Aspects of the immunobiology of *Dictyocaulus viviparus* infection

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To Mum, Dad, Janis and Iain for their love and encouragement

The research reported in this thesis
is my own original work, except where otherwise stated,
and has not been submitted for any other degree.

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Will someone please hand me the Oscar now.....

Summary

The irradiated larval vaccine for the control of bovine parasitic bronchitis is the only helminth vaccine widely used commercially and the continued success of this vaccine has limited the amount of research on the immunobiology of the *Dictyocaulus viviparus* host/parasite relationship. By examining some of the properties of the surface or released products of *D. viviparus*, it was hoped that the studies reported here would provide some insight into the immune mechanisms directed against this nematode.

The first experiments (Chapter 3) were designed to examine the surface of several developmental stages of *D. viviparus* using the IFAT. Most of the stages were found to express stage-specific surface antigens and the relevance of these in immune avoidance in the host is discussed. When adult parasites were maintained in mammalian tissue culture conditions, they were observed to shed surface-bound antibody, which was considered to be of relevance in survival of adult worms in the lungs. Another interesting finding was the apparent immunogenicity of the L3 sheath, a structure previously assumed to be lost in the rumen of the host. Further studies indicated that the L3 stage may, in fact, penetrate the host mucosa with its sheath intact and the possibilty that the sheath may act to divert immune effector mechanisms away from the immunogenic surface of migrating stages during a primary infection was proposed. In addition, a strong heterophile IgM reactivity was observed against the cuticle of the exsheathed L3 and it was suggested that this antibody may block, by steric hindrance, the binding of more effective isotypes to L3 surface epitopes whose recognition might be essential to the development of protective immunity.

In Chapter 4, the results of studies on parasite surface recognition by antibodies in sera from infected and vaccinated calves are presented. It was found that L3 surface-specific antibody did not increase in calves vaccinated twice with *D. viviparus* larvae irradiated at 400Gy or 1000Gy. These results confirmed previous studies, using the complement fixation and indirect haemagglutination tests, and indicated that immunity induced by vaccination with 400Gy-irradiated larvae was not necessarily accompanied by a measurable rise in specific antibody. On challenge of all vaccinated calves, a typical anamnestic response was observed which indicated that the calves had been

primed sufficiently against the surface of the L3 stages. Antibody responses directed against the surface of the adult stage in normal L3-infected calves followed classical antibody kinetics in that there was an increase in IgM by Day 52 of infection, followed by a higher and more sustained increase in IgG_1 and IgG_2 subclasses. This response to normal L3 infection perhaps reflected the longevity of the adult surface antigens presented to the host in the respiratory tract. Interestingly, the IgG isotypes appeared to increase at the presumed time of adult parasite expulsion and the relevance of these antibody increases are discussed.

In Chapter 5, immunisation experiments against *D. viviparus* in guinea pigs are presented. The results showed that a high degree of protection (86% mean reduction in worm burden) was obtained with adult ES products in the context of Freund's Complete adjuvant. The efficacy of these *in vitro* released products was compared with somatic extracts of adult and L3 stages, both of which failed to stimulate significant levels of protection. In a preliminary attempt to identify effector mechanisms induced by ES vaccination, immunity was passively transferred from ES-immunised guinea pigs to naive recipients which indicated the relevance of antibody in this response. However, the level of immunity conferred was less than that transferred with serum raised against normal L3 infection. These results indicated that adult ES materials could be a source of protective antigens and attempts were made to further fractionate this rather complex antigen preparation using SDS-PAGE and electroelution techniques. Subsequent immunisation with whole ES obtained following these procedures failed to protect against challenge but this may have been a result of denaturation of the protective epitopes during the preparative procedures. Unfortunately, in this second immunisation study native adult ES material also failed to induce a significant level of immunity and the possible reasons for this are discussed. No further attempts were made to fractionate the adult ES products using SDS-PAGE.

Since a considerable degree of heterogeneity in antigen recognition was found among individuals immunised with similar preparations, further experiments were undertaken to examine antigen recognition induced in ES-immunised inbred guinea pigs. While one inbred strain (strain 13) and outbred Dunkin-Hartley animals were significantly protected against challenge, another inbred strain (strain 2) showed no protection. In addition, the two inbred strains of guinea pigs showed differences in the antibody repertoires induced by adult ES immunisation. In all these protection experiments, specific antigen recognition patterns could not

be correlated with protection but the results indicated that the adult ES-induced antibody repertoire was under genetic control.

In the final chapter of this thesis (Chapter 6), *D. viviparus* was examined for the presence of an enzyme of possible immunomodulatory activity, acetylcholinesterase. The objective of this approach was to search for molecules which may be essential to parasite survival within the host. Five isoforms of AChE were found to be present in both larval and adult stages and all isoforms were released into culture by L4/5 and adult stages, but not by L3. Evidence for *in vivo* release was provided by the fact that antibodies in sera from infected calves bound the AChE isoenzymes. Antibodies specific for these parasite enzymes were also found in the serum of adult ES-immunised guinea pigs and this response appeared to be under genetic control. A preliminary investigation of the protective capacity of *D. viviparus* AChE was performed in guinea pigs using an adult ES fraction enriched for this enzyme. This preparation, in the context of FCA, stimulated a significant degree of protective immunity when compared with the adjuvant control group, but not when compared with the challenge controls. Again, the native adult ES products failed to confer a significant degree of protective immunity when administered with either FCA or a plurionic block copolymer adjuvant and the possible reasons for this failure are discussed. The fact that the AChE enzymes were recognised by infected hosts and could confer some level of protection when administered in an enriched form argues for an important role of AChE in the immunobiology of this host/parasite relationship.

Abbreviations

AChE	acetylcholinesterase
AdH	adult homogenate
$Al(OH)_3$	aluminium hydroxide
AP	ammonium persulphate
APC	antigen presenting cell
ATCI	acetylthiocholine iodide
$Be(OH)_2$	berylium hydroxide
^oC	degree Celsius
cAMP	cyclic 3', 5'-adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CFT	complement fixation test
cm	centimetre
CO_2	carbon dioxide
cpm	counts per minute
CTAB	cetyltrimethylammonium bromide
DNTB	dithiobis-2-nitrobenzoic acid
EDTA	ethylenediaminetetra-acetic acid
ES	excretory/secretory
FBS	foetal bovine serum
FCA/FIA	Freund's Complete/Incomplete adjuvant
FITC	fluorescein di-isothiocyanate
g	unit of gravity
Gy	Gray
HRP	horseradish peroxidase
IFAT	indirect fluorescent antibody test
IFN	interferon
Ig	immunoglobulin
IHA	indirect haemagglutination
ip	intraperitoneal
iv	intravenous
kDa	kiloDalton
kg	kilogram
Kr	kilorad
L	litre

L3S/H	L3 sonicate/homogenate
M	molar
mA	millampere
mAb	monoclonal antibody
MBq	megaBequerel
mf	microfilariae
mg	milligram
MHC	major histocompatibility complex
ml	millilitre
mM	milliMolar
µg	microgram
µl	microlitre
µm	micrometre
µM	microMolar
NBCS/NBS	newborn calf serum/normal bovine serum
NBT	nitroblue tetrazolium
OD	optical density
OPD	orthophenylene-diamine
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
psi	pounds per square inch
SB	sample buffer
sc	subcutaneous
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
TCA	tetrachloroacetic acid
TCR	T cell receptor
TEM	transmission electron microscopy
TEMED	N,N,N',N'-tetramethylethylenediamine
Th	T helper cell
TM	triethylene melamine
Tris	tris(hydroxymethyl)amino methane
UV	ultraviolet
w/v	weight per volume
V	volt

Chapter 1

Introduction

Section 1.1 General Introduction

Helminth parasites are a major cause of morbidity and production loss in domestic animals throughout the world, and over the last few decades, a great deal of research has been directed towards the development of immunoprophylactic strategies for diseases caused by these parasites. Despite this effort, only two commercial anti-helminth vaccines are currently available. These were developed for the control of the pulmonary nematodes of ruminants, *Dictyocaulus viviparus* and *D. filaria.*

For 30 years, the radiation-attenuated larval vaccine used for the prevention of bovine parasitic bronchitis has been widely used in Europe with considerable success. Unfortunately, this success has had a negative effect on support for research on the immunobiology of this fascinating host/parasite relationship. Using *Dictyocaulus viviparus* infection as a model in which successful immune responses can be stimulated, the work described in this thesis was designed to examine some of the potential interactions which may be occurring at the host/parasite interface.

1.2 Parasitic bronchitis: background and life history

Bovine parasitic bronchitis ("husk" or "hoose") is caused by the trichostrongylid nematode, *Dictyocaulus viviparus* (Bloch, 1782). The presence of worms within the main stem bronchi and trachea was first described by Ruysch in 1744 but he did not associate these parasites with any specific clinical entity. It was not until Nicholls presented a paper at the Royal Society, in 1755, that the link between disease and the presence of parasites was first established. The parasite was originally named *Gordius viviparus* by Bloch in 1782 and it subsequently underwent four name changes before being assigned to the genus *Dictyocaulus* by Railliet and Henry in 1907 (reviewed by Allan and Johnson, 1960). In addition to cattle, *D. viviparus* has been demonstrated in the respiratory tract of camels, deer and buffaloes and it has been suggested that deer are the preferred definitive hosts (Cobbold, 1886; Taylor, 1942).

