THE NOTTINGHAM TRENT UNIVERSITY
Department of Life Sciences

Development of a vaccine against the human hookworm, *Necator americanus*

by

Nadine GIROD

A thesis submitted in partial fulfilment of the requirements of The Nottingham Trent University for the Degree of Doctor of Philosophy.

June 2001

Funded by The Sir Halley Stewart Trust

DECLARATION

This work has not been accepted for any other degree and is not concurrently being submitted for any other degree.

We certify that the work submitted was carried out by the author. Due acknowledgement has been made of any assistance received.

Signed.....(Candidate)

Signed..... (Director of studies)

A mes Parents,

ACKNOWLEDGEMENTS

I would like to thank Dr Ellen Billett for giving me the opportunity to do a PhD, for her support, scientific expertise and for so much indefatigable support besides the writing up. Thanks also to Professor David Pritchard for his enthusiasm, endless ideas and for the ('dodgy') parasite.

I would also like to thank Sir Halley Stewart Trust for their financial support. Thanks to Bob Rees, June, Selman, Karen and Fiona for fruitful collaborations. Thanks to Mike Billett who not only gave me a bit of his time to discuss molecular biology but also gave a little bit of his arm! Many thanks to all the technical staff for their great help and support, particularly Maggie and Roger.

A very special thanks to everybody body in the lab - Maxine, Cheryl, Nico, Gino, Alan (who probably wonder after all this years who is the parasite!), Ghazi, Kevin - who taught me Science as well as English (or at least they tried!) and who made this PhD really enjoyable. A special thanks to Maxine and Cheryl for being there for me. Thanks also to James, Comell, Cecile, John, Claire, and Zita (for letting me win at badminton). Many thanks are also due to people from Nottingham University.

Merci également ă Claire, Sandrine, Emilie et Julia pour leur amitie et support. Et 'Merci France Telecom' pour ta douce sonnerie du telephone!

Et enfin un grand MERCI ă mes parents, ma grand-mére, mon frere, ma soeur et leur famille respective. Merci de votre soutient, encouragement, patience et amour.... Merci pour tout.

ABSTRACT

Necator americanus infections cause iron-deficiency anaemia contributing, after years of infection to a deficit in both physical and intellectual development, particularly in children. Despite the wide range of anthelminthic treatments available, the prevalence of *N. americanus* infection remains a major public health concern. Therefore, the development of a vaccine, providing life-long protection, represents a useful means of controlling hookworm infection. In this thesis, an attempt was made to identify candidate vaccine molecules and establish which immune responses can confer protection.

Since *N. americanus* infect their host by skin penetration, the migratory behaviour of infective larvae through human, hamster and mouse *ex vivo* skins was studied by incubation with proteinase inhibitors. Skin penetration was shown to involve serine, aspartyl and metallo proteinases. Pepstatin A, an aspartyl proteinase inhibitor, was the most potent inhibitor, significantly inhibiting by up to 50 % larval penetration through human and hamster skins. The aspartyl proteinase(s) was also important in the penetration of gamma-irradiated (40 krad) larvae through *ex vivo* skin. However the migration of irradiated larvae, through *ex vivo* skin, was delayed compared to that of live larvae, despite the production of more excretory/secretory proteins containing proteolytic activity.

A successful vaccination protocol was then established, using gamma-irradiated larvae as the vaccine. Indeed, two immunisations with 300 gamma-irradiated larvae (40 krad) were shown to induce a high level of protection against *N. americanus* infection in BALB/c mice, based on worm recovery in the lungs and intestines. Inflammatory foci and mast cell proliferation were present in the skin of vaccinated animals. In addition, analysis of cytokine mRNA in the skin by RT-PCR indicated that Th2 type cytokines were dominant in the vaccinated group. Reduced pathology was also observed in the lungs of vaccinated animals. Levels of IL4 and γ-IFN cytokines, determined in ConA stimulated lymphocytes, from the spleen, axillary and mesenteric lymph nodes, using ELISA, indicated a higher IL4/γ-IFN ratio in the vaccinated groups, especially in the skin draining lymph nodes. On the other hand, a

primary infection suppressed a Th2 response to induce a Th1 response. It was therefore clear that irradiated larvae promote a protective Th2 response against subsequent infection, initiated by the entrance of the parasite into the skin.

Based on the previous findings, excretory/secretory products from irradiated larvae and larval aspartyl proteinases were used as candidate vaccines. Mice were vaccinated with excretory/secretory products from irradiated larvae in alum, a Th2 adjuvant. This vaccination resulted in a significant reduction (50.8 %) in worm burden in the intestine of vaccinated animals. However, a comparative study showed that alum induced higher levels of protection than Freund's adjuvant, a Th1 adjuvant. In an attempt to improve antigen presentation, mice were vaccinated intradermally with mature dendritic cells pulsed with worm cell lysate. Finally, since the aspartyl protease appeared crucial for infection, a recombinant aspartyl proteinase was then used as a vaccine; unfortunately this was not successful.

It is therefore likely that a successful vaccine against *N. americanus* will contain several defined antigens targeted against different stages of the parasite's life cycle, together with an adjuvant capable of inducing a Th2 response.

CONTENTS

2.3. VACCINATION TECHNIQUES

2.4. IMMUNOLOGICAL TECHNIQUES

2.5. EX-VIVO TECHNIQUES TO STUDY SKIN PENETRATION BY L3 LARVAE

2.6. HISTOLOGICAL TECHNIQUES

2.7. MOLECULAR BIOLOGY TECHNIQUES

CHAPTER 4

4. CHARACTERISATION OF A SUCCESSFUL VACCINATION MODEL AGAINST *Necator americanus*

FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

TABLES

CHAPTER 1

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

ABBREVIATIONS

The following abbreviations and terminology are used throughout the text:

°C	Degrees centigrade
Ab	Antibody
Abs	Absorbance
ABTS	3-ethylbenzthiazoline-6-sulfonic acid
AChE	Acetylcholinesterase
ADCC	Antibody-dependent cell-mediated cytotoxicity
Adj	Adjuvant
Ag	Antigen
APC	Antigen presenting cell
APMSF	4-(amidinophenyl) methanesulphonyl fluoride
APS	Ammonium persulphate
ASP	*Ancylostoma*-secreted protein
BALs	Broncho-alveolar leucocytes
BCIP	5 Bromo 4 chloro 3 indolyl phosphate ρ-toluidine salt
bp	Nucleotide base pairs
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CFA	Complete Freund's adjuvant
Con A	Concanavalin A
CMI	Cell-mediated immunity
DC	Dendritic cells
De	Dermis
DEs	Dermal equivalent skin
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's minimum essential medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
Dnase	Deoxyribonuclease
DTT	Dithiothreitol
dNTP	Deoxyribonucleotide triphosphates
E	Epidermis

E64	L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane
EDTA	Ethylenediaminetetraacetic acid
EFA	Essential fatty acids
ELISA	Enzyme linked immunosorbent assay
ES	Excretory/secretory products
FA	Freund's adjuvant
FCA	Freund's complete adjuvant
FCS	Foetal calf serum
FIA	Freund's incomplete adjuvant
FITC	Fluorescein isothiocyanate
g	gramme
g	Unit of gravity (9.8 ms^{-2})
γ-IFN	Gamma interferon
ISCOMs	Immune-stimulating complexes
h	Hour
H	Hypodermis
HBSS	Hanks' balanced salt solution
H&E	Haematoxylin and eosin
HF	Hair follicles
HIV	Human Immunodeficiency Virus
HRP	Horse radish peroxidase
i.d.	Intradermal
IES	Excretory/secretory products from irradiated larvae
IF	Inflammatory foci
IFA	Incomplete Freund's adjuvant
Ig	Immunoglobulin
Ig GAM	Immunoglobulins G, A and M
IL	Interleukin
IL4	Interleukin 4
i.p.	Intraperitoneal
i.u.	International Unit
i.v.	Intravenous
kb	Kilobase
kDa	Kilodalton
L_3	Third stage larvae

L_4	Fourth larval stage of life-cycle
LC	Langerhans' cells
LN	Lymph node
m	meter
M	molar
MC	Mast cell
mA	milliampere
mAb	Monoclonal antibody
mg	milligram
min	minutes
ml	milliliter
mm	millimetre
mM	millimolar
mol wt	Molecular weight
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide
n	Number in study or group
Necepsin	*Necator americanus* pepsin-like proteinase
NBF	Neutral buffered formaldehyde
NBT	Nitro blue tetrazolium
NV	Non-vaccinated
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS/Tween	Phosphate buffered saline/0.05 % (v/v)Tween 20
PCR	Polymerase Chain Reaction
PD	Papillary dermis
Phe	1,10, phenanthroline
RD	Reticular dermis
RNA	Ribonucleic Acid
RNase	Ribonuclease
RT-PCR	Reverse Transcription PCR
s.c.	Subcutaneous
SC	Stratum corneum
SD	Standard deviation

SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SG	Sebaceous gland
Sm28 GST	Schistosome 28-kDa Glutathione-S-transferase
TAE	Tris acetate EDTA electrophoresis buffer
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TEMED	N'N'N-tetramethylethylenediamine
Tfl	*Thermus flavus*
Th1	T helper type 1
Th2	T helper type 2
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
µg	microgram
µl	microlitre
V	Volt
v/v	volume/volume
U	unit
WHO	World Health Organisation
W/v	weight/volume

Chapter 1

General Introduction

1. GENERAL INTRODUCTION

Hookworms are intestinal blood-feeding parasites, affecting over 1000 million individuals (Schad, 1991; Chan, 1997). Hookworm infection results in iron-deficiency anaemia, which in chronically infected individuals can severely impair the growth and development of children and significantly reduce the working capacity of adults (Hotez *et al.*, 1987; Hotez and Pritchard, 1995). Anthelminthic drug therapies are available to control hookworm prevalence; however re-infection from the environment occurs very rapidly after treatment. In addition, parasite resistance to anthelminthics is growing alarmingly and no human vaccines are yet available. The development of a vaccine, providing life-long protection, will therefore represent an attractive and necessary solution to eradicate hookworm infection.

1.1. HUMAN HOOKWORM

1.1.1. Species and morphology

Three different types of parasites have the ability to infect man and/or animals; the Protozoa which represent the unicellular microscopic animals including Plasmodium species responsible for malaria disease; the Arthropods constituting 80 % of all the animal species and representing highly organised invertebrate animals such as ticks and mites and finally the Helminths (worms) (table 1.1.).

Helminths are sub-divided into the Nematodes (roundworms; major classes: Phasmidia, Aphasmidia), the Platyhelminthes (flatworms; major classes: Cestoidea [tapeworms], Trematoda [flukes]) and the Acanthocephala ('spiny-headed worms').

Nematodes are cylindrical, non-segmented worms and are widely distributed. Approximately 25 % of all nematodes are parasitic and are of considerable economic and social importance. Infectious diseases caused by parasitic roundworms afflict over two billion people worldwide.

Table 1.1. Simplified classification of the main parasites

GROUP	PHYLUM	SUBCLASS	SUBORDER	NAME
Protozoa	Apicomplexa	Plasmodiida	Plasmodium	*Plasmodium falciparum*
	Mastigophora	Zoomastigophorea	Leishmania	*Leishmania tropica*
			Trypanosoma	*Trypanosoma cruzi*
Arthropoda	Arachnoidea	Arachnida	Acarina	Mites
				Ticks
Helminth	**Nematode**	**Phasmidia**	**Strongylata**	***Necator americanus***
				Ancylostoma duodenale
				Ancylostoma caninum
				Haemonchus contortus
			Rabditata	*Caenorhabditis elegans*
			Ascaridata	*Ascaris lumbricoides*
			Spirurata	*Brugia malayi*
				Wuchereria bancrofti
				Onchocerca volvulus
				Loa loa
		Aphasmidia	Trichurata	*Trichuridae Trichuris*
				Trichuris trichiura
	Platyhelminth	Cestoidea	Cyclophyllidea	*Taenia ovis*
				Taenia saginata
		Trematoda	Digenea	*Schistosoma mansoni*
			Monogenea	*Fasciola hepatica*
				Fasciolopsis buski
	Acantocephala	Archiacanthocephala	Moniliformis	*Moniliformis dubius*

Hookworms are intestinal parasitic roundworms that take their name from the hooked appendages that surround their mouths. Hookworms belong to the nematode of the subclass Phasmidia, suborder Strongylata, family Ancylostomidae (StGeorgiev, 1999). The two main hookworms responsible for human infection are *Necator americanus* and *Ancylostoma duodenale.* These two human hookworms are very similar in general appearance and in most details of their life cycle. Some other hookworm species, which are responsible for diseases in cats and dogs, such as *Ancylostoma caninum, A. ceylanicum* and *A. braziliense* also have the ability to penetrate human skin, but fail to complete their life cycle in the human host (Beaver, 1964; Caroll and Grove, 1986; Prociv and Croese, 1990; Chowdhury and Schad, 1972).

Male *N. americanus* measure only 7 to 9 mm by 0.3 mm in diameter compared to the female, of 9 to 11 mm long by 0.4 mm (figure 1.1.). The posterior tail of the adult female ends into a simple conical tail, contrasting with the complex copulatory bursa of the male's tail. *N. americanus* possess a simple digestive tract, starting from a mouth characterized by two pairs of semi-circular cutting plates aiding in the attachment of the worm to the mucosa and finishing by an anus (Banwell and Schad, 1978). *N. americanus* possess a well-developed secretory system, consisting of a glandular apparatus opening through an anterior pore and are covered by a very resistant cuticle, consisting of three acellular morphological distinct layers overlying the hypodermis (Pritchard, 1987).

Figure 1.1. ***Adult male and female*** **Necator americanus** *modified from Lee (1977).*

1.1.2. Geographical distribution

The distribution of *N. americanus* is rarely distinguished from the distribution of *A. duodenale*. However where mixed infections occur, one of the two species predominates. Although *N. americanus* was first discovered in America in 1902, it is likely that Necator is African in origin, exported to America via infected slaves. Despite reports of hookworm infection occurring in Europe, (e.g. in 1880 causing anaemia in Italian miners), the parasite has been largely eradicated in this area by high living standards and sanitary disposal of excrement (Banwell and Schad, 1978). Hookworms are found today mainly in the tropical and subtropical parts of the world (figure 1.2.), where living and climatic conditions (temperature, humidity) favour hookworm survival and transmission. Consequently, *N. americanus* predominates in India, South Pacific Islands (principally in Papua New Guinea), Central and South America and South Africa (Schad, 1991a; Hotez and Pritchard, 1995).

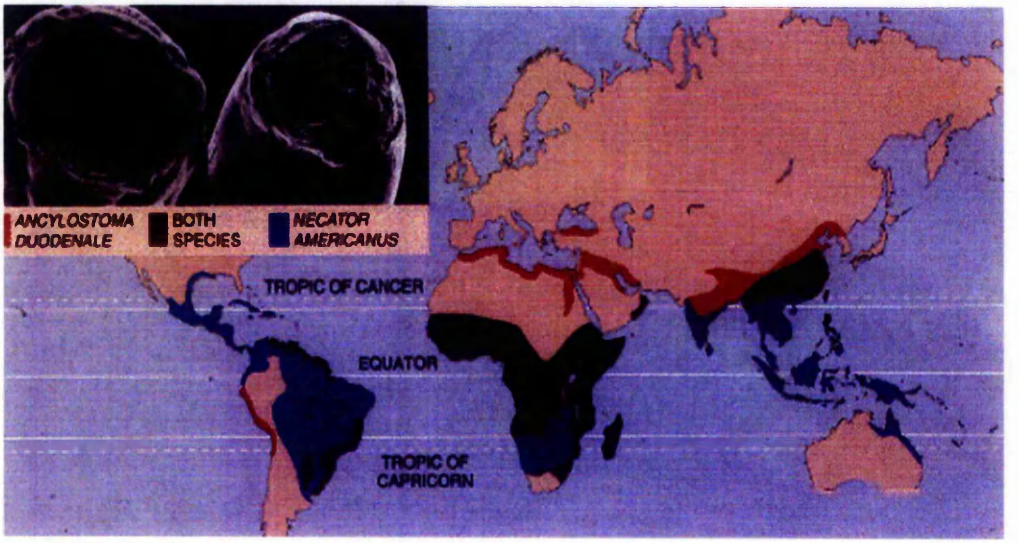


Figure 1.2. Distribution of hookworms. *World-wide distribution of hookworms which infect humans; Ancylostoma duodenale (red), Necator americanus (blue), both species (purple). (From Hotez and Pritchard, 1995).*

1.1.3. Life cycle

Hookworms have a direct life cycle, consisting of five stages and four moults (figure 1.3.). The adult worms live in the intestine, mainly in the duodenum and jejunum, where they are attached to the mucosa by the buccal capsule, acting as a suction pump. While in the intestine, adult worms mate and produce between 5 000 to 10 000 eggs per day which are then excreted at the four cells stage within the host faeces (Banwell and Schad, 1978).

Under favorable conditions (15 – 33 °C), the eggs hatch within 24 hours and undergo three moults (Rep, 1975; Miller, 1979; Udonsi and Atata, 1987). The first stage larvae, L_1, feed on faecal microflora, grow and moult to give the second-stage L_2 larvae. After several days, these free-living feeding larvae undergo a further period of growth and synthesise a new L_3 cuticule. The resulting non-feeding infective third-stage infective larvae (L_3) complete their development in 5 to 10 days. L_3 larvae, present in the upper half-inch of moist soil, then await for a host to approach. Even under optimal conditions, less than 1 % larvae survive for more than one month (Banwell and Schad, 1978).

L_3 larvae possess well-developed sensory structures, sensitive to temperature and carbon dioxide variations, which subsequently inform the larvae of the presence of a host. Stimulated larvae then migrate vertically to the soil surface or blades of grass towards their host. Once in contact with the host, L_3 larvae penetrate the skin, usually on the lower extremities. The infective larvae invade the epidermis and the dermis in two days. Unlike the obligate skin penetrator *N. americanus*, *A. duodenale* L_3 larvae have the additional option of oral infection (Schad *et al.*, 1973).

After percutaneous invasion, L_3 larvae are transported via the host venous circulation to the heart and reach the lungs one week post-infection. In the lungs, larvae break out of the capillaries into the alveoli. The larvae migrate up to the trachea and are coughed up into the mouth. The larvae are then swallowed and reach the small intestine where final development and maturation occurs. The intestinal stage consists of L_4 larvae and the final adult stage.

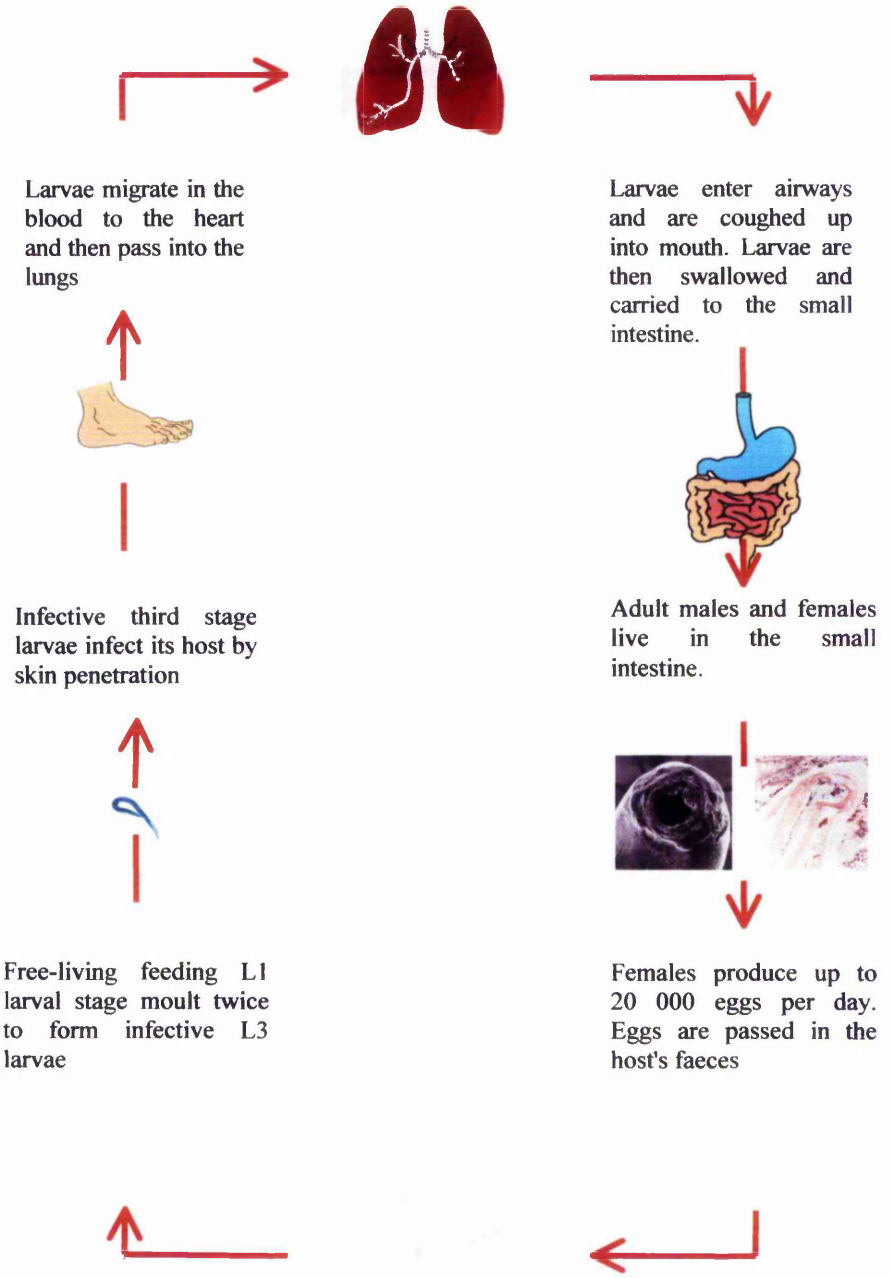


Figure 1. 3. Life cycle of *Necator americanus*. *Adult hookworms residing in the small intestine mate and fertilised eggs are passed out with the faecal material. Under favourable conditions the eggs hatch and develop into infective L_3 larvae.* N. americanus *infect their host by penetrating the skin barrier. Following entry into the host the larvae reach the intestinal tract via the circulation and lungs where they mature into adult hookworms, (adapted from www.biosci.ohio-state.edu/~parasite/life_cycles.html web page).*

The final moult is completed 5-6 weeks after infection. Under field conditions, adult worms have the ability to survive in the human intestine for about 3-4 years (Schad and Banwell, 1984).

1.1.4. Hookworm secretions

N. americanus excrete or secrete numerous products (referred to as 'excretory/secretory', ES, products) of which a few have been identified. However, the adult and larval stage of *N. americanus* have been shown to secrete a mixture of proteinases, essential in the maintenance of the hookworm life cycle (Salafsky *et al.*, 1990; Burleigh, 1992; Kumar and Pritchard 1992a; Kumar and Pritchard, 1992b; Brown *et al.*, 1995; Brown *et al.*, 1999). These proteinases belong to all four protease classes, namely aspartyl, serine, cysteine and metallo proteinases.

In order to survive in the intestine, adult *N. americanus* secrete a number of anti-haemostatic factors, capable of inhibiting both platelet aggregation and blood coagulation Factor Xa (Furmidge *et al.*, 1995; Pritchard and Furmidge, 1995). An aspartyl proteinase activity, degrading fibrinogen, is thought to be partly responsible for the inhibition of platelet activation (Brown, 2000). A similar anti-haemostatic activity has been shown to be present in soluble extracts of the dog hookworm *A. caninum* (Cappello *et al.*, 1993). The glutathione-S-transferase (GST, an aspartyl proteinase) and superoxide dismutase (SOD) also present in adult ES products have been described as defensive enzymes against the immune response, neutralizing reactive oxygen species released by eosinophils at the site of infection (Brophy and Barrett, 1990; Brophy *et al.*, 1995). SOD may also be acting as anti-haemostatic agents (Pritchard, 1996). Adult *N. americanus* also release considerable amounts of acetylcholine esterase (AChE) (Pritchard *et al.*, 1991; Pritchard *et al.*, 1994) but its biological function remains enigmatic.

N. americanus larval ES products are less well characterized. Acetylcholine esterase activity is not present in larval ES products (Pritchard *et al.*, 1991). The four classes of proteinases present in larval ES have been shown to degrade skin macromolecules, *in vitro* (Brown *et al.*, 1999) and it has been proposed that the enzymes play a role in

skin penetration by infective larvae. The importance of larval secretions will be discussed in more detail in Chapter 3.

1.2. HOOKWORM INFECTIONS

1.2.1. Prevalence

In 1996, parasitic infections (single or multiple) were affecting three-quarters of the global population, among which more than 1 000 million people suffered from hookworm infection (table 1.2.) (Chan, 1997).

Table 1.2. Prevalence of parasitic diseases caused by nematodes *(figures from Pan American Health Organisation home page, updated 1997).*

Species		**Prevalence (millions)**
Filarial nematodes	*Wuchereria bancrofti*	73
	Onchocerca volvulus	18
	Brugia malayi	6
Gastro-intestinal nematodes	***Ancylostoma duodenale*** *(hookworm)*	**1000**
	Necator americanus *(hookworm)*	
	Ascaris lumbricoides	1000
	Trichuris trichura	900
	Strongyloides stercoralis	100
Trematodes (flukes)	*Schistosoma mansoni*	
	Schistosoma haematobium	300
	Schistosoma japonicum	

Despite medical and chemical successes along with good environmental and social conditions, hookworm prevalence has been increasing for the last ten years, particularly in China (Labiano *et al.*, 1999; Liu, 1999). A study conducted in China between 1988 and 1992 showed that 17 % of China's population was infected with hookworm, reaching 87 % of the population in certain regions (Xu *et al.*, 1999). In 1995, an epidemiological study showed that India was also amongst the countries

with the highest number of hookworm cases (Hotez and Pritchard, 1995). While in Papua New Guinea, where most of the field studies are conducted, hookworm infections reached a prevalence of near 100 % in adults, each harboring a mean of 40 worms (Pritchard *et al.*, 1990a).

1.2.2. Pathology

1.2.2.1. Pathology in the skin

N. americanus infect their host by skin penetration and their migration through the skin induces an intense itching and stinging sensation, followed by a dermatitis, known as 'ground itch' (Miller, 1979). *N. americanus* stay in the skin for 2-4 days, but the skin pathology can last up to two weeks. The pathological changes associated with hookworm penetration are few and still poorly understood. The intensity of the symptoms may depend on bacteria and micro-organisms present on the larval surface. Indeed, *A. ceylanicum* larvae carefully cleaned with antibiotic treatment do not cause any symptoms during skin penetration (Carroll and Grove, 1986). Repeated exposure to infection leads to more intense responses. Despite the inflammatory response observed in man, no correlation has been made with protection against *N. americanus*.

1.2.2.2. Pathology in the lungs

One week after infection, hookworm larvae reach the lungs and undergo important morphological changes, inducing some extensive damage (Behnke *et al.*, 1986a). Indeed, the presence of larvae in the lungs induces respiratory distress, bronchitis, asthma and alveolar haemorrhages. Upper respiratory tract symptoms are, however, more common and these include pharyngitis and laryngitis (reviewed by Miller, 1979). In addition, difficulty in swallowing with associated pain has been described. Despite the severe pathology, the condition of the lungs normally improves in the week following larval departure.

1.2.2.3. Pathology in the intestines

The main pathology observed during hookworm infection is iron deficiency anaemia, associated with abdominal pain, diarrhoea and protein malnutrition (Hotez and Pritchard, 1995). Iron-deficiency anaemia is caused by intestinal blood loss caused by the attachment of the adult parasites to the surface and subsurface layers of the intestinal wall (figure 1.4.).

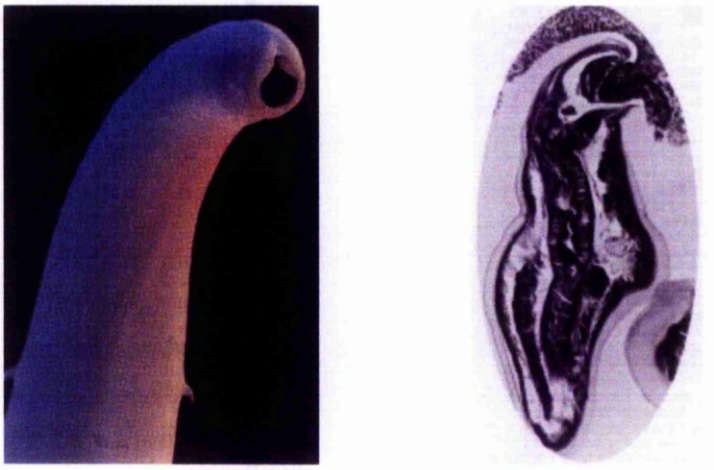


Figure 1.4. ***Necator americanus*** Electron microscope view of *N. americanus* (left) and cross section of adult hookworm in the small intestine shown sucking in superficial layers of the intestinal wall (right). (David Scharf and Wayne M. Meyers *Armed Forces Institute of Pathology* from Hotez and Pritchard, 1995).

The blood released is either ingested by the parasite or leaks at the site of hookworm attachment. Blood loss increases as the parasite moves and reattaches itself every 4-6 hours (Variyam and Banwell, 1982). This results in a new bleeding area as well as the incessant bleeding occurring at the old attachment site. This way, a single worm causes between 30 to 50 µl of blood loss per day in the case of *N. americanus*, and 120 to 230 µl for *A. duodenale*. Consequently, in heavily infected people (2500 worms/host), blood loss increases dramatically and can reach 100 ml per day.

After a variable period of time, symptoms of anaemia appear with lassitude, breathlessness, palpitations, mental apathy and depression. The damage created by hookworms directly to the mucosa appears to be minimal and limited at the feeding sites where the worms bite into the gut tissue. Regeneration and repair of damaged tissue are rapid and there is little evidence of persistence scars, atrophy or extensive general pathology in the intestine. However, in the long term, due to its chronic nature, even mild iron-deficiency anaemia can lead to intellectual and growth retardation and can even be fatal (Ogilvie *et al.*, 1978; Hotez and Pritchard, 1995). In China, for instance, infantile hookworm disease has a mortality rate of 3.6-6.0 % (Liu, *et al.*, 1999).

1.2.3. Diagnosis and treatment

Because most of the symptoms associated with hookworm infection correlate with other diseases such as depression or malnutrition, hookworm infection cannot be diagnosed only by symptoms. Furthermore, immunological methods of diagnosis based on the detection of parasite antigens are not yet in routine use. Therefore, accurate diagnosis still depends on the identification of hookworm eggs or larvae in human faeces (Banwell and Schad, 1978). These tedious methods are still used in population surveys and control programmes. The estimation of worm burden by counting eggs in faecal samples gives a rough value for the intensity of infection but does not give an indication of the exact numbers of adult worms present in a patient. An accurate measure of the adult worm load in a patient can be obtained after treatment by a vermifuge.

Once diagnosed, most parasitic diseases can be treated by chemotherapeutic agents. The main anthelminthics currently used are ivermectin, mebendazole, albendazole and pyrantel, albendazole being the most effective against hookworm infection (Sacko, 1999). The drugs are absorbed by the parasite either via its alimentary tract or its cuticule, and lead to damage to the parasite's neuromuscular function, resulting in the expulsion of the worms just a few days after drug administration (Wang *et al.*, 1989; Rossignol, 1998). One dose of albendazole leads to expulsion of 92-99 % of the worms, accompanied by a sustained improvement in the growth and academic performance of the host, particularly in children (Stephenson *et al.*, 1989). Mass

treatment of infected human populations with chemotherapeutic drugs has been effective in reducing the prevalence of disease. However all these drugs have limitations; they are expensive, have adverse effects, are hard to obtain in many places and notably don't prevent re-infection. Indeed re-infection usually occurs several months after specific anthelminthic administration (Gutteridge, 1982; Quinnell *et al.*, 1993). Furthermore, as indicated earlier, the potential for the eventual emergence of drug-resistant strains of parasite remains a persistent threat.

Therefore prevention could be a strategy to control the prevalence of hookworm infection. Improvements in the ultrastructure of sanitation (latrines, wells) and the standard of living will reduce if not eradicate infection, as observed in Europe. However the construction of a sanitation system is unlikely to happen in the near future in most developing countries, just as it is unlikely that people living in the hot tropics will always wear protective clothing to reduce exposure to parasites.

For all these reasons, the development of a vaccine will be the most efficient and cost-effective means to prevent infectious diseases. An ideal vaccine will have to be administered as a single dose before natural exposure to the parasite and provide a life-long protection. Unfortunately, the chronicity and the relatively low incidence of mortality with hookworm infection has often resulted in a low priority for programmes aimed at the control of hookworms and in the availability of limited funding for research.

1.3. IMMUNE RESPONSES TO *N. AMERICANUS* INFECTION

1.3.1. Natural immune response

The ability of helminths to survive in their host for a long time as well as their ability to rapidly re-infect them after treatment suggests an absence of innate and acquired immune responses to the parasite or the ability of the parasite to evade the host's immune response (Hotez and Pritchard, 1995). It has been shown that some people are predisposed to hookworm infection. Indeed, individuals infected by only a few worms could suffer from a severe infection (Schad and Anderson, 1985; Haswell *et*

al., 1988; Quinnell *et al.*, 1993). In addition, a survey carried out in North Carolina, USA, during the 1920s and 1930s showed that 22 % of the white population were infected with hookworm compared to 4 % in the black population (Banwell and Schad, 1978) and that the infected white population harboured heavier infection compared to those in the black population. Similarly, females have been shown to be more susceptible to hookworm infection (Banwell and Schad, 1978; Hotez and Pritchard, 1995). However, this difference in host susceptibility depends more on the genetic background than an adapted immune response.

The small evidence for the elaboration of an acquired immune response in man against hookworm infection resides in the disparity in prevalence and intensity between children and older age-groups. Indeed, in most endemic areas, the prevalence and intensity of hookworm infections have been shown to be higher in children than in adults (25-50 years old) (Liu *et al.*, 1999; Labiano Abello *et al.*, 1999). However it is important to note that the peak of prevalence and intensity noticed in hookworm infection, particularly for *N. americanus* infection, is very low compared to other parasitic infections such as *Ascaris lumbricoides* or *Trichuris trichiura* (Bundy, 1995). Children infected with *N. americanus* in their first 4 years of life and if further exposed, seem to accumulate parasites until the age of 20-30 years when worm burden appears to plateau (Schad *et al.*, 1975). This plateau can be attributed to the development of a protective immunity rather than to non-specific age-related changes in human behaviour. Indeed, no evidence for a change in the rate of exposure was noticed. The development of Necator specific antibody responses in human volunteers infected several times confirmed this concept. It has been demonstrated that the correlation between anti-larval IgG levels against worm burden declines consistently with host age (Quinnell *et al.*, 1995). Therefore, the host is able to develop an immune response against the parasite, but the response is not sufficient to expel the parasite, suggesting that the worm has evolved an effective strategy to evade host immune effector mechanisms.

1.3.2. Humoral immune responses

Following hookworm infection, as in all helminth infections, a strong antibody response has been observed, involving all antibody isotypes, particularly IgG and IgE. The effects and mechanisms by which antibodies could control parasitic infections are described figure 1.5.

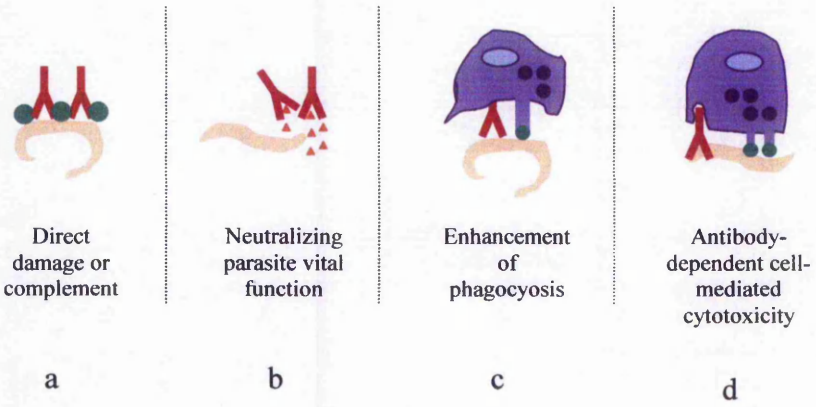


Figure 1.5. Mechanisms by which specific antibody could control parasitic infections. *The antibody can: a/ act directly on the parasite or can activate the classical complement pathway, causing damage to the parasite membrane; b/ neutralise the parasite by blocking its attachment or vital functions; c/ enhance phagocytosis by macrophages; d/ enhance antibody dependent cell mediated cytotoxicity, resulting in worm damage (adapted from Roitt, Fifth Edition, 1998).*

Human sera from infected individuals recognize antigens of each developmental stage of the parasite (Pritchard *et al.* 1988, Pritchard, 1990; Pritchard *et al.*, 1990; Wells and Behnke, 1988a). However, the principal antibody responses are directed against adult cuticular collagen (figure 1.5.a.). Such a humoral immune response has also been observed against the malarial parasite, *Plasmodium berghei*, damaging the outer membrane of the parasite, resulting in fluid leakage. However, no evidence of parasite damage has so far been observed against Necator. In *N. americanus* infection, the humoral response is also directed against acetylcholine esterase, and other components of the excreted/secreted material of the third stage larvae, suggesting that the antibody response is directed towards the vital function of the worm in order to neutralize its migration (figure 1.5.b.) (Wells and Behnke, 1988a). Although patients infected with *N. americanus* develop humoral antibody responses,

their antibody titres (with the possible exception of IgE, Pritchard and Walsh, 1995) do not correlate with resistance (Pritchard *et al.*, 1990a).

High levels of IgE is characteristic of helminth infections. However, the only evidence for an IgE effective immune response against *N. americanus* resides in the negative correlation observed between total IgE levels and parasite weight and fecundity (Pritchard *et al.*, 1995). A small proportion of IgE antibody has been shown to be Necator specific, IgE specific antibodies being directed against adult secretions (Pritchard and Walsh, 1995). Similarly, Dessaint *et al.,* (1975) showed that only 10 % of the IgE response was parasite specific following *S. mansoni* infection. The absence of convincing evidence for the protective role of IgE implies that IgE could be beneficial to the parasites and harmful to the host. Indeed, parasite non specific IgE antibody could saturate Fc_ε receptors present on mast cells, eosinophils and B lymphocytes, therefore preventing the attachment of parasite specific IgE and subsequently immunological effector mechanisms against the parasite. Furthermore, the binding of IgE on mast cells leads to the release of mast cell mediators which will then cause pathology such as allergy and asthma. The parasite accentuates this reaction by secreting some allergen like molecules such as calreticulin (Pritchard *et al.*, 1999).

1.3.3. Cell mediated responses

1.3.3.1. Mast cell responses

Mast cells are distributed in tissue exposed to the external environment, that is the skin, lungs and intestines. Mast cells, present where infection occurs, have been suggested to be major effector cells in parasite immunity (Lee *et al.*, 1986; Wakelin, 1993).

Accumulation of mast cells at the sites of parasitic infection has been observed in helminth infections (Miller *et al.*, 1993; Winter *et al.*, 1997, Cutts and Wilson, 1997). The release of antigens by the parasites stimulates the release of cytokines that recruit mast cells to the region of infection. The significance of this accumulation remains controversial although it has been shown that expulsion of the intestinal

nematode, *Trichinella spiralis,* is frequently associated with the local and systemic release of mast cell granule proteases (Woodbury *et al.*, 1984). Miller *et al.*, (1983) showed that mucosal mast cells are activated in response to *Nippostrongylus brasiliensis* challenge infections or to parasite antigens. The elimination of a primary schistosome infection in rats has also been shown to coincide with elevated IgE titres and mast cell degranulation (Cutts and Wilson, 1997). Once activated, mast cells release mediators such as histamine, prostaglandin D2, leukotriene, proteases and cytokines, including IL4 (Tunon de lara, 1996). The release of mast cell mediators could directly have an effect on the parasite survival. Thus pretreatment of *Trichostrongylus colubriformis* larvae *in vitro* with the mast cell mediator serotonin resulted in metabolic damage to the worms (Rothwell, 1989). Mast cell proteases have also been shown to digest adult Necator cuticular collagen, responsible for maintaining the structural integrity of the nematode cuticle (McKean and Pritchard, 1989). However, these collagens are unlikely to be directly available to the mast cells, being masked by the surface coat. Once the surface coat is disrupted by an immune response, the structural components of the cuticule become exposed to mast cells and will then be susceptible to mast cell protease degradation.

Mast cells not only appear to have a direct effect on parasite survival, they also have the ability to recruit eosinophils, neutrophils and platelets to the site of infection, increasing the host's defence. Although mast cell activation may benefit the host, mast cell degranulation is also the major source of pathology. The release of histamine, heparin and mast cell proteases by activated mast cells results in bronchoconstriction, increased vascular permeability and induction of tissue pathology (Mota, 1994). This immune response is amplified and triggered by the presence of high levels of IgE, as mentioned previously.

1.3.3.2. Eosinophil responses

An elevation of eosinophil numbers is also the hallmark of the immune response to parasite infections (Teixeira *et al.*, 1993). In human helminthiases, more than 50 % of circulating white blood cells may be eosinophil granulocytes, in contrast to their normal level of 2-5 % (Maizels *et al.*, 1983). There is a controversy as to the importance of eosinophils in the immune control of parasites. Wells and Behnke

(1988) clearly established that the invasive and migratory stages of *N. americanus* infections were capable of eliciting an eosinophilia in mice; however insufficient data - with respect to infection kinetics - were available to establish a correlation between resistance to infection and eosinophil response. In association with IgE these cells have the ability to kill helminth larvae. High peripheral blood eosinophil counts are associated in resistance to re-infection with *S. mansoni*, *S. haematobium* and *N. americanus* (Dent *et al.*, 1997; Culley *et al.*, 2000). Eosinophils are capable of secreting and generating many toxic products such as major basic protein and superoxide anions. It has been shown that eosinophils play an important role in the killing of cercariae schistosomes, (Dent *et al.*, 1997), acting on the infective larval stages, but not on the adult stages (Meeusen and Balic, 2000). On the other hand, Wakelin (1993) showed that injection of anti-IL5 antibody (which eliminates eosinophils in mice) did not affect *Trichinella spiralis* expulsion and had no effect on the development of protective immunity.

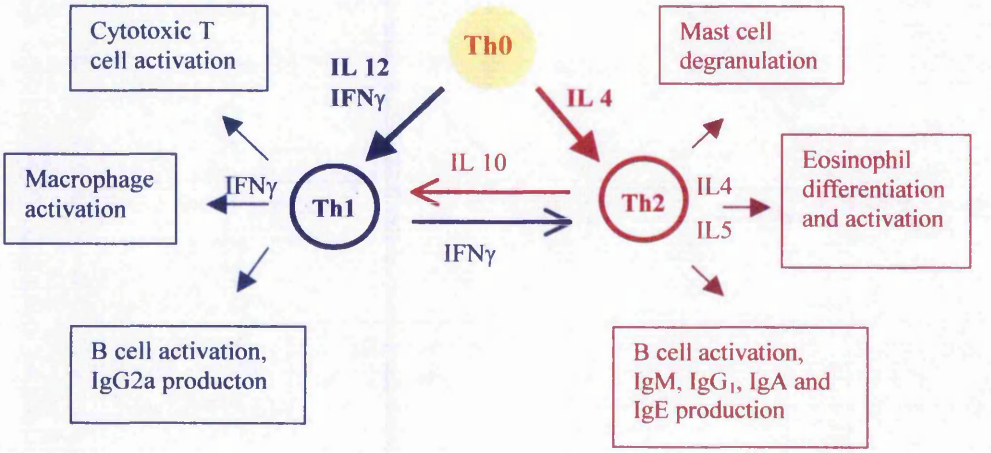
The elevation of eosinophil levels may protect the host but the products released from eosinophils also induce important immunopathology, such as allergy. The involvement of eosinophils in allergic reactions at mucosal surfaces is usually preceded by the crosslinking of IgE on the surfaces of mast cells resulting in a localized inflammatory response characterized by vasodilation and the infiltration of monocytes, more eosinophils and platelets.

1.3.3.3. T cell responses

In most parasitic infections, protection cannot be conferred experimentally in T cell deprived mice. Manipulation of the host by thymectomy, irradiation and adoptive or passive immunization has established that the thymus and T cells are crucial in the development of resistance during primary infections against infectious diseases. The type of T cell, namely $CD4^+$ and $CD8^+$ T cell, responsible for controlling an infection varies with the parasite and the stage of infection. In chronic infection, $CD4^+$ T cells seem to be essential in the development of immune protection against a parasite. $CD4^+$ T cells, called T helper (Th) cells, can be divided into T helper 1 subset (Th1) and T helper 2 subset (Th2). Some studies indicate that the balance between Th1 and Th2 cytokines regulate the immune response to infection. Th1 cytokines (IL2, IFN-

gamma) are inflammatory mediators and selectively activate macrophages and cytotoxic T cells, whereas Th2 cytokines (IL4, IL5, IL10) stimulate eosinophilia, antibody production and subsequent antibody-dependent cellular cytotoxicity reactions (figure 1.6.) (Mosmann and Coffman, 1989). These subsets are reciprocally cross-inhibitory. The balance between type 1 and type 2 responses is a central issue in all helminth infections studied (Bancroft and Grencis, 1998; Maizels *et al.*, 1999).

Figure 1.6. Differentiation and selection of effector mechanisms of CD4$^+$ T helper cells. *(adapted from Roitt, Fifth Edition, 1998).*



The presence of high levels of IgE and eosinophils during *N. americanus* infection is characteristic of a Th2 response. But it remains unclear whether the Th2 derived responses are important in the protective immune response to the parasite or are responsible for immune-mediated pathology (Finkerman *et al.*, 1991; Romagnani, 1997). In some worm infections, Th2 cell cytokines may contribute to host protection, while in others they may promote parasite survival. Parasites may manipulate the Th1/Th2 balance to promote their survival. In human schistosomiasis, both elevated eosinophilia and specific IgE responses correlate with resistance to reinfection after chemotherapy. In mice susceptible to *Trichuris muris*, the suppression of γ-IFN (Th1 cytokine) results in expulsion of the parasite; this is the first evidence for a role for γ-IFN in the establishment of a chronic helminth infection

(Wigzell *et al.*, 1993). Parasite specific IgE and eosinophils may be involved in anti-parasite effector mechanisms or may simply be markers of Th2 activation like non-specific IgE (Pritchard *et al.*, 1995).

Nematode infection in mouse models has shown that the protective Th2 response is only induced by the early larval stage while latter stages and adult worms induce a Th1 response (Grencis, 1993; Else *et al.*, 1993), suggesting that the early stages of an infection are important in the induction of immune protection against the parasite.

