Fasciola hepatica hemoglobin: isolation, characterisation and induction of protective immune response in cattle

Thesis presented for the degree of PhD

by

Sharon Mc Gonigle

under the supervision of

John P. Dalton, PhD.

School of Biological Sciences

Dublin City University

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

Signed: Sharon Mc Gonigle Date: 16/2/'96

Sharon Mc Gonigle

Date: _____

To Brigid & Jimmy Mc Gonigle

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Contents

Abstract

A hemoprotein released *in vitro* by *Fasciola hepatica* was purified by gel filtration chromatography on Sephacryl S200 followed by ion exchange chromatography on DEAE sepharose. Absorption spectra studies characterised the molecule as hemoglobin. N-terminal amino acid sequence analysis revealed no similarity between the *F. hepatica* hemoglobin and other vertebrate or invertebrate hemoglobins. Immunolocalisation studies demonstrate that the hemoglobin is present in the vitelline glands and excretory tubules of mature flukes. The hemoglobin was shown to be highly immunogenic in *F. hepatica* infected bovines.

Hemoglobin was included in a cattle vaccine trial to determine its immunoprophylactic potential. Vaccination with partially purified hemoglobin (Hf) yielded a significant level of protection (43.8%) against challenge infection. Protective immunity was increased to 72.4% when Hf was combined with the liver fluke cysteine protease CL2. Vaccination with Hf and CL2 also resulted in reduced liver damage as assessed by serum GLDH and γGT. Furthermore, eggs recovered from both vaccine groups showed reduced viability. This anti-embryonation effect of vaccination was particularly evident in the group that received Hf / CL2 where >98% of recovered eggs did not embryonate to miracidia. Although both vaccine preparations induced high antibody titres which were boosted following the challenge infection, there was no correlation between antibody titre and protection. The results of these trials demonstrate that Hf and CL2 could form the basis of a molecular vaccine that would not only reduce parasite burden but would also prevent transmission of liver fluke disease.

Using sera from cattle vaccinated with Hf to screen an adult *F. hepatica* cDNA library, genes encoding β tubulin and the novel antioxidant, peroxiredoxin were isolated. The presence of these proteins in the immunising fraction may have contributed towards the induction of the protective response. Peroxiredoxin activity was demonstrated in fluke extracts as the ability to protect glutamine synthetase from oxidative damage by mixed iron thiol oxidation systems. The antioxidant enzyme may play an important role in protecting against oxidative stress in the fluke.

Abbreviations

AhpC	Alkyl Hydroperoxide peroxidase
AhpF	Alkyl hydroperoxide reductase
ADP	Adenine diphosphate
ATP	Adenine triphosphate
BCA	Bicinchoninic acid
BCG	Bacille Calmette-Guerin
BCIP	5-bromo-5-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
cDNA	complementary DNA
CL2	Cathepsin L2
DAB	3,3'-diaminobenzidine hydrochloride
DEAE	Diethylaminoethyl
DEPC	Diethylpyrocarbonate
DTT	Dithiothreitol
E 64	L-trans-epoxysuccinyl-leucylamido-[4-guanidino]-butane
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
EPO	Eosinophil peroxidase
ES	Excretory / Secretory products
g	Acceleration due to gravity
FCA	Freunds complete adjuvant
FIA	Freunds incomplete adjuvant
FITC	Fluorescein isothiocyanate
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulphide
GST	Glutathione S-transferase
HEPES	N-[2-hydroxyethyl] piperazine-N'[2-ethanesulphonic acid]
Hf	Hemoglobin fraction
γ IFN	γ Interferon
IL	Interleukin

IPTG	Isopropyl-β-D-thiogalactopyranoside
LFH	Liver fluke homogenate
MPO	Myeloperoxidase
MSL	Muscle stage larvae
NBL	Newborn larvae
NBT	Nitro blue tetrazolium
NEJ	Newly excysted juvenile
NK	Natural killer
NKEF	Natural killer enhancing factor
PAG	Proliferation associated gene
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulphonylfluoride
PVDF	Polyvinylidine difluoride (Problott)
QAE	Quaternary aminoethyl
ROOH	Hydroperoxide
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TH	T helper cell
β TNF	β Tumour necrosis factor
T-PBS	0.1% Tween 20 in PBS
TPx	Thioredoxin peroxidase
TR	Thioredoxin reductase
Tris	Tris [hydroxymethyl]-aminomethane
TSA	Thiol-specific antioxidant
XGal	5-bromo-4-chloro-3-indolyl-β D-galactopyranoside

Chapter 1

Introduction

General Introduction: life cycle of *Fasciola hepatica*

The parasitic helminth, *Fasciola hepatica,* is the causative agent of fascioliasis, or liver fluke disease. The disease is most common in sheep, cattle and goats, but a wide range of mammalian hosts have been reported, including humans. The WHO estimates that 2 million people have become infected with this fluke due mainly to eating contaminated watercress and other aquatic plants (Maurice, 1994). Recent reports indicate that *F. hepatica* is a major human pathogen in South America and China (Mas-Coma and Barques, 1990), and isolated cases of human infection have also been reported in many European countries (Maurice, 1994). In Ireland fascioliasis occurs mainly in sheep and cattle and is estimated to cost the Irish agriculture industry over 20 million pounds annually, as a result of sheep death, condemnation of sheep and cattle livers, reduction in growth of animals, decreased milk yield from cattle, and poor sheep wool condition. *F. hepatica* and the related *F. gigantica* are the most widespread liver flukes in the world infecting more than 300 million cattle and 250 million sheep worldwide (Maurice, 1994); hence, these parasites cause huge economic losses to agricultural industry.

F. hepatica is a digenetic trematode with a two host life cycle, spending one stage of its life history in an invertebrate host, which is usually *Limnaea truncatula,* a snail common to fresh water or damp pastures. The life cycle, shown in Figure 1.1 is complex, beginning with the release of eggs from adult flukes in the bile duct of the infected mammalian host. Numerous eggs are passed onto pasture in the faeces, where they embryonate to miracidia within 14-17 days, depending on moisture levels and other environmental conditions. The miracidium, which is ciliated and free swimming has a life span of only *ca* 24 hours if it fails to reach its snail host. On finding a snail it attaches by suckers, digests the host epidermis and reaches the digestive gland via the lymph channels.

Within the host digestive gland, the miracidia develops into a second developmental stage called a sporocyst. Inside, the hollow sporocyst germinal cells give rise to a number of embryonic masses each of which develops into a daughter sporocyst or redia. Germinal cells within the redia further develop into a number of larvae called cercariae. These possess a digestive tract, suckers and a tail. A single miracidium can give rise to *ca*

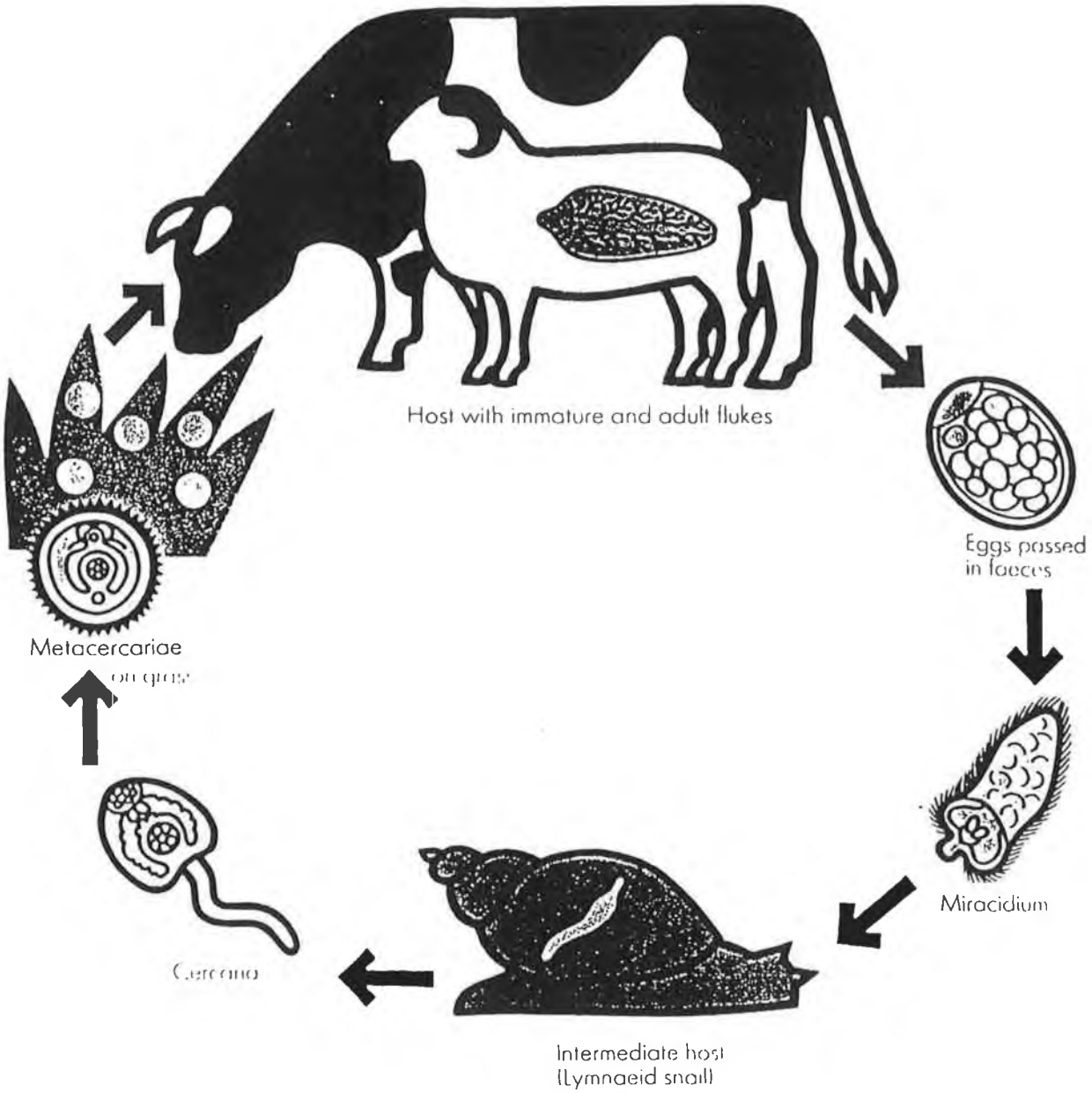

Figure 1.1 *Life cycle of Fasciola hepatica*

600 cercariae. The cercariae begin to emerge from the snail 5-6 weeks following the initial infection. On emergence, the cercaria anchors itself by means of its oral sucker to a suitable substrate such as grass or other vegetation. It loses its tail and secretes a cyst by means of its cystogenic glands. Encysted metacercariae have high resistance to low temperatures but are susceptible to dessication. In water they can remain quiescent for up to twelve months (Grove and Newell, 1979; Smyth, 1994).

Following ingestion of the metacercarial contaminated vegetation by the mammalian host excystment occurs in the small intestine. Excystment of metacercariae is an active process initiated in the stomach by high concentrations of carbon dioxide, reducing conditions, and a temperature of about 39^{o}C. Emergence occurs in the duodenum where bile stimulates active escape from the cyst (Sukhedo and Mettrick, 1986). The newly excysted juvenile fluke (NEJ) burrows through the intestinal wall, reaching the abdominal cavity within 6-8 hours of excystment (Burden *et al*, 1981). The liver is then penetrated and immature flukes spend 7-12 weeks migrating through the tissue causing extensive haemorrhaging and fibrosis before they move to the bile ducts and mature to adults. Mature flukes residing in the bile ducts produce thousands of eggs daily. These are carried to the intestine and leave the body in the faeces, thus beginning the cycle again.

Development of *F. hepatica* in the primary host: morphological, antigenic and biochemical changes

Within the definitive host, the fluke undergoes extensive migration, passing from the intestine to the bile ducts. The different environments encountered by the parasite during its migration necessitate morphological and biochemical adaptations (Barrett, 1981). Bennett and Threadgold, (1973), showed that the basic structure of the adult fluke; the caeca, tegument and parenchyma are present in NEJs, but in each case the fine structure is considerably different from the adult.

The caecum of NEJs contains only secretory cells unlike adult caeca which have both digestive and absorptive cells. NEJ caecum cells contain large secretory granules which are used up in gut wall penetration. Cells

associated with absorptive function do not appear until after *ca* 24 hours of development in the peritoneal cavity. As the juvenile fluke migrates through the liver the caeca continues to develop with the formation of diverticulae from the main caecal limbs and the maturation of cells involved in the secretory and absorptive cycle (Bennett, 1975).

The tegument of the migrating fluke also develops morphologically. The surface of the early migratory stages is smooth in contrast to the highly invaginated surface of mature parasites in the bile duct. The tegument of adult flukes also appears thicker and contains an increased number of mitochondria (Bennett and Threadgold, 1975).

The morphology of tegumental cells also changes during the fluke development. NEJs contain a single type of tegumental cell referred to as T0 cells which contain large T0 granules. These granules are observed in the immature fluke up to 3 weeks following excystment. They move to the syncytium and discharge at the apical membrane allowing continual turnover of the glycocalyx. During migration of the immature fluke through the liver, the T0 cells metamorphose into T1 type cells and embryonic cells in the parenchyma differentiate into T2 cells, both cells secreting their granules (T1 and T2) to the syncytium, where their contents become incorporated into the glycocalyx. The synthesis and migration to the syncytium of T0, T1 and T2 granules appears to be a response to alterations in the parasites environment. All three granules contribute to the glycocalyx but presumably the T0 granule-derived layer is physically, chemically and functionally different from that derived from T1 and T2 granules (Bennett and Threadgold, 1975).

The glycocalyx undoubtedly plays an important role in the protective, absorptive and immunological properties of the tegument, in the developing fluke. In NEJs the glycocalyx functions mainly in protection against hydrolytic enzymes, emulsification and lowering of surface tension. But as the juvenile fluke moves into the peritoneal cavity, immunological protection is vital due to the presence of numerous macrophages and the generous blood supply to the liver. Hanna, (1980), has shown that apart from morphological changes in the tegument, antigenic changes also occur during the flukes development. Exocrine secretion of the

immunologically related T0 and T1 bodies is involved in protecting the juvenile fluke against host responses, by continual glycocalyx turnover which causes sloughing of host antibody. T1 granule proteins elicit a massive antibody response in the early stages of infection, as the fluke migrates through the peritoneum and the liver parenchyma. These juvenile antigens appear to decrease in quantity and significance as the fluke becomes established in the bile duct. Here, the flukes are no longer under continual immunological bombardment and therefore the rate of glycocalyx turnover decreases. T2 antigens, though present in the glycocalyx from 1-2 weeks post infection, do not elicit an antibody response until shortly before entry into the bile ducts. This response declines 5-6 weeks later. T2 antigens may be responsible for maintaining the structural integrity and the nutritive function of the apical membrane of the tegument as the fluke changes from a tissue habitat to the highly surfactant environment of the bile (Hanna, 1980).

The metabolic pathways of *F. hepatica* also undergo substantial alterations as the fluke develops in the final host. As with all helminths studied, the primary source of energy metabolism in *F. hepatica* is carbohydrate breakdown. The parenchyma and muscle cells of the fluke contain large amounts of glycogen. This is rapidly depleted during starvation but readily synthesised as glucose becomes available. Glucose is fermented anaerobically by adult flukes, to propionate and acetate via glycolysis and a fumarate reductose pathway (Prichard, 1989). The anaerobic respiration of adults, necessitated by the low oxygen tension of the bile is in complete contrast to the fully aerobic metabolism of juvenile flukes (Tielens *et al,* 1981; Tielens *et al*, 1982). In the presence of oxygen NEJs metabolise glucose to carbon dioxide via glycolysis in the cytoplasm and Kreb's cycle in the mitochondria.

In early parenchymal stages the juvenile flukes have a fully aerobic metabolism, with the Kreb's cycle accounting for almost 100% of energy production (see Figure 1.2). After twelve days development the contribution of Kreb's cycle to energy production drops to 50%, with the other 50% coming from aerobic acetate production. In the late parenchymal stages, (24 days development), energy metabolism becomes partly anaerobic, with anaerobic dismutation accounting for 20% of total energy production;

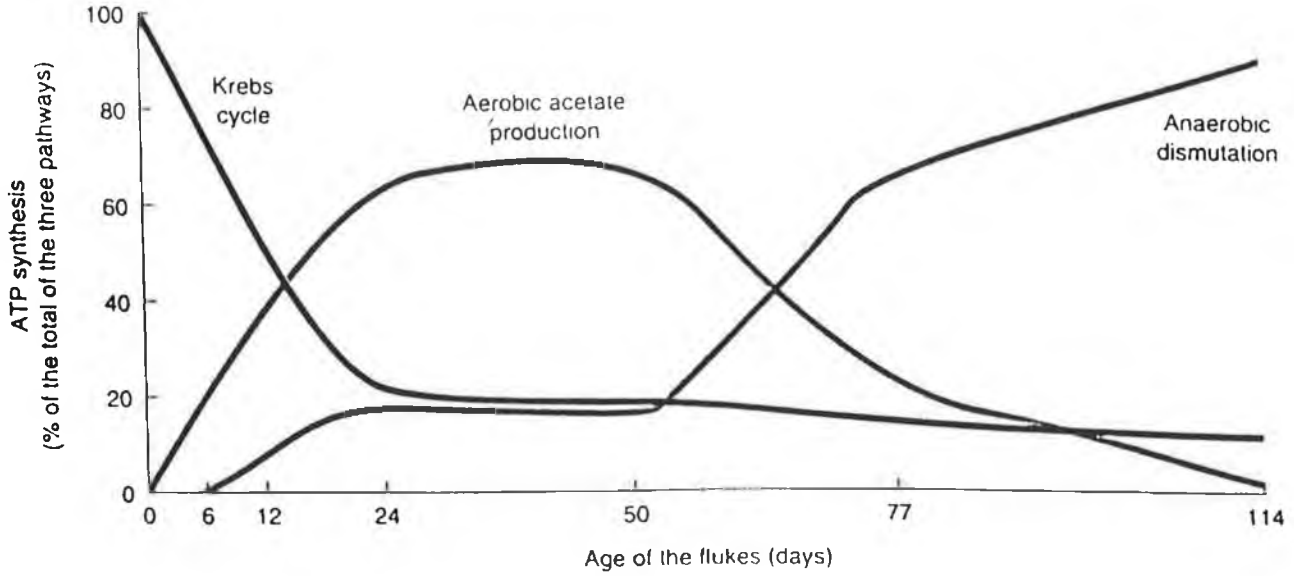

Figure 1.2 *Changes in energy metabolism during the development of F. hepatica in the host*

Kreb's cycle contributing only 17%, and the majority (60%) coming from aerobic acetate glucose breakdown (Figure 1.2). Thus, acetate formation from glucose is probably the most important source of energy for the developing fluke. Shortly after arrival into the bile duct, flukes still possess the capacity for aerobic acetate formation if oxygen is available. However, this capacity gradually decreases and the adult fluke becomes a true anaerobe (Tielens *et al*, 1984). The anaerobic potential of the fluke does not appear to develop as the fluke crosses the liver, rather aerobic capacity diminishes as a result of decreased oxygen availability to the fluke. Tielens *et al,* (1981), showed that although NEJs have an aerobic metabolism, they are fully equipped for anaerobic functioning, and, *in vitro,* they can survive prolonged periods of anaerobisis, where they catabolise glucose to acetate and propionate.

Tielens *et al,* (1984), also showed that Kreb's cycle activity in the developing fluke was directly proportional to its surface area. Thus, growth of the fluke appears to limit oxygen diffusion to its inner layers leading to

decreased cycle activity. The Kreb's cycle activity becomes limited to the outermost layer of the fluke as it develops in the liver. After arrival in the bile duct, the size of the fluke and the very low oxygen content of the bile force the parasite to a permanently anaerobic energy metabolism (Tielens *et al,* 1984).

Invertebrate hemoglobins: structural classification

Respiration of adult flukes in the bile duct is anaerobic, however, oxygen is still required for a number of other metabolic processes. Oxygen transport and storage is therefore vital to the adult flukes survival in the almost anaerobic conditions of the bile duct. Thus oxygen binding proteins of *F. hepatica* play an important role in both the adaption of the developing fluke to its changing environment and the survival of adults in the bile duct. In vertebrates, hemoglobin and myoglobin are the molecules responsible for oxygen transport and storage respectively. Hemoglobin is highly conserved throughout vertebrates and is almost invariably intracellular and tetrameric, with one heme group per subunit. Vertebrate myoglobins found in skeletal muscle cells are also highly conserved; these are monomeric, globular proteins with a molecular mass of 16 kDa, and a single heme group. In contrast, oxygen binding proteins have not been reported in many invertebrate groups and in the remaining groups some classes have one or more of the following; intracellular hemoglobin, extracellular hemoglobin, and copper-containing hemocyanin (Vinogradov, 1985). The next section of this review will focus on invertebrate hemoglobins, in an attempt to illustrate their wide diversity in structure and function. After a general discussion and classification of invertebrate hemoglobins, the hemoglobins of annelids, nematodes and platyhelminthes will be examined in greater detail.

The globin gene is found in a wide variety of species ranging from bacteria and plants to mammals (Trotman *et al,* 1994). Vertebrate globins are very similar in sequence and structure, and their oxygen affinities are all in the order of P_{50} = 0.5 - 25 mm Hg. Invertebrate hemoglobins exhibit great variability in their primary and quaternary structures, and in their oxygen binding properties (De Baere *et al*, 1992). Intracellular invertebrate hemoglobins may be monomeric, dimeric, tetrameric and sometimes

polymeric and are found among the nematodes, molluscs, annelids and echinoderms. Invertebrate extracellular hemoglobins display a much broader variation in molecular size ranging from monomeric molecules comparable in size to vertebrate globins, to highly aggregated molecules comparable in size to intracellular organelles. Vinogradov, (1985), classified the globins into the four following groups;

1. *Monomeric hemoglobins*

These molecules consist of a single polypeptide chain, containing one heme group and having a molecular mass similar to vertebrate myoglobin of *ca* 16 kDa. This hemoglobin has been reported in the tissues of many parasitic platyhelminthes and nematodes, and in the larvae of insects (Vinogradov, 1985)

2. *Two domain, multi subunit hemoglobins*

These hemoglobins range in size from 250-800 kDa and consist of aggregates of dimeric polypeptide chains, each having a molecular mass of 30-40 kDa and containing two heme binding domains. They are found predominantly in crustaceans such as *Caenestheria* and *Daphnia* (Daniel, 1983).

3. *Multi domain, multi subunit hemoglobins*

These consist of two or more polypeptide chains each comprising from 8-20 heme binding domains. The brine shrimp *Artemia* (Geelen *et al*, 1982) and some bivalve and gastropod molluscs (Terwilliger and Terwilliger, 1985) contain this type of hemoglobin molecule.

4. *Single domain, multi subunit hemoglobins*

These are aggregates of several monomeric subunits, some of which are disulphide bonded and some of which do not contain heme. They are usually homo-dodecameric with a highly characteristic two tiered hexagonal bilayer structure at electron microscope level, and are also referred to as erythrocrourins. They are found extensively among the annelids. A well studied example is the hemoglobin of *Lumbricus terrestris* (Vinogradov, 1985).

The structural themes common to invertebrate extracellular hemoglobins are also found among the hemocyanins, which are non-heme extracellular proteins containing copper (I) atoms. The molluscan hemocyanins consist of chains of *ca* 400 kDa, which contain 8 copper

binding sites, and aggregate into large cylindrical structures. The arthropod hemocyanins consist of variable aggregates of polypeptide chains of *ca* 75 kDa, each containing one copper binding site (Van Holde and Miller, 1982; Ellerton *et al*, 1983).

Approximately 140 amino acid sequences of non-vertebrate globins have been accumulated over the past several years, clearly revealing the span of their variability (Vinogradov *et al,* 1992). Sequence similarity to vertebrate globins is present though not extensive. However, investigation of the phylogenetic relationships between invertebrate globins and those of vertebrates has revealed that despite their differences there is compelling evidence for their common origin (Goodman *et al,* 1988). The crystal structures of several invertebrate hemoglobins have been determined. The inter-subunit interactions in the dimeric and tertrameric molecules differ from those observed in vertebrates, however, the secondary structure of all the globins retain the characteristic vertebrate myoglobin folding around the heme group, again suggesting a common ancestor (Vinogradov *et al*, 1993). The broad range of related globin like sequences probably reflects a deviation from the familiar oxygen storage and transport functions of vertebrate hemoglobins. Various functions have been proposed for many invertebrate hemoglobins, however, most remain as yet unelucidated. More detailed sequence and structural data is required before the complex architecture of invertebrate hemoglobins can be related to their functional properties (Blaxter 1993; Vinogradov, 1985).

Annelid Hemoglobins

Annelid extracellular hemoglobins are giant molecules which possess sediment coefficients of 60 S, a characteristic two tiered, hexagonal electron microscope appearance, and oxygen binding properties that vary widely with respect to affinity, cooperativity and pH dependency (Vinogradov *et al*, 1980). They are found extensively among all three classes of annelids; the polychaetes, the oligochaetes and the leeches (Vinogradov, 1985). The molecules consist of aggregates of several smaller subunits, not all of which contain heme (Vinogradov *et al*, 1986). Annelid hemoglobins are believed to have adapted to their extracellular environment in the circulating fluid, by evolving into these high molecular

weight aggregates. The adoption of a very high molecular weight is thought to minimise loss by excretion (Trotman *et al*, 1994).

The best studied annelid hemoglobin is that of the oligochaete, *Lumbricus terrestris*, the common North American earthworm. *Lumbricus* hemoglobin has a molecular mass of 3600-3800 kDa (Vinogradov and Kolodziej, 1988). It consists of four different globins a-d, whose primary structures have been determined (Shishikura *et al*, 1987; Fushitani *et al*, 1988).The hemoglobin also contains three linker polypeptide chains which lack heme. Examination of these linker chains reveals that they are similar to one another, but are either unrelated or very distantly related to the heme binding chains (Suzuki and Riggs, 1993). The quaternary structure consists of twelve dodecamers, held together by linker chains, accounting for two thirds and one third of the total mass respectively (Vinogradov *et al*, 1991). Studies on the oxygen binding characteristics of the molecule show that, unlike other invertebrate globins, oxygen affinity is modulated by cation binding (Fushitani *et al*, 1986).

Ebina *et al*, (1995), have recently reported the importance of carbohydrate in maintaining the architecture of the giant extracellular hemoglobins. The extracellular hemoglobin of *Perinereis aibuhitensis,* (a marine worm), was shown to be about 0.5% (mass) carbohydrate. The linker chains contain carbohydrates with both N-acetylglucosamine and N-acetylgalactosamine. Two of the four globin chains contain only N-acetyl glucosamine carbohydrates; the other globin chains have no carbohydrate moieties. Addition of monosaccharides to the hemoglobin reversibly dissociates the molecule. However, removal of attached carbohydrate with N-glycanase results in irreversible dissociation. The carbohydrate appears to act non-covalently to glue the components together, yielding the complete quaternary supramolecular structure of the giant hemoglobin. This carbohydrate gluing is thought to be mediated by lectin-like carbohydrate binding of the linker chains, which were shown to share significant sequence similarity with the lectin wheat germ agglutinin (Ebina *et al*, 1995).

The functions of annelid hemoglobins are not well understood. They are generally the most abundant proteins in the coelomic fluid and thus,

may be expected to possess functions other than the transport of oxygen. Among the possible roles for the non-heme carrying polypeptide chains the most plausible are; a structural and / or regulatory function, and a role as a met-hemoglobin reductase (Vinogradov *et al*, 1980).

Nematode Hemoglobins

Nematode hemoglobins, also known as "nemoglobins", are a distinct branch of the globin family, only distantly related to the globins of other invertebrates and their hosts. Information on nematode globins is sketchy, however, their distribution throughout the species suggests that all nematodes may have them (Blaxter, 1993). A wide range of quaternary structures are found within the nemoglobin family, and many nematodes contain more than one type of hemoglobin. The ascarids contain a monomeric globin usually located in the body wall and pharyngeal muscle and an extracellular pseudocoelomic fluid hemoglobin, which is octameric. The strongylids also contain two iso-forms; the monomeric body wall globin and a tetrameric globin located in the cuticle (Blaxter, 1993).

Among the nemoglobins, the extracellular hemoglobin from the perienteric fluid of *Ascaris suum,* an intestinal parasite of the pig, has been the most extensively studied. Interest in this hemoglobin was aroused by its exceptionally high oxygen binding affinity, owing to a decreased oxygen dissociation rate. *Ascaris* perienteric hemoglobin binds oxygen 25,000 times more tightly than that its mammalian homologue. The partial pressure of oxygen at which the hemoglobin is saturated is about 0.001 mm Hg for *Ascaris* hemoglobin, and 25 mm Hg for human hemoglobin (Gibson and Smith, 1965). Studies have established that this hemoglobin is an octamer (molecular mass 350 kDa) of eight identical subunits. The 37 kDa molecular mass subunits consist of two tandem domains, sharing a high sequence similarity and each containing a heme binding site, followed by a 22 amino acid C-terminal extension. The latter region, consisting of several repeats of the motif H K E E may be responsible for the octamer formation via tail to tail salt bridging (ie. polar zipper) (De Baere *et al*, 1992).

An extracellular hemoglobin from another nematode, *Pseudoterranova decipiens,* which parasitises the grey seal in the adult

stage and the Atlantic cod in the larval stage, has also been studied. This globin shares substantial sequence similarity with the *Ascaris* hemoglobin, and again, an exceptionally high oxygen affinity two orders of magnitude greater than that of any vertebrate hemoglobin was observed (Dixon *et al,* 1991).

Oxygen dissociation from these extracellular globins requires several minutes, thousands of times longer than that required for vertebrate hemoglobins. The oxygen reaction is highly specific as the dissociation rates for carbon monoxide and nitrous oxide fall within normal limits (Lee and Smith, 1965). Elucidation of the primary structure by protein sequencing (De Baere *et al*, 1992) and by gene cloning (Sherman *et al*, 1992a) did not reveal the molecular interactions responsible for the extreme oxygen avidity. The individual globin domains were expressed in *E. coli* (Kloek *et al*, 1993). Domain 1 was found to exist as a monomer, while domain 2 with its C-terminal extension was capable of forming octamers. Both recombinant domains retained oxygen avidity comparable to that of the native molecule, suggesting that oxygen affinity was probably derived from heme pocket composition and did not involve oligomer formation or intramolecular heme interactions (Kloek *et al,* 1993).