The most common clinical manifestations of infection are varying degrees of coughing and respiratory distress in calves, usually during their first year at grass. Prior to the development of the lungworm vaccine by Jarrett *et al.* (1958), this disease was a serious cause of morbidity and mortality in the U.K. and in a knackery survey carried out between August and October 1952, parasitic bronchitis was the main cause of death in over 50% of the carcasses examined (Jarrett, McIntyre and Urquhart, 1954).

The life cycle is typical of the Trichostrongyloidea in that it is direct and infection is by ingestion of third stage larvae. The third stage larva (L3) retains the cuticle, or

"sheaths", of one or both of the preceding stages. These afford the infective stage a degree of protection during its sojourn in the environment (Taylor, 1942).

Following ingestion, the L3 are assumed to rid themselves of their sheaths in the rumen of the host (Pfeiffer and Supperer, 1980), after which they travel through the forestomachs and abomasum to the small intestine and, from there, begin their migration to the lungs. After penetrating the small intestinal mucosa, the L3 are thought to moult to the next stage in the mesenteric lymph node complex (Jarrett, McIntyre and Urquhart, 1957) before migrating to the lungs via the lymphovascular route. A moult within the mesenteric lymph node complex, however, does not appear to be essential to the migratory phase of the life cycle since Wade and Swanson (1958) demonstrated that larvae given subcutaneously to calves could reach the lungs just as efficiently as those administered orally. Moreover, when large larval doses were orally administered to calves, third stage larvae were observed in the lungs within 48 hours of infection (Poynter *et al.*, 1960). In these experiments, a few L3 were obtained from the mesenteric lymph nodes, especially those draining the small intestine, suggesting that this was the principal site of larval penetration. Work performed in guinea pigs also demonstrated that third stage larvae could reach the lungs within 24 hours of infection (Douvres and Lucker, 1958; Poynter *et al.*, 1960).

Under more natural circumstances in calves, fourth stage larvae (L4) start to penetrate the alveoli from the pulmonary capillaries by Day 7 of infection (Jarrett and Sharp, 1963). From the alveoli, the L4 migrate up towards the main stem bronchi and trachea and by Day 15 post-infection most parasites have moulted to the fifth stage (L5). The parasites grow very rapidly from Day 17 onwards, the females reaching up to 8cm and the males, 5cm, in length (Smythe, 1937). Patency occurs between Days 21 and 25 of infection and the adults are thought to feed on the profuse local inflammatory exudate which they induce (Taylor, 1951).

Adult females produce embryonated eggs which hatch almost immediately to release the first stage larvae (L1) which measure between 390-450μm in length (Smythe, 1937). The majority of the L1 are coughed up and swallowed to be subsequently excreted in the faeces. Eggs and L1, however, may be aspirated into the alveoli where they stimulate a granulomatous reaction (Jarrett and Sharp, 1963). Patency lasts between 4 and 6 weeks, after which the adult parasites are thought to be expelled from the respiratory tract by host immune effector mechanisms.

In the faeces, the L1 develop to the infective L3 stage in as little as five days, under optimal conditions of temperature and humidity (Urquhart *et al.*, 1987). The free-living stages do not feed, but obtain their nutrition from food granules present within their intestinal cells. The L3 is the most environmentally-resistant of the free-living stages

and a proportion can survive over the winter to the next grazing season (Jarrett, McIntyre and Urquhart, 1954; Nelson, 1977; Duncan *et al.*, 1979; Oakley, 1979).

D. viviparus may enter a state of arrested development at the early L5 stage, when the parasites only attain 1-3mm length (Jarrett *et al.*, 1955b; Simpson *et al.*, 1957) and have a reduced metabolic turnover (Pfeiffer and Supperer, 1980). This event is thought to be induced when parasites are exposed to adverse environmental conditions, i.e. in autumn (Pfeiffer, 1976), or when parasites are undergoing development in hosts which have some degree of immunity (Jarrett *et al.*, 1955b). Cessation of development at this stage has been observed to last for up to 6 months (Pfeiffer and Supperer, 1980).

1.3 The epidemiology of parasitic bronchitis

The epidemiology is mainly dependent upon environmental conditions and the level of host susceptibility. In the case of parasitic bronchitis, there are several additional factors which introduce a degree of unpredictability into the epidemiology.

1.3.1 Environmental factors

Temperature and moisture have most influence on the development and survival of the free-living stages. *D. viviparus* larvae are susceptible to desiccation (Michel and Rose, 1954; Jørgensen, 1980) and have a higher rate of survival in a moist environment (Cobbold, 1886; Taylor 1942; Michel and Rose, 1954; Rose, 1960). Being relatively non-motile nematodes, they depend upon surface water for their escape from the intact faecal pat (Taylor, 1951).

Environmental temperature greatly influences the rate of development of the free-living stages and the critical level at which development occurs is low. In some areas development may occur throughout the year (Daubney, 1920). Development proceeds rapidly at higher temperatures, but larval mortality is high (Rose, 1956) and there must be sufficient water to enable the parasites to move onto the herbage. As animals will preferentially avoid areas contaminated with faeces (Oakley, 1982), it is important for the larvae to escape from the faecal pat. Michel and Rose (1954) demonstrated that larval migration from the faecal pat could be facilitated by spreading faeces thinly onto the pasture and these workers postulated that translation could, therefore, be increased by animals trampling in faeces or when faeces were loose or diarrhoeic, for example due to *Ostertagia* infection or in animals grazing on thick, leafy pastures. Under circumstances of high temperature and adequate moisture, larvae can build up very rapidly on pasture and precipitate the sudden onset of disease typical of parasitic bronchitis outbreaks in the West of Scotland. In winter, although there is plenty of moisture for translation of the larvae, their infectivity is lower (Rose, 1956).

Of the larvae excreted in the faeces, only a small proportion develop to the L3 stage with subsequent translation onto the herbage (Michel, 1969). In Denmark, Jørgensen (1980) noted that most L3 development and dispersal occurred during the first two weeks of excretion into the environment but that many of the larvae soon disappeared.

In order to perpetuate infection from one season to the next, *D. viviparus* third stage larvae can overwinter on pasture (Taylor, 1951; Jarrett, McIntyre and Urquhart, 1954; Jarrett *et al.*, 1955b; Rose, 1956; Baxter and Allan, 1977; Oakley, 1977; Duncan *et al.*, 1979; Oakley, 1979; Jørgensen, 1980). Furthermore, L3 have been observed to survive frost (Jørgensen, 1980). The larvae were assumed to overwinter in the faecal pat (Oakley, 1977) or in the soil (Duncan *et al.*, 1979). In early Spring, *D. viviparus* larvae have been detected more than 3 inches deep within the soil but not on the herbage (Nelson, 1977). *D. viviparus* larvae were also observed within earthworms by Cobbold (1886), who wrongly assumed that these acted as obligate intermediate hosts. In this context, an homogenate of earthworms removed from infected pastures was capable of inducing a patent infection in a naive calf (Oakley, 1981). This author suggested that although pasture contamination resulting from the earthworm reservoir was low, it could be sufficient to provide low level infections in calves in the Spring. In other countries such as Austria (Supperer, 1976) and the United States (Porter and Cauthen, 1942), it was observed that *D. viviparus* L3 were unable to overwinter in the external environment.

An additional factor in the epidemiology of parasitic bronchitis is the contribution made by the common faecal fungus, *Pilobolus*, found in areas of high rainfall. Third stage larvae mature simultaneously with the sporangia and their presence on bursting sporangia was first demonstrated by Robinson (1962). Jørgensen *et al.* (1982) subsequently observed that in pastures without *Pilobolus*, *D. viviparus* infectivity to grazing calves was significantly reduced. It was suggested that bursting sporangia may mediate airborne transmission of *D. viviparus* L3 away from faecal-contaminated areas of pasture. Doncaster (1981) filmed *D. viviparus* L3 climbing and invading sporangia and suggested that larvae which penetrate the sporangium may be protected from the damaging effects of desiccation and UV light. This fungus has also been implicated in the spread of the equine lungworm, *D. arnfieldi* (Jørgensen and Andersen, 1982).

1.3.2 Host factors

Another phenomenon which permits infection to perpetuate from one season to the next is the capacity of *D. viviparus* to persist, either as adult or inhibited L5, in the lungs of carrier animals. *D. viviparus* L3, ingested in the autumn, can enter a state of arrested development at the early L5 stage (Taylor and Michel, 1952; Oakley, 1979, Supperer,1976). The parasites resume their development in the spring and thus

provide a source of contamination for the succeeding grazing season (Pfeiffer, 1976). In a study by Oakley (1979), 52% of 38 previously-infected animals were observed to harbour inhibited L5 in their lungs during the winter period.

Small numbers of adult parasites have also been observed in the lungs of animals during the winter months (Jarrett *et al.*, 1954; Cunningham *et al.*, 1956). In a knackery survey carried out in Scotland, adult parasites were recorded in the lungs of up to 3.8% of adult animals and 31% of yearlings. It must be taken into account, however, that this study did not represent a random selection of cattle and that there would have been a disproportionate number of sick and debilitated animals examined. The results indicated, nevertheless, that adult cattle can harbour parasites, despite the fact that acquired immunity to *D. viviparus* is generally stimulated after an infection has reached patency (Jarrett *et al.*, 1959). As immunity following natural infection (Jarrett *et al.*, 1959; Cornwell and Berry, 1960) and vaccination (Cornwell and Berry, 1960; Jarrett *et al.*, 1961 a and b; Oakley, 1982; Armour *et al.*, 1987) is non-sterile, these animals may be regarded as potential sources of pasture contamination. The rate at which calves acquire immunity depends on the rate of their initial uptake of larvae (Michel and Parfitt, 1956).

