Fasciolosis: Immunodiagnosis and Immunology

Thesis presented for the degree of

DOCTOR OF PHILOSOPHY

by

Sandra M. O' Neill, B.Sc., RGN.

under the supervision of

John P. Dalton, Ph.D.

School of Biotechnology

Dublin City University

March 1999.

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my own work.

Signed: Sandra O' Neill Date: 26.2.99

Sandra O' Neill

To James and Mary

Acknowledgements

I wish to thank my supervisor Dr. John Dalton for giving me the opportunity to pursue my Ph.D. and particularly for all your patience, help, guidance and enthusiasm throughout the course of this work.

I would also like to thank all those people involved in the immunodiagnostic project; Dr. Wilma Strauss and Dr. Rene Angles for analysing the coprological samples and for making my trip to South America so enjoyable. In addition, I would like to acknowledge Dr. Jose Esteban and Dr. S. Mas-Coma for their collaboration on this project. I would particularly like to thank our statistician Dr. Michael Parkinson for all his hard work on the statistical analysis of this data.

To all the members past and present in JDs laboratory, Andrew, Bernie, Ciaran, Eamon, Jacinta, Paul, Pepe and Leda, thanks for all the support and for the famous laboratory outings. Thanks a million, Sharon for all the pleasant hours passed in O' Flaherty's pub and to Bernie for all your support and understanding particularly over the last few months.

I would like to thank Dr. Kingston Mills for giving me the opportunity to perform the immunological experiments in his laboratory and for your invaluable expertise and assistance over the past year. I would like to especially thank all the members of his lab., Aengus, Bernie, Christine, Fiona, Geraldine, Helen, Liz, Sheila, Patricia, Peter and Mags, for making me feel so welcome and for all your help. I would particularly like to thank Miriam for being such a great laboratory partner. Your assistance in the co-infection and vaccine experiments was greatly appreciated.

Finally I would like to thank all my friends and family for their support and encouragement. This PhD thesis is dedicated to the O' Neill Clann, James, Mary, Paul, Louise, Deirdre, James and Jennifer.

Contents

Abstract

Using enzyme linked immunosorbant assays (ELISA) we compared the suitability of liver fluke homogenate (LFH), excretory/secretory products (ES) and cathepsin L1 as a diagnostic agent for the serodiagnosis of human fasciolosis in the Bolivian Altiplano. Cathepsin L1 (CL1) proved significantly better at the discriminating between seropositive and seronegative *Fasciola hepatica* infected individuals when compared to the other antigen preparations. The sensitivity of the CL1-ELISA was further improved by using secondary antibodies directed against IgG4 only. This IgG4/CL1-ELISA was highly specific because sera obtained from individuals infected with other parasites were negative in the assay.

We also employed recombinant CL1 as antigen in this ELISA. A statistically significant correlation ($r^2 = 0.751$; $p<0.001$) was observed between recombinant and native proteins making the standardisation of this assay possible. The difficulty of obtaining blood from the indigenous population prompted us to explore the method of collecting samples of blood onto filter paper after lancet-pricking of the finger (blood-filter samples). Statistical analysis revealed that there was significant correlation between the results obtained with serum and blood-filter samples ($r^2 = 0.848$; $p<0.001$). The collective serological data revealed that 44.3% of the 360 individuals in the Bolivian Altiplano tested were diagnosed as serologically positive. In addition, our data shows that fasciolosis has been endemic in this region for at least a decade.

Fasciola hepatica infection in mice was associated with a type II immune response which results in a generalised Th-subset imbalance polarising towards a Th2 cytokine profile. An established type I immune response in mice vaccinated with *B. pertussis* whole cell vaccine was significantly down-regulated, in an IL-4 dependent process, when these mice were subsequently infected with *F. hepatica*. We also demonstrated that intravenously administered *F. hepatica* ES products could induce the down-regulation of the type I immune response in these vaccinated mice. In addition, mice injected intra-peritoneally with ES exhibit a predominant Th2 cytokine profile. Peritoneal exudate cells secrete IL-10 *in vitro* when stimulated with ES suggesting that this cytokine may be the involved in the initial stages of inducing a type II response.

Abbreviations

ABTS	2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
BCA	Bicinchoninic acid
Bisacrylamide	N,N-Methylene bisacrylamide
BP	*B. pertussis* antigen
BSA	Bovine serum albumin
CL1	Cathepsin L1
DTT	Dithiothreitol
ELISA	Enzyme linked immunosorbant assay
ES	Excretory / Secretory products
FCS	Foetal calf serum
IFNγ	Interferon-gamma
IL	Interleukin
LFH	Liver fluke homogenate
PAGE	Polyacrylamide gel eletrophoresis
PBMC	Peripheral blood mononuclear cells
PMA	Phorbal,12-myristate,13-acetate
PBS	Phosphate buffered saline
pNpp	p-nitrophenyl phosphate
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulphate
Temed	N,N,N',N'-tetramethylethylenediamine
Th	T helper cell
TNFβ	Tumour necrosis factor-beta
Tris	Tris [hydroxymethyl]-aminomethane
Tween 20	Polyoxymethylenesorbitan monolaurate

Chapter 1

Introduction

1.1 General introduction

Fasciola hepatica, an obligate, endoparasitic trematode is the causative agent of fasciolosis, or liver fluke disease. The disease has a worldwide distribution but is located primarily in the temperate zones (Europe, the Americas and Asia) and the most common definitive hosts are sheep and cattle. In Ireland alone over 30 million pounds are lost annually by the agricultural community as a result of the disease. Infection in livestock results in weight loss, an increase in mortality (particularly in sheep) and a reduction in reproduction rates. In addition, a decrease in milk yield is found in dairy cattle and reduced wool production in sheep.

The disease in humans has been reviewed in several publications (Maurice, 1994; Boray, 1981; Dawes and Hughes, 1970; Facey and Marsden, 1960). In particular an extensive review by Chen and Mott (1990), reported a total of 2594 cases from 42 countries over a period of 20 years. However, the incidence of the disease is believed to be much higher than that documented, particularly if one considers the number of cases misdiagnosed due to asymptomatic cases or incorrect diagnosis. In the review by Chen and Mott (1990) only 16 cases are documented in Iran compared to recent publications which estimate infection in some 30,000 individuals (Bahar *et al.*, 1990; Massound, 1990; Pourtaghva *et al.*, 1990). The number of cases reported does not therefore truly reflect the incidence of the disease in this region. In addition no reference has been made to Bolivia where a prevalence of 53% has been reported by Hillyer *et al.* (1992) in the Corapata community in the northwest Altiplano and an estimated 100,000 people are at risk of contracting liver fluke disease The morbidity and mortality in this region has yet to be assessed.

1.2 Life cycle of *Fasciola hepatica*

The life cycle of *Fasciola hepatica* is both cyclical and indirect, requiring a invertebrate intermediate host, the snail *Lymnaea truncatula*, and a definitive mammalian host (Fig. 1). The monoecious adult lives in the biliary tracts where it feeds on the lining of the bilary ducts. It is here that the sexually mature adult lays eggs which are carried by bile into the intestine. Although flukes are hermaphroditic and can self-fertilise, its preferred means of reproduction is by cross fertilisation. Eggs are passed with the faeces and embryonation will only occur in the presence of suitable conditions. Moreover eggs must be liberated from faecal matter as it inhibits embryonation.

In the absence of sufficient water eggs will desiccate rapidly. Precipitation of approximately 0.2mm in 24 hours (6mm per month) is a condition necessary for egg development. Eggs embryonate to form miracidia at temperatures greater than 10^0C and less than 37^0C (9-10 days at 26^0C, 14-17 days at 22^0C and 20 days at 20^0C) (Rowcliffe and Ollerenshaw, 1960). Other important factors which influence embryonation are oxygen tension and pH. Low oxygen tension inhibits embryonation (Rowcliffe and Ollerenshaw, 1960) and pH 7.0 is optimum for egg development (Al-habbib, 1974).

Liberation of miracidia from the egg is coupled with the secretion of a "hatching enzyme" (Rowan, 1956) in the presence of sunlight (Roberts, 1950) and ambient temperatures (Gold and Goldberg, 1976). The free swimming phototropic miracidia must invade the secondary host within 24 hours (Hope Cawdery *et al.*, 1978). It is believed that this adaptation has developed in order that the miracidia have the advantage of always continually migrating to the edge of the water source where

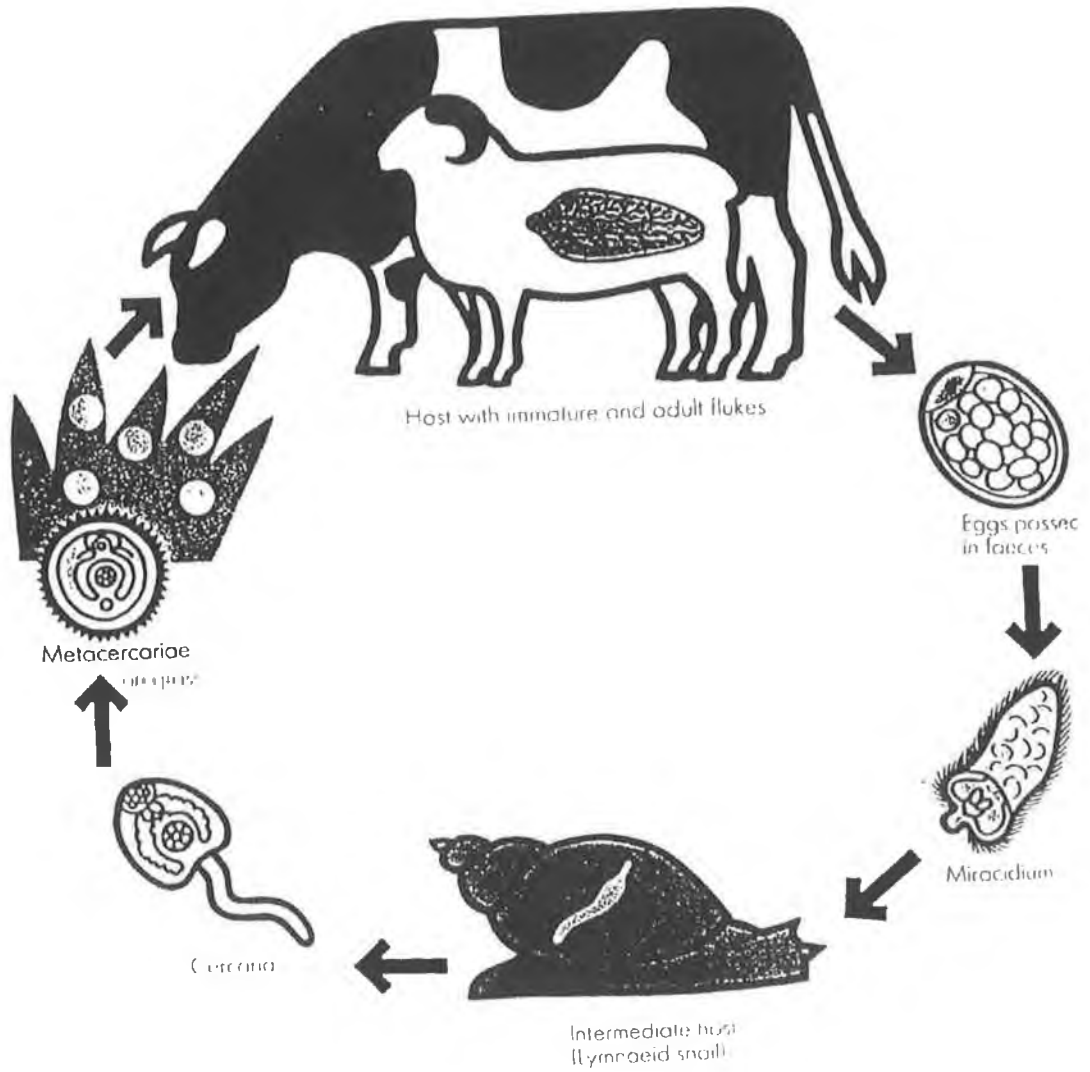


Figure 1. Life cycle of *Fasciola hepatica.*

the amphibious host resides. Once present in the host's habitat it is attracted to the snail by chemotaxsis. Transmission into the intermediate host is active and may require proteolytic enzymes to penetrate the body wall.

On reaching the snail the miracidia attach to the intermediate host by its apical gland. Cilia are cast off after the initial attachment. The final penetration is by a young sporocyst which migrates via the lymph or blood vessels to the digestive gland. In favourable conditions one sporocyst develops into 5-8 first generation rediae. First generation rediae in turn give rise to 40 daughter rediae. Further development occurs and the rediae develop into 600 cercariae which are shed via the birth pore (Andrew, 1998). The development of larval stages in the snail is temperature dependent. At environmental temperatures of 15^0C, 20^0C and 25^0C development takes 82, 40 and 25 days, respectively. The release of cercariae is stimulated by rainfall or a drop in ambient temperature (indicating a water source in which the cercariae can swim). The cercariae are not released at temperatures lower than 9^0C and the snail dies at temperatures greater than 26^0C; temperature is therefore an important limiting factor. In the absence of water, snails can burrow into mud and survive still infected for up to several months. Once in the presence of water and at suitable temperatures the snails will emerge and rapidly shed numerous cercariae (Schmidt and Roberts, 1989).

After the release of cercariae some will encyst at the waters surface but most attach to aquatic plants below the water level. The tail of the cecariae are shed and the cyst wall is secreted within two minutes to two hours to form metacercaria, the infectious stage of the life cycle (Smith and Halton, 1983). Transmission to the definitive host is passive, by the ingestion of aquatic plants or by drinking contaminated water. Survival of metacercaria is also dependent on moisture and temperature (Shaw,

1932) and once encysted on herbage 50% can survive a typical Irish winter (Ollerenshaw, 1967).

Metacecariae excystment is initiated in the stomach of the mammalian host in the presence of high concentrations of carbon dioxide, reducing conditions and high temperatures (39^0C) (Fried, 1994). Liberation of the newly excysed juvenile (NEJ) fluke is stimulated by bile salts secreted in the duodenum (Sukhedo and Mettrick, 1986). The NEJ migrates through the intestinal wall into the peritoneal cavity, subsequently penetrating the gilsons capsule and migrating across the liver parenchyma to the biliary passages. This process takes almost two months in cattle and sheep (Dawes and Hughes, 1964). Once present in the bile duct the worms become sexually mature in four-five weeks and commence producing eggs. Adults can successfully live in the bile ducts of cattle for nine months to several years and up to 11 years in sheep (Pantelouris, 1965; Dan *et al.*, 1981).

1.3 Pathology and clinical manifestation of human fasciolosis

The clinical manifestation of liver fluke disease in man is similar to that exhibited by animals and for ease of reference can be divided into three stages; the invasive or acute stage, the prepatent stage, and the chronic or obstructive stage (Chen and Mott, 1990; Bjorland *et al.*, 1997; Mas-Coma and Bargues, 1997; Arjona *et al.*, 1995). The invasive stage coincides with the migration of the juvenile flukes through the peritoneal cavity and across the liver parenchyma, where they mature on reaching the bile duct. The precise mechanism for reaching the liver is not known but once the liver capsule is penetrated it burrows through the liver parenchyma causing extensive haemorrhaging and inflammation. This stage is associated with the major pathology of the disease, the

extent of which is dependent upon the number of flukes that invade the liver. The symptomology is due to the mechanical destruction of liver tissue and the initial symptoms include fever, sweating, abdominal pain and urticaria. The symptoms may be vague or absent in light infections. In addition, respiratory symptoms have been reported, the most common are bronchial wheeze, dysponea, dry cough and chest pain. Upon physical examination individuals present with hepatomeagly, splenomegaly and ascites. Ascites is yellow with high a leucocyte count and eosinophilia. Anaemia is common in the acute stage and individuals may be pale, dizzy and weak. The duration of this stage is approximately 6-8 weeks. Granuloma formation occurs around trapped flukes in the liver parcenchyma.

The prepatent or latent stage is the period when the mature fluke is laying eggs and the individual is asymptomatic. This stage commences 6-8 weeks after the initial infection. The chronic or obstructive stage occurs after several months to several years. The fluke feeds on the lining of the bile ducts causing hyperplasia and inflammation of the epithelium. Thickening and dilation of the bile ducts and gallbladder ensue. Dilation of the common bile duct is reported to have increased by 2-3 fold (Chen and Mott, 1990). The occurrence of cholangitis or cholecystitis coupled with the presence of flukes may result in mechanical obstruction resulting in an enlarged and oedematous gall bladder. The clinical manifestations include right upper quadrant tenderness, epigastric pain, biliary colic, nausea, fatty food intolerance, jaundice and pruritus. Hepatic enlargement, splenomegaly and ascites are also common during this stage. The liver increases in size during the course of the disease and is more rigid on palpitation than in the acute stage. Splenomegaly occurs in a small number of individuals and ascites is more common in the acute stage. In the chronic stages individuals may appear jaundiced. Several reports of egg granuloma

have been reported but this is not extensive and does not contribute significantly to the pathogenesis. Development of cirrhosis and liver failure was reported in only a small proportion of cases. Ectopic fasciolosis is possible with flukes migrating to organs other than the liver, but is not common (Arjona *et al.*, 1985). The disease is mainly associated with morbidity but some mortalities have been reported. There is no worldwide figures available on morbidity or mortality rates but an estimated 2.5 million individuals are believed to be infected with fasciolosis (Maurice, 1994).

1.4 Treatment of human fasciolosis

There are several drugs that are very effective in the treatment of human fasciolosis; table 2 summarizes the most effective drugs available. However some of these drugs are highly toxic causing serious adverse effects. Emetine is used widely and although its therapeutic effect includes elimination of infection and improved symptoms, it can be highly toxic. Dehydroemetine an analogue to emetine, has similar effects but is less toxic. There have been reported incidences of resistance to both drugs (Chen and Mott, 1990). In addition, they are administered by intermuscular (IM) or subcutaneous (SC) injection for a period of over one week. This is the principal disadvantage of dehydroemetine as self administration would be the preferred means particularly in rural areas such as the Bolivian Altiplano. Bithinol and hetol have a good therapeutic index; that is, they are very effective in the treatment of individuals and exhibit only mild gastrointestinal adverse effects. There have been numerous studies on the efficacious use of both of these drugs in treatment of this disease (Rakhmanov, 1987; Wang *et al.*, 1981; Bacq *et al.*, 1991; Espino *et al.*, 1994.) Metranidozole, a drug more commonly employed in protozoan infections has been

used to treat fasciolosis but its success is dependent upon a high dosage and requires administration over a prolonged period of time. Small doses of the drug are not effective in eliminating an infection (Eckhardt and Heckers, 1981).

Table 2: Drug treatment of Human Fasciolosis.

Drug	Dose Mg/kg	Frequency	Route of Administration	Side effects
Emetine	1	OD/10 days	IM/SC	Toxic effects, Cardiovascular changes, Hypotension
Hetol	50-60	TDS/7 days	Oral	Gastro Intestinal Complaints Dizziness
Bithionol	50	10 days	Oral	Anorexia, Nausea, Vomiting Abdominal Pain
Metronidazole	1.4g	OD/13-28 days	Oral	Nausea, Vomiting, Anorexia
Triclabendazole	10	OD/ 1-2 days	Oral	Fever, Nausea, Liver damage

OD=once daily, TDS= three times daily, IM= Intramuscular, SC= Subcutaneous.

Triclabendazole, a drug more commonly used in veterinary medicine is now prescribed for the treatment of human liver fluke disease. Several publications have reported successful treatment of the infection with only single or double oral doses of the drug (Apt *et al.*, 1995; Laird and Boray, 1992). Toxic side effect are mild with some gastrointestinal adverse effect. Apt *et al.* (1995) reported the treatment of 24 cases of asymptomatic *F. hepatica* infected individuals in Chile with a single oral dose of 10mg/kg of body weight of triclabendazole. Nineteen individuals were coprologically negative after two months while three of the remaining five received a second treatment and subsequently tested coprologically negative within a two month period. Tolerance of the drug was excellent in all cases as there was no alteration in liver function tests post-treatment. All successfully treated individuals were serologically negative within a six month period after treatment. Due to the limited number of human drug trials further investigation into its use in human fasciolosis is required before it can be recommended for therapuesis. Other drugs examined for the

treatment of the disease include chloroquine, niclofolan, albendazole and praziquantel (Price *et al.*, 1993; Laird and Boray, 1992; Chen and Mott, 1990). The use of these drugs are not recommended because of their toxic effect or limited therapeutic value.

1.5 Diagnosis of human fasciolosis

Clinically, human fasciolosis is primarily diagnosed by coprological or serological analysis particularly in large scale epidemiological studies. However, other methods of diagnosis (both invasive and non-invasive) are employed and will be briefly discussed.

Non-invasive and invasive techniques.

The majority of non-invasive diagnostic techniques are expensive and would not be employed in large epidemiological studies, particularly in countries where financial resources are limited. Radiological techniques employed in the diagnosis of fasciolosis include abdominal and chest X-ray (Han *et al.*, 1993), cholangiography (Reidtmann *et al.*, 1995) and endoscopic retrograde cholangiopancreatography (ERCP) (Price *et al.*, 1993; Hauser *et al.*, 1984). Images obtained using these techniques show narrowing of the bile ducts, shadows and tracking in the liver tissue. Radioisotope scanning has also been employed as a useful tool in diagnosis (Han *et al.*, 1993). It illuminates the presence of "cold areas" in the liver tissue where the liver fluke is present. Ultrasound is also useful in diagnosis, but has limited value as individual flukes are not visualized (Abdel *et al.*, 1996). Computed tomography scans have diagnosed the disease by identifying multiple hypodense areas in the liver tissue. It is a particularly useful in monitoring an individual's response to treatment (Kodama *et al.*, 1991; Melero *et al.* 1991).

Invasive techniques for the diagnosis of fasciolosis include liver biopsy, laparoscopy and surgical intervention. Liver biopsy reveals eosinophilic abscess, granulation and charcot-leyden crystals. Eggs are sometimes present in the biopsy tissue (Kodama *et al.*, 1991; Comse *et al.*, 1990). Laparoscopy is performed by placing a scope through the abdominal wall facilitating examination of the abdominal and liver tissue. Lesions suggestive of liver fluke infection can be observed in the form of hepatic nodules of various shapes and sizes. Lesions may also be observed in Gilson's capsule and the peritoneal cavity (Cosme *et al.*, 1990; Croese *et al.* 1982). During exploratory laparatomy eggs may be found in the bilary ducts. Finally, the symptoms of fasciolosis may be mistaken for cholecystitis, cholelthiasis or obstructive jaundice. During a cholecystectomy or choledochostomy, liver flukes or ova may be observed in the biliary ducts or the gall bladder (Angehrn *et al.*, 1975; Moreau *et al.*, 1995). In a case reported by Chen and Mott (1990) over fifteen flukes were drained from the bile ducts during choledochostomy.

Abnormal laboratory findings are extremely useful in the diagnosis of all stages of human fasciolosis. The haematological signs include results of elevated white blood cell counts (greater than 10,000/mm^3) and eosinophilia. An eosinophilia count greater than 5% of total leucocytes is indicative of a helminth infection. Leucocytosis and eosinophilia are usually greater in the acute phase but remain elevated in all stages. Abnormal liver function has been reported in both the acute and chronic stages. In the acute stage patients have elevated glutamic pyruvic transaminase (GPT), glutamic oxalacetic transaminase (GOT) and raised alkaline phosphatase (AKP). Jaundice is prominent in the chronic stage of the disease resulting in high serum bilirubin, in addition to elevated GPT, GOT and AKP (Reidtmann *et al.*, 1995). In all stages of the disease raised immunoglobulins to *F. hepatica* antigens are found. IgM is associated

with early infection (Osman *et al.*, 1995) whereas elevated IgG is observed in all stages of infection (Hillyer *et al.* 1992). Specific IgE is detected in 48% of individuals (Sampaio Silva *et al.*, 1985).

Parasitological examinations.

Parasitological or coprological diagnosis is based upon the identification of *Fasciola hepatica* eggs in a patients stool and is widely employed for the diagnosis of human and animal fasciolosis. *Fasciola hepatica* eggs have a distinctive operculated shape and are 130-150μm in length and 63-90μm in width making it possible to distinguish them from other helminth eggs. Eggs are passed through the faeces only when flukes are mature which is approximately 2-3 months after initial infection. Coprological analysis is a rapid, reproducible and inexpensive means of diagnosis. However, there are several inherent flaws. Since eggs are released 2-3 months after infection, diagnosis of acute infection is impossible. Early diagnosis is imperative because it is at this stage that most of the pathogenicity of the disease occurs. In addition, eggs may be passed through the faeces from ingestion of raw or under cooked liver from infected livestock. Several stool samples are required on different times on different days as the release of eggs is sporadic.

There are numerous techniques to diagnose the presence of eggs in stool samples. However, the sensitivity of coprological analysis is dependent on the technique employed and its specificity is heavily dependent on the experience of the technician performing the technique. The most rudimentary technique is the simple direct smear, an insensitive but rapid means of diagnosis (Blagg *et al.*, 1955). Egg concentration techniques include the sedimentation technique, the egg flotation technique (Stork *et al.*, 1973) and the Kato-Katz or cellophane faecal thick-smear

technique (Katz *et al.*, 1972). These latter methods are generally more accurate and sensitive. A comparative study was performed by Munoz *et al.* (1987) comparing three different coprologically analysis. The techniques employed were the Fast Sedimentation Method (FSM), the teleman centrifugation technique (TM) and the gravity sedimentation technique (GS). Using TM only 33% of individuals were diagnosed correctly compared to 100% for the remaining two techniques. Several studies have been performed reporting different sensitivities for different techniques. The most widely used is the Kato-Katz technique and rapid sedimentation technique. The latter is highly sensitive but is expensive and laborious. The former is rapid, reproducible and economically viable making it suitable for large epidemiological surveys.

Immunological examinations.

Immunological techniques are invaluable in the diagnosis of human fasciolosis particularly since the diagnosis of acute infection is possible. Moreover, most immunological techniques are inexpensive, rapid and sensitive. Immunological techniques exploit one of two immunological phenomena, a hypersensitivity reaction to antigen (skin test) or the formation of an antigen-antibody immune complex. The latter involves the detection of circulating liver fluke antigen, circulating immune complexes or the detection of specific antibodies to liver fluke antigen.

Skin tests were one of the first immunological tests to diagnose human fasciolosis. The antigen utilised was prepared from crude somatic liver fluke antigen. Although this method is simple and cheap it is not highly sensitive and therefore has limited use in diagnosis (Stork. *et al.*, 1973; Smithers, 1982). The detection of circulating antigens (Ambroise-Thomas *et al.*, 1980) or circulating immune complexes

(CIC) (Sampaio Silva *et al.*, 1985) has been utilised to diagnose fasciolosis but only incorporated as part of an experimental design in animal and human studies. Low sensitivity means it can only be employed in conjunction with other techniques to confirm diagnosis (Knobloch, 1985; Sampaio Silva *et al.* 1981.).

One of the earliest immunological methods is the immunufluorescence assay (IFA) (Stork *et al.*, 1973; Wessely *et al.*, 1987). A cross section of liver fluke tissue is mounted on a slide and probed with serum. While it is a sensitive assay it is not very specific as cross reactivity has been observed with other helminth infections particularly schistosomiasis, ascariasis and filariasis. In addition, this method is cumbersome and impractical for use in large surveys. Other techniques for diagnosis of human fasciolosis include indirect haemataggIutination test (IHA), double diffusion (Garcia-Rodriguez *et al.*, 1985), complement fixation (CF)((Stork *et al.*, 1973; Wessely *et al.*, 1987) and counter electrophoresis (CEP) (Hillyer, 1975). Enzyme linked immunosorbent assay (ELISA) and enzyme linked immuno-electrotransfer blot (ETIB) have been found to be the most sensitive and specific methods of diagnosis (Hillyer *et al.,* 1992). However the ETIB method is cumbersome, time consuming and only allows the diagnosis of a limited number of patients at any one time. The degree of sensitivity of all these assays varies. Knobloch (1985) performed a study on 45 human subjects parasitologically diagnosed for liver fluke disease. Their sensitivities for ELISA, IHA and circulating antigen where 98%, 56% and 20%, respectively.

1.6 *Fasciola hepatica* cathepsin L proteinases

Proteases are a group of enzymes that cleave peptide bonds at specific locations in a polypeptide. They are a diverse group of enzymes that are involved in many

proteolytic processes important in cellular function. Exopeptidases, cleave amino acids from either the amino (aminopeptidases, dipeptidases) or carboxyl terminus of proteins (carboxypeptidases) while endoproteinases, cleave internal peptide bonds. The International Union of Biochemistry classifies proteases according to their physical structure, chemical properties, active site and biological properties. Accordingly proteases are divided into four main classes, namely serine proteinases, aspartic proteinases, metalloproteinases and cysteine proteinases.

The cysteine class of proteases include the plant proteinase papain, interleukin converting enzyme (ICE) and several mammalian lysosomal cathepsins (Cathepsin L, B, H and S). Catalysis proceeds via a thiol ester intermediate and is dependent upon an active cysteine residue site in the presence of reducing agents to stabilize or enhance its activity. The cathepsin L group of proteases are powerful lysosomal glycoproteins that can cleave extracellular matrix proteins such as collagen and elastin. In addition, they can cleave other biologically important proteins such as insulin B-chain, histones and haemoglobin. They are similar to that of papain and are believed to share a common evolutionary origin with this proteinase. This opinion is deduced from studies of their protein structure (particularly the catalytic site), amino acid sequence and biological properties. Papain-like cysteine proteases are synthesized as preproproteins. The enzyme is activated on cleavage of the N-terminal peptidase.

The molecular weight of a mammalian cathepsin L ranges between 21-28kDa. It's enzyme activity is optimum between pH 4-6 and is irreversible above pH 7.0. Classification of cathepsin L cysteine proteinases can be made using synthetic substrates. Three synthetic substrates used for this purpose include Bz-arg-NN2, Z-lys-OPhNO2 and Z-Phe-Arg-NHmec. Cathepsin L contains highly activated thiol groups and readily react with thiol blocking agents. Inhibitors therefore include

phenylmethylsulfonylfluoride (PMSF), Iodoacetamide, Leupeptin and L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane (E64) (Barret and Kirschke, 1981; Dalton and Brindley, 1997).

Papain-like proteases of parasites are reported to function in the destruction of host macromolecules, and in facilitating migration through the host tissue and in the acquisition of nutrients. In addition, they are involved in immune evasion mechanisms. An early publication by Howell *et al.* (1966) reported the presence of an enzyme in adult and juvenile fluke which had collagenase activity. Howell (1973) confirmed the proteolytic activity of the enzyme and demonstrated using incubating sections of fluke on photographic plates that the activity is restricted to the gut tissue. It is now generally accepted that the protease is secreted by vesicles in gut epithelium cells (Smith *et al.*, 1993) and that the protease may function in tissue migration and feeding. However, several papers have reported that these proteases may have an additional vital role in immunoprotection as they cleaved immunoglobulin splitting the molecule at the hinge region into Fab and Fc fragments in a papain-like manner (Chapman and Mitchell, 1982; Heffernan and Dalton, 1989).

Heffernan and Dalton (1989) showed that when immature and mature *Fasciola hepatica* were maintained in culture media for 16 hours they released a proteolytic enzyme. This enzyme was classified as a thiol protease as it was deactivated by thiol protease inhibitors and exhibited enhanced activity in the presence of reducing agents (cysteine and dithiothreitol). Subsequent biochemical and characterisation studies revealed that this proteolytical activity was derived from two cysteine proteinases, termed cathepsin L1 and cathepsin L2 (Smith *et al.*, 1993; Dowd *et al.* 1994). Both of these proteins accounts for 80% of the excretory-secretory products (ES) secreted by adult flukes (Dowd *et al.*, 1993).

Smith *et al.* (1983) was the first to purify and characterize the cathepsin L1 proteinase from the media in which liver flukes were cultured. Cathepsin L1 is a 27 kDa proteinase with optimum activity at pH 4.5 and is inhibited by cysteine proteinase inhibitors. N-terminal sequence of cathepsin L1 proteinase showed homology to cathepsin L proteinases of chicken liver (63%) and bovine (58%). Cathepsin L1 is synthesized as a preproenzyme which are processed to the proenzyme form. Further processing gives rise to the mature form. Immunolocalisation studies at light and electron microscope level demonstrated that cathepsin L1 was located in vesicles in the gut epithelial cells. This protein is secreted into the gut where it is regurgitated to the exterior by the fluke. It is postulated to be involved in three parasite functions, burrowing, feeding and immune evasion. In addition to cleaving host immunoglobulin, it was shown to prevent the antibody-mediated attachment of eosinophils to newly excysted juvenile (NEJ) flukes (Carmona *et al.*, 1993).

A second proteinase, cathepsin L2, was purified and characterized by Dowd *et al.* (1994) and was found also to be a lysosomal proteinase belonging to the papain superfamily. It differs from cathepsin L1 in its migration in zyograms, its affinity for binding to QAE-Sephadex, its enzyme specificity for various synthetic enzymes and N-terminal sequences. It was shown to hydrolyse a number of substrates such as collagen and elastin. In addition, it cleaves fibrinogen producing a novel type of fibrin clot (Dowd *et al.*, 1995). This may have evolved by the fluke to prevent haemorrhaging in the liver as the fluke migrate through the host. Both cathepsin L1 and cathepsin L2 are believed to be good targets for immunoprophylaxis, or chemotherapy against fasciolosis. Both proteins have shown potential as vaccine candidates in studies performed in bovines (Dalton *et al.*, 1996).

1.7 General introduction to immunology

Mammalian immune systems have evolved to protect against infection from harmful microbes (viruses and bacteria) and parasites (protozoa and helminths). Such systems consist of a complex network of cells and molecules that recognise foreign antigens (or non-self antigens) as potential pathogens. The detective agents elicit such information concerning these pathogens and relay their discoveries through a number of complex interactions resulting in an immune attack that eliminates the source of the antigen. The immune system is classically divided into innate (non-adaptive) immunity and adaptive immunity.

Innate immunity

Innate immunity responds rapidly in a non-specific manner to a foreign antigen. In essence, defence strategies consist of physical/chemical barriers to infection, extracellular chemical defences (complement system, interferons) and cellular defences (leukocytes). The skin and mucous membranes, which line the alimentary, gut and respiratory tract, act as physical barriers to foreign antigens. In addition, they have chemical properties, such as the low pH on the surface of the skin and the secretion of lysozyme, which delimit bacterial survival. Such physical and chemical barriers are generally regarded as the primary line of defence against pathogens. However pathogens have developed mechanisms to bypass these barriers. In these circumstances the secondary line of defence is crucial for the early recognition and elimination of a foreign antigen.

The complement system consists of 20 plasma proteins which perform in an enzyme cascade manner. Its functions include regulation of inflammatory reactions,

clearance of immune complexes, leukocyte activation and microbial defence. The initiation of complement may proceed via two pathways, the classical or alternate pathway. The classical pathway is activated by the specific binding of IgG or IgM antibody. The initiation of the alternative pathway is by the reaction of polysaccharides in the cell wall of the pathogen or by the specific binding to IgA antibody with complement components. Regardless of the route the common terminal pathway is the formation of the membrane attack complex (MAC) which occurs via the convergence of the intermediate component C3 to C3b. The MAC complex is a cylindrical molecule which forms a pore in the cell wall causing osmotic disruption resulting in cell death. C3b activates complement by covalently binding non-specifically to micro-organisms or immune complexes. Damage limitation is curtailed by the simultaneous production of RCA (regulators of complement activation) proteins, ensuring the prevention of C3b binding to host antigens so only cells expressing foreign antigen are opoinised. Therefore, C3b has a vital role in the early recognition of foreign antigen. Other intermediate components also exercise important functions in innate immunity. C3a and C5a stimulate macrophages to release oxidising agents. In addition, C5a is a chemotatic factor (attracts white blood cells to migrate into the area of infection). Other extracellular defences include interferons and acute phase proteins. Interferons, released by white blood cells, bind non-specifically to virally infected cells while acute phase proteins are believed to enhance phagocytosis.

Cellular defences activated during innate immunity can be divided into three functional groups: cellular phagocytic cells (macrophages, neutrophils), cytotoxic cells (eosinophils, naturals killer, γδ-T cells), and inflammatory cells (basophils, mast cells). Cellular phagocytic cells principally consist of macrophages and neutrophils. Phagocytes attach to the pathogen via opsonins (antibodies, acute phase proteins and

complement). The pathogen is engulfed by phagocytosis and is eliminated by the release of powerful oxidising agents. Similarly cytotoxic cells eliminate pathogens by releasing powerful toxins, however this process does not necessitate phagocytosis, the cells merely attach directly to the pathogen. This occurs in the circumstance where the pathogen is of such volume and density as to render this option redundant. Eosinophils are cytotoxic cells which play an important role in the direct damage of parasitic worms. These cells consist of granules containing degradative enzymes which when released onto the surface of the parasite inflict damage. Although attachment to the worm can occur in an antibody-independent manner, cell attachment is substantially enhanced by the presence of antibodies. Other cytotoxic cells include natural killer cells which perform a significant role in immune responses to viral antigens and K-cells which are antibody dependent cell mediated cells. Inflammatory white blood cells release a number of active molecules provoking an acute inflammatory reaction at the infection site. These cells degranulate liberating biologically active molecules which cause an inflammatory reaction (swelling, redness and heat at the infection site). The degranulation of mast cells and basophils is augmented after IgE binding, to highly specific IgE surface receptors found on these inflammatory cells.

Adaptive/Acquired immunity

The acquired immune response differs from the innate response since it can specifically recognise a diverse antigen range. In addition, it exhibits immunological memory, ensuring a more effective immune response during subsequent infections. The acquired immune system consists of two main cell populations, T-cells and B-cells. Precursor B-cells and T-cells originate from lymphoid stem cells found in the bone marrow.

B-cells

B-lymphocytes, or B-cells, mature and clonally expand in the presence of foreign antigen and activating signals from T-cells. Antigen adheres directly to B-cells via surface immunoglobulin (sIg- approximately 10^5 are found on the surface of B-cells). Sites in the sIg structure interact with complementary epitopes on the antigen. As soon as antigen-sIg binding occurs, the antigen is internalised and it is in the endosome that the antigen is proteolytically cleaved into smaller peptides. Subsequently these peptides are presented on the cell surface in association with class II major histocompatibility complex (MHC). The antigen is presented to T-helper cells which in turn release cytokines activating B-cells and causes them to undergo clonal expansion into memory cells and plasma cells.

Plasma cells produced antibodies which can be classified by their respective structures and functions. There are five different classes of antibody, IgG, IgA, IgM, IgD and IgE. All classes of antibody structures comprise of similar basic Y-shaped immunoglobulin structures. IgG immunoglobulin class has the simplest structure. It may be cleaved into two identical Fab regions and a single Fc region. The Fc region is the constant domain which attaches to complement or various cell surface receptors. The Fab region is the antigen-specific region. This region varies between different antibodies which exhibit different antigen specificity.

IgM is composed of five units of the basic antibody structure. Although not highly specific it has ten binding sites ensuring its effectiveness in entrapping pathogens and producing large aggregates of antigen, thereby increasing the efficacy of phagocytic cells. In addition, it triggers the classical complement system and is important in the early recognition of antigen during a primary infection until the more specific IgG subclass can function effectively. IgG can be further subdivided into four

isotypes, IgG1, IgG2, IgG3 and IgG4 (in humans). IgG1 and IgG3 are effective in activating complement; and enhance phagocytosis by readily opsonising bacteria. The function of IgG4 in humans is unknown since it appears neither to activate complement nor binds immune effector cells. In helminth infections IgG4 is the predominant antibody produced, and is believed to benefit the parasite by blocking the attachment of IgG1 and IgE and preventing IgG1 activation of the complement cascade.

IgE is normally present in serum in low concentrations and is also found attached to the surface of cells which bear the IgE receptor. The serum IgE concentration greatly increases during helminth infections (and allergic disease). Bound IgE acts as opsonins for eosinophils on parasites increasing their cytotoxic effect. IgA is associated with pathogens of the gut and lung as it is predominantly found in secretions of the alimentary, respiratory and genito-urinary tract. IgD, which accounts for about 1% of serum is expressed on the surface of B-cells. The function of IgD is unknown.

T-cells

T-cells undergo maturation and differentiation processes in the thymus before migrating into the spleen and the lymph nodes. At this juncture cells commit themselves to one of two main T-cell populations, cytotoxic T-cells (Tc) or helper-T cells (Th). Unlike B-cells, T-cells do not recognise antigen directly, instead they recognise antigen presented by the major histocompatibility complex I or II. T-cells are distinguished phenotypically by CD markers. Both populations express CD3 which is situated in the T-cell receptor. CD8 is expressed on Tc-cells while CD4 is

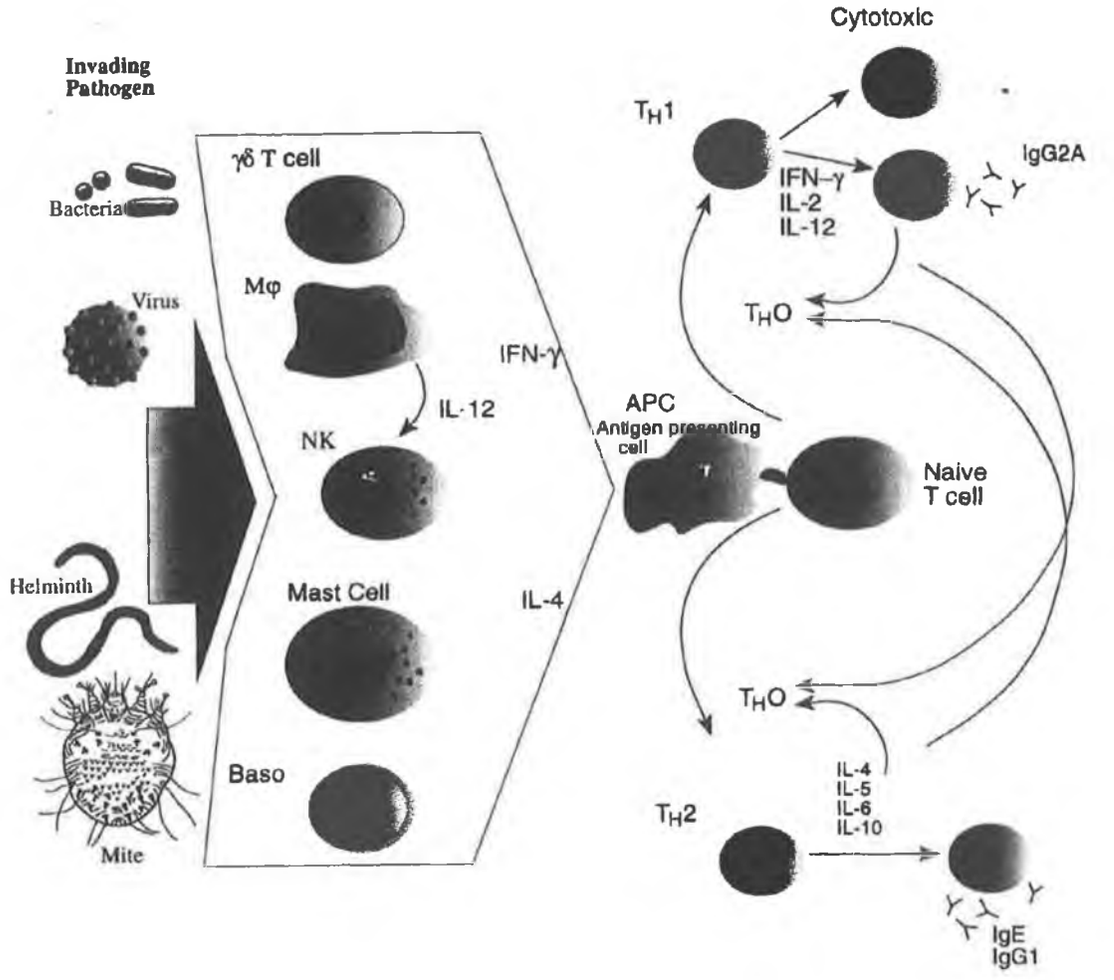


Figure 1.2 Overview of the immune response.

expressed on Th-cells. Tc-cells kill host cells that express foreign antigens. Cell killing is performed by the release of cytotoxic chemicals and pore forming molecules. Antigen recognition occurs via the Class I MHC molecule which is found on every cell in the body except activated leucocytes (macrophages, B-cells and T-cells). Activated leucocytes are unique in that they recognise antigens by utilising the Class II MHC molecule.

