGE gradient gel under reducing conditions.

6.3.2.3 IgG, IgG1 and IgG2 antibody responses to **F. hepatica** *excretory-secretory preparation*

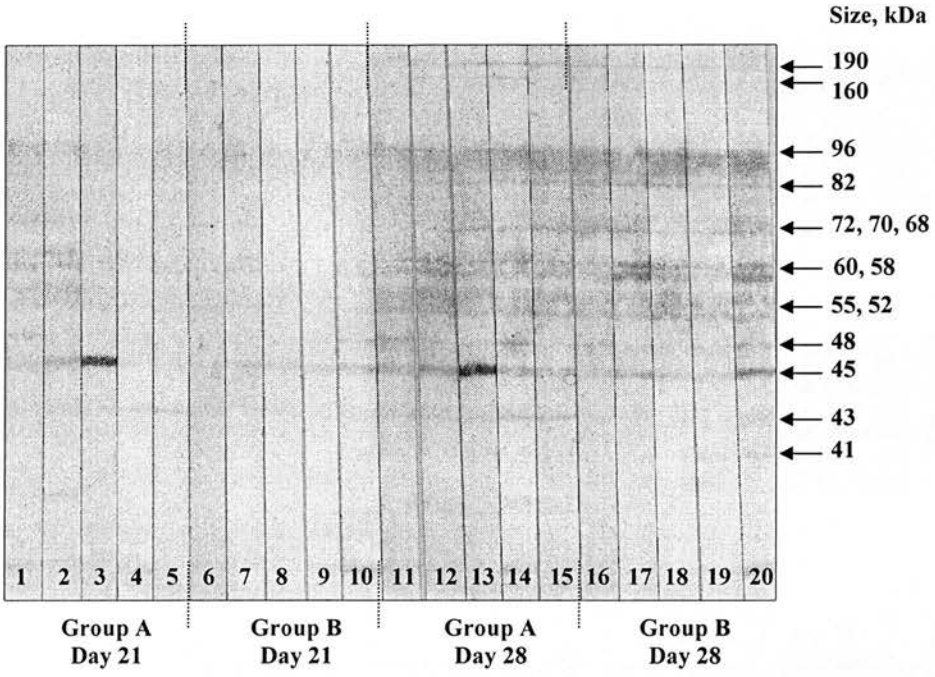
(a) Total IgG antibody responses

Antibody responses in groups A and B to the ES preparation were detected in all animals tested by day 28. The band recognition pattern in both groups was similar, with antibodies to proteins in the mid region of 96-82, 72-68, 60, 58, 55 and 52 kDa pre-dominating (Figure 6.7). At day 35, 7 days after secondary challenge, responses to 96-52 kDa bands were still detected in both groups A and B, but by day 42, the group A response to this region had begun to wane and a strong response to bands of 30 kDa and less was detected (Figure 6.8). In contrast, group B animals maintained a good response to the mid region bands for a longer period than group A: significant band reactivity was still present at days 49 and at day 56 (Figure 6.9). Both groups C and D detected bands of 96-82, 72-68, 60, 58, 55 and 52 kDa from days 42-49 onwards, showing a similar response to that observed in groups A and B prior to secondary challenge. Groups B, C and D only reacted weakly to bands of 30 kDa and less at day 56, in complete contrast to the group A response.

(b) IgG1/G2 antibody isotype responses

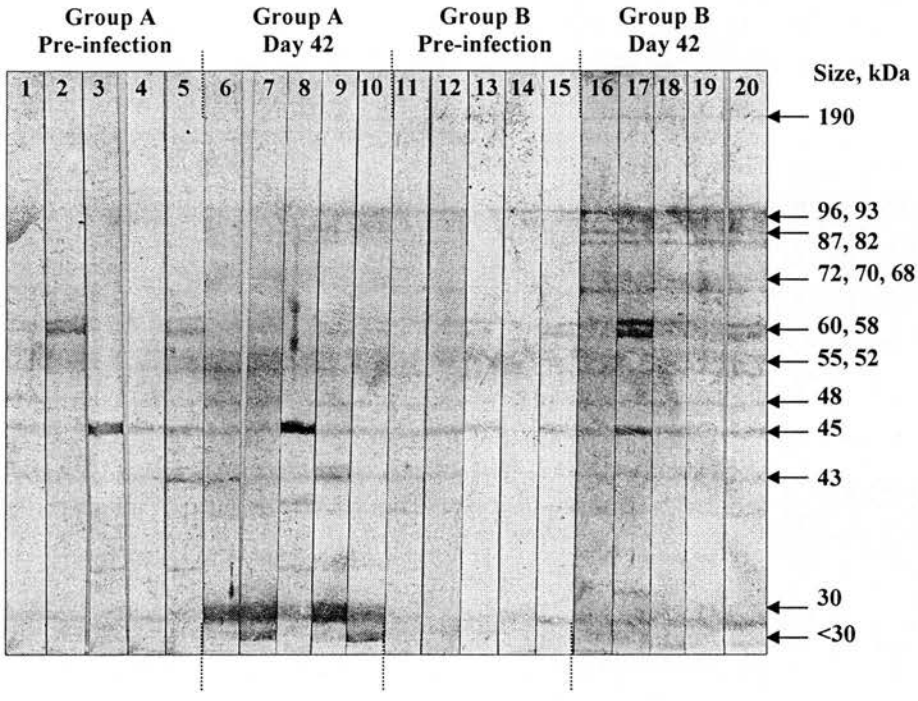
The IgG1/IgG2 isotype antibody response pattern to the ES preparation was examined at day 56 for groups A, B and D. The IgG1 banding pattern was identical to that obtained with the polyclonal anti-total IgG reagent, although recognition of bands of 30 kDa and less in group A was not as strong. However, the group A response to these protein bands was still clearly greater than that of groups B and D. As with the WFA preparation, the IgG2 response was minimal, although samples from three animals recognised bands of either 45, 60 and 58 and 41 kDa. No band specific trend for the IgG2 response was noted.

Figure 6.7: Experiment A; IgG antibody responses to *F. hepatica* excretory-secretory preparation for groups A and B at days 21 and 28.



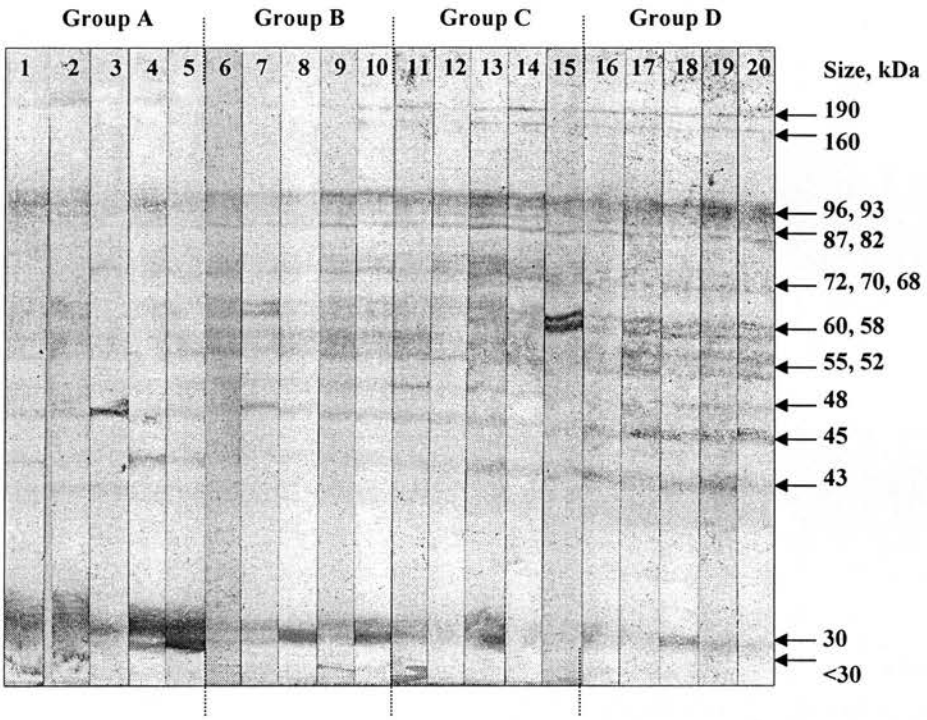
IgG antibody responses to the excretory-secretory preparation for individual animals in groups A (day 21: lanes 1-5, day 28: lanes 11-15) and B (day 21: lanes 6-10, day 28: lanes 16-20) The same individuals were used and were placed in order for each time-point. The major sera-reactive bands are marked with arrows, with band sizes given in kDa. ES was separated on a 10% SDS PAGE gel under reducing conditions.

Figure 6.8: Experiment A; IgG antibody responses to *F. hepatica* excretory-secretory preparation for groups A and B at day 42.



Pre-infection and day 42 IgG antibody responses to the excretory-secretory preparation for individual animals in groups A (pre-infection: lanes 1-5, day 42: lanes 6-10) and B (pre-infection: lanes 11-15; day 42: lanes 16-20). The position of major sera reactive bands are marked with arrows, with band sizes given in kDa. ES was separated on a 10% SDS PAGE gel under reducing conditions.

Figure 6.9: Experiment A; IgG antibody responses to *F. hepatica* excretory-secretory preparation for groups A, B, C and D at day 56.



Day 56 IgG antibody responses for individual animals in groups A (lanes 1-5), B (lanes 6-10), C (lanes 11-15) and D (lanes 16-20). Major sera-reactive bands are marked with arrows, with band sizes given in kDa. ES was separated on a 10% SDS PAGE gel under reducing conditions.

6.3.2.4 Comparison of antibody responses to whole fluke antigen and excretory-secretory preparations

Group antibody responses to the ES preparation followed a similar pattern to that observed with the WFA preparation. Early during infection, bands of 96-82, 70, 68 and 55-52 kDa common to both preparations were recognised by the chronic infection group, A, and the 5-day pre-exposed group, B. After secondary challenge, group A recognition of these mid region bands became less significant, and a response to bands of size 30 and 28 kDa became prominent. In contrast, group B maintained a strong antibody response to the mid region bands after secondary challenge. Group B recognition of the 30 and 28 kDa bands appeared 3-4 weeks later than that in group A, at a similar time to the response in the naïve group, D. Although the immediate post-secondary challenge response to the 96-82, 70, 68 and 55-52 kDa bands in the WFA preparation was stronger in group B than D, by day 70 there was no clear difference in band intensities between the two groups.

Reactive bands present in the ES preparation but absent in the WFA preparation included those of 72, 60 and 58 kDa. Antibody responses to these bands were present particularly during early infection in groups A and B and after secondary challenge in groups C and D. The response to these bands became less significant as the infection progressed. Reactive bands exclusive to the WFA fraction included those of 59, 57.5 and 54 kDa to which there was a strong response early during infection and bands of 120, 76, 34 and 33 kDa, which were only weakly positive at various time-points throughout infection.