Ascaris globin domains share 15% identity with vertebrate hemoglobins, and almost all the invariant residues throughout the globin phylogeny have been conserved (De Baere *et al*, 1992). A notable difference is the replacement of the E7 (globin sequences are divided into eight conserved helices, A to H, and residues are numbered by their position within the helix) distal histidine of vertebrate hemoglobins with a glutamine in the *Ascaris* hemoglobin domains. The distal histidine is a well studied residue known to stabilise the heme-bound oxygen through a hydrogen bond (Nagai *et al,* 1987). Recent studies on sperm whale myoglobin have brought attention to the B10 position as another residue important in oxygen binding affinity (Carver *et al*, 1992).In vertebrates leucine at position B10 is highly conserved, and when mutated to phenylalanine in sperm whale myoglobin, the oxygen dissociation rate slowed 10 fold (Carver *et al*, 1992). In both *Ascaris* domains the B10 residue is tyrosine. Evidence for the importance of these two residues in *Ascaris* hemoglobin comes from site directed mutagenesis studies of

recombinant domain 1 (De Baere *et al*, 1994, Kloek *et al*, 1994). Replacement of tyrosine at B10 in *Ascaris* with phenylalanine or leucine increases the dissociation rate about 200 fold and 600 fold respectively, whereas replacement of *Ascaris* glutamine at E7 with leucine or alanine increases the rate 5 fold and 60 fold respectively. Kloek *et al*, (1994) and De Baere *et al*, (1994), proposed that the increased oxygen affinity of the *Ascaris* heme pocket results from the formation of two hydrogen bonds with the liganded oxygen molecule; one at tyrosine B10 and one at glutamine E7. This was confirmed by solving the crystal structure of recombinant domain 1 (Yang *et al*, 1995). Resolution at 2.2 Å revealed a strong hydrogen bond between tyrosine at B10 and the distal oxygen of the ligand, combined with a weak hydrogen bond between glutamine at E7 and the proximal oxygen, which grip the ligand in the binding pocket. A third hydrogen bond between these two amino acids appears to stabilise the structure. The B helix of domain 1 is displaced laterally when compared with sperm whale myoglobin, shifting the tyrosine B10 hydroxyl far enough from the liganded oxygen to form a strong hydrogen bond without steric hindrance. Changes in the F helix contribute to a tilted heme group which may also be important for oxygen affinity. Studies on mutants chosen on the basis of this structure are now required to further explain the remarkable properties of these hemoglobins.

The functional role of nematode pseudocoelomic hemoglobins has been questioned ever since their high oxygen affinity was discovered. The avidity with which the globin hangs onto its bound oxygen would appear to preclude a role in oxygen transport, as the nematode would have to be experiencing severe oxygen depletion to bring the globin anywhere near letting go. Various possible functions have been proposed for these proteins, which are abundant in the perienteric fluid (Blaxter, 1993);

1. They may be simple osmoregulatory components of the perienteric fluid acting as non-diffusable ions to maintain the integrity of the tissues.
2. They may act as oxygen sinks to protect the essentially anaerobic metabolic activity of the nematode from free oxygen.
3. Another possibility is that globins supply heme rather than oxygen for metabolic functions. Each female *Ascaris* worm produces 0.5 g of eggs per day. Each egg contains a small amount of hematin, which may be supplied

by the extracellular hemoglobin.

4. Hemoglobin, like all heme compounds catalyses the decomposition of hydrogen peroxide. In *A. lumbricoides*, hydrogen peroxide is formed during respiration, but this parasite appears deficient in catalase. The pseudoperoxidase activity of its extracellular hemoglobin may prevent toxic levels of hydrogen peroxide accumulating (Barrett, 1981).
5. Nematodes in the intestines have to cope with high environmental carbon dioxide levels and consequent tissue acidification. These parasites also excrete large amounts of organic acids. Hemoglobins may have a buffering role to play, thus minimising pH changes (Barrett, 1981).
6. The globin may act as an enzyme, an enzyme co-factor or a carrier protein. In both yeast and bacteria, globins are found as part of a two domain hybrid where the second domain is enzymically active, for example, globins are found as part of flavoreductase in *S. cerevisiae,* where the aerobic enzyme reaction derives its oxygen from the globin directly rather than by diffusion (Zhu and Riggs, 1992).
7. Sherman *et al,* (1992b) proposed a role in sterol metabolism. The sterol precursor squalene was co-purified with the extracellular hemoglobin. The globin was able to epoxidate the squalene in a reaction linked to cytochrome reduction. This first step in sterol synthesis would have great significance for the nematode, since sterols are essential components of membranes, found in abundance in *Ascaris* eggs.

Although these and other alternative functions have been advanced, the problem of relating function to the high oxygen affinity of extracellular nemoglobins remains.

Ascaris also contains a monomeric hemoglobin which is distinct from extracellular globin in several ways. It is localised in cellular compartments belonging to the hypodermis, the dorsal, ventral and lateral cords, the nerve ring and body wall muscle. It is found as single domain subunits of 17 kDa molecular mass, which form dimers of 35 - 37 kDa native molecular mass. Similar molecules have been cloned from the strongylid nematode *Trichostrongylus colubriformis* (Frenkel *et al*, 1992) and the rhabitid *Caenorhabditis elegans* (Mansell *et al*, 1993). The *Ascaris* hemoglobin shares sequence similarity with these nemoglobins and with other invertebrate hemoglobins. Body wall hemoglobins have high oxygen

affinity, though 10 fold lower than their perienteric fluid counterparts. This means that the extracellular hemoglobin cannot deliver oxygen to the body wall hemoglobin, therefore, oxygen transport by facilitated diffusion does not occur. The body wall hemoglobin is thought to function in oxygen supply for muscle activity, as deoxygenation of live worms *in vivo* results in cessation of movement (Blaxter *et al*, 1994). A similar role has been demonstrated for the pharyngeal muscle globin of the marine free living nematode *Enopulus brevis*, which inhabits relatively anoxic tidal muds. Deoxygenation of this globin results in the cessation of pharyngeal pumping (Atkinson, 1975).

A unique function has been proposed for the nemoglobin of the adenophorean nematode, *Mermis nigrescens*. These are positively phototrophic and have an anterior hypodermal colouration. The abundant red pigment has been shown spectrophotometrically to be hemoglobin. The globin is thought to constitute a shading chromophore for the nematode's light sensing organs (Burr and Harosi, 1985).

Overall, studies of nemoglobins are providing information on the evolution of nematodes, especially in relation to the co-evolution of parasitic species with their hosts. They are also a group of molecules for which there are solid models of function, against which adaptations to parasitism can be measured.

Platyhelminth Hemoglobins

Platyhelminth hemoglobins are not as well studied as those of nematodes. Within the phylum there are some 25,000 species divided among three classes; Turbellaria, Cestoda and Trematoda. Hemoglobin has not been reported in the cestodes but is found in some enterosymbiotic turbellarians (Jennings and Cannon, 1985, 1987) and appears to be widespread among the parasitic trematodes. Studies on trematode hemoglobins are few and fragmentary. Several reports have demonstrated that the trematode hemoglobins are electrophoretically and spectrophotometrically distinct from their hosts (Lutz and Siddiqi, 1967; Cain, 1969; Haider and Siddiqi, 1976). However, relatively little is known about the physical and chemical properties of trematode hemoglobins in general.

The hemoglobin of *Dicrocoelium dendriticum,* a lanceolate fluke which infests the hepatic ducts of certain mammals, is the best studied trematode hemoglobin. It is a monomeric globin with an estimated molecular mass of 18-20 kDa, and a high oxygen binding affinity (Tuchschmid *et al*, 1978). The oxygen affinity, although an order of magnitude lower than that reported for *Ascaris* extracellular hemoglobin, is the highest reported for a monomeric hemoglobin, showing no cooperativity (Smit *et al*, 1986). Its primary structure which has largely been determined, shows low similarity with other globins (De Baere *et al*, 1992). Amino acid differences occur at residues B10 and E7 in the heme cavity. The importance of these residues in high oxygen affinity has recently been demonstrated in *Ascaris* globin (De Baere *et al*, 1994; Kloek *et al*, 1994). The B10 residue, which is leucine in vertebrate globins, is tyrosine in both the *Dicrocoelium* and *Ascaris* globins (Smit and Winterhalter, 1981). The E7 residue, which is leucine in vertebrate globins and glutamine in *Ascaris* hemoglobin, was shown by nuclear magnetic resonance studies to be tyrosine in the *D. dendriticum* hemoglobin (Lecomte *et al*, 1989). Further studies on the molecular interactions occurring within the heme cavity are required to determine whether the ionic interactions observed in the *Ascaris* globin are also responsible for the high oxygen affinity of the *Dicrocoelium* globin. The *Dicrocoelium* hemoglobin appears well adapted to the low oxygen environment of the bile duct, where it resides as an adult. However, since the parasites metabolism depends essentially on anaerobic glucose oxidation, the presence of hemoglobin in *D. dendriticum* suggests that oxygen is required for metabolic pathways other than energy providing routes.

An unusual property of the *Dicrocoelium* hemoglobin is its marked acid Bohr effect (Smit *et al*, 1986). This is the effect protons have on oxygen affinity. Vertebrate hemoglobins show alkaline Bohr effects; their oxygen affinity increases with increasing pH. Thus, when carbon dioxide reacts with water and carbonic acid is formed, the hemoglobin is induced to give up its oxygen easily. With *Dicrocoelium* hemoglobin the oxygen affinity is decreased 10 fold at alkaline pH. The biological significance of this inverse Bohr shift is as yet unknown

The most recent trematode hemoglobin studies have focused on the

hemoglobins of *Gastrothylax crumenifer* and *Paramphistomum epiclitum*, which parasitise the rumen of water buffalo, and *Isoparorchis hypselobagri* which parasitise catfish (Siddiqui and Siddiqi, 1987; Haque *et al*, 1992; Rashid *et al*, 1993). The *P. epiclitum* hemoglobin is a monomer of 16 kDa molecular mass (Haque *et al*, 1992). Monomeric (15 kDa molecular mass) and dimeric (30 kDa molecular mass) forms of globin have been observed in *G. crumenifer* (Haque *et al*, 1992) and *I. hypselobagri* contains three monomeric chains of molecular mass, 15-17 kDa. N-terminal sequencing of the *P. epiclitum* globin and the three globins of *I. hypselobagri* revealed no similarities with vertebrate or invertebrate hemoglobins.

The functions of trematode hemoglobins are largely undetermined (Lee and Smith, 1965). However, it is believed that their functions are inextricably linked with their location, and there may be specific adaptions of the oxygen supplying role in these cases. Many of the functions advanced for nemoglobins have also been proposed for the globins of trematodes, which like the nematode globins, possess oxygen affinities higher than those found in vertebrate globins.

An inherent property of hemoglobins and all hemoproteins is their ability to catalyse hydrogen peroxide breakdown. Hydrogen peroxide is a reactive oxygen species (ROS), with the potential for hydroxyl radical production, and as such, may contribute to oxidative stress. Thus, by effecting its removal, hemoglobin can be considered as an antioxidant. However, the *in vivo* role of hemoglobin as an antioxidant in parasites has not been elucidated.

Oxidative Damage and Antioxidants

Antioxidants in parasites may have two roles to play;
(a) protecting the parasite against its own reactive oxygen species, generated by metabolic processes involving oxygen
(b) protecting itself against oxygen radicals released by host immune effector cells in an attempt to destroy the parasite.
All parasitic helminths are believed to contain at least one of the following major antioxidant enzymes; superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx). The remainder of this discussion will focus

on the antioxidants of parasites, beginning with a general introduction to oxidation and antioxidants and with particular emphasis on the three aforementioned antioxidant enzymes and the ancillary enzyme, glutathione S-transferase (GST).

Oxidative reactions and antioxidants will be discussed in relation to mammalian systems which are well studied; it is believed that many of these processes are inherent to all organisms, including the invertebrates. For the past 2 x 10^9 years oxygen has served as the major electron sink in biological systems. The electronic structure of molecular oxygen permits the uptake of electrons to form a number of intermediate, partially reduced oxygen molecules, collectively termed reactive oxygen species (ROS). Reactive oxygen species include; hydrogen peroxide (H_2O_2), superoxide (O_2^-), the hydroxyl radical ($HO^\bullet$), the alkoxyl ($RO^\bullet$) and peroxyl ($ROO^\bullet$) radicals, singlet oxygen ($^1O_2$) and nitric oxide ($NO^\bullet$) (Riley, 1994).

The generation of reactive oxygen species is widespread in biological materials, even under basal conditions. They are the cytotoxic by-products of biological processes, including mitochondrial energy metabolism, respiration, prostaglandin synthesis, phagocytosis and detoxification. Hydrogen peroxide and the superoxide anion are products of normal aerobic metabolism. In addition, superoxide leaks from the electron transport chains of mitochondria and endoplasmic reticulum. Oxygen radicals are also formed in metal catalysed substrate oxidations and in the reduction of hydroperoxides by transition metals, such as, iron and copper. There is a continual requirement for inactivation of these reactive oxygen species by antioxidants. Disruption of the balance between ROS formation and removal i.e. "oxidative stress" can lead to damage to all types of biological molecules.

Although ROS do have beneficial actions, their presence is a source of oxidative stress, and much of the interest in them is related to their potential to cause cellular damage. Oxidative damage caused by ROS is inevitable in an oxygen environment and life only becomes possible by strategically placed antioxidants and the continual repair and replacement of damaged molecules. The most reactive and therefore potentially hazardous oxygen-

derived radical is the hydroxyl radical ($HO^{\bullet}$), which has a half life of 10^{-9} s. Its reactions are diffusion limited, which means they occur practically at the site of generation (Sies, 1993). In contrast, superoxide and hydrogen peroxide are relatively stable and their significance from the point of view of cellular damage is essentially connected with their potential ultimately to give rise to hydroxyl radicals as shown in the following reactions;

$$H_2O_2 + e^- \longrightarrow HO^{\bullet} + HO^-$$ single electron H_2O_2 reduction

$$H_2O_2 + O_2^{\cdot-} \longrightarrow HO^{\bullet} + HO^- + {}^1O_2$$ Haber Weiss reaction

Since superoxide is comparatively stable and unreactive, hydrogen peroxide is now thought to be the main source of initiating free radicals. The relative stability of some ROS means that these molecules can diffuse away from their site of generation and thus transport the radical or oxidant function to other target sites (Riley, 1994; Sies, 1993).

The formation of hydroxyl radicals from hydrogen peroxide and other hydroperoxides (ROOH) is greatly accelerated in the presence of suitable transition metals via the Fenton reaction. In the co-presence of a suitable reducing species to reconvert the Fe^{3+} to Fe^{2+} the production of hydroxyl radicals from hydrogen peroxide can proceed catalytically. A number of reducing species may take part in this reducing cycle, including ascorbate, thiols and superoxide;

$$O_2^{\cdot-} + Fe^{3+} \longrightarrow Fe^{2+} + O_2$$

$$H_2O_2 + Fe^{2+} \longrightarrow HO^{\bullet} + HO^- + Fe^{3+}$$ Fenton Reaction

$$H_2O_2 + O_2^{\cdot-} \xrightarrow{Fe^{2+} / Fe^{3+}} HO^{\bullet} + HO^- + O_2$$ Total Reaction

This radical generation process can also be catalysed by UV light and other chemical inducing agents, such as reducing agents.

Most complex organic molecules are susceptible to damage by reactive species such as the hydroxyl radical. One of the most studied of

these reactions is the peroxidation of lipid components of cell and organelle membranes. Peroxidation alters the membrane structure, causing increased permeability, and interferes with membrane function. Once started, peroxidation is amplified by chain branching reactions, thereby broadening cellular effects. The initial phase of lipid peroxidation is the abstraction of a hydrogen atom from an unsaturated lipid, forming an alkyl radical ($R^{\bullet}$).

$$RH + OH^{\bullet} \longrightarrow R^{\bullet} + H_2O$$ Initiation Reaction

Oxygen uptake rapidly follows, with the formation of the peroxyl radical ($ROO^{\bullet}$). This can then abstract a hydrogen atom from another unsaturated lipid, forming stable lipid hydroperoxide (ROOH). However, the reaction is maintained and propagated by the formation of another lipid radical $R^{\bullet}$.

$$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet}$$ Propagation Reactions

$$ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet}$$

The hydroperoxides formed may be reduced by glutathione peroxidase; if not they can give rise to alkoxyl and peroxyl radicals in the presence of iron and copper complexes.

$$2\ ROOH \xrightarrow{\text{Fe or Cu complexes}} ROO^{\bullet} + RO^{\bullet} + H_2O$$

Peroxyl and alkoxyl radicals, if not appropriately reduced, can inflict further peroxidative damage on membrane lipids and on a range of other macromolecules, including proteins and nucleic acids. The reaction of lipid peroxides with iron and copper complexes also results in the formation of a wide range of reactive carbonyls, including aldehydes, which can attack thiol and amino groups on proteins, causing inhibition of enzymes and crosslinking of membrane proteins. Other low molecular mass products of lipid peroxidation inhibit protein synthesis and exert chemotactic actions on neutrophils (Halliwell and Gutteridge, 1989).

Apart from lipid peroxidation, hydroxyl radicals produce a number of other adverse biological reactions by attacking structural and functional

molecules. Oxidative attack on proteins may significantly alter their properties and functions; damage to protein thiols causes impaired cell calcium homeostasis and alterations of enzymic, carrier or receptor functions may also have important consequences (Orrenius *et al*, 1985). Oxygen radicals have been shown to fragment certain structural proteins, increasing their susceptibility to enzymatic hydrolysis (Wolff and Dean, 1986). Oxidative damage to DNA binding proteins may result in profound alterations in gene expression, leading to changes in levels of certain oxidative-stress related proteins, which may have protective functions (Riley, 1994), or to the initiation of apoptosis (Carson and Ribeiro, 1993). DNA damage by hydroxyl and other oxygen radicals may lead to either mutational lesions or major chromosome derangement, which may be cytotoxic to proliferating cells (Imlay *et al*, 1988; Imlay and Linn, 1988).

Effects of Host Oxidants on Parasites

In parasites, as in mammalian cells, ROS are produced as a by-product of cell metabolism and from the metabolism of certain pharmacological agents. Many anti-parasitic drugs achieve killing through the production of oxygen radicals. The sensitivity of parasites to these radicals depends on their antioxidant defences (Docampo and Moreno, 1986). Another source of radicals in parasitic helminths is the effector arm of the host immune system. The host response is a potent source of oxidants, which can result in damage sufficient to kill cells and even whole organisms. Here again, the ability of a parasite to survive in its host has been directly related to its content of antioxidant enzymes (Batra *et al*, 1992). A brief discussion follows on the production of ROS by host immune cells and the effects these radicals have on parasites. The various responses of host cells to *Schistosoma mansoni* infection and the host self cure phenomenon in infections with the nematode *Nippostrongylus brasiliensis* are given as examples to illustrate the widespread use of oxygen radicals in defence against parasites.

Multicellular parasites present a unique challenge to mammalian host defence mechanisms because of their size and complex structure. Host phagocytes appear to be the most important effectors of parasite killing. Eosinophilia occurs in most helminth infections; in human helminthiases,

more than 50% of circulating white blood cells may be eosinophil granulocytes, in contrast to the normal 2-5% (Maizels *et al*, 1993). They have been shown to kill parasites *in vitro* either by secreting toxic proteins or by free radical-mediated mechanisms, usually in conjunction with antibody or complement (Callahan *et al,* 1988). All leucocytes, especially neutrophils, eosinophils and macrophages, when exposed to appropriate stimuli, or during phagocytosis become activated and undergo profound metabolic changes, including a dramatic increase in oxygen consumption and hexose monophosphate shunt activity. This "respiratory burst" results in the production of superoxide catalysed by NADPH oxidase. Other ROS including hydrogen peroxide, the hydroxyl radical and singlet oxygen are produced in subsequent reactions. Organisms vary in their ability to induce a respiratory burst and this may be one of the factors influencing their survival. The toxicity of the hydrogen peroxide produced may be increased many fold by peroxidases found in high concentrations in some phagocytes. The enzymes myeloperoxidase (MPO) and eosinophil peroxidase (EPO), produced by neutrophils and eosinophils respectively, bond ionically to the negatively charged surface of helminths and catalyse the conversion of hydrogen peroxide and halides to hypohalous acids, which can be converted into more damaging and stable molecules (Callahan *et al*, 1988).

The schistosomula of *Schistosoma mansoni* are killed by this EPO / MPO-hydrogen peroxide-halide system. EPO binds to the surface of the schistosome, and is toxic to the organism in the presence of hydrogen peroxide and halides. Further, schistosomula are more susceptible to neutrophil damage in the presence of specific antibody and complement, when coated with EPO (Jong *at al*, 1984). Neutrophil mediated destruction of schistosomula is inhibited by adding catalase- or hemoprotein-inhibitors, again, implicating the peroxidase system as the mode of killing (Kazura *et al*, 1981). However, it is believed that the toxic effect of neutrophils can only be mediated following the initial damage inflicted by eosinophils (Jong *et al*, 1984).

Another pathway has been implicated in the killing of larval and schistosomula stages of *S. mansoni*. The activated macrophage-mediated pathway is thought to be effected through the action of nitrogen

intermediates, which are produced independently of the respiratory burst (James and Glavern, 1989). Macrophage and endothelial cells, following stimulation with cytokines, especially g interferon, become activated to produce nitric oxide, which is toxic to schistosomula (Wynn *et al*, 1994). This production of nitric oxide is iron and arginine dependent. Incubation of schistosomula with activated macrophages results in the formation of lesions in the mitochondria, damage to DNA replication enzymes and inhibition of NADH-ubiquinone oxidoreductase and succinate-ubiquinone oxidoreductase, which are important respiratory enzymes, as a result of iron loss. Schistosomula have been shown to be dependent on aerobic respiration for at least 24 hours following transformation from cercariae, therefore this damage to the respiratory function appears to effect killing of the fluke (James and Hibbs, 1990). Damage was prevented by the addition of excess iron, which presumably stabilised the iron containing enzymes. (James and Glavern, 1989). The local production of nitric oxide by activated macrophages may be directly toxic to the schistosomula and the resulting inflammation in the host may impede the parasites migration, effectively increasing nitric oxide exposure (Wynn *et al*, 1994). To date there have been no reports of any specific antioxidant defence against this effector molecule (James, 1993). Nitric oxide may also interact with superoxide, leading to the generation of the hydroxyl radical, therefore SOD may have some role to play in the prevention of nitric oxide mediated toxicity (Brophy and Pritchard, 1992)

Kazura *et al*, (1985) showed that reactive oxygen species, especially superoxide can damage schistosomal eggs. These oxidants generated through the respiratory burst of the host leucocytes, may also play a role in the formation of *S. mansoni* granulomas. Macrophages from the acute phase of granulomas produce ten times more superoxide as those from non-egg induced granulomas. This superoxide interferes with the eggs metabolic activity, specifically the TCA cycle, and with hatching. The administration of superoxide dismutase (SOD) inhibits egg destruction, however, the eggs contain only moderate amounts of endogenous SOD (Kazura *et al*, 1985).

The related nematodes *Nippostrongylus brasiliensis* and *Nematospiroides dubius* are both intestinal parasites of rodents. Infections

with *N. brasiliensis* fail to establish; adult worms being expelled from the host within 10-12 days, whereas, adult *N. dubius* persist for several months. The rapid expulsion is due to host ROS production. *N. dubius* contains twice as much superoxide dismutase and 3-4 times as much catalase as *N. brasiliensis*, explaining its stronger resistance to the reactive oxidants and why its expulsion by the host is much slower (Smith and Bryant, 1986)

Infections of *N. dubius* fail to establish in the small intestine of rats, in contrast to long lasting infections which occur in mice. Comparison of rat and mouse leucocytes revealed that the rat cells produce large amounts of ROS in response to *N. dubius* infection, as opposed to the relatively low levels produced in mice, leading to the more rapid expulsion of the nematode from rats (Smith and Bryant, 1986). Spontaneous nematode expulsion is thought to involve T-lymphocytes, and intestinal levels of MPO are elevated at the time of expulsion in both *N. brasiliensis* (Mayberry *et al*, 1986) and *Trichinella spiralis* (Smith and Castro,1978).

Parasites are not always killed by host responses and killing may be the exception rather than the rule. Different parasite species and even different stages are susceptible to these radicals to differing extents, which could reflect the parasites basal level of antioxidant enzymes as well as an ability to secrete and / or boost production of antioxidant enzymes following stimulation (Callahan *et al*, 1988).

Protection against Oxidative Damage

Because of the potential damage that can be engendered by ROS through their ability to generate hydroxyl radicals, a number of cellular antioxidant defences have evolved. A first line of defence against ROS is protection against their formation i.e. prevention.

1. The packaging of DNA in chromatin, thus, shielding the genetic material.
2. Sensitive material may be compartmentalised away from sites of ROS generation. For example, the transfer of mitochondrial DNA to the nuclear genome away from the mitochondria, where ROS levels may reach concentrations high enough to cause significant DNA damage.
3. The prevention of initiation of chain reactions by metal chelation is a major means of controlling lipid peroxidation and DNA fragmentation.

Thus, the metal binding proteins ferritin, transferrin, ceruloplasmin and lactoferrin are of central importance in the control of potential radical generating reactions. Also, the binding of iron in hemoglobin is a simple and most efficient way of removing excess iron.

4. Prevention of oxidation due to incident radiation may occur through specialised pigments, such as melanins for UV radiation and carotenoids for electronically excited states such as singlet oxygen.

5. Many enzymic systems control levels of reactive species which might otherwise generate products leading to ROS. One important group of these enzymes is the glutathione S-transferases (GST), which catalyse reactions with glutathione and reactive electrophillic intermediates, formed in a variety of metabolic pathways, giving the more stable thioethers, or S-conjugates (Sies and Ketterer, 1988). The GSTs of parasites will be discussed in greater depth towards the end of this review.

Radicals, once formed, may be intercepted by antioxidants, preventing further damage. These chain breaking antioxidants are often phenolic compounds. The main reductant is α tocopherol, the most active form of Vitamin E, which is optimally positioned for peroxyl radical removal in the lipid membrane. Its antioxidation reaction yields α tocopherol radical;

$$ROO^{\bullet} + EOH \longrightarrow ROOH + EO^{\bullet}$$

The tocopherol radical is subsequently regenerated by external reductants including ascorbic acid and thiols (Wefers and Sies, 1988). The synergism involved in regeneration means that tocopherol is a highly efficient antioxidant, with only 1-3 antioxidant molecules required per 1000 target radicals (Sies, 1993).

ROS may also be intercepted by the antioxidant enzymes; catalase, superoxide dismutase and glutathione peroxidase. These enzymes, which will be discussed in detail later, appear to act in concert in vertebrate cells, to bring about the elimination of toxic oxygen intermediates (Halliwell and Gutteridge, 1989). In addition, there are several specialised antioxidant enzymes which act indirectly, for example, the replenishment of glutathione (GSH) from glutathione disulphide (GSSG), by the flavoprotein glutathione reductase (Sies, 1993).

Protection from the effects of oxidants can also occur by repair of damage once it has occurred. As prevention and interception processes are not completely effective, damage products are continuously formed in low yields and hence may accumulate. DNA damage, in the form of damaged bases, or single or double stranded breaks is monitored and repaired by a battery of DNA repair enzymes. Lipolytic and proteolytic enzymes function in the restitution and replenishment of damaged membranes.

By such antioxidant mechanisms cells are able to survive in an oxidising environment, but when the generation of ROS exceeds the ability of the cell to disarm or repair the damage, oxidative stress ensues.

Antioxidant Enzymes in Parasites

All protozoan and helminth parasites examined to date contain at least one of the three main antioxidant enzymes; superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) (Callahan *et al*, 1988). SOD dismutates superoxide to hydrogen peroxide, while catalase, the glutathione-dependent enzymes GPx and glutathione reductase, and other peroxidases such as cytochrome c peroxidase are involved in hydrogen peroxide detoxification. If these primary defence mechanisms are breached, the parasite can call upon back-up defences, including glutathione S-transferase (GST), which is involved in the detoxification of xenobiotics, including the products of lipid peroxidation (Nare *et al*, 1990). If this secondary defence mechanism fails, cytotoxic carbonyls (the breakdown products of lipid peroxidation), may be neutralised by NADPH / NADH carbonyl reductases or via glutathione conjugation catalysed by GST enzymes. Parasitic helminths don't always express a complete defence system. Parasitic nematodes, in general, have active free radical and peroxide defences and only a limited back-up defence against carbonyls. Digeans have a strong superoxide defence, in SOD and high levels of GSTs. Cestodes have GSTs, but only weak primary defences.

Many non-enzymic mechanisms such as α tocopherol, ascorbate and thiols (glutathione), shown to be highly effective in mammalian systems, may also be effective against reactive oxidants in parasites, however, apart

from the widespread occurrence of glutathione, levels of these compounds in helminths is not known (Brophy and Barrett, 1990).

Superoxide Dismutase

Superoxide dismutase (SOD) catalyses the dismutation of the superoxide radical to molecular oxygen and hydrogen peroxide;

$$O_2^- + O_2^- + H^+ \longrightarrow H_2O_2 + O_2$$

The hydrogen peroxide can diffuse and is potentially more damaging to parasites than superoxide. Therefore, if helminths produce SOD to protect against oxygen radicals, they also require a mechanism to detoxify hydrogen peroxide. It is normally decomposed to water, by either catalase, glutathione peroxidase or other peroxidases.

Superoxide dismutases are characterised by the metal they contain in their active site. Iron-SOD is found in protozoan parasites. Mitochondrial enzymes are tetrameric and contain manganese in their active site, whereas cytosolic SODs contain copper and zinc and are dimers of identical subunits of 16 kDa molecular mass. Extracellular SODs are tetrameric; each 30 kDa subunit containing Cu and Zn and they are synthesised with a 18 amino acid signal peptide that is cleaved upon secretion. Cu / Zn SODs are particularly abundant in tissues where metabolic activity is highest, and in helminths have been reported from muscles, nerve trunks, oesophagus and uterine accessory cells (Callahan *et al*, 1993). Many SODs are thought to be inducible by oxidant stress. In mammalian cells expression is induced by cytokines (James, 1994). In helminths, the adaption of SODs function from a strictly intracellular to an extracellular defence mechanism against superoxide radicals encountered in the worms environment may represent an essential role for the enzyme in the maintenance of the host-parasite relationship (Rhoads, 1983).

Schistosoma mansoni contain the antioxidant enzymes SOD, glutathione peroxidase (GPx) and cytochrome c peroxidase. *In vitro* studies have shown age specific differences in susceptibility in oxidant killing, adult worms being most resistant (Mkoji *et al*, 1988). The levels of the fluke antioxidant enzymes correlate with this difference, being much higher in adult worms than in schistosomula (Nare *et al*, 1990). Secreted (Simurda

et al, 1988) and cytosolic (DaSilva *et al*, 1992) forms of SOD have been cloned from *S. mansoni*. Simurda *et al*, (1988) described the secreted form as a Cu / Zn containing dimer of 20 kDa subunits, with a signal peptide and a very high pI. The SOD has been immunolocalised to the sub-tegumental region of the worms, therefore, secretion to the surface is thought to occur through cytons (Hong *et al*, 1993). The cytosolic SOD is a Cu / Zn enzyme with a molecular mass of 16 kDa (DaSilva *et al*, 1992). Both enzymes are immunogenic and are recognised by infection sera, implying that both the cytosolic and the extracellular enzymes are secreted. Henkle *et al* (1991), reported that both SODs of *Oncocherca volvulus* are also secreted. The secretion of the SODs is thought to be in response to host ROS production.

