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ADDENDUM

Under Table 1.3 after the text "Cattle refers to" add "Hereford-Ayreshire crosses and Jerseys"

P37 line 9: delete "(Appendix 1)" and read " carbon dioxide (CO_2) and taurocholic acid."

THE MOLECULAR BASIS FOR THE RESISTANCE OF *FASCIOLA HEPATICA* TO CELLULAR CYTOTOXICITY

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BAppSci (Hons) Swinburne University

A Thesis Submitted in Fulfillment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

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DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in this or any other university, and to the best of my knowledge and belief, contains no material previously published or written by another person, except when due reference is made in the text of the thesis.

[redacted]

Rhoda Prowse

December, 2002

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PUBLICATIONS

Paper

Prowse R.K., Chaplin P., Robinson H.C., and Spithill T.W. 2002. *Fasciola hepatica* cathepsin L suppresses sheep lymphocyte proliferation *in vitro* and modulates surface CD4 expression on human and ovine T cells. *Parasite Immunology* **24**, 57-66
(See Appendix)

Poster Presentations

Prowse R.K., Hansen D., Robinson, H.C., and Spithill, T.W.. Comparative Effect of *Fasciola hepatica* and *F. gigantica* Excretory/Secretory Products on the Production of Nitric Oxide by Rat Peritoneal Lavage Cells. *Annual Meeting of Australian Society of Parasitology*, 27th September – 1st October 1998. *This poster was awarded the Australian Society of Parasitology poster prize in 1998*

Prowse R.K., Robinson, H.C., and Spithill, T.W.. Suppression of the Proliferation of Sheep Lymphocytes *in vitro* by Cathepsin L proteases released by *Fasciola*. *Annual meeting of the Co-operative Research Centre for Vaccine Technology*, 3rd – 5th May 1999.

Prowse R.K., Chaplin, P., Robinson, H.C. and Spithill, T.W.. *Fasciola* Cathepsin L modulates surface CD4 expression on human and ovine T cells: suggested role in suppression of sheep lymphocyte proliferation. *Annual meeting of the Co-operative Research Centre for Vaccine Technology*, 30th August – 1st September 2000.

Prowse R.K., Chaplin P., Robinson H.C. and Spithill, T.W.. Title of Abstract Submitted: *Fasciola hepatica* cathepsin L suppresses sheep lymphocyte proliferation *in vitro* and modulates surface CD4 expression on human and ovine T cells. Title of Poster: *Fasciola* Cathepsin L modulates surface CD4 expression on human and ovine T cells: suggested role in suppression of

sheep lymphocyte proliferation. *Oxford 2000 – Joint meeting for the Royal Society of Tropical Medicine and Hygiene, the British Society of Parasitology, the American Society of Tropical Medicine and Hygiene, and the Japanese Societies of Parasitology and Tropical Medicine*, 18th September – 22nd September 2000

Abbreviations

ADCC	antibody dependent cell cytotoxicity
AOD	anti-oxidant defence
APC	antigen presenting cells
CDNB	1-chloro-2,4-dinitrobenzene
CMI response	cell mediated immune response
cNOS	constitutive nitric oxide synthase
Con A	concanavalin A
CTL	cytotoxic T lymphocytes
Cu/Zn	copper/zinc
dH_2O	distilled water
DMEM	dulbeccos modified eagles media
DMSO	dimethylsulphoxide
E64	L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane
EDTA	ethylenediamine tetracetic acid
ESP	excretory / secretory products
FACS	fluourescent activated cell sorter
FCS	foetal calf serum
FhESP	*Fasciola hepatica* excretory / secretory products
FhGST	*Fasciola hepatica* glutathione S-transferase
FR	free radicals
GSH	glutathione
GSH-Px	glutathione peroxidase
GSSG	glutathione disulfide
GST	glutathione S-transferase

H_2O_2	hydrogen peroxide
HIR	humoral immune response
iNOS	inducible nitric oxide synthase
ITT	Indonesian thin tailed sheep
L-NMMA	monomethyl L-arginine
LPS	lipopolysaccharide
MFI	mean fluourescent intensity
NEJ	newly excysted juvenile
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
O_2	oxygen
O_2-	superoxide radical
$^1O_2$	singlet oxygen
OH^-	hydroxyl radical
PBMC	purified blood mononuclear cell
PBS	phosphate buffered saline
PHA	phytohemagglutinin
PI	primary infection
PLC	peritoneal lavage cell
PMA	phorbol myristate acetate
rFhCatL	recombinant *F. hepatica* cathepsin L
rFhCatL5	recombinant *F. hepatica* cathepsin L5
rFhCatL69Y	recombinant *F. hepatica* cathepsin L69Y
rHuCD4	recombinant human CD4

RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
RPMI	Rosswell Park Memorial Institute culture media
RPMI-PR-	Rosswell Park Memorial Institute culture media, phenol red free
S6	superose 6
S12	superose 12
S75	superdex 75
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
Th	T helper
WBA	whole blood assay
WHO	world health organisation
WPI	week post infection
WPSI	week post secondary infection
WWE	whole worm extract

SUMMARY

Fasciola hepatica and *F. gigantica* are estimated to result in economic losses in excess of US $3 billion per year. In order to manufacture effective vaccines a thorough understanding of the parasite : host relationship is required. The aim of this thesis was to identify both evasion strategies employed by *Fasciola sp.* parasites and / or biochemical differences between *F. gigantica* and *F. hepatica* which would explain the variance in host susceptibility between the two species. We showed that *F. gigantica* infections suppressed Merino sheep lymphocyte proliferation at post infection weeks 3, 9 and 10. This is a similar to *F. hepatica* infections. We demonstrated that suppression of sheep lymphocyte proliferation *in vitro* was, at least partially attributable to excretory / secretory products (ESP) released by both *Fasciola sp.*. The active molecule within *Fasciola sp.* ESP was identified as the cysteine protease cathepsin L. The effect of *Fasciola sp.* ESP and recombinant *F. hepatica* cathepsin Ls on 29 sheep surface markers was investigated to identify a potential mechanism for suppression of sheep lymphocyte proliferation. Cysteine proteases within *Fasciola sp.* ESP selectively regulated only three surface markers of the 29 studied. CD4 and CD8 expression were down regulated on lymphocytes while CD1b expression was up regulated on dendritic cells. CD4 expression was also shown to be down regulated on both human and bovine lymphocytes. These are two new roles for *Fasciola sp.* cathepsin Ls. We next investigated the ability of *Fasciola sp.* to modulate nitric oxide (NO) production by both activated and resting rat peritoneal lavage cells (PLCs). Interestingly, both adult *Fasciola sp.* ESP induced NO production by resting rat PLCs. The active components within both parasites were identified as high molecular weight molecules by gel filtration. Anti-oxidant defence (AOD) enzyme levels were compared between *F. hepatica* and *F. gigantica* adult and newly excysted juvenile (NEJ) parasites. We observed lower levels of superoxide dismutase within *F. gigantica* NEJ whole worm extract (WWE) compared with *F. hepatica* NEJ WWE. We also observed significantly lower levels of two glutathione dependent enzymes, glutathione S-transferase and glutathione peroxidase, within adult *F. gigantica* WWE compared with adult *F. hepatica* WWE. These differences in AOD enzymes may partially explain the greater susceptibility of *F. gigantica* within the rat and sheep host when compared with *F. hepatica*.

CHAPTER 1: INTRODUCTION

1.1 Introduction

There are more kinds of parasites than non-parasitic organisms within the world, even when excluding viruses, rickettsias, parasitic bacteria and fungi. It would be unusual to examine a domestic or wild animal without finding at least one species of parasite on or within it (Schmidt & Roberts 1985). Due to the high incidence and nature of parasitic infections they have been shown to have an immense impact on both economic and social issues. Table 1.1 lists a range of human parasites along with the estimated number of people infected worldwide and deaths each year. These statistics have led to enormous research into vaccines against these diseases along with investigating host: parasite interactions.

While there are a reported 2-3 million people infected globally with the common liver fluke, *Fasciola* (WHO Figures 1995), it is the losses within the agricultural industry that have stimulated most of the research into this parasite. *Fasciola* infections are estimated to result in economic losses in excess of US$3 billion per year (Spithill *et al.* 1999). There are two predominant *Fasciola* species (sp.): *F. hepatica* and *F. gigantica* which cause temperate (e.g. South America, United States of America, Caribbean, Europe and Australia) and tropical (e.g. Africa, Asia and Hawaii) fasciolosis respectively. *Fasciola sp.* infect a range of mammalian hosts including: sheep, cattle, buffalo, pigs, goats, horses, kangaroos, wombats, rabbits, rats and mice (Smyth & Halton 1983, Arundal 1986, Roy & Reddy 1969).

1.2 *Fasciola sp.* Parasites

1.2.1 Life Cycle in Ruminants and Humans

Fasciola species belong to the Phylum Platyhelminthes, Class Trematoda, Subclass Digenea and Family Fasciolidae. *Fasciola sp.* have 2 hosts: an intermediate host (snail – eg. *Lymnaea sp.*) and a ruminant / human host. The life cycle of *Fasciola* parasites is shown in Figure 1.1.

Briefly, unembryonated eggs are released by the adult parasites within the bile ducts of their ruminant / human host where they subsequently pass through the small intestine before being released in stools. In water, the embryonated eggs hatch, releasing motile miracidia which have a

Table 1.1: Worldwide Infection / Death Statistics for some Parasites

Disease / Species	Number of People Infected Worldwide	Number of Yearly Deaths Worldwide
African trypanosomes	300,000-500,000	Perhaps 65,000
Amoebic dysentery	Unknown: 48 million with sever disease	70,000
Ascaris lumbricoides	1.47 billion	Perhaps 60,000
Brugia malayi *Brugia timori*	13 million	Unknown
Clonorchis sinensis	7 million	Unknown
Diphyllobothrium spp.	9 million	Unknown; few
Echinococcus granulosus *Echinococcus multilocularis*	2.7 million	Unknown
Echinostomes	150,000	Unknown; few
Fasciola hepatica *Fasciola gigantica*	2.4 million	Unknown; few
Fasciolopsis buski	210,000	Unknown; few
Giardiasis	350 million	Unknown; few
Heterophyids	900,000	Unknown; few
Hookworms	1.3 billion	Ca 70,000
Leishmaniasis	12 million	Unknown
Loa loa	13 million	Unknown; few
Malaria	300-500 million	1.5-2.7 million
Onchocercus volvulus	17.7 million	Unknown
Opisthorchis viverrini *Opisthorchis felineus*	10.3 million	Unknown
Paragonimus spp.	20.7 million	Unknown; few
Schistosomiasis	201 million	Perhaps 20,000
Strongyloides stercoralis	70 million	Unknown
Taenia saginatus	77 million	Unknown; few
Taenia solium	10 million	Unknown
Trichuris trichiura	1.05 billion	Unknown; few
Trypanosoma cruzi	16-18 million	Unknown
Vampirolepis nana	75 million	Unknown; few
Wuchereria bancrofti	107 million	Unknown

Modified from http://www.ksu.edu/parasitology/classes/545intro.html

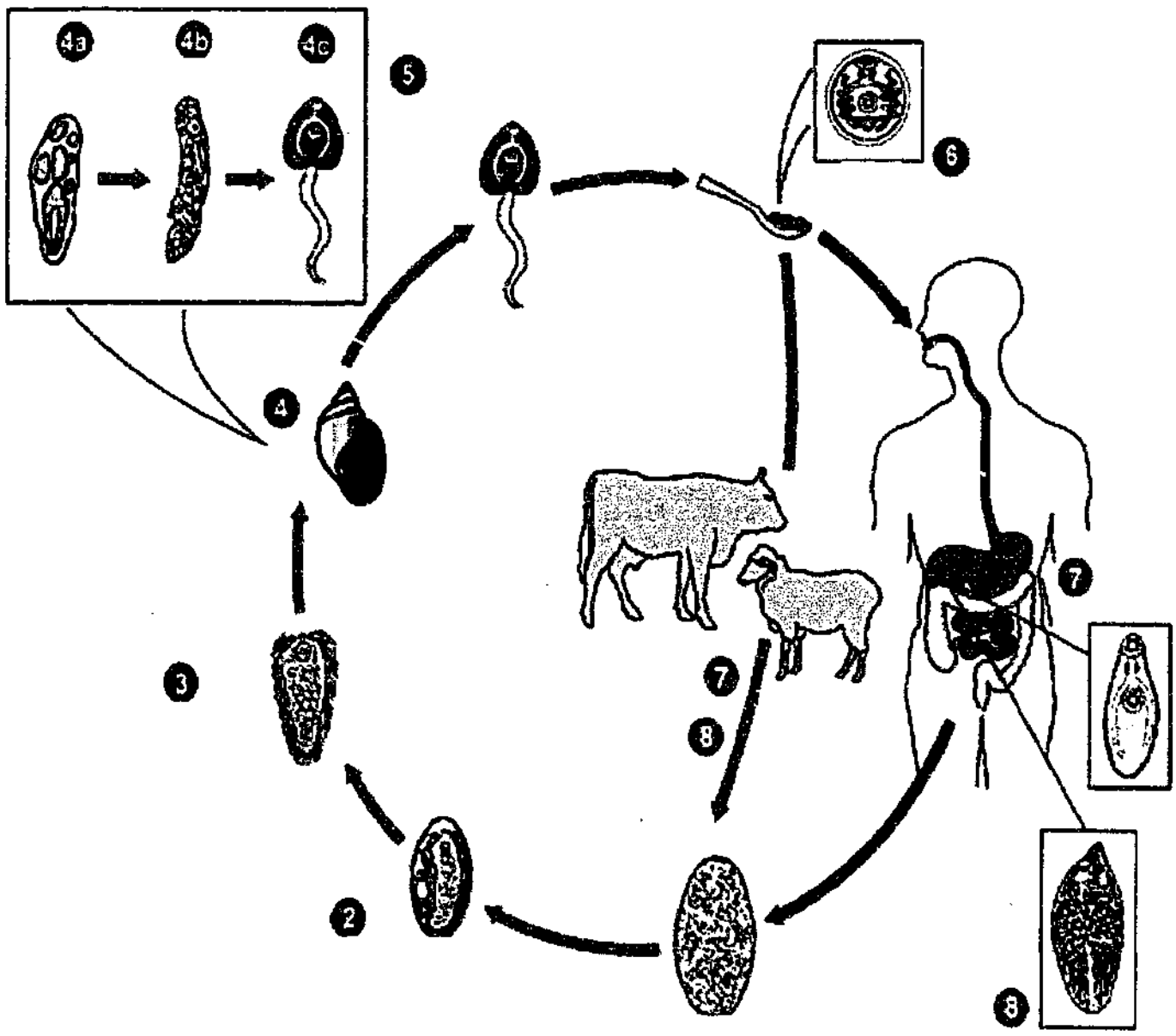


Fig 1.1 Life cycle of *Fasciola*. 1. Unembryonated eggs are passed in faeces, 2. Eggs are embryonated in water, 3. Miracidia hatch and penetrate snail, 4. Miracidia undergo several transformations within the intermediate snail host 4a. Miracidia transform into sporocysts, 4b. Sporocysts into Rediae, 4c. Rediae give rise to cercariae, 5. Cercariae are released by the snail and encyst in water or on water vegetation, 6. Metacercariae on infected vegetation are ingested by human or ruminants, 7. Juvenile flukes excyst in small intestine and migrate through to the liver, 8. *Fasciola* matures into adult parasite within the hepatic biliary ducts. This figure was modified from http://www.dpd.cdc.gov/dpdx/html/fascioliasis.htm.

maximum of 24 hrs to penetrate a suitable snail intermediate host. Once inside the snail, a series of maturation phases occur: miracidia transform into sporocysts, sporocysts develop into rediae and daughter rediae are then produced within the digestive gland of the snail. Finally germinal cells in rediae give rise to cercariae. The rate of development depends on the size of the infection and the available food reserves. Cercariae (up to 4000 per miracidium) (Boray 1969) are released 5 to 7 weeks post infection from the snail, and these swim and encyst on fresh water vegetation or remain in water if there is no vegetation. This reliance on water limits the distribution of *Fasciola* to wetter climates. Upon digestion of metacercariae on infected plant life by the ruminant / human host, juvenile flukes excyst in the small intestine, migrate through the intestinal wall, the peritoneal cavity, and the liver parenchyma. Here they feed for around 40 days before burrowing into the biliary ducts where they mature into adults and commence egg laying (Dawes & Hughes 1964). *Fasciola* has been estimated to produce in the range of 20,000-50,000 eggs per fluke per day (Boray 1969).

There are several variations within the lifecycle between *Fasciola sp.*. Firstly, the time taken to reach the bile ducts and subsequently mature is 7-10 weeks post infection (WPI) compared with 12-16 WPI for *F. hepatica* and *F. gigantica,* respectively (Dawes & Hughes 1964, Boray 1969, Grigoryan 1958, Guralp, Oxcan & Simms 1965, Sewell 1966). Secondly, the parasites occupy different *Lymnaea sp.* for their intermediate hosts. Finally, the *Fasciola* parasites also differ in size with *F. hepatica* and *F. gigantica* measuring up to 30 mm and 75 mm in length, respectively (http://aa.merial.com/produces/dairy/disease/fasci.html).

1.2.2 Pathology

Fasciola sp. infections can lead to chronic or acute disease within human and ruminant hosts (cattle, buffalo and sheep). Chronic infections, the result of a small intake of parasites over a long period of time, are the most common form of liver fluke infection in sheep (Boray 1969; 1986). Chronic infections are characterised by diminished appetite leading to a lower level of weight gain; reduced work performance as host mobility decreases; eosinophilia; poorer yield and quality of milk production; decrease in wool lustre (sheep); reduction in erythrocytes leading to anaemia; decreased fertility; and may lead to greater susceptibility to secondary bacterial infections (Spithill *et al.* 1999). Humans suffering chronic *Fasciola sp.* infections display clinical

features including abdominal pain, hepatic tenderness, fever, vomiting, diarrhoea, urticaria and eosinophilia (http://www.dpd.cdc.gov/dpdx/HTML/Fascioliasis.asp). Severe or acute *Fasciola sp.* infections can result in death of the host (Boray 1969). Acute infections are the product of a large intake of *Fasciola sp.* metacercariae in a short period of time.

1.3 The Parasite/Host Relationship

Research has primarily focussed on *F. hepatica* infections with parallel research into *F. gigantica* still in its early stages (eg a search of Pub Med reveals approximately ten *F. hepatica* articles are published for every *F. gigantica* article). From the current literature, it appears that *F. hepatica* and *F. gigantica* vary in resistance / susceptibility to attack by different hosts. This variation in host susceptibility along with the dominant host immune mechanisms expressed against *Fasciola sp.* will be expanded upon in the next sections.

1.3.1 Immunity in Sheep to Infection with *Fasciola* Parasites

There have been multiple studies characterising the relative survival efficiency of *F. hepatica* and / or *F. gigantica* in a range of sheep breeds. This has recently been reviewed by Spithill, Smooker & Copeman (1999). This section is going to be a brief overview of *Fasciola* infections in some sheep breeds.

(i) F. hepatica

Sheep are incapable of mounting a protective response against natural or experimental primary and secondary infections of *F. hepatica* (Boray 1969, Reid *et al.* 1970, Sinclair 1971, Kelly & Campbell 1979, Meek & Morris 1979, Knight 1980, Rickard & Howell 1982, Haroun & Hillyer 1986, Boyce, Courtney & Loggins 1987). Table 1.2 shows the infection efficiency of *F. hepatica* in primary and secondary infections in a range of sheep breeds (modified from Spithill, Smooker & Copeman 1999). While these results suggest that sheep do not acquire resistance to *F. hepatica* infections they also show a variation in susceptibility to *F. hepatica* between sheep breeds. These differences are likely to be determined by host genetic factors including heritable immunological mechanisms (reviewed in Wakelin 1978, 1991). It should be noted that there is a decrease in the severity of fasciolosis in sheep upon subsequent infections as measured by: decreased worm size; reduced egg production by adult worms, delayed onset of anaemia, an

Table 1.2: Percentage of *Fasciola sp.* flukes recovered after infection

	F. hepatica[a]		*F. gigantica*[a]	
	Primary	Secondary	Primary	Secondary
ITT	31[b]	39[b]	0.8 – 5[c]	0.32-1.1[d]
Sudanese Desert	nt	nt	5[e], 17[f]	3.4[f]
Merino	23-53[g,h]	23-53[h]	15-25[i]	12[i]
St Croix	16[j]	13.7[j]	6.3, 6.7[k]	nt
Finn / Rambouillet	24[j]	31[j]	nt	nt
Florida native	27[j]	16[j]	nt	nt
Barbados Blackbelly	38[j]	30[j]	nt	nt

nt: not tested

[a] Mean % recoveries of flukes

[b] Roberts *et al.* 1997a

[c] Wiedosari & Copeman 1990, Roberts *et al.* (1997 a,b,c);

[d] Roberts *et al.* (1997a,c)

[e] Ali *et al.* 1985

[f] A'Gadir *et al.* 1986

[g] Horak *et al.* 1972

[h] Boray 1969; Sexton *et al.* 1990; Wijffels *et al.* (1994b) Creaney *et al.* (1996)

[i] Roberts *et al.* (1996, 1997c)

[j] Boyce *et al.* (1987)

[k] Roberts *et al.* (1997a,b)

This table was modified from Spithill, Smooker and Copeman (1999, p489)

earlier rise and greater number of eosinophils and an earlier lymphocyte infiltration into the liver (Boray 1969, Sinclair 1971; 1973; 1975).

The immune responses generated by sheep following a *F. hepatica* infection include elevated parasite specific antibodies (IgG, IgM and IgE isotypes) and an infiltration of white blood cells including eosinophils and $CD4^+$ T cells into portal tract areas and liver tissue (Sinclair 1971; Boray 1967,1969, Movsesijan & Jovanovic 1975; Rushton & Murray 1977, Knight 1980; Sandeman & Howell 1980 a,b; Wedrychowicz *et al.* 1984, Boyce, Courtney & Loggins 1987, Chauvin, Bouvet & Boulard 1995, Meeusen *et al.* 1995, Hansen *et al.* 1999). The elevated IgG levels are most likely due to an increase in the production of IgG1. In work by Movsesijan and Jovanovic (1975), IgG1 levels increased post infection while IgG2 levels did not alter after infection with *F. hepatica.* The eosinophil response was biphasic with the first peak corresponding to the juvenile parasite's migratory phase and the second peak relating to the parasite's entry into the bile ducts (Chauvin, Bouvet & Boulard 1995). Due to the susceptibility of sheep to *F. hepatica* it is likely that both the specific IgG1 antibody and eosinophils are ineffective immune responses for controlling *F. hepatica* infection.

(ii) F. gigantica

Sheep have been shown to acquire resistance to *F. gigantica* infections (reviewed in Haroun & Hillyer 1986, Spithill, Smooker & Copeman 1999). Primary and secondary *F. gigantica* infection rates are displayed in Table 1.2. Significant reductions in parasitic burdens upon secondary infection, indicative of acquired resistance, have been observed in Indonesian Thin Tail (ITT), Merino and Sudanese Desert sheep. Further evidence for an immunological based role was shown through the administration of dexamethasone in ITT sheep (Roberts *et al.* 1997c). Dexamethasone, an immunosuppressant, was shown to reverse the acquired resistance of ITT sheep. The acquisition of resistance has not been observed for *F. hepatica* infections in ITT or Merino sheep pointing to a major difference between *Fasciola sp.*. This suggests that *F. hepatica* may express a parasite defence mechanism which is not a part of the *F. gigantica* defence strategy or that *F. hepatica* expresses mechanisms that allow it to resist the host responses (i.e. some form of immunosuppression). Alternatively, *F. gigantica* may release a molecule that induces a protective response in the sheep host for which there is no *F. hepatica* analogue.

An interesting phenomenon is the apparent natural resistance of ITT sheep to primary infections of *F. gigantica* (Wiedosari & Copeman 1990, Roberts *et al.* 1997a). This natural resistance is likely to be comprised of both innate and acquired resistance mechanisms. Innate resistance is based on biochemical, physiological or anatomical factors. Acquired resistance is based on immunological factors (Wakelin 1992).

A comparison of immunological factors induced by *F. gigantica* infections in ITT and Merino sheep has been made (Hansen *et al.* 1999). *F. gigantica* infections in ITT sheep produced higher eosinophilia, neutrophilia and lower parasite-specific IgG_2 levels. Both breeds of sheep produced similar levels of IgM, IgG_1 and a biphasic IgE response (Hansen *et al.* 1999). The authors proposed that IgG_2 may be a resistance blocking antibody. By producing less IgG_2, ITT sheep are capable of killing *F. gigantica* infections. Finally, work by Roberts *et al.* (1997b) suggests that resistance to *F. gigantica* infections in ITT sheep is likely to be controlled by a single gene that displays substantial dominance.

1.3.2 Immunity in Cattle to Infection with *Fasciola* Parasites

(i) F. hepatica

Several reports show that cattle acquire resistance to challenge infection following a single infective dose of *F. hepatica.* Cattle can also display resistance to primary infections of *F. hepatica* (Ross 1966, Boray 1969; Dawes & Hughes 1970; Doyle 1971; 1972; 1973a; Doy & Hughes 1984, reviewed in Haroun & Hillyer 1986). In a primary infection, *F. hepatica* parasites were expelled by 30 weeks post infection (Doyle 1971, 1972). Protection post secondary infections requires a minimum of 12 weeks of a primary infection as demonstrated by a lack of resistance acquired after only 7 weeks exposure (Doyle 1973). Other evidence for acquired resistance in cattle is the fact that cattle show a 56% and a 94% reduction in fluke burden after 18 weeks and 26 weeks of a primary infection, respectively (Doy & Hughes 1984). Collectively these results point to greater resistance against challenge infection relating to longer primary infections. In addition work by Clery, Torgerson and Mulcahy (1996) suggests that there is no acquired resistance against challenge infections in cattle which received a trickle infection. A trickle infection is representative of a slowly acquired chronic infection.

However, further studies suggested that this "resistance" is not immunologically based but rather is a result of the observed calcification of the liver which coincides with parasite rejection (Boray 1969; Doy & Hughes 1984; Hughes 1987). This indirectly supports more recent work that implies *F. hepatica* parasites induce immune mechanisms in cattle that aid in their survival (Bossaert *et al.* 2000a,b). The immune response post *F. hepatica* infections are characterised by an early short lived cell mediated immune (CMI) response as measured by significant increases in proliferation of mononuclear cells stimulated by *F. hepatica* somatic antigen (Oldham 1985, Oldham & Williams 1985). The humoral response results in high levels of IgG1 antibody production, low levels of IgG2 antibody levels and parasite specific IgE antibody from week 2 post infection are also observed (Oldham 1983, Bossaert *et al.* 2000a,b). This is similar to IgG1 and IgG2 levels observed during *F. hepatica* infections in sheep (Movsesijan & Jovanovic 1975). The antibody isotype profiles suggest a bias towards a Th2-like response (discussed in section 1.4.1). The authors propose that a Th2 response does not correlate with protection. This was supported by a vaccination regime with purified *Fasciola* enzymes Cathepsin L1 and Cathepsin L2, which resulted in the induction of a Th1- like isotype profile characterised by high IFN-γ production from peripheral blood lymphocytes, from which it was possible to infer that the vaccine induced protection to cattle (Mulcahy *et al.* 1999).

(ii) F. gigantica

F. gigantica parasites have been proposed to be better adapted to cattle than sheep because they are both more infective and live longer in cattle (Hammond & Sewell 1974). Acquired resistance to a secondary infection has been observed following a primary infection (reviewed in Haroun & Hillyer 1986). This supports current sheep experimental data, which implies that there are fundamental differences between *F. hepatica* and *F. gigantica.*

1.3.3 Rat Immunity to Infection with *Fasciola* Parasites

(i) F. hepatica

Rats develop immunologically-based resistance to *F. hepatica* mediated through a combination of humoral and cellular immune responses (Armour & Dargie 1974, Doy & Hughes 1982, Poitou, Baeza & Boulard 1992, Hughes 1987, Keegan & Trudgett 1992, van Milligen *et al.*

1998a,b). This resistance varies between strains, age and sex of rats utilised (Hayes, Bailer & Mitrovic 1974a; Hughes, Harness & Doy 1976; Rajasekariah and Howell 1977, 1981). Resistance levels are not dependent on the *F. hepatica* metacercariae clones used for challenge infection (Chapman, Rajasekariah & Mitchell 1981).

Protective immunity is proposed to be induced as the parasite migrates through either the peritoneal cavity or the liver of the rat (van Milligen, Cornelissen & Bokhout 1998a, 2000). The majority of the protective rat immune responses are directed against *Fasciola* newly excysted juveniles (NEJ) as they migrate through the gut (Doy & Hughes 1982, Burden *et al.* 1983, van Milligen, Cornelissen & Bokhout 1998, van Milligen *et al.* 1998).

F. hepatica infections in rats are characterised by early rises in parasite specific antibody of the IgM and IgE isotype, along with increased eosinophilia and antigen-specific and mitogen-induced lymphocyte proliferation (Pfister *et al.* 1983, Poitou, Baeza & Boulard 1992, Poitou, Baeza & Boulard 1993). There are some suggestions that induction of eosinophils may invoke resistance to challenge infections (Doy, Hughes & Harness 1978, Davies & Goose 1981, Milbourne & Howell 1990). Eosinophils were shown to attach to newly excysted juvenile *F. hepatica* that were injected intraperitoneally into the rat and this resulted in tegument damage (Davies & Goose 1981). It should however be noted that injection of *F. hepatica* ESP into a rat (resistant experimental model) or a mouse (susceptible experimental model) elicited a significant number of eosinophils but only resulted in protection for the rat experimental model (Milbourne & Howell 1990). Van Milligen and colleagues (van Milligen, Cornelissen & Bokhout 1998a, 1999, 2000 , van Milligen *et al.* 1998b), using an *ex vivo* loop of the jejunum in rats were able to show a strong correlation of protection against *F. hepatica* with eosinophil levels and IgG1. It may be that eosinophils are only protective in some models and may be a "smoke screen" evasion strategy utilised by *F. hepatica* in other hosts.

(ii) F. gigantica

Rats are not readily susceptible to infection with *F. gigantica* and rats reject a high proportion of an incoming infection (Mango, Mango & Esamel 1972, Gupta & Chandra 1987, Itagaki *et al.* 1994, reviewed in Spithill, Smooker & Copeman 1999). As *F. gigantica* are eliminated very

early after infection there have been no published studies examining the immune response generated by the rat against *F. gigantica in vivo*. These findings in the rat host are consistent with the observations in sheep and cattle that *F. gigantica* is more susceptible to host effector responses: since rats can acquire resistance to *F. hepatica* it is not surprising that they will also express resistance to *F. gigantica*.

Overall there are variations in the susceptibility of different hosts to *Fasciola* sp. infections (Table 1.3) as well as variations in the infection efficiency of *Fasciola sp.* within the same hosts (Table 1.2). This suggests that there are significant differences in the ways *Fasciola sp.* interact with, or are attacked by host immune cells. Such variations suggest that we should be cautious in making universal conclusions regarding the potential immune effector mechanisms that may be expressed against *Fasciola sp.* in different experimental hosts (Piedrafita *et al.* 2001). One of the potential reasons for these differences in pathogenesis may be related to more efficient evasion strategies existing within one parasite over the other. This introduction now presents a summary of known evasion strategies employed by parasites within different host species, including *Fasciola sp.*.

1.4 Broad Evasion Strategies Utilised by Parasites

Parasites are typically able to evade their natural host's defence mechanisms. If this was not the case there would be no need to research parasite evasion mechanisms or to develop drugs and biological vaccines to control harmful parasites. Common parasitic evasion mechanisms include release of excretory / secretory products (ESP) to modulate immune mechanisms, production of antioxidant defence (AOD) enzymes and, in the case of trematodes, the turnover of their tegument. Each of these will be expanded upon in the following sections.

1.4.1 Modulation of the Immune Response

As previously mentioned the immune system is an integrated unit comprising of two interconnected arms. Innate immune responses are the first line of defence mechanisms utilised to eliminate the invading pathogen. These mechanisms include the complement cascade, acute phase protein response, and phagocyte effector mechanisms such as free radical production. The second arm is the acquired immune response, also referred to as the adaptive immune response,

Table 1.3: Resistance of *Fasciola* sp. to host immune responses

	Host		
Fasciola sp. Parasite	Sheep (primary / secondary infections)	Cattle (primary / secondary infections)	Rat (primary / secondary infections)
F. hepatica	Yes	Yes/ ?	Yes/No
F. gigantica	No	Yes/ ?	No/No

- Sheep refers to ITT and Merino
- Cattle refers to
- Rats refers to Sprague Dawley and Wistar

? Unclear as to whether immunological resistance is developed or occurs due to physical barriers

specific for the invading pathogen. Activation of T and B cells, an integral part of the acquired immune response, is dependent on dendritic cells (DCs) which are part of the innate response. Briefly DCs interact with the invading pathogen within peripheral / infected organs, which results in the presentation of antigenic peptides on the DC surface. Antigen loaded DCs then migrate to the lymphoid organs where naïve T and B cells are activated through the recognition of the antigen peptides on the DC surface. These activated cells then migrate to the site of inflammation / infection and elicit their effector responses. Part of this response may involve the release of lymphokines to attract innate effector cells to the infection site, serving to amplify the adaptive immune response with innate immune effector cells (Luster 2002).

Acquired immunity is separated into the humoral immune response (HIR) and the cell mediated immune (CMI) response (reviewed in Abbas, Murphy & Sher 1996). Both of these responses are dependent upon effector cells supplied by the innate immune response (Luster 2002). The HIR is characterised by antibody mediated mechanisms, while the CMI response is characterised by cellular cytotoxicity. There have been several in depth reviews published (Abbas, Murphy & Sher 1996) and a brief overview of the CMI response will now be discussed.

The CMI responses are mediated through two classes of T lymphocytes. CD8 positive cytotoxic T lymphocytes (CTLs) are directly involved in the killing of infected target cells such as virus infected cells. CD4 positive helper T lymphocytes (Th) are broadly separated by their lymphokine expressing profiles into Th1 and Th2 subsets (reviewed in Abbas, Murphy & Sher 1996). Differentiation of Th subsets is based on the murine model. Th1 cells express IFN-γ, IL-2 and TNF-β and are commonly involved in mediating inflammatory responses, the CMI response against viruses and organisms growing within macrophages. Th2 cells are known for expressing IL-4, IL-5, IL-10 and IL-13 (reviewed in Romagnani 1999) and play a role in the HIR. The lymphokines released by Th1 and Th2 cells can have opposing effects, resulting in either the control of infection or promotion of disease (reviewed in Abbas, Murphy & Sher 1996, London, Abbas & Kelso 1998). It should be noted that there is not as clear a separation of Th clones into Th1/Th2 classes in the bovine, ovine, and human hosts (Yssel *et al.* 1992, Del Prete *et al.* 1993, Brown *et al.* 1994b). However a predominant Th1 or Th2 class response does develop in certain hosts *in vivo* and *in vitro,* with the overall response comprised of a heterologous population of

clonal T cells expressing a range of cytokines and activity levels (Kelso 1995, Brown, Rice-Ficht & Estes 1998).

The induction of either the Th1 or Th2 lymphocyte population firstly requires the recognition of foreign antigens released from parasites by T cells. More specifically a processed foreign peptide is displayed in association with MHC class I or MHC class II molecules on the surface of antigen presenting cells like DCs. These foreign peptides are then recognised by the T cell receptor : CD3 complex on $CD8^+$ and $CD4^+$ T lymphocytes, respectively (Miceli & Parnes 1991, Julius, Maroun & Haughn 1993). T cells are then activated by the release of lymphokines, like IL-2, stimulating proliferation. These expanded clones secrete other lymphokines which influence antibody production by B cells and the recruitment of phagocytic cells. Lymphokines can also induce innate defence mechanisms (James & Scott 1988).

Due to the significance of T lymphocytes within the immune system and that parasite infection can polarise $CD4^+$ T cell subset responses, it was not unexpected to discover that some parasites (eg *Echinococcus granulosus*, *Brugia malayi*, and *F. hepatica*) can modulate lymphocyte proliferation, most likely to improve their chances for survival (Rigano *et al.* 2001, Loke, MacDonald & Allen 2000, Brady *et al.* 1999b). Lymphocyte proliferation in response to parasitic infections has been used to measure the functional capacity of the infected host's immune system. The modulation of lymphocyte proliferation is a common characteristic observed in parasites from the Protozoan and Helminth classes and several mechanisms of suppression have been proposed. These will now be reviewed.

Protozoa

(i) Trypanosomes

Suppressed T lymphocyte proliferation is associated with *Trypanosoma cruzi* and *T. brucei rhodesiense* infection. *T. b. rhodesiense* infections suppress PHA-stimulated human lymphocyte proliferation. This suppression was associated with a reduction in expression of the IL-2 receptor on T cells (Kierszenbaum *et al.* 1991). The proposed mechanism of suppression stems from the requirement of IL-2 binding to the IL-2 receptor to activate T cell proliferation. A decrease in the expression of the IL-2 receptor is likely to result in a decrease in cell proliferation.

For human *T. cruzi* infections a decrease in proliferation of PMA-, anti-CD3 or anti-CD2 stimulated human lymphocytes was observed in the acute phase (Sztein, Cuna & Kierszenbaum 1990, Beltz, Sztein and Kierszenbaum 1988 and Kierszenbaum *et al.* 1990). Coinciding with this suppression was a reduction in IL-2 receptor expression on T cells, similar to *T. b. rhodesiense* infections, along with decreases in CD3, CD4 and CD8 expression. CD3, CD4 and CD8 are important molecules on T cells involved in activation of proliferation (Vignali 1994, Fleury, Croteau & Sekaly 1991). Suppressed cell proliferation may result from reduced expression of these four surface markers. These effects were only observed on activated lymphocytes i.e lymphocytes in the presence of a mitogen. There was no reduction in IL-1, IL-2 or IFN-γ production by activated human T cells with *T. cruzi* (Beltz, Sztein & Kierszenbaum 1988, Beltz, Sonnenfeld and Kierszenbaum 1989).

More recently, work has been published suggesting that the *T. cruzi*-induced suppression of human lymphocyte proliferation is due to a reduction in the competency of accessory cells (AC) (Motran *et al.* 1996). The proliferative capacity of lymphocytes alone and lymphocytes with AC was examined in the presence of mitogens and *T. cruzi* parasites. Lymphocyte proliferation was only affected in the presence of ACs. Furthermore, a decreased expression of HLA-DR and CD11b surface markers on ACs was observed. Both of these markers play important roles in human monocyte / macrophage adherence to T cells, activation and phagocytosis (Motran *et al.* 1996). Authors of both bodies of work point to suppressive mechanisms employed by *T. cruzi* as host evasion strategies.

(ii) *Leishmania*

Leishmania major contains a gene which, when translated, expresses a ribosomal protein termed LmS3a. LmS3a has been detected in *L. infantum, L. amazonensis* and *L. mexicana* (Cordeiro-Da-Silva *et al.* 2001). The authors showed that recombinant LmS3a had a suppressive effect *in vivo* and *in vitro* on murine T and B cells respectively. LmS3a was also shown to reduce IFN-γ, IL-2 and IL-12 secretion by T cells leading to the hypothesis that LmS3a plays an important role in the balance of Th1 and Th2 host immune responses (Cordeiro-Da-Silva *et al.* 2001). Another *Leishmania* parasite, *L. donovani,* also suppresses mouse and human mitogen-stimulated T

lymphocyte proliferation (Hviid *et al.* 1990, Saha *et al.* 1995). Both authors point to a different modulation mechanism to that described by Cordeiro Da-Silva *et al.* (2001). The first proposal is based on suppression requiring the presence of live parasites to cause a reduction in CD3 expression on T cells. Reduced CD3 levels are likely to decrease the antigenic recognition pathway mediated by T cell receptor : CD3 complex (Hviid *et al.* 1990). The second proposal was based on the production of prostaglandins which impaired the ability of macrophages to stimulate lymphocytes (Saha *et al.* 1995).

Helminths

(i) *Brugia* parasites

B. malayi and *B. pahangi* suppress lymphocyte prolifération from mice and jirds, respectively (Loke *et al.* 2000, MacDonald, Loke & Allen 1999, Leiva & Lammie 1989). For *B. malayi* this effect was induced through ESP which recruited an IL-4-dependent population of suppressor macrophages i.e alternatively activated macrophages (Stein *et al.* 1992, Bronte *et al.* 1998, Bronte *et al.* 1999, Apolloni *et al.* 2000), which suppressed murine proliferation (Loke *et al.* 2000). This was the first report of this novel suppression mechanism induced by parasites. Interestingly this population of macrophages could also suppress the proliferation of a range of human carcinoma lines, leading the authors to allude to the benefit of a parasitic infection in preventing tumour expansion (Loke *et al.* 2000). In contrast, a soluble extract from *B. pahangi* was responsible for suppression of Con A induced lymphocyte proliferation *in vitro* (Leiva & Lammie 1989). *B. pahangi* infections do not result in a generalised immunosuppression of lymphocyte proliferation, leading the authors to propose that this effect may be limited to the micro-habitat of the parasite in the host (Leiva & Lammie 1989).

(iii) *Schistosoma mansoni*

Lacto-N-fucopentaose III (LNFPIII) is a pentasaccharide expressed by *S. mansoni*. LNFPIII was conjugated to the carrier molecule dextran and injected into mice which led to suppression of the proliferation of naïve $CD4^+$ T cells. This suppression was mediated by LNFPIII inducing an IFN-γ-dependent suppressor macrophage population (Atochina *et al.* 2001). These macrophages fall into the classically activated macrophage class (Kusmartsev, Li & Chen 2000, Angulo *et al.* 2000). This mechanism is similar to that induced by the nematode *B. malayi* except with a

different population of suppressor macrophages. The authors of this *S. mansoni* paper conclude that LNFPIII may be a mechanism induced by the parasite to modulate host immune responses (Atochina *et al.* 2001).

The parasite examples presented here, while showing the common occurrence of modulating host lymphocyte responses, also point to an array of responsible mechanisms. Through cannulating lymphatic tissues of experimental models, it is possible to investigate host immune responses to invading pathogens. The sheep cannulation model will now be discussed.

1.4.2 Release of Excretory / Secretory Products

Parasitic antigens are derived from excretory / secretory products (ESP), along with surface and somatic components (Dea-Ayuelu & Bolas-Fernandez 1999). ESP contains a variety of molecules released by parasites into their host environment. The two major sources of ESP are products from the cuticular and tegumental surfaces of some helminths along with those molecules released by specialised excretory / secretory organs (Lightowlers and Rickard 1988).

ESP provides a source of antigens for host attack (Holland *et al.* 2000). In other cases, molecules within ESP have been identified as participating in numerous immunological roles aiding in parasitic survival and immune evasion. These roles include modulating the immune response through cleavage of host immune cell surface markers or exploiting the immune response through stimulation of specific cytokines which are capable of polarising an ineffective Th immune response (reviewed in Riffkin *et al.* 1996). Additional roles include shedding of surface bound ligands as well as regulating complement and other host inflammatory responses (Lightowlers & Rickard 1988). The importance of ESP in parasite survival mechanisms is highlighted in some vaccine studies. Vaccinating animals with ESP or ESP-derived molecules from parasites has conferred significant protection against *Trichostrongylus colubriformis* (Rothwell & Love 1974), *Nippostrongylus brasiliensis* (Day *et al.* 1979), *Toxocara canis* (Nicholas, Stewart & Mitchell 1984) and *Dictyocaulus viviparus* (Britton *et al.* 1993). It is these survival and evasion strategies utilised by helminth parasites through ESP that will be the focus of this section.

Helminths

Schistosoma mansoni

Different constituents of *S. mansoni* ESP have been characterised along with their immuno-modulatory effects (Brady *et al.* 1999a, Wasilewski *et al.* 1996, Yoshino *et al.* 1993, Duvuax-Miret *et al.* 1992, Ramaswamy *et al.* 1995, Trottein *et al.* 1999). *S. mansoni* release a number of cysteine proteases including cathepsin B, cathepsin L1 and cathepsin L2. Cathepsin L1, which differs from cathepsin L2 in both substrate specificity and location, has been proposed to be involved in digestion of host hemoglobin (Brady *et al.* 1999a). Hemoglobin is central to parasitic survival, as it is the parasite's main source of amino acids. Other experimental work has shown that inhibition of cysteine protease activity leads to reduction in parasite viability and this is related to the inability of *S. mansoni* to breakdown hemoglobin (Wasilewski *et al.* 1996, Yoshino *et al.* 1993).

Three other immunologically important *S. mansoni* ESP molecules are proopiomelanocortin (POMC) derived peptides (Duvaux-Miret *et al.* 1992), a molecule termed Sm16.8 (Ramaswamy *et al.* 1995) and lipophilic substances (Trottein *et al.* 1999). POMC derived peptides were shown to inhibit the migratory activity of immune cells from the parasite's definitive host (hamster) and its intermediate host (snail) (Duvaux-Miret *et al.* 1992). Sm16.8, as the name suggests, is a 16.8 kDa protein which can suppress antigen-induced lymphocyte proliferation *in vitro*. This phenomenon may coincide with the observed reduction in IL-2 secretion by T cells (Ramaswamy *et al.* 1995). The lipophilic substance/s in ESP from *S. mansoni* schistosomula are rich in polyunsatturated fatty acids and were shown to reduce expression of the adhesion molecules E-selectin and VCAM-1 by TNF-α-activated endothelial cells. This reduction occurred at the gene expression level and was not a result of cleavage of the markers from the surface. These adhesion molecules play an important immune role by interacting with counter receptors on circulatory immune cells causing transmigration of leukocytes at sites of inflammation. This is a necessary response for parasite elimination. Collectively each of these three ESP molecules play important roles in aiding *S. mansoni* evasion of host immune responses by hindering immune cell recruitment, preventing inflammatory responses and dampening antigen presentation mechanisms (Duvaux-Miret *et al.* 1992, Ramaswamy *et al.* 1995, Trottein *et al.* 1999).

Brugia malayi

B. malayi also releases ESP molecules into the host environment. Two classes of ESP modulatory molecules were recently reviewed in Maizels *et al.* (2001). The first molecule is a 15 kDa cystatin (cysteine protease inhibitor) Bm-CPI-2 found on the surface of both a larval stage (L3) and adult *B. malayi* parasites. Bm-CPI-2 has been shown to block papain activity *in vitro* and to inhibit the function of a legumain-like aspariginyl endopeptidase (reviewed in Maizels *et al.* 2001). The legumain-like protein is an alternate processing enzyme, found in lymphocytes, involved in the intracellular processing of antigens for subsequent presentation by MHC class II molecules (Manoury *et al.* 1998). Bm-SPN-2 is secreted by microfilariae and belongs to the serpin (serine protease inhibitor) class of compounds. Bm-SPN-2 prevents the activity of cathepsin G and neutrophil elastase (Zang *et al.* 1999, Zang & Maizels 2001), which are both involved in innate immunity and are mediators of the inflammatory response (Maizels *et al.* 2001).

1.4.3 Production of Anti-Oxidant Defence Enzymes Against ROI and RNI

The host has a range of defence mechanisms against invading pathogens including the complement cascade, acute phase protein response and effector mechanisms of inflammation and phagocytes. Phagocyte effector mechanisms include acidification of the phagolysosomal vacuole, hydrolytic lysosomal enzymes, nutrient deprivation, cytotoxic proteins, defensins and free radicals (Jones 1993, Cohen 1994, Reiner 1994). Free radicals are one of the most important host defence mechanisms identified to date. Due to the non-specific nature of free radicals they can cause harm to both the invading pathogen and the hosts cells. Over production of free radicals has been shown to lead to suppression of host immunity (reviewed in Piedrafita & Liew 1998). A brief overview is presented below on free radicals, their derivatives formed as part of a host's defence mechanism and the common defences shared by both the host and parasite.

While being essential to life, oxygen can also be extremely toxic when it is partially reduced to water during normal cellular metabolism, resulting in the addition of a single electron as opposed to four electrons (Halliwell & Gutteridge 1984). This leads to the formation of free radicals classed as reactive oxygen intermediates (ROI) such as the superoxide anion radical (O_2^-),

hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$). ROI are also produced during the respiratory burst by NADPH oxidase as a host defence mechanism against invading pathogens. Another important class of free radicals, reactive nitrogen intermediates (RNI which includes nitric oxide (NO)), are produced in the presence of nitric oxide synthase. Free radicals can be produced both intra- and extra- cellularly (Cross & Jones 1991). Free radicals have been shown to play a role in ischemia reperfusion injuries, exercise-induced muscular soreness, and in the pathophysiological changes during respiratory, gastrointestinal and other systemic diseases associated with ageing (Doelman & Bast 1990, Gutteridge & Halliwell 1990, Anaya-Prado *et al.* 2002). Free radicals are also a powerful host non-specific defence mechanism against parasites (reviewed in Miller & Britigan 1997, Brunet 2001). Free radicals can unfold or inactivate proteins, degrade nucleic acids, and lead to lipid peroxidation in cell membranes causing the release of lipid hydroperoxides that can further breakdown to yield a range of products such as cytotoxic aldehydes. These products can cause extensive damage which can ultimately kill cells and in some circumstances whole organisms (reviewed in Miller & Britigan 1997). Figure 1.2 shows the reactions involving free radicals. The enzymatic and spontaneous production of ROI and RNI have been extensively reviewed (Badwey & Karnovsky 1980, Fantone & Ward 1982, Freeman & Crapo 1982, Eagleson & Moriarty 1984, Clark, Hunt & Cowden 1986, James 1995, Eiserich, Patel & O'Donnell 1998, Turpaev 2002) and are briefly discussed below.

(i) Reactive Oxygen Intermediates

Reactive oxygen intermediates (ROI) are produced in significant quantities in biological systems by non-phagocytes (lymphocytes, fibroblasts, endothelial cells, mesangial cells) during normal cellular respiration. ROI are also produced by activated phagocytes (neutrophils, platelets, eosinophils and macrophages) (Table 1.4) for host defence against invading microbes (Callahan, Crouch & James 1988). It is thought that the binding of phagocytes to parasites stimulates the respiratory burst leading to increased oxygen consumption. This results in the formation of ROI which can then mediate damage / killing of parasites by these cells (Babior, Kipnes & Curnutte 1973, Curnutte, Whitten & Babior 1974, Salin & McCord 1974, reviewed in Babior 2000).

The first free radical produced is the superoxide anion radical ($O_2^{\bullet -}$) (reviewed in Babior 2000). The $O_2^{\bullet -}$ radical can play a role as either a mild reductant or oxidant. As it is not highly reactive

Table 1.4: Reactive primary and derived products of activated phagocytes (Callahan, Crouch & James 1988)

Cell	Oxyradicals
Platelets	H_2O_2, $O_2^{\cdot-}$, $^{\bullet}OH$,
Neutrophils	H_2O_2, $O_2^{\cdot-}$, $^{\bullet}OH$, HOCl,
Eosinophils	H_2O_2, $O_2^{\cdot-}$, $^{\bullet}OH$,HOCl, $^1O_2$
Macrophages	H_2O_2, $O_2^{\cdot-}$, $^{\bullet}OH$, HOCl

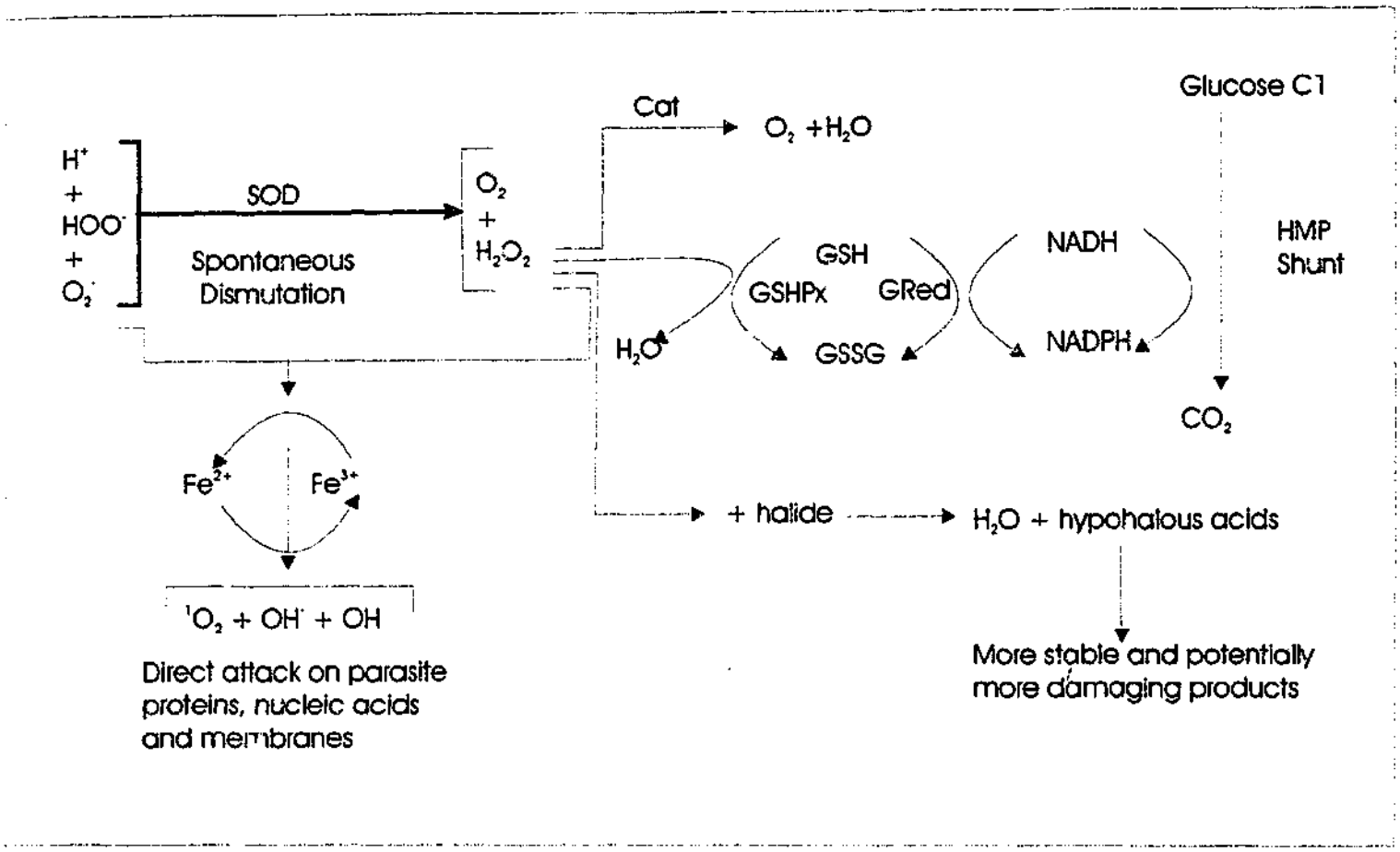


Fig 1.2 Reactions for producing free radicals and the involvement of anti-oxidant defence enzymes in their detoxification. The abbreviations were SOD: superoxide dismutase, GSHPx: glutathione peroxidase, GRed : gluthatione reductase, Cat: catalase and HMP: Hexose monophosphate. This figure was modified from Callahan, Crouch & James (1988).

it is capable of diffusing some distance before acting and this may involve crossing the cellular membrane (Roos *et al.* 1984). The superoxide anion has been shown to inactivate enzymes involved in the formation of branched amino acids (e.g. α,β-dihydroxy isovalerate dehydratase and NADH- bound lactic dehydrogenase) (Haas & Goebel 1992, Kuo, Mashino & Fridovich 1987). $O_2^{\bullet-}$ is the substrate in either an enzymatic or spontaneous dismutation reaction leading to the formation of H_2O_2 (Fig 1.2) (Fridovich 1975, Storz *et al.* 1990, reviewed in Babior 2000). $O_2^{\bullet-}$ radical is also the substrate in Fentons reaction which results in the formation of $OH^{\bullet-}$ and reactive singlet oxygen ($^1O_2$) formation (Fig 1.2, reviewed in Babior 2000).

H_2O_2 is more reactive as an oxidant than $O_2^{\bullet-}$ and has been implicated in the damage of cellular constituents through the oxidation of essential thiol groups (reviewed in Selkirk *et al.* 1998), the damage and mutagenesis of DNA and the inhibition of membrane transport processes (Miller & Britigan 1997, Weiss 1986). H_2O_2 can also diffuse across cell membranes like $O_2^{\bullet-}$. The result of the interaction of H_2O_2 and $O_2^{\bullet-}$ is the highly reactive $OH^{\bullet-}$ radical. Studies have shown that the rate of this reaction is only significant in the presence of Fe^{3+} (Haber & Weiss 1934).

$OH^{\bullet-}$ is involved in the oxidation of proteins, DNA and lipids (Miller & Britigan 1997). $OH^{\bullet-}$ has a strong oxidative nature which results in limited diffusion and a short half-life of 10^{-9} s (Selkirk *et al.* 1998). Due to these factors, $OH^{\bullet-}$ needs to be formed close to its target to provide any level of attack (Czapski 1984).

(ii) Reactive Nitrogen Intermediates

The most common RNI is nitric oxide (NO). When the terminal guanidino nitrogen of L-arginine and molecular oxygen interact, in an NADPH dependent reaction, the result is the formation of L-citrulline and NO (Hibbs *et al.* 1988, Abu-Soud & Stuehr 1993; Prince & Gunson, 1993). This reaction is catalysed by the enzyme nitric oxide synthase (NOS). To date, three isoforms of NOS have been characterised. Two of the isoforms are constitutive NOS (cNOS), and neuronal NOS (nNOS). Both of these enzymes respond to changes in the intracellular concentration of Ca^{2+}. In these circumstances, the NO produced is then involved in neurotransmission and blood pressure regulation (Miller & Britigan 1997). The other isoform is inducible NOS (iNOS) for which transcription is upregulated in response to cytokines (eg IFN-γ), microorganisms and / or

microbial products (eg LPS) (Moncada & Higgs 1993, Stuehr & Marletta 1985, Miller & Britigan 1997).

NO is a highly reactive compound produced in the greatest quantities by macrophages and macrophage-like cells. Neutrophils, platelets, adrenal cells and respiratory epithelial cells have also been shown to produce NO (Nussler & Billiar 1993, Billiar *et al.* 1992). NO can exist in three alternative redox states: nitric oxide ($NO^{\bullet}$), nitroxylanion (NO^{-}) and the nitrosonium cation (NO^{+}) (Stamler, Singel & Loscalzo 1992). NO is capable of reacting with other NO molecules, water and oxygen to generate other RNI (Stamler *et al.* 1992; Yoshida *et al.* 1993). RNI may cause tissue damage, impair growth and cellular function or decompose in aqueous solutions to form the stable end products nitrite (NO_2^-) and nitrate (NO_3^-) (Hibbs *et al.* 1988, Stuehr, Morris & Nathan 1988, Liew & O'Donnell 1993 James 1995). NO inactivates several key metabolic enzymes of the respiratory cycle like the aconitase enzyme of the Krebs cycle and glyceraldhyde-3-phosphate dehydrogenase which contain Fe moieties at their catalytic sites (Stamler, Singel & Loscalzo 1992, Drapier & Hibbs 1986, James 1995, Miller & Britigan 1997). NO can also cause mutations in DNA (Miller & Britigan 1997).

Finally NO and O_2^- also can interact with one another, with the potential for even stronger free radical production. One such molecule is peroxynitrite, a strong oxidant, that reacts with thiols, initiates iron independent lipid peroxidation and can also react with metallo-proteins (Miller & Britigan 1997).

In summary, free radicals can cause deleterious effects to parasites which may result in parasitic death. NO is one free radical shown to be involved with host attack against parasites (reviewed in Brunet 2001). *In vitro* experiments have shown the cytotoxic and / or cytostatic effects of NO on *Leishmania major* (Assreuy *et al.* 1994), *Mycobacterium bovis* (Flesch & Kaufmann 1991), *Toxoplasma gondii* (Adams *et al.* 1990), *Schistosoma mansoni* (James & Glaven 1989), *Trypanosoma musculi* (Vincendeau & Daulouede 1991), *F. hepatica* (Piedrafita *et al.* 2001) and *Trypanosoma cruzi* (Melo & Brener 1978). The effectiveness of NO *in vivo* within hosts against parasites is being investigated.

With free radicals being implicated in the host's non-specific armament against parasites, it seems only natural that, over time, hosts and parasites will have evolved free radical evasion strategies. These strategies include DNA and protein repair systems, free radical scavenging substrates (Hassett & Cohen 1989) like ascorbate (Rose & Bode 1993), hemoglobin (Giulivi & Davies 1990), serotonin (Jovanovic, Steenken & Simic 1990) and the production of anti-oxidant defence (AOD) enzymes. AOD enzymes have been shown to act directly on the free radicals forming less offensive products. To date all protozoan and helminth parasites tested have at least one AOD enzyme (Callahan, Crouch & James 1988, Selkirk *et al.* 1998, Henkle-Duhrsen & Kampkotter 2001) and these biochemical interactions of AOD enzymes have been reviewed (Callahan, Crouch & James 1988, Fridovich 1995). The level of expression of AOD enzymes has been shown to vary between parasite species, sex and life cycle stages of a single species (Ou *et al.* 1995, Ben-Smith, Lammas & Behnke 2002, Paramchuk *et al.* 1997, Ismail *et al.* 1997). In some systems, the levels of AOD enzymes have shown some correlation with the susceptibility of parasites to free radical damage (Nare, Smith & Prichard 1990, Batra, Chatterjee & Srivastava 1992).

AOD enzymes are generally present in low levels in cells, but are often up-regulated upon recognition of certain stimuli (Storz, Tartaglia & Ames 1990, Wu & Weiss 1991, Harris 1992). The noteworthy exception is GST which is often present at high levels within cells (reviewed in Jackoby 1985). The primary purpose of AOD enzymes is to protect the parasite against host mediated free radical damage. These enzymes are typically water soluble and are therefore able to function in the host plasma, in the parasite cytosol or in the periplasmic spaces of host cells (Harris 1992). There are four main AOD enzymes: Superoxide Dismutase (SOD), Catalase, Glutathione Peroxidase (GSH-Px) and Glutathione *S*-Transferase (GST).

(iii) SOD

SOD was first discovered in bovine erythrocytes (McCord & Fridovich 1969). The presence of SOD is essential in microbes for protection against free radical production by the host. SOD exists in three different isoforms according to the metal present within the active site (Keele, McCord & Fridovich 1970, McCord & Fridovich 1969, Weiseger & Fridovich 1973, reviewed in Fridovich 1995) and each SOD catalyses the same reaction.

Manganese-SOD (Mn-SOD): Found in prokaryotes and in the mitochondria of cells. Mn-SOD exists as a dimer with 20 kDa sub units, with one Mn molecule per subunit.

Iron-SOD (Fe-SOD): Found in protozoa (including parasitic protozoa), eukaryotic algae and in the periplasmic space of some higher plants (Fridovich 1975);

Copper/Zinc-SOD (CuZn-SOD): Exists in a range of eukaryotes including multicellular plants and animals including mammals, bacteria and helminths. The common cytosolic CuZn-SOD exists in a dimeric form comprised of 16 kDa subunits which contain one ion of each Cu and Zn (Fridovich 1975). The extracellular CuZn-SOD (EC-SOD) is a tetrameric, glycosylated compound of 30 kDa subunits (reviewed in Callahan, Crouch & James 1988, Fridovich 1995).

(iv) Catalase

Catalase exists as a tetramer with subunits of approximately 60 kDa, each containing haematin as a prosthetic group (reviewed in Callahan, Crouch & James 1988). Catalase activity has been identified in several nematodes (reviewed in Henkle-Duhrsen & Kampkotter 2001) but has not been shown in trematodes or cestodes.

(v) GSH-Px

GSH-Px is a tetramer with identical subunits of 75-100 kDa. Each subunit contains one atom of selenium which is covalently bound to cysteine in the active site. GSH-Px has been localised to the cytosol and mitochondrial matrix: to date GSH-Px activity has been shown in all mammalian tissues (reviewed in Callahan, Crouch & James 1988). GSH-Px acts on both hydrogen peroxide and hydro-peroxides unlike catalase which only detoxifies hydrogen peroxide.

(vi) GST

GSTs are commonly dimeric in structure with a subunit size between 23 and 29 kDa (Ketterer, Meyer & Clark 1988). There are a large number of GST isoenzymes which differ based on their subunit composition, substrate and inhibitor specificities and antigenic cross reactivity (reviewed in Ketterer 1988 and Ketterer, Meyer & Clark 1988). GSTs are either soluble or membrane

bound (Mannervik & Danielson 1988) and have been found in most mammalian tissues, as well as insects, protozoa, helminths, molluscs, algae, fungi and bacteria. (Smith *et al.* 1986, 1987, Stenersen *et al.* 1987). GST is differentiated from GSH-Px by the absence of selenium in each subunit and incapacity to detoxify H_2O_2 (reviewed in Callahan, Crouch & James 1988). GSTs are involved in ligand transport (Ketterer 1988) and the detoxification of DNA hydroperoxides, membrane phospholipids and lipid hydroperoxides by the conjugation of glutathione (GSH) (Ketterer 1988, Brophy & Barrett 1990). GSTs present in helminths protect against both cytotoxic aldehydes which are themselves products of lipid peroxidation and exogenously derived toxic compound (Brophy & Pritchard 1994). The rate of the reaction depends on the concentration of GSH (Ketterer 1988). The GSH conjugates formed have greater water solubility, thus aiding their eventual elimination from cells (Board *et al.* 1990).

The balance of the various AOD enzymes is crucial, as either GSH-Px or catalase are required to detoxify H_2O_2 produced by the activity of SOD. Without a balance, the products of the SOD catalysed reaction, which are toxic, may lead to host cell death (Harris 1992, Amstad *et al.* 1991, Amstad, Moret & Cerutti 1993). Finally, it is also necessary to emphasize the importance of the tri-peptide glutathione (GSH), as it is essential for the activity of both GSH-Px and GST (Arrick *et al.* 1982). Both of these enzymes use GSH in the reduction or conjugation of free radicals. Studies have shown that GSH deficiency leads to multi-organ failure and the subsequent death of rats and guinea pigs, related to the loss of an anti-oxidant system (Martensson, Meister & Martensson 1991, Martensson *et al.* 1991).

(vii) Anti Oxidant Defence Enzyme Levels in *Schistosoma mansoni*

There have been numerous studies looking at the vulnerability of different life stages of *S. mansoni* to host immune defences. The results point to the parasite's susceptibility to free radical killing decreasing as the parasite matures, with the adult being relatively resistant to free radical attack (Kazura *et al.* 1981, Jong, Mahmoud & Klebanoff 1981, Yazdanbakhsh *et al.* 1987, Yazdanbakhsh, Eckmann & Roos 1987, Smithers & Doenhoff 1982, Pearce & James 1986, Capron *et al.* 1987). A major proportion of the host attack is cell mediated through the release of toxic ROI. Based on the differences in susceptibility between life stages and the main host defence mechanism employed, work has focussed on AOD enzymes present in *S. mansoni*.

CuZn SOD (cytosolic and signal peptide containing), GSH-Px, GST and cytochrome c peroxidase (involved in H_2O_2 detoxification) expression levels all increase as *S. mansoni* matures from schistosomula through to the adult (Mkoji, Smith & Prichard 1988a,b, Nare, Smith & Prichard 1990, Mei *et al.* 1996, Mei & LoVerde 1997). Catalase has not been detected in any stage of *S. mansoni* (Mkoji, Smith & Prichard 1988a,b). Interestingly, adult male parasites were more effective at detoxifying H_2O_2 than adult female parasites (Mkoji, Smith & Prichard 1988b) and the reason for this is currently unclear. SOD and GSH-Px have been localised to the tegument and the gut epithelium (Mei & LoVerde 1997). The tegument is the host:parasite interface, suggesting that SOD and GSH-Px are likely to be involved in *S. mansoni* defence mechanism.

Each author reasons that these higher AOD enzyme levels and their key locations are likely to be responsible for the relative resistance of adult *S. mansoni* to host immune attack compared with schistosomula. If this is true, these findings emphasise the importance of AOD enzymes in parasitic protection. Finally, the phenomenon of increasing AOD enzyme levels as parasites mature correlating with an increased resistance to host attack has also been observed for *Trichinella spiralis* (Kazura & Meshnick 1984), *Leishmania donovani* (Murray 1982, Reiner & Kazura 1982, Channon & Blackwell 1985), *Nippostrongylus* and *Nematospiroides* (Smith & Bryant 1986). The role of AOD enzymes in *Fasciola* biology is discussed (Section 1.5).

1.4.4 Glycocalyx Turnover

The surface of any parasite plays at least two important roles. Firstly, it is across the surface that physiologically vital nutrients are acquired from the host and secondly it is expected that a large part of any host defence mechanism will be directed against the surface of the parasite (Lumsden 1975). Therefore, the helminth tegument potentially plays a crucial role in a parasite's evasion strategy.

One example is the *S. mansoni* tegument, which is proposed to participate in several evasion strategies. Firstly *S. mansoni's* tegument can incorporate host serum proteins with the theory that this helps mask parasite antigens thereby helping the parasite escape immune attack (Yong &

Das 1983, McLaren 1984). Secondly the cercarial tegument is mostly shed not long after the penetration of the host's skin. This is beneficial to the parasite in two ways. Firstly, the cercarial glycocalyx is highly antigenic and also stimulates complement by the alternative pathway so shedding the glycocalyx decreases parasite antigenicity. *S. mansoni* has been shown to be more susceptible to host attack during these first 3 days post infection. Studies have also shown that, compared to irradiated schistosomula, irradiated cercariae confer higher protection against subsequent challenge. The authors propose that the higher protection relates to the cercarial glycocalyx (Brouwers *et al.* 1999) and that by shedding this glycocalyx early on, the schistosomula may escape immune attack. Secondly, the rapid shedding of the glycocalyx by early developmental stages of *S. mansoni* (Brouwers *et al.* 1999) is proposed to prevent sustained attachment of effector cells and molecules. Hawn and Strand (1993) identified in *S. mansoni* the presence of phospholipase D activity which is capable of cleaving glycosylphosphatidyl inositol (GPI) anchored proteins, which provides a potential mechanism for the forementioned shedding.

1.5 Major Proposed Evasion Strategies of *Fasciola sp.*

As discussed in Section 1.3 *Fasciola* parasites have varying degrees of success at establishing within different hosts and this is also species dependent. While this observation does suggest differences between host immune responses it also suggests that *Fasciola* parasites may have evolved immune evasion strategies which vary in success in the different hosts. The next section will predominantly discuss some of the evasion strategies expressed by *Fasciola* that are proposed to be effective within the ruminant host before the parasite reaches the bile ducts. Bile ducts are an immunologically safe environment (Hughes, Hanna & Symonds 1981). Once inside the bile ducts resistance of adult parasites to immune killing may be due to their inaccessibility rather than the development of defence mechanisms.

1.5.1 Modulation of the Immune Response

F. hepatica infections, like other parasitic infections discussed previously, result in modulation of lymphocyte proliferative responses. A short lived, early increase in mitogen-stimulated lymphocyte proliferation is commonly observed during *F. hepatica* infections in cattle and rats (Poitou, Baeza & Boulard 1992, Oldham 1985, Oldham & Williams 1985, Clery & Mulcahy 1998). The work by Poitou, Baeza & Boulard (1992) showed significant increases in

proliferation of rat splenocytes between 2 – 4 weeks post infection (WPI), in the presence of Concanavalin A (Con A), pokeweed mitogen (PWM), adult *F. hepatica* ESP or metacercariae antigen. The stimulation with these mitogens suggests that the effect is induced on a range of lymphocyte populations and the results with parasite antigens suggest that this response is antigen specific. This work is partly supported by reports by Cervi *et al.* (1998) who observed a sustained increase in LPS-stimulated proliferation of splenocytes removed from *F. hepatica* infected rats, compared with uninfected control rat cells. In contrast, this paper and two other papers from the same group (Cervi *et al.* 1998, Cervi, Rubenstein & Masih 1996, Cervi & Masih 1997) reported a decrease in proliferation of splenocytes from *F. hepatica* infected animals when these rat cells were stimulated with Con A or *F. hepatica* ESP. This suppression was linked to NO and/or H_2O_2 production, since the addition of either aminoguanidine (nitric oxide synthase inhibitor) or catalase (decomposes H_2O_2, inhibits NO production) restored proliferation. Potential reasons for the variation in the response of lymphocytes to mitogen-stimulation were not alluded to. What is apparent is that *F. hepatica* infections modulate lymphocyte proliferation within the rat host. Overall, modulation of mitogen-stimulated proliferation of lymphocytes from cattle and rats were observed early after *F. hepatica* infection. The proposal is that these responses do not correlate with host resistance.

F. hepatica infections in sheep cause significant suppression of mitogen-stimulated lymphocyte proliferation *in vitro* (Zimmerman *et al.* 1983, Chauvin, Bouvet & Boulard 1995). Zimmerman *et al.* (1983) observed significant suppression of proliferation during *F. hepatica* infections with either Con A or PHA at 4 and 8-10 WPI. A similar trend in the suppression profile was also observed with PWM suggesting that the suppressive phenomenon is likely to be occurring with all lymphocyte populations (eg T and B cells). This was supported by Chauvin and colleagues (1995) who revealed a similar significant suppression of sheep lymphocyte proliferation at 4 and 11 WPI using Con A as the mitogen. Furthermore, the proliferative capacity of lymphocytes post secondary infection was examined in this study. There was a significant increase in proliferation during 1 and 2 weeks post secondary infection, followed by a return of proliferation levels to those observed in primary infected sheep. In conclusion, the significance of an effect on lymphocyte proliferation at 4 and 8-10 WPI, is that these times correspond to growth phases and migration of *F. hepatica* within the liver parenchyma (Zimmerman *et al.* 1983). This

suppressive response is proposed to aid in the parasites establishment within the host. Sheep have not been shown to acquire resistance to *F. hepatica* unlike infections in cattle and rat. The significance of increased cellular proliferation post a secondary infection with *F. hepatica* has yet to be decided. The effects of an *F. gigantica* infection on sheep lymphocyte proliferation in the presence of mitogens has to date not been studied.

1.5.2 Release of Excretory / Secretory Products

The invading *Fasciola* parasite continuously releases a group of molecules into the host environment, collectively referred to as excretory / secretory products (ESP). Several immune evasion strategies have been attributed to *F. hepatica* and *F. gigantica* ESP.

F. hepatica ESP have been shown to modulate accessory cell function, firstly through lowering phagocytic activity and the antigen presentation ability of rat peritoneal cells *in vitro* (Masih, Cervi & Casado 1996). Secondly *F. hepatica* ESP contains 4 non-lipid molecules which can cause chemokinesis in sheep neutrophils (Jefferies *et al.* 1996). Lastly an IL-5 like molecule within *F. hepatica* ESP stimulates eosinophil maturation and this consequently leads to a rise in eosinophil peroxidase (EPO) activity in mice (Milbourne & Howell 1993). This has added weight to the suggestion that *F. hepatica* induces an inappropriate immune response, as a high eosinophilia is observed post *Fasciola* infection in sheep that has not correlated with resistance in this host (Hansen *et al.* 1999).

As discussed in section 1.5.1 *F. hepatica* suppresses lymphocyte proliferation during infection (Zimmerman *et al.* 1983, Chauvin, Bouvet & Boulard 1995, Cervi *et al.* 1998, Cervi, Rubenstein & Masih 1996, Cervi & Masih 1997). *F. hepatica* ESP has been identified as potentially playing a role in dampening this immune response. 12-23 kDa glycoproteins within *F. hepatica* ESP were shown to stimulate a rat mononuclear cell population which upon adoptive transfer, suppressed the delayed type hypersensitivity response to parasite and non related antigens (Cervi, Rubinstein & Masih 1996). *F. hepatica* ESP could also suppress Con A or LPS-stimulated proliferation of rat spleen mononuclear cells *in vitro* (Cervi & Masih 1997). In other hosts, increasing concentrations of *F. hepatica* ESP resulted in decreasing levels of proliferation of Con A- or PHA- stimulated sheep and human lymphocytes (Jefferies, Barrett & Turner 1996).

F. hepatica ESP has also been shown to contribute to modulating host free radical levels (Cervi, Rossi & Masih 1999, Jefferies, Turner & Barrett 1997, Baeza, Poitou & Boulard 1993). Firstly, *F. hepatica* ESP decreases nitric oxide (NO) production by activated rat PLCs *in vitro* (Cervi, Rossi & Masih 1999) and inhibits NO production by activated sheep neutrophils while stimulating NO production by activated human neutrophils *in vitro* (Jefferies, Turner & Barrett 1997). Secondly, *F. hepatica* decreases superoxide (O_2^-) production by activated sheep and human neutrophils (Jefferies, Turner & Barrett 1997). Baeza and colleagues (1993) observed a similar finding with *F. hepatica* infection decreasing the metabolic burst of cattle neutrophils. Together, these findings suggest *F. hepatica* parasites have evolved strategies to avoid damage from host cell free radical production.

Significantly less work has investigated the role of *F. gigantica* ESP in immuno-modulation. The majority of the research into *F. gigantica* ESP has focussed on identifying immunodiagnositic antigens and the development of monoclonal antibodies specific for these antigens (Maleewong *et al.* 1999, Intapan *et al.* 1998, Krailas *et al.* 1999, Viyanant *et al.* 1997, Maleewong *et al.* 1997, Yadav & Gupta 1995, Fagbemi, Aderibigbe & Guobadia 1997).

One report, has shown that *F. gigantica* ESP can suppress the respiratory burst of sheep neutrophils, measured by a decrease in the release of ROI. There were at least two responsible molecules, both were non protein in behaviour, and were either less than 10 kDa or greater than 50 kDa in size (El Ghaysh *et al.* 1999). Neutrophils are an important component of the acute inflammatory cascade (reviewed in Haslett, Savill & Maegher 1989, Ward & Lentsch 1997). By dampening the neutrophil's ability to produce ROI, *F. gigantica* may evade oxidative damage.

