Immunochemical analysis of antigens of the bovine lungworm *Dictyocaulus viviparus*

A thesis presented for the degree of

Doctor of Philosophy

by

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ABBREVIATIONS

APS	ammonium persulphate
BZ	benzoyl
CBZ	carbobenzoxy
CF	complement fixation
Ci	Curie
cpm	counts (of radioactivity) per minute
CTAB	cetyltrimethyl ammonium bromide
°C	degrees Celsius
^{60}Co	Cobalt 60
CO_2	carbon dioxide
DOC	sodium deoxycholate
DTT	dithiothreitol
E-64	L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane
EDTA	ethylenediaminetetra acetic acid
ELISA	enzyme-linked immunosorbent assay
ES	excretory-secretory
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
FITC	fluorescein isothiocyanate
g	gram
g	relative centrifugal force
Gy	Gray(s)
h	hour(s)
4-HMB	4-hydroxymethoxybenzaldehyde
IHA	indirect haemagglutination
Ig	immunoglobulin
i.p.	intraperitoneal
IU	international unit
i.v.	intravenous
kDa	kiloDalton
kg	kilogram
krad	kilorad
L1,L2,..L5	larval stages first to fifth
M	molar

mA	milliampere
2ME	2-mercaptoethanol
MEM	Minimal Essential Medium
mg	milligram
MHC	major histocompatibility complex
min	minute(s)
ml	millilitre
mM	millimolar
M_r	relative molecular mass
NA	nitroanilide
NBCS	newborn calf serum
NEM	N-ethylmaleimide
nm	nanometre
NPE	nitrophenol ester
O.D.	optical density
OPD	orthophenyl diamine
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC	phosphorylcholine
Pms-F	phenylmethylsulphonyl fluoride
S.D.	standard deviation
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
TCA	trichloroacetic acid
TEM	triethylmaleimide
Tris	tris(hydroxymethyl)aminomethane
µM	micromolar
U.V.	ultraviolet
V	volt

SUMMARY

A successful irradiated larval vaccine against the cattle lungworm, *Dictyocaulus viviparus*, has been available for over thirty years. At the outset of the project, however, little was known of antigens of the parasite or of the mechanism of vaccine-induced immunity. This study aimed to characterise antigens of this parasite which may be involved in protective immunity and which could eventually lead to the development of a molecularly-defined vaccine against lungworm infection. The antibody responses to surface, somatic and excretory-secretory (ES) products of larval and adult stage parasites were examined following infection or vaccination of bovine hosts and of the guinea-pig model host in which infection proceeds only to the L5 stage.

No parasite proteins could be detected in ES products of infective stage larvae, despite containing substantial levels of proteinase activity. In contrast, a complex range of proteins were detected in the secretions of adult worms. Immunoprecipitation studies of radioiodinated adult ES products revealed that all of these, with the exception of two components, one of which was identified as bovine serum albumin, are antigenic to infected bovine hosts. Calves vaccinated with irradiated larvae, and therefore, not exposed to patent lungworm infection showed restricted recognition of adult ES products, thus demonstrating the stage-specific nature of *D. viviparus* released products.

Significant heterogeneity in the specificity of the antibody response to adult ES products was observed between individual bovine and guinea-pig hosts. This individual variability was examined in the model system using inbred strains of guinea-pig and was shown to have a genetic basis, possibly being controlled by the MHC class II region.

Examination of the antibody response to surface-exposed antigens of the egg, L1, L3 and adult stages of *D. viviparus* demonstrated both the antigenicity and stage-specificity of surface components. Immunofluorescence studies revealed significant recognition of the L3 sheath by antibody from infected and vaccinated bovine and guinea-pig hosts. In contrast, surface-exposed antigens of the adult, egg and L1 stages were recognised uniquely by calves infected with normal larvae and , therefore, exposed to patent lungworm infection. No binding of parasite specific IgG antibody was

observed on the exposed surface of exsheathed L3 (i.e. the L3 cuticle) with serum from infected calves. All bovine pre-infection sera examined showed a substantial degree of IgM antibody binding to the L3 cuticle and it is proposed that this non-specific IgM antibody may block immune recognition of parasite-specific surface antigens. IgG antibody recognition of exposed L3 cuticular antigens was observed, however, with sera from hosts exposed to irradiated larvae suggesting that this immunoevasive mechanism may be overcome to some extent by vaccination.

As well as differing antigenically, the L3 cuticle was found to differ biophysically from that of other stages of *D. viviparus* as demonstrated by its inability to bind the fluorescent lipid analogue 5-(*N*-octadecanoyl)aminofluorescein and to incorporate Iodine-125 into cuticular proteins. These findings may reflect changes in the surface properties of the parasite associated with host infection. Radioiodination of intact sheathed larvae identified a restricted set of proteins while a complex set of labelled proteins was observed following radioiodination of intact adult parasites. Many more adult components were labelled by the Bolton-Hunter than by the Iodogen technique, probably reflecting that labelling by the latter method is more surface-restricted. There was no turnover of the major adult surface-associated antigens suggesting that surface components do not contribute to adult ES products of this parasite.

Examination of the biological functions of larval and adult extracts and ES products revealed the presence of superoxide dismutase (SOD) and proteinase activities. Characterisation of the latter by pH optima, substrate specificity, inhibitor sensitivity and substrate gel electrophoresis identified multiple proteolytic activities. These enzymes may be involved in parasite invasion and survival within the host. The significant inhibition of both proteinase and SOD activities observed following incubation with immunoglobulin from immune calves may, therefore, be important in limiting parasite survival and consequently such enzymes may be of value as potential vaccine candidates.

Finally, a comparison of ^{35}S-methionine labelled polypeptides of normal and irradiated third stage larvae revealed no qualitative nor quantitative differences. It is, therefore, proposed that vaccine-induced immunity to *D. viviparus* may not depend on the expression of novel parasite antigens but on an enhanced immune recognition of larval stage antigens.

CHAPTER 1

GENERAL INTRODUCTION

1.1 HISTORY OF *DICTYOCAULUS VIVIPARUS* AND PARASITIC BRONCHITIS

Parasitic bronchitis in cattle was first described by Nicholls in 1755, who attributed the disease to "small tape worms". Since then several names have been given to the nematode, but it was not until 1907 that the genus *Dictyocaulus* was created, with the species *viviparus* affecting cattle and deer, *filaria* affecting sheep and goats, and *arnfieldi* affecting horses and donkeys (Raillet and Henry, 1907).

Parasitic bronchitis usually affects calves in their first grazing season and is characterised by rapid breathing and coughing. The disease has caused great economic losses in temperate climates and, consequently, many studies were carried out in the early 1950's to develop a method of controlling the disease. Researchers at the University of Glasgow found significant protection against infection could be achieved by the administration of X-ray attenuated infective-stage larvae (Jarrett *et al.,* 1959a). This vaccine was launched commercially in 1959 and is still widely used 30 years later. Since this time, many studies examining the pathology, epidemiology, diagnosis and treatment of parasitic bronchitis have been carried out.

The success of the vaccine has, however, been detrimental to the study of *Dictyocaulus viviparus* infection to some extent. As yet, there are no definitive reports on the mechanism of immunity to the parasite, nor on the mode of action of the vaccine. The present study aims, in the longterm, to identify antigens of the parasite which may be involved in protective immunity in order to provide a better understanding of how the irradiated vaccine operates. This could eventually lead to the development of a new synthetic vaccine against *D. viviparus* without the need for live parasites. Of perhaps greater significance, understanding vaccine-induced immunity to this parasite will have relevance to the design of other anti-helminth vaccines against parasites of humans and domestic animals.

1.2 LIFE CYCLE

Dictyocaulus viviparus has a direct life cycle with an external free-living stage. Infection is initiated by the ingestion of infective third-stage larvae (L3) from contaminated pasture (Daubney, 1920). These larvae are often enclosed within a sheath comprising the cast cuticles of the second, and sometimes the first, larval stages, which is considered to provide protection for the infective larvae (Michel, 1969). It is generally thought that the larvae exsheath in the rumen (Poynter *et al.*, 1960) although there is no direct evidence for this. They then penetrate the intestinal wall, and pass to the mesenteric lymph nodes, where they moult to the fourth stage (Jarrett, McIntyre and Urquhart, 1957b). The larvae continue their lymphatic migration, entering the thoracic duct and are carried to the right heart, pulmonary circulation and the pulmonary capillaries. They then break out of the capillaries and enter the alveoli. L4 migrate via the bronchioles to the bronchi where they make their final moult to L5 stage, and then mature to adult worms. The life cycle of *D. viviparus* is summarised in Figure 1.1.

In the bronchi, females lay larvated eggs which are coughed up, swallowed and hatch to release first stage larvae during passage down the gut. These are then excreted in the faeces. First stage larvae of *D. viviparus* are inactive and, in contrast to those of other strongyles, the pre-parasitic stages do not feed but instead rely on endogenous food granules which give the larvae a dark appearance.

While this developmental cycle is generally accepted, there has been much controversy about the finer details. Much of this has probably arisen from the fact that the development in the guinea-pig model is faster than in the bovine host and Jarrett and Sharp (1963) cautioned against the extrapolation of results in the model system. For example, Douvres and Lucker (1958) obtained L3 from the lungs of guinea-pigs 18 hours after infection with 120,000 to 450,000 infective larvae. However, a slower rate of development in the guinea-pig was reported by Soliman (1953) using an infecting dose of 7,000 larvae. He found that L3 were present in the mesenteric lymph nodes after 24 hours and in the lungs after 3 days.

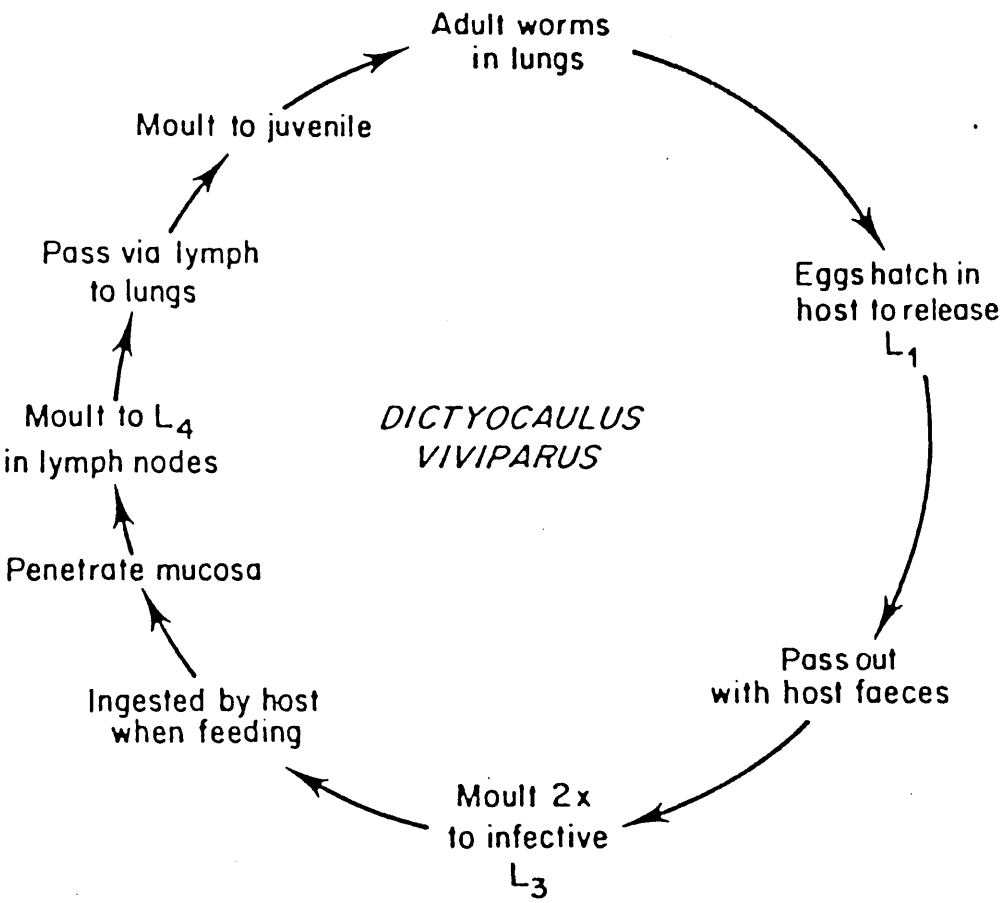


Figure 1.1 Life cycle of *Dictyocaulus viviparus* in cattle

A rapid migration from the bowel to the lungs of cattle was reported in 1960 by Poynter *et al.* (1960), who found L3 in the lungs 24 hours after the administration of 24,000 larvae. They considered that the majority of larvae reached the lungs much earlier than had previously been thought, and did not dwell in the lymph nodes as fourth stage larvae, as had been suggested by Jarrett *et al.* (1957b). However, Jarrett and Sharp (1963) reported that they were unable to recover larvae from the lungs of calves until the seventh day after infection with 1,000 larvae.

Histological examinations of organs from infected calves revealed no lesions one and two days after infection, while lesions in the mesenteric lymph nodes were found on the third day. It was not until 7 days after infection that lesions were found in lung tissue. Jarrett and Sharp (1963) found that under normal circumstances significant numbers of fourth-stage larvae did not appear in the lungs until 13 to 15 days after infection, and thereafter the number of parasites fell to a constant level which approximated the final worm burden expected from the infecting dose. These workers critisised the work of Poynter *et al.*(1960) on the grounds that the larval doses were excessive and the number of larvae recovered from the lungs in the first few days represented an extremely small percentage of the infecting dose. It, therefore, appeared that the results of such studies were dependent on the host species and the number of parasites administered.

It is now generally accepted that in the rabbit, mouse and guinea-pig (the latter being the most susceptible) L3 appear in the lungs after 24 hours. On the fourth day L4 are present, with L5 being found on the 7th day after infection. Around day 10, the mean worm burden decreases, with only a few parasites being found 13 days post infection (Poynter *et al.*, 1960; Wilson, 1966). In the guinea-pig and other laboratory animals, *D.viviparus* does not reach maturity, ceasing its development at the early fifth larval stage (Wade, Fox and Swanson, 1960).

In the bovine host, third stage larvae remain in the mesenteric lymph nodes for 3 to 8 days. Around the 7th day larvae begin to arrive in the lungs in the fourth

stage, although in heavy infections they reach the lungs earlier. Fifth stage larvae are present in the lungs by the 15th day and sexual maturity is reached on the 22nd day of infection (Soliman 1953; Rubin and Lucker 1956; Jarrett and Sharp 1963). In all studies carried out, no larvae were found in the livers of the infected hosts, and it was, therefore, concluded that larvae travel via the lymphatic route.

It has been stated that pre-natal infection with *D. viviparus* occurs. This presumably derives from the report of Kasparek (1900) who found parasites in calves only a few days old. However, Soliman (1953) examined more than 20 foetuses born of cows infected with *D.viviparus* and found no evidence of pre-natal infection. In the field, the epidemiological situation does not suggest the occurrence of pre-natal infection.

1.3 PATHOLOGY

A detailed picture of the development of lungworm disease was established by Jarrett *et al.* (1957b). By slaughtering calves at various times after single infections they were able to establish the serial pathology of parasitic bronchitis. These workers were able to reproduce all the lesions found in natural infections, and correlated these with the clinical condition of the animals.

The infection was divided into four phases (Jarrett *et al.,* 1960a): the penetration phase, which occurs 1 to 7 days after infection; the pre-patent phase, days 8 to 25; the patent phase, days 26 to 55; and the post-patent phase, days 56 to 70. These phases are characterised as follows:

a) Penetration phase

The penetration phase is of minimum clinical significance. Three days after infection with 5,000 larvae few or no lesions are observed in the alimentary tract, the mesenteric lymph nodes or the lungs. Five days after infection, giant cells are found in the mesenteric lymph nodes and although the lungs show no macroscopic lesions at this time, they show alveolar interstitial necrotic foci at the microscopic

level, associated with the breakthrough of larvae into the alveoli. These areas are surrounded by infiltrations of neutrophils, eosinophils, macrophages and multinucleate giant cells, while the bronchial glands show cortical hyperplasia, but no local eosinophilia. Similar lesions are observed with higher infecting doses, but are more intense and appear 1 to 2 days earlier. In mild infections, the lesions are rapidly resolved and do not produce any clinical effects.

b) Pre-patent phase

Clinical effects are first detected during the pre-patent phase, which is associated with the arrival of larvae within the alveoli. 8 days after infection there is an intense eosinophilic exudate into the lungs, resulting in blockage of the small bronchi and bronchioles. Such blockages lead to the collapse of alveoli distal to the block, since the air in the alveoli is absorbed into the blood and no more air can enter to replace it. In heavy infections, there is a marked increase in respiratory rate and coughing becomes more noticeable. Severe complications can arise during the pre-patent period, the most common being pulmonary oedema, emphysema and secondary bacterial infections. Pulmonary oedema results in a serious reduction in the lung surface area available for gas exchange and is the cause of the majority of fatal cases of parasitic bronchitis in the field (Jarrett, McIntyre and Urquhart, 1954).

c) Patent phase

The patent phase of the disease, from approximately 26 to 55 days after infection, is associated with adults in the bronchi and trachea. Mature adult worms are found on the surface of the bronchial mucosa in varying amounts of frothy mucous, containing eggs, larvae and cells. In the alveoli, macrophages and multinucleate giant cells attempt to engulf these eggs and hatched first stage larvae, which results in a marked consolidation of lung lobules. A great deal of the clinical effect of the infection is caused by this reaction (Jarrett *et al.*, 1960a). The respiratory rate

may rise to over 100 per minute, loss of appetite occurs, there is a reduction in growth, and coughing is frequent. Larvae are detected in the faeces of infected calves at the beginning of this phase.

d) Post-patent phase

By 50 days, the start of the post-patent phase, the majority of animals begin to recover as adult worms are eliminated. The respiratory rate gradually decreases, coughing becomes less frequent and weight increase is resumed. In some animals, however, there is a sudden exacerbation of the dyspnoea during the 7th and 8th week of infection, which can be fatal. This is associated with a marked increase of epithelialisation of the alveoli which may spread to involve whole lobes of the lung. The etiology of this lesion is not fully understood, but it has been observed that by the time it has become widespread, all or most of the worms have been eliminated from the lungs (Jarrett *et al.*, 1960a). It had previously been suggested that this reaction may have an immunological basis (Jarrett *et al.*, 1954).

Although calves surviving an infection develop strong immunity to the disease, they may, nevertheless, show clinical signs after a heavy challenge such as coughing and tachypnoea. This condition, known as the "reinfection syndrome", is due to ingested larvae reaching the lungs and being killed in the small bronchioles by the host's immune response. The resulting proliferation of lympho-reticular cells around dead larvae will de discussed in greater detail later. Deaths do not usually occur as a result of this reaction.

1.4 EPIDEMIOLOGY

1.4.1 Larval development, survival and translation

The transmission of *Dictyocaulus* infection depends on the development of excreted first stage larvae to the infective stage, their ability to survive on pasture, and the movement of larvae from the faeces to the pasture. All of these factors are affected by environmental conditions, particularly climate, by virtue of its direct effect on larval development and survival (Rose, 1960).

The rate of development of first stage helminth larvae to the infective stage depends on humidity and temperature. With *D. viviparus* this process can take from three days at 25 ^{o}C to twenty-six days at 5 ^{o}C (Taylor,1942). There are no resistant stages to this species comparable to the embryonated egg and Michel (1959) found that as many as 98% of larvae may be killed during the first fortnight. However, unlike larval development, survival of infective larvae is greatest at low temperatures.

It has been demonstrated (Taylor, 1951; Jarrett *et al.*, 1955b; Gupta and Gibbs, 1970; Oakley, 1977; Jorgensen, 1980) that in some parts of Europe larvae can survive on pasture during the winter in sufficient numbers to initiate infection in young animals in spring of the following year. This is one of the main mechanisms by which lungworm disease persists on endemic farms. There is also evidence that larvae can survive in the soil for periods of up to 12 months (Nelson, 1977; Armour *et al.*,1980a).

When climatic conditions are suitable for both larval development and survival, the movement of larvae onto the herbage then becomes important, a process known as translation (Michel and Parfitt, 1956). This is important as grazing animals are known to avoid the area around faecal pats (Michel, 1955a) and as infective larvae of *D. viviparus* are extremely inactive, with very few larvae in a faecal pat reaching the pasture (Michel and Rose, 1954), the dissemination and mechanical spreading of faeces by rain, tractor wheels or animal's hooves are necessary for translation. Earthworms are also thought to be involved in the transmission of lungworm infection by carrying larvae from the soil to the surface (Oakley, 1981b).

The consistency of the faeces is a major factor in translation (Rose and Michel, 1957). Fluid or semi-fluid faeces, spread over a wide area give rise to greater herbage infestation than dry faecal pats. Therefore, during winter when faeces are wet and disintegrate rapidly, high numbers of larvae are found on the herbage. However, as larval development is minimal at low temperatures, most

larvae recovered are non-infective. In summer, faeces dry quickly with no disintegration and few larvae are found on the herbage, although such faecal pats can serve as a reservoir of infection. The most favourable conditions for transmission occur in spring and autumn when the faeces are moist and disintegrate rapidly, and the temperature allows both larval development and survival. The overall effect of these conditions is to produce a seasonal fluctuation in the numbers of lungworm larvae on the pasture.

Another agent important in the translation of *D. viviparus* larvae from the faeces onto the pasture is a fungus of the genus *Pilobolus* which grows on the surface of faecal pats (Robinson, 1962). The larvae climb onto the sporangia of the fungus, which then explode to ensure dissemination of the fungal spores. This can propel the spores and larvae up to a distance of ten feet. A survey in Britain showed that this fungus is present on over 95% of faecal pats and, therefore, appears to play a significant role in the spread of lungworm infection.

1.4.2 Carrier animals

Carrier animals represent an important factor in the epidemiology of parasitic bronchitis. These can be divided into two categories: the silent carriers and those carrying developmentally-arrested parasites.

a) Silent carriers

Silent carriers were first described by Wetzel in 1948 as naturally infected animals which excreted larvae without showing signs of lungworm disease. These carriers were usually yearlings and he attributed the situation to a less efficient immune response in calves compared to older animals.

A study by Cunningham *et al.* (1956) indicated that carrier animals were ones which had not been exposed to a heavy infection and may not, therefore, have acquired a high degree of immunity. In contrast, animals which had recovered from a severe infection were immune to reinfection as well as being able to eliminate the primary parasite burden. Shedding of lungworm larvae by young animals after their

first grazing season has been reported in several countries (in Germany, Wetzel, 1948; in Scotland, Jarrett *et al.*,1955b; in Canada, Gupta and Gibbs, 1970; in Austria, Supperer and Pfeiffer, 1971) and may contribute to pasture contamination in spring.

As well as young animals, vaccinated cattle have been implicated as silent carriers of infection (Cornwell and Berry, 1960). Although vaccination does provide a high level of protection, a study by Menear and Swarbrich (1968) demonstrated low levels of patency in over 40% of healthy vaccinates at pasture. Vaccinates are of a similar immune status to young carriers: they are refactory to the low pasture challenge they themselves create, but are susceptible to high pasture challenges produced by susceptible calves. It is for this reason that vaccinated and non-vaccinated animals should not be grazed together.

b) Developmentally arrested larvae

Developmentally arrested larvae of *D.viviparus* were first observed in 1951 by Taylor, who found immature worms in the lungs of calves which had been witheld from infection for periods longer than the pre-patent period. Since then, this phenomenon has been observed in natural and experimental infections by several groups (Taylor and Michel, 1952; Supperer and Pfeiffer, 1971; Eisenegger and Eckert, 1975; Gupta and Gibbs, 1975; Inderbitzen, 1976). Arrested development of *D.viviparus* occurs in the early fifth stage, in contrast to gastrointestinal nematodes, in which it occurs at the early fourth stage.

Arrested development is thought to occur as a result of host immunity to the parasite (Michel 1955b; Jorgessen 1981). It was found that worms from a challenge infection given 9 months after the primary infection, developed normally for 10-11 days, after which time larval development was arrested or retarded (Michel *et al.*, 1965). From this it was presumed that the effects of resistance to early larval stages had been lost, while those against later stages were still effective.

Certain climatic conditions can also result in arrested development, a phenomenon known as hypobiosis (Gordon, 1970). As with several other nematodes, arrested development of *D. viviparus* in the Northern hemisphere occurs following the ingestion of infective larvae in autumn, as the temperature declines (Gupta and Gibbs, 1970; Inderbitzen, 1976). This appears to be an adaptive mechanism by the parasite in response to climatic conditions unfavourable to the development of the free-living stages. This ensures that infection is carried through the winter, with the next parasite generation contaminating the pasture in spring.

The climatic trigger appears to exert a direct effect on the parasite itself. Inderbitzen (1976) and Oakley (1979) demonstrated arrested development of *D.viviparus* following the administration of larvae which had been chilled for prolonged periods of time, and similar studies with *Ostertagia ostertagi* larvae (Armour, 1977) indicated that the host plays no part in inducing hypobiosis.

Whether arrested development results from resistance or partial resistance to infection, or an adaptive mechanism of the parasite, it appears that this phenomenon is important in ensuring the contamination of the pasture from one year to the next.

1.5 IMMUNISATION AGAINST *DICTYOCAULUS VIVIPARUS*

1.5.1 Naturally acquired immunity

It has been known for many years that cattle exposed to a natural infection with *D. viviparus* are subsequently resistant to reinfection (Porter and Cauthen, 1942; Jarrett *et al.*,1955a; Rubin and Lucker, 1956; Weber, 1958). Michel (1955b) observed that reinfection of calves which had recovered from an experimental infection did not result in patency, but immature worms could be found in the lungs for some months thereafter. Resistance developed in less than a fortnight after the first infection and was found to decline in the absence of reinfection (Michel and Shand, 1955; Michel and Coates, 1958). Michel (1962) suggested that the loss of protection he observed as early as five months after infection may have been associated with the loss of adult worms which occurred around this time.

There is thought to be a threshold level of primary infection which must be reached before effective immunity results. Michel (1969) observed that negligible resistance resulted from low level infections and protection was, therefore, dependent on subsequent exposure to a heavier infection. Generally, acquired immunity is of a high level, although it can break down under heavy challenge (Taylor, 1951; Michel and Shand, 1955; Rubin and Lucker, 1956), particularly when nutrition is poor and other diseases are present.

Although older animals, not previously exposed, remain relatively susceptible to lungworm infection (Sellman and Urquhart, 1979), it has been demonstrated that a degree of age-related immunity does exist. Several studies demonstrated that susceptible yearlings had greater resistance than calves as measured by the size and percentage of worms established in the lungs (Wade *et al.*, 1962; Taylor *et al.*,1988). Armour (1980) postulated that age may influence the course of helminth infections by extending the pre-patent period, reducing pathogenic effects and possibly increasing the ability to acquire immunity.

It has also been demonstrated that calves of the same age vary inherently in their capacity to acquire resistance following experimental infection (Rubin and Lucker, 1956, Weber, 1958). This is probably due to genetic differences between hosts, although no particular breed of cattle has been shown to be more resistant than another.

1.5.2 Passive immunisation

Evidence that a strong acquired immunity to *D. viviparus* infection exists in the field prompted Jarrett *et al.* (1955a) to determine whether this immunity could be transferred with serum. Prior to this, passive immunisation against other helminths had produced variable results with successful immunisation being reported for *Nippostrongylus* (Sarles and Taliaferro, 1936) and *Strongyloides* infections in rats (Lawlor, 1940), while less successful results had been obtained in ancylstome infections of dogs (Otto, 1938) and schistosome infections of mice (Stirewalt and Evans, 1953).

Jarrett *et al.* (1955a) prepared a globulin fraction from serum of recovered field cases of lungworm disease. The antibody titre of these animals had been boosted by experimental reinfection with 50,000 to 200,000 infective larvae thirty days previously. 1.5 litres of this was administered to susceptible calves and found to confer significant protection against a challenge of 4,000 larvae. Rubin and Weber (1955) also demonstrated protection using a lesser amount of whole serum and a greater challenge dose. It was concluded from these findings that the serum of animals recovering from parasitic bronchitis contained antibodies capable of conferring protection against challenge. However, this method was of little practical use in the prevention of lungworm infection.

1.5.3 Active immunisation

Attention then turned to methods of stimulating immunity to *D. viviparus* by the administration of dead parasite material. Jarrett *et al.*(1957a, 1960b) sensitised groups of calves with one or two doses of 100mg of dried whole adult homogenate in Freund's Complete Adjuvant. Immunity was then tested by a challenge of 4,000 larvae thirty days later. It was found that no protection was afforded by this method of immunisation although, in subsequent experiments, some degree of protection was obtained against a challenge dose of 2,000 larvae. However, there was no appreciable difference in the amount of damage to the lungs of the immunised and control groups, with immunised calves showing lesions typical of an exaggerated response to the parasite. Using adult worms and L3 homogenate together, Wade *et al.* (1962) achieved greater protection. However, Jarrett *et al.* (1960b) concluded that this method of immunisation would be expensive due to the requirement of adult worms from infected calves and would be of limited value as a prophylactic treatment against disease.

Cornwell (1962b) found that a 70% reduction in worm burden could be achieved by the parenteral administration of fourth stage larvae. However, he concluded that the level of immunity obtained by this method was not as effective as

that resulting from a natural infection, and he suggested that the L3 stage may contribute to the development of immunity.

1.5.4 Vaccination

One reason for the failure of the dead infective preparation to stimulate sufficient immunity was thought to be that the protective mechanism depended on exposure to antigens associated with live parasites. It was demonstrated in 1958 by workers at the University of Glasgow that successful immunisation could be achieved using infective stage larvae which had been attenuated by exposure to X-irradiation. These attenuated larvae were capable of stimulating a protective immune response, but incapable of completing their life cycle or causing significant damage to the lungs (Jarrett *et al.,* 1958). Experiments were carried out to determine the level of radiation and the number of larvae needed for effective immunisation. An irradiating dose of 60 krad was found to be too great, with larvae failing to penetrate the host and inducing protective immunity. Irradiating doses of 20 and 40 krad resulted in effective immunity without causing any appreciable disease (Jarrett *et al.,* 1960c). It was established that one dose of 4,000 larvae irradiated to 40krad resulted in 99% protection against a challenge of 4,000 normal larvae given 50 days later. However, this imunising dose was reduced to 1,000 larvae so that any failure of attenuation would not produce fatal disease. This gave a reduced but significant degree of protection.

Studies were carried out using double vaccination with larvae irradiated at 40krad (Jarrett *et al.,* 1959a). Three groups of calves were immunised with 1,000 irradiated larvae as the first dose and then with either 4,000, 2,000 or 1,000 larvae irradiated to 40krad as the second dose, 42 days later. Calves were challenged with 10,000 normal larvae after 50 days. There was no increase in respiratory rate and no worms were found when the calves were killed 25 days after challenge. A field trial using this double vaccination procedure was then carried out (Jarrett *et al.,* 1961). Five calves were vaccinated on two occasions, 30 days apart, with 1,000 larvae irradiated at 40krad. 20 days after the second

dose they were turned out to pasture which was known to be heavily contaminated. Another group of five calves acted as controls. Vaccinates had a lower respiratory rate and gained more weight than the controls. When the calves were killed 70 days later a total of 1,767 adults were found in the controls, while only 125 immature worms were recovered from the vaccinates.

This study demonstrated the effectiveness of the X-irradiated vaccine under practical farming conditions. Subsequent to this work, workers from several countries have confirmed the efficacy of the lungworm vaccine (Nelson, Jones and Peacock, 1961; Poynter, Peacock and Menear, 1970, in Britain; Pierre *et al.*, 1961, in France; Englebrecht, 1961, in the U.S.A.; Olson, 1962, in Sweden; Downey,1968, in Ireland).

A similar vaccine in which infective larvae were attenuated by exposure to the cytotoxic chemical agent triethylene melanine (TEM) was described by Cornwell and Jones (1970,1971). This proved to be effective against both experimental and natural challenge, when given in a similar regime to the X-irradiated vaccine but was not developed commercially.

More recently, larvae which had been gamma-irradiated from a ^{60}Co source were used to immunise calves and were found to be as efficient as the X-irradiated larvae. A vaccine was subsequently developed in the Netherlands using larvae attenuated by gamma-radiation (Nobivac lungworm, Intervet International, the Netherlands).

Immunisation has now been carried out successfully for over 30 years by the oral administration of 2 doses of 1,000 X-irradiated larvae, with an interval of 4 weeks between each dose. To allow a high level of immunity to develop, vaccinated calves should be protected from challenge until 2 weeks after the second dose. It is recommended that animals be vaccinated when aged 2 months or more, but it has been shown that calves develop effective immunity even when vaccinated at 1 and 4 weeks old (Benitez-Usher, Armour and Urquhart, 1976). This is in contrast to vaccination or infection with gastrointestinal nematodes where young animals show

a prolonged period of immunological unresponsiveness (Urquhart *et al.*, 1966a; Urquhart, 1981).

Since its introduction, several questions have been raised concerning the effectiveness of the lungworm vaccine (Poynter *et al.*, 1970). It is not known how long immunity lasts following vaccination, but it has been shown that the level of vaccine-induced immunity is not as high, nor as persistant, as naturally acquired immunity (Michel *et al.*, 1965). However, as natural immunity in the field cannot be controlled nor predicted, vaccination is obviously a safer method of controlling infection. As vaccination does not confer sterile immunity to challenge, vaccinates will be exposed to natural infection which stimulates their resistance. As mentioned previously, vaccinates can act as "carriers" of infection causing a small degree of pasture contamination which can result in clinical disease in susceptible calves. Therefore, Cornwell and Berry (1960) and Urquhart (1985) advised that all calves in a herd should be vaccinated before going out to pasture.

1.6 IMMUNITY TO *D. VIVIPARUS*

1.6.1 Site of protective immunity

The mechanism of action of immunity to lungworm infection has puzzled workers in this field for many years and is still largely unknown. Michel (1956) in a study of acquired immunity to *D. filaria*, the sheep lungworm, suggested several sites where immunity may operate: larvae may fail to penetrate the intestinal wall, or be destroyed there; they may be destroyed in the mesenteric lymph glands or fail to leave them; they may fail to break out of the blood vessels into the alveoli; or they may be destroyed or eliminated from the air spaces of the lungs. A comparison of the fate of *D. filaria* larvae in resistant and susceptible mice showed that fewer larvae were present in the lungs of resistant animals and larvae did not appear to be destroyed in this site. When the mesenteric lymph nodes were by-passed, equal numbers of larvae were recovered from the lungs of resistant and susceptible mice. From this model system, it was concluded that in the resistant host, larvae of *D. filaria* were destroyed in the mesenteric lymph nodes or the lymphatic vessels.

A study by Poynter *et al.* (1960) showed that, in contrast to *D. filaria,* normal larvae of *D. viviparus* reached the lungs of resistant and susceptible guinea-pigs at the same rate and in similar numbers. It was also demonstrated that X-irradiated larvae migrated to the lungs in the same way as normal larvae. It had previously been thought by Jarrett *et al.* (1957a) that X-irradiated larvae migrated as far as the mesenteric lymph nodes and that this was the site of protective antibody formation. However, it was apparent from the mild and transient clinical signs observed following vaccination that some larvae did reach the lungs (Jarrett *et al.*, 1959a).

To determine whether the mesenteric lymph nodes played any role in immunity to parasitic bronchitis in the bovine host, Poynter *et al.* (1960) administered 20,000 40krad irradiated larvae to a previously infected calf. After 48h, 3,151 larvae were recovered from the lungs and 1,082 were found in the mesenteric lymph nodes. It was concluded that irradiated larvae, even in the resistant host, were not held up in the mesenteric lymph nodes and the majority of larvae reached the lungs.

However, Jarrett and Sharp (1963) cautioned against assuming that larvae undergo the same fate in bovine and guinea-pig hosts. It was pointed out that the exact fate of irradiated larvae in the bovine was still uncertain as a repeatable method of larval recovery from the mesenteric lymph nodes of calves was not available. In their opinion the mesenteric lymph nodes were involved in immunity to some extent.

A more recent study, however, demonstrated that subcutaneous vaccination with irradiated larvae was as effective as the oral route of administration (Bain and Urquhart, 1988). This implied that the passage of larvae through the mesenteric lymph nodes was not essential for the production of immunity, although the mechanisms of immunity induced by these different routes of vaccination were not compared.

From these studies it was suggested that X-irradiated larvae in susceptible

hosts and normal larvae in resistant hosts migrated to the lungs and at some point their development was suppressed by the effects of X-irradiation and by the host's immune response, respectively. Using the guinea-pig system, Poynter *et al.* (1960) demonstrated that in both situations larval development was suppressed at, or immediately after the moult from the fourth to the fifth larval stage. This was also demonstraed by Cornwell and Jones (1971) using TEM-attenuated larvae. In the bovine host, larval development was also suppressed at this time and the behaviour of normal larvae in resistant calves was found to be similar to that of X-irradiated larvae in susceptible calves. However, the exact mechanism of this suppression is still unknown.

1.6.2 Mechanism of immunity

a) Lympho-reticular nodules

Jarrett and Sharp (1963) described the development of lympho-reticular nodules in the walls of the bronchi and bronchioles of calves following the administration of X-irradiated larvae to susceptible calves or normal larvae to resistant calves. These nodules had the morphological characteristics of antibody-producing sites and it was speculated that these may play a part in the formation of protective antibodies. The presence of lymphoid tissues in the lungs of naturally resistant calves killed following challenge had previously been reported by Simpson *et al.*(1957) and by Jarrett *et al.* (1960a). Identical lesions had also been found in calves challenged after being protected with immune serum (Jarrett *et al.,* 1955a) or with whole worm extract (Jarrett *et al.,* 1960b) and from infected calves treated with diethylcarbamazine (Jarrett, McIntyre and Sharp, 1962).

These nodules were 2 to 4 mm in diameter, varying in colour from grey to greenish. They were found throughout the lung, although they occurred most frequently in the diaphragmatic lobes. Microscopically, they were composed of lymphoreticular tissue surrounding a mass of degenerating eosinophils enclosing the remains of larvae situated within or beside the walls of the bronchioles. Jarrett and Sharp (1963) speculated that these nodules may be the sources of antibody to

"somatic" antigens as they were in close contact with disintegrating parasites.

The number of lymphoid nodules increased following challenge of vaccinated calves, while very few were observed in susceptible calves after challenge (Pirie *et al.,* 1971). This increase was thought to be due to larvae from the challenge infection being killed immunologically in the small bronchioles and the presence of nodules was, therefore, thought to be a good criterion of the host's immune status. However, Poynter *et al.* (1970) found that the presence of nodules was not always consistent with immunity and warned that although they appeared to have some relationship to immunity, the nature of this was not clear.

b) Eosinophil response

As with other helminth infections, *D. viviparus* infection is associated with blood and tissue eosinophilia. Several studies have demonstrated two peaks of blood eosinophilia following infection with normal larvae, the first at 9 to 15 days after infection and a higher peak at about 40 days post infection (Weber and Rubin, 1958; Cornwell, 1962a; MacKenzie and Michel, 1964). Following reinfection a higher and more rapid response was evident, with the first peak being higher.

Vaccination with X-irradiated larvae stimulated a single eosinophil response with a peak at 18 days after infection, while the second dose of vaccine gave a rise of similar level after 14 days. Following challenge with normal larvae, vaccinated calves developed an eosinophilia four times as great as the response to irradiated larvae, with peaks at 14 and 40 days after challenge, the second peak being smaller. It was suggested that the first peak was stimulated by developing larvae in the lungs and the second response by the egg production of adult females (Cornwell, 1962a). Only one peak was, therefore, observed following vaccination, as irradiated larvae reached the lungs but did not develop to the mature adult stage.

Djafard, Swanson and Baeker (1960) reported that calves which survived an outbreak of parasitic bronchitis had a greater eosinophil response than those which succumbed to the disease, suggesting a link between eosinophilia and resistance to *D. viviparus* infection. *In vitro* studies by Knapp and Oakley (1981) demonstrated

that eosinophils adhered to the surface of *D. viviparus* larvae earlier than did other blood cells. Cell adherence was associated with an antibody-mediated, complement-independent mechanism in hyperimmune sera and with a heat labile factor in normal serum, presumed to be complement. A similar mechanism has been shown to be involved in the killing of newborn larvae of *Trichinella spiralis* by eosinophils in the presence of immune sera (Bass and Szejda, 1979; MacKenzie *et al.*, 1980).

1.6.3 Measuring host immunity to *D. viviparus*

Several attempts have been made using serological techniques to assess the immune status of calves following infection or vaccination against *D. viviparus*. Using the complement fixation (C.F.) test with an adult whole worm antigen prepared at low temperature, Weber (1958) detected an initial antibody response 12 days after infection, which reached a peak 4 days later and remained at a high level until 63 days after infection. This response was observed in calves following their first infection and on subsequent infections.

However, Jarrett *et al.* (1959b) and Cornwell and Michel (1960), using a heated whole worm preparation, were unable to confirm the results of Weber. They first detected an antibody response 30-35 days after infection which reached a peak at 80-100 days. The titre then declined after 150-200 days and reinfection after this time produced a secondary response, which peaked at around 3 weeks. The pattern of response, as measured by the C.F. test, was essentially the same in calves exposed to natural or experimental infection. It was concluded that, as antibody titres developed more slowly than resistance to reinfection, there was no correlation between the two measurements.

The C.F. test was then used to study the antibody response of calves following vaccination with irradiated larvae (Cornwell, 1960a). The first dose of vaccine produced a low response which rose to a moderate level following the second dose. However, the response of individual calves varied widely. It was suggested that the

antibody response was due to irradiated larvae which succeeded in developing in the lungs and the extent of migration of irradiated larvae may have varied between individual calves. Following challenge, the majority of vaccinated calves showed an increase in antibody titre after two weeks (Cornwell, 1960b). However, it was concluded that the antibody titre measured by the C.F.test could not be relied upon to reflect the immune status of the host.

Also using the C.F. test, Sinclair (1964) found that sera from calves infected with *Ostertagia ostertagi* cross-reacted with *D. viviparus* antigen. It was suggested by Bokhout, Boon and Hendriks (1979) that non-denatured antigens would result in more specific reactions, which could be used in the diagnosis of lungworm infection. Using a whole worm soluble extract prepared at low temperature, they carried out an indirect haemagglutination (IHA) test on 46 farms in the Netherlands. No significant cross-reactivity with other bovine parasitic nematodes was observed. In addition, a positive correlation in 80% of calves between IHA results and clinical diagnosis was reported. Positive IHA titres were observed 14 days after infection which reached a peak at 45 days and were still present several months later.

Using an enzyme-linked immunosorbent assay (ELISA) Marius *et al.* (1979) detected antibodies to *D. viviparus* adult whole worm extract in both serum and nasal secretions of infected calves. Boon, Kloosterman and Van den Brink (1982) found a good correlation between the ELISA and IHA titres of experimentally and naturally infected calves and concluded that the ELISA technique was easier to carry out and was sufficiently specific and sensitive for survey work and for herd diagnosis. Using the ELISA as a serodiagnostic tool, Bos, Beekman-Boneschanscher and Boon (1986) were able to demonstrate stage-specific antibody responses to third stage larvae and adult antigen preparations. The response to larval antigens was detected one week earlier than the anti-adult response following challenge of vaccinated and non-vaccinated calves. They concluded that the anti-larval titres reflected the number of larvae ingested and the anti-adult titres indicated the subsequent level of infection. However, despite the usefulness of this technique in

the diagnosis of lungworm infections, there is still no serological test which can quantitatively detect the degree of immunity to *D. viviparus*.

1.7 DRUG TREATMENT OF PARASITIC BRONCHITIS

Of the drugs used in the treatment of parasitic bronchitis diethycarbamazine, levamisole, the benzimidazoles and ivermectin are the most effective. Many studies have been carried out to determine the parasite stages most susceptible to the action of each drug, so that an efficient system of treatment can be established. As well as being able to control current infections, immunity to reinfection following drug termination has also been examined. Such studies have been used to determine the relative immunogenic effects of various stages of the parasite.

1.7.1. Diethylcarbamazine

In 1957 Parker demonstrated the efficiency of diethylcarbamazine (DEC) against *D. viviparus*. In natural outbreaks of parasitic bronchitis, an oral dose of 2.2mg/kg of bodyweight prevented deaths in 37 calves, while 11 out of 37 untreated calves died. In experimental infections, daily doses of 25mg/kg were effective when administered 14-18 days after infection but had no effect against later parasite stages. Jarrett *et al.*, (1962), Cornwell (1963) and Kendall (1965) confirmed the greater effectiveness of DEC against 14-17 day old infections compared to the treatment of earlier or later stages of the disease.

Cornwell (1963) found that all the treated groups showed significant resistance to reinfection and concluded that by the time the parasite was susceptible to the action of the drug, the host had already been stimulated by parasite antigens giving rise to immunity. In contrast, Kendall (1965), using a larger dose of 300mg/kg, showed a reduced level of immunity in treated calves compared to controls in which infection had not been terminated. However, this massive dose greatly exceeded the recommended therapeutic level.

1.7.2. Levamisole

Levamisole has, for a long time, been considered to be the best treatment against parasitic bronchitis. Using the recommended dose rate of 5mg/kg several workers have shown activity of about 99% against fifth stage and adult parasites and 85% against younger larvae (Broome and Lewis, 1974; Oakley, 1980a; Vazquez *et al.*, 1980). Pouplard, Lekeux and Detry (1986) showed that 95% activity could be achieved against developing larvae at a dose rate of 10mg/kg and Oakley (1981a) demonstrated a significant effect on inhibited *D. viviparus* larvae.

Levamisole treatment of parasitic bronchitis is associated with fewer lung lesions and lower mortality than other anthelmintic drugs. This is thought to be for two reasons. Firstly, levamisole is rapidly absorbed and has been shown to exert an effect only 3 hours after administration (Oakley, 1980b). This is advantageous in the treatment of pre-patent infections by limiting the disease before it advances to the more damaging patent phase. Secondly, unlike DEC and the benzimidazoles which act by killing worms *in situ*, levamisole causes paralysis of nematodes which are then expelled from the host by physiological mechanisms (Coles, 1977).

Treatment of calves with levamisole 1 or 32 days after infection has been shown to result in significant protection against reinfection, but protection following treatment of pre-patent disease was found to be erratic (Downey, 1980; Oakley, 1980a; Urquhart *et al.*, 1981). Although reductions in worm burdens of 70%, 38% and 69% respectively were reported by these workers, there was found to be no significant amelioration of the severity of clinical signs. At necropsy, lesions were present which are usually associated with animals whose immunity is only partially developed.

1.7.3. Benzimidazoles

Following the description of thiabendazole as a anthelmintic in 1961 (Brown *et al.*), many derivatives with increased potency were developed. Of these, fenbendazole (Duwel and Kirsh, 1980; Malan, Roper and Shabangu, 1983),

oxfendazole (Downey, 1976; Chalmers, 1979), albendazole (Benz and Ernst, 1978; Downey, 1978) and febantel (Grelck, Horchner and Woehrl, 1978) have been used with almost 100% effectiveness against adults and developing larvae of *D. viviparus*. Fenbendazole has also been shown to be effective against developmentally-arrested larvae (Inderbitzen and Eckert, 1978; Pfeiffer, 1978). Following treatment of pre-patent infections with fenbendazole the number of worms developing from a challenge infection was reduced by 76% (Downey, 1980) and 68% (Urquhart *et al.*, 1981), although clinical signs of parasitic bronchitis were apparent.

Recently, an oxfendazole pulse release (OPR) bolus was developed which produces five therapeutic doses of the drug at intervals of around 3 weeks. This is administered to first year calves when turned out to graze and is used as a prophylaxis against lungworm, as well as preventing gastrointesinal nematodiasis. As the drug is released every 3 weeks, adult worms do not become established. Therefore, lung damage in infected calves is prevented and there is also little risk of subsequent pasture contamination which could result in lungworm disease at the end of the treatment period.

Although Jacobs *et al.* (1986) demonstrated immunity to challenge 5 months after treatment, other workers have found that adequate immunity to reinfection does not occur in all cases (Herbert and Probert, 1987; Jacobs *et al.*, 1987; Vercruysse *et al.*,1987).

1.7.4. Ivermectin

Another recent and very effective method of treating parasitic bronchitis is by the administration of ivermectin. Ivermectin is a chemically modified derivative of the avermectins, fermentation products of the actinomycete *Streptomyces avermitilus* (Burg *et al.*,1979). It has proved to be highly effective against adult worms and larval populations, including arrested and developing larvae of the most important cattle nematodes (Armour, Bairden and Preston, 1980b). In particular, *D.*

viviparus has been shown to be very susceptible to ivermectin (Egerton *et al.*, 1979). Using a dose rate of 200µg/kg almost 100% effectiveness was achieved against all stages of lungworm (Alva-Valdes *et al.*, 1984; Benz, Ernst and Egerton, 1984; Pouplard *et al.*, 1986).

Unlike most veterinary anthelmintics, which have a relativey short half-life, ivermectin persists at therapeutic concentrations against most gastrointestinal nematodes for two weeks and against *D. viviparus* for as long as three weeks (Armour *et al.*, 1985). The persistence of ivermectin has led to the development of an effective treatment regime against pulmonary and gastrointestinal nematodes, where animals are treated at 3, 8 and 13 weeks after being turned out to graze. This has proved to be highly effective against lungworm infection (Taylor, Mallon and Green, 1986; Armour *et al.*, 1987). Using this regime, lung damage due to adult parasites and subsequent pasture contamination are prevented as the longest infection times are 3 weeks at the start of the grazing season and 2 weeks before further treatments. Infections are, therefore, terminated before patency is reached.

It has been demonstrated that calves protected by this treatment develop considerable resistance to the parasite, although mild clinical signs were observed on reinfection. This immunity is thought to be a result of exposure to heavy pasture contamination before, between and following treatment.

1.7.5. Drug treatment or vaccination ?

It was stated by Taylor *et al.* (1986) that treatment with ivermectin over a period of 2-3 years might result in eradication of *D. viviparus* from selected fields or farms. However, due to the ubiquitious nature of lungworm infection, together with the persistence of *D.viviparus* larvae on pasture and the small number which need to be ingested before clinical disease results, this is unlikely to be possible in endemic areas. It is more likely that such treatment carried out for several years will result in a gradual decrease in the level of pasture contamination which may become so low that animals acquire little or no immunity to *D.viviparus* and gastrointestinal nematodes, while the immunity of older animals may decline. Any

sudden increase in the pasture larval population, by carrier animals or dispersion by the fungus *Pilobolus* would then result in severe outbreaks of disease. Many workers, therefore, agree that successful control of parasitic bronchitis can only be achieved by vaccination with irradiated larvae (Poynter *et al.*, 1970; Urquhart *et al.*, 1981; Armour and Bogan, 1982). As vaccination is not 100% effective, a low level of pasture contamination is maintained which boosts immunity, thus enabling vaccinates to withstand even severe infections.

Due to the susceptibility of drug-treated animals to disease in the absence of challenge infections, it would be dangerous to discontinue such treatment. However, drug treatment on a long-term basis can be expensive as well as being labour intensive. With repeated and frequent treatments there is also the possibility of drug-resistant strains of the parasite developing (Waller, 1987), particularly with the use of slow-release devices. The development of long-acting anthelmintics has also introduced the environmental problem of drug residues. For example, ivermectin, which is excreted in the faeces, has been reported to cause abnormal degradation of faecal pats due to the persistent insecticidal effect of the drug on dung beetles (Wall and Strong, 1987). The possibility of drug persistence in meat and milk products from drug-treated animals also presents a problem.

In contrast, vaccination requires to be carried out on only two occassions, one month after which, fully immune animals can be turned out to graze with no further treatment necessary, thus making it a simpler and less expensive method of protection. There is no problem of resistant strains developing, and the few breakdowns in vaccination which have been reported have, in most instances, been due to failure to vaccinate, or vaccination under unsuitable circumstances, and were not related to the vaccine itself. Unlike drug treatment, vaccination has no long term harmful effects on the host, nor on the environment.

However, on farms where lungworm infection is sporadic, anthelmitics are used to treat clinically affected animals. One of the difficulties in the treatment of natural outbreaks of this disease is that not all cattle in a herd will be infected to the same extent at the same time. Drug treatment of patent infections can be hazardous,

particularly when the worm burden is large. Lesions associated with the death of larvae or adults in the bronchi and bronchioles are often found at necropsy following anthelmintic treatment of parasitic bronchitis (Jarrett, Urquhart and Bairden, 1980). Such treatment can, therefore, exacerbate the clinical signs of infected animals and in some may result in death. On the other hand, it is pointless treating calves showing no clinical signs as they may be uninfected or may be carrying so few worms that protective immunity would not result. Following treatment of infected calves it must then be decided what should be done with them. Keeping treated animals inside is expensive, while leaving them out to graze could lead to further lung damage.

Therefore, although the anthelmintic drugs discussed act very effectively against *D.viviparus*, their failure to stimulate long-lasting immunity together with their effect on the larval population and on the environment, could have serious consequences. It is apparent then that the long term control of parasitic bronchitis can most effectively be achieved by vaccination.

1.8 CONTROL OF HELMINTH PARASITES

1.8.1 Introduction

Parasitic helminths cause some of the most widespread and debilitating diseases of man and domestic animals. It is apparent from the resulting clinical and economic problems that effective control of such diseases must be sought. Solutions based on improvements in hygiene and sanitation for the control of gastrointestinal and zoonotic helminths are expensive and difficult to implement, particularly in developing countries. Insecticide control of vector-transmitted diseases or intensive programmes of chemotherapy of infected hosts are also expensive and require much cooperation for their implementation, as well as introducing the problems of drug resistance and environmental pollution. Therefore, vaccines, giving effective and long-lasting protection, are very much needed. The problems encountered in the development of anti-parasite vaccines over the last few years have indicated,

however, that this will not be a quick nor simple solution and it is now apparent that vaccines against most helminth parasites will not be as simple to develop as that against *Dictyocaulus viviparus*.

In many of these infections effective immunity does not result from natural exposure to the parasite, as it does with *D. viviparus* (Anderson and May, 1982). Vaccination will, therefore, require an understanding of the mechanisms by which the parasite survives within the host, the immune responses it elicits and how effective responses can be induced or enhanced to produce long lasting protection. To some extent, the *D. viviparus* vaccine represents a reversal of current vaccine design. A successful vaccine is available but little is known of the immune reponses it induces or how these affect parasite survival within the host. These mechanisms may be elucidated by examining current knowledge of immune responses to other parasitic helminths and applying the techniques used in other parasite systems to *D. viviparus*. Some aspects of the immunological control of parasitic helminths will now be examined.

Unlike protozoan and microbial pathogens, parasitic helminths are too large to be phagocytosed by the host's immune cells. Instead, a full spectrum of effector mechanisms are often involved (Mitchell, 1979) with the most effective and dramatic response being observed when the host expels a worm population from the intestine. Antibody, T cells and non-specific effector mechanisms have all been implicated as playing a role in immunity to parasitic helminths (Ogilvie and Love, 1974; Wakelin, 1978), although studies in animal models have demonstrated that the exact mechanisms involved depend on both the host and parasite species and also on the site of infection (Vignali, Bickle and Taylor, 1989). As many species of parasites have both tissue and gastrointestinal stages, different effector mechanisms may be active during a single infection.

1.8.2 Antibody-mediated responses

The role of antibody in immunity to helminth infections was demonstrated by the passive immunisation of rats against *Nippostrongylus brasiliensis* infection with

serum from immune donors (Sarles and Taliaferro, 1936; Chandler, 1938). However, from the weak and variable effects of the antiserum on the parasites it was concluded that antibodies alone would result in a gradual loss of worms, while effective immunity required the presence of antibody and effector cells (Jones and Ogilvie, 1971; Ogilvie and Love, 1974). This was confirmed by MacKenzie, Preston and Ogilvie (1978) who demonstrated the *in vitro* killing of *N. brasiliensis* and *Trichinella spiralis* infective larvae in the presence of antibody and myeloid cells, with complement enhancing the killing effect. It was found that killing was achieved using effector cells from either uninfected or immune donors, showing that the effector response is immunologically non-specific, with only the initial step requiring antigen recognition. In some cases, cells adhered to and killed the parasites in the absence of antibody, requiring only complement to initiate this effect (MacKenzie *et al.*, 1980).

As cell adherence to parasites is mediated via the antibody Fc region, the antibody isotype will determine the effector function involved. For example, IgE and IgG1 are the only murine antibodies which are bound by mast cell Fc receptors (Bloch, 1967) and are, therefore, the only classes capable of mediating anaphylactic responses. Neutrophils, eosinophils and macrophages also vary in the affinity with which they bind different immunoglobulin isotypes (Capron *et al.*,1975; Capron *et al.*, 1978). With the exception of IgD, all immunoglobulin classes have been reported to be involved in immune responses to parasitic helminths. In particular, high levels of IgE antibody are associated with helminth infections (Ogilvie, 1967), although its contribution to effective immunity remains unclear. Rats rendered incapable of IgE synthesis by neonatal administration of anti-epsilon serum showed an impaired response to *T. spiralis* larvae with fewer eosinophils at the encystment sites in the muscles compared to control animals. IgE, therefore, appears to play a role in immunity but is not thought to be the only mechanism of resistance as these antibodies are present in many infections, yet the parasites are not destroyed (Pond, Wassom and Hayes,

1989).

IgA has also been implicated in helminth resistance, especially in response to gastrointestinal nematodes. In the mouse a peak IgA antibody response to surface antigens of adult *T. spiralis* occurred 10-20 days after infection in NIH mice, at which time adult worms were expelled from the intestine. This response was absent in the more susceptible C3H mice (Jungery and Ogilvie, 1982), suggesting that, in mice, rejection of *T. spiralis* adults was mediated by an IgA response to adult surface antigens. IgA is thought to contribute to protective immunity against *N. brasiliensis* (Jones, Edwards and Ogilvie, 1970) and *Haemonchus contortus* (Duncan, Smith and Dargie, 1978; Smith *et al.*, 1984) and is also thought to be involved in worm trapping by mucous in the intestine (Lee and Ogilvie, 1981).

IgG and IgM antibodies also play a role in protective immunity, with IgM being particularly important in complement fixation (Klaus *et al.*,1979). It has been speculated that the particular immunoglobulin class stimulated during infection is dependent on the mode of antigen presentation, with the effector mechanism being a consequence of this (Maizels, Philipp and Ogilvie, 1982).

1.7.3 Myeloid cells

The role of myeloid cells in parasite rejection was demonstrated by the ability of highly irradiated rats to expel antibody-damaged *N. brasiliensis* worms when restored with lymphocytes and bone marrow cells, but not with lymphocytes alone (Dineen and Kelly, 1974; Wakelin and Wilson, 1977).

Eosinophilia is a characteristic of a number of helminth infections. *In vitro,* eosinophils readily adhere to the worm surface in the presence of serum and release a variety of factors, such as peroxidases and phospholipases. Such release has been shown to mediate damage to schistosomula (Capron *et al.*, 1983; Butterworth and Hagan, 1987), *T. spiralis* (MacKenzie *et al.*,1981) and the filarial parasite *Wuchereria bancrofti* (Higashi and Chowdhury, 1970). Although levels of these cells do increase in helminth infections (Ottolengi *et al.*,1975), there is no direct evidence to implicate eosinophils in parasite rejection *in vivo.*

This is also true of macrophages, which have been shown to exert anti-parasite effects *in vitro* against microfilaria of *Dipetalonema viteae* (Tanner and Weiss, 1978), *Litomosoides carinii* (Subrahmanyam *et al.,* 1976) and *N. brasiliensis* larvae (MacKenzie *et al.,* 1980) although whether they are essential to rejection *in vivo* is unknown. Both macrophages (James and Sher, 1983; James and Cheever, 1985) and neutrophils (McLaren, Strath and Smithers, 1987) have been implicated as being important in schistosome infections in mice, as indicated by depletion studies with monoclonal antibodies and adoptive transfer experiments. *In vitro* studies demonstrated enhanced shedding of labelled surface proteins of *T. spiralis* in the presence of rat serum and neutrophils (Philipp, Parkhouse and Ogilvie, 1980), while the rate of release from *Toxocara canis* larvae decreased in the presence of neutrophils (Williamson *et al.,* 1990). Such studies demonstrate the difficulties in trying to assess the effects of particular cell types even using *in vitro* systems.

A similar situation exists in defining the role of mast cells in immunity to helminth infections. Such infections result in a local proliferation of this cell type, which has been studied in detail in gastrointestinal infections. *In vivo* perfusion of mast cell mediators demonstrated that prostaglandin E accelerated the expulsion of *N. brasiliensis* from rats (Kelly and Dineen, 1976) while perfusion of histamine and serotonin resulted in the expulsion of *Trichostrongylus colubriformis* in guinea-pigs (Rothwell, Pritchard and Love, 1974). However, histamine and serotonin had no effect on *N. brasiliensis* infection and there was little effect on *T. colubriformis* expulsion in the presence of prostaglandin E (Rothwell, Love and Goodrich, 1977). Histamine and serotonin result in increased mucosal pemeability which may allow specific anti-worm antibody to reach the parasite. It is also thought that this change in permeability may alter the environment to the detriment of the worms or, alternatively, that the factors released from these cells affect the worms directly. It has been demonstrated that although mast cell degranulation is immunologically specific, involving the binding of antigen to reaginic antibody, the

subsequent inflammatory changes are non-specific, causing the expulsion of other unrelated species of gastrointestinal parasites during concurrent infections (Bruce and Wakelin, 1977; Kennedy, 1980).

The role of mast cells was also questioned by the finding that worms of *N. brasiliensis* are not expelled from lactating rats, although mast cell invasion occurs at the same time and to the same extent as in non-lactating controls (Kelly and Ogilvie, 1972). It has been demonstrated *in vitro* that mast cells can adhere to, but do not kill schistosomula (Capron and Capron, 1986). However, it was found that killing of schistosomula by eosinophils was enhanced in the presence of mast cells, which seems likely to be due to the enhancement of eosinophil surface C3b and Fc receptors by the mast cell products ECF-A (eosinophil chemotactic factor of anaphylaxis) and leukotrienes.

1.8.4 T cell responses

T lymphocytes of the T helper subset play a central role in many parasitic infections, including helminth diseases, being involved in both the antibody response and the cell-mediated effector stage (Wakelin, 1978; Mitchell, 1987). The essential role of T cells has been demonstrated by the impaired immune response of athymic animals to many diseases, including schistosome infections, where reductions in immunoglobulin level and eosinophil and macrophage cytotoxicity have been reported in athymic mice (Capron *et al.*, 1983). T cells have also been shown to restore the ability to expel *N. brasiliensis* infection in athymic mice (Jacobson and Reed, 1976).

T helper cells mediate their effect on cellular immunity via the production of lymphokines, which control the activities of effector cells. This is a complex mechanism and it is now recognised that different subsets of T helper cells can mediate different effects (Mossman and Coffman, 1987). In the mouse, Type 2 helper cells (Th2) produce lymphokines such as IL-4 and IL-5 which, through facilitation of IgG1, IgE and IgA antibodies and eosinophils, seem to be the major participants in helminth infections. In contrast, the gamma interferon-producing

Th1 cells appear to be involved in resistance to intracellular protozoan parasites, as has been demonstrated with *Leishmania major* (Locksley *et al.*, 1987; Heinzel *et al.*, 1989). However, this pattern is not always conserved and it is thought that activation of Th1 cells, through the induction of IgG2a, which activates complement and is involved in macrophage-dependent ADCC, may be more useful against some helminth parasites (Mitchell, 1989; Pond *et al.*, 1989).

1.8.5 Immunopathology

Although the immune response acts to remove parasites from the host, such responses can often result in the pathological syndromes characteristic of helminth infections. This is exemplified by the severe dermatitis observed in *Onchocerca volvulus* infections. This reaction is often localised, for example to one leg, when it is referred to as Sowda. This condition is associated with a low density of skin microfilariae and humoral and cellular hyper-responsiveness (Lucius *et al.*, 1986). In contrast, generalised onchocerciasis is associated with greater microfilarial survival, possibly reflecting a lowered immune responsiveness. An examination of the antibody responses to *O. volvulus* antigens showed a similar pattern of recognition in patients suffering from generalised or Sowda-type oncocherciasis with the exception of the IgG3 isotype response. IgG3 in the sera of Sowda patients recognised two major antigens of 99kDa and 72kDa which were not recognised by any class of antibody from sera of generalised onchocerciasis patients (Cabrera, Buttner and Parkhouse, 1988). This demonstrates a possible correlation between antigen recognition and disease status.

The granuloma formation around schistosome eggs trapped in the liver or blood vessels presents another example of an immunopathological host response. Secretory antigens of mature miracidia are released through the egg shell (Hang, Warren and Borros, 1974) and stimulate a T cell response. The subsequent release of lymphokines initiates and regulates the granulomatous response, which involves macrophages, lymphocytes, eosinohils, epithelial cells and fibroblasts (Wyler and

Tracy, 1982).

The characteristic IgE response to helminth parasites, leading to allergic reactions against both parasite-derived and other allergens (Warren, 1982; Ogilvie and de Savigny, 1982), and the renal pathology due to deposition of circulating antigen and immune complexes (Van Marck, Deelder and Gigase, 1982) are other immunopathological consequences of helminth infections.

1.9 PARASITE EVASION MECHANISMS

It is apparent from the number of people and domestic animals infected worldwide with helminth parasites and the chronicity often associated with these diseases, that the immune mechanisms discussed do not always act effectively. Chronicity is often associated with gastrointestinal parasites although infections with systemic parasites, such as schistosome species and filarial nematodes, can also be extremely long lasting. The reasons for the long-term survival of such parasites *in vivo* are not fully known, but examination of the immune responses in animal models and of the properties of the parasites themselves have been useful in identifying some of the mechanisms which may be involved in this phenomenon.

1.9.1 Parasite adaption

It is important to consider the level and frequency of infection on subsequent immunity. In controlled experiments it is customary to administer a single, large infection to study the development of immunity. However, in the field this is unlikely to occur, with hosts usually being exposed to continuous or interrupted low level trickle infections. It was found that a single large infection with *Nippostrongylus brasiliensis* in rats resulted in spontaneous cure, while small daily infections gave rise to a large stable population of adult worms (Jenkins and Phillipson, 1972). Established worms were found to be stunted and showed a different acetylcholinesterase isoenzyme pattern to adult worms rejected from a single infection (Ogilvie and Hockley, 1968; Edwards, Burt and Ogilvie, 1971). It

was speculated that these stunted worms had adapted in some way, becoming less immunogenic and less susceptible to the host's immune reaction. In natural infections in the field, such adaptive mechanisms by the parasite may enable it to survive. It is also possible that with continuous low grade infections the host gradually becomes tolerant to the parasite.

1.9.2 Stage-specific antigens

The cuticle or tegument of helminths presents another potential evasion mechanism. Lumsden (1975) proposed that the nematode cuticle was an antigenically inert layer serving as a structural exoskeleton. It has since been demonstrated that the parasite surface is a dynamic and antigenic structure (Philipp and Rumjaneck, 1984), with a different set of surface antigens often being expressed by each parasite stage. Therefore, by the time the host responds to the surface antigens presented by one stage, the parasite will be expressing a different set of antigens. It has been speculated that this mechanism may allow *T. spiralis* newborn larvae to survive their migration from the intestine to the muscle (Almond and Parkhouse, 1985). However, this method of evasion will only be effective during a primary infection and in infections where exposure of stage-specific antigens is of a shorter duration than the development of an antibody response to them.

1.9.3 Antigen shedding

Release of parasite antigens is essential for the initiation of the host's immune response to large parasites, due to the necessity of lymphoid cells to be presented with antigen in the context of the host's major histocompatibility complex (MHC) antigens. The release of surface antigens which occurs with many parasitic helminths *in vitro* may, however, play an important role in evasion of the host's immune response. This may explain the reduced ability of host-derived schistosomula of *S. mansoni* to bind anti-schistosome antibody to their surface, compared to schistosomula developing *in vitro* (Pearce, Basch and Sher, 1986). This was found to be unrelated to the acquisition of host molecules by the parasite,

which had previously been shown to result in a reduction in surface antigenicity (McLaren, 1984).

Shed surface antigens can also act as immunosorbents, removing antibody specificities directed against the parasite surface. It has been demonstrated that excretory/secretory products of *T. canis* L2 contain proteins which are antigenically related to those found on the larval surface (Maizels, de Savingy and Ogilvie, 1984). As a result, no binding of antibody to the surface was observed in immunofluorescence studies, unless incubations were carried out in the cold or in the presence of antimetabollites to prevent shedding of surface antigens or, alternatively, to prevent the release of inhibitory factors preventing antibodies binding to the surface (Smith *et al.* 1981). Badley *et al.* (1987) also found that absorption of *T. canis* infection serum with larval ES products resulted in a loss of antibody reactivity to the larval surface and abrogation of antibody-mediated eosinophil adherence.

Once binding to the parasite surface has occurred, antibody can be removed by the shedding of antibody-surface antigen complexes. This has been demonstrated with *Fasciola hepatica,* with the glycocalyx-IgG complex being shed and the glycocalyx being rapidly replaced thus potentially preventing antibody-mediated binding of accessory cells and subsequent damage to the parasite surface (Hanna, 1980),

Following the release of surface antigens, new antigenic determinants may be exposed which are not present on the surface of intact worms. These new antigens may divert the immune response from the production of antibodies to antigens the host is normally exposed to. It has been found that monoclonal antibodies derived from lymphocytes of a mouse following normal infection with *T. spiralis* recognised detergent-extracted surface-labelled proteins in immunoprecipitation studies but did not bind to the surface of intact parasites (Ortega-Pierres *et al.,* 1984). However, the opposite view has been taken by Pritchard, McKean and Rogan (1988) who suggested that these "hidden" epitopes may be important to protective

immunity, with released antigens diverting the immune response.

1.9.4 Host mimicry

Acquisition of host materials is another effective escape mechanism used by parasites. The longevity of schistosome infections is thought to be due, in part, to the acquisition of a variety of host components including immunoglobulins, histocompatibility antigens and glycolipid antigens of the major blood groups, as well as the parasite's ability to synthesise molecules cross-reactive with host antigens (reviewed by McLaren, 1984). The presence of host components has also been demonstrated on the surface of circulating microfilariae (mf) of certain filarial worms. Radiolabelling studies of surface proteins of *Litomosoides carinii* and *Onchocerca gibsoni* showed a complex profile of proteins on the surface of *in vitro*-released mf, while only one component, identified as host serum albumin, was present on the surface of host-derived mf (Philipp *et al.,* 1984; Forsyth, Copeman and Mitchell, 1984). Human serum albumin has been identified on the surface of blood-derived mf of *Wuchereria bancrofti* (Maizels *et al.,* 1984), while blood-group determinants have been identified on circulating mf of *W. bancrofti* and *Loa loa* (Ridley and Hedge, 1977). Cat immunoglobulin has also been identified on the surface of circulating mf of *Brugia pahangi* (Premartne, Parkhouse and Denham, 1984), which, if bound non-specifically, may prevent the binding of anti-parasite antibodies, while binding of immunoglobulin to the surface via the Fc region would prevent cellular adherence.

1.9.5. Inactivation of host effector components

The release of enzymes by parasitic helminths can allow them to survive immune attack, as will be discussed in detail in Chapter 8. The hydrolysis of host IgG by 'fabulating' enzymes released by *S. mansoni* schistosomula (Auriault *et al.,* 1981) and immature *F. hepatica* (Chapman and Mitchell, 1982) prevents complement fixation and the adherence of granulocytes to the parasite surface, due to the removal of the immunoglobulin Fc region. The peptides produced by the

cleavage of IgG also inhibit macrophage stimulation and IgE mediated cytotoxicity against schistosomula *in vitro* (Auriault *et al.,* 1980).

The release of anti-oxidant enzymes has been demonstrated *in vitro* to protect parasites from toxic oxygen metabolites produced by host neutrophils and activated macrophages. The resistance of adult worms and the sensitivity of newborn larvae of *T. spiralis* to *in vitro* killing by granulocytes was reflected by differences in their superoxide dismutase (Rhoads, 1983) and glutathione peroxidase (Kazura and Meshnick, 1984) content. The finding that co-incubation of adult worms with newborn larvae partially protected the larvae against oxidant damage strongly suggested that secretion of these enzymes may be important in protection against oxidant-mediated damage *in vivo.*

1.9.6 Immunosuppression

Many parasitic infections are associated with suppression of host immune responses to both parasite derived and other unrelated antigens. It is thought that immunosuppression is mediated by suppressive factors produced by parasites which are beneficial to their survival. It has been demonstrated that suppression of host immune responses in *T. spiralis* infection can be mediated by material released by newborn larvae via the activation of cyclophosphamide-sensitive suppressor T cells and the administration of cyclophosphamide at specific times during infection was found to decrease the parasite burden (Faubert, 1982).

Immunosuppression is also associated with the gastro-intestinal nematode *Nematospiroides dubius.* Adult worms from a primary infection can survive for up to eight months and, in most mouse strains, subsequent infections proceed as successfully as the primary. However, immunity to challenge can be induced by repeated infections accompanied by drug treatment to reduce the worm burden (Behnke and Parish, 1980) or by the administration of irradiated larvae, which do not mature to the adult stage (Hagan *et al.,* 1981). It was found, however, that irradiated larvae stimulated no immunity when adult *N. dubius* were implanted in

the intestine before the larvae were administered (Behnke, Hannah and Pritchard, 1983). From this it was concluded that larvae stimulated protective immunity which could be depressed by adult worms. This immunosuppression is non-specific (Ali and Behnke, 1983) and is thought to involve suppressor T cells (Pritchard, Ali and Behnke, 1984) although the exact mechanism is unknown.