6.3.2.5 Total IgG antibody responses to the cathepsin-L pool fraction

The sera antibody IgG1 and IgG2 responses of animals in Experiment A to the cathepsin-L pool fraction were investigated by ELISA and are shown in section 5.3. Western blots of this fraction were also performed with a limited number of samples to support the ELISA analysis.

Sera collected at days 35, 49 and 77 from 5 animals in each of groups A, B and D was tested. At day 35, 7 days post-secondary challenge, group A showed only weak reactivity to the 29.5-28 kDa band doublet and no response was detected in groups B and D. At day 49 (Figure 6.10), the group A response was stronger, but only minimal band recognition was observed in groups B and D. By day 77, sera

from animals in all three groups recognised the cathepsin doublet, with group A showing the strongest response and group B the weakest.

6.3.2.6 Total IgG antibody responses to the metacercarial preparation

Sera samples from 5 animals in each of groups A and B were tested at day 21 (Figure 6.11a) for total IgG responses to the metacercarial preparation. 6 animals from groups A, B and D were also tested at day 49, 21 days after the secondary challenge. Due to scarcity of material, the minimum quantity of the preparation was loaded onto the gels and this is likely to account for the poor responses seen.

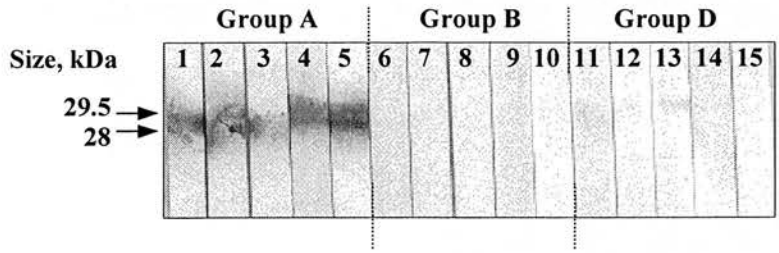
A weak response to 49, 45, 43, 41 and 37.5 kDa bands was present by day 21 with the majority of group A and B samples tested. Weak reactivity to the 37.5 kDa band was also observed with pre-infection samples, suggesting recognition was not infection specific. Some samples from each group also detected 85, 79 and 66 kDa bands, particularly those from group B. By day 49, group A samples showed moderate reactivity to 49, 45, 43, 41 and 37.5 kDa bands and a response to another band of 21 kDa was detected. In group B, 4/6 samples recognised the mid region bands of 85, 79 and 66 kDa, although no reaction to the 45, 43 and 41 kDa bands was detected. Group D sera samples were unreactive at day 49, except for that of one animal, which recognised the 85 and 79 kDa bands. As with the juvenile preparation, material was extremely limited, preventing further investigation.

6.3.2.7 Total IgG antibody responses to the 14 day juvenile preparation

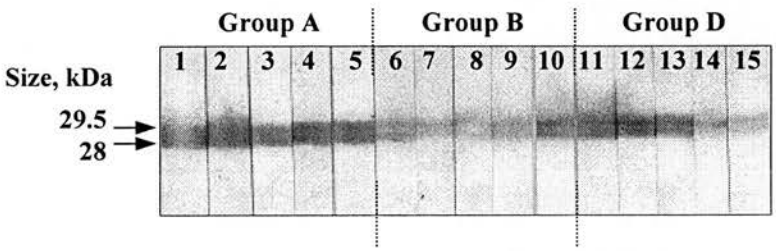
Group A and B sera samples were tested at day 14 for responses to the juvenile homogenate preparation. No specific band responses were detected for any of the samples tested at this time-point. However, a secondary challenge response to this extract was observed in group A at days 42 and 49 (Figure 6.11b). The recognition of two bands of 30 and 28 kDa was particularly prominent and a moderate response to 41, 38 and 38.5 kDa bands was also detected. Groups B, C and D showed a minimal response to the extract at days 42 and 49, although weak recognition of 48, 45, 38 and 30 kDa bands was observed with some samples. Unfortunately, adequate material was not available to allow further investigation of responses to this extract.

Figure 6.10: Experiment A; IgG antibody responses to the cathepsin-L pool fraction for groups A, B and D at days 49 and 77.

(a)



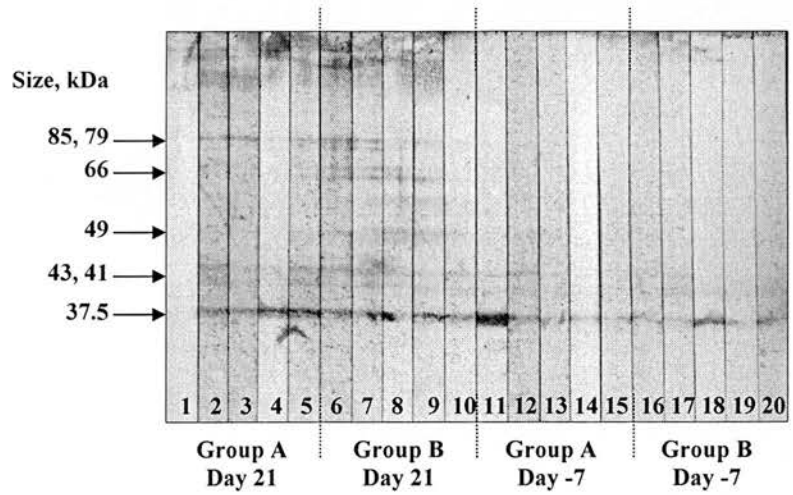
(b)



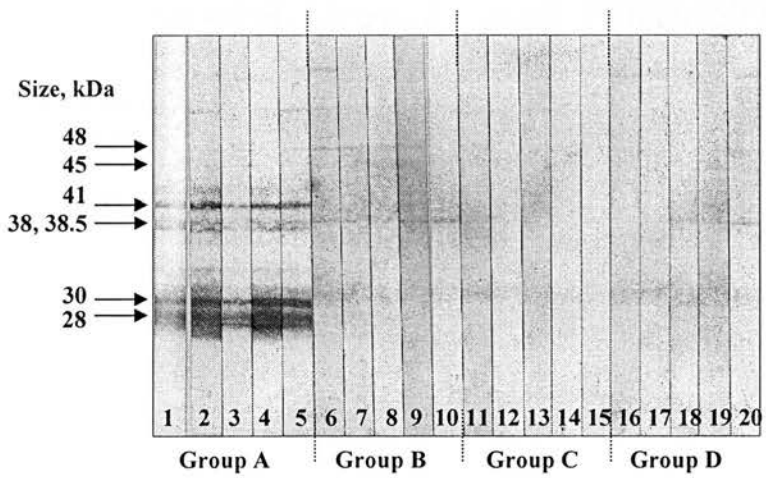
Lanes were incubated with sera taken from 5 animals in each of groups A (lanes 1-5), B (lanes 6-10) and D (lanes 11-15) on days 49 (a) and 77 (b), using the same animals at each time-point. Cathepsin was separated on a 14% SDS PAGE gel under reducing conditions.

Figure 6.11: Experiment A; IgG antibody responses to metacercarial and 14 day juvenile homogenate preparations for groups A, B, C and D.

(a) Metacercarial preparation



(b) Juvenile preparation



Metacercarial preparation (a), was incubated with sera taken at day 21 (group A: lanes 1-5 and group B: lanes 6-10) and day –7 (group A: lanes 11-15 and group B: lanes 16-20). Juvenile preparation (b) was incubated with day 49 sera from groups A (lanes 1-5), B (lanes 6-10), C (lanes 11-15) and D (lanes 16-20). Both preparations were separated on 12% SDS PAGE gels under reducing conditions.

6.3.3 Results of Western blots performed with samples from Experiment B

6.3.3.1 Sera IgG1 antibody responses to whole fluke antigen

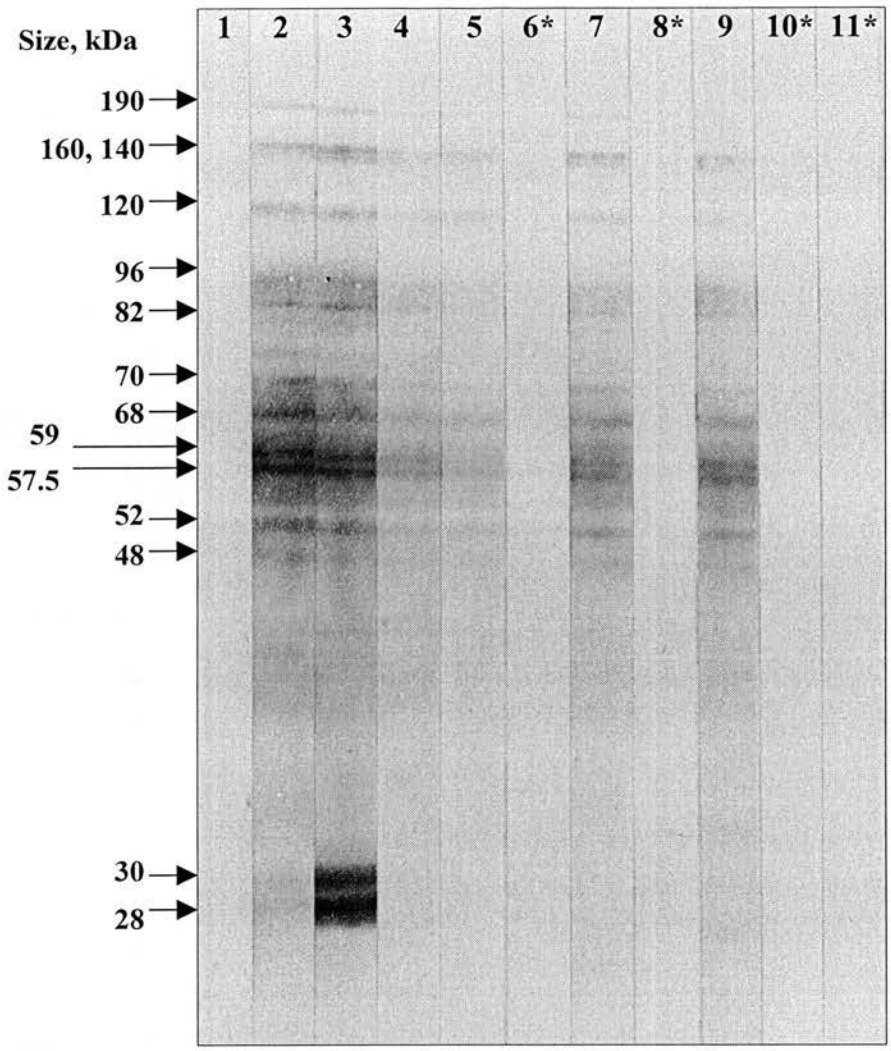
Antibody responses to WFA in the 5-6 day pre-exposed group of Experiment B (group B) were similar to those observed with samples from group B of Experiment A (section 6.3.2.2). At days 19 and 26 post-primary infection, 3/4 sera samples were strongly reactive to the 59 and 57.5 kDa bands and 2/4 samples were moderately reactive to 55, 54, 43, 41, 34 and 33 kDa bands. After secondary challenge at days 40/41, all pre-exposed group B animals reacted strongly to the 59, 57.5, 54, 43, 41 and 31 kDa bands. However, bands of 96-82 and 70-68 kDa were only weakly recognised by sera in this experiment, in contrast to the antibody response observed in Experiment A. No bands were detected by group D sera at day 40/41. Bands of 30 and 28 kDa were not detected by any group B sample at any time-point.

