To Flash and Granny

Necator americanus: characterisation of secreted proteinases and vaccine development.

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DECLARATION

The material contained within this thesis has not been used in any other submission for academic award and is entirely the author's individual contribution. Due acknowledgement has been made of any assistance received. The author has attended the appropriate lectures and seminars in partial fulfilment of the requirements of the degree.

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Signed.....
(Director of studies)

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ABSTRACT

The proteinases present in *Necator americanus* larval excretory/secertory (ES) products were characterised using FITC labelled casein and shown to have a pH optimum of pH 6.5 with lesser peak of activity at pH 8. At pH 6.5 the presence of an aspartyl proteinase was demonstrated for the first time. At pH 8 a serine proteinase activity was shown to predominate. Metallo-proteinase activity was present at both pH points while cysteinyl proteinase activity was observed at pH 6.5. The mixture of proteinases secreted by both the adult and larval stages of *N. americanus* was further characterised by the use of a number of fluorogenic substrates. Both life cycle stages were shown to possess 'trypsin-like' serine proteinase activity together with cathepsin B and L-'like' cysteinyl proteinase activities.

Larval ES products were shown to degrade the human skin macromolecules collagen (type I, III, IV and V), fibronectin, laminin and elastin. The aspartyl proteinase activity was capable of degrading all the skin macromolecules except type V collagen. The metallo-proteinase degraded collagen and elastin while the serine proteinase degraded elastin alone.

A number of larval and adult proteinases were purified from ES products by affinity chromatography. Cathepsin B and L 'like' cysteinyl proteinases with an approximate molecular mass of 55 kDa were purified using activated thiol Sepharose 4B, an aspartyl proteinase with a mass of 66 kDa was purified using pepstatin A agarose. A 42 kDa metallo-proteinase was purified from larval ES using Z-Gly-D-Leu-AH-Sepharose.

A mixture of larval aspartyl and cathepsin B and L 'like' enzymes were used to vaccinate BALB/c mice against challenge infection with live *N. americanus* larvae, resulting in an apparent reduction in worm burden of 37.7 % compared with non-vaccinated control animals. Immunity to *N. americanus* challenge infection was induced for the first time by vaccinating BALB/c mice with γ-irradiated larvae. ELISA analysis demonstrated that irradiated larvae induce a Th2 response characterised by IgG1 and IL5 production typical of *N. americanus* infection, but with no significant difference to that induced by the same vaccination protocol using normal larvae. Further study is required to discover the mechanism or molecule(s) responsible for this immunity.

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ABBRIEVIATIONS

°C	Degrees centigrade
Abs	Absorbance
AChE	Acetylcholinesterase
AMC	7-amino-4-methylcoumarin
APMSF	4-(amidinophenyl)methanesulphonyl fluoride
APS	Ammonium persulphate
BCIP	5 Bromo 4 chloro 3 indolyl phosphate ρ-toluidine salt
Con A	Concavalin A
CPTA	copper phthalocyanine 3, 4', 4", 4"'-tetrasulphonic acid tetrasodium salt
DMEM	Dulbecco's minimal essential medium
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DTNB	5,5'-dithiobis-(2-nitro-benzoic acid)
Dpm	Disintegrations per minute
E64	L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
EF	exsheathing fluid
ES	Excretory/secretory products
FDA	Fluorescein diacetate
FITC	Fluorescein isothiocyanate
FITC-caesin	Fluorescein isothiocyanate labelled caesin
g	Relative centrifugal force
H	Hydrogen
HBSS	Modified Hanks buffered saline solution
h	Hour
Ig	Immunoglobulin
Ig GAM	Immunoglobulins G, A and M
IgG1	Immunoglobulin, subclass G_1
IgG2a	Immunoglobulin, subclass G_{2a}
IgG2b	Immunoglobulin, subclass G_{2b}

IgG3	Immunoglobulin, subclass G_3
i.u.	International Unit
kDa	Kilodalton
mA	milliampere
mg	milligram
ml	millilitre
NBT	Nitro blue tetrazolium
nm	nanometre
PBS	Phosphate buffered saline
PBS/Tween	Phosphate buffered saline/0.05%Tween 20
Phe	1, 10-phenanthroline
SIS	Skin immune system
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
STI	Soybean trypsin inhibitor
TCA	Trichloroacetic acid
TEMED	N'N'N-tetramethylethylenediamine
TBS	Tris buffered saline
Tris	tris (hydroxymethyl)-aminomethane
µg	microgram
µl	microlitre
V	Volt
Z	benzyloxycarbonyl

CHAPTER 1

GENERAL INTRODUCTION

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1.1 HOOKWORM INFECTION

Over 1150 million people world wide, almost a fifth of the worlds population, are infected with hookworms (Pawlowski, 1991). Of all the hookworm species, *Necator americanus* and *Ancylostoma duodenale* are the most important from a public health standpoint that develop to patency in man (Pritchard *et al.*, 1990a). However, *Ancylostoma ceylanicum,* primarily a parasite of cats and dogs (Garside & Behnke, 1988), has been shown to infect humans both experimentally (Carroll & Grove, 1986) and in naturally exposed populations (Chowdhury & Schad, 1972). A number of cases of eosinophillic enteritis presenting at Townsville General Hospital in Queensland, Australia, have been attributed to the dog hookworm *Ancylostoma caninum* which now also appears to be able to complete its life cycle in man (Croese, 1988; Prociv & Croese, 1990; Croese, *et al.*, 1990). The larvae of the cat hookworm *Ancylostoma tubaeforme* and the zoonotic *Ancylostoma braziliense* may penetrate human skin but fail to complete their life cycle. Instead they undergo a prolonged migration in the skin causing larva migrans or creeping eruption (Beaver, 1956; Beaver, 1964).

Hookworm infection is prevalent in the poorer, developing tropical nations. *N. americanus* predominates in India, Asia, Papua New Guinea, Central and South America while *A. duodenale* predominates in areas of Africa, China, and South America. Both species may occur together in areas of South America, Africa, Asia and the north east of Australia (Hotez & Pritchard, 1995).

1.2 PATHOLOGY

Adult hookworms reside in the small intestine where they attach to the gastrointestinal mucosa. A portion of the mucosa is drawn under negative pressure into the buccal cavity of the worm allowing it to ingest host tissue fluid and blood. Hookworms frequently move around the gut and reattach themselves to new sites but leakage and blood loss from the old

site of attachment continues (Variyam & Banwell, 1982). Each hookworm extracts only a small amount of blood each day (0.15-0.23 ml per day in the case of *Ancylostoma duodenale* and 0.03ml per day for *Necator americanus* (Hoagland & Schad, 1978)). This may be negligible to a healthy host, but when individuals become infected with increasing numbers of worms and the diet of the infected individual is poor then iron deficiency anaemia and protein malnutrition occurs (Gilman, 1982; Pritchard, *et al.*, 1991a; Hotez & Pritchard, 1995). Patients suffering from hookworm associated anaemia may complain of being short of breath, easily tired, palpitations, dizziness, aching legs not associated with exercise and epigastric pain (Pawlowski, 1991). Severe infections (over 500 worms) can lead to profound anaemia and heart problems. Children chronically infected with hookworm suffer from impaired growth and mental retardation that can be irreversible (Hotez & Pritchard, 1995).

1.3 HOOKWORM MORPHOLOGY AND LIFECYCLES

Hookworms belong to the family *Ancylostomaidae*, a part of the super family *Strongyloidea.* Adult hookworms are small fusiform, grayish white nematodes. Female worms (9-13 mm by 0.35-0.6 mm) are larger than the males (5-11 mm by 0.3-0.45 mm). The posterior end of the male worms has a broad membranous copulatory bursa with riblike rays (spicules) which are used for attachment to the female during copulation. The posterior of the female tapers to a blunt point. The chief differences between the species lie in the buccal capsule, vulva, male copulatory bursa and the overall shape of the worm. *A. duodenale* has two ventral pairs of teeth, the vulva is located two thirds from the anterior end of the female worm and the spicules of the male copulatory bursa remain separate. The anterior end of *N. americanus* is more distinctly 'hooked' and the buccal capsule has a dorsal pair of cutting plates, a concave median tooth and a pair of subventral lancets. The vulva of the female worm is located anterior to the middle of the body and the two spicules of the copulatory bursa are fused unlike those of *A. duodenale.*

N. americanus and *A. duodenale* have differing life cycles (Figure 1.1). As previously mentioned the adults of both species live in the intestinal tract feeding on blood and mucosal tissue. *N. americanus* is usually found in the jejunum and duodenum while *A. duodenale* is more frequently found in the jejunum and proximal ileum. In heavy

infections *A. duodenale* may be found as high as the stomach or as far as the colon (Rep, 1975). While in the intestine the adult worms mate and the fertilised eggs undergo a number of cell divisions before they are passed out in the faeces. If conditions are favourable the embryonated eggs hatch within 1-2 days. *N. americanus* eggs have been show to hatch in the temperature range 15-33 °C (Rep, 1975) with an optimum of 25-28 °C (Miller, 1979). The resulting rhabditiform larvae (L_1) actively feed on the faecal microflora and, after a period of growth, moult shedding the old L_1 cuticle. This L_2 larva is morphologically similar to the L_1 and undergoes a further period of growth followed by the secretion of a further new L_3 cuticle. The L_2 cuticle is detached but retained forming a sheath around the now non-feeding but infective L_3 larva. Development to the infective L_3 stage takes between 5-10 days. Hookworm larvae exhibit negative geotaxis and will crawl to high points on vegetation, soil particles and rocks, providing moisture is present (Schad, 1991). Alternate wetting and drying of such surfaces causes the larvae to migrate frequently rapidly exhausting their lipid reserves and hastening larval death (Udonsi, 1984). Even under optimum conditions very few larvae survive for more than a month under field conditions (Banwell & Schad, 1978). *N. americanus* infects man by penetrating the skin barrier while *A. duodenale, A. ceylanicum,* and *A. caninum* are all capable of infecting percutaneously but they prefer the oral route of infection. Following infection, the larva enters the blood stream or lymphatic system, from which it migrates to the lungs and breaks out into the alveolar spaces. The larva migrates up the trachea and is swallowed finally arriving in the small intestine where final development and maturation occurs. The intestinal stage consist of an L_4 larva with a temporary buccal cavity, which is shed when the final moult to the adult occurs. *A. duodenale* after entering the host may also undergo a period of arrested development, the resumption of development being timed so that eggs are released into soil just before the arrival of the 'monsoon season' (Schad, *et al.*, 1973; Schad, 1991).

A. duodenale is often considered to be a more opportunist parasite than *N. americanus* and less well adapted to its host. *A. duodenale* has a relatively short life span of approximately 1 year (Nawalinski, *et al.*, 1978), but is more virulent causing severer symptoms. Transmission of *A. duodendale* infection is maximised by producing a greater number of eggs, having more robust larvae and having the option of being able to infect via the

percutaneous route (Hoagland & Schad, 1978). Conversely, *N. americanus* has a life span of 3 to 4 years (Nawalinski, *et al.*, 1978), causes less blood loss and is considered better accommodated to the human host (Hoagland & Schad, 1978). *N. americanus* is unable to infect via the oral route unless the larvae penetrate the oral mucosa (Nagahana, *et al.*, 1962).

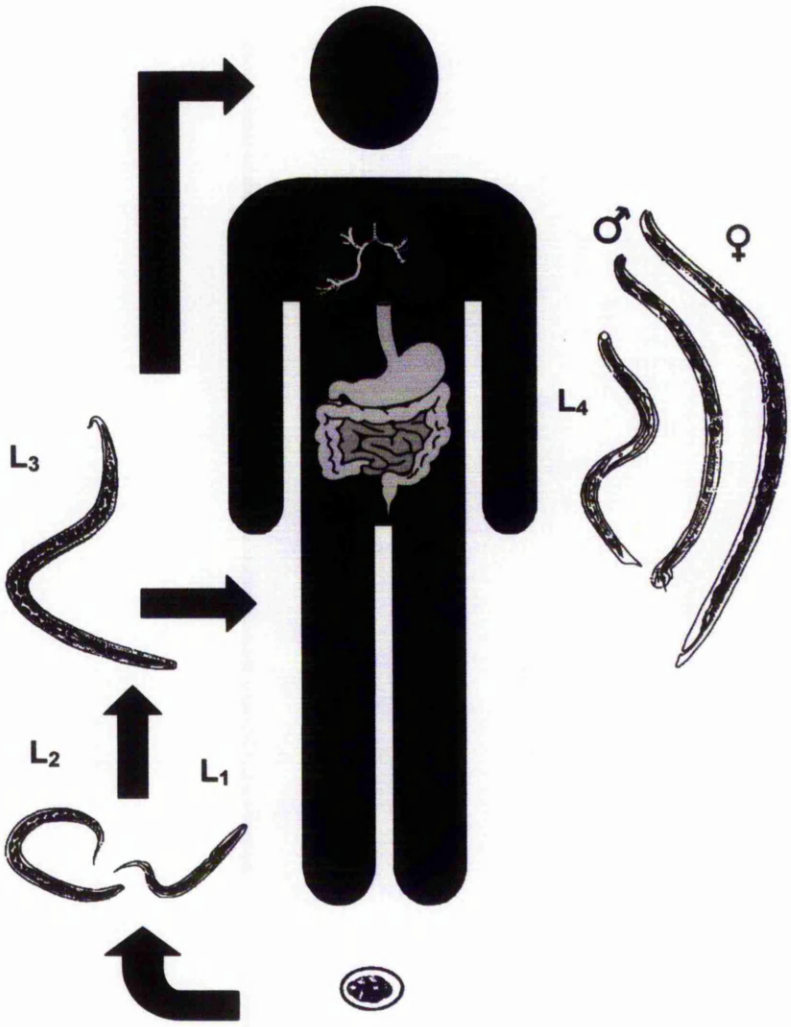

Figure 1.1

The life cycles of the hookworms *Necator americanus* and *Ancylostoma duodenale.*

Adult hookworms residing in the small intestine mate and fertilised eggs are passed out with the faecal material. Under favourable conditions the eggs hatch and develop into infective L_3 larvae. *N. americanus* infects by penetrating the skin barrier, *A. duodenale* whilst being capable of infecting percutaneously prefers the oral route of infection. Following entry into the host the larvae reaches the intestinal tract via the circulation and lungs where it matures into the adult hookworm (Adapted from Schad & Banwell, 1984).

1.4 IMMUNITY TO HOOKWORMS

There is considerable debate over the existence of immunity to hookworm infection (reviewed by Behnke, 1987). Hookworms have been shown to generate an immune response with antibodies being produced against hookworm antigens during natural and artificial infections (Ball & Bartlett, 1969; Ogilvie *et al.*, 1978; Carr & Pritchard, 1986; Carr & Pritchard, 1987). However, despite the presence of antibodies to *N. americanus* antigens, including acetylcholinesterase (Ogilvie *et al.,* 1978), these studies failed to correlate antibody levels to worm burden and no protective immunity was observed.

Early epidemiological evidence, however, may suggest the development of protective immunity. Hookworms are acquired steadily through life but between 20-50 years of age worm burdens remain stable. From 50 years of age onwards worm burdens increase suggesting that between the ages of 20 to 50 years the immune system is capable of controlling hookworm infection to some extent (Schad *et al.*, 1975). Populations of individuals have been identified that are predisposed to acquiring heavy or light hookworm infections despite constant re-infection (Schad & Anderson, 1985; Wakelin, 1985; Quinnell *et al.,* 1993).

Antibody responses to infection by *N. americanus* are characterised by a rise in all five antibody isotypes, in particular IgG and IgE (Ball & Bartlett, 1969; Maxwell *et al.*, 1987; Pritchard *et al.*, 1992). Epidemiological studies in Papua New Guinea have shown that IgG and IgM levels against adult worm secretions appear to reflect the current level of infection while IgG and IgM levels against adult cuticular collagen seem to be a reflection of exposure to cumulative hookworm infection (Pritchard *et al.*, 1990b). A year following chemotherapy levels of IgG and IgM against adult ES products fell while specific IgA and IgE levels returned to pre-treatment levels (Pritchard *et al.*, 1992). It is proposed that in the year following chemotherapy infection levels increased sufficiently to restore the IgA and IgE response but were not sufficient to restore the IgM and IgG reponses. This again suggests that antibody levels against adult ES products reflect the intensity of infection. IgD levels rose slightly following chemotherapy, an observation which currently remains unexplained.

Negative correlations between immune responses and parasite burden had not been recorded for *N. americanus* infection and it had been suggested that that there was no controlling immunity to *N. americanus*. However, using an age structured analysis of a study population in Papua New Guinea, Quinnell *et al.* (1995) demonstrated that the correlation coefficient between IgG levels against adult ES products and worm burden declined significantly with host age from positive in younger hosts (3-32 years), to negative in older hosts (33-48 years). Similar responses were observed for anti-ES IgM and IgE levels and anti-larval IgG levels. This may suggest the existence of a controlling protective immune responses against *N. americanus* infection in the 33-48 year age range.

Parasite infection is characterised by a rise in non-specific and specific IgE and a profound eosinophilia (Taylor & Turton, 1976; Knox-Macaulay, 1981; Maxwell *et al.*, 1987) associated with the release of interleukin 5 (White *et al.*, 1986). Pritchard *et al.* (1993) also observed levels of up to 1700 iu/ml total IgE in an infected population in Papua New Guinea. From the work of Dessaint *et al.*, (1975) with *S. mansoni* only 10 % of this IgE would be expected to be parasite specific. This excess of non-specific IgE has been suggested to be advantageous to the parasite, by blocking both the high affinity IgE receptor $Fc_{\varepsilon}R1$ on mast cells and antibody–dependant cytotoxicity (ADCC) reactions from both eosinophils and macrophages via the $Fc_{\varepsilon}R11$ receptor (Pritchard, 1993). Total IgE responses against adult ES products have also been shown to be negatively correlated with *N. americanus* weight and fecundity (Pritchard, 1995). Similar but non-significant correlations were observed when specific IgE responses were analysed. A negative correlation was also observed between eosinophil levels and worm weight and fecundity prior to antihelmintic treatment. These responses suggest that the immune system does have a controlling effect over *N. americanus* infection. IgE responses against *N.americanus* may also be of diagnostic value as there appears to be minimal cross reactivity of this isotype between *N. americanus* and other parasite species tested (Pritchard & Walsh, 1995).

1.5 HOOKWORM SECRETIONS

N. americanus has been shown to excrete or secrete numerous products (referred to as ES products) which may play important roles in the maintenance of the hookworm life cycle.

When resolved using 2 dimensional gel electrophoresis, the ES products of adult *N. americanus* may be resolved into over 200 polypeptide chains of which relatively few have been identified (Brown *et al.*, 1997). However, adult *N. americanus* has been shown to secrete a mixture of proteinases, which may play a role in feeding and evasion of the host's immune responses (Burleigh, 1993; Brown *et al.*, 1995). *N. americanus* also secretes a dimeric globular form of acetylcholinesterase (Pritchard *et al.*, 1994) for which a number of roles have been proposed from providing a biochemical holdfast (Philipp, 1984) to providing acetate and choline precursors for nematode metabolism. Studies have shown that AChE may act as an immunomodulant by interfering with the cholinergenic stimulation of leucocyte membrane receptors (Plaut, 1987). This may explain why parasites such as *N. americanus* that establish long term infections secrete large quantities of AChE in comparison to *A. duodenale* which, while being more pathogenic, is a more acute infection. Anticoagulant activities are also present in adult ES products, capable of inhibiting both platelet aggregation and blood coagulation Factor Xa (Furmidge *et al.*, 1995; Pritchard & Furmidge, 1995). A similar activity has also been shown to be present in soluble extracts of the dog hookworm *A. caninum* (Capello *et al.*, 1993). Glutathione-S transferase (GST) and superoxide dismutase (SOD) are also present in adult ES products. SOD has previously been described as a defensive enzyme against the immune response, neutralising reactive oxygen species released by eosinophils at the site of infection. SOD may also be acting as an anti-haemostatic (Pritchard, 1996). Some of these secreted proteins have been purified to homogeneity by a number of methods (Pritchard *et al.*, 1991b; Brophy *et al.*, 1995).

N. americanus larval ES products are less well characterised. Acetylcholinesterase activity is not present in larval ES products (Pritchard *et al.*, 1991b); however, the presence of a number of serine, cysteinyl and metallo-proteinase enzymes have been identified (Salafsky *et al.*, 1990; Kumar & Pritchard 1992a; Kumar & Pritchard 1992b). It has been proposed that these enzymes may play a role in skin penetration by infective larvae, which will be discussed in more detail in Chapter 3.

In addition to the secretion of proteinases both the adult and larval stages of *Ancylostoma doudenale* and *A. caninum* produce a hyaluronidase capable of degrading hyaluronic acid

found in the lamina propria of the gastrointestinal tract and in the dermal layer of the skin. It is proposed that this hyaluronidase activity may act as an additional virulence factor facilitating larval passage through the skin (Hotez *et al.*, 1992) or the invasion of the adult hookworm into the gut mucosa (Hotez *et al.*, 1994). To date this activity has not been detected in either *N. americanus* adult or larval ES products.

1.6 STRUCTURE OF THE SKIN

The skin is the largest organ of the body and presents a complex barrier through which *N. americanus* larvae must pass. Conventionally two major layers are recognised as constituting human skin. The outer layer or epidermis is a stratified epithelium that varies little in thickness (between 75 and 150 μm) over most of the body, except on the palm of the hands and the feet where it may increase to between 0.4 and 0.6 mm. Underlying the epidermis is a dense fibro-elastic connective tissue called the dermis. This constitutes the main mass of the skin and supports extensive vascular and nerve networks, specialised excretory/secretory glands and keratinized appendage structures such as hair and nails. Beneath the skin is the subcutaneous tissue or hypodermis, which is composed of connective and fatty tissue. Fibrous bands extend through the hypodermis attaching the skin to fibrous skeletal components.

1.6.1 THE EPIDERMIS

The epidermis is essentially a cellular sheet that encloses the entire body. It consists of a continuous stratified keratinizing epithelium which is only disrupted in its integrity by the pores of glandular structures and the follicles from which hairs emerge. The epidermis can be further split into two principal cell layers, an outer laminated sheet of dry anucleate cornified cells (the stratum corneum) and a living inner germinative cell layer from which the surface cells arise. The principal cells in the epithelium are called keratinocytes and have a cytoskeleton that is primarily composed of keratin. The epidermis also contains populations of melanocytes, Langerhans cells and Merkel cells whose function is unclear. The epidermis is in a constant state of turnover; as the basal keratinocytes divide the daughter cells are displaced outwards, undergo a number of differentiations and finally form the stratum corneum. Surface cells from the stratum corneum are lost into the environment as they are replaced from below. It has been estimated that the epidermis

completely replaces itself every two months (Odland, 1991). Keratinocytes are attached to each other by cellular attachments called desmosomes, the structure of which becomes degraded during the transition from germinative layer to stratum corneum. At the basal surface of the germinative layer, the cell membrane and desmosome structures abut upon a layer of approximately 20 nm thickness (the lamina lucidia) which in turn lies on the lamina densa of the dermis. This area of attachment between the epidermis and dermis is called the basement membrane. The basement membrane is composed primarily of collagen type IV, laminin (which adheres epithelial cells to type IV collagen), heparin sulphate and proteoglycans.

1.6.2 THE DERMIS

The dermis is a moderately dense connective tissue composed of collagen, elastin fibres and a gel of glycosaminproteoglycans, salts and water. The structural components of the dermis are synthesised by the dermal fibroblast. The main structural protein of the dermis is collagen, which gives the skin its strength and may account for up to 77 % of the dry weight of the skin. Collagen type I is the major collagen in the dermis along with type III (8-10 %) and lesser amounts of types V and VI. Woven between the collagen fibres is a network of elastin fibres that restore the dermis to its original position following deformation due to external mechanical forces. In addition to fibroblasts the dermis also contains mast cells and tissue macrophages. The dermis like the epidermis can be split into two regions. The papillary dermis (pars papillaris) is the thinner outermost area of dermis and is to be found closely moulded to the overlying epidermis. The papillary dermis contains smaller and more sparsely distributed collagen (0.3-3 μm in diameter) and elastin fibres than the reticular dermis and as a result, has a greater proportion of ground substance and connective tissue cells. Microcirculatory blood and lymph vessels as well as antigen presenting Langerhans cells (Bos and Kapsenberg, 1986) are also to be found in the papillary dermis.

The reticular dermis (pars reticularis) comprises the bulk of the dermis. It is a relatively acellular and avascular area containing dense collagen (10-40 μm) and elastin (10-12 nm) fibres. Collagen fibres are arranged as interwoven strands or bundles to form a lattice work in a plane parallel to the skin surface. There is no anatomical connection between the

collagen and elastin fibres. This arrangement of fibres is such that the skin shows a 'preferential direction of extensibility'. Should the skin be punctured then the fibres rearrange themselves so that most of them become aligned parallel to the direction of least extensibility. Smaller type III and V collagen fibrils are found in the regions of the epidermal-dermal junction and around blood vessels and nerves throughout both regions of the dermis. The reticular dermis also contains components of the skin immune system (SIS) such as mast cells, macrophages granulocytes and T cells (Bos and Kapsenberg, 1986).

1.6.3 COLLAGEN

Collagen is the major structural protein of the skin accounting for approximately 90 % of the protein present in the human dermis. Collagen consists of a family of related molecules with diverse biological roles including tissue repair, cellular adhesion, cellular migration, chemotaxis and platelet aggregation. Collagen has a unique molecular structure which renders it largely resistant to non-specific attack by proteolytic enzymes in the extra cellular matrix where it serves a number of important functions. Type I collagen is considered to be the prototypical collagen. The type I molecule consists of three polypeptide chains called α chains, each in turn composed of approximately 1000 amino acids. Except for the amino acid sequences found at either end of the molecule, every third amino acid is glycine. The general structure of the amino acid chain can be represented as $(Gly\text{-}X\text{-}Y)_n$ where X and Y are amino acids other than glycine. Collagen chains consist of 20 % proline and 4-hydroxyproline in approximately equal numbers, proline typically occurs in the X position while 4-hydroxyproline only occurs in the Y position. Each chain is coiled in a left handed helix with three amino acids per turn. The three chains are then wound around each other in a right handed super helix with the glycine residues orientated to the centre. The chains are bound to each other by hydrogen bonds approximately one per three amino acids (Traub & Piez, 1971). The presence of 4-hydroxyproline contributes to the stability of the triple helix as collagen with lower 4-hydroxyproline content is less stable and denatures at lower temperatures. (Rosenbloom *et al.*, 1979). Collagen microfibrils are formed by packing collagen molecules into a five stranded rope 'like' structure. Microfibrils formed in this way wrap around other microfibrils to form fibrils, which subsequently wrap around other fibrils to form larger fibres.

Thirteen types of collagen have been identified (Burgeson, 1988) all of which are composed of three polypeptide chains. Each component α chain within a collagen molecule is designated by an arabic numeral i.e. α1, α2. Each type of collagen is designated by a Roman numeral and may be either a homopolymer, $(\alpha I)_3$ or a heteropolymer $(\alpha 1)_2\alpha 2$. The types of collagen and their chain structure are summarised in Table 1.1

The dermis is composed predominately of type I (85-90 %), type III (8-10 %) and type V (2-4 %) collagen. Type I collagen, $[\alpha 1(I)]_2\,\alpha 2(I)$ is the major structural support for the dermis and indeed for almost all tissues. Type III collagen, $[\alpha 1(III)]_3$ is found closely associated with type I collagen and in the walls of the blood vessels in the skin.

Type IV collagen, $[\alpha 1(IV)]_2\ \alpha 2(IV)$ is a highly specialised, non fibrillar collagen that forms the basement membrane and anchoring plaques in the dermis which, together with type VII collagen $[\alpha 1(VII)]_3$ fibrils hold the basement membrane to the dermis. This is achieved by the type VII fibrils extending from the basement membrane and anchoring plaques and intertwining between the type I and III collagen fibres. Within the basement membrane type IV collagen molecules combine to form X-shaped tetramers, which join via their globular domains to form a 'chicken wire-like' network. Cells bind to type IV collagen through laminin and heparin sulphate proteoglycan (Figure 1.2), although in some cases keratinocytes may bind directly (Murray *et al.*, 1979).

Less information is known about type V collagen, and a number of structures have been proposed, e. g. $[\alpha 1(V)]_2\,\alpha 2(V)$, $\alpha I(V)\ \alpha 2(V)\ \alpha 3(V)$ and the homotrimers $[\alpha 1(V)]_3$ $[\alpha 2(V)]_3$. Type V collagen is found throughout the dermis, and occasionally in the basement membrane although it is not secreted by the cells resting upon it. Type V collagen may also extend into the epidermis (Woodley *et al.*, 1987). It has been suggested that, because of the association with the basement membrane, type V collagen may play an anchoring role.

Type VI collagen $\alpha 1(VI)\ \alpha 2(VI)\ \alpha 3(VI)$ is also found throughout the dermis where it forms a fine flexible fibrillary network that seems to anchor blood vessels, nerve cells and

fat cells into the dermis. Type VI collagen matrices have also been shown to demonstrate a non-fibronectin mediated spreading and adhesion of fibroblasts and may form a regulatory system for fibroblast cytoarchitectural organisation.

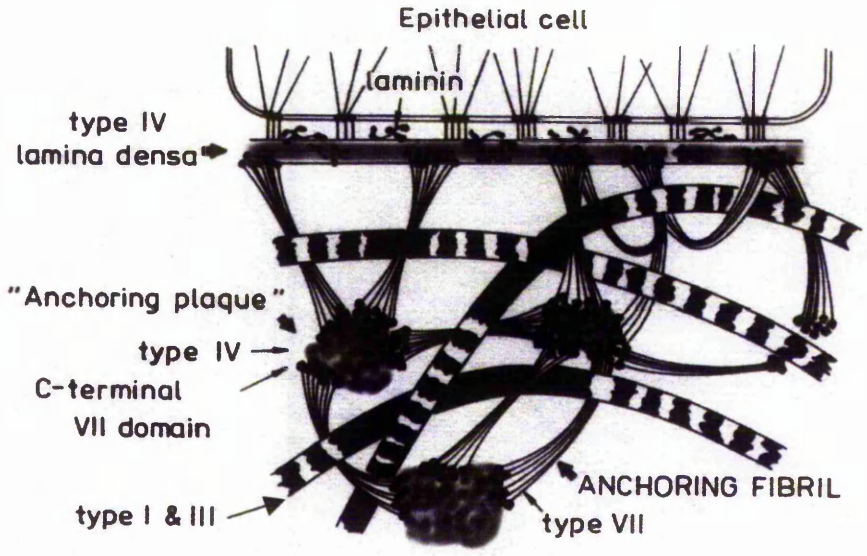

Figure 1.2

The anchoring fibril network

Fibrils of type VII collagen extend between the lamina densa and anchoring plaques in the dermis (both composed of type IV collagen). In this way they 'trap' fibers of type I and III collagen securing the basement membrane to the dermis. (Adapted from Kucharz (1992).

Table 1.1

Summary of the physical properties of the collagens found within the skin (adapted from Wenstrup *et al.*, (1991); Kucharz, (1992).

Type	Chain composition	Size	Structure	Tissue Distribution
I	$[\alpha1(I)]_2\ \alpha2(I)$	300 nm	Banded fibrils	Skin, tendon, bone, large vessels, intestine, uterus
III	$[\alpha1(III)]_3$	300 nm	Banded fibrils	Skin, muscle, uterus, blood vessels heart valves
IV	$[\alpha1(IV)]_2\ \alpha2(IV)$	390 nm	Non fibrillar network	Basement membranes
V	$[\alpha1(V)]_2\ \alpha2(V)$ $\alpha I(V)\ \alpha2(V)\ \alpha3(V)$ $[\alpha1(V)]_3$ $[\alpha2(V)]_3$ $\alpha I(V)\ \alpha'I(V)\ \alpha2(V)\ \alpha3(V)$	300 nm	Small fibres	Skin, placental membranes, cornea, bone blood vessels
VI	$\alpha1(VI)\ \alpha2(VI)\ \alpha3(VI)$	150 nm	Micro fibrils	Skin, heart muscle
VII	$[\alpha1(VII)]_3$	450 nm	Dimer	Skin (anchoring fibrils) placenta, lung cartlidge, cornea

1.6.4 ELASTIN

Elastin is a connective tissue component of a variety of tissues and organs. It is found in blood vessels and provides elasticity to the lungs, tendons, cartilage and skin. Within the dermal layers of the skin, elastic fibres are found forming a network interwoven between the collagen bundles. Elastin is secreted as soluble proelastin by fibroblasts within the skin. Proteolytic cleavage converts proelastin to insoluble tropoelastin, the monomer of elastin fibres (Smith & Wood, 1991). Crosslinking of tropoelastin occurs within the extracellular matrix to form elastin fibres. Like collagen almost a third of the amino acid residues are glycine with a high content of proline, alanine and lysine. Certain amino acid sequences are repeated often within the molecule (Lys-Ala-Ala, Lys-Ala-Ala-Lys and Lys-Ala-Ala-Ala-Lys) (Stryer, 1988). These sequences are substrates for the enzyme lysylamino-oxidase which crosslinks tropoelastin into elastin fibres. Structurally, elastin exists as random coils of monomer crosslinked in such a way that when stretched in any direction the coils become straight chains of elastin crosslinked to each other. When the tension is released the coils reform returning the skin into its original position. Interweaving the elastin fibres between collagen prevents overstretching and subsequent damage of the skin structure.

1.6.5 FIBRONECTIN

Fibronectin is a multifunctional extracellular and plasma protein that plays a role in cellular adhesion and crosslinking of the extracellular matrix. It occurs as a glycoprotein with an approximate molecular weight of 440 kDa, composed of two protein chains of 220 kDa linked together by disulphide bonds at their carboxyterminal ends. Fibronectin is capable of interacting with a number of molecules such as collagen (types I to V), heparin, hyaluronic acid and fibrinogen (McDonald & Kelly, 1980). Binding to these molecules has been shown to be associated with specific regions of the fibronectin molecule (Timpl & Martin, 1997). Fibronectin may be crosslinked by transglutaminase, but because the collagen binding site is distinct from the crosslinking site, crosslinks may be formed between fibronectin molecules and between fibronectin and collagen within the skin (Timpl & Martin, 1997). Fibronectin binding to collagen has been shown to be stronger when the collagen is in a denatured state and resistant to pepsin digestion (Kucharz, 1992).

The fibronectin molecule also contains a cell binding domain to which fibroblasts adhere within the extracellular matrix (Ruoslahti, 1988).

1.6.6 LAMININ

Laminin is a large basement membrane specific glycoprotein (containing 12-15 % carbohydrate) with a molecular weight of approximately 800 to 1000 kDa (Timpl & Martin, 1997). Laminin is a multifunctional protein with specific functions being associated with different regions of the molecule. Laminin has the ability to interact with heparin and heparin sulphate, specifically type IV collagen (Kucharz, 1992) and cells (Terranova *et al.*, 1980). Similarly, the cell binding ability of laminin is specific for endothelial and epithelial cells and not fibroblasts or chondrocytes. As such laminin is capable of specifically binding epithelial and endothelial cells to the basement membrane.

1.7 HOOKWORM LARVAE AND SKIN PENETRATION

The skin presents a complex barrier to the penetration process. Initially, it was considered that this barrier was so formidable that entry of hookworm larvae through the skin took place via routes such as hair shafts, pores or damaged skin. Hookworm larvae have since been shown to be able to penetrate the skin barrier at any point, entry being achieved by a combination of mechanical force, secreted proteinases and the ability to evade the cellular and humoral immune responses mounted by the skin against foreign material. It was originally thought that the infective larvae had to cast off the L_2 sheath prior to skin penetration (Matthews, 1977; Matthews, 1982). However, Kumar & Pritchard (1992c) demonstrated the presence of ensheathed larvae deep within the dermis, proving that exsheathment is not always a pre-requisite to skin penetration. It has also been demonstrated that the sera of infected individuals recognise the surface antigens of ensheathed larvae but not those of exsheathed larvae (Pritchard, 1990c). It is thought that this response against what eventually become 'cast' antigens diverts the immune response allowing the exsheathed larva to proceed essentially unrecognised.

Early evidence for the involvement of proteolytic secretions during skin penetration by *N. americanus* larvae was demonstrated by Matthews (1975, 1982). *N. americanus* larvae were shown to secrete an azocoll degrading activity with optimal activity at pH 8 and

capable of penetrating human skin from either the epidermal or dermal directions. Furthermore, their passage through human skin was accompanied by cellular destruction around the anterior end of the invading larva. However, larvae digested from human skin were unable to penetrate human skin a second time despite maintaining their motility, suggesting that the larval stage stores a finite amount of enzyme necessary for skin penetration. It has also been shown that secretory granules associated with the oesophageal glands of the L_3 reduce in size after skin penetration (Smith, 1976). This introduces the possibility that the proteases secreted during skin penetration are produced and stored by the L_2 larva. In contrast, the cat hookworm *A. tubaeforme* does not digest azocoll and the secretory granules associated with the oesophagus do not decrease in size after skin penetration (Smith, 1976). *A. tubaeforme* is able to penetrate skin membranes at least twice (Croll *et al.*, 1975) and it is proposed that skin penetration by *A. tubaeforme* relies completely on a mechanical process while in the case of *N. americanus* penetration into the stratum corneum is largely a mechanical process followed by the release of proteolytic enzymes which aid passage through the lower epidermal layers and dermis (Matthews, 1982).

1.8 THE PROTEINASE ENZYMES OF *N. AMERICANUS*

Necator americanus apparently needs proteinases to survive. They are secreted to facilitate tissue invasion (Matthews, 1982; Kumar & Pritchard, 1992a; Kumar & Pritchard, 1992b; Brown *et al.*, 1998), feeding, (Pritchard *et al.*, 1990d; Brown *et al.*, 1995), and to destroy components of the immune system (Kumar & Pritchard, 1992a; Pritchard, 1990d). Therefore, they present a potential target for neutralisation by the immune system and make feasible candidate molecules for vaccination. Proteinases are often allergenic and may be involved in the disregulation of the IgE network, which is a characteristic of hookworm infection (Hewitt, *et al.*, 1995; Pritchard *et al.*, 1992). Given the potential of the IgE response to regulate parasite infection (Ogilvie, 1964; Hagan, 1993) it may be of increasing importance to fully characterise and assess the vaccine potential of the hookworm proteinases

1.8.1 LARVAL PROTEINASES

Necator americanus invades the body by penetrating the skin barrier. As indicated earlier proteinase enzymes secreted by the larvae have been implicated in this process, some of the enzymes possibly involved have been partially characterised by a number of methods including, azocoll degradation (Matthews, 1982), penetration through a gelatin agar matrix (Salafsky *et al.*, 1990), gelatin substrate SDS-PAGE (Kumar & Pritchard, 1992a) and by monitoring the release of labelled peptides and amino acids from radio-labelled gelatin bound to a polystyrene plate (Kumar & Pritchard, 1992b). The results of these characterisation experiments have shown that *N. americanus* larvae secrete a mixture of serine, cysteinyl and metallo-proteinases.

Metallo-proteinase activity has been demonstrated in somatic extracts of *A. caninum* and *A. duodenale* larvae. This activity was shown to have a pH optimum of between 9 and 10 and molecular weights of 38 and 68 kDa (Hotez *et al.*, 1990). Live *A. caninum* and *A. duodenale* larvae are able to degrade fibronectin but unable to degrade the skin macromolecules elastin and laminin. Larvae of the human nematode *Strongyloides stercoralis* degrade all three skin macromolecules and the cercariae of *Schistosoma mansoni* have been shown to digest elastin and fibronectin (Marikovsky *et al.*, 1988). *S. stercoralis* and *S. mansoni*, like *N. americanus* are obligate skin penetrators, and it is proposed that the differences in connective tissue invasion may correlate with patterns of skin macromolecule degradation. The ability to degrade laminin may not, however, be a requisite for basal membrane penetration as metastatic tumour cells also release metallo proteases that facilitate passage but do not degrade laminin (Liotta *et al.*, 1986). Metallo-proteases found in the exsheathing fluid (EF) of *H. contortus* larvae mediate the breakdown of the L_2 sheath, subsequently allowing the escape of the L_3 larvae (Gamble *et al.*, 1989). As exsheathment is a prerequisite to further development it is possible that metallo-proteinases found in both *Ancylostoma spp* and *N. americanus* also perform a similar function.

1.8.2 ADULT PROTEINASES

The proteolytic activities present in the ES products of adult *N. americanus* have also been partially characterised using tritiated haemoglobin as the substrate (Burleigh, 1993).

Cysteinyl, serine, metallo and aspartyl proteases were shown to be present in adult ES products and proteolysis shown to be optimal at a number of pH points. A broad peak of activity was seen against haemoglobin in the pH range 5 to 7 with maximum activity at pH 6.6. Two smaller peaks of activity were also observed at pH 3.5 and 8.5. Proteolysis of haemoglobin was enhanced by the presence of $Ca,^{2+}$ Zn^{2+} and Fe^{2+} ions. 'Haemoglobinase' activity has been shown to resolve as a protein doublet of approximately 66 kDa using haemoglobin substrate SDS-PAGE (Pritchard *et al.*, 1990a).

1.9 CONTROL OF HOOK WORM; DRUG TREATMENT VERSUS VACCINES

The most effective way of controlling hookworm infection would be to improve sanitation to the point where the life cycle is broken and transmission from host to host is impossible. However, in most developing countries where hookworm is prevalent it is unlikely that these costly sanitation systems will be introduced in the near future. Hookworm infection may be reduced to some degree by wearing protective clothing such as shoes to prevent infective larvae penetrating through the soles of the feet. Again inhabitants of the tropics where hookworm is prevalent are often reluctant to wear such protective clothing.

A number of treatments are available for the treatment of hookworm infection including mebendazole, albendazole, pyrantal pamoate (Combantrin, Pfizer, Rossignol, 1998) and ivermectin (Wang *et al.*, 1989). Mebendazole and albendazole inhibit microtubule formation, while pyrantal and ivermectin interfere with neurotransmission (Wang *et al.*, 1989; Rossignol, 1998). Albendazole may be effective as a single dose, while mebendazole and pyrantal pamoate require repeated doses to be fully efficient. Ivermectin is effective against *Ancylostoma* hookworms while *N. americanus* has been shown to be up to 300 times more resistant to ivermectin in laboratory animals (Behnke *et al.*, 1993).

Hookworm disease is most prevalent in tropical developing nations where necessary treatments and iron supplements are often unavailable (Hotez & Pritchard, 1995). Furthermore, treatment by chemotherapy does not prevent re-infection by hookworm as infected individuals do not always develop a strong immunity capable of preventing further infection following chemotherapy (Quinnell *et al.*, 1993). The problems that arise due to

the lack of access to repeated doses of chemotherapy have led to an increasing interest in the possibility of developing a vaccine to prevent hookworm infection.

Vaccines to hookworms could in theory take a number of forms. Whole organisms, either killed or attenuated, may be administered in an attempt to generate an immune response enhanced enough to eliminate the parasite any time it attempts to enter the body. Similarly, one or more molecules of sufficient importance to the parasite; for example anticoagulants (Furmidge *et al.*, 1995), detoxification enzymes (Brophy *et al.*, 1995) or acetylcholinesterase (Pritchard, 1996) may be isolated and administered in a suitable form so that their neutralisation by the immune response causes the parasite to be expelled (reviewed by Hotez *et al.*, 1987). Although the immune response to natural hookworm infection does not appear to induce natural immunity it may be that vaccination using either whole organisms or individual molecules may still be successful (Hotez & Pritchard, 1995).

Miller (1971) demonstrated immunity to *A. caninum* infection in dogs following vaccination with live radiation attenuated larvae. He later hypothesised that a similar approach could be used to vaccinate humans against *N. americanus* (Miller, 1973). A commercial vaccine against *A caninum* based on irradiated larvae was developed but eventually withdrawn from the market after two years in the USA (Miller, 1978). This was because although it protected against the disease the vaccine suffered from a relatively short shelf life. Similarly, radiation attenuated cercariae have been used to immunise mice and baboons against *S. mansoni* (Smythies, 1996; Yole *et al.*, 1996; Pritchard & Wilson, 1997), *Fasciola hepatica* (Creaney *et al.*, 1995; Creaney *et al.*, 1996), and nematodes *Heligmosomoides polygyrus* (Hagan, *et al.*, 1981; Pleass & Bianco 1995).

It is unlikely that a human vaccine will be developed using live organisms. However, by studying the effects of irradiation on the parasite, or by isolating immunogenic products secreted following irradiation, it may be possible to start designing increase an effective vaccine against *N. americanus* infection. This will be discussed further in Chapter 5.

1.10 HOOKWORM PROTEINASES AS VACCINES

Chandler (1932) first proposed the concept of parasite enzymes as possible targets for immunological attack. This was applied to hookworms by Thorson (1956) who demonstrated that serum from infected dogs had the ability to neutralise the activity of an anticoagulant protease from *A. caninum*. Adult worms taken from immunised dogs had a significantly reduced capacity to feed, indicating the functional importance of this enzyme to the hookworm. Despite this observation a relatively small amount of research has been carried out to characterise the proteolytic secretions of these parasites. Furthermore, it has been shown that antibodies against adult ES products, which contain proteolytic enzymes, have the ability to affect both worm weight and fecundity (Pritchard, 1995). Similarly, it has been shown that vaccinating sheep with cysteinyl proteinases from *Fasciola hepatica* also reduces worm fecundity (Wijffels *et al.*, 1994). The larvae of *N. americanus* have also been shown to be immunogenic (Wells & Behnke, 1988a) and it has been suggested that, because of their proposed role in skin invasion, the proteinases secreted by *N. americanus* larvae provide a valid target for immune attack. Prior to this the secreted proteases must be fully characterised in order to define the relative importance of each of the enzymes and to design relevant purification protocols. Once purified, the individual proteases will be assessed for their potential as possible vaccines.

1.11 ANIMAL MODELS

Excluding man, the only hosts in which *N. americanus* can be successfully maintained are the hamster (Sen & Seth, 1967) and the rabbit (Bhopale *et al.*, 1977). Hamsters must be infected within 2-4 days of birth (Behnke *et al.*, 1986) making pre-challenge vaccination studies impossible. Mice, however, support the development of *N. americanus* to the L_4 stage and have been used as a model to study the invasive and migratory stage of the parasite (Timothy & Behnke, 1993; Wilkinson *et al.*, 1990; Wells & Behnke, 1988b). In addition, mice do not need to be infected as neonates, thus allowing vaccination studies that require multiple boosts possible. The *N. americanus* mouse model has been well characterised (Timothy, 1994) and currently represents the best laboratory hookworm animal model available for vaccination studies. This model will be used in the final stage of this project to study the effects, of vaccination with irradiated larvae or purified *N. americanus* larval proteinases.

1.12 AIMS

The aims of the work contained within this thesis are:

1) To complete the characterisation of the proteinases secreted by the larval and adult stages of the human hookworm *Necator americanus.*

2) To design relevant purification protocols to purify these enzymes.

3) To assess the potential of these enzymes as a vaccine against *Necator americanus* using a previously well characterised mouse model of hookworm infection.

CHAPTER 2

GENERAL MATERIALS AND METHODS

CHAPTER 2

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2.1 MATERIALS

General laboratory reagents were purchased from Sigma Aldrich, Poole, Dorset. Sticking plaster used to infect neonate hamsters was a generous gift from Johnson and Johnson Ltd. Sagatal (sodium pentobarbitone) was purchased from Rhone Merieux, Essex. Fluorescent proteinase substrates were purchased NovaBiochem Ltd, Nottingham and nitro cellulose (Optitran BA583) was purchased from Schluer and Schell.

2.2 PARASITE MAINTENANCE

Necator americanus was maintained in the Life Science Department of the University of Nottingham in syngeneic DSN hamsters (*Mesocricetus aureus*) using the method described by Sen & Seth, (1967). This hamster-adapted strain of the human hookworm was originally obtained in 1983 from Dr G. Rajasekariah of Hindustan, CIBA-GEIGY Ltd., Bombay, India.

2.2.1 PERCUTANEOUS INFECTION OF DSN HAMSTERS WITH *N. AMERICANUS* LARVAE

A breeding colony of DSN hamsters was maintained at the University of Nottingham under conventional animal house conditions. 2-4 day old neonate hamsters from this colony were infected percutaneously with 100 infective third stage larvae (Behnke *et al.*, 1986). To infect neonate hamsters, small pieces (approximately 5x 5 mm) of gauze were placed on strips of sticking plaster (approximately 5x 50 mm). 100 infective larvae in 10-20 µl of water were applied to the gauze and the sticking plaster wrapped around the abdomen of the hamster so that the larvae were applied to the ventral thorax. Plasters were left in position for 24 hours before being carefully removed to avoid tearing the delicate neonate skin.

The infection was allowed to proceed for 35 days after which the adult worms were harvested from the small intestine (Section 2.3.2) or the infection was allowed to proceed for longer periods (over 50 days) to enable the collection of fertilised *N. americanus* eggs.

2.2.2 CULTURE OF INFECTIOUS *N. AMERICANUS* LARVAE

From 50 days post-infection hamsters used specifically for the production of fertilised eggs were immunosupressed by treating them weekly with hydrocortisone 2-1 acetate (Appendix 2.1.14). Faecal material containing *N. americanus eggs* was collected on trays lined with moist tissue by housing infected hamsters overnight in cages with a wire grid base. Infective larvae were cultured from faecal material by a method modified from Harada & Mori (1955) and first described by Kumar & Pritchard (1992). Faecal material was mixed with activated charcoal, 1 % (w/v) amphotericin B (final concentration) and water to form a smooth paste which was applied to the upper half of a 5 x 30 cm strip of filter paper. The strips were then suspended in a large glass chromatography tank containing approximately 750 ml of distilled water. The tanks were sealed and incubated at 28 °C for 7-10 days, after which the filter paper strips were carefully removed and discarded. The water containing the larvae was transferred to a measuring cylinder and the larvae allowed to settle for 2 h. After this period the water was aspirated off and the larvae washed twice to remove any faecal contamination. Finally, washed larvae were resuspended in approximately 20 ml of storage buffer (50 mM Na_2HPO_4, 70 mM NaCl, 15 mM KH_2PO_4, pH 7.4; (Hawdon & Schad, 1991). Larvae were stored in the dark at room temperature until required, or for a maximum period of one month.

2.3 PREPARATION OF LARVAL AND ADULT *N. AMERICANUS* EXCRETORY/SECRETORY (ES) PRODUCTS

2.3.1 LARVAL EXCRETORY/SECRETORY PRODUCTS

ES products were collected as described by Kumar & Pritchard, (1992). Freshly collected, ensheathed larvae were resuspended in larval storage buffer and exsheathed by bubbling carbon dioxide through the suspension for 2 h at room temperature. Exsheathed larvae were allowed to settle and then washed extensively with RPMI 1640 containing 100 i.u. /ml penicillin, 100 µg/ml streptomycin and 1 % (w/v) amphotericin B under sterile

conditions. Following this 'sterilisation period' the larvae were cultured in RPMI 1640 containing the above additives for 72 h at 37 °C, changing the culture medium every 24 h. ES products collected over the 72 h period were pooled, dialysed against distilled water, lyophilised and stored at -20 °C until required.

2.3.2 ADULT EXCRETORY/SECRETORY PRODUCTS

Adult ES products were collected as described by Brown & Pritchard, (1993). Hamsters infected with *N. americanus* were killed with an overdose of chloroform at 35 days post - infection. The small intestine was removed, cut along its length and placed in a petri dish containing modified Hanks buffered saline solution (HBSS). The petri dishes were incubated at 37 °C to allow the adult worms to detach voluntarily from the intestine thus minimising the possibility of host tissue contaminating subsequent ES cultures. Adult worms were then removed from the petri dish using a wide bore, blunt ended pasteur pipette to avoid damage to the worms and washed extensively in RPMI 1640 containing 100 i.u./ml penicillin and 100 µg/ml streptomycin over a period of 2 h. Following this 'sterilisation' period adult worms were cultured in sterile RPMI 1640 for 24 h. Adult worm viability was assessed by monitoring worm motility and, during the early experimental work, acetylcholinesterase (AChE) secretion. AChE secretion has previously been used as a measure of worm 'well being' (Burt & Ogilvie, 1975) and has been shown to be cumulative over a 48h period (Pritchard *et al.*, 1991b). Although the AChE activity of every ES culture was not monitored, its cumulative (Pritchard *et al.*, 1991b) nature indicates that it is unlikely that the various enzymatic activities present in ES products is due to the death of the parasite and the subsequent leakage of intracellular enzymes. ES products obtained after 24 hours were dialysed against distilled water, freeze dried and stored at –20 °C.

2.4 PROTEIN ESTIMATION

The protein content of larval and adult ES products was determined using the BioRad protein assay kit (Bradford, 1976). Protein standards were prepared from a 1 mg/ml solution of bovine serum albumin (BSA) in distilled water, to give a range of 0-140 µg of protein in a final volume of 100 µl. Samples under test were used either neat or diluted if necessary in distilled water to a final volume of 100 µl. 5 ml of BioRad protein dye

reagent (diluted 1:4 with distilled water) was added to both the standards and samples and mixed thoroughly. After 30 minutes the absorbance of the standards and samples was measured at 595 nm. Protein concentrations of samples was then determined by plotting the absorbance of the standards against their protein content and reading the protein content of the sample from this standard curve.

2.5 ENZYME ASSAYS

2.5.1 DETERMINATION OF ACETYLCHOLINESTERASE ACTIVITY

Acetylcholinesterase activity was determined by monitoring the hydrolysis of acetylthiocholine iodide to thiocholine and acetic acid by AChE (Ellman *et al.*, 1961). Released thiocholine reacts with DTNB (5,5'-dithiobis-(2-nitro-benzoic acid)) to give a yellow colouration that can be followed spectrophotometrically. 793 µl of working solution (Section A.2.2.4) was pipetted into a 1 ml cuvette and 7 µl of substrate (75 mM acetylthiocholine iodide) added and mixed thoroughly. The reaction was started by adding 20 µl of adult ES products and the change in absorbance monitored at 412 nm over a 10 minute time period. AChE activity was calculated as described in section A 2.2.5.