Suppression of immune responsiveness to both parasite-specific and unrelated antigens is also characteristic of filarial infections. That this effect was parasite mediated was demonstrated by the ability of microfilarial antigen to induce suppression in normal cells *in vitro.* These cells then suppressed the proliferative response of normal cells (Piessens *et al.,* 1982), a phenomenon which is also mediated by serum from microfilaraemic patients (Piessens *et al.,* 1980).

Interference in the activity or production of lymphokines can also have profound consequences on the cellular components of the immune system. It has been found that a proteinase inhibitor of the cestode parasite *Taenia taeniaeformis* prevents IL-1 activation of host cells, as well as suppressing lymphocyte activity by the inhibition of IL-2 synthesis (Leid *et al.,* 1986).

Parasites can, therefore, interfere with a variety of immune mechanisms as demonstrated by *in vitro* assays. The chronicity of many helminth infections suggests that some of these, at least, are operational *in vivo.* However, host factors can also affect the outcome of infection, giving rise to the phenomenon of overdispersion, where the majority of parasites are harboured by a small proportion of the parasitised population (Shad and Anderson, 1985; Anderson, 1986). This uneven parasite distribution is influenced by several factors including the age of the host, the nutritional status, the presence of concurrent infections, level of hygiene and the host's genotype. It is particularly difficult to assign the effects of the latter to the outcome of parasitic infections in humans, with large sample sizes and detailed family studies being necessary. However, detailed investigations have been carried out in the murine system and have demonstrated associations between host MHC and non-MHC genes on the outcome of infection with *T. muris* (Else, Wakelin and Roach, 1989), *T. spiralis* (Wassom *et al.,* 1983),

H. polygyrus (Keymer *et al.*, 1990), *N. dubius* (Behnke and Robinson, 1985), *B. malayi* (Kwan-Lim and Maizels, 1991) and *S. japonicum* (Mitchell *et al.*, 1985). The importance of this phenomenom to the future control of parasitic helminths will be discussed further in Chapter 4.

The minority of individuals who are heavily and chronically infected are important in maintaining the transmission of the parasite and ensuring its survival, and it has been suggested that parasite-control programmes should be aimed at the treatment of these individuals (Anderson and Medley, 1985).

1.10 VACCINES AGAINST PARASITIC HELMINTHS

It is apparent from the above discussion that effective immunological control of parasite infections will require an understanding of protective host mechanisms, how the parasite evades or exploits such responses in chronic infections, and the effects of host factors on the efficiency of the immune response. Once these are understood, it should then be possible to design vaccines to specifically stimulate protective responses in all hosts.

1.10.1 Vaccine development

The success of the *D. viviparus* vaccine in 1960 led to the optimistic view that vaccines against other parasites would soon follow (Stoll, 1961; Thorson, 1963). However, the many problems involved in vaccine production and testing were not anticipated and, despite the significant advances in our knowledge of host/parasite interactions, Dictol remains the only commercially available vaccine against a parasitic helminth. Nevertheless, the advent of gene cloning has brought with it a great deal of enthusiasm in this field and, with the technology available to generate large amounts of parasite antigens without the need for the parasites themselves, the development of other anti-helminth vaccines is a more realistic possibility.

a) Attenuated parasites

The efficacy of the bovine lungworm vaccine has been matched by attenuated larval vaccines against the sheep lungworm, *Dictyocaulus filaria* (Tewari, Dhar and Singh, 1971) and the dog hookworm, *Ancylostoma caninum* (Miller, 1971). Although the *A. caninum* vaccine was effective in preventing hookworm disease it, like Dictol, did not prevent infection. The presence of hookworm eggs in the faeces of vaccinated dogs led to suspicion of vaccine failure and as effective anthelmintics were readily available, drug treatment was carried out in preference to vaccination, with the subsequent withdrawal of the vaccine.

Successful immunisations against *H. contortus* (Urquhart *et al.,* 1966b) and *T. colubriformis* (Greg and Dineen, 1978) in adult sheep have been achieved by the administration of irradiated infective larvae. However, such vaccines were unable to induce protective responses in young lambs, possibly due to their lack of cellular immunity or inability to mount secretory IgA responses (Duncan, Smith and Dargie, 1978).

Vaccination of sheep and cattle with X-irradiated schistosomula of *S. bovis* resulted in 60-70% protection against challenge (Bushara *et al.,* 1978). Similar studies with irradiated cercaria or schistosomula of *S. mansoni* produced variable results, with effective immunisation being achieved in mice while little protection was produced in baboons and chimpanzees (Taylor, 1980), perhaps indicating that a similar vaccine would be unsuccessful in humans.

Although vaccines based on live, attenuated infective forms have produced effective vaccines for veterinary use, it is unlikely that the use of such parasites will be permitted in humans. This, together with the difficulty of obtaining sufficient quantities of parasites for use in vaccination and the short shelf-life of live vaccines, make this form of immunisation unattractive. However, analysis of attenuated vaccine systems is important to our understanding of the mechanisms of immune killing.

b) Parasite extracts

Immunisation with crude extracts of helminths has produced limited and variable success with a number of parasites. With *Ascaris suum*, Kerr (1938) was unable to induce immunity in guinea-pigs with larval or adult extracts while Stromberg and Soulsby (1977) reported some degree of immunity with second-stage larvae and adult worms. In the natural host, the pig, Benkova (1982) obtained 89% immunity using L2 extract.

Attempts to vaccinate against *S. mansoni* with homogenates of cercaria, adult worms or eggs have also produced variable results. Thomson (1954) and Ritchie, Garson amd Erickson (1962) detected no protective immunity following immunisation of mice. In contrast, significant reductions in mortality were reported following immunisation of previously infected mice with *S. mansoni* extracts (Silva and Ferri, 1968). However, vaccination of rhesus monkeys with homogenate of *S. mansoni* stimulated no detectable resistance to challenge (Smithers, 1962). To determine whether parasite antigens had been destroyed during preparation of the extracts, adult worms, killed by snap freezing, were injected into the portal vein of rhesus monkeys. These worms induced no resistance, while a similar number of live adult schistosomes, injected in the same way, stimulated a high level of immunity (Smithers and Terry, 1967).

In most instances, normal or attenuated parasites induce greater protection than whole parasite homogenates, suggesting that antigens expressed or presented by live parasites are essential to immunity. Such antigens may be present in the whole extracts but in such small quantities that effective immunity is not induced, or perhaps the administration of a large number of parasite components may overload the host's immune system. This may explain the inability of larval and adult extracts of *T. spiralis* to protect rats (Berntzen, 1974), although alpha and beta granules isolated from stichocyte organelles induced significant protection (Despommier, Campbell and Blair, 1977).

c) Excretory-secretory antigens

Following the failure of most crude extracts to induce protective immunity attention then turned to products released by live parasites *in vitro*. That such material could induce immunity was demonstrated by Thorson (1953) who achieved 60% protection in rats following immunisation with *N. brasiliensis*. This has since been confirmed by Poulain *et al.* (1976) who demonstrated 94% protection using secretory antigens of *N. brasiliensis,* while soluble extracts of whole worms were relatively ineffective. Significant protection has also been achieved using ES products from *T. colubriformis* fourth stage larvae in guinea-pigs (Rothwell and Love, 1974); products released from *H. contortus* during the moult from L3 to L4 (Ozerol and Silverman, 1970); and significant, although variable, protection has been reported with *A. suum* ES products in mice and guinea-pigs (Clegg and Smith, 1978).

d) Surface antigens

Demonstration that the surface of helminth parasites is antigenic (Philipp and Rumjaneck, 1984) and is a potential target for host attack, has prompted assessment of surface extracts in generating immunity. This has met with varying success, with protection being achieved in mice against *T. spiralis* following immunisation with surface antigens of muscle larvae (Grencis *et al.,* 1986) and by passive immunisation with monoclonal antibodies directed against surface determinants (Ortega-Pierres, MacKenzie and Parkhouse, 1984). However, proteins purified from insoluble extracts of *S. mansoni,* thought to be surface proteins due to their ability to absorb antibody capable of killing intact schistosomula *in vitro,* gave no protection (Sher *et al.*, 1974) and more recent studies have reported only a limited degree of protection (26%) following vaccination with *S. mansoni* adult worm surface membranes (Smithers *et al.*, 1989).

e) Cloned antigens

Protection studies with parasite surface extracts have been limited by the lack of surface antigen preparations. However, such problems may now be overcome by the use of recombinant DNA technology. Assuming that protective antigens are proteins, then cloning of these into expression systems will allow the synthesis of antigens for use in vaccination. Difficulties can arise if the protein is glycosylated or if its conformation is important to its immunogenicity, whereby alternative mammalian or insect expression systems will be needed.

As yet, no recombinant helminth vaccine has been developed commercially. However, significant protection (94%) has been reported against *Taenia ovis* in sheep using a recombinant onchosphere antigen, produced as a glutathione-S-transferase fusion protein (Johnson *et al.*, 1989) and could form the basis of a commercial vaccine. With other parasitic helminths, DNA technology has allowed examination of purified parasite antigens without the need for large quantities of live parasites. However, the initial problem of identifying what antigen or antigens are essential to protective immunity and how these can be delivered to the host to induce an effective response still remain.

1.10.2 Identification of protective antigens

Several strategies have been employed to identify antigens involved in protective immunity. The first involves vaccination with a crude parasite extract or with fractionated extracts and identifying potential protective antigens by examining the response to the vaccine. Such antigens can then be purified by further fractionation or by preparative SDS-PAGE and their immunisation potential assessed in protection studies. This method was used in the identification of paramyosin as a potential vaccine candidate for *S. mansoni* infection (Pearce *et al.*, 1988).

A more rational approach is the identification of molecules which are essential to parasite survival, inactivation of which would be detrimental to the organism. This approach has led to parasite enzymes, such as proteinases thought to be involved in skin penetration, being selected as vaccine candidates (McKerrow *et*

al.,1985; McKerrow *et al.*, 1990). Although such enzymes are often not strongly immunogenic, possibly due to conservation of amino acid sequences between parasite and host enzymes, inactivation even by low levels of antibody may be harmful to the parasite, as demonstrated by vaccination with *S. mansoni* glutathione-S-transferase (Capron *et al.*, 1987).

Another approach is to identify antigens which are seen uniquely by naturally resistant hosts. It has been found that sera from amicrofilaraemic individuals living in a *Brugia malayi* endemic area recognise an antigen of molecular mass 75kDa which is not recognised by individuals with patent infections (Selkirk *et al.*, 1986; Kurniawan *et al.*, 1990). This strategy has been aided by the identification of animal strains which are resistant to infection and comparing their response to that of susceptible strains. For example, mice of the inbred 129/J strain, which are uniquely resistant to infection with *S. japonicum*, show an antibody response to a 26kDa antigen, identified as glutathione-S-transferase, which is not recognised by susceptible strains (Smith *et al.*, 1987). It has been suggested, however, that this resistance may be due to defects in the hepato-portal system of 129/J mice resulting in damage and, ultimately, in elimination of worms from the lungs rather than to differences in immune responsivess of different mouse strains (Coulson and Wilson, 1989; Mitchell *et al.*, 1990). Therefore, the implications of such studies must be carefully considered.

Finally, a widely utilised approach is the production of protective monoclonal antibodies allowing the identification of the relevant antigen. A monoclonal antibody which mediated eosinophil-dependent destruction of *T. spiralis* newborn larvae *in vitro* and reduced infectivity in mice following passive immunisation was found to recognise a 64kDa antigen on the surface of the larvae (Ortega-Pierres, MacKenzie and Parkhouse, 1984). Several protective mouse monoclonal antibodies have also been described for *S. mansoni* and their respective antigens identified (Smith *et al.*, 1982; Zodd and Philipps, 1982; Simpson and Cioli, 1987) while it has recently been demonstrated that IgA monoclonal antibodies directed against

stichocyte granules of *T. muris* confer protection against this parasite (Roach *et al.*, 1991). As well as identifying protective antigens, monoclonal antibodies can also be used as immunosorbents to purify the specific antigen for use in vaccination studies. They can also be used to screen DNA expression libraries to identify genes encoding the corresponding antigens, although monoclonal antibodies which recognise carbohydrate epitopes will not react with polypeptides expressed in bacterial systems.

Carbohydrate determinants can, however, be generated by using monoclonal antibodies to raise anti-idiotype antibodies, the antibody binding site of which will be similar to the epitope of the original antigen. This strategy has proved effective against *S. mansoni* using a monoclonal antibody to a 38kDa membrane glycoprotein to raise anti-idiotype antibodies, which in turn were used to immunise naive rats (Grzych *et al.,* 1985).

1.10.3 Optimising Vaccine Efficacy

Experimental immunisation using defined helminth parasite antigens has rarely produced protection of greater than 40-50%. While 100% efficiency is not essential for the control of these non-replicating organisms, it is likely that vaccines capable of higher levels of protection will be required to create a significant impact on disease. As stated by Murray (1987), there exists a "block between cloning genes for polypeptides and the development of practical vaccines". Rational approaches combining knowledge of the immune response to specific parasites with molecular techniques are necessary to improve the efficacy of helminth vaccines.

Recombinant DNA or synthetic peptide vaccines can be modified to enhance their immunising effect. For example, purified antigens induce antibody but little T cell response. T cell epitopes should, therefore, be included in subunit vaccines for the induction of cell mediated immunity and for immunological memory. The positioning of T cell epitopes, with respect to the relevant B cell epitopes is also thought to be important to its effectiveness (Zanetti, Sercarz and Salk, 1987).

Subunit vaccines can also be designed so that determinants detrimental to an effective immune response, such as those inducing immunosuppressive, immunopathological or immunoevasive reactions are not included.

As mentioned previously, the ability of an individual to respond to a particular antigen is dependent on the MHC antigens it expresses, due to the restriction of T cells to recognise antigen in association with either Class I (for cytotoxic T cells) or Class II (for helper T cells) antigens (Schwartz, 1985). As MHC antigens may vary in structure, the association between MHC and parasite antigen will vary for individuals in an outbred population. This MHC restriction is more apparent in the response to simple antigens such as peptides, as has been demonstrated using a peptide derived from the circumsporozoite protein of the malarial parasite, *Plasmodiium falciparum,* where only mice of certain MHC-haplotypes responded to vaccination (Del Giudice *et al.*, 1986). Incorporation of a T cell antigenic peptide broadened the MHC restricted response and also primed T helper cells for an anamnestic response (Good *et al.*, 1986). It was suggested that the more T cell inducing determinants in a vaccine, the less restricted the response to it will be (Good *et al.*,1987). However, it is not yet clear whether the algorithms used to define putative T and B cell epitopes (Rothbard and Taylor, 1988) apply to all MHC types or if they can be generalised at all. For example, studies on influenza haemagglutinin have indicated commonality of B and T cell epitopes which is thought to be relevant to B and T cell anamnestic responses (Barnett *et al.*, 1989)

Another problem which may arise in peptide vaccines is the nature of the B-cell epitope. As B cells often recognise conformational antigens it must be ensured that the synthetic vaccine induces antibodies capable of recognising epitopes on the native antigen.

As well as improvements to subunit vaccines themselves, immunisation in conjunction with adjuvants can increase their potency, particularly with peptide vaccines which are weakly immunogenic. Although a variety of adjuvants have been

used in laboratory models and in domestic animals, aluminium hydroxide remains the only one which is licensed for use in humans. It is thought to act by creating an antigen depot, allowing a longer time for the antigen to interact with antigen-presenting cells and lymphocytes and is also involved in the activation of complement. One of the most potent adjuvants is Freund's Complete Adjuvant which consists of a water in oil emulsion with killed mycobacteria in the oil phase (Freund, Casals and Hismer, 1937). The emulsion retains the antigen at the site of injection, while the mycobacteria activate macrophages and stimulate both humoral and cell-mediated immunity. Liposomes are also effective antigen carriers and their efficacy can be enhanced by the incorporation of other adjuvants, such as lipopolysaccharide or muramyl dipeptide. Their mode of action has been shown to depend on the route of administration, with intramuscular or intradermal injection resulting in the formation of an antigen depot (Kramp, Six and Kasel, 1982), while intravenous injection results in rapid delivery to the liver, spleen and lungs (Gregoriadis, 1976). Such differences may be utilised in the development of localised specific immunity to infections.

The use of live vectors for the delivery of recombinant parasite antigens has also proved an effective method of immunisation. Parasite genes can be inserted into viral or bacterial vectors without impairing their infectivity. Vaccinia virus is widely used because of its large genome, allowing the insertion of 20-25 kilobases of foreign DNA (Smith and Moss, 1983). The protein products of these genes may be glycosylated and processed for secretion or membrane insertion, while the persistence of antigen expression by the virus-infected cells will stimulate a vigorous immune response. This approach has been used to examine the immunogenicity of a cloned 28kDa surface antigen of *S. mansoni* schistosomula (Simpson and Cioli, 1987). Parasite genes can also be inserted into attenuated *Salmonella* and may be used in the future as oral vaccines (Dougan, Hormaeche and Maskell, 1987). Vectors can also be designed to express more than one foreign gene and may serve as polyvalent vaccines or enhancers of immune responsiveness by the insertion of genes coding for cytokines. However, vaccination with live infectious

agents does raise doubts concerning the safety of such recombinant vaccines, particularly in immunocompromised hosts. With recombinant vaccinia virus there is also the possibility that existing immunity to the vector may inhibit viral replication in the host, so limiting the effectiveness of the vaccine.

It is probable that successful parasite vaccines of the future will require an understanding of the immune responses to infection, identification of protective antigens, isolation and generation of such antigens, and optimisation of their immunogenicity. It is likely to be some time before these aims are achieved with most parasites, if at all. However, understanding aspects of immunity to parasite which can be successfully controlled by vaccination may be relevant to the future control of other related parasites.

To this end, an immunochemical characterisation of antigens of *D. viviparus* has now been carried out. Prior to this study there was little information on the antigens of this parasite or on the mechanism of vaccine-induced immunity. In view of the advances in immunology and molecular biology over the last thirty years, it now seems appropriate to define the antigens of this parasite involved in stimulating effective immunity.

This study may eventually lead to the development of a new molecularly-defined vaccine against bovine lungworm which will be cheaper and easier to produce than the current attenuated larval vaccine. More importantly, understanding immunity to this parasite and how this is successfully achieved by vaccination may have relevance to the control of other helminth diseases of livestock and humans.

CHAPTER 2

MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS AND ANTISERA

2.1.1 Calves.

Three month old Ayrshire or Friesian calves were obtained from local sources where they were reared under conditions in which infection with helminth larvae was unlikely. During experimentation they were kept indoors and maintained on a commercial pelleted diet and hay.

Calves were infected orally with normal or 40krad (equivalent to 400Gy) radiation-attenuated infective third stage larvae (L3), or intravenously with larvae irradiated to 100krad (1,000Gy). Blood samples were taken by venepuncture of the jugular vein prior to the experimental procedure and at intervals of approximately 4 weeks thereafter. After leaving the blood to clot at 4^{o}C overnight, the serum was removed following centrifugation at 7,000g for 20 min and stored at -20^{o}C. The exact number of larvae administered and the days of infection varied for each experiment and will be given in greater detail later.

For species-specificity experiments, calves were infected twice, 10 weeks apart, with 50,000 third stage larvae of *Cooperia oncophora* or *Ostertagia ostertagi,* common gastrointestinal nematodes of cattle.

2.1.2 Guinea-pigs.

Male albino guinea-pigs of the Dunkin Hartley outbred strain were purchased from Harlan Olac Ltd. (Blackthorn, Bicester, Oxon, U.K.) and used when 250-300g in weight. Inbred guinea-pigs of strain 2 and strain 13 were purchased from the National Institute for Medical Research (N.I.M.R.) (Mill Hill, London, U.K).

Guinea-pigs were infected orally with 5,000 normal or 40krad irradiated larvae, or intraperitoneally with 5,000 100krad irradiated larvae, on two occasions 28 days apart. After a further 28 days, blood was sampled by cardiac puncture under general anasthaetic (ether). Guinea-pigs were then challenged with 5,000 normal larvae and blood collected 7 days later.

For protection experiments, guinea-pigs were immunised intraperitoneally with 50μg of L3 extract, adult extract or adult ES in 1ml Freund's Complete Adjuvant (FCA: Sigma Chemical Company, F-4258, Fancy Road, Poole, Dorset, U.K.) and boosted by the same route in Freund's Incomplete Adjuvant (FIA: Gibco Europe Ltd, 660-5720, Paisley, Scotland, U.K.) 21 days later. Blood was sampled

21 days after the last dose of antigen in adjuvant and the guinea-pigs were then challenged orally with 5,000 normal larvae. Blood was collected 7 days after challenge and serum extracted as previously described.

Inbred guinea-pigs were immunised with 5,000 40krad irradiated larvae on two occasions, 21 days apart and blood sampled prior to challenge with 5,000 normal larvae after a further 21 days. Blood was collected 7 days after challenge.

2.1.3 Rabbits.

One male Lop Rabbit, weighing 3kg, was purchased from N.I.M.R. and immunised intramuscularly with 100μg adult ES material emulsified in 0.5ml FCA. This was repeated 7 days later using FIA. It was then boosted 28 and 56 days later with the same quantity of antigen in 0.5ml FIA, subcutaneously in five sites on the back and blood sampled after a further 7 days.

2.2 POST-MORTEM TECHNIQUES

2.2.1 Calves

Calves were killed by conventional methods and live lungworms recovered by opening the air passages from the trachea to the small bronchioles with fine, sharp pointed scissors. Worms were removed and placed in a petri dish containing warm saline, for counting.

2.2.2 Guinea-pigs

Guinea-pigs were killed by terminal anasthesia with trichloroethylene (Trilene, BDH Chemicals Ltd., Poole Dorset, U.K). The lungs and thoracic portion of the trachea were removed, placed in a stomacher plastic bag containing 20ml of 0.85% saline solution and homogenised in a Stomacher 80 device (A.J. Seward, UAC House, London, U.K.) until only very fine lung fragments remained. This suspension was filtered through gauze into a flask containing saline at 37^{o}C (Baermann aparatus) for 6-12h and the sedimented larvae collected and counted.

2.3 PARASITES

2.3.1 Third stage larvae

Infective and 40krad X-irradiated third stage larvae were provided by Glaxovet (Ware, U.K.) and later by Intervet U.K.(Cambridge, U.K.). For some studies larvae

were gamma irradiated in a Cobalt-60 source chamber (Nuclear Engineering, Berkshire, U.K.).

When needed, larvae were exsheathed using 5% Milton's fluid (Milton 2, Richardson-Vicks Ltd., U.K.) equivalent to 0.001% w/v sodium hypochlorite. The larvae were suspended in this solution for 15 min, then centrifuged at 550g for 5 minutes. This was repeated until larvae were microscopically free of sheaths when they were then washed three times in phosphate buffered saline (PBS), pH7.2 (see Appendix).

2.3.2 Adult parasites

Adult worms were collected from the lungs of calves 28-35 days after infection with 5,000 normal larvae, or from field cases of patent lungworm infection.

2.3.3 Eggs

Eggs were collected from cultures of adult parasites following centrifugation of spent culture medium at 900g for 5 min. These were then washed five times in PBS to deplete any adult material which may have attached to their surface.

2.3.4 First stage larvae

First stage larvae (L1) were recovered from fresh faeces of infected animals by passage through "Maxo" milk filters (Blow Ltd.) in tapwater at room temperature. The larvae were collected by sedimentation 5 and 24h later. Alternatively, eggs collected from adult cultures were incubated at 37^{o}C for 48h to permit completion of their embronyation, then hatched in a loosely fitting glass tissue homogeniser.

2.4 PARASITE ANTIGENS

2.4.1 Preparation of larval antigens

Infective stage larvae were washed in PBS before being cultured at a concentration of 10,000/ml in RPMI 1640 (Gibco, 041-02409 M) as used in the culture of *Ascaris* larvae (Kennedy and Qureshi, 1986) (see Appendix), at 37^{o}C, 5% CO_2/air. L3 survived for approximately two weeks, after which time the medium, termed excretory-secretory (ES) material, was filtered through low protein binding 0.22μm filters (Millex GV, Millipore S.A; Molsheim, France) and concentrated approximately 20 fold by centrifugal ultrafiltration at 4^{o}C using

Centicon 10 devices (Amicon Ltd., 4206, Stonehouse, U.K.) with a nominal molecular weight cut-off of 10 kiloDaltons (kDa). ES was then dialysed against PBS, and stored at -70°C until use. On some occasions medium was supplemented with 20% new born calf serum (Gibco, 021-060-10H) as this has previously been shown to enhance the development of L3 to L4 stage (Bos and Beekman, 1985). However, this complex medium was not used in the preparation of ES material.

Extracts of infective larvae were prepared by sonication in a MSE Soniprep 150 at 18μm amplitude. The container was cooled by ice and, to keep heating to a minimum, seven one minute bursts were interspersed with one-minute pauses. Microscopic examination was carried out to ensure complete disruption of larvae. The supernatant was collected following centrifugation of the homogenate at 7000g, 4°C for 30 minutes. Extracts were prepared in ice-cold PBS for use in immunisation studies and enzyme assays. For other studies, parasites were homogenised in ice-cold 10mM Tris, pH 8.3, containing a variety of proteinase inhibitors (Appendix). This is referred to as Tris Homogenisation Buffer (Tris HB).

2.4.2 Preparation of adult material

Following removal from calf lungs, live adult parasites were washed extensively in Hanks' Balanced Salt Solution (HBSS: Gibco, 070-012 00A), containing 12,000 units penicillin, 1,200μg/ml streptomycin, 125μg/ml gentamycin and 25μg/ml amphotericin B. They were then cultured at a concentration of 2 worms/ml in protein-free medium as used in the culture of L3. The medium was harvested every 24h and concentrated as detailed previously. Adult worms survived for 3 days in this medium.

PBS or Tris HB extracts were prepared by disruption of adult parasites in a 1ml glass tissue homogeniser (Jencons Scientific Ltd., Bedfordshire, U.K.) which was kept on ice throughout the procedure. The supernatant was collected following centrifugation as before.

The protein concentration of the samples was estimated using a Coomassie Blue-based assay (Pierce Chemical Co., 23200, Rockford, Illinois.). All antigen preparations were stored at -70°C until use.

2.5 METABOLIC LABELLING

2.5.1 ^{35}S-Methionine metabolic labelling of larvae

60,000 L3 were cultured in 6ml methionine-free Eagle's modified Minimum Essential Medium (MEM: Flow Laboratories, 221-49, Irvine, Scotland) supplemented with 200µg/ml L-glutamine, 100mg/ml glucose, 100mM sodium pyruvate, 2400 units/ml penicillin, 240µg/ml streptomycin 5µg/ml amphotericin B, 20% new born calf serum and 250µCi ^{35}S-methionine (Amersham International, SJ.1015, Aylesbury, Bucks., U.K.). ES was collected after 7 days and was concentrated and separated from unincorporated radiolabel by centrifugal ultrafiltration using Centricon 10 devices.

2.5.2 ^{35}S-Methionine metabolic labelling of adult parasites

Approximately 40 adult worms were cultured in 20 ml MEM supplemented as above to which 400µCi ^{35}S-methionine was added. Culture supernatant was harvested after 24h and replaced with fresh MEM with added ^{35}S-methionine. This was collected after a further 24h and replaced with RPMI with no added ^{35}S-methionine. Culture supernatant was collected after another 24h and adult ES preparations were concentrated and dialysed as previously described.

2.5.3 ^{3}H-Glucosamine labelling of adult parasites

This was carried out by culturing adult worms at a concentration of 2 worms/ml RPMI, supplemented as for *Ascaris* parasites and containing 10µCi/ml of ^{3}H-glucosamine. ES products were collected after 24 and 48h and concentrated as described previously.

2.5.4 Solubilisation of labelled proteins

At the end of the culture period, L3 and adult parasites were washed three times in PBS. They were then homogenised in Tris HB or Tris HB containing the anionic detergent sodium deoxycholate (NaDOC: Sigma, D-6750) at a final concentration of 1%. Glass tissue homogenisers were used for the disruption of both adults and larvae to avoid the possibility of producing radioactive aerosols during sonication. Extracted material was collected following centrifugation of the homogenate at 7000g for 30 minutes at 4^{o}C.

Further extraction was achieved by boiling the remaining pellet for 3 min in

PBS containing 5% sodium doedecyl sulphate (SDS: BDH, 44244), 5% 2-mercaptoethanol (2ME: Sigma, M-6250) and 8M urea (BDH, 45204), termed harsh extraction buffer, and incubating for one hour on ice. The harsh extract was collected after centrifugation for 30 min as described previously. To determine the amount of radiolabel incorporated into ES or present in extracted material, 50µl aliquots of each were dried onto filter paper and counted in a liquid scintillation counter (Pharmacia LKB Biotechnology, Milton Keynes, Bucks., U.K.) following the addition of 2ml scintillant (OptiScint Safe, LKB, FSA Laboratory Supplies, Loughborough, U.K.). Samples were stored at -20^{o}C until analysed by SDS-PAGE.

2.6 RADIO-IODINATION OF SOLUBLE PROTEINS

Iodine labelling of ES or homogenate material was carried out by the Bolton-Hunter method (Bolton and Hunter, 1973), where the iodinated ester reagent conjugates to amino groups, predominantly lysine residues, and by the Iodogen method (Markwell and Fox, 1978), which labels tyrosine groups.

2.6.1 Bolton-Hunter labelling

10µg protein was diluted in 0.1M borate buffer, pH 8.5, to a final volume of 200µl. This was added to a glass vial, which had been pre-coated with 200µCi Bolton-Hunter reagent (*N*-succinimidyl 3-(4-hydroxy,5-[^{125}I] iodophenyl)propionate) (Amersham IM.5861) by leaving the solvent to evaporate overnight in a fumehood. Following 10 min incubation on ice, excess iodine was consumed by the addition of 10% by volume of 2M glycine in 0.1M borate buffer and incubating for 5 min at room temperature, with occasional agitation. Labelled macromolecules were then isolated by gel filtration on Sephadex G-25 (PD-10, Pharmacia Ltd., 17-085-01, Milton Keynes, U.K.), washing with PBS/0.5% Triton X-100 (Sigma, T-6867). Fractions were collected at 30 second intervals and counts measured using a hand-held gamma counter (Mini-Instruments, Scintillation meter, 5.40). Fractions collected in the first peak of radioactivity, containing labelled antigen, were pooled and stored at -20^{o}C until use.

2.6.2 Iodogen labelling

Labelling by the Iodogen method was carried out by diluting 10µg ES or homogenate in PBS to a final volume of 200µl (Kennedy and Qureshi, 1986). This was

incubated with 200μCi Na ^{125}I (Amersham IMS.30) in a tube pre-coated with 200μg IODOGEN (1,3,4,6,-tetrachloro-3a,6a-diphenyl glycoluril, Pierce, 28600) prepared in chloroform at 1mg/ml. Incubation was carried out for 10 min on ice with occasional agitation after which time excess iodine was removed by incubation on ice for 5 min with 10% by volume of saturated tyrosine solution. Labelled proteins were collected as described previously.

2.7 RADIO-IODINATION OF SURFACE PROTEINS

Intact parasites were radiolabelled by the Iodogen and Bolton-Hunter methods as described by Marshall and Howells (1985).

2.7.1 Radiolabelling larval surface

50,000 L3 were suspended in 200μl PBS to be labelled by the Iodogen method, or 200μl 0.1M borate buffer for Bolton-Hunter labelling. Larvae were incubated as described for ES, with 200μCi Na ^{125}I in a tube pre-coated with 200μg IODOGEN, or 200μCi Bolton-Hunter reagent, for 10 min on ice with frequent agitation. Excess ^{125}I was consumed as before, and the parasites washed extensively in cold PBS or Tris HB by centrifugation at 550g for 5 min. This was repeated until the supernatant was free of radioactivity.

2.7.2 Radiolabelling adult surface

Five adult worms were washed in PBS and resuspended in 400μl of the appropriate buffer. They were incubated with 400μCi Na ^{125}I in a tube pre-coated with 400μg IODOGEN, or with 400μCi Bolton-Hunter reagent. All incubations were as described previously. Adult worms were washed in cold PBS or Tris HB by sedimentation, and the supernatant discarded. This was repeated approximately five times, with care being taken not to damage the labelled parasites during the procedure.

2.7.3 Extraction of surface-labelled proteins

The extraction method employed for removal of labelled surface-associated proteins of larval and adult stages varied slightly for different experiments, and exact details will be given later. The methods employed were based on those described by Maizels *et al.* (1989) and were similar to those used for the solubilisation of ^{35}S-and ^{3}H-labelled proteins (Section 2.5.4), with several additional steps being carried out.

Parasites were usually incubated for 2-4h at 4°C in Tris HB containing the cationic detergent cetlytrimethylammonium bromide (CTAB: Sigma, M-7635), at a final concentration of 0.25% as described by Pritchard *et al.* (1985). Extracted material was collected following centrifugation of L3 at 2000g for 10 min at 4°C, or removed directly from adult incubations. Parasites were then homogenised as described previously in Tris HB containing 1% DOC and the supernatant collected following centrifugation at 7000g for 30 min, 4°C. The remaining pellet was suspended in Tris HB/1% DOC/5% 2ME, incubated for 2 hours on ice, and the solubilised material collected as before. The pellet was then boiled in harsh extraction buffer and the supernatant collected following a further 1h incubation on ice.

On some occasions, intact surface-labelled parasites were cultured in *Ascaris* medium for 24h at 37°C to determine whether any shedding of surface proteins occurred. The culture medium was collected and concentrated as before.

2.8 TCA PRECIPITATION

Precipitation of the labelled ES or surface extracts with trichloroacetic acid (TCA: BDH) was carried out to estimate the amount of radiolabel which was bound to macromolecules. This eliminates the counts due to free ^{125}I, as only large molecules are precipitated by TCA. 5µl normal rabbit serum (Lop rabbit pre-bleed) and 2µl labelled antigen were added to 50µl PBS/0.5% Triton X-100 and the input counts per minute (cpm) measured in a gamma counter (Pharmacia LKB). 10% TCA was added and samples centrifuged at 2000g for 10 min at 4°C. The supernatant was removed and the output cpm of the pellet was measured. The percentage of counts precipitated by TCA was calculated as follows:

% TCA precipitate = 100% x output cpm/input cpm

2.9 IMMUNOPRECIPITATION

This was carried out using *Staphylococcus aureus* (Staph A) as a solid-phase absorbent (Kessler, 1975). 150-200 $x10^3$ cpm of ^{125}I or ^{35}S labelled parasite material was incubated with 2.5µl serum in 50µl PBS/0.5% Triton X-100 overnight at 4°C. To detect any radiolabelled host serum components, 2.5µl rabbit antiserum to bovine whole serum (Sigma, B-8270) or bovine serum albumin (B-1520) were used.

IgG immune complexes were precipitated with 50µl formalin-fixed *S. aureus* cell suspension (Gibco, 560-93215B) and unbound labelled antigen removed by washing the pellets at least three times. For immunoprecipitations with radiolabelled ES, washes were carried out using Lithium Wash Buffer (see Appendix), which was found to reduce non-specific binding of normal serum. Immunoprecipitations of CTAB or DOC extracts of surface-labelled parasites were washed with PBS/0.5% Triton X-100 containing 0.25% CTAB or 1% DOC to prevent any non-specific precipitation of the labelled extracts. Prior to incubation with serum these extracts were pre-cleared to prevent non-specific binding to Staph A. This was achieved by incubating the extracts with an equal volume of Staph A suspension at 4^{o}C for 1h, and collecting the supernatant following centrifugation at 2000g for 10 min at 4^{o}C. In some experiments Staph A pre-coated with anti-bovine IgG (Sigma, B-8395), was used to detect precipitation of all IgG and IgM subclasses. The radioactivity of the precipitated material was measured in a gamma counter and expressed as a percentage of the input cpm and as a percentage of the TCA precipitable cpm for the particular antigen in use, as follows:

% precipitation = 100 x output cpm/input cpm x 1/% TCA precipitate

Pellets were stored at -20^{o}C until analysed by SDS-PAGE.

2.10 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

2.10.1 Electrophoresis procedure

Separation of proteins was carried out by the method of Laemmli (Laemmli, 1970). 0.7mm thick 5-25% gradient gels, consisting of 120mm separating gel and 10mm stacking gel, were prepared by standard procedures (Appendix). Samples were mixed with SDS-PAGE sample buffer (see Appendix), and boiled for 10 min prior to loading. Sample buffer contained 5% 2ME or 1mg/ml iodoacetamide (Sigma) for reducing and non-reducing conditions, respectively. Usually 20µl sample buffer was added to 20µl protein samples and 40µl buffer added to immunoprecipitation pellets. Pellets were resuspended in buffer, boiled for 10 min, then centrifuged at 7000g for 5 min. The supernatant, containing free radio-labelled antigen, was then applied to the gel. Molecular weights of parasite proteins were estimated by reference to molecular weight marker proteins (Pharmacia 17-0446-01) (Mol Wt Range 14,000-94,000 Da). These were radiolabelled with ^{125}I by the Iodogen method for molecular weight determination of radiolabelled proteins on

autoradiographs and fluorographs.

Electrophoresis was carried out for 4h at a constant current of 40mA per gel, using Tris/glycine buffer (see Appendix). Gel tanks were cooled by circulating water at 4°C to minimise any gel distortion due to heating during electrophoresis.

2.10.2 Staining polyacrylamide gels for protein

Following electrophoresis, proteins were visualised by incubating gels in 0.1% Coomassie Brilliant Blue R-250 (Sigma, B-0149) in 25% methanol, 10% acetic acid, 1% glycerol for 2h, followed by destaining in the solvent until a clear background was obtained. Alternatively, proteins were detected by silver staining, which is a more sensitive detection method. This was performed using a silver stain kit (Quick Silver Kit, Amersham, RPN.17). Stained gels were dried onto filter paper on a gel slab drier (Bio-Rad, 1125B) at 80°C.

2.10.3 Autoradiograpy and fluorography

Following electrophoresis of radiolabelled proteins, gels were fixed for 30 min in 25% methanol, 7.5% acetic acid, 1% glycerol, then dried at 80°C, as above. To allow the detection of low energy beta particles of ^{35}S and ^{3}H, gels were rocked in scintillant (Amplify, NAMP.100, Amersham) for 30 min prior to drying. Gels were then exposed to flashed Fuji RX X-ray film (Technical Phot Systems, Cumbernauld, Scotland) with Du Pont Cronex Lightning-Plus intensifying screens (H.A. West, Edinburgh) in Harmer X-ray film cassettes at -70°C.

2.11 PROTEINASE DIGESTION

To ensure that radiolabelled material was proteinaceous, labelled ES and surface extracts were treated with broad specificity proteolytic enzymes, as described by Qureshi *et al.* (1987). 10µl diluted radiolabelled sample (50,000-100,000 cpm) was incubated for 30 min at 37°C with 10µl diluted pronase (Boehringer Mannheim, 165921, Lewes, U.K.) or proteinase K (Boehringer Mannheim, 161519, EC 3.4.21.14), to give final concentrations in the range 2.5µg/ml-25mg/ml enzyme. Digestion was terminated by the addition of 20µl sample buffer containing proteinase inhibitors. Samples were then boiled for 10min, and products analysed by SDS-PAGE and autoradiography.

2.12 COLLAGENASE DIGESTION

Surface labelled extracts of adult parasites were treated with high purity collagenase (Sigma, Type VII, C-0773) at final concentrations of 0, 10 and 100µg/ml, as described by Selkirk *et al.* (1989). 10µl labelled protein (ideally 50,000-100,000 cpm) was incubated at 37°C for 1h with 10µl collagenase, diluted in 10mM Tris/HCl pH 7.5 containing 10mM calcium chloride (BDH), 1mM phenylmethylsulfonyl fluoride (Pms-F: Sigma, P-7626) and 5mM N-ethylmaleimide (NEM: Sigma, E-3876). The proteinase inhibitors Pms-F and NEM were added to exclude any general proteolytic activity, rendering the digestion specific for collagenase. The reaction was terminated by the addition of 5µl of 25mM ethylenediaminetetra acetic acid (EDTA: Sigma, ED2SS), and samples immediately boiled with 20µl sample buffer, to be analysed by SDS-PAGE.

2.13 GLYCOSIDASE DIGESTION

Glycopeptidase F (PNGase F, Boehringer Mannheim, 913 782), which hydrolyses asparagine-linked oligosaccharides from glycoproteins (Tarentino, Gomez, and Plummer, 1984), was used to examine the glycosylation of surface and ES proteins as described by Maizels *et al.*(1989). 5µl ^{125}I-labelled protein (50,000-100,000 cpm) was boiled for 3 min in 5µl PBS containing 1% SDS and 1.6% 2ME. When cool, samples were mixed with 17µl of PNGase digestion buffer (Appendix). 3µl PNGase F, diluted in 50% glycerol/2.5mM EDTA, was then added to give final concentrations in the range 1mU/ml to 10 U/ml. A control containing diluting buffer only was also included. Samples were incubated at 37°C, for 24h, after which time digestion was terminated by the addition of 20µl sample buffer to the reaction mixtures, which were then boiled for 10min and analysed by SDS-PAGE and autoradiography.

2.14 LECTIN AFFINITY CHROMATOGRAPHY

The presence of glycoproteins in ES and adult surface extracts was also examined by the use of lectins, carbohydrate-binding proteins which interact with specific sugar structures (Goldstein and Hayes, 1979). A variety of lectins were used with different binding specificities, as detailed in Table 2.1. All were purchased from Sigma and were immobilised on 4% beaded agarose.

TABLE 2.1 Lectin binding specificities

LECTIN (Sigma Cat. No.) (Source organism)	BINDING SPECIFICITY	ELUTING SUGAR (Sigma Cat. No.)
lentil (L-4018) (Lens culinaris)	-D-mannose, -D-glucose	0.1M methyl-D-mannopyranoside (M-6882)
Concanavalin A (C-8402)	-D-mannose, -D-glucose	0.1M methyl-D-mannopyranoside followed by 0.1M HCl
wheat germ (L-1882) (Triticum vulgaris)	N-acetly glucosamine	0.1M N-acetyl D-glucosamine (A-8625)
snail (L-8639) (Helix pomatia)	N-acetyl galactosamine	0.1M N-acetyl D-galactosamine (A-2795)
lotus (L-3275) (Tetragonolobus purpureas)	-L-fucose	0.1M -L-fucose (F-2252)

Chromatography columns(Econo-column, Biorad) were packed with 5ml immobilised lectin and washed with running buffer (see Appendix). Radiolabelled sample (approximately 80,000 cpm) was diluted in running buffer to a volume of 100µl and loaded onto the column, followed by 400µl buffer. After 30 min, unbound proteins were removed by applying running buffer to the column and 0.5ml fractions collected until the radioactivity of the collected material fell to background level. Bound proteins were then eluted with running buffer containing 0.1M of the appropriate competing sugar (see Table 2.1). Due to the high affinity of Concanavalin A for mannose/glucose residues, 0.1M hydrochloric acid was applied to Con A columns to ensure complete elution of mannose/glucose containing glycoproteins, and fractions collected into tubes containing 25µl 2M Tris to neutralise the acid. Fractions containing the highest cpm were concentrated using Centricon 10 devices, as described previously, to a volume of approximately 100µl. 20µl of each fraction was boiled with 20µl sample buffer and analysed by SDS-PAGE.

2.15 IMMUNOBLOTTING

2.15.1 Blotting procedure

Immunoblotting was carried out by the method of Towbin, Staehelin and Gordon (1979). 5-25% SDS-gels were run as described previously, loading 100µg protein per well or using gels with one large single well. Proteins were electrophoretically transferred onto nitrocellulose paper (Hybond C-extra, 0.45µm, Amersham, RPN. 303E), using a Tris/glycine/SDS transfer buffer, pH 7.0 (Appendix) in a Trans-blot cell (Bio-Rad, 170-3910) at a constant current of 30mA for 16h at 4^{o}C.

2.15.2 Enzyme-linked antibody detection system

Following transfer, the nitrocellulose membrane was air dried and cut into strips of width 1cm. One strip was stained in 0.1% amido black (BDH, 44291) in 45% methanol, 10% acetic acid for approximately 15 min to visualise proteins, then destained in the solvent. The remaining strips were incubated for 1h in 20% soya milk/0.5% Tween-20 (Sigma) in wash buffer (10mM Tris buffered saline, pH 7.2, containing 0.5% Tween-20) to block non-specific binding of anti-serum to

the nitrocellulose. After this and all subsequent steps, the membrane was washed three times, each for 5 min, in wash buffer. Nitrocellulose strips were incubated for 2h with primary antibody, diluted 1/500 in wash buffer. The presence of the anti-oxidant enzymes superoxide dismutase (SOD) and catalase in parasite preparations was tested by incubating blotted strips in rabbit antiserum to bovine erythrocyte SOD (Sigma, C-10) or bovine liver catalase (Sigma, S-2515), also diluted 1/500 in wash buffer. (These antisera were kindly provided by Ms. Julie Healer, Department of Biology, Imperial College, London, U.K.) Biotinylated anti-species IgG, either anti-bovine (Serotec, AAI-03B, Kidlington, Oxford, U.K.), anti-guinea-pig (Serotec, AAI-02B) or anti-rabbit (Sigma, B-7389) was then incubated for 1h at a dilution of 1/500 in PBS, pH 7.2, as was the next layer, peroxidase-conjugated streptavidin (Serotec, ST AR5B). All steps were carried out at room temperature, and the nitrocellulose rocked in 4ml of the appropriate reagent.

Binding was visualised by incubating the membrane in a solution of the chromogen, 4-chloro-1-naphthol (Sigma, C-8890) containing H_2O_2 (BDH, 45202) at a final concentration of 0.01%. The reaction was terminated after 45 min by removing the substrate solution and washing the nitrocellulose in water. The stained blot was then air dried.

2.16 BIOTIN LABELLING OF SURFACE PROTEINS

Surface proteins of L3 parasites were labelled by the biotin technique which relies on the linkage of a reactive biotin moiety to lysine residues, the binding of which was detected by radiolabelled streptavidin (Hurley, Finkelstein and Holst, 1985).

2.16.1 Biotinylation procedure

20,000 L3 were suspended in 500µl 10mM Tris saline, pH 7.2 and incubated with 150µg *D*-biotin-*N*-hydroxy-succinimide-ester (NHS-biotin: Amersham, TRK 805) in dimethyl formamide, for 1h at room temperature, with occasional agitation. Labelling was terminated by washing the larvae extensively in Tris saline.

2.16.2 Solubilisation of labelled proteins

Surface proteins were extracted by previously descibed methods (Section 2.7.3), by incubation in 0.25% CTAB/ Tris HB followed by homogenisation in 1% DOC and

harsh extraction.

2.16.3 SDS-PAGE and electroblotting

20µl aliquots of each extract were boiled with 20µl sample buffer and proteins separated on 5-25% gradient gels as described in Section 2.10.1. Gels were then blotted onto nitrocellulose paper for 16h at 30mA. The nitrocellulose membrane was incubated for 1h at room temperature with 1% Tween-20 in 10mM Tris buffered saline, pH 7.4, to block non-specific binding. It was then probed with ^{125}I-streptavidin (Amersham, IM. 236) in 50ml Tris saline ($6x10^5$ cpm/ml) for 2h at room temperature. The blot was washed five times in Tris saline/0.05% Tween, to remove free ^{125}I-streptavidin, and exposed to X-ray film, using an intensifying screen.

2.17 INDIRECT IMMUNOFLUORESCENCE ON LIVE PARASITES

The binding of antibody to the surface of larval and adult parasites was studied by quantitative immunofluorescence. Usually 3,000 L3, L1 or eggs, or one adult worm, male or female, were used for each serum tested. Preliminary studies showed that there was no difference in antibody binding when normal or X-irradiated larvae were used as targets. Normal larvae were, therfore, used throughout.

2.17.1 Immunofluorescence procedure

Parasites were washed three times in PBS/0.1% sodium azide (Sigma) and incubated in test serum, 200µl being added to larvae and eggs and 400µl added to adults, at a final dilution of 1/30 in PBS/azide. When examining the binding of polyclonal antiserum against a 30kDa surface glycoprotein of *B. pahangi* (Devaney, 1987), this was also diluted 1/30 in PBS/azide. (This antiserum was kindly provided by Dr. E. Devaney, Department of Parasitology, Liverpool School of Tropical Medicine, U.K.). Incubation was carried out for 30 min on ice with occasional agitation. Parasites were then washed three times in PBS/azide and incubated for a further 30 min on ice with FITC-conjugated anti-species IgG (whole molecule), either anti-bovine (Sigma, F-7509), anti-guinea-pig (Sigma, F-6261) or anti-rabbit (Sigma, F-0382) at a final dilution of 1/30. Following a further three washes, parasites were mounted on microscope slides within a ring of

Vaseline to raise the coverslip from the surface of the parasites, and viewed under ultraviolet light.

To determine the class of bovine antibody binding to the parasite surface an additional step was performed. Following incubation with primary antibody, parasites were incubated for 30 min on ice with mouse monoclonal antibodies to bovine IgM, IgG1 and IgG2 (Centraal Diergeneeskundig Instituut, Lelystad, Netherlands) diluted 1/50 in PBS/azide, the binding of which was detected by subsequent incubation in FITC-conjugated antibody to mouse immunoglobulins (Sigma, F-1010) diluted to 1/30. Parasites were then viewed under U.V. light as before.

2.17.2 Quantitation of fluorescence

Fluorescence was quantified by photon counting using a Leitz MPV Compact 2 microscope photometer linked to an Olivetti H100 Computer. The photometer diaphragm was set to measure a rectangular field in the range 230-555μm^2 using a x40 objective for larvae and eggs, or 920-2220 μm^2 with a x10 objective for adult worms. For larvae and eggs, the area measured was in the centre of the parasite, avoiding edge fluoresence. For adults, which all displayed internal autofluorescence centrally, the edge fluorescence was measured, always keeping the edge line in the centre of the measuring field. This avoided most of the autofluorescence and, because the signal was stronger there, improved the signal to noise ratio. The fluorescence of one of the brightest specimens was set at the arbitrary fluorescence value of 650, and the fluorescence of the other samples measured relative to this. The means and standard deviations of at least 20 estimations per serum or pooled sample were recorded and the backgrounds automatically subtracted.

2.18 INSERTION OF LIPID ANALOGUES

The insertion of the fluorescent lipid analogue 5-(*N*-octadecanoyl)amino fluorescein (C_{18}-Fl) (Molecular Probes Inc., O-322, Junction City, OR, U.S.A.) into the surface membrane of various stages of *D. viviparus* was carried out essentially as described by Kennedy *et al.* (1987). 3,000 eggs, L1 or L3 stages or one adult worm were incubated, with frequent agitation, for 15 min at 37°C in 1ml *Ascaris* culture medium containing 10µl of C_{18}-Fl diluted 1/100 in PBS.

Parasites were then washed six times in medium to remove excess fluorescent lipid and mounted on microscope slides to be viewed under U.V. light. The level of fluorescence was measured as described above.

2.19 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

The ELISA technique was used to quantify the antibody response to larval and adult antigens. 96 well microtitre plates (Immulon A2, Dynatech Laboratories Ltd., Bilingshurst, U.K.) were coated with antigen in carbonate/bicarbonate buffer, pH 9.6 (see Appendix) at 4^{o}C, overnight. The antigens used were Tris HB extracts of infective larvae or adult worms, or adult ES prepared as described in Section 2.4. Plates were coated with 60µl/well of L3 extract at 4µg/ml, 1µg/ml adult extract or 0.5µg/ml adult ES. The optimum antigen and antibody concentrations for each ELISA system were determined using standard checker-board procedures. Following each incubation, wells were washed three times with ELISA wash buffer (Appendix) and blocked for 45 min with 60µl per well of 4% skimmed milk in wash buffer to reduce non-specific binding. 50µl/well of test serum diluted 1/500 in wash buffer was then added. This and all subsequent incubations were carried out at 37^{o}C for 30 min. Plates were then incubated with 50µl/well of anti-bovine peroxidase conjugate (Sigma, A-7414) or anti-bovine alkaline phosphatase conjugate (Sigma, A-7914) diluted in PBS at 1/250 and 1/100, respectively. When rabbit antisera was used as the primary antibody, anti-rabbit alkaline phosphatase conjugate (Sigma, A-8025) diluted 1/100, was added. 50µl/well of the appropriate enzyme substrate and chromogen solutions (see Appendix) were then added. Orthophenylenediamine (OPD: Sigma, P-1526) and hydrogen peroxide were used for peroxidase reactions, and phosphatase substrate tablets (Sigma, 104-105) used for phosphatase reactions. Reactions were terminated after 30 min by the addition of 40µl 4M sulphuric acid to the peroxidase substrate solution, or 3M sodium hydroxide to the phosphatase substrate solution, and their optical densities (O.D.) measured at 492nm and 405nm, respectively, using a Titertek Multiskan MC plate reader (Flow Laboratories Ltd., Uxbridge, U.K.). All plates included known positive and negative control wells, and the results were expressed as the means of the O.D. of duplicate samples.

2.20 PURIFICATION OF IgG

Immunoglobulin G (IgG) was purified from serum of normal, vaccinated and infected calves by affinity chromatography using Protein G-Agarose (GammaBind G, BDH Ltd., 39505 2D). Anti-*D. viviparus* serum was prepared from calves vaccinated four times with 5,000 irradiated third-stage larvae, or from field cases of patent infections, where the response was boosted by infection with 1,000 and 10,000 normal larvae administered 28 days apart. Serum was collected 21 days after the final vaccination or infection. This was microfuged to remove any particulate material, and 2ml was then applied to the column. Unbound material was removed with washing buffer (Appendix) and 1.5ml fractions collected until the optical density (O.D.) at 280nm of the collected material fell to zero. Bound IgG was then eluted with 0.5M ammonium acetate buffer, pH 3.0 and eluted fractions immediately adjusted to pH 7.0 with approximately 0.5ml 2M Tris. The column was then re-equlibrated with 20 column volumes of washing buffer, and the procedure repeated.

Eluted IgG from several column runs was pooled, concentrated by centrifugal ultrafiltration using Centricon 10 devices and dialysed against PBS. The protein concentration of purified IgG was estimated as described previously and was found to be approximately 5mg/ml for all serum types. The purity of preparations was examined by SDS-PAGE, followed by protein staining and the antigen-binding ability of purified IgG was tested by ELISA using adult homogenate as the target antigen, and by immunoprecipitation with ^{125}I-adult ES. Purified IgG was stored at -20^{o}C until used.

2.21 PROTEINASE ASSAYS

2.21.1 Measuring proteolytic activity

Proteolytic activities of larval and adult preparations were measured and characterised using the procedures of Knox and Kennedy (1988). Activity was measured spectrophotometrically in a colour release assay using azocasein as substrate (Sigma, A-2765). Incubations were carried out for 16h at 37^{o}C, in 0.1M phosphate buffer, pH 5.5 or 0.1M Tris buffer, pH 7.5. Undigested substrate protein was removed by precipitation with an equal volume of 1M perchloric acid and the absorbance of released dye in the supernatants was measured at 405nm in an I.L.Multistat III microcentrifugal analyser (Instrument Laboratory, Warrington, England). Activities of samples were calculated by subtraction of reagent blanks

containing no sample. Reaction mixtures comprised 20µl sample (ES or homogenate), 10µl azocasein (5mg/ml) and 100µl buffer containing 500 U/ml penicillin and 5mg/ml streptomycin.

2.21.2 Substrate specificities

To determine the type of enzyme activity present, a variety of low molecular weight substrates were used as follows: N-carbobenzoxy-L-tryptophan-4-nitrophenol ester (CBZ-L-Trp-4-NPE: C-5502), CBZ-L-arginine-4-NPE (CBZ-L-Arg-NPE), CBZ-L-lysine-4-NPE (CBZ-L-Lys-NPE: C-3637), CBZ-L-alanine-4-NPE (CBZ-L-Ala-NPE: C-4635), N-benzoyl-L-tyrosine p-nitroanilide (BZ-L-Tyr-NA: B-6760), BZ-L-arginine p-NA (BZ-L-Arg-NA; B-3133), all purchased from Sigma. These were prepared in dimethylsulphoxide (DMSO: Sigma), at a concentration of 1mg/ml. Reaction mixtures comprised 20µl sample, 100µl buffer, 50µl DMSO and 5µl substrate solution. Initial (0 min) and final (30 min) absorbance values were measured at 405nm. All activities were corrected for non-enzymic hydrolysis by subtraction of the appropriate reagent blank.

2.21.3 pH effects on enzyme activity

The effect of pH on activity against azocasein was tested over the range pH 4.5-10, using 0.1M citrate-phosphate buffer (pH 4.5-5), 0.1M phosphate buffer (pH 5.5-7), 0.1M Tris-HCl buffer (pH 7.5-9), and 0.1M glycine-NaOH buffer (pH 9-10).

2.21.4 Inhibitor studies

Low molecular weight inhibitors were tested for their effects on the hydrolysis of azocasein at pH 5.5 and 7.5. 20µl sample was pre-incubated with 3µl inhibitor in 100µl buffer to give the following final reaction concentrations: phenylmethlysulphonyl fluoride (Pms-F: P-7626), 1mM; N-ethylmaleimide (NEM: E-3876), 1mM; ethylenediaminetetra acetic acid (EDTA: ED2SS), 2mM; pepstatin (P-4265), 2µM; L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64: E-3132), 0.2mM; 4-hydroxymethoxybenzaldehyde (4-HMB: H-9752), 1mM; dithiothreitol (DTT: D-9779), 2mM, all purchased from Sigma. After 1h pre-incubation at room temperature, enzyme activities were determined as described above with azocasein as substrate.

2.21.5 Effect of antibody on proteinase activity

To determine whether IgG antibody from immune hosts could inhibit parasite proteolytic activity, 20µl sample was pre-incubated for 30 min at room temperature with 100µl buffer containing 5µl (25µg) IgG purified from normal, infected or vaccinated hosts as described in Section 2.20. 10µl azocasein solution was then added and enzyme activity monitored as before.

2.22 POLYACRYLAMIDE SUBSTRATE GELS

Proteolytic enzymes can be separated by SDS-PAGE and detected on gels co-polymerised with a suitable enzyme substrate, following protein staining. Several different substrates can be used to detect different types of enzyme activity (Andary and Dabich, 1974). In this study, gelatin was used to detect general proteolytic activity of L3 and adult preparations.

2.22.1 Electrophoresis procedure

Separation of parasite proteinases was performed on 5-25% gradient gels co-polymerised with gelatin (bacteriological grade, Gibco, 152-0221) at a final concentration of 0.1% (w/v). On some occasions proteinases were separated on 7.5% SDS-polyacrylamide gels (70x80x0.5mm) using a Mini Protean II Dual Slab Cell (BioRad, 165-2940) according to the manufacturer's instructions. 20µl sample was mixed with 15µl sample buffer, containing no proteinase inhibitors, and usually applied to gels without boiling. To test the effect of antibody on enzyme activity, 20µl sample was pre-incubated with 5µl (25µg) purified IgG for 30 min, prior to electrophoresis.

Gradient substrate gels were run for 2h at 400V, 55mA, and mini-gels run at 200V, constant voltage, for 45 min. The gel systems were kept on ice or cooled by circulating water at 4^{o}C.

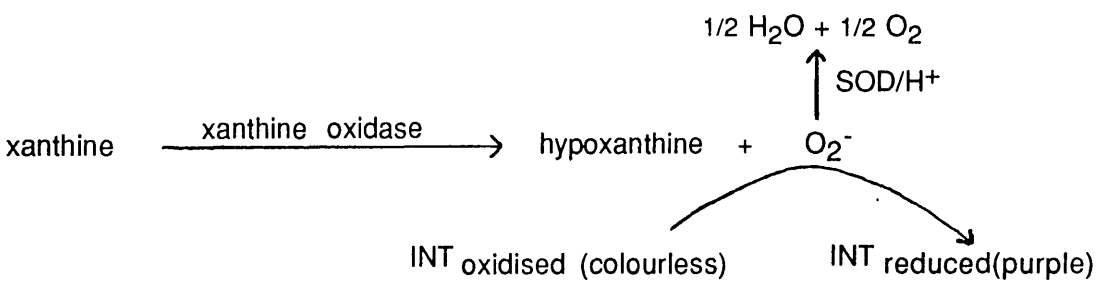
Following electrophoresis, gels were rocked in 2.5% Triton X-100 for 20 min, and incubated overnight at 37^{o}C in 0.1M phosphate buffer, pH 5.5, or 0.1M Tris/HCl, pH 7.5. To examine inhibition of enzyme activity by low molecular weight proteinase inhibitors, individual excised gel tracks were incubated in buffer containing inhibitor at the final concentrations described for proteinase assays (Section 2.21.4).

2.22.2 Detection of proteinase activity

Following overnight incubation, gels were stained in 0.1% Coomassie Brilliant Blue R-250 as previously described (Section 2.10.2) for 1h, then destained for 2h with gentle agitation. They were then placed on a light box to be photographed, or dried at 80°C onto cellophane. Positions of enzyme activity were visualised as clear zones against a stained background of unhydrolysed substrate. Molecular weights of proteinases were estimated by reference to prestained marker proteins (Gibco BRL, 6041LA) (Mol Wt Range 15-214 kDa), which were more clearly visible on substrate gels than unstained marker proteins.

2.23 SUPEROXIDE DISMUTASE (SOD) ASSAYS

Superoxide dismutase (SOD) activity can be detected indirectly by the inhibition of reactions directed by the superoxide anion (O_2^-). In the assay system utilised here, SOD activity was measured in terms of its ability to inhibit the reduction of iodonitrotetrazolium violet (INT, Sigma, I-8377) by superoxide generated by the xanthine oxidase system (Jones and Suttle, 1981) as follows:



The purple colour of reduced INT was measured spectrophotometrically at 500nm and the level of SOD activity was estimated by reference to standard SOD solutions of known activity.

2.23.1 Assay procedure

20µl parasite sample, SOD standard (EC 1.15.1.1, Boehringer Mannheim 567 680) or H_2O (control) was mixed with 175µl xanthine substrate solution (see Appendix). 5µl xanthine oxidase (EC 1.1.3.22, Boehringer Mannheim, 110 434), diluted to 0.3 U/ml in H_2O, was then added and the O.D. at 500nm was immediately read (time 0). The reaction was allowed to proceed for 30min at room temperature,

after which time the absorbance at 500nm was again read (time 30). The change in absorbance at 500nm (ΔO.D.500) during the assay was then calculated (O.D.500 time 30 -O.D.500 time 0).

2.23.2 Calculation of SOD activity

O.D.500nm plotted against SOD concentration is non-linear. However, % inhibition of INT reduction plotted against log $_{10}$ [SOD] showed a linear relationship. The % inhibition (%I) was calculated as follows:

$$\%I = \frac{\Delta O.D.500(control) - \Delta O.D.500\ (sample\ or\ standard)}{\Delta O.D.\ 500(control)} \times 100\%$$

The $\log_{10}$ SOD concentration (units/ml) was calculated and plotted against %I. Log_{10} [SOD] of unknown samples was then estimated by reference to the standard graph, using the measured values of %I. This estimated SOD activity in U/ml, which was converted to U/mg using the protein concentration of the samples. One unit of SOD is that which inhibits reduction of cytochrome c by 50% (McCord and Fridovich, 1969).

2.23.3 Effect of antibody on SOD activity

The effect of IgG antibody from immune hosts on parasite SOD activity was determined by incubating 20µl parasite preparation with 5µl purified IgG at room temperature for 10 min prior to assaying for SOD activity.

2.24 DETECTION OF SUPEROXIDE DISMUTASE ACTIVITY BY PAGE

In this system, SOD activity was detected by the inhibition in reduction of nitroblue tetrazolium (NBT) by O_2^- generated by photoreduced riboflavin upon reoxidation in the presence of O_2 and tetramethylethlenediamine (TEMED)(Beauchamp and Fridovich, 1971). To identify different SOD isoenzymes, electrophoretic separation of parasite proteins was performed prior to the detection of SOD activity.

2.24.1 Electrophoresis procedure

Proteins were separated on 7.5% polyacrylamide gels using the Mini Protean II

Dual slab cell. SDS was omitted from the separating and stacking gels, sample buffer and electrophoresis buffer, to limit any enzyme inactivation. 20µl sample was mixed with 20µl sample buffer, with no added proteinase inhibitors, and applied to gels without boiling. Electrophoresis was carried out as before at 4°C.

2.24.2 Detection of SOD activity

Following electrophoresis, gels were incubated at room temperature for 20 min in 2.45mM NBT (Sigma, N-6876) (0.1g in 50ml Tris/HCl, pH 8.5), with gentle agitation. Gels were then incubated in the dark for 15 min at room temperature in 100ml of 0.1M Tris/HCl, pH 8.5 containing 0.028mM riboflavin (Sigma, R-4500)/0.028M TEMED (Sigma, T-8133) (see Appendix). This solution was then discarded and gels illuminated for approximately 15 min, after which time SOD activity was detected as clear zones while the rest of the gel became blue due to reduced NBT.

CHAPTER 3

BIOCHEMICAL CHARACTERISATION OF *D.VIVIPARUS* EXCRETORY-SECRETORY PRODUCTS

3.1 INTRODUCTION

Together with surface antigens, materials released by living parasites represent the host-parasite interface and as such are receiving increasing attention because of their important role in the immunobiology of infection and as a source of potential protective antigens (Campbell,1955; Rothwell and Love, 1974; Silberstein and Despommier, 1984). Several functions for nematode excretory-secretory (ES) products have been postulated, for example they may be involved in evasion of host immune mechanisms by "mopping up" antibody specificities directed against surface antigens or by inducing a state of immunosuppression in the host, as mentioned in Chapter 1 (reviewed by Lightowlers and Rickard, 1988), while the release of enzymes may be essential to parasite invasion, nutrition and survival (see Chapter 8).

The value of ES antigens in diagnosis has also been demonstrated (de Savigny, 1975; Maizels, Denham and Sutanto,1985; Weil and Liftis, 1987; De Jonge and Deelder, 1988), being particularly useful in the early detection of infection where parasites may not be detected in the circulation, or in infections which stimulate low levels of antibody response (Glickman and Schantz, 1981). ES antigens are favoured over somatic extracts of whole parasites due to the relative simplicity of the composition of ES products, their enhanced species specificity (Voller and de Savigny, 1981), and the fact that they are products of living worms and, therefore, provide a better indication of current infection. Although it cannot be assumed that antigens released *in vitro* represent those expressed by parasites *in vivo*, it has been found that a monoclonal antibody which binds *T. canis* antigens released during culture, also detects circulating toxocaral antigen in infection serum (Robertson *et al.*, 1988).

As the precise nature of most released parasite antigens is unknown, they have been referred to as metabolic antigens, exoantigens, excretory-secretory antigens (Anders, Howard and Mitchell, 1982) or, more simply, as ' *in vitro*-released' antigens (de Savigny, 1981). Some ES products are clearly associated with secretory glands at parasite orifices or excretory pores, as demonstrated with *T. canis* infective larvae (Hogarth-Scott, 1966), or stichocyte cells surrounding the oesophagus in *T. spiralis* (Despommier and Muller, 1976) and *T. muris* (Jenkins and Wakelin,

1977), while ES products associated with moulting have been shown to be released from a region at the base of the oesophagus (Soulsby, Sommervillle and Stewart, 1959). It has also been demonstrated with several nematodes that antigens shed from the parasite surface may contribute to ES material (Philipp, Parkhouse and Ogilvie, 1980; Smith *et al.*, 1981; Maizels, Meghji and Ogilvie, 1983a; Maizels *et al.*,1984b). Release of surface antigens *in vivo* has not been demonstrated and it has been suggested that maintenance of parasites in suboptimal conditions *in vitro* may result in abnormal physiological alterations in the parasite, being reflected in the release of surface components (Lightowlers and Rickard, 1988).

As yet, there is no information on ES products of any stage of *D. viviparus*. Using a culture system devised for *Ascaris* nematodes (Kennedy and Qureshi, 1986), adult parasites of *D. viviparus* have been successfully maintained *in vitro* and ES products collected over a three day period. This adult ES material was found to protect guinea-pigs by up to 78% against challenge infection (G.J.Canto, C. Britton, G.M. Urquhart and M.W.Kennedy, unpublished; J.B. McKeand, C. Britton, G.M. Urquhart and M.W. Kennedy, unpublished), while soluble extracts of whole adult worms were less effective. This, therefore, implicates ES products as a potentially important source of protective antigens against *D. viviparus*.

The protection achieved with adult ES products in the guinea-pig host, in which the parasite does not mature to the adult stage (Wade *et al.*, 1960), suggests a significant degree of cross-reactivity between adult and larval antigens. We have been unable to confirm whether such antigens are also released by the infective third stage because of difficulty in obtaining sufficient quantities of L3 ES for analysis. Immunochemical characterisation of adult ES products may, therefore, be useful in identifying antigens common or specific to different developmental stages and in identifying potential protective antigens. As the pathology of lungworm disease is associated with the presence of adult worms in the lungs (Jarrett *et al.*, 1969), a study of products released by the adult stage may also be important in understanding how this damage is mediated.

This study characterises ES products of *D. viviparus* adult parasites detailing their SDS-PAGE profile, glycosylation pattern and synthesis by parasites *in vitro*. A comparison of the protein composition of adult ES and homogenate was carried out,

firstly, to examine the basis for the greater immunising efficacy of adult ES, and secondly, to identify antigens common to both soluble extracts and ES products which may be obtained in greater quantity from parasite extracts for use in immunisation studies. That *in vitro* released material of adult *D. viviparus* parasites may represent components exposed to the hosts during infection, is suggested by the recognition of almost all ES molecules by antibody from calves patently infected with lungworm.

3.2 RESULTS

3.2.1 Comparison of adult ES and somatic proteins

ES products were collected from cultures of adult parasites after 24 and 48 hours, with the medium being replenished after the first 24 hours. Components of adult ES were compared with those present in Tris-soluble extracts of fresh adult worms by separation on SDS-PAGE followed by Coomassie staining. Both the ES and extract were prepared from mixed sexes of adult worms and would, most likely, have contained egg and L1 components.

As shown in Figure 3.1, a complex mixture of proteins was present in both preparations, ranging in molecular mass from 14 to 78kDa in ES and from 14 to approximately 200kDa in the extract. Four proteins of low molecular mass (14-18kDa) were more abundant in ES. There were a number of proteins of identical molecular mass in both preparations, although two dimensional analysis will be necessary to confirm that these represent the same proteins. Some proteins, however, were present only in the extract, most notably those of molecular mass 19kDa, 65-68kDa and those of M_r greater than 78kDa. There appeared to be no significant qualitative difference in the composition of ES collected from adult cultures after 24h and that collected after 48h, although there was a decrease in the level of some proteins, most notably those of 30-33kDa and 75kDa.

3.2.2 Profile of radio-iodinated ES

SDS-PAGE analysis of adult ES radio-iodinated by both the Bolton-Hunter and Iodogen

techniques showed a similar profile to one another and to Coomassie stained ES. ES labelled by the Bolton-Hunter method showed less evidence of degradation as indicated by the sharper bands on SDS-PAGE than that labelled by the Iodogen method (Figure 3.2). The former was, therefore, the method of choice when characterising ES products. However, a component of molecular mass 290kDa, which could only clearly be observed on over-exposed gels (tracks d and e), was labelled only by the Iodogen method, indicating the absence of lysine residues in this protein, or, alternatively, reflecting the different labelling efficiencies of these two labelling techniques. It has been demonstrated with *Toxocara canis* L2 ES that the Iodogen method labels large components more efficiently than the Bolton-Hunter (Meghji and Maizels, 1986).

The broad band of radioactivity at 14-18kDa was resolved into four entities which could be clearly observed following exposure of the gel in the absence of an intensifying screen (Figure 3.2, track a).

All ES components radio-iodinated by either the Iodogen or Bolton-Hunter techniques were susceptible to cleavage by pronase and proteinase K as indicated by the degradation pattern on SDS-PAGE (Figure 3.3) and are, therefore, all proteinaceous.

3.2.3 Presence of a female-specific component

ES material was collected from separate cultures of male and female adult worms and labelled by the Iodogen method. SDS-PAGE analysis revealed that the 290kDa component, which was labelled only by the Iodogen technique, is female-specific (Figure 3.4). Another ES component of 67KDa which, although present in male ES, was more abundant in female ES. In contrast, a 30kDa component appeared to be present in greater quantity in male than female ES products. Subsequent studies were performed using ES from cultures of mixed sexes, to contain a full complement of ES components.

3.2.4 Effect of reduction on ES

The presence of disulphide bonds between or within ES components was examined by SDS-PAGE analysis of radioiodinated ES under non-reducing and reducing conditions. As can be seen in Figure 3.5 (Panel A), there was a decrease in mobility of two major components under reducing conditions, suggesting that these contain intrachain

disulphide bonds. The M_r of these proteins was estimated to be 39 and 42kDa under non-reducing conditions. Although showing a decrease in mobility under reducing conditions, their apparent M_r also decreased due to alterations in the mobility of the marker proteins ovalbumin (43kDa) and albumin (67kDa) in the presence of 2ME. The M_r of the two *D. viviparus* ES proteins reduced by 2ME will be referred to as 39 and 42kDa in subsequent studies.

There were no apparent alterations in the migration of other ES components under these conditions. Examination of immunoprecipitates formed between serum from immune bovine or guinea-pig hosts and adult ES in a Staph-A based assay indicated, however, two additional components which showed decreased mobility on reduction. These could not be clearly seen in analysis of whole ES, possibly due to other ES components of similar molecular mass masking their alteration in position. The importance of this effect is demonstrated by recognition of an antigen of 33kDa under non-reducing conditions which alters mobility to 40kDa on reduction, confusing it with the recognition of another, more dominant component of similar M_r (Figure 3.5, Panel B). Another antigen of molecular mass 26kDa showed an apparent mass of 30kDa when run in the presence of 2ME. The diagonal lines connecting the positions of antigens under each condition represent molecules in a transition state due to the lateral diffusion of the reducing agent during electrophoresis. It was observed that some ES proteins resolved better under reducing conditions and subsequent studies were, therefore, performed in the presence of 2ME.