1.4. OVERVIEW OF VACCINATION

1.4.1. General principles

Infectious diseases have always been the leading cause of human death around the world. For a time, it was widely assumed that most of the infectious diseases would have been brought under control thanks to the use of vaccine technology. However, today, no vaccine is yet available for any human parasite. The British physician Edward Jenner took the first rational approach to vaccination in 1798 when he used cowpox to immunize individuals against the devastating disease smallpox. Subsequently, Louis Pasteur proved that altered preparations of microbes could be used to generate immunity against the virulent organism. This discovery led to the production of a successful vaccine against rabies, in 1885.

The aim of a vaccine is to induce a faster and more effective immune response during second exposure to an immunogen, resulting in an accelerated elimination of the organism and long-lasting protection from the disease. The rapid secondary immune response is based on two key elements of the adaptive immune system, namely specificity and memory. The first exposure to a pathogen induces a clonal expansion of specific T and/or B cells, leaving behind a population of memory cells available in case of a second exposure.

In order to be used in a vaccine, the immunogen responsible for a disease must be altered in such a way that it becomes harmless without losing immunogenicity. Live

or killed pathogens are the most commonly used antigens, reproducing many of the features of wild type infection, without causing clinical disease. Live pathogens are attenuated by exposure to radiation (ultraviolet, X-ray or gamma source), treatment with chemicals or passage through intermediate hosts. At present, numerous live-attenuated vaccines are licensed for use in humans (table 1.3.).

Table 1.3. Range of antigenic preparations used as vaccines (from Roitt, Fifth edition, 1998).

Type of Antigen		*Vaccine examples*
Living organisms	*natural*	Smallpox vaccine
	attenuated	Measles, mumps, rubella, BCG
Killed organisms	*viruses*	Polio, influenza, hepatitis A
	bacteria	Pertussis, typhoid, cholera
Subcellular fragments	*capsular polysaccharides*	Pneumococcus, meningococcus
	surface antigen	Hepatitis B
Toxoids		Tetanus, diphtheria
rDNA	*cloned and expressed*	Hepatitis B
	naked DNA	Experimental

The first attenuated live vaccines was achieved against tuberculosis, by Calmette and Guerin with a bovine strain of *Mycobacterium tuberculosis*, which after 13 years of culture *in vitro* randomly mutated to a much less virulent but protective form now known as BCG (Bacille Calmette-Guerin). However less virulent pathogens are not always available and reversion to wild type could occur, consequently killed vaccine using whole non-living pathogens have been developed. Although not infectious and therefore relatively safe, killed vaccines can be unpredictable.

Subcellular fractions and recombinant proteins, associated with the pathogen's metabolic or developmental activity, were then considered as potential immunogens. The application of recombinant DNA technology to the field of vaccine development has led to remarkable progress over the past decade in the development of subunit

vaccines. Indeed, molecular biology helps not only in the identification of critical protective antigenic determinants but also in the production of high quantities of a vaccine. The first genetically engineered vaccine for human use was against hepatitis B where the surface antigen was cloned, produced in yeast and purified to homogeneity. However, these new antigens are usually of lower immunogenicity, requiring the use of an adjuvant. Traditionally adjuvants have been used to elicit an early, high and long lasting immune response. Adjuvants are now also used to selectively modulate the immune response with regards to major histocompatibility complex and T-helper cell types (Gupta *et al.*, 1996). The only adjuvants used routinely in human vaccines are the aluminum adjuvants, discovered in 1950 (Gupta and Siber, 1995).

1.4.2. General features of a parasite vaccine

As for any other vaccine, parasite vaccines must be safe, with minor side effects, inexpensive and efficient. Knowing that helminths do not replicate in the mammalian host, complete immunity is not required. A simple reduction in worm burden will decrease overall re-infection levels, prevent clinical disease and therefore have major consequences on the morbidity of hookworm infection (Maizels *et al.*, 1989). The vaccine should also induce a long-term protection with a single dose and be stable.

As the aim of a vaccine is to generate immunity that confers protection against a consecutive infection, ideally it should act on different stages of the parasite life cycle. For instance, immunization can be directed against the invasive stage, leading to the destruction of the parasite immediately upon entry into the host, thereby preventing development into the pathogenic form. The development of a vaccine against the lung stage will prevent pathology and further transmission, whilst a vaccine directed against the adult stage, responsible for pathology, will stop transmission. In order to interrupt the parasite life cycle, a commonly proposed strategy is to interfere with the activity of and/or the secretion of enzymes involved in the parasite survival, such as skin penetration and feeding (Pritchard *et al.*, 1991b).

1.4.3. Problems specific to parasite vaccines

One of the first limitations for vaccine development against parasites was the availability of a good animal model to study the host/parasite interaction in detail. Lots of progress has been made in this field and now a large number of parasites are maintained in the laboratory allowing a study of the immune response and the identification of important immunogens.

Although the size, structure and complex life cycle of the parasite provides multiple opportunities for immune intervention, they also make it difficult to identify effective immunogens (Mitchell, 1989). Immune serum has so far been the major tool for identifying candidate vaccine antigens but the complexity of the life cycle makes it generally impossible to select any particular antigens among the vast array of antigens recognised. In addition, many selected candidates present highly conserved proteins with similarities to mammalian or invertebrate antigens (Maizels *et al.*, 1999). Stimulated T lymphocytes are now used *in vitro* to identify specific antigens responsible for the induction of a T cell response. As mentioned previously, molecular biology will help identify and produce novel parasite specific antigens.

In addition, the parasites have developed various strategies to evade the host's immune response. Firstly, parasites which are motile and continuously moult can evade the localized cellular build up of the host' immune response (Kumar and Pritchard, 1992c). The parasite's rapid turn-over also suggests that whilst the surface proteins provide a target for host responses, they mask underlying molecules important in maintaining the structural integrity of the parasite (Pritchard, 1991). The larval sheaths L_2 of *N. americanus* might act as a smoke screen by diverting the host response away from the invading larva (Kumar and Pritchard, 1992c). Furthermore, parasites reduce the antigenic disparity between them and their host by producing their own host-like molecules, inducing tolerance (Wilson and Coulson, 1998). Hookworms can also have a direct regulatory effect on host cytokine production (Pritchard, 1995). Another fascinating parasite strategy to evade the immune system is the release of immunomodulating substances. Indeed, parasite-specific enzymes such as acetylcholine esterase (AChE) and glutathione-S-transferase (GST) are able to protect them against the damaging effects of oxygen radicals released (Brophy *et*

al., 1995). Some parasite proteases also have the ability to degrade host immunoglobulins (McKerrow and Doenhoff, 1988; Pritchard *et al.*, 1991). The development of a vaccine must therefore by-pass the parasite's evasive strategies.

Another serious problem is that much of the symptoms associated with parasitic diseases are immunopathologic. Therefore mis-directed or over-enthusiastic immune responses may thus damage or kill the host. Because immunopathology can be a major aspect of parasitic disease, immunization with crude antigen mixtures may have entirely undesirable consequences. The problem of immunopathology explains why almost all the existing vaccines currently available relate to diseases of domestic animals. By dealing with animals, adverse effects are more acceptable and a greater degree of vaccine-induced pathology may be acceptable (Gutteridge, 1989).

The development of a vaccine against many parasites represents a considerable challenge. However after many years of research, some potential vaccines have now progressed to clinical and field trials. Much progress has been made with the malicious protozoan organism, *Plasmodium falciparum*, causing malaria. Malaria is one of the leading causes of morbidity and mortality in the tropics and numerous scientists are concentrating on this parasite. Malaria kills one person - often a child under five - every 12 seconds, affecting 300-500 million individuals each year (Butler *et al.*, 1997). A vaccine, based on the major surface protein of sporozoites, which helps to infect hepatocytes, has been shown to protect six out of seven volunteers subjected to repeated bites by infected mosquitoes (Butler, 1997). This vaccine along with others represents great hope for parasitic vaccine development. With advance under way in parasite genomics and new vaccine delivery systems offering more rapid assessment and development, there are now excellent opportunities for new anthelminth vaccines.

1.5. CURRENT MODELS FOR HELMINTH VACCINES

1.5.1. Lungworm vaccine in cattle

Dictyocaulus viviparus is a nematode affecting cattle, sheep and other ruminants. The adult worms inhabit the bronchial tubes and cause severe bronchitis and even death. Animals which survive the disease become highly resistant to infection but the responsible antigens have not been identified. The stage involved in the induction of immunity is the fourth stage, preceding the pathogenic stage. In order to artificially induce immunity, avoiding pathology, irradiated larvae were used to immunize cattle and complete protection was obtained. This vaccine was then commercialized and has been successfully used for over 30 year, against the lung-worm *D. viviparus.* This vaccine is a success partly because it satisfies the pragmatic requirements of farming; avoiding animal mortality and reducing failure to thrive. Furthermore, each generation of offspring can be immunised at one time with a fresh batch of vaccine.

Research on irradiated larval vaccines has led to the commercialisation of a vaccine for the lungworm of sheep and goats, *Dictyocaulus filarial*, in 1965 and also for the canine hookworm *Ancyslostoma caninum* in 1973, of which the production ceased in 1975, because of incomplete protection and manufacturing problems. Irradiation has been successful in animal models against a range of parasites, such as *Plasmodium berghei* (Nussenweig *et al.*, 1998), *Leishmania major* (Rivier *et al.*, 1993), *Toxoplasma gondii* (Dubey *et al.*, 1996), *Schistosoma mansoni* (Smythies *et al.*, 1996) and the nematodes *Heligmosomoides polygyrus* (Hagan *et al.*, 1981) and *Strongylus vularis* (Clifton *et al.*, 1997). Despite this success, irradiated vaccines won't be used for a variety of commercial and safety reasons to provide protection against intestinal nematodes in man. However, lessons may be learnt from using irradiated larvae as vaccines such as the development of a protective immune response.

1.5.2. *Schistosoma mansoni* vaccine models

Schistosoma mansoni is a flatworm parasite (table 1.1.) with a complex life cycle involving an intermediate snail host. Human schistosomiasis is estimated to afflict more than 200 million people worldwide. *S. mansoni* cercariae infect their human host by skin penetration and migrate to the lungs, similar to *N. americanus* infection, but then, in contrast to Necator, schistosomes finish their life in the blood vessels of the hepatic portal system where they remain for many years. The principal stimulus for schistosomiasis is caused by the eggs produced by the adult schistosome; these can either be trapped in local tissue sites or break free within the blood vessels. This entrapment leads to an intense delayed type hypersensitivity reaction, which can cause splenomegaly and hepatomegaly.

One of the most successful vaccines developed so far against schistosome infection is an attenuated vaccine, efficient in mice (Dean, 1983; Mountford *et al.*, 1988), guinea pigs (reviewed by Ritcher *et al.*, 1995) and baboons (Yole *et al.*, 1996). C57B6 mice vaccinated once with 500 cercariae irradiated with 20 krad ^{60}Co present up to 80 % protection against *S. mansoni* infection and no pathological symptoms. It has been demonstrated that the dose of irradiation, the number of exposures and the number of attenuated parasites used to vaccinate affect the level of resistance. Indeed, the level of resistance was found to increase when irradiation dose increased from 24 to 50 krad and maximal protection was achieved with as few as 50 to as many as 30 000 cercariae irradiated larvae using percutaneous exposure (Ritcher *et al.*, 1995). In general, additional vaccinations do not boost the level of resistance and the means by which attenuated larvae induce protection is still unclear.

A detailed analysis of attenuated schistosome vaccination showed that the level of resistance displayed by vaccinated mice depends on the presence of $CD4^+$, particularly Th1 subsets, rather than on an antibody response (Pemberton *et al.*, 1991; Coulson, 1997). Indeed higher levels of γ-IFN, a marker of the Th1 subset, were expressed in the vaccinated group compared to the non-vaccinated animals (Pemberton *et al.*, 1991). γ-IFN is thought to promote intercellular adhesion between leukocytes in an effector focus, promoting its ability to block parasite migration in the lungs (Wilson *et al.*, 1996). Furthermore, irradiated cercariae, compared to

normal larvae, have been shown to spend an extended time in the skin, lymph nodes and lungs and fail to mature in the host, avoiding the pathological adult stage (Mangold and Dean, 1984; Ritcher *et al.*, 1995). Constant *et al.,* (1990) showed that the cellular response observed in vaccinated animals coincided with the kinetics of parasite migration, with marked changes in cell number and γ-IFN expression essentially in the draining lymph nodes of the skin and lungs. Therefore, it was proposed that irradiated schistosomes induce the production of γ-IFN by T lymphocytes in the skin draining lymph nodes. These γ-IFN producing T cells are then recruited to the lungs following infection triggering inflammatory foci, that trap the migrating challenge larvae (Mountford *et al.*, 1992; Coulson *et al.*, 1993). Mountford *et al.,* (1995) claim that antigens from the lung stage are an important source of potentially protective molecules, inducing a major Th1 like response.

These studies undertaken in a mouse model would suggest that a vaccine for human use against *S. mansoni* should solicit a Th1 response. However, the immune effector mechanisms responsible for immune protection in the mouse model of schistosome is not in agreement with those found in human studies. Indeed, immunity in human schistosomiasis is associated with high levels of IgE, dependent on Th2 cytokines. The great variety in the life cycle and the prolonged co-evolution of schistosomes with their hosts must therefore be taken into account when designing a vaccine.

Researchers are now therefore working towards the development of a subunit vaccine requiring the isolation and synthesis of protective antigen(s). Consequently, with the help of the helminth genome project, nearly 3600 Schistosoma genes have been identified (Williams and Johnston, 1999). Among them, putative vaccine antigens for *Schistosoma mansoni* have been identified over the past decade, six of which (Sm97, GST, rIrV5, Sm23, TPI and Sm14) were selected by the World Health Organisation (WHO) for a series of independent trials (Bergquist, 1995). Unfortunately, none reached a consistent induction of 40 % protection.

1.5.3. *Haemonchus contortus* vaccine models

Progress in the development of a vaccine against economically important nematodes of livestock is particularly important with *Haemonchus contortus*. *H. contortus* is a parasite of sheep and goats, and lives in the intestine where they feed on blood, damaging the abdominal mucosa. The effects of infection range from mild anaemia to mortality, particularly in younger animals. Repeated infection with irradiated *H. contortus* larvae results in high levels of protection in older sheep, but young lambs, which are most at risk, are not protected (Smith and Angus, 1980). Therefore other vaccines were considered, using natural or hidden antigens. Natural antigens, such as cuticular collagens, larval ES antigens have given little protection (Neilson and Van de Walle, 1987). However hidden antigens, hidden from the afferent immune system and therefore inducing no immune response during infection, induce high level of protection. One of these hidden antigens, H11, is approaching a commercial product (Newton and Munn, 1999). This antigen is an integral membrane protein from the brush border of the parasites intestinal lumen. This protein has an aminopeptidase activity and has shown high level of immunogenicity. Based on the same strategy, a commercial vaccine (TickGARD™) based on the gut membrane structural protein Bm86 was released in Austalia in 1994 against a cattle tick, *Boophilus microplus* (Willadsen, 1997).

1.5.4. Hookworm vaccine models

Despite the limited acquired immune response developed in the host against hookworm, the success of irradiated larval vaccine against *A. caninum* was the proof that vaccination against hookworm could be possible. Beside attenuated vaccines, most of the work on hookworm vaccines has been carried out on the dog hookworm, *A. caninum*, focusing essentially on excretory and/or secretory (ES) products of L_3 larvae. Hookworm ES products have been considered for two reasons. Firstly, circulating antibodies have been shown to form immune precipitates around the oral and excretory openings of the parasite (Hawdon and Hotez, 1996). Secondly, L_3, exposed *in vitro* to canine serum, secretes protein antigens in greater abundance, suggesting therefore that these proteins are important for the maintenance of the parasite (Hawdon and Schad, 1990). Therefore the neutralisation of these proteinase

activities by the immune system could aim at early destruction of the parasite and reduce infection and pathology. Thorson (1956) showed that immmunization against *A. caninum* with oesophageal extracts could partially protect against infection.

Subsequently two *Ancylostoma*-secreted proteins (ASP-1 45 kDa and ASP-2, 24 kDa) were identified and used as candidate vaccines. Recently mice immunized with alum precipitated recombinant ASP-1 exhibited reduced burdens compared to naïve mice after *A. caninum* challenge infection (Sen *et al.*, 2000). In contrast, no protection was obtained with ASP-2. Alum precipitated recombinant ASP-1 vaccine protection was largely antibody mediated (mainly IgG1) and probably dependent on T helper 2 (Th2) type immune responses, as it was associated with anti-Ac-ASP-1 specific IgG1 and mildly elevated IgE (Ghosh and Hotez, 1998).

1.6. IMPORTANT CONSIDERATIONS FOR THE DEVELOPMENT OF A VACCINE AGAINST *N. AMERICANUS*

1.6.1. Animal model

Almost immediately after *N. americanus'* discovery in 1902, the search began for a suitable model host in which the parasite developed normally and in which the host-parasite relationship could be studied. The suitability of a large number of species has been investigated in the quest for a model host for *N. americanus.*

Chronic infection - analogous to those in humans - was achieved by infecting 2-4 day neonate hamsters (Sen and Seth, 1967), rabbits (Bhopale *et al.*, 1977) and adult primates (Miller, 1979). Although primates are phylogenetically more closely related to the definitive host than mice, their size and the cost of housing and maintenance prohibit extensive study. Hamsters subsequently represented the most widely utilized model host (Behnke *et al.*, 1986; Behnke and Pritchard, 1987; Rose and Behnke, 1990). Although this model supports a chronic infection, the necessity for neonatal infection prevented any vaccination study prior to infection. Hamsters were therefore used to maintain the *N. americanus* life cycle, but another animal model was required to study vaccination. Mice were subsequently considered.

N. americanus has the ability to infect adult mice but has an incomplete life cycle, stopping at the L_4 stage (Wells and Behnke, 1988; Timothy and Behnke, 1993; Wilkinson *et al.*, 1990). Additionally, it has been demonstrated that, both primary and secondary *N. americanus* infections elicit an immune response (Wells and Behnke, 1988; Wilkinson *et al.*, 1990) and that functional resistance may operate at each of the three tissue sites, i.e. skin, lungs and intestine (Timothy and Behnke, 1993; Timothy, 1994).

Despite the discontinuous nature of this model, mice remain the only easy laboratory-based system in which the immunological interactions between the host and larval stages of *N. americanus* can be studied. Therefore, BALB/c mice have been used to dissect the protective responses elicited by irradiated larvae as well as for the screening of candidate vaccine preparations.

1.6.2. Irradiated larval vaccine model

The same approach, based on *A. caninum* (Miller, 1971) and schistosome (Dean, 1983) attenuated vaccines, has been used to vaccinate BALB/c mice against *N. americanus* in our laboratory (Brown, PhD thesis, 2000).

Mice were immunized three times at three weekly intervals with 300 gamma-irradiated larvae (40 krad) and then infected with 300 non-irradiated infective larvae (challenge). The level of protection acquired with vaccination was determined with the number of worms recovered in the lungs and intestines of vaccinated and non-vaccinated animals, 3 and 8 days post-infection respectively. The time point for worm recovery was based on the time course obtained following the migration of larvae during a primary infection (Brown, PhD thesis, 2000). Following vaccination, complete protection was obtained in BALB/c mice against subsequent challenge infection, providing the proof that effective vaccination for *N. americanus* may be possible. The same degree of protection was obtained when mice were immunized either percutaneously or subcutaneously. However fewer worms were recovered in the lungs of animals vaccinated subcutaneously, suggesting that the skin, as in the schistosome vaccine, plays an important role in the induction of a protective immune

response. The percutaneous route was therefore chosen to immunize BALB/c mice against *N. americanus*.

A high antibody response, particularly IgG1 antibody, was observed in the vaccinated animals and a preliminary cytokine analyses demonstrated that vaccination induces a Th2 response, characterised by the production of IL5 (Brown, 2000). Il5 and γ-IFN levels were analyzed in the spleen of vaccinated and non-vaccinated animals on days 3 and 8 post-infection only. Cytokine production by activated T-cells in the lymph nodes draining the site of vaccination and their subsequent recruitment in the lungs may be pivotal to the generation of immunity and therefore will be analysed in detail in chapter 4.

1.6.3. Potential Necator immunogens

Although irradiated larvae induce a high level of protection, such vaccines (as discussed previously) cannot be used in man and a more defined vaccine is required. Most of the work has focused on the excreted/secreted products of adult and larval *N. americanus*.

Necator adult and larval stages have been shown to release a substantial amount of soluble antigens which induce a strong humoral immune response and appear to be essential for parasite survival. Indeed, the adult worms secrete a mixture of proteinases which may play a role in feeding and immune evasion (Burleigh, 1992; Brown *et al.*, 1995). They also secrete acetylcholine esterase (AChE) which may prevent the aggregation of inflammatory cells in the intestine (Pritchard *et al.*, 1991; Pritchard and Furmidge, 1995). Glutathione-S-transferase (GST) has been shown to neutralize the damaging effects of oxygen radicals released by accessory cells (Brophy and Barrett, 1990; Brophy *et al.*, 1995). *N. americanus* also produces proteases which can degrade host immunoglobulin IgA, present in large quantity in the intestinal walls during infection (Pritchard *et al.*, 1993). Anticoagulant activities are also present in adult ES products, capable of inhibiting both platelet aggregation and blood coagulation factor Xa and XIIIa (Furmidge *et al.*, 1995; Pritchard and Furmidge 1995). Larval ES products, on the other hand, are less well characterized, although the presence of a number of serine, cysteinyl, metallo and aspartyl

proteinase enzymes have been identified (Salafsky *et al.*, 1990; Kumar and Pritchard, 1992; Kumar and Pritchard, 1992b; Brown *et al.*, 1999). Early attempts to understand the mechanisms underlying the effective immune response show the development of humoral immunity directed against antigens secreted around the opening and excretory pore of the infective larvae. It has also been shown that the four protease classes may play an important role in skin penetration, particularly the aspartyl proteases (Brown *et al.*, 1999).

Another class of antigens has recently been noted as a potential vaccine candidate identified in the L4 and adult stages. Glycoprotein neutrophil inhibitory factor (NIF) which binds to macrophages antigens on the surface of leukocytes could be of interest as well as a hookworm allergen that resembles calreticulin (Moyle *et al.*, 1994; Pritchard *et al.*, 1999; Kasper *et al.*, 1999, Kasper *et al.*, 2001).

1.7. PROJECT AIMS

The overall aim of this project is to work towards the development of a vaccine against *N. americanus.* This is achieved by:

- Analysing in detail the migration behaviour of *N. americanus* through *ex vivo* skin, in an attempt to understand how larvae penetrate the skin.

- Establishing a successful attenuated vaccine against Necator in order to characterize the immunological phenotype following vaccination, focusing on the skin and the lungs of vaccinated animals at different times of infection.

- Using more defined antigens as vaccines and assessing how the adjuvant used can influence the level of protection obtained. In addition, an attempt is made to achieve optimum antigen presentation, using antigen presenting cells.

Chapter 2

Materials and Methods

2.1. ANIMALS

2.1.1. Mice

Pathogen free, 4 week old male BALB/c mice were obtained from Harlan Ltd, Bicester, Oxon. Mice were housed under conventional conditions in the Department of Life Sciences at The Nottingham Trent University and allowed to rest for one week before the beginning of any experiments. Mice were given pelleted food and water *ad libitum*. All animal experiments were carried out in accordance with the Animals (Scientific Procedures) Act, 1986.

2.1.2. Hamsters

Breeding pairs of syngeneic DSN hamsters *(Mesocricetus auratus)* were originally purchased from Shamrock (G.B.) Ltd. and bred at the School of Biological Sciences, University of Nottingham. All hamsters were provided with food and water *ad libitum* and housed in conventional conditions. Hamsters used for the passage of hookworms were isolated before the infection reached patency.

2.2. *NECATOR AMERICANUS* LARVAE

The life cycle of *N. americanus* was maintained at the School of Biological Sciences, University of Nottingham, using the hamster-adapted strain of *N. americanus* obtained, in 1983, from Dr. G. Rajasekariah of Hindustan, CIBA-GEIGY Ltd., Bombay, India. Disposable gloves were worn each time the parasite was handled.

2.2.1. Maintenance of the life cycle

Percutaneous infections with the third stage infective larvae were carried out on 2-4 day old neonate hamsters in accordance with the method described by Behnke, Wells and Brown (1986b). Strips of masking tape (one strip per mouse), measuring about 5 x 50 mm, were cut and 5 x 5 mm gauzes were placed centrally onto the strips. 100 infective live larvae, held in no more than 10 µl of distilled water, were applied using a 20 µl pipette to the gauze. The gauze was then placed on the under-belly of the

neonate hamster, in such a way that the larvae were directly in contact with the skin. The gauze was secured by wrapping the plaster around the thorax. Neonates were immediately returned to the nest after completion of this procedure. Plasters were left in place for 24 hours.

2.2.2. Culture of third stage infective larvae

N. americanus L_3 were obtained by *in vitro* culture of infected hamster faeces using the technique described by Harada and Mori (1955). From 50 days post infection onwards, the infected hamsters were housed in cages with a metal grid base and the faeces collected overnight in trays lined with wet tissue paper.

The faeces were then collected in a beaker and mixed with Fungizone (amphotericin B 250 µl/ml, GIBCO, Grand Island, New York; approximately 3 % (v/v) at an approximate ratio of 1:4 (v/v)) to reduce fungal contamination. Activated charcoal granules (untreated, granular, 16-40 mesh, Sigma) were added to the mixture to form a thick paste. The faecal mixture was then spread with a spatula, in a thin, even layer, in the center of 25 x 7 cm Whatman filter paper strips suspended in a tank containing distilled water. The tank was sealed with silicone (Sigma) and then incubated at 28 °C for 7-10 days, allowing the eggs present in the faeces to hatch into larvae.

After the incubation time, the filter papers were discarded and the third-stage larvae, which had accumulated in the water, were harvested into a 1 l measuring cylinder. Larvae were allowed to settle overnight. The excess water was aspirated and larvae were subsequently washed twice in distilled water to remove any faecal contamination. The number of larvae was determined as described in section 2.2.3. Fresh harvested larvae were used for each infection and the remaining larvae were stored in plastic flasks and kept in the dark for one week at room temperature until required or frozen at - 20 °C.

2.2.3. Larval count

10 µl of the concentrated larvae were spread over a slide and the number of larvae was counted under a Gallenkamp light microscope. The count was repeated 3 times, and an average taken to determine the number of larvae.

2.2.4. Gamma irradiation of L3 infective larvae

Fresh harvested larvae were extensively washed in distilled water then irradiated with 40 krad of Gamma radiation from a sealed ^{137}caesium source for 44 minutes (Nottingham University). The attenuated larvae were used within two hours of irradiation.

2.3. VACCINATION TECHNIQUES

2.3.1. Route of immunisation

2.3.1.1. Percutaneous infection

Percutaneous infections with *N. americanus* larvae were carried out in accordance with the method described by Behnke *et al.*, (1986b).

2.3.1.1.1. Anaesthetic

Mice were anaesthetised, by intraperitoneal injection (25 G needle, 1 ml syringe) (section 2.3.1.2.) of Sagatal (sodium pentabarbitone, M & B Veterinary Products). The drug was administered at 0.1 ml / 10 g body weight after dilution of the stock solution (60 mg/ml), at a ratio of 1:9 (v/v), in sterile phosphate buffered saline (PBS); appendix 2.9.1.

2.3.1.1.2. Infection of mice

While the mice were anaesthetised, the back of their necks were shaved using an electric clipper. Strips of self-binding horse bandage (International Market Supply,

Dane Mill, Broadhurst Lane, Congleton, Cheshire, CW12 1LA), about 10 x 120 mm, were cut and 10 x 10 mm gauzes were placed one third of the way along the length of the bandage. 300 normal or irradiated infective larvae, in an aliquot of no more than 25 µl, were then applied to the gauze which was then secured by wrapping the plaster around the shaved thoracic cavity. The infective dose was always applied to the animal' s back, thus making it difficult for the mice to remove the bandage. Mice were then returned to their cages and left to recover. Most of the mice recovered 45 minutes after the Sagatal injection. After 24 hours, plasters were removed with a blunt scissors and soaked overnight in bleach (Haz-TABS, Guest Medical, appendix 2.9.1.) to kill any remaining larvae.

2.3.1.2. Other routes of immunisation

1 ml syringe and 25 G needle were used for all routes of administration. Mice were handled in accordance with the Animals (Scientific Procedures) Act, 1986 and all the procedures undertaken were described in the personal project licence and project licence.

2.3.1.2.1. Intraperitoneal injection

Mice immunised intraperitoneally were injected with 0.1 ml of antigen in the lower left and right quadrant of the abdomen making sure that the 25 G needle penetrated the peritoneal wall.

2.3.1.2.2. Subcutaneous injection

Mice were injected with 0.1 ml of antigen under the skin of each side of their back.

2.3.1.2.3. Intradermal injection

The backs of mice were shaved and wiped with 70 % (v/v) ethanol. The mice were injected with 0.05 ml of antigen under the superficial layer of the epidermis. This administration was carried out by Dr Selman Ali (Nottingham Trent University).

2.3.2. Immunogen preparation

2.3.2.1. Preparation of irradiated larval excretory/secretory (IES) products

Irradiated third stage larvae (section 2.2.4.) were stimulated by bubbling 100 % carbon dioxide through the larval suspension for 1 hour at room temperature. The following procedures were achieved under sterile conditions in a sterile laminar flow hood. Exsheathed larvae were allowed to settle, the water was aspirated and replaced by 5 ml of phenol-red free RPMI 1640 medium containing 100 IU/ml of penicillin, 100 µg/ml streptomycin, and 1 % (v/v) amphotericin B. The stimulated larvae were transferred into a 25 ml tissue culture flask and cultured for an additional 72 hours in a 5 % (v/v) CO_2 Jouan incubator at 37 °C. Every 24 hours, the larvae were left to settle down in the bottom of the flask, the culture medium containing irradiated larval ES products was then pipetted and replaced with 5 ml of fresh complete RPMI. Larval IES products collected were pooled and dialysed against water. 15 cm dialysis tubing (Sigma) was boiled for one hour and a knot was made at one extremity of the tube, secured by a clip. IES products were then transferred into the tube with a Pasteur pipette. The tube, tied by a second knot, was placed in 2.5 l of distilled water and stirred overnight at 4 °C. The water was changed three times. After dialysis IES products were then precipitated by adding acetone (1:4 (v/v) Sigma) overnight at - 20 °C. IES solution was centrifuged (MSE Centaur 2 centrifuge) for 30 minutes at 12 000 x g and the pellet resuspended in water. The protein concentration of irradiated larval ES products was estimated using the micro Bio-Rad protein assay kit with bovine serum albumin standards (section 2.8.1.2.). IES products were stored at - 20 °C until required. Non-irradiated larval ES was obtained similarly using non-irradiated larvae.

2.3.2.2. Larval homogenate

Third stage infective larvae, suspended in a minimal volume of distilled water, were homogenised (Polytron PT3000) mechanically on ice three times for 2 minutes at 17000 rpm. After the third homogenisation, 5 µl of the homogenate were placed on a microscope slide and observed under a light microscope to ensure that all the sheaths and larvae were broken. The homogenate was then lysed by five freeze (on liquid

nitrogen) and thaw cycles (room temperature). The protein concentration of the homogenate was estimated using the micro Bio-Rad protein assay kit with bovine serum albumin standards (section 2.8.1.2.) and then the homogenate stored in - 20 °C until required.

2.3.2.3. Recombinant necepsin II

Two different *N. americanus* aspartyl proteases (necepsin I and necepsin II) have been cloned by Karen Girdwood, at Cardiff University (PhD student supervised by Dr C. Berry, Cardiff School of Biosciences, data unpublished). The recombinant proteins, expressed in *Escherichia. coli*, were stored in 6 M urea, 0.1 M sodium-phosphate and 0.01 M tris-HCl, pH 4.5. Contrary to necepsin I, expressed only by the L_4 and adult stages, necepsin II is present in L_3, L_4 and adult stages and therefore was used as an antigen. A first necepsin II sample was sent on ice at a concentration of 20 µg/ml and a second, one month later, at a concentration of 8 µg/ml. The recombinant protein was stored at – 4 °C until required.

2.3.3. Adjuvant preparation

2.3.3.1. Freund's adjuvant

The volume of antigen containing the required amount of protein for immunisation was mixed with an equal volume of Freund's complete adjuvant (FCA) or incomplete (FIA) adjuvant (Sigma). An emulsion with the aqueous phase containing the antigen was obtained by thoroughly mixing on a vortex (Fisons Whirlimixer) for 1 or 2 minutes. The emulsion was immediately administered either subcutaneously or intraperitoneally.

2.3.3.2. Alum adjuvant

The volume (v) of antigen containing the required amount of protein for immunisation was mixed with an equal volume of aluminium hydroxide (Superfos Biosector, Denmark). The solution was mixed thoroughly on a vortex (Fisons Whirlimixer) for 1 or 2 minutes and used immediately for immunisation.

2.3.3.3. Dendritic cells

Dentritic cells were cultured as described by Inaba *et al.*, (1992) and supplied by June Lynam (PhD student, supervised by Prof R. Rees, Department of Life Sciences, Nottingham Trent University). Dendritic cells were obtained from BALB/c bone marrow and cultured for 7 days at a concentration of 1 x 10^6 cells per well per ml of medium. Phenotypic changes were monitored by flow cytometric analyses using CD40, CD80 and MHC class II antibodies. Larval homogenate lysate was then added to the DCs at a concentration of 10 µg/ml. After 2 days of incubation, the DCs were washed three times in sterile PBS and resuspended in a total volume of 0.5 ml of sterile PBS. The antigen-loaded dendritic cells were then intradermally administered into BALB/c mice.

2.3.4. Recovery of *N. americanus* from infected mice

2.3.4.1. Worm recovery from the lungs

Mice were killed by excess inhalation of chloroform (Merck Ltd.) and the 5 lung lobes were excised. The number of larvae present in the lungs was determined as described by Wells and Behnke (1988b). The lungs were transferred into 45 mm petri dishes, finely minced with fine scissors and immersed in 5 ml of Hanks Balanced Salt solution (HBSS, appendix 2.9.1.) for 6 hours in an incubator at 37 °C, allowing larvae to migrate from the tissue. Every two hours, the HBSS was collected avoiding any lung tissue and replaced with fresh warm HBSS. Following the addition of fresh media, the lungs were chopped even finer and left incubated further at 37 °C. This procedure was repeated twice and the worms recovered from each lung counted as described in section 2.3.3.3. When all the worms couldn't be counted on the same day, the medium was kept at - 20 °C until required. This last procedure did not affect larval integrity.

2.3.4.2. Worm recovery from intestine

Larvae were recovered from the intestine by a modification of the Baermann method (Wells and Behnke, 1988b). Mice were killed by chloroform (Merck Ltd.) inhalation

and the intestine excised. The intestine was then placed on a nylon gauze and opened longitudinally with scissors. The gauzes were suspended in a 50 ml beaker containing 20 ml of HBSS and incubated for 6 hours in a 37 °C incubator. Every two hours, intestines were gently agitated with a micro spatula. At the end of the incubation time, the gauzes were removed and HBSS containing the parasites was collected. The worms were then counted as described in section 2.3.3.3.

2.3.4.3. Worm count

The medium collected from each lung and intestine was transferred into a 45 mm graduated petri dish. The petri dish was then placed on a dissecting binocular microscope, and moved carefully from right to left; top to bottom. The number of worms was counted using a hand counter.

2.3.4.4. Statistics

Statistical analysis of larval number was carried out using a nonparametric one-way analysis of variance with $P \leq 0.05$ being considered statistically significant.

2.3.5. Serum collection

The thoracic cavity of mice killed by excess inhalation of chloroform was exposed and the aorta sectioned. The blood was immediately pipetted with a Pasteur pipette and transferred before clotting into a 1.5 ml Eppendorf tube. The blood was left to clot for 2 hours at 4 °C and then centrifuged for 15 minutes at 13 000 g. The serum was then collected into a 0.5 ml Eppendorf tube and stored at - 20 °C until required.

2.4. IMMUNOLOGICAL TECHNIQUES

2.4.1. Lymphocyte culture

2.4.1.1. Tissue isolation

BALB/c mice were killed by excess inhalation of chloroform. Their under-bellies were swabbed with 70 % (v/v) ethanol and then opened. An incision was made in the skin without puncturing the peritoneum wall and slit longitudinally. The skin was then loosened from the peritoneum and thoracic wall, without tearing the axillary region in order to prevent any bleeding. The axillary lymph nodes were isolated pulling away the associated fatty tissue. Then the peritoneum was opened and the spleen located below and to the left of the stomach was removed. The intestines were unfolded to expose the sheet-like mesentery and within the mesentery, the mesenteric lymph nodes were isolated. Immediately after isolation, lymph nodes and spleen were transferred into individual universal tubes containing 5 ml of RPMI containing 10 % (v/v) foetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine, and maintained on ice until required. The skin sites of infection (or dorsal skin patches in the case of naïve controls) from 3 mice were also removed, placed into cryovials and immediately submerged into liquid nitrogen.

2.4.1.2. Lymphocyte culture

The following procedures were undertaken under sterile conditions in a sterile laminar flow hood. For each mouse, the lymph nodes and the spleen were transferred into individual 45 mm sterile petri-dishes containing 5 ml of complete RPMI. The tissue was mashed using the rigid end of a 2 ml sterile syringe plunger. The cells were then collected with a Pasteur pipette avoiding any large particles and transferred into a 25 ml sterile universal tube. The tubes were filled with culture medium up to 25 ml and centrifuged for 7 minutes at 1300 g. The supernatant was discarded and the cells were resuspended in 1 ml of complete RPMI for the lymphocytes and 5 ml for the splenocytes.

2.4.1.3. Cell count

The number of viable cells was determined using Trypan blue (Sigma). Trypan blue was used at a dilution of 1:2 with the cell suspension. Cells were loaded onto a haemocytometer (Weber Scientific International) and counted. The number of cells was then adjusted to the number required and plated out.

2.4.1.4. Cell plating and mitogen activation

5 x 10^5 cells were dispensed in each well of a 96-well flat bottom plate (Corning). Concanavalin A (ConA, Sigma, 5 μg/ml final concentration) was added to half of the wells and the final volume of each well adjusted to 200 μl with complete RPMI. The plates were then incubated in a humidified, 37 °C, 5 % (v/v) CO_2 incubator for 3 days. The mitogen was reconstituted in culture medium to make a concentrate stock solution, aliquoted and stored in - 20 °C.

2.4.2. Cytokine Enzyme Linked Immunosorbent Assay (ELISA)

IL4 and γ-IFN cytokine ELISAs were performed using a DuoSET ELISA Development System kit (R&D Systems, Minneapolis). Protocols for each cytokine were similar except for the buffers and diluents used. Details of each solution are given in the appendix. 96-well microplates were coated with 100 μl per well of the diluted capture antibody (appendix 2.9.2.). The plates were sealed with an adhesive strip and stored at room temperature overnight. The capture antibody was removed by inverting the plate and washed by filling each well with wash buffer using a squirt bottle, repeating the process twice. After the last wash, the plates were inverted onto a clean paper towelling in order to remove any remaining wash buffer. 300 μl of blocking buffer (appendix 2.9.2.) were added to each well and incubated at room temperature for 1 hour. The plates were then washed three times as described above. 100 μl of each standard dilution and samples were added per well. Cytokine standards (appendix 2.9.2.) were diluted in reagent diluent in 1.5 ml Eppendorf tubes, using a 2 fold dilution series. The samples tested were lymphocyte and splenocyte supernatant obtained after 3 days of culture, in the presence and absence of ConA (section 2.4.3.). The plates were covered and incubated for 2 hours at room

temperature. The plates were washed three times and then 100 µl of the detection antibody, diluted in reagent diluent were added to each well. The covered plates were incubated for 2 hours at room temperature and washed again three times. 100 µl of the working dilution of Streptavidin-horseradish-peroxidase (Streptavidin-HRP; appendix 2.9.2.) were added to each well, incubated for 20 minutes at room temperature and washed three times with wash buffer. After three washes, 50 µl of stopping solution (appendix 2.9.2.) were added and finally absorbance was measured at 450 nm in a microplate reader.

2.4.3. Dot Blot

2.4.3.1. Dot blotting

Gloves were worn for all procedures involving nitrocellulose and filter paper. A piece of nitrocellulose (Nitropure, 0.22 µm, GRI) and 3MM filter paper (8 x 12 cm) were cut and wetted in tris buffered saline (TBS; appendix 2.9.2.) in a glass staining tray. The piece of wetted 3MM filter paper was placed on to the membrane support (Dot blotter, Schleicher and Schuell), and the nitrocellulose carefully layed on the top of the filter paper avoiding any air bubbles. The sample template was placed on top of the membrane and the Dot blotter closed using a diagonal crossing pattern. Unused sample wells were covered with tape to ensure a complete vacuum. 250 µl of antigen or TBS (blank) were applied onto the appropriate wells, ensuring that no air bubbles were present. The vacuum was then turned on to dry the wells. 250 µl of TBS was added to each well while the vacuum was off, and then the vacuum was turned on to dry the wells.

2.4.3.2. Immunoprobing

The nitrocellulose was removed from the apparatus and blocked in 20 ml of 3 % (w/v) TBS/marvel for 1 hour at room temperature. After 1 hour incubation, primary antibodies diluted in 3 % (w/v) TBS/marvel at the appropriate dilution were added and incubated overnight at 4 °C while shaking. The nitrocellulose was then washed 3 times 20 minutes each in 0.1 % (v/v) TBS/tween 20. The nitrocellulose was then incubated with the secondary antibody, alkaline phosphatase goat anti mouse Ig

(Dako), used at a dilution 1/1000 in 3 % (w/v) TBS/Marvel, for 2 hours at room temperature. The membrane was washed for 1 hour with TBS/tween (3 x 20 min) and was rinsed in distilled water for 5 minutes. After equilibration for 5 minutes in substrate buffer (appendix 2.9.2.), the nitrocellulose was developed in substrate solution with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) (appendix 2.9.2.). The development was stopped by extensive washing in distilled water. The nitrocellulose was dried between clean pieces of filter paper.

2.5. *EX VIVO* TECHNIQUES TO STUDY SKIN PENETRATION BY L3 LARVAE

2.5.1. Preparation of skin samples

2.5.1.1. Skin samples

2.5.1.1.1. Animal skin

Skin tissue was taken from non-infected adult DSN hamsters and/or BALB/c mice. The animals were killed by excess inhalation of CO_2. Immediately after death, the skin was carefully removed with fine scissors excluding the adipose tissue.

2.5.1.1.2. Human skin

White human skin was obtained from breast surgery. The tissue was delivered fresh, without medium in a sealed plastic bag, on ice. The tissue was not tested for HIV, Hepatitis B or syphilis but no suspect tissue was supplied. The skin was treated as a pathological specimen within Health and Safety Guidelines. Therefore all the manipulations were done in containment level II laboratory and in a class I hood. The work was done with the help of Dr Ellen Billett, Nottingham Trent University.

2.5.1.1.3. Dermal equivalent skin (DEs)

The following procedures were conducted under sterile condition in a laminar flow hood.

2.5.1.1.3.1. Culture of human fibroblasts

Human fibroblast cultures were established from normal forearm skin of healthy volunteers by standard techniques, and donated by Dr M. Billett, Department of Biochemistry, Queens Medical Centre (Q.M.C), Nottingham. Fibroblasts were cultured in 75 ml flasks in Dulbecco's Minimal Essential Medium (DMEM) completed with 10 % (v/v) foetal bovine serum (FBS), 1 % (v/v) of L-glutamine and 1 % (v/v) of penicillin and streptomycin and maintained at 37 °C in an atmosphere of 5 % (v/v) CO_2 : 95 % (v/v) air. When the fibroblasts were confluent (approximately after 5 days of culture), the medium was removed and the cells were rinsed with 5 ml sterile PBS (appendix 2.9.1.) to remove excess calcium. 2 ml of 0.1 % (v/v) trypsin/EDTA were added to the cells and incubated for 2-3 minutes at 37 °C in 5 % (v/v) CO_2 incubator. When all the fibroblasts were detached from the bottom of the flask, the cells were transferred into a sterile tube containing 20 ml of complete DMEM and centrifuged for 5 minutes at 1300 g. The supernatant was discarded and the pellet was resuspended in 5 ml of complete DMEM. The fibroblasts were counted as described in section 2.3.1.3. The remaining fibroblasts, not used for the DE formation, were transferred into a flask and maintained in culture.

2.5.1.1.3.2. Collagen extraction

The collagen extraction procedure was based on the method described by Rowling *et al.,* (1990). Rat tail tendons, donated by the Department of Life Sciences of Nottingham Trent University, were used as a relatively pure source of type I collagen. All manipulations were carried out under sterile conditions in a class II microbiological cabinet. Using a sterile scalpel, the outer skin of 7 rat tails was scored down and removed. Tendons containing 98 % of type I collagen were clearly visible and were removed by gripping one end with sterile forceps and pulling the tendons until they came away from the tail. Tendons were minced finely with

scissors in a sterile petri dish, then placed in 50 ml of 17.4 mM sterile acetic acid and stirred for 48 hours at 4 °C. The resulting mixture was centrifuged at 30 000 g for 90 minutes at 4 °C. The supernatant was neutralised to pH 7 with 0.1 M NaOH, stirred at 4 °C for 90 minutes to allow the collagen to precipitate. Precipitated collagen was then removed by centrifugation at 10 000 g at 4 °C for 20 minutes, resuspended in 50 ml of acetic acid and stirred at 4 °C for 48 hours and stored at 4 °C. The protein content of the purified collagen was estimated using the Lowry (Lowry *et al.*, 1951) protein assay using commercial type I collagen (Sigma) as the protein standard (section 2.9.1.1.).

2.5.1.1.3.3. Components of Dermal Equivalent skin

2.5×10^5 fibroblasts, 3 ml of collagen (adjusted to 1.7 mg/ml in DMEM) and 1 ml of 0.1 M NaOH (Sigma) were mixed in a sterile universal tube and then adjusted to a final volume of 6 ml with complete DMEM. The mixture was kept on ice until required (maximum of 15 minutes).

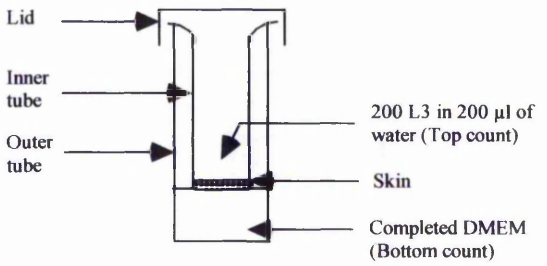
2.5.1.2. Skin assemblies

2.5.1.2.1. Animal and human skin

The apparatus (figure 2.1.a.) used to mount the skin was previously described by Kumar and Pritchard in 1992 (reference 1992c). Excised skin was cut into small square pieces (2 x 2 cm) and mounted on the open ends of a 7 cm long by 1.4 cm diameter plastic tube such that the outer skin surface faced the inside of the tube. The skin was secured tightly to the inner tube by an elastic band. The tube was then placed in a 20 ml tube (9 cm long by 2.5 cm wide), containing 5 ml of complete DMEM. Care was taken to ensure that the inner skin surface was fully immersed and that no upward leakage occurred.