Th-cells can only bind to an antigen when it is coupled with a class II MHC molecule. Helper T-cells activate and enhance innate and acquired immunity by synthesising and secreting cytokines which act as immunoregulators. Direct cell to cell

interactions between T-cells and B-cells also enable B-cells to undergo maturation and to secrete antibody. Th-cells can be further divided into two sub-populations, Th1 and Th2 cells.

The Th-cell dichotomy

Mossman and Coffman (1980) were the first to identify and describe two functionally polarised Th-cell sub-populations in mice $CD4^+$ T-cell clones. To date, no phenotypic difference has been identified. The cells can be differentiated by their secretion of distinct cytokine profiles. Th1 cells secrete type I cytokine profile, IFNγ, IL-2, IL-12 and lymphotoxin. These cytokines are involved in the regulation of cell-mediated inflammatory reactions and in the induction of delayed type hypersensitivty reactions. The type I cytokine IFNγ, controls the production of IgG2a by B-cells, which activates the classical complement pathway and hence phagocytosis. IFNγ has a direct effect upon the regulation of nitric oxide production from macrophages. Th2 cells secrete type II cytokines, IL-4, IL-5, IL-6, IL-13, and IL-10 and are largely associated with responses to helminth infections and allergic diseases. Th2 cells augment the antibody response, particularly IgE and enhance eosinophil proliferation and function.

Both Th1 and Th2 cells are derived from a common precursor, Th0. Environmental and genetic factors during antigen presentation influence Th0 cells to clonally expand into either type I or type II CD4+ sub populations. Type I and Type II Th-cells counter regulate each other negatively. IFNγ, which is secreted by Th1 cells, selectively inhibits proliferation of type II CD4+ cells and IL-10, which is secreted by Th2 cells, inhibits cytokine synthesis by type I cells. Such counter regulation tends to invoke a polarisation of the immune response towards a type I or type II bias. Polarisation of the immune response influences the susceptibility of the host to an

infectious agent. Different pathogens require different immune responses for their elimination. In general, bacteria are eliminated by a type I immune response while gut parasites are eliminated by a type II immune response.

1.8 Immunological evasive strategies of *Fasciola hepatica*:

In common with other successful parasites *Fasciola hepatica* has developed a number of immunological evasive mechanisms which are critical for it's survival in the definitive host. In order to reach the bile duct the fluke must burrow through the mesenteric wall and migrate across the liver parenchyma thus exposing itself to the full force of the hosts immune response. Immune evasion strategies have been developed by *Fasciola hepatica* to counteract this immune attack and include, *inter alia*, such mechanisms as antigen switching, antigen shedding, parasite motility, destruction of innate immunity and modulation of the immune response. These mechanisms facilitate the completion of the parasites life cycle by allowing the subject to achieve sexual maturity. Survival in the host continues over the course of several years, causing morbidity rather than mortality; the former being more beneficial for its survival.

As the fluke migrates across the liver parenchyma towards the bile duct its glycocalyx undergoes antigen switching. This process occurs twice during fluke development. The first switch occurs when the newly excised juvenile (NEJ) burrows through the mucosal wall. During each switch the vesicles within the tegument wall secrete, synthesize and package antigenically distinct glycocalyx components transforming the cells from TO cells to T1 cells. The final transformation occurs when the fluke enters the bile duct (T1 cells to T2 cells) (Hanna, 1980; Dalton and Joyce, 1987). This process is believed to avoid an immune attack at a time when the parasite

is most immunologically vulnerable. In addition to antigen switching the glycocalyx is continuously sloughed off and quickly replaced every three hours thus hindering antibody binding (Duffus and Franks, 1981). The shed glycocalyx acts as a decoy mopping up circulating antibodies and effector cells. The final destination for the mature liver fluke is the immunologically safe environment of the bile ducts (Duffus and Franks, 1980). At this juncture, the turnover of the glycocalyx is much slower and accordingly there are diminished antibody responses.

The migration of the parasite acts as a mechanism to avoid the host immune response as it "leaves behind" the immune effector cells. Immunohistochemical studies of liver tissue have shown that migratory tunnels were infiltrated by immune leukocytes but the flukes were not surrounded by these cells (Meeusen *et al.*, 1995). In addition the flukes migration through these distinct anatomical regions was shown to influence the pattern of antibodies which were secreted from the local lymph nodes (mesenteric and hepatic lymph nodes) and the spleen in rats (Meeusen and Brandon, 1994). Expression of IgM isotype was found in the spleen whereas IgE and IgA were expressed in the hepatic and mesenteric lymph nodes respectively. The different isotype expression in different regions would suggest that cytokine secretion was regulated in each of these microenvironments. Migration through the tissue would expose the liver fluke to different immune responses rather than a single response that would become increasingly efficient during parasite migration (Meeusen and Brandon, 1994).

The alternative complement pathway is inhibited by the presence of sialic acids in the glycocalyx components (Baeza *et al.*, 1994). Similarly antibody shedding is thought to prevent the activation of the classical pathway. Antibody cleaving by cathepsin L proteinases released by the fluke also blocks this pathway (Carmona *et al.*,

1995). Newly excysed juveniles are thought to be highly resistant to complement destruction as no deposition of the complement component C3 was observed on the surface of the glycocalyx, in studies performed *in vitro* or *in vivo* (Duffus and Franks, 1980; Davies and Goose, 1981). The activation of both complement pathways is thus prevented. In addition both processes may be blocked by the release of decay accelerating factor (DAF), which regulates complement and is found on the surface of other helminths such as schistosomes (Pearce *et al.*, 1990).

The neutralisation of the reactive oxygen products from the respiratory burst of leukocytes and macrophages is employed by liver fluke to evade the host immune attack. Studies performed *in vivo* have shown undamaged liver flukes in the liver of cattle surrounded by immune effector cells. An experiment performed by Piedrafieta (1995) demonstrated that NEJ cultured *in vitro* with macrophages exhibited resistance to reactive oxygen intermediates.

Experimental evidence in sheep suggest a lack of anamestic antibody response following challenge infection and a lack proliferative responses of lymphocytes during primary infection (Sandeman and Howell, 1981). This evidence suggests that liver flukes secrete factors that suppress or modulate the immune response. There are several molecules which are thought to be involved in immunomodulation or suppression. Sloan *et al.* (1991) suggest that glyco-conjugates released from the glycocalyx may interfere with antigen processing by macrophages or suppress T-lymphocyte responses. A Kunitz-type (Fh-KTM) serine proteinase inhibitor expressed in the gut and tegument of adult liver fluke may have a role in inhibiting the activity of elastases released by neutrophils or may interfere with cytokine production (Bozas *et al.*, 1995). Milbourne and Howell (1993) demonstrated that eosinophilia in mice and

rats was observed two weeks after intravenous injection of ES products. They hypothesized that ES may have a similar activity to IL-5 cytokine.

1.9 Immunology to *Fasciola hepatica* infections, a general review

Cattle rarely die from fasciolosis and can habour an infection from several months to several years (Dan *et al.*, 1981). The morbidity associated with disease in cattle includes weight loss, an increase in spontaneous abortion and a decrease in milk yields. In contrast, sheep frequently die during the acute stages of infection as a result of the pathological sequale. However, infection in sheep can also persist for many years, the longest duration reported being eleven years (Pantelouris, 1965).

Sheep and cattle are considered the natural host of *F. hepatica* but other animals can become infected accidentally. In the accidental hosts such as pigs and horses liver flukes do not reach maturity as the flukes are trapped in the liver parenchyma. The ability of the host to inhibit fluke migration by its encapsulation in the liver tissue is believed to be an important factor in controlling infection. The susceptibility of sheep to fasciolosis may be explained by their failure to trap flukes in the liver parenchyma, thus extensive pathological damage occurs as the flukes migrate uninhibited.

Evidence suggests that resistance in cattle to fasciolosis is not completely immunologically based as the "self cure" is believed to be related to the calcification and thickening of the bile duct wall a phenomenon not observed in sheep. The level of acquired resistance in cattle following a primary infection is significantly high, ranging from 60%-84% (Boray, 1967; Doyle *et al.*, 1971). Resistance to infection is observed following anti-helminth chemotherapy suggesting that the presence of liver flukes in

the bile ducts is not a necessary requirement (Boray, 1967; Kendall *et al.*, 1978). Hepatic fibrosis is believed to contribute to resistance of both a primary and secondary infection in cattle. During a challenge infection Doyle *et al.* (1973) established a correlation between the extent of resistance and the degree of pathological damage in the liver. In addition development of fibrosis was associated with resistance during primary infection (Ross *et al.*, 1966). Cattle infected orally with a small (200 to 1300 metacercariae) or large number (1500-2500) of metacecariae resulted in a 30% or 3% recovery rate of parasites in the bile duct, respectively.

Since sheep exhibit more extensive liver damage than cattle, one would hypothesize that there would be a higher degree of resistance during a challenge infection in sheep compared to cattle. In contrast to cattle the number of worms recovered during a challenge infection in sheep is the same as that recovered during a primary infection (Ross, 1967; Sinclair, 1962; 1970). However, during a challenge infection, egg production is reduced and fluke migration and development is retarded (Siclair, 1960; 1970; 1971). Therefore, sheep lack some other factor important in establishing resistance. Different levels of resistance and susceptibility have been reported in sheep with different genetic backgrounds. Japanese thin tailed sheep are highly resistant to infection with *F. gigantica* where other breeds of sheep are highly susceptible (Roberts *et al.*, 1997).

Immunological evidence suggests that in both the natural host and laboratory host a type II immune response is associated with fasciolosis. Antibody responses in cattle are predominantly of the IgG1 isotype; levels peaking at eight-ten weeks post initial infection. The type of CD4+ cell type in cattle has yet to be determined, however Browne *et al.* (1994) isolated TH2 and TH0 clones but not TH1 clones in two chronically infected cattle. Clery *et al.* (1996) determined the cytokine profile in

cattle infected with *Fasciola hepatica* by measuring mRNA from the hepatic lymph nodes during a chronic infection. Both IFNγ and IL-4 mRNA was expressed in the month following initial infection but IFNγ mRNA production declined after one month where IL-4 mRNA was detected throughout the course of the infection. The levels of IL-4 mRNA were several fold higher in the hepatic lymph node when compared to the precapsular lymph nodes. This data suggests that during the acute stages of infection the CD4+ cells express both IFNγ and IL-4 but as the infection progresses the cells differentiate and commit themselves to a more dominant type II profile (Clery *et al.*, 1996).

Although sheep do not demonstrate immunological resistance to liver fluke, anti-fluke antibodies are detected in serum and are predominantly of the IgG1 subtype. This immunoglobulin subtype peaks five-six weeks following initial infection (Sexton *et al.*, 1994; Chauvin *et al.*, 1995). The lack of acquired immunity in sheep is thought to be related to the lack of cellular responses as ovine antibodies passed to rats passively transfer protection (Mitchell *et al.*, 1981; Boyce *et al.*, 1995). Sheep lack an anamnestic antibody response following challenge infection and peripheral blood lymphocytes from infected sheep are suppressed in infected animals when challenged *in vitro* with a mitogen (Chauvin *et al.*, 1995). In the acute stages of disease type II cells, eosinophils and $CD4^+$ T-cells are found in the liver tissue of sheep, in the chronic stage the cellular responses are quite different with infiltration of $CD8^+$ and γδ TCR^+ cell type (Meussen *et al.*, 1995; Chauvin and Boulard, 1996).

In the laboratory, both mice and rats can be experimentally infected with liver fluke. Rats may harbour an infection for as long as their natural life whereas mice die four-five weeks after an infection with more than a single parasite (Boray, 1969) The

antibody isotype profile detected in serum from *F. hepatica* infected rats is predominantly IgE, IgG1, IgM and IgG2a subclasses (Pfister *et al.*, 1983; Poitou *et al.*, 1983). Immunoglobulin-E antibody responses are biphasic, peaking at five and nine weeks following initial infection. Titres of IgG1 isotype increase over a period of five weeks whereas IgG2a increases gradually peaking at ten weeks following initial infection. This data suggests that both type I and type II cytokines are involved with primary infection of rats (Pfister *et al.*, 1984; Poitou *et al.*, 1993).

Eosinophilia is a characteristic feature of liver fluke disease in rats. Doy *et al.* (1978) reported eosinophil infiltration within the lamina propia. This study also demonstrated that the infiltration of eosinophils increased significantly during a challenge infection. The peritoneum of rats is infiltrated with eosinophils, neutrophils and lymphocytes when implanted with mature parasites. This data indicates that eosinophils and neutrophils may have an important role to play in the antibody-mediated immune protection against liver flukes at both the level of the gut and liver. Evidence of these cells being involved in parasite killing *in vitro* and *in vivo* has yet to be established. However, recent studies indicate that peritoneum lavage cells are able to mediate killing of NEJ *in vitro* by the production of nitric oxide (Spithill *et al.*, 1997).

Mueeusen and Brandon (1994) suggest that there are two sites of immune attrition in rats. Rats cured of a 10 day old infection by drug treatment resisted an oral, but not an intraperitoneal challenge with *F. hepatica*. If rats were not drug treated and the primary infection allowed to develop, resistance to both oral and intreperitoneal challenge was observed. Resistance at the level in the liver must require the parasites to develop for a period of time within the hepatic tissue. In addition they investigated antibody secretion in serum and compared it to the antibody production in

the local tissue. Antibody secreting cell probes were obtained from lymphocytes from mesenteric lymph nodes (MLN), hepatic lymph nodes (HLN) and the spleen. During a primary infection only the HLN are stimulated to secrete IgE. This stimulation occurs as the flukes migrate across the liver parenchyma. However during a challenge infection both the MLN and the HLN are stimulated to secrete IgA and IgE, respectively. In drug treated mice followed by challenge infection there was no antibody secretion observed in the HLN, MLN or the spleen, although serum antibodies were detected. Therefore, it was suggested that the responses in the HLN and the MLN are responsible for protection in the liver and gut, respectively.

Milbourne and Howell (1990) reported an increase in peripheral blood eosinophils and an increase in production of eosinophils by bone marrow cells *in vitro* in both rats and mice. The onset of eosinophila in rats is more rapid compared to that observed in mice. Moreover, the eosinophil number in rats following primary and challenge infection was 32% and 45%, respectively, where eosinophil counts in mice is only 5% for both primary and secondary infection. The production of free radicals by rat peritoneal exudate cells is 3.5 more per cell and 30 times more per animal when compared to murine cells. Fadiel (1996) demonstrated that unlike rat intestines mice do not exhibit an increase in mast cells and eosinophils during infection. This data may explain why mice are more susceptible than rats to liver fluke disease and suggests that these cells may play a role in immune protection.

There is controversy as to whether mice can develop acquired resistance to *F. hepatica* infection. Mice infected with two liver fluke metacecariae demonstrated some resistance to re-infection and resistance differed between mice strains (Lang *et al.*, 1967; Harness *et al.*, 1976). This protection was dependent on exposure to the fluke rather than the stage of fluke development (Lang and Dronen, 1972; Lang,

1974). In contrast, others have demonstrated that mice are susceptible to infection (Chapman and Mitchell, 1982).

There a few reports on immune mechanisms in mice. When adult flukes were implanted into the peritoneum of sensitised mice eosinophils, neutrophils and lymphocytes attached to their surface within 4 hours. Recently, a report by Van der Heijden *et al.* (1997) indicating the production of IL-5 and IL-4 mRNA but not IFNγ in the lamina propia of mice infection with fasciolosis.

Chapter 2

Materials and Methods

2.1 Materials:

Aldrich-Sigma Chemical Company (Poole, Dorset, England)

Ammonium persulphate, avidin-alkaline phosphatase, avidin-peroxidase, 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)(ABTS), bovine serum albumin, dithiothreitol, Ethidium bromide, gentamicin, HEPES, 2-mercaptoethanol, peroxidase-conjugated anti-human immunoglobulin, peroxidase-conjugated avidin, biotin-conjugated anti-human monoclonal antibodies, phorbal,12-myristate,13-acetate (PMA), p-nitrophenyl phosphate (pNpp), sodium dodecyl sulfate, temed (N,N,N',N'-tetramethylethylenediamine), triton X-100.

Amicon (Danvers, MA, USA)

8400 Ultrafiltration Unit.

Bachem (Bubendorf, Switzerland)

Z-phe-arg-AMC.

BioRad (BioRad Lab. GmbH, Munchen, Germany)

Bradford assay.

Crompton Paddock Laboratories (Berkshire, UK)

Fasciola hepatica metacercariae.

GibcoBRL, Life Technologies Ltd (Paisley, UK)

Roswell park memorial institute-1640 medium (RPMI-1640), fetal calf serum (FCS), penicillin/streptomycin (1000 IU/10000 μg), L-glutamine

Nuclon (Kamstrup, Roskilde, Denmark)

Microtitre plates.

Pierce and Warriner (Chester, England)

BCA (bicinchoninic acid), protein assay reagent kit.

Pharmacia LKB Biotechnology (Uppsala, Sweden)

QAE Sephadex A50, Sephacryl S-200HR

Pharminogen (San Diego, USA)

Alkaline phosphatase-conjugated rat anti-mouse IgG1, IgG2a, Ig2b and IgG3, biotin conjugated rat anti-mouse IFNγ, IL-5, IL-4, IL-10 and IL-12, mouse purified IFNγ, IL-5, IL-4, IL-10 and IL-12, purified anti-mouse IFNγ, IL-5, IL-4, IL-10 and IL-12.

Reidel-de-Haen (Seelze, Germany):

Bromophenol blue, citric acid, dimethyl formamide (DMF), ethylenediaminetetraacetic acid (EDTA), di-sodium hydrogen phosphate, glucose, glycerol, glycine, hydrochloric

acid, potassium dihydrogen phosphate, protogel, sodium acetate, sodium chloride, sodium dihydrogen phosphate, tris-HCl, glycin

Schliecher and Schull (Dassel, Germany)

Nitrocellulose paper.

Wallac (Turka, Finland)

BetaScint, fibre filter mats, microbeta scintillation counter.

2.2 Subjects and serum samples

During three expeditions to Bolivia carried out by this laboratory serum samples were obtained from 29 individuals in the village of Chijipata Alta (February 1996). In addition, blood samples stored on filter paper were obtained from 10 individuals in the village of Chijipata Alta (February 1996) and from 67 individuals living in the village of Cutusuma (March 1997). Blood collected by venepuncture was allowed to clot and the serum obtained following centrifugation at 2000 x *g* for 5 minutes, was subsequently stored at -20^{0}C. Blood on filter paper was obtained by soaking drops of blood onto whatmann filter paper. Filter paper samples were stored at 4^{0}C.

Serum samples were also obtained from the serum library of the Insitituto Nacional de Laboratorios de Salud (INLASA) in Bolivia. One hundred and forty four serum samples and 64 filter paper samples were obtained from individuals living in the village of Cutusuma (1987), 95 serum samples were obtained from individuals residing in Calasaya (December, 1991) and 32 serum samples from individuals residing in Santa Ana (December 1991). A summary of the serum samples obtained were shown in Table 2.1.

All samples were accompanied by data concerning the donor age, gender, location and date of sample collection. Coprological analysis for *F. hepatica* eggs was performed on faecal samples obtained from all individuals as outlined in section 2.4. Serum samples from patients infected with schistosomiasis (20), cysticercosis (15), hydatidosis (15) and Chagas disease (15) were obtained from the INLASA serum library. Five serum samples from patients infected with filariasis were donated by Dr. Alex Loukas at the Institute of Cell, Animal and Population Biology, University of Edinburgh. These samples were used

to test for cross reactivity in the assay. Control serum samples were provided by eight volunteers at Dublin City University.

Table 2.1 Summary of serum samples obtained:

Location	Year	Number of Samples		Total Number of Samples	Serum Y/N	Filter Y/N	Coprolgical analysis Y/N	Source
		<16 years	>16 years					
Cutusuma	1987	93	51	144	Y	Y	Y	INLASA
Calasaya	1991	22	67	89	Y	N	Y	INLASA
Santa Ana	1991	\	\	32	Y	N	Y	INLASA
Chijipata	1996	15	14	29	Y	Y	Y	DCU EXPEDITION
Cutusuma	1997	66	\	66	N	Y	Y	DCU EXPEDITION

All studies performed with samples from humans were approved by the Ethics Committee of INLASA and by the Department of health, Bolivia. The nature of the study was explained to each individual and a signed document of consent was obtained prior to commencement of the studies. In addition, a record of each volunteers personal details were obtained. All individuals which were positive for fasciolosis were offered treatment with triclabendazole.

2.3 Elution of human antibodies from filter paper

The method employed was previously described by Coltorti *et al.* (1988). Antibodies were obtained from blood stained filter paper by cutting one circle of 5 mm in diameter and soaking it in 250 µl of PBS for one hour at room temperature. An antibody dilution of 1:100 was assumed for all eluates.

2.4 Coprological analysis of human faecal samples

All coprological analysis was performed by Dr. Rene Angles (Unidad de Parasitologia, INLSA, Bolivia) and by Dr. Jose Esteban (Faculty of Pharmacy, University of Valencia, Spain) using either the Kato-Katz technique or the MIF sedimentation method. The Kato-Katz technique (Katz *et al.*, 1972) is a simple faecal thick smear technique. The faecal sample was smeared on a slide and a positive diagnosis made by the presence of *F. hepatica* eggs in the sample.

The MIF sedimentation Method (Esteban *et al.*, 1997) was employed to determine egg number in addition to diagnosis. Two grams of faeces were suspended in water plus an emulsifying agent, merthiolate-iodine-formaldehyde (MIF). The eggs were separated by sedimentation. A standard technique of three series of repeated washing and sedimentation was employed.

2.5 Preparation of liver fluke antigens

2.5.1 Preparation of liver fluke homogenate

Adult liver flukes were obtained from infected livers of condemned cattle at an abattoir in Ballyjamesduff, Co Cavan. In order to remove bile and debris the flukes were washed six times in sterile phosphate buffered saline (PBS) (0.14 M NaCl, 2.7 mM KCL, 8.1 mM Na_2PO_4H and 1.5mM KH_2PO_4), pH 7.3. Five mature liver flukes were homogenized in a Thyrister Regler TR homogenizer with 10 mls of sterile PBS. The liver fluke homogenate was centrifuged at 13,000 X *g* for 30 minutes. The supernatant containing soluble

antigens liver fluke homogenate (LFH) was removed, aliquoted in 1 ml vials and stored at -20^{0}C. Protein concentration of all liver fluke antigens was determined using BCA assay (section 2.6).

2.5.2 Preparation of liver fluke excretory/secretory products

Excretory/secretory (ES) products were prepared as described by Dalton and Heffernan (1989). Briefly, adult liver flukes (approximately 40) were cultured *in vitro* for 24 hours in 150 mls of RPMI-1640, pH 7.3, containing 2% glucose, 30 mM Hepes and 25 mg/ml gentamycin at 37^{0}C. The culture media was changed eight hours later and the flukes were incubated for a further 12 hours. The culture media were pooled and centrifuged at 13,000 X *g* for 30 minutes to remove debris (particularly eggs). The ES products were filter-sterilised and concentrated to 10 mls using an Amicon 8400 Ultrafiltration Unit (Danvers, MA, USA) with a Ym3 membrane (3000 mw cut-off). Aliquots of 1 ml were then stored at -20^{0}C.

2.5.3 Purification of cathepsin L cysteine proteinases

Cathepsin L1 was purified from ES products as described previously (Dowd *et al.*, 1994; Smith *et al.*, 1984). Culture media in which mature flukes were maintained *in vitro* were concentrated from 500 mls to 10 mls as previously described (section 2.2.2.). The concentrated ES was applied to a Sephacryl S200HR gel filtration column (2.6cm X 74.5cm) equilibrated in 0.1 M Tris-HCl, pH 7.0, at 4^{0}C. The column was eluted with 0.1M Tris-HCl, pH 7.0, and after a void volume of 110 mls had passed, 5 ml fractions were collected. The column eluate was monitored for protein concentration at 280 nm

using a LKB Uvicord monitor. Fractions were analyzed for cathepsin L activity using the fluorogenic substrate, Z-phe-arg-NHMec.

Fractions containing cathepsin L activity were pooled and applied to a 50 ml QAE-Sephadex column (2.5 cm X 10.0 cm) equilibrated in 1M Tris-HCl, pH 7.0. Cathepsin L1 was collected in the column run-through. This was then passed over the column once more and the second run-through collected. The elution containing CL1 was concentrated using an Amicon 8400 Ultrafiltration Unit (Danvers, MA, USA) with a Ym3 membrane (3000 mw cut-off). Aliquots of 1 ml were stored at -20^{0}C. Cathepsin L1 analyzed by sodium dodecyl sulfate (SDS)-PAGE for purity (section 2.8) and enzyme activity was measured using gelatin substrate (GS)-PAGE (section 2.9) and fluorgenic assay with Z-phe-arg-NHMec (section 2.7).

2.5.4 Purification of recombinant yeast-expressed cathepsin L1 proteinase

The culturing of pFheCL1Y29-transformed DBY746 yeast expressing active cathepsin L1 cysteine proteinase was performed by Dr. Andrew Dowd as previously described (Dowd *et al.*, 1997; Roche *et al.*, 1998). Twenty mls of concentrated yeast supernatant was provided and purified using gel filtration chromatography as described in section 2.5.3. Fractions were assayed for cathepsin L proteinase activity and the fractions containing activity, pooled and concentrated. The purity and activity of the recombinant cathepsin L was analysed by SDS-PAGE gel (section 2.8), GS-PAGE gel (section 2.9) and by assay using the fluorogenic peptide Z-phe-arg-NHMec (section 2.7).

2.6 Measurement of protein concentrations

Protein concentrations of all liver fluke antigens were measured using bicinchoninic protein assay (Smith *et al.*, 1985). This assay was used to quantitatively determine protein concentrations within the range of 20 μg to 2,000 μg/ml. Bovine serum albumin at concentrations from 25 μg/ml to 2000 μg/ml were used to plot a standard curve.

Concentration of all *Bordetella pertussis* antigens were measured using the Bradford assay (Bradford *et al.*, 1976). This assay was used to determine protein concentrations within the range of 10 μg-1000 μg. The protein standards employed was bovine serum albumin at concentrations of 10 μg-1000 μg/ml..

2.7 Measurement of the cathepsin L activity using the fluorogenic substrate Z-phe-arg-NHMec

Proteinase activity was measured fluorometrically using Z-phe-arg-NHMec as substrate (Barrett and Kirschke, 1980). Assays (1 ml volume) were performed with substrate at a final concentration of 10 μm in 0.1 M, Tris-HCl, pH 7.0, containing 0.5 mM dithiothreitol. Each mixture was incubated at 37^0C for 30 minutes and the reaction stopped by the addition of 200 μl of 1.7 M acetic acid. The activity of each sample was measured in duplicate and at three different dilution's. The amount of 7-amino-4-methylcoumarin (NHMec) released was measured using a Perkin-Elmer fluorescence spectrophotometer with excitation set at 370 nm and emission at 440 nm. One unit of enzyme activity was defined as that amount which catalyzed the release of one μmole of NHMec per minute at 37^0C.

2.8 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

All antigens were analyzed by one dimensional, 12% SDS-PAGE according to the method of Laemmli (1970). The running gel was prepared using 12% (w/v) acrylamide, 0.27% (w/v) bisacrylamide, 0.373 M Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 0.03% (w/v) ammonium persulphate and 0.008% TEMED. The stacking gel contained 3% (w/v) acrylamide, 0.08% (w/v) bisacrylamide, 0.125 M Tris-HCl, pH6.8, 0.075% (w/v) ammonium persulphate, 0.1% (w/v) SDS and 0.023% (w/v) TEMED. Samples were prepared in non-reducing (0.12 M Tris-HCl, pH 6.8, containing 5% (w/v) SDS, 10% (w/v) glycerol and 0.01% (w/v) bromophenol) and reducing sample buffer (same as above except that 5% 2-mercaptoethanol was included, and the sample boiled for two minutes). Gels were run in a vertical slab gel apparatus in electrode buffer (25 mM tris-HCl, 192 mM glycine and 0.1% SDS, pH 8.3) at 25 mA at room temperature. A voltage of 8V/cm2 was applied and the gel was run until the bromophenol blue dye reached the bottom of the gel. Proteins were visualized by soaking the gel in a solution containing 0.1% (w/v) Coomassie Brilliant Blue R, 20% (v/v) methanol and 10% acetic acid for one hour at room temperature. The gel was destained with 20% (v/v) methanol and 10% (v/v) acetic acid.

2.9 Gelatin-substrate polyacrylamide gel electrophoresis (GS-PAGE)

GS-PAGE was performed as described by Dalton and Heffernan (1989). The preparation of the gel was similar to that of SDS-PAGE except that 0.1% gelatin was added. Samples were applied in non-reducing sample buffer to maintain biological activity. After electrophoresis the gels were washed for 60 minutes in 0.1M sodium citrate, pH 4.5, containing 2.5% triton X-100 with one change to remove the SDS. The gels were subsequently incubated in 0.1 sodium citrate, pH 4.5 for 12 hours at 37^0C, and stained in Coomassie Brilliant Blue R solution as previously described.

2.10 Enzyme linked immunosorbant assay (ELISA)

The optimal dilutions of antigen, serum and secondary antibodies for the ELISA method described below was determined using a chequerboard titration method. One hundred µl of LFH, ES, CL1 or YCL1 antigen (50 µg/ml, 20 µg/ml, 5 µg/ml and 5 µg/ml, respectively) was dispensed into wells of a microtitre plate which were subsequently incubated overnight at 37^0C. Excess protein binding sites were blocked at 37^0C for 30 minutes by adding 200 µl 2% bovine serum albumin (BSA) diluted in PBS/0.1% Tween 20 to each well. After the wells were washed three times with PBS/0.1% Tween 20, human sera (diluted 1:125) were added and the plates incubated for 30 minutes at 37^0C. Following another wash, 100 µl of peroxidase-conjugated anti-human IgG (diluted 1:4000) or 100 µl of biotin-conjugated anti-human IgG1, IgG2, IgG3 or IgG4 monoclonal antibodies (diluted at 1:1000, 1:4000, 1:4000 and 1:1000, respectively) were added to

each well and the plates incubated for a further 30 minutes at 37^{0}C. The washing step was repeated. The binding of the biotin-conjugated antibodies was then detected by the addition of 100 µl of avidin-conjugated peroxidase (diluted to 1:4000) into each well. The binding of the secondary antibody was visualized by the addition of 100 µl of ABTS (25 mg/100 mls) in phosphate citrate buffer, pH 5.0 (0.2 M Na_2HPO_4 0.1 M citric acid, mixed in a ratio of 25.7:24.3). After the colour had developed for 10 minutes the plates were read on a Titreteck multiscan at 405 nm. All samples were assayed in triplicate.

2.11 Preparation of formaldehyde treated sonic extract of *B. pertussis* antigen

Formalin treated *B. pertussis* antigen was prepared as previously described (Redhead *et al.*, 1993). In brief, a bacterial suspension of *B. pertusis* at 5 X 10^7 - 5 X 10^9 was sonicated in sterile PBS. The sample was centrifuged at 10,000 X *g* for 15 minutes at 4^{0}C. The supernatant was removed and the protein concentration determined using the Bradford assay. The concentration of sample was adjusted to 100 µg/ml. Gelatin (0.02%) and Tween 20 (0.05%) were added before treating with 0.02-0.04% formaldehyde for 7 days. After 7 days the sample was exhaustedly dialysed against PBS for seven days at 4^{0}C. The concentration was measured as described in section 2.6 and the sample stored at 4^{0}C.

2.12 Assessment of cell viability

A mixture of 500 µl of ethidium bromide/acridine orange (ETBR) (10X stock; dissolve 10 mg of ethidium bromide and 10 mg of acridine orange in 100 mls of sterile PBS) and 10 µl of cell suspension was made to count the number of viable spleen cells (1:10 dilution for lymph node cells). The number of viable cells (green) or non-viable cells (red) were counted by using direct microscopic counts with a haemocytometer and a Olympus TO41 fluorescent microscope.

2.14 Stimulation of murine spleen and lymph node cells with antigen

In vitro culturing of cells was performed as previously described (Redhead *et al.*, 1993). Briefly, spleens from individual mice or lymph nodes pooled for four mice were removed aseptically and the cells crushed in a wire grid to form a single cell suspension in RPMI 1640 medium containing 8% heat inactivated FCS (fetal calf serum), penicillin (100 U/ml), streptomycin (100 µg/ml) glutamine (2 nM) and 2-ME ($5X10^5$ M). Cell suspensions were then washed in RPMI medium and resuspended in 0.5 mls or 2 mls of RPMI for lymph nodes and spleen cells, respectively. The number of viable cells per ml were counted as described in section 2.13 and the concentration adjusted to $2 X 10^6$ /ml for spleen cells and $1 X 10^6$ /ml for lymph node cells. The cells were stimulated *in vitro* in a 96 well plate by the addition of varying concentrations of antigen (LFH, 100 µg/ml -0.03µg/ml; ES 20-100 µg/ml; BP 0.2-5 µg/ml). PMA/α-CD3 and RPMI was added to additional wells as a positive and negative controls, respectively. The cells were incubated in a CO_2 incubator for three days at 37^0C. After 24 hours 50 µl of supernatant was removed to measure IL-2

production and after 72 hours supernatant was removed to measure all other cytokines. All tests were performed in triplicate.

2.14 Measurement of murine IL-2 by CTLL Bioassay

Cytotoxic T-cell lymphocytes (CTLL's) were maintained in RPMI 1640 medium containing heat inactivated FCS, penicillin (100 U/ml), streptomycin (100 μg/ml) glutamine (2nM) and 2-ME ($5X10^5$ M). Recombinant IL-2 was added to give a final concentration of 3 μg/ml to promote cell proliferation. Every 2-3 days when cell growth was confluent the cells were split by taking 2 mls of the cell suspension and adding it to 8 mls of fresh media.

IL-2 was assayed by testing the ability of the supernatant from antigen stimulated T-cells to support the proliferation of a IL-2 sensitive, CTLL cell line. Nine mls of CTLLs were spun down at 1100 X *g* for 6 mins at room temperature (the remaining 1 ml is added to fresh media and incubated for three days at 37^0C before cryopreservation.) The supernatant was pipetted off and the cells were resuspended in 10 mls of fresh warm media in order to remove the recombinant IL-2. This washing step was repeated twice and the cells were placed in a CO_2 incubator at 37^0C. After 1 hour the washing step was repeated and the number of cells counted as described in section 2.13. The cell concentration was adjusted to a concentration of 1 X 10^5cells/ml. To each well containing CTLL's 50 μl of supernatant or standard were added and the plates incubated at 37^0C overnight in a CO_2 incubator. After 24 hours the cells were pulse labelled with 25 μl/ml of [^{3}H]-thymidine, 25 μl per well (50 μl per 2.5 ml plate) and incubated for 4 hours at 37^0C.

The cells were subsequently harvested onto glass-fiber filter mats with an automatic cell harvestor. The filters were dried and sealed in sample bags with non-aqueous scintillation fluid, betascint. [^{3}H]-thymidine incorporation was measured using a microbeta scintillation counter. The results were expressed as the mean counts per minute (cpm) of [3H]-thymidine incorporation for triplicate cultures. The concentration of each sample was determined using the standard curve and the concentration was expressed as international units per ml.

2.15 Measurement of murine cytokine by capture ELISA

The cytokines IL-5, IL-4, IL-10, IL-12 and IFNγ were measured by capture ELISA as described by Barnard *et al.* (1996). Approximately 50 µl of capture antibody at a concentration of 1 µg/ml was dispensed into each well of a microtitre plates which were subsequently incubated overnight at 4^{0}C. Excess protein binding sites were blocked at room temperature for two hours by adding 200 µl of skimmed milk at a protein concentration of 1 mg/ml. After the wells were washed six times, 50 µl of supernatant or standard were added in triplicate and the plates incubated overnight at 4^{0}C. The washing step was repeated and the biotin labeled detector antibody (1 µg/ml), was added to each well and the plates incubated at room temperature. After one hour the plates were washed and avidin-alkaline phosphatase at a 1/2500 dilution was added to each well for 30 minutes at room temperature. Bound antibodies were detected by the addition of 100 µl pNPP (1mg/ml) in diethanolamine buffer, pH 9.8 (97mls of diethanolamine, 0.2g NaN_2 and 100 mg of $MgCL_2.6H_20$). The plates were allowed to develop in the dark until the

top standard exhibited an absorbance of 1 absorbance unit at 405nm. Standard curves were used as a reference to determine concentration. Concentration of IL-5, IL-4, IL-10 and IL-12 were expressed as pg/ml, whereas IFNγ was expressed as ng/ml.

2.16 Culturing of murine peritoneal exudate cells

Peritoneal exudate cells were obtained from BALB/c mice by performing pertinoneal lavage with 10-15 mls of cold sterile serum free media. The cells were washed two times with serum free media, the cell number calculated and the concentration of the cells adjusted to 1 X 10^6 cells/ml. The cells were cultured in triplicate in the presence of varying concentrations of ES and LFH antigen (20-100 μg/ml) for 72 hours, then the supernatant was removed after 72 hours and the concentration of IL-10 present in the supernatant was measured.

2.17 Measurement of IgG1 and IgG2a isotypes

Approximately 100 μl of ES (10 μg/ml) was dispensed into wells of a microtitre plate which were subsequently incubated overnight at 4^0C. Excess protein binding sites were blocked for two hours at room temperature by adding 200 μl of skimmed milk (1 mg/ml) into each well. The serum samples were titred at a dilution of 1:100 - 1:218,700. After the wells were washed six times with PBS/0.1% Tween 20 the serum was added in triplicate and the plates incubated overnight at 4^0C. After 24 hours the wash step was repeated and alkaline phosphatase conjugated rat IgG1 and IgG2a, (diluted at 1:500, 1:1000 respectively) were added to each well and the plates incubated for one hour at

room temperature. After a final washing step, bound antibodies were detected by the addition of pNpp (1 mg/ml) in diethanolamine buffer, pH 9.8. The plates were read on a Titreteck multiscan at 405nm. The antibody titre was expressed as log titre.

2.18 Mice models

Mice were maintained under the guidelines of the Irish Department of Health. All mice were female and between 2-3 months old at the initiation of the experiment. (3.7.3).

2.19 Statistical analysis

Cluster analysis, regression analysis, chi-squared analysis and student *t* test were performed using SPSS for Windows™. K-Means Cluster analysis was carried out to separate the ELISA data into two sub-populations. Euclidian distances between pairs of data points were first calculated using the combined, unweighted absorbances due to two experimental groups. Two initial cluster centres were derived from the Euclidian distances and class assigned to clusters by a process of iteration and classification using a maximum of 10 iterations, and a convergence criterion of 0.02. These parameters correspond to the default settings for the process in SPSS. The statistical analysis for the immunodiagnostic data was carried out in collaboration with Dr. Michael Parkinson.

Chapter 3

Immunodiagnosis of *Fasciola hepatica* infection (fasciolosis) in a human population in the Bolivian Altiplano.

3.1 Introduction

Several methods have been developed for the immunological diagnosis of human fasciolosis. Enzyme linked immunosorbant assay is both a sensitive and reliable means of diagnosing the acute and chronic stages of human fasciolosis (Hillyer *et al.*, 1992). Previously developed ELISA methods have employed crude somatic antigen or liver fluke excretory/secretory (ES) products to detect anti-fluke antibodies in serum (Knobloch *et al.*, 1985). These antigen preparations are complex and may result in reduced specificity of the assay since many parasites share similar antigens (Capron *et al.*, 1971). Previous work in this laboratory led to the isolation and characterisation of cathepsin L1, a major molecule of ES products. This molecule has been shown to be highly immunogenic in infected animals (Smith *et al.*, 1993; Dowd *et al.*, 1994).

In this study we analysed and compared the respective suitability of liver fluke crude somatic antigen (LFH), excretory/secretory products (ES) and purified cathepsin L1 cysteine proteinase (CL1) as coating antigens in an indirect ELISA for the serological detection of human fasciolosis. Initially, the detecting antibody employed was rabbit anti-human total immunolglobulin but since we discovered that the predominant IgG isotype elicited by liver fluke in humans is IgG4, an ELISA was developed with enhanced sensitivity based upon the detection of IgG4 responses to ES or CL1. Finally, the specificity of the assay was determined by assaying sera from individuals infected with other parasitic diseases.

3.2 Experimental design

Serum samples obtained on December the 16th, 1991, from 95 native Aymara (47 males and 48 females) living in Calysaya, a small village northwest of La Paz, Bolivia, were used in this study. The age of the volunteers ranged between one and 85 years, with a mean +/- standard deviation (SD) age of 31.4+/- 19.9 years. Coprological analysis for *F. hepatica* eggs was available in respect to 58 individuals in this group. Serum samples from ninety one individuals were serologically analysed by enzyme linked immunosorbant assay for cysticercosis using soluble extracts of *Taenia solium*. The latter two tests were performed at the Instituto Nacional de laboratorios de Salud (INLASA) in 1991.

The serum samples were analysed by ELISA for total antibody responses against LFH, ES and CL1 and the absorbance data was statistically analysed. In order to increase the sensitivity of the immunodiagnostic assay we investigated the application of secondary antibodies directed against the different subclasses of human IgG antibodies namely anti-fluke total IgG, IgG1, IgG2, IgG3 and IgG4. The specificity of the ELISA for detecting fasciolosis was examined by testing sera obtained from patients with other infections including schistosomiasis mansoni (20), cysticercosis (15), hydatidosis (15) and Chagas disease (15).

3.3 RESULTS

3.3.1 Analysis of human serum samples by ELISA using rabbit anti-human total IgG as detecting antibody.

3.3.1.1 Comparison of LFH, ES and CL1 in ELISA.

As previously noted serum samples were obtained in December (1991) from 95 Aymarans living in the village of Calasaya in the Bolivian Altiplano. These samples were analysed by ELISA for total antibody responses against LFH, ES and CL1. The population was then examined by plotting the frequency of absorbancy measurements, grouped into blocks of 0.05 absorbance units, as histograms (Fig. 3.1A, B and C). The frequency distribution of the absorbances obtained with all three antigens was bimodal. The largest mode situated to the left of the histograms represents the seronegative population and the second smaller mode located to the right represents the seropositive population.

The histogram displaying LFH absorbance shows a single broad peak at 0.175 absorbance units (A) and a long tail stretching up to 0.7 A (Fig. 3.1A). There is a second poorly defined peak at approximately 0.5A. In this histogram it is difficult to distinguish between the seronegative and seropositive population as there is no clear division between the peaks in the histogram. In contrast, the frequency distribution obtained with ELISA, using ES as antigen exhibits two peaks, at 0.175A and 0.675A, although a small peak at 0.375A may represent the points where these seronegative and seropositive populations bisect (Fig. 3.1B). ELISAs performed with purified CL1 as antigen further discriminated between the seronegative and seropositive populations, with absorbance peaks at 0.15A and 0.475A (Fig. 3.1C). For both ES and CL1

antigens, the low absorbance peak follows a bell-shaped curve characteristic of a normal distribution. In a normally distributed populations, 99.9% of all individuals should lie within 3.09 standard deviations of the mean. Therefore, a cut-off point that defines the seronegative from the seropositive population can be calculated. The calculated values for ES and CL1 antigens are 0.35A and 0.32A, respectively (dashed line in Fig. 3.1B and C).