Stage specific susceptibility to reactive oxidants also occurs in the nematode *Trichinella spiralis*. Adult worms and muscle stage larvae (MSL) are much more resistant to toxicity then newborn larvae (NBL), (Rhoads, 1983). *In vitro* studies, using enzymically produced oxidants, showed 10% killing of adult worms and MSL as compared to almost 90% of NBL (Kazura and Meshnick, 1984). NBL have 3-5 times less SOD and 5-10 times less GPx than adult worms or MSL, suggesting that these two enzymes are responsible for the relatively high resistance to killing in adults and MSL. The SOD, which is immunogenic, is actively secreted by the worm and when isolated from *in vitro* culture media, has a similar specific activity to the worm extract enzyme (Kazura and Meshnick, 1984).

SOD activity has also been detected in the excreted and secreted (ES) products of *Dictyocaulus viviparus*, *Trichostrongylus vitrinus, Teladorsagia circumcincta, Taenia taeniformis* and *Dirofilaria immitis* (James, 1994). SOD is thought to be secreted in response to an extracellular source of superoxide, most likely generated by host phagocytes. The SOD would also prevent the generation of neutrophil chemotactic factors produced by the interaction of superoxide and serum. However, the importance of SOD as a parasite defence against host ROS is still speculative. It is not known what effect depletion of a parasites SOD might have on its survival, although for bacterial mutants, this depletion leads to an increased clearance rate from the host. Depletion of parasite SODs may lead to enhanced effectiveness of host leucocytes (James, 1994). A recent report shows some evidence that SOD depletion may be detrimental to helminths

(Britton *et al*, 1992). Sera from cattle immunised against *D. viviparus* by infection, neutralises the parasite SOD. This SOD-neutralising ability of the immune serum may contribute to the observed protection against challenge in these animals (Britton *et al*, 1992).

Brophy and Pritchard (1995) have proposed an alternative, more aggressive role for secreted SOD. Rather than functioning solely in parasite defence, they suggest that secreted SOD from the hookworm *Necator americanus* is used to damage host tissues, assisting evasion, feeding and immune modulation. The hookworm secretes SOD, probably in response to superoxide production by host immune cells. SOD converts the superoxide into hydrogen peroxide. However, catalase and peroxidase activity are not secreted by the hookworm, thus, it is proposed that the hydrogen peroxide and ultimately the hydroxyl radical is produced by *N. americanus* to decompose host proteins at its surface. Hydrogen peroxide can also diffuse from its site of generation and encounter host elements at a relatively large distance. Gastrointestinal nematodes are relatively resistant to hydrogen peroxide and hookworms express endogenous catalase activity, thus protecting the worm from the externally produced hydrogen peroxide (Brophy and Pritchard,1995). Extracellular secondary lipid peroxidation defence systems, such as glutathione S-transferases (GST) may neutralise cytotoxins arising from either immune attack or oxidant induced damage. Therefore, hookworm secretory SOD, with a GST shield could be described as a pro-active defence strategy for host tissue destruction (Brophy and Pritchard, 1995).

Catalase

Catalase catalyses the reduction of hydrogen peroxide to water.

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

It is a tetrameric enzyme, each subunit (molecular mass 60 kDa) containing a single heme group. The active site of the enzyme appears conserved throughout the species, however, there is substantial variation in the remainder of the molecule. The mode of action of catalase is catalytic at high hydrogen peroxide concentrations and peroxidatic at low concentrations. It is located in the peroxisomes of cells and occurs in most tissues, but is notably absent in anaerobes. In mammals, the greatest

amount is found in the liver and kidney, but red blood cells also contain a substantial amount (Calabrese and Canada, 1989).

Unlike SOD, catalase has not been isolated or characterised from any helminths to date. However, catalase activity has been reported in several nematodes (Callahan *et al*, 1988). It occurs at low concentrations in most parasitic species except *N. brasiliensis* and *N. dubius*, where it is present in considerable quantity and in the free living *Turbatix aceti* and *Caenorhabditis elegans* where it is also present in relatively high concentrations. Catalase and SOD levels in *C. elegans* appear important in determining the nematodes life span (Vanfleteren, 1993). A mutation which doubles *C. elegans* lifespan, also results in an increase in catalase and SOD levels, showing that oxidative stress defence and potential life span are closely correlated in this organism. These results are compatible with the general hypothesis that the balance between oxidative stress and antioxidant defence determines longevity (Vanfleteren, 1993).

Catalase activity has not been reported in either trematodes or cestodes, therefore the toxic hydrogen peroxide of these parasites must be removed by other means. Catalase is not detected in *S. mansoni*, however it does contain glutathione peroxidase to decompose hydrogen peroxide (Nare *et al*, 1990). Peroxidase activity which may be cytochrome c linked has been identified in *Fasciola hepatica* (Barrett, 1980). Cytochrome c linked peroxidase appears solely responsible for peroxide metabolism in the cestodes *Hymenolepis diminuta* and *Moniezia expansa* (Paul and Barrett, 1980). The function of catalase in defence against host ROS has not yet been demonstrated and to date there are no reports of catalase secretion by helminths.

Glutathione Peroxidase

Glutathione peroxidase (GPx), catalyses the reduction of hydroperoxides (ROOH) to water, using glutathione (GSH) as the reductant.

$$ROOH + 2\,GSH \longrightarrow ROH + GSSG + H_2O$$

Glutathione (GSH) becomes reduced during the reaction. Reduced glutathione (GSSG), is converted back to GSH by the enzyme glutathione

reductase (GR). GPx is a tetramer of 75-100 kDa molecular mass, composed of four identical subunits, each containing one atom of selenium covalently bonded to cysteine in the active site. The enzyme can act on hydrogen peroxide as well as hydroperoxides and like catalase, it is extremely efficient, capable of degrading 42,000 molecules of hydrogen peroxide per second at $0^{o}C$. The enzyme, found in all mammalian tissues is localised in the cytosol and mitochondrial matrix. GPx has been detected in most helminths studied, but is always present in low concentrations, and like catalase, it has not yet been isolated or characterised from these organisms (Callahan *et al*, 1988). GPx is important in enzymatic cellular defence against free radical damage. If the first line of defence (the antioxidant enzymes SOD and catalase), is breached, then the second line of defence is provided by the glutathione dependent enzymes; GPx and glutathione S-transferase (GST). These detoxify lipid peroxides before they decompose into secondary products, such as damaging cytotoxic carbonyls.

The GPx of *S. mansoni* has been cloned (Williams *et al*, 1992; Mei and LoVerde, 1995). It is approximately 40% similar to the mammalian GPx and the GPx of the nematode *Brugia pahangi*. The native enzyme is predicted to have a selenocysteine active site. GPx activity in *S. mansoni* increases significantly as worms mature in their host and this increase in activity is positively correlated to an increase in resistance to antioxidants (Nare *et al*, 1990).

The GPx of *B. pahangi*, a causative agent of lymphatic filariasis, has been identified and cloned (Cookson *et al*, 1992). It is a major protein of the adult cuticle and its expression is up-regulated following infection of its mammalian host. The GPx is synthesised in the synctial hypodermis and exported to the cuticle where its secretion results in constant turnover (Cookson *et al*, 1992). Further characterisation of the enzyme by Tang *et al*, (1995), revealed it to be a homotetrameric glycoprotein, which lacks selenocysteine at its active site. The enzyme shows remarkable similarity to mammalian plasma GPx, which contains selenocysteine, and to another mammalian GPx from epididymal tissues, which like the nematode enzyme has cysteine at its active site. The enzyme has activity for various

hydroperoxides, especially fatty acid hydroperoxides and phospholipid hydroperoxides, but not hydrogen peroxide. The specific activity of the *B. pahangi* enzyme is less than that of selenium-containing enzymes (Tang *et al*, 1995). It is unlikely that glutathione serves as the enzymes natural substrate, since collagen, the major component of the cuticle, is cross linked by disulphide bonds and secretion of thiols into this environment would be expected to have deleterious consequences due to the formation of mixed thiols (Tang *et al*, 1995).

The role of GPx in protecting the nematode against host ROS, may be highly significant in the parasites ability to persist in the host lymphatic system for a long time, despite a vigorous host response. Cookson *et al* (1992), proposed that *Brugia* GPx catalyses the formation of cross linking residues such as, dityrosine, trityrosine and isotrityrosine which have been identified in cuticular collagen and "cuticilin", a protein complex that forms the epicuticle, the structural external cortex of the cuticle. The expression of GPx corresponds with a dramatic growth phase in the life of the parasite and an increased level of cross linking of collagens may be necessary to maintain the tensile strength of the cuticle. This postulated function may not be mutually exclusive with an antioxidant activity, as a highly cross linked external cortex may also protect the parasite from immune attack (Cookson *et al*, 1992).

Glutathione S-Transferase

Glutathione S-transferases are a family of multifunctional enzymes, which recognise a broad spectrum of molecular targets and can bring about cellular detoxification of a wide range of xenobiotics. They catalyse the conjugation of organic molecules containing an electrophillic centre with the thiol group of glutathione. The effect of this reaction is generally to convert a reactive lipophilic molecule into a water soluble, non reactive conjugate which may easily be excreted after further processing if necessary (Clark, 1989). Although structurally unrelated to GPx, GSTs may also possess peroxidase activity for organic peroxides. Thus, GSTs have the potential to detoxify exogenously derived toxic compounds (xenobiotics) and endogenously derived toxic compounds, including the secondary products of ROS-induced lipid peroxidation and DNA damage,

using both their enzymatic and binding capabilities (Ketterer and Meyer, 1989). A number of GSTs may have more specific endogenous functions in metabolism, including a role in prostaglandin synthesis and in transport by binding ligands such as heme, steroids and bile acids (Brophy and Barrett, 1990). In mammals four GST multigene families have been characterised and designated alpha, mu, pi and theta. GST iso-enzymes within each class show a high level of sequence conservation, but can be distinguished by differences in iso-electric point, molecular mass, immunological properties and specific substrate affinities (Tsuchida and Sato, 1992). In mammals the iso-enzymes differ in their tissue distribution, which may reflect the detoxification needs and physiological stresses encountered either by the whole organism or by specific tissues within the organism (Creaney *et al*, 1995).

A great deal of interest has been shown in parasite GSTs, principally because of their potential as antihelmintic vaccines (Brophy and Pritchard, 1994). GST iso-enzymes have been identified in all helminths analysed to date (Brophy and Barrett, 1990) and it may be one of the major detoxification enzymes of these parasites, which appear to lack the important cytochrome P-450 detoxification system (Precious and Barrett, 1989). The level of GST activity varies and generally, helminths with a "naked" tegument such as digeans and intestinal cestodes appear to have relatively high activity compared to intestinal and tissue parasitic nematodes (Brophy and Barrett, 1990).

GST iso-enzymes have been resolved from the cestodes *Moniezia expansa* and *Schistocephalus solidus* (Brophy *et al*, 1989a; Brophy *et al*, 1989b). Both cestode GSTs are dimers of subunits of 24-25 kDa molecular mass and appear to be closely related to the mammalian mu class of GSTs (Brophy and Pritchard, 1994). GSTs have also been detected in the nematode *Onchocerca volvulus,* a causative agent of human filariasis (Liebau *et al* , 1994). Four GST iso-enzymes have been purified, none of which possesses peroxidase activity using hydrogen peroxide as substrate. The GSTs appear to belong to the pi class of GSTs, in contrast to trematode GSTs, which are mostly mu GSTs (Salinas *et al*, 1994). The enzymes appear to be peripherally associated with the outer membrane of the hypodermis which may facilitate exchange of material with the cuticle

and the environment, and active secretion of the enzyme *in vitro* has been reported (Liebau *et al*, 1994).

GSTs have been identified from a number of trematodes; *Schistosoma mansoni* (Smith *et al*, 1986), *Schistosoma japonicum* (Taylor *et al*, 1988), *Schistosoma bovis* and *Schistosoma haematobium* (Trottein *et al*, 1992) and *Fasciola hepatica* (Howell *et al*, 1988). At least four cytosolic GST iso-enzymes have been identified in *S. mansoni.* These include a family of three major iso-enzymes of subunit size approximately 28 kDa, which show similar immunological properties and similar but distinguishable biochemical properties (O'Leary and Tracy, 1988; O'Leary *et al*, 1992) and a minor iso-form of subunit molecular mass 26 kDa, which differs in immunological and biochemical properties (O'Leary *et al*, 1992). Native GSTs are dimers and the principal enzyme in *S. mansoni* is a homodimer of a 28 kDa antigen. The enzyme has both conjugation and peroxidase activities, mainly directed against fatty acid hydroperoxides (Taylor *et al*, 1988). Iso-enzymes of 26 kDa and 28 kDa molecular mass have also been described in adult *S. japonicum* . Four recombinant GST proteins have been expressed from the cDNAs of *S. mansoni* and *S. japonicum;* Sm26, Sm28, Sj26 and Sj28. Characterisation revealed that the 28 kDa proteins are related to the mammalian mu class, whereas the 26 kDa proteins consist of an apparent hybridisation of mu and alpha class features. Schistosome GST activity has been immunolocalised to the tegument and its extensions into the parenchyma and excretory epithelial cells, but unlike F. *hepatica* GST is absent from the parasite gut. GSTs are also present in the tegument and associated structures of the schistosomules (Taylor *et al*, 1988) and the activity of the enzyme appears to increase with fluke development (Nare *et al,* 1990).

Schistosome GSTs may function in protection against lipid peroxidation-derived carbonyls (Brophy and Pritchard, 1994). They can also bind with high affinity to various endogenous molecules, such as bilirubin and hematin, thereby solubilising them. Hematin is a breakdown product of metabolised host hemoglobin and is secreted in large amounts by the parasite, eventually becoming lodged in the host liver. Schistosomal GSTs may contribute to this process by binding hematin, preventing the formation of large hematin crystals which might otherwise block the

parasites gut (Smith *et al*, 1986). GSTs also have the potential to bind exogenously derived toxins such as antihelmintics. The mode of action of the anti schistosomal drug Oltripraz is linked with glutathione metabolism. Oltripraz inhibits *S. mansoni* activity *in vitro* and treatment of worms with the drug lowers *in vivo* GST levels (Nare *et al*, 1991). The external location of the schistosome GST may be a consequence of these GSTs being part of a crude "dumping" mechanism for attached toxins, such as antihelmintics (Brophy and Pritchard, 1994).

GSTs have also been identified as important protective antigens in schistosomiasis. Vaccination of mice with recombinant Sj26GST resulted in up to 50% protection (Mitchell, 1989). Immunisation using both native and recombinant Sm28GST induced protection levels of 40-70% in rodents (Balloul *et al*, 1987a; Balloul *et al*, 1987b), and vaccination with the recombinant molecule in baboons affected worm viability (38% reduction in worm burden) and worm fecundity (Boulanger *et al*, 1991). *S. bovis* recombinant GST immunisation of goats resulted in a 50% reduction in worm burden (Boulanger *et al,* 1994). The success of the GSTs as vaccine candidate molecules probably reflects the essential functions they perform in schistosomes. Furthermore, because of their apparent roles in protection against immune-mediated lipid peroxidation, the inhibition of GSTs offers the possibility of combining chemotherapy with immunotherapy.

GSTs in *Fasciola hepatica* represent about 4% of the total soluble protein (Brophy *et al,* 1990). This is about 4-18 times the levels found in human liver and the high concentration in the fluke suggests that the enzymes play an important role in the parasites metabolism (Howell *et al*, 1988). *F. hepatica* GSTs show catalytic activity with reactive carbonyl compounds, indicating that they may provide protection against these toxic products of lipid metabolism (Brophy *et al*, 1990). Several of the GSTs also have associated peroxidase activity, which could provide initial protection by neutralising lipid hydroperoxides prior to their decomposition to secondary products, such as, carbonyls. *F. hepatica* GSTs, through their binding capacity, may also function as intracellular receptors for bile acids (Brophy and Barrett, 1990), which have a number of key functions in helminth metabolism, including acting as developmental triggers and influencing the rate of uptake of glucose and amino acids (Barrett, 1981).

GST activity was inhibited *in vitro* by hematin (Brophy *et al*, 1990). This interaction which may detoxify hematin by passive binding, preventing crystal formation, is also proposed for schistosome GSTs (Smith *et al,* 1986).

Immunolocalisation studies in adult flukes show the GSTs in the cytoplasm of parenchymal cells, the tegument and sub tegument and on the surface of lamellae of the intestinal epithelium (Howell *et al*, 1988; Wijffels *et al*, 1992). In NEJs, GSTs are present in the cytoplasm, not in the tegument but in the sub tegument and also in the excretory system, suggesting their involvement with excretion of waste products formed during the excystment stage (Wijffels *et al*, 1992). Localisation to the gut in the adult stage suggests a possible absorptive role for the GSTs. In juvenile fluke the digestive system is underdeveloped and performs a secretion function only (Bennett, 1975).

Two classes of GSTs have been characterised from *F. hepatica*; one alpha GST, with low affinity for glutathione and multiple forms of mu classes with high affinity for glutathione (Brophy and Barrett, 1990). The mu class GSTs resolve into at least seven iso-forms, four of which have been cloned, sharing 60-90% amino acid identity (Panaccio *et al*, 1992). The recombinants show a range of different catalytic activities and different substrate specificities (Salvatore *et al*, 1995). Immunolocalisation studies show that the iso-enzymes are differentially expressed within the tissues of both adult and juvenile flukes (Creaney *et al*, 1995). The expression of several mu class GSTs by *F. hepatica* may suffice to generate the repertoire of substrate specificities required by the adult fluke for survival in the harsh conditions of its natural habitat, the bile duct.

Like schistosome GSTs *F. hepatica* GSTs appear to bind a number of commercially available antihelmintics including substituted phenol-based compounds and benzimidazoles (Brophy and Barrett, 1990). This binding may potentiate the antihelmintic effect through intracellular transport or alternatively it may passively detoxify the compound. Conjugated glutathione products are often potent inhibitors of GSTs and there is little evidence that helminths can metabolise further or excrete these potentially inhibitory compounds. Thus, the synthesis of toxic glutathione conjugates

is a potential for pro-drug design (Brophy and Barrett, 1990). Despite low natural immunity, vaccination of sheep with *F. hepatica* GSTs resulted in a 57% reduction in liver fluke burden (Sexton *et al*, 1990). The efficacy of GST vaccination presumably reflects that the GST function is essential to the parasites survival.

It appears that many parasites apparently possess sufficient quantities of antioxidant enzymes to protect against oxidants generated by activated phagocytes *in vivo*. However, many of the same helminths have been shown to be susceptible to killing by both phagocytes and oxidants *in vitro* . There are numerous explanations for this apparent discrepancy, for example, the levels of oxidants used *in vitro* are often much greater than those found *in vivo* and also helminths may be able to move from away the site of attack *in vivo*. Helminth parasites must survive for extended periods of time in their definitive host, to ensure propagation of the next generation. There does appear to be a link between survival in the host and the level of antioxidant enzymes in the parasite. However, the enzyme levels may reflect an adaption to stresses other than the host response. Different parasite species at different stages of development are susceptible to radicals to differing extents, possibly reflecting the basal level of antioxidants as well as an ability to secrete and / or boost production of antioxidants following stimulation. There is, of course, evidence that parasite killing is not always mediated through ROS of host immune cells. However, the evidence to date does suggest that antioxidant enzymes may play a protective role in the down regulation of the host response and future studies in the area will be of importance in designing methods to enhance control of parasite infections.

Novel Antioxidant family

A novel family of antioxidant enzymes has recently been discovered (Chae *et al*, 1994a). These antioxidants were originally termed thiol-specific antioxidants, but are now known as the peroxiredoxins and they appear to be present in a wide variety of organisms, though not yet reported in parasites.

Autooxidation of thiols in the presence of iron or copper generates ROS, including hydrogen peroxide (H_2O_2), superoxide (O_2^-), the hydroxyl radical ($HO^•$), and the thiyl radical ($RS^•$). In addition, the disulphide radical anion ($RSSR^•$) and peroxysulphonyl radical ($RSSO^•$) have been suggested as intermediates during the thiol autooxidation. Hydroxyl radicals, formed through the Fenton reaction inflict damage on a wide range of biological molecules including protein. Sulphur radicals have been identified in biological fluids, however, their potential damaging effect is unknown.

Several enzymes lose their activity in the presence of oxygen, iron and a reducing agent, such as dithiothreitol (DTT), mercaptoethanol or ascorbate (Kim *et al*, 1985). The presence of EDTA, catalase or GPx protects the enzyme by removing either iron or hydrogen peroxide, thus, preventing the damage (Kim *et al*, 1985). Kim *et al* (1988), reported the isolation and purification of a *Saccharomyces cerevisiae* protein which specifically inhibited the inactivation of various enzymes by the oxygen, iron, thiol system. If thiol in the reaction was replaced by another reducing agent such as ascorbate, the protein was no longer able to protect the enzyme (Kim *et al*, 1988). The protein with a subunit molecular mass of 25 kDa has no detectable catalase, GPx, SOD or iron chelation activities. The protective activity appeared specific for oxidative reactions containing thiols and the protein was named thiol-specific antioxidant (TSA), (Chae *et al*, 1993), though now termed thioredoxin peroxidase (TPx), (Chae *et al*, 1994b).The application of oxidative pressure to yeast, by incubation under 100% oxygen, or by the addition of iron or mercaptoethanol, results in an increase in TPx synthesis (Kim *et al*, 1989). Yeast mutants lacking TPx genes remain viable in air, suggesting that the TPx is not essential for viability, however, the mutants growth rate is significantly reduced suggesting that it is a physiologically important antioxidant (Chae *et al*, 1993).

Yeast and rat TPx genes have been cloned and sequenced and the sequences show no significant similarity with other antioxidant enzymes (Chae *et al*, 1993; Chae *et al*, 1994a). Both TPx genes are 40% similar with the AhpC component of *Salmonella typhimurium* alkyl hydroperoxide reductase, which converts alkyl hydroperoxides into their corresponding

alcohols. *S. typhimurium* alkyl hydroperoxide reductase has been shown to consist of a 22 kDa AhpC component and a 57 kDa AhpF, FAD-containing NAD(P)H dehydrogenase (Jacobson *et al*, 1989; Storz *et al* 1989). The AhpC decomposes the substrate and is subsequently regenerated by the AhpF coupled to either NADH or NADPH oxidation. AhpF was shown to have considerable similarity to thioredoxin reductase and the purified AhpC contains TPx activity (Chae *et al*, 1994a).

Twenty six additional protein sequences are similar to the TPxs and AhpC. These similar proteins, with the exception of AhpC are not associated with known biochemical functions and appear to represent a new, widely distributed family of antioxidants. Alignment of the amino acid sequences of the antioxidant family members revealed two highly conserved cysteine sites, corresponding to cys 47 and cys 170 in the yeast protein (Chae *et al*, 1994a). The oxidised form of the 25 kDa protein exists mainly in a dimeric form, linked by two disulphide bonds between cys 47 and cys 170. Site directed mutagenesis studies showed that cys 47, but not cys 170, is essential for antioxidation *in vitro*, showing that the antioxidation reaction does not involve the formation of intermolecular disulphide bonds (Chae *et al*, 1994c).

Lim *et al*, (1993), showed that high concentrations of TPx could decompose hydrogen peroxide in the presence but not in the absence of DTT. They proposed that the inactive form of the TPx is converted to the active form by DTT. They also demonstrated TPx protection of DNA cleavage by the specific removal of the hydroxyl radical. Chae *et al*, (1994b), discovered that inactive yeast TPx was regenerated *in vivo* by the thioredoxin system. Thioredoxin and thioredoxin reductase (TR) together mediate the flow of electrons from NADPH to the oxidised form of TPx. This physiological hydrogen donor system was shown to be almost ten times more efficient than regeneration with thiols (DTT). The previously ascribed thiol specific antioxidant activity was attributed to the fact that thiols but not ascorbate are able to reduce the TPx disulphide. In the presence of the thioredoxin system, TPx reduces hydrogen peroxide, cys 47 being the primary site of reduction, with thioredoxin as the immediate hydrogen donor and protects proteins by the elimination of hydrogen peroxide, thus preventing formation of the damaging hydroxyl radical (Chae *et al,* 1994b).

Chae *et al,* (1994b), speculate that the additional 26 proteins of the antioxidant family are peroxidases and propose to name them the peroxiredoxin family. The diversity in the amino acid sequences may reflect several different mechanisms in the regeneration of the reduced peroxiredoxin. The peroxiredoxin family thus, probably represents a widely distributed class of enzymes that directly reduce hydrogen peroxide and various alkyl hydroperoxides with hydrogens derived from NAD(P)H via various routes. TPx, which appears to be ubiquitous and abundant in mammalian tissues, together with catalase and GPx, would provide a major pathway of hydrogen peroxide elimination.

Chapter 2

Isolation and characterisation of *Fasciola hepatica* hemoglobin

2.1 Materials

Biological Laboratories Europe Ltd.
Wistar rats (male)

Gibco, Life Technologies Ltd.
RPMI-1640 (10X) w/o L-glutamine

Pharmacia LKB Biotechnology
Sephacryl S-200 HR
DEAE Sepharose

University of Cambridge Protein Sequencing Facility
Polyvinylidine difluoride (PVDF, Problott)

Schleicher and Schuell
Nitrocellulose (0.4 µm pore size)

Sigma Chemical Company
Anti-rat IgG conjugated to alkaline phosphatase (rabbit), anti-rat IgG (goat) conjugated to fluoroisothiocyanate (FITC), bovine serum albumin (BSA), 5-bromo-5-chloro-3-indolyl phosphate (BCIP), coomassie brilliant blue R, 3,3'-diaminobenzidine hydrochloride (DAB), gentamycin, N-[2-hydroxyethyl] piperazine-N'[2-ethanesulphonic acid] (HEPES), iodoacetamide, L-trans-epoxysuccinyl-leucylamido-[4-guanidino]-butane (E-64), leupeptin, nitro-blue tetrazolium (NBT), phenylmethylsulphonyl fluoride (PMSF), Tween 20.

2.2 Methods

Preparation of Excretory / Secretory (ES) products from mature liver fluke

Mature *F. hepatica* flukes were removed from the bile ducts of infected livers from cattle at a local abattoir. The flukes were washed six times in phosphate buffered saline (PBS), pH 7.3, and then maintained in RPMI-1640, pH 7.3, containing 2% glucose, 30 mM HEPES and 25 mg ml^{-1} gentamycin at 37^{o}C for 18h. Following the incubation period, the culture medium (ES products) was removed, centrifuged at 12,000 x*g* for 30 min and the supernatant collected and stored at -20^{o}C.

Purification of hemoprotein from ES

The ES supernatant was concentrated to 15 ml in an Amicon 8400 Ultrafiltration unit (Danvers, MA, USA) with a YM3 membrane (3,000 mw cut-off). The concentrated sample was centrifuged at 12,000 x*g* for 30 min and applied to a 340 ml Sephacryl S-200 column equilibrated in 0.1M Tris-HCl, pH 7.0, at 4^{o}C. Fractions, each of 5 ml, were collected after the void volume (110 ml) had been passed. The absorbance of the eluate was monitored at 280 nm using an Atto UV monitor. Those fractions containing hemoprotein (yellow coloured) were pooled and concentrated in an Amicon 8050 ultrafiltration unit to 5 ml *. This was dialysed against 0.025 M HEPES, pH 6.8, and applied to a 10 ml DEAE Sepharose column equilibrated in the same buffer. Proteins were eluted using a linear gradient from 0 to 0.4 M sodium chloride in 0.025 M HEPES, pH 6.8. The hemoprotein-containing fractions, detected by absorbance at 415 nm, were pooled, concentrated by ultrafiltration, and stored at -20^{o}C until required.

The Sephacryl S200 column was calibrated with the following standards; mouse immunoglobulin, 150 kDa; albumin, 68 kDa; ovalbumin, 45 kDa; b-lactoglobulin, 18.4 kDa.

** This concentrate was termed hemoglobin fraction (Hf) in chapter 3, and was used in the cattle vaccine trial.*

Extraction of hemoprotein from mature liver flukes

Mature liver flukes were removed from the medium at the end of the culture period and washed three times with PBS. An equal volume of PBS was then added to the flukes, and the parasites were homogenised on ice with a teflon homogeniser. Following centrifugation of the homogenate at 12,000 x*g* for 30 min, the supernatant was decanted and stored at -20^{o}C.

Liver flukes were extracted in the presence and absence of the following proteinase inhibitors, phenylmethylsulphonylfluoride (PMSF), 10 mM; iodoacetamide, 10 mM; leupeptin, 5 μg ml^{-1}; and L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane (E-64), 5 μg ml^{-1}.

Polyacrylamide gel analysis of hemoprotein

Non-denaturing 10% polyacrylamide gel electrophoresis (PAGE) was performed using the buffer system of Laemmli (1970). Gels were stained for protein with Coomassie brilliant blue R, and for hemoproteins using 3,3'-diaminobenzidine hydrochloride (DAB) and hydrogen peroxide as described by McDonnel and Staehelin (1981). Briefly, the gel was fixed in 7% acetic acid for 20 min, washed with several changes of 0.5 M tris-HCl, pH 7.0, and incubated for 15 min in 0.5 mg ml^{-1} DAB, 0.5 M tris-HCl, pH 7.0. It was then transferred to 0.5 mg ml^{-1} DAB in 50 mM sodium citrate, pH 4.0 containing 0.1% hydrogen peroxide and incubated at 4^{o}C for 12 h. Heme-containing proteins appeared as reddish-brown bands that were stable over time.

Bovine hemoglobin, used as a positive control in these gels, was obtained by lysing bovine erythrocytes in water followed by centrifugation at 10,000 x*g* for 30 min in order to remove cell debris.

N-Terminal sequence determination

F. hepatica hemoprotein, purified from ES products as described above, and total mature liver fluke proteins extracted in the presence of inhibitors, were separated in a non-denaturing 10% polyacrylamide gel. Following electrophoresis the gel was incubated in transfer buffer (25 mM Tris-HCl, 190 mM glycine and 10% methanol) and then electrophoretically transferred to PVDF membrane (Problott) using a semi-dry electroblotting

apparatus (Atto Corporation, Tokyo, Japan). The membrane was stained with Coomassie brilliant blue R and the bands corresponding to the liver fluke hemoprotein were sequenced at the Protein Sequencing Facility, Department of Biochemistry, Tennis Court Rd., Cambridge,CB2 IQW or at BioResearch Ireland, University College Cork,using an Applied Biosystems 477A protein sequencer.

Light absorption spectra

The absorption spectra of mature *F. hepatica* homogenate hemoprotein and hemoprotein purified from ES were measured with a recording spectrophotometer (Shimadzu UV-160A). Derivatives of liver fluke hemoglobin were prepared as described by Tsuneshige *et al.* (1989). Briefly, oxy-hemoglobin in mature fluke extracts was reduced to deoxyhemoglobin by adding five milligrams of solid sodium dithionite to 200 mg extract. To obtain met-hemoglobin (oxidised), an excess of potassium ferricyanide crystals was added to the liver fluke extract. The cyanomet derivative was obtained by including 1 mM potassium cyanide in the above oxidation reaction.

Preparation of hemoprotein antiserum, immunoblotting and ELISA

Polyclonal antisera against the hemoprotein purified from adult fluke ES products was prepared by immunising male Wistar rats subcutaneously with 20 μg purified protein (500 μl). The initial injection was prepared in Freunds complete adjuvant and the four subsequent injections, given at three week intervals, in Freunds incomplete adjuvant.