In order to have a double layer system, the previous apparatus was fitted onto a similar open ended tubes of higher diameter. The excised skin was mounted on each tube as described previously and only the bottom end of the tube was submerged in complete DMEM.

a. *Ex vivo* skin



b. DE skin

Figure 2.1: Basic apparatus used to study the penetration of third stage infective larvae through a/ *ex vivo* skin, b/ DE skin.

2.5.1.2.2. DE skin

The Dermal Equivalent skin was established in a glass sinter described in figure 2.1.b. The apparatus consisted of an inner tube with small holes allowing the larvae to get through the DE skin and to fall into a bigger tube containing DMEM. Prior to use, the glass sinter and 1 cm^2 square pieces of parafilm were immersed in 70 % (v/v) ethanol, and then dried under the hood air flow. The tubes were assembled and a piece of parafilm was applied to the bottom of the inner tube. The above procedures were carried out under sterile conditions. 300 µl of the homogenous cold DE mixture were transferred into each tube, mixing immediately to ensure that the base of the tube was completely covered. The collagen lattices were left to polymerise for 1 hour at 37 °C in the incubator. 300 µl of complete DMEM were then added on the top of the DE mixture and kept at 37 °C with 5 % (v/v) CO_2 for 5 days. The medium was changed twice a week by adding 300 µl of medium to each tube and then removing 300 µl. The DEs were used after 5 days of culture.

2.5.2. Skin penetration by infective larvae

2.5.2.1. Time course of skin penetration

Two hundred fresh normal or irradiated larvae were placed over the outer surface of the skin (natural or artificial) in a 200 µl final volume of distilled water. The universal tubes were then capped and placed in a water bath at 37 °C. After different incubation times (3, 24, 48 hours), the inner tubes were carefully taken out of the universal tube and their contents transferred into a plastic tube by Pasteur pipetting with repeated rigorous washing of the outer surface of the skin. The inner tube contained the larvae that had failed to penetrate the skin (top counts). The contents of the larger tube which contained larvae that had fully traversed the skin (bottom counts) were left until they were counted. The larvae present in each tube were carefully counted as described in section 2.3.3.3.

2.5.2.2. Effect of proteases

The differential involvement of proteinase classes during skin penetration was investigated using class specific inhibitors. The different inhibitors were purchased from Sigma. The proteinase inhibitors were used at final concentrations of 50 µM 4-(amidinophenyl) methane sulfonyl fluoride (APMSF), 1 µM pepstatin A (Pep A), 1 µM E64 (L-trans-epoxysuccinyl-leucycla--mide-[4-guanidine]-butane), 1 mM 1,10-phenanthroline (Phe) and 10 mM EDTA (ethylenediamine tetracetic acid). The APMSF was always freshly prepared due to its short half-life (6 min). A water and ethanol control (0.1 % (v/v) final concentration) were also included. The inhibitors were applied onto the upper surface on the skin and then two hundred untreated or attenuated larvae were applied to the skin surface. The tubes were incubated at 37 °C for 24 hours, after which the number of larvae were determined (section 2.3.3.3.).

2.5.2.3. Effect of lipids

Human skin lipids were donated by Dr J. Hales, Department of Dermatology, University of Leicester. Lipids were collected from skin scrapings and extracted with chloroform and methanol (2:1 (v/v)). The surface of the DEs was coated for one hour

with 0, 0.5, 1, 2 µl of the lipid mixture. 200 larvae, in a final volume of 200 µl of distilled water, were then placed onto the centre of the skin and incubated for 24 hours at 37 °C. At the end of the incubation, the surface of the DE was washed gently several times with distilled water to collect larvae that had not penetrated. The number of larvae which had completely penetrated the DEs and those which remain on the surface was determined as described in section 2.3.3.3.

2.6. HISTOLOGICAL TECHNIQUES

2.6.1. Skin Sections

Following infection, mice were killed by excess inhalation of chloroform. The skin exposure site was removed, placed on 1 cm square filter paper, to prevent tissue distortion and then fixed in 10 % (v/v) neutral buffered formaldehyde (NBF). Tissues were processed using the Shandon Hypercentre and then embedded in paraffin wax using conventional techniques. Transverse skin sections of 10 µm thickness were then produced using a rotary Leitz microtome.

2.6.2. Staining

Staining was performed using conventional techniques described by Freeman W. H., (1979).

2.6.2.1. Hydration

Before staining, the slides were warmed to not more than 50 °C for 10-15 minutes. The slides were then placed on a staining rack, and washed for 5 minutes in two separate xylene baths, then twice for 3 minutes in absolute ethanol. The rack was then transferred to 90 % (v/v) ethanol for 2 minutes and 80 % (v/v) ethanol for 3 minutes before a final rinse in distilled water for 2 minutes.

2.6.2.2. Staining steps

2.6.2.2.1. Gills Haematoxylin and Eosin

After hydration, the slides were immersed in Harris' Haematoxylin (Sigma) solution for 2 minutes. The slides were then gently rinsed in running tap water for 2 minutes, dipped in 1 % (w/v) Borate (Sigma) for 45 minutes to remove excess of stain and rinsed again gently in running tap water for 2 minutes. The rack was then transferred to 1 % (w/v) eosin for 3 minutes and rinsed quickly in tap water.

2.6.2.2.2. Thionine staining

The rehydrated slides were immersed in a 0.6 % (v/v) aqueous thionin solution for 30 minutes, rinsed in tap water and dipped (until the nuclei and mast cell granules are selectively stained) for 45 seconds in 0.2 % (v/v) acetic acid before a final rinsed in water.

2.6.2.3. Dehydration

After staining, the slides were immediately immersed for 1 minute in each of a graded series of ethanol baths (80, 90, 100, 100 % (v/v)) and in two different xylene baths. The rack was left in the last xylene bath until the mounting step.

2.6.2.4. Mounting

One by one, the slides were removed from the last xylene bath, the back and the side of the slide wiped by a tissue to remove excess xylene. Then a XAM (Merck Ltd) drop was added onto the section, covered by a coverslip and left to set. The sections were then observed under a light microscope (Laborlux 12 Leitz) and photographs were taken if necessary.

2.7. MOLECULAR BIOLOGY TECHNIQUES

Prior to each molecular biological procedure, the bench was cleaned thoroughly with ethanol. Gloves and steriles tubes were used to avoid any RNase contamination.

2.7.1. RNA extraction

The following steps were carried out in order.

2.7.1.1. Extraction

RNA STAT-60™ (Biogenesis) solution was used to extract RNA. It is a single-step method which includes phenol and guanidinium thiocyanate in a monophase solution. The following steps were undertaken on ice. Skin samples (section 2.4.1.1.) were removed from liquid nitrogen and placed on ice. Tissues were quickly weighed and then homogenised in 1 ml of RNA STAT-60™ per 100 mg of tissue in a glass-Teflon homogeniser. The homogenate was then stored at 4 °C for 5 minutes to permit the complete dissociation of nucleoprotein complexes. 200 µl of chloroform (ACS grade, Merck Ltd) per ml of the RNA STAT-60™ were then added to each tube and the mixture was shaken vigorously for 15 seconds and left on ice for 2-3 minutes. The homogenate was then centrifuged at 12 000 g for 15 minutes at 4 °C. Following centrifugation, the homogenate separated into two phases: a lower red phenol/chloroform phase containing DNA and proteins and a colourless upper aqueous phase containing the RNA.

2.7.1.2. Precipitation

The aqueous phase was transferred to a sterile 1.5 ml Eppendorf tube and mixed with 0.5 ml of isopropanol (ACS grade, Sigma) per 1 ml of the RNA STAT-60™ used for homogenisation. The samples were stored on ice for 10-15 minutes and then centrifuged at 12 000 g for 10 minutes at 4 °C.

2.7.1.3. Solubilisation and estimation of RNA

The supernatant was removed and the RNA pellet was washed once with 75 % (v/v) ethanol by vortexing and subsequent centrifugation at 7 500 g for 5 minutes at 4 °C. 1 ml of 75 % (v/v) ethanol was added per 1 ml of the RNA STAT-60™ used for the initial homogenisation. At the end of the procedure the RNA pellet was briefly air-dried before resuspension in 50 µl of diethylpyrocarbonate (DEPC) treated water and the RNA concentration determined spectrophotometrically by measuring the absorbance at 260 and 280 nm and using the following formula:

Absorbance 260 nm x 40 x d = concentration in µg/ml

where d is the dilution factor of the bulk preparation to give the test solution, and 40 is the concentration of RNA in µg/ml that gives an OD of 1 at 260 nm. The relative purity of the RNA was estimated by calculating the OD 260/OD 280 ratio. A ratio > 1.8 was desirable. The rest of the RNA is aliquoted out and stored in 100 % (v/v) ethanol and frozen at –70 °C until required.

2.7.2. Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) preparation

2.7.2.1. Sample preparation

The Promega Access RT-PCR System (Promega, 2800 Woods Hollow Road, Madison, WI53711-5399 USA) was used. This system used the Avian Myeloblastosis Virus (AMV) reverse transcriptase for the first strand DNA synthesis and the thermostable *Thermus flavus* (Tfl) DNA polymerase for the second strand cDNA synthesis and DNA amplification. Once the concentration of RNA was known, 0.01 µg RNA was used to synthesise cDNA. Working with different primers (IL4, γ-IFN and ß-actin primers), a Master-Mix was prepared in a sterile 0.5 ml reaction tube on ice, combining appropriate multiples of each of the components indicated below:

10 µl AMV/Tfl 5 x Reaction Buffer
1 µl dNTP (10 mM each dATP, dCTP, dGTP and dTTP)
25 mM $MgSO_4$

The appropriate amount of nuclease-free water was added into each tube to have a final volume of 50 µl and the specific primers were added. The components were mixed by pipetting. Then 1 µl of AMV Reverse Transcriptase (5 u/µl) and 1 µg of Tfl DNA polymerase (5 u/µl) were added to each tube and gently vortexed for a few seconds. The reaction was initiated by the addition of the RNA sample. For each RT-PCR experiment, a positive and a negative control (1 µM) were run with the samples, the negative control being all the components except the template. ß-actin mRNA (Promega) was used as a positive control. Once the reagents were distributed, 50 µl of nuclease-free mineral oil were added to each tube to prevent any condensation or evaporation.

2.7.2.2. RT-PCR cycles

The tubes were placed in the Perkin Elmer Cetus - DNA thermal cycler. The program used for the RT-PCR was the following:

First strand cDNA synthesis

1 cycle	48 °C for 45 minutes	Reverse transcription
1 cycle	94 °C for 2 minutes	AMV RT inactivation RNA/cDNA/primer denaturation

Second Strand cDNA Synthesis and PCR Amplification

40 cycles	94 °C for 30seconds 60 °C for 1 minute 68 °C for 2 minutes	denaturation annealing extension
1 cycle	68 °C for 7 minutes	final extension
1 cycle	4 °C	soak

The reaction products were stored at - 20 °C until analysed.

2.7.2.3. Agarose gel electrophoresis

2 % (w/v) agarose gels (Sigma) were used to visualise the RT-PCR end products. The two extremities of the electrophoretic chamber were sealed with tape and the comb placed in the correct orientation across the end of the chamber. The agarose was melted in tris-acetate with EDTA (TAE, appendix 2.9.4.) using a microwave oven. The agarose was allowed to cool down to hand hot temperature before addition of ethidium bromide to a final concentration of 0.5 µg/ml. The agarose was then poured into the electrophoretic chamber and allowed to set. The comb and tape were then carefully removed and the gel holder was placed in the electrophoretic apparatus (Bio-Rad). TAE buffer was poured into the apparatus until the gel was covered. The samples were prepared as followed: 10 µl of PCR products were mixed with 2 µl of loading buffer (x10, Promega.), 2 µl of ethidium bromide (0.5 µg/ml, Promega), and 6 µl of DEPC water. 2 µl of DNA marker (pGEM DNA markers, Promega) were added to 1 µl of ethidium bromide, 1 µl of loading buffer and 6 µl of DEPC water. The samples and standard were then loaded into the wells and the apparatus was connected to a power supply (Bio-Rad, EPS 500/400). The gel was run at 100 volts until the dye had migrated approximately three quarters of the length of the gel. The gel was visualised on a trans-illuminator and photographed. Eyes and skin were protected when viewing the gel.

2.8. BIOCHEMICAL TECHNIQUES

2.8.1. Protein estimation

2.8.1.1. Lowry assay

The protein concentration of antigen preparations was determined by the method detailed by Lowry *et al.*, (1951). Protein standards were prepared from a 1 mg/ml solution of BSA (Sigma) in distilled water, to give a range of 0-250 µg of protein in final volumes of 250 µl. 10 µl of test sample were made up to 250 µl with distilled water and 1.25 ml of working Lowry solution (appendix 2.9.5.) added to each of the standards and test samples. After 10 minutes incubation at 37 °C, 125 µl of Folin and Ciocalteaus' phenol reagent (diluted 1:1 (v/v) in distilled water, Sigma) were added

and the samples incubated for a further 15 minutes at 37 °C. 200 µl of each of the standards and test samples were then transferred, in triplicate, to a microtitre plate and the absorbance read at 620 nm. The protein concentrations of test samples could then be calculated from a calibration curve of absorbance versus standard (known) protein concentrations.

2.8.1.2. Biorad Assay

The protein concentration of antigen preparations was determined by the Bio-Rad method. Protein standards were prepared from a 1 mg/ml solution of BSA in phenol-free RPMI (Sigma), to give a range of 0-25 µg of protein in final volumes of 800 µl. Different volumes of the test sample were made up to 800 µl with RPMI. 0.2 ml of Dye Reagent Concentrate (Bio-Rad) was added to each of the standards and test samples, vortexed and incubated at room temperature for at least 5 minutes but no longer than one hour. In the hour following the addition of the dye, 200 µl of each standard and test sample were transferred to a 96 well-microtitre plate and read in a spectrophotometer at 595 nm. The protein concentrations of test samples could then be calculated from the standard curve obtained.

2.8.2. Sodium Dodecyl Sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE procedures were carried out in accordance with the method described by Laemmli (1970).

2.8.2.1. Sample preparation

The samples were diluted 1:1 (v/v) with sample buffer in 1.5 ml Eppendorf tubes to obtain a final protein concentration of 1-2 mg/ml. The samples and standards were boiled for 10 minutes ensuring that all the tops of Eppendorf were pierced with a needle. The samples and standards were centrifuged for 10 seconds to ensure that all condensation was incorporated into the samples. Cooled samples and standards were then loaded onto the gel, loading 20-100 µg of protein per lane.

2.8.2.2. SDS-PAGE

The glass plates (Bio-Rad Mini Protean II Electrophoresis kit) were carefully cleaned with ice cold acetone and assembled according to the manufacturers instructions. 12 % (v/v) acrylamide resolving gel (appendix 2.9.5.) was poured into the gel mould to a height of 1.5 comb depths from the top edge of the plate sandwich. The gel was carefully overlayed with water saturated butanol to exclude air and ensure a flat surface and left to polymerise ar room temperature for approximately 45 minutes. Once set, the butanol was removed and the surface of the gel washed extensively with electrode buffer (appendix 2.9.5.) and then dried with filter paper. The stacking gel mix (appendix 2.9.5.) was poured on to the top of the gel and the comb placed slowly into the stacking mixture avoiding any air bubbles. The stacking gel was left to polymerise for 30 minutes at room temperature. The comb was then removed and wells were washed with electrode buffer. The plates were placed into the electrophoretic tank that was filled to the top with electrode buffer. The prepared samples and standards (section 2.8.2.1.) were applied in the appropriate well using a microlitre pipette. The gel, connected to the power supply EPS 500/400, was run at a constant current of 20 mA/gel, until the tracking dye was at the bottom of the gel. To visualise the band, the gel was immersed in Coomassie Blue stain (appendix 2.9.5.) for 1-2 hours and then destained until the gel background was clear or blotted onto nitrocellulose (section 2.8.3.).

2.8.3. Western Blotting

Western blotting procedures were carried out in accordance with the method described by Towbin *et al.*, (1979). After electrophoresis, the unstained gel was washed in 100 ml of electroblotting buffer (appendix 2.9.5.) to remove SDS. Meanwhile 18 pieces of 8 x 12 cm 3MM filter paper and nitrocellulose membrane were cut and soaked in electroblotting buffer. Nine pieces of filter papers were placed on the top of each other onto the wetted anode of a semi dry blotting apparatus, removing all air bubbles by rolling a clean glass rod over. The nitrocellulose membrane was then applied on top of the filter papers excluding any air bubbles and the gel carefully placed on the top. The gel was first covered with the remaining 9 filter papers, again excluding air bubbles and then with the wetted cathode. The gel

was blotted for 1 hour at 45 mA/gel at room temperature and then immunoprobed as described in section 2.4.5.2.

2.8.4. Proteinase assays using FITC-labelled casein

2.8.4.1. Preparation of FITC-labelled casein

Fluorescein isothiocyanate (FITC) labeled casein was labeled as described by Beynon and 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 FITC was added and the solution stirred for 1 hr at room temperature. The FITC labeled casein was then extensively dialysed against distilled water over a period of 48 hours at 4 °C. Finally, the protein content was adjusted to 5 mg/ml and the labeled casein aliquoted and stored at - 20 ° until required.

2.8.4.2. Proteinase assays using FITC-labelled casein

Proteinase activity in larval ES products was detected by monitoring the release of FITC-labeled casein amino acids from FITC labeled casein. Typically, 10 µg of ES products were mixed with 10 µl of FITC-casein (final concentration 250 µg/ml) and 140 µl of 0.1 M phosphate buffer, pH 6-8 in a microfuge tube. Reactions were incubated at 37 °C for 3 hours after which 120 µl of 5 trichloroacetic acid was added to stop the reaction. The tubes were allowed to stand at room temperature for 1 hour 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 µl Tris-HCl pH 8.5 and the fluorescence measured (excitation 490 nm, emission detection 525 nm). Proteinase activity was characterized using a number of proteinase inhibitors as described in section 2.5.2.2.

2.9. APPENDIX

2.9.1. Solutions required for vaccination procedures

Bleach

Haz-TABS, Chlorine release tablets, Guest Medical
Dissolve 2 Haz-TABS in 1l of water giving 5 000 ppm.

Phosphate Buffered Saline, PBS, pH 7.4

NaCl	8.0 g
KCl	0.2 g
KH_2PO_4	0.2 g
$Na_2HPO_4.12H2O$	1.15 g

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

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.

Hanks' Balanced Salt Solution (modified)

HBSS was modified by excluding glucose and $NaHCO_3$ and increasing the remaining salts to an osmotic pressure of 300 mosmole.

Solution 1:

NaCl	168.0g
KCl	8.0g
KH_2PO_4	4.0g
Na_2HPO_4	4.0g
0.2% (w/v) phenol red	200 ml

Made up to 2 litres with deionized water.

Solution 2:

$CaCl_2.2H_2O$	3.92g
$Mg\ Cl_2.6H_2O$	2.0g

Made up to 2 litres with deionized water.

110 ml of solution 1 were added to 110 ml of solution 2 and 780 ml of distilled water. The pH was adjusted to 7.2 with 1 M NaOH.

2.9.2. Immunological solutions

•Mouse γ-IFN ELISA (R&D Systems)

Capture antibody: 1080 μg/ml of rat anti-mouse γ-IFN was reconstituted with 1.0 ml of PBS. After reconstitution, the capture antibody was aliquoted and stored at - 70 °C until required. The antibody was diluted to a working concentration of 4 μg/ml in PBS.

Detection antibody: 72 μg/ml of biotinylated goat anti-mouse γ-IFN was reconstituted with 1.0 ml of reagent diluent. The antibody was then aliquoted and stored at - 70 °C until required. The antibody was diluted to a working concentration of 400 ng/ml in reagent diluent.

Standard: 100 ng/ml of recombinant mouse γ-IFN was reconstituted with 0.5 ml of reagent diluent. The standard was then aliquoted and stored at - 70 °C until required. Dilution of the standard in reagent diluent started at a concentration of 2000 pg/ml.

Streptavidin-HRP (1.0 ml/vial): streptavidin conjugated to horseradish-peroxidase was diluted to the working concentration specified on vial label using reagent diluent.

Wash buffer PBS containing 0.05 % (v/v) Tween 20, pH 7.4

Block Buffer: 0.1 % (w/v) BSA, 0.05 % (w/v) sucrose in PBS with 0.05 % (w/v) NaN_3

Reagent Diluent: 0.1 % (w/v) BSA, 0.05 % (v/v) Tween 20 in TBS

Substrate Solution: 1:1 mixture of Color Reagent A (H_20_2) and Color Reagent B (Tetramethylbenzidine) (R&D Systems) pH 7.3, 0.2 µm filtered

Stop Solution: 2 M H_2SO_4

• **Mouse IL4 ELISA (R&D Systems)**

Capture antibody: 720 µg/ml of rat anti-mouse IL4 was reconstituted with 1.0 ml of PBS. After reconstitution, the capture antibody was aliquoted and stored at - 70 °C until required. The antibody was diluted to a working concentration of 4 µg/ml in PBS.

Detection antibody: 36 µg/ml of biotinylated goat anti-mouse IL4 was reconstituted with 1.0 ml of reagent diluent. The antibody was then aliquoted and stored at -70°C until required. The antibody was diluted to a working concentration of 200 ng/ml in reagent diluent.

Standard: 66 ng/ml of recombinant mouse IL4 was reconstituted with 0.5 ml of reagent diluent. The standard was then aliquoted and stored at – 70 °C until required. Dilution of the standard in reagent diluent started at a concentration of 1000 pg/ml.

Wash buffer: 0.05 % (v/v) PBS Tween pH 7.4

Block Buffer: 1 % (w/v) BSA, 5 % (w/v) sucrose in PBS with 0.05 % (w/v) NaN_3

Reagent Diluent: 1 % (w/v) BSA in PBS, 0.2 µm filtered

• **Immunoprobing solutions**

Alkaline phosphatase substrate buffer: 0.75 M Tris dissolved in 330 ml distilled water, pH adjusted to 9.5 with concentrated HCl, made up to a final volume of 500 ml

BCIP: 5-Brom-4-Chloro-3-Indolyl Phosphate, 50 mg/ml in dimethyl formamide (DMF)

NBT: Nitroblue tetrazolium 75 mg/ml in 70 % (v/v) DMF

Substrate buffer: 20 ml of alkaline phosphatase substrate buffer containing 33 µl BCIP and 44 µl NBT

2.9.3. Staining solutions

Neutral Buffered Formalin (10 % v/v)

Formaldehyde (40 % (v/v) solution, Sigma)	100 ml
$NaH_2PO_4.H_2O$	4.0 g
Na_2HPO_4	6.5 g

Made up to 1 litre with distilled water.

Acid Alcohol

Concentrated HCl	1 ml
Absolute ethanol	63.9 ml
Distilled water	29.7 ml

2.9.4. Biomolecular solutions

Tris-acetate with EDTA (TAE)

Tris (Sigma)	40 mM
Acetic acid (Sigma)	40 mM
EDTA (Sigma)	1 mM

pH was adjusted to 8.0

2.9.5. Biochemical solutions

'Lowry' Technique

Solution 1

Na_2CO_3	5.0 g
NaOH	1.0 g
Distilled water	250 ml

Solution 2

$CuS0_4.5H_2O$	1.0 g
Distilled water	100 ml

Solution 3

NaK Tartrate	2.0 g
Distilled water	100 ml

1 ml of each of solutions 2 and 3 added to 50 ml of solution 1 to make working reagent.

Electrode buffer

Tris base (Sigma)	25.6 mM
Glycine (Sigma)	192 mM
SDS	1%

12 % Resolving gel for one gel

30 % (v/v) acrylamide stock	4.0 ml
1.5 M tris buffer, pH 8.8	2.5 ml
10 % (w/v) SDS (SDS, Sigma)	100 µl
Distilled water	3.4 ml

To polymerise 10 ml, add 5 µl of TEMED (N,N,N',N'-Tetramethylethylenediamine, Sigma) and 50 µl of 10 % (w/v) APS (Ammonium persulphate, Sigma)

Stacking gel (4 %) to make 10 ml

30 % (v/v) acrylamide stock	1.33 ml
1.5 M tris buffer, pH 8.8	2.5 ml
10 % (w/v) SDS (SDS, Sigma)	100 µl
Distilled water	6.07 ml

To polymerise 10 ml, add 20 µl of TEMED and 50 µl of 10 % APS

Reducing sample buffer

10 % (w/v) sodium dodecyl sulphate (SDS, Sigma)	40 ml
Glycerol	20 ml
0.5 M tris-HCl, pH 6.8	25 ml
Bromophenol blue	0.02 g
Dithiothreitol (Final concentration, 100 mM)	1.54 g

Made up to 100 ml with distilled water

Chapter 3

Characterisation of *N. americanus* infection process

3.1. INTRODUCTION

N. americanus infects its host by skin penetration. This first contact between the host and the parasite in the skin may be crucial for the outcome of the infection and therefore essential for the development of a vaccine against *N. americanus*.

The skin is the largest organ of the body (surface area of 2 m^2) varying in thickness from about 0.5 to 4 mm in adults (Edelson and Fink, 1985). The skin is composed of two main layers: the epidermis and the dermis, each layer acting as a physical and immunological barrier to the parasite (figure 3.1.).

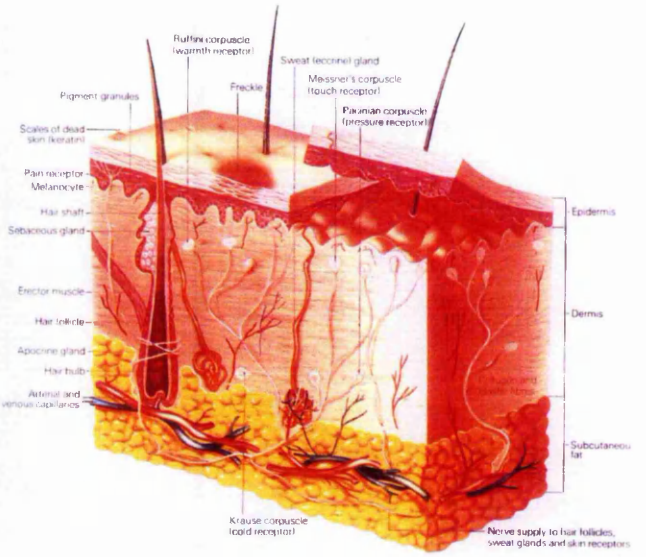


Figure 3.1. **Structure of the skin.** Adapted from Seeley *et al.*, (1999).

The epidermis has an overall thickness of 75 to 150 μm, increasing to 400-600 μm on the palm of the hands and feet, where most of the larvae reach their host. The epidermis is essentially non-vascular and made of four distinct cell types; keratinocytes, melanocytes, Langerhans' cells and merkel cells. The keratinocytes are the principal cell type of the epidermis, involved in its physical and

immunological property. They differentiate continuously, originating from an inner germinative layer and die at the surface of the skin, to form an impermeable barrier; the stratum corneum layer. The dead superficial keratinized cells are constantly lost by friction and replaced by newly differentiated keratinocytes. It has been estimated that the epidermis completely replaces itself every two months (Odland, 1991). In addition to generating a protective layer, the keratinocytes produce cytokines such as interleukin 1 (IL1) affecting T lymphocyte differentiation and circulation in the skin (Choi and Sauder, 1986). The Langerhans' cells also play an active role in modulating and mediating immune responses in the skin (Bos and Kapsenberg, 1986). Indeed, the Langerhans' cells (LCs) belong to a family of potent antigen presenting cells (APCs), and are widely distributed in the epidermis (Edelson and Fink, 1985). LCs have the ability to capture and process an antigen present in the skin (e.g. parasitic antigens) resulting in the stimulation of specific T helper cells. The epidermis is also traversed by pores of glandular structures and follicles from which hairs emerge. The epidermis is separated from the dermis by the basal membrane, composed primarily of collagen, laminin, heparin sulphate and proteoglycans.

In contrast to the epidermis, the dermis consists mainly of connective tissue composed of elastin and collagen, the collagen accounting for about 90 % of the protein in the dermis. Collagens are composed of three polypeptide chains which associate and coil in a right-handed direction to form a long rope-like molecule. Nineteen types of collagen have been identified, each distinguished by some variations in their non-collageneous domains and disulphide bond. In the dermis, collagen type I (85-90 %), III (8-10 %) and V (2-4 %) predominate, while collagen types IV and VII are found in the basal membrane (Burgeson, 1988). The difference in the condensation of these collagen fibres results in two distinct dermal layers; the papillar and reticular layer. The papillar layer is the outer most layer, formed of more sparsely distributed collagen and elastin fibres. The papillary layer contains predominantly type III collagen, while the reticular layer contains mostly type I collagen. The fibroblasts, principal cells of the dermis are responsible for the synthesis of the collagen and elastin which provide the strength and elasticity property of the skin. Fibroblasts adhere to the extracellular matrix via the fibronectin (Ruoslahti, 1988), an extracellular protein capable of interacting with collagen and

cell adhesion proteins (McDonald and Kelly, 1980). In addition to fibroblasts, the dermis contains mast cells, Langerhans' cells and macrophages. While the LCs play a key role in the development of a specific immune response as described before, mast cells and macrophages represent the first line of defence against pathogens. Nerve endings, hair follicles, smooth muscles, glands (sebaceous and sweat glands), blood and lymphatic vessels extend into the dermis (figure 3.1.). Beneath the dermis is the hypodermis, composed of loose adipose tissue, serving as an attachment to deeper structures.

N. americanus larval penetration through the human skin barrier has been shown to be associated with cellular destruction covering a region of about 100 μm in diameter, in an *in vitro* assay, suggesting the involvement of parasite-derived enzymes (Matthews, 1982). Subsequently, Matthews showed that an undefined enzymatic process, optimally active at pH 8, mediated the cellular destruction. Salafsky *et al.,* (1990) using a gelatin-agar membrane as a model for hookworm skin penetration, then showed that serine, and possibly cysteinyl proteinase activities were responsible for the penetration of larvae through this type of membrane. Parasite secretions have since been shown to contain all four protease classes, namely aspartyl, serine, cysteine and metallo proteinases (Kumar and Pritchard, 1992), (Burleigh, 1992; Brown *et al.*, 1999). Using specific proteinase inhibitors, Alan Brown in our laboratory recently demonstrated that these proteinases are able to degrade human skin macromolecules, in an *in vitro* assay (Brown *et al.*, 1999). A metalloproteinase(s) was shown to hydrolyse collagen and elastin while a serine proteinase(s) hydrolysed elastin. An aspartyl proteinase(s) was able to degrade all intact proteins tested, namely collagen types I, III, and IV (but not type V), fibronectin, laminin and elastin. No role was attributed to the larval cysteinyl proteinase as it fails to degrade any of the skin macromolecules tested. Larval proteinases could therefore play a critical role in the infection process and may represent a suitable vaccine target. Indeed a humoral immunity directed against larval ES has also been observed.

Irradiated larvae have been shown to induce protection in a BALB/c mice model against *N. americanus*, irradiated larvae being more protective than non-irradiated larvae (Chapter 4). The effect of irradiation on infective larvae is as yet unknown. It

was proposed that radiation alters antigen conformation or synthesis, rendering attenuated larvae more immunogenic than infective parasite (Wales and Kusel, 1992). However, there are no data available to support this theory. Nonetheless, irradiated schistosome cercariae migration was delayed following administration while non-irradiated larvae migrate more slowly through the skin of vaccinated animals than non-vaccinated animals (Ritcher *et al.*, 1995). This delay has so far been explained only, by an alteration in schistosome motricity due to radiation, but suggests that the interaction between the irradiated parasite and the skin is important in the induction of a protective immune response. In order to gain a better understanding of how Necator gamma-irradiated larvae confer protection in BALB/c mice, a comparison will be made of irradiated versus non-irradiated larval migration.

In order to survive and complete its life cycle, the infective larvae must then overcome the skin immune system. Indeed, larval penetration induce an immune-inflammatory response characterised by an intense itching rash, followed by dermatitis (Miller, 1979). Great variation in the duration of these skin reactions has been recorded, i.e. from approximately 7 days to about 4 weeks. However, larval contamination with faecal and environmental bacteria may be responsible for exacerbation of the cutaneous response. Indeed, in self-infection experiments with sterile cultured *N. americanus* larvae, a much less intense response was observed and this lasted only 12-24 hours (Miller, 1973). Histopathological studies of skin samples, following percutaneous infection with hookworms, have revealed local accumulations of inflammatory cells, such as macrophages, monocytes and granulocytes, peaking 36 hours post-infection (Timothy, 1994). Despite this immune reaction, no damage has been observed against the worms. However, since helminth infection depends essentially on T helper cell subset, the cell mediated immune response may play an important part against parasite in the skin. Indeed, the skin equipped with keratinocytes and Langerhans' cells have the potential to react to any pathogen invasion.

3.1.1. Aims of Chapter 3

Since the interaction between the skin and the parasite may be crucial for the initiation of the development of an immune response against the parasite and therefore for the development of a vaccine, the migration of *N. americanus* through the skin was characterize in detail in this Chapter by:

- Assessing the role of proteinases excreted/secreted by the larval stage in skin penetration. The effects of class specific proteinase inhibitors on larval penetration through *ex vivo* skin were studied.

- Gaining a better understanding of how gamma-irradiated larvae confer protection in the host, a comparison will be made of irradiated versus non-irradiated larval migration.

Since *N. americanus* is a human pathogen, white human skin, obtained from breast surgery, was firstly used to study larval penetration. However, due to its unpredictablelity and limited availability, limited experiments were undertaken using human skin and other skin models were subsequently used to further the current knowledge on hookworm infection. Hamster, mouse and dermal equivalent skin (DEs) were thus used and the results compared within the human skin. *N. americanus'* life cycle has been maintained in hamster for the last 17 years at Nottingham University, implying therefore that hamster skin represents a relevant substitute to human skin. However, since vaccination required the use of BALB/c mice, mouse skin was also used. The limitation of animal use by legislation led to the development of *in vitro* skin models. "Tissue engineering" skin has been useful as a skin replacement and has been shown to be a very useful tool to assess cutaneous irritation, phototoxicity and photoprotection (Damour *et al.*, 1998). Artificial skin has also been used to study *S. mansoni* penetration and was proved to be a suitable model (Fusco *et al.*, 1993). Living skin equivalent generally consists of a dermal equivalent reconstituted with collagen and dermal fibroblasts and a differentiated epidermis that arises from cultured keratinocytes plated onto the surface of the dermal equivalent (Bell *et al.*, 1983). Since the synthesis and deposition of the epidermal layer is a

complex and highly regulated process, only the dermal layer was reconstituted in this project. The dermal equivalent skin (DE) was composed of human dermal fibroblasts embedded in a rat tail collagen type I matrix. In addition, to complete the study, human skin lipids (ceramides, cholesterol and free fatty acids) were added on the surface of the skin in order to mimic the epidermal layer.

3.2. METHODS

The procedure used to study larval penetration through the various skin models was similar for each experiment and described in detail in section 2.5. Briefly, while the dermal equivalent skins (section 2.5.1.1.3.) were mounted in a glass sinter tube (section 2.5.1.2.2.), human, hamster and mouse skins were mounted on the open ends of a tube (outer skin surface facing the inside of the tube), secured with an elastic band and placed in an universal tube containing 5 ml DMEM (section 2.5.1.2.1.). Two hundred larvae were then placed over the skin in the presence and absence of inhibitors and incubated in a water bath at 37 °C. After different incubation times (3, 24 and 48 hours), the larvae remaining on the surface of the skin ('top counts') and the larvae which had completely penetrated the skin ('bottom counts') were counted. To characterize the role of proteinases in skin penetration, fresh inhibitors were added every 24 hours to maintain an optimal concentration.

The study of irradiated larval penetration was further characterized by the study of larval penetration through two layers of skin. The apparatus used is described in section 2.5.1.2.1. and the methods used were as described above. In addition, biochemical techniques such as SDS-PAGE, Western blot and enzyme activity assay, described in sections 2.8.2.2., 2.8.3.and 2.8.4 respectively, were also carried out.

3.3. RESULTS

3.3.1. Characterisation of *N. americanus* penetration through human skin

3.3.1.1. Time course of migration

The time course of larval penetration through human skin is presented in figure 3.2. The 'top counts', representing the number of larvae remaining on the surface of the skin, indicated that 70 % of larvae (140 out of a total of 200) have entered the skin after 3 hours, increasing to 79 % after 24 hours and remaining at this level until 48 hours.

The 'bottom counts', representing the larvae that manage to completely traverse the skin, showed that only 2 % of larvae have completely penetrated the skin after 48 hours. Therefore, about 97 % of the larvae that had entered the skin remained within the human skin after 48 hours. It was also interesting to observe that the larvae that had penetrated the skin had kept their L_2 sheath. Based on this profile, a 48 hours incubation period was chosen to assess the effect of proteinases on L_3 penetration (section 3.3.1.2.).

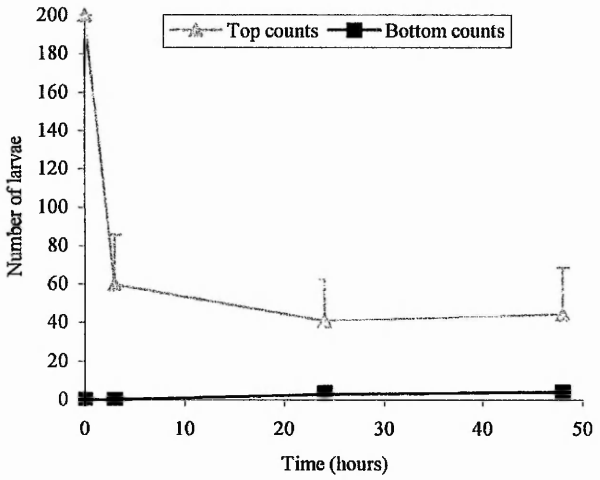


Figure 3.2. Penetration of *N. americanus* infective larvae through human skin. *The 'top counts' represent the larvae that remain on the surface of the skin. The 'bottom counts' represent the larvae which have completely traversed the skin. The results are expressed as the mean of 3 experiments + SD number of larvae failing to enter the skin or the mean + SD number of larvae fully penetrating the skin.*

3.3.1.2. Role of proteinases in human skin penetration

Figure 3.3. shows the effect of proteinase inhibitors on larval skin penetration. In this experiment, 46 % of larvae had migrated into the human skin after 48 hours (water control, 'top counts'), while 71 % of larvae had entered the skin in the ethanol control. The 'top counts' revealed that pepstatin A, an aspartyl proteinase inhibitor, was the only inhibitor having an effect on the migration of larvae into the skin, reducing it by 56.6 % compared to the ethanol control (figure 3.3.a).

However when considering the 'bottom counts' (which were again low), all the inhibitors, except E64, a cysteinyl proteinase inhibitor, reduced larval penetration to a certain extent. Among the inhibitors dissolved in water, APMSF, a serine proteinase inhibitor, was the most potent one, reducing the number of larvae that had completely penetrated the skin by 91 %, compared to the water control (figure 3.3.b). Ethanol also reduced complete larval penetration by 39 % compared to the water control, where 3.3 larvae out of 200 had successfully penetrated the skin. Both of the inhibitors dissolved in ethanol, that is phenanthroline, a metallo proteinase inhibitor and pepstatin A, reduced the 'bottom count', with phenanthroline having the greatest effect (65 %).

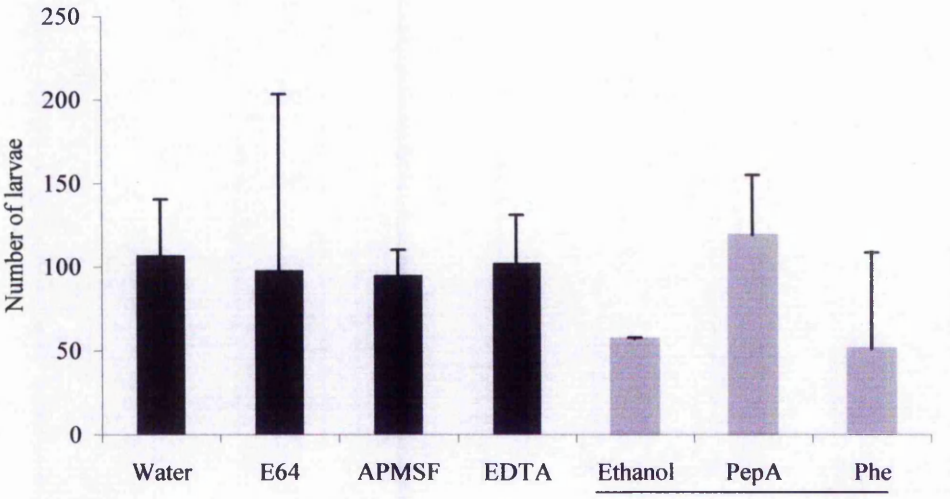
3.3.2. Evaluation of a suitable membrane to study larval penetration

3.3.2.1 Hamster skin model

3.3.2.1.1. Time course of migration

The time course of larval migration through hamster skin is represented in figure 3.4. The 'top counts' indicated that, after 3 hours, 98 % of the initial larvae number entered the skin, increasing to 99 % after 48 hours. However, after 3 hours only 1 % of the larvae which had entered the skin, completely traversed the skin, reaching 28.7 % after 24 hours (bottom counts). After 48 hours, 42 % of the larvae (ensheathed and sheathed) had reached the other side of skin. Thus, 57 % of the larvae remained within the skin at 48 hours. From this graph, 24 hours incubation was chosen as the time point to assess the effect of proteases on L_3 penetration through hamster skin.

a- Top counts



b- Bottom counts

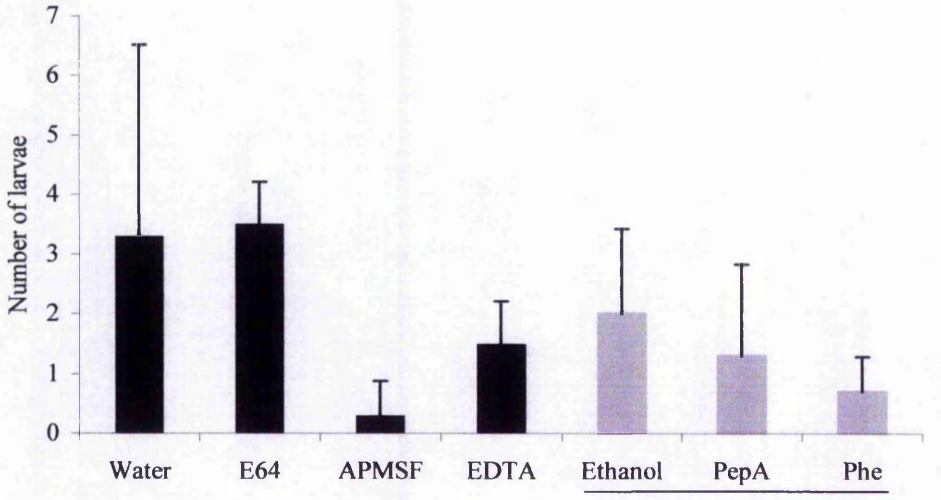


Figure 3.3. Effects of proteases on the penetration of *N. americanus* infective larvae through human skin. *The number of larvae was determined by counting the larvae which remain on the surface of the skin after 48 hours. Each value represents the mean of 3 experiments. The ethanol control should be used for phenanthroline and pepstatin A. The results are expressed as the mean + SD number of larvae failing to enter the skin (Top counts, a) or the mean + SD number of larvae fully penetrating the mouse (Bottom counts, b).*

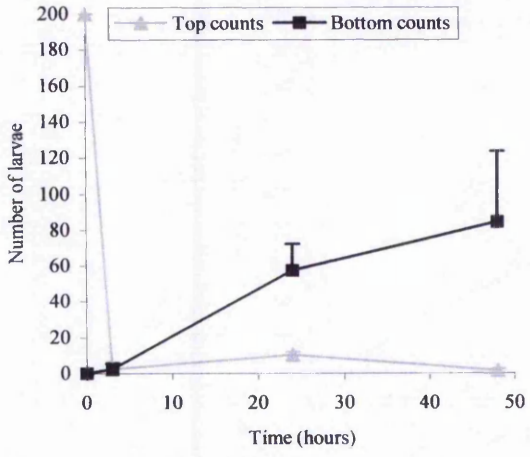


Figure 3.4. Penetration of *N. americanus* infective larvae through hamster skin. *The bottom counts represent the larvae which have completely traversed the skin. The top counts represent the larvae which remain on the surface of the skin. Each time point represents the mean of 4 experiments. The results are expressed as the mean + SD number of larvae failing to enter the skin or the mean + SD number of larvae fully penetrating the skin.*

3.3.2.1.2. Role of proteinases

The effects of proteinase inhibitors on complete penetration of Necator larvae through hamster skin are presented in figure 3.5.

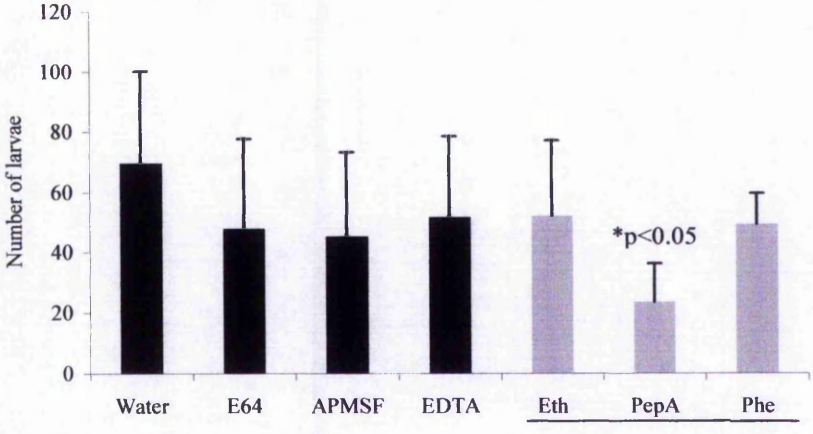


Figure 3.5. Effects of proteinases on the penetration of *N. americanus* infective larvae through hamster skin. *The number of larvae was determined by counting the larvae which have completely traversed the skin after 24 hours (bottom counts). Each value represents the mean of 9 experiments. The ethanol control should be used for phenanthroline and pepstatin A. The results are expressed as the mean + SD number of larvae fully penetrating the mouse (data published in Brown* et al., *1999).*

After 24 hours incubation, 35 % of larvae (70 out of 200) had penetrated the skin (see water control), while only 26 % of larvae had penetrated the skin in presence of ethanol. All the inhibitors studied reduced larval skin penetration to a certain degree. However, pepstatin A had the greatest effect, reducing significantly larval penetration by 54.8 % ($P \leq 0.05$) compared to the ethanol control.