1.4. Pathogenesis and clinical signs

In 1960, Jarrett stated that parasitic bronchitis was a disease made up of weeks (cited in Poynter *et al.*, 1960). By killing calves at various times following infection, Jarrett, McIntyre and Urquhart (1957) and Simpson *et al.* (1957) built up a picture of the developing histopathological and clinical signs during the course of parasitic bronchitis.

Clinical and pathological signs are not seen until after Day 7 of infection, when the parasites start to penetrate the lungs in large numbers and begin their migration up the respiratory passages inducing an intense inflammatory exudate. This consists of eosinophils, neutrophils, lymphocytes and giant cells and may be sufficient to produce check valve lesions resulting in the collapse of associated alveoli. In severe cases, pulmonary oedema and interstitial emphysema develop which further exacerbate the clinical signs and, if extensive enough, may result in death.

By Day 25 of infection, adult parasites are present in the main stem bronchi and trachea, in which they exist in a sea of exudate mostly consisting of eosinophils, eggs and newly-hatched L1. The L1 or eggs can be aspirated back into the alveoli where they are associated with a granulomatous, foreign body type reaction resulting in parasitic pneumonia. During the patent phase, the respiratory signs worsen and animals become debilitated. A small proportion of animals within each infected group

may display severe dyspneoa and may die. Surviving animals enter a recovery period which can last several months.

Approximately 20 % of severely affected animals may develop sudden onset respiratory distress during the post-patent phase and may die. This syndrome is associated with a pathological phenomenon termed epithelialisation (Jarrett, McIntyre and Urquhart, 1954) in which there is a massive proliferation of Type 2 pneumocytes within the alveoli. This lesion is sometimes complicated by emphysema and diffuse alveolar oedema (Jarrett *et al.*, 1960a). Peacock *et al.* (cited by Poynter, 1963) observed that, in a group of experimentally-infected calves, 98 out of 250 animals died in the post-patent phase and the majority of these animals exhibited alveolar epithelialisation. An infrequent complication of the post-patent phase is secondary bacterial pneumonia (Jarrett *et al.*, 1960a; Poynter, 1963).

When previously infected or vaccinated animals are exposed to challenge, they invariably develop peribronchiolar lymphoid hyperplasia (Simpson *et al.*, 1957; Jarrett and Sharp, 1963). These lesions have also been observed in calves challenged following passive transfer of immune serum (Jarrett *et al.*, 1955a; Canto, 1990). The typical lesion consists of a central dead or dying larva, surrounded immediately by eosinophils and polymorphs. Outwith these cells, lie plasma cells and macrophages. Due to their characteristic morphology, Jarrett and Sharp (1963) suggested that these nodules may be sites of local antibody production. In immune animals subsequently exposed to heavy challenge, mild clinical signs may be observed (Jarrett *et al.*, 1960a; Michel, 1969). Pathologically, there is a marked local immune response to dying and dead larvae and lymphoid nodules are obvious throughout the lungs (Jarrett *et al.*, 1960a).

As immunity to *D. viviparus* is generally accepted to be acquired following infection, adult animals, which have never been exposed to the parasite, or those in which immunity has waned due to lack of challenge, can develop the disease.

1.5 Diagnosis, treatment and control of parasitic bronchitis

1.5.1 Diagnosis

The local history and the relatively sudden onset of varying degrees of coughing and respiratory distress in a group of calves at pasture is suggestive of parasitic bronchitis. Confirmation may be made on detection of L1 in rectal faecal samples by the modified Baermann technique (Henrikson, 1965), keeping in mind that results may be negative if animals are in the pre-patent phase of infection.

1.5.2 Treatment

Treatment should be initiated as soon as clinical signs are seen in order to reduce further lung damage by the parasites (Duncan and Urquhart, 1982). It may, however, result in the exacerbation of clinical signs which is thought to be associated with overwhelming responses in the lung to the dead or dying parasites in partially immune animals. Four major drug groups are available for the treatment of parasitic bronchitis in calves.

Dyethylcarbamazine citrate (DECC) was the first recommended for use (Parker and Roberts, 1958). This drug was later demonstrated to exhibit poor efficacy against adult stage parasites (Jarrett, McIntyre and Sharp, 1962) and was therefore not recommended for the treatment of infections which had reached patency.

Levamisole has very high efficacy against all stages of *D. viviparus* (Broome and Lewis, 1974; Oakley, 1982; Vazquez *et al.*, 1980), including inhibited L5 (Oakley, 1981). This drug has the added advantage that it can be administered parenterally or as a "pour on".

Of the modern benzimidazoles, fenbendazole (Saad and Rubin, 1977), oxfendazole (Downey, 1976) and febantel (Grelck, Horchner and Wohrl, 1978) have all been shown to be almost 100% active against developing larval and adult stages. In addition, fenbendazole was shown to have activity against inhibited stages of *D. viviparus* (Pfeiffer, 1978).

Ivermectin, at a dose rate of 200µg/kg, exhibits almost 100% efficacy against all developmental stages of *D. viviparus* (Alva-Valdes *et al.*, 1984). Like levamisole, it is administered by subcutaneous injection or as a "pour on". Ivermectin has residual activity against lungworm, lasting 21 days after treatment and, where cases cannot be kept indoors or moved to other pasture, this permits animals to be returned to the same pasture following treatment (Armour *et al.*, 1985).

1.5.3 Control: chemoprophylaxis vs vaccination.

The only reliable method for the control of parasitic bronchitis in the longterm is by the vaccination of calves using the radiation-attenuated larval vaccine (Urquhart *et al.*, 1987). In a outbreak recorded by a practitioner in 1983 (Allison, 1983), of eight unvaccinated calves treated immediately clinical signs were observed, one died from parasitic bronchitis and two more had to euthanased because of chronic respiratory problems. Fifty vaccinates which were on the same pasture as the eight unvaccinated animals, exhibited no clinical signs of parasitic bronchitis.

In recent years, chemoprophylactic strategies developed for the control of gastrointestinal nematodes, have also been applied to the control of parasitic bronchitis. One of the suggested options involved grazing management combined with a single

anthelmintic treatment (dose and move). This has been successful in the control of parasitic gastroenteritis but, due to the inherent variability in the survival of *D. viviparus* larvae on pasture (Nelson, 1977; Oakley, 1977; Duncan *et al.*, 1979), in addition to the relatively small numbers of parasites required to induce clinical disease, this strategy is not to be recommended for the control of parasitic bronchitis.

Other control methods include the sequential treatment of animals with anthelmintic up until mid-summer (Armour *et al.*, 1987), or the administration of intra-ruminal devices which release anthelmintic in pulses or in a sustained manner. By using these highly-efficacious drugs in chemoprophylactic strategies, the degree of exposure to parasite antigens may be very restricted. Successive use of such strategies may eventually eliminate pasture contamination, with the consequence that animals would not be exposed to sufficient parasite antigen to stimulate immunity. Eventually a whole herd could become susceptible and, considering the degree of unpredictability in the appearance of lungworm larvae on the pasture, severe outbreaks could occur. The high degree of susceptibility present within a herd would also necessitate annual anthelmintic treatments which would be costly, labour-intensive and raise questions regarding the environmental consequences (Strong and Wall, 1990).

1.6 Immunity to *D. viviparus*

That infection with *D. viviparus* induces a strong degree of immunity has been accepted for many years. In 1942, Porter and Cauthen experimentally infected calves and, on subsequent infection, noted that the parasites took longer than normal to reach patency and were soon expelled. Michel (1955) observed that on re-infection, calves did not produce L1 in their faeces and the parasites that developed in their lungs were immature. These parasites existed in this state for several months prior to being expelled. Michel and Shand (1955) also observed that, under field conditions, resistance could be induced in less than a fortnight after infection and that the acquired immunity was strong. This immunity could occasionally break down, especially when animals were subjected to heavy challenge. This was in agreement with observations made previously by other workers (Taylor, 1951; Rubin and Lucker, 1956).

By infecting calves with 2,500 L3, followed by a second dose of 4,500 L3 and a subsequent challenge of 13,000 L3, Jarrett *et al.* (1959) demonstrated that only four of ten calves developed patent infections on secondary challenge. On tertiary challenge, three out of the ten calves harboured low numbers of parasites, all of which were small or immature. Immunity was also induced by administering 25 infections of 300 L3 (Jarrett *et al.*, 1959).

Although acquired immunity appears to be of primary importance, some workers have suggested that age immunity may also influence resistance to *D. viviparus*

infection. Younger animals were found to be more susceptible than older animals to infection (Porter and Cauthen, 1942; Wade *et al.*, 1962; Taylor *et al.*, 1988). It has been suggested that the difference in the apparent immunity between calves of different ages is related to improved local cellular and antibody responses in the lungs of older animals (Urquhart, 1988). Armour (1980) suggested that the age of an animal may indirectly influence its susceptibility to parasitic infection through increasing the prepatent period and reducing the pathogenic effects.

It also emerged from the early immunisation experiments that there was significant variation in parasite burdens obtained from individual animals after challenge, indicating that different levels of immunity had developed (Poynter, 1963).