For immunoblotting experiments, liver fluke homogenates (extracted in the presence and absence of proteinase inhibitors), and purified hemoprotein were electrophoresed under non-denaturing conditions and transferred to nitrocellulose paper using an Atto semi-dry blotting system. Non-specific binding sites were blocked with 1% BSA and 0.1% Tween 20 in PBS. Nitrocellulose filters were then incubated in rat anti-hemoprotein serum or normal rat serum (1:400 dilution). Bound antibody was visualised using alkaline phosphatase conjugated anti-rat IgG. Nitro-blue tetrazolium (NBT) and 5-bromo-5-chloro-3-indolyl phosphate (BCIP) were used as substrates for alkaline phosphatase.

For enzyme linked immunosorbant assays (ELISA), 50 µl (1 µg) of purified hemoprotein was dispensed into wells of a 96-well plate and incubated overnight at 37^{o}C. The plates were then blocked with 2% BSA and 0.1% Tween 20 in PBS for one hour before the antisera were added. Bound antibodies were detected using alkaline phosphatase conjugated rabbit anti-bovine IgG serum, with p-nitrophenyl phosphate as substrate.

Immunolocalisation studies

The immunolocalisation studies were carried out in collaboration with Dr A. Trudgett's laboratory in the Medical Biology Centre, Queens University Belfast, Northern Ireland. Immunolocalisation studies at the light microscope level were carried out on 3 µM JB-4 plastic embedded sections using the rat antiserum prepared against purified liver fluke hemoprotein. FITC-conjugated goat anti-rat serum was used to detect bound antibody according to the procedure described previously (Hanna, 1980).

2.3 Results

Purification of hemoprotein

Liver fluke hemoprotein was purified from ES products of mature liver flukes by a procedure involving two chromatographic columns. A large protein peak elutes at the void volume of the gel filtration column, followed by a second peak which contains the hemoprotein. The fractions containing the hemoprotein were easily identified by their yellowish colour due to the presence of heme (Figure 2.1A). These fractions were pooled, concentrated and dialysed before being applied to a DEAE Sepharose ion exchange column. The hemoprotein was eluted with a 0 to 400 mM NaCl gradient and was detected by absorbance at 415 nm. The hemoprotein elutes at approximately 200 mM NaCl (Figure 2.1B).

Analysis of the pooled hemoprotein-containing fraction by non-denaturing PAGE revealed the presence of a single protein with a minor slower migrating protein (some protein is also seen at the top of the separating gel) (Figure 2.1C, lane 2). The predominant protein co-migrates with a major component of mature fluke ES products (Figure 2.1C lane 1).

The hemoprotein elutes on the Sephacryl S200 column just before the standard protein mouse IgG (150 kDa), and was estimated to have a molecular mass of > 200 kDa.

Analysis of hemoprotein by electrophoresis

Mature liver fluke homogenates (prepared in the presence and absence of proteinase inhibitors) and purified hemoprotein were analysed by non-denaturing PAGE. Gels were stained for protein or for hemoproteins (McDonnel & Staehelin, 1981). The mature fluke extracts contain multiple proteins; however, the extract prepared in the absence of inhibitors contained more faster-migrating proteins than the extract prepared in the presence of inhibitors (Figure 2.2A, lanes 2 & 3). A single protein, which is also the most predominant protein in both of these extracts, stains positive for the presence of a heme group. The heme staining protein in the homogenates prepared in the absence of proteinase inhibitors migrates faster than that in homogenates prepared in the presence of proteinase inhibitors (Figure 2.2B, lanes 2 & 3). This observation suggests that the hemoprotein is susceptible to proteinase digestion.

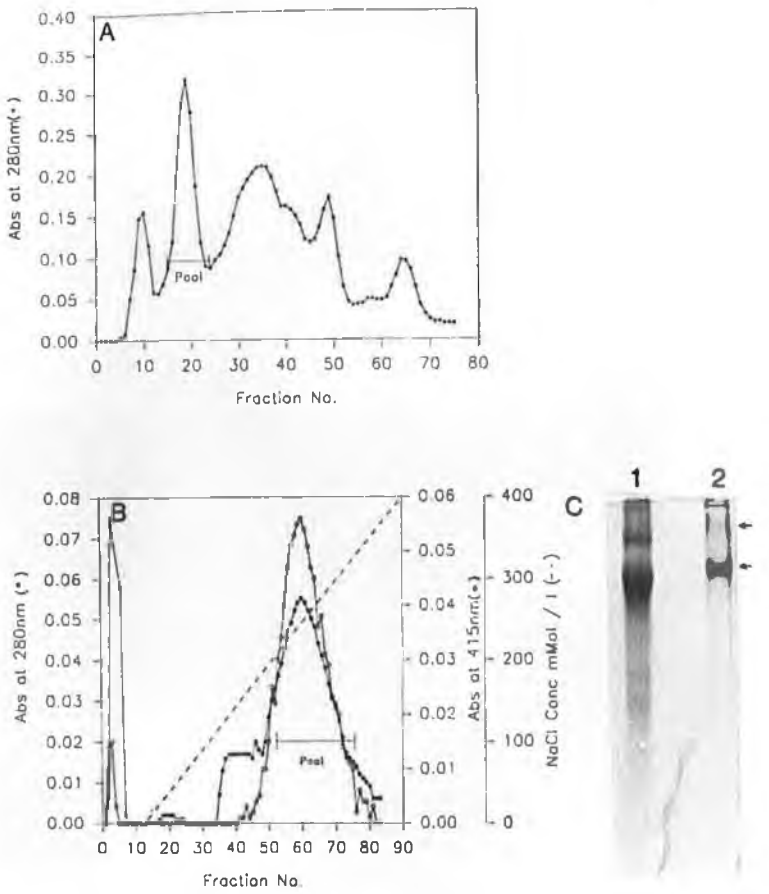

Figure 2.1

Purification of liver fluke hemoprotein

A. Sephacryl S200HR Chromatography. Concentrated *F. hepatica* ES products were applied to the gel filtration column and eluted using a 0.1 M Tris-HCl, pH 7, mobile phase. Eluted protein (-●-) was monitored by absorbance at 280 nm. The hemoprotein eluted in the second major protein peak. Fractions containing the hemoprotein were identified by their yellowish colour and were pooled and concentrated in an Amicon Ultrafiltration unit.

B. DEAE-Sepharose Ion Exchange Chromatography. The concentrated post-gel filtration sample was applied to an ion-exchange column. Proteins, monitored by absorbance at 280 nm (-●-), were eluted using a 0 to 400 mM NaCl gradient (indicated by broken lines). The hemoprotein was detected by absorbance at 415 nm (-O-) and eluted at approximately 200 mM NaCl. The hemoprotein-containing proteins were pooled and concentrated as before.

C. Non-denaturing polyacrylamide gel electrophoresis. Total mature fluke ES products (lane 1) and purified hemoprotein (lane 2) were analysed by non-denaturing native polyacrylamide electrophoresis. Arrows indicate the position of the major and minor components in the purified fraction.

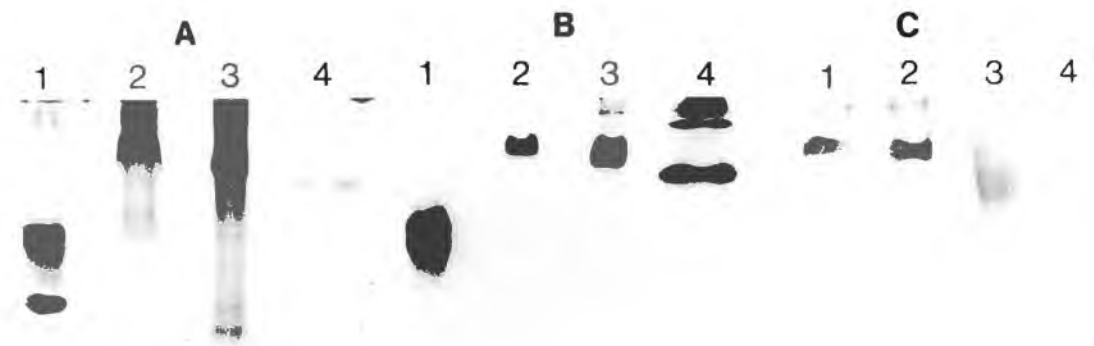

Figure 2.2

Non-denaturing polyacrylamide gel analysis

Gels were stained for protein with Coomassie Blue (panel A) and and for heme-containing proteins with DAB and hydrogen peroxide (panel B). Mature adult liver fluke proteins, extracted in the presence (lanes 2) and absence (lanes 3) of proteinase inhibitors, were compared to purified hemoprotein (lanes 4). Bovine hemoglobin (lanes 1) served as a control for heme staining and to demonstrate that the fluke hemoprotein was not derived from bovine erythrocytes.

Immunoblot analysis

Antiserum prepared against hemoprotein purified from adult ES products was used to probe nitrocellulose filters containing mature liver flukes proteins (panel C), extracted in the presence (lane 1) and absence (lane 2) of proteinase inhibitors, and purified hemoprotein (lane 3). Normal rat serum was used to probe fluke extracts in a control experiment (lane 4).

Similar analysis of the purified hemoprotein demonstrated that both the predominant and minor bands in this preparation contain a heme group (Figure 2.2A & B, Lane 4). Immunoblotting experiments using antiserum prepared against this purified preparation showed that antibodies bound to both of these components (although the binding to the minor band is very faint, Figure 2.2C, lane 3), and to a single band in extracts of mature fluke that co-migrated with the heme-containing protein (Figure 2.2C, lane 1 & 2). Antibodies in control serum did not bind to any molecules in immunoblots (Figure 2.2C, lane 4)

Bovine hemoglobin which appears as a doublet when stained for heme, migrates much further into the gel than the liver fluke hemoprotein. Coomassie blue staining reveals the presence of many contaminating bands in the bovine hemoglobin preparation (Figure 2.2A & B, lane 1). This observation confirms that the hemoprotein identified in mature fluke extracts and ES products did not originate from bovine erythrocytes.

N-Terminal sequence determination

The ES purified hemoprotein preparation and proteins of mature liver flukes, extracted in the presence of inhibitors, were transferred to PVDF membrane. The bands identified as containing a heme group in each preparation were subjected to N-terminal sequencing. No sequence was obtained for the minor component in the ES purified hemoprotein. However, a 26 amino acid sequence was obtained for the major band (Figure 2.3). A 20 amino acid N-terminal sequence was obtained for the major hemoprotein in the total fluke extract and this showed only one amino acid difference to that obtained for the ES hemoprotein (arginine at position thirteen is glycine in the ES hemoprotein).

These sequences showed no significant similarity to other invertebrate or vertebrate hemoproteins when entered into the GenBank database. Furthermore, no protein containing an amino acid sequence with significant similarity to the *F. hepatica* hemoprotein was found.

Spectral properties

The absorption spectra of the hemoprotein in mature fluke extracts and its deoxy-, met-, and cyanomet- derivatives were examined over the visible light range, 325-650 nm (Figure 2.4A). The hemoprotein in the fluke extract

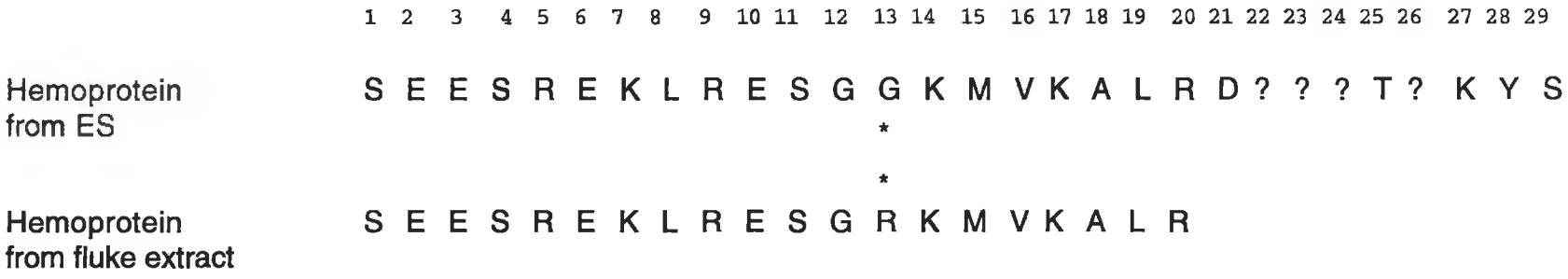

Figure 2.3

N-terminal sequence determination

N-terminal amino acid sequence of *Fasciola hepatica* hemoprotein, from ES and from mature liver fluke homogenate extracted in the presence of inhibitors. The single amino acid difference at position 13 is highlighted by asteriks.

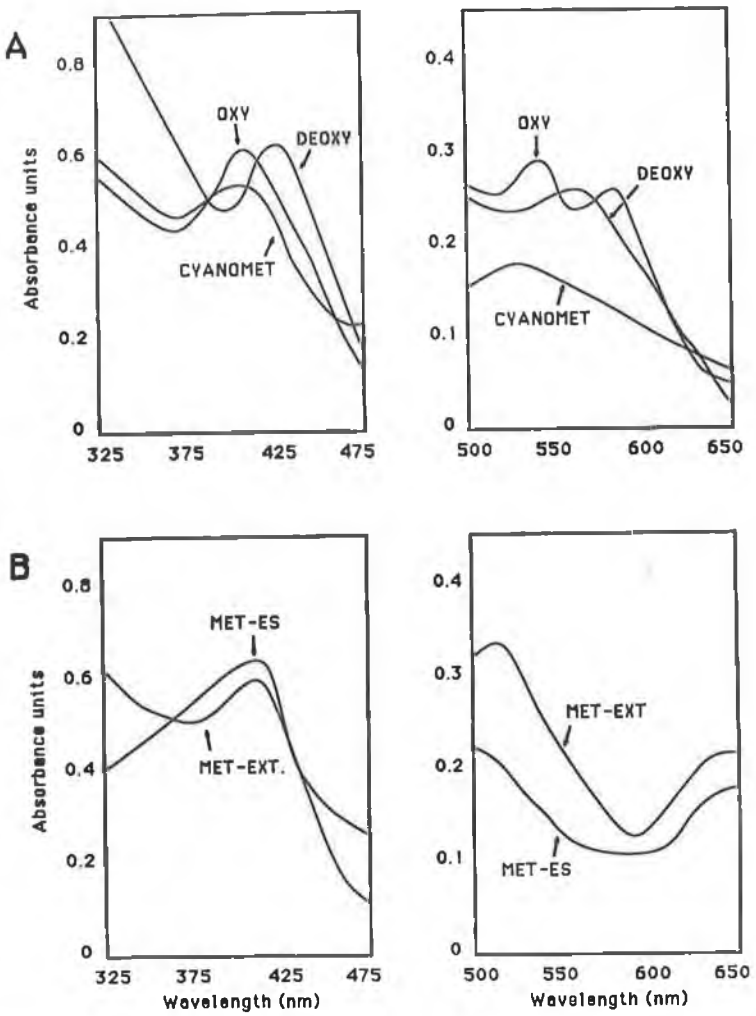

Figure 2.4

Absorption spectra analysis

A. The absorption spectrum, over the range 325- 650 nm, for the hemoprotein in mature liver fluke extracts. The spectra for the hemoprotein and its derivatives are characteristic of hemoglobins. Derivatives of the fluke hemoglobin were prepared as described in Methods 2.2.
OXY, oxy-hemoglobin; DEOXY, deoxy-hemoglobin; CYANOMET, cyanomet-hemoglobin.

B. Absorption spectrum of the purified hemoprotein compared to that of the oxidised (met-) derivative of the hemoglobin in mature fluke extracts. The spectra show that the purified hemoprotein is a hemoglobin in the oxidised or met- form. MET-EXT, oxidised mature fluke hemoglobin; MET-ES, purified hemoprotein.

showed a spectrum with maxima at 578 nm and 540 nm which is characteristic of the a and b peaks, respectively, of oxy-hemoglobins. The b peak is clearly higher than the a peak, a property which is unusual in mammalian vertebrates, but which is found in many parasite hemoglobins (Lee and Smith, 1965). In addition, a Soret peak (maxima 415 nm) which is characteristic of heme-containing proteins was also observed (Figure 2.4A). When the fluke hemoprotein was reduced to deoxy-hemoprotein the a and b peaks were no longer evident; however a flattened peak between 550 and 560 nm was seen. Furthermore, the Soret peak showed a maximum at 430 nm in the deoxy-hemoprotein (Figure 2.4A). The cyanomet derivative of the hemoprotein also lacked the a and b peaks but showed a peak at 540 nm and a Soret band at 420 nm (Figure 2.4A). The absorption spectra obtained confirmed that the liver fluke extract contains a hemoprotein with characteristics of a hemoglobin (Tsuneshige, *et al.*, 1989).

The purified hemoprotein shows an absorption spectrum with a slight peak around 500 nm, a second broad peak between 630 and 640nm and a Soret band at 400 nm (Figure 2.4B). This spectra was similar to that obtained for the hemoprotein in mature fluke extracts when it was oxidised to met-hemoglobin (Figure 2.4B). The oxidation of the purified hemoprotein to met-hemoglobin could not be reversed with the reducing agents sodium dithionite and iron (II) sulphate (data not shown as the spectrum was similar to that in Figure 2.4B).

ELISA studies

Using purified hemoglobin as antigen, ELISAs were carried out with serum obtained from cattle experimentally infected with metacercariae of *F. hepatica* (Figure 2.5). Anti-hemoglobin antibodies could be detected in all animals as early as one week after infection using serum dilutions of 1:100. Anti-hemoglobin antibodies were not detected in sera obtained from the cattle on the day of infection. The immune responses to the hemoglobin were highly variable between the four animals examined. Antibody titres rose in the first four weeks of infection and then remained steady until the final bleeding date of eleven weeks after infection. The titre of antibodies in the serum from week 4 to 11 after infection were >8,000 (data not shown).

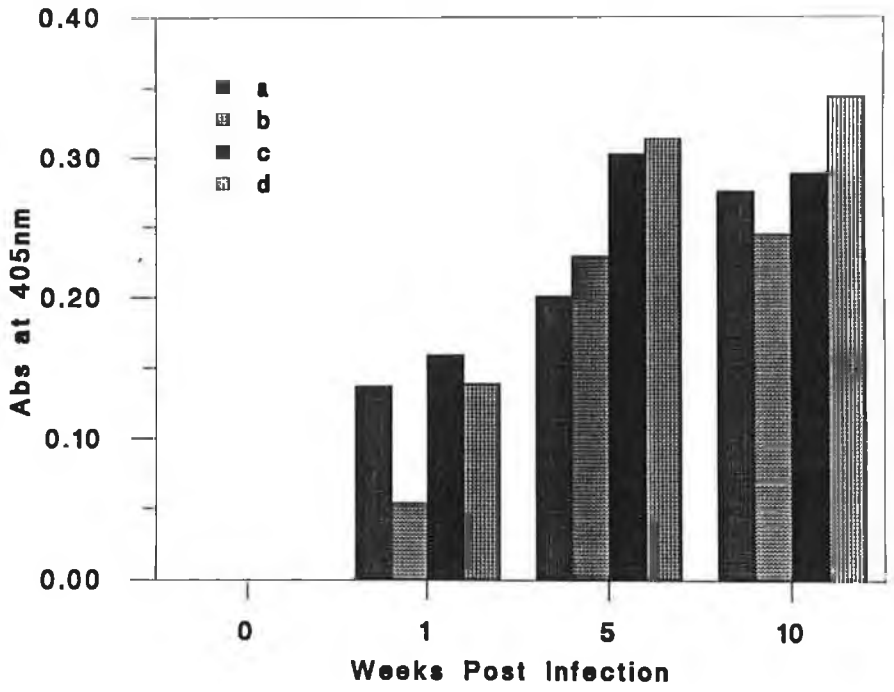

Figure 2.5

Enzyme linked immunosorbant assays (ELISA)

ELISAs were performed in 96-well tissue-culture plates using purified hemoprotein as antigen (1 μg/well) and 1:100 dilutions of sera. Serum was obtained weekly for up to 11 weeks from four cattle (a-d) experimentally infected with *ca.* 500 metacercariae of *Fasciola hepatica.* Results using sera obtained at weeks 1, 5 and 10 post-infection are shown.

Immunolocalisation studies

Immunolocalisation studies using antiserum prepared against the purified hemoglobin were carried out on sections of plastic embedded immature (three weeks old) and mature (23 weeks old) liver flukes obtained from infected rats. Antibody binding on sections of immature flukes was distributed throughout the parasite tissues including the tegument, sub-tegumental cells, parenchyma and within tubules that penetrated the parenchyma (Figure 2.6B). No antibody binding was observed with control rat serum (Figure 2.6A). The distribution of antibody binding on sections of mature flukes was located to tubules, parenchyma tissue and vitelline glands, but was absent from the tegument (Figure 2.6C & D).

A
B
C
D
V
+
I
I

Figure 2.6

Immunolocalisation studies

Antiserum prepared against the purified hemoglobin was used to probe sections of plastic embedded (JB4) immature and mature liver flukes.

A. Control serum used to probed sections of immature fluke containing tegument and parenchyma. No staining of tissues is observed.

B. Immunostaining of sections of immature flukes, as in A, with anti-hemoglobin serum. Fluorescent staining is observed in the tegumental syncytium (T) and in the sub-tegumental cells that lie below the longitudinal muscle layer (M) and connect to the tegument. These connections can be observed surrounding the muscle blocks. In the parenchyma staining is associated with tubules of the secretory system.

C. Immunostaining of section of ventral surface of mature flukes with anti-hemoglobin serum. Staining is seen associated with the tubules (t) which merge to form the excretory tubules (E).

D. Staining on sections taken in the caudal region of a mature fluke is associated with the excretory ducts (E) and vitelline glands (V) involved in the production of egg components. Bar, ~ 40 μm.

Chapter 3

Efficacy of hemoglobin as a vaccine against liver fluke infection

3.1 Materials

Compton Paddock Laboratories, UK
Fasciola hepatica metacercariae

Pierce Chemical Company
BCA protein assay kit

Sigma Chemical Company
Anti-bovine IgG conjugated to alkaline phosphatase (rabbit), Freund's complete and incomplete adjuvants, gelatin, horse spleen ferritin, p-nitrophenyl phosphate, prestained molecular weight markers, sodium dodecyl sulphate (SDS).

3.2 Methods

Preparation of antigens for vaccine trial

The hemoglobin fraction (Hf) was prepared by passing concentrated ES products over a Sephacryl S200HR gel filtration column, then pooling and concentrating the yellow coloured peak as described in methods 2.2.

F. hepatica cathepsin L2 (CL2) was isolated to homogeneity from ES products by gel filtration chromatography on Sephacryl S200HR followed by ion exchange chromatography on QAE Sephadex as previously detailed (Dowd *et al* , 1994). Briefly, CL2 is eluted from the gel filtration column in a broad protein peak which also contains another cysteine protease characterised by Smith *et al*, (1993a), as Cathepsin L1 (CL1). The two proteins are separated by ion exchange chromatography on QAE Sephadex; CL1 does not bind to the resin and is carried in the column run-through, whereas, CL2 binds to the Sephadex and is subsequently eluted with 400 mM NaCl. Purified CL2 is then concentrated and stored at -20^{o}C until required.

Both antigens were analysed by polyacrylamide gel electrophoresis prior to use in the trial; Hf was compared to total ES by native PAGE and CL2 was compared to ES using reducing SDS PAGE. Protein concentration of antigen preparations was determined by the MicroBCA protein assay (Redinbaugh and Turley, 1986), using bovine serum albumin as a standard. The purified antigens were dialysed against distilled water, freeze dried in aliquots of 200 µgs and stored at -20^{o}C.

Formulation and administration of vaccines

On the day prior to vaccine administration, freeze dried antigens were reconstituted in PBS and the vaccines formulated by mixing 1 ml of the reconstituted antigen with an equal volume of Freunds complete adjuvant (FCA) or Freunds incomplete adjuvant (FIA). The mixture was emulsified by sonicating (7 x 30 sec bursts, duty cycle 0.7 sec) on ice.

Vaccination of cattle

Twenty one male (castrated and entire) Holstein-Freisian calves, aged one year, were randomly allocated into three groups, each comprising seven

calves. Calves in Group 1 received 200 μg hemoglobin fraction (Hf) per injection; Group 2 received 200 μg of both Hf and cathepsin L2 per injection and Group 3 (control) received 200 μg horse spleen ferritin per injection. The first immunisation with two ml of antigen preparation, formulated in an equal volume of FCA, was injected into the *biceps femoris* of the left hind leg on day 0. Booster injections into the *biceps femoris* of the right hind leg on day 35 (week 5) and into the *gluteus medius* on day 63 (week 9) were formulated in FIA.

Parasite challenge

Metacercariae of *F. hepatica* (less than four weeks old) were counted and placed in a gelatin capsule which was inserted into a gelatin / agar bolus (Andrews *et al*, 1995) and administered *per os* to each animal using an oesophageal balling gun. All animals received *ca.* 600 metacercariae, 16 days following the third immunisation (week 11).

Analysis of antibody responses by enzyme linked immunosorbant assay (ELISA)

Antibody responses were also examined by ELISA. Microtitre plates were coated with *F. hepatica* ES products, 2 μg per well, by adding the antigen in PBS to the wells and incubating overnight at 37^{o}C. The excess binding sites were blocked with 2% BSA and 0.1% Tween 20 in PBS at 37^{o}C for one hr. After washing three times with 0.1% Tween in PBS (T-PBS) the bovine sera (100 μl, 1:8000 dilution) was added to the wells and the plates incubated at 37^{o}C for one hr. Bound antibody was detected using alkaline phosphatase conjugated rabbit anti-bovine IgG and the substrate p-nitrophenyl phosphate.

Immunoblot analysis of cattle antibody responses

Mature *F. hepatica* ES products were separated on 10 % native polyacrylamide gels for analysis of Hf responses (Laemmli *et al* 1970). Gel proteins were transferred to nitrocellulose paper and non-specific sites were blocked as previously detailed in Methods 2.2. The nitrocellulose filters were used to analyse the specificity of the immune responses of vaccinated and control cattle. Serum for immunoblots was taken on week

11, prior to challenge and week 17, six weeks post-challenge. Filters were probed with pooled group serum (1:500 dilution), and bound immunoglobulin (IgG) was visualised using alkaline phosphatase conjugated anti-bovine IgG. NBT and BCIP were used as substrates.

Liver enzyme analysis

Liver damage in all vaccinated and control animals was assessed by measuring the serum levels of the liver enzymes glutamic dehydrogenase (GLDH, EC 1.4.1.3.) and γ glutamyl transferase (γGT, EC 2.3.2.2.) in blood samples taken weekly from the time of challenge infection to the day of slaughter. Liver enzyme analysis was carried out by the Veterinary Research Laboratory, Abbotstown, Co. Dublin.

Assessment of protection

All cattle were humanely slaughtered thirteen weeks after challenge. The duodenum, 6 inches each side of the common bile duct, was ligated and removed with the liver and gall bladder. The fluke burdens of the livers were assessed by Drs Grace Mulcahy and Diane Cleary at UCD Veterinary college, Ballsbridge, Dublin 4. The total number of flukes in the liver was estimated by standard techniques (MAFF) and the number of flukes < and > 6 mm long was also recorded. Total worm burdens were summarised for the vaccinated and control groups using arithmetic means. Statistical differences between vaccinates and control were tested using analysis of variance. Any differences found to be significant at a 95% level (P = 0.05) or more were further investigated using a multiple range test.

Estimation of egg viability

The viability of recovered liver fluke eggs was estimated by Drs Grace Mulcahy and Diane Cleary at UCD Veterinary College. The gall bladders were separated from the livers, sliced open and the bile decanted into a conical vessel. After one hour the supernatant was siphoned off and the deposit containing the *F. hepatica* eggs, was washed five times with water. The eggs were then incubated in darkness at 22^{o}C for 14 days. After this incubation period they were examined daily for miracidial development. Viability was assessed by estimating the percentage of eggs that had embryonated and developed to miracidia. In cases where low numbers of

eggs were recovered from the gall bladder all eggs were considered in the calculation, and in those where many eggs were recovered 300 eggs was taken as representative.

3.3 Results

Purification of vaccine components

Hemoglobin fraction (Hf) only and Hf in combination with cathepsin L2 (CL2) were tested in the vaccine trial. Both antigens were compared to ES by polyacrylamide gel electrophoresis (PAGE) prior to formulation and administration. Native PAGE of total ES products shows a major protein secreted by these parasites (Figure 3.1A lane 1). This molecule has been characterised as liver fluke hemoglobin and is not observed in reducing SDS PAGE. Hf, isolated by gel filtration appears 80 - 90% pure (Figure 3.1A lane 2). The hemoglobin is susceptible to proteolytic cleavage in ES and therefore migrates as a single band preceded by a smear.

When ES products are resolved by reducing SDS PAGE two major proteins are observed (Figure 3.1B lane 1). The larger of these, cathepsin L2 (molecular mass 29.5 kDa) was purified to homogeneity by gel filtration followed by ion exchange chromatography, and can be seen as a single band in Figure 3.1B, lane 2.

Studies of immune response by ELISA

The antibody response of vaccinated and control animals, during immunisations and following challenge, was analysed by ELISA using adult *F. hepatica* ES products as antigen. Figure 3.2A shows mean antibody levels of each group prior to parasite challenge, using serum dilutions of 1:8000. Antibodies were observed in all vaccinated animals two weeks after the first immunisation. A boosting of antibody responses was observed in the vaccinates (Groups 1 & 2) following both the second and third immunisations (Figure 3.2A). The pre-challenge sera of control animals (Group 3) did not contain antibodies reactive with ES antigens (Figure 3.2A).

An increase in the antibody titres in all vaccinated animals was observed within one week following challenge (Figure 3.2B). Since the animals were challenged two weeks after the final immunisation this observed increase in antibody titre may be due to the maturation of the immune response to the vaccination and to boosting by invading parasites. Antibodies in all vaccinated groups remained high throughout the infection. The level of antibodies in the sera of the control group (Group 3) increased

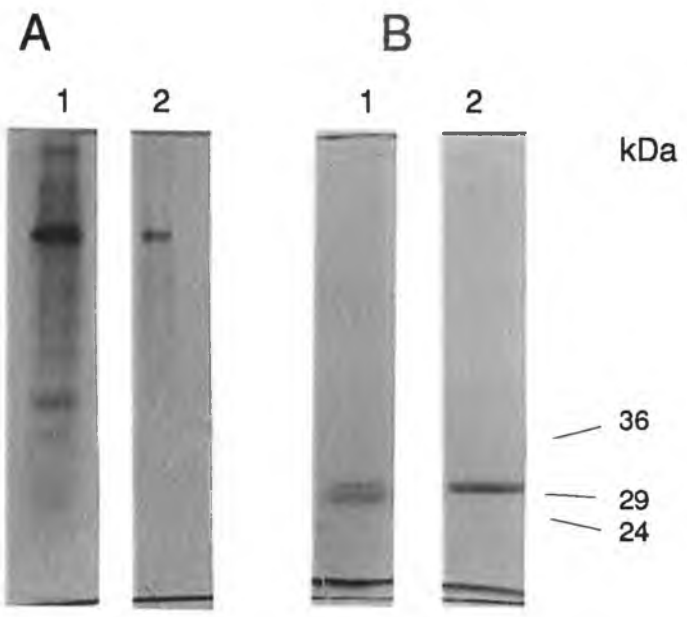

Figure 3.1

Polyacrylamide gel electrophoresis of vaccine components

A. Analysis of ES products (lane 1) and hemoglobin fraction (lane 2) by non denaturing PAGE reveals that the hemoglobin band is the major protein in both these preparations.