2.5.2 PROTEINASE ASSAYS USING FLUORESCEIN ISOTHIOCYANATE-LABELLED CASEIN

2.5.2.1 PREPARATION OF FITC-LABELLED CASEIN

Fluorescein isothiocyanate labelled casein was labelled as described by Beynon & Bond, (1989). 1 g of casein was dissolved in 100 ml of 50 mM sodium carbonate buffer, 150 mM NaCl pH 9.5. 40 mg of solid fluorescein isothiocyanate (FITC) was added and the solution stirred for 1 hr at room temperature. The FITC labelled casein was then extensively dialysed against distilled water over a period of 48 h. Finally, the protein content was adjusted to 5 mg/ml and the labelled casein aliquoted and stored at -20 °C until required.

2.5.2.2 PROTEINASE ASSAYS USING FITC-LABELLED CASEIN

Proteinase activity in larval and adult ES products was detected by monitoring the release of fluorescein isothiocyanate labelled amino acids from FITC labelled casein. Typically

10 µg (50 µl) of ES products were mixed with 10 µl of FITC-casein (final concentration 250 µg/ml) and 140 µl of buffer suitable for the pH required (0.1 M citric acid/sodium citrate buffer, pH 3-5.5, 0.1 M phosphate buffer, pH 6-8 or 0.05 M 2-amino-2-methyl-1:3-propanediol-HCl buffer, pH 8-10) in a microfuge tube. Reactions were incubated at 37 °C for 3 h after which 120 µl of 5 % w/v trichloroacetic acid was added to stop the reaction. The tubes were allowed to stand at room temperature for 1 h and the undigested, precipitated protein removed by centrifugation at 13 000 g for 10 minutes. Triplicate, 20 µl aliquots of the supernatant were added to 980 µl of 0.5 M Tris-Cl pH 8.5 and the fluorescence measured (excitation 490 nm, emission detection 525 nm).

Proteinase activity was characterised using a number of proteinase inhibitors as described in Table 2.1. The exact concentrations used for individual experiments are described in each chapter.

2.5.3 PROTEINASE ASSAYS USING FLUORESCENT SYNTHETIC PEPTIDE SUBSTRATES

Proteinase activity was also assessed by monitoring the release of 7-amino-4-methylcoumarin (AMC) from a range of synthetic peptide substrates. 10 µg of larval or adult ES products (50 µl) were incubated with 10 µl of substrate (5 mM stock in DMSO) in a final volume of 940 µl of buffer at the required pH. Samples were incubated at 37 °C for 1 h after which the reaction was stopped by the addition of glacial acetic acid to 300 mM (Dowd *et al.*, 1994) and the fluorescence measured (excitation 365 nm, emission detection 465 nm). Any variations to the assay and details of proteinase inhibitors used to characterise the activity are again described in each individual chapter.

2.6 SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Polyacrylamide gel electrophoresis was carried out using a discontinous gel system as described by Laemmli (1970). All gels were run using ATTO mini gel kits supplied by Genetic Research Instruments Ltd.

2.6.1 PREPARATION OF RESOLVING AND STACKING GELS

Glass plates and spacers were thoroughly cleaned with detergent and then washed with distilled water, ethanol and finally acetone prior to assembly according to the manufacturers instructions. Unless otherwise stated proteins were separated on 12 % acrylamide resolving gels under reducing conditions. 50 µl of 10 % ammonium persulphate (APS) and 5 µl of N'N'N'-tetramethylethylenediamine (TEMED) were added to 10 ml of degassed resolving gel solution (Appendix A.2.3.6). The gel solution (approximately 7 ml) was then quickly poured between the gel plates avoiding the formation of air bubbles. An overlay of water saturated butanol was applied to the surface of the gel and the gel left to polymerise for approximately 1 hour. Following polymerisation the overlay was removed, the gel washed with distilled water six times and the surface of the gel dried carefully with filter paper, prior to pouring the stacking gel.

For all gel electrophoresis 4 % acrylamide stacking gels were used. 25 µl of 10 % APS and 10 µl of TEMED was added to 5 ml of stacking mix (Appendix A.2.3.7). A Teflon well forming comb was inserted between the glass plates at an angle of approximately 45 degrees and the stacking gel mix (approximately 2-3 ml depending on the size of the well forming comb), poured between the glass plates. The comb was then inserted fully, thus avoiding the formation of air bubbles. The stacking gel was then left to polymerise for approximately 30 minutes.

2.6.2 SAMPLE PREPARATION

A maximum volume of 20 µl was loaded per lane. If the protein concentration of the sample was sufficient, then the sample was prepared by adding equal amounts of sample buffer (Appendix A.2.3.8). If the protein concentration of the sample was too low, then the sample was concentrated by acetone precipitation (Appendix A.2.3.11), and the precipitate resuspended in 20 µl of loading buffer. Samples were reduced by boiling for 10 minutes and loaded into the wells of the stacking gel. Gels were run at 20 mA constant current per gel for 90 minutes using an ATTO SJ-1082 mini powerpack. The temperature was kept low by refrigerating the SDS-PAGE running buffer prior to running the gel. Protein samples were run concurrently with protein standards of known molecular mass,

(Sigma SDS 6H, high molecular mass standards for Coomassie staining or Sigma SDS 7B pre-stained molecular mass standards for Western blots). Molecular mass standards were prepared according to the manufacturer's instructions and boiled for 10 minutes before loading onto the gel.

2.6.3 PROTEIN VISUALISATION

Gels were stained by either Coomassie brilliant blue R250 or Colloidal Coomassie blue G250 (Neuhoff *et al.*, 1988).

2.6.3.1 COOMASSIE BRILLIANT BLUE R250

Gels were fixed (Appendix A.2.3.13) for 30 minutes before being stained in Coomassie Brilliant Blue (Appendix A.2.3.12) at room temperature for 2-3 hours. Proteins were visualised by destaining the gels for 2-3 hours with repeated changes of destain.

2.6.3.2 COLLOIDAL COOMASSIE BLUE G250

Gels were fixed in destain (Appendix A.2.3.13) for 30 minutes before being stained in Colloidal Coomassie Blue G 250 according to the manufacturers instructions. Proteins were visualised by washing for a few minutes with destain.

2.7 WESTERN BLOTTING

Proteins separated by SDS-PAGE were transferred onto nitrocellulose (Optitran BA583 reinforced nitrocellulose, 0.2 μm pore size) by the method of Towbin *et al* (1979) using a Bio-Rad Mini Protean II Transfer Cell. Transfer was carried out at 100 V constant voltage for 60 minutes or 20 mA constant current overnight. Following transfer, Western blots were stained with 0.05 % copper phthalocyanine 3, 4', 4", 4"'-tetrasulphonic acid tetrasodium salt (CPTA, Section 2.7.1) to ensure protein transfer, prior to immunoprobing. The differing conditions used for the immuno probing of Western blots are described in each chapter.

2.7.1 VISUALISATION OF PROTEIN ON WESTERN BLOTS

Proteins transferred onto Western blots were visualised by staining with CPTA (Bickar & Reid, 1992). Western blots were immersed into 0.05 % CPTA (Appendix 2.4.4) for 1

minute and then destained with distilled water in order to visualise bound protein. Prior to immuno probing copper phthalocyanine 3, 4', 4", 4"'-tetrasulphonic acid tetrasodium salt was removed from Western blots by washing with 12 mM sodium hydroxide.

APPENDIX 2

A.2.1 GENERAL BUFFERS AND REAGENTS

A.2.1.1 PHOSPHATE BUFFERED SALINE (PBS)

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g

Dissolved in approximately 500 ml of deionised water, pH adjusted to 7.4 and made up to a final volume of 1 litre.

A.2.1.2 TRIS BUFFERED SALINE (TBS)

NaCl	9.0 g
Tris	6.21 g

Dissolved in approximately 200 ml of deionised water, pH adjusted to 7.4 and made up to a final volume of 1 litre.

A.2.1.3 PBS/0.05 %TWEEN 20

Tween 20 (polyoxyethylene sorbitan monolaurate)	250 µl
PBS	500 ml

A.2.1.4 TBS/0.5 % TWEEN

Tween 20	2.5 ml
TBS	500 ml

A.2.1.5 0.1 M CITRATE/SODIUM CITRATE BUFFER

Sodium citrate	14.7 g
Citric acid	10.52 g

Each made up to 500 ml in distilled water then titrated against each other to the required pH.

A.2.1.6 0.1 M SODIUM PHOSPHATE BUFFER

$NaH_2PO_4.2H_20$	7.8 g
Na_2HPO_4	7.09 g

Each made up to 500 ml in distilled water then titrated against each other to the required pH.

A.2.1.7 2-AMINO-2-METHYL-1:3-PROPANEDIOL-HCL BUFFER

2-amino-2-methyl-1:3-propanediol	2.6 g

Made up to 500 ml with distilled water, adjusted to the required pH with concentrated HCl

A.2.1.8 MODIFIED HANKS BUFFERED SALINE SOLUTION (HBSS)

A.2.1.8.1 Solution 1

NaCl	168 g
KCl	8.0 g
KH_2PO_4	4.0 g
Na_2HPO_4	4.0 g
0.2 % Phenol red	200 ml

Made up to 2 litres with deionised water.

A.2.1.8.2 Solution 2

$CaCl_2.2H_20$	0.92 g
$MgCl_2.6H_20$	2.0 g

Made up to 2 litres with deionised water.

110 ml of solution 1 was added to 110 ml of solution 2 and 780 ml of deionised water and the pH adjusted to 7.2 with 1 M NaOH.

A.2.1.9 0.05 M CARBONATE/BICARBONATE BUFFER pH 9.6

$Na_2 CO_3$	0.80 g
$Na HCO_3$	1.47 g

Dissolved in 100 ml deionised water and adjusted to pH 9.6 with 1 M HCl and made up to a final volume of 500 ml.

A.2.1.10 GLYCINE BUFFER pH 10.4

Glycine	3.151 g
$MgCl_2$	0.047 g
$ZnCl_2$	0.068 g

Dissolved in 300 ml distilled water, pH adjusted to 10.4 with NaOH. Made up to a final volume of 500 ml.

A.2.1.11 0.1 M SODIUM ACETATE pH 6

Sodium acetate	8.2 g

Dissolved in 800 ml distilled water, pH adjusted to 6 with glacial acetic acid and made up to a final volume of 1 litre.

A.2.1.12 2.5 M SULPHURIC ACID

Concentrated sulphuric acid	18 ml
Distilled water	82 ml

A.2.1.13 LARVAL STORAGE BUFFER

Na_2HPO_4	7 g
NaCl	4 g
KH_2PO_4	3 g

Dissolved in 1 litre of distilled water

A.2.1.14 HYDROCORTISONE-2, 1-ACETATE

Hydrocortisone-2, 1-acetate	500 mg
Deionised water	20 ml

0.1 ml administered per animal, weekly, subcutaneously in the nape of the neck.

A.2.1.15 SAGATAL (SODIUM PENTOBARBITONE)

Sagatal (60 mg/ml) was diluted 1:9 v/v in pyrogen free water and used to anaesthetise mice by intraperitoneal injection (0.1 ml/100 g body weight).

A.2.1.16 FLUORESCEIN DIACETATE

Fluorescein diacetate	5 mg
Acetone	1 ml

Store at –20 °C until required.

Working solution is 1:50 dilution in PBS.

A.2.1.17 RED CELL LYSIS SOLUTION

Ammonium chloride	0.85 g

Dissolved in 50 ml distilled water, pH adjusted to 7 with concentrated ammonia and made up to a final volume of 100 ml.

A.2.2 ELLMAN ASSAY (AChE) REAGENTS

A.2.2.1 **Solution 1** 50 mM sodium phosphate buffer pH 7.4

A.2.2.2 **Solution 2** 10 mM DTNB (39.6 mg/ml) in 50 mM sodium phosphate buffer pH 7

A.2.2.3 **Solution 3** 10 % Triton X-100 in 5 0mM sodium phosphate buffer pH 7

A.2.2.4 **Working solution** 20 ml of solution 1, 700 µl of solution 2 and 200 µl of solution 3

A.2.2.5 Substrate 75 mM (21.67 mg/ml) acetylthiocholine iodide in 50 mM sodium phosphate buffer pH 7)

Under these conditions $c = A / \varepsilon l$

Where: A = change in absorbance at 412 nm

l = light path of 1 cm

ε= molar extinction coefficient =1.36×10^4/(cm. M)

c = concentration of product

Taking into account the natural breakdown of the substrate at pH 7 (0.0016 abs/min)

$c = A - 0.0016 / 1.36 \times 10^4$ moles/min

1 unit of AChE is defined, as being the activity required to hydrolyse 1 mM of substrate per minute at 25 °C. Specific AChE activities are expressed as units per mg of protein.

A.2.3 SDS-PAGE SOLUTIONS

The following recipes were used to prepare 12 % acrylamide gels for the separation of proteins.

A.2.3.1 STOCK ACRYLAMIDE/BIS-ACRYLAMIDE SOLUTION (30 %: 0.8 % w/v)

Acrylamide	146 g
Bis-acrylamide	4 g

Dissolved in water to 500 ml, filtered and stored at 4 °C in the dark.

A.2.3.2 1.5 M TRIS-HCL pH 8.8

Tris	90.83 g

Dissolved in water, pH adjusted to 8.8 with concentrated HCl and made up to a final volume of 500 ml

A.2.3.3 0.5 M TRIS-HCL pH 6.8

Tris	30.3 g

Dissolved in water, pH adjusted to 6.8 with concentrated HCl and made up to a final volume of 500 ml

A.2.3.4 10 % AMMONIUM PERSULPHATE

Ammonium persulphate	1 g

Dissolved in distilled water to a final volume of 10 ml. Stored at -20 °C in 200 µl aliquots.

A.2.3.5 10 % SODIUM DODECYL SULPHATE

Sodium dodecyl sulphate	10 g

Dissolved in distilled water to a final volume of 100 ml.

A.2.3.6 RESOLVING GEL

Acrylamide/Bis-acrylamide (30 %: 0.8 % w/v)	8.0 ml
1.5 M Tris-HCl pH 8.8	5.0 ml
Distilled water	6.69 ml
10 % SDS	0.2 ml
10 % Ammonium persulphate	0.1 ml
TEMED	0.01 ml

A.2.3.7 STACKING GEL

Acrylamide/Bis-acrylamide (30 %: 0.8 % w/v)	0.65 ml
0.5 M Tris-HCl pH 6.8	1.25 ml
Distilled water	2.98 ml
10 % SDS	0.05 ml
10 % Ammonium persulphate	0.05 ml
TEMED	0.02 ml

A.2.3.8 2x REDUCING SAMPLE BUFFER

0.5 M Tris-HCl pH 6.8	2 ml
Glycerol	2 ml
10 % SDS	4 ml
1 % Bromophenol Blue	0.2%
Dithiothreitol (100 mM)	0.154 g
Distilled water	2 ml

A.2.3.9 2x NON REDUCING SAMPLE BUFFER

0.5 M Tris-HCl pH 6.8	2 ml
Glycerol	2 ml
10 % SDS	4 ml
1% Bromophenol Blue	0.2 %
Distilled water	2 ml

A.2.3.10 SDS-PAGE ELECTRODE BUFFER

Tris	30 g
Glycine	144 g
SDS	10 g

Distilled water to 10 litres

A.2.3.11 ACETONE PRECIPITATION OF PROTEIN SAMPLES PRIOR TO ELECTROPHORESIS

Proteins were precipitated by the addition of acetone (-20 °C) to a final concentration of 80 %. Samples were held at –20 °C for 20 minutes before being centrifuged at 13000 g for 15 minutes. The acetone was aspirated off and the pellet resuspended in 20 µl of SDS-PAGE loading buffer.

A.2.3.12 COOMASSIE BRILLIANT BLUE R250 STAIN

Coomassie Brilliant Blue R250	0.5 g
Ethanol	125 ml
Glacial Acetic acid	50 ml

Distilled water to 500 ml. Filter before use and store in the dark at room temperature.

A.2.3.13 COOMASSIE BLUE FIXATIVE AND DESTAIN

Ethanol	250 ml
Glacial Acetic acid	100 ml
Distilled Water	650 ml

A.2.4 WESTERN BLOTTING SOLUTIONS

A.2.4.1 TRANSFER BUFFER

Glycine	14.41 g
Tris	3.025 g
SDS	1 g
Methanol	200 ml

Made up to 1 litre with distilled water and stored at room temperature.

A.2.4.2 ALKALINE PHOSPHATASE SUBSTRATE BUFFER

Tris	45.41 g

Dissolved in distilled 330 ml distilled water, pH adjusted to 9.5 with concentrated HCl, made up to a final volume of 500 ml.

A.2.4.3 ALKALINE PHOSPHATASE SUBSTRATE

A.2.4.3.1 NITRO BLUE TETRAZOLIUM

Nitro blue tetrazolium	75 mg

Dissolved in 1 ml 70 % dimethylformamide, stored at -20 °C

A.2.4.3.2 5 BROMO 4 CHLORO 3 INDOLYL PHOSPHATE *p*-TOLUIDINE SALT (BCIP)

5 bromo 4 chloro 3 indolyl phosphate *p*-toluidine salt	50 mg

Made up to 1 ml with 100 % dimethylformamide, stored at –20 °C

A.2.4.3.3 ALKALINE PHOSPHATE SUBSTRATE WORKING SOLUTION

Substrate buffer	20 ml
NBT	44 µl
BCIP	33 µl

A.2.4.4 0.05% COPPER PHTHALOCYANINE 3, 4', 4'', 4'''-TETRASULPHONIC ACID TETRASODIUM SALT

Copper phthalocyanine 3, 4', 4'',4'''-tetrasulphonic acid tetrasodium salt	0.05 g

Dissolved in 100 ml of 12 mM HCl.

Table 2.1.

Proteinase inhibitors used to characterise proteinase activity secreted by *N. americanus* (adapted from Beynon & Bond, 1989)

Inhibitor	Proteinase inhibited	Stock solution	Working concentration
E64 L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane	Cysteine proteinases	1 mM in distilled water	1-10 µM
APMSF 4-(amidinophenyl)methanesulphonyl fluoride	Trypsin 'like' serine proteinases	50 mM in distilled water	10-100 µM
1, 10-phenanthroline (ortho-phenanthroline)	Metallo-proteinases	1 M in ethanol	1-10 mM
EDTA ethylenediaminetetraacetic acid	Metallo-proteinases and metal dependant cysteine proteinases	0.5 M in distilled water	1-10 mM
Pepstatin A	Aspartyl proteinases	1 mM in ethanol	1-10 µM

CHAPTER 3

FURTHER CHARACTERISATION OF PROTEINASES SECRETED BY THE LARVAL AND ADULT STAGES OF *NECATOR AMERICANUS*.

CHAPTER 3

FURTHER CHARACTERISATION OF PROTEINASES SECRETED BY THE LARVAL AND ADULT STAGES OF *NECATOR AMERICANUS.*

3.1 INTRODUCTION

Numerous papers (reviewed by McKerrow, 1989) have described and characterised the proteolytic enzymes secreted by the adult and larval stages of a wide range of parasites. Proteinases from all four mechanistic classes have been described and are no doubt crucial to parasite survival. A number of roles have been suggested for these enzymes including the invasion of host tissue (Hamajima *et al.*, 1985; Yamakami & Hamajima, 1986; Knox & Kennedy, 1988; Aldape *et al.*, 1994); feeding (Rosenthal *et al.*, 1993; Hawthorne *et al.*, 1994); evasion of the immune response (Auriault *et al.*, 1981; Verwaerde *et al.*, 1988; Bontempi & Cazzulo, 1990; Hamajima *et al.*, 1994) and the prevention of blood coagulation (Thorson, 1956; Hotez & Cerami, 1983).

Some of the most widely studied proteinases are those produced by the trematode parasite *Schistosoma mansoni.* This parasite like *N. americanus* infects its host by passing through the skin barrier. Early work implicated proteinases in this process (Stirewalt, 1978) and demonstrated the presence of a serine proteinase in extracts of infective cercariae (Gazzinelli *et al.*, 1966; Dresden & Asch, 1972). Secretion of proteinases by *S. mansoni* cercariae is inducible by skin lipids (Stirewalt, 1978) and subsequently a 30 kDa serine proteinase has been purified from these secretions (McKerrow *et al.*, 1985). This enzyme is capable of degrading elastin, azocoll, gelatin, laminin, fibronectin, keratin and types IV and VIII collagen (McKerrow *et al.*, 1985). No activity was observed against type I, III or V collagen. A larger 47 kDa serine protease with activity against elastin and type VI collagen has also been purified from *S. mansoni* cercarial secretions (Chavez-Olortegui *et al.*, 1992).

As far as adult *S. mansoni* is concerned the digestive enzymes have been the most extensively studied. Adult schistosomes feed on red blood cells and it has been estimated that they may digest up to 330 000 red blood cells per day (Dalton *et al.*, 1995). Early work demonstrated the presence of an acidic, globin digesting enzyme in extracts of adult *S. mansoni* (Timms & Bueding, 1959; Grant & Senft, 1971). This enzyme activity was shown to be enhanced by the reducing agents dithiothreitol and 2-mercaptoethanol and inhibited by N-ethylmaleimide and leupeptin (inhibitors of cathepsin B 'like' proteinases) (Dresden & Deelder, 1979). The enzyme was also shown to cleave the cathepsin B peptide substrates CBz-Arg-Arg-pNA and CBz-Arg-Arg-AFC (Dresden *et al.*, 1981; Chappell & Dresden, 1986). In 1987, Chappell & Dresden, purified two cathepsin B-'like' cysteinyl proteases from adult *S. mansoni* with molecular weights of 32 and 28 kDa. These enzymes were termed Sm 32 and Sm 28 respectively. Sm 32 is immunogenic during infection (Ruppel, *et al.*, 1985) and has since been shown to consist of two proteins, Sm31/32. Sequence analysis has shown Sm 31 to be the cathepsin B 'like' enzyme (Klinkert *et al.*, 1989) while Sm 32 is an asparaginyl endoproteinase (Takeda, 1994). Both enzymes are capable of degrading haemoglobin.

The cysteinyl proteinase activity of *S. mansoni* has been further defined with the demonstration of a cathepsin L 'like' activity (active at pH 4.5) in extracts of adult worms (Smith *et al.*, 1994b). This enzyme may also play a role in haemoglobin digestion. Cathepsin L 'like' enymes are distinguished from cathepsin B 'like' enzymes by their ability to cleave the peptide substrate Z-Phe-Arg-AMC.HCl. Cathepsin L enzymes prefer the hydrophobic amino acid phenylalanine in the P_2 position, whereas cathepsin B proteinases prefer the basic amino acid arginine. Cathepsin B and L activities have also been described in the liver fluke *Fasciola hepatica* (McGinty *et al.*, 1993), and the protozoan parasite *Leishmania mexicana* (Bontempi & Cazzulo, 1990; Robertson & Coombs, 1990), while cathepsin L 'like' activities have been described in *Trypanosoma cruzi* (cruzipain, Cazzulo *et al.*, 1990) and the malarial parasite *Plasmodium falciparum* (Rosenthal *et al.*, 1988).

Strongyloides stercoralis, like *S. mansoni* and *N. americanus,* is an obligate skin penetrating parasite capable of penetrating skin at rates of up to 10 cm per hour

(McKerrow *et al.*, 1990). This process is mediated by the secretion of proteinases including an acidic cysteinyl proteinase capable of degrading collagen (Rege & Dresden, 1987) and a potent histolytic metallo-proteinase capable of degrading fibronectin, elastin and laminin but not type I collagen (McKerrow *et al.*, 1990). This metallo-proteinase has since been shown to be both zinc dependant and immunogenic during infection (Brindley *et al.*, 1995).

As far as hookworms are concerned the proteinases from *N. americanus* and a number of *Ancylostoma* species have been studied. Matthews, (1982) demonstrated that during skin penetration by *N. americanus,* cellular destruction of the skin was accompanied by an undefined enzymatic process, capable of degrading azocoll and optimally active at pH 8. Subsequently, Salafsky *et al.*, (1990) developed a gelatin-agar membrane as a model for *N. americanus* skin penetration and showed that serine, and possibly cysteinyl, proteinase activities were responsible for larval penetration through this type of membrane. Cysteinyl proteinases with molecular weights of 219, 200, 166, 137, 92, 72, and 62 kDa and a serine proteinase of 195 kDa have been identified in *N. americanus* larval ES products using gelatin substrate SDS-PAGE (Kumar & Pritchard, 1992a). Metallo-proteinase activity associated with live *N. americanus* larvae was demonstrated by monitoring the release of labelled peptides and amino acids from radio-labelled gelatin bound to a polystyrene plate (Kumar & Pritchard, 1992b).

The proteinases present in adult *N. americanus* have also been characterised using natural, fluorogenic and chromogenic substrates. Haemoglobin and fibrinogen degradation has been demonstrated by monitoring the release of labelled amino acids from tritiated haemoglobin (Burleigh, 1993; Brown *et al.*, 1995). Haemoglobin degradation was shown to be optimal at pH 6.5 and accomplished primarily by aspartyl and metallo-proteinases with some serine and cysteinyl proteinase activity. When analysed using substrate SDS-PAGE, haemoglobin degradation was associated with a protein doublet of approximately 66 kDa (Pritchard *et al.*, 1990a). Fibrinogen hydrolysis however, was mediated at acid pH by aspartyl proteinase activity while at alkaline pH serine proteinase activity predominated (Brown *et al.*, 1995). All three subunits of fibrinogen (Aα, Bβ, and γ) are degraded by *N. americanus* ES products and this degradation may interfere with platelet-fibrinogen

interactions and so prevent platelet aggregation (Furmidge *et al.*, 1995). Using synthetic fluorogenic substrates, cathepsin L and cathepsin B 'like' cysteinyl proteinase activities and a trypsin 'like' serine proteinase activity have been demonstrated. Proteinase activity was also observed against the collagen 'like' substrate Suc-Gly-Pro-Leu-Gly-Pro-AMC. This activity was only inhibited by the metallo protease inhibitor 1, 10-phenanthroline (Brown *et al.*, 1995). Interestingly, although adult ES products were shown to contain an aspartyl proteinase activity inhibitable with pepstatin A, no activity was seen against the chromogenic pepsin A substrate Pro-Thr-Glu-Phe-(NO_2-Phe)-Arg-Leu (Dunn *et al.*, 1984).

Metallo-proteinases with molecular masses of 68 and 38 kDa have been shown to be present in larval extracts of both *Ancylostoma caninum* and *A. duodenale*. These enzymes have also been implicated in the skin penetration process, being shown to be capable of degrading human fibronectin but not bovine elastin or human laminin (Hotez *et al.*, 1990). They may also play a role in larval nutrition as the release of metallo-proteinase activity correlates with the activation and resumption of feeding by *A. caninum* larvae *in vitro* (Hawdon *et al.*, 1995). The 38 kDa metallo-proteinase present in *A. caninum* and *A. duodenale* has properties similar to the metallo-proteinase activity found in the exsheathing fluid of *H. contortus* (Gamble *et al.*, 1989) and may also mediate larval exsheathment and further development (Hotez *et al.*, 1990). The proteinases present in the exsheathing fluid of *N. americanus* larvae have also been examined but to date only a 116 kDa cysteinyl proteinase has been identified (Kumar & Pritchard, 1992a).

A 37 kDa metallo-proteinase has been purified from adult *A. caninum* worm extracts and characterised as a metallo-proteinase with activity against elastin, fibrin and an extracellular matrix secreted by rat vascular smooth muscle cell line (Hotez *et al.*, 1985). As the purified enzyme appears to have activity against elastin it is presumably different to the 38 kDa metallo-proteinase found in *A. caninum* larvae. It was hypothesised that the adult proteinase may play a role in tissue invasion and the prevention of blood clotting during feeding. Using substrate SDS-PAGE, this 37 kDa *A. caninum* proteinase also appeared to be capable of degrading haemoglobin optimally at pH 8.5. This may suggest that the enzymatic process by which *N. americanus* and *A. caninum* degrade haemoglobin are due to different proteinases. Haemoglobinase activity in *N. americanus* adult extracts

analysed by monitoring the degradation of tritiated haemoglobin is optimal at pH 6.5 with smaller peaks of activity at pH 3.5 and 8.5 (Burleigh, 1993; Brown *et al.*, 1995). Haemoglobin substrate SDS-PAGE at pH 4.2 shows haemoblobin degradation to be mediated by a protein doublet with an apparent molecular mass of approximately 66 kDa (Pritchard *et al.*, 1990a).

In 1977 a cysteinyl proteinase activity capable of degrading haemoglobin was described in adult *A. caninum* (Oya & Noguchi, 1977); this activity has been shown to be cathepsin L 'like' (Dowd *et al.*, 1994), while the genes for two cathepsin B 'like' proteinases, with predicted molecular masses of 28.83 and 28.85 kDa have been cloned (Harrap *et al.*, 1995). This cathepsin B 'like' activity has been localised in the oesophageal, amphidial and excretory glands and demonstrated by Western blotting to be present in the ES products of the worm. Similarly, a gene encoding a cathepsin D 'like' aspartyl proteinase has also been cloned from adult *A. caninum* (Harrap *et al.*, 1996).

While the proteinases secreted by both the adult and larval stages of *N. americanus* have been partially characterised definite roles for some of them have yet to be assigned. It has been suggested that the serine proteinase secreted by *N. americanus* larvae may cleave IgM, IgG and IgA at the site of infection and so evade one of the hosts immune responses (Kumar & Pritchard, 1992a). Adult ES products also have the capability to degrade IgG to some extent but a greater activity is seen against IgA, an antibody found in the gut mucosa (Pritchard, 1990d). This chapter describes experiments designed to further characterise the proteinases secreted by both larval and adult *N. americanus* and to assign a more defined role for these enzymes during skin penetration, feeding and evasion of the immune response. The hydrolysis of collagen types I, III, IV, V, elastin, fibronectin and laminin by *N. americanus* larvae has been studied and for each substrate, the enzymes responsible for its degradation have been characterised using a range of proteinase inhibitors. Similarly, the enzymes responsible for haemoglobin and fibrinogen degradation in adult ES were also studied. The data obtained using individual proteins was confirmed by studying the effects of proteinase inhibitors on the penetration of live larvae through excised hamster skin.

3.2 MATERIALS AND METHODS

N. americanus larval and adult ES products were prepared and their protein content determined as described in Section 2.1.4. Proteinase activity present in larval and adult ES products was determined using either FITC-labelled casein (Section 2.5.2.2) or synthetic fluorogenic substrates (Section 2.5.3)

3.2.1 DEGRADATION OF SKIN MACROMOLECULES

3.2.1.1. HYDROLYSIS OF ELASTIN

Elastinolytic activity was measured by monitoring the release of dye from elastin-orcein (Knox & Kennedy, 1988). 1 mg of elastin-orcein was mixed with 20 µg (100 µl) of larval ES products and the reaction volume made up to 1 ml with buffer (0.1 M citric acid/sodium citrate buffer, pH 3-5.5, 0.1 M phosphate buffer, pH 6-8 or 0.05 M 2-amino-2-methyl-1:3-propanediol-HCl buffer, pH 8-10). The reaction was incubated at 37 °C for 16 hr with constant mixing after which undigested elastin was removed by centrifugation (13000 g, 10 min) and the absorbance of the sample measured at 550 nm. Proteinase inhibitors and effectors were added to individual assays to give a final concentration of 1 µM E64, 50 µM APMSF, 1 mM 1, 10-phenanthroline, 1 µM pepstatin A and 5 mM cysteine.

3.2.1.2 HYDROLYSIS OF COLLAGEN

3.2.1.2.1 TRITIUM LABELLING OF COLLAGEN

A mixture of human placental type I and III collagen was tritiated by reductive methylation of exposed amino groups (Tack *et al.*, 1980). 25 mCi (specific activity 9.21 mCi/mg) of tritiated sodium borohydride (Amersham International) were carefully resuspended in 25 µl of 0.1 M sodium hydroxide, aliquoted into 5 µl volumes (each containing 5 mCi) and stored at –70 °C. 1 mg of purified collagen was first solublised in 50 µl of acetic acid (diluted 1:1000 from glacial) and then made up to a final volume of 200 µl with 0.2 M sodium borate buffer pH 9 to give a final concentration of 5 mg/ml and maintain an alkaline pH. The protein was activated by the addition of 30 µl ice cold 0.2 M formaldehyde and kept on ice for 2 minutes. 5 mCi of tritiated sodium borohydride were added to the activated protein and the reaction allowed to proceed on ice for a further 2

minutes before being stopped by the addition of 600 µl of 0.4 M Na H_2PO_4, 0.1 M glycine, pH 5.8. After a further five minutes, labelled protein was separated from free tritium using a Sephadex G-25 column. Prior to use the G-25 column was equilibrated with 150 mM phosphate buffered saline (PBS), pH 7.4. Any available binding sites on the column were blocked by washing the column with PBS containing 1 mg/ml bovine serum albumen. (BSA). Unbound BSA was removed by further washing with PBS and the absorbance monitored at 280 nm to ensure the complete removal of unbound BSA. Tritiated collagen was applied to the top of the column which was then washed with PBS. 1 ml fractions eluting from the column were collected and assayed for radioactivity by liquid scintillation counting and protein content. Fractions containing labelled collagen were pooled, dialysed against distilled water and stored in 50 µl aliquots at -20 °C (specific activity 1.36 x 10^6 dpm/mg). Proteins labelled in this way are very similar to the unmodified protein and are suitable for use as natural substrates.

3.2.1.2.2 ASSAY OF COLLAGEN HYDROLYSIS

Collagen hydrolysis was assessed using either a liquid phase assay adapted from the haemoglobinase assay described by (Burleigh, 1992) or a solid phase assay designed to monitor the release of labelled amino acids from tritiated collagen bound to the wells of a microtitre plate.

3.2.1.2.3 LIQUID PHASE ASSAY

Larval ES products (10 µg, 50 µl) were incubated with 16.6 µl (20 000 dpm) of tritiated collagen and 384.4 µl of 0.1 M sodium phosphate, pH 6.5, for 3h at 37 °C. Proteinase inhibitors and effectors were added to individual assays to give a final concentration of 1 µM E64, 50 µM APMSF, 1 mM 1, 10-phenanthroline, 1 µM pepstatin A and 5 mM cysteine. The reaction was stopped by the addition of 50 µl of 100 % w/v trichloroacetic acid (TCA) and the tubes kept on ice for 1h, after which they were centrifuged at 13 000 g for 10 minutes. Duplicate aliquots of the TCA soluble supernatant were then removed for liquid scintillation counting.

3.2.1.2.4 SOLID PHASE ASSAY

Collagen hydrolysis was also assessed by monitoring the release of labelled peptides and amino acids from tritiated collagen previously bound to the wells of a 96 well polystyrene microtitre plate. 20 000 dpm (specific activity as described above) of tritiated collagen (diluted to 50 µl in PBS) was bound to the wells by baking the plate at 55 °C overnight. The plate was then washed with distilled water to remove any unbound protein. 10 µg of ES products were added to the wells in a final volume of 200 µl 0.1 M sodium phosphate buffer pH 6.5 for larval ES products. Proteinase inhibitors and effectors were added to individual wells to give a final concentration of 1 µM E64, 50 µM APMSF, 1 mM 1, 10-phenanthroline, 1 µM pepstatin A and 5 mM cysteine. Plates were incubated at 37 °C for 3 h after which 150 µl of the reaction mix was removed from the wells for scintillation counting.

3.2.1.3 DETECTION OF SKIN MACROMOLECULE HYDROLYSIS BY *N. AMERICANUS* LARVAE USING WESTERN BLOTTING

10 µg of human collagen types I, III, IV and V, fibronectin and laminin were incubated in 0.1 M phosphate buffer pH 6.5 with 10 µg of larval ES products in the presence and absence of a number of protease inhibitors. After 4 h incubation at 37 °C undigested protein and any breakdown products were precipitated with ice cold acetone (appendix A.2.3.10) and the pellet resuspended in 20 µl of SDS-PAGE reducing sample buffer (Laemmli, 1970). In the case of collagen, 1 unit of collagenase (Sigma) was incubated with each type of collagen tested as a positive control. Breakdown products were separated using a 12 % SDS-PAGE gel (7% for laminin) and transferred onto nitrocellulose (Towbin *et al.*, 1979). Western blots were blocked for 1 hour with 5% milk powder in Tris buffered saline (TBS) before being probed with rabbit antisera (Sigma) raised to the relevant skin protein (dil 1:500 in blocking solution), and incubated overnight at 4°C. After washing with TBS/ 0.05% Tween 20, the blots were incubated with alkaline phosphatase conjugated goat anti rabbit IgG (Sigma, dil 1:1000 in blocking solution). Antibody binding was visualised using the alkaline phosphate substrate 5-bromo-4-chloro-3-indolyl phosphate /nitroblue tetrazolium as the substrate as described in section A2.4.3.3.

3.2.2 DETECTION OF PROTEINASE ACTIVITY USING SUBSTRATE SDS-PAGE

Substrate SDS-PAGE was carried out as using a method modified from Kumar & Pritchard (1992a). 12 % (w/v) SDS-PAGE gels were prepared as described in Chapter 2 with the inclusion of either 0.1 % (w/v) haemoglobin or fibrinogen in the resolving gel. 100 µg (100 µl) of adult *N. americanus* ES products were mixed with an equal volume of non reducing sample buffer (0.5 M Tris, pH 6.8, 5 % SDS (w/v), 20 % glycerol (w/v), 0.01 % bromophenol blue) and incubated at 37 °C for 30 minutes. The sample was then applied to a preparative well formed in the stacking gel and the sample electrophoresed at a constant current of 20 mA. Following electrophoresis, the gels was washed in 2.5 % Triton X-100 for 1 h at room temperature to re-nature the enzymes as described by Lacks & Sringhorn (1980). The gels were then washed in water for 30 minutes, cut into individual strips and incubated for 48 hours at 37 °C in 0.1 M sodium phosphate buffer pH 6.5 in the presence or absence of a number of proteinase inhibitors. Proteinase activity was detected by staining gels with Coomassie brilliant blue R250 (Appendix 2.3.4), and is observed as areas of clear banding against a blue background.

3.2.3 DETECTION OF HAEMOGLOBIN DEGRADATION BY *N. AMERICANUS* ADULT ES PRODUCTS USING WESTERN BLOTTING

10 µg of *N. americanus* adult ES products were incubated with 10 µg of human haemoglobin (Sigma) under the same conditions as those described for collagen, fibronectin and laminin (Section 3.2.1.3). Breakdown products were separated using a 15 % SDS-PAGE gel and transferred onto nitrocellulose. The subsequent Western blot was blocked as described earlier and probed with a polyclonal rabbit antiserum (diluted 1:1000 in blocking agent) raised against human haemoglobin (Sigma). The blot was then washed and antibody binding revealed as described previously.

3.2.4 PENETRATION OF *N. AMERICANUS* LARVAE THROUGH EXCISED HAMSTER SKIN

3.2.4.1 PREPARATION OF HAMSTER SKIN

The penetration of *N. americanus* through hamster skin was assessed using a method modified from Kumar & Pritchard (1992c). This work was carried out in collaboration with Miss Nadine Girod as part fulfilment of her European MSc in Biotechnology (awarded July 1997). The abdomen of freshly killed adult DSN hamsters was wetted, shaved and the skin carefully removed (6 pieces of skin were taken from one animal). The skin was mounted on the open ends of an 8 cm long by 1.4 cm diameter plastic tube such that the outer skin surface faced the inside of the tube, and the skin secured tightly by an elastic band. The tube was then placed in a 20 ml universal tube, containing 5 ml of Dulbecco's minimal essential medium (DMEM) supplemented with 1 % amphotericin B, 100 i.u/ml penicillin, and 100 µg/ml streptomycin. Care was taken to ensure that the inner skin surface was completely in contact with the medium and that no leakage occurred back across the skin to the upper surface.

3.2.4.2 TIME COURSE OF SKIN PENETRATION

200 freshly harvested live and active larvae were placed on the upper surface of the hamster skin (in a final volume 200 µl of water) and the apparatus incubated at 37 °C. Three tubes were removed, after 3, 24 and 48 h, and the number of larvae, which had failed to penetrate, and had successfully penetrated the skin carefully counted.

3.2.4.3 EFFECT OF PROTEINASE INHIBITORS ON LARVAL PENETRATION THROUGH HAMSTER SKIN

200 larvae were applied to the upper surface of the hamster skin in the presence of proteinase inhibitors at the concentrations described earlier. An ethanol control was also included. The tubes were incubated at 37 °C for 24 h after which the number of larvae fully penetrating the skin were counted. Statistical analysis of the number of larvae fully penetrating the skin was carried out using ANOVA with $p \leq 0.05$ being considered significant.

3.3 RESULTS

3.3.1 CHARACTERISATION OF THE PROTEINASE ACTIVITIES SECRETED BY *N. AMERICANUS* LARVAE USING FITC-CASEIN

Proteinases secreted by the larvae of *N. americanus* were initially examined using FITC-casein as the substrate. Total proteinase activity was found to be optimal at pH 6.5 with a peak of lesser activity at pH 8 (Figure 3.1). Proteinase activity at these two pH points was characterised by the use of class differentiating inhibitors (Table 3.1). At pH 6.5 proteinase activity was markedly inhibited (78.9 %) by pepstatin A, indicating that an aspartyl proteinase was primarily present. At pH 8 inhibition by pepstatin A was reduced to 24.6 % but APMSF inhibited proteolytic activity by 81.5 % indicating the predominant involvement of a serine proteinase at this pH. Inhibition by the metallo-protease inhibitors 1, 10-phenanthroline and EDTA was lower (32 % and 10.8 % respectively at pH 6.5 increasing to 41.2 % and 18.6 % at pH 8). At pH 6.5 the cysteinyl protease inhibitor E64 inhibited activity by 23.3 %, this being reduced to 13.2 % at pH 8. Similarly, cysteine enhanced activity by 11.4 % at pH 6.5 and only 7.4 % at pH 8.

3.3.2 FURTHER CHARACTERISATION USING SYNTHETIC PEPTIDE SUBSTRATES

The proteinase activity in *N. americanus* adult and larval ES products was further characterised by the use of a number of fluorescent substrates (Table 3.2). All assays were carried out at pH 6.5 as this was the overall pH optimum for the proteolytic activities of both larval and adult ES products. The substrates Tosyl-Gly-Pro-Arg-AMC.HCl and H-Pro-Phe-Arg-AMC.2HCl were cleaved by both larval and adult ES products confirming the presence of a trypsin 'like' serine proteinase activity. However, none of the chymotryptic substrates tested were readily cleaved by ES products from either stage of the life cycle except for Glutaryl-Gly-Gly-Phe-AMC which was hydrolysed to a small degree by larval ES products. The dual trypsin/papain substrates Z-Arg-AMC.HCl and Z-Phe-Arg-AMC.HCl were readily cleaved by adult and larval ES products. In both cases the substrates were hydrolysed to a higher degree than the solely tryptic substrates, indicating the combined activity of serine and cysteinyl proteinases against these substrates. The cathepsin B substrate Z-Arg-Arg-AMC.2HCl was cleaved by both larval and adult ES

products indicating the presence of a cathepsin B 'like' cysteinyl proteinase. Larval ES products failed to cleave the elastase substrate Suc-Ala-Ala-Ala-AMC; adult ES products on the other hand demonstrated an ability to cleave this substrate. Conversely, larval ES products showed a small degree of activity against the cathepsin H/aminopeptidase substrate H-Arg-AMC.2HCl and the urokinase substrate Glutaryl-Gly-Arg-AMC.HCl, while adult ES failed to hydrolyse either of these substrates.

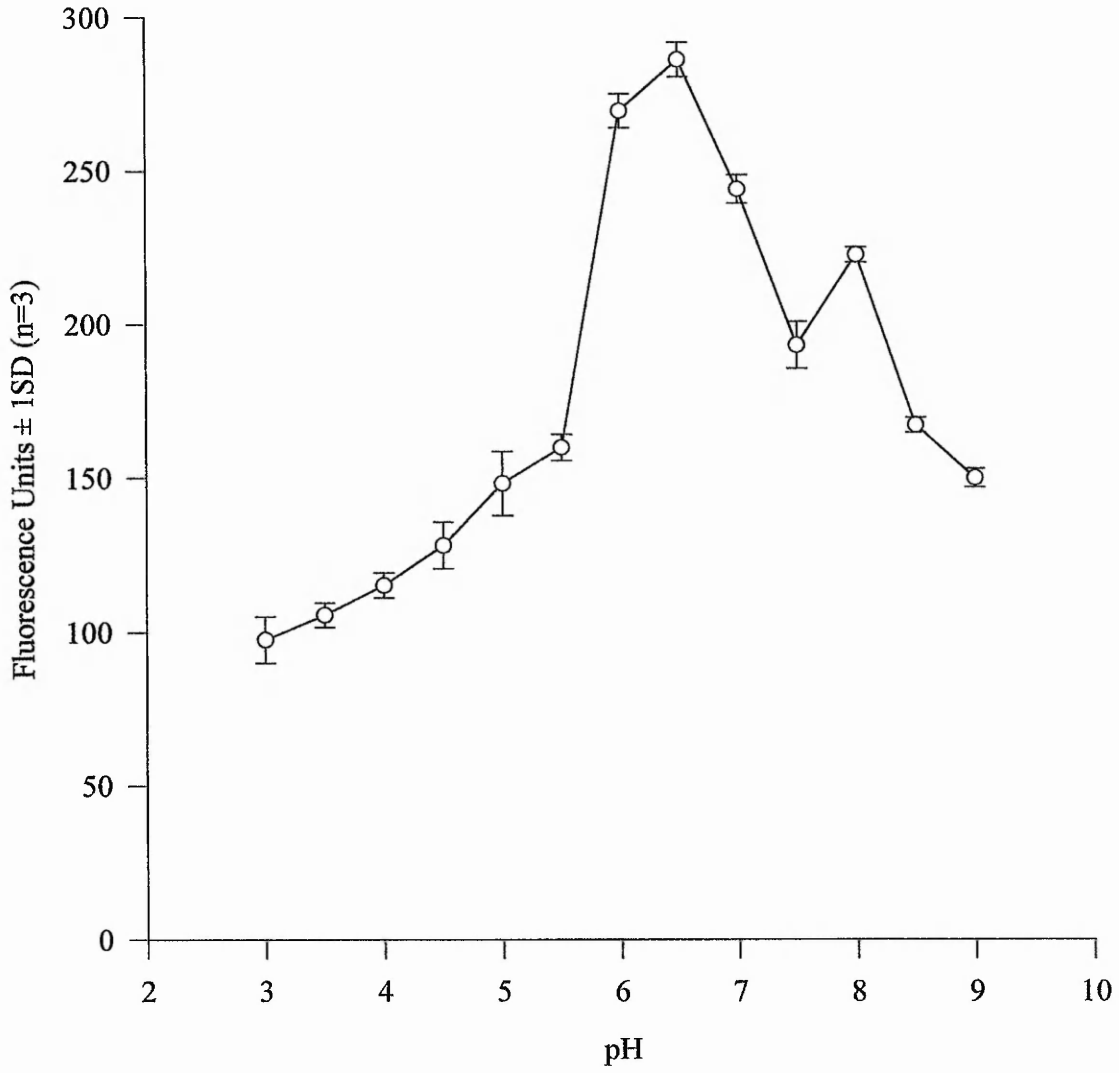

Figure 3.1

The pH optima of the proteinase activities present in the ES products of *N. americanus* larvae.

Larval ES products were incubated with FITC-casein over a range of pH points as described in the Materials and Methods. Proteinase activity is expressed as the mean ± 1 SD number of fluorescence units (n=3) emitted over a 1h period following the subtraction of a non enzymatic buffer control.

Table 3.1.

The characterisation of the proteinase activities present in the ES products of *N. americanus* larvae.

	pH 6.5	pH 8
ES products alone (Fluorescence units/h)	269.7±19.2	199.5±9.1
Inhibitor/enhancer (% change)		
5 mM cysteine	+ 11.4	+ 7.4
1 μM E64	- 22.3	- 13.2
50 μM APMSF	- 14.7	- 81.5
1 mM 1, 10-phenanthroline	- 32.0	- 41.2
1 mM EDTA	- 10.8	- 18.6
1 μM pepstatin A	- 78.9	- 24.6

10 μg of larval ES products were incubated with FITC-casein at 37 °C for 3 h as described in the Materials and Methods. Proteinase activity is expressed as the mean number of fluorescence units emitted (n=4) ±1SD. Inhibition of proteinase activity by a number of inhibitors is shown as the percentage inhibition (-) or enhancement (+) of proteinase activity compared with an uninhibited control following the subtraction of a non enzymatic blank.

Table 3.2

The further characterisation of the proteinases present in *N. americanus* adult and larval ES products, using fluorogenic substrates.

Substrate	Substrate for	Proteinase activity (FU/h) Larval ES	Adult ES
Tosyl-Gly-Pro-Arg-AMC.HCl	Thrombin and plasmin	324	143
Glutaryl-Gly-Arg-AMC.HCl	Urokinase	60.6	0
H-Pro-Phe-Arg-AMC.2HCl	Kallikreins and elastase	263.8	108
Suc-Ala-Ala-Pro-Phe-AMC	Chymotrypsin	59.4	0
Suc-Leu-Leu-Val-Tyr-AMC	Chymotrypsin	12.8	0
Glutaryl-Gly-Gly-Phe-AMC	Chymotrypsin and cathepsin G	18.36	0
Suc-Ala-Ala-Ala-AMC	Elastase	14.64	250
Z-Arg-AMC.HCl	Trypsin and papain	750	650
Z-Phe-Arg-AMC.HCl	Kallikreins, papain, cathepsin B and cathepsin L	423.3	768
H-Arg-AMC.2HCl	Cathepsin H and aminopeptidase B	78.96	0
Z-Arg-Arg-AMC.2HCl	Cathepsin B	217.8	157.8

10 µg of larval and adult ES products were incubated with each substrate as described in the Materials and Methods. Proteinase activity is expressed as the mean number of fluorescence units (n=3) emitted over a 1 hour period after the subtraction of a non enzymatic control.

3.3.3 DEGRADATION OF SKIN MACROMOLECULES

3.3.3.1 ELASTIN

Elastin hydrolysis was shown to be optimal at pH 7.5 with a lesser peak of activity at pH 8.5 (Figure 3.2). No hydrolysis of elastin was observed between pH 3 and 6 and above pH 10. Elastin hydrolysis was characterised at pH 7.5 and 8.5 using proteinase inhibitors (Table 3.3). No enhancement of activity was seen in the presence of cysteine and no inhibition was observed with E64 indicating that cysteinyl proteinase activity plays no part in the degradation of elastin. At pH 7.5, elastin hydrolysis was primarily inhibited by 1, 10-phenanthroline (100%) and pepstatin A (28.8 %). EDTA and APMSF also inhibited elastin breakdown to a lesser degree. At pH 8.5, 1, 10-phenanthroline only inhibited elastin degradation by 63.2 % while APMSF inhibited elastin hydrolysis by 46.7 % again indicating increased serine proteinase activity at this pH. Inhibition by pepstatin A remained at a similar level (33.3 %) to that observed at pH 7.5. These data suggest that the hydrolysis of elastin is accomplished by a mixture of metallo, serine and aspartyl proteinases with the metallo-proteinase being the most important for this substrate.

3.3.3.2 COLLAGEN

Figures 3.3-3.6 show the breakdown of types I, III, IV and V by larval ES products. All types of collagen tested were degraded by larval ES products. Type I collagen hydrolysis was characterised by the degradation of protein bands at 93 and 69 kDa and the appearance of a breakdown product at 45 kDa (Figure 3.3). The 69 kDa protein may be a breakdown product as collagen may degrade upon storage. Type III collagen breakdown was demonstrated primarily by the degradation of a protein band of 91 kDa and the appearance of breakdown products at 73 and 69 kDa (Figure 3.4). Similarly, Type IV collagen breakdown was characterised by the degradation of protein bands at 95, 62 and 54 kDa and the appearance of degradation products at 75, 72, 45 and 37 kDa (Figure 3.5). Finally, Type V collagen was degraded to proteins of 73, 64, 52 and 37 kDa (Figure 3.6). All types of collagen tested were completely degraded by collagenase.

No inhibition of collagen breakdown was seen with E64 or APMSF. 1, 10-phenanthroline partially inhibited the degradation of collagen types III, IV and V. EDTA partially inhibited the degradation of type IV collagen and completely inhibited the breakdown of

type V collagen. Pepstatin A completely inhibited the degradation of collagen types I and III and partially inhibited the breakdown of collagen type IV. However, pepstatin A failed to inhibit the breakdown of type V collagen.