The constituents of ESP change as the parasite matures but NEJ and adult flukes also contain common proteins (Dalton & Heffernan 1989, Tkalcevic, Ashman & Meeusen 1995, Tkalcevic, Brandon & Meeusen 1996). In sheep infected with *F. hepatica* the immunogenic bands detected by western blotting decrease in size as the parasite develops, suggesting that antigens expressed by the fluke varies with maturation: the high molecular weight bands recognised early in infection may be the shed tegument (Chauvin, Bouvet & Boulard 1995). Both immature and

mature *Fasciola* ESP contain cysteine proteases (cathepsin L or B) with immature ESP exhibiting at least 2 cathepsin Ls while adult ESP has at least 7 isoenzymes (Wijffels *et al.* 1994a).

Cathepsin Ls are major abundant constituents of *Fasciola* ESP. There are at least 12 Cathepsin Ls in *F. hepatica* ESP based on cDNA sequences. The non-redundant BLAST database contains 23 cathepsin L-like sequences, all derived from parasite mRNA; 17 of the sequences encode *F. hepatica* proteins (FhCatL) and 6 encode *F. gigantica* proteins (FgCatL) (Irving *et al.* in press). Cathepsin Ls have been detected within *F. hepatica* ESP collected from immature and mature flukes (Dalton and Heffernan 1989, Carmona *et al.* 1993). Cathepsin Ls are likely to be detected in all life stages and measurable activity levels have been recorded within NEJ, 3 wk, 5 wk and mature flukes whole worm extracts (Carmona *et al.* 1993) although no cathepsin L like sequence was detected in ESP from NEJ (Wilson *et al.* 1998). Cathepsin L activity levels increase as the parasite matures (Carmona *et al.* 1993) which may suggest an increased reliance on Cathepsin L in immune evasion or a general role in fluke development and maturation. There have been 15 proteases identified in *F. gigantica* whole worm extract (WWE) and the majority of these were cysteine proteases along with some serine proteases (Fagbemi & Hillyer 1991, 1992).

F. hepatica cathepsin Ls are purported to play a couple of key roles in tissue invasion and immune evasion (reviewed in Mulcahy & Dalton 2001). Cathepsin Ls can degrade both the extracellular matrix (fibrillar types I and II collagen) and the basement membrane (type IV collagen) (Berasain, *et al.* 1997). The authors proposed that this may aid in *Fasciola* invasion of the tissues. Cathepsin Ls also degrade haemoglobulins *in vitro*, pointing to the possible digestion of host haemoglobin for nutritional purposes (Dalton & Heffernan 1989, Wilson *et al.* 1998). A similar affect is observed for *S. mansoni* Cathepsin B (Felleisen & Klinkert Mo Quen 1990).

Finally cathepsin Ls have been shown *in vitro*, to cleave immunoglobulins from mice and humans (all IgG subclasses) in the hinge region of the heavy chain *in vitro* (Carmona *et al.* 1993, Smith *et al.* 1993a, Berasain, *et al.* 2000). To cleave human IgG1, IgG2 and IgG4, Cathepsin L required the presence of a reducing reagent. This suggests the necessity of prior reduction of disulphide bonds in the middle hinge region to allow Cathepsin L access to it's cleavage site

(Berasain *et al.* 2000). The importance of this cleavage was demonstrated when the addition of *F. hepatica* ESP along with *Fasciola* immune sera prevented the antibody-mediated attachment of eosinophils to NEJ (Carmona *et al.* 1993). This was reversed when the cysteine protease inhibitor leupeptin was added to the incubation. This is an important potential evasion mechanism considering *in vitro* work which showed the high toxicity of the Major Basic Protein (at μM concentrations) released by bovine eosinophils to *F. hepatica* NEJ (Duffus, Thorne & Oliver 1980). It should be noted in this work by Carmona and colleagues (1993) that there was no cytotoxicity mechanism shown in the absence of Cathepsin L. There is also no direct evidence that eosinophils do actually kill *F. hepatica* NEJ. The cleavage of host immunoglobulin by Cathepsin L may be the reason why, *in vitro* work has been unable to demonstrate effective antibody-dependent attack of *F. hepatica* NEJ by eosinophils (Carmona *et al.* 1993). In addition the cysteine proteases isolated from *F. gigantica* WWE have also been shown to digest both bovine IgG and bovine globin (derived from bovine haemoglobin) *in vitro* (Fagbemi and Hillyer 1991, 1992). These findings imply that *F. gigantica* may evoke similar evasion strategies to *F. hepatica*.

1.5.3 Anti-Oxidant Defence Enzymes as a Defence Mechanism

There have been several studies, including direct and indirect evidence, suggesting a role for AOD enzymes in protecting *Fasciola* against free radical attack (Piedrafita *et al.* 2000, El-Ghaysh *et al.* 1999, Cervi, Rossi & Masih 1999, Cervi *et al.* 1998, Jefferies, Turner & Barrett 1997, Baeza, Poitou & Boulard 1993). Piedrafita and colleagues (2000) compared the cytotoxicity of chemically generated ROI and RNI on *S. mansoni* schistosomula and *F. hepatica* NEJ *in vitro*. *S. mansoni* schistosomula were shown to be more susceptible than *F. hepatica* juveniles to killing by free radicals. Furthermore when AOD enzyme levels were measured *F. hepatica* AOD enzyme levels were up to 10 times higher than in schistosomula suggesting these enzyme levels were linked to the relative resistance of *F. hepatica* to free radical attack. Jefferies and colleagues (1997) showed *in vitro*, that increasing concentrations of ESP released by *F. hepatica* correlated with increasing suppression of superoxide and hydrogen peroxide production from sheep neutrophils. In other work from this group (El Ghaysh *et al.* 1999) *F. gigantica* ESP was also shown to inhibit ROI production from sheep neutrophils. The authors proposed that

both observations were linked to SOD, however both reports measured little or no SOD within the respective *Fasciola sp.* ESP.

The majority of *F. hepatica* AOD enzyme work has focussed on *F. hepatica* GSTs (FhGST). FhGST exist as a mix of homo- and hetero-dimers with subunit sizes ranging from 24-29 kDa (Hillyer, Soler De Galanes & Battisti 1992, Howell, Board & Boray 1988, Wijffels *et al.* 1992). FhGSTs are expressed in a range of tissues including the parenchyma, gut and tegument (Howell, Board & Boray 1988, Creaney *et al.* 1995, Wijffels *et al.* 1992). FhGST exists as at least six isoenzymes as detected by agarose/starch gel electrophoresis (Howell, Board & Boray 1988), chromatofocussing (Brophy, Crowley & Barrett 1990) and 2D gel electrophoresis (Wijffels *et al.* 1992, Jefferies *et al.* 2001) and cDNA cloning (Panaccio *et al.* 1992, Muro, Rodriquez-Medina & Hillyer 1993). The level of GST expression varies between NEJ and adult *F. hepatica* (Piedrafita *et al.* 2000). Interestingly, Miller, Howell & Boray (1993) showed variations in isoenzyme expression and activity of FhGSTs in flukes recovered from different hosts. Lower FhGST activity levels were observed for flukes removed from resistant hosts (cattle and rats) as opposed to susceptible hosts (sheep and mice).

FhGSTs are proposed to play three roles. Firstly FhGSTs are involved in detoxifying cytotoxic aldehydes produced during lipid peroxidation (Brophy, Crowley & Barrett 1990). Secondly FhGSTs are involved in the absorptive function of the adult parasite's gut (Creaney *et al.* 1995) and thirdly, FhGST interacts with haematin and this is proposed to prevent blockage of the parasite's gut by haematin crystal formation (Brophy, Crowley & Barrett 1990). Noteworthy is the fact that there is a lack of cellular responses against FhGSTs during *F. hepatica* infections in sheep (Moreau, Chauvin & Boulard 1998) as opposed to infections in rats (Howell, Board & Boray 1988). There are conflicting reports of antibody responses to FhGST in sheep (Sexton *et al.* 1990, Hillyer, Soler de Galanes & Battisti 1992) and not all hosts generate anti-FhGST antibodies during fluke infections (Hillyer, Soler de Galanes & Battisti 1992). This is unexpected, as GST is a major constituent of *F. hepatica* ESP (Jefferies *et al.* 2001, Cervi, Rossi & Masih 1999). However, when sheep are injected with GST as a vaccine, high antibody titres are observed along with a reduction in worm burdens (Sexton *et al.* 1990).

SOD is another AOD enzyme expressed by *F. hepatica* (Sanchez-Moreno *et al.* 1987, Piedrafita *et al.* 2000). Three isoenzymes have been observed (Sanchez-Moreno *et al.* 1987) and in work by Kim *et al.* (2000), two identical subunits for cytosolic CuZn SOD of 17.5 kDa were identified. SOD activity levels have been detected in *F. hepatica* ESP, detergent soluble and somatic fractions of *F. hepatica* with the highest activities observed within *F. hepatica* ESP (Piacenza *et al.* 1998). Within *F. hepatica* ESP, 2-3 bands for CuZn SOD were measured with sizes of 16 and 60 kDa (Piacenza *et al.* 1998, Jefferies *et al.* 2001). In contrast to other parasites like *B. malayi* (Ou *et al.* 1995) and *S. mansoni* (Nare, Smith & Prichard 1990), *F. hepatica* SOD activity decreased in ESP as the parasite develops to adulthood (Piacenza *et al.* 1998) but increased in somatic tissues (Piedrafita *et al.* 2000). The authors proposed that this may relate to the parasite moving from an aerobic to an anaerobic environment and becoming less exposed to toxic oxygen intermediates.

CuZn SOD does invoke a cellular immune response within bovine and human hosts (Kim *et al.* 2000). The main function of SOD is to catalyse the spontaneous dismutation of the superoxide anion radical to H_2O_2 and molecular oxygen (Piacenza *et al.* 1998). However H_2O_2 is still toxic to parasites, hence other AOD enzymes are needed to break this down into less harmful products. Typically catalase and GSH-Px are involved in this second stage. Catalase activity has not been detected in *F. hepatica* (Sanchez-Moreno *et al.* 1987, Piedrafita *et al.* 2000) and only low activity levels of a selenium dependent GSH-Px have been detected in the cytosol of *F. hepatica* (Brophy, Crowley & Barrett 1990). Work by McGonigle *et al.* (1997) identified a cDNA from *F. hepatica* which encoded a protein from the most recently identified anti-oxidant family Peroxiredoxin (Chae *et al.* 1994). Peroxiredoxins remove the toxicity of H_2O_2 and could be the "missing link " for *F. hepatica*. This was the first report of a peroxiredoxin in a trematode. The cDNA was isolated using anti-sera raised against adult *F. hepatica* ESP to screen a cDNA expression library, suggesting that the protein is likely to be released and may be involved in the parasite's defence mechanism.

In contrast there is only one published report examining the presence / activity of AOD enzymes in *F. gigantica*. As discussed in Section 1.5.2, *F. gigantica* releases two products (less than 10 kDa and greater than 50 kDa) which were able to suppress the release of toxic oxygen

intermediates by neutrophils (El Ghaysh *et al.* 1999). This result implies the presence of factors, possible AOD enzymes, in *F. gigantica* effective against reactive oxygen intermediates. Interestingly in our laboratory, *F. gigantica* has been found to be susceptible to free radical killing by chemically generated ROI, ROI from sheep PLCs and NO (Piedrafita *et al.* 2002 - submitted). In contrast *F. hepatica* is not killed by this mechanism. This could imply differences between AOD enzymes between the two species, which could result in different susceptibility to free radical killing.

Overall, the literature points to a variation in AOD enzymatic activities between *F. hepatica* isolates and among flukes removed from different hosts (eg Miller, Howell & Boray 1993). To date, there has been no published reports of direct comparisons of AOD enzyme activity levels between *Fasciola* sp..

1.5.4 Glycocalyx Turnover

The surface of *F. hepatica* is covered by a syncytial epithelium, called the tegument. The tegument contains three types of bodies (T0, T1 and T2) which secrete the glycocalyx (Threadgold 1963, 1967, Bennett & Threadgold 1973, 1975). In contrast, preliminary work examining adult *F. gigantica* suggests the presence of only one type of body secreting the glycocalyx (Sobhon *et al.* 1994). The glycocalyx consists of two layers, a continuous layer, lying next to the apical plasma membrane and another layer of filaments arising from the continuous layer (Threadgold 1976). The glycocalyx is rich in glycoproteins with ganglioside and oligosaccharide side chains (Threadgold 1976).

The glycocalyx, as the interface between the parasite and the host, is likely to be the site of important biochemical and physiological interactions (Threadgold 1976). Possibly due to this, the glycocalyx has been proposed to contribute to immune evasion and three mechanisms have been suggested. Firstly the composition of the glycocalyx changes as the parasite matures (Threadgold 1963, 1967, Bennett & Threadgold 1973, 1975). Juvenile flukes contain only Type 0 (T0) bodies within the tegument secreting T-0 granules (Bennett and Threadgold 1973, 1975). As *F. hepatica* matures, T0 bodies are replaced by T1 and T2 bodies secreting T-1 and T-2 granules respectively (Threadgold 1963, 1967). Such compositional changes are likely to delay

relevant antibody production specific for the ever changing glycocalyx. Secondly the glycocalyx is continuously sloughed off and replaced by molecules released by secretory vesicles (Hanna 1978). By sloughing the glycocalyx off, components within the immune system which attach themselves to the parasite are being shed such as antibody mediated attachment of eosinophils, neutrophils or macrophages (Hanna 1980a, Burden, Hughes & Hanmet 1982, Piedrafita *et al.* 2001) resulting in prevention of successful cellular attacks (Duffus and Franks 1980, Hanna 1980b). Similarly to *S. mansoni*, Hawn and Strand (1993) identified in *F. hepatica* extracts the presence of phospholipase C activity. This phospholipase, like phospholipase D found in S. mansoni, is capable of cleaving glycosylphosphatidyl inositol (GPI) anchored proteins, which provides a potential mechanism for the forementioned shedding.

Aims of Study

The objective of this Project was to identify both evasion strategies utilised by *Fasciola sp.* parasites and / or possible biochemical differences between *F. gigantica* and *F. hepatica*, which could contribute to the variance in host susceptibility to infection with *F. hepatica* and *F. gigantica* observed between these species. We set out to test four hypotheses:

1. that *F. gigantica* parasites are unable to inhibit nitric oxide (NO) production by phagocytes resulting in the high resistance of the rat host to *F. gigantica*;
2. that parasite antioxidant AOD enzymes are important determinants of the resistance of *F. hepatica* to antibody dependent cellular cytotoxicity (ADCC);
3. that *F. hepatica* has an enhanced capacity relative to *F. gigantica* to suppress host cellular proliferation responses *in vivo* and *in vitro* and that this contributes to the infectivity of *F. hepatica*;
4. that the interaction of *Fasciola sp.* ESP with specific host immune effector cell surface markers may regulate/suppress host immune responses.

CHAPTER 2. MATERIALS AND METHODS

2.1 General Techniques

Unless otherwise stated, all experimental work was carried out within the Department of Biochemistry and Molecular Biology, Monash University, Australia. The Monash University Biochemistry and Molecular Biology Animal Ethics Committee under A.E.C. Number Bioc/1998/07 approved all experimental procedures.

2.1.1 15% SDS PAGE Gels

F. hepatica ESP and S75 fractions were qualitatively analysed on 7.5, 12 and 15% SDS PAGE gels. Gels were prepared according to the manufacturer's instructions (BioRad). Briefly, samples were diluted in sample loading buffer and boiled for 4 min and then loaded into the gel lanes. Gels were electrophoresed for approximately 1 hr at a constant voltage (170 V). Three different staining techniques were used:

Coomassie Stain: The gel was briefly rinsed in H_2O and then immersed in 0.1% Coomassie Brilliant Blue R-250 (in methanol:$H_2$0:acetic acid in a 5:5:1 ratio) for 15 min at RT with agitation. Gels were destained in 7.5% acetic acid.

Silver Stain: The method of Morrissey (1981) was used for visualising proteins with a greater sensitivity than standard Coomassie stain. The gel was briefly rinsed in H_2O and then fixed in 10 % ethanol, 7.5 % acetic acid for 15 min followed by 10 % ethanol. The gel was rinsed with 3 changes in H_2O and then soaked in DTT (0.6 μg/ml) for 20 min. The gel was incubated with silver nitrate (0.15 %) for 20 min followed by developing in 3 % sodium carbonate, 0.05 % formaldehyde. Staining was stopped by acidification with solid citric acid.

Silver Periodic Acid Stain: This stain is a highly sensitive technique for staining 1,2-diol groups of carbohydrates (Dubray & Bezard 1982). SDS PAGE gels were rinsed briefly in H_2O and then fixed overnight at RT in 25% v/v iso-propyl alcohol and 10% glacial acetic acid followed by a 30 minute rinse in 7.5% acetic acid at RT. The gel is then soaked for 1 hour at 4°C in 0.2% periodic acid and then rinsed with three 1 hr changes in distilled water. The gel was incubated in

fresh 0.1% $AgNO_3$ for 30 min, rinsed once in water and then immersed in silver stain developer solution (3% w/v Na_2CO_3, 0.05% formalin). Staining was stopped by acidification with solid citric acid.

2.1.2 Western Blot Analysis

Western blot analysis using 15% SDS PAGE gels was used to identify the presence of *F. hepatica* Cathepsin L as previously described (Wijffels *et al.* 1994b). The membrane was washed and incubated for 30 min at RT with Sheep anti *F. hepatica* Cathepsin L antibody diluted to 1/1000 (Wijffels *et al.* 1994b) and developed for 30 min at RT with Rabbit anti Sheep IgG (H+L) AP conjugate (1/1000 dilution)(Silenus).

2.2 Reagents

2.2.1 General Reagents

Polyethylene glycol 20000, L-cysteine.HCL, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E64), sodium pyruvate, concanavalin A, phytohaemagglutinin, silver nitrate, imidazole, z-Phe-Arg-NHMec, β-mercaptoethanol, trypan blue and dithiothreitol (DTT) were purchased from Sigma Chemical Co. (Louis, MO, USA). Dextrose, glycerol and calcium chloride were purchased from BDH Chemicals (Kilsyth, Australia). Yeast extract and peptone were purchased from Oxoid Ltd., (Basingstoke, Hampshire, England). Gentamycin and DMEM were purchased from Life Technologies (Rockville, MD, USA). RPMI-PR, penicillin/streptomycin and foetal calf serum were purchased from Trace Scientific Ltd., (Melbourne, Australia). ELISA plates, 24 well plates and 96 well U bottomed plates were purchased from Greiner Labortechnik (Kremsmuenster, Austria). Ficoll paque, ^{3}H-thymidine, Superdex 75 FPLC column, Histrap column and the Mono S column were all purchased from Amersham Pharmacia Biotech (Sydney, Australia). Nitrocellulose membranes were purchased from Schleicher and Schuell (Germany). Lithium heparin vacutainers were purchased from Becton Dickinson (United Kingdom). Recombinant human CD4 was supplied courtesy of Dr Garry Lynch from the Centre for Virus Research, Westmead Institutes of Health Research, Westmead Hospital Sydney, NSW.

2.2.2 Monoclonal Antibodies

A range of murine monoclonal antibodies (mAb) to sheep leukocyte surface antigens, which had been compared within the International Ruminant Workshops (Larson *et al.* 1990), were used to monitor effects elicited by *F. hepatica* ESP on leukocytes. I have listed the mAbs as: clone (isotype) surface marker. These mAbs were: CC20 (IgG2a) CD1b; MUC2A (IgG2a) CD2; GC1A (IgG2a) CD4; CC17 (IgG1) CD5; CC63 (IgG1) CD8α; ILA99 (IgG1) CD11a; MM12A (IgG1) CD11b; BAQ153A (IgM) CD11c; CC-G33 (IgG1) CD14; CC21 (IgG1) CD21; CACT116A (IgG1) CD25; GS5A (IgG1) CD45R; CC32 (IgG1) L-selectin; H58A (IgG2a) MHC I; H42A (IgG2a) MHC II (DP); TH14B (IgG2a) MHC II (DR); TH81A5 (IgG2a) MHC II (DQ); 86D (IgG1) γδ TCR; CC98 (IgG2b) WC6; CC28 (IgG1) WC10; CC81 (IgG1) ALVC subset (Howard *et al.* 1997); ILA24 (IgG1) MyD-1 (Brooke, Parsons & Howard 1998). Antibodies for CD45Ra, CD58, sIgG, WC1, VLA4 and TcR1-N7 were supplied by Dr P Chaplin.

Three mAbs to human lymphocytes were used: mAb α Human CD4 (OKT4) (WEHI, Melbourne, Australia), PE mAb α Human CD4 (PharMingen, Becton Dickinson) and PE mAb α Human CD3 (PharMingen, Becton Dickinson). One mAb to bovine lymphocytes was used: mAb α Bovine CD4 (Serotec, UK). The non fluorescent labelled antibodies were used with PE α mouse Ig F(ab')2 fragment (Silenus, AMRAD, Australia).

2.3 *Fasciola* Parasites and Products

2.3.1 Collection of Adult *Fasciola* Parasites

Living adult *F. hepatica* parasites were obtained from naturally infected bovine livers at a meat processing plant in Tongala (Victoria, Australia), and washed twice briefly at 37°C in phosphate buffered saline solution pH 7.2 (PBS) to remove blood and bile. Living, intact parasites were immediately incubated at 37°C for about 45 min in RPMI PR^- + 50 U/ml penicillin/streptomycin (Pen/Strep) until all visible blood products and bile had been regurgitated. These "clean" flukes were then snap frozen on dry ice and stored at –80C. Living, adult *F. gigantica* parasites were obtained from bovine livers in Indonesia using the same protocol.

F. hepatica metacercariae were purchased from either Compton Paddock Laboratories (Surrey U.K.) or Baldwin Aquatics (Oregon, U.S.A.). *F. gigantica* metacercariae were obtained from

Balitvet Research Institute for Veterinary Science (Bogor, Indonesia) or purchased from the Indian Veterinary Research Institute (Izatnagar, India).

2.3.2 Excystment of Metacercariae

The excystment protocol for *F. hepatica* and *F. gigantica* metacercariae was based on that of Smith and Clegg (1981) as modified by Wilson *et al.* (1998) and Piedrafita *et al.* (2000). The required number of viable cysts were removed either from cellophane sheets or from H_2O, transferred to a 15 ml tube and held in 10 mls of H_2O for 1hr at 37°C. The subsequent treatments required to activate the metacercariae to excyst were incubation at 37°C, a low oxidation-reduction (redox) potential, carbon dioxide (CO_2) and taurocholic acid (Appendix 1). At the end of the incubation period, NEJ, empty cysts and unexcysted metacercariae were washed twice with phenol red-free RPMI 1640 media (RPMI-PR$^-$) at 37C to remove the taurocholic acid and placed in an "exystment tower" (height 2 cm, internal diameter 1 cm) within a well of a 24 well tissue culture plate. The excystment tower was a modification of that of Tielens *et al.* (1981) and consisted of two halves of an inverted small tube (Nunc cryotubes, USA) separated by small-mesh plastic netting (100 µm holes)(Swiss Screens, Australia). The excysted parasites actively passed through the mesh and settled at the bottom of the well. The mesh netting allowed separation of the empty cyst walls and the unexcysted metacercariae from NEJ. NEJ used in experiments were recovered at RT and washed 3-4 times with RPMI-PR$^-$.

2.3.3 Whole Worm Extracts

Adult parasites were homogenised in 0.1% Triton X in 90 mM HEPES + 5 mM EDTA (0.05g parasite / 500 µl of buffer) using an Ultra Turrax at 4°C. Homogenates were rocked on ice at 4°C for 1 hr and then the supernatant was collected after centrifuging at 13000 rpm for 20 seconds. Protein concentration for each preparation was determined using BioRad D_C Protein Assay Kit according to manufacturer's instructions. The supernatant was stored in small aliquots at –80°C until required.

NEJ *Fasciola* parasites were suspended in 0.1% Triton X in 90 mM HEPES + 5 mM EDTA (5000 NEJ / 50 µl of buffer). Homogenates were prepared using a conical hand held homogeniser which fitted tightly into an eppendorf tube. Complete homogenisation was

determined by viewing the lysate under a microscope and observing only a few intact parasites out of the initial 5000. Homogenates were spun at 13000 rpm for 20 seconds, the supernatant was collected and the protein concentration was determined as described above. These homogenates were used immediately.

2.3.4 ESP Collection

Living, intact adult *F. hepatica* parasites were treated and "cleaned" as described above. "Clean" parasites were then incubated in fresh RPMI PR^- + 50 U/ml Pen/Strep (50 worms/100 mls) for approximately 4 hr at 37°C. The regurgitant was immediately frozen on dry ice and stored at –80°C until use. The ESP was placed in a dialysis bag (cutoff of 1000 Da) concentrated 10- fold using polyethylene glycol 20000 and then dialysed overnight at 4°C against 3 L of PBS. The protein concentration was estimated using the Bio-Rad D_C Protein Assay Kit as described above. This ESP preparation was used in all assays. ESP from adult *F. gigantica* parasites was collected using the same protocol in Indonesia.

2.3.5 Treatment of ESP

Periodate cleavage

Periodate is a glycol cleavage reagent that reacts with vicinal hydroxyl groups to cleave the carbon to carbon bond between them (Kennedy 1988). 10mM periodate was incubated with 100 µg/ml of *Fasciola* ESP for 2 hr at RT in the dark. Periodate cleavage of ESP was stopped by dialysing the reaction against PBS overnight at 4°C. Samples were stored at –20°C (Yamashita *et al.* 1993, Cervi *et al.* 1996).

Proteinase K degradation

Proteinase K is an endopeptidase from the serine protease family, which predominantly cleaves peptide bonds after the carboxyl group of N-substituted hydrophobic aliphatic and aromatic amino acids and it also cleaves peptide amides (Corbalan-Garcia *et al.* 1994). 60 µg/ml proteinase K was incubated with 1 mg/ml of *Fasciola* ESP at RT for 30 min. ESP degradation by proteinase K was stopped by a 10 min incubation at RT with 1 mM PMSF (Protease Inhibitors: Properties and Applications). Samples were stored at –20°C.

2.3.6 Fractionation of ESP

Superose 12 Column

ESP molecules were fractionated by chromatography on a FPLC column of Superose 12 (S12) HR 10/30. S12 is 12% highly cross-linked agarose, which yields the greatest separation for proteins in the range of 1-300 kDa. 1 ml samples were loaded and 1 ml fractions were eluted using sterile PBS (filtered through a 0.22 μm filter) at a flow rate of 0.5 ml/min at room temperature. Collected proteins were estimated to have been diluted approximately 3 fold during fractionation.

Superose 6 Column

ESP molecules were fractionated by chromatography on a FPLC column of Superose 6 (S6) HR 10/30. S6 is 6% highly cross-linked agarose, which yields the greatest separation for proteins in the range of 5-5000 kDa. 1 ml samples were loaded and 1 ml fractions were eluted using sterile PBS (filtered through a 0.22 μm filter) at a flow rate of 0.5 ml/min at room temperature. Collected proteins were estimated to have been diluted approximately 3 fold during fractionation.

Superdex 75 Column

ESP molecules were fractionated by chromatography on a FPLC column of Superdex 75 (S75) HR 10/30. S75 is a composite of cross-linked agarose and dextran and yields the greatest separation for components in the range of 3 – 70 kDa. 1 ml samples were loaded and 1 ml fractions were eluted using sterile PBS (filtered through a 0.22 μm filter) at a flow rate of 0.5 ml/min at room temperature. Collected proteins were estimated to have been diluted approximately 3 fold during fractionation.

2.3.7 Recombinant Cathepsin L Proteases

Two recombinant *F. hepatica* Cathepsin L (rFhCatL) proteases were used: rFhCatL5 and a mutant of rFhCatL5 termed rFhCatL69Y in which the leucine at position 69 was mutated to tyrosine. Their expression and purification have been previously described (Smooker *et al.* 2001). Briefly, glycerol stocks of transfected yeast (expressing rFhCatL) were grown in minimal media for 48 hr at 28°C. This minimal culture was then added to YPHSM media (1 % Dextrose,

3 % Glycerol, 20 mM $CaCl_2$, 1 % Yeast Extract, 8 % Peptone) for a further incubation at 28°C for 72 hr. The supernatant was collected after centrifugation of the culture medium (2000 rpm for 10 min) and then dialysed for 16 hr at 4°C against 4 L of HisTrap starting buffer pH 7.6 (10 mM Imidazole, 20 mM NaH_2PO_4, 0.5 M NaCl) with 2 changes. The supernatant was loaded onto a HisTrap column at 2 ml/min at 4°C and bound protein was eluted using increasing 10 ml step gradients of Imidazole (100 mM, 250 mM) in HisTrap starting buffer. The eluate was collected from each step, fractions containing eluted protein were pooled and analysed for protein integrity (using SDS PAGE gels). The final yield of rFhCatL was approximately 4 mg per litre of culture. This rFhCatL preparation was dialysed for 16 hr at 4°C against 1 L of ion exchange starting buffer pH 5.0 (25 mM CH_3COONa, 1 mM EDTA, Buffer A). rFhCatL was activated using 10 mM L-cysteine.HCl for 2 hr at 37°C and then loaded onto a FPLC column of Mono S HR 5/5, equilibrated in Buffer A, at 1 ml/min and eluted using a gradient over 15 mls from 0–0.5 M NaCl in Buffer A. Fractions containing rFhCatL were pooled following identification by analysing on SDS PAGE gels. The proteolytic activity of the rFhCatL was determined by titration with the inhibitor E64 using the dipeptide z-Phe-Arg-NHMec as the substrate at 37°C using the method from Barrett and Kirschke (1981). Approximately 10% of the purified recombinant protease preparations were enzymatically active.

2.4 Collection / Purification of Immune Cells

2.4.1 Collection of Rat Peritoneal Lavage Cells (PLCs)

Monocyte/macrophage rich cell populations were obtained by peritoneal cavity lavage of naïve Wistar Rats. Resident lavage cells from *F. hepatica* and *F. gigantica*-naïve rats, were collected by simple lavage using sterile PBS containing 6 mM EDTA. The linea alba was incised and 50 mls of PBS poured directly into the peritoneal cavity followed by gentle massage for 1 min and recovery of the peritoneal wash fluid using 20 ml syringes. PLCs were obtained by centrifuging the PLC wash fluid at 1500 r.p.m. at 25°C for 6 minutes. If there was red blood cell contamination then the pellet was resuspended in red cell lysis buffer (0.17 M Tris Ammonium Chloride pH 7.2) for 10 min at 37°C. This treated cell pellet or uncontaminated cell preparations were then washed three times with PBS, and the resulting pellet was resuspended in 5-10 mls of sterile RPMI-PR$^-$ at 4°C containing 10 % heat inactivated foetal calf serum, 2 μg/ml amphotericin B and 10 μg/ml gentamycin.

2.4.2 Isolation of Human, Ovine and Bovine Peripheral Blood Mononuclear Cells (PBMCs)

Blood was collected from either naïve humans, mature Merino ewes or Fresian cross cattle into 10 ml sterile lithium heparin vacutainers and centrifuged for 20 min at 1700 rpm at RT. The buffy coat was removed manually using a pasteur pipette, diluted 1:5 in PBS, layered onto 10 mls of 100% Ficoll Paque, then centrifuged for 30 min at 2400 rpm at RT. The cells at the interface (PBMCs) were removed and washed 3 times in PBS by 6 min centrifuge spins at 1300 rpm at RT. PBMCs were counted and resuspended at the desired concentration in RPMI complete media.

2.4.3 Isolation of Sheep Afferent and Efferent Lymphocytes

Sheep afferent lymph was obtained by cannulation of the re-anastomosed afferent ducts following removal of the prefemoral lymph node (Emery, Mac Hugh & Ellis 1987). Efferent lymph was obtained from the prescapular lymph node of a mature Merino ewe which had been previously cannulated (Hall & Morris 1962). Lymph was collected into sterile plastic bottles containing 5000 IU heparin (DBL, Australia). Lymph was pelleted at 1700 rpm for 7 min at RT and then washed once in PBS at RT. Cells were resuspended in Dulbecco's Modified Eagle Media (containing 10 % FCS, 50 μg/ml gentamycin, 50 U/ml pen/strep, 50 μM β-mercaptoethanol and 20 μM sodium pyruvate) and cell counts were performed.

2.5 Infecting Merino Sheep with *F. gigantica*

Six random bred Merino ewes (Group A) and six *Haemonchus contortus* resistant ewes (Group B – descendents from the Golden Ram) were orally infected with one dose of 250 *F. gigantica* metacercariae. Five random bred Merino ewes composed the uninfected control group. A week prior to *F. gigantica* infection, sheep were treated with Closantel to kill any existing *H. contortus* infections. Sheep were not included in the experiment if they tested positive for any specific antibodies for *Fasciola* antigens. Sheep were killed 10 weeks post infection.

2.6 Whole Blood Assays

Blood was collected from naïve sheep into 10 ml sterile lithium heparin vacutainers. This blood was diluted 1:10 into RPMI Complete Media (10 % foetal calf serum (FCS), 50 μg/ml

gentamycin, 50 U/ml pen/strep, 50 μM 2-mercaptoethanol and 20 μM sodium pyruvate). 200 μl was added to each well of a 96 well ELISA plate containing either: 10 μg/ml Concanavalin A (Con A); Con A + adult *F. hepatica* ESP; Con A + S75 *F. hepatica* ESP fractions and Con A + rFhCatL. These incubations were performed in quadruplicate and repeated in the presence of E64 (10 μM final concentration). The cultures were incubated for 72 hr at 37°C in an atmosphere containing 5 % CO_2. 24 hr prior to harvesting, ^{3}H-thymidine (0.2 μCi/well) was added. At completion of the incubation, the plates were frozen at –20°C. Cells were harvested onto multiscreen filtration system 96 well microplates (Millipore) which were analysed for ^{3}H-thymidine activity using a Packard TopCount Microplate Scintillation Counter. The viability of the cells after the 72 hr incubation was assessed by the exclusion of trypan blue on a control plate. Statistics were performed on all data using the program INSTAT with the specific statistical measurement detailed in the figure/table legends.

Sheep PBMC proliferation assays were performed using the above protocol for whole blood proliferation assays at a final concentration of 2 x 10^5 cells/200 μl/well.

2.7 FACS

FACS analysis was used to identify the effect of *F. hepatica* and *F. gigantica* ESP or rFhCatL on a broad range of surface markers on both sheep PBMCs and efferent lymphocytes. 5 x 10^5 cells were plated into each well (100 μL) of a 96 well U bottomed plate. Plates were centrifuged at 1200 rpm for 3 min at RT, the supernatant was decanted and cells were resuspended in medium alone or medium containing *F. hepatica* or *F. gigantica* ESP (50-500 μg/ml) or rFhCatL (50-300 μg/ml), with and without E64 (10 μM). Since active site titration assays showed that only 10% of these recombinant protease preparations were active, the concentration of active recombinant protease was 20-30 μg/ml. These cultures were incubated for 5 hr at 37°C in an atmosphere containing 5 % CO_2. Cells were washed 3 times in PBS at RT by centrifuging of the plates at 1300 rpm, then incubated with the appropriate mouse anti-sheep surface marker antibody (1/200 – 1/1000 dilution, supplied by CSIRO – Division of Animal Health) for 15 min at RT. Controls were cells with medium alone and cells incubated with mouse anti-sheep Ig isotype antibody alone. Non-specific binding of these mouse antibodies (mouse isotypes IgG1, IgG2a, IgG2b,

IgM) to T cells was not observed (data not shown). Cells were washed 2 times in PBS at RT and then incubated with goat anti-mouse Ig antibody with a FITC tag (1/200 dilution, Silenus) at room temperature for 15 min. Cells were washed 2 times in PBS at RT, resuspended in 100 µl PBS and analysed using a Becton Dickinson FACSsort. Results were based on 10,000 – 100,000 events. In one experiment, this method was modified as follows: Cells were plated out as above and then fixed in 1 % paraformaldehyde for 15 min at RT. The cells were washed 3 times in PBS at RT and the assay performed as usual using incubations with either media or *F. hepatica* ESP, with and without E64 (10 µM). In another experiment, cells were plated out and incubated with either media or *F. hepatica* ESP for 5 hr and then washed as described above. These cells were then incubated for a further 18 hr at 37°C in media alone, washed 2 times in PBS and then stained as described above. FACS analysis was performed on human PBMCs using the same method described above, using antibodies to human CD4 and CD3. Statistics (using statistical measurements within Cell Quest from Becton Dickinson) were performed on all cell populations. The data was presented in either histogram format or bar graphs which displayed the mean fluorescent intensity (X geometric mean) of the CD4+ T cell populations.

2.8 *In vitro* Cleavage of Recombinant Human CD4

Individual incubations of 4 µg of rHuCD4 were digested with 20 ng of rFhCatL5 or rFhCatL69Y, incubated for 0, 10, 20, 30 60 and 90 min at 37°C. These reactions were stopped by the addition of reducing sample buffer and immediately snap frozen. The individual samples were then analysed on silver stained 15% SDS PAGE gel.

2.9 *In vitro* Nitric Oxide Assays

The levels of nitrite in culture supernatants of lavage cells were determined at the end of an incubation period and used as an indicator or nitric oxide production by lavage cells (Hibbs *et al.* 1988; Ignarro *et al.* 1993). Incubations were carried out at 37°C in 96 well, flat bottom ELISA plates, in a humidified incubator in the presence of 95% air and 5% CO_2. 2 x 10^5 viable rat PLCs were cultured for 3 days in 200 µl RPMI PR- containing 10% heat-inactivated foetal calf serum, 2 µg/ml amphotericin B, 10 µg/ml gentamycin, with or without 0.5 µg/ml LPS, *F. hepatica* ESP, *F. gigantica* ESP, or combinations of these stimulants. The ESP was either treated, unfractionated or fractions collected following gel filtration chromatography as described above.

In addition 0.5 mM monomethyl L-arginine (L-NMMA, Sigma, USA) was included in some incubations as a competitive inhibitor of nitric oxide synthase (Hibbs et. al. 1988).

Nitrite (NO_2^-) concentration in the culture media was assayed by a standard Greiss reaction (Green *et al.* 1982). At the end of the incubation period, 100 µl of media was added to 50 µl of 1% sulphanilamide (Sigma, USA) in 2.5% H_3PO_4 pH 1.0 (0.85%, BDH Chemicals, England) and 50 µl of 1% naphthylenediamine dihydrochloride (Sigma, USA). After 15 min, the concentration was determined with reference to a standard curve generated using concentration from 10 µM to 280 µM sodium nitrite (Sigma, USA) in culture media. Under these conditions, the detection limit was 1 µM.

2.10 Anti-Oxidant Defence Enzyme Assays

Glutathione peroxidase

The glutathione peroxidase (GSH-Px) assay was based on the method of Hopkins and Tudhope (1973). Glutathione peroxidase catalyses the reduction of organic peroxides (ROOH) and hydrogen peroxide by glutathione (GSH). These assays were based on GSH-Px catalysing the reduction of hydrogen peroxide by glutathione. The oxidised glutathione (GSSG) produced was reduced back to glutathione by glutathione reductase, measured spectrophotometrically as a decrease in absorbance at 340nm due to the accompanied oxidation of NADPH to $NADP^+$.

$$H_2O_2 + 2GSH \xrightarrow{GSH\text{-}Px} 2H_2O + GSSG + NADPH \xrightarrow{GR} GSH + NADP^+$$

650 µl of 90 mM HEPES pH 7.4, + 5 mM EDTA was used as the blank. To this buffer, in this order, glutathione reductase (5µl), NADPH (25µl), NaN_3 (5µl), GSH (25µl) and H_2O (265µl) were added. Measurements were commenced and after 2 minutes of equilibration, 25µl of H_2O_2 was added and the solution was allowed a further minute to equilibrate. Parasite homogenate was added to render a final concentration of approximately 50 µg/ml. The specific activity was expressed as nmoles of GSH oxidised per mg of protein.

Glutathione S-Transferase

The glutathione *S*-transferase (GST) assay was based on that of Habig, Pabst and Jackoby (1974). This assay monitors the oxidation of reduced glutathione as observed by an increase in absorbance at 340nm.

The spectrophotometer was blanked after the addition of 25 µl of CDNB (8.1 mg/ml) to 970 µl of 1mM GSH in 0.1M Phosphate buffer (pH 6.5). Measurements were commenced and after the stabilisation of a baseline, parasite homogenate was added to yield a final concentration of approximately 0.05 mg/ml. The specific activity of glutathione S-transferase was defined as the amount (nmoles) of CDNB conjugated per minute per mg of protein.

Catalase

The catalase assay was based on the method of Ganschow and Schimke (1969) and measured the dissociation of hydrogen peroxide (H_2O_2) into water and oxygen as a decrease in absorbance at a wavelength of 240 nm. 1 unit of catalase activity was defined as the amount of enzyme necessary to decompose 50% of 20mM H_2O_2 in 1 min.

Catalase

$$2H_2O_2 \rightarrow\rightarrow\rightarrow\rightarrow\rightarrow 2H_2O + O_2$$

900 µl of 0.1% Triton X 100 in 2.5mM Phosphate buffer pH 7.0 was added to a cuvette and this was used as the blank. Reading commenced after the addition of 100 µl of H_2O_2. Once the baseline had stabilised sample was added to give a final concentration of approximately 0.05mg/ml. Bovine liver catalase was used as a positive control in all experiments. One unit of catalase activity is defined as that amount of enzyme required to decompose 50% of 20 mM hydrogen peroxide in 1 minute at 25C. The specific activity of catalase was expressed as units of catalase per mg of protein.

SOD

The superoxide dismutase (SOD) assay was based on the cytochrome c reduction method of Flohe and Otting (1984) using bovine erythrocyte SOD as a standard. Cytochrome c is reduced

by the superoxide radical generated by the xanthine-xanthine oxidase enzyme system. The reduction of cytochrome c causes an increase in absorbance at a wavelength of 550 nm. In the presence of SOD, cytochrome c reduction is inhibited and, thus, no increase in absorbance at 550 nm is observed.

Xanthine Oxidase

Xanthine →→→→→→→→→ O_2^-

O_2^-

Cytochrome c →→→→ reduced cytochrome c

Solution A consisted of 50mM Phosphate buffer + 0.1mM EDTA (pH 7.8) (655µl), H_20 (45µl), xanthine (250µl), cytochrome c (25µl). Solution B was xanthine oxidase from cow's milk. The spectrophotometer was blanked at 550 nm against Solution A. Maximum reduction of cytochrome c was determined by adding 20 µl of Solution B to Solution A and observing the incline in absorbance. The presence and activity of SOD was determined by adding the appropriate amount of parasite homogenate (final concentration approximately 0.05mg/ml) or a commercial source of SOD (bovine erythrocyte) being added to Solution A, followed by the addition of 20 µl of Solution B and recording the incline in absorbance. One unit of SOD activity is defined as the amount of enzyme necessary to inhibit the rate of reduction of cytochrome c by 50%.

CHAPTER 3: SUPPRESSION OF SHEEP CELLULAR PROLIFERATION

3.1 Introduction

Fasciola hepatica, the temperate liver fluke, is capable of attenuating the mammalian immune system (Mulcahy, Joyce & Dalton 1999). In sheep, *F. hepatica* infections exert immunosuppressive effects at specific times post infection. Significant suppression, as measured by the lymphocyte proliferative response to different mitogens during parasitic infection, was observed 4 and 10 weeks after infection of sheep (Zimmerman *et al.* 1983, Chauvin, Bouvet & Boulard 1995). Selective suppression of the host's immune response may be to the parasite's advantage since these times correspond to phases of rapid parasite growth within the liver parenchyma and establishment within the bile ducts (Zimmerman *et al.* 1983).

Immunomodulatory effects of excretory / secretory products (ESP) released by the adult parasite have been observed *in vitro*. These ESP- related activities include dose-dependent inhibition of mitogen-stimulated proliferation of lymphocytes from naïve sheep (Jefferies, Barrett & Turner 1996); induction of mononuclear spleen cell populations which, upon adoptive transfer, suppress delayed type hypersensitivity responses to parasite antigen (Cervi, Rubinstein & Masih 1996); inhibition of the superoxide burst by phorbol myristate acetate-stimulated sheep and human neutrophils (Jefferies, Turner & Barrett 1997); and inhibition of nitric oxide production by LPS-stimulated rat peritoneal cells in the first two weeks following infection (Cervi *et al.* 1998). However, the active components within *F. hepatica* ESP responsible for this immunosuppression have not yet been elucidated.

A major component of *F. hepatica* ESP is a group of cathepsin L cysteine proteases which are 27-30 kDa in size (Smith *et al.* 1993b, Wijffels *et al.* 1994a, Dowd *et al.* 1994). These proteases are expressed in the intermediate to late phase of parasite development and at least 7 different cathepsin L isoenzymes have been identified by 2D gel electrophoresis and by cDNA cloning (Wijffels *et al.* 1994a, Tort *et al.* 1999, Smooker *et al.* 2000). *F. hepatica* cathepsin L has already been shown to initiate a range of effects including: prevention of antibody-mediated attachment of eosinophils to juvenile flukes *in vitro* (Carmona *et al.* 1993); the formation of blood clots

induced through clotting of fibrinogen (Dowd, McGonigle & Dalton 1995) and cleavage of the Fc portion of immunoglobulin (Berasain *et al.* 2000). This was reviewed in Chapter 1.5.

The first section of this study investigated the effect of *F. gigantica* infection on sheep lymphocyte proliferation, in order to determine whether the immunosuppresive effect on whole blood proliferation was not limited to *F. hepatica* infection alone but a characteristic of infections with *Fasciola sp.* in the ovine host. The second section of this study attempted to identify the molecule(s) within ESP responsible for the *in vitro* suppression of ovine whole blood cellular proliferation.

3.2 Results

3.2.1 The Effect of a *F. gigantica* Infection in Sheep on Subsequent Lymphocyte Proliferation *ex vivo* in Response to Mitogen Stimulation

A primary infection experiment with *F. gigantica* was conducted at University of Sydney (Camden, New South Wales) by Professer H. Raadsma. Permission was obtained from AQIS to perform the trial for up to 10 weeks post infection (i.e.. before eggs are released into faeces) to prevent possible contamination of the environment with *F. gigantica* eggs. We investigated the resistance of two Merino Sheep groups to *F. gigantica*. Group A were unselected outbred Merino sheep and Group B were descendents from the "Golden Ram" which have a genetic predisposition to *Haemonchus* resistance (Woolaston, Barger & Piper 1990).

Whole blood from both Merino sheep groups were stimulated with the mitogens Concanavalin A (Con A) and Phytohemagglutinin (PHA) to investigate the effect on cellular proliferation *ex vivo*, at weekly intervals from 3 to 10 WPI with *F. gigantica*. Data for week 0-2 are unfortunately not available because of a technical failure during transport of blood from University of Sydney to Monash University (Clayton, Victoria). Significant suppression of cellular proliferation *ex vivo* between mononuclear cells from *F. gigantica* infected Group B Merino and *F. gigantica*-naïve Merino sheep were observed at 3 and 10 WPI and 3,4,5 and 9 WPI when cells were stimulated with Con A and PHA, respectively (Fig 3.1, 3.2). There were no significant differences in cellular proliferation observed between cells from *F. gigantica* infected Group A Merino and *F. gigantica*-naïve Merino sheep (Fig 3.3, 3.4). The two groups did show similar proliferation profiles suggesting an overall trend for suppression around 3&4 and 9&10 WPI. Although only one experiment was performed, due to the constraints imposed by AQIS and the cost of meeting quarantine requirements, these results suggest that a *F. gigantica* infection can suppress lymphocyte proliferation in sheep.

3.2.2 Identification of *Fasciola* ESP Components that Suppress Lymphocyte Proliferation *in vitro*

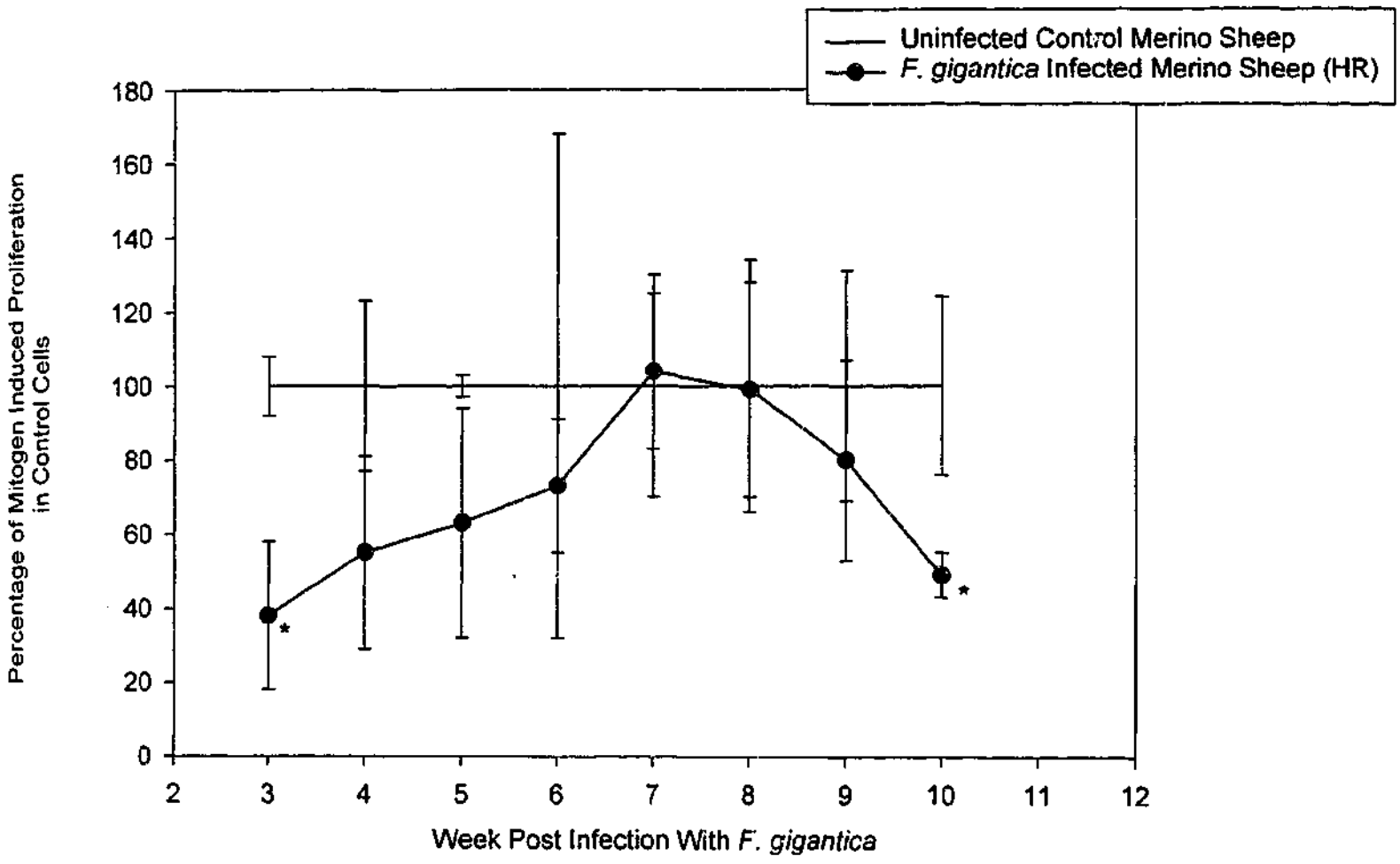


Fig 3.1 Whole blood proliferation during an *F. gigantica* infection in Group B Merino sheep stimulated with Concanavalin A. The results are expressed as a percentage of ^{3}H-thymidine uptake for the control uninfected sheep cells with Con A collected at the corresponding week. Blood from 6 *F. gigantica* infected sheep and from 4 uninfected controls was assayed in quadruplicate and the mean and standard deviation values are shown. Significant differences ($P<0.05$) were calculated using the parametric Bonferroni test and are shown by *.

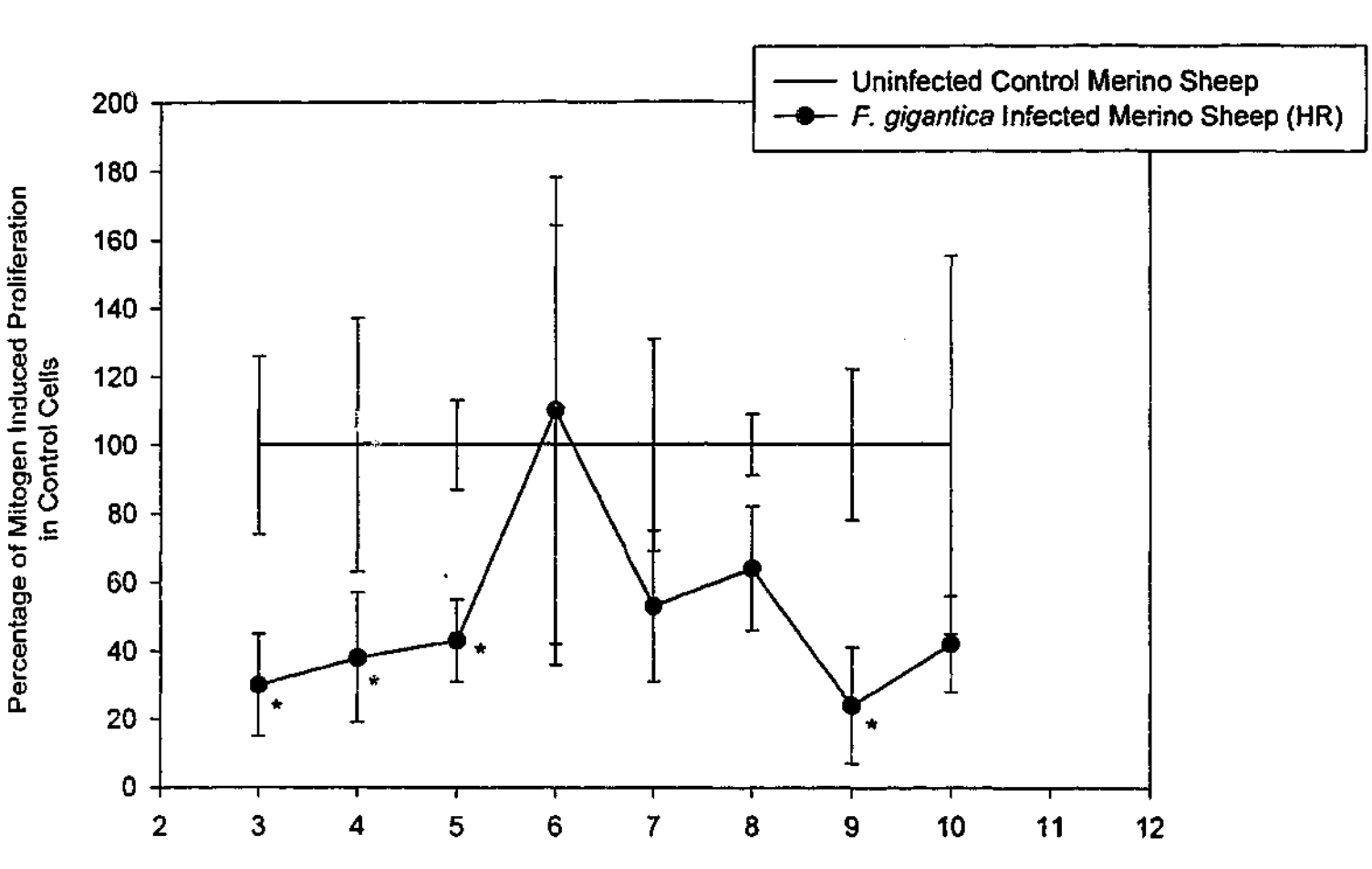


Fig 3.2 Whole blood proliferation during an *F. gigantica* infection in Group B Merino sheep stimulated with PHA. The results are expressed as a percentage of ^{3}H-thymidine uptake for the control uninfected sheep cells with PHA collected at the corresponding week. Blood from 6 *F. gigantica* infected sheep and from 4 uninfected controls was assayed in quadruplicate and the mean and standard deviation values are shown. Significant differences ($P<0.05$) were calculated using the Bonferroni parametric test and are shown by *.

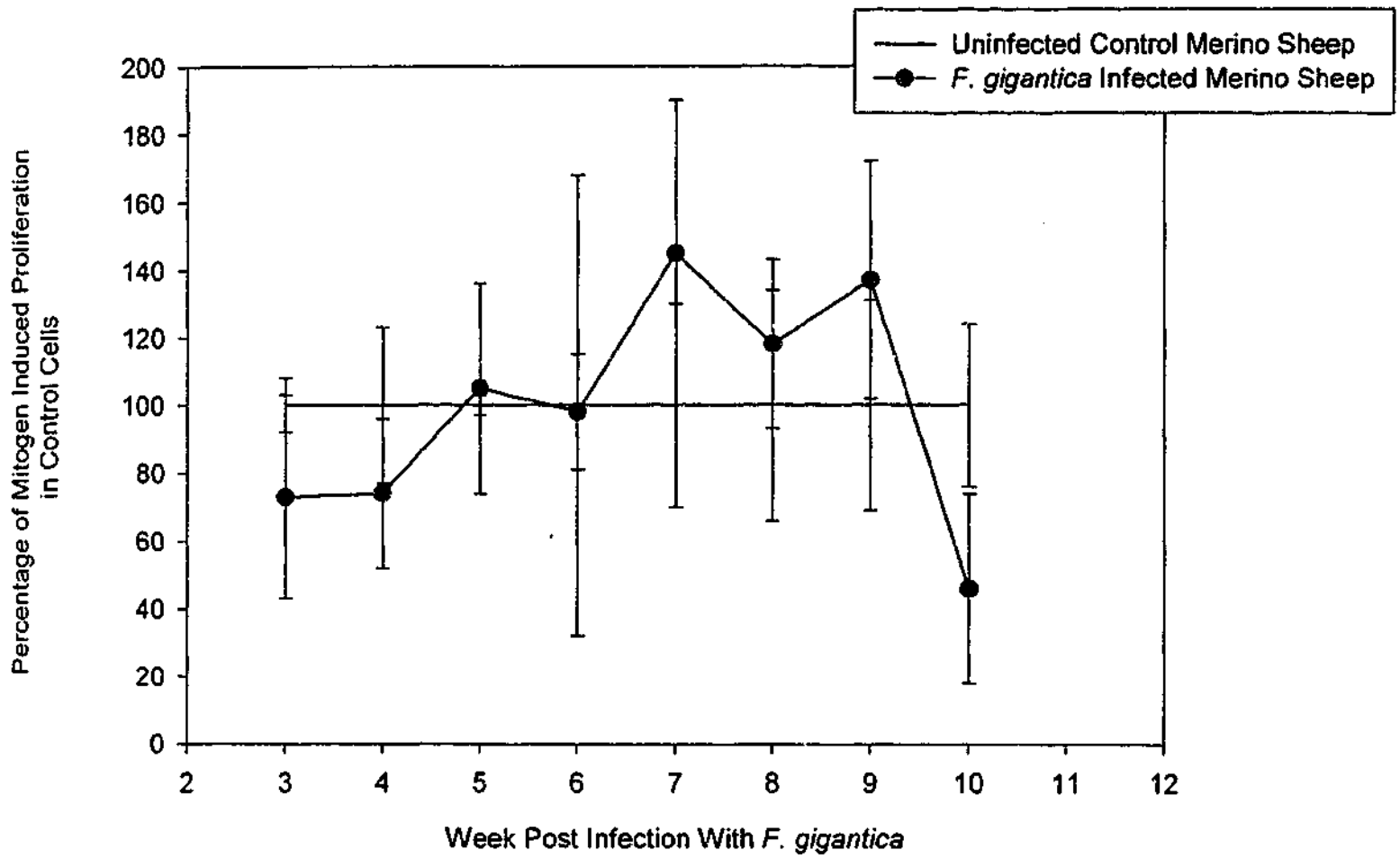


Fig 3.3 Whole blood proliferation during an *F. gigantica* infection in Group A Merino sheep stimulated with Concanavalin A. The results are expressed as a percentage of ^{3}H-thymidine uptake for the control uninfected sheep cells with Con A collected at the corresponding week. Blood from 6 *F. gigantica* infected sheep and from 4 uninfected controls was assayed in quadruplicate and the mean and standard deviation values are shown. There were no significant differences ($P<0.05$) calucated using the parametric Bonferroni test.

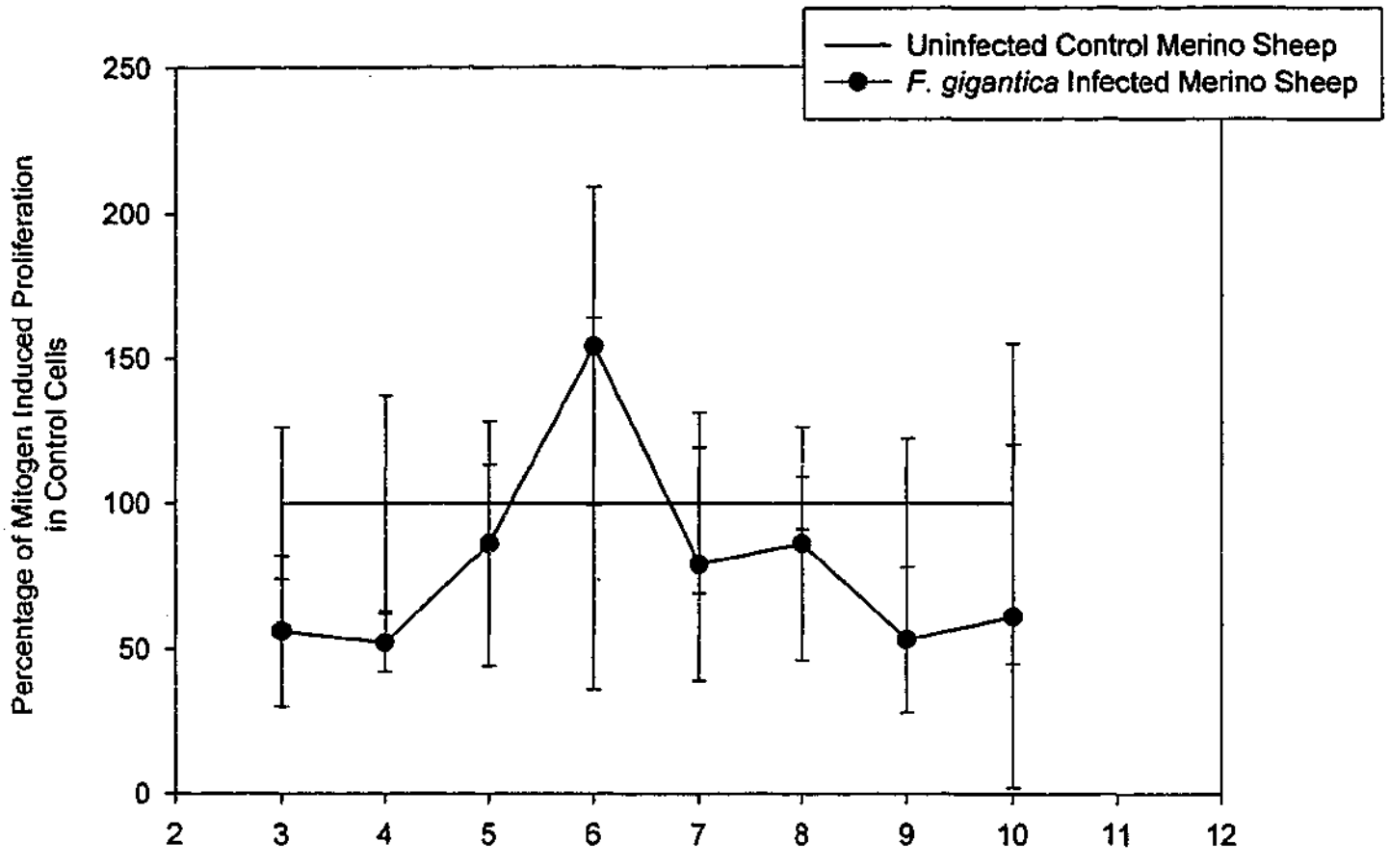


Fig 3.4 Whole blood proliferation during an *F. gigantica* infection in Group A Merino sheep stimulated with PHA. The results are expressed as a percentage of ^{3}H-thymidine uptake for the control uninfected sheep cells with PHA collected at the corresponding week. Blood from 6 *F. gigantica* infected sheep and from 4 uninfected controls was assayed in quadruplicate and the mean and standard deviation values are shown. There were no significant differences ($P<0.05$) calculated using the parametric Bonferroni test.

We next investigated whether *Fasciola sp.* ESP was associated with the suppression of mitogen-induced whole blood cellular proliferation *in vitro*, using blood from *Fasciola* naïve outbred Merino sheep. Significant levels of suppression of Con A or PHA-stimulated cellular proliferation were observed when mononuclear cells were incubated with 15 µg/ml and 50 µg/ml of either *F. hepatica* or *F. gigantica* ESP, when compared to control cells incubated with Con A or PHA alone (Fig 3.5, 3.6). Lymphocyte viability was not affected at these concentrations of *Fasciola* ESP as determined by trypan blue exclusion (data not shown). The exact mechanisms of action for Con A and PHA on T cells are unknown. It is thought that these mitogens will both act through indirectly cross linking the T cell receptor (TCR) surface molecule. This is due to the observation that both mitogens are ineffective on cells which do not express the TCR (Kruisbeek & Shevach 1999). Similar effects on cellular proliferation were observed with both mitogens and, therefore, subsequent experiments in this section used only Con A.

In order to identify the suppressive components in *Fasciola sp.* ESP, ESP was fractionated by molecular sieving on a Superose 6 (S6) column and the individual fractions were analysed for their effect on Con A-induced cellular proliferation from control *Fasciola*-naïve sheep whole blood. Significant suppression was observed for both *F. hepatica* and *F. gigantica* ESP fractions 17-25 and 21-22 respectively (Fig 3.7, 3.8). These fractions were isolated using an S6 column which predominantly separates components in the 5 - 5000 kDa range and corresponded to proteins in the <70 kDa range. To further characterise the suppressive constituents we then proceeded to run Superdex 75 (S75) columns as these give optimal separation for components in the 3 – 70 kDa range.

F. hepatica and *F. gigantica* ESP were each fractionated on a S75 column and the fractions collected were subsequently analysed for their effect on Con A-induced cellular proliferation *in vitro*, using blood from *Fasciola*-naïve outbred Merino sheep. Significant suppression of proliferation was observed with fractions 6-14 and 7-13&15-17 collected from the S75 column using *F. hepatica* and *F. gigantica* ESP, respectively (Fig 3.9, 3.10). This level of suppression was comparable to or greater than that seen with unfractionated *F. hepatica* or *F. gigantica* ESP (50 µg/ml) respectively (Fig 3.9, 3.10).

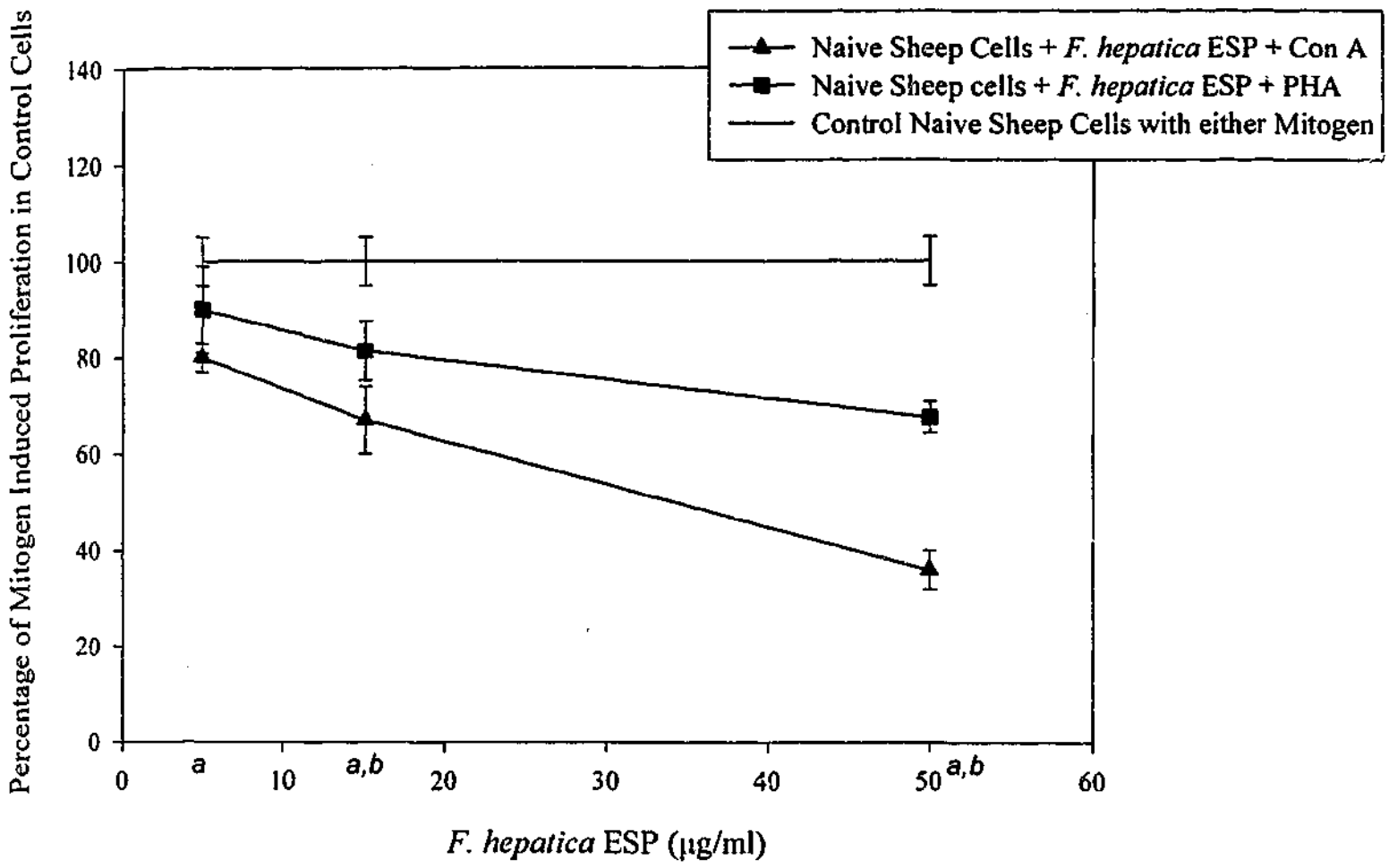


Fig 3.5 Whole blood proliferation assay for *Fasciola*-naive sheep cells stimulated with different mitogens at increasing concentrations of *F. hepatica* ESP. The results are expressed as a percentage of ^{3}H-thymidine uptake for the control naive sheep cells with either mitogen but with no *F. hepatica* ESP present. Values represent the means ± SD of quadruplicate wells. Significant differences ($P<0.05$), compared to control cells, were calculated using the Dunnett Multiple Comparison test and are shown by an *a* or *b* for the Con A and PHA stimulated cells respectively. Similar results were obtained in two different experiments.

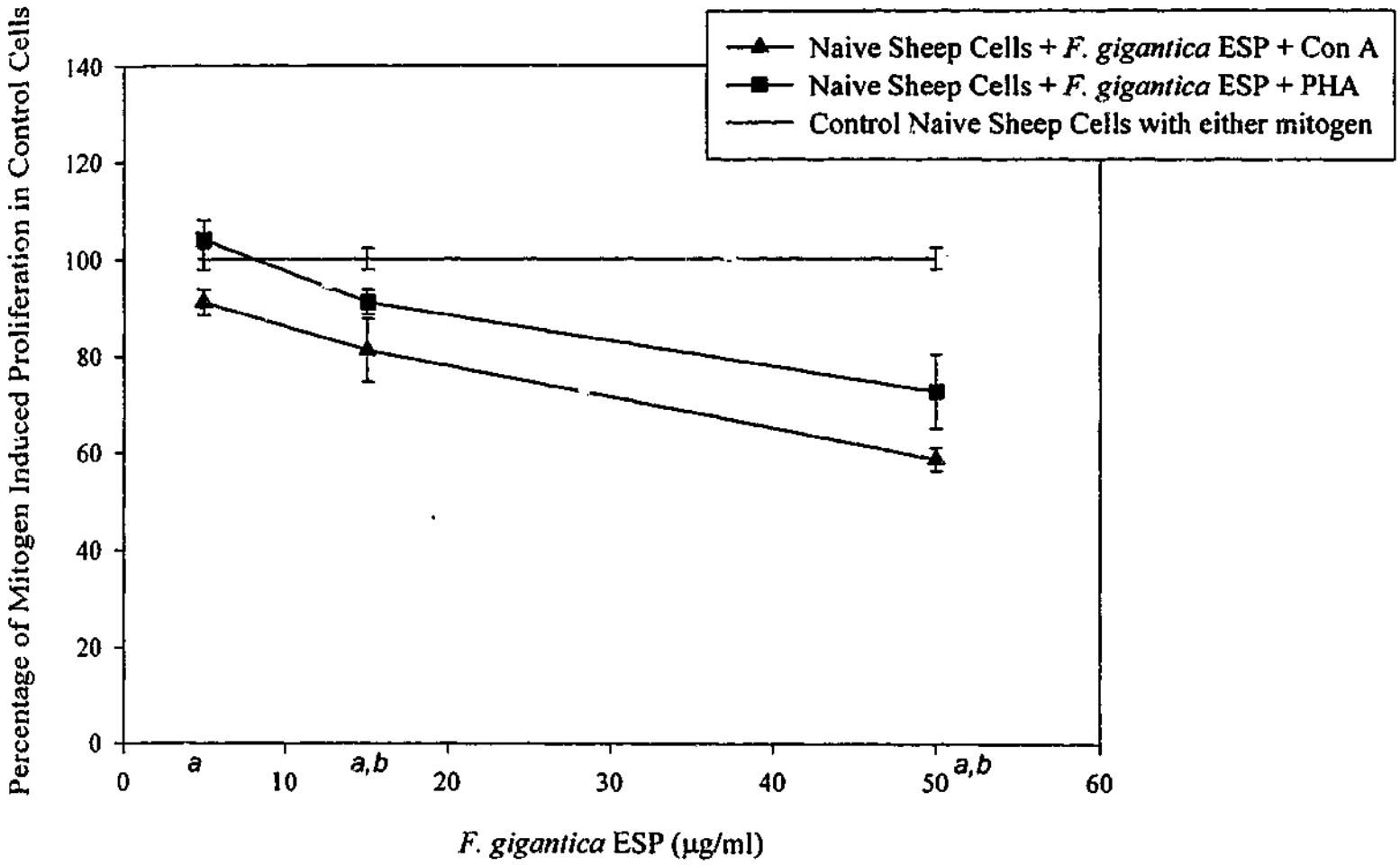


Fig 3.6 Whole blood proliferation assay for *Fasciola*-naive sheep cells stimulated with different mitogens at increasing concentrations of *F. gigantica* ESP. The results are expressed as a percentage of ^{3}H-thymidine uptake for the control naive sheep with either mitogen but with no *F. gigantica* ESP present. Values represent the means $\pm$ SD of quadruplicate wells. Significant differences ($P<0.05$), compared to control cells, were calculated using the Dunnett Multiple Comparison test and are shown by an *a* or *b* for the Con A and PHA stimulated cells respectively. Similar results were obtained in two different experiments.

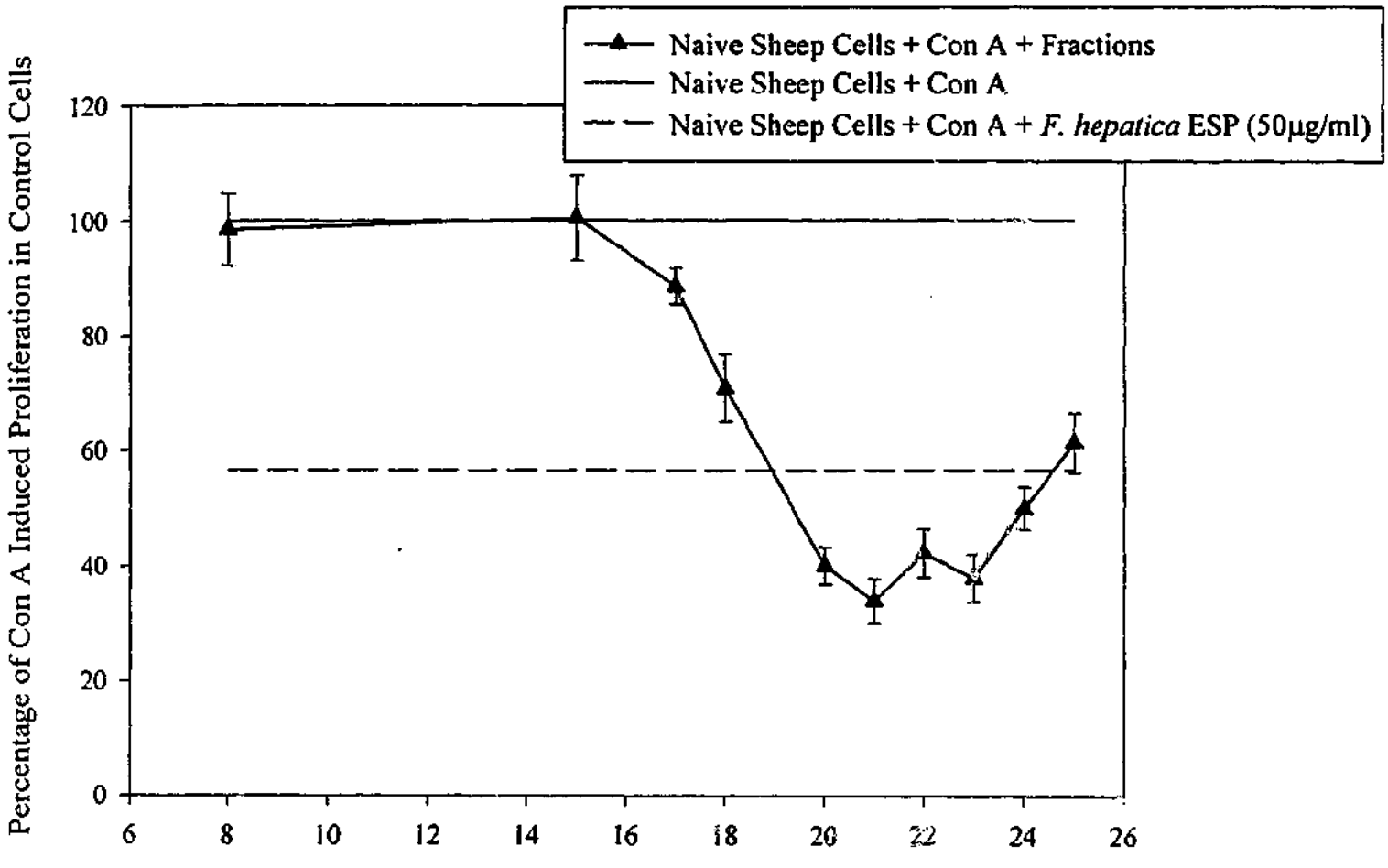


Fig 3.7 Whole blood proliferation assay for *Fasciola*-naive sheep cells stimulated with Con A and individual fractions of *F. hepatica* ESP separated on a Superose 6 FPLC column. The results are expressed as a percentage of ^{3}H-thymidine uptake for the control naive sheep cells with Con A alone. Values represent the means ± SD of quadruplicate wells. Significant differences ($P<0.05$), compared to cells with Con A alone, were calculated using the Dunnett Multiple Comparisons test. Significant differences were observed for cells + Con A and fractions 17-25.