B. Reducing SDS PAGE analysis of ES products and cathepsin L2 shows that the CL2 migrates as a single band of molecular mass 29.5 kDa. Molecular size markers are indicated.

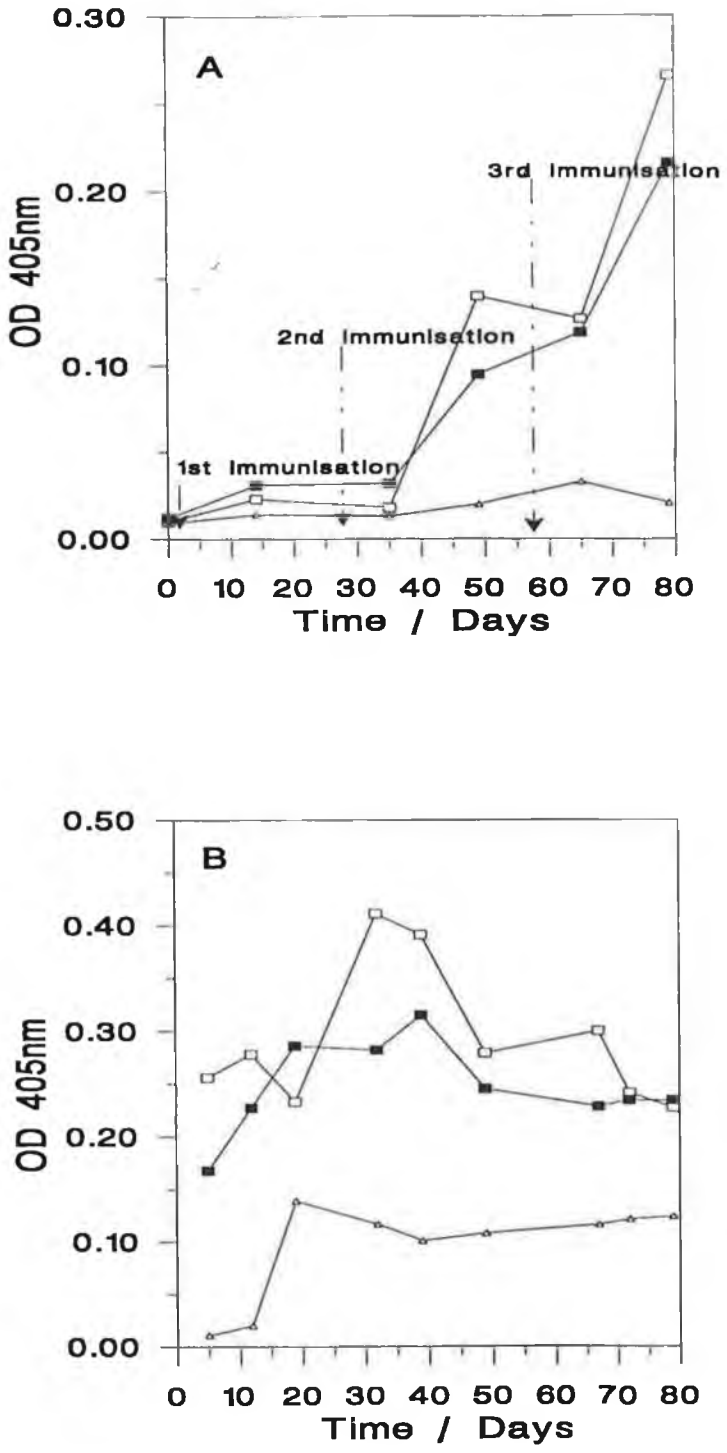

Figure 3.2

Analysis of antibody responses in vaccinates and control animals by ELISA

Sera was taken during the course of vaccination (Panel A) and following the challenge infection (Panel B). Adult liver fluke ES products was used as antigen and was probed with sera from each animal (1:8000 dilution). Each point represents the mean responses in animals immunised with Hf (Group 1 □), Hf and cathepsin L2 (Group 2, ■) or horse spleen ferritin (Group 3, controls, △) per immunisation.

within the first two to three weeks after infection and then remained constant throughout the subsequent weeks (Figure 3.2B).

Immunoblot studies of antibody response

The specificity of antibody responses of animals prior to and after parasite challenge were analysed by immunoblotting using adult *F. hepatica* ES products as antigen. Anti-Hf responses were analysed by transfer of ES proteins from native 10% polyacrylamide gels to nitrocellulose filters. These blots showed that the sera taken on the day of parasite challenge (week 11) and six weeks post-challenge (week 17) from animals vaccinated with Hf (Groups 1&2), contained antibodies reactive with the Hf in the liver fluke ES products (Figure 3.3A & B lanes 1 & 2). Antibodies were not detected in the sera obtained from animals in the control group (Group 3) at the time of and six weeks after infection (Figure 3.3A & B lane 3).

The sera taken on the day of challenge and at six weeks post-challenge from animals vaccinated with Hf and cathepsin L2 (Group 2), contains antibodies reactive with the 27.5 kDa cathepsin L1 and 29.5 kDa cathepsin L2 on immunoblots of ES transferred from 10% reducing SDS polyacrylamide gels (unpublished results, Dowd and Dalton). This reaction of the sera with both cathepsins is not surprising, since it has been previously shown that the two molecules share common epitopes (Dowd *et al* 1994). Sera obtained from the control animals (Group 3) contained antibodies reactive with CL1 and CL2 at 6 weeks post challenge, but not at the time of challenge.

Liver enzyme analysis

Since extensive liver damage is a pathological consequence of liver fluke infection, the extent of the damage in vaccinates and control animals was examined by measuring their serum levels of glutamate dehydrogenase (GLDH) and γ glutamyl transferase (γGT), throughout the course of the infection. Increased GLDH serum levels is indicative of liver damage, particularly to the liver parenchyma cells. In liver fluke infections this enzyme is observed in the serum as immature flukes enter the liver tissue, and its concentration rises as damage ensues. In all vaccinated and control animals a rise in serum GLDH was observed within six weeks of the

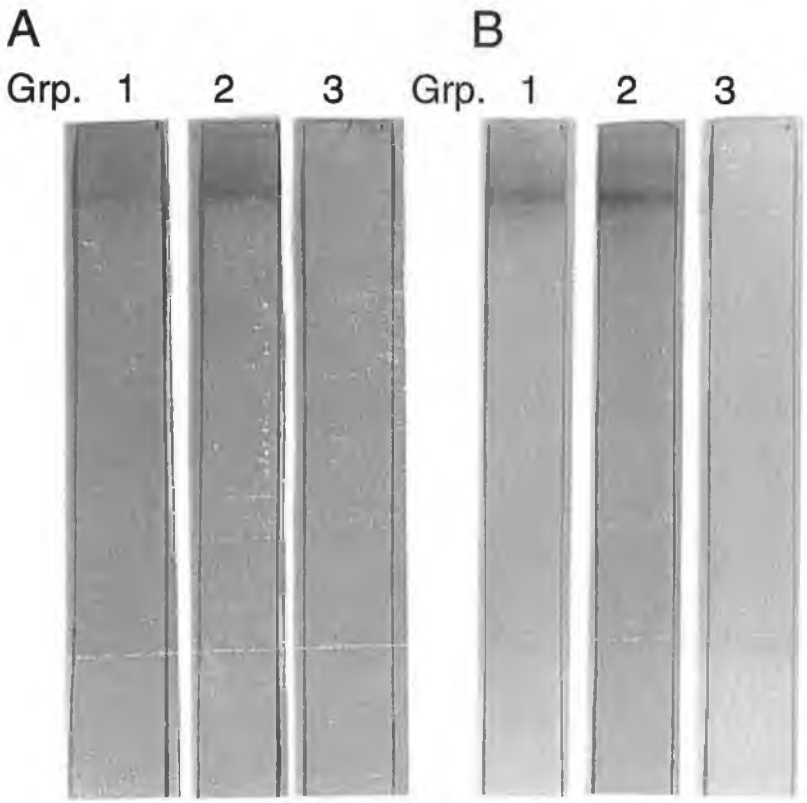

Figure 3.3

Immunoblot analysis of sera from vaccinated and control animals

Adult liver fluke ES products was separated by 10% native PAGE and transferred to nitrocellulose. Pools of sera obtained from animals in each group at the time of challenge (A) and six weeks following infection (B) were used to probe the nitrocellulose filters. Animals were immunised with Hf (Group 1), Hf and cathepsin L2 (Group 2), or horse spleen ferritin (Group 3, controls).

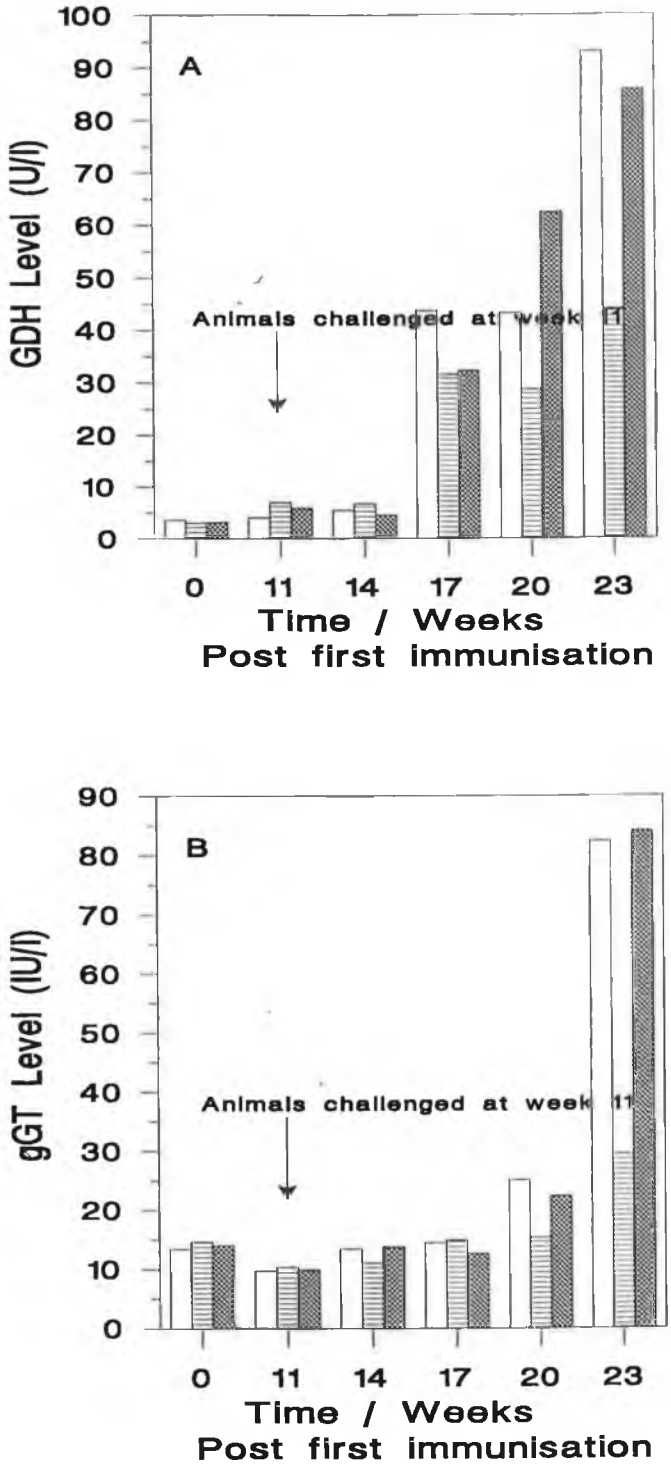

Figure 3.4

Liver enzyme analysis

Analysis of the levels of glutamic dehydrogenase (GDH, Panel A) and γ glutamyl transferase (γGT, Panel B) in the sera of vaccinated and control cattle. Sera was obtained three-weekly, before and after challenge, from all animals immunised with Hf (Group 1, clear bars), Hf and cathepsin L2 (Group 2, bars with horizontal lines) or horse spleen ferritin (Group 3, controls, hatched bars).

challenge infection; at this time point there was no significant difference between the vaccine groups and the control group (Figure 3.4A). As the infection progressed the mean serum GLDH levels increased in the animals in Group 1 and in the control group animals. However, from six weeks after infection to the day of slaughter the mean serum GLDH levels in the animals that were vaccinated with Hf and cathepsin L2 (Group 2) did not increase significantly. The serum GLDH levels in this group from six weeks after infection to the week of slaughter were significantly lower ($P < 0.05$) than that observed in animals vaccinated with Hf alone (Group 1), and the control animals (Group 3) (Figure 3.4A).

Increased serum γGT is indicative of hyperplasia in the bile ducts, and in liver fluke infections it is associated with the arrival and residence of flukes in the bile ducts, which begins at approximately 9 weeks after infection. The mean serum γGT levels in the control group animals (Group 3) increased sharply from nine to twelve weeks after infection (Figure 3.4B). The mean γGT levels in the animals vaccinated with Hf alone (Group 1) showed a similar increase to the control group. The γGT levels of animals vaccinated with a combination of Hf and cathepsin L2 (Group 2) had not increased above the pre-infection levels at nine weeks after infection and were only slightly increased at 12 weeks after infection. The γGT levels of this group were significantly lower than that observed for Group 1 and the control group (Group 3) ($P < 0.05$) (Figure 3.4B).

Assessment of Protection

Fluke burdens were assessed in all animals thirteen weeks after the metacercariae challenge (Table 3.1). The mean recovery of liver flukes from the control animals (Group 3) was 152.1 which represents 25.4% of the infection dose. Both vaccinated groups (Groups 1&2) exhibited fluke burdens which were significantly lower than the control group ($P<0.05$). Vaccination with Hf alone (Group 1) induced a high level of protection (43.8%). However, vaccination with the cocktail of Hf and cathepsin L2 (Group 2) induced a protection level of 72.4% which was significantly higher ($P < 0.05$) than that obtained with Hf alone (Group 1).

We also examined whether vaccination had an effect on the growth of flukes that were recovered from the livers of animals vaccinated with Hf

Table 3.1

*Assessment of protection following vaccination of cattle with Hf and cathepsin L2**

Group (Ag)	*Flukes >6 mm*	*Flukes <6 mm*	*Total flukes*	*%Reduction vs control*
Group 1 (Hf)	26.4 +/- 3	59.1 +/- 8	85.5 +/- 8.2	43.8
Group 2 (Hf and CL2)	8.0 +/- 5	34.0 +/- 11	42.0 +/- 16	72.4
Group 3 (HSF, control)	78.4 +/- 16	73.7 +/- 14	152.1 +/- 20	-

* all vaccinations formulated in Freunds complete and incomplete adjuvant.

alone (Group 1) and Hf and CL2 together (Group 2). For this reason the recovered parasites were separated into size groups of < and > 6 mm (Table 3.1). This size was chosen as an indicator of development since liver fluke parasites reach approximately 6 mm before migrating into the bile duct and developing to full maturity. However, flukes of <6 mm may not necessarily have been recovered from the liver parenchyma nor have undeveloped internal organs. In the control animals the mean number of recovered flukes that were <6 mm was approximately equal to the number that were >6 mm. However in the vaccinates the recovered parasites tended to be smaller. In group 1 animals (Hf only) *ca* 30% of recovered flukes reached >6mm and in animals vaccinated with Hf and CL2 the mean number of recovered parasites developing beyond 6 mm was only *ca* 20% (Table 3.1).

Viability of recovered fluke eggs

The effect of vaccination on the viability of eggs produced by worms that developed to maturity in the vaccinated animals was examined. The viability data is presented in Table 3.2 for eggs recovered from each animal in the trial. Eggs with a viability of 96-100% were recovered from the gall bladder of all animals in the control group (Group 3). The viability of eggs recovered from animals vaccinated with Hf (Group 1) ranged between 30 - 75% (mean 35%). Eggs were not recovered from three of the seven animals vaccinated with Hf and cathepsin L2 (Group 2) probably because the flukes did not develop to maturity. Eggs were recovered from the other four animals in this group, however, for three of these no eggs embryonated to miracidia, while only 7% of the eggs from the remaining animal were viable. This data shows that vaccination with a combination of Hf and cathepsin L2 elicited an overall anti-embryonation effect of >98% (Table 3.2).

Table 3.2

Viability of F. hepatica eggs recovered from gall bladders of cattle

Group (Ag)	*Animal no. (%)*	*Egg Viability*
Group 1 (Hf)	4053	75
	4080	35
	4108	30
	4071	nd
	4109	42
	4138	partial*
	4105	30
Group 2 (Hf and Cl2)	4058	7
	4110	no eggs
	4124	no eggs
	4076	partial
	4068	partial
	4125	partial
	4136	no eggs
Group 3 (HSF, control)	4142	98
	4113	98
	4143	100
	4147	100
	4137	96
	4057	100
	4114	100

* partially embryonated, containing a blastocyst but no miracidium.

Chapter 4

Immunoscreening of *Fasciola hepatica* cDNA library and identification of clones

4.1 Materials

Amersham

Alpha ^{32}P dATP

AMS Biotechnology Ltd.

RNAzol™ B

Kodak

X-Omat X-ray film, FX 40 liquid Fixer, LX 24 developer
667 Polaroid film

Promega

Agarose, Anti-β galactosidase antibody labelled with alkaline phosphatase (mouse), Apa I, 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal), dNTP's, EcoR I, Hind III, isopropylthio-β-D-galactoside (IPTG), pGem DNA markers, pGem®T vector system, Prime-a-Gene® system, Sac I, Taq DNA Polymerase, Wizard™ λ preps, Wizard™ DNA clean-up system.

Sigma Chemical Company

Adenine diphosphate (ADP), anti-bovine IgG conjugated to alkaline phosphatase (rabbit), diethylpyrocarbonate (DEPC), dithiothreitol (DTT), glutamine, glutamine synthetase, lysozyme, proteinase K, salmon sperm DNA

4.2 Methods

Immunoscreening of F. hepatica λ gt11 cDNA expression library

1. *Preparation of λ gt11 cDNA library*

A λ gt11 cDNA library was prepared in our laboratory by the following standard method (Promega Handbook). Total RNA was isolated from mature adult flukes using RNAzol™. From this, mRNA was isolated by binding to an oligo dT column. Double stranded cDNA was generated from the mRNA using the Riboclone® cDNA synthesis kit. EcoR I linker arms were added to the cDNA, which was then ligated to gt11 arms and packaged into λ heads using the Packagene® system. The packaged phage was titred and then amplified by infecting phage competent *E. coli* Y1090 cells (overnight culture grown in LB media with 0.2% maltose and 10 mM $MgSO_4$) with dilutions of the phage, incubating at room temperature for 20 min and then plating the bacteria in top agar onto LB agar plates with 100 μg ml^{-1} ampicillin.

2. *Preparation of sera for immunoscreening*

The cDNA library was immunologically screened using a pool of sera from animals vaccinated with hemoglobin fraction (Group 1, see section 3.2). The sera was obtained following three vaccinations with Hf and prior to parasite challenge. Before use the sera was pre-adsorbed to remove all antibodies reactive with *E. coli* proteins. This was achieved by incubating the sera with nitrocellulose discs containing bound *E. coli* proteins at room temperature for 6 h. This adsorption procedure was repeated three times. The discs were prepared by incubating the discs in a sonicated extract of *E. coli* cells (10 x 30 sec bursts, duty cycle 0.7 sec) for 24 h at 4°C and then blocking the excess sites with 1% BSA / T-PBS. Sera was incubated with discs, removed, centrifuged and stored at 4°C until required.

3. *Immunoscreening of λ library*

Phage competent *E. coli* Y1090 were infected with 1:50 dilution of phage.

Following an incubation for 20 min at room temperature the cells were plated in top agar on LB ampicillin plates and incubated at 42°C until plaques were visible (*ca* 3 h). Nitrocellulose discs which had been soaked in 10 mM IPTG and air dried, were carefully placed on the plates and their orientation was marked by three needle stabs. The plates were incubated for 4 h at 37°C, the discs were then carefully removed and blocked overnight in 1% BSA / T-PBS, before probing with the pre-adsorbed bovine antisera (1:500 dilution). Following washing in T-PBS bound antibody was detected using alkaline phosphatase labelled anti-bovine IgG, with NBT and BCIP as substrate. Positive plaques appeared as purple rings. These plaques were removed as an agar plug using a sterile pasteur pipette, transferred to 1 ml phage buffer (10 mM $MgSO_4$, 100 mM NaCl, 20 mM Tris-HCl, pH 7.4) and allowed to diffuse at 4°C overnight. Individual phage were re-plated and the antibody screening repeated two additional times or until pure plaques were obtained *i.e.* when all plaques on a plate were reactive with the antibody.

Preparation of λ lysates and isolation of DNA

Isolated plaques were picked into 200 µl 0.1XSM buffer (0.01% gelatin, 8 mM $MgSO_4$, 100 mM NaCl, 50 mM Tris-HCl, pH7.5) and incubated overnight at 4°C. One hundred µl was used to infect competent Y1090 cells, which were plated as before and incubated at 42°C until confluent lysis was observed (*ca* 5 h). Four ml 0.1X SM buffer was added to the plate and after an overnight incubation at 4°C the buffer was removed. Chloroform was added (0.5% final concentration) and the lysate was stored at 4°C until required.

PCR analysis of λ DNA

Polymerase Chain Reaction (PCR) was employed to size and isolate inserts from the phage library, using universal λ primers. These primers are derived from the sequence flanking the EcoR I cloning site of the λ gt11 vector. Twenty µl of stock λ lysates was added to 180 µl water and boiled for 10 minutes and then 1µl was used per 50 µl PCR. Each PCR vial

consisted of the following mix:

10X Polymerase buffer	5.0 µl
dNTP's (1 mM each)	5.0 µl
$MgCl_2$ (25 mM)	6.0 µl
Sterile distilled water	30.7 µl
λ forward primer (50 ng μl^{-1})	1.0 µl
λ reverse primer (50 ng μl^{-1})	1.0 µl
Taq Polymerase (5U μl^{-1})	0.3 µl
λ lysate DNA	1.0 µl

Each mix was overlaid with 70 µl mineral oil, placed in the Hybaid Omnigene Thermal Cycler, and the PCR carried out as follows:

Stage 1	(Denaturation)	94°C for 4 min
Stage 2	(Denaturation)	94°C for 30 sec
	(Annealing)	55°C for 1 min
	(Extension)	74°C for 1 min 30 sec
-stage 2 was repeated for 35 cycles		
Stage 3	(Extension)	74°C for 4min

25 µl of PCR reactions were analysed by agarose gel electrophoresis as detailed in Sambrook *et al* (1989).

Sub Cloning of PCR fragments

PCR amplified gene fragments were excised from the gel. The agarose was disrupted using glass beads, and the recovered DNA was purified using the Wizard™ DNA clean-up system (Promega). The fragments were then sub cloned directly into the pGem®-T plasmid,as follows:

1 µl (2 5 ng) pGem®-T vector, 8 µl ligase buffer (10 mM ATP, 100 mM $MgCl_2$, 100 mM DTT, 300 mM tris-HCl, pH 7.8), 1 U T4 DNA ligase and 100 ng insert DNA were mixed gently and the ligation was allowed to proceed overnight at 4°C.

Competent cells were prepared using one of the following methods:

(a) calcium chloride transformation

A log phase culture of *E. coli* JM109 cells was aliquoted, placed on ice for 5 min, centrifuged at 12,000 x*g* for 2 min and the supernatant removed. The cells were gently resuspended with 1 ml of cold $CaCl_2$ and incubated on ice for 30 min. The cells were spun again and resuspended in 0.5 ml cold $CaCl_2$. 10 µl ligation mix was carefully added to 50 µl aliquots of cells and placed on ice for a further 30 min. The cells were then heat shocked at 42°C for 90 sec and returned to ice for 2 - 5 min. Immediately after transformation 950 µl pre-warmed LB media was added and the cells incubated at 37°C for 1 h. Cells were concentrated by centrifugation and spread on LB plates containing 100 µg ml^{-1} ampicillin, 0.5 mM IPTG and 40 µg ml^{-1} X-Gal (for blue white selection).

(b) electroporation

A log phase culture of *E. coli* XL1-blue electrocompetent cells was concentrated by centrifugation and aliquoted. 2.5 µl ligation reaction was added to 300 µl cells, gently mixed and placed in 0.2 µm electroporation cuvettes. The cells were then transformed by electroporating under the following conditions: the pulse generator was set at 25 µF, 2.48 kV, and 200 Ω. One pulse at these settings results in a pulse of 12.5 kV cm^{-1} with a time constant of *ca* 4sec. 1 ml pre-warmed SOC (containing 20 mM glucose) medium was added immediately and the cells were incubated for 1 h at 37°C, before concentrating and plating as before.

Plates spread with transformed cells were incubated overnight at 37°C.

Screening of recombinant plasmids

With X-Gal and IPTG colour screening, recombinant colonies should be white and colonies with no insert DNA blue. White colonies were picked into 2 ml LB with 100 µg ml^{-1} ampicillin (and 15 µg ml^{-1} tetracycline for XL blue cells), and incubated overnight at 37°C. Plasmid DNA from 1 ml of this mini prep culture was isolated by either the boiling or alkali lysis method described by Sambrook *et al* (1989). The DNA was double digested with the restriction enzymes Sac I and Apa I and the inserts observed on agarose gel electrophoresis.

Sequencing of Plasmid DNA

Purified plasmid DNA from positive clones was further cleaned up using Wizard™ λ preps. The DNA was sent for sequence analysis to the Department of Biological Sciences, Durham University or BioResearch Ireland, Trinity College Dublin.

Preparation of fusion protein

Sequence analysis revealed that clone B1 was a novel fluke antioxidant protein (peroxiredoxin). We therefore further characterised this clone. Fusion protein from the λ B1 clone was prepared by the plate wash supernatant method. Phage competent *E. coli* Y1090 were infected with 10,000 pfu recombinant phage and incubated for 20 min at room temperature, before pouring onto LB ampicillin plates in top agar. The plates were incubated at 42°C for 3 h (lysis almost confluent), then 5 ml phage buffer containing 1 mM EDTA, 1 mM PMSF, 1 mM iodoacetamide and 10 mM IPTG was added to the plates which were incubated at 37°C overnight. The buffer was recovered and the top agar was also scraped into a centrifuge tube. This was vortexed for 20 sec before centrifuging at 10,000 x*g* for 10 min at 4°C. The supernatant was removed to microfuge tubes which were spun again at 12,000 x*g* . Supernatants were stored at -20°C until required.

The fusion protein was analysed by reducing SDS polyacrylamide gel electrophoresis followed by silver staining, and by immunoblotting using an anti-β galactosidase primary antibody.

Preparation of radiolabelled DNA probe

A 400 bp fragment was PCR amplified from clone B1 I DNA using the following consensus primers,designed from comparing the protein sequences of the peroxiredoxin antioxidant family. These primers crossed the regions that code for the conserved active site regions, cys 47 (VCP 47) and cys 168 (VCP 168).

VCP 47 forward primer (Shem F)

5' GAT TTY ACW TTY GTN TGT CCW ACW GAR -3'

VCP 168 reverse primer (SmTSAR)

5' GGW CAN ACY TCW CCA TGY TC -3'

where Y =T or C, W= A or G and N = T, C, A or G

The PCR product was excised from an agarose gel and cleaned as before. The fragment was labelled with Alpha ^{32}P by random priming using the Promega Prime-a-Gene® system. The reaction mix was as follows:

5X labelling buffer	10 µl
(250 mM tris-HCl, pH 8.0, 25 mM $MgCl_2$, 10 mM DTT, 1 mM HEPES, pH 6.6, 26 A_{260}units ml^{-1} random hexadeoxyribonucleotides)	
mixture of dCTP, dGTP, dTTP (100 mM each)	2 µl
acetylated BSA 10 mg ml^{-1}	2 µl
denatured DNA probe	25 ng
sterile water	25 µl
alpha ^{32}P dATP (50 µCi, 3,000 Ci $mMol^{-1}$)	5 µl
Klenow enzyme	5 U

The reaction tube was mixed gently and incubated at room temperature for 1 h. 200 µl 0.5 M EDTA was added and the reaction terminated by boiling for 2 min. The probe was now ready for use in hybridisation reactions.

Isolation of RNA and northern blotting

1. *Isolation of adult fluke RNA*

Mature flukes were cultured overnight as previously described (Methods 2.2), to allow clearing of the gut contents which could contain host cells. Appproximately 10 flukes (1 gram tissue) were placed in a centrifuge tube, 5 ml RNAzol™ was added and the flukes were homogenised at top speed for 30 sec using a Thyristor Regler TR50 homogeniser. One ml chloroform was added and the solution was shaken vigorously for 15 sec and placed on ice for 5 min. After aliquoting into microfuge tubes the solution was centrifuged at 13,000 x*g* for 15 min at 4°C and two layers formed. The upper aqueous phase was removed to a new tube, an equal volume of isopropanol was added and the samples were incubated at

4°C for 15 min (or aliquoted for long term storage at -80°C). They were recentrifuged for 15 min, the supernatant was removed and the RNA pellet washed with 75% ethanol before drying and reconstitution with 200 µl 0.1% DEPC treated water. Bovine RNA was isolated using the same procedure with 1 g fresh bovine liver as starting material. The RNA was analysed by electrophoresis on agarose gels containing formaldehyde as detailed in Sambrook *et al* (1989).

2. *Northern blotting*

Following electrophoresis the gel was rinsed with DEPC treated water to remove the formaldehyde and the RNA was transferred onto nitrocellulose membrane by the capillary transfer method outlined by Sambrook *et al,* (1989). RNA fragments are carried from the gel in a flow of buffer and deposited on the surface of the nitrcellulose. Following transfer, the RNA was fixed onto the membrane by baking for 2 h at 80°C in an oven.

3. *Hybridisation with radiolabelled probe*

The nitrocellulose filter was soaked in 6X SSC (0.9 M NaCl, 90 mM sodium citrate pH 7.0) until thoroughly wetted and placed in a heat-sealable bag. Then, 200 ml prehybridisation solution (6X SSC, 5X Denhardt's reagent, 0.5% SDS, 100 µg ml^{-1} denatured, fragmented salmon sperm DNA) was added to the bag. As much air as possible was squeezed from the bag which was sealed and incubated overnight at 68°C. Following incubation the bag was opened by removing a corner and the radiolabelled probe carefully added. The resealed bag was then placed in a second sealed bag and incubated again at 68°C for 24 h. The hybridisation solution was carefully poured into a suitable container and the filters were removed and immediately submerged in 300 ml 2X SSC and 0.1% SDS. The filters were incubated with gentle agitation at room temperature for 15 min. The wash solution was replaced twice and the incubation repeated. Then 0.1X SSC and 0.5% SDS was added to the filters which were further incubated at 68°C for 1 h. Filters were rinsed with 0.1X SSC to remove the SDS, blotted briefly on paper towels and wrapped in clingfilm, and then exposed to X-ray film at -80°C to obtain an autoradiographic image. Exposure for 24 h at 80°C with an intensifying screen was required to obtain an image.

