Immune responses to *Fasciola hepatica* infection, and *Fasciola hepatica* derived antigens

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MASTERS OF SCIENCE

by

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I hereby certify that this material, which I submit for assessment on the program leading to the award of MSc 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

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Abbreviations

BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CL1	Cathepsin L1
CL2	Cathepsin L2
ELISA	Enzyme linked immunosorbant assay
ES	Excretory / Secretory products
FCS	Foetal calf serum
IFN-γ	Interferon-gamma
IL	Interleukin
LFH	Liver fluke homogenate
PAGE	Polyacrylamide gel electrophoresis
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,-tetramethylenediamine
Th	T helper cell
Tris	Tris hydroxymethy-aminomethane
Tween 20	Polyoxymethylenesorbatin monolaurate
recFheCL1	recombinant cathepsin L1
mutFheCL1	mutant cathepsin L1

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Abstract

The aim of this study was to investigate immune responses as a result of infection with the parasitic helminth, *Fasciola hepatica* Analysis of IL-4 and Interferon-γ cytokines described a predominant type 2 immune response in BALB/c mice infected with metacercaria of *F hepatica* Levels of IL-4 mRNA assessed by reverse transcription-polymerase chain reaction (RT-PCR) provide the first evidence that the immune response becomes polarised 1 day post infection We also investigated immune responses to *F hepatica* derived antigens IL-4 and Interferon-γ cytokine production revealed a polarised type 2 response in BALB/c mice immunised with *F hepatica* excretory/secretory (ES) products As type 1 immune responses have been associated with protection against infection with *F hepatica* (Mulcahy *et al*, 1998), we established a polarised type 1 immune response in BALB/c mice by immunising with cathepsin L in combination with various adjuvants

1.1

Introduction

1 1 1 Introduction

Parasitic helminthic worms comprise a diverse group of metazoan organisms, which represent an enormous burden on human and ruminant health in most tropical countries and can cause serious disease in infected populations (Allen & Maizels, 1997) The impact of helminthic infections is more as a result of the large numbers of individuals infected, than that of the severity of the disease (Allen & Maizels, 1996) While clinical symptoms of infection may not always be displayed by the infected individual, disease may arise from an overwhelming burden of infection, or as a result of an mappropriate immune response Individuals may become infected concurrently with multiple helminth species, and can accommodate parasites for several years (Allen & Maizels, 1997) Among the parasitic helminths, *Fasciola hepatica,* the causative agent of fascioliasis, exhibits a wide range of distribution, with humans, livestock and wild animal infections being reported on the five continents (Rondelaud *et al* , 2000)

Fascioliasis is one of the most common helminth infections of cattle and sheep, and can result in productivity losses impacting on the economy of the livestock industry Economic losses include costs of anti-helminthics and land drainage, and losses in productivity as a result of mortality, including reduction in meat, milk and wool production (Salehaa, 1991) It has been estimated that losses due to fascioliasis may amount to more than $200 million dollars annually (Spithill & Dalton, 1998) Fascioliasis is widespread in Ireland and is a particular problem in areas where high rainfall and poor draining soils combine to exacerbate the situation However, the prevalence of infection is significantly higher in developing countries (MacDonald *et al* , 2002)

Fascioliasis is also recognised as an important disease in humans, with an estimated 17 million people considered to be infected (Hopkins, 1992) and a further 180 million at risk of infection Humans may contract infection via the consumption of raw vegetables or the consumption of contaminated water Infections are hypoendemic in areas of South America, Iran, Egypt, Portugal and France (Esteban *et al*, 1997) In Bolivia and Peru, prevalence of infection is considered to be hyperendemic, and fascioliasis is considered to be a serious health problem The most striking levels of infection are recorded in the Bolivian Altiplano, in which prevalences between 72 and 100% have been observed (Mas Comma *et al*, 1999) It has been estimated that over a quarter of a million humans are infected in the Bolivian altiplano alone (Hillyer & Apt 1997, O'Neill *et al*, 1998) Human fasciolosis has also been observed in the European countries of France and Spam (Arjona *et al*, 1995)

1 2 1 Life-Cycle of *F hepatica*

The complex life cycle of *F hepatica* involves two distinct stages within two different hosts, the primary or definitive host and the secondary or intermediate host *F hepatica*, in general, persists for years in the bile duct of its definitive host (e g sheep, cattle or human) where it undergoes sexual reproduction The life cycle entails passage from the primary host, into the secondary intermediate host or vector, an invertebrate (lymnaeid snail) *F hepatica* utilises the intermediary host to increase its numbers by asexual reproduction before re-establishment in a definitive host, where it reproduces sexually thus completing its life cycle

Eggs produced by the mature fluke are passed from the bile duct into the duodenum and into the faeces of the definitive vertebrate host Embryonation of

the eggs occurs once they have exited the definitive host A temperature of between 10-30°C is required for embryonation In the absence of water eggs will desiccate rapidly Eggs can remain viable in faeces from three weeks to several months before they are liberated by the action of rain, deposition of faeces in water and the trampling of faeces by animals A "hatching" enzyme (Rowan, 1956) aids in the hatching of the egg, and liberation of the miracidia Hatching occurs in the presence of light (Roberts, 1950) and ambient temperatures (Gold & Goldberg, 1976) The free-swimming miracidia need to find a secondary host within 24 hours of hatching (Hope Cawdery *et al* , 1978)

The secondary host is usually *Lymnea truncatula* (Boray, 1966), although other lymnaeid snails may also be infected The snails' habitat is usually close to the edge of small ponds or marshy land The miricidium is photosensitive, and tends to move towards light sources This ensures that it will not waste time exploring the deeper areas of ponds where *L truncatuala* does not reside Stimulant molecules exist in the mucus of snails (Wilson *et al* , 1971) to which miracidia are attracted A positive chemotactic response by the miricidia occurs up to a distance of 15 cm (Nehanus, 1953) Specific attachment and subsequent penetration in *L truncatuala* is due to the texture of the epidermis (Mattes, 1936) Penetration of the snail is achieved by mechanical boring by the miricidial anterior papilla, which are aided by the secretion of proteolytic enzymes (Smith & Halton, 1983) Tissue at the point of penetration is observed to be degraded (Wilson *et al* , 1971)

Upon entry the mericidium transforms to the next larval stage, termed the sporocyst and migrates to the digestive gland where it proceeds to develop into the next larval stage, the redia The existence of *F hepatica* in the secondary host

results in several detrimental effects on the intermediate host, including castration or decrease in fecundity, increased mortality, destruction of the digestive gland, metabolic changes (re-allocation of energy from reproduction and growth, inducing gigantism), increased sensitivity to environmental stress (Gutierrez *et al*, 2000) Experimental infections have demonstrated that the *F hepatica* induces higher mortality in snails originating from populations with low natural prevalences than those with high prevalences (Bargues *et al*, 1997), indicating that co-adaptation between host and parasite may occur The redia move actively in the hosts tissue and cause considerable damage Redia multiply to form germinal cell balls from which the final larval stage, the cercaria, is produced Fully developed cercaria emerge from the snail by way of the birth pore The mobile, tadpole-like cercaria usually leave the snail 4 -7 weeks after infection

Between a few minutes and 2 hours after emergence from the snail, the cercaria attaches by means of a vertical sucker to various object including blades of grass Once attached the body contracts inwards and the outer layer of the cyst is formed Simultaneously, as the embryonic "epithelium" is shed and the outer layer is established, the tail separates from the body The cyst is immediately infective to the definitive host Longevity of the encysted metacercaria, while waiting to be consumed by the definitive host, depends on various climatic conditions Survival for long periods (several weeks-several months) is mainly dependant on sufficient moisture and moderate temperatures Major sources of infection for the definitive hosts are plants associated with water, such as watercress (Roneldaud *et al*, 2000)

Within an hour of infection, metacercaria begin to excyst in the small intestine Metacercarial excystation involves intrinsic factors such as secretions by the fluke, and extrinsic factors including elevated temperature, pCo^2, reducing conditions, pH and the presence of bile salts There are two stages of excystation , a passive active stage and an active emergence stage (Smith, 1981) The activation stage occurs in the stomach prior to emergence, and is stimulated by high levels of Co_2 temperature of about 39°C and reducing conditions The miricidia empties its caecal contents (Sukhedo & Mettrick, 1986), which contain secretions which affect the inner cyst wall, aiding emergence Bile may be influential in the emergence phase, and may activate an enzyme secreted by the parasite enhancing muscular movements of the young fluke (Dixon, 1966) Within two hours of infection the juvenile liver flukes have bored through the wall of the intestine by breaking down epithelial cells, connective tissue and muscle fibres, causing extensive haemorrhage, and can be found in the abdominal cavity *en route* to the

liver Once through the liver capsule, the flukes burrow through the liver tissue for 5-6 weeks causing extensive haemorrhage and fibrosis The flukes reach the bile ducts within 7 weeks of infection where they develop into sexually mature adults Flukes become established for a considerable time in the liver Flukes residing for a period of 11 years have been recorded in sheep by Durbin (1952) The fluke is hermaphroditic and self-mating may occur Eggs are produced by each fluke after approximately one more week of development The embryonated eggs are passed from the bile duct into the duodenum and subsequently into the faeces, thus completing the life cycle

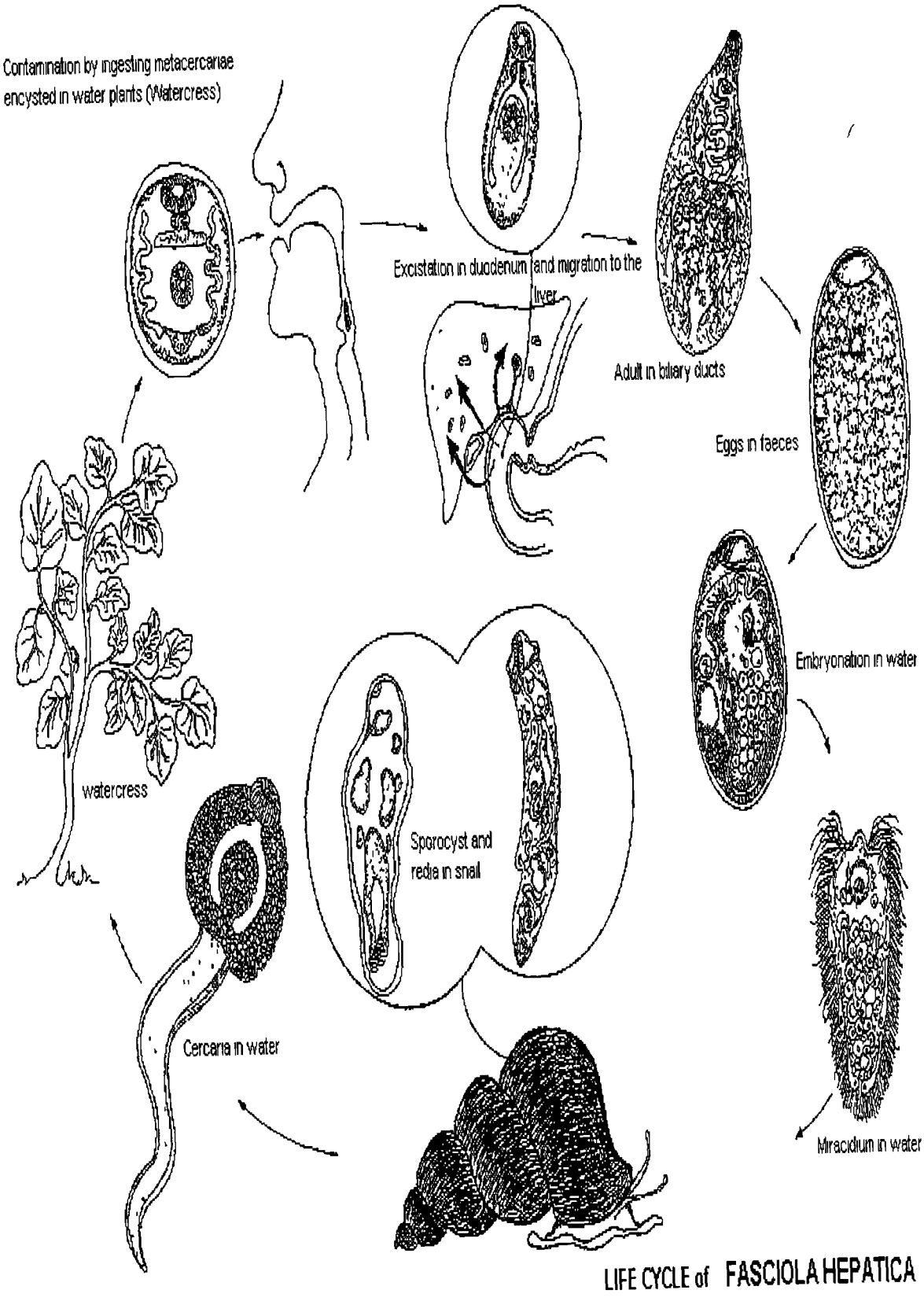


Fig 1 1 Life-cycle of *F hepatica* (Andrews, 1999)

1 3 1 Pathology of *F hepatica* infection

Flukes travel within and between organs, for a period of 12-16 weeks as they develop An individual fluke may pass trough the same area of the liver several times, and as a result, fresh lesions caused by sequential damage may be found in the same section of tissue The size of the tracts and the extent of the resulting damage will increase as the fluke grows to maturity Tracts have been observed composed of blood cells, cellular debris and infiltrate of numerous eosinophils, macrophages and $CD3^+$ lymphocytes, plasma cells and proliferation of fibrous connective tissue (Martinez-Moreno *et al*, 1999) Eosinophilia is observed at the early stages, and thereafter during *F hepatica* infections Eosinophilia increases rapidly at the parenchymal stage, and persists at an elevated level after the flukes have entered the bile ducts (Ross *et al*, 1966) Experiments involving sheep have demonstrated how juvenile flukes induce the formation of granulomatous lesions in hepatic parenchyma (Chauvin & Boulard, 1996) Fluke migration leads to immune mediated damage of liver tissue as infiltrates of immune cells replace wide areas of hepatic parenchyma (Martinez-Moreno *et al*, 1996) The extent of the inflammatory response increases as the fluke increases in size The level of infection also affects the pathology, with heavy burdens causing more severe pathology and earlier termination by death, especially in the case of sheep Secondarily infected hosts exhibit more severe hepatic damage than that of primarily infected animals (Martinez-Moreno *et al*, 1996)

The severity of disease varies depending on the level of infection, the nutritional plane of the animals and also varies between animals in a group Acute fascioliasis may cause sudden death of stock, especially in sheep An early indication of infection may be the presence of abdominal pain or ascites Sub-

acute disease is a haemorrhagic anaemia which is slightly more protracted than the acute disease Acute haemonchosis also causes a fatal anaemia Calves may suffer from acute fasciolosis in heavy infestations Pallor of mucous membranes, ventral oedema, wool break, and weight loss are associated with chronic disease Chronic disease is uncommon in cattle but may manifest in production loss, however sheep with chronic disease die with obvious signs including the presence of eggs in faeces

Changes in blood constitution is observed in infected hosts *F hepatica* infection is known to cause anaemia in infected individuals, and anaemic responses have been observed in the presence of late immature and mature flukes in the bile duct of infected hosts (Martinez-Moreno *et al*, 1996) Direct feeding on host blood results in blood loss at a rate of 0 2-0 5ml per day per fluke (Dawes & Hughes, 1964) Anaemia is not usually associated with the parenchymal stage of the disease, except in mice (Dawes, 1963), unless the infection burden is severe, in which case significant mortality coincides with hepatic haemorrhages when the flukes are migrating to the bile duct

Albumin and immunoglobulins are the major protein components of plasma Serum albumin is only produced by the liver whereas leucocytes are produced at a variety of sites in the body Therefore, hypoalbuminaemia and hyperglobulinaemia regularly occur in liver fluke infections in all hosts Liver damage caused by migrating flukes at the parenchymal stage of infection compromises liver function Studies by Dalton & Heffernan (1989) demonstrated that migrating flukes secrete endo-proteinases which may function in parasite migration Bersain *et al* (1997) described Cathepsin L1, secreted by *F hepatica* as being capable of degrading extracellular matrix and membrane components and

thus aids in parasite migration through the tissue of the host In sheep and calves this parenchymal damage is reflected in the decline in plasma albumin concentrates (Anderson *et al*, 1977) During the biliary stage of the infection, loss of blood is so severe that the functional capability of the liver is insufficient to replace lost albumin Although the liver parenchymal tissue has healed by this stage, more metacercariae from subsequent infections further damage the liver Plasma albumin is progressively diminished in infected hosts An elevation in the levels of immunoglobulins occurs several weeks after infection and immunoglobulins including IgM, IgG1, and IgE persist throughout the infection (Holmes *et al*, 1968)

Activities in the serum of the hepatocyte enzymes glutamate dehydrogenase and glutamate-oxaloacetate aminotransferase increase markedly during early infection, peaking towards the end of the parenchymal stage (Thorpe, 1965) Damage to the bile duct can be indicated by the presence of γ-glutamyl transferase, an enzyme produced by the epithelium of the bile duct, in blood The peak of γ-glutamyl transferase activity follows the peak of hepatocyte enzymes (Anderson *et al*, 1977) The extent to which hepatic enzymes found in the blood as a result of damage to the liver tissuehas been used to monitor the progress of infection

Most of the damage caused to the liver tissue appears to be as a direct consequence of the spines and prehensile sucker action of the liver fluke Haemorrhaging induced by this damage may result in the death of the host In infections of mice (Dawes, 1963c), cattle (Dow *et al*, 1967) and sheep (Sinclair, 1967) desquamation and ulceration were observed in liver tissue close to the spiny body of flukes In some cases indentation of spines in the tissue was observed

The fluke obtains the majority of its nutrition by means of its oral sucker, in a process which can cause considerable damage to the liver Oral suckers are the main organ involved in tissue damage It has been observed by Sukhedo *et al* (1988), that chronic ulceration and haemorrhage were associated with areas of tissue adjacent to oral suckers The ventral sucker which the fluke uses as a hold-fast organ has also been observed to inflict damage on host tissue (Dawes, 1963) Enlargement of the bile duct wall and lumen as a result of hyperplasia of the epithelial and sub-epithelial cells occurs long before the maturing fluke enters the bile ducts (Dawes, 1963a) Increased concentrations of the amino acid proline appear to be an important factor in this process Infusion of proline in rats mimicked in part, enlargement of the bile duct by flukes (Modavi & Isseroff, 1984)

An inflammatory response mounted by the host coincides with mechanical damage caused by the migrating fluke (Dawes, 1963) Studies in sheep (Sinclair, 1968, 1975) demonstrated that an inflammatory response plays a protective role against damage caused by the invading fluke Fluke tracks fill with cellular debris and damage to the cells surrounding the tracks is evident Macrophages and fibroblasts accumulate in older areas of tracks forming scar tissue In hosts with a heavy burden, fibrosis of the liver becomes severe, and is more evident in cattle than in other hosts Fibrosis may restrict movement of the fluke Once flukes have entered the bile ducts the parenchymal tissue recovers and inflammation is restricted to the epithelia of the bile ducts Treatment of infected sheep with dexamethasone, an anti-inflammatory agent that kills lymphocytes, permitted more rapid development of the fluke, and resulted in increased physical damage to the liver In treated sheep there was extensive haemorrhaging, little hepatic

fibrosis, and no thickening of the bile duct wall The sheep displayed clinical signs of illness such as pallor, weakness, weight loss and anaemia These clnical signs were not observed in infected controls

While the inflammatory response has an important role in the response of the host to the invading parasite there is also evidence that this response also leads to hepatic dysfunction Many aspects of liver dysfunction, including bio-energetic abnormalities, accumulation of non-esterified fatty acids and depletion of phospholipids do not occur in fluke infection where the hosts T-cell function was inhibited (Hanisch *et al*, 1991) Oxidative stress imposed on infected rat livers is as a result of inflammatory cells such as neutrophils, macrophages and eosinophils, which produce oxygen free radicals, nitric acid and their products It is important that the host strikes a balance between the protective inflammatory response against fluke damage to the liver, and the inflammatory response which leads to dysfunction of the liver

The liver has many functions including metabolism of ammo acids, carbohydrate and lipid balance, urea synthesis, ketogenesis and detoxification Therefore, liver fluke infection and subsequent damage to the liver may induce many systemic changes, leading to reduced productivity in livestock The magnitude of the systemic changes generally depends on the extent of the infection Reduced weight gain in cattle and sheep have two main causes reduced feed conversion and anorexia Fluke burdens <200 in sheep do not induce severe anorexia (Sinclair, 1975), indicating that loss of weight is due to compromised feed conversion In sheep with a higher fluke burden anorexia is a consistent feature of chronic fasciolosis Animals on poorer diets display more severe disease symptoms than those on a higher level of nutrition (Berry & Dargie,

1976) Experiments (Dargie *et al*, 1979) involving heavy fluke burdens of 1000 metacercaria in sheep, showed that nitrogen retention was lower after week 8 of infection, which could account for the difference in body weight, and that the loss of nitrogen was as a result of increased urinary excretion rather than decreased intestinal absorption

The liver is important in controlling the concentration of blood glucose This function is especially important in ruminants as glucose is not acquired directly from the diet, but is manufactured from a glucose precursor, by the process of gluconeogenesis Dysfunction in hepatic carbohydrate metabolism may occur as a direct result of infection with *F **hepatica*** Infected rats show lower levels of glycogen throughout infection than that of control animals (Gameel, 1982) A study of infected sheep (Lenton *et al*, 1996) showed that levels of glucogenesis in the left lobe was impaired, while that of the right lobe functioned as normal Therefore in low to moderate infections of sheep, carbohydrate metabolism, while compromised in the left lobe, may be compensated for by activity in the less affected area

1 4 1 Introduction to Immunology

Immunity refers to the mechanisms employed by the body to protect against environmental agents which are foreign to the body The primary function of the immune system is to eliminate infectious agents and to minimise the damage they cause Animals evolved immune defences to protect against viruses, bacteria, fungi, protozoa and helminths The immune responses can be classified as either innate immunity or adaptive immunity

1 5 1 Innate immunity

Innate immunity is a product of evolution Its function does not require a learning process or active recollection of previous encounters by the individual The effectiveness of the innate protection is determined by such features as the species of the individual and the non-specific activity of its tissue cells Innate immunity is referred to as the defensive elements with which an individual is born and which are always present and available at short notice to protect the individual from challenges by foreign invaders These elements include the skin, surface mucous layers, and the cough reflex, which present effective barriers to environmental agents Chemical influences such as pH and secreted fatty acids provide effective barriers against invasion by many micro-organisms

Innate immunity is also concerned with the early phase of immune responses during which the body employs phylogenitically conserved receptors to identify and respond to a wide range of components of organisms (Medzhitov & Janeway Jr , 2000) This early response results in a rapid activation of immune system cells and the subsequent release of a variety of inflammatory mediators Components of the innate immune system include dendritic cells (DCs), macrophages and natural killer (NK) cells Immature DCs are among the first cells to detect invading microbes (Pulendran *et al* , 2001) DCs utilize various receptors to detect potential pathogens and respond by secreting a number of cytokines (Banchereau *et al* , 2000) Cytokines are molecules involved in signalling between cells during immune responses All are proteins or peptides, some with sugar molecules attached (glycopeptides) There are a number of categories of cytokines including, (i) Interferons (IFNs), which are involved in immune responses to certain bacterial and viral infections Interferons are involved in the early stages of

immune responses and are considered to be the first line of resistance to many viruses (ii) Interleukins (IL), are a large group of cytokines produced mainly by T-cells, and have a variety of functions, most of which involve the direction of other cells to divide and differentiate Each interleukin acts specifically on a limited group of cells which express the appropriate receptor for that interleukin, and (iii) Tumour necrosis factors (TNF) have several functions, including mediating inflammation and cytotoxic reactions Cytokines such as Interleukin-1 (IL-1), IL-6, IL-12, and IL-18 stimulate the growth of T-helper cells to differentiate along the type 1 immune response

Cellular components of the innate immune system can detect microbes via cell-surface receptors Stimulation of macrophage receptors for lipopolysaccharides in the membrane surface of gram-negative bacteria, induces the synthesis of chemical signals or cytokines Macrophages also release regulatory and effector molecules that can influence the innate response including IL-12 and NO production (Mittrucker & Kauffmann, 2000), and are a major component of infiltrate tumors, and promote tumor progression (Mantovani *et al*, 1992) Resident macrophages which reside in tissue, and neutrophils which migrate in blood to sites of infection, and are crucial in the innate defence against bacterial pathogens through their removal and destruction (Aderhem & Underhill, 1999) NK cells are employed early in the immune response as they are the major source of Interferon-γ, a critical macrophage activating cytokine (Schwacha *et al*, 1998) Also, NK cells have been shown to produce NO (Cifone, 1999), which is functional in the innate immune response as a regulator of IL-12 mediated activation of NK cells (Diefenbach *et al*, 1999) The critical elements characteristic of the innate immune responses are controlled by intracellular

interactions between DCs, NK cells and macrophages (al-Ramadi *et al*, 2003) Mast cells and basophils can be activated to secrete cytokines such as IL-4, thus playing a role in the innate immune response (Wedemeyer *et al*, 2000) IL-5 secretion stimulates the release of eosinophils (Behm & Ovington, 2000) Eosinophils travel in blood to the site of infection where they become activated and secrete cytokines, degranulate to release cytotoxic products, and phagocytose particulate material Their primary function is in the defence against organisms too large to be phagocytosed (Behm & Ovington, 2000)

The complement system is the major soluble protein component of the innate immune system It consists of a group of serum proteins that activate each other in an enzyme cascade system, where the product of one system is the enzymatic catalyst of the next, to generate biologically active molecules capable of lysing cells by attacking and forming pores in membranes, inducing inflammatory responses and opsonising targets for phagocytosis by granulocytes and macrophages Complement can be activated in one of three pathways (i) the classical pathway which is initiated by antigen-antibody complexes, (ii) the alternative pathway, in which complement components become activated by the cell walls of bacteria or yeast, and (iii) the lectin pathway that activates the classical pathway of complement in the absence of the C1q component (Mastellos & Lambris, 2002) Once activated, the complement system generates peptides which have the following effects, (i) oponisation of micro-organisms for uptake by phagocytosis (ii) attraction of phagocytes to sites of infection (iii) increased blood flow to the site of activation and elevated levels of permeability in capillaries to plasma molecules and (iv) damage in plasma membranes of cells Complement acts as an effecter system in host defence against invading pathogens, contributes

through its activation products to the release of inflammatory mediators, promotes tissue injury at sites of inflammation, and has been implicated in the pathogenesis of several autoimmune and vascular diseases (Arlaud *et al.,* 1998). Complement has been identified as providing a link between innate and acquired immunity by augmenting the humoral response to T-cell dependant antigens and affecting the threshold of B-cell activation (Dempsey *et al.*, 1996).