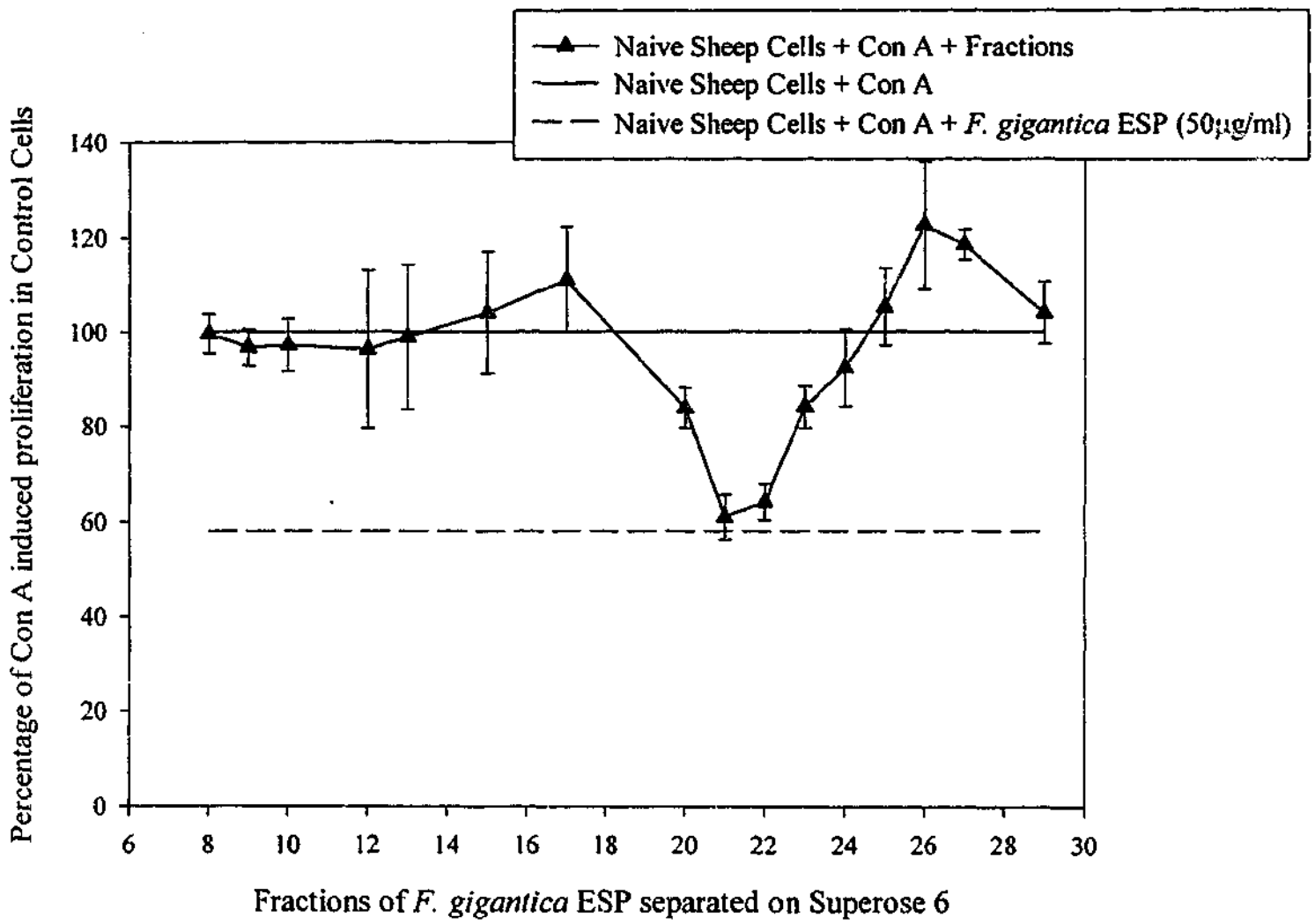


Fig 3.8 Whole blood proliferation assay for *Fasciola*-naive sheep cells stimulated with Con A and individual fractions of *F. gigantica* ESP separated on a Superose 6 FPLC column. The results are expressed as a percentage of ^{3}H-thymidine uptake for the control naive sheep cells with Con A alone. Values represent the means $\pm$ SD of quadruplicate wells. Significant differences ($P<0.05$), compared to naive sheep cells with Con A alone, were calculated using the Dunnett Multiple Comparisons test. Significant differences were observed for cells + Con A and fractions 21,22 and 26.

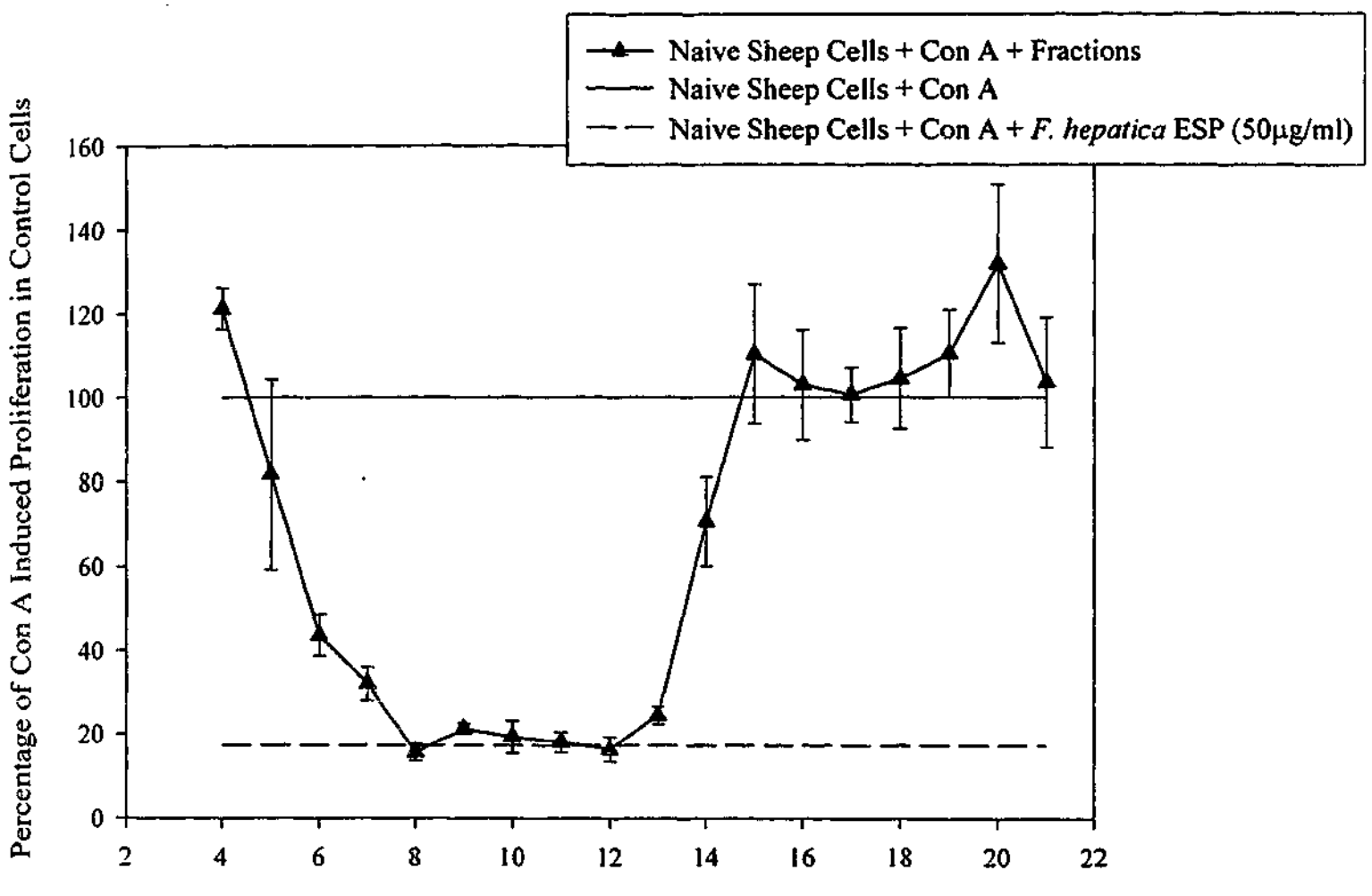


Fig 3.9 Whole blood assay for *Fasciola*-naive sheep cells stimulated with Con A in the presence of individual fractions of *F. hepatica* ESP separated on a Superdex 75 FPLC column. The results are expressed as a percentage of ^{3}H-thymidine uptake for the control naive sheep cells with Con A alone. Values represent the means $\pm$ SD of quadruplicate wells. Significant differences ($P<0.05$), compared to naive sheep cells with Con A alone, were calculated using the Dunnett Multiple Comparisons test. Significant differences were observed for cells + Con A and fractions 6-14 and 20. Similar results were obtained in two different experiments.

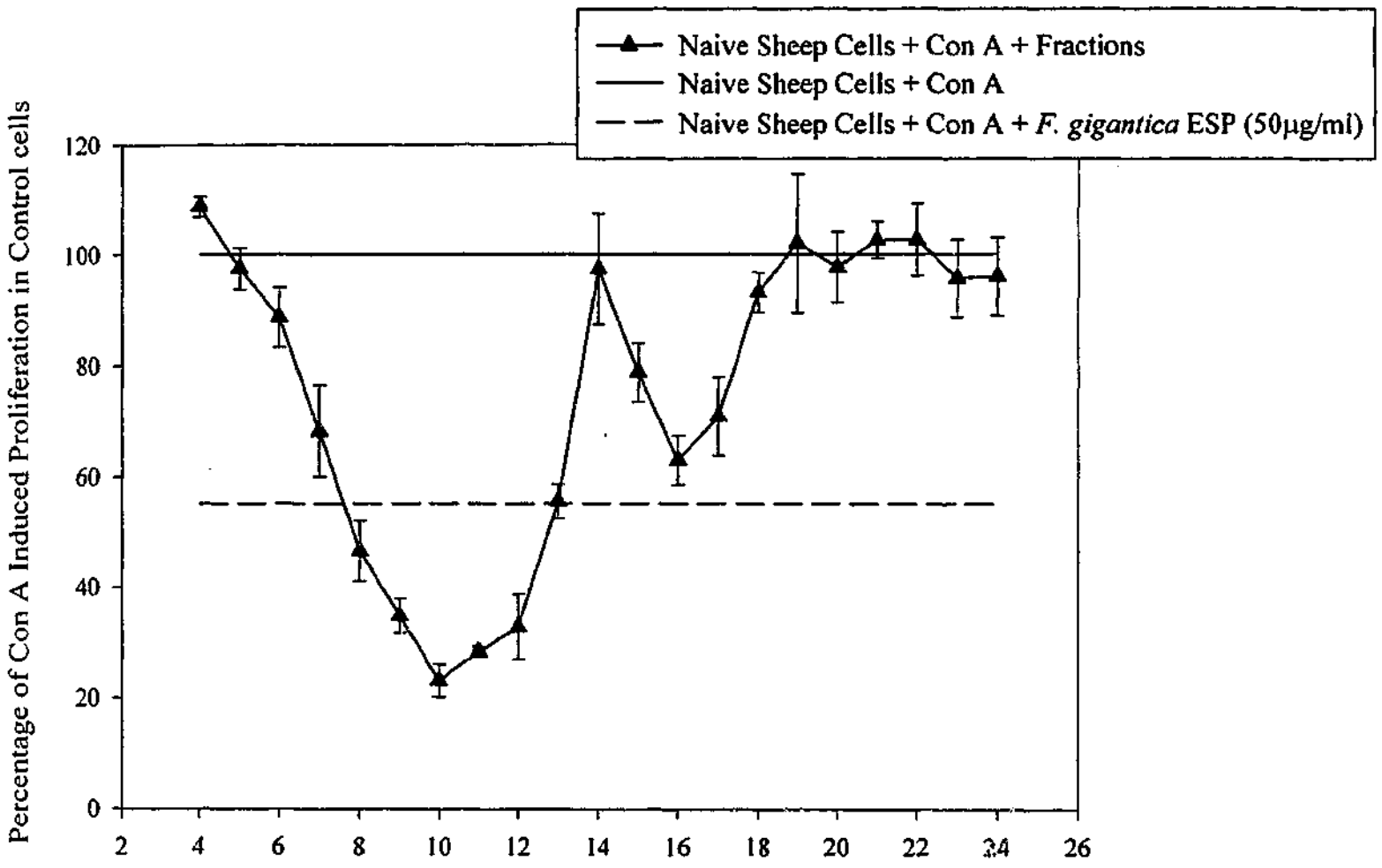


Fig 3.10 Whole blood assay for Fasciola-naive sheep cells stimulated with Con A in the presence of individual fractions of *F. gigantica* ESP separated on a Superdex 75 FPLC column. The results are expressed as a percentage of ^{3}H-thymidine uptake for the control naive sheep cells with Con A alone. Values represent the means ± SD of quadruplicate wells. Significant differences ($P<0.05$), compared to naive sheep cells with Con A alone, were calculated using the Dunnett Multiple Comparisons test. Significant differences were observed to cells + Con A and fractions 7-13 and 15-17. Similar results were obtained in two separate experiments.

The greatest suppressive effect on Con A- induced whole blood cellular proliferation *in vitro* was observed in the presence of fractions 7-13 of *F. hepatica* ESP and fractions 8-12 of *F. gigantica* ESP. These ESP fractions were analysed by 15% SDS PAGE gels and compared with whole *Fasciola* ESP and fractions 4 and 14 from their respective S75 column, where minimal suppression was observed. As shown in Fig 3.11 A and B, a major component within suppressive fractions 8-11 was a 28 kDa doublet known to represent cathepsin L (Wijffels *et al.* 1994a), although there were also some other lower molecular weight bands present.

In order to confirm whether the 28 kDa doublet was cathepsin L, Western blot analysis of the S75 suppressive fractions, using sheep anti-*F. hepatica* cathepsin L antibody, was performed. A band corresponding to the 28 kDa cathepsin L doublet in both *Fasciola sp.* S75 suppressive fractions (Fig 3.12 A, B) was identified. Two controls were included on each gel to further confirm the identification of cathepsin L: unfractionated *F. hepatica or F. gigantica* ESP, in which the characteristic 28 kDa cathepsin L doublet was identified and rFhCatL5, which was detected in its unprocessed proenzyme form (46 kDa). This unprocessed form was also detected in fractions 7 and 8.

To confirm that suppression of cellular proliferation was due to the cathepsin L activity within *F. hepatica* and *F. gigantica* ESP, the whole blood cell proliferation assay was repeated with the fractions in the presence of the cysteine protease inhibitor, E64 (Barrett *et al.* 1982). Incubation of whole *F. hepatica* ESP with E64 restored proliferation by approximately 50 % (Fig 3.13). Incubation of whole blood cells with *F. hepatica* ESP fractions 7-12, collected from the S75 column, in the presence of E64 restored their proliferation to control levels (Fig 3.13). With *F. gigantica* ESP fractions addition of E64 also restored proliferation to control levels although the level of suppression seen in this batch of ESP was lower than observed above (Fig 3.14 compare Fig 3.10). However addition of E64 to cells incubated with whole *F. gigantica* ESP only partially restored proliferation (Fig 3.14). An additional control for the experiment was whole blood cells incubated with Con A + E64 in the absence of either *Fasciola sp.* ESP, which showed that E64 had no effect on Con A-induced proliferation (data not shown).

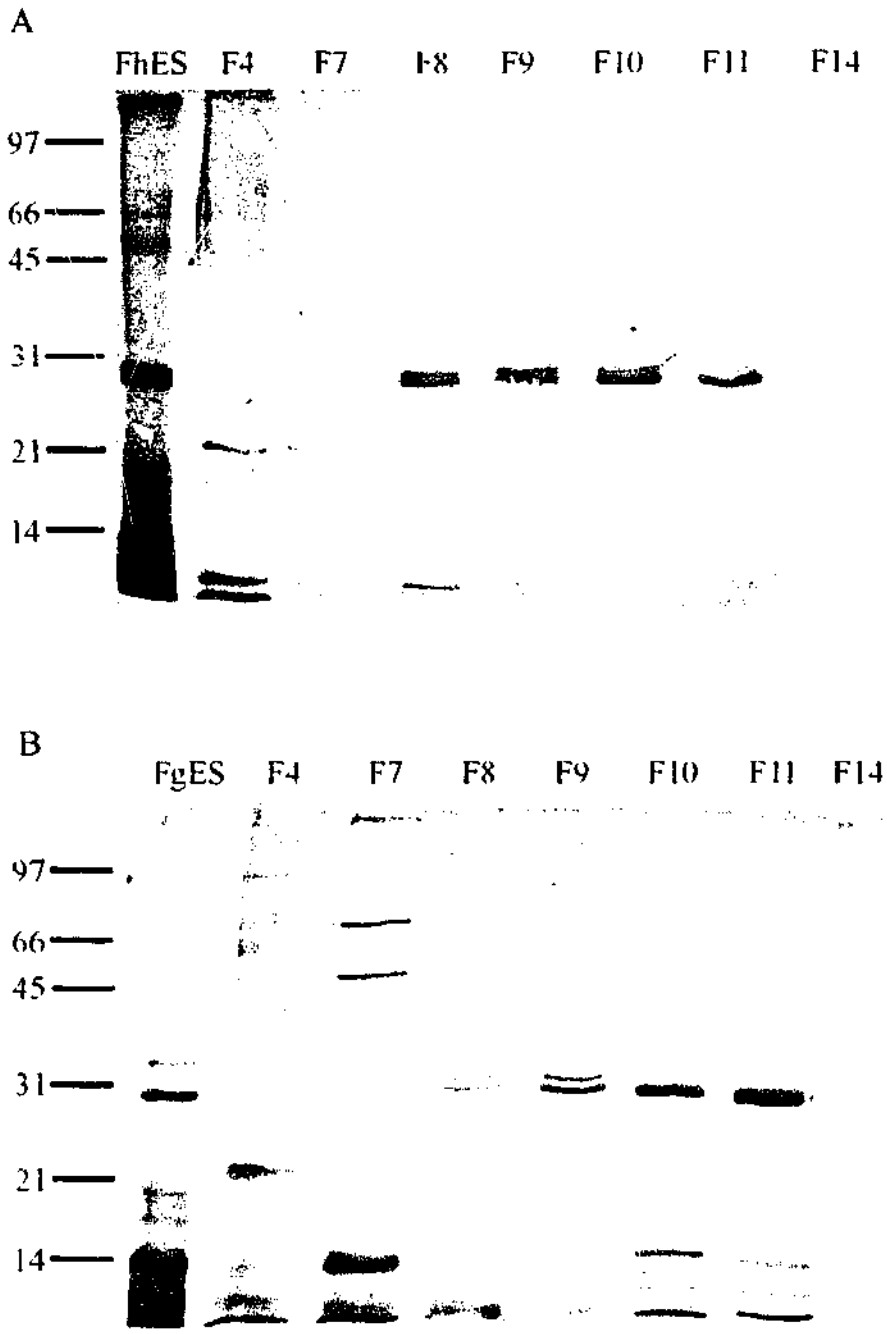
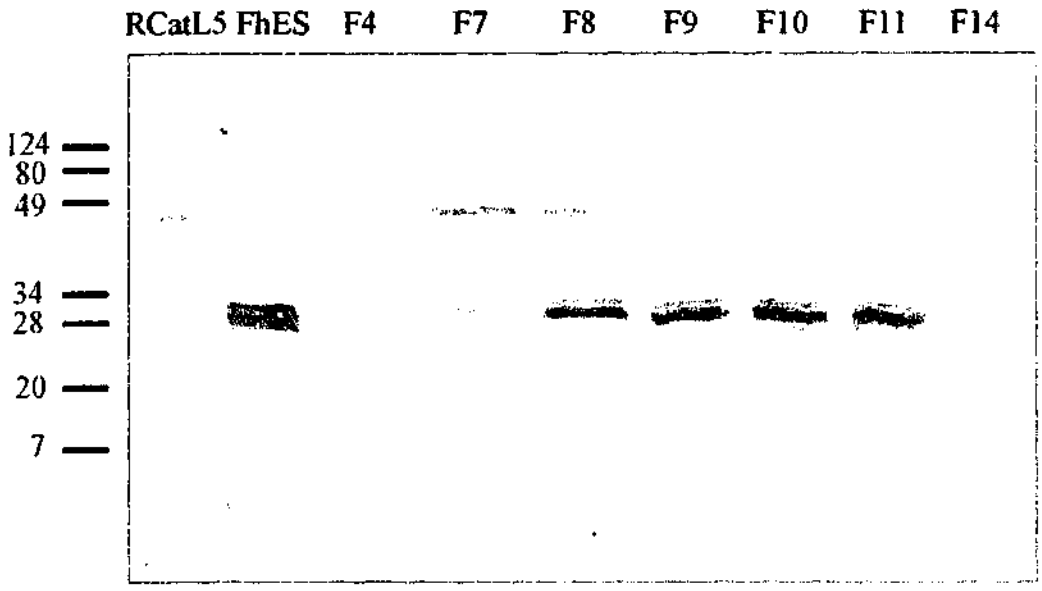


Fig 3.11 Silver stained 15% SDS PAGE gels analysing *F. hepatica* ESP and *F. hepatica* Superdex 75 fractions (A) and *F. gigantica* ESP and *F. gigantica* Superdex 75 fractions (B). *F. hepatica* and *F. gigantica* ESP (10µl) and respective fractions (30µl) were fractionated on SDS PAGE gels. The migration of the standards is shown on the left.

A



B

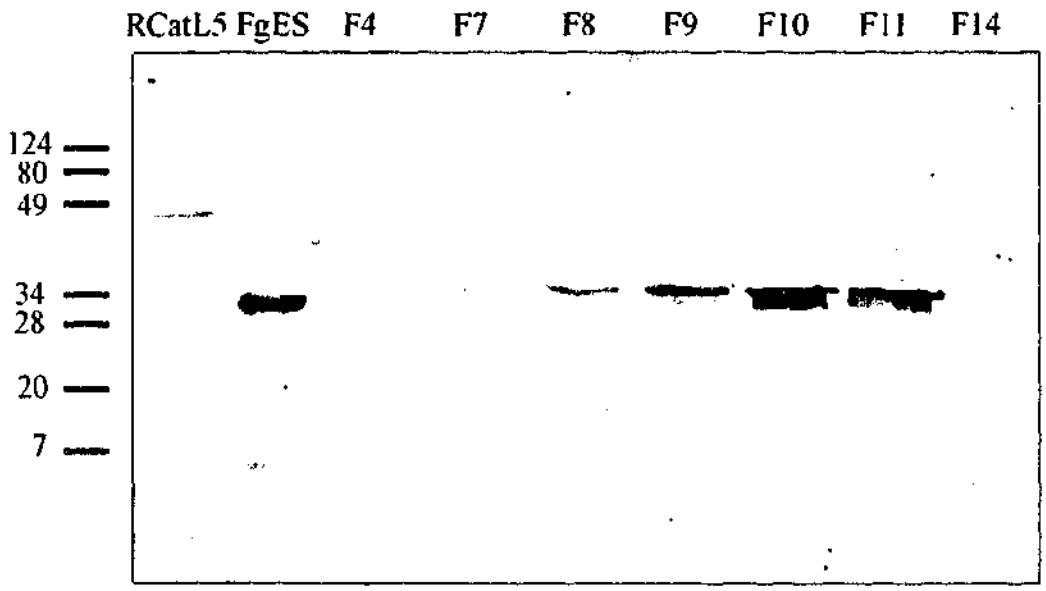


Fig 3.12 Western blot analysis of *F. hepatica* ESP and *F. hepatica* Superdex 75 fractions (A) and *F. gigantica* ESP and *F. gigantica* Superdex 75 fractions (B). *F. hepatica* and *F. gigantica* ESP (10µl), pro rFhCatL5 (20µl) and respective fractions (30µl) were fractionated by SDS PAGE, blotted and probed with sheep anti *F. hepatica* Cathepsin L antibody. The migration of the standards is shown on the left.

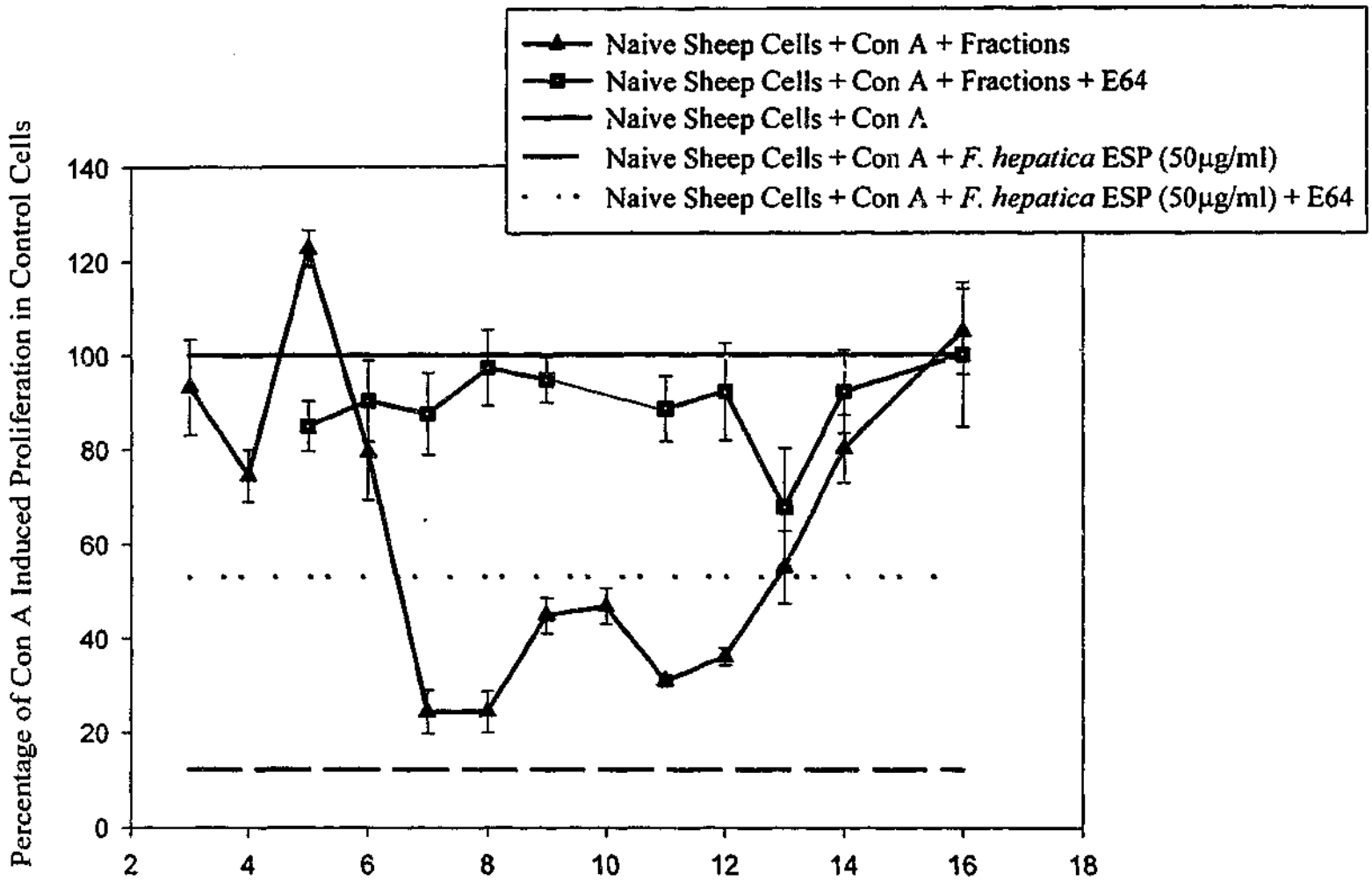


Fig 3.13 Whole blood assay for *Fasciola*-naive sheep cells stimulated with Con A in the presence of individual Superdex 75 *F. hepatica* fractions with and without E64. The results are expressed as a percentage of ^{3}H-thymidine uptake for the control naive sheep cells with Con A alone. Values represent the means ± SD of quadruplicate wells. Significant differences ($P<0.05$), compared to naive sheep cells with Con A alone, were calculated using the Dunnett Multiple Comparisons test. Significant differences were observed for cells + Con A and fractions 4-14. Significant differences ($P<0.05$), compared to naive sheep cells with Con A, E64 and fractions were calculated using the Bonferroni Multiple Comparisons Test. Significant differences were observed for cells + Con A + fractions 5 and 7-12. Similar results were obtained in two separate experiments.

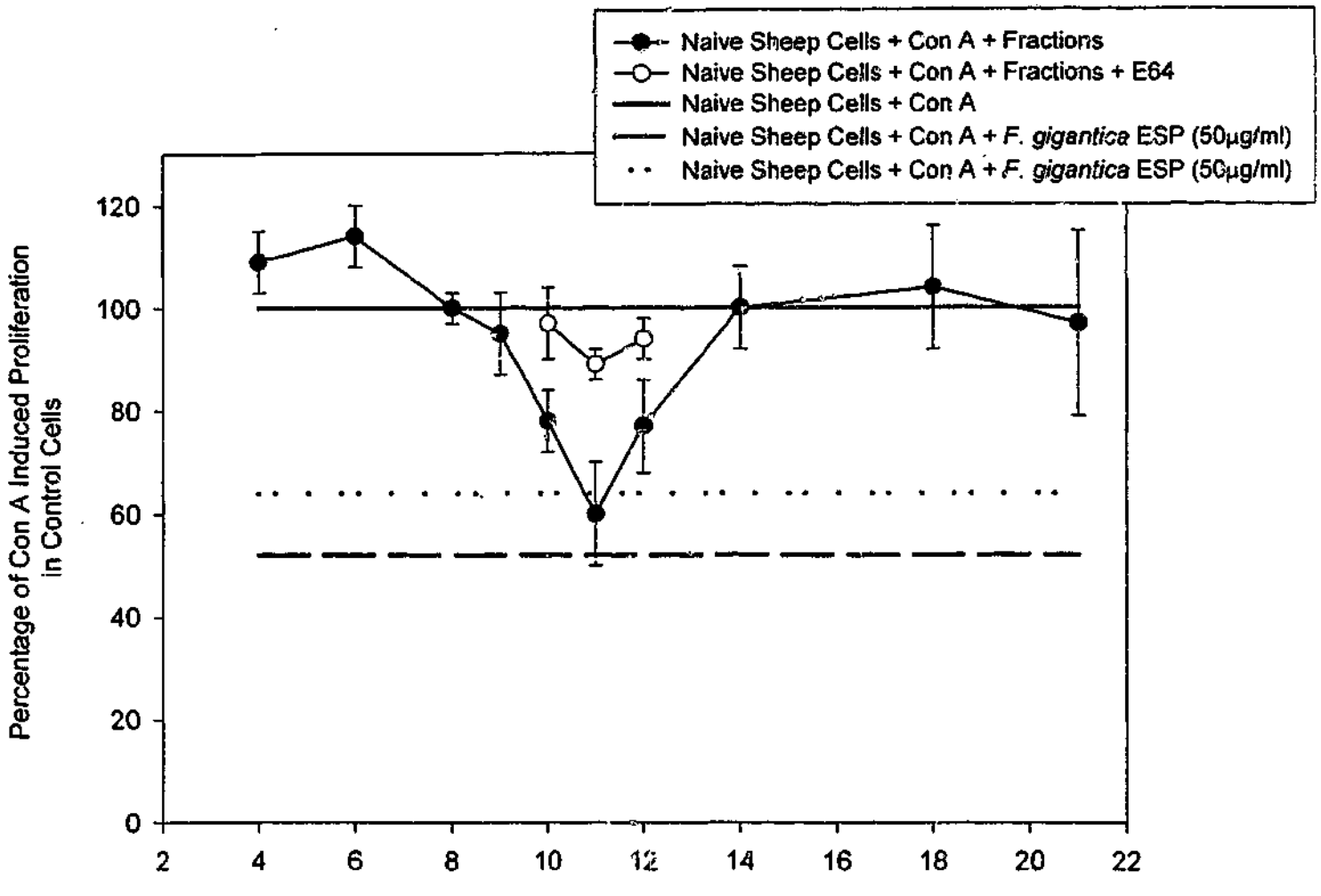


Fig 3.14 Whole blood assay for *Fasciola*-naive sheep cells stimulated with Con A in the presence of individual Superdex 75 *F. gigantica* fractions with and without E64. The results are expressed as a percentage of 3H-thymidine uptake for the control naive sheep cells with Con A alone. Values represent the means + SD of quadruplicate wells. Significant differences ($P<0.05$), compared to naive sheep cells with Con A alone, were calculated using the Dunnett Multiple Comparisons test. Significant differences were observed for cells + Con A and fractions 10, 11 and12. Significant differences ($P<0.05$), compared to naive sheep cells with Con A, E64 and fractions were calculated using the Bonferroni Multiple Comparisons Test. Significant differences were observed for cells + Con A and fractions 10 and 11. Similar results were obtained in two separate experiments.

To further confirm that cathepsin L was mediating the suppressive effects observed, we examined the effects on cell proliferation of two different recombinant *F. hepatica* cathepsin L isoenzymes (rFhCatL5, rFhCatL69Y), that were available in our laboratory (Smooker *et al.* 2000). Two concentrations were investigated and significant suppression of cell proliferation was observed at both 5 and 25 μg/ml of rFhCatL5 and 5 and 10 μg/ml of rFhCatL69Y (Fig 3.15). Correspondingly, E64 was able to restore proliferation when included with 5 μg/ml of rFhCatL5. These results add further evidence for a cathepsin L role in suppressing sheep cellular proliferation.

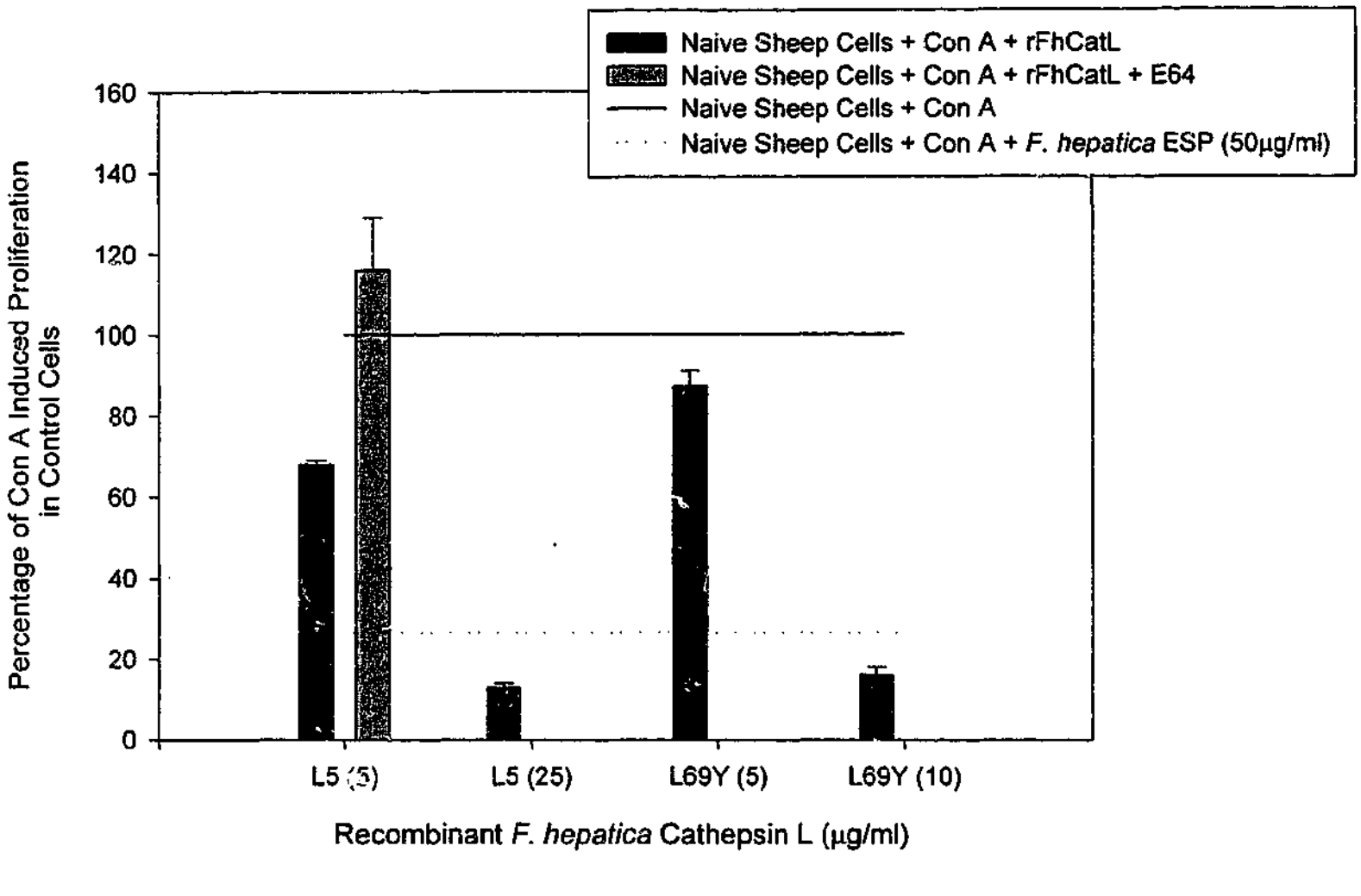


Fig 3.15 Whole blood assay for *Fasciola*-naive sheep cells stimulated with Con A in the presence of recombinant *F. hepatica* Cathepsin L (rFhCatL5 or rFhCatL69Y), with and without E64. the results are expressed as a percentage of 3H-thymidine uptake for the control naive sheep cells with Con A alone. Values represent the means + SD of quadruplicate wells. Significant differences ($P<0.05$), compared to the naive sheep cells with Con A alone, were calculated using the Dunnett Multiple Comparisons test. Significant differences were observed for cells + Con A and any concentration of rFhCatL5 or rFhCatL69Y investigated. A significant difference ($P<0.05$) was observed for naive sheep cells with Con A, E64 and rFhCatL5 (5µg/ml) compared to naive sheep cells with Con A and rFhCatL5(5µg/ml). Similar results were obtained in two separate experiments.

3.3 Discussion

To date, the suppression of proliferative responses due to fasciolosis in sheep has only been demonstrated in *F. hepatica* infected sheep (Zimmerman *et al.* 1983 and Chauvin, Bouvet & Boulard 1995). In this study we examined the effect of a current *F. gigantica* infection in two different strains of Merino sheep on cellular proliferation in response to two different mitogens *ex vivo*, in order to determine whether inhibition of proliferative responses was a general phenomenon in *Fasciola* infections within sheep. It was also of interest whether *F. gigantica* infections in Merino sheep with a genetic predisposition to resistance to *Haemonchus* parasites would display different proliferative characteristics compared with outbred Merino sheep. Significant suppression for Con A or PHA induced cellular proliferation was only observed for descendents from the "Golden Ram" at 3 &10 and 3, 4, 9 & 10 WPI respectively. With outbred Merino sheep cellular proliferation was not significantly suppressed during the infection trial but the proliferation profiles showed a similar trend during the experiment to those obtained for the Golden Ram descendents. Variations were expected with the different sheep genotypes in relation to the extent of their immune response. Several papers report higher eosinophilia in *H. contortus*-infected lambs that are genetically selected for resistance to *H. contortus* compared with outbred Merino sheep (Dawkins, Windon & Eagleson 1989, Buddle *et al.* 1992, Rothwell *et al.* 1993). Hence the observed differences in level of suppression observed between breeds, infected with *F. gigantica*, was expected. Overall these results suggested that *F. gigantica* infections, like *F. hepatica* infections, can exert an immunosuppressive effect on cellular responses. However the magnitude of suppression varied between the two different genetic populations of Merino sheep investigated and this variation is also likely to occur between sheep species. The preliminary data presented here suggests that *F. gigantica* can suppress cellular proliferative responses during the first 10 weeks of infection in the ovine host. This is the first report of *F. gigantica* suppressing host cellular proliferative responses (not published).

The life cycle within sheep of *F. gigantica* does vary from that of *F. hepatica* (Spithill, Smooker & Copeman 1999). *F. gigantica* starts moving from the liver parenchyma and establishing in the bile ducts around 12 –16 WPI (Grigoryan 1958, Guralp, Oxcan & Simms 1964, Sewell 1966, Prasitirat *et al.* 1996). In contrast *F. hepatica*, moves into the bile ducts around 7-10 WPI (Dawes

& Hughes 1964, Boray 1969). Due to AQIS requirements that *F. gigantica* trials conclude by 10 WPI, we were prevented from extending the time course to determine if current *F. gigantica* infections also induce suppression of cellular proliferation at the time adults enter the bile ducts as seen for *F. hepatica.*

We then investigated the potential molecule (s) responsible for causing this suppression within both *F. hepatica* and *F. gigantica* ESP using whole blood from naïve sheep as a source of lymphocytes for proliferation assays *in vitro*. *F. hepatica* ESP has been shown to suppress mitogen-induced naïve sheep cellular proliferation at increasing concentrations of ESP products (Jefferies, Barrett & Turner 1996). Our first experiment repeated this work, and extended the work to show that *F. gigantica* ESP is also capable of suppressing cellular proliferation from *Fasciola*-naïve sheep *in vitro*. These results indicate a role for both *F. hepatica* and *F. gigantica* ESP in suppressing lymphocyte proliferation responses suggesting that these two parasites may express similar suppression mechanisms.

We subsequently identified macro-molecules within both *F. hepatica* and *F. gigantica* ESP capable of suppressing mitogen-induced sheep cellular proliferation *in vitro*. ESP was fractionated on both S6 and S75 columns and S75 separated fractions with suppressive activity on Con A- induced cellular proliferation were identified. This experiment was confirmed using two different preparations of ESP collected from adult *F. hepatica* and a single batch of ESP from *F. gigantica.* This is due to the difficulty in collecting ESP from *F gigantica* in Indonesia. SDS PAGE gels showed that the main component of these forementioned suppressive fractions (separated on the S75 column) was a 28 kDa doublet characteristic of cathepsin L (Wijffels *et al.* 1994a). The SDS PAGE gels also revealed some bands of < 28kDa in size. These lesser constituents may be breakdown products of cathepsin L since cathepsin L is known to self cleave into components of 14kDa (Wijffels *et al.* 1994a,b) ; alternatively, the components may be other proteins which co-eluted with cathepsin L. Western blot analysis using specific anti-sera identified the 28 kDa doublet as cathepsin L.

To confirm a role for cathepsin L in suppressing sheep cellular proliferation *in vitro* E64 (a cysteine protease inhibitor) was utilized (Barrett *et al.* 1992). E64 is a specific cysteine protease

inhibitor, which will reverse any suppressive effects caused by the activity of cysteine proteases. The addition of E64 to the suppressive fractions ablated their suppressive activity, confirming the involvement of an active cysteine protease in suppression. Interestingly E64 only partially reversed the suppressive effects of whole *Fasciola sp.* ESP. This result suggests that there are at least two components in *Fasciola* ESP responsible for suppressing whole blood cellular proliferation, but only one of these being a cysteine protease. Future work could involve the identification of this other component by purification and sequencing individual proteins that promote suppression *in vitro*.

In order to directly demonstrate a role for cathepsin L in causing suppression of cellular proliferation, recombinant *F. hepatica* cathepsin Ls were evaluated. Two different recombinant *F. hepatica* cathepsin Ls were shown to be capable of suppressing naïve sheep whole blood cellular proliferation *in vitro*. The addition of E64 suppressed the activity of recFhCatL5 at the concentration tested (5 μg/ml). Together, these results identified cathepsin L proteases as major immunosuppressive molecules released by *F. hepatica*.

Cathepsin L cysteine proteases are 27-30 kDa in size and represent the major protein component of *Fasciola* ESP (Smith *et al.* 1993b, Wijffels *et al.* 1994a, Dowd *et al.* 1994). These proteases are expressed in the intermediate to late phase of parasite development. At least 7 different cathepsin L isoenzymes have been identified by 2D gel electrophoresis of ESP from adult *F. hepatica* flukes (Wijffels *et al.* 1994b) and cDNA cloning (Smooker *et al.* 2001 and Tort *et al.* 1999). As discussed above, *F. hepatica* cathepsin L has already been shown to initiate a range of effects including prevention of antibody-mediated attachment of eosinophils to juvenile flukes *in vitro* (Carmona *et al.* 1993), the formation of blood clots induced through clotting of fibrinogen (Dowd, McGonigle & Dalton 1995) and cleavage of F region of IgG (Carmona *et al.* 1993, Smith *et al.* 1993a, Berasain, *et al.* 2000). These were reviewed in Chapter 1.5.

The seven cathepsin L isoenzymes can be separated into two main groups based on their substrate specificity at the P_2 position (Smooker *et al.* 2001). rFhCatL5 belongs to group 1 which prefers a phenylalanine at P_2. Group 2 cathepsin Ls such as rFhCatL69Y prefer a proline at P_2. Despite these subtle differences in substrate specificity, both recombinant cathepsins suppressed

proliferation *ex vivo*. It remains to be seen whether all the cathepsin L isoenzymes from both *Fasciola sp*. are capable of suppressing sheep whole blood proliferation *in vitro*. Further work would address this question by purification of the individual isoenzymes and testing these individually. In addition to this, mass spectrometry and N-terminal sequencing could be utilised to confirm which enzymes are being released by the different developmental stages of *Fasciola* parasites at the observed times of suppression.

In summary, we have provided preliminary evidence that an *F. gigantica* infection can suppress the mitogen induced proliferation of sheep lymphocytes at similar time periods to those observed with *F. hepatica* infections in Merino sheep *ex vivo*. We have also demonstrated for the first time that both *F. hepatica* and *F. gigantica* cathepsin Ls present in ESP are involved in suppressing mitogen-induced whole blood cellular proliferation by naïve sheep immune cells *in vitro* (Prowse *et al.* 2002). In the following Chapter the mechanism of suppression induced by exposure to cathepsin L is investigated.

CHAPTER 4: IDENTIFICATION OF SURFACE MARKERS ON T CELLS AND DENDRITIC CELLS CLEAVED BY CYSTEINE PROTEASES OF *FASCIOLA*

4.1 Introduction

In the previous Chapter we showed that suppression of sheep whole blood cellular proliferation *in vitro* was associated with cathepsin L protease activity in both *F. hepatica* and *F. gigantica* ESP. This was a novel immunomodulatory role for *Fasciola* cathepsin L proteases. Previously identified roles of *F. hepatica* cathepsin L were reviewed in Chapter 1.5. Together these results demonstrate that cathepsin L proteases exhibit a range of immunomodulatory activities. However, to date the mechanism by which *Fasciola* cathepsin Ls inhibit mitogen induced cellular proliferation was unknown.

Other parasite infections have also been shown to suppress lymphocyte proliferation *in vitro*. This was reviewed in Chapter 1, section 1.4. An example was *Trypanosoma cruzi* infections in humans, which result in reduced immune responsiveness and several studies have investigated the underlying mechanism/s operating in this infection (Motran *et al.* 1996, Beltz, Sztein & Kierszenbaum 1988, Kierszenbaum *et al.* 1990, 1991, Sztein, Cuna & Kierszenbaum 1990). One *in vitro* study revealed diminished T cell proliferation in response to a range of different mitogens due to a deficiency at the level of accessory cells (Motran *et al.* 1996). Further investigation revealed a decrease in accessory cell surface marker expression of HLA-DR (MHC class II gene product) and CD11b antigens. The authors proposed that infection by *T. cruzi* reduced the level of expression of these markers, such that accessory cells can no longer induce normal levels of T lymphocyte proliferation (Motran *et al.* 1996). Other *in vitro* studies, have shown that a secretion product released by *T. cruzi* can reduce surface expression of the IL-2 receptor, CD3, CD4 and CD8 (Beltz, Sztein & Kierszenbaum 1988, Kierszenbaum *et al.* 1990, Sztein, Cuna & Kierszenbaum 1990). All of these markers play a key role in lymphocyte activation and a reduction in their expression may lead to suppression of T cell proliferation.

Lymphatic cannulation sheep models have allowed significant insights to be made into lymphocyte trafficking along with the physiology of cell movements through lymphoid and non-

lymphoid tissues (reviewed in Young 1999). An advantage of cannulating sheep, a larger experimental model, is that it is possible to isolate lymphatic vessels draining individual lymph nodes. The surgical model for cannulating sheep efferent and afferent lymphatics were first described by Hall and Morris (1962) and Emery, MacHugh & Ellis (1987), respectively. From these models a local draining node is isolated and cell populations are monitored entering (afferent lymph) or leaving (efferent lymph) the node.

This technology has been used in experiments analysing immune responses to different antigens and adjuvants, as induced immune responses are measurable at the lymph node draining the site of vaccination (Dresser, Taub & Krantz 1970, Trnka & Cahill 1980, Hopkins, McConnell & Pearson 1981). More recently, cannulation models have been used for collection of different cell populations for *in vitro* experimentation of the effect of antigens on individual cell populations, cytokine responses and cellular surface markers (Emery, Rothel & Wood 1990, Rothel *et al.* 1997, 1998, Windon *et al.* 2000). Advances in the depth of analysis are achievable because of the increasing production / availability of monoclonal antibodies to sheep surface markers and cytokines (Mackay, Maddox & Brandon 1987, Mackay 1988, Hein & Mackay 1991).

In this Chapter, we attempted to identify the mechanism of action exhibited by *Fasciola sp.* cathepsin Ls in suppressing sheep cellular proliferation. It was hypothesised that suppression of sheep cellular proliferation may be mediated by cleavage of proteins on the surface of immune cells involved in the initiation / activation of cellular proliferation. Therefore, the aim of this Chapter was to identify potential effector cells and surface protein targets of *Fasciola sp.* ESP on immune cells collected from sheep afferent and efferent lymph.

4.2 Results

The FACS data presented in this Chapter are the results from a single experiment which was representative of at least two other experiments conducted with sheep cells. The Mean Fluorescent Intensity (MFI) values for the surface marker expression on cells did vary between experiments. This was most likely due to acquiring cells from different animals for experimentation, or may have resulted from minor variations in incubation times, room temperature and the fact that different batches of *Fasciola* ESP were used during the course of this study. However consistent trends were observed between experiments, for the relative level of expression of surface markers under the conditions examined and it is these trends which are discussed.

4.2.1 Effect of *Fasciola sp.* ESP on Expression of Sheep Cell Markers *in vitro*

In order to begin to identify surface markers on cells that could be a target for cathepsin L the effect of *Fasciola sp.* ESP on the expression of a range of surface markers (Table 4.1 – in total 29 surface markers) on cells from afferent and efferent lymph was analysed. Antibodies to these markers were available through our collaboration with CSIRO Animal Health, Parkville. These markers are found on a range of immune cells, as shown in Table 4.1. We studied both afferent and efferent lymph as each contains different immune cells. The cell types found in afferent and efferent lymph are displayed in Table 4.2. In the initial experiments afferent and efferent lymph from cannulated sheep were analysed for possible shifts in each of the forementioned cell markers. Gates for lymphocyte populations were based on FACS data analyzing size and granularity of either afferent or efferent lymph. Gates for dendritic cells in afferent lymph were based on size and expression of surface CD1b.

Preliminary experiments centered on the minimal concentration of *Fasciola* ESP required for a change in expression of any of the sheep cell surface markers. 50, 100 and 250 μg/ml of *F. hepatica* ESP and 50, 100, 250 and 500 μg/ml of *F. gigantica* ESP were incubated for 5 hrs with sheep afferent or efferent lymph (5×10^5 cells / well). Since cathepsin L represents approximately 40% of total protein in ESP (Wijffels *et al.* 1994a), these concentrations of ESP

Table 4.1: Leukocyte Markers Analysed

Marker	Cell/Tissue Distribution
CD1b	Dendritic cells; cortical thymocytes
CD2	CD4 and CD8 T cells; 60-70% thymocytes; dendritic cells;
CD4	Subset of α/β TCR expressing cells
CD5	Pan-T cells; subset of B cells
CD8α	Subset of α/β TCR expressing cells
CD11a	Lymphocytes; granulocytes; monocytes; macrophages
CD11b	Alveolar macrophages; blood mononuclear cells; granulocytes
CD11c	Alveolar macrophages; afferent lymph dendritic cells; eosinophils
CD14	Monocytes, macrophages and langerhans cells
CD21	B cells; dendritic cells
CD23	B cells; monocytes; weakly on T cells, dendritic cells and eosinophils
CD25	Activated T cells; 30-40% $CD4^+$ T cells in blood
CD45R	All lymphocytes; macrophages; granulocytes
CD45Ra	Same as CD45R
CD58	Mature and immature haematopoietic cells; erythrocytes
L-selectin	T cells[a]
MHC I	Expressed on most nucleated cells
MHC II dp	Dendritic cells; B cells; monocytes; macrophages; activated T cells
MHC II dr	Same as MHC II dp
MHC II dq	Same as MHC II dp
MyD-1	Dendritic cells, monocytes and neutrophils[b]
sIgG	B lymphocytes[c]
WC1	γδ T Cells
WC6	30% lymphocytes from the blood, afferent and efferent lymph, majority of afferent lymph dendritic cells[d]
WC10	Some afferent lymph veiled cells (ALVC)[e]
γδTCR	γδ T cells
Unknown	MAb cc81 – specific for an undefined marker on a subset of ALVCs[e]

VLA4	Mononuclear leukocytes, lymphocytes[f,g]
TcR1-N7	γδ T cells[h]

Unless otherwise indicated this table was complied from Barclay *et al.* 1997

[a]Howard, Sopp & Parsons 1992; [b]Brooke, Parsons & Howard 1998; [c]Da Costa *et al.* 1992; [d]Dutia, Ross & Hopkins 1993; [e]Howard *et al.* 1997; [f]Tanaka *et al.* 1994; [g]Stoltenborg *et al.* 1993; [h]Davis *et al.* 1996

Table 4.2: Cell types found in peripheral blood and afferent and efferent lymph

Cell Type	Peripheral Blood (%)	Afferent Lymph (%)	Efferent Lymph (%)
Lymphocytes	40 - 75	70 - 95	95 - 100
- CD4 T cells	14.3 ± 3.0	38.5 ± 3.7	38.8 ± 7.7
- CD8 T cells	8.5 ± 0.5	12.8 ± 1.1	14.0 ± 1.8
- γδ T cells	11.8 ± 2.1	27.8 ± 2.2	10.0 ± 3.1
- B cells	17.5 ± 5.3	7.3 ± 0.3	32.0 ± 7.0
Neutrophils	10 - 50	0 – 10	< 1
Eosinophils	0 - 15	0 – 10	< 1
Basophils	0 - 3	0 – 10	< 1
Macrophages, monocytes, dendritic cells	1-6	5-20	< 1

This table was compiled from Peterhans & Carter 1998, Smith *et al.* 1970 and Mackay *et al.* 1988

represent about 20-200 µg/ml of native cathepsin L however it should be noted that only a proportion of this cathepsin L protein is enzymatically active, suggesting that the actual level of active cathepsin L in the incubations is < 20 – 200 µg/ml. Significant shifts in CD1b, CD4 and CD8 expression were observed with 250 and 500 µg/ml of *F. hepatica* and *F. gigantica* ESP respectively (Fig 4.1a & 4.1b). The raw results for the other 26 surface markers analysed are displayed in Appendix 1. This Chapter will only focus on the three forementioned markers where consistently significant shifts were observed. CD4 and CD8 expression was reduced about 10 fold whereas CD1b expression was up-regulated. The broad profile of CD1b positive cells suggests that there is a range of CD1b expression levels on afferent dendritic cells. This experiment was repeated twice confirming consistent differences in surface expression for these three markers alone. All future experiments, unless otherwise stated, used 250 µg/ml of *F. hepatica* ESP or 500 µg/ml *F. gigantica* ESP with 5×10^5 cells / well.

4.2.2 Effect of Incubation Time of *Fasciola sp.* ESP on CD4, CD8 and CD1b Expression on Sheep Cells

In the preliminary experiments CD4, CD8 and CD1b were shown to have altered expression after a 5 hr incubation with either *F. hepatica* ESP or *F. gigantica* ESP. In this set of experiments the effect of incubation time on the expression of these surface markers was examined.

(a) CD4

CD4 expression on dendritic cells and afferent and efferent lymphocytes decreased over time following incubation in the presence of *F. hepatica* or *F. gigantica* ESP (Fig 4.2a). A notable down regulation in the mean fluorescent intensity (MFI) of the CD4 positive sheep cells was observed after a 3 hr incubation with either *Fasciola sp.* ESP (Table 4.3a). After a 16 hr incubation, the MFI of the CD4-positive cells had decreased along with a reduction of cells gated within this range (Table 4.3a) indicating an elimination of the CD4 surface marker on the majority of lymphocytes and all dendritic cells. Exposure to *Fasciola* ESP thus induced a down regulation of CD4 levels on sheep immune cells *in vitro*.

(b) CD8

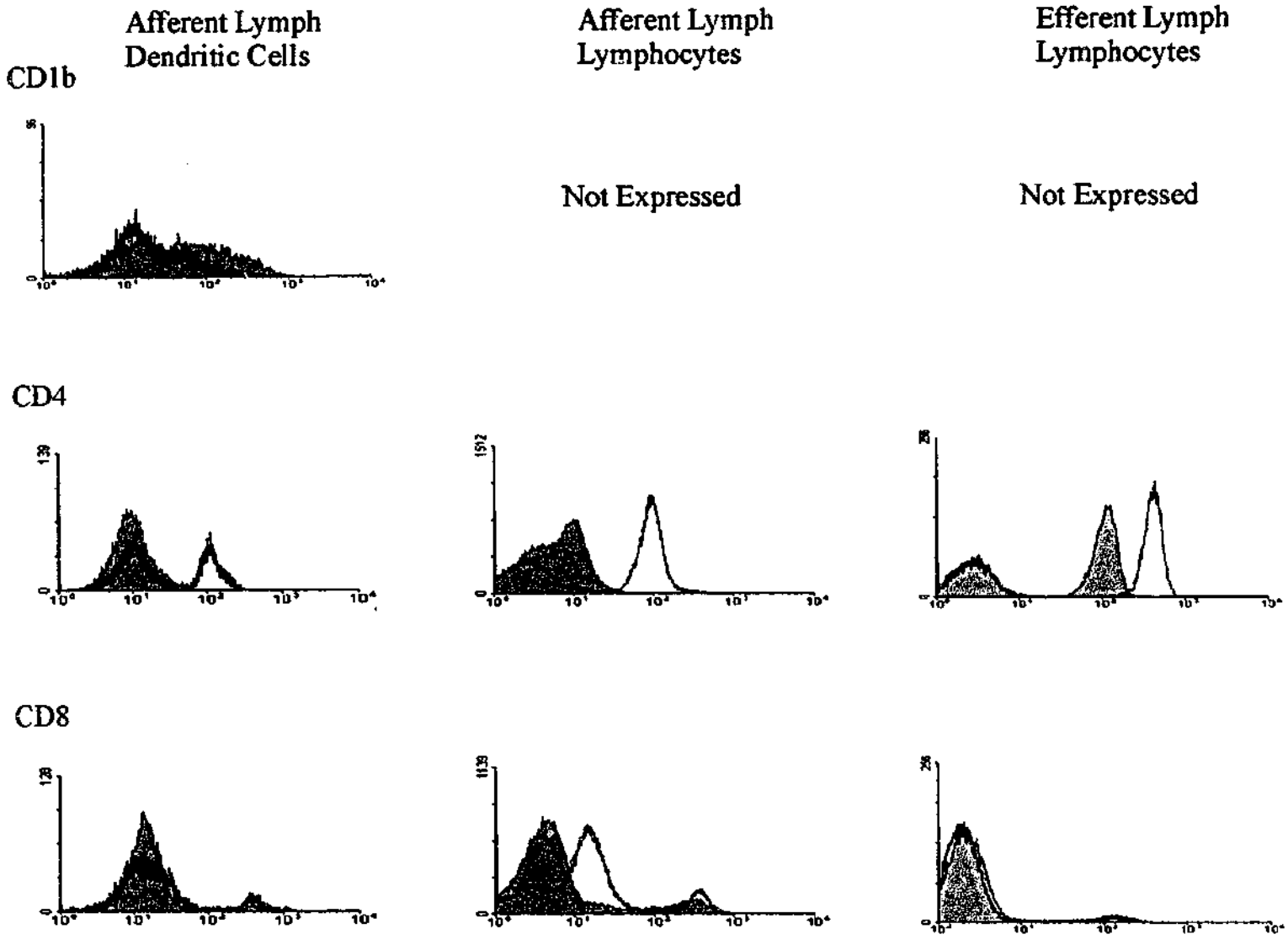


Fig 4.1a: The effect of *F. hepatica* ESP (250 µg/ml) on the expression of sheep cell surface markers CD1b, CD4 CD8 on either afferent (gated for dendritic cells or lymphocytes) or efferent lymph (gated for lymphocytes) after a 5 hr incubation. The grey shaded area represents the surface marker expression on sheep cells in the presence of *F. hepatica* ESP after a 5 hr incubation while the black overlay shows the normal surface marker expression on the identical population of sheep cells incubated for 5 hrs in media alone. Data in each plot represent a minimum of 2,500 events for dendritic cells and a minimum of 20,000 events for lymphocytes stained with mouse anti-sheep surface marker and FITC anti-mouse immunoglobulin. Histograms show the fluorescent intensity (X axis) versus cell number (Y-axis). Similar results were obtained in two separate experiments.

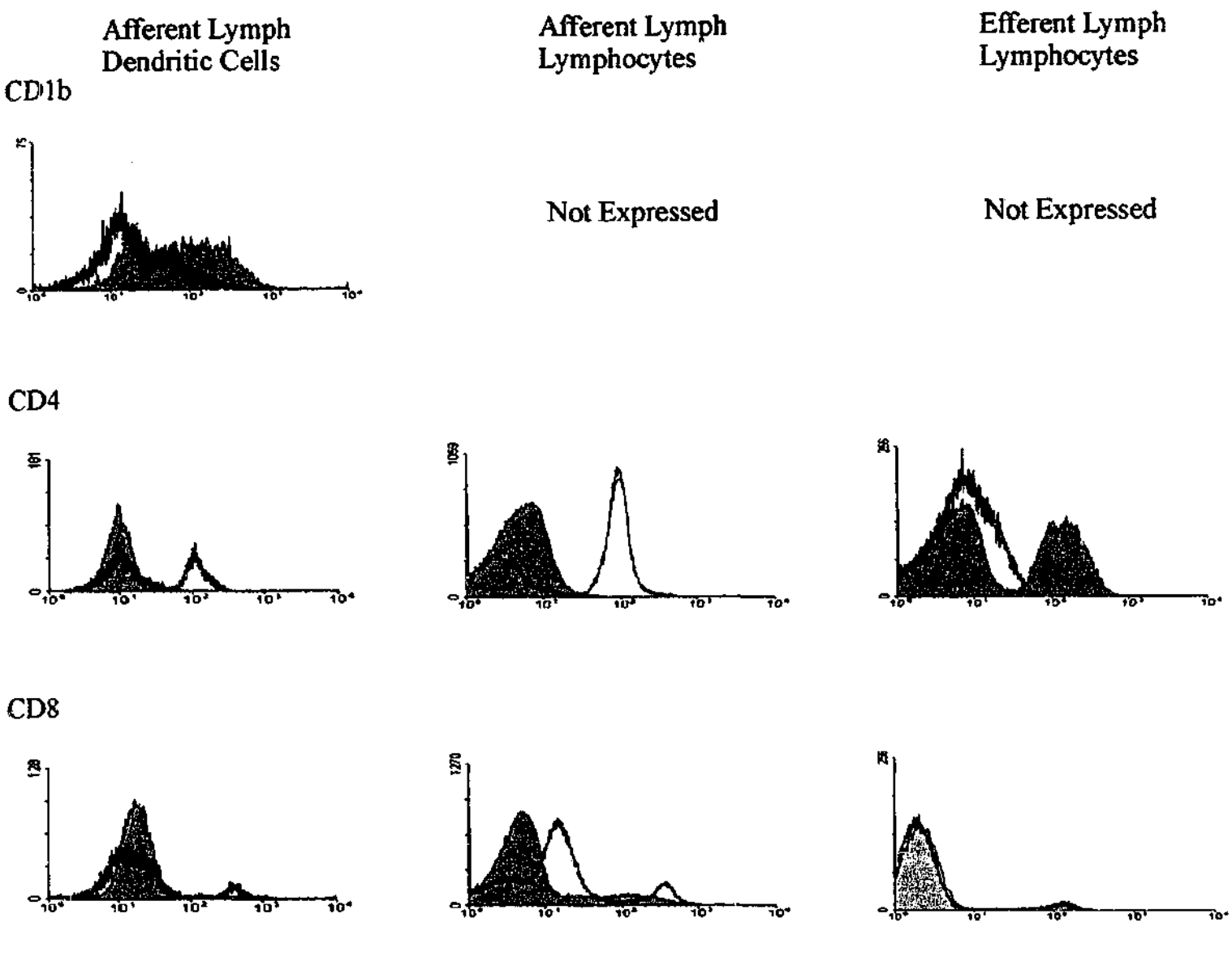
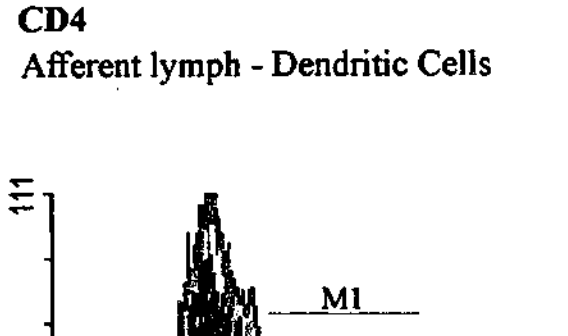
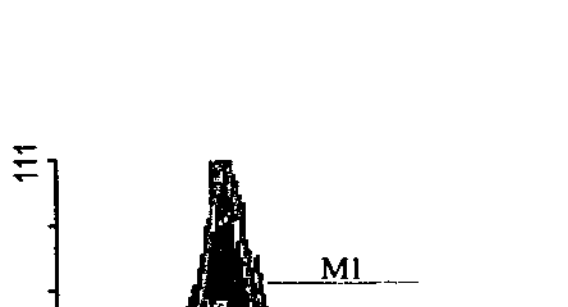


Fig 4.1b: The effect of *F. gigantica* ESP (500 μg/ml) on the expression of sheep cell surface markers CD1b, CD4 and CD8 on either afferent (gated for dendritic cells or lymphocytes) or efferent lymph (gated for lymphocytes) after a 5 hr incubation. The grey shaded area represents the surface marker expression on sheep cells in the presence of *F. gigantica* ESP after a 5 hr incubation while the black overlay shows the normal surface marker expression on the identical population of sheep cells incubated for 5 hrs in media alone. Data in each plot represent a minimum of 2,500 events for dendritic cells and a minimum of 20,000 events for lymphocytes stained with mouse anti-sheep surface marker and FITC anti-mouse immunoglobulin. Histograms show the fluorescent intensity (X axis) versus cell number (Y-axis). Similar results were obtained in two separate experiments.

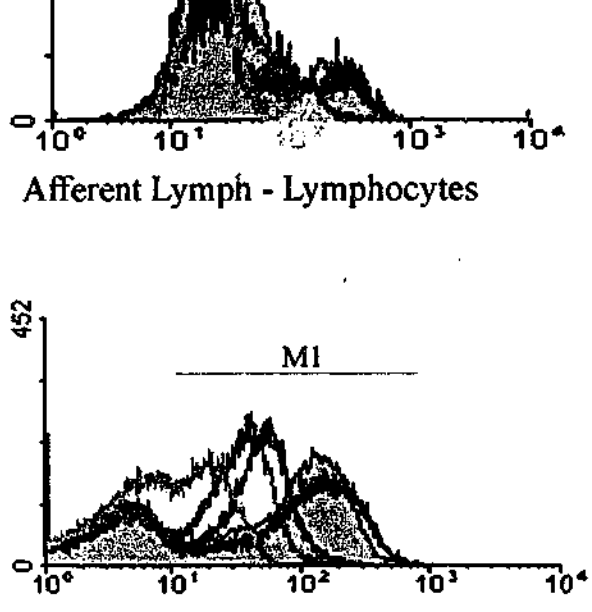
CD4

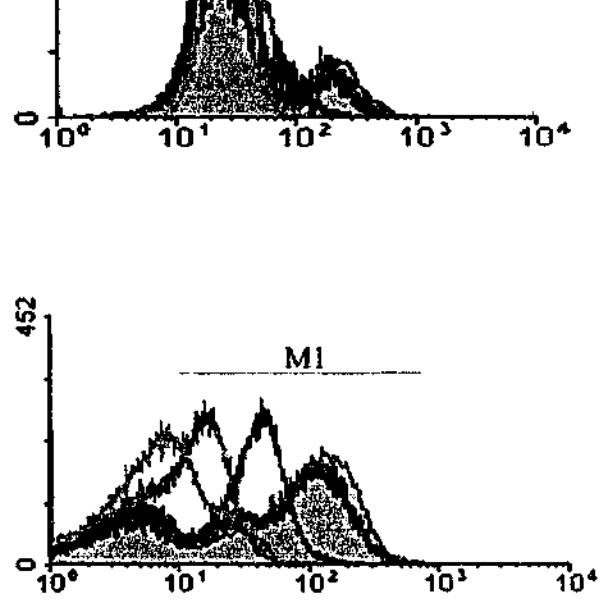
Afferent lymph - Dendritic Cells



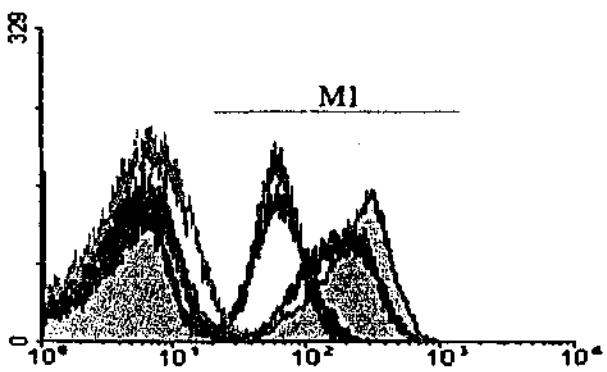


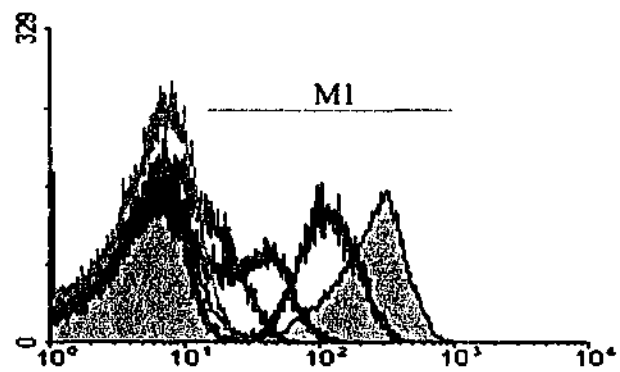
Afferent Lymph - Lymphocytes





Efferent Lymph - Lymphocytes





F. hepatica ESP

F. gigantica ESP

Fig 4.2a: The effect of *F. hepatica* ESP (250 µg/ml) and *F. gigantica* ESP (500 µg/ml) on the expression of the CD4 surface marker on sheep efferent lymph over a 16 hr period. The grey shaded area represents the normal CD4 expression on sheep cells after a 1 hr incubation in media alone. The brown, blue, red, and orange overlays represent the CD4 expression levels on sheep efferent lymph after 1, 3, 5, or 16 hr incubations respectively with antigen. Data in each plot represent a minimum of 2,500 events for dendritic cells and a minimum of 20,000 events for lymphocytes stained with mouse anti-sheep CD4 and FITC anti-mouse immunoglobulin. Histograms show the fluorescent intensity (X-axis) versus cell number (Y-axis). M1 represents the region for the MFI values displayed in Table 4.3a. Similar results were obtained in two separate experiments.

Sample	Events Gated	% Gated	Mean Fluorescent Intensity
Afferent Lymph – Dendritic Cells			
Media 1 hr	4451	33.8	165.5
F. hepatica ESP 250 µg/ml 1 hr	6488	36.7	195.5
F. hepatica ESP 250 µg/ml 3 hr	4989	26.9	104.5
F. hepatica ESP 250 µg/ml 5 hr	3340	18.0	91.3
F. hepatica ESP 250 µg/ml 16 hr	10	0.1	67.4
F. gigantica ESP 500 µg/ml 1 hr	5001	28.57	160.67
F. gigantica ESP 500 µg/ml 3 hr	4116	19.2	93.12
F. gigantica ESP 500 µg/ml 5 hr	849	3.8	90.4
F. gigantica ESP 500 µg/ml 16 hr	5[a]	0.1	131.6
Afferent Lymph - Lymphocytes			
Media 1 hr	51617	55.4	144.2
F. hepatica ESP 250 µg/ml 1 hr	61293	55.8	164.1
F. hepatica ESP 250 µg/ml 3 hr	65586	53.6	59.6
F. hepatica ESP 250 µg/ml 5 hr	68672	52.8	45.5
F. hepatica ESP 250 µg/ml 16 hr			
F. gigantica ESP 500 µg/ml 1 hr	61145	55.7	127.9
F. gigantica ESP 500 µg/ml 3 hr	76670	53.0	48.6
F. gigantica ESP 500 µg/ml 5 hr	52425	31.4	23.2
F. gigantica ESP 500 µg/ml 16 hr			
Efferent Lymph - Lymphocytes			
Media 1 hr	20006	44.0	198.1
F. hepatica ESP 250 µg/ml 1 hr	19215	42.3	155.5
F. hepatica ESP 250 µg/ml 3 hr	18351	40.1	64.0
F. hepatica ESP 250 µg/ml 5 hr	18269	41.7	59.6
F. hepatica ESP 250 µg/ml 16 hr	4817	10.2	19.6
F. gigantica ESP 500 µg/ml 1 hr	18634	41.3	111.9
F. gigantica ESP 500 µg/ml 3 hr	14596	31.2	34.8
F. gigantica ESP 500 µg/ml 5 hr	9990	20.9	22.6
F. gigantica ESP 500 µg/ml 16 hr	2488	5.2	18.2

Table 4.3a: CD4 expression levels on sheep immune cells after exposure to *Fasciola* ESP over a time course. Data were collected from the experiment shown in Fig 4.2a. MFI values were measured on the events within the M1 region shown in Fig 4.2a. Similar results were obtained in two separate experiments. [a]Cells autofluouresced

The effect of *Fasciola sp.* ESP on CD8 expression varied on each cell type. CD8 expression on efferent lymphocytes with either *Fasciola* ESP was up-regulated at 1 and 3 hr after exposure to ESP (Fig 4.2b, Table 4.3b). However after the 5 hr incubation with either ESP, CD8 was down regulated on efferent lymphocytes and this down regulation increased following overnight incubations with either *Fasciola sp.* ESP (Fig 4.2b,Table 4.3b). In contrast, on afferent lymphocytes CD8 levels were upregulated after 1 –3 hr exposure to ESP and in the presence of *F. hepatica* ESP remained higher than control CD8 levels after 16 hr incubations with ESP. CD8 expression levels on afferent lymphocytes in the presence of *F. gigantica* ESP decreased after 5 hours and, after the 16 hr incubation, the percentage of CD8 positive cells had decreased (Fig 4.2b,Table 4.3b).

On dendritic cells, up-regulation of CD8 expression was observed in the presence of either *Fasciola* ESP after 1-3 hr exposure to ESP (Fig 4.2b, Table 4.3b). A decrease in the % of cells expressing CD8 was observed after both the 5 hr and overnight incubations with either *Fasciola sp.* ESP (Fig 4.2b, Table 4.3b).

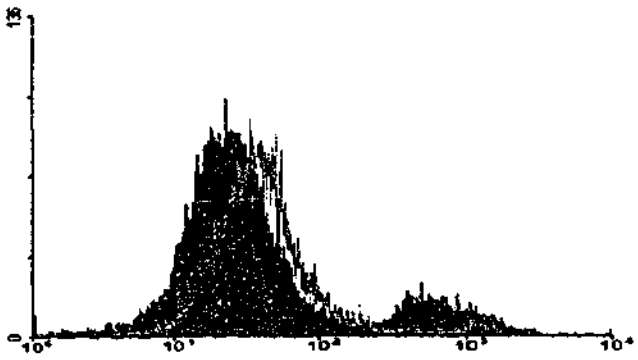
Overall, these results suggest that the effect of *Fasciola sp.* ESP on CD8 expression is complex. There is an early up-regulation of CD8 expression on immune cells after 3hrs incubation with either *Fasciola sp.* ESP. However, a down regulation in CD8 expression is observed on sheep efferent lymphocytes and afferent dendritic cells in the presence of either *Fasciola sp.* ESP over 5-16 hr incubation period. *F. gigantica* ESP also down regulates CD8 expression on afferent lymphocytes over 5-16 hr incubation period. The down regulation of CD8 expression on sheep immune cells after a 5 hr incubation with *Fasciola sp.* ESP is focussed on in the following experiments.