Assay of mature fluke extract for novel antioxidant activity

Antioxidant activity in mature liver fluke extract was measured by monitoring its ability to inhibit the thiol / iron / oxygen mediated inactivation of glutamine synthetase. Assays were performed in microtitre plates in a 100 µl reaction volume containing 0.5 U glutamine synthetase (*E. coli*), in the presence or absence of inactivation solutions and protector protein (liver fluke homogenate). Inactivation solutions consisted of 15 µM $FeCl_3$ and either 5 mM DTT or 14 mM 2-mercaptoethanol (final concentrations). After incubation for 10 min at 37^{o}C remaining glutamine synthetase activity was measured by adding 100 µl of γ glutamyl transferase assay mixture. This contained 0.4 mM ADP, 150 mM glutamine, 10 mM potassium arsenate, 0.4 mM manganese chloride, 20 mM hydroxylammonium chloride in 50 mM imidazole-HCl, pH 7.0. The reaction was incubated at 37^{o}C for 30 min and terminated by the addition of 50 µl stop mixture, consisting of 55 g $FeCl_3 \cdot 6H_2O$, 20 g trichlororacetic acid and 21 ml concentrated HCl per litre. An absorbance resulting from the γ glutamyl hydroxamate-Fe^{3+} complex was measured at 540 nm. In the absence of "protector protein" under these conditions 70 to 100% of glutamine synthetase activity was lost.

Time course analysis of antioxidant protection of glutamine synthetase from inactivation by thiol iron oxidation systems

Glutamine synthetase (0.5 U) was incubated in the presence of 15 µM $FeCl_3$, with 5 mM DTT or 14 mM 2-mercaptoethanol at 30^{o}C for various times from 1 to 60 min. The reaction mixes in a total volume of 100 µl included various amounts of LFH. Following incubation, the mixtures were analysed for remaining glutamine synthetase activity using the γ glutamyl transferase assay as before.

4.3 Results

Immunoscreening of F. hepatica cDNA library and analysis of isolated clones by PCR and restriction digestion

Bovine sera from the vaccine trial was used to screen a *Fasciola hepatica* cDNA library constructed in λ gt11 phage. The serum pool used was obtained on the day of parasite challenge (week 11) from animals immunised with hemoglobin fraction (Group 1). These animals showed a mean level of protection from parasite challenge of 43.8%. This sera should contain antibodies reactive with hemoglobin and any other antigens present in the immunising fraction.

Ten plates with *ca* 2,000 pfu on each were used in the primary screening with a 1:500 dilution of pre-adsorbed sera. Thirty positive plaques were chosen and these were subjected to three or four further rounds of screening until all plaques on the plates were positive indicating pure clones. Lysates of positive plaques were then prepared and the DNA analysed by PCR using λ forward and reverse primers. Of the thirty positives selected only twenty produced PCR products; the remaining ten were therefore disregarded. Clones were classified into groups on the basis of the size of the PCR fragment (Figure 4.1).

Group	*Size of PCR fragment*	*Clones*
1	~1700 bp	D6
2	~1600 bp	B5 & D5
3	~1400 bp	A1, A4, A5, B1, B4, B6, E3
4	~1100 bp	C4
5	~1000 bp	C2, D1, D7, E2
6	~ 900 bp	D8
7	~ 700 bp	C1 & D3
8	~ 650 bp	A8
9	~ 550 bp	E4

Figure 4.1

PCR amplified inserts of immunoselected λ gt11 clones

Positive clones were amplified by PCR using universal λ forward and reverse primers. Samples of each PCR reaction were analysed by agarose gel electrophoresis.

lanes 1, 12, 23	pGem DNA markers	
lane 2	clone	D6
lanes 3 & 4	clones	B5, D5
lanes 5 - 11	clones	A1, A4, A5, B1, B4, B6, E3
lane 13	clone	C4
lanes 14 - 17	clone	C2, D1, D7 E2
lane 18	clone	D8
lanes 19 & 20	clones	C1, D3
lane 21	clone	A8
lane 22	clone	E4

Sub cloning of phage inserts

λ clones of groups 1, 2 & 3 were chosen for further investigation as these contained the largest DNA inserts. To increase the amount of DNA available for characterisation, the inserts were first subcloned into a plasmid. Subcloning was successful with D6 of clone Group 1 (1700 bp) and B1 of Group 3 (1400 bp). The λ PCR products of these two clones were subcloned directly into the pGem®-T plasmid. White colonies were picked and screened by double digestion with Sac I and Apa I restriction enzymes. A clone with a 1600-1700 bp insert was isolated from D6 and a 1400-1500 bp insert was obtained from clone B1.

Sequence analysis of clone D6

DNA from the recombinant plasmids was sequenced commercially following purification using Wizard™ λ preps. From clone D6 a partial sequence of *ca* 420 bases was obtained. The deduced 141 amino acid sequence was compared to sequences from available databases and was found to show significant homology with the C-terminal end of β tubulins from various organisms. β tubulins are proteins of 440 - 450 amino acids in length, corresponding to *ca* 1320 bases, therefore clone D6 of *ca* 1700 bases may contain the entire *F. hepatica* β tubulin gene. Figure 4.2 shows the alignment of the partial D6 sequence with β tubulin from *Toxoplasma gondii*. In the region of overlap the D6 sequence shows 64% identity and 73% similarity with the C-terminus of the protozoan tubulin.

Sequence analysis of clone B1

Clone B insert was estimated to be *ca* 1400 bp in length by PCR amplification using λ primers. Approximately 1200 bases of the insert were sequenced in the 5' to 3' direction. This revealed a start codon TAG and an open reading frame of *ca* 580 bases ending with the in-frame termination codon TAG. Downstream from the termination codon was stretch of about 20 adenine residues (Poly A tail), preceded by two poly adenylation sequences, AAAATAAA and AATA, indicating that the clone was complete at its 3' end. The DNA has a 5' untranslated region of *ca* 200 bases and a 3' untranslated region of *ca* 700 bases.

```
clone D6                1    ----------------------------------
ß Tubulin Toxoplasma    1    MREIVHVQGGQCGNQIGAKFWEVISDEHGIDPTG

clone D6                1    ----------------------------------
ß Tubulin Toxoplasma    35   TYCGDSDLQLERINVFYNEATGGRFVPRAILMDL

clone D6                1    ----------------------------------
ß Tubulin Toxoplasma    69   EPGTMDSVRAGPFGQLFRPDNFVFGQTGAGNNWA

clone D6                1    ----------------------------------
ß Tubulin Toxoplasma    103  KGHYTEGAELIDSVLDVVRKEAEGCDCLQGFQIT

clone D6                1    ----------------------------------
ß Tubulin Toxoplasma    137  HSLGGGTGSGMGTLLISKVREEYPDRIMETFSVF

clone D6                1    ----------------------------------
ß Tubulin Toxoplasma    171  PSPKVSDTVVEPYNATLSVHQLVENADEVQVIDN

clone D6                1    ----------------------------------
ß Tubulin Toxoplasma    205  EALYDICFRTLKLTTPT-YGDLNHLVSAAMSGVT

clone D6                1    ----------------------------------
ß Tubulin Toxoplasma    238  CCLRFPGQLNSDLRKLAVNLVPFPRLHFFLIGFA

clone D6                1    ----------------------------------
ß Tubulin Toxoplasma    272  PLTSRGSQQYRALSVPELTQQMFDAKNMMCASDP

clone D6                1    -------------RCJMCZNKEF----QATLSNZ
ß Tubulin Toxoplasma    306  RHGRYLTASAMFRGRMSTKEVDEQMLNVQNKNSS

clone D6                18   ----IPNNVKTAVCDIPPRGLKMSVTFVGNSTAI
ß Tubulin Toxoplasma    340  YFVEWIPNNMKSSVCDIPPKGLKMSVTFVGNSTA

clone D6                48   QELFKRVSEQFTAMFRRKAFLHWYTGEGMDEMEF
ß Tubulin Toxoplasma    374  IQEMFKRVSDQFTAMFRRKAFLHWYTGEGMDEME

clone D6                82   TEAESNMNDLVSEYQQYQZATAEEEGEFQLZAGA
ß Tubulin Toxoplasma    408  FTEAESNMNDLVSEYQQYQDATAEEEGEFDEEEG

clone D6                116  TITSWSGVKSQHGAGASTZPNRPZBZL
ß Tubulin Toxoplasma    442  EMGAEEGA-------------------
```

Figure 4.2

Alignment of predicted amino acid sequence of clone D6

The deduced amino acid sequence of the partial D6 sequence was aligned with that of β tubulin from *Toxoplasma gondii* (GenBank accession no. P10878, Nagel and Boothroyd, 1988). Boxes surround homologous regions and gaps have been introduced to give maximum alignment. Z = not determined.

Clone B1 is predicted to encode a protein of 194 amino acids with a calculated molecular mass of 21,646 Da. When used to screen protein sequence databases, the predicted amino acid sequence shows a highly significant identity with a novel family of antioxidant proteins, the peroxiredoxin family. Alignment of clone B1 with rat thiol specific antioxidant (TSA, GenBank accession no. P35704), human natural killer cell enhancing factor B, (NKEF B, accession no. P31945), human proliferation associated gene, (PAG, accession no. X67951), human TSA (Lim *et al,* 1994, accession no. P35701), and *Oncocherca volvulus* TSA (accession no. U09385) is shown in figure 4.3.

The protein with the highest identity is rat TSA; 57.0 % and 74.6% similar. The other identities are as follows human NKEF B 56.9%, (71.5% similar) human PAG 53.8% (73.0% similar), human TSA 53.7% (71.0% similar) and *Oncocherca volvulus* TSA 2.0% (33.7% similar). Similarity was observed over the entire length of the sequences and two highly conserved domains were observed. The first of these is a sixteen amino acid stretch at *ca* positions 40 - 60, - F Y P L D F T F V C P T E I I A -. The second shorter domain - H G E V C P A - is found at *ca* positions 165 -175.

Having identified B1 as a novel antioxidant protein in *Fasciola hepatica,* which may play an important role in the flukes defence against reactive oxygen species, we decided to continue its characterisation, and assay for the presence of the antioxidant activity in flukes.

Expression of fusion protein by clone B1

The plate wash supernatant method was used to make fusion proteins from clone B1 phage. The resulting supernatant and supernatant from *E. coli* infected with wild type phage were analysed on reducing SDS PAGE (Figure 4.4A). In the wild type preparation a protein with the same molecular mass as β galactosidase was observed (lane 1). In B1 supernatants this protein was absent but a larger protein of molecular mass *ca* 160 kDa, not found in wild type, was observed (lane 2). To determine if this was the fusion protein, the gel was blotted onto nitrocellulose paper and probed with anti-β galactosidase antibody (Figure 4.4B). Binding of the antibody to the large protein confirmed its identity as a β galactosidase fusion protein (lane 2). The antibody also bound the wild

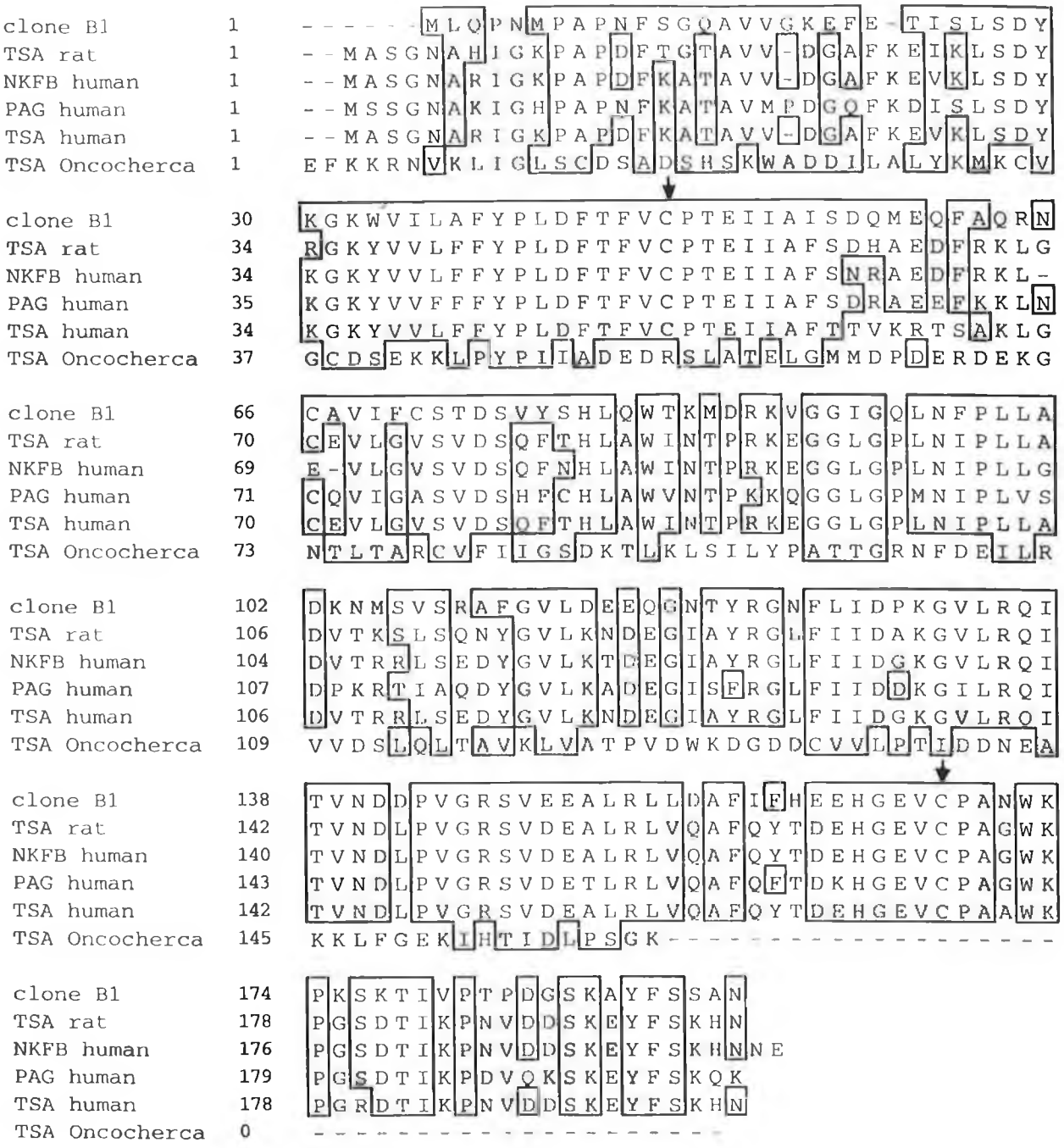

Figure 4.3

Alignment of predicted amino acid sequence of clone B1

The deduced amino acid sequence of clone B1 was aligned with that of rat thiol-specific antioxidant (TSA, GenBank accession no. P35704), human natural killer cell enhancing factor B, (NKEF B, accession no. P31945), human proliferation associated gene, (PAG, accession no. X67951), human TSA (Lim *et al*, 1994, accession no. P35701), and *Oncocherca volvulus* TSA (accession no. U09385). Boxes denote conserved residues and gaps have been introduced to maximise alignment. The active site cysteine residues are indicated by arrows.

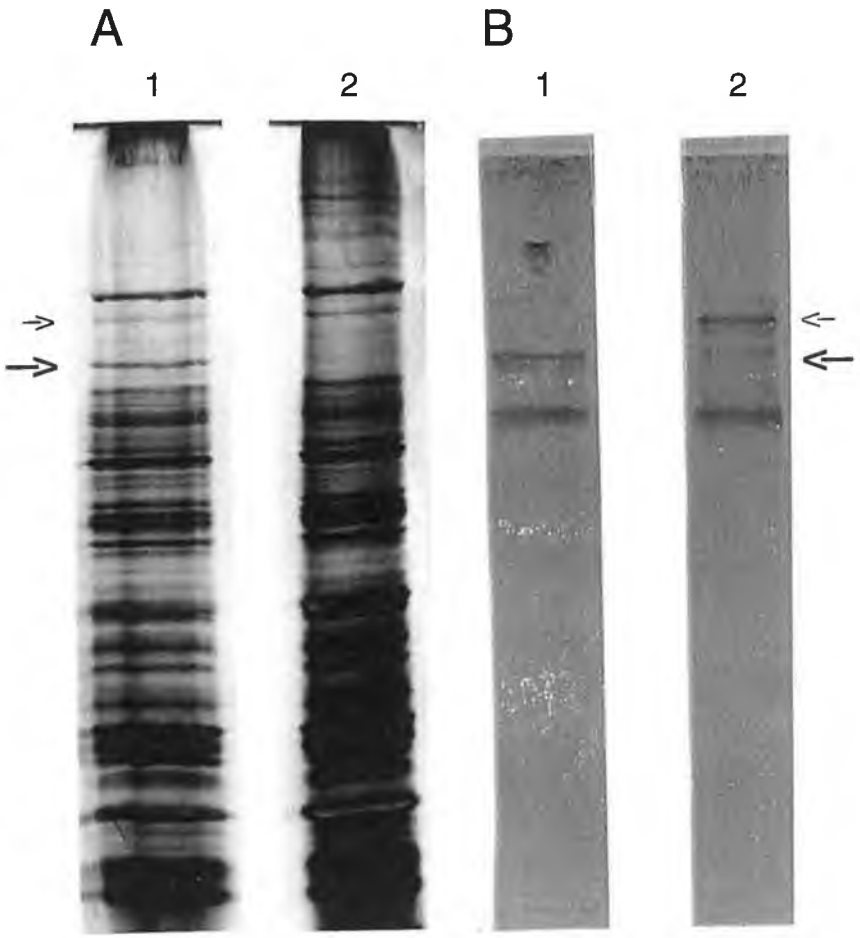

Figure 4.4

Expression of clone B1 fusion protein

A. Plate wash supernatants of wild type phage (lane 1) and clone B phage (lane 2) were subjected to reducing SDS PAGE and silver staining.

B. Following electrophoresis, SDS gels were blotted onto nitrocellulose and probed with anti-β galactosidase antibody. Lane 1 contains wild type phage supernatant and lane 2 contains clone B1 supernatant.

Large arrows indicate the position of β galactosidase. Small arrows indicate the position of B1 recombinant fusion protein.

type β galactosidase molecule (lane 1) and a number of other proteins common to both supernatants.

Northern analysis

Primers designed from the conserved domains of the antioxidant proteins (around the VCP motifs at *ca* positions 50 & 170), were used to amplify a DNA fragment of *ca* 400 bp in length. This was ^{32}P labelled and used to probe both *F. hepatica* and bovine RNA, which were analysed on an agarose gel prior to blotting. A single transcript of *ca* 750 kb was found in the *F. hepatica* RNA (Figure 4.5 lane 1). No peroxiredoxin-similar binding was observed in the bovine RNA (Figure 4.5 lane 2).

Antioxidant activity of mature fluke extract

Antioxidant activity was measured as the ability of liver fluke extract to prevent the inactivation of glutamine synthetase by a mixed iron thiol inactivation system. Figure 4.6 shows the inactivation of glutamine synthetase by iron and DTT in the presence of various levels of liver fluke homogenate (LFH). Incubation of glutamine synthetase with iron and DTT results in a 70% loss of the enzymes activity. The presence of LFH provides dose dependent protection, with 0.3 mg, 0.6 mg and 0.9 mg LFH restoring 50%, 61% and 75% glutamine synthetase activity, respectively

Time course analysis of antioxidant activity

The time dependent inactivation of glutamine synthetase by iron and DTT (Figure 4.7) and iron and 2-mercaptoethanol (Figure 4.8) in the presence of increasing levels of LFH was examined. Incubation of glutamine synthetase with inactivating solution containing either DTT or 2-mercaptoethanol for 10 min results in a 70% loss of enzyme activity. Prolonged incubations (60 min) destroyed almost 95% of the enzymes activity (Figure 4.7 and 4.8). Enzyme inactivation occurred in the presence of iron and either DTT or 2-mercaptoethanol, showing that both thiol compounds can provide reducing equivalents for the generation of ROS. When LFH was included in the reaction mixes, protection of glutamine synthetase was observed. In DTT / iron inactivations the presence of 0.6 mg LFH in the reaction resulted in 50% protection of the enzyme, even under prolonged incubations of up to 45 min (Figure 4.7). When

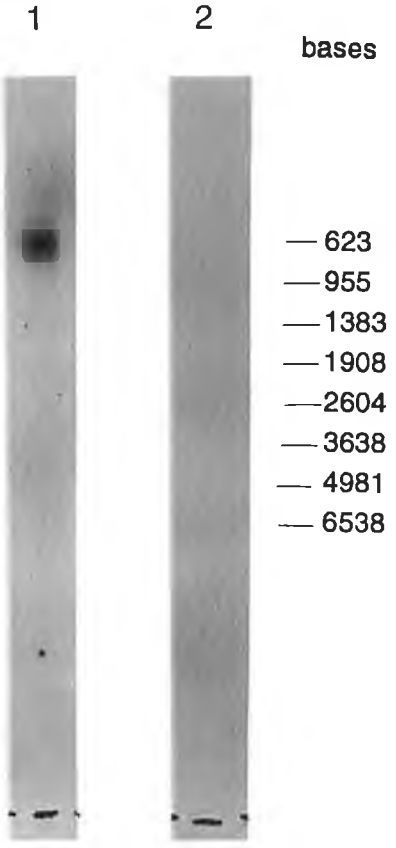

Figure 4.5

Northern blot analysis of total RNA from Fasciola hepatica and bovine liver

Total RNA from *F. hepatica* (lane 1) and from bovine liver (lane 2) was electrophoresed in a formaldehyde agarose gel, transferred to a nitrocellulose filter and probed with ^{32}P labelled 400bp fragment. RNA size markers are indicated.

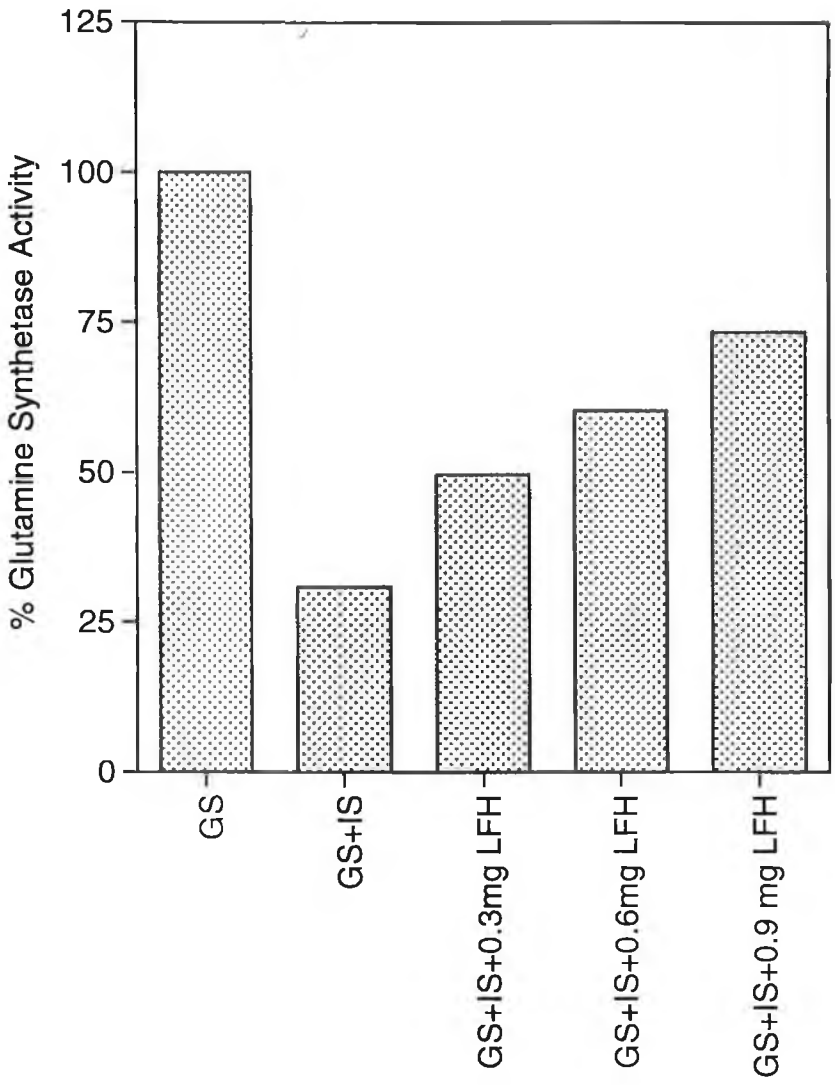

Figure 4.6

Protection of glutamine synthetase by liver fluke homogenate against the DTT / Fe^{3+} system

0.5 U glutamine synthetase (GS) was incubated in the presence of the inactivating solution (IS): 15 μM $FeCl_3$ and 5 mM DTT, with 0.3 mg, 0.6 mg and 0.9 mg liver fluke homogenate (LFH), for 10 min at 37°C. Reactions were then assayed for remaining glutamine synthetase activity.

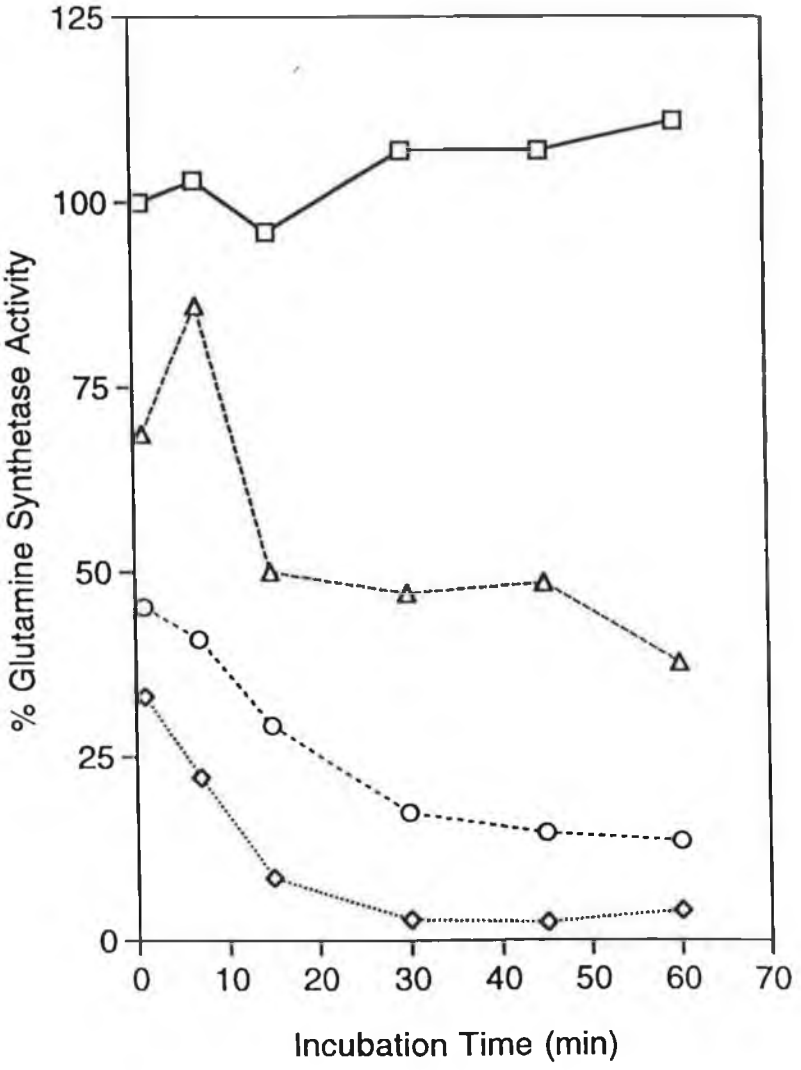

Figure 4.7

Time course analysis of glutamine synthetase protection from inactivation by DTT and iron

0.5 U glutamine synthetase was incubated with 5 mM DTT and 15 µM Fe Cl_3 in a total volume of 100 µl at 30°C for various times. Reaction mixes included no LFH (---◇---), 0.3 mg LFH (-○--), 0.6 mg LFH (--△--). Glutamine synthetase in the absence of inactivating solution served as control (—□—).

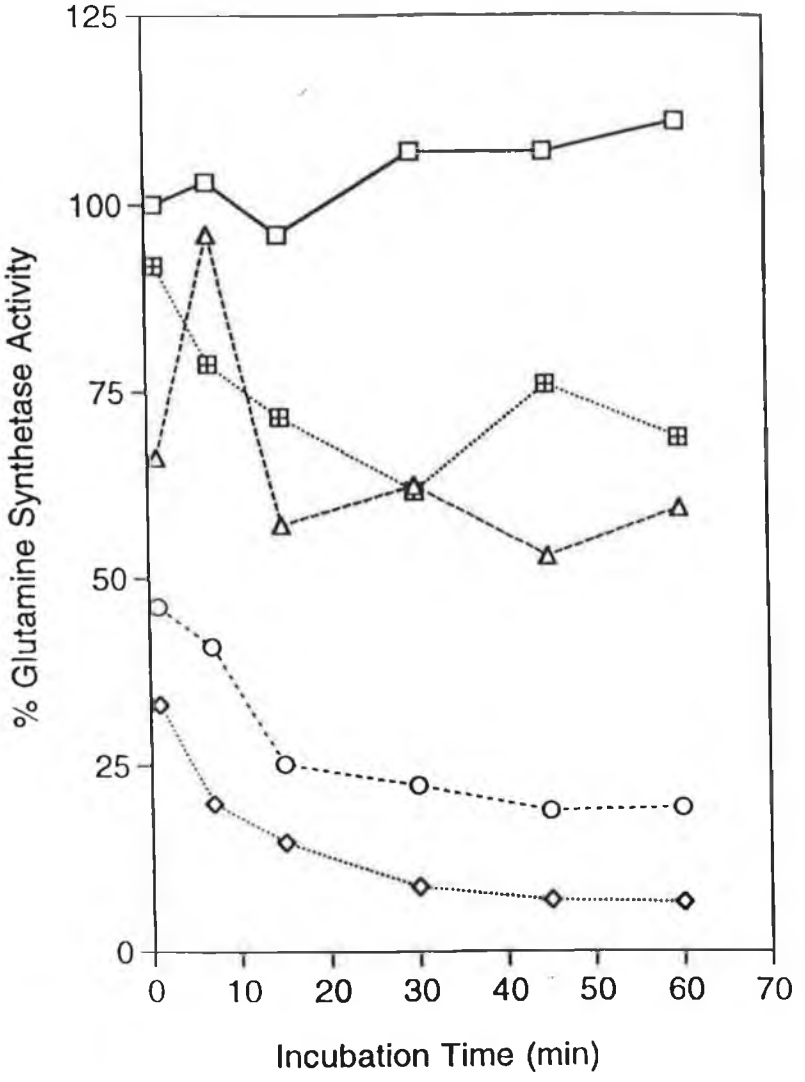

Figure 4.8

Time course analysis of glutamine synthetase protection from inactivation by 2-mercaptoethanol and iron

0.5 U glutamine synthetase was incubated with 14 mM 2-mercaptoethanol and 15 µM Fe Cl_3 in a total volume of 100 µl at 30°C for various times. Reaction mixes included no LFH (---◇---), 0.3 mg LFH (--○--), 0.6 mg LFH (--△--) and 0.9 mg LFH (---⊞---). Glutamine synthetase in the absence of inactivating solution served as control (—□—).