6.3.3.2 Hepatic lymph node IgG1 and IgG2 antibody responses to whole fluke antigen

The cell culture fluid IgG1 response to WFA is shown in Figure 6.12. Samples from the trial animals, calves 813 and 827 (section 4.2.1), reacted strongly to 190-120, 96-82, 76, 70, 68, 59, 57.5 and 52 kDa bands. Calf 827 also reacted very strongly to the 30 and 28 kDa bands. Group B animals showed a response pattern to the high and mid region bands which was identical to that of calves 813 and 827, although the response was weaker overall. However, the 30 and 28 kDa bands were not recognised by any of the group B samples. The most immunoreactive bands in the preparation were those of 160-140, 70, 68, 59 and 57.5 kDa. There was no reaction to any band with cell culture fluid from group D animals. Cell culture fluid from calves 813 and 827 was tested with a polyclonal anti-IgG2 reagent and culture fluid from all other animals was tested with the monoclonal anti-IgG1 and IgG2 antibodies. No IgG2 response was detected with any sample.

Both sera and cell culture fluid detected the same bands in the WFA preparation, with antibody responses to 59 and 57.5 kDa bands predominating. However, the cell culture fluid also recognised higher region bands of 190–120, 96-82 and 76-68 kDa, which were not detected by sera samples.

Figure 6.12: Experiment B; hepatic lymph node cell culture supernatant fluid IgG1 antibody responses to the whole fluke antigen (WFA) preparation.



Lanes were incubated with hepatic lymph node cell culture fluid (undiluted) as follows: culture media negative control (lane 1), trial calves 813 and 827 (lanes 2-3) and main experiment calves 1, 2, 6*, 9, 12*, 13, 16* and 17* (lanes 4-11). Lanes incubated with group D calves are shown by an asterisk *. WFA was separated on a 10-15% SDS PAGE gradient gel under reducing conditions.

6.3.3.3 Sera IgG antibody responses to the excretory-secretory preparation

The IgG antibody response of the 5-6 day pre-exposed group, to proteins in the ES preparation was again very similar to the pre- and post-challenge responses of group B animals of Experiment A (section 6.3.2.3). At day 19, sera reacted strongly to 96-82, 60, 58, 55 and 52-45 kDa bands. After secondary challenge at day 40/41, the response to the above bands was maintained and further bands of 72-68 kDa were also detected. There was no detectable antibody response to bands in the region of 30 kDa or less. Samples from group D animals were negative prior to the secondary challenge and only showed a weak response to 60, 58, 55 and 52-45 kDa bands at days 40/41.

6.3.3.4 Hepatic lymph node IgG antibody responses to the excretory-secretory preparation

Cell culture supernatant fluid from all pre-exposed animals (group B) recognised bands of 96-82, 72-68, 60, 58, 55 and 52 kDa (Figure 6.13). The responses of calves 9 and 13 were particularly strong. Only one animal in the naïve group, D, (calf 12) showed any reaction to these bands, and this was minimal. Control media, and hepatic node cell culture fluid from an uninfected animal, calf 241, were negative.

Both sera and cell culture fluid samples recognised the same bands in the ES preparation. In all cases the culture fluid responses were much weaker than the sera responses.

6.3.3.5 Hepatic lymph node IgG antibody responses to the cathepsin and high molecular weight fractions

An attempt to investigate the presence of antibodies in hepatic node cell culture fluid to the high molecular weight and cathepsin fractions by ELISA were unsuccessful, due to the high background response induced by the culture media. Western blots were therefore performed. Figure 6.14 shows the IgG response of cell culture samples to the cathepsin and high molecular weight fractions. All samples, except that from calf 827, were negative for antibody to the cathepsin fraction. The positive control sample of sera from calf 95 at day 69 gave a very strong response to the cathepsin preparation showing that the blot transfer and incubation steps were

successful. In contrast, samples from the trial calves 813 and 827, calves 1, 2 and 13 (group B) and calf 12 (group D) showed weak reactivity to the high molecular weight fraction.

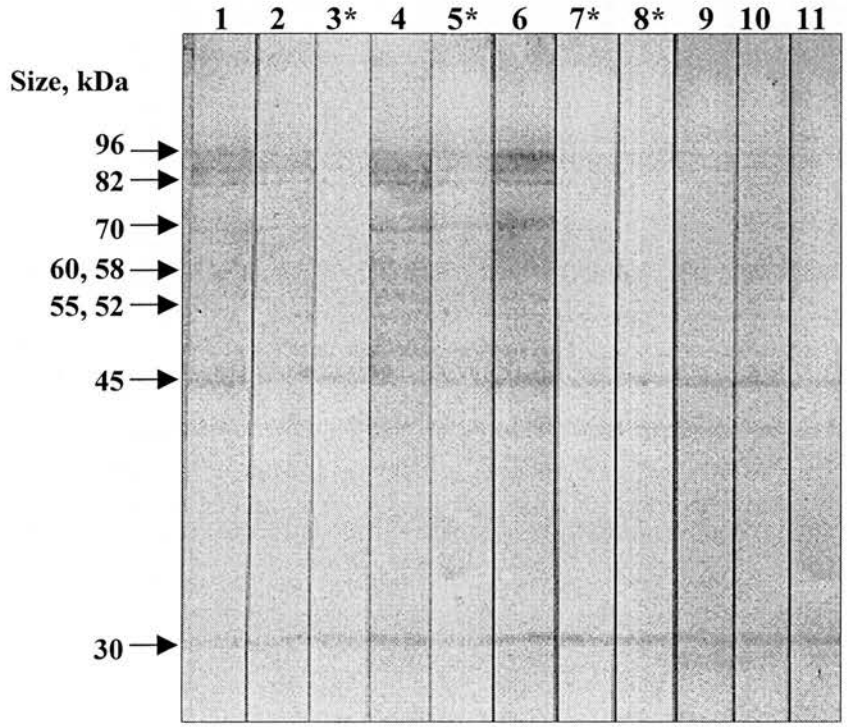
6.3.3.6 Sera total IgG antibody responses to the metacercarial preparation

Sera responses to the metacercarial preparation were tested on both large gradient and small mini-blots. Results of the mini-blot tested with samples from the pre-exposed group, B, is shown in Figure 6.15a. Most group B animals showed a weak non-specific response to the 37.5 kDa band with negative pre-infection and early infection samples. However, after challenge at days 40/41, the response to the 37.5 kDa band and those of 49, 43 and 41 kDa was very strong with all animals except calf 2. Moderate reactivity to 85, 79, and 66 kDa bands was also present. Sera from group D animals showed only a minimal response to the 49 and 43-37.5 kDa bands after challenge.

6.3.3.7 Sera total IgG antibody responses to the 14 day juvenile preparation

A strong post-challenge response to the juvenile preparation was present at day 40/41 with 3 out of 4 animals in group B. The greatest response was to bands of 57.5, 48, 45, 38 and 30 kDa (Figure 6.15b). Two animals in group B also showed a weak response to these bands during the primary infection period at day 26. Group D animals showed no reaction to any bands at any of the time-points studied.

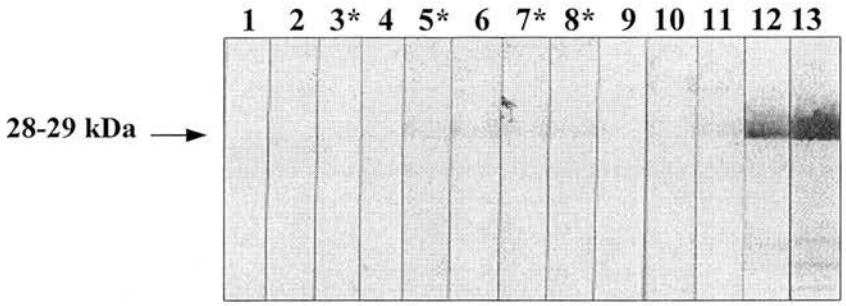
Figure 6.13: Experiment B; hepatic lymph node cell culture supernatant fluid IgG antibody responses to *F. hepatica* excretory-secretory preparation.



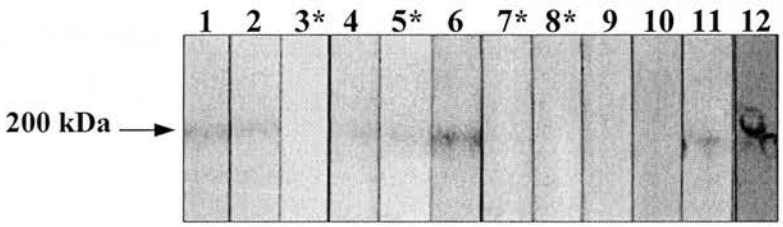
Lanes were incubated with hepatic lymph node cell culture fluid (undiluted) as follows: calves 1, 2, 6*, 9, 12*, 13, 16* and 17* (lanes 1-8); negative controls: culture fluid from hepatic nodes of two uninfected calves, 237 and 241 (lanes 9 and 10) and culture media alone (lane 11). Lanes incubated with group D calves are marked by an asterisk *. ES was separated on a 12% SDS PAGE gel under reducing conditions.

Figure 6.14: Experiment B; hepatic lymph node cell culture supernatant fluid IgG antibody responses to the cathepsin-L pool and high molecular weight fractions.

(a) Cathepsin-L pool fraction



(b) High molecular weight fraction



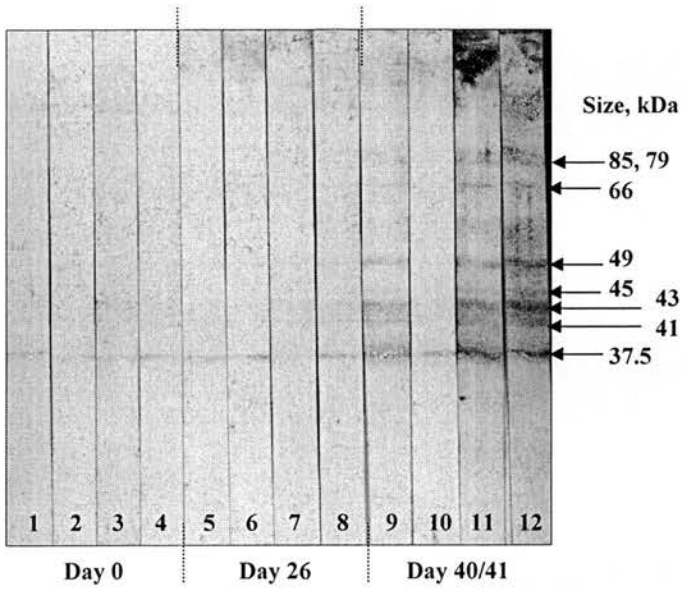
In both (a) and (b) lanes were incubated with hepatic lymph node cell culture fluid (undiluted) as follows: calves 1, 2, 6*, 9, 12*, 13, 16* and 17* (lanes 1-8); negative control samples of culture media alone (lane 9) and culture fluid from an uninfected calf, 241 (lane 10). Lanes incubated with group D calves are marked by an *.