3.2.5 Presence of carbohydrate components

Glycosylation of ES proteins was examined by lectin affinity chromatography using a variety of lectins to encompass a wide range of sugar specificities. Those used are listed in Table 2.1. A component of 42kDa bound to lentil lectin and Con A, indicating the presence of mannoside residues (Figure 3.6). No other ES components bound to these lectins, nor was binding of radiolabelled ES molecules observed to any other of the listed lectins.

The glycosylation of this 42kDa protein was confirmed by treatment of ES with the exoglycosidase Peptide-F-glycanase (PNGase F) which cleaves asparagine-linked

carbohydrate groups at the peptide-carbohydrate junction (Tarentino *et al.*, 1984). The 42kDa protein was digested, with no apparent intermediates, to 39kDa, indicating the presence of only one N-linked sugar per molecule (Figure 3.7 Panel A).

No other ES proteins were susceptible to degradation by PNGase F and treatment of ES radio-iodinated by the Iodogen method showed no N-linked glycoslylation of the 290kDa female-specific component (Figure 3.7 Panel B).

3.2.6 Synthesis of ES components *in vitro*

Active synthesis of adult ES products in culture was demonstrated by incorporation of ^{35}S-methionine and ^{3}H-glucosamine into parasite components. SDS-PAGE analysis revealed a complex pattern of ^{35}S-labelled ES and somatic material (Figure 3.8). Labelled ES components ranged from approximately 14 to 210kDa, with a cluster of heavily labelled proteins between 27 and 33kDa. The profile of labelled ES was consistent during the first and second days, with the exception of a protein of approximately 130kDa which was detected only during the first 24h. The profiles are identical to that of ES collected following chasing with cold, complete medium for 24h.

No newly synthesised proteins were extracted following incubation of biosynthetically labelled adult worms for 3h, 4^{o}C in CTAB, a detergent which has been shown to selectively remove surface components from a number of nematodes (Pritchard *et al.*, 1985; Pritchard, McKean and Rogan, 1988b). Not surprisingly, DOC extraction of biosynthetically labelled parasites revealed a large number of components. Several ^{35}S-labelled components were solubilised by incubation of DOC-insoluble material in 2ME, most notably components of 44 and 58kDa. Treatment of the remaining pellet in 2ME/SDS/ 8M urea resulted in further extraction of proteins previously solubilised in DOC or 2ME alone.

In contrast to the complex pattern observed with ^{35}S-methionine labelling, there was limited incorporation of ^{3}H-glucosamine into both ES and somatic components, confirming the paucity of glycosylation of *D. viviparus* molecules (Figure 3.9). Radiolabel was incorporated into 3 bands of 34, 42 and 50kDa in ES, although whether the 42kDa component represents the previously described glycoprotein awaits further analysis. A labelled component of approximately 285kDa was also observed in ES and in adult extracts. This appears to be of similar molecular

mass to the female-specific component. Detergent extraction of biosynthetically labelled worms also revealed material of approximately 90kDa and two components, possibly glycolipids, running at the dye front. No additional labelled components were extracted in the presence of SDS and urea (track d).

3.2.7 Antigenicity of *D. viviparus* adult ES and identification of host albumin.

To examine the antigenicity of adult ES products in the bovine host, radioiodinated ES was immunoprecipitated in a Staph A-based assay with pooled serum from calves infected on two occasions with *D. viviparus* normal larvae. Examination of immunoprecipitates by SDS-PAGE revealed that all labelled ES molecules are antigenic to the bovine host, with the notable exception of two components of 27 and 67kDa (Figure 3.10, track b). An identical recognition profile was observed using Staph A which had been pre-coated with anti-bovine IgG (whole molecule), to precipitate all IgG classes (not shown).

The failure of bovine immune sera to precipitate the 67 and 27kDa components raised the possibility that these were of host origin. This was confirmed for the 67kDa molecule which was precipitated by antiserum to bovine whole serum (Figure 3.10, track d) and antiserum to bovine albumin. However, the component of 27kDa was precipitated by neither of these antisera. In subsequent studies this component was never precipitated by serum from infected or vaccinated calves or adult cattle, yet rabbit antiserum to *D. viviparus* adult ES did precipitate this molecule (Figure 3.11, track b) indicating that it is not intrinsically non-antigenic.

3.2.8 Species-specific recognition of *D. viviparus* ES products

The species-specificity of *D. viviparus* ES antigens was examined by incubating ^{125}I-ES with serum from calves experimentally infected on two occasions with *Cooperia oncophora* or *Ostertagia ostertagi*, common gastrointestinal nematodes of cattle. Cross-reactivity of an antigen of approximately 290kDa was indicated by its recognition by all three infection sera, but not by normal bovine serum (Figure 3.12). The remaining ES antigens were recognised only by antiserum to *D. viviparus* infection. A slight degree of non-specific binding of proteins of 21 and 46kDa was

observed with all serum types, including normal bovine serum. This was not a consistent finding, but occurred only on prolonged storage of radiolabelled antigens.

Figure 3.1 SDS-PAGE profile of adult ES and somatic proteins. ES products were collected from adult parasites after 24 (a) and 48 (b) hours in culture, concentrated approximately 20 fold, and compared with a Tris-soluble extract of fresh adult worms (c). Electrophoresis was carried out on 5-25% SDS-gradient gels under reducing conditions. Following electrophoresis proteins were stained with Coomassie blue. The relative molecular mass (M_r) of marker proteins (M) are indicated in kiloDaltons (kDa).

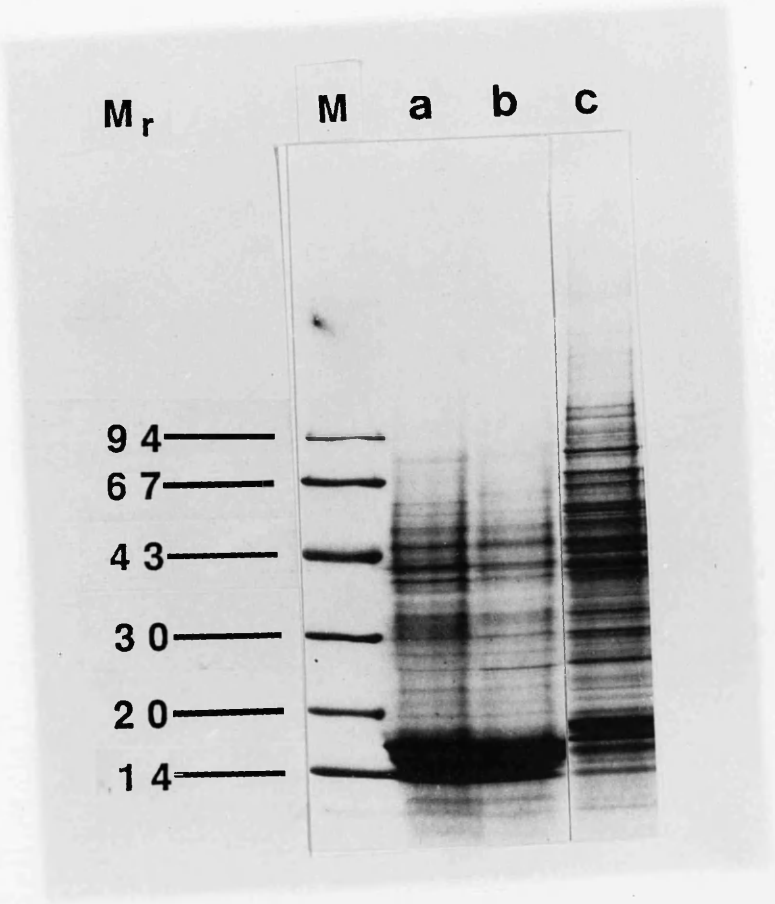

Mr
M
a
b
c
94
67
43
30
20
14

Figure 3.2 SDS-PAGE profile of radioiodinated adult ES. Adult ES products were radioiodinated by the Bolton-Hunter (tracks a, b, d) and Iodogen (tracks c, e) methods and analysed by SDS-PAGE under reducing conditions. Tracks d and e were over-exposed to show the high M_r proteins present and track a was exposed in the absence of an intensifying screen to allow better resolution of proteins of low M_r. The M_r of marker proteins (M) are indicated, expressed in kDa.

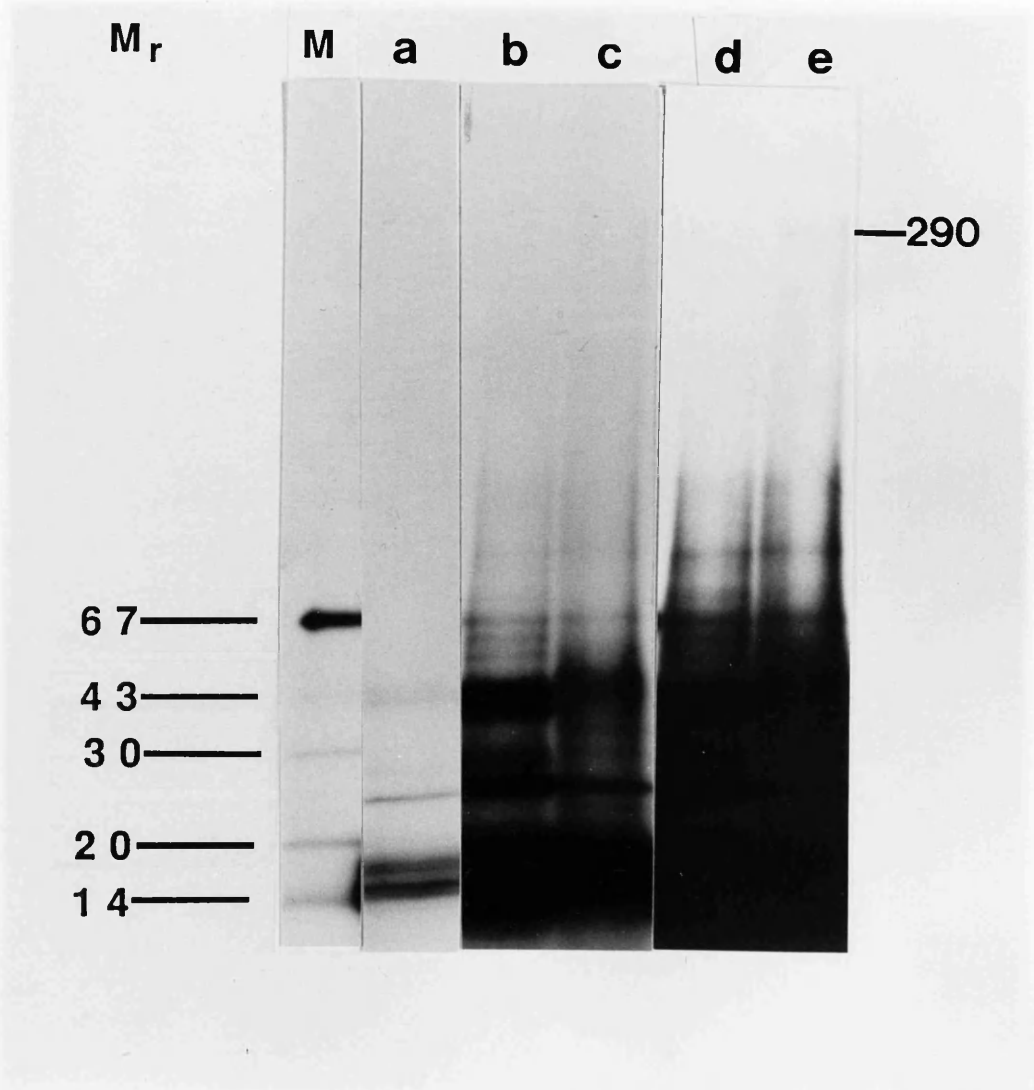

Mr
M
a
b
c
d
e
290
67
43
30
20
14

Figure 3.3 Sensitivity of ES products to pronase treatment. ^{125}I-ES was incubated with 0 (a), 2.5μg/ml (b), 25μg/ml (c), 250μg/ml (d), 2.5mg/ml (e), 12.5mg/ml (f) and 25mg/ml (g) of pronase as described in Chapter 2. Treated ES was analysed by SDS-PAGE under reducing conditions. The M_r of marker proteins (M) are indicated in kDa.

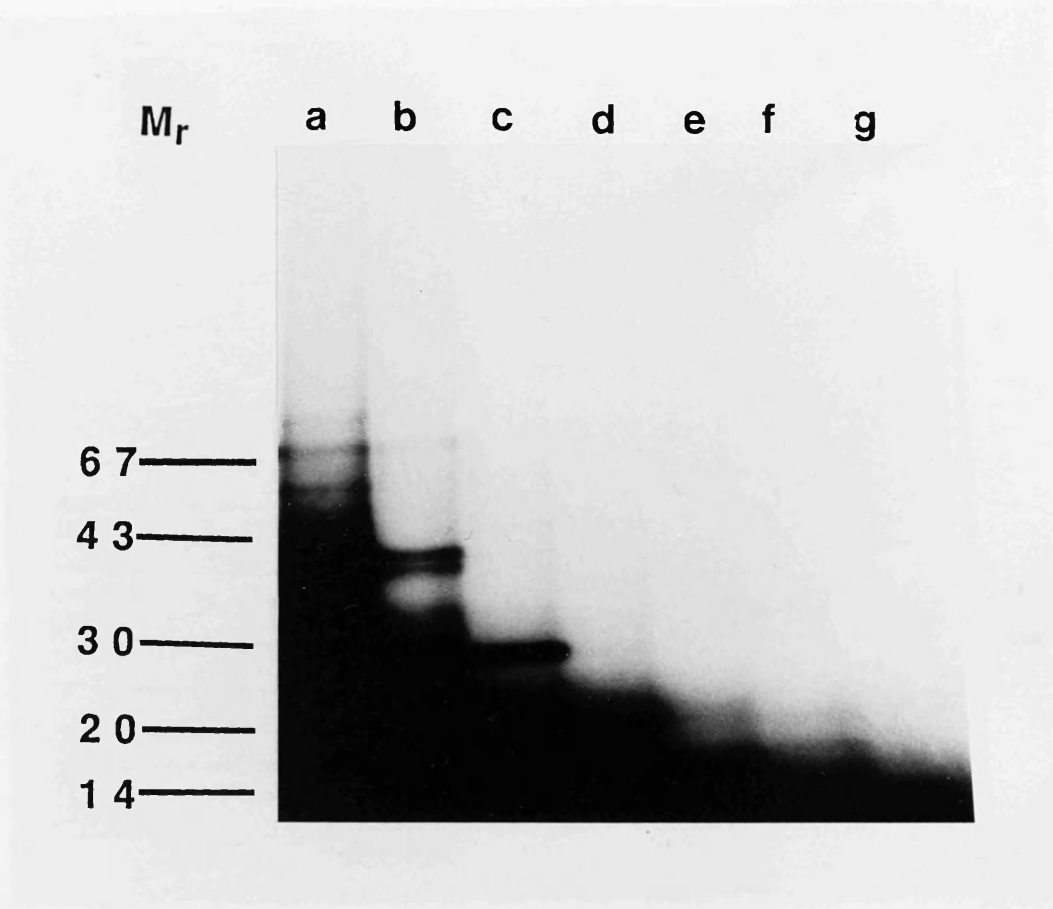

Mr
a
b
c
d
e
f
g
67
43
30
20
14

Figure 3.4 Comparison of male and female ES products. Male (a) and female (b) ES components were radioiodinated by the Iodogen method and analysed by SDS-PAGE under reducing and non-reducing conditions as indicated. The M_r of marker proteins are indicated in kDa.

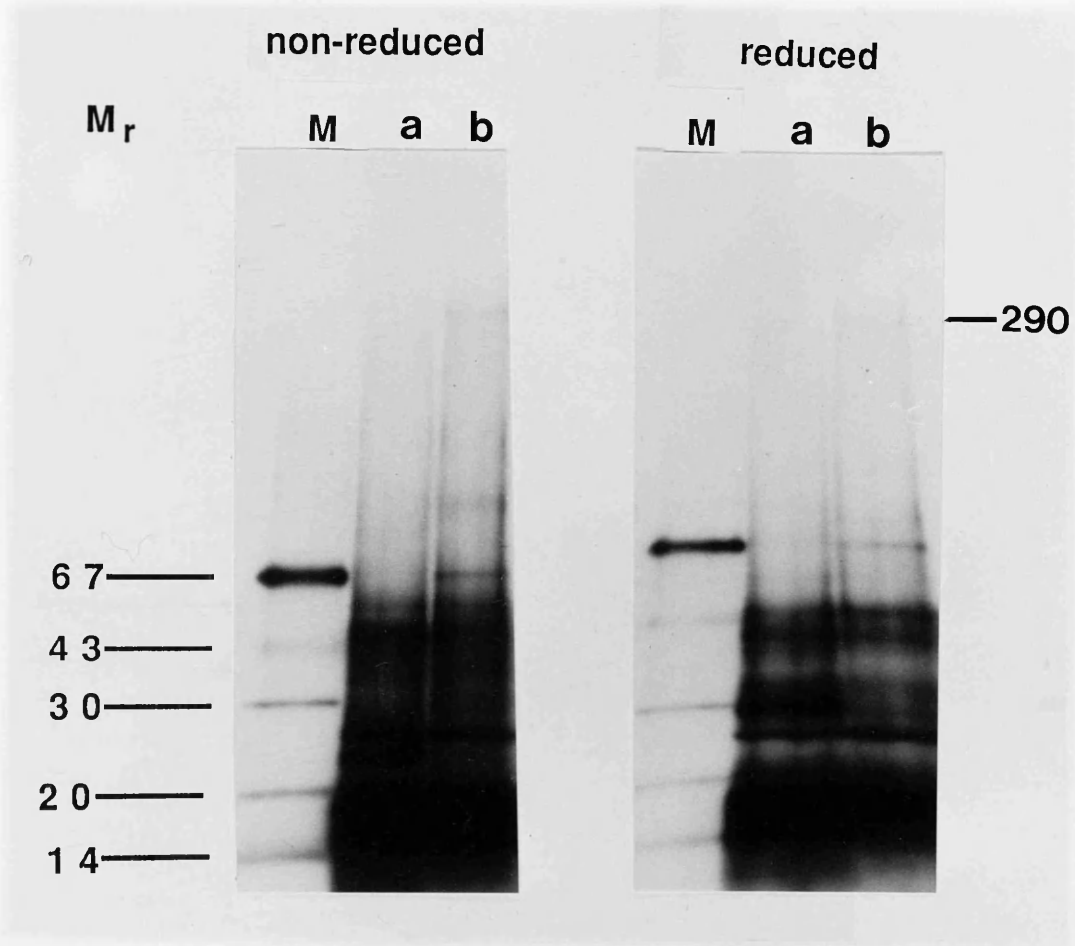
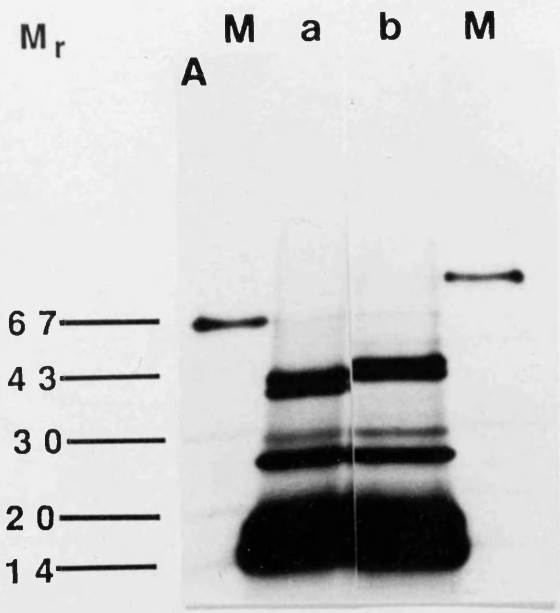

non-reduced
reduced
Mr
M
a
b
M
a
b
290
67
43
30
20
14

Figure 3.5A Effect of reducing agent on adult ES proteins. Adult ES products, radiolabelled by the Bolton-Hunter method, were analysed under non-reducing (a) and reducing (b) conditions.

Figure 3.5B Radioiodinated ES (R) was immunoprecipitated in a Staph A-based assay with normal guinea-pig serum (N) and pooled serum from guinea-pigs infected on two occasions with normal *D. viviparus* L3 (I). Immunoprecipitated antigens were analysed by SDS-PAGE under non-reducing and reducing conditions as shown. The M_r of marker proteins (M) are shown in kDa.


Mr
M a b M
A
67
43
30
20
14

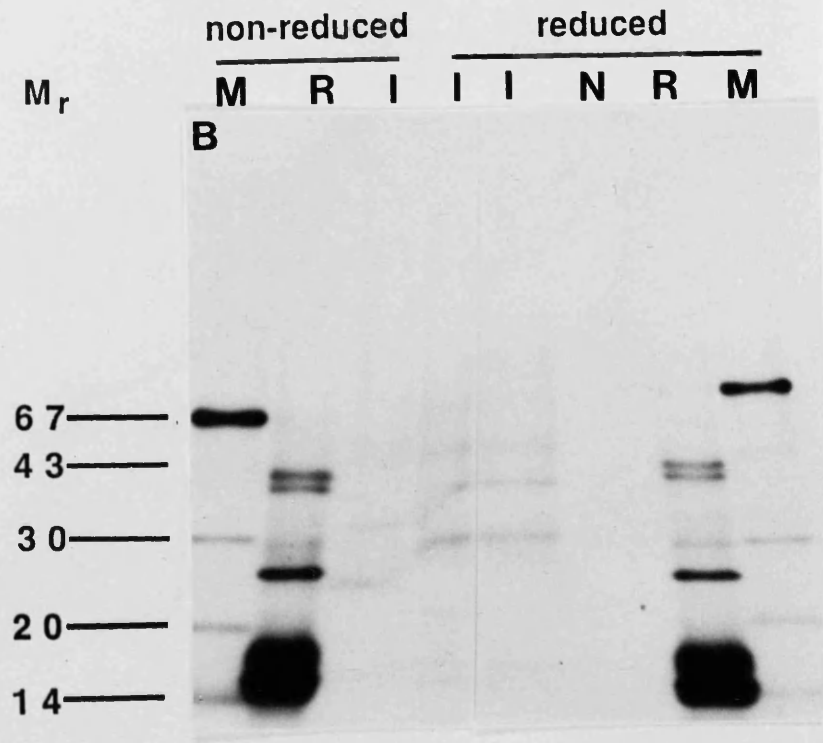

non-reduced
reduced
Mr
M R I I I N R M
B
67
43
30
20
14

Figure 3.6 Glycosylation of ES products. Bolton-Hunter iodinated adult ES (a) was applied to a lentil lectin affinity column. Unbound (b) and bound (c) components, the latter eluted with methyl mannoside, were analysed by SDS-PAGE under reducing conditions.

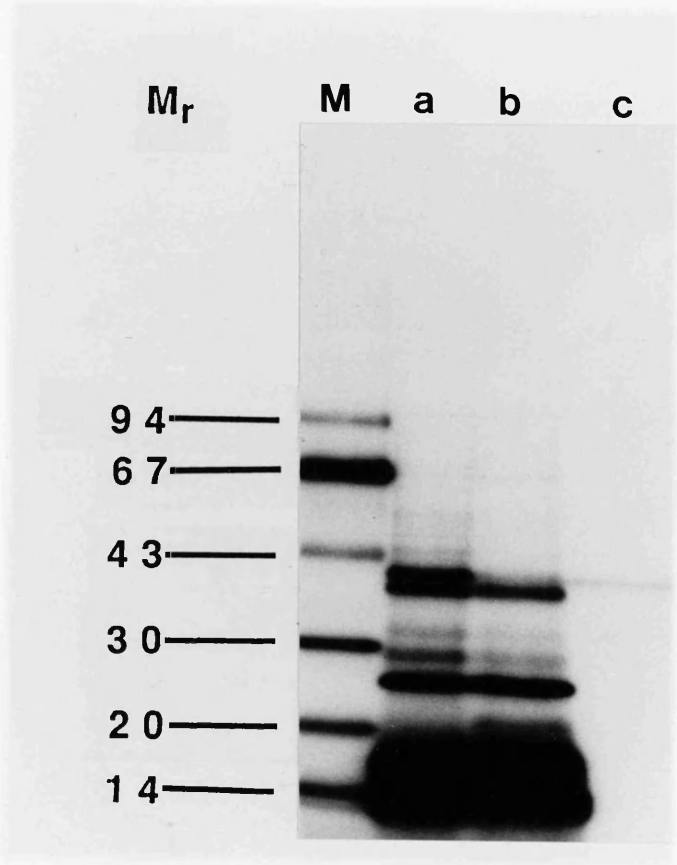
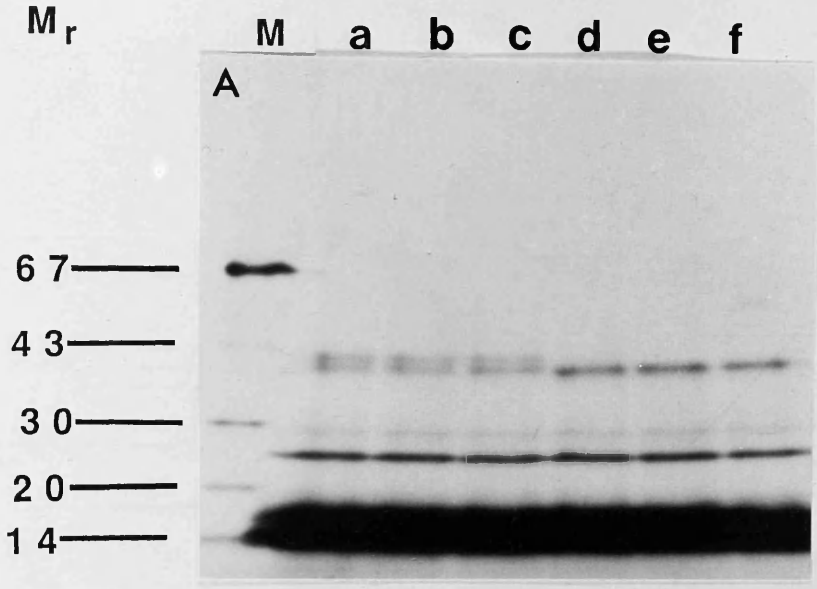

Mr
M
a
b
c
94
67
43
30
20
14

Figure 3.7 Exoglycosidase treatment of adult ES. ES radioiodinated by the Bolton-Hunter (Panel A) and Iodogen (Panel B)(track R) techniques were incubated with 0 (a), 1mU/ml (b), 10mU/ml (c), 100mU/ml (d), 1U/ml(e) and 10U/ml (f) of peptide-N-glycanase (PNGase F) as described in Chapter 2. Following incubation, ES products were analysed by SDS-PAGE under reducing conditions. The M_r of marker proteins (M) are indicated in kDa.


Mr
M a b c d e f
A
67
43
30
20
14

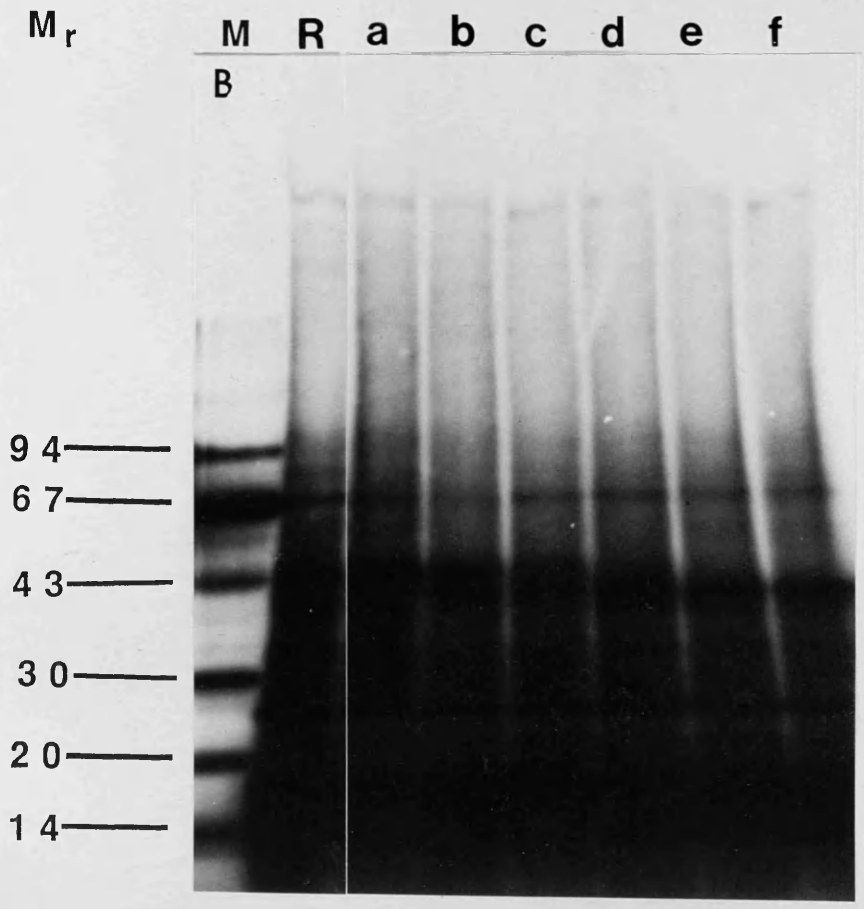

Mr
M R a b c d e f
B
94
67
43
30
20
14

Figure 3.8 ^{35}S-methionine *in vitro* labelling of adult *D. viviparus*. Mixed sexes of adult parasites were cultured in MEM-methionine-free medium supplemented with ^{35}S-methionine. ES was collected after 24 (a) and 48 (b) hours, with the medium being replaced and another 400µCi ^{35}S-methionine being added after the first 24 hours. Parasites were then chased with cold complete RPMI medium and ES products collected after a further 24 hours (c). Labelled parasites were then incubated in 0.25% CTAB at 4^{o}C (d) prior to homogenisation in Tris homogenisation buffer/1% DOC (e). Remaining ^{35}S-labelled proteins were extracted in 1% DOC/5% 2ME (f) and 5% 2ME/1%SDS/8M urea (g). SDS-PAGE was performed under reducing conditions and gels were incubated in scintillant prior to autoradiography. The M_r of marker proteins (M) are shown in kDa.

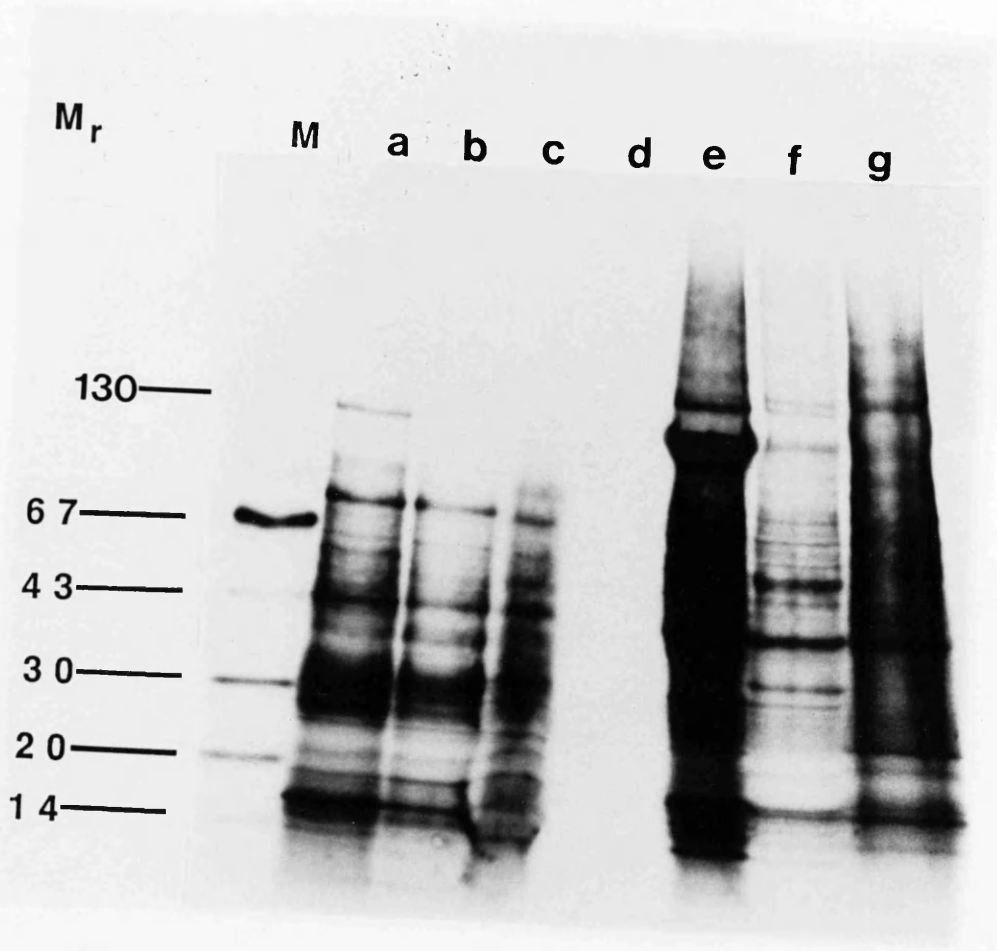

Mr
M
a
b
c
d
e
f
g
130
67
43
30
20
14

Figure 3.9 ^{3}H-glucosamine biosynthetic labelling of adult parasites. Adult worms were incubated in complete medium with added ^{3}H-glucosamine. After 24 hours the culture fluid was collected (a) and parasites homogenised in Tris homogenisation buffer (b), followed by extraction in Tris homogenisation buffer/1% DOC (c) and 5% 2ME/1% SDS/8M urea (d). Labelled components were analysed by SDS-PAGE under reducing conditions.

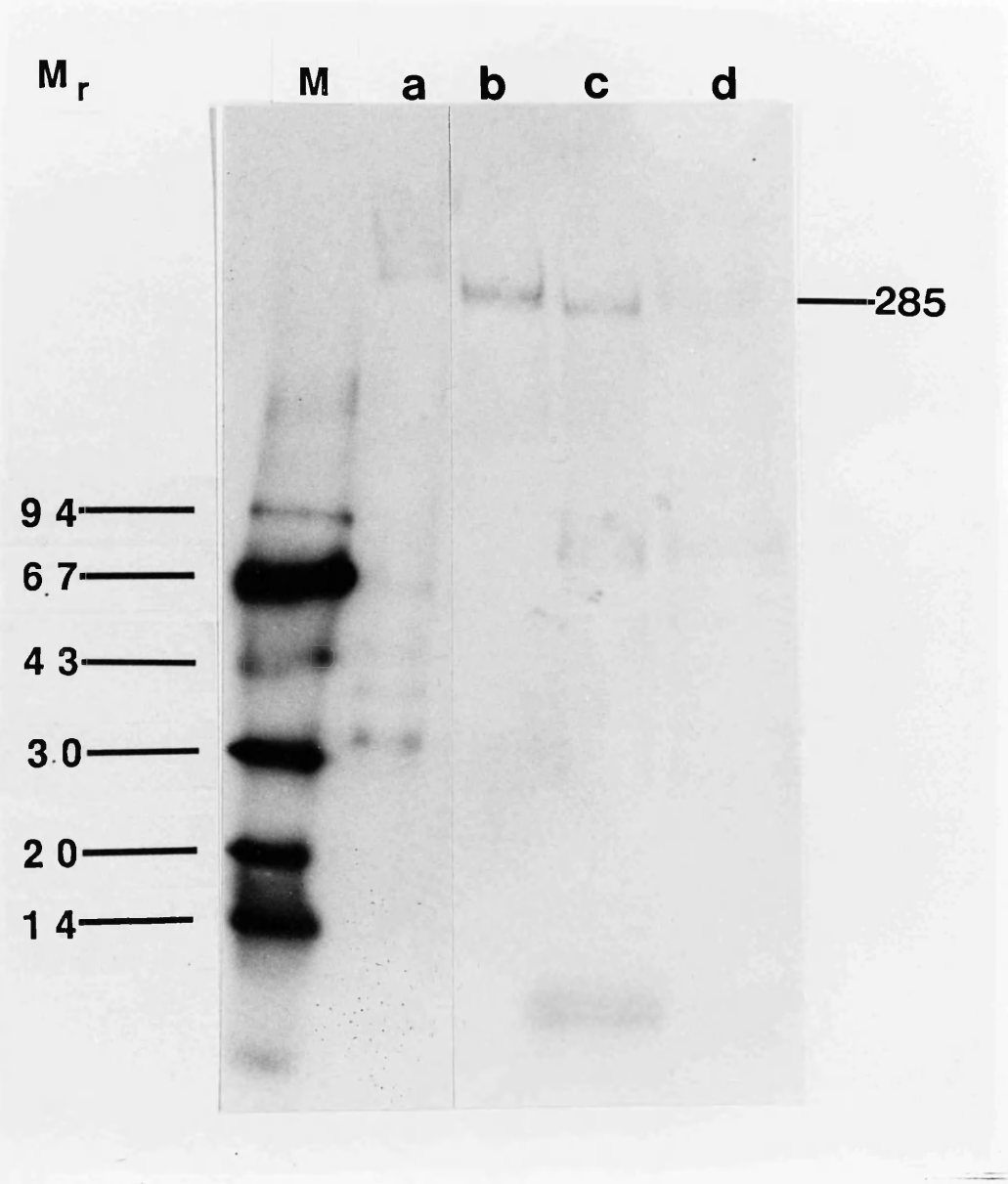

Mr
M
a
b
c
d
285
94
67
43
30
20
14

Figure 3.10 Antigenicity of adult ES and identification of bovine serum albumin. ^{125}I-ES (R) was immunoprecipitated in a Staph A-based assay with serum from calves patently infected with *D. viviparus* (b) and with rabbit antiserum to bovine whole serum (c). Tracks a and d show immunoprecipitation with control normal bovine and normal rabbit serum, respectively.

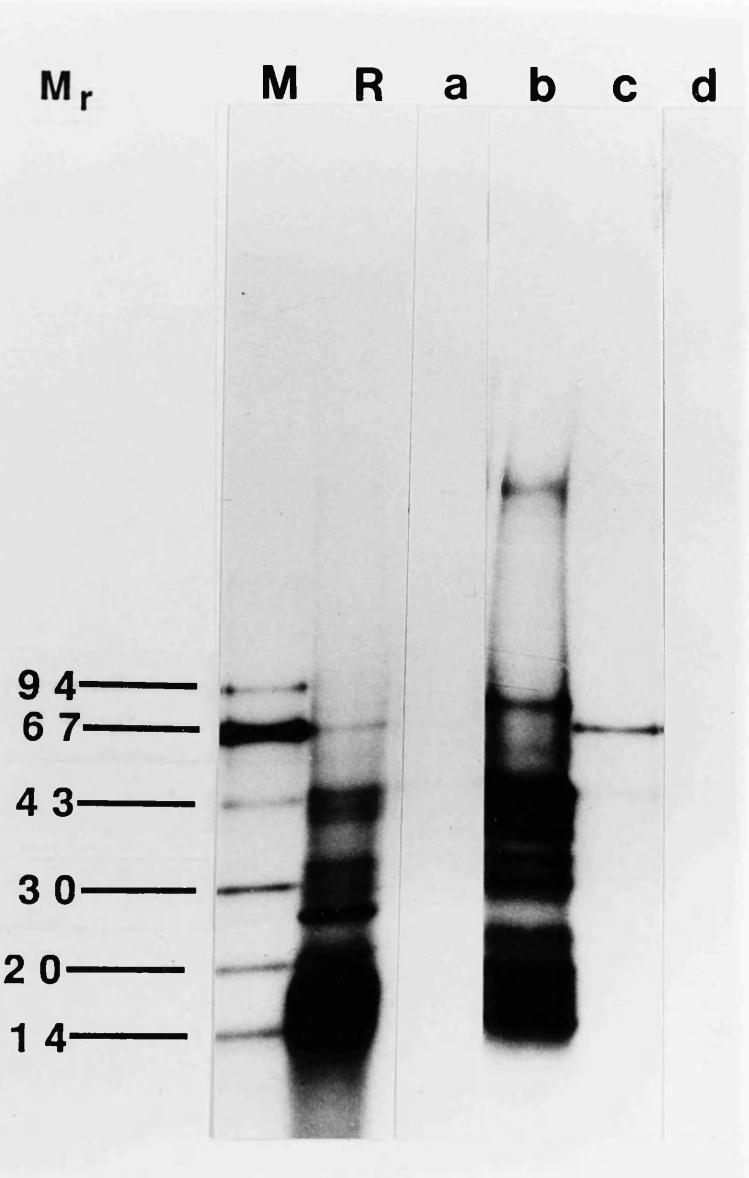

Mr
M
R
a
b
c
d
94
67
43
30
20
14

Figure 3.11 Recognition of a 27kDa ES component by anti-ES antiserum. Radiolodinated ES (R) was incubated with rabbit antiserum to *D. viviparus* adult ES products and antigens precipitated by Staph A were analysed by SDS-PAGE (b). Immunoprecipitation with control normal rabbit (a) and normal bovine (c) serum are shown. The recognition profile of serum from lungworm infected bovine hosts is shown for comparison (d).

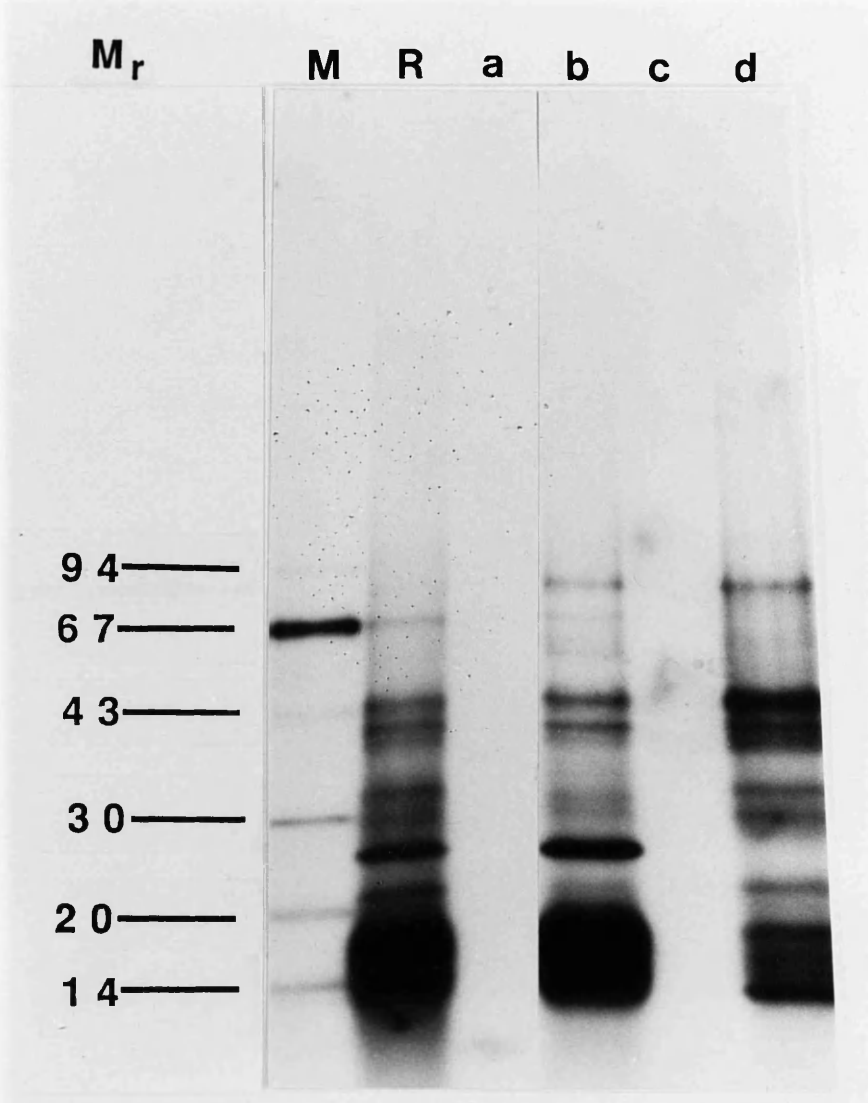
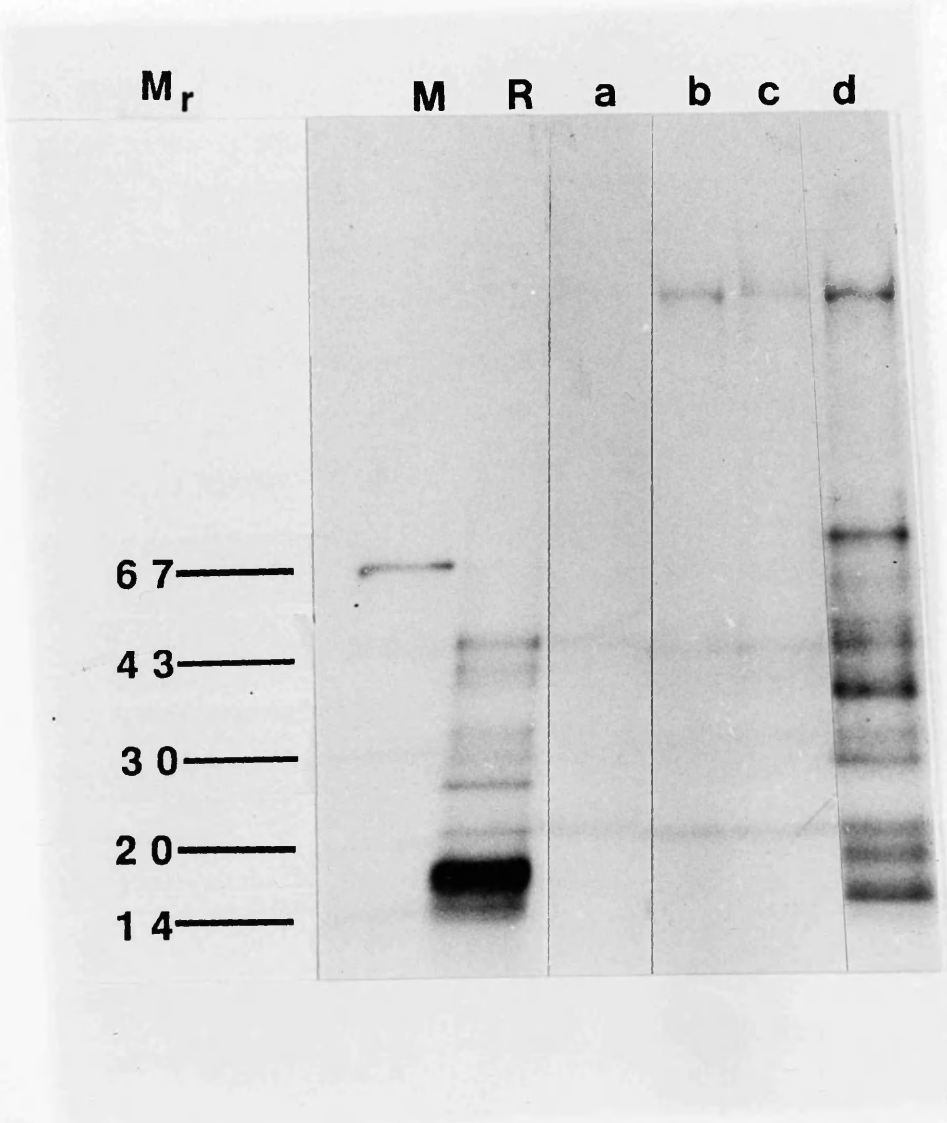

Mr
M
R
a
b
c
d
94
67
43
30
20
14

Figure 3.12 Species-specific recognition of *D. viviparus* ES antigens. Radioiodinated ES (R) was immunoprecipitated with normal bovine serum (a) and serum from calves infected on two occasions with *C. oncophora* (b), *O. ostertagi* (c) or *D. viviparus* (d). Immunoprecipitated antigens were analysed by SDS-PAGE under reducing conditions. The M_r of marker proteins (M) are indicated in kDa.


Mr
M
R
a
b
c
d
67
43
30
20
14

3.3 DISCUSSION

The identification of protective antigens from parasite materials will most likely require a knowledge of the components present and an understanding of the immunological reaction to each. To identify potential protective antigens of *D. viviparus*, an immunochemical characterisation was carried out of ES products of the adult stage, previously shown to induce significant protection against infection in the guinea-pig model (G.J.Canto, C.Britton, G.M.Urquhart and M.W. Kennedy, unpublished; J.B. McKeand, C. Britton, G.M. Urquhart and M.W. Kennedy, unpublished). This demonstrated that *D. viviparus* adult ES is a heterogenous preparation, containing many components, most of which are antigenic to the bovine host.

Comparison of adult somatic and ES material showed that proteins of identical molecular mass appear to be present in each, although two dimensional analysis will be necessary to show whether these represent the same molecules. At this time, it is, therefore, not clear why adult ES products induce significantly greater protection in guinea-pig hosts than adult somatic antigens. The greater abundance of components of 14-18kDa in ES might be taken to suggest that these may be involved in protective immunity. These antigens were recognised strongly by calves infected with *D. viviparus*, thus demonstrating their immunogenicity (Figures 3.10-3.12).

Alternatively, antigens common to both ES and homogenate preparations may be capable of stimulating protective immunity to *D. viviparus* infection. The response to these may, however, be lower in hosts sensitised with parasite extract due to the presence of a greater number of proteins which may diminish the stimulation of specific immune responses due to antigenic competition (Neilson and Van de Walle, 1987). If the latter is true it should be possible to isolate potential protective components from somatic extracts from which they can be prepared in greater quantity, as has previously been achieved for ES products of *T. spiralis* (Despommier *et al.*, 1977) and *T. muris* (Jenkins and Wakelin, 1977).

The significant number of proteins labelled during *in vitro* synthesis and the similarity of these, with respect to molecular mass, to extrinsically labelled ES might indicate that ES proteins are actively synthesised and released from live parasites. The

profile of ES proteins is consistent throughout the culture period, showing that the parasites are active throughout this time, and also that there is no degradation of proteins from day to day.

Surprisingly, no ^{35}S-labelled proteins were extracted from the worm surface following treatment with CTAB, which has been shown to remove ^{125}I-labelled surface-associated proteins from *D. viviparus* (see Chapter 6) and from other nematodes (Pritchard *et al.*, 1985 and 1988b). This suggests that no newly synthesised methionine-containing proteins are present on the surface, or if they are, they are rapidly shed into the culture medium. This observation might indicate that ES products of this parasite are not adsorbed onto the epicuticle following their synthesis and secretion as is thought to occur with other nematodes. Monoclonal antibodies specific to *T. spiralis* ES antigens of 48 and 50-55kDa bind to both the cuticle and gut lining (Silberstein and Despommier, 1984). Similarly, monoclonal antibodies to *T. canis* ES products, which bind the parasite surface in immuno-electron microscopy, do not localise to the cuticular matrix, but in the oesophageal and excretory glands (Maizels and Page, 1990). The relationship between surface and ES antigens of *D. viviparus* will be discussed further in Chapter 6.

Precipitation of radio-labelled ES with serum from infected calves demonstrated that all ES components are antigenic, with the exception of two molecules, one of which has been identified as bovine serum albumin. Host serum albumin has been found on the surface and in ES products of other parasitic nematodes (Maizels *et al.*, 1984a; Forsyth, Copeman and Mitchell, 1984; Kennedy and Qureshi, 1986; Egwang *et al.*, 1988a), and is thought to be involved in evasion of host immune mechanisms by masking parasite antigens, particularly those exposed on the surface. As surface components of these parasites may be shed *in vitro* (Philipp *et al.*, 1980; Maizels *et al.*, 1983a) albumin present in ES may be derived from this source. However, bovine serum albumin was not detected on the surface of *D. viviparus* adult worms in radiolabelling studies nor by immunofluorescence with anti-bovine albumin antiserum (Chapter 6). It may, therefore, be excreted following ingestion *in vitro*, or may be present on the surface in such small quantities that it is not detected. Host albumin was more abundant in female than male ES products. This may reflect ingestion of greater amounts of this protein by female worms, perhaps associated

with egg production or release. Alternatively, this could be a result of more albumin being absorbed onto the female worm due to its greater surface area.

Another ES component of a 27kDa also failed to be recognised by bovine infection sera. It was, however, recognised by rabbit antiserum to adult ES, indicating that this molecule is potentially immunogenic. At present, it is thought to be of host origin, although the possibility that it is a parasite protein which is non-immunogenic to the bovine host cannot be excluded. If the latter proves to be true, this component could form the basis of an ideal diagnostic test for *D. viviparus* infection, as it would not be rapidly cleared by the bovine humoral immune system (Parkhouse and Clark, 1983). This component may be released *in vitro* from the parasite surface as radioiodination studies have identified a major surface component of similar molecular mass (see Chapter 6).

Radio-iodination of ES by the Iodogen method revealed a protein of 290kDa which is present only in the culture fluid of adult female worms and may be involved in the production, hatching or development of the egg and first stage larvae. Indeed evidence that egg related antigens are present in adult ES products has been provided by the observation that antiserum raised against adult ES binds to the egg surface in immunofluorescence studies (Chapter 6).

This female-specific component was recognised by serum from calves infected with *D. viviparus* and also by that of calves infected with the common bovine gastrointestinal nematodes *C. oncophora* and *O. ostertagi*. This may represent a component conserved between these different nematode species or may be due to recognition of cross-reacting epitopes. Cross-reactivity between parasite antigens is often associated with the presence of carbohydrate (Campbell, 1936; Maizels *et al.*,1987) or phosphorylcholine (PC) determinants (Forsyth *et al.*, 1985; Maizels, Burke and Denham, 1986), structures which are found in a range of parasite components, while peptide epitopes are generally thought to be more species-specific. However, the 290kDa component does not appear to contain PC as indicated by its lack of reactivity with a monoclonal antibody to PC (Bp-1) (Sutanto, Maizels and Denham, 1985) in immunoblotting and immunoprecipitation studies (data not shown). Nor does it appear to be glycosylated as suggested by its failure to bind to a variety of lectins and

its resistance to Peptide-N-Glycanase, although the presence of O-linked sugars could explain the latter observation. Interestingly, a component of very similar molecular mass was biosynthetically labelled with ^{3}H-glucosamine, indicating the synthesis of sugar groups. Although it has not been confirmed that this glycosylated component represents the cross-reacting antigen, periodate oxidation of ES proteins prior to immunoprecipitation may demonstrate whether the observed cross-reactivity of the 290kDa antigen is due to the recognition of carbohydrate epitopes.

Although only one *D. viviparus* ES component was recognised by circulating antibody from calves infected with gastrointestinal nematodes the possibility that infection with such parasites induces a local secretory antibody response to cross-reactive epitopes cannot be excluded in the present study. Therefore, while immunoprecipitation of *D. viviparus* adult ES products is a specific indicator of *D. viviparus* infection it does not demonstrate that ES antigens of this parasite are species-specific. This may be examined by carrying out immunoprecipitation studies of ES products of gastrointestinal nematodes with *D. viviparus* immune sera.

Surprisingly, the 290kDa antigen was not labelled following *in vitro* incubation of parasites with ^{35}S-methionine, nor by iodination by the Bolton-Hunter method, and was only weakly labelled by the Iodogen method, although a protein of such a size would be expected to contain a significant number of the amino acids labelled by these techniques. Similar characteristics have been described for a 400kDa component of *T. canis* L2 ES (Meghji and Maizels, 1986), which is thought to be a proteoglycan with a relatively small peptide component. The same may also be true of the 290kDa component of *D. viviparus* ES.

In contrast to ES products of other parasitic nematodes (Meghji and Maizels, 1986; Kwan-Lim *et al.*,1989), *D.viviparus* ES appears to contains only one N-linked oligosaccharide, of M_r 42kDa. The deglycosylated form of this protein showed identical molecular mass to a 39kDa non-glycosylated protein. Both of these proteins showed decreased mobility on reduction and it is possible that the 42kDa protein is a glycosylated form of the 39kDa molecule. Indeed, in a preliminary study, peptide mapping with *Staphylococcus aureus* V8 protease of each of these proteins purified from preparative SDS-PAGE indicated that both contained the same protein backbone (not shown). Both the glycosylated and non-glycosylated forms of this protein were

found to be antigenic to the bovine host.

The present study has indicated that PC determinants are not present in the ES products of *D. viviparus* adult parasites. In view of the proposed role of PC in diversion and suppression of host immune responses (Mitchell *et al.*, 1977, Butterworth *et al.*,1987), the absence of this structure in *D. viviparus* ES may in part explain its relatively rapid elimination from the lungs of infected calves, while parasites which secrete or express on their surface large quantities of this determinant survive for long periods causing chronic infections. However, the present immunoblotting and imunoprecipitation studies have not excluded the possibility of PC being linked to polysaccharide and phospholipid structures. The use of ELISA to detect PC determinants irrespective of the nature of the carrier molecule may provide more definitive information on the presence of PC in *D. viviparus* ES products (Sutanto *et al.*, 1985)

The recognition of almost all *D. viviparus* adult ES products by sera from infected calves might suggest that *in vitro*-released material may be a true representation of ES molecules produced *in vivo*. This, therefore, presents an opportunity to examine the immune responses to such antigens following infection with this parasite.

CHAPTER 4

KINETICS AND GENETIC CONTROL OF ANTIBODY RESPONSE TO *D. VIVIPARUS* ES PRODUCTS

4.1 INTRODUCTION

Dissection of the immune repertoire of individual hosts to helminth infections is important for several reasons. Firstly, differences in the specificity of the immune response between hosts may be related to susceptibility or resistance to infection and may, therefore, be useful in identifying potential protective antigens (Else and Wakelin, 1989; Kurniawan *et al.*, 1990). Secondly, serodiagnosis will be affected by variations between subjects in their reactivity to parasite components, particularly where purified antigens are used. Finally, the success of parasite vaccines will depend on their ability to elicit a protective response in all individuals. This is extremely important in view of the move towards synthetic and recombinant vaccines which, as mentioned previously, are likely to comprise a single or limited number of parasite antigens. Genetic differences in the immune response of vaccinates may seriously impair the efficiency of vaccination. For example, there is already evidence that the success of vaccination of humans with purified surface antigen of Hepatitis B virus varies substantially between individuals, and that this is associated with certain alleles of the major histocompatibility complex (MHC) (Varla-Leftherioto, 1990).

The MHC has been found to be a group of closely linked genes in all species examined to date. Its structure and function are best understood in mice and humans in which the gene complexes are referred to as the H-2 and the HLA, respectively (Klein, 1975). The cell surface glycoproteins encoded by these genes were first identified by transplantation studies as the body's means of distinguishing self from non-self. MHC genes can be divided into distinct regions, the most important of which, in terms of T cell reactivity, are the class I and class II genes. Products of class I genes are generally found on all nucleated cells and are involved in the regulation of cytotoxic T cell (T_C) responses. Class II region genes are expressed on T and B lymphocytes, on antigen presenting cells (APC's) and on other tissues under conditions of immunological or infection stress (Daar *et al.*, 1984). Products of these genes are primarily asociated with antigen presentation and co-operation between T and B lymphocytes in the induction of antibody responses (Schwartz, 1985; Davis and Bjorkman, 1988).

Individuals within an outbred population of most mammals show considerable polymorphism at MHC gene loci (Klein, 1986). The MHC genes expressed by an individual can affect its reactivity to particular antigens due to the differing affinities of MHC molecules for processed antigen fragments and also by influencing the presentation of antigen to T cells (Allen *et al.*, 1987; Townsend and McMichael, 1987). Individuals of an outbred population will, therefore, show differences in their immunological reactivity to foreign molecules. Hosts will not respond to molecules cross-reactive with their MHC gene products, nor with antigen fragments which will not associate with MHC molecules expressed on their APC's. MHC polymorphism is thought to be beneficial to the population by preventing pathogens exploiting these gaps in the host immune repertoire (Klein, 1986). However, this can also give rise to considerable variation between individuals in their recognition of parasite antigens and can influence the outcome of infection in individual hosts (see Chapter 1). It has also to be remembered that polymorphism at other gene loci, such as the immunoglobulin and T cell receptor genes, can also influence the host immune response (Epstein *et al.*, 1986).

The MHC is known to control responses in mice to *S. mansoni*, following infection (Kee *et al.*, 1986) or vaccination with irradiated larvae (Sher, Hieny and James, 1984), and to determine the specificity of the antibody response to particular nematode antigens presented in the context of infection or with adjuvant (Else and Wakelin, 1989; Kennedy, 1989; Kennedy, Fraser and Christie, 1991a; Kennedy *et al.*, 1991b; Kwan-Lim and Maizels, 1991). This region is also thought to play a role in genetic resistance of sheep to *H. contortus* (Gray, 1987). It is not yet known, however, whether the observed individual variability in resistance of calves to *D. viviparus* is under MHC control or if, indeed, this has a genetic basis (Rubin and Lucker, 1956; Weber, 1958; Poynter *et al.*, 1970).

In the present study the immune recognition of adult ES antigens by infected and vaccinated bovine hosts was compared. Significant differences both in the specificity and kinetics of antibody response were observed between hosts exposed to normal or irradiated larvae. Moreover, considerable heterogeneity in antibody repertoire was found between individual animals, regardless of the infection regime. The possibility that this was due to genetic differences between hosts was examined using inbred

guinea-pigs of strains 2 and 13, which share the same class I region alleles but disparate class II alleles (Klein, 1986). The present findings indicate that the antibody response to *D. viviparus* ES antigens is under genetic control, possibily being influenced by the class II region of the MHC.

4.2 RESULTS

4.2.1 Kinetics of antibody response to adult ES antigens following experimental infection

The recognition of ES antigens following repeated exposures to normal, 40krad irradiated or 100krad irradiated larvae was examined by immunoprecipitation of ^{125}I adult ES. Sequential recognition of ES antigens was observed following infection with normal larvae. Experimental infection with 1,000 larvae induced a significant antibody response to an antigen of M_r 30kDa, which in some calves was first detected 30 days after infection (Figure 4.1). Strong recognition of antigens of M_r 14-18, 39, 42 and 76 kDa was observed after 45 days. In some calves a decrease in the level of antibody response could be observed by 52 days post infection.

In other hosts infected with 2,000 larvae, as detailed in Table 4.1 (Group A), antibodies directed against antigens of 14-18kDa were the first to be detected (Figure 4.2). After two infections, antigens of molecular masses 30, 39 and 42kDa were also precipitated. Although it is not clear in Figure 4.2, antigens of approximately 72 and 76 kDa and another doublet at 125 and 135kDa were also precipitated by serum taken on day 76. This is shown in track 3^{I}, using a different batch of radio-iodinated ES. The absence of these high molecular mass bands in Figure 4.2 may have been due to disintegration of labelled components during storage. After 4 infections with normal larvae the response to adult ES antigens gradually diminished and was not boosted following subsequent heavy infections (Figure 4.2, tracks 7, 8, a, b).

4.2.2 Antibody response to 40krad irradiated larvae

Following vaccination with two doses of 40krad irradiated larvae only one antigen, of 30kDa, was stongly precipitated. This can be seen in Figure 4.3 showing the recognition profiles of two individual calves (Panels A and B) vaccinated as described in Table 4.1 (Group B). Weak recognition of a 48kDa antigen also occurred at this time. Two other antigens of 18kDa and 76kDa were precipitated after 3 vaccinations in one calf and after 4 vaccinations in the other. Maximum precipitation occurred after 5 vaccinations in both hosts. With the exception of a 14kDa antigen which was transiently recognised by one host (Panel A), the response to ES antigens remained constant even after challenge infection with normal larve with no additional antigens being recognised (tracks a, b, c).

4.2.3 Kinetics of antibody response following repeated vaccination with 100krad irradiated larvae

It has previously been shown that calves vaccinated with larvae irradiated to 100krad are immune to reinfection although such larvae do not develop beyond the L3 stage (Canto, 1990). We examined the antibody recognition of adult ES antigens by two such hosts, vaccinated as described in Table 4.1 (Group C). In contrast to 40krad vaccinates, calves vaccinated with larvae irradiated to 100krad recognised a wide range of adult ES antigens after two vaccinations (Figure 4.4, track 3). In one calf (Panel A) the level of this response was maximal after 3 vaccinations, while in the other a maximal response was observed after 4 vaccinations. In both hosts the level of response to all antigens gradually decreased, although a significant boost in the response to antigens of 30kDa and 76kDa was observed following challenge with normal larvae.

4.2.4 Kinetics of antibody response to ES as measured by precipitated cpm

The level of radioactivity present in immunoprecipitates formed between radio-iodinated ES and immune serum demonstrated quantitatively the gradual increase in antibody response to ES antigens. This is shown in Figure 4.5 using sera from another group of calves. The % TCA precipitable cpm was used as a measure of the IgG antibody

response in a Staph A-based assay. Following exposure to 1,000 normal larvae on day 0, there was a gradual increase in the counts precipitated, rising most sharply between 28 and 56 days after infection i.e. during the patent period. Serum from one calf showed an increase in the level of precipitated counts following a second similar infection on day 63, while in the other the level decreased slightly.

In contrast to infected hosts, serum from calves vaccinated with 1,000 40krad irradiated larvae on days 0 and 28 precipitated very little radiolabelled ES material, even after challenge with normal larvae (30 L3/ kg bodyweight) on day 63. Slight variability was observed between individual calves, with serum from one showing a maximal increase in precipitated counts following the first vaccination, while in the other a maximal increase was observed after challenge with normal larvae. This variability did not reflect any difference in the immune status of the vaccinates, both of which harboured less than 5 adult worms at necropsy on day 90.

4.2.5 Heterogeneity of bovine antibody response to ES antigens

Following the slight but significant differences observed in kinetics and recognition of ES antigens by individual hosts, sera from a larger number of calves was examined. This revealed significant heterogeneity in the recognition profiles of different individuals. This is demonstrated in Figure 4.6, showing immunoprecipitates of radioiodinated ES with serum from nine individual calves exposed on 2 occasions to normal *D. viviparus* larvae (tracks g, h, i), vaccinated with 2 doses of 1,000 40krad irradiated larvae (tracks a, b, c) or 2,500 100krad irradiated larvae (tracks d, e, f) and exposed to challenge infection. Serum from all calves was sampled 28 days after challenge. Consistent with Figure 4.3, calves vaccinated with irradiated larvae showed restricted recognition of ES antigens, while infected calves recognised almost all ES components. Considerable variability was observed, however, between animals in the same treatment groups.

4.2.6 Heterogeneity of antibody response to ES antigens in outbred guinea-pigs

To determine whether the observed heterogeneity in antibody response to *D. viviparus* also occurred in the guinea-pig model, the immune recognition of adult ES products by guinea-pigs of the Duncan-Hartley outbred strain was examined. Guinea-pigs were vaccinated with 2 doses of 40krad irradiated larvae 21 days apart and serum sampled 21 days after the second dose was immunoprecipitated with ^{125}I-ES (Figure 4.7). This revealed restricted recognition of adult ES antigens in guinea-pigs exposed to irradiated larvae, with only 3-4 antigen species being recognised. The recognition profile was similar to that of guinea-pigs infected with normal larvae (see Figure 3.4B). Moreover, a significant degree of heterogeneity was observed in the recognition patterns of individual hosts. This pattern was unaltered using serum taken 7 days after challenge with normal larvae.

4.2.7 Genetic control of the antibody repertoire in inbred guinea-pigs

If the variability observed between guinea-pigs was due to genetic differences, then such variability would not be expected to occur between individuals of an inbred strain. The responses of inbred guinea-pigs of strains 2 and 13 to vaccination with 40krad irradiated larvae were, therefore, examined. As demonstrated in Figure 4.8, guinea-pigs of the same strain showed similar, although not identical, recognition profiles. Significant differences were observed, however, in the response patterns of the two strains. In particular, a 14kDa antigen was recognised only by animals of strain 2, while recognition of an antigen of 30kDa was restricted to strain 13. The differential recognition of ES antigens did not reflect any difference in host resistance to the parasite as indicated by the absence of parasites in the lungs of guinea-pigs of both strains at necropsy 7 days later.

TABLE 4.1 Infection regime of bovine hosts

All calves were challenged orally with 30 normal L3/kg bodyweight on 17/10

DATE	GROUP A normal L3 orally	GROUP B 40krad L3 orally	GROUP C 100krad L3 i.v.
9/2	2,000		
29/3	2,000		
6/4			5,000
26/4	10,000		10,000
23/5	20,000	5,000	10,000
16/6	20,000	10,000	10,000
10/7		10,000	20,000
28/7		20,000	
18/8	10,000	20,000	10,000
13/9	20,000	20,000	20,000
17/10	30 L3/kg	30 L3/kg	30 L3/kg

Figure 4.1 Kinetics of antibody response to adult ES following experimental infection. Bolton-Hunter iodinated ES (R) was immunoprecipitated in a Staph A-based assay with serum from a calf infected with 1,000 normal infective larvae. Serum was sampled immediately before infection (a) and at 12 (b), 26 (c), 30 (d), 38 (e), 45 (f) and 52 (g) days post infection. Immunoprecipitated antigens were analysed by SDS-PAGE under reducing conditions. The M_r of marker proteins (M) are indicated in kDa.

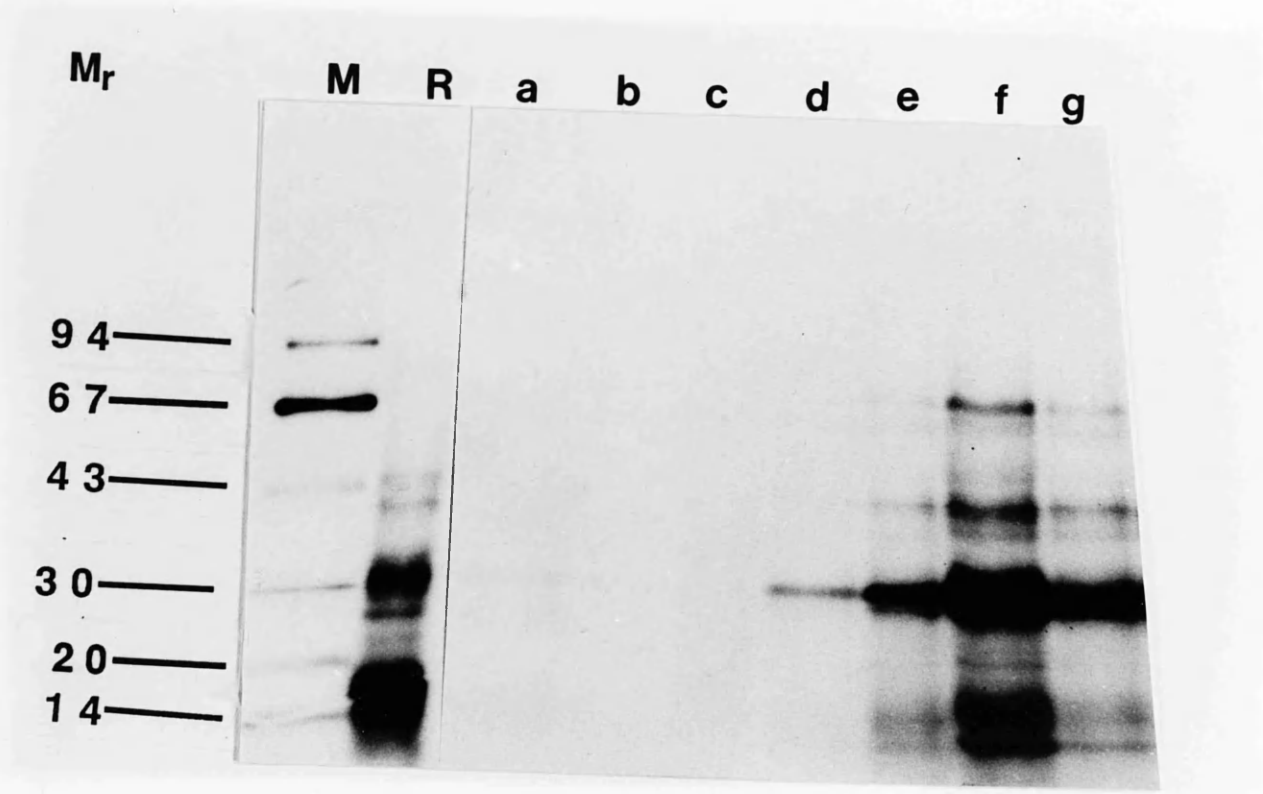

Mr
M
R
a
b
c
d
e
f
g
94
67
43
30
20
14

Figure 4.2 Recognition of adult ES antigens following multiple infections. Radioiodinated ES (R) was incubated with serum from a calf experimentally infected with normal larvae of *D. viviparus* on eight occasions as detailed in Table 4.1 (Group A). The track numbers refer to the infection administered. Serum was sampled prior to each infection (i.e. immunoprecipitation with serum sampled prior to the first infection is shown in track 1) and at 7 (track a) and 14 (track b) days after the final infection. Staph A-precipitated antigens were analysed by SDS-PAGE under reducing conditions. Track 3^{I} shows an immunoprecipitation carried out with the same serum as track 3, but with a different batch of ^{125}I-ES.