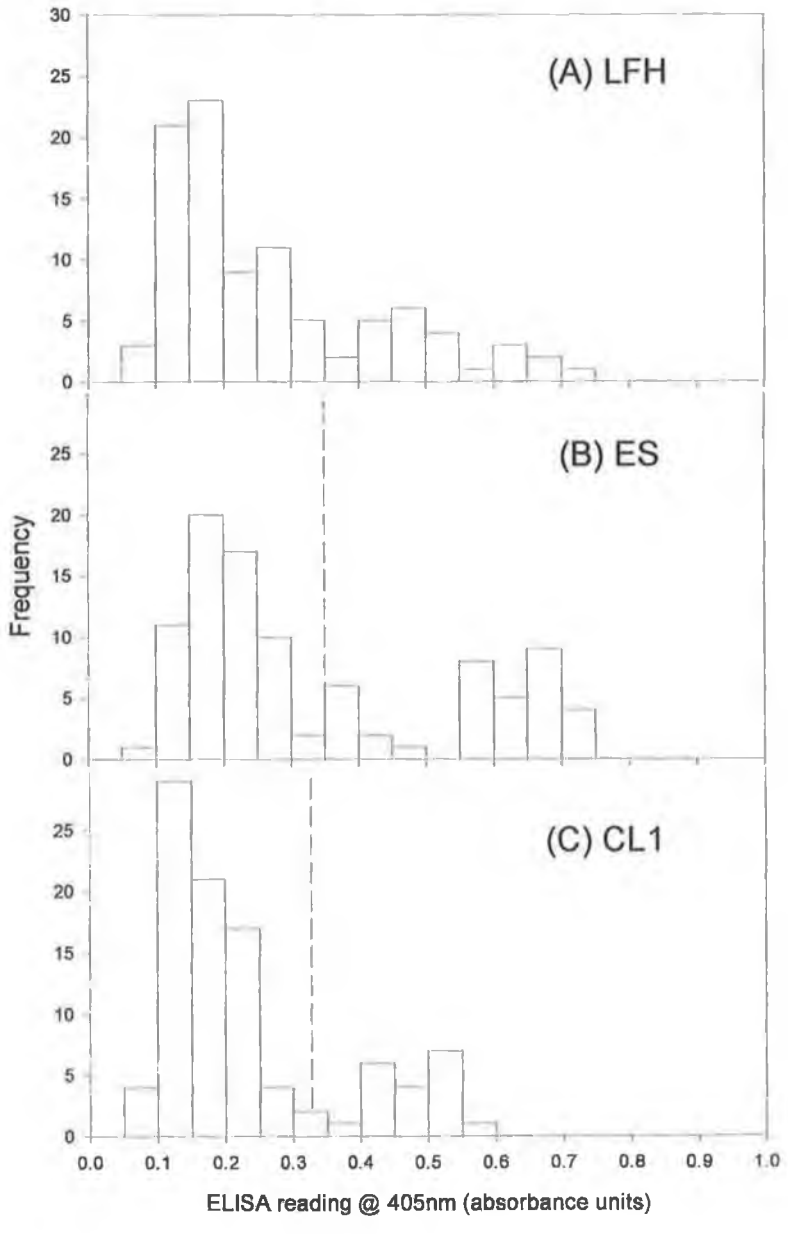


Figure 3.1. Analysis of sera obtained from 95 Aymarans by total IgG-ELISA utilising liver fluke homogenate, LFH (Panel A), excretory/secretory products, ES (Panel B) and purified cathepsin L1, CL1 (Panel C) as antigen. The frequency of the absorbance readings obtained were grouped into 0.05 absorbance units and plotted as histograms.

The vertical dashed lines in Panel B and Panel C represent the cut-off point between the seronegative and seropositive populations which were calculated as 3.09 standard deviations from the mean of the seronegative population.

3.3.1.2 Scattergram comparison of ELISA results obtained with ES and CL1.

When the absorbances obtained for ES and CL1 antigens were plotted against each other in a scattergram a scatter of points centred at a low absorbance and another at a high absorbance was observed (Fig. 3.2A). The data was sorted into two clusters using K-means cluster analysis. This analysis provides an objective and unbiased method for separating the data into two sub-populations based on a range of measured parameters (in this case, antibody responses to ES and CL1). This analysis designated 26 individuals in the high absorbance cluster as seropositive and 69 individuals in the low absorbance cluster as seronegative (Fig. 3.2A, indicated by solid and open circles respectively). While all of these 26 individuals were seropositive when ES alone was employed for detecting antibody, 20 were seropositive when CL1 was utilised (above horizontal dashed line in Fig. 3.2A). However, nine individuals determined as being seronegative by cluster analysis were deemed positive when ES was used as antigen in ELISA (open circles right of vertical dashed line, Fig. 3.2A).

3.3.1.3 Coprological analysis of *F. hepatica* eggs.

Coprological analysis for *F. hepatica* was performed on 58 individuals in this population. Six individuals were diagnosed as being coprologically positive for *F. hepatica* by the Kato-Katz technique. Chi-squared statistical analysis of the observed and expected numbers revealed a very highly significant ($P<0.001$) over-representation of individuals with fluke eggs in the seropositive cluster (all 6 grouped with the

seropositive cluster). Seven individuals that were coprologically negative were among the 26 serologically positive individuals. All remaining coprologically negative individuals were among the seronegative sub-population (Fig. 3.2A).

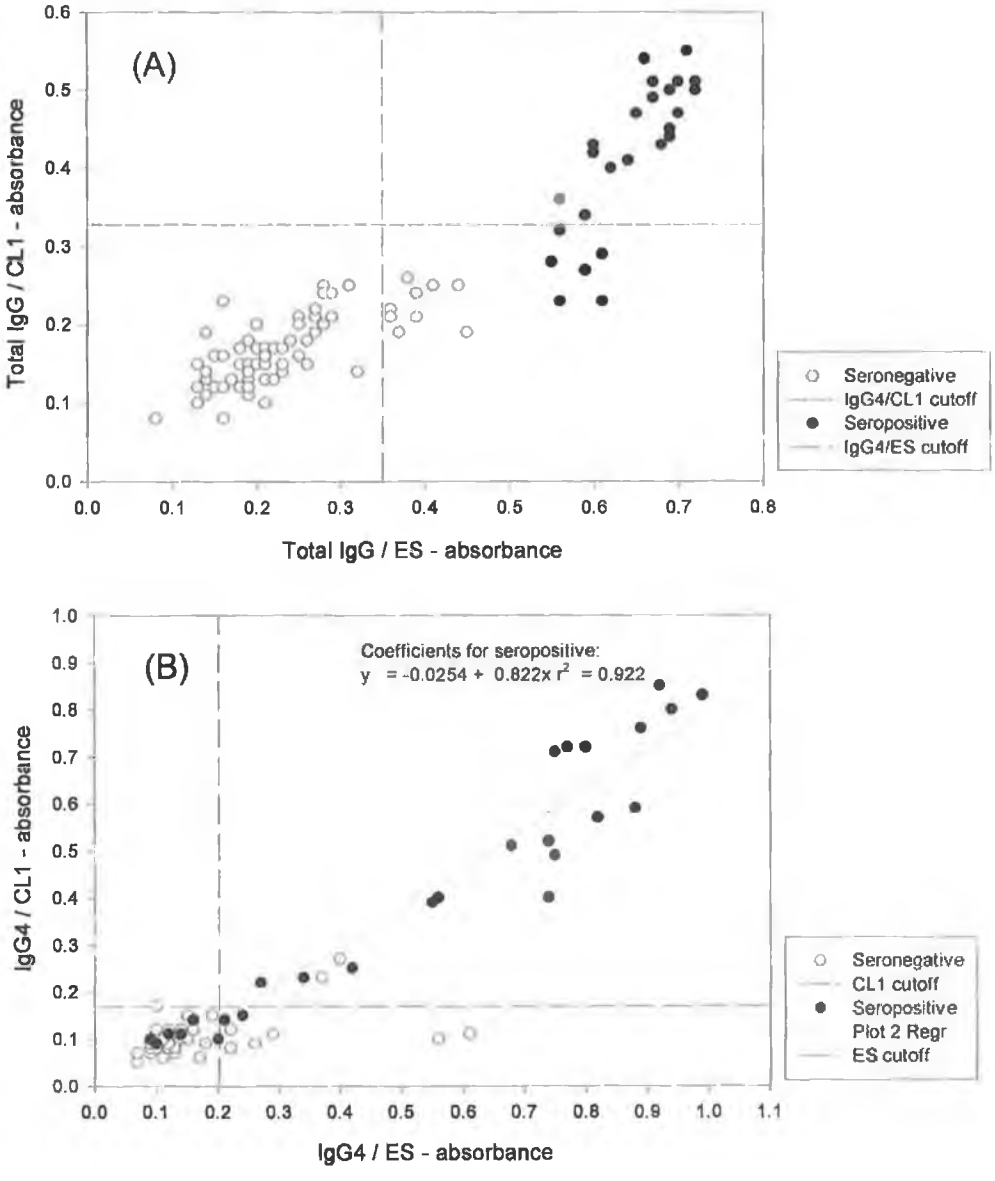


Figure 3.2. Scatter graphs of the combined data obtained in the total IgG-ELISA (Panel A) and IgG4-ELISA (Panel B) using ES and CL1 antigens were plotted. K-means cluster analysis using the combined data obtained for ES and CL1 in the total IgG-ELISA separated the population into seronegatives (indicated by open circles) and seropositives (indicated by solid circles) sub-populations (Panel A). The performance of the individuals in these sub-populations in the IgG4-ELISA is also indicated by the open and closed circles (Panel B). The vertical and horizontal dashed lines indicate the calculated cut-off points for ES and CL1 antigens in the total IgG-ELISA and IgG4-ELISA.

3.3.1.4 ELISA for cysticercosis

Ninety one sera samples were also analysed for cysticercosis at the INLASA, La Paz using the ELISA technique. Fifteen individuals were found to be seropositive for cysticercosis using this method. These positive individuals were evenly distributed throughout the population. Chi-squared statistical analysis of the observed and expected numbers showed no significant over-representation in either cluster (Fig. 3.3).

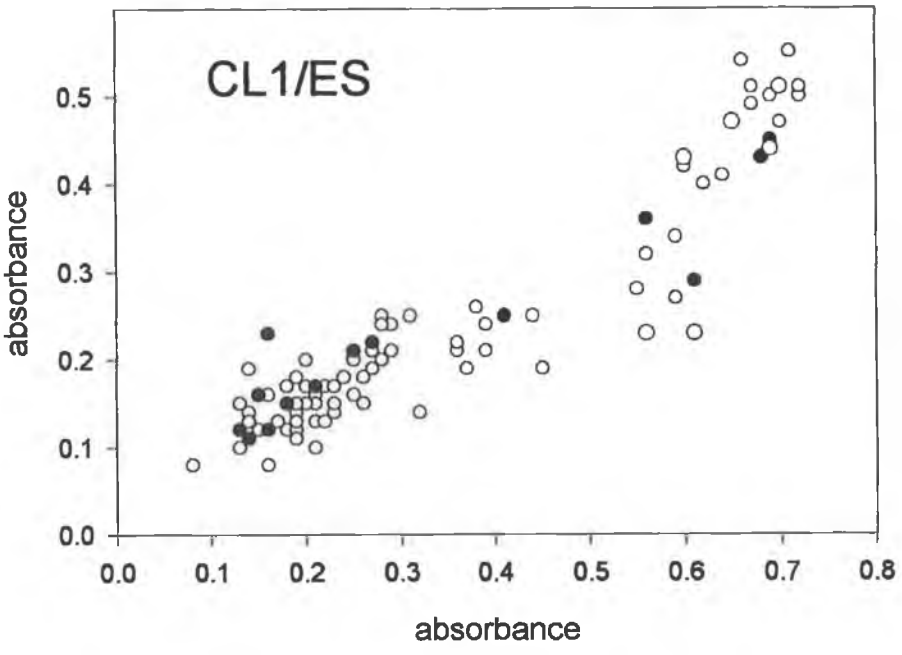


Figure 3.3 This graph is identical to that shown in Figure 3.2. Panel B. However, the individuals in this population who tested positive for cysticercosis are indicated by the closed circles. This graph illustrates that the cysticercosis positive individuals are evenly distributed throughout the population.

3.3.2. Analysis of isotypic responses of humans.

The 26 individuals deemed seropositive by cluster analysis using ES as antigen were selected for an analysis of the IgG1, IgG2, IgG3 and IgG4 antibody responses to LFH, ES and CL1 (Fig. 3.4A, B and C, respectively).

When LFH was used as a coating antigen in this ELISA the highest mean absorbance above the mean of the negative controls was obtained when anti-IgG1

conjugated secondary antibody was employed (mean = 0.292). Similarly, high absorbances were also obtained with anti-IgG4 conjugated secondary antibodies (mean = 0.129). In contrast, the mean absorbances values obtained for IgG2 and IgG3 were low (0.004 and 0.009, respectively) (Fig. 3.4A). The lowest absorbance readings obtained against LFH with the seropositive sera overlapped with the highest absorbance readings obtained with sera from the negative controls.

When the coating antigen was ES, the highest mean absorbances were also obtained with anti-IgG1- (mean = 0.304) and anti-IgG4- (mean = 0.477) conjugated secondary antibodies, although the latter of the two exhibited the highest absorbance readings (Fig. 3.4B). With these two isotypes there was no overlap of the absorbance readings for the positive and negative controls.

Using CL1 as a coating antigen the highest mean absorbances were obtained with anti-IgG1- (mean = 0.262) and anti-IgG4- (mean = 0.540) conjugated secondary antibodies and similarly to ES antigen the latter of the two exhibited the highest mean absorbance readings. There was no overlap of absorbance readings for the positive and negative controls when CL1 was employed (Fig. 3.4C).

Fig 3.4A LFH

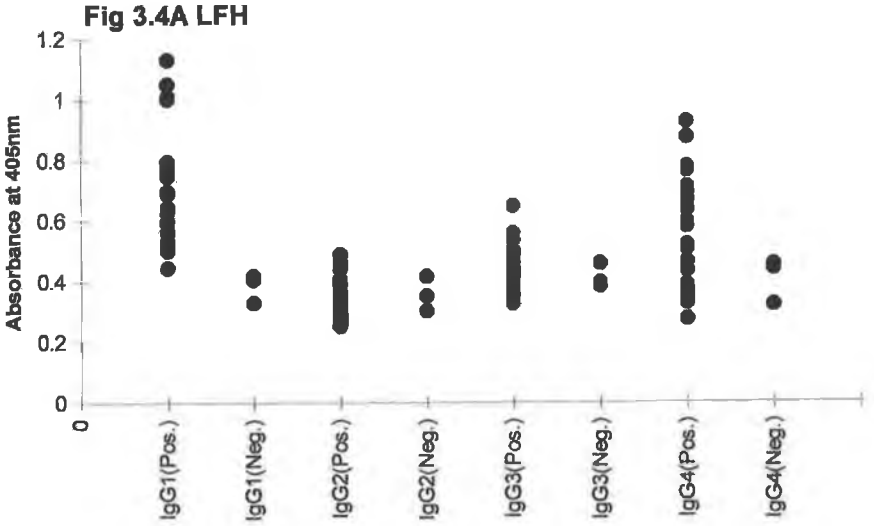
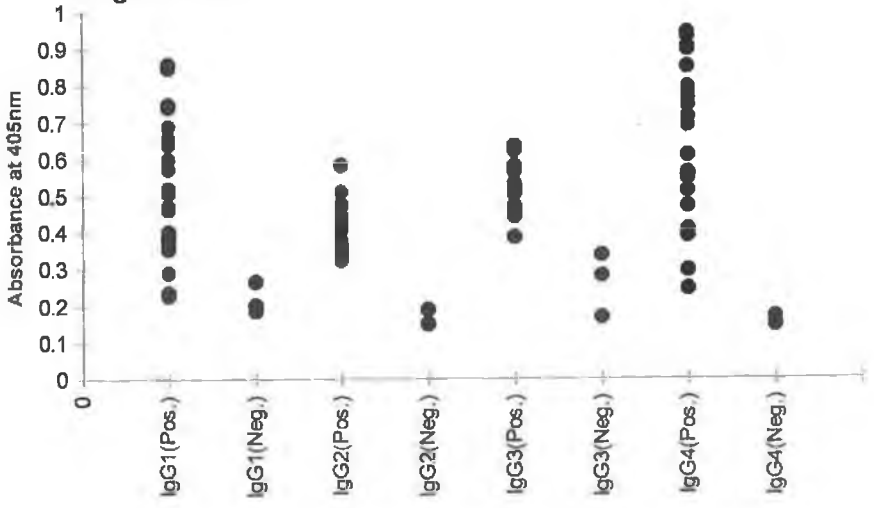


Fig 3.4B. ES



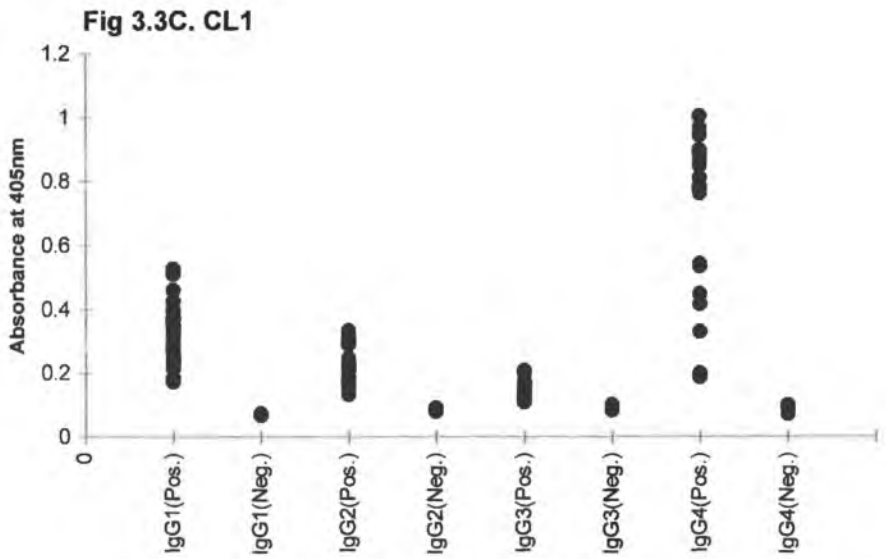


Figure 3.4. Analysis of the isotypic responses of the 26 Aymaran individuals deemed seropositive by the K-means cluster analysis (Pos.). Sera were analysed by ELISA utilising LFH (A), ES (B) and CL1 (C) as antigen. Isotype responses were detected using avidin-conjugated rabbit anti-human IgG1, anti-human IgG2, anti-human IgG3 and anti-human IgG4. Sera obtained from eight volunteers at Dublin City University served as background negative controls (Neg.).

3.3.3 Analysis of human serum samples by ELISA using rabbit anti-human IgG4 as detecting antibody.

Since we established that IgG4 was the more effective isotype for distinguishing between seropositive and seronegative individuals, we measured the IgG4 responses of all 95 individuals to ES and CL1 by ELISA. The absorbance frequencies were then plotted as a histogram. The seronegative sub-population was represented by a bell-shaped curve with a sharp single peak (Fig. 3.5A and B) for both antigens. The cut-off point between the seronegative and seropositive sub-populations was calculated as

previously described (3.09 standard deviations of the mean); values of 0.202A and 0.169A were obtained for ES and CL1, respectively (vertical lines in Fig. 3.5). These cut-off values were much lower than those calculated for the total IgG ELISA (Section 3.3.2.1; Fig. 3.1B and C). From this analysis it was observed that using CL1 allowed the identification of a more defined separation between the two populations than ES.

A comparison of scatter graphs of the absorbances obtained for total IgG-ELISA and IgG4-ELISA responses to ES and CL1 revealed that there is a more sharply defined difference between the seronegative and seropositive sub-population in the IgG4-ELISA (Fig. 3.2, compare A and B). In the IgG4-ELISA the seronegative sub-population formed a tight cloud in the low absorbancy region (Fig. 3.2B). Therefore, in ELISAs using ES as antigen 29 individuals were deemed seropositive (right of vertical dashed line in Fig. 3.2B). However, five individuals of the 26 identified as seropositive in the total IgG-ELISA were seronegative using this analysis; and eight of the previously deemed seronegatives were now seropositive. When CL1 was used as the antigen for IgG4-ELISA, 20 individuals were deemed seropositive. Eighteen of these were also deemed positive by total IgG-ELISA, while two had been previously deemed seronegative (above horizontal dashed line, Fig. 2B). Also, two individuals previously deemed to be seropositive were now seronegative.

Linear regression analysis of the ELISA results obtained for all the seropositive individuals detected with ES or CL1 showed a linear relationship between both antigens (Fig. 3.2B, $r^2 = 0.922$, dotted line indicates regression).

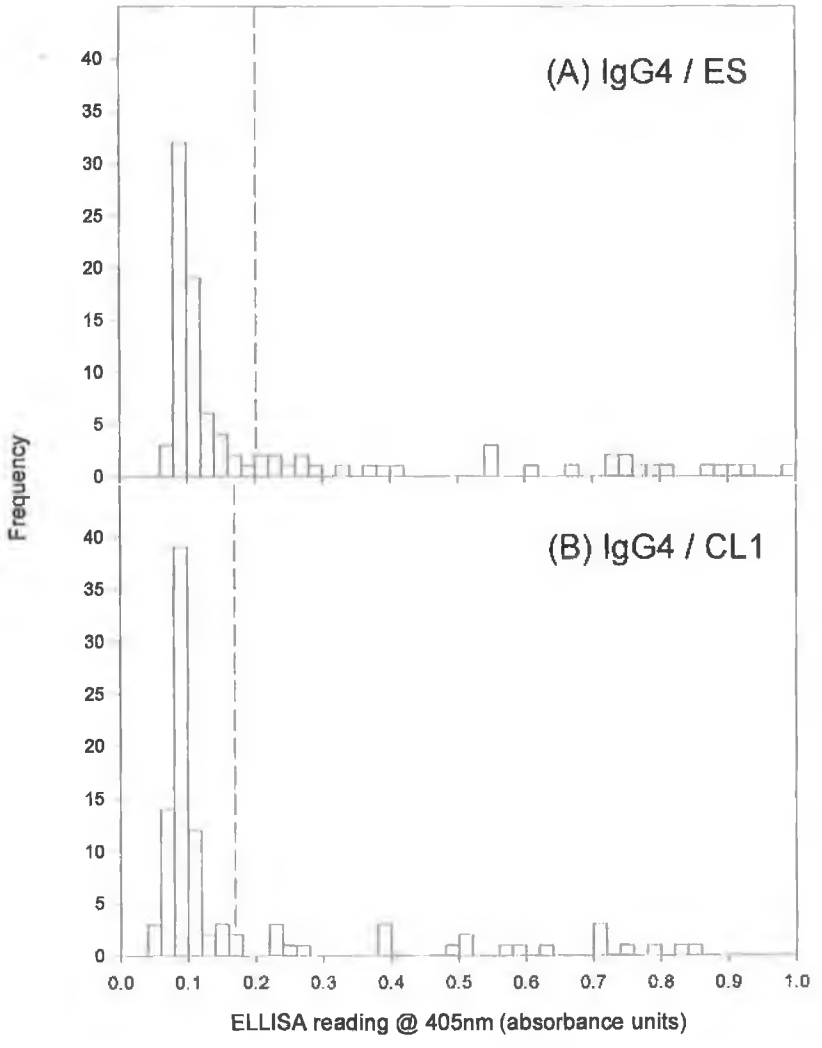
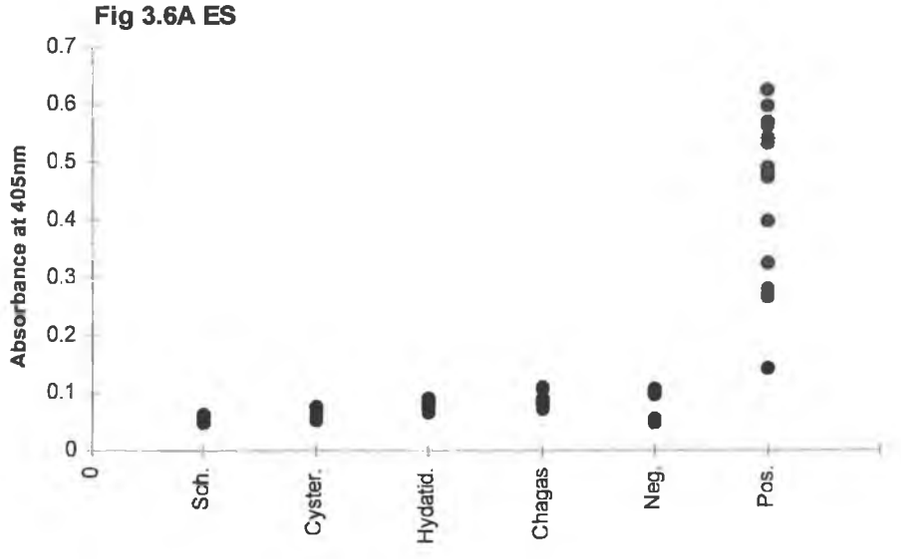


Figure 3.5. Analysis of sera obtained from 95 Aymarans by IgG4-ELISA utilising ES (Panel A) and CL1 (Panel B) as antigen. The frequency of the absorbance readings obtained was plotted as histograms. The horizontal lines represent the cut-off point between the seronegative and seropositive populations which were calculated as 3.09 standard deviations from the mean of the seronegative population.

3.3.4 Analysis of serum from humans infected with other parasites in the IgG4-ELISA for fasciolosis.

Sera obtained from individuals with schistosomiasis mansoni, cysticercosis, hydatidosis and Chagas disease were analysed by IgG4-ELISA and compared to sera from individuals that were serologically and coprologically positive for fasciolosis.

For both ES and CL1 antigens individuals seropositive for fasciolosis gave significantly higher absorbance readings than those obtained from individuals infected with other parasites (Fig. 3.6A and B, respectively). The absorbance readings obtained from the sera provided by individuals with these various infections did not significantly differ in comparison with the sera obtained from control individuals.



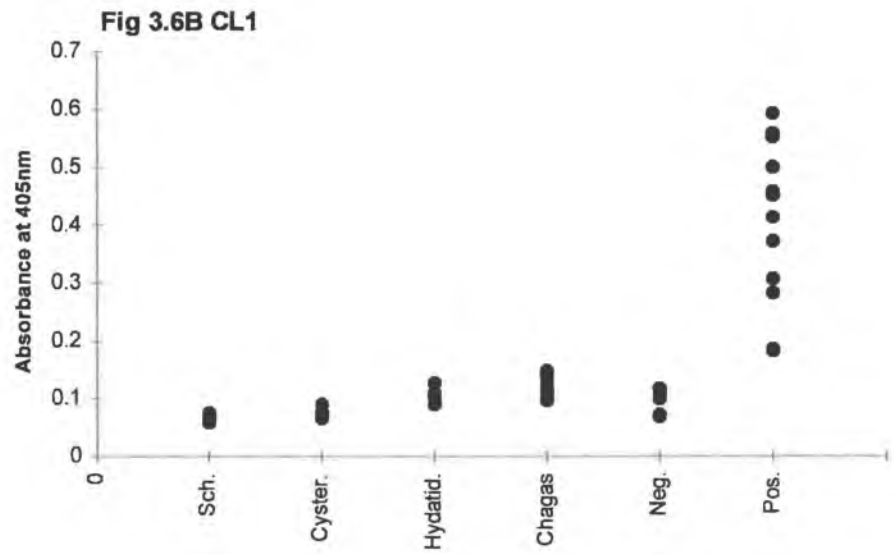


Figure 3.6. Analysis of sera from individuals with various infections by IgG4-ELISA utilising *F. hepatica* ES (Fig. 3.6A) and CL1 (Fig. 3.6B) as antigen. Serum samples obtained from individuals with schistosomiasis (20), cysticercosis (15), hydatidosis (15) and Chagas disease (15) were compared to control sera obtained from volunteers at Dublin City University (negative controls, neg.) and with sera obtained from Aymaran individuals infected with *F. hepatica* (positive controls, pos.).

3.4 Discussion

Enzyme linked immunosorbent assay has been successfully employed for the diagnosis of many parasitic diseases (Cook, 1990). In ELISAs previously developed for diagnosing human fasciolosis crude antigen preparations, such as somatic antigen or excretory/secretory antigens, were employed (reviewed by Chen and Mott, 1990). The principal disadvantage of using such a heterogeneous mixture of antigens is that it compromises the specificity of the ELISA. Reduced specificity occurs because cross

reactivity with similar antigens expressed by other parasites is a common feature (Capron *et al.*, 1971). Several reports have identified and described common cross-reactive antigens between trematodes such as *F. hepatica* and *Schistosoma mansoni* (Fagbemi and Obarisiagbon, 1991; Hillyer *et al.*, 1995; Tendler *et al.*, 1996). In addition, the use of crude antigen makes standardisation of the ELISA problematic since variation in antibody responses may be observed in different laboratories due to different methods of antigen preparation and storage conditions (Sampaio Silva *et al.*, 1996). Previous studies have reported an increase in the sensitivity and specificity of ELISA employing a single purified *Schistosoma mansoni* egg antigen (CEF6) in comparison to an ELISA using a more complex soluble egg antigen (SEA) (Doenhoff *et al.*, 1993; Ghandour *et al.*, 1997). In an attempt to enhance the specificity and sensitivity of the serological diagnosis of human fasciolosis we examined the use of a single antigen, cathepsin L1 (CL1), in these ELISA assays and compared its performance with crude somatic extract (LFH) and with a less crude preparation, ES.

Cathepsin L1 cysteine proteinase was initially purified from liver fluke ES antigens by Smith *et al.* (1993). It is synthesised by the intestinal cells in the gut lining of the liver fluke and released by vesicles into the gut lumen where it is hypothesized to have a number of functions. Cathepsin L1 is believed to function by assisting in the migration of the maturing fluke through the liver tissue. In addition it was shown to block antibody-mediated host immune responses by cleaving immunoglobulin into the Fab and Fc regions (Dalton and Heffernan, 1989; Smith *et al.*, 1993; Smith *et al.*, 1993; Dowd *et al.*, 1994). The enzyme was considered as a potential candidate for the serological detection of acute fasciolosis since it is secreted by all stages of fluke development in the mammalian host, including newly excysted juveniles. Also, the

enzyme elicits a humoral response in cattle as early as two weeks post initial infection facilitating diagnosis during the acute stage (Dowd *et al.*, 1994).

In this study we investigated the ability of ELISA for its ability to identify infected individuals in an endemic area. The serum samples employed were obtained from 95 Aymaran individuals residing in Calasaya, a region in the Bolivian Altiplano where a high prevalence of human fasciolosis has been previously reported (Hillyer *et al.*, 1992; Bjorland *et al.*, 1995). The performance of all three antigen preparations, LFH, ES and CL1, was compared in order to determine which antigen exhibited the most discrimination between those individuals that were seropositive and seronegative for fasciolosis.

The frequency of OD absorbances for all three antigens using total anti-human IgG were presented as histograms. In a given population when the absorbance readings are plotted, the presence of infection should be represented by two distinct bell shaped peaks reflecting the serologically negative (non-infected) and serologically positive (infected) individuals, respectively. ELISA analysis demonstrated that utilizing LFH antigen did not adequately discriminate between seronegative and seropositive individuals within the population because the peaks in the LFH histograms have a large region of overlap. When ES, a less complex antigen mixture, was employed the sensitivity of the assay was improved because the seronegative and seropositive populations were more clearly defined. However, there was a marked similarity to the LFH histogram in that the peaks were broad. In contrast, to both ES and LFH antigens, the histogram generated using CL1 had more clearly defined bell-shaped peaks and a more significant separation between the seropositive and seronegative sub-populations.

A common problem when using ELISA as a diagnostic tool in a given population is that it is often difficult to determine the exact cut-off point in the absorbance readings separating the seronegative from seropositive individuals. In an ELISA previously developed by Hillyer *et al.* (1992) to diagnose human fasciolosis, the positive individuals were described as those samples from individuals with an absorbance value greater than the mean plus three standard deviations of 20 negative controls samples. In similar studies, such as that performed by Espino *et al.* (1994), an absorbance reading greater than four standard deviations of the mean absorbance values obtained for 100 coprologically negative individuals was defined as the cut-off absorbance value. Clearly different parameters are defined by different laboratories in identifying the positive population. This creates the risk that they will not identify a number of individuals in the acute stages of infection. This would significantly alter the mean absorbance reading of the cut-off value. Espino *et al.* (1998) recently described the development of a sandwich ELISA that detects circulation *F. hepatica* antigen in serum. An absorbance value of 0.25 absorbance units was chosen as an arbitrary cut-off point because it separates seropositives from seronegatives in the initial assay. Using this arbitrary value, the intra-batch variation resulting from different specificity of monoclonal antibodies, time of blood collection during the course of infection and species specific antigen, was not considered. Standarisation of an ELISA to diagnose human fasciolosis will require a statistically valid way of determining the cut-off absorbance value between seropositive and seronegative individuals rather than arbitrarily chosen ones. Accordingly, in this study the ELISA data was sorted by K-means cluster analysis, a method now widely used to identify distinct sub-populations within whole populations (Ferguson *et al.*, 1995; Prokpowicz Bobrowsta *et al.*, 1995; Shiddo *et al.,* 1995).

The primary objective of cluster analysis is to classify data into relatively homogenous groups or clusters based on the variables considered. Each cluster contains variables which tend to be similar in each cluster but dissimilar to variables in other clusters. The similarity of the cluster is determined by measuring the distance between the pair of variables. This is known as the euclidean distance and is defined as the square root of the sum of the squared difference in values for each variable (Malhotra, 1996).

This study employed K-means cluster analysis, a statistical method widely employed in a diverse number of applications. This statistical method can be employed to predict the survival of infants in the third world (Harrell *et al.*, 1998) or determine the prevalence of antibodies against an infection, such as lyme disease, in non-symptomatic individuals in an endemic zone (Kimura *et al.*, 1995). Using this method the number of clusters is pre-specified and in this study the absorbance data for seropositive and seronegative individuals is divided into two clusters based on the measure of similarity in terms of distance between the absorbance readings. When the data is divided into two clusters the cut-off points are calculated as 3.09 standard deviations from the mean of the low absorbance cluster which contains 99.9% of the seronegative individuals.

Using this statistical method the ELISA data obtained for ES and CL1 were combined and the seropositive and seronegative populations identified. This analysis revealed that 26 individuals out of 95 clearly separated into a distinct high absorbance cluster (27.3%). All six individuals coprologically positive for fasciolosis associated with this seropositive population, supporting the use of this method for identifying those individuals harbouring infection in the latent or chronic stage. Seven coprologically negative individuals were also associated with this seropositive

population. These individuals may have harboured acute infections (i.e., parasites which had not yet entered and matured in the bile ducts) or alternatively, they were coprologically misdiagnosed. The remaining 45 coprologically negative individuals all associated with the seronegative sub-population supporting the use of our IgG4 ELISA as a sensitive means of diagnosing human fasciolosis.

The K-means cluster analysis shows that the ES detected all 26 individuals in the seropositive cluster. However the nine individuals that were deemed seronegative by cluster analysis were identified as positive when ES was employed. In comparison, CL1 identified 20 of the 26 individuals in the seropositive cluster, all of which were well separated from the seronegative cluster. The higher proportion of individuals reacting to ES was possibly a reflection of its more complex antigenic make-up. Moreover, it is unsurprising that CL1 detects a subset of the individuals identified by the ES since CL1 is one of the major components of ES.

An analysis of the isotypic responses of the individuals to ES and CL1 revealed that the predominant antibodies elicited by infection with *F. hepatica* were IgG1 and IgG4 isotypes. The highest mean absorbance was associated with IgG4 isotype. These observations led to the development of an ELISA that specifically detects anti-IgG4 responses of humans to ES and CL1.

Histograms of the frequency of OD values obtained (using IgG4-ELISA employing either ES or CL1 as antigen) showed that the seronegative population formed a sharper bell-shaped curve than that obtained with the total IgG-ELISAs. In addition, the definition between the seronegative and seropositive populations was substantially sharper. This difference was particularly obvious when the data was compared in scatter graphs (see Fig. 3.2A and B). In the IgG4-ELISAs the seronegative sub-population was found to cluster tightly together at the low

absorbance readings, whereas the individuals forming part of the seropositive sub-population was dispersed in a wide range of high absorbance readings. Consequently, the calculated cut-off points were much lower and the ratio of the positive/negative absorbance readings much higher in the IgG4-ELISA compared to the total IgG-ELISA. Therefore, by limiting the detection of the ELISA to IgG4 isotypic responses the sensitivity of the assay was increased.

Five of the 26 individuals deemed seropositive by total IgG-ELISA employing ES as antigen were deemed seronegative by the IgG4-ELISA. Conversely, eight of the seronegative individuals by total IgG-ELISA were deemed seropositive by IgG4-ELISA. The differences in the composition of the seropositive population observed in the two ELISA methods may again reflect the complexity of the ES as different immunogens would elicit different isotypic responses. In contrast the results obtained in both ELISA methods that employed CL1 antigen were more consistent. Eighteen of the 20 individuals deemed seropositive by total IgG-ELISA were also found to be seropositive by the IgG4-ELISA. These particular eighteen individuals were always found within the seropositive sub-population, even when LFH was used as antigen. However the clearest distinction between these and the seronegative population was obtained when CL1 was employed in the IgG4-ELISA assay. Therefore whilst CL1 identifies fewer individuals as seropositive compared to ES, this antigen provides a more reliable and confident diagnosis of the individuals that have been exposed to liver fluke infection.

Fifteen individuals in our sample population were serologically positive for cysticercosis, a disease caused by *Taenia solium,* which is also highly prevalent in the Bolivian Altiplano. Chi-squared analysis revealed that these individuals did not cluster with those that were serologically positive for liver fluke disease but were randomly

distributed throughout the entire population. Thus, our IgG4-ELISA assay using liver fluke ES or CL1 does not pick up false positives due to cross-reactivity with antibodies induced by cysticercosis infections.

In order to examine the issue of specificity further, we analysed sera obtained from individuals diagnosed with single infections, including schistosomiasis mansoni, cysticercosis, hydatidosis and Chagas disease, in the IgG4-ELISA tests. Hydatidosis and cysticercosis are prevalent throughout the region where schistosomiasis mansoni, and Chagas disease are more common in other regions throughout South America. There are several studies reporting cross reactive antigens between schistosomiasis mansoni and fasciolosis (Fagbemi and Obarisiagbon, 1991; Tendler *et al.*, 1996). However none of the sera cross-reacted with ES or CL1 in these assays which provides support for their use in the specific diagnosis of fasciolosis particularly in schistosomiasis endemic regions.

The results outlined in this chapter demonstrate that both ES and CL1 antigens can be used for the specific diagnosis of human fasciolosis using ELISA. The sensitivities of these ELISA assays were greatly improved by the use of conjugated secondary antibodies that detect IgG4 rather than total serum antibodies. CL1 provided a more conclusive diagnosis compared to ES as it discriminated more sharply between the seropositive and seronegative sub-populations. This assay also demonstrated a 100% specificity as no cross-reaction was observed with schistosomiasis mansoni, cysticercosis, hydatidosis and Chagas disease. Finally this study which identifies 20 of the 95 individuals positive for fasciolosis (21%, by CL1 IgG4-ELISA) confirms the earlier reports (Hillyer *et al.*, 1992; Bjorland *et al.*, 1995) of the high prevalence of human fasciolosis in the Bolivian Altiplano.

Chapter 4

Immunodiagnosis of human fasciolosis using recombinant *Fasciola hepatica* cathepsin L1 cysteine proteinase.

4.1 Introduction

Results presented in Chapter 3 demonstrated that CL1 can be employed as an antigen in ELISA for the sensitive and specific diagnosis of human fasciolosis. The sensitivity of this ELISA was improved by the use of conjugated second antibodies that detect IgG4 anti-CL1 antibodies rather than total IgG anti-CL1 serum antibodies. Recently, in our laboratory functionally active recombinant *F. hepatica* CL1 was expressed in the yeast *Saccharomyces cerevisiae*. This recombinant protein exhibited similar molecular size and physicobiochemical properties to the native CL1 (Roche *et al.*, 1997). In this chapter we examine the potential of this recombinant protein as a diagnostic reagent for human fasciolosis in our IgG4-ELISA and compare it's performance with native CL1 antigen.

4.2 Experimental Design

In this study we used blood samples obtained in January 1987 from 64 native individuals residing in the village of Cutusuma and in February 1996 from 25 individuals living in Chijipata Alta. IgG4-ELISA was performed on all serum samples using recombinant and native CL1 antigen. Coprological analysis for *F. hepatica* eggs was performed at INLASA on faecal samples obtained from all individuals using the sedimentation method in 1987 and the Kato-Katz method in 1996.

The specificity of recombinant CL1 was analysed by performing IgG4-ELISA on serum samples from individuals infected with schistosomiasis (20), cysticercosis (15), hydatidosis (15) and Chagas disease (15). These serum samples were obtained from the INLASA serum library. Serum samples obtained from individuals infected

with filariasis (5) were also analysed. These latter samples were donated by Dr. Alex Loukas at the Institute of Cell, Animal and Population Biology, University of Edinburgh. Control serum samples were provided by eight volunteers at Dublin City University.

4.3 Results

4.3.1 Comparison of native and recombinant CL1 using IgG4 ELISA.

4.3.1.1 Analysis of human serum samples from Cutusuma (1987).

IgG4-ELISA was performed on serum samples from 64 individuals residing in Cutusuma (1987) using recombinant and native CL1 antigen. The absorbance readings obtained were plotted as scattergrams (Fig. 4.1). Using cluster analysis we identified a compact low absorbance cluster which represents the seronegative individuals and a diffuse high absorbance cluster which represents the seropositive individuals. Cut-off points between the clusters were calculated at 3.09 standard deviations from the mean of the low absorbance cluster which contains 99.9% of the seronegative sub-population and were 0.117A and 0.121A for the recombinant and native CL1, respectively. Using the cut-off points, thirty eight individuals (58%) were deemed seropositive and twenty six individuals were seronegative by IgG4-ELISA using both the recombinant and native CL1 (Fig. 4.1 and Table 4.1).

Twenty six of the 64 individuals were coprologically positive for *F. hepatica* eggs. These individuals were also seropositive in the IgG4-ELISA using either the recombinant or native CL1 as antigen. Chi-squared analysis showed that it was highly unlikely ($p < 0.001$) that the coprological and serologically positive individuals would group together by chance, thus reinforcing the designation of the high absorbance

cluster as the seropositive sub-population. However, twelve coprologically-negative individuals were identified as seropositive. These individuals may harbour early (pre-patent) infections, since the appearance of eggs in their faeces only follows the maturation of the parasite in the bile duct, approximately 12 weeks post initial infection. Importantly, the remaining twenty six individuals that were coprologically negative were also seronegative by our IgG4-ELISA (This data is summarised in Table 4.1). Using correlation analysis a statistically significant correlation ($r^2 = 0.898$; $p < 0.001$) was demonstrated between the absorbance readings obtained using the recombinant and native proteins.

4.3.1.2 Analysis of human serum samples from Chijipata Alta (1996).

IgG4-ELISA was performed on 25 serum samples obtained in 1996 from individuals residing in Chijipata Alta (Table 4.1). The results obtained employing either native or recombinant CL1 were identical and statistical analysis revealed a significant correlation between recombinant and native CL1 ($r^2 = 0.81$; $p < 0.001$). Sixteen (64%) individuals were identified as seropositive. Fifteen of these individuals were harbouring *F. hepatica* eggs in their faeces. There was only one individual that was coprologically positive but serologically negative. The remaining eight individuals that were coprologically negative were also seronegative by our IgG4-ELISA. A summary of the data obtained in Chijipata Alta is presented in Table 4.1.