Positive controls in (a) were trial calves 813 and 827 (lanes 11 and 12) and day 68 infection sera from a chronically infected calf, 95 (lane 13). The latter sera sample was used at a 1/200 dilution. Positive controls in (b) were trial calves 813 (lane 12) and 827 (lane 11). In (b), supernatant fluid for group B and D calves was obtained from cells cultured at 1 x 10^6 cells/ml, whereas all other samples were cultured at 2 x 10^6 cells/ml.

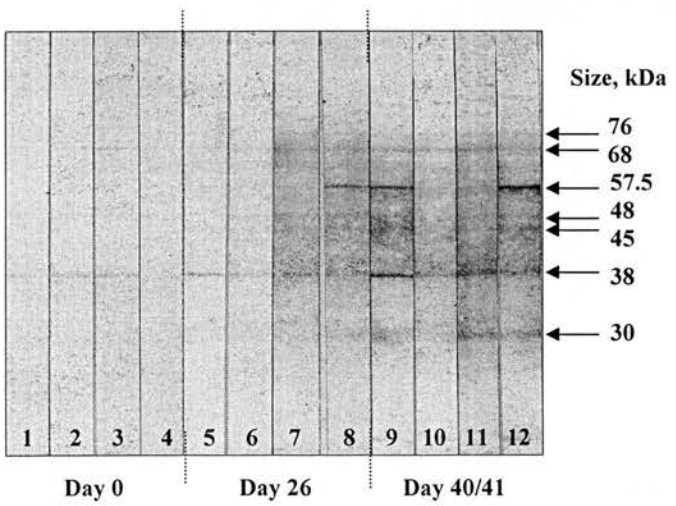
Cathepsin was separated on a 14% SDS PAGE gel under reducing conditions and the high molecular weight fraction was separated on a 7.5% SDS PAGE gel under non-reducing conditions.

Figure 6.15: Experiment B; IgG sera antibody responses to metacercarial and 14 day juvenile homogenate preparations for group B at days 0, 26 and 40/41.

(a) Metacercarial preparation



(b) Juvenile preparation



Lanes 1-4, 5-8 and 9-12 were incubated with sera taken from group B calves 1, 2, 9 and 13 at days 0, 26 and 40/41 post-primary infection, respectively. Both preparations were separated on 12% SDS PAGE gels under reducing conditions.

6.4 DISCUSSION

6.4.1 Distinct banding patterns for the early and late phases of infection

One of the most striking differences in the band response pattern to WFA and ES preparations with the four groups in Experiment A, was the immunodominance of high to mid weight protein bands during early infection and the subsequent switch to recognition of lower weight proteins as infection progressed to chronicity.

A strong antibody response to the 96-82 and 70-52 kDa protein bands was detected in group A, and to a lesser extent in group B, during the primary infection period at day 28 and in groups B and D, after the secondary challenge infection. Antibody responses to bands of 96-82, 70, 68, 55 and 52 kDa common to both ES and WFA preparations were especially distinctive. With the WFA preparation, antibody responses to these bands were particularly strong in group B between days 42 to 56, at a point when the group A response appeared to have waned and that of group D was yet to become substantial. This trend differs slightly from the response pattern observed with the ES preparation, where the timing and strength of the group B response to the high to mid range proteins was similar to that of the naïve control group, D, after the secondary challenge infection.

As infection progressed in the chronic group A and after the secondary challenge, the antibody response switched from the recognition of the high to mid weight bands to those of 30-28 kDa. This switch became apparent at day 42, and by day 70 the response to the 30-28 kDa region in group A was extremely strong. In contrast, group B and C animals did not show any reactivity to 30-28 kDa bands until much later, at around day 70, and at the same time as antibody responses were detected in group D.

The antibody response to proteins exclusive to either of the two preparations, the doublet bands 60 and 58 kDa present in ES, and 59 and 57.5 kDa present in WFA, were also predominant during early, but not late infection. Antibody recognition of the 59 and 57.5 kDa bands was particularly strong in group B after the secondary challenge infection. Although these bands may be merely markers of the juvenile stages of infection, their prominence in the pre-exposed group B, which displayed signs of reduced liver damage after secondary challenge infection (section 3.3), is significant. Antibody responses to these bands should be examined in more

detail using a quantitative method such as ELISA and any possible correlations with infection parameters investigated.

Why did the secondary challenge infection not boost the response to the high to mid region proteins in group A, as it had with group B? This observation is similar to the situation demonstrated by the IgG1/IgG2 ELISA analysis of antibody responses to the high molecular weight fraction (section 5.3.3), in which the antibody response in group A was dampened immediately after secondary challenge. In order to clarify whether the waning response was a result of the superimposed secondary challenge infection, an additional group could have been run alongside group A, receiving only a single primary infection. Alternatively, the response to the high to mid region bands may not have been boosted because the secondary challenge flukes were unable to establish in the presence of a primary infection. However, infection parameters did provide indirect evidence that the secondary infection flukes were able to invade and cause damage to the liver (sections 3.3.1-3.3.3).

Other studies, using sera from naturally and experimentally *F. hepatica* infected cattle have detected antibody responses to proteins of similar size to the high and mid weight proteins reported in this study. The methods used for separating proteins by SDS PAGE and for calculating band sizes vary between different investigators. Therefore, actual band sizes in kDa may not be comparable between different studies. However, a comparison of antibody reactivity to regions of the protein profile and similarities in the binding patterns can be made. Diffuse binding to a 65-52 kDa region in adult whole fluke preparation has been observed with sera from naturally and experimentally infected cattle in Japan (Itagaki *et al.*, 1995). Another study also detected antibody to proteins of 56, 64 and 69 kDa at 4-6 weeks post-infection in cattle (Santiago and Hillyer, 1988). Immunoreactive proteins of 95, 75, 70, 66, 55 and 50 kDa in adult whole fluke and excretory-secretory preparations have also been detected with sera from infected Peruvian cattle (Oblitas, 1997). The latter study also detected antibodies to various proteins less than 45 and greater than 100 kDa in size. Bovine IgG antibody responses to proteins of 76-68 and 60-52 kDa are therefore consistently detected in studies conducted in different countries, using different parasite and host stocks.

It is likely that the naturally exposed cattle from Japan and Peru were being continually super-infected throughout the fluke season, and yet antibody to these high

and mid weight proteins was still detected in field sera samples, probably obtained from animals with mature infections. In contrast, in this experiment, the chronically infected group, A, showed a diminished antibody response to this region after secondary challenge, with the 30-28 kDa region becoming immunodominant within 6 weeks of primary infection. A possible explanation for this difference between the studies could be the method of super-infection and the size of infective dose: naturally infected animals probably ingest low doses of metacercariae over a prolonged period, whereas in this study, animals were given moderate bolus doses. The host immune response may react differently in the two situations.

The change in the antigen recognition profile, from the detection of protein bands in the high and mid weight region during early infection, to recognition of bands of less than 30 kDa during late infection, has also been observed in other ruminant studies (Chauvin *et al.,* 1995, Itagaki *et al.,* 1995). However, this study is unique because a dominant antibody response to these proteins was demonstrated after secondary challenge in 5-6 day pre-exposed animals, which had showed evidence of a degree of functional protection to challenge, when compared to naïve animals.

6.4.2 Antibody responses to metacercarial and juvenile protein preparations

IgG antibody responses to the 14 day juvenile preparation were not detected in any of the groups of Experiment A until after the secondary challenge infection. Given the presence of certain antigens common to this and the adult fluke preparations, this observation is unusual and could be due to poor optimisation of the assay for this particular protein preparation. As with responses to the WFA and ES preparations, there was a clear difference between antibody responses after secondary challenge in the chronically infected group, A and the pre-exposed group, B. Group A showed a strong response to the 30–27 kDa bands, which were not detected by sera from any of the other groups at this time-point. Instead, all of the group B animals tested and some animals in groups C and D recognised bands of 48, 45, 41 and 38 kDa. Group B animals from the second experiment (B), showed a similar but stronger reaction to these bands, additionally recognising those of 76, 68 and 57.5 kDa.

One difference between antibody responses to the juvenile preparation and adult preparations, was the fact that the majority of the mid weight proteins of 70-55 kDa were not detected in the juvenile homogenate by antibody, whilst antibody responses to proteins of 48-38 kDa, which were poorly recognised in adult WFA and ES preparations, were detected in the juvenile homogenate. IgG antibody responses to bands of 66, 45, 38 and 25-30 kDa, present in a 21 day homogenate preparation, have previously been detected in experimentally infected cattle (Oblitas, 1997).

The metacercarial preparation contained a different set of proteins, with the exception of a 45 kDa band, to those found in the adult and juvenile preparations. Unlike the juvenile preparation, an early antibody response to primary infection was observed at days 14 and 21, with sera from groups A and B of Experiment A, even though a smaller quantity of the metacercarial extract was used. During the primary infection antibody responses to 49, 45, 43, 41 and 37.5 kDa proteins were detected. After secondary challenge, group A maintained a reaction to these bands and a further band of 21 kDa was detected. However, sera from group B was poorly responsive after challenge, although a weak reaction to bands of 85, 79 and 66 kDa was observed. Group B animals from the second experiment (B) showed a stronger antibody reaction, recognising 85, 79, 66, 49, 45, 43, 41 and 37.5 kDa bands. Oblitas (1997), detected antibody responses to metacercarial proteins of 40-38 and 22-18 kDa with infected cattle sera, which may correspond to some of the bands recorded in this study.

6.4.3 Nature of reactive bands

A variety of proteins have now been isolated from *F. hepatica* adult flukes. The majority of these are of low weight, including the cathepsin-L and B proteases, 28-30 kDa (Smith *et al.,* 1993a; Heussler and Dobbelaere, 1994; Wilson *et al.,* 1998), glutathione S-transferase, 26-28 kDa (Howell *et al.,* 1988; Muro *et al.,* 1993), fatty acid binding protein, 12-14 kDa (Hillyer, 1985), calcium binding protein, 22 kDa (Ruiz de Eguino *et al.,* 1999) and cytochrome c peroxidase, 31 kDa (Campos *et al.,* 1999). Some greater than 200 kDa have also been identified such as the high molecular weight haem-containing fraction (McGonigle and Dalton, 1995) and a dipeptidylpeptidase (Carmona *et al.,* 1994). One or more of the proteins of 30-28 kDa detected in the WFA and ES preparations may correspond to a cathepsin protease.

Antibody to these bands was first detected at a similar time-point to that of antibody to the purified cathepsin-L pool fraction. The use of 2 dimensional electrophoresis or specific antisera to the cathepsin fraction would be necessary to demonstrate this unequivocally. It should be noted that cathepsin proteases have also been isolated from juvenile flukes (Creaney *et al.,* 1996; Wilson *et al.,* 1998) and so it is possible that the 30-28 kDa bands in the juvenile preparation also correspond to a cathepsin protease. Other possible proteins that could be present in this region of the WFA or ES preparations include cytochrome c peroxidase and glutathione S-transferase.