2-mercaptoethanol was used as reducing agent for inactivations, 0.6 mg and 0.9 mg LFH protect the enzyme to a similar level; 60 - 70% following 60 min incubation (Figure 4.8).

Chapter 5

Discussion

Fasciola hepatica is an invasive organism that penetrates the host intestinal wall and liver tissue during its migration to the bile duct. Our laboratory has been interested in characterising the molecules secreted by migrating and mature flukes, since these may be involved in various important biological functions, such as, tissue invasion, immunoevasion and nutrition. Hence, we have focused on the isolation of molecules secreted by the fluke when it is maintained in tissue culture media (excretory / secretory (ES) products). To date, two molecules, termed cathepsin L1 and cathepsin L2 have been purified, characterised and their genes isolated. The present study focuses on novel molecules secreted by adult flukes.

Purification and characterisation of liver fluke hemoglobin

When the ES products of *F. hepatica* were analysed by non-denaturing PAGE a protein possessing a yellowish colour was observed migrating through the gel. In the present study, the purification of this protein by gel filtration and ion exchange chromatography is reported. The molecule was characterised as an oxygen binding hemoglobin. Several data indicate that the hemoglobin molecule is not of bovine origin; these include its high molecular mass, migration in polyacrylamide gels, novel N-terminal sequence and immunogenicity in bovines. This is the first characterisation of a hemoglobin from *F. hepatica*.

On gel filtration chromatography the *F. hepatica* hemoglobin elutes just after the void volume and before the molecular weight standard; mouse immunoglobulin (150 kDa) and is therefore estimated to have a molecular mass of approximately 200 kDa. The hemoprotein was detected on the basis of its yellow colour; it has a high absorbance at 415 nm which indicates the presence of a heme group. It is eluted from DEAE Sepharose ion exchange chromatography with 200 mM NaCl and the purified protein resolves as two bands in non-denaturing PAGE. The faster migrating band is the most predominant and co-migrates with a predominant band in the ES products. Both of these bands were shown to contain a heme group by staining with DAB and hydrogen peroxide.

A single heme-containing protein was also detected in non-denaturing

PAGE of mature fluke extracts, prepared both in the presence and absence of protease inhibitors. The hemoprotein is predominant in both extracts and appears to be abundant in the adult fluke. The hemoprotein migrated further into the gel in the extracts prepared without inhibitors, suggesting that it had been proteolytically cleaved. Neither of the two bands in the ES-purified protein co-migrated with the heme-containing protein present in the liver fluke extracts. Antibodies raised in rats against the ES-purified hemoprotein bound to both components of the ES hemoprotein and to the single hemoprotein of the adult fluke extracts, demonstrating that the protein found in the culture medium was liberated from the flukes. Only one heme-containing protein was detected in the extracts of liver fluke, and this protein is susceptible to proteolytic cleavage. When it is liberated into the culture medium, proteolytic attack occurs, possibly giving rise to the two ES hemoprotein bands observed in the non-denaturing gels. Secreted cathepsin L cysteine proteases, previously characterised in our laboratory, (Dowd *et al,* 1994), are abundant in ES products and may be responsible for this proteolytic activity.

Bovine hemoglobin was included as a control in the PAGE and immunoblotting experiments. In non-denaturing PAGE, bovine hemoglobin appears as a doublet when stained for heme and it migrates much further into the gel than the liver fluke hemoprotein. Bovine hemoglobin has a molecular mass of 66 kDa, so its faster migration is in agreement with the higher molecular mass of the fluke hemoprotein found on gel filtration (*ca* 200 kDa). Furthermore, antibodies raised against the ES-purified hemoprotein do not react with bovine hemoglobin, showing that the two proteins are not immunologically related.

All hemoproteins have characteristic absorption spectra and the exact position and intensity of the various absorption peaks depends on the nature of the protein, the valence of the iron and the nature of any group combined with the heme. The heme group gives rise to all the colour in the hemoproteins. Absorption of light occurs in two main regions; between 390 nm and 440 nm, which is referred to as the Soret region and between 490 nm and 650 nm, generally referred to as the visible region. The heme group consists of a chelate complex of protoporphyrin with iron and in hemoglobin the four ligand groups of the protoporphyrin form a complex

with the iron; the fifth position is usually occupied by an imidazole group of histidine and the sixth is either unoccupied as in deoxy-hemoglobin or occupied by oxygen or other ligands. Hemoglobin derivatives normally present in living tissue are oxy-hemoglobin and deoxy-hemoglobin. Demonstration of the conversion of oxy-hemoglobin to other known derivatives makes identification of hemoglobin certain (Lehninger, 1975; Lee and Smith, 1965).

The absorption spectrum of the hemoprotein in extracts of mature flukes shows similar wavelength maxima to that of human oxy-hemoglobin, and is very close to those observed for the hemoglobins of the trematodes *Dicrocoelium dendriticum, Fasciolopsis buski*, and *Gastrothylax crumenifer* . The spectra obtained for the deoxy-, met- and cyanomet-hemoprotein derivatives are also similar to the derivatives of the above hemoglobins (Tuchschmid *et al*, 1978; Haider & Siddiqi, 1976). Thus, it can be concluded that the hemoprotein identified in the extracts of liver fluke is a hemoglobin. Although crude extracts were used in this study, the qualitative characterisation of the pigment can still be made out safely on the basis of spectral characteristics.

The relative intensities of absorption of the α and β peaks can be used to infer the affinity of hemoglobins for oxygen. When the absorption is greater in the β than α peak, it indicates that the hemoglobin molecule has a high affinity for oxygen and many parasite hemoglobins, including *Ascaris* hemoglobin, which has the highest oxygen affinity of any known hemoglobin, show this relationship (Lee and Smith, 1965). Consistent with these studies, in *F. hepatica* oxy-hemoglobin the β peak is higher than the α peak in the visible light range. In contrast, the reverse is true for most vertebrate hemoglobins.

The absorption spectrum studies on the ES-purified hemoprotein showed that it was irreversibly oxidised to the met-hemoglobin form. This was the only spectrum that could be obtained for the ES hemoprotein. Oxy-hemoglobin is oxygenated, not oxidised; the ability to form a stable ferrous-oxygen complex is the characteristic, specific property of all hemoglobins. In the normal function of hemoglobin the iron atom does not undergo

change in valence as oxygen is bound and lost; it remains in the ferrous (Fe(II)) state. However, it can be oxidised to the ferric (Fe(III)) state by oxidising agents to form met-hemoglobin which cannot function reversibly as an oxygen carrier. Hemoglobin is normally found in the ferrous state, being maintained against a tendency to auto-oxidise by reducing systems present in the tissues (Lehninger, 1975). The oxidation of the secreted ES hemoglobin most likely occurred during the culturing period or during the purification scheme. The hemoglobin in extracts of mature flukes also became quickly oxidised to met-hemoglobin on standing at 4^{o}C; auto-oxidation was slower when stored -20^{o}C. For human hemoglobin variants with deletions or substitutions of heme contact residues, instability of the hemoglobin towards auto-oxidation is well documented. Thus, as suggested for *Dicrocoelium dendriticum* hemoglobin (Tuchschmid *et al*, 1978), the observed instability in the ferrous form of the *F. hepatica* hemoglobin might be interpreted as reflecting alterations in the heme contact region.

A 26 amino acid N-terminal sequence was obtained for the ES-purified hemoglobin and a 20 amino acid sequence for the liver fluke extract hemoglobin. The two sequences showed only one amino acid difference; glycine at position 13 in the ES hemoglobin was arginine in the extract protein. The hemoglobin of the fluke extract was prepared in the presence of protease inhibitors, indicating that the sequence obtained is likely to be the true N-terminal sequence. In addition, this observation suggests that the proteolytic cleavage of ES hemoglobin, observed by PAGE, must have occurred at the C-terminus of the protein. The hemoglobin gave a single N-terminal sequence suggesting that it consists of a single globin chain. Furthermore, its N-terminal sequence is different from the N-terminal sequences of the two chains of bovine hemoglobin shown below (Kleinschmidt and Sgouros, 1987). Thus, the fluke hemoglobin appears not to be contaminated with host hemoglobin.

Bovine hemoglobin

α chain	V L S A A D K S
β chain	M L T A E E K A A V

Fluke hemoglobin

ES	S E E S R E K L R E S G G K M V K A L R D
extract	S E E S R E K L R E S G R K M V K A L R D

When entered into the GenBank database the hemoglobin showed no significant similarity with other invertebrate or vertebrate hemoglobins or with any other protein. The best alignment was with the alpha chain of *Xenopus* globin, between regions D2 and E17; however, the scoring is not significant. The only other trematode hemoglobin N-terminal sequences yet published are from *Paramphistomum epiclitum* which parasitises the rumen of water buffalo and *Isoparorchis hypselobagri* which parasitises catfish (Haque *et al*, 1992; Rashid *et al*, 1993). *P. epiclitum* hemoglobin consists of a single chain of molecular mass 16 kDa. The hemoglobin of *I. hypselobagri* consists of three monomeric globin chains termed a, b and c, all of molecular mass 17 kDa. Chains a and c share 27 N-terminal amino acid residues and differ in their primary structure at other locations. The b chain differs at ten positions in its N-terminal sequence to the a and c chains. The *F. hepatica* hemoglobin sequence shows no significant similarity with any of these N-terminal sequences.

Many invertebrate hemoglobins contain leader sequences before the A helix of the hemoglobin. If *F. hepatica* hemoglobin contains a leader, then, arginine at position 9 in the N-terminal sequence is the start of the A helix and the sequence contains hemoglobin consensus residues at positions A6, 8, 10, 12 and 13, as shown below. This gives a leader sequence that compares favourably with the composition of the leaders from *Parasponia* and *Ascaris* , shown below, (personal communication, Dr. Clive Trotman, University of Otago, New Zealand, 1995). *Ascaris* extracellular hemoglobin is an octamer of identical subunits each having a molecular mass of 40.6 kDa, and two globin domains (De Baere *et al*, 1992). *Parasponia* hemoglobin is a dimer of identical subunits of 19 kDa molecular mass (Wittenberg *et al*, 1986).

	Leader	A Helix
		1 2 3 4 5 6 7 8 9 10 11 12 13
Fluke Hb	S E E S R E K L	R E S G G K M V K A L R D
Consensus		K V A R D
Ascaris	N K T - R E L C M	
Parasponia	S S S E V N K V F	

Therefore, whilst the *F. hepatica* N-terminal sequence does not match well with any other vertebrate or invertebrate hemoglobins, certain structural characteristics are similar to the large extracellular hemoglobin of the parasitic nematode *Ascaris* and the hemoglobin found in nitrogen fixing nodules of the non-leguminous plant *Parasponia* (a tropical tree of the elm family), (Wittenberg *et al*, 1986).

Invertebrate hemoglobins have been classified according to their size, number of domains and number of subunits (Vinogradov, 1985). The *F. hepatica* >200 kDa hemoglobin differs from the earlier described hemoglobins of related trematodes including *Fasciolopsis buski* (Cain, 1969), *Dicrocoelium dendriticum* (Tuchschmid *et al,* 1978) and *Isoparorchis hypselobagri* (Rashid *et al*, 1993) which are single-domain, single-subunit hemoglobins of approximately 16 kDa (Vinogradov, 1985). Whilst information concerning trematode hemoglobins is lacking, a number of nematode hemoglobins (nemoglobins) have been characterised and classified into three groups including high molecular sized octameric globins (>350 kDa native molecular mass) found extracellularly in the perivascular or pseudoceolomic fluid (Blaxter, 1993). The *F. hepatica* hemoglobin may consist of aggregates of smaller subunits; however, the molecule could not be visualised on reducing SDS-PAGE.

F. hepatica hemoglobin is the predominant protein in both ES and adult fluke extracts when analysed by non denaturing PAGE, thus, it appears to be an abundant protein in the fluke. *F. gigantica* removed from the bile ducts of buffaloes, where oxygen tension is very low, was reported to contain 10 mg hemoglobin per gram wet tissue and *Cotylophoron indicum* removed from the buffalos reticulum, where oxygen tension is much higher contains 16 mg hemoglobin per gram wet tissue (Goil, 1961). The high concentration of hemoglobin in these parasites, despite their location in the host, is thought to reflect the important biological function of hemoglobin in the provision of oxygen for diverse metabolic functions, vital to the survival of the organism.

In contrast to vertebrate hemoglobins which function solely in the transport of oxygen for respiration, parasite hemoglobins may perform a range of different functions. These include acting as myoglobin-like oxygen-stores, as pools of hematin and facilitating the movement of oxygen through the tissues of the parasite (Lee & Smith 1965; Blaxter, 1993). Other proposed roles include functioning as an oxygen sink, protecting the essentially anaerobic respiration of the parasite from free oxygen and acting as a pseudo-peroxidase, preventing the build up of toxic hydrogen peroxide (Blaxter, 1993). The only well characterised trematode hemoglobin is that of the liver fluke *D. dendriticum* . This hemoglobin has an extremely high oxygen binding affinity and thus appears well adapted to living in the oxygen depleted environment of the bile duct (Smit *et al* , 1986). The avidity with which *D. dendriticum* hemoglobin binds its oxygen would appear to preclude a role in oxygen transport, suggesting that the hemoglobin plays a different and as yet undefined role in the flukes metabolism.

Immuno-flourescence studies localised the *F. hepatica* hemoglobin throughout many tissues of immature flukes including the tegument, sub-tegumental cells and parenchyma tissue. Immature *F. hepatica* have a predominantly aerobic energy metabolism, and in the presence of oxygen they oxidise glucose to carbon dioxide, via glycolysis in the cytoplasm and Kreb's cycle in the mitochondria (Tielens *et al*, 1981). A functioning Kreb's cycle is a characteristic of the early liver stage, but this activity slowly decreases during the development of the fluke, and energy production

from aerobic acetate metabolism increases (Tielens *et al*, 1982). While the young fluke is migrating through the liver it is constantly increasing in size, and this growth limits oxygen diffusion to its inner layers. Kreb's cycle activity is directly proportional to the flukes surface area, leading to a decrease in cycle activity as the fluke develops. The diminishing cycle activity occurs exclusively in the outermost layer of the fluke, as it moves through the liver. Upon arrival in the bile ducts the very low oxygen content of the bile, and possibly also the size of the fluke, forces it to a permanently anaerobic respiration, although aerobic potential does still exist (Tielens *et al,* 1984). The tegumental hemoglobin of the immature fluke may be used solely for aerobic respiration. As the fluke matures on entering the bile ducts, and respiration becomes anaerobic, this tegumental hemoglobin is no longer required. Immunofluorescence studies on mature fluke sections showed the absence of hemoglobin in the tegument.

While respiration in the mature fluke is anaerobic, oxygen is still required for other metabolic processes, such as egg production. A mature liver fluke is reported to produce an average of 2,500 eggs daily (Bjorkman and Thorsell, 1963), and therefore requires a constant supply of oxygen. Mansour (1958), showed that oxygen uptake by *F. hepatica* increased during phenolase tanning of egg shell proteins. The hemoglobin localised to vitelline glands and tubules within the mature fluke tissues may indicate a role for this molecule in transporting oxygen to the vitelline glands for egg production, and to various other tissues.

Antigens in the ES products of liver flukes can induce high levels of protection in rats and cattle (Rajasekariah *et al*, 1979). The hemoglobin molecule described in this study was identified during the systematic characterisation of the antigens in these ES products. In an earlier study it was shown, using reducing SDS PAGE, that the major components in adult fluke ES products were the cysteine proteases termed cathepsin L1 and cathepsin L2 (Dowd *et al*, 1994). However, in reducing SDS PAGE the fluke hemoglobin could not be visualised even when anti-hemoglobin antibodies were used as a probe in immunoblotting experiments. The fluke hemoglobin possibly either aggregates or degrades in the presence of SDS and mercaptoethanol.

In ELISA studies anti-hemoglobin antibodies were detected in the sera of experimentally infected cattle as early as one week post infection. Thus, the hemoglobin molecule is immunogenic and must be liberated from the parasite *in vivo* soon after infection begins. Although the immune response was highly variable between the four animals examined, they all contained antibodies to hemoglobin within one week of the infection and the antibody level increased for the first five weeks, then remaining steady until the tenth week of infection. The ELISAs were carried out a 1 / 100 dilution, however titres of greater than 1 / 8000 were recorded at both 5 and 10 weeks post infection, indicating a significant increase in antibody levels over the course of the infection.

Immunogenic hemoglobins have been described in two nematodes (Dixon *et al*, 1991; Frenkel *et al*, 1992). The hemoglobin of *Pseudoterranova decipiens* (codworm) is immunogenic in grey seals, the nematodes definitive host. Dixon *et al* (1991), used seal infection serum to screen a cDNA library and subsequently clone the hemoglobin. Frenkel *et al*, (1992), purified an 18 kDa hemoglobin from the ES products of *Trichostrongylus colubriformis*, an economically important gastrointestinal parasite of sheep. This hemoglobin does not appear to be immunogenic in natural infections of sheep or guinea pigs, however, when used to immunise guinea pigs, it stimulates an immune response conferring protection on the guinea pigs. It is not known in this case, or in the case of the *F. hepatica* hemoglobin, whether the hemoglobin is actively secreted by the fluke or is liberated from dying parasites, possibly from the pseudocoelom. Liver fluke tegumental hemoglobin although shown by immunofluorescence studies in immature flukes was absent in adult flukes removed from the bile ducts. This hemoglobin may be liberated from the developing fluke as it migrates through the liver.

Cattle vaccine trials with liver fluke hemoglobin

The hemoglobin molecule appears vital for the flukes development and survival, performing critical metabolic functions, ranging from oxygen transport for aerobic respiration in immature flukes, to oxygen supply for egg production in the adult fluke. Its important biological role, together with its localisation within the tegument of the immature fluke and its high

immunogenicity in infected bovines made it an ideal candidate for inclusion in an anti-fluke vaccine trial performed in cattle. Accordingly, vaccine trials were carried out to test the immunoprophylactic potential of *F. hepatica* hemoglobin alone and in combination with the fluke cysteine protease cathepsin L2.

For most helminths, vaccine strategies are very different from those for viral, bacterial or protozoal infections, since sterile immunity is unlikely to be attainable. This is because the infective stages of helminths are large, antigenically complex and actively migratory within the host. Natural immunity itself takes months or years to develop in the face of repeated, often daily challenge. Helminths have evolved a diverse array of specific and general adaptations to evade the host immune system. These include avoiding the initial induction of damaging immune responses, compromising selected arms of the immune system and disabling short range offensives mounted by various effector molecules. Any putative liver fluke vaccine would be given to newborns in order to prevent severe infection in the following years of high risk. Ideally, booster doses of the vaccine would not be necessary, since subsequent trickle infections could provide continuous restimulation of immunity. Since the goal is prevention of disease through reduction of intensity, the stimulation of a complete, sterilising immunity is not a necessity.

High levels of protection were induced in cattle against a challenge of *F. hepatica*, by immunisation with hemoglobin fraction and cathepsin L2 purified from liver fluke ES products. Besides eliciting immunoprotection, vaccination also affected fluke growth and egg viability. The hemoglobin fraction (Hf) used for immunisation was a concentrated pool of fractions from the gel filtration chromatography of fluke ES products. Non-denaturing PAGE of Hf revealed the hemoglobin to be 80 - 90% pure, therefore, minor contaminating proteins were present, the identity of which was unknown. Cathepsin L2 (CL2) was purified to homogeneity as demonstrated by reducing SDS PAGE. CL2 is the larger (molecular mass 29.5 kDa) of two cathepsin L proteases (CL1 and CL2) identified in liver fluke ES products (Dowd *et al*, 1994). These proteases are secreted by the fluke in all stages of the parasite that exist in the mammalian host and immunolocalisation studies localised the enzymes to the secretory granules of the intestinal

epithelial cells. The enzymes are packaged in vesicles within the columnar epithelial cells that line the gut wall of the parasite. The cells undergo phases of secretion when the vesicles exude their contents into the gut lumen; the proteases are released and most probably degrade ingested host proteins (Dalton and Heffernan, 1989; Smith *et al*, 1993a).The gut contents of the liver are voided regularly by regurgitation, therefore, it is presumed that as the fluke migrates through the host liver the proteases are excreted to the exterior of the parasite.

The cathepsins have the ability to cleave immunoglobulin at or near the hinge region in a papain-like manner. Therefore, secreted cathepsins may play a role in immunoevasion by cleaving host immunoglobulin and thus preventing antibody mediated immune effector cell attachment (Smith *et al*, 1993b). Secreted CL2 may play an important role in the flukes nutrition, as degradation of host tissue would provide peptides, that, following further digestion to amino acids and dipeptides by exoproteases could be assimilated by the parasite for growth. CL2 may also be a prime mechanism by which the parasite penetrates host tissue, assisting the fluke in burrowing through the gut wall and liver of its host, before taking up residence in the bile duct. CL2 plays a crucial role in the survival of the parasite, thereby representing an additional vulnerable target to which a vaccine could be directed.

Vaccination of the control group of cattle with horse spleen ferritin resulted in the mean fluke recovery of 152.1, corresponding to 25.4% of the infection dose, which is consistent with other bovine liver fluke infection studies (Anderson *et al*, 1977). Immunisation with Hf alone elicited a significant protective response; 43.8% reduction in total worm burden. The immune response to Hf may have interfered with oxygen metabolism in the fluke. Juvenile flukes have 100% aerobic respiration and hemoglobin may be essential for the required oxygen supply. As the immature fluke migrates through the liver, TCA cycle activity becomes limited to the fluke tegument and anti-hemoglobin antibodies are localised in the immature fluke tegument.

When cattle were immunised with a combination of Hf and CL2, a higher level of protection was obtained; 72.4% reduction in total worm

burden. This boosting of the protection by including CL2 may be the result of the immune responses effect on the immunoevasion, nutrition and tissue penetration activities of the protease. The incorporation of both the surface antigen (tegumental hemoglobin) and the secreted antigen (CL2) in a composite vaccine proved more effective than immunisation with Hf alone. Increased protection may be due to the stimulation of different effector arms of the immune system and the provision of more targets for immune attack in the parasite.

Flukes recovered from vaccinated animals tended to be smaller in size than those recovered from control animals. Thus, the vaccination preparations appeared to be exerting their effects on the migratory stages of the flukes, before they took up residence in the bile ducts. Anti-hemoglobin antibodies are detected in the serum of experimentally infected cattle as early as one week post challenge. In addition, the expression of cathepsin proteases has been demonstrated in all fluke stages that parasitise the mammalian host, including juveniles that excyst in the intestines (Carmona *et al*, 1993). Therefore, both hemoglobin and CL2 may be accessible to vaccine-induced immune responses at the early stages of infection, resulting in the inhibited growth and development of surviving flukes.

Serum liver enzyme analysis revealed that Group 2 which received the highly effective combination of Hf and CL2, had a significantly lower level of γ glutamyl transferase (γGT) than control group animals. γGT is an indicator of bile duct hyperplasia, levels rising in infection upon the arrival and residence of flukes in the bile duct. Therefore, the very low levels in Group 2 is consistent with the low numbers of flukes recovered from the bile ducts of these animals. Group 2 animals also had reduced levels of serum glutamate dehydrogenase (GLDH), as compared to control, indicating that these animals suffered less damage to the liver parenchyma. The GLDH levels in Group 2 animals increased in the first 6 weeks of infection, similar to the control animals. However, from week 6 to the day of slaughter (week 13) the enzyme levels in Group 2 remained constant, while control animal levels continued to rise. The initial increase in GLDH levels in group 2 animals was probably caused by the migration

of flukes which survived the host immune response. Liver fluke infections generally cause morbidity rather than mortality, with disease severity typically related to worm burden. The morbidity of the infection is a direct consequence of liver pathology caused by migrating flukes, therefore, a vaccine will only be effective if it prevents the development of these early stages, which vaccination with Hf and CL2 appears to achieve.

An important feature of the vaccines was their effect on the viability of eggs recovered from the gall bladders of the vaccinated animals. Most remarkable was the almost complete anti-embryonation effect (> 98%) of vaccinating with a combination of Hf and CL2 (Group 2). Anti-embryonation effects mediated by immune responses are well established in schistosomiasis, a disease caused by the related trematode *Schistosoma sp.*. The pathology associated with schistosomiasis is a direct consequence of inflammatory responses to eggs trapped in the liver. Granulomas form around the eggs, however, as the infection proceeds, the size of the granulomas decrease with a concurrent abatement of the disease (Mitchell, 1991). Liver granuloma modulation is believed to involve anti-egg antibodies. The primary effect of these antibodies is the inhibition of maturation of eggs, leading to the destruction of the embryo before it matures into a miracidium. The antigens of immature and maturing eggs responsible for inducing these antibodies have not yet been identified (Mitchell, 1991). However, Day *et al*, (1996), detected cathepsin L proteases in schistosome eggs and suggested that release of these enzymes may be associated with the egg granulomatous response.

In addition, immunisation of both rodents and baboons against *S. mansoni* with recombinant *S. mansoni* 28 kDa glutathione S-transferase (rSm28GST) results in reduced female worm fecundity and reduced egg viability (Boulanger *et al*, 1991). Passive immunisation of *S. mansoni* infected mice with a monoclonal antibody which inhibits Sm28GST activity leads to decreased worm fecundity and egg hatching ability, without having an effect on worm burdens (Xu *et al*, 1991). Epitope mapping has localised this anti-fecundity effect to C- and N-terminal peptides of the GST and vaccination with these peptides gives high anti-embryonation effects (Xu *et al*, 1993). While eggs are not associated with pathology in liver fluke disease, blocking of miracidial development and hatching would have a

profound effect on the extent of pasture contamination, and hence disease transmission.

The mechanism(s) by which immune responses to Hf and CL2 exert the anti-embryonation effects are not understood but would be of interest to elucidate. Adult flukes have an anaerobic respiration, however, oxygen is required for egg production in the phenolase tanning of eggshell proteins (Mansour, 1958) and hemoglobin may be involved in transporting oxygen to this tissue. Furthermore, immunohistochemical studies have demonstrated the presence of cathepsin L proteinases in the oocytes and vitelline glands of flukes (Wijffels *et al*, 1994). Possibly, antibodies interact directly with hemoglobin and CL2 in the vitelline or Mehlis glands, interfering with their function. Alternatively, the production of non-viable eggs may be a favourable, but indirect, consequence of the effect of the vaccine on the parasites nutrition and development.

The total IgG antibody response of all animals, during immunisation and following challenge, was analysed by ELISA, using fluke ES products as antigen. High antibody titres were induced by both vaccine preparations and these were boosted following the challenge infection. Therefore, these vaccines formulated in Freunds adjuvant could induce memory B-cells that are stimulated by antigens released from the parasite. However, in the present study, no correlation was found between antibody responses, assessed by ELISA and immunoblotting, and the level of protection obtained.

A more detailed analysis of the antibody isotypes induced by vaccination may reveal correlations, although Sexton *et al,* (1994), found no correlation between IgG1 and IgG2 responses to linear peptide epitopes and the level of protection induced in sheep by vaccination with *F. hepatica* GST. Isotype analyses of schistosomal infections in humans have revealed the production of IgE and its role as a mediator of protective immunity (Hagan, 1993). Where patients are susceptible to re-infection, levels of IgM and IgG were equal to, or greater than, those of the resistant group, whereas IgE levels were 6-8 fold higher in the most resistant individuals (Dessein *et al*, 1992). High levels of IgG4 were found in the least resistant individuals and it has been suggested that IgG4 antibodies

serve to block the binding and action of other protective isotypes, hence, preventing the expression of immunity (Rihet *et al*, 1992). The expression of resistance was shown to be dependent on the balance between protective IgE and blocking IgG4 antibodies to larval antigens (Hagan *et al*, 1991). Although studies point to IgE as the principal mediator of resistance other mechanisms may be involved. Vaccination of rats with rSm28GST leads to a 59% decrease in worm burden (Grzych *et al*, 1993). High levels of IgE and IgA are elicited and both are effective in the *in vitro* killing of newly transformed schistosomula using eosinophils as the effector molecule. The conclusion is that a key feature of the expression of protection is not so much the presence or absence of an antibody response but rather the balance between the isotypes of antibodies produced. Therefore, isotype mapping of the sera from the cattle in the trial may supply information as to how immunisation with Hf and CL2 was so effective at protecting cattle against a challenge infection.

Following infection with parasitic helminths a clonal expansion of antigen-specific T cells occurs and the subsequent and concentrated immune response is regulated by a given set of cytokines secreted differentially by subsets of T helper cells; TH1 and TH2. The products of TH1 are involved in cell mediated immunity; γ interferon (γ IFN) and β tumour necrosis factor (β TNF), are inflammatory mediators and selectively activate macrophages. TH2 cytokines, which include interleukins (IL) 4, 5 and 10, stimulate B cell and eosinophil development and antibody production. These subsets are reciprocally cross-inhibitory; one cell type gains at the expense of the other, which may explain the profoundly unbalanced TH1 / TH2 ratio in most helminth infections (Maizels *et al*, 1993). The critical T cell population may differ in each parasite combination and in each tissue site. Schistosome cercariae induce a TH1 response in mice, however, the onset of egg deposition after worm maturation results in a dramatic shift to a TH2 profile. Thereafter responses to schistosome infection are dominated by TH2 characteristics, such as eosinophilia and IgE production (Sher *et al*, 1992). The ratio between helminth induced TH1 and TH2 responses is instrumental in determining the extent of pathology and / or immunity induced by these parasites. Studies on both TH1 and TH2 immune responses in the immunised cattle may be required for

understanding both the anti-fluke and anti-embryonation effects of the Hf / CL2 vaccine.

Systematic studies to compare the use of different commercially approved adjuvants are also required. A variety of adjuvants are currently available for experimental use, but relatively few have been approved for either veterinary or human use. Freunds complete adjuvant (FCA) consists of killed mycobacteria in mineral oil and an emulsifier. Soluble antigen when mixed with FCA forms an emulsion which effectively restrains the antigen at the injection site, facilitating contact of antigen presenting cells. The mycobacteria seem to preferentially elicit TH1 activity characterised by the production of IL2 and IL3, γ IFN, lymphotoxin and granulocyte / macrophage colony stimulating factor. In contrast Freunds incomplete adjuvant (FIA), which lacks mycobacteria stimulates only the humoral response. Unfortunately, the toxic side effects associated with both FCA and FIA, including chronic granuloma formation and ulceration, induction of tuberculin sensitivity and the possible carcinogenic potential of mineral oil and Arlacel A, have precluded their use in even veterinary medicines (Smith, 1992).