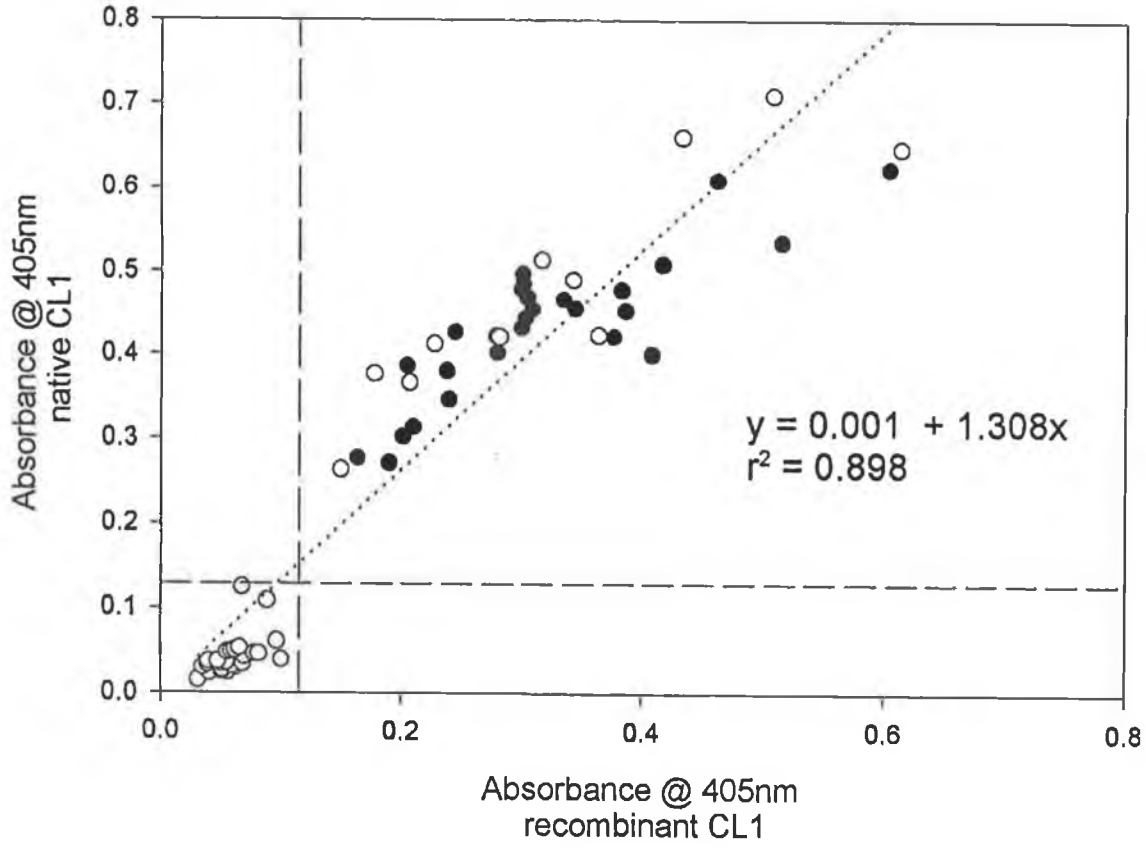


Figure 4.1 Scattergram of IgG4-ELISA data obtained for the sera of 64 individuals from Cutusuma (1987) using yeast-expressed recombinant CL1 (YCL1) and native CL1 (NCL1) antigens. Cluster analysis, using combined data obtained for both proteins, separated the population into low-absorbance (seronegative) and high absorbance (seropositive) subpopulations. The vertical and horizontal dashed lines indicate the calculated cut-off points for each antigen. The closed circles represent the individuals that were coprologically positive and the open circles represent those that were coprologically negative. Chi-squared analysis on whether individuals were seronegative or seropositive showed a very high significant relationship between the two antigens ($r^2 = 0.898$ with $p<0.001$)

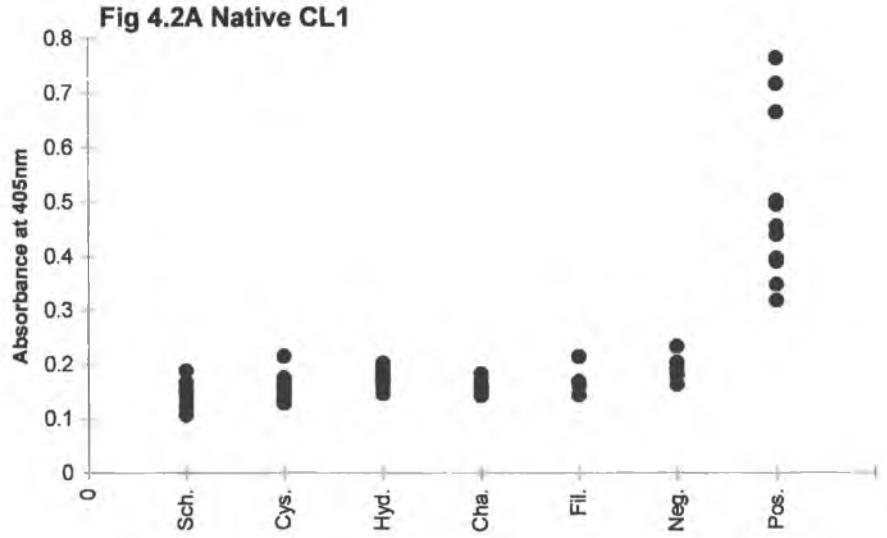
Table 4.1. Summary of the serological and coprological data obtained for individuals living in Cutusuma (1987) and Chijipata Alta (1997) in the Bolivian Altiplano.

	Cutusuma '87		Chijipata Alta '97	
Group	**Recombinant CL1**	**Native CL1**	**Recombinant CL1**	**Native CL1**
Copro+/Sero+	26 (40.6%)	26 (40.6%)	15 (60%)	15 (60%)
Copro-/Sero+	12 (18.7%)	12 (18.7%)	1 (4%)	1 (4%)
Copro+/Sero-	0 (0%)	0 (0%)	1 (4%)	1 (4%)
Copro-/Sero-	26 (40.6%)	26 (40.6%)	8 (32%)	8 (32%)
Total	64	64	25	25

Copro = Coprologically; Sero = Serologically

4.3.2 Analysis of serum from humans infected with other parasites using native and recombinant CL1.

Sera obtained from individuals infected with various parasites were analysed by IgG4-ELISA and compared to results obtained from Aymara individuals that were both serological and coprological positive and nine healthy volunteers from Dublin City University. Sera from individuals infected with schistosomiasis mansoni, cysticercosis, hydatidosis, Chagas disease and filariasis were negative by IgG4-ELISA that employed either native (Fig. 4.2A) or recombinant CL1 (Fig. 4.2B). The mean absorbance for these sera was not significantly different from those obtained for the negative control sera. The serum samples from *F. hepatica* seropositive/coprologically positive individuals had significantly higher absorbance readings than those obtained from individuals infected with other parasites ($p<0.05$).



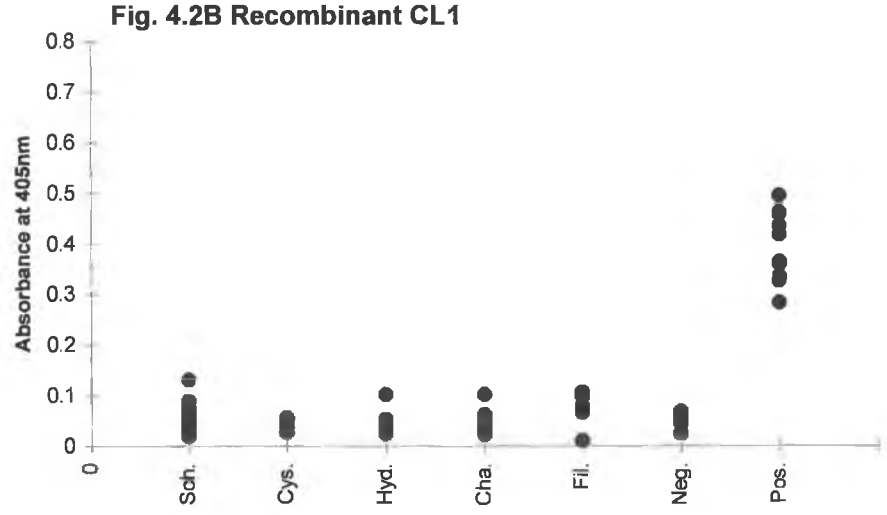


Figure 4.2. Analysis of individuals with various infections by IgG4-ELISA using *Fasciola hepatica* native (Panel A) and recombinant (Panel B) CL1 antigens. Serum samples were obtained from individuals with schistosomiasis mansoni (Sch.), cysticercosis (Cys.), hydatidosis (Hyd.), Chagas disease (Cha.) and filariasis (Fil.) and compared with control sera obtained from volunteers at Dublin City University (negative controls, neg.) and sera obtained from Aymara individuals, serologically and coprologically positive for *F. hepatica* infection (positive controls, pos.).

4.4 Discussion

In the previous chapter it was demonstrated that the CL1 antigen in an IgG4-ELISA discriminated between seropositive and seronegative individuals for fasciolosis to a markedly greater extent than crude antigens such as LFH or total ES products. This antigen was also highly specific and sensitive and was useful in diagnosing both acute and chronic fasciolosis. The fact that a single antigen is useful for the diagnosis of human fasciolosis allows the development of an ELISA method employing recombinant protein. The principal advantage of using recombinant protein is that large quantities of antigen can be produced. In addition, the ELISA can be standardised facilitating the comparison of diagnostic data from different regions.

Several studies have reported the use of recombinant proteins for the serodiagnosis of parasitic diseases in humans such as schistosomiasis mansoni, onchocerciasis, toxoplasmosis and amebiasis (Klinkert *et al.*, 1988; Ogunrinade *et al.*, 1993; Martin *et al.*, 1998; Myung *et al.*, 1992). The assays employed have functioned with varying degrees of success. Ogunrinade *et al.* (1993) developed a diagnostic assay for the specific and sensitive diagnosis of onchocerciasis. The development of a recombinant assay was essential because of the difficulty in obtaining sufficient quantities of native antigen; since previously employed native antigens were found to be highly non-specific. This study evaluated the use of two recombinant *Onchocerca volvulus* antigens, OC 3.6 and OC 9.3, in an assay based on the measurement of total IgG or IgG4 antibodies with sera from patients with onchocerciasis. Similarly to the findings in our study the sensitivity of the assay was significantly improved by the use of second conjugated antibodies that detect IgG4 anti-onchocerical antibodies (OC 3.6, 95% sensitive; OC 9.3 83%, sensitive). This assay was also highly specific as no

cross reactivity was observed when these recombinant antigens were tested by IgG4-ELISA with sera from individuals with other parasitic diseases (bancroftian filariasis, brugian filariasis, loiasis, ascariasis, schistomiasis and dracunculiasis).

Many of the recombinant ELISAs developed have limited use in the clinical field given the common factors of reduced specificity and sensitivity. The development of a recombinant ELISA to diagnose schistosomiasis, using two cDNA sequences from the C-terminus of the *Schistosoma mansoni* heat shock protein (HSP 70), was only useful in detecting the chronic stages of infection. Using this assay cross-reactive antibodies were elicited in donors with other parasitic disease such as filariasis and malaria (Moser *et al.*, 1990). In contrast, the development of an ELISA to diagnose toxoplasmosis with recombinant polypeptides termed H4/GST and H11/GST was only useful when diagnosing individuals in the acute stage of infection. Using this assay, only 14% of 159 individuals tested in the chronic stage of infection were diagnosed correctly (Tenter and Johnson, 1991).

This study assesses the value of utilising recombinant CL1 antigen in the IgG4-ELISA for the specific and sensitive diagnosis of human fasciolosis. The development of a recombinant ELISA was possible because large quantities of functional recombinant CL1 were expressed in the yeast *S. cerevisiae* in our laboratory (Roche *et al.*, 1997). Serum samples obtained from the villages of Cutusuma (1987) and Chijipata Alta (1996) were analysed in an IgG4-ELISA against both native and recombinant CL1. Statistical analysis revealed a significant correlation between the native and recombinant CL1 in both Cutusuma and Chijipata Alta ($r^2 = 0.898$, $p < 0.001$; $r^2 = 0.81$, $p<0.001$, respectively).

Using cluster analysis the populations were divided into seronegative and seropositive individuals. All coprologically positive individuals with the exception of

one in the Chijipata Alta region, were also serologically positive in the IgG4-ELISA when either native or recombinant CL1 was employed. Therefore, the sensitivity of the ELISA for both recombinant and native antigen was 97.6%. Recombinant CL1 appears to be useful in diagnosing acute infections, as twelve individuals in Cutusuma (18.7%) and one individual in Chijipata Alta were serologically positive and coprologically negative. All seronegative individuals, with the exception of one in Chijipata Alta were also coprologically negative which adds support to the use of recombinant CL1 in the sensitive diagnosis of human fasciolosis.

A higher percentage of individuals (18.7%) were seropositive/coprologically negative in the Cutusuma survey when compared with individuals with a similar diagnosis in the Chijipata Alta survey (4%). These individuals are most likely to be in the acute stage of infection but some of these individuals may be in the chronic stage of infection where coprological analysis failed to detect *F. hepatica* eggs in their stool. Accurate diagnosis by coprological analysis is highly dependent upon the technique employed. In Cutusuma (1987) only one stool sample was obtained for each individual and coprological analysis was performed using the less sensitive sedimentation method. In contrast, in Chijipata Alta (1996) the more sensitive Kato-Katz method was employed to analyse the stool samples and two stool samples were analysed for each individual in this group.

In order to examine the specificity of the recombinant protein we analysed sera obtained from individuals with single infections, including schistosomiasis mansoni, cysticercosis, hydatidosis, Chagas disease and filariasis. Sera from these individuals did not react with either the native or recombinant CL1 in the IgG4-ELISA supporting its use for the specific diagnosis of human fasciolosis.

In summary the IgG4-ELISA using recombinant CL1 as antigen is highly sensitive and specific in the diagnosis of human fasciolosis. In addition, this assay is useful in diagnosing both at the acute and chronic stages of disease. This recombinant protein was not only functionally active in *S. cerevisiae* but also exhibited similar physico-chemical properties to the native CL1, sharing similar molecular size, pH profile of activity, stability and substrate specificity. It is not overly surprising that the recombinant antigen performed similarly to the native antigen in the IgG4-ELISAs described in this study. However, the important development in this study was the demonstration of the possibility of producing sufficient quantities of antigen to provide all diagnostic centres with a standardised serological test for human fasciolosis. Finally, the incidence of infection in Cutusuma (1987) and Chijipata Alta (1996) determined by IgG4-ELISA was 59% and 64%, respectively, confirming reports of the high incidence of infection in the Bolivian Altiplano.

Chapter 5

Diagnosis of human fasciolosis: detection of anti-cathepsin L antibodies in blood samples collected on filter paper and analysis of serological and coprological data from individuals between 1987-1997.

5.1 Introduction

The studies presented in Chapter 3 and Chapter 4 using the CL1/IgG4-ELISA confirmed earlier reports of the high incidence of human fasciolosis in the Bolivian Altiplano. Further studies to examine the extent of disease in this region were hampered by the reluctance of the indigenous Aymaran population to provide blood by venepuncture. This difficulty prompted us to explore a simpler and less invasive method of collecting samples onto filter paper after lancet-pricking of the finger. This innovation has already been successfully employed by the Nacional de Laboratorios de Salud, (INLASA) for obtaining blood for the serological diagnosis of Chagas Disease. In the present study, we employed IgG4-ELISA to analyse blood samples obtained on filter paper in the following three surveys a) Cutusuma (1987), samples obtained from INLASA, b) Chijipata Alta (1996) during an expedition by our laboratory and c) Cutusuma 1997 during a final expedition by our laboratory. Finally in this chapter we collated and analysed the data obtained by our laboratory and INLASA using serum, blood-filter samples and faecal samples during the period from 1987 to 1997.

5.2 Experimental Design

In this study serum and faecal samples were obtained from three separate surveys. In 1987, 57 serum samples and blood/filter samples were obtained from the region of Cutusuma. Coprological analysis was performed on all individuals using the sedimentation technique. In February 1996, serum samples and blood filter samples were obtained from 11 individuals residing in Chijipata Alta. Coprological diagnosis

was performed using the Kato-Katz method. In 1997 blood/filter samples were obtained from 67 children (all under 13 years old) residing in Cutusuma. Faecal samples were also obtained and tested for *F. hepatica* eggs by the Kato-Katz method. All serum and blood/filter samples were analysed by IgG4-ELISA which employed purified native CL1 antigen. All coprological analysis was performed at INLASA.

5.3 Results

5.3.1 A comparative analysis of serum and blood/filter samples in Cutusuma (1987) and Chijipata Alta (1996) using the CL1/IgG4 ELISA.

5.3.1.1 Cutusuma (1987).

Serum samples and blood/filter samples obtained from 57 individuals residing in Cutusuma in 1987 were analysed by IgG4-ELISA using purified CL1 as antigen. The absorbance readings obtained were plotted against each other on a scattergram (Fig. 5.1). Cluster analysis revealed that the scatter of points divided into two clusters, one compact cluster of low absorbance and a diffuse cluster of high absorbance. The cut-off points were set at 3.09 standard deviations from the mean of the low absorbance readings and were 0.2035 and 0.1733 for the serum and blood/filter samples, respectively. Using these cut-off points 41 individuals were deemed seropositive for fasciolosis and 16 individuals deemed seronegative when following sera analysis (Table 5.1). IgG4-ELISA using blood deposited onto filter paper identified 36 individuals as seropositive and 21 individuals as seronegative (Table 5.1). A highly significant relationship was observed between the results obtained with serum and blood/filter samples (r^2=0.848, $p < 0.001$). Chi-squared analysis showed that it is highly unlikely

that such a coincidence in results using the two methods occurred merely by chance ($P< 0.001$).

All coprologically positive individuals, with the exception of two, associated with the seropositive cluster (Fig. 5.1, indicated by solid circles). Chi-squared analysis verified that the individuals with a positive coprological diagnosis were significantly ($P< 0.001$) concentrated in the high absorbance cluster reinforcing the designation of this group as the infected individuals. On the other hand, 22 (38.6%) and 17 (29.8%) individuals that were coprologically negative proved positive by IgG4-ELISA using the serum and blood-filter samples, respectively (Table 5.1).

5.3.1.2 Chijipata Alta (1996).

Serum and blood-filter samples were obtained from eleven individuals at Chijipata Alta and analysed by IgG4-ELISA against CL1. There was a highly significant relationship between the two sampling methods for these patients ($r^2 = 0.983$, $p > 0.001$, Fig. 5.2). Five individuals were identified as seropositive using both serum and blood-filter samples (Table 5.1). Three of these were also coprologically positive for *F. hepatica* eggs, but no faecal samples were obtained from the remaining two individuals. Of the six coprologically negative individuals, five were also serologically negative and the remaining individual did not provide a faecal sample. The results are summarised in Table 5.1.

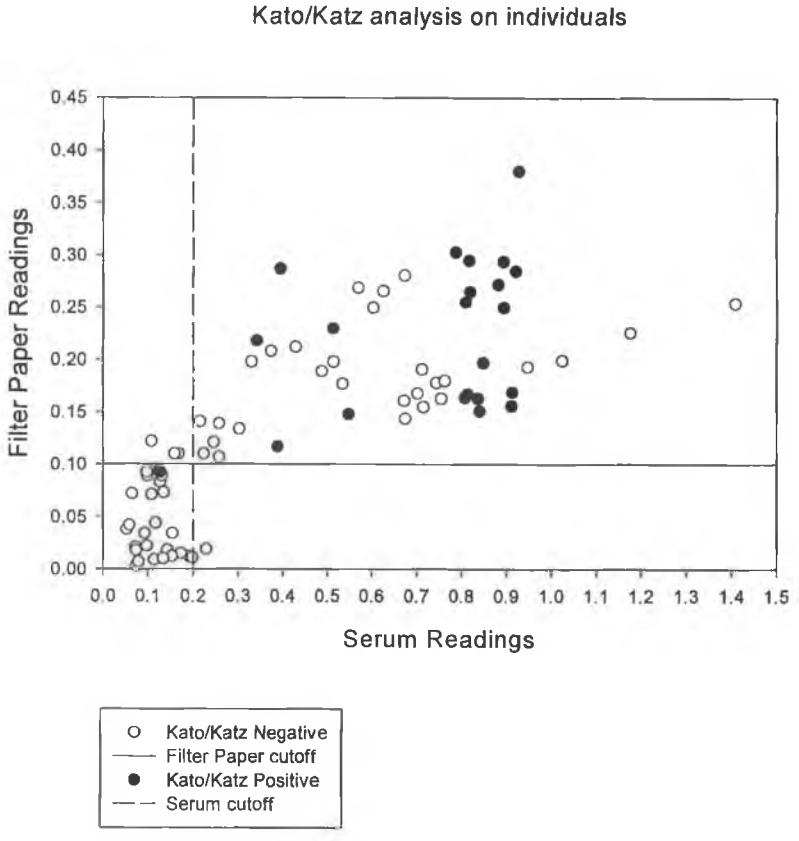


Figure 5.1. Scattergrams of the data obtained by IgG4-ELISA using serum and blood/filter samples obtained from individuals from the village of Cutusuma (1987). Cluster analysis using the combined data obtained with serum and blood-filter samples separated the Cutusuma population into low absorbance (seronegative) and high absorbance (seropositive) sub-populations. The vertical and horizontal lines indicate the calculated cut-off points for the serum and blood/filter samples, respectively. Closed circles represent individuals that were coprologically positive and open circles represent those that were coprologically negative. The triangles represent the five individuals that were seropositive by analysis with serum and seronegative by analysis with blood/filter samples. The results are summarised in Table 5.1.

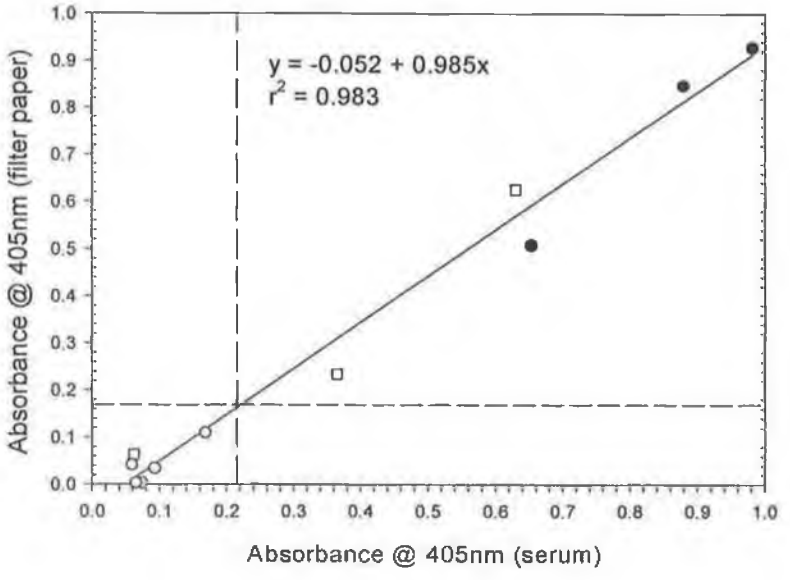


Fig. 5.2. Scattergrams of the data obtained by IgG4-ELISA against CL1 using serum and blood-filter samples obtained from individuals from the village of Chijipata Alta (1996). The vertical and horizontal lines indicate the calculated cut-off points for the serum and blood/filter samples, respectively. Closed circles represent individuals that were coprologically positive and open circles represent those that were coprologically negative. The squares represent the three individuals for which no coprological analysis was available

Table 5.1. Summary of coprological and serological (serum and blood/filter samples) data for individuals from Cutusuma (1987), Chijipata Alta (1996) and Cutusuma (1997).

	Cutusuma '87		Chijipata Alta '96		Cutusuma '97
Group	Serum sample	Filter sample	Serum sample	Filter sample	Filter sample
Copro+/Sero+	19 (33%)	19 (33%)	3	3	34 (51.5%)
Copro-/Sero +	22 (38.6%)	17 (29.8%)	0	0	8 (12.1%)
Copro+/Sero-	2 (3.5%)	2 (3.5%)	0	0	0 (0%)
Copro-/Sero -	14 (24.6%)	19 (33.3%)	5	5	24 (36%)
Total	57	57	8	8	66

Copro = Coprologically; Sero = Serologically.

5.3.2 Analysis of blood/filter samples obtained in Cutusuma (1987).

Blood/filter samples obtained from 67 individuals residing in Cutusuma 1997 were analysed by IgG4-ELISA against native CL1. Using cluster analysis the population was divided into seropositive and seronegative individuals. All coprologically positive individuals were also serologically positive. Eight individuals were identified as being serologically positive and coprologically negative. The remaining twenty four individuals were coprologically and serologically negative. A summary of the data obtained is presented in Table 5.1.

5.3.3 Collated data on the incidence of human fasciolosis in the Bolivian Altiplano from 1987-1997.

Analysis of the sera obtained from the Altiplano region from 1987 to 1997 revealed that 44.3% of the entire sample population tested were serologically positive for fasciolosis (Table 5.2). The seropositive individuals, represented 54.2% at Cutusuma

(1987), 20.2% at Calasaya (1991), 34.4% at Santa Ana (1991), 69.0% at Chijipata Alta (1996) and 63.6% at Cutusuma (1997). The coprological examinations, which detect only chronic infections, were positive for 36.4% of the total population tested. The coprologically positive individuals for each region were 31.7% at Cutusuma (1987), 11.5% at Calasaya (1991), 64.0% at Chijipata Alta (1996) and 49.2% at Cutusuma (1997) (Table 5.2).

The age of the individuals in our population sample ranged between 1-85 years, with a mean of 21+/-17 (Santa Ana was excluded as the age and gender of all individuals were not recorded). The number of serologically and coprologically positive individuals in different age groups were examined. In all communities there was no statistical relationship between the proportion of infected individuals and the age of these individuals. However, if the entire population is divided into two age groups (excluding Cutusuma 1997 since all individuals were children under 13 years) i.e. individuals above and below 16 years, it was shown that using both serological and coprological analysis there is an over-representation ($P<0.001$) of infected individuals in the <16 years old group compared to the ≥16 years old group. In this study the proportion of males infected was not significantly different from that of females infected for any age range studied (data not shown).

Table 5.2. Summary of coprological and serological data from the indigenous population residing in the Bolivian Altiplano from 1987-1997.

Year		Cutusuma 1987	Calasaya 1991	Santa Ana 1991	Chijipata Alta 1996	Cutusuma 1997	Total
Serology	<16 years	57\93 61.3%	7\22 31.8%	\ \	10\15 86.7%	42/66 63.6%	116\196 55.8%
	>16 years	21\51 41.2%	11\67 16.4%	\ \	7\14 50.0%		39\132 29.5%
	Total	78\144 54.2%	18\89 20.2%	11\32 34.4%	17\29 69.0%	42/66 63.6	166\360 44.3%
Coprology	<16 years	24\68 35.3%	5\13 38.5%	\ \	9\12 75.0%	34/66 51.5%	72\159 44.3%
	>16 years	2\14 14.3%	1\39 2.6%	\ \	7\13 53.8%		10\66 15.2%
	Total	26\82 31.7%	6\52 11.5%	\ \	16\25 64.0%	34/66 51.5%	82\225 36.4%

5.4 Discussion

Our IgG4-ELISA is a specific and sensitive method of serologically diagnosing human fasciolosis. In order to develop the ELISA further it was hypothesized that a less invasive means of obtaining blood samples, by lancet-pricking of the finger with subsequent collection of blood onto filter paper and elution of antibodies, would be as empirically accurate as using serum samples.

Serum and blood/filter samples were obtained from individuals in Cutusuma (1987) and Chijipata Alta (1996) and were analysed by IgG4-ELISA using CL1 as antigen. Using cluster analysis the seropositive and seronegative individuals were identified for both communities. In the Cutusuma population the same 19 individuals

were identified as coprologically and serologically positive by both methods. Similarly two individuals were diagnosed as serologically negative and coprologically positive. Using the blood/filter samples, 17 individuals were serologically positive/coprologically negative and 19 individuals were coprologically negative/serologically negative compared to 22 and 14 individuals when serum samples were employed. The long term storage of the sera and blood-filter samples (10 years) may have contributed to the differences observed in diagnosis between both methods of sample collection; the blood/filter samples were stored at 4^0C whereas the sera samples were stored frozen at -20^0C. Nevertheless, our analysis of blood/filter samples from Cutusuma (1987) reveals that blood samples taken onto filter paper can be stored for up to ten years at 4^0C without much deterioration. In the Chijipata Alta survey the IgG4-ELISA using either serum and blood/filter samples provided a similar diagnosis for all individuals. Statistical analysis revealed that there was significant correlation between the results obtained with serum derived from whole blood samples or eluates from blood samples dried onto filter paper ($r^2 = 0.848$, $p < 0.001$ for Cutusuma; $r^2 = 0.983$, $p < 0.001$ for Chijipata).

The method of collecting blood onto filter paper for the serological diagnosis of human fasciolosis is an excellent innovation. It could prove a useful tool for a large scale epidemiological study to determine the extent of human fasciolosis in the Bolivian Altiplano and in other countries where a high prevalence is reported. This method is not only cost effective, an important consideration with such a study, but also allows large scale screening within a relatively short time. However there are several disadvantages associated with this method for example, there is a limit to the number of assays that can be performed and blood titres cannot be measured accurately using

this method (data not shown). Therefore the depletion of circulating anti-fluke antibodies following chemotherapy cannot be monitored.

The development of this method of collecting blood onto filter paper made it possible for a survey to be performed in Cutusuma in 1997 to compare the incidence of human fasciolosis with that which existed in 1987. Using the IgG4-ELISA with native CL1 as antigen the prevalence of fasciolosis in this region was calculated to be 63.6%. The proportion of serologically positive similarly-aged (<16 years) children in Cutusuma in 1987 was 61.3%. These results indicate that human fasciolosis has been endemic in Cutusuma for at least 10 years. This survey also underlines the advantages of using the blood/filter method as a means of obtaining blood samples in a large epidemiological study.

Hillyer *et al.* (1992) and Bjorland *et al.* (1995) have argued that the incidence of human fasciolosis in the Bolivian Altiplano occurs in sporadic outbreaks. However the collective coprological and serological data collated from INLASA and this laboratory suggests that human fasciolosis has been endemic in the Bolivian Altipalno for at least a decade. The studies performed by our laboratory using serological analysis reveal a high prevalence of human fasciolosis of approximately, 54.2% at Cutusuma (1987), 20.2% at Calasaya (1991), 34.4% at Santa Ana (1991), 69% at Chijipata Alta (1996) and 56% at Cutusuma (1997). Over a period of ten years in the entire region 44.3% of all individuals tested were diagnosed as serologically positive.

Coprological analysis, which can detect only chronic infections, was positive for 36.4% of the total population tested. Therefore it provides a false picture of the actual prevalence of human fasciolosis in this region. In this study approximately 8% of individuals diagnosed using coprological analysis were not detected using this method. Therefore in the entire region where an estimated 100,000 individuals are at

risk of infection, an estimated 8,000 individuals would not be correctly identified. This is the principal disadvantage of using coprological analysis in large epidemiological surveys.

In this study, the greater risk from human fasciolosis was statistically associated with children under 16 years. This may be related to an increased exposure to infection as children commonly work in the fields minding livestock where the intensity of transmission is expected to be higher. Children may also be more likely to consume aquatic plants which are contaminated with *F. hepatica* metacecariae. Alternatively, our data could indicate the development of an age related immunological resistance to infection as observed for schistosmiasis haematobium infection in Kenya (Hagan *et al.*, 1991).

In conclusion, analysis of both serum and faecal samples showed a high incidence of human fasciolosis in all communities studied. The highest incidence of infection was observed in the Cutusuma and Chijipata Alta region (Table 5.2). When we consider only those individuals for which we have both serological and coprological data 78% were similarly diagnosed (either as infected or non infected) by both techniques. Seventeen percent were serologically positive but coprologically negative (individuals that may be acutely infected) and the remaining five percent (11 individuals) were coprologically positive but serologically negative. Collectively this IgG4-ELISA employing CL1 as antigen is 95% sensitive which further supports the use of this assay in a large epidemiological survey.

Chapter 6

Cytokine and antibody profile in wild-type and knockout mice infected with *F. hepatica*.

6.1 Introduction

We have demonstrated that anti-fluke antibodies circulating in humans infected with *F. hepatica* are predominantly of the IgG4 subtype. This immunoglobulin subtype is regulated by type II cytokines. To date there have been no reported studies quantitatively measuring cytokine secretion from T-cells in human or animal models infected with fasciolosis. The Bolivian Altiplano is an ideal location to study the immunology of fasciolosis on a human population but this was not logistically possible because of the reluctance of the indigenous population to donate blood. Accordingly, we considered that the utilisation of the murine model would afford us some insight into the immune responses associated with fasciolosis.

6.2 Experimental design

Three to four month old female BALB/c, C57BL/6 and $129^{SV/EV}$ wild-type mice and IFNγR$^{-/-}$ and IL-4$^{-/-}$ knockout mice were employed in this study. The IL-4$^{-/-}$ knockout mice cannot synthesize any IL-4 cytokine therefore it cannot drive a Th2 response. The wild-type mouse for the IL-4$^{-/-}$ knockout mice is the C57BL/6 strain. The IFNγ$^{-/-}$ knockout mice lacks the IFNγ receptor and accordingly while it can produce IFNγ this cytokine cannot drive the Th1 response. The wild-type mouse for the IFNγ$^{-/-}$ knockout is the $129^{SV/EV}$ strain.

In the first experiment four mice from each group were infected orally with five *F. hepatica* metacercariae. Two non-infected mice from each group served as controls.

Three weeks post infection all mice were sacrificed by cervical dislocation and the spleens removed. Isolated spleen cells were stimulated *in vitro* with LFH. Supernatant samples were removed after 24 hours to measure IL-2 whereas IL-4, IL-5 and IFNγ were measured after 72 hours. Serum was obtained via cardiac puncture and circulating anti-fluke IgG1 and IgG2a antibodies were measured.

In the second experiment, eight mice of each strain were infected with 15 *F. hepatica* metacercariae. In addition, two mice from each group were included in the study as controls. Three weeks later all mice were sacrificed, and the spleen cells isolated and stimulated *in vitro* with LFH. Similar to the previous experiment IFNγ, IL-2, IL-4 and IL-5 cytokines in supernatant were quantified and circulating anti-fluke IgG1 and IgG2a antibodies were measured.

In the third experiment, four BALB/c mice were infected with 10 metacercariae. Four additional mice were included in the study as controls. Three weeks post initial infection all mice were killed and their spleens, hepatic lymph nodes (HLN), mesenteric lymph nodes (MLN) and superficial lymph nodes (SLN) removed. Cells isolated from these tissues were then stimulated *in vitro* with LFH and then IFNγ, IL-2, IL-4 and IL-5 cytokines in the supernatant measured.

6.3 RESULTS

6.3.1 Cytokine production by spleen cells of mice infected with five metacercariae of *F. hepatica*.

Four female, C57BL/6, $129^{SV/EV}$, BALB/c, IFNγR$^{-/-}$ knockout and IL-4$^{-/-}$ knockout mice were infected with five *F. hepatica* metacercariae. Three weeks later all mice were sacrificed and their livers examined for signs of infection i.e. migratory tracts and pathology. Spleen cells were isolated and stimulated with LFH *in vitro*. Subsequently, the amount of Th1 cytokines, IFNγ and IL-2, and Th2 cytokines, IL-4 and IL-5, secreted into culture supernatants were quantified (Fig. 1A -D).

Spleen cells from C57BL/6 mice produced IL-5, IFNγ and low levels of IL-2 and IL-4 cytokines. Cells of $129^{SV/EV}$ mice produced higher levels of IL-4, IL-5 and IL-2 cytokines but no significant levels of IFNγ. The cells from BALB/c mice produced high levels of IL-4, medium levels of IL-5, low levels of IL-2 and no significant IFNγ (Fig. 1A-D). As expected, the cells from IFNγR$^{-/-}$ knockout mice secreted medium levels of IL-4, high levels of IL-5 but low levels of IL-2 and IFNγ. In contrast, and also as expected, the cells of IL-4$^{-/-}$ knockout mice produced IFNγ and IL-2 cytokines but low levels of IL-5 and no IL-4 (Fig. 1A-D).

When spleen cells from mice infected in 5 *F. hepatica* metacercariae were stimulated *in vitro* with PMA/anti-CD3 there was no generalised polarisation of the immune response towards a Th1 or Th2 response in the C57BL/6, $129^{SV/EV}$, BALB/c or IFNγR$^{-/-}$ ($129^{SV/EV}$) knockout mouse strain. However, when spleens cells from IL-4$^{-/-}$

(C57BL/6) knockout mice were stimulated *in vitro* with PMA/anti-CD3 the cytokine response was skewed towards a Th1 cytokine response.

For all groups of mice, the *in vitro* cytokine production by spleen cells was higher when the cells were stimulated with 100 μg/ml LFH as compared with 20 μg/ml. Spleen cells obtained from non-infected mice of each strain did not produce cytokines in response to stimulation with LFH.

Fig. 6.1A IL-4

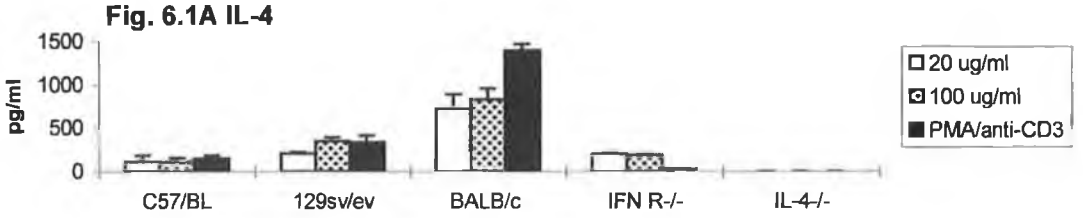


Fig. 6.1B IL-5

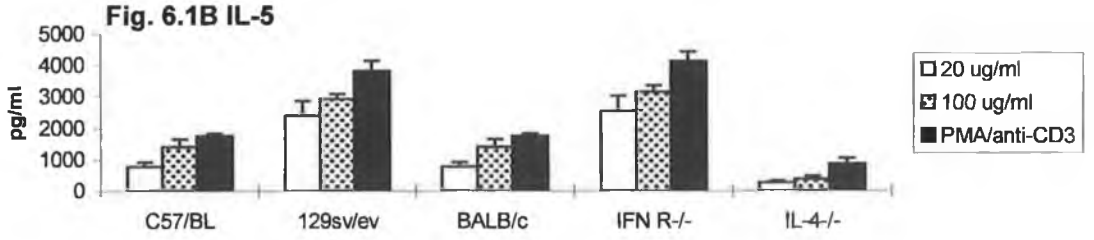


Fig 6.1C IFNγ

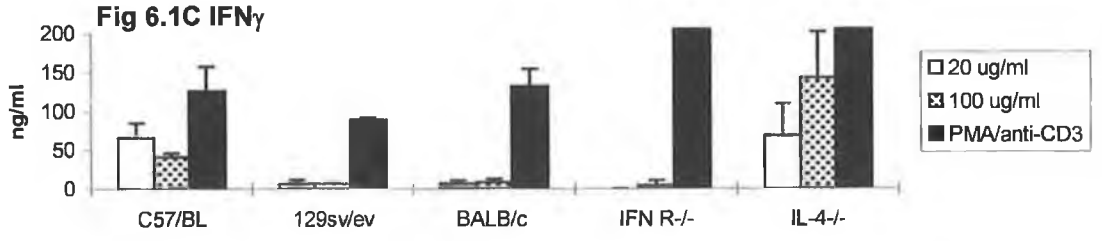


Fig. 6.1D IL-2

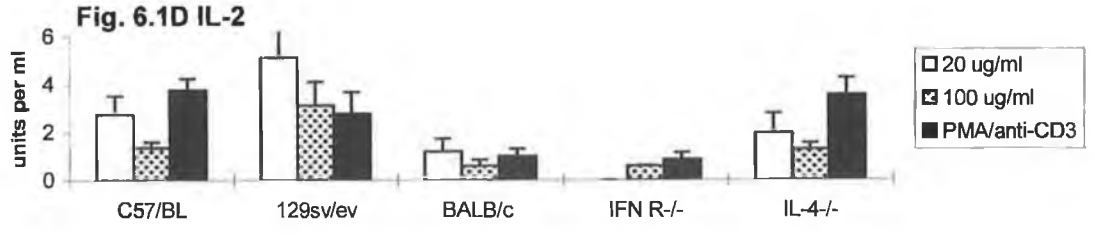


Fig. 6.1 Cytokine release from spleen cells after *in vitro* stimulation with 20 and 100 μg/ml of LFH and a positive control PMA/anti-CD3. IL-4 (Panel A), IL-5 (Panel B), IFNγ (Panel C) and IL-2 (Panel D) were measured in supernatants following stimulation of spleen cells from five different strains of mice (BALB/c, C57BL/6 and $129^{SV/EV}$, $IFN\gamma R^{-/-}$ and $IL\text{-}4^{-/-}$ mice strains) infected three weeks previously with 5 *F. hepatica* metacercariae. The bars represent the standard error of the means of four mice.

6.3.2 Cytokine production by spleen cells of mice infected with 15 *F. hepatica* metacercariae.

Spleen cells were isolated from eight female C57BL/6, $129^{SV/EV}$, BALB/c, $IFN\gamma R^{-/-}$, $IL\text{-}4^{-/-}$ mice three weeks post infection with 15 *F. hepatica* metacercariae. The cytokines released *in vitro* following stimulation with LFH were then quantified. The most striking difference observed in the cytokine production by spleen cells obtained from these mice as compared to cells from those infected with five metacercariae was the almost complete lack of secretion of the type I cytokines, IL-2 or IFNγ. This suppression was apparent in all mouse strains used but was particularly evident in the $IL\text{-}4^{-/-}$ mice which are only capable of producing these cytokines. Moreover, the suppression of type I cytokine production was observed even when the spleen cells were stimulated with the mitogen PMA/anti-CD3. While the higher infection dose also suppressed the production of IL-4, this suppression was less marked than that observed for IL-2 or IFNγ. In contrast, production of the type II cytokine IL-5 by the spleen cells of mice given a high infection

dose was not significantly different from that modulated by cells of mice given a low-dose infection.

Fig. 6.2A IL-4

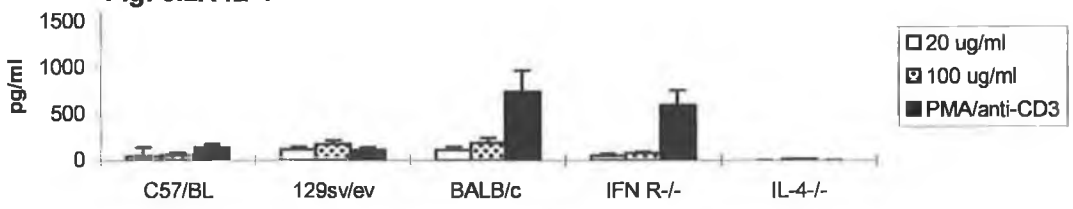


Fig. 6.2B IL-5

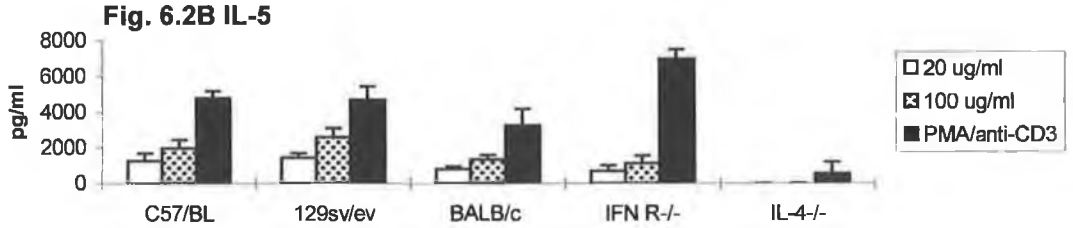


Fig. 6.2C IFNγ

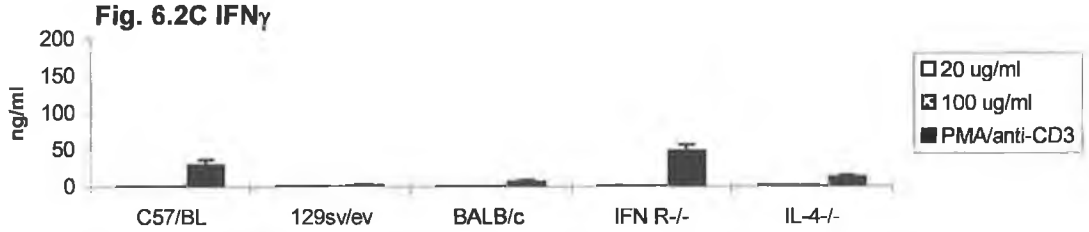


Fig. 6.2D IL-2

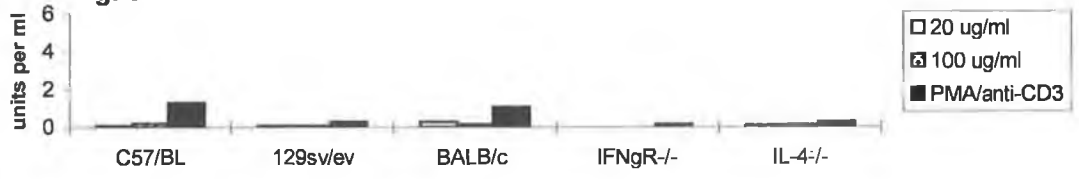
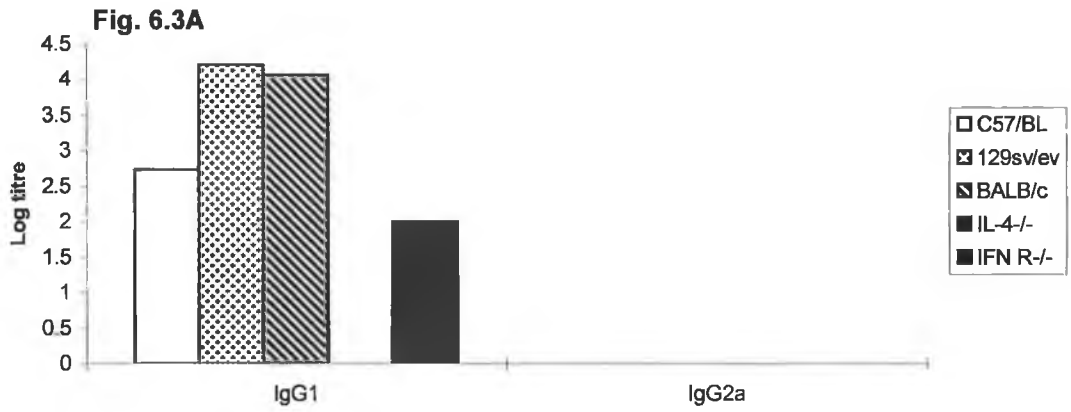


Fig. 6.2 Cytokine production was assessed in the supernatants of spleen cells derived from five different strains of mice (BALB/c, C57BL/6 and $129^{SV/EV}$, $IFN\gamma R^{-/-}$ and $IL\text{-}4^{-/-}$) infected three weeks previously with 15 *F. hepatica* metacercariae. IL-4 (Panel A), IL-5 (Panel B), IFNγ (Panel C) and IL-2 (Panel D) cytokines were measured from supernatant from spleen cells stimulated *in vitro* with 20 and 100 μg/ml of liver fluke homogenate and a positive control PMA/anti-CD3. The bars represent the standard error of the means of eight mice.