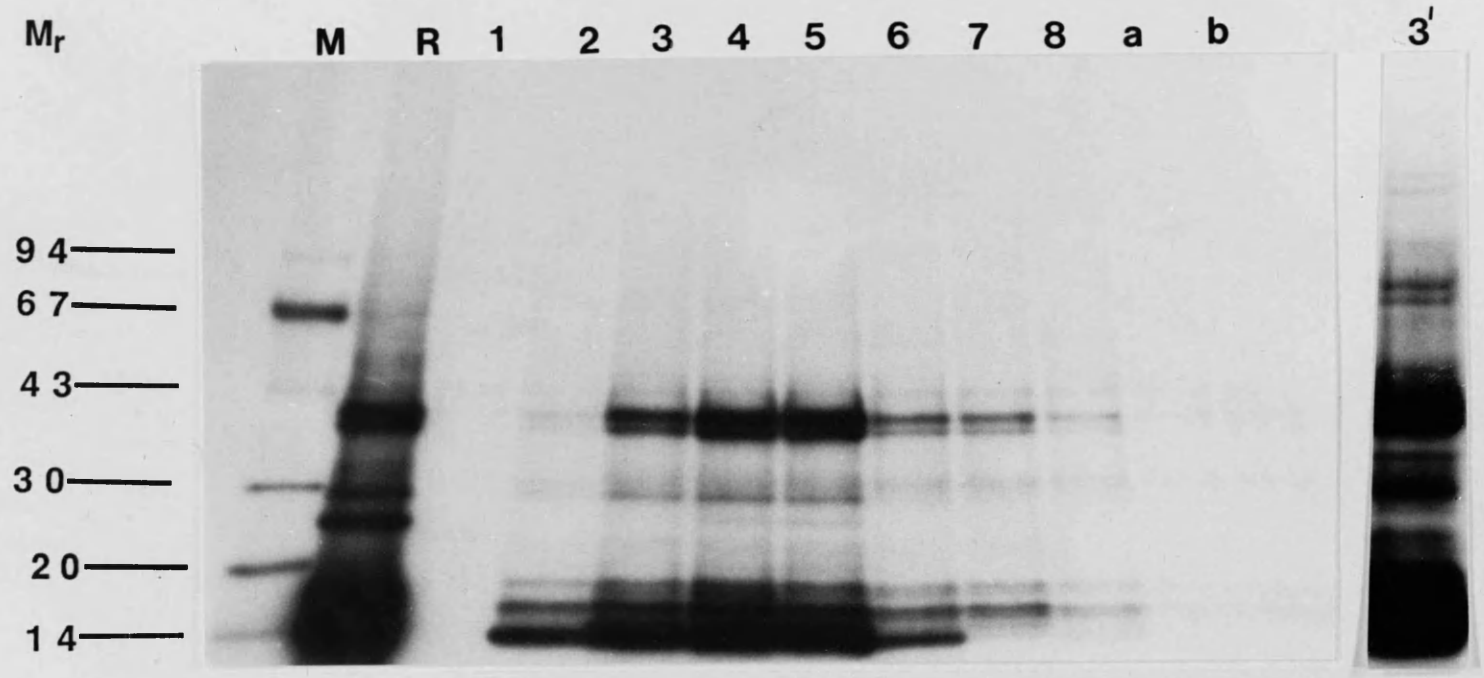
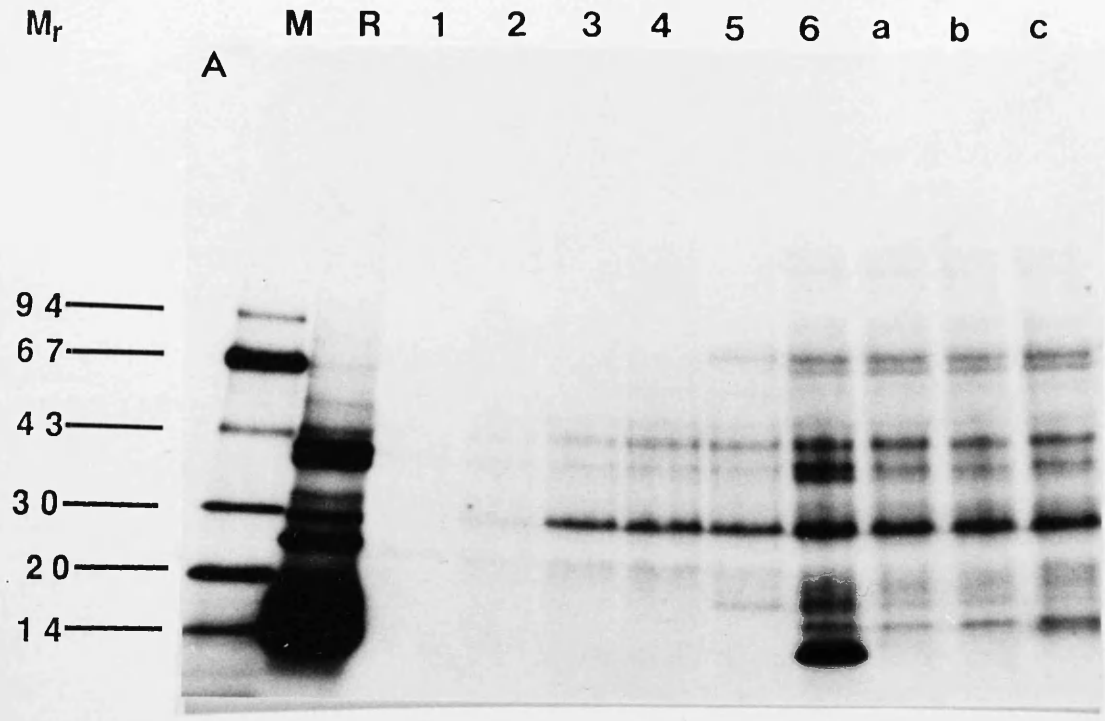

Mr
M
R
1
2
3
4
5
6
7
8
a
b
3'
94
67
43
30
20
14

Figure 4.3 Response to adult ES antigens following vaccination with 40krad irradiated larvae. Serum from two calves, vaccinated on six occasions with larvae irradiated to 40krad, was immunoprecipitated with radioiodinated ES (R). Serum was sampled prior to each vaccination, the number of which is indicated by the track numbers (See Table 4.1 for details of vaccine dose). Panels A and B show the recognition profiles of individual calves. After the sixth vaccination (track 6) calves were challenged with normal *D. viviparus* larvae and serum sampled 7 (track a), 14 (track b) and 21 (track c) after challenge. Immunoprecipitated antigens were analysed by SDS-PAGE under reducing conditions.


Mr
M R 1 2 3 4 5 6 a b c
A
94
67
43
30
20
14

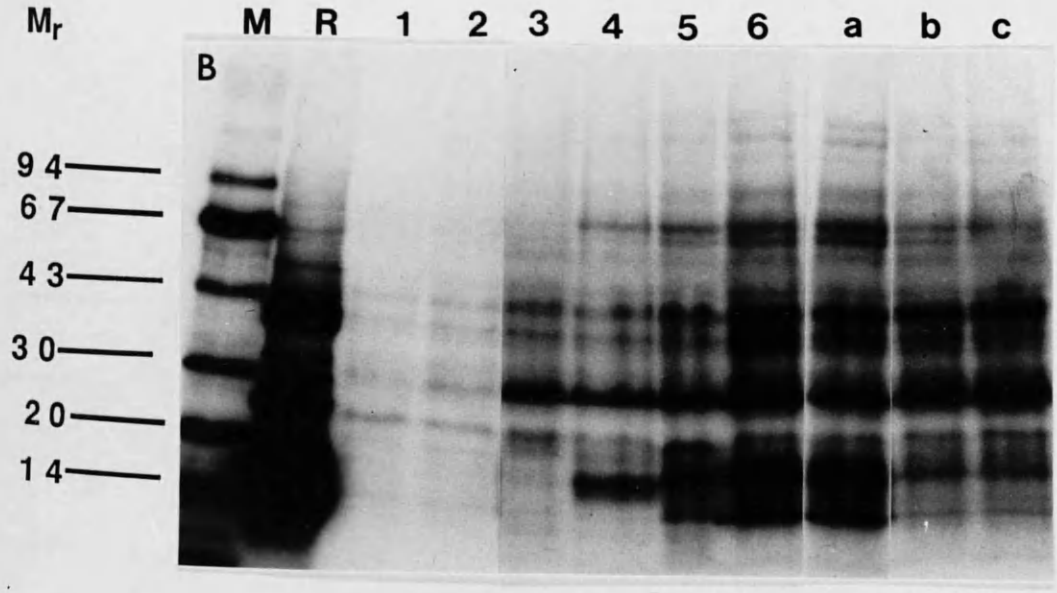

Mr
M R 1 2 3 4 5 6 a b c
B
94
67
43
30
20
14

Figure 4.4 Kinetics of adult ES recognition following multiple vaccination with 100krad irradiated larvae. Calves were vaccinated on seven occasions, as indicated by the track numbers, with larvae irradiated to 100krad. Details of the larval doses are shown in Table 4.1 (Group C). Serum was sampled prior to each vaccination and at 7 (track a), 14 (track b) and 21 (track c) days after challenge infection with normal larvae. This was immunoprecipitated in a Staph A-based assay with ^{125}I-ES (R) and precipitated antigens analysed by SDS-PAGE.

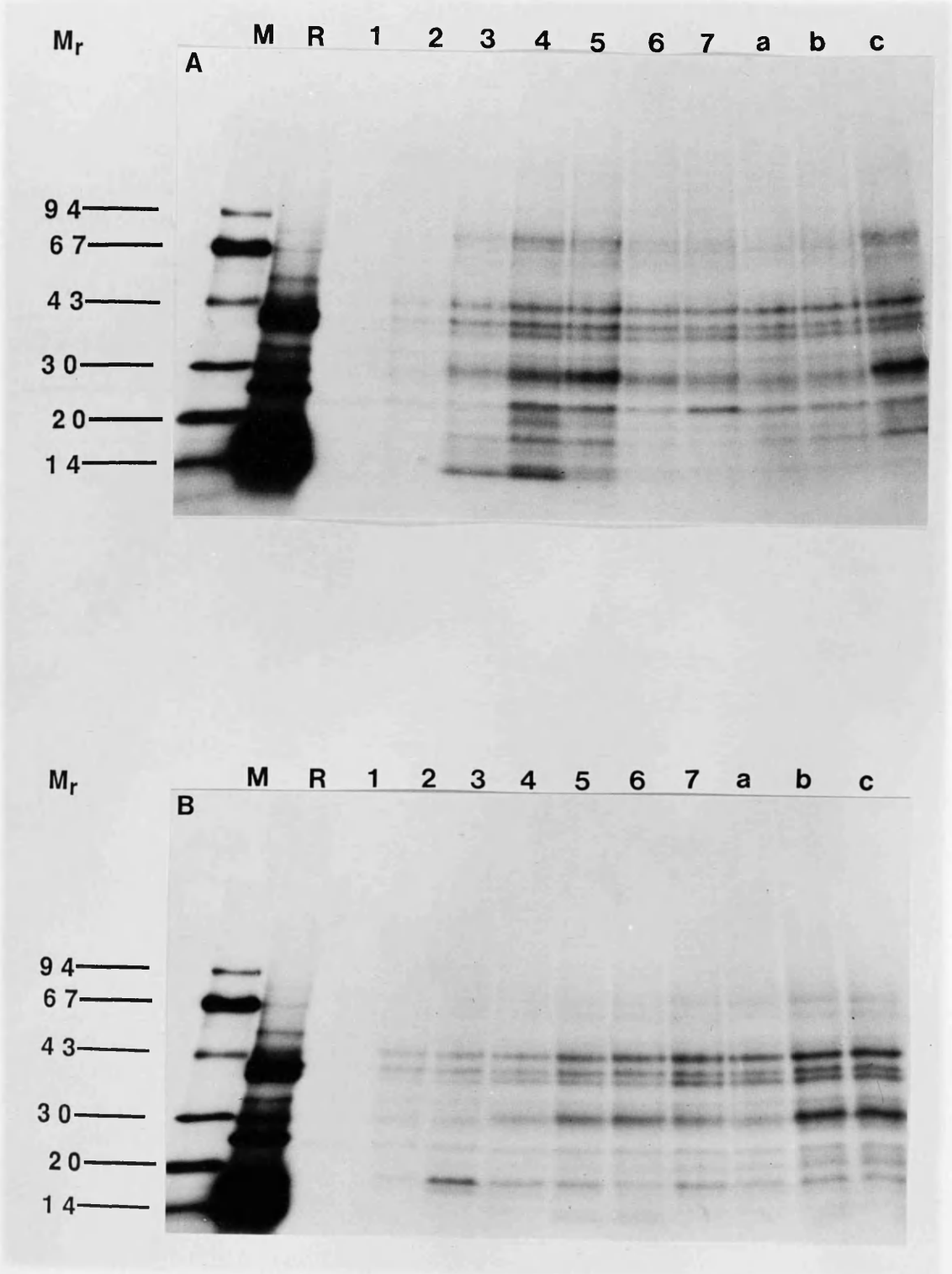

Mr
M R 1 2 3 4 5 6 7 a b c
A
94
67
43
30
20
14
Mr
M R 1 2 3 4 5 6 7 a b c
B
94
67
43
30
20
14

Figure 4.5 Kinetics of IgG antibody response to adult ES following infection and vaccination. Radiolabelled ES was immunoprecipitated with serum from calves exposed to *D. viviparus* L3 as follows: infected with 1,000 and 2,000 normal larvae on days 0 and 63, respectively (open symbols indicate the responses of two such calves) or vaccinated with 1,000 40krad irradiated larvae on days 0 and 28 and challenged with 2,000 normal larvae on day 63 (shaded symbols). Serum was sampled from both groups on days 0, 28, 56 and 21 days after the final infection. The mean % TCA-precipitable cpm is used as a measure of IgG antibody level. Immunoprecipitation was carried out on three occasions and the graph shows the typical results of one such assay using serum from two calves in each group.

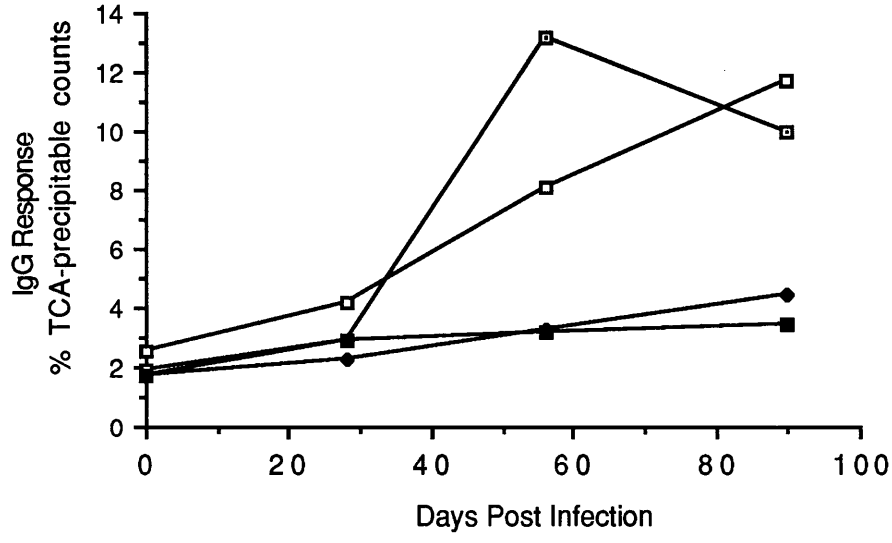

IgG Response
% TCA-precipitable counts
14
12
10
8
6
4
2
0
0
20
40
60
80
100
Days Post Infection

Figure 4.6 Heterogeneity of bovine antibody response to *D. viviparus*. Radiolabelled adult ES (R) was immunoprecipitated with serum from calves exposed to *D. viviparus* L3 as follows: vaccinated orally with two doses of 1,000 40krad irradiated larvae 21 days apart and challenged with 2,000 normal larvae after a further 21 days (tracks a, b, c): vaccinated on two occasions with 2,500 100krad irradiated larvae, 21 days apart and challenged with normal larvae as described above (tracks d, e, f); or infected on two occasions, 48 days apart, with 2,000 normal L3 (tracks g, h, i). Serum was sampled 28 days after the final exposure to larvae and the antibody responses of three individuals in each group are shown. Track N shows immunoprecipitation with bovine pre-infection serum.

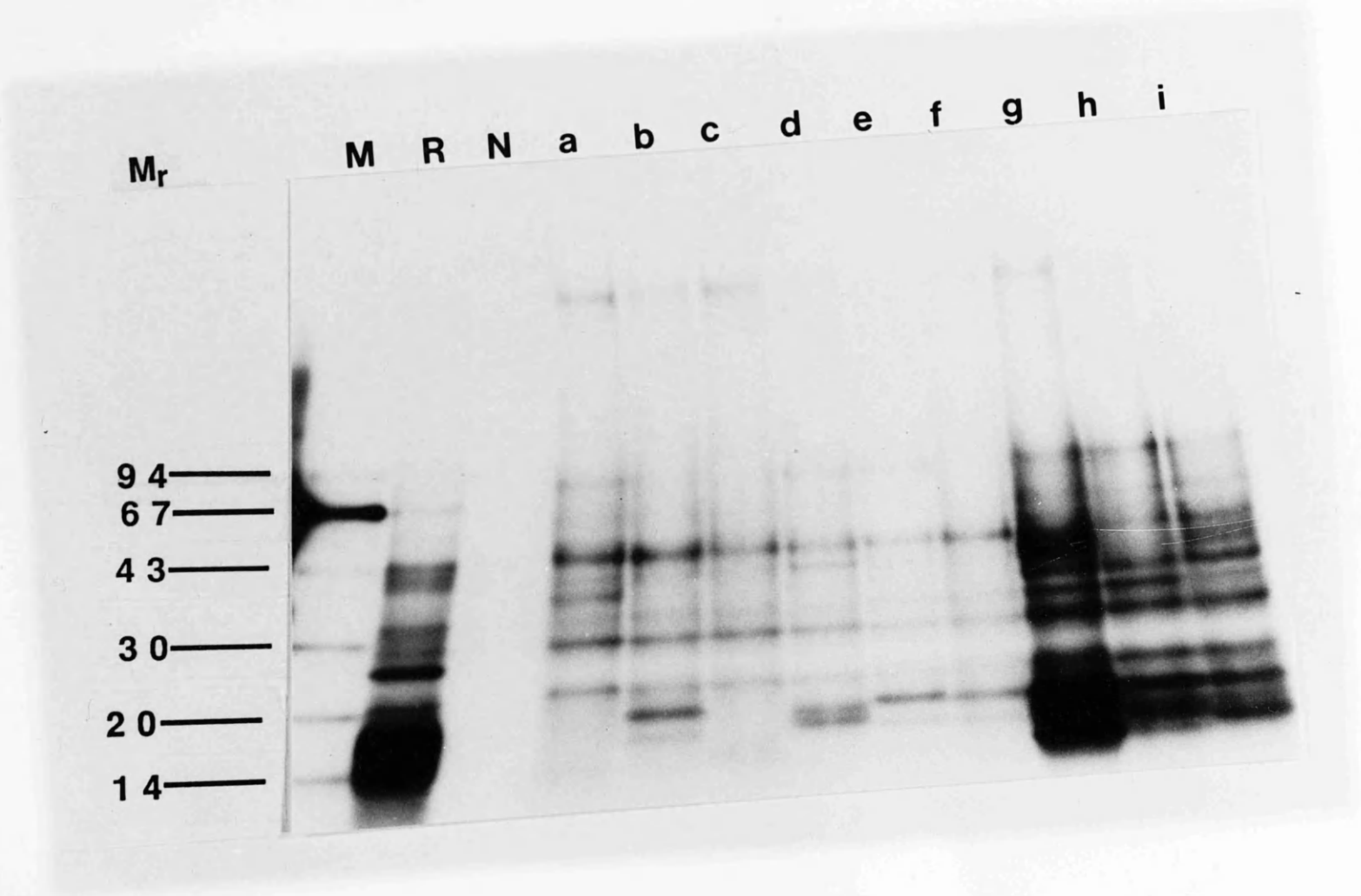

Mr
M
R
N
a
b
c
d
e
f
g
h
i
94
67
43
30
20
14

Figure 4.7 Heterogenous recognition of ES antigens by outbred guinea-pigs. Guinea-pigs of the Dunkin-Hartley outbred strain were vaccinated on two occasions, 21 days apart, with 5,000 *D. viviparus* larvae irradiated to 40krad. Serum from individual hosts (numbered tracks) was sampled 21 days later and immunoprecipitated with ^{125}I-ES (R). Immunoprecipitation with normal guinea-pig serum is shown (N).

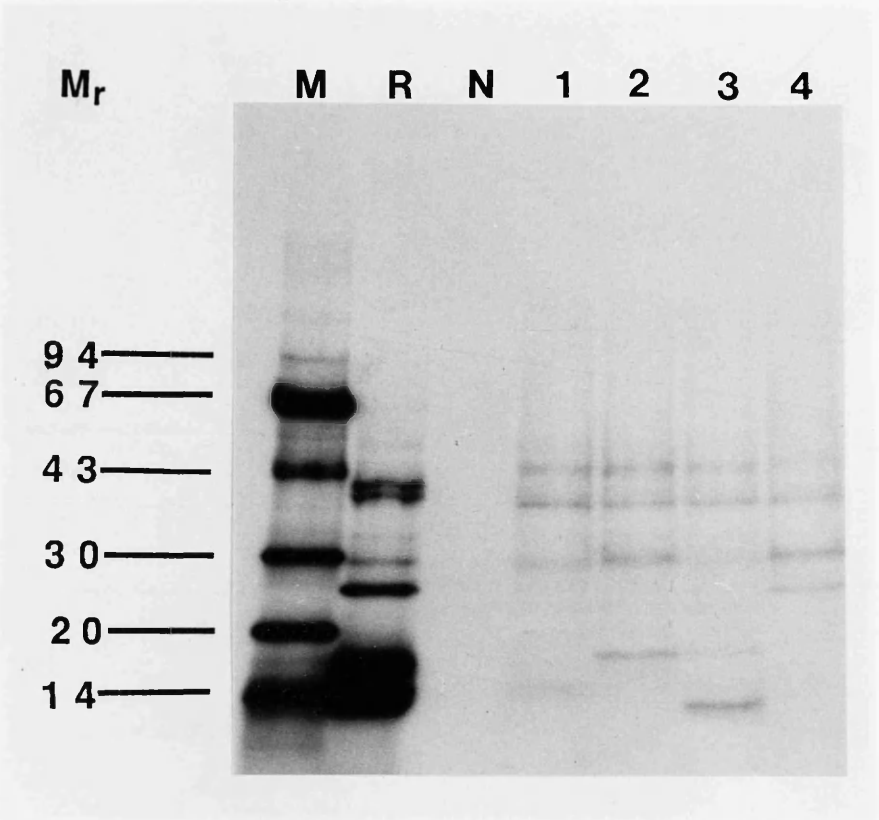
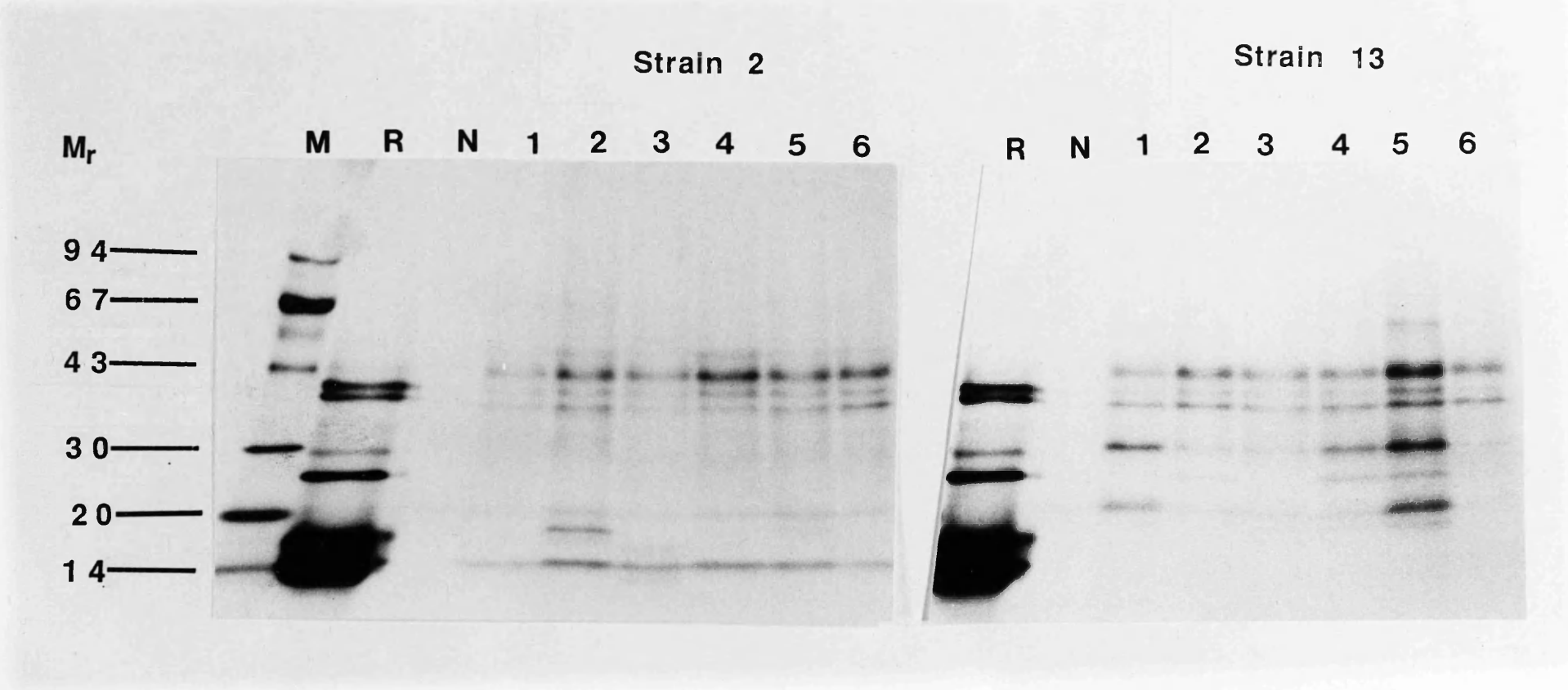

Mr
M
R
N
1
2
3
4
94
67
43
30
20
14

Figure 4.8 Genetic control of antibody repertoire to *D. viviparus* ES in inbred guinea-pigs. Guinea-pigs of strains 2 and 13 were vaccinated as in Figure 4.7 and serum from individual hosts (numbered tracks) was immunoprecipitated with radioiodinated ES (R) in a Staph A-based assay. Track N shows imunoprecipitation with normal guinea-pig serum.


Strain 2
Strain 13
Mr
M R N 1 2 3 4 5 6
R N 1 2 3 4 5 6
94
67
43
30
20
14

4.3 DISCUSSION

Examination of the kinetics and specificity of the immune response to ES antigens of adult *D. viviparus* revealed significant differences between vaccinated and infected hosts. Calves infected with normal larvae gradually recognised almost all ES antigens, as did those vaccinated with high doses of 100krad irradiated larvae. In contrast, 40krad vaccinates showed restricted recognition of ES antigens

It has previously been demonstrated that larvae irradiated to 100krad do not develop beyond the L3 stage (G.J.Canto, 1990). The recognition of a significant number of adult ES antigens by such vaccinated hosts, therefore, indicates a significant degree of cross-reactivity between L3 and adult antigens. It is unlikely that these antigens represent L3 ES products as we have found that this stage releases very little parasite material, at least *in vitro*. They may, however, be L3 somatic components released following parasite destruction.

The detection of cross-reacting antibody only in serum from 100krad vaccinated hosts may be explained by differences in the viability of 40krad and 100krad irradiated larvae. Canto (1990) has demonstrated a reduction of 80% in the number of 100krad irradiated larvae reaching the lungs of guinea-pig hosts two days after vaccination relative to 40krad irradiated larvae, irrespective of the route of administration. It may, therefore, be speculated that 100krad irradiated larvae are destroyed *in situ* before reaching the lungs, thus exposing the host immune system to parasite somatic antigens. In contrast, larvae irradiated to 40krad may be expelled or killed in the air spaces of the lungs and perhaps stimulate a local secretory antibody response but no circulating antibody response to somatic components.

The presence of homologous or cross-reacting antigens between adult ES and larval components was also suggested by the recognition of a number of adult ES antigens by infected and vaccinated guinea-pig hosts, in which larvae do not develop to the mature adult stage (Poynter *et al.*, 1960). This was previously suggested by the ability of adult ES to protect guinea-pigs against reinfection with *D. viviparus*, although the immunity induced was active against the larval stages of the parasite.

Some adult ES components, particularly those of 14-18kDa, appeared to be stage-specific as indicated by the significant response to these by hosts exposed to

patent lungworm infections, but not by those vaccinated with two doses of 40krad irradiated larvae as used in the commercial vaccine. This suggests that these antigens are not exposed to the host during the standard vaccination procedure and may, therefore, be irrelevant to vaccine-induced immunity. The greater immunising efficacy of adult ES compared to adult homogenate may, therefore, not be due to the greater abundance of these low molecular mass antigens in adult ES as suggested in Section 3.3, although it is, of course, possible that immunisation with adult ES might operate by a different mechanism to the irradiated vaccine.

An important observation in these studies was the very restricted recognition profile of antigen recognition by 40krad vaccinates. These hosts showed strong recognition of only one ES antigen, of 30kDa, after two vaccinations, by which time they are immune to reinfection. This suggests that the antibody response to other adult ES antigens is unlikely to be of any functional significance to vaccine-induced immunity. The recognition of a wide range of ES components by these hosts following five vaccinations is possibly due to a gradual accumulation of L4 and L5 stages developing from irradiated larvae and the possible induction of an immune response to components of these stages cross-reactive or homologous to adult ES products.

We have found that this 30kDa antigen is recognised by all bovine hosts so far examined and in some naturally infected calves is the first ES antigen to be recognised, with an antibody response to it being detected as early as 30 days after infection (Figure 4.1). This finding, together with its strong recognition by calves vaccinated with 40krad irradiated larvae and, therefore, not exposed to the mature adult stage of the parasite, suggests that this 30kDa antigen is expressed by an earlier stage, possibly by the L4 or L5 stages. It does not appear to be expressed as strongly in the L3 stage as indicated by its slightly weaker recognition by 100krad vaccinates (Figure 4.4). This may explain the greater protection achieved by vaccination with two doses of 40krad irradiated larvae (91%) compared to that with larvae irradiated to 100krad (77%) (Canto, 1990) and indicate the importance of this antigen in protective immunity. 100krad vaccinates did, however, show a boost in antibody response to the 30kDa antigen following challenge infection with normal larvae, possibly due to its expression by later parasite stages.

In calves exposed repeatedly to normal, 40krad or 100krad irradiated larvae

the antibody response reached a maximal level then decreased, both in the number of antigens recognised and in the level of response. This indicated that there was no correlation between the response to most ES antigens and protective immunity. However, components of 30kDa, the doublet at 39 and 42kDa and a 48kDa antigen were all strongly recognised throughout the course of infection with normal or 40krad irradiated larvae. Interestingly, the 30 and 48kDa antigens were also recognised by outbred guinea-pigs protected by exposure to normal (Figure 3.4B) or irradiated (Figure 4.7) larvae, suggesting the involvement of these antigens in protective immunity in both the bovine and guinea-pig hosts. It should, therefore, be possible to examine the protection-inducing potential of these antigens using the guinea-pig model system. Such studies should, however, be exercised with caution due to variability in antibody specificity between different host strains. For example, vaccination of guinea-pigs of strain 2 with irradiated larvae induces no antibody response to the 30kDa ES antigen suggesting that in these hosts recognition of this antigen is not essential to protective immunity (Figure 4.8).

The observed differences between guinea-pig strains in their antibody response suggests that the immune repertoire to *D. viviparus* antigens is under genetic control in these rodents. This may also be the cause of the significant heterogeneity in the specificity of the antibody response in infected or vaccinated calves (Figure 4.6). At present, other possible causes such as variability in the number of larvae which penetrated and developed, or cross-reactivity with other infectious agents cannot be formally eliminated. While a limited degree of cross-reactivity has been found between antigens of *D. viviparus*, *C. oncophora* and *O. ostertagi* (Section 3.2.9), calves were raised under conditions in which infection, at least with other parasitic helminths, was unlikely. Nor do differences in the kinetics of antibody response appear to be responsible for individual variability as differences in antigen recognition were observed between hosts even after multiple exposures to larvae (Figures 4.3 and 4.4). A similar degree of heterogeneity was reproduced in outbred, but not in inbred guinea-pigs, suggesting that the antibody repertoire to *D. viviparus* antigens is genetically controlled.

From this study it is not possible to identify the genetic locus/loci in control,

but the findings of other studies on nematode infections would strongly suggest the involvement of the MHC (referred to as the 'GPLC' in the guinea-pig and 'BoLA' in cattle)(Else and Wakelin, 1989; Kennedy, 1989; Kennedy *et al.*, 1990). To demonstrate this rigorously would require inbred MHC congenic strains of guinea-pig which are not currently available. As guinea-pigs of strains 2 and 13 bear identical class I but disparate class II genes (Klein, 1986), this suggests that if the GPLC region were involved in the differential recognition of ES antigens then this is controlled by class II loci. Class II control of antigen recognition has been demonstrated in mice following infection with *A. suum* and *T. spiralis* (Kennedy *et al.*, 1991a; Kennedy *et al.*, 1991b) in which the region involved can be implicated with greater certainty by the use of MHC congenic and recombinant animals.

The class II region is classically associated with antibody immune response (Ir) genes (Klein, 1986) which were originally defined using synthetic antigens containing highly repetitive epitopes or readily available natural products (such as insulin or myoglobin) which have mammalian homologues. With synthetic antigens, limited epitope composition would readily lead to limited immune recognition and complete non-recognition by animals of certain genotypes, as has been found to occur with a *P. falciparum* circumsporozoite peptide vaccine (Del Giudice *et al.*, 1986). In the case of vertebrate proteins homology with self constituents might lead to non-responsiveness. However, the present study was concerned with large parasite-derived molecules which are unlikely to fall into the above classifications. Although we have no structural or sequence information for any *D. viviparus* ES antigens, an allergen of *A. suum* (ABA-1), which appears to have no repeated motifs in its sequence, nor any homology with other protein sequences, is subject to MHC control (Tomlinson *et al.*, 1989; Christie *et al.*, 1990).

Although it is too early to assign a mechanism for the genetic effects observed in this study, several possibilities exist. Firstly, class II molecules may fail to bind and present processed fragments of particular ES antigens (Schwartz, 1985; Germain, 1990). Secondly, cross-reactivity between self and parasite components might lead to deletion of particular specificities from the host immune repertoire (Schwartz, 1978; Matzinger, 1981; Vidovic and Matzinger, 1988). Finally, as this study was restricted to examining IgG responses, we cannot eliminate the possibility that other Ig

isotypes are involved.

Surprisingly, a degree of heterogeneity, albeit limited, was observed in the recognition of ES antigens between individuals of each inbred strain of guinea-pig. This could be due to differences in T cell receptor or immunoglobulin variable region genes, heterogeneity in T cell epitope recognition (Barnett *et al.*, 1981), or residual heterozygosity in non-MHC loci affecting the immune repertoire through cross-tolerance (Schwartz, 1978; Matzinger, 1981). This, therefore, demonstrates that knowledge of the MHC of a particular animal will not precisely predict its immune repertoire.

If differential recognition of parasite antigens proves to be a general phenomenon in nematode infections of man and domestic animals, this could contribute to heterogeneity in resistance to infection and may, partly, explain the characteristic overdispersion of parasitic infections mentioned in Chapter 1. The specificity of antibody responses could also be directly related to variations in pathology of parasitic infections, particularly to hypersensitivity reactions if only particular parasite antigens elicit reaginic antibody responses (Tomlinson *et al.*, 1989; Christie *et al.*, 1990). Finally, and perhaps of greatest importance at the current time, the application of new-generation vaccines using recombinant or synthetic parasite antigens could be compromised if the polypeptides concerned were subject to differential immune recognition in the host population. It would be essential, therefore, to select antigens for which this did not apply or to use antigen mixtures, at least one component of which is recognised and protective in all individuals. It may also be possible to overcome restrictions to responsiveness by the use of an appropriate adjuvant, or designing an antigen sequence containing both B and T cell epitopes (Good *et al.*, 1987; Leclerc *et al.*, 1987).

Finally, although products of adult parasites may seem an inappropriate target for investigating the immune responses to vaccination or infection with *D. viviparus*, we have demonstrated cross-reactivity between adult and larval antigens. It is probable that the principle of antibody heterogeneity established here will also apply to other antigen compartments of *D. viviparus*.

CHAPTER 5

ANTIBODY RESPONSE TO SURFACE EXPOSED ANTIGENS OF THIRD STAGE LARVAE

5.1 INTRODUCTION

The nematode cuticle was at one time considered to be a robust, antigenically inert layer serving as a structural exoskeleton and to protect the organism against destruction by host immune effector mechanisms (Lumsden, 1975). It has, however, been demonstrated that import of components occurs across the cuticle (Howells and Chen, 1981) and that antigens can be presented on (Ogilvie *et al.*, 1980; Maizels *et al.*, 1982) or shed from (Philipp *et al.*,1980; Smith *et al.*,1981; Forsyth *et al.*,1981; Maizels *et al.*, 1984b) the external surface of the cuticle, referred to as the epicuticle (Bird, 1980). These findings have suggested that the nematode surface is an antigenic and dynamic structure and have led to increasing interest in the relevance of the parasite surface to the immunobiology of parasitic infections.

The antigenic nature of the exposed nematode cuticle was first demonstrated in 1963 by Sadun using immunofluorescent techniques to demonstrate antibody binding to the exposed parasite surface. It was later shown that antibody directed against surface antigens could recruit granulocytes which bound to the parasite and mediated parasite killing *in vitro* (MacKenzie *et al.*, 1978).

Evidence that surface antigens might be involved in protective immunity has come from the successful immunisations with parasite surface preparations of *T. spiralis* (Grencis *et al.*, 1986) and *S. mansoni* (Harn, 1987) and the passive protection obtained in experimental animals given monoclonal antibodies directed against surface determinants of these parasites (Ortega-Pierres *et al.*, 1984b; Smith *et al.*, 1982; Grzych *et al.*, 1982).

Recent work has moved away from immunofluorescence to focus on radiolabelling techniques to characterise surface antigens (Philipp and Rumjaneck, 1984). Although the sensitivity of this method makes it extremely useful in the identification and characterisation of surface-associated molecules, as will be demonstrated in Chapter 6, it does have several disadvantages. Firstly, molecules such as collagens, which are not exposed on the surface, have been found to be labelled following radioiodination of intact parasites (Pritchard, McKean and Rogan, 1988a; Cox, Shamansky and Boisvenue, 1989; Selkirk *et al.*, 1989). Secondly, most of the methods employed have focussed on iodinatable polypeptides, while other

components, such as glycolipids and polysaccharides have been excluded from analysis. The presence of such materials may explain the difficulties encountered in identifying antigens on the surface of infective stage larvae of species such as *N. brasiliensis* (Maizels *et al.*, 1983a) and *B. pahangi* (Devaney and Jecock, 1991). Finally, it has been found that lipids present on the surface can interfere with radiolabelling of helminth surface proteins (Hayunga *et al.*, 1979). To avoid these problems in our initial studies antibody-binding techniques were used to detect surface-exposed antigens of *D. viviparus* regardless of their biochemical nature. The antibody reponse to surface antigens of third stage larvae was examined by quantitative immunofluorescence.

Like most trichostrongyles, the infective larva of *D. viviparus* is enclosed within a sheath which comprises the cast cuticle of the second stage larvae. This sheath may be shed spontaneously by the parasite on storage, or can be removed by treatment with sodium hypochlorite, to expose the L3 cuticle. In this study the antibody responses to both sheathed and exsheathed larvae were examined and revealed that epitopes exposed on the sheath of infective larvae are strongly recognised by hosts following infection and vaccination with irradiated larvae. In contrast, there was no specific IgG antibody recognition of antigens exposed on the surface of exsheathed larvae by naturally infected hosts. Vaccination with 40krad-irradiated larvae induced a slight IgG antibody response, while immunisation with 100krad-irradiated larvae stimulated a significant specific IgG response to the L3 cuticle. A significant degree of non-specific binding of IgM antibody to the surface of exsheathed larvae was observed with pre-infection serum from all hosts examined. The contribution this may have to immune evasion and how this may be overcome by vaccination are discussed.

5.2 RESULTS

5.2.1 Surface-exposed antigens of third stage larvae

The antibody response of infected and vaccinated calves to the surface of sheathed larvae was examined by indirect immunofluorescence. This showed a significant level of antibody binding, which was evenly distributed over the surface. As can be seen in Figure 5.1, antibody binding was restricted to the L3 sheath with no penetration and binding to the L3 cuticle being observed. The bright regions of

fluorescence in the figure are due to overlapping layers of parasite tissue resulting from the coiled position of the larvae and are not due to regional specialisation of antibody binding. Fluorescence was always measured on single layers of the larval body. No fluorescence was detected with normal bovine serum.

Using X-irradiated larvae as targets there was no difference in the distribution nor level of antibody response with serum from infected or vaccinated calves. Subsequent experiments were carried out on normal larvae.

5.2.2 Kinetics of antibody response to L3 surface antigens

Calves were infected with normal larvae or vaccinated with 40krad or 100krad irradiated larvae on several occasions, as previously outlined in Table 4.1. The level of the antibody response to the L3 sheath was measured by immunofluorescence using serum collected immediately prior to each exposure to larvae. This is shown in Figure 5.2 using pooled serum from two calves in each group. In all groups the antibody level rose gradually, reaching a maximum after 2 exposures. It remained at this level after several more infections or vaccinations, then decreased gradually. Only in calves vaccinated with 100krad irradiated larvae (Panel C) did the antibody response to L3 surface antigens increase slightly after challenge infection (columns a, b, c). The kinetics of antibody response to the L3 sheath are similar to those previously observed against adult ES antigens (Chapter 4).

5.2.3 Species-specific recognition of L3 surface antigens

To examine any cross-reactivity between surface antigens of sheathed *D. viviparus* and antigens of the gastrointestinal nematodes *C. oncophora* and *O. ostertagi*, immunofluorescence was carried out using sera from calves infected with these parasites. As shown in Figure 5.3, fluorescence was detected only with serum from lungworm-infected calves. This suggests that antigens expressed on the surface of *D. viviparus* are species-specific at least between these gastrointestinal nematodes or, alternatively, that infection with these gastrointestinal nematodes does not induce a circulating antibody response to the larval sheath. There is now some evidence that the sheaths of *D. viviparus*, *H. contortus* and *C. oncophora* are antigenically similar, suggesting that the latter may be true (J. Gilliard, personal communication).

5.2.4 Antibody response to exsheathed L3

In contrast to the findings with sheathed larvae, larvae which had exsheathed spontaneously or had been exsheathed by treatment with sodium hypochlorite solution bound bovine immunoglobulin in a non-specific manner. Strong fluorescence was observed on the surface of exsheathed larvae following incubation in normal bovine serum (Figure 5.4 Panel A) and serum from calves infected (Panel B) or vaccinated with *D. viviparus* larvae.

Significant levels of fluorescence were also observed following incubation of exsheathed third stage larvae with newborn calf serum (from calves less than 10 days old) and serum from calves infected with *C. oncophora* or *O. ostertagi* (Figure 5.5). No binding was observed, however, with foetal calf serum, normal guinea-pig serum nor with FITC-anti bovine IgG conjugate alone. These findings suggested that bovine sera contained antibody which cross-reacted with determinants exposed on the surface of the L3 cuticle of *D. viviparus.* The non-specific binding observed with newborn calf serum indicated that calves were exposed to this cross-reactive antigen soon after birth or that heterophile antibody was acquired via the colostrum.

5.2.5 Presence of phos phorlycholine on L3 cuticle

Phosphorlycholine (PC) determinants on parasite surfaces have often been implicated as a cause of cross-reactivity between parasites themselves and with other infectious agents (Pery *et al.*, 1974; Maizels, Burke and Denham, 1987a). To determine whether this epitope was exposed on the surface of exsheathed L3 and was perhaps responsible for the high level of non-specific binding observed with pre-infection bovine sera, immunofluorescence was carried out using a mouse monoclonal antibody to PC (Bp-1) (Sutanto *et al.*, 1985). As can be seen in Figure 5.6, Bp-1 bound strongly to the L3 cuticle, indicating that the PC determinant is indeed present. This antibody did not bind to the surface of sheathed larvae (not shown).

To confirm whether the non-specific binding of bovine sera was due to recognition of the PC determinant, immunofluorescence was carried out in the presence of free PC or choline chloride. Both PC and choline chloride inhibited the binding of Bp-1 to the exsheathed surface at a concentration of 10^{-7}M but had no effect on the binding of normal bovine serum, even at a concentration of 10^{-1}M

(Figure 5.7). This suggested that the recognition of PC is not responsible for the binding of bovine pre-infection serum, although, of course, the difference in inibition patterns observed here may have been due to different affinities of the antibodies for the phosphorylcholine epitope.

Using a range of FITC-conjugated lectins (FITC-conjugated concanavalin A, lentil lectin, wheat germ agglutinin, peanut agglutinin and glycine max were used) no binding was observed on the surfaces of sheathed nor exsheathed larvae. This might suggest that cross-reactivity was not due to recognition of carbohydrate residues, although further studies perhaps using different lectins or treating larvae with periodate to oxidise diol rings of carbohydrate structures will be necessary to confirm this.

5.2.6 Examination of immunoglobulin isotype response to L3 cuticle

Examination of the immunoglobulin isotypes involved in the non-specific binding of bovine antibody to the surface of exsheathed larvae was carried out in an isotype-specific immunofluorescence assay using mouse monoclonal antibodies to different bovine Ig classes.The binding of these monoclonal antibodies was detected with FITC anti-mouse Ig.

Pre-treatment sera from a number of calves were assayed and significant binding of bovine IgM was observed in all cases (Figure 5.8). No binding of any other bovine Ig isotype was detected, nor was there any binding of mouse monoclonal antibodies in the absence of bovine serum.

Immunofluorescence was then carried out using sera from calves exposed to infection with normal larvae or vaccinated with larvae irradiated to 40 or 100krad. Following infection there was a slight increase in the level of IgM antibody, but no significant IgG1 nor IgG2 responses to the L3 cuticle (Figure 5.8 Panel A).

Vaccination with 40krad irradiated larvae also resulted in a slight increase in IgM above the non-specific background level. In contrast to experimentally infected calves, an IgG1 response was observed in these hosts following challenge infection with normal larvae (Figure 5.8 Panel B).

The absence of any significant response to the L3 cuticle following infection with normal larvae and limited response observed in vaccinates raised the possibility that treatment with sodium hypochlorite may have damaged the surface, destroying its antigenicity. However, calves vaccinated with 100krad irradiated

larvae and exposed to challenge infection showed a strong specific IgG1 response to the surface of exsheathed larvae (Figure 5.8 Panel C) thus demonstrating the antigenicity of the exposed L3 cuticle.

5.2.7 Immunoglobulin isotype response to L3 sheath antigens

In contrast to exsheathed larvae, there was no non-specific binding of any bovine Ig isotype to the L3 sheath. Infection with normal larvae and vaccination with 40 or 100krad irradiated larvae induced a significant IgG1 response to the surface of sheathed larvae. No significant IgM or IgG2 responses were observed following these treatments (Figure 5.9).

In the guinea-pig host, the pattern of antibody response to sheathed and exsheathed larvae was similar to that observed with bovine hosts. Infection or vaccination induced very little response to the L3 cuticle, while a significant and specific antibody response to the L3 sheath was detected (Figure 5.10).

5.2.8 Differential insertion of lipid probe

That the surface of exsheathed larvae is different not only antigenically but biophysically from that of sheathed larvae, was demonstrated by the differential insertion of the fluorescent lipid analogue 5-(*N*-octadecanoyl)aminofluorescein (AF18) into the parasite cuticle. This 18 carbon aliphatic chain lipid was taken up by all stages of *D. viviparus* examined, with the exception of the exsheathed L3 stage (Figure 5.11). This pattern was unchanged when tested on exsheathed larvae which had been maintained in culture for one week.

Although first stage larvae showed an affinity for AF18, this was lower than that of the egg and sheathed L3 stages.

TABLE 5.1 Abbreviations for antisera used in immunofluorescence

SOURCE	IMMUNISATION	ROUTE	ABBREVIATION
Calf	none (normal serum)	-	NBovS
	normal D. viviparus L3	oral	BovNL3
	40krad D. viviparus L3	oral	Bov40
	100krad D. viviparus L3	intraperitoneal	Bov100
	O.ostertagi L3	oral	BovOo
	C.oncophora L3	oral	BovCo
Guinea-pig	none (normal serum)	-	NGPS
	normal D. viviparus L3	oral	GPNL3
	40krad D. viviparus L3	oral	GP40
	100krad D. viviparus L3	intraperitoneal	GP100
Rabbit	none (normal serum)	-	NRS
	adult ES material	adjuvant	RâES
Mouse	none (normal serum)	-	NMS
	adult B. pahangi (Sutanto et al., 1985)		Bp-1

Figure 5.1 Antigenicity of sheathed L3 surface. The bovine antibody response to the surface of sheathed larvae following two experimental infections was detected by a FITC-anti-bovine IgG conjugate. Larvae were viewed under bright field (A) and U.V. light (B) with a x40 objective lens.

A

B

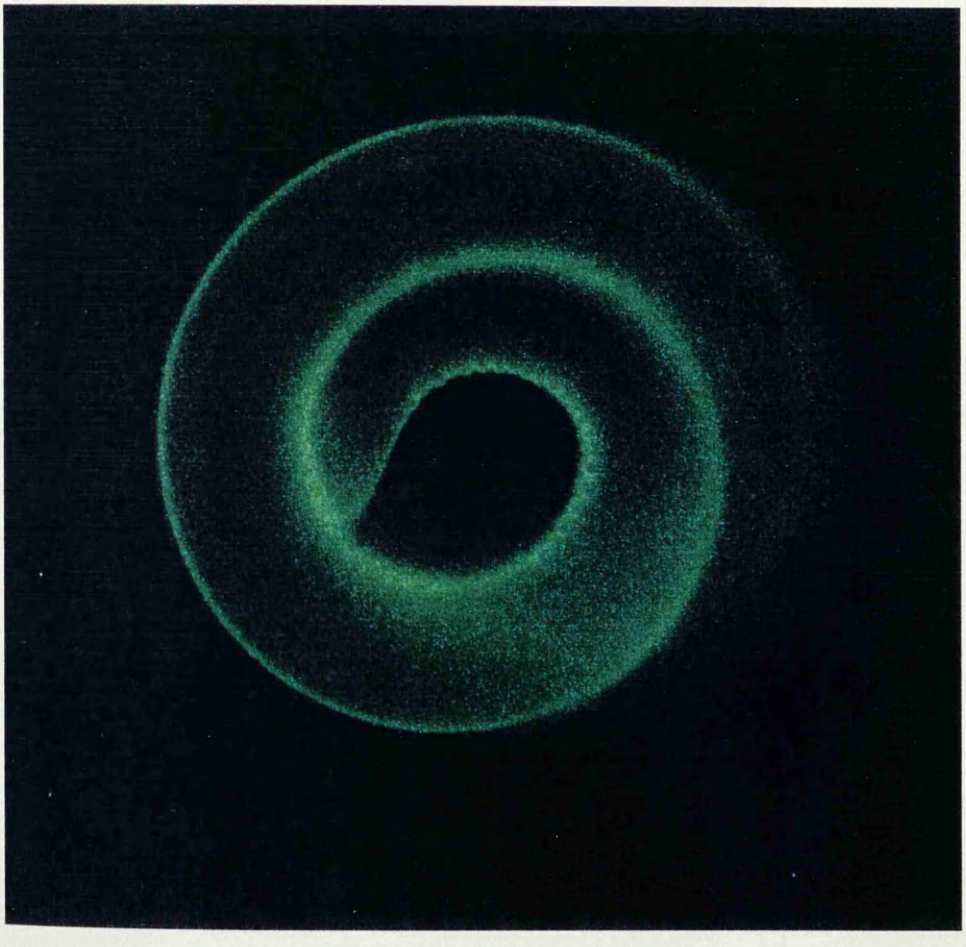
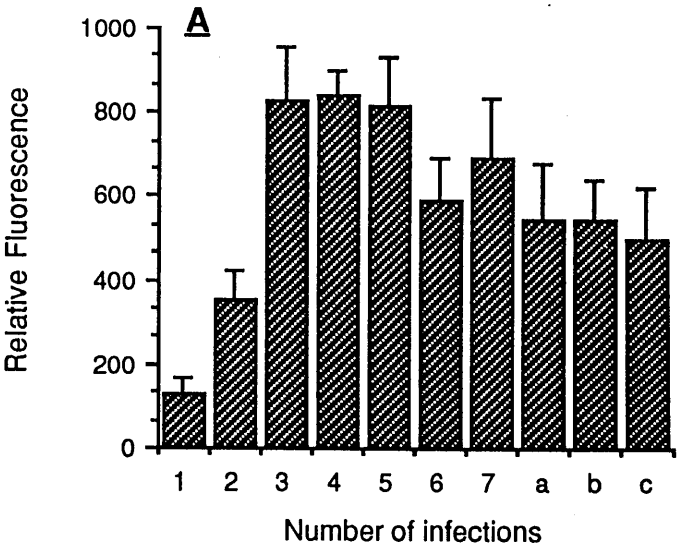
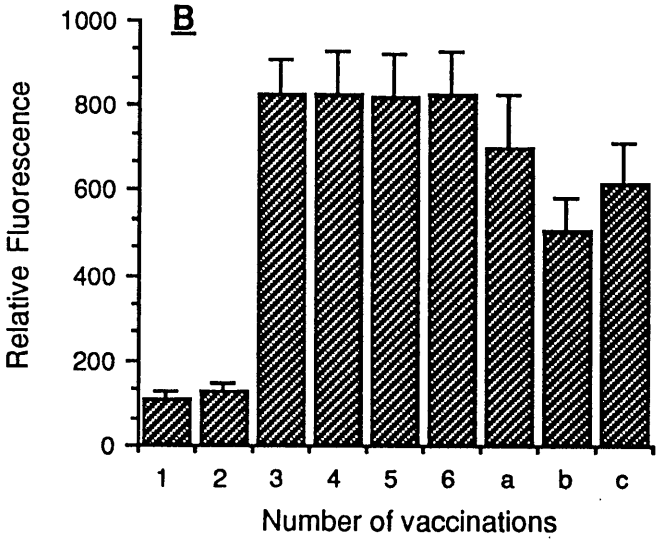


Figure 5.2 Kinetics of antibody response to L3 surface-exposed antigens. Calves were infected with normal (A), 40krad irradiated (B) or 100krad irradiated (C) larvae, as described in Table 4.1. Larvae were administered at each of the numbered points. Serum was sampled prior to infection or vaccination and at 7 (column a), 14 (column b) and 21 (column c) days after a challenge infection, administered 24 days after the previous infection or vaccination. The level of fluorescence was quantitated as described in Chapter 2. These graphs show the response to L3 surface antigens using pooled serum from two calves in each group. The mean fluorescence of 25 measurements is shown per data point, with error bars representing the S.D. of the observations.


A
Relative Fluorescence
1000
800
600
400
200
0
1 2 3 4 5 6 7 a b c
Number of infections


B
Relative Fluorescence
1000
800
600
400
200
0
1 2 3 4 5 6 a b c
Number of vaccinations

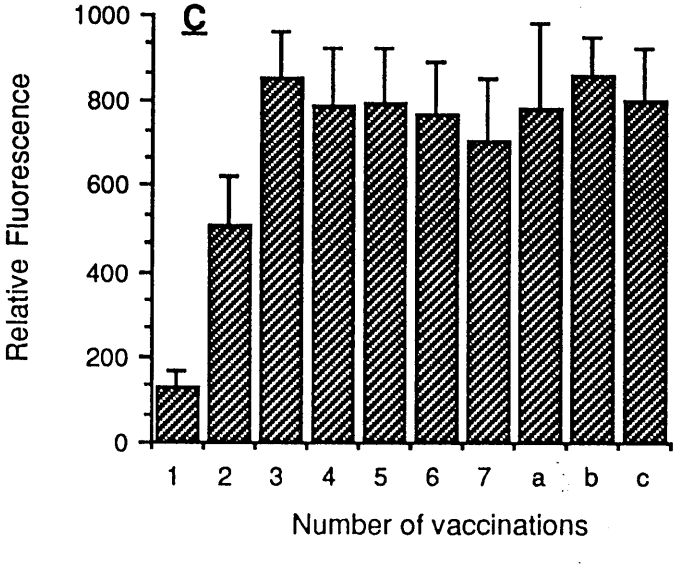

C
Relative Fluorescence
1000
800
600
400
200
0
1 2 3 4 5 6 7 a b c
Number of vaccinations

Figure 5.3 Species-specific recognition of L3 surface antigens. Immunofluorescence was carried out on sheathed larvae using normal bovine serum, or serum from calves infected on two occasions with third stage larvae of *C. oncophora*, *O. ostertagi* or *D. viviparus*. Abbreviations for the antisera tested are explained in Table 5.1.

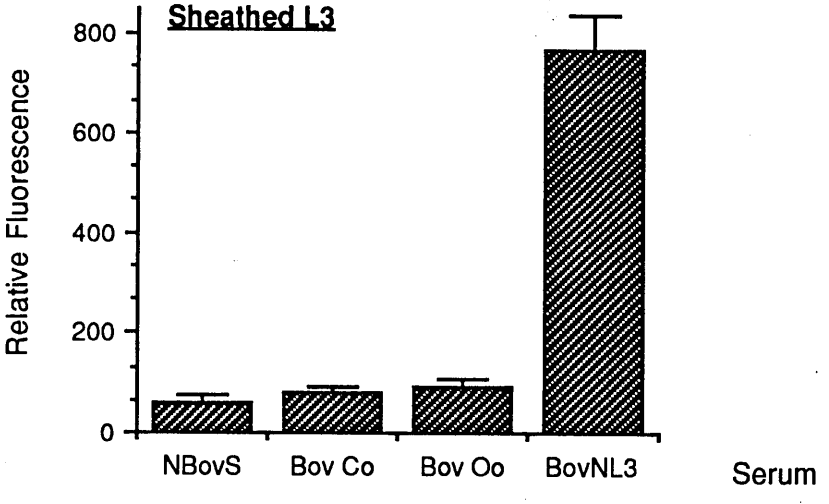
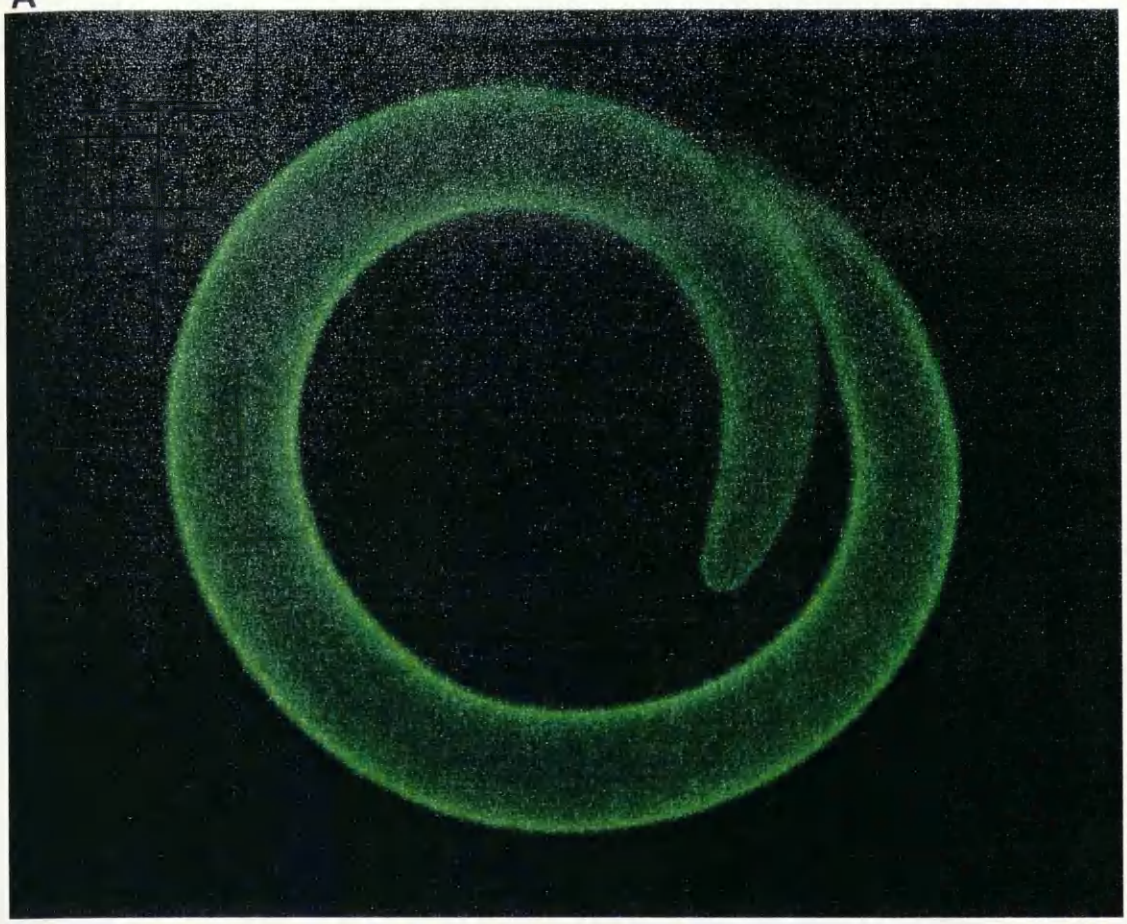

Sheathed L3
Relative Fluorescence
800
600
400
200
0
NBovS
Bov Co
Bov Oo
BovNL3
Serum

Figure 5.4 Fluorescence on the L3 cuticle with normal and anti-*D. viviparus* bovine serum. L3 parasites were exsheathed by treatment with sodium hypochlorite and incubated with pre-infection serum (Panel A) and serum from calves infected on two occasions with 1,000 normal larvae (B). Antibody binding was detected by further incubation in FITC-conjugated anti-bovine IgG. Larvae were viewed under U.V. light at x40 magnification.

A



B

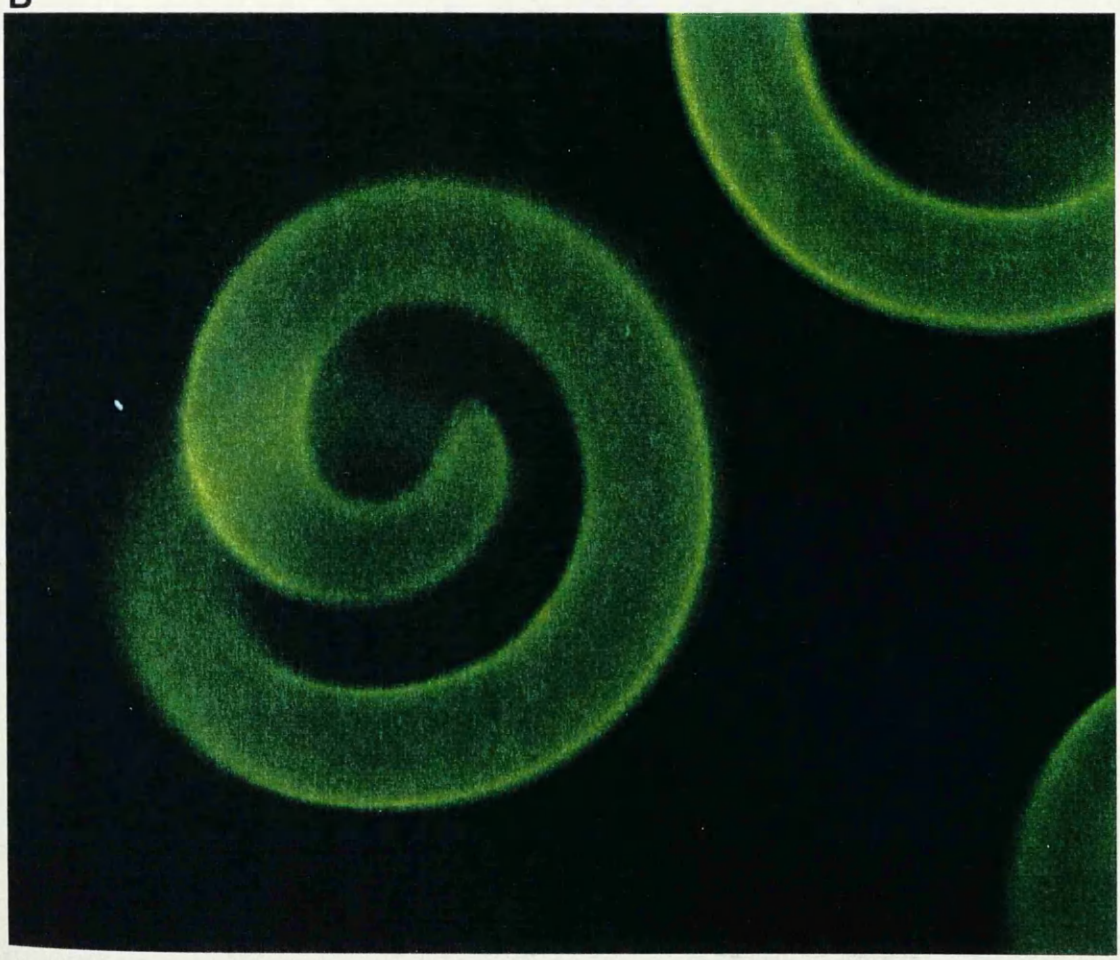


Figure 5.5 Non-specific binding of antibody to the L3 cuticle. Immunofluorescence was carried out on exsheathed larvae using no serum (FITC anti-bovine conjugate alone), normal guinea-pig serum, foetal calf serum, normal bovine serum or serum from calves infected on two occasions with *O. ostertagi*, *C. oncophora* or *D. viviparus*. The level of fluorescence was measured by photon counting. The means of 25 observations are shown with error bars representing the S.D. of these. See Table 5.1 for abbreviations of antisera tested.

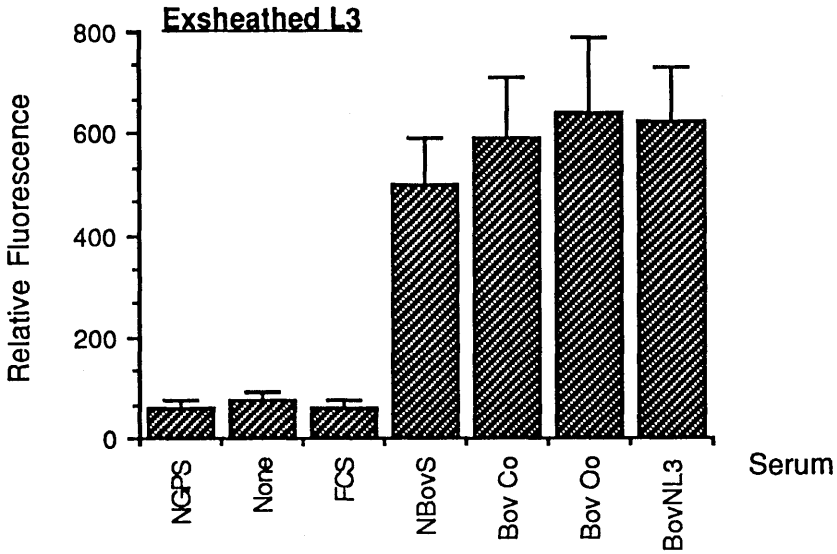

Exsheathed L3
Relative Fluorescence
800
600
400
200
0
NGPS
None
FCS
NBovS
Bov Co
Bov Oo
BovNL3
Serum

Figure 5.6 Presence of phosphorylcholine on the L3 cuticle. Exsheathed larvae were incubated with normal mouse serum, normal bovine serum and a mouse monoclonal antibody to phosphorylcholine (Bp-1). Antibody binding was detected with FITC anti-species IgG conjugates and quantitated as before. See Table 5.1 for abbreviations.

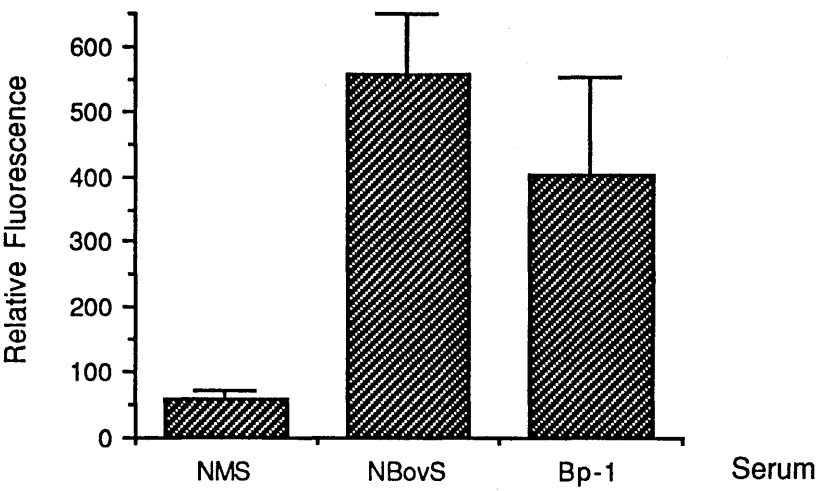

Relative Fluorescence
600
500
400
300
200
100
0
NMS
NBovS
Bp-1
Serum

Figure 5.7 Inhibition of Bp-1 binding to the L3 cuticle. Immunofluorescence was carried out on exsheathed larvae using normal bovine serum (NBovS) and Bp-1 in the absence (a) and presence of 10^{-1}M (b) and 10^{-3}M (c) choline chloride.

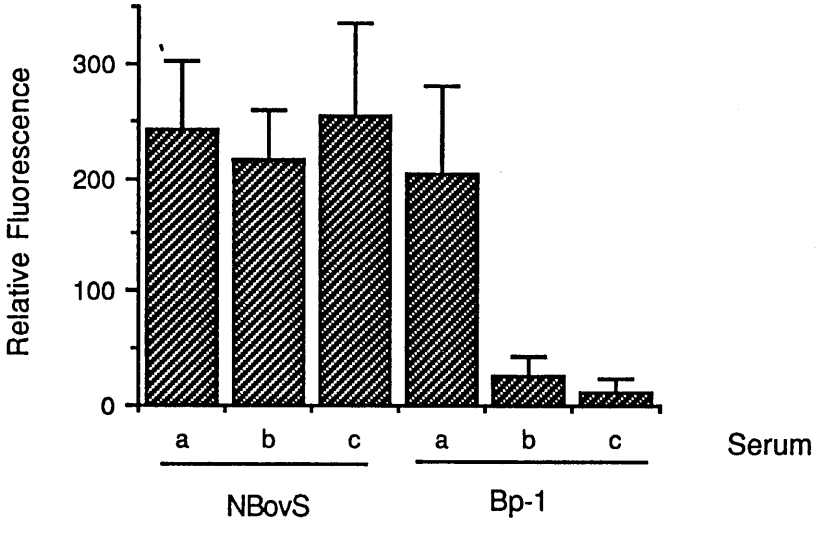
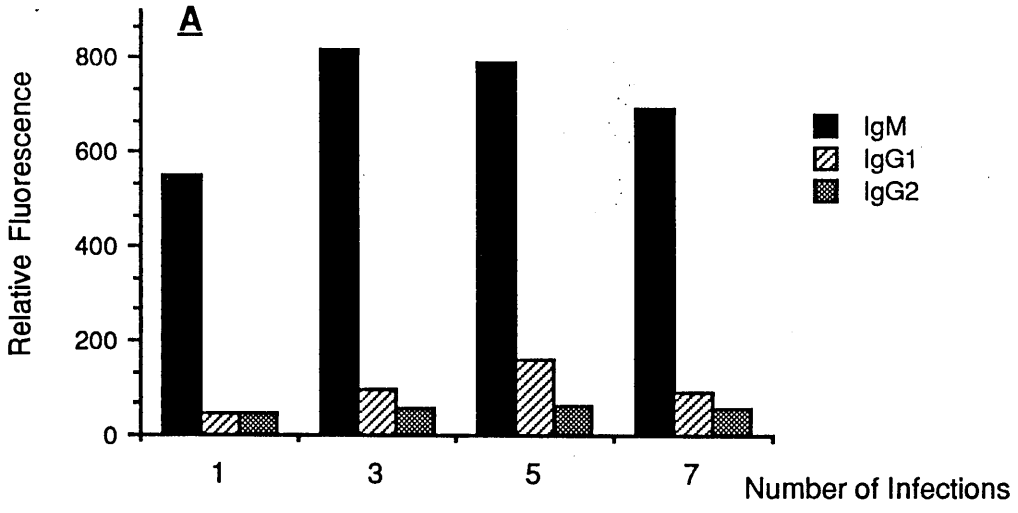
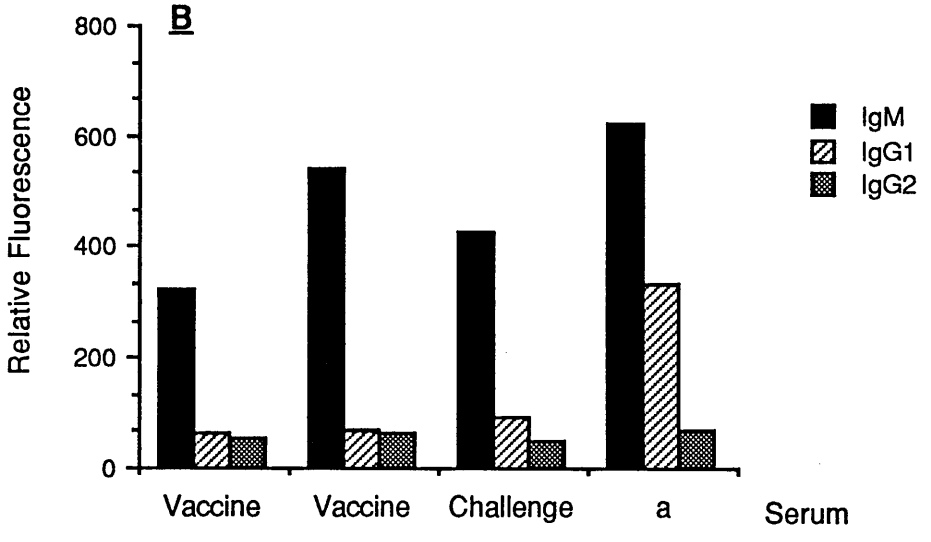

Relative Fluorescence
300
200
100
0
a
b
c
a
b
c
Serum
NBovS
Bp-1

Figure 5.8 Non-specific binding of IgM to the exsheathed L3 surface. Pooled serum from two calves exposed to normal (A), 40krad irradiated (B) or 100krad irradiated (C) larvae was incubated with exsheathed larvae. Calves in Panel A were infected as described in Table 4.1. and the numbers refer to the infection number (i.e. first, third etc.). Calves in Panels B and C were vaccinated on two occasions, 21 days apart, with 1,000 40krad irradiated larvae orally (Panel B) or 2,500 100krad irradiated L3 i.v. (Panel C) and challenged with 2,000 normal larvae 21 days later. Serum was sampled prior to each infection or vaccination, immediately prior to challenge infection (challenge) and 28 days after challenge (a). Binding of different bovine Ig isotypes was detected using mouse monoclonal antibodies to bovine Ig classes and FITC anti-mouse IgG.