1.6.1 Acquired immunity

Acquired immunity is more specialised than innate immunity, and it supplements the protection provided by the innate system. In contrast to the innate immune system the acquired immune system displays specificity, diversity, memory and discrimination between self and non-self. The efficiency of acquired immunity depends on the recognition of foreign or new material by specialised cells of the lymphoid system which once activated differentiate into effector cells which synthesise functional molecules and memory cells which can be activated specifically in the event of a second encounter. Central to the adaptive immune response are a subset of leucocytes, the lymphocytes. Lymphocytes specifically recognise individual pathogens, whether they are inside the host cells or in the tissue fluids or blood. Lymphocytes can be categorised as B lymphocytes (B cells) or T lymphocytes (T cells). Immunity is acquired by contact with the invader and is specific to that invader only. The initial contact with the foreign agent leads to the activation of lymphocytes and the synthesis of proteins which exhibit specific reactivity towards the invading agent. In this manner the individual acquires the immunity to defend against a subsequent attack by the same invading agent. An adaptive immune response is initiated when T cells

recognise foreign peptides bound to self-MHC molecules expressed on antigen-presenting cells (APCs), with the aid of co-stimulatory molecules such as CD80 (Barton & Medzhitov, 2002) T-lymphocytes kill their targets by secreting cytokines that can ligate pro-apopotic receptors on the target cell, or by recruiting inflammatory cells into the area of infection (Santamaria, 2001) DCs recognise signs of infection and serve as antigen presenting cell for the activation of naive T-cells (le Bon & Tough, 2002), which is a critical event in the induction of an adaptive immune response Also, DCs can detect indirect indicators of infections such as the expression of cytokines by infected cells (Banchereau *et al*, 2000)

Lymphocytes circulate in the blood and migrate to sites of entrapped antigen in secondary lymphoid tissue such as the spleen, lymph nodes and Peyers patches (Clark & Ledbetter, 1994) B lymphocytes combat extra cellular pathogens and their products They are capable of recognising a broad range of foreign antigens while ignoring self antigens (Kelly & Cahn, 2000) They possess a receptor which allows them to bind to antigen on pathogens or to secreted pathogen products Following binding, the antigen-receptor complex is internalised and the antigen is processed by proteolytic cleavage in the endosomes

Basophils and mast cells are associated with acquired immunity involving antibody-associated immune responses (Wedemeyer *et al*, 2000) They are involved in production and secretion of cytokines (Kinet, 1999), and are regulated by IgE antibody (Galli & Lantz, 1999) Also, basophils are involved in acute-IgE-associated allergic reactions and contribute to the expression of aspects of acquired immune responses that develop over hours, or days to weeks, for example chronic allergic inflammation (Galli & Lantz 1999)

There is a considerable amount of interaction between the innate and the acquired immune responses and as a result most immune responses to infectious organisms consist of a combination of innate and acquired responses Innate responses predominate in the earlier stages of infection, before the lymphocytes begin to generate an adaptive immune response Co-stimulatory molecules, needed for the initiation of an adaptive immune response are regulated by receptors for microbial products, thereby linking innate recognition of non-self with induction of adaptive immunity (Janeway, 1989) The magnitude and quality of the adaptive immune response depends on signals derived from the innate response to infection (Medzhitov & Janeway, 1997) The principal cell type associated with the translation of information from the innate immune response to that of the acquired system is the dendritic cell (DC)

1 7 1 Th-cell dichotomy

Regulation of immune response is multi-factorial involving appropriate activation, co-stimulation and the presence of specific soluble factors Mossman and Coffman (1989) observed that Th clones differentiated into two distinct populations (Th1 & Th2), according to the type of cytokines they produced The Th1/Th2 paradigm subdivides T cell immune responses into those specialised for defence against intracellular pathogens such as viruses and bacteria (Th1), and a second involved in the defence against larger extra-cellular pathogens such as helminths (Th2) Although both populations are derived from a common pre curser (Th0 cells), they exhibit phenotypic and functional differences The selective differentiation of CD4+ T cells into effector Th1 and Th2 cells is established during the early stimulation of these cells and is manipulated by

various extra-cellular influences including the dose of antigen, the source of co-stimulation and the cytokine environment (Constant & Botorrly, 1997) Among these the most influential polarising factor is the cytokine environment

Th1 responses employ the cytokines IL-12 and IFN-γ to mediate a range of biological effects designed for intracellular immunity Interferon-γ promotes germline transcription at the IgG2a locus and increases the overall frequency of IgG2a expressing cells (Severinson *et al*, 1990) Interferon-γ stimulates the production of IgG2a and IgG3 by B cells which may activate the classical complement pathway, and induces phagocytosis of microbes *via* binding to the Fc receptor on macrophages Interferon-γ may also promote the cytocidal and microbicidal activity of macrophages and hence their ability to produce nitric oxide This antibody-independent type of immune defence by activated macrophages is associated with cell-mediated immunity to intracellular organisms It can also be employed against extracellular parasites such as helminths (James *et al*, 1982) However the killing is non-specific and host cells in the vicinity of the reaction may also be damaged hence macrophage activation by type 1 responses is often associated with pathological conditions (delayed type II hypersensitivity reaction) such as those observed in chronic infections

In contrast, Th2 responses are characterised by the production of IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 These cytokines aid in B-cell proliferation and the secretion of IgG1 and IgE IL-4 influences the differentiation of naive T-helper cells into Th2 cells, and has also been shown to play a major role in the expression of IgG1 (Finkelman *et al*, 1988), and it has been demonstrated (Kuhn *et al*, 1991) that IgG1 is produced at a much lower level in mice lacking IL-4 Th2 responses cause inflammatory processes designed to expel larger parasites and promote the

mobilisation of eosinophils which can release toxic cationic compounds that are important in the control of helminth infections IL-3 plays a crucial role in the promotion of allergic inflammatory eosinophilia reactions through IgE isotype switching (Levy *et al*, 1997) However, the stimulation of these cells may also induce allergic and atopic manifestations, which correlates with the findings that Th2 derived cytokines may induce airway hyperactivity as well as the production of IgE (Sher & Coffman, 1992) IL-13 is also functional in the production of inflammatory cytokines, the induction of B-cell proliferation and differentiation, inducing IgE production and in the enhancement of the expression of CD23 and MHC class 11 molecules (major histocompatability complex class 2) (De Vries & Zurawski, 1995) The Th2 cytokine IL-10 has been associated with the down-regulation of cellular immune responses, and as a result affects the outcome of infection of bacterial and viral infections by the inhibition of Th1-associated cytokines

Th1 and Th2 immune responses tend to counter-regulate one another through the action of the cytokines that are specific to each type of response IFN-γ down regulates Th2 responses, and conversely IL-4, IL-10 and IL-3 can inhibit the effects of IFN-γ, and the development of Th1 responses As a result of the counter-regulation of the opposing cytokines, a polarisation of the immune response occurs

1 8 1 Immunology of helminth infection

It has been established that in terms of T-cell responses to helminth infection, Th2 activation correlates with disease (Heinzel *et al*, 1998) with a subsequent down regulation of Th1 responses It remains unclear whether Th2 response provide

protective immunity against the invading parasite, or are responsible for immune related pathology, or both (Allen & Maizels, 1996). For example IL-4 knockout mice generally do not produce protective immunity to helminth infections (Brunett *et al.*, 1999). In murine schistosomiasis, an early Th1 response is superseded by a more prevalent Th2 response after egg production.

The development of an immune response to helminth infection may result in pathological alterations which lead to the primary signs of the disease (MacDonald *et al.,* 2002). For example, eggs produced by *Schistosoma mansoni* may become trapped in the sinusoids of of the liver inducing a Th2 response, which results in the development of granulomatous lesions (Cheever *et al.*, 2000). However, infected mice, incapable of producing granulomatous lesions die as a result of toxic effects of egg protein on hepatocytes (Amiri *et al.,* 1992). The granulomata function by segregating the egg and allowing continued function of liver tissue. After the egg has been destroyed, the granulomata resolve, and fibrosis develops (Cheever *et al.*, 2000). This may lead to the formation of varices, the bleeding of which is the most common cause of death in schistosomiasis (MacDonald *et al.*,2002).

Elevation of immunoglobulin IgE and tissue eosinophilia are characteristic of immune responses to helminth infections (Sher & Colley, 1989), and these responses are regulated by the Th2 subset cytokines, IL-5, IL-4 and IL-3. Infection with the nematode *Nippostrongylus brasiliensis* increases IgE serum levels 100 fold over 14 days in rodents (Maizels *et al.*, 1993). Helminth parasites can be killed *in vitro* by IgE–regulated mechanisms, involving platelets, mast cells, basophils, eosinophils and macrophages (Capron & Dessaint, 1985). IgE-mediated

hypersensitivity is associated with pathology rather than resistance in human gut trichuriasis infections (Cooper *et al*, 1991)

The primary function of eosinophils is thought to be in the immune defence against large organisms such as helminths This observation is based on the facts that (i) eosinophils can degranulate and kill helminths *in vitro* (ii) eosinophils aggregate in areas of helminth infection and (iii) degranulate in the vicinity of invading helminths (Butterworth, 1984) However, direct evidence for eosinophils in host protection *in vivo* is lacking (Meeusen & Balic, 2000)

1 9 1 Immunology of *Fasciola hepatica* infection

Although many mammalian species may be infected with *Fasciola,* there is variation in the degree of susceptibility to infection, and in the ability to mount an effective immune response For example, sheep often die from acute fasciolosis, while some infections may last for as long as 11 years (Pantelouris, 1965) Different genetic backgrounds may be causative in the differing levels of susceptibility to infection (Boyce *et al*, 1978) In contrast, cattle rarely die from infection with liver fluke, and display a " self cure" between 9 and 26 months after infection This self-cure may be due to the observed thickening by calcification of the bile duct walls, in chronically infected cattle This immune strategy employed by cattle is not observed in sheep, and may explain the higher mortality rates associated with infection of sheep In general, infection in humans tends to cause high morbidity, and persists in hosts for lengthy periods (Maizels *et al*, 1993), rather than causing high mortality rates

Infection with *F hepatica* induces a predominant Th2 response It has been observed that Th cell clones specific for *F hepatica* enhanced IgG synthesis

through IL-4 expression (Brown *et al*, 1999), a characteristic Th2 cytokine response The capacity to produce IgG2 is associated with the production of IFN-γ (Estes *et al*, 1994), and as a result of a polarised Th2 response, the production of IFN-γ, and consequently IgG2 is inhibited This observation is consistent with the polarised Th2 response observed in chronically infected animals (Clery *et al*, 1996), where IgG1 was shown to be the dominant isotype produced in response to *Fasciola* infection Elevated levels of protection against experimental challenge have been associated with IgG2 antibodies (Mulcahy *et al*, 1998), however this protective response is down-regulated in the polarised Th2 response characteristic of infection with *F hepatica*

Susceptibility to a secondary infection and chroincity is a common feature of *Fasciola* infection For example, the relationship between pathogenesis of disease and host immune responses was observed in primary and secondary *F hepatica* infections of goats (Martinez-Moreno *et al*, 1999) The extent to which infection had been established, measured as the percentage of recovered flukes at the necropsy, was similar in animals during primary and secondary infections, however liver damage was much more severe in secondarily infected animals Primary infection was observed to evolve to chronic fasciolosis that did not induce the development of resistance, as animals were highly susceptible to secondary infection, exhibiting severe and acute hepatic lesions that ultimately led to the death of some of the animals (Martinez-Moreno *et al*, 1999) It was also observed that secondary infection failed to induce any difference in either IgG response or in the composition of cellular infiltrate of hepatic lesions, although lesions were more extended in the secondarily infected animals There was no significant correlation between the level of antibody titres and the number of flukes recovered

at necropsy, suggesting that antibodies have no protective function against Fasciola infection in primary or secondary infection (Martinez-Moreno *et al*, 1999), this correlates with the observations by Dalton *et al*, (1996) who reported that no correlation was observed between antibody titres and protection against *F hepatica* Animals chronically infected with *F hepatica* do not acquire a protective immune response (Clery *et al*, 1996), and it has been suggested (Ortiz *et al*, 2000) that animals with chronic infections remain as susceptible to *Fasciola* infection as naive animals A similar response to re-infection has also been observed in sheep (Chauvin *et al*, 1995) in experiments in which infected animals did not develop resistance against secondary infection

While immunohistochemical features of *Fasciola* infection appear to suggest vigorous cellular responses against the invading parasite (Martmez-Moreno *et al*, 1999), these responses are not observed to be protective, as there is no evidence of effective destruction of *Fasciola* flukes at any stage of development The effector mechanism of protective immunity has not been clearly established, however reported data suggests that it may occur at the early stage of infection m three different sites, the wall of the intestine (Charbon *et al*, 1991), the peritoneal cavity (Burden *et al*, 1983) and the liver surface of the parenchyma (Keegan & Trudgett, 1992) The effector response is believed to be nitric oxide-mediated killing which involves attachment of the effector cells (eosinophils, neutrophils and macrophages) to the tegument (Spithill *et al*, 1997)

Eosinophilia is a common feature of *Fasciola* infection, and eosinophils have been observed in close association with the surface of damaged newly excysted juveniles (NEJ), suggesting a role for this cell type in resistance to *Fasciola* infection (Burden *et al*, 1983) However, Hughes (1987) remarked that

there is only circumstantial evidence which shows eosinophils are functional in the killing of *F hepatica* NEJ's Furthermore, it has been demonstrated *in vitro* that eosinophils fail to induce irreversible damage on NEJ of *F hepatica* (Glauert *et al*, 1985) The fact that the immune responses are induced, but are ineffective against *Fasciola* implies that the immune response is ineffective due to a defensive capability of the parasite (O'Neill *et al* , 2001)

1 10 1 Immunological evasive strategies of *F hepatica*

Flukes may persist in their definitive hosts for extensive periods of time and therefore must possess means of evading prolonged attack from the hosts' immune system *F hepatica* has developed various mechanisms of immune modulation allowing its establishment and survival in the liver causing a severe hepatic disease (Meussen *et al* ,1995)

While the parasite ultimately resides in the bile duct of the liver, it must first find safe passage as it migrates through the intestinal wall and liver tissue Adult worms are generally more resistant to immune effector mechanisms than the earlier larva stages, suggesting that it has developed more efficient mechanisms for evasion of the hosts' immune response As the tegument of the liver fluke is involved in most of the interactions between the parasite and the host, the liver fluke surface plays an important role in protection against immune attack Liver fluke tegumental membrane is covered by a polyanionic glycocalyx consisting of ganglioside terminating in sialic acids (Threadgold, 1976) Two experimental approaches have demonstrated the significance of glycosylation in helminth infections First, immunodominant glycosylated epitopes are often the major targets of natural and experimental host humoral responses, as demonstrated by

the loss of antibody recognition through deglycosylation of the parasitic glycoprotein antigens or destruction of the glyco-epitopes by periodate oxidation Second, immuno-staining by glycan-recognising monoclonal antibodies or lectins against whole parasite or parasite derived extracts may show developmental stage-specific expression profiles of glycosylation

The tegumental glycocalyx may aid in immune evasion in several ways, (i) Antigen switching, composition of the glycocalyx changes during the development of the parasite in the host, thus presenting the hosts' immune system with a changing target For example, the glycocalyx coat changes in composition from T1-type tegumental cells to T2-type tegumental cells as the fluke migrates from liver tissue to that of the bile duct Changes in the fluke surface are reflected in changes in the immune system Host antibodies specific to the T1-derived components peak between 3 and 5 weeks after infection, and following their decline, anti-T2 antibodies can be observed Anti-T2 antibody production in infected rats declines after the parasite has entered the bile duct (Hanna, 1980) Various isotypic responses are observed as a result of parasite–induced stimulation of different lymphoid compartments IgE responses are significantly greater in the hepatic lymph nodes in comparison with that of the mesenteric lymph nodes or the spleen, while IgA responses are higher in the mesenteric lymph nodes This provides evidence of a unique regulation of the cytokines secreted by T cells in each of these micro-environments It has been suggested (Meeussen & Brandon, 1994) that by migrating between different tissue types, which are predisposed to a specific type of immune response, the flukes may be protected from tackling a single immune response that would otherwise become increasingly efficient as the parasite migrates

(ii) Antigen shedding, as a result of the flukes altering glycocalyx, antibody-bound immune effector cells, such as eosinophils and neutrophils may not bind sufficiently with the parasite to allow degranulation and damage to the to the surface, but are shed with the glycocalyx (Duffus & Franks, 1980, Hanna 1980) Glycocalyx turnover slows down once the bile duct is reached, as migration is completed and the fluke is no longer under such vigorous attack (iii) Antigen decoy, shed products of the glycocalyx may act to "mop up" circulating anti-fluke antibodies preventing their participation in direct attack on the fluke (Duffus & Franks, 1980)

Newly excysted juveniles are highly resistant to complement Terminal sialic acids in the glycocalyx prevent the activation of complement by the alternative pathway (Baeza *et al*, 1994a) The shedding of antibody from the flukes surface may prevent activation of complement by the classical pathway

It has been observed (Maarinez-Moreno *et al*, 1999), that immune inflammatory cells are rarely found in close association with the flukes, which would otherwise be expected to be instrumental in mounting a destructive strategy towards the invading pathogen This suggests an evasive strategy employed by the fluke in avoiding contact with the immune inflammatory cells This may be explained by the lack of CD3+ T cells in the infiltrate surrounding tracts made by migrating parasites inhibits immune inflammatory cells from migrating through the liver parenchyma This hypothesis is supported by the involvement of *Fasciola* excretory/secretory products in the suppression of peripheral blood lympocytes (PBL) proliferation (Martinez-Moreno *et al*, 1996) A further possible evasion strategy employed by the fluke is the rapid migration by the parasite through the liver, as has been previously reported in goats (Maarinez-Moreno *et al*, 1999) and

sheep (Meeusen *et al*, 1995), which makes it impossible for the leucocytic infiltration around the parasite (Chauvin *et al*, 1995)

Liver flukes also possess an ability to disable immune effector cells, for example by inactivating the toxic reactive oxygen products of the respiratory burst of leukocytes (eosinophila and neutrophils) and macrophages or reactive nitrogen intermediates generated by macrophages (Piedrafita *et al*, 2000) Oxygen scavenging enzymes such as superoxide dismutase (SOD) may be involved in the inactivation of oxygen species (Brophy *et al*, 1990) Piedrafita (PhD Thesis, 1995) observed increased activity of SOD in extracts of newly excysted juveniles SOD has also been detected in the excretory/secretory product of adult flukes (Tang *et al*, 1994) It has been observed (McGonigle *et al*, 1997) that adult flukes release a peroxiredoxin-like enzyme which may protect flukes against hydrogen peroxidase and other reactive oxygen intermediates

Goose (1978) observed that medium in which liver fluke had been cultured in was toxic to spleen cells He observed that these excretory/secretory ES products could prevent *in vitro* killing of newly excysted juveniles by peritoneal-inflammatory cells in the presence of immune serum by inhibiting the binding of effector cells to parasites Dalton and Heffernan (1989) demonstrated that liver flukes secrete two cysteine proteinase activities which are involved in host tissue penetration and feeding as well as immune evasion Subsequent studies showed these enzymes were cathepsin L proteinases, termed cathepsin L1 and cathepsin L 2 These molecules can specifically cleave immunoglobulins (Dowd *et al*, 1994) It was also demonstrated that purified cathepsm L could inhibit the antibody mediated attachment of eosinophils to newly excysted juveniles (Carmona *et al*, 1993)

1 11 1 *F hepatica* excretory/secretory products

Proteases catalyse the cleavage internal peptide bonds between peptides and proteins and are involved in a wide range of eukaryotic processes Proteases are also known to required for the virulence of pathogenic agents including helminth infections It has been demonstrated (Dalton & Heffernan, 1989) that immature and mature flukes secrete endo-proteinases into culture medium when maintained *in vitro* Several functions have been suggested for the role of these enzymes including functioning in migration through host tissue (Dalton & Heffernan, 1989), the acquisition of nutrient (Smith *et al*, 1993) and evasion of host immune responses (Dalton & Heffernan, 1989) Two cysteine proteases were isolated and characterised as having physiochemical properties similar with the mammalian lysosomal cathepsin L proteinases (Dowd *et al* , 1994) The two enzymes were observed to differ in their specificities for hydrolysing peptide bonds (Dowd *et al* , 1994) and as a result were termed cathepsin L1 and cathepsin L2

McGonigle and Dalton (1995) isolated another antigen containing a haem group from flukes maintained in culture medium, which was shown to be a liver fluke haemoglobin (Hb) (Dalton and McGonigle, 1995) Hb is involved in the aerobic respiration of immature flukes and egg production m adult flukes (Bjorkman and Thorsell, 1963) Because the cathepsin L1, cathepsm L1 and Hb molecules are involved m processes functional in the survival of the parasite in the host, they have been used as potential targets for liver fluke Investigations have been carried out to test the viability of these molecules for use as vaccines (Dalton *et al* , 1996), where individual molecules, and combinations of the molecules were given to cattle, to investigate their immunoprophylactic potential It was observed that cattle immunised with cathepsm L1 induced protection against

experimental challenge of 53 7%, while animals vaccinated with cathepsin L2 and Hb were also protected A combinational vaccine containing cathepsin L2 and Hb induced the highest level of protection (72 4%) Flukes recovered from this group were smaller in size than that of control groups, indicating that vaccination had stunted fluke growth, and as a result less liver damage was observed

In a similar study (Wijffels *et al*, 1994) in sheep it was observed that animals immunised with a thiol-cathepsin-related proteinase of M(r) 28,000, developed antibodies to the cysteine ptoteinase prior to infection with metacercaria of *F hepatica* On completion of the trial, there was no difference in worm burden between animals which had been immunised prior to infection and that of infected animals which did not received the proteinase However, faecal egg counts and therefore worm fecundity was significantly decreased in the immunised animals

Diagnosis of fasciohasis in the human host is achieved by the observation of eggs in faeces As flukes begin to release eggs 8 weeks after infection, diagnosis of the disease by coprological methods can not be achieved prior to this time point Purified protemases secreted by the parasite have recently been used in the diagnosis of human fascioliasis An IgG4-ELISA has been established (O'Neill *et al*, 1998) which uses purified cathepsin L1 or recombinant protein expressed in yeast as antigen The result of this report demonstrated the potential for the development of a standardised assay for the diagnosis of fascioliasis in humans

Cathepsin L1, one of the major molecules of fluke excretory/secretory product, is secreted at each stage in the development of the parasite, and has shown to be highly immunogenic in infected animals This molecule has the

ability to cleave host immunoglobulin and can inhibit in vitro attachment of eosinophils to newly excysted juveniles (Carmona *et al*, 1993) Cathepsin L1 is also capable of degrading extracellular matrix and basal membrane components and thus aids in parasite migration through the tissue of the host (Berasain *et al* 1997) The ability of cathepsin L1 and cathepsin L2 to produce vasoactive kinnins in alkaline pH may qualify them as factors of virulence in fascioliasis, since the intrinsic vasodilation activity exhibited by kinins, associated with endothelial leakage and anti aggregation platlet activity might assist in the migration and survival of the parasite in the tissue of hosts

Adult flukes secrete a cysteine protease capable of cleaving host IgG close to the papain binding site, and this hampers the hosts immune response to the invading parasite Immature flukes also secrete a papain or cathepsin B-like proteolytic enzyme which cleaves immunoglobulins of mice, rats and sheep in vitro (Chapman & Mitchell, 1982)

2.1

Materials and Methods

2 1 1 Materials

Sigma-Aldrich Ireland Ltd (Tallaght, Dublin)

Di-sodium Hydrogen phosphate, Ethanol, Ethidium bromide, Extra Avadin, Foetal Calf Serum, Isopropanol, Mercaptho-ethanol, p-Nitrophenyl Phosphate Tablet Sets, PCR master mix, Potassium Chloride, Phorbol Myristyl Acetate, RNAse free water, Sodium Chloride, N,N,N',N'-tetramethylethylenediamme, Tri-reagent, Trypan Blue

Stratagene (La Jolla, California, U S A)

IL-4 β-Actin and Interferon-γ forward and reverse primers

Bachem Limited (Merseyside, England)

Z-phe-arg-MHMec

Becton Dickinson & Co (Oxford, England)

Purified rat anti-mouse IL-4, Purified rat anti-mouse IL-5 Purified rat anti-mouse IL-10, Purified rat anti-mouse Interferon-γ, Biotin conjugated rat anti-mouse IL-4, Biotin conjugated rat anti-mouse Interferon-γ, Biotin conjugated rat anti-mouse IL-5, Biotm conjugated rat anti-mouse IL-10, Anti-Mouse IgG1, Anti-mouse IgG2a

Harlan U K Limited (Blackthorn, England)

BALB/c Mice

Campton Paddock Laboratıes (Thatcham, England)

Fascıola hepatıca Metacercarıae

GıbcoBRL, Lıfe Technologıes (Paısley, England)

Penıcıllın-Streptomycın(5000μ/5000μg),L-Glutamme(200mM),

Roswel Park Memorıal Instıtute(RPMI) 1640 Medıum

Pıerce and Warrıner (Chester, England)

Amv Reverse transcrıptase, Bıcınıchonınıc acıd (BCA) Protein Assay Reagent

Kıt, Mıxed set nucleotıdes(dATP, dGTP, dCTP, dTTP), 100bp DNA Ladder

Amersham Bıoscıences U K. Lımıted (Bucks, England)

Hıgh Resolutıon Sephacryl Gel S-300

Methods

2 1 2 Preparation of *F hepatica* whole somatic antigen

Adult flukes were obtained from the infected livers of condemned cattle at a local abbattoir Flukes were washed six times in phosphate-buffered saline (PBS) (0 14 M NaCl, 2 7MKCL, 1 5mM KH_2PO4 and 8 1mM Na2PO4H), pH 7 3 in order to remove debris They were then homogenised in a Thyrister Regler homogeniser with 10 ml of sterile PBS The homogenate was centrifuged at 13,000 X g for 30 minutes The supernatant containing soluble antigen, termed liver fluke homogenate (LFH) products was removed, ahquoted into 1ml vials and stored at 20°C (Dalton & Heffernan, 1989) Protein concentration of the liver fluke antigen was calculated using BCA protein assay reagent kit (Section 2 2)

2 1 2 1 Preparation of excretory-secretory products

Excretory–secretory products were prepared as described by Dalton and Heffernan, (1989) Adult flukes were cultured *in vitro* for 24 hours in 150 ml of RPMI-1640, pH 7 3, containing 2% glucose, 30mM Hepes and 25µg/ml gentamycin at 37°C The culture media was renewed after eight hours, and the flukes were incubated for a further 12 hours The culture media from both incubations were pooled and centrifuged at 13,000 X g for 30 minutes to remove eggs The supernatent (E S products) was filter sterilised and concentrated to 10 ml using an Amicon 8500 Ultrafiltration unit Amicons containing Ym3 filtration membranes with a 3,000 mw cut-off point Aliquots of 1 ml of concentrated antigen were then stored at -20°C

2 1 2 2 Separation of ES antigens by molecular weight using a high performance sephacryl gel filtration Column.