The identity of many of the high to mid weight proteins to which antibody responses were observed in this study remains unknown. As the response to this region was first seen during early infection, they must be associated with juvenile fluke stages, although held in common with mature flukes. Two isolated proteins fall within the 96-52kDa region: a leucine aminopeptidase, which runs at 65 kDa on reducing SDS PAGE, with minor bands of 43-30 kDa (Acosta *et al.,* 1998) and paramyosin, of approximately 94 kDa (Spithill *et al.*, 1998). Some of the unknown proteins may be surface antigens. The tegument of the fluke contains granules, called T0, T1 or T2 granules. Proteins within the granules are incorporated into the surface glycocalyx covering of the tegument (Hanna, 1980b). T0 granules are only found in newly excysted juveniles, whilst T1 granules appear soon after liver penetration, at around day 5 (Bennett and Threadgold, 1975). T2 granule proteins do not appear on the surface until the flukes begin to enter the bile ducts, at around 6 weeks post-infection. The protein composition of the T1 granules is therefore of most interest in this study, particularly regarding the responses of the 5-6 day pre-exposure group, B. Monoclonal antibodies have identified an antigen of 50 kDa and minor components of 40 and 25 kDa in the T1 granule (Hanna and Trudgett 1983). In this study, proteins of size 48 and 52 kDa were detected, which could be equivalent to one of the components of the T1 granule. Several surface proteins of 78, 46 and less than 32 kDa have been identified on juvenile flukes of up to 14 days old, by immunoprecipitation with sera from infected animals (Lammas and Duffus, 1985). 21 day old flukes show similar surface glycoprotein profiles to mature flukes, although the expression of a group of proteins of approximately 70 kDa is greatest in the juvenile, whilst 85, 94 and 110 kDa proteins are not detected in the adult (Dalton *et al.,* 1985). The 70 kDa cluster and those of 85 and 94 kDa identified in the latter

study could be equivalent to the proteins eliciting strong antibody responses during early infection in this experiment. Juvenile and mature fluke surface preparations also contained a 52 kDa antigen, which again could be equivalent to bands detected in this experiment.

6.4.4 Isotype specific antibody responses

With both WFA and ES preparations, an IgG1 antibody isotype response dominated in all groups, and only a minimal IgG2 response was observed. The weak IgG2 response had a similar antigen recognition profile as that of the IgG1 isotype, which may have been due to background binding of the anti-IgG2 monoclonal antibody to IgG1 immunoglobulin. In contrast, a small number of bands in the ES preparation were detected that exhibited an intensity greater than that seen with the IgG1 monoclonal, suggesting the presence of a specific IgG2 response. However, no consistent pattern with the IgG2 response was noted. Ideally, other time-points should have been tested for isotypic antibody responses, particularly during the first 4 to 5 weeks of infection. Although the day 56 time-point allowed both a chronic infection in the early stages (group D) and late stages (group A) to be examined, early isotype responses in animals receiving abbreviated primary infections (groups B and C) were not investigated. Previous results from the ELISA study with the high molecular weight fraction showed that some animals in group B had a definite IgG2 response at day 28 (section 5.3.3). However, from the results obtained in this Western blot study, it can be concluded that infection in all groups stimulated a predominantly IgG1 antibody response, typical for *F. hepatica* infection (Clery *et al.*, 1996; Bossaert *et al.*, 2000).

Other antibody isotypes responses were not studied: an effective antisera to bovine IgE is still not available and there was inadequate time available to investigate IgM and IgA responses. An investigation of specific IgE responses to fluke proteins would be very useful. In some human schistosomiasis studies, IgE antibody levels correlate with resistance to reinfection (Hagan *et al.*, 1991; Rihet *et al.*, 1991; Dunne *et al.*, 1992; Demeure *et al.*, 1993). It should also be noted that the group B animals of Experiment A tended to show an overall increase in strip background after the secondary challenge response, a reaction not noted with the other three groups. The

presence of an excess of antibody of another isotype binding non-specifically to the nitrocellulose membrane could have been responsible for this effect.

6.4.5 Antibody in hepatic lymph node cell culture fluid

Culture of unstimulated hepatic node cells to investigate local antibody production was successful. Cell culture supernatant fluid from all pre-exposed animals (trial and main study) detected various proteins in the WFA and ES preparations, whilst that from naïve challenged animals did not. This suggests that at 10 days post-infection, a single primary infection was unable to stimulate a detectable local antibody response to the adult-derived WFA and ES preparations. The antibody produced by the hepatic lymph node cells of pre-exposed animals may have been in response either to the primary, drug-abbreviated infection received at day 0, or to the secondary challenge infection evoking a memory response. A control group receiving only the primary pre-exposure should have been run alongside the challenged group, in order to determine whether antibody was induced locally by the primary drug-abbreviated infection, which was still detectable by day 40.

Hepatic node antibody response profiles were similar to those obtained with sera, with the exception of 190, 160 and 140 kDa proteins. These high molecular weight proteins were weakly and inconsistently detected by sera, but consistently recognised by antibody in cell culture fluid. The trial calf, 827, unlike all other pre-exposed animals, gave a strong reaction to the 30-28 kDa bands. This suggested that a positive response to the purified cathepsin-L fraction might also be obtained. Calf 827 did react strongly to the cathepsin fraction, showing a response similar to that of chronic infection sera. This sample therefore acted as a good positive control, indicating that the negative response to the cathepsin fraction obtained with all other cell culture fluids was genuine. The reason that calf 827 gave a positive response to this region is not clear, although it is possible that the primary infection was not completely terminated by triclabendazole treatment and maturing flukes were present in the liver at the time of secondary challenge. The absence of such a response with the other pre-exposed animals in this experiment (B) and with sera from group B and C animals of Experiment A prior to day 70, suggests that the termination of the primary infection by triclabendazole treatment was successful in all other cases.

Cell culture supernatant fluid from some group B animals gave a weak IgG antibody response to the high molecular weight protein fraction. This supports the observations of cell culture studies (section 4.3.5), in which hepatic lymph node cells showed a strong proliferative response after stimulation with the high molecular weight fraction 10 days after infection. In contrast, the same cells showed only a weak proliferative response to culture with the cathepsin-L pool fraction. In addition, sera antibody responses to the high molecular weight fraction were seen early during the primary and secondary challenge periods in Experiment A animals, whilst antibody to cathepsin did not appear for a further few weeks (sections 5.3.3-5.3.4).

6.4.6 Limitations of the analysis

Interpretation of antibody responses to proteins by Western blot analysis is limited by the detection capabilities of the reagents available. A trade-off between sensitivity and effective blocking of non-specific background responses must be carefully balanced. In this study, the polyclonal anti-IgG1 and total IgG reagents did show a degree of background response that was not ideal, which meant low concentrations of sera and reagents had to be used. This is likely to have affected the detection of weak band responses during the early days of primary infection, with the result that little reaction was noted during the first 21 days of infection, particularly in the drug-abbreviated groups B and C. The monoclonal antibodies that became available later in the study resulted in a lower background response, although they were less sensitive. A full analysis of samples with these reagents may have produced clearer blots, however time was limiting and repetition of blots was not possible. Not all time-points were investigated with all the various sera, particularly with the juvenile and metacercarial extracts. A fuller investigation of the latter responses could be useful, however material was severely limited due to the cost of metacercarial cysts, making this impossible for the current project.

Previous work comparing antigenic profiles of juvenile flukes during the first 10 days of infection, has demonstrated that protein expression differs markedly with age, but also with fluke location (Tkalcevic *et al.*, 1996). Up to 6 days old, juvenile flukes recovered from different locations, i.e. peritoneum or liver, showed different antigenic profiles, even if of the same age. However, by day 8, flukes recovered from either site had the same protein profiles, similar to that of adult flukes. A major

switch in antigen expression appeared to occur at 3-4 days post-infection. However, a common 49 kDa antigen was present in all fluke stages, which could be equivalent to the 49 kDa band present in the metacercarial preparation, or the 48 kDa bands in the other preparations used in this study. Given that the antibody responses during very early infection were of particular interest in this study, the use of a protein preparation from much younger flukes would have been appropriate. In particular, responses to a 5-7 day old fluke preparation should have been investigated, as this is the age at which infection was terminated in the group B pre-exposed animals.

6.4.7 Conclusions

The results displayed in this chapter clearly illustrate the recognition of a group of high to mid weight proteins of 96-82, 76-68 and 60-52 kDa, of unknown identity, by antibody present in early infection sera from animals receiving drug-abbreviated and chronic infections. Pre-exposed animals maintained a strong antibody response to these proteins after secondary challenge, whilst chronically infected animals did not. Lower weight proteins of 30 kDa and less, were not reactive with sera from infected animals until later in infection, from approximately day 40 onwards. Western blots also confirmed the absence of an IgG antibody response to cathepsin-L protease during the early stages of infection.

This study has also demonstrated for the first time the presence of a strong local hepatic IgG1 antibody response to fluke proteins, particularly those of 190-120, 96, 87, 70, 68, 59 and 57.5 kDa, during early infection. Although there was no difference in the nature of proteins recognised by sera and hepatic lymph node culture fluid, the analysis was useful in emphasising the particular bands most reactive with locally-derived antibody.

Further investigation into the identity and nature of the high to mid weight proteins, especially those of 190-120, 96-82, 72-68 and 60-54 kDa, is necessary to fully understand the immune responses that occur in infected animals during the early migratory stages of *F. hepatica* infection.

CHAPTER 7

GENERAL DISCUSSION & CONCLUSIONS

7.1 Summary of experimental results

This study has demonstrated that pre-exposure to juvenile flukes during the first 5-6 days of infection is beneficial to cattle. Upon subsequent re-challenge, pre-exposed animals showed evidence of reduced liver damage (lower levels of the enzymes γ-GT and GLDH), had a lower final eosinophilia and a transient reduction in early egg output levels, when compared to naïve animals.

The presence of a fluke-specific immune response in pre-exposed animals was detected in peripheral lymphocytes and in hepatic lymph node cells, but not in cells from mesenteric lymph nodes. No difference in the magnitude of proliferative responses of peripheral blood mononuclear cells or hepatic lymph node derived cells to fluke proteins was observed between pre-exposed or naïve challenged animals. However, a difference in the cytokine profile of hepatic node cells was detected, with pre-exposed animals producing less IFN-γ than naïve animals after stimulation with whole fluke antigen (WFA), excretory-secretory (ES) and cathepsin-L preparations.

The IgG antibody response to crude fluke protein preparations (WFA and ES) and to the high molecular weight and cathepsin-L fractions was qualitatively similar in pre-exposed and naïve animals after secondary challenge. In contrast, a distinct difference in the antibody response of chronically infected and pre-exposed animals was observed. Chronically infected animals showed a reduction in peripheral cellular reactivity to fluke proteins after challenge, which was greater than the reduction observed in proliferative responses of cells from 5-6 day pre-exposed animals. A shift in the antibody recognition profile from protein bands of high to mid molecular weight, to those of less than 30 kDa was also observed after challenge in chronically infected animals. Furthermore, the antibody response to the high molecular weight fraction was significantly reduced in chronically infected animals, compared to naïve or pre-exposed animals, following secondary challenge.