6.3.3 IgG1 and IgG2a responses in *F. hepatica*-infected mice

The titres of IgG1 and IgG2 antibodies were measured in all mice three weeks after infection with 5 or 15 *F. hepatica* metacercariae. Antibodies of the IgG1 subtypes were detected in all mouse strains with the exception of the $IL\text{-}4^{-/-}$ knockout mice which cannot produce IgG1 because they lack IL-4. There was no IgG2a antibodies detected in the serum of any mouse strain (Figure 3). The IgG1 titres observed in the groups infected with five metacercariae (Fig. 3A) were similar to those observed in mice infected with 15 metacercariae (Fig. 3B).



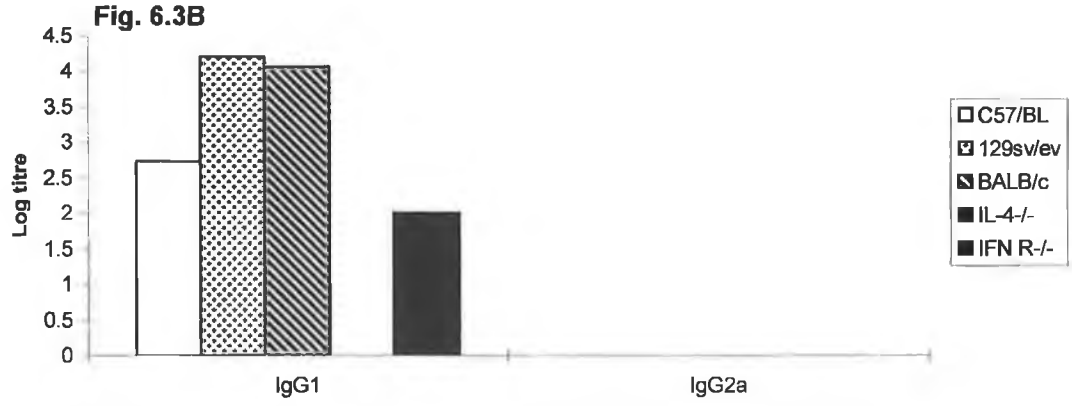
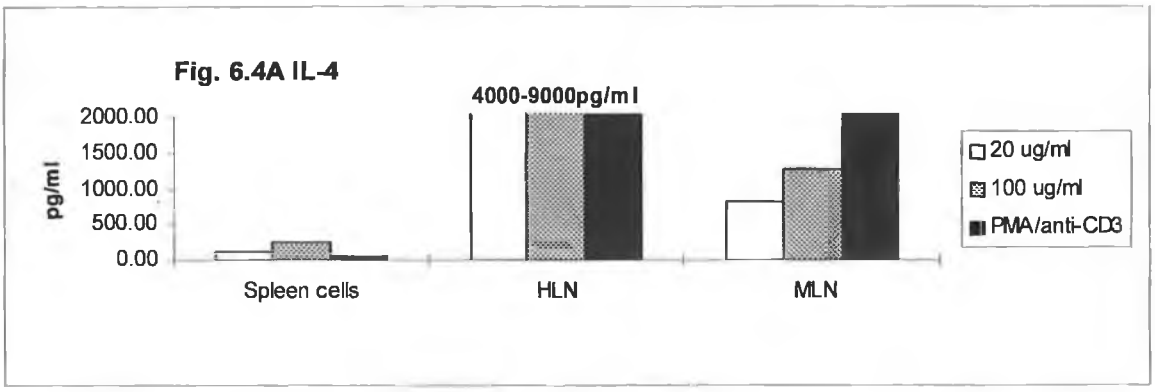
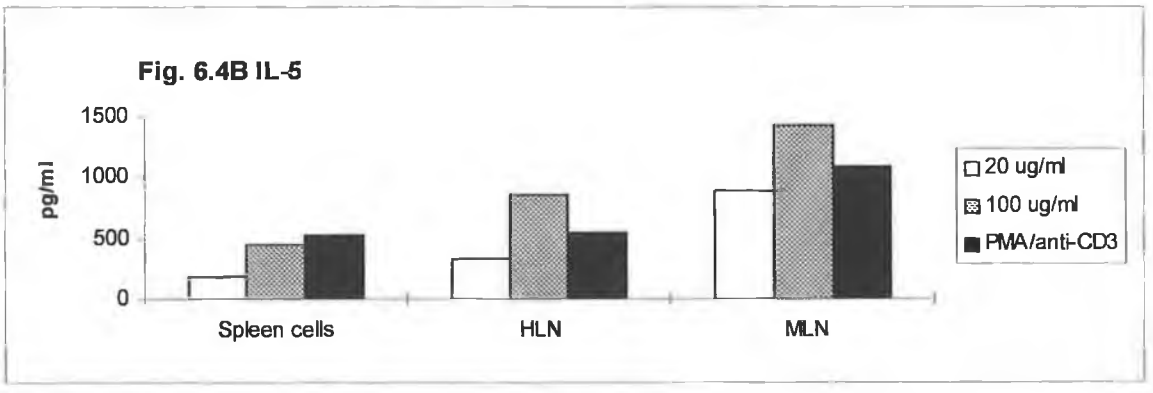


Fig. 6.3 Serum IgG1 and IgG2a antibodies specific for liver fluke excretory /secretory products, in mice infected with *F. hepatica*. Antibodies were measured three weeks after infection with a dose of 5 (6.3A) or 15 *F. hepatica* metacercariae (6.3B). A minimum of four mice from the low dose experiment and eight mice from the high dose experiment were assayed.

6.3.4 *In vitro* production of cytokines by T-cells of the peripheral lymph nodes and spleens of BALB/c mice infected with *F. hepatica*.

Cells were isolated from the spleens, HLN, MLN and SLN of BALB/c mice three weeks after an infection with 10 *F. hepatica* metacercariae. Isolated spleen cells were stimulated *in vitro* with LFH and PMA/anti-CD3 and the secretion of cytokines IL-4, IL-5 and IFNγ in the supernatant were quantified. Spleen, HLN and MLN cells secreted a predominant Th2 cytokine profile i.e. high levels of IL-4 and IL-5 and no secretion of IFNγ was observed. The amount of IL-4 cytokine secreted was significantly higher ($p > 0.5$) in the HLN and MLN when compared to the amount secreted by spleen cells also, HLN produced almost a nine-fold greater amount of IL-4 than the MLN. In contrast, cells from the MLN secreted significantly greater amounts of IL-5 when compared to that secreted by cells from the HLN and spleen. When spleen and lymph node cells were stimulated *in vitro* with PMA/anti-CD3 a predominant Th2 cytokine profile was exhibited. There was no significant cytokine production observed in the SLN in response to stimulation with LFH or PMA/anti-CD3.





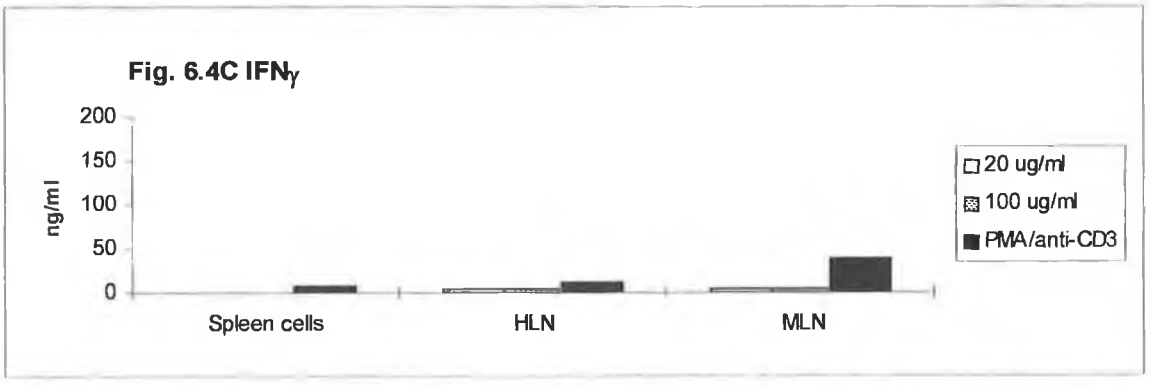


Fig. 6.4. Liver Fluke specific production of cytokines measured from supernatant stimulated *in vitro* with 4 μg/ml, 20 μg/ml and 100 μg/ml of liver fluke homogenate and PMA/anti-CD3. IL-4 (Panel A), IL-5 (Panel B), and IFNγ (Panel C) were measured in supernatants following stimulation of spleen or lymph node cells from BALB/c mice infected three weeks previously with 10 *F. hepatica* metacercariae. The spleen cells from each mouse were assayed individually, whereas lymph node cells were pooled.

6.4 Discussion

In most helminth infections a type II immune response predominates with the secretion of IL-4, IL-5, IL-6, IL-10 and IL-13 cytokines. The associated immunological elements include eosinophilia, masocytosis, elevated sera IgE antibodies and IgG4 (Humans) /IgG1 (Rodents) (Mulcahy, Joyce and Dalton, in press; Pearce *et al.*, 1996). In the murine model evidence supporting the association of a Th2 cytokine profile with fasciolosis is limited to a few studies. Van de Heijden *et al.* (1995) reported a type II cytokine response in lamina propria lymphocytes of mice infected with *F. hepatica*. In this study secretion of type II cytokine profile, IL-4 and IL-5, was associated with elevated total IgE antibodies in serum. However, cytokine secretion was not quantitatively measured and was limited to the study of local gut mucosal tissue. Other studies have identified eosinophilia in both the peripheral blood and bone marrow cells in *F. hepatica* infected mice (Milbourne and Howell, 1990; 1993). In addition, eosinophil attachment was observed on the surface of adult flukes in mice within four hours after implantation into the peritoneum (Rajasekariah *et al.* 1979). Since a Th2 class of CD4+ T-cells are involved in eosinophil differentiation and maturation, these studies indicate that a Th2 cytokine response would be associated with *F. hepatica* infection in mice.

Type II CD4+ T-cells produce IL-4, IL-5, IL-13 and IL-10 resulting in a strong antibody-mediated immune response. In contrast, type I T-cells produce IL-2, IFNγ and lymphotoxin resulting in a delayed hypersensitivity reaction. These two subsets counter-regulate each other through cytokines unique to each subset. The Th1 cytokine, IFNγ

inhibits the proliferation of Th2 cells whereas IL-10 inhibits the synthesis of Th1 cytokines. In the present chapter five different groups of mice (BALB/c, C57BL/6 $129^{SV/EV}$, $IFN\gamma R^{-/-}$ and $IL\text{-}4^{-/-}$ mice strains) were infected with 5 and 15 *F. hepatica* metacercariae to determine the immune response associated with fasciolosis. In addition we compared local and systemic immune responses in BALB/c mice infected with 10 *F. hepatica* metacercariae.

Spleen cells from both BALB/c and $129^{SV/EV}$ mice infected with five metacercariae elicited a strong Th2 cytokine response when stimulated *in vitro* with LFH. When spleen cells from infected C57BL/6 mice were stimulated *in vitro* with LFH a cytokine profile characteristic of a Th0 cytokine response was observed (high IL-4, IL-5 and IFNγ). C57BL/6 mice have been described as a "type I mouse" whereas BALB/c mice have been described as a "type II mouse" because each mouse strain was shown to be genetically predisposed to produce a more type I or type II immune response, respectively. Therefore it is unsurprising that BALB/c mice secreted the greatest amount of IL-4 cytokine or that C57BL/6 mice exhibit a Th0 cytokine profile. When spleen cells from all three strains of infected mice were stimulated *in vitro* with PMA/anti-CD3 there was no generalised polarisation of the immune response towards a predominant Th2 cytokine profile.

Spleen cells from all three wild-type mice infected with 15 *F. hepatica* metacercariae elicited a strong Th2 cytokine response; secretion of IL-2 and IFNγ cytokines from these cells was totally inhibited. Spleen cells from these mice also exhibited a generalised suppression of IL-4 secretion but no significant difference in the secretion of IL-5 was observed. Mitogen stimulated spleen cells from these infected mice exhibited a generalised polarisation of the Th-subset towards a Th2 cytokine profile.

These observation may indicate that *F. hepatica* secretes molecules that down-regulate a Th1 response. This would explain the difference observed in the cytokine profile released by spleen cells of mice that received 5 metacercariae as compared to those cells of mice that received 15 metacercariae. The increase in the number of flukes present correlates with increased suppression of Th1 cytokines. Moreover, the spleen cells from the "type I" C57BL/6 mice switched from producing a mixed Th0 cytokine in the lower infection dose to a predominant Th2 cytokine profile in the higher infection dose.

IFNγR$^{-/-}$ mice, which cannot drive a Th1 response, were infected and the cytokine profile quantified. As expected these mice produced IL-4 and IL-5, but the level of these cytokines did not differ from the wild-type 129$^{sv/ev}$ controls. This was observed when mice were infected with 5 or 15 *F. hepatica* metacercariae. However, when the spleen cells of the mice that were given the high infection dose were stimulated with PMA/anti-CD3 we observed that they produced significantly higher levels of the Th2 cytokines than the wild-type controls. These observations are as expected in an infection that causes Th2 immune responses.

In a reciprocal-like experiment we infected IL-4$^{-/-}$ knockout mice that are unable to synthesize IL-4 cytokine. As anticipated there was no production of IL-4 cytokine by these mice and reduced levels of IL-5, compared to wild-type controls, were produced. This result was in agreement with experiments performed by Pearce *et al.* (1996) and Lawrence *et al.* (1994) which showed that whilst IL-5 production is not dependent on IL-4, it's production is enhanced when this cytokine is present. Moreover, it stresses that Th2 responses are not totally dependent on IL-4. The other interesting observation was the fact that IFNγ production in the IL-4$^{-/-}$ knockout mice was significantly higher than in

the wild-type when these were infected with five *F. hepatica* metacercariae suggesting that a down-regulation of Th1 responses is dependent upon IL-4.

When the infection dose was increased to 15 *F. hepatica* metacercariae we observed total inhibition of IFNγ and IL-2 cytokine production. Therefore, liver fluke may secrete immunosuppressive molecules that protect it from immune attack. The secretion of immunosuppressive molecules that block lymphocyte population was previously reported by Chauvin *et al.* (1995) and Chervi and Masih (1997). However, it is clear that infection had no effect on IL-5 production suggesting that this cytokine may be produced by cells other than those cell populations that produce IFNγ, IL-2 and IL-4 (which are produced by CD4+ cells). Several reports have demonstrated that non-CD4+ cells can secrete either Th1 or Th2 cytokines and can contribute to the nature of the *in vivo* immune response. An IL-5 response which is Th2-like may result from activation and expansion of other cell populations such as CD8+ cells, B-cells and eosinophils (Reiner and Locksley, 1993). The significance of the lack of IL-5 blocking is not yet understood but it is interesting to note that this cytokine is involved in eosinophil production. To date there has been no clear demonstration in fasciolosis of the susceptibility of parasites to eosinophils and therefore suppression of these cells may not be necessary.

The regulation of immunoglobulin subclasses by subsets of antigen specific helper T-cells has been previously reported by Stevens *et al.* (1988) and Purkerson and Isakson (1992). Studies performed in the murine model infected with helminth parasites such as schistosomiasis (Caulada-Benedetti *et al.*, 1991) and filariasis (Lawrence *et al.*, 1994) have demonstrated that IL-4 is associated with the secretion of IgG1 and IgE whereas IFNγ is associated with secretion of IgG2a. In agreement with these studies we found that

all mice exhibiting a predominant Th2 cytokine response had anti-fluke IgG1 antibodies present in their serum. There was no significant difference in the antibody production in mice that received 5 or 15 *F. hepatica* metacercariae.

In our final experiment we investigated if the immune response in the local lymph nodes is different from that found in the spleen cells of mice infected with *F. hepatica*. We chose to examine both HLN and MLN nodes since newly excysted juvenile liver flukes burrow through the gut wall, which is drained by the MLN, before migrating across the liver parenchyma for several weeks, which is drained by the HLN. We found that HLN, MLN and spleen cells all secreted Th2 cytokines. The induction of Th2 cytokines is highest at the intestine and hepatic tissue site as the draining lymph nodes (MLN and HLN, respectively) secrete significantly higher amounts of IL-4 and IL-5 when compared to that secreted by spleen cells. However the HLN produced a nine-fold greater amount of IL-4 when compared to the MLN. In contrast, cells from the MLN produces significantly greater amounts of IL-5. Therefore, as the liver fluke progresses through its developmental stages it may secrete molecules that induce a Th2 cytokine profile in the draining lymph nodes, although the pattern of the cytokines produced by these differs. This difference may reflect the secretion of different antigens as the parasite matures and develops. It is well known that changes takes place in the surface tegument as the parasite migrates. The main tegumental secretory antigens which make up the surface glycocalyx are derived from T0 cells in the newly excysted juvenile flukes whereas the glycocalyx is synthesised by T1 cells in the juvenile fluke during the hepatic stages. It would be of future interest to take MLN and HLN at various times during infection and determine at what point these lymphoid cells are stimulated to produce a Th2 cytokine profile. In this

study we only examined the cells at a single time point (i.e. 21 days after infection) and therefore we were looking at the "composite" response in the lymph nodes.

Studies on other helminth parasites have investigated the responses in the lymph nodes that are close to the infection or antigen-immunisation site. Pearce *et al.* (1996) demonstrated that the injection of *S. mansoni* egg antigen into the foot pad of mice induced a Th2 response in the local popliteal lymph node. Furthermore infection of mice with *B. malayi* induced Th2 responses in the lymph nodes close to the injection site, although a Th0 response was observed in the spleens (Pearlman *et al.*, 1993). These studies suggest that the early induction of Th2 responses in the local lymph nodes may be important in dictating the overall response to infection. In this study we found that once a Th2 response has developed the spleen cells respond in a Th2-like fashion even to the mitogen PMA/anti-CD3.

The role of type II cells in the protection of the host against helminth infections is controversial. A type II response is important for the control of the intestinal nematode, *Trichuris muris*, in mice (Bancroft *et al.*, 1998), whereas Type I $CD4^+$ T-cells are involved with protection in mice vaccinated with irradiated schistosoma larvae (Pearce *et al.*, 1996). The functional role of type II cells in fasciolosis has yet to be identified. However, recent evidence from a vaccine study performed in cattle has described the involvement of IgG2 antibody subtypes in protection while IgG1 secretion has been linked to susceptibility (Mulcahy *et al.*, 1997). Therefore the study of Mulcahy *et al.* (1997) provides evidence that a type I response may have an important role in the protection of *F. hepatica* infected cattle (refer to section 9.1).

In conclusion, this investigation is the first to quantitatively measure cytokine production of spleen cells, lymph node cells and circulating IgG subtypes in the murine model infected *F. hepatica* metacercariae. We have observed that a liver fluke infection induces a Th2 response in mice. The molecules involved in inducing this type II response has not been identified but we hypothesis that molecules of ES products may be important. In the following chapters we have exploited the murine model for fasciolosis to investigate this hypothesis.

Chapter 7

The effect of an infection with *Fasciola hepatica* on the Th1 responses induced in mice vaccinated with *Bordetella pertussis.*

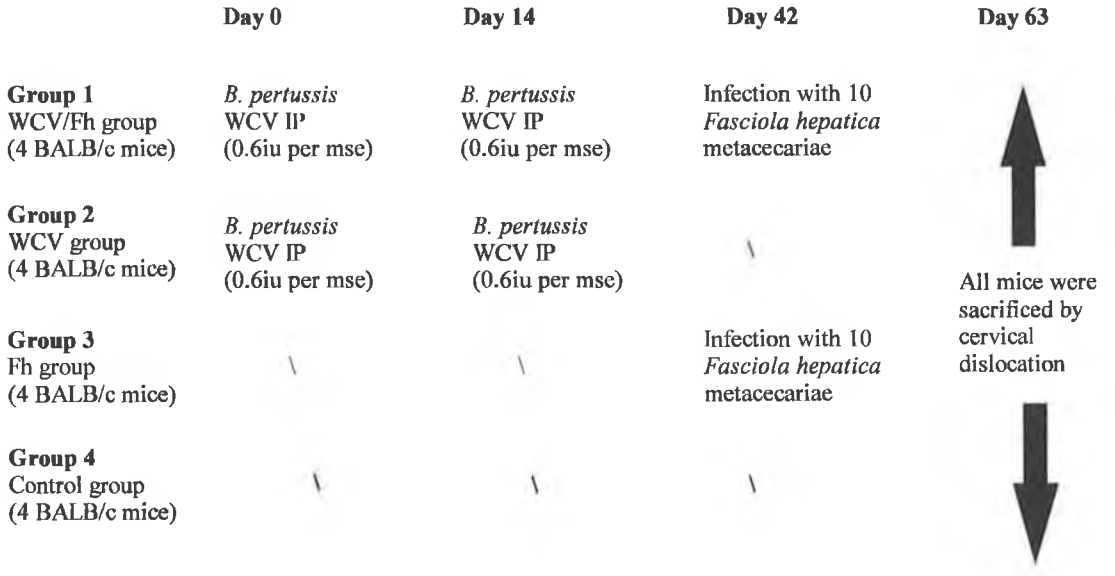
7.1 Introduction

The control of disease and the efficacy of vaccines is dependent upon the type of CD4+ subset response induced. Protection in humans and animals against bacterial, viral and unicellular parasitic diseases is primarily associated with the induction of a type I immune response whereas helminth infections are predominantly associated with a type II immune response. Several reports have demonstrated that, in both human and animal studies, a prior infection which induces a strong type II cytokine profile has an impact on the immune response elicited against a non-parasitic antigen (Kullberg *et al.*, 1992; Sabin *et al.*, 1996). A corollary of this observation could be that helminth infections which involve type II responses can effect the immune responses to vaccines that require type I responses.

In Chapter 6 we demonstrated that spleen cells from mice infected with *F. hepatica* secrete the type II cytokines, IL-4 and IL-5, on stimulation *in vitro* with LFH antigen. In contrast, T-helper cells from mice vaccinated with *B. pertussis* vaccine secrete the type I cytokines, IFNγ and IL-2, on stimulation with *B. pertussis* antigen (BP) *in vitro* (Mills *et al.*, 1993; Redhead *et al.*, 1993). In this chapter we investigated the effect of an *F. hepatica* infection on an established type I response in mice vaccinated with *B. pertussis* whole cell vaccine. Since IL-4 is a key cytokine that promotes type II cytokine secretion we investigated the role of IL-4 by repeating this experiment in IL-4 knockout C57BL/6 mice.

7.2 Experimental Design

7.2.1 Experimental design 1



In this experiment eight 3-4 month old female BALB/c mice were vaccinated with an intra-peritoneal injection of *B. pertussis* whole cell vaccine on day 0 and day 14 (0.8 IU/ mouse). On day 42, four of these mice were infected with 10 *F. hepatica* metacecariae (Group 1) while the other four mice were left uninfected (Group 2). Four additional mice did not receive any vaccinations but were infected on day 42 with *F. hepatica* (Group 3). Four mice acted as background controls, receiving no vaccination and no liver fluke infection (Group 4). All mice were sacrificed on day 63 and their spleens removed. Isolated spleen cells were stimulated *in vitro* with LFH and BP and the cytokines secreted into the supernatant quantified.

7.2.2 Experimental design 2

Wild-type controls:	**Day 0**	**Day 14**	**Day 42**	**Day 63**
C57BL/WCV/ (4 mice)	*B. pertussis* WCV IP (0.6iu per mse)	*B. pertussis* WCV IP (0.6iu per mse)	Infection with 10 *Fasciola hepatica* metacecariae	
C57BL/WC (4 mice)	*B. pertussis* WCV IP (0.6iu per mse)	*B. pertussis* WCV IP (0.6iu per mse)		
IL-4 knockout mice:				
IL-4$^{-/-}$WCV/Fh (4 mice)	*B. pertussis* WCV IP (0.6iu per mse)	*B. pertussis* WCV IP (0.6iu per mse)	Infection with 10 *Fasciola hepatica* metacecariae	
Il-4$^{-/-}$WCV (4 mice)	*B. pertussis* WCV IP (0.6iu per mse)	*B. pertussis* WCV IP (0.6iu per mse)		

In this experiment eight C57BL/6 IL-$4^{-/-}$ knockout mice and their wild-type controls were vaccinated with an intra-peritoneal injection of *B. pertussis* whole cell vaccine (0.8 IU/mouse) on day 0 and day 14. On day 42, four mice from each group were infected with 10 *F. hepatica* metacercariae. All mice were killed by cervical dislocation on day 63 and their spleens removed. Isolated spleen cells were stimulated *in vitro* with LFH and BP and the cytokines released into the supernatant quantified.

7.3 Results

7.3.1 *In vitro* cytokine production by spleen cells of BALB/c mice previously vaccinated with *B. pertussis* whole cell vaccine and infected with *F. hepatica*.

To examine whether an infection of *F. hepatica* had an effect on an established type I response in mice, we vaccinated 3-4 month old female BALB/c mice with *B. pertussis* whole cell vaccine and followed it with an *F. hepatica* infection. Mice were sacrificed 21 days following a challenge infection and isolated spleen cells were stimulated *in vitro* with LFH or BP antigen and the amount of IL-4, IL-5, IL-2 and IFNγ cytokines that were secreted into the culture media measured.

Spleen cells from naïve mice (Controls, Group 4) did not secrete either TH1 (IL-2, IFNγ) or TH2 (IL-4, IL-5) cytokines in response to stimulation with LFH or BP antigen (Fig. 7.1A-D and Fig. 7.2A-D, respectively). Spleen cells of BALB/c mice that only received liver fluke infection (Group 3) exhibited a predominant type II response to LFH antigen, where significant amounts of IL-4 and IL-5 cytokine were secreted and there was no secretion of IFNγ or IL-2 cytokines (Fig 7.1A-D). Spleen cells of these mice did not secrete any cytokines in response to stimulation with BP antigen (Fig. 7.2A-D).

Spleens cells from the mice that received the *B. pertussis* whole cell vaccine only (Group 2) did not secrete any cytokines following stimulation with LFH (Fig. 7.1A-D). However, these mice secreted IL-2 and IFNγ cytokines with no significant amount of IL-4 and IL-5 cytokine in response to BP antigen (Fig. 7.2A-D).

Spleen cells of mice that were vaccinated with *B. pertussis* whole cell vaccine and then infected with *F. hepatica* (Group 1) showed no statistical difference in their cytokine production following stimulation with LFH antigen to that of spleen cells from mice that were infected with *F. hepatica* only (Group 3) (i.e. high IL-4 and IL-5 production and no IFNγ or IL-2, Fig. 7.1A-D). However, most importantly, the spleen cells of these *B. pertussis* whole cell vaccinated and fluke infected mice (Group 1) showed a significant reduction ($P < 0.05$) of type 1 cytokines, IFNγ and IL-2, following stimulation with BP antigen when compared to spleen cells from mice that received the vaccine only (Group 2) (Fig. 7.2A-D). When spleen cells from vaccinated infected mice were stimulated *in vitro* with PMA/anti-CD3 a predominant Th2 cytokine profile was observed.

Fig. 7.1A IL-4

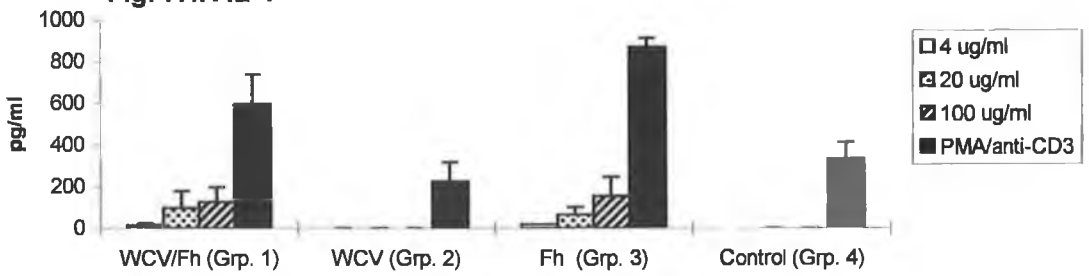


Fig. 7.1B IL-5

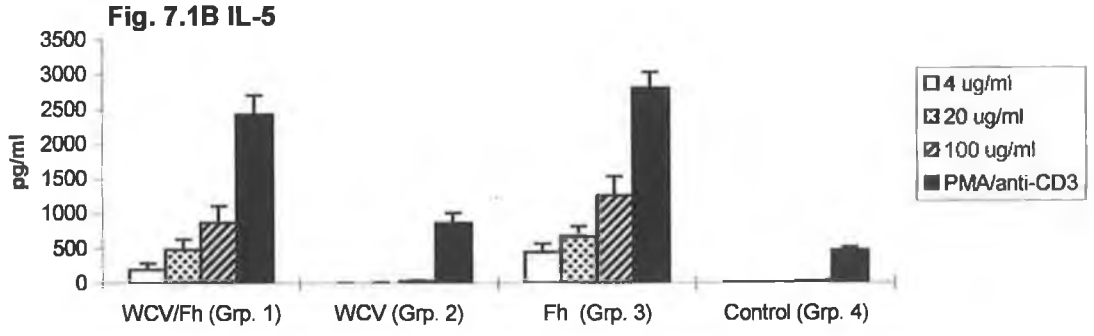


Fig. 7.1C IFNγ

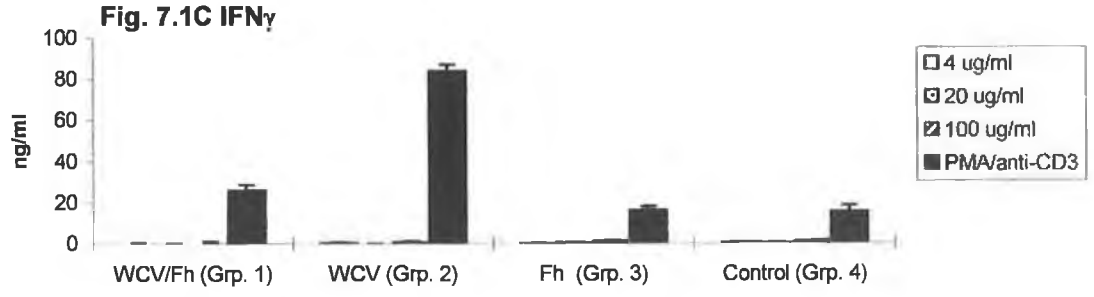


Fig. 7.1D IL-2

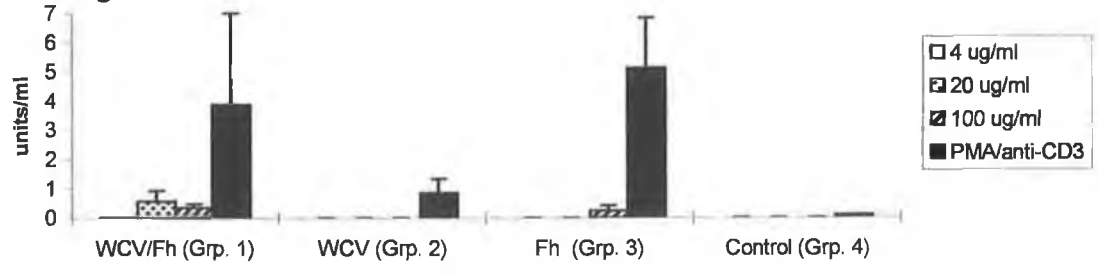


Figure 7.1 Measurement of the cytokine production of spleen cells from four different groups of BALB/c mice following stimulation with LFH (Group 1: mice that were vaccinated with *B. pertussis* whole cell vaccine and infected with *F. hepatica* (WCV/Fh); Group 2: mice that received *B. pertussis* whole cell vaccine only (WCV); Group 3: mice that were infected with *F. hepatica* only (Fh); Group 4: mice receiving neither *B. pertussis* whole cell vaccine or *F. hepatica* infection (Control)). Spleen cells were cultured with LFH (4 μg/ml, 20 μg/ml, 100 μg/ml) and PMA/anti-CD3. IL-2 was measured after 24 hours and IL-5, IL-4 and IFNγ were measured after 72 hours. Results are the means of assays from four individual mice.

Fig. 7.2A IL-4

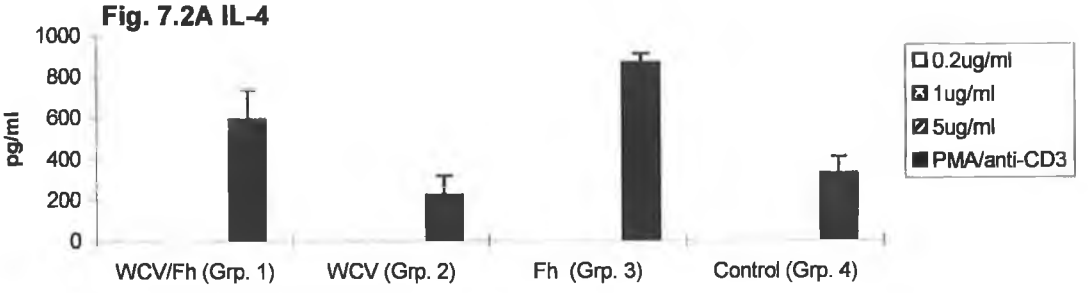


Fig. 7.2B IL-5

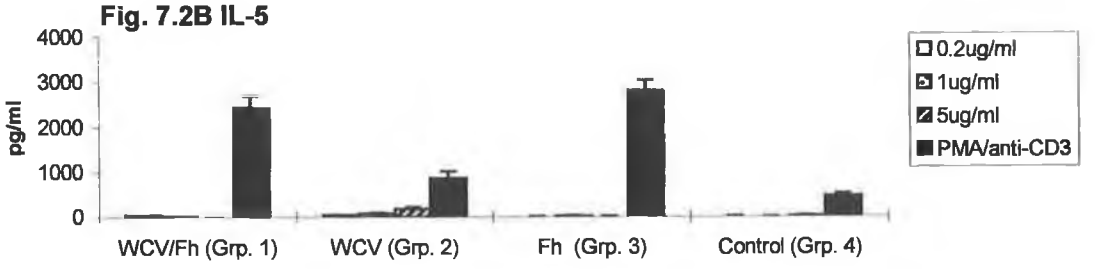


Fig. 7.2C IFNγ

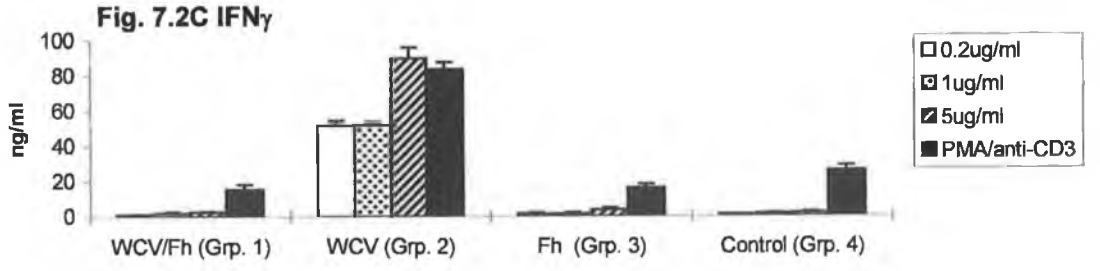


Fig. 7.2D IL-2

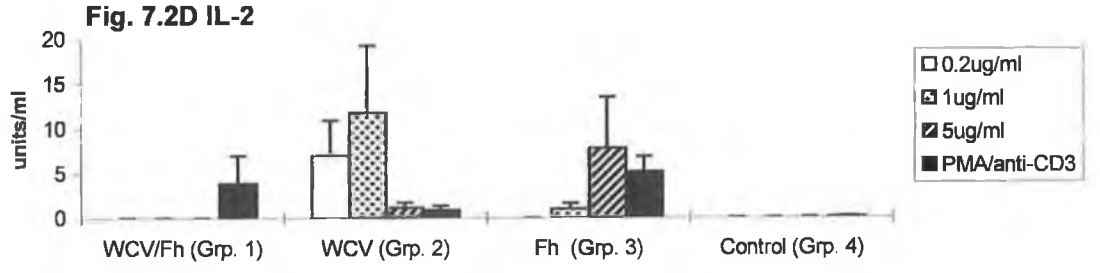


Figure 7.2 Measurement of the cytokine production of spleen cells from four different groups of BALB/c mice following stimulation with BP (Group 1: mice that were vaccinated with *B. pertussis* whole cell vaccine and infected with *F. hepatica* (WCV/Fh); Group 2: mice that received *B. pertussis* whole cell vaccine only (WCV); Group 3: mice that were infected with *F. hepatica* only (Fh); Group 4: mice receiving neither *B. pertussis* whole cell vaccine or *F. hepatica* infection (Control)). Spleen cells were cultured with BP (0.2 μg/ml, 1 μg/ml, 5 μg/ml) and PMA/anti-CD3. IL-2 was measured after 24 hours and IL-5, IL-4 and IFNγ were measured after 72 hours. Results are the means of assays from four individual mice.

7.3.2 Cytokine production by spleen cells of wild-type and IL-4 knockout C57/BL mice vaccinated with *B. pertussis* whole cell vaccine and infected with *F. hepatica*.

We have demonstrated in the first experiment that a type I response elicited in mice by vaccination with *B. pertussis* whole cell vaccine is suppressed by a subsequent infection with *F. hepatica*. To investigate the role of IL-4 in the up-regulation of type II immune responses, we repeated the experiment in wild-type and IL-4 knockout C57BL/6 mice. In this study we vaccinated eight IL-4 knockout mice and eight wild-type controls with *B. pertussis* whole cell vaccine. Four mice in each group were then subsequently infected with 10 *F. hepatica* metacecariae. All mice were sacrificed 21 days after challenge infection and their spleen cells cultured *in vitro* with LFH and BP antigen. Cytokine production in the supernatants was quantified. Interleukin 2 cytokine was measured after 24 hours whereas IL-4, IL-5 and IFNγ cytokines were measured after 72 hours.

Spleen cells of wild-type and IL-4 knockout C57BL/6 mice that received the BP vaccine only did not produce any cytokines in response to stimulation with LFH (Fig. 7.3 A-D). The wild-type C57BL/6 mice that were vaccinated with BP and then infected with *F. hepatica* displayed a type II response to LFH antigen i.e. production of IL-4 and IL-5 and no production of IFNγ and IL-2. The IL-4 knockout mice that were vaccinated and infected, as expected, did not produce IL-4, but did produce IL-5; these mice did not produce IFNγ or IL-2 (Fig. 7.3A-D).

Spleen cells of wild-type and IL-4 knockout C57BL/6 mice that received the *B. pertussis* whole cell vaccine only, produced no IL-4, low levels of IL-5 but high levels of

the Th1 cytokines IFNγ and IL-2 in response to stimulation with BP (Fig. 7.4A-D). Wild-type C57BL/6 mice that were vaccinated with *B. pertussis* whole cell vaccine and then infected with *F. hepatica* produced significantly higher levels of IL-4 ($P < 0.05$) and about the same levels of IL-5 as the group that received the vaccine only (Fig. 7.4A and B). However, these mice produced no IFNγ and reduced amounts of IL-2 compared to the vaccine only group (Fig. 7.4 C and D). When spleen cells from vaccinated infected C57BL/6 mice were stimulated *in vitro* with PMA/anti-CD3 a predominant Th2 cytokine profile was observed. The spleen cells of IL-4 knockout mice that were vaccinated with *B. pertussis* whole cell vaccine and infected with *F. hepatica* did not produce IL-4 as expected, and did not show any significant differences in the amount of IL-5, IFNγ or IL-2 production compared to the mice that received the vaccine only (Fig. 7.4A-D). When spleen cells from vaccinated infected IL-4 knockout mice were stimulated *in vitro* with PMA/anti-CD3 a cytokine profile similar to mice that were vaccinated only was observed.

Fig. 7.3C IFNγ

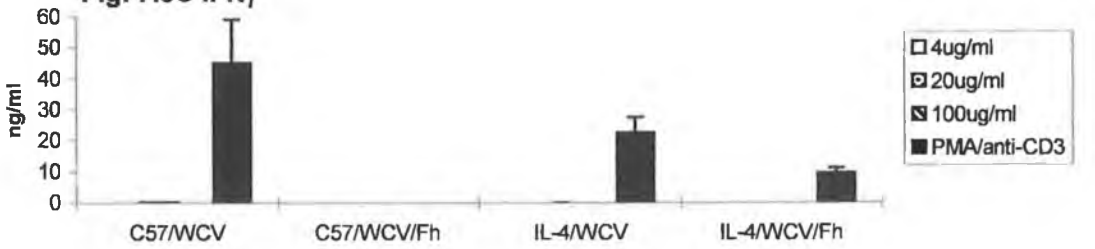


Fig. 7.3D IL-2

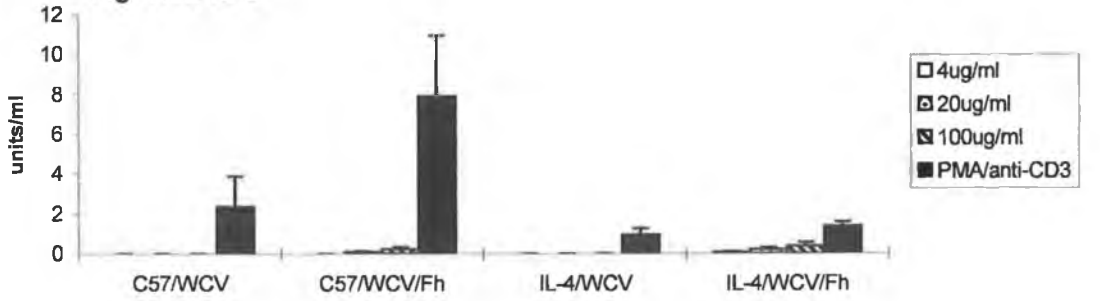


Fig. 7.3A IL-4

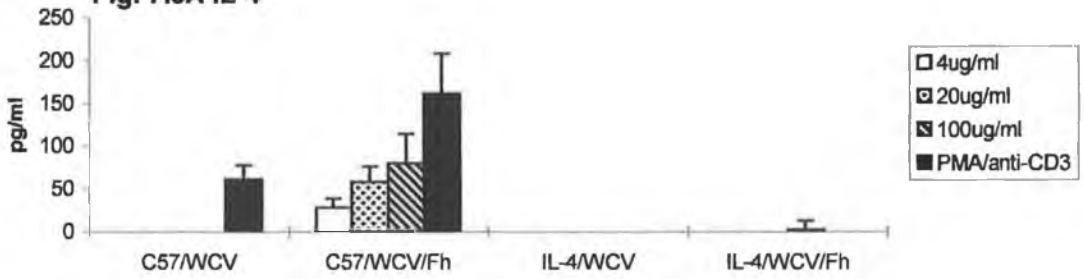


Fig. 7.3B IL-5

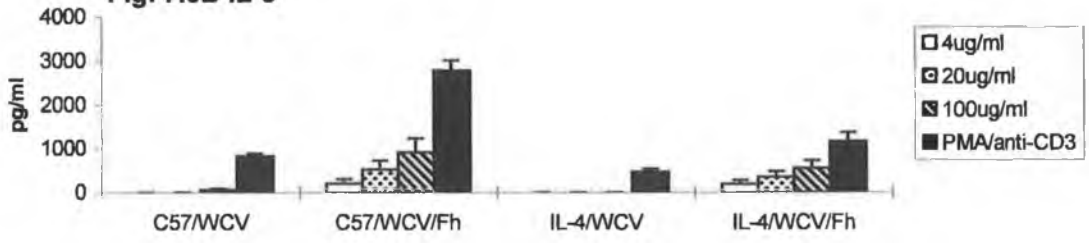


Figure 7.3 Cytokine production by spleen cells of wild-type (C57) and IL-4 knockout C57BL/6 mice (IL-4) that were vaccinated with *B. pertussis* whole cell vaccine (WCV) or vaccinated and then infected with *F. hepatica* (WCV/Fh). Spleen cells were stimulated *in vitro* with LFH antigen (4 μg/ml, 20 μg/ml, 100 μg/ml) and PMA/antiCD3. Samples of supernatant were removed for measurement of IL-4, IL-5, IFNγ and IL-2 cytokines (7.3 A-D). Results were expressed as the mean cytokine concentration of four mice per group tested in triplicate.