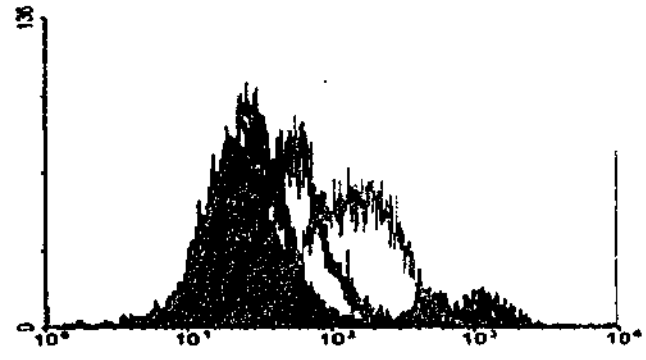
(c) CD1b

CD1b expression on afferent dendritic cells increased over time in the presence of either *Fasciola* ESP (Fig 4.2c). A notable positive shift in the MFI of CD1b positive sheep cells was apparent after incubation for 1 hr with *Fasciola* ESP and this increased over the time course studied. Although the extent of up-regulation of CD1b levels varied between experiments, the up-regulation of expression was repeatable (Fig 4.2c, Table 4.3c). Cells incubated in the

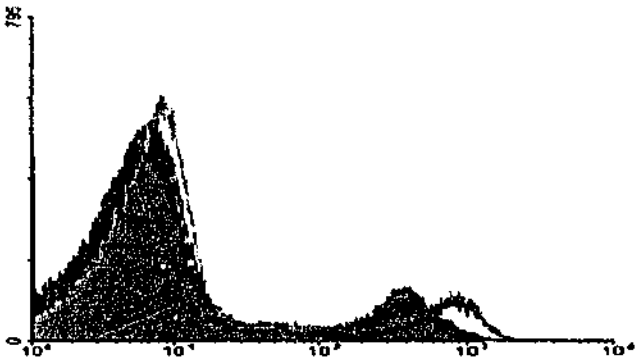
CD8

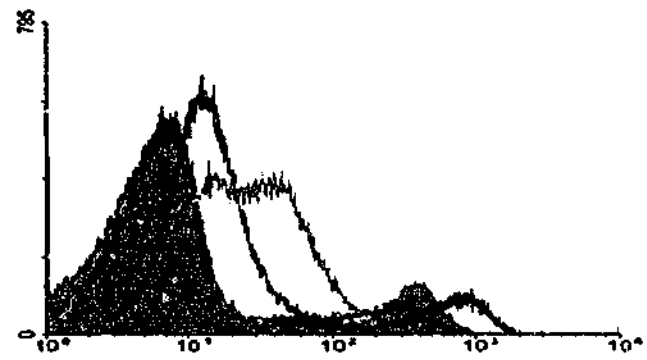
Afferent lymph - Dendritic Cells



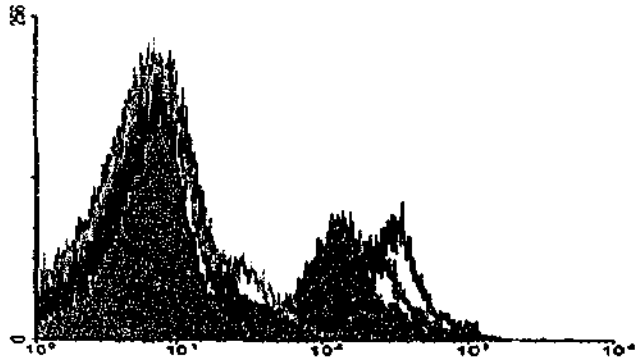


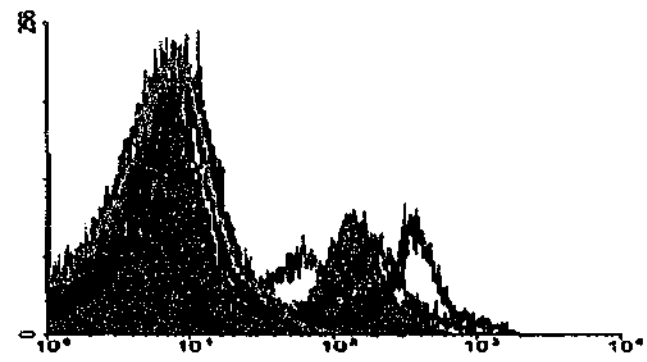
Afferent Lymph - Lymphocytes





Efferent Lymph - Lymphocytes





F. hepatica ESP

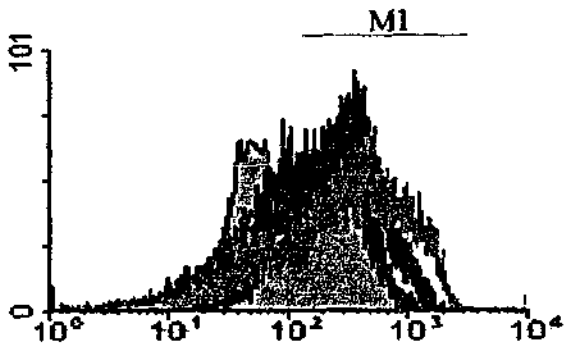
F. gigantica ESP

Fig 4.2b: The effect of *F. hepatica* ESP (250 µg/ml) and *F. gigantica* ESP (500 µg/ml) on the expression of the CD8 surface marker on sheep efferent lymph over a 16 hr period. The grey shaded area represents the normal CD8 expression on sheep cells after a 1 hr incubation in media alone. The brown, blue, red, and orange overlays represent the CD8 expression levels on sheep efferent lymph after 1, 3, 5, or 16 hr incubations respectively with antigen. Data in each plot represent a minimum of 2,500 events for dendritic cells and a minimum of 20,000 events for lymphocytes stained with mouse anti-sheep CD8 and FITC anti-mouse immunoglobulin. Histograms show the fluorescent intensity (X-axis) versus cell number (Y-axis). M1 represents the region for the MFI values displayed in Table 4.3b. Similar results were obtained in two separate experiments.

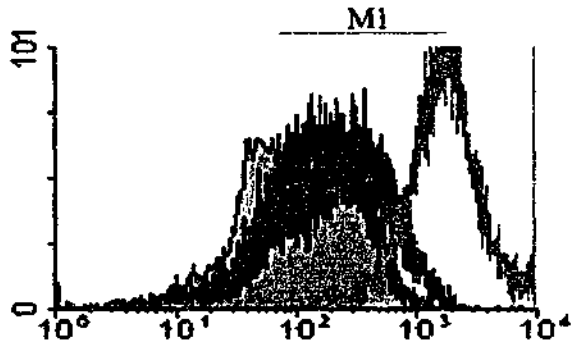
Sample	Events Gated	% Gated	Mean Fluorescent Intensity
Afferent Lymph – Dendritic Cells			
Media 1 hr	1062	8.5	508.4
F. hepatica ESP 250 µg/ml 1 hr	1678	13.3	640.9
F. hepatica ESP 250 µg/ml 3 hr	1165	7.9	1009.9
F. hepatica ESP 250 µg/ml 5 hr	457	2.6	663.4
F. hepatica ESP 250 µg/ml 16 hr	881	5.61	661.7
F. gigantica ESP 500 µg/ml 1 hr	1459	12.5	883.6
F. gigantica ESP 500 µg/ml 3 hr	973	5.6	913.8
F. gigantica ESP 500 µg/ml 5 hr	227	1.12	449.8
F. gigantica ESP 500 µg/ml 16 hr	6586	37.5	350.0
Afferent Lymph - Lymphocytes			
Media 1 hr	12513	11.6	325.6
F. hepatica ESP 250 µg/ml 1 hr	10187	10.4	370.2
F. hepatica ESP 250 µg/ml 3 hr	16102	12.6	577.5
F. hepatica ESP 250 µg/ml 5 hr	18366	12.6	339.4
F. hepatica ESP 250 µg/ml 16 hr	19706	12.4	592.1
F. gigantica ESP 500 µg/ml 1 hr	11285	12.0	549.4
F. gigantica ESP 500 µg/ml 3 hr	20335	13.0	362.9
F. gigantica ESP 500 µg/ml 5 hr	19651	10.8	262.6
F. gigantica ESP 500 µg/ml 16 hr	7069	4.3	374.5
Efferent Lymph - Lymphocytes			
Media 1 hr	10660	23.3	122.3
F. hepatica ESP 250 µg/ml 1 hr	10930	24.3	153.7
F. hepatica ESP 250 µg/ml 3 hr	11840	25.9	200.4
F. hepatica ESP 250 µg/ml 5 hr	11511	25.0	120.6
F. hepatica ESP 250 µg/ml 16 hr	5570	11.7	40.6
F. gigantica ESP 500 µg/ml 1 hr	10637	23.6	149.5
F. gigantica ESP 500 µg/ml 3 hr	12163	26.2	206.7
F. gigantica ESP 500 µg/ml 5 hr	10066	21.0	69.0
F. gigantica ESP 500 µg/ml 16 hr	3354	7.0	35.5

Table 4.3b: CD8 expression levels on sheep immune cells after exposure to *Fasciola* ESP over a time course. Data were collected from the experiment shown in Fig 4.2b. MFI values were measured on the events within the M1 region shown in Fig 4.2b. Similar results were obtained in two separate experiments.

CD1b



F. hepatica ESP



F. gigantica ESP

Fig 4.2c: The effect of *F. hepatica* ESP (250 µg/ml) and *F. gigantica* ESP (500 µg/ml) on the expression of the CD1b surface marker on sheep afferent lymph dendritic cells over a 16 hr period. The grey shaded area represents the normal CD1b expression on sheep cells after a 1 hr incubation in media alone. The brown, blue, red, and orange overlays represent the CD1b expression levels on sheep efferent lymph after 1, 3, 5, or 16 hr incubations respectively with antigen. Data in each plot represent a minimum of 2,500 events for dendritic cells stained with mouse anti-sheep CD1b and FITC anti-mouse immunoglobulin. Histograms show the fluorescent intensity (X-axis) versus cell number (Y-axis). M1 represents the region for the MFI values displayed in Table 4.3c. Similar results were obtained in two separate experiments.

Sample	Events Gated	% Gated	Mean Fluorescent Intensity
Media 1 hr	8385	49.3	290.6
F. hepatica ESP 250 µg/ml 1 hr	7637	45.5	290.4
F. hepatica ESP 250 µg/ml 3 hr	11251	69.0	364.2
F. hepatica ESP 250 µg/ml 5 hr	11095	67.8	364.7
F. hepatica ESP 250 µg/ml 16 hr	12545	72.3	486.3
F. gigantica ESP 500 µg/ml 1 hr	8225	47.4	269.4
F. gigantica ESP 500 µg/ml 3 hr	10664	62.8	381.2
F. gigantica ESP 500 µg/ml 5 hr	10721	60.0	361.5
F. gigantica ESP 500 µg/ml 16 hr	14927	93.0	1491

Table 4.3c: CD1b expression levels on sheep dendritic cells after exposure to *Fasciola* ESP over a time course. Data were collected from the experiment shown in Fig 4.2c. MFI values were measured on the events within the M1 region shown in Fig 4.2c. Similar results were obtained in two separate experiments.

presence of *F. gigantica* ESP appeared to autofluoresce after the overnight incubation (Fig 4.2c, Table 4.3c). This opinion was based on the excessively high fluorescence along with the change in the structure of the CD1b expression profile. Exposure to *Fasciola* ESP thus induced a prolonged up-regulation of expression of CD1b on sheep dendritic cells over the time course analysed *in vitro*.

Overall, these results suggest that *Fasciola sp.* ESP induces a down regulation in CD4 expression on immune cells, an up regulation of CD1b expression levels on dendritic cells and a bipolar effect on CD8 expression levels on immune cells. A 5 hr incubation of sheep immune cells with *Fasciola sp.* ESP was deemed the optimal time to observe notable shifts for these markers and this incubation time was used in subsequent experiments.

4.2.3 Mechanism of Action of *Fasciola sp.* ESP in Determining the Changes in CD4 and CD8 Expression on Sheep Cells

To ascertain whether CD4 and CD8 were being cleaved from sheep afferent lymphocytes, afferent lymph was fixed with 1 % paraformaldehyde and then incubated with *F. hepatica* (250 μg/ml) or *F. gigantica* ESP (500 μg/ml) for 5 hrs. If CD4 or CD8 were being actively down-regulated in the presence of *Fasciola sp.* ESP by an indirect effect of ESP on some other T cell component there would be little change of CD4 or CD8 expression on the fixed T cells. However, if CD4 and/or CD8 were being directly cleaved from the surface by protease activity in ESP, a reduction in the expression of these surface makers on T cells would still occur on fixed cells.

The results show a notable decrease in the MFI for CD4 on fixed afferent lymph dendritic cells and afferent lymphocytes in the presence of either *F. hepatica* ESP or *F. gigantica* ESP compared with CD4 expression on the same fixed cells incubated in media alone (Fig 4.3a, Table 4.4a). This implies that CD4 was being cleaved from the cell surface. CD8 expression was also decreased on fixed afferent lymph cells incubated with either *Fasciola sp.* ESP for 5 hrs (Fig 4.3b, Table 4.4b). The MFI for CD8 expressing cells decreased while the percentage of cells

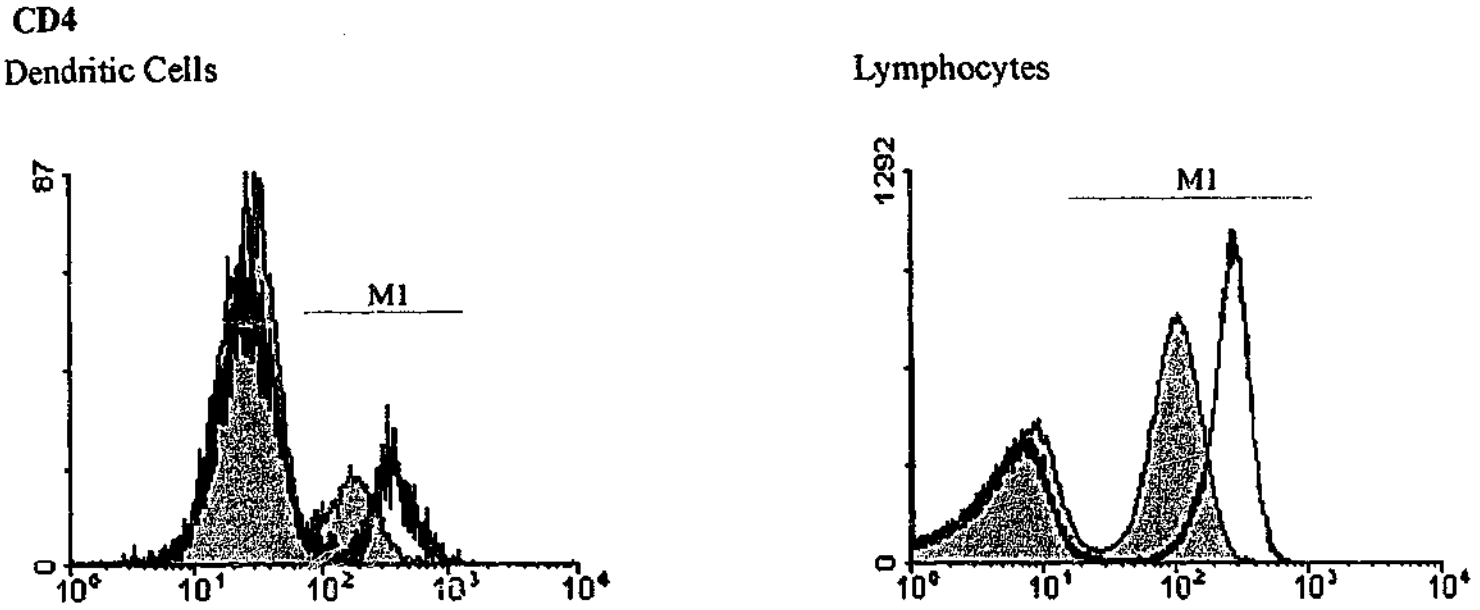


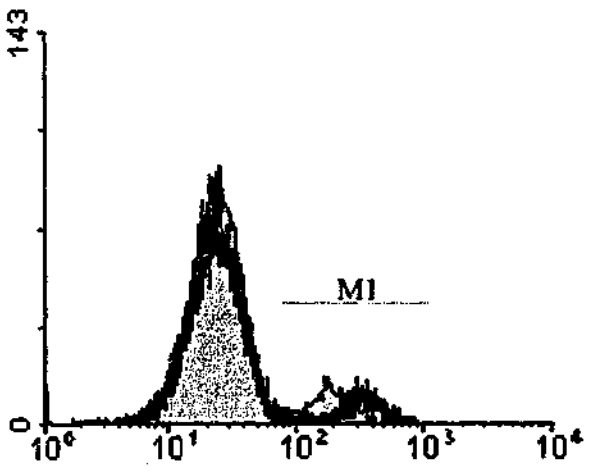
Fig 4.3a: The effect of *F. hepatica* ESP (250 µg/ml) and *F. gigantica* ESP (500 µg/ml) on the expression of CD4 on fixed sheep afferent lymph (gated for dendritic cells or lymphocytes) after a 5hr incubation. Sheep immune cells were firstly fixed with 1 % paraformaldehyde and then incubated for 5 hrs with either *Fasciola sp.* ESP. The grey shaded area represents CD4 expression on fixed sheep cells in the presence of *F. hepatica* or *F. gigantica* ESP after a 5 hr incubation while the black overlay shows CD4 expression on the identical population of fixed sheep cells incubated for the same incubation in media alone. Data in each plot represent a minimum of 2,500 events for dendritic cells and a minimum of 20,000 events for lymphocytes stained with mouse anti-sheep CD4 and FITC anti-mouse immunoglobulin. Histograms show the fluorescent intensity (X-axis) versus cell number (Y-axis). M1 represents the region for the MFI values displayed in Table 4.4a. Similar results were obtained in three separate experiments.

Sample	Events Gated	% Gated	Mean Fluorescent Intensity
Afferent Lymph – Dendritic Cells			
Media 1 hr	3017	22.7	337.4
F. hepatica ESP 250 µg/ml	1703	16.3	178.8
F. gigantica ESP 500 µg/ml	2032	18.8	201.2
Afferent Lymph - Lymphocytes			
Media 1 hr	89469	56.3	240.5
F. hepatica ESP 250 µg/ml	89778	56.4	96.5
F. gigantica ESP 500 µg/ml	98614	55.2	102.3

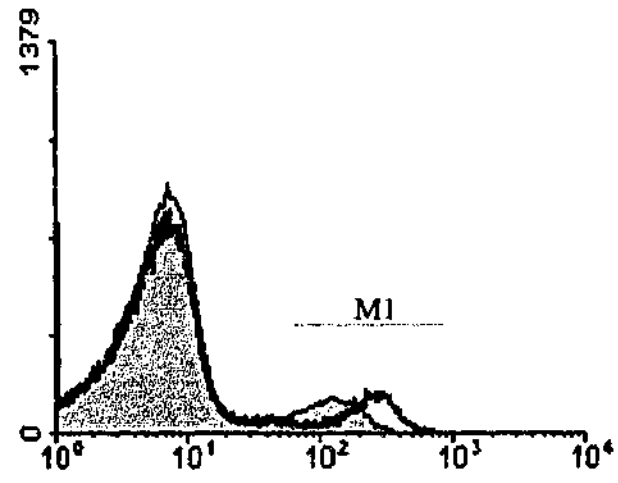
Table 4.4a: CD4 expression levels on sheep immune cells fixed in 1% paraformaldehyde and then exposed to *Fasciola sp.* ESP. Data were collected from the experiment shown in Fig 4.3a. MFI values were measured on the events within the M1 region shown in Fig 4.3a. Similar results were obtained in three separate experiments.

CD8

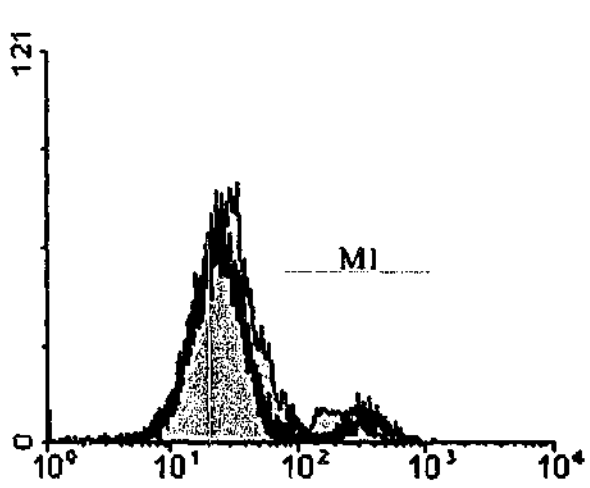
Dendritic Cells

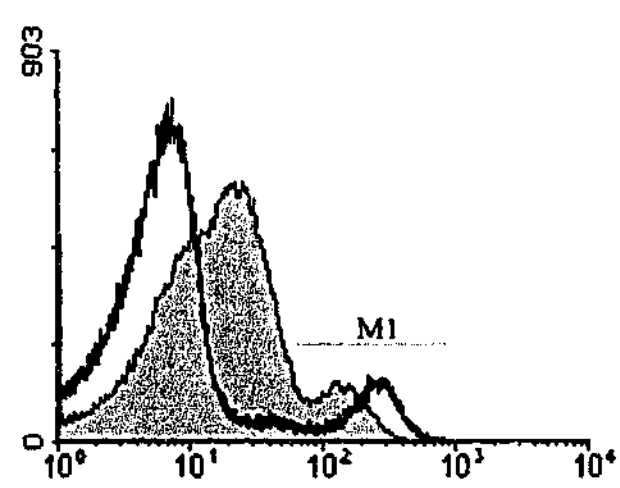


Lymphocytes



F. hepatica ESP





F. gigantica ESP

Fig 4.3b: The effect of *F. hepatica* ESP (250 µg/ml) and *F. gigantica* ESP (500 µg/ml) on the expression of CD8 on fixed sheep afferent lymph (gated for dendritic cells or lymphocytes) after a 5hr incubation. Sheep immune cells were firstly fixed with 1 % paraformaldehyde and then incubated for 5 hrs with either *Fasciola sp.* ESP. The grey shaded area represents CD8 expression on fixed sheep cells in the presence of *F. hepatica* or *F. gigantica* ESP after a 5 hr incubation while the black overlay shows CD8 expression on the identical population of fixed sheep cells incubated for the same incubation in media alone. Data in each plot represent a minimum of 2,500 events for dendritic cells and a minimum of 20,000 events for lymphocytes stained with mouse anti-sheep CD8 and FITC anti-mouse immunoglobulin. Histograms show the fluorescent intensity (X-axis) versus cell number (Y-axis).M1 represents the region for the MFI values displayed in Table 4.4b. Similar results were obtained in three separate experiments.

Sample	Events Gated	% Gated	Mean Fluorescent Intensity
Afferent Lymph – Dendritic Cells			
Media 1 hr	1561	11.8	306.3
F. hepatica ESP 250 µg/ml	902	9.2	196.5
F. gigantica ESP 500 µg/ml	880	10.0	186.0
Afferent Lymph - Lymphocytes			
Media 1 hr	17798	11.9	188.2
F. hepatica ESP 250 µg/ml	15756	10.3	114.2
Media 1 hr	15038	10.1	237.8
F. gigantica ESP 500 µg/ml	12900	8.4	149.8

Table 4.4b: CD8 expression levels on sheep immune cells fixed in 1% paraformaldehyde and then exposed to *Fasciola sp.* ESP. Data were collected from the experiment shown in Fig 4.3b. MFI values were measured on the events within the M1 region shown in Fig 4.3b. Similar results were obtained in three separate experiments.

expressing CD8 remained rather constant (Table 4.4b) This suggests that there was a reduction in the number of CD8 surface molecules on each individual cell.

In order to determine whether T cells were capable of up regulating CD4 expression levels following exposure to *F. hepatica* ESP, sheep efferent T cells were incubated with *F. hepatica* ESP for 5 hours, washed to remove ESP components, and then incubated for a further 18 hr in medium alone. This cell population exhibited a notably higher proportion of $CD4^+$ T cells (Fig 4.4) than cells incubated with *F. hepatica* ES for 5 hr (Fig 4.4). Relative levels of CD4 expression was estimated by comparison of MFI values (82 and 22 respectively, Fig 4.4). These results suggest that CD4 expression can be up-regulated on sheep T cells when *F. hepatica* ESP is removed from the system, although the level of CD4 expression did not return to control levels (Fig 4.4).

These observations imply that CD4 and CD8 are being cleaved from the surface of sheep immune cells and that CD4 can be up-regulated upon the removal of *F. hepatica* ESP, but do not demonstrate which component in ESP is responsible for the down regulation.

4.2.4 Identification of the Active Component Within *Fasciola sp.* ESP Responsible for Changes in CD4, CD8 and CD1b Expression on Sheep Lymphocytes and Dendritic Cells

As cathepsin L was shown to cause suppression of cellular proliferation in the previous chapter, we firstly investigated whether cathepsin L activity was responsible for the effects observed on CD4, CD8 and CD1b surface expression through the use of the cysteine inhibitor E64. Efferent lymph was incubated with either *F. hepatica* ESP (50, 100, 200 or 250 μg/ml) or *F. gigantica* ESP (50, 100, 250, or 500 μg/ml) with and without E64 (10 μM) for 5 hr at 37 °C and then stained for CD4 expression (Fig 4.5a). The addition of E64 to *F. hepatica* or *F. gigantica* ESP at any of the concentrations investigated restored the expression of CD4 to levels on efferent T lymphocytes (Fig 4.5a, Table 4.5a).

To further characterise the role of cysteine proteases in CD4 cleavage, the effect of rFhCatL proteases on efferent $CD4^+$ T cells was analysed. Two rFhCatL were available. rFhCatL5

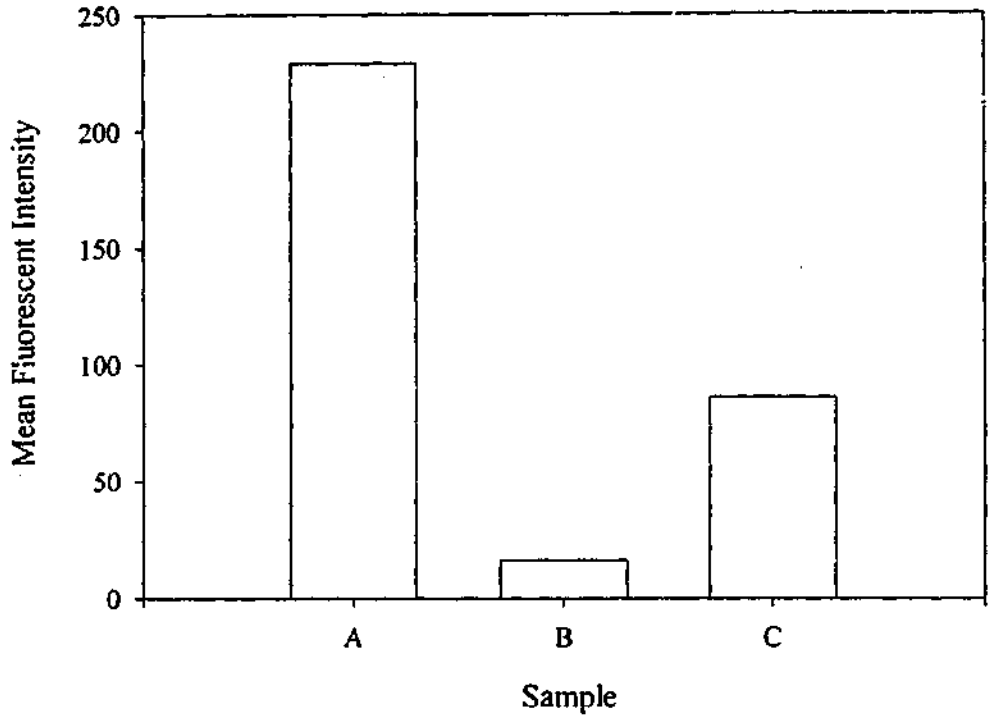
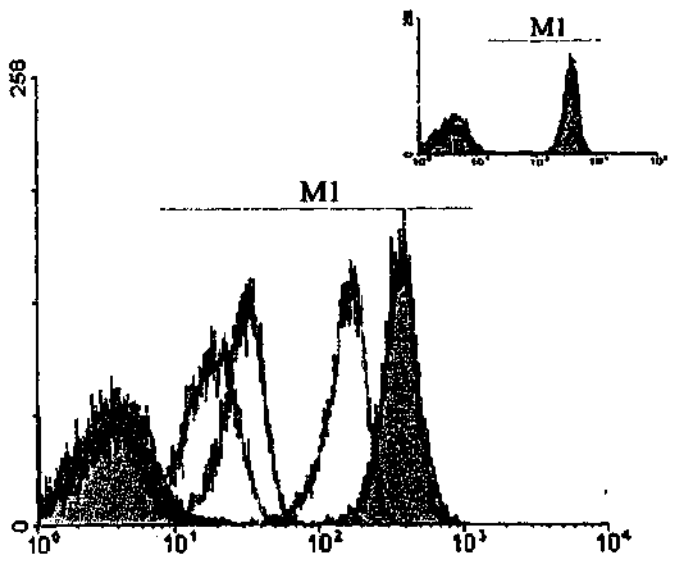
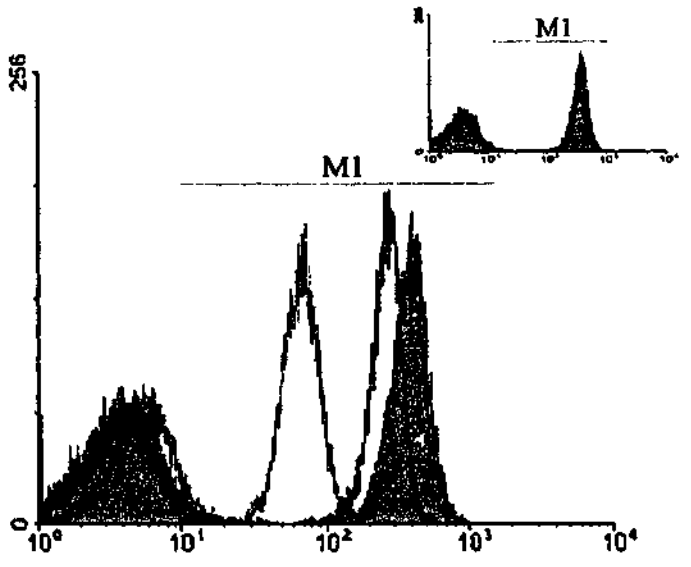


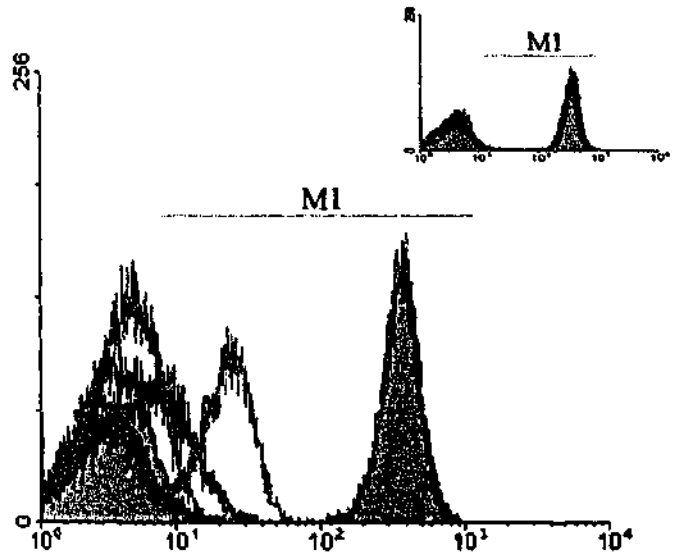
Figure 4.4: CD4 expression levels on sheep efferent lymph T lymphocytes after various incubations with *F. hepatica* ES at 250 μg/ml. Cells were incubated as follows: media alone (Fig 4.4A), *F. hepatica* ES for 5 hr (Fig 4.4B), or were incubated with *F. hepatica* ES for 5 hr, washed 3 times and then incubated for 18 hr in media alone (Fig 4.4C). Data in each plot represent 20,000 events for cells stained with mouse anti-sheep CD4 and FITC anti-mouse immunoglobulin. Histograms show the fluorescent intensity of the CD4 positive peak (X-axis) versus cell number (Y-axis). Similar results were obtained in two separate experiments with samples assayed in duplicate.



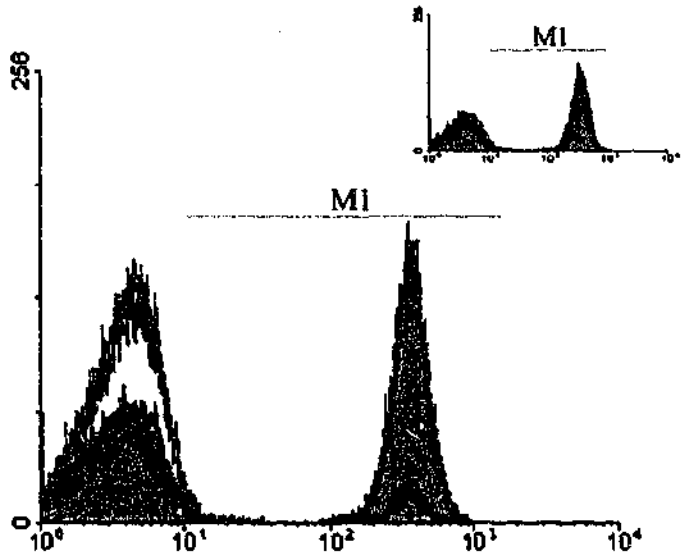
F. hepatica ESP



F. gigantica ESP



rFhCatL69Y



rFhCatL5

Fig 4.5a: The effect of E64 on the down regulation of CD4 on sheep immune cells after exposure to *Fasciola* ESP. *F. hepatica* ESP (50, 100, 200, or 250 μg/ml), *F. gigantica* ESP (50, 100, 250, or 500 μg/ml), rFhCatL69Y (5, 10, 15 or 20 μg/ml) and rFhCatL5 (5, 10, 15 or 20 μg/ml) were incubated with or without E64 with sheep efferent lymph (gated for lymphocytes) for 5hr. The grey shaded area represents CD4 expression on sheep cells in the presence of media, the brown, green, blue and red overlays represent increasing concentrations of *F. hepatica* ESP, *F. gigantica* ESP, rFhCatL69Y and rFhCatL5 respectively after a 5 hr incubation. The inserted graphs represent CD4 expression on the identical population of sheep cells incubated with the same concentrations of antigen in the presence of E64 (10 μM). Data in each plot represent a minimum of 20,000 events for lymphocytes stained with mouse anti-sheep CD4 and FITC anti-mouse immunoglobulin. Histograms show the fluorescent intensity (X-axis) versus cell number (Y-axis). M1 represents the region for the MFI values displayed in Table 4.5a. Similar results were obtained in three separate experiments.

Sample	Events Gated	% Gated	Mean Fluorescent Intensity
Media 1 hr	10982	54.0	289
F. hepatica ESP 50 µg/ml	11307	56.3	296.2
F. hepatica ESP 100 µg/ml	11019	55.2	141.4
F. hepatica ESP 200 µg/ml	11014	55.9	29.6
F. hepatica ESP 250 µg/ml	10657	53.4	18.7
F. hepatica ESP 50 µg/ml + E64	10924	54.2	295.2
F. hepatica ESP 100 µg/ml + E64	10905	54.7	277.1
F. hepatica ESP 200 µg/ml + E64	11885	59.8	323.9
F. hepatica ESP 250 µg/ml + E64	11968	59.1	326.5
F. gigantica ESP 50 µg/ml	11448	57.8	299.1
F. gigantica ESP 100 µg/ml	11257	56.5	320.1
F. gigantica ESP 250 µg/ml	11266	56.4	230.4
F. gigantica ESP 500 µg/ml	12656	63.2	53.8
F. gigantica ESP 50 µg/ml + E64	10895	54.2	319.4
F. gigantica ESP 100 µg/ml + E64	11237	56.5	266.6
F. gigantica ESP 250 µg/ml + E64	10433	52.7	325.1
F. gigantica ESP 500 µg/ml + E64	11203	59.6	220.7
recFhCatL5 5 µg/ml	11316	57.4	310.1
recFhCatL5 10 µg/ml	11652	58.9	302.5
recFhCatL5 15 µg/ml	2454	12.5	69.8
recFhCatL5 20 µg/ml	1128	5.6	10.3
recFhCatL5 5 µg/ml + E64	10617	53.6	265.7
recFhCatL5 10 µg/ml + E64	11678	58.2	274.7
recFhCatL5 15 µg/ml + E64	11880	59.9	281.3
recFhCatL5 20 µg/ml + E64	9560	55.0	236.5
recFhCatL69Y 5 µg/ml	11215	56.2	305.2
recFhCatL69Y 10 µg/ml	10502	52.5	23.1
recFhCatL69Y 15 µg/ml	5104	25.9	12.6
recFhCatL69Y 20 µg/ml	2020	10.1	10.4
recFhCatL69Y 5 µg/ml + E64	10209	51.4	249.9
recFhCatL69Y 10 µg/ml + E64	10634	53.6	256.1
recFhCatL69Y 15 µg/ml + E64	11273	56.0	291.0
recFhCatL69Y 20 µg/ml + E64	11606	58.2	308.7

Table 4.5a: CD4 expression levels on sheep efferent lymphocytes after exposure to *Fasciola sp.* ESP and recombinant *F. hepatica* cathepsin Ls, with or without E64 for 5 hr. Data were collected from the experiment shown in Fig 4.5a. MFI values were measured on the events within the M1 region shown in Fig 4.5a. Similar results were obtained in three separate experiments.

belongs to group 1 which prefers a phenylalanine at P_2 and rFhCatL69Y belongs to group 2 which prefers a proline at P_2 (Smooker *et al.* 2000). Due to this difference in substrate specificity both these enzymes were analysed for their effect on CD4 expression levels since it was feasible that substrate specificity at the P2 position could affect activity against CD4. Efferent lymphocytes were incubated with rFhCatL5 (5, 10, 15 and 20 μg/ml) and rFhCatL69Y (5, 10, 15 and 20 μg/ml). 20 μg/ml of enzymatically active protein for either rFhCatL protease, resulted in total down regulation / cleavage of CD4 from the surface of efferent T lymphocytes (Fig 4.5a). The addition of E64 to either of the active recombinant proteases at any of the forementioned concentrations investigated resulted in normal expression levels of CD4 on efferent T lymphocytes (Fig 4.5a, Table 4.5a).

Afferent lymph was incubated with *F. hepatica* ESP (250 μg/ml) or *F. gigantica* ESP (500 μg/ml) with and without E64 and stained for CD4, CD8 or CD1b expression (Fig 4.5b, c, d). The down regulation of CD4 and CD8 on dendritic cells and afferent lymphocytes in the presence of either *Fasciola sp.* ESP after 5 hr was blocked in the presence of E64 (10 μM) (Fig 4.5b,c and Table 4.5b,c). The up-regulation of CD1b on dendritic cells by *Fasciola* ESP after 5 hr was also blocked in the presence of E64 (Fig 4.5d, Table 4.5d). It should be noted the positive control shift in CD1b expression was generally small making conclusions difficult.

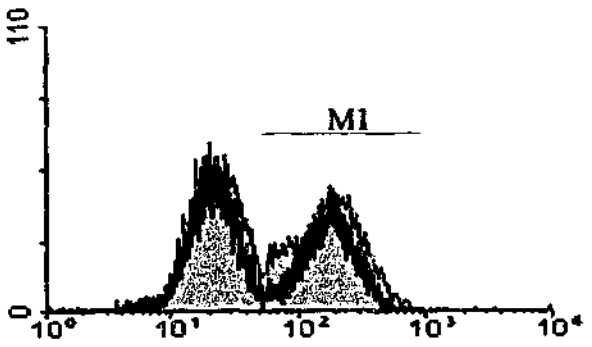
This is the first demonstration that *Fasciola sp.* ESP cysteine proteases have a direct effect on CD4, CD8 and CD1b expression on sheep immune cells. Cysteine protease activity, within *Fasciola sp.* ESP, down regulates CD4 and CD8 expression and up regulates CD1b expression on sheep immune cells. It was also shown that two recombinant *F. hepatica* cathepsin Ls could down regulate CD4 expression on sheep efferent lymphocytes, substantiating that it is the cysteine proteases within ESP that are most likely responsible for the down regulation activity.

4.2.5 Effect of *Fasciola sp* ESP on Expression of CD3 and CD4 on Human T cells and CD4 on Bovine T cells

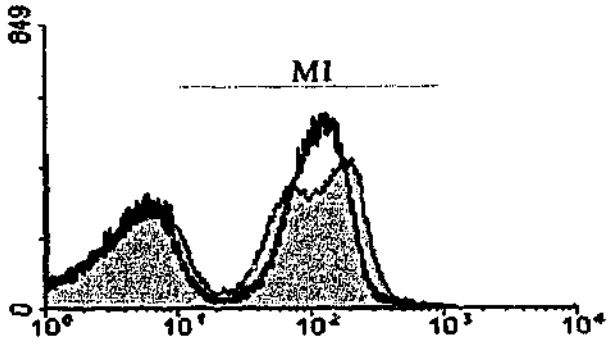
Fasciola parasites infect a range of mammalian hosts and it is therefore of interest to determine whether the effect of *Fasciola* cysteine proteases on CD4 expression levels was specific for

CD4

Dendritic cells



Lymphocytes



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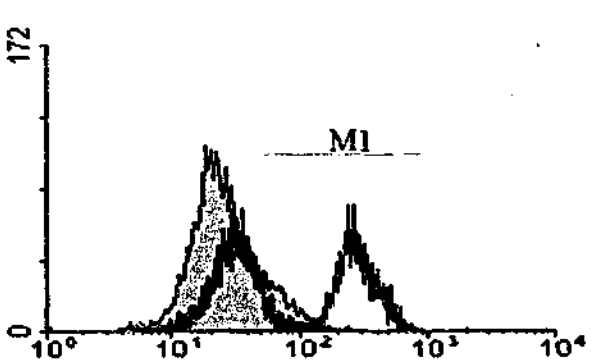
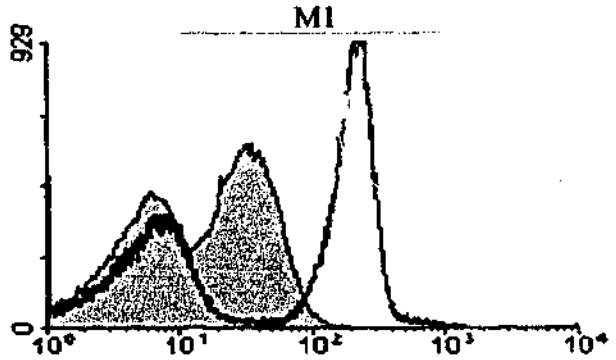
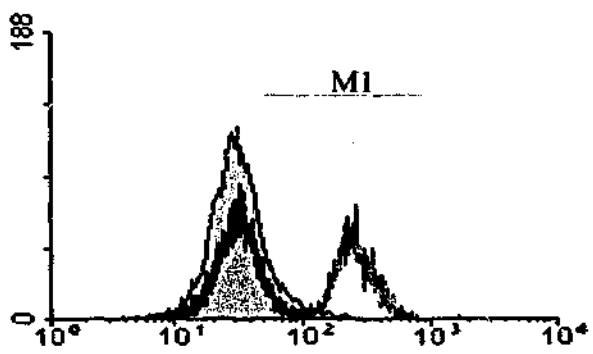
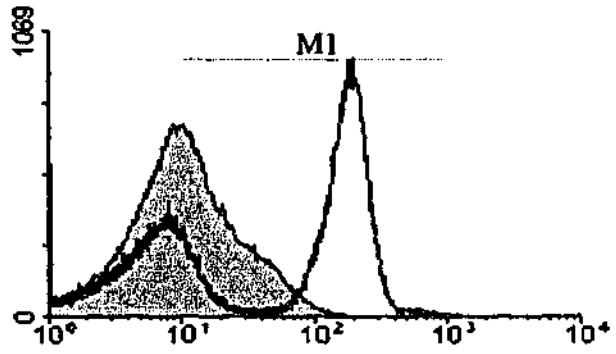




F. hepatica ESP





F. gigantica ESP

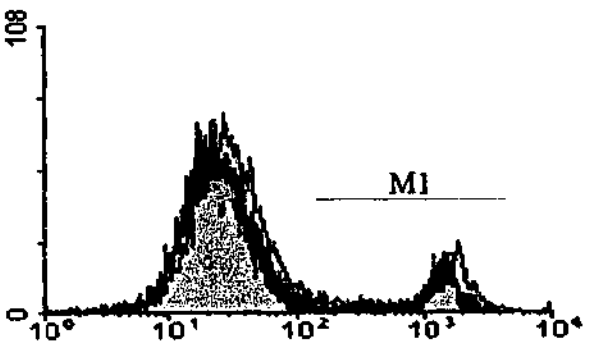
Fig 4.5b: The effect of E64 on the down regulation of CD4 on sheep immune cells after exposure to *Fasciola* ESP. *F. hepatica* ESP (250 μg/ml) and *F. gigantica* ESP (500 μg/ml) were incubated with or without E64 with sheep afferent lymph (gated for dendritic cells or lymphocytes) for 5hr. The grey shaded area represents CD4 expression on sheep cells in the presence of media, *F. hepatica* or *F. gigantica* ESP after a 5 hr incubation while the black overlay shows CD4 expression on the identical population of sheep cells incubated for the same incubation in the presence of E64 (10 μM). Data in each plot represent a minimum of 2,500 events for dendritic cells and a minimum of 20,000 events for lymphocytes stained with mouse anti-sheep CD4 and FITC anti-mouse immunoglobulin. Histograms show the fluorescent intensity (X-axis) versus cell number (Y-axis). M1 represents the region for the MFI values displayed in Table 4.5b. Similar results were obtained in three separate experiments.

Sample	Events Gated	% Gated	Mean Fluorescent Intensity
Afferent Lymph – Dendritic Cells			
Media	4968	50.0	170.3
Media + E64	4395	43.2	170.7
F. hepatica ESP 250 µg/ml	1578	14.7	74.6
F. hepatica ESP 250 µg/ml + E64	4275	50.2	229.8
F. gigantica ESP 500 µg/ml	1538	14.4	72.3
F. gigantica ESP 500 µg/ml + E64	4720	50.0	170.3
Afferent Lymph – Lymphocytes			
Media	83884	58.7	135.4
Media + E64	75340	56.9	166.1
F. hepatica ESP 250 µg/ml	73378	53.8	28.3
F. hepatica ESP 250 µg/ml + E64	80200	60.6	106.8
F. gigantica ESP 500 µg/ml	43919	31.9	20.3
F. gigantica ESP 500 µg/ml + E64	82151	62.3	145.5

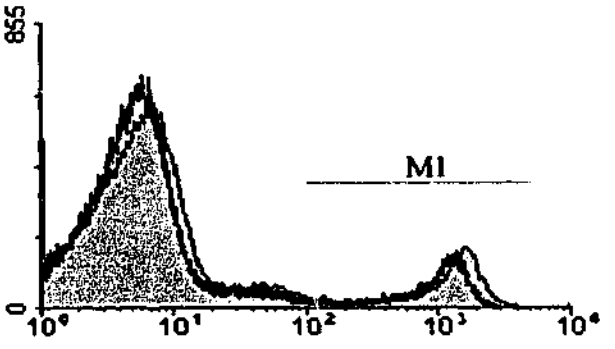
Table 4.5b: CD4 expression levels on sheep immune cells exposed to *Fasciola sp.* ESP with or without E64 for 5 hr. Data were collected from the experiment shown in Fig 4.5b. MFI values were measured on the events within the M1 region shown in Fig 4.5b. Similar results were obtained in three separate experiments.

CD8

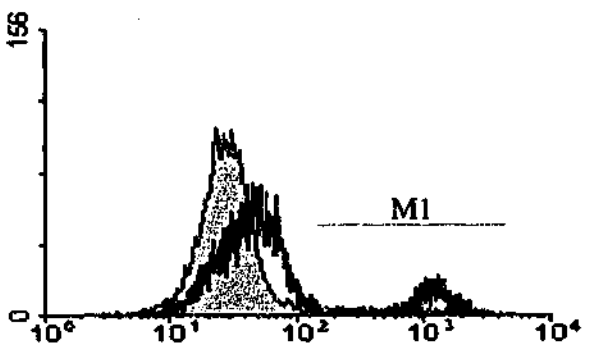
Dendritic cells

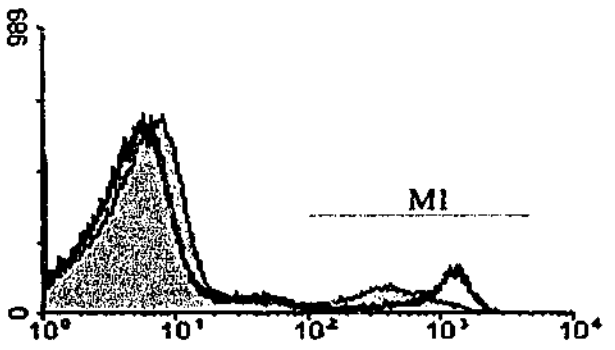


Lymphocytes

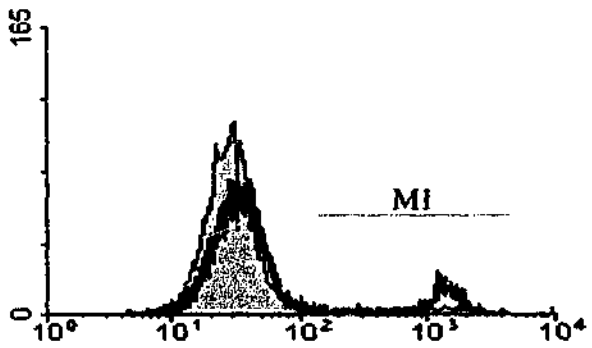


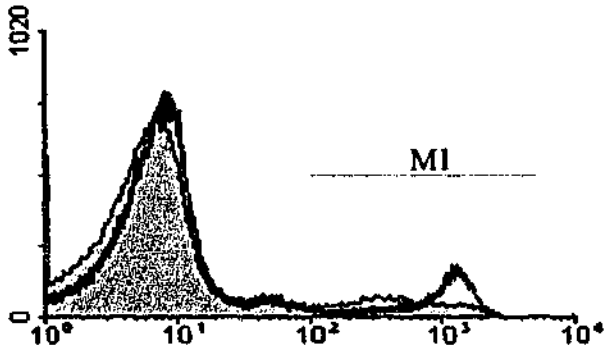
Control: Media Alone





F. hepatica ESP





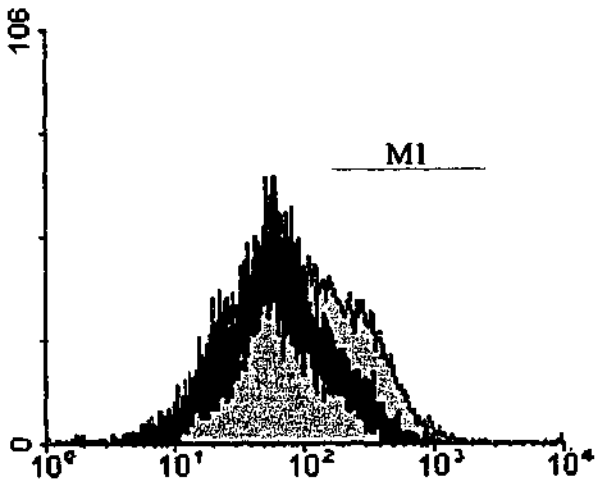
F. gigantica ESP

Fig 4.5c: The effect of E64 on the down regulation of CD8 on sheep immune cells after exposure to *Fasciola* ESP. *F. hepatica* ESP (250 μg/ml) and *F. gigantica* ESP (500 μg/ml) were incubated with and without E64 with sheep afferent lymph (gated for dendritic cells or lymphocytes) for 5hr. The grey shaded area represents CD8 expression on sheep cells in the presence of media, *F. hepatica* or *F. gigantica* ESP after a 5 hr incubation while the black overlay shows CD8 expression on the identical population of sheep cells incubated for the same incubation in the presence of E64 (10 μM). Data in each plot represent a minimum of 2,500 events for dendritic cells and a minimum of 20,000 events for lymphocytes stained with mouse anti-sheep CD8 and FITC anti-mouse immunoglobulin. Histograms show the fluorescent intensity (X-axis) versus cell number (Y-axis). M1 represents the region for the MFI values displayed in Table 4.5c. Similar results were obtained in three separate experiments.

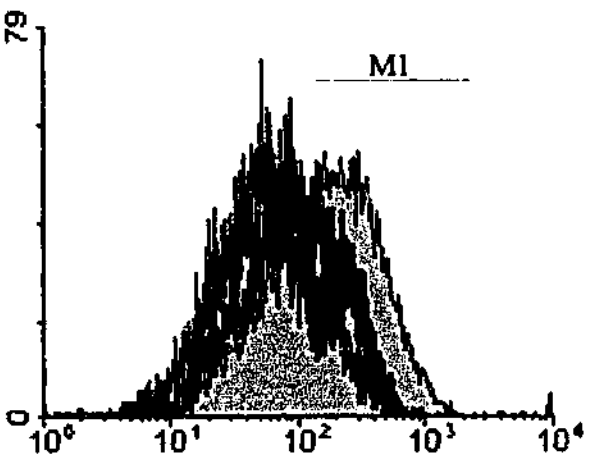
Sample	Events Gated	% Gated	Mean Fluorescent Intensity
Afferent Lymph – Dendritic Cells			
Media	1697	17.3	1056.5
Media + E64	1304	13.8	981.2
F. hepatica ESP 250 µg/ml	435	4.5	490.9
F. hepatica ESP 250 µg/ml + E64	1591	18.0	809.0
F. gigantica ESP 500 µg/ml	424	4.4	660.1
F. gigantica ESP 500 µg/ml + E64	1582	15.4	951.7
Afferent Lymph – Lymphocytes			
Media	16860	12.5	889.8
Media + E64	16017	12.5	780.4
F. hepatica ESP 250 µg/ml	15568	11.4	394.3
F. hepatica ESP 250 µg/ml + E64	15907	12.6	658.4
F. gigantica ESP 500 µg/ml	16283	11.5	431.9
F. gigantica ESP 500 µg/ml + E64	17150	12.4	774.8

Table 4.5c: CD8 expression levels on sheep immune cells exposed to *Fasciola sp.* ESP with or without E64 for 5 hr. Data were collected from the experiment shown in Fig 4.5c. MFI values were measured on the events within the M1 region shown in Fig 4.5c. Similar results were obtained in three separate experiments.

CD1b



F. hepatica ESP



F. gigantica ESP

Fig 4.5d: The effect of E64 on the up regulation of CD1b on sheep immune cells after exposure to *Fasciola* ESP. *F. hepatica* ESP (250 μg/ml) and *F. gigantica* ESP (500 μg/ml) were incubated with and without E64 with sheep afferent lymph (gated for dendritic cells) 5 hr. The grey shaded area represents CD1b expression on dendritic cells in the presence of *F. hepatica* or *F. gigantica* ESP after a 5 hr incubation while the black overlay shows CD1b expression on the identical population of sheep cells incubated for the same incubation in the presence of E64 (10 μM). The blue overlay represents CD1b expression on the identical cells population incubated in media alone. Data in each plot represent a minimum of 2,500 events for dendritic cells stained with mouse anti-sheep CD1b and FITC anti-mouse immunoglobulin. Histograms show the fluorescent intensity (X-axis) versus cell number (Y-axis). M1 represents the region for the MFI values displayed in Table 4.5d. Similar results were obtained in three separate experiments.

Sample	Events Gated	% Gated	Mean Fluorescent Intensity
Afferent Lymph – Dendritic Cells			
Media	2112	22.1	204.5
Media + E64	739	7.4	174.8
F. hepatica ESP 250 µg/ml	4709	47.9	250.7
F. hepatica ESP 250 µg/ml + E64	2310	24.2	203.7
F. gigantica ESP 500 µg/ml	6768	64.5	279.8
F. gigantica ESP 500 µg/ml + E64	4314	40.6	211.7

Table 4.5d: CD1b expression levels on sheep immune cells exposed to *Fasciola sp.* ESP with or without E64 for 5 hr. Data were collected from the experiment shown in Fig 4.5d. MFI values were measured on the events within the M1 region shown in Fig 4.5d. Similar results were obtained in three separate experiments.

sheep T cells or whether a similar effect of these proteases would be observed with T cells from other hosts. For this purpose, the effect of *Fasciola sp.* ESP and recombinant *F. hepatica* cathepsin Ls on CD4 expression on human T lymphocytes was examined. CD3 expression on human lymphocytes in the presence of *Fasciola sp.* ESP was also investigated, since CD4, in association with the CD3:TCR complex is required for T cell activation and subsequent proliferation (Janeway 1989, Janeway and Bottomly 1994). Experiments with ovine CD3 were not possible since, when these experiments were performed, there was no anti-ovine CD3 antibody available. Three different sources of human and bovine purified blood lymphocytes were examined in these studies.

Human T cells

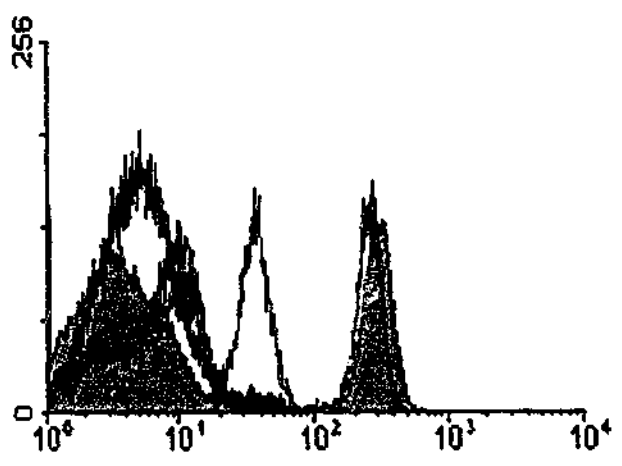
(a) CD3

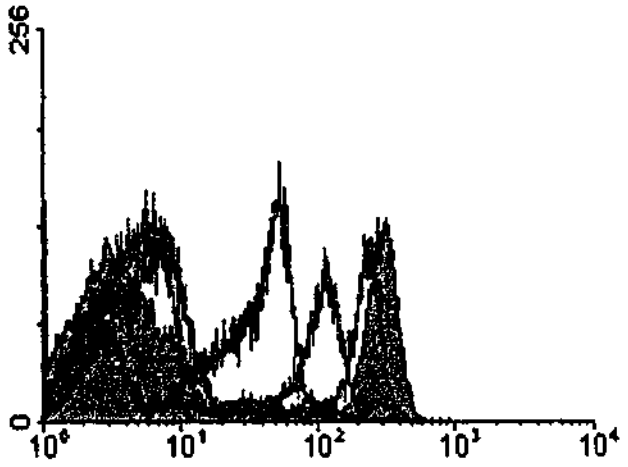
Human T lymphocytes were isolated from blood collected from volunteers and purified as described in Chapter 2.4. The effect of incubation of *F. hepatica* ESP (250 μg/ml) and *F. gigantica* ESP (400 μg/ml) on CD3 expression levels on human T lymphocytes was examined during a 5 hr incubation. *Fasciola sp.* ESP showed no effect on CD3 expression (Fig 4.6b). This would suggest that even though CD3 and CD4 are both necessary for T cell activation, *Fasciola* ESP is selective for the CD4 surface marker.

(b) CD4

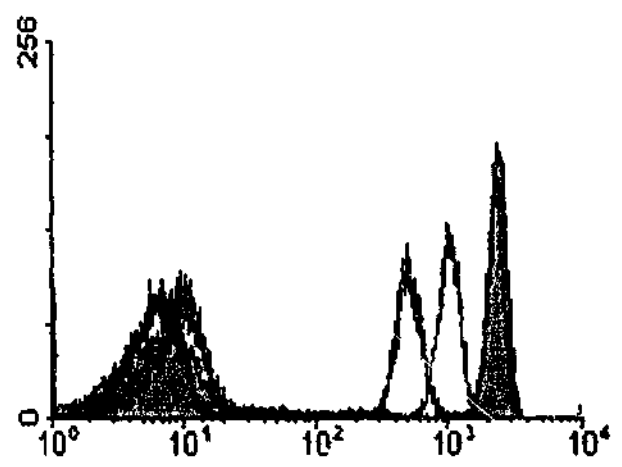
We used two different monoclonal antibodies specific for human CD4 which recognise different epitopes on the extracellular portion of CD4. The commercial antibody (T114) recognises epitope/s in domain 1 (D1) whereas the OKT4 antibody recognises epitope/s in domain 4 (D4). CD4 has four extra-cellular domains, with D4 being the closest to the membrane and D1 being the furthest away. *F. hepatica* ESP (50, 100 and 200 μg/ml) and *F. gigantica* ESP (100, 250 and 400 μg/ml) were incubated with 5×10^5 cells / well for 5 hrs (Fig 4.6a). The commercially available antibody (T114) and the OKT4 antibody recognised similar percentages of CD4 positive expressing cells (38 and 40 % respectively). Both antibodies revealed a down regulation of CD4 on human T lymphocytes following exposure to either *Fasciola sp.* ESP. As the concentration of *Fasciola* ESP increased the level of expression of CD4 on human lymphocytes

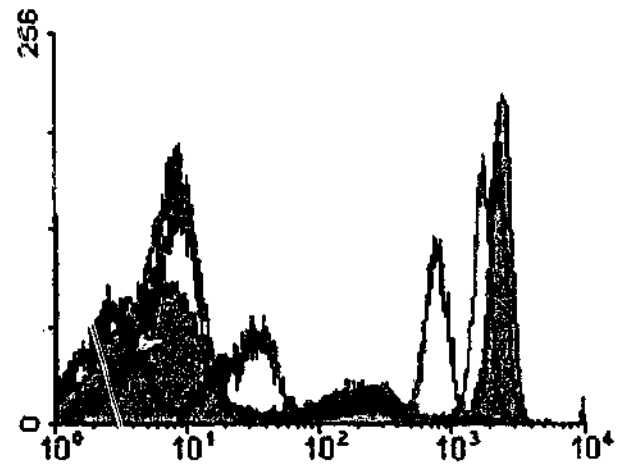
CD4 expression using PE mouse anti-human CD4 (T114)





CD4 expression using mouse anti-human CD4 (OKT4) and PE anti-mouse immunoglobulin





F. hepatica ESP

F. gigantica ESP

Fig 4.6a: The effect of *Fasciola* ESP on the expression of CD4 on human lymphocytes. *F. hepatica* ESP (50, 100, or 200 μg/ml) and *F. gigantica* ESP (50, 250 or 400 μg/ml) were incubated for 5 hours with human PBMCs (gated for lymphocytes). The grey shaded area represents CD4 expression on human lymphocytes in the presence of media while the brown, green and blue overlays represent the effects on CD4 expression of increasing concentrations of *F. hepatica* ESP and *F. gigantica* ESP respectively after 5 hr. Data in each plot represent a minimum of 20,000 events for lymphocytes stained with either PE mouse anti-human CD4 (T114) or mouse-anti human CD4 (OKT4) and PE anti mouse immunoglobulin. Histograms show the fluorescent intensity (X-axis) versus cell number (Y-axis). Similar results were obtained in two separate experiments.

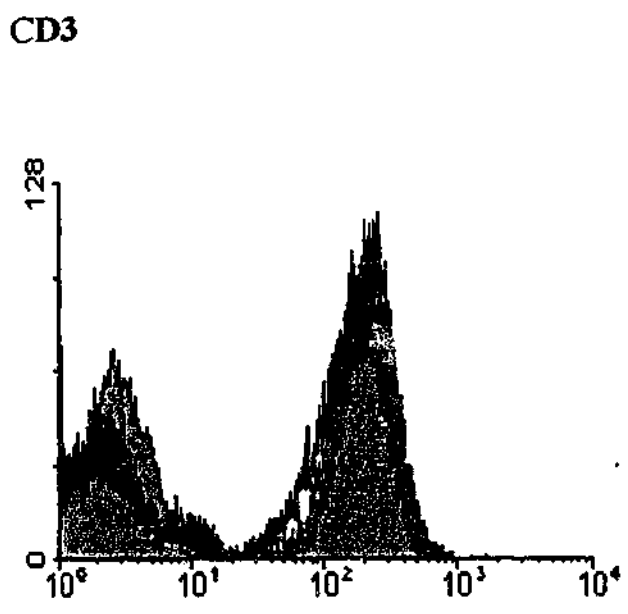


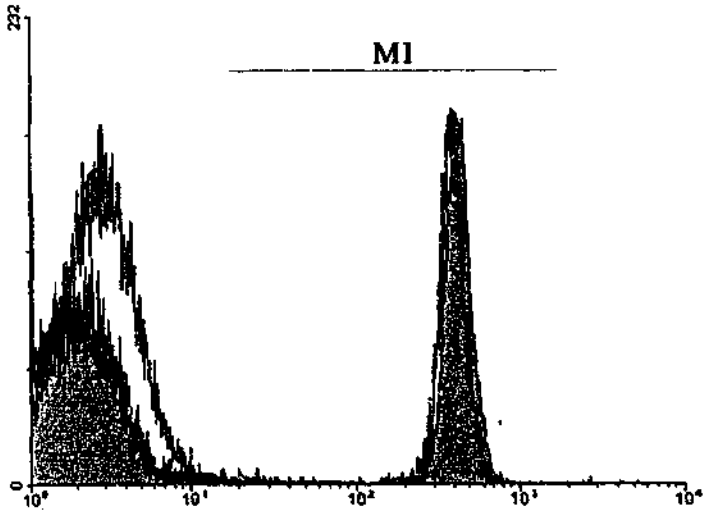
Fig 4.6b: The effect of *Fasciola* ESP on the expression of CD3 on human lymphocytes. *F. hepatica* ESP (250 μg/ml) and *F. gigantica* ESP (400 μg/ml) were incubated for 5 hours with human PBMCs (gated for lymphocytes). The grey shaded area represents CD3 expression on human lymphocytes in the presence of media while the green and blue overlays represent the effects of *F. hepatica* ESP and *F. gigantica* ESP respectively after 5 hr. Data in each plot represent a minimum of 20,000 events for lymphocytes stained with PE mouse anti-human CD3. Histograms show the fluorescent intensity (X-axis) versus cell number (Y-axis). Similar results were obtained in two separate experiments.

lymphocytes decreased (Fig 4.6a). Differing degrees of down regulation of CD4 expression levels were observed when comparing the results obtained with the two antibodies. Since these antibodies recognise different epitopes on the extracellular portion of CD4, these results suggest that cathepsin L may have multiple cleavage sites on CD4, resulting in CD4 expression being more markedly reduced when using the T114 available antibody that recognises epitopes on D1: these epitope/s will be "removed" from cells when cathepsin L cleaves any of the 4 extracellular domains of CD4 whereas D4 epitope/s recognized by OKT4 are only "removed" when cathepsin L acts directly upon the D4 domain. Subsequent experiments described below use the commercially available PE anti-CD4 antibody (T114) as it showed a greater decrease in the MFI of CD4 positive lymphocytes.

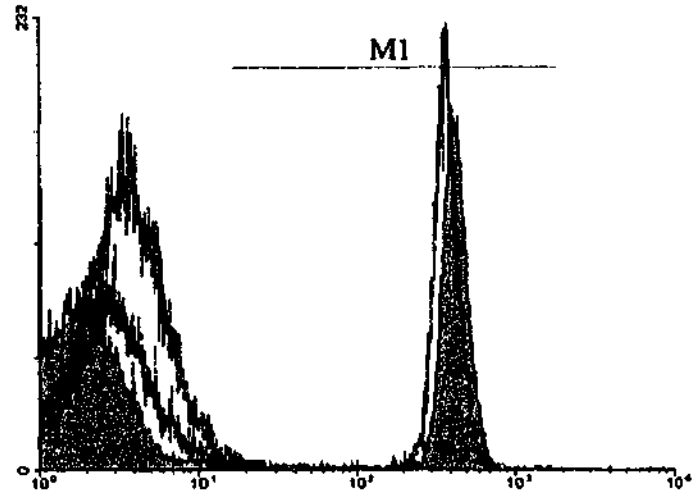
CD4 expression, on human T lymphocytes, was examined after a 5 hr incubations with *F. hepatica* ESP (250 μg/ml), rFhCatL5 (25 μg/ml), rFhCatL69Y (25 μg/ml) or *F. gigantica* ESP (400 μg/ml) with and without E64 (10 μM, Fig 4.7). A reduction in CD4 expression was observed on human lymphocytes in the presence of both *Fasciola* ESP and the two recombinant *F. hepatica* cathepsin L proteases at the concentrations investigated (Fig 4.7). The addition of E64 (10 μM) resulted in normal CD4 expression on human T lymphocytes (Fig 4.7, Table 4.6). These results strongly suggest a role for *Fasciola* cysteine proteases in reducing CD4 expression on human lymphocytes, as observed above with sheep lymphocytes.

Bovine T cells

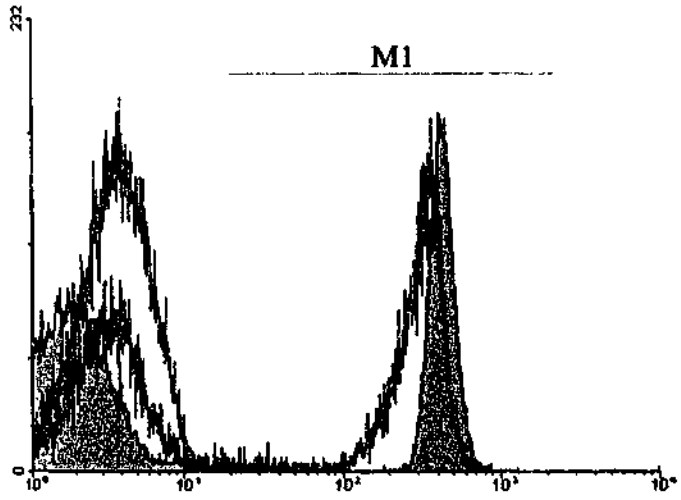
CD4 expression on bovine T lymphocytes was examined after a 5 hr incubation in the presence of *F. hepatica* ESP (100, 200 and 250 μg/ml) or *F. gigantica* ESP (50, 100, 200 and 400 mg/ml), with and without E64 (10 μM, Fig 4.8). As the concentration of *Fasciola sp.* ESP increased the level of expression of CD4 expression on bovine T cells was reduced (Table 4.7). The *F. hepatica* ESP showed higher activity with bovine CD4 than that shown by *F. gigantica* ESP (Fig 4.8). This is consistently observed between experiments and also within the three hosts examined (ovine, human and bovine). A preliminary experiment was performed and revealed comparable amounts of E64 were required to suppress cysteine protease activity within *F. hepatica* and *F. gigantica* (data not shown). Further work is therefore required to identify the



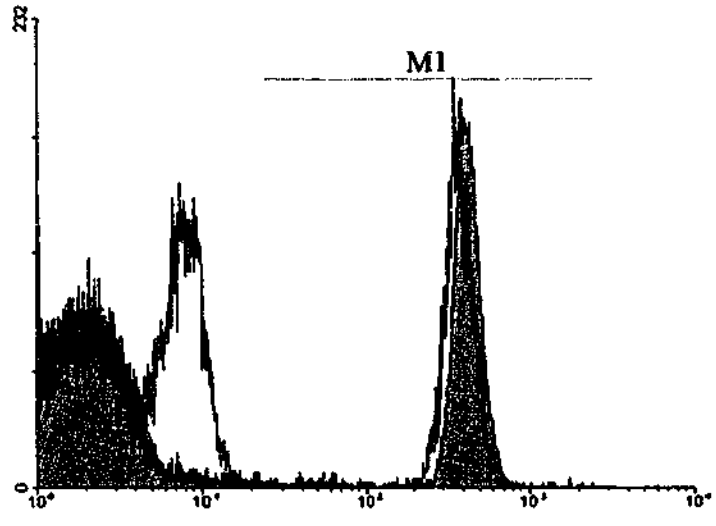
F. hepatica ESP



F. gigantica ESP



rFhCatL5



rFhCatL69Y

Fig 4.7: The effect of E64 on the down regulation of CD4 by *Fasciola* ESP and recombinant *F. hepatica* cathepsin Ls on human lymphocytes. Human PBMCs (gated for lymphocytes) were incubated with *F. hepatica* ESP (250 μg/ml), *F. gigantica* ESP (400 μg/ml), rFhCatL5 (25 μg/ml) or rFhCatL69Y (25 μg/ml) with and without E64for 5 hours. The grey shaded area represents CD4 expression on human lymphocytes in the presence of media, the brown overlay represents the effect of the antigens on CD4 expression while the blue overlay shows CD4 expression on the identical population of human lymphocytes incubated under the same conditions in the presence of E64 (10 μM) for 5 hr. Data in each plot represent a minimum of 20,000 events for lymphocytes stained with PE mouse anti-human CD4. Histograms show the fluorescent intensity (X-axis) versus cell number (Y-axis). M1 represents the region for the MFI values displayed in Table 4.6. Similar results were obtained in three separate experiments.

Sample	Events Gated	% Gated	Mean Fluorescent Intensity
Media	10220	52.3	355.5
Media + E64	6458	52.9	353.1
F. hepatica ESP 250 µg/ml	1474	7.4	8.0
F. hepatica ESP 250 µg/ml + E64	5225	50.0	317.52
F. gigantica ESP 400 µg/ml	2838	15.9	8.0
F. gigantica ESP 400 µg/ml + E64	7694	50.9	277.1
recFhCatL5 250 µg/ml	4820	26.4	7.7
recFhCatL5 250 µg/ml + E64	6817	52.6	162.2
recFhCatL69Y 250 µg/ml	6183	41.3	8.9
recFhCatL69Y 250 µg/ml + E64	4786	48.8	315.5

Table 4.6: CD4 expression levels on human T lymphocytes after exposure to *Fasciola* ESP or cathepsin L, with or without E64 for 5hr. Data were collected from the experiment shown in Fig 4.7. MFI values were measured on the events within the M1 region shown in Fig 4.7. Similar results were obtained in three separate experiments.

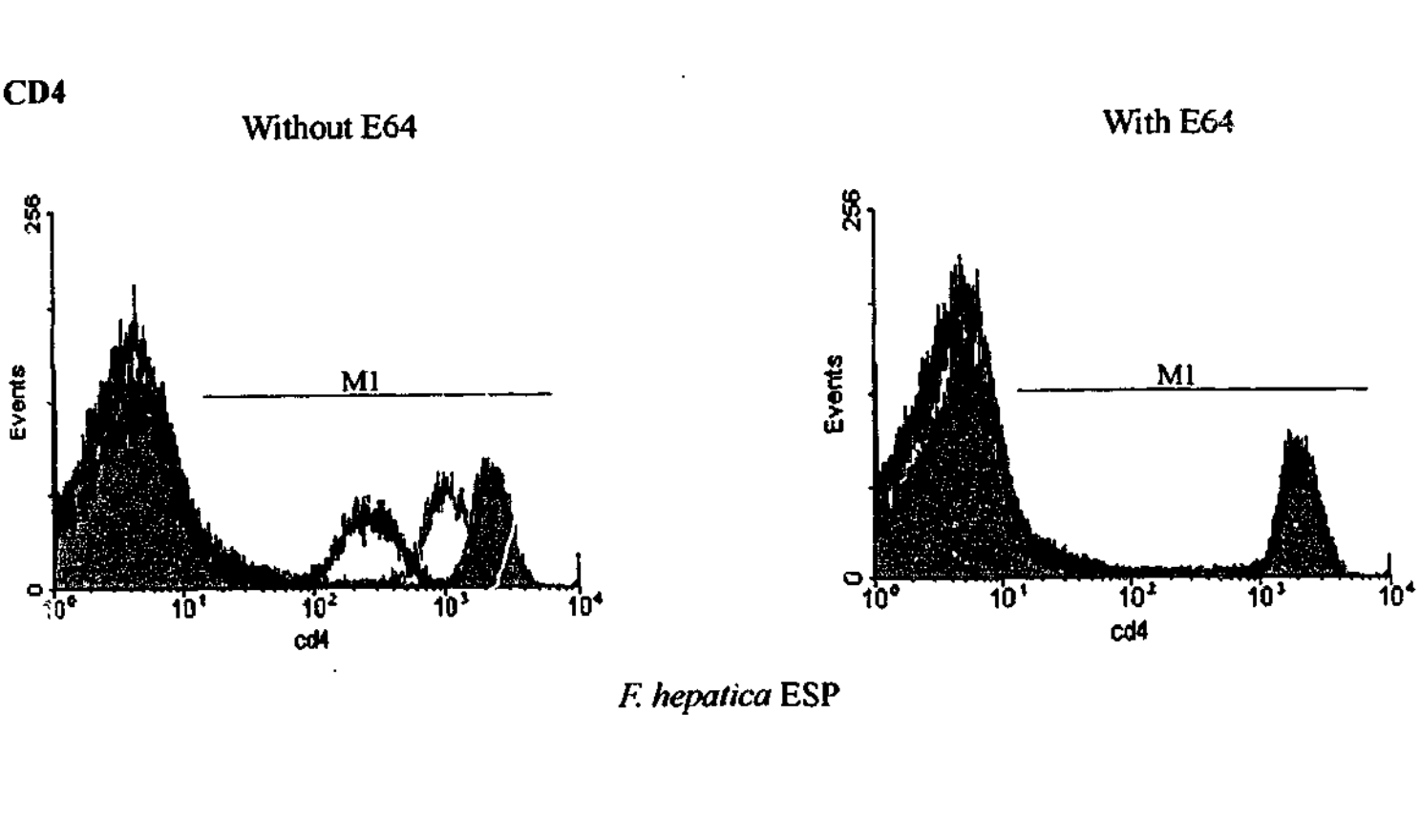


Fig 4.8: The effect of E64 on the down regulation of CD4 by *Fasciola* ESP and recombinant *F. hepatica* cathepsin Ls on bovine lymphocytes. Bovine PBMCs (gated for lymphocytes) were incubated with *F. hepatica* ESP (100, 200, or 250 μg/ml), *F. gigantica* ESP (50, 100, 200, or 400 μg/ml) with and without E64for 5 hours. For the histograms on the left hand side, the grey shaded area represents CD4 expression on human lymphocytes in the presence of media, the brown, green, blue and red overlays represent the effect of increasing concentrations of antigens on CD4 expression. While the graphs on the right hand side show CD4 expression on the identical population of human lymphocytes incubated under the same conditions in the presence of E64 (10 μM). Data in each plot represent a minimum of 20,000 events for lymphocytes stained with PE mouse anti-bovine CD4. Histograms show the fluorescent intensity (X-axis) versus cell number (Y-axis). M1 represents the region for the MFI values displayed in Table 4.7. Similar results were obtained in three separate experiments.