3.3.2.2. Mouse skin model

3.3.2.2.1. Time course of migration

The time course of Necator infection through mouse skin is represented in figure 3.6. The 'top counts' show that 94 % of the larvae entered the skin after 3 hours, increasing to 99 % after 48 hours. Among these larvae, 55 % had penetrated entirely the skin after 48 hours ('bottom counts'). Thus, about 45 % of the larvae were within the skin at 48 hours. Once again a mixture of sheathed and ensheathed larvae were recovered in the 'bottom counts'.

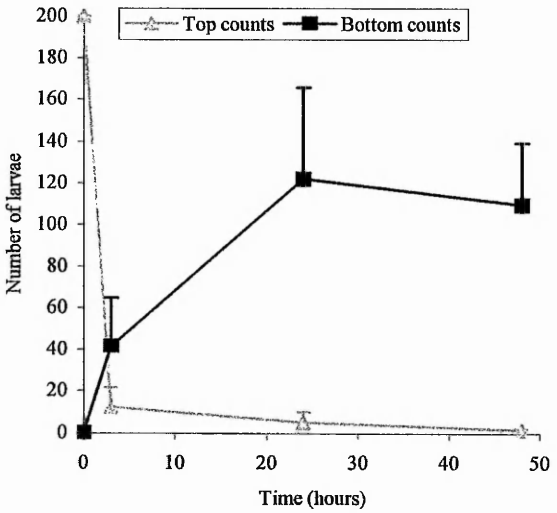


Figure 3.6. Penetration of *N. americanus* infective larvae through mouse skin. *The 'top counts' represent the larvae which remain on the surface of the skin. The 'bottom counts' represent the larvae which have completely traversed the skin. Each time point represents the mean of 6 experiments. The results are expressed as the mean + SD number of larvae failing to enter the skin or the mean + SD number of larvae fully penetrating the skin.*

3.3.2.2.2. Role of proteinases in penetration

Figure 3.7. shows the effects of proteinase inhibitors on larval penetration through mouse skin. In the presence of water, 73 % of larvae had fully penetrated the skin, while 63 % larvae had penetrated the skin in the presence of ethanol. Of all the inhibitors studied, only APMSF and E64 reduced larval penetration by about 20 % compared to the water control.

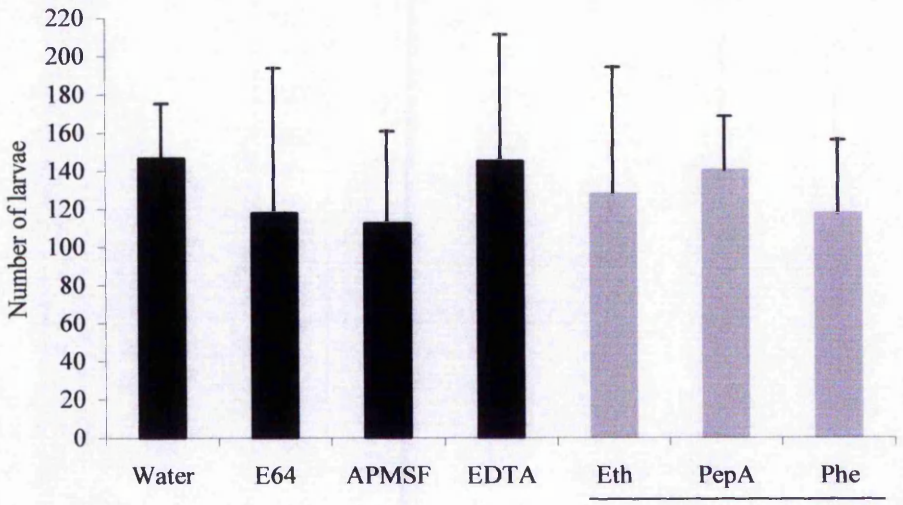


Figure 3.7. Effects of proteinases on the penetration of *N. americanus* infective larvae through mouse skin. *The number of larvae was determined by counting the larvae which have completely traversed the skin after 24 hours. Each value represents the mean of 6 experiments. The ethanol control should be used for phenanthroline and pepstatin A. The results are expressed as the mean + SD number of larvae fully penetrating the skin.*

3.3.2.3. Dermal equivalent skin (DEs)

The dermal equivalent skin consisted of human dermal fibroblasts embedded in a rat tail collagen type I lattice. After 5 days *in vitro*, the DE was tissue-like in its consistency. The membrane was 2-3 mm thick, slightly opaque and elastic. Before the application of the larvae, some medium was added on the surface of the membrane to make sure that no leakage occurred.

3.3.2.3.1. Time course of migration

The data (figure 3.8.) shows that *Necator* larvae had entered and penetrated the DEs. In this experiment, more sheaths were observed on the surface of DEs compared with human or animal tissue. Out of the 200 initial larvae, 58 % of the larvae had entered the skin after 3 hours, increasing to around 96 % after 24 hours. After 3 hours, 11 % had fully traversed the DEs, increasing to 53 % after 24 hours and 67 % after 48 hours. Thus, only 20 % of the larvae which had entered the skin remained within the skin at 48 hours.

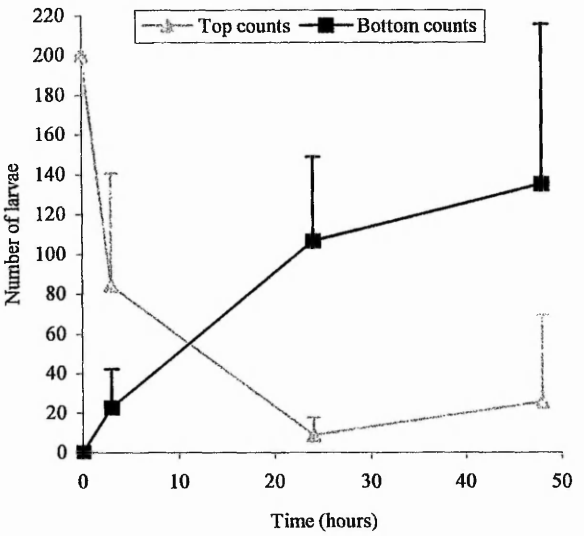


Figure 3.8. Penetration of *N. americanus* infective larvae through dermal equivalent skin. *The bottom counts represent the larvae which have completely traversed the skin. The top counts represent the larvae which remain on the surface of the skin. Each time point represents the mean of 4 experiments. The results are expressed as the mean + SD number of larvae failing to enter the skin or the mean + SD number of larvae fully penetrating the skin.*

3.3.2.3.2. Effect of human lipids on larval migration through DEs

The addition of human lipids on the surface of the artificial skin increased larval penetration as shown figure in 3.9. The number of larvae entering the skin ('top counts') as well as the number of larvae completing their migration ('bottom counts') increased in a dose-dependent manner. For instance, the number of larvae penetrating the skin is 10 fold higher with the addition of 2 µl of human lipids.

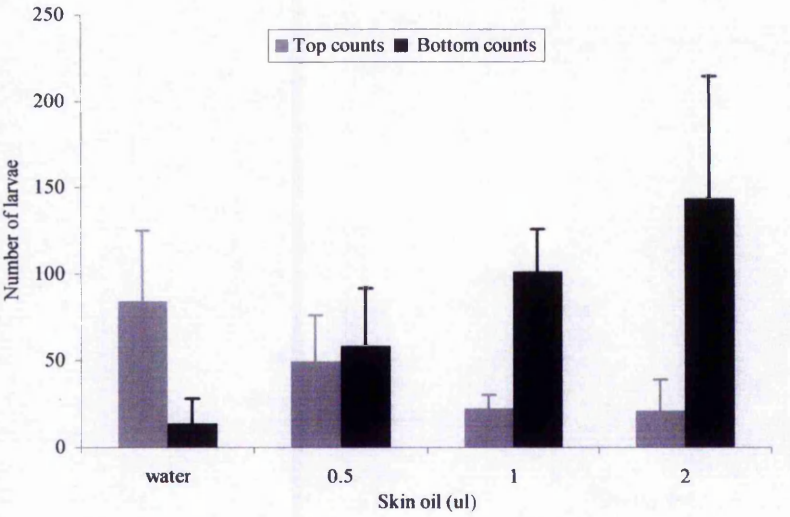


Figure 3.9. Effects of lipids on the penetration of *N. americanus* infective larvae through DE skin. *The number of larvae was determined by counting the larvae which have completely traversed the skin after 24 hours (bottom counts). Each value represents the mean of at least 6 experiments. The results are expressed as the mean + SD number of larvae failing to enter the skin or the mean + SD number of larvae fully penetrating the skin.*

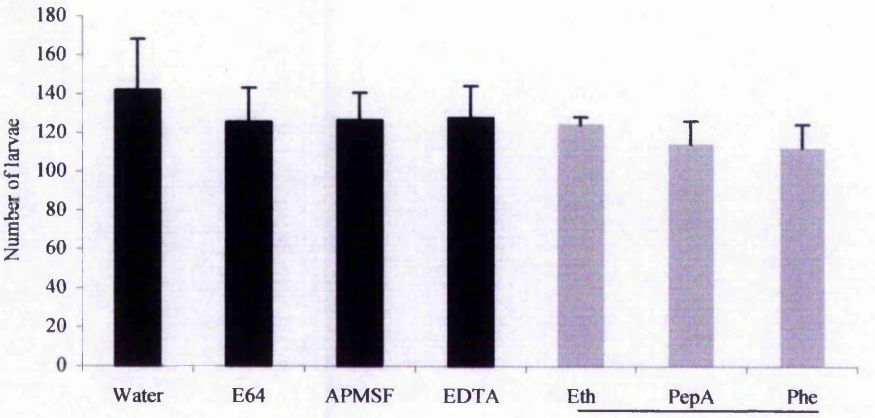


Figure 3.10. Effects of proteinases on the penetration of *N. americanus* infective larvae through DE skin. *The number of larvae was determined by counting the larvae which have completely traversed the skin after 24 hours (bottom counts). Each value represents the mean of 4 experiments. The ethanol control should be used for phenanthroline and pepstatin A. The results are expressed as the mean + SD number of larvae fully penetrating the skin.*

However it should be noted that the number of larvae penetrating the DEs ('top counts': 58 %; 'bottom counts': 7 %) are surprisingly low compared to the number obtained in the time course experiment after 24 hours incubation ('top counts': 96 %; 'bottom counts': 53 %).

3.3.2.3.3. Role of proteinases in the penetration of DEs by larvae

The effect of the protease inhibitors on larval penetration through DEs is presented in figure 3.10. After 24 hours, 71 % of larvae had fully traversed the DE skin in the water control, compared to 62 % in the ethanol control. The inhibitors had little or no effect on larval penetration through DEs.

3.3.3. Characterisation of irradiated larval penetration through mouse skin

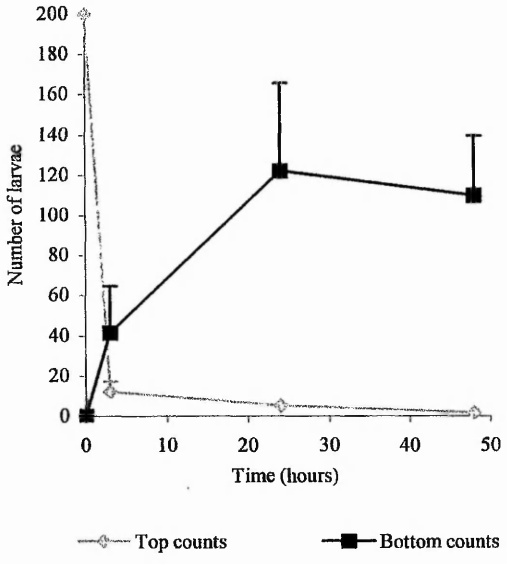
3.3.3.1. Time course of irradiated larval penetration

In order to compare the effect of gamma irradiation on larval penetration, the penetration of normal and irradiated larvae through mouse skin were carried out at the same time using the same batch of larvae (half of which was irradiated). The data are represented in figure 3.11.a. and 3.11.b.

After 3 hours incubation, no significant difference was observed in the number of larvae entering the skin ('top counts) between non-irradiated (figure 3.11.a.) and irradiated larvae (figure 3.11.b.), up to 98 % of larvae entered the skin. However, the bottom counts showed that only 3 % of irradiated larvae had completely traversed the skin (figure 3.11.b.), compared to 20 % with non-irradiated larvae (figure 3.11.a.). This difference between irradiated and non-irradiated larvae is even more accentuated after 24 hours. After 24 hours, only 26 % of irradiated larvae completely traversed the skin compare to 61 % of normal larvae ($p < 0.05$).

Thus, 67 % of irradiated larvae were within the skin after 48 hours compared to 36 % of non-irradiated larvae.

a- Penetration of non-irradiated larvae through *ex vivo* mouse skin



b- Penetration of irradiated larvae through *ex vivo* mouse skin

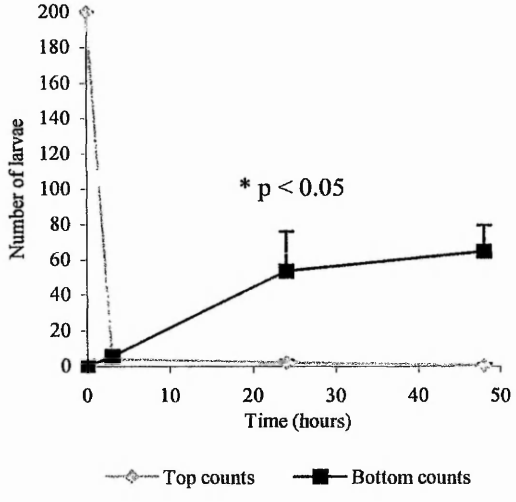


Figure 3.11. Penetration of *N. americanus* infective larvae through mouse skin. *The bottom counts represent the larvae which have completely traversed the skin. The top counts represent the larvae which remain on the surface of the skin. Each time point represents the mean of 4 experiments. The results are expressed as the mean + SD number of larvae failing to enter the skin or the mean + SD number of larvae fully penetrating the skin.* ***a*** *= penetration of non-irradiated larvae;* ***b*** *= penetration of irradiated larvae through mouse skin.*

3.3.3.2. Effect of proteinase inhibitors on irradiated larval penetration

The effect of proteinase inhibitors on the penetration of irradiated larvae was studied on both mouse and hamster skin. The results are shown in figure 3.12.

Using *ex vivo* mouse skin, no inhibitor had a significant effect on irradiated larval penetration (3.12.a.) although APMSF reduced larval penetration by approximately 30 %. Similar results were previously obtained with non-irradiated larvae through mouse skin (figure 3.7.). Phenanthroline, a metalloproteinase inhibitor, slightly enhanced irradiated larval penetration compared to the ethanol control, but this was not significant.

Among the inhibitors dissolved in water, EDTA (33 %) but mainly APMSF (53 %) inhibited irradiated larval penetration through hamster skin (3.12.b.). The penetration of irradiated larvae was also significantly inhibited by pepstatin A inhibitor (54 %) compared to the ethanol control.

3.3.3.3. Penetration of larvae through two mouse skin layers

The penetration of irradiated and non-irradiated layer through two layers of mouse skin is represented in figure 3.13. These results showed for the first time that normal larvae as well as irradiated larvae are able to penetrate the skin twice.

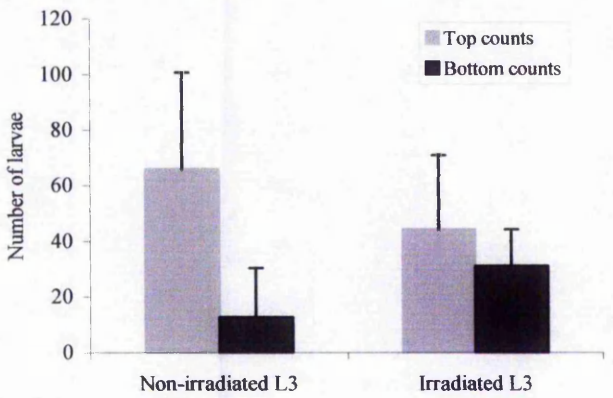
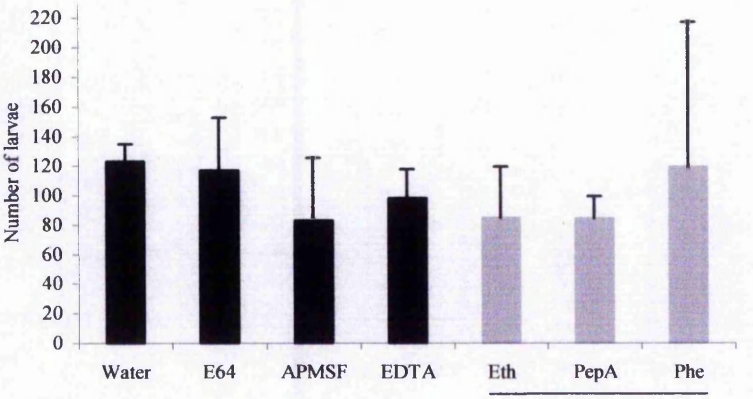


Figure 3.13. Penetration of irradiated and non-irradiated *N. americanus* infective larvae through two layers of mouse skin. *The bottom counts represent the larvae which have completely traversed the two skins. The top counts represent the larvae which remain on the surface of the skin. Each time point represents the mean of 3 experiments. The results are expressed as the mean + SD number of larvae failing to enter the skin or the mean + SD number of larvae fully penetrating the skin.*

a-Mouse skin



b- Hamster skin

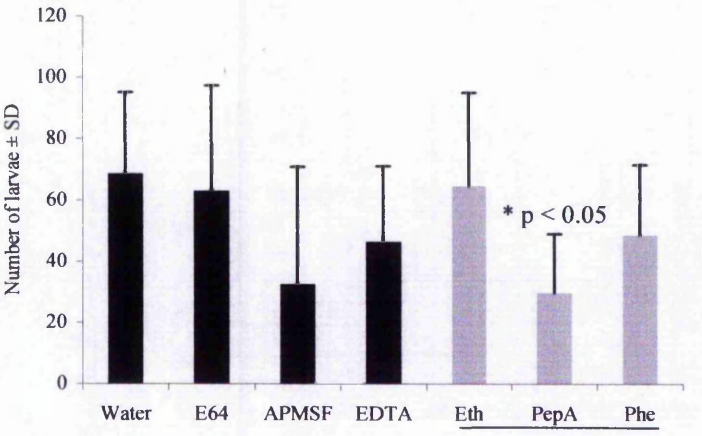


Figure 3.12. Effects of proteinases on the penetration of irradiated *N. americanus* larvae through mouse (a) and hamster (b) skin. *The number of larvae was determined by counting the larvae which have completely traversed the skin after 24 hours (bottom counts). Each value represents the mean of 4 experiments. The ethanol control should be used for phenanthroline and pepstatin A. The results are expressed as the mean + SD number of larvae fully penetrating the skin*

3.3.3.4. Effect of gamma irradiation on larval excretory/secretory products (ES)

In order to check why irradiated larvae are delayed in the skin, the effect of radiation on the secretion and activity of ES products were studied. The results are represented in figure 3.14. The production of ES by normal larvae increased constantly from 0 to 144 hours. Normal larvae produced about 5.5 µg/ml of protein every 24 hours, the concentration of protein secreted/excreted reaching 22.8 µg/ml after 144 hours.

Time zero represents the time between the CO_2 bubbling and the beginning of the culture; at this point irradiated larvae have already produced 6 µg/ml of ES products compared to 0 µg/ml with normal larvae. Subsequently the rate of ES products in the irradiated and non-irradiated larvae was very similar over a 75 hour time course. The secretion pattern of irradiated larvae seems to be modified by the effect of radiation. Indeed, irradiated larvae appear to secrete ES products by pulse every 24 hours. Despite the fluctuation of ES production, the cumulative amount of ES produced by irradiated larvae was at all times greater than the amount secreted by non-irradiated larvae.

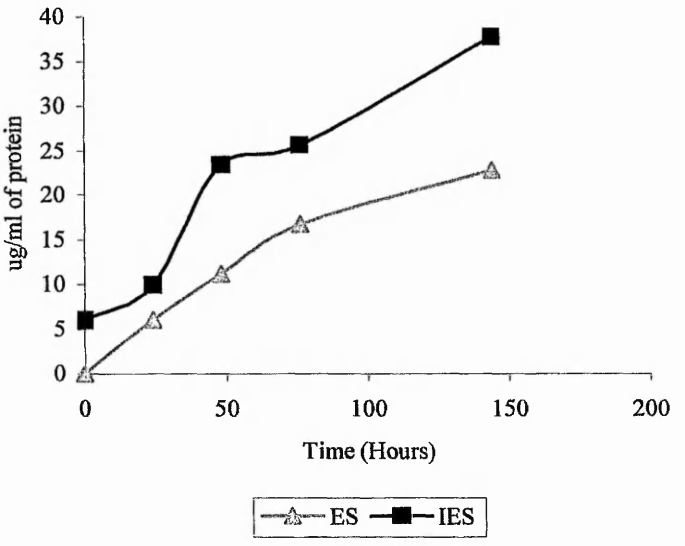


Figure 3.14. Effect of gamma-radiation on larval excretory/secretory products (ES). *Non-irradiated and irradiated larvae were activated for 1 hour with CO_2 and then incubated at 37 °C for 144 hours. The protein concentration of ES products from normal (ES) and irradiated (IES) larvae was estimated for each time point using Bio-Rad protein assay kit with bovine serum albumin standards.*

3.3.3.5. Characterisation of ES products secreted by non-irradiated and irradiated larvae

Figure 3.15. shows the SDS-PAGE profile of ES products from non-irradiated (ES) and irradiated (IES) larvae after 74 hours. Six bands are identified in ES secreted by non-irradiated larvae, varying in intensity and size from 33 to 80 kDa. The most prevalent proteins have a molecular weight around 33 kDa. The protein pattern of IES was similar to the pattern found in ES.

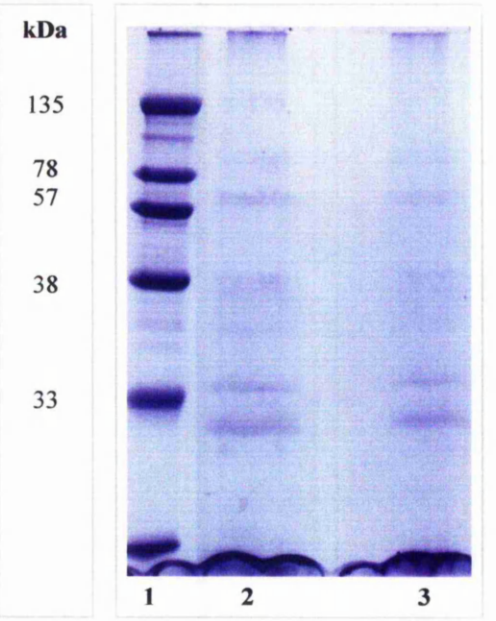


Figure 3.15. Characterisation of ES products secreted by non-irradiated and irradiated larvae after 72 hours of culture. *20 µg of ES and IES were loaded in each well of a 12 % SDS PAGE. The gel run at 20 mA/gel and then stained with coomassie blue. Lane 1: molecular weights, lane 2: 20 µg ES and lane 3: 20 µg IES.*

The proteinase classes present in non-irradiated and irradiated larval ES were then assessed by monitoring the hydrolysis of FITC-labeled casein. The only inhibitor presenting a differential effect between irradiated and normal ES was E64, being 10 times more active on irradiated than non-irradiated (table 3.1.). Significantly, pepstatin A is the most effective inhibitor in both larvae.

Table 3.1. Characterisation of the proteinase activities presents in normal and irradiated larval ES. *50 µl of larval ES products (20 µg/ml) were incubated with fluorescein isothiocyanate (FITC) casein at 37 °C for 3 hours as described in section 2.8.4. Proteinase activity is expressed as the mean of fluorescence emitted (n=3). Inhibition of proteinase activity by a number of inhibitors is shown as the percentage inhibition/enhancement of activity compared with uninhibited control following the substraction of non-enzymatic blanck.*

Inhibitors	Non-irradiated larval		Irradiated larvae	
	FITC unit/mg	%	FITC unit/mg	%
None	1574		1975	12.6
E64	1546	1.7	1726	6.7
APMSF	1725	9.5	1841	15.3
EDTA	1388	11.8	1672	40
Phenanthroline	57	37.6	0	91.8
Pepstatin A	309	80.3	160	

3.4. DISCUSSION

3.4.1. Migration of infective larvae through skin models

N. americanus larvae were able to penetrate *ex vivo* human, hamster and mouse skins and also DE skin, but variations in larval vitality and motility were observed between batches. Although *N. americanus* larvae was always cultured in the same conditions, the quality of the larvae may have been a function of the age of the larvae. Indeed, following culture, infective larvae were sometimes stored for a few days before use; this may have affected worm behaviour. Each batch of larvae derives from the faeces of 12 to 24 hamsters and since each hamster responds differently to the infection, this also may be responsible for the difference in motility. Furthermore, the 200 infective larvae applied to the skin surface were an undetermined mixture of males and females which may result in variations since female worms have been shown to secrete more enzymes than males (A. Brown, personnal communication).

Larval migration characteristics were shown to vary between skin species. Indeed, although minor variations were observed between individuals of one species, important differences were observed between species. Whilst mouse and hamster skin structure appeared very similar, human skin was thicker (at least twice as thick as the others) and less elastic. The human skin also shows more and larger pores, as well as adipose tissue compared to the other two species. DE skin, consisting only of human fibroblasts embedded in collagen type I, was obviously the simplest model, and was also the most reproducible. It is also important to mention that significant handling of skin was required in order to fix it to the apparatus (section 2.5.1.2.), and this appeared to make the skin more greasy. However, this does not apply to DEs. Overall, this Chapter demonstrated that *ex vivo* hamster skin is the closest analogy to human skin.

In most cases, ethanol was shown to decrease larval penetration. However short chain alcohols, such as ethanol, have previously been shown to increase the permeability of the skin, having an effect on the phase behaviour of the stratum

corneum lipids (Krill *et al.*, 1992; Goates and Knutson, 1994). These results suggest that the physiological property of the *ex vivo* skin is different from that *in vivo*.

3.4.1.1. Time course of migration

The results of the present investigation confirm that exsheathment is not required for larval penetration, as demonstrated by Kumar *et al.,* (1992). In addition, the time courses of larval migration through the different skin models indicate that the majority (at least 70 %) of larvae had entered the skin after 3 hours incubation. These data are in agreement with field data showing that most larvae enter the host's skin in the first three hours of skin contact (Salafsky *et al.*, 1990).

Using DEs, less than 70 % of the larvae entered the skin after 3 hours contact, suggesting a role for the epidermis in larval penetration. Similar results were obtained by Matthews (1982) who noted that, when skin was delipidated with chloroform, larval penetration was decreased. Human lipids were therefore added onto the surface of DE skin and an increase in the number of larvae entering the skin was subsequently noted. It is likely, therefore, that the epidermis plays an essential role in enhancing larval migration and that epidermal lipids are at least partly responsible for this. Indeed Salafsky *et al.*, (1990) showed that migration of Necator larvae through an artificial membrane (10 % gelatin) is stimulated by essential fatty acids (EFA). In the present work, infective larvae were shown to invade animal skin more rapidly than human skin. This may in part be due to skin handling and a difference in lipid composition between the skins; however no literature is available to support these suggestions.

More larvae had fully penetrated DE skin (80 %) than human skin (3 %) after 48 hours. These results therefore suggested that DEs did not present any obstacle to larvae penetration, confirming the importance of the epidermal layer as a barrier. These data are in agreement with Matthews (1977) who showed that once the epidermis has been passed, passage through the dermis is relatively easy. However, Fusco *et al.* (1993) using a 10 % gelatin/agar membrane showed that schistosome cercarial penetration was considerably reduced, this membrane being a more formidable barrier than human skin.

Whist 3 % of larvae fully penetrated human skin, 50 % of larvae fully penetrated animal skins. Even when the time of incubation was extended to 6 days for the human skin study, the same number of penetrated larvae was recovered (data not shown). However, Behnke *et al.*, (1986) showed that infective larvae remained in the skin of infected patients for only 3-4 days; this would still allow high interaction between the host immune system and the parasite. The prolonged arrest observed in *ex vivo* human skin compared to animal skin may be due to its thickness and complexicity. In addition, at the beginning of the time course, the isolated skin was already one day old and might have lost some of its physical property. It is also possible that the *N. americanus* strain, maintained for the last 17 years in hamster, has become increasingly adapted to the rodent, rendering it more difficult for the larvae to penetrate human skin. Indeed, A. Brown (2000) has suggested this adaptation in his PhD thesis, noticing a decrease in the time taken for *N. americanus* larvae to reach both the lungs and intestine compared to that previously reported (Wells and Behnke, 1988; Timothy and Behnke, 1993).

3.4.1.2. Role of proteinases in skin penetration

The effect of specific proteinase inhibitors on larval penetration through *ex vivo* intact skin established that all 4 classes of proteinases (cysteinyl, aspartyl, serine and metallo proteinases) are involved to a certain extent in the larval infection process (table 3.3.). In order to simplify the interpretation, the results obtained with human skin, representing the most relevant system to hookworm infection, will be analysed in detail.

Table 3.3. Summary of the role of various proteinase classes secreted by infective larvae in skin penetration.

Skin models	Serine proteinases	Cysteine proteinases	Metallo-proteinases	Aspartyl proteinases
Human	++	-	+	+++
Hamster	+	+	+	+++
Mouse	+	+	-	-
DEs	-	-	-	-

First of all, it is important to note that the proteinase inhibitors had no effect on larval penetration through DEs, suggesting that the larvae do not require proteolytic enzymatic activities to penetrate DEs. The larvae therefore appeared to penetrate DEs by ondulatory movement only. However, since the DEs is an over simplified model, this result does not necessarily mean that infective larvae penetrate the dermis of human skin in the same way.

The absence of effect of the cysteinyl proteinase inhibitor, E64, on larval penetration through human skin is in agreement with the finding that cysteinyl proteinases play no part in the degradation of human collagen, fibronectin, elastin and laminin (Brown *et al.*, 1999). The cysteinyl proteinase activity is a cathepsin B and L-like activity (Brown *et al.*, 1999). Although not statistically significant, the effect of E64 on the infection process through hamster and mouse skin suggested that cysteinyl proteinases digest host skin macromolecules not yet studied or have a role in the parasite itself. Indeed, cysteinyl proteinases have previously been shown to be involved in the ensheathment rather than the migration of infective larvae (McKerrow *et al.*, 1985; Salafsky and Fusco, 1987).

The serine proteinase inhibitor, APMSF, was shown to have a potent effect on larval migration through all three skin types. Serine proteinase activity is a trypsin-like activity, optimum at pH 8, and has been shown to degrade elastin but not collagen, fibronectin and laminin (Brown *et al.*, 1999). Elastin is present in great quantity in the dermis; however APMSF had no effect on the penetration of the larvae through DEs. The serine proteinase may have another function not yet characterised. For instance, a serine proteinase is thought to facilitate the initial step in human infection by *S. mansoni*. A serine proteinase has also been identified in adult *N. americanus* and it is thought that the enzyme may induce apoptosis in activated T cells (Chow *et al.*, 2000; Talinan *et al.*, 1997).

Both the metalloproteinase inhibitor, EDTA and phenanthroline, also inhibited larval penetration. Larval Necator metalloproteinases have been shown to degrade collagen and elastin, present in the dermis and basal membrane (Brown *et al.*, 1999). These metalloproteinase enzymes are calcium dependent and are optimally active at pH 7.5. Previous enzyme substrate specificity assays demonstrated the existence of two

metalloproteinases with different inhibitor sensitivity. One enzyme appears to degrade collagen types III, IV and elastin and is sensitive to phenanthroline while the other degrades types I and V collagen and appears to be sensitive to EDTA. Metalloproteinases have also been observed in adult ES products and have been shown to degrade haemoglobin (Burleigh, 1992) and to cleave eotaxin, inhibiting eosinophil recruitment *in vivo* (Culley *et al.*, 2000). Production of these metalloproteinases, inactivating eotaxin, may be a strategy employed by the hookworm to prevent recruitment and activation of eosinophils at the site of infection. Indeed, eosinophils have been shown to damage parasites such as schistosomes by releasing the contents of their intracellular granules (Butterworth *et al.*, 1975).

Pepstatin A, an aspartyl proteinase inhibitor, had the most potent effect on larval penetration through both human and hamster skins. Pepstatin A inhibited the entry of larvae in the human skin by 56.6 % and complete larval penetration by 35 %. In hamster skin, larval penetration was inhibited significantly by 54.8 %. The latter results have been published in the *American Society of Tropical Medicine and Hygiene*, volume 60, number 5, pages 840-847, 1999.

Aspartyl proteinases have been identified in larval and adult ES products (Burleigh, 1992; Brown *et al.*, 1999). The presence of an aspartyl proteinase has also been documented in *Haemonchus contortus* (Karanu *et al.*, 1993) and a cDNA encoding a cathepsin D-like aspartyl proteinase has been identified from the dog hookworm *Ancylostoma caninum* (Harrap *et al.*, 1996). However no function has yet been allocated to the aspartyl proteinase secreted by the larvae. The aspartyl proteinase activity present in adult ES can degrade haemoglobin and fibronectin, a key element for parasite survival (Burleigh, 1992; Brown, 2000).

In this chapter, larval aspartyl proteinases have been shown for the first time to play an important role in skin penetration by Necator larvae. In a previous *in vitro* study, A. Brown showed that pepstatin A is capable of inhibiting the degradation of all intact skin proteins tested, namely collagen (except type V collagen), fibronectin, laminin and elastin (Brown *et al.*, 1999). The combined results indicate that aspartyl proteinases play a key role in the infection process and could represent an excellent

vaccine candidate in humans. Necator aspartyl proteinases have subsequently been cloned by Dr K. Girdwood, Cardiff School of Biosciences, Cardiff University, and have been used in a vaccine against Necator infection. These results are presented in Chapter 5.

3.4.2. Effect of irradiation on the ability of third stage larvae to penetrate the skin.

Irradiated larvae successfully traversed mouse skin; however their migration was delayed compared to non-irradiated larvae. Indeed, after 48 hours incubation, 67 % of irradiated larvae remained within the skin compared to 44 % of normal larvae. Irradiated schistosome cercariae have also been shown to be delayed in the skin and to have a truncated migration (Coulson, 1997). However, some irradiated *N. americanus* parasites have previously been found in mouse intestine following percutaneous injection (Brown, 2000). The prolonged arrest in the skin increased the period of contact between the parasite and the skin immune system, which may explain why irradiated larvae are more immunogenic than non-irradiated larvae.

In order to further characterize the effect of radiation on larval penetration, mouse skin was initially used to study the effect of proteinases inhibitors. None of the proteinase inhibitors studied had a significant effect on the penetration of irradiated larvae. However, similar results were observed with non-irradiated larvae. Hamster skin, where specific proteinase inhibitors had an effect on larval penetration, was subsequently used. Pepstatin A and APMSF were the most potent inhibitors, inhibiting irradiated larval penetration by up to 50 %. Aspartyl proteinases were therefore as important for irradiated as non-irradiated larvae in the infection process. These data confirmed the important role of the aspartyl proteinases in skin penetration, supporting their potential as a good vaccine candidate.

It has been accepted for some time that hookworm larvae have a limited amount of proteinases stored in the oesophageal glands and that larvae use all these reserves to penetrate the skin (Smith, 1976). In addition, Matthews (1982) suggested that infective larvae were able to penetrate human skin only once. The theory was that irradiated larvae secrete their oesophageal contents all at once, having none left to

migrate further. However, this study demonstrates that non-irradiated as well as irradiated larvae can successfully penetrate two layers of mouse skin. It could be said that the *ex vivo* system used was not such a good barrier, allowing the larvae to keep some ES products for the second infection process. However even with hamster skin, the best human skin analogy as demonstrated by the similar effect obtained with the proteinase inhibitors, infective larvae were able to penetrate the skin twice (data not shown). It was also interesting to note that even though migration of irradiated larvae was delayed, the ability of these larvae to penetrate the skin twice was higher than non-irradiated larvae.

The subsequent question was whether irradiated larvae secrete less ES than normal larvae. Indeed, treatment of newly excysted *Fasciola hepatica* with 40 krad of γ-irradiation resulted in a reduction in the secretion of cathepsin B and a reduced carbohydrate expression on the parasite surface (Creaney *et al.*, 1996). On the contrary, irradiating *Trichinella spiralis* larvae resulted in an increase in the release of antigens in a dose dependent manner (Agyei-Frempong and Catty, 1983). Necator irradiated larvae were shown in this study to secrete more ES products than non-irradiated larvae at a determined time point. The result suggested that the difference observed between irradiated and non-irradiated larvae occured during CO_2 bubbling, however the effect of the CO_2 on the irradiated larvae secretion is unknown. In addition, 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 FITC-casein. The delay in migration through the skin cannot be explain by less enzymatically active secretions, and can only be due to the damage of neuromuscular functions caused by irradiation. 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 and Wilson, 1993). The combination of reduced larval mobility and increased ES concentration may therefore be responsible for enhancing the immune response against the parasite. Indeed, the pool of ES secreted by irradiated larvae is probably perceived as highly immunogenic by the host's immune system and represents a good source of proteinases to be included in a vaccine.

To conclude, the penetration of infective larvae through skin has been characterized in Chapter 3 and the main findings are summarized below.

- The infection process varies between skin species, underlying the importance of a relevant animal model for vaccination.
- The epidermis is the principal barrier to larval penetration and its lipid constituents play an essential role in stimulating larval penetration.
- Serine, cysteinyl, aspartyl and metallo proteinases contribute to larval penetration. However, the aspartyl proteinase(s) is the main proteinase involved in the infection process.
- Irradiated larvae present a pool of IES products for an extended amount of time in the skin, which may be responsible for enhancing the immune response against *N. americanus*.
- Finally, this Chapter has identified components for inclusion in a test vaccine, namely ES from irradiated larvae and the aspartyl proteinase.

Chapter 4

Characterisation of a 'successful' vaccination model against *Necator americanus*

4.1. INTRODUCTION

Prior to this study, work on the development of a vaccine against *Necator americanus* was limited to that undertaken by Dr A. Brown, working in our laboratory, in collaboration with Prof. D. Pritchard (School of Pharmaceutical Sciences, University of Nottingham). Dr A. Brown established that three immunisations with 300 irradiated (40 krad) *N. americanus* larvae, in male BALB/c mice, stimulate complete protection against further infection (300 larvae), irradiated larvae being more protective than non-irradiated larvae (Brown, 2000). The antibody response occurring in vaccinated animals was subsequently analysed in detail (Brown, 2000). Higher antibody titres were observed in animals vaccinated with either live larvae or irradiated larvae. A difference in IgG3 and IgG2a (marker of the Th1 response) levels was observed in animals vaccinated with irradiated larvae, but IgG1 (marker of a Th2 response) dominated in both groups. Furthermore, no apparent difference in antibody specificity was observed between animals vaccinated with irradiated or non-irradiated larvae. A preliminary study indicated also that resistance to Necator infection was associated with a Th2 response, characterised by an elevated level of IL5, at least in the spleen of animals vaccinated with both normal and irradiated larvae, 3 and 8 days post-infection (Brown, 2000). Thus, further work was required to understand how gamma irradiated larvae induce an immune response against Necator infection and which immune compartment was involved in the induction of protection.

In the previous chapter, it was demonstrated that irradiated larvae persisted for a longer period in the skin and also released more antigens compared to normal larvae. Knowing that the skin represents a favourable environment for the processing and presentation of antigens, this prolonged period of contact between the host and the attenuated parasite may therefore be crucial for the development of immune protection and may be at least partly responsible for the success of the irradiated vaccine. The nature of the immune response induced in the skin by the attenuated vaccine will therefore be studied in detail in this Chapter.

In addition, the immune response occurring in the lungs of vaccinated animals will also be analysed. Indeed, *N. americanus* larvae undergo important morphological changes in the lungs, essential for further development and subsequent establishment in the intestine (section 1.2.2) (Behnke *et al.*, 1986). This development and passage through the lungs has previously been associated with an important increase in cell numbers, such as neutrophils, eosinophils and lymphocytes, the evidence of a lung immune response (Timothy, 1994). In addition, it has been reported that during a secondary *N. americanus* infection in mice more larvae are found entrapped in the lungs than in the primary infection, confirming the fact that the lungs play a significant role in immunity in the mouse model, important in the development of a vaccine (Wells and Behnke, 1988; Wilkinson *et al.*, 1990). Since *N. americanus* larvae reach the intestine in mice but do not develop into the adult stage, the immune response induced in the intestine will not be studied in detail.

4.1.1. Aims of Chapter 4

This Chapter describes the establishment of a successful irradiated larval vaccine model for *Necator americanus*. The schedule initially used was the same as that established by Dr A. Brown (2000), that is BALB/c mice vaccinated with 300 irradiated (40 krad) larvae three times at two week intervals and challenged with 300 larvae, two weeks after the last immunisation. However, it has been shown in other parasites such as schistosomes that three immunisations are not necessary to stimulate an efficient immune response (Dean, 1983). This has obvious advantages with respect to time gains and use of less larval material. The number of vaccinations required to obtain a good immune protection against challenge larvae in mouse was thus checked. To assess the level of protection induced by the vaccine, vaccinated animals were killed on day 3 and 8 post-infection, and the number of worms recovered in the lungs (day 3) and in the small intestine (day 8) counted. These time points were determined by Brown (2000), to be optimum for obtaining the highest worm numbers in the non-vaccinated animals.

Having established a successful vaccination model, the immunological mechanisms associated with this vaccine were then analysed in detail in the infected sites. Sections from skin and lungs of vaccinated animals were specifically stained to study

morphological and mast cell changes. To investigate which T helper subset was involved in protection, the γ-IFN (Th1) and IL4 (Th2) cytokine profiles were assessed in the spleen, axillary and mesenteric lymph nodes using ELISA. Since, the level of cytokines expressed in the skin were too small to be detectable by ELISA, γ-IFN and IL4 mRNAs expressions were monitored in the skin using RT-PCR.

4.2. RESULTS

Statistical analyses were carried out for each experiment (section 2.3.3.4.) and unless otherwise stated (* = $p < 0.05$), the results were not significant. All the results are expressed ± standard deviation.

4.2.1. Induction of immunity by gamma-irradiated larvae

4.2.1.1. Protection induced by one, two and three immunisations with gamma-irradiated larvae

BALB/c mice were vaccinated once (I), twice (II) or three (III) times with irradiated larvae and then infected as described in figure 4.1. and detailed in section 2.3.1. Each vaccination experiment also includes a group of mice infected with 300 larvae (C) and a group of non-infected/non-vaccinated naive mice. Following vaccination and infection, the numbers of worms recovered from the lungs (3 days post challenge) and from the small intestine (9 days post challenge) are presented in table 4.1. Three mice (one mouse from groups C, III and II) were found to be blind and dead one day after challenge. No apparent correlation was noticed between the dose of vaccination and blindness or death.

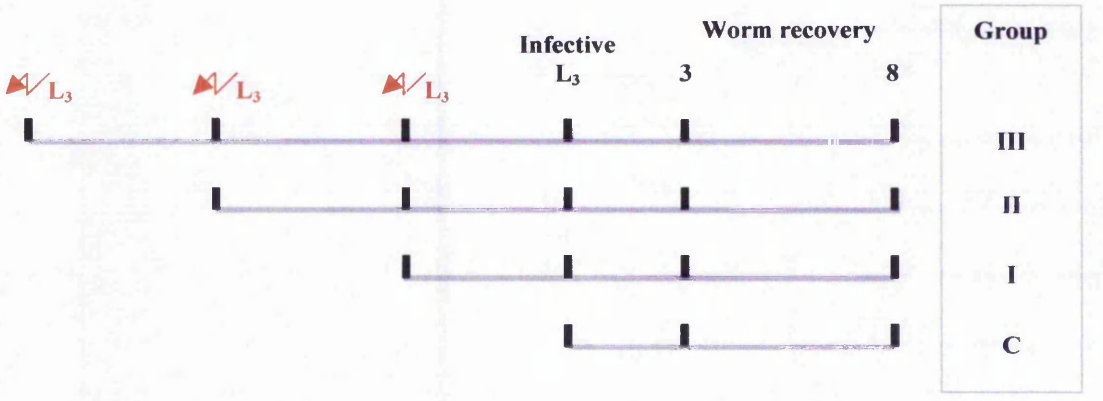


Figure 4.1. Schematic description of vaccination schedule. *Male BALB/c mice were vaccinated three (III), two (II) or one (I) times with 300 irradiated larvae at three week intervals, and infected two weeks after the last immunisation with 300 normal larvae. The group C represents non-vaccinated but infected with 300 larvae animals. The number of worms was recovered 3 days post-infection in the lungs and 8 days post-infection in the small intestine of each group. The mice were vaccinated with gamma-irradiated larvae as described in the materials and method section 2.2.4.*

Following infection, 55.7 ± 16.9 larvae (out of 300 larvae) were found in the lungs of non-vaccinated animals (group C). A reduction in larval number was only observed in the lungs of animals vaccinated twice with irradiated larvae (group II), resulting in 47 % protection. The reduction observed in larval numbers (55 ± 22.5 larvae) of animals vaccinated three times (group III) was not significant compared to the non-vaccinated group (group C). Unexpectedly, the number of worms recovered from the lungs of animals vaccinated once with irradiated larvae (group I) was twice the number of larvae recovered from the lungs of non-vaccinated animals (group C). In each group, the standard deviation was relatively large.

Table 4.1. Number of worms recovered from the lungs (3 days post-infection) and small intestine (8 days post-infection) following vaccination with 300 irradiated larvae. *C = mice non-vaccinated but challenged with 300 normal larvae, I = mice vaccinated once with 300 irradiated larvae and challenged, II = mice vaccinated twice with irradiated larvae and challenged, III = mice vaccinated three times with irradiated larvae and challenged. The results are expressed as the mean number of worms recovered from the lungs and small intestine ± standard deviation (SD) (six mice per group except where indicated # where n = 5). % Protection = % reduction of worm recovery in the vaccinated group compared with the mean of the non-vaccinated control group (C).*

Group	Lungs (Day 3)		Intestine (Day 8)	
	Worms recovered ± SD (n = 6)	Protection (%)	Worms recovered ± SD (n = 6)	Protection (%)
C	55.7 ± 16.9		6.8 ± 7.2 #	
I	111.4 ± 23.5	-	0	100
II	29.5 ± 9.5	47 *	0 #	100
III	55.0 ± 22.5	1	2.6 ± 4.7#	61

Eight days post-infection, a small number of larvae (6.8 ± 7.2 larvae) was recovered in the intestines of non-vaccinated animals. Since only 2 % of the initial dose (300 larvae) were recovered from the intestine of group C, the intestines were therefore left in the incubator overnight to ensure that all the worms were removed; no more worms were recovered. Despite the low worm recovery, 61 % protection was obtained in animals vaccinated three times with irradiated larvae (group III) and

complete protection was achieved in mice vaccinated once (group I) or twice (group II) with irradiated larvae.