7.2 The early immune response to *F.hepatica* infection

One of the aims of this study was to examine the cellular and antibody responses occurring during the early stages of infection with *F. hepatica*. This was performed by examining the immune responses present within the chronic group (A) prior to secondary challenge and within the naïve group (D) after secondary challenge, throughout the course of Experiment A. Several conclusions can be drawn from the results.

Western blot analysis of WFA and ES fluke preparations found that a predominantly IgG1 antibody response was directed against a group of proteins sized 96-54 kDa during the first 4 to 5 weeks of infection. The identity of these proteins is not known, however they do not appear to correspond to any of the proteins that have been previously used in vaccination trials (Spithill and Dalton, 1998), with the exception of a 65 kDa leucine aminopeptidase (Acosta *et al.*, 1998; Piacenza *et al.*, 1999) and a 94 kDa paramyosin (Spithill *et al.*, 1998). As flukes matured within the host, the pattern of antigenic recognition changed. Antibody responses directed against the 96-54 kDa proteins diminished, whilst those to several proteins of less than 30 kDa became more prominent.

Antibody responses to cathepsin-L, an antigen considered to be a prime vaccine candidate (Dalton *et al.*, 1996), examined by ELISA and Western blot methods, were not prominent during the first 5 weeks of infection. A possible explanation for this observation is that expression of this protein by juvenile flukes did not occur in quantities able to stimulate a detectable antibody response. Additionally, whilst hepatic lymph node cells were moderately responsive to the haem-containing high molecular weight fraction, also a vaccine candidate (Dalton *et al.*, 1996), cellular proliferation in response to cathepsin 10 days after fluke challenge, was poor. However, cells did respond to cathepsin during early infection by the production of IFN-γ. Perhaps cathepsin released by juvenile flukes modulates or downregulates specific antibody production during early infection. These results suggest that a cathepsin vaccine may not be efficient at generating an immune response to the early invasive stages of the fluke. In contrast, antibody to the high molecular weight fraction appeared within the first 7-14 days of infection, even in the 5-6 day and 24-48 hour pre-exposure groups. The high molecular weight protein

must therefore be expressed by flukes of less than 7 days old. Further investigation into the role of this protein fraction in the juvenile fluke and the host response generated during early infection is therefore warranted.

7.3 The effect of pre-exposure upon subsequent infections

The second objective of the study was to investigate whether pre-exposure to juvenile flukes stimulated resistance to secondary challenge infections. Pre-exposure to flukes over the first 5-6 days of infection did induce a degree of protection against challenge, but exposure to flukes during the first 24-48 hours of migration did not. Host exposure to the gut and peritoneal migratory fluke stages only, therefore, did not appear to stimulate a protective effect. In rats, in which resistance to challenge is effected at both the level of the gut and peritoneum/liver, pre-exposure to the gut migratory stages alone does not induce resistance to subsequent challenge (van Milligan *et al.*, 1998c). However, priming animals by implanting newly excysted juveniles into the peritoneal cavity and allowing the infection to progress, does result in resistance. These observations suggest that exposure to the presence of flukes in the peritoneal cavity or liver is required for the development of effective resistance in the rat, a mechanism which is undoubtedly immune-mediated. A similar process in cattle may have been operating in this study.

Resistance to reinfection after a primary, drug abbreviated infection has been observed previously in cattle (Doy and Hughes, 1984). Infection was terminated by the administration of rafoxanide at 16-24 weeks post-infection, by which time mature flukes would have been present in the bile ducts for several weeks. As a result of juvenile migration through the liver and the presence of adults in the bile ducts, it is likely that considerable fibrotic changes in the parenchyma and around the bile ducts would have been induced. Such changes may have resulted in the development of a physical barrier to fluke migration. In the experiments described in this thesis, the contribution of fibrotic changes in the parenchyma to the inhibition of fluke migration are likely to be much less significant. This is because juvenile flukes would have been killed whilst still small, and so before substantial damage occurred. This theory is supported by the results of liver enzyme analysis: little change was observed in GLDH levels above pre-infection values, in pre-exposed animals during the first few weeks of the experiment. The protective effect of pre-exposure to 5-6 day old

flukes may therefore be immunologically based, although definitive evidence supporting this theory is still lacking.

Peripheral and local hepatic cellular immune responses in 5-6 day pre-exposed animals were similar to those found in naïve challenged animals. Furthermore, although antibody from pre-exposed animals recognised various fluke proteins of 96-54 kDa on Western blots with greater intensity than that of naïve animals shortly after the challenge infection, no antigens exclusive to the pre-exposed group were detected. Two issues follow from these observations.

Firstly, a distinctive antibody response in the pre-exposed group may not have been detected because responses to an appropriate antibody isotype or fluke protein preparation were not investigated. Maturation of the antibody response in pre-exposed animals may have also led to the development of protective antibodies of the same isotype, directed against the same protein, but of greater affinity for particular antigenic epitopes. This issue requires further exploration.

The second issue is the question of the significance of antibody in mediating protective immune responses to parasite helminths. Distinct differences in the antibody response between pre-exposed and naïve animals may not have been observed simply because antibody was not involved in the development of the protection observed in the pre-exposed group.

Substantial evidence does exist to support the concept that antibody-dependent immune protective mechanisms are involved in host resistance to trematode and cestode parasites. Epidemiological studies support the view that antibody plays an important role in the resistance to schistosomiasis during natural infections in humans. IgG1 and IgE antibody titres have been found to correlate significantly with resistance to reinfection after parasites have been cleared by drug treatment (Hagan *et al.*, 1991; Rihet *et al.*, 1991: Dunne *et al.*, 1992; Demeure *et al.*, 1993). *In vitro* studies have also demonstrated the ability of eosinophils to kill schistosomula in the presence of specific antisera (Butterworth, 1984; Veith and Butterworth, 1983). The most effective recombinant antigen parasite vaccines that have been produced so far are those used to protect sheep against infection with *Taenia ovis* (Johnson *et al.*, 1989, Harrison *et al.*, 1996) and *Echinococcus granulosus* (Lightowlers *et al.*, 1996a; 1999). The protective effect with both these vaccines appears to be mediated by antibody and can be transferred in colostrum to

the neonate (Lightowlers *et al.*, 1996b; 2000).

A clear association between antibody and resistance to reinfection was not apparent with the cathepsin, high molecular weight and glutathione S-transferase *F. hepatica* vaccines. An initial study found no correlation between IgG antibody titres and the level of protection obtained by inoculating cattle with a combined cathepsin and haem-containing high molecular weight fraction vaccine (Dalton *et al.*, 1996). In a further investigation, a negative correlation was observed between the degree of fluke burden and IgG2 or IgG1 titres in vaccinated animals (Mulcahy *et al.*, 1998). However, the level of protection induced in the vaccinated group was not significant and animals receiving adjuvant alone also displayed a negative correlation of antibody titre against fluke burden. Similarly, studies investigating the protective effect of vaccination with the antigen glutathione S-transferase found no association between antibody titre and the level of protection in sheep (Sexton *et al.*, 1994) or in the ability of sera from vaccinated cattle to inhibit GST activity *in vitro* (Morrison *et al.*, 1996). A possible explanation for these observations is that protection may have been mediated by different antibody isotypes to those studied. In *S. mansoni* infections in humans, a positive association has been detected between IgA antibody specific to the recombinant antigen Sm28GST, a trial schistosomiasis vaccine antigen, and resistance to reinfection after parasite elimination by chemotherapy (Grzych *et al* 1993). IgA present in the sera from these individuals was also able to inhibit GST activity *in vitro*. The presence of any association between antibody titres of other isotypes and the degree of protection induced by the trial *F. hepatica* vaccines should be investigated.

An alternative reason for the absence of specific antibody associated with protection in the *F. hepatica* vaccination studies and in this experiment, could be that an antibody independent immune response, yet to be identified, was involved in resistance.

7.4 The role of strategic drug delivery in the control of fascioliasis

Is it possible to utilise the benefit obtained in this experiment by drug-abbreviated pre-exposure to juvenile flukes in the control of fascioliasis in the field? The timing of drug treatment may be critical, with administration during the first week or two of exposure perhaps being necessary to obtain significant benefit. In

Experiment A, a 4 week old infection (chronic group, A), resulted in substantial depression of the antibody response to a variety of antigens after secondary challenge, as well as a reduction in peripheral cellular reactivity to fluke proteins. Treating animals carrying old juveniles and adult fluke may therefore be ineffective, because they may exert a modulating effect on the host response to challenge. Strategic early drug treatment would not be practical for stock kept on pasture all year, because it would not be possible to accurately identify the point of first infection. However, early chemotherapy could be useful in the management of cattle overwintered indoors, which are subsequently turned out during the early spring onto contaminated pasture.

Interaction between drugs and the immune response has been demonstrated with the drug praziquantel, which is used in the treatment of schistosomiasis. Praziquantel acts synergistically with antibody directed against schistosomula tegumental antigens, to accelerate parasite clearance in animal models (Brindley and Sher, 1987; Fallon *et al.*, 1994; Fallon and Doenhoff, 1995). Evidence for an alteration in the immune response in humans after drug treatment has also been demonstrated in the field by numerous workers (section 3.4.5). Furthermore, these changes are maintained for some time after chemotherapy: an alteration in the antibody response to specific *S. mansoni* proteins has been demonstrated up to 36 weeks following treatment (Mutapi *et al.*, 1998). Whether a similar process occurs with triclabendazole treatment during the early stages of *Fasciola* infection, which could be exploited in strategic drug control, remains to be demonstrated.

7.5 Limitations of the study

The results of this study are particularly useful because it has been conducted in cattle, one of the natural hosts for *F. hepatica*. However, several problems are associated with experimental work involving cattle. These include the lack of available reagents for examining immune responses, specifically an anti-IgE antibody and methods for measuring cytokine production by bovine cells *in vitro*. The cost and logistics of housing and dealing with large animals is also an issue, restricting the size of experimental groups.

Another problem that became apparent during this study was the degree of variability in the response to infection, both physiological and immune-mediated, that

was observed between different animals receiving the same treatments. The source of variability may be two-fold: that as a result of variability between individual animals, but also variability associated with the "take" of infection.

Host variability is influenced by both genetics and environment: the calves in the first study (Experiment A) were obtained from different farms, forming a distinctly outbred population, which would also have experienced different environmental conditions during their first weeks of life. Responses of calves in the second experiment, B, showed a lower level of variability, and were obtained from a single closed farm. Using laboratory bred animals reduces the effect of both genetics and environment, because inbred strains of animals are available and living conditions can be closely regulated (Gärtner, 1999). The recent advances in the development of large animal clones (Wilmut *et al.*, 1997; Cibelli *et al.*, 1998), brings the possibility of the production of genetically identical animals on a relatively large scale. However, it has been demonstrated that variability in a series of physiological characteristics measured in a group of cloned calves (cloned by splitting a blastocyst and implanting cells into enucleated oocysts), was greater that that observed for monozygotic twins calves (Gärtner *et al.*, 1998). It was hypothesised that oocyst signals during very early development may have a significant impact on the development of variability. The use of monozygotic twins may still, therefore, be the best available method of reducing the effects of genetic variability in experimental studies.