A
800
600
400
200
0
Relative Fluorescence
1
3
5
7
Number of Infections
IgM
IgG1
IgG2


B
800
600
400
200
0
Relative Fluorescence
Vaccine
Vaccine
Challenge
a
Serum
IgM
IgG1
IgG2

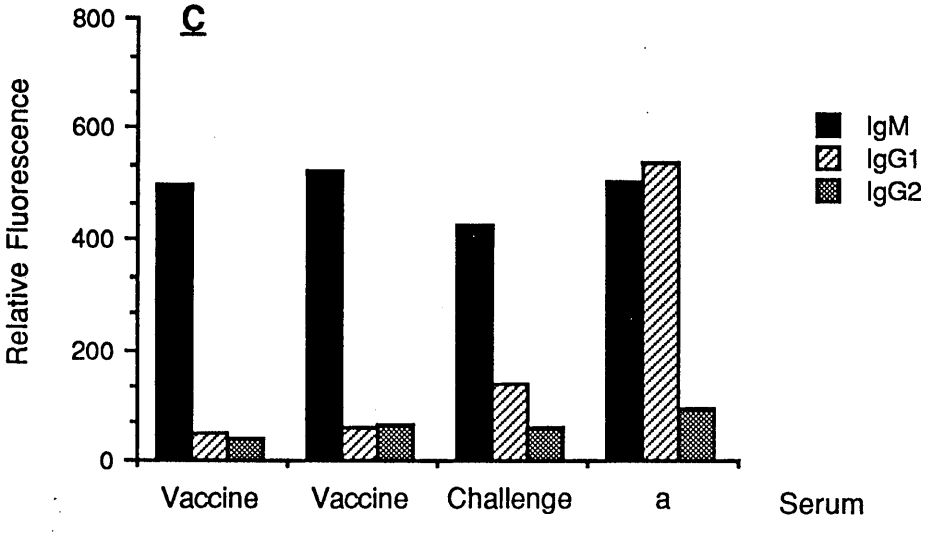
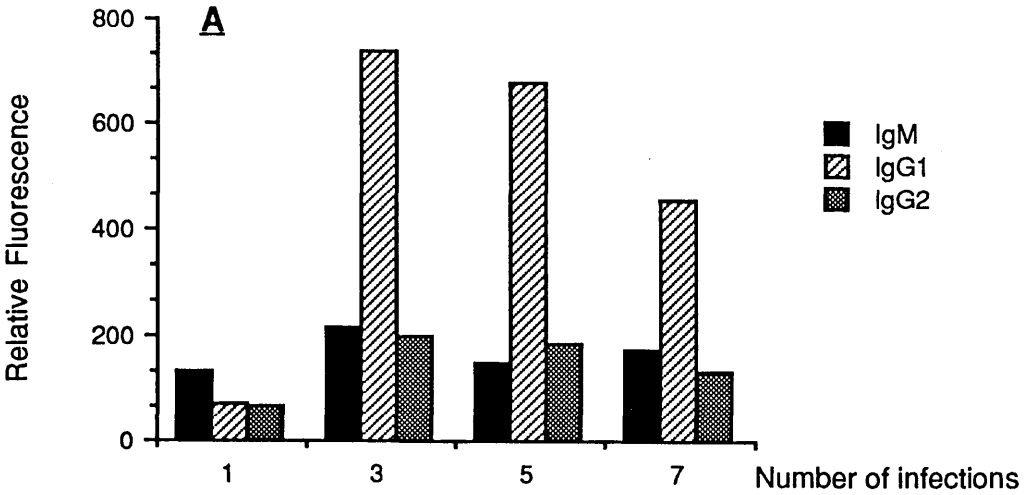
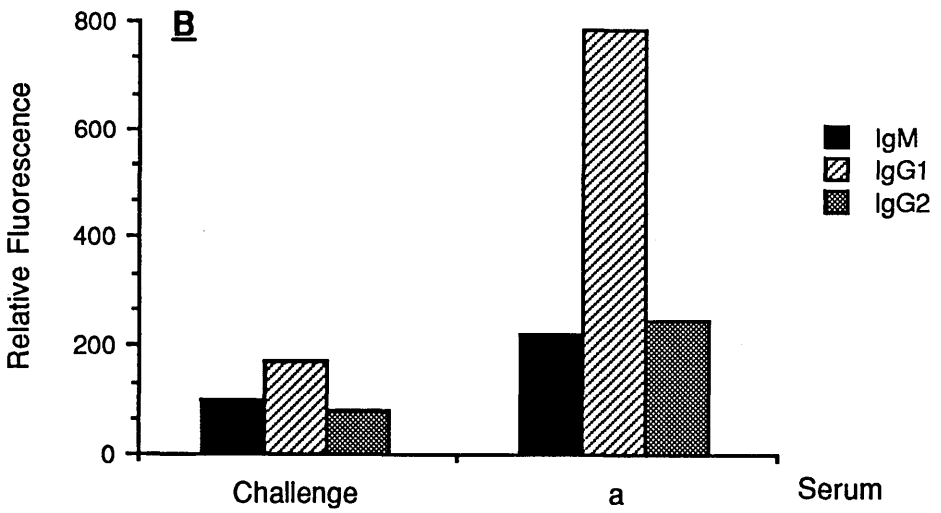

C
800
600
400
200
0
Relative Fluorescence
Vaccine
Vaccine
Challenge
a
Serum
IgM
IgG1
IgG2

Figure 5.9 Specific recognition of sheath antigens following infection and vaccination. The binding of different bovine isotypes to the surface of sheathed larvae was examined using serum from calves exposed to normal (A), 40krad irradiated (B) or 100krad irradiated (C) larvae and fluorescence quantitated as before. Infections, vaccinations and challenge infections were carried out as described in Figure 5.8 and serum was sampled prior to each exposure to larvae.


A
800
600
400
200
0
Relative Fluorescence
IgM
IgG1
IgG2
1
3
5
7
Number of infections


B
800
600
400
200
0
Relative Fluorescence
IgM
IgG1
IgG2
Challenge
a
Serum

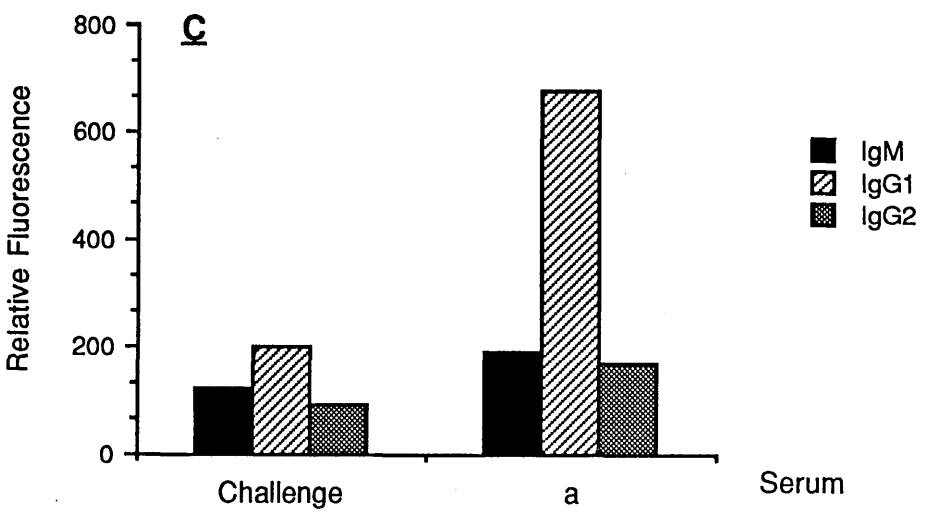

C
800
600
400
200
0
Relative Fluorescence
IgM
IgG1
IgG2
Challenge
a
Serum

Figure 5.10 Recognition of surface-exposed antigens of sheathed and exsheathed larvae by guinea-pigs following infection and vaccination. Immunofluorescence was carried out on sheathed and exsheathed larvae using normal guinea-pig serum and serum from guinea-pigs exposed on 2 occasions, 21 days apart, to normal or 40krad irradiated larvae. They were challenged with normal larvae 21 days later and serum sampled 7 days after challenge. See Table 5.1 for abbreviations of serum used.

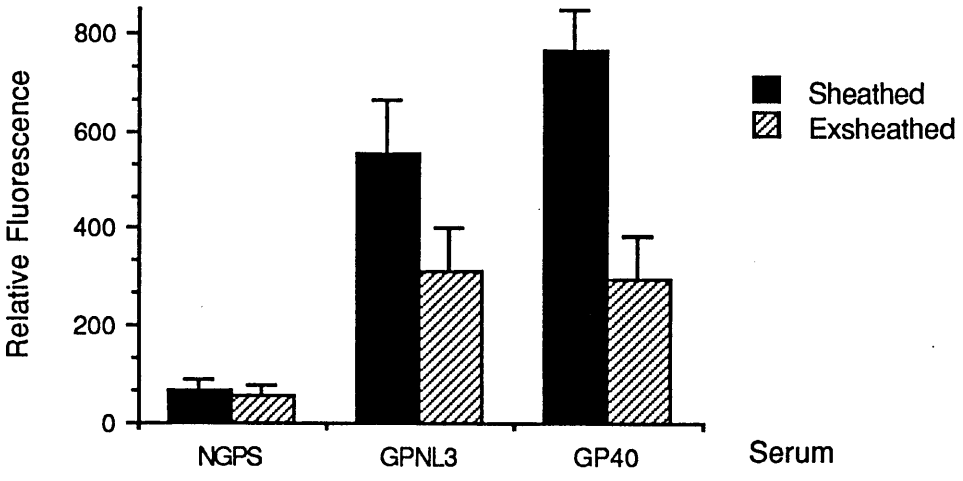
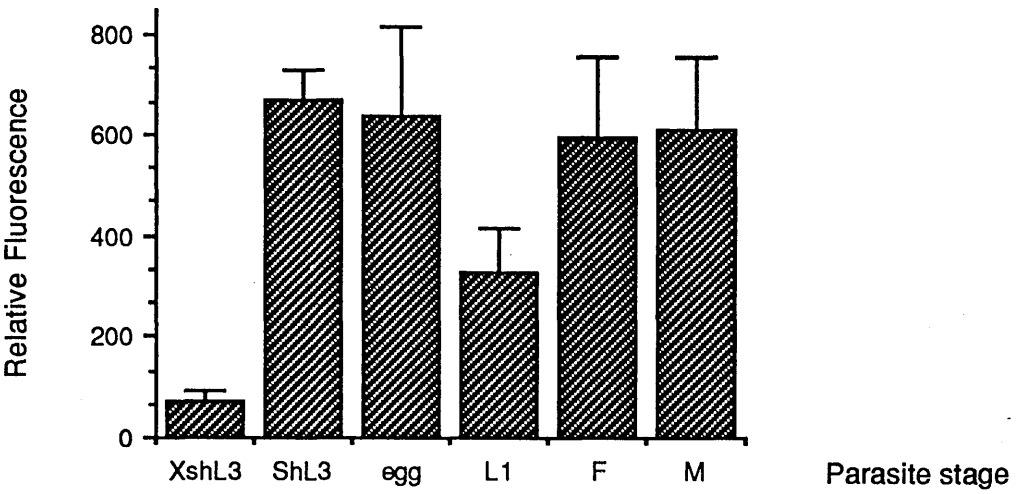

Relative Fluorescence
800
600
400
200
0
NGPS
GPNL3
GP40
Serum
Sheathed
Exsheathed

Figure 5.11 Differential uptake of lipid probe AF18 by different stages of *D. viviparus*. An aminofluorescein labelled C18 lipid probe (AF18) was incubated with exsheathed larvae (XshL3), sheathed L3 (ShL3), egg, L1, and male (M) and female (F) adult stages of *D. viviparus* as described in Chapter 2. The insertion of the lipid probe into the parasite surface was measured by photon counting, as before. The L3, egg and L1 stages were viewed at x40 magnification and adult parasites viewed at x10


Relative Fluorescence
800
600
400
200
0
XshL3
ShL3
egg
L1
F
M
Parasite stage

5.3 DISCUSSION

The exposed parasite surface represents the interface between the parasite and the host environment. Current thinking on its dynamic and antigenic nature has led to increasing attention in defining its role in host-parasite interactions. Immune recognition of surface antigens may be important in parasite killing (Mackenzie *et al.*, 1978) and identification of these antigens will, therefore, be potentially relevant to the design of anti-parasite vaccines. Alternatively, shedding of surface antigens or masking of surface determinants by the acquisition of host components (McLaren, 1984) may represent important parasite evasion mechanisms. In the present study the exposed surfaces of sheathed and exsheathed third stage larvae of *D. viviparus* were examined.

By immunofluorescence it has been demonstrated that the sheath of *D. viviparus* infective larvae is antigenic and exposed antigens are strongly recognised by both infected and vaccinated hosts. It is surprising that a structure which is generally thought to be cast from the surface of these larvae prior to their penetration of the intestinal wall and entry into the circulation, should elicit such a substantial antibody response. However, there is, as yet, no evidence as to where exsheathment of *D. viviparus* larvae occurs. Several studies have demonstrated the penetration of host skin tissue by ensheathed larvae of *Ancylostoma tubaeforme* (Matthews, 1972; Matthews, 1975) and , more recently, it has been shown that *A. duodenale* sheathed larvae can penetrate skin tissue to some extent (Hotez *et al.*, 1990). Therefore, the possibility that *D. viviparus* larvae may also be able to penetrate host tissue with the sheath still attached cannot be eliminated.

It has been speculated that nematode exsheathment may be associated with the release of enzymes involved in the penetration of host tissue (B.E. Matthews, personal communication). This was suggested by studies of the human hookworm *N. americanus* which exsheathes prior to skin penetration and also releases enzymes thought to be necessary for host invasion. In contrast, the aforementioned hookworm of cats, *A. tubaeforme*, does not exsheath prior to host invasion nor does it appear to depend on the release of proteolytic enzymes for skin penetration. Although it has been shown that proteolytic activity is released by sheathed *D. viviparus in vitro* (see Chapter 8) examination of the biological role of this enzyme activity and its

association with the exsheathing process have been limited by the difficulty in obtaining sufficient quantities of secreted products of this stage.

An alternative explanation for the substantial anti-sheath antibody response may be cross-reactivity of antigens released by later parasite stages with antigens exposed on the L3 sheath. Evidence that this may be true has come from our studies in guinea-pigs, where those infected with exsheathed larvae were capable of recognising antigens on the L3 sheath at a similar level to those infected with sheathed larvae (data not shown). However, it was difficult to ensure that all the "exsheathed" larvae administered were fully exsheathed or that no fragments of sheath were present in the infecting preparation. Monoclonal antibodies have now been prepared against exposed sheath antigens and will be useful in demonstrating whether such antigens are restricted to the sheath alone.

A significant antibody response to the sheath of *D. viviparus* infective larvae was observed soon after vaccination or infection (Figure 5.2), but whether this response is relevant to immunity has not yet been established. Grencis *et al.* (1986) demonstrated immunity to *T. spiralis* could be induced by immunisation of mice with material selectively removed from the surface of muscle stage larvae with the cationic detergent CTAB. However, we have been unable to remove surface antigens of sheathed *D. viviparus* as indicated by the absence of components on silver-stained SDS-gels following incubation of larvae in a variety of detergents including CTAB, DOC and Triton X-100. Passive immunisation studies using anti-sheath monoclonal antibodies may, therefore, be useful in assessing the role of sheath antigens in immunity to *D. viviparus*.

It has been demonstrated that the acquisition of anti-sheath antibodies correlates with the clearance of *Brugia malayi* microfilariae from the host circulation (McGreevy *et al.*, 1980). However, the mechanism underlying this parasite clearance has not been defined and could be a result of protective mechanisms against other parasite stages (Haque *et al.*, 1978).

In contrast to sheath antigens, there is almost no recognition of L3 cuticular antigens following infection (Figure 5.8). No difference in binding was observed using larvae which had been exsheathed and maintained *in vitro* for one week. This suggests that the lack of antibody binding to the surface of freshly exsheathed larvae is not due to a delay in expression of antigens on the parasite surface, as has been demonstrated with infective larvae of *Toxocara canis* and *T. cati* (Kennedy *et al.*,

1987b; Maizels *et al.*, 1987b), although it does not exclude the possibility that expression of L3 cuticular antigens is activated by entry into the vertebrate host. However, the specific binding of antibody to the L3 cuticle observed using serum from calves exposed to 40krad and particularly to 100krad irradiated larvae and challenge infection indicates that antigens are present and exposed on the surface of exsheathed L3. This was also indicated by the specific binding of guinea-pig immune serum to exsheathed larvae, although the level of binding was significantly lower than that observed against sheathed larvae (Figure 5.10).

Why then does vaccination with larvae irradiated to 100krad stimulate a significant response to the L3 cuticle while 40krad irradiated larvae induce only a slight response and normal larvae apparently stimulate no antibody reaction? This may be explained by differences in the properties of normal and irradiated larvae. It has previously been found that 100krad irradiated larvae do not develop beyond the L3 stage (Canto, 1990). It is possible that death of such larvae results in the release of antigens which cross-react with those exposed on the L3 cuticle. The lower antibody response to the L3 surface following vaccination with 40krad irradiated larvae and the absence of any such response following natural infection could then be due to decreasing levels of these cross-reactive antigens in the L4, L5 and adult stages.

An alternative explanation for the greater response in 100krad vaccinates may be prolonged exposure of surface antigens by irradiated larvae. As 100krad larvae do not develop beyond the L3 stage antigens present on their surface will be exposed to the host for a longer period of time than L3 antigens of normal or 40krad irradiated larvae which are capable of further development. As no difference was observed in the level of anti-sheath antibody between the different groups, this suggests that 100krad larvae exsheath at the same rate as normal larvae and it is the L3 cuticle which is exposed.

The lower, but significant, response to L3 cuticular antigens following challenge infection of 40krad vaccinates may also be explained by a slower rate of development of 40krad irradiated larvae to the L4 stage relative to normal larvae. While we have no evidence that irradiation to 40krad affects the rate of *D. viviparus* development, the rate of development of other nematodes has been reported to be affected by irradiation (Oothuman *et al.*,1978). The greater recognition of L3 cuticular antigens by vaccinated hosts observed in this study

might suggest that the success of the X-irradiated vaccine may be due to prolonged exposure to the exsheathed L3 stage. As such antigens are poorly recognised following exposure to normal larvae they are unlikely to be responsible for the protective immunity resulting from natural infection. Recognition of L3 cuticular antigens may, however, be relevant to the success of the irradiated larval vaccine by limiting the number of larvae reaching the lungs and so reducing the pathogenicity of lungworm infection.

The absence of any boost in antibody response to exsheathed L3 surface antigens even after six infections with normal larvae suggests that naturally infected hosts are never exposed or are exposed too briefly to L3 cuticular antigens. This would, therefore, indicate that throughout the normal duration of the L3 stage, host responses to L3 surface antigens are suppressed. One of the ways in which this immune evasion may be mediated has been indicated by the binding of IgM antibody from bovine pre-treatment sera to the L3 cuticle. Non-specific binding occurred with all bovine sera examined, including newborn calf serum, indicating the presence of antigen(s) on the surface of *D. viviparus* L3 which cross-react with an antigen to which calves are commonly exposed. It was speculated that non-specific antibody binding may have been due to the presence of PC on the L3 cuticle. This determinant is widely distributed in nature (Crandall and Crandall, 1971; Pery *et al.*, 1974; Gutman and Mitchell, 1977; Maizels *et al.*, 1987a; Shepherd and McManus, 1987) and is thought to be responsible for much of the cross-reactivity observed amongst nematodes and other organisms. However, binding of normal bovine sera to the L3 cuticle was not reduced in the presence of free phosphorylcholine or choline chloride, at least at the concentrations used here. Therefore, the non-specific binding of bovine antibody cannot be explained by the presence of phosphorylcholine. At present the nature of this cross-reactivity is unknown.

PC may, nevertheless, be involved in suppressing the antibody response to surface antigens of exsheathed larvae. It has been suggested that this determinant is produced by parasites to induce a state of immunosuppression, through its ability to inhibit the production of high affinity antibodies to parasite antigens but promote the production of antibodies of low affinity, usually of the IgM isotype (Mitchell *et al.*,1977).

It is possible that both PC and non-specific binding of IgM operate to prevent

recognition of *D. viviparus* L3 cuticular antigens. IgM antibody may mask antigenic epitopes exposed on the surface or prevent antibodies of other specificities from binding due to steric hinderance. It has been found that "blocking" antibodies of the IgM and IgG2 classes prevent cell adherence to the schistosomula surface of *S. mansoni* and are associated with susceptibility to reinfection in humans (Butterworth *et al.*, 1987). The levels of these blocking antibodies were found to decrease with time, thus allowing the expression of protective immune responses. A similar mechanism may operate in the immune recognition of *D. viviparus* L3 cuticular antigens. We have found that naturally infected hosts, which showed a consistently high IgM antibody level, displayed the lowest IgG antibody response to the L3 cuticle. In contrast, the slight decrease in IgM level observed during the course of vaccination may have been responsible for the induction of the IgG1 response to L3 cuticular antigens in these hosts. Whether IgM blocking antibody is induced by phoshorylcholine is as yet unkown, but may indicate the involvement of this determinant in immune evasion by *D. viviparus* infective stage larvae.

A simple explanation for the observed differences in Ig isotype response to the L3 cuticle could be competitive binding of different immunoglobulins in the immunofluorescence assay. This seems unlikely, however, as the IgM antibody levels of calves exposed to normal or irradiated larvae were very similar (Figure 5.8), yet the level of IgG1 antibody response to L3 cuticle was significantly greater in vaccinated hosts. This suggests that IgM does not inhibit the binding of IgG1 to the larval surface. This could be confirmed by performing immunofluorescence assays with individual immunoglobulin isotypes purified from bovine antiserum.

That the surface of exsheathed larvae differs not only antigenically but also biophysically from that of sheathed larvae and of other stages of *D. viviparus* was demonstrated by its lack of affinity for the lipid analogue AF18. Although this study was limited to examining the insertion of only one lipid analogue into the parasite surface, it has been demonstrated that the exsheathed infective stages of other parasitic nematodes display the same phenomenon (Proudfoot *et al.*, 1990). It is speculated that this is due to changes in the lipid composition or reorganisation of the surface of this parasite stage. It would be interesting to determine whether these alterations are necessary in the transition to the parasitic state or represent a parasite mechanism to avoid immune recognition. A better understanding of these changes in surface structure may provide important information on parasite

survival within the vertebrate host. Although first stage larvae showed some affinity for AF18, this was lower than that of the egg and sheathed L3 stage. This suggests that changes in the structure of the parasite surface may also occur in the transition from the parasitic to the free-living stage. First stage larvae were collected from adult parasites *in vitro* and maintained at 37°C, indicating that changes in surface properties were not directly caused by alterations in environmental conditions but may be controlled by the parasite itself.

In conclusion, significant antibody responses to antigens exposed on the sheath of *D. viviparus* infective stage larvae have been demonstrated. In contrast, exsheathed larvae appear to avoid immune recognition by the binding of non-specific antibody and perhaps also by immune suppression, possibly mediated by PC determinants. It is speculated that vaccination with irradiated larvae may overcome this evasion by allowing prolonged exposure of L3 surface antigens. Examination of the antigens present on the L3 cuticle and further studies on the biophysical properties of this surface may, therefore, be important in understanding how this immune evasion is mediated and in identifying antigens which may be responsible for limiting parasite survival in vaccinated hosts.

CHAPTER 6

STAGE-SPECIFICITY AND CHARACTERISATION OF *D. VIVIPARUS* SURFACE ANTIGENS

6.1 INTRODUCTION

The exterior covering, or cuticle, of nematode parasites is a highly complex acellular structure. The cuticle is composed of three layers, or zones, referred to as the basal, median and cortical in order of distance from the hypodermis (Bird, 1971, 1984; Inglis, 1983). It is difficult to characterise the layers in terms of functional, structural or biochemical properties as layers with the same characteristics may not always occupy the same topographical position, or several zones may show identical properties (Rudin, 1990). However, it is generally thought that collagens, the major structural components of the cuticle (Cox and Hirsh, 1985), are localised primarily to the basal and internal cortical layers which often appear highly ordered in structure (Leushner, Semple and Pasternak, 1979; Cox, Kusch and Edgar, 1981a). The epicuticle is the layer of the cortical zone exposed to the external environment (Bird, 1980) and appears to be composed of covalently cross-linked insoluble proteins, termed 'cuticulin' (Fujimoto and Kanaya, 1973). In some nematode species additional material, referred to as the glycocalyx or coat, has been observed to be attached to the epicuticle (Bird, 1980; Franz, 1988; Maizels and Selkirk, 1988).

The nematode cuticle is thought to fulfil many different functions which depend on the stage of the life-cycle and on the environmental conditions, as reflected by changes in the cuticle as the parasite develops and migrates to different sites. Nematode species shed their entire cuticle four times in their life cycle at each moult, with a new cuticle being synthesised by the underlying hypodermis (Bird, 1971).

That the expression of antigens exposed on the nematode cuticle could change during development, was first demonstrated by MacKenzie *et al.* (1978), who found that adsorption of infection sera with *T. spiralis* adult worms removed antibody to this stage while antibody adherence to infective larvae was unaffected. This stage-specific recognition of surface antigens was reflected by the completely different set of antigens present on the surface of different stages of the parasite, as indicated by radiolabelling studies (Philipp *et al.*, 1980; Philipp *et al.*, 1981; Parkhouse, Philipp and Ogilvie, 1981.)

Stage-specific antigens have now been identified on the surface of a number of

nematodes, including *Nippostrongylus brasiliensis* (Maizels *et al.*,1983a), *Nematospiroides dubius* (Pritchard *et al.*, 1984b), *Necator americanus* (Pritchard *et al.*, 1986) and *Haemonchus contortus* (Cox *et al.*, 1989). While surface antigens from different stages of filarial nematodes have been shown to cross-react (Maizels *et al.*, 1983b), a degree of stage-specificity has been observed with some species (Abraham, Grieve and Mika-Grieve, 1988; Lustigman *et al.*, 1990; Selkirk *et al.*, 1990). In contrast to nematode parasites, schistosomes are thought to change the composition of surface lipids, not surface proteins, as they grow from the schistosomula to the adult stage (Rumjaneck and McLaren, 1981).

It is now apparent that changes in nematode surface proteins can also occur within a single stage. Modifications in the surface protein composition of *T. spiralis* newborn larvae (Jungery, Clark and Parkhouse,.1983), *N. brasiliensis* infective larvae (Maizels *et al.*, 1983a) and microfilariae (Furham *et al.*, 1987) and infective larvae (Carlow *et al.*, 1987) of *Brugia malayi* have been demonstrated in the absence of any moult.

As mentioned previously, changes in surface antigen expression appear to occur at the same time a⌣ alterations in parasite environment and may reflect shifts in parasite metabolism as their needs and environment change. This can be demonstrated by comparing the changes in *N. brasiliensis* surface antigens as it migrates from the lungs to the intestine (Maizels *et al.*, 1983a) with the more static expression of surface antigens of *Litomosoides carinii* whose development is confined to the pleural cavity of the host (Philipp *et al.*, 1983). In view of this, it might be expected that migration of *D. viviparus* from the intestine to the lungs would be accompanied by changes in the expression of surface proteins. It has already been established that the exposed surfaces of the L3 sheath and cuticle differ markedly (Chapter 5). In this study the surface antigenicity of sheathed L3 and adult stages of *D. viviparus* are compared. Such studies may be useful in identifying which parasite stages are involved in immunity, as well as being relevant to the developmental control of *D. viviparus* antigens in general.

Surface antigens of the egg and L1 stages are also examined. *D. viviparus* lay larvated eggs which hatch either in the respiratory or alimentary tracts. It might be predicted that as the egg and L1 stages do not penetrate host tissue there would be little likelihood of an antibody response to their surfaces. However, part of the

pathology of lungworm infection involves the formation of granulomatous nodules around eggs and first stage larvae which become trapped in the alveoli and an immune response to antigens of these stages may, consequently, be relevant to the development of this granulomatous reaction.

By immunofluorescence the antigenicity of the exposed surface of various developmental stages of *D. viviparus* was demonstrated. The differences in immune recognition of surface-exposed antigens by infected and vaccinated hosts indicated that these are expressed in a stage-specific manner. Surface antigens of the infective and adult stages were subsequently identified and characterised by radiolabelling techniques.

6.2 RESULTS

6.2.1 Antibody response to surface-exposed antigens of adult *D. viviparus*

The antibody response of infected and vaccinated calves to antigens exposed on the surface of adult worms was examined by immunofluorescence. Adult worms were cultured in RPMI overnight to deplete any host material which may have attached to their surface. They were then incubated with sera from calves infected on three occasions with normal larvae or vaccinated three times with 40krad or 100krad irradiated larvae as detailed in Table 4.1. Antibody binding to the parasite surface was detected by incubating the worms in FITC-anti-bovine IgG and viewing them under U.V. light. Following incubation in sera from infected calves, strong fluorescence was observed on the parasite surface (Figure 6.1A). No fluorescence was detected on the surface of the worm following incubation in normal bovine serum or serum from hosts vaccinated with irradiated larvae (Figure 6.1B) although the parasites showed a degree of autofluorescence internally even in the absence of host antibody or FITC conjugate. Fluorescence was quantitated and compared with that observed on the surface of sheathed infective stage larvae using the same serum (detailed in Chapter 5 and shown in Figure 6.2 Panel A). As can be seen in Figure 6.2 Panel B, only those calves infected with normal larvae and exposed to patent lungworm infections recognised the adult surface, while an antibody response to the surface of the L3 stage was observed with serum from both infected and vaccinated calves. This, therefore, argues that surface exposed antigens

of the infective and adult stages differ.

Further evidence for stage-specific expression of *D. viviparus* surface antigens was obtained by examining the antibody responses of infected guinea-pigs, in which larvae do not mature beyond the early L5 stage (Wade *et al.*, 1960). Such animals mounted a strong antibody response to the L3 surface (Figure 6.3, Panel A), but not to adult surface antigens (Figure 6.3, Panel B). This not only demonstrated that surface antigens of the L3 and adult stages are different, but also that those of the early L5 and adult stage must differ. A similar pattern was obtained using serum from guinea-pigs vaccinated with irradiated larvae.

6.2.2 Species-specific recognition of *D. viviparus* adult surface antigens

This was examined by carrying out immunofluorescence on *D. viviparus* adult worms with sera from calves infected with *C. oncophora*, *O. ostertagi* or *D. viviparus*. As shown in Figure 6.4, only hosts infected with *D. viviparus* recognised adult surface antigens, indicating the species-specific recognition of *D. viviparus* surface components, at least between these common bovine nematodes. As previously mentioned, however, the possibility that this is due to the absence of circulating anti-surface antibody in hosts infected with gastrointestinal parasites cannot be excluded. It would, therefore, be informative to examine any binding of *D. viviparus* immune sera to the surface of these gastrointestnal nematodes.

6.2.3 Antiserum to adult ES products does not bind to the adult surface

It has been demonstrated that surface antigens of a number of nematodes are shed *in vitro*, thereby contributing to ES products, and that antibody directed against ES antigens also binds to the parasite surface (Badley *et al.*, 1987). To determine whether this was also true of *D. viviparus* the ability of rabbit antiserum raised against adult ES to bind to the adult worm surface was examined by immunofluorescence. This antiserum has previously been shown to precipitate all radio-iodinated ES components in immunoprecipitation studies (Chapter 3). It was quite unexpected, therefore, that it showed no detectable binding to the adult worm surface (Figure 6.5), nor to that of the L3 stage (not shown).

6.2.4 Phosphorylcholine is not present on the adult surface

It has previously been shown that phosphorylcholine is exposed on the surface of exsheathed infective stage of *D. viviparus* (Chapter 5). To examine whether this determinant is also present on the adult surface an immunofluorescence assay was carried out using the anti-PC monoclonal antibody, Bp-1. As also shown in Figure 6.5, Bp-1 did not bind to the exposed adult surface.

6.2.5 Antibody binding to the egg surface

The antibody response to egg surface antigens was examined by immunofluorescence using the various bovine, guinea-pig and rabbit sera described in Table 5.1. Some non-specific binding to the egg surface was observed with serum from uninfected calves, but to a much lesser extent than that found with exsheathed L3. As previously observed with adult worms, only calves infected with normal larvae showed significant recognition of egg surface antigens (Figure 6.6). Guinea-pigs exposed to normal or irradiated larvae showed slight specific recognition of egg surface antigens. As these hosts would not have been exposed to this parasite stage, this indicated a limited degree of cross-reactivity between egg and larval antigens. In contrast to the adult surface, significant binding of anti-ES antiserum was observed on the egg surface.

6.2.6 Antibody response to L1 surface antigens

Immunofluorescence was carried out on first-stage larvae using the same sera as in Section 6.2.5. As previously observed with the egg and adult surfaces, significant binding of antibody to the L1 surface occurred only with serum from calves exposed to patent lungworm infection (Figure 6.7). As there was no recognition of L1 antigens by vaccinated hosts, which showed significant recognition of L3 surface antigens, this indicated that antigens expressed on the surfaces of the L1 and L3 stages differed. This was also demonstrated by the absence of any fluorescence with sera from infected or vaccinated guinea-pig hosts. Differences between the exposed surfaces of the egg and L1 stages were demonstrated by the lack of binding by anti-ES serum to the L1 stage.

The reactivity of antisera to different developmental stages of *D. viviparus*, as demonstrated by immunofluorescence, is summarised in Table 6.1

6.2.7 Identification of L3 surface antigens

Having demonstrated that antigens are exposed on the surface of different stages of *D. viviparus*, radiolabelling studies were carried out to characterise these. Surface antigens of an increasing number of nematodes have been identified by labelling of intact parasites with ^{125}Iodine (Parkhouse *et al.*, 1981; Forsyth, Copeman and Mitchell, 1984; Marshall and Howell, 1985; Devaney, 1987; Maizels *et al.*, 1988). This technique was, therefore, applied to the L3 and adult stages of *D. viviparus*.

Sheathed and exsheathed third stage larvae were labelled by the Iodogen method, which has been shown to be surface-restricted when applied to other nematodes (Forsyth *et al.*, 1981; Baschong and Rudin, 1982; Marshall and Howells, 1985). Labelled components were recovered following sequential treatments as follows: incubation for 1h at 4^{o}C in 0.25% CTAB/Tris homogenisation buffer; sonication of larvae in 1% DOC/Tris homogenisation buffer; and incubation of the DOC-insoluble pellet at 100^{o}C for 2 min in harsh extraction buffer (5% 2ME/1% SDS/8M urea). Soluble fractions were then separated on SDS-PAGE under reducing conditions. This revealed a limited number of labelled proteins (Figure 6.8, Panel A), with only one labelled component of 67kDa being extracted from radioiodinated sheathed L3 following incubation in CTAB, a detergent which has been shown to selectively strip surface components from a number of other nematodes (Pritchard *et al.*, 1985; 1988b). Two components of molecular mass 70 and 155kDa were extracted following homogenisation in DOC and six proteins of approximately 23, 36, 65, 85, 119, 180kDa and 227kDa were solubilised in harsh extraction buffer. Approximately 40% of the total radiolabel remained in the pellet. Microscopic examination showed that this insoluble material was composed of cuticle fragments with no somatic material being observed.

Exsheathed larvae appeared to take up very little ^{125}I (Figure 6.8, Panel B). Labelled components which could be detected appeared to be of similar molecular mass to those present in sheathed larval extracts, with no additional molecules being labelled. Labelling of exsheathed L3 with NHS-biotin showed the same pattern, while Bolton-Hunter mediated iodination of exsheathed L3 revealed no labelled components. No difference was found using larvae which had been exsheathed and cultured in RPMI for five days prior to labelling.

6.2.8 Antigenicity of L3 surface proteins

Serum collected from calves following two infections with normal *D. viviparus* infective larvae or two vaccinations with 40krad irradiated larvae was immunoprecipitated with the DOC-soluble extract of iodinated sheathed L3 in a Staph A-based assay. SDS-PAGE analysis of immunoprecipitates revealed that both the 70 and 155kDa components were recognised by infected and vaccinated bovine hosts (Figure 6.9). No precipitation was observed with pre-treatment serum.

6.2.9 Radioiodination of adult worms

Intact adult worms were iodinated by both the Bolton-Hunter and Iodogen methods. Extraction of labelled components was carried out as described for the L3 stage with additional incubation in 1% DOC/2ME at 4°C for 1h prior to harsh extraction. Similar treatment of other parasitic nematodes with 2ME has been shown to solubilise cuticular collagens (Cox *et al.*, 1989; Selkirk *et al.*, 1989).

SDS-PAGE separation of adult surface-labelled components showed a much more complex pattern than that observed with the L3 stage (Figure 6.10). A number of low molecular mass components were observed in CTAB and DOC extracts of adult parasites which were not present in labelled L3 extracts. However, adult surface-labelled molecules of 65 and 119kDa, solubilised in the presence of 2ME, appeared to be of similar molecular mass to those found following harsh extraction of radiolabelled third stage larvae (see Figure 6.8 Panel A).

A greater number of adult molecules were labelled by the Bolton-Hunter than by the Iodogen method. Moreover, there were significant differences in the particular proteins labelled by the two methods, indicating considerable differences in their lysine versus tyrosine content or, alternatively, their accessibility to the radiolabel. In particular, a component of 30kDa was strongly labelled by the Iodogen but not by the Bolton-Hunter method.

In contrast to that obtained following extraction of iodinated L3 fractions, the pellet remaining following extraction of adult worms contained only 1-2% of the total activity.

6.2.10 Female-specific surface proteins

Iodogen labelling of male and female worms revealed both qualitative and quantitative differences in the surface proteins of the two sexes (Figure 6.11). In

particular, DOC-soluble components of 30 and 45kDa, although present in both sexes, were more abundant in male than female extracts. In contrast, a DOC-soluble component of 119kDa and several high molecular mass components of the DOC, DOC/ME and harsh extracts of female worms were not detected in male extracts.

6.2.11 Examination of DOC-soluble proteins under non-reducing conditions

SDS-PAGE analysis of surface labelled extracts was usually carried out under reducing conditions to allow examination of fractions extracted in the presence of 2ME. When analysed under non-reducing conditions the major Iodogen-labelled 30kDa component showed a decrease in apparent M_r to 27kDa. A slight decrease in M_r of a DOC-soluble protein of 45kDa was also observed under non-reducing conditions (Figure 6.12). These alterations in apparent M_r suggest the presence of intrachain disulphide linkages in these components which may be destroyed in the presence of reducing agent. This may cause the proteins to form a more linear configuration with a subsequent decrease in mobility on SDS-PAGE.

6.2.12 Solubility characteristics of labelled proteins

Extraction of surface-labelled components of most nematode parasites is usually achieved in the presence of detergents, such as DOC or n-octyl glucoside (Marshall and Howells, 1985). To examine whether this was true of *D. viviparus*, surface-labelled adult worms were incubated or homogenised in the presence or absence of detergent. As shown in Figure 6.13 Panel A, almost all components labelled by the Bolton-Hunter method were solubilised by homogenisation in Tris buffer alone. Extraction in 2ME in the absence of detergent resulted in only partial extraction of 2ME-soluble proteins (track c).

In contrast to components labelled by the Bolton-Hunter method, there was no solubilisation of Iodogen-labelled proteins in the absence of detergent (Figure 6.13 Panel B). Extraction in 2ME alone resulted in the release of three components, thought to be collagens (see 6.2.15), while molecules of 17, 30 and 45kDa were extracted only in the presence of detergent.

Curiously, a collagen component of approximately 40kDa was not observed when 2ME was used subsequent to DOC extraction, but appeared to form a dimer as suggested by the appearance of collagen component of approximately 80kDa in the

presence of DOC. This did not occur with Bolton-Hunter labelled collagens.

6.2.13 Antigenicity of adult surface proteins

Immunoprecipitation studies were performed to examine the antigenicity of adult surface-associated molecules. SDS-PAGE analysis of immunoprecipitates showed that calves infected twice with normal larvae mounted a strong antibody response to the 30 and 45kDa DOC soluble proteins of Iodogen-surface labelled adult worms (Figure 6.14). Calves vaccinated with 2 doses of 40 or 100krad irradiated larvae showed no response to any adult surface antigens. This antigen recognition pattern was unchanged following another dose of vaccine. In contrast to bovine hosts, guinea-pigs exposed to normal or 40krad irradiated larvae on two occasions showed an antibody response to adult surface antigens (Figure 6.14, tracks h and i). Vaccinated guinea-pig hosts showed a weak response to the 30kDa antigen. This antigen was more strongly recognised by infected guinea-pigs, which also recognised the 45kDa component. Although not clearly visible in Figure 6.14, both the 30 and 45kDa antigens were also precipitated by rabbit antiserum to adult ES products (track f).

As with Iodogen-labelled proteins, immunoprecipitation of Bolton-Hunter surface labelled extracts occurred only with serum from calves infected with normal larvae, while vaccinated bovine hosts showed no response (Figure 6. 15). As well as a response to the dominant 13kDa antigen, infected calves recognised a series of minor components. Although bovine hosts showed no response to the major 27kDa molecule, this was strongly recognised by rabbit anti-ES antiserum, together with low molecular mass antigens of 13, 14 and 16kDa (Figure 6.15, track f).

6.2.14 *In vitro* release of surface-labelled proteins

The recognition of labelled surface-associated molecules by anti-ES antiserum suggested that these components are shed *in vitro* and contribute to adult ES material. To examine this possibility radioiodinated adults were cultured in RPMI for 24 hours, after which time the culture fluid was collected and analysed by SDS-PAGE, as were extracts of the cultured parasites (Figure 6.16). A wide range of labelled proteins were released *in vitro* from adults labelled by the Bolton-Hunter method (Panel B track a). In contrast, only three minor components of 14, 16 and

28 kDa were detected at very low levels in the culture fluid of adults labelled by the Iodogen method. Surprisingly, there was no release of the major 30kDa antigen in the absence of detergent, although this was strongly immunoprecipitated by anti-ES antiserum.

The one-dimensional SDS-PAGE profile of *in vitro* released material from Bolton-Hunter surface-labelled adults showed greater similarity to extrinsically labelled ES than to extracts of surface-labelled adults (Figure 6.17). In particular, molecules of 13-18kDa and a doublet of M_r 39 and 42kDa were more abundant in both ES preparations than in DOC-extracts of radiolabelled worms.

6.2.15 Identification of adult cuticular collagens

Collagens were identified by treatment of soluble extracts of surface-labelled worms with collagenase from *Clostridium histolyticum*, which specifically hydrolysis the X-Gly bond in the triple helical region of collagens, where X is any amino acid (Seifter and Harper, 1971). Molecules released in detergent alone were resistant to collagenase (Figure 6.18 Panel A), while those extracted in the presence of 2ME were susceptible to digestion (Panel B). Although not clearly reproduced in this figure, all three components present in the 2ME extract were digested, indicating the collagenous nature of all of these. Identical results were obtained when 2ME extracts of Bolton-Hunter or Iodogen surface-labelled adults were treated.

6.2.16 Antigenicity of *D. viviparus* cuticular collagens

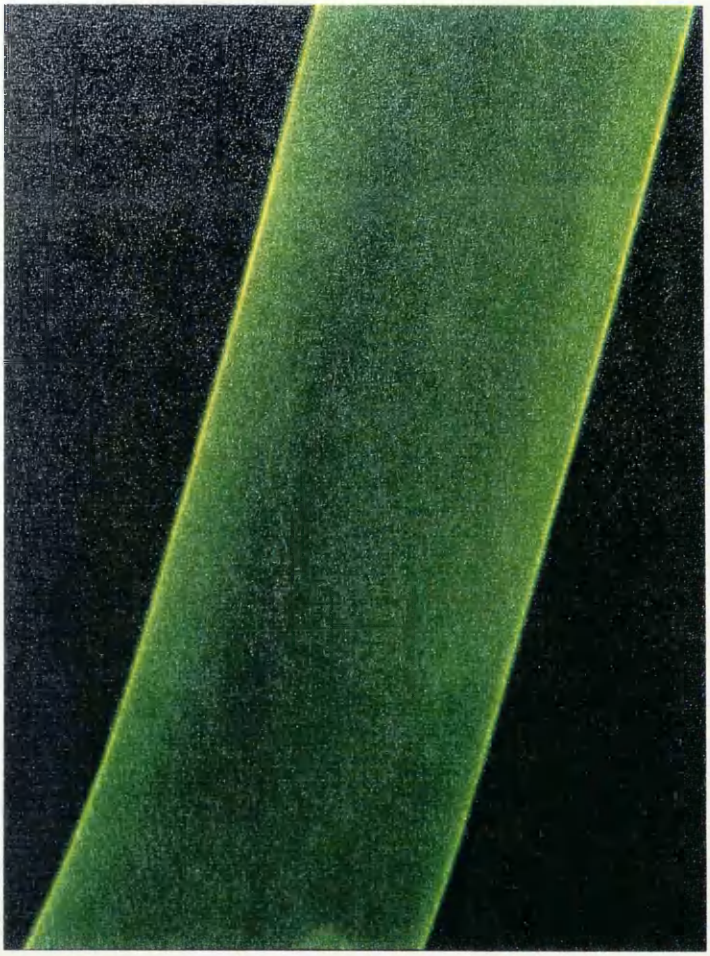
Immunoprecipitation studies were carried out to determine whether adult cuticular collagens are antigenic to the bovine host. The 2ME extracts were extensively dialysed prior to incubation with serum from calves which had been infected or vaccinated on two occasions. Once again, vaccinates showed no response to the adult surface antigens, while calves exposed to patent infections showed a low, but detectable antibody response to all three collagen components, thus demonstrating both their antigenicity and stage-specificity (Figure 6.19). Identical patterns were observed using either Bolton-Hunter or Iodogen-labelled 2ME extracts.

TABLE 6.1 Summary of antisera reactivity to different developmental stages *D. viviparus*

Serum source	Immunisation	Level of flourescence			
		L3	Adult	Egg	L1
Calf	Normal L3	+++	+++	+++	+++
	40krad L3	+++	-	-	-
	100krad L3	+++	-	-	-
Guinea-pig	Normal L3	+++	-	+	-
	40krad L3	+++	-	+	-
	100krad L3	+++	-	+	-
Rabbit	adult ES	-	-	++	-

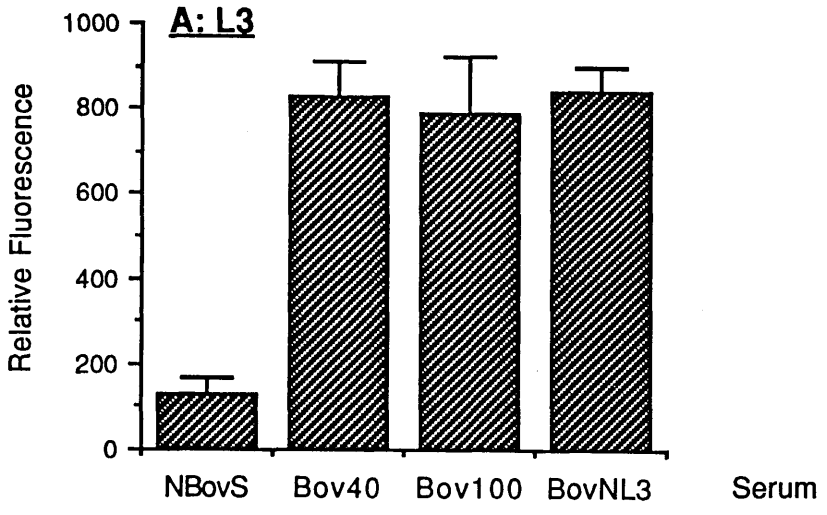
Figure 6.1 Fluorescence on the surface of *D. viviparus* adult parasites following incubation with infection serum. *D. viviparus* adult worms were incubated with serum from calves infected on three occasions with normal infective larvae (A) and with serum collected prior to infection (B). Antibody binding to the parasite surface was detected by further incubation in FITC-conjugated anti-bovine IgG and parasites viewed under U.V. light with a x10 objective lens.

A



B

Figure 6.2 Stage-specificity of surface-exposed antigens of *D. viviparus*. Immunofluorescence was carried out on third-stage larvae (Panel A) and adult parasites (Panel B) using normal bovine serum or serum from calves exposed on three occasions to normal, 40krad irradiated or 100krad irradiated larvae. Sera was sampled approximately 3 weeks after the third infection or vaccination, as detailed in Table 4.1. The level of fluorescence was quantitated by photon counting as detailed in Chapter 2. The mean fluorescence of 25 measurements is shown per data point, with error bars representing the S.D. of the observations. See Table 5.1 for abbreviations of antisera examined.


A: L3
Relative Fluorescence
1000
800
600
400
200
0
NBovS
Bov40
Bov100
BovNL3
Serum

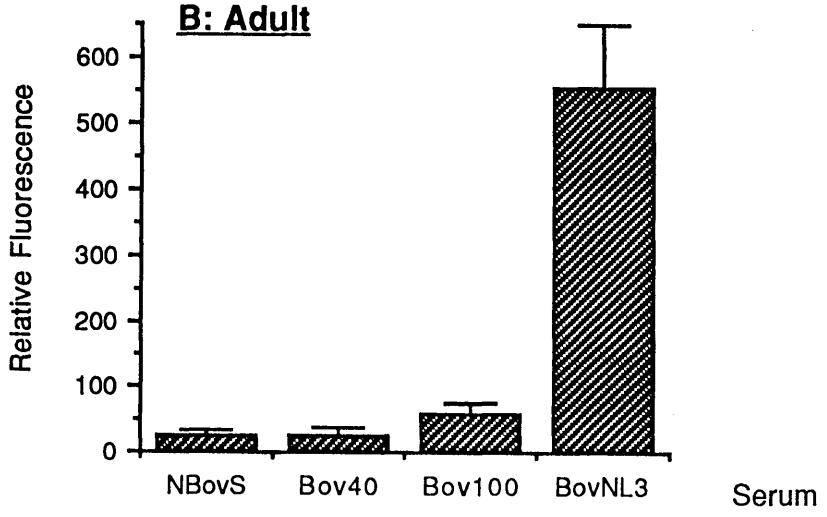
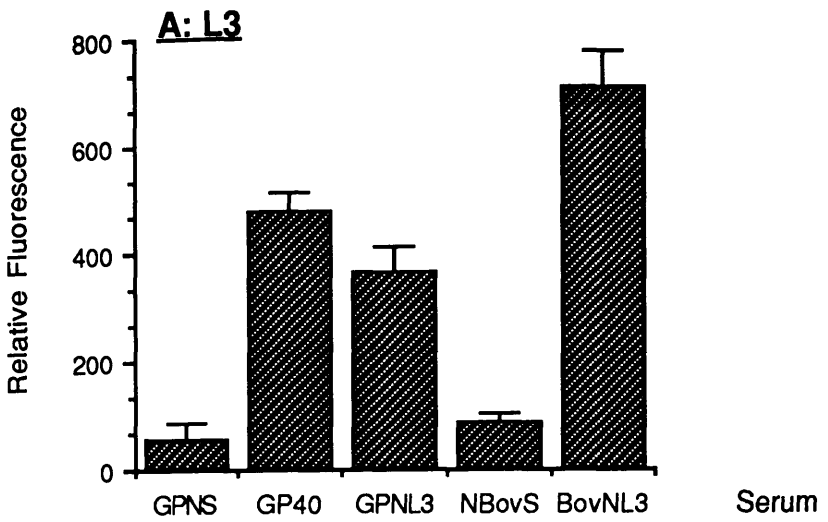

B: Adult
Relative Fluorescence
600
500
400
300
200
100
0
NBovS
Bov40
Bov100
BovNL3
Serum

Figure 6.3 Recognition of stage-specific surface antigens by guinea-pig hosts. Normal guinea-pig serum and sera collected from guinea-pigs 28 days after two exposures, 28 days apart, to normal or 40krad irradiated larvae, was incubated with third stage (A) and adult (B) parasites. The level of antibody binding was measured by immunofluorescence following further incubation in FITC anti-guinea-pig IgG. The level of fluorescence observed with bovine infection serum is shown for comparison. Abbreviations of sera used are shown in Table 5.1.


A: L3
Relative Fluorescence
800
600
400
200
0
GPNS
GP40
GPNL3
NBovS
BovNL3
Serum

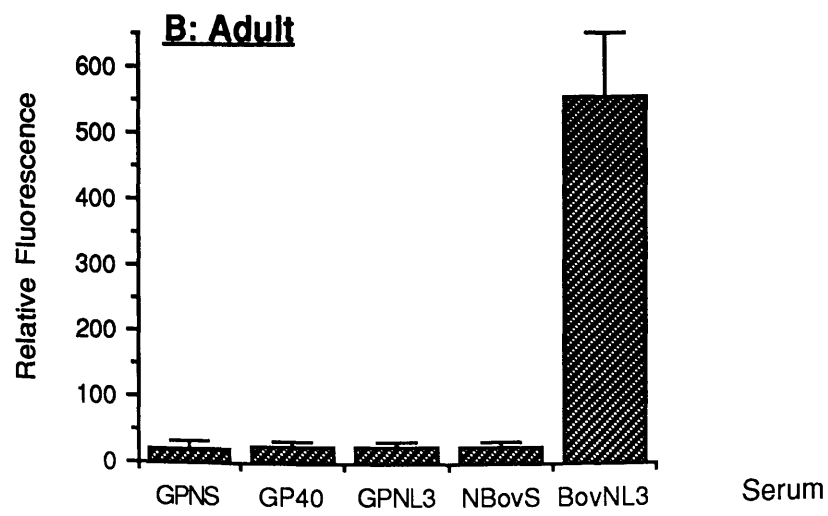

B: Adult
Relative Fluorescence
600
500
400
300
200
100
0
GPNS
GP40
GPNL3
NBovS
BovNL3
Serum

Figure 6.4 Species-specific recognition of *D. viviparus* adult surface antigens. Adult *D. viviparus* parasites were incubated with normal bovine serum or sera from calves infected on 2 occasions with *C. oncophora*, *O. ostertagi* or *D. viviparus* and antibody binding detected with FITC-conjugated antibovine IgG. See Table 5.1 for abbreviations of antisera used.

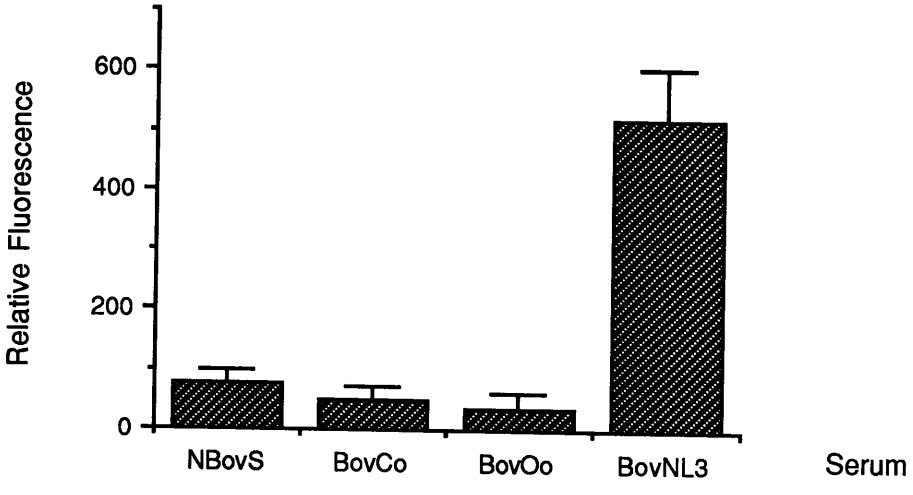

Relative Fluorescence
600
400
200
0
NBovS
BovCo
BovOo
BovNL3
Serum

Figure 6.5 Anti-adult ES antiserum and Bp-1 do not bind to the adult surface. Rabbit antiserum to *D. viviparus* adult ES products and Bp-1, a mouse anti-phosphorylcholine monoclonal antibody, were assayed in immunofluorescence against adult worms. The level of fluorescence observed with bovine anti-*D. viviparus* infection serum is shown for comparison, as are controls of normal rabbit, mouse and bovine sera. Antibody binding was detected using the appropriate FITC-conjugated anti-species IgG. See Table 5.1 for abbreviations.

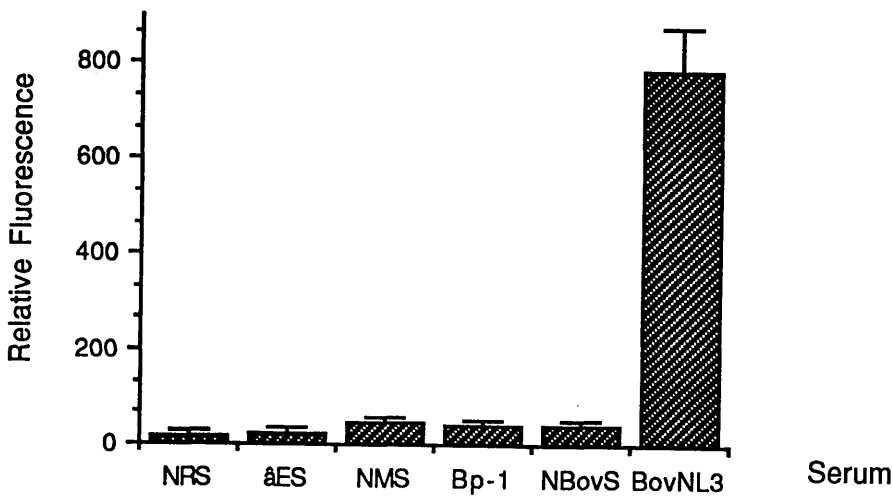
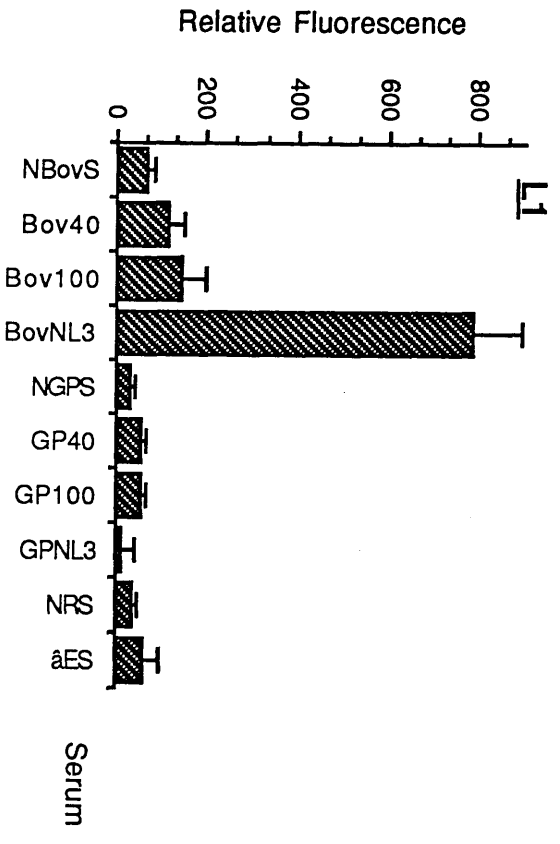

Relative Fluorescence
800
600
400
200
0
NRS
âES
NMS
Bp-1
NBovS
BovNL3
Serum

Figure 6.6 Antibody response to egg surface antigens. Immunofluorescence was carried out on *D. viviparus* eggs using no serum (PBS control), sera from uninfected bovine and guinea-pig hosts and hosts infected or vaccinated with *D. viviparus* L3 on two occasions. The binding of rabbit anti-ES antiserum was also assayed. See Table 5.1 for abbreviations of antisera used.

Figure 6.7 Antibody recognition of surface-exposed antigens of first stage larvae. L1 parasites were subjected to immunofluorescence using the range of bovine, guinea-pig and rabbit antisera as described in Figure 6.6 and the level of fluorescence measured by photon counting.


Relative Fluorescence
0
200
400
600
800
NBovS
Bov40
Bov100
BovNL3
NGPS
GP40
GP100
GPNL3
NRS
âES
L1
Serum

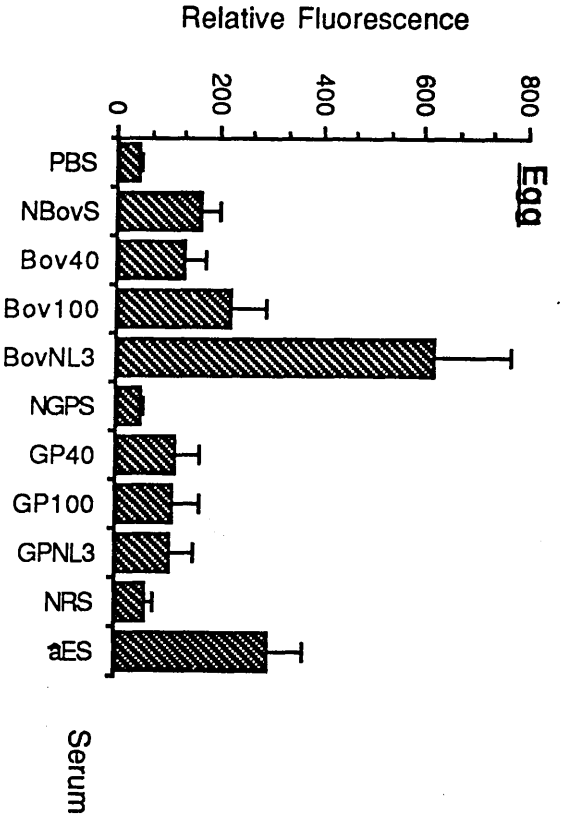

Relative Fluorescence
0
200
400
600
800
PBS
NBovS
Bov40
Bov100
BovNL3
NGPS
GP40
GP100
GPNL3
NRS
âES
Egg
Serum

Figure 6.8 Identification of L3 surface proteins by radioiodination. Intact sheathed (A) and exsheathed (B) third stage larvae were iodinated by the Iodogen method and proteins solubilised by incubation in 0.25% CTAB (track a), homogenisation in 1% DOC (track b) followed by extraction in 5% 2ME/1% SDS/8M urea (track c) as described in Chapter 2. Extracted labelled proteins were analysed by SDS-PAGE under reducing conditions. The M_r of marker proteins (M) are shown in kDa.

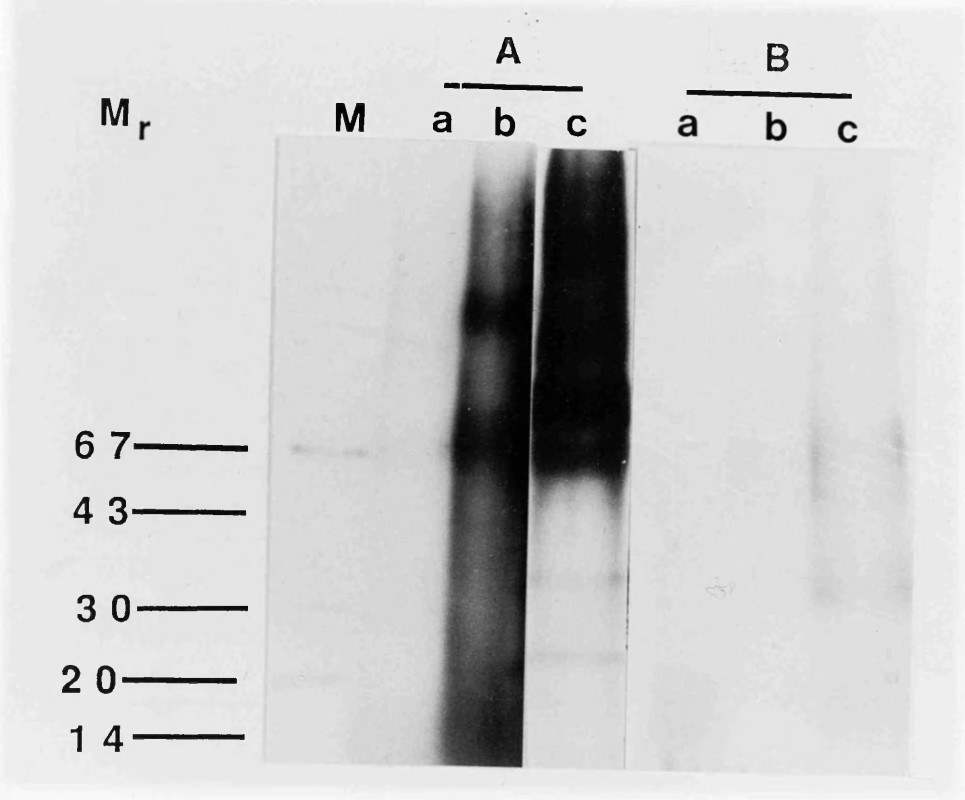

A
B
M_r
M
a
b
c
a
b
c
67
43
30
20
14

Figure 6.9 Antigenicity of L3 surface components. DOC-soluble surface-labelled components of sheathed L3 (R) were immunoprecipitated with normal bovine serum (N), sera from calves infected on two occasions, 56 days apart, with normal larvae (I) or vaccinated with two doses of 40krad-irradiated larvae 28 days apart (V). Serum was sampled 28 days after the last infection or vaccination.

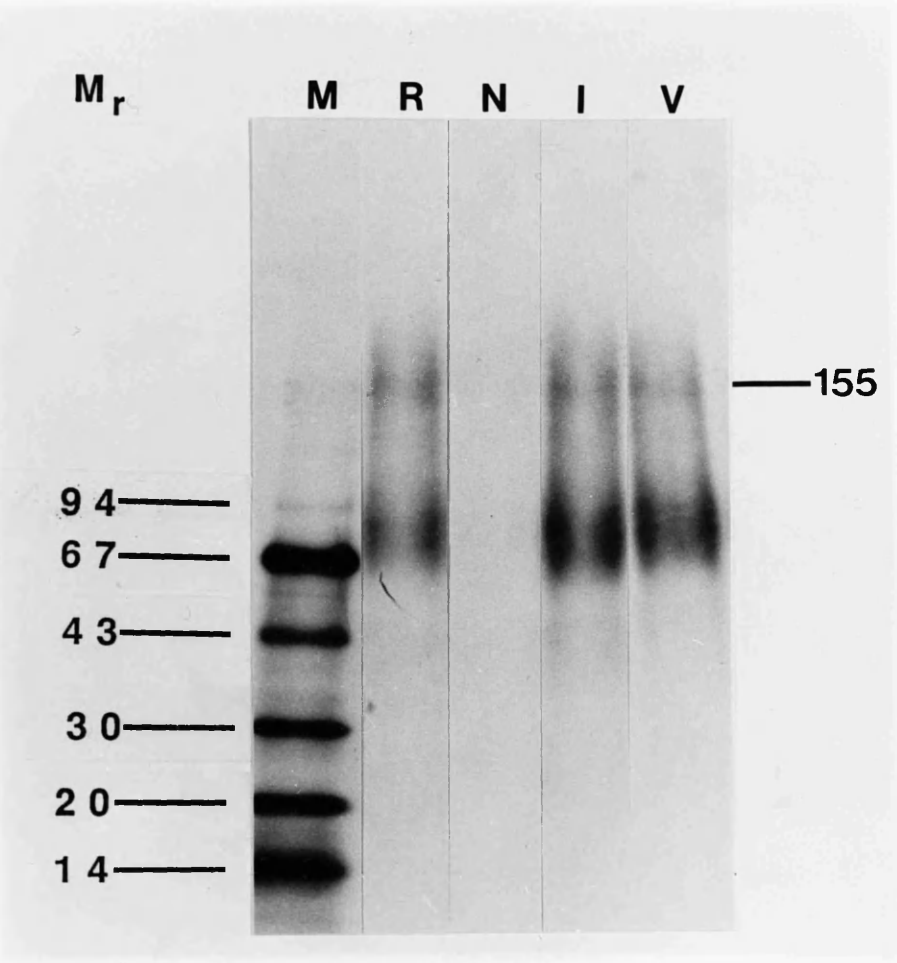

Mr
M
R
N
I
V
155
94
67
43
30
20
14

Figure 6.10 Surface proteins of adult *D. viviparus*. Mixed sexes of adult parasites were radioiodinated by the Bolton-Hunter and Iodogen techniques. Proteins were sequentially solubilised by incubation in 0.25% CTAB (a), homogenisation in 1% DOC (b), extraction in 1% DOC/5% 2ME (c) followed by 5% 2ME/1% SDS/8M urea (d). Labelled extracts were analysed by SDS-PAGE under reducing conditions. The M_r of marker proteins (M) are indicated in kDa.

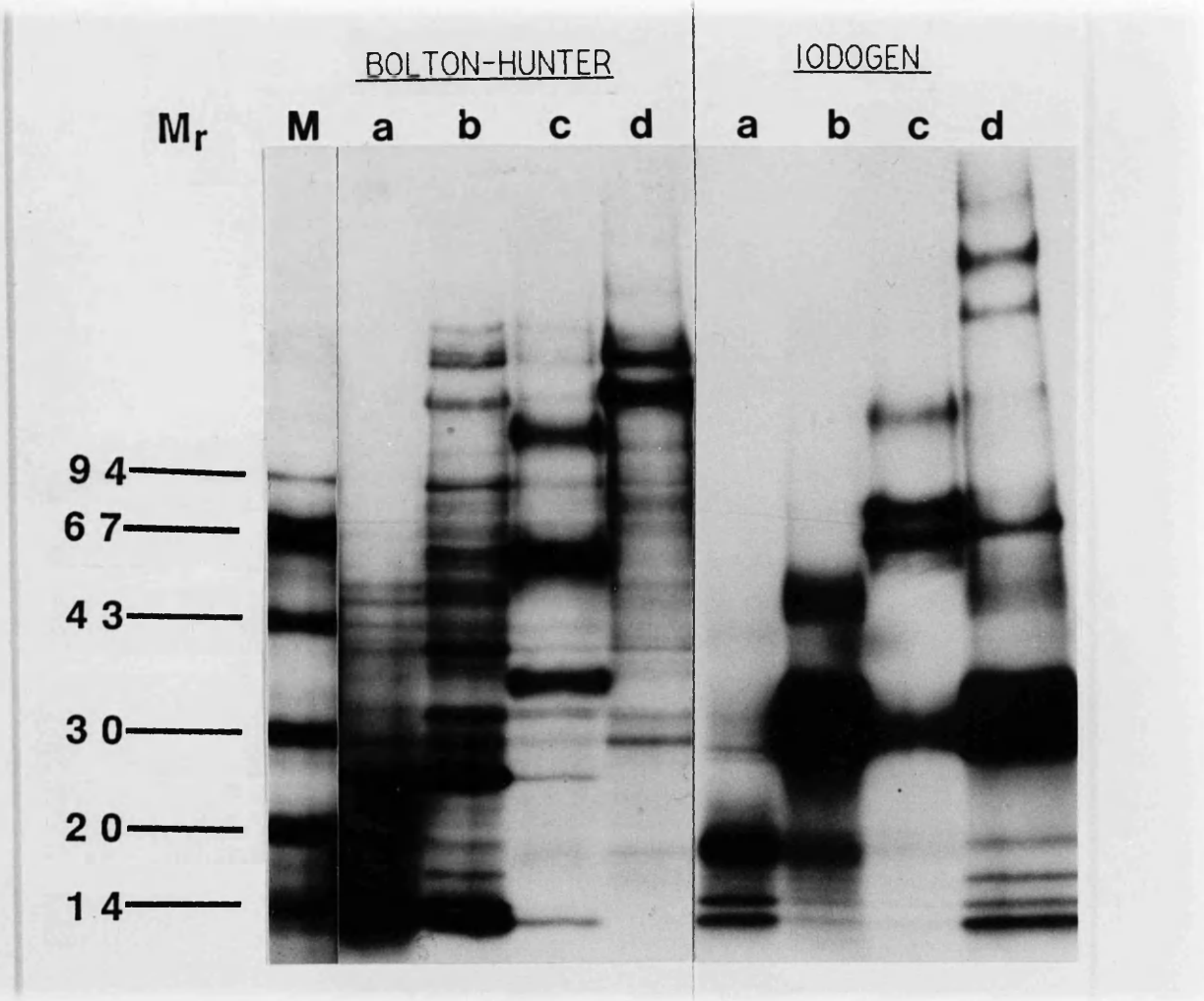

BOLTON-HUNTER
IODOGEN
Mr
M
a
b
c
d
a
b
c
d
94
67
43
30
20
14

Figure 6.11 Comparison of surface-labelled proteins of male and female adult parasites. Female (Panel A) and male (Panel B) worms were iodinated by the Iodogen method. Proteins were extracted by homogenisation in 1% DOC (a), followed by 5% 2ME (b) and 5% 2ME/ 1% SDS/8M urea (c) and analysed by SDS-PAGE under reducing conditions.