1.6.1 Site of immune effector mechanisms against *D. viviparus*

When one examines the migratory cycle of *D. viviparus* there are obviously a number of sites where mechanisms of immunity could act to prevent parasite migration or to mediate parasite killing. These sites include the intestinal mucosa or wall; the mesenteric lymph node complex; the vascular system and the respiratory tract and associated lymph nodes.

Local responses in the intestine may be important in preventing larval penetration and migration in animals with an established infection, i.e. concomitant immunity. In *D. viviparus* infection, one could postulate that antibody actively secreted into the bile or locally-released and transported across the epithelium into the intestinal mucus may be of importance in preventing the penetration of third stage larvae. Specific antibody levels against other parasite species, in particular IgA, have been measured in intestinal and gastric secretions. Local release of IgA has been measured against *Ostertagia circumcincta* (Wedrychowicz *et al.*, 1992) and *Haemonchus contortus* (Smith, 1977; Duncan, Smith and Dargie, 1978, Smith *et al.*, 1984) in sheep. The results regarding the correlation of these antibody levels with protective immunity, however, have been variable. The existence of concomitant immunity in *D. viviparus* infection does, however, appear unlikely in light of the fact that larvae can reach the lungs of immune animals (Jarrett *et al.*, 1959; Cornwell and Berry, 1960; Michel, 1969; Oakley, 1982). Moreover, the immune effector mechanisms directed against the adult stages in the lung appear to be very successful.

That the mesenteric lymph nodes are involved in the expression of resistance to *D. viviparus* has been a major point of discussion for many years. Jarrett *et al.* (1961) initially suggested that x-irradiated larvae migrated as far as the mesenteric lymph nodes and that this was the site of production of protective antibodies. It was subsequently noted, however, that animals did display respiratory signs following vaccination which suggested that at least some of the irradiated larvae were reaching the lungs (Jarrett and

Sharp, 1963). Poynter *et al.* (1960) subsequently infected immune calves and guinea pigs with large numbers of larvae and demonstrated parasites in the lungs within 48 hours of infection. It was therefore concluded that, in immune animals, larvae could migrate to the lungs without being subjected to host effector mechanisms in the mesenteric lymph nodes. Later this was disputed by Jarrett and Sharp (1963), who drew attention to the fact that the fate of larvae within in the guinea pig should not be directly compared with the situation in the natural host and that, because there was no reliable method of obtaining larvae from mesenteric lymph nodes, the involvement of these structures should not be entirely overlooked.

Several studies have subsequently indicated that the mesenteric lymph nodes are not essential to the development of protective immunity against *D. viviparus*. Wade and Swanson (1958) and Sweitlikowski (1969) were able obtain parasites from the lungs after administering subcutaneous injections of normal L3 to calves. In addition, Sweitlikowski (1969) demonstrated that L3 injected subcutaneously were able to successfully immunise calves against re-infection. Also, Bain and Urquhart (1988) vaccinated calves orally and subcutaneously with irradiated larvae and found similar degrees of protection in both groups.

There is, as yet, no evidence that the migration of lungworm larvae is halted in the circulatory system. By employing several immunoassay techniques, *D. viviparus* -specific antibody has been detected in infected (Michel and Cornwell, 1959; Jarrett *et al.*, 1959; Cornwell and Michel, 1960, Boon, Kloosterman and van der Brink, 1982; Bos and Beekman, 1985; Canto, 1990) and vaccinated (Cornwell, 1960a; Cornwell, 1960b; Jarrett *et al.*, 1960c; Cornwell, 1963) calves. Despite this work, no correlation has been observed between *D. viviparus* -specific antibody levels and the development of protective immunity (Duncan, Gilleard and McKeand, 1992).

The most likely location of successful immune effector mechanisms in parasitic bronchitis is the respiratory tract. The immune expulsion of adults is mediated here (Urquhart *et al.*, 1987); irradiated larvae appear to invade the lungs prior to the development of immunity (Cornwell, 1960b; Poynter *et al.*, 1960) and, in animals which are re-challenged, normal larval development occurs until it is terminated in the lungs from Day 11 onwards (Michel, 1969). Despite these findings, little attention has been paid to the possible effector mechanisms that may be in action in the lungs during infection or vaccination.

Jarrett and Sharp (1963) stated that the development of immunity to *D. viviparus* was associated with the "pulmonary part of the helminth's life cycle" and that thoracic and mediastinal lymphoid hyperplasia was "always" associated with immunity to parasitic bronchitis. Moreover, dead parasites have been observed in the mediastinal lymph nodes (Simpson *et al.*, 1957; Jarrett and Sharp, 1963). On several occasions,

accumulations of inflammatory and plasma cells have been observed to surround dead larvae in the bronchioles (Simpson *et al.*, 1957; Jarrett and Sharp, 1963). The presence of these "lymphoid nodules" was correlated with the development of immunity following vaccination and, it was suggested, that these may be local antibody-producing sites (Jarrett and Sharp, 1963). The nodules have also been observed in animals challenged following the passive transfer of immune serum (Jarrett *et al.*, 1955a; Canto, 1990); vaccination with whole worm homogenate (Jarrett *et al.*, 1960b) and in the lungs of DECC-treated calves (Jarrett, McIntyre and Sharp, 1962). The relationship of these lesions with the development of immunity was contested by Poynter, Peacock and Menear (1970), who found that, in animals challenged after vaccination, the presence of nodules bore no relation to the numbers of adult worms obtained in the lungs.

1.6.2 Stage of *D. viviparus* affected by successful immune responses

As vaccination with 400Gy-irradiated L3 successfully induces immunity to re-infection without the development of significant numbers of adult stages, potential protective antigens must be present in the L3, L4 or early L5 stages. When guinea pigs immunised with normal or irradiated larvae were re-challenged with normal larvae, the parasites were suppressed at the transition from L4 to L5 stage (Poynter *et al.*, 1960; Canto, 1990). Parenteral immunisation with *D. viviparus* L4 has also been successfully demonstrated in calves and pulmonary lesions suggested that appreciable numbers of the challenge larvae had reached the lungs prior to elimination (Cornwell, 1962). The immunity induced by administration of the L4 stages did not, however, produce as high an immunity as that observed following normal or 400Gy-irradiated L3 infection.

Robinson (cited by Poynter, 1969) observed that antigens prepared from *in vitro* cultured L4 stages were inferior in stimulating protective immunity to those extracted from third stage larvae. Subsequently, Canto (1990) was able to induce protection to re-infection in both guinea pigs and calves by parenterally administering third stage larvae irradiated to 1000Gy. These larvae did not develop beyond the third stage and, following two immunisations, 94-98% protection was induced in guinea pigs and 76.6% in calves. Interestingly, serum from calves and guinea pigs, immunised on several occasions with 1000Gy-irradiated L3, could not confer protection when passively transferred to naive animals of the homologous species. This suggested that cell-mediated mechanisms were of relevance in the stimulation of immunity by third stage larval antigens. Canto (1990) also demonstrated that L3 homogenate preparations, in the context of Freund's Complete adjuvant (FCA), could induce protection against re-

infection in guinea pigs. Therefore, it appeared that the L3 stage contained potential host-protective antigens.

Adult homogenate preparations have also been shown to stimulate a degree of immunity in calves (Jarrett *et al.*, 1960b), but the protection obtained (less than 50%) was not significant when compared with the challenge control group. The level of immunity obtained largely depended upon the immunisation protocol. An antigen preparation containing extracts of adult and third stage parasites was also shown to induce significant protection in 8 month-old, but not 2 month-old, calves (Wade *et al.*, 1962). This apparent superior resistance in older calves was not, however, reflected in clinical manifestations as they exhibited coughing, pyrexia and weight loss comparable with the younger vaccinates. These authors suggested that immunity was stimulated by later developmental stages in the lung, as significantly more parasites were present in a vaccinated calf which died on Day 25 post-challenge compared with the main group of calves, killed on Day 35.

The L1 stage was also demonstrated as being capable of stimulating a degree of immunity when Sweitlikowski (1969) used live L1 to partially immunise calves against re-infection.

It therefore appears that several stages of *D. viviparus* individually or, in combination with other stages, can induce successful immune responses in both guinea pigs and calves. Whether or not the same antigens and mechanisms are involved with each developmental stage is, at present, unknown. The type of immunity induced by the various stages may differ and it could be that all are effective, to some extent, on their own. In natural infection, where several stages develop, the different mechanisms may act in concert to produce the most effective level of immunity. Interestingly, Downey (1976), found that calves treated with anthelmintic on Day 19 of a primary infection were more susceptible to re-challenge than infected animals that remained untreated. He suggested that there had been a higher or continued level of antigenic stimulus in the animals which had not been treated.

1.6.3 Possible mechanisms of immunity

That antibodies could be involved in protection against *D. viviparus* was first demonstrated by Jarrett *et al.* (1955a) by the successful passive transfer of serum from immune to naive recipient calves. The serum transferred was demonstrated to contain *D. viviparus* -specific antibody by the complement-fixation test. Rubin and Weber (1955) also obtained some degree of immunity by passive transfer but as two of their five recipients died following transfer, the results were difficult to interpret. Despite these encouraging early studies, no correlation has ever been made between specific antibody levels and the immune status in *D. viviparus* infection.

In common with other helminths, infection with *D. viviparus* appears to induce tissue and peripheral eosinophilia (Weber and Rubin; 1958). Following primary infection with normal L3, two peaks of peripheral eosinophilia were observed which were reinforced on secondary infection (Weber and Rubin, 1958). Djafard, Swanson and Becker (1960) subsequently observed that circulating eosinophil levels were lower in animals that died of parasitic bronchitis, compared with animals which made a full recovery. Increases in peripheral eosinophils have also been observed following lungworm vaccination (Cornwell, 1962; Mackenzie and Michel, 1964). On secondary vaccination the eosinophilia peaked more rapidly and, on subsequent challenge, it was four times that induced by vaccination.