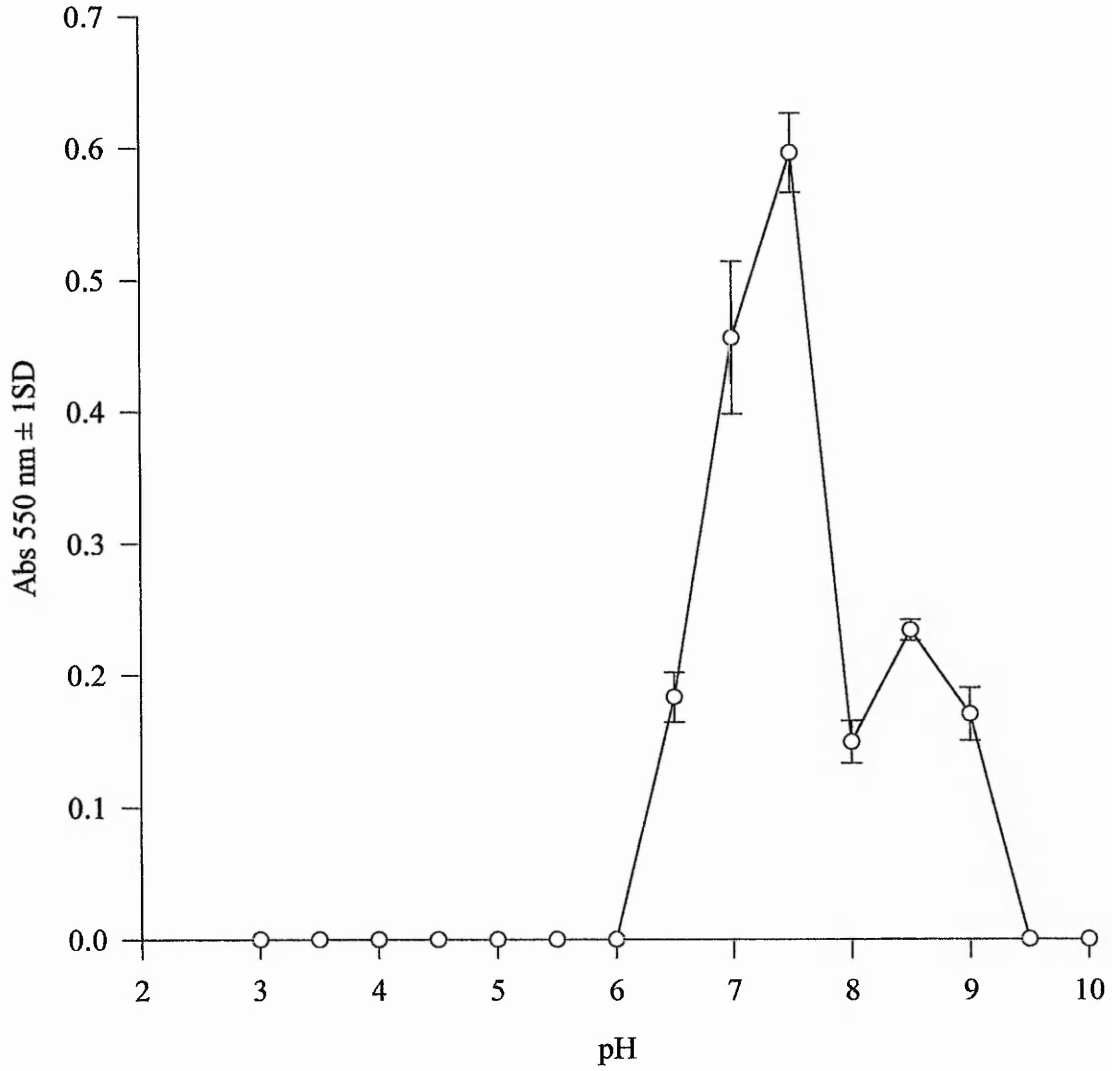

Figure 3.2

The pH optima of the proteinase activities responsible for elastin degradation in the ES products of *N. americanus* larvae.

Larval ES products were incubated with 1mg of elastin-orcein over a range of pH points. Proteinase activity is expressed as the mean ± 1 SD (n=3) absorbance at 550 nm after 16 hours incubation following the subtraction of a non enzymatic buffer control.

Table 3.3.

The characterisation of the proteinase activities responsible for elastin degradation in the ES products of *N. americanus* larvae.

	pH 7.5	pH 8.5
ES products alone (Abs 550 nm)	0.124±0.006	0.111±0.001
Inhibitor/enhancer (% change)		
5 mM cysteine	-6.4	0
1 μM E64	- 1.6	+1.0
50 μM APMSF	- 9.4	- 46.7
1 mM 1, 10-phenanthroline	- 100	- 63.2
1 mM EDTA	- 10.8	- 18.6
1 μM pepstatin A	- 28.8	- 33.3

Larval ES products were incubated with 2 mg of elastin-orcein in the presence of proteinase inhibitors. Table 2 shows the mean elastinolytic activity ± 1SD of 3 batches of larval ES products and the percentage inhibition (-) or enhancement (+) of elastinolytic activity compared to an uninhibited control following the subtraction of a non enzymatic blank.

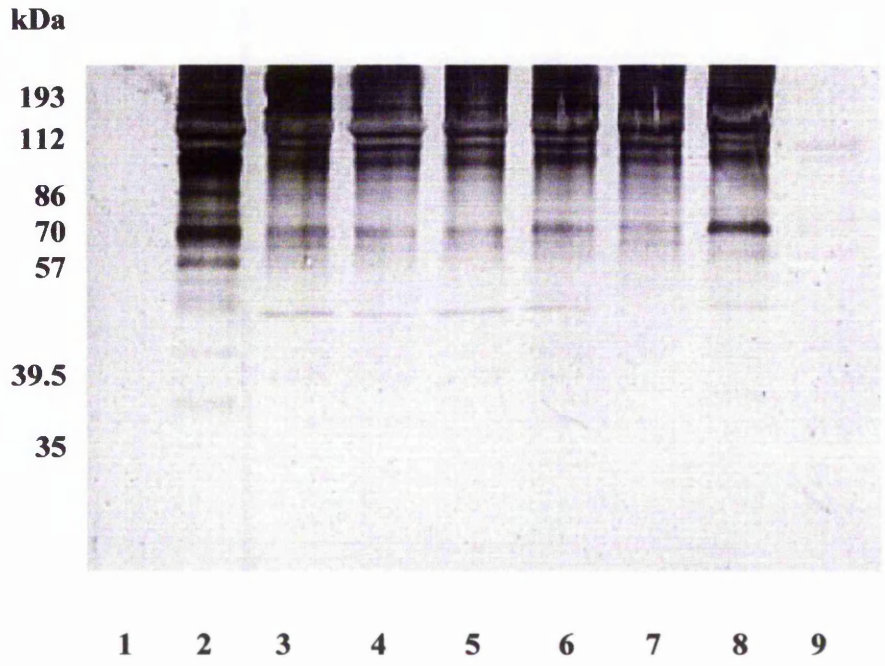

Figure 3.3.

The breakdown of collagen type I by *N. americanus* larval ES products

Lane 1	Larval ES products
Lane 2	Type I collagen
Lane 3	Type I collagen + larval ES products
Lane 4	1 μM E64
Lane 5	50 μM APMSF
Lane 6	1 mM 1, 10-phenanthroline
Lane 7	1 mM EDTA
Lane 8	1 μM pepstatin A
Lane 9	Collagenase

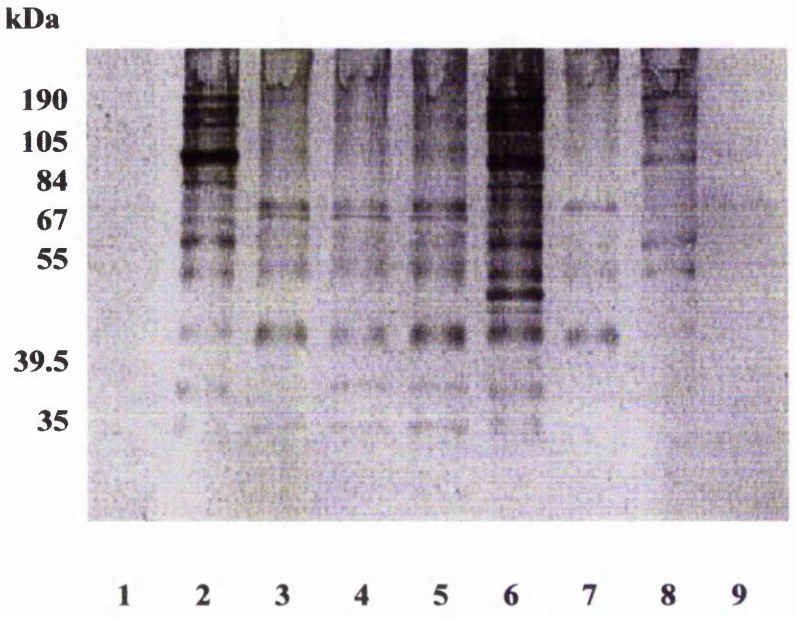

Figure 3.4.

The breakdown of collagen type III by *N. americanus* larval ES products

Lane 1	Larval ES products
Lane 2	Type III collagen
Lane 3	Type III collagen + larval ES products
Lane 4	1 μM E64
Lane 5	50 μM APMSF
Lane 6	1 mM 1, 10-phenanthroline
Lane 7	1 mM EDTA
Lane 8	1 μM pepstatin A
Lane 9	Collagenase

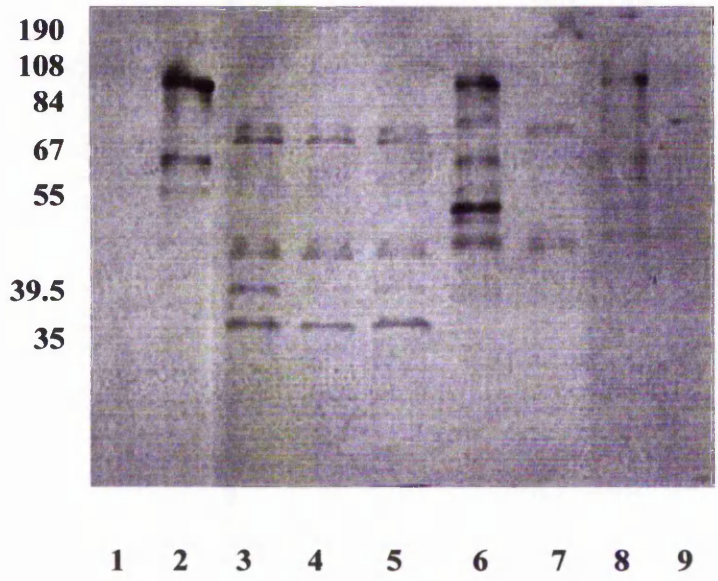

Figure 3.5.

The breakdown of collagen type IV by *N. americanus* larval ES products

Lane 1	Larval ES products
Lane 2	Type IV collagen
Lane 3	Type IV collagen + larval ES products
Lane 4	1 μM E64
Lane 5	50 μM APMSF
Lane 6	1 mM 1, 10-phenanthroline
Lane 7	1 mM EDTA
Lane 8	1 μM pepstatin A
Lane 9	Collagenase

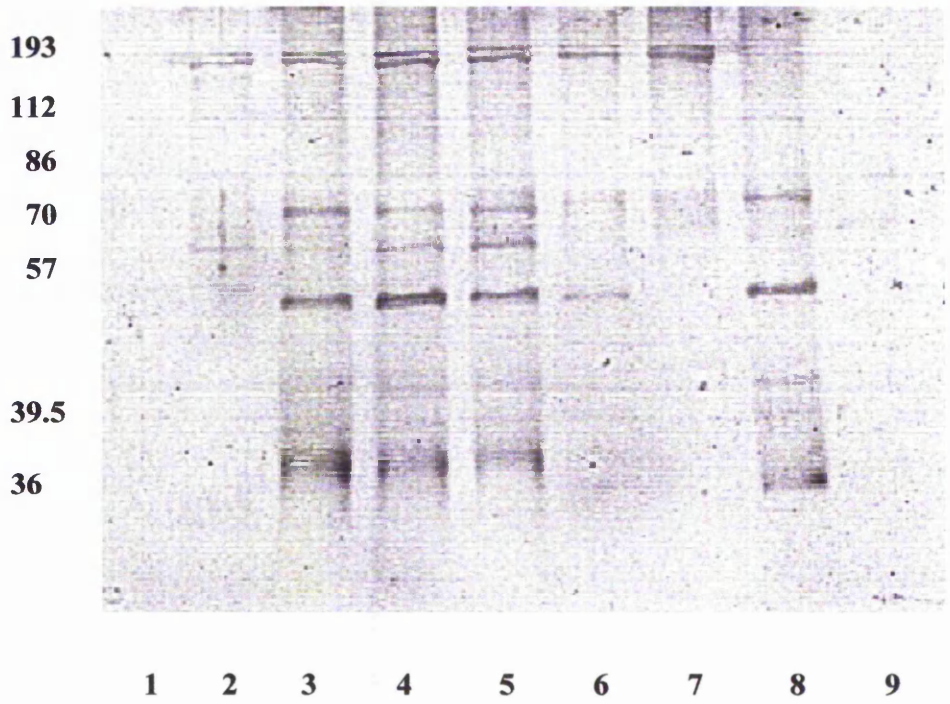

Figure 3.6.

The breakdown of collagen type V by *N. americanus* larval ES products

Lane 1	Larval ES products
Lane 2	Type V collagen
Lane 3	Type V collagen + larval ES products
Lane 4	1 μM E64
Lane 5	50 μM APMSF
Lane 6	1 mM 1, 10-phenanthroline
Lane 7	1 mM EDTA
Lane 8	1 μM pepstatin A
Lane 9	Collagenase

3.3.3.3 FIBRONECTIN

Fibronectin was also hydrolysed by larval ES products, the most predominant degradation product being a protein of 40 kDa (Figure 3.7). Unlike collagen, only pepstatin A inhibited the hydrolysis of fibronectin indicating that only the aspartyl proteinase is capable of degrading fibronectin. Interestingly, when proteolysis was inhibited by pepstatin A, some cross-linking of fibronectin, occurred resulting in the formation of a higher molecular mass protein of 145 kDa (Lane 8).

3.3.3.4 LAMININ

When laminin (Figure 3.8) was incubated with larval ES products a degradation product of approximately 90 kDa was observed, except in the presence of EDTA where the degradation of laminin was almost complete (Lane 7). As for fibronectin, laminin degradation was inhibited only by pepstatin A, again indicating the presence of an aspartyl proteinase.

3.3.4 TIME COURSE OF LARVAL PENETRATION THROUGH HAMSTER SKIN AND THE EFFECT OF PROTEINASE INHIBITORS

Figure 3.9 shows the time course of larval penetration through hamster skin. The results are expressed as the mean number of larvae ±1SD, failing to enter the skin or the mean number of larvae ±1SD, fully penetrating the hamster skin (six determinations were carried out at each time point). The larvae entered the skin rapidly and after 3 hours 196 (98 %) larvae had entered the hamster skin increasing to 198 (99 %) after 48 hours. 3 hours after the application of larvae to the hamster skin only 2 (1 %) of the larvae had completely traversed the skin increasing to 57.7 (28.8 %) after 24 hours and 84 (42 %) by 48 h. It was necessary to obtain proteinase inhibitor data while the skin was still in as natural condition as possible. As the percentage of larvae fully penetrating the skin only increased by a further 13.2 % between 24 and 48 h, the effects of proteinase inhibitors was studied after 24 h incubation

Figure 3.10 shows the effect of proteinase inhibitors on larval skin penetration. The figure represents the mean ± 1SD of nine determinations for each control and inhibitor tested. When the number of larvae fully penetrating the skin after 24h was determined, all the inhibitors tested inhibited skin penetration to some degree. The only significant inhibition

of penetration was observed in the presence of pepstatin A (54.8%, $p \leq 0.05$) when compared to the ethanol control. Interestingly E64 (36.7 %) and APMSF (36.9 %) inhibited skin penetration to a greater degree than 1, 10-phenanthroline (5.3 %) and EDTA (27.9 %)

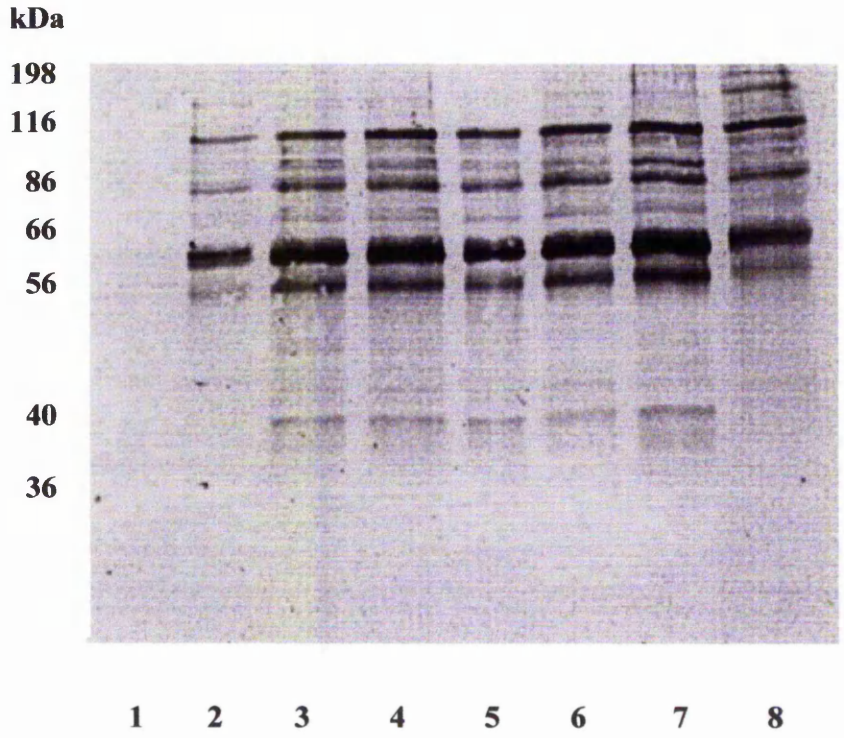

Figure 3.7

The breakdown of fibronectin by *N. americanus* larval ES products

Lane 1	Larval ES products
Lane 2	Fibronectin
Lane 3	Fibronectin + larval ES products
Lane 4	1 μM E64
Lane 5	50 μM APMSF
Lane 6	1 mM 1, 10-phenanthroline
Lane 7	1 mM EDTA
Lane 8	1 μM pepstatin A

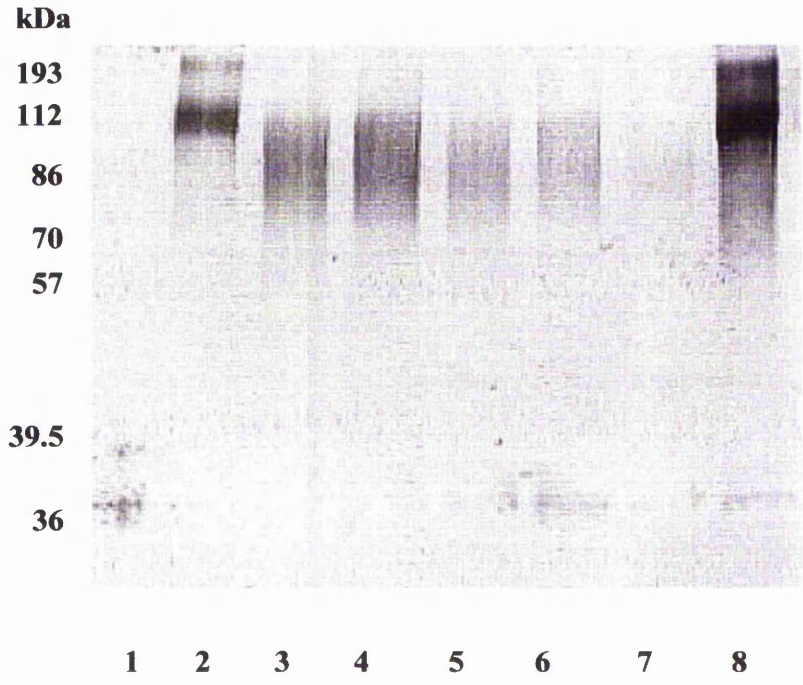

Figure 3.8

The breakdown of laminin by *N. americanus* larval ES products

Lane 1	Larval ES products
Lane 2	Laminin
Lane 3	Laminin + larval ES products
Lane 4	1 μM E64
Lane 5	50 μM APMSF
Lane 6	1 mM 1, 10-phenanthroline
Lane 7	1 mM EDTA
Lane 8	1 μM pepstatin A

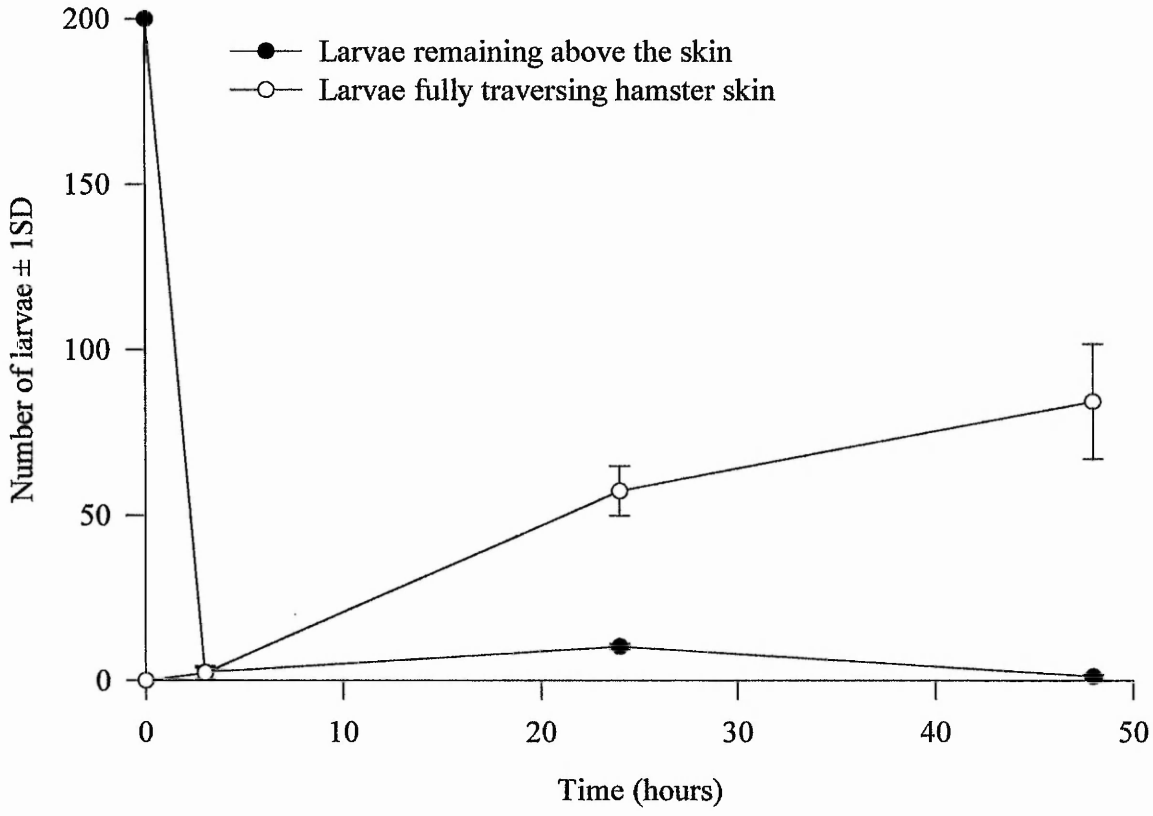

Figure 3.9.

The time course of larval penetration through hamster skin

200 *N.americanus* larvae were placed on the surface of excised hamster skin. After 3, 24 and 48 h incubation at 37 °C, triplicate samples were removed from the incubator and the number of larvae remaining above the skin and fully penetrating the skin determined.

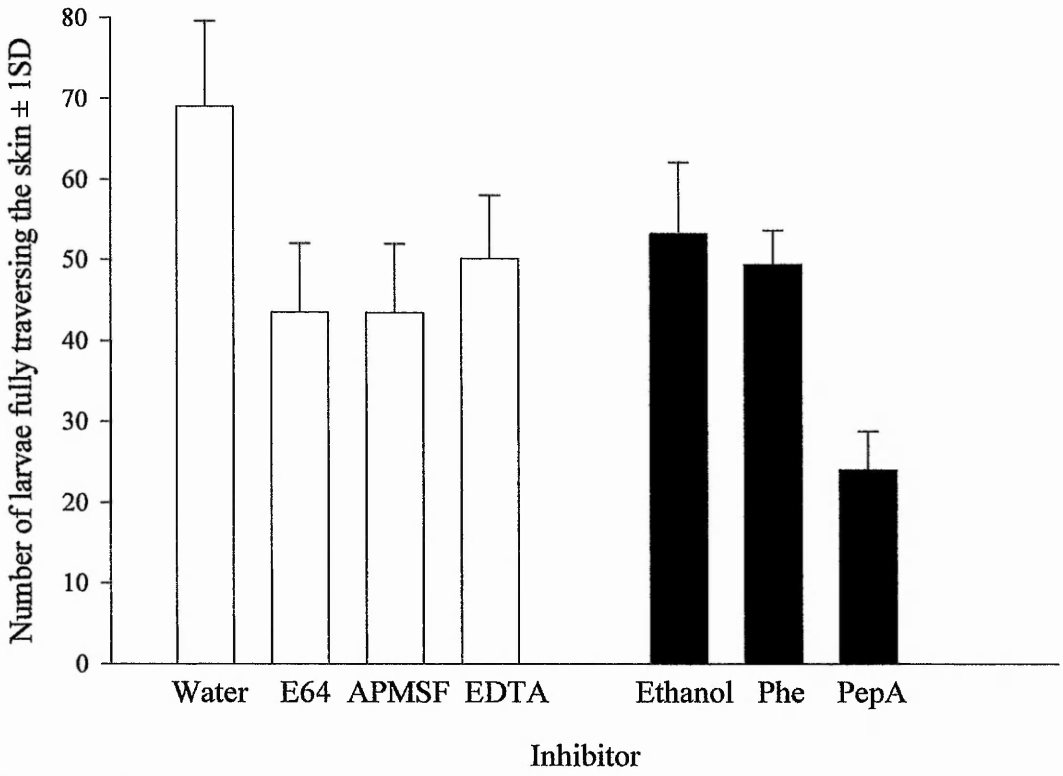

Figure 3.10.

The effect of proteinase inhibitors on the penetration of *N. americanus* larvae through hamster skin

200 *N. americanus* larvae were placed on excised hamster skin in the presence and absence of proteinase inhibitors. The number of larvae fully penetrating the skin was determined after 24 h incubation at 37 °C. White bars = water control and water soluble inhibitors. Black bars = ethanol control and ethanol soluble inhibitors.

3.3.5 DEGRADATION OF HAEMOGLOBIN AND FIBRINOGEN BY ADULT *N. AMERICANUS* ES PRODUCTS

3.3.5.1 HAEMOGLOBIN

Under denaturing conditions haemoglobin is reduced from its normal protein subunit struture of $\alpha_2\beta_2$ to its individual protein chains, each with an approximate molecular mass of 16 kDa (Goldberg *et al.*, 1990). Western blot analysis (Figure 3.11) demonstrated the degradation of this 16 kDa protein by adult *N. americanus* ES products. Haemoglobin degradation was primarily inhibited by pepstatin A, although this inhibition was not complete. Some inhibition was also seen in the presence of EDTA. No inhibition was seen with E 64 or APMSF or 1, 10-phenanthroline.

When the degradation of haemoglobin was examined using substrate SDS-PAGE (Figure 3.12) a zone of proteolysis was observed at approximately 66 kDa. Under these conditions proteolysis was only inhibited by pepstatin A.

3.3.5.2 FIBRINOGEN

Similarly when the degradation of fibrinogen was examined using substrate SDS-PAGE (Figure 3.13), proteolysis was observed at approximately 66 kDa. Again degradation of fibrinogen was only inhibited by pepstatin A.

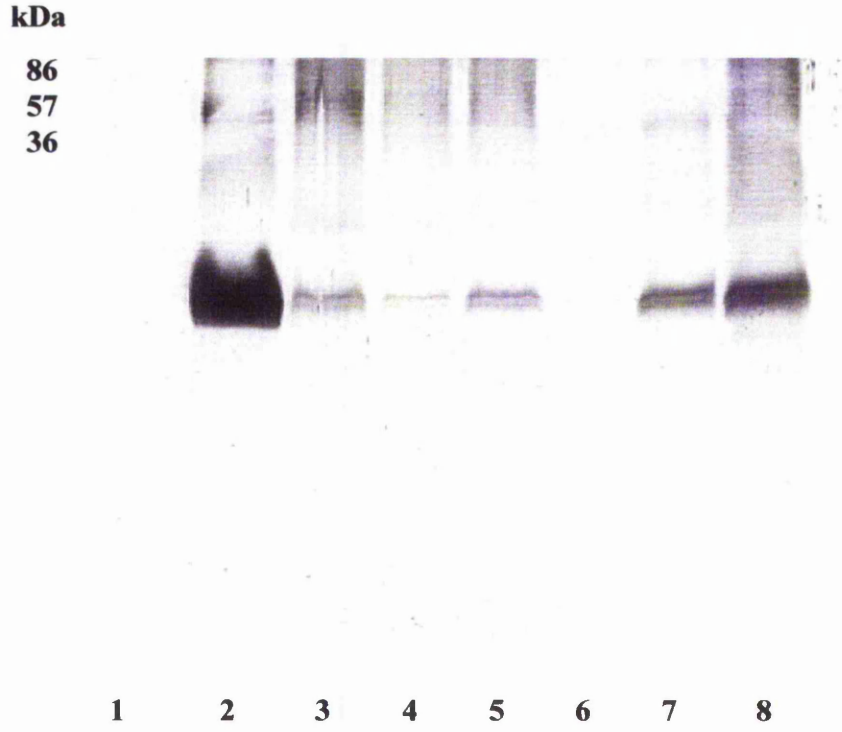

Figure 3.11

The breakdown of haemoglobin by *N. americanus* adult ES products

Lane 1 Adult ES products

Lane 2 Haemoglobin

Lane 3 Haemoglobin + adult ES products

Lane 4 1 μM E64

Lane 5 50 μM APMSF

Lane 6 1 mM 1, 10-phenanthroline

Lane 7 1 mM EDTA

Lane 8 1 μM pepstatin A.

Molecular mass markers (kDa) are indicated.

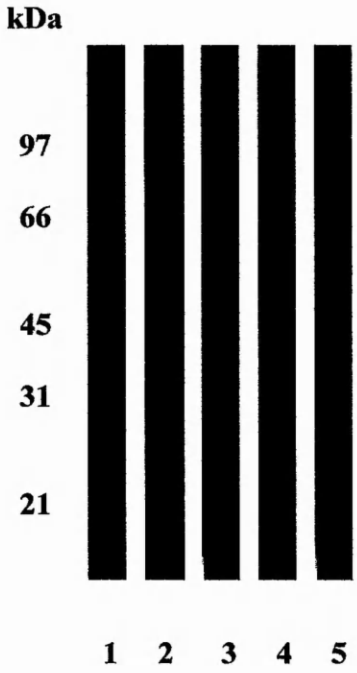

Figure 3.12

Haemoglobin degradation by *N.americanus* adult ES products using substrate SDS-PAGE

Lane 1	Adult ES products
Lane 2	10 μM E64
Lane 3	100 μM APMSF
Lane 4	10 mM 1, 10-phenanthroline
Lane 5	10 μM pepstatin A

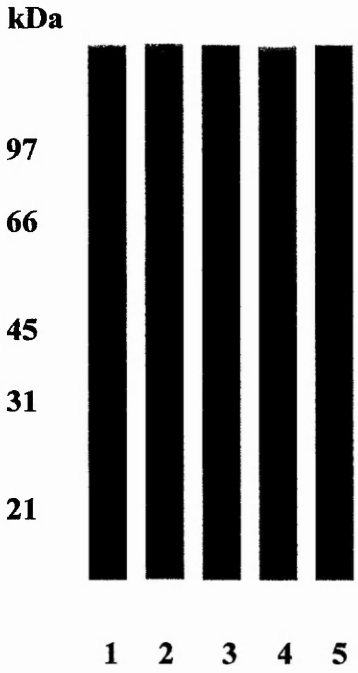

Figure 3.13

Fibrinogen degradation by *N. americanus* adult ES products using substrate SDS-PAGE

Lane 1	Adult ES products
Lane 2	10 μM E64
Lane 3	100 μM APMSF
Lane 4	10 mM 1, 10-phenanthroline
Lane 5	10 μM pepstatin A

3.4 DISCUSSION

Using FITC labelled casein as the substrate the pH optimum of the proteinase activity secreted by *N. americanus* larvae was shown to be pH 6.5 with lesser peak of activity at pH 8 (Figure 3.1). The peak of activity observed at pH 8 may be the azocoll degrading activity first described by Matthews (1982). At pH 6.5 and pH 8 the proteinase activities present were characterised using a number of protease inhibitors (Table 3.1). At pH 6.5 proteinase activity was primarily inhibited by the aspartyl protease inhibitor pepstatin A, indicating that at this pH the predominant activity is aspartyl in nature. All the other inhibitors tested inhibited proteolysis to some degree indicating that a mixture of cysteinyl, serine, and metallo proteolytic enzymes are also active at this pH. At pH 8 the activity of the aspartyl proteinase was significantly reduced as demonstrated by the reduction in inhibition by pepstatin A. Inhibition by the serine proteinase inhibitor APMSF at pH 8 increased to 81.5 % indicating that at this pH the secreted serine proteinase is the most active proteinase. Metallo-proteinase activity as defined by inhibition by 1, 10-phenanthroline and EDTA also increased from pH 6.5 to pH 8. Proteinase activity was also slightly enhanced by the presence of cysteine confirming the low levels of one or more cysteinyl proteinases. Cysteinyl proteinase activity was reduced at pH 8 and this was reflected by the reduced enhancement of activity by 5 mM cysteine. The mixture of proteinases demonstrated here is reminiscent of the mixture of proteinase activities secreted by adult *N. americanus* (Brown *et al.*, 1995). However, unlike adult ES products, no peak of activity was seen at pH 3.5. With respect to larval ES products the data presented here confirm and extend the findings of Kumar & Pritchard (1992a; 1992b) who, using gelatin substrate SDS-PAGE, demonstrated that larval secretions contain cysteinyl and serine proteinases. Metallo-proteinase activity was demonstrated by monitoring the release of radiolabelled peptides and amino acids from radio labelled gelatin bound to polystyrene plates (Kumar & Pritchard, 1992b). In addition to these activities previously described, the presence of an aspartyl protease in larval ES products has now been demonstrated for the first time.

The mixture of proteinases secreted by both the adult and larval stages of *N. americanus* was further characterised by the use of a number of fluorogenic substrates (Table 3.2). Serine proteinase activity for both life cycle stages was shown to be 'trypsin-like' in its nature, failing to significantly cleave any of the chymotryptic substrates when compared

with the tryptic substrates tested. 'Tryptic-like' activities have been previously described and purified from the secretions of *S. mansoni* (Chavez-Olortegui *et al.*, 1992). *N. americanus* larval ES products failed to hydrolyse the elastase substrate Suc-Ala-Ala-Ala-AMC but degraded the kallilkrein and elastase substrate H-Pro-Phe-Arg-AMC.HCl. Adult *N. americanus* ES products while hydrolysing both substrates, demonstrated a preference for Suc-Ala-Ala-Ala-AMC compared with H-Pro-Phe-Arg-AMC.HCl, hydrolysing over twice the amount of H-Pro-Phe-Arg-AMC.HCl over the same time period. This would seem to indicate a preference for different amino acid sequences within elastin, by one or more of the enzymes in adult and larval ES products, responsible for elastin degradation (Table 3.3).

Cathepsin B and L-'like' cysteinyl proteinase activities in both adult and larval ES products were detected by the hydrolysis of the cathepsin B substrate Z-Arg-Arg-AMC.2HCl and the cathepsin B and L substrate Z-Phe-Arg-AMC.HCl. Activity for both larval and adult ES products was enhanced against the substrate Z-Phe-Arg-AMC.HCl compared to the substrate Z-Arg-Arg-AMC.2HCl. If only a cathepsin B 'like' proteinase was present in adult and larval ES products then it would be reasonable to assume that activities against these substrates would be similar. Therefore the increased activity against Z-Phe-Arg-AMC.HCl may indicate the presence of a cathepsin L 'like' proteinase. Cathepsin L 'like' activity has also been identified in the adult ES products of the hookworm *A. caninum* (Dowd *et al.*, 1994) and a cDNA encoding a cathepsin B 'like' cysteinyl proteinase has been cloned and proposed as a possible allergen inducing human eosinophillic enteritis (Harrap *et al.*, 1995).

As indicated in the introduction, the cathepsin B 'like' activity of *S. mansoni* is one of the most characterised activities; a cathepsin L 'like activity has also been described and two cDNAs encoding a cathepsin L 'like' enzyme have been sequenced (Smith *et al.*, 1994b). Indeed it has been shown that the cathepsin L activity is greater than the cathepsin B activity and may play a more important role in haemoglobin degradation (Dalton *et al.*, 1995). This may be the case as, whilst cathepsin L and B are both associated with mammalian lysosomes and have roles in protein degradation, the cathepsin L enzymes are

generally accepted to be the more powerful proteolytic enzymes (Barrett & Kirschke, 1981).

Cathepsin L activity has been shown to be secreted by *Fasciola hepatica*. This activity is thought to be immunosupressive hydrolysing host immunoglobulin close to the papain cleaving site (Smith *et al.*, 1993a) and, in the case of newly excysted juveniles, preventing antibody mediated attachment of eosinophils (Carmona *et al.*, 1993). Inhibition of this activity with anti cathepsin L antibodies reverses this effect (Smith *et al.*, 1994a).

Previous work (Brown *et al.*, 1995), and the characterisation experiments in this chapter clearly demonstrate the presence of an aspartyl proteinase activity inhibitable by pepstatin A in larval and adult ES products. At the pH closest to that of the normal skin surface (pH 5.5) the predominant and possibly most important proteinase activity in larval ES products as far as skin penetration is concerned is the aspartyl proteinase and this is confirmed by the inhibition of larval penetration through hamster skin by pepstain A. However, further characterisation has proved difficult due to the lack of activity of this enzyme against a number of commercially available chromogenic substrates. No activity could be detected in adult ES products against the pepsin substrate Pro-Thr-Glu-Phe-(NO_2)-Phe-Arg-Leu (Dunn *et al.*, 1984; Brown *et al.*, 1995). Similarly, no activity was demonstrated in larval ES products against the cathepsin D substrate H-D-Phe-Ser-(Bzl)-Phe-Phe-Ala-Ala-p-aminobenzoate (Orlowski *et al.*, 1984), the cathepsin D and pepsin substrate Pyr-His-Phe(NO_2)-Phe-Ala-Leu-NH_2 (Pohl *et al.*, 1983; 1984), the pepsin substrate Pro-Thr-Glu-Phe-(NO_2)-Phe-Arg-Leu and the chymosin substrate H-Leu-Ser-Phe-Nle-Ala-Leu-OMe (Martin *et al.*, 1980, own data not shown). Aspartyl proteinases often have more complex requirements from a synthetic substrate and these frequently have to be designed for the proteinase in question (North, 1997). Indeed, the only parasite aspartyl protease successfully analysed using a chromogenic synthetic substrate has been the plasmepsin II enzyme of *Plasmodium falciparum*, assayed using the substrate Ala-Leu-Glu-Arg-Thr-Phe-Nph-Ser-Phe-Pro-Thr. The design of this substrate was based on the amino acid sequence of α globin around the Phe-Leu bond initially cleaved by the enzyme (Hill *et al.*, 1994). As haemoglobin is a proposed substrate for the adult aspartyl proteinase it could be that a

substrate designed around a similar sequence may be more suitable, at least for the assay of the adult enzyme.

Elastase activity in larval ES products measured by the release of dye from elastin-orcein was optimal at pH 7.5 with a smaller peak of activity at pH 8.5 (Figure 3.2). The difference in pH optima against elastin compared with FITC-casein probably reflects the pH optima of the type of enzymes involved in elastin breakdown. At pH 7.5, elastin degradation was completely inhibited by 1, 10-phenanthroline while at pH 8.5 inhibition by APMSF increased to 46.7 % (Table 3.3). Elastin degradation would appear to be achieved by two proteinases (serine and metallo) with more alkaline pH optima. The combination of these enzymes shifts the pH optima of the combined ES products against elastin to pH 7.5 compared the that seen using FITC-casein (pH 6.5). The peak of elastin hydrolysis at pH 8.5 is reflective of the enhanced serine activity seen at pH 8 against FITC-casein. No inhibition or enhancement of elastase activity was seen with 1 μM E64 or 5 mM cysteine indicating that the cathepsin B and L 'like' activities play no role in elastin degradation.

The ability of *N. americanus* larval ES products to degrade human collagen, fibronectin and laminin was also tested. No collagenolytic activity was detected using two assays designed to detect the release of labelled amino acids or small fragments of labelled peptide (Section 3.2.1.2.3 and 3.2.1.2.4). However, breakdown products from all types of collagen tested were observed using SDS-PAGE and Western blotting (Figures 3.3 to 3.6). Similarly, breakdown products were observed with fibronectin and laminin (Figures 3.7 and 3.8). For all skin macromolecules examined, no inhibition of proteolysis was seen with E64 or APMSF suggesting that the cysteinyl and serine proteinases play no part in the degradation of these skin macromolecules during penetration. The degradation of gelatin by larval cysteinyl and serine proteinases as demonstrated by substrate SDS-PAGE (Kumar & Pritchard, 1992a) may be explained by the denatured state of the collagen in these gels. This may allow the secreted cysteinyl and serine enzymes access to susceptible peptide bonds not normally available in the protein's natural state.

The results shown in Figures 3.3 to 3.8 demonstrate the importance of the aspartyl proteinase to the invading larvae during skin penetration. This proteinase was capable of

degrading all the skin macromolecules tested with the exception of type V collagen. The secreted metallo-proteinase may also be important to the skin penetration process although its substrate specificity appears to be limited to collagen and elastin. Indeed it may be possible to speculate on the possibility of two metallo-proteinases with different inhibitor sensitivities and substrate specificities. One enzyme appears to degrade collagen types III, IV, V and elastin. This proteinase is sensitive to 1, 10-phenanthroline while the other degrades Types IV and V collagen and appears to be sensitive to EDTA. Type I collagen was undegraded by either metallo-proteinase and its degradation was only inhibited by pepstatin A. Similarly, fibronectin and laminin breakdown was only inhibited by pepstatin A. When fibronectin degradation was inhibited by pepstatin A, a 145 kDa protein was observed indicating that in the absence of the aspartyl proteinase some degree of fibronectin cross linking occurs. This could possibly be due to the presence of a tranglutaminase in larval secretions the function of which requires further study.

When the proteinases present in adult ES products were tested for activity against fibronectin and laminin no activity was observed (data not shown), again indicating a possible difference in the substrate specificity of the adult enzymes, despite the similarity of the enzyme classes present within adult and larval ES products.

To successfully penetrate the skin barrier *N. americanus* larvae must pass through the epidermis, basement membrane and dermis before finally entering the blood or lymphatic system. It has been suggested in the case of the cat hookworm *Ancylostoma tubaeforme* that penetration through the skin is a purely mechanical process (Matthews, 1975). It may also be the case that penetration of *N. americanus* through the epidermal layer of the skin may to some degree be mechanical as Matthews (1982) only demonstrated cellular destruction by *N. americanus* larvae at the germinal layer of the epidermis. If this is the case then the first major barrier to skin penetration is the basement membrane. The basement membrane as described earlier is composed primarily of collagen type IV, laminin and fibronectin, all of which were cleaved by the aspartyl proteinase present in larval ES products. Once through the basement membrane the larvae must penetrate the dermis until it reaches the blood or lymphatic system. The dermis is composed of types I, III, V and VII collagen and elastin fibres within a 'gel' of glycosaminproteolglycans, salt

and water (Section 1.6.2). To penetrate the dermis a mixture of aspartyl, metallo and serine proteinases are employed. It was impossible to detect collagenolytic activity in assays designed to detect the release of amino acids and small peptide fragments and only relatively large breakdown products of collagen (75-32 kDa) were observed on Western blots. From these results it is tempting to postulate that during the infection process the complete degradation of skin macromolecules is unnecessary and only the hydrolysis of a small number of peptide bonds is sufficient to allow *N. americanus* larvae to successfully penetrate the skin barrier. The final barrier to the larva is entry into the blood and lymph vessels, the walls of which contain predominantly type III collagen, which was again degraded by larval aspartyl and metallo-proteinases. The results obtained from the Western blot analysis of collagen degradation (Figures 3.3 to 3.6) would suggest that the collagens that present the greatest barrier; i.e. types III and IV are more readily degraded than types I and V.

The degradation profile of skin macromolecules shown by *N.americanus* larvae is consistent with the proposal put forward by Hotez *et al.*, (1990) that obligate skin penetrating larvae should be capable of degrading elastin, fibronectin and laminin. However, the breakdown of these macromolecules by an aspartyl proteinase is unusual and has not been described previously, although aspartyl proteinase activities have been demonstrated in the secretions of adult *Haemonchus contortus* (Karanu *et al.*, 1993) and the dog hookworm *Ancylostoma caninum* (Harrap *et al.*, 1996). Similarly, *Strongyloides stercoralis*, an obligate skin penetrator, also degrades elastin, fibronectin and laminin (Hotez *et al.*, 1990) and secretes a potent histolytic metallo-proteinase (McKerrow *et al.*, 1990). Inhibition of this enzyme with a metallo-protease inhibitor prevents larval penetration of the skin. This enzyme has also been shown to be both immunogenic and allergenic during infection (Brindley *et al.*, 1995).

In addition to proteolytic enzymes *Ancylostoma* hookworms secrete a hyaluronidase which hydrolyses hyaluronic acid found between keratinocytes of the epidermis and in the dermis (Hotez *et al.*, 1992). Levels of hyaluronidase secretion appear to correlate with the degree of larval migrans caused by different *Ancylostoma* species and the enzyme has been proposed as an additional virulence factor. To date hyaluronidase activity has not been

demonstrated in the larvae of *N. americanus* (data not shown) and it would appear that they rely solely on the use of proteolytic enzymes to penetrate the skin barrier.

The importance of the aspartyl proteinase during skin penetration was most evident in the experiments carried out using excised hamster skin. Larval penetration was significantly inhibited by pepstatin A (54.8 %, $p< 0.05$). All the other inhibitors tested had some effect on larval penetration. Possible roles for the serine and metallo enzymes have already been discussed, but it is interesting to note the inhibition by E64 (36.7 %), implying a role for cysteinyl proteinases in skin penetration, perhaps degrading a skin protein not yet studied. A summary of the proteinase activities present in larval ES products and their possible substrates can be found in Table 3.4.

N. americanus adult ES products contain a number of different proteinases capable of degrading haemoglobin (Brown *et al.*, 1995). In this work haemoglobin degradation was studied at pH 6.5 using Western blotting and substrate SDS-PAGE. On Western blots, in the presence of adult ES products the 16 kDa subunits of haemoglobin were degraded (Figure 3.11). Pepstatin A inhibited this degradation although this inhibition was not complete. EDTA also inhibited haemoglobin degradation to some extent. Using substrate SDS-PAGE, haemoglobin degradation was mediated by an enzyme with a molecular mass of approximately 66 kDa (Figure 3.12). This proteolytic activity was inhibited by pepstatin A defining it as aspartyl in nature. Haemoglobin degradation by *S. mansoni* is achieved by a mixture of cathepsin B and L 'like' proteinase activities, however, only the malarial parasite *Plasmodium falciparum* has been described as having an aspartyl proteinase capable of degrading haemoglobin (Goldberg *et al.*, 1990; Goldberg & Slater, 1992). In this parasite haemoglobin degradation is an ordered process, taking place in specialised digestive vacuoles. Two haemoglobin degrading aspartyl proteinases have been identified (Gluzman *et al.*, 1994). Plasmepsin I makes an initial cleavage that leads to the unravelling of haemoglobin, exposing further sites for proteolysis. Plasmepsin II is also capable of cleaving native haemoglobin but is more active against denatured haemoglobin. Following the action of these aspartyl proteinases, which produce relatively large protein fragments, a cysteinyl proteinase then cleaves these fragments into small peptides and amino acids. The latter enzyme is sensitive to the antimalarial agent, 4-aminoquinoline and may suggest a

possible mode of action for the drug (Gluzman *et al.*, 1994). In the case of *N. americanus* haemoglobin degradation is primarily inhibited by pepstatin A and it is possible to envisage a similar scenario, where haemoglobin is initially clipped by the aspartyl enzyme followed by further cleavage by cysteinyl, serine and metallo enzymes. No breakdown products were observed on the Western Blot of haemoglobin degradation (Figure 3.11); however it is possible that the small size of any breakdown products may not be detected using this particular gel system.

The aspartyl proteinase was also shown to hydrolyse fibrinogen when analysed using substrate SDS-PAGE (Figure 3.13). *N. americanus* has developed a number of antihaemostatic strategies to facilitate feeding and promote blood loss from the host. These include the inhibition of Factor Xa and the inhibition of platelet aggregation (Furmidge *et al.*, 1995). Fibrinogen plays an important role in platelet-platelet interactions and the degradation of fibrinogen by adult ES products would significantly interfere with this process. However, the time course for fibrinogen degradation is longer than required for the inhibition of platelet aggregation by ES products. This suggests that the hydrolysis of fibrinogen only accounts for part of the process and it is likely that a further component of ES products has a more direct role in the inhibition of platelet aggregation.

Table 3.4

Summary of the substrates degraded by the proteinases present in larval and adult ES products.

	Proteinase Class	Substrate
Larvae	Aspartyl	Collagen Types I, III and IV Elastin, Fibronectin and laminin
	Cysteinyl	Unknown
	Metallo	Collagen Types III, IV and V
	Serine	Elastin
Adult	Aspartyl	Haemoglobin, Fibrinogen
	Cysteinyl	Haemoglobin
	Metallo	Haemoglobin
	Serine	Haemoglobin

Having further assessed the role of the proteinases present in larval and adult ES products and established the relative importance of some of these enzymes it is clear that immune responses against a number of them, but particularly the aspartyl enzyme, may significantly interfere with parasite invasion and feeding. Chapter 4 describes attempts to purify a number of these enzymes with a view to assessing their potential as candidate vaccines.

CHAPTER 4

THE PURIFICATION OF PROTEINASES FROM *NECATOR AMERICANUS* ADULT AND LARVAL EXCRETORY/ SECRETORY PRODUCTS.

CHAPTER 4

THE PURIFICATION OF PROTEINASES FROM *NECATOR AMERICANUS* ADULT AND LARVAL EXCRETORY/ SECRETORY PRODUCTS.

4.1 INTRODUCTION

The characterisation experiments described in Chapter 3 have demonstrated the presence of proteinases from all four mechanistic classes in larval ES products and have further confirmed the importance of the aspartyl proteinase in adult ES products. These enzymes appear to play an important role in larval skin penetration and possibly nutrition of the adult hookworm and are particularly attractive as candidate vaccine molecules. Neutralisation of these proteinase activities by the immune system could possibly prevent larval passage through the skin and/or disrupt adult feeding in the small intestine and so lead to expulsion of the adult worm. In order to test this hypothesis a supply of purified proteinase is essential.

This chapter describes a number of attempts to purify proteinase activities from both larval and adult ES products. Proteolytic enzymes have been purified from other parasites by a number of methods (Hotez *et al.,* 1985; Yamakami & Hamajima. 1987; Smith *et al.,* 1993b). In all these cases purification has been achieved using multiple step procedures. However, *N. americanus* is difficult to maintain in the laboratory environment and the production of large quantities of ES products is almost impossible, particularly in the case of larval ES. With this in mind, purification of proteinases was attempted using one-step protocols with specific affinity matrices, in an attempt to avoid the protein loses associated with multiple step procedures. For example, purification of the aspartyl proteinase was attempted using a matrix to which the reversible aspartyl inhibitor pepstatin A had been bound (Slater, 1981). Similarly, metallo proteinase purification was attempted using a matrix containing the metallo-proteinase inhibitor N-Benzyloxycarbonylglycyl-D-

leucylaminohexyl-Sepharose (Inouye *et al.,* 1985) while serine proteinase purification was attempted using matrices with bound soybean trypsin inhibitor (Reeck *et al.,* 1971) and *p*-aminobenzamidine (Grant *et al.,* 1978). In all protocols ES products were applied to the matrix in a suitable buffer and non-specifically bound protein removed by extensive washing. Elution of bound protein was achieved by the application of either excess free inhibitor (pepstatin A column) or by a change in buffer pH (soybean trypsin inhibitor, *p*-aminobenzamidine and N-Benzyloxycarbonylglycyl-D-leucylaminohexyl-Sepharose columns). Cysteinyl proteinase purification was attempted using activated thiol Sepharose 4B; in this case the reactive thiol group immobilised on Sepharose 4B reacts specifically with the cysteine residue in the active site of the proteinase. Following extensive washing, specific elution was achieved by washing the column with a reducing agent such as cysteine, dithiothreitol or reduced glutathione. Metallo-proteinase purification was also attempted using the matrix bacitracin-Sepharose; bacitracin is an antibiotic that has been shown to inhibit all classes of proteinase (Irvine *et al.,* 1993). In this case elution of class specific proteinase activities can be achieved by the use of different elution buffers.

For each of these purification protocols eluted proteinase activity was characterised using a number of proteinase inhibitors and the purity of the enzyme examined by SDS-PAGE. Where possible purification was attempted from both adult and larval ES products, in order to assess the purification potential of these protocols and to assess if similar enzymes are found in both adult and larval ES products. In addition, the immunogenicity of the purified cysteinyl and aspartyl proteinases was examined by probing Western blots of the purified enzymes with human post-hookworm infection serum. Activated thiol Sepharose 4B essentially consists of glutathione immobilised on Sepharose 4B. This gives the matrix the potential to bind non proteolytic 'glutathione binding proteins', which have been demonstrated in a number of parasites (Brophy & Barrett, 1990; Brophy *et al.*, 1994). To examine this Western blots of purified cysteinyl proteinases were also probed with a rabbit antiserum raised to *N. americanus* glutathione-S-transferase purified using glutathione-Sepharose. This antiserum has been shown to recognise a 66 kDa glutathione binding protein in addition to *N. americanus* glutathione-S-transferase (rabbit anti *N. americanus* glutathione-S-transferase and information supplied by Dr P. M. Brophy).

Following the establishment of these affinity purification protocols sufficient quantities of larval aspartyl and cysteinyl proteinase were purified and used to vaccinate mice against *N. americanus* infection (Chapter 5).

4.2 MATERIALS AND METHODS

Larval and adult ES products were prepared and their protein content determined as described in Chapter 2 (Section 2.3). All protein purifications were carried out using the Bio-Rad Econosystem.