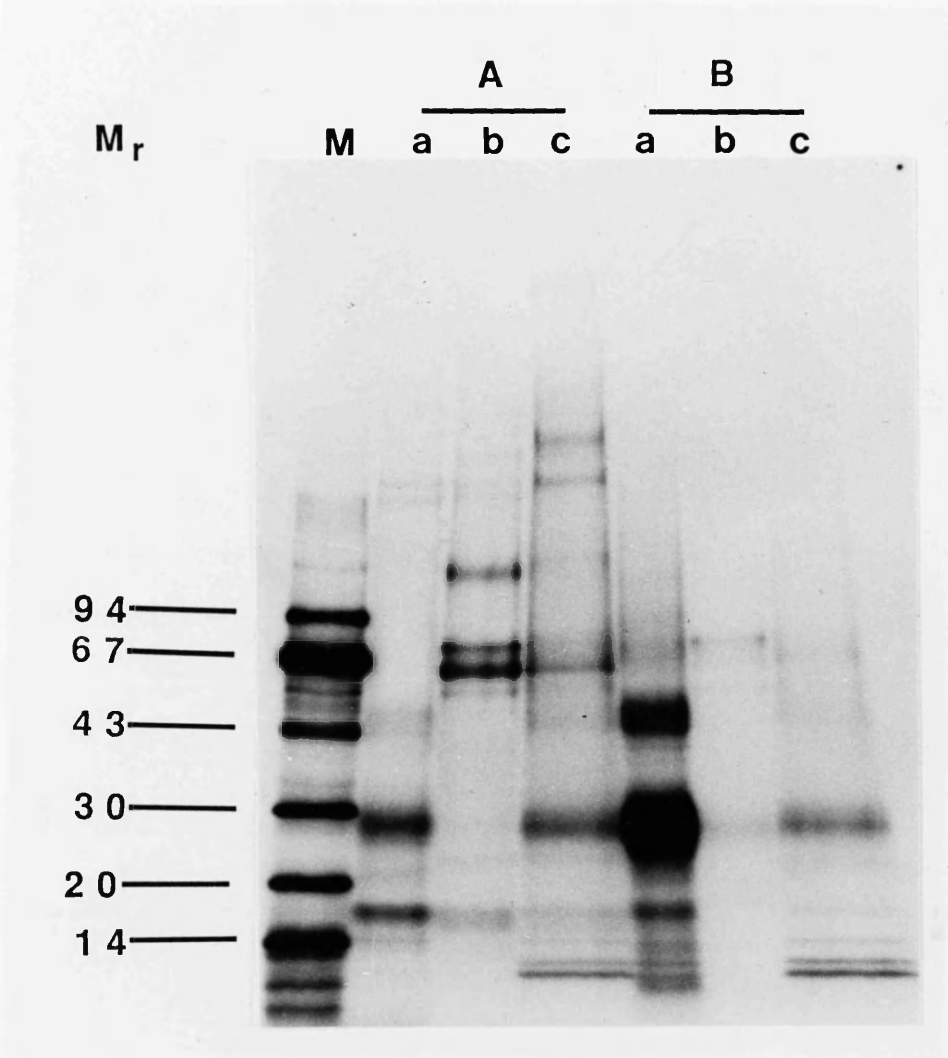

A
B
Mr
M
a
b
c
a
b
c
94
67
43
30
20
14

Figure 6.12 Comparison of DOC-soluble surface-labelled extracts under non-reducing and reducing conditions. Iodogen-mediated labelled DOC-soluble adult extract was analysed by SDS-PAGE under non-reducing (tracks a, b) and reducing (track c) conditions. The M_r of marker proteins run under non-reducing (M) and reducing conditions (M') conditions are shown in kDa.

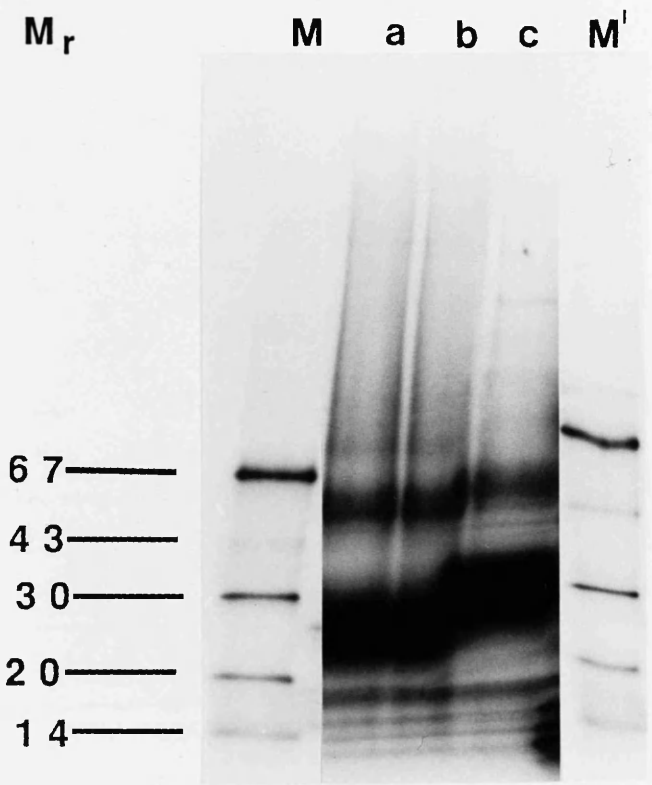
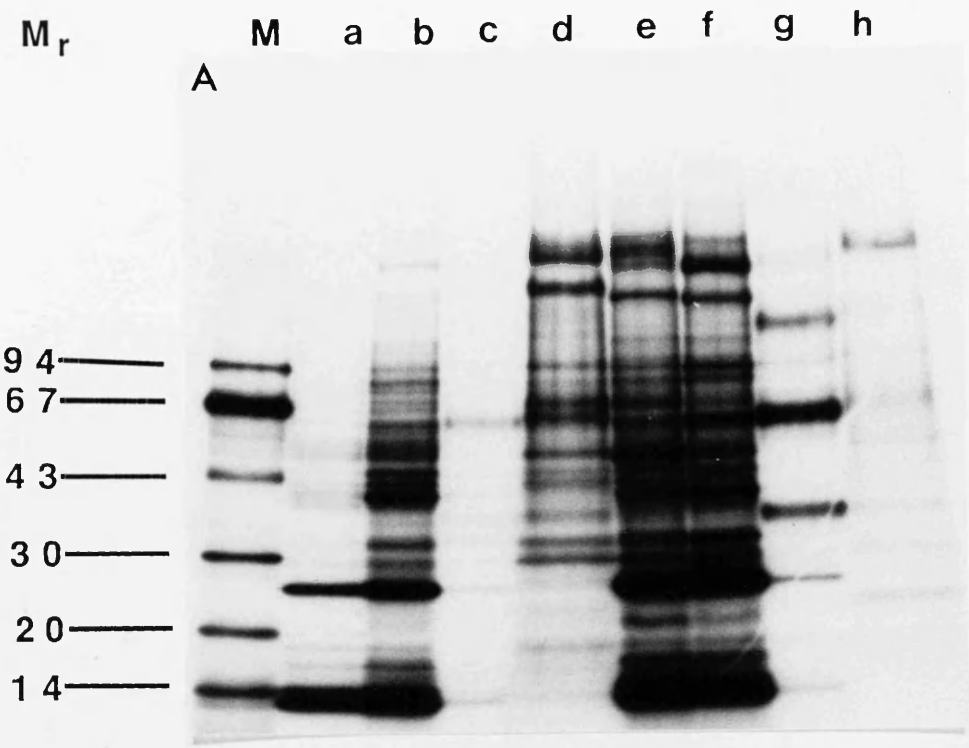

Mr
M
a
b
c
M'
67
43
30
20
14

Figure 6.13 Solubility characteristics of adult surface-labelled proteins. Mixed sexes of adult worms were labelled by the Bolton-Hunter (Panel A) and Iodogen (Panel B) techniques. Proteins were solubilised by incubation in Tris buffer for 2h, 4^{o}C (a), homogenisation in Tris buffer (b), extraction with 5% 2ME (c) followed by 5% 2ME/1% SDS/8M urea (d). Proteins extracted by identical procedures carried out in the presence of 1% DOC are shown in tracks e-h.


Mr
M a b c d e f g h
A
94
67
43
30
20
14

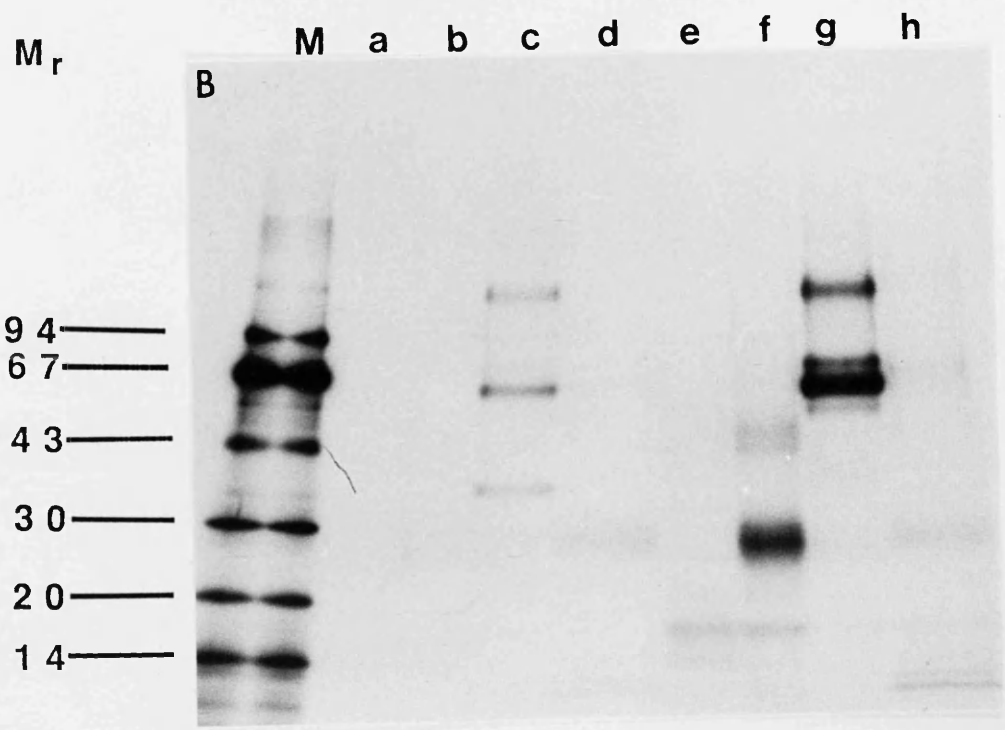

Mr
M a b c d e f g h
B
94
67
43
30
20
14

Figure 6.14 Stage-specificity of *D. viviparus* adult surface antigens. DOC-soluble proteins (R) of Iodogen surface-labelled adult parasites were immunoprecipitated with a variety of sera as follows: normal bovine serum (a); sera from calves exposed to 40krad irradiated (b), 100krad irradiated (c) or normal (d) larvae; normal rabbit serum (e); rabbit antiserum to adult ES products (f); normal guinea-pig serum (g) and serum from guinea-pigs exposed to 40krad irradiated (h) or normal (i) larvae. Antisera were collected from calves three weeks after two infections or vaccinations administered 56 and 28 days apart, respectively. Guinea-pig antisera were collected four weeks after two infections or vaccinations, 28 days apart. Precipitated antigens were analysed by gradient SDS-PAGE under reducing conditions.

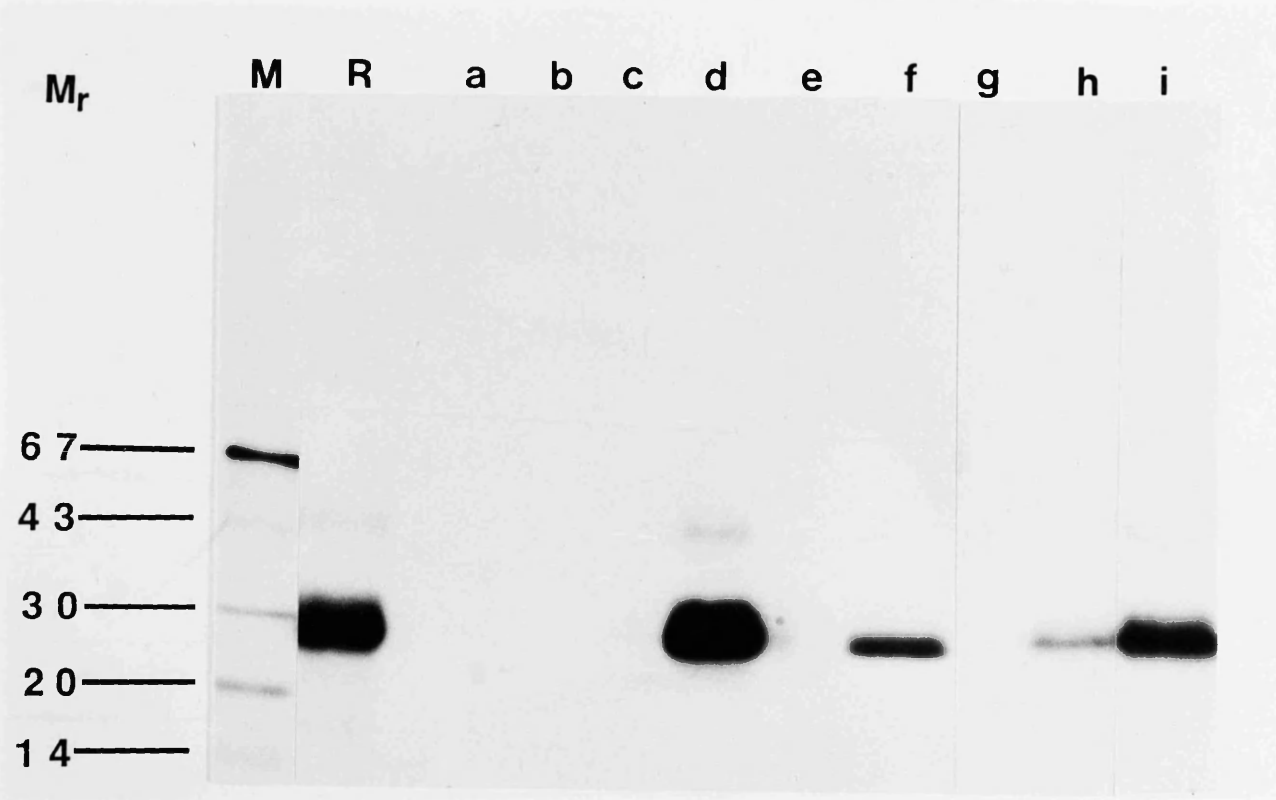

Mr
M
R
a
b
c
d
e
f
g
h
i
67
43
30
20
14

Figure 6.15 Immunoprecipitation of Bolton-Hunter labelled adult surface-associated antigens. DOC-soluble proteins (R) of adults labelled by the Bolton-Hunter method were immunoprecipitated with sera as described in Figure 6.14 (tracks a-f). Precipitated antigens were analysed by SDS-PAGE under reducing conditions.

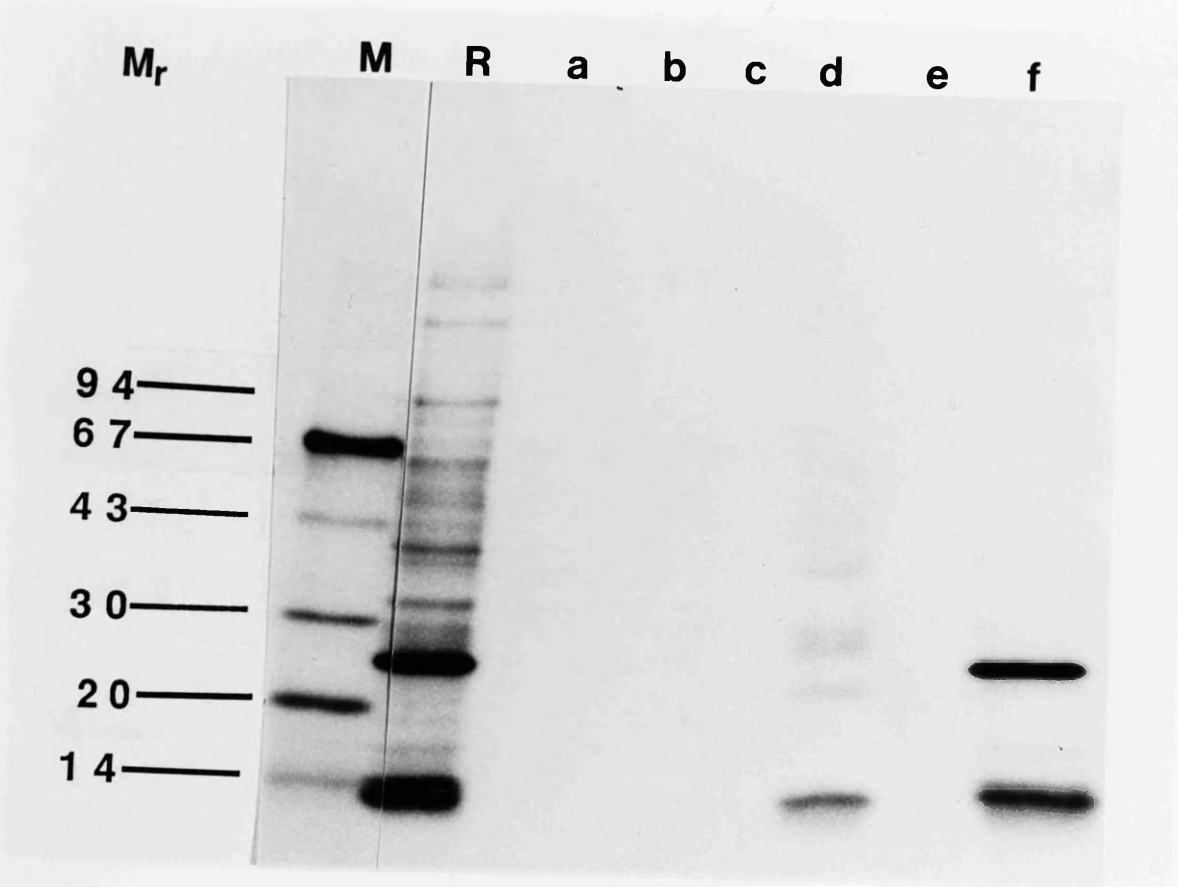

Mr
M
R
a
b
c
d
e
f
94
67
43
30
20
14

Figure 6.16 *In vitro* release of surface-labelled proteins. Adults labelled by the Bolton-Hunter (Panel A) and Iodogen (Panel B) techniques were cultured in RPMI at 37^{o}C and proteins released into the medium after 24 hours (a) were analysed by SDS-PAGE under reducing conditions. Proteins solubilised from the cultured parasites by incubation in 0.25% CTAB (b), homogenisation in 1% DOC (c) followed by extraction in 5% 2ME (d) and 5% 2ME/1% SDS/8M urea (e) were also analysed.

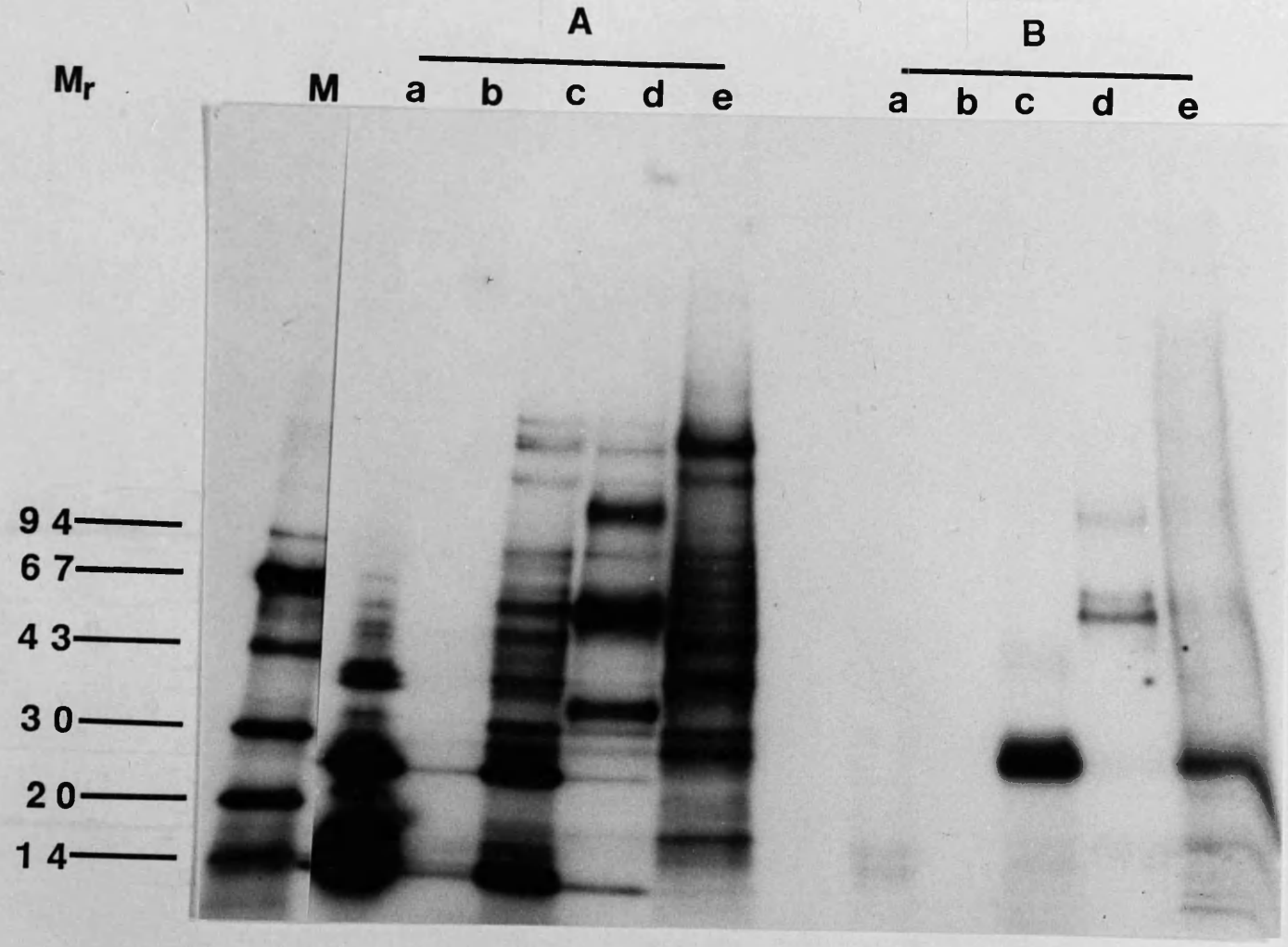

A
B
Mr
M
a
b
c
d
e
a
b
c
d
e
94
67
43
30
20
14

Figure 6.17 Comparison of adult ES products and *in vitro*-released surface-labelled proteins. ES material collected from adult worms maintained in culture was radioiodinated by the Bolton-Hunter technique (a). This was compared on SDS-PAGE with a DOC extract of adult surface-labelled proteins (b) and with components released *in vitro* from Bolton-Hunter surface-labelled adults (c).

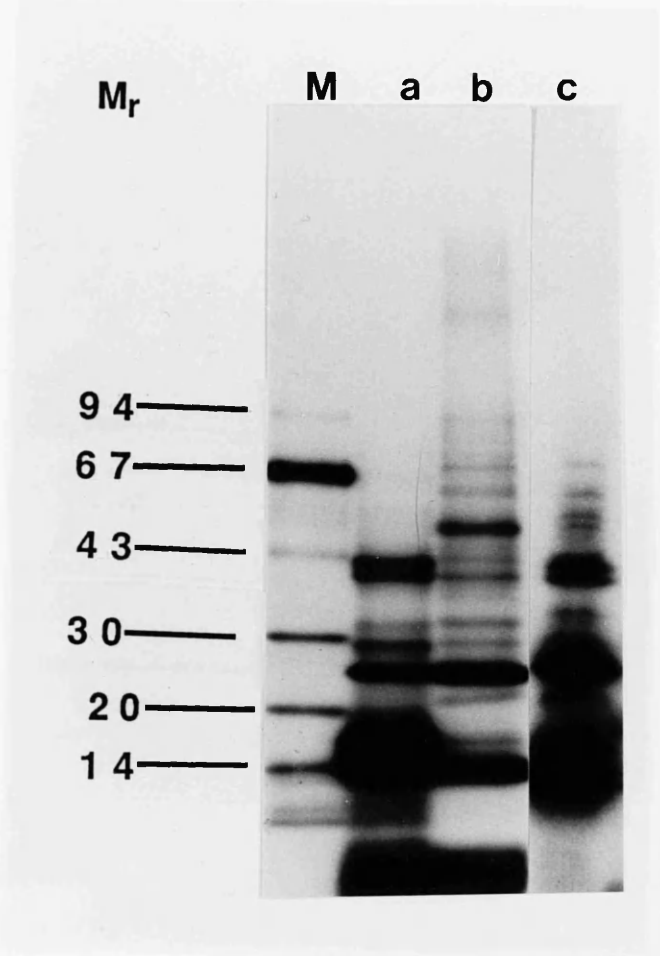

Mr
M
a
b
c
94
67
43
30
20
14

Figure 6.18 Sensitivity of adult surface-labelled proteins to collagenase. DOC- (Panel A) and 2ME- (Panel B) soluble proteins of Iodogen labelled adult worms were incubated at 37°C with 0 (a), 10 (b) and 100 (c) µg/ml of clostridial collagenase. Digestion was terminated after 30 min and proteins analysed by SDS-PAGE under reducing conditions.

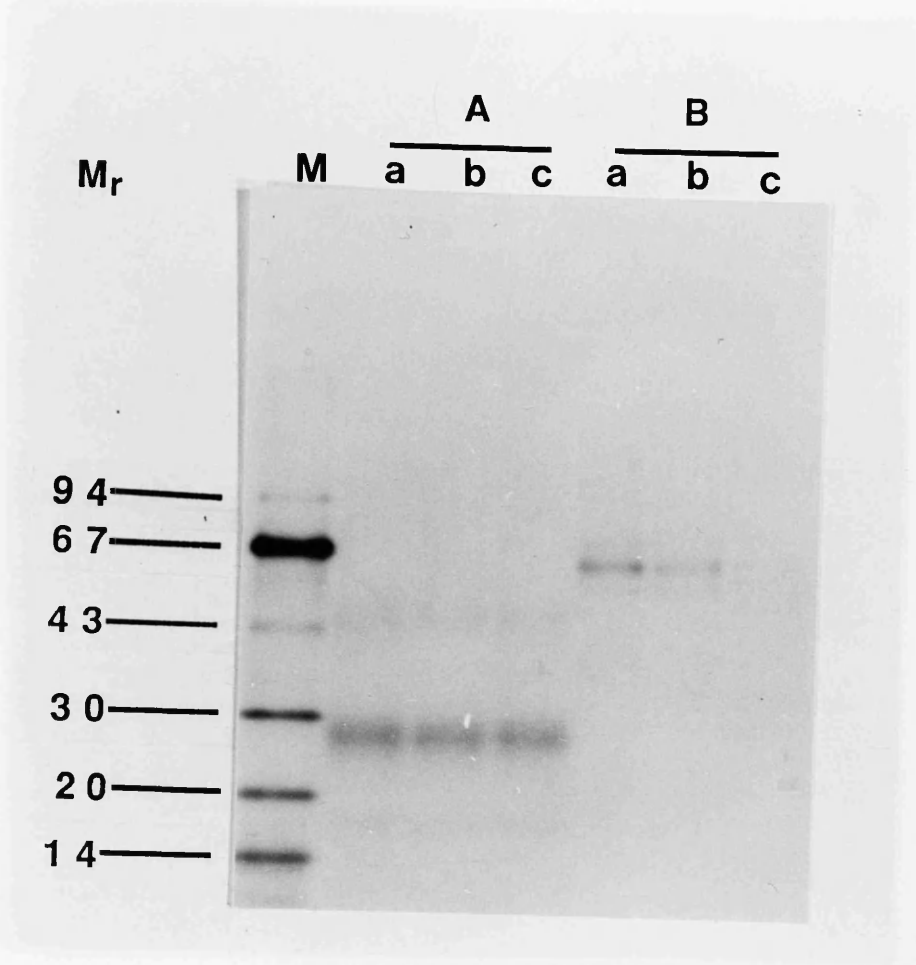
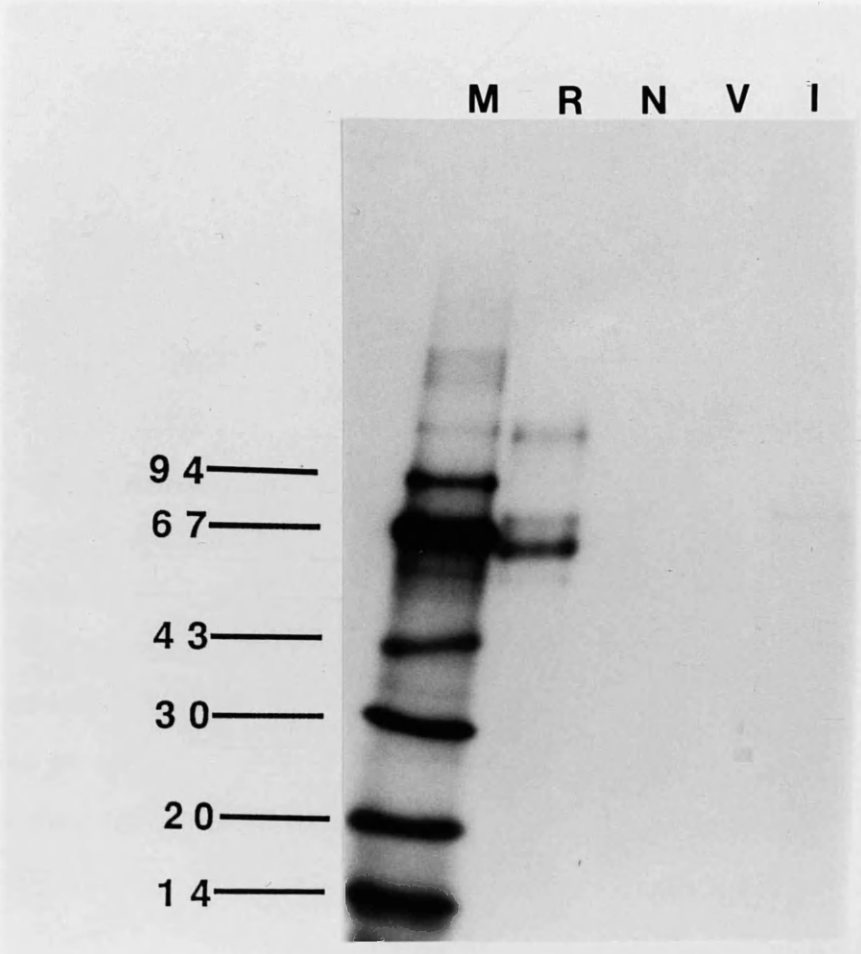

A
B
Mr
M
a
b
c
a
b
c
94
67
43
30
20
14

Figure 6.19 Antigenicity and stage-specificity of *D. viviparus* cuticular collagens. 2ME-soluble proteins (R) of Iodogen-labelled adult worms were immunoprecipitated with normal bovine serum (N) or serum from calves exposed on 2 occasions to 40krad irradiated (V) or normal larvae (I). Serum was collected 28 days after the second infection or vaccination. Antigens precipitated in a Staph A-based assay were analysed by SDS-PAGE under reducing conditions.


M
R
N
V
I
94
67
43
30
20
14

6.3 DISCUSSION

Stage-specificity of surface antigens has been demonstrated for a number of a nematodes. This phenomenon is thought to be associated with changes in the parasites' environment within the host, and it was, therefore, speculated that *D. viviparus*, being a migratory parasite, may demonstrate stage-specific expression of surface antigens. Examination of stage-specific antigen expression may be important in identifying which parasite stages are involved in protective immunity as well as giving an insight into the developmental control of parasite antigens in general.

Stage-specificity of *D. viviparus* surface antigens was first inferred from the failure of antibody from cattle vaccinated with irradiated larvae and from guinea-pigs exposed to normal or irradiated larvae to bind to the adult surface in immunofluorescence studies, while the same antibody bound strongly to the surface of infective larvae. As these hosts would have been exposed to the L5 stage this also suggests that the exposed surfaces of the L5 and mature adult stages differ.

Antigens exposed on the surface of the egg and L1 stages were also examined for two reasons. Firstly, antigens of these stages may be involved in the pathology of lungworm disease, through the generation of granulomatous reactions in the lungs of infected cattle. Secondly, surface antigens of the egg and L1 may be important in limiting the output of larvae onto pasture, through immune trapping in the respiratory or alimentary tracts. We have found that the surfaces of both stages are antigenic, with antigens being detected on the surface of first-stage larvae which were submitted to immunofluorescence within 1h of hatching *in vitro*. It appears, therefore, that antigen expression by *D. viviparus* larvae occurs far sooner after hatching than for the infective larvae of *Toxocara canis*, *T. cati* (Kennedy *et al.*, 1987b; Maizels *et al.*, 1987b) and *Ascaris suum* (Fraser and Kennedy, 1991).

Alternatively, larvae may emerge from the egg coated in hatching fluids which are themselves immunogenic and that we are not examining the true larval surface. However, such fluids might be expected to contribute to adult ES products due to the release and hatching of eggs during the culture of adult females. As we detected no binding of anti-ES antiserum to the L1 surface this might indicate that the latter does not occur. Binding of anti-ES antiserum was, however, observed on

the egg surface. This is most likely due to eggs being coated in adult material, possibly uterine fluids, which may be secreted by adults *in vitro*.

Both the egg and L1 surfaces were strongly recognised by antibody from patently infected bovine hosts but not by serum from vaccinated calves nor guinea-pig hosts. This demonstrates that the surfaces of the egg and L1 differ from that of the L3 stage. Differences between surface antigens of the adult, egg and L1 stages could not be observed as antibody from patently infected hosts bound to all three. This may be resolved by antibody absorption studies as performed in the original studies by MacKenzie *et al.* (1978) or, alternatively, by raising antibody to individual parasite stages.

Stage-specificity of *D. viviparus* surface proteins was demonstrated at the molecular level by comparing the profiles of radiolabelled proteins following iodination of intact L3 and adult parasites. A restricted set of molecules was observed following labelling of the L3 stage, while adult parasites showed a more complex pattern of labelled components. There appeared to be little similarity, between L3 and adult surface proteins with respect to molecular mass, although some components extracted in the presence of 2ME appeared to be common to both of these stages by one dimensional electrophoresis.

Surface labelling of exsheathed L3 provided inconsistent results. On some occasions no labelled components could be detected, while at other times the SDS-PAGE profile was similar to that observed following labelling of sheathed larvae. The latter finding may have been due to incomplete exsheathment or the presence of sheaths in the larval suspension. The L3 cuticle of other parasitic nematodes have also been difficult to analyse by radiolabelling techniques, perhaps due to the presence of non-proteinacious glycoconjugates (Maizels *et al.*,1983a; Rhoads and Fetterer, 1990), or the presence of components derived from an intermediate host which may inhibit radiolabelling or mask parasite proteins. The latter, however, cannot be applied to parasites such as *D. viviparus* where no intermediate host is involved. An increase in surface labelling of *B. pahangi* L3 is observed following incubation at room temperature for 2 hours in Hank's Balanced Salt Solution, presumably due to changes in the surface properties of the larvae, although such changes have not been identified at the biochemical level (Devaney and Jecock, 1991). Following incubation of *D. viviparus* at 37^{o}C for one week in RPMI there was no enhancement in the efficiency of L3 cuticular labelling, although the larvae

were motile throughout this time. This suggests that changes in the surface properties occur more slowly in this parasite or will not occur *in vitro.* It would be interesting to discern whether the inability of exsheathed L3 to take up radiolabel is in some way associated with the previously observed changes in surface lipid organisation and is common to infective stage larvae of all parasitic nematodes (see Chapter 5).

As mentioned above, a more complex pattern was observed following surface labelling of adult parasites than with third stage larvae. The wide range of components labelled by the Bolton-Hunter technique might suggest, however, that the radiolabel had penetrated the parasite cuticle and labelled somatic components, as has previously been reported with other nematodes (Marshall and Howell, 1985; Maizels *et al.*, 1989). Although no autoradiographic analysis has been carried out, this possibility would seem likely, particularly in view of the more restricted number of components labelled by the Iodogen method, which has been shown to be more surface-restricted (Forsyth *et al.*, 1981; Baschong and Rudin, 1984; Marshall and Howell, 1985; Sutanto *et al.*, 1985).

Stage-specific surface antigens were identified by immunoprecipitation studies with sera from infected and vaccinated hosts. While all hosts showed a response to L3 surface-labelled components, only those infected with normal larvae recognised adult surface antigens. Surprisingly, although somatic molecules are thought to be labelled by the Bolton-Hunter technique, vaccinated hosts showed no recognition of any labelled adult proteins. This may be due to low antigenicity of these components or to stage-specificity of the labelled somatic antigens.

The recognition of the major Iodogen labelled 30 and 45kDa adult surface antigens by serum from guinea-pigs exposed to normal and irradiated larvae indicated that these molecules are also present in the L5 stage and are expressed by irradiated larvae. It is, therefore, not clear why calves vaccinated with irradiated larvae showed no response to these surface antigens. This could be due to differences in the level of exposure as a greater number of irradiated larvae were administered to guinea-pig hosts. Our findings do, however, suggest that the 30 and 45kDa surface-associated antigens are not present in L3 and L4 parasite stages which vaccinated bovine hosts would have been exposed to in significant numbers.

Although serum from infected and vaccinated guinea-pigs recognised adult surface antigens in immunoprecipitation studies, this serum did not bind to the

adult surface in immunofluorescence. This suggests that the antigens identified by immunoprecipitation are not exposed on the adult surface. It is possible that the immunofluorescence observed was a result of antibody binding to glycolipids or polysaccharides which would not have been identified by the radioiodination/SDS-PAGE methods used. Alternatively, immunoglobulin isotypes other than those immunoprecipitated by *S. aureus* may mediate binding to the exposed surface. Treatment of iodinated intact parasites with proteases, such as trypsin which has been used to demonstrate the surface location of the 29kDa glycoprotein of adult *Brugia malayi* (Maizels *et al.*, 1989) may indicate whether the surface-associated molecules identified here are also surface exposed.

A similar pattern of antibody binding was observed with anti-ES antiserum which recognised adult surface antigens in immunoprecipitation studies but did not bind to the intact surface. It has similarly been found that monoclonal antibodies to surface-associated glycoproteins of *T.* canis, *T. cati* (Kennedy *et al.*, 1987b, Maizels, *et al.*, 1987b) and *T. spiralis* (Ortega-Pierres *et al.*, 1984) do not necessarily bind to the living parasites, suggesting the epitopes recognised are not surface exposed.

We found that anti-adult ES antiserum precipitated a 27kDa molecule which was not recognised by bovine infection serum. This recognition pattern is similar to that of a 27kDa component present in adult ES which was postulated to be of host origin (Chapter 3). Identification of this molecule following surface labelling might suggest that its presence in ES is due to surface turnover. However, as it was identified only by Bolton-Hunter mediated labelling, which is thought to penetrate and label internal components, the 27kDa protein present in ES products may be derived from an internal store, rather than being released from the surface.

Surface labelling was useful not only in identifying stage-specific antigens, but also in providing an insight into the organisation of these molecules within the cuticle. With other parasitic nematodes, the cationic detergent CTAB has been shown to cause disruption of the outermost layer of the cuticle, the glycocalyx, leaving the underlying cortical layers intact (Pritchard *et al.*, 1985, 1988b). CTAB treatment of *D. viviparus* adults labelled by the Iodogen method resulted in the release of three molecules of 13, 14 and 16kDa. Their solubility characteristics suggested that these components are expressed on the epicuticle and might, therefore, be expected to elicit an immune response. However, we could detect no

antibody recognition of CTAB extracted molecules (data not shown). It is not known whether this is perhaps due to disruption of antigenic epitopes by the detergent or if these components do not stimulate an antibody response.

In contrast to these components, molecules of 30 and 45kDa could only be released following homogenisation in detergent, suggesting that these are integral cuticular proteins (Helenius and Simons, 1975). The 30kDa component is a major surface-associated antigen of *D. viviparus* and is similar in molecular mass to a major adult surface antigen of a number of filarial nematodes (Maizels *et al.*, 1983c; Maizels *et al.*, 1985; Devaney, 1987; Morgan *et al.*, 1986; Egwang *et al.*, 1988b). This antigen is exposed on the surface of *Brugia* parasites (Devaney, 1987), is solubilised following homogenisation in PBS and is glycosylated with binding to Concanavalin A being observed (Devaney, 1988, Maizels *et al.*, 1989). While preliminary studies have indicated that the *D. viviparus* 30kDa surface antigen also binds to Con A, the difference in solubility characteristics and the failure of polyclonal antiserum to the 30kDa antigen of *B. pahangi* to recognise the *D. viviparus* 30kDa surface antigen in immunoprecipitation assays or bind to the surface of *D. viviparus* adult worms (not shown) might suggest that the *D. viviparus* antigen is unlikely to be another 30kDa homologue. It is possible, however, that the latter observations could be a consequence of the *D. viviparus* antigen being too distantly related to the filarial homologues to be antibody cross-reactive. Information on the sequence or function of these surface antigens would be useful in determining any homology.

The *D. viviparus* 30kDa surface antigen displays similar characteristics to an antigen of similar molecular mass present in adult ES products, being more abundant in male worms and showing decreased mobility on reduction. Vaccinated bovine hosts, however, showed no response to the 30kDa surface molecule, while the 30kDa ES antigen is the most dominant ES component recognised by these hosts (see Chapter 3). This suggests that the two antigens differ, although further biochemical and immunological studies will be necessary to confirm this.

There was no release of the 30kDa antigen nor of any major Iodogen surface-labelled components *in vitro*, although a limited degree of turnover of minor components of 13, 14 and 28kDa was observed. Rhoads and Fetterer (1990) reported only limited release of surface-labelled proteins of adult *H. contortus in vitro* and also found that no labelled proteins were extracted from cultured worms.

However, four labelled proteins were present in the culture medium of dead adult parasites and they concluded from this that a protease secreted by live parasites hydrolysed released cuticular proteins. It has been found in the present study that the major *D. viviparus* surface labelled proteins of 30 and 45kDa can be extracted from adult worms following culture, indicating that the absence of these components in culture medium is not due to their degradation following release from the surface.

Our findings indicate that release of the major surface proteins of *D. viviparus* does not occur. Surface turnover has most often been demonstrated with larval and microfilarial parasite stages (Philipp and Rumjaneck, 1984; Marshall and Howells, 1986; Abfel and Meyer, 1990). Release of surface molecules of adult *T. spiralis* has, however, been reported (Phillip, Parkhouse and Ogilvie, 1980) and while Marshall and Howells (1986) reported no release of radioactivity by surface-labelled adults of *B. pahangi*, this was questioned by Selkirk *et al.* (1990), as the majority of ^{125}I would have been complexed to cuticular collagens and cuticulin, which are not released *in vitro*, and to surface lipids. If surface turnover is a general phenomenon amongst adult stage parasites, our findings suggest that *D. viviparus* differs from other nematodes.

Although not shed from the surface of labelled worms during culture, the 30kDa surface-associated antigen was immunoprecipitated by adult anti-ES antiserum. This suggests that this antigen or a cross-reacting component are released from an internal source. It would, therefore, be informative to examine the localisation of this major surface antigen by immunoelectron microscopy using specific antiserum to this component.

In contrast to Iodogen-mediated labelled worms, adults labelled by Bolton-Hunter method showed significant release of labelled molecules during culture. That release was not due to damage to the nematode cuticle was suggested by the SDS-PAGE profile of released molecules. This bore greater resemblance to ES products labelled following their release from live adults in culture than to extracts of surface-labelled adults, indicating the selective release of labelled components. The large number of components released from Bolton-Hunter but not from Iodogen labelled adults further indicates that internal components were labelled by the former method. Significant loss of radiolabel after Bolton-Hunter mediated labelling of adult *B. pahangi* has similarly been attributed to the penetrating

nature of the Bolton-Hunter reagent (Marshall and Howells, 1986).

Three components of adult parasites were solubilised only following treatment with 2ME, which has been shown with other parasitic nematodes to dissociate collagenous proteins which are cross-linked by disulphide bonds (McBride and Harrington, 1967; Cox *et al.*, 1981a; Adams, 1978). The sensitivity of these components to *Clostridium histolyticum* collagenase confirmed their collagenous nature. It is generally thought that cuticular collagens are not exposed on the nematode surface, but are localised primarily to the basal and inner cortical layers of the cuticle. The identification of *D. viviparus* collagens following Iodogen-mediated iodination indicates that although this method is thought to be surface restricted the radiolabel can penetrate the cuticle of adult *D. viviparus*, to some extent. Although cuticular collagens are thought to be inaccessible to the immune system in intact worms, infected calves showed an antibody response to collagens of adult worms. This demonstrated that *D. viviparus* adult cuticular collagens are antigenic and are exposed to the host during natural infection. However, adult cuticular collagens were not recognised by hosts exposed to 40krad irradiated larvae on three occasions, suggesting that there is no cross-reactivity between larval and adult cuticular components or, alternatively, that cuticular collagens are not exposed to the immune system of vaccinated hosts.

Collagens are probably exposed to the host during moulting or following damage to the outer cortical layer. These components have tended to be ignored, with most studies concentrating on the identification of "surface" antigens. It would be interesting to determine whether antibodies directed against collagenous proteins are damaging to the worms, as suggested by Pritchard *et al.* (1988a), thus being relevant to protective immunity. Such antibodies may also be involved in the pathology of infection due to cross-rectivity between parasite and host collagens as demonstrated with the human filarial nematode *Brugia malayi* (Selkirk *et al.*, 1989). Alternatively, it has been postulated that the ability of collagen to promote platelet aggregation and activate their cyclo-oxygenase and lipoxygenase pathways may be relevant to the pathogenesis of parasitic infections (McCord and Leid, 1988; Selkirk and Blaxter, 1990).

Several surface-associated components of both the L3 and adult stages were only extracted following treatment with 2ME/SDS/urea and are presumably involved in maintaining the structure of the cuticle. Following such treatment of

third stage larvae, approximately 40% of the radiolabel was associated with the insoluble fraction. Similar findings have been reported for the sheaths of *H. contortus* L3 (Fetterer and Rhoads, 1990) and for adult stages of *Dipetalonema viteae* (Betschart and Jenkins, 1987), *Dirofilaria immitis* (Scott *et al.*, 1988), and *Brugia* species (Selkirk *et al.*, 1989). The insoluble material, termed 'cuticulin' (Fujimoto and Kanaya, 1973) is thought to represent the parasite epicuticle. It is insensitive to collagenase, but can be partially digested by a number of proteolytic enzymes, notably elastase (Selkirk *et al.*, 1989; Betschart, Marti and Glaser, 1990). Although insoluble surface antigens have been identified (Murrell and Graham, 1982) the difficulties in analysing such material may mean that the predominant "surface" antigens of most nematode parasites have yet to be defined.

CHAPTER 7

ANTIBODY RESPONSE TO *D. VIVIPARUS* SOMATIC ANTIGENS AND EFFECTS OF IRRADIATION ON PARASITE PROTEIN SYNTHESIS

7.1 INTRODUCTION

Attempts to vaccinate against parasitic helminths with extracts of whole parasites have been carried out since the 1930's. As mentioned in Chapter 1, this method has, in most cases, met with little success. It was speculated that interaction between the host and living parasites is essential to the induction of protective immunity and that this could not be achieved with dead parasite material.

Significant protection was achieved, however, with soluble extracts of fourth stage larvae of *Trichostrongylus colubriformis* in guinea-pigs although the molecular complexity of this extract made it impossible to identify the antigens responsible for immunity (Rothwell and Love, 1974). More recent work, involving DOC extraction of PBS-insoluble somatic components of *T. colubriformis* L4, has resulted in the isolation of a potentially protective 41kDa antigen (O'Donnell *et al.*, 1989). Surprisingly, antibodies to this antigen, which is associated with the muscle layer of the parasite, are not present in sera from naturally infected guinea-pigs or sheep. Therefore, although immunisation with this antigen induces approximately 50% protection, it does not appear to be exposed to the host during normal infection. This is reminiscent of the findings of Pritchard *et al.* (1988a) with cuticular collagens of *Necator americanus* which are potentially immunogenic but are not recognised by infected humans. This may indicate that parasite somatic antigens are potentially protective but must be presented to the host in an appropriate way. This has previously been demonstrated to be true of *S. mansoni* paramyosin, which was found to induce significant protection in mice when administered intradermally with adjuvant but to be ineffective when adaministered intravenously or intramucscularly, or in the absence of adjuvant (James, 1985).

Following the success of ES products in immunisation against some helminth infections (reviewed by Lightowlers and Rickard, 1988) it was speculated that ES antigens were synthesised and secreted by living parasites, but not present in any appreciable quantity in homogenate. Later studies, however, demonstrated that secretory granules could be isolated from soluble extracts of *T. spiralis* (Despommier and Muller, 1970a). Vaccination with α and ß secretory granules derived from the nematode stichosome induced significant protection in mice,

reducing the number of muscle larvae resulting from a challenge infection by up to 98% (Despommier and Muller, 1970b; Despommier, Campbell and Blair, 1977).

Similarly, soluble antigens derived from the stichosome of *T. muris* have been used to vaccinate mice effectively (Wakelin and Selby, 1973; Jenkins and Wakelin, 1977). These antigens showed immunological identity with material secreted by worms *in vitro*. Therefore, where stored ES antigens can be readily obtained from homogenates of whole parasites, the latter may be used as an alternative source of protective antigens. This is particularly useful with parasites such as *D. viviparus* third stage larvae which release little material *in vitro* or with parasite stages or species which are difficult to maintain in culture.

It has been found that PBS-soluble extracts of *D. viviparus* can induce protection against lungworm infection in guinea-pigs with 66% protection being achieved with L3 extract and 30% with that of the adult stage (G.J. Canto, C. Britton, G.M. Urquhart, M.W. Kennedy, unpublished; J.B. McKeand, C.Britton, G.M. Urquhart, M.W. Kennedy, unpublished). This protection may be induced by antigens normally exposed to the host, namely ES or surface components which may be present in the soluble extract. *D. viviparus* extracts may, therefore, act as a source of ES and surface antigens which are difficult to isolate in significant amounts, particulary from the L3 stage. In view of the work of Pritchard *et al.* (1988a), however, it is also possible that somatic components exposed to the host during moulting or following parasite damage are also involved in this protection. To identify potentially protective antigens of *D. viviparus* present in soluble extracts an examination of the antibody response to L3 and adult somatic antigens in naturally infected and vaccinated bovine hosts has now been carried out. The response to such antigens may also be relevant to the immunopathology of dictyocaulosis which is associated with parasite death in the lungs (Jarrett *et al.*, 1960a).

This is the first qualitative study of antibody recognition of *D. viviparus* somatic antigens. Previous quantitative assays (Boon *et al.*, 1982; Bos and Beekman, 1985; Bos *et al.*, 1986; Boon, Ploeger and Raaymaker, 1986) have indicated that such studies may be useful in the serodiagnosis of lungworm disease in cattle, but give no indication of immunity to infection. In the present study these responses were examined at the molecular level. Also presented here are the results of a preliminary examination of somatic proteins synthesised by normal

and irradiated infective larvae during *in vitro* maintenance. Any differences in antigen expression between these larval forms may be relevant to the mechanism of vaccine-induced immunity.

7.2 RESULTS

7.2.1 SDS-PAGE profile of larval and adult somatic proteins

Somatic components of third stage larvae and adult parasites were compared by examining the Coomassie stained SDS-PAGE profiles of Tris-soluble parasite extracts under reducing and non-reducing conditions. A complex range of proteins was extracted as shown if Figure 7.1. A number of components in the range 26-67kDa appeared to be common to larval and adult stages. In contrast, the migration on SDS-PAGE of low molecular mass proteins of approximately 14-20kDa differed between the the two stages, indicating the stage-specificity of these components.

Differences in migration between Tris-soluble extracts of male and female adults were also detected (tracks b and c). In particular proteins of 16 and 19kDa were more abundant in extracts of male parasites, while components of 110-120kDa appeared to be female-specific. These female-specific proteins were not present in L3 homogenate suggesting that they are not derived from developing larvae, but may be involved in egg production or release. Soluble extracts of mixed sexes of adult worms, containing a full complement of adult somatic proteins, were used in subsequent studies.

7.2.2 Antigenicity of larval somatic components

Tris homogenates of third stage larvae were radiolabelled with ^{125}Iodine by the Bolton-Hunter and Iodogen techniques. Immunoprecipitation of labelled extracts with sera from calves and guinea-pigs infected or vaccinated on two occasions was performed in a Staph A-based assay. SDS-PAGE analysis of immunoprecicitates with iodinated L3 homogenate revealed that components of 15, 29-33 and 45kDa were strongly immunogenic (Figure 7.2). Moreover, identical recognition profiles were observed with sera from infected or vaccinated bovine and guinea-pig hosts.

A significant degree of precipitation of radio-labelled L3 components was observed, however, with normal bovine serum. This did not occur to the same extent with normal guinea-pig serum. Moreover, pre-incubation of iodinated

antigen with an equal volume of Staph A prior to immunoprecipitation did not appear to ameliorate this binding. This may, therefore, be due to specific recognition of radioiodinated L3 antigens by normal bovine serum.

7.2.3 Antibody recognition of L3 somatic antigens by immunoblotting

Because of the high level of binding of normal bovine serum to radioiodinated L3 homogenate and the smearing often observed even with freshly labelled homogenate, the response to *D. viviparus* somatic antigens was examined by immunoblotting. Tris-soluble extracts of L3 parasite were separated by SDS-PAGE, electro-blotted onto nitrocellulose and probed with bovine and guinea-pig antisera to infection or vaccination with irradiated larvae. As can be seen in Figure 7.3, there was very little binding of normal bovine serum to L3 components on immunoblots. There was also better resolution of individual components, with recognition of a large number of antigens being clearly observed, particularly with bovine antiserum. The majority of these antigens had a molecular mass of approximately 23-110kDa.

Examination of the kinetics of antibody repertoire against L3 somatic antigens indicated quantitative differences between infected and vaccinated hosts. Naturally infected calves showed a slight decrease in antibody level with repeated exposure to larvae, as indicated by the decreasing density of recognised bands. In contrast, calves vaccinated with 40krad irradiated larvae showed an increase in antibody response following subsequent vaccinations and challenge infection, while the response of 100krad vaccinates remained constant. These findings are paralleled by the antibody responses measured by ELISA, as will be shown later. With the exception of an L3 antigen of approximately 17kDa, a response to which was not observed in sera from 40krad vaccinates, there were no qualitative differences in L3 antigen recognition by infected and vaccinated bovine hosts.

The recognition profile of infected and vaccinated guinea-pigs was similar to that of bovine hosts, although guinea-pigs showed stronger recognition of antigens of 23-24kDa and a weaker response to higher molecular mass components (also shown in Figure 7.3). Guinea-pigs immunised with adult Tris homogenate showed significantly weaker recognition of these antigens, but did respond to an antigen of approximately 18kDa, not recognised by guinea-pig or bovine antisera to infection or vaccination.

7.2.4 Antibody recognition of adult homogenate

In contrast to the findings with L3 homogenate, iodinated adult somatic antigens were recognised only by naturally infected bovine hosts (Figure 7.4). All labelled adult somatic components were immunoprecipitated, with the notable exception of a major component of 26kDa. This is of identical M_r to an adult ES component which also fails to be recognised by bovine immune sera (see Chapter 3). Calves vaccinated on two occassions with 40krad irradiated larvae or guinea-pigs exposed to normal larvae showed no recognition of adult somatic antigens. This failure to detect any antigen recognition may, however, have been a consequence of the low level of radiolabelled antigen used in immunoprecipitation due to the poor labelling efficiency of parasite extracts.

7.2.5 Antibody recognition of adult somatic antigens on immunoblots

The resolution of adult somatic antigens was also improved by immunoblotting. As can be seen in Figure 7.5, calves infected with normal larvae recognised a wide range of adult antigens. These appeared to correlate with antigens present in immunoprecipitates of radiolabelled adult homogenate, with components of 12-16kDa being strongly recognised, as well as antigens of 23, 40-50 and 100kDa. There was also specific recognition of high molecular mass antigens (210-400kDa), observed as a smear at the top of the separating gel.

In contrast to the previous observations (Figure 7.4), where calves vaccinated with two doses of irradiated larvae showed no recognition of radio-iodinated adult antigens, the calves examined here, which had been vaccinated on three occasions with a greater number of irradiated larvae (see Table 4.1) showed significant recognition of adult somatic antigens on immunoblots. Their recognition profile was similar to that of naturally infected hosts, with the exception of low molecular mass antigens of approximately 12 and 14kDa which were not recognised by hosts vaccinated with 40krad irradiated larvae. Guinea-pigs infected with normal larvae, or vaccinated with adult homogenate also showed no recognition of antigens of low molecular mass, but did respond to high molecular mass adult antigens, although less strongly than did bovine hosts. Identical recognition profiles were observed with antiserum from guinea-pigs exposed to normal or X-irradiated larvae or vaccinated with adult homogenate, although the level of response to a 23kDa antigen was lower in guinea-pigs vaccinated with X-

irradiated larvae.

Following repeated infections or vaccinations, qualitative and quantitative changes in the antibody response to adult antigens were observed. Repeated exposure of bovine hosts to normal larvae resulted in a decrease in antibody response to antigens of 12-16kDa and 40-50kDa, while there was no longer any recognition of the 23kDa antigen. Repeated vaccination and challenge infection of 100krad vaccinates resulted in a loss of reactivity to the 16kDa antigen. Recognition of this component also decreased following repeated vaccination with 40krad irradiated larvae and challenge infection, while the level of response to antigens of 40-50kDa and 110kDa increased slightly in these hosts.

7.2.6 Detection of antibody response to *D. viviparus* somatic antigens by ELISA

The level of antibody response of calves exposed on several occasions to normal, 40krad or 100krad irradiated larvae was measured by ELISA against both L3 and adult Tris-soluble extracts, with the O.D. at 492nm used as a measure of IgG antibody response. Sera were sampled from calves prior to infection or vaccination carried out as described in Table 4.1.

A single infection with normal larvae induced a significant response to L3 somatic antigens. As shown in Figure 7.6, this was first detected 14-21 days after infection with 1,000 larvae. The antibody level increased slightly following subsequent infections, remained constant, then decreased gradually after 4 infections (Figure 7.7, Panel A). Some heterogeneity in the antibody level of individual calves was observed, although the overall pattern of antibody responses were identical.

In contrast to natural infection, a single vaccination with 40krad irradiated larvae (as detailed in Table 4.1) resulted in no substantial antibody response to L3 somatic antigens (Figure 7.7, Panel B). Subsequent vaccinations induced a gradually increasing response, which reached a maximal level after 5 exposures to irradiated larvae. The level of response decreased slightly after subsequent vaccinations, but was boosted following challenge infection with normal larvae.

The kinetics of antibody recognition of L3 somatic antigens by calves vaccinated with 100krad irradiated larvae differed from that of both naturally infected and 40krad vaccinated hosts. A single vaccination with 100krad larvae

induced a substantial antibody response to L3 antigens (Figure 7.7, Panel C), but this was not as great as that observed with sera from naturally infected hosts. The response increased gradually, reaching a peak after 4 vaccinations, then decreased slightly. As observed with 40krad vaccinates, there was a slight increase in antibody level following challenge infection of 100krad vaccinates.

The pattern of response to adult homogenate was identical to that measured against L3 antigens. Naturally infected hosts again showed a sharp and significant rise in antibody level 14-21 days after infection (Figure 7.8 and Figure 7.9, Panel A), while the response of vaccinated hosts developed more slowly and did not rise to as high a level, particularly in 100krad vaccinates (Figure 7.9, Panels B and C).

7.2.7 Recognition of antigens common to L3 and adult stages

The similarity in kinetics of the antibody response to L3 and adult somatic antigens suggested that hosts may be recognising antigens common to both stages. This was examined by plotting the response to larval homogenate antigens against the corresponding anti-adult value, for each observed time point during infection (Panel A) or vaccination with 40krad (Panel B) or 100krad (Panel C) irradiated larvae. A linear relationship was found between the responses to the two parasite stages in naturally infected and vaccinated hosts (Figure 7.10) suggesting recognition of antigens common to both stages.

7.2.8 Protein synthesis by normal and irradiated third stage larvae

To examine any differences in the synthesis of parasite components by normal and irradiated larvae, exsheathed third stage larvae were biosynthetically labelled with ^{35}S-methionine. Irradiated larvae were cultured immediately after irradiation in a ^{60}Co source. Culture medium was collected after 6 days and parasite proteins extracted sequentially in Tris homogenisation buffer, then in 1% DOC followed by 1% SDS/5% 2ME/8M urea (harsh extraction). SDS-PAGE analysis and fluorography showed the presence of one heavily labelled component of 67kDa in the culture medium (data not shown). This was probably due to the uptake of ^{35}S-methionine by albumin present in the newborn calf serum used to supplement the culture medium, as has previously been observed (Parkhouse *et al.*, 1985). Approximately 1% of the total incorporated radioactivity was present

in the culture medium, while the Tris-soluble extract, DOC homogenate and harsh extract contained approximately 9%, 4% and 86% of the total counts, respectively. Identical results were obtained following extraction of normal or irradiated larvae.

Separation of DOC-soluble extracts by SDS-PAGE revealed a complex array of labelled components. Identical profiles of ^{35}S-labelled proteins were observed with normal, 40krad or 100krad irradiated larvae (Figure 7.11). Tris-soluble labelled proteins of normal or irradiated larvae also showed identical profiles on SDS-PAGE (not shown). Individual labelled proteins present in the harsh extract were not clearly resolved on SDS-PAGE due to the high level of ^{35}S-methionine incorporation into proteins present in this fraction.

To examine whether protein synthesis was altered in the 24 hour period immediately after irradiation, normal and 40krad irradiated L3 parasites were cultured in medium with added ^{35}S-methionine and labelled components extracted as before. Following SDS-PAGE analysis no ^{35}S-labelled proteins were detected in parasite extracts or ES products of normal or irradiated parasites after 2 or 4 hours in culture. After 24 h, however, identical labelled components were detected in harsh extracts of normal and irradiated larvae but no labelled components were present in Tris or DOC extracts at this time. The high level of radioactivity incorporated into SDS/2ME/urea-soluble proteins may have been due to the synthesis of cuticular proteins prior to the moulting of larvae to the L4 stage which occurred after approximately 2-3 days in culture.

Figure 7.1 SDS-PAGE profile of L3 and adult somatic proteins. 30μg of L3 (a), male (b) and female (c) adult Tris-soluble extracts were electrophoresed on 5-25% gradient SDS-gels under non-reducing (A) and reducing (B) conditions and proteins stained with Coomassie blue. The relative molecular mass (M_r) of marker proteins are shown in kiloDaltons(kDa).

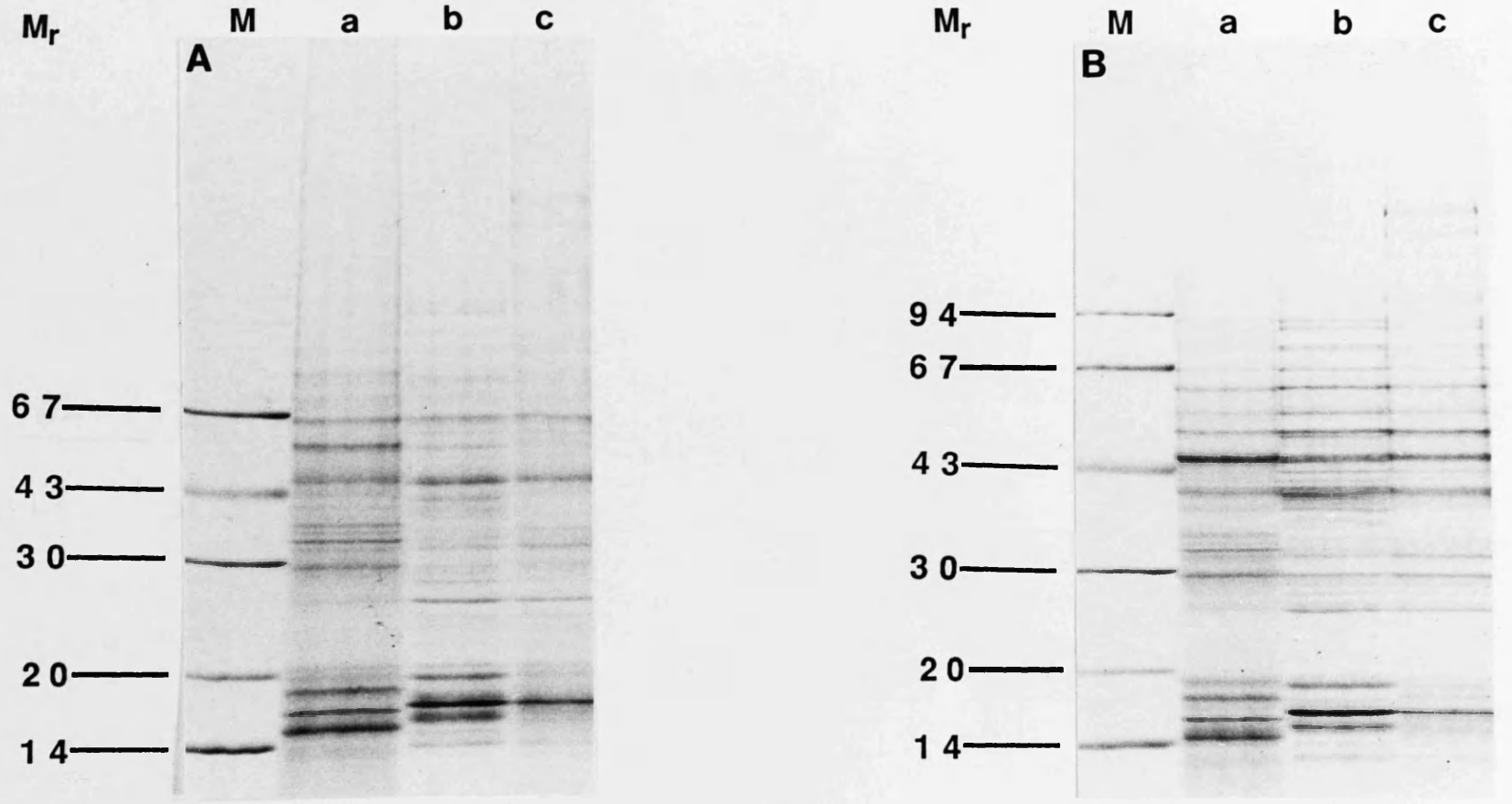

Mr
M
a
b
c
A
67
43
30
20
14
Mr
M
a
b
c
B
94
67
43
30
20
14

Figure 7.2 Antigenicity of L3 extract. L3 homogenate (R), radioiodinated by the Iodogen method, was immunoprecipitated with sera from bovine and guinea-pig hosts which had been exposed to the parasite as follows: vaccinated on 2 occasions with 40krad irradiated larvae and challenged 28 days later with normal larvae (V), or infected on 2 occasions with normal larvae (I). Serum was collected from bovine and guinea-pig hosts 25 and 7 days after challenge, respectively. Track N shows immunoprecipitation of L3 homogenate with pre-infection bovine and guinea-pig serum. The M_r of marker proteins (M) are shown in kDa.

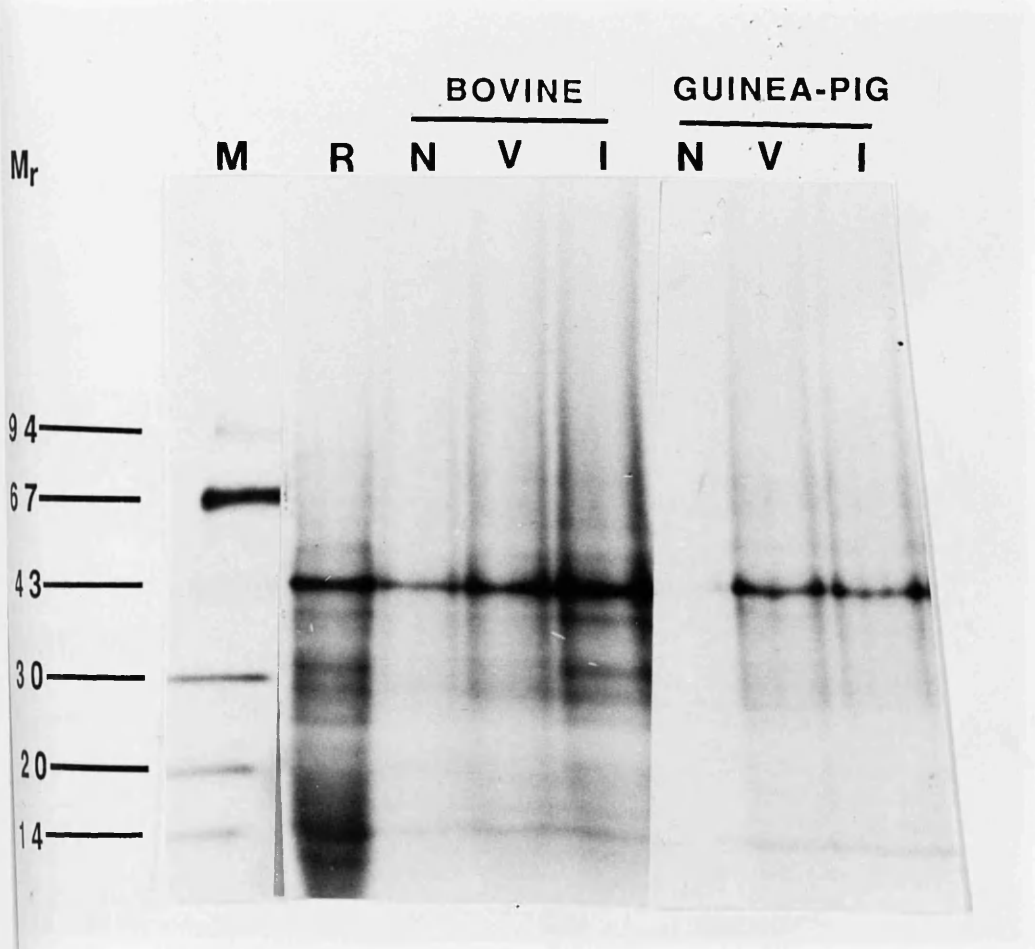

BOVINE
GUINEA-PIG
Mr
M
R
N
V
I
N
V
I
94
67
43
30
20
14

Figure 7.3 Recognition of L3 somatic antigens by bovine and guinea-pig hosts. L3 Tris-soluble extract was electrophoresed on 5-25% gradient SDS-PAGE and electro-blotted onto nitrocellulose. This was probed with serum from calves exposed to normal (I), 40krad irradiated (V) or 100krad irradiated (X) larvae. Serum was collected approximately 20 days after 3 infections or vaccinations (tracks a) and after 7 infections with normal larvae or challenge infection of calves repeatedly vaccinated with irradiated larvae (tracks b) (see Table 4.1 for details of infections and vaccinations). Blotted proteins were also probed with serum from guinea-pigs infected with 2 doses of normal (I) or 40krad irradiated (V) larvae, or immunised on 2 occasions, 28 days apart, with 100μg adult PBS-soluble extract in FCA (H). Guinea-pig hosts were challenged after 28 days with normal larvae and serum collected 7 days later. L3 homogenate probed with normal bovine or guinea-pig serum is shown (tracks N). Transferred proteins stained with amido black are shown in track R. The M_r of pre-stained marker proteins are indicated in kDa.