A haemoprotein fraction and cathepsin L were purified from ES products as described by Dowd *et al* , (1994) and Smith *et al* , (1983) The ES sample (4ml) was applied to a Sepacryl S300HR gel filtration column (2 5cm x 55cm) equilibrated in phosphate buffered saline (PBS), pH 7 2 Fractions of 3 mls were collected after a void volume of 45mls was passed Each of the fractions were monitored for protein concentration using a BCA protein assay reagent kit and an Amicon 2001 micro-titre plate reader set to 560nm Fractions were monitored for cathepsin L activity using the fluorogenic substrate, Z-phe-arg-NHMec (Section 2 3) Fractions containing the higher molecular weight bands and no cysteine protease activity were pooled and concentrated to 5ml using an Amicon ultrafiltration unit and termed peak 1 Fractions containing cysteine protease activity were pooled, concentrated and termed peak 2

2 1 3 Recombinant CL1 and Mutant CL1

recFheCL1 and mutFheCL1 were purified from yeast medium using affinity chromatography for the His_6 tag using Ni-NTA agarose (Qiagen) Gene sequences for both recFheCL1 and mutFheCL1 were cloned into a *Pichia pastoris* vector containing the α-factor yeast signal sequence After transformation into GS115 strain of *P pastoris*, clones were grown in YPD (yeast extract, peptone, dextrose) media and secretion of the cathepsin proteins induced by addition of 1% methanol (Collins *et al* , in press)

A 1 ml column was equilibrated by passing 10ml 50mM sodium phosphate buffer, pH 8 0, containing 300mM NaCl and 10mM imidazole, through the

column A 40ml sample of the same buffer/NaCl/imidazole mix with yeast media supernatant (10ml) was then added to the column The column was washed with 15ml 50mM sodium phosphate buffer, pH 8 0, containing 20mM imidazole and 300mM NaCl The purified protein was eluted using 50mM sodium phosphate buffer, pH 7 0, containing 250mM imidazole and 300mM NaCl Purified recombinant proteins were then dialysed into 1X Phosphate Buffered Saline (PBS)

2 2 1 BCA measurement of protein concentration

Protein concentrations (0 2-2mg/ml) were calculated using a BCA Protein Assay Reagent Kit according to the manufacturers instructions Briefly, 10µl of sample of unknown concentration and standard was added to wells on a 96-well micro-titre plate Bovine serum albumin was used as a protein standard (0 2-2 0mg/ml) BCA reagent (1 part reagent B 50 parts reagent A) was added m 200µl volumes, and the plates were incubated at room temperature for 30 minutes The absorbance of the reaction solution was measured at 560nm on an Anthos 2001 micro-titre plate reader Protein concentrations were determined by comparison of the absorbance of the unknown samples to the standard curve prepared using the protein standards

2 2 2 Bradford assay measurement of protein concentration

Protein concentrations within the range of 10µg/ml-1000µg/ml were measured using a Bradford protein assay (Bradford *et al*, 1976) Briefly, 10µl of unknown sample was brought to a final volume of 800µl with water The protein standards

employed were bovine serum albumin at concentrations of 10µg/ml-1000ug/ml 200µl of concentrated Biorad reagent was added and the sample was allowed to incubate for 5 minutes Samples were measured at 595 nm on an Anthos 2001 micro-titre plate reader Protein concentration was determined by comparison of the absorbance of the unknown samples to the standard curve prepared using the BSA standards

2 3 1 Measurement of cathepsin-L activity using the fluorogenic substrate Z-phe-arg-NHMec

Cathepsin L activity was measured flourometrically using Z-phe-arg-MHMec as substrate (Barrett & Kirschke, 1980) Assays (210µl volume) were performed with substrate at a final concentration of 10µm in 0 1 M, Tris-HCL, pH 7 0, containing 0 5mM dithiothreitol on a 96 well micro-titre plate Plates were incubated at 37°C for 30 minutes and the reaction stopped by the addition of 50µl of 10% acetic acid The amount of 7-amino-4-methylcouramin (NHMec) released was measured using a Perkin-Elmer fluorescence spectrophotometer with excitation set at 370 nm and emission at 440nm One unit of enzyme activity was defined as the amount which catalysed one µmole of NHMec per minute at 37°C

2 4 1 Sodium dodecyl sulfate polyacrylamide gel electrophesis (SDS-PAGE)

ES, peak 1 and peak 2 were analysed by one dimensional, denaturing 12% SDS-PAGE, using the buffer system 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, 0 1% (w/v) SDS, 0 03% (w/v) ammonium persulphate and 0 08% TEMED The stacking gel contained 3% (w/v) acrylamide, 0 08% (w/v)

bisacrylamide, 0 125 M Tris-HCL, pH 6 8, 0 075% (w/v) ammonium persulphate, 0 1% (w/v) SDS and 0 023% (w/v) TEMED Samples were prepared in reducing sample buffer (0 12 M Tris-HCL, pH 6 8, containing 5% (w/v) SDS, 10% (w/v) glycerol, 0 01% (w/v) bromophenol and 5% 2-mercapthoethanol) Samples were boiled for two minutes Gels were run in a vertical slab gel apparatus in electrode buffer (25 mM Tris-Hcl, 192mM glycine and 0 1% SDS, pH 8 3) at 25 mA at room temperature A voltage of $8V/cm^2$ was applied and the gel was run until the bromophenol blue dye reached the bottom of the gel Proteins were visualised by soaking the gel in a solution containing 0 1% (v/v) Coomaissie Brilliant Blue R, 20% (w/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 5 1 Infection and immunisation of Mice

Female BALB/c mice (aged 8-12 weeks) were purchased from Harlan U K Ltd (Blackthorn, Engalnd) All mice were maintained under the guidelines of the Department of Health and Children and were 8-12 weeks old at the initiation of each experiment Animals were infected with metacercaria of *F hepatica* purchased from Campton Paddock Laboratories (Thatchem, England), 15 metacercaria were administered orally to individual animals Immunised animals were injected intraperitoneally with antigen derived from *F hepatica* excretory/secretory products The mice were sacrificed by cervical dislocation, and the spleens, hepatic lymph nodes and mesenteric lymph nodes removed Blood was collected by cardiac puncture and serum was obtained following centrifugation at 2,000 X g for 5 minutes

2 6 1 Stimulation of murine spleen and lymph node cells with antigen

Spleens and lymph nodes were removed aseptically Cells were crushed on a wire grid to form single cell suspensions in RPMI 1640 medium containing 8% heat inactivated foetal calf serum, penicillin (100ug/ml), streptomycin (100ug/ml) glutamine (2mM) and 2ME (5X105 M) Cell debris was allowed to settle for 10 minutes, after which time the supernatants were removed and centrifuged at 12,000 revolutions per minute The cells were then washed with 2ml of RPMI The number of viable cells was determined by creating a mixture of 96µl Bromophenol Blue, 100µl RPMI 1640 medium (containing penicillin (100 U/ml), streptomycin (100ug/ml) glutamine (2nM) and 2ME (5X105 M) 2% glucose), and 4µl of cell suspension The number of viable cells (transparent) or non-viable cells (blue) were counted by using direct microscopic counts with a haemocytometer and an Olympus B201 microscope, and the concentration adjusted to 1 $X10^6$/ml for spleen cells and 2 X 10^6 /ml for lymph nodes Spleen and lymph nodes were stimulated *in vitro* in 96 well plates by the addition of varying concentrations of antigen (ES 1-10µg/ml, LFH 1-10µg/ml, peak 1 1-10µg/ml, peak 2 1-10µg/ml, Peak 3 1-10µg/ml, mutFheCL1 1-10µg/ml and recFheCLI 1-10µg/ml PMA/α-CD3 and sterile PBS were added to additional wells as positive and negative controls, respectively The cells were incubated in a CO_2 incubator for three days at 37°C 50µl of supernatant was removed after 72 hours to measure IL-4, IL-5, IL10 and IFN-γ cytokine production All tests were performed in triplicate and experiments repeated 2-3 times

2 7 1 Measurement of murine cytokine by capture ELISA

Antigen-specific and non-specific IL-4 and IFN-γ were measured by capture ELISA Plates were coated with 50µl of capture antibody (Beckton Dickenson & Co) (1µg/ml), and allowed to incubate at 4°C overnight Plates were washed six times in PBS /0 1% Tween 20 Excess protein binding sites were blocked with 200µl of skimmed milk (1mg/ml) The wash step was repeated and 50µl of supernatant or standard (recombinant IL-4/IFN-γ) (Beckton & Dickenson & Co) were added in triplicate and the plates incubated overnight at 4°C The washing step was repeated and the biotin labelled anti-IL-4/IFN-γ monoclonal detector antibody (1µg/ml) (Beckton & Dickenson & Co) in 1X PBS was added to each well and the plates incubated at room temperature for an hour After a further washing step 100µl of avidin-alkaline phosphatase (0 4µl/ml) in 1X PBS, was added to each well and the plates incubated at room temperature for 30 minutes The washing step was repeated and 100µl of p-Nitrophenyl Phosphate (pNpp) (Sigma Aldrich) (1mg/ml) in 0 2 M Tris buffer was added to each well to detect bound antibody The plates were allowed to develop in the dark until the top standard displayed an absorbance value of 1 absorbance unit at 405nm Standard curves were used to determine cytokine concentrations Concentration values for IL-4 and IFN-γ were expressed as pg/ml and ng/ml, respectively

2 8 1 Measurement of IgG1 and IgG2a isotypes

Microtitre-plates were coated with 100µl of ES or CL1 (5µg/ml) and allowed to incubate overnight at 37°C The plates were washed and excess protein binding sites were blocked by adding 200µl of skimmed milk (1mg/ml) to each well for two hours at room temperature After a further wash step, serum samples were titred at a dilution of 1 100- 1 218,700, and left to incubate for one hour at 37°C The wash step was repeated and alkaline phosphatase conjugated anti-mouse IgG1 and IgG2a (Beckton Dickenson & Co) (diluted at 1 500, 1 1000 respectively in 1X PBS) were added The plates were incubated at 37°C for one hour After a final washing, 100µl of p-Nitrophenyl Phosphate (pNpp) (1mg/ml) in 0 2 M Tris buffer was added to the plates to detect bound antibodies The plates were read on an Anthos 2001 microtitre plate reader at 405nm The antibody titre was expressed as log titre

2 9 1 Examination of liver pathology

At post mortem examination, hepatic tissue was fixed in 10% neutral-buffered-formahn Following fixation the tissues were paraffin embedded, cut at 4µm and stained with haematoxylin and eosin

2 10 1 Reverse transcriptase Polynuclear chain reaction (rtPCR)

Total RNA was extracted from cell preparations of spleen and lymph node tissue using the one step method as described by Chomczynski and Sacchi (1987) Briefly, cells (1X 10^6) were lysed in Tri-Reagent (Sigma) Nucleic acid was separated from proteins by the addition of chloroform (200µl), and incubated at room temperature for 15 minutes, followed by centrifuging at 12,000 X g for 15

minutes The aqueous layer was removed and RNA precipitated by incubation at room temperature for 10 min in the presence of isopropanol, followed by a centrifugal step at 12,000 X g for 15 min The supernatent was removed and the RNA pellet washed by the addition of 75% ethanol and centrifuged at 7,500 X g for 10 min The resultant was resuspended in 50µl of RNAse free water and stored at –20°C

Complementary DNA was prepared by reverse transcription using the following components in sterile 0 5ml microfuge tubes 2 µl 10X reaction buffer, 4 µl 5mM $MgCl_2$, 2 µl 1mM DNTP, 3 µl OhgoDT(1ug/ml), 50 units RNAse inhibitor, 1 µl, 20 units AMV Reverse transcriptase and 1µg RNA cDNA reaction mixtures were incubated for 10 mm at room temperature to facilitate binding of oligodT primer, and cDNA was transcribed at 42°C for 1h The AMV reverse transcriptase was heated to 99°C for 5 mm

A 5µl aliquot of cDNA was subjected to PCR with forward and reverse primers specific to IL-4, IFN-γ, and β-Actin (Table 2 1) Each PCR reaction contained the following 1X PCR Buffer, 0 2mMdNTP, 1 5mM $MgCl_2$ 25 units taq and RNAse free water to a final volume of 25µl DNA was amplified using a thermocycle (PCR Express-Thermo Hybrid), under the following conditions, 95°C for five minutes, followed by five cycles at 95°C, 55°C and 72°C, respectively each for a duration of one minute, and a final extension step of 72°C for 7 min PCR products were separated on a 1% agarose gel (w/v m 1X TAE) by electrophoresis, and visualised using ethidium bromide

Table 2 1 PCR primers

Primer	Sequence 5'-3'	Product size (bp)
β-Actin forward	ATGGATGACGATATCGCT	600
β-Actin reverse	ATGAGGTAGTCTGTCAGGT	
IL-4 forward	ACGGAGATGGATGTGCCAAA CG	279
IL-4 reverse	CGAGTAATCCATTTGCATGA TGC	
IFN-γ forward	TATTGCCACGGCACAGTCAT TGA	405
IFN-γ reverse	GCAGCGACTCCTTTCCGCTTC CT	

2 11 Statistical Analysis

All statistical analysis was performed using SPSS for windows (version 11 0) Analysis of the effects of antigen concentration, antigens and time were performed using factorial analysis of variance Post-hoc significance testing was by Tukey's HSD

2 12 Determination of cellular profiles

Intra-peritoneal cellular profiles were determined by counting cells in three fields of vision per sample An average number of cells was determined per sample

Results

3.1.1

Cytokine, antibody and pathology profile in BALB/c mice infected with *F. hepatica.*

Introduction

3 1 1 1 Fasciolosis is associated with the induction of T-cell responses polarised towards the Th2 subtype Th2 clones, but not Th1 clones have been isolated from chronically infected cattle (Brown *et al*, 1994, Clery *et al*, 1996, Mulcahy *et al*, 1999) Previous investigations performed in our laboratory, by O'Neill *et al*, (2001) demonstrated a polarised type 2 immune response as a result of *F hepatica* infection in rodents, with significant levels of the type 2 cytokine, IL-4 recorded 21 days post infection, while production of the type 1 cytokine, IFN-γ was not observed

In the present study we investigated the T-cell and antibody production of BALB/c mice exposed to infection of *F hepatica* over a period of 21 days We also sought to determine at what stage of infection the immune response becomes polarised towards a type 2 response, by measuring early IL-4 mRNA levels Our data confirms that a polarised type 2 immune response is induced by infection with *F hepatica*, and that this response can be detected as early as day 1 post infection Histological investigations of liver tissue taken from *F hepatica* infected mice, demonstrated the extent of tissue damage as a result of infection

Experimental design

3 1 2 1 To examine the cytokine profile of BALB/c mice infected with *F hepatica* over a period of three weeks, 16 female BALB/c mice (8-10 weeks) were orally infected with 10 metacercaria of *F hepatica* Four animals were sacrificed by cervical dislocation on days 7, 10, 14 and 21 Groups of four non-infected mice were used as controls at each time point Isolated spleen cells ($5x10^6$/ml) were

stimulated *in vitro* at 37°C with ES (5μg/ml, 1μg/ml), LFH (5μg/ml, 1μg/ml) and PMA/anti-CD3 as a positive control and medium as a negative control Supernatant samples were removed after 72 hours and the amount of IL-4 and IFN-γ cytokines that were secreted into culture media was measured (Fig 3 1) Serum was obtained via cardiac puncture, and circulating anti-fluke IgG1 and IgG2a antibodies were measured Liver tissue samples were obtained and examined using haematoxylin and eosin Liver tissue damage and cellular infiltration during *F hepatica* infection was measured at days 7, 10, 14 and 21 Groups of four non-infected (control) mice were included at each time point as negative controls Isolated livers were fixed in formalin, cut at 4 μm and stained with haemotoxylin and eosin

In order to measure early cytokine responses to infection with *F hepatica*, the levels of mRNAs associated with cytokine production were assessed by reverse transcription-polymerase chain reaction (rtPCR), at 0, 1, 2, 4 and 8 days post infection in lymph node tissue of *F hepatica* infected mice An infection of 10 metacercaria of *F hepatica* was administered orally to 15 BALB/c mice aged 8-10 weeks Groups of three animals were sacrificed by cervical dislocation on each of days, 0, 1, 2, 4, and 8 Groups of three non-infected mice were used as controls at each time point RNA was extracted from cells of mouse hepatic lymph nodes and mesenteric lymph nodes at each time point, and mRNA production with specificity for IL-4 and IFN-γ was investigated The positive control, β-Actin proved positive for each sample at each of the time points

Results

3 1 3 1 1 IL-4 and interferon-γ cytokine production by spleen cells of BALB/c mice infected with 10 metacercaria of *Fasciola hepatica* at day 7, 10, 14 and 21

The results (Fig 3 1) demonstrate that spleen cells of infected mice stimulated with ES and LFH, exhibit a predominant type 2 immune response, with significant amounts of IL-4 cytokine being produced The amount of IL-4 cytokine produced increased with time from infection, with the greatest cytokine response observed at day 21 Also, cells stimulated with the higher concentration of antigen (5μg/ml) produced a greater cytokine response than that of cells stimulated with the lower concentration (1μg/ml) Cells stimulated with LFH produced a greater response than that of cells stimulated with ES Levels of IFN-γ cytokine secretion was lower than that expected of a positive response Spleen cells from naive mice (controls) did not secrete either IL-4 or IFN-γ cytokines in response to *Fasciola* antigen Stimulation of spleen cells with PMA and anti-CD3 demonstrated that all cells were capable of producing both Th1 and Th2 cytokines (data not shown) Analysis of the effects of antigens, antigen concentration and time were carried out by factorial analysis of variance Post-hoc significance was by Tukey HSD Cytokine production was expressed as the mean cytokine concentration of four mice per group tested in triplicate

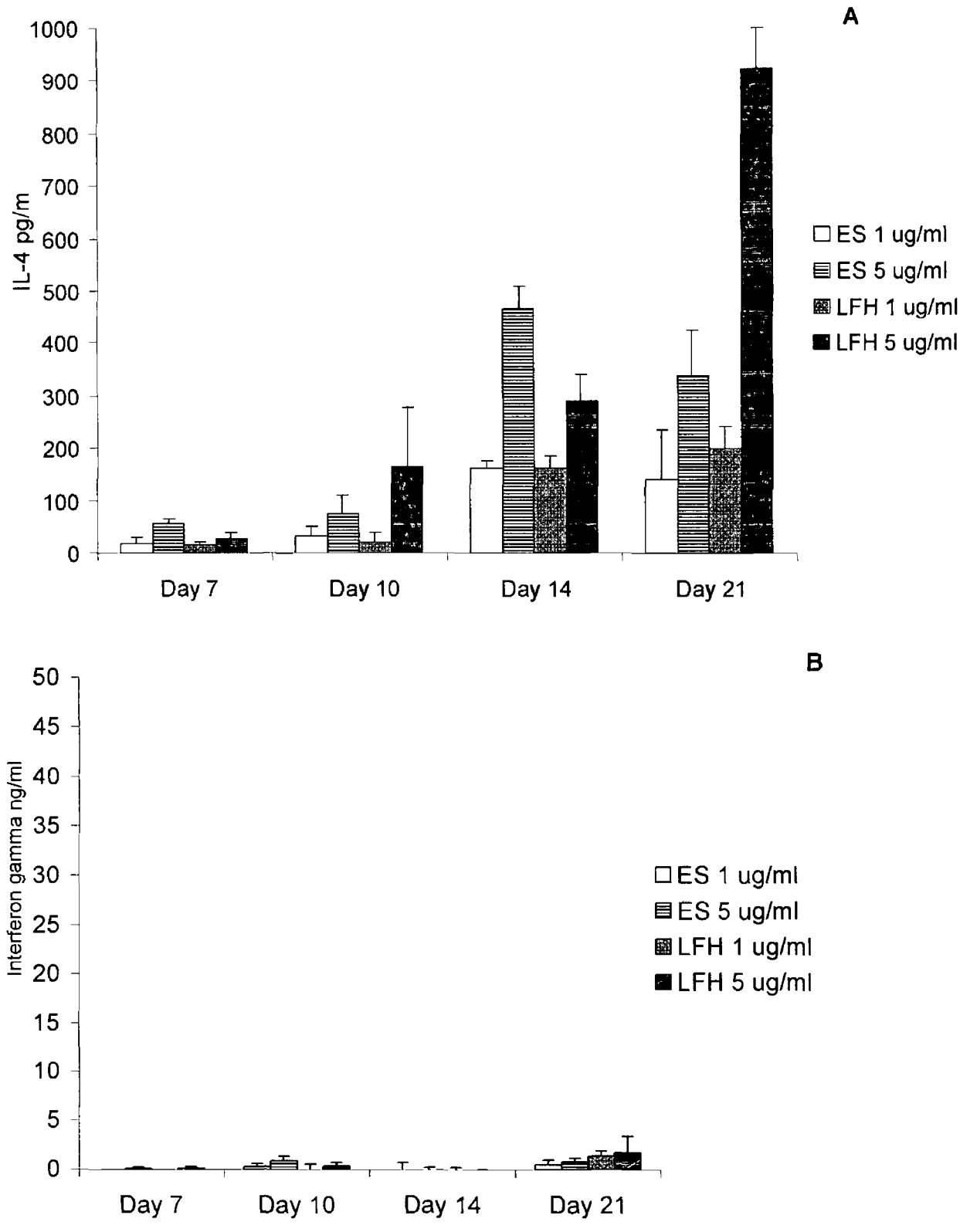


Fig 3 1

Fig 3 1Antigen-specific IL-4 (A) and IFN-γ (B) cytokine released from spleen cells obtained from BALB/c mice infected with 10 *I hepatica* metacercaria

Spleen cells were culture *in vitro* with 1µg/ml and 5µg/ml LFH and ES antigen, and the supernatant removed after 72 hours for measurement of cytokine production by ELISA All tests were carried out in triplicate, and the experiment repeated twice

3 1 3 1 2 IgG1 and IgG2a antibody production in serum of *F hepatica* infected mice