Variability in the progress of fluke migration is another component which may have influenced the responses observed during this study. The proportion of metacercariae establishing as mature flukes is low and can vary considerably between animals (Dawes and Hughes, 1964; Ross *et al.*, 1966). Metacercariae may not excyst within the intestine, may become trapped in the intestinal wall or even the pancreas and have been found in the diaphragm, pleurae and lungs, suggesting that migration is random (Boray, 1969). The actual burden of flukes reaching the target organ, the liver, is therefore not predictable and may well have a significant impact on host immune and physiological responses during infection.

7.6 Further work

Whilst the results of this project have provided an important insight into early and local immune responses during *F. hepatica* infection in cattle, many areas of the host immune response to infection remain to be investigated.

The nature of peripheral and local antibody responses present during early infection require further investigation, specifically those to metacercarial and early juvenile preparations. An analysis of IgE and IgA isotype responses to adult and immature preparations may reveal further differences in the immune responses of the experimental groups used in this study. Immunohistochemical investigation of isotype antibody responses to flukes using resin-embedded fluke sections (Hanna, 1980b) would only require a small number of flukes and would be very useful for examining antibody responses to the early juvenile stages, for which material is limited. An alternative approach for identifying *F. hepatica* immunoreactive proteins is by screening cDNA expression libraries with sera from infected animals or from animals vaccinated with ES products. Various proteins, including a calcium-binding protein (Ruiz de Eguino *et al.*, 1999), peroxiredoxin (McGonigle *et al.*, 1997) and thioredoxin (Shoda *et al.*, 1999) have been identified by this method. Screening cDNA libraries of juvenile flukes with pooled sera from animals in pre-exposed, naïve and chronically infected groups may result in differential recognition of immunoreactive fluke proteins.

The response of peripheral and lymph node cells to culture with fluke proteins provides useful information regarding the particular protein fractions which stimulate an immune response and the type of cytokine profile induced. This in turn provides an indication of the nature of the immune effector mechanisms operating during infection. In this study, it would have been appropriate to have examined hepatic and mesenteric lymph node cell responses to metacercarial, newly excysted juvenile and 7 day juvenile protein preparations. The response to these fractions may differ to that seen after stimulation with adult fluke preparations, and this may be particularly relevant regarding the immune response of the 5-6 day pre-exposed animals. Analysis of cell phenotypes present after culture with fluke preparations, or the use of selected cell population depletion prior to culture could give an indication of the cell types responsible for the observed responses. Previous work has demonstrated that both $CD4^+$ and $CD8^+$ T cells contribute to cell proliferation after antigenic stimulation

during *F. hepatica* infection (McCole *et al.*, 1999a). Phenotype analysis may also provide an insight into the reason for the depression of cellular reactivity, observed after secondary challenge infection with chronically infected animals (section 3.3.9).

Another cell type that may be involved in the downregulation of lymphocyte responses in this study is the $\gamma\delta^+$ T cell subset. $\gamma\delta^+$ T cells are found at mucosal and epithelial surfaces, as well as in the peripheral circulation. Various studies have supported the idea that they may play a role in regulating the host immune response to environmental proteins, such as ingested or inhaled antigens, inducing a degree of tolerance (reviewed by Kaufmann, 1996). $\gamma\delta^+$ T cells are able to react quickly to pathogen stimulation by producing a variety of cytokines including IFN-γ and IL4 (Ferrick *et al.*, 1995). Such cells have been implicated in the downregulation of specific $\alpha\beta^+$ T cell responses (McMenamin *et al.*, 1995; Szczeparik *et al.*, 1996), although there is also evidence for a positive stimulatory role of $\gamma\delta^+$ T cells in cell-mediated immune responses (Askenase *et al.*, 1995; Ptak *et al.*, 1996). Additionally, $\gamma\delta^+$ T cells may have a positive role in the stimulation of antibody-mediated immune responses (Szczeparik *et al.*, 1998).

Depletion of $\gamma\delta^+$ T cells prior to cell culture appears to have little effect on lymphocyte proliferative responses during *F. hepatica* infection (McCole *et al.*, 1999a), however, the role of this phenotype in cell cultures where the proliferative response is reduced after secondary challenge has not been investigated. The proportion of $\gamma\delta^+$ T cells present in bovine peripheral blood varies with age, being high in the neonate and declining as animals mature (Wilson *et al.*, 1996; Hein, 1994). In this study, calves of 4-6 months old were used; the contribution of $\gamma\delta^+$ T cells to *F. hepatica* infection in calves and adult cattle may therefore vary and is a point of consideration when comparing cellular responses between different studies.

The use of alternative methods for measuring cytokine production by lymphocytes in response to stimulation with fluke preparations would have been helpful in confirming the cytokine responses observed in this study. Standard or semiquantitative RT-PCR (Brown *et al.*, 1995; McInnes *et al.*, 1998) or multiplex PCR (McKeever *et al.*, 1997), would have allowed investigation of a greater number of cytokines than were examined in this study. These techniques could also be used to examine the cytokine profiles of fresh, unstimulated cells, particularly lymph node

derived cells. It was unfortunate that although RNA was isolated from unstimulated and cultured cells, inadequate time was available to perform these assays.

An area that was not investigated during this study was the histological and immunohistochemical appearance of the liver parenchyma and gut mucosa during infection. Differences in the nature and frequency of immune cell phenotypes present within hepatic tissue could provide an insight into the underlying reasons for the disparate responses observed between pre-exposed, naïve and chronically infected animals. Recently, the presence of distinct hepatic lymphocyte populations present in normal human liver tissue, compared to those present in the peripheral circulation, has been demonstrated (Norris *et al.*, 1998). The majority of human hepatic-derived lymphocytes were found to consist of natural killer (NK) cells, $\gamma\delta^+$ T cells and T cells co-expressing NK molecules (NKT), with $CD4^+$ or $CD8^+$ cells comprising less than 40% of the total population.

NK cells, $\gamma\delta^+$ T cells and NKT cells express invariant type receptors that respond to a variety of antigens, often non-peptide, such as lipids or glycoproteins and are involved in the innate immune response (reviewed Doherty and O'Farrelly, 2000). $\gamma\delta^+$ T cells are also able to bind specific peptides in combination with antigen presenting molecules, via the highly variant regions of the γδ TCR (Kaufmann, 1996). Although there is no evidence that such cells can kill juvenile flukes by direct cytotoxic actions, these cells are able to produce a variety of cytokines in response to receptor or cytokine stimulation and may be important in defining the local cytokine environment within the liver (Doherty and O'Farrelly, 2000). This in turn could regulate the $CD4^+$ helper T cell response to fluke specific antigens and products. The presence of a surface lipoglycan on *F. hepatica* has recently been demonstrated (Singh and Semprevivo, 1999) and it is possible that this molecule may be recognised by receptors on NK, NKT or $\gamma\delta^+$ T cells. Meeusen *et al.* (1995) found large numbers of $CD8^+$ and γδ cells within the hepatic parenchyma during chronic infections in sheep and speculated a possible role for these cells in immune regulation. If similar cells were identified within the hepatic tissue of chronically infected animals shortly after challenge infection (group A) in greater numbers than in pre-exposed or naïve animals, this may provide an explanation for the depression in cellular responses observed in this study.

The production of cytokines locally, within the liver, could be investigated by extraction of total mRNA from sections of hepatic tissue. In a study using normal mouse liver, resting levels of cytokine mRNA were found to be low and mRNA encoding IL4 and IFN-γ was not detected (Diehl, 2000). If this is also the case in cattle, any cytokine mRNA detected in liver samples is likely to be a specific response to liver infection. This method of analysing local cytokine expression could be extremely useful in the context of the current study and may be important in defining the differences in host response to challenge between pre-exposed and naïve animals. Lymphocytes could also be extracted directly from the liver and cell and cytokine responses to fluke proteins examined in culture.

The role of the hepatic antigen presenting cells during fluke infection, the sinusoidal endothelial cells and Kupffer cells, also requires investigation. Unusually, Kupffer cells from *Schistosoma mansoni* infected mice are able to produce IL4, IL6, IL10 and IL13 in response to schistosome proteins, an atypical response for macrophage cells (Hayashi *et al.*, 1999). Kupffer cells may also be involved, therefore, in influencing the local cytokine environment and so T cell response to infection. Alternatively, Kupffer cells could respond to fluke infection by the production of toxic metabolites, such as nitric oxide. As Kupffer cells exist as a resident macrophage population within the liver, their role during *F. hepatica* infection should be investigated further.

There is therefore great scope for a detailed and thorough investigation of the local liver and hepatic lymph node immune responses occurring during the early stages of *F. hepatica* infection in cattle. Given the lack of mesenteric response observed after challenge infection in both the pre-exposed and naïve animals, understanding the local hepatic response could be crucial for determining the immune mechanisms operating in this study.

7.7 Conclusions

This study has provided a unique insight into the early immune response and in particular, the local lymph node responses, to *F. hepatica* infection in cattle. In summary, this study has demonstrated that:

- Pre-exposure to 5-6 day old fluke by drug abbreviated infections results in a reduction in liver damage, eosinophilia and in faecal egg counts during the early patent period, upon secondary challenge, when compared to naïve animals.

- The predominant immune response to whole fluke antigen and excretory-secretory protein preparations 10-11 days following challenge infection is present at the level of the liver and not the gut, in both 5-6 day pre-exposed and naïve animals.

- A mixed IgG1/IgG2 antibody response was observed to the haem-containing high molecular weight fraction during the first few weeks of infection. Hepatic lymph node cells also showed a strong proliferative reaction to culture with this fraction at 10 days post-infection. Antibody and cellular immune responses to cathepsin-L were less significant during the early infection period, with IgG1 antibody responses absent until the 5th week of infection.

- The early, peripheral and local IgG1 antibody response to infection is directed against a variety of proteins present in adult fluke preparations of 96-82, 72-68 and 60-54 kDa. The identity and function of these antigens is not known and merits further investigation.

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APPENDIX A

Recipes for solutions and culture media

All chemicals were obtained from Sigma or BDH, unless stated otherwise.

A.1 Solutions used for protein preparation, SDS PAGE and Western blots

A.1.1 Phosphate buffered saline solution, PBS, pH 7.4 (1X)

NaCl	8g
KCl	0.2g
$Na_2HPO_4.7H_2O$	1.5g
KH_2PO_4	0.2g

Distilled water to 1000 mls

A.1.2 PBS-T

0.5 ml of Tween 20 per 1000 ml of PBS.