Sample	Events Gated	% Gated	Mean Fluorescent Intensity
Media	6744	19.5	1680.8
Media + E64	6966	17.9	1694.7
F. hepatica ESP 50 µg/ml	6753	19.0	1832.0
F. hepatica ESP 100 µg/ml	7318	20.3	880.13
F. hepatica ESP 200 µg/ml	6930	11.5	288.0
F. hepatica ESP 50 µg/ml + E64	7887	18.9	1641.5
F. hepatica ESP 100 µg/ml + E64	8024	21.0	1693.8
F. hepatica ESP 200 µg/ml + E64	8298	16.3	1529.0
F. gigantica ESP 50 µg/ml	7262	19.1	1682.7
F. gigantica ESP 100 µg/ml	7015	18.9	1754.4
F. gigantica ESP 250 µg/ml	7917	18.4	1531.1
F. gigantica ESP 500 µg/ml	12423	21.2	803.7
F. gigantica ESP 50 µg/ml + E64	7402	15.8	1593.3
F. gigantica ESP 100 µg/ml + E64	8333	19.9	1619.9
F. gigantica ESP 250 µg/ml + E64	8848	18.6	1501.6
F. gigantica ESP 500 µg/ml + E64	9192	19.5	1090.6

Table 4.7: CD4 expression levels on bovine blood T lymphocytes after exposure to *Fasciola* ESP or cathepsin L with or without E64 for 5 hr. Data were collected from the experiment shown in Fig 4.8. MFI values were measured on the events within the M1 region shown in Fig 4.8. Similar results were obtained in three separate experiments.

reason for this difference in CD4 down regulation. It may be that different cathepsin Ls are expressed in the two *Fasciola* ESP with a more active cathepsin L for CD4 cleavage expressed within *F. hepatica* ESP. The addition of E64 (10 μM) blocked the reduction in CD4 expression on bovine T lymphocytes reduced by exposure to *Fasciola sp.* ESP (Fig 4.8, Table 4.7). These preliminary findings imply that the cysteine protease activity within both *Fasciola sp.* ESP is responsible for the down regulation of CD4 expression on bovine T lymphocytes.

This is the first demonstration of *Fasciola* cysteine proteases down regulating CD4 expression on human and bovine lymphocytes.

4.2.6 Cleavage of Recombinant Human CD4 by Recombinant *F. hepatica* Cathepsin Ls *in vitro*

As discussed in the previous sections, cysteine protease activity within ESP of both *Fasciola sp.* was responsible for the down regulation of CD4 from the surface of sheep, cattle and human immune cells. These results were extended to show that two recombinant *F. hepatica* cathepsin L proteases could also down regulate CD4 on the surface of sheep and human immune cells. In this last section, we present preliminary findings on whether *F. hepatica* cathepsin Ls are likely to cleave recombinant human CD4 (rHuCD4).

rHuCD4 was incubated for different times up to 1.5 hr at 37°C with both rFhCatL5 and rFhCatL69Y. These samples were then analysed on a silver stained 15% SDS PAGE gel (Fig 4.9). rHuCD4 was totally broken down (represented by absence of observable bands) after 1.5 hr incubation with either recombinant protease. From the cleavage patterns obtained after 10, 20 and 30 min incubations, the generation of multiple bands is observed indicating cleavage is occurring in more than one position within rHuCD4. Further work using mass spectrometry or N-terminal amino acid sequencing is required to precisely identify cleavage sites on rHuCD4. Overall, these results imply that cathepsin L does cleave the extracellular portion of CD4 adding further weight to the earlier suggestions that cathepsin L is cleaving CD4 from the cell surface of human T cells.

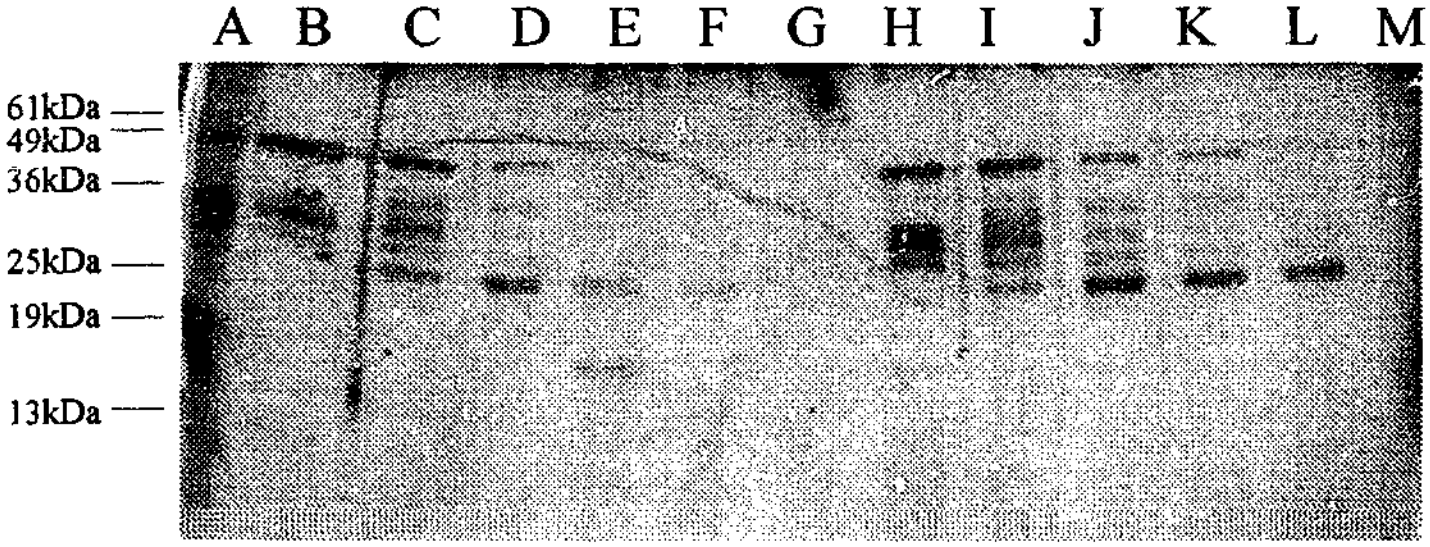


Fig 4.9: The cleavage of recombinant human CD4 (rHuCD4) by recombinant Cathepsin Ls over time.. rHuCD4 (4 μg/ml) was incubated at 37°C with 20 ng/ml of rFhCatL5 and rFhCatL69Y for a set time. Reactions were stopped by the addition of reducing sample buffer and were snap frozen on dry ice. These samples were then analysed on a silver stained 15% SDS PAGE gel. The individual lanes were rHuCD4 (A); rHuCD4 + rFhCatL5, t=0 (B); rHuCD4 + rFhCatL5, t=10min (C); rHuCD4 + rFhCatL5, t=20min (D); rHuCD4 + rFhCatL5, t=30min (E); rHuCD4 + rFhCatL5, t=1hr (F); rHuCD4 + rFhCatL5, t=1.5 hr (G); rHuCD4+ rFhCatL69Y, t=0 (H), rHuCD4 + rFhCatL69y, t=10 min (I); rHuCD4 + rFhCatL69Y, t=20 min (J); rHuCD4 + rFhCatL69Y, t=30min (K); rHuCD4 + rFhCatL69Y, t=1 hr (L); rHuCD4 + rFhCatL69Y, t=1.5 hr (M). This result is representative of two separate experiments.

4.3 Discussion

In these studies we attempted to identify the mode of action for cathepsin L in suppressing cellular proliferation by studying the interaction of *Fasciola sp.* ESP with a wide range of surface markers on sheep cells. It was hypothesised that such interactions may be involved in initiating suppression of cellular proliferation.

Two different sources of cells were employed using sheep with either an afferent or efferent cannula draining from the preformal and prescapular lymph nodes, respectively. Afferent lymph, migrating from the tissues into the local lymph node, contains lymphocytes and antigen-processing accessory cells such as DCs. Efferent lymph which is draining out of the lymph node, predominantly contains lymphocytes (>99%) (Table 4.2). Under physiological conditions, there are profound differences between afferent and efferent lymph flow rates, cell content and activation status of the cells (Haig, Hopkins & Miller 1999). One major difference is that efferent lymphocytes do not have an activated phenotype, unlike afferent lymphocytes (Haig, Hopkins & Miller 1999). So, the use of these cell populations created the potential for two different lymphocyte populations to be examined along with other immune cells such as dendritic cells. The cannulated sheep used in these studies were healthy, uninfected animals.

We examined 29 sheep cell surface markers for alterations in expression after 5 hr incubations with *Fasciola sp.* ESP *in vitro*. *Fasciola sp.* ESP selectively altered CD4, CD8 and CD1b expression on sheep immune cells.

Fasciola sp. ESP was shown to significantly down regulate the level of CD4 expression on sheep dendritic cells and T lymphocytes *in vitro*. Experiments with fixed cells confirmed that CD4 was most likely cleaved from the surface of sheep dendritic cells and T cells and that CD4 expression could at least partially be restored once *Fasciola sp.* ESP was removed from the system. The addition of E64 to the incubations with *Fasciola sp.* ESP ablated the suppressive effect on CD4 expression levels, implying that cysteine protease activity in *Fasciola sp.* ESP is responsible for the forementioned effects. To extend these findings, two recombinant cathepsin L proteases that show different substrate specificity (Smooker *et al.* 2000) were incubated with sheep T

lymphocytes and, in both cases, CD4 expression was down-regulated. This effect was also inhibited in the presence of E64. These results show that *F. hepatica* cathepsin L selectively removes CD4 from the surface of sheep T lymphocytes.

It was also shown that *Fasciola sp.* ESP and *F. hepatica* cathepsin L down regulated CD4 from the surface of human T cells and that *Fasciola sp.* ESP down regulated CD4 from the surface of bovine T cells. These two results suggested that this effect of cathepsin L is not specific to ovine CD4. Lastly the two recombinant proteases were shown to cleave rHuCD4 *in vitro* adding further weight to the argument that *Fasciola sp.* cathepsin Ls cleave CD4 from the surface of immune cells.

The effect of cathepsin L activity on CD8 was more complex. CD8, like CD4, was shown to be down regulated on the surface of efferent lymphocytes after 5 hrs in the presence of either *F. hepatica* or *F. gigantica* ESP. CD8 expression was however transiently upregulated between 1 and 3 hrs after the commencement of incubation with either *Fasciola sp.* ESP. This modulation of CD8 may suggest that CD8 is up-regulated early by other ESP components and the subsequent down regulation of CD8 may be indicative of a delay in the cathepsin L activity in this function. The stimulus for this early up regulation will be investigated in future experiments using recombinant cathepsin L and by the addition of E64 to a concurrent set of incubations as a negative control. This would identify whether cathepsin L or another component of ESP was involved in the up regulation of CD8. Experiments with fixed cells confirmed that CD8 was most probably cleaved from the surface of afferent lymph T cells. The addition of E64 to the incubations with *Fasciola sp.* ESP ablated the suppressive effect on CD8 expression levels on sheep immune cells, implying that cysteine protease activity in *Fasciola sp.* ESP is responsible for the forementioned effects. Future work will focus on recombinant cathepsin Ls and extending this work to include human and bovine $CD8^+$ T cells.

We also showed that CD1b expression on the surface of sheep dendritic cells was upregulated over time in the presence of either *Fasciola sp.* ESP *in vitro*. E64 inhibited the effect on CD1b expression caused by either *Fasciola sp.* ESP, implying cysteine proteases are responsible for this up regulation. CD1b is considered a marker for early activation (personal communication K.

Walsh). ESP contains multiple constituents and some of these are likely to be inducing activation. Due to the reversal of this activation in the presence of E64 it suggests cathepsin L may be acting on dendritic cells in some way which then induces an activated state measurable by increased CD1b expression. This may be the result of cathepsin L cleaving another surface molecule which sends a signal into the cell for increased CD1b expression. Another possibility is that as CD4 and CD8 are down regulated, CD1b is upregulated to provide an alternative antigen presentation pathway. As expanded upon below CD1b presented antigen interacts with TCR on $CD4^{-}CD8^{-}$ T cells. Future work will extend these findings of cysteine protease involvement in the up regulation of CD1b, through the use of recombinant *F. hepatica* cathepsin Ls that we have access to in our laboratory.

CD4 is a 65 kDa surface marker on T cells and current research suggests that CD4 acts as a co-receptor along with the TCR: CD3 complex in antigenic recognition of MHC II (Janeway 1989, Janeway and Bottomly 1994). This co-receptor function of CD4 potentiates signalling by 10-100 fold, greatly reducing the density of ligand required for T cell activation (Marrack *et al.* 1983, Hampl, Chien & Davis 1997, Madrenas *et al.* 1997). The bivalent interaction of CD4 with the TCR: CD3 complex has been shown to be necessary for T helper cell activation and development (Janeway 1989, Janeway and Bottomly 1994). Considering the importance of CD4 in regulating host T cell activation it is possible that cathepsin L-induced cleavage of CD4 may be one of the mechanisms utilised by *Fasciola* parasites *in vivo* to suppress lymphocyte proliferation in sheep, contributing to evasion of the host's immune system and establishment of infection. Further studies are required to establish this, which could be tested in experiments where animals are treated with a parasite specific cysteine protease inhibitor (currently not available) during a *Fasciola* infection and determining whether this prevents the suppressive effects seen *in vivo*. CD4 expression levels on sheep lymphocytes during infection, could also be monitored by cannulation of these sheep. The fact that cleavage of CD4 by these fluke cathepsins also occurs with human T cells suggests that this may be a general mechanism used by this parasite to establish infection and further work will determine this by examining the effects of cathepsin L on proliferation of human T cells. Infection of mice with *F. hepatica* has been shown to suppress Th1 like responses, to reverse the Th1 response to the *Bordetella pertussis* vaccine and to induce changes in cytokine profiles in spleen cells (Brady *et al.* 1999b, O'Neill *et al.*

2000). Whether these effects of fluke infection are linked to the down regulation of CD4 levels on murine T cells is yet to be determined. Future work will focus on characterisation/ identification of the proposed site(s) of cleavage on human recombinant CD4 and determining whether *F. hepatica* cathepsin L also affects $CD4^+$ T cells in other hosts such as rats. Ovine CD4 has recently been cloned in our laboratory and this will allow direct analysis of cathepsin L activity using recombinant sheep CD4.

While this is the first example of a cysteine protease regulating ovine CD4 expression levels on T cells, two metallo-proteases have been shown to exhibit similar effects on CD4 expression. The major surface glycoprotein (gp63) from the protozoa *Leishmania major* and *L. donovani* was also shown to selectively cleave CD4 from human T cells (Hey *et al.* 1994). Similarly, the bacterial *Legionella pneumophilia* protease was shown to degrade IL-2 and cleave CD4 from human T cells (Mintz *et al.* 1993). Both of these reports suggest that the cleavage of CD4 may be the method utilised by both *Leishmania sp.* and *Legionella sp.* to impede T cell activation, resulting in disease progression.

CD8 is expressed either as a homodimer of two α- chains on natural killer (NK) cells or intestinal T cells or as a heterodimer of α- and a β- chain on the surface of mature MHC class I restricted T cells (Zamoyska 1994 and Zamoyska 1998). CD8, similarly to CD4, is a receptor for MHC molecules. CD4 and CD8 bind to the constant domains of MHC II and MHC I molecules respectively (Cammarota *et al.* 1992, Konig, Huang & Germain 1992, Norment *et al.* 1988 and Salter *et al.* 1990). It is thought that CD8 acts as a co-receptor with TCR for MHC I molecules, resulting in an increase in the avidity of the interaction. It has subsequently been shown that co-ligating CD8 to the TCR provides a more potent stimulus for T cell activation than simply ligating TCR alone (Boyce *et al.* 1988, Jonsson, Boyce & Eichmann 1989). CD8 co-receptor involvement has also been shown to broaden the recognition of T cells to antigens and their ability to respond to related antigens (Blok *et al.* 1992). Finally the CD8 β- chain has been shown to enhance IL-2 production in response to stimulator cells (Wheeler, von Hoegen & Parnes 1992). While $CD8^+$ cells have not yet been studied for their role in *Fasciola* infections, cleavage of CD8 on cytotoxic T lymphocytes will suppress proliferation of an important component of Th1 defence strategies. This suppression may by one contributor to the

polarisation towards a Th2 dominated immune response characteristic of *F. hepatica* infections. Current theory proposes that Th2 responses are induced as part of *F. hepatica's* evasion strategy.

To date there has only been one published report of a protease reducing CD8 expression on the cell surface. The warble-fly, an endoparasite of cattle, secretes the serine protease hypodermin A which has been shown to down regulate a range of surface markers on bovine cells including CD8 (Moire *et al.* 1997). While this protease does not appear to be specific for only a few markers it is interesting to note that hypodermin A has also been shown to suppress proliferation of bovine lymphocytes activated by different mitogens (Chabaudie & Boulard 1992, Nicolas-Gaulard, Moire & Boulard 1995). These results have led to the hypothesis that cleavage of the surface markers investigated may be one of the mechanisms the parasite utilises to suppress lymphocyte proliferation (Moire *et al.* 1997).

In summary CD4 and CD8 participate in: lymphocyte adhesion to antigen presenting cells involving MHC II and I products respectively, lymphocyte activation through interactions with TCR-CD3 complex, and modulation of late events during lymphocyte activation (Weiss & Imboden 1987). The results presented in this section show that *Fasciola* parasites affect the expression of both CD4 and CD8, which play important roles in T lymphocyte activation. This selective modulation of these two markers is likely to play a key role in *Fasciola sp.* evasion strategies during infection.

The cluster of differentiation 1 (CD1) cell surface glycoproteins have a domain organisation similar to that of MHC Class I molecules and consist of a heterodimer on an α chain (43-49 kDa) associated with β2 microglobulin (Calabi *et al.* 1991, Porcelli 1995). The number of CD1 genes expressed varies between hosts with five genes in Humans (CD1A, CD1B, CD1C, CD1D and CD1E), two in mouse, eight in rabbit (Porcelli 1995) and seven in sheep (Ferguson *et al.* 1996, Hopkins, Dutia & Rhind 2000). CD1 genes have been separated based on sequence analysis of the leader, α1, and α2 domains into group I CD1 genes comprising of human CD1A, CD1B and CD1C genes and group II CD1 genes consisting of CD1D genes (Ferguson *et al.* 1996, Naidenko, Koezuka & Kronenberg 2000). To date, humans express 4 CD1 proteins (CD1a, CD1b, CD1c and CD1d) on specialised antigen-presenting cells, including dendritic cells

(Porcelli & Modlin 1999), in a wide variety of lymphoid and non lymphoid tissue (Jackman *et al.* 1998). More specifically group I protein products CD1a, CD1b and CD1c molecules are expressed by professional antigen presenting cells such as Langerhans cells, dermal and lymphoid dendritic cells, cytokine activated monocytes and cortical thymocytes (Small *et al.* 1987, Amiot *et al.* 1988, Cattoretti *et al.* 1989, Porcelli *et al.* 1992). Human CD1d is expressed by epithelial cells and at low levels on cortical thymocytes (Blumberg, Terhost & Bleicher 1991, Canchis *et al.* 1993).

Unlike rodents which only express group II genes, but similar to humans and cattle, sheep express genes belonging to both groups (Rhind Hopkins & Grant 2000). Current literature shows sheep express four CD1B like genes and one CD1D like gene (Ferguson *et al.* 1996, Rhind, Hopkins & Dutia 1999). The CD1b molecule has a unique role in the immune system in its ability to present non-peptide antigen to T cells. Specifically, CD1b displays microbial lipid and glycolipid antigens on the antigen presenting cells surface (Moody *et al.* 1997, Jullien *et al.* 1997) allowing for specific responses of subpopulations of TCR (T cell receptor) $\alpha\beta^{+}CD4^{-}CD8^{-}$ T cells or $CD4^{-}CD8^{+}$ T cells (Beckman *et al.* 1994, 1996). These responses have been hypothesised to include: secretion of pro inflammatory lymphokines, killing of infected cells and aiding in antibody production by specific B cells (reviewed in De Libero *et al.* 2001).

One published link between CD1 expression and parasitic disease relates to *Trypanosoma* infection in a susceptible sheep breed. There was an increase in B cells during a *Trypanosoma evansi* infection as evidenced by an increase in the $CD1^{+}$ surface marker (Onah, Hopkins & Luckins 1999). Interestingly CD4 and CD8 were both down regulated following infection. This is similar to our *in vitro* results with *Fasciola* ESP and may alude to a phenomenon common to several parasites.

There have been no reported cases of a parasitic protease affecting CD1b or any CD1 protein expression levels. Due to this our results showing an apparent increase in CD1b levels on afferent lymph dendritic cells following exposure to *Fasciola* ESP are unique. This up regulation of CD1b was prevented by the addition of E64, suggesting cathepsin L activity being involved. The parasite may induce up-regulation of CD1b expression as a "smoke screen" effect to protect

itself from a more effective defensive pathway. Alternatively CD1b up regulation may be an immune response mounted following recognition of the shed glycocalyx of the parasite which is rich in glycolipids. However, no functional consequence to this phenotype has yet been ascribed and remains for future determination.

As discussed in Chapter 3, at least seven cathepsin L isoenzymes have been identified on SDS PAGE gels or by cDNA cloning (Wijffels *et al.* 1994a; Tort *et al.* 1999; Smooker *et al.* 2000). Analysis of cDNA sequences for cathepsin L identifies 3 clades and these can be separated into at least two functional groups based on their substrate specificity at the P_2 position (Smooker *et al.* 2001). rFhCatL5 belongs to group 1 which prefers a phenylalanine at P_2 and rFhCatL69Y belongs to group 2 which prefers a proline at P_2. The results presented here suggest that down regulation of CD4 may be characteristic of enzymes from at least two of the clades. Further work is needed to confirm a role for all cathepsin L isoenzymes in regulating CD4 expression on sheep T cells. This could involve fractionation of ESP to prepare different cathepsin L isoenzymes and testing their activity on CD4, CD8 and CD1b.

The effect of cathepsin L on six murine surface markers (CD4, CD8, B220, CD3ε, CD11a and CD28) has since been investigated (Personal communication Baz and Law 2002). Cathepsin L was shown to reduce CD4 and CD8 expression on murine lymphocytes. B220 and CD28 were only partially affected while no effect on CD3ε and Cd11a were observed. These results are similar to those we observed on sheep immune cells and add further evidence for the selective down regulation of CD4 and CD8 on the surface of immune cells.

The importance of cathepsin L isoenzymes in liver fluke biology has been demonstrated in vaccine trials in ruminants. Cathepsin L molecules have shown efficacy as vaccines in cattle, sheep and rats (Wijffels *et al.* 1994b, Dalton *et al.* 1996b, Piacenza *et al.* 1999, Mulcahy *et al.* 1998, Mulcahy *et al.* 1999b, Kofta *et al.* 2000, reviewed in Spithill *et al.* 1999). Protection in cattle with cathepsin L vaccines against a challenge infection has varied from 38-69%. The greatest protection (72%) was observed for a combination vaccine which included the fluke derived antigens cathepsin L2 and fluke haemoglobin (Dalton *et al.* 1996b, Mulcahy *et al.* 1998). Protection in cattle appears to correlate with induction of IgG2 antibodies to cathepsin L

(Mulcahy *et al.* 1998). These results suggest that IgG2 antibodies against cathepsin L induce partial protection but the mechanism of protection is unknown. Anti-cathepsin L antibodies have been shown to neutralise cathepsin L activity *in vitro*, which prevents antibody- mediated attachment of eosinophils to the parasite (Smith *et al.* 1994). Cathepsin L has also been shown to cleave the Fc region of human IgG at specific points (Berasain *et al.* 2000) and a role for cathepsin L in blocking antibody- dependent cell mediated killing of *F. hepatica* is feasible since, in rats, killing of juvenile *F. hepatica in vitro* has been shown to be mediated by antibodies and macrophages (Piedrafita *et. al.* 2001). Exposure of mice to cathepsin L has recently been shown to suppress IFN-γ expression and this is proposed to prevent a Th-1 like response (O'Neill, Mills & Dalton 2001). Future work will involve analysing the effect of vaccine-induced anti-cathepsin L antibodies or treatment with E64, on the down regulation of CD4 levels on sheep T cells to confirm that CD4 is actually cleaved *in vivo*.

CHAPTER 5: FREE RADICALS AND DEFENCE ENZYMES

5.1 Introduction

Two main classes of free radicals are reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI). Free radical generation by host immune cells is one of the important defence mechanisms against invading parasites. In some circumstances, free radicals have been implicated in parasite evasion, where the stimulation of nitric oxide (NO) by host cells enables parasite establishment (Haswell-Elkins *et al.* 1994, Saeji *et al.* 2002).

Circumstantial evidence suggests free radicals may be involved in the elimination of juvenile *F. hepatica* parasites from the host. In work by Smith, Ovington and Boray (1992), free radical production levels in response to *F. hepatica* were compared between the resistant rat and the susceptible mouse hosts. The authors showed significantly higher free radical production (measured by a chemiluminescence assay) from rat leukocytes when compared to mice leukocytes. It was further shown that upon secondary infection with *F. hepatica*, rats produced even larger quantities of free radicals and this was not observed in mice. However this is only circumstantial evidence but does support a potential role for free radicals in parasite elimination in resistant hosts. This is supported by other *in vitro* work showing that cell-mediated cytotoxicity by rat peritoneal lavage cells (PLCs) towards *F. hepatica* NEJ is dependent on both the production of NO by rat PLCs and the addition of immune sera (Piedrafita *et al.* 2001).

In work by Cervi and colleagues (1998) NO levels produced by LPS-stimulated peritoneal cells decreased in the first two weeks post *F. hepatica* infection in rats. From these results it was proposed that *F. hepatica* NEJ may have evolved mechanisms to down regulate the NO production by rat PLCs. One method used by *Fasciola sp.* to modulate host immune responses is through the production of excretory / secretory products (ESP). Adult *F. hepatica* ESP caused a reduction in NO production by LPS-stimulated rat peritoneal cells *in vitro* (Cervi, Rossi & Masih 1999). This reduction in NO production by activated rat peritoneal cells was partially attributed to GST, one of the constituents of *F. hepatica* ESP (Cervi, Rossi & Masih 1999). Interestingly

inhibition of GST increased the susceptibility of *F. hepatica* NEJ to NO production by LPS-stimulated rat PLCs (Piedrafita *et al.* 2000).

Laboratory experiments have shown both the ability and inability of *F. hepatica* and *F. gigantica* respectively, to establish within the rat host during a primary infection (Mango, Mango, Esamel 1972, Itagaki *et al.* 1994, Piedrafita *et al.* 2000). We hypothesised that the ability to modulate NO production by the rat host could play an important role in resistance to *Fasciola* infection. We further hypothesised that molecules within *Fasciola* ESP would in part mediate this modulation.

As discussed in Chapter 1.4 parasites release a range of molecules to detoxify free radicals such as anti-oxidant defence (AOD) enzymes. AOD enzymes catalyse the detoxification of ROI and RNI into less harmful products. We hypothesised that the difference in susceptibility of *Fasciola sp.* to host responses is related to the expressed levels of AOD enzymes. *F. hepatica* has been shown to excrete or produce the following AOD enzymes: glutathione S-transferase (Howell, Board & Boray 1988, Wijffels *et al.* 1992), superoxide dismutase (Piacenza *et al.* 1998, Sanchez-Moreno *et al.* 1987), glutathione peroxidase (Brophy, Crowley & Barrett 1990, Piedrafita *et al.* 2000), peroxiredoxin (Barrett 1980, McGonigle, Curley & Dalton 1997), thioredoxin peroxidase (Salazar-Calderon *et al.* 2000) and cytochrome c peroxidase (Campos *et al.* 1999). While not as well studied, *F. gigantica* has been shown to excrete or produce GST and be capable of detoxifying ROI, suggesting the presence of other AOD enzymes (El-Ghaysh *et al.* 1999, Estuningsih *et al.* 1997).

This Chapter firstly compares the ability of adult ESP from *F. hepatica* and *F. gigantica* to alter NO production by *Fasciola*-naïve rat peritoneal lavage cells (PLCs) *in vitro* and investigates the active components released by both parasites. The second section of this Chapter compares the relative levels of AOD enzymes expressed by NEJ and adult parasites of both *F. hepatica* and *F. gigantica.*

5.2 Results

Nitrite levels in culture supernatants of rat peritoneal lavage cells (PLCs) were measured by a standard Greiss reaction (Green *et al.* 1982). Nitrite levels were used as an indicator of NO production from PLCs (Hibbs *et al.* 1988, Ignarro *et al.* 1993). In addition, monomethyl L-arginine (L-NMMA) was included in some incubations as a competitive inhibitor of nitric oxide synthase (Hibbs *et al.* 1988). Wistar Rat PLCs were chosen as the cell source as they are rich in monocytes/macrophages (60-70%, Piedrafita *et al.* 2001) and these cells are known to produce NO.

5.2.1 The effect of a Dose Response of *Fasciola sp.* ESP on NO production by rat PLCs

Increasing amounts of either adult *Fasciola sp.* ESP (0.0025 - 15 µg/ml)in the presence of LPS-stimulated rat PLCs did not significantly inhibit or stimulate NO production (as measured by nitrite levels) when compared with rat PLCs in the presence of LPS alone (Fig 5.1, 5.2). These findings are in contrast with a previously published experiment which showed adult *F. hepatica* ESP caused a reduction in NO production by LPS-activated rat peritoneal cells (Cervi, Rossi & Masih 1999). This will be examined in the discussion.

The effect of *Fasciola* ESP on resting rat PLCs has not yet been reported. In our experimental system both adult *F. hepatica* and *F. gigantica* ESP were capable of stimulating NO production in the presence of resting rat PLCs. This was inhibited by the addition of L-NMMA, a specific inhibitor of nitric oxide synthase (Fig 5.1, 5.2). Rat PLCs appear to have a threshold for NO production. This proposal is based firstly on the absence of additional NO production by LPS-activated rat PLCs in the presence of increasing concentrations of adult *Fasciola* ESP. Secondly, NO production by resting rat PLCs in the presence of increasing concentrations of *Fasciola* ESP also reaches a maximum, which in this series of experiments, was around 30-40 µM. Maximal levels of NO production by rat PLCs varied between experiments and this is likely to reflect a difference between each individual outbred rat. To minimise these differences, Wistar rats of a certain weight and age were used for all experiments. This is the first report of ESP from either adult *Fasciola* parasite being capable of stimulating NO production by resting rat PLCs. Due to

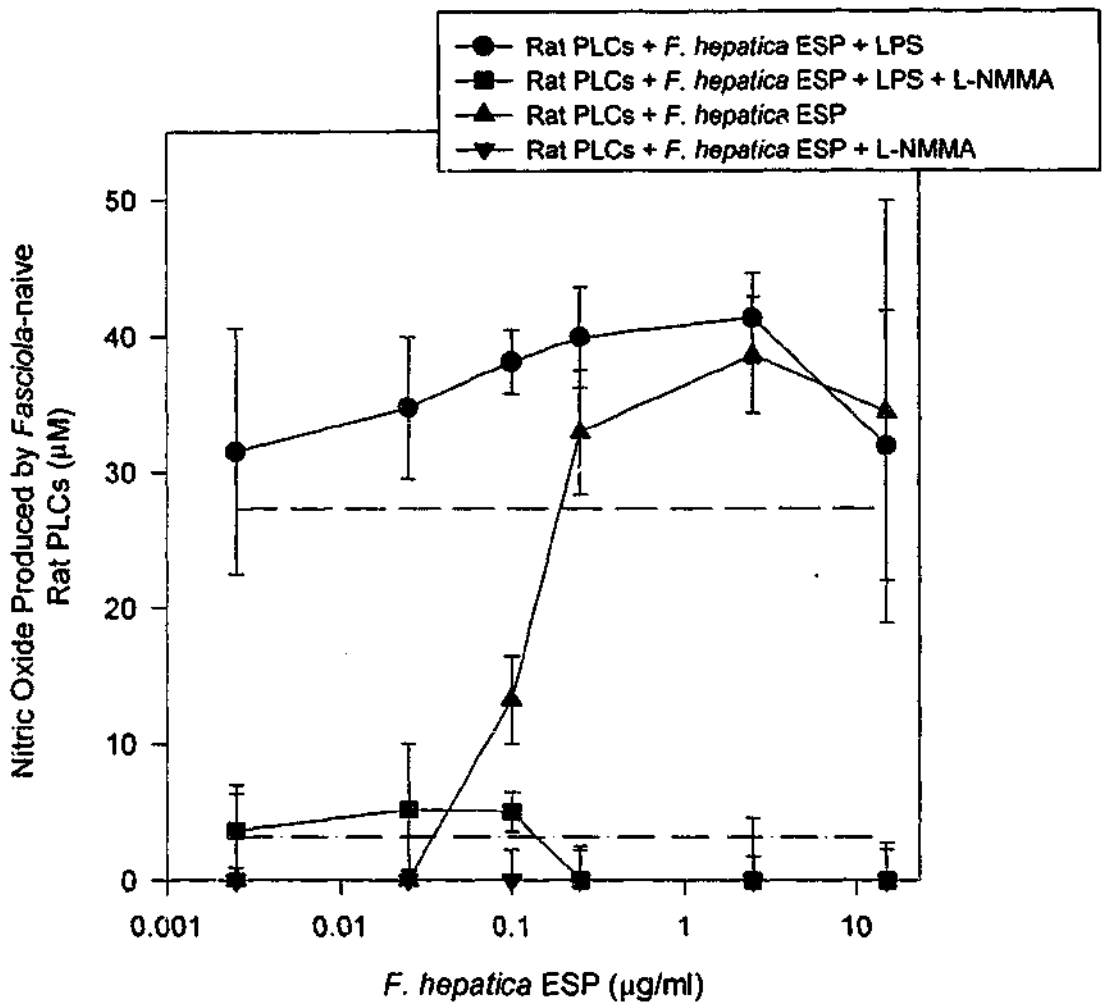


Fig 5.1 NO produced by *Fasciola*-naïve rat PLCs in the presence of *F. hepatica* ESP. Rat PLCs were stimulated with increasing concentrations of *F. hepatica* ESP with and without LPS and or L-NMMA. The dashed and the dash-dot lines show the amount of NO produced by Rat PLCs with LPS alone (27.3 $\pm$ 11.5 μM) and L-NMMA alone (3.2 $\pm$ 2.5 μM) respectively. Values represent the means $\pm$ SD of triplicate wells. The results shown are representative of two separate experiments performed. Significant differences ($P<0.01$) for PLCs with *F. hepatica* ESP, compared to PLCs with L-NMMA alone, were calculated using the Dunnett Multiple Comparisons test. Significant differences were observed for PLCs incubated with 0.1 μg/ml and greater conentrations of *F. hepatica* ESP. Significant differences, calculated using the Dunnett Multiple Comparisons test, were not observed for PLCs with LPS alone compared with PLCs with *F. hepatica* ESP and LPS.

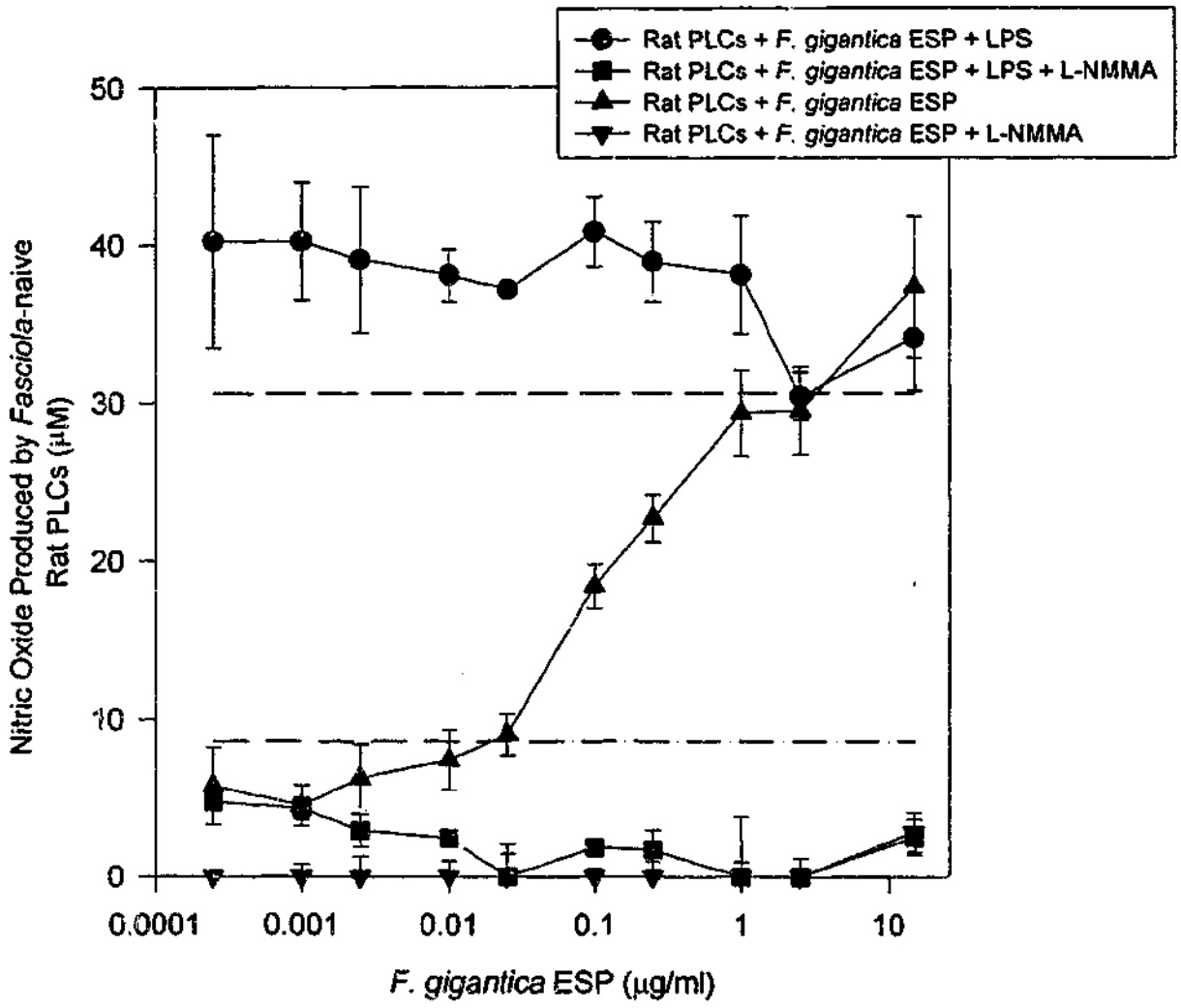


Fig 5.2 NO produced by *Fasciola*-naïve rat PLCs in the presence of *F. gigantica* ESP. Rat PLCs were stimulated with increasing concentrations of *F. gigantica* ESP with and without LPS and or L-NMMA. The dashed and the dash-dot lines show the amount of NO produced by Rat PLCs with LPS alone (30.6 $\pm$ 7.0 µM) and L-NMMA alone (8.6 $\pm$ 1.2 µM) respectively. Values represent the means $\pm$ SD of triplicate wells. The results shown are representative of two separate experiments performed. Significant differences ($P<0.01$) for PLCs with *F. gigantica* ESP, compared to PLCs with L-NMMA alone, were calculated using the Dunnett Multiple Comparisons test. Significant differences were observed for PLCs incubated with 0.1 µg/ml and greater concentrations of *F. gigantica* ESP. Significant differences, calculated using the Dunnett Multiple Comparisons test, were not observed for PLCs with LPS alone compared with PLCs with *F. gigantica* ESP and LPS.

this observed stimulation of NO production the remainder of this section will attempt to elucidate the NO stimulatory molecules within adult *Fasciola sp.* ESP.

5.2.2 Identification of NO Stimulatory Molecules within Adult *Fasciola sp.* ESP

To identify the biological class of the NO stimulatory molecules, *Fasciola sp.* ESP underwent treatment with either periodate or proteinase K. Periodate oxidation will denature carbohydrates present within ESP while proteinase K is a serine protease that degrades native and denatured proteins.

Fasciola sp. ESP were treated on the same day using the identical reagents with either periodate or proteinase K as described in section 2.3.5 of the Materials and Methods Chapter. Following treatment, periodate was removed by dialysis and proteinase K activity was blocked by addition of PMSF (see Chapter 2.3.5). To ascertain the effectiveness of both treatments, untreated *Fasciola sp.* ESP and ESP treated by either method were analysed on a 12% SDS PAGE gel stained with silver periodic acid (Fig 5.3). The silver periodic acid staining technique will detect carbohydrate groups as well as proteins (Dubray & Bezard 1982). *F. hepatica* and *F. gigantica* ESP showed similar patterns with *F. gigantica* ESP appearing to contain more material below 28 kDa. Proteinase K treatment of *F. hepatica* ESP decreased ESP components at approximately 45 – 66 kDa, while there were no obvious effects of periodate treatment on *F. hepatica* ESP (Fig 5.3). In the case of *F. gigantica* ESP, both proteinase K and sodium periodate treatment decreased material at approximately 45 kDa. Periodate treatment also considerably decreased material less than 28 kDa in *F. gigantica* ESP (Fig 5.3). These treatments were deemed successful due to the degradation of proteins and carbohydrates within *F. gigantica* ESP. These treated *Fasciola sp.* ESP were used in subsequent experiments.

The periodate treated *F. hepatica* ESP stimulated significantly lower levels of NO (about 80% lower) by resting rat PLCs than that observed with native *F. hepatica* ESP (Fig 5.4). As periodate is a glycol cleavage reagent (Kennedy 1988), the loss of activity observed for treated *F. hepatica* ESP suggested that the active molecules may be carbohydrate moieties. This result also confirms that the periodate treatment was successful, even though there was no observable change in the periodate-treated *F. hepatica* ESP profile on the 12% SDS PAGE gel (Fig 5.3).

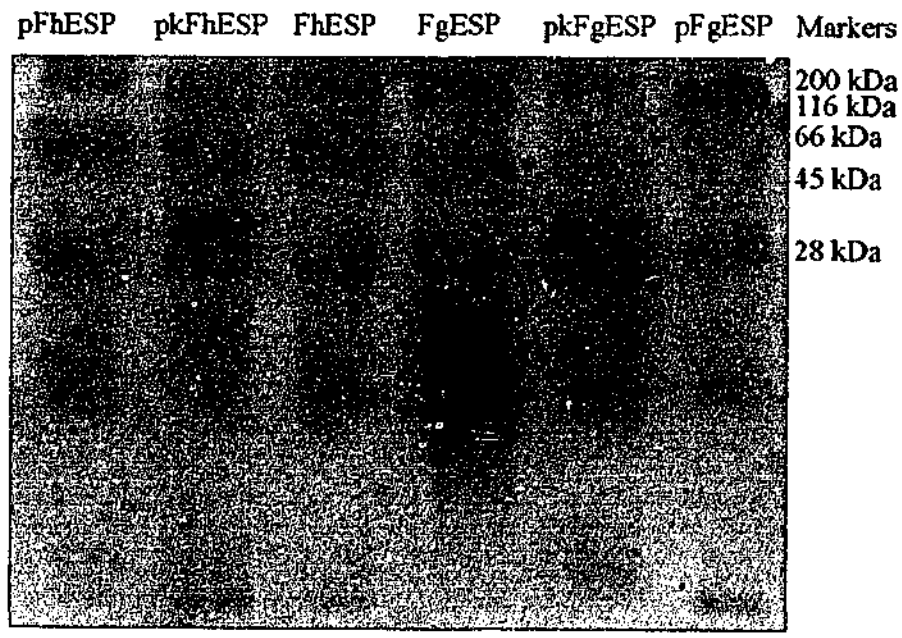


Fig 5.3 Silver periodic acid stained 12% SDS PAGE gel analysing treated *Fasciola sp.* ESP. *F. hepatica* and *F. gigantica* ESP were incubated with either 0.01M periodate (p) for 2 hr or 60 µg/ml proteinase K (pk) for 30 min at RT. These treated samples (30 µl) were fractionated on SDS PAGE gels and stained with silver periodic acid. The migration of the standards is shown on the right. The #,*, and + mark the location of protein bands present in the native ESP but absent following one of the treatment methods. The results presented are representative of two separate experiments.

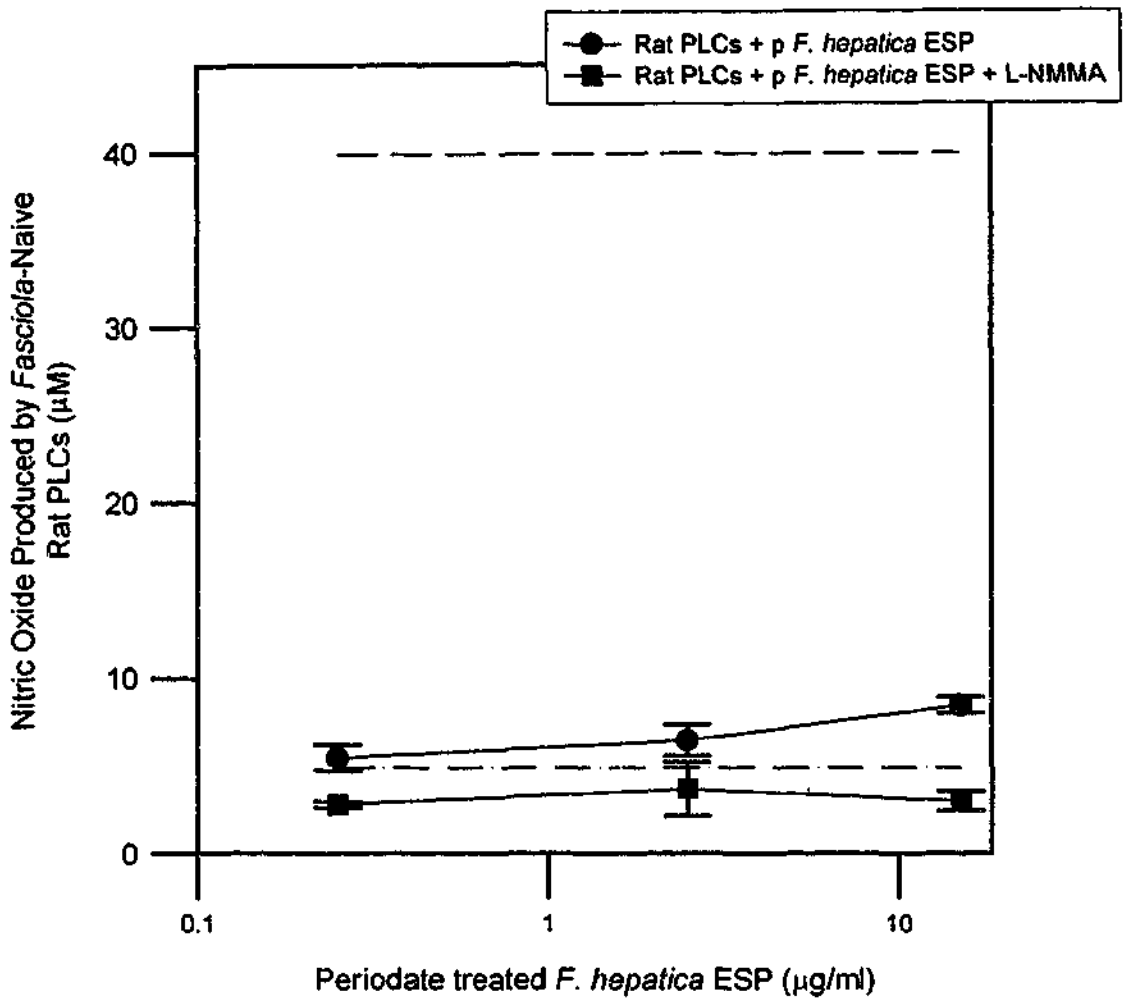


Fig 5.4 NO produced by *Fasciola*-naïve rat PLCs in the presence of periodate treated *F. hepatica* ESP (p*F. hepatica* ESP). Rat PLCs were stimulated with increasing concentrations of p*F. hepatica* ESP with and without L-NMMA. The dashed and the dash-dot lines show the amount of NO produced by Rat PLCs with 15 mg/ml of untreated *F. hepatica* ESP (39.9 $\pm$ 2.4 µM) and PLCs alone (4.9 $\pm$ 0.5 µM) respectively. Values represent the means $\pm$ SD of triplicate wells. The results shown are representative of two separate experiments performed. Significant differences ($P<0.01$) for PLCs with p*F. hepatica* ESP, compared to PLCs alone, were calculated using the Dunnett Multiple Comparisons test. A significant difference was observed, for PLCs incubated with 15 µg/ml of p*F. hepatica* ESP.

When *F. hepatica* ESP was treated with proteinase K, the NO-stimulatory effect was reduced by approximately 50% compared to 15 µg/ml of untreated *F. hepatica* ESP (Fig 5.5). The level of NO production from rat PLCs in the presence of *F. hepatica* ESP previously treated with 15 µg/ml of proteinase K was significantly greater than that produced by resting rat PLCs alone (Fig 5.5). Proteinase K cleaves peptide bonds within proteins (Corbalan-Garcia *et al.* 1994). By diminishing the activity of *F. hepatica* ESP by approximately 50% and combined with data showing that periodate treatment reduces NO production by 80%, these results suggest that the active molecules within *F. hepatica* ESP are likely to be both glycoproteins and carbohydrates. The addition of L-NMMA to these incubations eliminated NO production suggesting the induction of NO was dependent on nitric oxide synthase.

In contrast to the results with *F. hepatica* ESP, *F. gigantica* ESP continued to stimulate NO production by rat PLCs after treatment with either periodate or proteinase K (Fig 5.6, 5.7). Significantly greater levels of NO were produced by rat PLCs incubated with 2.5 and 15 µg/ml of either treated *F. gigantica* ESP, compared to that for resting rat PLCs alone (Fig 5.6, 5.7). Interestingly, levels of NO produced by 15 µg/ml of either treated *F. gigantica* ESP were comparable to those produced by rat PLCs in the presence of 15 µg/ml untreated *F. gigantica* ESP, suggesting both treatments had no effect on NO stimulatory molecules. These results imply that the predominant active components within *F. gigantica* ESP do not belong to either the protein or carbohydrate classes. This was not expected as the main classes of molecules found within ESP were expected to be from the protein or carbohydrate class. The active molecule/s may belong to the lipid biological class. The other possibilities are that the active molecules have folded structures or are protected in some way, that the carbohydrate residues are resistant to periodate making them resistant to these two treatment methods or that there is a large excess of carbohydrates within ESP.

The next step in identifying the active molecules within *Fasciola sp.* ESP was to determine their size through the use of gel filtration chromatography. *Fasciola sp.* ESP components were fractionated by molecular sieving on a Superose 6 column, which has a broad separation range of molecular components (5 – 5000 kDa). The individual *Fasciola sp.* ESP fractions were analysed for NO stimulatory ability on resting rat PLCs. For both *F. hepatica* and *F. gigantica* the NO

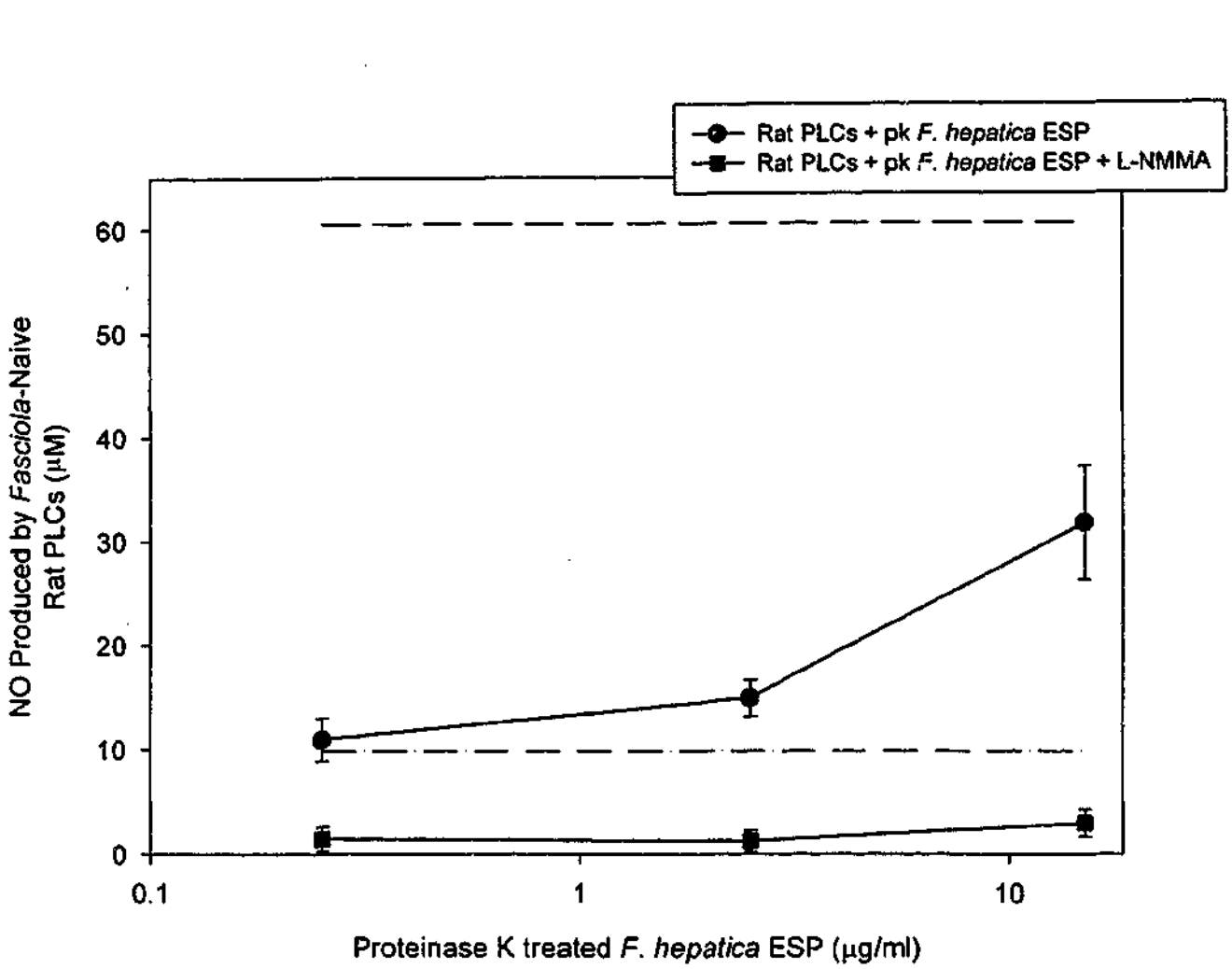


Fig 5.5 NO produced by *Fasciola*-naïve rat PLCs in the presence of proteinase K treated *F. hepatica* ESP (pk*F. hepatica* ESP). Rat PLCs were stimulated with increasing concentrations of pk*F. hepatica* ESP with and without L-NMMA. The dashed and the dash-dot lines show the amount of NO produced by Rat PLCs with 15 mg/ml of untreated *F. hepatica* ESP (60.5 $\pm$ 0.5 µM) and PLCs alone (9.8 $\pm$ 2.1 µM) respectively. Values represent the means $\pm$ SD of triplicate wells. The results shown are representative of two separate experiments performed. Significant differences ($P<0.01$) for PLCs with pk*F. hepatica* ESP, compared to PLCs alone, were calculated using the Dunnett Multiple Comparisons test. A significant difference was observed, for PLCs incubated with 15 µg/ml of pk*F. hepatica* ESP.

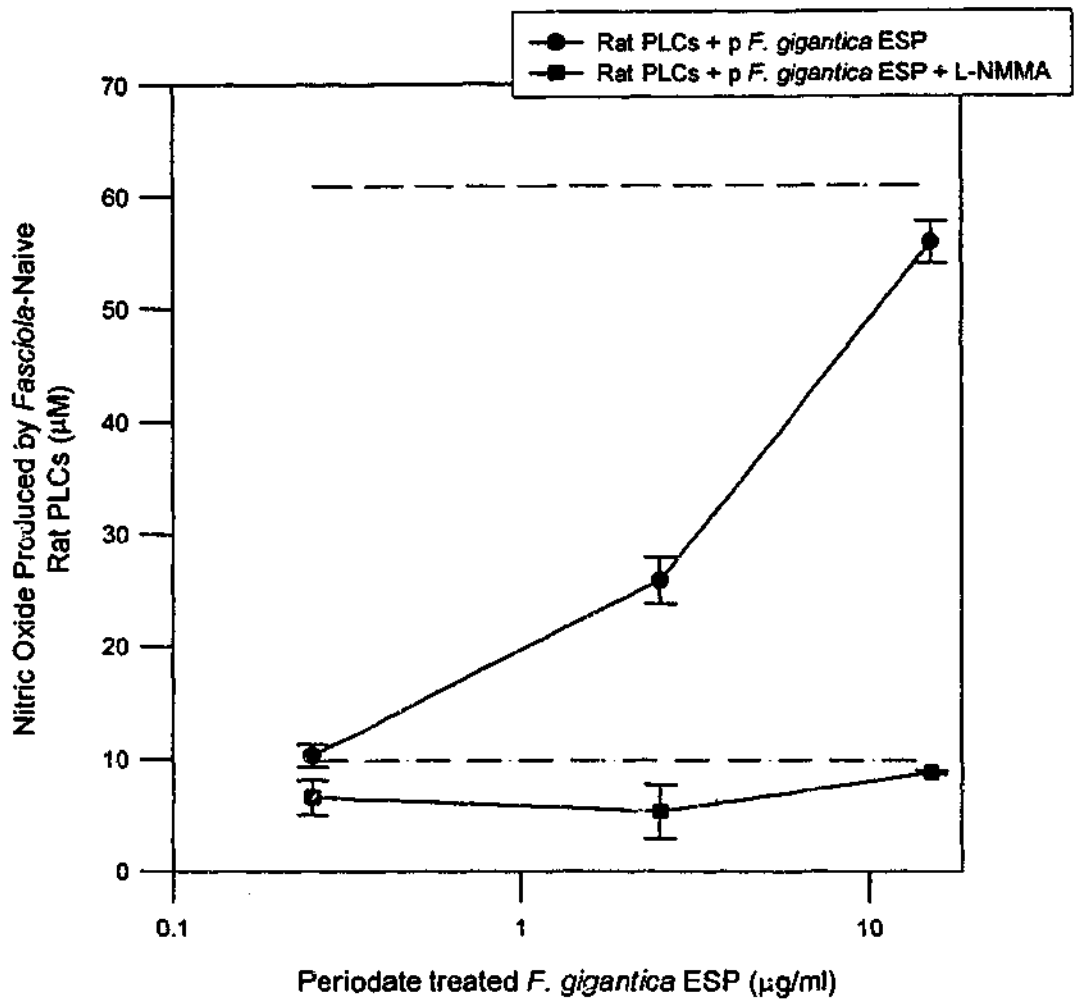


Fig 5.6 NO produced by *Fasciola*-naïve rat PLCs in the presence of periodate treated *F. gigantica* ESP (p*F. gigantica* ESP). Rat PLCs were stimulated with increasing concentrations of p*F. gigantica* ESP with and without L-NMMA. The dashed and the dash-dot lines show the amount of NO produced by Rat PLCs with 15 mg/ml of untreated *F. gigantica* ESP (60.9 $\pm$ 1.8 µM) and PLCs alone (9.9 $\pm$ 1.1 µM) respectively. Values represent the means $\pm$ SD of triplicate wells. The results shown are representative of two separate experiments performed. Significant differences ($P<0.01$) for PLCs with p*F. gigantica* ESP, compared to PLCs alone, were calculated using the Dunnett Multiple Comparisons test. Significant differences were observed, for PLCs incubated with 2.5 and 15 µg/ml of p*F. gigantica* ESP.

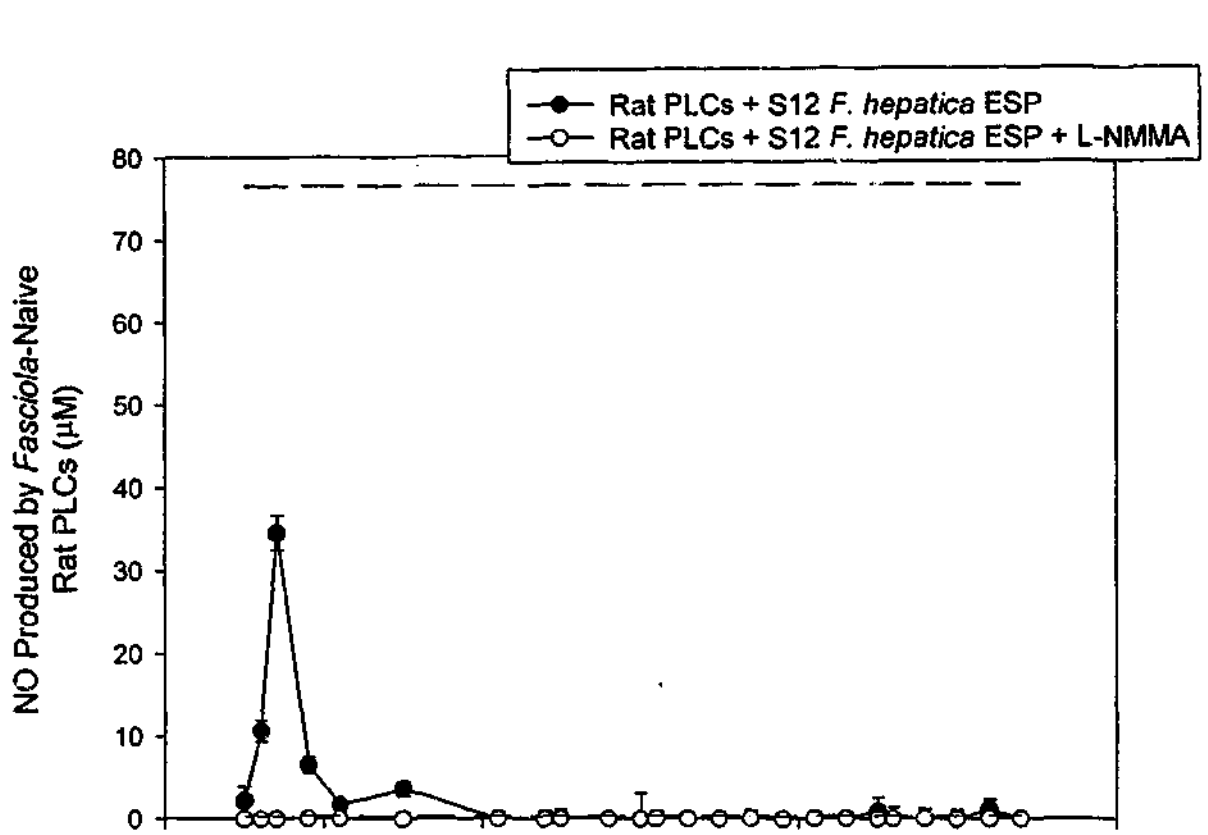


Fig 5.10 NO produced by *Fasciola*-naïve rat PLCs in the presence of *F. hepatica* ESP fractions collected from a Superose 12 (S12) column. Rat PLCs were stimulated with S12 *F. hepatica* ESP fractions with and without L-NMMA. The dashed and the dash-dot lines show the amount of NO produced by Rat PLCs with 15 mg/ml of untreated *F. hepatica* ESP (76.5 $\pm$ 1.7 μM) and PLCs alone (0.35 $\pm$ 0.4 μM) respectively. Values represent the means $\pm$ SD of triplicate wells. The results shown are representative of two separate experiments performed. Significant differences ($P<0.01$) for PLCs with S12 *F. hepatica* ESP fractions, compared to PLCs alone, were calculated using the Dunnett Multiple Comparisons test. Significant differences were observed, for PLCs incubated with S12 *F. hepatica* ESP fractions 16-17.

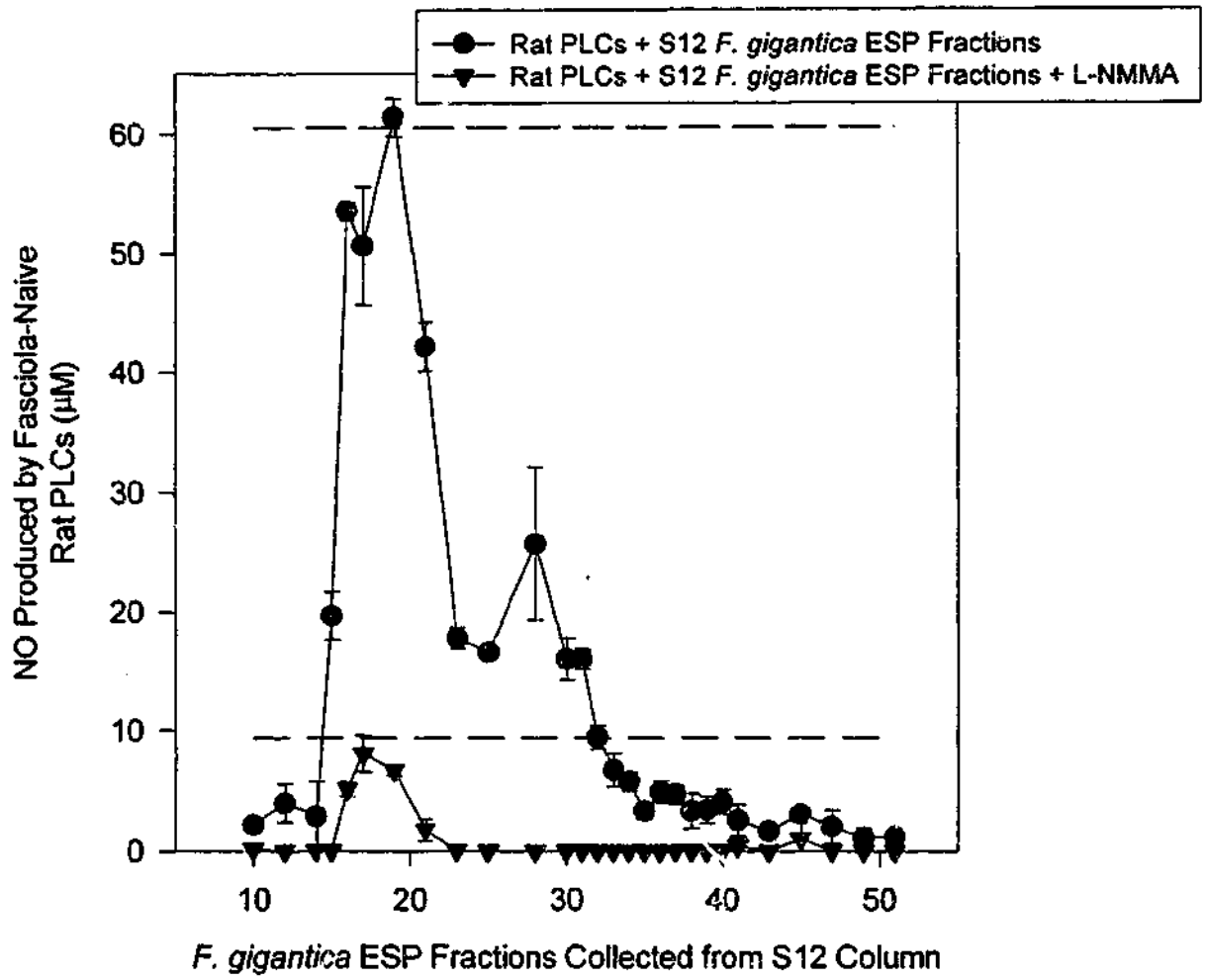


Fig 5.11 NO produced by *Fasciola*-naïve rat PLCs in the presence of *F. gigantica* ESP fractions collected from a Superose 12 (S12) column. Rat PLCs were stimulated with S12 *F. gigantica* ESP fractions with and without L-NMMA. The dashed and the dash-dot lines show the amount of NO produced by Rat PLCs with 15 mg/ml of untreated *F. gigantica* ESP (60.4 $\pm$ 2.1 µM) and PLCs alone (9.4 $\pm$ 0.5 µM) respectively. Values represent the means $\pm$ SD of triplicate wells. The results shown are representative of two separate experiments performed. Significant differences ($P<0.01$) for PLCs with S12 *F. gigantica* ESP fractions, compared to PLCs alone, were calculated using the Dunnett Multiple Comparisons test. Significant differences were observed, for PLCs incubated with S12 *F. gigantica* ESP fractions 15-21 and 28.

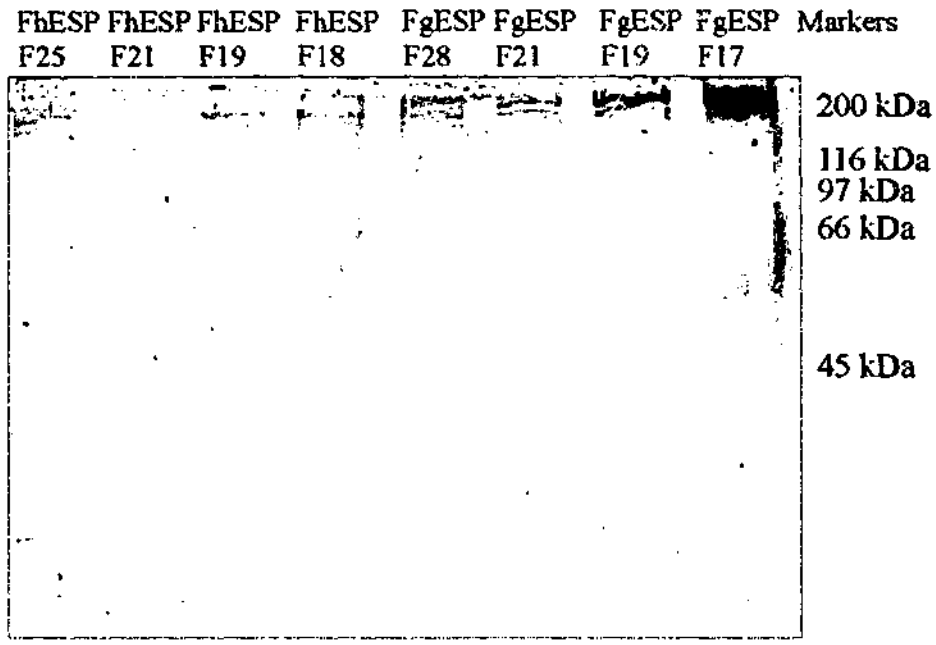


Fig 5.12 Silver periodic acid stained 7.5% SDS PAGE gel analysing gel filtration fractions of *Fasciola sp.* ESP. *F. hepatica* and *F. gigantica* ESP were fractionated on a Superose 12 (S12) column. These fractions (30 µl) were fractionated on 7.5% SDS PAGE gels and stained with silver periodic acid. The migration of the standards is shown on the right. The results presented are representative of two separate experiments.

by the methods used. The results suggest that different molecules were employed by the two, adult *Fasciola sp.* to stimulate NO production by resting Rat PLCs *in vitro*.

5.2.3 Anti-Oxidant Defence Enzyme Levels in *Fasciola* Whole Worm Extracts

To counterattack free radicals, which are involved in the host's arsenal of defence mechanisms, parasites have evolved a range of AOD enzymes to protect themselves. The next series of experiments compared the levels of AOD enzymes within adult and NEJ *F. hepatica* and *F. gigantica* whole worm extract (WWE). We hypothesised that, since *F. gigantica* is more susceptible to both the rat and sheep defence system, this parasite would not be capable of producing the range and quantity of defence enzymes produced by *F. hepatica*. Adult *F. hepatica* WWE were prepared from parasites collected from the bovine host and snap frozen. Adult *F. gigantica* WWE were prepared from parasites collected from the ovine host and snap frozen. These WWEs were used to detect the levels of glutathione S-transferase (GST), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase in adult *F. hepatica* and *F. gigantica* WWE.

The optimised protocols are displayed in Chapter 2.10. GST, GSH-Px and SOD levels were measured in adult *F. hepatica* and *F. gigantica* (Table 5.1). From these experiments adult *F. hepatica* WWE had significantly higher levels ($P<0.001$) of both GST and GSH-Px compared to adult *F. gigantica* WWE. Similar SOD levels were measured in both *Fasciola sp* WWE. Catalase was unable to be detected in either *Fasciola sp.* WWE. Catalase activity has not previously been detected in *F. hepatica* WWE (Prichard & Schofield 1971, Sanchez-Moreno *et al.* 1987, Piedrafita *et al.* 2000). The data presented here agree with these reports and also provide preliminary evidence for undetectable expression of catalase activity in *F. gigantica* WWE. Overall these results imply, that from our samples there is significantly greater expression of the glutathione dependent AOD enzymes, GST and GSH-Px, within adult *F. hepatica* WWE compared with adult *F. gigantica* WWE while SOD expression levels are similar.

The next experiments compared AOD enzyme levels in *F. hepatica* NEJ and *F. gigantica* NEJ. To assure that meaningful comparisons could be obtained WWEs were prepared from 5000 NEJ of *F. hepatica* and *F. gigantica* excysted on the same day and used immediately. In addition,

Enzyme Activity	Adult *Fasciola* Parasite	
	F. hepatica	*F. gigantica*
SOD	43 ± 9	43 ± 8
GST	9165 ± 1247	2924 ± 616
GSH-Px	535 ± 39	294 ± 8
CAT	nd	nd

nd – not detectable.

Table 5.1 AOD enzyme activities in whole worm extract (WWE) from adult *Fasciola sp.* WWEs were prepared from adult *F. hepatica* parasites collected from 3 different bovine hosts and adult *F. gigantica* parasites collected from three different ovine hosts. Values represent the mean enzyme activity ± SD from 3 experiments. Significant differences ($P<0.001$) for AOD enzyme activities between adult *F. hepatica* and *F. gigantica* WWE were calculated using the unpaired alternate t-test. Significantly higher levels of GST and GSH-Px were present in adult *F. hepatica* WWE compared to adult *F. gigantica* WWE.

each AOD enzyme assay was performed under the identical conditions on the same day. Only GST, GSH-Px and SOD activity levels were measured since Piedrafita *et al.* (2000) had shown undetectable catalase activity in *F. hepatica* NEJ (Table 5.2). Both GST and SOD enzymes, assayed in duplicate, were detected. GST expression levels were not significantly different between the two parasites. SOD expression levels were greater with *F. hepatica* NEJ WWE compared to *F. gigantica* NEJ WWE. Statistics could not be performed on the two *Fasciola sp.* NEJ measurements. However these values were representative of WWE prepared from 5000 NEJ of each species. Multiple experiments were limited due to the difficulty and associated costs in obtaining these large quantities of NEJ.

GSH-Px was not detected in single sample analyses of either NEJ *Fasciola* WWE. This was possibly due to the limited amount of sample available. Previous reports have detected GSH-Px in NEJ *F. hepatica* WWE (Piedrafita *et al.* 2000, Callahan *et al.* 1988). Future experiments therefore are still needed to address whether there is a difference in GSH-Px levels between *Fasciola sp.* NEJ.

Overall, our results showed comparable levels of SOD and significantly greater levels of GST and GSH-Px in adult *F. hepatica* WWE compared to adult *F. gigantica* WWE. In NEJ SOD expression was greater in *F. hepatica* NEJ WWE compared to *F. gigantica* NEJ WWE, while both *Fasciola sp.* NEJ WWE expressed comparable levels of GST. These results suggest a potential for *F. hepatica* to mount a more effective response against free radical attack by host immune cells.

Enzyme Activity	NEJ *Fasciola* Parasite	
	F. hepatica	*F. gigantica*
SOD	44 46	33 32
GST	486 503	512 491
GSH-Px	nd	nd
CAT	na	na

SOD – Superoxide dismutase; GST – Gluthatione S-transferase; GSH-Px – Glutathione Peroxidase; CAT – Catalase.

1 unit of superoxide dismutase activity is defined as that amount of enzyme required to inhibit the rate of reduction of cytochrome c by 50%.

The specific activity of glutathione S-transferase was defined as the amount of CDNB conjugated per min per mg protein.

1 unit of glutathione peroxidase activity is defined as that amount of enzyme necessary to oxidase 1 mmol of reduced glutathoine in 1 min.

1 unit of catalase activity is defined as that amount of enzyme necessary to decompose 50% of 20mM H_2O_2 in 1 min.

Specific activities of GSH-Px and GST are expressed as nmol min^{-1} mg^{-1} protein. Sod activity was expressed as U mg^{-1} protein.

NEJ - newly excysted juvenile liver fluke; nd – not detectable; na – not assayed.

Table 5.2 AOD enzyme activities in whole worm extract (WWE) from *Fasciola sp.* NEJ. WWEs were prepared from 5000 *F. hepatica* and *F. gigantica* NEJ parasites excysted overnight.. Values represent the enzyme activity from two determinations.