Specific antibody isotypes characteristic of Th1 (IgG2a) and Th2 (IgG1) responses were measured in serum samples taken from mice infected with 10 *F hepatica,* metacercariae at days 7, 10, 14 and 21 Titres were measured against ES in all samples Antibodies of the IgG1 subtype were detected in all infected animals at days, 10, 14 and 21 (Fig 3 2) Antibody production observed at days 14 and 21 was higher than levels recorded on day 0, 7 or 14 The most significant levels of antibody production were observed at days, 14 and 21 No anti-ESIgG2a antibody production was detected at any stage over the course of infection No specific IgG1 nor IgG2a antibodies were detected in non-infected mice which were established as controls at each time point

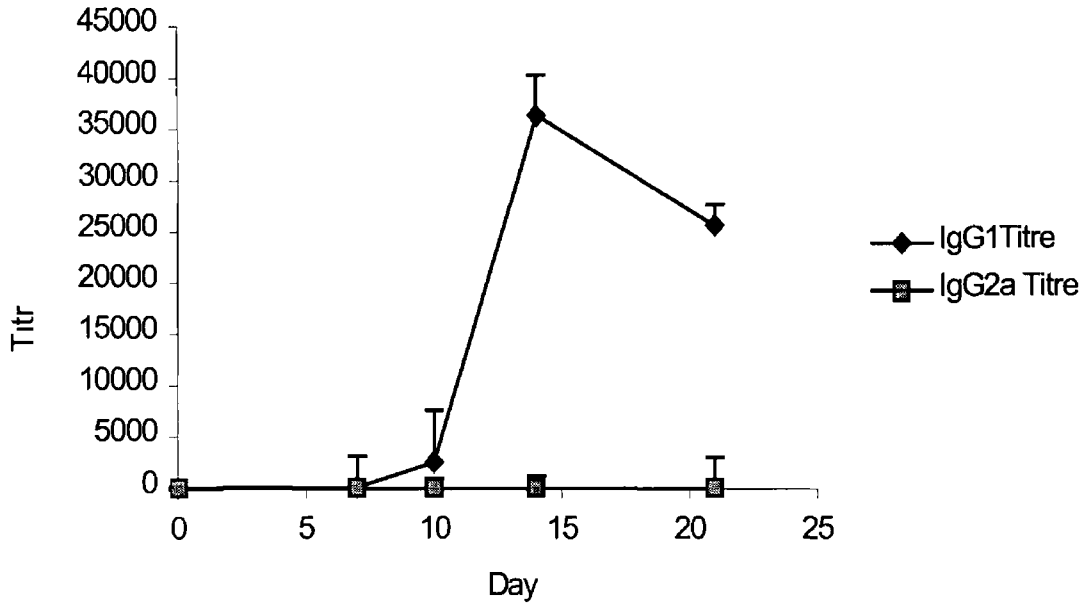


Fig 3 2 Titres of IgG1 and IgG2a antibody production specific for ES, in serum of BALB/c mice infected with 10 metacercaria of *F hepatica* Titrations were performed on days 7, 10, 14 and 21 All tests were performed in triplicate

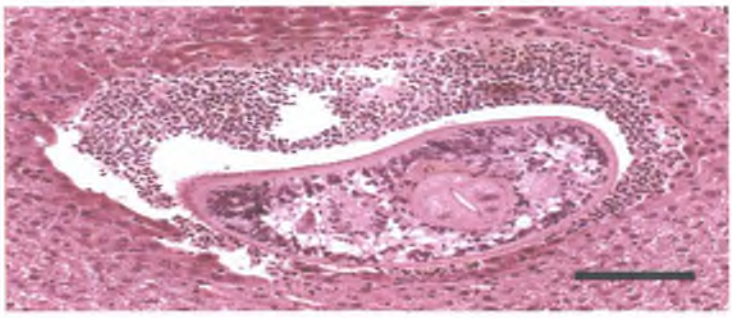
3 1 3 1 3 Pathology of liver tissue of mice infected with metacercariae of *F hepatica*

A summary of the pathology observed in the liver is described in Table 2 1 Tracts were observed in all infected mice at each time point, with the number of tracts increasing as the course of infection progressed Multiple tracts were observed in the livers of mice sacrificed at days 14 and 21 As expected no tracts were observed m control mice Neutrophils were present in each of the infected mice at each time point with dense aggregations observed on day 14 and day 21 (Fig 3 6 (A)) In contrast no neutrophils were recorded in control mice Mononuclear cells were present in tracts of infected mice on day 7, and were present in surrounding tissue at later time points, but were not observed in the tracts Mononuclear cells were observed m the livers of all control animals Damaged hepatocytes were observed in livers at all time points (Figs 3 4 (D), 3 5, (A), and 3 6 (B)) however no hepatocytes were observed in the tracts after day 14 As expected no damaged hepatocytes were observed in non-infected mice No eosinophils were observed in the livers of infected or control mice

Table 3 1 Liver pathology recorded in mice infected with 10 metacercaria of ***F hepatica.*** Liver tissue was isolated on days 7, 10, 14 and 21

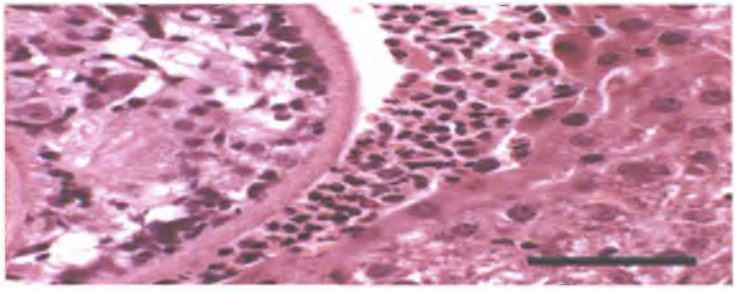
	Infected	**Control**
Tracts	Tracts were observed in all samples at each time point Multiple tracts were observed on day 14 and day 21	Normal architecture was observed in all samples
Neutrophils	Observed in all samples at each time point Dense aggregations were noted on day 14 and day 21	None observed
Mononuclear cells	Observed in 100%, of animals on day 7 with cells not observed in tracts after this time point but could be observed in surrounding tissue	Observed in all control animals
Hepatocytes	Damaged hepatocytes observed in 60% and 50% of animals on day 7 and day 10 respectively Were absent at later time points	None observed
Eosinophils	None observed	None observed

A.



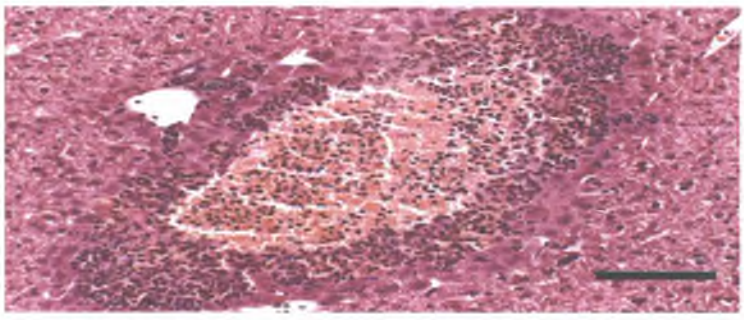
(H & E) (X 200) Bar 50µm

B.



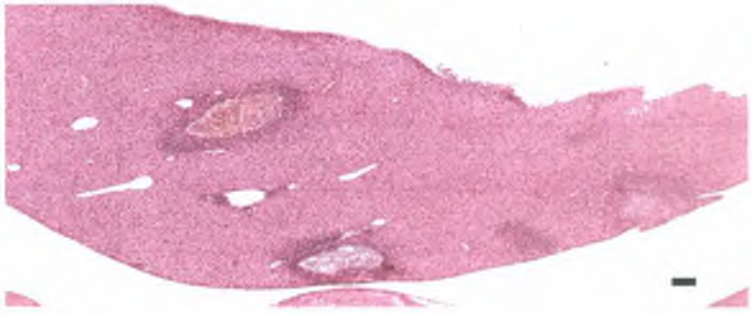
(H & E) (X 600) Bar 50µm

C.



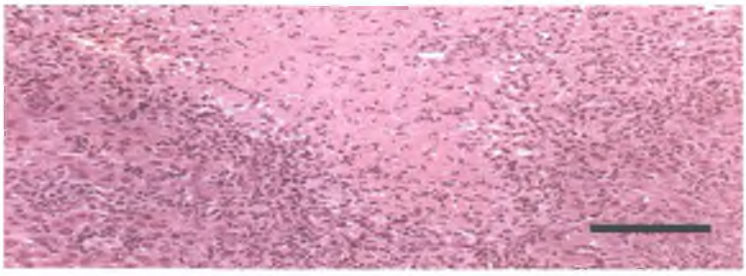
(H & E) (X200) Bar 50µm

D.



(H & E) (X40) Bar 50µm

E.

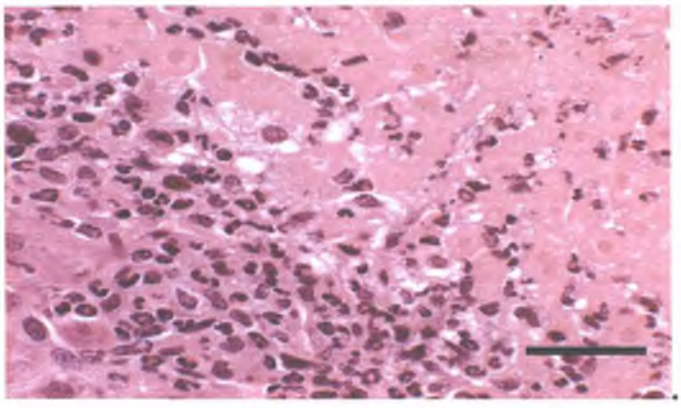


(H & E) (X 200) Bar 50µm

Fig. 3.4 Liver pathology day 7 post infection.

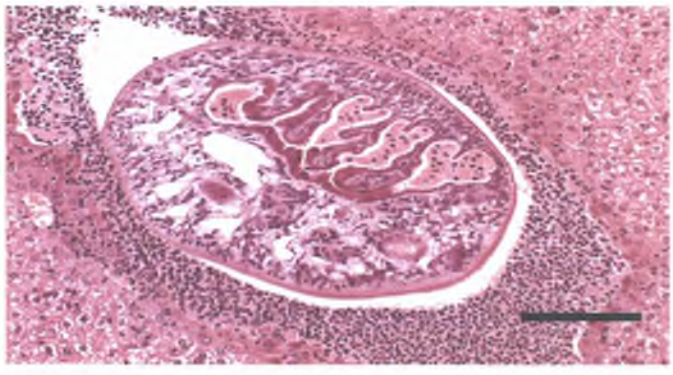
Fig 3 4 Liver pathology day 7 post infection Photomicrograph representing a section of liver from BALB/c mice 7 days post infection with 10 metacercariae of *F hepatica* Photomicrograph illustrates **(A)** a migrating parasite, **(B)** a section of the *F hepatica* adjacent to an aggregation of degenerating neutrophils, **(C)** a parasitic tract, **(D)** three parasitic tracts within the parenchyma, **(E)** a region of acute coagulative necrosis of hepatocytes

A.



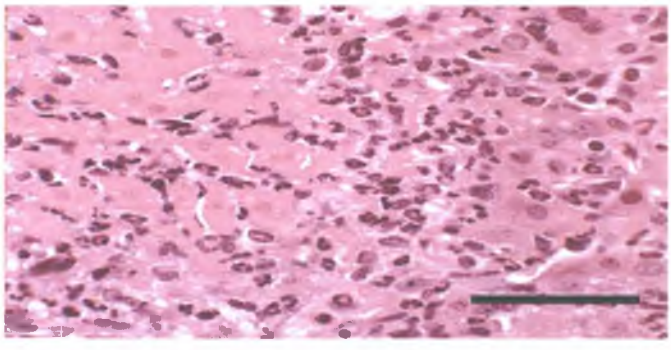
(H & E) (X 200) Bar 50µm

B.



(H & E) (X 200) Bar 50µm

C.



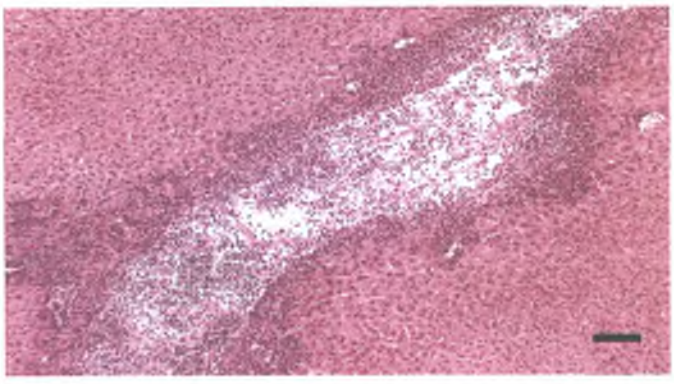
(H & E) (X 600) Bar 50µm

Fig. 3.5 Day 10 post infection.

Fig. 3.5 Day 10 post infection.

Photomicrograph representing a section of liver from BALB/c mice 10 days post infection with 10 metacercariae of *F hepatica* Photomicrograph illustrates **(A)** a region of acute coagulative necrosis of hepatocytes This region is bordered by a neutrophil-rich inflammatory cell infiltration, **(B)** a parasite in a tract surrounded by a large number of neutrophils, **(C)** a region of acute coagulative necrosis of hepatocytes in which there are infiltrations of degenerating hepatocytes

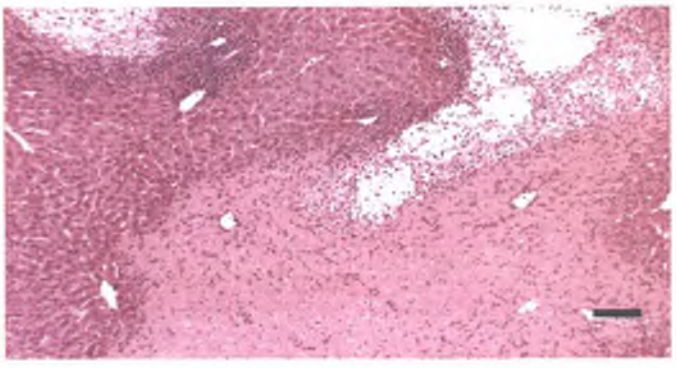
A.



(H & E) (X 100) Bar 50μm

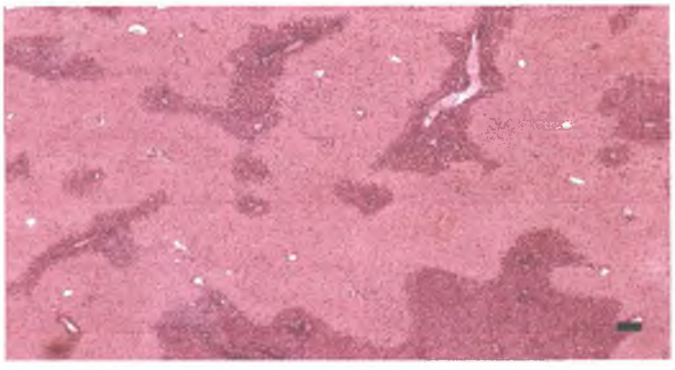
Fig. 3.6: Photomicrograph representing a section of liver from BALB/c mice 14 days post infection with 10 metacercaria of *F. hepatica.* Photomicrograph illustrates **(A)** a parasitic tract containing neutrophils, fibrin and some red blood cells. At the periphery of this tract neutrophil-rich infiltrates are noted in the sinuses.

A.



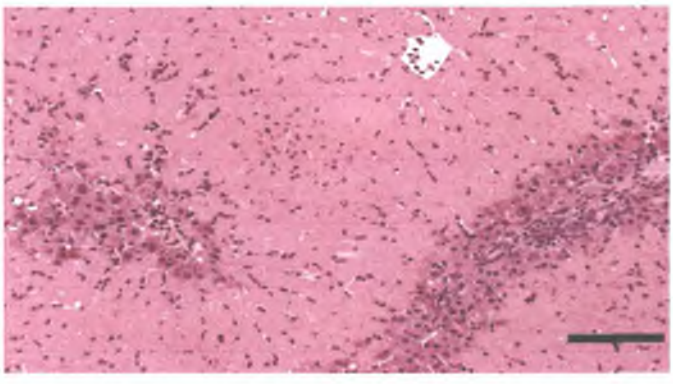
(H & E) (X100) Bar 50µm

B.



(H & E) (X40) Bar 50µm

C.



(H & E) (X200) Bar 50µm

Fig. 3.7 Day 14 & 21 post infection.

Fig 3 7 Photomicrograph representing a section of liver from BALB/c mice 21 days post infection with 10 metacercariae of *F hepatica* Photomicrograph illustrates **(A)** a parasitic tract which is bordered by a region of acute coagulative necrosis of hepatocytes **(B)** illustrates massive and bridging coagulative necrosis **(C)** illustrates extensive acute coagulative necrosis of hepatocytes Small regions adjacent blood vessels of the periportal regions are spared

3 1 3 1 4 Early cytokine profile in BALB/c mice infected with *F hepatica*

mRNA production in the mesenteric lymph nodes showed a dominant IL-4 response as early as day 1 post infection, with strong responses also observed on days 4 and 8 (Fig 3 7) No significant IFN-γ specific mRNAwas observed, although faint bands were visible on days 4 and 8 No significant levels of IL-4 or IFN-γ mRNA were observed at any time point in the non-infected animals The experiment was repeated thrice with similar results in each case

A dominant IL-4 response was observed in the hepatic lymph nodes on day 2 post infection, with similar bands observed on days 4 and 8 (Fig 3 8) No IFN-γ mRNAwas observed at any time point No significant levels of IL-4 or IFN-γ mRNA were observed at any time point in the non-infected animals mRNA production in spleen cells of *F hepatica* infected mice was also investigated However, as control levels of mRNA production was similar to that of mRNA representing IL-4 and IFN-γ the data could not be presented with this experimental data Also, as systemic infections influence cytokine production in the compartment of the spleen, and therefore may not represent cytokine production as a direct result of *Fasciola* infection, the data was not relevant to this study The experiment was repeated thrice with similar results in each case

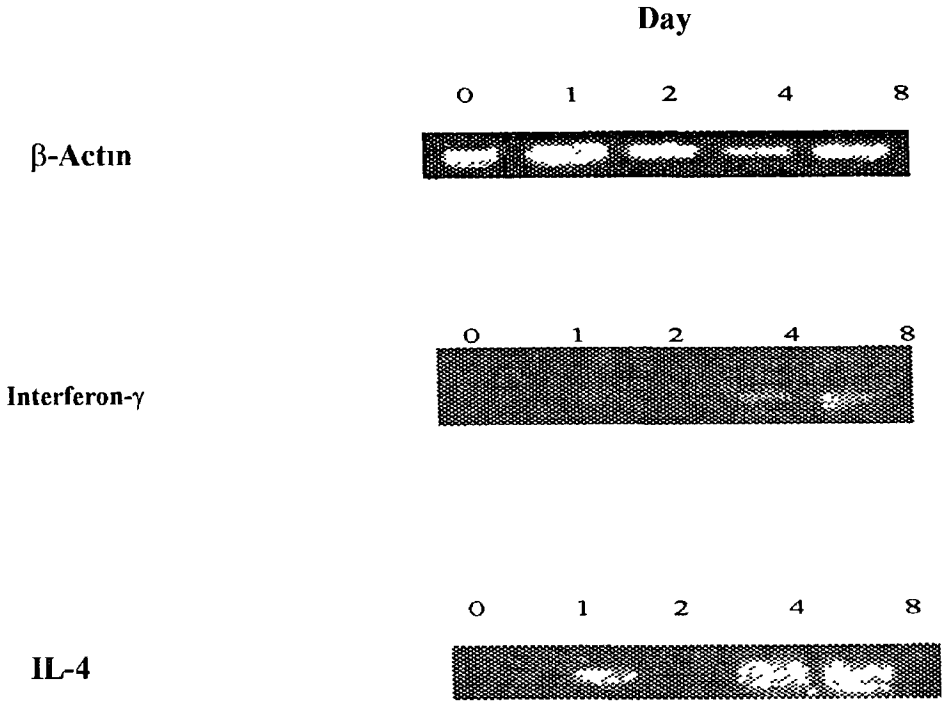


Fig 3.7. Expression of mRNA in the mesenteric lymph nodes obtained from BALB/c mice infected with 10 metacercariae of *F hepatica* Lymph node cells were isolated on days 0, 1, 2, 4 and 8 post infection Experiments were repeated thrice and the figure represents the mRNA expression per time point

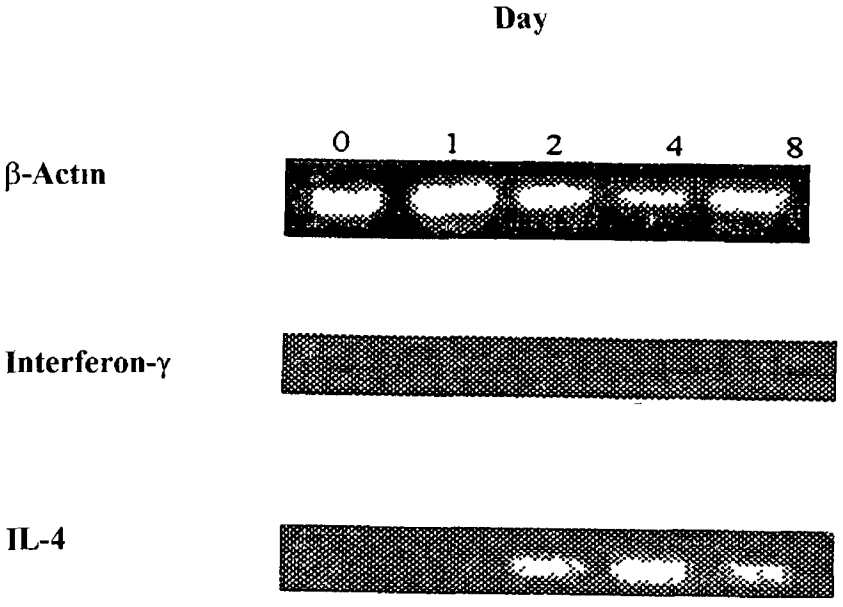


Fig 3 8 Expression of mRNA in the hepatic lymph nodes obtained from BALB/c mice infected with 10 metacercariae of *F hepatica* Lymph node cells were isolated on days 0, 1, 2, 4 and 8 of infection Experiments were repeated thrice with similar results in each case

3 1 4 Discussion

Fasciolosis is associated with the induction of T-cell responses polarised towards the Th2 subset Previous studies (O'Neill *et al*, 1999), demonstrated a dominant type 2 immune response in BALB/c mice as a result of infection with *F hepatica,* with high levels of the type 2 cytokine, IL-4, and undetectable IFN-γ production by spleen cells Studies in sheep (Chauvin *et al*, 1995) and cattle (Brown *et al*, 1994) in which animals were exposed to infection *F hepatica* also describe a type 2 response Correlating with previous data, results from the present study defines a polarised type 2 immune response as a result of *Fasciola* infection Type 1 and type 2 immune responses counter-regulate one another through the action of specific cytokines (Sher & Coffman, 1992), and in the context of this experiment, type 1 immune responses, measured by quantification of IFN-γ production, were not observed at any time point investigated Significant levels of IL-4 were recorded in the spleens of mice infected with metacercariae of *F hepatica*, as early as day 7 post infection Increased levels of IL-4 were recorded as infection progressed, with the greatest IL-4 response recorded on day 21 post infection Spleen cells were stimulated *in vitro* with (1μg/ml and 5μg/ml) ES and LFH Both stimulating antigens produced similar levels of IL-4, with the greatest cytokine production observed as a result of stimulation with the greater antigen concentration No significant levels of IFN-γ production was observed at any time point Similar suppressive effects on T cell proliferation have been observed in infected sheep (Zimmerman *et al*, 1983 and rats (Poitou *et al*, 1992) Studies investigating cytokine production in *F hepatica* infected rats by Tliba *et al*, (2002), describe a mixed response at day 7, with increased levels of both IFN-γ

and IL-4 cytokines observed However, by day 14, IL-4 levels were more pronounced while no IFN-γ production was observed

Antibody responses to *F hepatica* illustrate a marked predominance of IgG1 (type 2) over IgG2a (type 1) isotypes (Clery *et al*, 1996) A degree of protection has been associated with IgG2 antibodies (Mulcahy *et al*, 1998), however this protective response is down-regulated in the polarised Th2 response characteristic of infection with *F hepatica* In conjunction with the polarised type 2 cytokine response observed in the current study, antibody profiles of serum samples obtained from *F hepatica* infected mice exhibited a predominant type 2, IgG1 response While a significant level of IgG1 was not recorded at day 7, IgG1 antibody was observed on days, 10, 14 and 21 Production of the type 1 antibody, IgG2a was not observed at any time point under investigation Studies by Clery *et al*, (1996), also showed IgG1 to be the dominant immunoglobulin isotype in cattle infected with *F hepatica* These observations indicate that *F hepatica* actively inhibits a type 1 immune response Regulation of immunoglobulin subclasses by antigen specific helper T-cells was observed in studies by Purkerson & Isakson (1992) Studies investigating helminth infections such as, Schistosomiasis (Caulada-Benedetti *et al*, 1998) observed that IL-4 is associated with secretion of IgG1 whereas IFN-γ is associated with secretion of IgG2a In accordance with these results, we observed that all mice observed eliciting a predominant type 2 cytokine profile also exhibited anti-fluke IgG1 antibodies in their serum