A.1.3 Sample loading buffer (non-reducing and reducing)

0.5M Tris-HCl pH 6.8	1.2 ml
Glycerol	1.0 ml
10% w/v SDS	2.0 ml
0.1% w/v Bromophenol blue	0.5 ml
Distilled water	4.8 ml

For a reducing buffer, 25 µl of β-mercaptoethanol was added to 475 µl aliquots of the above sample buffer and used within 2 weeks (stored at 4°C).

A.1.4 SDS-PAGE running buffer (1X)

Tris	9g
Glycine	43.2g
SDS (Lauryl sulphate)	3g

Distilled water to 1000 mls

A.1.5 Western blot transfer buffer

Tris	5.82g
Glycine	2.93g
10% SDS (w/v)	3.75 ml
Methanol	200 ml

Distilled water to 1000 mls

A.1.6 Tris buffered saline, TBS, pH 7.6 (1X)

Tris	3.15g
NaCl	8g

Distilled water to 1000 mls

A.1.7 TBS-T

1 ml of Tween 20 per 1000 ml of TBS.

A.2 Solutions and media used in tissue culture procedures (* indicates material obtained from Life Technologies)

A.2.1 PBS, pH 7.4 for all cell culture techniques was prepared from tablets (Sigma P-4417) and sterilised by autoclaving.

A.2.2 Acid citrate dextrose anticoagulant

2.5% w/v D-glucose

2.05% w/v Di-sodium hydrogen citrate

in distilled water; sterilised through a 0.22 μm filter.

A.2.3 Tissue culture complete media

RPMI 1640 (Sigma R-7388)	500 ml
L-glutamine*	5 ml
2-mercaptoethanol*, 50 mM	0.5 ml
Foetal calf serum (FCS)*	50 ml
Gentamicin 10 mg/ml*	5 ml

A.2.4. Lymph node media

HBSS[a]	500 ml
Foetal calf serum (FCS)*	25 ml
Gentamicin 10 mg/ml*	5 ml

Heparin (Sigma H-3149) to a final concentration of 10 IU/l

[a] = Hank's Buffered Saline Solution without Ca^{2+} and Mg^{2+} (Sigma H-6648)

A.2.5 0.83% ammonium chloride red cell lysis solution

Buffer A

Tris	20.6g

pH 7.65, to 1000ml with distilled water

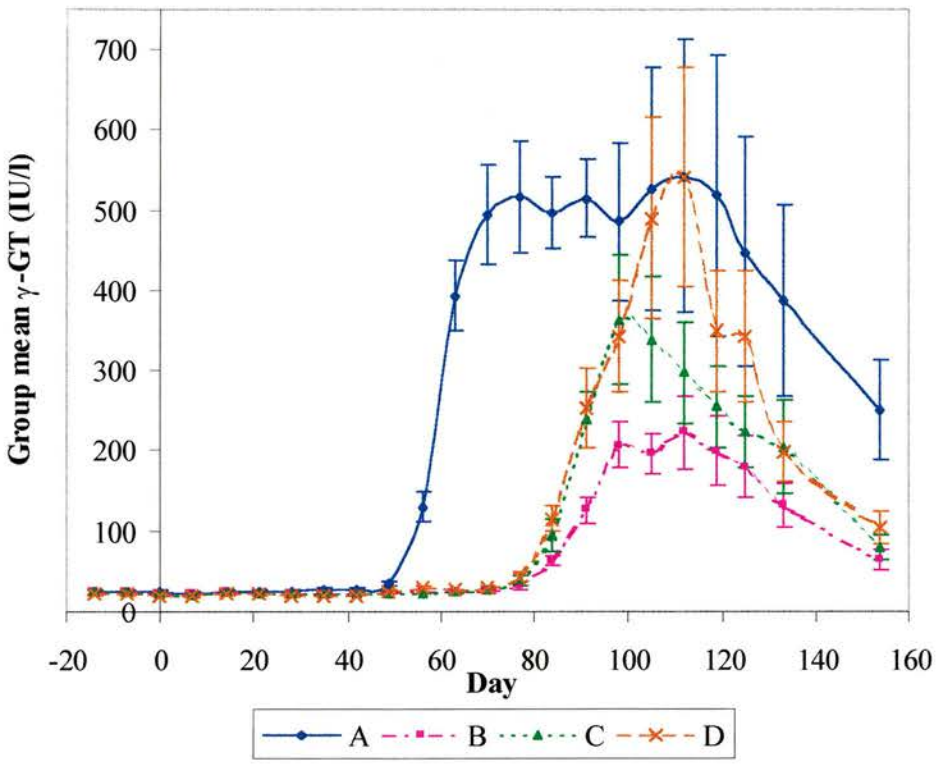
Buffer B

0.83% w/v NH_4Cl in distilled water

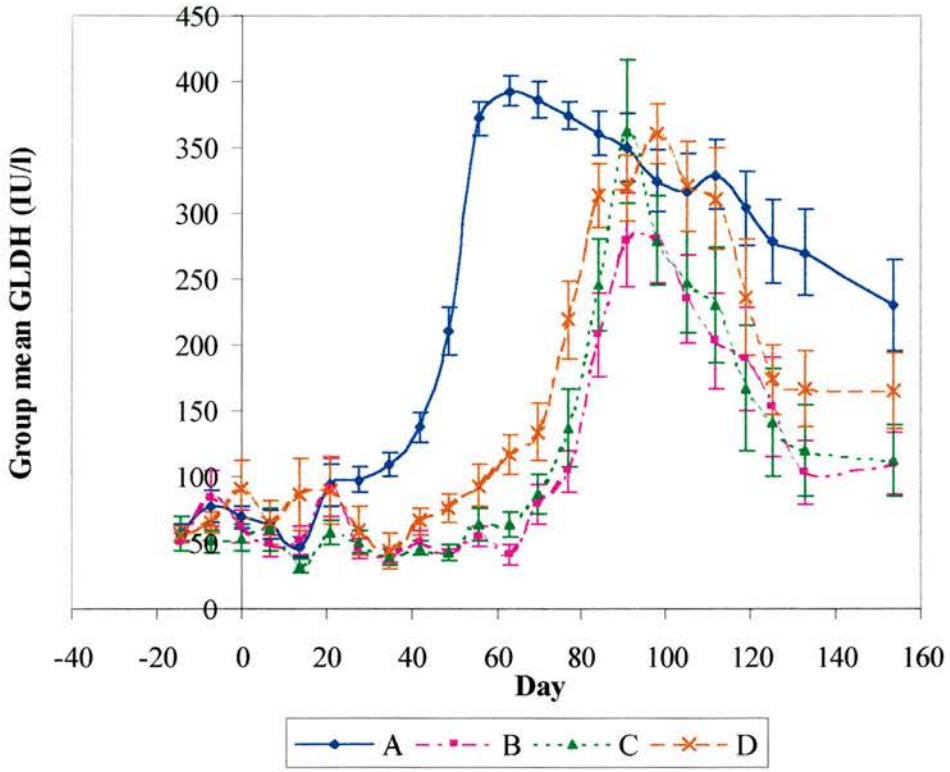
Buffers A and B were sterilised by autoclaving. The working solution was prepared immediately prior to use by mixing 1 ml of buffer A with 9 ml of buffer B.

APPENDIX B

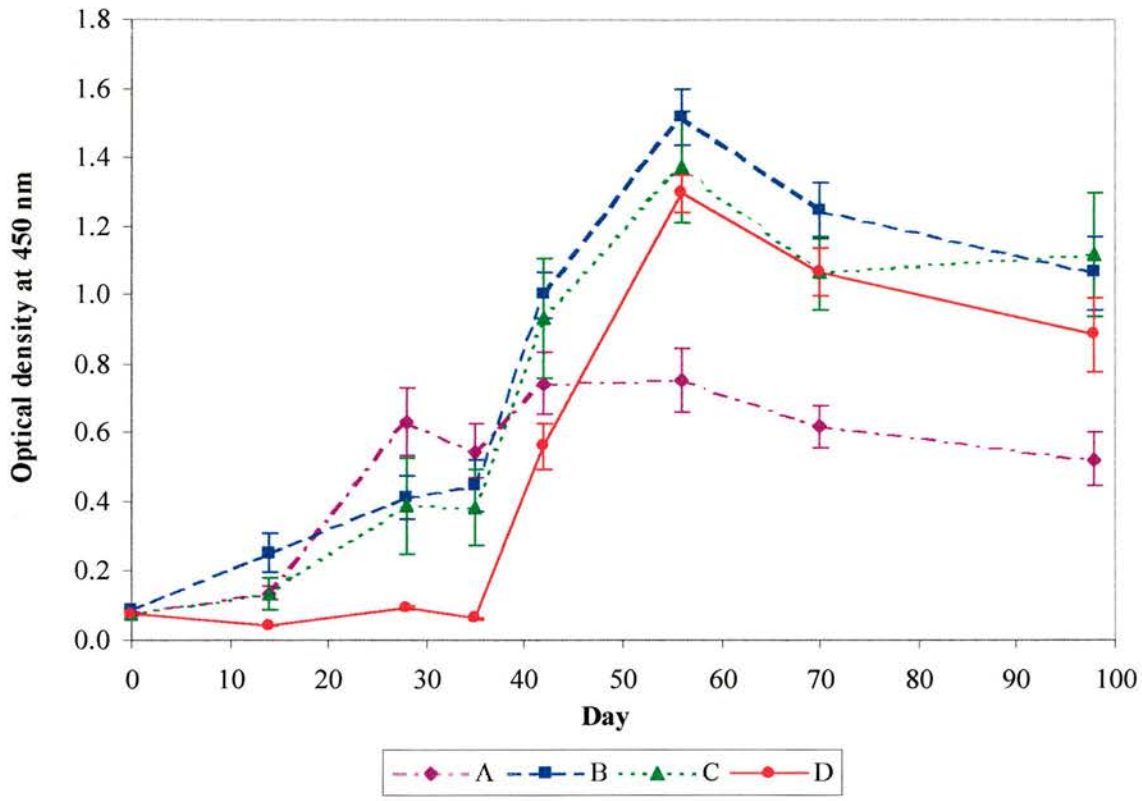
B1: Longitudinal profile of group mean serum levels of the enzyme gamma glutamyl transferase (γ-GT) over the entire period of study, for groups A, B, C and D (Experiment A), in international units per litre (IU/l). Error bars show the standard error of the mean. Equivalent to Figure 3.4, page 58.



B2: Longitudinal profile of group mean serum levels of the enzyme glutamate dehydrogenase (GLDH) over the entire period of study, for groups A, B, C and D (Experiment A), in international units per litre (IU/l). Error bars show the standard error of the mean. Equivalent to Figure 3.5, page 59.



B3: Longitudinal IgG1 antibody response to the high molecular weight fraction over the period days 0-98. Group mean optical density values for groups A, B, C and D (Experiment A) are shown, with error bars representing the standard error of the mean. Equivalent to Figure 5.3, page 130.



B4: Longitudinal IgG1 antibody response to the cathepsin-L pool fraction over the period days 0-112. Group mean optical density values for groups A, B, C and D (Experiment A) are shown, with error bars representing the standard error of the mean. Equivalent to Figure 5.5, page 133.