Fig. 7.4C IFNγ

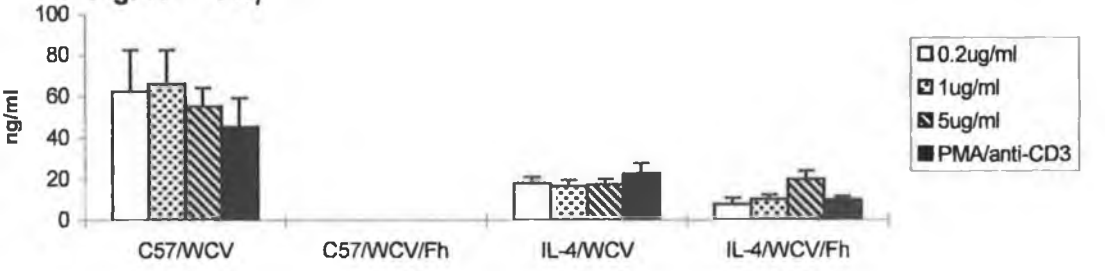


Fig. 7.4D IL-2

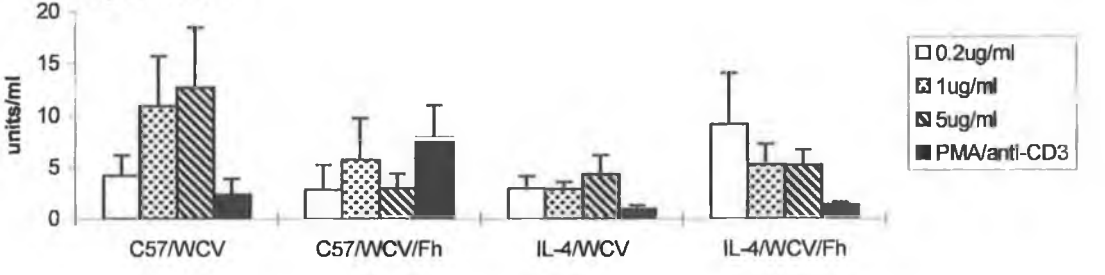


Fig. 7.4A IL-4

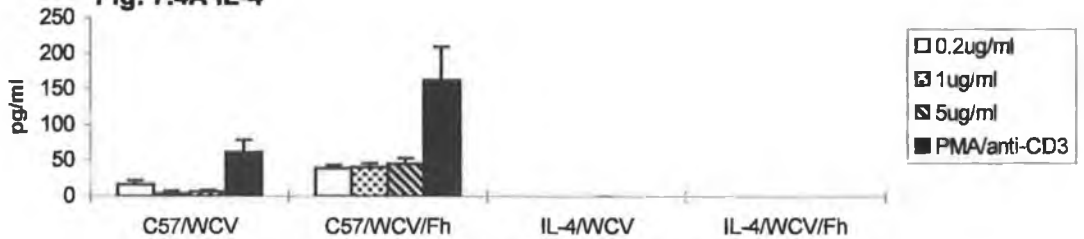


Fig 7.4B IL-5

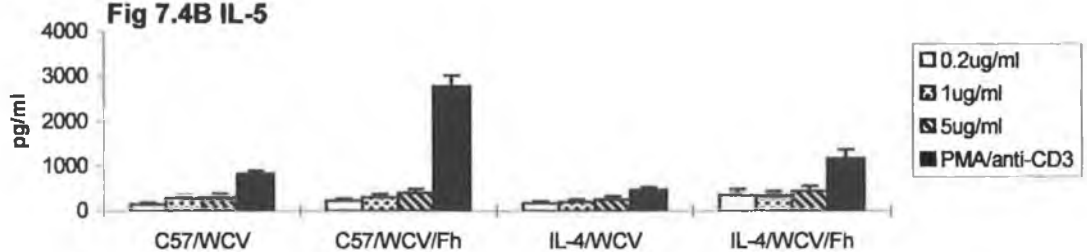


Figure 7.3 Cytokine production by spleen cells of wild-type (C57) and IL-4 knockout C57BL/6 mice (IL-4) that were vaccinated with *B. pertussis* whole cell vaccine (WCV) or vaccinated and then infected with *F. hepatica* (WCV/Fh). Spleen cells were stimulated *in vitro* with BP antigen (0.2 μg/ml, 1 μg/ml, 5 μg/ml) and PMA/anti-CD3. Samples of supernant were measured for IL-4, IL-5, IFNγ and IL-2 cytokines (7.4 A-D). Results were expressed as the mean cytokine concentration of four mice per group tested in triplicate.

7.4 Discussion

Empirical evidence gleaned from the previous chapter demonstrates that spleen cells of *F. hepatica* infected mice secrete a cytokine pattern characteristic of a Th2 cytokine profile (high IL-4 and IL-5); but do not secrete IFNγ and IL-2 cytokines which are characteristic of a Th1 cytokine profile. Furthermore, the same cytokine secretion pattern was elicited when spleen cells of infected mice were stimulated *in vitro* with non-specific mitogens such as, PMA/anti-CD3. This is perhaps indicative of a general imbalance in Th-cell subset response, an immunological phenomenon observed with other trematode infections (Bassily *et al.*, 1992; Sabin *et al.*, 1995). This provoked an interest in elucidating if this Th-cell subset imbalance would influence the outcome of the immune response to a Th1 inducing antigen.

Brito *et al.* (1976) was the first to report the immunosuppressive effects of a trematode infection in mice vaccinated with tetanus toxoid. In this study, mice infected with *S. mansoni* were unable to mount the same antibody response to the vaccine when compared with uninfected control mice. Moreover, the immunosuppression of antibody response was only observed during the patent stage of infection (six to eight weeks after initial infection when mature females produce eggs). It is now well established that oviposition in mice infected with *S. mansoni* is the major stimulus for the induction of Th2 cytokine producing CD4+ cells (Grzych *et al.*, 1991). However, in the pre-patent stage of infection spleen cells from *S. mansoni*-infected mice produce Th1 cytokines in response to SWAP antigen (Schistosoma Worm Adult Protein). The secretion of Th1 cytokines declines after egg production commences. This confirms that egg production is associated

with the down regulation of Th1 cytokine secretion in schistosomiasis (Vella and Pearce, 1992).

In an experiment performed by Kullberg *et al.* (1992) it was demonstrated that the generalised imbalance of Th-cell function during an infection with *S. mansoni* alters the Th1 cytokine response elicited in BALB/c mice vaccinated with sperm whale myoglobulin (SwMb). Immunisation of mice eight weeks after an infection with *S. mansoni* cercariae resulted in the reduction of IFNγ and IL-2 secretion (79% and 71%, respectively) by spleen cells stimulated with SwMb when compared with uninfected animals. In the same group of mice the secretion of IL-4 from cultured spleen cells was three times as high as that observed in non-infected animals. The production of Th1 and Th2 cytokines was CD4+ cell dependent and the addition of IL-10 antibodies to the cultured spleen cells restored the down-regulation of IFNγ to SwMb observed in the infected and vaccinated mice.

Several other studies such as that by Actor *et al.* (1993) reported similar findings. In this study BALB/c mice were vaccinated with the vaccinia virus, a Th1 inducing antigen, and then were co-infected with *S. mansoni*. In comparison to vaccinated mice only, spleen cells of infected and vaccinated mice exhibited a reduction in the secretion of Th1 cytokines. More importantly, there was a down-regulation of virus specific CD8+ cytotoxic T-cells. When these mice were challenged with vaccinia virus there was delayed clearance of the virus for up to three weeks in mice infected with *S. mansoni*. This observation indicates that helminth infections may influence the immune response to concurrent infections.

This present study employed mice vaccinated with *B. pertussis* whole cell vaccine as a model to study the effects of *F. hepatica* on an established Th1 immune response. *B. pertussis* is a gram-negative bacterium that causes whooping cough, a respiratory disease that accounts for 300,000 deaths of young children annually. The whole cell vaccine is effective in preventing disease in children and Th1 cells are believed to play a role in protective immunity generated following infection with *B. pertussis* (Ryan *et al.*, 1997). The pathogenicity and immunology of *B. pertussis* infection in mice has been widely studied (Redhead *et al.*, 1993; Barbic *et al.*, 1997). Similar to investigations in humans, cell mediated immune responses are associated with clearance of a challenge infection and subsequent protection against recurrent infections (Redhead *et al.*, 1993). Immunisation of mice with whole cell vaccine induces a high level of protection against respiratory challenge and this protection is associated with a type I immune response (Barnard *et al.*, 1996).

In the present study mice were vaccinated with *B. pertussis* whole cell vaccine (a type I inducing antigen) (Redhead *et al.*, 1993) and then infected with *F. hepatica* to identify if an established type I immune response is altered by a dominant type II response. The data presented in this chapter for mice infected with *F. hepatica* only or vaccinated with *B. pertussis* whole cell vaccine supports results previously reported. Spleen cells from BALB/c mice infected with *F. hepatica* is associated with a predominant type II immune response when stimulated *in vitro* with LFH antigen (Chapter 6). As previously described by Redhead *et al.* (1993) spleen cells from BALB/c mice vaccinated with *B. pertussis* whole cell vaccine exhibited a predominant type I response to BP antigen. Spleen cells of vaccinated and liver fluke infected mice when challenged with LFH antigen

exhibited no significant differences in cytokine production when compared to mice infected with *F. hepatica* infection only. In contrast, spleen cells from these vaccinated and infected mice when stimulated *in vitro* with BP antigen exhibited a significant down regulation of Th1 cytokine production (IFNγ and IL-2) compared to immunised uninfected control mice. Furthermore, in this group there was a significant increase in IL-5 although no difference was observed in amount of IL-4 cytokine secretion.

This observed down-regulation in Th1 cytokines with the simultaneous increase in Th2 cytokines in vaccinated and infected BALB/c mice may be due to the cross-regulation of Th-subsets (Grzych *et al.*, 1991; Pearce *et al.*, 1991). Mice infected with *F. hepatica* induce spleen cells to secrete IL-4 and perhaps another important regulatory cytokine, IL-10. Interleukin-10 was not measured in this study but was shown to be secreted in *F. hepatica* infected mice (data not shown). Interleukin 4 is a growth factor of Th2 cells and therefore is important in regulating Th2 cytokine production while IL-10 inhibits the synthesis of IFNγ, IL-2, IL-3, lymphotoxin and granulocyte/ macrophage - CSF by Th1 cells (Mosmann and Moore, 1991). In this present study the role of IL-4 was investigated by repeating the previously described experiment in wild-type and IL-4 knockout C57BL/6 mice.

In agreement with the data presented for BALB/c mice, wild-type C57BL/6 mice vaccinated with *B. pertussis* whole cell vaccine and then infected with *F. hepatica*, exhibited a Th2 response to LFH antigen. Spleen cells from IL-4 knockout mice that were vaccinated and then infected with the liver fluke did not secrete any IL-4 to LFH antigen as expected but did produce significant levels of IL-5. In agreement to that described in

Chapter 6 IL-4 knockout mice exhibited a significant decrease in IL-5 production when compared with their wild-type controls.

Similar to that reported for BALB/c mice, spleen cells of wild-type and IL-4 knockout C57BL/6 mice vaccinated with *B. pertussis* whole cell vaccine secreted high levels of IL-2 and IFNγ, low levels of IL-5 and no IL-4 when stimulated with BP antigen. Spleen cells from vaccinated and infected wild-type C57BL/6 mice stimulated with BP antigen produced no IFNγ and reduced amounts of IL-2 compared to the vaccine only group. In contrast to the BALB/c mice, spleen cells from these mice produced higher levels of IL-4 but the same levels of IL-5. The spleen cells of vaccinated and infected IL-4 knockout mice did not show any significant difference in the amount of IL-5, IFNγ or IL-2 production compared to mice that received vaccine only (as expected these mice did not produce any IL-4). These results indicate that a down regulation of Th1 cytokines by spleen cells from vaccinated and infected mice is dependent upon the production of IL-4.

In agreement with the data presented in Chapter 6, when spleen cells of *F. hepatica* infected BALB/c and C57BL/6 mice were stimulated with a non-specific mitogen (PMA/anti-CD3) a predominant Th2 cytokine subset was exhibited. The predominance of Th2 cytokines in mitogen-stimulated spleen cells was also observed when mice were vaccinated with *B. pertussis* whole cell vaccine and infected with *F. hepatica*. This again illustrates the generalised imbalance in the Th-cell subsets associated with *F. hepatica* infection. When spleen cells from vaccinated and infected IL-4 knockout mice are stimulated with mitogen a Th0 cytokine profile was exhibited. This indicates that the Th-subset imbalance observed in *F. hepatica* infected mice is a IL-4 dependent process.

Interleukin-4 is a positive growth factor for Th2 cells, in addition it suppresses the development of IL-2, a positive growth factor for Th1 cells. The levels of IL-4 and IL-2 determine both the levels of effector cells developed in response to mitogen or antigen and the patterns of lymphokines they secrete when stimulated *in vitro* (Swain *et al.*, 1990). Kullberg *et al.* (1992) suggested that IL-4 produced *in vivo* in *S. mansoni* infected mice may promote the outgrowth of Th2 precursor cells in these animals immunised with a foreign antigen. In this study the number of units of IL-4 secreted per CD4+ cell in response to SwMb, from *S. mansoni*-infected mice was measured. These CD4+ cells produced over three times as much IL-4 as did cultured CD4+ cells from uninfected animals. This indicates that either higher levels of IL-4 are produced by cells or that there is an increase in the number of Th2 cells in infected mice. This hypothesis may also explain the down regulation of BP stimulated spleen cells from *F. hepatica* infected mice vaccinated with *B. pertussis* whole cell vaccine.

In conclusion, this is the first study to demonstrate the down regulation of Th1 cytokines to a non-parasitic antigen in *F. hepatica* infected mice. These results merit an examination of the efficacy of immunisation programs in the Bolivian Altiplano where human fasciolosis is endemic (Bjorland *et al.*, 1992). Infection with *F. hepatica* may make individuals susceptible to other infections that require Th1 responses to resist them. Polyparasitosis was described in the Amaryan population of the Bolivian Altiplano and a positive correlation was observed between individuals with fasciolosis and the protozoan, *Giardia intestinalis* (MasComa *et al.*, 1999). Finally the molecules responsible for the induction of Th2 response or the down-regulation of Th1 response have yet to be identified. The experimental system described in this chapter would be an excellent model

to identify these molecules; and possibly investigate the role of another important Th2 regulatory cytokine, IL-10.

Chapter 8

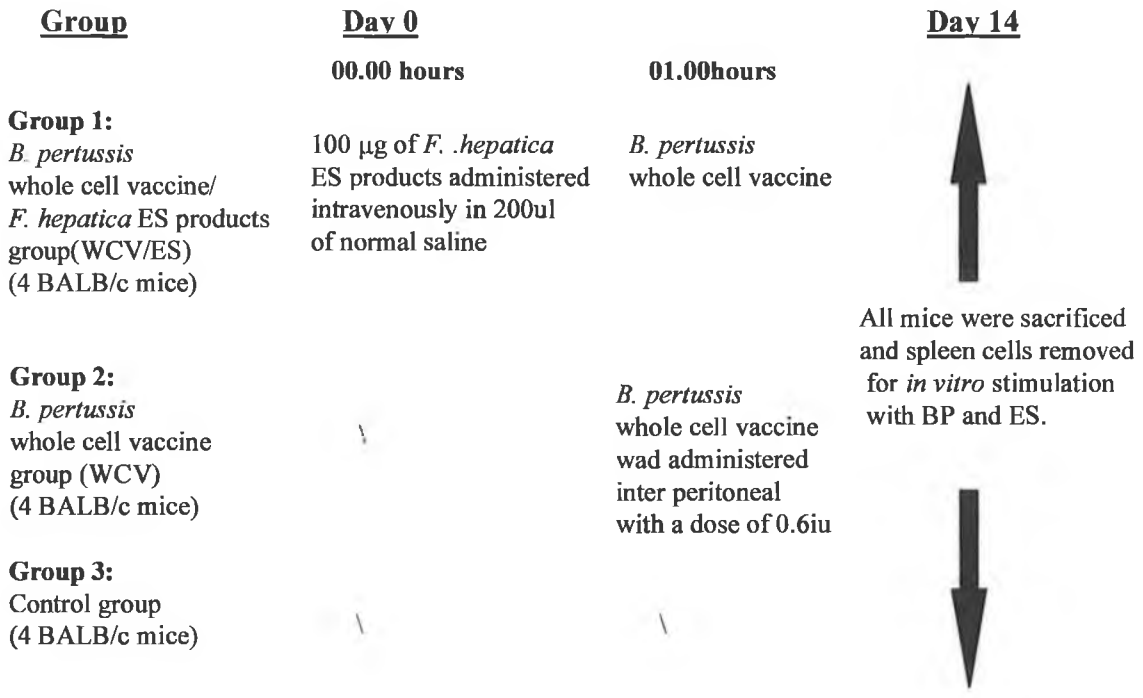
Down-regulation of type I responses in BALB/C mice to *B. pertussis* whole cell vaccine elicited by a co-injection of *F. hepatica* ES products.

8.1 Introduction

In the previous chapter we demonstrated that an established type I immune response in mice vaccinated with *B. pertussis* whole cell vaccine was down regulated when these mice were subsequently infected with *F. hepatica*. This was an IL-4 dependent process because when this experiment was repeated in IL-4 knockout mice this down regulation was not observed. Since *F. hepatica* ES products are actively secreted by the parasite we hypothesize that these molecules may have a vital role in the suppression of type I immune responses by actively enhancing the production of Th2 like cytokines. In the following study we have performed both *in vitro* and *in vivo* studies to investigate this hypothesis.

8.2 Experimental design

8.2.1 Experimental design 1



Four 3 to 4 month old female BALB/c mice were administered 100 μg (in 100 μl) of *F. hepatica* ES products intravenously via the tail vein (Group 1). One hour later these four BALB/c mice and an additional four mice that did not receive ES products (Group 2) were vaccinated with *B. pertussis* whole cell vaccine (0.8 iu/mouse). Four untreated BALB/c mice were included in the study as controls (Group 3). On day 14 all mice were sacrificed by cervical dislocation and the spleens removed. Isolated spleen cells were stimulated *in vitro* with BP antigen and IFNγ, IL-2, IL-4 and IL-5 cytokines in the supernatant measured.

8.2.2 Experimental design 2

Spleen cells obtained from mice vaccinated with a single dose of *B. pertussis* whole cell vaccine were stimulated *in vitro* with BP (0.1 μg/ml, 2 μg/ml and 5 μg/ml) in the presence or absence of 100 μg/ml of ES antigen. The spleen cells were cultured at 37^0C and supernatant samples removed after 24 hours to measure IL-2 and after 72 hours to measure IL-4, IL-5 and IFNγ.

8.2.3 Experimental design 3

Spleen cells and peritoneal exudate cells from BALB/c mice were cultured *in vitro* with *F. hepatica* ES products (4 μg/ml, 20 μg/ml and 100 μg/ml). Spleen cells were cultured at 37^0C and supernatant samples were removed after 24 hours to measure IL-2 and after 72 hours to measure IL-4, IL-5, IL-10, IFNγ and IL-12. Peritoneal exudate cells were cultured at 37^0C and the supernatant samples removed after 48 hours to measure IL-10 and IL-12.

8.2.4 Experimental Design 4

Four BALB/c mice were administered *F. hepatica* ES products by intra-peritoneal injection (20 μg/mse) on alternate days. Four untreated BALB/c mice were included in the study as controls. On day 10 all mice were sacrificed by cervical dislocation and the spleens removed. Isolated spleen cells were stimulated *in vitro* with ES antigen and IFNγ, IL-4 and IL-5 cytokines in supernatant were measured after 72 hours.

8.3 Results

8.3.1 Cytokine production by spleen cells of mice injected intravenously with *F. hepatica* ES products vaccinated with *B. pertussis* whole cell vaccine.

To identify the molecules involved in the down regulation of Th1 cytokines as described in the previous chapter, BALB/c mice were first injected intravenously with ES antigen and then vaccinated with *B. pertussis* whole cell vaccine. Mice were sacrifice 14 days later and the spleens removed. Isolated spleen cells stimulated *in vitro* with BP antigen and IL-4, IL-5, IL-2 and IFNγ cytokines secreted into culture media were measured.

Spleen cells of mice that received *B. pertussis* whole cell vaccine only (Group 2) produced the type I cytokines, IFNγ and IL-2, when stimulated with BP antigen (8.1A and B); no secretion of either IL-4 or IL-5 was observed (data not shown). Spleen cells of mice that received an intra-venous injection of ES products, previous to vaccination with the *B. pertussis* whole cell vaccine (Group 1) produced significantly reduced levels of IFNγ and IL-2 ($p<0.0001$) when compared to cells of the vaccinated mice only (Group 2) (8.1A and B). Similarly, in group 2 there was no secretion of either IL-4 or IL-5 cytokines (data not shown). Spleen cells of control mice (Group 3) did not secrete any cytokines in response to stimulation with BP.

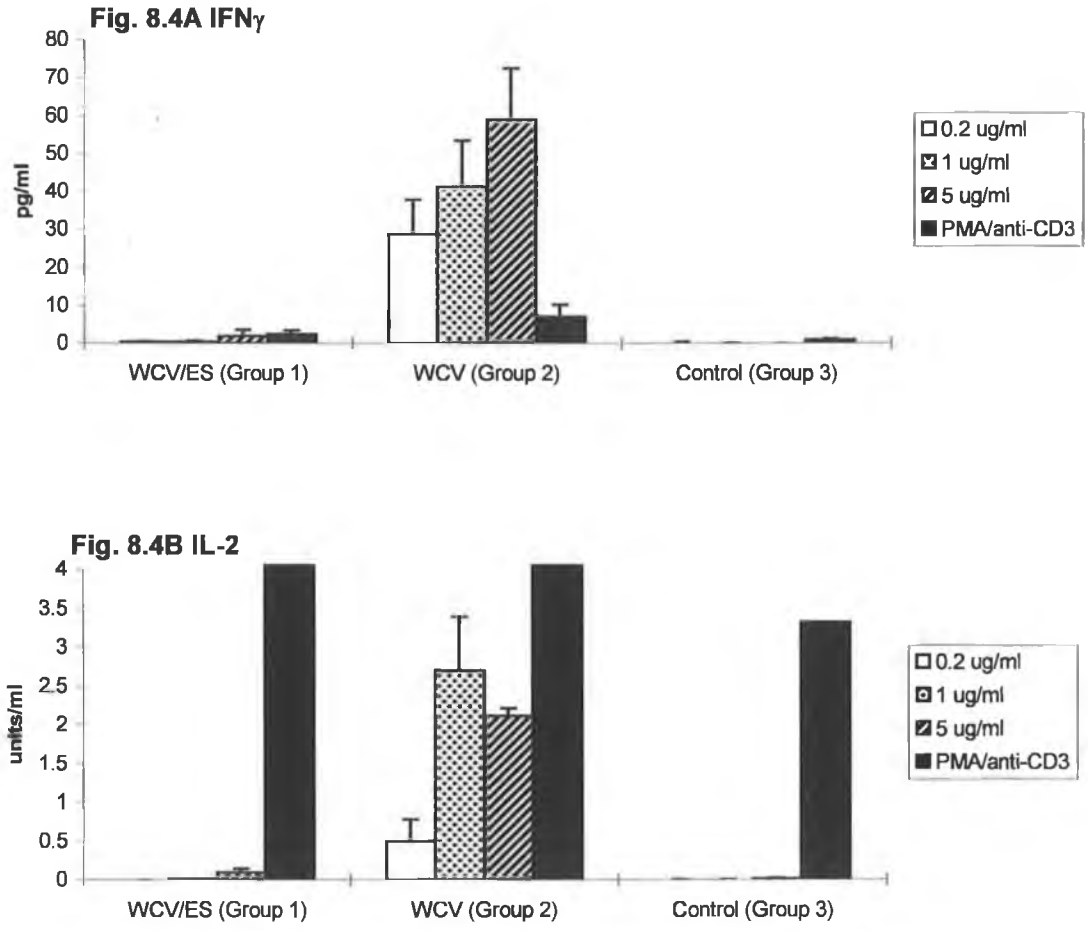
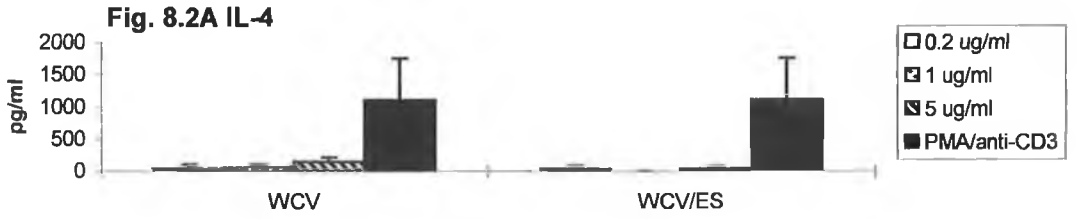


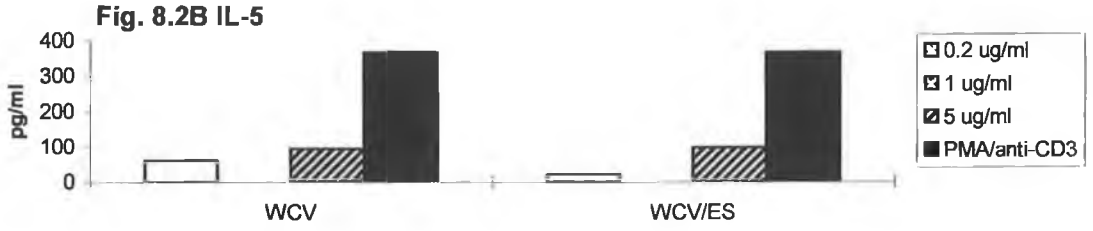
Fig. 8.1 Measurement of IFNγ (Panel A) and IL-2 (Panel B) cytokine production of spleen cells from three different groups of BALB/c mice following stimulation with BP. (Group 1: mice that were injected with ES products intravenously and vaccinated with *B. pertussis* whole cell vaccine (WCV/ES); Group 2: Mice that received *B. pertussis* whole cell vaccine only (WCV); Group 3: untreated control mice (Control)). Results were the mean of four individual mice for triplicate cultures of spleen cells stimulated with BP antigen and PMA/anti-CD3.

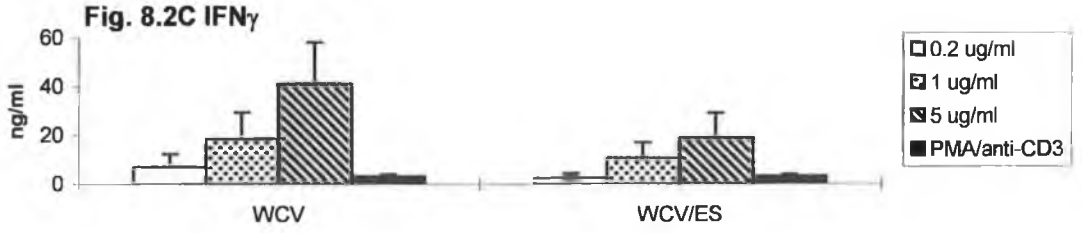
8.3.2 Cytokine production by spleen cells of mice vaccinated with *B. pertussis* whole cell vaccine in response to stimulation *in vitro* with BP antigen in the presence or absence of *F. hepatica* ES products.

It was demonstrated in the first experiment (section 8.3.1) that a Th1 cytokine response elicited by mice vaccinated with *B. pertussis* whole cell vaccine was blocked when these mice received a prior injection of ES antigen. We investigated if this down regulation can be observed *in vitro*. To this end spleen cells from mice vaccinated with *B. pertussis* whole cell vaccine were stimulated with BP in the presence and absence of ES antigen.

Spleen cells of BALB/c mice that were vaccinated with *B. pertussis* whole cell vaccine secreted IFNγ and IL-2 cytokines when stimulated with BP antigen (Fig. 8.2C and D); a low level of IL-4 and IL-5 secretion was also observed (Fig. 8.2A and B). When these spleen cells were stimulated with BP antigen in the presence of *F. hepatica* ES products a significantly lower level of IFNγ was produced ($P < 0.05$) (Fig. 8.2C); no significant changes in the production of IL-2, IL-4 and IL-5 were observed (Fig. 8.2A, B and D).







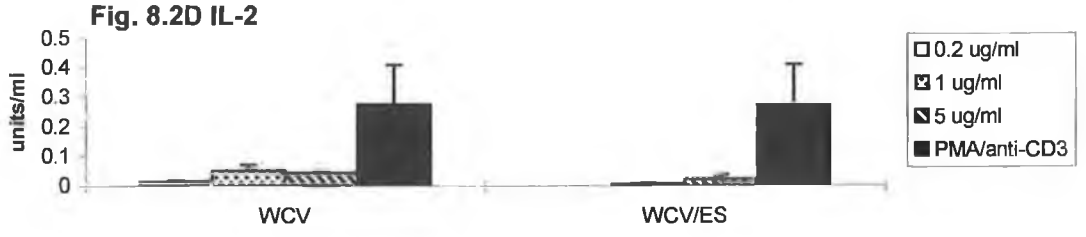


Fig. 8.2 Cytokine production by spleen cells of mice vaccinated with a single dose of *B. pertussis* whole cell vaccine. Spleen cells were stimulated *in vitro* with BP antigen in the presence (WCV/ES) or absence (WCV) of ES antigen (100 μg/ml) and PMA/anti-CD3.

Supernatant samples were removed after 24 hours to measure IL-2, whereas IL-4, IL-5 and IFNγ cytokines were quantified after 72 hours. Results were the mean of four individual mice for triplicate cultures.

8.3.3 Cytokine production by spleen cells and peritoneal exudate cells stimulated *in vitro* with ES antigen.

This experiment investigated if ES antigen can directly stimulate cytokine production of naive spleen and peritoneal exudate cells, by stimulation of these cells *in vitro* with ES antigen. We found that spleen cells from BALB/c mice did not secrete IL-4, IL-5, IL-10, IFNγ or IL-12 cytokines when stimulated *in vitro* with ES (data not shown). Peritoneal exudate cells from BALB/c mice were also stimulated with ES antigen and the production of IL-12 and IL-10 quantified, while these cells did not produce any IL-12 (data not shown) they did secrete significant amounts of IL-10 cytokine (Fig. 8.3). The concentration of IL-10 cytokine produced correlated with the concentration of ES antigen present in the culture medium.

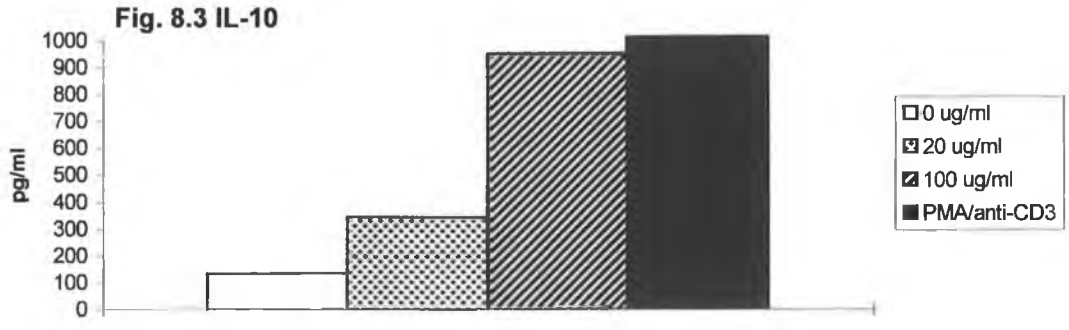


Fig. 8.3 Measurement of IL-10 production by peritoneal exudate cells from BALB/c mice stimulated *in vitro* with ES antigen (0 μg/ml, 20 μg/ml and 100 μg/ml) and PMA/anti-CD3. Results were expressed as the mean cytokine concentration of a pool of peritoneal exudate cells from four mice tested in triplicate.

8.3.4 Cytokine production by spleen cells from mice vaccinated on alternate days with ES antigen.

To examine whether ES products can induce a Th2 cytokine response, 3-4 month old BALB/c mice were injected intraperitonally with ES antigen on alternate days. Mice were sacrificed after 10 days and isolated spleen cells were stimulated *in vitro* with ES. The amount of IL-4, IL-5 and IFNγ present in culture media was measured.

Spleen cells from control BALB/c mice did not secrete either Th1 (IFNγ) or Th2 (IL-4 and IL-5) cytokines in response to stimulation with ES antigen (data not shown). Spleen cells of mice that were injected with ES exhibited a predominant type II response when stimulated *in vitro* with ES, with significant secretion of IL-4 and IL-5 cytokines; no secretion of the Th1 cytokine, IFNγ, was observed.

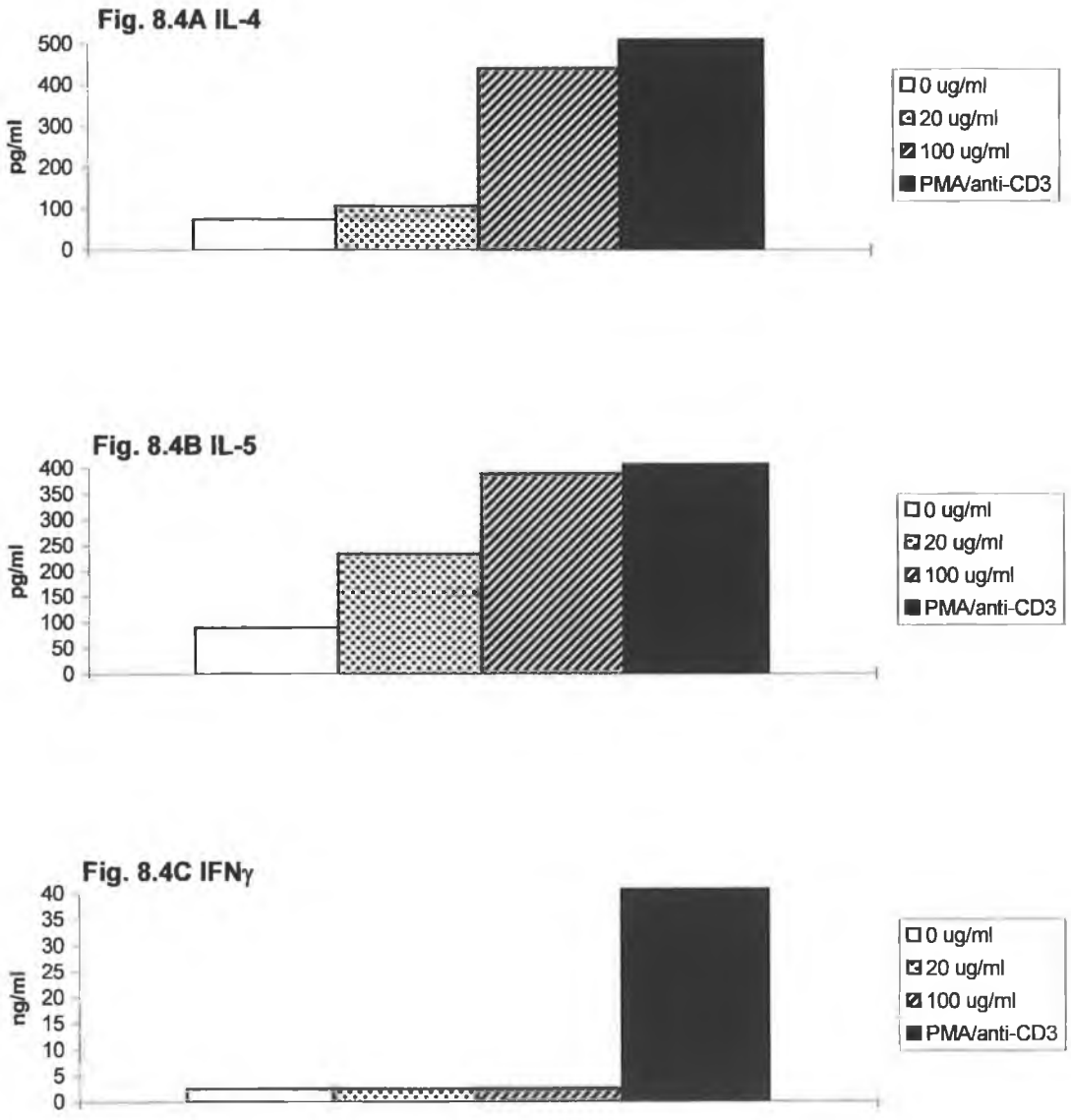


Fig. 8.4 Measurement of cytokine production by spleen cells from BALB/c mice that were injected intraperitoneally with *F. hepatica* ES products on alternate days. Spleen cells were stimulated *in vitro* with ES antigen (0 μg/ml, 20 μg/ml, 100 μg/ml) and PMA/anti-CD3. After 72 hours supernatant samples were obtained and IL-4, IL-5 and IFNγ were quantified. Results were expressed as the mean cytokine concentration of four mice per group tested in triplicate.

8.4 Discussion

Information gleaned from the previous chapter suggested that fasciolosis is associated with a generalised Th-subset polarisation towards a Th2 cytokine profile. Antigen stimulated spleen cells from infected mice secrete a predominant Th2 cytokine profile. More importantly an infection with *F. hepatica*, down-regulates a type I immune response to a bystander non-parasitic antigen. Similar to other parasites, these processes are dependent upon IL-4 (Sabin and Pearce, 1995; Mac Donald *et al.*, 1998). The molecules of ES products involved in the induction a type II immune response or in the suppression of Th1 cytokines has yet to be identified. Since *F. hepatica* continuously secretes ES products throughout all stages of development in the definitive host we hypothesise that these molecules may function by inducing the secretion of the cross-regulatory cytokine, IL-10 or the Th2 cell growth factor, IL-4. The early induction of Th2 cytokines would inhibit the development of a type I immune response. In this present chapter a number of preliminary studies were performed to investigate these matters.

Successful infection of a potential host by parasites requires the release of molecules which disarm the host defence system. The release of immunomodulatory molecules is not unique to parasitic infections. The Epstein-Barr virus genome encodes a homologue of IL-10 which shares many of the cellular cytokine's biological activities. It plays a central role in the host-virus interactions by down-regulating the protective type I immune response (Moore *et al.*, 1990). Similarly, the bacterium *B. pertussis* inhibits the development of cell mediated immunity by down-regulating IL-12 cytokine production (McGuirk *et al.*, in press).

Several studies have been performed to determine the immunomodulatory role of ES products in parasitic infections such as that reported by MacDonald *et al.* (1998), Fukumoto *et al.* (1997) and Ramaswamy *et al.* (1995). When mice were infected with the larval and adult stages of *Brugia malayi* high levels of parasite-specific IL-4 were produced by spleen cells on stimulation *in vitro* (Lawrence *et al.*, 1994). Both stages of infection are capable of inducing profound peritoneal exudate cell mediated suppression. The development of this proliferative suppression is dependent upon IL-4 and could not be reversed by the addition of nitric oxide inhibitors. In these studies there was no apparent defect in either antigen-specific processing or co-stimulation. In contrast, when mice were infected with *B. malayi* microfilariae, which induces a Th1 response, a moderate suppressive effect that can be fully reversed with inhibitors of nitric oxide was observed (MacDonald *et al.*, 1998). In a subsequent study they investigated whether the molecules of excretory/secretory products were involved in the generation of these suppressor antigen presenting cells. ES was found to generate suppressive peritoneal exudate cells as effectively as the live parasite itself. Moreover, this process was also dependent upon IL-4 and could not be reversed by the addition of nitric oxide inhibitors. The molecules present in ES which are involved in augmenting these suppressor cells have not been identified. In this experiment it was also demonstrated that ES from Toxocara- and Nippostronylus-infected mice also generate a suppressive peritoneal exudate cell population. These results indicate that the immunomodulaory phenomenon may be a common feature of the Nematode phylum (Allen and MacDonald, 1998).

Fukumoto *et al.* (1997) investigated the anti-inflammatory properties of ES products of plerocercoids of *Spirometra erinacei*. In this experiment murine peritoneal

exudate cells were stimulated with IFNγ and/or lipopolysaccharides (LPS) in the presence or absence of ES products. Both IFNγ and LPS stimulate the expression of iNOS (inducible nitrous oxide synthase) and JE mRNA. The release of nitrate oxide from mononuclear phagocytes is important in host defence functions, whilst JE, a chemokine, exhibits chemoattractant properties triggering leukocyte trafficking and migration. This study demonstrated that ES products of plerocercoids suppressed iNOS and JE mRNA expression. In addition nitrate production of macrophages was inhibited in a dose-dependent manner. These may explain why plerocercoids found in the peritoneal cavity of infected animals are not harmed by bound inflammatory leucocytes. Similar to filarial worms the molecules present in ES involved in these processes have not been identified.

Schistosomulae of *S. mansoni* actively penetrate the skin to gain entry into the definitive host by releasing a number of proteinases in their excretory/secretory products (Dalton and Brindley, 1998). The ES products of schistosomulae also exhibit anti-inflammatory properties. Ramaswamy *et al.* (1995) demonstrated that ES products induced the anti-inflammatory cytokine IL-1ra, from human keratinocytes. This IL-1ra inducing molecule was identified as a 16.8 kDA protein (SM16.8). Addition of SM16.8 to cultured spleen or lymph node cells also results in a significant reduction in cell proliferation and IL-2 secretion.

In this chapter we performed several experiments to determine if the ES products of *F. hepatica* contained molecules that could modulate immune responses. Spleen cells from *B. pertussis* whole cell vaccinated mice exhibit a predominant type I immune response (high IFNγ and IL-2) when stimulated *in vitro* with BP antigen. When these mice received a co-injection of intravenous ES antigen one-hour prior to vaccination there

was a significant decrease in both IFNγ and IL-2 production ($P < 0.001$); no significant secretion of IL-4 or IL-5 cytokines was observed. Therefore, molecules of ES products can down-regulate the production of the Th1 cytokines, IFNγ and IL-2.

In contrast to results obtained in the previous chapter there was no generalised Th-subset imbalance associated with an intravenous injection of ES products. When spleen cells of mice co-injected with *B. pertussis* whole cell vaccine and ES products were stimulated *in vitro* with PMA/anti-CD3 there was no significant difference in the cytokine profile observed when compared to that of control mice or of mice that received vaccine only. A single dose of ES antigen may be enough to down-regulate the Th1 response but is not sufficient to induce a strong Th2 response which would result in this generalised Th-subset imbalance. This may explain why spleen cells from co-injected mice when stimulated *in vitro* with ES did not secrete either Th1 (IFNγ and IL-2) or Th2 (IL-4 and IL-5) cytokines (data not shown).