During the migratory period of infection, both natural (sheep, cattle), and experimental (mice) hosts develop a cellular response against the parasite The most striking feature of tissue architecture in liver tissue, obtained from infected animals, is the occurrence of migratory tracts In a study investigating liver

pathology as a result of infection with *F hepatica* in goats, Martinez-Moreno *et al*, (1999), recorded tracts in the hepatic surface and parenchyma In the present study, tracts were observed at each time point, with multiple tracts observed as infection progressed Tracts are the result of coagulative necrosis followed by the dissolution of hepatocytes In some of the liver tissue obtained from *F hepatica* infected mice, parasites were visible in the lumina of tracts

Neutrophils have been suggested to play a role in the immune process or in tissue repair (Meeusen *et al*, 1995) During acute primary infections in sheep, neutrophils have been observed infiltrating to the tracts produced by the migrating flukes Martinez-Moreno *et al*, (1999) also observed neutrophils in the tracts of migrating parasites in infected goats Lloyd and Oppenheim, (1992) observed that neutrophils release a range of immunomodulatory cytokines and can aid significantly in the initiation and amplification of cellular and humoral immune responses In the present study neutrophils were found in the liver parenchyma at all time points At day 7, acute inflammatory cells, mainly neutrophils, were observed both in the lumnia of tracts, on the periphery and within adjacent sinuses By day 10, neutrophil-rich aggregations were also noted, usually adjacent or encompassing vascular structures Dense neutrophil aggregations were observed at days 14 and 21 Jefferies *et al*, (1996) observed neutrophils surrounding or invading hepatocytes that appeared either still normal or had become non-viable If cell death is due to the surrounding neutrophils, then neutrophil proliferation in the current study may be responsible for some of the pathology associated with the disease, particularly at the later time points, where dense aggregations of neutrophils and increased liver damage are observed Also, in this context, the

dense aggregation of neutrophils observed at days 14 and 21 post infection may explain the lack of damaged hepatocytes in the tracts of migrating flukes

Eosinophilia is defined as an increase in the number of eosinophils in the blood or tissues and has historically been recognised as a distinctive feature of helminth infections in mammals (Behm & Ovington, 2000) Blood and tissue eosinophilia are generally associated with helminth infection During helminth infections eosinophils are released more rapidly from the bone marrow, their survival in tissue is enhanced, and the rate of entry of eosinophils into infected and inflamed tissues is considerably upregulated, this results in tissue eosinophilia Evidence that eosinophils are capable of killing many different species of metazoan parasites, including *Schistosoma mansoni* by an antibody or complement dependant mechanism, suggests a role for eosinophils in defence against these organisms The hypothesis that the primary function of eosinophils is to protect the host from infection by relatively large organisms, such as parasitic helminths is based on the accumulation of observations that (i) eosinophils degranulate and kill helminths *in vivo* (ii) they aggregate in the vicinity of helminths in vivo (iii) they are observed to degranulate in the vicinity of, or on the surface of helminths *in vivo* (Butterworth, 1984)

The type 2 immune response cytokine, IL-5 plays a key role in the development and maturation of the eosinophil population (Behm & Ovmgton, 2000) IL-5 controls or influences the development, maturation and survival of eosinophils during a Th2 cytokine response However direct evidence of a role for eosinophils in host protection against helminths *in vivo* is lacking Studies in which IL-5 cytokine production was inhibited, reduced the development of eosinophils in response to helminth infection, but had little affect on the survival

or reproduction of a number of nematodes and trematodes Studies by Piedrafita *et al*, (2000), were unable to demonstrate irreversible damage to juvenile *F hepatica* flukes by eosinophils These results suggest that either eosinophic attack is an ineffective means of combating helminth infection, or that helminths possess the ability to evade or inhibit eosinophilic attack

One hypothesis to account for the different immune responses to eosinophilia is that helminths with rapid transit through host tissues do not usually encounter large populations of activated eosinophils, as it may take the host seven days or more post infection to mount an eosinophilopoietic response Therefore, rapidly migrating parasites would not have been under evolutionary pressure to develop protective mechanisms against attack from eosinophils, as a result of the speed of migration For example when larvae of rapid transit parasites such as *Nippostrongylus brasiliensis* encounter large populations of eosinophils within hours of inoculation into IL-5 transgenic mice, they have inadequate protective mechanisms and are damaged or killed If the "rapid transit" theory is true, one prediction would be that helminths that reside in the host tissue for longer periods, such as *F hepatica*, would be the ones selected during evolution to express protective mechanisms that allow them to survive eosinophilic attack, and thus would not be adversely affected by hypereosinophilic mice One such protective mechanism against eosinophilic attack is demonstrated by *F hepatica* derived cysteine proteases, which have been observed to cleave immunoglobulins *in vitro,* and inhibit the *in vitro* adherence of eosinophils to juvenile flukes in the presence of immune serum (Carmona *et al*, 1993) Studies by Cully *et al*, (2000), suggested that helminths may employ mechanisms to inhibit eosinophil recruitment, to prolong survival in the host Eotaxin is a potent eosinophil

chemoattractant, which acts through the receptor CCR3, expressed on eosinophils (Sallusto *et al*, 2001), and is involved in the stimulation of eosinophils from bone marrow, mediating their selective recruitment at sites of inflammation (Ugucciori *et al*, 1997) Culley *et al*, (2000), suggested an immuno-evasive strategy employed by helminths, in which production of enzymes inactivate eotaxin prevents recruitment and activation of eosinophils at the site of infection Inhibition of eosinophilic responses were also observed in murine studies by Lima *et al*, (2002), in which mice immunised with helminth worm extract had a profound inhibitory effect on eotaxin production, resulting in reduction of eosinophil numbers Studies by Carmona *et al*, (1993) demonstrated that cathepsin L1 contained in ES products cleaved Ig at the hinge region and could prevent the antibody mediated attachment of eosinophils to juvenile flukes

In the present study, eosinophilia was not observed in the compartment of the liver of *F hepatica* infected BALB/c mice Mice were infected with 10 metacercariae of *F hepatica*, and liver samples were investigated 7, 10, 14 and 21 days post infection Eosinophilia was not observed at any time point in the liver tissue, in either control or infected animals This observation coincides with the "rapid transit" theory, in so far as *F hepatica* resides in host tissue for relatively extensive periods of time, and as a result may have developed a method of surviving eosinophilic attack

Another explanation as to the lack of eosinophils in the livers of *Fasciola*-infected mice is that the parasitic tracts are the result of coagalative necrosis followed by the disolution of hepatocytes As this is primarily a necrotic reaction, it has the potential to draw neutrophils to the site as the first cell population

Therefore the local reaction observed is possibly not driven by a systemic response to parasites but by the parasite's ability to cause hepatocyte necrosis

We also studied the early development of the cellular response with respect to type 1 and type 2 cytokine mRNA levels Early cytokine production was measured in the hepatic lymph nodes (HLN) and mesenteric lymph nodes (MLN) of BALB/c mice during the first 8 days of experimental infection with *F hepatica* This study was performed with the aim of determining at what time point the immune response becomes predisposed towards a type 2 immune response Also, the early immune responses are the most likely to provide insight, and to determine the outcome of the immune response initiated locally to the area containing the parasite

As the ELISA method for analysing cytokine production was not sufficiently sensitive to measure cytokine production in this early infection time-course, we assessed cytokine production by reverse transcription-polymerase chain reaction (rtPCR) We investigated the levels of mRNA for cytokine markers in the HLN and MLN, at 0, 1, 2, 4 and 8 days post infection Preliminary tests were carried out on early cytokine production in spleens of mice experimentally infected with *F hepatica* (data not shown) However, as the immune responses observed in the spleen may not be specific, in the context of *Fasciola* infection, and may represent other peripheral immune responses, results were disregarded

A polarised type 2 response was observed in the HLN as early as 2 days post infection with elevated levels of the mRNA encoding IL-4 observed at 2 days post infection, with levels remaining constant throughout the study No detectable mRNA encoding IFN-γ was observed at any time point A predominant type 2 response in the MLN was detected at 1 day post infection with similar elevated

levels recorded on days 4 and 8 post infection No significant levels of IFN-γ were present at any time point, although minor levels were observed at days 4 and 8 post infection Results from the current study, consistent with the data from the previous experiment, illustrate a polarised type 2 immune response as a result of infection with *F hepatica* The induction of a type 1 response appears to be down-regulated as early as 1-2 days post infection It appears logical that a polarised type 2 response was observed in the MLN 1 day prior to detection in the HLN, as the migratory path of the fluke involves the fluke entering the intestine, and therefore stimulating a response in the local MLN, before migrating towards the hepatic tissue, and the subsequent stimulation of the HLN A study in rats by Tliba *et al*, (2002) described significant levels of IL-4 and IFN-γ in the HLN at 4 days post infection, which remained constant throughout the 14 day infection

Results presented in this study provide the first evidence that the immune response to infection with *F hepatica* become polarised towards the type 2 sub-type as early as 1 day post infection Once initiated, the production of the type 2 cytokine, IL-4 acts as a potent stimulus of Th2 responses (Allen & MacDonald, 1998), and progression towards a type 2 immune response is induced IL-4 production is also believed to play a crucial role in the down-regulation of IFN-γ, and therefore in the inhibition m a type 1 immune response (Brady *et al*, 1999) This result is consistent with studies by O'Neill *et al*, (2000) in which the importance of IL-4 in driving the polarised type 2 immune response is demonstrated in IL-4 deficient mice IL-4 deficient mice exposed to *F hepatica* produce significantly higher levels of IFN-γ, while the type 2 cytokines, IL-5 and IL-10 are downregulated, suggesting that the polarisation of the immune response

towards the type 2 sub-set in fascioliasis, is dependant on IL-4 (O'Neill *et al*, 2000)

In this study we demonstrated that infection with *F hepatica* induces a pre dominant type 2 immune response as early as 1 day post infection, and therefore provides a model for studying type 2 immune responses Other helminthic infections, such as schistosomiasis, also induce a pre dominant type 2 response, but only at later stages of infection when eggs are produced (Baulada-Benedetti *et al*, 1991) Sher *et al*, (1991), describe eggs as the major stimulus of the strong type 2 cytokine production in response to infection with *Schistosoma mansoni Filariasis* also induces a pre dominant type 2 immune response, but elements of a type 1 responses are also observed Therefore, with the early polarisation towards type 2 immune responses, and the significant levels of type 2 cytokines recorded, infection with *F hepatica* provides an ideal model for the study of type 2 immune responses Other benefits of this model include a) laboratory rodents are relatively inexpensive, b) can be easily infected and c) many reagents are available for examination of immunological responses in these hosts

While data derived from these experiments have demonstrated a polarised type 2 response as a result of infection with *F hepatica*, the next logical step is to determine the source of the type 2 immune response The following experiments, investigate immune responses to elements of antigens derived from *F hepatica*

3.2

Comparison of immune responses to *F. hepatica* and that of *F. hepatica* excretory/secretory (ES) products.

Introduction

3.2 1 Several investigators (Jefferies *et al*, 1997, Milbourne & Howell, 1997, O'Neill *et al*, 2001) have demonstrated the immnogenicity of Fasciola excretory/secretory products (ES) Previous studies performed in our laboratory by O'Neill *et al* (2001), demonstrated that *F hepatica* derived (ES) products were capable of mimicking the suppressive effect of infection with *F hepatica* on type 1 immune responses, by the production of a type 2 cytokine repertoire Also, ES products have been shown to induce eosinophilia in mice and rats (Milbourne & Howel, 1997)

In this experiment we compared immune responses of *F hepatica* infected mice to that of mice immunised with *F hepatica* excretory secretory products Our results show that both mice infected with *F hepatica*, and mice immunised with ES, induce a polarised type 2 immune response We also investigated cellular proliferation of mice injected with *F hepatica* derived ES Significant increases in proliferation of neutrophils, eosinophils and monocytes were observed in animals injected with ES

Experimental design

3 2 2 Experimental groups were established to compare immune responses between *F hepatica* infected mice and that of mice immunised with ES Four BALB/c mice aged 8-10 weeks were infected with 10 metacercariae of *F hepatica*, and a further 4 mice were immunised with ES A control group of 4 non-infected mice was established All animals were sacrificed by cervical dislocation 14 days post-infection Spleens were removed and cultured *in vitro*

with ES and PMA/antiCD3 Cytokine production in the supernatants was quantified by ELISA (Fig 3 9)

Intra-peritoneal cellular profiles in response to injection with *F hepatica* excretory/secretory (ES) products was examined in a group of four mice, each receiving ES (100µg) A control group of four non-infected (control) mice was also used All mice were sacrificed by cervical dislocation 24 hours after administration of ES Peritoneal lavages were performed on each mouse by flushing out the peritoneal cavity with 10mls of sterile phosphate buffered saline Cells samples of 100µl were placed on a glass slide and spun on a cytospin Cells were counted as described in section 2 6

Results

3 2 3 1 Comparison of IL-4 and interferon-γ cytokine production by spleen cells of BALB/c mice infected with 10 metacercariae of *Fasciola*, and that of mice immunised with ES

The greatest levels of IL-4 cytokine was observed in mice immunised with ES (Fig 3 9) Infected animals also produced significant levels of IL-4 No IFN-γ response was observed in either the infected or immunised mice Stimulation of spleen cells with PMA and anti-CD3 demonstrated that all cells were capable of producing both IL-4 and IFN-γ cytokines Neither IL-4 nor IFN-γ production was recorded in non-mfected/immunised mice Results were the mean of four individual mice for triplicate cultures of spleen cells

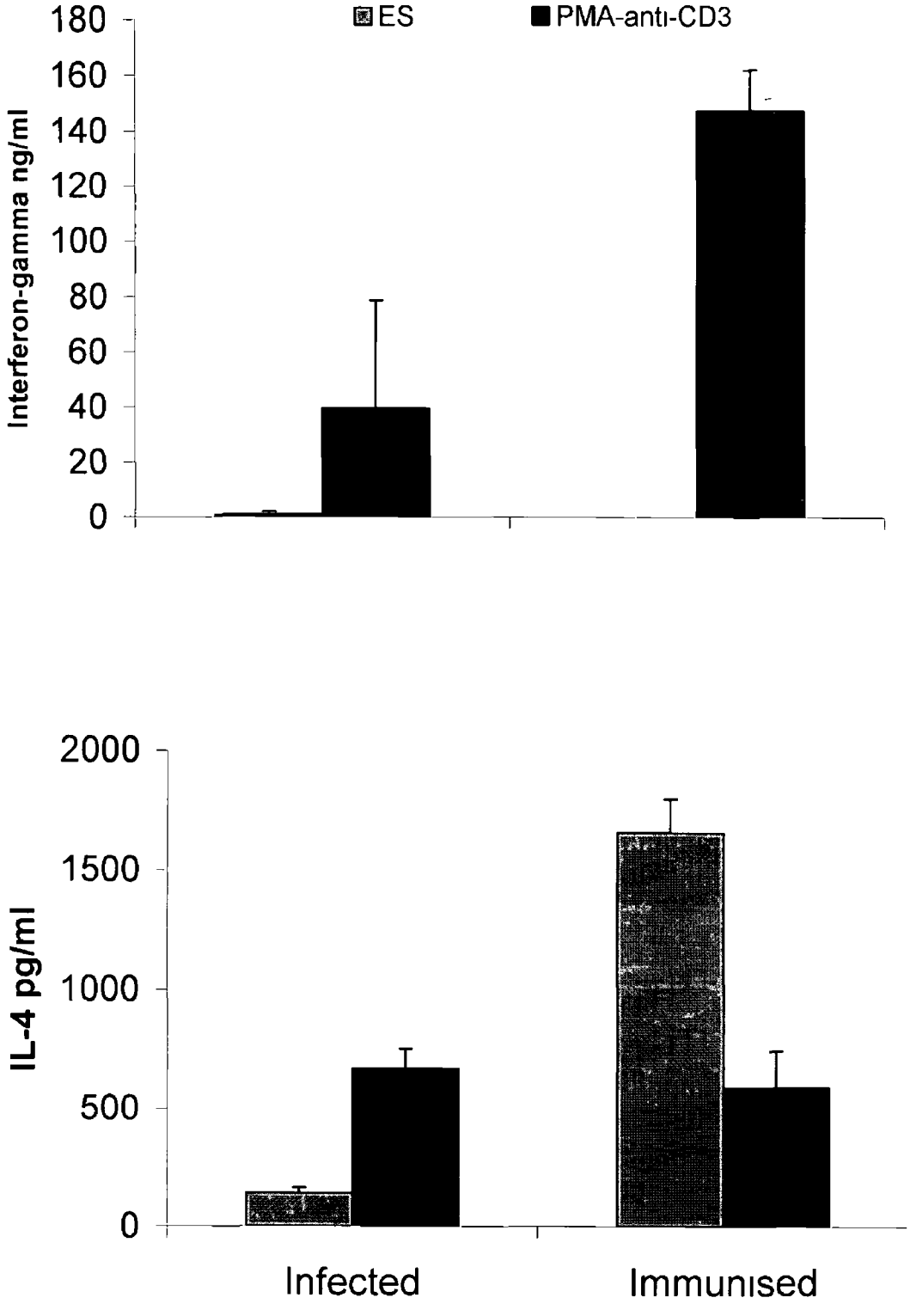

ES
PMA-anti-CD3
Interferon-gamma ng/ml
180
160
140
120
100
80
60
40
20
0
IL-4 pg/ml
2000
1500
1000
500
0
Infected
Immunised

Fig 3 9

Fig 3 9 Comparison of IL-4 and IFN-γ production in spleen cells of BALB/c mice infected with 10 *F hepatica metacercaria,* and that of mice immunised with *F hepatica* derived ES Cells were isolated 14 days post infection or immunisation and stimulated *in vitro* at 37°C for 72 hours with 5μg/ml ES products and PMA/antiCD3 Tests were carried out in triplicate

3 2 3 2 Intra-peritoneal cellular profiles in response to administeration of *F hepatica* excretory/secretory products

Cell counts show lymphocyte, monocyte, mast-cell, eosinphil and neutrophil proliferation as a percentage of all cells recorded (Fig 3 10) The total number of peritoneal exudate cells counted m infected mice after 72 hours was almost twice that recorded in control animals The total number of peritoneal exudate cells in control animals was 3 5 x 10^6, compared to 6 0 x 10^6 in infected animals This result demonstrates that ES is instrumental in the change in cellular profiles observed in *Fasciola* infections Between 80% and 95% of cells observed were lymphocytes, with similar levels of proliferation observed in control and infected mice, although a higher percentage was observed in control mice Significantly higher levels of monocytes were observed in infected mice than in controls (P>0 0001) Eosinophil profiles were significantly greater in infected than in control mice (P>0 0001) Levels of neutrophils were significantly greater in infected mice than control animals (P>0 00001) No mast cells were observed in either control or infected mice

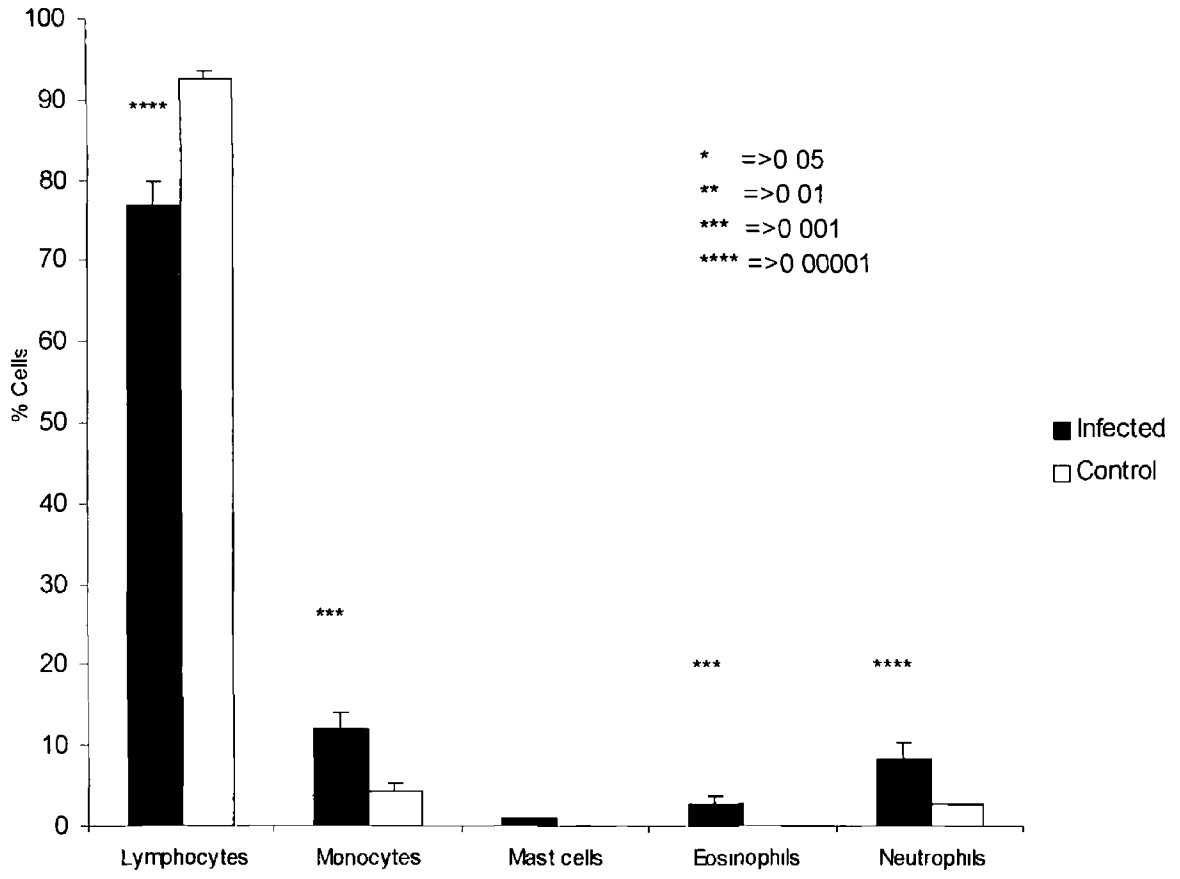


Fig 3 10 Percentage of cells present in peritoneal lavage sample from BALB/c mice immunised with *F hepatica* excretory/secretory product (100µg) Control mice received PBS Samples were obtained 72 hours post infection

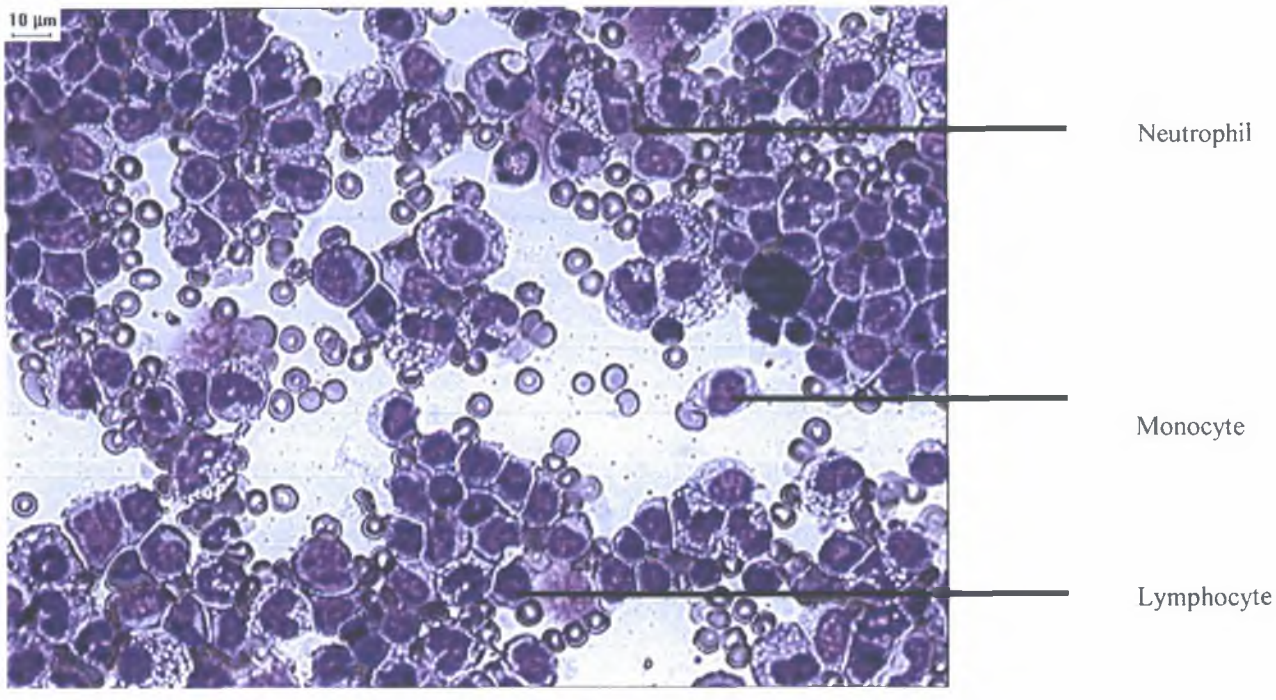


Fig. 3.11: Intra-peritoneal cellular proliferation of BALB/c mouse infected with 100µg *F. hepatica* excretory/secretory product. Cells were isolated 72 hours post infection. Tests were carried out thrice with similar results in each case. (H & E)