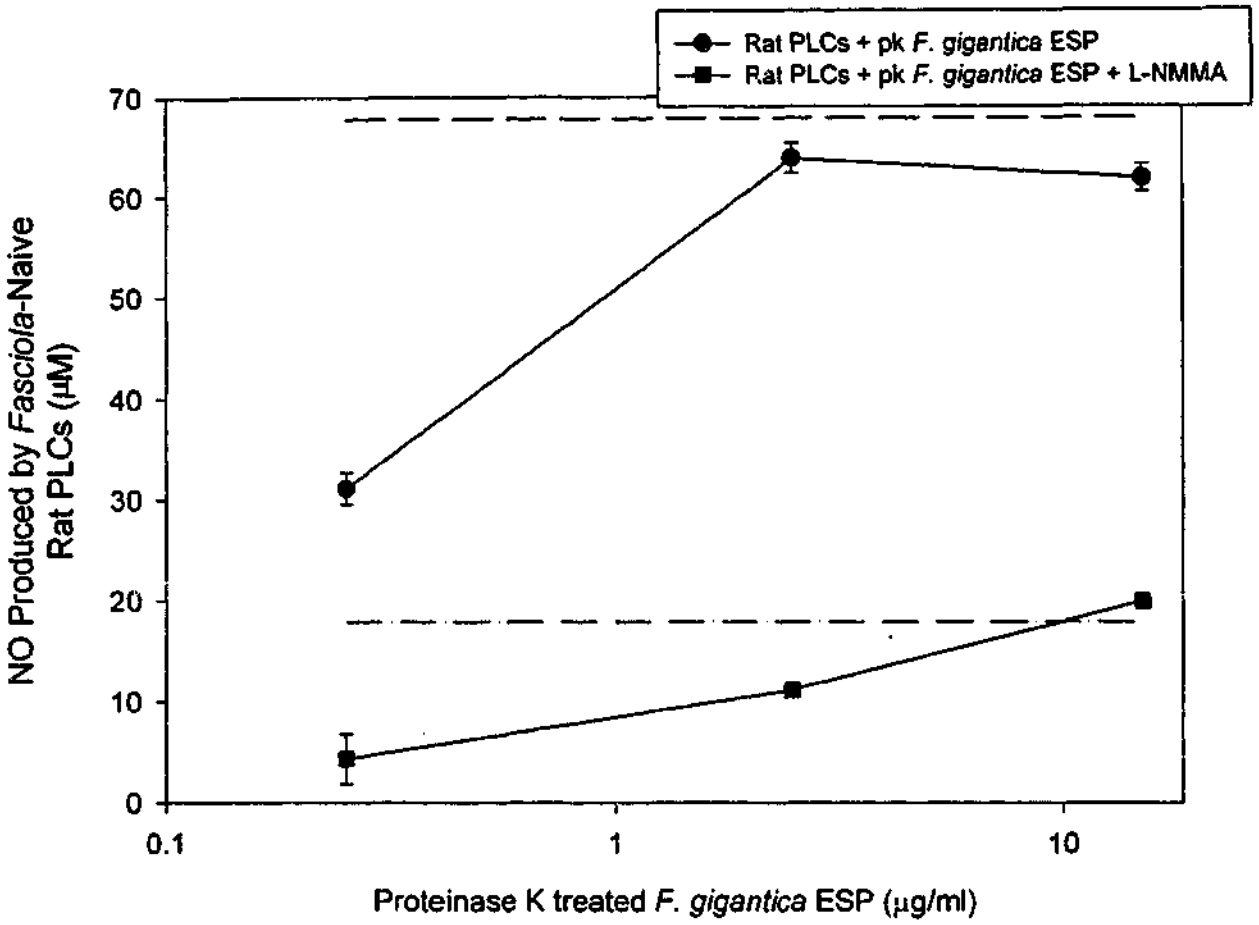


Fig 5.7 NO produced by *Fasciola*-naïve rat PLCs in the presence of proteinase K treated *F. gigantica* ESP (pk*F. gigantica* ESP). Rat PLCs were stimulated with increasing concentrations of pk*F. gigantica* ESP with and without L-NMMA. The dashed and the dash-dot lines show the amount of NO produced by Rat PLCs with 15 mg/ml of untreated *F. gigantica* ESP (67.8 $\pm$ 0.3 µM) and PLCs alone (17.9 $\pm$ 0.3 µM) respectively. Values represent the means $\pm$ SD of triplicate wells. The results shown are representative of two separate experiments performed. Significant differences ($P<0.01$) for PLCs with pk*F. gigantica* ESP, compared to PLCs alone, were calculated using the Dunnett Multiple Comparisons test. Significant differences were observed, for PLCs incubated with 0.25, 2.5 and 15 µg/ml of pk*F. gigantica* ESP.

stimulatory ability appeared to be predominantly localised in the early eluted fractions (Fig 5.8, 5.9). Significant differences compared to resting PLCs alone were observed for rat PLCs in the presence of *F. hepatica* and *F. gigantica* fractions 17-19 and 16-29 respectively (Fig 5.8 and 5.9). These fractions contain high molecular weight ESP components. In addition, *F. gigantica* fractions 37-42 also showed significantly greater NO production compared to resting rat PLCs alone (Fig 5.9). This suggests that there are at least two active components of NO-stimulatory molecules within *F. gigantica* ESP.

Due to the observed high molecular weight nature of the NO-stimulatory molecules, *Fasciola sp.* ESP were fractionated using gel filtration chromatography on a Superose 12 (S12) column which has a condensed optimal separation range, potentially yielding greater separation for the NO stimulatory molecules (1 – 300 kDa). The individual *Fasciola sp.* ESP fractions from the S12 column were also analysed for their effect on NO stimulatory ability. Significant differences compared to resting rat PLCs alone were observed in NO production from rat PLCs incubated with *F. hepatica* and *F. gigantica* S12 fractions 16-17 and 15-21 & 28 respectively (Fig 5.10, 5.11). These results again point to high molecular components being responsible for this mechanism. They also confirmed the likelihood of at least two NO stimulatory molecules within *F. gigantica* ESP based on consistently observing the two peak profile.

These *Fasciola sp.* ESP-stimulatory fractions, collected from the S12 column were analysed on a 7.5% SDS PAGE gel to visualise the size of these components. The NO-stimulatory fractions from the S12 column, for both *Fasciola sp.* ESP, contained molecular weight components greater than 200kDa (Fig 5.12).

Based on the observed size of the active components on 7.5 % SDS PAGE gels of *Fasciola sp.* ESP separated by gel filtration chromatography and the affect of either periodate or proteinase K treatment of *Fasciola sp.* ESP the following conclusions were drawn. Molecule/s within *F. hepatica* ESP capable of stimulating resting rat PLCs to produce NO appear to be high molecular weight glycoproteins and carbohydrates. In contrast, there were at least two molecules within *F. gigantica* ESP capable of stimulating resting rat PLCs to produce NO and these appear to be high molecular weight components resistant to either periodate oxidation or proteinase K degradation

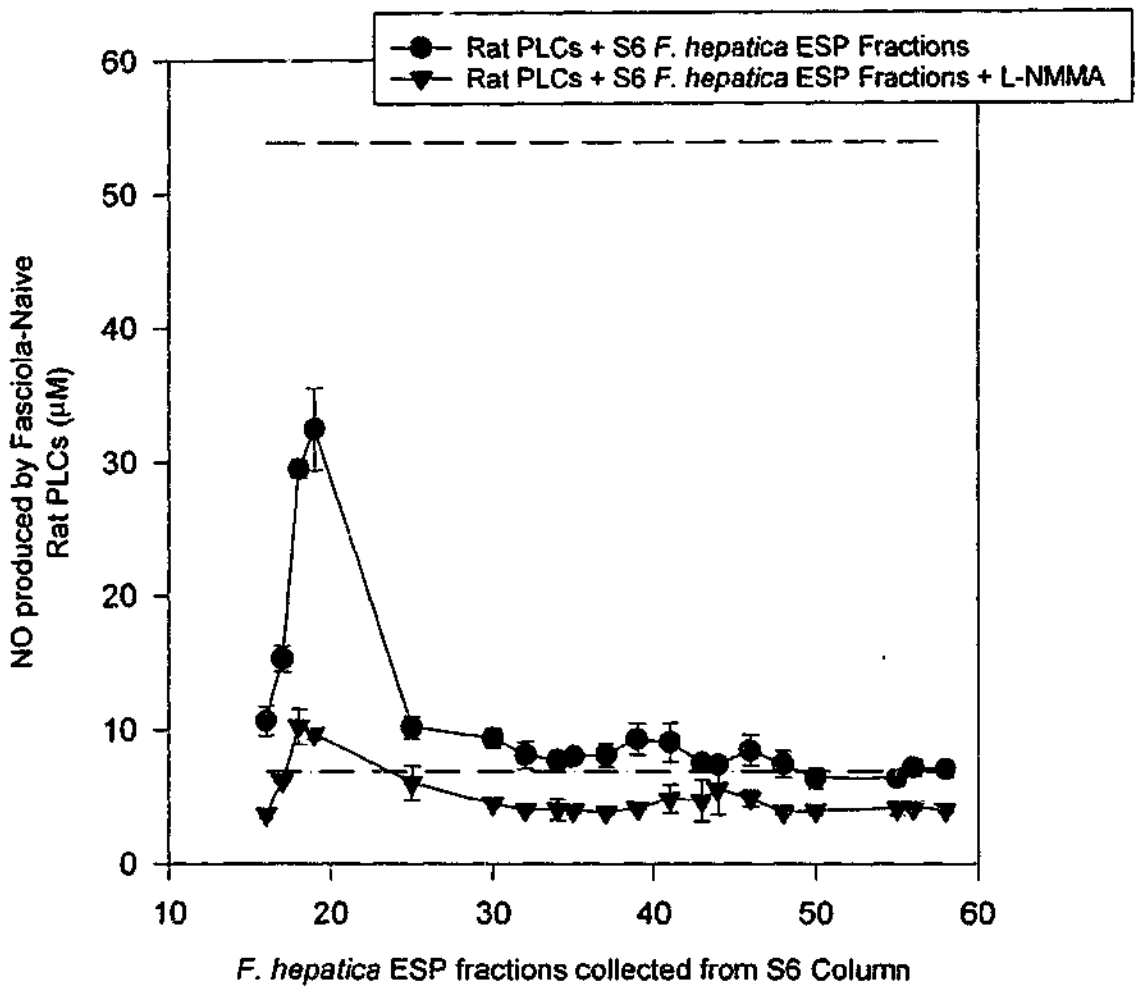


Fig 5.8 NO produced by *Fasciola*-naïve rat PLCs in the presence of *F. hepatica* ESP fractions collected from a Superose 6 (S6) column. Rat PLCs were stimulated with S6 *F. hepatica* ESP fractions with and without L-NMMA. The dashed and the dash-dot lines show the amount of NO produced by Rat PLCs with 15 mg/ml of untreated *F. hepatica* ESP (53.8 $\pm$ 2.0 μM) and PLCs alone (6.9 $\pm$ 0.3 μM) respectively. Values represent the means $\pm$ SD of triplicate wells. The results shown are representative of two separate experiments performed. Significant differences ($P<0.01$) for PLCs with S6 *F. hepatica* ESP fractions, compared to PLCs alone, were calculated using the Dunnett Multiple Comparisons test. Significant differences were observed, for PLCs incubated with S6 *F. hepatica* ESP fractions 16-17.

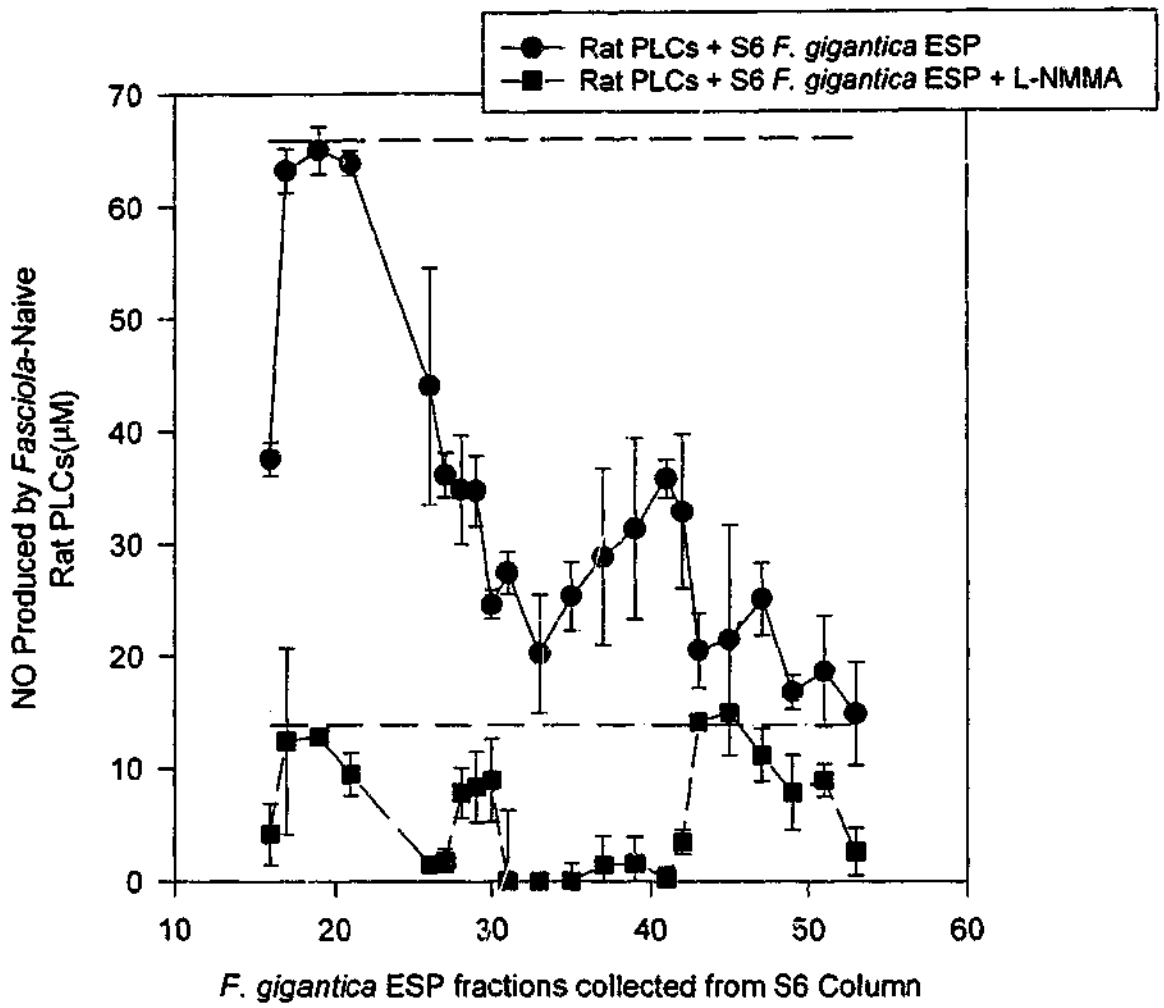


Fig 5.9 NO produced by *Fasciola*-naïve rat PLCs in the presence of *F. gigantica* ESP fractions collected from a Superose 6 (S6) column. Rat PLCs were stimulated with S6 *F. gigantica* ESP fractions with and without L-NMMA. The dashed and the dash-dot lines show the amount of NO produced by Rat PLCs with 15 mg/ml of untreated *F. gigantica* ESP (65.9 $\pm$ 3.5 μM) and PLCs alone (13.8 $\pm$ 1.6 μM) respectively. Values represent the means $\pm$ SD of triplicate wells. The results shown are representative of two separate experiments performed. Significant differences ($P<0.01$) for PLCs with S6 *F. gigantica* ESP fractions, compared to PLCs alone, were calculated using the Dunnett Multiple Comparisons test. Significant differences were observed, for PLCs incubated with S6 *F. gigantica* ESP fractions 16-29, and 37-42.

5.3 Discussion

In this study we firstly examined the influence of adult *Fasciola sp.* ESP on NO production from both activated and resting rat PLCs. There was no observed effect, with either adult *Fasciola sp.* ESP, on NO production levels by LPS-activated rat PLCs. However, incubation of either adult *Fasciola sp.* ESP with resting Rat PLCs stimulated NO production. Further investigation of adult ESP revealed the active component(s) within *F. hepatica* ESP was sensitive to periodate oxidation and partially sensitive to proteinase K treatment, whereas the active components within *F. gigantica* ESP were insensitive to either treatment. Untreated ESP was fractionated utilizing gel filtration chromatography. The NO stimulatory fractions contained high molecular weight components. Collectively, these results suggest that the active components within *F. hepatica* ESP are high molecular weight proteins, glycoproteins or carbohydrates whereas within *F. gigantica* ESP the high molecular weight components are resistant to periodate and proteinase K treatment.

Our original hypothesis was that *F. gigantica* parasites, unlike *F. hepatica* parasites, are unable to inhibit NO production by activated rat PLCs. We investigated whether this could play a role in the high resistance of the rat host to primary infection by *F. gigantica*. This was based on the observation of Cervi *et al.* (1998) that *F. hepatica* ESP inhibits NO production. Whilst we did not observe adult *F. gigantica* ESP inhibiting the NO burst by activated rat PLCs, we also did not observe this for adult *F. hepatica* ESP. The obtained results for adult *F. hepatica* ESP do differ with previously published work showing a reduction in NO production by LPS activated rat PLCs in the presence of adult *F. hepatica* ESP, collected from fluke recovered from bovine livers (Cervi, Rossi and Masih 1999). The work by Cervi and colleagues observed suppression of NO production by LPS stimulated rat PLCs at higher concentrations (20 and 30 μg/ml) of *F. hepatica* ESP than we presented in this section (maximum of 15 μg/ml). When using 20-30 μg/ml of ESP we observed a decrease in NO production by LPS activated rat PLCs but also a decrease in cell viability which Cervi, Rossi and Masih (1999) did not observe. A reduction in cell viability will lead to reduced levels of NO production. Therefore, our results do not show a difference between the two adult parasites in suppressing NO production by activated rat PLCs under conditions where viability is high.

From experiments examining *F. gigantica* establishment within the rat host, it appears that *F. gigantica* are killed not long after infecting the rat (Mango, Mango & Esamel 1972, Itagaki *et al.* 1994). In contrast *F. hepatica* show greater resistance to the early attack by rat immune cells in naïve rats. *F. hepatica* NEJ have been previously shown to be relatively resistant *in vitro* to free radicals from LPS-stimulated rat cells or chemically generated free radicals when compared with *Schistosoma mansoni* schistosomula (Piedrafita *et al.* 2000). One possibility is that a component within *F. hepatica* NEJ ESP is interacting with the immune system, but that this component is absent within *F. gigantica* NEJ ESP. Adult *Fasciola sp.* ESP was used in our NO experiments due to the ease of collecting large quantities for the experiments. NEJ and adult ESP do share some common antigens (Sandeman & Howell 1981,McGinty *et al.* 1993), with the thought being that NO-suppressive molecules within NEJ ESP may also be present in adult ESP. While a molecule that suppresses NO production in rat cells was not observed, a novel NO-stimulatory pathway was identified for adult *Fasciola* parasites. Piedrafita *et al.* (2001) showed that living NEJ release molecules that also stimulate NO and ROI production by rat PLCs. Future work will focus on comparing the effects of both *Fasciola sp.* NEJ ESP on NO production by activated rat PLCs or PLCs from other ruminant hosts.

Interestingly, we observed that different molecules within each of the *Fasciola sp.* ESP were able to stimulate NO production from resting rat PLCs *in vitro.* Due to both the size of the active component in ESP and the difficulty in inhibiting the activity of these molecules using standard techniques, the molecules were not identified, nor their structure established. Future work would focus on alternative treatments for cleaving carbohydrates such as N-, O- glycosidases and lipid moieties with the aim of identifying the NO stimulatory molecules in *Fasciola sp.* ESP.

There are a couple of published reports of a parasite stimulating NO production to aid in parasite survival. *Trypanoplasma borreli* parasites have been shown to stimulate host immune phagocytic cells to produce NO (Saeji *et al.* 2002). NO production was shown to be detrimental to the cyprinid fish, by inhibiting the proliferative response of blood and spleen cells. The authors propose that by inducing NO, *T. borreli* may be able to evade host immune mechanisms leading to longevity of infection (Saeji *et al.* 2002). The second report is for the human liver fluke

Opisthorchis viverrini which is also associated with excess nitric oxide generation with the authors suggesting these excessive levels are associated with the development of cancer of the biliary tract in humans (Haswell-Elkins *et al.* 1994). NO production is not thought to be detrimental to the parasite as *O. viverrini* is considered to have a long life span. The same group in subsequent papers proposes that NO aids in supplying *O. viverrini* with serum nutrients in the hepatic bile ducts by stimulating vasodilation of the biliary epithelium (Sithithaworn *et al.* 1994, Satarug *et al.* 1998).

Whether NO production favours the adult *Fasciola* parasite by breaking down tissue within the liver and/or bile ducts, aiding in the establishment and/or sustenance of the parasite, is unknown. The toxicity of NO with other co-factors is limited by: its short half life (3-50 sec); the need to be in close contact with the pathogen (Liew & Cox 1991, Brophy & Pritchard 1992); and the large quantities (>50 μM)needed to kill *F. hepatica* NEJ (Piedrafita *et al.* 2000, 2001). Based on these findings, cells surrounding the adult *Fasciola* parasite may not be able to produce enough NO to cause harm to the parasite and therefore NO production might aid adult *Fasciola* parasites by damaging the bile duct lining.

Our second working hypothesis was that *F. gigantica* mount a less effective defence against host free radical attack, through production of lower levels of AOD enzymes and that this would lead to increased susceptibility of *F. gigantica* to free radical attack. Our preliminary results supported this hypothesis. The results showed significantly higher activities of GST and GSH-Px within adult *F. hepatica* compared to adult *F. gigantica*. Higher levels of SOD activities were present in *F. hepatica* NEJ WWE compared to *F. gigantica* NEJ WWE. In addition, higher levels of GST were observed in adult parasites compared with NEJ parasites. Catalase activity was not detectable in adult *F. hepatica* nor *F. gigantica*; other groups have also not detected catalase in *Fasciola* parasites (Prichard and Schofield 1971, Piedrafita *et al.* 2000). In recent work by our laboratory it has been shown that *F. gigantica* NEJ are more susceptible to chemically generated ROI, ROI from sheep PLCs and NO than *F. hepatica* NEJ (Piedrafita *et al.* 2002 - submitted). This susceptibility of *F. gigantica* to killing by sheep PLCs was reversed when SOD was added, suggesting that the active component responsible for killing is ROI. This supports the hypothesis that *F. gigantica* is sensitive to free radical attack due to lower SOD

expression in *F. gigantica* NEJ compared with *F. hepatica* NEJ. The production of lower levels of AOD enzymes may be one of a number of factors contributing to the greater susceptibility of *F. gigantica* to host attack.

GST activities in several parasites appear to be higher when recovered from susceptible hosts compared to parasites from resistant hosts (Miller, Howell & Boray 1993, Brophy *et al.* 1995). The adult *F. hepatica* WWE were prepared from flukes collected from a resistant host (cattle) and the adult *F. gigantica* WWE were prepared from flukes collected from a resistant host (ITT sheep). Even though both hosts display resistance to the respective parasite, in future it would be beneficial to compare AOD enzyme activity levels between adult *F. hepatica* and adult *F. gigantica* WWE recovered from the same host. Thus although our results are likely to be indicative of a phenomenon of lower expression levels of GST and GSH-Px in *F. gigantica* compared with *F. hepatica* this hypothesis needs further evaluation.

The observation of higher GST enzyme activities for adult parasites compared to NEJ for both *Fasciola* parasites were expected. A previous report showed that as *F. hepatica* matured, the activity of GST increased (Piedrafita *et al.* 2000). Also, during the development of *S. mansoni* there is an increased resistance to killing by ROI, in a cell free system that corresponds with an increase in the activity of several AOD enzymes (Nare, Smith & Prichard 1990). There was a change in profile of AOD enzyme activities between the parasites as they develop to adulthood with higher levels of GST and GSH-Px in adult *F. hepatica* compared with adult *F. gigantica*: higher levels of SOD were observed in NEJ *F. hepatica* relative to *F. gigantica*. This may be one of the contributing reasons for the greater resistance of *F. hepatica* to immune attack by host cells. Piedrafita and colleagues (2000) showed that NEJ *F. hepatica* are more susceptible to free radical killing *in vitro* after treatment with inhibitors of glutathione metabolism and peroxidases. Whether these enzymes play an important role in the resistance to oxidative killing by the host *in vivo* has yet to be determined. This could be addressed by *in vivo* experiments treating animals with selective inhibitors of parasite GST or SOD. Currently there are no such inhibitors on the market.

CHAPTER 6. GENERAL DISCUSSION

The original aim of this thesis was to identify both evasion strategies utilised by *Fasciola sp.* parasites and / or possible biochemical differences between *F. gigantica* and *F. hepatica*, which could contribute to the variance in host susceptibility to infection with *F. hepatica* and *F. gigantica* observed between these species. We set out to test four hypotheses:

1. that *F. gigantica* parasites are unable to inhibit nitric oxide (NO) production by phagocytes resulting in the high resistance of the rat host to *F. gigantica*;
2. that parasite antioxidant AOD enzymes are important determinants of the resistance of *F. hepatica* to antibody dependent cellular cytotoxicity (ADCC);
3. that *F. hepatica* has an enhanced capacity relative to *F. gigantica* to suppress host cellular proliferation responses *in vivo* and *in vitro* and that this contributes to the infectivity of *F. hepatica*; and
4. the corollary was that this suppression would involve an interaction of *Fasciola sp.* ESP with specific host immune effector cell surface markers.

Accordingly, this thesis has evaluated the ability of *Fasciola sp.* ESP to influence the defense pathways involving NO production, compared the levels of AOD enzymes in *F. hepatica* and *F. gigantica* and investigated in detail the factors in ESP involved in immunosuppression.

The host's immune response generated after parasitic infection is a decisive factor in whether the parasite will be eliminated or will survive. In many cases the host is able to induce the appropriate effector mechanism and destroy the parasite (Abbas *et al.* 1996). This response may consist of both innate and acquired defence mechanisms. These responses are connected due to their co-dependency on one another in their immunological functions as discussed in Chapter 1.4. The toxic effect of free radicals released by monocytes / macrophages or chemically generated towards parasites *in vitro* and *in vivo* has been well studied. Free radicals have been shown to play a role in the host response against a range of protozoan and helminth parasites including *Trypanosoma cruzi*, *Toxoplasma gondii*, *Leishmania sp.*, *Plasmodium sp.*, *Dirofilaria immitis*, *Nippostronglyus brasiliensis*, *Trichinella spiralis* and *Schistosoma mansoni* (cited in

Smith 1989). These toxic effects of free radicals were in some instances only observed after the inhibition of parasite AOD enzymes, suggesting the crucial role of AOD enzymes in the parasites' survival.

In published work by Piedrafita and colleagues (2000) NEJ *F. hepatica* parasites were shown to be less susceptible to NO-mediated damage than the juvenile stage of *S. mansoni*. In contrast *F. gigantica* are highly susceptible to NO (personal communication with Dr David Piedrafita). This may explain why *F. gigantica* has a poor survival rate within the rat as rat PLCs produce high quantities of NO (Smith, Ovington & Boray 1992, Piedrafita *et al.* 2001). Our original hypothesis for this body of work was that *F. gigantica* when compared to *F. hepatica* parasites would be ineffective at down regulating NO production by rat immune cells. Considering this, it was of interest when we identified molecules released by adult *F. hepatica* and *F. gigantica* ESP which actually stimulated NO production by resting rat PLCs. The NO-stimulatory components within adult *F. hepatica* ESP were high molecular weight glycoproteins and carbohydrates and, within adult *F. gigantica* ESP were high molecular weight components resistant to periodate and proteinase K treatment. These molecules stimulated NO production without the presence of normal iNOS stimulators like LPS or cytokines, suggesting the complete identification of these fluke molecules will add additional classes to these known iNOS stimulators. Furthermore the identification of these molecules may allow the elucidation of their stimulatory mechanism.

It is evident that NO plays many roles besides being toxic to invading pathogens (reviewed in Chapter 1.4) and that alternative explanations for the stimulation of NO production by *Fasciola* ESP need to be made. Precedents exist for the ability of trematode parasites to stimulate NO production during infection. The human parasite, *Opisthorchis viverrini*, is associated with increased levels of nitric oxide production by host cells during infection (Haswell-Elkins *et al.* 1994). NO is not thought to cause any detrimental effects to the parasite. The same group has published work suggesting that this production of NO is associated with the increased risk of cancer of the biliary tract through the nitrosation of amines, resulting in the formation of N-nitroso compounds like N-nitrosodimethylamine (Haswell-Elkins *et al.* 1994). In addition NO levels were implicated in the suppression of mitogen-induced lymphocyte proliferation *in vitro*, which is similar to observations during infection with Malaria and Trypanosomes (Satarug *et al.*

1998). Whether the elevated levels of NO produced by PLCs stimulated with adult *F. hepatica* ESP *in vitro* can be associated with the specific suppression of rat cellular proliferation observed late in infection is not yet known. What is of interest to this current *Fasciola* work is the suggestion that NO aids in supplying *O. viverrini* with serum nutrients in the hepatic bile ducts by coercing vasodilation of the biliary epithelium (Sithithaworn *et al.* 1994 and Satarug *et al.* 1998). The fish parasite *Trypanoplasma borelli*, was shown to induce NO production by cyprinid fish cells (Saeji *et al.* 2002). The NO production was shown to cause detrimental effects to the fish, leading the authors to propose that NO production favoured the parasite and not the host. Following on from these experimental findings, we propose one function of induction of NO by adult *F. hepatica* parasites *in vitro*, may elude to a role for NO in aiding the establishment of the adult parasite within the liver parenchyma of rats and in supplying the parasite with nutrients.

Protective immunity within the rat is proposed to be induced as the parasite migrates through either the peritoneal cavity or the liver of the rat (van Milligen, Cornelissen & Bokhout 1998a, 2000). It would be of interest to examine whether NEJ ESP also stimulates NO production by rat PLCs. Piedrafita and colleagues (2001) have shown that rat PLCs incubated with *F. hepatica* NEJ also produce NO and ROI, suggesting that *F. hepatica* NEJ ESP also stimulates NO production. The collection of NEJ ESP from both *Fasciola* sp. would allow for further experiments to be conducted to compare the ability of NEJ ESP from the *Fasciola sp.* to modulate free radical production. If both *Fasciola sp.* NEJ stimulate NO and NO is shown to be involved in the resistance of rats to *F. gigantica* infections it may suggest the presence of an opposing unknown factor within *F. gigantica* NEJ which reduces the parasites resistance to NO. Circumstantial evidence has pointed to a NO role when *F. gigantica* NEJ were shown to be highly susceptible to NO cytotoxicity *in vitro* when compared to *F. hepatica* NEJ (personal communication with Dr Piedrafita).

Work in our laboratory has shown that *F. gigantica* NEJ are more susceptible to chemical or sheep PLC generated ROI than *F. hepatica* NEJ (Piedrafita *et al.* 2002). This lead to another hypothesis to be explored in this thesis, whether *F. gigantica* when compared with *F. hepatica*, would express lower levels of AOD enzymes, thereby making the parasite more susceptible to host free radical attack. This appears to be true with *F. gigantica* NEJ expressing lower levels of

SOD. In recent work, *F. gigantica* NEJ were able to survive antibody-dependent cell cytotoxicity (ADCC) in the presence of sheep PLCs only when exogenous SOD was added to the *in vitro* culture incubations (Piedrafita *et al.* 2002 submitted). This mechanism of ADCC was ineffective against *F. hepatica* NEJ. This may suggest that *F. hepatica* produces sufficient quantities of SOD to evade attack by ROI and that *F. gigantica* is deficient in this AOD enzyme. This is consistent with our preliminary findings of lower SOD expression in *F. gigantica* NEJ compared with *F. hepatica* NEJ. Whether this is one of the factors for the relative resistance of rats and sheep to *F. gigantica* infections is yet to be proved.

We also showed that adult *F. gigantica* expressed lower levels of both GST and GSH-Px when compared to defence enzyme expression from the same life stage of *F. hepatica*. The higher levels of GSH-dependent AOD enzymes within adult *F. hepatica* compared with adult *F. gigantica* was interesting. In work by Miller, Howell & Boray (1993) it was shown that *F. hepatica* produced different quantities of GST depending on the host from which the parasites were isolated. Within resistant hosts *F. hepatica* produced lower levels of GST whereas in susceptible hosts *F. hepatica* produced higher quantities of GST. Whether this implies that GST is an important part of *F. hepatica*'s evasion strategies and that a resistant host is able to suppress this production of GST has yet to be determined. It does, however, suggest that future comparison of defence enzyme levels between adult *F. hepatica* and *F. gigantica* should be made from parasites recovered from the same host and preferentially of the same age as it has also been shown that GST levels increase as the parasite ages.

In the relatively resistant cattle host, an early-induced proliferation of mononuclear cells is observed in response to *F. hepatica* somatic antigen (Oldham 1985, Oldham & Williams 1985). There are suggestions that resistance in the cattle host is not completely immunologically determined (Boray 1969; Doy and Hughes 1984; Hughes 1987). However, the results of Oldham and Williams (1985) may suggest that the capability of host lymphocytes to respond to fluke antigens may be one of the defence arms needed in the elimination of *Fasciola* parasites.

Our third hypothesis was that *F. gigantica*, unlike *F. hepatica*, would not be able to suppress lymphocyte proliferation at key times post infection. We showed however that infections with *F.*

gigantica, like *F. hepatica* infections, are also capable of suppressing mitogen-induced Merino sheep cellular proliferation, thereby suggesting repression of the sheep immune system. Suppression occurred at 3 and 9-10 weeks post infection corresponding with proposed crucial times in the parasite's life cycle where a dampening of the immune response aids in parasitic establishment. In Merino sheep the difference in the survival rate of *Fasciola sp.* is not that large (Table 1.2). Considering this perhaps it was not surprising that both parasites were able to suppress lymphocyte proliferation in a *Fasciola sp.* susceptible sheep breed suggesting a common evasion mechanism. What has not yet been answered is whether the extent of suppression differs between *F. hepatica* and *F. gigantica* infections and whether this suppression leads to a functional impairment of the immune system. If suppression leads to functional impairment, it would be expected that *F. gigantica* would suppress proliferation to a lesser extent as sheep tend to show greater resistance to *F. gigantica*. This will be answered in future experiments, by infecting the same population of sheep with either *F. hepatica* or *F. gigantica* and proliferation profiles can then be compared statistically.

The definitive experiment for whether lymphocyte proliferation is a measure of the functional capacity of the immune system could be performed by infecting the relatively resistant ITT sheep (Table 1.2) with *F. gigantica* in parallel with Merino sheep. ITT sheep are not currently permitted into Australia.

In the work presented in this thesis we discovered two additional capabilities for cathepsin L from both *F. hepatica* and *F. gigantica*. Previous work had alluded to a role for *F. hepatica* ESP in suppressing immune responses in rats and sheep (Masih, Cervi & Casado 1996, Cervi & Masih 1997, Jefferies, Barrett and Turner 1996). We extended this by showing cysteine protease activity within *Fasciola* ESP plays a key role in suppressing cellular proliferation *in vitro* (Prowse *et al.* 2002). Cathepsin L, *in vitro*, was shown to be able to suppress mitogen-induced sheep cellular proliferation. Interestingly, cathepsin L activity is detected in all life stages of *F. hepatica* (Dalton and Heffernan 1989, Carmona *et al.* 1993, Tkalcevic *et al.* 1995). This has not yet been shown for *F. gigantica* (genes encoding 6 cathepsin Ls have been detected in adult *F. gigantica*, Grams *et al.* 2001). If cathepsin L is the suppressor molecule, and is released by all stages of *F. hepatica* why is suppression only observed at certain times post infection? There are

many possibilities and one of these may be that there is increased expression of cathepsin L at these suppression times. This could be addressed by quantifying levels of cathepsin Ls mRNAs in the different parasite life stages. Another possibility is based on the fact that *Fasciola* express different isoenzymes of cathepsin L. As mentioned in Chapter 3 there are at least seven different isoenzymes. Both their expression during parasite development and their immunological roles may vary for each isoenzyme. Indeed, NEJ of *F. hepatica* express different cathepsin L sequence classes to those found in adult *F. hepatica* (Heussler & Dobbelaere 1994, Tkalcevic *et al.* 1995). We attempted to address this by examining the effect of different recombinant cathepsin Ls, on T cell proliferation, each enzyme belonging to a different cathepsin L class based on substrate specificity. The two recombinant cathepsin Ls were each able to suppress sheep T cell proliferation *in vitro*. Further work is needed to purify the cathepsin Ls being expressed at different stages post-infection and examine each cathepsin L in the *in vitro* proliferation system.

While the importance of cathepsin L involvement in suppressing cellular proliferation *in vitro* was demonstrated, this has not been shown *in vivo*. There are two possible approaches to address this. The first is to treat animals with a parasite specific cysteine protease inhibitor (currently not on the market) during *Fasciola* infection and the second approach is to induce neutralising cathepsin L antibody by vaccinating animals with cathepsin L and then infecting with *Fasciola*. Proliferation profiles between uninfected animals, *Fasciola*-infected animals and treated animals infected with *Fasciola* could then be compared. If feasible both of these experiments would add additional evidence for a role of cathepsin L in suppressing sheep cellular proliferation *in vivo*.

Fluke cathepsin Ls are also released during cattle infections where there is no observed suppression of cellular proliferation. Some possible explanations for this observation are that cattle either release an inhibitor of *Fasciola* cathepsin L, that cathepsin L is unable to suppress cattle cellular proliferation through the same pathway utilised within sheep or that cathepsin L is suppressing lymphocyte proliferation locally and this is not being detected systemically due to a global increase in proliferation. These experiments need further investigation.

In some cases the host generates ineffective immune responses which may result from the parasite modulating the host's immune response to favour its own survival. There is no one set of

immune mechanisms that will eliminate any type of parasite (Abbas, Murphy & Sher 1996). Previously published work has shown that different parasites require different immune responses to be killed and that different parasites elicit different host immune responses. Many intracellular parasites have been shown to be more susceptible to a cell mediated immune response (*Toxoplasma gondii*, *Trypanosoma cruzi* and *Leishmania major*; cited in Locksley R.M *et al.* 1999). In contrast many extracellular parasites are more susceptible to a polarised Th2 response mediated by humoral immune mechanisms (Finkelman F.D *et al.* 1991, Garside *et al.* 2000). While this is effective in some cases it has been proposed that in some circumstances the elicited immune response is a "smoke screen" to allow the parasite to survive (reviewed in Kelso 1995, Riffkin *et al.* 1996).

In the case of *F. hepatica* infections, a dominant Type 2 like response is elicited post-infection within mice, rats, cattle and sheep. These responses include: strong / high levels of eosinophilia, high IgG1 levels, and a biphasic production of IgE. Work by O'Neill and colleagues (2000, 2001) also points to *F. hepatica* overriding the normal induction of a Th1 response in mice post-infection with *Bordetella pertussis* in favour of a Th2 response. The polarisation of the immune response was shown to be dependent on cathepsin L within *F. hepatica* ESP inducing an IL-4 dependent suppression of IFN-γ which is a key Th1 cytokine. It is proposed that these Th2 dominant responses are likely to be induced by the parasite in order to evade an effective host immune response. In contrast, *F. gigantica* infections in ITT sheep were shown to induce a stronger Th2 like response than in Merino infected sheep (Hansen *et al.* 1999). These responses were characterised by higher levels of IgM, IgG1 and IgE antibodies. ITT sheep also produced lower levels of IgG2 (Th1 like antibody in sheep). As ITT sheep are resistant to *F. gigantica* infections, these results highlight that the generated immune response within sheep is a complex system that is not yet fully understood.

Our results showing that both *F. hepatica* and *F. gigantica* cathepsin L suppresses mitogen-induced cellular proliferation add to a growing body of potential roles for cathepsin L in evading host immune responses. As reviewed in Chapter 1.5, *F. hepatica* cathepsin Ls can degrade the extracellular matrix (fibrillar types I and II collagen), the basement membrane (type IV collagen) (Berasain, Goni et al 1997)and haemoglobulins *in vitro* (Dalton & Heffernan 1989). Cathepsin

Ls can cleave immunoglobulins released by mice and humans *in vitro* (all IgG subclasses) in the hinge region of the heavy chain (Carmona *et al.* 1993, Smith *et al.* 1993, Berasain, Carmona *et al.* 2000). Finally *F. hepatica* cathepsin L polarises the murine immune response to a Th2 profile by decreasing IFN-γ production in an IL4-dependent manner (O'Neill, Mills & Dalton 2001). In addition to cathepsin L's role in suppression of cellular proliferation *in vitro*, we also showed that *Fasciola sp.* cathepsin L selectively down regulate CD4 and CD8 on lymphocytes along with up regulating CD1b expression on dendritic cells.

CD4 acts as a co-receptor along with the TCR: CD3 complex in antigenic recognition of MHC II on the surface of antigen presenting cells (Janeway 1989, Janeway and Bottomly 1994). This bivalent interaction has been shown to be necessary for T helper cell activation and development (Janeway 1989, Janeway and Bottomly 1994). The importance of $CD4^+$ T cells in regulating helminth infections has been well established in several hosts. For example, genetic resistance of lambs to *Haemonchus contortus* could be abrogated after $CD4^+$ T cell depletion (Gill, Watson & Brandon 1993). In mice, the $CD4^+$ T cell subset was demonstrated, through depletion studies, to mediate expulsion of *Trichinella spiralis* from the gut (Grencis, Riedlinger & Wakelin 1985). In *Heligmosomoides polygyus* challenge infections, mice are capable of inducing a protective response to the infection, which significantly reduces development of new adult worms by 70-80%. However, when mice were administered an anti-CD4 antibody this resistance was reversed (Urban, Katona & Finkelman 1991). In primary infections with *F. hepatica*, increased lymphocyte proliferation attributable to $CD4^+$ T cells has been observed in cattle (McCole *et al.* 1999, Brown *et al.* 1994a). In direct contrast, the more susceptible sheep host shows diminished T lymphocyte proliferation *ex vivo* during infection with *F. hepatica* (Zimmerman *et al.* 1983, Chauvin, Bouvet & Boulard 1995). This is unlikely to be the only factor governing acquired resistance to fasciolosis as rats also exhibit a decrease in $CD4^+$ lymphocytes levels during a primary infection (Poitou, Baeza & Boulard 1993). It now appears likely that $CD4^+$ T cells will be crucial players in host immunity to *Fasciola sp.* as has been shown for other helminth infections.

Based on our work showing cleavage of CD4 from the surface of lymphocytes by cathepsin L and the work by O'Neill and colleagues (2000, 2001) showing cathepsin L involvement in

biasing the immune response towards a Th2 response in mice the following scenario is envisaged. Following fluke infection, CD4 is cleaved from Th1 subsets thereby reducing their proliferation and reducing the Th1 cytokine output aiding in Th2 polarisation. While we showed that CD4 is cleaved *in vitro* from ovine, human and bovine cells, CD4 is also cleaved from the surface of mouse lymphocytes (Personal communication Ruby Law and Adriana Baz). In work by Xu and colleagues (1998) a Th2 specific marker (ST2L) was identified. Therefore, this hypothesis could be examined in future work by a dual staining experiment using CD4 and ST2L labelled with different fluorescent tags. This will allow the visualisation of the subset, from which CD4 is being cleaved. However it is very likely that cathepsin L will cleave CD4 from both T cell subsets to achieve a general dampening of the immune activation in response to foreign antigens.

CD8 was also shown to be down regulated on lymphocytes in the presence of cysteine protease activity within *Fasciola* sp. ESP. CD8 is expressed as a hetero-dimer of an α- and β- chain on the surface of mature MHC class I restricted T cells (CTLs). CTLs are one of the arms of Th1 mediated defence mechanisms. CD8, similarly to CD4, participates in lymphocyte adhesion to antigen presenting cells, lymphocyte activation through interactions with TCR-CD3 complex, and is involved in modulation of late events during lymphocyte activation (Weiss & Imboden 1987). This was reviewed in Chapter 4. Down regulation of CD8 on CTLs will suppress proliferation of an important component of Th1 defence strategies. This suppression may also contribute to the polarisation towards a Th2-dominated immune response. The mechanism for this suppression may relate to the decreased expression of IL-2 from cells expressing the hetero-dimer of CD8. Whether cleavage of CD8 by cysteine protease activity within *Fasciola* ESP correlates with lower IL-2 production has yet to be seen. If it does, this will have two effects on the immune response. Firstly, IL-2 stimulates proliferation of lymphocytes. By *Fasciola* cysteine proteases decreasing IL-2 production this may result in suppressed proliferation. As discussed previously *F. hepatica* infections bias the immune system toward a Th2 response. IL-2 is a Th1/Th0 cytokine. Th1 and Th2 cytokines negatively modulate the production of the other cytokine subset (Sher & Coffman 1992, reviewed in Abbas, Murphy & Sher 1996). By decreasing the production of IL-2, Th2 cytokines may be up regulated. This may be an additional mechanism for the biasing of the Th2 response to add to the IL-4 dependent suppression of IFN-

γ production reported by O'Neill *et al.* (2001). Whether the cleavage of CD4 and CD8 from lymphocytes is the reason for the suppressed proliferation remains to be proved. Future work will look at antigen-specific proliferation assays and whether cathepsin L suppresses proliferation in these assays.

The work presented in this thesis only assessed the effect of *Fasciola* ESP on a multiplicity of sheep surface markers and whether the addition of E64, a cysteine protease inhibitor, affected these changes in expression. Future work will utilise recombinant cathepsin L proteases and analyse their effects on CD8, CD1b and other markers expressed on a range of host cells (such as mice, humans and cattle). This will provide new insights into the generality of the observations made in sheep. Future experiments will also examine the effect of *Fasciola sp.* ESP on ITT sheep surface markers.

In conclusion we have shown that *Fasciola* parasites affect the expression of both CD4 and CD8 on Merino T cells, molecules known to play important roles in T lymphocyte activation: down regulation of CD4 and CD8 levels may result in suppression of lymphocyte proliferation. This selective modulation of these two T cell markers is likely to play a key role in *Fasciola sp.* evasion strategies *in vivo*.

In summary we have identified potential evasion mechanisms *Fasciola sp.* may utilise to survive in receptive hosts. This included the suppression of sheep lymphocytes by *F. gigantica* at proposed key times post infection, previously demonstrated for *F. hepatica*, along with the cleavage of CD4 and CD8 on the surface of sheep lymphocytes. In addition CD4 was shown to be down-regulated by *Fasciola sp.* cysteine proteases on the surface of human and bovine lymphocytes. Further circumstantial evidence for cleavage of CD4 was obtained by recombinant *F. hepatica* cathepsin Ls cleaving recombinant human CD4 *in vitro*. Both of these mechanisms were new roles for *Fasciola sp.* cathepsin L. Whether these two phenomena are linked requires further investigation. Interestingly both adult *Fasciola sp.* ESP were shown to stimulate the production of NO by resting rat PLCs. The immunological relevance of this stimulation is yet to be determined. Finally we have acquired preliminary evidence that AOD enzymes levels expressed by *Fasciola sp.* may be important determinants in their susceptibility within the rat

and sheep host. However all of these findings need to be further studied and characterised by future investigators with a keen interest in the immunology of the parasite: host relationship.

CHAPTER 7. REFERENCES

Abbas A.K., Murphy K.M., and Sher A. 1996. Functional diversity of helper T lymphocytes. *Nature* **383**, 787-793

Abu-Soud H.M., and Stuehr D.J. 1993. Nitric oxide synthases reveal a role for calmodulin in controlling electron transfer. *Proceedings of the National Academy of Sciences USA* **90**, 10769-10772

Adams L.B., Hibbs J.B. Jr., Taintor R.R., and Krahenbuhl J.L. 1990. Microbiostatic effect of murine-activated macrophages for *Toxoplasma gondii*. Role for synthesis of inorganic nitrogen oxides from L-arginine. *Journal of Immunology* **144**, 2725-2729

A'Gadir H., Haroun E.M., and Gameel A.A. 1987. The protective effect of irradiated metacercariae of *Fasciola gigantica* against homologous challenge in sheep. *Journal of Helminthology* **61**, 137-142

Ali B.H. Hassan T., Haroun E.M. and Abu Samra M.T. 1985. The effect of niclofolan on desert sheep experimentally infected with immature *Fasciola gigantica*. *Journal of Veterinary Pharmacology and Therapeutics* **8**, 398-403

Amiot M., Dastot H., Degos L., Dausset J., Bernard A., and Boumsell L. 1988. HLA class I molecules are associated with CD1a heavy chains on normal human thymus cells. *Proceedings of the National Academy of Sciences USA* **85**, 4451-4454

Amstad, P., Moret R., and Cerutti P. 1994. Gluathione Peroxidase compensates for the hypersensitivity of Cu,Zn-superoxide dismutase overproducers to oxidant stress. *The Journal of Biological Chemistry* **269**, 1606-1609

Amstad P., Peskin A., Shah G., Mirault M.E., Moret R., Zbinden I., and Cerutti P. 1991. The balance between Cu,Zn-superoxide dismutase and catalase affects the sensitivity of mouse epidermal cells to oxidative stress. *Biochemistry* **30**, 9305-9313

Anaya-Prado R., Toledo-Pereyra L.H., Lentsch A.B., and Ward P.A. 2002. Ischemia/reperfusion injury. *Journal of Surgical Research* **105**, 248-258

Angulo I., de las Heras F.G., Garcia-Bustos J.F., Gargallo D., Munoz-Fernandez M.A., Fresno M. 2000. Nitric oxide-producting CD11b(+)Ly-6G(Gr-1)(+)CD31(ER-MP12)(+) cells in the spleen of cyclophosphamide-treated mice: implications for T-cell responses in immunosuppressed mice. *Blood* **95**, 212-220

Apolloni E., Bronte V., Mazzoni A., Serafini P., Cabrelle A., Segal D.M., Young H.A., and Zanovello P. 2000. Immortalized myeloid suppressor cells trigger apoptosis in antigen-activated T lymphocytes. *Journal of Immunology* 165, 6723-6730

Armour J., and Dargie J.D. 1974. Immunity to *Fasciola hepatica* in the rat. Successful transfer of immunity by lymphoid cells and by serum. *Experimental Parasitology* **35**, 381-388

Arrick B.A., Nathan C.F., Griffith O.W., and Cohn Z.A. 1981. Glutathione depletion sensitises tumor cells to oxidative cytolysis. *Journal of Experimental Medicine* **153**, 766-782

Arundal J.H. 1986. Liver fluke. In: *Sheep and wool refresher course*. Department of Agriculture, Tasmania, Australia, 17-25

Assreuy J., Cunha F.Q., Epperlein M., Noronha-Dutra A., O'Donnell C.A., Liew F.Y., and Moncada S. 1994. Production of nitric oxide and superoxide by activated macrophages and killing of *Leishmania major*. *European Journal Of Immunology* **24**, 672-676

Atochina O., Daly-Engel T., Piskorska D., McGuire E., and Harn D.A. 2001. A schistosome-expressed immunomodulatory glycoconjugate expands peritoneal Gr1(+) macrophages that

suppress naive CD4(+) T cell proliferation via an IFN-gamma and nitric oxide-dependent mechanism. The *Journal of Immunology* **167**, 4293-4302

Babior B.M. 2000. Phagocytes and oxidative stress. *American Journal of Medicine* **109**, 33-44

Babior B.M., Kipnes R.S., and Curnutte J.T. 1973. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *The Journal of Clinical Investigation* **52**, 741-744

Badwey J.A., and Karnovsky M.L. 1980. Active oxygen species and the functions of phagocytic leukocytes. *Annual Review of Biochemistry* **49**, 695-726

Baeza E., Poitou I., and Boulard C. 1993. *In vitro* effects of *Fasciola hepatica* on the main functions of polymorphonuclear leukocytes: chemotaxis and free radical generation induced by phagocytosis. *International Journal for Parasitology* **23**, 1077-1081

Barclay A.N., Brown M.H., Law S.K.A., McKnight A.J., Tomlinson M.G., and van der Merwe P.A. 1997. In *The Leucocyte Antigen Facts Book.* Academic Press, London, UK. p 1-613

Barrett J. 1980. Peroxide metabolism in the liver fluke, *Fasciola hepatica. The Journal of Parasitology* **66**, 697

Barrett A.J., Kembhavi A.A., Brown M.A., Kirschke H., Knight C.G., Tamai M., Hanada K. 1982. L-trans-Epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) and its analogues as inhibitors of cysteine proteinases including cathepsins B, H and L. *Biochemical Journal* **210**, 189-198

Barrett A.J., and Kirschke H. 1981. Cathepsin B, Cathepsin H, and cathepsin L. *Methods in Enzymology* **80**, 535-561

Batra S., Chatterjee R.K., and Srivastava V.M.L. 1992. Antioxidant system of *Litomosoides carinii* and *Setaria cervi*: effect of a microfilarial agent. *Veterinary Parasitology* **43**, 93-103

Beckman E.M., Melian A., Behar S.M., Sieling P.A., Chatterjee D., Furlong S.T., Matsumoto R., Rosat J.P., Modlin R.L., and Porcelli S.A. 1996. CD1c restricts responses of mycobacteria-specific T cells: evidence for antigen presentation by a second member of the human CD1 family. *The Journal of Immunology* **157**, 2795-2803

Beckman E.M., Porcelli S.A., Morita C.T., Behar S.M., Furlong S.T. and Brenner M.B. 1994. Recognition of a lipid antigen by CD1-restricted $\alpha\beta^+$ T cells. *Nature* **372**, 691-694

Beltz L.A., Sonnenfeld G., and Kierszenbaum F. 1989. Inhibition by *Trypanosoma cruzi* of interferon-gamma production by mitogen-stimulated mouse spleen cells. *International Journal for Parasitology* **19**, 55-559

Beltz L.A., Sztein M.B., and Kierszenbaum F. 1988. Novel mechanism for *Trypanosoma cruzi*-induced suppression of human lymphocytes. Inhibition of IL-2 receptor expression. *The Journal of Immunology* **141**, 289-294

Bennett C.E. and Threadgold L.T. 1973. Electron microscope studies of *Fasciola hepatica* XIII. Fine structure of newly excysted juvenile. *Experimental Parasitology* **34**, 85-89

Bennett C.E. and Threadgold L.T. 1975. *Fasciola hepatica*: Development of tegument during migration in mouse. *Experimental Parasitology* **38**, 38-55

Ben-Smith A., Lammas D.A., and Behnke J.M. 2002. Effect of oxygen radicals and differential expression of catalase and superoxide dismutase in adult *Heligmosomoides polygyrus* during primary infections in mice with differing response phenotypes. *Parasite Immunology* **24**, 119-129

Berasain P., Carmona C., Frangione B., Dalton J.P., and Goni F. 2000. *Fasciola hepatica*: Parasite-secreted proteinases degrade all human IgG subclasses: Determination of the specific cleavage sites and identification of the immunoglobulin fragments produced. *Experimental Parasitology* **94**, 99-110

Berasain P., Goni F., McGonigle S., Dowd A., Dalton J.P., Frangione B., and Carmona C. 1997. Proteinases secreted by *Fasciola hepatica* degrade extracellular matrix and basement membrane components. *The Journal of Parasitology* **83**, 1-5

Billiar T.R., Curran R.D., Harbrecht B.G., Stadler J., Williams D.L., Ochoa J.B., Di Silvio M., Simmons R.L., and Murray S.A. 1992. Association between synthesis and release of cGMP and nitric oxide biosynthesis by hepatocytes. *American Journal of Physiology* **262**, C1077-1082

Blok R., Marguiles D., Pease L., Ribaudo R., Schnect J., and McCluskey J. 1992. CD8 expression alters the fine specificity of an alloreactive MHC class I-specific T hybridoma. *International Immunology* **4**, 455-466

Blumberg R.S., Terhost C., and Bleicher P. 1991. Expression of a non polymorphic MHC class-I-like molecule, CD1d, by human intestinal epithelial cells. *The Journal of Immunology* **147**, 2518-2524

Board P., Coggan M., Johnston P., Ross V., Suzuki T., and Webb G. 1990. Genetic heterogeneity of the human glutathione transferases: a complex of gene families. *Pharmacology and Therapeutics* **48**, 357-369

Boray J.C. 1967. Studies on experimental infections with *Fasciola hepatica*, with particular reference to acute fascioliasis in sheep. *Annals of Tropical Medicine and Parasitology* **61**, 439-450.

Boray J.C. 1969. Experimental fascioliasis in Australia. *Advances in Parasitology* **7**, 95-210

Boray J.C. 1986. The epidemiological control of fascioliasis. *In* Preceedings of the Sheep and Wool Seminar and Refresher Course, Armidale. Department of Agriculture, NSW, Australia, 12-1 – 12-14

Bossaert K., Farnir F., Leclipteus T., Protz M., Lonneux J.F., and Losson B. 2000a. Humoral immune response in calves to single-dose, trickle and challenge infections with *Fasciola hepatica. Veterinary Parasitology* **87**, 103-123

Bossaert K., Jacquinet E., Saunders J., Farnir F., and Losson B. 2000a. Cell-mediated immune response in calves to single-dose, trickle, and challenge infections with *Fasciola hepatica. Veterinary Parasitology* **88**, 17-34

Boyce W.M., Courtney C.H., and Loggins P.E. 1987. Resistance to experimental infection with *Fasciola hepatica* in exotic and domestic breeds of sheep. *International Journal for Parasitology* **17**, 1233-1237

Boyce N.W., Jonsson J.I., Emmrich F., and Eichmann K. 1988. Heterologous cross-linking of Lyt-2 (CD8) to the αβ-T cell receptor is more effective in T cell activation than homologous αβT cell receptor cross-linking. *The Journal of Immunology* **141**, 2882-2888

Brady C.P., Dowd A.J., Brindley P.J., Ryan T., Day S.R., and Dalton J.P. 1999a. Recombinant expression and localisation of *Schistosoma mansoni* cathepsin L1 support its role in the degradation of host hemoglobin. *Infection and Immunity* **67**, 368-374

Brady M.T., O'Neill S.M., Dalton J.P., and Mills K.H. 1999b. *Fasciola hepatica* suppresses a protective Th1 response against *Bordetella pertussis. Infection and Immunity* **67**, 5372-5378

Britton C., Canto G.J., Urquhart G.M., and Kennedy M.W. 1993. Characterisation of excretory-secretory products of adult *Dictyocaulus viviparous* and the antibody response to them in infection and vaccination. *Parasite Immunology* **15**, 163-174

Brooke G.P., Parsons K.R., and Howard C.J. 1998. Cloning of two members of the SIRP alpha family of protein tyrosine phosphatase binding proteins in cattle that are expressed on monocytes and a subpopulation of dendritic cells and which mediate binding to CD4 T cells. *European Journal of Immunology* **28**, 1-11

Bronte V., Chappell D.B., Apolloni E., Cabrelle A., Wang M., Hwu P. and Restifo N.P. 1999. Unopposed production of granulocyte-macrophage colony-stimulation factor by tumors inhibits CD8+ T cell responses by dysregulating antigen-presenting cell maturation. *Journal of Immunology* **162**, 5728-5737

Bronte V., Wang M., Overwijk W.W., Surman D.R., Pericle F., Rosenberg S.A., and Restifo N.P. 1998. Apoptotic death of CD8+ T lymphocytes after immunisation: induction of a suppressive population of Mac-1+/Gr-1+ cells. *Journal of Immunology* **161**, 5313-5320

Brophy P.M., and Barrett J. 1990. Strategies for detoxification of aldehydic products of lipid peroxidation in Helminths. *Molecular and Biochemical Parasitology* **42**, 205-212

Brophy P.M., Ben-Smith A., Brown A., Behnke J.M., and Pritchard D.I. 1995. Differential expression of glutathione S-transferase (GST) by adult *Heligomosomoides polygyrus* during a primary infection in fast and slow responding hosts. *International Journal for Parasitology* **25**, 641-645

Brophy P.M., Crowley P., and Barrett J. 1990. Detoxification reactions of *Fasciola hepatica* cytosolic glutathione transferases. *Molecular and Biochemical Parasitology* **39**, 155-161

Brophy P.M., and Pritchard D.I. 1992. Immunity to helminths: ready to tip the biochemical balance? *Parasitology Today* **8**, 419-422

Brophy P.M., and Pritchard D.I. 1994. Parasitic Helminth glutatione s-transferases: an update on their potential as targets for immuno- and chemotherapy. *Experimental Parasitology* **79**, 89-96

Brouwers J.F., Skelly P.J., Van Golde L.M., and Tielens A.G. 1999. Studies on phospholipid turnover argue against sloughing of tegumental membranes in adult *Schistosoma mansoni*. *Parasitology* **119**, 287-294

Brown W.C., Davis W.C, Dobbelaere D.A., and Rice-Ficht A.C. 1994a. CD4+ T-cell clones obtained from cattle chronically infected with *Fasciola hepatica* and specific for adult worm antigen express both unrestricted and Th2 cytokine profiles. *Infection and Immunity* **62**, 818-827

Brown W.C., Woods V.M., Chitko-McKown C.G., Hash S.M., and Rice-Ficht A.C. 1994b. Interleukin-10 is expressed by bovine type 1 helper, type 2 helper, and unrestricted parasite-specific T-cell clones and inhibits proliferation of all three subsets in an accessory-cell-dependent manner. *Infection and Immunity* **62**, 4697-4708

Brown W.C., Rice-Ficht A.C., and Estes D.M. 1998. Bovine type 1 and type 2 responses. *Veterinary Immunology and Immunopathology* **63**, 45-55

Brunet L.R. 2001. Nitric oxide in parasitic infections. *International Jounal of Immunopharmacology* **1**, 1457-1467

Buddle B.M., Jowett G., Green R.S., Douch P.G.C. and Ridson P.L. 1992. Association of blood eosinophilia with the expression of resistance in Romney lambs to nematodes. *International Journal for Parasitology* **22**, 955-960

Burden D.J., Bland A.P., Hammet N.C., and Hughes D.L. 1983. *Fasciola hepatica*: Migration of newly excysted juvenilesin resistant rats. *Experimental Parasitology* **56**, 277-288

Burden D.J., Hughes D.L., and Hammet N.C. 1982. *Fasciola hepatica*: antiobdy coatingof juvenile flukes in the intestinal lumen of resistant rats. *Research in Veterinary Science* **32**, 44-47

Calabi R., Yu C.Y., Bilsland C.A.G., and Milstein C. 1991. Immunogenetics of the Major Histocompatibility Complex. VCA Publishers pg ref

Callahan H.L., Crouch R. K., and James E.R. 1988. Helminth anti-oxidant enzymes: a protective mechanism against host oxidants? *Parasitology Today* **4**, 218-225

Cammarota G., Schierle A., Takacs B., Doran D., Knorr R., Bannwort W., Guardiola j., and Sinigaglia F. 1992. Identification of a CD4 binding site on the b2 domain of HLA-DR molecules. *Nature* **356**, 799-801

Campos E.G., Hermes-Lima M., Smith J.M., and Prichard R.K. 1999. Characterisation of *Fasciola hepatica* cytochrome c peroxidase as an enzyme with potential antioxidant activity *in vitro*. *International Journal for Parasitology* **29**, 655-662

Canchis P.W., Bhan A.K., Landau S.B., Canchis P.W., Bhan A.K., Landau S.B., Yang L., Balk S.P., and Blumberg R.S. 1993. Tissue distribution of the non-polymorphic major histocompatibility complex class I-like molecule, CD1d. *Immunology* **80**, 561-565

Capron A., Dessaint J.P., Capron M., Ouma J.H., and Butterworth A.E. 1987. Immunity to schistosomes: Progress towards vaccine. *Science* **238**, 1065-1072

Carmona C., Dowd A.J., Smith A.M., and Dalton J.P. 1993. Cathepsin L proteinase secreted by *Fasciola hepatica in vitro* prevents antibody-mediated eosinophil attachment to newly excysted juveniles. *Molecular and Biochemical Parasitology* **62**, 9-18

Cattoretti G., Berti E., Manusco A., D'Amato L., Schiro R., Soligo D., and Delia D. 1987. Leukocyte Typing III. Oxford University Press, Oxford

Cervi L. and Masih T. 1997. Inhibition of spleen cell proliferative response to mitogens by excretory-secretory antigens of *Fasciola hepatica*. *International Journal for Parasitology* **27**, 573-579

Cervi L., Rossi G., Cejas H., and Masih D.T. 1998. *Fasciola hepatica*-induced immune suppression of spleen mononuclear cell proliferation: role of nitric oxide. *Clinical Immunology and Immunopathology* **87**, 145-154

Cervi L., Rossi G., and Masih D.T. 1999. Potential role for excretory-secretory forms of glutathione-S-transferase (GST) in *Fasciola hepatica*. *Parasitology* **119**, 627-633

Cervi L., Rubinstein H., and Masih D.T. 1996. Involvement of excretion-secretion products from *Fasciola hepatica* inducing suppression of the cellular immune responses. *Veterinary Parasitology* **61**, 97-111

Chabaudie N., and Boulard C. 1992. Effect of hypodermin A, an enzyme secreted by *Hypoderma lineatum* (*insect, Oestridae*), on the bovine immune system. *Veterinary Immunology and Immunopathology* **31**, 167-177

Chae H.Z., Robison K., Poole L.B., Church G., Storz G., and Rhee S.G. 1994. Cloning and sequencing of thiol-specific antioxidant from mammalian brain: alkyl hydroperoxide reductase and thiol-specific antioxidant define a large family of antioxidant enzymes. *Proceedings of the National Academy of Sciences U S A.* **91**, 7017-7021.

Channon J.Y., and Blackwell J.M. 1985. A study of the sensitivity of *Leishmania donovani* promastigotes and amastigotes to hydrogen peroxide. I. Differences in susceptibility correlate with parasite removal of hydrogen peroxide. *Parasitology* **91**, 197-204

Chapman C.B., Rajasekariah G.R., and Mitchell G.F. 1981. Clonal parasites in the analysis of resistance to reinfection with *Fasciola hepatica*. *The American Journal of Tropical Medicine and Hygiene*. **30**, 1039-1042

Chauvin A., Bouvet G., and Boulard C. 1995. Humoral and cellular immune responses to *Fasciola hepatica* experimental primary and secondary infection in sheep. *International Journal for Parasitology* **25**, 1227-1241

Clark I.A., Hunt N.H. and Cowden W.B. 1986. Oxygen-derived free radicals in the pathogenesis of parasitic disease. *Advances in Parasitology* **25**, 1-44

Clery D.G., and Mulcahy G. 1998. Lymphocyte and cytokine responses of young cattle during primary infection with *Fasciola hepatica*. *Research in Veterinary Science* **65**, 169-171

Clery D., Torgerson P., and Mulcahy G. 1996. Immune responses of chronically infected adult cattle to *Fasciola hepatica*. *Veterinary Parasitology* **62**, 71-82

Cocude C., Pierrot C., Cetre C., Fontaine J., Godin C., Capron A., and Khalife J. 1999. Identification of a developmentally regulated *Schistosoma mansoni* serine protease homologous to mouse plasma kallikrein and human factor I. *Parasitology* **118**, 389-396

Cohen M.S. 1994. Molecular events in the activation of human neutrophils for microbial killing. *Clinical Infectious Diseases* **18** Suppl 2: S170-179

Corbalan-Garcia S., Teruel J.A., Villalain J., and Gomez-Fernandex J.C. 1994. Extensive proteolytic digestion of the (Ca^{2+} and Mg^{2+})-ATPase from sarcoplasmic reticulum leads to a highly hydrophobic proteinaceous residue with a mainly alpha-helical structure. *Biochemistry* **33**, 8247-8254

Cordeiro-Da-Silva A., Borges M.C., Guilvard E., and Ouaissi A. 2001. Dual role of the *Leishmania major* ribosomal protein S3a homologue in regulation of T- and B-cell activation. *Infection and Immunity* **69**, 6588-6596

Creaney J., Wilson L., Dosen M., Sandeman R.M., Spithill T.W., and Parsons JC. 1996. *Fasciola hepatica*: irradiation-induced alterations in carbohydrate and cathepsin-B protease expression in newly excysted juvenile liver fluke. *Experimental Parasitology* **83**, 202-215

Creaney J., Wijffels G.L., Sexton J.L., Sandeman R.M., Spithill T.W., and Parsons J.C. 1995. *Fasciola hepatica*: localisation of glutathione S-transferase isoenzymes in adult and juvenile liver fluke. *Experimental Parasitology* **81**, 106-116

Cross A.R., and Jones O.T.G. 1991. Enzymic mechanisms of superoxide production. *Biochimica et Biophysica Acta* **1057**, 281-298

Curnutte J.T., Whitten D.M., and Babior B.M. 1974. Defective superoxide production by granulocytes from patients with chronic granulomatous disease. *The New England Journal of Medicine* **290**, 593-597

Czapski G. 1984. Reaction of .OH. *Methods in Enzymology* **105**, 209-215

Da Costa B., Djilali S., Levy D., Kessler J.L., Cribiu E.P., Parodi A.L. 1992. Partial characterisation of a familial B lymphosarcoma with a thymic localisation in cattle. *Leukemia* **6**, 696-702

Dalton J.P., Clough K.A., Jones M.K., and Brindley P.J. 1996a. Characterization of the cathepsin-like cysteine proteinases of *Schistosoma mansoni*. *Infection and Immunity* **64**, 1328-1334

Dalton J.P. and Heffernan M. 1989. Thiol proteases released *in vitro* by *Fasciola hepatica*. *Molecular and Biochemical Parasitology* **35**, 161-166

Dalton J. P., McGonigle S., Rolph T.P., and Andrews S.J. 1996a. Induction of protective immunity in cattle against infection with *Fasciola hepatica* by vaccination with cathepsin L proteinases and with hemoglobin. *Infection and Immunity* **64**, 5066-5074

Davies C., and Goose J. 1981. Killing of newly excysted juveniles of *Fasciola hepatica* in sensitized rats. *Parasite Immunology* **3**, 81-96

Davis W.C., Brown W.C., Hamilton M.J., Wyatt C.R., Orden J.A., Khalid A.M., and Naessens J. 1996. Analysis of monoclonal antibodies specific for the gamma delta TcR. *Veterinary Immunology and Immunopathology* **52**, 275-283

Dawes B., and Hughes D.L. 1964. Fascioliasis: the invasive stages of *Fasciola hepatica* in mammalian hosts. *Advances in Parasitology* **2**, 97-168

Dawes B., and Hughes D.L. 1970. Fascioliasis: the invasive stages in mammals. *Advances in Parasitology* **8**, 259-274

Dawkins H.J.S., Windon R.G., and Eagleson G.K. 1989. Eosinophil responses in sheep selected for high and low responsiveness to *Trichostrongylus colubriformis*. *International Journal for Parasitology* **19**, 199-205

Day K.P., Howard R.J., Prowse S.J., Chapman C.B., and Mitchell G.F. 1979. Studies on chronic versus transient intestinal nematode infections in mice. I. A. comparison of responses to excretory/secretory (ES) products of *Nippostrongylus brasiliensis* and *Nematospiroides dubius* worms. *Parasite Immunology* **1**, 217-239

Dea-Ayuelu M.A., and Bolas-Fernandez F. 1999. Trichinella antigens: a review. *Veterinary Research* **30**, 559-571

De Libero G., Donda A., Mori L., Manolova V., and Shamshiev A. 2001. Immunity to glycolipid antigens in microbial infections. *Journal of Biological Regulators and Homeostatic Agents* **15**, 249-256

Del Prete G., De Carli M., Almerigogna F., Giudizi M.G., Biagiotti R., and Romagnani S. 1993. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. *The Journal of Immunology* **150**, 353-360

Doelman C.J., and Bast A. 1990. Oxygen radicals in lung pathology. *Free Racical Biology and Medicine* **9**, 381-400

Donelson J.E., Hill K.L., El-Sayed N.M. 1998. Multiple mechanisms of immune evasion by African trypanosomes. *Molecular and Biochemical Parasitology* **91**, 51-66

Dowd A. J., McGonigle S., and Dalton J.P. 1995. *Fasciola hepatica* cathepsin L proteinase cleaves fibrinogen and produces a novel type of fibrin clot. *European Journal of Biochemistry* **15**, 241-246

Dowd A.J., Smith A.M., McGonigle S., and Dalton J.P. 1994. Purification and characterisation of a second cathepsin L proteinase secreted by the parasitic trematode *Fasciola hepatica. European Journal of Biochemistry* **223**, 91-98

Doy T.G., Hughes D.L., and Harness E. 1978. Resistance of the rat to reinfection with Fasciola hepatica and the possible involvement of intestinal eosinophil leucocytes. *Research in Veterinary Science* **25**, 41-44

Doy T.G., and Hughes D.L. 1982. Evidence for two distinct mechanisms of resistance in the rat to reinfection with *Fasciola hepatica. International Journal for Parasitology* **12**, 357-361

Doy T.G., and Hughes D.L. 1984. *Fasciola hepatica*: site of resistance to reinfection in cattle. *Experimental Parasitology* **57**, 274-278

Doyle J.J. 1971. Acquired immunity to experimental infection with *Fasciola hepatica* in cattle. *Research in Veterinary Science* **12**, 527-534

Doyle J.J. 1972. Evidence of an acquired resistance in calves to a single experimental infection with *Fasciola hepatica. Research in Veterinary Science* **13**, 456-459

Doyle J.J. 1973. The relationship between the duration of a primary infection and the subsequent development of an acquired resistance to experimental infections with *Fasciola hepatica* in calves. *Research in Veterinary Science* **14**, 97-103

Drapier J.C., and Hibbs J.B. Jr. 1986. Murine cytotoxic activated macrophages inhibit aconitase in tumor cells: inhibition involves the iron-sulfur prosthetic group and is reversible. *Journal of Clinical Investigations* **78**, 790-797

Dresser D.W., Taub R.N., and Krantz A.R. 1970. The effect of localized injection of adjuvant material on the draining lymph node: II circulating lymphocytes. *Immunology* **18**, 663-670

Dubray G., and Bezard G. 1982. A highly sensitive periodic acid-silver stain for 1,2-diol groups of glycoproteins and polysaccharides in polyacrylamide gels. *Analytical Biochemistry* **119**, 325-329.