4.2.1 PURIFICATION OF CYSTEINYL PROTEINASES USING ACTIVATED THIOL-SEPHAROSE 4B.

Activated thiol-Sepharose 4B (Sigma) was re-swollen in 50 mM phosphate buffer pH 6.5 according to the manufacturers instructions and poured into a 5 ml glass chromatography column. The column was then equilibrated using 50 mM phosphate buffer pH 6.5 (flow rate 0.2 ml/min) before applying ES products (resuspended in equilibration buffer) to the column. Unbound protein was eluted using sequential washes of 50 mM phosphate buffer pH 6.5 (15 ml) and 50 mM phosphate buffer pH 6.5 containing 1 M sodium chloride (15 ml). Finally bound protein was eluted using 30 ml of 50 mM phosphate buffer pH 6.5, containing 25 mM reduced glutathione. Protein eluting from the column was monitored by measuring absorbance at 280 nm. Fractions of 1.5 ml were collected and assayed for protein content and proteolytic activity using FITC-casein and the synthetic fluorogenic substrates Z-Phe-Arg-AMC.HCl (Morita *et al.*, 1977) and Z-Arg-Arg-AMC.HCl (Mason *et al.*, 1985) as described in Chapter 2. Used activated thiol-Sepharose was regenerated by washing extensively with 5 mM 2'2'dipyridyldisulphide followed by 50 mM phosphate buffer pH 7.

4.2.2 PURIFICATION OF ASPARTYL PROTEINASES USING PEPSTATIN A-AGAROSE

Aspartyl proteinases were purified from larval and adult ES products using pepstatin A agarose (Sigma) by a method modified from Slater (1981). A 5 ml pepstatin A agarose column was equilibrated with 50 mM sodium acetate pH 5.5. Larval and adult ES products were resuspended in 50 mM sodium acetate pH 5.5 and applied to the column at a flow rate of 0.2 ml/min. The column was washed sequentially with 50 mM sodium acetate pH 5.5 (10 ml) followed by 50 mM sodium acetate, 0.5 M sodium chloride pH 5.5 (10 ml). Bound protein was eluted from the column with 500 μM pepstatin A dissolved in 50 mM sodium acetate pH 5.5 (15 ml). 1 ml fractions were collected and analysed for protein content

(Section 2.3) and proteolytic activity using FITC-casein (Section 2.4.2.1). Fractions eluted from the column with 500 μM pepstatin A were dialysed against distilled water prior to analysis of proteolytic activity. Used pepstatin A agarose was regenerated by washing with ten column volumes of 6.5 M urea and 10 column volumes of 0.1 M sodium borate prior to re-equilibration in 50 mM sodium acetate pH 5.5. Pepstatin A-agarose columns were re-used a maximum of 3 occasions.

4.2.3 PURIFICATION OF METALLO-PROTEINASES

4.2.3.1 PURIFICATION OF METALLO-PROTEINASES USING BACITRACIN-SEPHAROSE

Bacitracin-Sepharose was prepared as described by van Noort *et al.* (1991). 4g of cyanogen bromide activated Sepharose 4B (Sigma) was mixed with 300 mg of bacitracin (Sigma) in 30 ml of 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.3. The mixture was incubated overnight at 4 °C after which any uncoupled bacitracin was removed and any remaining binding sites blocked by washing with 0.1 M Tris-HCl, pH 8 for 150 minutes at room temperature. The gel was then washed extensively with 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.3. Finally the gel was equilibrated in 0.1 M Tris-HCl, pH 8 and stored at 4 °C until required.

Affinity chromatography on bacitracin-Sepharose was carried out as described by Markaryan *et al.* (1994). 5 ml of bacitracin-Sepharose was poured into a column and equilibrated with 50 mM Tris-HCl, pH 7.2. Larval ES products were resuspended in 50 mM Tris-HCl, pH 7.2 (buffer 1) and applied to the column at a flow rate of 0.2 ml/min. The column was washed with 10 ml of buffer 1 to remove any unbound protein. Bound protein was eluted sequentially with 10 ml, 50 mM Tris-HCl, 1 M NaCl, 1 mM $CaCl_2$, 7 % iso-propanol, pH 7.2 (buffer 2) and finally 15 ml of 50 mM Tris-HCl, 1 M NaCl, 1 mM $CaCl_2$, 25 % iso-propanol, pH 7.2 (buffer 3). 1 ml fractions were collected and analysed for protein content (Section 2.3), elastase activity using the substrate elastin-orcein (Section 3.2.1.1) and for purity by SDS-PAGE (Section 2.5).

4.3.2.2 PURIFICATION OF METALLO-PROTEINASES USING N-BENZYLOXYCARBONYLGLYCYL-D-LEUCYLAMINOHEXYL-SEPHAROSE

N-Benzyloxycarbonylglycyl-D-leucylaminohexyl-Sepharose (Z-Gly-D-Leu-AH-Sepharose) was prepared as described by Inouye *et al.* (1985). AH-Sepharose (Pharmacia) was re-swollen in deionised water. 20 ml of the re-swollen gel was mixed with 20 ml of 40 % dimethylformamide (pH 5.0) containing 456 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.HCl and 150 mg Z-Gly-D-Leu (Sigma) overnight at room temperature. The resulting gel was then washed with 1 M NaCl in 40 % dimethylformamide followed sequentially by 1 M $NaHCO_3$, deionised water, 1 M acetic acid and final wash with deionised water. The gel was stored in deionised water until use. Prior to chromatography, the gel was packed into a 5 ml glass column and equilibrated with 50 mM phosphate buffer pH 7.0. Larval ES products were resuspended in 50 mM phosphate buffer pH 7.0 and applied to the column (flow rate 0.2 ml/min). The column was washed with 50 mM phosphate buffer pH 7.0 (10 ml) followed by 50 mM phosphate buffer pH 5.6, 2 M urea (10 ml). Finally, bound protein was eluted with 0.1 M sodium acetate pH 4.1, 2 M urea (10 ml). 950 µl fractions were collected into 50 µl of 1 M NaOH to neutralise the acidity of the elution buffer. The fractions collected were analysed for protein content (Section 2.3), proteinase activity using FITC-casein (Section 2.4.2.2) and purity by SDS-PAGE (Section 2.5).

4.2.4 PURIFICATION OF SERINE PROTEINASES

4.2.4.1 SOYBEAN TRYPSIN INHIBITOR-SEPHAROSE CHROMATOGRAPHY

Serine proteinase purification was also attempted using STI-Sepharose as described by Reeck *et al.* (1971). 5 ml of STI-Sepharose was equilibrated with 20 mM Tris, 0.5 M NaCl, pH 7.5 and packed into a column. Adult *N. americanus* ES products were resuspended in 1 ml of 20 mM Tris, 0.5 M NaCl, pH 7.5 and applied to the column (flow rate of 0.2 ml/min). The column was washed with 20 mM Tris, 0.5 M NaCl, pH 7.5 (20 ml). Bound protein was eluted from the column with 0.1M sodium acetate, pH 3 (20 ml). 0.9 ml fractions were collected into tubes containing 0.1 ml 500 mM Tris-Cl pH 8.5 to neutralise the acidic pH. The fractions collected were analysed for protein content (Section

2.3) and proteinase activity using the substrate H-Pro-Phe-Arg-AMC.HCl (Lottenberg *et al.,* 1981).

4.2.4.2 *p*-AMINOBENZAMIDINE CHROMATOGRAPHY

Serine proteinase purification using the affinity matrix *p*-aminobenzamidine was carried out as described by Grant *et al.* (1978). *p*-aminobenzamidine (Sigma) was equilibrated with 50 mM Tris, pH 8.35 and packed into a 5 ml column. Adult and larval *N. americanus* ES products were resuspended in 50 mM Tris, pH 8.35 and applied to the column (flow rate 0.2 ml/min). The column was washed with 50 mM Tris, pH 8.35 (20 ml), followed by 50 mM Tris, 1 M NaCl, pH 8.35 (20 ml). Bound proteinase was eluted by washing with 70 mM sodium acetate, 0.5 M KCl, pH 5.6 (20 ml). 0.9 ml fractions were collected into microfuge tubes containing 0.1 ml 500 mM Tris-Cl pH 8.5 to neutralise the acidic pH. The fractions collected were analysed for protein content, proteolytic activity using the substrate Tosyl-Gly-Pro-Arg-AMC.HCl (Novabiochem) and purity by SDS-PAGE Section 2.5).

4.2.5 WESTERN BLOTTING

Purified proteinases were separated by SDS-PAGE and transferred onto nitrocellulose as described in Chapter 2 (Section 2.6). Blots were then blocked with 5 % Marvel milk powder dissolved in Tris buffed saline pH 7.4 (blocking agent) for 1 hour before being probed with pooled human *N.americanus* post infection sera (diluted 1:400 in blocking agent) overnight at 4 °C. The blot was then washed thoroughly with TBS/ 0.1% Tween 20 (TBS/Tween) and then incubated with sheep anti human Ig GAM-alkaline phosphatase conjugated or goat anti rabbit IgG-alkaline phosphatase conjugated (Sigma), diluted 1:1000 in blocking agent, for 2 h at room temperature. Blots were again washed thoroughly (3 x 20 minutes) with TBS/Tween. Antibody binding was revealed by the addition of BCIP/NBT prepared in substrate buffer as described in section A.2.4.3.3.

4.3 RESULTS

4.3.1 PURIFICATION OF CYSTEINE PROTEINASES

4.3.1.1 LARVAL ES PRODUCTS

Figure 4.1a shows the purification of cysteine proteinases from *N. americanus* larval ES products. The protein profile and the proteolytic activity against FITC-casein of the fractions obtained from an activated thiol-Sepharose 4B column are shown. Two peaks of proteolytic activity were eluted from the column by 25 mM reduced glutathione. The fractions obtained were also tested for proteolytic activity against Z-Phe-Arg-AMC.HCl and Z-Arg-Arg-AMC (Figure 4.1b). Both peaks of proteolytic activity eluted by 25 mM glutathione were capable of hydrolysing Z-Phe-Arg-AMC.HCl while only one peak was capable of hydrolysing Z-Arg-Arg-AMC. The proteinase activity capable of hydrolysing Z-Phe-Arg-AMC.HCl represents a yield of 10.6 % i.e. the protein content of the fractions corresponding to an elution volume of 39-42 ml compared to the total protein loaded. Similarly, the proteinase activity capable of hydrolysing Z-Arg-Arg-AMC represents a yield of 11 % comparing the protein corresponding to an elution volume of 52.5-55.5 ml to the total protein loaded.

4.3.1.2 ADULT ES PRODUCTS

Figure 4.2a shows the protein profile and proteolytic activity against FITC casein obtained when *N.americanus* adult ES products were applied to an activated thiol-Sepharose column. Again, two peaks of proteolytic activity were eluted by 25 mM glutathione but these were very small. Both these peaks of proteolytic activity were capable of hydrolysing Z-Phe-Arg-AMC while only the later eluting peak only hydrolysed Z-Arg-Arg-AMC (Figure 4.2b). The proteinase activity capable of hydrolysing Z-Phe-Arg-AMC.HCl represents a yield of 2.2% i.e. the protein content of the fractions corresponding to an elution volume of 42-43.5 ml compared to the total protein loaded. Similarly, the proteinase activity capable of hydrolysing Z-Arg-Arg-AMC represents a yield of 2.1 % comparing the protein corresponding to an elution volume of 52.5 ml to the total protein loaded.

4.3.1.3 CHARACTERISATION OF THE PURIFIED CYSTEINYL PROTEINASES

All the proteolytic activities eluted from the activated thiol-Sepharose column by 25 mM glutathione were analysed for their inhibitor sensitivity, pH optima, metal dependency and purity by SDS-PAGE. Table 4.1 shows the characterisation of the purified proteinases. Both proteinases purified from larval ES products had a pH optimum of 7 while the proteinases purified from adult ES products both had pH optima of 6.5. All the purified proteases were completely inhibited by 1 μM E64. Enhancement by 5 mM cysteine only occurred with the cathepsin L 'like' activities from larval and adult ES products. This would seem to identify the proteinases primarily as cysteinyl in nature and indicate that both cathepsin L and B 'like' activities have been eluted from the thiol-Sepharose column. Inhibition was also seen with 1, 10-phenanthroline against all the purified proteinases. This may be a reflection of the metal ion enhancement seen in figures 4.6 and 4.7, proteinase activity being inhibited due to the chelating activity of 1, 10-phenanthroline. The larval cysteinyl proteinases were also inhibited by APMSF. APMSF has been shown to have the ability to inhibit some cysteinyl proteinase activities (Dr L McDonald) and this may reflect yet another difference between the adult and larval enzymes.

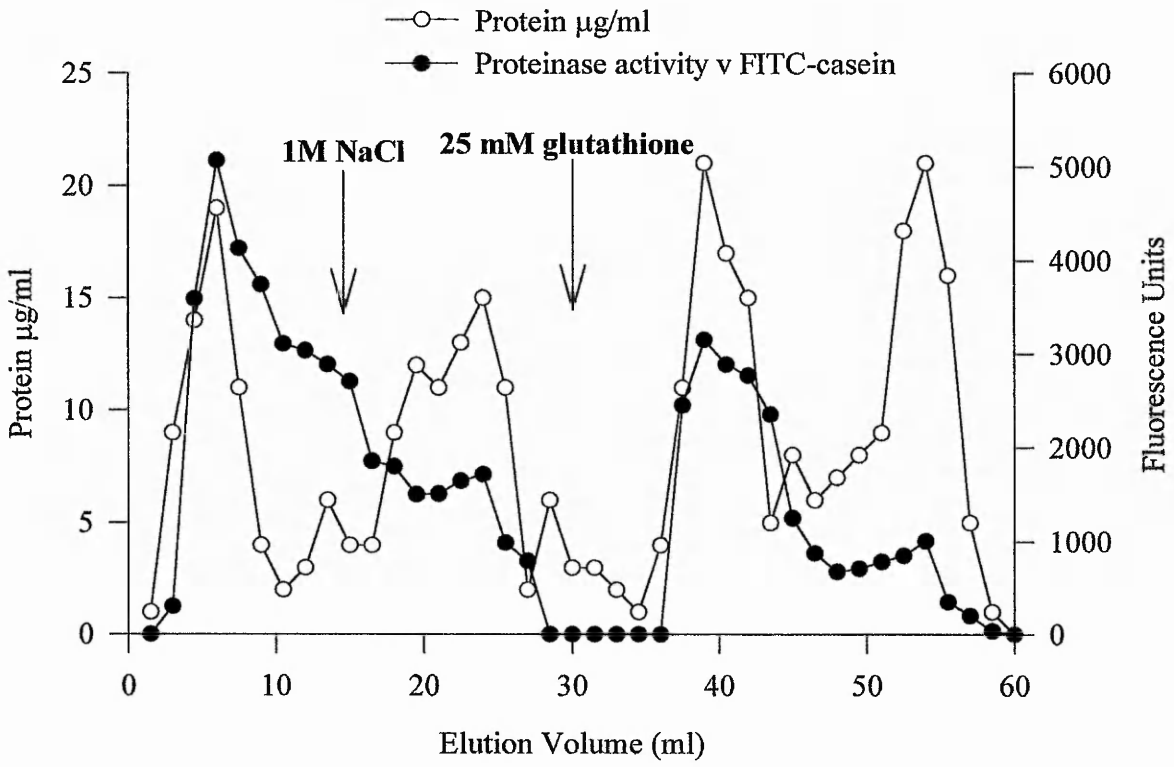

Figure 4.1a

Purification of cysteinyl proteinases from *N. americanus* larval ES products.

A 5 ml activated thiol-Sepharose 4B column was equilibrated with 50 mM phosphate buffer pH 6.5. Approximately 0.5 mg of larval ES products were resuspended in 50 mM phosphate buffer pH 6.5 and applied to the column. Unbound protein was eluted using sequential washes of 50 mM phosphate buffer pH 6.5 (flow rate 0.2 ml/min) and 50 mM phosphate buffer pH 6.5 plus 1 M NaCl. Finally, bound protein was eluted using 50 mM phosphate buffer pH 6.5 plus 25 mM reduced glutathione. Arrows indicate points of buffer changes. Fractions eluting from the column were assayed for protein content (Section 2.3) and proteolytic activity at pH 6.5 using FITC-casein (Section 2.4.2.2).

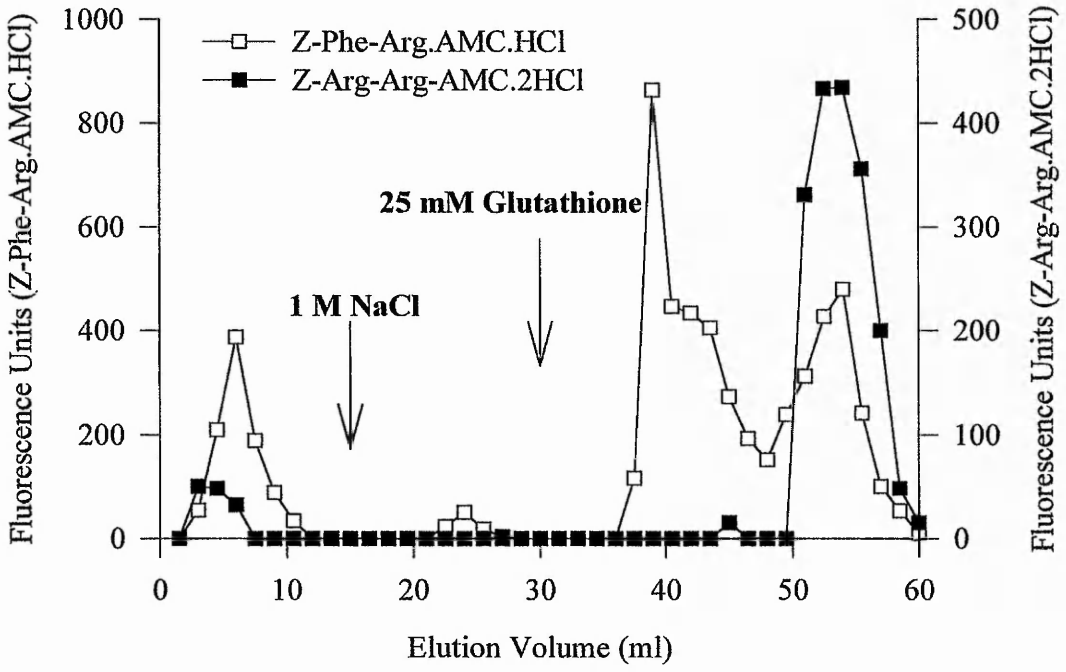

Figure 4.1b

Analysis of the proteinase activity eluting from activated thiol-Sepharose following the application of *N. americanus* larval ES products using the substrates Z-Phe-Arg-AMC.HCl and Z-Arg-Arg-AMC.2HCl

Fractions eluting from Activated thiol-Sepharose (Figure 4.1a) were further characterised using the fluorogenic substrates Z-Phe-Arg-AMC.HCl and Z-Arg-Arg-AMC.2HCl (Section 2.4.3). All assays were carried out at pH 6.5.

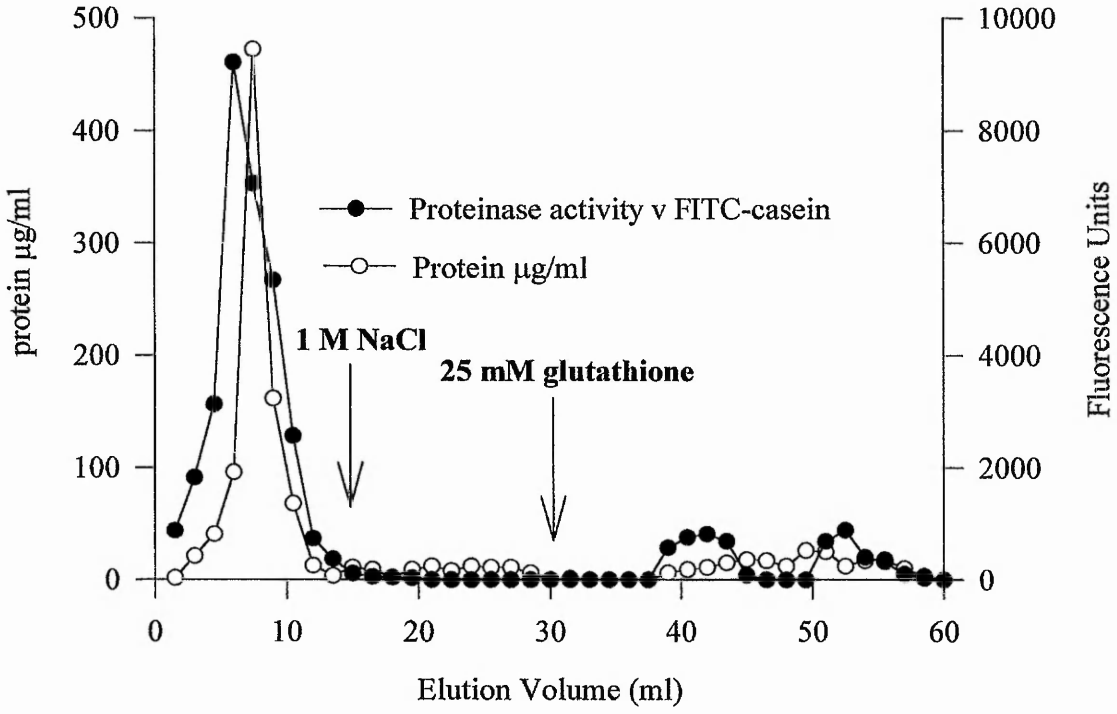

Figure 4.2a

Purification of cysteinyl proteinases from *N. americanus* adult ES products.

A 5 ml activated thiol-Sepharose 4B column was equilibrated with 50 mM phosphate buffer pH 6.5. Approximately 1.16 mg of adult ES products were resuspended in 50 mM phosphate buffer pH 6.5 and applied to the column. Unbound protein was eluted using sequential washes of 50 mM phosphate buffer pH 6.5 (flow rate 0.2 ml/min) and 50 mM phosphate buffer pH 6.5 plus 1 M NaCl. Finally, bound protein was eluted using 100 mM phosphate buffer pH 6.5 plus 25 mM reduced glutathione. Arrows indicate points of buffer changes. Fractions eluting from the column were assayed for protein content (Section 2.3) and proteinase activity at pH 6.5 using FITC-casein (Section 2.4.2.2).

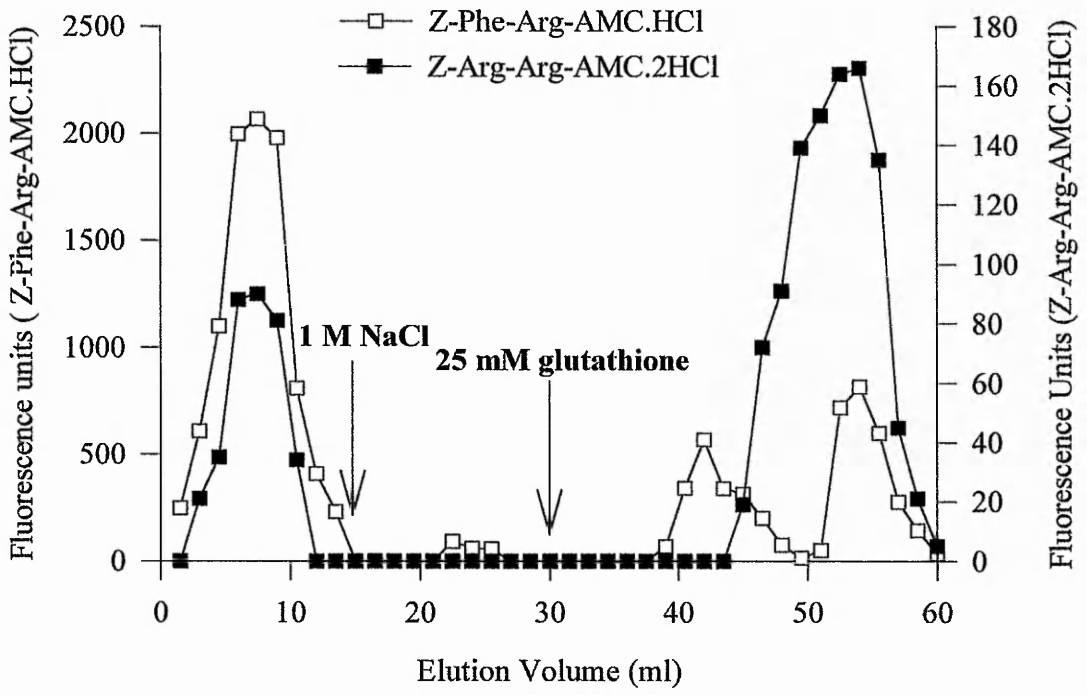

Figure 4.2b

Analysis of the proteinase activity eluting from activated thiol-Sepharose following the application of *N. americanus* adult ES products using the substrates Z-Phe-Arg-AMC.HCl and Z-Arg-Arg-AMC.2HCl

Fractions eluting from Activated thiol-Sepharose (Figure 4.1a) were further characterised using the fluorogenic substrates Z-Phe-Arg-AMC.HCl and Z-Arg-Arg-AMC.2HCl (Section 2.4.3). All assays were carried out at pH 6.5.

Table 4.1

The pH optima and inhibitor profile of the cysteinyl proteinases purified from larval and adult ES products by affinity chromatography on activated thiol-Sepharose 4B

Proteinase	pH optima	5mM cysteine	1 µM E64	50µM APMSF	1mM 1, 10-phenanthroline	1µM pepstatin A
Larval cathepsin L-'like' Z-Phe-Arg-AMC.HCl	**7**	**+96**	**-100**	**-28**	**-98**	**0**
Larval cathepsin B-'like' Z-Arg-Arg-AMC.2HCl	**7**	**0**	**-100**	**-33**	**-60**	**-15.6**
Adult cathepsin L 'like' Z-Phe-Arg-AMC.HCl	**6.5**	**+41.1**	**-100**	**+1.1**	**-54**	**-48**
Adult cathepsin B 'like' Z-Arg-Arg-AMC.2HCl	**6.5**	**0**	**-100**	**+5**	**-31**	**-13**

The proteinases eluting from an activated thiol-Sepharose 4B column were assessed for their pH optima and inhibitor sensitivity using the substrate Z-Phe-Arg-AMC.HCl to analyse the cathepsin L 'like' activity and Z-Arg-Arg-AMC.2HCl for the cathepsin B 'like' activity. The results are expressed as the percentage inhibition (-) or enhancement (+) of the individual proteinases compared with an uninhibited control.

4.3.1.4 SDS-PAGE AND WESTERN BLOT ANALYSIS

All the purified proteinases from both adult and larval ES products resolved on SDS-PAGE as a protein doublet with an estimated molecular masses of approximately 66 and 55 kDa (Figures 4.3a and 4.3b). All the purified proteinases were also shown to be immunogenic as assessed by Western blots probed with pooled human post-infection sera (Figures 4.4a and 4.4b).

When Western blots of the purified proteinases from adult and larval ES products were probed with rabbit antiserum to *N. americanus* glutathione-S-transferase only the 66 kDa band was recognised (Figure 4.5). This may imply that the cysteinyl proteinase activities eluting from the activated thiol-Sepharose column are associated with the 55 kDa protein.

4.3.1.5 THE EFFECT OF METAL IONS ON CYSTEINYL PROTEINASE ACTIVITY

Figures 4.6 to 4.7 show the effects of a number of different metal ions on the activities of the cysteinyl proteinases purified from larval and adult *N. americanus* ES products. All assays were carried out using the substrate FITC-casein. It was not possible to use fluorogenic substrates as the presence of Fe^{2+} and Fe^{3+} ions quenched the fluorescence emitted by these substrates. The larval cysteinyl proteinases exhibited different inhibition/enhancement profiles. The cathepsin B 'like' proteinase was enhanced in the presence of Zn^{2+}, Mg^{2+}, Fe^{2+} and Fe^{3+} ions but inhibited by Ca^{2+} ions (Figure 4.6). The cathepsin L 'like' proteinase (Figure 4.6) was enhanced by the presence of Fe^{2+} and Fe^{3+} ions, and no significant inhibition/enhancement was seen with Zn^{2+}, Mg^{2+} or Ca^{2+} ions.

The adult cathepsin B 'like' proteinase was enhanced makedly by $Fe^{2+}$and Fe^{3+} ions with some inhibition of the cathepsin B 'like activity by Zn^{2+}. Mg^{2+} and Ca^{2+} ions (Figure 4.7). The adult cathepsin L 'like' proteinase was only significantly enhanced by Fe^{2+} and Fe^{3+} ions (Figure 4.7).

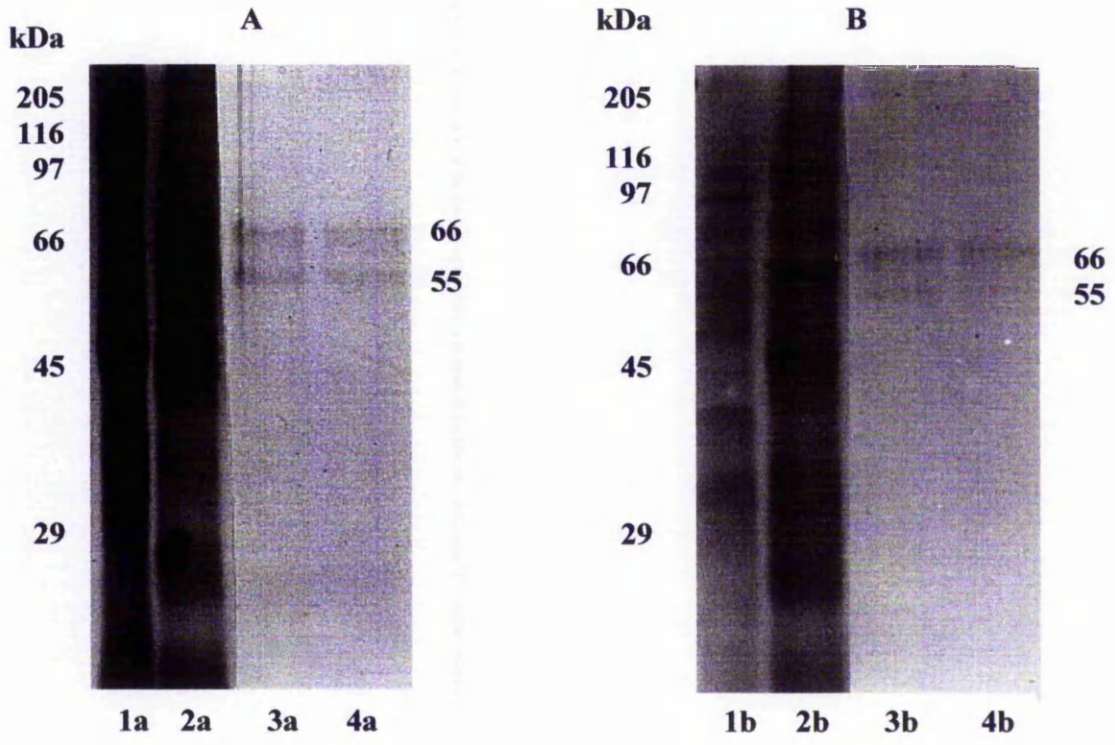

Figure 4.3

The SDS-PAGE profile of the cysteinyl proteinases purified from *N.americanus* larval (A)and adult (B) ES products using activated thiol-Sepharose 4B.

100 µl of the peak fraction from each column wash buffer indicated was concentrated by acetone precipitation (Section A.2.3.11) and resolved by SDS-PAGE under reducing conditions.

Lane 1a) Larval ES products, Lane 2a) 50 mM phosphate buffer pH 6.5 wash, Lane 3a) 25 mM glutathione elution, cathepsin L 'like' proteinase. Lane 4a) 25 mM glutathione elution, cathepsin B 'like' proteinase. Lane 1b) Adult ES products, Lane 2b) 50 mM phosphate buffer pH 6.5 wash, Lane 3b) 25 mM glutathione elution, cathepsin L 'like' proteinase. Lane 4b) 25 mM glutathione elution, cathepsin B 'like' proteinase. Gels were stained using either the BioRad silver stain (larval ES), according to the manufacturers instructions or Coomassie Brilliant Blue R250 (Section A.2.3.12). Molecular mass markers are indicated.

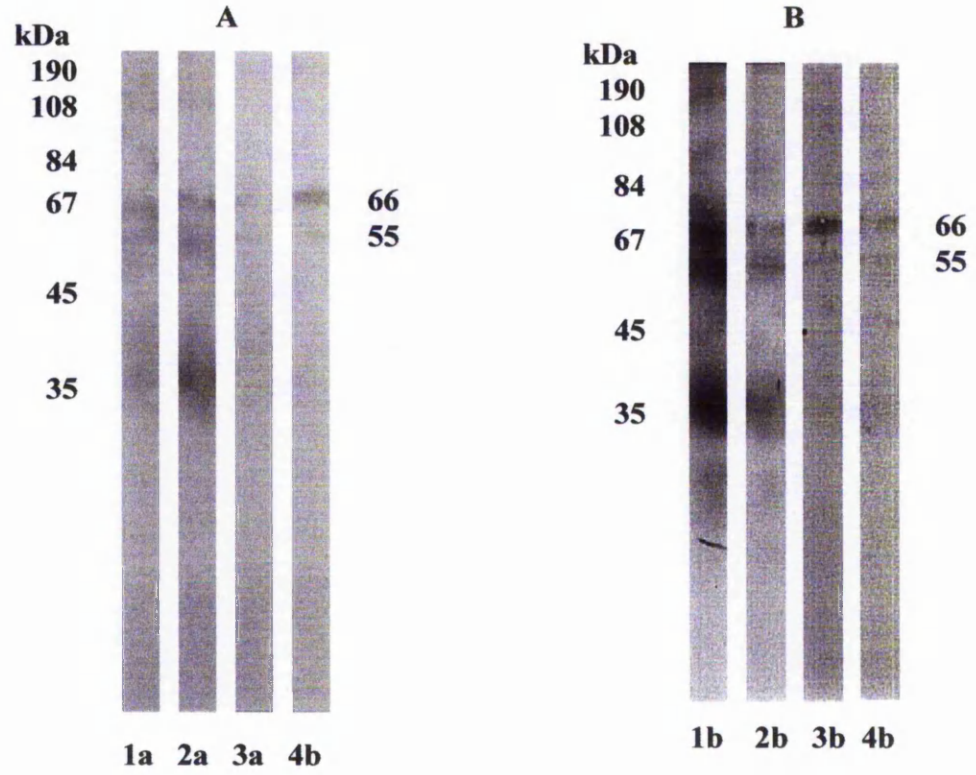

Figure 4.4

The immunogenicity of the cysteinyl proteinases purified from larval (A) and adult (B) ES products using activated thiol-Sepharose.

100 µl of the peak fraction from each column wash buffer indicated was concentrated by acetone precipitation (Section A.2.3.11), resolved by SDS-PAGE under reducing conditions and transferred onto nitrocellulose (Section 2.6). The Western blots were probed with pooled human post *N.americanus* infection sera as described in section 4.2.5.

Lane 1a) Larval ES products, Lane 2a) 50 mM phosphate buffer pH 6.5 wash, Lane 3a) 25 mM glutathione elution, cathepsin L 'like' proteinase. Lane 4a) 25 mM glutathione elution, cathepsin B 'like' proteinase. Lane 1b) Adult ES products, Lane 2b) 50 mM phosphate buffer pH 6.5 wash, Lane 3b) 25 mM glutathione elution, cathepsin L 'like' proteinase, Lane 4b) 25 mM glutathione elution, cathepsin B 'like' proteinase. Molecular mass markers are indicated.

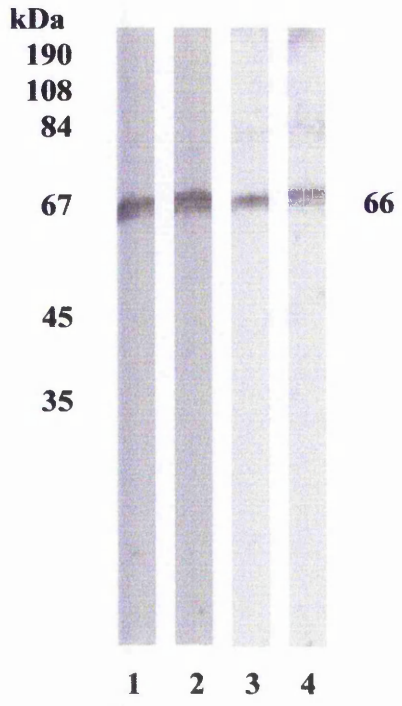

Figure 4.5

Recognition of a 66 kDa glutathione binding protein by rabbit anti *N.americanus* glutathione-S-transferase serum.

100 µl of the cathepsin L and B 'like' proteinases purified from larval and adult ES products using activated thiol-Sepharose 4B was concentrated by acetone precipitation (Section A.2.3.11), resolved by SDS-PAGE under reducing conditions and transferred onto nitrocellulose (Section 2.6). The Western blots were probed with rabbit anti *N.americanus* glutathione-S-transferase serum as described in section 4.2.5.

Lane 1) Larval cathepsin L 'like' proteinase, Lane 2) Larval cathepsin B 'like' proteinase, Lane 3) Adult cathepsin L 'like' proteinase, Lane 4) Adult cathepsin B 'like' proteinase. Molecular mass markers are indicated.

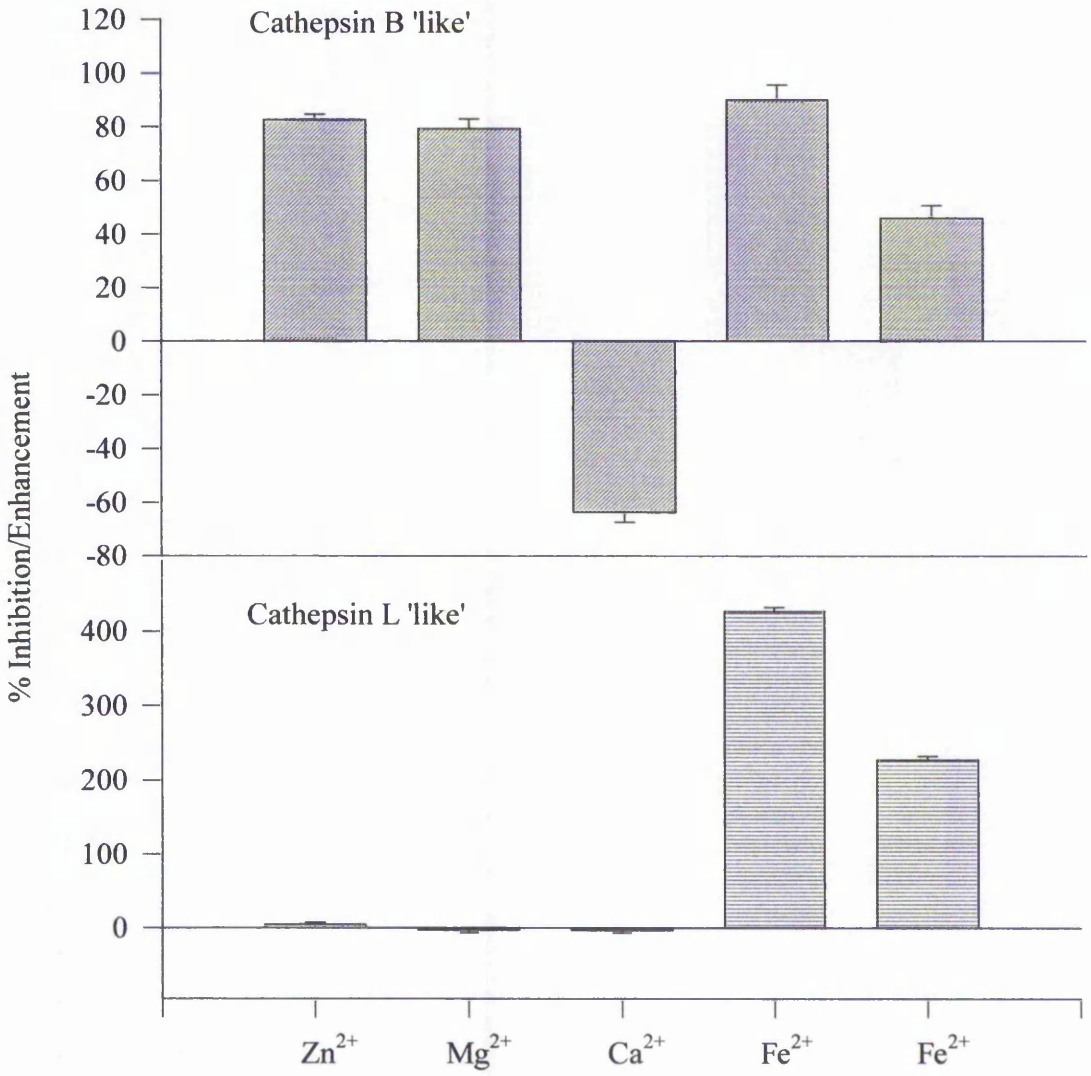

Figure 4.6

The effects of metal ions on the cathepsin B and L 'like' activities purified from larval ES products

The effects of a number of metal ions at a concentration of 1mM on the proteinase activity of the purified larval cathepsin B and L 'like' proteinases were studied using the substrate FITC-casein at pH 7. The results are expressed as the percentage inhibition/enhancement of proteinase activity by each metal ion compared to a control following the subtraction of a non enzymatic blank.

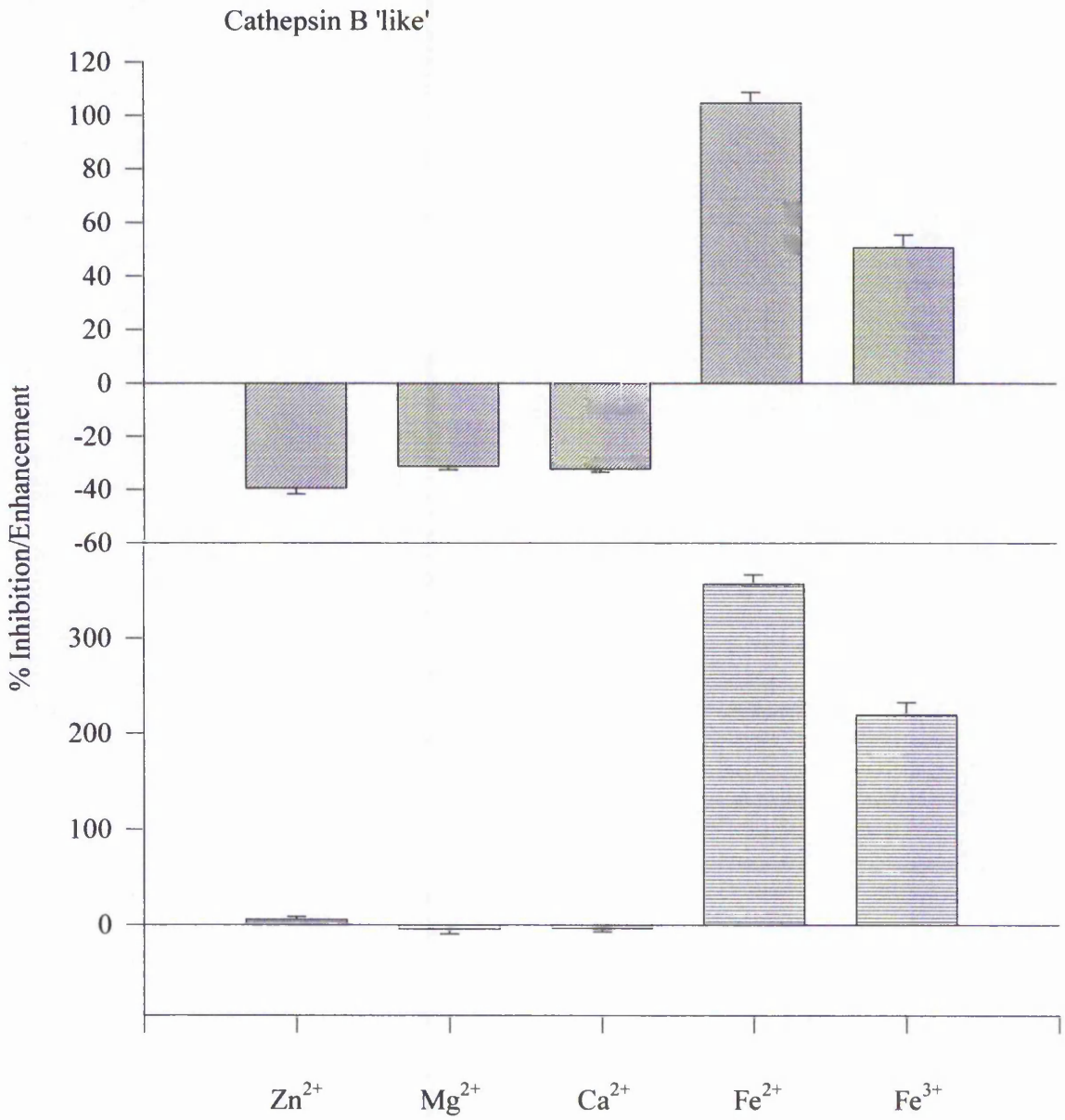

Figure 4.7

The effects of metal ions on the cathepsin B and L 'like' activities purified from adult ES products

The effects of a number of metal ions at a concentration of 1mM on the proteinase activity of the purified adult cathepsin B and L 'like' proteinases were studied using the substrate FITC-casein at pH 7. The results are expressed as the percentage inhibition/enhancement of proteinase activity by each metal ion compared to a control following the subtraction of a non enzymatic blank.

4.3.2 PURIFICATION OF ASPARTYL PROTEINASES FROM LARVAL AND ADULT ES PRODUCTS

Aspartyl proteinases were purified using Pepstatin A-agarose as described in section 4.2.2. Figure 4.8 shows the elution profile and proteolytic activity against FITC-casein following the application of larval ES products to the column. Most of the proteolytic activity present in larval ES products bound loosely to the column and was removed by washing with 50 mM sodium acetate, 0.5 M NaCl pH 5.5. Specific elution using 500 μM pepstatin A resulted in a further peak of proteolytic activity representing a yield of 7 % (protein content corresponding to an elution volume of 29-30 ml compared to the total protein loaded). A similar profile was observed for adult ES products (Figure 4.9) except that more proteolytic activity was washed from the column by 50 mM sodium acetate alone. The protein eluted from the column following the application of adult ES products represents an approximate yield of 1.7 % (protein content corresponding to an elution volume of 32 ml compared to the total protein loaded). Fractions eluted by from the column were analysed for protein content (Section 2.3) proteinase activity using FITC-casein (Section 2.4.2.2) and purity by SDS-PAGE (Section 2.5).

4.3.2.1 CHARACTERISATION OF THE PURIFIED ASPARTYL PROTEINASES

The proteinases eluted by 500 μM pepstatin A from a pepstatin A-agarose column following the application of larval and adult ES products were tested for their pH optima and inhibitor sensitivity using FITC-casein as the substrate. The larval and adult aspartyl proteinases were both optimally active at pH 6.5 (Figure 4.10). The adult aspartyl proteinase also demonstrated a further peak smaller peak of activity at pH 4. The larval and adult aspartyl proteinases were tested for their inhibitor sensitivity at pH 6.5. The results are shown in Table 4.2. Both the larval and adult proteinases were primarily inhibited by pepstatin A, confirming the activities eluting from the column to be aspartyl in nature.

4.3.2.2 SDS-PAGE AND WESTERN BLOT ANALYSIS

The purified enzymes from adult and larval ES products again resolved on SDS-PAGE as a predominant band of approximately 66 kDa (Figures 4.11) although and fainter band of

approximately 55 kDa was also observed. Both the larval and adult purified proteinases were shown to be immunogenic during infection when Western blots of the purified proteinases were probed with pooled human post *N. americanus* infection sera (Figure 4.12).

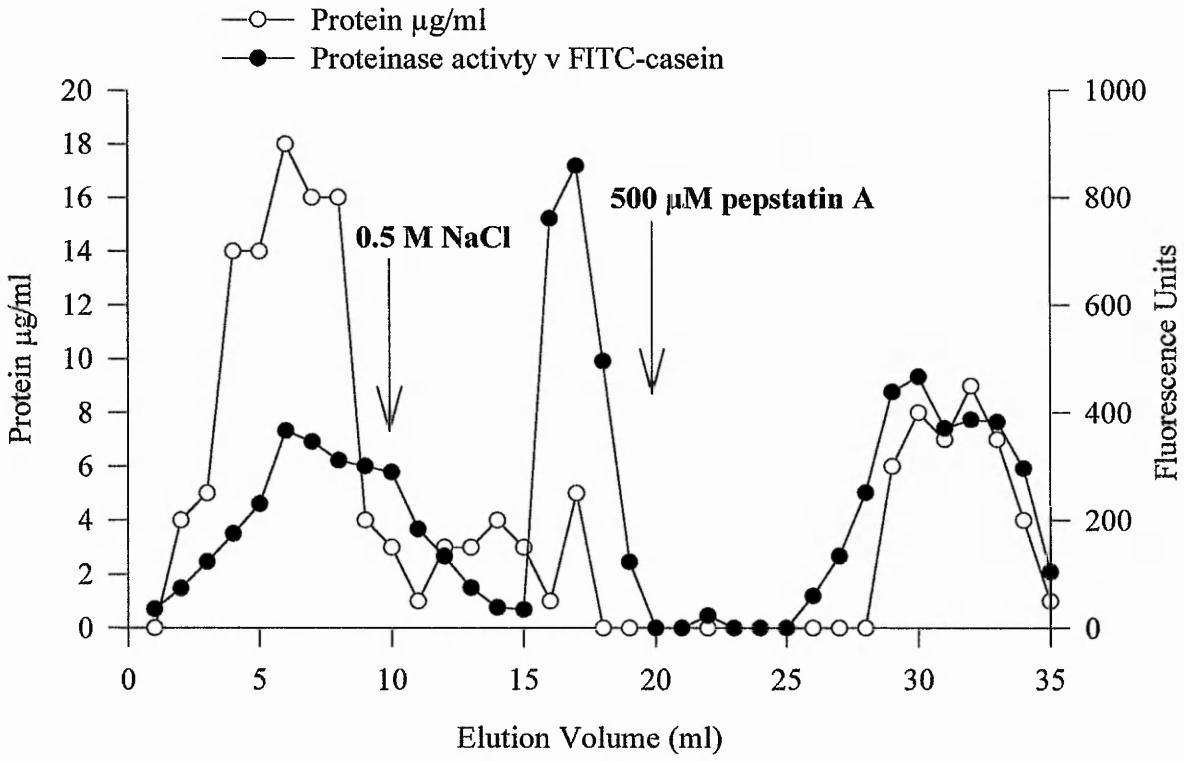

Figure 4.8

The purification of an aspartyl proteinase from *N. americanus* larval ES products using pepstatin A agarose.

A 5 ml pepstatin A agarose column was equilibrated with 50 mM sodium acetate pH 5.5. Approximately 200 µg of larval ES products were resuspended in 50 mM sodium acetate pH 5.5 and applied to the column. The column was washed sequentially with 50 mM sodium acetate pH 5.5 (flow rate of 0.2 ml/min) followed by 50 mM sodium acetate, 0.5 M sodium chloride pH 5.5. Bound protein was eluted from the column with 500 µM pepstatin A. Arrows indicate the point of buffer change. Fractions eluting from the column were analysed for protein content and proteinase activity at pH 6.5 using FITC-casein.

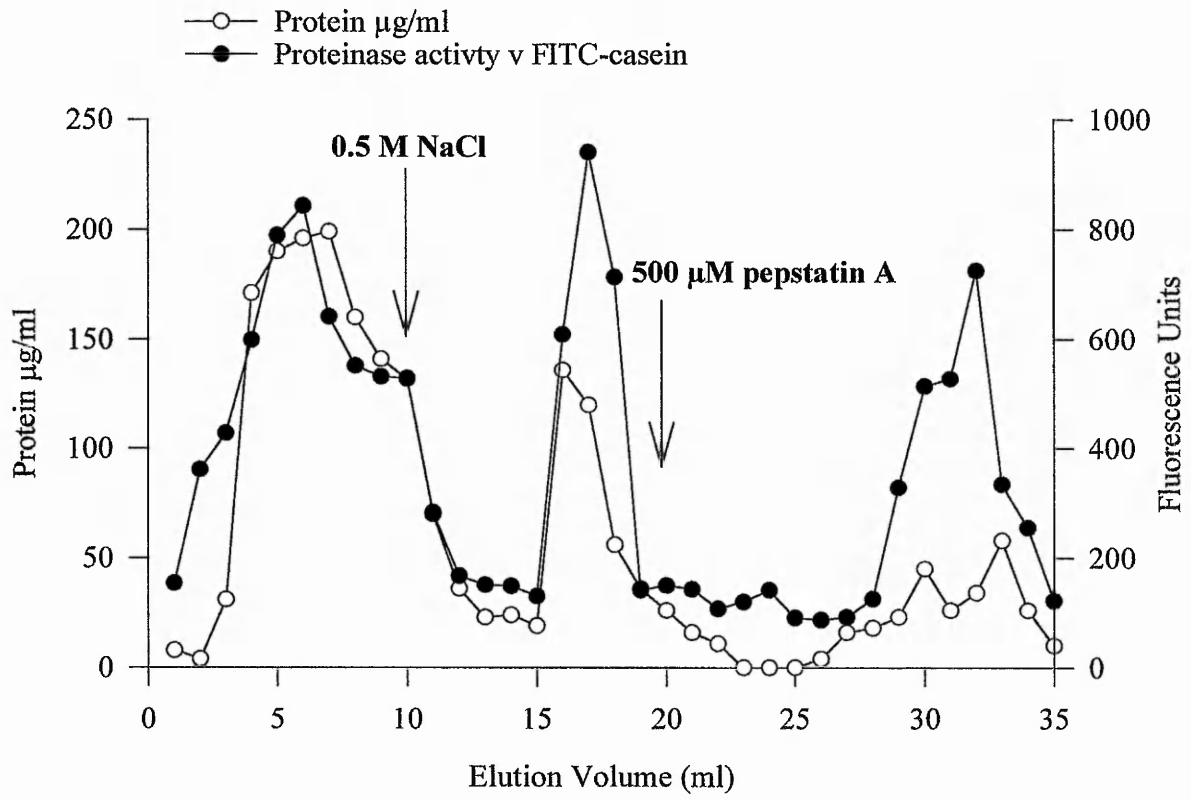

Figure 4.9

The purification of an aspartyl proteinase from *N. americanus* adult ES products using pepstatin A agarose.