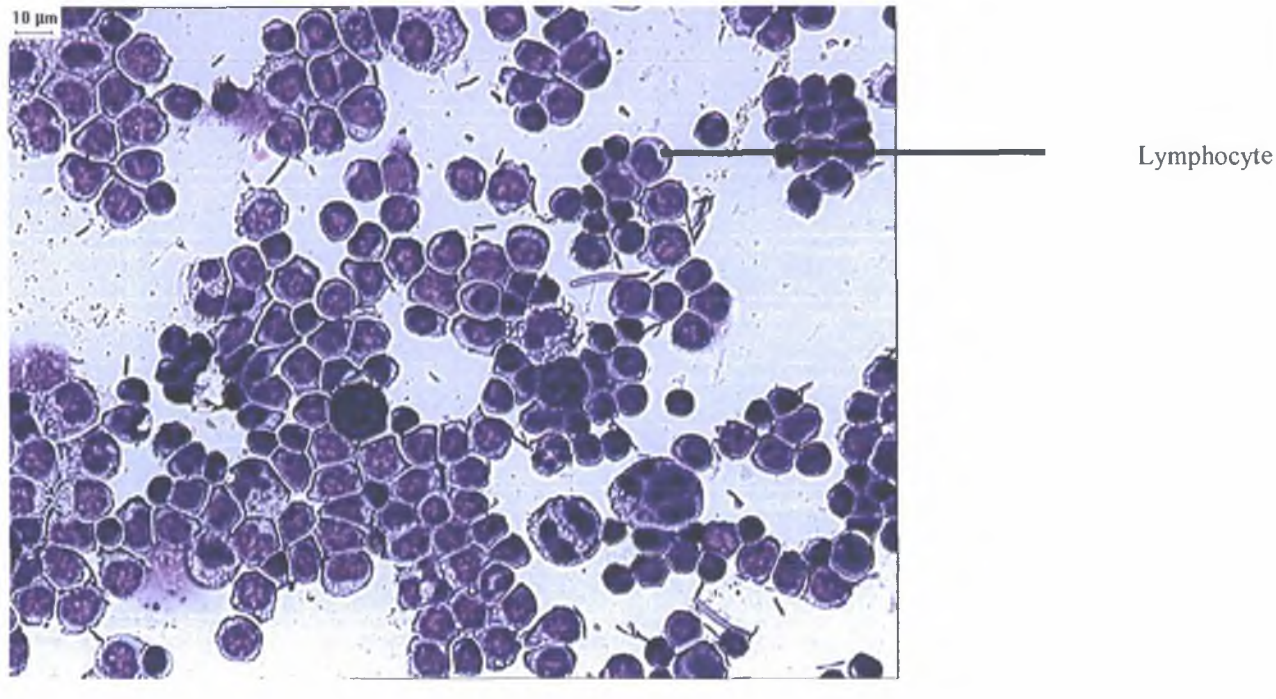


Fig. 3.12: Intra-peritoneal cellular proliferation of non-infected (control) mouse. Cells were isolated 24 hours post infection. Tests were carried out thrice with similar results in each case. (H & E)

3.2 4 Discussion

Fasciola excretory/secretory (ES) products are believed to be involved in several aspects of tissue penetration, immune evasion and pathogenesis (Dalton & Heffernan, 1989), and many studies have investigated the immunomodulatory role of ES products in parasitic infections (Fukumoto *et al*, 1997) Additionally, ES has been observed to inhibit superoxide output in human neutrophils (Jefferies *et al*, 1997) Therefore, we would expect ES products to be involved in the stimulation of the hosts' immune response In light of experiments performed by Milbourne and Howell (1997), in which ES products were suggested to induce eosinophilia, it has been suggested that ES antigens are the main source of immune stimulatory material in the host Data from the previous studies demonstrated that a type-2 immune response is observed in response to infection with *F hepatica* In this study we sought to investigate the immune response induced by antigens excreted and secreted by the parasite, and to compare any resulting response to that induced by infection with *F hepatica* With this in mind, BALB/c mice were injected with ES antigens, and the resulting immune response compared to that of mice infected with metacercariae of *F hepatica*

As in the previous experiments, a polarised type 2 immune response was observed in infected mice Cytokine production was monitored 14 days post infection, with significant levels of IL-4 production recorded in all infected mice BALB/c mice immunised with ES also produced a polarised type-2 immune response, with elevated levels of IL-4 observed 14 days post immunisation with ES Significantly more IL-4 production was observed in mice immunised with ES than that of infected mice No IFN-γ cytokine production was detected in either infected or immunised mice No significant levels of IL-4 or IFN-γ were recorded

in control mice O'Neill *et al*, (2001), demonstrated that the suppression of a type 1 immune response by *F hepatica* is mediated by molecules liberated by the parasite The data presented in this study show that *F hepatica* infection stimulates a type 2 immune response and that ES antigens can act in a similar manner Therefore, it may be deduced that the type 2 response observed in fascioliasis may not be as a direct result of the presence of the parasite, but may be stimulated by antigens secreted or excreted by the invading fluke

The effects of *F hepatica* excretory/secretory products on cellular profiles in the peritoneal cavity were also investigated Studies by Meeuseen, *et al*, (1995) and Jefferies, *et al*, (1996) demonstrated that during acute infections in sheep, neutrophils infiltrate into the primary tracts produced by the migrating fluke, suggesting that neutrophils may be involved in the immune response to infection These observations are supported by those of Lloyd and Oppenheim, (1992), who also implicated neutrophils in an immuno-responsive role to *F hepatica* infection In the present study a significantly greater number of neutrophils were recorded in mice injected with ES than that of control mice In infected mice, 21% of total cells counted were neutrophils, compared to 4% in non-immunsed control mice, indicating an up-regulation of neutrophil proliferation in response to *Fasciola* infection or a migration of neutrophils to the site of infection

Several studies have demonstrated that *F hepatica* can have a suppressive effect on the immune system of the host in which they reside (Chauvin, *et al*, 1995) It has also been observed that lymphocyte populations may be affected by infection with *F hepatica* (Brady, *et al*, 1999) Work by Jefferies *et al*, (1996) observed that ES products decreased lymphocyte proliferation in sheep and human

cells In the present study, lymphocytes were the most numerous cell type observed in both control and immunised animals However, significantly fewer lymphocytes were recorded in ES-immnuised mice than in the non-immunised controls thus implying a down-regulation of lymphocyte proliferation in mice immunised with ES products

As described in section (3 1) eosinophha is generally associated with helminth infection Although, eosinophils were not observed in the compartment of the liver in our studies, significant increases in the number of eosinophils observed as a result of immunisation with ES were detected in the peritoneal cavity While no eosinophils were detected in the peritoneal cavity of non-immunised mice, 8% of the total cells counted in mice immunised with ES were eosinophils This observation is in contrast to observations in the previous section, in which no eosinophils were observed m the compartment of the liver in mice infected with metacercaria of *F hepatica* The occurrence of eosinophils in the peritoneal cavity, in response to injection with ES, indicates a difference in the local and systemic immune response

Significantly greater numbers of monocytes were observed in the peritoneal cavity of BALB/c mice immunised with ES than that of control mice Monocytes are the pre-cursor of macrophages, which may act to phagocytose parasites

In the current study, the immune response as a result of immunisation with ES products were investigated Our data confirms that ES induces a type-2 immune response, similar to that of animals infected with *F hepatica* In the next section, experiments will describe immune responses to antigen derived from ES, m order to evaluate further the immunostimulatory effects of ES products

3.3

Immune responses to *F. hepatica* derived antigens.

Introduction

3.3 1 ES contains molecules which are are secreted by all stages of liver fluke (Dalton & Heffernan, 1989) and have important roles in facilitating parasite migration (tissue degration), feeding and immunoevasion For example studies by Jefferies *et al*, (1997), observed ES products involved in the inhibition of superoxide output by neutrophils As oxygen scavenging enzymes such as superoxide dismutase have been suggested to have a protective role against invading parasites, inhibition of superoxide output may provide a means of immune evasion for an invading helminth As demonstrated in experiments by Milbourne & Howell (1997) and O'Neill *et al*, (2001), ES products are known to illicit a stimulatory effect on immune responses, which are similar to immune responses observed in infection with *F hepatica* For example Milbourne and Howell (1997), recorded eosinophilia in rats as result of injection with ES, and also as a result of infection with *F hepatica* Therefore, it may be assumed that immune responses to *Fasciola* infection may in part be due to the production of ES by the invading parasite Various fractions of ES were purified in order to compare various components of ES in terms of induced immune responses, and to investigate elements of *F hepatica* derived antigen which stimulate type 2 immune responses

In this study, spleen cells taken from *F hepatica*-infected BALB/c mice, were analysed for T-cell cytokine production (IL-4 & Interferon-γ) in response to stimulation with various *F hepatica*-derived antigen Also, T-cell cytokine and antibody production was investigated in BALB/c mice immunised with various *F hepatica*-derived antigen

Experimental design

3 3 2 ES was purified on a gel filtration column, and the resulting fractions were isolated in terms of molecular weight, protein concentration and cysteine protease activity ES was separated on a Sephacryl S300, high resolution gel ultra-filtration column "peak 1" and "peak 2" were established by monitoring fractions for protein concentration and cathepsin-L activity Fractions containing a high protein concentration and no cysteine protease were pooled and termed peak 1, while fractions containing cysteine protease activity were pooled and termed peak 2 (Fig 3 13) *Fasciola* antigens are shown in Fig 3 14

To investigate the immune response of cells of *F hepatica* infected mice, stimulated with *F hepatica* derived antigen, four mice aged 8-10 weeks, were infected with 10 metacercaria of *F hepatica* A control group of four non-infected mice was established All mice were sacrificed by cervical dislocation 14 days post infection Spleen cells were stimulated *in vitro* at 37°C with ES (10 μg/ml), peak 1 (10 μg/ml), and peak 2 (10μg/ml), Stimulation of spleen cells with PMA and anti-CD3 demonstrated that all cells were capable of producing both Th1 and Th2 cytokines Cells were stimulated with culture media to act as a negative control The amount of IL-4 and IFN-γ secreted into the culture media was measured by bio-assay (Fig 3 13)

In order to establish the type of immune response to mice immunised with *F hepatica* derived antigen, three groups of four BALB/c mice aged 8-10 weeks were immunised with ES, peak 1, and peak 2, respectively A control group of four non-immunised mice was established Spleens were removed 14 days post immunisation and stimulated with (5μg/ml) ES, peak 1 and peak 2 Cells were stimulated with PMA and anti-CD3 as a positive control and culture media as a negative control Supernatants were removed and the levels of IL-4 and IFN-γ

cytokines secreted into the culture media was recorded by bio-assay Results show a polarised Th2 (IL-4 cytokine) response in all immunised groups (Fig 3 14) No IFN-γ was recorded in any of the immunised groups (Figs 3 15) Results were expressed as the mean cytokine concentration of four mice per group tested in triplicate

To investigate antibody production in response to immunisation with *F hepatica* derived antigen, serum samples were analysed for IgG1 and IgG2a isotype production from three different groups of BALB/c mice following intra-peritoneal immunisation with *F hepatica* derived antigen (Group 1 mice that were immunised with ES, Group 2 mice which received peak 1, Group 3 mice immunised with peak 2, and a control group of non-immunised mice) Serum samples were obtained 14 days post immunisation Analysis of IgG1 and IgG2a antibody production was performed on each mouse Antibody titrations were performed using each of the immunising antigen as antigen for the titrations, including PMA/antiCD3 and media as positive and negative controls, respectively Fig 3 16 shows the mean antibody levels of each group obtained with serum dilutions of 1 25000

3 3 3 Results

3 3 3 2 Purification of *F hepatica* derived antigen

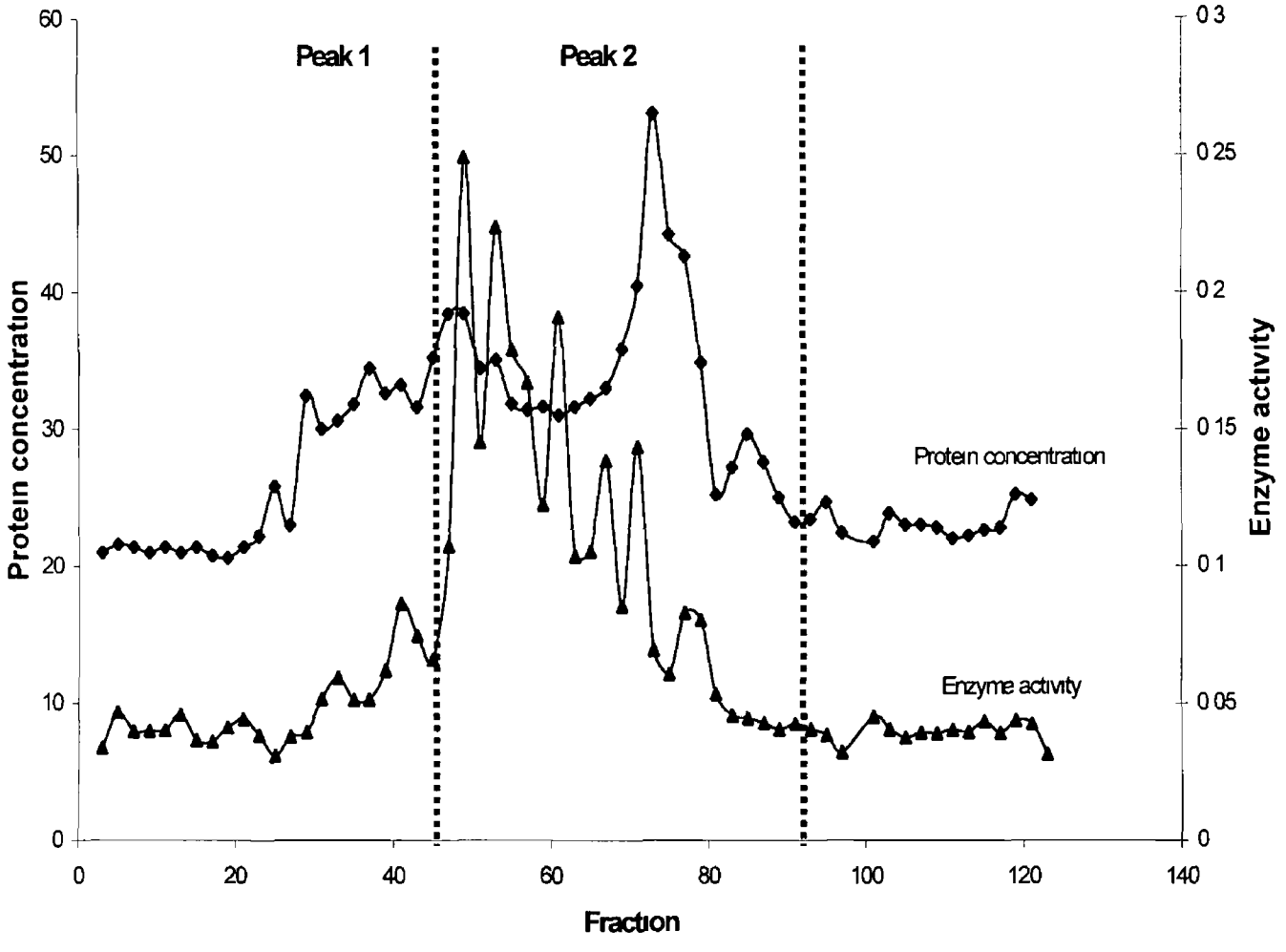


Fig 3 13 Purification profile of peak1 and peak 2 from *F hepatica* excretory/secretory products on a high resolution gel-filtration column Protein concentration and Cathepsin-L activity of ES fractions were recorded Fractions containing a high protein concentration and no cysteine protease activity (fractions, 22-43) were pooled and termed peak 1 Fractions containing a high protein concentration and cysteine protease activity (fractions, 44-96) were pooled and termed peak 2

SDS PAGE analysis of the ES antigen

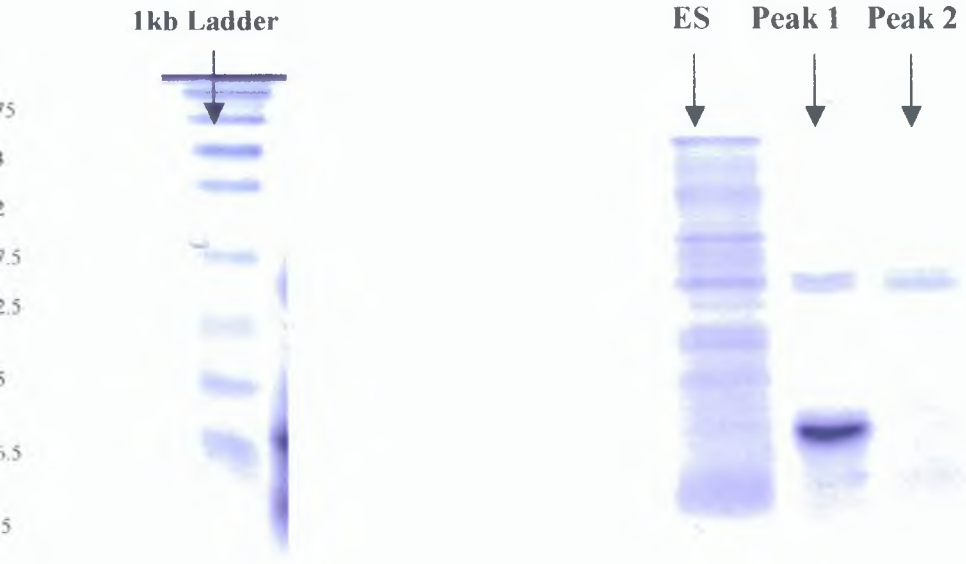


Fig. 3.14: Separation of *F. hepatica* derived antigen by SDS gel (15% running gel, 5% stacking gel), showing the major proteins secreted by the parasite. 5µl (10µg/ml) samples were applied with 10µl of 1kb molecular marker. The haemoprotein is visible in peak 1, while a Cathepsin-L band is visible in peak 2.

3 3 3 3 Antigen specific cytokine production in spleen cells of mice infected with metacercariae of *F hepatica*

A predominant type 2 cytokine response was observed in infected mice with cells stimulated by ES, peak 1, and peak 2 (Fig 3 15) Cells stimulated with ES produced the greatest cytokine (IL-4) response No significant levels of IFN-γ was detected in infected mouse cells stimulated with *F hepatica* derived antigen Spleen cells from the non-infected (control) mice did not secrete either Th1 (IFN-γ) or Th2 (IL-4) cytokines in response to stimulation with *F hepatica* derived antigen Results were the mean of four mice tested in triplicate

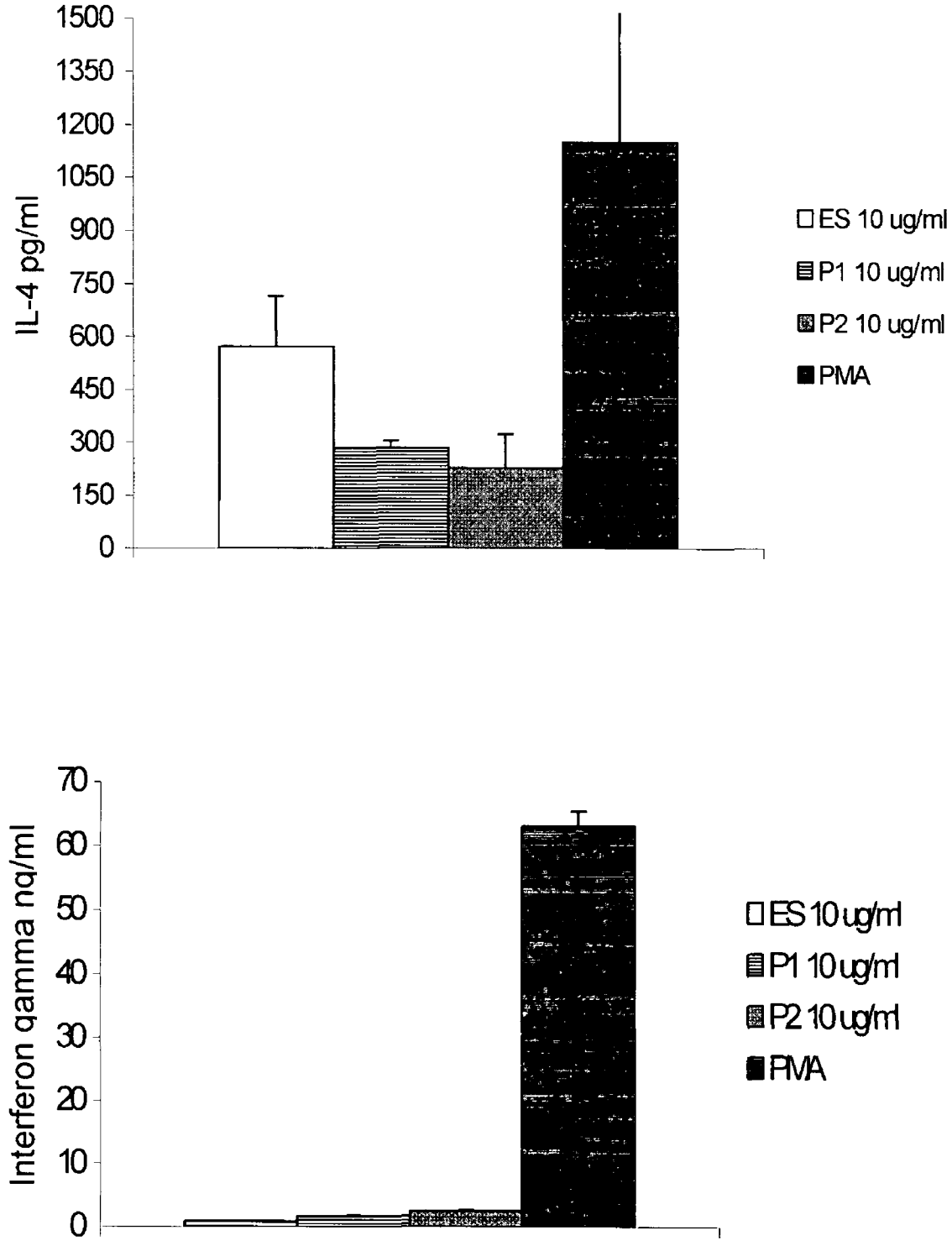

1500
1350
1200
1050
900
750
600
450
300
150
0
IL-4 pg/ml
ES 10 ug/ml
P1 10 ug/ml
P2 10 ug/ml
PMA
70
60
50
40
30
20
10
0
Interferon gamma ng/ml
ES 10 ug/ml
P1 10 ug/ml
P2 10 ug/ml
PMA
Antigen

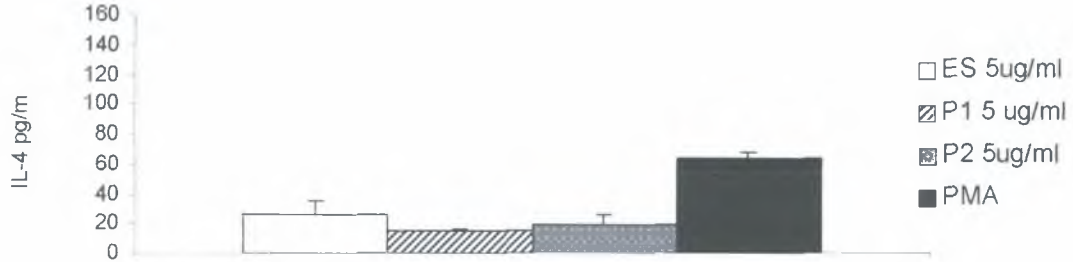
Fig 3 15

Fig 3 15 IL-4 and IFN-γ cytokine production by spleen cells of BALB/c mice infected with 10 metacercariae of *F hepatica* Cells were isolated 14 days post infection and stimulated *in vitro* for 72 hours at 37°C, with 10μg/ml ES, 10μg/ml peak 1, 10μg/ml peak 2, 10μg/ml and PMA/antiCD3 All tests were carried out in triplicate

3.3.3.4 : Antigen-specific cytokine production in spleen cells of mice immunised with *F. hepatica* derived antigen.

Results show a polarised type 2 (IL-4 cytokine) response in all immunised groups (Fig. 3.16). No IFN-γ was recorded in any of the immunised groups (Fig. 3.17). Results are expressed as the mean cytokine concentration of four mice per group tested in triplicate.