To identify the mechanism involved in blocking Th1 cytokines it would be advantageous to develop an *in vitro* model to illustrate the effects of *F. hepatica* ES products on Th1 cells. To this end, spleen cells from *B. pertussis* vaccinated mice were stimulated *in vitro* in the presence or absence of ES products. As previously reported spleen cells from *B. pertussis* whole cell vaccinated mice when stimulated *in vitro* with BP antigen, secrete high levels of IFNγ and IL-2 but very low levels of IL-4 and IL-5 cytokine. When these cells were stimulated with BP in the presence of ES there was a significant down-regulation of IFNγ; no significant difference in the secretion of IL-2, IL-4 or IL-5 cytokines was observed when compared to cells stimulated with BP only. However, the suppression of IFNγ secretion by ES was only statistically significant when

spleens cells were stimulated with the highest concentration of BP antigen. The molecules of ES products may inhibit IFNγ secretion *in vitro* by several different mechanisms such as a) the induction of IL-10 secretion, b) the blocking of IL-12 secretion or c) by directly cleaving IFNγ. The inability to down-regulate IL-2 may reflect the concentration of ES employed. However the amount added to the cultured spleen cells was the same as the dose administered *in vivo* by intravenous injection. Therefore these results may indicate that parasite derived factors act in concert with host immune components that are not present in the *in vitro* system.

When peritoneal exudate cells of naïve BALB/c mice were stimulated *in vitro* with ES a significant production of IL-10 was produced. Interleukin-10 is a multifunctional cytokine that was first described as the synthesis inhibitory factor because it inhibits proliferation, effector function and development of Th1 cells. In the murine model IL-10 is expressed by mouse CD4+ Th2 clones, T-cells, activated mast cell lines, activated macrophages, keratinocytes and Ly-1 B-cells. It is an important cross regulatory cytokine inhibiting Th1 cytokines at the mRNA and protein level. *In vitro* it exhibits a number of immunostimulatory properties as it enhances the growth and differentiation of B-cells, thymocytes, peripheral T-cells, mast cells and cytotoxic T-cells. Thus, the early production of IL-10 in infected mice may inhibit the synthesis of Th1 cytokines allowing the type II immune responses to develop. The cellular components present in the peritoneal exudate which secrete IL-10 in response to ES antigen *in vitro* have not been identified. Peritoneal exudate cells from naïve mice consist primarily of macrophages, neutrophils and lymphocytes (predominantly Ly-1 B-cells). Both macrophages and Ly-1

B-cells are a rich source of IL-10. It is possible that these cells are the early source of IL-10 during the initial stages of infection.

The production of IL-10 by CD4+ T-lymphocytes was shown by Sher *et al.* (1991) to down-regulate Th1 cytokine secretion. In their experiments spleen cells from chronically infected mice were stimulated for 24 hours with soluble egg antigen (SEA). The supernatant was then tested for its ability to alter IFNγ synthesis by a Th1 cell line. Spleen cells from infected mice inhibited the production of IFNγ by Th1 cells. This inhibition was totally neutralised by the addition of IL-10 monoclonal antibodies. In our experiment we have demonstrated that the down regulation of Th1 cytokines is exhibited in mice infected with liver fluke and vaccinated with *B. pertussis* whole cell vaccine. Although this process was shown to be dependent upon IL-4, IL-10 may also be important. IL-10 may mediate it's effects by the induction of early IL-4 production. The use of monoclonal antibodies to inhibit IL-10 molecules in these vaccinated infected mice may help elucidate the role of this cytokine.

Velupillai and Harn (1994) have identified a carbohydrate ligand LNFP-III on *S. mansoni* eggs which induces B-cells to secrete IL-10 and Prostaglandin E_2, two molecules known to down-regulate Th1. In addition this molecule induces the proliferation of splenic B-cells but not CD4+ cells. The nature of the LHFP-III interaction with B-cells is unknown. *F. hepatica* and *S. mansoni* are both digenic trematode worms that share similar physiological and morphological characteristics. Several experiments have demonstrated that these worms express similar molecules. It is possible that *F. hepatica* secretes structurally and functionally related oligosaccarides to LHFP III. Unlike schistosomiasis the induction of a type II immune response by *F. hepatica* is not

associated with egg production. The production of Th2 cytokines by spleen and lymph node cells of infected mice was detected as early as one week post initial infection (data not shown). These LNFP-III-like oligosaccharides may be present in the glycoprotein rich surface glycocalyx. When liver flukes are cultured *in vitro* these oligosaccharides could be released into the culture media on antigen shedding, explaining their presence in ES products. Further work is necessary to determine if this hypothesis is correct.

In our final experiment mice were injected with ES antigen (20 μg/mse) on alternate days for 10 days. Spleen cells from these mice exhibited a predominant type II response when stimulated *in vitro* with ES. Previously in our laboratory we showed that spleen cells of mice that received only one intra-peritoneal injection of ES secreted neither Th1 or Th2 cytokines when stimulated *in vitro* with ES (data not shown). Therefore, a continuous injection of ES was necessary to induce the Th2 response. This would reflect the *in vivo* situation as liver flukes continuously secretes ES products. It was estimated by our laboratory that each fully mature liver fluke can secrete approximately 16-32 μg/hour of ES products. This is in an agreement with an experiment by Allen *et al.* (1998) who demonstrated that the suppressive effects of filaria ES antigen was only observed when mice received several injections.

In conclusion, the data from these preliminary studies demonstrates that *F. hepatica* ES down-regulates a type I immune response to a bystander non-parasitic antigen. More importantly, ES induces peritoneal exudate cells to secrete IL-10 *in vitro*. Intra-peritoneal injection of ES on alternate days for 10 days induced a predominant Th2 cytokine profile.

Chapter 9

General Discussion

Human fasciolosis is becoming recognised as a major public health problem, particularly in such diverse countries as Bolivia and Iran. In this study the region of interest is the Bolivian Altiplano, a high plain region situated between two Andean mountain ranges, approximately 3,700 metres above sea level. The Altiplano is the largest expanse of arable land in the Andes and is principally inhabited by the indigenous Amaryan population. The subjects in this study reside in a region located between the capital city, La Paz and lake Titicaca where a high prevalence of human fasciolosis was previously reported by Hillyer *et al.* (1992) and Bjorland *et al.* (1995). The contributory factors causing this high regional prevalence include, *inter alia*, climatic conditions, human dietary habits, life style, presence of intermediate host and animal reservoirs.

The appearance of liver fluke disease is influenced by climatic factors. In Europe the disease generally appears in late summer and early Autumn (Ripert *et al.*, 1988). Although human infection is rare in Europe it is also at this time that cases appear. In contrast, the Bolivian climate appears to promote transmission of the disease all year round (Stork *et al.*, 1973). Temperature does not vary significantly in this region averaging from 18^0C to 22^0C during the day and dropping at night from 0^0C to 6^0C in the wet (October-July) and dry seasons (May-September), respectively. Accordingly, temperature would not appear to be an important limiting constraint in disease transmission in this region. However, during the period of May to August the drop in temperature can retard the development of the larval stages in the molluscan host (Fuentes *et al.*, 1998)

The bar chart in Fig. 9.1 displays the monthly precipitation in the Altiplano over a period of three years (data provided by INLASA). The minimal rainfall for successful

transmission is six mm per month. The bar chart illustrates that in the Bolivian Altiplano during a typical year sufficient rainfall occurs for eight months of the year. During the height of the rainy season lake Titicaca and its tributaries overflow and subsequent extensive flooding producing optimum conditions for transmission. A positive relationship can be observed between the proximity to the lake and the prevalence of the disease in cattle (Buchon *et al.*, 1997). During the dry season successful completion of the life cycle is still possible because animals and humans tend to collect around water sources. The Lymanaeidae snail introduced to the region during colonization by Spanish explorers has adapted to the climatic conditions in the Bolivian Altiplano. This snail is reported to reside almost wholly sub-aqua in comparison to its European counterpart; therefore they are not dependent to the same extent upon the amount of precipitation levels per month. In addition, they are observed on aquatic plants during the dry season (Oviedo *et al.*, 1995). The incidence of infection is believed to be clustered in particular regions, depending upon the distribution of the molluscan host which is predominantly found in the north west of the Altiplano.

Over the course of the last two decades local health care officials have become increasingly aware of the problems associated with the high incidence of human fasciolosis in the Bolivian Altiplano (Angles *et al.*, 1992). Esteban *et al.* (1997) published a review focusing upon the incidence of human liver fluke disease from 1962 to 1992. The earliest paper published by Hartmann and Patino (1962) featured a clinical report concerning two medical cases. A further 26 years elapsed before subsequent papers were published by Vela Pacheco (1988) and Flores Serna and Estevez Martini (1988). In common with the initial reports, these papers focused upon clinical cases and did not report on the

prevalence of the disease in the region. Caceres Vega (1989) marked a new departure by reporting upon the incidence of disease in four different locations in the Bolivian Altiplano (Table 9.1). Several epidemiological surveys have been published since 1989 and Table 1 summarizes the reported recorded incidence of the disease in humans in this region over the last 17 years.

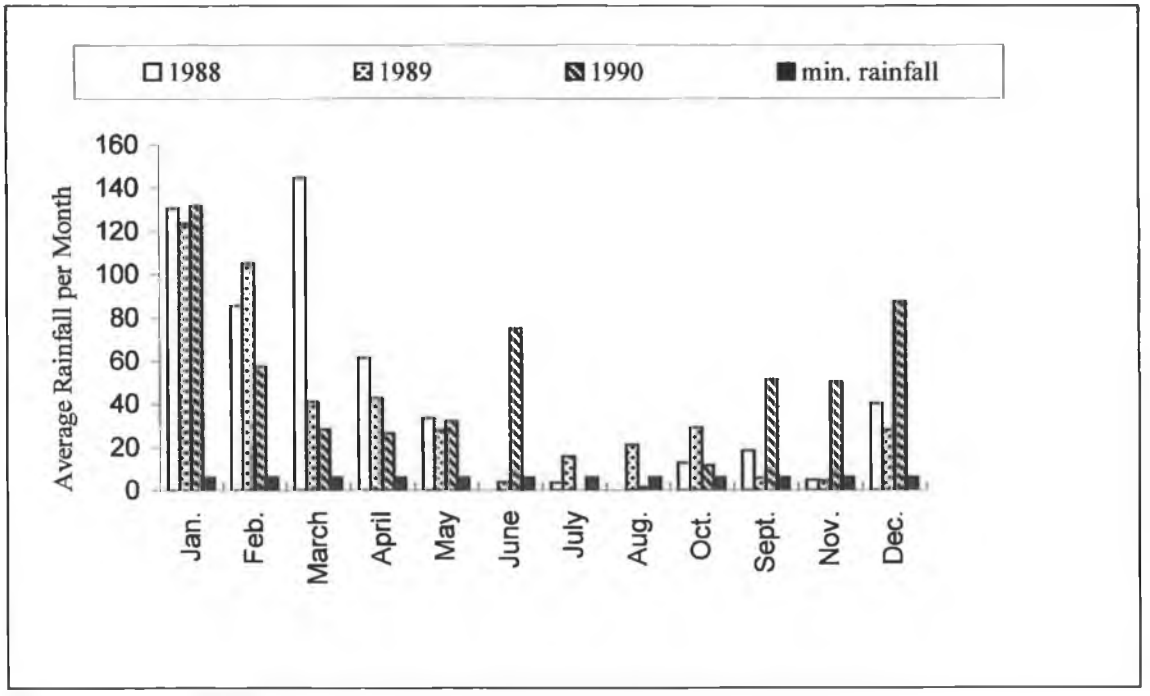


Figure 9.1: Official rainfall from Battalas (2km from the city of La Paz) from 1988-1989. The minimal rainfall required for transmission is 6 mm per month (black bars).

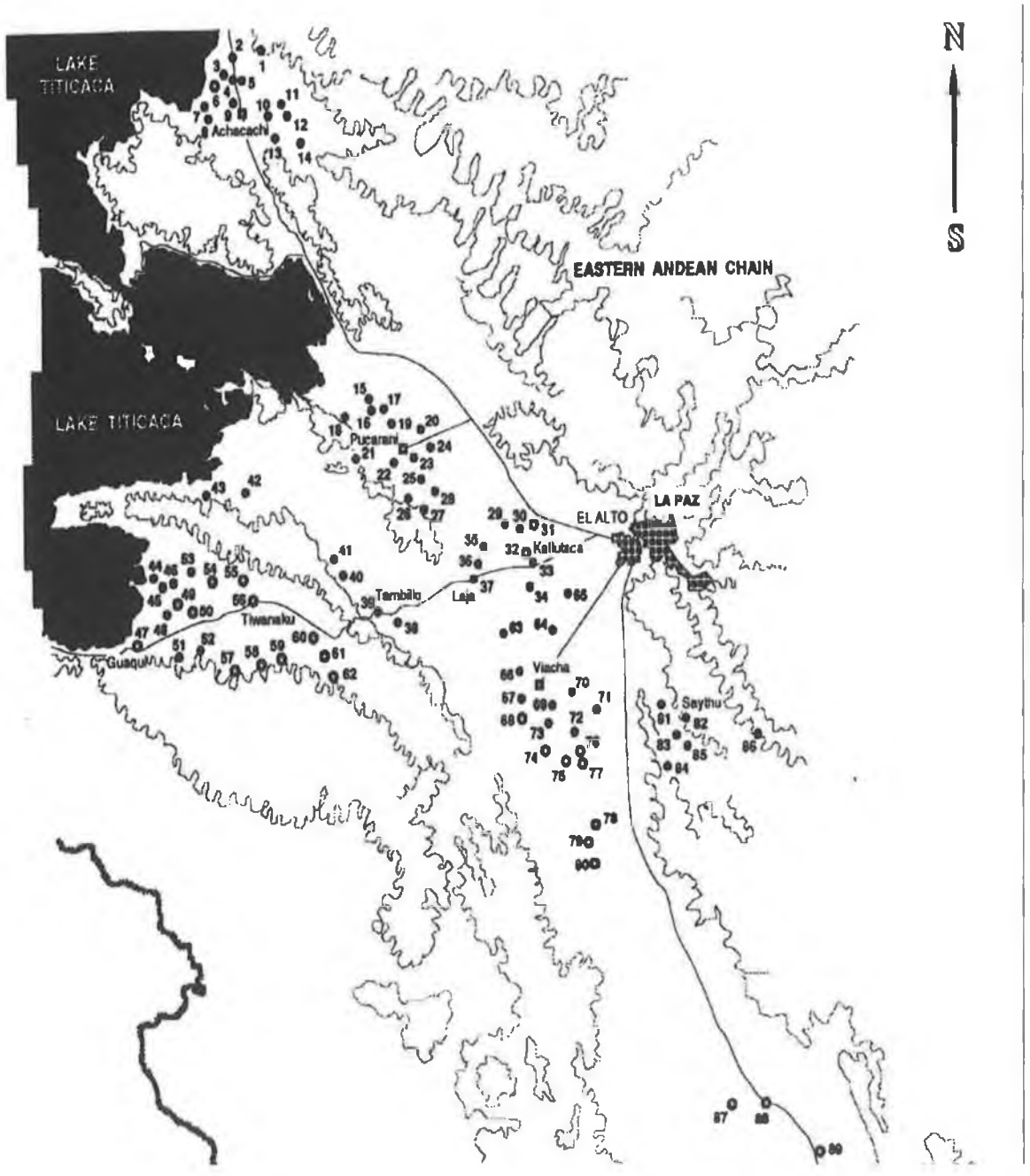


Fig. 9.2 A map of the Bolivian Altiplano illustrating the zones and communities where cattle were surveyed for *F. hepatica* infection (Buchon *et al.* 1997). A total of 107 communities were surveyed (89 communities numbered on the map) in this study. The

communities where bovine fasciolosis was present were indicated by closed circles whereas communities where fasciolosis was not detected were indicated by open circles.

The majority of the studies performed by this laboratory and by others were carried out in the Pucarani and Tambillo region (Fig. 9.2). In the village of Cutusuma four surveys were performed in Cutusuma between the period of 1984-1989. Each survey reported a high incidence of infection in this region. Our laboratory surveyed serum samples obtained in Cutusuma (1997) and demonstrated that human fasciolosis was present in this region in 1986 and 1997. These results and the data presented in this table supports our findings which highlights that fasciolosis has been prevalent in these regions for over a decade.

The prevalence of liver fluke disease varies throughout the Altiplano (Table 9.1). In the south of la Paz and in the Tiwanaku corridor the incidence of infection is relatively rare compared to the highly prevalent zones in the north west of La Paz (Fig. 9.2). Our hypothesis postulates that this is due to the proximity of the southern region from Lake Titicaca. In addition, the distribution of the molluscan host is mainly restricted to the north west of the Altiplano and has not yet been found in the Timanaku corridor.

In our study we found that one of the highest reported incidence of human fasciolosis was in a small village, Chijipata Alta, situated north of the city La Paz. In this random study performed on 29 individuals 69% tested serologically positive in our IgG4/CL1 ELISA. A similar study was performed by Esteban *et al.* (1997) one year later. This study was performed on school children who were diagnosed by coprological analysis. In agreement with our results 67% of individuals tested were positive for liver fluke disease. In the study performed by this laboratory we observed that coprological

analysis underestimated the number of infected individuals by 8%. Therefore the true incidence of infection in this survey may be as high a 72%.

Table 1: The incidence of human fasciolosis from 1981 - 1998.

No.	Year	Town	Number Examined	% Infected				
				Copro.	Sero.			
					CF	ELISA	IFA	IDR
1	1981	Tambillo	12	0				
2	1984	Guaqui	126	0				
3	1984	Battallas	92	3				
4	1984	Pucarani	73	3				
5	1984	Cutusuma	92	21				
6	1986	Rio Seco	816	0				
7	1986	Tauca	93	1				
8	1987	Cutusuma	144	31.7		54.2		
9	1987	Santiago de Huata	71	0				
10	1987	Viacha	272	0				
11	1987	Cutusuma	183	37			92	80
12	1987	Tauca	79	0				18
13	1987	Batallas	164	7				58
14	1989	Kallutaca	90	47.7				
15	1989	Cutusuma	\	55.6				
16	1989	Cutusuma	\	92.2				
17	1989	Cullucachi	\	13.5	71.9			
18	1991	Calasaya	89	11.5		20.2		
19	1991	Calasaya	71	21		49		
20	1991	Santa Ana	32			34.4		
21	1992	Coropata	100	27		53		
22	1992	Huacullani	123	38.2				
23	1993	Huacullani	256	31.2				
24	1993	Quinpajo	43	20.9				
25	1993	Caleria	51	5.9				
26	1993	Coropata	85	17.6				
27	1996	Chijipata	29	64		69		
28	1997	Chijipata	48	66.7				
29	1997	Cutusuma	67	46		49.2		

Cf=Complement fixation, IFA=Indirect immunofluorecence, IDR=intradermal reaction to fasciolosis.
Copro. = coprological analysis, Sero. = Serological analysis.
(Angles *et al.*. 1992[1-7,9-13]; Caceres Vega. 1989[14-17]; Bjorland *et al.*. 1995[18]; Hillyer *et al.* 1992[21]; Esteban *et al.* 1997[22-26,28]; O' Neill *et al.*, 1998, this thesis[12,19,20,27,29])

The coprological analysis performed by our laboratory on the individuals in Chijipata Alta (1996) revealed the highest global intensities of eggs per gram (epg) of faeces is to be found in the Bolivian Altiplano. In a"typical" infection recorded in France or Portugal an average intensity of 1 to 4 epg is anticipated. The highest reported European incidence was recorded in Portugal, an estimated 2100 epg (Chen and Mott, 1990) but this egg count is not normally associated with a typical infection. In contrast, the intensities in Bolivia ranged between 24 epg to 4446 epg and the highest intensity reported was 5064 epg in Chijipata Alta. Therefore individuals in this region are either a) exposed to a higher level of infection, b) are genetically more susceptible to infection c) are immunologically more susceptible to infection as a result of concurrent infections or d) the parasite has evolved to survive within the human host.

Many individuals in the Bolivian Altiplano carrying acute or chronic fasciolosis are asymptomatic or present with 'vague' symptoms rendering clinical diagnosis problematic (Bjorland *et al.*, 1989). A significant number of individuals are in the latent stage of infection which can last from a period extending from several months to several years and clinical diagnosis is difficult during this stage. Accordingly, the development of a simple, sensitive and cost effective diagnostic assay which can identify individuals at all stages of disease is necessary. While coprological analysis may fulfill some of these requirements it has several disadvantages. Firstly, it provides a definitive parasitological diagnosis only when eggs are found in the patients faeces. Therefore, early infections cannot be detected using this method. An early diagnosis of fasciolosis is vital since it is the juvenile stages of the parasite which cause extensive damage to the liver tissue (perforations and haemorrhaging) as they migrate towards the bile ducts. Secondly misdiagnosis of

individuals can occur since eggs are irregularly released with the bile juices into the intestines. Definitive coprological analysis requires the examination of at least two faecal samples taken at different times on consecutive days. In the 1986 survey of Cutusuma it was possible to obtain only a single faecal sample from each patient. This may explain why a higher proportion of individuals were coprologically negative but serologically positive in this region in comparison to surveys where two stool samples were obtained. Thirdly, the use of different methods of coprological analysis also may result in different estimates of the prevalence of infection (Munoz *et al.*, 1987). A significant variation was observed between the sedimentation method and the Kato-Katz technique (Chapter 4). This is particularly important when diagnosing patients with low intensity infections since only 2-3 epg may found in the faecal sample. Finally applying this method in the field may be difficult as children are reluctant to provide stool samples. In the Cutusuma 1997 survey we suspected that some children may have provided us with stool samples which they had obtained from other children.

The CL1/IgG4-ELISA was shown to be a useful tool in diagnosing human fasciolosis and is both highly sensitive and specific. This immunological assay eliminates many of the problems associated with coprological analysis. One sample of blood is required for diagnosis and this sample is taken directly from the subject participating in the study. ELISA can diagnose infection in both the acute and chronic stages of disease. Using our IgG4/CL1 assay large numbers of samples can be analysed within a relatively short time. Provision of diagnostic laboratories with purified cathepsin L would standardise the assay thus facilitating comparison of results throughout the Altiplano and

in other regions where fasciolosis is endemic. If 3 mg of recombinant protein is given to the INLASA by our laboratory approximately 2000 individuals can be tested.

Using out newly developed ELISA the present report reveals that human fasciolosis has been endemic in the Bolivian Altiplano for at least a decade and a total of 44.3% of individuals from 361 surveyed were infected with human fasciolosis. There are approximately 1.5 million people resident in the Bolivian Altiplano with approximately 100,000 individuals at risk to infection. If our study reflects the overall incidence in this region then approximately 44,300 individuals are infected. A more comprehensive study of fasciolosis, in humans in the Altiplano, is therefore essential to determine the full extent of infection, the limits to which it extends and the epidemiological factors that influence transmission.

Some of the factors which contribute to the high incidence of human fasciolosis include living in close proximity to their livestock, the consumption of aquatic plant and contaminated water, the outdoor defaecation habits and the underdeveloped health care system. The majority of individuals residing in the Bolivian Altiplano are subsistence farmers relying on the land and their livestock to survive. Animals are primarily fed on aquatic plants and algae which may be contaminated with *F. hepatica* metacercarial cysts. In some regions of the Altiplano pasturing is free or mixed and the absence of pasturing zones leads to contaminated pastures. Transmission in humans is promoted by animal reservoirs such as sheep, cattle (Hillyer, 1996), pigs (Apt, 1993), llamas and alpacas (Ueno, 1975) because of the high prevalence of fasciolosis in livestock in this region. Fig. 9.2 highlights the prevalence of Bovine fasciolosis. Similar to the studies performed in humans the incidence of infection is high in the Pucarani and Tambillo region. However,

the incidence is also high in other regions that have not been surveyed for human fasciolosis. Similar to humans the incidence of bovine fasciolosis is low in the south of La Paz and in the Tiwanaku corridor. These result would support the close association of bovine and human fasciolosis (Buchon *et al.*, 1997).

Transmission in humans is believed to occur because of their dietary habits. Individuals supplement their diet with aquatic plants during daily animal husbandry. The main types of aquatic plants are "berro berro" (watercress), "algas" (algae), kjosco and totora (Bjorland *et al.*, 1995; Angles, personal communication, 1996). Whilst drinking water is not believed to be associated with infection, the consumption of surface water while working in the fields is believed to be a source of infection. Caceres Vegas (1989) was the first author to suggest that drinking untreated water may be a source of infection. He suggested that the strain of *F. hepatica* had evolved to produce free floating metacercarial cysts. Vegetables washed in contaminated water may also become a source of infection. The incidence of infection is almost inevitably aggregated within familial groups which share contaminated food and drink from a common water source (Bjorland *et al.*, 1995).

The data collated by this laboratories and by others in collaboration with INLASA have underlined the extent of human fasciolosis in this region. Presently the Bolivian government are seeking to address this problem with the formation of a committee, tasked with the development of a health care policy specific to this region. Although it is apparent that this disease is endemic to this region, it should be possible with the combination of an extensive regional survey and the provision of primary preventive health care to eradicate this disease. Firstly, the incidence of infection throughout the entire

region should be determined. This is to identify specific target areas. The use of our IgG4 ELISA would provide a cheap simple and quick diagnostic method to do this. The provision of recombinant protein would standardise this assay making it possible to directly compare data from different regions. In this study individuals were reluctant to provide blood samples. However, the method of collecting blood onto filter paper makes a large epidemiological survey possible. This method would also allow us to obtain a large number of samples within a relatively short period of time. It is vital that once individuals are drug treated primary preventive steps are introduced to curtail re-infection. The installation of a number of water pumps throughout the region are vital to stop the consumption of contaminated water. In addition, drinking water should be filtered or boiled. This would not only limit the spread of infections such as liver fluke disease but also other infections that are transmitted through water contamination.

If feasible cattle should be provided with water from troughs which are supplied with pumped water. This innovation, coupled with the herding of cattle into areas divorced from the water sources, prevents the consumption of pond algae, would protect the cattle by controlling consumption, it would have the further advantage of preventing pollution of the water source by ruminants defecating directly into the system. This would prevent transmission of the life cycle.

Health education programmes in schools are essential as we and others (Mas-Coma *et al.*, 1997) have shown that children have a higher risk of infection. Children should be aware of the *F. hepatica* life cycle and the mode of transmission and be encouraged to use toilets or latrines away from water source. *F. hepatica* infection in children may be related to an increased exposure to infection as children commonly work

in the fields minding livestock where the intensity of transmission would be expected to be higher. Children are more likely to consume the aquatic plants. If algae is used as a supplement to their diet it should be washed and the eating of algae directly from the ponds or rivers should be discouraged.

It is possible that the higher incidence of infection in children observed in this study may indicate the existence of an age related immunological resistance to infection by *F. hepatica* in operation, a phenomenon that is well documented for the related digenetic trematode of the spp. *Schistosoma.* Studies performed by Hagan *et al.* (1991) demonstrated age-related resistance to schistosomiasis haematobium in Kenya. The intensity of schistosomiasis peaked in the second decade of life and fell to lower levels as the subject aged. They also observed that after treatment with praziquantel the frequency and intensity of re-infection was greatest in children under 15 years of age compared to low frequency and intensity subjects older than 15 years. Contact with water was monitored during this study and it is apparent that some adults were as intensely exposed to possible infection as the children. Specific IgE antibody against adult worms was higher in the older age group whereas higher levels of IgG4 antibodies to either worm or egg antigens were present in the younger age group. It is thought that IgE and IgG4 compete for the same antigenic sites where IgG4 blocks binding of the more specific IgE. It is apparent that as the subject ages a switch from IgG4 to IgE occurs. The development of high levels of specific IgE is associated with protection.

Immunological investigations were not logistically possible in the Bolivian Altiplano because of the reluctance of the Aymarans to provide blood samples. However, analysis of the serum obtained from individuals of all age groups showed that these elicited

high titres of IgG4 but not IgG1 isotype in response to infection. This result suggests that a type II immune response may be associated with human fasciolosis. Fundamental questions remain concerning the mechanisms of immunity to infection with *F. hepatica* in humans. To this end we employed the mouse as a model to gain some insight into the immunological mechanisms associated with infection. Although the Th1/Th2 cytokine network described for mice is not identical to that of humans the murine model provides a basis through which we can begin to understand these immune responses. In addition, information acquired in this study may also prove useful in understanding the immunological mechanisms in other animal models such as sheep and cattle since infection in these animals is a source of economic loss to farmers throughout the world.

The ability of helminth infected animals to determine the outcome of disease is dependent upon whether a type I or type II immune response is mounted for example, *T. spiralis* infection in resistant AKR mice is associated with a Th1 cytokine profile whereas the susceptible B10.BR strain predominantly secretes IL-5. This suggests that a type I response is associated with protection (Pond *et al.* 1989). However, T-cells from susceptible strains of mice infected with *T. muris* secrete IFNγ while resistant mice strains secrete the IL-5 cytokine suggesting that in this parasitic infection Th2 responses are protective (Pearce *et al.*, 1990).

Schistosomiasis in mice induces a type II response which initiates the immune-mediated granulomatous reaction to entrapped eggs. Granuloma formation is associated with the gross pathology and manifests itself clinically by portal hypertension and extensive hepatic fibrosis. SCID mice infected with *S. mansoni* are unable to generate a granulomatous response and die rapidly from severe hepatitis as a result of eggs trapped in

the hepatic tissue. Therefore, in murine schistosomiasis a type II response does not eliminate the worms but is important in limiting the immunopathology (Pearce *et al.*, 1996). In humans a more complex immunological phenomena occurs as a type II response is associated with both resistance and susceptibility to disease. However, differential type II antibody responses are associated with resistance and susceptibility; high levels of IgG4 antibodies are associated with susceptibility whereas high levels of IgE antibodies are associated with resistance (Hagan *et al.*, 1991).

B. malayi, the causative agent of lymphatic filariasis, is another important example of the balance that exists between the immune mediated expulsion of the parasite and the immunopathology associated with disease. Transmission of *B. malayi* occurs by the inoculation of the host with the infectious larvae (L3) via the intermediate host, the mosquito. The larvae migrates to the lymphatic system where mature male and female worms mate to produce microfilariae. Maizels *et al.* (1993) postulated that the Th2 responses observed in the asymptomatic individuals ultimately results in the expulsion of the microfilariae. However, expulsion of the worms is associated with the induction of a Th1 response which is believed to cause the immunopathology observed in elephantiasis.

The present study has clearly demonstrated that fasciolosis induces type II responses in mice. Antibody isotype responses in rats (Poitou *et al.*, 1993), sheep (Sexton *et al.*, 1994) and cattle (Clery *et al.*, 1996) indicate that these species also develop type II responses to infection. Immunological studies investigating cytokine responses in animals infected with *F. hepatica* have been limited to a few studies which were predominantly performed in cattle. In a study by Brown *et al.* (1994) it was demonstrated that antigen specific T-cell clones isolated from cattle chronically infected with *F. hepatica* express

Th0 and Th2 CD4+ cells but not Th1 CD4+ cells. Clery *et al.* (1996) measured mRNA from peripheral blood mononuclear cells (PBMC) lymphocytes of chronically infected cattle with *F. hepatica*. In this experiment it was demonstrated that cattle failed to produce IFNγ but produced both IL-4 and IL-5 mRNA. The proliferation of peripheral blood lymphocytes correlated with IgG1 titres to liver fluke antigen in infected cattle which confirms that a type II response is associated with *F. hepatica* infection.

Although type II responses appear to predominate in liver fluke infection it is not known whether this offers protection to the host or is responsible for the pathological sequale that is associated with disease. Various hosts have exhibited differences in their susceptibility to infection and in their ability to develop resistance. Although some studies have shown protection in mice (Lang *et al.*, 1974), they are generally considered susceptible to infection as they fail to develop resistance to a challenge infection either following light infection or immunisation with *F. hepatica* antigens. Even drug abbreviated infections also fail to induce any significant resistance to re-infection (Chapman and Mitchell, 1982). Many studies have also demonstrated a lack of resistance in sheep to liver fluke infection (Ross 1967; Sinclair, 1962; 1970). In rats and cattle resistance to re-infection is observed (Hayes *et al.*, 1973; Doyle *et al.*, 1971). Recently, Clery *et al.* (1997) have shown that cattle are not resistant to recurrent infections. In contrast, rats appear to show resistance to recurrent infections and protection in rats was shown to occur at the level of the gut mucosa (Meussen and Brandon, 1994). In conclusion these studies would suggest that Th2 responses do not protect against liver fluke infection and that rats, unlike other infected animals, are somehow unique in their response.

Vaccine studies in cattle and sheep using purified *F. hepatica* antigens have been successfully employed in reducing worm burden and egg fecundity. There are several vaccine candidates which show potential for commercial use such as the fatty acid binding protein (FABP) (Hillyer *et al.,* 1985; 1987) and the glutathione S-transferase (GST) vaccine (Sexton *et al.,* 1990). The FABP antigen elicited 31-55% protection in cattle (Hillyer *et al.*, 1985; 1987) while the GST vaccine elicited 18-69% protection in cattle (Morrison *et al.*, 1996) and 57% protection in sheep, respectively (Sexton *et al.*, 1990). The most successful vaccine to date which is presently undergoing vaccine trails was developed by this laboratory (Dalton *et al.*, 1996; Mulcahy *et al.*, 1998). This vaccine is composed of two molecules, cathepsin L2 and heme protein, which were purified from *F. hepatica* ES products (Smith *et al.*, 1993; McGonigle and Dalton, 1995). The function of cysteine proteins in immunoevasion strategies, migration and feeding have already been discussed (section 1.6). The heme protein is believed to function in the aerobic respiration of immature flukes as they migrate within the liver mass (McGonigle and Dalton, 1995). The combination of these proteins elicited 72.4% protection in cattle. Moreover, 98% of eggs recovered from these infected cattle were non-viable thus preventing transmission of the life cycle.

In experiments carried out during these vaccine trials a positive correlation in non-vaccinated animals between fluke-specific serum IgG1 levels and fluke burden was observed. In contrast, a correlation was observed between IgG2 and low fluke burden. Cattle vaccinated with the combination cathepsin L heme protein vaccine exhibited > 8 fold titre of IgG1 and > 200 fold titre of IgG2 than non-vaccinated cattle. The Th-subset response in cattle has not been clearly defined but evidence suggests that IgG1 is

associated with a type II immune response and IgG2 with a type I response. Both IgG1 and IgG2 antibodies are observed in vaccinated cattle which indicates that a Th0 cytokine response may be observed in these cattle. However, since IgG2 is only observed after vaccination and because there is a positive correlation between low fluke burden and IgG2 tires it was concluded that a IgG2 confers protection in vaccinated cattle (Mulcahy *et al.*, 1997). This would indicate that a type I response is associated with protection.

In this study we demonstrated that *F. hepatica* infection shifts the balance in the T-helper cytokine response towards a Th2 cytokine profile. This generalised Th-subset polarisation results in the down-regulating of Th1 cytokines to a bystander antigen. Several other studies have also reported the immunomodulatory effects of *F. hepatica* infection. Oldam and Williams (1985) demonstrated that T-cell proliferation and IL-2 production in cattle was suppressed by *F. hepatica* infection. Observations of immunosuppression were also made in studies performed in rats and sheep (Cervi and Masih, 1997; Chauvin *et al.*, 1995). The suppression of Th1 responses may make animals susceptible to infection that requires this arm of the immune system for protection. Moreover, this may also effect the efficacy of vaccines where the induction of a Th1 responses is required for protection.

Polyparasitosis is common in developing countries. For example, in the Bolivian Altiplano Mas-Coma *et al.* (1999) found that besides being infected with liver fluke many patients also harboured infections such as *Giardia intestinalis*, *Cryptosporidium* sp., *Ascaris lumbricoides* and *Trichuris trichiura*. Moreover, they found a direct correlation between fluke infection and susceptibility to *Giardia intestinalis*. There is also a number of reports that have correlated the susceptibility of individuals with schistosomiasis to

other infections such as hepatitis B and septicaemia salmonella (Neues and Martin, 1967; Ghaffar *et al.* 1989; Bassily *et al.* 1992). These observations raise the question of how does the immune response directed to one infection influence the immune response to another. It is known, as demonstrated in the study by Neues and Martin (1967), that because helminths induce strong Th2 responses they cause a down-regulation of Th1 specific CTL responses. Accordingly, it is believed that this makes helminth-infected patients susceptible to infections that require CTL responses for protection. Indeed, it has been shown that the successful treatment of salmonella in schistosome-infected patients first requires the removal of the schistosome parasite with praziquantel (Butterworth, 1993).

Salmonella infection has also been associated with bovine schistosomiasis and in this case treatment of the schistosome parasite is required before the salmonella infection can be successfully treated (Butterworth, 1993). A parallel in fasciolosis may be infection with *Clostridium novyi*, known as black disease of the liver. It is well established that liver fluke-infected cattle are susceptible to this disease; one theory suggests that the parasite itself carries the bacterium into the liver while another suggests that the bacterium propagates in the necrotic tissues that result from damage caused by the liver flukes. In the light of the discussion above it would be interesting to determine whether the expression of black disease in liver fluke-infected cattle is associated with the down-regulation of Th1 responses.

Recently, there has been much discussion on the similarity of the immune responses observed in helminth infections and in other hypersensivity states such as atopy (response to allergens, asthma). The clinical manifestations of both include the production

of high levels of IgE and appearance of eosinophilia which are clearly Th2 driven. To explain this similarity it has been suggested that the part of the immune system which evolved to tackle parasites, i.e. the type II responses, is now triggered by certain chemicals, proteins or particles in our environment (Cookson and Moffall, 1997). Of more fundamental relevance, the increase in atopic disorders in the developed world has now been attributed to the lack of exposure of individuals to Th1-inducing infections (Cookson and Moffall, 1997; Shirahawa *et al.* 1998). Cookson and Moffall (1997) believe that exposure to viral or bacterial infections during childhood is important in providing protection to atopy. There are several experimental findings that support this idea. Shaheen *et al.* (1996) showed that there was a 50% decrease in the number of positive atopy skin-tests during a measles epidemic in Guinea-Bissau. Skirakam *et al.* (1997) found and inverse relationship between immune response to *Mycobacterium tuberculosis* and atopy in children and Cookson and Moffal (1997) found that children with eczema underwent a spontaneous remission after severe bacterial or viral infectious. Wang and Rook (1998) also demonstrated that an established allergic response to ovalbumin in BALB/c mice was inhibited by an injection of *Mycobacterium vaccae.* Although it seems contradictory, it has also been indicated that helminth infections can protect children against asthma; however, it was suggested that the saturation of IgE binding sites on effector cells by parasite-induced IgE prevents them from reacting to aeroallergens (Cookson and Moffal, 1997).

Allergens are a heterogeneous class of molecules. One of the most common aeroallergen is Der p1 which is found in the excrement of the house dust mice, *Dermatophagoides pteronyssimus.* Der p1 is a 30 kDa cysteine proteinase which is

involved in digestion in the mite. This molecule is thought to induce atopic immune responses by cleaving the surface molecules CD23 and CD25 on B cells and lymphocytes, respectively (Hewitt *et al.,* 1995; Skakib *et al.,* 1998). CD23 is involved in the regulation of IgE; the binding of IgE to this membrane-bound molecule results in the down-regulation of IgE secretion by B cells. However, when not occupied by IgE CD23 is cleaved releasing soluble-CD23 which can enhance IgE production. It is thought that Der p1 up-regulates IgE by cleaving CD23 from B cells (Hewitt *et al.,* 1995). CD25 is a sub-unit of the IL-2 receptor found on peripheral blood mononuclear cells which, following cleavage, results in diminished lymphocyte proliferation and IFNγ secretion. Therefore, cleavage of this receptor by Der p1 would block the proliferation of Th1 cells and thereby facilitate the augmentation of Th2 responses (Shakib *et al.*, 1998).

In the context of the above discussions the findings of this study are of particular interest. We have demonstrated that *F. hepatica* can induce Th2 responses that are strong enough to overcome the Th1 responses induced by injection of an unrelated antigen, in this case *B. pertussis* whole cell vaccine. As mentioned above liver fluke infection could therefore influence the outcome of other infections. But, more importantly, liver fluke infection could negate the protection provided by Th1-inducing vaccines. Work is underway to test this hypothesis and data already obtained demonstrates that a concurrent infection of liver fluke inhibits the ability of mice to clear an active infection of *B. pertussis* (Brady T., O'Neill S.M., Dalton J.P. and Mills K.H.G., unpublished data).

Clearly, the mechanism by which *F. hepatica* induces the polarisation of the immune response to a Th2 type needs to be understood. We believe that the molecules responsible for this are most likely to be found in the ES products of the parasite.

Accordingly, we demonstrated that an intravenous injection of ES products prior to vaccination with *B. pertussis* whole cells vaccine can down-regulate the Th1 responses to the vaccine. Whilst the nature of the molecules that induce this down-regulation has yet to be established we suggest that they may well be the cathepsin L proteinases (CL1 and CL2) characterised by Smith *et al.* (1993) and Dowd *et al.* (1994) (and used in the first section of this thesis as immunodiagnostic reagents). These molecules play a digestive role in the fluke and together represent 80% of the total protein in ES products (Dowd *et al.,* 1994). Most interestingly, the two enzymes are cysteine proteinases that are highly homologous to the dust mite cysteine proteinase Der p1. Other helminths such as schistosomes and hookworms also secrete cathepsin L cysteine proteinases (Brady *et al.*, 1999; Hewitt *et al.*, 1995). The obvious question therefore arises, could helminth cysteine proteinases be responsible for inducing atopic-like reactions (high IgE and eosinophilia) in a similar manner to Der p1 i.e. the cleavage of CD23 and CD25 ? It is the intention of this laboratory to answer this question in the near future.

So how does *F. hepatica* - and indeed other helminths - drive a Th2 immune response ? Our findings suggest that the cytokines that are important in initiating the type II responses include IL-4, IL-5 and IL-10. The specific production of this IL-10 was induced when peritoneal exudate cells were stimulated with ES. Although the role of this cytokine in humans is less clear, in mice it is involved in blocking the synthesis of Th1 cytokines. It is possible that juvenile flukes migrating through the peritoneal cavity of the host may induce the production of IL-10. At the same time secreted antigens (cysteine proteinases and/or other antigens) may then cleave surface-bound molecules like CD23

and CD25 on B-cells and other lymphocytes triggering the production of IL-4 and IL-5 which would then augment the type II response.

The experiments carried out in this thesis show that *F. hepatica* is a very useful model for the study of the immune responses to helminths. The advantages of this model include a) laboratory animals, such as mice and rats, can be easily infected, and many reagents are available for studying immunological responses in these hosts b) the molecules secreted from the liver fluke are easily obtained and purified for analysis of their effects on the hosts immune effector mechanisms and c) unlike in schistosomiasis, liver fluke eggs are not trapped in the liver and hence we observe very polarised Th2 responses rather than a mixture of responses. The knowledge gained from studies performed in laboratory animals can then be applied to the more relevant hosts like cattle, sheep and humans.

9.2 Conclusions and future prospects

In this study we have confirmed that human fasciolosis has been hyper-endemic in the Bolivian Altiplano for at least a decade. Prior to the planning of a health care programme a large scale regional survey is required to fully establish the distribution of the disease throughout the entire region. To this end our highly sensitive and specific standardised diagnostic assay could be employed. The reluctance of the indigenous population to provide blood samples makes studying human fasciolosis in this region very difficult. However, taking blood samples onto filter paper provided a means of overcoming this problem.

Due to the difficulty in obtaining sufficient blood samples to explore the immunological responses of humans to fasciolosis we employed the mouse as a model to investigate the immune response associated with fasciolosis. We have demonstrated that *Fasciola hepatica* generates a highly polarised type II immune response. In addition we showed that the molecules of ES products are involved in triggering type II immune responses and in down-regulating a type I immune response. Although some progress has been made towards understanding the immunology of fasciolosis in mice there are many fundamental questions which remain unanswered. In future experiments it is important that we a) identify the cytokines and cellular components involved in the production of type II cytokines by the molecules of the ES, b) identify the molecules of ES products and the regulatory cytokines involved in the suppression of Th1 responses to the gram negative bacterial pathogen, *B. pertussis* and c) establish the role of these molecules in the molecular pathway resulting in the enhancement of type II responses or in the suppression of type I responses.

Chapter 10

Bibliography

Abdel Wahab M.F., Younis T.A., Fahmy I.A. and El Gindy I.M. 1996. Parasitic infections presenting as prolonged fevers. *Journal of Egyptian Society Parasitology*, 26(2): 509-516.

Al-habbib W.M.S. 1974. The effects on constant and changing temperatures on the development of larval stages of *Fasciola hepatica*. Ph.D Thesis, University of Dublin, Ireland.