Duffus W.P.H. and Franks D. 1980. *In vitro* effect of immune serum and bovine granulocytes on juvenile *Fasciola hepatica*. *Clinical and Experimental Immunology* **41**, 430-440

Duffus W.P.H., Thorne K., and Oliver R. 1980. Killing of juvenile *Fasciola hepatica* by purified bovine eosinophil proteins. *Clinical and Experimental Immunology* **40**, 366-344

Dutia B.M., Ross A.J., and Hopkins J. 1993. Analysis of the monoclonal antibodies comprising WC6. *Veterinary Immunology and Immunopathology* **39**, 193-199

Duvaux-Miret D., Stefano G.B., Smith E.M., Mallozzi L.A., and Capron A. 1992. Proopiomelanocortin-derived peptides as tools of immune evasion for the human trematode *Schistosoma mansoni*. *Acta Biologica Hungaria* **43**, 281-286

Eagleson J.S. and Moriarty K.M. 1984. Review article: neutrophils, oxidative killing mechanisms and chemiluminescence. *The New Zealand Veterinary Journal* **32**, 41-43

Eiserich J.P., Patel R.P., and O'Donnell V.B. 1998. Pathophysiology of nitric oxide and related species: free radical reactions and modification of biomolecules. *Molecular Aspects of Medicine* **19**, 221-357

El Ghaysh A., Turner R.J., Brophy P.M., and Barrett J.1999. Effect of *Fasciola gigantica* somatic extracts and excretory/secretory products on superoxide production by activated neutrophils. *Veterinary Parasitology* **84**, 91-100

Emery D.L., MacHugh N.D., Ellis J.A. 1987. The properties and functional activity of non-lymphoid cells from bovine afferent (peripheral) lymph. *Immunology* **62**, 177-183

Emery D. L., Rothel J.S., and Wood P.R. 1990. Influence of antigens and adjuvants on the production of gamma-interferon and antibody by ovine lymphocytes. *Immunology of Cellular Biology* **68**, 127-136

Estuningsih S.E., Smooker P.M., Wiedosari E., Widjajanti S., Vaiano S., Partoutomo S., and Spithill T.W. 1997. Evaluation of antigens of *Fasciola gigantica* as vaccines against tropical fasciolosis in cattle. *International Journal for Parasitology* **27**, 1419-1428

Fagbemi B.O., Aderibigbe O.A., and Guobadia E.E. 1997. The use of monoclonal antibody for the immunodiagnosis of *Fasciola gigantica* in cattle. *Veterinary Parasitology* **69**, 231-240

Fagbemi B.O. and Hillyer G.V. 1991. Partial purification and characterisation of the proteolytic enzymes of *Fasciola gigantica* adult worms. *Veterinary Parasitology* **40**, 217-226

Fagbemi B.O., and Hillyer G.V. 1992. The purification and characterisation of a cysteine protease of *Fasciola gigantica* adult worms. *Veterinary Parasitology* **43**, 223-232

Fantone J.C. and Ward P.A. 1982. Role of oxygen-derived free radicals and metabolites in leukocyte-dependent inflammatory reactions. *American Journal of Pathology* **107**, 395-418

Felleisen R. and Klinkert Mo-Quen. 1990. *In vitro* translation and processing of cathepsin B of *Schistosoma mansoni*. *EMBO Journal* **9**, 371-377

Ferguson E.D., Dutia B.M., Hein W.R., and Hopkins J. 1996. The sheep CD1 gene family contains at least four CD1B homologues. *Immunogenetics* **44**, 86-96

Flesch I.E., and Kaufmann S.H. 1991. Mechanisms involved in mycobacterial growth inhibition by gamma interferon-activated bone marrow macrophages: role of reactive nitrogen intermediates. *Infection and Immunity* **59**, 3213-3218

Fleury S.G., Croteau G., and Sekaly R.P. 1991. CD4 and CD8 recognition of class II and class I molecules of the major histocompatability complex. *Seminars in Immunology* **3**, 177-185

Flohe L., and Otting F. 1984. Superoxide dismutase assays. *Methods in Enzymology* **105**, 93-104

Freeman B.A., and Crapo J.D. 1982. Biology of disease: free radicals and tissue injury. *Lab Invest* **47**, 412-426

Fridovich I. 1975. Superoxide dismutases. *Annual Review of Biochemistry* **44**, 147-159

Fridovich I. 1995. Superoxide radical and superoxide dismutases. *Annual Reviews in Biochemistry* **64**, 97-112

Ganschow R.E., and Schimke R.T. 1969. Independent genetic control of the catalytic activity and the rate of degradation of catalase in mice. *Journal of Biological Chemistry* **244**, 4649-4658

Garside P., Kennedy M.W., Wakelin D., and Lawrence C.E. 2000. Immunopathology of intestinal helminth infection. *Parasite Immunology* **22**, 605-612

Gill H. S., Watson D.L., and Brandon M.R. 1993. Monoclonal antibody to $CD4^+$ T cells abrogates genetic resistance to Haemonchus contortus in sheep. *Immunology* **78**, 43-49

Giulivi C., and Davies K.J. 1990. A novel antioxidant role for hemoglobin. The comproportionation of ferrylhemoglobin with oxyhemoglobin. *Journal of Biological Chemistry* **265**, 19453-19460

Graham S.P., Trees A.J., Collins R.A., Moore D.M., Guy F.M., Taylor M.J., and Bianco A.E. 2001. Down-regulated lymphoproliferation coincides with parasite maturation and with the collapse of both gamma interferon and interleukin-4 responses in a bovine model of onchocerciasis. *Infection and Immunity* **69**, 4313-4319

Grams R., Vichasri-Grams S., Sobhon P., Upatham E.S., and Viyanant V. 2001. Molecular cloning and characterisation of cathepsin L encoding genes from *Fasciola gigantica*. *Parasitology International* **50**, 105-114

Green L.C., Wagner D.A., Glogowski J., Skipper P.L., Wishnok J.S., and Tannenbaum S.R. 1982. Analysis of nitrate, nitrite, and [^{15}N]nitrite in biological fluids. *Analytical Biochemistry* **126**, 131-138

Grencis R. K., Riedlinger J., and Wakelin D. 1985. L3T4 positive lymphoblasts are responsible for transfer of immunity to *Trichinella spiralis* in mice. *Immunology* **56**, 213-218

Grigoryan G.A. 1958. Experimental study of *Fasciola gigantica* infestation in sheep. *Trudy Armyansk Inst Zhivotnovod i Veterinariya* **3**, 155-168

Gupta S.C. and Chandra R. 1987. Susceptibility of some laboratory animals to infection with *Fasciola gigantica*. *Journal of Veterinary Parasitology* **1**, 19-21

Guralp N., Oxcan C., & Simms B.T. 1964. *Fasciola gigantica* and fascioliasis in Turkey. *American Journal of Veterinary Research* **25**, 196-210

Gutteridge J.M., and Halliwell B. 1990. Reoxygenation injury and antioxidant protection: a tale of two paradoxes. *Archives of Biochemistry and Biophysics* **283**, 223-226

Haas A., and Goebel W. 1992. Microbial strategies to prevent oxygen-dependent killing by phagocytes. *Free Radical Research Communications* **16**, 137-157

Haber F., and Weiss J. 1934. The catalytic decomposition of hydrogen peroxide by iron salts. *Proceedings of the Royal Society of London Series A* **147**, 332-351

Habig W.H., Pabst M.J., and Jakoby W.B. 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry* **249**, 7130-7139

Haig D.M., Hopkins, J., and Miller H.R.P. 1999. Local immune responses in afferent and efferent lymph. *Immunology* **96**, 155-163

Hall J.G., and Morris B. 1962. The output of cells in lymph from the popliteal node of sheep. *Quarterly Journal of Experimental Physiology* **47**, 360-369

Halliwell B., and Gutteridge J.M. 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochemical Journal* **219**, 1-14

Hammond J.A., and Sewell M.M. 1974. The pathogenic effect of experimental infections with *Fasciola gigantica* in cattle. *British Veterinary Journal* **130**, 453-465

Hampl J., Chien Y.H. and Davis M.M. 1997. CD4 augments the response of a T cell to agonist but not to antagonist ligands. *Immunity* **7**, 379-385

Hanna R.E.B. 1978. A possible immunolgical role for the tegument of *Fasciola hepatica*. *Parasitology* **77**, part 3 ix

Hanna R.E.B. 1980a. *Experimental Parasitology* **80**, 103-114

Hanna R.E.B. 1980b. *Fasciola hepatica*: an immunofluorescent study of antigenic changes in the tegument during development in the rat and the sheep. *Experimental Parasitology* **50**, 155-170

Hansen D.S., Clery D.G., Estuningsih S.E., Widjajanti S., Partoutomo S., and Spithill T.W. 1999. Immune responses in Indonesian thin tail and Merino sheep during a primary infection with *Fasciola gigantica*: lack of a specific IgG2 antibody response is associated with increased resistance to infection in Indonesian sheep. *International Journal for Parasitology* **29**, 1027-1035

Haroun E.T., and Hillyer G.V. 1986. Resistance to fascioliasis--a review. *Veterinary Parasitology* **20**, 63-93

Harris E.D.1992. Regulation of antioxidant enzymes. *Journal of the Federation of American Societies for Experimental Biology* **6**, 2675-2683

Haslett C., Savill J.S., and Maegher L. 1989. The neutrophil. *Current Opinion In Immunology* **2**, 10-18

Hassett D.J., and Cohen M.S. 1989. Bacterial adaptation to oxidative stress: implications for pathogenesis and interaction with phagocytic cells. *Journal of the Federation of American Societies for Experimental Biology* **3**, 2574-2582

Haswell-Elkins M.R., Satarug S., Tsuda M., Mairiang E., Esumi H., Sithithaworn P., Mairiang P., Saitoh M., Yongvanit P., and Elkins D.B. 1994. Liver fluke infection and cholangiocarcinoma: model of endogenous nitric oxide and extragastric nitrosation in human carcinogenesis. *Mutational Research* **305**, 241-252

Hawn T.R., and Strand M. 1993. Detection and partial characterization of glycosylphosphatidylinositol-specific phospholipase activities from *Fasciola hepatica* and *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **59**, 73-81

Hayes T.J., Bailer J., and Mitrovic M. 1974. Acquired immunity and age resistance in rats with chronic fascioliasis. *The Journal of Parasitology* **60**, 247-250

Hein W.R., and Mackay C.R. 1991. Other surface antigens identified on sheep leukocytes. *Veterinary Immunology and Immunopathology* **27**, 115-118

Henkle-Duhrsen K., and Kampkotter A. 2001. Antioxidant enzyme families in parasitic nematodes. *Molecular and Biochemical Parasitology* **114**, 129-142

Heussler V.T., and Dobbelaere D.A. 1994. Cloning of a protease gene family of *Fasciola hepatica* by the polymerase chain reaction. *Molecular Biochemistry and Parasitology* **64**, 11-23

Hey A.S., Theander T.G., Hviid L., Hazrati S.M., Kemp M., and Kharazmi A. 1994. The major surface glycoprotein (gp63) from *Leishmania major* and *Leishmania donovani* cleaves CD4 molecules on human T cells. *The Journal of Immunology* **152**, 4542-4548

Hibbs J.B. jr., Taintor R.R., Vavrin Z., and Rachlin E.M. 1988. Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochemical and Biophysical Research Communications* **157**, 87-94

Hillyer G.V., Soler de Galanes M., and Battisti G. 1992. *Fasciola hepatica*: host responders and nonresponders to parasite glutathione S-transferase. *Experimental Parasitology* **75**, 176-186

Holland M.J., Harcus Y.M., Riches P.L., and Maizels R.M. 2000. Proteins secreed by the parasitic nematode *Nippostrongylus brasiliensis* act as adjuvants for Th2 responses. *European Journal of Immunology* **30**, 1977-1987

Hopkins J., Dutia B.M., and Rhind S.M. 2000. Sheep CD1 genes and proteins. *Veterinary Immunology and Immunopathology* **31**, 3-14

Hopkins J., McConnell I., and Pearson J.D. 1981. Lymphocyte traffic through antigen-stimulated lymph nodes: II. Role of prostoglandin E_2 as a mediator of cell shutdown. *Immunology* **42**, 225-231

Hopkins J., and Tudhope G.R. 1973. Glutathione peroxidase in human red cells in health and disease. *British Journal of Haematology* **25**, 563-575

Horak I.G., Snijders A.J. and Louw J.P. 1972. Trials with rafoxanide. 5 Efficacy studies against *Fasciola hepatica, Fasciola gigantica, Paramphistomum microbothridium* and various nematodes in sheep. *Journal of the South African Veterinary Association* 43, 397-403

Howard C.J., Sopp P., Brownlie J., Kwong L.S., Parsons K.R., and Taylor G. 1997. Identification of two distinct populations of dendritic cells in afferent lymph that vary in their ability to stimulate T cells. *The Journal of Immunology* **159**, 5372-5382

Howard C.J., Sopp P., and Parsons K.R. 1992. L-selectin expression differentiates T cells isolated from different lymphoid tissues in cattle but does not correlate with memory. *Immunology* **77**, 228-234

Howell M.J., Board P.G., and Boray J.C. 1988. Glutathione S-transferases in *Fasciola hepatica*. *The Journal of Parasitology* **74**, 715-718

Hughes D.L. 1987. *Fasciola* and fascioloides. *In*: Immune reponses in parasitic infections: immunology, immunopathology, and immunoprophylaxis. Volume II: Trematodes and cestodes. E.J.L. Soulsby, ed. CRC Press, Florida, USA. 91-114

Hughes D.L., Harness E., and Doy T.G. 1976. The establishment and duration of *Fasciola hepatica* infections in two strains of rats and the development of acquired resistance. *Research in Veterinary Science* **20**, 207-211

Hughes D.L., Hanna R.E., and Symonds H.W. 1981. *Fasciola hepatica*: IgG and IgA levels in the serum and bile of infected cattle. *Experimental Parasitology* **52**, 271-279

Hviid L., Sorensen A.L., Kharazmi A., and Theander T.G. 1990. Functional and phenotypic changes in human lymphocytes after coincubation with *Leishmania donovani in vitro*. *Infection and Immunity* **58**, 3163-3167

Ignarro L.J., Fukuto J.M., Griscavage J.M., Rogers N.E., and Byrns R.E. 1993. Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine. *Proceedings of National Academy of Science U.S.A.* **90**, 8103-8107

Intapan PM, Maleewong W, Wongkham C, Tomanakarn K, Ieamviteevanich K, Pipitgool V, Sukolapong V. 1998. Excretory-secretory antigenic components of adult *Fasciola gigantica* recognized by infected human sera. *Southeast Asian Journal of Tropical Medicine and Public Health* **29**, 579-583

Irving J.A., Spithill T.W., Pike R.N., Whisstock J.C., and Smooker P.M. The evolution of enzyme specificity in *Fasciola spp*. *Journal of Molecular Evolution*, In Press

Ismail S.O., Paramchuk W., Skeiky Y.A., Reed S.G., Bhatia A., and Gedamu L. 1997. Molecular cloning and characterization of two iron superoxide dismutase cDNAs from *Trypanosoma cruzi*. *Molecular and Biochemical Parasitology* **86**, 187-197

Itagaki T., Sakamoto T., Tsutsumi Y., and Itagaki H. 1994. Infectivity of three species of *Fasciola* to Wistar rats. *Journal of Veterinary Medicine and Science* **56**, 977-979

Jakoby W.B. 1985. Glutathione Transferases: An overview. *Methods in Enzymology* **113**, 495-499

Jackman R.M., Stenger, S., Lee A., Moody D.B., Rogers R.A., Niazi K.R., Sugita M., Modlin R.L., Peters P.J., and Porcelli S.A. 1998. The tyrosine-containing cytoplasmic tail of CD1b is essential for its efficient presentation of bacterial lipid antigens. *Immunity* **8**, 341-351

James S.L. 1995. Role of nitric oxide in parasitic infections. *Microbiological Reviews* **59**, 533-547

James S.L., and Glaven J. 1989. Macrophage cytotoxicity against schistosomula of *Schistosoma mansoni* involves arginine-dependent production of reactive nitrogen intermediates. *Journal of Immunology* **15**, 4208-4212

James S.L. and Scott P. 1988. Induction of cell-mediated immunity as a strategy for vaccination against parasites. *In*: The biology of parasitism. Eds. P.T. Englund and A. Sher. Alan R. Liss Inc., New York. 249-264

Janeway C. A. 1989. The role of CD4 in T-cell activation: accessory molecule or co-receptor? *Immunology Today* **10**, 234-238

Janeway C.A. and Bottomly K. 1994. Signals and signs for lymphocyte responses. *Cell* **76**, 275-285.

Jefferies J.R., Campbell A.M., van Rossum A.J., Barrett J., and Brophy P.M. 2001. Proteomic analysis of *Fasciola hepatica* excretory-secretory products. *Proteomics* **1**, 1128-1132

Jefferies J.R., Barrett J. and Turner R.J. 1996. Immunomodulation of sheep and human lymphocytes by *Fasciola hepatica* excretory-secretory products. *International Journal for Parasitology* **26**, 1119-1121

Jefferies J.R., Corbett E., Barrett J., and Turner R.J. 1996. Polarisation and chemokinesis of ovine and human neutrophils in response to *Fasciola hepatica* excretory-secretory products. *International Journal for Parasitology* **26**, 409-414

Jefferies J.R. Turner R.J. and Barrett J. 1997. Effect of *Fasciola hepatica* excretory-secretory products on the metabolic burst of sheep and human neutrophils. *International Journal for Parasitology* **27**, 1025-1029

Jones O.T. 1993. The mechanism of the production of superoxide by phagocytes. *Molecular Chemistry and Neuropathology* **19**, 177-184

Jong E.C., Mahmoud A.A.F., and Klebanoff S.J. 1981. Peroxidase-mediated toxicity of schistosomula of *Schistosoma mansoni*. *Journal of Immunology* **126**, 468-471

Jonsson J.I., Boyce N.W., and Eichmann K. 1989. Immunoregulation through CD8 (Ly-2); state of aggregation with the αβ/CD3 T cell receptor controls interleukin 2-dependent T cell growth. *European Journal of Immunology* **19**, 253-260

Jovanovic S.V., Steenken S., and Simic M.G. 1990. One-electron reduction potentials of 5-indoxyl radicals. A pulse radiolysis and laser photolysis study. *Journal of Physical Chemistry* **94**, 3583-3588

Julius M., Maroun C.R., and Haughn L. 1993. Distinct roles for CD4 and CD8 as co-receptors in antigen receptor signalling. *Immunology Today* **14**,177-183

Jullien D., Stenger S., Ernst W.A., and Moldin R.L. 1997. CD1 presentation of microbial nonpepetide antigens to T cells. *The Journal of Clinical Investigation* **99**, 2071-2074

Kazura J.W., Fanning M.M., Blumer J.L., and Mahmoud A.A.F. 1981. Role of cell generated hydrogen peroxide in granulocyte-mediated killing of schistosomula of *Schistosoma mansoni in vitro*. *Journal of Clinical Investigation* **67**, 93-102

Kazura J.V., and Meshnick S.R. 1984. Scavenger enzymes and resistance to oxygen mediated damage in *Trichinella spiralis*. *Molecular Biochemical Parasitology* **10**, 1-10

Keegan P.S., and Trudgett A. 1992. *Fasciola hepatica* in the rat: immune responses associated with the development of resistance to infection. *Parasite Immunology* **14**, 657-669

Keele B.B.Jr., McCord J.M., and Fridovich I. 1970. Superoxide dismutase from *Escherichia coli B*. A new manganese-containing enzyme. *The Journal of Biological Chemistry* **245**, 6176-6181

Kelly J.D., and Campbell N.J. 1979. The effect of route of infection on acquired resistance to *Fasciola hepatica* in the rat and sheep. *Research in Veterinary Science* **27**, 205-209

Kelso A. 1995. Th1 and Th2 subsets: paradigms lost? *Immunology Today* **16**, 374-379

Kendall S.B., Sinclair I.J., Everett G., and Parfitt J.W. 1978. Resistance to *Fasciola hepatica* in cattle I. Parasitological and serological observations. *Journal of Comparative Pathology* **88**, 115-122

Kennedy J.F. ed 1988. *Carbohydrate Chemistry*. Clarendon Press, Oxford 1988 p53-57

Ketterer B. 1988. Protective role of glutathione and glutathione transferases in mutagenesis and carcinogenesis. *Mutation Research* **202**, 343-361

Ketterer B., Meyer D.J., and Clark A.G. 1988. Soluble Glutathione Transferase Isozymes. In Glutathione Conjugation. Ed. Academic Press. 73-135

Kierszenbaum F., Cuna W.R., Beltz L.A., Sztein M.B. 1990. Trypanosomal immunosuppressive factor: a secretion product(s) of *Trypanosoma cruzi* that inhibits proliferation and IL-2 receptor expression by activated human peripheral blood mononuclear cells. *The Journal of Immunology* **144**, 4000-4004

Kierszenbaum F., Muthukkumar S., Beltz L.A., and Sztein M.B. 1991. Suppression by *Trypanosoma brucei rhodesiense* of the capacities of human T lymphocytes to express interleukin-2 receptors and proliferate after mitogenic stimulation. *Infection and Immunity* **59**, 3518-3522

Kim T.S., Jung Y., Na B.K., Kim K.S., and Chung P.R. 2000. Molecular cloning and expression of CuZn-containing superoxide dismutase from *Fasciola hepatica*. *Infection and Immunity* **68**, 3941-3948

Knight R.A. 1980. Relationship of globule leucocytes to infections with *Fasciola hepatica* in sheep. *The Journal of Parasitology* **66**, 844-845

Kofta W., Mieszczanek J., Pluciennicak G., and Wedrychowicz H. 2000. Successful DNA immunisation of rats against fasciolosis. *Vaccine* **18**, 2985-2990

Konig R., Huang L-Y., and Germain R. 1992. MHC class II interaction with CD4 mediated by a region analogous to the MHC class I binding site for CD8. *Nature* **356**, 796-798

Krailas D., Viyanant V., Ardseungnoen P., Sobhon P., Upatham E.S., and Keawiam R. 1999. Identification of circulating antibodies in fasciolosis and localization of 66 kDa antigenic target using monoclonal antibodies. *Asian Pacific Journal of Allergy and Immunology* **17**, 53-58

Kruisbeek A.M., and Shevach E.M. 1999. Section 3.12.2. In *Current Protocols in Immunology*, ed. Coligan J.E., Kruisbeek A.M., Margulies D.H., Shevach E.M., and Strober W.: John Wiley & Sons.

Kuo C.F., Mashino T., and Fridovich I. 1987. alpha, beta-Dihydroxyisovalerate dehydratase. A superoxide-sensitive enzyme. *Journal of Biological Chemistry* **262**, 4724-4727

Kusmartsev S.A., Li Y., and Chen S.H. 2000. Gr-1+ myeloid cells derived from tumor-bearing mice inhibit primart T cell activation induced through CD3/CD28 costimulation. *Journal of Immunology* **165**, 779-785

Larsen H.J. 1979. A whole blood method for measuring mitogen-induced transformation of sheep lymphocytes. *The Journal of Immunology* **122**, 2340-2344

Larson R., Monaghan M., Park Y., Hamilton M., Ellis J., and Davis W. 1990. Identification and characterisation of monoclonal antibodies reactive with bovine, caprine and ovine T-lymphocyte determinants by flow microfluorimetry. *Veterinary Immunology and Immunopathology* **25**, 195-208

Lederer J.A., Perez V.L., DesRoches L., Kim S.M., Abbas A.K., and Lichtman A.H. 1996. Cytokine transcriptional events during helper T cell subset differentiation. *Journal of Experimental Medicine* **184**, 397-406

Leiva L.E., and Lammie P.J. 1989. Modulation of lymphocyte activation by soluble *Brugia pahangi* extracts. *Tropical Medicine and Parasitology* **40**, 327-331

Liew F.Y., and Cox F.E.G. 1991. Nonspecific defence mechanism: the role of nitric oxide. *Parasitology Today* **7**, A17-A21

Liew F.Y., and O'Donnell C.A. 1993. Immunology of leishmaniasis. *Advances in Parasitology* **32**, 161-259

Lightowlers M.W., and Rickard M.D. 1988. Excretory-secretory products of Helminth parasites: effects on host immune responses *Parasitology* **96**, S123-126

Loke P., MacDonald A.S., and Allen J.E. 2000. Antigen-presenting cells recruited by *Brugia malayi* induce Th2 differentiation of naïve CD4 (+) T cells. *European Journal of Immunology* **30**, 1127-1135

Loke P., MacDonald A.S., Robb A., Maizels R.M., and Allen J.E. 2000. Alternatively activated macrophages induced by nematode infection inhibit proliferation via cell-to-cell contact. *European Journal of Immunology* **30**, 2669-2678

London C.A., Abbas A.K., and Kelso A. 1998. Helper T cell subsets: heterogeneity, functions and development. *Veterinary Immunology and Immunopathology* **63**, 37-44

Lumsden R.D. 1975. Surface ultrastructure and cytochemistry of parasitic helminths. *Experimental Parasitology* **37**, 267-339

Luster A.D. 2002. The role of chemokines in linking innate and adaptive immunity. *Current Opinions in Immunology* **14**, 129-135

McCole D. F., Doherty M.L., Baird A.W., Davies W.C., McGill K., and Torgerson P.R. 1999. T cell subset involvement in immune responses to *Fasciola hepatica* infection in cattle. *Parasite Immunology* **21**, 1-8

McCord J.M., and Fridovich I. 1969. Superoxide dismutase: An enzymic function for erythrocuprein (hemocuprein). *Journal of Biological Chemistry* **244**, 6049-6055

McGinty A., Moore M., Halton D.W., and Walker B. 1993. Characterisation of the cysteine proteinases of the common liver fluke *Fasciola hepatica* using novel, active-site directed affinity labels. *Parasitology* **106**, 487-493

McGonigle S., Curley G.P., and Dalton J.P. 1997. Cloning of peroxiredoxin, a novel antioxidant enzyme, from the helminth parasite *Fasciola hepatica*. *Parasitology* **115**, 101-104.

McLaren D.J. 1984. Disguise as an evasive stratagem of parasitic organisms. *Parasitology* **88**, 597-611

MacDonald A.S., Loke P., and Allen J.E. 1999. Suppressive antigen-presenting cells in Helminth infection. *Pathobiology* **67**, 265-268

Mackay C. 1988. Sheep leukocyte molecules: a review of their distribution, structure and possible funtion. *Veterinary Immunology and Immunopathology* **19**, 1-20

Mackay C.R., Kimpton W.G., Brandon M.R., and Cahill R.N. 1988. Lymphocyte subsets show marked differences in their distribution between blood and the afferent and efferent lymph of peripheral lymph nodes. *Journal of Experimental Medicine* **197**, 1755-1765

Mackay C.R., Maddox J.F., and Brandon M.R. 1987. Lymphocyte antigens of sheep: identification and characterisation using a panel of monoclonal antibodies. *Veterinary Immunology and Immunopathology* **17**, 91-102

Madrenas J., Chau L.A., Smith J., Bluestone J.A. and Germain R.N. 1997. The efficiency of CD4 recruitment to ligand-engaged TCR controls the agonist/partial agonist properties of peptide-MHC molecule ligands. *Journal of Experimental Medicine* **185**, 219-229

Maizels R.M., Gomez-Escobar N., Gregory W.F., Murray J., and Zang X. 2001. Immune evasion genes from filarial nematodes. *International Journal for Parasitology* **31**, 889-898

Maleewong W., Intapan P.M., Wongkham C., Sripa B., Sukolapong V., and Ieamviteevanich K. 1997. Specific monoclonal antibodies to *Fasciola gigantica*. *Asian Pacific Journal of Allergy and Immunology* **15**, 49-54

Maleewong W., Wongkham C., Intapan P.M. and Pipitgool V. 1999. *Fasciola gigantica*-specific antigens: purification by a continuous-elution method and its evaluation for the diagnosis of human fascioliasis. *The American Journal of Tropical Medicine and Hygiene* **61**, 648-651

Mango A.M., Mango C.K., and Esamal D. 1972. A preliminary note on the susceptibility, prepatency and recovery *of Fasciola gigantica* in small laboratory animals. *Journal of Helminthology* **46**, 381-386

Mannervik B., and Danielson U.H. 1988. Glutathione transferases--structure and catalytic activity. *CRC Crit Rev Biochem* **23**, 283-337

Manoury B., Hewitt E.W., Morrice N., Dando P.M., Barrett A.J., and Watts C. 1998. An asparaginyl endopeptidase processes a microbial antigen for class II MHC presentation. *Nature* **396**, 695-699

Marrack P., Endres R., Shimonkevitz R., Zlotnik A., Dialynas D., Fitch F. and Kappler J. 1983. The major histocompatibility complex-restricted antigen receptor on T cells. II. Role of the L3T4 product. *Journal of Experimental Medicine* **158**, 1077-1091.

Martensson J., Jain A., Stole E., Frayer W., Auld P.A., and Meister A. 1991. Inhibition of glutathione synthesis in the newborn rat: a model for endogenously produced oxidative stress. *Proceedings of the National Academy of Sciences U S A.* **88**, 9360-9364.

Martensson J, Meister A, Martensson J. 1991. Glutathione deficiency decreases tissue ascorbate levels in newborn rats: ascorbate spares glutathione and protects. *Proceedings of the National Academy of Sciences U S A.* **88**, 4656-4660

Masih D.T., Cervi L., and Casado J.M. 1996. Modification of accessory activity of peritoneal cells from *Fasciola hepatica* infected rats. *Veterinary Immunology and Immunopathology* **53**, 257-268

Meek A.H., and Morris R.S. 1979. The effect of prior infection with *Fasciola hepatica* on the resistance of sheep to the same parasite. *Australian Veterinary Journal* **55**, 61-64

Meeusen E., Lee C.S., Rickard M.D., and Brandon M.R. 1995. Cellular responses during liver fluke infection in sheep and its evasion by the parasite. *Parasite Immunology* **17**, 37-45

Mei H., and Lo Verde P.T. 1997. *Schistosoma mansoni*: The developmental regulation and immunolocalization of antioxidant enzymes. *Experimental Parasitology* **86**, 69-78

Mei H., Thakur A., Schwartz J., and Lo Verde P.T. 1996. Expression and characterization of glutathione peroxidase activity in the human blood fluke *Schistosoma mansoni*. *Infection and Immunity* **64**, 4299-4306

Melo R.C. and Brener Z. 1978. Tissue tropism of different *Trypanosoma cruzi* strains. *Journal of Parasitology* **64**, 475-482

Miceli M.C., and Parnes J.R. 1991. The roles of CD4 and CD8 in T cell activation. *Seminars in Immunology* **3**, 133-141

Michel A., Ghoneim H., Resto M., Klinkert M-Q., and Kunz W. 1995. Sequence, characterisation and localisation of a cysteine proteinase cathepsin L in *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **73**, 7-18

Milbourne E.A., and Howell M.J. 1990. Eosinophil responses to *Fasciola hepatica* in rodents. *International Journal for Parasitology* **20**, 705-708

Milbourne E.A., and Howell M.J. 1993. Eosinophil differentiation in response to *Fasciola hepatica* and its excretory/secretory antigens. *International Journal for Parasitology* **23**, 1005-1009

Miller C.M.D., Howell M.J., and Boray J.C. 1993. Host effects on glutathione S-transferase activity in *Fasciola hepatica*. *International Journal for Parasitology* **23**, 1073-1076

Miller R.A., and Britigan B.E. 1997. Role of oxidants in microbial pathophysiology. *Clinical Microbiology Reviews* **10**, 1-18

Mintz C.S., Miller R.D., Gutgsell N.S., and Malek T. 1993. *Legionella pneumphila* protease inactivates interleukin-2 and cleaves CD4 on human T cells. *Infection and Immunity* **61**, 3416-3421

Mkoji G.M., Smith J.M., and Prichard R.K. 1988a. Antioxidant systems in *Schistosoma mansoni*: correlation between susceptibility to oxidant killing and the levels of sc ngers of hydrogen peroxide and oxygen free radicals. *International Journal for Parasitology* **18**, 661-666

Mkoji G.M., Smith J.M., and Prichard R.K. 1988b. Antioxidant systems in *Schistosoma mansoni*: Evidence for their role in protection of the adult worms against oxidant killing. *International Journal for Parasitology* **18**, 667-673

Moire N., Nicolas-Gaulard I., Le Vern Y., and Boulard C. 1997. Enzymatic effect of hypodermin A, a parasite protease, on bovine lymphocyte membrane antigens. *Parasite Immunology* **19**, 21-27

Moncada S., and Higgs A. 1993. The L-arginine-nitric oxide pathway. *New England Journal of Medicine* **329**, 2002-2012

Moody D.B., Reinhold B.B., Guy M.R., Beckman E.M., Frederique D.E., Furlong S.T., Ye S., Reinhold V.N., Sieling P.A., Modlin R.L., Besra G.S., and Porcelli S.A. 1997. Structural requirements for glycolipid antigen recognition by CD1b-restricted T cells. *Science* **278**, 283-288

Moreau E., Chauvin A., and Boulard C. 1998. Lack of humoral and cellular responses against glutathione S-transferases in *Fasciola hepatica* experimentally infected sheep. *Veterinary Research* **29**, 585-591

Morrissey J.H. 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Analytical Biochemistry* **117**, 307-310

Motran C., Gruppi A., Vullo C.M., Pistoresi-Palencia M.C., and Serra H.M. 1996. Involvement of accessory cells in the *Trypanosoma cruzi*-induced inhibition of the polyclonal response of T lymphocytes. *Parasite Immunology* **18**, 43-48

Movsesijan M., and Jovanovic. 1975. Immune response of sheep to *Fasciola hepatica* infection. *Research in Veterinary Science* **18**, 171-174

Mulcahy G., Joyce P., and Dalton J.P. 1999. Immunology of *Fasciola hepatica* Infection. In *Fasciolosis*. J. P. Dalton, eds. CABI Publishing, Oxon, UK, p. 349

Mulcahy G., and Dalton J.P. 2001. Cathepsin L proteinases as vaccines against infection with *Fasciola hepatica* (liver fluke in ruminants. *Research in Veterinary Science* **70**, 83-86

Mulcahy G., O'Connor F., Clery D., Hogan S.F., Dowd A.J., Andrews S.J., and Dalton J.P. 1999. Immune responses of cattle to experimental anti-*Fasciola hepatica* vaccines. *Research in Veterinary Science* **67**, 27-33

Mulcahy G., O'Connor F., McGonigle S., Dowd A., Clery D.G., Andrews S.J., & Dalton J.P. 1998. Correlation of specific antibody titre and avidity with protection in cattle immunised against *Fasciola hepatica. Vaccine* **16**, 932-939

Muro A., Rodriguez-Medina J.R., and Hillyer G.V. 1993. Sequence analysis of a *Fasciola hepatica* glutathione S-transferase cDNA clone. *American Journal of Tropical Medicine and Hygiene* **48**, 457-463

Murray H.W.1982. Cell mediated immune response in experimental visceral leishmaniasis. II. Oxygen-dependent killing of intracellular *Leishmania donovani* amastigotes. *The Journal of Immunology* **129**, 351-357

Naidenko O.V., Koezuka Y.,and Kronenberg M. 2000. CD1-mediated antigen presentation of glycophingolipids. *Microbes and Infection* **2**, 621-631

Nare B., Smith J.M., and Prichard R.K. 1990. *Schistosoma mansoni*: Levels of antioxidants and resistance to oxidants increase during development. *Experimental Parasitology* **70**, 389-397

Nicholas W.L., Stewart A.C., and Mitchell G.F. 1984. Antibody responses to *Toxocara canis* using sera from parasite-infected mice and protection from toxocariasis by immunisation with ES antigens. *Australian Journal of Experimental Biology and Medical Science* **62**, 619-626

Nicolas-Gaulard I., Moire N., and Boulard C. 1995. Effect of the parasite enzyme, hypodermin A, on bovine lymphocyte proliferation and interleukin-2 production via the prostaglandin pathway. *Immunology* **84**, 160-165

Norment A.M., Salter R.D., Parham P., Engelhard V.H., and Littman V.H. 1988. Cell-cell adhesion mediated by CD8 and MHC class I molecules. *Nature* **336**, 79-81

Nussler A.K., and Billiar T.R. 1993. Inflammation, immunoregulation, and inducible nitric oxide synthase. *Journal of Leukocyte Biology* **54**, 171-178.

Oldham G. 1983. Antibodies to *Fasciola hepatica* antigens during experimental infections in cattle measured by ELISA. *Veterinary Parasitology* **13**, 151-158

Oldham G. 1985. Immune responses in rats and cattle to primary infections with *Fasciola hepatica*. *Research in Veterinary Science* **39**, 357-363

Oldham G., and Williams L. 1985. Cell mediated immunity to liver fluke antigens during experimental *Fasciola hepatica* infection of cattle. *Parasite Immunology* **7**, 503-516

Onah D.N., Hopkins J., and Luckins A.G. 1999. Changes in peripheral blood lymphocyte subpopulations and parasite specific antibody responses in *Trypanosoma evansi* infection of sheep. *Parasitology Research* **85**, 263-260

O'Neill S.M., Brady M.T., Callanan J.J., Mulcahy G., Joyce P., Mills K.H., and Dalton J.P. 2000. *Fasciola hepatica* infection down regulates Th1 responses in mice. *Parasite Immunology* **22**, 147-155

O'Neill S.M., Mills K.H., and Dalton J.P. 2001. *Fasciola hepatica* cathepsin L cysteine proteinase suppresses *Bordetella pertussis*-specific interferon-gamma production *in vivo*. *Parasite Immunology* **23**, 541-547

Osman M.M., Shehab A.Y., el Masry S.A., Helmy M.H., and Farag H.F. 1995. Evaluation of *Fasciola* excretory-secretory (E/S) product in diagnosis of acute human fasciolosis by IgM ELISA. *Tropical Medicine and Parasitology* **46**, 115-118

Ou X., Tang L., McCrossan M., Henkle-Duhrsen K., and Selkirk M.E. 1995. *Brugia malayi*: localisation and differential expression of extracellular and cytoplasmic CuZn superoxide dismutases in adults and microfilariae. *Experimental Parasitology* **80**, 515-529

Outteridge P.M., Licence S.T., and Binns R.M. 1981. Characterisation of lymphocyte subpopulations in sheep by rosette formation, adherence to nylon wool and mitogen responsiveness. *Veterinary Immunology and Immunopathology* **2**, 3-18

Panaccio M., Wilson L.R., Crameri S.L., Wijffels G.L., and Spithill T.W. 1992. Molecular characterization of cDNA sequences encoding glutathione S-transferases of *Fasciola hepatica*. *Experimental Parasitology* **74**, 232-237

Paramchuk W.J., Ismail S.O., Bhatia A., and Gedamu L. 1997. Cloning, characterization and overexpression of two iron superoxide dismutase cDNAs from *Leishmania chagasi*: role in pathogenesis. *Molecular Biochemistry and Parasitology* **90**, 203-221

Pearce E.J., and James S.L. 1986. Post lung-stage schistosomula of *Schistosoma mansoni* exhibit transient susceptibility to macrophage-mediated cytotoxicity *in vitro* that may relate to late phase killing *in vivo*. *Parasite Immunology* **8**, 513-527

Peterhans E., Carter S.D. 1998. Sheep immunology and goat peculiarities. In *Handbook of Vertebrate Immunology*. P.P. Pastoret eds. Academic Press, London, UK p. 493

Pfister K., Turner K., Currie A., Hall E., and Jarrett E.E. 1983. IgE production in rat fascioliasis. *Parasite Immunology* **5**, 587-593

Piacenza L., Acosta D., Basmadjian I., Dalton J.P., and Carmona C. 1999. Vaccination with cathepsin L proteinases and with leucine aminopeptidase induces high levels of protection against fascioliasis in sheep. *Infection and Immunity* **67**, 1954-1961

Piacenza L., Radi R., Goni F., and Carmona C. 1998. CuZn superoxide dismutase activities from *Fasciola hepatica*. *Parasitology* **117**, 555-562

Piedrafita D. 1995. Immune mechanisms of killing of juvenile *Fasciola hepatica*. PhD Thesis, Latrobe University

Piedrafita D., Estuningsih S. E., Suharyanta, Widjajanti S., Partoutomo S., Raadsma H.W., and Spithill T.W. 2002 submitted. Peritoneal lavage cells of Indonesian thin tailed sheep mediate antibody-dependent superoxide radical cytotoxicity to newly excysted juvenile *Fasciola gigantica*.

Piedrafita D., and Liew F.Y. 1998. Nitric oxide: a protective or pathogenic molecule? *Reviews in Medical Microbiology* **9**, 179-189

Piedrafita D., Parsons J.C., Sandeman R.M., Wood P.R., Estuningsih S.E., Partoutomo S., and Spithill T.W. 2001. Antibody-dependent cell-mediated cytotoxicity to newly excysted juvenile

Fasciola hepatica in vitro is mediated by reactive nitrogen intermediates. *Parasite Immunology* **23**,473-82

Piedrafita D., Spithill T.W., Dalton J.P., Brindley P.J., Sandeman M.R., Wood P.R., and Parsons J.C. 2000. Juvenile *Fasciola hepatica* are resistant to killing *in vitro* by free radicals compared with larvae of *Schistosoma mansoni*. *Parasite Immunology* **22**, 287-295

Poitou I., Baeza E., and Boulard C. 1992. Humoral and cellular immune responses in rats during a primary infestation with *Fasciola hepatica*. *Veterinary Parasitology* **45**, 59-71

Poitou I., Baeza E., and Boulard C. 1993. Kinetic responses of parasite specific antibody isotypes, blood leucocyte pattern and lymphocyte subsets in rats during primary infestation with *Fasciola hepatica*. *Veterinary Parasitology* **49**, 179-190

Porcelli S.A. 1995. The CD1 family: a third lineage of antigen-presenting molecules. *Avances in Immunology* **59**,1-98

Porcelli S.A., and Modlin R.L. 1999. The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids. *Annual Reviews of Immunology* **17**, 297-329

Porcelli S., Morita C.T., and Brenner M.B. 1992. CD1b restricts the response of human $CD4^-$ $CD8^-$ lymphocytes to a microbial antigen. *Nature* **360**, 593-597

Prasitirat P., Thammasart S., Chompoochan T., Nithiuthai S., & Taira S. 1996. The dynamics of antibody titres and faecal egg output in cattle and buffalo following infection with 500 and 1000 *Fasciola gigantica* metacercariae. *Thai Journal of Veterinary Medicine* **26**, 85-89

Prichard R.K., and Schofield P.J. 1971. *Fasciola hepatica*: cytochrome c oxidoreductases and effects of oxygen tension and inhibitors. *Experimental Parasitology* **29**, 215-222

Prince R.C., and Gunson D.E. 1993. Rising interest in nitric oxide synthase. *Trends in Biochemical Sciences* **18**, 35-36

Prowse R.K., Chaplin P., Robinson H.C., and Spithill T.W. 2002. *Fasciola hepatica* cathepsin L suppresses sheep lymphocyte proliferation *in vitro* and modulates surface CD4 expression on human and ovine T cells. *Parasite Immunology* **24**, 57-66

Rajasekariah G.R., and Howell M.J. 1977. *Fasciola hepatica* in rats: effects of age and infective dose. *International Journal for Parasitology* **7**, 119-121

Rajasekariah G.R., and Howell M.J. 1981. Age-associated responses in susceptible and resistant rats to infection with *Fasciola hepatica*. *International Journal for Parasitology* **11**, 59-65

Ramaswamy K., Salafsky B., Potluri S., He Y.X., Li J.W., and Shibuya T. 1995. Secretion of an anti-inflammatory, immunomodulatory factor by Schistosomulae of *Schistosoma mansoni*. *Journal of Inflammation* **46**, 13-22

Reid J.F., Armour J., Urquhart G.M., and Jennings F.W. 1970. Studies on ovine fascioliasis. I. Observations on the sequential development of the naturally-acquired disease. *The Veterinary Record* **86**, 242-249.

Reiner N.E. 1994. Altered cell signaling and mononuclear phagocyte deactivation during intracellular infection. *Immunology Today* **15**, 374-381

Reiner N.E., and Kazura J.W.1982. Oxidant-mediated damage of *Leishmania donovani* promastigotes. *Infection and Immunity* **36**, 1023-1030

Renshaw H.W., Eckblad W.P., Everson D.O., Tassinari D., and Amos D. 1977. Ontogeny of immunocompetence in cattle: evaluation of phytomitogen-induced *in vitro* bovine fetal lymphocyte blastogenesis using a whole blood culture. *American Journal of Veterinary Research* **38**, 1141-1150

Rhind S.M., Hopkins J., and Dutia B.M. 1999. Amino-terminal sequencing of sheep CD1 antigens and identification of a sheep CD1D gene. *Immunogenetics* **49**, 225-230

Rhind S.M., Hopkins J., and Grant E.S. 2000. Differential expression of ovine CD1. *Immunology* **101**, 452-457

Rickard M.D. and Howell M.J. 1982. Comparative aspects of immunity in fascioliasis and cysticercosis in domesticated animals. In: Symons I.E.A., Donald A.D. and Dineen J.K. (eds) *Biology and Control of Ectoparasites*. Academic Press, Sydney, p. 343-374

Riffkin M., Seow H.F., Jackson D., Brown L., and Wood P. 1996. Defence against the immune barrage: helminth survival strategies. *Immunology and Cell Biology* **74**, 564-574

Rigano R., Profumo E., Bruschi F., Carulli G., Azzara A., Ioppolo S., Buttari B., Ortona E., Margutti P., Teggi A., and Siracusano A. 2001. Modulation of human immune response by *Echinococcus granulosus* antigen B and its possible role in evading host defenses. *Infection and Immunity* **69**, 288-296

Roberts J.A., Widjajanti S., and Estuningsih E. 1996. Acquired resistance of merino sheep against *Fasciola gigantica. Parasitology Research* **82**, 743-746

Roberts J.A., Estuningsih E., Widjayanti S., Wiedosari E., Partoutomo S. and Spithill T.W. 1997a. Resistance of Indonesian Thin Tail sheep against *Fasciola gigantica* and *F. hepatica. Veterinary Parasitology* **68**, 69-78

Roberts J.A., Widjajanti S., Estuningsih E., and Hetzel D.J. 1997b. Evidence for a major gene determining the resistance of Indonesian Thin Tail sheep against *Fasciola gigantica. Veterinary Parasitology* **68**, 309-314

Roberts J.A., Estuningsih E., Wiedosari E., and Spithill T.W. 1997c. Acquisition of resistance against *Fasciola gigantica* by Indonesian Thin Tail sheep. *Veterinary Parasitology* **73**, 215-224

Romagnani S. 1999. Th1/Th2 cells. *Inflammatory Bowel Diseases* **5**, 285-294

Roos D., Eckmann C.M., Yazdanbakhsh M., Hamers M.N., and DeBoer M. 1984. Excretion of superoxide by phagocytes measured with cytochrome c entrapped in resealed erythrocyte ghosts. *Journal of Biological Chemistry* **259**, 1770-1775

Rose R.C., and Bode A.M. 1993. Biology of free radical scavengers: an evaluation of ascorbate. *Journal of the Federation of American Societies for Experimental Biology* **7**, 1135-1142

Ross J.G. 1966. Experimental infections of cattle with *Fasciola hepatica*. *Nature* **212**, 1464-1465

Ross J.G. 1967. Studies of immunity to *Fasciola hepatica*. Acquired immunity in cattle, sheep and rabbits following natural infection and vaccine procedures. *The Journal of Helminthology* **41**, 393-399

Rothel J.S., Corner L.A., Lightowlers M.W., Seow H.F., McWaters P., Entrican G. and Wood P.R. 1998. Antibody and cytokine responses in efferent lymph following vaccination with different adjuvants. *Veterinary Immunology and Immunopathology* **63**, 167-183

Rothel J.S., Hurst L., Seow H.F., Pepin M., Berthon P., Corner L.A., and Wood P.R. 1997. Analysis of ovine IL-production in vivo and in vitro by ELISA and immunohistochemistry. *Veterinary Immunology and Immunopathology* **57**, 267-278

Rothwell T.L., and Love R.J. 1974. Vaccination against the nematode Trichostrongylus colubriformis. I. Vaccination of guinea-pigs with worm homogenates and soluble products released during *in vitro* maintenance. *International Journal for Parasitology* **4**, 293-299

Rothwell T.L.W., Windon R.G., Horsburgh B.A., and Anderson B.H. 1993. Relationship between eosinophilia and responsiveness to infection with *Trichostrongylus colubriformis* in sheep. *International Journal for Parasitology* **23**, 203-211

Rouse B.T., and Babiuk L.A. 1974. Host responses to infectious bovine rhinotracheitis virus. III. Isolation and immunologic activities of bovine T lymphocytes. *The Journal of Immunology* **113**, 1391-1398

Roy R.M., and Reddy N.R. 1969. Studies on the activity of nitroxynil against *Fasciola gigantica* in naturally infected buffaloes, cattle and sheep. *The Veterinary Record* 85-87

Rushton B. and Murray M. 1977. Hepatic pathology of a primary experimental infection of *Fasciola hepatica* in sheep. *Journal of Comparative Pathology* **87**, 459-470

Saeji J.P., Van Muiswinkel W.B., Groeneveld A., and Wiegertjes G.F. 2002. Immune modulation by fish kinetoplastid parasites: a role for nitric oxide. *Parasitology* **124**, 77-86

Saha B., Das G., Vohra H., Ganguly N.K., and Mishra G.C. 1995. Macrophage-T cell interaction in experimental visceral leishmaniasis: failure to express costimulatory molecules on *Leishmania*-infected macrophages and its implication in the suppresion of cell-mediated immunity. *European Journal of Immunology* **25**, 2492-2498

Salazar-Calderon M., Martin-Alonso J.M., Ruiz de Eguino A.D., Casais R., Marin M.S., and Parra F. 2000. *Fasciola hepatica*: heterologous expression and functional characterization of a thioredoxin peroxidase. *Experimental Parasitology* **95**, 63-70

Salin M.L., and McCord J.M. 1974. Superoxide dismutases in polymorphonuclear leukocytes. *The Journal of Clinical Investigation* **54**, 1005-1009

Salter R.D., Benjamin R.J., Wesley P.K., Buxton S.E., Garrett T.P., Clayberger C., Krensky A.M., Norment A.M., Littman D.R., and Parham P. 1990. A binding site for the T-cell co-receptor CD8 on the alpha 3 domain of HLA-A2. *Nature* **345**, 41-46

Sanchez-Moreno M., Leon P., Salas-Peregrin J.M., Garcia-Ruiz M.A., and Monteoliva M. 1987. Superoxide dismutase in trematodes. Isoenzymatic characterisation and studies on inhibition by a series of benzimidazoles and by pyrimidines of recent syntheses. *Arzneimittelforschung* **37**, 903-905

Sandeman R.M. and Howell M.J. 1980a. *In vitro* studies of the response of sheep to infection with *Fasciola hepatica*. *Veterinary Parasitology* **6**, 347-357

Sandeman R.M. and Howell M.J. 1980b. An attempt to vaccinate sheep against *Fasciola hepatica* using a juvenile fluke antigen sheep antibody complex. *Research in Veterinary Science* **29**, 255-259

Sandeman R.M., and Howell M.J. 1981. Precipitating antibodies against excretory secretory antigens of *Fasciola hepatica* in sheep serum. *Veterinary Parasitology* **9**, 35-46

Satarug S., Haswell-Elkins M.R., Sithithaworn P., Bartsch H., Ohshima H., Tsuda M., Mairiang P., Mairiang E., Yongvanit P., Esumi H., and Elkins D.B. 1998. Relationships between the synthesis of N-nitrosodimethylamine and immune responses to chronic infection with the carcinogenic parasite, *Opisthorchis viverrini*, in men. *Carcinogenesis* **19**, 485-491

Schmidt G.D., and Roberts L.S. 1985. Introduction to parasitology. *In*: Foundations of Parasitology. D.L. Bowen editor. Times mirror/mosby college publishing, Missouri. 1-5.

Sewell M.M.H. 1966. The pathogenesis of fascioliasis. *The Veterinary Record* **78**, 98-105

Selkirk M.E., Smith V.P., Thomas G.R., and Gounaris K. 1998. Resistance of filarial nematode parasites to oxidative stress. *International Journal for Parasitology* **28**, 1315-1332

Sexton J.L., Milner A.R., Panaccio M., Waddington J., Wijffels G.L., Chandler D., Thompson C., Wilson L., Spithill T.W., Mitchell G.F., and Campbell N.J. 1990. Glutathione *S*-transferase. Novel vaccine against *Fasciola hepatica* infection in sheep. *The Journal of Immunology* **145**, 3905-3910

Sher A. and Coffman R.L. 1992. Regulation of immunity to parasites by T cell-derived cytokines. *Annual Review of Immunology* **15**, 553-558

Sinclair K.B. 1970. Observations on the clinical pathology of ovine fascioliasis. *British Veterinary Journal* **118**, 37-53

Sinclair K.B. 1971. Acquired resistance to *Fasciola hepatica* in sheep. *British Veterinary Journal* **129**, 236-250

Sinclair K.B. 1973. The resistance of sheep to *Fasciola hepatica*: studies on the development and pathogenicity of challenge infections. *British Veterinary Journal* **129**, 236-250

Sinclair K.B. 1975. The resistance of sheep to *Fasciola hepatica*: studies on the pathophysiology of challenge infections. *Research in Veterinary Science* **19**, 296-303

Sithithaworn P., Haswell-Elkins M.R., Mairiang P., Satarug S., Mairiang E., Vatanasapt V., and Elkins D.B. 1994. Parasite associated morbidity: liver fluke infection and bile duct cancer in Northeast Thailand. *International Journal for Parasitology* **24**, 833-843

Small T.N., Knowles R.W., Keever C., Dernen N.A., Collins N., O'Reilly R.J., Dupont B., and Flomenberg N. 1987. M241 (CD1) expression on B lymphocytes. *The Journal of Immunology* **138**, 2864-2868

Smith A. M., Carmona C., Dowd A.J., McGonigle S., Acosta D., and Dalton J.P. 1994. Neutralization of the activity of a *Fasciola hepatica* cathepsin L proteinase by anti-cathepsin L antibodies. *Parasite Immunology* **16**, 325-328

Smith A.M., Dowd A.J., Heffernan M., Robertson C.D., and Dalton J.P. 1993a. *Fasciola hepatica*: A secreted cathepsin L-like proteinase cleaves host immunoglobulin. *International Journal for Parasitology* **23**, 977-983

Smith A.M., Dowd A.J., McGonigle S., Keegan P.S., Brennan G., Trudgett A., and Dalton J.P. 1993b. Purification of a cathepsin L-like proteinase secreted by adult *Fasciola hepatica*. *Molecular Biochemistry and Parasitology* **62**, 1-8

Smith D.B., Davern K.M., Board P.G., Tiu W.U., Garcia E.G., and Mitchell G.F. 1986. M,26000 antigen of *Schistosoma japonicum* recognised by resistant WEHI 129/J mice is a parasite glutathione S-transferase. *Proceedings of the National Academy of Sciences USA* **83**, 8703-8707

Smith D. B., Rubira M.R., Simpson R.J., Davern K.M., Tiu W.U., Board P.G., and Mitcherll G.F. 1987. Expression of an enzymatically active parasite molecule in *Escherichia coli*: *Schistosoma japonicum* glutathione S-transferase. *Molecular and Biochemical Parasitology* **27**, 249-256

Smith J.B., McIntosh G.H., and Morris B. 1970. The traffic of cells through tissues: a study of peripheral lymph in sheep. *Journal of Anatomy* **107**, 87-100

Smith M.A., and Clegg J.A. 1981. Improved culture of *Fasciola hepatica in vitro*. *Z Parasitenkd.* **66**, 9-15

Smith N.C., and Bryant C. 1986. The role of host generated free radicals in Helminth infections: *Nippostrongylus brasiliensis* and *Nematospiroides dubius* compared. *International Journal for Parasitology* **16**, 617-622

Smith N.C., Ovington K.S., and Boray J.C. 1992. *Fasciola hepatica*: free radical generation by peritoneal leukocytes in challenged rodents. *International Journal for Parasitology* **22**, 281-286

Smithers S.R., and Doenhoff M.J. 1982. Schistosomiasis. In: Immunology of Parasitic Infections. 2nd Edition (Edited by Cohen S. & Warren K.S). p 527-607. Blackwell Scientific Publications, Oxford.

Smooker P.M., Whisstock J.C., Irving J.A., Siyaguna S., Spithill T.W., and Pike R.N. 2000. A single amino acid substitution affects substrate specificity in cysteine proteinases from *Fasciola hepatica*. *Protein Science* **9**, 2567-2572

Smyth J.D. and Halton D.W. 1983. The Physiology of Trematodes, 2nd edn. Cambridge University Press. Cambridge p. 446

Sobhon P. Anantavara S., Dangprasert T., Viyanant V., Krailas D., Upatham E.S., Wanichanon C., and Kusamran T. 1994. *Fasciola gigantica*: Studies of the tegument as a basis for the developments of immunodiagnosis and vaccine. *Southeast Asian Journal of Tropical Medicine and Public Health* **29**, 387-400

Spithill T.W., Smooker P.M., and Copeman D.B. 1999. *Fasciola gigantica*: Epidemiology, Control, Immunology and Molecular Biology. In *Fasciolosis*. J. P. Dalton, eds. CABI Publishing, Oxon, UK, p. 465-525

Spithill T.W., Smooker P.M., Sexton J.L., Bozas, E., Morrison C.A., Creaney J., and Parsons J.C. 1999. Development of vaccines against *Fasciola hepatica*. In *Fasciolosis*. J. P. Dalton, eds. CABI Publishing, Oxon, UK, p. 377-410

Stadnyk A.W. and Gauldie J. 1991. The acute phase protein response during parasitic infection. *Parasitology Today* **7**, A7-A12

Stamler J.S., Simon D.I., Osborne J.A., Mullins M.E., Jaraki O., Michel T., Singel D.J., Loscalzo J. 1992. S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds. *Proceedings of the National Academy of Sciences USA* **89**, 444-448

Stamler J.S., Singel D.J., and Loscalzo J. 1992. Biochemistry of nitric oxide and its redox activated forms. *Science* **258**, 1898-1902

Stenersen J., Kobro S., Bjerke M., and Arend U. 1987. Glutathione transferases in aquatic and terrestrial animals from nine phyla. *Comparative Biochemistry and Physiology* **86**, 73-82

Stein M., Keshav S., Harris N., and Gordon S. 1992. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *Journal of Experimental Medicine* **176**, 287-292

Stoltenborg J.K., Straney R.A., Tritch R.J., Mackin W.M., and George H.J. 1993. Expression and functional characterization of recombinant human vascular cell adhesion molecule-1 (VCAM1) synthesized by baculovirus infected insect cells. *Protein Expression and Purification* **4**, 585-593

Storz, G., Tartaglia L. A., and Ames B.N. 1990. Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. *Science* **248**, 189-194

Storz G., Tartaglia L.A., Farr S.B., and Ames B.N. 1990. Bacterial defenses against oxidative stress. *Trends in Genetics* **6**, 363-368

Stuehr D.J. and Marletta M.A. 1985. Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. *Proceedings of the National Academy of Sciences USA* **82**, 7738-7742

Stuehr D.J., Morris C., and Nathan C.F. 1988. Cytostasis from nitrite, a product of activated macrophages. *Journal of the Federation of American Societies for Experimental Biology* **2,** A1452

Sugita M., and Brenner M.B. 2000. T lymphocyte recognition of human group 1 CD1 molecules: implications for innate and acquired immunity. *Seminars in Immunology* **12,** 511-516

Sztein M.B., Cuna W.R., and Kierszenbaum F. 1990. *Trypanosoma cruzi* inhibits the expression of CD3, CD4, CD8, and IL-2R by mitogen activated helper and cytotoxic human lymphocytes. *The Journal of Immunology* **144**, 3558-62

Tanaka H., Saito S., Sasaki H., Arai H., Oki T., and Shioya N. 1994. Morphological aspects of LFA-1/ICAM-1 and VLA4/VCAM-1 adhesion pathways in human lymph nodes. *Pathology International* **44**, 268-279

Threadgold L.T. 1963. The tegument and associated structures of *Fasciola hepatica*. L. *Quaterly Journal of Microscopical Science* **104**, 505-512

Threadgold L.T. 1967. Electron-microscope studies of *Fasciola hepatica*. III. Further observations on the tegument and associated structures. *Parasitology* **57**, 633-637

Threadgold L.T. 1976. *Fasciola hepatica*: Ultrastructure and histochemistry of the glycocalyx of the tegument. *Experimental Parasitology* **39**, 119-134

Tkalcevic J., Ashman K., and Meeusen E. 1995. *Fasciola hepatica*: rapid identification of newly excysted juvenile proteins. *Biochemical and Biophysical Research Communications* **213,** 169-174

Tkalcevic J., Brandon M.R., and Meeusen E. N. 1996. *Fasciola hepatica*: rapid switching of stage specific antigen expression after infection. *Parasite Immunology* **18,** 139-147

Tort J., Brindley P.J., Knox D., Wolfe K.H., and Dalton J.P. 1999. Proteinases and associated genes of parasitic helminths. *Advances in Parasitology* **43**, 161-266

Trnka Z., and Cahill R.N.P. 1980. Aspects of the immune response in single lymph nodes. *Monogr. Allergy* **16**, 245-259

Trottein F., Nutten S., Angeli V., Delerive P., Teissier E., Capron A., Staels B., ande Capron M.1999. *Schistosoma mansoni* schistosomula reduce E-selectin and VCAM-1 expression in TNF-a-stimulated lung microvascular endothelial cells interfering with the NF-xB pathway. *European Journal of Immunology* **29**, 3691-3701

Turpaev K.T. 2002. Reactive oxygen species and regulation of gene expression. *Biochemistry (Moscow)* **67**, 281-292

Urban J. F., Katona I.M., and Finkelman F.D. 1991. Heligosomoides polygyrus: $CD4^+$ but not $CD8^+$ T cells regulate the IgE response and protective immunity in mice. *Experimental Parasitology* **73**, 500-511

van Milligen F.J., Cornelissen J.B.W.J. and Bokhout B.A. 1998a. Location of induction and expression of protective immunity against *Fasciola hepatica* at the gut level: A study using an *ex vivo* infection model with ligated gut segments. *Journal of Parasitology* **84**, 771-777

van Milligen F.J., Cornelissen J.B.W.J., Gaasenbeek C.P.H. and Bokhout B.A. 1998b. A novel *ex vivo* rat infection model to study protective immunity against *Fasciola hepatica* at the gut level. *Journal of Immunological Methods* **213**, 183-190

van Milligen F.J., Cornelissen J.B.W.J. and Bokhout B.A. 1999. Protection against *Fasciola hepatica* in the intestinge is correlated with eosinophil and immunoglobulin G1 responses against newly excysted juveniles. *Parasite Immunology* **21**, 243-251

van Milligen F.J., Cornelissen J.B.W.J. and Bokhout B.A. 2000. *Fasciola hepatica*: An antigen fraction derived from newly excysted juveniles, containing an immunoreactive 32-kDa protein, induces strong protective immunity in rats. *Experimental Parasitology* **94**, 163-171

Vignali D.A. 1994. The interaction between CD4 and MHC class II molecules and its effect on T cell function. *Behring Inst Mitt* **94**, 133-147

Vincendau au P., and Daulouede S. 1991. Macrophage cytostatic effect on *Trypanosoma musculi* involves dependent mechanism. *Journal of Immunology* **146**, 4338-4343

Viyanant V., Upatham E.S., Sobhon P., Krailas D., Ardseungnoen P., and Anatawara S. 1997. Development and characterization of monoclonal antibodies against excretory-secretory antigens of *Fasciola gigantica*. *The Southeast Asian Journal of Tropical Medicine and Public Health* **28**, 128-133

Wakelin D. 1978. Genetic control of susceptibility and resistance to parasitic infection. *Advances in Parasitology* **16**, 219-308

Wakelin D. 1991. Immunity and genetics: their relation to control of parasitic zoonoses. *Parassitologia* **33**, 61-66

Ward P.A., and Lentsch A.B. The acute inflammatory response and its regulation. *Archives of Surgery* **134**, 666-669

Wasilewski M.M., Lim K.C., Phillips J., McKerrow J.H. 1996. Cysteine protease inhibitors block schistosome hemoglobin degradation in vitro and decrease worm burden and egg production *in vivo*. *Molecular and Biochemical Parasitology* **81**, 179-189

Wedrychowicz H., Turner K., Pfister K., Holmes P.H., and Armour J. 1984. Local antibody responses in the bile and faeces of sheep infected with *Fasciola hepatica*. *Research in Veterinary Science* **37**, 44-51

Weidosari E. and Copeman D.B. 1990. High resistance to experimental infection with *Fasciola gigantica* in Javanese thin-tailed sheep. *Veterinary Parasitology* **37**, 101-111

Weisiger R.A., and Fridovich I. 1973. Superoxide dismutase: organelle specificity. *The Journal of Biological Chemistry* **248**, 3582-3592

Weiss S.J. 1986. Oxygen, ischemia and inflammation. *Acta Physiol Scand Suppl* **548**, 9-37

Weiss A., and Imboden J.B. 1987. Cell surface molecules and early events involved in human T lymphocyte activation. Advances in Immunology 41, 1-38

Wheeler C.J., P. von Hoegen, and Parnes J.R. 1992. An immunological role for the CD8 beta-chain. *Nature* **357**, 247-249

Wijffels G. L., Panaccio M., Salvatore L., Wilson L., Walker I.D., and Spithill T.W. 1994a. The secreted cathepsin L – like proteinases of the trematode, *Fasciola hepatica*, contain 3-hydroxyproline residues. *Biochemical Journal* **299**, 781-790

Wijffels G. L., Salvatore L., Dosen M., Waddington J., Wilson L., Thompson C., Campbell N., Sexton J., Wicker J., Bowen F., Friedel T., and Spithill T.W. 1994b. Vaccination of Sheep with Purified Cysteine Proteinases of *Fasciola hepatica* Decreases Worm Fecundity. *Experimental Parasitology* **78**, 132-148

Wijffels G.L., Sexton J.L., Salvatore L., Pettitt J.M., Humphris D.C., Panaccio M., and Spithill T.W. 1992. Primary sequence heterogeneity and tissue expression of glutathione S-transferases of *Fasciola hepatica*. *Experimental Parasitology* **74**, 87-99

Wilson L.R., Good R. T., Panaccio M., Wijffels G.L., Sandeman R.M., and Spithill T.W. 1998. *Fasciola hepatica*: characterization and cloning of the major cathepsin B protease secreted by newly excysted juvenile liver fluke. *Experimental Parasitology* **88**, 85-94

Windon R.G., Chaplin P.J., Beezum L., Coulter A., Cahill R., Kimpton W., Drane D., Pearse M., Sjolander A., Tennent J., Scheerlinck J-P.Y. 2000. Induction of lymphocyte recruitment in the absence of a detectable immune response. *Vaccine* **19**, 572-578

Woolaston R.R., Barger I.A., and Piper L.R. 1990. Response to helminth infection of sheep selected for resistance to *Haemonchus contortus*. *International Journal for Parasitology* **20**, 1015-1018

Wu J., and Weiss B. 1991. Two divergently transcribed genes, sosR and sosS, control a superoxide response regulon in *Escherichia coli*. *Journal of Bacteriology* **173**, 2864-2871

Xu D., Chan W.L., Leung B.P., Huang F., Wheeler R., Piedrafita D., Robinson J.H., and Liew F.Y. 1998. Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. *The Journal of Experimental Medicine* **187**, 787-794

Yadav S.C., and Gupta S.C. 1995. Immunodiagnostic moieties in somatic and excretory/secretory antigens of *Fasciola gigantica*. *Indian Journal of Experimental Biology* **33**, 824-828

Yamashita T., Watanabe T., Saito S., Araki Y., and Sendo F. 1993. *Schistosoma japonicum* soluble egg antigens activate naïve B cells to produce antibodies: definition of parasite mechanisms of immune deviation. *Immunology* **79**, 189-195

Yazdanbakhsh M., Eckmann C.M., and Roos D. 1987. Killing of schistosomula by taurine chloramine and taurine bromamine. *American Journal of Tropical Medicine and Hygiene* **37**, 106-110

Yazdanbakhsh M., Tai P.-C., Spry C.J.F., Gleich G.J., and Roos D. 1987. Synergism between eosinophil cationic protein and oxygen metabolites in killing of schistosomula of *Schistosoma mansoni*. *The Journal of Immunology* **138**, 3443-3447

Yong W.K., and Das P.K. 1983. Acquisition of host proteins by the tegument of *Schistosoma mansoni* recovered from rats. *Z Parasitenkd* **69**, 53-60

Yoshida K., Akaike T., Doi T., Sato K., Ijiri, S., Suga M., Ando M., and Maeda H. 1993. Pronounced enhancement of NO-dependent antimicrobial action by an NO-oxidising agent, imidazolineoxyl N-oxide. *Infection and Immunity* **61**, 3552-3555

Yoshino T.P., Lodes M..J., Rege A.A., and Chappell C.L. 1993. Proteinase activity in miracidia, transformation excretory-secretory products, and primary sporocysts of *Schistosoma mansoni*. *The Journal of Parasitology* **79**, 23-31

Young A.J. 1999. The physiology of lymphocyte migration through the single lymph node *in vivo*. *Seminars in Immunology* **11**,73-83

Yssel H., De Waal Malefyt R., Roncarolo M.G., Abrams J.S., Lahesmaa R., Spits H., and de Vries J.E. 1992. IL-10 is produced by subsets of human CD4+ T cell clones and peripheral blood T cells. *Journal of Immunology* **149**, 2378-2384

Zamoyska R. 1994. The CD8 coreceptor revisited: one chain good, two chains better. *Immunity* **1**, 243-246

Zamoyska R. 1998. CD4 and CD8: modulators of T-cell receptor recognition of antigen and of immune responses? *Current Opinion in Immunology* **10**, 82-87

Zang X., and Maizels R.M. 2001. Serine proteinase inhibitors from nematodes and the arms race between host and pathogen. *Trends in Biochemical Sciences* **26**, 191-197

Zang X., Yazdanbakhsh M., Jiang H., Kanost M.R., Maizels R.M. 1999. A novel serpin expressed by blood-borne microfilariae of the parasitic nematode *Brugia malayi* inhibits human neutrophil serine proteinases. *Blood* **94**, 1418-1428

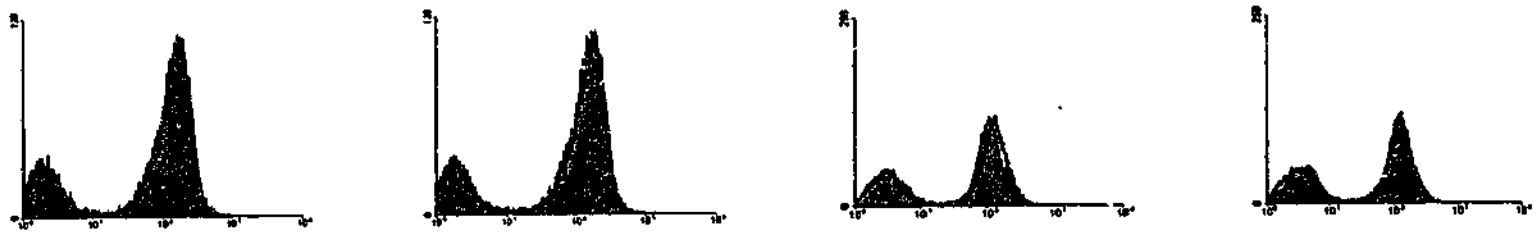
Zimmerman G.L., Kerkvliet N.I., Brauner J.A., and Cerro J.E. 1983. Modulation of host immune responses by *Fasciola hepatica*: responses of peripheral lymphocytes to mitogens during liver fluke infections of sheep. *The Journal of Parasitology* **69**, 473-477

APPENDIX

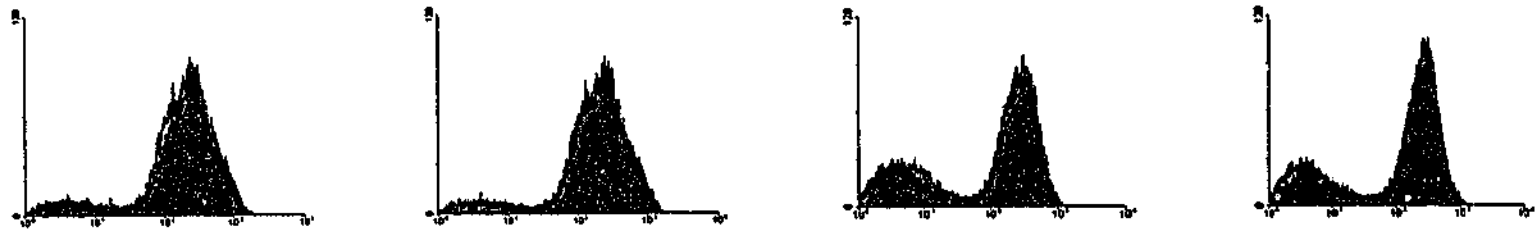
Afferent Lymph

Efferent Lymph

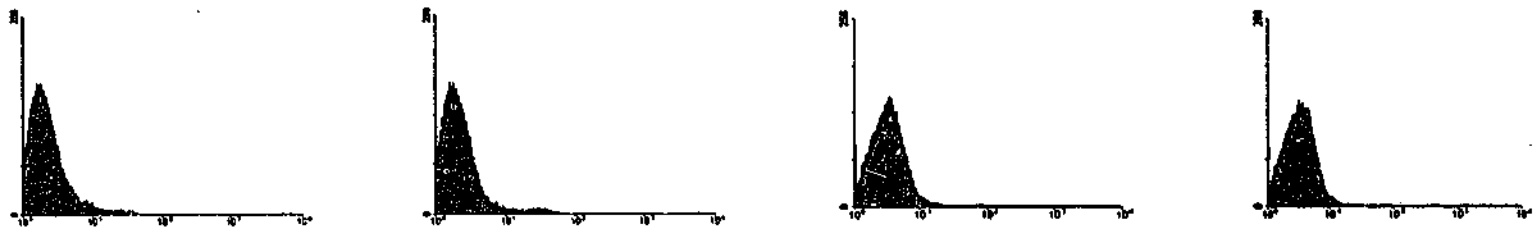
CD2



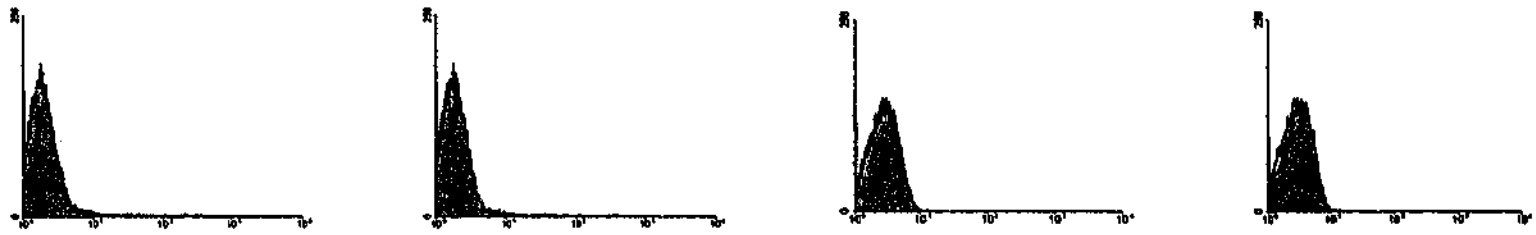
CD5



CD11a



CD11b



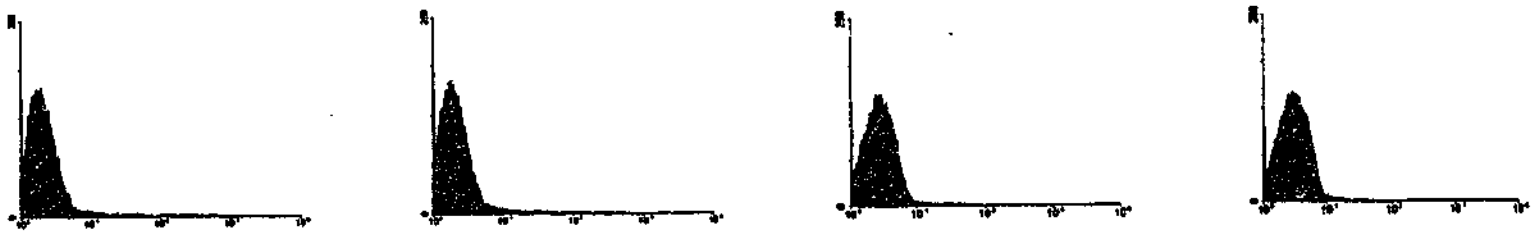
F. hepatica ES | *F. gigantica* ES | *F. hepatica* ES | *F. gigantica* ES

Appendix 1a: The effect of *F. hepatica* ES (250 µg/ml) and *F. gigantica* ES (250 µg/ml) on the expression of sheep cell surface markers CD2, CD5, CD11a and CD11b on either afferent or efferent lymph after a 4 hr incubation. The grey shaded area represents the surface markers expression on sheep cells in the presence of antigen after a 4 hr incubation while the black overlay shows the normal surface marker expression on the identical population of sheep cells incubated for 4 hrs in media alone. Histograms display 20000 events for each sample.

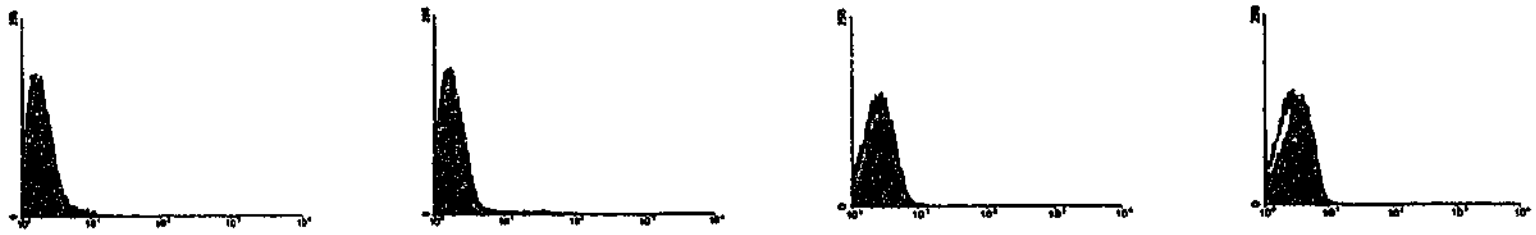
Afferent Lymph

Efferent Lymph

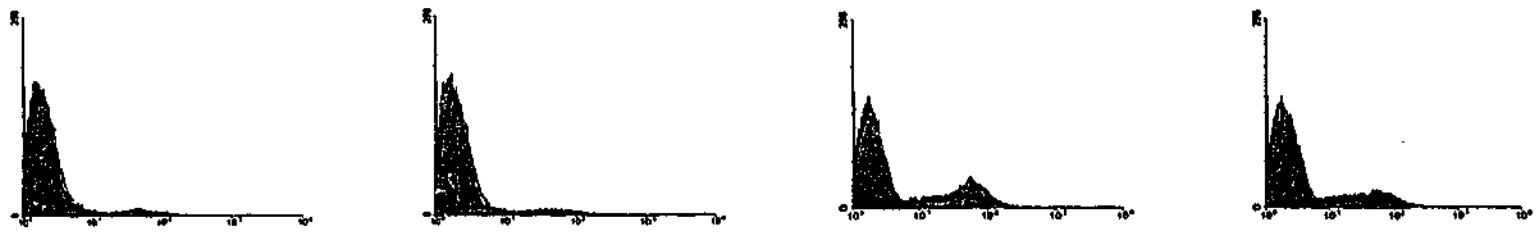
CD11c



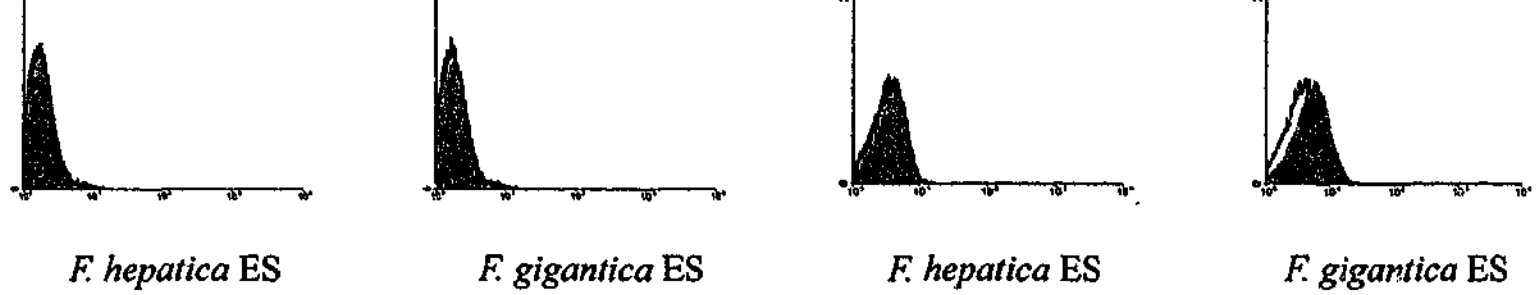
CD14



CD21



CD23



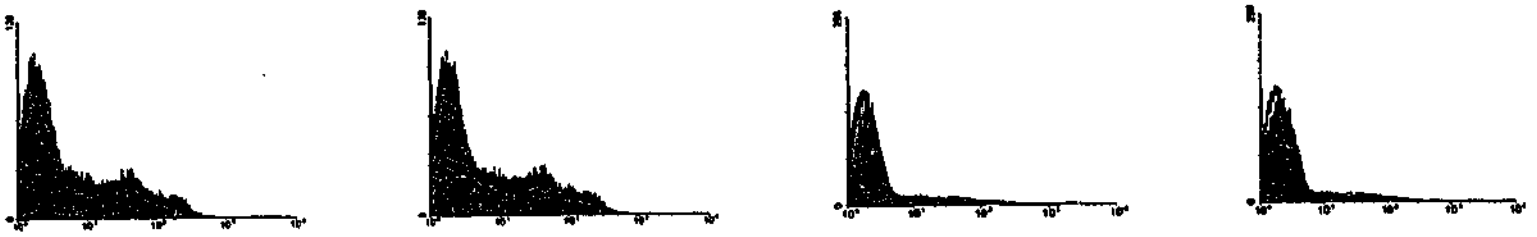
F. hepatica ES | *F. gigantica* ES | *F. hepatica* ES | *F. gigantica* ES

Appendix 1b: The effect of *F. hepatica* ES (250 µg/ml) and *F. gigantica* ES (250 µg/ml) on the expression of sheep cell surface markers CD11c, CD14, CD21 and CD23 on either afferent or efferent lymph after a 4 hr incubation. The grey shaded area represents the surface markers expression on sheep cells in the presence of antigen after a 4 hr incubation while the black overlay shows the normal surface marker expression on the identical population of sheep cells incubated for 4 hrs in media alone. Histograms display 20000 events for each sample.