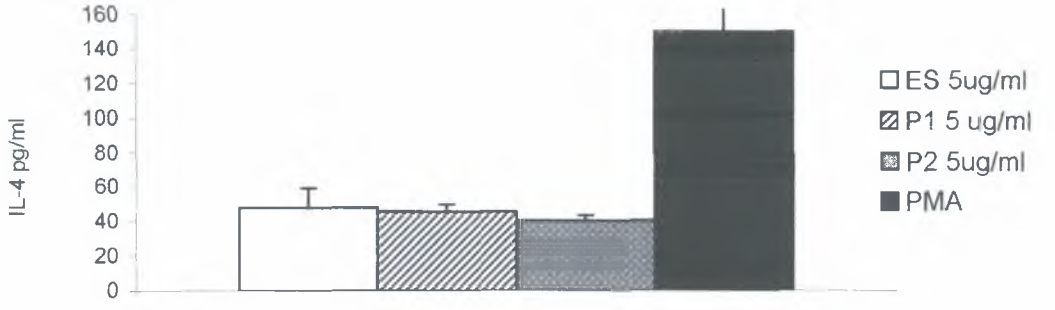
C.
Peak 2



D.
Control

Antigen

Fig. 3.16


160
140
120
100
80
60
40
20
0
IL-4 pg/ml
ES 5ug/ml
P1 5 ug/ml
P2 5ug/ml
PMA

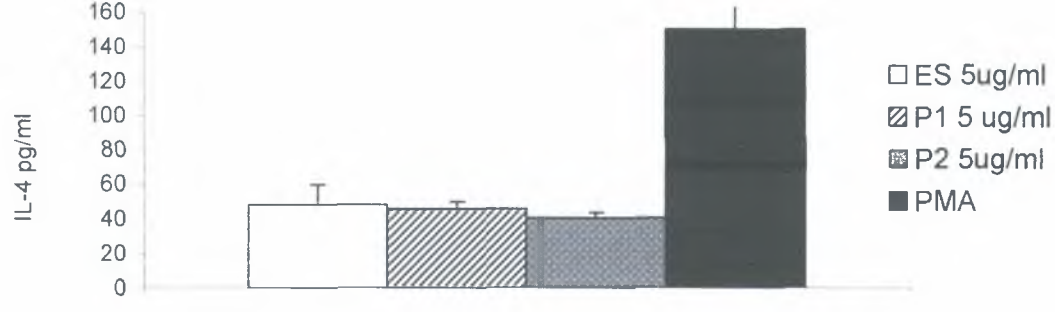

160
140
120
100
80
60
40
20
0
IL-4 pg/ml
ES 5ug/ml
P1 5 ug/ml
P2 5ug/ml
PMA

Fig 3.16: IL-4 production in spleen cells taken from mice immunised with (A) ES, (B) peak 1, and (C) peak 2. A non-immunised control group (D) was established. Cells were isolated 14 days post-infection and stimulated *in vitro* for 72 hours at 37°C, with (5μg/ml) ES, peak 1, peak 2, PMA/antiCD3. Results were expressed as the mean cytokine concentration of four mice per group tested in triplicate.

eak 2

Control

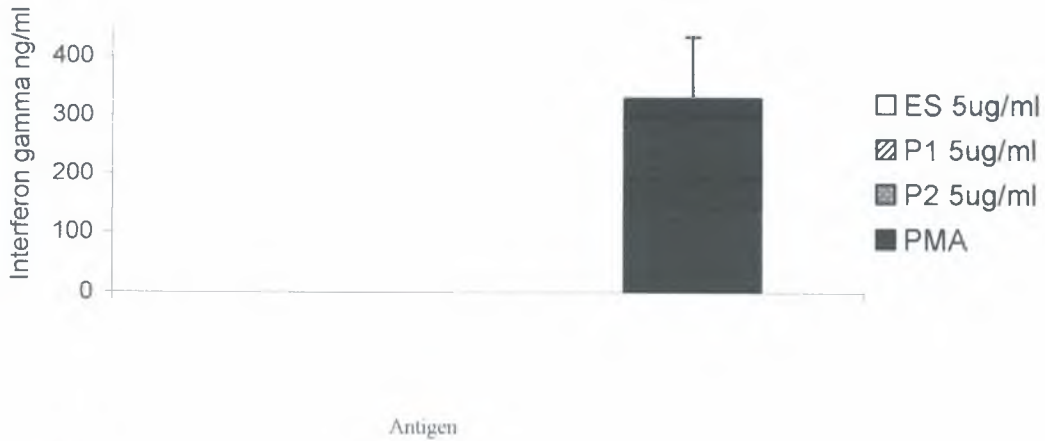


Fig. 3.17

A.
ES


Interferon gamma ng/ml
500
400
300
200
100
0
ES 5ug/ml
P1 5ug/ml
P2 5ug/ml
PMA

.
eak 1


Interferon gamma ng/m
400
300
200
100
0
ES 5ug/ml
P1 5ug/ml
P2 5ug/ml
PMA

Fig. 3.17: IFN-γ production in spleen cells taken from mice immunised with (A) ES, (B) peak 1, and (C) peak 2. A non-immunised control group (D) was established. Cells were isolated 14 days post infection and stimulated *in vitro* for 72 hours at 37°C, with (5μg/ml) ES, peak 1, peak 2, PMA/antiCD3. Results were expressed as the mean cytokine concentration of four mice per group tested in triplicate.

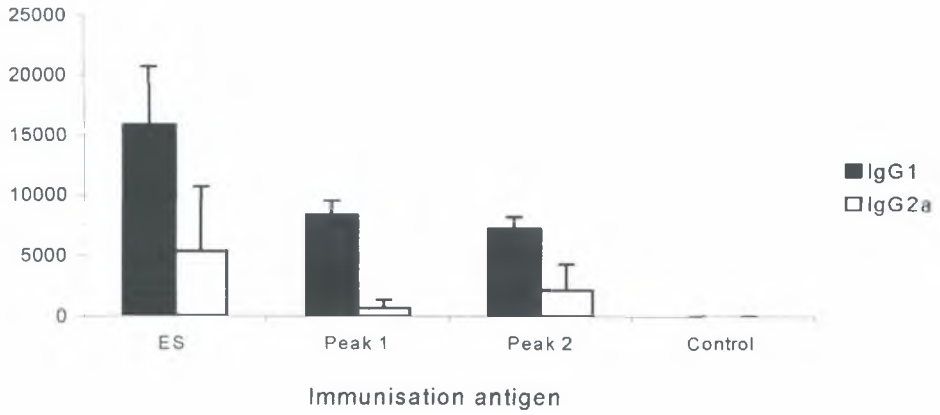
3.3.3.5 IgG1 and IgG2a antibody production in serum of mice immunised with *F. hepatica* derived antigens

Antibody analysis of immunised groups, stimulated with ES (Fig. 3.17-A) demonstrated a pre dominant IgG1 response to immunisation. Mice immunised with ES produced the highest levels of IgG1. Animals immunised with peak 1 and peak 2, also produced significant levels if IgG1. IgG2a production was also observed in animals immunised with ES, peak 1 and peak 2. No antibody production was recorded in control animals.

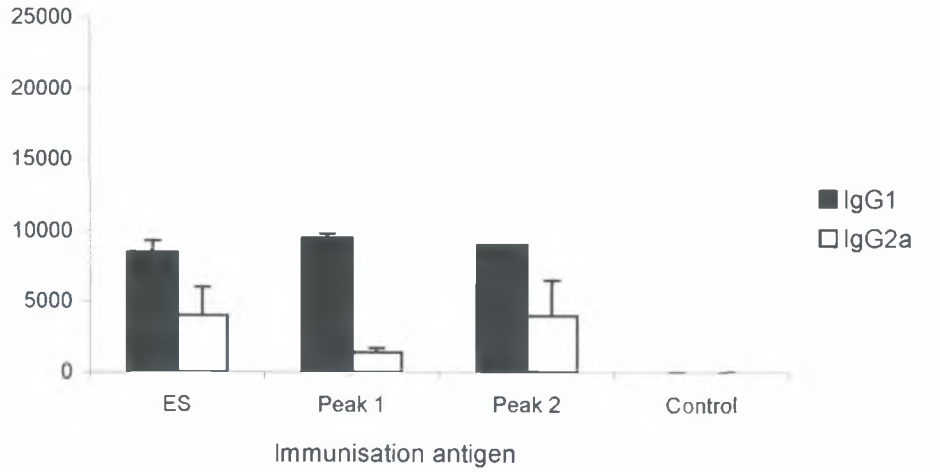
Serum stimulated with peak 1 (Fig. 3.17-B) displayed a polarised Th2 response in all immunised groups. Low levels of IgG2a was observed in mice immunised with ES, peak 1 and peak 2. No antibody response was observed in control mice.

Analysis of serum samples stimulated with peak 2 (Fig. 3.17-C) demonstrated a polarised Th2 response with the greatest antibody production observed in the group immunised with ES. Low levels of IgG2a were observed in mice immunised with ES and peak 2. No antibody production was observed in control mice. Results are the mean of four individual mice, tested in triplicate.

A.
ES



B.
Peak 1



C.
Peak 2

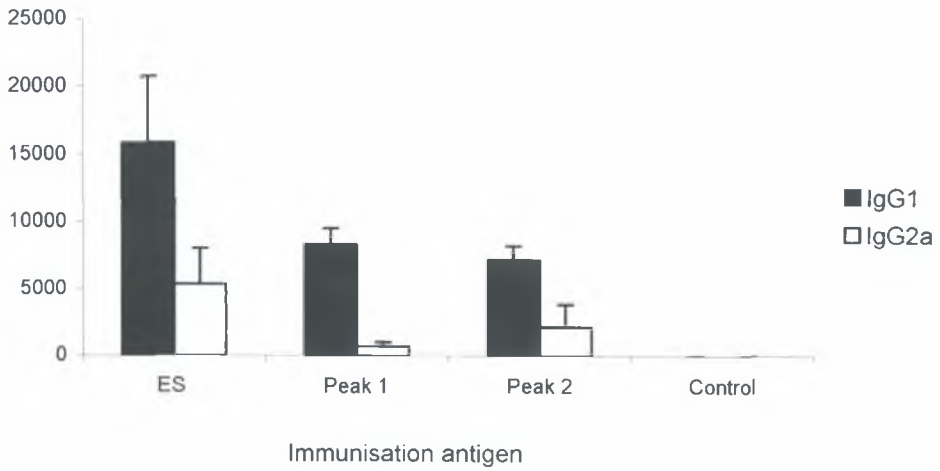


Fig. 3.18

Fig. 3.18: (A) IgG1 and IgG2a antibody production with specificity for *F. hepatica* excretory/secretory products, in serum taken from immunised BALB/c mouse cells. Mice were immunised with excretory/secretory products, peak 1, and peak 2. All tests were performed in triplicate. **(B)** IgG1 and IgG2a antibody production with specificity for peak 1, in serum taken from immunised BALB/c mouse cells. Mice were immunised with excretory/secretory products, peak1, and peak 2. All tests were performed in triplicate. **(C)** IgG1 and IgG2a antibody production with specificity for peak 2, in serum taken from immunised BALB/c mouse cells. Mice were immunised with excretory/secretory products, peak 1, and peak 2. All tests were performed in triplicate.

3.3.4 : Discussion

Sections 3.1 and 3.2 described the polarised type 2 immune response observed in response to infection with *F. hepatica.* Also investigated, was the predominant type 2 response induced by immunisation with *F. hepatica* derived ES. In the current study we investigated immune responses to isolated fractions of purified ES (peak 1 and peak 2), in order to further investigate the source of the type 2 immune response. Studies involving various helminth antigens including antigens contained in ES have been performed by several investigators. A type 2 response was observed in work by Cervi *et al* (1999) in which rats were immunised with *F. hepatica* derived glutathione-S-transferase. Mice immunised with recombinant fatty acid binding protein from *F. hepatica* exhibit antibody production characteristic of type 1 immune responses (Abane *et al.*, 2000). A high molecular sized antigen secreted by adult flukes into culture medium was isolated by McGonigle and Dalton (1995). This molecule contained a heme group, and was classified as a liver fluke haemoglobin (Hb) (McGonigle & Dalton, 1995). Hb is involved in the aerobic respiration of juvenile flukes when present in the liver. In adult flukes, it aids in oxygen–independent functions such as egg production (Bjorkman & Thorsell, 1963). This hemeprotein (a conjugated protein linked to an iron-porphyrin compound), is present in the current study contained in peak 1. Studies by Dalton *et al.,* (1996) demonstrated that two cathepsin L proteinases, cathepsin L1 (CL1), and cathepsin L2 (CL2), which are contained in peak 2, in the context of this experiment, may be involved in host tissue penetration, nutrition and immune evasion. Bentancor *et al.,* (2002) observed a type 1 immune response in rats immunised with cathepsin L1 and cathepsin L2. CL1 and CL2 differ in their specificities for hydrolysing peptide bonds (Dowd, *et al.,* 1994). In studies by Dalton *et al.*, (1996), Cathepsin L2 was observed to cleave peptide bonds with greater efficiency than that of cathepsin L1. For example, cathepsin L2 cleaved several

peptide bonds with a proline in the P-2 position that were poorly cleaved by cathepsin L1 (Dowd *et al.*, 1994). The difference in observed specificities may indicate that the two cysteine proteases have different roles in digestion, tissue penetration or immune evasion (Dalton *et al.*,1996).

In the previous experiments, all cytokine and antibody production as a result of *Fasciola* infection, was measured with specificity for *F. hepatica* ES. In the current studies cytokine and antibody production stimulated by Fasciola infection was measured with specificity to ES and ES-derived antigen. These experiments aimed to determine and compare the immunogenicity of the various Fasciola derived antigens.

Spleen cells obtained from *F. hepatica* infected BALB/c mice were stimulated with *F. hepatica* derived antigen. As in our previous experiments, high levels of ES-specific IL-4 production were observed in spleen cells of infected mice. The ES-derived peak 1 and peak 2 produced similar levels of IL-4, although production was not as great as that observed by ES-stimulated spleen cells.

The heme protein contained in peak 1 may be in part, responsible for the polarisation of the immune response towards the type 2 sub-type, observed with stimulation with peak 1. The type 2 response elicited by spleen cells obtained from mice infected with *F. hepatica* and stimulated with peak 2, is in contrast to work by Bentancor e*t al*, (2002), in which a type 1 response was observed in response to immunisation with CL1 and CL2 cysteine proteinases, present in the context of this experiment, in peak 2. (Fig. 3.16). The type-2 immune response observed in the current study may be as a result of the combination of the two proteases, the immune response to which was not investigated by Bentancor, e*t al.*, (2002). Also, other molecules present in peak 2, which are yet to be identified, may be involved in the stimulation of a type 2 response.

This study also investigated the immune responses of BALB/c mice to *F. hepatica* derived ES, peak 1 and peak 2. A similar type 2 response was observed in mice immunised with ES antigens to that of *F. hepatica* infected mice stimulated with ES antigens. Cytokine production was measured in immunised mice, with specificity for each of the Fasciola antigens. Each of the immunising antigens produced a predominant type 2 response. Similar levels of IL-4 cytokine were observed in mice immunised with ES and peak 1. Lower, levels of IL-4 production was recorded in mice immunised with peak 2. Stimulation of immunised mouse spleen cells with each of the antigens had similar effects on IL-4 cytokine production. As expected, no IFN-γ production was detected in any of the immunisation groups.

Antigen-specific antibody production in *F. hepatica*-derived antigen-immunised mice was also investigated (Fig. 3.18). A polarised type 2 antibody profile was observed in response to immunisation with each of the antigens. Antigen specific IgG1 antibody production was recorded in each of the immunised groups for each of the stimulating antigens.

3.4

Immunisation with *F. hepatica*-derived antigen and various adjvuvants.

Introduction

3.4.1: While a type 2 immune response is associated with *F. hepatica* infection, a type 1 response has been suggested as being involved in protection against the parasite. Studies by Mulcahy *et al.*, (1998) demonstrate a protective function for type 1 immune response associated antibody isotypes (IgG2). The aim of this experiment was to establish a predominant type 1 immune response in BALB/c mice by immunising with various combinations of adjuvants and antigen (mutant CL1). Adjuvants help antigen to elicit an early, high and long-lasting immune response with less antigen (Gupta & Siber, 1995). The adjuvants used in the current experiment were alum (aluminium hydroxide), heptavac (A *Clostridium perforingens* vaccine) and oligodeoxynucleotides with cpg motifs (unmethylated CpG motifs).

Experimental design

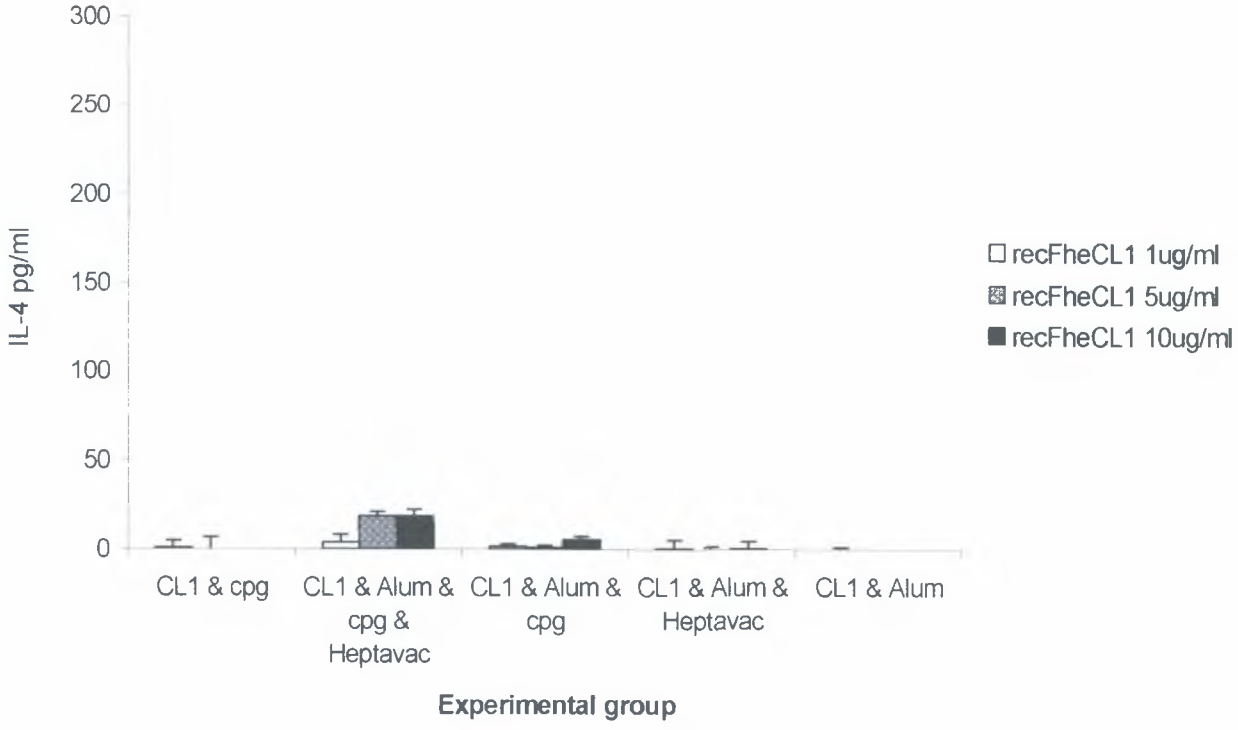
3.4.2: Experimental groups of 4 BALB/c mice were established. (Group 1: mice that were immunised with mutFheCL1 and cpg; Group 2: mice immunised with mutFheCL1, alum, cpg and heptavac; Group 3: mice received mutFheCL1, alum and cpg; Group 4; mice that received mutFheCL1, alum and heptavac; Group 5 : mice immunised with mutFheCL1 and alum. A control group of four non-immunised mice was established. All immunised mice received booster immunisations at days 21 and 42 post initial immunisation. All animals were sacrificed after 61 days by cervical dislocation. Isolated spleen cells were stimulated *in vitro* at 37°C with recFheCL1 (10μg/ml, 5μg/ml, 1μg/ml) and mutFheCL1 (5μg/ml, 1μg/ml). Supernatants were removed and the amount of IL-4 and interferon-γ secreted into the culture media was determined by means of ELISA.

The antibody responses to immunisation were investigated 61 days post-immunisation. The titre of IgG subclasses was determined using mutFheCL1 and *Clostridium perfringens* as antigen in antibody titrations (Fig. 3.21-22).

3.4.3 Results

3.4.3.1: IL-4 and Inteferon-γ cytokine production in mice immunised with *F. hepatica*-derived antigen and various adjuvants

The results demonstrate that predominant type-1 response can be induced in spleen cells of mice stimulated with recFheCL1, with significant amounts of IFN-γ cytokine being produced (Fig. 3.19). The greatest amount of IFN-γ produced was observed in mice immunised with mutFheCL1, alum, cpg and heptavac. Generally, cells stimulated with a higher concentration of antigen resulted in a higher level of cytokine production. No significant level of IL-4 cytokine production was recorded (Fig. 3.36). Low levels of IL-4 was observed in mice immunised with mutFheCL1, alum, cpg and heptavac. No significant levels of interferon-γ or IL-4 were observed in immunised mice stimulated with mutFheCL1 (Figs. 3.38 and 3.39). Results were expressed as the mean cytokine concentration of four mice per group tested in triplicate.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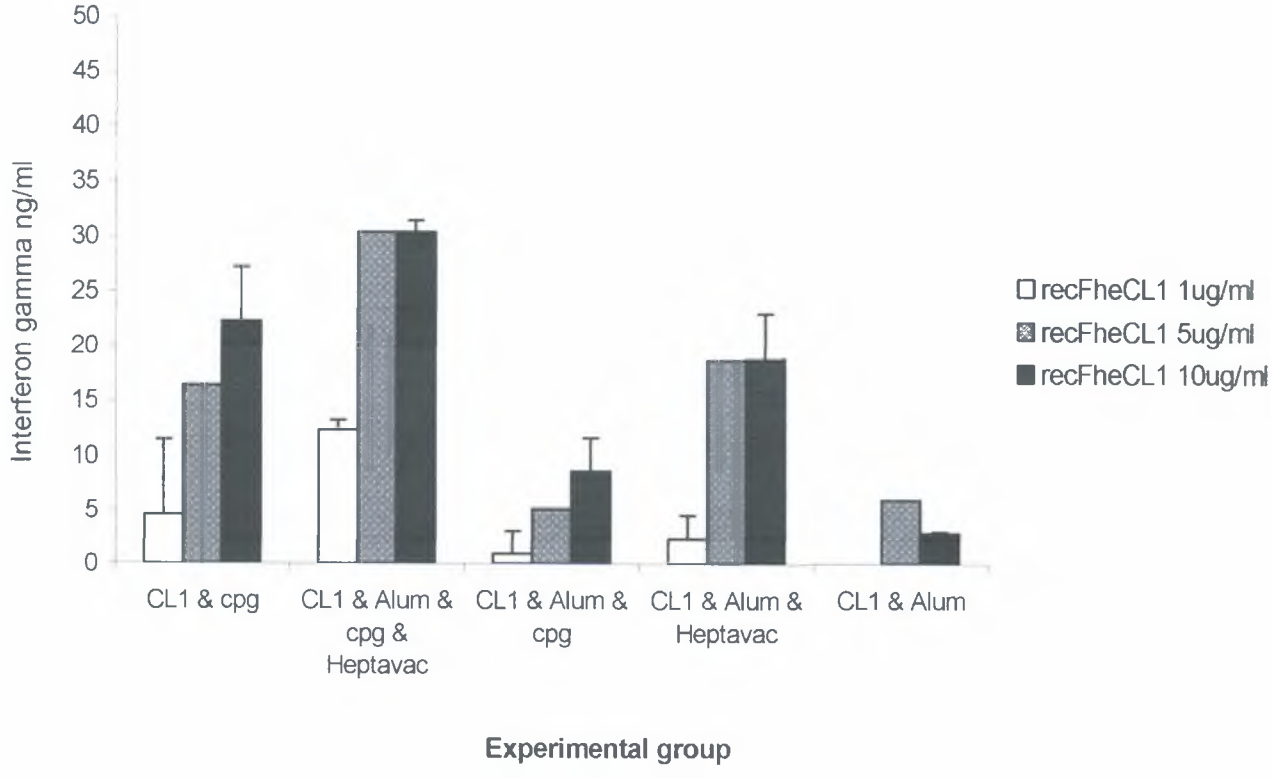
250
200
150
100
50
0
IL-4 pg/ml
recFheCL1 1ug/ml
recFheCL1 5ug/ml
recFheCL1 10ug/ml
CL1 & cpg
CL1 & Alum & cpg & Heptavac
CL1 & Alum & cpg
CL1 & Alum & Heptavac
CL1 & Alum
Experimental group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40
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30
25
20
15
10
5
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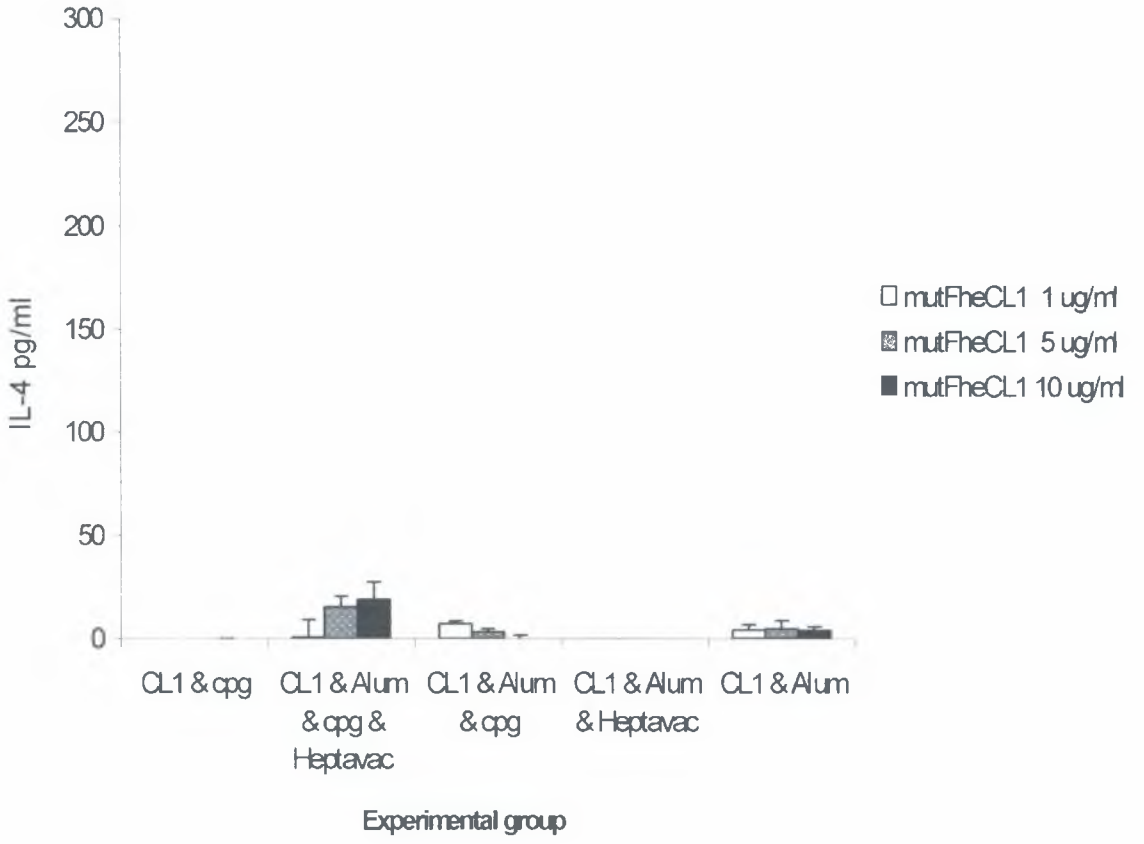
recFheCL1 1ug/ml
recFheCL1 5ug/ml
recFheCL1 10ug/ml
CL1 & cpg
CL1 & Alum & cpg & Heptavac
CL1 & Alum & cpg
CL1 & Alum & Heptavac
CL1 & Alum
Experimental group