4.2.1.2. Lung clearance before infection

In order to check if any irradiated larvae from the vaccination remained in the lungs or intestine prior to challenge, mice were vaccinated once (group I) or twice (group II) with 300 irradiated larvae as described section 2.3.1. and worms were recovered in the lungs and intestines of vaccinated animals two weeks after the last immunisation. The results are shown table 4.2. No parasites were recovered from the lungs and small intestine of animals vaccinated once or twice with irradiated larvae, on the day of challenge.

Table 4.2. Number of worms recovered from the lungs and small intestine 14 days after the last immunisation with 300 irradiated larvae. *I = mice vaccinated once with 300 irradiated larvae, II = mice vaccinated twice with irradiated larvae. The results are expressed as the mean number of worms recovered from the lungs and small intestine (n = 2).*

Group	Worms recovered 14 days after last immunisation (n = 2)	
	Lungs	Intestine
I	0	0
II	0	0

4.2.1.3. Protection induced by one or two immunisations with irradiated larvae

In order to validate the results obtained with one/two immunisations, the experiment was repeated. Table 4.3. shows the numbers of larvae recovered from the lungs and the small intestine of animals vaccinated once (group I) or twice (group II), 3 and 8 days post-infection, respectively. The number of worms recovered from the non-vaccinated group (group C) was small in both the lung (18.2 ± 11.6) and small intestine (3.7 ± 3.9) counts. However, larval numbers in the lungs of both vaccinated groups were greater than in the control group (C), reaching 26.7 ± 16.2 and 31 ± 21.5 respectively. Despite this, no worms were recovered in the intestines of either

vaccinated group, suggesting 100 % protection; this was checked in the next experiment.

Table 4.3. Number of worms recovered from the lungs (3 days post-infection) and small intestine (8 days post-infection) following one or two immunisations with 300 irradiated larvae. *C = mice non-vaccinated but challenged with 300* N. americanus *larvae, I = mice vaccinated once with 300 irradiated larvae and challenged, II = mice vaccinated twice with irradiated larvae and challenged. The results are expressed as the mean number of worms recovered from the lungs and small intestine ± SD (n = 4). % Protection = % reduction compared with the mean of non-vaccinated control group (C).*

Group	Lungs (Day 3)		Intestine (Day 8)	
	Worm recovered ± SD (n = 6)	**Protection** (%)	Worm recovered ± SD (n = 6)	**Protection** (%)
C	18.2 ± 11.6		3.7 ± 3.9	
I	26.7 ± 16.2	-	0	**+ 100**
II	31.0 ± 21.5	-	0	**+ 100**

4.2.1.4. Protection induced by two immunisations with irradiated larvae

The numbers of larvae recovered from the lungs (3 days post-infection) and small intestine (8 days post-infection) in animals vaccinated twice with gamma-irradiated larvae (group II) are shown in table 4.4. In this experiment, it is important to mention that the first immunisation was performed on the back on the mouse whilst the second vaccination was undertaken on the belly of the mouse. Worm recovery in both the lungs and small intestine of non-vaccinated animals (group C) was better than the previous experiment, with 79.5 ± 57.6 larvae being recovered from the lungs and 20.5 ± 17.5 recovered from the small intestine. Furthermore, based on the lung and intestine counts, respectively, 57 % and 98 % protection was significantly achieved in the vaccinated group ($p < 0.05$). As this experiment gave almost complete protection against further infection, various samples taken from the animals were analysed further.

Table 4.4. Number of worms recovered from the lungs (3 days post-infection) and small intestine (8 days post-infection) following two vaccinations with 300 irradiated larvae. *C = non-vaccinated mice but challenged with 300* N. americanus *larvae, II = mice vaccinated twice with irradiated larvae and challenged with 300 larvae. The results are expressed as the mean number of worms recovered from the lungs and small intestine ± SD (n = 6). % Protection = % reduction compared with the mean of the non-vaccinated control group; * data significant, p < 0/05.*

Group	Lungs (Day 3)		Intestine (Day 8)	
	Worms recovered ± SD (n = 6)	**Protection** (%)	Worms recovered ± SD (n = 6)	**Protection** (%)
C	79.5 ± 57.6		20.5 ±17.5	
II	34.2 ± 16.8	**+ 57 ***	0.3 ± 0.5	**+ 98 ***

4.2.2. Nature of the immune responses induced in the skin of animals vaccinated twice with irradiated larvae

4.2.2.1. Morphological changes observed in the skin of vaccinated/infected animals

The cutaneous response following infection with *N. americanus* was studied in vaccinated and non-vaccinated BALB/c mice. Age-matched non-vaccinated/infected, vaccinated/infected and naïve animals were killed 3 days post-infection. The skin sites of vaccination and/or infection from 4 mice of each group (or dorsal skin patches in the case of naïve controls) were excised and processed as described in section 2.6. All animals in each group showed the same morphological changes; a representative of each is presented in figure 4.2. No larvae were identified in either group.

Dorsal skin from naive control BALB/c mice presented a typical morphology (see figure 4.2.a.) with three principal layers; the external epidermis (E; purple thin superficial layer), the dermis (De; light pink loose layer) and the hypodermis (H). The epidermis was overlaid by a layer of devitalized cells forming the stratum corneum (SC). The external surface of the epidermis was fairly smooth and flat. Within the dermis hair follicles (HF) and sebaceaous glands (SG) were evident.

The skin from the non-vaccinated/infected animals (figure 4.2.b.) showed a slightly thicker epidermis than in naïve, control samples. There were no significant changes in the thickness of the dermis, in non-vaccinated/infected animals compared to the naïve animals. The two layers of dermis were identified: the papillary dermis (PD) just beneath the epidermis and the reticular dermis (RD), the latter layer consisting of less condensed fibres.

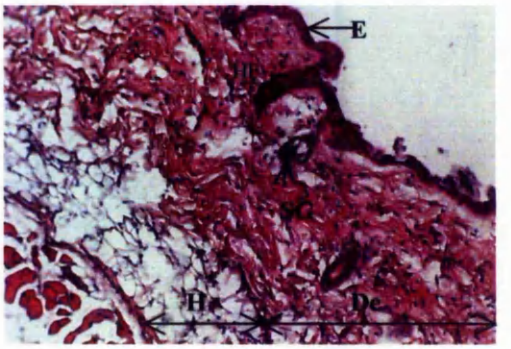
In the vaccinated/infected group, important morphological changes are evident in the epidermal and dermal layer (figure 4.2.c.). More nuclei were observed in the whole section, explaining the darker colour observed. The epidermis thickness was 2 fold greater than in the non-vaccinated mouse skin, with both the stratum basal layer (SB), localised above the dermis, as well as the stratum corneum (SC), being considerably thicker. Furthermore, the epidermal ridge system was much more prominent than in the non-vaccinated skin. In the dermis of the vaccinated animals, more sebaceous glands and hair follicles were observed. Numerous inflammatory foci (IF) were also identified in the vaccinated animals, particularly in the inner-most layer of the dermis. In addition, numerous lymphocytes and neutrophils were observed in the reticular dermis layer.

4.2.2.2. Mast cell changes in the skin of vaccinated animals

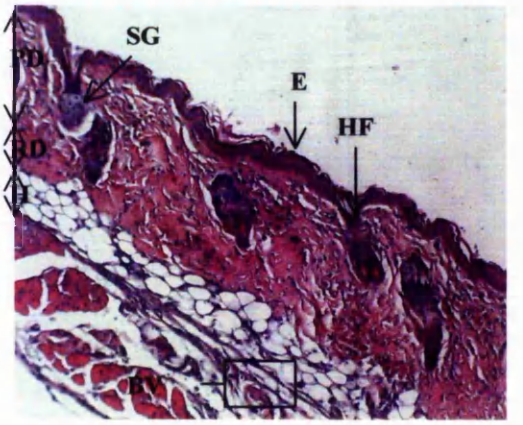
In order to identify mast cells in naïve, non-vaccinated/infected and vaccinated/infected animals 3 days post-infection, transverse sections of mouse skin were stained with thionin blue as described in section 2.6.2.2.2. Skin sections from each group (consisting of 4 individuals) were found to show the same changes with respect to mast cells. A representative of each is presented in figure 4.3.

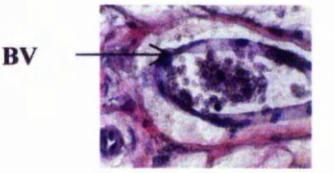
Following infection of non-vaccinated animals, a small increase in mast cell number was observed (M; figure 4.3.b.) compared to that of the naive tissue (figure 4.3.a.), where mast cells were sparse. Mast cell granules were also observed in naïve and non-vaccinated tissue. In the vaccinated tissue, an increased number of mast cells (figure 4.3.c.) was observed compared to the non-vaccinated and naïve animals. The mast cells in the vaccinated animals accumulated mainly in the innermost part of the dermis and were degranulated.

a- Naïve tissue

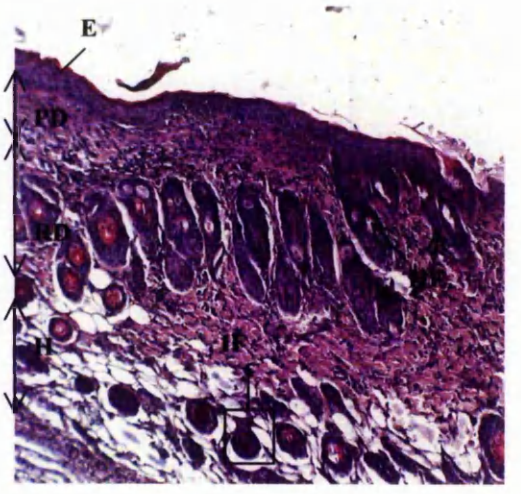


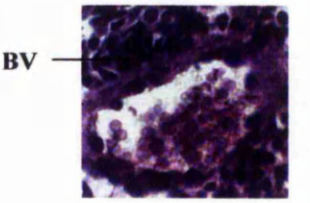
b- Non –vaccinated/infected tissue





c- Vaccinated/infected tissue





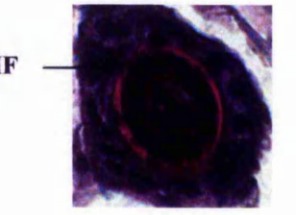
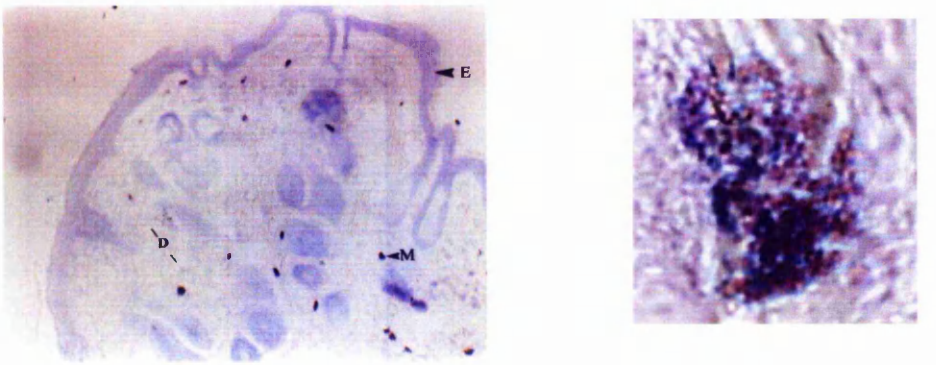
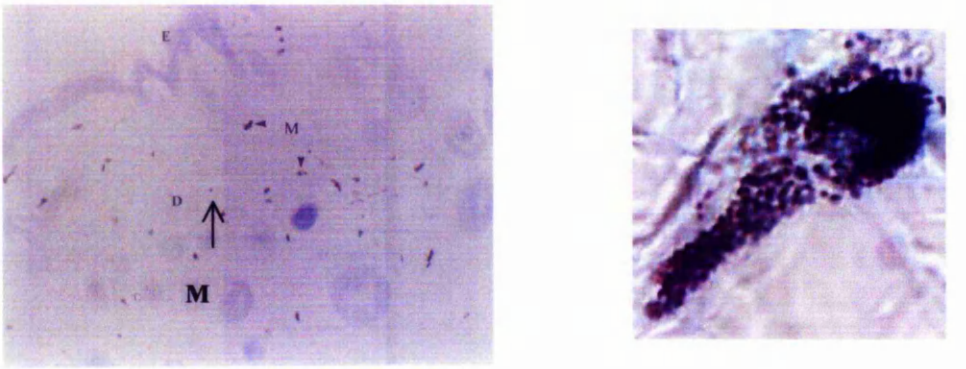


Figure 4.2. Morphological changes occurring in vaccinated and non-vaccinated skin following infection. *10 μm transverse sections of mouse skin was stained with haematoxylin and eosin after formalin fixation (x200 and x400).* ***a****: skin from a naïve animal,* ***b****: skin from a non-vaccinated/infected animal and* ***c****: skin from a vaccinated/infected animal. E = epidermis, SC = stratum corneum, SB = stratum basale, D = dermis, PD = papillary dermis, RD = reticular dermis, HF = hair follicle, H = hypodermis, SG = sebaceous gland, IF = inflammatory foci, BV = blood vessel.*

a- Naïve tissue



b- Non-vaccinated/infected tissue



c- Vaccinated/infected tissue

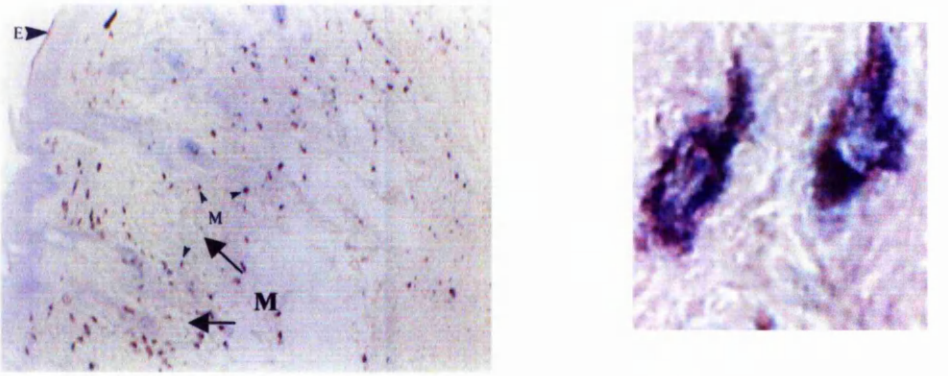


Figure 4.3. Presence of mast cells in vaccinated and non-vaccinated skin 3 days post-infection. *10 μm transverse sections of mouse skin were stained with thionin after formalin fixation (x200 and x400).* ***a****:skin from a naïve animals,* ***b****: skin from a non-vaccinated/infected animal and c: skin from a vaccinated/infected animal. E = epidermis, D = dermis, M = mast cell*

Mast cell numbers were subsequently quantified on days 1, 3 and 8 post–infection, using the thionin blue stained samples. The results, represented in figure 4.4., showed that the number of mast cells in the three different groups remained generally constant from day 1 to 8 following infection. No significant changes in mast cell numbers were observed in the non-vaccinated/infected group compared to the naïve animals. The main difference in mast cell numbers was observed in the vaccinated/infected group, where the number of mast cells was always greater than in the non-vaccinated/infected animals. Indeed, the number of mast cells in the vaccinated/infected animals was 3 and 5 times that of the non-vaccinated/infected tissue on day 1 and 3 post-infection respectively ($p < 0.05$).

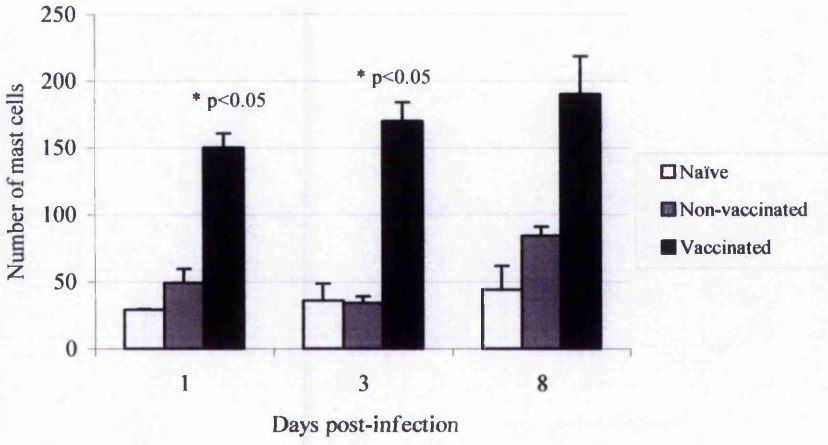


Figure 4.4. Mast cell involvement in the skin of vaccinated and non-vaccinated animals following infection. *Skin sections of naïve, non-vaccinated and twice vaccinated animals were stained with thionin blue after formalin fixation. The mast cell numbers (day 1, 3 and 8 post-infection) were then counted using a light microscope. Each point represents the mean of five different fields.* * *= $p < 0.05$*

4.2.2.3. Cytokine mRNA levels in the skin using RT-PCR

4.2.2.3.1. Preliminary work using control primers

In order to check that the RT-PCR system was working, control primers in the kit were used to amplify the positive control RNA supplied in the kit (section 2.7.2.). Furthermore, to check if the RNA prepared from tissue was intact, commercial β-

actin primers, house keeping gene, were used with RNA samples from naïve skin (section 2.7.1.).

Before amplification, the yield of extracted total RNA was estimated (section 2.7.1.3.). Approximately 0.6 μg RNA was extracted from 0.3 g of skin in a total volume of 100 μl DEPC water. Its 260/280 ratio was greater than 1.8, indicating acceptable RNA purity. The results of the RT-PCR are presented in figure 4.5.

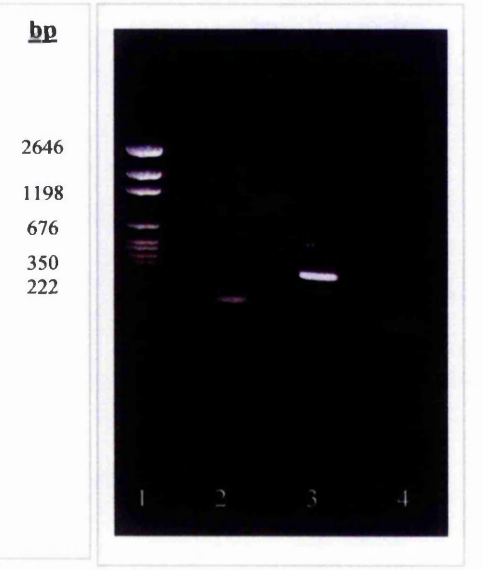


Figure 4.5. Amplification of positive control RNA and skin RNA templates using RT-PCR. *The positive control RNA was amplified using the control oligonucleotide primers supplied in the kit and the skin RNA template was amplified using commercial β-actin primers. Products were fractionated on a 2 % (w/v) agarose gel and visualised under ultra-violet light. Lane 1: pGEM® DNA molecular weight markers, Lane 2: β-actin primers on naïve skin RNA template, Lane 3: positive control, Lane 4: negative control with no RNA.*

Lane 3 revealed one major amplification product of about 350 base pair (bp) corresponding to the amplification product (predicted size 323 bp) of the positive control RNA using upstream and downstream control primers. A faint amplification product of about 220 bp was also observed, this product arises from the amplification of a sequence in the carrier RNA added to the control RNA from the kit. Lane 2 showed a faint amplification product of about 170 bp, corresponding to the β-actin mRNA. Lane 4 represents the negative control of the RT-PCR reaction and showed no amplification products as expected.

4.2.2.3.2. Preliminary RT-PCR on RNA extracted from spleen

In order to optimise the results, preliminary amplifications were undertaken on spleen samples which contain higher levels of IL4 - and γ-IFN - mRNAs than the skin. The spleen from non-vaccinated/infected and vaccinated/infected animals was treated in the same manner as the skin (section 2.7.1.). The yield and purity of extracted RNA were then checked (table 4.5.).

Table 4.5. Yield and purity of total RNA extracted from mouse spleen. *Total RNA was extracted from snap frozen spleen using RNA STAT-60™ and resuspended in 100 µl of diethylpyrocarbonate (DEPC) treated water. The absorbance of the sample (diluted 1/10) was measured at 260 and 280 nm.*

Skin Samples	Weight (g)	Absorbance (260 nm)	Absorbance (280 nm)	Ratio 260/280	[RNA] µg/ml
Non vaccinated/infected	0.166	0.894	0.466	1.92	7.15
Vaccinated/infected	0.1616	0.674	0.349	1.93	5.4

As the 260/280 ratio was greater than 1.8, the spleen RNA was subjected to RT-PCR using β-actin (internal positive control), IL4 and IFNγ primers following the protocol described in section 2.7.2.2. The results are presented in figure 4.6.

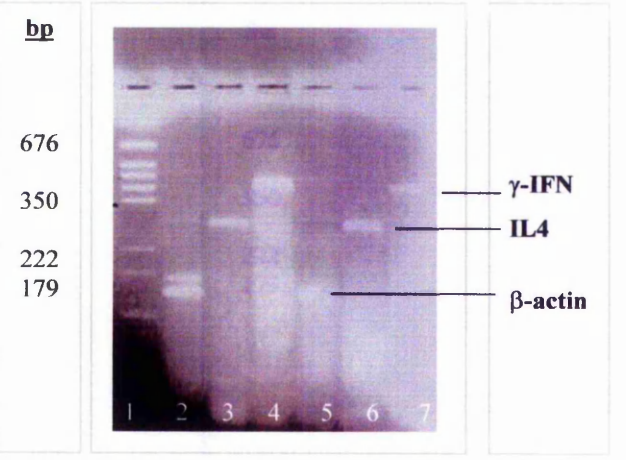


Figure 4.6. Amplification of non-vaccinated/infected and vaccinated/infected spleen RNA templates. *The spleen RNA templates of non-vaccinated/infected and vaccinated/infected animals were amplified with β-actin, Il4 or γ-IFN primers. Products were fractionated on a 2 % (w/v) agarose gel and visualised under ultra-violet light. Lane 1: pGEM® DNA molecular weight markers, Lanes 2, 3, and 4 represent products of RT-PCR conducted on non-vaccinated/infected spleen RNA using β-actin, IL4 and γ-IFN primers respectively. Lane 5, 6 and 7 represent products of RT-PCR conducted on vaccinated/infected spleen RNA using β-actin, IL4 primer, IFNγ primers respectively.*

Figure 4.6. shows the level of IL4 and γ-IFN in the spleen. The band appearing in lanes 2 and 5 corresponds to a 170 bp fragment. This amplification product, representing the ß-actin mRNA, was higher in the non-vaccinated/infected group (lane 2) compared to the vaccinated/infected group (lane 5). This difference had to be considered when comparing the level of cytokine mRNAs in the two different groups. The 279 bp product observed in lanes 3 and 6 correspond to the IL4 mRNA (300 bp). Considering ß-actin mRNA expression in both tissues, the intensity of the IL4 product was higher in the vaccinated group (lane 6) than in the non-vaccinated group (lane 3). In lanes 4 and 7, a product of 400 bp was observed, corresponding to the γ-IFN mRNA. No major differences in γ-IFN product intensity between the non-vaccinated/infected (lane 4) and vaccinated/infected animals (lane 7) was observed. In the non-vaccinated/infected animals, the intensity of the γ-IFN product (lane 4) was higher than the intensity of the IL4 product (lane 3); the opposite was observed in the vaccinated animals.

4.2.2.3.3. RT-PCR on RNA extracted from non-vaccinated and vaccinated mouse skin

RNA was extracted from snap frozen samples of skin from non-vaccinated/infected and vaccinated/infected groups as described in section 2.7.2.1. A representative example of the yield and purity of extracted RNA are shown in table 4.6.

Table 4.6. Yield and purity of total RNA extracted from mouse skin. *Total RNA was extracted from snap frozen skin using RNA STAT-60™ and resuspended in 100 µl of DEPC water. The absorbance of the sample (diluted 1/10) was measured at 260 and 280 nm.*

Skin Samples	Weight (g)	Absorbance (260 nm)	Absorbance (280 nm)	Ratio 260/280	[RNA] µg/ml
Non vaccinated/infected	0.165	0.453	0.229	1.977	1.8
Vaccinated/infected	0.307	1.078	0.562	1.918	4.3

The 260/280 ratio being greater than or equal to 1.8 indicated that the final preparations of total RNA were free of DNA and proteins (table 4.6). The RNA was

then subjected to RT-PCR using β-actin (internal positive control), IL4 and IFNγ primers following the protocol described in section 2.7.2.2. RT-PCR reactions were undertaken for a set of animals (non-vaccinated/infected and vaccinated/infected) using β-actin, IL4 and γ-IFN primers. However, few sets gave fully satisfactory results. The result of the best set is presented in figure 4.7.

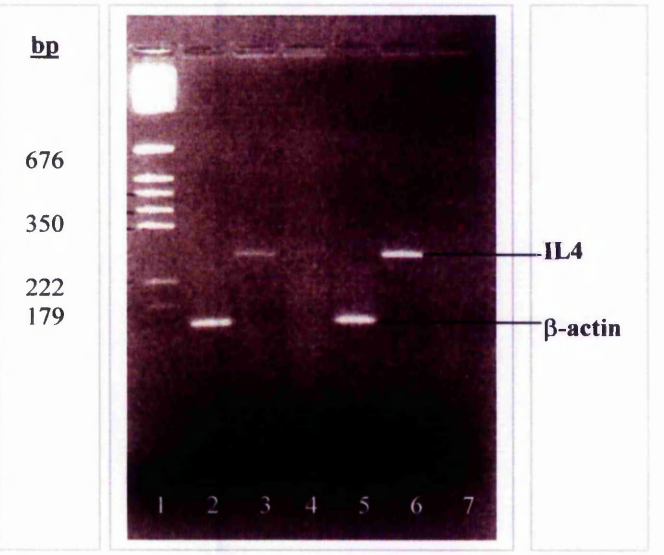


Figure 4.7. Amplification of non-vaccinated/infected and vaccinated/infected skin RNA templates. *The skin RNA templates of non-vaccinated/infected and vaccinated/infected animals were amplified with β-actin, Il4 or γ-IFN primers. Products were fractionated on a 2 % (w/v) agarose gel and visualised under ultra-violet light. Lane 1: pGEM® DNA molecular weight markers, Lanes 2, 3, and 4 represent products of RT-PCR conducted on non-vaccinated skin RNA using β-actin, IL4 and γ-IFN primers respectively. Lane 5, 6 and 7 represent products of RT-PCR conducted on vaccinated skin RNA using β-actin, IL4 primer, IFNγ primers respectively.*

The band appearing in lanes 2 and 5 corresponds to a 170 bp fragment. This amplification product represents the ß-actin mRNA product. The intensity of this fragment in the non-vaccinated and vaccinated groups was similar, suggesting that a direct comparison of cytokine mRNA levels between the two different skin samples is possible. The 279 bp band observed in lanes 3 and 6 correspond to the IL4 gene (300 bp). The intensity of this band was higher in the vaccinated/infected group than in the non-vaccinated/infected group. The γ-IFN product could not be detected in either skin samples (lanes 4 and 7).

4.2.3. Nature of the immune response induced by the irradiated larval vaccine in the lungs

4.2.3.1. Effect of vaccination on larval migration through the lungs

The effect of vaccination on larval migration was studied by monitoring the number of worms recovered from the lungs of non-vaccinated/infected and vaccinated/infected animals on days 1, 2, 3, 4, 5, 7, 8, 9 and 10 following infection with normal Necator larvae. The results are presented in figure 4.8.

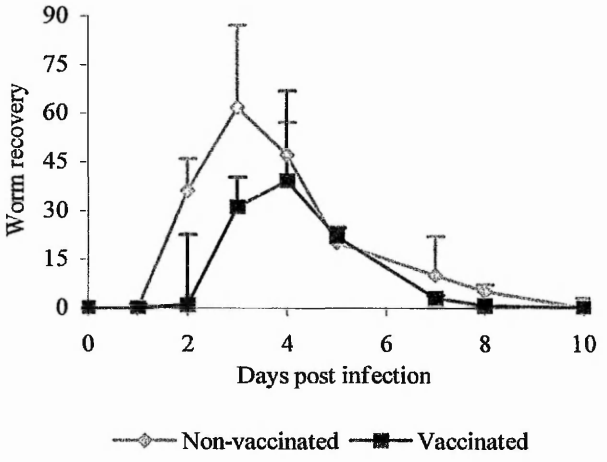


Figure 4.8. Number of larvae recovered from the lungs of non-vaccinated/infected and vaccinated/infected BALB/c mice. *The number of worms recovered from the lungs of non-vaccinated and twice vaccinated animals determined on days 1, 2, 3, 4, 5, 8, 9 and 10 following infection. Results are expressed as the mean + SD of the number of worms recovered, (n = 6 for non-vaccinated/infected animals and n = 4 for vaccinated/infected animals).*

Whilst lung worm burdens peaked 3 days post-infection in the non-vaccinated animals, worm recovery peaked on day 4 post-infection in the lungs of vaccinated animals. Furthermore, the maximum number of worms recovered from the vaccinated animals (39 worms) was reduced compared to the number of worms recovered from the lungs of non-vaccinated animals (62 worms).

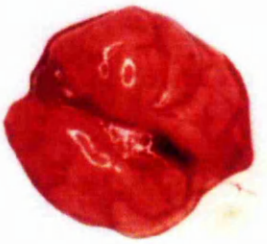
4.2.3.2. Morphological changes in the lungs

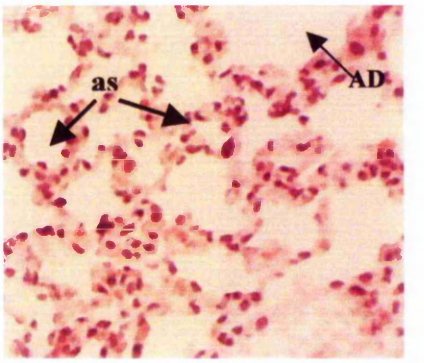
Figure 4.9. (a-c) shows images of intact and sections of lungs from naive, non-vaccinated/infected and vaccinated/infected animals, 3 days post-infection. Intact lungs removed from a healthy mouse are uniformally pink in color. The naïve lungs (4.9.a) showed a typical morphology with the bronchioles dividing into several alveolar ducts (AD) ending in alveolar sac (AS). Lungs from non-vaccinated/infected animals (4.9.b) are dark red, suggesting an accumulation of red blood cells (Acrb) in the entire tissue and therefore haemorrhage associated with the damaging effects of infective larvae migrating through the lungs. These results were confirmed by the transverse section showing an accumulation of red blood cells in the alveolar spaces. Lungs from vaccinated/infected animals (4.9.c) showed little haemorrhage compared to the non-vaccinated tissue. In the transverse section of the lungs of vaccinated/infected animals, red blood cells were much less abundant than in the non-vaccinated/infected tissue. Pulmonary foci (PC) were also observed in the lungs of vaccinated/infected animals, however no worms could be detected.

4.2.3.3. Mast cell changes in the lungs

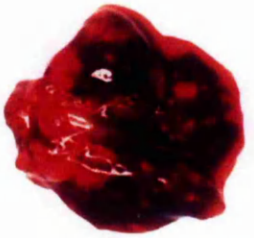
Figure 4.10. (a-c) shows transverse sections of mouse lungs, 3 days post-infection, stained with thionin blue, the specific mast cell stain. In the naïve lungs (4.10.a.), no mast cells were found in the tissue, and only a few were observed in the non-vaccinated/infected animals. In the section presented here, two mast cells were identified in the non-vaccinated/infected lungs section (4.10.b.). In the lungs of vaccinated/infected animals, although the number of mast cells was still low, a greater number of mast cells (4.10.c.) was observed compared to the non-vaccinated/infected group.

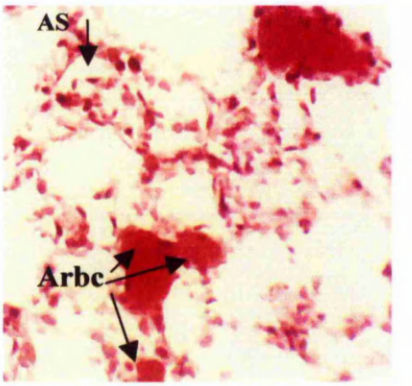
a-Naïve tissue





b- Non-vaccinated/infected tissue





c- Vaccinated/infected tissue

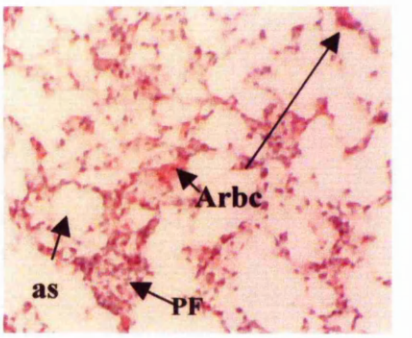
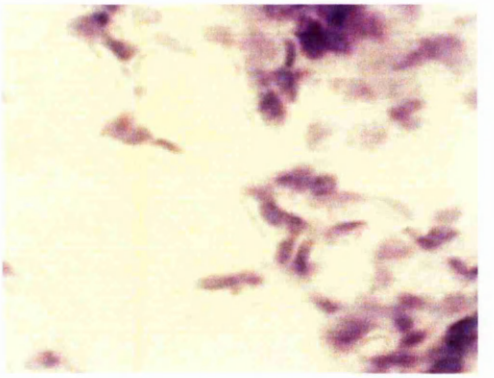
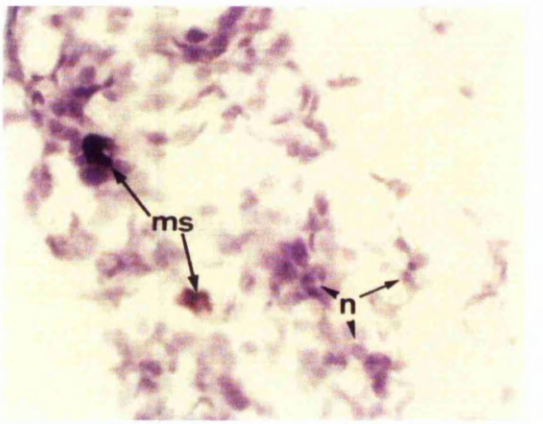


Figure 4.9. Morphological changes in the whole and transverse sections of lungs following vaccination. ***a****; lungs from naïve animals,* ***b****: lungs from non-vaccinated/infected animals and* ***c****: lungs from vaccinated/infected animals. Lung sections were stained with haematoxylin and eosin (sections magnification x 400). AD = alveolar duct, AS = alveolar sac, Arbc = accumulation of red blood cells, PF = pulmonary foci.*

a- Naïve lungs



b- Non-vaccinated/infected lungs



c- Vaccinated/infected lungs

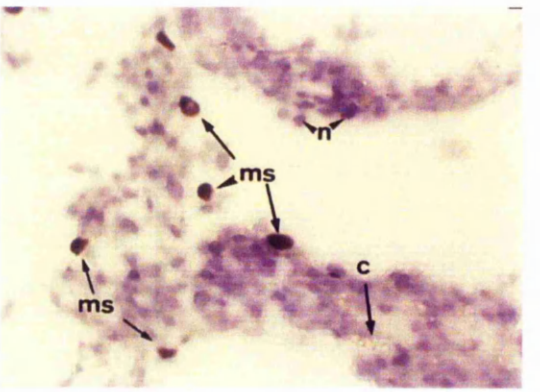


Figure 4.10. Presence of mast cells in naïve, non-vaccinated/infected and vaccinated/infected lungs. *10 µm transverse sections of mouse lungs were stained with thionin (x 400).* ***a****: lungs from naïve animals,* ***b****: lungs from non-vaccinated/infected animals and* ***c****: lungs from vaccinated/infected animals. ms = mast cells, n = nuclei of lung cells, c = connective tissue.*

4.2.4. Lymphocyte proliferation and cytokine responses in twice vaccinated animals

Note: In all these assays, individual animals were processed, it was not feasible to analyse 6 animals, so 4 animals were randomly chosen.

4.2.4.1. Effect of vaccination

4.2.4.1.1. Cell number

The cellular events occurring in the spleen, axillary and mesenteric lymph nodes of vaccinated animals were assessed two weeks after the last immunisation; i.e. on the day of infection, and compared to those of non-vaccinated animals. The results are presented in figure 4.11. The number of cells present in the spleen and the axillary lymph nodes of vaccinated animals was higher than that present in the non-vaccinated animals; particularly in the axillary lymph node where the number of cells was 1.8 fold higher than in the non-vaccinated animals ($p < 0.05$). Similarly, before infection, the spleen of vaccinated animals had 1.5 times more splenocytes than the non-vaccinated animals. The number of cells present in the mesenteric lymph nodes, however, was not significantly different between vaccinated and non-vaccinated animals.

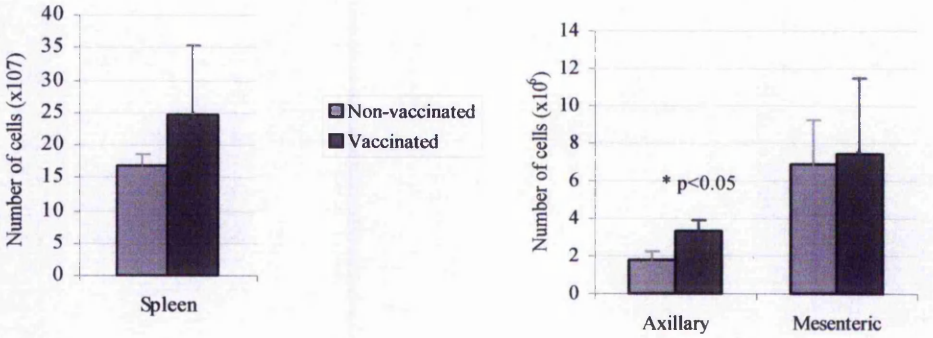


Figure 4.11. Changes in total cell number in the lymph nodes and spleen of mice vaccinated twice with irradiated larvae prior to infection compared to non-vaccinated mice. *n = 4, * = p < 0.05.*

4.2.4.1.2. Cytokine responses in the vaccinated animals before infection

γ-IFN and IL4 levels in non-vaccinated and vaccinated animals were analysed in the spleen, axillary and mesenteric lymph nodes, before infection (section 2.4.2.). The results are represented in table 4.7.

Table 4.7. Levels of IL4 and γ-IFN secreted by *in-vitro* Con-A stimulated lymphocytes from non-vaccinated and twice vaccinated BALB/c mice. *The levels of cytokines were determined by ELISA in the spleen, axillary and mesenteric lymph nodes (LN) in each group, two weeks after the last immunisation. Values represent the mean from 4 of individual mice.*

	Non-vaccinated animals			Vaccinated animals		
	IL4 (pg/ml)	γ-IFN (pg/ml)	IL4/γ-IFN	IL4 (pg/ml)	γ-IFN (pg/ml)	IL4/γ-IFN
Axillary LN	1 ± 2	63± 31	**0.016**	6 ± 3	14 ± 20	**0.428**
Mesenteric LN	1 ± 2	82± 67	**0.017**	8 ± 10	105 ± 32	**0.076**
Spleen	444.± 453	663 ± 132	**0.669**	577 ± 421	410.± 381	**1.407**

The ratio IL4/γ-IFN was consistently higher in the three lymphoid organs of animals vaccinated twice with irradiated larvae compared to the non-vaccinated animals. The change in ratio was particularly accentuated in the axillary lymph nodes, where not only the IL4 level was 6 times higher in the vaccinated group compared to the naive, but the level of γ-IFN was reduced in the non-vaccinated group from 63 ± 31 μg/ml to 14 ± 20 μg/ml. A similar trend was observed in the spleen. In the mesenteric lymph nodes, IL4 and γ-IFN levels were higher in the vaccinated group.

4.2.4.2. Effect of infection

The immune response occurring following infection with 300 larvae was then assessed in both the twice vaccinated and the non-vaccinated animals. The immune response was also monitored in the lymphoid organs of 4 naïve animals, as a control. The effect of infection was be studied by comparing the naïve group with non-vaccinated/infected animals. The effect of vaccination following infection was studied by comparing non-vaccinated/infected and vaccinated/infected animals.

4.2.4.2.1. Cell number

The cellular events occurring in the spleen, axillary and mesenteric lymph nodes, in each group, were monitored on day 3, 8 and 15 post-infection. The results are shown in figure 4.12.

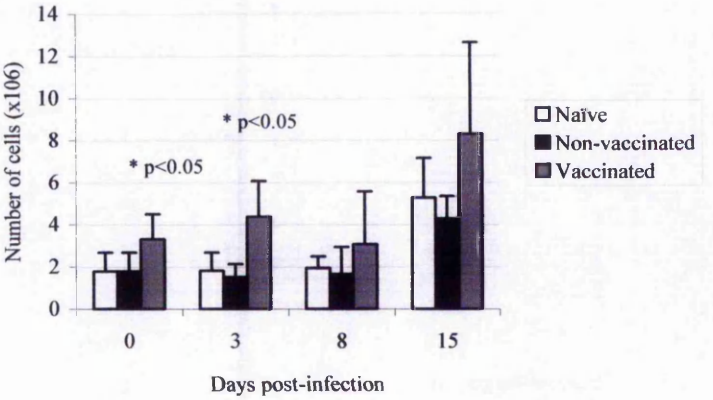
Following infection, no difference in cell number was generally observed between naïve and non-vaccinated/infected animals in the three lymphoid organs. The cell number profile of the non-vaccinated/infected animals essentially followed that of the naïve mice, except on day 3 in the spleen where the number of cells in the non-vaccinated/infected animals was significantly reduced compared to that of the naïve animals.

In the vaccinated/infected animals, the number of cells present in the axillary lymph node was always higher (50 – 100 %) than those of the non-vaccinated/infected animals, particularly on day 3 post-infection when vaccinated/infected animals had 2.9 times more cells than non-vaccinated/infected animals ($p < 0.05$). The number of cells increased further by day 15 post-infection, however, no significant increase was observed over time in the vaccinated/infected group.

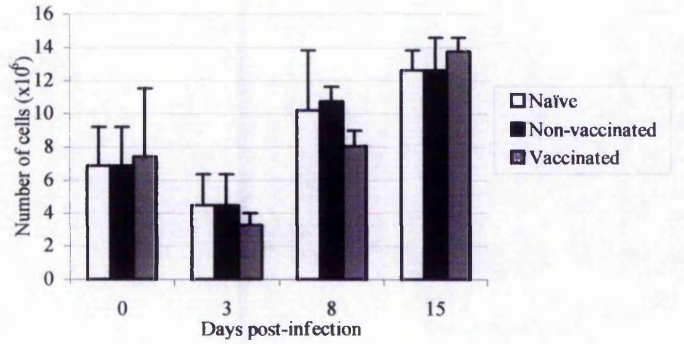
In the mesenteric lymph nodes, no significant differences were observed between the number of cells in the three different groups. The overall trend was similar, increasing from day 0 to day 15 post-infection.

In the spleen, vaccinated/infected animals presented higher cell numbers compared to non-vaccinated/infected animals, except on day 8 post-infection. The number of splenocytes was at least two fold higher in the vaccinated/infected group compared to the non-vaccinated/infected animals on day 3 and 15 post-infection ($p < 0.05$). No significant increase in cell number was observed over time following infection in the vaccinated group.

a- Axillary lymph nodes



b- Mesenteric lymph nodes



c- Spleen

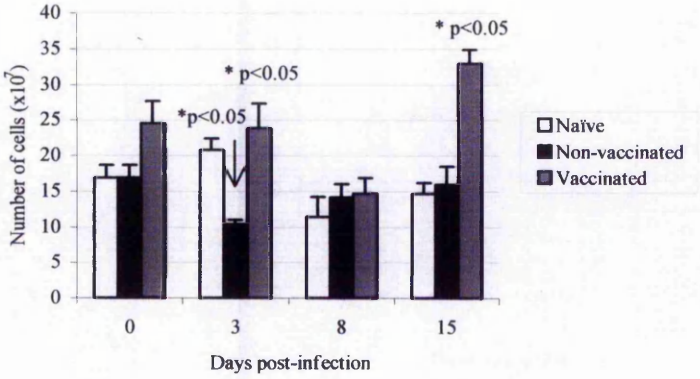


Figure 4.12. Changes in the total cell number from the lymph nodes and spleen of mice infected and vaccinated twice with irradiated/ larvae compared to non-vaccinated/infected animals. ***a****: Axillary lymph node;* ***b****: mesenteric lymph node and* ***c****: spleen, (n = 4).*

4.2.4.2.2. Cytokine responses induced in vaccinated animals following infection

The levels of γ-IFN and IL4 production in response to infection were analysed in the spleen, axillary and mesenteric lymph nodes of non-vaccinated/infected and vaccinated/infected animals, on day 3, 8 and 15 post-infection. The results are presented in table 4.8.

Prior to analysing the cytokines individually, it was important to note the variation in cytokine levels occurring in the naïve animals. In addition, following infection, the IL4/γ-IFN ratio was generally smaller in the three lymphoid tissues of the non-vaccinated/infected group compared to the naïve animals. However, this ratio was higher in the three lymphoid tissues of vaccinated/infected mice compared to the non-vaccinated/infected group. The ratio difference was the highest in the axillary lymph nodes.

In the axillary lymph nodes, levels of Il4 were very low in the non-vaccinated/infected animals compared to naïve and vaccinated/infected animals. Levels of IL4 in the vaccinated/infected groups reached a peak at 8 days post-infection (69 ± 21 pg/ml), this peak being 10 times higher than the non-vaccinated/infected group ($p < 0.05$). Similarly, post-infection, levels of γ-IFN were higher in the vaccinated/infected animals compared to the non-vaccinated/infected and naïve groups.

In the mesenteric lymph nodes, non-vaccinated/infected animals produced more γ-IFN and less IL4 than naïve animals. However, the vaccinated/infected animals generally secreted higher levels of both cytokines compared to the non-vaccinated/infected and naïve animals.

In the spleen, although the Il4/γ-IFN ratio was generally higher in the vaccinated/infected animals, there were only small differences in cytokine secretions between the non-vaccinated/infected and vaccinated/infected groups. Similarly, no significant differences were observed following infection in the naïve and non-vaccinated/infected animals.