A 5 ml pepstatin A agarose column was equilibrated with 50 mM sodium acetate pH 5.5. Approximately 2 mg of adult ES products were resuspended in 50 mM sodium acetate pH 5.5 and applied to the column. The column was washed sequentially with 50 mM sodium acetate pH 5.5 (flow rate of 0.2 ml/min) followed by 50 mM sodium acetate, 0.5 M sodium chloride pH 5.5. Bound protein was eluted from the column with 500 µM pepstatin A. Arrows indicate the point of buffer change. Fractions eluting from the column were analysed for protein content and proteinase activity at pH 6.5 using FITC-casein.

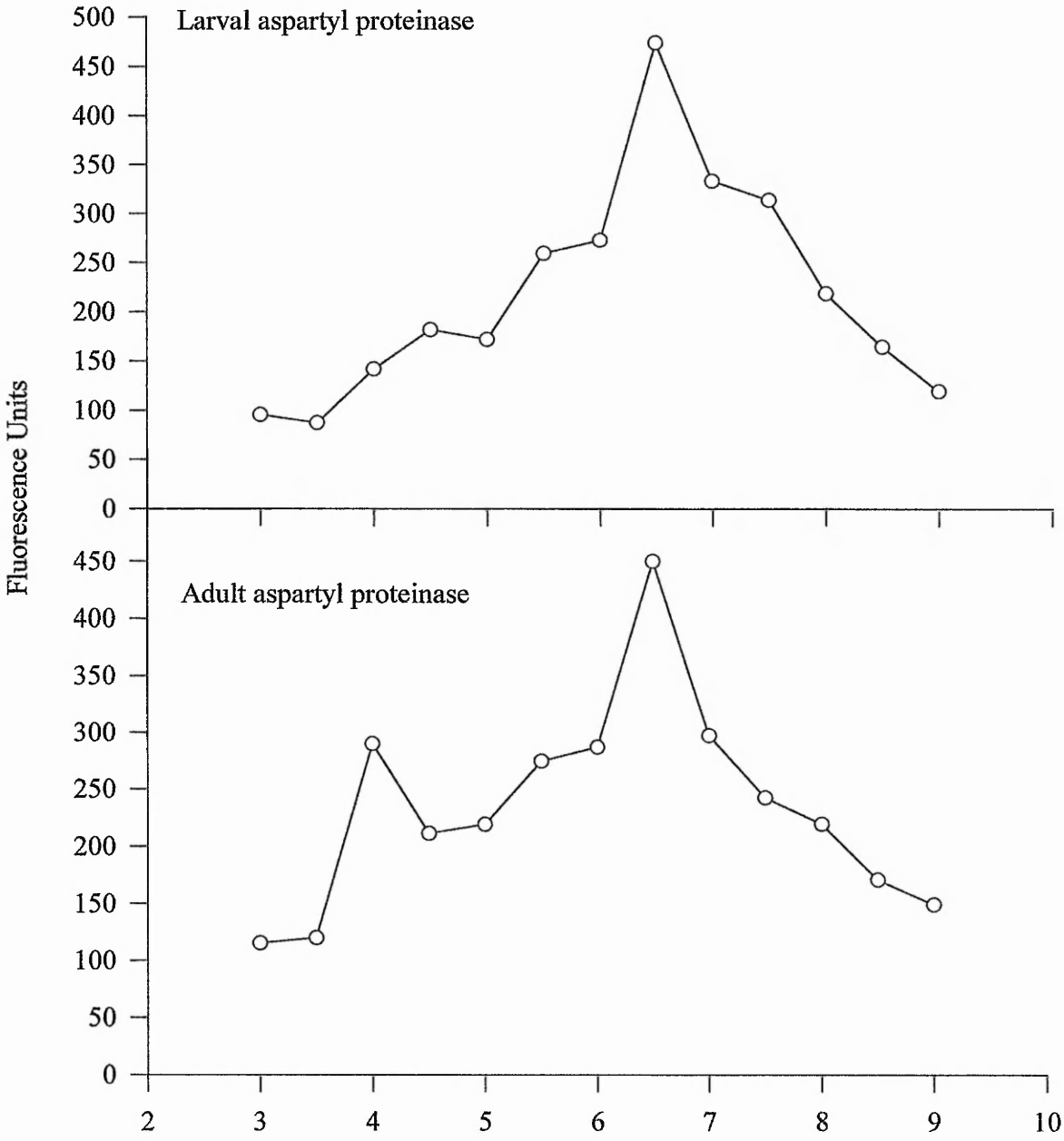

Figure 4.10

The pH optima of the aspartyl proteinases purified from *N. americanus* larval and adult ES products

The proteinase activity eluted by 500 μM pepstatin A from a pepstatin A-agarose column following the application of *N. americanus* larval and adult ES products was assessed for its pH optimum using the substrate FITC-casein. Proteinase activity is expressed as the number of fluorescence units released after 1 h incubation at 37 °C.

Table 4.2

Characterisation of the proteinase activity eluted by 500 μM pepstatin A from a pepstatin A-agarose column following the application of larval and adult ES products

	Larval ES products pH 6.5	Adult ES products pH 6.5
Inhibitor	% Inhibition/Enhancement	% Inhibition/Enhancement
5 mM cysteine	0	0
1 μM E64	+17.9	+6.4
50 μM APMSF	-34.6	-17.7
1 mM 1, 10-phenanthroline	-23.6	-26.7
1 μM pepstatin A	-95.8	-74.0

The proteinase activity eluted by 500 μM pepstatin A from a Pepstatin A-agarose column was tested for inhibitor sensitivity using the substrate FITC-casein. Results are expressed as the percentage inhibition (-) or enhancement (+) of activity compared to an uninhibited control following the subtraction of a non enzymatic blank.

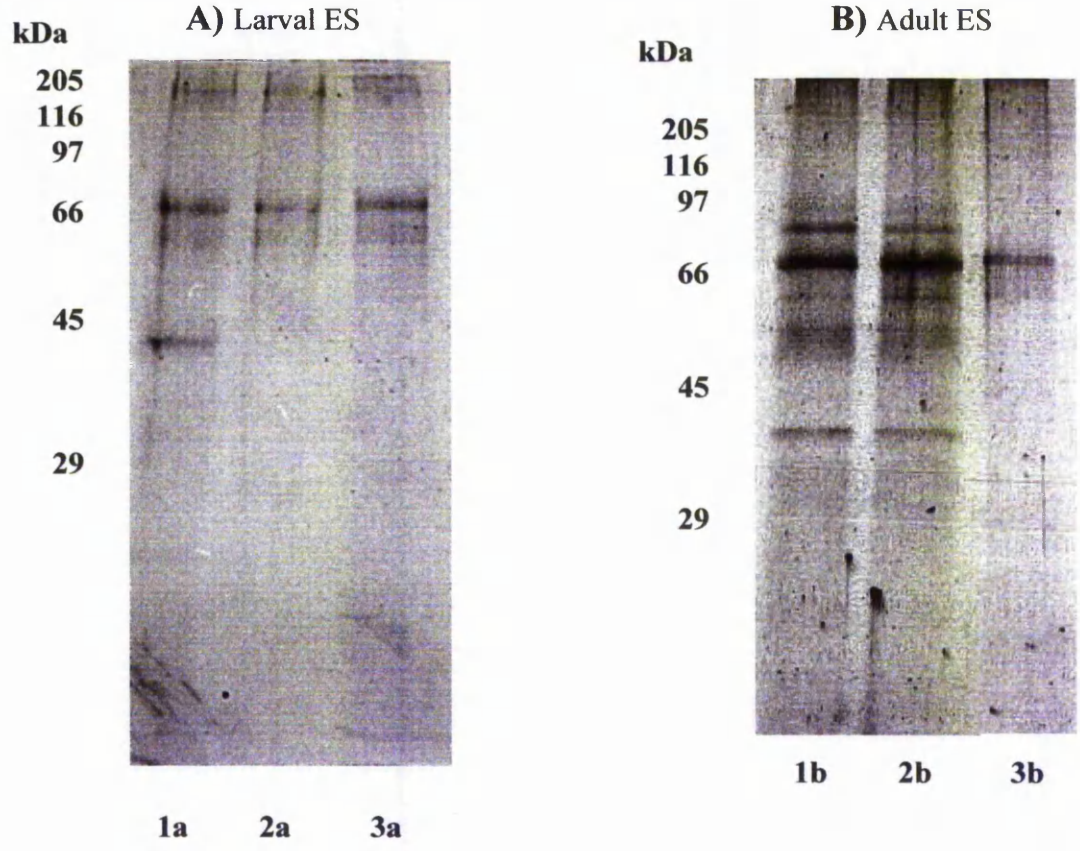

Figure 4.11

The SDS-PAGE profile of the aspartyl proteinases purified from larval ES products using pepstatin A-agarose.

100 µl of the peak fraction from each column wash buffer indicated was concentrated by acetone precipitation (Section A.2.3.11) and resolved by SDS-PAGE under reducing conditions.

Lane1a) Larval ES products, Lane 2a) 50 mM sodium acetate pH 5.5 wash, Lane 3a) 500 µM pepstatin A elution.

Lane1b) Adult ES products, Lane 2b) 50 mM sodium acetate pH 5.5 wash, Lane 3b) 500 µM pepstatin A elution.

Proteins were stained with colloidal Coomassie blue G250. Molecular mass markers are indicated.

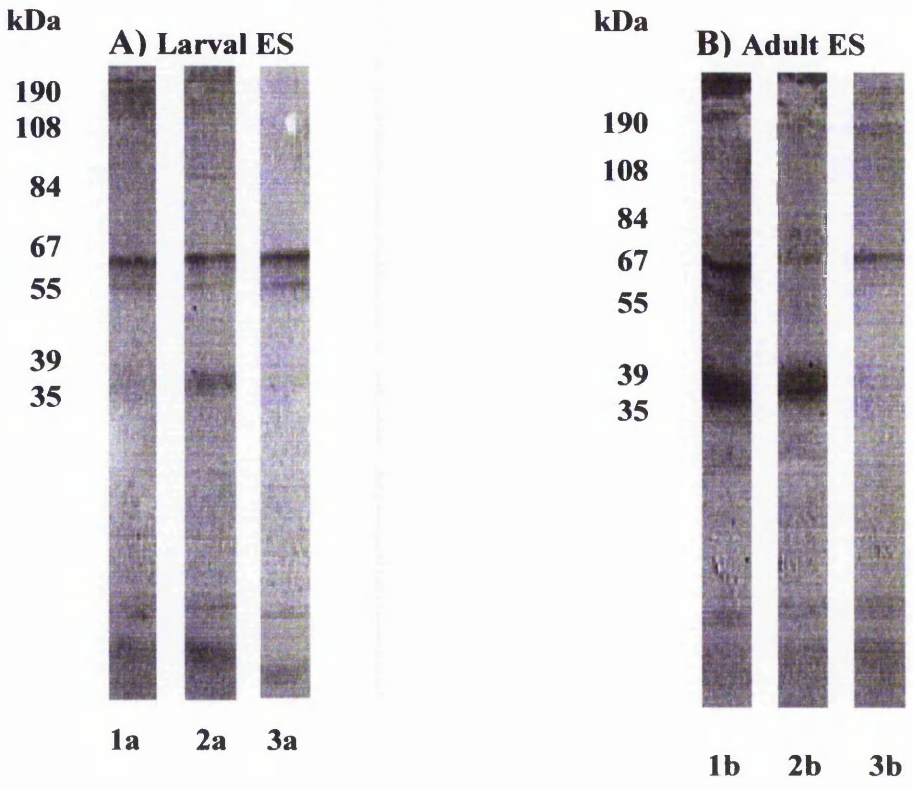

Figure 4.12

The immunogenicity of the aspartyl proteinases purified from larval and adult ES products using pepstatin A-agarose.

100 µl of the peak fraction from each column wash buffer indicated was concentrated by acetone precipitation (Section A.2.3.11), resolved by SDS-PAGE under reducing conditions and transferred onto nitrocellulose (Section 2.6). The Western blots were probed with pooled human post *N.americanus* infection sera as described in section 4.2.5.

Lane1a) Larval ES products, Lane 2a) 50 mM sodium acetate pH 5.5 wash, Lane 3a) 500 µM pepstatin A elution.

Lane1b) Adult ES products, Lane 2b) 50 mM sodium acetate pH 5.5 wash, Lane 3b) 500 µM pepstatin A elution.

Molecular mass markers are indicated.

4.3.3 PURIFICATION OF METALLO-PROTEINASES

4.3.3.1 BACITRACIN-SEPHAROSE

Bacitracin-Sepharose has previously been used to purify elastinolytic metallo-proteases (Markaryan *et al.*, 1994). Fractions obtained from this column were tested for elastase activity using the substrate elastin orcein. Figure 4.13 shows the elastase activity and protein profile eluted when larval ES products were applied to the bacitracin-Sepharose column. Elastase degrading activity was seen to elute with each change of elution buffer. The fractions containing the highest elastase activity associated with each change of buffer were analysed for inhibitor sensitivity (Table 4.3) and for purity by SDS-PAGE. Only the activity eluted by buffers 1 and 2 contained proteolytic activity inhibitable by 1, 10-phenanthroline indicating metallo-proteinase activity to be present in these fractions. However, the proteolytic activity eluted by buffer 1 also contained activity inhibitable by pepstatin A (Table 4.3). The fractions containing the peak of metallo-proteinase activity eluted by buffer 2 (fractions 14 and 15, [yield uncalculatable due to low amounts of protein]) were analysed by SDS-PAGE followed by staining with Coomassie brilliant blue R250. This resulted in a band of 42 kDa being observed (Figure 4.14). However, when the same gel was silver stained a number of contaminating bands were also seen (data not shown).

4.3.3.2 N-BENZYLOXYCARBONYLGLYCYL-D-LEUCYLAMINOHEXYL-SEPHAROSE

Figure 4.15 shows the protein profile and the proteinase activity eluting from a N-benzyloxycarbonylglycyl-D-leucylaminohexyl-Sepharose column following the application of larval ES products. Bound proteinase activity was eluted from the column by 0.1 M sodium acetate pH 4.1 plus 2 M urea. The proteinase activity eluted from the column by sodium acetate pH 4.1 plus 2 M urea (yield 13.9 %) was analysed for inhibitor sensitivity (Table 4.4) and purity by SDS-PAGE (Figure 4.16). Proteinase activity was completely inhibited by 10 mM EDTA indicating that a metallo proteinase is indeed eluting from the column. However, proteinase activity was not inhibited by 1, 10-phenanthroline. When analysed by SDS-PAGE followed by staining with Colloidal Coomassie Brilliant blue G250 a 42 kDa protein was observed (Figure 4.16).

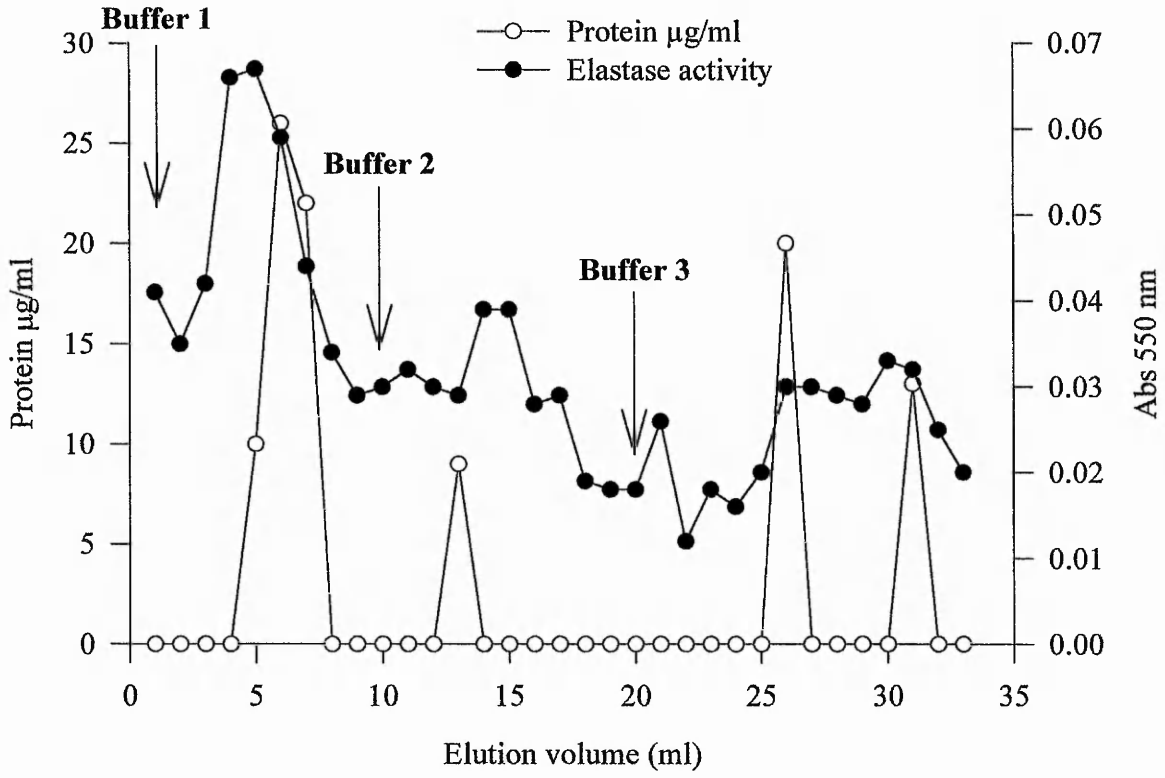

Figure 4.13

The purification of metallo-proteinase activity from larval ES products using bacitracin-Sepharose

5 ml of bacitracin-Sepharose was packed into a column and equilibrated with 50 mM Tris-HCl, pH 7.2. Approximately100 µg of larval ES products were resuspended in 50 mM Tris-HCl, pH 7.2 (buffer 1) and applied to the column at a flow rate of 0.2 ml/min. The column was washed sequentially with 50 mM Tris-HCl, pH 7.2 (buffer 1), 50 mM Tris-HCl, pH 7.2, 1 M NaCl, 1 mM $CaCl_2$, 7 % iso-propanol (buffer 2) and finally 50 mM Tris-HCl, pH 7.2, 1 M NaCl, 1 mM $CaCl_2$, 25 % iso-propanol (buffer 3). Arrows indicate the points of buffer change. 1 ml fractions were collected and analysed for protein content (Section 2.3), elastase activity (Section 3.2.1.1) and purity by SDS-PAGE (Section 2.5).

Table 4.3

Characterisation of the proteinase activities eluting from a bacitracin-Sepharose column following the application of larval ES products.

	Buffer 1	Buffer 2	Buffer 3
Inhibitor	% Inhibition	% Inhibition	% Inhibition
1 μM E 64	0	0	0
50 μM APMSF	0	0	25.5
1 mM 1, 10-phenanthroline	36.2	31.9	0
1 μM pepstatin A	33.1	0	34.5

Larval ES products were applied to a bacitracin-Sepharose as described in section 4.3.3.1. The peak proteolytic activity from each buffer wash was analysed for inhibitor sensitivity using elastin orcein as the substrate. The results are expressed as the percentage inhibition of proteolytic activity compared with an uninhibited control following the subtraction of a non enzymatic blank.

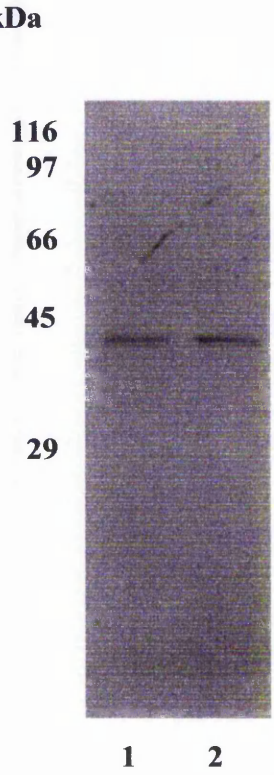

Figure 4.14

The SDS-PAGE profile of the peak fractions (14 & 15) eluted by buffer 2 from a bacitracin-Sepharose column following the application of larval ES products.

100 µl of fractions 14 and 15 were concentrated by acetone precipitation (Section A.2.3.11) and resolved by SDS-PAGE under reducing conditions.

Lane 1), Fraction 14, Lane 2), Fraction 15

Molecular mass markers are indicated.

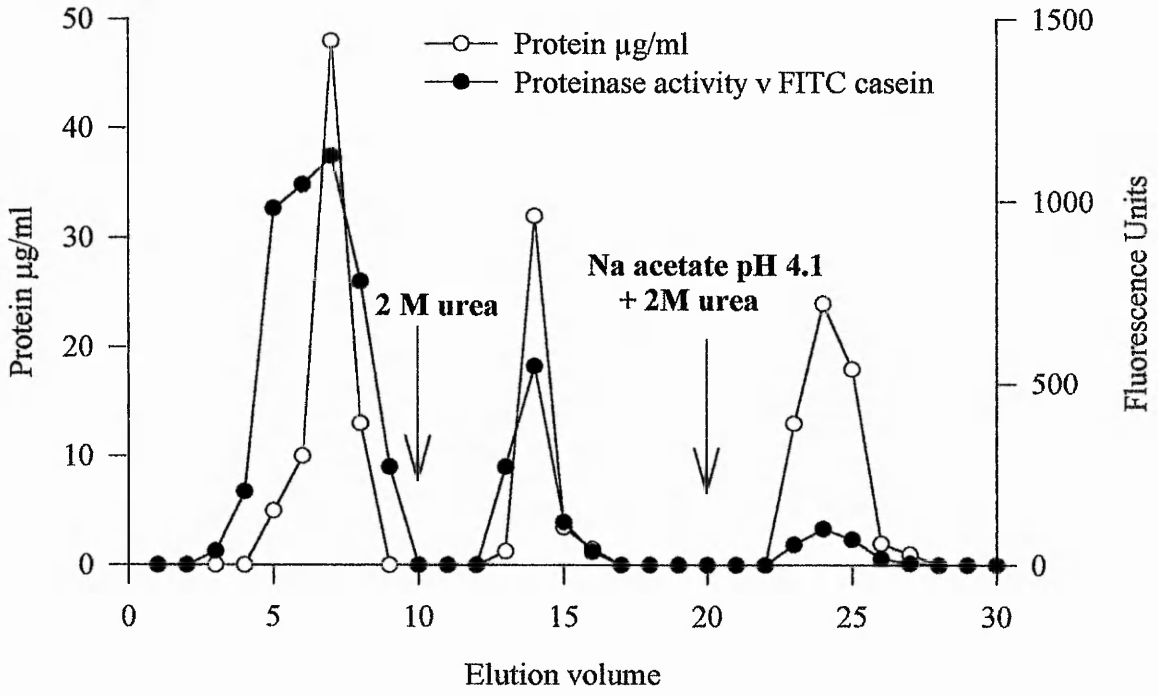

Figure 4.15

The purification of a metallo proteinase from larval ES products using N-benzyloxycarbonylglycyl-D-leucylaminohexyl-Sepharose

A 5 ml N-benzyloxycarbonylglycyl-D-leucylaminohexyl-Sepharose column was equilibrated with 50 mM phosphate buffer pH 7.0. Approximately 200 µg of larval ES products were resuspended in the same buffer and applied to the column (flow rate 0.2 ml/min). The column was washed with 50 mM phosphate buffer pH 7.0 followed by 50 mM phosphate buffer pH 5.6 plus 2 M urea. Finally, bound protein was eluted with 0.1 M sodium acetate pH 4.1 plus 2 M urea. 950 µl fractions were collected into 50 µl of 1 M NaOH to neutralise the acidity of the elution buffer. The arrows indicate the points of buffer change. Fractions eluting from the column were analysed for protein content (Section 2.3), proteinase activity using FITC-casein (Section 2.4.2.2) and purity by SDS-PAGE (Section 2.5).

Table 4.4

Characterisation of the proteinase activity eluted from N-benzyloxycarbonylglycyl-D-leucylaminohexyl-Sepharose column by sodium acetate pH 4.1 plus 2 M urea following the application of larval ES products.

Inhibitor	% Inhibition/Enhancement
1 μM E 64	-13.9
50 μM APMSF	-12.7
1 mM 1, 10-phenanthroline	-24
10 mM EDTA	-100
1 μM pepstatin A	-10.9

The proteinase activity eluting from a N-benzyloxycarbonylglycyl-D-leucylaminohexyl column was characterised with a number of protease inhibitors using FITC-casein as the substrate. Results are expressed as the percentage inhibition of proteinase activity compared with an uninhibited control following the subtraction of a non enzymatic blank.

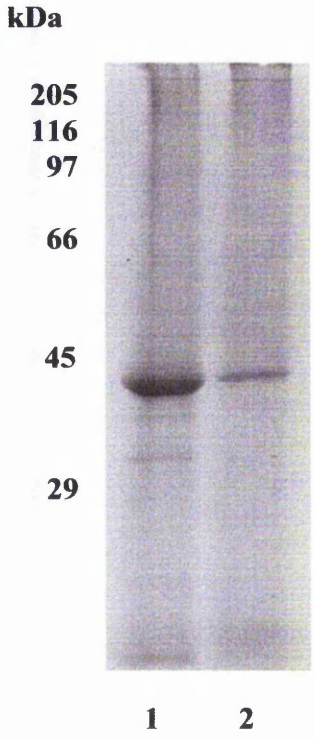

Figure 4.16

The SDS-PAGE profile of the proteinase eluting from a N-benzyloxycarbonylglycyl-D-leucylaminohexyl-Sepharose column.

10 μg of larval ES products and 100 μl of the protein peak eluted by 0.1 M sodium acetate, 2 M urea, pH 4.1 were concentrated by acetone precipitation (Section A.2.3.11) and resolved by SDS-PAGE under reducing conditions.

Lane 1) Larval ES products, Lane 2) Proteinase activity eluted by 0.1 M sodium acetate, 2 M urea, pH 4.1

Molecular mass markers are indicated.

4.3.4 PURIFICATION OF SERINE PROTEINASES

4.3.4.1 SOYBEAN TRYPSIN INHIBITOR-AGAROSE

When larval and adult ES products were applied to a soybean trypsin inhibitor-agarose column no protein binding was observed and all serine proteinase activity (as monitored by the substrate H-Pro-Phe-Arg-AMC.HCl) was washed off the column with 20 mM Tris, 0.5 M NaCl pH 7.5 (data not shown). As no proteinase activity bound to the column no further purifications were attempted using this matrix.

4.3.4.2 *p*-AMINOBENZAMIDINE-AGAROSE

Following the application of larval and adult ES products to a *p*-aminobenzamidine-agarose column, serine proteinase activity (as detected using the substrate Tosyl-Gly-Pro-Arg-AMC.HCl) was eluted by 70 mM sodium acetate, 0.5 M KCl pH 5.6. The fraction containing the peak of serine proteinase activity from this elution was analysed for inhibitor sensitivity using FITC casein as the substrate (Section 2.4.2.2) and purity by SDS-PAGE (Section 2.5). Proteinase activity eluted from the *p*-aminobenzamidine column was inhibited by all the inhibitors tested, except 1, 10-phenanthroline (data not shown). When the fractions were analysed by SDS-PAGE a large number of protein bands were observed, indicating that although the specific activity of the serine proteinase had been increased the method had failed to purify the enzyme to homogeneity.

4.4 DISCUSSION

This chapter describes a number of different attempts to purify proteinases from the (ES) products of the adult and larval stage of the human hookworm *Necator americanus*. Proteinases have been purified from other parasites using a variety of methods. For example, a combination of gel filtration and QAE-Sephadex chromatography has been used to purify a cathepsin L 'like' proteinase from adult *Fasciola hepatica* (Smith *et al.*, 1993b) and a neutral thiol proteinase has been purified from *Paragonimus westermani* cercariae using arginine-Sepharose, Ultrogel AcA-54 and DEAE-toyopearl (Yamakami & Hamajima, 1987). Similarly a metallo proteinase has been purified from *Ancylostoma caninum* using ion exchange chromatography, gel filtration and hydrophobic interaction chromatography (Hotez *et al.*, 1985). For *N. americanus* the greatest hindrance to the purification of proteins has always been the availability of very limited amounts of starting material. As a result of this, purification was attempted by a number of one step affinity chromatography protocols in a manner similar to that used to purify acetylcholinesterase (AChE) from adult *N. americanus* ES products (Pritchard *et al.*, 1991b).

The purification of the cysteinyl proteinase activity from larval and adult ES products was attempted using the affinity matrix activated thiol-Sepharose 4B (Figures 4.1a and 4.2a). Larval and adult ES products were applied to the column, and loosely bound protein was washed from the matrix by 50 mM phosphate buffer, pH 6.5, followed by a more stringent wash with 1 M sodium chloride in 50 mM phosphate buffer, pH 6.5. Bound protein could only be eluted by 25 mM glutathione in 50 mM phosphate buffer, pH 6.5, as the weaker reducing agents cysteine and dithiothreitol, commonly used to elute cysteinyl proteinases from this matrix, failed to remove bound protein from the column.

For both adult and larval ES products two peaks of proteolytic activity were eluted from the column by 25 mM glutathione. Both peaks were capable of hydrolysing FITC-casein and the fluorogenic cathepsin B and L substrate Z-Phe-Arg-AMC.HCl. However, only the second peak eluting from the column was capable of hydrolysing the cathepsin B substrate Z-Arg-Arg-AMC indicating that the proteolytic activity eluting in the first peak is probably due to a cathepsin L 'like' proteinase and the activity in the second peak a cathepsin B 'like' proteinase. The activity of both peaks was completely inhibited by E64, a specific

inhibitor of cysteinyl proteinases; however, in some cases inhibition was high with the metallo proteinase inhibitor 1, 10-phenanthroline. As all the purified proteinases exhibited different inhibition/enhancement profiles in the presence of a number of metal ions it is possible that the inhibition by 1, 10-phenanthroline reflects this. The activity of the cathepsin L 'like' proteinases was enhanced by the presence of cysteine but the activity of the cathepsin B 'like' proteinases was not enhanced, a property uncharacteristic of typical cathepsin B 'like' cysteine proteinases. The true significance of this data remains to be discovered; however, it could be possible that as cysteine was unable to elute proteinase activity from the column that activation of the cathepsin B 'like' enzyme also require a stronger reducing agent. This data however, does confirm the presence of cathepsin B-and L-'like' activities demonstrated previously during the initial characterisation of the proteinases present in adult ES products (Brown *et al.*, 1995).

The proteolytic activity of the purified adult cathepsin 'like' cysteinyl proteinases was enhanced only by the presence of Fe^{2+} and Fe^{3+} ions. The initial characterisation of the proteinases present in adult ES products, using tritiated haemoglobin as the substrate, demonstrated that proteinase activity in general was also enhanced by the presence of Ca^{2+} and Zn^{2+} ions. At the time it was unclear if the metal ions tested were enhancing cysteinyl proteinase activity or metallo proteinase activity. It would now appear that Fe^{2+} and Fe^{3+} ions enhance the activity of the adult cysteinyl proteinases whilst Ca^{2+} and Zn^{2+} ions (and possibly that Fe^{2+} and Fe^{3+}) enhance the metallo-proteinase activity in adult ES products althought this has not yet been proven. Fe^{2+} and Fe^{3+} ions will undoubtedly be present in the gut of the feeding adult worm and the enhancement of proteinase activity by these ions lends weight to the argument that they may be feeding enzymes and play a role in haemoglobin breakdown. Similarly, the larval cathepsin L 'like' proteinase was enhanced only by the presence of Fe^{2+} and Fe^{3+} ions, whilst the cathepsin B 'like' proteinase was enhanced by Zn^{2+}, Mg^{2+}, Fe^{2+} and Fe^{3+}. As described in Chapter 3 a role for the larval cysteinyl proteinases in the hookworm life cycle has yet to be defined as they appear to play no part in the degradation of any of the skin macromolecules tested, although penetration of hamster skin by live larvae was inhibited 36.7 % by E64 (Brown *et al.*, 1999), suggesting they may still play a role in skin penetration.

All the purified cysteinyl proteinases resolved on SDS-PAGE as a doublet with molecular masses of 66 and 55 kDa. This doublet has been previously shown to exhibit proteolytic activity using haemoglobin substrate gels (Pritchard *et al.*, 1990a) although at the time no inhibitor studies were carried out to characterise the proteinase activity observed. Western blots of the purified proteinases were probed with antiserum raised against *Necator americanus* glutathione-S-transferase. This antiserum has also been shown to recognise a 66 kDa glutathione binding protein with no associated proteinase activity (P. M. Brophy personal communication). A 66 kDa protein eluting from the column was also recognised by this antiserum indicating that this may be the same glutathione binding protein. If this is the case then it would appear that the cysteinyl proteinase activity is associated with the 55 kDa protein while the remaining proteinase activity associated with the 55-66 kDa doublet belongs to a different class of enzyme. The yields from the activated thiol-Sepharose column were not particularly high and this may be due to competition from this glutathione binding protein for binding sites on the column. Similar 'blocking factors' have been demonstrated in the tapeworm *Hymenolepis diminuta* and the mouse parasite *Helimosomoides polygyrus* (Brophy & Barrett, 1990; Brophy *et al.*, 1994). Presumably the removal of this binding protein would enhance purification yields.

The molecular mass observed on SDS-PAGE is somewhat large for a cysteinyl proteinase. For example the classical cysteinyl proteinase, papain, has a molecular mass of 27 kDa, human cathepsin B1 is a protein of 24 kDa (Barrett, 1973) while the cysteinyl proteinase and major allergen *Der p* I of the house dust mite *Dermatophagoides pteronyssinus* has a molecular weight of 25-27 kDa (Hewitt *et al.*, 1995). Cysteinyl proteinases purified from parasites also fall within a similar range of molecular masses e.g. *F. hepatica* cathepsin L, 27 kDa (Smith *et al.*, 1993b), *P. westermani* neutral cysteinyl proteinase, 22 kDa (Yamakami & Hamajima, 1987) and the cathepsin B of *S. mansoni* which has a mass of 32 kDa (Klinkert *et al.*, 1989). Recently the cathepsin B 'like' enzyme of *N. americanus* has been cloned (Pritchard *et al.*, manuscript in press) The predicited molecular mass of the mature enzyme is 29 kDa, approximately half the molecular mass of the proteins that elute from an activated thiol-Sepharose 4B column. The reason for this difference has yet to be determined, however, Kumar & Pritchard, (1992a) have previously shown using gelatin substrate gel analysis that a cysteinyl proteinase (inhibitable by N-ethylmaleimide) with an

approximate molecular mass of 62 kDa is present in larval ES products. Kumar & Pritchard, (1993) have also suggested that the proteinases of *N. americanus* may oligermerise under the conditions necessary for their purification and this may be the case here.

The purification of aspartyl proteinase activity was attempted using the affinity matrix pepstatin A-agarose. The method was adapted from one described by Slater (1981) for the purification of renin. In Slater's method the proteinase activity was eluted using 0.1 M acetic acid pH 3.2. However, under these elution conditions *N. americanus* proteinase activity was lost, despite being able to elute protein from the column. Proteinase activity could also be eluted competitively from the column by the use of 500 μM pepstatin A. Following elution the pepstatin A was removed from the fractions by dialysis against water prior to analysis, yielding active enzyme. The larval enzyme was optimally active at pH 6.5 (Figure 4.10), the same pH optimum as that described for larval ES products against FITC-casein (Figure 3.1). The adult enzyme was also optimally active at pH 6.5 (Figure 4.10) but also demonstrated a smaller peak of activity at pH 4. This is reminiscent of the pH profile of adult ES products, which show peaks of activity against tritiated haemoglobin at pH 3.5, 6.5 and 8.5 (Brown *et al.*, 1995). Both larval and adult ES products demonstrated a small alkaline peak of proteolytic activity at pH 8 and 8.5 respectively, which was inhibitable by pepstatin A. The lack of activity of the purified enzymes at these pH points may indicate that another more unusual aspartyl proteinase, which does not bind to the column under the conditions used, may be present in larval and adult ES products.

The activities eluted by 500 μM pepstatin A following the application of larval and adult ES products to a pepstatin A column were characterised with a number of proteinase inhibitors (Table 4.2). At pH 6.5 proteinase activity was primarily inhibited by pepstatin A, characterising the enzymes as aspartyl in nature. SDS-PAGE analysis revealed a predominant protein of molecular mass 66 kDa and a minor band of 55 kDa was observed. Again these molecular masses are larger than typical aspartyl proteinases. ACASP, the aspartyl proteinase of *A. caninum*, has a predicted molecular mass of 49.3 kDa (Harrap *et al.*, 1996) while the partial purification of the aspartyl proteinases of *Plasmodium*

falciparum has demonstrated the presence of three proteins of 36, 37 and 38 kDa (Dame *et al.*, 1994). The significance of these apparent high molecular masses still remains unresolved. Thus it would appear that the two different matrices have purified two proteinase activities from larval and adult ES products with differing inhibitor sensitivities, yet these proteinases appear to have identical molecular masses when analysed by SDS-PAGE. The genes for two aspartyl proteinases have been cloned (Girdwood *et al.*, Manuscript in press). Nasp1 is an aspartyl proteinase form the adult hookworm while Nasp2 has been isolated from the larval stages. The sequence for Nasp1 would appear to encode for a protein with a molecular mass of 45 kDa and Nasp 2 for a protein of molecular mass 42 kDa. While again there would appear to be some descrepency between the predicted and actual molecular weights the sequence data makes no allowances for potential glycosylation sites.

Haemoglobin and fibrinogen substrate gels have demonstrated in adult ES products, the presence of aspartyl proteinase activity at approximately 66 kDa (Figure 3.12 and 3.13). To date it has not been possible to demonstrate a similar activity in larval ES products, although as already mentioned Kumar & Pritchard, (1992a) have demonstrated, using gelatin substrate gels, a cysteinyl proteinase with a molecular mass of 62 kDa. This would seem to indicate that proteinases of a high molecular mass do occur within larval and adult ES products. N-terminal sequencing of the purified aspartyl and cysteinyl enzymes may solve this dilemma, but so far attempts to obtain sequence data have been unsuccessful due to insufficient material.

Metallo-proteinase purification was attempted using two different affinity matrices (Figures 4.20 and 4.22). Initial attempts at purifying the metallo enzyme from larval ES products concentrated on the use of bacitracin-Sepharose. This matrix has previously been used to purify an elastinolytic metallo-proteinase from *Aspergillus fumigatus* (Markaryan *et al.*, 1994). Only the peak of proteinase activity eluted by 50 mM Tris-HCl, 1 M NaCl, 1 mM $CaCl_2$, 7 % isopropanol, pH 7.2, showed specific inhibition of activity with 1, 10-phenanthroline (Table 4.3). When this peak was analysed by SDS-PAGE followed by staining with Coomassie Brilliant Blue R250 a band of 42 kDa was observed (Figure 4.21). However, when the same gel was silver stained a number of contaminating bands were

observed. Metallo-proteinase purification from larval ES products was then attempted using N-Benzyloxycarbonylglycyl-D-leucylaminohexyl-Sepharose (Z-Gly-D-Leu-AH-Sepharose) as described by Inouye *et al.*, (1985). Proteinase activity was eluted from the column by 50 mM phosphate buffer pH 5.6 containing 2 M urea (Figure 4.22). This activity was completely inhibited by EDTA but only weakly inhibited by 1, 10-phenanthroline (Table 4.4). When analysed by SDS-PAGE a protein of 42 kDa was again identified (Figure 4.23). To date this purification protocol has not been evaluated using adult ES products. Chapter 3 discusses the possibility that there may be two metallo-proteinases present in larval ES products. The 42 kDa metallo-proteinase purified here is only weakly inhibited by 1, 10-phenanthroline while being strongly inhibited by EDTA. Thus it would seem likely that this is the metallo-proteinase with a preference for Types IV and V collagen although this hypothesis has not been tested using the purified enzyme. Nor to date has the metal ion enhancement profile for this enzyme been examined. A molecular mass of 42 kDa for the metallo-proteinase of *N. americanus* is similar to that described for other parasite species. For example a 44 kDa zinc metallo-proteinase has been purified from the larvae of *Haemonchus contortus* (Gamble *et al.*, 1989), while a 45 kDa enzyme has been purified from *Trichuris suis* ES products and implicated in tissue invasion (Hill *et al.*, 1993). Similarly, the invasive larvae of *Strongyloides stercoralis, Ancylostoma duodenale* and *A. caninum* secrete metallo-proteinases of 40 kDa (Brindley *et al.*, 1995) and 38 kDa respectively (Hotez *et al.*, 1990).

The purification of serine proteinase activity using affinity matrices has so far proved unsuccessful. Early work prior to the commencement of this project using the affinity matrix aprotinin-agarose failed to demonstrate any binding of serine proteinase activity (data not shown). Similarly, no serine proteinase activity bound to soybean trypsin inhibitor-agarose. Both soybean trypsin inhibitor and aprotinin are inhibitors of trypsin 'like' serine proteinases (Beynon & Bond, 1989). As the serine proteinase activity present in larval and adult ES products has been characterised as trypsin 'like' in nature (Table 3.2), the lack of binding to this matrices is surprising. This could be due to either an unusual and yet to be defined property of these serine proteinases, or the conditions used so far are unsuitable for binding the serine proteinases from *N. americanus* ES products.

Serine proteinase activity did however, bind to the affinity matrix *p*-aminobenzamidine-agarose and could be eluted from the column by 70 mM sodium acetate pH 5.6 as demonstrated by the ability of the peak eluted to hydrolyse the substrate Tosyl-Gly-Pro-Arg-AMC.HCl (data not shown). To preserve the activity of the proteinase, the acidity of the buffer was immediately neutralised by eluting into 500 mM Tris pH 8.5. Characterisation of the proteinase activity eluting from the column was carried out using the non-specific proteinase substrate FITC-casein (Section 2.4.2.2). All the inhibitors tested strongly inhibited proteinase activity except for 1, 10-phenanthroline and the SDS-PAGE analysis revealed a large number of protein bands eluting from the column for both adult and larval ES products. This included the protein doublet at approximately 66 and 55 kDa associated with cysteinyl and aspartyl proteinase activities. This may explain the inhibition by both E64 and APMSF. It would appear from the data obtained so far that the purification of serine proteinase activity will probably not be achieved by a one step affinity method and future attempts to purify the enzymes will involve a number of procedures. To achieve this efforts will have to be made to generate much larger amounts of adult and larval ES products.

To fully characterise the enzymes at the molecular level, provide material for vaccination purposes, further determine their roles in the life cycle (feeding, immune evasion) or for possible use as diagnostic reagent (in ELISA at the IgE level should any of the proteinase enzymes prove to be allergenic), will require much larger quantities of enzyme than can ever be reasonably generated using these protocols. Larger amounts of hookworm proteinase will only be generated by cloning these enzymes and expressing them in an active form in *E. coli* or a yeast (*Saccharomyces cerevisiae*) expression system, as has been achieved for the cathepsin B of *Schistosoma mansoni* (Lipps *et al.*, 1996). While the ultimate aim of this project is to assess the potential of purified proteinases as candidate vaccines against hookworm infection (Chapter 5), a useful by product of these purification experiments could be to provide enough proteinase material to generate either; N-terminal sequence data, from which suitable PCR primers can be designed to generate a proteinase specific DNA probe, or to raise a proteinase specific antibodies. Both of these tools can then be used to screen larval and adult cDNA libraries with a view to the further identification and cloning of *N. americanus* proteinase genes.

CHAPTER 5

VACCINATION AGAINST THE HUMAN HOOKWORM *NECATOR AMERICANUS*

CHAPTER 5

VACCINATION AGAINST THE HUMAN HOOKWORM *NECATOR AMERICANUS*

5.1 INTRODUCTION

Chapter 3 described the characterisation of the proteinases secreted by *Necator americanus* and discusses the possible roles they may play in maintaining the hookworm life cycle. Whether an immune response that neutralises the activity of one or more of these proteinases would be an effective vaccine against hookworm infection is unresolved. For example, if immune responses in the skin were to neutralise the activity of the larval aspartyl proteinase, would the passage of larvae through the skin be prevented?

The idea of using proteinases as a vaccine is not a new one. As early as 1932 Chandler suggested the possibility of their use and, in 1956, Thorson demonstrated that serum from dogs infected with *A. caninum* had the ability to neutralise the activity of an anticoagulant protease produced by the worm. Subsequent vaccination of dogs against *A. caninum* with extracts from oesophageal glands (containing proteolytic activity) failed to reduce worm fecundity but did, however, result in a small reduction in the number of worms recovered from the small intestines of challenged dogs (Thorson, 1956b). Similarly, Carroll and Grove (1985) injected *A. ceylanicum* soluble worm extracts into dogs (again containing proteinase activity) and demonstrated a 74 % reduction in worm recovery in vaccinated dogs.

Vaccination of sheep with a cathepsin B 'like' cysteinyl proteinase from *Fasciola hepatica* has been shown to reduce worm fecundity (Wijffels *et al.*, 1994). Similarly, it has been shown that vaccination with papaya latex, a rich source of the cysteinyl proteinase papain has the ability to protect mice against *Heligmosomoides polygyrus* (Satrija *et al.*, 1995). High antibody levels in cattle against the cysteine proteinase 'congopain' from *Trypanosoma congolense* have been implicated in resistance to trypanosome infection (Authié *et al.*, 1993).

Some of the most successful vaccines are attenuated whole organism vaccines produced by the use of irradiation. Irradiation is a simple method of disabling organisms and has been successful in animal models against a number of parasites such as *Plasmodium berghei* W (Nussenweig, 1969), *Leishmania major* (Rivier *et al.*, 1993), *Toxoplasma gondii* (Dubey *et al.*, 1996), *Schistosoma mansoni* (Smythies, 1996), *Fasciola hepatica* (Creaney *et al.*, 1995) and the nematodes *Heligmosomoides polygyrus* (Hagan *et al.*, 1981), *Strongylus vularis* (Clifton *et al.*, 1997) and *Ancylostoma caninum* (Miller, 1971). However, despite success in animal model systems there are relatively few irradiation attenuated vaccines against parasites currently on the market. The most commercially successful of these is the irradiated vaccine against the lungworm *Dictyocaulus viviparus* (Smith, 1998). This vaccine, like many of the irradiated larval vaccines, relies on the larvae only developing to fourth-stage larvae and juveniles and failing to mature into adults. However, during this process they provide enough antigenic stimulus to allow development of a protective immune response.

Vaccines based on radiation attenuated larvae suffer from a number of problems. The shelf life of these vaccines is relatively short only (≤ 2 weeks) and the cost of production is high (Smith, 1998). Problems of this nature resulted in the withdrawal in 1975 (after only two years), of the irradiated larval vaccine against the dog hookworm *A. caninum* (Miller, 1978). However, lessons may be learnt from using irradiated larvae as vaccines, by for example examining the effects of irradiation on the molecule(s) responsible for conferring immunity or by studying the type of immune response induced by the irradiated parasite.

This chapter describes attempts to vaccinate BALB/c mice against *N. americanus* using either a mixture of purified proteinase enzymes or (in separate experiments) radiation attenuated *N. americanus* larvae.

In the first experiment using irradiated larvae mice were vaccinated subcutaneously as described by Miller (1971) who, using the dog hookworm, *A. caninum*, demonstrated that, despite the oral route being the preferred method of entry, subcutaneous vaccination with irradiated larvae gave greater degrees of protection than oral vaccination. To investigate if a similar scenario existed for *N. americanus*, the vaccination protocol in the second

experiment included additional groups in which the mice were vaccinated percutaneously (to simulate the natural route of infection) with either pyrogen free water or 300 γ-irradiated *N. americanus* larvae. The protocols used for individual vaccination experiments are summarised in Tables 5.1 to 5.5.

During the course of some of the vaccination experiments worm recoveries were less than expected based on previous studies (Wells & Behnke, 1988b; Timothy & Behnke, 1993). One explanation could be that this particular strain of *N. americanus* had become rodent adapted as a consequence of continuous passaging through hamsters since 1983. This may have resulted in an altered time course of infection and hence assessment of worm burdens at inappropriate times. To check this, an experiment was also designed to re-assess the time course of primary infection in BALB/c mice (Section 5.2.1).

5.2 MATERIALS AND METHODS

5.2.1 ANIMALS

All vaccination experiments were carried out using specific pathogen free 5-6 week old male BALB/c mice purchased from Harlan Olac Ltd, Bicester, Oxon. The mice were housed separately and allowed to rest for one week after delivery prior to experiments commencing.

5.2.2 PERCUTANEOUS INFECTION OF MICE WITH *N. AMERICANUS* LARVAE

Mice were infected with *N. americanus* by a method similar to that used for hamsters. Mice were immobilised using 'Sagatal' (M&B Vetinary Products, Section A.2.1.15) and a band of fur shaved all around the thoracic cavity. The required number of larvae in a volume of no greater than 20 µl were placed onto a gauze (approximately 8 mm x 8 mm). The gauze was then secured to the back of the neck by means of a strip of sticking plaster wrapped around the shaved thoracic cavity. The plasters were removed after 24 h. However, to ensure the gauzes remained in place for at least 3 h, they were applied to the back of the neck to make it as difficult as possible for the mouse to remove the gauze and to avoid unintentional ingestion of the larvae. During the initial vaccination experiments normal non-medicated sticking plaster (Johnson and Johnson) was used. However, in later experiments, a self-binding horse bandage (International Market Supply, Dane Mill, Broadhurst Lane, Congleton, Cheshire, CW12 1LA) was used to secure the gauzes to the mice.

5.2.3 PREPARATION OF RADIATION ATTENUATED LARVAE

Larvae were harvested as described in section 2.2.1 and then washed extensively in pyrogen free water. Larvae were then exposed to 40 kilorads of γ-radiation from a sealed ^{137}caesium source. Irradiated larvae were used in the vaccination experiments within 2 h of irradiation.

5.2.4 RECOVERY OF ACTIVELY MIGRATING LARVAE FROM THE LUNGS

The number of actively migrating larvae present in the lungs was determined as described by Wells & Behnke (1988b). Mice were killed by inhalation of excess chloroform, the lungs removed and placed into 45 mm petri dishes containing modified Hanks buffered salt solution (HBSS) pre-warmed to 37 °C on a bench incubator. The surface of the lungs was examined for larvae using a binocular dissecting microscope and then the lungs were finely chopped using dissecting scissors and incubated at 37 °C. After 2 h incubation the HBSS was removed into another petri dish and replaced with fresh warm HBSS. Following the addition of fresh HBSS the lungs were chopped even finer and left to incubate further at 37 °C. An equal volume of water was added to the HBSS removed from the lungs to lyse any red blood cells present and the number of migrating larvae counted. This was repeated after 4, 6 and 24 h incubation and the cumulative total of migrating larvae determined for each mouse.

5.2.5 RECOVERY OF L4 STAGES FROM THE SMALL INTESTINE

L4 stages were recovered from the small intestine by a modified Baermann technique (Wells & Behnke, 1988b). Mice were killed by excess chloroform inhalation and the small intestine removed and opened along its length on a nylon gauze. The gauze was suspended in a 50 ml beaker containing HBSS pre-warmed to 37 °C and incubated for a minimum of 4 h, thus allowing any L4 larvae in the gut to sediment under gravity in the bottom of the beaker. Following this period, the gauze was removed, the excess HBSS aspirated off and the sedimented L4s counted.

5.2.6 COLLECTION OF MOUSE PLASMA

Mice were anaesthetised by inhalation of ether and bled from the retro orbital plexus into heparinised micro-haematocrit tubes. The tubes were centrifuged at 13 000g for 10 minutes and the plasma collected into 0.5 ml eppendorf tubes.

At time points when lung and gut worm burdens were being determined mice were killed by excess chloroform inhalation, the thoracic cavity opened and the aorta severed. Blood was collected into 1.5 ml eppendorf tubes and allowed to clot. The tubes were then

centrifuged at 13 000 g for 15 minutes and the serum collected into fresh eppendorf tubes. Sera from all experiments were stored at - 20 °C until required.

5.2.7 ANALYSIS OF CYTOKINE PRODUCTION

5.2.7.1 COLLECTION OF LEUCOCYTES

Spleens from individual animals of each group were pooled and placed into 5 ml of RPMI culture medium containing 10 % foetal calf serum, 100 i.u./ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 0.1 % sodium pyruvate and 0.002 % monothioglycerol. Under sterile conditions, spleens were 'dissociated' by pushing the organ through a sterile wire gauze using a sterile syringe plunger, the resulting cells were collected into a sterile Petri dish and left to stand for 2 minutes before being transferred into a sterile Universal. The medium in the Universal was topped up to 25 ml and centrifuged at 500 g for 3 minutes. The pellet of cells was resuspended in 2 ml of red cell lysis buffer (0.85 % ammonium chloride, pH 7.0) and allowed to stand for 30 seconds, before being reconstituted with 25 ml of medium, and re-centrifuged at 500 g for 3 minutes. The cells were washed a further three times before being resuspended in a final volume of 2 ml of culture medium. The cells were then stored on ice until required.

5.2.7.2 LEUCOCYTE CELL COUNTS

The number of viable cells was determined by staining with fluorescein diacetate (FDA-Sigma). FDA (5mg/ml stock) was diluted 1:50 with culture medium and then further diluted 1:10 with the cell suspension. Cells were loaded onto a haemocytometer slide (Hawksley) and counted using a fluorescence microscope. The number of leucocytes was adjusted to $5x10^6$ cells per/ml and 200 µl aliquots added to the wells of a flat bottomed 96 well plate. The harvested leucocytes were stimulated by the addition of 5 µg/ml Concavalin A and incubated at 37 °C in an atmosphere supplemented with 5% CO_2 for 48 hours after which the supernatants were removed and stored at –80 °C until required.

5.2.7.3 ELISA ASSAY FOR IL-5 AND γ- IFN

Levels of IL-5 and γ-IFN in culture supernatants were determined by a capture ELISA using a method developed by Else & Grencis (1991) and modified by Robinson *et al.*,

(1994). 96 well flat bottomed polystyrene plates (Dynatech) were coated overnight at 4 °C with 50 µl per well of the capture antibody diluted to 2 µg/ml in 0.05M carbonate/bicarbonate buffer, pH 9.6. The monoclonal antibodies R46A2 (Pharmingen, San Diego, CA.) and TRFK-5 (Pharmingen) were used for γ-IFN and IL-5 assays respectively. The plate was then washed 3 times with phosphate buffered saline, 0.05 % Tween (PBS/Tween) and blocked with 200 µl per well of 10% foetal calf serum (FCS) in PBS/Tween 20 for 1 hour at 37 °C. The plate was washed again prior to the addition of the samples.