More recently, Knapp and Oakley (1981) observed that eosinophils adhered to the surface of third stage *D. viviparus* larvae more readily than other myeloid cells. This adherence was associated with an antibody-dependent, complement-independent mechanism in immune serum. Adherence was also observed in the presence of normal bovine serum and was associated with a heat labile factor which was presumed to be complement.

The self cure mechanism postulated for the elimination of several intestinal nematodes, was suggested as being involved in rejection of *D. viviparus* from the lungs of immune animals (Michel, 1969; Downey, 1976). It was noted that most of a secondary challenge was rapidly expelled and, because of the heavy inflammatory infiltrate and oedema, this response was considered comparable to the "self-cure" mechanism.

1.7 Immune mechanisms against helminth parasites

Metazoan parasites stimulate an array of host effector mechanisms and, with regard to the IgE-eosinophil axis, may have specific immunological components directed against them (Mitchell, 1991). The level of immunity which can be induced can range from total deficiency to complete resistance. During helminth infection, both humoral and cell-mediated responses develop, in addition to an inflammatory reaction which involves several types of granulocytes and biochemical mediators.

The immune mechanisms which have been most intensively studied are those directed against intestinal parasites in rodents, such as *Nippostrongylus brasiliensis*, *Trichinella spiralis*, *Trichuris muris* and *Heligmosomoides polygyrus* (*Nematospiroides dubius*). The study of *in vivo* responses to helminths, together with techniques developed to examine host/parasite interactions *in vitro*, have produced a plethora of information

regarding the possible components of the effector mechanisms but, despite this, the exact elements which are involved during natural infection remain to be elucidated.

1.7.1 The contribution of antibodies in helminth infections

Evidence that antibodies are involved in immunity against helminth infections has been provided by several studies in which serum has been used to transfer resistance from infected to naive individuals. Chandler (1938) was one of the first to passively immunise naive rats using serum taken from rats infected with *N. brasiliensis* . The degree of protection obtained, however, was not as high as that induced by natural infection.

In several helminth infections, it appears that antibody acts to direct the specificity of later, cell-mediated responses which act in concert to eliminate the parasite from the host (Jones, Edwards and Ogilvie, 1970; Ogilvie and Parrott, 1977). Antibodies can mediate the killing of parasites by fixing complement, promoting phagocytosis and triggering mast cells and basophils to degranulate and secrete compounds that are capable of damaging parasites. Specific antibody directs basophils, mast cells, eosinophils, macrophages and natural killer cells via their Fc receptors in responses against nematodes (reviewed by Askenase, 1982). Both complement activation and granulocyte binding are highly class-dependent and the effector mechanisms are therefore governed by the antibody isotypes which predominate (Capron *et al.*, 1976; Maizels, Philipp and Ogilvie, 1982). Separately, antibodies may act by blocking parasite receptors or the active site of enzymes essential to parasite survival. The humoral mechanisms, themselves, are orchestrated by T-helper cell activity.

Complement-independent, antibody-dependent cellular cytotoxicity was first described against schistosomulae of *S. mansoni* by Butterworth *et al.* (1974) using serum from infected patients in combination with normal human peripheral blood leucocytes. IgE was demonstrated as an important *in vitro* mediator of antibody-dependent cellular cytotoxicity (ADCC) reactions against these stages (Butterworth *et al.*, 1982) and, more recently, elevated levels of adult worm-specific IgE have been shown to correlate with the development of protective immunity against *S. haematobium* (Hagan *et al.*, 1991; Hagan,1992). Evidence of the importance of IgE in responses to nematodes was provided by Askenase (1982) who showed that *T. spiralis* -infected animals which received anti-epsilon antibodies, harboured significantly more muscle larvae than challenge controls. A known role of IgE antibody is the stimulation of mast cells in which activation and subsequent degranulation appears to be of relevance to effective immune responses directed against several gastrointestinal nematodes. Paradoxically, elevated IgE levels have often been described in infections where hosts were incapable of

expelling their worm burdens, so the contribution made by this antibody class to protective immunity remains to be elucidated.

IgG_1 has also been demonstrated to mediate *in vitro* ADCC against *Schistosoma mansoni* schistosomulae (reviewed by Butterworth *et al.*, 1982), while antibody and complement on their own have also been shown to damage these developmental stages (Clegg and Smithers, 1972). There is, as yet, limited information regarding the importance of these mechanisms *in vivo*.

In vitro ADCC has also been demonstrated against newborn larvae and infective stages of *T. spiralis* and the infective stages of *N. brasiliensis* (Mackenzie, Preston and Ogilvie, 1978). More than 66% of newborn *T. spiralis* larvae were killed when incubated with immune serum and leucocytes from normal mice and this degree of parasite killing was amplified in the presence of complement, where 93% of the parasites were killed. Furthermore, in the presence of cells from immune animals, antibody directed more than 98% cytotoxicity against the newborn larvae. Pre-incubation of the serum with live parasites abrogated the parasite killing in a stage-specific manner and indicated the importance of surface antigens in the stimulation of successful effector mechanisms.

1.7.2 The contribution of T-cells in helminth infections

During the course of infection, lymphocytes are stimulated by macrophage-derived interleukin-1 (IL-1) to express receptors for IL-2 which is released from activated lymphocytes. IL-2 subsequently induces the clonal expansion of parasite-specific T helper (Th) cells which release mediators involved in the orchestration of the overall immune response. Th cells direct the proliferation and activation of B cells, goblet cells, eosinophils, mast cells and basophils (Kassai, 1989). Little is known about the cytotoxic activity of T cells during helminth infection (Capron *et al.*, 1976).

The preferential activation of Th sub-populations, as delineated by Mosmann *et al.* (1986), has previously been demonstrated in protozoal infections such as *Leishmania*, where activation of the Th1 subset is correlated with immunity (Scott *et al.*, 1988). In *T. muris* infection, the stimulation of Th2-type responses appears to be strongly associated with the development of resistance to re-infection in specific mouse strains (Else and Grencis, 1991b). These strains displayed elevated levels of interleukin-4 (IL-4), IL-5 and IL-9 which, in turn, appeared to determine IgE, IgG_1 and eosinophil levels (Else, Hultner and Grencis, 1991, unpublished; cited in Else and Grencis 1991b). In contrast to this, low responder strains exhibited increased interferon-gamma, IL-2 and increased serum IgG_{2a}, all of which are indicative of Th1-controlled responses.

A straightforward pattern has not, however, been demonstrated in studies carried out with other helminth species and it appears that the sub-population of Th cells which is

induced depends upon several factors, including the parasite and host species involved, the route of antigen administration, the antigen preparation and the time point of infection (Finkelman *et al.*, 1991, Else, Hultner and Grencis, 1992). Exemplifying this was the situation observed with *T. spiralis* infection in mice, where it has been demonstrated that both Th1 (Pond, Wassom and Hayes, 1989 and 1992) and Th2 (Grencis, Hultner and Else, 1991 and 1992) populations appear to proliferate at the time of parasite expulsion.

This paradox has also been observed in immunisation studies with *S. mansoni* where immunity associated with normal infection was related to Th2-type responses (reviewed by Finkelman *et al.*, 1991; Finkelman and Urban, 1992), while the immunity induced by vaccination using irradiated cercariae was associated with Th1-type responses (Sher *et al.*, 1991, cited by Finkelman *et al.*, 1991). These studies underline the complexity of the immune response even within a single host/parasite relationship.

The factors determining which helper subset will predominate during helminth infection remain to be elucidated. It has been suggested, however, that products released or present within particular parasite groups may stimulate a specific Th subpopulation. Injection of BALB/c mice subcutaneously with 100μg of papain increased mRNA transcription for IL-4, IL-5 and IL-9 and induced a tenfold increase in serum IgE (Finkelman and Urban, 1992). The authors suggested that cysteine proteinases, which are released by many helminth parasites, may be involved in the stimulation of Th2 responses.

1.7.3 The contribution of accessory cells in helminth infection

Eosinophils represent specialised granulocytes which can interact with, and damage, large non-phagocytosable particles (McLaren, 1980). They have been incriminated in immune effector mechanisms by their ability to induce parasite damage *in vitro*, especially against schistosomulae of *S. mansoni.* Of the granulocytes, eosinophils appear to be the most capable of mediating parasite damage and killing (Butterworth *et al.*, 1974, 1975; MacKenzie *et al.*, 1980 and 1981) and binding of these cells to the parasite surface can be directed by either complement or antibody (reviewed by Butterworth *et al.*, 1982). Mast cell products, such as eosinophil chemotactic factor and heparin, also promote eosinophil-mediated ADCC and motility (Kay, 1979; Anwar *et al.*, 1980). Following contact, eosinophils release granules containing substances, such as major basic protein and cationic protein A (McLaren, 1980), which can directly damage the parasite (Glauert *et al.*, 1978). *In vitro* studies have indicated that following eosinophil-mediated damage, a number of other granulocytes may interact sequentially to achieve elimination of the parasite (Mackenzie, Preston and Ogilvie, 1978).