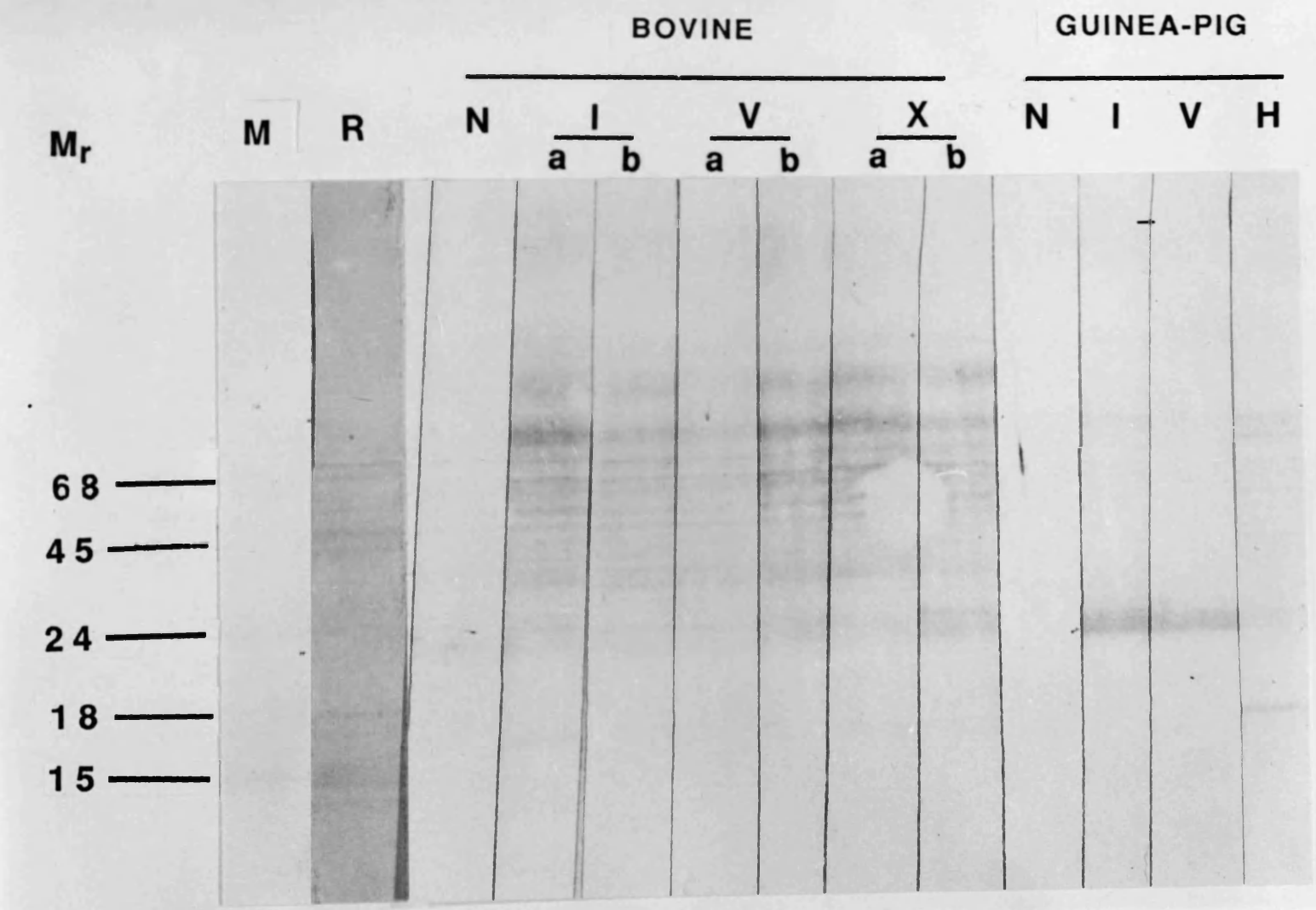

BOVINE
GUINEA-PIG
Mr
M
R
N
I
a
b
V
a
b
X
a
b
N
I
V
H
68
45
24
18
15

Figure 7.4 Antigenicity of adult extract. Bolton-Hunter radioiodinated adult extract (R) was immunoprecipitated with sera from bovine and guinea-pig hosts which had been vaccinated with irradiated larvae and exposed to challenge infection (V) or infected on two occasions with normal larvae (I), as described in Figure 7.2. Immunoprecipitation with bovine and guinea-pig pre-infection serum (N) is shown. The M_r of marker proteins (M) are indicated in kDa.

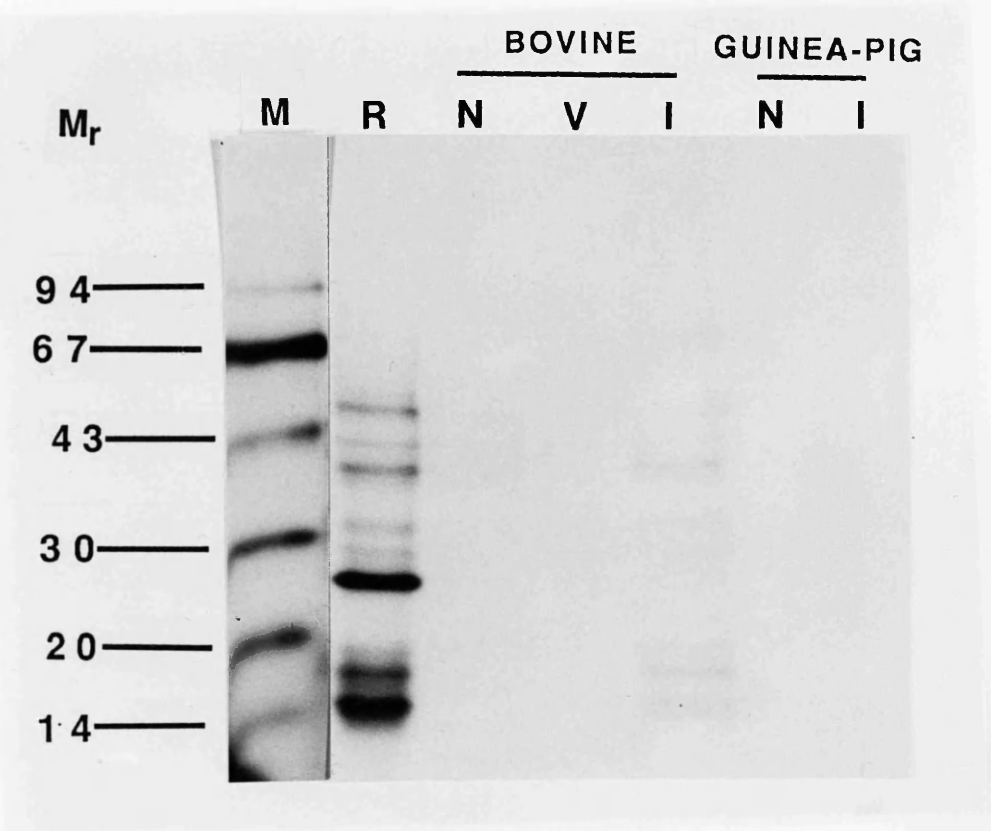

BOVINE
GUINEA-PIG
Mr
M
R
N
V
I
N
I
94
67
43
30
20
14

Figure 7.5 Antibody recognition of adult somatic antigens by bovine and guinea-pig hosts. Adult Tris-soluble extract was blotted onto nitrocellulose and stained with amido black (R) or probed with serum from calves exposed to normal (I), 40krad irradiated (V) or 100krad irradiated (X) L3, as described in Figure 7.3. Serum was sampled 20 days after three infections or vaccinations (tracks a) and 22 days after the final infection or after challenge infection of vaccinated hosts (tracks b). Tracks probed with serum from guinea-pig hosts exposed to normal (I) or 40krad irradiated (V) larvae, or immmunised with adult homogenate (H) and challenged with normal larvae, as described in Figure 7.3 are also shown. Tracks N show blottted proteins probed with normal bovine or guinea-pig serum.

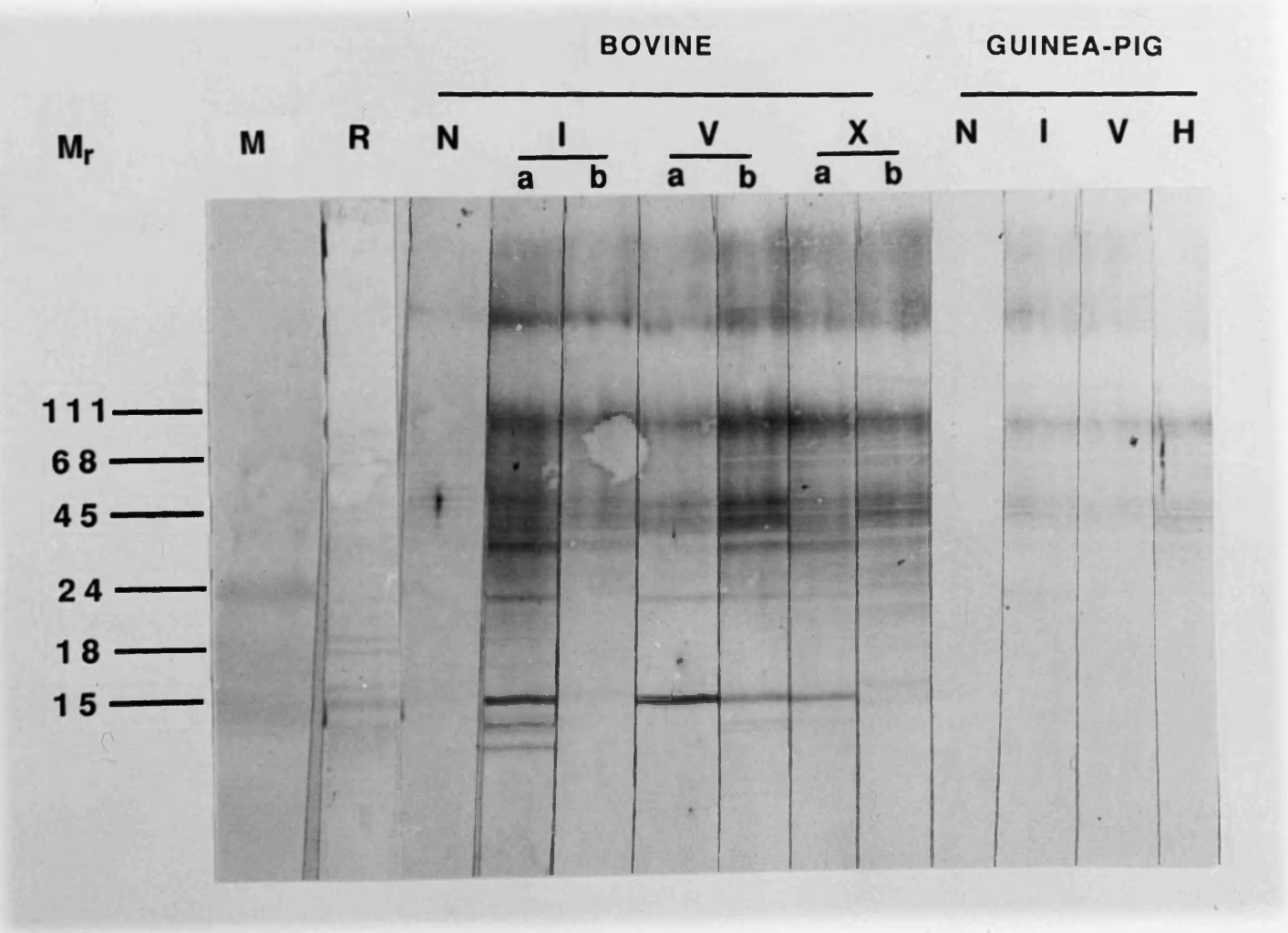

BOVINE
GUINEA-PIG
Mr
M
R
N
I
a
b
V
a
b
X
a
b
N
I
V
H
111
68
45
24
18
15

Figure 7.6 Recognition of L3 somatic antigens after a single infection. Serum was sampled from calves prior to infection with 1,000 L3 on day 0, and at weakly intervals thereafter. The O.D. 492nm was used as a measure of antibody response to L3 extract in ELISA studies. This shows the level of antibody response using pooled serum from 3 calves.

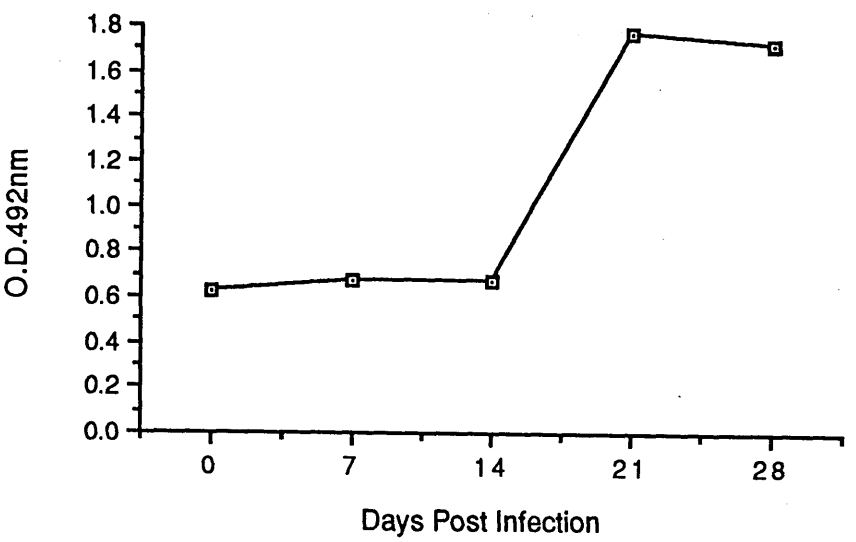
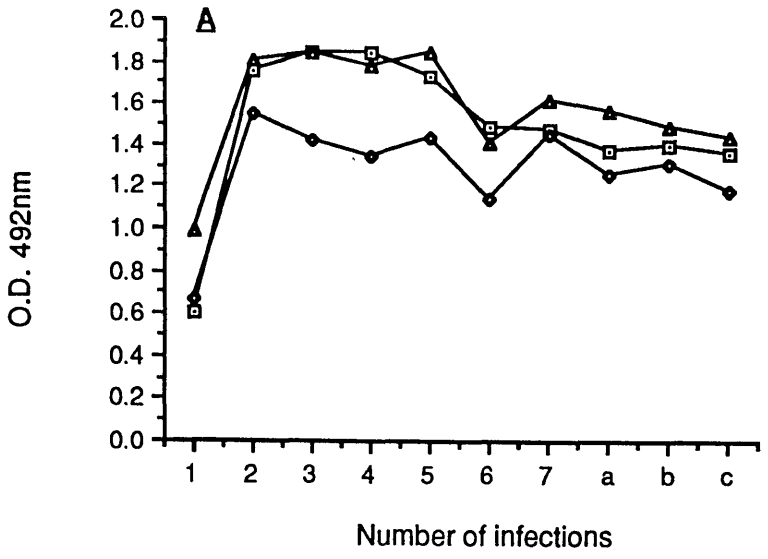
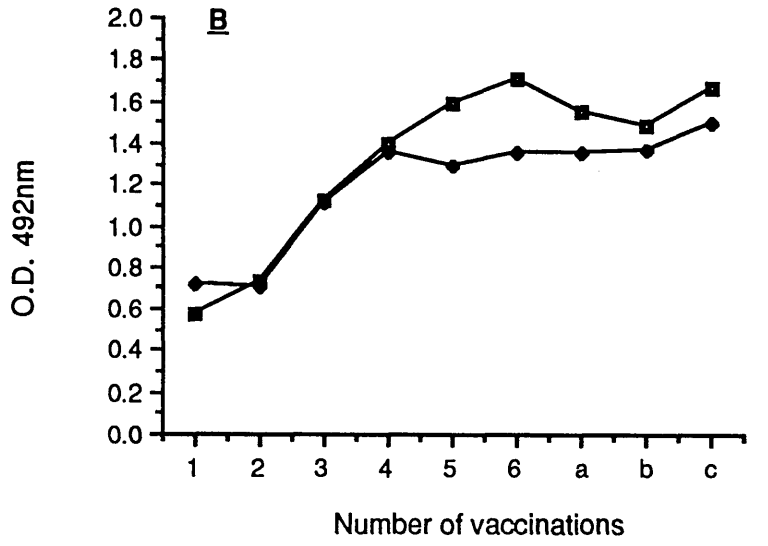

O.D.492nm
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
7
14
21
28
Days Post Infection

Figure 7.7 Kinetics of antibody response to L3 homogenate. Calves were repeatedly exposed to normal (A), 40krad irradiated (B) or 100krad irradiated (C) L3, as detailed in Table 4.1. Serum was sampled prior to each infection or vaccination and at 7 (a), 14 (c) and 21 (b) days after the final infection (Panel A) or after challenge with normal larvae administered 24 days after the final vaccination (panels B and C). The O.D. at 492nm was used as a measure of antibody response to L3 antigens in ELISA and the means of duplicate samples are shown. The antibody response of individual calves in each group is shown.


A
O.D. 492nm
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 a b c
Number of infections


B
O.D. 492nm
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1 2 3 4 5 6 a b c
Number of vaccinations

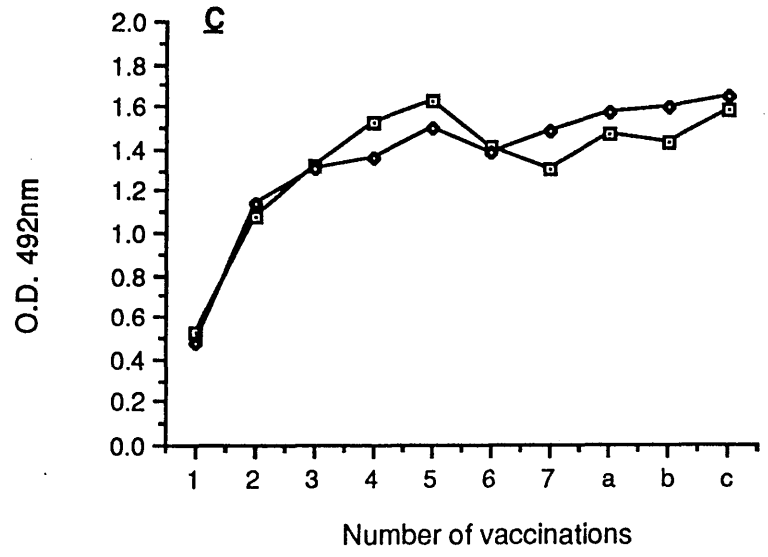

C
O.D. 492nm
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 a b c
Number of vaccinations

Figure 7.8 Antibody response to adult somatic antigens after a single infection. Calves were infected with 1,000 normal larvae and serum sampled prior to infection on day 0 and at weekly intervals therafter. Serum was pooled from 3 individuals and tested in ELISA against adult homogenate. The means of duplicate samples are shown.

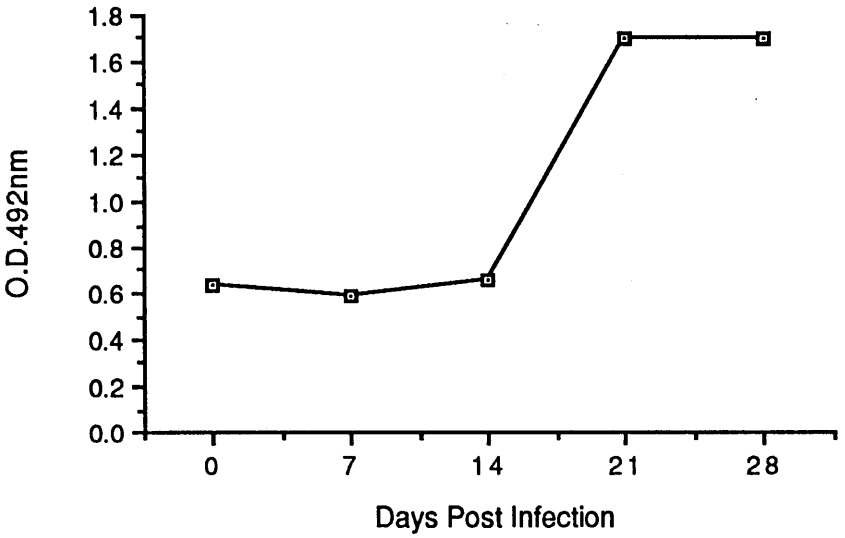
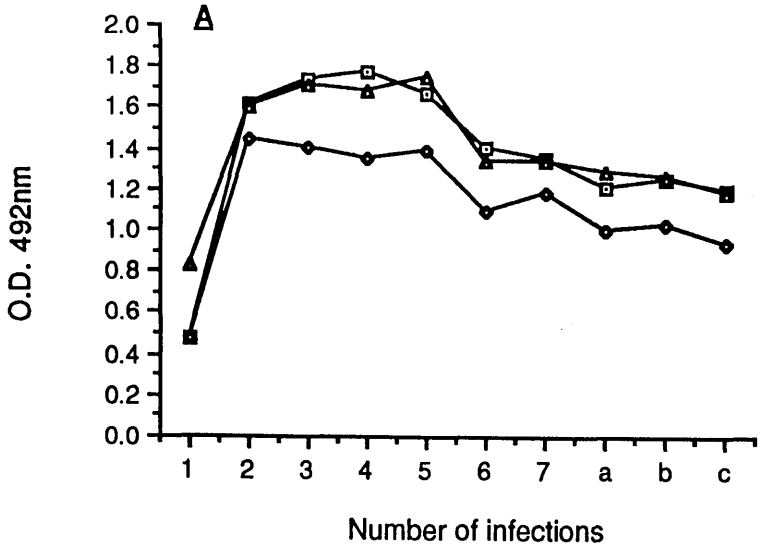
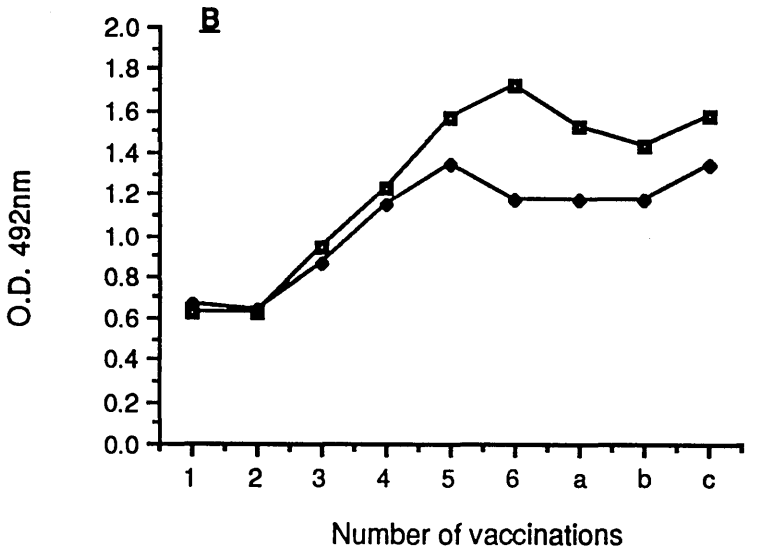

O.D.492nm
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
7
14
21
28
Days Post Infection

Figure 7.9 Kinetics of antibody response to adult somatic antigens. Calves were exposed to normal (A), 40krad irradiated (B) and 100krad irradiated (C) larvae on several occasions as detailed in Table 4.1. Serum was sampled prior to each exposure and at 7 (a), 14 (b) and 21 (c) days after challenge infection. The O.D. at 492nm measured in ELISA against adult homogenate is shown for individual calves in each group.


A
O.D. 492nm
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 a b c
Number of infections


B
O.D. 492nm
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1 2 3 4 5 6 a b c
Number of vaccinations

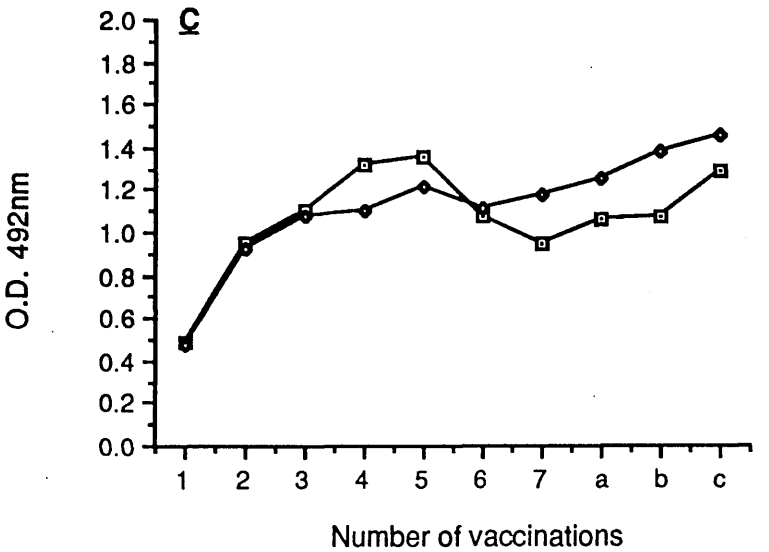
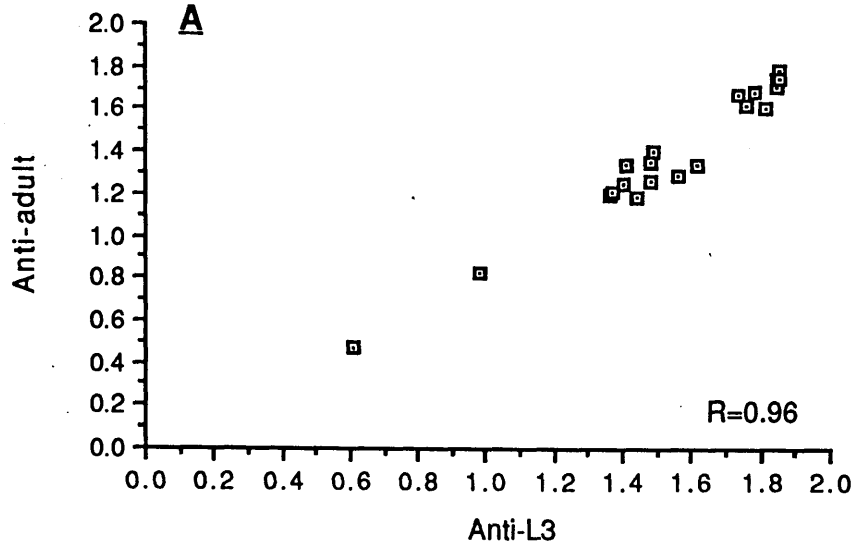
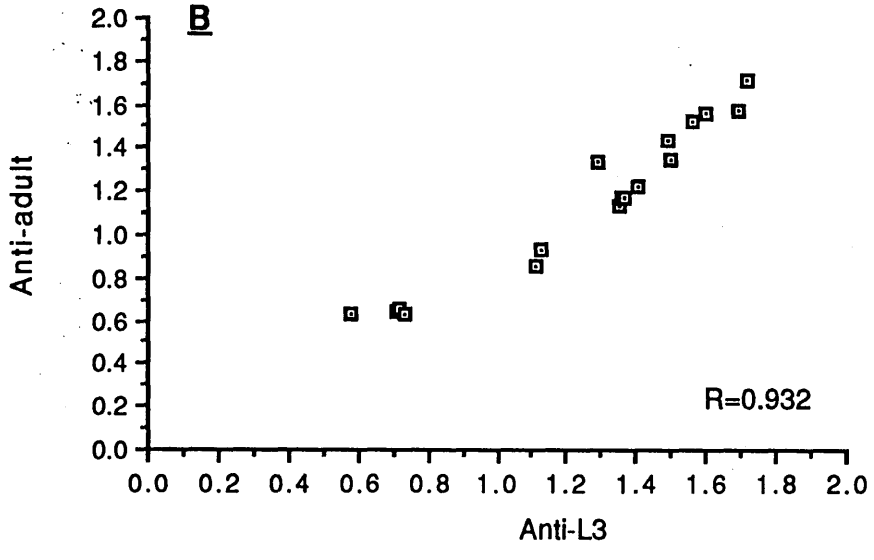

C
O.D. 492nm
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 a b c
Number of vaccinations

Figure 7.10 Correlation between level of responses to larval and adult somatic antigens. The absorbance at 492nm measured against L3 homogenate in ELISA was plotted against the corresponding anti-adult ELISA value. Sera were tested from calves exposed on several occasions to normal (A), 40krad irradiated (B) or 100krad irradiated (C) larvae as described in Table 4.1. A linear relationship between the responses was found with correlation coefficients as shown.


A
Anti-adult
Anti-L3
R=0.96
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0


B
Anti-adult
Anti-L3
R=0.932
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0

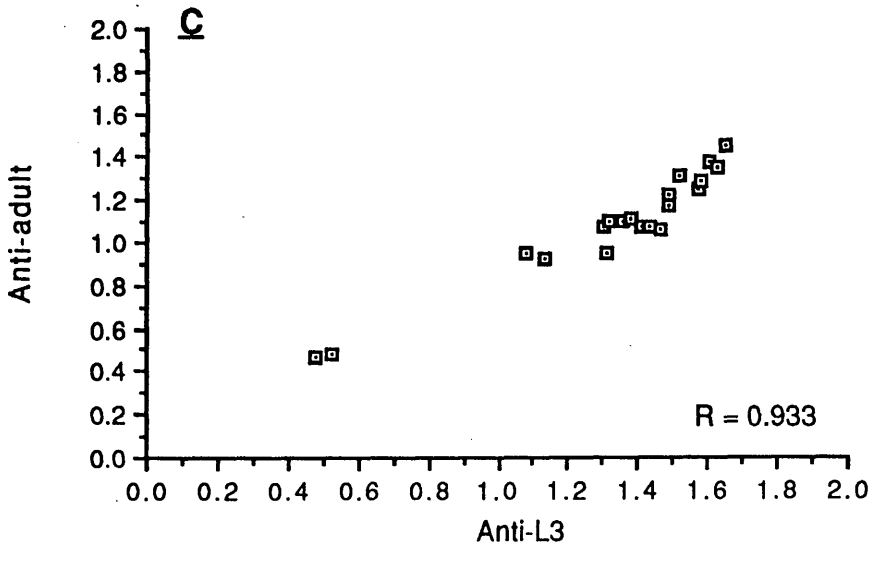

C
Anti-adult
Anti-L3
R = 0.933
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0

Figure 7.11 *In vitro* synthesis of somatic proteins by normal and gamma-irradiated larvae. Normal (a), 40krad irradiated (b) and 100krad irradiated (c) exsheathed third stage larvae were biosynthetically labelled with ^{35}S-methionine at 37^{o}C in MEM supplemented with 20% (v/v) NBCS. DOC-soluble proteins, extracted from parasites which had been cultured for 6 days, were analysed by SDS-PAGE under reducing conditions.

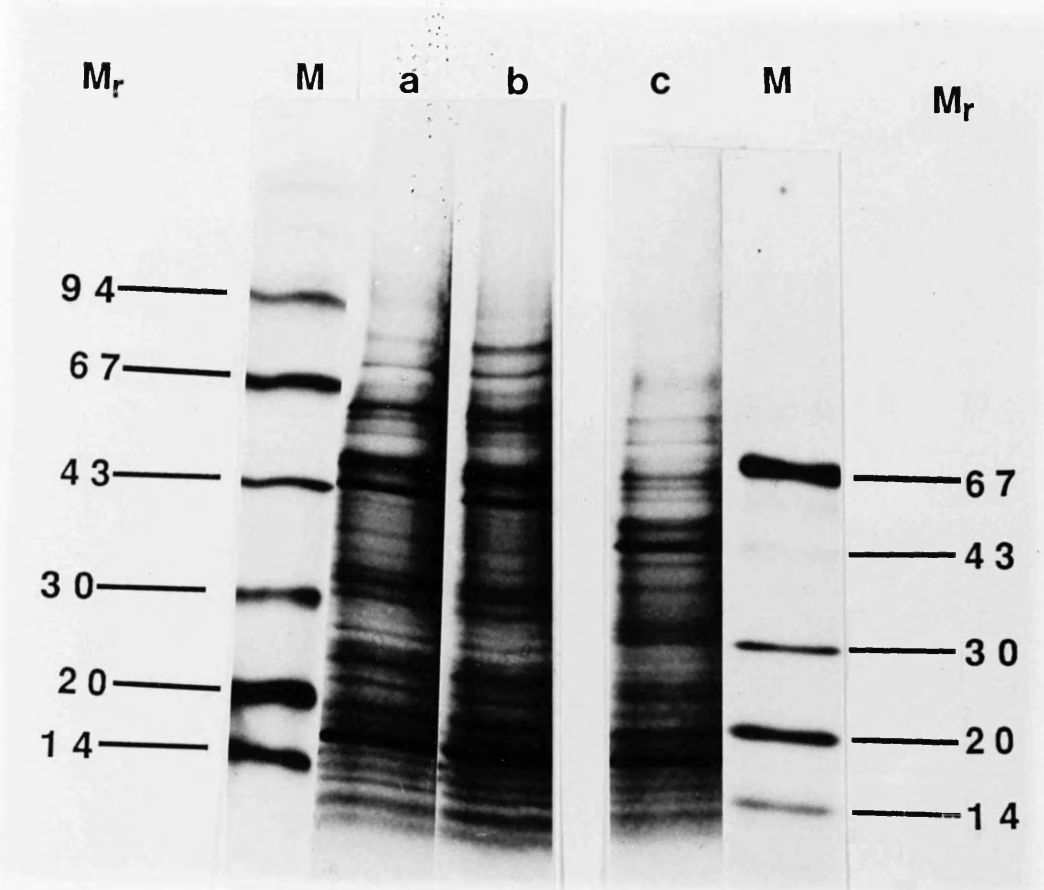
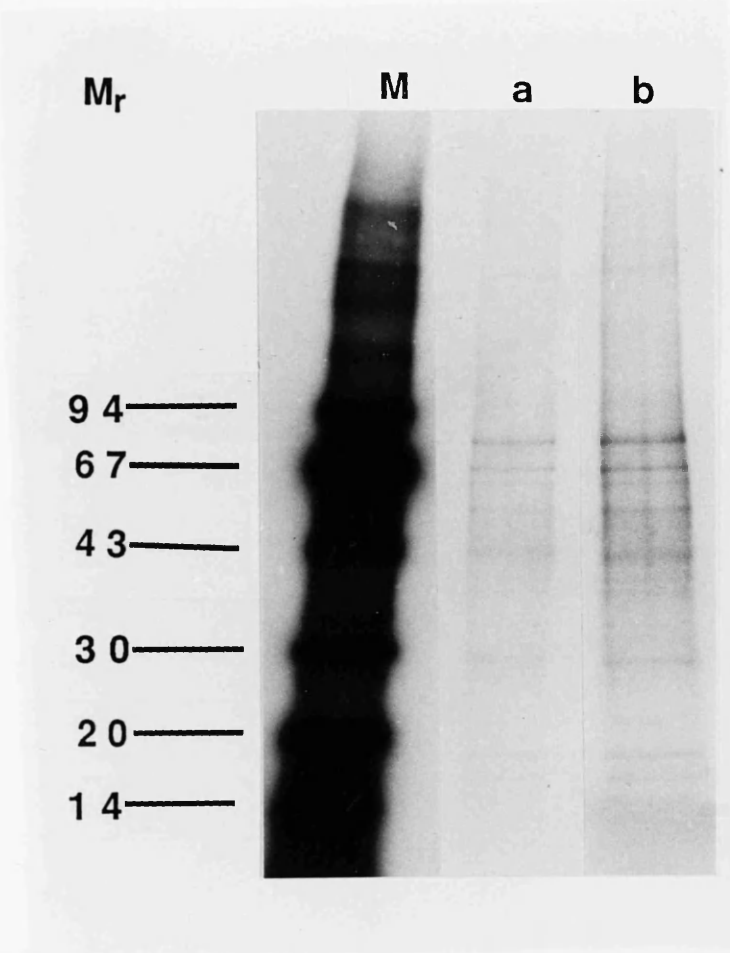

Mr
M
a
b
c
M
Mr
94
67
43
30
20
14
67
43
30
20
14

Figure 7.12 Proteins synthesised by normal and irradiated larvae during 24 hours. Normal (a) and 40krad irradiated (b) exsheathed larvae were biosynthetically labelled with ^{35}S-methionine as in Figure 7.11. After 24h in culture, parasites were homogenised and proteins sequentially extracted as described in Chapter 2. This shows the profile of SDS/urea-soluble labelled proteins analysed by SDS-PAGE under reducing conditions.


Mr
M
a
b
94
67
43
30
20
14

7.3 DISCUSSION

It has previously been shown that somatic antigens of L3 and adult stages of *D. viviparus* induce significant protection in the guinea-pig model (G.J. Canto *et al.*, unpublished; J.B. McKeand *et al.*, unpublished). This study characterised the proteins present in these parasite extracts and demonstrated their antigenicity in the context of infection and vaccination with irradiated larvae in both bovine and guinea-pig hosts.

The profiles of larval and adult extracts on SDS-PAGE revealed a wide range of proteins, which is not surprising in view of the complexity of these multicellular organisms. More importantly, there appeared to be a considerable degree of similarity between proteins of the L3 and adult stages, particularly those greater than approximately 30kDa, although further examination, such as by 2D-electrophoresis, will be necessary to confirm this. These may represent 'housekeeping' gene products, conserved between different stages. If such components prove to be important to protective immunity, then they may be obtained in greater quantity from adult worms for use in immunisation studies.

In contrast, the SDS-PAGE profile of somatic proteins of less than 30kDa indicated that these may be expressed in a stage-specific manner. Although both L3 and adult homogenates protect against challenge infection, the L3 extract does so with greater effectiveness. The stage-specificity of low molecular mass somatic proteins may, therefore, be useful in identifying L3 antigens responsible for this greater protection. These may represent L3 surface or sheath antigens, previously shown to be stage-specific (Chapter 6).

The presence of both common and stage-specific components in L3 and adult extracts was also demonstrated by their recognition by infected and vaccinated hosts in immunoprecipitation and immunoblotting studies. Immunoblotting allowed clearer resolution and identification of individual antigens than immunoprecipitation of radiolabelled material and was, therefore, the preferred method of analysis. Successful radioiodination of L3 somatic material was often difficult to achieve with a low level of radiolabel being incorporated and a significant degree of smearing being observed even with material freshly labelled by the Iodogen or Bolton-Hunter methods. In immunoprecipitation studies there

was a high level of precipitation of L3 somatic antigens with normal bovine serum, thereby limiting the value of immunoprecipitation analysis. Pre-clearing the antigen by incubation in a Staph A suspension did not appear to eliminate this binding. Specific recognition of radiolabelled L3 somatic antigens by normal bovine serum, as previously observed with L3 cuticular antigens (Chapter 5), may be responsible for this effect although binding might then have been expected to occur with L3 homogenate on immunoblots.

The recognition profile of radioiodinated L3 somatic antigens was similar in both infected and vaccinated calves and guinea-pigs. In contrast, immunoprecipitation of adult antigens was observed only with serum from naturally infected bovine hosts. Similarly, several low molecular mass antigens of adult extract were recognised on immunoblots only by calves exposed to a patent infection, not by vaccinated calves or guinea-pigs, thus demonstrating the stage-specificity of these antigens.

As well as identifying stage-specific antigens, examination of the antigen recognition profiles of immune hosts was useful in identifying potentially protective antigens. As all vaccinated and infected bovine hosts showed identical recognition patterns of L3 homogenate, it was not possible to identify any somatic antigen(s) which was more strongly recognised or recognised uniquely by vaccinated hosts. Vaccinated and infected guinea-pigs did, however, show a greater response to antigens of 23-24kDa, than to antigens of higher molecular mass. Interestingly, these 23-24kDa antigens were not as strongly recognised by guinea-pigs vaccinated with adult homogenate suggesting, firstly, that such antigens are not present to as great a level in adult parasites, and, secondly, as guinea-pigs vaccinated with adult extract are not protected as efficiently as those vaccinated with normal or irradiated L3, that these antigens may be important to immunity. Vaccination with these components following their purification from L3 homogenate by preparative SDS-PAGE will important in determining this.

Similarly, no adult somatic antigen was recognised specifically by vaccinated hosts. As previously mentioned, however, some adult antigens were recognised only by naturally infected calves, suggesting that these are adult-specific and are possibly irrelevant to immunity, at least that induced by the irradiated larval vaccine. Again, examination of the reactivity of guinea-pig sera was useful in identifying potentially protective adult antigens. Vaccinated or infected guinea-pig

hosts recognised adult antigens of 40-50kDa, 100kDa and those at the top of the separating gel (210-400kDa). There was no recognition of antigens of 12-16kDa by these hosts, yet the 16kDa antigen is present in the L3 stage as indicated by its recognition by bovine hosts vaccinated with 100krad irradiated larvae. This might, therefore, suggest that guinea-pig hosts are not exposed to this antigen or are incapable of mounting an antibody response to it.

By immunoblotting it could be observed that the level of response to some antigens decreased with increasing exposure to infection or vaccination, thus suggesting that recognition of such antigens is not essential to the maintenance of protective immunity. Although immunoblotting is not generally used as a quantitative measure of antibody response, these findings were paralleled by those obtained by the ELISA technique.

By ELISA clear quantitative differences in the responses of infected and vaccinated bovine hosts were demonstrated. Following infection with normal larvae, a rapid response to L3 and adult somatic antigens was observed. The response to both stages was first detected 14-21 days after infection with 1,000 larvae. Bos *et al.* (1986) also reported a response to L3 antigens 15-20 days after infection, but did not detect any anti-adult response until 20-30 days post-infection. The reason for this difference is unclear, particularly as the previous workers used an infecting dose of 10,000 larvae which might have been expected to stimulate a greater and perhaps earlier detectable response than the infecting dose used in our studies. The response to adult somatic antigens at 14-21 days after infection, prior to the patent period, suggests recognition of antigens common to larval and adult stages. This was previously suggested by Bos *et al.* (1986) to explain the rapid anti-adult response of vaccinated calves following challenge infection.

In contrast to naturally infected hosts, calves vaccinated with two doses of 40krad irradiated larvae, as used in the commercial vaccine, showed only a slight response to L3 and adult antigens, despite being given a greater number of larvae (see Table 4.1). The recognition of adult antigens by vaccinated hosts, in which larvae do not reach the mature adult stage, again suggests cross-reactivity between larval and adult somatic antigens. The level of antibody response of vaccinated hosts increased on subsequent vaccinations, reached a plateau, and was boosted following challenge infection. This was previously reported by Bos and Beekman (1985) and

Bos *et al.* (1986), but an explanation for this increase in antibody level was not given. It may be speculated that by measuring the response to somatic antigens by ELISA, we are measuring the response to larvae developing in the lungs. As irradiated larvae may not reach the lungs as rapidly or in as great numbers as normal larvae (Jarrett and Sharp, 1963) they may induce a lower antibody response. The boosting in antibody level observed following challenge infection of vaccinates may be due to a significant number of normal larvae rapidly reaching the lungs.

A similar pattern of antibody response to L3 and adult extract was observed following vaccination with 100krad irradiated larvae, although the level of response rose more rapidly than in 40krad vaccinates. This may be explained by the intravenous administration of 100krad larvae, possibly increasing the speed with which they reached the lungs. It is thought, however, that irradiation to such a high level affects larval viability resulting in lower numbers of such larvae reaching the lungs (Jarrett *et al.*, 1960). A recent study has demonstrated that lower numbers of larvae are recovered from the lungs of guinea-pigs immunised with larvae irradiated to 100krad than those exposed to normal or 40krad irradiated larvae, irrespective of the route of administration of these larvae (Canto, 1990). This may explain the lower level of antibody response to somatic antigens in hosts vaccinated with 100krad irradiated larvae compared to those infected with normal larvae.

In all bovine hosts examined the antibody response measured against both L3 and adult somatic antigens gradually decreased although immunity to reinfection remained high, thus indicating no correlation between the antibody level measured by ELISA and protective immunity. This is to be expected in examining the response to a complex mixture of antigens, many of which will be irrelevant to immunity. From their studies Bos and Beekman (1985) also concluded that the ELISA technique with parasite extracts may be useful in the diagnosis of lungworm infection, but clinical and parasitological data would be necessary to assess the degree of resistance. The kinetics of antibody response to somatic antigens are similar to those measured against adult ES and L3 sheath antigens, suggesting that the antibody responses to these are also not indicative of resistance to *D. viviparus* infection. It may, therefore, be necessary to measure the response to more defined parasite antigens, such as subfractions or individual components of

homogenates or ES products, to gain information on the level of immunity to *D. viviparus*.

Finally, the synthesis of proteins by normal and gamma-irradiated infective stage larvae was examined. Any differences between proteins expressed by these parasites may have relevance to the mechanism of vaccine-induced immunity. For example, a reduction in protein synthesis, particularly of heat shock proteins (HSP's) of the HSP-70 family has been observed following U.V.or gamma-irradiation of *S. mansoni* schistosomula (Wales, 1989). HSP's are produced by both prokaryotic and eukaryotic cells in response to elevations in temperature and other environmental stresses, and, among other functions, play an essential role in the maintenance of protein conformation and in the recognition and removal of denatured proteins (Linquist, 1986; Pelham, 1988). The reduced amounts of HSP'S synthesised by irradiated schistosomula may, therefore, give rise to parasite components of modified conformation which may be processed and presented to antigen presenting cells more effectively than native antigens, thus stimulating an enhanced immune response. Modified components must, however, be sufficiently similar to the native antigens to elicit a secondary response upon challenge with normal parasites. This may explain why the efficiency of vaccination with U.V.- or gamma-attenuated schistosomula is highly dependent on the radiation dose (Dean *et al.*, 1983).

In the present study no qualitative nor quantitative differences were observed in the proteins synthesised by normal or irradiated infective larvae of *D. viviparus* during the initial 24 h after irradiation nor during the following six days, even in larvae irradiated to 100krad. It would, however, be important to confirm this by comparing the antigenicity and conformation of proteins synthesised by these larvae. Nevertheless, similar observations have been made following ^{35}S-methionine labelling of normal and irradiated third stage larvae of *B. pahangi.* It is speculated that although irradiation appears to have no effect on the L3 stage it may affect the synthesis of cuticular components of the L4 stage (Devaney and Bancroft, 1991). It would therefore be worthwhile examining whether irradiation has a similar effect on protein synthesis in post-infective stages of *D. viviparus*.

The success of the *D. viviparus* vaccine is thought to depend on sufficient exposure of the host to parasite antigens despite termination of parasite

development prior to the pathogenic adult stage (Jarrett *et al.*, 1958). Parasite death may be a direct consequence of the effects of irradiation on parasite components, such as an inhibition in the synthesis of essential proteins. On the other hand, irradiation may act indirectly on parasite survival, with irradiated parasites stimulating a greater immune response than normal worms resulting in immune-mediated parasite destruction. This may be due to the expression of aberrant parasite components of enhanced immunogenicity, as previously described for *S. mansoni* (Wales, 1989). The identical profiles of ^{35}S-labelled polypeptides of normal *D. viviparus* larvae and larvae irradiated to 100krad, and incapable of developing beyond the L3 stage, might suggest, however, that vaccine-induced immunity to *D. viviparus*, at least with 100krad irradiated larvae, is not due to the recognition of novel parasite antigens. Alternatively, irradiation may affect the rate of parasite growth and development perhaps resulting in prolonged exposure of the host to parasite antigens (as discussed in Chapter 5) and leading to immune-mediated destruction of the parasite prior to development to the adult stage. There is some evidence from a study in the guinea-pig host that the rate of development of fourth stage larvae from irradiated L3 is slower than that from normal larvae (Canto, 1990). To examine these possibilities it would be worthwhile examining the rate of ^{35}S-methionine incorporation by post-infective parasite stages recovered from infected and vaccinated hosts. This may indicate whether radiation affects only one particular parasitic stage or affects the development of all post-parasitic stages, if at all.

If the rate of parasite development is reduced by irradiation this could result in the expression of parasite antigens in abnormal sites. For example, larval stages normally confined to the intestinal mucosa or mesenteric lymph nodes may be found in the lymphatic or blood circulations or in the lungs. Antigens released from such parasites may interact with different antigen presenting cells (APC's) to those involved in normal infections. This could influence the subsequent immune response due to the activation of different T cell subsets by different APC's (Allen *et al.*, 1985). This may explain the surprising results obtained in passive immunisation studies with *D. viviparus* immune sera. Serum from hosts exposed to normal infection was almost 100% effective in passive immunisation, while that of hosts vaccinated with 40krad irradiated larvae conferred only modest protection (40%)(Canto, 1990). No protection was conferred with serum from

calves vaccinated with 100krad irradiated larvae which were administered intravenously and, therefore, present in an abnormal site. These findings might suggest that irradiated larvae, particularly when administered intravenously, induce a greater cell-mediated immune response than normal larvae although the specificity of the response may be the same. This possibility may be examined both *in vitro* and *in vivo*. Antigen presenting cells could be removed from different sites, for example the lungs or mesenteric lymph nodes, of infected and vaccinated hosts and their reactivity to larvae or individual parasite antigens examined *in vitro*. *In vivo* radiotracking studies using normal and irradiated parasites metabolically labelled with ^{35}S-methionine or surface labelled with ^{125}I may also be informative in determining the fate of such larvae in susceptible hosts. APC's could also be recovered from these hosts and processed parasite antigens identified by immunoprecipitation of extracts of these cells with anti-*D. viviparus* serum. This may be useful in indicating whether the same parasite antigens are recognised by hosts exposed to normal or irradiated larvae.

CHAPTER 8

ENZYMATIC ACTIVITY OF *D. VIVIPARUS* ANTIGENS

8.1 INTRODUCTION

During the last few years there has been increasing interest in defining the biological function of parasite antigens. A wide range of enzyme activities, including proteinases, anti-oxidant enzymes and acetylcholinesterase (AChE), have been identified in extracts and excretory-secretory (ES) products of a number of helminths. Parasite proteolytic enzymes are thought to play a role in host invasion (Matthews, 1977; McKerrow *et al.*, 1990; Wertheim *et al.*, 1983), parasite feeding (Hotez and Cerami, 1983; Chappell and Dresden, 1986) and in immune evasion (Auriault *et al.*, 1981; Chapman and Mitchell, 1982; Leid, 1987) as well as being implicated in the pathology of parasitic infections (McKerrow, 1988).

All helminth parasites examined to date also appear to contain at least one of the major anti-oxidant enzymes: superoxide dismutase (SOD)(Rhoads, 1983; Sanchez-Moreno *et al.*, 1987), catalase (Smith and Bryant, 1986) and glutathione peroxidase (Kazura and Meshnick, 1984, Smith and Bryant, 1986). These enzymes are thought to protect parasites against oxidant-mediated damage which can result from normal cellular processes, the action of certain anti-parasite drugs (Do Campo and Moreno, 1986), and by activated host phagocytic cells through the respiratory burst (Babior, Kipness and Curnutte, 1973).

High activities of AChE have been detected in ES products of the intestinal nematodes, *Nippostrongylus brasiliensis* (Lee, 1970), *Trichostrongylus colubriformis*, *Oesophagostorum radiatum* (Ogilvie *et al.*, 1973) and *Necator americanus* (McLaren, Burt and Ogilvie, 1974). Although the function of AChE in the host-parasite relationship is not clearly defined, it may act as a 'biological holdfast', allowing the worms to maintain their position in the host intestinal mucosa (Ogilvie and Jones, 1971).

Parasite enzyme activities are often expressed in a stage-specific manner, possibly reflecting differences in the requirements of the different developmental stages. For example, AChE is secreted by the L4 and adult stages, but not by the infective larval stage of the intestinal parasites examined (Ogilvie *et al.*, 1973). Similarly, the level of activity and substrate specificity of parasite proteolytic enzymes has been found to vary between different stages (Newport *et al.*, 1987; Knox and Kennedy; 1988; Pratt *et al.*, 1990).

Identification of parasite enzymes may be useful in the serodiagnosis of

helminth infections. A difficulty in the application of this detection system is that such enzymes, like other parasite molecules, can provoke antibody responses and, therefore, be rapidly cleared from the circulation by the host. The functional activity of some parasite enzymes may, however, be unimpaired when complexed with antibody and could provide the basis of a serodiagnostic assay. This is true of *T. colubriformis* AChE which stimulates an antibody response in infected sheep but whose activity is unaffected by antibody interaction (Rothwell and Merrit, 1974). The level of intestinal mucosal AChE activity has been found to correlate with the worm burden of lambs chronically infected with *T. colubriformis* (Jones, 1983) and could, therefore, be used as a serodiagnostic marker of the level of infection. Differences in AChE isoenzymes and antigenicity between diffferent genera and species have been observed (Rothwell, Ogilvie and Love, 1973) and may be useful in specific diagnostic immunoassays.

As well as a potential role in diagnosis, parasite enzyme systems may be exploited in the development of anti-parasite drugs. Further definition of nematode biochemistry may allow identification of novel enzymes or functional pathways through which specific inhibition of parasite activities may be achieved (Wang, 1984).

There is also a need to define, at the molecular level, the effects of the immune response on parasite enzyme activity. It has long been speculated that in some parasite infections host antibodies may act as anti-enzymes, blocking parasite invasion and impairing survival within the host (Chandler, 1932, 1936). It has been demonstrated that proteolytic activity of oesophageal extracts from adult *Ancylostoma caninum* is inhibited by sera from dogs immune to reinfection, but not by non-immune hosts (Thorson, 1956). Similarly, antibody from rabbits infected with *Ascaris suum* was found to inhibit the activity of *in vitro* released proteinases of *A. suum* larval stages (Knox and Kennedy, 1988). The observation that host antibody can inhibit parasite enzymes offers an important opportunity not only to examine their function *in vivo*, but also to design novel anti-parasite vaccines to selectively inhibit essential enzyme-mediated processes.

As yet, there is only limited information on enzymes of *D. viviparus*. An acidic thiol proteinase has been identified in extracts of adult *D. viviparus* (Rege *et al.*, 1989) and it is speculated that this enzyme may be involved in the nutrition of the parasite and/or contribute to the pathologic lesions found in the lungs of

infected hosts, due to its ability to degrade host collagen and haemoglobin. It has also been proposed that it may have value as a potential protective antigen (Bos *et al.*, 1989). However, it is not known whether this proteinase is secreted by the parasite and, therefore, available as a target for immune attack.

Presented here is the first detailed characterisation of proteolytic enzymes of *D. viviparus* infective and adult stages. Proteinase activities of extracts and ES products have been characterised according to pH optima, substrate specificity and inhibitor sensitivities. Moreover, the effect of immunoglobulin from infected and vaccinated hosts on proteolytic activity was examined. Inactivation of enzyme activity may be important in elucidating the basis of immunity to *D. viviparus* as well as being relevant to the development of new synthetic vaccines against this and other parasites.

No studies on anti-oxidant enzymes of *D. viviparus* have yet been undertaken. Such enzymes may be important in the survival of the parasite within host tissues and against oxidants generated by phagocytes and granulocytes in response to lungworm infection. Also presented are the results of preliminary studies on SOD activity of the L3 and adult stages of *D. viviparus*.

8.2 RESULTS

8.2.1 pH optima of proteinase activity

Proteolytic activity of L3 and adult material against azocasein was observed over a wide pH range (Figure 8.1). L3 homogenate, adult homogenate and adult ES all showed dominant peaks of activity at pH 5.5 and 7.5, with some activity at pH 9.0. The dominant activity of L3 ES differed slightly from this, with peaks at pH 5.0 and 6.5 in addition to those at pH 7.5 and 9.0. The level of activity in L3 ES, as measured by absorbance, was similar to that of L3 homogenate, yet there was at least twenty fold less protein in the ES sample (see Figure 8.1). This was also true of adult ES compared to adult homogenate, where ES showed substantially greater activity per unit protein. The pH profiles of adult ES and homogenate were similar, although ES showed greater activity at the lower pH peak.

Separation of proteinase activities by electrophoresis on gelatin substrate gels under non-reducing conditions confirmed the pH optima of general proteinase activity and also indicated that different enzymes were active at the different pH peaks. When incubated at pH 5.5, L3 homogenate showed a clear zone of proteolysis of M_r 57kDa and a second zone was faintly observed at 47kDa (not shown). At pH 7.5, these zones were more intense, with additional activity of approximately 110-120kDa and 275kDa being present (Figure 8.2 Panel A). This parallels the findings using azocasein in the test-tube assay, where greater L3 activity was observed at pH 7.5.

The greater activity of adult ES at pH 5.5 using azocasein was also confirmed by substrate gel electrophoresis. An intense zone of proteolytic activity of approximate M_r of 57kDa was observed at pH 5.5, while at pH 7.5, weaker activity of approximately 110kDa could be seen (Figure 8.2, Panel B). Curiously, these activities resolved poorly on SDS-PAGE, with broad zones of activity being observed. That this was not a fault of the gel conditions was indicated by the sharp, clear zones of proteolysis observed with collagenase (subunit molecular masses of 57.4kDa and 105kDa)(track 3).

Despite showing activity in the test-tube assay using azocasein as substrate, no enzyme activity was detected with adult homogenate on substrate gels. L3 ES showed enzyme activity at pH 7.5 but this did not resolve into clear zones. As L3 ES is extremely difficult to obtain in sufficient quantities for analysis, it was not examined further, despite having high levels of proteinase activity.

8.2.2 Substrate specificities

The proteolytic activities of L3 homogenate, adult homogenate and adult ES were examined at pH 5.5 and 7.5 using a range of low molecular weight substrates (Table 8.1). The results indicated that the activity of L3 homogenate was predominately tryptic in nature, while adult ES and homogenate showed strong enzyme activity against chymotrypsin, trypsin and elastase substrates, the latter activity predominating. The activity of L3 and adult homogenates was generally greater at pH 7.5 than at 5.5. In contrast, the tryptic and chymotryptic activities of adult ES did not increase significantly at higher pH, indicating a disparity between adult ES and homogenate.

8.2.3 Inhibitor sensitivities

The effects of various inhibitors on the proteolytic activity of L3 homogenate, adult homogenate and adult ES against azocasein as substrate are shown in Figure 8.3. The differing effects of inhibitors on the activities of L3 and adult proteinases suggested the presence of stage-specific activities. At pH 5.5 the carboxyl proteinase inhibitor, pepstatin, markedly inhibited the activity of adult homogenate and ES, while only having a mild effect on the L3 stage. In contrast, L3 activity was inhibited by the metallo-proteinase inhibitor EDTA and the alkylating agent NEM, while adult activity was either unaffected by these or, in the presence of EDTA, was enhanced.

At pH 7.5 there was a markedly different pattern of inhibition, further confirming that multiple proteolytic activities are present. L3 homogenate activity was significantly reduced by the serine proteinase inhibitor, Pms-F, the thiol inhibitor E-64 and the metallo inactivator, EDTA. Additionally, activity was inhibited by DTT, while NEM had less effect on L3 activity at this pH. In contrast to the findings at pH 5.5, adult ES and homogenate activities were markedly reduced by NEM and EDTA, and in the case of adult ES by DTT, but unaffected by pepstatin.

These findings were confirmed by substrate gels incubated in the presence of inhibitors. Although total inhibition was not always observed, it was possible to attribute zones of activity to a particular class of proteinase. In particular, the level of activity of a 275kDa proteinase present in L3 homogenate was significantly reduced by EDTA indicating it is a metallo-proteinase (Figure 8.4). This inhibitor had no effect on L3 proteinases of 47 and 57kDa. Following incubation in NEM or Pms-F, there was no detectable alteration in the level of activity of any L3 proteinases on

gelatin gels.

8.2.4. Effect of reducing agent on proteinase activity

To examine whether the activity of *D. viviparus* proteolytic enzymes was dependent on their conformation, L3 homogenate and adult ES were heated to 100°C or mixed with reducing agent (5% 2ME) prior to electrophoresis on gelatin substrate gels. No proteinase activity was observed following heat-denaturation of L3 and adult preparations (Figure 8.5). Similarly, destruction of inter- or intra- disulphide bonds in adult ES products by treatment with 2ME resulted in the destruction of proteolytic activity at 7.5. An adult proteinase active at pH 5.5 showed a significant reduction in activity in the presence of 2ME, while its mobility on SDS-PAGE increased. In contrast, treatment with reducing agent had no effect on the activity of L3 proteinases of 47 and 57kDa at either pH. The activity of other L3 proteinases could not be clearly seen on this gel, even under control (non-reduced and unheated) conditions.

8.2.5 Inhibition of proteinase activity by antibody

Extracts of L3 and adult parasites as well as adult ES are known to be antigenic to the infected bovine host (Chapters 7 and 3, respectively), and immunisation using these preparations has been shown to induce significant protection in guinea-pigs (G.J.Canto, C.Britton, G.M.Urquhart and M.W.Kennedy, unpublished; J.B. McKeand, C. Britton, G.M. Urquhart and M.W. Kennedy, unpublished). The effect of serum antibody on the proteolytic activity of these antigens was determined using Protein G-purified immunoglobulin from infected and vaccinated calves in proteinase assays using azocasein and on substrate gels.

At pH 5.5, hydrolysis of azocasein by L3 homogenate was reduced to 52% and 65% in the presence of antibody from infected and vaccinated hosts, respectively, relative to a control with no added antibody (Figure 8.6 Panel A). At pH 7.5, L3 activity fell to 41% and 46% with infection and vaccinate antibody, respectively. IgG from normal bovine serum had no effect indicating that it does not compete with azocasein as a substrate for parasite proteolytic enzymes. This might suggest that bovine IgG is not a substrate for *D. viviparus* proteolytic enzymes or, alternatively, that too little IgG was present to observe any substrate competition.

Antibody from immune hosts had a similar effect on proteinase activity of

adult ES (Figure 8.6 Panel B). At pH 5.5, adult activity was reduced to 45% and 22% of control activity by infection and vaccinate antibody, respectively. At pH 7.5, the same IgG caused activity to fall to 50% and 51% of control activity, while IgG from normal bovine serum had no effect.

In contrast, antibody from immune hosts had little effect on proteinase activity of adult homogenate (Figure 8.6 Panel C). A reduction in activity to 84% was observed following incubation at pH 5.5 with IgG from vaccinated hosts, while activity was enhanced by antibody from infected or uninfected animals, relative to a control with no added IgG. At pH 7.5, proteinase activity of adult homogenate was reduced to 84% and 86% by IgG from infected and vaccinated hosts respectively, while that of uninfected animals had no effect.

Antibody inhibition of adult ES enzyme activity was visualised on substrate gels in which samples were pre-incubated with host IgG prior to electrophoresis. At pH 5.5, the activity of an adult ES proteinase of M_r 57kDa was reduced by antibody from infected and vaccinated hosts (Figure 8.7), as observed by a decrease in the density of the zone of activity. Activity was unaffected by IgG from normal serum. In contrast, an adult proteinase of Mr 98kDa active at pH 7.5, was unaffected by antibody from animals immunised by either method (not shown). Inhibition of L3 homogenate enzyme activity could not be seen clearly on gelatin substrate gels.

8.2.6 Superoxide dismutase activity of *D. viviparus*

Significant superoxide dismutase (SOD) activity was detected in PBS-soluble extracts of L3 and adult stages and in adult ES products. The specific activity, measured by the inhibition of iodonitrotetrazolium violet (INT) reduction was estimated to be 39.4 ± 0.8 U/mg (mean ± standard deviation of 2 observations) for L3 extract; 27.4 ± 9.8 U/mg for adult extract and 8.9 ± 4.4 U/mg for adult ES. Although a low level of activity was detected in L3 ES, the specific activity could not be calculated accurately due to the low protein concentration of the preparation (40µg/ml).

SOD activity was visualised by polyacrylamide gel electrophoresis. Clear zones of activity, resulting from the inhibition in reduction of NBT, were observed with L3 homogenate, adult homogenate and adult ES (shown diagramatically in Figure 8.8). In addition to the broad zone of activity observed at the top of the separating gel with all three parasite preparations, a sharp band of activity towards the cathode was observed in tracks containg adult homogenate (track d). On some occasions, a second

diffuse zone of activity, on the cathodic side of the first, could also be seen with L3 homogenate (track b). No activity was observed with L3 ES on SOD gels.

8.2.7 Detection of *D. viviparus* SOD by immunoblotting

The presence of SOD in *D. viviparus* infective and adult stages was further indicated by the recognition of parasite components on immunoblots by rabbit antiserum to bovine erythrocyte SOD (Figure 8.9). This antiserum showed strong recognition of a component of approximately 19kDa in L3 and adult extracts. There was also weak recognition of molecules of approximately 21 and 55kDa.

Anti-SOD antiserum also recognised a 55kDa component of adult ES. Although no other ES molecules were recognised this could have been due to the poor transfer of ES proteins onto the nitrocellulose, as can be seen in Figure 8.9. The reason for this poor transfer will require further investigation. There appeared to be no correlation with respect to molecular mass between parasite components recognised by anti-SOD antiserum and antigens recognised by bovine sera to natural infection with *D. viviparus*.

There was no recognition of *D. viviparus* adult or L3 components by rabbit antiserum to bovine catalase (not shown).

8.2.8 Effect of antibody on SOD activity

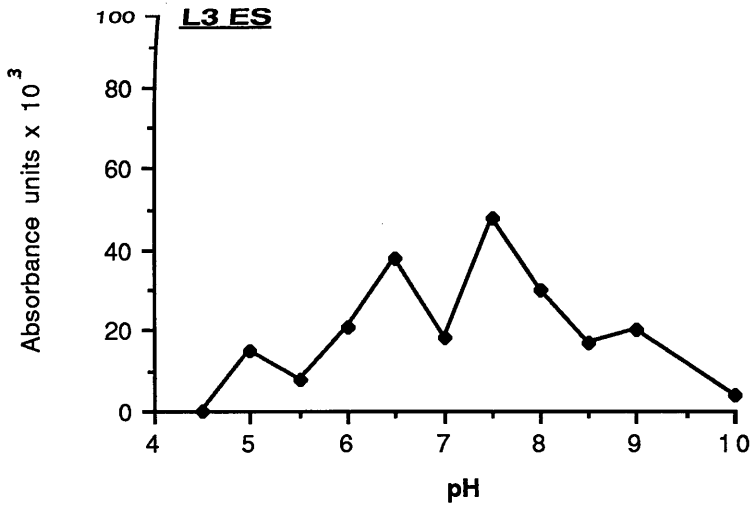
To examine the effect of antibody on parasite SOD activity, *D. viviparus* L3 and adult preparations were pre-incubated with 25µg of IgG purified from serum from infected and vaccinated calves. The level of activity remaining was assayed as before using the xanthine-xanthine oxidase system. IgG from vaccinated, but not from infected or uninfected hosts, had a slight inhibitory effect on SOD activity of L3 homogenate, with activity falling to 84% relative to a control pre-incubated with PBS (Figure 8.10). Antibody from both infected and vaccinated hosts had an inhibitory effect on SOD activity of adult homogenate, with activity falling to 83% and 65% with infection and vaccinate IgG respectively, while IgG from normal bovine serum had no effect. Similarly, SOD activity of adult ES was reduced to 80% and 56% of control activity following pre-incubation with IgG from infected and vaccinated calves, respectively. Antibody from normal bovine serum also had a slight inhibitory effect on adult ES SOD activity with 90% remaining relative to a control pre-incubated with PBS.

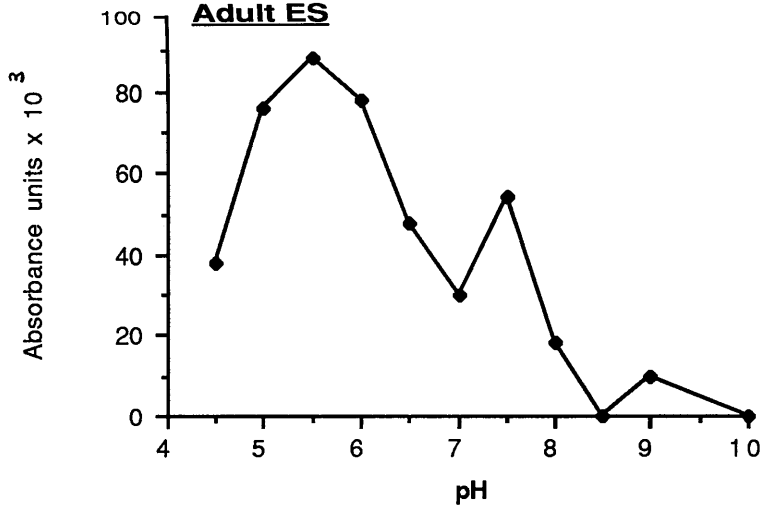
TABLE 8.1 Proteolytic activity of *D.viviparus* L3 homogenate, adult homogenate and adult ES against a variety of substrates

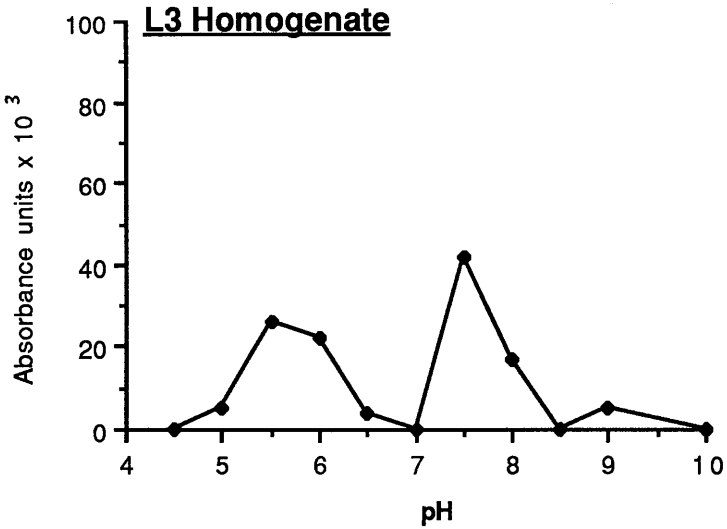
Type of enzyme activity indicated	Substrate	L3 homogenate		Adult Homogenate		Adult ES	
		pH 5.5	pH 7.5	pH 5.5	pH 7.5	pH 5.5	pH 7.5
Chymotryptic (esterolytic)	CBZ-L-Trp-4-NPE	17	32	93	199	47	47
Tryptic (esterolytic)	CBZ-L-Arg-4-NPE	67	381	87	242	56	60
	CBZ-L-Lys-4-NPE	29	150	92	154	48	20
Elastolytic (esterolytic)	CBZ-L-Ala-4-NPE	15	25	136	810	64	199
Chymotryptic (amidolytic)	BZ-L-Tyr-NA	16	15	15	159	15	45
Tryptic (amidolytic)	BZ-L-Arg-NA	20	10	17	26	15	19

Assays were carried out on two occassions and yieled similar results. The results of one assay are shown.
Data expressed as change in absorbance (Absorbance units x 10^{3}) / 30 min

Figure 8.1 The effect of pH on proteinase activity of L3 ES, L3 homogenate, adult ES and adult homogenate against azocasein as substrate. Absorbance of reaction mixture supernatant was measured at 405nm. The protein concentrations of parasite preparations were as follows: L3 ES, 40μg/ml; L3 homogenate, 1.0mg/ml; adult ES, 2.5mg/ml; adult homogenate, 3.4mg/ml.


L3 ES
Absorbance units x 10 3
100
80
60
40
20
0
4
5
6
7
8
9
10
pH


Adult ES
Absorbance units x 10 3
100
80
60
40
20
0
4
5
6
7
8
9
10
pH


L3 Homogenate
Absorbance units x 10 3
100
80
60
40
20
0
4
5
6
7
8
9
10
pH

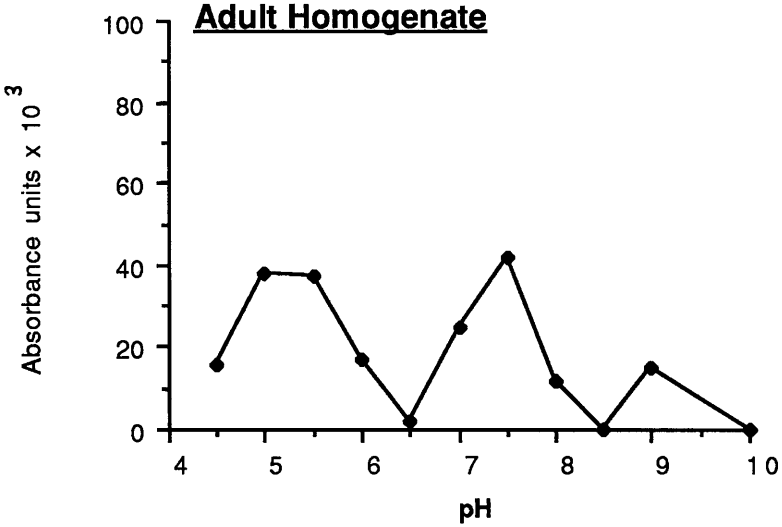

Adult Homogenate
Absorbance units x 10 3
100
80
60
40
20
0
4
5
6
7
8
9
10
pH

Figure 8.2A Separation of L3 homogenate proteinases by SDS-PAGE. L3 PBS extract was electrophoresed on 7.5% SDS-mini gels containing 0.1% gelatin. The gel was incubated overnight in buffer at pH 7.5 and stained with Coomassie blue. M_r are shown in kDa.

Figure 8.2B Proteinase activity of adult ES on gelatin substrate gels. Adult ES was electrophoresed on 5-25% gradient substrate gels and incubated overnight in buffer at pH 5.5 (a) and 7.5 (b). Activity of collagenase incubated at pH 5.5 is shown in track c. The M_r of marker proteins (M) are indicated in kDa.