Table 4.8. Levels of IL4 and γ-IFN secreted by *in vitro* Con-A stimulated lymphocytes from vaccinated and non-vaccinated BALB/c mice, 0, 3, 8 and 15 days post-infection. *5 x 10^5 lymphocytes were stimulated for 3 days with a final concentration of 5μg/ml of ConA in a final volume of 200 μg. The values were determined by ELISA using 100 μl of samples (n = 4). (a) Axillary lymph nodes, (b) Mesenteric lymph nodes and (c) spleen.*

a- Axillary lymph nodes

Days post-infection	Naive			Non-vaccinated/infected			Vaccinated/infected		
	Il4 (pg/ml)	γ-IFN (pg/ml)	IL4/γ-IFN	Il4 (pg/ml)	γ-IFN (pg/ml)	IL4/γ-IFN	Il4 (pg/ml)	γ-IFN (pg/ml)	IL4/γ-IFN
0	1± 2	63 ± 31	**0.016**				6 ± 3	14 ± 20	**0.428**
3 (lungs)	7 ± 8	0	-	2 ± 4	57.5 ± 3.5	**0.035**	14 ± 13	58 ± 33	**0.241**
8 (intestine)	9 ± 8	35 ± 24	**0.257**	1 ± 1	30 ± 15	**0.033**	69 ± 21	52 ± 36	**1.326**
15	9 ± 7	29 ± 29	**0.310**	1.7 ± 2.7	22 ± 32	**0.080**	30 ± 39	98 ± 57	**0.306**

b- Mesenteric lymph nodes

Days post-infection	Naive			Non-vaccinated/infected			Vaccinated/infected		
	Il4 (pg/ml)	γ-IFN (pg/ml)	IL4/γ-IFN	Il4 (pg/ml)	γ-IFN (pg/ml)	IL4/γ-IFN	Il4 (pg/ml)	γ-IFN(pg/ml)	IL4/γ-IFN
0	1± 2	82 ± 67	**0.018**				8 ± 10	105 ± 32	**0.076**
3 (lungs)	7 ± 10	32 ± 52	**0.218**	0	26 ± 19	-	37 ± 8	78 ± 19	**0.474**
8 (intestine)	15 ± 22	33 ± 29	**0.454**	0	103 ± 56	-	14 ± 10	159 ± 61	**0.088**
15	0 ± 0	39 ± 12	**0**	10 ± 8	100 ± 48	**0.100**	21 ± 10	447±139	**0.046**

c-Spleen

Days post-infection	Naive			Non-vaccinated/infected			Vaccinated/infected		
	Il4 (pg/ml)	γ-IFN (pg/ml)	IL4/γ-IFN	Il4 (pg/ml)	γ-IFN (pg/ml)	IL4/γ-IFN	Il4 (pg/ml)	γ-IFN (pg/ml)	IL4/γ-IFN
0	443±452	663±132	**0.668**				577±421	410±381	**1.407**
3 (lungs)	604±242	146±133	**4.136**	4 ± 5	208±310	**0.019**	53 ± 9	145±127	**0.365**
8 (intestine)	240±259	481±558	**0.499**	362±214	328±189	**1.104**	136±105	385±169	**0.353**
15	962±702	287±201	**3.351**	812±427	348±263	**2.333**	539±293	138±26	**3.906**

4.3. DISCUSSION

4.3.1. General problems with the vaccination model

Irradiated larvae have been shown to be particularly effective vaccines, inducing protective immunity against *N. americanus* in BALB/c mice. Whilst being well characterised, the BALB/c mouse model is not ideal for vaccination studies and has a number of problems. *N. americanus* is an obligate human parasite which in mouse will only proceed to the L4 larval stage, failing to reach maturity in the small intestine. However, it should be noted that infection of fully immunocompetent adult mice as opposed to the neonate hamsters provides a relatively good model to study the immune response to *Necator americanus*.

It was also noticed on occasions that the number of worms recovered from the infected control animals in the intestines and lungs was too low to assess the level of resistance induced by any vaccination. Timothy and Behnke (1993) demonstrated that 9 days following infection worm recovery in the small intestine peaked at only 5.5 % of the infective dose. As a result of this, an infection of 300 larvae will at best only result in approximately 16 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 this experiment. Variation in worm numbers can depend on numerous factors such as the vitality of the worms and the efficiency of the radiation. A variation in worm vitality has also been observed between parasite cultures as discussed in chapter 3, therefore infective larvae were used as fresh as possible for each vaccination and infection procedure.

4.3.2. Induction of immunity: number of vaccinations

This chapter confirmed that the irradiated larval vaccine induced a high level of protection against *N. americanus* infection in BALB/c mice and also demonstrated, for the first time, that three immunisations are not necessary to achieve high level of protection against challenge infection. In the first experiment, complete protection

was indeed achieved with both one and two immunisations based on worm counts in the intestine 8 days post-infection.

It was thought that one immunisation could be enough to induce a high level of protection against Necator infection. However in the first vaccination experiment (table 4.1.), although 100 % protection was achieved in the intestine of animals vaccinated once, the number of worms recovered from the lungs was greater than in the non-vaccinated animals. The concern was that the worms counted in these vaccinated animals were a mixture of irradiated larvae (residual from the vaccination step) and normal parasites (from the infection step). However, two weeks after the last immunisation (at the time of challenge), no irradiated parasites were recovered from the lungs or gut of animals vaccinated once (table 3.2.). Therefore, the number of worms recovered in the lungs of animals vaccinated once represented only non-irradiated larvae introduced as a challenge infection. Infective larvae could be trapped in the skin of the vaccinated animals, which would explain the difference in the lung recovery between the non-vaccinated and the once vaccinated mice. These results also suggested that infective larvae were trapped in the lungs of animals vaccinated once with irradiated larvae, preventing further migration.

Further work was undertaken to confirm the immune protection induced by one and two immunisations with irradiated larvae, but another difficulty was encountered in this vaccination experiment. The number of worms recovered from the challenge control in the intestines and lungs was too low to confirm the level of resistance induced by one or two immunisations.

In the last vaccination experiment, mice were only immunised twice with irradiated larvae and then challenged with infective larvae. Two immunisations with irradiated larvae lead to 98 % protection in the intestine and 57 % protection in the lungs following infection. The vaccination experiments therefore demonstrated that two immunisations with attenuated larvae were sufficient to induce protection. The level of protection obtained following two or three immunisations with irradiated larvae suggested that additional vaccinations did not boost the level of resistance to *N. americanus*. Subsequently, it will have been interesting to confirm the level of protection induced by one immunisation. However, due to the time limitation of this

project, this could not be checked. Indeed, it has previously been shown that a single exposure to 500 optimally attenuated cercariae induces 60-70 % protection in C57BL/6 mice against schistosome (Dean, 1983). However, Hsu *et al.* (1981) using X-irradiated cercariae larvae reported a progressive increase in resistance to levels greater than 90 % when the number of immunizing exposures was raised from one to five.

In order to characterise the immune responses involved in conferring protection, animals vaccinated twice with attenuated larvae were analysed.

4.3.3. Induction of immunity: skin responses

Having established that two immunisations with irradiated larvae confer a high level of protection, the effect of vaccination was first studied in the skin. At the vaccination site, the skin from vaccinated animals developed significant morphological changes. The thickness of the epidermis was increased in the vaccinated animals. A proliferation of keratinocytes was observed from the stratum basal layer. The friction caused by the larvae during vaccination may have resulted in desquamation and subsequently keratinocyte proliferation (Seeley *et al.*, 1999). Indeed, the rate of mitosis has been shown to equal the rate of desquamation of keratin from the outer surface. The epidermis has been shown in Chapter 3 to be the main physical barrier to larval penetration. This observation suggested therefore that the first protection induced by irradiated larval vaccine is physical. Indeed, the increase in epidermal thickness should impair subsequent larval migration. Furthermore, *Arlian et al.,* (1996) showed that keratinocytes have the ability to produce IL1 cytokines in response to *Sarcoptes scabiei* mite skin penetration, which then induces inflammation and stimulates T cells. Indeed, IL1 cytokines have been shown to bind to receptors on the surface of T cells. Therefore, following administration of the irradiated vaccine, a stimulated T cell population could then be recruited against further infective larvae. It would have been interesting to confirm this hypothesis by monitoring the level of IL1 in the skin of vaccinated animals using RT-PCR. Vaccination also induced an immune inflammation following infection. Numerous inflammatory foci were observed in the vaccinated skin 3 days post-infection. In order to check if any worms were trapped in these foci, the skin sections

were stained with Giemsa stain, specific for parasites, but no worms could be observed (data not shown). It was interesting to note that such foci have not been identified in the skin of non-vaccinated animals following infection. The presence of these inflammatory foci in response to larval infection may therefore be responsible for the elimination of challenge parasites and subsequently responsible for the protection induced by the irradiated larval vaccine.

Similar foci were observed in mouse skin following schistosome infection (McLaren, 1997) and following *A. caninum* infection where these granulomas were a major component of the post-vaccination murine host immune response against challenge (Yuanqing *et al.*, 1998). Granuloma-like foci in the subdermal tissues were associated with the elimination of challenge schistosomula in mice which had previously been vaccinated with either γ-irradiated (Ward & McLaren, 1988) or X-irradiated (Hsu *et al.*, 1983) *S. mansoni* cercariae. Such foci have not, however, been observed in mice chronically infected with *S. mansoni* (Incani & McLaren, 1984).

In addition, mast cell proliferation was observed in the skin of vaccinated animals. Infection did not, however, affect the number of mast cells in the non-vaccinated animals, suggesting that vaccination was responsible for mast cell recruitment. It was also interesting to note that the mast cells in the non-vaccinated tissue showed some granules while the mast cells in the vaccinated tissue were degranulated. The degranulation could be caused by the binding of parasite specific IgE to Fc receptors on the mast cell surface. The released mediators could then stimulate an inflammatory response, protective for the host, as suggested by the level of protection acquired with the irradiated larval vaccine. These results are in agreement with McKean *et al.*, (1989) who suggested that mast cells were major effector cells in the immune response to infection with helminths. For instance, the killing of *S. mansoni* by eosinophils has been shown to be enhanced by mast cell products (McLaren, 1989). However, the significance of this accumulation has remained controversial because mast cells could also enhance pathology. Although pretreatment of *Trichostrongylus olubriformus* larvae *in vitro* with the mast cell mediator serotonin resulted in metabolic damage to the worms, no correlation was observed in the peak levels of hyperplasia and the onset of nematode expulsion (Lee

et al., 1986). In addition, maximum effects might not necessarily correlate with the highest mast cell numbers.

Mast cell recruitment following vaccination suggested that Th2 activation leads to a protective immune response to *Necator americanus*. To confirm the involvement of a Th2 response in immune protection against Necator infection, γ-IFN - (Th1) and IL4 - (Th2) mRNAs expression were monitored in the skin of vaccinated/infected and non-vaccinated/infected animals, using RT-PCR. The use of the RT-PCR method to detect cytokine levels in the skin proved to be difficult and the results were limited. Nevertheless, the RT-PCR results indicated an activation of a Th2 cell subset in the vaccinated animals, confirming that the immune protection induced by irradiated larvae is mediated by a Th2 response. High levels of IL5 (Th2 marker) were also observed in mice vaccinated with attenuated Necator larvae (Brown, 2000). This supports the previous analyses done in human populations by Pritchard *et al.*, (1995) on IgE production which showed a negative relationship between IgE (Th2 marker) and parasite weight and fecundity. However, care must be taken when considering the results because mast cells are also able to secrete IL4. Therefore the elevated Th2 response observed in the vaccinated animals may be linked to mast activation rather than a T cell response, but regardless of the source, IL4 can switch on a Th2 response.

4.3.4. Induction of immunity: lung responses

The passage of infective larvae into the lungs of twice vaccinated and infected animals was delayed compared to that in non-vaccinated/infected animals. Furthermore, a smaller number of larvae reached the lungs of vaccinated/infected, compared to non-vaccinated/infected, animals. These combined data are evidence of the development of an acquired immunity in the vaccinated/infected animals.

Three days post-infection, the lungs of non-vaccinated/infected animals presented severe generalised haemorrhage, caused by the passage of Necator larvae into the lungs, rupturing pulmonary capillaries. Rupture of the pulmonary capillaries, as larvae enter the alveoli, has been shown to be sufficient to cause transient anaemia in BALB/c mice (Wilkinson *et al.*, 1990; Timothy, 1994). Despite the severe pathology

observed in the lungs of non-vaccinated/infected animals, very few mast cells accumulated in response to infection. Wells and Behnke (1988) also found very few mast cells in the lungs of BALB/c mice following primary and secondary *N. americanus* infections and speculated that the parasite either failed to elicit pulmonary mast cell hyperplasia or suppressed such a response. However, mastocytosis were observed in the skin following infection, this may suggest that mast cells are stimulated by the third stage infective larvae and not by the lung stage larvae. Another hypothesis is that on day 3 post-infection, all mast cells were recruited into the skin and were not available for passage into the lungs.

However, in the lungs of vaccinated animals, a significant reduction in haemorrhage was observed. Therefore vaccination, which reduces the number of worms reaching the lungs as discussed above, results in less pathology. A reduction in pathology has previously been shown between primary and secondary Necator infection (Wilkinson *et al.*, 1990), suggesting that the reduction in pathology is due to repeated exposure rather than being a function of using irradiated larvae. In addition, at the time of infection (two weeks after the last immunisation), the likely pathology induced by irradiated larvae in the lungs would have disappeared, haemorrhage disappearing 9 days post-infection (Wilkinson *et al.*, 1990). Furthermore, in the lungs of mice vaccinated with attenuated larvae, slightly higher numbers of mast cells were present. Thus there is some evidence that the irradiated vaccine elicited mast cell accumulation in the lungs, and may also be responsible for protection against Necator. It would be necessary to carry out further histological examinations of lung tissue over a full time course to verify the occurrence of a mast cell response. Pulmonary foci were also observed in the lungs 3 days post-infection, suggesting that the attenuated vaccine induced cell recruitment into the lungs, forming foci which can subsequently isolate the parasite. In schistosome, infiltrating cells have also been observed in mice vaccinated with attenuated cercariae where they surrounded the lung stage larvae as early as 24 hours after intravenous challenge infection (Smythies *et al.*, 1996).

Eosinophilia is an omnipresent characteristic of hookworm infections and has been described in naturally occurring human infections, human volunteer infections and in experimental animal models. The pulmonary eosinophil response in response to *N.*

americanus vaccine was assessed using broncho-alveolar leucocytes (BALs) by Dr Fiona Culley (Department of Applied Pharmacology, Imperial College, London). The BALs were taken from mice vaccinated three times with irradiated larvae (first vaccination experiment) where the level of protection was of 61 % in the intestine and 2 % in the lungs following infection. An increase in eosinophil number and serum IL5 level was observed in the lungs of vaccinated animals (Culley et al., in press 2001). The administration of a neutralizing antibody to IL5 was shown to reverse the vaccination effect. The combined data therefore confirmed the induction of a protective Th2 response in the vaccinated animals. The accumulation of eosinophils may be the result of the secretion of mast cells mediators.

4.3.5. Lymphocyte proliferation and cytokine responses in vaccinated animals

Following infection, no changes in cell numbers were observed in the three lymphoid organs in the naïve and non-vaccinated/infected animals. Infection did not therefore stimulate lymphocyte proliferation and the significant reduction in cell numbers observed in the spleen of non-vaccinated/infected animals on day 3 post-infection suggested that primary infection suppressed proliferation. This immunomodulation may reflect a strategy by which the parasite could evade the host immune response. Previous mast cell data supported this theory. Garside *et al.,* (1989) investigated spontaneous cell activity in the spleen and mesenteric lymph nodes of hamsters, at various time points, after infection with *A. ceylanicum*. Cell activity, in both secondary lymphoid organs, was depressed compared to cells from uninfected control animals and it was suggested that parasite-mediated immunomodulation may have been responsible.

Before and after infection, the study of cell proliferation in the three different lymphoid organs revealed that the spleen and particularly the axillary lymph nodes, are highly stimulated by the vaccination process, suggesting that they were the main effectors involved in the development of immune protection against Necator infection. The number of axillary lymphocytes increased as early as three days post-infection in the vaccinated/infected animals; activated lymphocytes could then trap the parasite at an early stage. The number of cells in the mesenteric lymph nodes of vaccinated/infected animals was similar to that observed in the non-

vaccinated/infected animals. Although infective larvae are able to reach the intestine in mice, they do not mature in the intestine, are rapidly expelled and therefore do not induce pathology (Wells and Behnke, 1988). This truncated migration may explain the lack of immune response in the mesenteric lymph nodes. In order to induce protection, an attenuated vaccine must therefore stimulate an immune response well before the intestinal stage.

In order to define a Th1 or Th2 phenotypes following infection, lymphocytes from the spleen, axillary and mesenteric lymph nodes were stimulated *in vitro* with ConA (a T cell mitogen) and the level of cytokines determined using a specific ELISA. This result demonstrated that infection correlated with the production of γ-IFN and a negligible level of IL4. Following infection, the level of γ-IFN peaked on day 3 in the skin draining lymph nodes, on day 8 in the intestine draining lymph nodes and day 15 in the spleen. The passage of the parasite into the skin, lungs and intestine appeared to promote the production of γ-IFN. γ-IFN is a typical Th1 type cytokine, secreted by stimulated T cells activating cytotoxic T cells, inflammation, delayed hypersensitivity and macrophage activity. These cytokine profiles were not consistent with a high level of IgE and eosinophils observed in infected individuals and vaccinated animals (Chapter 4) but were consistent with the immune response observed in the skin following infection. Indeed, mast cell numbers (Th2 marker) have been shown to remain constant following infection. Furthermore, Timothy *et al.*, (1994) showed that the number of neutrophils and mononuclear cells was increased in primary infected animals. These results suggested therefore that Th1 type responses are important in promoting primary infections rather than protection. Th1 responses have also been shown to promote *Trichuris muris* infections. Brancroft *et al.*, (1994) showed that if IL 12, a Th1 promoting cytokine, was administered to normally resistant mice then a dominant Th1 response was generated and parasite expulsion was completely prevented.

In the vaccinated animals, cytokine analyses demonstrated that the irradiated vaccine induced the production of high levels of IL4 and less γ-IFN. The difference in the IL4/γ-IFN ratio between non-vaccinated and vaccinated animals was particularly accentuated in the axillary lymph nodes prior to infection, where IL4 levels were 10

times higher in the vaccinated group compared to the non-vaccinated group. A Th2 response was therefore induced by the irradiated larval vaccine, agreeing with recruitment of mast cell and the RT-PCR results. These results are also in agreement with epidemiological studies undertaken in humans after several infections. Indeed, as indicated earlier, immunity in humans infected by *N. americanus* has been associated with a high level of IgE, characteristic of a Th2 response (Pritchard *et al.*, 1995). The IL4/γ-IFN ratio remained higher in the vaccinated group following infection. The axillary lymph nodes therefore play an important role in the development and maintenance of the immune protection against *N. americanus*. The rapid response observed in the axillary lymph nodes stems from an initial stimulation in skin keratinocytes, mast cells and probably antigen presenting cells. These cells subsequently stimulated T cells in the axillary lymph nodes which then migrated to the infected tissue, i.e. the lungs and induced protection.

One concern regarding the development of a Th2 response was that it could be responsible for immune-mediated pathology (Finkelman and Urban, 1992). However, the absence of pathology in vaccinated lungs observed previously proved that the Th2 response to attenuated vaccine is responsible for the development of an immune protection and not the development of immuno-pathology.

In vaccination experiment with schistosome attenuated vaccines, it was 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 against arrival of challenge larvae (Mountford *et al.*, 1992; Coulson *et al.*, 1993). When challenge larvae reach the primed lungs, this triggers inflammatory foci that trap the migrating larvae (Mountford *et al.*, 1995). A similar concept is proposed for the *N. americanus* attenuated vaccine.

In summary, the use of irradiated larvae as a vaccine model demonstrated that:

- Two immunisations with irradiated larval are sufficient to generate complete immunity against *N. americanus* infection in BALB/c mice.
- The success of attenuated vaccine depends on a localised Th2 response, characterised by high levels of IL4 and IL5, occurring principally in the axillary lymph nodes of vaccinated animals.
- The protective Th2 response is initiated in the skin, following the secretion of a pool of IES by irradiated larvae for an extended amount of time.
- The stimulated Th2 population is then recruited into the lungs, resulting in a reduction in both worm numbers and lung pathology, following infection.
- Mast cells are recruited in the skin of vaccinated animals following infection.

Chapter 5

Use of soluble molecules as a vaccine

5.1. INTRODUCTION

Irradiated larval vaccines have been shown to induce high levels of protection against Necator infection in BALB/c mice, associated with the early development of a Th2 response (Chapter 4). However, attenuated vaccines present a number of problems such as a short shelf life and the establishment of a degree of infection. It is therefore unlikely that such a vaccine will ever be a completely safe and successful human vaccine. Thus, the use of soluble, more defined components as a vaccine will be studied in this Chapter.

The third stage infective larvae have been shown to secrete a number of immunogenic proteinases essential for skin penetration, the first step in *N. americanus* infection (Chapter 3; Brown *et al.*, 1999). A vaccine which induces an immune response neutralizing these proteinase activities, may possibly prevent larval infection and also pathology and transmission (McKerrow and Doenhoff, 1988). These proteinases therefore represent a potential candidate vaccine. The idea of using proteinases was first suggested by Chandler (1932). Thorson (1956) then demonstrated that serum from dogs infected with *A. caninum* had the ability to neutralize the activity of an anticoagulant proteinase produced by the worm (Thorson, 1956). Dogs were then vaccinated with proteolytic hookworm enzymes (contained in an *A. caninum* oesophageal extracts) and a reduction in worm burden was observed in the intestine of infected dogs (Thorson, 1956). Subsequently, sheep vaccinated with *Fasciola hepatica* cysteinyl proteinases showed a reduction in worm fecundity (Wijffels *et al.*, 1994). Similarly, vaccination with a rich source of a cysteinyl proteinase has lead to protection in mice against *Heligmosomoides polygyrus* (Satrija *et al.*, 1995). Concerning *N. americanus*, BALB/c mice vaccinated with a mixture of semi-purified cysteinyl and aspartyl proteinases presented a promising level of protection against further infection, confirming that larval proteinases are good candidate vaccines (Brown, 2000). However, irradiated larvae have been shown to be more immunogenic and to secrete more immunlogically active ES products than non-irradiated larvae (Chapter 3). Irradiated larval ES (IES) products were therefore used as a potential vaccine in this Chapter.

Among the proteinases present in larval ES products, aspartyl proteinases have been shown to be the most important proteinases involved in skin penetration and consequently represent a potent vaccine candidate (Chapter 3). However, the purification of the aspartyl proteinases has been found to be difficult and has not yet been successful (Brown, 2000). Recombinant expression of the protein was therefore undertaken by Dr K. Girdwood, from Cardiff School of Biosciences, Cardiff University (Girdwood, 2000). Gene fragments encoding aspartic proteinases were amplified from a *N. americanus* cDNA library by PCR. The desired genes were then cloned into an expression vector, pET16b, leading to the expression of two aspartic proteinases, necepsin I and II (for *Necator americanus* pepsin-like proteinase). The necepsin II gene was expressed in all life cycle stages, i.e. L_3, L_4 and adult stages. In contrast, the necepsin I expression was restricted to the late L_4 and adult stages of the parasite. The refolded proteins were of a molecular weight consistent with the pro-enzyme forms of necepsin I and necepsin II that had been produced in a recombinant form (around 46 kDa). Each recombinant proteinase presented low levels of proteolytic activity, active at pH 4.8 and 6.5 for necepsin I and pH 6.5 for necepsin II (Girdwood, 2000). The recombinant necepsin II was supplied by Dr K. Girdwood and was used to immunise BALB/c mice against *Necator americanus*.

Vaccination with recombinant antigens has been successful in animal models, particularly in shistosomiasis where several recombinant antigens have been extensively studied (Williams and Johnston, 1999). The most successful one was a vaccination with a recombinant form of the *S. mansoni* fatty acid binding protein, rSm14, which gave up to 67 % protection against subsequent infection with *S. mansoni* cercariae in mice, in the absence of adjuvant (Tendler *et al.*, 1996). Similarly, mice vaccinated with alum-precipitated recombinant Ancylostoma secreted protein 1 (ASP1) from *A. caninus* were protected against hookworm infection, this protection was associated with an elevation of all antibody subclasses, particularly IgG1 and IgG2b which interfere with parasite larval migration (Ghosh and Hotez, 1999).

However, it is generally accepted that subunit vaccines are weak immunogens and are unable to induce effective cellular responses. This limitation has forced workers to consider the development of several new approaches for vaccine delivery, notably

the use of various adjuvants (Singh and O'Hagan, 1999). A number of adjuvants are now available such as mineral salts (alum), immunostimulatory adjuvants (cytokines, saponins, lipopolysaccharide, immunostimulating complexes), lipid particles (liposomes), microparticulate adjuvants and mucosal adjuvants (cholera toxin). Adjuvants, not only, help the antigen to elicit an early, high and long-lasting immune response with fewer antigens but can also selectively modulate Th1 or Th2 type responses (Gupta and Siber, 1995). The choice of adjuvant is therefore essential to elicit the desired immune response. Indeed, Jacobs *et al.,* (1999) demonstrated that sheep immunized with a purified antigen (Hc-sL3) expressed on the surface of *H. contortus* L3 larvae induced protection when the antigen was associated with aluminium hydroxide but no protection was observed when the same antigen was associated with QuilA. Two common adjuvants were used in this Chapter, Freund's adjuvant (FA) and aluminium hydroxide (alum). Freund's adjuvant is an oil-based adjuvant with a potent immunostimulator, *Mycobacterium,* known to stimulate antibody-producing cells and to induce a Th1 response (Ada, 1990). Aluminium hydroxide, with aluminum phosphate (referred to as alum) is a mineral adjuvant and is the most commonly used in human vaccines today. The constituents of alum activate complement, eosinophil and macrophages and induce a Th2 response (Gupta and Siber, 1995).

Another approach to increasing the immunogenicity of a given vaccine is the delivery of the antigen using natural adjuvants such as dendritic cells (DCs) (Nestle and Burg, 1999). Such approach was used in this Chapter. DCs are potent antigen presenting cells and are localized in the skin, lung and intestine, zones of parasite infection. When in contact with an antigen, DCs are able to capture and process the antigen (e.g. parasitic antigens) resulting in the presentation of an antigen-peptide associated with a MHC class I or II on their surface (Nestle and Burg, 1999). Activated DCs then migrate to the adjacent lymph nodes where they stimulate resting T cells by presenting the respective antigenic peptide associated with an MHC. The DCs are particularly effective in stimulating T helper cells, with one DC activating 100 - 3 000 T cells (Banchereau and Steinman, 1998). This unique capacity to recruit resting T cells into the immune response has led to the use of DCs as an adjuvant (Melief, 1989; Metlay *et al.*, 1990). DCs generated from peripheral blood has then been shown to be safe and to represent a promising approach in the treatment of metastatic

melanoma (Nestle *et al.*, 1998). Indeed, Nestle *et al.*, (1998) showed that 5 out of 16 patients vaccinated with DCs pulsed with tumor lysate presented a regression of metastases in various organs. DCs could also play an important role in the immune response directed against *N. americanus*. Indeed, the data presented in Chapter 4 suggested that a functional immune response was induced early in the skin, especially in the epidermis where the DCs (also called Langerhans' cells, LCs) are widely distributed (Choi and Sauder, 1986). This Chapter therefore describes the use of DCs pulsed with *N. americanus* homogenates as a vaccine, the aim being to promote a Th2 response at the skin level. This work was undertaken in collaboration with J. Lynam (PhD student, supervised by Professor R. Rees, Department of Life Sciences, The Nottingham Trent University) who kindly supplied the pulsed DCs. DCs were grown to maturity from the bone marrow of naïve animals and in the absence of a specific parasite antigen, were pulsed with larval homogenate. BALB/c mice were then immunized intradermally with pulsed DCs.

5.1.1. Aims of Chapter 5

The general aim of this Chapter was to use soluble molecules as a vaccine. Consequently, irradiated larval ES and necepsin II were used against *N. americanus* infection. Their efficiency was assessed by counting the number of worms recovered from the lungs and intestines, 3 and 8 days post-infection, respectively.

Having established a successful subunit vaccine, the host immune response was then modulated. A Th2 adjuvant (alum) was used in association with IES products to enhance the level of protection developed against Necator infection and compared to the efficiency of a Th1 adjuvant (Freund's adjuvant).

Since the importance of developing an early protective Th2 response in the skin had been established, a preliminary experiment using dendritic cells pulsed with homogenate lysate was also undertaken.

5.2. RESULTS

The different vaccination schedules used in this chapter are summarized in table 5.1. and further described in Chapter 2 sections 2.3.1. and 2.3.2. Two weeks after the last immunization, BALB/c mice were infected with 300 larvae and the number of worms from the lungs and the small intestine 3 and 8 days post-infection respectively was monitored as described 2.3.3.

Table 5.1. Summary of the different vaccinations used against *N. americanus*, in male BALB/c mice

Expt n°	*Immunogens*	*Adjuvant*	*Doses (μg)*	*Time*	*Route*	*Sites*
1	IES	FA	0.5, 1, 1	3	i.p.	1
2	IES	FA	2, 1, 1	3	s.c.	2
3	Necepsin II	FA	10, 5, 5	3	s.c./i.p.	2
4	IES	FA/ Alum	2, 1, 1	3	s.c	2
5	Homogenate	DC	10	3	i.d.	1

Statistical analyses were carried out for each experiment (section 2.3.3.4.). Unless otherwise stated (* = $p < 0.05$), the results were not significant.

5.2.1. Use of irradiated larval ES (IES) as a vaccine

5.2.1.1. Preliminary study of the use of irradiated larval ES as a vaccine (experiment 1)

BALB/c mice were vaccinated with IES as outlined in table 5.1., experiment 1. In order to monitor the effects of vaccination, worms were recovered from the lungs on day 2, 3 and 4 post-infection. The results are presented in table 5.2.

Table 5.2. Number of worms recovered from the lungs in IES vaccinated animals 2 (a), 3 (b) and 4 (c) days post-infection. Non-vaccinated (NV) = mice infected with 300 larvae, Freund's control (FA) = mice vaccinated three times at three week intervals with Freund's adjuvant and infected with 300 larvae, IES vaccinated = mice vaccinated three times at three week intervals with irradiated larval ES (0.5, 1 and 1 μg) and infected. The results are expressed as the mean number of worms recovered from the lungs ± standard deviation (SD) (n = 6 except when indicated # n = 5). % Protection = % reduction compared with the mean of non-vaccinated control group (* = $p < 0.05$). (Experiment 1)

a- Number of worms recovered 2 days post-infection

Group	Lungs (Day 2)		
	Worm recovered ± SD (n = 6)	% Protection compared to	
		NV	FA
Non-vaccinated	17 ± 11		
FA control	31 + 8 #	- 79 *	
IES vaccinated	17 ± 7 #	3	46 *

b- Number of worms recovered 3 days post-infection

Group	Lungs (Day 3)		
	Worm recovered ± SD (n = 6)	% Protection compared to	
		NV	FA
Non-vaccinated	72 ± 16		
FA control	63 ± 14	12	
IES vaccinated	50 ± 16	31*	21

c- Number of worms recovered 4 days post-infection

Group	Lungs (Day 4)		
	Worm recovered ± SD (n = 6)	% Protection compared to	
		NV	FA
Non-vaccinated	55 ± 18		
FA control	48 + 11 #	12	
IES vaccinated	36 + 15	34	25

The percentage of worms recovered from the lungs of non-vaccinated animals was within the expected range, allowing a study of the effect of vaccination on Necator infection.

Two days post-infection, the number of worms recovered from the lungs of the Freund's adjuvant control group was significantly higher than the number of worms recovered from the non-vaccinated animals. Vaccination with IES induced 3 % protection compared to the non-vaccinated animals and 46 % protection compared to the FA control group ($p < 0.05$).

Three days post-infection, control animals vaccinated with Freund's adjuvant showed a reduction in worm burden of 12 % compared to the non-vaccinated group. Mice vaccinated with IES showed a reduction in the number of worms recovered in the lungs of 21 % compared to the Freund's adjuvant control group and a significant reduction of 31 % compared to the non-vaccinated group.

Four days post-infection, similar levels of protection to day 3 were observed in each group, with 34 % protection induced in animals vaccinated with IES compared to non-vaccinated animals.

5.2.1.2. Further analysis of the use of irradiated larval ES as a vaccine (experiment 2)

In order to optimize the previous experiment, BALB/c mice were vaccinated three times subcutaneously and with more immunogens as described in table 5.1., experiment 2. The number of worms recovered from the lungs (3 days post challenge) and small intestine (8 days post challenge) are represented in table 5.3.

Table 5.3. Number of worms recovered from the lungs (3 days post-infection) and small intestines (8 days post-infection) following vaccination with irradiated larval ES. Non-vaccinated (NV) = mice infected with 300 larvae, FA control (FA) = mice vaccinated three times at three week intervals with Freund's adjuvant and infected with 300 larvae, IES vaccinated = mice vaccinated three times at three week intervals with irradiated larval ES (2, 1 and 1 μg) and challenged. The results are expressed as the mean number of worms recovered from the lungs and small intestine ± standard deviation (SD) (n = 6 except where indicated # n = 5). % Protection = % reduction compared with the mean of non-vaccinated control group. (Experiment 2).

Group	Lungs (Day 3)			Intestine (Day 8)		
	Worm recovered ± SD	Protection to		Worm recovered ± SD	Protection to	
		NV (%)	FA (%)		NV (%)	FA (%)
Non-vaccinated	103 ± 39			25 ± 13		
FA control	73 ± 32	**29**		31 ± 13		
IES vaccinated	76 ± 29#	**26**		17 ± 12 #	**32**	**45**

Three days post-infection, 103 ± 39 larvae were found in the lungs of non-vaccinated animals. In the Freund's adjuvant control group, a protection of 29 % was observed in the lungs, whilst 26 % protection was achieved in the lungs of the vaccinated animals.

Eight days post-infection, 25 ± 13 larvae were recovered from the intestines of the non-vaccinated mice. This number decreased to 17 ± 12 larvae in the intestine of vaccinated mice, resulting in a 32 % protection. No reduction in the worm count was observed in the intestine of mice vaccinated with only Freund's adjuvant.

5.2.2. Use of an aspartyl protease vaccine

5.2.2.1. Level of protection induced by an aspartyl protease vaccine, necepsin II (experiment 3)

Male BALB/c mice were immunized with the recombinant necepsin II as described in table 5.1. (experiment 3) and then infected with 300 infective larvae. After the first subcutaneous immunisation, mice presented nodules and inflammation at the site of

injection and subsequent immunizations were therefore administered intraperitoneally. The number of worms recovered from the lungs (3 days post-infection) of vaccinated animals are presented in table 5.4.

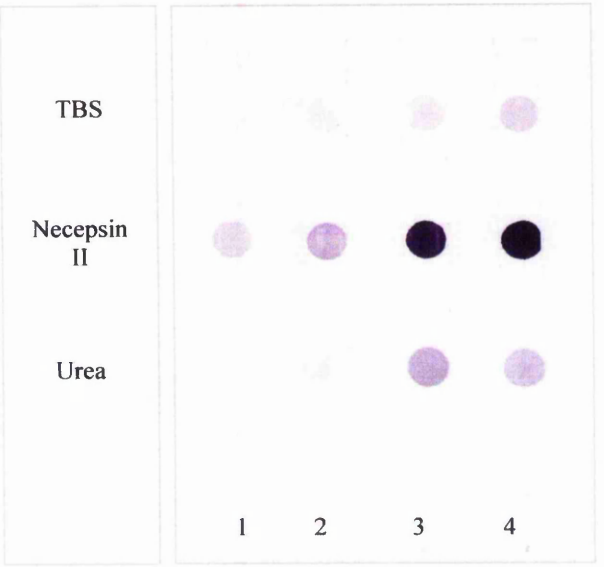
Table 5.4. Number of worms recovered from the lungs (3 days post-infection) following vaccination with necepsin II. Non-vaccinated (NV) = mice infected with 300 larvae, FA control = mice vaccinated three times at three week intervals with Freund's adjuvant and buffer and then infected, necepsin II vaccinated = mice vaccinated three times at three week intervals with (10, 5 and 5 μg) of necepsin II and infected. The results are expressed as the mean number of worms recovered from the lungs ± standard deviation (SD) (n = 6 except where indicated # where n=5). % Protection = % reduction compared with the mean of non-vaccinated control group.

Group	Lungs (Day 3)	
	Worm recovered ± SD (n = 6)	**Protection to NV (%)**
Non-vaccinated	111 ± 50#	
FA control	71 ± 60	**36**
Necepsin II vaccinated	110 ± 35	**1**

Three days post infection, 111 ± 50 larvae were found in the lungs of non-vaccinated control group. A protection of 36 % was achieved in animals vaccinated with just Freund's adjuvant compared to the non-vaccinated animals. Vaccination with necepsin II had no effect in the number of larvae recovered in the lungs, 3 days post-infection.

5.2.2.2. Immunogenicity of necepsin II.

The absence of an immune protection in mice vaccinated with necepsin II may suggest that the recombinant aspartyl proteinase is a weak immunogen and unable to induce effective cellular responses. Therefore, necepsin II immunogenicity was determined using a dot blot method as described in section 2.3.5., using individual sera from the previous experiments. The dot blot results are presented in figure 5.1.



Strip 1	No primary antibody
Strip 2	Serum from naïve mice (Dilution 1/50)
Strip 3	Serum from Freund's adjuvant control mice (1/50)
Strip 4	Serum from mice vaccinated with necepsin II (1/50)

Figure 5.1 Dot blot using serum from vaccinated animals against necepsin II to determine the immunogenicity of necepsin II antigen. *TBS and urea were used as control.*

Serum from naïve mice (negative control) did not react with necepsin II. Serum from mice vaccinated with necepsin II detected necepsin II antigen. However serum from mice vaccinated with Freund's adjuvant and necepsin II buffer also gave positive binding to necepsin II antigen. Nevertheless, serum from mice vaccinated with necepsin II had a higher affinity for necepsin II compared to the Freund's adjuvant control animals.

5.2.3. Vaccine delivery

5.2.3.1. Use of a Th2 adjuvant with larval IES (experiment 4)

The vaccination schedule using Freund's (Th1) and alum (Th2) adjuvants is described in table 5.1., experiment 4 (sections 2.3.2.1 and 2.3.2.2.). The numbers of worms recovered from the lungs and small intestines of BALB/c mice following vaccination are shown in table 5.5.

Table 5.5. Comparison of Th1 (Freund's) and Th2 (alum) adjuvant in the induction of the immune protection against infective larvae. *The table shows the number of worms recovered from the lungs (3 days post-infection) and small intestines (8 days post-infection) of BALB/c mice following vaccination with irradiated larval ES mixed with either Freund's or alum adjuvant. Non-vaccinated (NV) = mice infected with 300 larvae, adjuvant control (AC) = mice vaccinated three times at three week intervals with either Freund's or alum adjuvant and infected with 300 larvae, IES = mice vaccinated three times at three week intervals with irradiated larval ES (2, 1 and 1 μg) presented by Freund's or Alum adjuvant and infected. The results are expressed as the mean number of worms recovered from the lungs and small intestine ± standard deviation (SD) (n= 5 except when indicated # n = 4). % Protection = % reduction compared with the mean of primary infection control group, * = $p < 0.05$*

Group	Lungs (Day 3)			Intestine (Day 8)		
	Worm recovered ± SD (n = 5)	% Protection		Worm recovered ± SD (n = 5)	% Protection	
		NV	AC		NV	AC
Non-vaccinated	132 ± 40			35 ± 7		
Freund's control	114 ± 40	**13**		15.± 2	**57 ***	
IES in Freund's	131 ± 41#	**0.2**		17 ± 7 #	**50 ***	
Alum control	133 ± 16#			17 ± 8	**51 ***	
IES in alum	99 ± 16	**25**	**26 ***	17 ± 10	**51***	

Lung counts taken 3 days post-infection showed that 132 ± 40 worms were recovered in the non-vaccinated animals. Freund's adjuvant control induced 13 % protection while the alum adjuvant control has no effect on the worm recovery compared to non-vaccinated group. Whilst vaccination with IES in Freund's adjuvant induced no protection, IES in alum induced a significant level of protection (26 %) compared to the alum control group.

On day 8, no significant difference in worm recovery was observed between the two adjuvant controls and no effect of IES was shown.

To conclude, IES vaccine adsorbed on alum was the only vaccine to induce a significant level of protection in both the lungs (26 %) and intestine (51 %) of vaccinated mice.

5.2.3.2. Preliminary data using dendritic cells (experiment 5)

DCs were cultured as described in section 2.3.3.3. After 7 days of culture, the DCs were analysed by flow cytometry and presented some phenotypic changes, such as higher levels of MHC class II, CD40 and CD80, characteristic of DCs maturation (data not shown). DCs were then pulsed with homogenate lysate (section 2.3.2.2.) for 2 days and BALB/c mice were subsequently vaccinated as described in table 5.1. The level of protection following vaccination is shown in table 5.6.

Table 5.6. Number of worms recovered from the lungs (3 days post-infection) and small intestines (8 days post-infection) of BALB/c mice vaccinated with larval homogenate presented by mature dendritic cells (DC). Non-vaccinated = mice infected with 300 larvae, Homogenate = mice vaccinated three times with homogenate and then challenged, DC = mice vaccinated three times with non-pulsed DC and then challenged, DC + homogenate = mice vaccinated three times with DC pulsed with homogenate. The results are expressed as the mean number of worms recovered from the lungs and small intestine ± standard deviation (SD) (n = 3). % Protection = % reduction compared with the mean of primary infection control group.

Group	Lungs (Day 3)		Intestine (Day 8)	
	Worm recovered ± SD (n = 3)	**Protection (%)**	Worm recovered ± SD (n = 3)	**Protection (%)**
Non-vaccinated	70 ± 26		17 ± 3	
Homogenate	46 ± 7	**34**	11 ± 5	**32**
DCs	82 ± 20	-	17 ± 9	-
DC + homogenate	85 ± 1	-	19 ± 5	-

In the non-vaccinated group, 70 ± 26 and 17 ± 3 worms were recovered in the lungs and small intestines, respectively. Mice vaccinated with the homogenate showed a reduction in worm recovery compared to non-vaccinated animals, with 34 % protection shown in the lungs and 32 % protection in the intestine. The use of dendritic cells had no effect on the number of worms recovered from the lungs and intestines compared to the non-vaccinated animals.

5.3. DISCUSSION

5.3.1. Protection induced by irradiated larval ES vaccine

In the first experiment, BALB/c mice were vaccinated intraperitoneally with ES from irradiated larvae mixed with Freund's adjuvant, three times at three weekly intervals and then challenged with 300 *N. americanus* infective larvae. The doses of IES used for each immunization were 0.5, 1 and 1 μg per mouse. Before considering the effect of the IES vaccine, it was interesting to note that the Freund's adjuvant control significantly increased the number of worms recovered from the lungs, compared to the non-vaccinated animals, 2 days post-infection. This highest number of worms observed in the lungs of mice vaccinated with Freund's adjuvant suggested that Necator larvae migrated more rapidly into the lungs of animals immunized with Freund's adjuvant compared to non-vaccinated animals, implying that on day 3, most of the worms had migrated into the intestine. Freund' s adjuvants have been shown to induce a Th1 response (Ada, 1990), which has been associated with Necator infection in Chapter 4. This concept was confirmed by the number of worms recovered from the lungs on day 3. Indeed, the number of worms recovered from the lungs of animals vaccinated with the Freund's adjuvant control was smaller than in the non-vaccinated animals, in agreement with the hypothesis that their migration was facilitated. Despite the effect of Freund's adjuvant, vaccination with larval IES products generally reduced the number of worms recovered from the lungs of vaccinated animals. The effect of IES vaccination was observed in the lungs as early as day 2 post-infection, leading to a significant 46 % protection compared to Freund's adjuvant control. The levels of protection induced by IES vaccination, on days 3 and 4 respectively, were 31 % and 34 %. However, the protection was only significant compared to non-vaccinated group on day 3. These results may therefore suggest that IES induced a Th2 response counterbalancing the Th1 response induced by the adjuvant. Nevertheless, irradiated larval ES represents a good vaccine against *N. americanus* infection.

In order to enhance the level of protection obtained previously with larval IES, mice were vaccinated with 2, 1 and 1 μg of IES adsorbed on Freund's adjuvant. In addition, considering the importance of the skin in the development of the immune

response against Necator (Chapter 4), mice were immunized subcutaneously in order to simulate the initial immune response taking place in the skin. Freund's adjuvant caused a depot formation of IES in the skin at the site of injection mimicking the situation when irradiated larvae are delayed in the skin. As before on day 3, the use of Freund's adjuvant reduced the number of worms recovered from the lungs, but the number of worms reaching the intestine on day 8 was slightly higher than the control. The intestine data confirmed the previous concept that a Th1 response is not associated with protection but infection. Following vaccination, the number of worms was reduced in both the lungs and intestine of vaccinated animals compared to non-vaccinated mice, resulting in 26 % and 32 % protection, respectively. Thus modifications to the dose and route of immunization did not to improve the level of protection. Similarly Tavernor *et al.*, (1992) demonstrated that an increase in the dose of membrane glycoprotein from *Haemonchus contortus* did not increase the level of protection against *H. contortus* infection.

5.3.2. Protection induced by necepsin II vaccine

Larval aspartyl proteinases have been shown to be highly implicated in the penetration of the skin by Necator third stage larvae and therefore represent a potent candidate vaccine. Although *N. americanus* infective larvae can be cultured *in vitro*, the production and purification of sufficient amount of aspartyl proteinase is still a limitation. Therefore, there are distinct advantages in using *N. americanus* proteins in recombinant form. Necepsin; a recombinant aspartyl proteinase, was consequently produced and supplied by Dr K. Girdwood (Cardiff). Mice were vaccinated three times with 10, 5 and 5 μg of necepsin II, the proteinase expressed in the third, fourth and adult stages of the *N. americanus* life cycle. The recombinant protein was solubilised in 6 M urea, 0.1 M tris pH 8.0, 1 mM glycine; this buffer was used in the Freund's adjuvant control. Severe inflammation and nodules were observed at the site of injection following immunisation with both necepsin II and Freund's adjuvant control, suggesting that the buffer was toxic to the mice. Vaccination with necepsin II protein did not induce protection against Necator infection; this may at least be partly due to the toxic nature of the buffer. However, an antibody response was detected against necepsin II in vaccinated mice. The denatured configuration of the recombinant protein may suggest that the epitope recognised by the necepsin II

antibody may not be displayed by the native Necator aspartyl proteinases. Furthermore, Chapter 3 demonstrated that although the aspartyl proteinase was essential for the penetration of infective larvae through human and hamster skins, it seemed not to be involved in larval penetration through mouse skin. Consequently, the antibody response observed in vaccinated mice may be neutralising an aspartyl proteinase(s) non-vital for larval infection in the mouse model. Necepsin II might, of course, have been successful in humans. Having determined in Chapter 4 that protection was associated with a Th2 response, it would have been interesting to vaccinate mice with necepsin II presented with alum. K. Girdwood has demonstrated that necepsin I is very similar to the aspartic Pep1 protein from *H. contortus*, located on the luminal surface of intestinal cells in the adult parasite (Longbottom *et al.*, 1997; Girdwoood, 2000). A gut membrane protein complex with aspartic proteinase activity has also been shown to be highly protective in sheep against *H. contortus* challenge (Smith *et al.*, 1994). Similarly, necepsin I protein may be located on the surface of intestinal cells. In addition, the adult Necator aspartyl proteinase(s) has been shown to degrade haemoglobin and fibrinogen, a property essential for a blood-feeding parasite (Burleigh, 1992; Brown, 2000). Therefore, the use of a vaccine containing both necepsin I and necepsin II may be successful in humans.