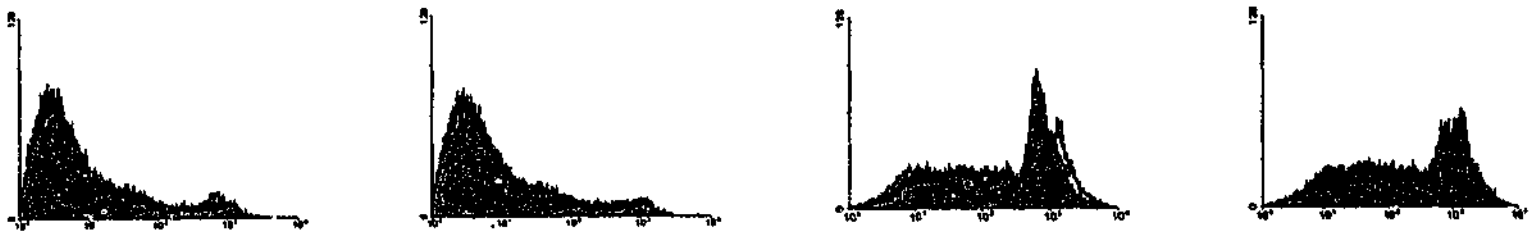
Afferent Lymph

Efferent Lymph

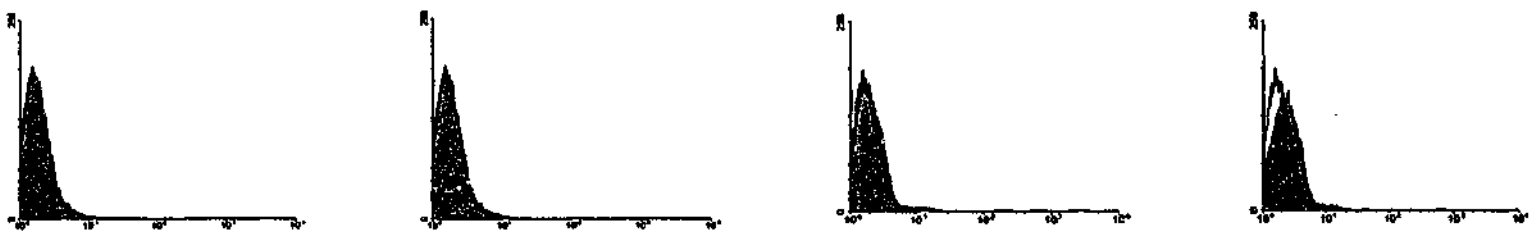
CD25



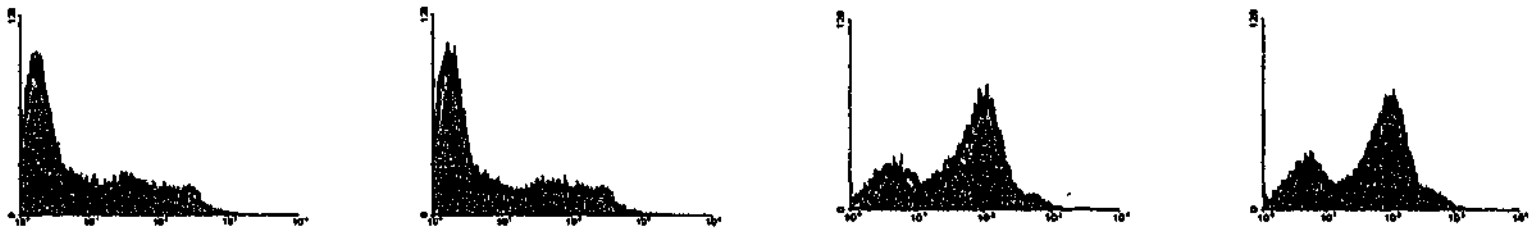
CD45R



CD45Ra



L-selectin



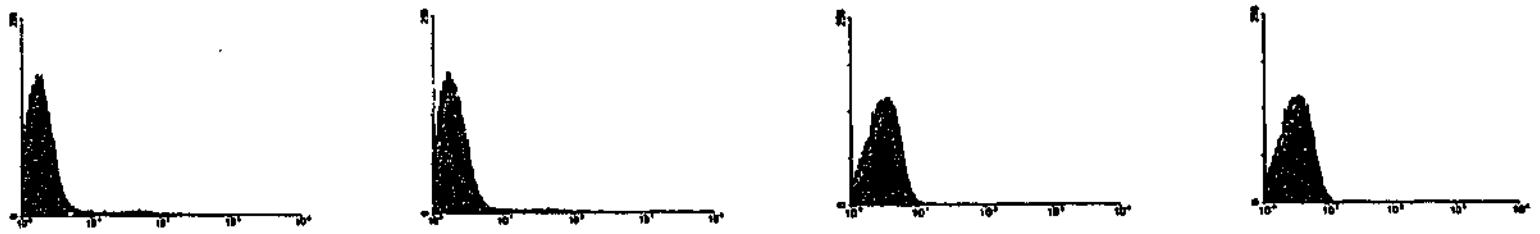
F. hepatica ES | *F. gigantica* ES | *F. hepatica* ES | *F. gigantica* ES

Appendix 1c: The effect of *F. hepatica* ES (250 µg/ml) and *F. gigantica* ES (250 µg/ml) on the expression of sheep cell surface markers CD25, CD45R, CD45Ra and L-selectin on either afferent or efferent lymph after a 4 hr incubation. The grey shaded area represents the surface markers expression on sheep cells in the presence of antigen after a 4 hr incubation while the black overlay shows the normal surface marker expression on the identical population of sheep cells incubated for 4 hrs in media alone. Histograms display 20000 events for each sample.

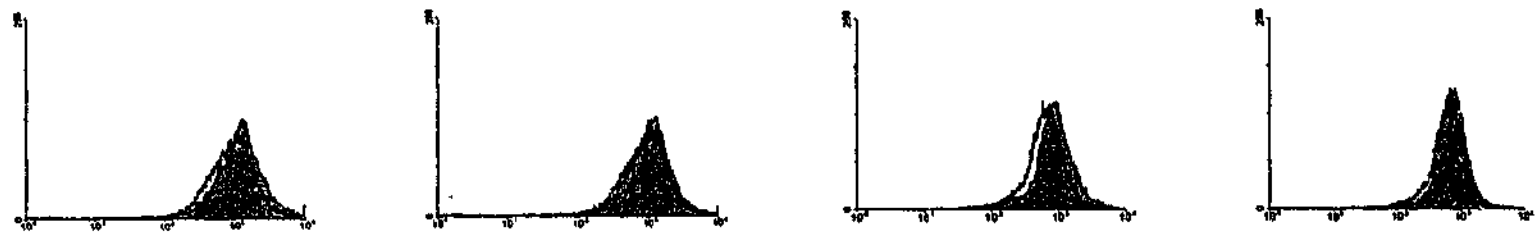
Afferent Lymph

Efferent Lymph

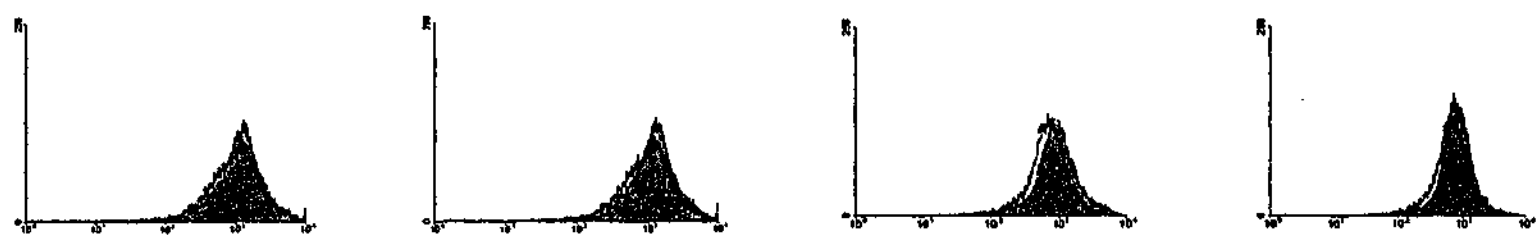
MyD-1



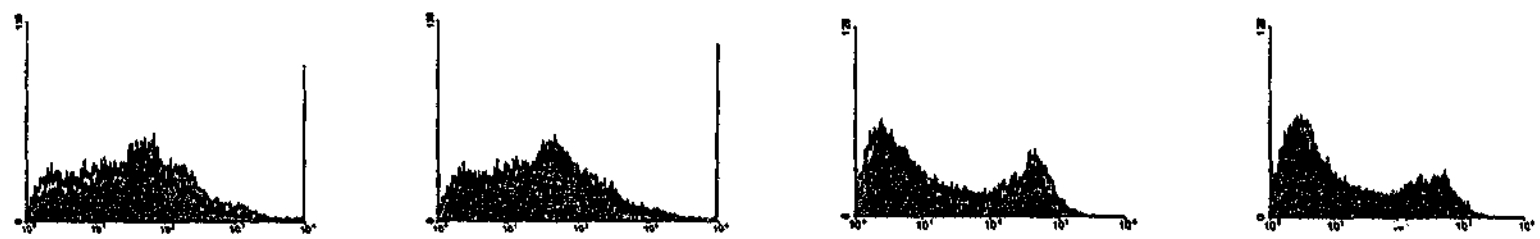
MHC I



MHC II (DP)



MHC II (DQ)



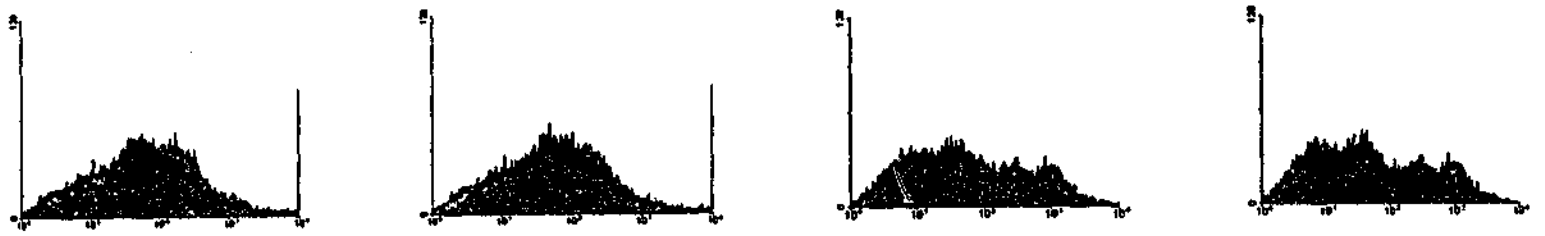
F. hepatica ES | *F. gigantica* ES | *F. hepatica* ES | *F. gigantica* ES

Appendix 1d: The effect of *F. hepatica* ES (250 µg/ml) and *F. gigantica* ES (250 µg/ml) on the expression of sheep cell surface markers MyD-1, MHC I, MHC II (DP) and MHC II (DQ) on either afferent or efferent lymph after a 4 hr incubation. The grey shaded area represents the surface markers expression on sheep cells in the presence of antigen after a 4 hr incubation while the black overlay shows the normal surface marker expression on the identical population of sheep cells incubated for 4 hrs in media alone. Histograms display 20000 events for each sample.

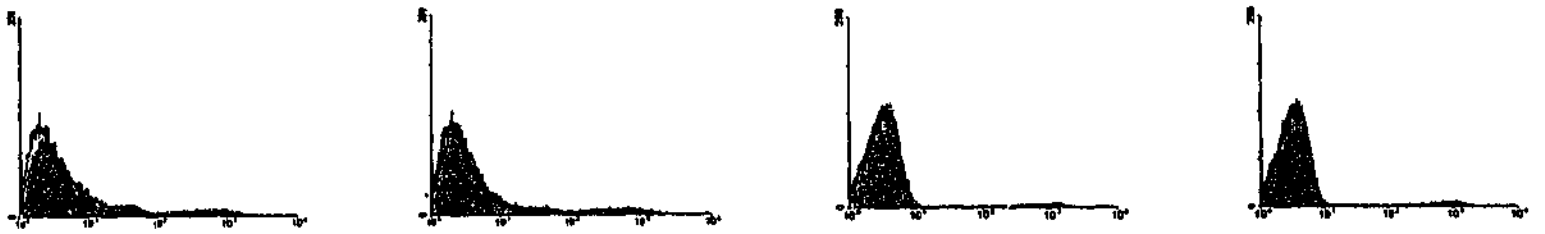
Afferent Lymph

Efferent Lymph

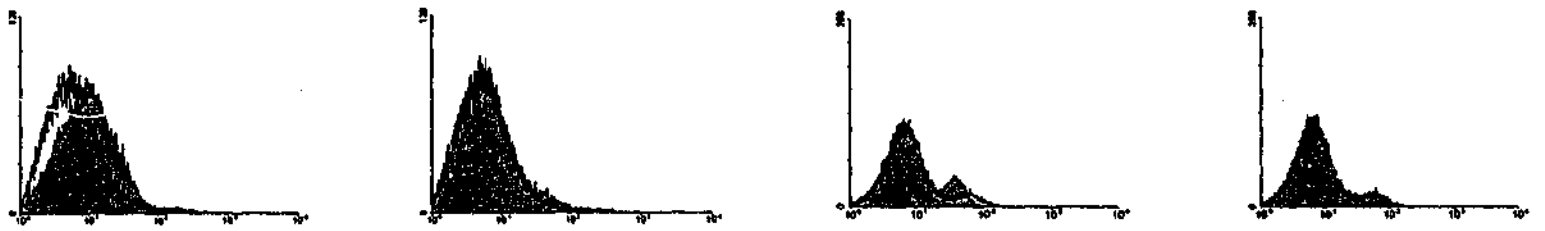
MHC II (DR)



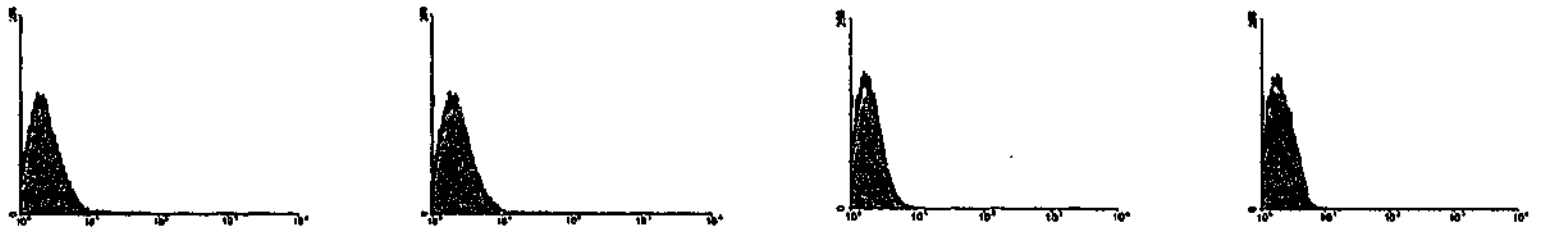
WC1



WC6



WC10



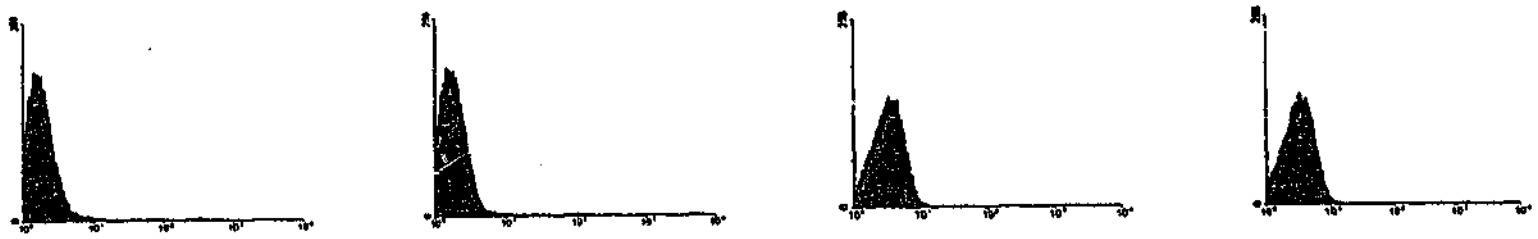
F. hepatica ES | *F. gigantica* ES | *F. hepatica* ES | *F. gigantica* ES

Appendix 1e: The effect of *F. hepatica* ES (250 µg/ml) and *F. gigantica* ES (250 µg/ml) on the expression of sheep cell surface markers MHC II (DR), WC1, WC6 and WC10 on either afferent or efferent lymph after a 4 hr incubation. The grey shaded area represents the surface markers expression on sheep cells in the presence of antigen after a 4 hr incubation while the black overlay shows the normal surface marker expression on the identical population of sheep cells incubated for 4 hrs in media alone. Histograms display 20000 events for each sample.

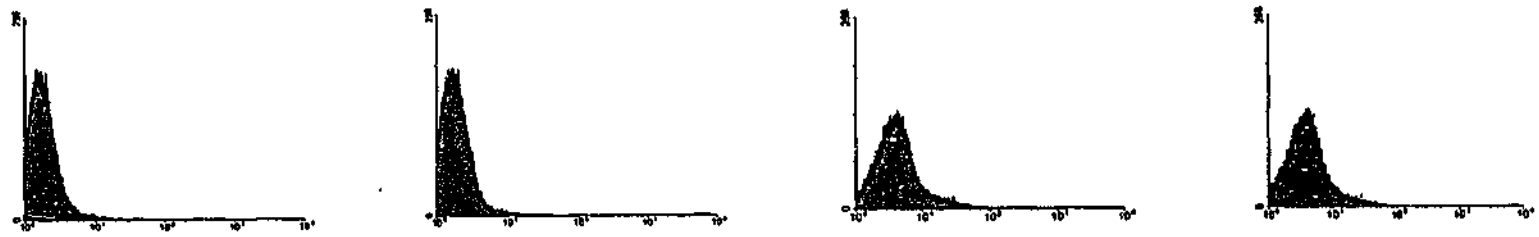
Afferent Lymph

Efferent Lymph

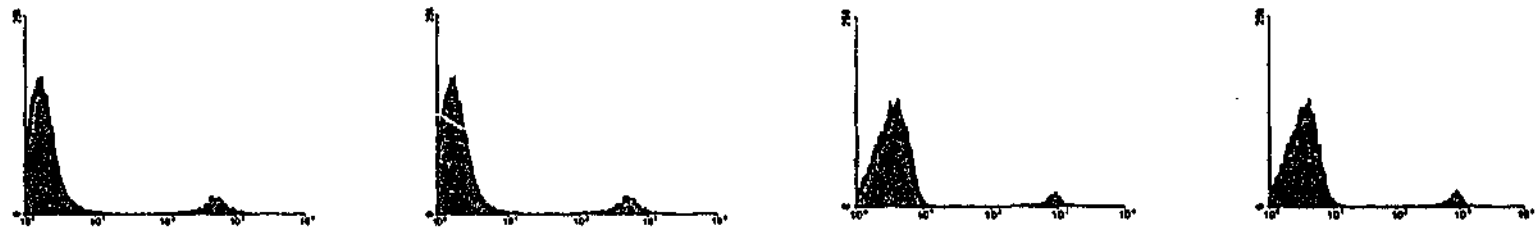
cc81



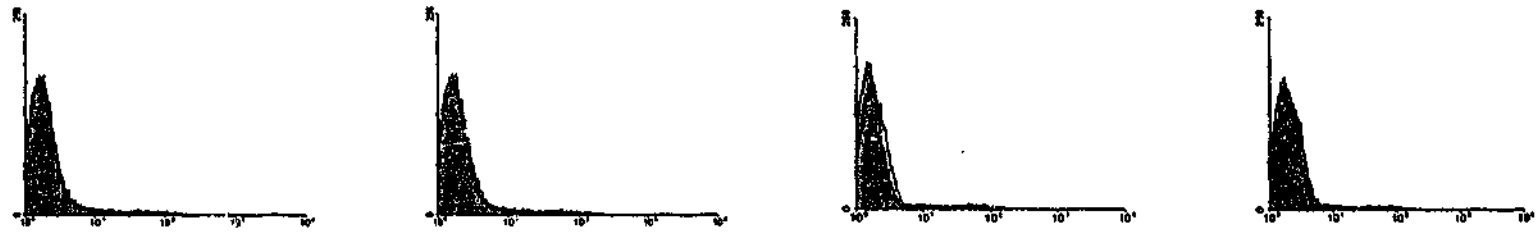
sIgG



TcR1N7



λδ TCR



F. hepatica ES | *F. gigantica* ES | *F. hepatica* ES | *F. gigantica* ES

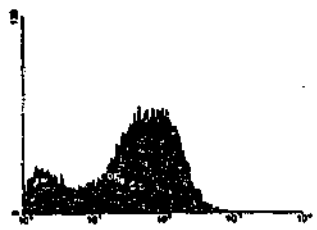
Appendix 1f: The effect of *F. hepatica* ES (250 µg/ml) and *F. gigantica* ES (250 µg/ml) on the expression of sheep cell surface markers cc81, sIgG, TcR1N7 and λδ TCR on either afferent or efferent lymph after a 4 hr incubation. The grey shaded area represents the surface markers expression on sheep cells in the presence of antigen after a 4 hr incubation while the black overlay shows the normal surface marker expression on the identical population of sheep cells incubated for 4 hrs in media alone. Histograms display 20000 events for each sample.

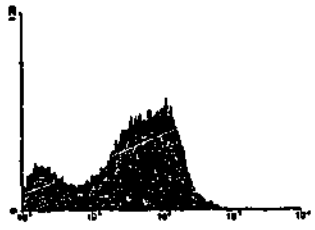
Afferent Lymph

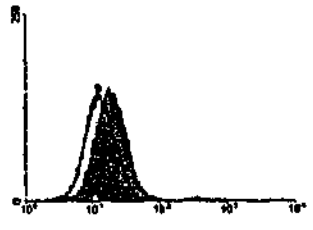
Efferent Lymph

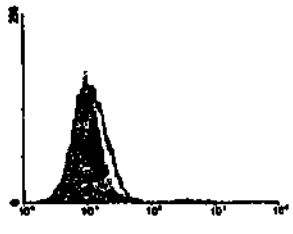
VLA4

CD58









F. hepatica ES

F. gigantica ES

F. hepatica ES

F. gigantica ES

Appendix 1g: The effect of *F. hepatica* ES (250 µg/ml) and *F. gigantica* ES (250 µg/ml) on the expression of sheep cell surface markers VLA4 and CD58 on either afferent or efferent lymph after a 4 hr incubation. The grey shaded area represents the surface markers expression on sheep cells in the presence of antigen after a 4 hr incubation while the black overlay shows the normal surface marker expression on the identical population of sheep cells incubated for 4 hrs in media alone. Histograms display 20000 events for each sample.

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Fasciola hepatica cathepsin L suppresses sheep lymphocyte proliferation *in vitro* and modulates surface CD4 expression on human and ovine T cells

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SUMMARY

Fasciola hepatica *infection has been shown to suppress sheep lymphocyte proliferation* in vitro *and this is at least partially attributable to excretory/secretory products (ES) released by* F. hepatica *parasites. We identified a suppressive component in ES by analysing the effect of ES fractions, separated by gel filtration, on the proliferation of sheep T cells* in vitro. *A major proportion of the suppressive activity in ES was shown to coelute with the cathepsin L proteases: E64, a cysteine protease inhibitor, blocked the suppressive activity of cathepsin L. In order to identify possible mechanisms by which cathepsin L could suppress T cell proliferation, the effect of ES and* F. hepatica *recombinant cathepsin L (rFhCatL) on the expression of 22 different sheep T cell surface markers was analysed by flow cytometry. Incubation of sheep T cells with ES or two rFhCatL signicantly reduced surface CD4 expression and this effect was prevented in the presence of E64. In similar experiments with human T lymphocytes, ES and rFhCatL were shown to down regulate surface CD4 expression. These results show that* F. hepatica *cathepsin L both suppresses sheep T cell proliferation and reduces surface CD4 expression on both human and ovine T cells.*

Keywords Fasciola hepatica, *cathepsin L, cysteine protease, T cells, CD4, sheep, human, suppression, FACS*

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INTRODUCTION

Fasciola hepatica, the temperate liver fluke, is capable of attenuating the mammalian immune system (1). In sheep, *F. hepatica* infections exert immunosuppressive effects at specific times postinfection (2). Following infection of sheep, significant suppression of the lymphocyte proliferative response to different mitogens was observed at week 4 and around weeks 7–11 (2). Selective suppression of the host's immune response may be to the parasite's advantage since these times correspond to phases of rapid parasite growth within the liver parenchyma and establishment within the bile ducts (2).

Immunomodulatory effects of excretory/secretory products (ES) released by the adult parasite have been observed *in vitro*. These ES related activities include dose-dependent inhibition of mitogen-stimulated proliferation of lymphocytes from naïve sheep (3); induction of mononuclear spleen cell populations which, upon adoptive transfer, suppress delayed type hypersensitivity responses to parasite antigen (4); inhibition of the superoxide burst by phorbol myristate acetate-stimulated sheep and human neutrophils (5); and inhibition of nitric oxide production by lipopolysaccharide-stimulated rat peritoneal cells in the first 2 weeks following infection (6). However, the active components within *F. hepatica* ES responsible for this immunosuppression have not yet been elucidated.

A major component of *F. hepatica* ES is a group of cathepsin L cysteine proteases which are 27–30 kDa in size (7–9). These proteases are expressed in the intermediate to late phase of parasite development and at least seven different cathepsin L isoenzymes have been identified by two-dimensional gel electrophoresis and by cDNA cloning (8,10); these seven isoenzymes comprise three clades (11) *F. hepatica* cathepsin L has already been shown to initiate a range of effects, including prevention of antibody-mediated attachment of eosinophils to juvenile flukes *in vitro* (12); the formation of

blood clots induced through clotting of fibrinogen (13) and cleavage of the Fc portion of immunoglobulin (14).

The purpose of this study was to identify immunosuppressive molecules in adult *F. hepatica* ES and to study potential suppressive mechanisms utilized by these molecules to down regulate host immune responses. This was achieved by studying the effect of ES components on both lymphocyte proliferation and the expression of cell surface markers on sheep lymphocytes.

MATERIALS AND METHODS

Reagents

Polyethylene glycol 20000, L-cysteine, HCL, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E64), sodium pyruvate, Concanavalin A (Con A), silver nitrate, imidazole, z-Phe-Arg-NHMec, β-mercaptoethanol, trypan blue and dithiothreitol (DTT) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Dextrose, glycerol and calcium chloride were purchased from BDH Chemicals (Kilsyth, Australia). Yeast extract and peptone were purchased from Oxoid Ltd (Basingstoke, Hampshire, UK). Gentamycin and DMEM were purchased from Life Technologies (Rockville, MD, USA). RPMI-PR$^-$, penicillin/streptomycin (Pen/Strep) and Foetal calf serum (FCS) were purchased from Trace Scientific Ltd (Melbourne, Australia). ELISA plates and 96-well U-bottomed plates were purchased from Greiner labourtechnik (Kremsmuenster, Austria). Ficoll paque, ^{3}H-thymidine, Superdex 75 (S75) fast protein liquid chromatography (FPLC) column, Histrap column and the Mono S column were all purchased from Amersham Pharmacia Biotech (Sydney, Australia). Nitrocellulose membrane was purchased from Schleicher and Schuell (Dassel, Germany). Lithium heparin vacutainers were purchased from Becton Dickinson (Oxford, UK).

Monoclonal antibodies

A range of murine monoclonal antibodies (mAb) to sheep leucocyte surface antigens, which had been compared within the International Ruminant Workshops (15), were used to monitor effects elicited by *F. hepatica* ES on leucocytes. These mAbs were: CC20 (IgG2a) CD1b; MUC2A (IgG2a) CD2; GC1A (IgG2a) CD4; CC17 (IgG1) CD5; CC63 (IgG1) CD8α; ILA99 (IgG1) CD11a; MM12A (IgG1) CD11b; BAQ153A (IgM) CD11c; CC-G33 (IgG1) CD14; CC21 (IgG1) CD21; CACT116A (IgG1) CD25; GS5A (IgG1) CD45R; CC32 (IgG1) L-selectin; H58A (IgG2a) MHC I; H42A (IgG2a) MHC II (DP); TH14B (IgG2a) MHC II (DR); TH81A5 (IgG2a) MHC II (DQ); 86D (IgG1) γδ TCR; CC98 (IgG2b) WC6; CC28 (IgG1) WC10; CC81 (IgG1) ALVC subset (16); and ILA24 (IgG1) MyD-1 (17).

Two mAbs to human lymphocytes were used: PE mAb α human CD4 and PE mAb α human CD3 (Pharmingen, San Diego, CA, USA).

ES collection

ES was collected from living adult *F. hepatica* parasites obtained from bovine livers at a meat processing plant in Tongala (Victoria, Australia) as previously described (18). The regurgitant was concentrated 10-fold using polyethylene glycol 20 000 and then dialysed for 24 h at 4°C against 3 l of phosphate-buffered saline (PBS). The protein concentration was estimated using the Bio-Rad D_C Protein Assay Kit (Bio-Rad, Richmond, CA, USA) according to manufacturer's instructions. This ES preparation was used in all assays.

Fractionation of ES

ES molecules were fractionated by chromatography on a S75 FPLC column. 1 ml samples of *F. hepatica* ES were loaded and 1 ml fractions were eluted using sterile PBS (filtered through a 0·22-μm filter) at a flow rate of 0·5 ml/min at room temperature. Collected proteins were estimated to have been diluted approximately three-fold, from the initial *F. hepatica* ES concentration, during fractionation.

Recombinant cathepsin L proteases

Two recombinant *F. hepatica* cathepsin L (rFhCatL) proteases were used: rFhCatL5 and a mutant of rFhCatL5 termed rFhCatL69Y in which the leucine at position 69 was mutated to tyrosine. Their expression, purification and substrate specificity have been previously described (11). The two rFhCatL were active site titrated with the inhibitor E64 using the dipeptide z-Phe-Arg-NHMec as the substrate at 37°C (19). Approximately 10% of the purified recombinant protease preparations were enzymatically active.

Isolation of human and sheep peripheral blood mononuclear cells (PBMCs)

Blood was collected from either naïve humans or mature Merino ewes into 10 ml sterile lithium heparin vacutainers and centrifuged at room temperature for 20 min at 1700 r.p.m. The buffy coat was removed, diluted 1 : 5 in PBS, layered onto 10 ml of Ficoll Paque, then centrifuged at room temperature for 30 min at 2400 r.p.m. The cells at the interface were removed and washed three times at room temperature in PBS by 6 min centrifuge spins at 1300 r.p.m. PBMCs were counted and resuspended at desired concentration in RPMI complete media.

Isolation of shee

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temperature. Cells
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Pen/Strep, 50 μM
pyruvate) and cel

Whole blood pro

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by (21). Blood w
sterile lithium hep
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(FCS), 50 μg/ml
mercaptoethanol
added to each w
either: 10 μg/ml C
A + S75 *F. hepati*
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repeated in the pr
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atmosphere conta
to harvesting, [^{3}H
completion of the
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well microplates (
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cells alone repres
was typically betw
control was cells i
ally less than 200
72 h incubation w
on a control plate
using an ordinary

15% SDS PAGE

F. hepatica ES and
on 15% SDS PAG
(18). Western blot
of *F. hepatica* cath
analyses were perf

Flow cytometry:

Flourescence-activ
to identify the effe

Isolation of sheep efferent lymphocytes

Efferent lymph was collected from the right lymph node of a mature Merino ewe which had been previously cannulated (20). The lymph was centrifuged at 1700 r.p.m. for 7 min at room temperature and then washed once in PBS at room temperature. Cells were resuspended in Dulbecco's Modified Eagle Media (10% FCS, 50 μg/ml gentamycin, 50 U/ml Pen/Strep, 50 μM β-mercaptoethanol and 20 μM sodium pyruvate) and cell counts were performed.

Whole blood proliferation assays

Whole blood proliferation assays were conducted as described by (21). Blood was collected from naïve sheep into 10 ml sterile lithium heparin vacutainers. This blood was diluted 1 : 10 into RPMI complete media (10% foetal calf serum (FCS), 50 μg/ml gentamycin, 50 U/ml Pen/Strep, 50 μM 2-mercaptoethanol and 20 μM sodium pyruvate). 200 μl was added to each well of a 96-well ELISA plate containing either: 10 μg/ml Con A; Con A + adult *F. hepatica* ES; Con A + S75 *F. hepatica* ES fractions; *F. hepatica* ES and PBS. These incubations were performed in quadruplicate and repeated in the presence of E64 (10 μM final concentration). The cultures were incubated for 72 h at 37°C in an atmosphere containing 5% CO_2. Twenty-four hours prior to harvesting, [^{3}H]-thymidine (0·2 μCi/well) was added. At completion of the incubation, the plates were frozen at −20°C. Cells were harvested onto multiscreen filtration system 96 well microplates (Millipore Corp., Bedford, MA, USA) which were analysed for ^{3}H-thymidine activity using a Packard TopCount Microplate Scintillation Counter. Con A with cells alone represented the positive control and proliferation was typically between 12 000 and 20 000 c.p.m. The negative control was cells incubated with PBS and counts were typically less than 200 c.p.m. The viability of the cells after the 72 h incubation was assessed by the exclusion of trypan blue on a control plate. Statistics were performed on all assays using an ordinary ANOVA parametric Bonferroni test.

15% SDS PAGE gels and Western blot analysis

F. hepatica ES and S75 fractions were qualitatively analysed on 15% SDS PAGE gels stained with silver stain as described (18). Western blot analysis was used to identify the presence of *F. hepatica* cathepsin L as previously described (18). These analyses were performed twice.

Flow cytometry: sheep lymphocyte surface markers

Flourescence-activated cell sorter (FACS) analysis was used to identify the effect of *F. hepatica* ES or rFhCatL on a broad range of surface markers on both sheep PBMCs and efferent lymphocytes. 5×10^5 cells were plated into each well (100 μl) of a 96-well U-bottomed plate. Plates were centrifuged at 1200 r.p.m. for 3 min at room temperature, the supernatant was decanted and cells were resuspended in medium alone or medium containing *F. hepatica* ES (200–250 μg/ml) or enzymatically active rFhCatL (20–30 μg/ml), with and without E64 (10 μM). These cultures were incubated for 5 h at 37°C in an atmosphere containing 5% CO_2. Cells were washed three times in PBS at room temperature then incubated with the appropriate mouse anti-sheep surface marker antibody 1 : 200 to 1 : 1000 dilution, supplied by CSIRO (Division of Animal Health) for 15 min at room temperature. Controls were cells with medium alone and cells incubated with mouse immunoglobulin (Ig) isotypes IgG1, IgG2a, IgG2b and IgM. Non-specific binding of these mouse antibodies to T cells was not observed (data not shown). Cells were washed twice in PBS at room temperature and then incubated with goat anti-mouse Ig antibody with a FITC tag (1 : 200 dilution, Silenus, Melbourne, Australia) at room temperature for 15 min. Cells were washed twice in PBS at room temperature, resuspended in 100 μl PBS and analysed using a Becton Dickinson FACSsort. Results were based on 20 000 events. In the experiment investigating CD4 expression levels on sheep efferent lymph T lymphocytes after various incubations with *F. hepatica* ES, this method was modified as follows: Cells were plated out as above and then fixed in 1% paraformaldehyde for 15 min at room temperature. The cells were washed three times in PBS at room temperature and the assay performed as usual using incubations with either media or *F. hepatica* ES, with and without E64 (10 μM). In another experiment, cells were plated out and incubated with either media or *F. hepatica* ES for 5 h and then washed as above. These cells were then incubated for a further 18 h at 37°C in media alone, washed twice in PBS and then stained as described above. FACS analysis was performed on human PBMCs using the same method described above, using antibodies to human CD4 and CD3. All experiments were performed at least twice and each sample was performed in duplicate. Statistics (using statistical measurements within Cell Quest from Becton Dickinson) were performed on all cell populations. The data was presented in either histogram format or bar graphs which displayed the mean fluorescent intensity (*x* geometric mean) of the $CD4^+$ T cell populations.

RESULTS

Identification of *F. hepatica* ES components that suppress lymphocyte proliferation

The suppressive effect of *F. hepatica* ES on the Con A-induced proliferation of sheep whole blood cells *in vitro* was

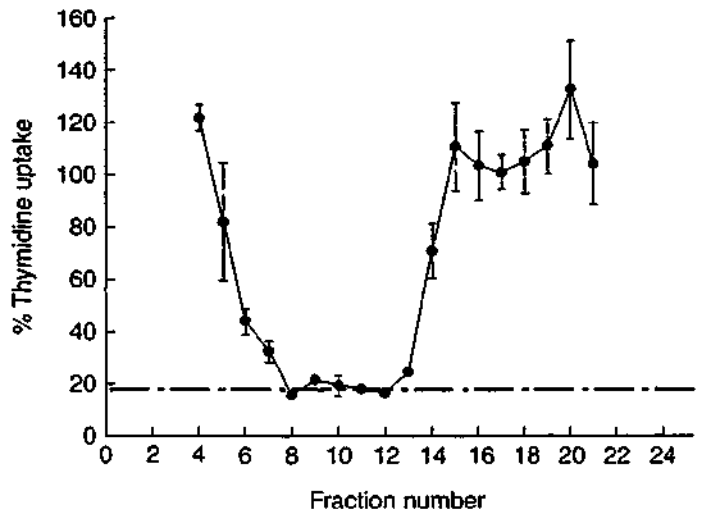

Figure 1 Suppressive effect of *F. hepatica* ES fractions on sheep lymphocyte proliferation *in vitro*. *F. hepatica* ES was fractionated on a Superdex 75 column. 20 µl of individual fractions was analysed for suppressive activity on Con A-induced proliferation. Significant suppression was observed for fractions 6–13 ($P < 0{\cdot}005$ for each fraction). A significant stimulation was observed for fraction 20 ($P < 0{\cdot}05$). The control (Cells + Con A) proliferation value is set at 100%. The dashed line shows the level of Con A stimulation in the presence of whole *F. hepatica* ES at 50 µg/ml. This experiment was performed twice with samples assayed in quadruplicate.

analysed as reported previously (3). Significant levels of suppression were observed *in vitro* at 15 µg/ml and 50 µg/ml of *F. hepatica* ES, compared to cells treated with Con A alone (data not shown). These results indicate a role for *F. hepatica* ES in suppressing lymphocyte proliferation responses and correlate with earlier data (3). Incubation of cells with *F. hepatica* ES without Con A at corresponding concentrations did not induce naïve sheep cellular proliferation above that of the PBS negative control (results not shown). Cell viability as estimated by Trypan Blue exclusion was not affected at these concentrations of *F. hepatica* ES (data not shown).

In order to identify the suppressive components in *F. hepatica* ES, ES was fractionated by molecular sieving on a S75 column and the individual fractions were analysed for their effect on Con A-induced proliferation of sheep cells. Significant suppression of proliferation was observed for fractions 6–13, at a level of suppression comparable to that seen with the whole unfractionated *F. hepatica* ES (50 µg/ml) (Figure 1). Fraction 20 appeared to induce cellular proliferation. This fraction may contain a 'mitogenic' component which acts to neutralize/offset some of the suppressive nature of *F. hepatica* ES. This fraction was not studied further in these experiments.

The fractions (7–11) which induced peak suppression were analysed by SDS PAGE and compared with whole *F. hepatica* ES and fractions 4 and 14 where minimal suppression was

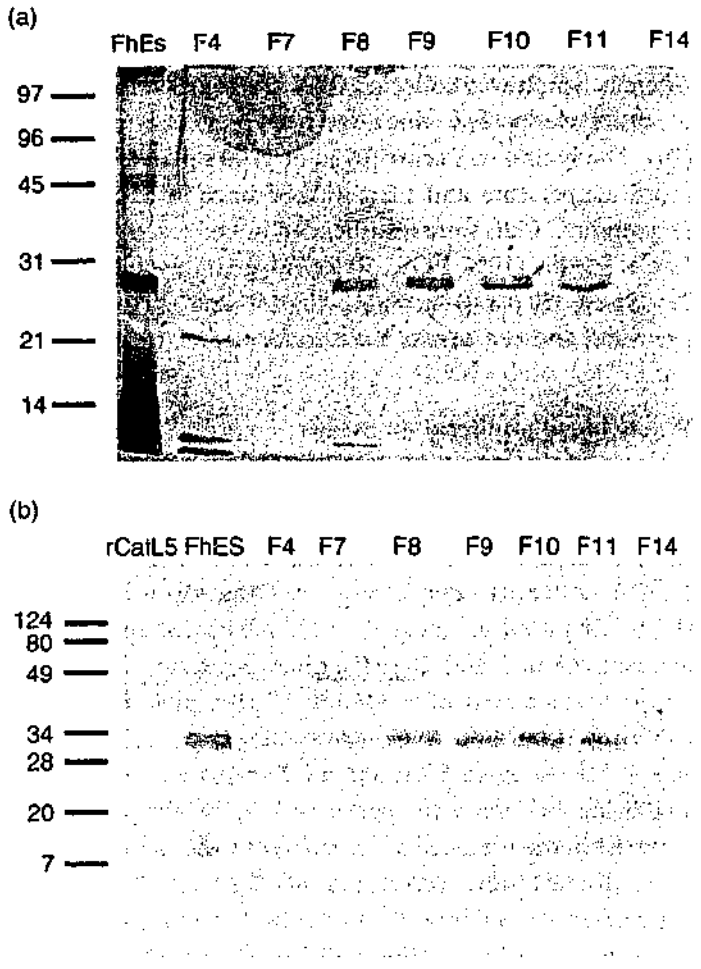

Figure 2 (a) Silver-stained 15% SDS PAGE gel analysing *F. hepatica* ES and *F. hepatica* Superdex 75 fractions. *F. hepatica* ES (10 µl) and fractions 4–14 (30 µl) were fractionated on SDS PAGE gels. The migration of the standards is shown on the left. Similar results were obtained in two separate experiments. (b) Western blot analysis of *F. hepatica* ES and *F. hepatica* ES Superdex 75 fractions. *F. hepatica* ES (10 µl), rFhCatL5 (20 µl) and fractions 4–14 (30 µl) were fractionated by SDS PAGE, blotted and probed with sheep anti-*F. hepatica* Cathepsin L antibody. The migration of the standards is shown on the left. Similar results were obtained in two separate experiments.

observed (Figure 2a). From the gel, a major component in the suppressive fractions is the 28 kDa doublet known to represent cathepsin L (8). Suppressive fractions 8–11 consisted predominantly of this cathepsin L doublet, although there were also other lower molecular weight bands present. These lesser constituents include breakdown products of cathepsin L, since cathepsin L is known to self cleave into components of 14 kDa, and other proteins which coeluted with cathepsin L (8,18).

Western blot analysis of the suppressive fractions using sheep anti-*F. hepatica* cathepsin L antibody was performed to confirm the identity of cathepsin L in the fractions. This analysis identified the 28 kDa doublet as cathepsin L (Figure 2b). Two positive controls were included for the

identification of the presence of cathepsin L: unfractionated *F. hepatica* ES, in which only the characteristic 28 kDa cathepsin L doublet was identified and rFhCatL5 which was detected in its pro form (46 kDa, Figure 2b). The proform of native cathepsin L was also detected in fractions 7 and 8. These results demonstrate that the major component of the ES fractions, which induce suppression of lymphocyte proliferation, is cathepsin L.

To confirm that suppression of lymphocyte proliferation was due to the protease activity of *F. hepatica* cathepsin L, the whole blood proliferation assay was repeated with S75 fractions in the presence of the inhibitor, E64 (10 μM). E64 is a specific cysteine protease inhibitor which will reverse any suppressive effects caused by the activity of cysteine proteases. Previous studies have shown that 92% inhibition of *F. hepatica* cysteine protease activity is observed with E64 at a concentration of 0·5 μM (8). The concentration chosen in our experiments should therefore inhibit all enzyme activity. Incubation of whole *F. hepatica* ES with E64 restored proliferation by approximately 50% (Figure 3). This may suggest that there are at least two components in *F. hepatica* ES responsible for suppressing proliferative responses, one of these being a cysteine protease that contributes approximately one-half of the total suppressive activity. Incubation of cells with fractions 7–12 in the presence of E64 completely restored their proliferation (Figure 3). This confirmed that it was the cysteine protease activity within fractions 7–12 that is responsible for inducing suppression of Con A-induced proliferation. An additional control for the experiment, in which whole blood cells were incubated with Con A + E64 in the absence of *F. hepatica* ES, showed that E64 had no effect on the Con A-induced proliferation (Figure 3).

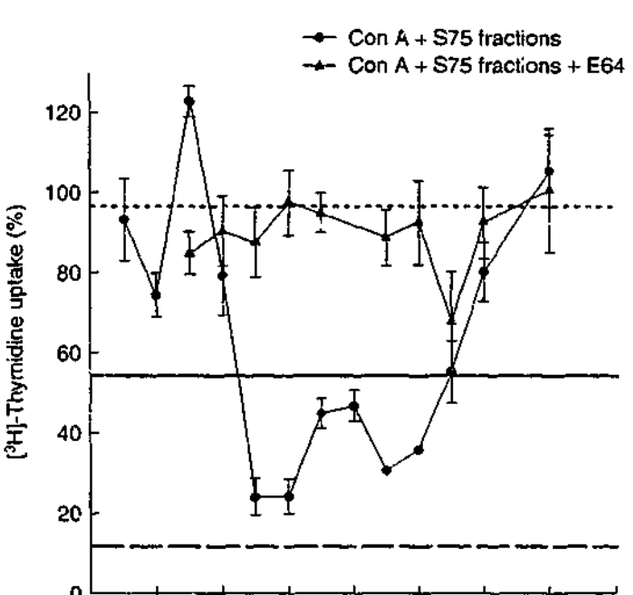

Figure 3 E64 reverses the suppressive effect of *F. hepatica* ES fractions on sheep lymphocyte proliferation *in vitro*. Addition of E64, a cysteine protease inhibitor, at 10 μM to either whole *F. hepatica* ES or the fractions resulted in 50% and 100% restoration of the Con A-induced proliferation, respectively. The control (Cells + Con A) proliferation value is set at 100%. The dashed line shows the level of proliferation in the presence of whole *F. hepatica* ES; the solid line shows the level of proliferation in the presence of whole *F. hepatica* ES plus E64. The dotted line shows the control level of proliferation in the presence of Con A and E64. Significant suppression was observed for fractions 7–13 ($P < 0{\cdot}001$). Significant stimulation was observed for fraction 5 ($P < 0{\cdot}01$). The S75 fractions analysed here were collected from a separate S75 fractionation experiment to that shown in Figure 1. Similar results were obtained in two separate experiments with samples assayed in quadruplicate.

Effect of *F. hepatica* ES on expression of sheep T cell surface markers

The fact that suppression of proliferation of whole blood cells was associated with cathepsin L protease activity suggested that suppression may be mediated by cleavage of proteins on the surface of T cells. In order to characterize possible protein targets on cells that could be substrates for cathepsin L, the effect of *F. hepatica* ES on the expression of a range of surface markers on sheep cells was analysed. Cell surface markers investigated were CD1b, CD2, CD4, CD5, CD8α, CD11a, CD11b, CD11c, CD14, CD21, CD25, CD45R, L-selectin, MHC I, MHC II (DP), MHC II (DQ), MHC II (DR), γδ TCR, WC6, WC10, ALVC subset and MyD-1. Cell populations used for these initial experiments included PBMCs and efferent lymph collected from cannulated sheep (20). When naïve sheep cells were incubated with *F. hepatica* ES at the optimal concentration (250 μg/ml, results not shown), we observed a significant change, measured as a major shift in the positive peak in the presence of *F. hepatica* ES, in the expression of the CD4 marker on T lymphocytes in all cell populations (see below). As CD4 was the marker of interest, the results presented below focus on cells from efferent lymph which contains 95–100% lymphocytes.

Effect of *F. hepatica* ES and recombinant cathepsin L on expression of CD4 on sheep T cells

The effect of *F. hepatica* ES and cathepsin L proteases on expression of CD4 on sheep T cells was studied in detail using efferent lymph consisting of 95–100% lymphocytes (35–40% of these being $CD4^+$ T lymphocytes. Viability of cells was not effected by incubation with ES or recombinant cathepsin L proteases as determined by propidium iodide staining (data not shown). Approximately 40% of the efferent

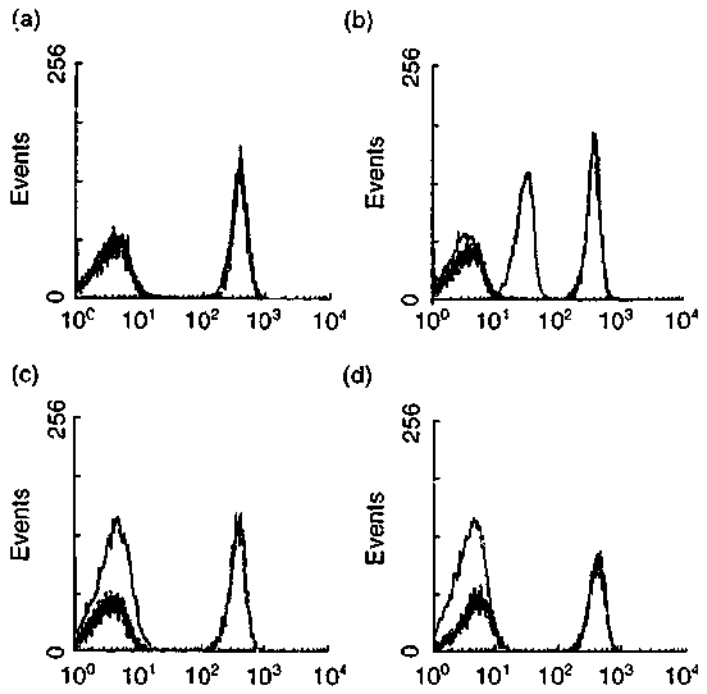

Figure 4 Effect of *F. hepatica* ES and recombinant cathepsin L on the expression of the CD4 surface marker on sheep efferent lymph T cells. CD4 expression on control T cells is shown (a, shaded area). CD4 expression was substantially reduced on T cells preincubated with *F. hepatica* ES (250 μg/ml) for 5 h before staining (b, shaded area). Expression of CD4 on sheep T cells was ablated after a 5-h incubation with either rFhCatL5 (30 μg/ml) (c, shaded area) or rFhCatL69Y (30 μg/ml) (d, shaded area). The black overlay on each plot shows the CD4 expression on T cells for the same incubation performed in the presence of E64 (10 μM). Data in each plot represent 20 000 events for cells stained with mouse anti-sheep CD4 and FITC anti-mouse immunoglobulin. Histograms show the fluorescent intensity (*x*-axis) versus cell number (*y*-axis). Similar results were obtained in three separate experiments with samples assayed in duplicate.

lymph cells used in this work were $CD4^+$ T cells (Figure 4a). The average mean fluorescent intensity (MFI) of this entire cell population using anti-sheep CD4 antibodies was 230 (Figure 4a). Cells were incubated with *F. hepatica* ES (50–200 μg/ml) for 5 h at 37°C and then stained for CD4 expression. A 5-h incubation with 200 μg/ml ES had been determined as the optimal conditions for a significant shift in the CD4 profile in earlier experiments (data not shown). A marked shift in the $CD4^+$ T cell population to the left was observed, with a resulting MFI value of 22 (Figure 4b). This significant level of reduction in fluorescence signifies a down regulation or cleavage of CD4 from the surface of sheep T cells.

To further characterize the role of cysteine proteases in CD4 cleavage, the effect of rFhCatL proteases on $CD4^+$ T cells was analysed. rFhCatL was used at a concentration of 30 μg/ml of enzymatically active protease. Both rFhCatL5 and rFhCatL69Y totally down-regulated/cleaved CD4 from the surface of T lymphocytes (Figure 4c,d) and this effect was completely reversed by the addition of E64 (Figure 4c,d, overlay).

This is the first demonstration that *F. hepatica* cathepsin L proteases have a direct affect on CD4 expression levels. We therefore tested whether this alteration was caused by the direct cleavage of CD4 from the surface of sheep T cells or an indirect down-regulation of CD4 expression by cathepsin L.

To ascertain whether CD4 was being down-regulated or cleaved, sheep T cells were fixed with 1% paraformaldehyde and then incubated with *F. hepatica* ES. If CD4 was being actively down-regulated in the presence of *F. hepatica* ES by an indirect effect of ES on some other T cell component, there would be little change of CD4 expression on the fixed T cells. However, if CD4 was being directly cleaved from the surface by protease activity in ES, a reduction in CD4 expression on T cells would still occur on fixed cells. The results show a notable shift in the $CD4^+$ T cell population for fixed cells in the presence of *F. hepatica* ES (Figure 5c). These observations imply that CD4 is cleaved from the surface of T cells by protease activity in *F. hepatica* ES. After fixation, the shift in the $CD4^+$ T cell population resulting from incubation with *F. hepatica* ES was not as great as that for unfixed cells (Figure 5b) but this may be due to an alteration of the CD4 structure on the cell surface during the fixing process, such that the active components in *F. hepatica* were not capable of recognizing CD4 to the same extent as on unfixed cells, thereby limiting the degree of cleavage. The addition of E64 to *F. hepatica* ES prior to either incubation with unfixed or fixed cells resulted in normal expression levels of CD4 on lymphocytes (Figure 5b,c). This again implicates the action of a cysteine protease in *F. hepatica* ES.

In order to determine whether T cells were capable of up-regulating CD4 expression levels following exposure to *F. hepatica* ES, sheep T cells were incubated with *F. hepatica* ES for 5 h, washed to remove ES components, and then incubated for a further 18 h in medium alone. This cell population exhibited a higher proportion of $CD4^+$ T cells (Figure 5d) than cells incubated with *F. hepatica* ES for 5 h (Figure 5b). Relative levels of CD4 expression were estimated by comparison of MFI values (101 for Figure 5d and 16·4 for Figure 5a, respectively, Table 1). These results suggest that CD4 expression can be up-regulated on sheep T cells when *F. hepatica* ES is removed from the system, although the level of CD4 expression did not return to control MFI levels (216, Table 1) within the 18 h incubation. Again, incubation of cells with *F. hepatica* ES in the presence of E64 completely prevented the reduction in CD4 expression levels on sheep T cells, confirming that this effect is a direct result of cathepsin L protease activity present in *F. hepatica* ES (Figure 5b and Table 1).

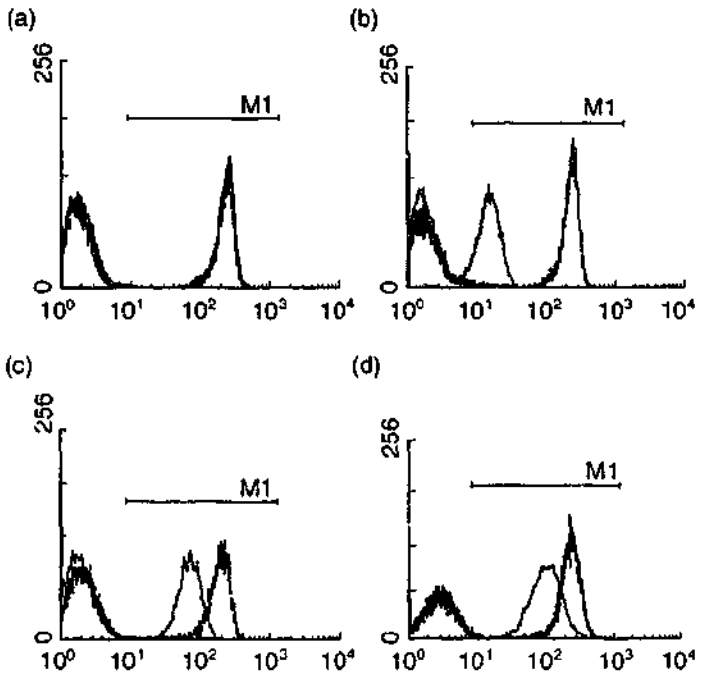

Figure 5 CD4 expression levels on sheep efferent lymph T lymphocytes after various incubations with *F. hepatica* ES at 250 μg/ml. Cells were incubated as follows: media alone (a, shaded area); *F. hepatica* ES for 5 h (b, shaded area); cells were firstly fixed with 1% paraformaldehyde and then incubated with *F. hepatica* ES for 5 h (c, shaded area); or were incubated with *F. hepatica* ES for 5 h, washed three times and then incubated for 18 h in media alone (d, shaded area). The black overlay on each plot represent the same incubations as forementioned with the addition of E64 (10 μM) prior to the incubation. Data in each plot represent 20 000 events for cells stained with mouse anti-sheep CD4 and FITC anti-mouse immunoglobulin. Histograms show the fluorescent intensity (*x*-axis) versus cell number (*y*-axis). Similar results were obtained in two separate experiments with samples assayed in duplicate.

Effect of *F. hepatica* ES and recombinant *F. hepatica* Cathepsin L on expression of CD4 on human T cells

F. hepatica infects a range of mammalian hosts and it is therefore of interest to determine whether this effect of *F. hepatica* cathepsin L was specific for sheep T cells or whether a similar effect of these proteases would be observed with T cells from other hosts. For this purpose, the effects of *F. hepatica* ES and recombinant cathepsin Ls on CD4 expression on human T lymphocytes was examined.

CD4 expression on three different sources of human purified blood lymphocytes was approximately 44% (Figure 6a). CD4 expression on human T lymphocytes was totally suppressed after incubation in the presence of *F. hepatica* ES (200 μg/ml) (Figure 6b), rFhCatL5 (30 μg/ml) (Figure 6c) or rFhCatL69Y (20 μg/ml) (Figure 6d). When E64 was added to the proteases prior to incubation with the cells this suppression was eliminated (Figure 6b–d, overlays). Control experiments were performed looking at the effect of *F. hepatica* on CD3 expression levels on human T lymphocytes. No effect was detected, suggesting that the proteases are selectively targeting CD4 (data not shown). This is the first report of a cysteine protease cleaving CD4 from either human or ovine T cells.

DISCUSSION

We have identified macromolecules within *F. hepatica* ES capable of suppressing mitogen-induced sheep whole blood cellular (lymphocyte) proliferation *in vitro*. ES was fractionated and the suppressive activity in ES was shown to correlate with the presence of cathepsin L proteases in the proliferation assay and identification of the characteristic cathepsin L doublet on SDS PAGE gel and Western blot. Furthermore, the addition of E64 (a cysteine protease inhibitor) to the suppressive fractions ablated their suppressive activity, confirming the involvement of a cysteine protease activity in mediating suppression. The addition of E64 to whole *F. hepatica* ES resulted in approximately 50% inhibition of its suppressive activity, suggesting the involvement of another molecule within ES capable of suppressing whole blood cellular proliferation. It was also observed that some S75 fractions contained 'mitogenic' properties with respect to whole blood cellular proliferation, and the characterization of these components would be of interest. Together, these results identified cathepsin L proteases as major immunomodulatory molecules released by *F. hepatica*.

Table 1 CD4 expression levels on sheep efferent lymph T lymphocytes after exposure to ES of *F hepatica*

Sample	Events gated	% Gated	Mean fluorescent intensity	SD
Media	9400	47·0	216·4	3·2
Media + E64	9236	46·2	215·3	2·7
F. hepatica ES	8714	43·6	16·4	1·6
F. hepatica ES + E64	10154	50·8	206·1	4·5
Fixed – *F. hepatica* ES	8997	45·0	67·7	2·2
Fixed – *F. hepatica* ES + E64	9110	45·6	177·3	3·6
F. hepatica ES for 5 h, media O/N	12064	60·3	101·5	2·7
F. hepatica ES + E64 for 5 h, media O/N	11618	58·1	220·30	4·1

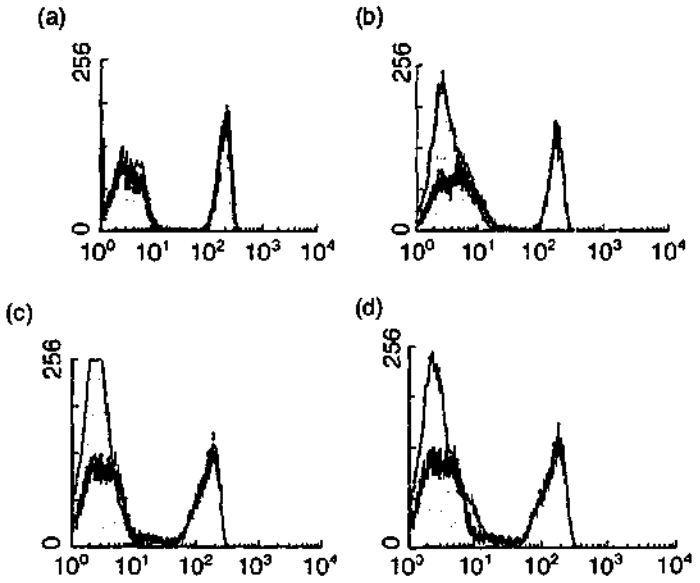

Figure 6 Effect of *F. hepatica* ES and recombinant cathepsin L on the expression of the CD4 surface marker on human PBMCs. CD4 expression on control T cells is shown (a, shaded area). CD4 expression was substantially reduced on human PBMCs preincubated with *F. hepatica* ES (200 μg/ml) for 5 h before staining (b, shaded area). Expression of CD4 on human T lymphocytes was ablated after a 5-h incubation with either rFhCatL5 (30 μg/ml) (c, shaded area) or rFhCatL69Y (20 μg/ml) (d, shaded area). The black overlay on each plot shows the CD4 expression on T cells for the same incubation performed in the presence of E64 (10 μM). Data in each plot represent 20 000 events for cells stained with a PE mouse anti-human CD4 immunoglobulin. Histograms show the fluorescent intensity (*x*-axis) versus cell number (*y*-axis). This experiment was performed five times in total (using PBMCs from three different volunteers) with duplicate incubations.

We then attempted to identify the mode of action for cathepsin L in suppressing cellular proliferation by studying the interaction of *F. hepatica* ES and *F. hepatica* cathepsin L with a wide range of surface markers on sheep cells. It was hypothesized that such interactions may be involved in initiating suppression of cellular proliferation. *F. hepatica* ES was shown to significantly down regulate the level of CD4 expression on T lymphocytes. Experiments with fixed cells confirmed that CD4 was cleaved from the surface of sheep T cells and that CD4 expression could at least partially be restored once *F. hepatica* ES was removed from the system. The addition of E64 to the incubations with *F. hepatica* ES completely ablated the suppressive effect on CD4 expression levels, implying that cysteine protease activity in *F. hepatica* ES is totally responsible for the forementioned effects. To extend these findings, two recombinant cathepsin L proteases that show different substrate specificity (11) were incubated with sheep T lymphocytes and, in both cases, CD4 expression was totally down-regulated. This effect was also inhibited in the presence of E64. These results show that *F. hepatica* cathepsin L selectively removes CD4 from the surface of sheep T lymphocytes. It was also shown that *F. hepatica* cathepsin L down-regulated CD4 from the surface of human T cells, suggesting that this effect of cathepsin L is not specific to ovine CD4.

We propose that this may be a major mechanism by which *F. hepatica* can mediate immunomodulation of host T cell responses. CD4 is a 65-kDa surface marker on T cells and current research suggests that CD4 acts as a coreceptor along with the TCR : CD3 complex in antigenic recognition of MHC II (22,23). This coreceptor function of CD4 potentiates signalling by 10–100-fold, greatly reducing the density of ligand required for T cell activation (24–26). The bivalent interaction of CD4 with the TCR : CD3 complex has been shown to be necessary for T helper cell activation and development (22,23). Considering the importance of CD4 in regulating host T cell activation, it is likely that cathepsin L-induced cleavage of CD4 may be one of the mechanisms utilized by *F. hepatica* to suppress lymphocyte proliferation in sheep contributing to evasion of the host's immune system and establishment of infection, but further studies are required to establish this. The fact that cleavage of CD4 by these fluke cathepsins also occurs with human T cells suggests that this may be a general mechanism used by this parasite to establish infection and further work will determine this by examining the effects of cathepsin L on proliferation of human T cells. Infection of mice with *F. hepatica* has been shown to suppress Th1-like responses and to reverse the Th1 response to the *Bordetella pertussis* vaccine (27,28). Whether these effects of fluke infection are linked to the down-regulation of CD4 levels on murine T cells is yet to be determined. Future work will focus on characterization/identification of the proposed site(s) of cleavage on human recombinant CD4 and determining whether *F. hepatica* cathepsin L also affects $CD4^+$ T cells in other hosts such as cattle and rats.

While this is the first example of a cysteine protease regulating CD4 expression levels on ovine T cells, two metalloproteases have been shown to exhibit similar effects. The major surface glycoprotein (gp63) from the protozoa *Leishmania major* and *L. donovani* was also shown to selectively cleave CD4 from human T cells (29). Similarly, the bacterial *Legionella pneumophilia* protease was shown to degrade interleukin-2 and cleave CD4 from human T cells (30). Both of these reports suggest that the cleavage of CD4 may be the method utilized by both *Leishmania* sp. and *Legionella* sp. to impede T cell activation, resulting in disease progression.

The importance of $CD4^+$ T cells in regulating helminth infections has been well established in several hosts. For example, genetic resistance of lambs to *Haemonchus contortus* could be abrogated after $CD4^+$ T cell depletion (31). In mice, the $CD4^+$ T cell subset was demonstrated, through depletion studies, to mediate expulsion of *Trichinella spiralis*

from the gut (32). In *Heligmosomoides polygyrus* challenge infections, mice are capable of inducing a protective response, which significantly reduces development of new adult worms by 70–80%. However, when mice were administered an anti-CD4 antibody, this exquisite resistance was reversed (33). In primary infections with *F. hepatica*, increased lymphocyte proliferation attributable to $CD4^+$ T cells has been observed in cattle (34,35). In direct contrast, the more susceptible sheep host shows diminished T lymphocyte proliferation during infection with *F. hepatica* (2). This is unlikely to be the only factor governing acquired resistance to fasciolosis as rats also exhibit a decrease in $CD4^+$ lymphocytes levels during a primary infection (36). Further work is aimed at examining the effect of *F. hepatica* cathepsin L on cattle $CD4^+$ T cells. It now appears likely that $CD4^+$ T cells will be crucial players in host immunity to *F. hepatica* as has been shown for other helminth infections.

To date, at least seven cathepsin L isoenzymes have been identified on SDS PAGE gels or by cDNA cloning (8,10,11). Analysis of cDNA sequences for cathepsin L identifies three clades and these can be separated into at least two functional groups based on their substrate specificity at the P_2 position (11). rFhCatL5 belongs to group 1 which prefers a phenylalanine at P2 and rFhCatL69Y belongs to group 2 which prefers a proline at P2. The results presented here suggest that down-regulation of CD4 may be characteristic of enzymes from at least two of the clades. Further work is needed to confirm a role for all cathepsin L isoenzymes in regulating CD4 expression on sheep T cells.

The importance of cathepsin L isoenzymes in liver fluke biology has been demonstrated in vaccine trials in ruminants. Cathepsin L molecules have shown efficacy as vaccines in cattle, sheep and rats (1,18,37–41). Protection in cattle with cathepsin L vaccines against a challenge infection has varied from 38% to 69%. The greatest protection (72%) was observed for a combination vaccine which included the fluke derived antigens cathepsin L2 and fluke haemoglobin (37,39). Protection in cattle appears to correlate with induction of IgG2 antibodies to cathepsin L (39). These results suggest that IgG2 antibodies against cathepsin L induce partial protection but the mechanism of protection is unknown. Anti-cathepsin L antibodies have been shown to neutralize cathepsin L activity *in vitro*, which prevents antibody-mediated attachment of eosinophils to the parasite (42). Cathepsin L has been shown to cleave the Fc region of human IgG at specific points (14) and a role for cathepsin L in blocking antibody-dependent cell mediated killing of *F. hepatica* is feasible since, in rats, killing of juvenile *F. hepatica in vitro* has been shown to be mediated by antibodies and macrophages (43). Future work will involve analysing the effect of vaccine-induced anticathepsin L antibodies on down-regulation of CD4 levels on sheep T cells.

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REFERENCES

1 Mulcahy G, O'Connor F, Clery D *et al.* Immune responses of cattle to experimental anti-*Fasciola hepatica* vaccines. *Res Vet Sci* 1999; **67**: 27–33.
2 Zimmerman GL, Kerkvliet NI, Brauner JA, Cerro JE. Modulation of host immune responses by *Fasciola hepatica*: responses of peripheral lymphocytes to mitogens during liver fluke infections of sheep. *J Parasitol* 1983; **69**: 473–477.
3 Jefferies JR, Barrett J, Turner RJ. Immunomodulation of sheep and human lymphocytes by *Fasciola hepatica* excretory-secretory products. *Int J Parasitol* 1996; **26**: 1119–1121.
4 Cervi L, Rubinstein H, Masih DT. Involvement of excretion-secretion products from *Fasciola hepatica* inducing suppression of the cellular immune responses. *Vet Parasitol* 1996; **61**: 97–111.
5 Jefferies JR, Turner RJ, Barrett J. Effect of *Fasciola hepatica* excretory-secretory products on the metabolic burst of sheep and human neutrophils. *Int J Parasitol* 1997; **27**: 1025–1029.
6 Cervi L, Rossi G, Cejas H, Masih DT. *Fasciola hepatica*-induced immune suppression of spleen mononuclear cell proliferation: role of nitric oxide. *Clin Immunol Immunopathol* 1998; **87**: 145–154.
7 Smith AM, Dowd AJ, McGonigle S *et al.* Purification of a cathepsin L-like proteinase secreted by adult *Fasciola hepatica*. *Mol Biochem Parasitol* 1993; **62**: 1–8.
8 Wijffels GL, Panaccio M, Salvatore L, Wilson L, Walker ID, Spithill TW. The secreted cathepsin L-like proteinases of the trematode, *Fasciola hepatica*, contain 3-hydroxyproline residues. *Biochem J* 1994; **299**: 781–790.
9 Dowd AJ, Smith AM, McGonigle S, Dalton JP. Purification and characterisation of a second cathepsin L proteinase secreted by the parasitic trematode *Fasciola hepatica*. *Eur J Biochem* 1994; **223**: 91–98.
10 Tort J, Brindley PJ, Knox D, Wolfe KH, Dalton JP. Proteinases and associated genes of parasitic helminths. *Adv Parasitol* 1999; **43**: 161–266.
11 Smooker PM, Whisstock J, Irving J, Siyaguna S, Spithill TW, Pike RN. A single amino acid substitution affects substrate specificity in cysteine proteinases from *Fasciola hepatica*. *Protein Sci* 2000; **9**: 2567–2572.
12 Carmona C, Dowd AJ, Smith AM, Dalton JP. Cathepsin L proteinase secreted by *Fasciola hepatica in vitro* prevents antibody-mediated eosinophil attachment to newly excysted juveniles. *Mol Biochem Parasitol* 1993; **62**: 9–17.
13 Dowd AJ, McGonigle S, Dalton JP. *Fasciola hepatica* cathepsin L proteinase cleaves fibrinogen and produces a novel type of fibrin clot. *Eur J Biochem* 1995; **15**: 241–246.
14 Berasain P, Carmona C, Frangione B, Dalton JP, Goni F. *Fasciola hepatica*: parasite-secreted proteinases degrade all human IgG subclasses: determination of the specific cleavage sites and identification of the immunoglobulin fragments produced. *Exp Parasitol* 2000; **94**: 99–110.

15 Larson R, Monaghan M, Park Y, Hamilton M, Ellis J, Davis W. Identification and characterisation of monoclonal antibodies reactive with bovine, caprine and ovine T-lymphocyte determinants by flow microfluorimetry. *Vet Immunol Immunopathol* 1990; **25**: 195–208.
16 Howard CJ, Sopp P, Brownlie J, Kwong LS, Parsons KR, Taylor G. Identification of two distinct populations of dendritic cells in afferent lymph that vary in their ability to stimulate T cells. *J Immunol* 1997; **159**: 5372–5382.
17 Brooke GP, Parsons KR, Howard CJ. Cloning of two members of the SIRP alpha family of protein tyrosine phosphatase binding proteins in cattle that are expressed on monocytes and a subpopulation of dendritic cells and which mediate binding to CD4 T cells. *Eur J Immunol* 1998; **28**: 1–11.
18 Wijffels GL, Salvatore L, Dosen M *et al.* Vaccination of sheep with purified cysteine proteinases of *Fasciola hepatica* decreases worm fecundity. *Exp Parasitol* 1994; **78**: 132–148.
19 Barrett AJ, Kirschke H. Cathepsin B, Cathepsin H and Cathepsin L. *Meth Enzymol* 1981; **80**: 535–561.
20 Hall JG, Morris B. The output of cells in lymph from the popliteal node of sheep. *Quart J Exp Physiol* 1962; **47**: 360–369.
21 Clery D, Torgerson P, Mulcahy G. Immune responses of chronically infected adult cattle to *Fasciola hepatica*. *Vet Parasitol* 1996; **62**: 71–82.
22 Janeway CA. The role of CD4 in T-cell activation: accessory molecule or co-receptor? *Immunol Today* 1989; **10**: 234–238.
23 Janeway CA, Bottomly K. Signals and signs for lymphocyte responses. *Cell* 1994; **76**: 275–285.
24 Marrack P, Endres R, Shimonkevitz R *et al.* The major histocompatibility complex-restricted antigen receptor on T cells. II. Role of the L3T4 product. *J Exp Med* 1983; **158**: 1077–1091.
25 Hampl J, Chien YH, Davis MM. CD4 augments the response of a T cell to agonist but not to antagonist ligands. *Immunity* 1997; **7**: 379–385.
26 Madrenas J, Chau LA, Smith J, Bluestone JA, Germain RN. The efficiency of CD4 recruitment to ligand-engaged TCR controls the agonist/partial agonist properties of peptide-MHC molecule ligands. *J Exp Med* 1997; **185**: 219–229.
27 Brady MT, O'Neill SM, Dalton JP, Mills KH. *Fasciola hepatica* suppresses a protective Th1 response against *Bordetella pertussis*. *Infect Immun* 1999; **67**: 5372–5378.
28 O'Neill SM, Brady MT, Callanan JJ *et al.* *Fasciola hepatica* infection down regulates Th1 responses in mice. *Parasite Immunol* 2000; **22**: 147–155.
29 Hey AS, Theander TG, Hviid L, Hazrati SM, Kemp M, Kharazmi A. The major surface glycoprotein (gp63) from *Leishmania major* and *Leishmania donovani* cleaves CD4 molecules on human T cells. *J Immunol* 1994; **152**: 4542–4548.
30 Mintz CS, Miller RD, Gutgsell NS, Malek T. *Legionella pneumphila* protease inactivates interleukin-2 and cleaves CD4 on human T cells. *Infect Immun* 1993; **61**: 3416–3421.
31 Gill HS, Watson DL, Brandon MR. Monoclonal antibody to $CD4^+$ T cells abrogates genetic resistance to *Haemonchus contortus* in sheep. *Immunology* 1993; **78**: 43–49.
32 Grencis RK, Riedlinger J, Wakelin D. L3T4 positive lymphoblasts are responsible for transfer of immunity to *Trichinella spiralis* in mice. *Immunology* 1985; **56**: 213–218.
33 Urban JF, Katona IM, Finkelman FD. *Heligosomoides polygyrus*: $CD4^+$ but not $CD8^+$ T cells regulate the IgE response and protective immunity in mice. *Exp Parasitol* 1991; **73**: 500–511.
34 McCole DF, Doherty ML, Baird AW, Davies WC, McGill K, Torgerson PR. T cell subset involvement in immune responses to *Fasciola hepatica* infection in cattle. *Parasite Immunol* 1999; **21**: 1–8.
35 Brown WC, Davis WC, Dobbelaere DA, Rice-Ficht AC. $CD4^+$ T-cell clones obtained from cattle chronically infected with *Fasciola hepatica* and specific for adult worm antigen express both unrestricted and Th2 cytokine profiles. *Infect Immun* 1994; **62**: 818–827.
36 Poitou I, Baeza E, Boulard C. Kinetic responses of parasite specific antibody isotypes, blood leucocyte pattern and lymphocyte subsets in rats during primary infestation with *Fasciola hepatica*. *Vet Parasitol* 1993; **49**: 179–190.
37 Dalton JP, McGonigle S, Rolph TP, Andrews SJ. Induction of protective immunity in cattle against infection with *Fasciola hepatica* by vaccination with cathepsin L proteinases and with hemoglobin. *Infect Immun* 1996; **64**: 5066–5074.
38 Piacenza L, Acosta D, Basmadjian I, Dalton JP, Carmona C. Vaccination with cathepsin L proteinases and with leucine aminopeptidase induces high levels of protection against fascioliasis in sheep. *Infect Immun* 1999; **67**: 1954–1961.
39 Mulcahy G, O'Connor F, McGonigle S *et al.* Correlation of specific antibody titre and avidity with protection in cattle immunised against *Fasciola hepatica*. *Vaccine* 1998; **16**: 932–939.
40 Kofta W, Mieszczanek J, Pluciennicczak G, Wedrychowicz H. Successful DNA immunisation of rats against fasciolosis. *Vaccine* 2000; **18**: 2985–2990.
41 Spithill TW, Smooker PM, Sexton JL *et al.* Development of vaccines against *Fasciola hepatica*. In *Fasciolosis*, ed. Dalton JP. Oxon: CABI Publishing; 1999: 377–410.
42 Smith AM, Carmona C, Dowd AJ, McGonigle S, Acosta D, Dalton JP. Neutralization of the activity of a *Fasciola hepatica* cathepsin L proteinase by anti-cathepsin L antibodies. *Parasite Immunol* 1994; **16**: 325–328.
43 Piedrafita D, Parsons JC, Sandeman RM, *et al.* Antibody-dependent cell-mediated cytotoxicity to newly excysted juvenile *Fasciola hepatica in vitro* is mediated by reactive nitrogen intermediates. *Parasite Immunol* 2001; **23**: 473–482.