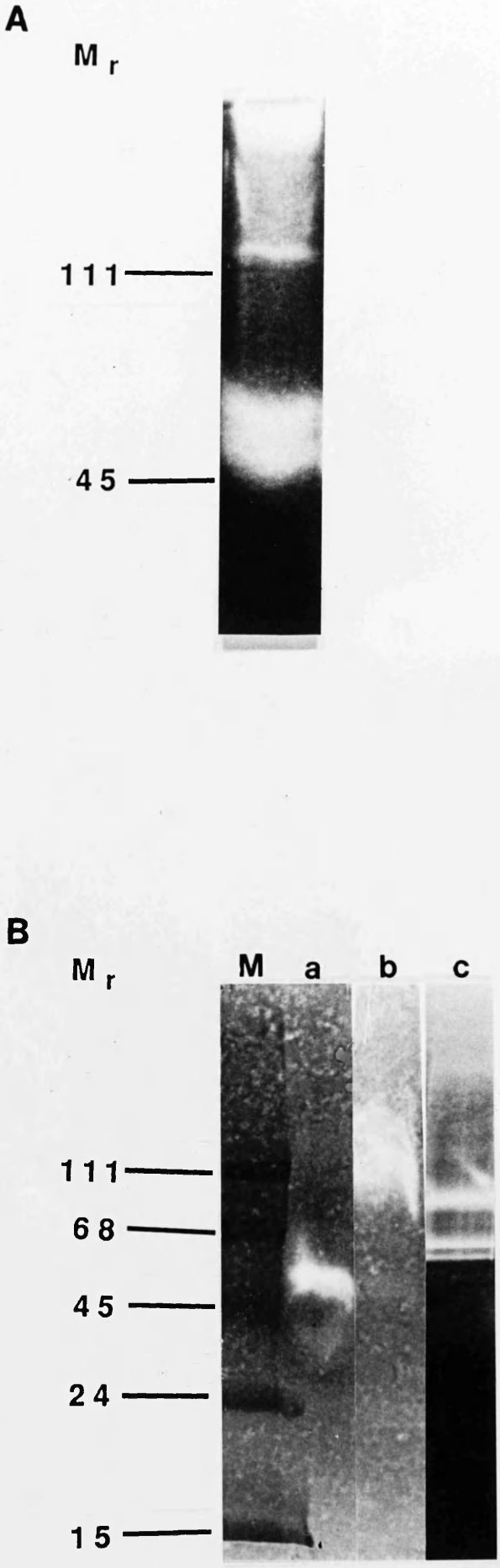
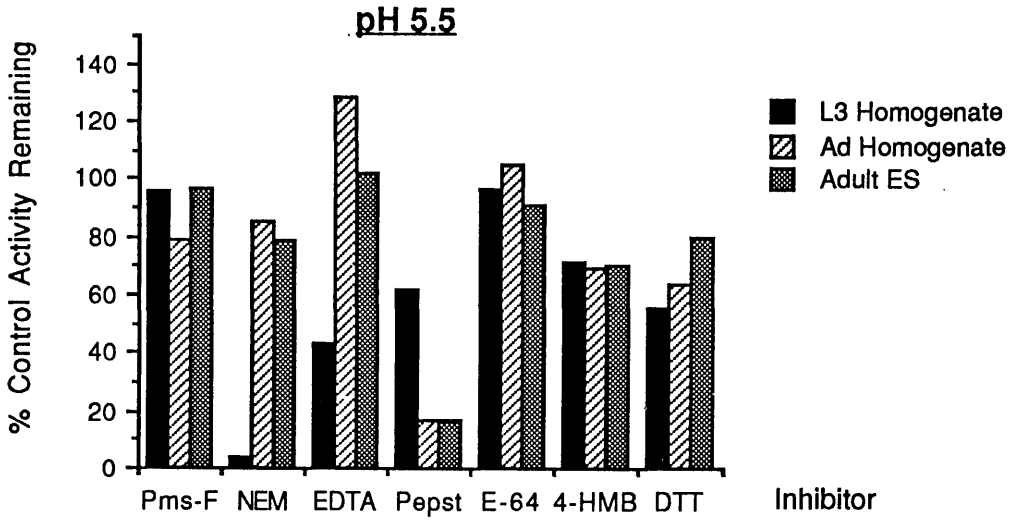

A
Mr
111
45
B
Mr
M
a
b
c
111
68
45
24
15

Figure 8.3 The effect of various inhibitors on the hydrolysis of azocasein by L3 homogenate, adult homogenate and adult ES. The final concentrations of inhibitor in the reaction mixture were as described in Materials and Methods. The degree of inhibition is expressed as the percent activity remaining relative to a control sample run in the absence of inhibitor. Proteinase assays were carried out on one occasion following pre-incubation with E-64, 4-HMB and DTT and on three occasions with Pms-F, NEM, EDTA and pepstatin in which case the means of the results are shown.


pH 5.5
% Control Activity Remaining
140
120
100
80
60
40
20
0
Pms-F
NEM
EDTA
Pepst
E-64
4-HMB
DTT
Inhibitor
L3 Homogenate
Ad Homogenate
Adult ES

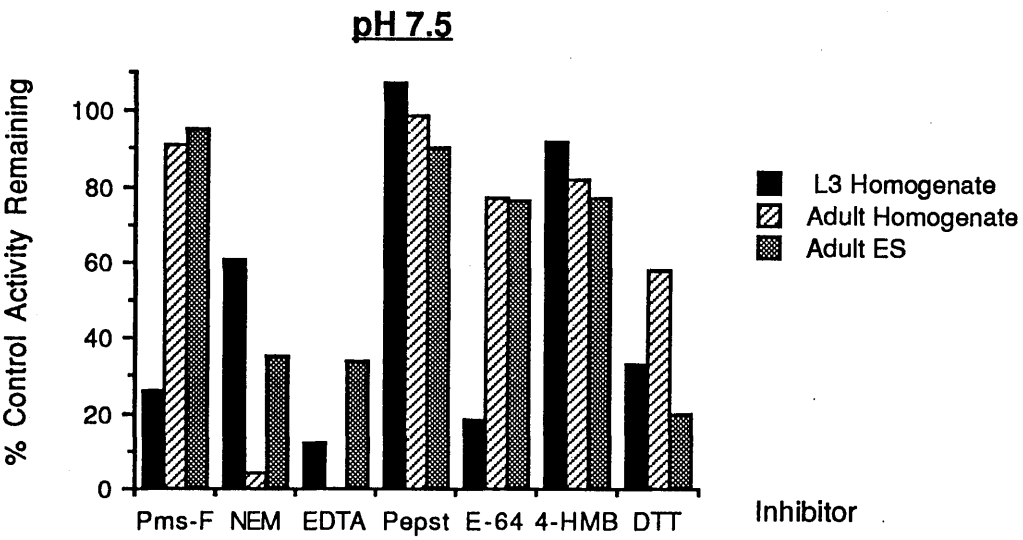

pH 7.5
% Control Activity Remaining
100
80
60
40
20
0
Pms-F
NEM
EDTA
Pepst
E-64
4-HMB
DTT
Inhibitor
L3 Homogenate
Adult Homogenate
Adult ES

Figure 8.4 Effect of low molecular weight inhibitors on L3 proteinase activity. L3 homogenate was electrophoresed on 7.5% SDS-mini-gels containing 0.1% gelatin and incubated overnight in buffer at pH 7.5 containing no inhibitor (track a), EDTA (track b), NEM (track c) and Pms-F (track d) at the final concentrations described in Chapter 2. M_r are indicated in kDa.

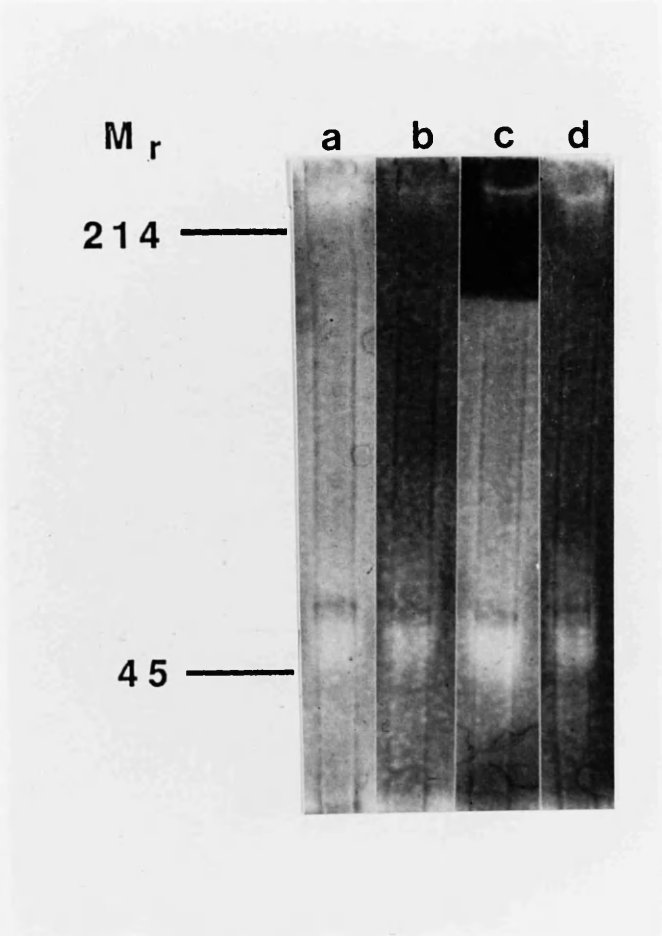
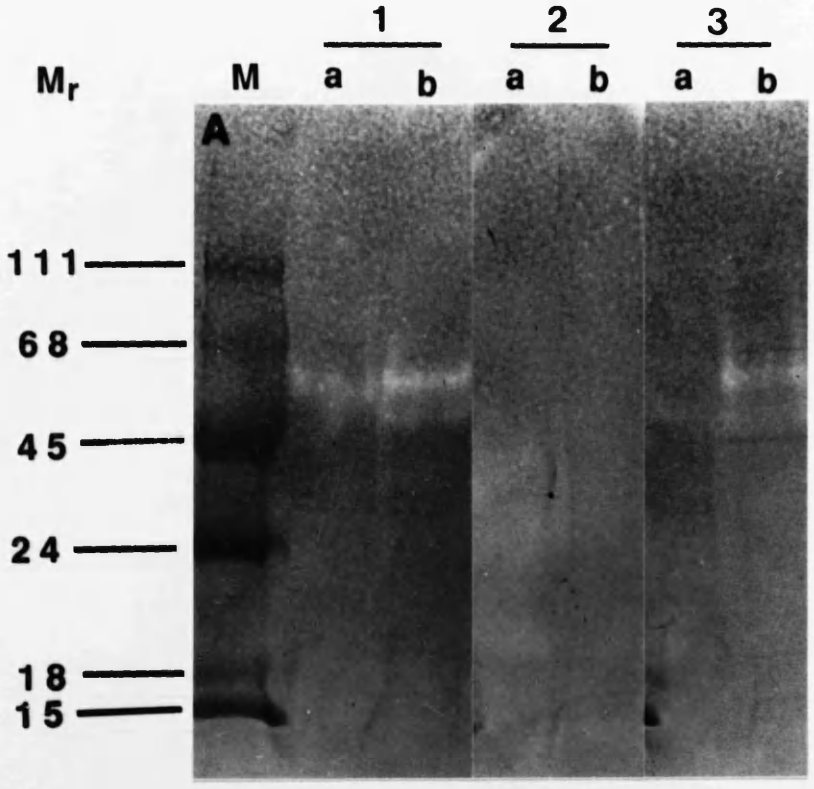

M r
a
b
c
d
214
45

Figure 8.5 Effect of heat and reducing agents on proteinase activity. Adult ES (tracks a) and L3 homogenate (tracks b) were electrophoresed on 5-25% gelatin substrate gels under control conditions (non-reduced and not pre-heated) (1), following incubation at 100°C for 10 min (2) or run in the presence of 5% 2ME (3). Gels were incubated overnight in buffer at pH 5.5 (A) or pH 7.5 (B), then stained with Coomassie blue. M_r of marker proteins (M) are shown in kiloDaltons.


1
2
3
Mr
M
a
b
a
b
a
b
A
111
68
45
24
18
15

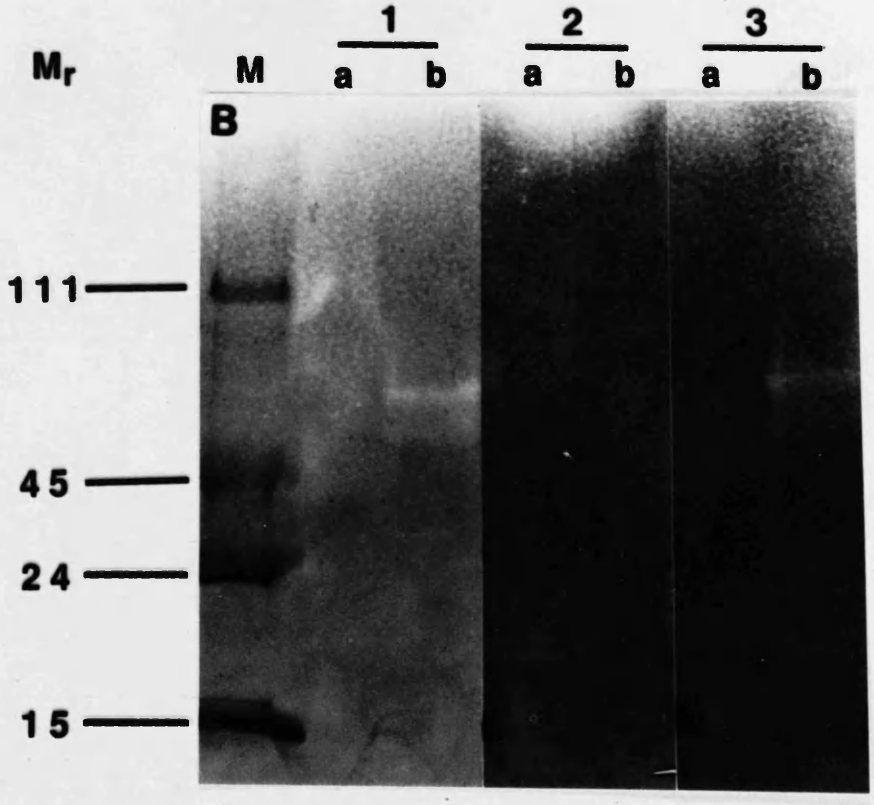
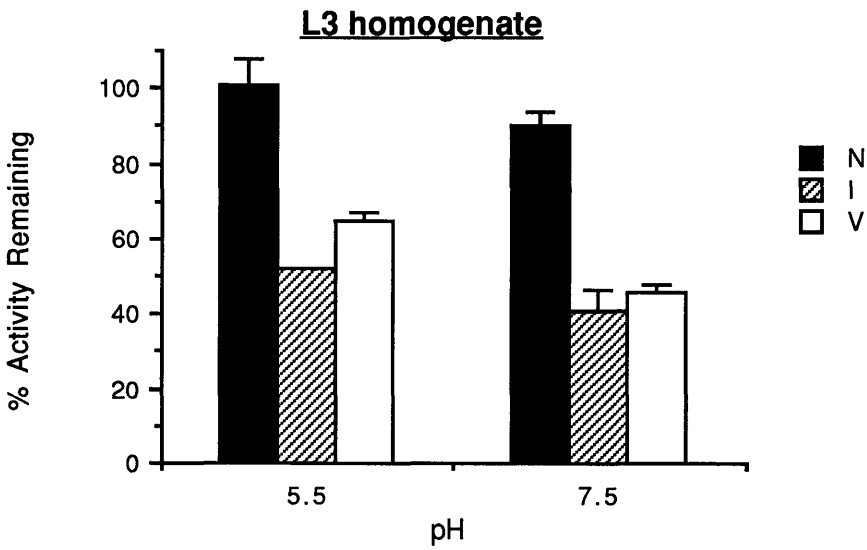
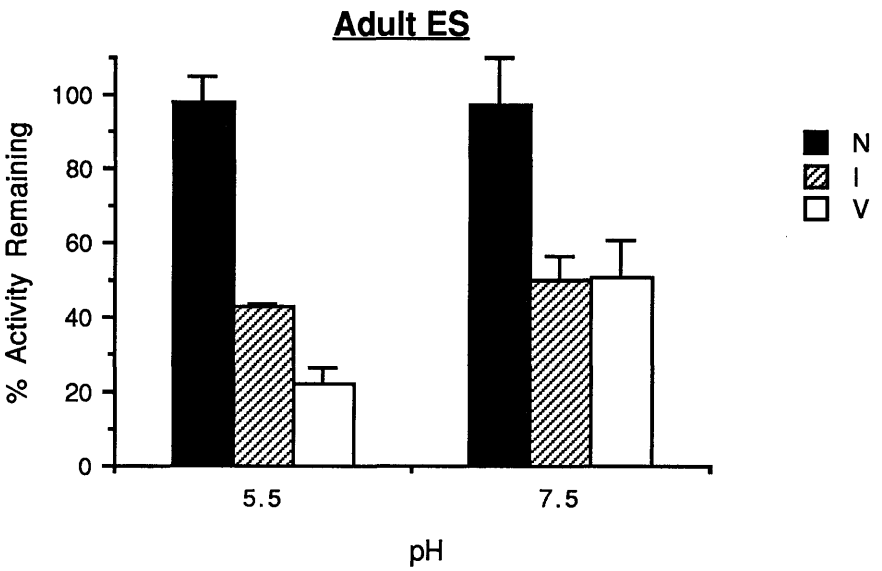

1
2
3
Mr
M
a
b
a
b
a
b
B
111
45
24
15

Figure 8.6 The effect of IgG antibody on the hydrolysis of azocasein. L3 homogenate, adult homogenate and adult ES were incubated with 25µg IgG purified from serum from normal (N), *D.viviparus*-infected (I), and vaccinated (V) calves, prior to overnight incubation with azocasein. The degree of inhibition is expressed as the percent activity remaining, relative to a control with no added IgG. The means of two observations are shown, with error bars representing the S.D. of the observations.


L3 homogenate
% Activity Remaining
100
80
60
40
20
0
5.5
7.5
pH
N
I
V


Adult ES
% Activity Remaining
100
80
60
40
20
0
5.5
7.5
pH
N
I
V

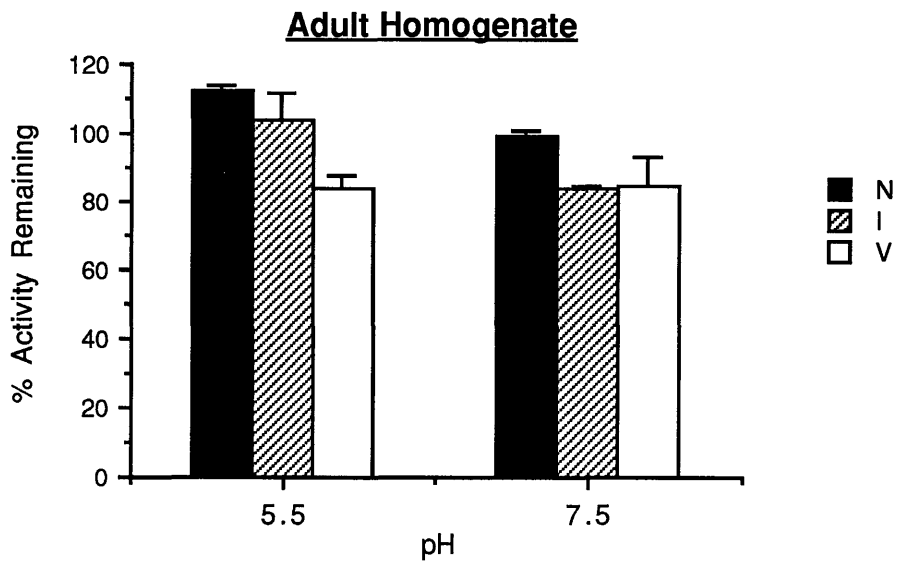

Adult Homogenate
% Activity Remaining
120
100
80
60
40
20
0
5.5
7.5
pH
N
I
V

Figure 8.7 Inhibition of adult ES proteinase activity by purified IgG. Adult ES was incubated with 25µg IgG purified from serum from normal (N), *D. viviparus*-infected (I), and vaccinated (V) calves, prior to substrate gel electrophoresis. A control track (C) shows the proteinase activity of adult ES pre-incubated with PBS only. Overnight incubation was carried out in buffer at pH 5.5.

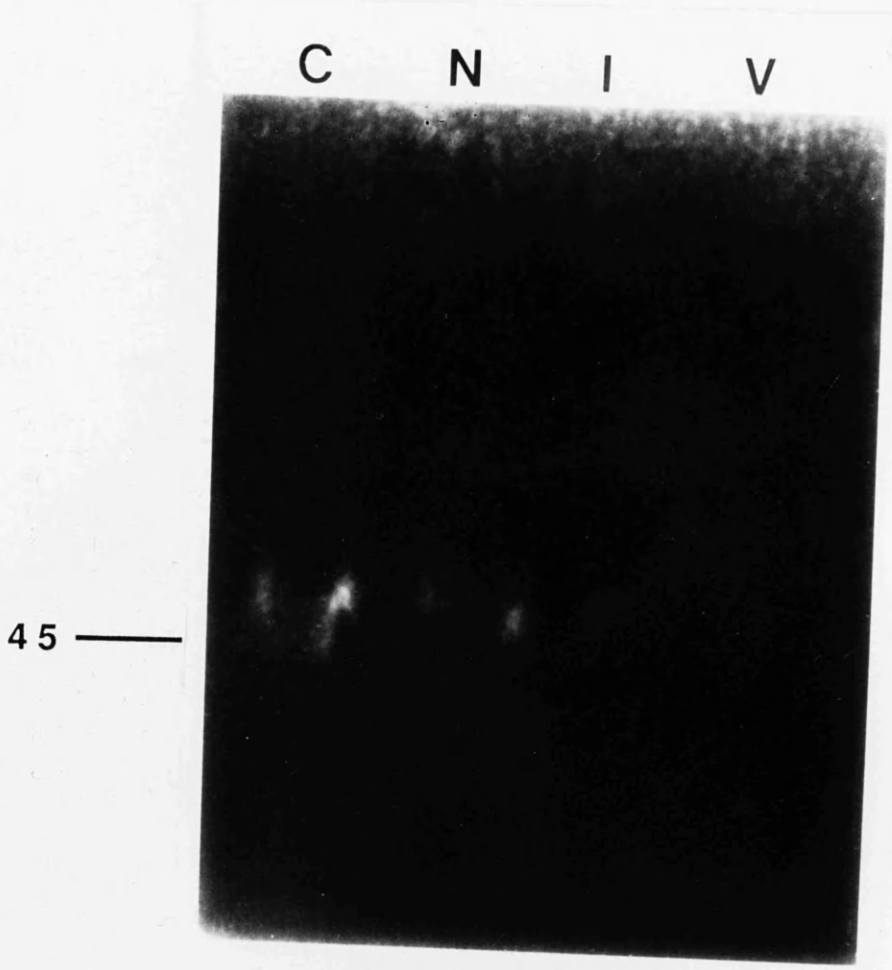

C
N
I
V
45

Figure 8.8 Isoenzymes of *D. viviparus* superoxide dismutase (SOD). L3 ES (track a), L3 homogenate (track b), adult ES (track c) and adult homogenate (track d) were electrophoresed on 7.5% mini-gels which were subsequently stained for SOD activity. Clear zones of SOD activity are represented diagramatically.

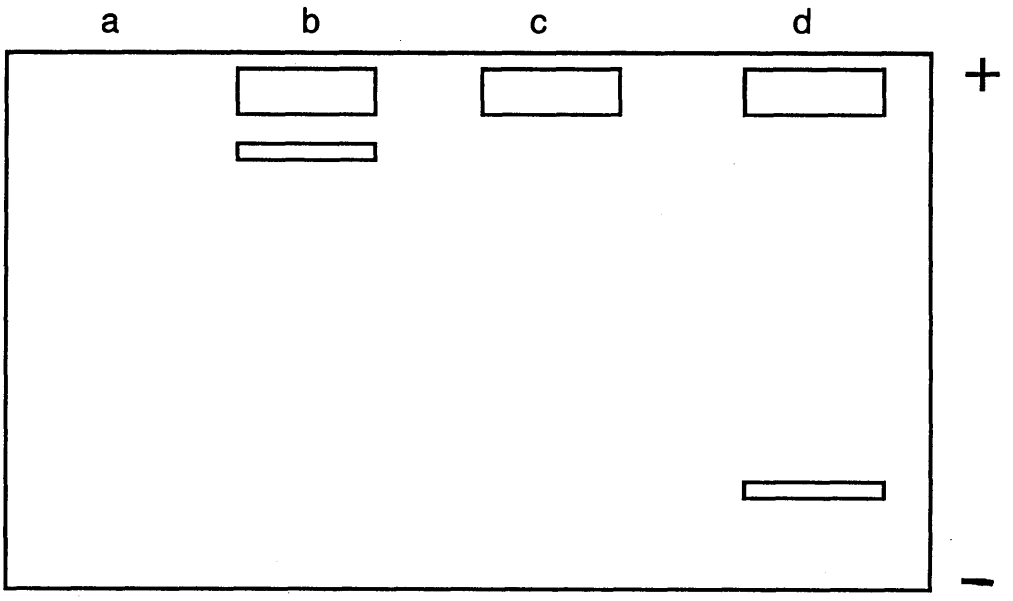

a
b
c
d
+
−

Figure 8.9 Recognition of *D. viviparus* proteins by anti-bovine SOD antiserum. 75μg of adult ES (a), adult homogenate (b) and L3 homogenate (c) were electrophoresed on 5-25% SDS-gradient gels under reducing conditions. Proteins were then blotted onto nitrocellulose and probed with rabbit antiserum to bovine erythrocyte SOD (panel A) and serum from calves exposed to 3 infections with *D. viviparus* L3 as detailed in Table 4.1 (Panel B). Tracks NA and NB show L3 homogenate probed with normal rabbit and normal bovine sera, respectively. The M_r of marker proteins (M) are shown in kDa.

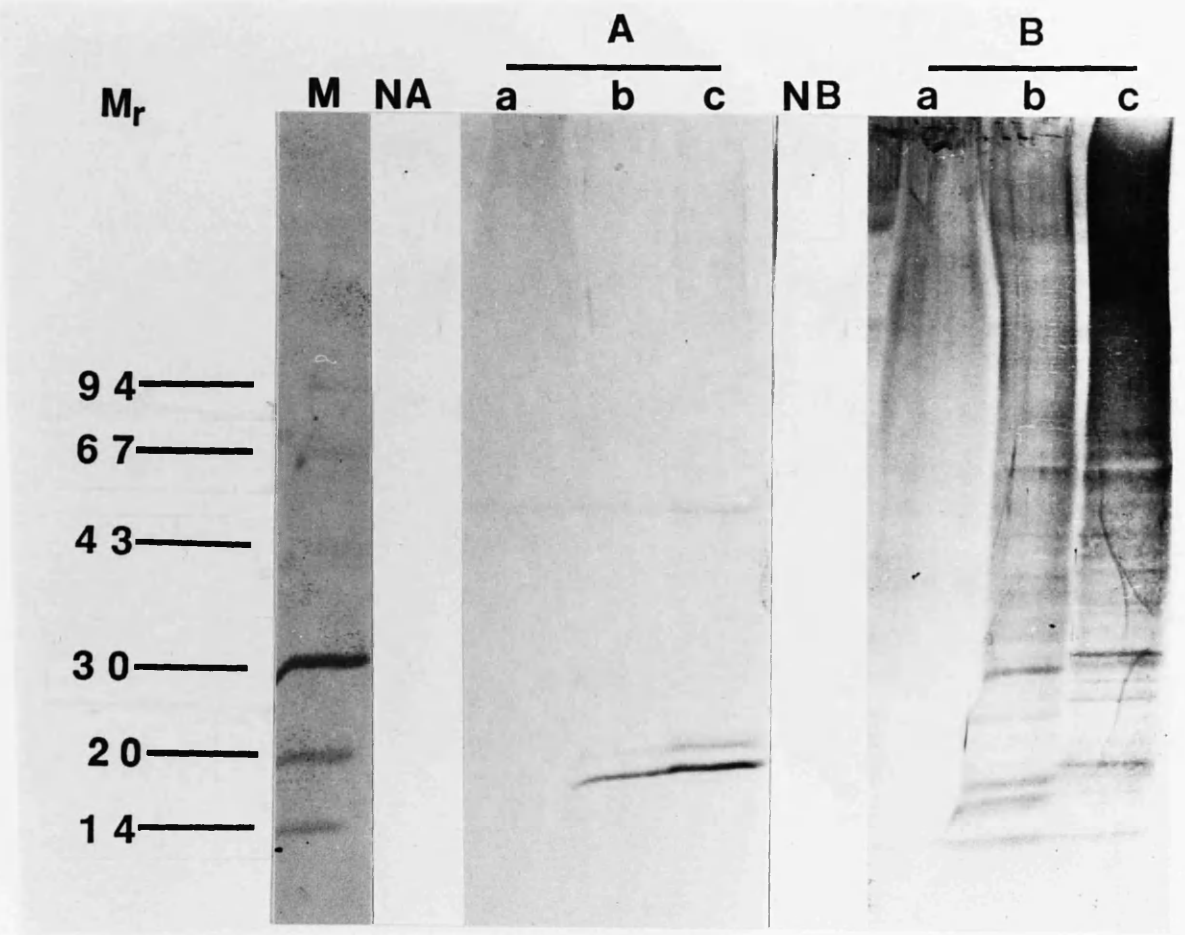
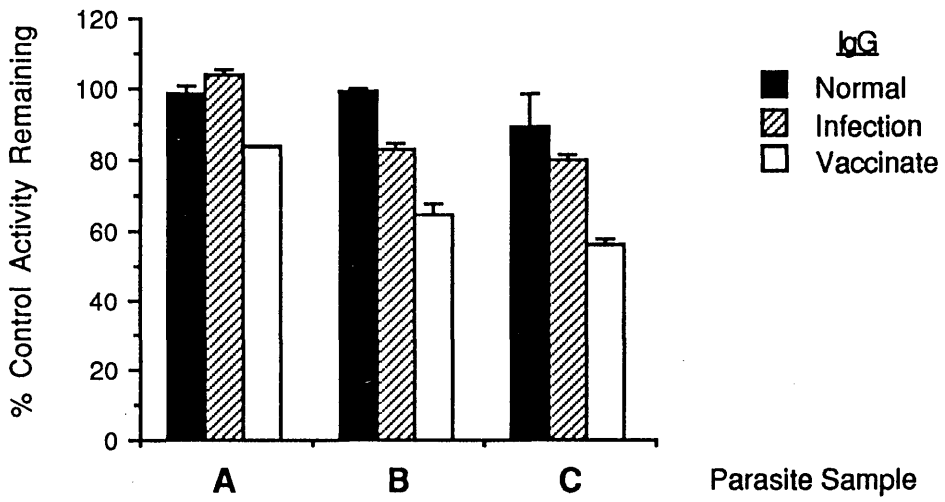

A
B
Mr
M
NA
a
b
c
NB
a
b
c
94
67
43
30
20
14

Figure 8.10 Effect of antibody on SOD activity. L3 homogenate (A), adult homogenate (B) and adult ES (C) were pre-incubated for 10 min with 25µg IgG purified from normal bovine serum and serum from calves infected with normal *D. viviparus* larvae or vaccinated with 40krad irradiated larvae on three occasions. SOD activity of parasite samples were then assayed as described in Chapter 2. The degree of inhibition is expressed as the percent activity remaining relative to a control pre-incubated with PBS. The means of two observations are shown, with error bars representing the S.D. of the observations.


% Control Activity Remaining
120
100
80
60
40
20
0
A
B
C
Parasite Sample
IgG
Normal
Infection
Vaccinate

8.3 DISCUSSION

A recent report (Rege *et al.*, 1989) described a 'cysteine' proteinase in extracts of adult *D. viviparus* and the authors speculated that it might contribute to both parasite nutrition and the pathology of lungworm infection. On the basis of postulated, but undefined, release by the parasite it was proposed that this enzyme might have value as a potential protective antigen (Bos *et al.*, 1989). The data presented here considerably expand on the previous observations. It has been clearly shown that proteolytic enzymes are released by adult and larval parasites during *in vitro* maintenance and definition by pH optima, substrate specificity and inhibitor sensitivity has revealed that parasite extracts and ES products contain multiple proteolytic activities. Clear differences in the proteinase activities of the L3 and adult stages were demonstrated indicating that such enzymes are developmentally regulated in this parasite. It has also been shown that SOD activity is present in extracts of L3 and adult parasites and is released at a low level from adult worms *in vitro*. Finally, of potential importance to the development of immunity, it has been demonstrated that the levels of proteinase and SOD activity in *D. viviparus* extracts and ES products are reduced in the presence of antibody from infected and vaccinated hosts but not from uninfected controls.

Extracts and ES material from both larval and adult parasites showed both acidic and alkaline proteinase activity (Figure 8.1). Tissue migratory parasites have been previously shown to express a variety of proteinases, often in a stage-specific manner (Wertheim *et al.*, 1983; Newport *et al.*, 1987; Knox and Kennedy, 1988) and these enzymes facilitate parasite feeding and penetration of host tissue barriers. This study provides stong evidence that this is also true of *D. viviparus* enzymes. In addition, the present observations of multiple activities argue that it is probably an over-simplification to suggest that the pathological lesions found in the lungs of infected hosts can be attributed to a single enzyme activity, as has been postulated previously (Rege *at al.*, 1989)

Using a range of substrates (Table 8.1), proteinase activity was differentiated into chymotryptic-, tryptic- or elastolytic-like. L3 homogenate exhibited tryptic activity, with very little activity against chymotryptic or elastase substrates. This lack of elastase activity, despite the fact that only L3 homogenate was available, is

consistent with parasite stages which invade only the intestinal wall. Such parasites release histolytic proteinases, but these are not elastases (McKerrow *et al.*, 1990). Elastase activity was detected, however, in adult ES and homogenate and may play a role in parasite penetration and nutrition. It was previously suggested (Rege *at al.*, 1989) that *D. viviparus* adult worms feed by penetrating and digesting lung tissue and our present findings that proteinases are released by the adult stage would be consistent with such a process.

Despite containing comparatively small amounts of protein, both adult and larval ES had similar or higher proteinase activities per unit protein than the corresponding homogenates against the protein substrate azocasein. By contrast, adult homogenate appeared to contain greater enzyme activity than ES against low molecular weight peptide substrates. This anomaly could be caused by endogenous parasite proteinase inhibitors, released during homogenisation, blocking access to the enzyme active site by high molecular weight substrates, but not small peptide substrates. An example of this inhibition pattern is provided by the mammalian proteinase inhibitor, alpha-2-macroglobulin (Starkey and Barrett, 1979).

Parasite proteinase inhibitors may play a role in protecting *D. viviparus* from damage by host proteinases. It has been demonstrated with *Ascaris suum* that such inhibitors are not secreted by the parasite, but act on host enzymes which are taken up by the worms (Peanasky *et al.*, 1987) and may protect the parasite from digestion by exogenous proteinases. It was also speculated that *D. viviparus* proteinase inhibitors might play a role in protecting parasites against host mast cell proteinases in view of the very high numbers of mast cells present in the lungs of calves infected with *Dictyocaulus* (H.R.P. Miller, personal communication). It was found, however, that adult homogenate has no effect on the activity of Rat Mast Cell Proteinases I and II *in vitro* (not shown) although it might be more pertinent to examine ES products for inhibition of mast cell proteinase activity.

It was clearly demonstrated by the use of low molecular weight substrates that stage-specific enzyme activities are present, and this was further indicated by the differing effects of inhibitors on the activity of the L3 and adult stages. At pH 5.5, L3 activity was greatly reduced by inhibitors of metallo proteinases, while adult ES and homogenate were affected only by the carboxyl proteinase inhibitor, pepstatin. At pH 7.5, L3 activity was inhibited by Pms-F, a serine proteinase inactivator, E-64, a thiol inhibitor and the chelating agent, EDTA. Activity of the adult stage was inhibited

only by metallo-proteinase inhibitors. In contrast to the previous findings (Rege *et al.*, 1989) we detected no significant thiol proteinase activity in adult homogenate and ES. This is possibly due to the acidic extraction conditions used by the previous authors and the presence of DTT in the reaction mixtures which would have protected thiol activity specifically. Full definition of parasite proteinases will, therefore, probably require the development of more sophisticated extraction procedures.

The identification of serine proteinase activity in L3 homogenate at pH 7.5, at which pH tryptic activity was dominant, suggests *D. viviparus* may express trypsin-like serine proteinases similar to those found in other parasitic helminths (McKerrow *et al.*, 1989). Recently, gene fragments of such a protease, thought to be involved in the invasion of the intestinal wall by *Anisakis simplex* larvae, were isolated using oligonucleotide probes based on the consensus sequence of the conserved active site (Sakanari *et al.*, 1989). Using this method, it may be possible to isolate the gene(s) encoding the serine proteinase expressed by larval *D. viviparus*.

Separation of proteinases by substrate gel electrophoresis confirmed that both adult ES and L3 homogenate contain a heterogenous mixture of enzymes. The diversity of these was indicated by the selective inhibition of particular zones of activity by inhibitors, although more detailed characterisation of each proteinase species awaits purification of individual enzymes. For example, adult ES proteinase activity is significantly reduced in the presence of the reducing agent 2ME, although purification of the enzyme(s) involved will be necessary to show whether this is due to disruption of intra-chain or inter-chain disulphide bonds, which may hold enzyme subunits together. It will be important to bear in mind the importance of tertiary structure to the activity of *D. viviparus* proteinases, particularly in future work involving the expression of such enzymes in bacterial expression systems.

As well as identifying and characterising proteinase activity, SOD activity of *D. viviparus* larval and adult stages was examined. The specific activity of SOD in L3 and adult extracts is comparable to that of other parasitic helminths studied, including *T. spiralis* (Kazura and Meshnick, 1984), *Nematospiroides dubius* (Smith and Bryant, 1986), *Onchocerca cervicalis* (Callahan, James and Crouch, 1988), and *Taenia taniaeformis* (Leid and Suquet, 1986), while greater activity has been observed with *Ascaris suum* (Sanchez-Moreno *et al.*, 1988). The similarity in SOD levels may indicate a common role of this enzyme in different helminth species.

The level of SOD activity in *D. viviparus* adult ES was approximately one

third of that detected in adult extracts. Secretion of SOD by *T. spiralis* infective stage larvae has been observed (Rhoads, 1983) and is thought to play a role in protecting the parasites from immune attack as well as ameliorating tissue damage (McCord, 1974). The measurable, but limited levels of SOD activity in *D. viviparus* adult and L3 ES products might suggest, however, that in this parasite the main activity of SOD is to protect the parasite from intracellular superoxide anions produced by the worms' metabolic processes. Further characterisation and purification of SOD from extracts and ES products will be required to define the function of this enzyme in *D. viviparus*.

Antiserum to bovine SOD showed strong recognition of a 19kDa molecule present in L3 and adult homogenates. This is comparable in molecular mass to SOD purified from *T. spiralis* (Rhoads, 1983), *D. immitis* and *O. cervicalis* (Callahan *et al.*, 1988), suggesting conservation of this enzyme between different nematode species. Anti-bovine SOD antiserum also recognised a protein of approximately 55kDa in adult and L3 extracts and in adult ES.

Separation and staining of SOD activity on polyacrylamide gels revealed a broad zone of activity near the anode with L3 and adult homogenate and adult ES. On some occasions additional activity could be seen with L3 and adult homogenates. The inconsistency of these findings was probably due to the activity being below the sensitivity level of the system. Different SOD isoenzymes have been described for *T. spiralis* (Rhoads, 1983) and *A. suum* (Sanchez-Moreno *et al.*, 1987) while Knox and Jones (1989) have reported considerable polymorphism in SOD isoenzymes of *N. brasiliensis* 'adapted' worms, which develop from secondary or tertiary infections. It has been suggested that, as with AChE isoenzymes (Jones and Ogilvie, 1972), this polymorphism is important to the survival of *N. brasiliensis* 'adapted' parasites. It would be interesting to determine whether the few *D. viviparus* parasites which develop from challenge infections in hosts immunised by natural infection or vaccination also synthesise a greater number of isoenzymes than those recovered from primary infections.

No catalase was identified in *D. viviparus* extracts or ES products, using antiserum to bovine liver catalase. Although this does not definitively indicate the absence of this enzyme in *D. viviparus*, catalase activity has been reported to be low or absent from a number of helminth parasites (Paul and Barrett, 1980; Rhoads, 1983) and it has been suggested that catalase activity (i.e. decomposition of hydrogen

peroxide) may be the function of parasite haemoglobins (Laser, 1944).

The present study demonstrated that antibody raised in the natural host to vaccination or infection can inhibit proteinase and SOD activity of L3 and adult stages. By substrate gel electrophoresis, we have identified an adult ES proteinase of 57kDa which is a target for immune attack. In contrast, the recognition of *D. viviparus* enzymes of 19, 21 and 55kDa by rabbit antiserum to bovine erythrocyte SOD in immunoblotting indicates homology between parasite and host SOD. This might suggest that the reduction of SOD activity by antibody from infected and vaccinated calves is due to the inhibition of SOD isoenzymes other than those identified by immunoblotting, although the possibility that bovine antibody is directed against non-conserved sites of parasite SOD cannot be excluded.

As enzyme activity was reduced by serum from immune hosts, this established that both SOD and proteolytic enzymes were of parasite and not host origin. The use of purified IgG also demonstrated that this reduction was mediated by antibody and not by host proteinase or anti-oxidant inhibitors. Further studies using greater amounts of antibody will be necessary to demonstrate whether complete inhibition of enzyme activity can be achieved and that the observed residual activity is not due to parasite enzymes which are not inhibited by host antibody or, alternatively, to host enzymes present in the parasite preparations. The latter is, however, unlikely to be true of enzyme activity of the pre-parasitic L3 stage.

It is interesting to note that adult proteinase and SOD activities were inhibited by antibody from vaccinated hosts, in which adults do not develop to full maturity. This suggests that adult enzymes are expressed by earlier stages, perhaps by the L4 and early L5 stages which can develop from radiation-attenuated larvae (Poynter *et al.*, 1960). Also of interest is the observation that bovine immune serum had a greater inhibitory effect on the proteinase activity of adult ES than adult extract, indicating differences in the enzymes present in each. This might suggest that there is only a limited endogenous store of ES proteinases or, alternatively, that secreted enzymes are produced in an inactive form, perhaps complexed to parasite proteinase inhibitors.

We have previously found that L3 homogenate, adult homogenate and adult ES can induce significant protection in guinea-pigs, and the effect of antibody on the proteinase and SOD activities of these materials suggests that these enzymes could be of potential importance in protective immunity. Interestingly, antiserum from hosts

vaccinated with irradiated larvae had a greater inhibitory effect on SOD activity than those exposed to normal larvae. Whether this is due to greater antigenicity of SOD from irradiated parasites or to destruction of such larvae resulting in greater exposure to internal SOD is, as yet, unknown, but may be relevant to understanding the effectiveness of vaccine-induced immunity.

It has been speculated that the mesenteric lymph nodes are the site of protective antibody formation in *D. viviparus* infection (Jarrett and Sharp, 1963), possibly associated with the moult from the L3 to the L4 parasite stage. Whether enzymes are released during this process is unknown. This present study was limited to the analysis of infective and adult stages of the parasite and analysis of L3/L4 moulting fluids must, therefore, await the development of improved parasite culture conditions.

Inactivation of enzymes of other parasitic nematodes by antibody has been reported, but whether such enzymes can induce protective immunity *in vivo* remains to be seen. However, an enzyme present in *Babesia bovis*-infected erythrocytes has been shown to elicit protective responses (Commins, Goodger and Wright, 1985), and, more recently, extracts from *Haemonchus contortus* adults, purified on the basis of anticoagulant activity, conferred significant protection in sheep (Boisvenue *et al.*, 1990). It will be of particular interest to discern whether such enzymes are targets for vaccine-induced immunity to *D. viviparus.* If so, this could contribute to the identification of the working principle behind the *Dictyocaulus* vaccine and could lead to the development of a new synthetic vaccine against this parasite.

CHAPTER 9

GENERAL DISCUSSION

The aim of much of the current medical and veterinary parasitological research is the development of effective vaccines. This is likely to require an understanding of host-parasite interactions and the identification of potential protective antigens which can then be chemically synthesised or the respective genes cloned for use in vaccination studies. *D. viviparus* and *D. filaria*, the lungworms of cattle and sheep, respectively, are currently the only parasitic nematodes against which succesful vaccines have been developed. These irradiated larval vaccines are, however, expensive to produce, requiring a continual supply of first stage larvae from infected calves, the culturing of these to the infective stage and their subsequent irradiation. Being live vaccines they have short shelf-lives and, therefore, cannot be easily transported or stored. With the increase in our understanding of host immune mechanisms and the advances in DNA technology over the last few years, it now seems appropriate to develop a moleculary defined vaccine against these lungworm parasites which, in the longterm, would be cheaper to produce, easier to distribute and ethically more acceptable. An immunochemical analysis of antigens of *D. viviparus* has, therefore, been carried out with a view to identifying components which may be involved in protective immunity and which could form the basis of further work at the molecular level. It was not expected that the results of this study would have any direct application in the short term but could eventually lead to the development of a defined vaccine against this parasite, as well as being relevant to the design of other anti-helminth vaccines.

The antibody responses to antigens of *D. viviparus* the host is exposed to, namely surface and ES antigens, were examined in most detail. There was no detectable secretion of parasite proteins by infective stage larvae during *in vitro* maintenance in RPMI alone or supplemented with new born calf serum. Whether this reflects the *in vivo* situation or is an *in vitro* phenomenon due to the absence of stimuli provided by the host will require further investigation. This will most likely require the development of better culture conditions for the maintenance of third stage larvae and which will also allow their development to the L4 stage, which it has so far been unable to examine. It may also be possible to examine the release of L3/L4 products by culturing post-infective parasites recovered from infected hosts. It is important to examine the release of parasite components from developing larvae as it has been suggested that the mesenteric lymph nodes are an important site in the production of protective antibody, which may be directed against antigens secreted by infective stage larvae or released during the moult

from the L3 to the L4 stage (Jarrett *et al.*, 1957a; Jarrett and Sharp, 1963).

Although no parasite components were observed following Coomassie or silver staining of L3 ES products on SDS-PAGE, high levels of proteinase activity were detected in ES products of this stage using azocasein as substrate. It was not possible, however, to characterise L3 proteolytic enzymes due to the limited quantity of L3 ES available. It would seem important, however, to examine this activity further and determine whether released proteinases play a role in parasite moulting or in penetration of host tissue, as has been speculated for proteinases of other parasitic helminths (Gamble, Purcell and Fetterer, 1989; Hotez *et al.*, 1990; McKerrow *et al.*, 1985).

In contrast to infective stage larvae, a complex mixture of proteins was definable in the *in vitro*-released products of adult parasites. The recognition of most of these by infected and vaccinated bovine hosts demonstrated both their antigenicity and possible release by parasites *in vivo*. The limited glycosylation of ES antigens, indicated by the low level of ^{3}H-glucosamine uptake and lectin binding, suggested that antibody is directed against protein epitopes. It should, therefore, be possible to produce recombinant ES antigens by cloning of the genes involved.

An adult ES component of 30kDa was strongly recognised by antibody from all bovine hosts examined and although we have not shown whether recognition of this antigen is associated with protective immunity to *D. viviparus*, it is a good candidate for further studies. This antigen was recognised soon after infection and was the only adult ES component strongly recognised by sera from calves vaccinated with two doses of 40krad irradiated larvae and, therefore, not exposed to mature adult worms. These findings suggested that this adult ES antigen is also expressed by larval parasites and it would be interesting to determine the function of this component which appears to be conserved between different parasite stages. Using specific antiserum to the 30kDa component to screen *D. viviparus* expression libraries it should be possible to isolate and sequence the gene encoding this antigen.

A significant antibody response was detected to antigens exposed on the sheath of infective stage larvae of *D. viviparus*. This is surprising in view of the fact that such larvae are thought to exsheath prior to their invasion of host tissue and that the sheath plays no part in infection or immunity. The evidence for this is, however, circumstantial being based on the observations that the majority of L3 recovered from the mesenteric lymph nodes are exsheathed (Poynter *et al.*, 1960) and that exsheathed larvae are as infective and immunogenic as sheathed

larvae (Bain and Urquhart, 1988). The present findings suggest, however, that the site of larval exsheathment should be re-addressed and the role of anti-sheath antibody defined. *In vitro* studies using fluorescent- or radio-labelled larvae, where the label is restricted to sheath components, may be useful in demonstrating whether larval exsheathment occurs before, during or after passage through host intestinal tissue.

Interestingly, it has been found that a monoclonal antibody directed against antigens exposed on the sheath of *D. viviparus* also binds to that of the bovine gastrointestinal nematodes *C. oncophora*, *O. ostertagi* and *H. contortus* as does anti-*D. viviparus* infection serum (J. Gilliard, personal communication). In the present study it was observed, however, that antibody from calves infected with these parasites does not bind to sheathed larvae of *D. viviparus*. This might further suggest that an immune response to *D. viviparus* sheath antigens is induced by the presence of sheathed larvae within the host tissue while parasite species which are restricted to the gut induce no circulating anti-sheath antibody, although the possibility that they induce a local secretory antibody response has not been investigated.

It is not yet known whether anti-sheath antibodies are involved in protective immunity to *D. viviparus*. This may now be examined by passive immunisation with anti-sheath monoclonal antibody or, alternatively, by immunisation with purified sheaths. The latter has proved impossible with *D. viviparus* as exsheathment of this parasite can only be achieved by incubation in Milton's fluid (0.001% w/v sodium hypochlorite) which results in solubilisation of the sheath. However, the occurence of common or cross-reactive epitopes on the sheaths of *D. viviparus* and gastrointestinal trichostrongyles may allow immunisation studies to be performed with sheaths of these parasites, which can be easily recovered following Milton treatment or incubation in $C0_2$.

These differences in CO_2 exsheathment and solubility of L3 sheaths in hypochlorite between *D. viviparus* and gastrointestinal trichostrongyles may reflect differences in the mechanism of exsheathment of these parasites. A metalloproteinase isolated from exsheathing fluid of *H. contortus* has been shown to mediate exsheathment of this and other ruminant trichostrongyles including *T. colubriformis*, *O. ostertagi* and *C. oncophora* (Gamble *et al.*, 1989). This enzyme induces refractile ring formation in the L3 sheath which allows the cuticular cap to fall off, providing an opening for the parasite to emerge. This

proteinase was found to have no effect on the remainder of the sheath suggesting that the ring region differs in composition from the rest of the sheath. In contrast, no refractile ring is observed during exsheathment of *D. viviparus* L3, albeit in hypochlorite, nor does any region of the sheath appear to be uniquely susceptible to hypochlorite treatment (C. Britton and J. Gilliard, unpublished). This might suggest that exsheathment of *D. viviparus* requires a weakening of the entire cuticular structure, rather than the formation of an escape opening.

At present it is not known whether exsheathment of *D. viviparus* is enzyme-mediated. This may be determined by examining the effects of enzyme inhibitors on spontaneous or hypochlorite-induced larval exsheathment. It would also be interesting to determine whether the enzyme involved in the exsheathment of a number of other trichostrongyles (Gamble *et al.*, 1989) can mediate exsheathment of *D. viviparus* larvae. Alternatively, exsheathment of *D. viviparus* may be a mechanical process induced by penetration of larvae through host tissue as is thought to occur with infective larvae of *A. tubaeforme* (Matthews, 1972, 1977 and personal communication).

In contrast to the sheathed larval surface, there was no specific IgG antibody recognition of surface-exposed antigens of exsheathed larvae of *D. viviparus* by infected bovine hosts. There was, however, a high level of binding of IgM antibody to the L3 cuticle with all bovine pre-infection sera examined. It is proposed that this is due to the expression on the parasite surface of an antigenic determinant to which bovine hosts are commonly exposed. Non-specific binding of IgM antibody may represent an immunoevasive parasite mechanism with IgM masking surface-exposed epitopes or preventing antibody of other specificities from binding due to steric hinderence, as previously speculated for 'blocking' antibodies to the surface of *S. mansoni* schistosomula (Butterworth *et al.*, 1987). At present the determinant recognised by this non-specific IgM antibody has not been identified. No binding of fluorescent-labelled lectins to the exposed L3 cuticle was observed, suggesting that non-specific IgM binding was not due to the recognition of sugar residues. It may, however, be worthwhile examining this in greater detail using a wider range of lectins or by determining the effect of periodate on the reactivity of bovine pre-infection sera to the L3 surface. It would also be interesting to determine whether recognition of periodate-sensitive residues is responsible for inhibiting an antibody response to parasite-specific cuticular antigens *in vivo.* This may be achieved by examining host immune recognition of the L3 cuticle following infection with exsheathed L3 pre-treated with periodate although it

would, of course, be necessary to ensure that periodate treatment has no adverse effects on larval viability.

Although phosphorylcholine was detected on the L3 cuticle, this epitope did not appear to be responsible to any substantial degree for the non-specific binding of IgM antibody. Nevertheless, this determinant may be involved in maintaining the high level of IgM antibody in infected hosts through induction of low affinity IgM responses (Mitchell, 1977). It may be possible to examine the involvement of surface-exposed phosphorylcholine on immunosuppression *in vivo* by removing this epitope prior to infection as previously suggested for periodate-sensitive epitopes, although removal of phoshorylcholine by treatment of larvae with, for example, alkali and borohydride (Maizels *et al.*, 1987a) may have a damaging effect on parasite proteins.

In contrast to calves infected with normal larvae, those vaccinated with X-irradiated larvae showed a substantial IgG1 response to the exposed L3 cuticle, thus demonstrating that parasite-specific antigens are present on this surface. It is speculated that induction of this anti-cuticular antibody response in vaccinated hosts is due to irradiation-mediated suppression of larval development resulting in such hosts being exposed to the L3 cuticle for a greater length of time than those infected with normal larvae. This may stimulate an enhanced immune response to parasite specific cuticular antigens in vaccinated hosts, thereby allowing them to overcome the proposed immunoevasive mechanism of infective larvae. This anti-cuticular response may be involved in limiting the number of irradiated larvae reaching the lungs and it would, therefore, be interesting to determine whether the transfer of anti-L3 cuticular antibody has any effect on parasite burden.

In the present study it was not possible to identify exposed L3 cuticular antigens due to the poor radiolabelling of cuticular components of this parasite stage. This phenomenon has been reported with infective stage larvae of other parasitic nematodes, but can be overcome to some extent by incubating larvae at room temperature prior to radiolabelling (Devaney and Jecock, 1991). This increase in radiolabelling is thought to be due to changes in surface composition as the parasites move to a different environment. However, the labelling efficacy of *D. viviparus* larvae did not increase following *in vitro* maintenance, suggesting that changes in surface properties may occur more slowly in this parasite. Alternatively, the exposed surface of exsheathed larvae of *D. viviparus* may be composed of proteoglycan or glycolipid structures which would not be detected by radiolabelling. As yet, we have been unable to examine the nature of the L3 cuticle

with anti-cuticular monoclonal antibodies as those produced against the exsheathed surface following natural infection were all directed against the phosphorylcholine determinant (J. Gilliard, personal communication).

In contrast, surface-associated antigens of sheathed larvae could be identified by radiolabelling studies, thus demonstrating differences in the biophysical properties of sheathed and exsheathed larvae. This was also demonstrated by the insertion of a C18 lipid analogue into the cuticle of sheathed but not exsheathed L3. Examination of the molecular basis for these differences in surface properties may provide a better understanding of the changes in parasite metabolism and antigen expression during the infection process. Such studies may reveal whether these changes are essential to host invasion or are associated with immune evasion. By carrying out similar studies on L3 and L4 stages recovered from infected hosts it will be possible to determine whether such alterations in surface composition are maintained in the post-infective stages.

To understand the success of the irradiated vaccine it is important to determine the effects of irradiation on parasite metabolism. The present study examined whether irradiation had any effect on protein synthesis by infective stage larvae of *D. viviparus* which could limit their survival within the host, perhaps due to a reduction in the synthesis of essential proteins or the production of new components of enhanced immunogenicity. The identical profiles of ^{35}S-labelled proteins observed following biosynthetic labelling of normal, 40krad and 100krad irradiated larvae suggested that irradiation has no effect on protein synthesis in the L3 stage. This might indicate that vaccine-induced immunity to *D. viviparus* is not dependent on the synthesis of aberrant parasite proteins of enhanced immunogenicity as is thought to occur with irradiated schistosomula of *S. mansoni* (Wales, 1989) and may explain why, in contrast to *S. mansoni*, effective immunity to *D. viviparus* can be induced by exposure to normal or X-irradiated larvae. It would, however, be important to examine the ^{35}S-protein profiles by 2-dimensional gel electrophoresis and to compare the antigenicity and conformation of proteins synthesised by normal and irradiated larvae.

It may be speculated that the success of the irradiated larval vaccine may depend on a reduction in the number of larvae reaching the lungs and on termination of parasite development prior to the pathogenic adult stage. Inhibition in the development of irradiated parasites within the host may be reflected in a reduction in protein synthesis and it would, therefore, be important to compare the

uptake of ^{35}S-methionine by post-infective normal and irradiated parasites recovered from infected and vaccinated hosts. It would also be interesting to examine and compare the effects of drug-attenuation (TEM) (Cornwell and Jones, 1970, 1971) on parasite metabolism with those induced by irradiation.

It is probable that protective immunity to *D. viviparus* cannot be attributed to any single parasite stage and although the L3 and L4 stages have been implicated (Cornwell, 1962a; Oakley, 1981a) exposure to adult parasites also appears to be important to protection (Michel 1962; Downey, 1980). Although vaccinated hosts are not exposed to patent infections it is speculated that they are efficiently protected against disease due to an enhanced response to antigens expressed by developing larval stages. Irradiated larvae may develop more slowly than normal larvae and vaccinated hosts may, therefore, be exposed to antigens expressed by these stages for a greater length of time, possibly resulting in the induction of a greater immune response. It is also suggested that larval antigens are expressed in abnormal sites and processed differently to antigens of normal larvae, perhaps leading to an enhanced immune response. Passive immunisation studies with immune sera from naturally infected and vaccinated calves has suggested that irradiated parasites may induce a greater cell-mediated response than normal parasites (Canto, 1990). The fate of normal and irradiated parasites and processed antigens may be examined by radiotracking studies. For practical reasons this will most likely be performed in guinea-pig hosts, although it cannot be assumed that larvae undergo the same fate as in the bovine host.

Characterisation of the antibody response by immunoblotting and immunoprecipitation studies revealed no unique recognition of ES or somatic antigens by hosts vaccinated with irradiated larvae. Our studies were, however, restricted to examining responses of the IgG and IgM isotypes and examination of other Ig isotype responses should be carried out. It is well documented that helminth infections induce significant IgE responses, while local secretion of mucosal or bronchial IgA may be important to immunity. As X-irradiated larvae may induce greater cell-mediated responses than normal larvae it would also be worthwhile comparing the T-cell reactivities of infected and vaccinated hosts to whole parasites or to individual parasite antigens.

There is now increasing interest in defining the biological function of parasite antigens which appear to be involved in protective immunity. Demonstration that host effector mechanisms can, in some host-parasite interactions, inhibit essential parasite functions may allow a better understanding

of the mechanism of host-resistance to infection, as well as being relevant to the identification of potential protective antigens of related parasites. Consequently, parasite enzymes are now widely considered to be potential vaccine candidiates (Capron *et al.*, 1987; Commins, Goodger and Wright, 1985; Boisvenue *et al.*, 1990; McKerrow *et al.*, 1990). The present study has identified multiple proteolytic enzyme activities in ES products and somatic components of infective and adult stages of *D. viviparus*. The specificity of these proteinases was characterised as tryptic, chymotryptic or elastolytic according to their activity against low molecular weight substrates. However, examination of the ability of these parasite proteinases to degrade biological substrates such as bovine immunoglobulin, haemoglobin, fibrinogen or basement membrane collagen may provide important information on the function of such enzymes *in vivo*. It has previously been demonstrated that a cysteine proteinase present in extracts of *D. viviparus* adult worms is capable of degrading host haemoglobin and collagen and may, therefore, be involved in parasite nutrition (Rege *et al.*, 1989), although secretion of this enzyme was not demonstrated. The present study demonstrated clearly that proteinases are secreted by adult worms of *D. viviparus* and further studies will be carried out to examine their role in nutrition and in the lung pathology associated with *D. viviparus* infection.

It has long been speculated that host antibodies may act as anti-enzymes, blocking parasite invasion and inhibiting parasite survival (Chandler, 1932, Thorson, 1956). It has now been established that the activity of proteolytic enzymes present in extracts and ES products of *D. viviparus* L3 and adult stages is substantially reduced by host antibody *in vitro*. It will now be important to assess the immunising potential of such enzymes *in vivo*. Purification of these enzymes will be carried out by chromatographic techniques or by separation and elution of parasite enzymes from substrate gels. It is unlikely that sufficient quantities of enzymes will be purified is this way to allow large scale vaccination studies. The generation of antisera to purified parasite enzymes will, however, allow the screening of expression libraries and the identification of clones expressing such enzymes, thus enabling future immunisation studies to be performed with recombinant proteinases. Additionally, both serine and thiol proteinase activities were identified in L3 extract. The use of oligonucleotide probes based on the consensus sequence of the conserved active site of these enzyme classes will allow the synthesis of the genes encoding these enzymes, by the polymerase chain

reaction, and their subsequent expression in pro- or eukaryotic expression vectors (Sakanari *et al.*, 1989; Miller and Knox, 1991; Tighe and Pritchard, 1991).

As well as further studies on proteolytic activities of *D. viviparus* extracts and ES products it would be important to examine the expression of proteinase inhibitors by the parasite which may be important to its survival in the host. Proteins secreted by the cestode parasites *T. taeniaformis* and *E. granulosus* have been shown to inhibit elastase, chymotrypsin and trypsin activities, neutrophil chemotaxis, and complement activation *in vitro* and are thought to be important to parasite survival *in vivo* (Leid, Grant and Suquet, 1987; Shepherd, Aitken and McManus, 1991; Suquet, Green-Edwards and Leid, 1984). It would be particularly interesting to determine whether products secreted by adult parasites of *D. viviparus* have any effect on mast cell proteinase activities in view of the significant increase in the number of such cells in the lungs of *D. viviparus* infected calves (H.R.P. Miller, personal communication).

As well as examining the expression of parasite proteinase inhibitors it would be important to investigate further the antioxidant activity, superoxide dismutase (SOD), which was detected in extracts and ES products of the L3 and adult stages. SOD may be involved in protecting parasites from the toxic effects of superoxide produced by activated host phagocytic cells, particularly eosinophils, during infection. It would, therefore, be interesting to examine the effects of superoxide generating systems or host phagocytic cells on the survival of *D. viviparus in vitro* (Callahan, Crouch and James, 1988) and examine any subsequent increase in parasite SOD levels or SOD isoenzyme expression which may be induced by the superoxide anion (Michelson, 1977).

It would also be interesting to examine both SOD and proteolytic enzyme expression by the few *D. viviparus* adult parasites which develop in immune hosts and compare these with the enzyme activities of parasites recovered from primary infections. Any differences in the level of activity or isoenzyme patterns between these parasite populations might indicate modification of enzyme expression in response to immune stress and perhaps lead to a better understanding of the mechanisms by which parasites adapt to enhance their survival within the host.

The expression of other enzymic activities, such as acetylcholinesterase and glutathione-S-transferase, which has been selected as a vaccine candidate against schistosome infections (Capron *et al.*, 1987), should also be examined in *D. viviparus*, as should glutathione peroxidase to which the major 29kDa surface

glycoprotein of *B. malayi* has been reported to show sequence homology (Cookson, Baxter and Selkirk, 1991). The observation that other filarial nematodes express an analogous surface-associated antigen suggests that this molecule may play an important role in parasite survival. A major antigen of similar molecular mass has also been identified following surface labelling *D. viviparus* adult worms and it would be interesting to discern whether this represents another 29/30kDa anologue.

An important observation in this study was the significant heterogeneity in the specificity of the immune response of outbred bovine and guinea-pig hosts to antigens of *D. viviparus*. This did not occur to the same extent in guinea-pigs of inbred strains, suggesting that the immune response to *D. viviparus* is under genetic control. Studies on other nematode infections would suggest the antibody repertoire to *D. viviparus* is controlled by genes of the MHC region (Else and Wakelin, 1989; Kennedy, 1989; Kwan-Lim and Maizels, 1991; Kennedy *et al.*, 1991b). As the guinea-pigs studied here bore identical MHC class I but disparate class II region genes, our findings might suggest the involvement of the MHC class II region. Further examination of this will require the availability of MHC congenic or recombinant strains of guinea-pigs.

It may also be possible to examine genetic control of the immune response to *D. viviparus* antigens in the bovine host as a result of increasing knowledge on the bovine MHC class I (BoLA-A) and class II (BoLA-D) regions (Hoang-Xuan *et al.*, 1982; Emery *et al.*, 1987; Zijpp and Egberts, 1989) and to relate host MHC gene expression to the recognition of parasite antigens and perhaps also to susceptibility to infection, as has previously been demonstrated in infections with other helminth parasites including *T. muris* (Else and Wakelin, 1989), *T. spiralis* (Kennedy *et al.*, 1991) and *B. malayi* (Kwan-Lim and Maizels, 1991). Examination of host resistance to infection and the specificity of the immune response may be relevant to the identification of potential protective antigens. The heritable nature of host responsiveness has also raised the possibility of selectively breeding animals which are less susceptible to parasitic infections or which develop a strong protective immune response to parasite antigens, thus limiting parasite burden (Albers and Gray, 1986; Piper and Barger, 1988; Barger, 1989). While this may be of potential importance to the control of other parasitic diseases of livestock, the selective breeding of animals less susceptible to lungworm infection is unlikely to be justified in view of the successful vaccine available against this parasite.

Finally, it will be important to consider genetic restriction of immune responsiveness to antigens of *D. viviparus* in the design of a molecularly defined vaccine against this parasite. It will be essential to select antigens for which this does not apply or to use a mixture of antigens, at least one component of which is recognised and protective in all individuals. It may be possible to overcome restriction of immune responsiveness by linkage of antigens to carrier proteins or use of an appropriate adjuvant (Bomford, 1989; Warren and Chedid, 1988). With regard to the latter, however, it has recently been found that Freund's adjuvant-assisted immunisation of guinea-pigs with *D. viviparus* adult ES products results in restrictions to the antibody repertoire similar to those seen in the context of infection (J.B. McKeand, C. Britton, G.M Urquhart and M.W. Kennedy, unpublished).

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APPENDIX

PHOSPHATE BUFFERED SALINE (PBS), pH 7.2

10g	NaCl	(BDH)
0.25g	KCl	(Hopkin and William)
1.437g	Na_2HP0_4	(BDH)
0.25g	KH_2PO_4	(BDH)
	Made to 1 litre with deionised water	

***ASCARIS* CULTURE MEDIUM**

100ml	RPMI 1640	(Gibco, 041-02409M), supplemented with:
240µg	L-glutamine	(Flow, 16-801-49)
100mg	D-glucose	(Formachem)
40µg	Tripeptide (Glycl-L-histidyl-L-lysine)	(Sigma, G-1887)
50ng	glutathione	(Sigma, G-4251)
10,000 IU	penicillin	(Fow, 16-700-49)
10,000µg	streptomycin	(Flow, 16-700-49)
1mM	sodium pyruvate	(Flow, 16-820-49)
1mg	sodium bicarbonate	(Flow, 16-883-49)
2ml	amphotericin B	(Flow, 16-723-46)
0.25ml	gentamycin	(Sigma, G-7632)
1tablet	Cephalexin selectatab	(Mast, MS 10)
1 tablet	VCNT selectatab	(Mast, MS 6, Mast Laboratories Ltd., U.K.)

TRIS HOMOGENISATION BUFFER

100ml	10mM Tris base (Boehringer Mannheim)/HCl pH 8.3 containing:
2mM	ethylenediaminetetra acetic acid (EDTA: Sigma, ED2SS)
1mM	phenylmethylsulphonyl fluoride in isopropanol (Pms-F: Sigma, P-7626)
5µM	pepstatin in methanol (Sigma, P-4265)
2mM	1,10 phenanthroline in ethanol (Sigma, P-9375)
5µM	leupeptin (Sigma, L-2884)
5µM	antipain (Sigma, A-6271)
25µg/ml	N-*p*-tosyl-L-lysine chloromethyl ketone (TLCK: Sigma, T-7254)
50µg/ml	N-tosyl-L-phenyl alanine chloromethyl ketone (TPCK: Sigma T-4376)

LITHIUM WASH BUFFER

5ml	10%(w/v) SDS	(BDH)
10ml	1M LiCl	(BDH)
25ml	10% (v/v) Triton X-100	(Sigma)
25ml	1M Tris pH 7.2	
14.5g	NaCl	
	Made to 500ml with deionised water	

SDS-PAGE REAGENTS

Stock solutions for SDS-polyacrylamide gels

Solution

N	250g	Acrylamide	(BDH)
	3.875g	N,N'-methylene-bis-Acrylamide	(BDH)
		H_2O to final volume of 625ml	
		Stored at 4°C with 'Amberlite' monobed resin (MB-1, BDH, 55007)	

AA	480ml	1M HCl	(May and Baker, Dagenham, U.K.)
	366g	Tris base, pH 8.9	
	8g	SDS	
	2.3ml	N,N,N',N'-Tetramethylethlenediamine (TEMED: Sigma, T-8133)	
		H_20 to final volume 1 litre	

Solution

S	121.1g	Tris base/HCl, pH 6.8
	8g	SDS
	4ml	TEMED
		H_20 to final volume 1 litre

APS	100mg/ml	ammmonium persulphate (BDH)

Stock Acrylamide/bis-Acrylamide solution for SOD detection gels

29.9g	Acrylamide
0.8g	N,N'-methylene-bis-Acrylamide
	H_2O to final volume 100ml

Working solutions for SDS-polyacrylamide gels

i. Volumes for 2 x 0.7mm thick gradient gels

Solution	5% gel	25% gel	Stacking gel
N	3ml	15ml	2.5ml
AA	3ml	3ml	-
dH_20	18ml*	6ml	15ml
S	-	-	2.5ml
APS	50µl	33.35µl	200µl

ii. Volumes for 4x0.5mm thick 7.5% mini-gels

Solution	Separating gel	Stacking gel
N	4.5ml	2.5ml
AA	3ml	-
dH_2O	16ml*	15ml
S	-	2.5ml
APS	82µl	200µl

* for substrate gels dH_2O contained gelatin to give a final concentration of 0.1%

iii. Volumes for 4x0.5mm thick 7.5% mini-gels for SOD detection

Solution	Separating gel	Stacking gel
stock Acryl/bis	2.5ml	1.3ml
1.5M Tris/HCl, pH 8.8	2.5ml	-
0.5M Tris/HCl, pH 6.8	-	2.5ml
dH_2O	5ml	6ml
APS	50µl	50µl
TEMED	10µl	10µl

PAGE-sample buffer

5g	SDS
5ml	1M Tris, pH 7.5
2ml	100mM Pms-F
1ml	100mM EDTA
10ml	glycerol (May and Baker)
2ml	0.2% (w/v) Bromophenol blue (BDH, 44305)
	H_20 to final volume 95ml

Pms-F and EDTA omitted for polyacrylamide substrate gels

Pms-F, EDTA and SDS omitted for SOD detection gels

Electrophoresis buffer

1440g	glycine
300g	Tris
50g	SDS
	H_20 to final volume 10 litres. Dilute 1/5 with H_20 to use. SDS omitted for electrophoresis of SOD gels.

LECTIN AFFINITY CHROMATOGRAPHY RUNNING BUFFER

0.605g	Tris/HCl, pH 7.4
4.09g	NaCl
10mg	$MnCl_2$ (BDH)
74mg	$CaCl_2$ (BDH)
2.5ml	Triton X-100
0.5g	NaN_3
	H_2O to final volume 500ml

BLOTTING TRANSFER BUFFER

9.09g	Tris
43.26g	glycine (Sigma)
3g	SDS
600ml	methanol (May and Baker)
	H_20 to final volume 3 litres

BLOTTING SUBSTRATE SOLUTION

24mg	4-chloro-1 naphthol (Sigma, C-8890)
8ml	methanol
40ml	10mM Tris buffered saline, pH 7.2
16µl*	hydrogen peroxide (30% w/v) (BDH, 45202)

* Add immediately before use

ELISA REAGENTS

Coating buffer (Carbonate/bicarbonate, pH 9.6)

0.75g	Na_2CO_3 (BDH)
1.465g	$NaHCO_3$ (BDH)
0.1g	NaN_3 (Sigma)
	H_2O to 500ml. Stored at 4°C

ELISA wash buffer

1.07g	Na_2HPO_4	
0.39g	NaH_2PO_4	
8.5g	NaCl	
0.5ml	Tween-20	(Sigma P-1379)
	H_2O to final volume 1 litre	

OPD buffer

5.11g	Citric acid	(BDH)
9.15g	NaH_2PO_4	
	H_2O to final volume 1 litre	

OPD substrate solution

4mg	OPD	(Sigma, P-1526)
10ml	OPD buffer	
5µl	H_2O_2 (add immediately before use)	(BDH, 45202)

Alkaline phosphatase buffer (Diethanolamine buffer, pH 9.8)

9.7ml	diethanolamine	(BDH, 10393)
0.5mM	$MgCl_2$	(BDH)
0.2g	NaN_3	
	1M HCl to pH 9.8	
	H_20 to final volume 1 litre	

Alkaline phosphatase substrate solution

1 (5mg)	phosphatase substrate tablet	(Sigma, 104-105)
5ml	alkaline phosphatase buffer	

PNGase DIGESTION BUFFER

300µl	100mM	1,10 phenanthroline
500µl	7.5%	Nonidet P-40 (Sigma, N-6507)
900µl	550mM	sodium phosphate, pH 8.6

IgG PURIFICATION WASH BUFFER

1.38g	NaH_2PO_4
8.77g	NaCl
	NaOH to pH 7.0
	H_2O to final volume 1 litre

XANTHINE SUBSTRATE SOLUTION

Buffer 1 (50mM carbonate buffer, pH 10.2)

5.3g	Na_2CO_3
0.35g	EDTA
	H_2O to 1 litre
	$NaHCO_3$ to pH 10.2

Solution 2 (5mM Xanthine)

0.76g	xanthine, dissolved in 0.1M NaOH (Sigma, X-0626)
	H_2O to 100ml

Solution 3

0.253g	iodonitrotetrazolium (Sigma, I-8377)
15ml	75% ethanol
	H_2O to 100ml

Xanthine substrate solution

20ml	Solution 2
5ml	Solution 3
	Buffer 1 to 1 litre

RIBOFLAVIN/TEMED FOR SOD STAINING

Riboflavin stock solution

3.2mg	riboflavin (Sigma, R-4500)
	H_2O to final volume 3.9ml

Staining Solution

1ml	riboflavin stock solution
99ml	Tris/HCl, pH 8.5
400µl	TEMED