Other workers have been encountered similar difficulties with the use of recombinant vaccines in different parasite models. Vaccination with alum-precipitated recombinant ASP1 (Ancylostoma secreted protein 1) from *A. caninus* resulted in protection against this hookworm infection (Ghosh and Hotez, 1999). Reduction in hookworm burden was associated with elevation of all antibody subclases, particularly IgG1 and IgG2b, which interfered with parasite larval migration (Ghosh and Hotez, 1999). However, no protection was observed in dogs vaccinated with ASP 2, probably due to a difference in protein conformation (Hawdon *et al.*, 1999; Sen *et al.*, 2000). Another consideration in using protein vaccines is the similarity between the human and parasitic proteins. This possible sequence similarity raises the issue of the balance between exacerbation of allergic reactions and induction of immunity. However, so far no adverse autoimmune consequences have been reported in animal models, but such questions will require careful consideration prior to human trials.

5.3.3. Vaccine presentation

The use of ES from irradiated larvae in Freund's adjuvant previously induced a promising level of protection against Necator infection in mice. Since the immune protection generated by the irradiated larval vaccine was a Th2 response, it was thought that the level of protection following vaccination with larval IES could be enhanced with the use of a Th2 adjuvant such as alum. The effects of Freund's (Th1) and alum (Th2) adjuvants were subsequently compared by monitoring the number of worms recovered from the lungs and intestine of vaccinated animals. In this experiment, Freund's adjuvant vaccination by itself resulted in a reduction in worm recovery in both the lungs and intestine compared to non-vaccinated animals, which is in disagreement with previous results. Furthermore, the presence of larval IES antigen did not enhance the level of protection but increased the number of worms recovered compared to the Freund's adjuvant control. However, a high rate of mortality was observed in IES in Freund's adjuvant vaccinated mice and consequently the mean number of larvae obtained from the lung resulted only from two mice. These results did not agree with the two previous vaccination experiments. It is possible that a new bottle of adjuvant may have contributed to this unexpected result and the high rate of mortality.

The alum control had an effect on worm recovery in the intestine where 51 % protection was achieved compared to the non-vaccinated group. Although all four vaccination schedules induced protection in the intestine compared to non-vaccinated animals, only animals vaccinated with IES in alum presented a reduction in worm burden in both the lungs (25 %) and the intestine (51 %) following infection, compared to the non-vaccinated group. The level of protection induced in the lungs of IES/alum vaccinated mice was also significant compared to the alum control group. These results confirmed that the choice of adjuvant is very important and that a Th2 adjuvant is likely to be a beneficial component of a vaccine against *N. americanus*.

In *Trichinella spirali*, comparative adjuvant experiments revealed that vaccination with excretory/secretory material from *T. spirali* using adjuvants such as Freund's, alum and immune-stimulating complexes (ISCOMs) did not appear to modify the

immune response qualitatively but the magnitude of the response was affected greatly (Robinson *et al.*, 1994). The mechanisms by which alum operates are not well-known but some differences have been noticed between Freund's adjuvant and alum in other studies. Alum has been shown to be a good adjuvant for weak antigens in mice while Freund's adjuvant is a more potent adjuvant for strong antigens (Bomford, 1989). Alum has also been shown to induce higher antibody responses compared to the use of Freund's complete adjuvant (Ada, 1990). In animals as well as humans, alum increased the levels of specific and total IgE antibodies (Gupta *et al.*, 1995; Gupta and Siber, 1995). In addition, injections with *H. contortus* Hc-sL3 antigen in alum in sheep have been shown to induce a Th2 response, characterised by an increase in Il4 production and increased number of eosinophils (Jacobs *et al.*, 1999). The results reported in this Chapter were promising but more work is required, such as; monitoring the phenotype response; studying the effects of adjuvant dose and antigen dose, and establishing the number of boosts required. Indeed, the dose of adjuvant has been shown to affect the overall immunogenicity (Gupta *et al.*, 1995). Three boosts may not be required to induce the same level of protection. Indeed, similar protection was observed in mice vaccinated 1, 2, 3 or 4 times with Ac ASP1 against *A. caninum* (Sen *et al.*, 2000).

Dendritic cells are potent antigen-presenting cells and were used for Necator antigen delivery in BALB/c mice. Using flow cytometry, cultured DCs were shown to be matured, suggesting that the homogenate could be captured and processed. Pulsed DCs were then injected intradermally in order to initiate the immune response at the infection site. The idea was that pulsed DCs would stimulate B and T lymphocytes, particularly Th2 lymphocytes ready for the subsequent parasitic infection. In this preliminary study, pulsed DCs did not induce protection against *N. americanus*; indeed they had no effect on the infection process. This does not necessary mean that there was no immune response directed against the parasite, but this was not checked. Several explanations could be given for the absence of protection. Pulsed DCs may have stimulated MHC class I restricted (cytotoxic) rather than MHC class II restricted (helper) T cells. However, pulsed DCs have been shown by flowcytometry analysis to present essentially MHC-class II complexes, suggesting that T helper cells would indeed have been activated (data not shown). The selective activation of a Th2 immune response required for Necator protection, however, was not definite.

Furthermore, the use of cell homogenates to pulse DCs may have resulted in the presentation of a multitude of peptides, which could divert the immune response. DCs have also been use against the parasite *T. muris.* Mice vaccinated with DCs pulsed with *T. muris* antigens were successfully immunized against subsequent infection, as shown by a high level of Ag specific IgG1 prior to infection and accelerated worm expulsion on day 10 post-infection.

It was also interesting to note that the use of whole cell lysates induced around 30 % protection against infection, based on both the lungs and intestine worm counts. Whole worm preparations have generally failed in other systems to induce good immune protection, probably due to the complexity of the parasite and its distinct evasion mechanisms (Mitchell, 1989). However, some crude (non-antigenically defined) vaccines are in use in the veterinary field against *Dictyocaulid* nematodes. For human use, vaccines clearly need to be more antigenically defined, to allow the production of consistent preparations, ease of manufacture and quality assurance and giving a more predictable pattern of adverse reactions.

In conclusion, Chapter 5 demonstrated that:

- Vaccination with excretory/secretory products from irradiated larvae in alum induces high level of protection against Necator infection, demonstrating therefore that a vaccine targeting the infective stage can be efficient.
- A Th2 adjuvant is required to enhance immune protection against *N. americanus*.
- A Th1 adjuvant used by itself facilitates parasite migration from the skin into the lungs.
- A single recombinant aspartyl proteinase fails to induce protection against infection, but this may partly be due to the choice of adjuvant.
- A combined vaccine targeting several critical parasite functions may need to be use to be successful.

Chapter 6

General Discussion

6. GENERAL DISCUSSION

The overall aim of this project was to work towards the development of a vaccine against the human hookworm, *N. americanus.* Research in this field has been limited for several reasons, including the lack of suitable animal models. In this project, two animal models were required; DSN hamsters to maintain the *N. americanus* life cycle and BALB/c mice for the vaccination experiments. Although *N. americanus* does not reach maturity in the intestine of BALB/c mice, infection of adult, fully immunocompetent mice, compared to neonate hamsters, allows to study the immune response following vaccination. The development of an immune response in mice following vaccination, in agreement with human epidemiological data, suggested that the BALB/c mouse is a good animal model. However, when studying larval penetration in Chapter 3, the mouse model was shown to give different results from humans on several occasions. Nevertheless, no matter how good the model is, experimental conditions will always differ from the multi-factorial natural infection of the human population. Indeed, contrary to the human situation, the animals used are bred from uninfected mothers, are pathogen free and are infected at a determined time point (Fallon, 2000). Therefore the BALB/c mouse to date represents the best animal model available to study vaccination.

It was interesting to note that hamster skin gave comparable results to human skin (Chapter 3). It was recently observed that adult hamsters can be infected with Necator third stage larvae on their cheek pouch (unpublished work by Brown and Girod), suggesting that in the future hamsters could replace mice for vaccination studies. However, hamster reagents for subclass and cytokine analyses would need to be developed. The marmoset (a primate) has also been considered as an animal model, and research on these animals is currently in progress (Prof D. Pritchard, Nottingham University).

The other limitation encountered during this project was the quality and reproducibility of larvae. Although the methods for *N. americanus* maintenance were standardised, important variations have been observed between larval batches, namely differences in vitality, motility and enzyme secretions. By optimising the quality and activity of the

larvae, the efficiency of the irradiated larval vaccine and IES vaccine could be optimised. Although the quality of the larvae may depend on individual hamsters, a factor out of human control, larval variation may mainly result from larval storage. Indeed, at the beginning of this project, infective larvae were sometimes stored for up to one week prior to immunisation or infection. Since variations in worm behaviour were observed, fresh larvae were then used and subsequently reproducible results and higher worm recovery were obtained. Furthermore, it is thought that *N. americanus*, maintained in the DSN hamster for the last 17 years at Nottingham University, is too well adapted to hamsters. Indeed, enzyme activities required for the parasite survival in humans may not be required in hamsters and may not be expressed by the *N. americanus* strain anymore. Indeed, whilst *N. americanus* ES products were shown to inhibit platelet aggregation 6 years ago (Furmidge *et al.*, 1995), today this activity appears not to be present. There are plans to bring a new strain of *N. americanus* larvae from Papua New-Guinea at the end of the year 2001 and it will be interesting to monitor its evolution over time.

This project demonstrated on several occasions that the skin played an important role in the outcome of the infection. Although this study characterised the elements implicated in the infection process and the immune response, occurring in the skin, the mechanisms by which the immune response affects the parasites, remain unknown. The DEs could represent a great tool to extend our knowledge. The addition of a keratinocyte layer on the top of the DEs will demonstrate the involvement of these cells on larval penetration. Furthermore, dendritic cells and specific cytokines, such as IL1 produced by keratinocytes, could be added individually into the DEs and their effect on larval penetration could be analysed.

A successful attenuated vaccine against *N. americanus* infection has been established in this project. Indeed two immunisations with irradiated larvae have been shown to be sufficient to induce high levels of protection in BALB/c mice against subsequent Necator infection. Although the number of immunisations required to induce protection has been reduced in this project, the efficiency of a single immunisation remains to be confirmed. Furthermore, it would have been useful to determine the minimum dose, meaning the number of larvae used per immunisation, required for protection. The longevity of the protective effect is also unknown; memory, of course,

is a key element for the development of a vaccine, particularly when this vaccine is intended for the developing countries. Furthermore, although an immune protection was observed in vaccinated animals, no evidence of worm damage was demonstrated in this study. It is therefore not yet clear how a Th2 response affects the parasites. Future studies should check how lymphocytes from the different lymphoid organs of vaccinated animals respond to stage specific antigens. The role of mast cells should also be studied further; this could be achieved using mast cell animal knock outs.

Candidate vaccines were also identified in this project. The use of ES products from irradiated larvae presented in alum induced a significant reduction in worm burden. Although, this vaccine presents several limitations such as the amount of antigen required, it would have been useful to determine the best injection route and the dose required. In the future, a comparison of the effect of a vaccine using ES from non-irradiated larvae and a vaccine using ES from irradiated larvae should be conducted in order to establish whether irradiation is beneficial.

Although aspartyl proteinase has been shown to be important in larval penetration, the use of the recombinant aspartyl proteinase, necepsin II produced by K. Girdwood, as a vaccine did not induce protection against Necator infection. However, an antibody response was induced in mice vaccinated with necepsin II and it would have been interesting to see if sera from vaccinated animals had a direct effect on the penetration of infective larvae using *ex vivo* skin. Protection may have been obtained if necepsin II had been mixed with alum instead of Freund's adjuvant. Indeed, efforts should be directed to the delivery and presentation of the vaccine. Furthermore, necepsin II was presented in *E. coli* and a better immune response might have been observed if a mammalian expression system was used. In addition, considering the problem with the choice of animal models, Necepsin II, although not successful in the mouse, may have been successful in humans.

The fact that the IES vaccine induced protection was promising but may suggest that a cocktail vaccine is required to enhance the immune protection obtained with the recombinant vaccine. A combination vaccine should be targeted to neutralise different functions of the parasite. Indeed, the use of a mixture of purified cysteinyl and aspartyl proteinases has been shown to induce protection in BALB/c mice (Brown, 2000).

Different stages of the parasite life cycle could also be targeted, which may suggest the use of necepsin I and II in combination. The possibility of synergistic action could also be increased by incorporating antigens from different parasite strains or an antigen from one strain could protect from several different parasite infections. Indeed Necator ASP-1 has been shown to be protective against *A. caninum* (Hotez *et al.*, 1999). Similarly, it has been shown that recombinant *S. mansoni* fatty acid binding protein, Sm14, could be protective against *S. mansoni* and also *F. hepatica* (Tendler *et al.*, 1996). A successful cross-reactive vaccine would, of course, simplify immunization schedules. However, problems including antigenic competition and carrier-specific epitope suppression should be taken into account when developing a cocktail vaccine.

The application of recombinant DNA technology to the field of vaccine development has led to remarkable progress over the past decade. Indeed, molecular biology is today used to identify potential protective antigenic determinants. For instance, the immunoscreening of a *A. caninum* cDNA library has resulted in the identification of several potent antigens (listed table 6.1.), such as ASP-1, ASP-2 and nematode anticoagulant peptides (NAPs) (Hotez, *et al.*, 1999).

Table 6.1. Recombinant Ancylostoma antigen *(from Hotez* et al., *1999). ASP = Ancylostoma secreted protein, MEP = metalloprotease, NAP =nematode anticoagulant peptide, NIF = neutrophils inhibitory factor,*

Molecule	*Nature of molecule*	*Mol. weight*	*Life cycle stage*	
			L3	Adult
ASP-1	Secreted antigen	45 kDa	+	?
ASP-2	Secreted antigen	24 kDa	+	+
MEP	Metalloprotease	?	+	+
NAP	Anticoagulant	8 kDa	-	+
NIF	Integrin inhibitor	45 kDa	?	+
Cathepsin	Protease	28 kDa	?	+

Following cloning, these antigens can then be produced in high quantities in an appropriate expression system. Such a genetically engineered vaccine is now available for humans against hepatitis B. Similarly, recent work has been carried out to screen a *N. americanus* cDNA library and 161 new hookworm genes have been

identified (Daub *et al.*, 2000). Although, the antigenic properties of most of these genes remains to be studied, molecules such as necepsin I and II could result in a successful vaccine in the future. However, it must not be forgotten that hookworm infections occur only in developing countries and it will be a challenge to find commercial partners who will assist in vaccine research, production and clinical trials. The announcement of the Sabin Vaccine Institute Hookworm Vaccine initiative is encouraging for the future development of a vaccine against hookworms.

Chapter 7

References

7. REFENCES

Ada, G. L. 1990. The immunological principles of vaccination. The Lancet, **335**, 523-526.

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

Arlian, G. G., Vyszenski-Moher, D. L., Rapp, C. M. and Hull, B. E. 1996. Production of IL1and IL1 by human skin equivalents parasitized by *Sarcoptes scabiei*. Journal of Parasitology, **82**(5), 719-723.

Banchereau, J. and Steinman, R. M. 1998. Dendritic cells and the control of immunity. Nature, **392**, 245-252.

Bancroft, A. J., Else, K. J., Sypek, J. P. and Grencis, R. K. 1994. Il12 promotes a chronic intestinal nematode infection. European Journal of Immunology, **27**, 866-870.

Bancroft, A. J. and Grencis, R. K. 1998. Th1 and Th2 cells and immunity to intestinal helminths. Chemical Immunology, MacDonald T. T., Basel, Karger, **71**, 192-208.

Banwell, J. G. and Schad, G. A. 1978. Intestinal Parasites (Hookworm). Clinics in Gastroenterology, Marsden. P., 129-156.

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

Behnke, J. M., Paul, V. and Rajasekariah, G .R. 1986a. The growth and migration of *Necator americanus* following infection of neonatal hamsters. Transactions of the Royal Society of Tropical Medicine and Hygeine, **80**, 146-149.

Behnke, J. M., Wells, C. and Brown, J. 1986b. An improved technique for experimental infections with skin penetrating nematode larvae (*Necator americanus*). International Journal for Parasitology, 16(5), 461-464.

Behnke, J. M. and Pritchard, D. I. 1987. *Necator americanus* in neonatally infected hamsters. The time course of infection and antibody response to the surface antigens of L4 and adult worms. Transactions of the Royal Society of Tropical Medicine and Hygiene, **81**, 967-792.

Bell, E., Sher, S., Hull, B., Merrill, C., Rosen, S., Chamson, A., Asselineau, D., Dubertret, L., Coloumb, B., Lapiere, C., Nusgens, B. and Neveux, Y. 1983. The reconstitution of human skin. The Journal of Investigative Dermatology, **81**(1), 1s-10s.

Bergquist, R. 1995. Controlling Schistosomiasis by vaccination: a realistic option? Parasitology Today, **11**, 191-194.

Betts, C. J. and Wilson, R. A. 1998. Th1 cytokine mRNA expression dominates in the skin-draining lymph nodes of C57BL/6 mice following vaccination with irradiated *Schistosoma mansoni* cercariae, but is down-regulated upon challenge infection. Immunology, **93**(1), 49-54.

Beynon, R. J. and Bond, J. S. 1989. Proteolytic enzymes-A practical approach. Oxford, IRL Press.

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

Bin, Z., Hawdon, J., Qiang, S., Sen, L., Hainan, R., Huiqing, Q., Wei, H., Shuhua, X., Tiehua, L., Xing, G., Zheng, F. and Hotez, P. 1999. Ancylostoma secreted protein 1 (ASP-1) homologues in human hookworms. Molecular and Biochemical Parasitology, **98**, 143-149.

Bomford, R. 1989. Aluminium salts: perspectives in their use as adjuvants. Immunological Adjuvants and Vaccines, Gregoriadis, G., Allison, A. C. and Poste, G. New York, Plenum Press, 35-41.

Bos, J. D. and Kapsenberg, M. L. 1986. The skin immune system. Immunology Today, **7**(7), 235-248.

Brophy, P. M. and Barrett, J. 1990. Glutathione transferase in helminths. Parasitology, **100**, 345-349.

Brophy, P. M. and Pritchard, D. I. 1993. Immunity to helminths reply. Parasitology Today, **9**(4), 128-128.

Brophy, P. M., Patterson, L. H., Brown, A. and Pritchard, D. I. 1995. Glutathione S-transferase (GST) expression in the human hookworm *Necator americanus*: potential roles for excretory-secretory forms of GST. Acta Tropica, **59**, 259-263.

Brown, A., Burleigh, J. M., Billett, E. E. and 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., Girod, N., Billett, E. E. and Pritchard, D. I. 1999. *Necator americanus* (human hookworm) aspartyl proteinases and digestion of skin macromolecules during skin penetration. American Journal of Tropical Medicine and Hygiene, **60**, 5, 840-847.

Brown, A. 2000. *Necator americanus*: characterisation of secreted proteinases and vaccine development. Ph.D. thesis, The Nottingham Trent University.

Bundy, D. A. P. 1995. Epidemiology and transmission of intestinal helminths. London, Chapman and Hall Medical.

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

Burleigh, J. M. 1992. An analysis of the externally presented molecules of the human hookworm *Necator americanus*. PhD thesis, The Nottingham Trent University.

Butler, D., Maurice, J. and O'Brien, C. 1997. Time to put malaria control on the global agenda. Nature, **386**, 535-541.

Butterworth, A. E., Sturrock, R. F. and Houba, V. 1975. Eosinophils as mediators of antibody-dependent damage to schistosomula. Nature, **256**, 727-731

Cappello, M., Clyne, L. P., McPhedran, P. and 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.

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

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

Chan, M. S. 1997. The global burden of intestinal nematode infections - fifty years on. Parasitology Today, **13**(11), 438 - 443.

Chandler, A. C. 1932. Susceptibility and resistance to helminthic infections. The Journal of Parasitology, **18**, 135-152.

Choi, K. L. and Sauder, D. N. 1986. The role of Langerhans' cells and keratinocytes in epidermal immunity. Journal of Leukocyte Biology, **39**, 343-358.

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.

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

Clifton, M., Monohan, H., Taylor, W., Chapman, M .R. and 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**(6), 911-923.

Constant, S. L., Mountford, A. P. and Wilson, R. A. 1990. Phenotypic analysis of the cellular responses in regional lymphoid organs of mice vaccinated against *Schistosoma mansoni*. Parasitology, **101**, 15-22.

Coulson, P. S., Smythies, L. E. and 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**, 271-323.

Creaney, J., Wilson, L., Dosen, M., Sandeman, R. M., Spithill, T. W. and 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.

Culley, F. J., Brown, A., Conroy, D. M., Sabroe, I., Pritchard, D. I. and Williams, T. J. 2000. Eotaxin is specifically cleaved by hookworm metalloproteases preventing its action *in vitro* and *in vivo*. Journal of Immunology, **165**(11), 6447-6453.

Culley, F. J., Brown, A., Girod, N., Pritchard, D. I. and Williams, T. J. Submitted. Innate and cognate mechanisms of pulmonary eosinophilia in helminth infection.

Cutts, I. and Wilson, R. A. 1997. Elimination of a primary schistosome infection from rats coincides with elevated IgE titres and mast cell degranulation. Parasite Immunology, **19**, 91-102.

Damour, O., Augustin, C. and Black, A. F. 1998. Applications of reconstructed skin models in pharmaco-toxicological trials. Medical and Biological Engineering and Computing, **36**(6), 825-832.

Daub, J. , Loukas, A., Pritchard, D. I. and Blaxter, M. 2000. A survey of genes expressed in adults of the human hookworm, *Necator americanus*. Parasitology, **120**(2), 171-184.

Dean, D. A. 1983. Schistosoma and related genera: aquired resistance in mice. Experimental Parasitology, **55**, 1.

Dent, L. A., Munro, G. H., Piper, K. P., Sanderson, C. J., Finlay, D. A., Dempster, R. K., Bignol, L. P., Harkin, D. G. and Hagan, P. 1997. Eosinophilic interleukin 5 (IL-5) transgenic mice: eosinophil activity and impaired clearance of *Schistosoma mansoni*. Parasite Immunology, **19**, 291-230.

Dessaint, J. P., Capron, M., Bout, D. and Capron, A. 1975. Quantitative determination of specific IgE antibodies to schistosome antigens and serum IgE levels in patients with schistosomiasis (*Schistosoma mansoni* and *Schistosoma haematobium*). Clinical and Experimental Immunology, **20**, 427-433.

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

Edelson, R. L. and Fink, J. M. 1985. The Immunologic function of skin. Scientific American, **252**(6), 34-41.

Else, K. J., Entwistle, G. M. and Grencis, R. K. 1993. Correlations between worm burden and markers of Th1 and Th2 cell subset induction in an inbred strain of mouse infected with *Trichuris muris*. Parasite Immunology, **15**, 595-600.

Fallon, P. G. 2000. Immunopathology of schistosomiasis: a cautionary tale of mice and men. Immunology Today, **21**(1), 29-35.

Finkelman, F. D., Pearce, E. J., Urban Jr, J. F. and Sher, A. 1991. Regulation and biological function of helminth-induced cytokine response. Immunology Today, **6**, A 62-A66.

Finkelman, F. D. and Urban, J. F. 1992. Cytokines: Making the right choice. Parasitology Today, **8**(9), 311-314.

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

Fusco, A. C., Cassioppi, L., Salafsky, B. and Shibuya, T. 1993. Penetration of *Schistosoma mansoni* cercarae into a living skin equivalent. The Journal of Parasitology, **79**, 3, 444-448.

Garside, P., Behnke, J. M. and Rose, R. A. 1989. The immune response of male DSN hamsters to a primary infection with *Ancylostoma ceylanicum*. Journal of Helminthology, **63**(3), 251-260.

Ghosh, K. and Hotez, P. J. 1998. Mechanisms of anti-rASP-1 antibody mediated immunity against *Ancylostoma caninum* L3 challenge infection in a murine model. American Journal of Tropical Medicine and Hygiene, **59**, 300-307.

Ghosh, K. and Hotez, P. 1999. Antibody-dependent reductions in mouse hookworm burden after vaccination with *Ancylostoma caninum* secreted protein 1. The Journal of Infectious Diseases, **180**, 1674-1681.

Girdwood, K. 2000. Expression, purification and characterisation of aspartic proteinases and aspartic proteinase inhibitors. Ph.D. thesis, Cardiff University.

Goates, C. Y. and Knutson, K. 1994. Enhanced permeation of polar compounds through human epidermis. I. Permeability and membrane structural changes in the presence of short chain alcohols. Biochimica et Biophysica Acta, **1195**, 169-179.

Gupta, R. K., Rost, B. E., Relyveld, E. and Siber, G. R. 1995. Adjuvant properties of aluminum and calcium compounds. Vaccine design: the subunit approach, Powell, M. F. and Newman, M. J. New York, Plenum Publishing Corporation, 229-248.

Gupta, R. K. and Siber, G. R. 1995. Adjuvants for human use: current status, problems and future prospects. Vaccine, **13**, 1263-1276.

Gupta, R. K., Griffin, P., Chang, A-C., Rivera, R., Anderson, R., Rost, B., Cecchini, D., Nicholson, M. and Siber, G. R. 1996. The role of adjuvants and delivery systems in modulation of immune response to vaccines. Novel Strategies in Design and Production of Vaccines, **15**, 105-113.

Gutteridge, W. E. 1982. Chemotherapy. Modern Parasitology. Cox, F. E. G. Oxford, Blackwell Scientific, 287-293.

Gutteridge, W. E. 1989. Parasite vaccines versus antiparasite drugs: rivals or running mates. Parasitology, **98**, S87-S97.

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

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

Harrap, S. A., Prociv, P. and Brindley, P. J. 1996. ACASP, a gene encoding a cathepsin D-like proteinase from the hookworm *Ancylostoma caninum*. Biochemical and Biophysical Research Communications, **227**(1), 294-302.

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

Haswell, E. M., Elkins, D. B., Manjula, K., Michael, E. and Anderson, R. M. 1988. An investigation of hookworm infection and re-infection following mass anthelmintic treatment in the South Indian fishing community of Vairavankuppam. Parasitology, **23**, 234-278.

Hawdon, J. M. and Schad, G. A. 1990. Serum stimulated feeding *in vitro* by third stage infective larvae of the canine hookworm *Ancylostoma caninum.* The Journal of Parasitology, **76**, 394-398.

Hawdon, J. M. and Hotez, P. J. 1996. Hookworm: developmental biology of the infectious process. Current Opinion In Genetical Development, **6**, 618-623.

Hawdon, J. M., Narasimhan, S. and Hotez, P. J. 1999. Ancylostoma secreted protein 2: cloning and characterisation of a second member of a family of nematode secreted proteins from *Ancylostoma caninum*. Molecular and Biochemical Parasitology, **99**, 149-165.

Hotez, P. J., Le Trang, N. and Cerami, A. 1987. Hookworm antigens: the potential for vaccination. Parasitology Today, **3**(8), 247-249.

Hotez, P. J. and Pritchard, D. I. 1995. Hookworm Infection. Scientific American, **272**(6), 42-48.

Hotez, P. J. 1997. Vaccines for hookworm infection. Pediatric infectious Disease Journal, **16**, 935-940.

Hotez, P. J., Ghosh, K., Hawdon, J. M., Narasimhan, S., Jones, B., Shuhua, X., Sen, L., Bin, Z., Haechou, X., Hainan, R., Heng, W. and Koski, R. A. 1999. Experimental approaches to the development of a recombinant hookworm vaccine. Immunological Review, **171**, 163-172.

Hsu, S., Hsu, H. F., Xu, S. T,. Shi, F. H., Yx, H. C., Clarke, W. R. and Johnson, S. C. 1983. Vaccination against bovine schistosomiasis japonica with highly X-irradiated schistosomula. American Journal of Tropical Medicine and Hygiene,**32**(2), 367-370.

Inaba, K., Inaba, M., Romani, N., Deguchi, M., Ikehara, S., Muramatsu, S. and Steinman, R. M. 1992. Generation of large numbers of dendritic cells from mouse bone-marrow cultures supplemented with granulocyte macrophage colony-stimulating factors. Journal of Experimental Medicine, **176**(6), 1693-1702.

Incana, R. N. and McLaren, D. J. 1984. Histopathological and ultrastructural studies of cutaneous reactions elicited in naïve and chronically infected mice by invading schistosomula of *Schistosoma mansoni*. International Journal for Parasitology, **14**(3), 259-276.

Jacobs, H. J., C. Wiltshire, C., Ashman, K. and Meeusen, E. N. T. 1999. Vaccination against the gastrointestinal nematode *Haemonchus contortus* using a purified larval surface antigen. Vaccine, **17**, 362-368.

Kalinski, P., Hilkens, C. M. U., Wierenga, E. A. and Kapsenberg, M. L. 1999. T-cell priming by type-1 and type-2 polarized dentritic cells: the concept of a third signal. Immunology Today, **20**(12), 561-567.

Karanu, F. N., Hilkens, C. M. U., Wierenga, E. A. and Kapsenberg, M. L. 1993. *Haemonchus contortus*: identification of proteases with diverse characteristics in adult worm excretory-secretory products. Experimental Parasitology, **77**, 362-371.

Kasper, G., Brown, A., Eberl, M., Vallar, L., Kieffer, N., Berry, C., Girdwood, K., Eggleton, P., Quinnell, R. and Pritchard, D. I. 2001. A calreticulin-like molecule from the human hookworm *Necator americanus* interacts with C1q and the cytoplasmic signalling domains of some integrins. Parasite Immunology, **23**, 141-152.

Krill, S. L., Knutson, K. and Higuchi, W. I. 1992. Ethanol effects on the stratum corneum lipid phase behavior. Biochimica et Biophysica Acta, **1112**, 273-280.

Kumar, S. and Pritchard, D. I. 1992a. The partial characterisation of proteases present in the Excretory/Secretory products and exsheathing fluid of the infective (L3) larva of *Necator americanus*. International Journal for Parasitology, **22**, 5, 563-572.

Kumar, S. and Pritchard, D. I. 1992b. Secretion of metalloproteases by living infective larvae of *Necator americanus*. Journal of Parasitology, **78**(5), 917-919.

Kumar, S. and 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 of Parasitology, **22**(5), 573-579.

Labiano-Abello, N. Canese, J. and Velazquez, M. E. 1999. Epidemiology of hookwom infection in Itagua, Paraguay: a cross sectional study. Memorias Do Instituto Oswaldo Cruz, **94**(5), 583-586.

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

Lee, D. L. 1977. The nematode epidermis and collagenous cuticule, its formation and ecdysis. Symposium of the zoological Society of London, **39**, 145-170.

Lee, T. D. G., Swieter, M. and Befus, A. D. 1986. Mast cell response to helminth infection. Parasitology Today, **2**(7), 186-191.

Longbottom, D., Redmond, D. L., Russell, M., Liddell, S., Smith, W. D. and Knox, D. P. 1997. Molecular cloning and characterisation of a putative aspartate proteinase associated with a gut membrane protein complex from adult *Haemonchus contortus*. Molecular and Biochemical Parasitology, **88**(1-2), 63-72.

Lowry, O., Rosebrough, N., Farr, A. and Randall, R. 1951. Protein measurement with the Folin-phenol reagent. Journal of .Biology and Chemistry, **193**, 265-275.

Liu, C. H., Zhang, X. R., Qiu, D. C., Xia, S. H., Hotez, P. J., Zhen, D. F., Zhen, H. L., Li, M. D., Ren, H. N., Zhan, B., Xue, H. C., Hawdon, J. and Feng, Z. 1999. Epidemiology of human hookworm infections among adult villagers in Hejiang and Santai Counties, Sichuan Province, China. Acta Tropica, 73(3), 243-249.

Maizel, R. M., Meghji, M. and Ogilvie, B. M. 1983. Restricted sets of parasite antigens from the surface of different stages and sexes of the nematode parasite *Nippostrongylus-brasiliensis*. Immunology, **48**(1), 107-121.

Maizels, R. M., Selkirk, M. E. and Agnew, A. 1989. Prospects for new vaccines against helminth parasites. Trends in Biotechnology, **7**, 316-321.

Maizels, R. M., Holland, M. J., Falcone, F. H., Zang, X. X. and Yazdanbakhsh, M. 1999. Vaccination against helminth parasites - the ultimate challenge for vaccinologists. Immunological Reviews, **171**, 125-147.

Mangold, B. L. and Dean, D. A. 1984. The migration and survival of gamma irradiated *Schistosoma mansoni* larvae and the duration of host parasite contact in relation to the induction of resistance in mice. Parasitology, **88**, 249-266.

Matthews, B. E. 1977. The passage of larval helminths through tissue barriers. Cambridge University Press.

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

McDonald, J. A. and Kelly, D. G. 1980. Degradation of fibronectin by human leukocyte elastase. The Journal of Biological Chemistry, **255**(18), 8848-8858.

McKean, P. G. and Pritchard, D. I. 1989. The action of mast cell protease on the cuticular collagens of *Necator americanus*. Parasite Immunology, **11**(3), 293-297.

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

McKerrow, J. H. and Doenhoff M. J. 1988. Schistosome proteases. Parasitology Today, **4**(12), 334-340.

McLaren, D. J. 1989. Will the real target of immunity to schistosomiasis please stand up. Parasitology Today, **5**(9), 279-282.

McLaren, D. J. 1997. The cutaneous inflammatory response to parasite infection. Parasites: Infection and Immunity.

Melief, C. J. M. 1989. Dendritic cells as specialized antigen-presenting cells. Research Immunology, **140**(9), 902-906.

Metlay, J. P., Pure, E. and Steinman, R. M. 1990. Control of immune response at the level of antigen-presenting cells: A comparison of the function of dendritic cells and lymphocytes. Advance in Immunology, **47**, 45-116.

Meeusen, E. N. T. and Balic, A. 2000. Do eosinophils have a role in the killing of helminth parasites? Parasitology Today, **16**(3), 95-101.

Michel, M., L'Heureux, N., Pouliot, R., Xu, W., Auger, F. A. and Germain, L. 1999. Characterization of a new tissue-engineered human skin equivalent with hair. *In vitro* Cellular and Developmental Biology-Animal, **35**(6), 318-326.

Miller, H. R. P. 1973. The possibilites for application of the canine hookworm vaccine technology to the prevention and control of hookworm infection and disease in man. Nuclear Techniques in Helmintology Research, Vienna, 61-68.

Miller, H. R. P., Woodbury, R. G., Huntley, J. F. and Newlands, G. 1983. Systemic release of mucosal mast-cell protease in primed rats challenged with *Nippostrongylus brasiliensis*. Immunology, **49**, 471-495.

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

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

Mitchell, G. F. 1989. Problems specific to parasite vaccines. Parasitology, **98**, S19-S28.

Mosmann, T. and Coffman, R. 1989. Th1 and Th2 cells: Different patterns of lymphokine secretion lead to different functional properties. Annual Review of Immunology, **7**, 145-173.

Mota, I. 1994. The discovery of the relationship between mast cells, histamine and IgE. Immunology Today, **15**(5), 242-245.

Mountford, A. P., Coulson, P. S. and Wilson, R. A. 1988. Antigen localisation and the induction of resistance in mice vaccinated with irradiated cercariae of *Schistosoma mansoni*. Parasitology, **97**, 11-25.

Mountford, A. P., Coulson, P. S. and Pemberton, R. M. 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. and Wilson, R. A. 1995. Antigens derived from lung stage larvae 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.

Moyle, M., Foster, D. L., McGrath, D. E., Brown, S. M., Laroche, Y., De Meutter, J., Stanssens, P., Bogowitz, C. A., Fried, V. A., Ely, J. A., Soule, H. R. and Vlasuk, G. P. 1994. A hookworm glycoprotein that inhibits neutrophil function is a ligand of the integrin CD11b/CD18. Journal of Biological Chemistry, **269**(13), 1008-1015.

Neilson, J. T. M. and Van de Walle, M. J. 1987. Partial protection of lambs against *Haemonchus contortus* by vaccination with a fractionated preparation of the parasite. Veterinary Parasitology, **23**, 211-221.

Nestle, F. O., Alijagic, S., Gilliet, M., Sun, Y., Grabbe, S., Dummer, R., Burg, G. and Schadendorf, D. 1998. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nature Medicine, **4**(3), 328-332.

Nestle, F. O. and Burg, G. 1999. Dendritic cells: role in skin diseases and therapeutic applications. Clinical and Experimental Dermatology, **24**, 204-207.

Newton, S. E. and Munn, E. A. 1999. The development of a vaccines against gastrointestinal nematode parasites, particularly *Haemonchus contortus*. Parasitology Today, **15**(3), 116-122.

Nussenweig, R. S., Vanderberg, J. P. and Most, H. 1998. Protective immunity produced by the injection of -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. Physiology, Biochemistry and Molecular Biology of the Skin. S. A. Goldsmith. London, Oxford University Press.

Ogilvie, B. M., Bartlett, A., Godfrey, R. C., Turton, J. A. and Worms, M. J. 1978. Antibody responses in self infections with *Necator americanus*. Transactions of the Royal Society of Tropical Medicine and Hygiene, **72**(1), 66-71.

Pemberton, R. M., Smythies, L. E., Mountford, A. P. and Wilson, R. A. 1991. Patterns of cytokine production and proliferation by T lymphocytes differ in mice vaccinated or infected with *Schistosoma mansoni*. Parasitology, **73**, 327-333.

Pritchard, D. I., McKean, P. G. and Rogan, M. 1988. Cuticle preparations from *Necator americanus* and their immunogenicity in the infected host. Molecular and Biochemical Parasitology, **28**(3), 275-283.

Pritchard, D. I. 1990. *Necator americanus* antigens and immunological targets. Hookworm disease: current status and new directions, Schad, G. A. and Warren, K. S., London, Taylor and Francis, 340-350.

Pritchard, D. I., R. J. Quinnell, Slater, A. F. G., McKean, P. G., Dale, D. D. S., Raiko, A. and Keymer, A. E. 1990a. 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., McKean, P. G., Rogan, M. T. and Schad, G. A. 1990b. The identification of a species-specific antigen from *Necator americanus*." Parasite Immunology, **12**, 259-263.

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

Pritchard, D. I. 1991. There is immunity to hookworms (but you have to know where to look!). Transactions of the Royal Society of Tropical Medicine and Hygiene, **85**, 419-423.

Pritchard, D., Brown, A. and 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. The survival stragegies of hookworms. Parasitology Today, **11**(7), 255-259.

Pritchard, D. I. and Furmidge, B. A. 1995. The anti-haemostatic strategies of the human hookworm *Necator americanus*. Thrombosis and Haemostasis, **73**(3), 546.

Pritchard, D. I., Quinnell, R. J., Slater, A. F. G., McKean, P. G., Dale, D. D. S., Raiko, A. and Keymer, A. E. 1995. Immunity in humans to *N. americanus*: IgE, parasite weight and fecundity. Parasite Immunology, **71**, 71-75.

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

Pritchard, D. I. 1996. Do haematophagous parasites secrete SOD and promote blood flow? International Journal for Parasitology, **26**(11), 1339-1340.

Pritchard, D. I., Brown, A., Kasper, G., McElroy, P., Loukas, A., Hewitt, C., Berry, C., Fullkrug, R. and Beck, E. 1999. A hookworm allergen which strongly resembles calreticulin. Parasite Immunology, **21**, 439-450.

Pritchard, D. I. and Brown, A. 2001. Submitted. Is *Necator americanus* approaching a mutualistic symbiotic relationship with humans?

Prociv, P. and Croese, J. 1990. Human eosinophilic enteritis caused by dog hookworm *Ancylostoma caninum*. Lancet, **335**(8701), 1299-1302.

Quinnell, R. J., Slater, A. F. G., Tighe, P. J., Walsh, E. A., Keymer, A. E. and 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. and Pritchard, D. I. 1995. Immunoepidemiology of human nectoriasis: correlations between antibody responses and parasite burdens. Parasite Immunology, **17**, 313-318.

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

Ritcher, D., Harn, D. A. and Matuschka, F. R. 1995. The irradiated cercariae vaccine model. Looking on the bright side of radiation. Parasitology Today, **11**(8) 288-293.

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

Robinson, K., Bellaby, T. and 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.

Roitt, I., Brostoff, J. and Male, D. 1998. Immunology, Fifth Edition. London, Mosby International Limited.

Romagnani, S. 1997. The Th1/Th2 paradigm. Immunology Today, **18**(6), 263-267.

Rose, R. and Behnke, J. M. 1990. *Necator americanus* in the DSN hamster: density-dependent expulsion of adult worms during primary infection. Parasitology, **100**, 469-478.

Rossignol, J. M. 1998. Chemotherapy: present status. Hookworm Disease, Current staus and new directions. Schad, G. A. and Warren, K. S. London, Taylor and Francis, 281-290.

Rothwell, T. L. W. 1989. Immune expulsion of parasitic nematodes from the alimentary tract. International Journal for Parasitology, **19**(2), 139-168.

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

Sacko, M. De Clercq, D., Behnke, J. M., Gilbert, F. S., Dorny, P. and Vercruysse, J. 1999. Comparison of the efficacy of mebendazole, albendazole and pyrantel in treatment of human hookworm infections in the Southern Region of Mali, West Africa. Transactions of the Royal Society of Tropical Medicine and Hygiene, **93**(2), 195-203.

Salafsky, B. and Fusco, A. 1987. Eicosanoids as immunomodulators of penetration by *Schistosome cercariae*. Parasitology Today, **3**(9), 279-281.

Salafsky, B., Fusco, A. C. and Siddiqui, A. 1990. *Necator americanus* : factors influencing skin penetration by larvae. Hookworm Disease: Currrent status and new directions. Schad, G. A. and Warren, K. S. London, Taylor and Francis, 326-329.

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

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

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

Schad, G. A. and Banwell, J. G. 1984. Hookworms. Tropical and Geographical Medicine. Warren, K. S. and Mahmoud, A. A. F., McGraw- Hill, 359-372.

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

Schad, G. A. 1991a. Hooked on hookworm: 25 years of attachment. Journal of Parasitology, **77**(2), 177-186.

Schad, G. A. 1991b. The parasite. Hookworm Infections. Gilles, H. M. and Ball, P. A. J. Amsterdam, Elsevier, 15-49.

Seeley, R. R., Stephens, T. D. and Tate, P. 1999. Essentials of Anatomy and Physiology, Wheatley, C. H.

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

Sen, H. G. and Deb, B. N. 1973. Effect of cortisone upon serial passage with the human hookworm, *Necator americanus* in golden hamsters, *Mesocricetus auratus*. Indian Journal of Medical Research, **61**(4), 486-494.

Sen, L., K., Ghosh, K., Bin, Z., Qiang, S., Thompson, M. G., Hawdon, J., Koski, A., Shuhua, X. and Hotez, P. 2000. Hookworm burden reductions in BALB/c mice vaccinated with recombinant *Ancylostoma* secreted proteins (ASPs) from *Ancylostoma duodenale*, *Ancylostoma caninum* and *Necator americanus*. Vaccine, **18**, 1096-1102.

Singh, M. and O'Hagan, D. 1999. Advances in vaccine adjuvants. Nature, Biotechnology, **17**, 1075-1080.

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

Smith, W. D. and Angus, K. W. 1980. *Haemonchus contortus*: attempts to immunise lambs with irradiated larvae. Research in Veterinary Science, **29**, 45-50.

Smith, W. D., Smith, S. K. and Murray, J. M. 1994. Protection studies with integral membrane fractions of *Haemonchus contortus*. Parasite Immunology, **16**, 231-241.

Smyhties, L. E., Coulson, P. S. and Wilson, R. A. 1992. Monoclonal antibody to IFN-γ modifies pulmonary inflammatory responses and abrogates immunity to *Schistosoma mansoni* in mice previously vaccinated with irradiated cercariae." Journal of Immunology, **149**, 3654-3662.

Smythies, L. E., Betts, C., Coulson, P. S., Dowling, M. and 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.

Souza, J. B. and Playfair, J. H. L. 1995. A novel adjuvant for use with a blood-stage malaria vaccine. Vaccine, **13**(14),1316-1319.

Stephenson, L. S., Latham, M. C., Kurz, K. M., Kinoti, S. N. and Brigham, H. 1989. Treatment with a single dose of albendazole improves growth of Kenyan schoolchildren with hookworm, *Trichuris trichiura*, and *Ascaris lumbricoides* infections. American Journal of Tropical Medicine and Hygiene, **41**(1), 78-87.

StGeorgiev, V. 1999. Parasitic infections. Treatments and developmental therapeurics. 1- Necatoriasis. Current Pharmaceutical Design, **5**(7), 545-554.

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

Tavernor, A., Smith, T. S., Langford, C. F., Graham, M. and Munn, E. A. 1992. Immune response of Clun Forest sheep to vaccination with membrane glycoproteins from *Haemonchus contortus*. Parasite Immunology, **14**, 671-675.

Teixeira, M. M., Doenhoff, M. J., McNeice, C., Williams, T. J. and Hellewell, P. G. 1993. Mechanisms of the inflammatory response induced by extracts of *Schistosoma mansoni* larvae in guinea pig skin. Journal of Immunology, **151**(10), 5255-5534.

Tendler, M., Brito, C. A., Vilar, M. M., Serra-Freire, N., Diogo, C., Almeida, M. S., Delbem, A. C. B., Figueiredo da Silva, J., Savino, W., Garratt, R., Katz, N. and Simpson, A. J. G. 1996. A *Schistosoma mansoni* fatty acid-binding protein, Sm14, is the potential basis of a dual-purpose anti-helminth vaccine. Proceedings of the National Academy of Science USA, **93**, 269-273.

Thorson, R. E. 1956. 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. 1956. The effects of extracts of the amphidial glands, excretory gland and the esophagus of adults of *Acylostoma caninium* on the coagulation of dog's blood. Journal of Parasitology, **42**, 26-30.

Timothy, L. M. 1994. Characterisation of the *Necator americanus* mouse model of human hookworm infection. Ph.D. thesis, University of Nottingham.

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

Towbin, H., Staehelin, T. and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocelluose sheets: Procedure and some applications. Proceedings of the National Academy of Science, **76**(9), 4350-4354.

Tunon de lara, J. M. 1996. Immunoglobulines E et cellules de l'inflammation. Revue des Maladies Respiratoires, **13**(1), 27-36.

Udonsi, J. K. and Atata, G. 1987. *Necator americanus* : temperature, pH, light, and larval development, longevity, and desication tolerance. Experimental Parasitology, **6**, 136-142.

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

Wakelin, D. 1993. Allergic inflammation as hypothesis for the expulsion of worms from tissues. Parasitology Today, **9**(4), 115-116.

Wales, A. and Kusel, J. R. 1992. Biochemistry of irradiated parasite vaccines: Suggested models for their mode of action. Parasitology Today, **8**(11), 358-363.

Wang, C., Huang, X X., Zhang, Y. Q., Yen, Q. Y. and Wen, Y. 1989. Efficacy of ivermectin in hookworms as examined in *Ancylostoma caninum* infections. Journal of Parasitology, **75**(3), 373-377.

Ward R. E. M. and McLaren, D. J. 1988. *Schistosoma mansoni* - evidence that eosinophils and or macrophages contribute to skin -phase challenge attrition in vaccinated CBA/CA mice. Parasitology, **96**, 63-84.