In addition to eosinophils, macrophages, neutrophils and mast cells have all been observed to bind to the surface of infective and newborn *T. spiralis* larvae but eosinophils were the only cells shown to degranulate upon contact with the parasites (Mackenzie *et al.*, 1981). Unlike the new born larval stages, adult *T. spiralis* did not appear to be readily killed by ADCC although binding and subsequent cuticular damage was observed to occur (McLaren, 1980).

The precise role of neutrophils in helminth infection is unclear as these cells appear to be able to attach to the parasite surface but cannot kill (Mackenzie *et al.*, 1981). Neutrophils fail to bind closely to the parasite surface and do not degranulate (Askenase, 1982) but it may be that they invade the parasite following eosinophil-mediated damage.

Macrophages, on the other hand, can be activated to kill schistosomulae of *S. mansoni* (Askenase, 1982) and, in mouse strains in which there is a defect in macrophage activity, resistance following infection is poor (Askenase 1982). In addition, antibody or complement-mediated cytotoxicity against microfilariae (mf) of *Dipetalonema viteae* (Tanner and Weiss, 1978) was most effectively carried out by macrophages *in vitro*.

The relevance of all these experiments to what actually occurs during infection remains to be shown but Weiss and Tanner (1979) have demonstrated the presence of eosinophils, neutrophils and macrophages on the surface of nematodes which have undergone cell attachment in infected hosts.

Mast cells can also adhere to the surface of helminths and this has been demonstrated with *T. spiralis* and *N. brasiliensis* (Mackenzie *et al.*, 1981), but these cells do not appear to be directly cytotoxic and tend to express their activity via a range of chemical mediators. Mast cell-derived mediators include heparin, histamine, 5 hydroxytryptamine (5HT), platelet-activating factor and various proteinases, leukotreines and prostaglandins which are released following activation of mast cells by bound IgE. These mediators have a spectrum of activities which may be involved in the local anaphylactic responses often observed during the course of gastrointestinal helminth infections.

Recently, mast cells, have been divided into sub-populations depending upon their host tissue location and the type of proteinases which they release following activation (Kassai, 1989). The proteinases can be detected in the serum during periods of mast cell activation and degranulation and it was observed that the reduction in mucosal mast cell proteinases in rats (RMCPII), following corticosteroid treatment, correlated with loss of resistance (King *et al.*, 1985). This was not observed to be the case in mice where Mouse Intestinal Mast Cell Proteinase (MIMCP) and mucosal mast cell (MMC) numbers did not appear to decline following corticosteroid treatment of *Nippostrongylus*-infected animals (Newlands, MacKellar and Miller, 1990). In agreement with this, other workers have found it difficult to block rapid expulsion of *N. brasiliensis* in mice (Bell,

McGregor and Adams, 1982). As corticosteroid is thought to induce its effects via T cells, it was implied that murine MMC's were less dependent on T cells for their stimulation (Newlands, MacKellar and Miller, 1990). Work carried out in sheep infected with *H. contortus* has indicated a pronounced mucosal mastocytosis in multiple-infected immune animals and six weeks following the final infection, these animals were still immune to challenge (Huntley *et al.*, 1992). Despite this, their mucosal mast cell numbers had declined significantly so it was difficult to directly correlate the levels of mast cells with parasite expulsion Immune expulsion of nematodes in the absence of measurable mast cell numbers has also been observed (Dehlawi, Wakelin and Benhke, 1987).

Mast cells have also been recognised as potent sources of several cytokines including tumour necrosis factor-alpha and interleukin-4 (reviewed by Gordon,Burd and Galli, 1990) and Romagnani (1992) suggested that these cells may provide the initial source of IL-4 for the preferential stimulation of Th 2 subpopulations in the initial stages of helminth infection.

1.7.4 The contribution of microenvironmental changes in helminth infection

During infection with several species of gastrointestinal parasite, it has been observed that incoming larvae can be very rapidly expelled from animals that are immune (Levy and Frondoza, 1983). Although a wealth of information has been accumulated on the various facets of this event, some of the data so far obtained is conflicting (reviewed by Rothwell, 1989).

Rapid expulsion appears to be associated with the development of local anaphylaxis within the gastrointestinal tract resulting in the generation of a hostile environment which is detrimental to parasite survival. In contrast to the initial antibody recognition, the final effector mechanisms of this expulsion may be non-specific. The initiating step appears to involve the interaction of antigen with specific IgE which then binds and activates mucosal mast cells and mediates their degranulation on subsequent exposure to the antigen (Murray, 1972). The mediators which are subsequently released by the mast cells may then stimulate goblet cell hyperplasia, increased mucus secretion and gut hypermotility. Antibodies, inflammatory cells and various mast cell mediators may be found within the profuse amounts of mucus secreted. Some of the many cell mediators released have the capacity to directly damage parasites and reduce their motility.

Specific IgA in the mucus may bind to the parasites preventing their establishment within the lumen, a phenomenon known as mucus trapping which has been observed in *N. brasiliensis* infection in mice (Miller, Huntley and Wallace, 1981). In this case, 17-23% of the challenge worm population were observed to be present in the intestinal mucus of immune animals several hours following challenge and the bulk of the parasite

burden was subsequently expelled five hours after challenge. Increases in antibody and mediator-containing mucus may therefore precipitate "mucus trapping" of nematodes which, in addition to increases in gut motility, may result in the rapid expulsion of parasites from the intestine. Nematodes do not appear to be killed by the local inflammatory responses, but their metabolism is reversibly depressed as evidenced by their low ATP levels and reduced motility (Kassai, 1989). Changes have been observed in the gut cells of "antibody-damaged" *N. brasiliensis* which were thought to be related to the parasites moving from their mucosal niche to a more hostile environment with a reduced oxygen potential (Ogilvie and Hockley, 1968; Lee, 1969). This movement of the worms was suggested to follow the stimulation of antibodies directed against parasite acetylcholinesterases which were thought to reduce gut motility and function as a "biological holdfast" (Lee, 1971; Jones and Ogilvie, 1972). It also appears that some gastrointestinal nematode infections are capable of influencing expulsion of concurrent heterologous infections (Colwell and Westcott, 1973; Jenkins, 1975).

1.7.5 Concomitant and stage-specific immunity

In helminth infections, immunity may be stage-specific, while in others some developmental stages may persist and stimulate immune mechanisms which are active against other stages, i.e. concomitant immunity. In *S. mansoni* infection, resident adult parasites were thought to stimulate concomitant immunity which acted against incoming larval stages. This theory has recently been contested, however, by Hagan *et al.* (1991) who suggested that there was the development of active immunity against the adult stages. In contrast, stage-specific immunity may be encountered where mechanisms directed against one developmental stage have no effect on other parts of the life cycle. This has been observed with *T. colubriformis* infection in sheep immunised with anthelmintic-truncated infections which could eliminate incoming larval stages but could not eliminate adult parasites to which they had not been previously exposed (Emery *et al.*, 1992).

1.8 Parasite Evasion Strategies

The adaptation by parasites to life in the mammalian host appears to have involved the development of specific immune evasion strategies.

1.8.1 Antigenic variation

This is a striking example of successful parasite adaptation and most of the knowledge so far obtained relates to the variant surface antigens of protozoa, especially salivarian trypanosomes. These parasites have the ability to change the expression of their entire surface glycoprotein coat several times during the course of infection so that,

by the time the animal has mounted an effective immune response to the surface-expressed antigens of one population, the parasites have changed the presented epitopes (Børst, 1991). There is a spontaneous switching of genes which encode the surface antigens and this occurs at a constant rate so that there is always a proportion of heterotypes within any one population.

Antigenic variation, as such, is not a feature of helminth parasites but they do appear to be polymorphic in the epitopes that they present to the host. For example, within a population of *Ascaris suum* larvae, the surface antigens displayed amongst individual parasites was observed to be highly heterogeneous (Fraser and Kennedy, 1991). This would obviously enhance the survival of parasites encountering immune responses in a population of genetically-variable hosts and this phenomenon was likely to be a manifestation of "antigenic drift" which invariably occurs in all parasite populations due to random errors in the genome. Compared with antigenic variation, however, this is a much slower process.

A more obvious alteration in the expression of parasite components observed during the course of helminth infection, was demonstrated in *N. brasiliensis* -infected rats in which the parasites underwent a change in their acetylcholinesterase profiles as rats developed immunity (Jones and Ogilvie, 1972).

1.8.2 Stage-specificity

The capacity to change the surface epitopes which are expressed between one developmental stage and the next is a property exhibited by several nematode species. For example, Mackenzie, Preston and Ogilvie (1978) demonstrated that antibody-directed granulocyte binding to *T. spiralis* or *N. brasiliensis* was specific to different stages in the life cycle. This stage-specificity of these two species was later confirmed by radio-iodination of the surface of *T. spiralis* (Philipp, Parkhouse and Ogilvie, 1980) and *N. brasiliensis* (Maizels, Meghji and Ogilvie, 1983). Stage-specificity, in terms of surface antigens, has since been demonstrated in several other parasitic nematodes such as *Ostertagia circumcincta* and *O. ostertagi* (Keith *et al.*, 1990), *Onchocerca volvulus* (Lustigman *et al.*, 1990; Titanji, Mbacham and Sakwe, 1990) and *H. contortus* (Cox, Shamansky and Boisvenue, 1989; Rhoads and Fetterer, 1990).

Surface epitope changes without the intervention of a moult have also been demonstrated in *N. brasiliensis* (Maizels, Meghji and Ogilvie, 1983), *Toxocara canis* (Maizels *et al.*, 1987) and *Toxocara cati* (Kennedy *et al.*, 1987b).