Fig. 3.19

Fig. 3.19: Cytokine production by spleen cells of BALB/c mice immunised with various antigen and adjuvants. Mice received booster immunisations on days 21 and 42. All animals were sacrificed on day 61. Spleen cells were isolated and stimulated *in vitro* for 72 hours at 37°C with 1μg/ml, 5μg/ml and 10μg/ml recFheCL1. Samples of supernatant were measured for IL-4 (Fig. 3.36) and interferon-γ (Fig. 3.37) cytokines. Results were expressed as the mean cytokine concentration. Tests were carried out in triplicate.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250
200
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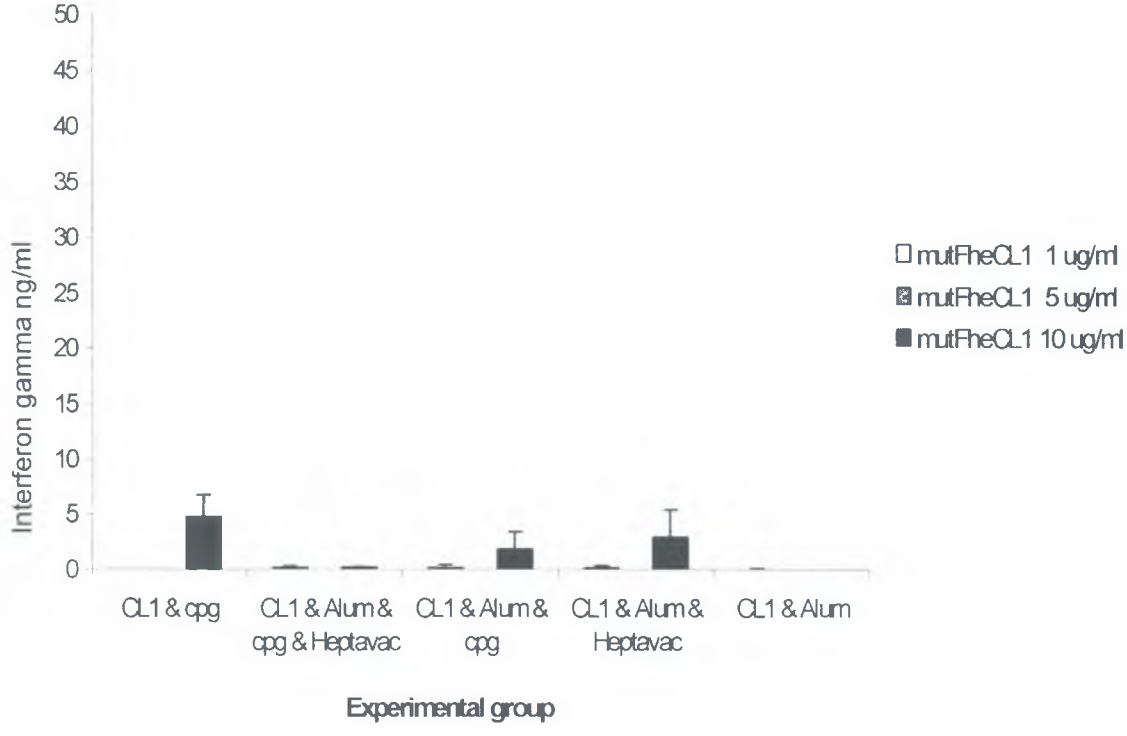
50
0
IL-4 pg/ml
mutFheCL1 1 ug/ml
mutFheCL1 5 ug/ml
mutFheCL1 10 ug/ml
CL1 & cpg
CL1 & Alum & cpg & Heptavac
CL1 & Alum & cpg
CL1 & Alum & Heptavac
CL1 & Alum
Experimental group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mutFheCL1 5 ug/ml
mutFheCL1 10 ug/ml
CL1 & cpg
CL1 & Alum & cpg & Heptavac
CL1 & Alum & cpg
CL1 & Alum & Heptavac
CL1 & Alum
Experimental group

Fig. 3.20.

Fig. 3.20 : IL-4 (Fig. 3.38) and Interferon-γ (3.39) cytokine production in spleen cells taken from mice immunised with various antigen and adjuvants. Mice received booster immunisations an days 21 and 42. All animals were sacrificed on day 61. Spleen cells were isolated and stimulated in vitro for 72 hours at 37°C with 1μg/ml, 5μg/ml and 10μg/ml mutFheCL1. Results were expressed as the mean cytokine. Tests were carried out in triplicate.

3.4.3.2: IgG1 and IgG2a antibody production in serum of immunised mice.

A pre-dominant IgG1 antibody response was observed in response to stimulation with mutFheCL1 (3.21). Significant levels of IgG1 were observed in all experimental groups with the exception of mice immunised with heptavac where low levels of antibody production were observed. There were no significant mutFheCL1-specific IgG2a antibodies detected in the serum of immunised mice. No antibody production was observed in control non-immunised mice. Results were expressed as the mean antibody titration of four mice per group tested in triplicate.

In serum stimulated with *C. perforingens* a predominant IiI1 response was observed in response to immunisation (3.22). The highest levels of IgG1 antibody production were observed in mice immunised with (i) mutFheCL1, alum and heptavac, (ii)mutFheCL1, alum, cpg and heptavac and (iii) cpg and heptavac. No antibody production was observed in control, non-infected mice. Results were expressed as the mean antibody titre of four mice per group tested in triplicate.

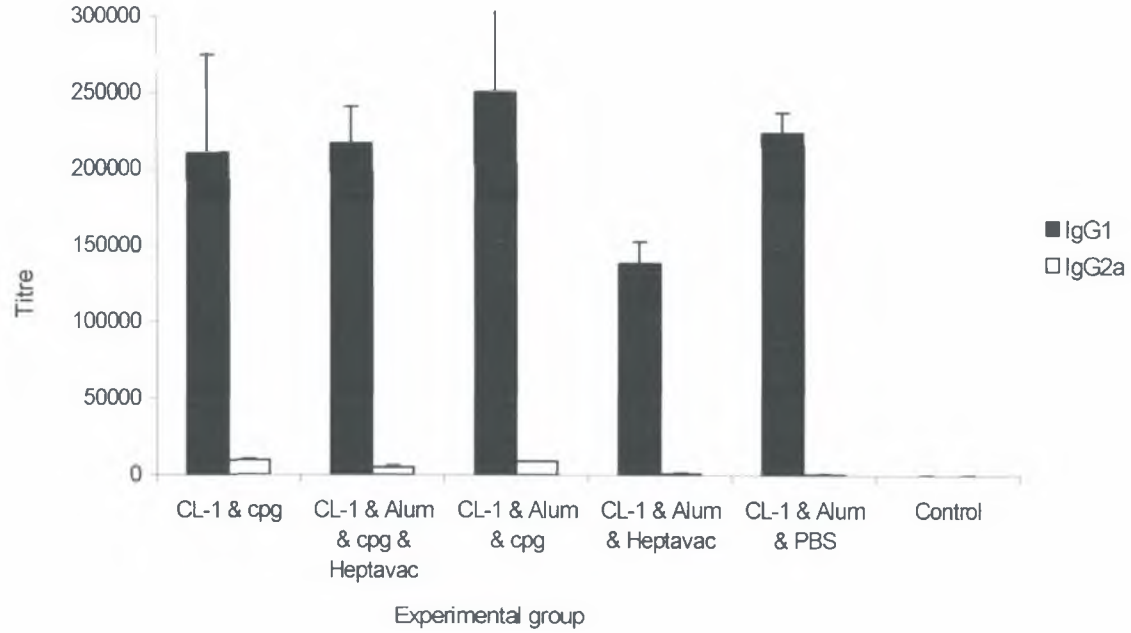

Fig. 3.21

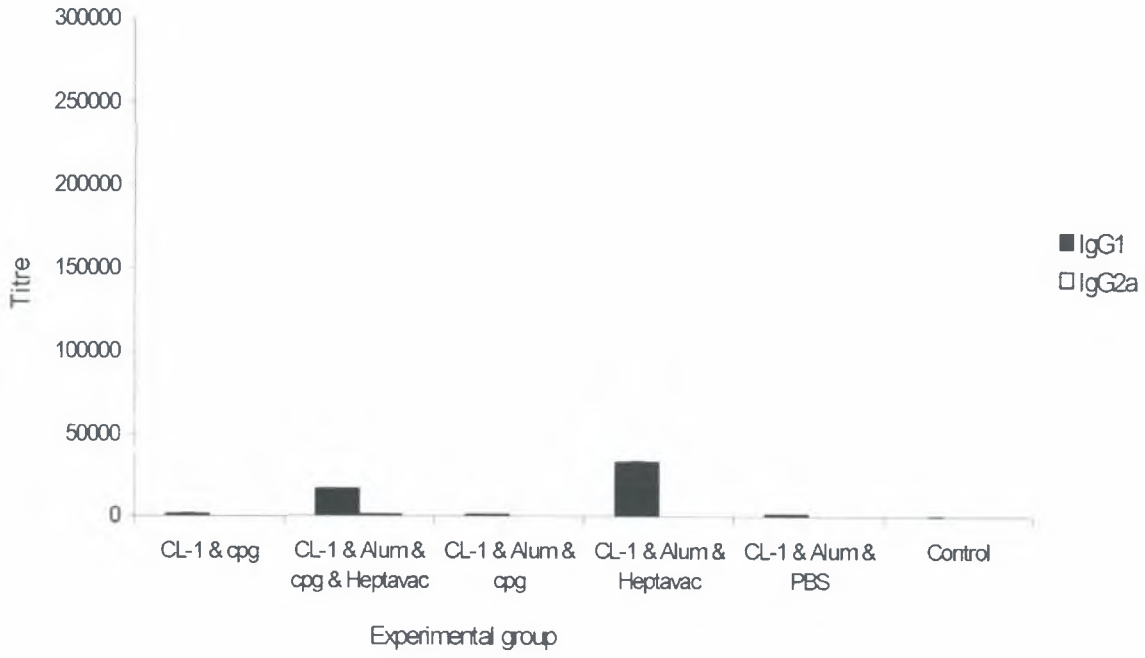

Fig. 3.22

Figs. 3.21-3.22: IgG1 and IgG2a antibody production in serum taken from mice immunised with various antigen and adjuvants. Mice were sacrificed 61 days post infection and serum obtained. Results in fig. 3.21 show antibody production specific for *Fasciola* mutFheCL1. Results in fig. 3.22 show antibody production specific for *C. perforingens* bacteria. Results are expressed as the mean antibody titre of four mice per group tested in triplcate.

3.4.4 : Discussion

Observations by Berquist, *et al.,* (2002) in relation to immune responses in helminth infections suggest that there are desirable, antigen-specific immune responses that would be valuable in a vaccine, but that there are also immune responses that should be avoided. The polarised type 2 immune responses in helminthic infections, and subsequent inhibition of type 1 immune responses may be advantageous to the parasite, as type 1 responses have been suggested to have a protective role in infections such as schistosomiasis (Cjaja, *et al.,* 1989). Studies by Clery *et al.*, (1996) showed the type 2 antibody, IgG1 to be the dominant isotype in *F. hepatica*-infected cattle. However, this type 2 immune mechanism appeared to be a non-protective response. While natural infections in cattle and sheep appear to elicit non-protective type 2 immune responses, studies by Mulachy *et al,* (1998) indicate that the protection induced by vaccination involves elements of a type 1 response. Capron and Capron (1994), suggested that T-cell mediated immunity involving IFN-γ and IL-12-dependant mechanisms (type 1 response) might be more advantageous in helminth infections. This observation is in agreement with studies by Wynn and Hoffmann (2000), which demonstrate that immunity to helminth infections is highly dependant on the production of IFN-γ from $CD4^+$ T-cells. Also, Smythies *et al* (1992) demonstrated decreased immnity to helminth infections in mice deficient in IFN-γ. A positive correlation between fluke-specific serum IgG1 levels and fluke burden was recorded in work by Mulcahy *et al.*, (1998), while a strong IgG2 (type 2) response was associated with low fluke burdens. The results of this study are further evidence of the non-protective status of specific immune responses in cattle following infection with *F.*

hepatica, and demonstrate that vaccination may induce a protective response (Mulcahy *et al.*, 1998).

Studies by Dalton, *et al.*, (1996), demonstrated that a significant degree of protection could be achieved in cattle against infection with *F. hepatica,* by immunising with various *F. hepatica* derived antigens. Cathepsin L proteases, cathepsin L1, cathepsin L 2, secreted by liver flukes, and liver fluke haemoglobin (Hb), were used in conjunction with Freunds complete and Freunds incomplete adjuvants. The initial trial demonstrated that cattle vaccinated with cathepsin L1 induced 53.7% protection against a heterologous challenge with *F. hepatica.* A subsequent trial showed that a combination of cathepsin L1 and Hb could also elicit a protective immune response. The most significant level of protection (72.4%) was observed in animals vaccinated with a combination of cathepsin L2 and Hb. Animals vaccinated in theses studies, displaying a level of protection against fascioliasis, exhibited an antibody profile predisposed towards a type 1 subset, with significant levels of IgG2 antibody recorded. The fact that the immune response of these vaccinated animals exhibited a polarised type 1 immune response may in part be due to the use of Freunds adjuvants, which are known to influence immune responses in favour of the type 1 sub type.

With these observations in mind, the current study sought to establish a type 1 immune response in BALB/c mice by immunising with *F. hepatica* derived antigens and 2 types of adjuvants. Investigations were performed in mice as fewer reagents would by required to perform the investigation than that required in cattle, and also due to the lesser cost of mice. As Freunds adjuvant has been known to cause pathology in humans we investigated the possibility of using a different adjuvant which would still provide type 1 immune response-inducing

properties. Studies by Near *et al.*, 2002 observed that oligodeoxynucleotides with cpg motifs (cpg), used as an adjuvant aided in the promotion of a type 1 immune response, with elevated levels of IFN-γ recorded. Cpg's were employed in the current study as an adjuvant.

Heptavac is commercially available as a vaccine against the bacterial infection, C. *Perfoingens.* Preliminary studies (data not shown) investigated the ability of heptavac to stimulate a type 1 immune response in immunised mice, however no polarised response was observed. Heptavac with a combination of other adjuvants and antigen, was established as an adjuvant in the current study. Alum hydroxide was also used as a comparative adjuvant.

Animals were immunised with a combination of various adjuvants and recFheCL1. Cytokine production was monitored with specificity for two forms of cathepsin L, the active form, recFheCL1 and inactive form, mutFheCL1 (Collins, *et al* in press).

IL-4 and IFN-γ cytokine analysis in spleen cells of immunised BALB/c mice, stimulated with recFheCL1 exhibited a polarised type 1 immune response in mice immunised with (i) CL1 and cpg, (ii) CL1, alum, cpg and heptavac, (iii) CL1, alum and cpg and (iv) CL1, alum and heptavac. As expected, lower levels of IFN-γ were recorded in mice immunised with CL1 and alum. No significant levels if IL-4 were detected. Cytokine analysis of immunised mouse spleen cells, stimulated with mutFheCL1 failed to produce significant levels of IL-4 or IFN-γ. These results demonstrate that the inactive form of cathepsin L, mutFheCL1 fails to elicit a t-cell stimulatory effect (Fig. 3.20), while the active form, recFheCL1 induces a polarised type 1 response (Fig. 3.19) when employed in conjunction with the appropriate adjuvants.

Antibody analysis of serum obtained from immunised mice with specificity for mutFheCL1, also exhibited a polarised type 1 immune response (Fig. 3.21). Significant levels of IgG2a antibody were detected in animals immunised with (i) CL1 and cpg, (ii) CL1, alum, cpg and heptavac, (iii), CL1, alum and cpg, (iv) CL1, alum and heptavac, (v) CL1, alum and PBS. We also investigated antibody production with specificity for clostridium bacteria. Only mice immunised with (i) CL1, alum and heptavac and (ii) CL1, alum, heptavac and cpg, produced clostridium specific IgG2a. However clostridium-specific antibody production was significantly lower than that of antibody production with specificity for mutFheCL1.

4.1

General Discussion

Discussion

4.1.1: Immune responses to most helminths elicit similar responses, which are characterised by the production of type 2 associated cytokines (IL-4, IL-5, IL-9, IL-10, IL-13), and antibodies (IgG1 in mice, IgG4 in humans and IgE in both species). Once initiated type 1 and type 2 immune responses tend to counter-regulate one another through the action of the cytokines that are specific to each type of response. Several helminthic antigens have been associated with the induction of type 2 responses. Cervi *et al.*, (1999) observed a type 2 response in rats immunised with *F. hepatica*-derived glutathione-S-transferase, while Milbourne and Howell (1997) recorded type 2 responses in rats immunised with ES products of *F. hepatica.* In contrast Abane *et al.*, (2000) observed an antibody profile characteristic of type 1 immune responses in mice immunised with recombinant fatty acid binding protein.

In the current study we compared the immune responses of mice infected with *F. hepatica* to that of mice immunised with *F. hepatica* ES. Results show that ES is capable of mimicking the type 2 immune response observed in Fasciola infection, and as such, may be in part responsible for immunopathology associated with *Fasciola* infection (Milbourne & Howell, 1997). Mice immunised with various *F. hepatica* derived antigens displayed a type 2 response as a result of immunisation with each of the antigens. Cytokine and antibody profiles indicated a predominant type 2 immune response in mice immunised with each of ES, peak (1) and peak (2). The most significant response was observed in mice immunised with ES. ES, peak 1 and peak 2 specific responses were also observed in *Fasciola* infected mice.

ES injected into the peritoneal cavity of BALB/c mice was also observed to stimulate the production of eosinophilia, a cellular response characteristic of helminth infections (Behm & Ovington, 2000). However, an eosinophilic response was not observed in the compartment of the liver in BALB/c mice infected with metacercariae of *F. hepatica.* This difference in eosinophilic responses suggests that mature flukes in the liver may possess an immuno-evasive strategy that inhibits the recruitment of eosinophils which might otherwise be harmful to the parasite. Injected ES products were also observed to have a stimulatory effect on neutrophils, with increased numbers of neutrophils recorded in injected mice. Jefferies *et al.,* (1997), observed stimulation of neutrophil populations in sheep injected with ES.

As both infection with *F. hepatica* and immunisation with *F. hepatica* derived antigens result in the polarisation of the immune responses towards the type 2 subtype, it has been suggested that parasite products act to divert a possible protective type 1 response towards a type 2 phenotype in order to prolong survival within the host.

In the current study we also investigated immune responses to mice infected with *F. hepatica.* Our findings are consistent with previous studies in cattle (Brown *et al.*, 1994; Clery *et al.,* 1996; Mulcahy *et al*, 1999) and mice (O'Neill *et al.,* 2001), in demonstrating a polarised type 2 immune response in animals infected with *F. hepatica.* Our results show a type 2 immune response in both cytokine and antibody profiles of infected mice. Studies in rats by Tliba *et al.,* (2002), observed polarisation of the immune response 14 days post infection, with IL-4 mRNA recorded in the hepatic lymph nodes. In the current study, IL-4 and IFN-γ mRNA levels in mice infected with metacercaria of *F. hepatica* suggest that

the immune response becomes polarised 1 day post infection, and as such fascioliasis may be considered an excellent model for use in the study of type 2 immune responses.

In contrast, immune responses associated with other helminths such as *Schistosoma mansoni* show mixed and elevated type 1 and type 2 associated immune responses at the early stages of infection, before the induction of a polarised type 2 immune response (Baulada-Benedetti *et al.*, 1991). These results suggest that potential vaccines against schistosomiasis should be linked to induction of both type 1 and type 2 associated immune responses. Conversely, as type 2 responses in fascioliasis appear to be linked with pathology, our current studies investigated the induction of a polarised type 1 response, with the aim of providing protection against infection with *F. hepatica.*

The relationship between pathology and immune expulsion in parasitic infections remains controversial. A fundamental obstacle to vaccine development in parasitic infections is the lack of understanding as to what type of immune response should be evoked. It is unclear in many parasitic infections which type of immune response is required by the host in order to achieve protection against an invading parasite. Also, an effective response elicited by a particular parasite may not provide similar protection against another parasitic infection. For example, type 2 responses are generally associated with expulsion of *Trichinella spiralis* (Lawrence *et al.*, 2000), and in infections involving a number of gastrointestinal nematodes, Th2 responses are generally associated with protection, while Th1 responses are associated with susceptibility (Garside *et al.*, 2000). Similarly immune expulsion of gastrointestinal helminths is generally associated with type 2 responses (Lawrence *et al.,* 1998). Girod *et al.*,(2003),

suggested that a localised type 2 response might be important in a protective response against *Neactor americanus.* In contrast, intestinal pathology observed in many other disease models is similar to that observed in helmnith infections, but has been associated with type 1 cytokines. For example, studies by Mulcahy *et al.*, (1998), suggest a protective role for type 1 responses against infection with *F. hepatica.* Also, type 2 responses are not considered effective in expulsion of *Brugia malayi* (Lawrence *et al.,* 1995).

Current methods of controlling helminth diseases employ the use of anti-helminthic drugs. However, issues in relation to the evolution of drug resistant parasites, and the presence of pesticides in food products has motivated research into investigating the possibility of employing molecular vaccines against these parasites (Dalton *et al.*, 2003). The ability to purify mRNA from different parasite life cycle stages and the development of cDNA expression libraries from mRNA has proven essential to the identification of immunogenic parasite proteins, which may be used as vaccine candidates (Knox *et al.,* 2001). Several anti-parasite vaccines have been previously developed, e.g., the recombinant EG95 oncosphere proteins against *Echinococcus granulosis* and the Bm86 vaccine against *Boophilus microplus.* The cysteine proteases, cathepsin L1 and cathepsin L2, which have important roles in host-parasite interactions, have been two of the most promising *F. hepatica* vaccine candidates to date. Dalton *et al.,* (1996), observed levels of protection up to 72.4% protection against infection with *F. hepatica* when these cathepsins were used in conjunction with liver fluke haemoglobin, the resulting immune response being predisposed towards a type 1 immune response. Antibodies associated with type 1 immune responses have also been associated with a degree of protection against infection with *F. hepatica* (Mulcahy *et al.*,

1998). Similarly, Bossaert *et al.*, (2000) suggested that IgG1 production has no significant protective effect against infection with *F. hepatica.* In the current study we investigated the ability of recombinant cathepsin L, combined with various adjuvants to establish a type 1 immune response in BALB/c mice. A polarised type 1 response with specificity for recFheCL1, was observed in five experimental groups. The most significant type 1 response was observed in mice immunised with CL1, Alum, cpg, and heptavac. This study also suggested that the recombinant cathepsin L, and not the inactive mutant cathepsin L is involved in immunostimulation.

Future recommendations

4.2.1: Further investigations related to our current studies should be performed in order to provide a better understanding of the immune responses in individuals infected with *F. hepatica*. (i)While type 2 immune responses were observed in response to ES, peak 1 and peak 2, further purification on these products should be performed to investigate their immunostimulatory properties. SDS PAGE analysis of peak 2 demonstrated several unidentified bands which should be isolated and investigated with reference to their immunostimulatory properties. (ii) Our experiments involving the use of cathepsin L with various adjuvants established a predominant type 1 response. Future investigation should determine whether this response provides protection against *Fasciola* infection. (iii) Immunisation with *Fasciola* antigens attached to agarose beads in order to facilitate the prolonged release of antigen over time, and thus simulate the release of ES products, should provide a useful insight into the effects of *F. hepatica* excretory/secretory products released during infection.

5.1

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