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

Wells, C. and Behnke, J. M. 1988b. The course of primary infection with *Necator americanus* in syngeneic mice. International Journal for Parasitology, **18**(1), 47-51.

Wigzell, H. 1993. The immune system as a therapeuric agent. Scientific American, **269**(3), 126.

Wijffels, G. L., Salvatore, L., Dosen, M., Waddington, J., Wilson, L., Thompson, C., Campbell, N., Sexton, J., Wicker, J. and Bowen, F. 1994. Vaccination of sheep with

purified cysteine proteinases of *Fasciola hepatica* decreases worm fecundity. Experimental Parasitology, **78**, 132-148.

Wilkinson, M. J., Wells, C. and 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.

Willadsen, P. 1997. Novel vaccines for ectoparasites. Veterinary Parasitology, **71**, 209-222.

Williams, S. A. and Johnston, D. A. 1999. Helminth genome analysis: the current status of the filarial and schistosome genome project. Parasitology, **118**, S19-S38.

Wilson, R. A., Coulson, P. S., Betts, C., Dowling, M. A. and Smythies, L. E. 1996. Impaired immunity and altered pulmonary responses in mice with a disrupted interferon- receptor gene exposed to the irradiated *Schistosoma mansoni* vaccine. Immunology, **87**, 275-282.

Wilson, A. and Coulson, P. S. 1998. Why don't we have a schistosomiasis vaccine. Parasitology Today, **14**(3), 97-99.

Winter, M. D., Wright, C. and Lee, D. L. 1997. The mast cell and eosinophil response of young lambs to a primary infection with *Nematodirus battus*. Parasitology, **114**, 189-193.

Wolowczuk, I., Delacre, M., Roye, O., Giannini, S. L. and Auriault, C. 1997. Interleukin-7 in the skin of *Schistosoma mansoni*- infected mice is associated with a decrease in interferon-gamma production and leads to an aggravation of the disease. Immunology, **91**, 35-44.

Woodbury, R. G., Miller, H. R. P., Huntlry, J. F., Newlands, G. F. J., Palliser, A. C. and Wakelin, D. 1984. Mucosal mast cells are functionally active during spontaneous expulsion of intestinal nematode rat infections. Nature, **312**, 450-452.

Wynn, T. A., Cheever, A. W., Jankovic, D., Poindexter, R. W., Caspar, P., Lewis, F. A. and Sher, A. 1995. An IL12 based vaccination method for preventing fibrosis induced by schistosome infection. Nature, **376**, 594-596.

Xu, L. Q., Yu, S., Yang, J. L., Lai, C. Q., Zhang, X. J. and Zheng, C. Q. 1999. Soil transmitted helminthiases: nationwide survey in China. Bull World Health, **73**, 507-513.

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

Yuanqing, Y., Shushua, X., Hainan, R., Jiatong, W. and Hotez, P. J. 1998. Cutaneous and subcutaneous granulomata formation in mice immunized and challenged with third stage infective hookworm (*Ancylostoma caninum*) larvae. Acta Tropica, **69**, 229-238.

PUBLICATIONS

Brown A., Girod N., Billett E. E. and Pritchard D. I. 1999. *Necator americanus* (human hookworm) aspartyl proteinases and digestion of skin macromolecules during skin penetration. The American Journal of Tropical Medicine and Hygiene, 60, 5, 840-847.

Culley, F. J., Brown, A., Girod, N., Pritchard, D. I. and Williams, T. J. Submitted. Innate and cognate mechanisms of pulmonary eosinophilia in helminth infection.

PRESENTATIONS

Girod, N. 2000. Development of a vaccine against *N. americanus*. Oral presentation, The Nottingham Trent University.

Girod N., Pritchard D. I. And Billett E. E. 1999. Identifying targets for a *N. americanus* vaccine. Poster presented at The British Society for Parasitology, Spring Meeting, Warwick.

Brown A., Girod N., Billett E. E. and Pritchard D. I. 1997. Hydrolysis of human skin proteins by secretions from the infective larvae of *N. americanus*. Poster presented at the conference of Parasitic Helminths from Genomes to Vaccines, Edinburgh.

Am. J. Trop. Med. Hyg., 60(5), 1999, pp. 840–847

NECATOR AMERICANUS (HUMAN HOOKWORM) ASPARTYL PROTEINASES AND DIGESTION OF SKIN MACROMOLECULES DURING SKIN PENETRATION

ALAN BROWN, NADINE GIROD, ELLEN E. BILLETT, AND DAVID I. PRITCHARD

Department of Life Sciences, Nottingham Trent University, Nottingham, United Kingdom; School of Biology, Nottingham University, Nottingham, United Kingdom

Abstract. The infective larvae of *Necator americanus* were shown to secrete all mechanistic classes of proteolytic enzymes with two overall pH optima of 6.5 and 8.5 using fluorescein isothiocyanate-labeled casein as the substrate. Since infective larvae are obligate skin penetrators, the effect of each of these enzyme classes against macromolecules derived from human skin was examined. Larval secretions were shown to degrade collagen types I, III, IV, and V, fibronectin, laminin, and elastin. All the skin macromolecules tested were hydrolyzed by aspartyl proteinase activity, which was inhibitable by pepstatin A. Collagen and elastin was also hydrolyzed by metalloproteinase activity, while the serine proteinase activity hydrolyzed only elastin. As a consequence of these experiments, the effect of proteinase inhibitors on the penetration of live larvae through hamster skin was tested. Larval penetration was significantly inhibited only by pepstatin A, confirming the importance of the aspartyl proteinase activity during the skin penetration process.

Necator americanus is a human pathogen that invades the body by penetrating the skin. In 1982 Matthews[1] showed that cellular destruction of the skin during larval penetration through the epidermis was mediated by an undefined enzymatic process, optimally active at pH 8. Subsequently, Salafsky and others[2] developed a gelatin-agar membrane as a model for hookworm skin penetration and showed that serine, and possibly cysteinyl, proteinase activities were responsible for larval penetration through this type of membrane. The presence of cysteinyl and serine proteinases in larval secretions was confirmed using gelatin substrate sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), [3] and it has been further demonstrated that live *N. americanus* larvae secrete a gelatinolytic metalloproteinase proteinase.[4]

Despite the obvious presence of proteolytic enzymes in the excretory-secretory (ES) products of *N. americanus* larvae, an exact role for each of these proteinases has yet to be defined. Consequently, and given the fact that the larvae are obligate skin penetrators, we now describe attempts to assign a role during skin penetration for the previously described proteinases secreted by *N. americanus* larvae. The hydrolysis of a number of skin macromolecules has been studied and the enzymes responsible for their degradation have been implicated using standard proteinase inhibitors. Despite being a human parasite, *N. americanus* can also be maintained in the golden hamster.[5] Therefore, to confirm the data obtained using individual skin macromolecules, the effects of proteinase inhibitors on the penetration of live larvae through excised hamster skin was also studied.

MATERIALS AND METHODS

Preparation of *N. americanus* larval ES products. *Necator americanus* was maintained in syngeneic DSN hamsters as described by Sen and Seth.[5] Infective larvae were cultured from fecal material by a method modified from Harada and Mori[6] and previously described by Kumar and Pritchard.[3] Cultured larvae were stored in 50 mM Na_2HPO4, 70 mM NaCl, 15 mM KH_2PO_4, pH 7.4, until used.[7]

Ensheathed larvae in 20 ml of storage buffer were exsheathed by bubbling carbon dioxide through the larval suspension for 2 hr at room temperature. Exsheathed larvae were allowed to settle and then washed extensively in RPMI 1640 medium containing 100 IU/ml of penicillin, 100 μg/ml streptomycin, and 1% amphotericin B under sterile conditions. Following this sterilization period, the larvae were cultured for an additional 72 hr at 37°C; the culture medium was changed every 24 hr. The ES products collected were dialyzed against water, lyophilized, and stored at −20°C until used. The protein concentration of ES products was estimated using the Bio-Rad (Hercules, CA) protein assay kit[8] with bovine serum albumin standards.

Assay of proteinase activity. *Fluorescein isothiocyanate (FITC)–labeled casein.* The proteinase activities present in larval ES products were examined over a range of pH values by monitoring the hydrolysis of FITC-labeled casein.[9] Larval ES products (50 μl, 10 μg) were mixed with 10 μl of FITC-casein (final concentration = 250 μg/ml) and 140 μl of 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) in a microfuge tube. Reactions were incubated at 37°C for 3 hr after which 120 μl of 5% trichloroacetic acid was added to stop the reaction. The tubes were allowed to stand at room temperature for 1 hr and the undigested, precipitated protein was removed by centrifugation at 13,000 × *g* for 10 min. Triplicate, 20-μl aliquots of the supernatant were added to 980 μl of 500 mM Tris-Cl, pH 8.5, and the quantity of fluorescence was measured (excitation detection = 490 nm and emission detection = 525 nm). The differential involvement of proteinase classes was investigated using class differentiating enhancers and inhibitors. These were added to the reaction in 20-μl volumes to give final concentrations of 5 mM cysteine, 1 μM E64, 50 μM 4-(amidinophenyl)methane sulfonyl fluoride (APMSF), 1 mM 1,10-phenanthroline, 10 mM EDTA, and 1 μM pepstatin A. Ethanol (0.1% final concentration), used to dissolve the pepstatin A and 1,10-phenanthroline, had no effect on proteinase activity. The APMSF was always freshly prepared due to its short half-life (6 min). In all assays where inhibitors were used, ES products were preincubated with inhibitor for 30 min before the addition of the substrate.

Synthetic peptide fluorogenic substrates. Proteolytic activ-

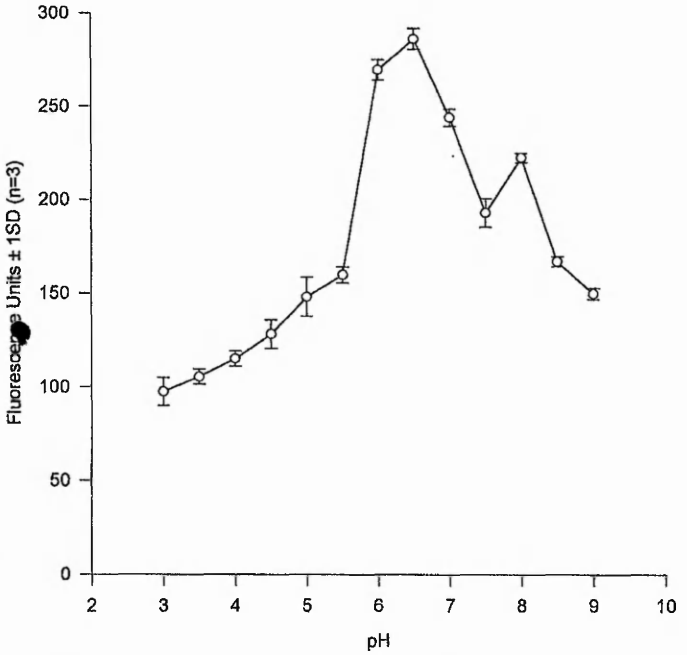


FIGURE 1. The pH optima of the proteinase activities present in the excretory-secretory (ES) products of *Necator americanus* larvae. Larval ES products were incubated with fluorescein isothiocyanate–casein over a range of pH points as described in the Materials and Methods. Proteinase activity is expressed as the mean ± SD number of fluorescence units (n = 3) emitted over a 1-hr period following the subtraction of a nonenzymatic buffer control.

ity was assessed by monitoring the release of 7-amino-4-methylcoumarin (AMC) from the synthetic peptide substrates Tosyl-Gly-Pro-Arg-AMC.HCl (Novabiochem, Nottingham, United Kingdom), Glutaryl-Gly-Arg-AMC.HCl and H-Pro-Phe-Arg-AMC.2HCl,[10] Suc-Ala-Ala-Pro-Phe-AMC,[11] Suc-Leu-Leu-Val-Tyr-AMC,[12] Glutaryl-Gly-Gly-Phe-AMC,[13] H-Arg-AMC.2HCl and Z-Arg-Arg-AMC.2HCl,[14] Z-Phe-Arg-AMC.HCl,[15] and Z-Arg-AMC.HCl.[16] The substrate was first dissolved in 100% dimethylsulfoxide to give a 5 mM stock solution and then further diluted to an assay concentration of 50 μM. Larval secretions (50 μl, 10 μg) were incubated with 10 μl of substrate in a final volume of 1 ml of the relevant buffer (as described previously) at the required pH. Samples were incubated at 37°C for 1 hr, after which the reaction was stopped by the addition of glacial acetic acid to a final concentration of 300 mM,[17] and the fluorescence was measured (excitation detection = 365 nm, emission detection = 465 nm). Inhibitors and enhancers used in synthetic substrate assays were prepared and used at the concentrations described previously. Dimethylsulfoxide (1% final concentration), used to solubilize the substrates, had no effect on proteinase activity.

Degradation of skin macromolecules. *Hydrolysis of elastin.* Elastinolytic activity was measured by monitoring the release of dye from elastin-orcein.[18] One milligram of elastin-orcein was mixed with 20 μg of larval ES products and the reaction was adjusted to a volume of 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 (13,000 × *g* for 10 min), and the absorbance of the sample was measured at 550 nm. Inhibitors and enhancers were used as described previously.

TABLE 1

Characterization of the proteinase activities present in the excretory-secretory (ES) products of *Necator americanus* larvae*

ES products alone (fluorescence units/hr)	pH 6.5 269.7 ± 19.2	pH 8 199.5 ± 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 fluorescein isothiocyanate–casein at 37°C for 3 hr as described in the Materials and Methods. Proteinase activity is expressed as the mean ± 1 SD number of fluorescence units emitted (n = 4). Inhibition of proteinase activity by a number of inhibitors is shown as the percentage inhibition/enhancement of activity compared with an uninhibited control following the subtraction of a nonenzymatic blank. APMSF = 4-(amidinophenyl)methane sulfonyl fluoride.

Hydrolysis of collagen, fibronectin, and laminin. Ten micrograms 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 incubation for 4 hr at 37°C, undigested protein and any breakdown products were precipitated with ice-cold acetone (final concentration = 80%) and the pellet was resuspended in 20 μl of SDS-PAGE reducing sample buffer.[19] In the case of collagen, one unit of collagenase 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.[20] Western blots were blocked for 1 hr with 5% milk powder in Tris-buffered saline (TBS) before being probed with rabbit antisera raised to the relevant skin protein (diluted 1:500 in blocking solution) and incubated overnight at 40°C. After washing the blots with TBS/0.05% Tween 20, they were incubated with alkaline phosphatase–conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO; diluted 1:1,000 in blocking solution). Antibody binding was visualized using 5-bromo-4chloro-3-indolyl phosphate/nitroblue tetrazolium as the substrate.

Penetration of *N. americanus* through hamster skin. *Preparation of hamster skin.* The penetration of *N. americanus* through hamster skin was assessed using a method previously described by Kumar and Pritchard.[21] The abdomen of freshly killed adult DSN hamsters was wetted, shaved, and the skin was carefully removed (six pieces of skin were taken from one animal). The skin was mounted on the open ends of a 7-cm long by 1.4-cm diameter plastic tube such that the outer skin surface faced the inside of the tube. The skin was secured tightly to the tube by an elastic band. The tube was then placed in a plastic bottle (9-cm long by 2.5-cm wide), containing 5 ml of Dulbecco's minimal essential medium supplemented with 1% amphotericin B, 100 IU/ml of penicillin. and 100 μg/ml of streptomycin. Care was taken to ensure that the inner skin surface was

TABLE 2

Further characterization of the proteinases present in the excretory-secretory (ES) products of *Necator americanus* larvae using fluorogenic substrates*

Substrate	Substrate for	Proteolytic activity (fluorescence units/hr)
Tosyl-Gly-Pro-Arg-AMC.HCl	Thrombin and plasmin	324
Glutaryl-Gly-Arg-AMC.HCl	Urokinase	60.6
H-Pro-Phe-Arg-AMC.2HCl	Kallikreins and elastase	263.8
Suc-Ala-Ala-Pro-Phe-AMC	Chymotrypsin	59.4
Suc-Leu-Leu-Val-Tyr-AMC	Chymotrypsin	12.8
Glutaryl-Gly-Gly-Phe-AMC	Chymotrypsin and cathepsin G	18.36
Suc-Ala-Ala-Ala-AMC	Elastase	14.64
Z-Arg-AMC.HCl	Trypsin and papain	750
Z-Phe-Arg-AMC.HCl	Kallikreins, papain, cathepsin B, and cathepsin L	423.3
H-Arg-AMC.2HCl	Cathepsin H and aminopeptidase B	78.96
Z-Arg-Arg-AMC.2HCl	Cathepsin B	217.8

* 10 μg of larval 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-hr period after the subtraction of a negative control value. AMC = 7-amino-4-methylcoumarin.

completely in contact with the medium and that no leakage occurred back across the skin to the upper surface.

Time course of skin penetration. Two hundred 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 hr, and the number of larvae that had failed to penetrate the skin and had successfully penetrated the skin carefully were counted.

Effect of proteinase inhibitors on larval penetration through hamster skin. Two hundred 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 hr, 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 a nonparametric one-way analysis of variance with $P \leq 0.05$ being considered statistically significant.

All animal experiments were carried out in accordance with the Animals (Scientific Procedures) Act, 1986.

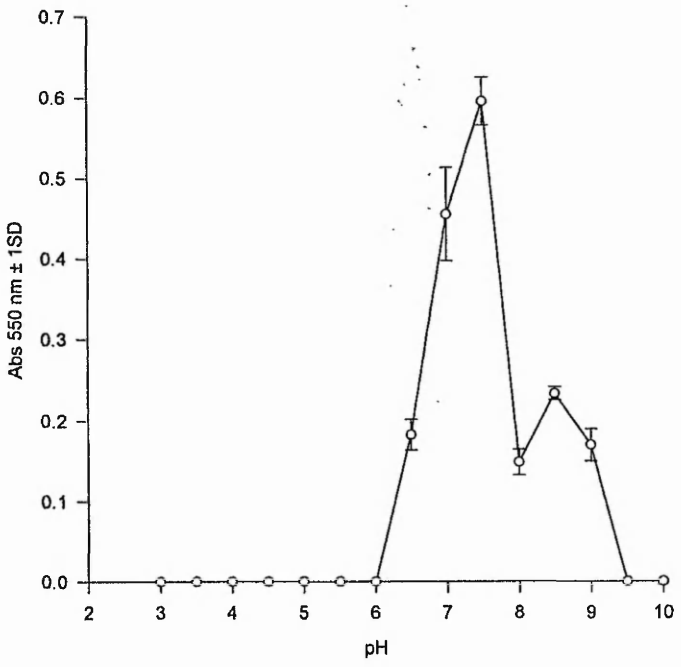


FIGURE 2. The pH optima of the proteinase activities responsible for elastin degradation in the excretory-secretory (ES) products of *Necator americanus* larvae. Larval ES products were incubated with 1 mg of elastin-orcein over a range of pH points. Proteinase activity is expressed as the mean ± SD (n = 3) absorbance (Abs) at 550 nm after 16 hr incubation following the subtraction of a nonenzymatic buffer control.

RESULTS

Characterization 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 1). Proteinase activity at these two pH points was characterized by the use of class differentiating inhibitors (Table 1). At pH 6.5, proteinase activity was primarily inhibited (78.9 %) by pepstatin A, indicating the presence of an aspartyl proteinase. 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 protease at this pH. Inhibition by the metalloprotease inhibitors 1,10-phenanthroline and EDTA was lower and remained similar (approximately 32% and 10.8%, respectively) over the two pH points tested. At pH

TABLE 3

Characterization of the proteinase activities responsible for elastin degradation in the excretory-secretory (ES) products of *Necator americanus* larvae*

ES products alone (abs at 550 nm)	pH 7.5 0.124 ± 0.006	pH 8.5 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. Values are the mean ± 1 SD elastinolytic activity of three batches of larval ES products and the percentage inhibition/enhancement of elastinolytic activity compared with an uninhibited control following the subtraction of a nonenzymatic blank. Abs = absorbance; APMSF = 4-(amidinophenyl)methane sulfonyl fluoride.

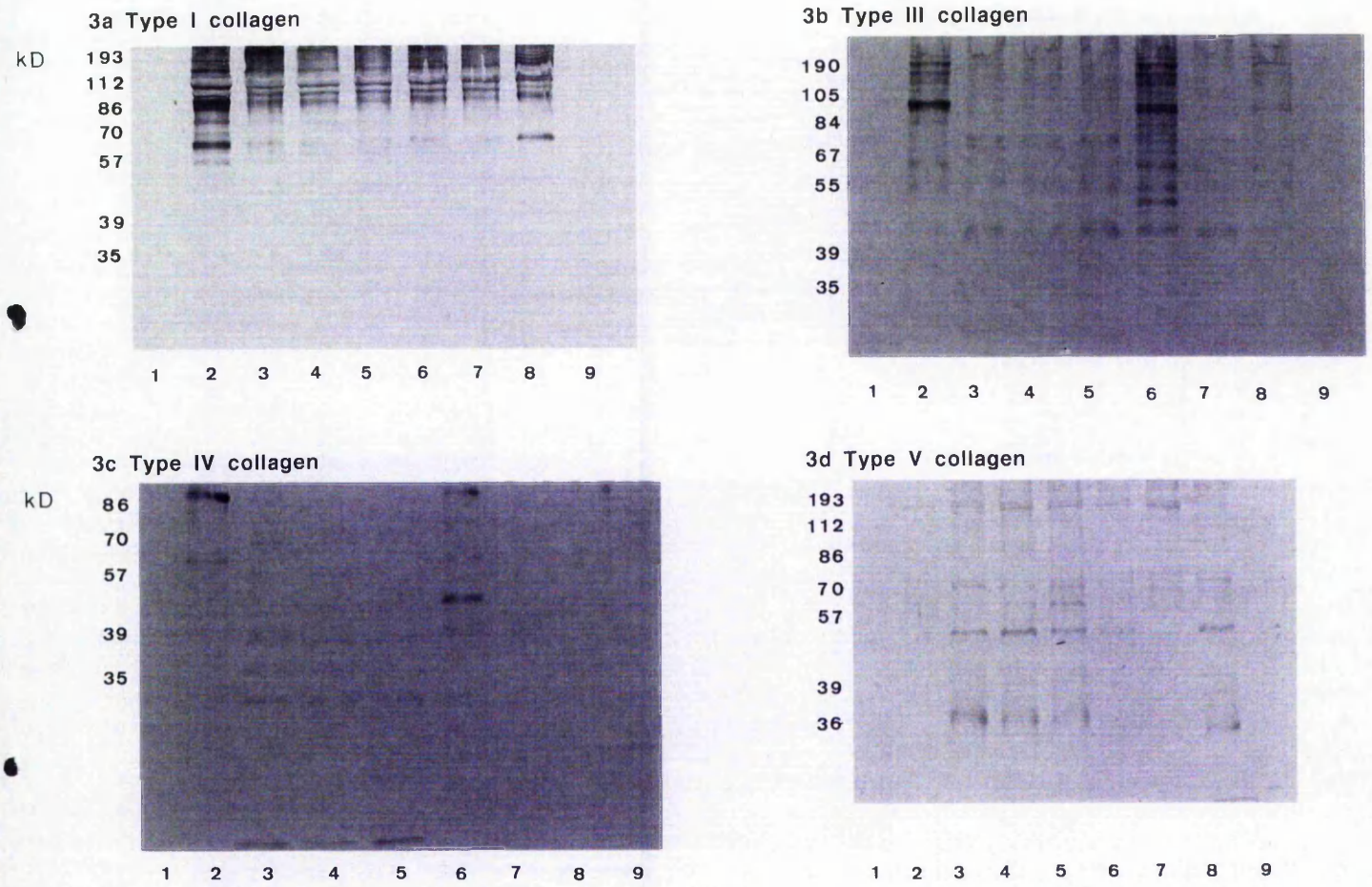


FIGURE 3. The breakdown of collagen types I, III, IV, and V by *Necator americanus* larval excretory-secretory (ES) products. Lane 1, larval ES products; lane 2, collagen alone; lane 3, collagen plus larval ES products; lane 4, 1 μM E64; lane 5, 50 μM 4-(amidinophenyl)methane sulfonyl fluoride; lane 6, 1 mM 1,10-phenanthroline; lane 7, 1 mM EDTA; lane 8, 1 μM pepstatin A; lane 9, collagenase. Molecular mass markers are indicated in kilodaltons (kD).

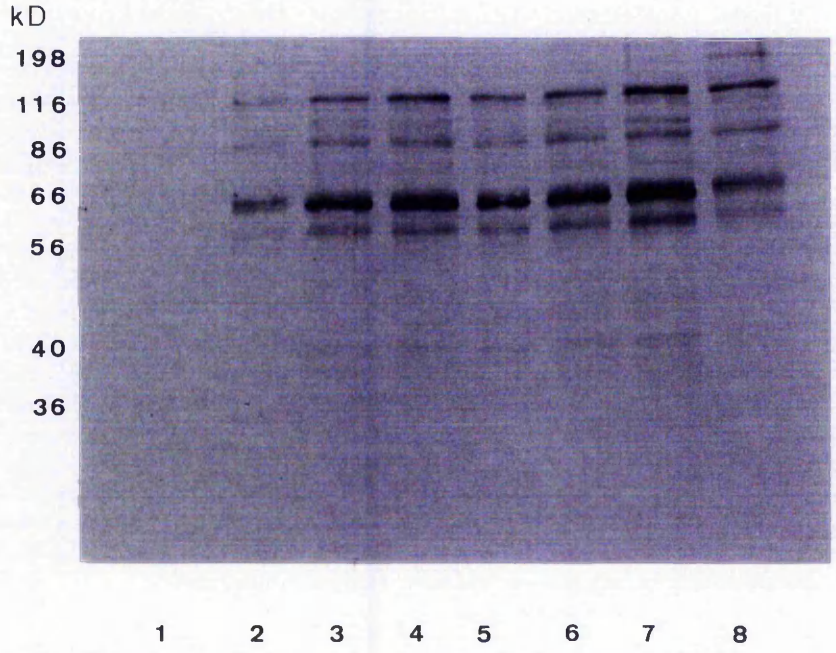


FIGURE 4. The breakdown of fibronectin by *Necator americanus* larval excretory-secretory (ES) products. Lane 1, larval ES products; lane 2, fibronectin; lane 3, fibronectin plus larval ES products; lane 4, 1 μM E64; lane 5, 50 μM 4-(amidinophenyl)methane sulfonyl fluoride; lane 6, 1 mM 1,10-phenanthroline; lane 7, 1 mM EDTA; lane 8) 1 μM pepstatin A. Molecular mass markers are indicated in kilodaltons (kD).

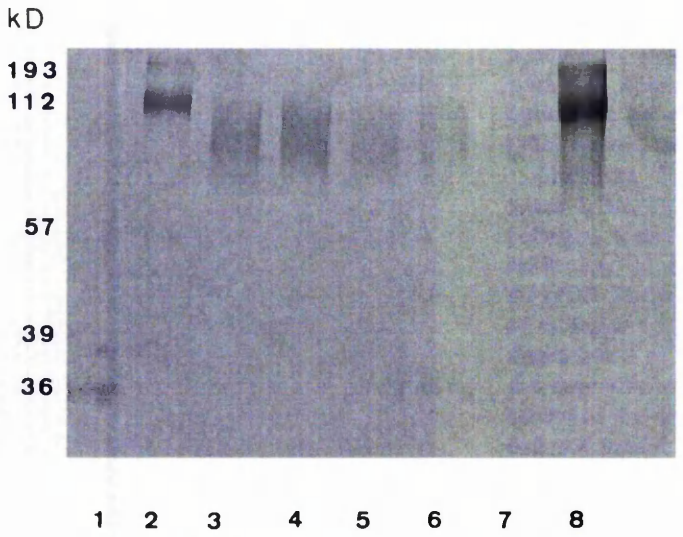


FIGURE 5. The breakdown of laminin by *Nector americanus* larval excretory-secretory (ES) products. Lane 1, larval ES products; lane 2, laminin; lane 3, laminin plus larval ES products; lane 4, 1 μM E64; lane 5, 50 μM 4-(amidinophenyl)methane sulfonyl fluoride; lane 6, 1 mM 1,10-phenanthroline; lane 7, 1 mM EDTA; lane 8, 1 μM pepstatin A. Molecular mass markers are indicated in kilodaltons (kD).

6.5, the cysteinyl protease inhibitor E64 inhibited activity by 23.3%, with this being reduced to 13.2% at pH 8. Similarly, cysteine enhanced activity by 11.4% at pH 6.5 and by only 7.4% at pH 8. These data suggest that at the pH closest to that of normal skin (pH 5.5), the predominant proteinase activity is aspartyl in nature.

Further characterization using synthetic peptide substrates. The proteinase activity in larval ES products was further characterized by the use of a number of fluorescent substrates (Table 2). All assays were carried out at pH 6.5 because this was the pH optimum closest to that of normal skin. These data further confirm the presence of cysteinyl and serine proteinase activities in larval secretions. None of the chymotryptic substrates tested were readily cleaved by larval ES products. The ES products also failed to cleave the elastase substrate Suc-Ala-Ala-Ala-AMC and the cathepsin H/aminopeptidase substrate H-Arg-AMC.2HCl. The ES products did, however, cleave the tryptic substrates Tosyl-Gly-Pro-Arg-AMC.HCl, H-Pro-Phe-Arg-AMC.2HCl, the dual trypsin/papain substrates Z-Arg-AMC.HCl and Z-Phe-Arg-AMC.HCl, and the cathepsin B substrate Z-Arg-Arg-AMC.2HCl.

Degradation of skin macromolecules. *Elastin.* Elastin hydrolysis was shown to be optimal at pH 7.5 with a lesser peak of activity at pH 8.5 (Figure 2). No hydrolysis of elastin was observed between pH 3 and 6 and above pH 1 0. Elastin hydrolysis was characterized at pH 7.5 and 8.5 using proteinase inhibitors (Table 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 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%) with 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.

Collagen. Figure 3 shows the breakdown of Collagen types I, III, IV, and V by larval ES products. All types of collagen tested were degraded by larval ES products. No inhibition of collagen breakdown was seen with E64 or APMSF. Phenanthroline partially inhibited the degradation of collagen types III and IV. EDTA completed inhibited the degradation of types I and V collagen and partially inhibited the degradation of type IV collagen. Pepstatin A completely inhibited the degradation of collagen types I, III, and IV but did not inhibit the breakdown of type V collagen. The inhibition of collagen hydrolysis by phenanthroline and EDTA may indicate a role for the secreted metalloproteinase, and the inhibition by pepstatin A again provides evidence for the importance of the aspartyl proteinase during skin penetration.

Fibronectin. Fibronectin (Figure 4) was hydrolyzed to a protein of 40 kD. Unlike collagen, only the aspartyl proteinase was capable of degrading fibronectin. Interestingly, when proteolysis was inhibited by pepstatin A, some cross-linking of fibronectin occurred, resulting in the enhanced formation of a higher molecular mass protein of 145 kD.

Laminin. Laminin (Figure 5) was also degraded by larval ES products. A breakdown product of approximately 90 kD was observed. As was the case for fibronectin, this degradation was only inhibited by pepstatin A.

Time course of third-stage larval penetration through hamster skin and the effect of proteinase inhibitors. Figure 6A shows the time course of larval penetration through hamster skin. The results are expressed as the mean ± SD number of larvae failing to enter the skin or the mean ± SD number of larvae fully penetrating the hamster skin (six determinations were carried out at each time point). The larvae entered the skin rapidly and after 3 hr 196 (98%) larvae had entered the hamster skin increasing to 198 (99%) after 48 hr. Three hours after the application of larvae to the hamster skin, only two (1%) of the larvae had completely traversed the skin, increasing to 57.7 (28.8%) after 24 hr and 84 (42%) by 48 hr. It was necessary to obtain proteinase inhibitor data while the skin was still in as natural a condition as possible. Since the percentage of larvae fully penetrating the skin only increased by an additional 13.3% between 24 and 48 hr, the effects of proteinase inhibitors were studied after incubation for 24 hr.

Figure 6B shows the effect of proteinase inhibitors on larval skin penetration. This figure represents the mean ± SD of nine determinations for each control and inhibitor tested. When the number of larvae fully penetrating the skin after 24 hr was determined, all the inhibitors tested inhibited skin penetration to some degree. However, significant inhibition of skin penetration was observed only in the presence of pepstatin A (54.8%; $P \leq 0.05$) when compared with the ethanol control.

DISCUSSION

These data confirm the presence of cysteinyl, serine, and metalloproteinases in larval ES products as described by Ku-

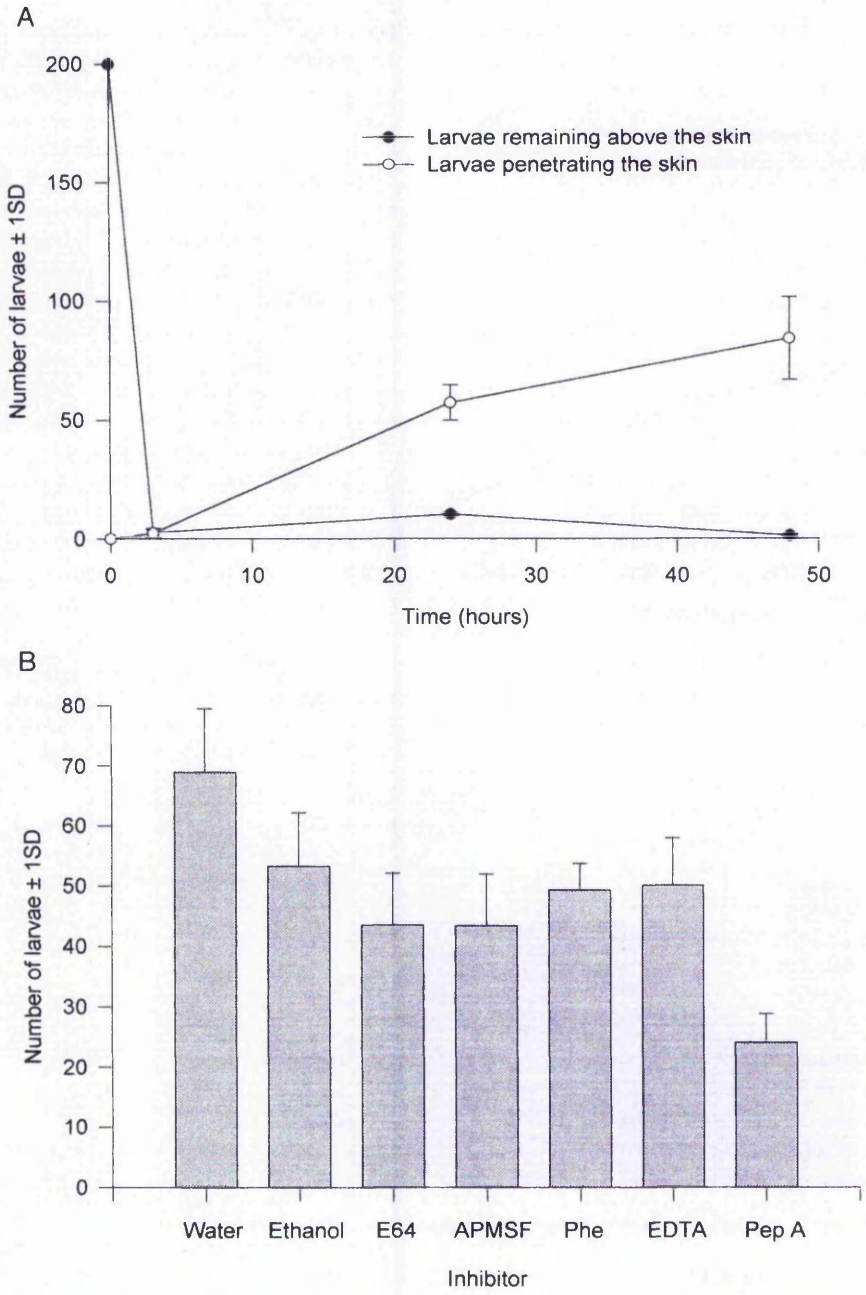


FIGURE 6. **A,** the time course of larval penetration through hamster skin. Two hundred *Necator americanus* larvae were placed on the surface of excised hamster skin. After 3, 24, and 48 hr incubation at 37°C, triplicate samples were removed from the incubator and the mean ± SD number of larvae remaining above the skin and fully penetrating the skin was determined. **B,** the effect of proteinase inhibitors on the penetration of *N. americanus* larvae through hamster skin. Two hundred *N. americanus* larvae were placed on excised hamster skin in the presence and absence of proteinase inhibitors. The mean ± SD number of larvae fully penetrating the skin was determined after 24 hr incubation at 37°C. APMSF = 4-(amidinophenyl)methane sulfonyl fluoride; Phe = 1,10-phenanthroline; Pep A = pepstatin A.

mar and Pritchard.[3,4] In addition to these activities, the presence of aspartyl proteinase activity has been demonstrated for the first time Using FITC-labeled casein as the substrate, the pH optimum of the overall proteinase activity secreted by *N. americanus* larvae was shown to be pH 6.5, with a lesser peak of activity at pH 8 (Figure 1). At pH 6.5 and pH 8, the proteinase activities were characterized using a number of proteinase inhibitors (Table 1) At pH 6.5, the predominant proteinase activity was shown to be aspartyl in nature. At pH 8, the activity of the aspartyl proteinase was greatly reduced, with the serine proteinase activity becoming predominant. This is reminiscent of the mixture of proteinase activities secreted by adult *N. americanus.*[22] However, unlike adult ES products, little activity was seen at pH 3.5.

The serine protease activity was shown to be trypsin-like in its nature failing to cleave any of the chymotryptic substrates tested (Table 2). Interestingly, although larval ES products were shown to be capable of degrading elastin, the elastase substrate Suc-Ala-Ala-Ala-AMC was not hydrolyzed. This may indicate that a true elastase activity is not present in *N. americanus* larval ES products, and that elastin degradation is a result of metallo, serine, and aspartyl pro-

teinases with a more general substrate specificity. The cysteinyl proteinase activity was shown to be cathepsin B- and L-like, hydrolyzing both the cathepsin B substrate Z-Arg-ArgAMC.2HCI and the cathepsin B and L substrate Z-Phe-Arg-AMC.HCI. Similar activities have also been described in the ES products of the adult hookworm.[22]

Elastase activity, measured by the release of dye from elastin orcein, was shown to be optimal at pH 7.5, with a smaller peak of activity at pH 8.5 (Figure 2). This shift in the pH optima against elastin compared with FITC-casein reflects 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 this inhibition was reduced to 63.2%. Conversely, inhibition by APMSF increased from 9.4% to 46.7% (Table 2). This suggests that the metalloproteinase capable of elastin degradation is optimally active around pH 7.5, shifting the peak of activity seen against FITC-casein (containing thiol and mainly aspartyl activities) from pH 6.5 to 7.5. The peak of elastin hydrolysis at pH 8.5 is reflective of the enhanced serine activity seen at pH 8 against FITC-casein.

The ability of *N. americanus* larval ES products to degrade human collagen, fibronectin, and laminin was also examined using SDS-PAGE and Western blotting. No inhibition was seen with E64 or APMSF, implying that the cysteinyl and serine proteinases play no part in the degradation of these skin macromolecules during skin penetration. The degradation of gelatin observed by Kumar and Pritchard[3] by larval cysteinyl and serine proteases may be explained by the denatured state of the collagen, which allows these secreted proteolytic enzymes access to susceptible peptide bonds not normally available.

The results shown in Figures 3–5 demonstrate the possible 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 metalloproteinase may also be important to the skin penetration process, although its substrate specificity appears to be limited to collagen and elastin. Indeed, it is possible to speculate that there may be two metalloenzymes with different inhibitor sensitivities and substrate specificities. One enzyme appears to degrade collagen types III, IV, and elastin and is sensitive to phenanthroline while the other degrades types I and V collagen and appears to be sensitive to EDTA.

The degradation profile of skin macromolecules shown by *N. americanus* larvae is consistent with the proposal put forward by Hotez and others[23] 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 an aspartyl proteinase activity has been demonstrated in the secretions of adult *Haemonchus contortus,*[24] and a cDNA encoding a cathepsin D-like aspartyl proteinase has recently been identified from the dog hookworm *Ancylostoma caninum.*[25]

These data suggest that *N. americanus* larvae primarily facilitate skin penetration by the use of a mixture aspartyl and metalloproteases, with a serine protease contributing to the breakdown of elastin. Similarly, a potent histolytic metalloprotease capable of degrading elastin and fibronectin but not type I collagen has been described in the secretions of *Strongyloides stercoralis.*[26] Inhibition of this enzyme with a metalloprotease inhibitor prevented larval penetration of the skin. The importance of the aspartyl proteinase during skin penetration was further confirmed by the experiments carried out using excised hamster skin. Larval penetration was significantly inhibited by pepstatin A (54.8%). All the other inhibitors tested had some effect on larval penetration. Possible roles for the serine and metalloenzymes have already been discussed but it was interesting to note the inhibition of larval penetration by E64 (36.7%). While not being statistically significant, this may imply that in this system the cysteinyl proteinases could still play a role in skin penetration, perhaps degrading a skin protein not yet studied.

In conclusion, although it has been known for a long time that the larvae of *N. americanus* secrete a range of proteinases, the relevance of these to pathogenesis has not been determined. From the data presented here, it is highly likely that a secreted aspartyl proteinase is a major virulence factor in the host parasite relationship.

Financial support: We acknowledge the financial support of The Wellcome Trust, The Sir Halley Stewart Trust, The Nottingham Trent University, and Nottingham University. Nadine Girod was supported by a grant from the European Social Fund.

Authors' addresses: Alan Brown and David I. Pritchard, School of Biology, Nottingham University, University Park, Nottingham, NG7 2RD, United Kingdom. Nadine Girod and Ellen E. Billett, Department of Life Sciences, Nottingham Trent University, Clifton Lane, Nottingham, NG11 8NS, United Kingdom.

Reprint requests: Alan Brown, School of Biology, Nottingham University, University Park, Nottingham, NG7 2RD, United Kingdom.

REFERENCES

1. Matthews BE, 1982. Skin penetration by *Necator americanus* larvae. *Z Parasitenkd 68:* 81–86.
2. Salafsky B, Fusco AC, Siddiqui A, 1990. *Necator americanus:* factors influencing skin penetration by larvae. Schad GA, Warren KS, eds. *Hookworm Disease: Current Status and New Directions.* London: Taylor and Francis, 326–329.
3. Kumar S, Pritchard DI, 1992. The partial characterisation of proteases present in the excretory/secretory products and exsheathing fluid of the infective (L3) larva of *Necator americanus. Int J Parasitol 22:* 563–572.
4. Kumar S, Pritchard DI, 1992. Secretion of metalloproteases by living infective larvae of *Necator americanus. J Parasitol 78:* 917–919.
5. Sen HG, Seth D, 1967. Complete development of the human hookworm, *Necator americanus,* in golden hamsters, *Mesocricetus auratus. Nature 214:* 609–610.
6. Harada Y, Mori O, 1955. A new method of culturing hookworms. *Y Acta Med 1:* 177–179.
7. Hawdon JM, Schad GA, 1991. Long term storage of hookworm infective larvae in buffered saline solution maintains larval responsiveness to host signals. *J Helminthol Soc (Wash) 58:* 140–142.
8. Bradford MM, 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem 72:* 248–254.
9. Beynon RJ, Bond JS, 1989. *Proteolytic Enzymes—A Practical Approach.* Oxford, United Kingdom: IRL Press.
10. Lottenberg R, Christensen U, Jackson CM, Coleman PL, 1981. Assay of coagulation proteases using peptide chromogenic and fluorogenic substrates. *Methods Enzymol 80:* 341–361.
11. Graf L, Cralk CS, Patthy A, Roczniak S, Fletterick RJ, Rutter

WJ, 1987. Selective alteration of substrate specificity by replacement of aspartic acid with lysine in the binding pocket of trypsin. *Biochemistry 26:* 2616–2623.
12. Ishura S, Sano M, Kamakura K, Sugita H, 1985. Isolation of two forms of the high molecular mass serine protease, ingensin, from porcine skeletal muscle. *FEBS Lett 198:* 119–123.
13. Hardy MF, Mantle D, Pennington RJT, 1983. Characteristics of a new muscle proteinase. *Biochem Soc Trans 11:* 348–349.
14. Barrett AJ, 1980. Fluorometric assays for cathepsin B and cathepsin H with methylcoumarylamide substrates. *Biochem J 187:* 909–912.
15. Morita T, Kata H, Iwanaga S, Takada K, Kimura T, Sakakibara S, 1977. New fluorogenic substrates for α-thrombin, factor Xa, kallikreins, and urokinase. *J Blochem 82:* 1495–1498.
16. Zimmerman M, Ashe BM, Yurewicz EC, Patel G, 1977. Sensitive assays for trypsin, elastase and chymotrypsin using new fluorogenic substrates. *Anal Biochem 78:* 47–51.
17. Dowd AJ, Dalton JP, Loukas AC, Prociv P, Brindley PJ, 1994. Secretion of cysteine proteinase by the zoonotic hookworm *Ancylostoma caninum. Am J Trop Med Hyg 51:* 341–347.
18. Knox DP, Kennedy MW, 1988. Proteinases released by the parasitic larval stages of *Ascaris suum* and their inhibition by antibody. *Mol Biochem Parasitol 28:* 207–216.
19. Laemmli UK, 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227:* 680–685.
20. Towbin H, Staehelin T, Gordon J, 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications *Proc Natl Acad. Sci USA 76:* 4350–4354.
21. Kumar S, Pritchard DI, 1992. Skin penetration by ensheathed third-stage infective larvae of *N. americanus,* and the host's immune response to larval antigens. *Int. J Parasitol 22:* 573–579.
22. Brown A, Burleigh, Billett EE, Pritchard DI, 1995. An initial characterisation of the proteolytic enzymes secreted by the adult stage of the human hookworm *Necator americanus. Parasitology 110:* 555–563.
23. Hotez P, Haggerty J, Hawdon J, Milstone L, Gamble HR, Schad G, Richards F, 1990. Metalloproteinases of infective *Ancylostoma* hookworm larvae and their possible functions in tissue invasion and ecdysis. *Infect Immun 58:* 3883–3892.
24. Karanu FN, Rurangirwa FR, McGuire TC, Jasmer DP, 1993. *Haemonchus contortus:* identification of proteases with diverse characteristics in adult worm excretory-secretory products. *Exp Parasitol 77:* 362–371.
25. Harrap SA, Prociv P, Brindley PJ, 1996. ACASP, A gene encoding a cathepsin D-like protease from the hookworm *Ancylostoma caninum. Biochem Biophys Res Comm 227:* 294–302.
26. McKerrow JH, Brindley P, Brown M, Gam AA, Staunton C, Neva F, 1990. *Strongyloides stercoralis:* identification of a protease that facilitates penetration of skin by infective larvae. *Exp Parasitol 70:* 134–143.