1.8.3 Shedding of surface antigens

This has been reported for several helminth species including *S. mansoni* (Kusel, Mackenzie and McLaren, 1975; Pearce, Basch and Sher, 1986), *Ancylostoma caninum*

(Vetter and Klaver-Wesseling, 1978), *Fasciola hepatica* (Duffus and Franks, 1981), *T. canis* (Smith *et al.*, 1981), *Dirofilaria immitis* (Ibrahim *et al.* ,1989) and *Acanthocheilonema (Dipetalonema) viteae* (Apfel and Meyer, 1990).

The ability of parasites to replace the outer cuticle or glycocalyx (in the case of the trematodes) would appear to be an advantage in evading immune responses, as any eosinophils or antibody bound to the parasite surface could be rapidly shed. The release of surface products not only permits the shedding of bound antibody, complement and granulocytes, but the released material itself may act to divert host effector mechanisms away from the surface of the live parasite. Such dynamic surface properties of some nematode species, notably *T. canis,* have been attributed to the presence of a "surface coat" which lies approximately 20nm outside the cuticle and which is thought to be synthesised by oesophageal and excretory glands (Blaxter *et al.*, 1992).

1.8.4 Excretory /secretory products

Many helminth nematodes have been observed to release excretory/secretory (ES) materials while maintained in culture and these products may be derived from the surface or specific secretory apparatus of the parasite.

ES molecules are soluble and thus, by their nature, may be tolerogenic even within a competent, mature immune system. Soluble ES antigens can be presented by cells other than conventional antigen presenting cells (APCs) and, when presented by these cells, a relative lack of accessary signals is imparted to T cells (Robey and Urbain, 1991). For full T cell activation, secondary signals are required from APCs such as the binding of an APC ligand to the T cell CD28 receptor and this does not appear to occur with unsuitable APCs (Robey and Urbain, 1991). T cells involved in low avidity interactions are said to be anergized, and once in this state, they are refractory to specific antigen, even if it is subsequently presented with a co-stimulatory signal such as IL-2 (Blackman, cited by Robey and Urbain, 1991). Soluble antigens, such as those present in the ES, have long been recognised as being tolerogenic and may act via this mechanism. This phenomenon has been suggested to occur in human filariasis where lymphocytes were thought to undergo clonal anergy (Maizels and Lawrence, 1991). Filarial parasites may conserve tolerogenic molecules in order to mediate evasion and this is borne out by the fact that such antigens, e.g. phosphorylcholine, are highly conserved by various filarial species (Maizels, Burke and Denham, 1987).

Materials released by parasites may also act to deplete complement and antibody directed against surface antigens on the live parasite and this has been observed with *T. canis* ES materials (Badley *et al.*, 1987). Furthermore, ES products may have a direct affect on components of host immunity, e.g. material released by *S. mansoni* adults in culture were capable of inhibiting mitogen-induced proliferation of mouse spleen cells

(Capron *et al.*, 1976). High molecular weight extracts of *Brugia malayi* mf and phosphorylcholine-containing antigens from adult stages were also demonstrated to non-specifically inhibit proliferation of lymphocytes by mitogens (King and Nutman, 1991).

The release of enzymes, capable of modifying host effector functions, has been observed in many helminth species. These enzymes include acetylcholinesterase, superoxide dismutase, glutathione peroxidase and proteinases. An example of a direct effect on host effector function was demonstrated with *S. mansoni* schistosomulae (Auriault *et al.*, 1981) and adult stage *Fasciola gigantica* (Fagbemi and Hillyer, 1991) which released material capable of breaking down surface-bound immunoglobulin. Furthermore, the peptides released from immunoglobulin cleavage by *S. mansoni* schistosomulae were observed to inhibit macrophage activity and IgE-mediated cytotoxicity (Auriault *et al.*, 1980). *Echinococcus granulosus* cyst stages secrete a proteinase inhibitor capable of preventing neutrophil chemotaxis (Shepherd, Aitken and McManus, 1991), while *Taenia taeniaeformis* larvae secrete a proteinase inhibitor, taeniaestatin, which can inhibit endogenous IL-2 production by murine lymphocytes and IL-1 induced proliferation of murine thymocytes (Leid *et al.*, 1986). As proteinases appear to be important for the activation and differentiation of various host leucocytes (Kramer and Simon, 1987), secretion of their inhibitors may provide the parasite with a mechanism for suppressing effector cell function.

Parasites may also secrete scavenging enzymes which may render ineffective, damaging oxidants produced by phagocytes (reviewed by Callahan, Crouch and James, 1988). Virtually all species of nematode so far studied contain and/or secrete these enzymes which include superoxide dismutase (SOD), catalase and glutathione peroxidase. A correlation has been observed between scavenging enzyme levels within certain parasite species and their relative susceptibility to immune-mediated attack. This was exemplified by measurements of SOD activity in the intestinal parasites *N. brasiliensis* and *T. muris* (Smith and Bryant, 1986). The former which had low levels of anti-oxidant was observed to be expelled from the host within 10 days of infection, while the latter parasite contained high levels of these enzymes and persisted for a longer period . In agreement with these results, it was noted that SOD activity and the range of isoforms were greater in "adapted" *N. brasiliensis* parasites which could survive longer than normal parasites in immune rats (Knox and Jones, 1992).

1.8.5 Resistance to complement-mediated mechanisms

Resistance to complement-mediated damage has been observed in several parasite species. The most detailed work has been carried out on *Leishmania* and *Trypanosoma* species which appear to ensconce molecules on their surface which interfere with the activation of the complement cascade (reviewed by Cooper, 1991). Of the helminths,

anti-complement activity has been demonstrated, for example, by *S. mansoni.* Pierce, Hall and Sher (1990) showed that the outer lipid bilayer of the adult stage of this parasite contains a molecule with decay accelerating factor (DAF) activity. This host-derived enzyme binds C3b to impede alternative pathway activation and its isolation lent support to previous suggestions that schistosomulae rapidly lost their ability to bind C3b *in vivo* (Ruppel *et al.*, 1984).

1.8.6. Stimulation of non-productive effector mechanisms

The type of immune response which is stimulated by certain parasites may also have an effect on the overall outcome of infection. *H. polygyrus* infection which develops to chronicity in some strains of mice can mediate local immunosuppression. For example, mice infected with *H. polygyrus*, which are subsequently infected with *N. brasiliensis*, cannot expel the latter parasite whose fecundity and longevity is therefore prolonged (Jenkins, 1975). When *N. brasiliensis* parasites were taken from animals co-infected with these two species and transplanted into naive individuals, the fecundity and longevity was still extended and so it appeared that *N. brasiliensis* had become adapted as a result of immune responses stimulated against the other parasite. It has since been observed that *H. polygyrus* stimulate Th1 type responses which may act to down-regulate more effective Th2 responses which would normally be directed against the *N. brasiliensis* parasites in single infections (Monroy and Enriquez, 1992).

In *T. muris* infection in mice, it has been suggested that antigen specific to the later developmental stages may exert an immunomodulatory influence on the expression of resistance (Else *et al.*, 1990; Else, Hultner and Grencis, 1992). An extensive analysis was made of the helper subset responses during the course of infection with *T. muris* in susceptible mouse strains and the results indicated that early successful Th2 responses were down-regulated by later, inappropriate Th1 responses (Else and Grencis, 1991b). There was further evidence for this phenomenon when it was shown that susceptible and resistant mouse strains, whose responses were modified by either steroid treatment or immunisation with ES products, had altered cytokine profiles which bore relation to the change in the type of immunity which these animals expressed (Else, Hultner and Grencis, 1992).

In *Schistosoma* infections it has been suggested that specific immunoglobulin isotypes, stimulated by egg antigens, may block more effective isotypes such as IgG_1 and IgG_3. For example, in humans, re-infection appears to occur more readily in individuals exhibiting high levels of IgG_4 antibody directed against the adult or egg stages (Hagan, 1992) and IgG_1 and IgG_3-mediated killing by eosinophils can be blocked by IgG_4 which is inefficient at activating complement and in binding monocytes and macrophages (Hagan *et al.*, 1991). A similar phenomenon has also been observed in

human trichuriasis in which high levels of IgG_4 were associated with a preponderance to high worm burdens (Bundy *et al.*, 1991).

Parasites may also express antigens which act to over-stimulate B-cell responses. For example, *Plasmodium* species contain immunodominant tandem repeat antigens which provide a complex network of cross-reacting epitopes which stimulate a wide range of B cells and may result in clonal exhaustion (Mitchell, 1987).

1.8.7. Host mimicry

Yet another feature which parasites appear to possess is the ability to passively acquire host molecules or make antigens which act to mimic those of the host. *S. mansoni* adult parasites can acquire glycolipids of human blood group antigens (Harrison and Ridley, 1975) and murine MHC molecules (Harn *et al.*, 1985) on their surface. Host blood group antigen has also been demonstrated on the surface of *Loa loa* mf (Harrison and Ridley, 1975) and the surface and ES products of second stage *T. canis* larvae contain parasite-derived molecules which mimic blood-group antigens (Smith *et al.*, 1983). Also, the surface of skin stage mf of *Onchocerca volvulus* are recognised by antibodies specific to mammalian collagen Type IV (Titanji, Mbacham and Sakwe, 1990).