Alternative adjuvants include aluminium salts, particularly aluminium hydroxide gels, referred to as alum. These are non-toxic efficacious adjuvants and represent the only adjuvant approved for human use. Antigen is absorbed onto the surface of the gel and is slowly released. Alum forms inflammatory foci and attracts immunocompetent monocytes, improving antigen contact with antigen presenting cells. It may also preferentially stimulate TH2 cells to produce IL4, 5, 6 and 3 and granulocyte / macrophage colony stimulating factor, thereby favouring a humoral response to antigen and an increase in IgG and IgE antibodies. The major drawback to using alum as adjuvant is its inability to stimulate a cell mediated response. Attenuated *Mycobacterium bovis*, more commonly referred to as Bacille Calmette-Guerin (BCG), is possibly the best inducer of cell mediated immune responses, stimulating systemic and local activation of macrophages as well as stimulation of CD4, CD8 and T cells (Frommel and Lagrange, 1989). The development of immune granulomas is also a feature of injection of BCG. Surprisingly, it has found only a limited

application in the development of anti-parasite vaccines, unlike saponin, a polysaccharide from the bark of a South American tree, which has been used in a number of anti-parasite vaccines. The efficacy of saponin vaccination appears to be related to its ability to enhance cell mediated immunity, as indicated by a vigorous delayed hypersensitivity reaction and production of IgG2 as well as IgG1 antibodies. Unfortunately at high concentrations it is toxic, having a propensity to lyse cells, promote inflammation and act as an irritant. A purified form of saponin, Quil A forms micelle-like structures when combined with membrane antigens. These present the antigen to the immune system in particulate form and have the added advantage of greatly decreasing the concentration of Quil A required for effective adjuvanticity (Smith, 1992).

Other adjuvants include proteosomes and liposomes, viral and bacterial vectors, cytokines and gut bacteria. The wide variety of available adjuvants means that they can be selected to preferentially enhance specific arms of the immune response appropriate to the parasitic disease. This further illustrates the need for a solid basic understanding of the immune response which effects parasite killing. An understanding of the hosts anti-fluke and anti-embryonation responses will be important in the selection of a suitable means of vaccine presentation to replace Freunds adjuvant. Both FCA and FIA were used in our immunisation protocol, therefore it is likely that both cell mediated and humoral responses were elicited in the vaccinated cattle.

Serious economic losses are caused by infection of both cattle and sheep by liver fluke. While high levels of protection were obtained in cattle it still has to be determined whether these vaccine candidates will induce similar responses in sheep. Cattle show a high level of natural resistance to infection whereas sheep do not (Haroun and Hillyer, 1986). Furthermore, vaccination of sheep with crude antigen preparations have been generally unsuccessful. Vaccination of sheep with purified liver fluke GST induced highly variable results, with protection levels ranging from 0 - 57% (Sexton *et al*, 1990; Spithill and Morrison, 1995). Wijffels *et al,* (1994), reported that vaccination of sheep with a mixture of *F. hepatica* cysteine proteinases had no effect on the parasite burden following a challenge infection but did reduce the egg output of the parasites by up to 70%.

The only other report of a protective hemoglobin is the 18 kDa hemoglobin of the nematode *Trichostrongylus colubriformis*, which parasitises the intestines of sheep (Frenkel *et al*, 1992). The hemoglobin, which was purified from the nematode ES products, is not naturally immunogenic in sheep or guinea pig infections. However, in vaccine trials a single dose of antigen without adjuvant conferred high levels of protection (60 - 84% reduction in worm burden) against challenge with *T. colubriformis.* The basis of the immunising activity is not yet understood, but it may preferentially stimulate the cellular arm of the immune system. Three peptides in the hemoglobin molecule conformed with the sequence motifs proposed for T cell epitopes (Frenkel *et al*, 1992).

Determination of the response of related trematodes to the Hf / CL2 vaccination will also be of interest. Infection of cattle and buffalo with the tropical liver fluke *F. gigantica* causes serious economic losses to agricultural communities throughout Asia and Africa and currently there are no vaccines for its control. Nor is there a vaccine presently available against schistosomiasis, which is a major human parasitic disease, with an estimated 200 million people currently infected. Previous studies have shown that mice and hamsters inoculated with subcellular fractions of *F. hepatica* worms acquired resistance to challenge infection with *S. mansoni* cercariae (Hillyer *et al*, 1977). Isolated *F. hepatica* antigens were shown to confer high levels of protection in mice against challenge infections with both *S. mansoni* and *F. hepatica* (Hillyer, 1979; Hillyer, 1985; Hillyer *et al*, 1988). The *Fasciola* protective antigens (hemoglobin and CL2) could be used to search for the analogues in these related trematodes.

In summary, we have demonstrated that a molecular vaccine against bovine fascioliasis is feasible. The efficacy of Hf and CL2 is close to some fasciolicides. A comparative study of triclabendazole and nitroxynil showed that these drugs have efficacies against fluke infection of 96.9% and 76.4%, respectively (Rapic *et al*, 1988). The reduction of worm burdens observed with this vaccine was reflected in a reduction of the severity of liver damage. Of particular significance, however, was the observed anti-embryonation effects. A commercial vaccine capable of inducing anti-embryonation effects of >98% would undoubtedly have major

epidemiological implications since pasture contamination would be negligible, leading to a great amelioration of the disease. Future work will require optimisation of protection levels through an investigation of the immune response. Bearing in mind that a large scale field trial with appropriate controls is the only way to give a reliable estimate of the real impact of a control programme, the results obtained in this study bring strong support to a vaccine based strategy against fascioliasis of veterinary importance.

A commercial vaccine based on native hemoglobin and CL2 antigens would not be feasible due to technical and logistical limitations on the quantity of antigen available. Recombinant DNA technology offers the potential for large scale production of vaccine antigens. Accordingly, a cDNA CL2 clone has been isolated and research on the yeast expression of recombinant cathepsin L2 is in progress.

Isolation of cDNA clones encoding liver fluke antigens

An adult *F. hepatica* cDNA library was screened with pooled sera from animals vaccinated with Hf (Group 1; protection level 43.8%) with the aim of isolating a cDNA encoding the fluke hemoglobin. This sera should contain antibodies reactive with hemoglobin and any other proteins present in the immunising fraction. Thirty immunopositive clones were identified, of which twenty contained PCR fragments when amplified with universal λ primers. These twenty clones formed nine groups when classified on the basis of size and restriction mapping. Due to time constraints only clones from groups 1, 2, and 3, containing the largest DNA inserts were selected for further characterisation. Clones with large inserts were chosen as these were thought most likely to encode the large molecular mass hemoglobin. Of these, subcloning into pGem®-T plasmid was successful only with clone D6 of group 1, which contained the largest DNA insert (*ca* 1700bp) and clone B1 of group 3 which contained inserts of *ca* 1400bp.

A partial sequence of *ca* 420 bases was obtained for clone D6, the only member of Group 1. Comparison with sequences from combined databases revealed significant similarity to the sequence of β tubulin from

various organisms. Sequencing of *ca* 1200 bases of clone B1, one of seven similar clones from Group 3, revealed an open reading frame of *ca* 580 bases. Screening of combined databases with the predicted amino acid sequence revealed a highly significant identity with a novel family of antioxidant proteins, recently named the peroxiredoxin family (Chae *et al*, 1994b). A cDNA encoding the fluke hemoglobin was not identified, however, characterisation of the remaining seven groups of clones may reveal its presence.

The identification of clones encoding proteins other than the hemoglobin highlighted the polyspecificity of the anti-Hf bovine sera. This polyspecificity was expected as the Hf used for immunisation of the cattle, was a crude fraction, 80 - 90% pure. Vaccination of cattle with Hf stimulated an immune response to both β tubulin and the fluke peroxiredoxin, resulting in the production of antibodies reactive with Group 1 and Group 3 clones. Therefore, these proteins were present in Hf, though probably only as minor contaminants. Liver fluke peroxiredoxin appears to be highly immunogenic; seven of the twenty immunopositive clones contained this antioxidant gene. The demonstration of β tubulin- and peroxiredoxin-specific antibodies in the bovine sera means that both proteins may have contributed to the 43.8% protection observed in these animals following challenge infection.

The deduced amino acid sequence from clone D6 shows significant similarity with the C-terminal end of β tubulins from a variety of organisms. The D6 sequence overlaps with the C-terminal 130 amino acids of *Toxoplasma gondii* β tubulin and within this region of overlap is 72% similar to the protozoan tubulin. β tubuiins are generally 440 - 450 amino acids in length, therefore, continuation of the sequence in the 3' to 5' direction will yield the entire liver fluke β tubulin gene.

Tubulins are highly conserved proteins present in all eukaryotic cells. They exist as two non-identical forms α and β, that co-polymerise in a helical fashion to form microtubules. Microtubules are the hollow, tube-like filaments detected in a number of different cellular arrangements, in plant

and animal cells, by electron microscopy. Microtubules, which are of variable and transient length, begin as a flat sheet made of many tubulin units, which then rolls into a tube. Once formed, the tubule can elongate by the addition of tubulins at one end. The binding units are not single tubulin molecules, but dimers, each consisting of one molecule of α tubulin and one molecule of β tubulin. A dynamic equilibrium exists within the cell between the pool of free tubulin dimers and the structural, polymerised form of tubulin within the microtubule.

Microtubules form part of the cytoskeleton, the network of fibres throughout the cytoplasm of the cell, that functions in giving mechanical support to the cell and helps maintain its shape. Organelles and even cytoplasmic enzymes can be held in place by anchoring to the cytoskeleton and microtubules serve as tracks along which organelles can move. Components of the cytoskeleton enable muscle cells to contract and are involved in the movement of ciliated and flagellated cells. They also form the axons of nerve fibres and are thought to play a role in nutrient absorption. Within the microtubule organising centre of cells, an amorphous mass located near the nucleus, there are two centrioles, each composed of microtubules. The centrioles are involved in the organisation of microtubule assembly for mitosis and meiosis, where the microtubules of the cytoskeleton are partially disassembled to provide material for constructing the spindle. While the role of microtubules within the mitotic spindle is fundamental and ubiquitous to all cell division, it is perhaps the subtler, metabolic effects of these cytoskeleton proteins that control, by direct or indirect means, the primary functions essential to cell homeostasis.

Microtubules have been visualised in nematodes, cestodes and trematodes, using electron microscopy. Using a labelled anti-b tubulin antibody, microtubules in *F. hepatica* have been identified throughout the fluke tegument and sub-tegument and in associated cell bodies, where, as in other organisms, the tubules of *F. hepatica* are believed to form part of the centrioles, mitotic and meiotic spindles and the cytoskeleton (Stitt *et al*, 1992). The presence of tubulin was also demonstrated in sensory nerve endings and in the nerves innervating the sub-tegumental musculature.

Within the vitelline follicle, tubulin was localised to the cytoplasmic extensions that ramify throughout the follicle between the vitelline cells. The microtubules probably provide support for these long processes and serve to supply nutrients to the developing vitelline cells (Stitt *et al*, 1992).

It follows that the Hf used in the vaccine trial, which was purified from total ES products by gel filtration, contained *F. hepatica* tubulin. The presence of tubulin, a structural protein, in ES products is unusual. It may have been released from dead or dying flukes, or from tegumental turnover. The Hf is a high molecular mass fraction (>200 kDa), however, fluke tubulin has a molecular mass of *ca* 53 kDa (Stitt *et al*, 1992), therefore, the tubulin either forms aggregates or interacts non-specifically with the column during gel filtration chromatography.

Conservation of tubulin is observed throughout the species. Nevertheless, an anti-tubulin response was elicited in the immunised cattle, indicating that fluke tubulin must contain epitopes foreign to the mammalian host. Tubulin is a structural protein necessary for maintaining cellular architecture and homeostasis in the fluke and as such, the immune response directed against it may have contributed to the protection of cattle observed in the vaccine trial. The potential of tubulin as a vaccinogen is strengthened by an additional important feature; the therapeutic application of microtubule inhibitors in helminth infections.

Helminth microtubules and their tubulin components represent an important target for anti-helmintic action, specifically the action of benzimidazoles. Triclabendazole, the most potent of the benzimidazoles was first introduced in 1961 and this more than any other drug heralded the commencement of anti-parasitic chemotherapy (Lacey, 1988). The primary site of interaction of benzimidazoles is within the microtubule matrix, however, the current understanding of this biochemical action is still limited. Tubulin in microtubules has intracellular associations with most organelles and it is via these interactions and the probable direct association of tubulin with the physiological mechanisms of hormone, neurotransmitter, nutrient, enzyme and receptor actions, that the cellular responses of microtubular inhibition by benzimidazoles are manifested. The disruption of the tubulin-microtubule equilibrium can be seen as

leading to a cascade of direct and indirect biochemical and physiological changes resulting in the loss of cellular homeostasis. Conditions of cellular disequilibrium, if maintained are lethal and these lethal effects are most apparent in actively dividing or growing cells (Lacey, 1988).

Triclabendazole (Fasinex®) was shown to be highly effective against the whole age spectrum of *F. hepatica* in cattle (Fuhui *et al*, 1989). The effectiveness of microtubule inhibitors in fluke killing suggests that tubulin represents a possible target to which an anti-fluke vaccine could be directed. The advantage of a successful anti-tubulin vaccine over chemotherapeutic agents would be the reduction in the number and frequency of doses required. Further studies will involve the expression of recombinant tubulin to obtain the antigen in quantities sufficient for inclusion in a vaccine trial.

Clone B1 contains the entire gene encoding liver fluke peroxiredoxin. The predicted amino acid sequence shows high similarity with the members of this newly discovered, widely distributed, antioxidant family. The highest similarities are with rat thiol-specific antioxidant (TSA), (TSA is the name by which the peroxiredoxins were previously known), human natural killer cell enhancing factor B (NKEF B), human proliferation associated gene (PAG) and human TSA. Similarity is observed over the length of the sequences and two highly conserved domains are observed. The first is a sixteen amino acid stretch from *ca* positions 40 - 60. The second is a shorter stretch of seven amino acids at *ca* positions 165 - 175. The two conserved regions are found in the B1 sequence and in all the aforementioned sequences. This high degree of conservation suggests that these regions are important to the proteins function. Both domains contain the - V C P - motif (VCP 47 and VCP 168), which has been proposed as the cysteine active site of the protein.

Organisms living in an aerobic environment require mechanisms that prevent or limit cell damage caused by reactive oxygen species (ROS), generated during the sequential univalent reduction of oxygen to water. These deleterious species, including superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^\bullet$), arise from the incomplete reduction of oxygen during respiration, or from exposure to external agents such as

light, radiation, redox cycling drugs or stimulated host phagocytes. In addition, cellular processes generate reactive sulphur species ($RS^{\bullet}$, $RSSR^{\bullet}$ and $RSOO^{\bullet}$) from thiol compounds. The interaction of these oxygen and sulphur species with proteins, nucleic acids and lipids is implicated in the etiology or manifestation of several pathological processes. The most potent oxidant and therefore, potentially damaging of the ROS, is the hydroxyl radical, which is known to cause membrane lipid peroxidation and the oxidation of proteins. To protect against destructive oxidative processes, organisms have developed a variety of antioxidant defences, including the enzymes, catalase, superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S-transferase (GST) and several small molecules, such as α tocopherol, ascorbate, carotenoids and thiols such as glutathione.

Iron and copper reduce oxygen, generating ROS in the presence of an electron donor such as thiol compounds, ascorbate, NAD(P)H / NAD(P) oxidase or xanthine / xanthine oxidase. In addition to ROS, thiyl radicals are also produced in autooxidations where thiols act as reducing equivalents. Several enzymes lose their activity in the presence of these oxidation systems (Kim *et al*, 1985). Iron chelating agents such as EDTA, and the antioxidant enzymes catalase and GPx protect the enzymes by removing either iron or hydrogen peroxide, thus preventing formation of the damaging hydroxyl radical. Peroxiredoxin enzymes can also protect proteins from this oxidative damage (Kim *et al*, 1988).

The protein protection ability of peroxiredoxins was first reported for yeast thioredoxin peroxidase (TPx) (Kim *et al*, 1988). TPx was originally thought to be specific for oxidation reactions using thiols and the enzyme was proposed to have a thiyl radical scavenging role. (Kim *et al*, 1988). However, it is now known that TPx acts as a peroxidase, reducing hydrogen peroxide and alkyl hydroperoxides with the use of hydrogens provided by thioredoxin, thioredoxin reductase (TR) and NADPH (Chae *et al*, 1994b). In this mechanism reduction of hydrogen peroxide is accompanied by the oxidation of the cys 47 sulphydryl groups on the two dimer subunits, which then react with the cys 170 groups to form an intermolecular disulphide. The sulphydryl groups are regenerated by the

transfer of reducing equivalents from NADPH by thioredoxin reductase to thioredoxin and finally to the disulphide of TPx (Chae *et al*, 1994b). The apparent thiol specificity observed, was attributable to the fact that the TPx disulphide could be reduced by a thiol but not by other reducing agents, such as ascorbate.

A DNA protection role has also been attributed to yeast TPx. Lim *et al*, (1993), showed that nicks in DNA formed as a result of oxygen radical generation were prevented by the addition of TPx, in the presence of a thiol compound. The hydroxyl radical is responsible for DNA cleavage and these studies indicate that TPx protection of the DNA may be due to direct removal of this radical, rather than by hydrogen peroxide breakdown, shown to be responsible for protein protection. There is no previously identified antioxidant enzyme which removes the hydroxyl radical directly. Thus, the ability of TPx and the other peroxiredoxin antioxidants to scavenge hydroxyl radicals *in vivo* would be of significant physiological importance.

Yeast TPx is a 25 kDa enzyme, the oxidised form of which exists mainly in a dimeric form linked by two disulphide bonds between the conserved cysteine sites (Chae *et al*, 1994b). In the present study, the fluke peroxiredoxin molecular mass calculated from the predicted amino acid sequence is 21, 646 Da and from SDS PAGE analysis of β galactosidase fusion protein is *ca* 30 kDa. These values are quite similar to the yeast values of 21,458 Da from amino acid sequence and 25 kDa from SDS PAGE of the purified protein (Chae *et al*, 1993).

Glutamine synthetase protection activity attributable to the antioxidant action of peroxiredoxin was demonstrated in adult liver fluke homogenate (LFH). The level of protection appears dose dependent, with higher levels of protection observed with increasing extract levels. Protection of the enzyme is sustained over prolonged incubation periods, although at slightly decreased levels. Catalase and glutathione peroxidase are also capable of protecting glutamine synthetase in this system, but neither enzyme has been reported in *F. hepatica.*

Peroxiredoxins become oxidised upon peroxide elimination and must

be regenerated. This can be achieved by the addition of a chemical reductant, such as DTT, or reductase proteins, such as the thioredoxin system which regenerates yeast TPx (Chae *et al*, 1994b). The thioredoxin system reduces TPx *in vivo* and is a much more potent hydrogen donor for TPx than DTT, on the basis of its ability to support TPx antioxidant activity. In the glutamine synthetase protection studies, fluke extract was used as a source of peroxiredoxin, at protein concentrations in the range 0.3 mg - 0.9 mg per 100µl reaction. Although the peroxiredoxin probably constitutes only a fraction of this protein, significant protection of glutamine synthetase activity was achieved with these crude extracts. The extract is likely to contain the specific peroxiredoxin regeneration system inherent to the fluke, the identity of which is unknown.

The novel fluke antioxidant appears to be a member of peroxiredoxin family, which is highly conserved from bacteria to mammals. Among the thirty members of the family, similarity ranges between 23 and 98% identity (Chae *et al*, 1994a). Most of these proteins are not associated with known biochemical functions, however the antioxidant activity of some members has been demonstrated. Fluke peroxiredoxin is 25% similar to the AhpC component of *Salmonella typhimurium* and *Escherichia coli* alkyl hydroperoxide reductase, which converts alkyl hydroperoxides into their corresponding alcohols. These bacterial alkyl hydroperoxide reductases have been shown to consist of a 22 kDa AhpC component and a 57 kDa FAD-containing NAD(P)H dehydrogenase (Jacobson *et al*, 1989; Storz *et al*, 1989). The AhpC component decomposes the substrate and is subsequently regenerated by AhpF coupled to NAD(P)H reduction.

At least eight of the prokaryotic genes encoding family homologues are found in close proximity to genes encoding proteins with other oxidation / reduction activities. In *S. typhimurium* and *E. coli* the AhpC gene is directly upstream of AhpF. In *Bacillus alcalophilus* and *Amphibacillus xylanus* the open reading frames encoding the homologue are just upstream of the genes encoding the NADH dehydrogenase and NADH oxidase proteins respectively (Chae *et al*, 1994a). These gene locations and the demonstration of antioxidant functions for some homologues have led to the proposal that the peroxiredoxin family members act as general reductants within the cell. The proteins may perform general reducing

functions in the cell analagous to the general folding or chaperoning functions carried out by the highly conserved heat shock proteins (Chae *et al*, 1994a).

Many of the family proteins are gene products from transformed mammalian cells. High levels of human proliferation associated gene (PAG), which shares 54% identity with fluke peroxiredoxin, are expressed in *ras* transformed cells (Prosperi *et al*, 1993). PAG is also transcribed at low levels in most human tissues suggesting that a constituitive level is necessary for life (Prosperi *et al*, 1993). MER 5, a mouse homologue having 52% identity with the fluke peroxiredoxin, is preferentially expressed in murine erythroleukemia cells and may be linked to cell differentiation (Yamamoto *et al*, 1989). Antioxidant activity has recently been ascribed to MER 5, which is now termed antioxidant protein 1 (aop 1) (Tsuji *et al*, 1995). The isolation of these genes suggests that redox regulation may be involved in the control of proliferation and differentiation.

Another homologue found in mice is MSP23; synthesis of MSP23 is induced in mouse peritoneal macrophages by treatment with sulphydryl reactive species or hydrogen peroxide (Ishii *et al*, 1993). Activated macrophages produce ROS and since these cells themselves resist the ROS and subsequent toxic by-products, they must possess a powerful defence system, which is likely to involve MSP23, to protect against their effects. Human natural killer cell enhancing factors A and B share extensive homology with each other and other members of the peroxiredoxin family (52 and 56% identity with fluke peroxiredoxin, respectively) (Shau *et al*, 1994). The two factors are found in red blood cells and enhance the activity of natural killer cells against tumour cells. Peroxiredoxin family members have also been identified in nine pathogenic organisms; in the three gastrointestinal pathogens, *Entamoeba histolytica, Cryptosporidium parvum* and *Helicobacter pylori*, in *S. typhimurium, E. coli* and *Bacillus fragilis* which also inhabit the gut and can be pathogenic and in *Mycobacterium avium* which causes tuberculosis-like pulmonary disease in immunocompromised patients and *Mycobacterium leprae* which is also a major human pathogen (Chae *et al*, 1994a). The homologues may not be the direct cause of pathogenesis but may be essential for the organisms to defend against oxidants generated

by macrophages and neutrophils. Diversity in the amino acid sequences of peroxiredoxin family members probably reflects several different mechanisms involved in the regeneration of reduced antioxidant. The fact that the genes are so well conserved and that their protein products are so abundant in some of the species where they are identified, indicates that they serve a very fundamental and essential function for the survival of cells throughout evolution.

Specific functions have been ascribed to very few of the peroxiredoxins, however, a striking feature of many of these proteins is that oxidative stress induces their hyperexpression. Yeast TPx synthesis is induced by the application of oxidative pressure (Kim *et al*, 1989). Yeast mutants lacking TPx remained viable, although their growth rate was significantly reduced, suggesting that TPx is a physiologically important antioxidant in yeast (Chae *et al*, 1993). Immature, migrating flukes have aerobic energy metabolism, which switches to anaerobic metabolism when the fluke matures and becomes resident in the bile ducts of the host, where oxygen tension is very low (Tielens *et al*, 1981; Tielens *et al*, 1982). Antioxidants protect against ROS generated intracellularly during respiration and other metabolic processes involving oxygen. Adult flukes are anaerobes, therefore, peroxiredoxin may function in protection against oxidants produced externally. Peroxiredoxin homologues are also found in the anaerobic bacteria *Methanobacterium thermoautotrophicum* and *Clostridium pasteurianum* (Chae *et al*, 1994a). It would be of interest in future studies to determine whether the fluke peroxiredoxin is inducible, by culturing in conditions of oxidative stress.

So far, only mammals have been found to contain more than one of the peroxiredoxin family genes, while bacteria and yeast carry only one copy. Alignment studies suggest that human TSA and NKEF B are the same protein and that human PAG and NKEF A are identical (Sauri *et al*, 1995). Both NKEF A and B are abundant in the cytosol of red blood cells and their protein protecting antioxidant function has been demonstrated (Shau and Kim, 1994). Natural killer cells are a subset of spontaneously cytotoxic lymphocytes that lytically destroy tumour cells and virus infected cells, without apparent antigen specificity or restriction from histocompatibility molecules. They are proposed to function as natural surveillance to deter

cancer development in the body and are also important in controlling viral infection and the regulation of hematopoiesis. Given their importance in maintaining host well being, it is not surprising that their activity is stringently controlled *in vivo.* Natural killer (NK) cell cytotoxicity is enhanced by IFN and IFN inducers and suppressed by cyclic AMP inducing agents. Adherent monocytes and diffusates from these cells also inhibit cytotoxicity, which is reversed in the presence of histamine (Hellstrand *et al*, 1990). The inhibition of NK cell function by monocytes is a consequence of oxidative injury caused by products of the respiratory burst. Histamine enhances NK cytotoxicity by preventing ROS generation, protecting NK cells from oxidative damage. *In vitro* studies have shown that hydrogen peroxide is the NK cell inhibitory agent and that addition of catalase, which decomposes hydrogen peroxide, results in NK activation (Hellstrand *et al*, 1994). It could therefore, be postulated that the peroxiredoxins NKEF A and B function *in vivo* in a manner similar to catalase. Thus, the NK cell enhancing ability of these proteins may be due to their antioxidant activity, in the down regulation of monocyte inhibition and subsequent activation of NK cytotoxicity.

F. hepatica peroxiredoxin may play a role in the flukes defence against ROS, generated as a by-product of intracellular metabolism, from the metabolism of certain pharmacological agents and from the effector arm of the host immune system. Newly excysted and juvenile flukes have a predominantly aerobic metabolism which generates a basal level of oxygen radicals, which must continually be removed to enable the fluke to survive. Another source of intracellular ROS may be the generation of oxygen radicals during digestion of host hemoglobin by the fluke. Iron-containing heme released during digestion, in the presence of oxygen and a reducing agent can generate ROS. The host response to parasite infection is also a potent source of oxidants which can result in damage sufficient to kill cells and even whole organisms. The ability of a parasite to survive this attack is directly linked to its content of antioxidant enzymes (Batra *et al*, 1992). When stimulated, aii leucocytes become activated and undergo a respiratory burst, leading to the production of ROS. The toxicity of the hydrogen peroxide produced may also be increased by peroxidases found in many phagocytes (Callahan *et al*, 1988). The location of

peroxiredoxin in the fluke is not yet known. However, the protein is present in ES products, therefore, it may be actively secreted by the fluke or it may be a surface antigen,which is released by tegumental turnover. The 29 kDa peroxiredoxin homologue of *Entamoeba histolytica* is located on the surface of trophozoites. Whether a surface antigen or secreted by the fluke, the peroxiredoxin may represent a powerful line of defence against host generated hydrogen peroxide.

The putative function of fluke peroxiredoxin in host defence may be further evidenced by recent studies which show that *F. hepatica* NEJs, in comparison to *S. mansoni* schistosomula are highly resistant to killing by ROS generated *in vitro* (unpublished results D. Piedrafita, J. P. Dalton and P. Brindley, Queensland Institute for Medical Research, Australia, 1994). In addition, Baeza *et al*, (1993), have demonstrated the *in vitro* inhibition of ROS generation and phagocytosis in bovine polymorphonuclear leucocytes by adult *F. hepatica* ES products, which contain fluke peroxiredoxin. The apparent inhibition of ROS generation and resistance to killing is likely due to fluke antioxidants. *F. hepatica* has been reported to possess relatively low levels of the enzymes which detoxify free radicals (Barrett, 1980; Callahan *et al*, 1988), therefore, the newly discovered peroxiredoxin may play a significant role in ROS removal and thus, in the flukes survival in the host.

The system employed for regeneration of the fluke peroxiredoxin *in vivo* is not known. It may have a cascade system like the yeast TPx which requires the flow of reducing equivalents from NADPH to thioredoxin reductase to thioredoxin and finally to TPx (Chae *et al*, 1994b), or it may require only a small endogenous thiol, such as glutathione to achieve regeneration. Thiols are also necessary for the activation of cysteine proteases within the fluke. *F. hepatica* cathepsin L1 and L2 are found in all stages of the fluke and are thought to have functions vital for the flukes development and survival. They may function in providing nutrition for the fluke by degrading ingested host proteins; they cleave immunoglobulin and may thus, play a role in immunoevasion and they are believed to assist in penetration of host tissue (Smith *et al*, 1993a; Smith *et al,* 1993b). The probable involvement of thiols with peroxiredoxin and the cathepsins, both proteins believed to be vital for the flukes survival, reflects the general

importance of sulphydryl groups and disulphides in biochemical mechanisms and cellular regulation. Further evidence of interaction between the antioxidant and protease molecules, is indicated by studies showing that cysteine protease inhibitors block nitric oxide production in activated macrophages, through interfering with nitric oxide synthase expression (Griscavage *et al*, 1995). If this were the case in the fluke, then it could be proposed that in periods of oxidative stress, cysteine proteases become inhibited, thiols required for peroxiredoxin activity would be more readily available for antioxidation and the protease inhibitors by preventing nitric oxide production, would also play a role in the flukes defence.

As in the case of the fluke tubulin, peroxiredoxin was present in the Hf preparation used to immunise cattle in the vaccine trial. The protein has a predicted molecular mass of *ca* 22 kDa and therefore, must have aggregated during purification to elute in the Hf fraction. Aggregation of these proteins during purification has been reported for both the yeast TPx (Kim *et al*, 1988) and the 26 kDa peroxiredoxin homologue of *Helicobacter pylori* (O'Toole *et al*, 1991). The presence of peroxiredoxin in the Hf may have contributed to the 43.8% protection achieved following challenge infection. The protein is highly immunogenic as seven of the twenty immunopositive clones appeared to contain this gene. So, like tubulin, the peroxiredoxin molecule has potential as a vaccinogen.

Future studies should include purification of the antioxidant and the elucidation of its natural regeneration system. It would also be of interest to determine the precise mechanism by which proteins and DNA are protected. Expression of recombinant peroxiredoxin may be necessary to obtain sufficient amounts of material for these studies and for vaccine trials. In order to understand the precise mechanism by which *F. hepatica* evades the host effector system, the parasites endowment with the range of antioxidant enzymes also needs to be examined and its susceptibility to each of the oxygen radicals produced determined.

Conclusion

In conclusion, a high molecular mass protein from *F. hepatica* has been isolated and characterised as hemoglobin. We have demonstrated that a preparation (Hf), containing this molecule can induce high levels of protection (43.8%) in cattle against a challenge infection. The protective immunity is increased to 72.4% when Hf is combined with another vaccine candidate, cathepsin L2. Using sera from the cattle vaccinated with Hf to screen an adult fluke cDNA library, genes encoding β tubulin and peroxiredoxin were isolated. These data indicate the presence of the two molecules in the Hf preparation and raises the possibility that they may have contributed towards the induction of protective responses. A cDNA clone encoding the hemoglobin was not isolated, however, this gene may be among several clones isolated but as yet uncharacterised. The production of recombinant hemoglobin, peroxiredoxin and β tubulin will allow the assessment of the protective ability of these molecules in the future.

Chapter 6

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Appendix

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