A doubling dilution of cytokine standards was constructed in the range 500 to 3.9 Units/ml for IL-5 and 307 to 2.4 Units/ml for γ-IFN (standards diluted in culture medium). Triplicate 50 µl volumes of each standard, a culture medium control and culture supernatant under test were added to the washed plate which was then incubated for 2 hours at room temperature. The plate was washed again and 50 µl of secondary antibody (for IL-5, 4 µg/ml biotinylated XMG2.1 and for γ-IFN, 4 µg/ml biotinylated TRFK-4, both from Pharmingen) diluted in 1% BSA, PBS-Tween was added to each well. Plates were incubated for 1 hour at room temperature, washed, and 75 µl of streptavidin peroxidase conjugate (2 µg/ml, diluted in 1% BSA in PBS-Tween, Sigma) added to each well. The plates were incubated for a further hour at room temperature after which they were washed again. Finally, 100 µl of substrate (15 ml 0.1 M sodium acetate, pH 6, 100 µl 3, 3'5, 5'-tetramethylbenzidine [TMB, 10 mg/ml stock in DMSO], 10 µl of 30%, v/v, hydrogen peroxide) was added to each well. The reaction was stopped by the addition of 50 µl of 2.5 M sulphuric acid and the absorbance of each well measured on a MR-700 microplate reader (Dynatech) at 450 nm. IL-5 and γ-IFN concentrations were determined following the construction of a standard curve using relevant cytokine standards.

5.2.8 ELISA ASSAYS FOR MOUSE IMMUNOGLOBULINS G, A AND M AND IMMUNOGLOBULIN G SUBCLASSES

N. americanus purified larval proteinases, ES products or somatic extracts were diluted to 5 µg/ml in 0.05 M sodium carbonate/bicarbonate buffer pH 9.6; 50 µl aluiquots were used to coat the wells of a 96 well polystyrene plate overnight at 4 °C. The plate was then washed 3 times with Tris buffered saline, 0.05% Tween 20 pH 7.4 (TBS/Tween) and the

wells blocked with 200 µl of 5% Marvel milk powder/TBS (blocking agent) for 1 hr at 37 °C. The plate was washed as before and 50 µl of mouse post-vaccination serum, diluted 1:50 in blocking agent was added to individual wells and the plate incubated at 4 °C overnight. The plate was washed again and 50 µl of secondary antibody labelled with alkaline phosphatase [sheep anti-mouse immunoglobulins G, A and M (Sigma) or sheep anti-mouse IgG1, IgG2a, IgG2b or IgG3 (Seralab, Crawley Down, Sussex UK)], diluted 1:1000 in blocking agent was added to individual wells and the plate incubated for a further 90 minutes at 37 °C. The plates were washed again and antibody binding visualised by the addition of 100 µl of *p*–nitro–phenol-phosphate substrate (1 mg/ ml in 0.1 M glycine, 1 mM calcium chloride, 1 mM magnesium chloride, pH 10.4). All assays were carried out in triplicate. ELISA values are expressed as the absorbance at 405 nm after the subtraction of a negative buffer control.

5.2.9 WESTERN BLOT ANALYSIS

Western blots of *N. americanus* larval ES products and somatic extracts were prepared as described in section 2.6 and probed as described in section 4.2.5. In this case the primary antibody was mouse post vaccination serum (diluted 1:50 in blocking agent) and the secondary antibodies sheep anti mouse IgGAM, IgG1, IgG2a, IgG2b or IgG3 - alkaline phosphatase (diluted 1:1000 in blocking agent). Antibody binding was revealed using BCIP/NBT substrate (Section A.2.4.3.3).

5.2.10 VACCINATION AGAINST *N. AMERICANUS* INFECTION USING PURIFIED PROTEINASES OR IRRADIATED LARVAE

5.2.10.1 VACCINATION OF BALB/c MICE WITH A MIXTURE OF PURIFIED LARVAL ASPARTYL AND CATHEPSIN B AND L 'LIKE' PROTEINASES

SPF BALB/c mice were divided into 5 groups (12 mice per group). Group A, (primary infection control) received only the challenge infection of 300 larvae. Groups B and C were vaccinated 3 times at 14 day intervals with respectively either adjuvant (Alhydrogel, Superfos Biosector, Denmark) or a mixture containing 10 µg each of larval aspartyl and cathepsin L and B 'like' proteinases (total 30 µg) in adjuvant. The mice in Group D

remained naive throughout the experiment while group E (secondary infection control) was infected twice with 250 normal *N. americanus* larvae, once on the day of the first vaccination and again on the day of challenge. Fourteen days after the final vaccination the mice in all groups (except group D) were challenged with 250 *N. americanus* larvae. Four days post challenge 6 mice from each group were sacrificed, bled from the thoracic cavity and the number of actively migrating larvae in the lungs determined. Nine days post infection the remaining 6 mice in each group were sacrificed, bled from the thoracic cavity and the number of L4 larvae present in the small intestine determined. A summary of the vaccination protocol can be found in Table 5.1

5.2.10.2 VACCINATION AGAINST *NECATOR AMERICANUS* USING γ–IRRADIATED LARVAE

SPF BALB/c mice were again divided into 5 groups (12 mice per group) and allowed to settle in the animal house prior to vaccination. The groups were the same as those described for the vaccination with purified larval proteinases. Group C was vaccinated subcutaneously on 3 occasions, 14 days apart with 300 γ–irradiated *N. americanus* larvae suspended in pyrogen free water (Section 5.2.3). Group B was vaccinated with pyrogen free water alone at the same time points. Group E was infected twice with 300 normal *N. americanus* larvae on day 0 and on the day of challenge. 14 days after the final vaccination all the mice except for those in group D were challenged with 300 normal *N. americanus* larvae. Lung worm burdens were assessed 4 days post-challenge and gut worm burdens were assessed 9 days post-challenge (Experiment 1). In later vaccination experiments with γ–irradiated larvae, two additional groups of mice were added to the protocol (Experiments 3 & 4). Group F was vaccinated percutaneously with pyrogen free water and Group G was vaccinated percutaneously with 300 γ–irradiated larvae. A summary of the γ–irradiated larvae vaccination protocols can be found in Tables 5.2 to 5.5.

5.2.11 A RE-EVALUATION OF THE TIME COURSE OF PRIMARY INFECTION WITH *N. AMERICANUS*

162 SPF BALB/c mice were divided into 3 groups of 54 and infected percutaneously with either 300 normal *N.americanus* larvae (Group A), 300 irradiated larvae (Group B) or subcutaneously with 300 irradiated larvae (Group C). 6 mice from each group were killed on days 1, 2, 3, 4, 5, 7, 8, 9, and 10 post-infection, bled from the thoracic cavity and the number of actively migrating larvae in the lungs determined. On days 5, 7, 8, 9 and 10 the number of worms present in the small intestine was also determined.

Table 5.1 Summary of the protocol used to vaccinate Balb/c mice with purified *N. americanus* larval proteinases

Day	Procedure
0	Group B vaccinated subcutaneously with 230 µl of Alhydrogel diluted 1:1 with pyrogen free water. Group C vaccinated subcutaneously with 230 µl of adjuvant/proteinase mixture (equivalent to 10 µg each the larval aspartyl cathepsin B and L 'like' proteinases) Group E infected with 250 *N. americanus* larvae
14	Groups B, C and D bled from the retro orbital plexus. Groups B and C vaccinated with adjuvant alone and adjuvant proteinase mix respectively
28	Groups B, C and D bled from the retro orbital plexus. Groups B and C vaccinated with adjuvant alone and adjuvant proteinase mix respectively
42	Groups B, C, D and E bled from the retro orbital plexus. All groups except group D challenged with 250 *N. americanus* larvae
46 4 days post challenge	6 mice from each group killed (5 in group C), bled and the number of actively migrating larvae in the lungs determined
51 9 days post challenge	6 mice from each group killed (5 in group C), bled and the number of worms present in the small intestine determined

Table 5.2 Summary of the protocol used to vaccinate Balb/c mice with *N. americanus* γ–irradiated larvae (Experiment 1).

Day	Procedure
0	Groups C vaccinated subcutaneously with 300 γ–irradiated larvae resuspended in pyrogen free water. Groups B vaccinated subcutaneously with an equivalent volume of pyrogen free water. Group E infected percutaneously with 300 normal *N. americanus* larvae.
14	Groups B, C and D bled from the retro orbital plexus. Groups C vaccinated subcutaneously with 300 γ–irradiated larvae resuspended in pyrogen free water. Groups B vaccinated subcutaneously with an equivalent volume of pyrogen free water.
28	Groups B, C and D bled from the retro orbital plexus. Groups C vaccinated subcutaneously with 300 γ–irradiated larvae resuspended in pyrogen free water. Groups B vaccinated subcutaneously with an equivalent volume of pyrogen free water.
42	Groups B, C, D and E bled from the retro orbital plexus. All groups except group D challenged with 300 normal *N. americanus* larvae.
46 4 days post challenge	6 mice from each group killed (5 in groups C and E, 4 in group D), bled and the number of actively migrating larvae in the lungs determined.
51 9 days post challenge	6 mice from each group killed, bled and the number of worms present in the small intestine determined.

Table 5.3 Summary of the protocol used to vaccinate Balb/c mice with *N. americanus* γ–irradiated larvae (Experiment 2).

DAY	PROCEDURE
0	Group C vaccinated subcutaneously with 300 irradiated larvae resuspended in pyrogen free water. Group B vaccinated subcutaneously with an equivalent volume of pyrogen free water. Group E infected percutaneously with 300 normal *N. americanus* larvae. Group G vaccinated percutaneously with 300 irradiated larvae resuspended in pyrogen free water. Group F vaccinated percutaneously with an equivalent volume of pyrogen free water.
14	Groups B, C, D, F and G bled from the retro orbital plexus. Group C vaccinated subcutaneously with 300 irradiated larvae resuspended in pyrogen free water. Group B vaccinated subcutaneously with an equivalent volume of pyrogen free water. Group G vaccinated percutaneously with 300 irradiated larvae resuspended in pyrogen free water Group F vaccinated percutaneously with an equivalent volume of pyrogen free water.
28	Groups B, C, D, F and G bled from the retro orbital plexus. Group C vaccinated subcutaneously with 300 irradiated larvae resuspended in pyrogen free water. Group B vaccinated subcutaneously with an equivalent volume of pyrogen free water. Group G vaccinated percutaneously with 300 irradiated larvae resuspended in pyrogen free water Group F vaccinated percutaneously with an equivalent volume of pyrogen free water.
42	Groups B, C, D, E, F and G bled from the retro orbital plexus. All groups except group D challenged with 300 normal *N. americanus* larvae.
46 4 days post challenge	6 mice from each group killed (5 in groups C, E, and G, 3 in group F), bled and the number of actively migrating larvae in the lungs determined.
51 9 days post challenge	6 mice from each group killed (5 in group E, 4 in group F), bled and the number of worms present in the small intestine determined.

Table 5.4 Summary of the protocol used to vaccinate Balb/c mice with *N. americanus* γ–irradiated larvae (Experiment 3).

DAY	PROCEDURE
0	Group C vaccinated subcutaneously with 300 irradiated larvae resuspended in pyrogen free water. Group B vaccinated subcutaneously with an equivalent volume of pyrogen free water. Group E infected percutaneously with 300 normal *N. americanus* larvae. Group G vaccinated percutaneously with 300 irradiated larvae resuspended in pyrogen free water. Group F vaccinated percutaneously with an equivalent volume of pyrogen free water.
14	Groups B, C, D, F and G bled from the retro orbital plexus. Group C vaccinated subcutaneously with 300 irradiated larvae resuspended in pyrogen free water. Group B vaccinated subcutaneously with an equivalent volume of pyrogen free water. Group E infected percutaneously with 300 normal *N. americanus* larvae. Group G vaccinated percutaneously with 300 irradiated larvae resuspended in pyrogen free water Group F vaccinated percutaneously with an equivalent volume of pyrogen free water.
28	Groups B, C, D, F and G bled from the retro orbital plexus. Group C vaccinated subcutaneously with 300 irradiated larvae resuspended in pyrogen free water. Group B vaccinated subcutaneously with an equivalent volume of pyrogen free water. Group G vaccinated percutaneously with 300 irradiated larvae resuspended in pyrogen free water Group F vaccinated percutaneously with an equivalent volume of pyrogen free water.
42	Groups B, C, D, E, F and G bled from the retro orbital plexus. All groups except group D challenged with 300 normal *N. americanus* larvae.
45 3 days post challenge	6 mice from each group killed (5 in groups C and G, 4 in group F), bled and the number of actively migrating larvae in the lungs determined.
50 and 51 8 & 9 days post challenge	6 mice from each group killed (Days 8 and 9, 5 in group E, 4 in group F), bled and the number of worms present in the small intestine determined.

Table 5.5 Summary of the protocol used to vaccinate Balb/c mice with *N. americanus* γ-irradiated larvae (Experiment 4).

DAY	PROCEDURE
0	Group C vaccinated subcutaneously with 300 irradiated larvae resuspended in pyrogen free water. Group B vaccinated subcutaneously with an equivalent volume of pyrogen free water. Group E infected percutaneously with 300 normal *N. americanus* larvae. Group G vaccinated percutaneously with 300 irradiated larvae resuspended in pyrogen free water. Group F vaccinated percutaneously with an equivalent volume of pyrogen free water.
14	Groups B, C, D, F and G bled from the retro orbital plexus. Group C vaccinated subcutaneously with 300 irradiated larvae resuspended in pyrogen free water. Group B vaccinated subcutaneously with an equivalent volume of pyrogen free water. Group E infected percutaneously with 300 normal *N. americanus* larvae. Group G vaccinated percutaneously with 300 irradiated larvae resuspended in pyrogen free water Group F vaccinated percutaneously with an equivalent volume of pyrogen free water.
28	Groups B, C, D, F and G bled from the retro orbital plexus. Group C vaccinated subcutaneously with 300 irradiated larvae resuspended in pyrogen free water. Group B vaccinated subcutaneously with an equivalent volume of pyrogen free water. Group E infected percutaneously with 300 normal *N. americanus* larvae. Group G vaccinated percutaneously with 300 irradiated larvae resuspended in pyrogen free water Group F vaccinated percutaneously with an equivalent volume of pyrogen free water.
42	Groups B, C, D, E, F and G bled from the retro orbital plexus. All groups except group D challenged with 300 normal *N. americanus* larvae.
45 (3 days post challenge)	6 mice from each group killed, bled, spleens removed and the number of migrating larvae in the lungs determined.
50 (8 days post challenge)	6 mice from each group killed, bled, spleens removed and the number of worms in the small intestine determined.

5.3 RESULTS

5.3.1 WORM RECOVERY FOLLOWING VACCINATION WITH LARVAL PROTEINASES

The numbers of worms establishing in the lungs and small intestines of BALB/c mice following vaccination with a mixture of purified larval proteinases are shown in Table 5.6. Four days post challenge 16.5 ± 10.33 larvae were found in the lungs of the primary infection control group (Group A). This number was reduced to 9.5 ± 4.3 larvae in the secondary infection control group (Group E). No apparent reduction in larval numbers (17 ± 9.6) was observed in the group vaccinated with purified larval proteinases (Group C) compared with the primary infection control group.

Nine days post challenge infection only 4.5 ± 3.14 worms were found in the small intestine of the mice in group A. No worms were found in the secondary infection control group and 2.8 ± 2.5 worms were found in the group vaccinated with *N. americanus* larval proteinases, an apparent reduction in worm burden of 37.7 %. It should be noted here that the numbers of larvae recovered from the lungs and small intestine of the primary and adjuvant control groups were significantly lower than expected (see discussion).

5.3.2 WORM RECOVERY FOLLOWING VACCINATION WITH γ-IRRADIATED LARVE (EXPERIMENT 1)

Table 5.7 shows the number of larvae recovered from the lungs (4 days post challenge infection) and from the small intestine on day 9. In the lungs the number of larvae recovered was reduced in the water control group, (Group B) compared to the primary infection control (Group A), while no reduction in larval recovery was observed in either the vaccinated mice (Group C), or Group E (multiple infection control).

Nine days after challenge infection, however, a significant reduction in worm numbers was observed in both the vaccinated animals (Group C) and the multiple infection control group (Group E) resulting in 92.7 % and 86.9% protection respectively. However, once again the number of worms recovered from the lungs and guts was still considerably lower than expected, given the number of larvae used in the challenge infection.

Table 5.6 The number of worms recovered from the lungs (4 days post infection) and small intestines (9 days post infection) of BALB\c mice following vaccination with a mixture of purified larval proteinases

	Lung recovery		Small intestine recovery	
Group	**Mean ±1SD (n=6)**	**% Protection**	**Mean ±1SD (n=6)**	**% Protection**
1° Infection (Group A)	16.5 ± 10.3	0	4.5 ± 3.1	0
Adjuvant Control (Group B)	23.3 ± 18.5	-41.2	5.5 ± 4.6	-22.2
Vaccinated (Group C)	17 ± 9.6 (n=5)	-1.03	2.8 ± 2.5 (n=5)	+37.7
Multiple Infection Control (Group E)	9.5 ± 4.3	+40.6	0	+100

SPF BALB\c mice were vaccinated with a mixture of purified larval proteinases as described in section 5.2.10.1, and the number of worms establishing in the lungs and small intestine assessed four and nine days post challenge infection respectively. The results are expressed as the mean number of worms recovered from the lungs ± 1SD (six mice per group except where indicated) and the percentage protection conferred, compared with the primary infection control group.

Table 5.7 The number of worms recovered from the lungs (4 days post infection) and small intestines (9 days post infection) of BALB\c mice following vaccination with γ-irradiated larvae (Experiment 1)

	Lung recovery		Small intestine recovery	
Group	**Mean ±1SD (n=6)**	**% Protection**	**Mean ±1SD (n=6)**	**% Protection**
1° Infection (Group A)	33.6 ± 9.9	0	11.5 ± 2.4	0
Water Control (Group B)	22.3 ± 9.2	+33.6	5.2 ± 1.4	+54.7
Vaccinated (Group C)	35.2 ± 13.9 (n=5)	-4.7	0.83 ± 2.5	+92.7
Multiple Infection Control (Group E)	38.5 ± 8.8 (n=5)	-14.5	1.5 ± 1.2	+86.9

SPF BALB\c mice were vaccinated with γ-irradiated larvae as described in section 5.2.10.2, and the number of worms establishing in the lungs and small intestine assessed four and nine days post challenge infection respectively. The results are expressed as the mean number of worms recovered from the lungs ± 1SD (six mice per group except where indicated) and the percentage protection conferred, compared with the primary infection control group.

5.3.3 WORM RECOVERY FOLLOWING VACCINATION WITH γ-IRRADIATED LARVE (EXPERIMENT 2)

The number of worms recovered from the small intestine of the primary infection control group (Group A) 9 days post challenge were again disappointingly low (0.5 ± 0.8) given the number of larvae used in the challenge infection. Therefore, while worm burdens appeared to be reduced in the vaccinated groups C and G (0.33 ± 3.2 and 0 respectively) it was impossible to determine the significance of this result, given the low numbers of worms found in the primary infection control and the data from this experiment are not shown here.

5.3.4 THE TIME COURSE OF PRIMARY INFECTION WITH *N. AMERICANUS*

Figure 5.1 shows the number of worms recovered from the lungs and small intestines of BALB/c mice following infection with normal and irradiated larvae. Lung worm burdens (Figure 5.1a) were shown to peak at day 3 following percutaneous infection with normal larvae. The progress of irradiated larvae administered percutaneously and subcutaneously appeared to be retarded and the number of worms recovered reduced. Following percutaneous infection the number of irradiated worms recovered peaked between days 4 and 7, while following subcutaneous administration the number of worms recovered peaked on day 7.

The number of worms recovered from the small intestine of BALB/c mice infected with normal and irradiated larvae are shown in Figure 5.1b. Following infection with normal larvae the number of worms recovered peaked 8 days following infection and not 9 days as previously reported although this difference appeared to be not statistically significant. The number of worms recovered following percutaneous infection with irradiated larvae also peaked on day 8 although the number of worms recovered was reduced. Worm recovery following subcutaneous administration was greatly reduced with only a small peak occurring at day 7. The results obtained from this experiment would seem to suggest that the time course of primary infection for *N. americanus* has changed and that assessment of the number of worms present in the small intestine should be carried out 8

days following infection. It should also be noted that even on day 8 the number of worm recovered was still highly variable and lower than expected following infection with 300 larvae.

5.3.5 WORM RECOVERY FOLLOWING VACCINATION WITH γ-IRRADIATED LARVAE (EXPERIMENT 3)

Following the re-evaluation of the time course of primary infection the vaccination using γ-irradiated larvae was repeated again. BALB\c mice were divided into the same groups as those described for Experiment 2 and the protocol used was the same with the exception that Group E (multiple infection control) was given a further infection with 300 normal larvae on day 14. The number of worms in the small intestine was assessed on both days 8 and 9 to ensure that the peak worm burden was observed. The protocol is summarised in Table 5.4

Table 5.8 shows the number of actively migrating larvae recovered from the lungs 3 days post challenge infection and the number of worms recovered from the small intestine on days 8 and 9 post challenge infection.. In the lungs larval numbers were reduced in both groups of vaccinated animals, 89.3 % in group C (subcutaneous vaccination) and 97.8 % in group G (percutaneous vaccination). Larval numbers were also reduced by 67.5 % in the group of animals vaccinated twice with normal larvae prior to challenge (Group E). On day 8 the number of worms recovered from the small intestine of mice in the primary infection control group were closer to the numbers expected given the size of the challenge infection. No worms were recovered in either of the groups vaccinated with irradiated larvae while worm burdens were reduced by 58.3 % in the multiple infection control group (Group E). On day nine no worms were recovered from either group of vaccinated animals; however, the number of worms recovered from the primary infection control group (Group A) were significantly lower than those observed on day 8 confirming the validity of the trend observed in the study of the re-evaluation of the primary infection time course.

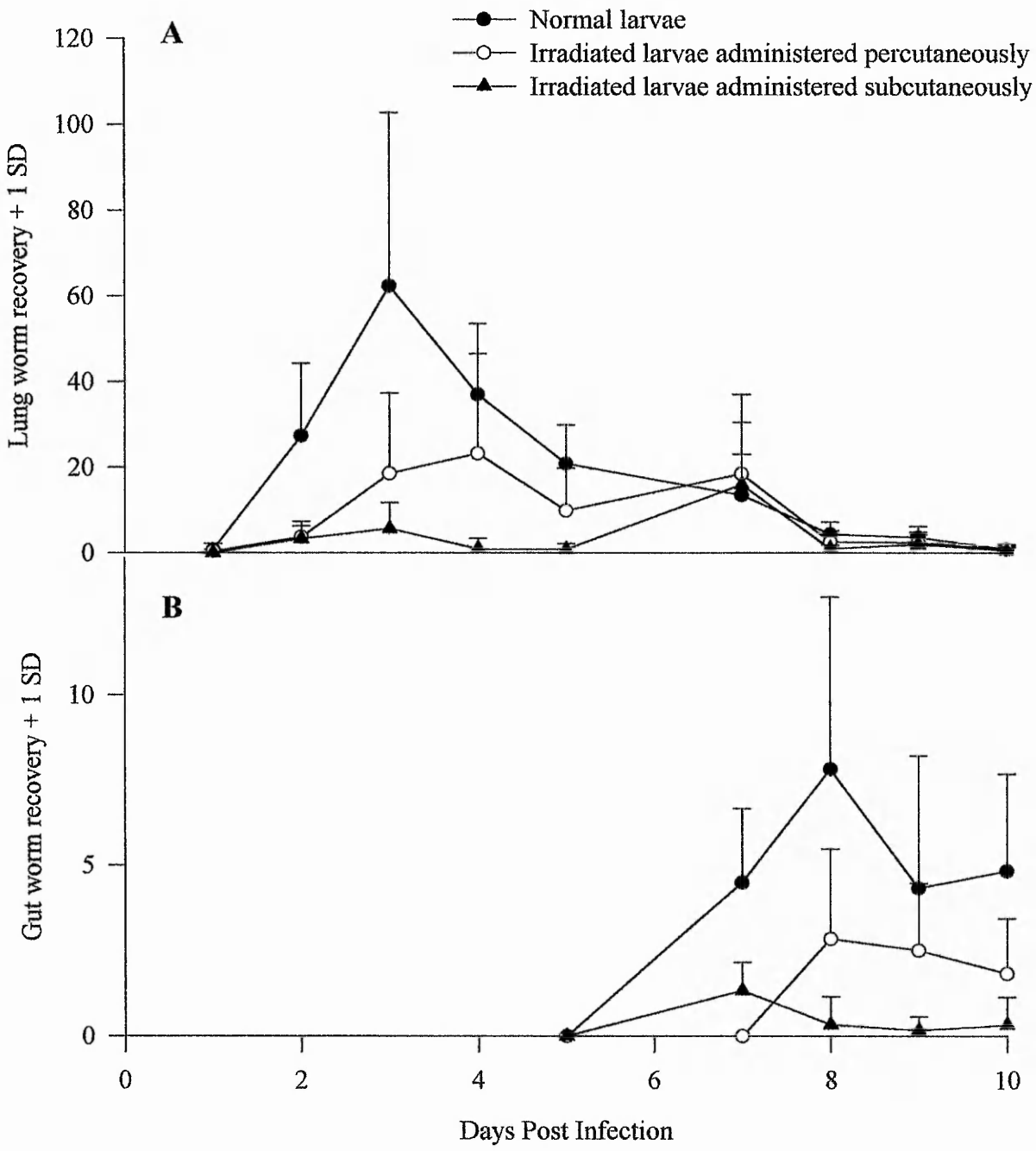

Figure 5.1 The number of actively migrating larvae recovered from the lungs and small intestine of BALB/c mice following infection with 300 normal or irradiated *N. americanus* larvae.

3 groups of mice were infected with normal or irradiated larvae and the number of actively migrating larvae in the lungs (A) and the number of worms in the small intestine (B) determined on days 1,2,3,4,5,7,8,9 and 10 following infection. Results are expressed as the mean (n=6) + 1SD of the number of worms recovered.

Table 5.8 The number of worms recovered from the lungs (3 days post infection) and small intestines (8 and 9 days post infection) of BALB\c mice following vaccination with γ-irradiated larvae (Experiment 3)

Group	Lung recovery		Small intestine recovery (Day 8)		Small intestine recovery (Day 9)	
	Mean ±1SD (n=6)	% Protection	Mean ±1SD (n=6)	% Protection	Mean ±1SD (n=6)	% Protection
1° Infection (Group A)	94.3 ± 17.5	0	18 ± 7.5	0	2.5 ± 1	0
Subcutaneous water control (Group B)	82.3 ± 9.2	+12.7	14.2 ± 4.7	+21.1	2.8 ± 1.2	-12
Vaccinated subcutaneously (Group C)	10 ± 5.1 (n=5)	+89.3	0	+100	0	+100
Multiple infection control (Group E)	30.6 ± 6 (n=5)	+67.5	7.5 ± 1.4 (n=5)	+58.3	2 ± 0.6 (n=5)	+20
Percutaneous water control (Group F)	100.5 ± 12.1	-6.5	14.5 ± 4.7 (n=4)	+19.4	2.7 ± 1.2 (n=4)	-8
Vaccinated percutaneously (Group G)	2 ± 2.8	+97.8	0	+100	0	+100

SPF BALB\c mice were vaccinated with γ-irradiated larvae as described in Section 5.2.10.2, and the number of worms establishing in the lungs and small intestine assessed three, eight and nine days post challenge infection respectively. The results are expressed as the mean number of worms recovered from the lungs ± 1SD (six mice per group except where indicated) and the percentage protection conferred, compared with the primary infection control group.

Experiment 3 demonstrated that complete immunity to *N. americanus* may be induced in BALB/c mice using γ-irradiated larvae. However, it was also observed that worm burdens were also reduced by vaccinating mice with normal larvae on two occasions although this immunity was not complete (Table 5.8). As this group of animals had only been vaccinated twice with normal larvae the experiment was repeated one further time to compare the ability of three doses of normal larvae to confer immunity with three doses of γ-irradiated larvae. Despite the improved worm recovery on day 8, there were still some concerns over the efficiency of the sticking plaster used to infect the mice therefore, in the final experiment the mice were vaccinated and challenged using a vetinary bandage obtained from International Market Supply (Section 5.2.2).

5.3.6 WORM RECOVERY FOLLOWING VACCINATION WITH γ-IRRADIATED LARVAE (EXPERIMENT 4)

The protocol for experiment 4 was identical to that used for Experiment 3 with the exception that the 'multiple infection' control group was given a further vaccination with 300 normal larvae on day 28 and the mice were infected and challenged using the new self binding bandage (Section 5.2.2). The protocol is summarised in Table 5.5.

Table 5.9 shows the number of actively migrating larvae recovered from the lungs 3 days post infection and from the small intestine 8 days post infection. On day 3 vaccination with three doses of normal larvae resulted in a 74.2 % reduction in larval recovery from the lungs. However, larval recovery was further reduced following vaccination with γ-irradiated larvae by either the subcutaneous (92 %) or percutaneous route (95.6 %). On day 8 complete immunity was observed in the mice vaccinated with γ-irradiated larvae (Groups C and G) and no worms were recovered from the small intestines of either group of mice. Vaccination with 3 doses of normal larvae (Group E) also resulted in a reduction in worm burden (77 %). However, it was also observed on day 3 the lungs of the mice in this group were damaged showing areas of haemorrhaging compared to the mice vaccinated with γ-irradiated larvae.

Table 5.9 The number of worms recovered from the lungs (3 days post infection) and small intestine (8 days post infection) of BALB\c mice following vaccination with γ-irradiated larvae (Experiment 4)

Group	Lung recovery		Small intestine recovery	
	Mean ±1SD (n=6)	% Protection	Mean ±1SD (n=6)	% Protection
1° Infection (Group A)	104 ± 20.7	0	19.3 ± 3.07	0
Subcutaneous water control (Group B)	121.6 ± 36.1	-16.9	16.6 ± 4.7	+13.9
Vaccinated subcutaneous (Group C)	8.3 ± 4.5	+92	0	+100
Multiple Infection Control (Group E)	26.8 ± 13.2	+74.2	4.3 ± 2.5	+77.7
Percutaneous Water control (Group F)	91.5 ± 28.7	+12	16.8 ± 3.4	+15.4
Vaccinated Percutaneous (Group G)	4.6 ± 2.6	+95.6	0	+100

SPF BALB\c mice were vaccinated with γ-irradiated larvae as described in section 5.2.10.2, and the number of worms establishing in the lungs and small intestine assessed 3 and 8 days post challenge infection respectively. The results are expressed as the mean number of worms recovered from the lungs ± 1SD (six mice per group except where indicated) and the percentage protection conferred, compared with the primary infection control group.

5.3.7 ELISA ANALYSIS OF ANTIBODY RESPONSES AGAINST *N. AMERICANUS* FOLLOWING VACCINATION WITH PURIFIED LARVAL PROTEINASES OR γ–IRRADIATED LARVAE

5.3.7.1 ELISA ANALYSIS FOLLOWING VACCINATION WITH PURIFIED LARVAL ASPARTYL, CATHEPSIN B AND L 'LIKE' PROTEINASES

Levels of combined mouse immunoglobulins G, A and M and IgG subclasses (IgG1, IgG2a and IgG3) following vaccination with purified larval aspartyl, cathepsin B and cathepsin L 'like' proteinases (see Table 5.1 for a summary of the protocol and Table 5.6 for the worm recovery data) were determined by ELISA as described in section 5.2.8. Figure 5.2 shows the IgGAM signal recorded against the individual purified proteinases. IgGAM signals were highest in the vaccinated mice (Group C) at all time points tested. On the day of challenge (day 42) antibody signals were greater against the cathepsin L 'like' proteinase (Abs 0.461 ± 0.153) compared with the aspartyl and cathepsin B 'like' proteinases (Abs 0.263 ± 0.106 and 0.286 ± 0.111 respectively). Following challenge infection with 250 *N. americanus* larvae, the Ig GAM signals against the cathepsin L 'like' proteinase was elevated at four days post challenge (day 46) infection but had fell back to pre-challenge levels by day nine (day 51). IgGAM antibody signals against the aspartyl and cathepsin B 'like' proteinases remained unchanged throughout the period of challenge infection.

IgG1, IgG2a, IgG2b and IgG3 signals against larval ES products following vaccination with purified proteinases are shown in Figure 5.4. The production of IgG1 was the predominant antibody response observed with little IgG2a and IgG3 being detected. IgG1 signals were however, elevated in the multiple infection control group (Group E) in comparison with the vaccinated group of animals (Group C). Similarly, the IgG2a response observed, while being reduced compared with IgG1 was higher in Group E compared with Group C. An IgG3 response in Group C was observed on day 14 following the first vaccination with larval proteinases but appeared to decline following subsequent vaccinations and challenge infection. No detectable signal for IgG2b was observed.

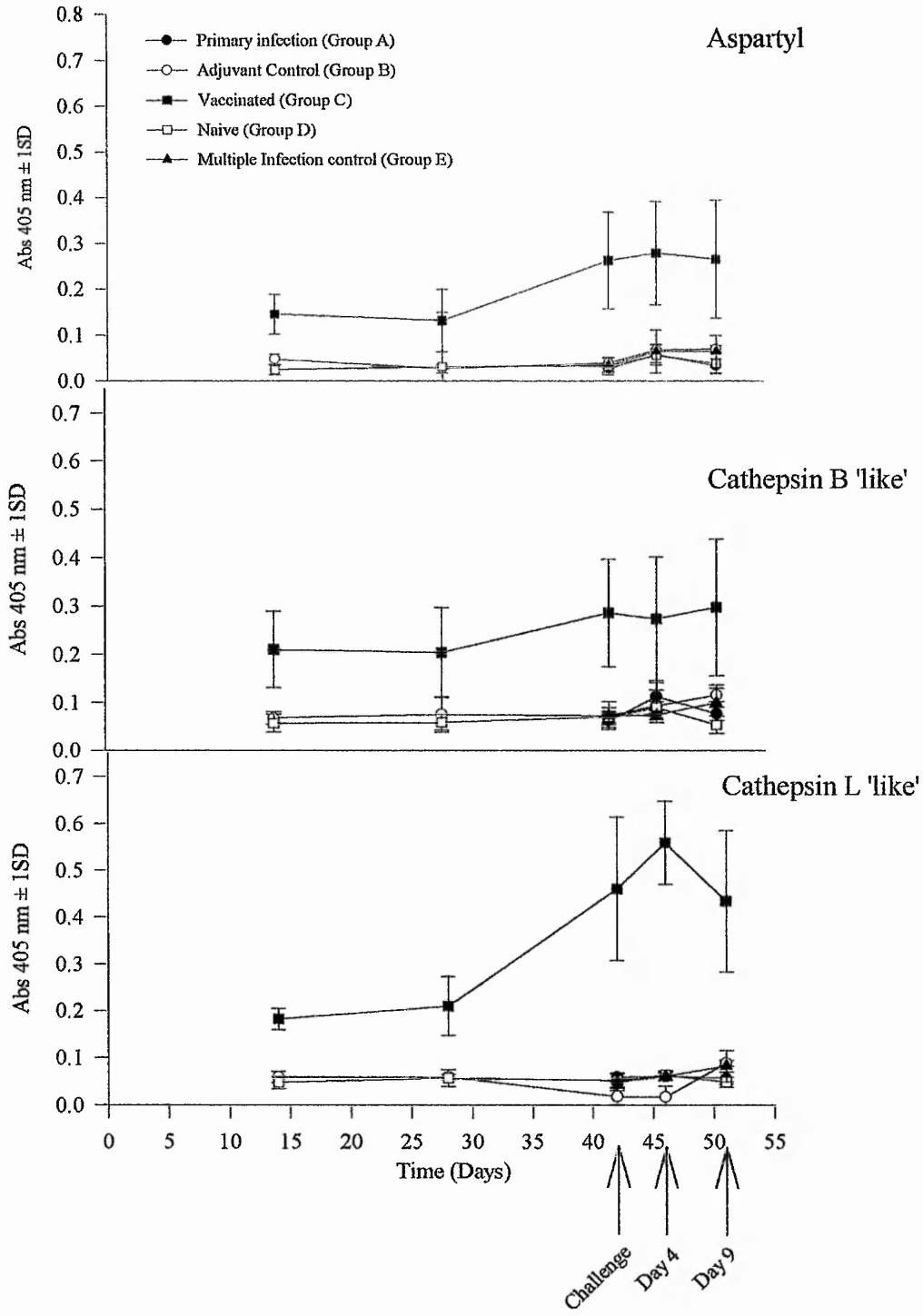

Figure 5.2 Combined immunoglobulin G, A and M levels in BALB\c mice against the larval aspartyl and cathepsin B and L 'like' proteinases following vaccination with a mixture of purified larval proteinases

IgGAM levels in vaccinated mice were determined by ELISA as described in section 5.2.8. Results at each time point are expressed as the mean absorbance of individual mouse serum samples (6 mice per group except where indicated in the vaccination protocol, all assays carried out in triplicate) ± 1SD following the subtraction of a negative control.

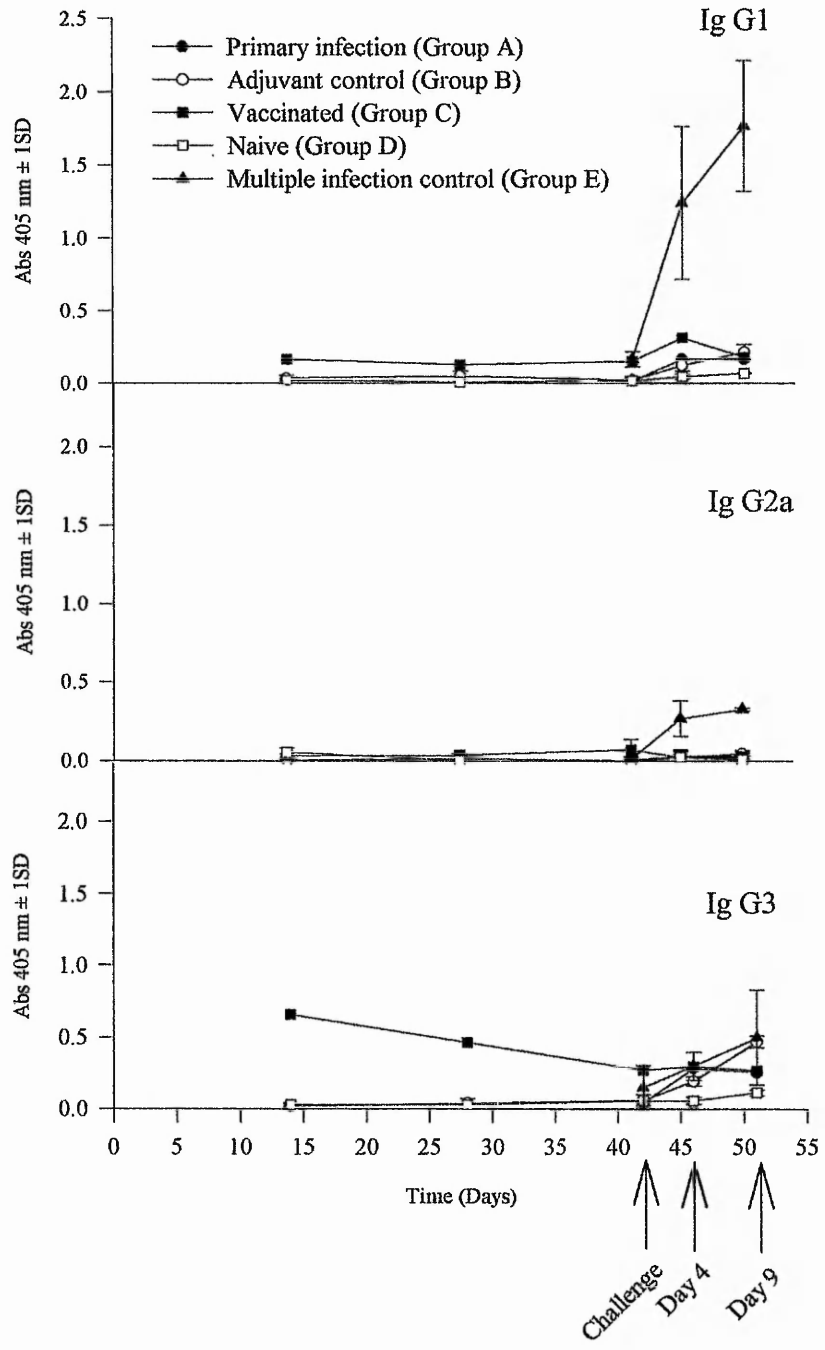

Figure 5.3 IgG1, IgG2a and IgG3 levels in BALB\c mice against larval ES products following vaccination with a mixture of purified larval proteinases

IgG1, IgG2a and IgG3 levels in vaccinated mice were determined by ELISA as described in section 5.2.8. Results at each time point are expressed as the mean absorbance of individual mouse serum samples (6 mice per group except where indicated in the vaccination protocol, all assays carried out in triplicate) ± 1SD following the subtraction of a negative control.

5.3.7.2 ELISA ANALYSIS FOLLOWING VACCINATION WITH γ-IRRADIATED LARVAE (EXPERIMENT 1)

Figure 5.4 shows the combined Ig GAM signal against normal larval ES products observed in BALB\c mice following vaccination with *N. americanus* irradiated larvae (Worm burden data in Table 5.7). Mice vaccinated with irradiated larvae (Group C) showed an increase in antibody signal throughout the vaccination protocol. On challenge (day 42) these signals increased until day 46 (4 days post challenge) and remained at his level up to day 56 (9 days post challenge). Mice vaccinated with normal larvae also showed a sharp increase in antibody signal following the final challenge infection peaking on day 46 and falling by day 51. Groups A and B (primary infection and water controls respectively) showed antibody levels similar to naïve mice up until the day of challenge infection, after which antibody levels peaked on day 46 and fell again by day 51.

A similar response was observed when antibody levels were determined against irradiated larval ES products (data not shown).

5.3.7.3 ELISA ANALYSIS OF IgG SUBCLASS RESPONSE (EXPERIMENT 3)

Individual serum from vaccination experiment 3 using irradiated larvae (worm burden data in Table 5.8) was used to examine the IgG subclass response to vaccination and subsequent challenge with *N. americanus*. Figures 5.5A-C show the IgG subclass signals as determined by ELISA (Section 5.2.8).

On day 42, prior to challenge infection, IgG1 levels in the animals vaccinated with both irradiated and normal larvae (Groups C, G and E) had increased above those of the relevant control animals (Figure 5.5A). IgG1 levels were particularly enhanced in those animals vaccinated percutaneously. IgG2a levels were raised in both groups (C and G) of animals vaccinated with irradiated larvae. IgG2b signals were extremely low. IgG3 was elevated in the group of animals vaccinated percutaneously with irradiated larvae (Group G).

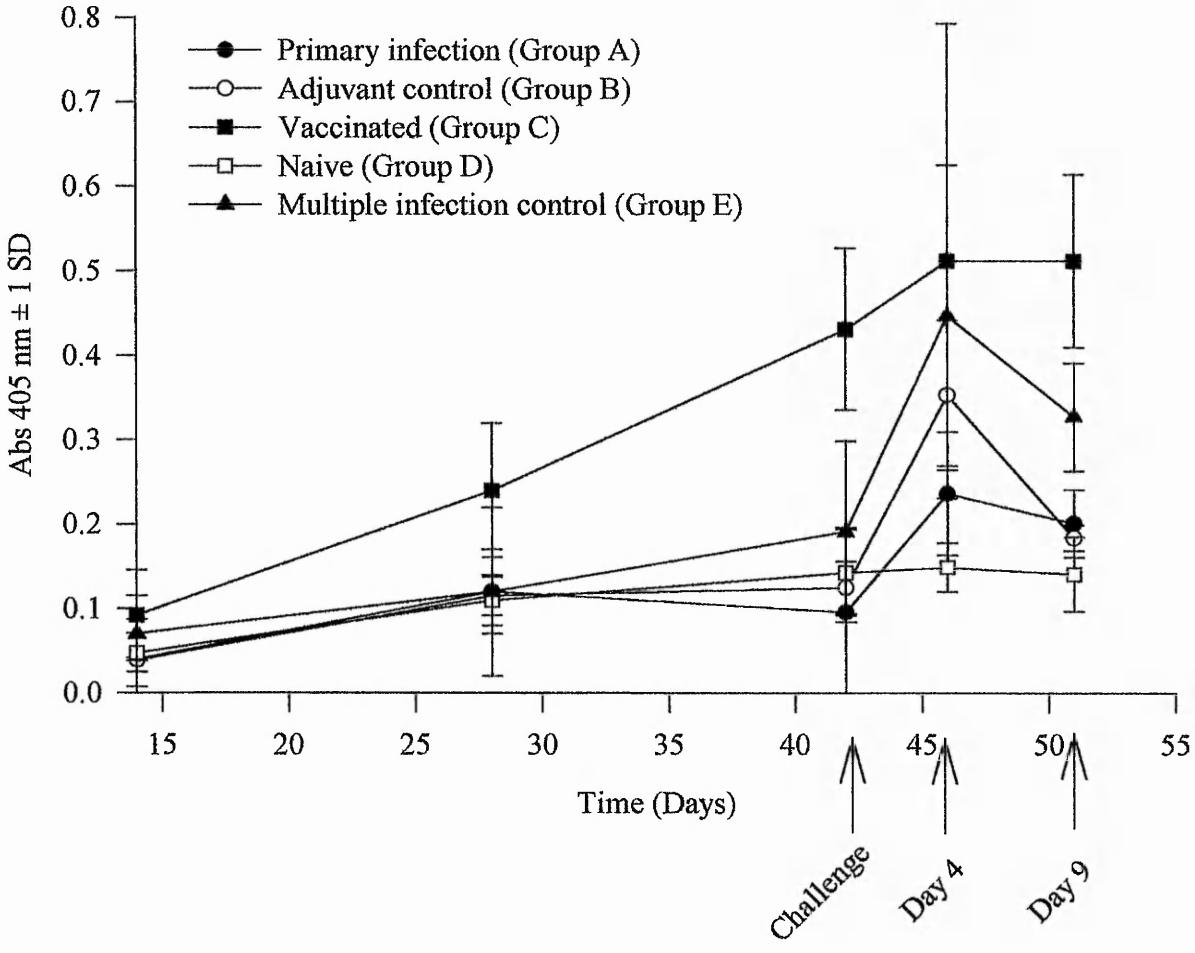

Figure 5.4 Immunoglobulin G, A and M levels in BALB\c mice against normal larval ES products following vaccination with γ-irradiated larvae (Experiment 1).

IgGAM levels in vaccinated mice were determined by ELISA as described in section 5.2.8. Results at each time point are expressed as the mean absorbance of individual mouse serum samples (6 mice per group except where indicated in the vaccination protocol, all assays carried out in triplicate) ± 1SD following the subtraction of a negative control.

Overall, it is worth noting the apparent differences in the IgG1 responses of the animals vaccinated with irradiated larvae via the subcutaneous (Group C) and percutaneous (Group G) routes. Both groups of animals were ultimately immune to challenge infection (Table 5.8) however, IgG1 responses in Group C increased throughout the period of challenge infection while IgG1 levels in Group G fell between days 42 and 45 and remained at a similar level between days 45 and 50. IgG1 responses in Group E (vaccinated with normal larvae) mimicked those in Group C (although at a lower level) despite being vaccinated by the same route (percutaneous) as the animals in Group G. Similar results were obtained for IgG2a.

Following challenge infection IgG3 levels increased on day 45 in both groups of animals vaccinated with irradiated larvae (Groups C and G) in comparison with the group of animals vaccinated with normal larvae. These antibody levels are reflective of larval recovery in the lungs (Group C, 10 ± 5.1, Group E, 30.6 ± 6, Group G, 2 ± 2.8). On day 50 IgG3 levels fell in both groups of animals vaccinated with irradiated larvae but remained higher than Group E whose IgG3 levels remained similar. Both Groups C and G demonstrated complete immunity to challenge infection while Group E showed a worm recovery of 7.5 ± 1.4.

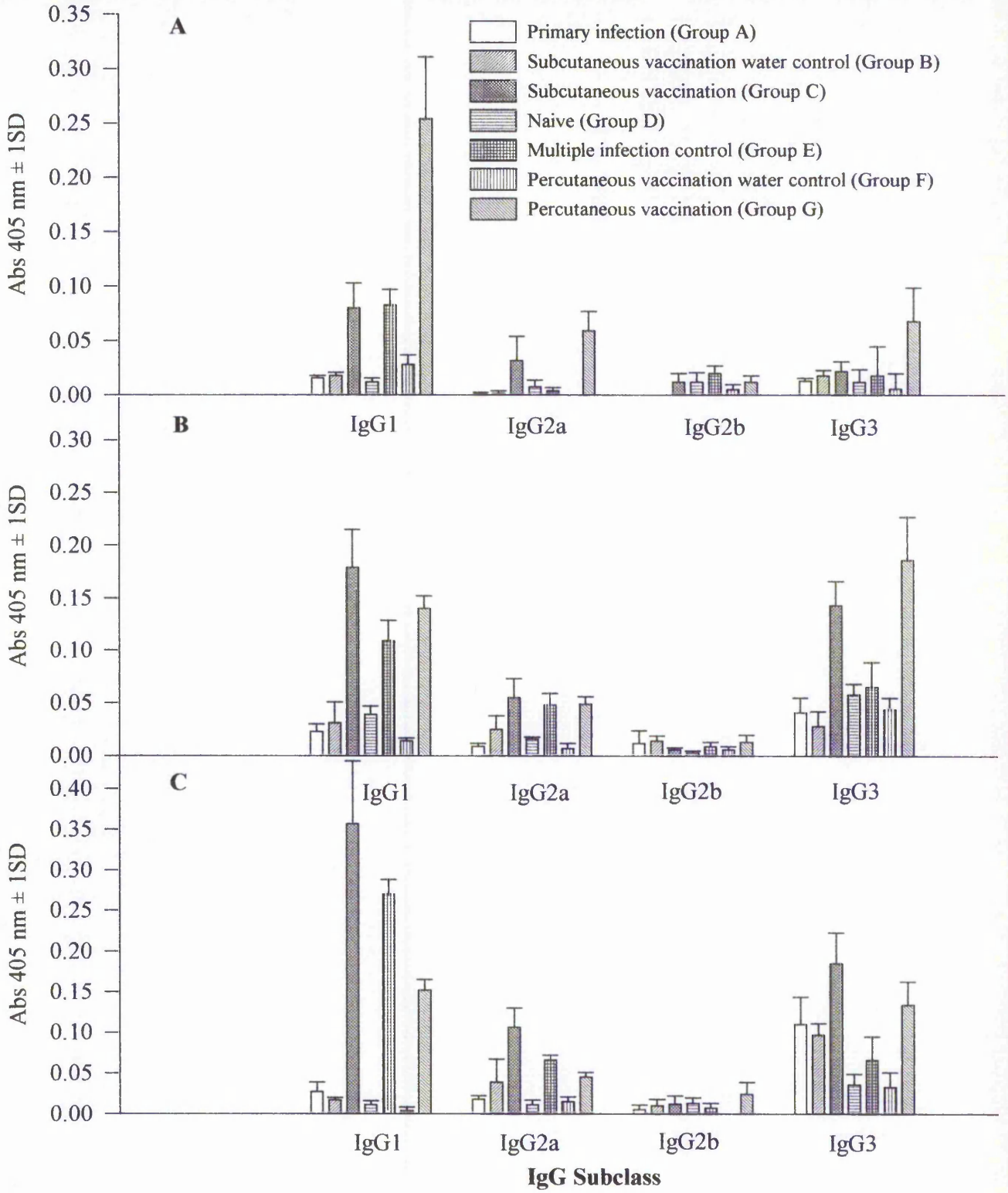

Figure 5.5 Characterisation of the IgG subclass response on (A) day 42 (pre-challenge) (B) day 45 and (C) day 50 following vaccination with irradiated *N. americanus* larvae.

Ig G subclass levels were determined as described in section 5.2.8. Results are expressed as the mean absorbance of sera obtained from individual mice ± 1 SD, (each assay carried out in triplicate) following the subtraction of a negative control.

5.3.7.4 WESTERN BLOT ANALYSIS FOLLOWING VACCINATION WITH γ-IRRADIATED LARVAE

The results obtained from the Western blot analysis of the antibody responses during the first attempt at vaccinating mice with irradiated larvae were of poor quality and are not shown here. A more detailed Western blot analysis of the antibody response in the third vaccination is presented.

5.3.7.4.1 WESTERN BLOT ANALYSIS OF IgG SUBCLASS RESPONSES

The serum samples collected during the third vaccination experiment were used to attempt to detect a difference in the IgG1 and IgG2a responses between vaccinated and non-vaccinated mice. These studies concentrated on the time of challenge infection (day 42, Figure 5.6) and during the time course of infection at the points when larval numbers peak in the lungs (day 45, Figure 5.7) and the small intestine (day 50, Figure 5.8).

Figures 5.6, 5.7 and 5.8 show the binding profiles of IgG1 and IgG2a against larval ES products on days 42, 45 and 50 respectively. In animals vaccinated with normal or irradiated larvae a predominant IgG1 response was seen against a ladder of proteins ranging between approximately 30-40 kDa, although this response was not always seen in every vaccinated animal. Binding of IgG1 appeared to reflect the ELISA data to some degree. Binding of IgG1 in the mice vaccinated subcutaneously with irradiated larvae appeared to increase during the period of challenge infection while IgG1 binding in the mice vaccinated percutaneously with irradiated larvae (Group G) diminished over the same time period. IgG1 binding in the mice vaccinated with normal larvae (Group E) deviated from the ELISA data showing a decrease in binding on day 45 compared to day 42, followed by an increase in binding on day 50.