Actor J.K., Shirai M., Kullberg M.C., Mark R., Buller L., Sher A. and Berzofsky J.A. 1993. Helminth infection results in decreased virus-specific CD8+ cytotoxic T-cell and Th1 cytokine responses as well as delayed clearance. *Proceedings of the National Academy of Science*, USA, 90: 948-952.

Allen J.E. and MacDonald A.S. 1998. Profound suppression of cellular proliferation mediated by the secretions of nematodes. *Parasite Immunology*, 5: 241-247.

Andrew S. 1999. The Life cycle of *Fasciola hepatica*. *In fasciolosis* (ed. John P. Dalton) CAB International, Wallingford, UK, 1-30.

Angehern W. and Wegmann T. 1975. Infestation of the ductus choledochus with *Fasciola hepatica*. Study of human Fasciola. *Schwiez med. Wochenschr*, 105(22): 715-718.

Angles R. 1992. Control de fascioliasis en el Altiplano Boliviano. Grant proposal.

Ambroise-Thomas P., Desgeorges P.T. and Bouttaz M. 1980. Diagnosis of human and bovine fascioliasis by means of Enzyme-Linked Immunosorbent assay (ELISA). Detection of circulating antibodies and/or antigens. *Annales de la Societe belge de Medecine Tropicale*, 60: 47-60.

Apt W., Aguilera X., Vega F., Miranda C., Zulantay I., Perez., Gabor M. and Apt P. 1995. Treatment of human chronic fasciolosis with triclabendazole; drug efficacy and serological response. *American Journal of Tropical Medicine and Hygiene*, 52(6): 532-535.

Apt W. 1993. Prevalence of Human Fascioliasis in humans, horses, pigs and wild rabbits in three Chilean provinces. *Bol. Oficina Sanit. Panam.*, 115(5): 405-414.

Arjona R., Riancho J., Aguado J., Salesa R. and Gonzalez-Macias J. 1995. Fascioliasis in developed countries: A review of classic and aberrant forms of the disease. *Medicine*, 74: 13-23.

Baeza E., Poitou I., Villejoubert C. and Boulard C. 1994. Complement depletion in rats infected with *Fasciola hepatica*: *in vivo* and *in vitro* studies. *Veterinary Parasitology*, 51: 219-230.

Bacq Y., Besnier J.M., Duong T.H., Pavie G., Metman E.H. and Choutet P. 1991. Successful treatment of acute fascioliasis with Bithionol, *Hepatology*, 14(6): 1066-1069.

Bahar K., Solymanlou F., Pourtaghva M., Rahimi F.B. and Nikbin B. 1990. A modified antigen currently used in diagnosis and follow up of recent epidemy of distomatosis in Iran. *Bulletin de la Societe Francaise de Parasitologie*, 8(2): 985.

Barbic J., Leef M.F., Burns D.L. and Shahin R.D. 1997 Role of gamma interferon in natural clearance of *Bordetella pertussis* infection. *Infection and Immunity*, 65(12): 4904-4908.

Barnard A., Mahon B.P., Watkins J., Redhead J. and Mills K.H.G. 1996. Th1/Th2 cell dichotomy in acquired immunity to *Bordetella pertussis*; variables in the *in vivo*

priming and *in vitro* cytokine detection techniques affect the classification of T-cell subsets as Th1, Th2 or Th0. *Immunology*, 87: 372-380.

Barret A.J. and Kirschke H. 1980. Cathepsin B, cathepsin H and cathepsin L. *Methods in Enzymology*, 80: 535-561.

Bassily G., Strickland M.F., Wahab A., Esmat E.G., Narooz S., El Masry N.A., Constantiene N.T. and Struewing J.P. 1992. Efficacy of hepatitis B vaccine in primary school children from a village endemic for *Schistsoma mansoni*. *The Journal of Infectious Diseases*, 166: 265-268.

Bjorland J., Byran R.T., Strauss W., Hillyer G.V. and McAuley J.B. 1995. An outbreak of acute fascioliasis among Aymara Indians in the Bolivian Altiplano. *Clinical Infectious diseases*, 21: 1228-1233.

Blagg W., Schloegel E.L., Mansour N.S. and Khalaf G.I. 1995. A new concentation technique for the demonstration of protozoa and helminth eggs in faeces. *American Journal of Tropical Medicine and Hygiene*, 4: 32-33.

Boray J.C. 1981. Fascioloiasis and other trematode infections. I. recent advances in research on Fasciola and other trematodes of animals. In: Slusarski W., ed. *Review of Advances in Parasitology*, Polish Scientific Publishers, 317-339.

Boray J.C. 1969. Experimental fascioliasis in Australia. *Advances in Parasitology*, 7: 195-210.

Boray J.C. 1967. The effect of host reaction to experimental *Fasciola hepatica* infections in sheep and cattle. In: Soulsby, E.J.L. (ed) *The reaction of the Host to Parasitism.* Proceedings of the third International Conference of the world association for the Advancement of Veterinary Parasitology. Elwert, Marburg, Germany.

Boyce W.M., Courtney C.H. and Thibideau M. 1986. Heterologous resistance to *Fasciola hepatica* conferred upon rats by passive transfer of serum from different breeds of sheep. *Veterinary Parasitology*, 22: 259-266.

Bozas S.E., Panaccio M., Creaney J., Dosen M., Parsons J.C., Vlasuk G.V., Walker I. and Spithill T.W. 1995. Characterisation of a novel Kunitz-type molecule from trematode *Fasciola hepatica*. *Molecular and Biochemical Parasitology*, 74: 19-24.

Bradford M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72: 248-254.

Brady C.P., Dowd A.M., Day S.R., Ryan T., Brindley P.J. and Dalton J.P. 1999. Functional expression and localisation of *Schistosoma mansoni*, cathepsin L1 supports its role in the degradation of host hemoglobin. *Infection and Immunity*, in press.

Brito I.V., Peel M.M. and Ree G.H. 1976. Immunological response to tetanus toxoid during a schistosomal infection in mice. *Journal of Tropical Medicne and Hygiene*, 79(7): 161-3.

Brown W.C., Woods V.M., Chitko-McKown C.G., Hash S.M. and Rice-Ficht A.C. 1994. 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. 1994. 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.

Buchon P., Cuenca H., Quiton A.M., Camacho A.M. and Mas-Coma S. Fascioliasis in cattle in the human endemic regions of the Bolivian Northern Altiplano. *Research and Reviews in Parasitology*, 57(2): 71-83.

Butterworth A.E. 1993. Immunology of Schistosomiasis. In Jordan P., Webbe G. and sturock R.F. (eds) Human Schistosomiasis. CAB international Wallingford, UK, pp 331-366.

Caceres-Vega E. 1989. *Fasciola hepatica* - "Enfermedad y Pobreza campesina". Accion un Maestro Mas, La Paz, Bolivia: imprenta Metodista: 202.

Carmona C., Dowd A., Smith A. 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.

Chapman C.B. and Mitchell G.F. 1982. Proteolytic cleavage of immunoglobulin by enzymes released by *Fasciola hepatica*. *Veterinary Parasitology*, 11: 165-178.

Chapman C.B. and Mitchell G.F. 1982. *Fasciola hepatica*: Comparative studies on fascioliasis in rats and mice. *International Journal for Parasitology*, 12: 81-91.

Caulada-Benedetti Z., al-Zamel F., Sher A. and James S. 1991. Comparison of Th1- and Th2-associated immune reactivities stimulated by single versus multiple vaccination of mice with irradiated *Schistosoma mansoni* cercariae. *Journal of Immunology*, 146(5): 1655-1660.

Chauvin A. and Boulard C. 1995. Local immune responses to experimental *Fasciola hepatica* infection in sheep. *Parasite*, 3: 209-215.

Chauvin A., Bouvet C. and Boulard C. 1995. Humoral and cellular immune responses to *Fasciola hepatica* experimental primary and secondary infection in sheep. *Tropical Diseases Bulletin*, 87: 1227-1241.

Chen M.G. and Mott K.E. 1990. Progress in assessment of morbidity due ot *Fasciola hepatica* infection: A reveiw of recent literature. *Tropical Diseases Bulletin*, vol. 87, No. 4: R1-R38.

Cervi L. and Masih D.T. 1997. Inhibition of spleen cell proliferative response to mitogens by excretory-secretory antigens of *Fasciola hepatica*. *International Journal of Parasitology*, 27(5): 573-579.

Clery D., Torgerson P. and Mulcahy G. 1996. Immune responses of chronically infected adult cattle to *Fasciola hepatica*. *Veterinary Parasitology*, 62(1-2):71-82.

Clery D., Torgerson P.R. and Mulcahy G. 1996 Immune responses of chronically infected adult cattle to *Fasciola hepatica*. *Veterinary Parasitology*, 62: 71-72.

Colin R.A., Hewitt R.A., Brown A.P., Hart B.J. and Pritchard D.I. 1995. A major house dust mite allergen disrupts the immunoglobulin E network by selectively cleaving CD23: innate protection by antiproteases. *Journal of Experimental Medicine*, 182: 1537-1544.

Coltorti E., Guarnera E., Larrieu L., Santillan G. and Aquino A. 1988. Seroepidemiology of human hydatidosis: use of dried blood sample on filter paper. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 82: 607-610.

Cook G.C. 1990. "Exotic" parasitic infections: recent progress in diagnosis and management. *Journal of Infection*, 20: 95-102.

Cookson W.O.C.M. and Moffatt M.F. 1997. Asthma: An epidemic in the absence of infection?. *Science*, 275: 41-42.

Comoy E.E., Pestel J., Duez C., Stewart G.A., Vendeville C., Fournier C., Finkelman F., Capron A. and Thyphronitis G. 1998. The house dust mite allergen, dermatophagoides pteronyssinus, promotes type II response by modulation the balance between IL-4 and IFNγ. *The Journal of Immunology*, 160: 2456-2462.

Cosme A., Alzate L., Orive V., Recasens M., Torrado J., Ruiz I. and Arenas J. 1990. Larparospic findings in liver fascioliasis. Study of 13 cases. *Rev. Esp. Enferm. Dig.*, 78(6): 359-362.

Croese J., Chapman G. and Gallagher N.D. 1982. Evolution of fascioliasis after eating wild watercress. *Australian and New Zealand Journal of Medicine*, 5: 525-527.

Dalton J.P. and Brindley P.J. 1997. Proteases of trematodes. *Advances in Trematode Biology*, 265-307.

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

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

Dalton J.P. and Joyce P. 1987. Characterisation of surface glycoproteins and proteins of different developmental stages of *Fasciola hepatica* by surface radiolabeling. *Journal of Parasitology*, 73: 1281-1284.

Dan M., Lichtenstein D., Lavochkin J., Stavorowsky M., Jedwab M. and Shibolet S. 1981. Human Fascioliasis in Israel, an imported case. *Israel Journal of Medical Sciences*, 17: 430-432.

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

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

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

Doenhoff M.J., Butterworth A.E., Hayes R.J., Strock R.F., Ouma J.H., Koech D., Prentice M. and Bain J. 1993. Seroepidemiology and serodiagnosis of schistosomiasis in Kenya using crude and purified egg antigens of *Schistosoma mansoni* in ELISA. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 87(1): 42-48.

Dowd A.J., Tort J., Roche L. and Dalton J.P. 1997. Isolation of cDNA encoding *Fasciola hepatica* cathepsin L2 and functional expression in *Saccharomyces cerevisiae*. *Molecular and Biochemical Parasitology*, 88: 163-174.

Dowd A.J., McGonigle S.M. and Dalton J.P. 1995. *Fasciola hepatica* cathepsin L proteinase cleaves fibrinogen and produces a novel type of fibrin clot. *European Journal of Biochemistry*, 232: 241-246.

Dowd A.J., Smith A.M., McGonigle S.M. and Dalton J.P. 1994. Purification and charcterisation of a second cathepsin L proteinase secreted by the parasite 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.

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

Duffus W.P.H. and Franks D. 1981. The interaction *in vitro* between bovine immunoglobulin and juvenile *Fasciola hepatica*. *Parasitology*, 82: 1-10.

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.

Eckhardt T. and Heckers H. 1981. Treatment of human fasciolosis with niclofolan. *Gastroenterology*, 81: 795-798.

Engvall E. and Perlmann P. 1974. Enzyme linked immunosorbant assay (ELISA): Quantitative assay of immunoglobulin G. *Immunochemistry*, 8: 871-874.

Espino A.M. and Finlay C.M. 1994. Sandwich enzyme-linked immunosorbent assay for detection of excretory-secretory antigens in humans with fascioliasis. *Journal of Clinical Microbiology*, 32: 190-193.

Espino A.M., Diaz A., Perez A. and Finlay C.M. 1998. Dynamics of Antigenemia and coproantigens during a human *Fasciola hepatica* outbreak. *Journal of Clinical Microbiology*, 36(9): 2723-2726.

Esteban J.G., Flores A., Aguirre C., Strauss W., Angles R. and Mas-Coma S. (in press) Presence of a very high prevalence and intensity of infection with *Fasciola hepatica* among Aymara children from the Northern Bolivian Altiplano. *Acta Tropica*.

Esteban J.G., Flores A., Angles R., Strauss W., Aguirre C. and Mas-Coma S. (in Press). A population-based coprological study of human fascioliasis in a hyperendemic area of the Bolivian Altiplano. *Tropical Medicine Institute of Health*.

Esteban J.G., Angles R., Oviedo J.A., Strauss W. and Mas-Coma S. 1996. High prevalence of fascioliasis in a children coprological study in a locality of the Bolivian Northern Altiplano. *Society for Tropical Medicine and Hygiene*.

Fagbemi B.O. and Obarisiagbon I.O. 1991. Common antigens of *Fasciola gigantica, Dicrocelium hospes* and *Schistosoma bovis* and their relevance to serology. *Veterinary Quarterly*, 13(2): 81-87.

Facey R.V. and Marsden P.D. 1960. Fascioliasis in man: an outbreak in Hamshire. *British Medical Journal*, 619-625.

Fadiel M.M. 1996. Mast cells, eosinophils and ion transport in the small intestine from experimental animals infected with *Fasciola hepatica*. PhD thesis, National University of Ireland, Dublin.

Ferguson F.G., Wikby A., Maxson P., Olsson J. and Johansson B. 1995. Immune parameters in a longitudinal study of a very old population of Swedish people: a comparison between survivors and nonsurvivors. *Journal of Biological Scientific Medicine*, 50: 378-382.

Fried B. 1994. Metacercarial excystment of trematodes. *Advances in Parasitology*, 33: 91-141.

Flores Serna A.F. and Estevez Martini R. 1988. Fascioliasis en la ciudad de la paz. *Cuadernos del Hospital de Clinicas, La Paz*, 34: 14-18.

Fukumoto S., Hirai K., Tanihata T., Ohmori Y., Stuehr D.J. and Hamilton T.A. 1997. Excretory/secretory products from plerocercoids of *Spirometra erinacei* reduce iNOS and chemokine mRNA levels in peritoneal macrophages stimulated with cytokines and/or LPS. *Parasite Immunology*, 19(7): 325-332.

Garcia-Rodriguez J.A., Martin Sanchez A.M., Fernandez Gerostarzu J.M. and Garcia Luis E.J. 1985. Fascioliasis in Spain: A review of literature and personal observations. *European Journal of Epidemiology*, 1:121-126.

Ghandour A.M., Tricker K., Doenhoff M.J., al-Robai A.A. and Banaja A.A. 1997. An enzyme-linked immunosorbent assay using *Schistosoma mansoni* purified egg antigen for the diagnosis of schistosomiasis in Saudi Arabia. *Transactions of the Royal Society for Tropical Medicine and Hygiene*, 91(3): 287-289.

Ghaffar Y.A., Kamel M., Abdel Wahab L.S., Dorgham L.S., Saleh M.S. and El Deeb A.S. 1990. Hepatitus B vaccination in children infected with *Schistosoma mansoni*: correlation with ultrasonographic data. *American Journal of Tropical Medicine and Hygiene*, 43(5): 516-519.

Grzych J.M., Pearce E., Cheever A., Caulada Z.A., Caspar P., Heiny S., Lewis A. and Sher A. 1991. Egg deposition is the major stimulus for the production of Th2 cytokines in murine schistosomiasis mansoni. *The Journal of Immunology*, 146(4): 1322-1327.

Gold D. and Goldberg M. 1976. Effect of light and temperature on hatching in *Fasciola hepatica*. *Israel Journal of Parasitology*, 25: 178-185.

Haseeb M.A. and Craig J.P. 1997. Suppression of the immune response to diphtheria toxoid in murine schistosomiasis. *Vaccine*, 15(1): 45-50.

Hagan P., Blumenthal U.J., Dunn D., Simpson A.J. and Wilkins H.A. 1991. Human IgE, IgG4 and resistance to re-infection with *Schistosoma haematobium*. *Nature*, 349: 243-245.

Han J.K., Choi B.I., Chung K.B., Han M.C. and Kim C.W. 1993. Radiolgical findings of human fascioliasis. *Abdominal Imaging*, 18(3): 261-264.

Hanna R.E.B. 1980. *Fasciola hepatica*: glycocalyx replacement in the juvenile as a possible mechanism for protection against host immunity. *Experimental Parasitology*, 50: 103-114.

Harness E., Hughes D.L. and Doy T.G., 1996. The demonstraton of pre-hepatic immune response to *Fasciola hepatica* in the mouse model. *International Journal for Parasitology*, 6:15-17.

Harrell F.E., Margolis P.A., Gove S., Mason K.E., Mulholland E.K., Lehmann D., Muhe L., Gatchalian S. and Eichenwald H.F. 1998. Development of clinical prediction model for an ordinal outcome: the world health organization multicentre study of clinical signs and etiological agents of pneumonia, sepsis and meningitis in young infants. WHO/ARI young infant multicentre study group. *Statistical Medicine*, 17(8): 909-944.

Hartmann L.F. and Patino F. 1962. Distomatosis extrahepatica. *Prensa Medica, La Paz*, 14: 104-106.

Hauser S.C. and Bynum T.E. 1984. Abnormalities on ERCP in a case of human fascioliasis. *Gastrintestinal Endoscopy*, 30: 80-82.

Hayes T.J., Bailer A.P. and Mitrovic M. 1973. Immunity to *Fasciola hepatica* in rats: the effect of two different levels of primary exposure on superinfection. *Journal of Parasitology*, 60: 930-934.

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

Hewitt C.R., Brown A.P., Hart B.J. and Pritchard D.I. 1995. A major house dust mite allergen disrupts the immunoglobulin E network by selectively cleaving CD23: innate protection by antiproteases. *Journal of Experimental Medicine*, 182(5): 1537-1544

Hillyer G.V., Soler de Galanes M., Buchon P. and Bjorland J. 1996. Herd evaluation by Enzyme Linked immunosorbant assay for the determination of *Fasciola hepatica* infection in cattle and sheep from the Alltiplano of Bolivia. *Veterinary Parasitology*, 61(3-4): 211-220.

Hillyer G.V. 1995. Comparison of purified 12 KDa and recombinant 15 kDa *Fasciola hepatica* antigens related to *Schistosoma mansoni* fatty acid binding protein, *Mem. Inst. Oswaldo Cruz*, 90 (2): 249-253.

Hillyer G.V., Soler de Galanes M., Rodriguez-Perez J., Bjorland J., Silva de Lagrava M., Ramirez Guzman S. and Bryan R. T. 1992. Use of the Falcon assay screening test-Enzyme-Linked Immunsorbent Assay (fast-ELISA) and the Enzyme-linked Immunoelectrotransfer Blot (ETIB) to determine the prevalence of human Fasciolosis in the Bolivian Altiplano. *American Journal of Tropical Medicine and Hygiene*, 46: 603-609.

Hillyer G.V., Haroun E.M., Hernandez A. and De Galanes M.S. 1987. Acquired resistance to *Fasciola hepatica* in cattle using a purified adult worm antigen. The *American Journal of Tropical Medicine and Hygiene*, 37: 363-369.

Hillyer G.V. 1985 Induction of immunity in mice to *Fasciola hepatica* with a Fasciola/Schistosoma cross-reactive defined immunity antigen. *The American Journal of Tropical Medicine and Hygiene*, 34: 1127-1131.

Hillyer G.V. 1975. Use of counterelectrophoresis to detect infections of *Fasciola hepatica*. *Journal of Parasitology*, 63: 557-559.

Hope-Cawdery M.J., Gettinby G. and Grainger J.N.R. 1978. Mathematical models for predicting the prevalence of liver fluke disease and its control from biological and meteorological data. In: *Weather and Parasitic Animal Disease*, World Meteorological Organisation Technical Note, No. 159: 21-38.

Howell R.M. 1966. Collagenase activity of immature *Fasciola hepatica*. *Nature*, 209: 713-714.

Howell M.J. 1973. Localization of proteolytic activity in *Fasciola hepatica*. *Journal of Parasitology*, 59: 454-456.

Jeannin P., Delneste Y., Lecoanet-Henchoz S., Gauchat J.F., Ellis J. and Bonnefoy J.Y. 1997. CD86 (B7-2) on human B cells. A functional role in proliferation and selective differentiation into IgE- and IgG4-producing cells. *Journal of Biological Chemistry*, 272(25): 15613-15619.

Katz N., Chaves A. and Pellegrino J. 1972. A simple device for quantitative stool thick smear technique in Schistosomiasis mansoni. *Review of the Institute of Medicine and Tropical diseases of Sao Paulo*, 14: 397-402.

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.

Kelch W.J., Coles B.M., Reynolds G.E. and Bailey D.E. 1977. Suspected infectious necrotic hepatitis (black disease) in Oregon cattle. *Journal of American Veterinary Association*, 151(5): 431-432.

Kimura K., Isogai E., Isogai H., Nishikawa T., Masuzawa T., Yoshikawa K., Kotake S., Ohno S. and Fujii N. 1995. Prevalence of antibodies against Borrelia species in patients with unclassified uveitis in regions in which Lyme disease is endemic and non-endemic. *Clinical Diagnostic Laboratory Immunology*, 2: 53-56.

Klinkert M.Q., Ruppel A., Felleisen R., Link G. and Beck E. 1998. Expression of diagnostic 31/32 kilodalton proteins of *Schistosoma mansoni* as fusions with bacteriophage MS2 polymerase. *Molecular Biochemical Parasitology*, 27(2-3): 233-239.

Knobloch J. 1985. Human fascioliasis in Cajamarca/Peru II: Humoral antibody response and antigenaemia. *Tropical and Medical Parasitology*, 36: 91-93.

Knobloch J., Delgado A.E., Alvarez A., Reymann U. and Bialek R. 1985. Human fascioliasis in Cajamarca/Peru I: Diagnostic methods and treatment with praziquantel. *Tropical Medical Parasitology*, 36: 88-90.

Kodama K., Ohnishi H., Matsuo T. and Matsumura T. 1991. Three cases of human fascioliasis. *Kansenshogaku Zasshi*, 65(12): 1620-1624.

Kullberg M.C., Pearce E.J., Hieny S.E., Sher A. and Berzofsky J.A. 1992. Infection with *Schistosoma mansoni* alters Th1/Th2 cytokine responses to a non-parasite antigen. *Journal of Immunology*, 148 (10): 3264-3270.

Laemmli U.K. 1970. Cleavage of structural proteins during the assembly of the head of a bacteriophage T4. *Nature*, 227: 680-685.

Laird P.P. and Boray J.C. 1992. Human fascioliasis successfully treated with triclabendazole. *Australian and new Zealand National Journal of Medicine*, 1:45-47.

Lang B.Z. 1974. Host-parasite relationships of *Fasciola hepatica* in the white mouse V. Age of fluke responsible for the induction of acquired immunity. *The Journal of Parasitology*, 60: 90-92.

Lang B.Z. and Dronen N.O. 1972. Host-parasite relationships of *Fasciola hepatica* in the white mouse. IV. studies on the worm transfer and the induction of acquired immunity by worms of different ages. *The Journal of Parasitology*, 58: 84-87.

Lang B.Z. 1967. Host parasite relationships of *Fasciola hepatica* in the white mouse II. Studies on acquired immunity. *The Journal of Parasitology*, 53: 21-30.

Lawrence R.A., Allen J.E., Osborne J. and Maizels R.M. 1994. Adult and microfilarial stages of the filarial parasite *Brugia malayi* stimulate contrasting cytokine and Ig isotype responses in BALB/c mice. *Immunology*, 153(3): 1216-1224.

Locatelli A. and Berretta C. 1969. Detection of amino acid N in saline or serum incubated with *Fasciola hepatica* and Proteolytic activity exerted by liver fluke *in vitro*. Archivo. Veterinario Italiano., 20: 385-391.

MacDonald A.S., Maizels R.M., Lawrence R.A., Dransfield I. and Allen J.E. 1998. Requirement for *in vivo* production of IL-4, but not IL-10, in the induction of proliferative suppression by filarial parasites. *Journal of Immunology*, 160(3): 1304-12; Corrected and republished in *Journal of Immunology*, 160(8): 4124-32

Malhotra N.K. 1996. Marketing Research. Prentice Hall International Editions.

Martin V., Arcavi M., Santillan G., Amendoeira M.R., De Souza Neves E., Griemberg G., Guarnera E., Garberi J.C. and Angel S.O. 1998. Detection of human Toxoplasma-specific immunoglobulins A, M, and G with a recombinant *Toxoplasma gondii* rop2 protein. Clinical *Diagnostic Laboratory Immunology*, (5): 627-631.

Mas-Coma S., Bargues M.D. and Esteban J.G. 1999. Human fasciolosis. *In fasciolosis* (ed. John P. Dalton) CAB International, Wallingford, UK, 411-434.

Mas-Coma S. and Bargues M.D. 1997. Human Liver Flukes. *Atlas of Infectious diseases, US Armed forces*. In press.

Massound J. 1990. Fascioliasis outbreak of man and drug test (Triclabendazole) in caspian Littoral, northern part of Iran, 1989. *Bulletin de la Societe Francaise de Parasitologie*, 8(1): 438.

Maurice J. 1994. Is something lurking in your liver?. *New scientist*. 1917: 26-31.

Maizel R.M., Bundy D.A., Selkirk M.E., Smith D.F. and Anderson R.M. 1993. Immunological modulation and evasion by helminth parasites in human populations. *Nature*, 365: 797-805.

McGonigle S. and Dalton J.P. 1995. Isolation of *Fasciola hepatica* haemoglobin. *Parasitology*, 111: 209-215.

Mc Guirk P., Mahon B.P. and Mills K.H.G. (in press) Binding of the *B. pertussis* virulence factor, filamentous haemagglutinin, to the C3R intergrin inhibits IL-12 production by macrophages. *Journal of Immunology*.

Melero M., Rigou R.C., Lloveras J. and Gennaro O. 1991. Hepatic fascioliasis. uncommon cause of prolonged febrile syndrome with hypereosinophilia and hypodense images on the comuted tomography of the liver. *Medicine*, 51(3): 244-248.

Meussen E.N.T. and Brandon M.R. 1994. The use of antibody-secreting cell probes to reveal tissue-restricted immune responses during infection. *European Journal of Immunology*, 24: 469-474.

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

Milbourne E.A. and Howell M.J. 1990. Eosinophils responses to *Fasciola hepatica* in rodents. *International Journal for Parasitolgy*, 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.

Mills K.H.G., Barnard A.L., Watkins J. and Redhead K. 1993. Cell mediated immunity to *Bordetella pertussis*: role of Th1 cells in bacterial clearance in a murine respiratory infection model. *Infection and Immunity*, 61: 399- 410.

Mitchell G.B.B, Armour J., Ross J.G. and Halliday W.G. 1981. Successful passive transfer of resistance to *Fasciola hepatica* infection in rats by immune serum and transfer factor. *Research in Veterinary Science*, 39: 246-247.

Moore K.W., Vieira P., Fiorentino D.F., Trounstine M.L., Khan T.A. and Mosmann T.R. 1990. Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRFI. *Science*, 248(4960): 1230-1234.

Moreau J.A., Fernandez J., Recco P., Seguela J.P. and Frexinos J. 1995. Efficacy and tolerance of praziquantel (Biltricide) in the treatment of distomatosis caused by *Fasciola hepatica*. *Gastroenterological Clinical Biology,* 5: 514-519.

Morrison C.A., Colin T., Sexton J.L., Bowen F., Wicker J., Friedel T. and Spithill T.W. 1996. Protection of cattle against *Fasciola hepatica* infection by vaccination with glutathione S-transferase. *Vaccine*, 14: 1603-1612.

Moser D., Doumbo O. and Klinkert M.Q. 1990. The humoral response to heat shock protein 70 in human and murine *Schistosomiasis mansoni*. *Parasite Immunology*, 12(4): 341-352.

Mosmann T.R. and Moore K.W. 1991. The role of IL-10 in crossregulation of Th1 and Th2 responses. *Immunology Today*, 12: 49-53.

Mulcahy G., Joyce P. and Dalton J.P. 1999. Immunolgy of *Fasciola hepatica* infection. *In fasciolosis* (ed. John P. Dalton) CAB International, Wallingford, UK, 341-376

Mulcahy G., O' Connor F., McGonigle S., Dowd A., Clery D.G., Andrews S.J. and Dalton J.P. 1998. Correlation of specific antibody titre and avidity with protection in cattle immunized against *Fasciola hepatica*. *Vaccine*, 16(9-10): 932-939.

Myung K., Burch D., Jackson T.F. and Stanley S.L. 1992. Serodiagnosis of invasive amebiasis using a recombinant *Entamoeba histolytica* antigen-based ELISA. *Archives of Medical Research*, 23(2): 285-288.

Neves J. and Martins N.R.L. 1967. Long duration of septicaemic salmonellosis: 35 cases with 12 implicated speices of salmonella. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 61: 541.

Ollerenshaw C.B. 1967. Some observations on the epidemiology and control of fascioliasis in Wales. In: *Proceedings of the Second International Liverfluke Colloquium*, Merck Sharp and Dohme International, Wageningen, 103-125.

Ogunrinade A.F., Chandrashekar R., Eberhard M.L. and Weil G.J. 1993. Preliminary evaluation of recombinant *Onchocerca volvulus* antigens for serodiagnosis of onchocerciasis. *Journal of Clinical Microbiology*, 7: 1741-1745.

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) products in diagnosis of acute human fasciolosis by IgM ELISA. *Tropical Medicine Parasitology*, 46: 115-118.

Oveido J.A., Bargues M.D. and Mas-Coma S. 1995. Lymnaeid snails in the Human fascioliasis high endemic zone of the Northern Bolivian Altiplano. *Research and Reviews in Parasitogy*, 55 (1): 35-43.

Pantelouris EM. 1965 The Common Liver Fluke. Pergamon Press Ltd, Oxford, London, New York, Paris and Frankfurt.

Pearce E.J., Vasconcelos J.P., Brunet L.R. and Sabin E.A. 1996. IL-4 in Schistosomiasis. *Experimental Parasitology*, 84: 295-299.

Pearce E.J. and Sher A. 1991. Functional Dichotomy in the CD4+ T-cell response to *Schistosoma mansoni*. *Experimental Parasitology*, 73: 110-116.

Pearce E.J., Caspar P., Grzych J.M. and Cheever A.W. 1991. Down-regulation of Th1 cytokine production accompanies induction of Th2 responses by a parasitic helminth, *Schistosoma mansoni*. *Journal of Experimental Medicine*, 173:159-163.

Pearlman E., Kroeze W.K., Hazlett F.E. Jr, Chen S.S., Mawhorter S.D., Boom W.H. and Kazura J.W. 1993. *Brugia malayi*: acquired resistance to microfilariae in BALB/c mice correlates with local Th2 responses. *Experimental Parasitology*, 76(2): 200-208.

Pfister K. 1984. Role of IgE in fascioliasis. *Preventative Veterinary Medicine*, 2: 359-370.

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

Piedrafita D. 1995. Immune mechanisms of killing of juvenile *Fasciola hepatica*. *PhD thesis, LaTrobe University , Melbourne, Australia.*

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. 1992. Kinetic responses of parasite-specific antibody isotypes, blood leukocyte pattern and lymphocyte subsets in rats during primary infestation with *Fasciola hepatica*. *Veterinary Parasitology*, 49: 179-190.

Pond L., Wassom D.L. and Hayes C.E. 1989. Evidence for differential induction of helper T cell subsets during *Trichinella spiralis* infection. *Journal of Immunology*, 143 (12): 4232-4237.

Purkerson J. and Isakson P. 1992. A two-signal model for regulation of immunoglobulin isotype switching. *Federation of European Biochemical Society Journal*, (14): 3245-3252.

Pourtaghva M., Shafi A., Saberi A., Bahar K. and Solymanlou F. 1990. Fasciolase en Iran. *Bulletin de la Societe Francaise de Parasitologie*, 8(1): 404.

Price T.A. Tuazon C.U. and Simon G.L. 1993. Fasciolosis: case reports and review. *Clinical Infectious Diseases*, 3: 426-430.

Pritchard D.I., Kumar S. and Edmond P. 1993. Soluble (s)CD23 levels in a parasitized population from papua new guinea. *Parasite Immunology*, 15: 205-208.

Prokpowicz D., Bobrowska E., Bobrowski M. and Grzeszcuk A. 1995. Prevalence of antibodies against tick-borne encephalitis among residents of north-eastern Poland. *Scandinavian Journal of Infectious Diseases*, 27: 15-16.

Rajasekariah G.R. and Howell M.J. 1979. *Fasciola hepatica* in rats: transfer of immunity by serum and cells from infected to *F. hepatica* naive animals. *Journal of Parasitology*, 65(4): 481-487.

Rakhamanov E.R. 1987. Complex treatment of patients with chronic fasciolosis complicated by bacterial infection of the biliary tract. Medicinskaja parazitologiya I parazitarnye bolezni, 2: 32-34.

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(1): 13-22.

Redhead K., Barnard A., Watkins J. and Mills K.H.G. 1993. Effective immunization against *Bordetella pertussis* is dependent on the induction of cell mediated immunity. *Infection and Immunity*, 61: 3190-3195.

Rege A.A., Herrera P.R., Lopez M. and Dresden M.H. 1989. Isolation and Characterisation of cysteine proteinase from *Fasciola hepatica* adult worms. *Molecular and Biochemical Parasitology*, 35: 89-96.

Reiner S.L. and Locksley R.M. 1993. The worm and the Protozoa: Stereotyped Responses or distinct antigens. *Parasitology Today*, 9(7): 258-260.

Riedtmann H.J., Obeid T., Aeberhard P. and Sakmann P. 1995. *Fasciola hepatica*- a unusual cause of acute cholecytitis with cholestatic jaundice. *Schweiz Med Wochenscher*, 125(35): 1642-1648.

Ripert C., Tribouley J., Loung Dinh Giap G., Combes A., Laboride M. and Bourianne C. 1988. Epidemiologie de la fasciolse humanie dans le sud ouest de la France. *Medecine et chirurgie digestives*, 17: 355-358.

Roberts E.W. 1950. Studies on the life cycle of *Fasciola hepatica* (Linnaeus) and its snail host, *Limnaea truncatula*, in the field and under controlled conditions in the laboratory. *Annuals of Tropical Medicine and Parasitology*, 44:187-206.

Roberts J.A., Widjayanti S., Estuningsih E. and Hetzel D.J. 1997. Evidence for a major gene determining the resistance of Indonesian thin tailed sheep against *Fasciola gigantica*. *Veterinary Parasitology*, 68: 309-314.

Roche L., Dowd A.J., Tort J., McGonigle S., McSweeney A., Curley P., Ryan T. and Dalton J.P. 1997. Functional expression of *Fasciola hepatica* cathepsin L1 in *Saccharomyces cerevisiae*. *European Journal of Biochemistry*, 497: 1620-1628.

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.

Rowan W.B. 1956. The mode of hatching of the egg of *Fasciola hepatica*. *Experimental Parasitology*, 5: 118-137.

Rowcliffe S.A. and Ollerenshaw C.B. 1960. Observations of the bionomics of the egg of *Fasciola hepatica*. *Annuals of Tropical, Medicine and Parasitology*, 54: 172-181.

Ryan M., Murphy G., Gothefors L., Nilsson L., Storsaeter J. and Mills K.H.G. 1997. *Bordetella pertussis* respiratory infection in children is associated with preferential activation of type I T-helper cells. *The Journal of Infectious Diseases*, 175: 1246-1250.

Sabin E.A., Araujo M.I., Carvalho E.M. and Pearce E.J. 1996. Impairment of tetanus toxoid-specific Th1-like immune responses in humans infected with *Schistosoma mansoni*. *Journal of Infectious Diseases*, 173(1): 269-272.

Sandman R.M. and Howell M.J. 1981. Response of sheep to challenge infection with *Fasciola hepatica*. *The Journal of Parasitology*, 74: 818-820.

Sampio Silva M.L., Vindimian M., Wattre P. and Capron A. 1985. IgE antibodies in human *Fasciola hepatica* distomiasis. *Pathologie et Biologie*(Paris), 33: 746-750.

Schmidt G.D. and Roberts L.S. 1989. Foundations of Parasitology. 4th edition. Times Mirror/Mosby College Publishing.

Sexton J.L., Milner A.R., Panaccio M., Waddington J., Wijffels G., Chandler D., Thompson C., Wilson L., Spithill T.W., Mitchell G.F. and Cambell N.J., 1990. Glutathione S-transferase. Novel vaccine against *Fasciola hepatica* infection in sheep. *The Journal of Immunology*, 145: 3905-3910.

Sexton J.L., Wilce M.C., Colin T., Wijffels G.L., Salvatore L., Feil S., Parker M.W., Spithill T.W. and Morrison C.A. 1994. Vaccination of sheep against *Fasciola hepatica* with glutathione S-transferase. Identification and mapping of antibody epitopes on a three-dimensional model of the antigen. *The Journal of Immunology*, 152: 1861-1872.

Shaheen S.O., Aaby P., Hall A.J., Barker D.J., Heyes C.B., Shiell A.W. and Goudiaby A. 1996. Measles and atopy in Guinea-Bissau. *Lancet*, 347(9018): 1792-1796.

Shakib F., Schulz O. and Sewell H. 1998. A mite subversive: cleavage of CD23 and CD25 by Der pI enhances allergenicity. *Immunology Today*. 19(7): 313-316.

Shaw J.N. 1932. Studies of liver fluke, *Fasciola hepatica*. *Journal of the American Veterinary Association*, 34: 76-82.

Sher A., Fiorentino D., Caspar P., Pearce E. and Mosmann T. 1991. Production of IL-10 by CD4+ T lymphocytes correlates with down-regulation of Th1 cytokine synthesis in helminth infection. *Journal of Immunology*, 147(8): 2713-2716.

Shiddo S.A., Aden Mohamed A., Akuffo H.O., Mohamud K.A., Herzi A.A., Herzi Mohamed H., Huldt G., Nilsson L.A., Ouchterlony O. and Thorstensson R. 1995. Visceral leishmaniasis in Somalia: prevalence of markers of infection and disease manifestations in a village in an endemic area. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 89: 361-365.

Shirakawa T., Enomoto T., Shimazu S. and Hopkin J.M. 1997. The inverse association between tuberculin responses and atopic disorder. *Science*, 275: 77-79.

Silva Sampaio M.L., Correia Da Costa J.M., Viana Da Costa A.M., Pires M.A., Lopes S.A., Castro A.M. and Monjour L. 1996. Antigenic components of excretory-secretory products of adult *Fasciola hepatica* recognised in human infections. *American Journal of Tropical Medicine and Hygiene*, 54: 146-148.

Sinclair K.B. 1971. Acquired resistance to *Fasciola hepatica* in sheep. *British Veterinary Journal*, 129: 236-250.

Sinclair K.B. 1970. The pathogenicity of *Fasciola hepatica* in previously infected, corticosteroid-treated lambs. *British Veterinary Journal*, 127: 125-136.

Sinclair K.B. 1962. Observations on the clinical pathology of ovine fascioliasis. *British Veterinary Journal*, 118: 37-53.

Sloan T., Dooge D. and Joyce P. 1991. Identification of phosphorylcholine containing antigens of *Fasciola hepatica* - successful tolerization against this epitope in experimental animals. *Parasite Immunology*, 13: 447-455.

Smith A., Dowd A.J., Heffernan M., Robertson C. and Dalton J.P. 1993. *Fasciola hepatica*: a secreted cathepsin L-like proteinase cleaves host immunoglobulin *International Journal of Parasitology*, 23: 977-983.

Smith A., Dowd A., McGonigle S.M., Keegan P., Brennan G., Trudgett A. and Dalton J.P. 1993. Purification of a cathepsin L-like proteinase secreted by adult *Fasciola hepatica*. *Molecular Biochemical Parasitology*, 62: 1-8.

Smith P.K., Krohn R.I., Hermanson G.T., Mallia G.T., Gartner F.H., Provenzano M.D., Fujimoto E.K., Goeke N.M., Olson B.J. and Klenk D.C. 1985. Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*. 150: 76-85.

Smithers S.R. 1982. Fascioliasis and other trematode infections. In: Immunology of Parasitic Infrctions. 2nd edition (S. Cohen & K.S. Warren) Blackwell Scientific Publications, Oxford, 608-621.

Smyth J.D. and Halton D.W. 1983. The physiology of trematodes. 2nd edition. Cambridge University Press, Cambridge, 446.

Spithill T.W., Piedrafita D. and Smooker P.M. 1998. Immunological approaches for the control of fasciolosis. *International Journal of Parasitology*, 27(10): 1221-1235.

Stevens T.L., Bossie A., Sanders V.M., Fernandez-Botran R., Coffman R.L., Mosmann T.R. and Vitetta E.S. 1988. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature*, 334(6179): 255-2588.

Stork M.G., Venables S.M., Jennings S.M.F., Beesley J.R., Bendezu P. and Capron A. 1973. An investigation of endemic fasciolosis in peruvian village children. *Journal of Tropical Medicine and Hygiene*, 76: 231-235.

Sukhedo M.V.K. and Mettrick D.F. 1987. Parasite behaviour: Understanding platyhelminth responses. *Advances in Parasitology*, 26: 73-144.

Wang J.Y., Ye H.Z., Lu M.L. and Znang C.X. 1981. Treatment of *Fasciola hepatica* infection with hexachloroparaxylol (hetol); a case report. *Chinese Journal of Pediatrics*, 19: 246.

Chapter 11

Appendix

PUBLICATIONS:

O' Neill SM., Parkinson M., Strauss W., Angles R. and Dalton JP 1998. Immunodiagnosis of *Fasciola hepatica* infection in a human population in the Bolivian Altiplano using purified cathepsin L cysteine proteinase. *American Journal of Tropical Medicine and Hygiene* 58(4): 417-423.

O' Neill SM., Parkinson M., Strauss W., Dowd AJ,. Angles R. and Dalton JP 1998. Immunodiagnosis of human fasciolosis using recombinant Fasciola hepatica cathepsin L1 cysteine proteinase. *American Journal of Tropical Medicine and Hygiene* (in press).

Strauss W., **O' Neill SM.**, Parkinson M., Angles R. and Dalton JP. Diagnosis of human fasciolosis: detection of anti-cathepsin L antibodies on blood samples collected on filter paper. *American Journal of Tropical Medicine and Hygiene* (in press).

O' Neill SM., Mills KHG., Joyce P., Mulcahy G., Brady MT., Murphy G. and Dalton JP (1999) Cytokine and antibody profile in mice infected with *Fasciola hepatica*. in preparation.

Brady MT., **O' Neill SM.**, Dalton JP. and Mills KHG., IL-4 dependent down-regulation of *Bordetella pertussis* specific Th1-like immune response in mice infected with *Fasciola hepatica*. (in preparation).

PRESENTATIONS

O' Neill SM, Parkinson M, Strauss W, Angles R and Dalton JP. (1996) Immunodiagnosis of *fasciola hepatica* infection (fasciolosis) in a Human population in the Bolivian Altiplano using purified Cathepsin L cysteine proteinase. Presented to the Irish society for Parasitology, UCG, Ireland.

O' Neill SM, Parkinson M, Strauss W, Angles R and Dalton JP. (1997) Immunodiagnosis of Human fasciolosis using purified Cathepsin L cysteine proteinase. Presented to the British Society for Parasitology, manchester university

O' Neill SM, Mills K, Brady M and Dalton JP. IL-4 dependent down-regulation of *Bordetella pertussis* specific Th1-like immune response in mice infected with *Fasciola hepatica*. Presented to the Irish society for Parasitology, UCD, Ireland.