IgG2a from vaccinated animals also bound to the same protein ladder in larval ES products although the response was weaker than that seen for IgG1. IgG2a binding appeared to diminish between days 24-45 with a slight increase by day 50.

Western blots of *N. americanus* larval homogenate were also probed with individual sera from vaccination experiment 3. Figure 5.9 shows the IgG1 response against larval homogenate on days 42 and 50. Again the predominant response on day 42 in the vaccinated animals was against a protein ladder between approximately 30-40 kDa, with some response to higher molecular mass antigens although this response resulted in smearing at the top of the blot. On day 50 following challenge infection the response was enhanced in all vaccinated groups unlike the response seen against larval ES products, with some response against the protein ladder also becoming apparent in groups A and B. On day 42 no IgG2a binding to larval homogenate was observed (not shown), however, on day 50 some weak binding to higher molecular mass proteins was observed in the vaccinated groups of animals (Figure 5.10).

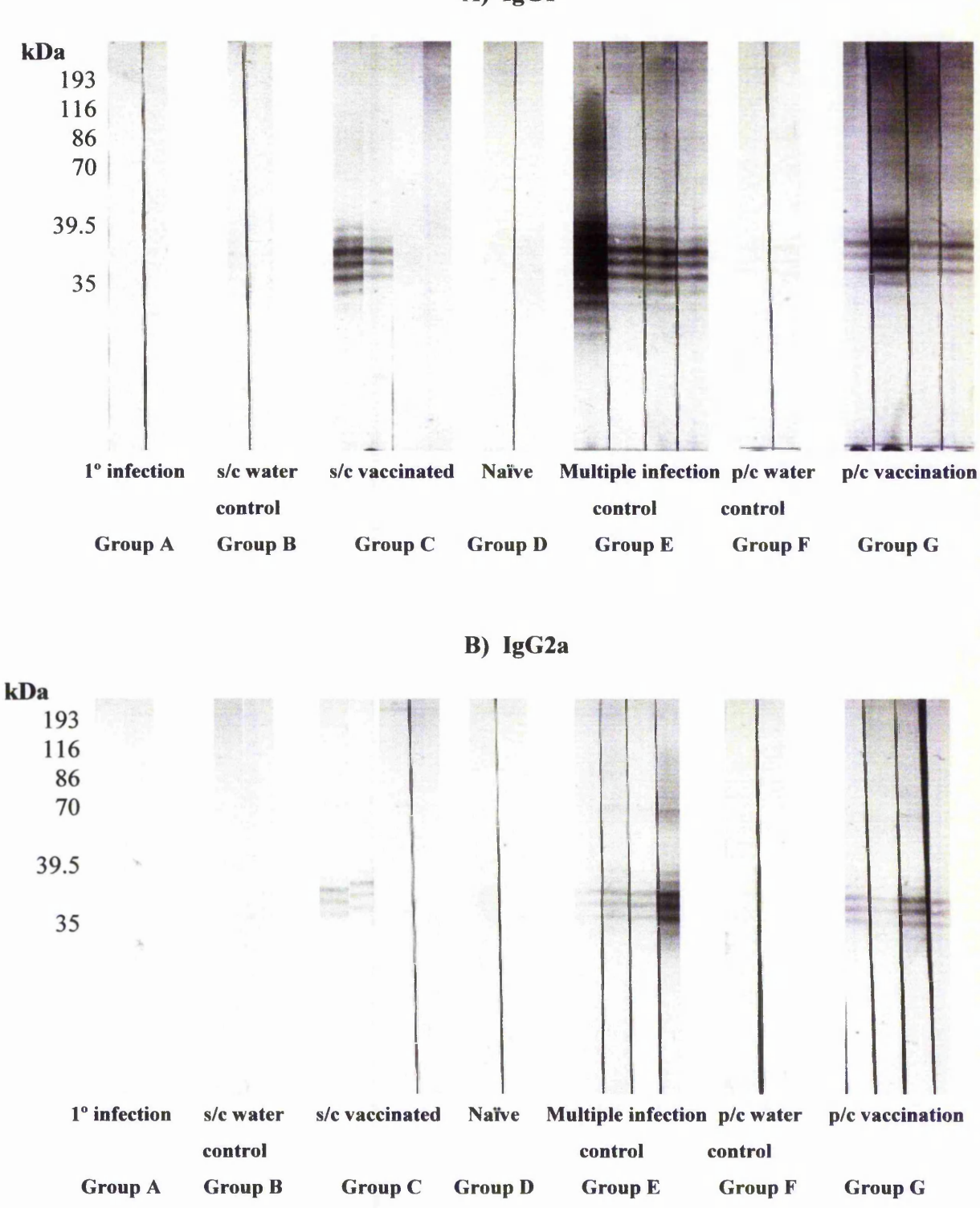

Figure 5.6 Western blot analysis of IgG1 (A) and IgG2a (B) subclass responses on day 42 against *N. americanus* larval ES products following vaccination with irradiated larvae.

Western blots of *N. americanus* larval ES products were probed as described in section 5.2.9 with serum from individual mice following vaccination with irradiated larvae Molecular mass markers are indicated.

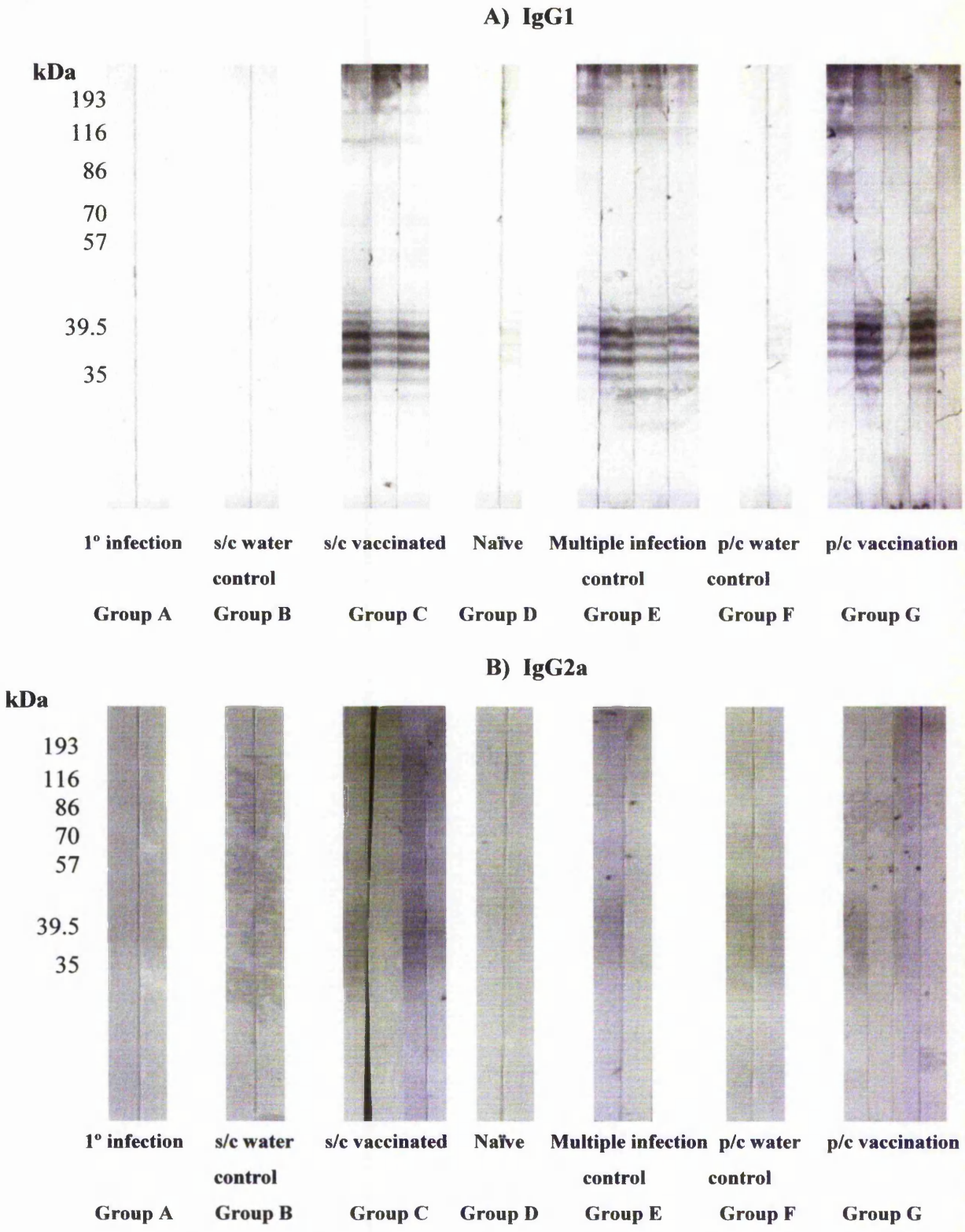

Figure 5.7 Western blot analysis of IgG1 (A) and IgG2a (B) subclass responses on day 45 against *N. americanus* larval ES products following vaccination with irradiated larvae.

Western blots of *N. americanus* larval ES products were probed as described in section 5.2.9 with serum from individual mice following vaccination with irradiated larvae Molecular mass markers are indicated.

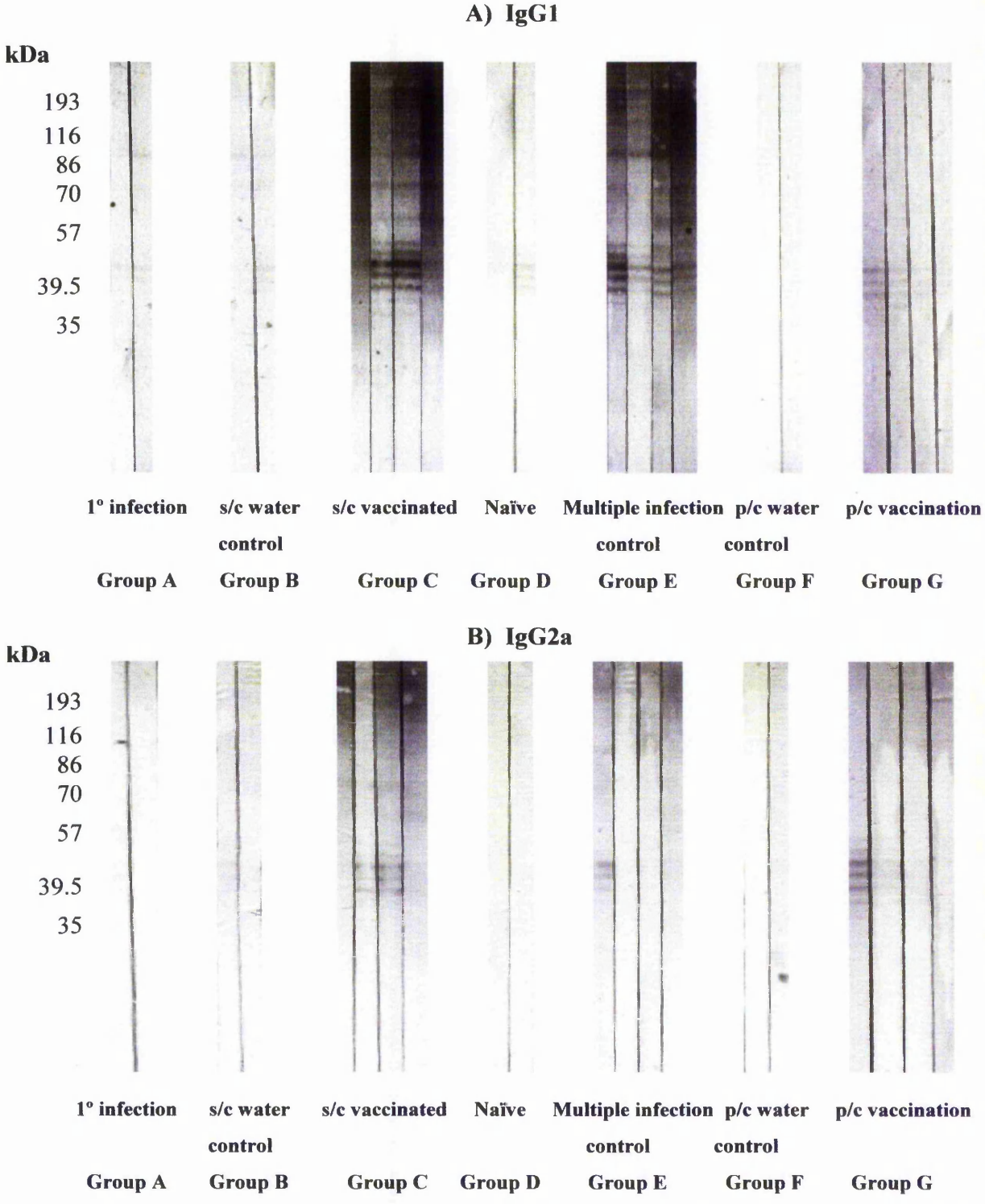

Figure 5.8 Western blot analysis of IgG1 and IgG2a subclass responses on day 50 against *N. americanus* larval ES products following vaccination with irradiated larvae.

Western blots of *N. americanus* larval ES products were probed as described in section 5.2.9 with serum from individual mice following vaccination with irradiated larvae Molecular mass markers are indicated.

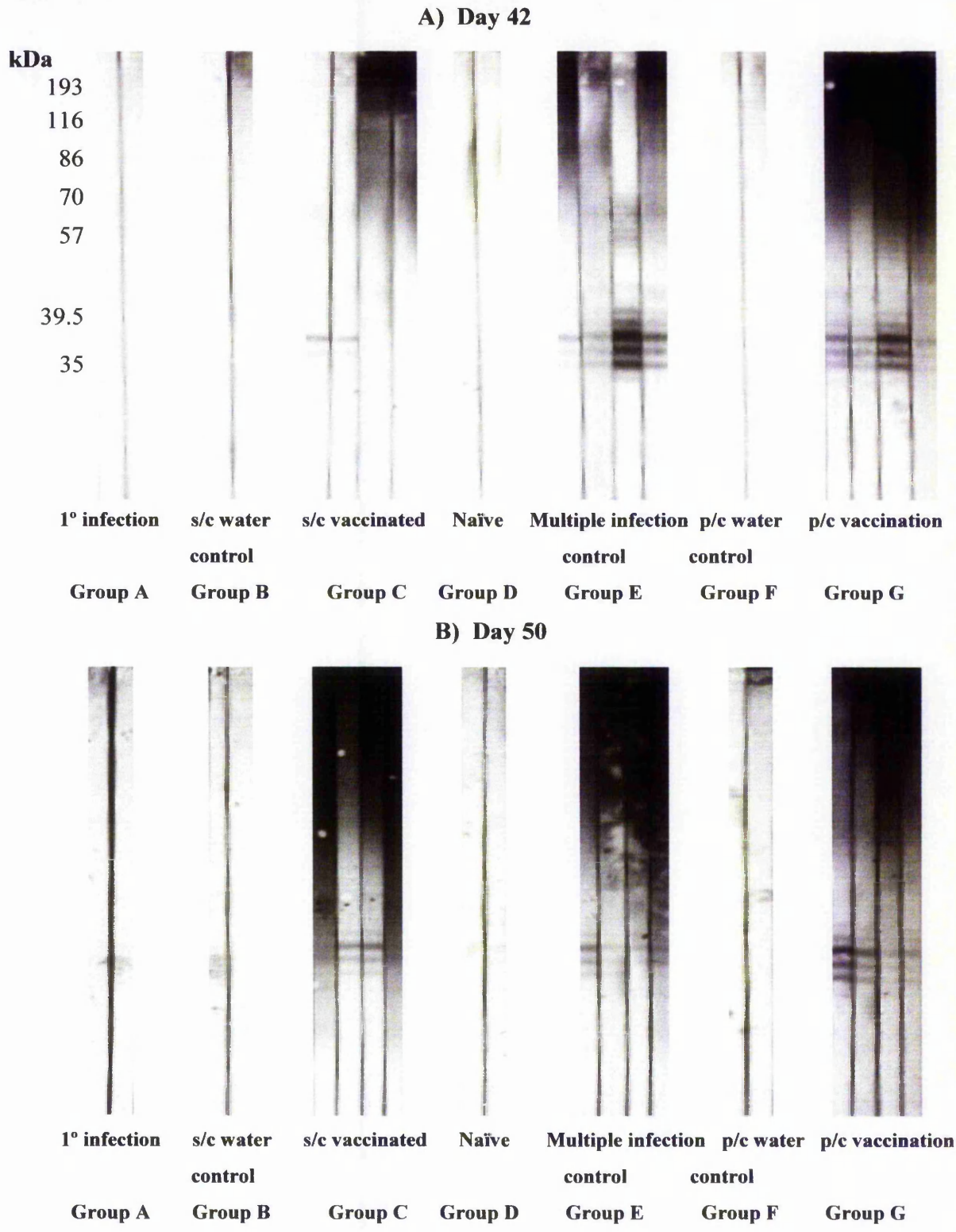

Figure 5.9 Western blot analysis of IgG1 responses on day 42 (A) and 50 (B) against *N. americanus* larval homogenate following vaccination with irradiated larvae.

Western blots of *N. americanus* larval homogenate were probed as described in section 5.2.9 with serum from individual mice following vaccination with irradiated larvae Molecular mass markers are indicated.

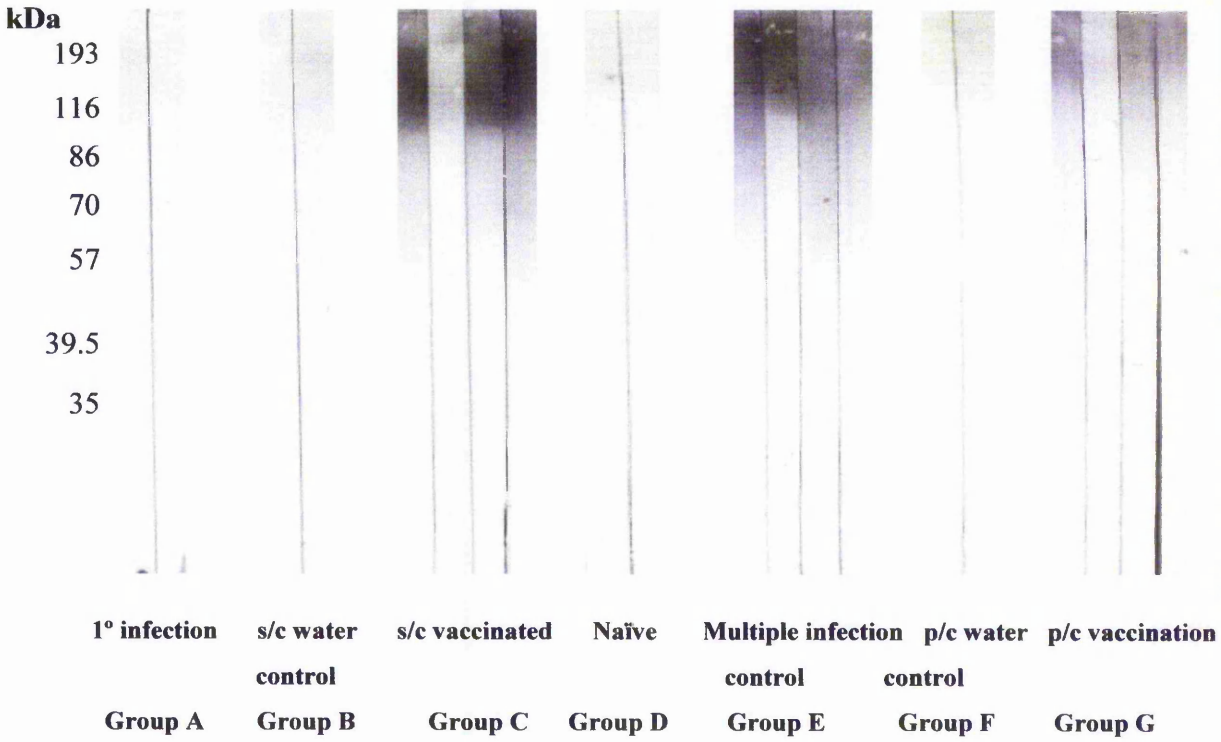

Figure 5.10 Western blot analysis of IgG2a responses on day 50 against *N. americanus* larval homogenate following vaccination with irradiated larvae.

Western blots of *N. americanus* larval homogenate were probed as described in section 5.2.9 with serum from individual mice following vaccination with irradiated larvae Molecular mass markers are indicated.

5.3.8 CYTOKINE ANALYSIS FOLLOWING VACCINATION WITH IRRADIATED LARVAE

Figures 5.11 and 5.12 show γ–IFN and IL-5 levels secreted by leucocytes harvested from the spleens of animals from the fourth experiment with irradiated larvae on days 45 and 50 (3 days and 8 days post-challenge infection respectively). Spleens were removed from the mice and cytokine secretion stimulated using Conacavalin A as described in section 5.2.7. On day 45 (3 days post challenge infection, Figure 5.11) γ–IFN was detected in all experimental groups but appeared slightly depressed in those animals either vaccinated or challenged with *N. americanus*. IL-5 levels were elevated in animals vaccinated with both normal and irradiated larvae. On day 50 (8 days post challenge, Figure 5.12) γ–IFN levels were reduced in all groups with no differences being detected between vaccinated and non vaccinated animals. IL-5 levels were again elevated in all animals vaccinated with either normal or irradiated larvae.

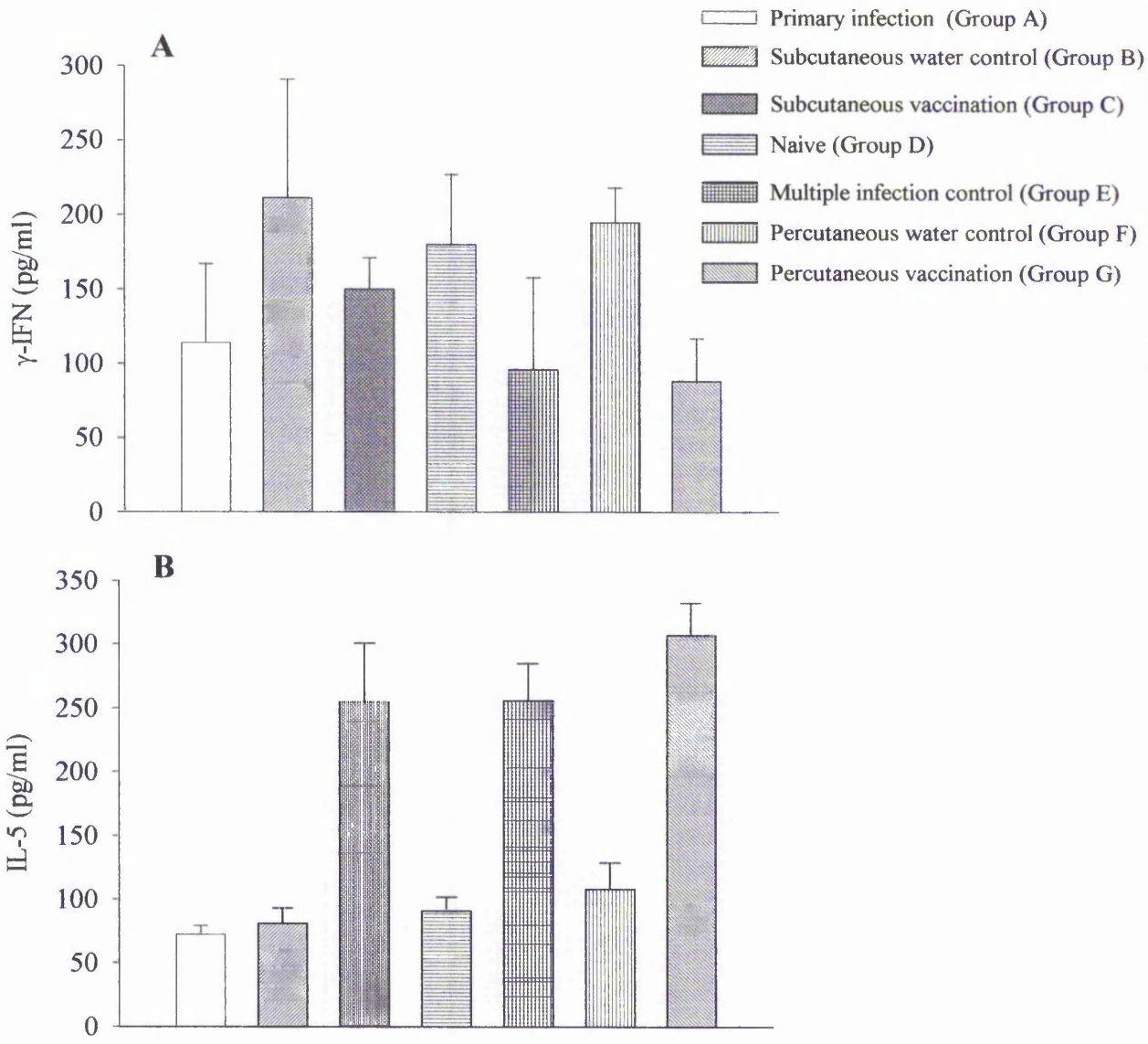

Figure 5.11 γ-IFN (A) and IL-5 (B) levels on day 45 (3 days post challenge) following vaccination with irradiated larvae.

Leucocytes were harvested from the spleen and cultured as described in section 5.2.7. Following culture γ-IFN and IL-5 levels were determined by ELISA as described in section 5.2.7.3.

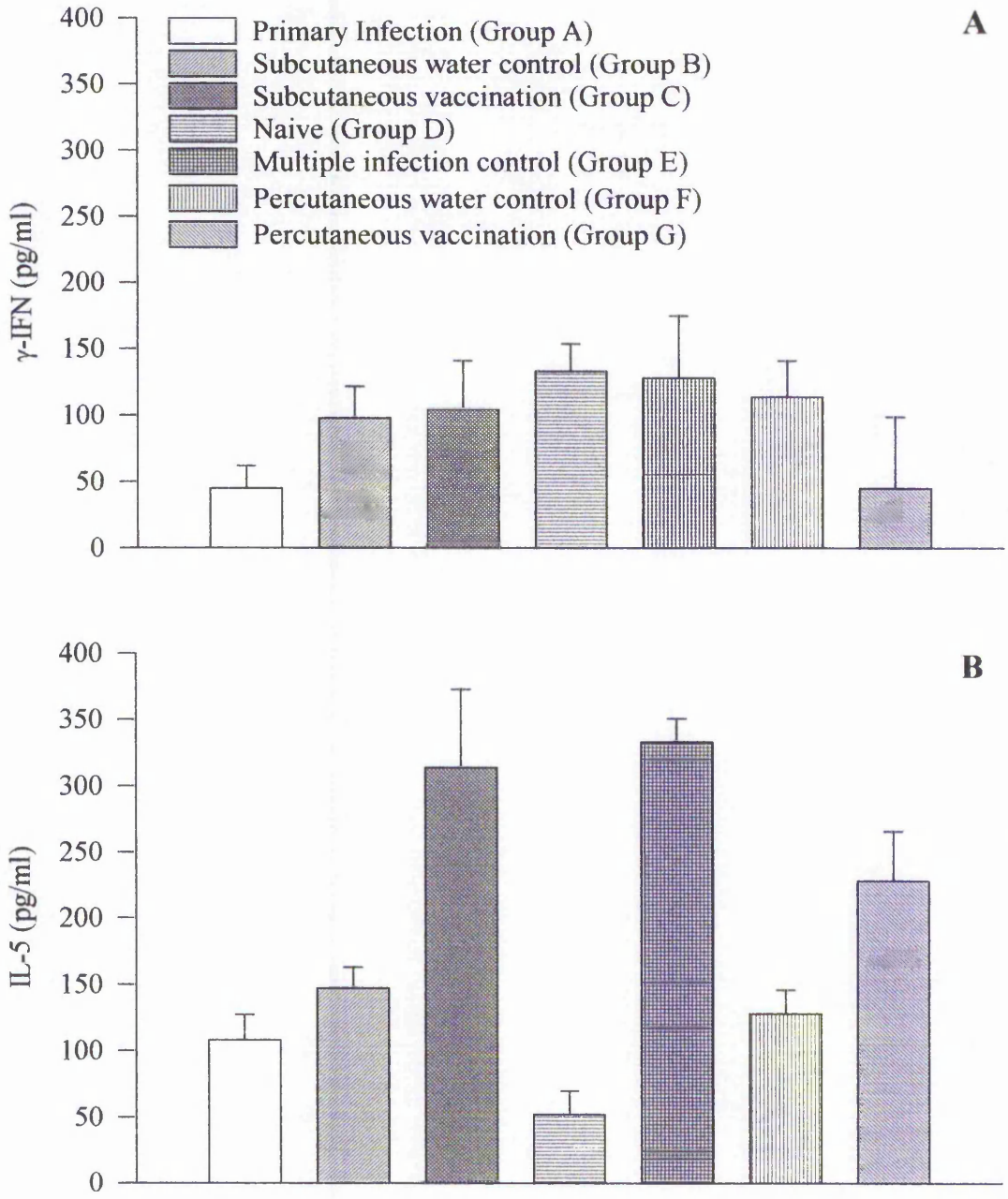

Figure 5.12 γ-IFN (A) and IL-5 (B) levels on day 50 (8 days post challenge) following vaccination with irradiated larvae.

Leucocytes were harvested from the spleen and cultured as described in section 5.2.7. Following culture γ-IFN and IL-5 levels were determined by ELISA as described in section 5.2.7.3.

5.4 DISCUSSION

The experiments presented in this chapter represent attempts to generate protective immunity in BALB\c mice against *N. americanus* infection using either a mixture of purified larval proteinases or γ-irradiated larvae.

5.4.1. SUCCESS OF VACCINATION PROTOCOLS

Following vaccination with purified larval proteinases and subsequent challenge infection, no reduction in larval recovery from the lungs was apparent on day 46. On day 51, worm recovery from the small intestine was apparently reduced by 37.7 %; however, the number of worms recovered from the primary infection control group was very small (4.5 ± 3.14) rendering the difference in worm recoveries between the primary infection control group and the vaccinated group insignificant. This experiment needs to be repeated to establish if the apparent 37.7 % reduction in worm burden is a true result. However, at this period of the study new batches of purified aspartyl and cathepsin 'like' proteinases were not available and the purification of the metallo-proteinase and serine proteinase was still ongoing making these unavailable for assessment as candidate vaccines.

As indicated earlier (Section 5.1), irradiated larvae have been shown to be particularly effective vaccines, inducing protective immunity in laboratory animals against a number of parasites e.g. *Strongylus vulgaris* (Monahan *et al.*, 1994), *Trichinella spiralis* (Agyei-Frempong & Catty. 1983), the dog hookworm *Ancylostoma caninum* (Miller. 1971) and in particular, *Schistosoma mansoni* (Dean. 1983). Due to these observations the potential of irradiated larvae to induce protective immunity against *N. americanus* infection in the Balb/c mouse model was examined. An irradiation dose of 40 krad was chosen as this was the equivalent radiation dose used by Miller (1971) to generate immunity in dogs to *A. caninum*. Similar doses have been shown to be effective in generating immunity against *Nematosporoides dubius* (Hagan *et al.*, 1981), *F. hepatica* (Creaney *et al.*, 1996) and *S. mansoni* (Mountford *et al.*, 1992). Immunity to *S. japonicum* has also been generated using ultraviolet radiation attenuated cercariae (Shi *et al.*, 1992). In addition dogs have been immunised against *A. caninum* with larvae attenuated with 5-fluorouracil (Vinayak *et al.*, 1981). However, chemical attenuation of *N. americanus* larvae has already been shown to fail to induce protective immunity in mice (Timothy, 1994). The initial

experiments (1 and 2) using irradiated larvae, despite implying that almost complete immunity was being induced, were again marred by low worm recoveries in the primary infection control group (Group A).

Whilst being well characterised (Wells & Behnke, 1988a & b; Timothy & Behnke, 1993; Timothy, 1994), the *N. americanus*/BALB/c mouse model is still less than ideal for vaccination studies and has a number of problems. *N. americanus* is an obligate human parasite which in the mouse will only proceed to the L_4 stage, failing to establish as adults in the small intestine (Wells & Behnke, 1988b). Timothy & Behnke, (1993) demonstrated that 9 days following infection worm recoveries in the small intestine peaked at only 5.5 % of the infective dose. As a result of this an infection of 300 larvae will only at best result in approximately 16.5 worms establishing in the small intestine. Therefore, should the infection not reach the expected maximum efficiency then worm recoveries from the small intestine will be very low, as demonstrated in the experiment using larval proteinases and the early experiments with γ-irradiated larvae. As described in section 5.2.2, mice are infected by securing a gauze with a strip of sticking plaster containing the infective dose to the back of the shaved neck, which is left in place for 24 h. This plaster is a source of irritation to the mouse and while it is placed in a position where it is least accessible, it is not uncommon for the mouse to remove it through constant scratching. The majority of the plasters did however, remain in place for at least 3 hours after which it is reported that the majority of larvae will enter mouse skin *in vitro* (Kumar & Pritchard, 1992c). It was also considered possible that the strain of *N.americanus* used may have become increasing rodent adapted having been maintained in hamsters for 26 years at the time these vaccination experiments were carried out. Figure 5.1 follows the time course of primary infection with normal and irradiated larvae administered percutaneously and irradiated larvae administered subcutaneously. The re-assessment of the time course of primary infection with normal *N.americanus* larvae revealed a decrease in the time taken for *N.americanus* larvae to reach both the lungs and small intestine compared with that previously reported (Wells & Behnke. 1988b; Timothy & Behnke. 1993). Larval recovery peaked in the lungs on day 3 post infection instead of day 4, while worm recovery from the small intestine was shown to peak on day 8 instead of day 9. The passage of irradiated larvae was retarded through the lungs peaking on day 7 for both routes of administration.

The number of worms recovered from the small intestine following infection with irradiated larvae was reduced compared with normal larvae peaking on day 8 for larvae administered by the percutaneous route and day 7 for the subcutaneous route. This would seem to indicate that while some irradiated larvae are capable of reaching the small intestine the passage of others is retarded either due to a direct effect of the irradiation rendering them incapable of movement, or trapping of the larvae in either the skin or the lungs.

Following this re-evaluation of the time course of primary infection two subsequent vaccination experiments (3 and 4) demonstrated that complete immunity could be induced against *N.americanus* infection by irradiated larvae. Immunity was induced regardless of whether the animals were vaccinated by the percutaneous or subcutaneous route. However, examination of the data (Table 5.7-5.8) from experiments 3 and 4 with irradiated larvae reveals that those animals vaccinated percutaneously with irradiated larvae (Group G) had slightly lower larval recoveries in the lungs than those vaccinated subcutaneously (Group C). This may suggest that immune responses in the skin following vaccination are capable of preventing larvae reaching the lungs therefore decreasing lung pathology.

It should be noted here that the assessment of worm burdens for the vaccination using larval proteinases was apparently carried out at the wrong time point and should be repeated before concluding that vaccination cannot be achieved by using larval enzymes. This work may be of increasing importance as a serine proteinase from *S. mansoni* cercariae, which is vital to the skin penetration process is now being considered as a possible vaccine against schistosomiasis (Doenhoff, 1998).

5.4.2. CHARACTERISATION OF IMMUNE RESPONSES

Antibody responses were determined by both ELISA and Western blotting during the course of all vaccination experiments carried out. Following vaccination with purified larval proteinases, IgGAM levels as determined by ELISA showed all the proteinases used to be immunogenic in vaccinated mice, with the cathepsin L 'like' proteinase being the most immunogenic (Figure 5.2). These levels persisted to varying degrees throughout the course of challenge infection. When the IgG subclass response was examined against

larval ES products from which the proteinases were purified, a predominant IgG1 response was observed but only in the animals vaccinated with normal larvae (Figure 5.3). No significant response was seen against larval ES products in the animals vaccinated with purified larval proteinases. As the production of IgG1 is accepted as an indicator of a Th2 response in mice (Bellaby *et al.*, 1996), a response which is believed to be protective in humans, these results indicate the stimulation of a protective Th2 response in animals vaccinated with normal larvae. It is also possible that the lack of an IgG1 response in the vaccinated mice indicates that the purified larval proteinases are incapable of inducing a sufficient response and are therefore fail to be protective. The apparent reduction in worm burden in mice vaccinated with larval proteinases (Table 5.6) may therefore not be real as a consequence of the low worm recovery in the control groups.

Unlike the IgG1 response following vaccination with purified larval proteinases which appeared to be unsustainable over the period of challenge infection (Figure 5.4), IgG1 production induced by vaccinating with irradiated larvae increased throughout the period of challenge infection (Figures 5.5 A-C). This enhanced Th2 response may account for the resistance of mice to challenge infection following vaccination with irradiated larvae.

Western blotting also demonstrated a predominant IgG1 (Th2) response against a 30-40 kDa ladder. The nature of this 30-40 kDa has yet to be elucidated. This protein ladder has been shown to be absent from Western blots of larval homogenate prepared following the culture of larvae in RPMI for 72 hours prior to extraction (not shown), implying that these proteins does form part of the ES products of *N. americanus* larvae. Similar responses against a protein ladder have been observed following multiple infection of mice with *Strongyloides ratti* (Northern & Grove, 1988) although again the nature of these proteins has not been elucidated.

Leucocytes were harvested from the spleens of the animals from experiment 4 stimulated with concavalin A and their supernatants assayed for γ-interferon, a Th1 cytokine, and IL-5, a Th2 cytokine controlling the production of eosinophilia (Figures 5.11 and 5.12). These experiments confirmed the induction of a Th2 response by vaccinating with both normal

and irradiated larvae as IL-5 levels were enhanced in all the vaccinated groups on both days 45 and 50.

These experiments demonstrated that complete immunity can be induced against *N. americanus* infection by the use of irradiated larvae. Immunity can be induced by vaccinating by either the subcutaneous of percutaneous route. Vaccinating with normal larvae could also induce high levels of immunity, but increased pathology was observed in the lungs of these mice. Antibody and cytokine analysis has demonstrated that vaccination with both normal and irradiated larvae induces a Th2 response characterised by the production of IgG1 and IL5. However, ELISA, Western blot and cytokine analysis has failed to show any difference in responses between mice vaccinated with irradiated larvae and normal larvae except the induction of IgG2a (a marker for the Th1 response) by vaccinating with irradiated larvae prior to challenge infection (day 42, Figure 5.5A). IgG3 levels (usually directed against polysaccharides) were raised (prior to challenge) in Group G vaccinated percutaneously with irradiated larvae. Three days post-challenge these levels remained high in Group G and became elevated in Group C. Eight days post-challenge IgG3 levels in these groups still remained highest in these groups. While it is too early to say whether the IgG3 response is responsible for increased immunity following vaccination with irradiated larvae it represents another area for further study in the future.

The use of irradiation to induce protection against parasite infection has been most extensively studied for *S. mansoni*, reviewed by Richter *et al.*, (1995). Exposure to irradiated cercariae has been shown to induce immunity in mice Dean, *et al.*, 1995, Mountford, *et al.*, 1995 and Guinea pigs (Sat and Kamiya, 1995) and while the vaccine has not been assessed against humans, immunity has been demonstrated in the baboon (Yole, *et al.*, 1996).

In the *S. mansoni* irradiated cercariae model both the skin and the lungs are proposed as being responsible for the elimination of challenge infections following vaccination (Mclaren, 1988, Wilson & Coulson, 1988). In both these sites inflammatory foci are observed which may be responsible for the elimination of challenge parasites (Sato & Kamiya, 1995, Ward & McLaren, 1988, Dean & Mangold, 1992). It has been proposed

that T lymphocytes in the skin draining lymph nodes are induced to produce γ–IFN by vaccination. These γ–IFN producing T cells are then recruited to the lungs prior to challenge infection which 'arms' the lungs against arrival of challenge larvae (Mountford *et al.*, 1992, Ratcliffe & Wilson. 1992; Coulson & Wilson. 1993). When challenge larvae reach the primed lungs, this triggers an inflammatory foci that 'traps' the migrating larvae. γ–IFN would appear to be essential to the generation of immunity to *S. mansoni* (Mountford, *et al.*, 1992). Neutralisation of γ–IFN at the time of larval migration through the lung virtually abolishes immunity and alters the composition of these inflammatory foci (Coulson, *et al.*, 1993). The success of the irradiated vaccine appears to revolve around this ability to generate schistosome specific T cells in the peripheral lymphoid tissue and subsequently recruit these cells to the lungs (Mountford & Harrop, 1998). The apparent failure of the six antigens chosen by the WHO/TDR as candidate vaccines (Berquist & Colley, 1998) appears to be due to their dispersed stimulation of T cells rather than concentrating them in the lungs. As an attenuated vaccine will never be used in the field it has been proposed that recombinant molecules delivered directly to the lungs may result in a suitable population of T cells. Antigens may be delivered intranasally incorporated into liposomes or micelles or using a live expression system such as attenuated *Salmonella typhimurium* (Coulson, 1998). Possibly a suitable antigen delivered in a similar manner may induce immunity against *N. americanus*.

Irradiation has been shown to have a number of differing effects on parasite larvae. 40 krad of γ–irradiation of newly excysted *F. hepatica* results in the reduction in the secretion of cathepsin B and reduced carbohydrate expression on the parasite surface (Creaney *et al.*, 1996). However, preliminary studies on the proteinase activities released by irradiated *N. americanus* larvae failed to show any change in either specific activity or inhibitor profile using the substrate FITC-casein (not shown). In the irradiated vaccine against *S. mansoni* it has been suggested that irradiated schistosomula may be potent immunogens as irradiation induces them to express antigens (either protein or carbohydrate) in disrupted or abnormal conformations (Wales & Kusel. 1992, Wales, *et al.*, 1993). It has also been suggested that irradiation may result in the inhibition of new protein synthesis, causing abnormal proteins to persist longer and therefore, be presented to the immune system for a longer period of time (Wales *et al.*, 1992). Irradiation of *S. mansoni* larvae with a dose (20

krad) that causes optimal immunity also appears to impair neuromuscular function, retarding larval movement resulting in larval (and antigenic) persistence in the skin and lungs (Harrop & Wilson, 1993). For *N. americanus* larvae the effects of irradiation need to be studied further; however, irradiation does seem to cause the larvae to secrete the contents of their secretory granule in an uncontrolled burst (Lightfoot, 1997). This may result in either an abnormal concentration of secreted antigen at the site of infection or larval persistence in the skin because of the inability of the larvae to penetrate further due to the lack of enzymatic activity required for further penetration. The data presented in Figure 5.1 possibly confirms this demonstrating that following irradiation larval passage to the lungs and gut is delayed. This may account for any increased immunogenicity of irradiated larvae. Similarly, irradiation of *Trichinella spiralis* larvae results in an increase in the release of antigens in a dose dependant manner (Agyei-Frempong & Catty, 1983).

In summary, irradiated larvae can be used to generate complete immunity to *N. americanus* challenge infection in BALB/mice. Immunologically, responses characteristic of the Th2 response, i.e. high levels of IgG1 and IL5 have been observed following vaccination. There was no evidence of γ–IFN secretion by splenocytes from vaccinated animals following stimulated by Con A. Furthermore, to date it has not been possible to demonstrate any apparent significant difference (by ELISA or Western blotting) in the immune responses of mice vaccinated with normal or irradiated larvae that may definitely account for the complete immunity generated by irradiated larvae. Further study is required to elucidate either the nature of any parasite molecule(s) that induce this immunity and the immune responses involved.

CHAPTER 6

GENERAL DISCUSSION

CHAPTER 6

6.1 GENERAL DISCUSSION

The original aims of this project were to complete the characterisation of the proteinases secreted by the larval and adult stages of the human hookworm *Necator americanus* and establish possible roles for these enzymes in the hookworm life cycle. Following these characterisation experiments the intention was to purify as many of these enzymes as possible and finally to assess the potential of these enzymes to vaccinate against *N. americanus* infection in a mouse model of hookworm infection.

The characterisation experiments described in Chapter 3 confirm and extend the observations of Kumar & Pritchard (1992a; 1992b), Burleigh (1993) and Brown *et al.* (1995). With respect to larval ES the presence of cysteinyl, serine and metallo-proteinases was confirmed. In addition to these activities, the presence of an aspartyl proteinase (optimally active at pH6.5) in larval ES products has been demonstrated for the first time (Table 3.1). This activity would appear to be particularly important during the skin penetration process as it appears to be able to degrade a number of structurally important skin macromolecules such as collagen types I, III and IV, fibronectin and laminin.

The serine and metallo-proteinase activities in larval ES would appear to be chiefly responsible for elastin degradation, optimally active at a higher pH than the aspartyl proteinase. The role of the larval cysteinyl proteinase currently remains elusive as it fails to degrade any of the skin substrates tested. However, in experiments with excised hamster skin, larval penetration was inhibited (37.6 %) by the cysteinyl proteinase inhibitor E64, suggesting that this activity does still play a role (albeit undefined) in the skin pentration process. The experiments with excised hamster skin also confirmed the importance of the aspartyl proteinase to the skin penetration process, larval penetration being inhibited by 54.8 % by the aspartyl proteinase inhibitor pepstatin A.

A summary of the proteinase activities present in larval ES products and their possible substrates is presented in Table 3.4.

With reference to adult ES products, previous work has demonstrated the presence of proteinases from all mechanistic classes capable of degrading haemoglobin (Burleigh, 1993; Brown *et al.*, 1995). Haemoglobin degradation was confirmed using Western blotting and substrate SDS-PAGE. On Western blots haemaglobin degradation was shown to be mediated by both aspartyl and metallo-proteinases (Figure 3.11). Substrate SDS-PAGE demonstrated that both haemoglobin and fibrinogen degradation to be mediated by an enzyme with a molecular mass of approximately 66 kDa, inhibitable by pepstatin A, defining it as aspartyl in nature (Figures 3.12 & 3.13).

The role of the adult serine proteinase still remains relatively undefined. However, to speculate, adult *N. americanus* ES products have been shown to induce apoptosis in activated T cells (Chow *et al.*, 1999) but the mechanism behind this phenomenon is unclear. Granzymes are serine proteinases secreted by cytotoxic T and natural killer (NK) cells which induce apoptosis in target cells by activating the cell death proteinase family known as caspases (Talanian *et al.*, 1997). Granzymes gain entry into the target cell by means of a peforin and the molecular chaperone calreticulin. Calreticulin has already been shown to be present in the secretions of *N. americanus* (Pritchard *et al.*, 1999) and it is possible that the serine proteinase in adult ES may induce apoptosis in activated T cells in a manner similar to that described for granzymes. Experiments are currently underway to establish if this is the case..

Chapter 4 describes a number of different attempts to purify proteinases from the ES products of the adult and larval stage of the human hookworm *N. americanus*. For *N. americanus* the greatest hindrance to the purification of proteins has always been the limited availability of starting material. As a result of this, purification was attempted by a number of one step affinity chromatography protocols in a manner similar to that used to purify acetylcholinesterase (AChE) from adult *N. americanus* ES products (Pritchard *et al.*, 1991b). Using these protocols a number of observations were made.

From both adult and larval ES products two cysteinyl proteinases were apparently purified. Both enzymes were capable of hydrolysing the fluorogenic cathepsin B and L substrate Z-Phe-Arg-AMC.HCl. However, only the second enzyme was capable of hydrolysing the cathepsin B substrate Z-Arg-Arg-AMC, possibly indicating that both a cathepsin L 'like' and a cathepsin B 'like' proteinase have been purified.

All the cysteinyl proteinases purified from larval and adult ES products resolved on SDS-PAGE as a doublet with molecular masses of 66 and 55 kDa (Figure 4.3), although Western blot experiments indicated that cysteinyl proteinase activity is more likely to be associated with the 55 kDa protein. The molecular mass observed on SDS-PAGE is somewhat large for a cysteinyl proteinase compared with the classical cysteinyl proteinase, papain (27 kDa) or human cathepsin B1 (24 kDa, Barrett, 1973). The reason for this difference has yet to be determined. However, Kumar & Pritchard, (1992a) have previously shown using gelatin substrate gel analysis that a cysteinyl proteinase with an approximate molecular mass of 62 kDa is present in larval ES products. Kumar & Pritchard, (1993) have also suggested that the proteinases of *N. americanus* may oligermerise under the conditions necessary for their purification and this may be the case here. Since the completion of this work the cathepsin B 'like' enzyme of *N. americanus* has been cloned (Pritchard *et al.*, 2000 manuscript in press). The predicted molecular mass of the mature enzyme is 29 kDa, approximately half the molecular mass of the proteins that elute from an activated thiol-Sepharose 4B column, again suggesting that the purified enzyme may have dimerised. A rabbit antiserum has been raised against the purified recombinant enzyme. This antiserum while being raised against a 29 kDa protein reacts on Western blots of adult homogenate with a band of approximately 55 kDa (unpublished data). Furthermore, IgG from this antiserum has been purified using protein G Sepharose. The IgG was immobilised on Affiprep hydrazide support (BioRad) and the resulting matrix used to affinity purify a cysteinyl proteinase from adult homogenate capable of hydrolysing Z-Phe-Arg-AMC.HCl. This cysteinyl proteinase resolves on SDS-PAGE as a single band of approximately 55 kDa (unpublished data). Confirmation of the 55 kDa protein as a cysteinyl proteinase could be obtained by N-terminal sequencing. However, despite a number of attempts no sequence data has been obtained to date.

The data obtained from the pepstatin A affinity column were again somewhat atypical. While the proteinases purified from larval and adult ES products had inhibitor profiles characteristic of aspartyl proteinases, SDS-PAGE analysis revealed a predominant protein of molecular mass 66 kDa and a minor band of 55 kDa. Again these molecular masses are larger than typical aspartyl proteinases; for example ACASP, the aspartyl proteinase of *A. caninum*, (49.3 kDa, Harrap *et al.*, 1996). The significance of these apparent high molecular masses still remains unresolved. However, in support of the purification data, haemoglobin and fibrinogen substrate gels have demonstrated the presence of aspartyl

proteinase activity at approximately 66 kDa in adult ES products (Figure 3.12 and 3.13). Again since the completion of this work the genes for two aspartyl proteinases (termed Nasp 1 and Nasp 2) have been cloned (Girdwood *et al.*, 2000, Manuscript in press). Nasp1 is an aspartyl proteinase from the adult hookworm while Nasp2 has been isolated from the larval stages. The Nasp1 sequence would appear to encode for a protein with a molecular mass of 45 kDa and Nasp 2 for a protein of molecular mass 42 kDa. Antiserum has again been raised in a rabbit against purified recombinant Nasp 1 and used to probe Western blots of adult homogenate. Similar results to those obtained using the rabbit anti cathepsin B serum were observed, with a band of approximately 66 kDa being recognised (unpublished data). Experiments are currently underway to isolate IgG from this anti serum to use for affinity purification with a view to sequencing the purified protein.

Metallo-proteinase purification was attempted using two different affinity matrices (Figures 4.20 and 4.22), the more successful of these being the purification of a metallo-proteinase from larval ES products using N-benzyloxycarbonylglycyl-D-leucylaminohexyl-Sepharose (Inouye *et al.*, 1985). Proteinase activity eluting from the column was completely inhibited by EDTA but only weakly inhibited by 1, 10-phenanthroline (Table 4.4). When analysed by SDS-PAGE a protein of 42 kDa was identified (Figure 4.23). To date this purification protocol has not been evaluated using adult ES products. Chapter 3 discusses the possibility that there may be two metallo-proteinases present in larval ES products. The 42 kDa metallo-proteinase purified by N-benzyloxycarbonylglycyl-D-leucylaminohexyl-Sepharose is only weakly inhibited by 1, 10-phenanthroline while being strongly inhibited by EDTA. Thus it would seem likely that this is the metallo-proteinase with a preference for Types IV and V collagen (Section 3.4). However, this hypothesis has not been tested using the purified enzyme; and, to date the metal ion enhancement profile for this enzyme has not been examined.

The purification of serine proteinase activity using affinity matrices has so far proved unsuccessful. Serine proteinase activity failed to bind to either aprotinin-agarose (data not shown) or soybean trypsin inhibitor-agarose. Serine proteinase activity did however, bind to the affinity matrix *p*-aminobenzamidine- agarose and could successfully be eluted by 70 mM sodium acetate pH 5.6. However, despite having the ability to hydrolyse the trypsin 'like' substrate Tosyl-Gly-Pro-Arg-AMC.HCl, the inhibitor profile observed using the substrate FITC caesin was uncharacteristic for a serine proteinase (data not shown) and

SDS-PAGE analysis revealed a large number of contaminating protein bands eluting from the column.

Chapter 5 describes experiments designed to attempt to confer immunity to *N. americanus* infection by vaccinating Balb/c mice with purified larval proteinases or γ-irradiated larvae. Early experiments with larval proteinases and irradiated larvae were marred by low worm recoveries from the primary infection controls, rendering any apparent reduction in worm burden in the vaccinated animals insignificant. For example, following vaccination with larval proteinases only 2.8 ± (2.5) worms were recovered from the small intestine of vaccinated animals, while 4.5 ± (3.14) worms were recovered from the primary infection control, an apparent reduction of 37.7 %. However, realistically the difference between the groups is too small to be significant. In an attempt to discover why the worm burdens in the primary infection control groups were unexpectedly small a re-evaluation of the time course of primary infection was carried out (Figure 5.1). The results demonstrated that the number of worms is the small intestine was peaking a day earlier than previously reported (Timothy & Behnke. 1993). The results also demonstrated that the passage of larvae through the skin and lungs is delayed following irradiation in a manner similar to that described for *S. mansoni* (Mountford & Harrop, 1998).

Following the re-evaluation of the time course of primary infection two more vaccination experiments (3 and 4) were carried out with irradiated larvae. These experiments showed good worm recovery in the small intestine or the primary infection groups and demonstrated the induction of complete immunity to *N. americanus* challenge infection. The same degree of protection was observed regardless of whether vaccination was carried out by the subcutaneous or percutaneous route although vaccination via the subcutaneous route resulted in fewer worms reaching the lungs, demonstrating that immune responses in the skin are capable of retarding larval progress to the lungs. Vaccination using purified larval proteinases thus needs to be repeated and gut count data obtained on day 8 in order to establish whether these enzymes can be used as vaccines. Indeed it may become more important to collect data on the larval serine proteinase. Should this enzyme be shown to play a role in T cell apoptosis or prove to be non-immunogenic during infection as is the case for the *S. mansoni* serine proteinase (Doenhoff, 1998) it may be an ideal candidate vaccine according to Waksmans postulate, which states: "rather than examining parasite antigens recognised by infected animals and patients as potential vaccine immunogens we

should focus on those molecules against which little or no response was directed", (Sher, 1988).

The ELISA and Western blot data obtained are discussed in depth in Chapter 5. In summary, following vaccination with larval proteinases all the enzymes were shown to be immunogenic. However, they appeared to be unable to induce a significant IgG1 levels (an indicator of the protective Th2 response) against larval ES products (Figure 5.3). Vaccination with irradiated larvae demonstrated that complete immunity can be induced against *N. americanus* challenge infection. High levels of immunity can also be induced by repeated exposure to normal larvae, but increased pathology was observed in the lungs of these mice. Antibody and cytokine analysis has demonstrated that vaccination with both normal and irradiated larvae induces a Th2 response characterised by the production of IgG1 and IL5. However, ELISA and Western blot analysis failed to show significant differences in responses between mice vaccinated with irradiated larvae and normal larvae except the induction of IgG2a (a marker for the Th1 response) by irradiated larvae on day 42 prior to challenge infection (Figure 5.5a).

The most widely studied irradiated larval vaccine is that of *S. mansoni* (reviewed by Coulson, 1997). In comparison the *N. americanus* irradiated larval vaccine is still in its infancy and several questions remain, such as the number of vaccinations required to generate immunity and the length of time this immunity lasts. Despite the wealth of information on the radiation attenuated *S. mansoni* vaccine model, the antigens responsible for immunity to *S. mansoni* have still to be identified. The nature of the immune responses, however, have been well studied and the production of activated T-cells in the lymph nodes draining from the site of vaccination, and their subsequent recruitment in the lungs along with γ-interferon and IL12 secretion would appear to be pivotal to the generation of immunity. No evidence of enhanced γ-interferon secretion by Con A stimulated splenocytes was observed following vaccination with *N. americanus* irradiated larvae. These studies were only carried out 3 and 8 days post challenge infection and not at the time of challenge when low levels of IgG2a in mice vaccinated with irradiated larvae was observed. *N. americanus* is a potent inducer of the Th2 response and has evolved a number of defences to evade this response e.g. the secretion of a metallo-proteinase capable of degrading eotaxin (a chemokine responsible for the recruitment of eosinophils (Elsner, 1996)). To conclude, it may be that as suggested by Wilson & Sher

(1993), the employment of immune mechanisms not normally elicited by a parasite may be the way forward to inducing immunity against parasite infection.

CHAPTER 7

REFERENCES

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REFERENCES

Agyei-Frempong, M. & Catty, D. (1983) The measurement of antigens released by radiation attenuated *Trichinella spiralis* larvae. Parasite Immunology, **5**, 289-304.

Aldape, K., Huizinga, H., Bouvier, J. & McKerrow, J. (1994) *Naegleria fowleri*: Characterisation of a secreted histolytic cysteine protease. Experimental Parasitology, **78**, 230-241.

Auriault, C., Ouaissi, M.I., Torpier, G., Eisen, H. & Capron, A. (1981) Proteolytic cleavage of IgG bound to the Fc receptor of *Schistosoma mansoni* schistosomula. Parasite Immunology, **3**, 33-44.

Authié, E., Duvallett, G., Robertson, C.Williams, D.J.L. (1993) Antibody response to a 33 kDa cysteine protease of *Trypanosoma congolense*: relationship to trypanotolerance in cattle. Parasite Immunology, **15,** 456-474.

Ball, P.A.J. & Bartlett, A. (1969) Serological reactions to infection with *Necator americanus.* Transactions of the Royal Society of Tropical Medicine and Hygiene, **63**, 362-369.

Banwell, J.G., & Schad, G.A. (1978) Intestinal Parasites (Hookworm). In Clinics in Gastroenterology, pp.129-156.

Barrett, A.J. (1973) Human cathepsin B1. Purification and some properties of the enzyme. Biochemical Journal, **131**, 809-822.

Barrett, A.J., & Kirschke, H. (1981) Methods in Enzymology, **80**, 535-560.

Beaver, P.C. (1956) Larva migrans. Experimental Parasitiology, **5**, 587-617.

Beaver, P.C. (1964) Cutaneous larva migrans. Industrial Medicine and Surgery, **33**, 319-321.

Behnke, J.M., Wells, C. & Brown, J. (1986) An improved technique for experimental infections with skin penetrating nematode larvae (*Necator americanus*). International Journal for Parasitolgy, **16**, 461-464.

Behnke, J.M. (1987) Do hookworms elicit protective immunity in man? Parasitology Today, **3**, 200-206.

Behnke, J., M., Rose, R. & Garside, P. (1993) Sensitivity to Ivermectim and Pyrantel of *Ancylostoma ceylanicum* and *Necator americanus*. International Journal for Parasitology, **23**, 945-952.

Bellaby, T., Robinson, K. & Wakelin, D. (1996) Induction of differential T-cell helper responses in mice infected with variants of the parasitic nematode *Trichuris muris*. Infection and Immunity, **64**, 791-795.

Berquist, N.R. & Colley, D.G. (1998) Schistomiasis vaccines: Research to development. Parasitology Today, **64** (3), 99-104.

Beynon, R.J. & Bond, J.S. (1989) Proteolytic enzymes-A practical approach, Oxford: IRL Press.

Bhopale, M.K., Menon, S. & Renapurkar, D.M. (1977) Infant rabbit-a suitable laboratory model for human hookworm (*N. americanus*) infection. Bulletin of the Haffkine Institute, **5** (1), 38-41

Bickar, D. & Reid, P.D. (1992) A high affinity protein stain for Western blots, tissue prints, and electrophoretic gels. Analytical Biochemistry, **203**, 109-115.

Bontempi, E., & Cazzulo, J.J. (1990) Digestion of human immunoglobulin G by the major cysteine proteinase (cruzipain) from *Trypanosoma cruzi*. FEMS Microbiology Letters, **70**, 337-342.

Bos, J.D. & Kapsenberg, M.L. (1986). The skin immune system. Immunology Today, **7** (7), 235-240.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. Analytical Biochemistry, **72**, 248-254.

Brindley, P.J., Gam, A.A., McKerrow, J.H., & Neva, F.A. (1995). Ss 40: The zinc endopeptidase secreted by infective larvae of *Strongyloides stercoralis*. Experimental Parasitology, **80**, 1-7.

Brophy, P.M. & Barrett, J. (1990) Blocking factors and the isolation of glutathione transferases from *Hymenolepis diminuta* (Cestoda: Cyclophyllidea). Parasitology, **100**, 137-141.

Brophy, P.M., Ben-Smith, A., Brown, A., Behnke, J.M. & Pritchard, D.I. (1994). Glutathione binding proteins in the gastrointestinal nematode *Heligmosomoides polygyrus*. Journal of Parasitology, **81**, 302-304.

Brophy, P.M., Patterson, L.H., Brown, A. & Pritchard, D.I. (1995) *Necator americanus*: GST expression: possible role of excretory secretory forms of GST. Acta Tropica, **59**, 259-263.

Brown, A. & Pritchard, D.I. (1993) The immunogenicity of hookworm (*Necator americanus*) acetylcholinesterase (AChE) in man. Parasite Immunolgy, **15**, 195-203.

Brown, A., Burleigh, J.M., Billett, E.E. & Pritchard, D.I. (1995) An initial characterisation of the proteolytic enzymes secreted by the adult stage of the human hookworm *Necator americanus*. Parasitology, **110**, 555-563.

Brown, A., Griffiths, G., Brophy, P.M., Furmidge, B.A. & Pritchard, D.I. (1997) The production and analysis of helminth Excretory Secretory (ES) products. In: Analytical Parasitology (Rogan, M.R., ed), pp. 83-132, Springer Verlag, Heidelberg.

Brown, A., Girod, N., Billett, E.E. & Pritchard, D.I. (1999). *Necator americanus* (human hookworm) uses aspartyl proteinases to digest skin macromolecules and invade the host. American Journal of Tropical Medicine and Hygiene (In Press).

Burgeson, R.E. (1988) New collagens, new concepts. Annual Review of Biochemistry, **4**, 551-557.

Burleigh, J.M. (1992). Ph.D. An analysis of the externally presented molecules of the human hookworm *Necator americanus*. Nottingham Trent University;

Burt, J.S. & Ogilvie, B.M. (1975) In vitro maintenance of nematode parasites assayed by acetylcholinesterase and allergen secretion. Experimental Parasitology, **38**, 75-82.

Capello, M., Clyne, L.P., McPhedran, P. & Hotez, P.J. (1993) Ancylostoma Factor Xa inhibitor: Partial purification and its identification as a major hookworm derived anticoagulant *in vitro*. Journal of Infectious Diseases, **167**, 1474-1477.

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

Carr, A. & Pritchard, D.I. (1986) Identification of hookworm (*Necator americanus*) antigens and their translation *in vitro*. Molecular and Biochemical Parasitology, **19**, 251-258.

Carr, A. & Pritchard, D.I. (1987) Antigen expression during development of the human hookworm, *Necator americanus* (Nematoda). Parasite Immunology, **9**, 219-234.

Carroll, S.M. & Grove, D.I. (1985) Resistance of dogs to re-infection with *Ancylostoma ceylanicum* following anthelminthic therapy. Transactions of the Royal Society of Tropical Medicine and Hygiene, **79**, 519-523.

Carroll, S.M. & Grove, D.I. (1986) Experimental infection of humans with *Ancylostoma ceylanicum*: clinical, parasitological, haematological and immunological findings. Tropical and Geographical Medicine, **38**, 38-45.

Cazzulo, J.J., Franke-Cazzulo, M.C., Martinez, J., & Franke de Cazzulo, B.M.F. (1990) Some kinetic properties of a cysteine proteinase (cruzipain) from *Trypanosoma cruzi*. Biochemica and Biophysica Acta, **1037**, 186-191.

Chandler, A.C. (1932) Susceptability and resistance to helmithic infections. The Journal of Parasitology, **18**, 135-152.

Chappell, C.L., & Dresden, M.H. (1986) *Schistosoma mansoni*: Proteinase activity of 'Hemoglobinase' from the digestive tract of adult worms. Experimental Parasitology, **61**, 160-167.

Chappell, C.L., & Dresden, M.H. (1987) Purification of cysteine proteinases from adult *Schistosoma mansoni*. Archives of Biochemistry and Biophysics, **256**, 560-568.

Chavez-Olortegui, C., Resende, M. & Tavares, C.A.P. (1992) Purification and characterisation of a 47 kDa protease from *Schistosoma mansoni* cercarial secretion. Parasitology, **105**, 211-218.

Chowdhury, A.B. & Schad, G.A. (1972) *Ancylostoma ceylanicum*: a parasite of man in Calcutta and environs. American Journal of Tropical Medicine and Hygiene, **21**, 300-301.

Chowdhury, A.B., Dean, C.G., Kochar, V.K., Nawalinski, T.A., Thomas, J. & Tonasica, J.A. (1973) Arrested development in human hookworm infection: An adaptation to a seasonally unfavourable external environment. Science, **180**, 502-504.

Chow, S. C., Brown, A. and Pritchard, D. I. (2000) The human hookworm pathogen *Necator americanus* induces apoptosis in T lymphocytes. Parasite Immunology, **22**, 21-29.

Clifton, M., Monohan, H., Taylor, W., Chapman, M.R. & Klie, T.R. (1997) Experimental immunization of ponies with *Strongylus vulgaris* radiation-attenuated larvae or crude soluble extracts from larval or adult stages. Journal of Parasitology, **80**, 911-923.

Coulson, P.S. & Wilson, R.A. (1993). Pulmonary T helper cells are $CD44^{hi}$, CD 45RB^{-} effector/memory cells in mice vaccinated with attenuated larvae of *Schistosoma mansoni*. Journal of Immunology, **151**, 3663-3671.

Coulson, P.S., Smythies, L.E. & Wilson, R.A. (1993) Pulmonary granulomatous type sensitivity: cell-mediated responses to embolised schistosome larvae and eggs. Regulatory Immunology, **5**, 165-173.

Coulson, P.S. (1997) The radiation-attenuated vaccine against schistosomes in animal models: Paradigm for a human vaccine? Advances in Parasitology **39**, 270-323.

Creaney, J., Spithill, T.W., Thompson, C.M., Wilson, L.R., Sandeman, R.M. & Parsons, J.C. (1995) Attempted immunisation of sheep against *Fasciola hepatica* using γ-irradiated metacercariae. International Journal of Parasitolgy, **56**, 209-218.

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

Croese, T.J. (1988) Eosinophilic enteritis-A recent North Queensland experience. Austrailian and New Zealand Journal of Medicine, **18**, 848-853.

Croese, T.J., Prociv, P., Maguire, E.J. & Crawford, A.P. (1990) Eosinophillic enteritis presenting as surgical emergencies: a report of six cases. Medical Journal of Australia, **153**, 415-417.

Croll, N.A., Matthews, B.E. & Smith, J.M. (1975) Hookworm behaviour: larval movement patterns after entering hosts. International Journal for Parasitology, **5**, 551-556.

Dalton, J.P., Smith, A.M., Clough, K.A. & Brindley, P.J. (1995) Digestion of haemoglobin by Schistosomes: 35 years on. Parasitology Today, **11**, 299-303.

Dame, J.B., Reddy, G.R., Yowell, C.A., Dunn, B.M., Kay, J. & Berry, C. (1994) Sequence, expression and modelled structure of an aspartic proteinase from the human malaria parasite *Plasmodium falciparum*. Molecular and Biochemical Parasitology, **64**, 177-190.

Dean, D.A. (1983) Schistosoma and related genera: acquired resistance in mice. Experimental Parasitology, **55** (1), 1-104

Dean, D.A. & Mangold, B.L. (1992) Evidence that normal and immune elimination of *Schistosoma mansoni* take place at the lung stage of migration prior to parasite death. American Journal of Tropical Medicine and Hygiene, **47** (2), 238-248.

Dean, D.A., Mangold, B.L. & Lewis, F.A. (1995) Comparison of *Schistosoma mansoni* with respect to the sites and kinetics of immune elimination in irradiated cercariae-immunised mice. Journal of Parasitology, **81** (1), 43-47.

Dessaint, J., Capron, M., Bout, D. & Capron, A. (1975) Quantitative determination of specific IgE antibodies to schistosome antigens and serum IgE levels in patients with schistosomiasis (*S. mansoni* and *S. haematobium*). Clinical and Experimental Immunology, **20**, 427-436

Doenhoff, M.J. (1998) A vaccine for schistosomiasis: Alternative approaches. Parasitology Today, **64** (3), 105-109.

Dowd, A.J., Dalton, J.P., Loukas, A.C., Prociv, P. & Brindley, P.J. (1994) Secretion of cysteine proteinase by the zoonotic hookworm *Ancylostoma caninum*. American Journal of Tropical Medicine and Hygiene, **51**, 341-347.

Dresden, M.H., & Asch, H.L. (1972) Proteolytic enzymes in extracts of *Schistosoma mansoni* cercariae. Biochim Biophys Acta, **289**, 378-384.

Dresden, M.H. & Deelder, A.M. (1979) *Schistosoma mansoni*: Thiol proteinase properties of adult worm 'Hemoglobinase'. Experimental Parasitology, **48**, 190-197.

Dresden, M.H., Rutledge, M.L. & Chappell, C.L. (1981) Properties of the acid thiol proteinase from *Schistosoma mansoni* adults. Molecular and Biochemical Parasitology, **4**, 61-66.

Dubey, J.P., Jenkins, M.C., Thayer, D.W., Kwock, O.C.H., & Shen, S.K. (1996). Killiing of *Toxoplasma gondii* oocysts by irradiation and protective immunity induced by vaccination with irradiated oocysts Journal of Parasitology, **82** (5), 724-727.

Dunn, B.M., Kammermann, B. & McCurry, K.R. (1984) The synthesis, purification, and evaluation of a chromomorphic substrate for pepsin and other aspartyl proteases: Design of a substrate based on subsite preferences. Analytical Boichemistry, **138**, 68-73.

Ellman, G.L., Courtney, K.D., Andrew, V. & Featherstone, R.M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology, **7**, 88-95.

Else, K. & Grencis, R.K. (1991) Cellular immune responses to the murine nematode parasite *Trichuris muris*. I. Differential cytokine production during acute or chronic infection. Immunology, **72**, 508-513

Elsner, J., Hochstetter, R., Kimmig, D. & Kapp, A. (1996) Human eotaxin represents a potent activator of the respiratory burst of human eosinophils. European Journal of Immunology, **26**(8), 1919-1925.

Furmidge, B.A., Horn, L.A. & Pritchard, D.I. (1995) The anti-haemostatic strategies of the human hookworm *Necator americanus*. Parasitology, **112**, 81-87.

Gamble, H.R., Purcell, J.P., & Fetterer, R.H. (1989) Purification of a 44 kilodalton protease which mediates the ecdysis of infective *Haemonchus contortus* larvae. Molecular and Biochemical Parasitology, **33**, 49-58.

Garside, P. & Behnke, J.M. (1988) *Ancylostoma ceylanicum* in the hamster: observations on the host parasite relationship during primary infection. Parasitology, **2**, 283-289.

Gazzinelli, G., Ramalho-Pinto, F.J. & Pellegrino, J. (1966) Purification and characterisation of the proteolytic enzyme complex of cercarial extract. Comparative Biochemistry and Physiology, **18**, 689-700.

Gilman, R.H. (1982) Hookworm disease: Host-pathogen biology. Reviews of Infectious diseases, **4**, 824-829.

Girdwood, K., Berry, C., Brown, A & Pritchard, D.I. (2000) The molecular characterisation of an aspartyl proteinase from the human hookworm pathogen *Necator americanus* that may be involved in larval entry into the host. (Manuscript in Press).

Gluzman, I.Y., Francis, S.E., Oksman, A., Smith, C.E., Duffin, K.L. & Goldberg, D.E. (1994) Order and specificty of the *Plasmodium falciparum* hemoglobin degradation pathway. Journal of Clinical Investigation, **93**, 1602-1608.

Goldberg, D.E., Slater, A.F.G., Cerami, A. & Henderson, G.B. (1990) Haemoglobin degradation in the malarial parasite *Plasmodium falciparum*: An ordered process in a unique organelle. Proceedings of the National Academy of Science USA, **87**, 2931-2935.

Goldberg, D.E. & Slater, A.F.G. (1992) The pathway of haemoglobin degradation in malaria parasites. Parasitology Today, **8**, 280-283.

Grant, A.W., Magee, A.I. & Hermon-Taylor, J. (1978) Optimisation of conditions for the affinity chromatography of human enterokinase on immobilised *p*-aminobenzamidine. Journal of Biochemistry, **88**, 183-189.

Grant, C.T., & Senft A.W. (1971) Schistosome proteolytic enzyme. Comparative Biochemistry and Physiology, **38B**, 663-678.

Hagan, P., Behnke, J.M., & Parish, A. (1981). Stimulation of acquired immunity to *Nematospiroides dubius* in mice using larvae attenuated by cobalt 60 irradiation. Parasite Immunology, *3*, 149-156.

Hagan, P. (1993) IgE and protective immunity to helminth infections. Parasite Immunology, **15**, 1-4.

Hamajima, F., Yamakami, K. & Fujino, T. (1985) Localisation of a thiol protease in metacercarial lung fluke. Japanese Journal of Parasitology, **34**, 507-508.

Hamajima, F., Yamamoto, M., Tsuru, S., Yamakami, K., Fujino, T., Hamajima, H. & Katsura, Y. (1994) Immunosupression by a neutral thiol protease from parasitic helminths in mice. Parasite Immunolgy, **16**, 261-273.

Harada, Y. & Mori, O. (1955).A new method of culturing hookworms. Yonago Acta Medica, **1**, 177-179.

Harrap, S.A., Sawangjaroen, N., Prociv, P. & Brindley, P.J. (1995) Characterisation and localisation of cathepsin B proteinases expressed by adult *Ancylostoma caninum* hookworms. Molecular and Biochemical Parasitology, **71**, 163-171.

Harrap, S.A., Prociv, P. & Brindley, P.J. (1996) ACASP, a gene encoding a cathepsin D-like proteinase from the hookworm *Ancylostoma caninum*. Biochemical and Biophysical Research Communications, **227**, 294-302

Harrop, R. & Wilson, R.A. (1993) Irradiation of *Schistosoma mansoni* cercariae impairs neuromuscular function in developing schistosomula. Journal of Parasitology, **79** (2), 286-289.

Hawdon, J.M. & Schad, G.A. (1991) Long term storage of hookworm infective larvae in buffered saline solution maintains larval responsiveness to host signals. Journal of the Helminthological Society of Washington, **58**, 140-142.

Hawdon, J.M., Volk, S.W., Rose, R., Pritchard, D.I., Behnke, J.M. & Schad, G.A. (1993) Observations on the feeding behavior of parasitic third stage hookworm larvae. Parasitology, **106**, 163-169.

Hawdon, J.M., Jones, B.F., Perragaux, M.A. & Hotez, P.J. (1995) *Ancylostoma caninum*: Metalloprotease release coincides with activation of infective larvae *in vitro*. Experimental Parasitology, **80**, 205-211.

Hawthorne, S.J., Halton, D.W. & Walker, B. (1994) Identification and charaterisation of the cysteine and serine proteinases of the trematode, *Haplometra cylindracae* and determination of their haemoglobinase activity. Parasitology, **108**, 595-601.

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

Hill, D.E., Gamble, H.R., Rhoads, M.L., Fetterer, R.H. & Urban, J.F. (1993) *Trichuris suis*: A zinc metalloprotease from culture fluids of adult parasites. Experimental Parasitology, **77**, 170-178.

Hill, J., Tyas, L., Phylip, L.H., Kay, J., Dunn, B.M. & Berry, C. (1994). High level expression and characterisation of plasmepsin II, an aspartic proteinase from *Plasmodium falciparum*. FEBS Letters, **352**, 155-158.

Hoagland, K.E. & Schad, G.A. (1978) *Necator americanus* and *Ancylostoma duodenale*: Life history parameters and epidemiological implications of two sympatiric hookworms on humans. Experimental Parasitology, **44**, 36-39.

Hotez, P.J. & Cerami, A. (1983) Secretion of a proteolytic anticoagulant by Ancylostoma hookworms. Journal of Experimental Medicine, **157**, 1594-1603.

Hotez, P.J., Le Trang, N., McKerrow, J.H. & Cerami, A. (1985) Isolation and characterisation of a proteolytic enzyme from adult hookworm *Ancylostoma caninum*. The Journal of Biological Chemistry, **260**, 7343-7348.

Hotez, P.J., Le Trang, N. & Cerami, A. (1987) Hookworm antigens: the potential for vaccination. Parasitology Today, **3**, 247-249.

Hotez, P.J., Haggerty, J., Hawdon, J., Milstone, L., Gamble, H.R., Schad, G. & Richards, F. (1990) Metalloproteinases of infective *Ancylostoma* hookworm larvae and their possible functions in tissue invasion and ecdysis. Infection and Immunity, **58**, 3883-3892.

Hotez, P.J., Narasimhan, S., Haggerty, J., Milstone, L., Bhopale, V., Schad, G.A. & Richards, F.F. (1992) Hyaluronidase from infective *Ancylostoma* larvae and its possible function as a virulance factor in tissue invasion and in cutaneous larva migrans. Infection and Immunity, **60**, 1018-1023.

Hotez, P., Capello, M., Hawdon, J., Beckers, C. & Sakanari, J. (1994) Hyaluronidases of the gastrointestinal invasive nematodes *Ancylostoma caninum* and *Anisakis simplex*: Possible functions in the pathogenesis of human zoonoses. The Journal of Infectious Diseases, **170**, 918-926

Hotez, P.J. & Pritchard, D.I. (1995) Hookworm Infection. Scientific American, **272**, 42-48.

Inouye, I., Kawaguchi, Y. & Nakamura, S. (1985) Affinity chromatography of neutral metalloendopeptidase produced by Streptomyces mauvecolor on N-benzyloxycarbonylglycyl-D-leucylaminohexyl-Sepharose. Chemistry and Parmacology Bulletein, **33**, 1544-1551.

Irvine, J.W., Coombs, G.H. & North, M.J. (1993). Purification of cysteine proteinases from trichomonads using bacitracin-Sepharose. FEMS Microbiology Letters, **110**, 113-120.

Karanu, F.N., Rurangirwa, F.R., McGuire, T.C. & Jasmer, D.P. (1993) *Haemonchus contortus*: Identification of proteases with diverse characteristics in adult worm excretory-secretory products. Experimental Parasitology, **77**, 362-371.

Klinkert, M.-Q., Felleisen, R., Link, G., Ruppel, A. & Beck, E. (1989) Primary structure of Sm31/32 diagnostic proteins of *Schistosoma mansoni* and their identification as proteases. Molecular and Biochemical Parasitology, **33**, 113-122

Knox, D.P., & Kennedy, M.W. (1988) Proteinases released by the parasitic larval stages of *Ascaris suum* and their inhibition by antibody. Molecular and Biochemical Parasitology, **28**, 207-216.

Knox-Macaulay, H.H.M. (1981) Transient hyper-eosinophilia in *Necator americanus* and *Schistosoma mansoni* infections. Transactions of the Royal Society of Tropical Medicine and Hygiene, **75**, 906-907.

Kucharz, E.J. (1992) The Collagens: Biochemistry and Pathophysiology, Springer-Verlag, Berlin.

Kumar, S. & Pritchard, D.I. (1992a) The partial characterisation of proteases present in the Excretory/Secretory products and exsheathing fluid of the infective (L_3) larva of *Necator americanus*. International Journal for Parasitology, **22**, 563-572.

Kumar, S. & Pritchard, D.I. (1992b) Secretion of metalloproteases by living infective larvae of *Necator americanus*. Journal of Parasitology, **78**, 917-919.

Kumar, S. & Pritchard, D.I. (1992c) Skin penetration by ensheathed third stage infective larvae of *Necator americanus*, and the host's immune response to larval antigens. International Journal for Parasitology, **22**, 573-579.

Kumar, S. & Pritchard, D.I. (1993) New observations on proteases of the human hookworm *Necator americanus*. Biochemistry and Molecular Biology International, **30**, 13-19.

Lacks, S.A. & Sringhorn, S.S. (1980) Renaturation of enzymes after polyacrylaminde gel electrophoresis in the presence of sodium dodecyl sulphate. Journal of Biological Chemistry, **255**, 7467-7473.

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

Lightfoot, C. D. (1997) The effect of gamma irradiation on the immunogenicity of *Necator americianus* L_3 infective larvae. M.Sc dissertation, University of Salford.

Liotta, L.A., Rao, C.N. & Wewer, U.M. (1986) Biochemical interactions of tumour cells with the basesment membrane. Annual Review of Biochemistry, **55**, 1037-1057.

Lipps, G., Fullkrug, R. & Beck, E. (1996) Cathepsin B of *Schistosoma mansoni*: Purification and activation of the recombinant pro-enzyme secreted by *Saccharomyces cerevisiae*. The Journal of Biological Chemistry, **271**, 1717-1725.

Lottenberg, R., Christensen, U., Jackson, C.M. & Coleman, P.L. (1981) Assay of coagulatuion proteases using peptide chromogenic and fluorogenic substrates. Methods in Enzymology, **80**, 341-365.

Marikovsky, M., Arnon, R. & Fishelson, Z. (1988) Proteases secreted by transforming schistosomula of *Schistosoma mansoni* promote resistance to killing by complement. The Journal of Immunology, **141**, 273-278.

Markaryan, A., Morozova, I., Yu, H. & Kolattukudy, P.E. (1994) Purification and characterisation of an elastinolytic metalloprotease from *Aspergillus fumigatus* and immunoelectro microscopic evidence of secretion of this enzyme by the fungus invading the murine lung. Infection and Immunity, **62**, 2149-2157.

Martin, P., Raymond, M.N., Bricas, E. & Ribadeau Dumas, B. (1980) Kinetic studies on the action of *Mucor pusllus, Mucor miehei* acid proteases and chymosins A and B on a synthetic chromamorphic hexapeptide. Biochimica and Biophysica Acta, **612**, 410-420.

Mason, R.W., Green, G.D.J. & Barrett, A.J. (1985) Human liver cathepsin L. Biochemical Journal, **226**, 133-241.

Matthews, B.E. (1975) Mechanisms of skin penetration by *Ancylostoma tubaeforme* larvae. Parasitology, **70**, 25-38.

Matthews, B.E. (1982) Skin penetration by *Necator americanus* larvae. Zeitschrift fur Parisitenkunde, **68**, 81-86.

Maxwell, C., Hussain, R., Nutman, T.B., Poindexter, R.W., Little, M.D., Schad, G.A. & Ottesen, E.A. (1987) The clinical and immunological responses of normal human volunteers to low dose hookworm (*Necator americanus*) infection. American Journal of Tropical Medicine and Hygiene, **37**, 126-134.

McDonald, J.A. & Kelly, D.G. (1980) Degradation of fibronectin by human leukocyte elastase. The Journal of Biological Chemistry, **255**, 8848-8858.

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

McKerrow, J.H., Jones, P. & Pino-Hess, S. (1985) Proteinases from invasive larvae of the trematode parasite *Schistosoma mansoni* degrade connective tissue and basement membrane macromolecules. Biochemical Journal, **231**, 47-51.

McKerrow, J.H., Pino-Heiss, Linquist, R. & Werb, Z. (1985) Purification and characterisation of an elastinolytic proteinase secreted by cercariae of Schistosoma mansoni. The Journal of Biological Chemistry, **260**, 3703-3707.

McKerrow, J.H. (1989) Parasite proteases. Experimental Parasitology, **68**, 111-115.

McKerrow, J.H., Brindley, P., Brown, M., Gam, A.A., Staunton, C. & Neva, F. (1990) *Strongyloides stercoralis*: Identification of a protease that facilitates penetration of skin by infective larvae. Experimental Parasitology, **70**, 134-143.

McLaren, D. J. (1988) Will the real target of immunity to schistsomiasis please stand up. Parasitology Today, **(5)**9, 279-282.

Miller, H.R.P. (1973) The possibilities for application of the canine hookworm vaccine technology to the prevention and control of hookworm infection and disease in man. In: Nuclear Techniques in Helminthology Research, 61-68, Vienna Atomic Energy Agency.

Miller, T.A. (1971) Vaccination against the canine hookworm diseases. Advances in Parasitology, **9**, 153-183.

Miller, T.A. (1978) Industrial development and field use of the canine hookworm vaccine. Advances in Parasitology, **16**, 333-342.

Miller, T.A. (1979) Hookworm infection in man. Advances in Parasitology, **17**, 315-383.

Morita, T., Kata, H., Iwanaga, S., Takada, K., Kimura, T. & Sakakibara, S. (1977) New fluorogenic substrates for a-thrombin, factor Xa, kallikreins, and urokinase. Journal of Biochemistry, **82**, 1495-1498.

Monahan, C. M., Taylor, H. W., Chapman, M. R. & Klei, T.R. (1994) Experimental immunization of ponies with *Strongylus vulgaris* radiation attenuated larvae or crude soluble somatic extracts from larval or adult stages. Journal of Parasitology, **80** (6), 911-923.

Mountford, A.P., Coulson, P.S. & Pemberton, R.M., Smythies, L. E. & Wilson, R. A. (1992) The generation of interferon-gamma producing T lymphocytes in skin-draining lymph nodes, and their recruitment into the lungs, is associated with protective immunity to *Schistosoma mansoni*. Immunology, **75**, 250-256.

Mountford, A. P., Harrop, R. & Wilson, R. A. (1995) Antigens derived from the lung stage of *Schistosoma mansoni* are efficient stimulators of proliferation and gamma interferon secretion by lymphocytes from mice vaccinated with attenuated larvae. Infection and Immunity, **63** (5) 1980-1986.

Mountford,A.P. & Harrop, R. (1998) Vaccination against schistosomiasis: The case for lung stage antigens. Parasitology Today, **14** (3), 109-114.

Murray, J.C., Stingl, G., Kleinman, H.K., Martin, G.R. & Katz, S.I. (1979) Epidermal cells bind preferentially to type IV (basement membrane) collagen. Journal of Cell Biology, **80**, 179-202.

Nagahana, M., Yoshida, Y., Tanabe, K., Kondo, K., Okamoto, K., Okada, S., Sato, K., Shigeyoshi, I., Fukotome, S. & Ishikawa, M. (1962) Experimental studies on the oral infection of *Necator americanus*. 1. Per stomach infection of puppies and guinea pigs with *N. americanus* larvae. Japanese Journal of Parasitology, **11**, 454-460.

Nawalinski, T., Schad, G.A. & Chowdury, A.B. (1978) Population biology of hookworms in children in rural West Bengal: I. General Parasitological observations. American Journal of Tropical Medicine and Hygiene, **27**, 1152-1161.

Neuhoff, V., Arold, N., Taube, D. & Ehrhardt, W. (1988) Improved staining of proteins in polyacrylamide gels including isoeletric focusing gels with clear background at nanogram sensitivty using Coomassie Brilliant blue G 250 and R 250. Electrophoresis, **9**, 255-262.

North, M.J. (1997). Parasite proteinases. In: Analytical Parasitology (Rogan, M.R., ed), pp. 133-186, Springer Verlag, Heidelberg.

Northern, C. & Grove, D. I. (1988) Western blot analysis of reactivity to larval and adult *Srogyloides ratti* antigens in mice. Parasite Immunology, **(10)**, 681-691.

Nussenweig, R. S., Vanderberg, J. P. & Most, H. (1998) Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei* IV. Dose response, specificity and humoral immunity. Military Medicine, **134**, 1176-1182.

Odland, G.F. (1991) Structure of the skin. In: Physiology, Biochemistry and Molecular Biology of the Skin (Goldsmith, S.A. ed.), 3-17, Oxford University Press, London.

Ogilvie, B.M. (1964) Reagin like antibodies in animals immune to helminth parasites. Nature, **204**, 91-92.

Ogilvie, B.M., Bartlett, A., Godfrey, R.C., Turton, J.A., Worms, M.J. & Yeates, R.A. (1978) Antibody responses in self infections with *Necator americanus*. Transactions of the Royal Society of Tropical Medicine and Hygiene, **72**, 66-71.

Orlowski, M., Orlowski, R., Chang, J.C., Wilk, E. & Lesser, M. (1984) A sensitive procedure for determination of cathepsin D: activity in alveolar and peritoneal macrophages. Molecular and Cellular Biochemistry, **64**, 155-162.

Oya, Y. & Noguchi, I. (1977) Some properties of hemoglobin protease from *Ancylostoma caninum*. Japanese Journal of Parasitology, **26**, 307-313.

Pawlowski, Z.S. (1991) Hookworm infection and anaemia. Approaches to prevention and control. World Health Organisation Geneva,

Philipp, M. (1984) Acetylcholinesterase secreted by intestinal nematodes: a reinterpretation of its putative role of 'biochemical holdfast'. Transactions of the Royal Society of Tropical Medicine and Hygeine, **78**, 138-139.

Plaut, M. (1987) Lymphocyte hormone receptors. Annual Review of Immunology, **5**, 621-669.

Pleass, R.J. & Bianco, A.E. (1995) The effects of γ-irradiation on the development of *Heligmosomoides polygyrus bakeri* in mice. International Journal for Parasitology, **25**, 1099-1109.

Pohl, J., Baudys, M. & Kostka, V. (1983) Chromomorphic peptide substrates for activity determination of animal aspartic proteinases in the presence of their zymogens: A novel assay. Analytical Biochemistry, **133**, 104-109.

Pohl, J., Zaoral, M., Jindra, A. & Kostka, V. (1984) Purification of pepsins and cathepsin D by affinity chromatography on Sepharose 4B with an imobilised synthetic inhibitor. Analytical Biochemistry, **139**, 265-271.

Pritchard, D.I., McKean, P.G. & Schad, G.A. (1990a). An Immunological and Biochemical Comparison of Hookworm Species. Parasitology Today, **6**, 154-156.

Pritchard, D.I., Quinnell, R.J., Slater, A.F.G., McKean, P.G., Dale, D.D.S., Raiko, A. & Keymer, A.E. (1990b) Epidemiology and immunology of *Necator americanus* infection in a community in Papua New Guinea: humoral reponses to excretory-secretory and cuticular collagen antigens. Parasitology, **100**, 317-326.

Pritchard, D.I. (1990c) *Necator americanus* antigens and immunological targets. In: Hookworm disease: Current status and new directions, 340-350, Taylor and Francis, London.

Pritchard, D.I., McKean, P.G. & Tighe, P.J. (1990d) Recent and predicted advances in hookworm biology. In: Parasite Genes, Menbranes and Antigens, 140-169, Taylor and Francis, London.

Pritchard, D.I., Quinnell, R.J., Moustafa, M., McKean, P.G., Slater, A.F.G., Raiko, A., Dale, D.D.S. & Keymer, A.E. (1991a) Hookworm (*Necator americanus*) infection and storage iron depletion. Transactions of the Royal Society of Tropical Medicine and Hygiene, **85**, 235-238.

Pritchard, D.I., Leggett, K.V., Rogan, M.T., McKean, P.G. & Brown, A. (1991b) *Necator americanus* secretory acetylcholinesterase and its purification from excretory-secretory products by affinity chromatography. Parasite Immunolgy, **13**, 187-199.

Pritchard, D.I., Walsh, E.A., Quinnell, R.J., Raiko, A., Edmonds, P. & Keymer, A.E. (1992) Isotopic variation in antibody responses in a community in Papua New Guinea to larval and adult antigens during infection, and following re-infection, with hookworm *Necator americanus*. Parasite Immunology, **14**, 617-631.

Pritchard, D.I., Kumar, S. & Edmonds, P. (1993). Soluble (s) CD23 levels in a parasitised population from Papua New Guinea. Parasite Immunology, **15**, 205-208.

Pritchard, D.I. (1993) Immunity to helminths: is too much IgE parasite-rather than host-protective? Parasite Immunology, **15** (1), 5-9.

Pritchard, D.I., Brown, A., & Toutant, J-P. (1994) The molecular forms of acetylcholinesterase from *Necator americanus* (Nematoda), a hookworm parasite of the human intestine. European Journal of Biochemistry, **219**, 317-323.

Pritchard, D.I. (1995) Immunity in humans to *N. americanus*: IgE, parasite weight and fecundity. Parasite Immunology, **71**, 71-75.

Pritchard, D.I. & Walsh, E.A. (1995) The specificity of the human IgE response to *Necator americanus*. Parasite Immunology, **17**, 605-607.

Pritchard, D.I., & Furmidge, B.A. (1995). The anti-haemostatic strategies of the human hookworm *Necator americanus*. Thrombosis and Haemostasis, **73**, 546.

Pritchard, D.I. (1996) Do haematophagous parasites secrete SOD and promote blood flow? International Journal for Parasitology, **26**, 1339-1340.

Pritchard, D.I. & Wilson, R.A. (1997) Helminth Infections. In: Pulmonary Defences, 305-319, John Wiley & Sons, New York.

Pritchard, D.I., Brown, A., Kasper, G., McElroy, P., Loukas, A., Hewitt, C., Berry, C., Füllkrug, R. & Beck, E. (1999) A hookworm allergen that strongly resembles calreticulin. Parasite Immunology. **21**, 439-450.

Prociv, P. & Croese, J. (1990) Human eosinophilic enteritis caused by dog hookworm *Ancylostoma caninum*. Lancet, **335**, 1299-1302.

Quinnell, R.J., Slater, A.F.G., Tighe, P.J., Walsh, E.A., Keymer, A.E. & Pritchard, D.I. (1993) Reinfection with hookworm after chemotherapy in Papua New Guinea. Parasitology, **106**, 379-385.

Quinnell, R.J., Woolhouse, M.E.J., Walsh, E.A. & Pritchard, D.I. (1995).Immunoepidemiology of human nectoriasis: correlations between antibody responses and parasite burdens. Parasite Immunology, **17**, 313-318.

Ratcliffe, E.C., & Wilson, R.A. (1992). The role of mononuclear-cell recruitment to the lungs in the development and expression of immunity to *Schistosoma mansoni*. Parasitology, **104**, 299-307.

Reeck, G.R., Walsh, K.A., Hermodson, M.A., & Neurath, H. (1971). New forms of bovine carboxypeptidase B and their homologous relationships to carboxypeptidase A. Proceedings of the National Academy of Science USA, **68**, 1226-1230.

Rege, A.A. & Dresden, M.H. (1987) Strongyloides spp: Demonstration and partial characterisation of acidic characterisation of acidic collagenolytic activity from infective larvae. Experimental Parasitology, **64**, 275-280.

Rep, B.H. (1975) The topographic distribution of *Necator americanus* and *Ancylostoma duodenale* in the human intestine. Tropical and Geographical Medicine, **27**, 169-176.

Richter, D. Harn, D. A. & Matuschka, F-R. (1995) The irradiated cercariae vaccine model. Parasitology Today, **11**(8), 288-293.

Rivier, D., Shah, R., Bovay, P. & Mauel, J. (1993) Vaccine development agaonst cutaneous leishmaniasis. Subcutaneous administration of radioattenuated parasites protects CBA mice against virulent *Leishmania major* challenge. Parasite Immunology, **15**, 75-84.

Robertson, C.D. & Coombs, G.H. (1990) Characterisation of three groups of cysteine proteinases in the amastigotes of *Leishmania mexicana mexicana*. Molecular and Biochemical Parasitology, **42**, 269-276.

Robinson, K., Bellaby, T. & Wakelin, D. (1994) Vaccination against the nematode *Trichinella spiralis* in high- and low- responder mice: effects of different adjuvants upon protective immunity and immune responsiveness. Immunology, **82**, 261-267.

Rosenbloom, J., Feldman, G., Freundlich, B. & Jimenez, S. (1979) Hydroxyproline content determines the denaturation temperature of chick tendon. Archives of Biochemistry and Biophysics, **158**, 478-484.

Rosenthal, P.J., McKerrow, J.H., Aikawa, M., Nagasawa, H. & Leech, J.H. (1988). A malarial cysteine proteinase is necessary for hemoglobin degradation by *Plasmodium vivax*. Journal of Clinical Investigation, **82**, 1560-1566.

Rosenthal, P.J., Ring, C.S., Chen, X. & Cohen, F.E. (1993) Characterisation of a *Plasmodium vivax* cysteine proteinase gene identifies uniquely conserved amino acids that may mediate the substrate specificity of malarial haemoglobinases. Journal of Molecular Biology, **241**, 312-316.

Rossignol, J.M. (1990) Chemotherapy: present status. In: Hookworm Disease: Current status and new directions, 281-290, Taylor and Francis, London.

Ruoslahti, E. (1988) Fibronectin and its receptors. Annual Review of Biochemistry, **57**, 375-413.

Ruppel, A., Diesteld, A.J. & Rother, O. (1985) Immunoblot analysis of *Schistosoma mansoni* antigens with sera of schistosomaisis patients: dianostic potential of an adult schistosome polypeptide. Clinical and Experimental Immunology, **62**, 499-506.

Salafsky, B., Fusco, A.C.,& Siddiqui, A. (1990) *Necator americanus*: factors influencing skin penetration by larvae. In: Hookworm Disease: Current status and new directions, 326-329, Taylor and Francis, London.

Sato, H & Kamiya, H. (1995) Role of epidermal Langerhans' cells in the induction of protective immunity to *Schistosoma mansoni* in guinea pigs. Immunology, **84**, 233-240.

Satrija, F., Nansen, P., Murtini, S. & He, S. (1995) Anthelmintic activity of papya latex against patent *Heligmosomoides polygyrus* infections in mice. Journal of Ethnopharmacology, **48**, 161-164.

Schad, G.A., Soulsby, E.J.L. & Chowdhury, A.B. (1975) Epidemiological and serological studies of hookworm infectionin endemic areas in India and West Africa. In: Nuclear Techniques in Helminthology Research, 41-54, Vienna Atomic Energy Agency.

Schad, G.A. & Banwell, J.G. (1984) Hookworms. In: Tropical and Geographical Medicine, 359-372, McGraw-Hill, New York.

Schad, G.A. & Anderson, R.M. (1985) Predisposition to hookworm infection in humans. Science, **228**, 1537-1540.

Schad, G.A. (1991) Hooked on hookworm: 25 years of attachment. Journal of Parasitology, **77**, 177-186.

Sen, H.G. & Seth, D. (1967) Complete development of the human hookworm, *Necator americanus*, in golden hamsters, *Mesocricetus auratus*. Nature, **214**, 609-610.

Sher, A. (1998) in The Biology of Parasitism, Englund, P.T. & Sher, A., eds, 169-182.

Shi, Y. E., Jiang, C. F., Han, J. J., Li, Y. L. & Ruppel, A. (1993) Immunisation with pigs against infection with *Schistosoma japonicum* using ultraviolet-attenuated cercariae. Parasitology, **106**, 459-462.

Slater, E.E. (1981). Renin. Methods in Enzymology **80**, 427-442.

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

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

Smith, A.M., Carmona, C., Dowd, A.J., McGonigle, S., Acosta, D. & Dalton, J.P. (1994a) Neutralisation of the activity of a *Fasciola hepatica* cathepsin L proteinase by anti cathepsin L antibodies. Parasite Immunology, **16**, 325-328.

Smith, A.M., Dalton, J.P., Clough, K.A., Kilbane, C.L., Harrop, S.A., Hole, N. & Brindley, P.J. (1994b) Adult *Schistosoma mansoni* express cathepsin L proteinase activity. Molecular and Biochemical Parasitology, **67**, 11-19.

Smith, C.A., & Wood, E.J. (1991). Fibrous proteins. In Biological Molecules. pp. 65-82. London: Chapman and Hall.

Smith, J.M. (1976) Comparative ultrasructure of the oesophageal glands of third stage larval hookworms. International Journal for Parasitology, **6**, 9-13.

Smith, N.C. (1998) Concepts and strategies for anti-parasite immunoprophylaxsis and therapy. International Journal for Parasitology, **22**, 1047-1082.

Smythies, L.E., Betts, C., Coulson, P.S., Dowling, M. & Wilson, R.A. (1996). Kinetics and mechanism of effector focus formation in the lungs of mice vaccinated with irradiated cercariae of *Schistosoma mansoni*. Parasite Immunology, **18**, 359-369.

Stirewalt, M. (1978) Quantitative collection and proteolytic activity of preacetabular gland enzyme(s) of cercariae of *Schistosoma mansoni.* American Journal of Tropical Medicine, **27**, 548-553.

Stryer, L. (1995). Biochemistry, Freeman, New York.

Tack, B.F., Dan Eilat, J.D., Lorenz, P.E. & Schecter, A.N. (1980) Tritium labelling of proteins to high specific radioactivity by reductive methylation. The Journal of Biological Chemistry, **255**, 8842-8847.

Talinan, R.V., Yang, X., Turbov, P., Seth, T., Ghayur, T., Casiano, C., Orth, K. & Froelich, C.J. (1997) Granule mediated cell killing pathways for granzyme B initiated apoptosis. Journal of Experimental Medicine. **186**, 1323-1331.

Takeda, O., Miura, Y., Mitta, M., Matsusita, H., Kato, I, Abe, Y., Yokosawa, H. & Ishii, S. (1994) Isolation and analysis of cDNA encoding a precursor of *Canavalia ensiformis* asparaginyl endopeptidase (legumain). Journal of Biochemistry, **116** (3), 541-546.

Taylor, M.M. & Turton, J.A. (1976) Antigen-induced lymphocyte blastogenesis in a hookworm (*Necator americanus*) infection in man. Tropenmed Parasit, **27**, 89-92.

Tellam, R.L. & Eisemann, C.H. (1994) Vaccination of sheep with purified serine proteases from the secretory and excretory material of *Lucilla cuprina* larvae. International Journal for Parasitology, **24**, 757-764.

Terranova, V.P., Rohrbach, D.H. & Martin, G.R. (1980) Role of laminin in the attachment of PAM 212 (epithelial) cells to basement membrane collagen. Cell, **22**, 719

Thorson, R.E. (1956a) The stimulation of acquired immunity in dogs by injections of extracts of the esophagus of adult hookworms. The Journal of Parasitology, **42**, 501-504.

Thorson, R.E. (1956b). Proteolytic activity in extracts of the esophagus of adults of Ancylostoma caninum and the effect of immune serum on this activity. Journal of Parasitology, **42**, 21-25.

Timms, A.R. & Bueding, E. (1959) Studies of a proteolytic enzyme from *Schistosoma mansoni*. British Journal of Pharmacology, **14**, 68-73.

Timothy, L.M. & Behnke, J.M. (1993) *Necator americanus* in inbred mice: a re-evaluation of primary infection kinetics. Parasitology, **107**, 425-431.

Timothy, L.M. (1994). PhD. Characterisation of the *Necator americanus* mouse model of human hookworm infection. University of Nottingham;

Timpl, R., & Martin, G.R. (1997). Components of basement membranes. In: Immunochemistry of the extracellular matrix, 119-150,

Towbin, H., Staehelin, T. & Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocelluose sheets: Procedure and some applications. Procedings of the National Academy of Science, **76**, 4350-4354.

Traub, W. & Piez, K.A. (1971) The chemistry and structure of collagen. Advances in Protein Chemistry, **25**, 243-352.

Udonsi, J.K. (1984) Changes in lipid contents of infective third-stage larvae of *Necator americanus* during desiccation and revival. Zeitschrift fur Parasitenkunde, **70**, 81-85.

van Noort, J.M., van den Berg, P. & Mattern, I.E. (1991) Visualisation of proteases within a complex sample following there selective retention on immobilised bacitracin, a peptide antibiotic. Analytical Biochemistry, **198**, 385-390.

Variyam, E.P. & Banwell, J.G. (1982) Hookworm disease: Nutritional implications. Reviews of infectious diseases, **4**, 830-835.

Verwaerde, C., Aurialt, C., Neyrinck, J.L. & Capron, A. (1988) Properties of serine proteases of *Schistosoma mansoni* schistosomula involved in the regulation of IgE synthesis. Scandinavian Journal of Immunology, **27**, 17-24.

Vinayak, V.K., Gupta, N. K., Chopra, A. K., Sharma, G. L. & Kumar, A. (1981) Effacies of vaccines against canine hookworm disease. Parasitology, **82**, 375-382.

Wakelin, D. (1985) Genetic control of immunity to helminth infections. Parasitology Today, **1**, 17-23.

Wales, A. & Kusel, J.R. (1992) Biochemistry of irradiated parasite vaccines: Suggested models for their mode of action. Parasitology Today, **8**, 358-363.

Wales, A., Kusel, J. R. & Jones, J. T. (1992) Inhibition of protein synthesis in irradiated larvae of *Schistosoma mansoni*. Parasite Immunology, **14**, 513-530.

Wales, A., Fukumoto, S.-I., Otieno, M. F. & Kusel, J. R. (1993) Effects of irradiation on surface carbohydrates of larvae of *Schistosoma mansoni*. Parasitology, **106**, 117-125.

Wang, C., Huang, X.X., Zhang, Y.Q., Yen, Q.Y. & Wen, Y. (1989) Efficacy of ivermectin in hookworms as examined in *Ancylostoma caninum* infections. Journal of Parasitology, **75**, 373-377.

Ward, R. E. M. & McLaren, D. J. (1988) *Schistosoma mansoni*: evidence that eosinophils and/or macrophages contribute to skin-phase challenge attrition in vaccinated CBA/Ca mice. Parasitology, **6**, 63-84.

Wells, C. & Behnke, J.M. (1988a) Acquired resistance to the human hookworm *Necator americanus* in mice. Parasite Immunology, **10**, 493-505.

Wells, C. & Behnke, J.M. (1988b) The course of primary infection with *Necator americanus* in syngeneic mice. International Journal for Parasitology, **18**, 47-51.

Wenstrup, R.J., Murad, S. & Pinnell, S.R. (1991) Collagen. In: Physiology, Biochemistry and Molecular Biology of the Skin. (Goldsmith, S.A. ed.), 481-508, Oxford University Press, London.

White, C.J., Maxwell, C.J. & Gallin, J.I. (1986) Changes in the structural and functional properties of human eosinophils during experimental hookworm infection. Journal of Infectious Diseases, **154**, 778-783.

Wijffels, G.L., Salvatore, L., Dosen, M., Waddington, J., Wilson, L., Thompson, C., Campbell, N., Sexton, J., Wicker, J., Bowen, F., Friedel, T. & Spithill, T.W. (1994) Vaccination of sheep with purified cysteine proteinases of *Fasciola hepatica* decreases worm fecundity. Experimental Parasitology, **78**, 132-148.

Wilkinson, M.J., Wells, C. & Behnke, J.M. (1990) *Necator americanus* in the mouse: histopathological changes with the passage of larvae through the lungs of mice exposed to primary and secondary infection. Parasitology Research, **76**, 386-392.

Wilson, R. A. & Coulson, (1988) Lung phase immunity to schistosomes: a new perspective on an old problem. Parasitology Today, **5** (9), 274-278.

Wilson, A. & Sher, A. (1993) Vaccines against schistosomes-an alternative view. Transactions of the Royal Society of Tropical Medicine and Hygiene, **87**(2), 237.

Woodley, D.T., Scheidt, V.J., Reese, M.J., Paller, A.S., Manning, T.O., Tosiike, T. & Briggaman, R.A. (1987) Localisation of the alpha 3 (V) chain of type V collagen in human skin. Journal of Investigative Dermatology, **88**, 246-252.

Yamakami, K. & Hamajima, F. (1986) Proteolysis of collagen by a thiol protease from *Paragonimus westermani* metacercariae. Japanese Journal of Parasitology, **35**, 145-148.

Yamakami, K. & Hamajima, F. (1987) Purification and properties of a neutral thiol protease from larval trematode parasite *Paragonimus westermani* metacercariae. Comparative Biochemistry and Physiology, **87B**, 643-648.

Yole, D.S., Pemberton, R. & Reid, G.D.F. (1996) Protective immunity in the olive baboon *Papio anubis* by the irradiated cecariae vaccine. Parasitology, **112**, 3663-3671.