1.9 Vaccination against parasitic helminths

Since the discovery of the effectiveness of cowpox virus in immunisation against small pox by Jenner in 1798, vaccination has been responsible for the control or eradication of some of the world's most notorious scourges of man and domestic animals. Such diseases include yellow fever, poliomyelitis and German measles, in man, and rabies, rinderpest, Foot and Mouth disease and swine colibacillosis in domestic animals. Unfortunately, the success achieved with vaccines against bacterial and viral diseases has not been accomplished for the control of parasitic diseases which constitute a major cause of morbidity and mortality in man and domestic animals.

1.9.1 The necessity for anti-helminth vaccines

Nearly 10 million cattle probably die every year as a result of parasitic infection (Duffus, 1989). Although many of the helminth parasites affecting domestic animals can be controlled using anthelmintics, their application is labour-intensive and often too costly for use in developing countries (Urquhart, 1988). While the development of longer-acting anthelmintic preparations has effectively reduced the labour input required, the possible detrimental effects of such drugs upon the environment gives cause for

concern (Strong and Wall, 1990). For example, the most recently-developed broad spectrum anti-parasitic drug, ivermectin, has been shown to have deleterious effects on the dung-degrading community of the faecal pat (Wall and Strong, 1987) but the impact of this in the field remains to be fully elucidated.

The development of drug resistance in nematodes, especially to two of the major anthelmintic drug groups, for example, the benzimidazoles and levamisole, is being increasingly recognised throughout the world. This is now a significant problem, especially in the warmer climates where higher frequency, sub-optimal treatments have been carried out extensively (Waller, 1987). In the U.K., Australia and other countries, specific guidelines have been recommended for the treatment and control of helminth parasites in sheep in an attempt to reduce the rate of development of anthelmintic resistance. Despite this, it appears to be an increasing problem and, although some benzimidazole-resistant strains can revert to susceptibility after a period without treatment, resistance is soon re-established following further drug exposure (Coles and Rouch, 1992). A disturbing development has been the recent observation of ivermectin-resistant strains of nematode in sheep (Van Wyck and Malan, 1988) and goats (Jackson *et al.*, 1991). Since the development and marketing of new chemotherapeutic compounds for use against helminth parasites is very expensive and takes many years (Murray, 1989), the generation of helminth vaccines would be of great benefit for the future control of these economically-important diseases.

Despite decades of intensive research, only two commercial anti-helminth vaccines are available, both of which were developed for the control of lungworm infections in ruminants. An attenuated vaccine based on the oral administration of 400Gy gamma-irradiated third stage larvae was developed for the control of parasitic bronchitis in cattle by Jarrett *et al.* (1958).

1.9.2 A radiation-attenuated larval vaccine against *D. viviparus*

A strong acquired immunity to *D. viviparus* is acquired following infection under field conditions (Taylor, 1951; Jarrett, McIntyre and Urquhart, 1954) and protective immunity was subsequently demonstrated following experimental infections with infective larvae (Porter and Cauthen,1942; Taylor and Michel, 1952). In more detailed studies, it was observed that acquired immunity could result from a single infection with a sub-lethal dose of larvae or from a series of smaller doses (Jarrett *et al.*, 1959). The successful passive transfer of immunity was subsequently achieved using serum from animals which had been exposed to field infection followed by a single experimental infection (Jarrett *et al.*, 1955a).

A high level of protection against *D. viviparus* infection was obtained in calves following a single oral immunisation with third stage larvae, x-irradiated at 20Kr

(200Gy) or 40kr (400Gy) (Jarrett *et al.*, 1960c). These animals, which received 4,000 irradiated L3, displayed moderate clinical signs of parasitic bronchitis following vaccination. When the two groups were subsequently challenged, clinical signs were not observed and they exhibited a level of immunity comparable with that of animals previously exposed to natural infection. Following irradiation at levels of 60Kr, larvae did not stimulate significant levels of protection against challenge and the parasites did not appear to penetrate the lungs (Jarrett *et al.*, 1960c).

In view of the clinical response observed after vaccination with 4,000 40Kr-irradiated larvae, calves were subsequently immunised with 1000 of these larvae and it was observed that the calves had reduced degree of protection against challenge than in the preceding experiment (Jarrett *et al.*, 1958). As a high anamnestic response had previously been observed on secondary challenge with normal larvae (Jarrett *et al.*, 1959), it was decided to administer two doses of 40Kr-irradiated larvae and it was observed that animals vaccinated in this manner were highly immune to challenge with 10,000 normal L3 (Jarrett *et al.*, 1959). Animals which received the double vaccination neither excreted L1 in their faeces nor had adults in their lungs by Day 33 post-challenge and weight gains in the vaccinates were significantly higher than those of the challenge controls. When animals were vaccinated twice in a similar manner and then subjected to a natural pasture challenge, L1 excretion did not occur and, at necropsy, the vaccinates harboured only a few immature parasites in their lungs (Jarrett *et al.*, 1961a). The results of these experiments suggested that two doses of 40Kr-irradiated larvae, administered orally, could induce a significant degree of protection against both experimental or pasture challenge (Jarrett *et al.*, 1958).

In investigations of alternative methods of parasite attenuation, *D. viviparus* third stage larvae were exposed to the cytotoxic agent, triethylene melamine (TM), at various concentrations and significant levels of protective immunity were obtained following vaccination with L3 attenuated with 0.7% TM (Cornwell and Jones, 1970a). In the animals used in this experiment, clinical signs were mild and the L1 output was low following normal L3 challenge (Cornwell and Jones, 1970b). When TM-attenuated L3 were compared with x-irradiated L3 in a vaccination experiment, the levels of protection achieved were similar (Cornwell and Jones, 1970c).

Subsequent experiments demonstrated that when the irradiated larval vaccine was given subcutaneously, more than 95% protection against challenge was stimulated (Bain and Urquhart, 1988). This was comparable with that induced by oral vaccination but had no commercial advantage and the sterility required for commercial exploitation of the parenteral vaccine could not be achieved. Gamma-irradiation, which has similar effects on infective L3, but is more accurately calibrated than x-irradiation (Miller, 1978), is now used for attenuating *D. viviparus* larvae for commercial vaccine production.

1.9.3 Vaccination against parasitic bronchitis in the field

Since its commercial introduction, the lungworm vaccine ("Dictol") has been widely used on calf-rearing farms within the U.K, Denmark, Holland, France and Belgium and its value in reducing the number of clinical outbreaks of parasitic bronchitis was reviewed by Poynter *et al* (1960), Jones and Nelson (1960) and Nelson, Jones and Peacock (1961). Out of 4,000 farms where the vaccine was used, problems directly related to parasitic bronchitis were present on only 16 (Poynter, 1963) although problems with mycoplasmal pneumonia were often misdiagnosed as parasitic bronchitis by farmers. Transient coughing in animals that had recently been vaccinated or in animals that had been vaccinated and subsequently exposed to a heavy pasture challenge were the commonest complaints. The inherent biological variation among calves, which was reflected as a failure to respond to the vaccine, was considered as a factor in vaccine breakdowns involving small numbers of animals.

After 20 years of commercial use, the influence of vaccination on the incidence of parasitic bronchitis was reviewed by Peacock and Poynter (1979). True loss of immunity due to lack of challenge was only observed twice in their survey and the prevalence of husk on vaccinating farms was very low (0.13%). Reported post-vaccinal pneumonias were assumed to merely represent the on-going and existing background of respiratory disease in housed calves (Peacock and Poynter, 1979). Most of these were seen in the first week following vaccination, whereas if the vaccine itself was involved, outbreaks would be expected during the second week of vaccination when larvae are invading and stimulating responses in the lungs. Perhaps the stress resulting from handling housed calves for oral vaccination stimulated the proliferation of bacterial or viral respiratory pathogens.

Vaccinated animals at pasture subjected to any other diseases, such as ostertagiasis, are considered more likely to succumb to *D. viviparus* infection especially if under heavy challenge (Menear and Swarbrick 1968; Peacock and Poynter 1979 and Armour *et al.*, 1987).

1.9.4 Radiation-attenuated vaccines against other helminths

The success achieved with *D. viviparus* stimulated numerous attempts to develop irradiated vaccines against other parasitic helminths. A 500Gy-irradiated larval vaccine has been developed to assist in the control of ovine dictyocaulosis due to *D. filaria* in India. This has made a significant contribution to the control of this disease and, by 1988, vaccination had been successful in the majority of 470,000 lambs (Sharma, Bhat and Dhar, 1988).

An anti-parasite vaccine consisting of radiation-attenuated third stage larvae of *Ancylostoma caninum* successfully immunised dogs against hookworm disease (Miller, 1971). This parasite vaccine was, however, discontinued in 1975 because of reduced demand from veterinary practitioners (Miller, 1978). This failure was attributed to financial and distribution factors including the availability of alternative anthelmintic therapy, the limited shelf-life and the fact that a considerable number of vaccinated animals, although protected against disease, were observed to pass hookworm eggs upon re-infection (Miller, 1978).

A 40Kr-irradiated vaccine against *H. contortus*, which stimulated protection against challenge in eight month-old lambs (Urquhart *et al.*, 1966b, Benitez-Usher *et al.*, 1977), failed to induce immunity in three month-old animals (Urquhart *et al.*, 1966a). Changes in the vaccination protocol which included the intraperitoneal injection of larvae and immunisation, in the context of FCA, failed to induce protective immunity in young lambs (Urquhart *et al.*, 1966b). Even when lambs were trickle-infected with normal L3 they did not develop immunity. It was later suggested by Duncan, Smith and Dargie (1978) that the unresponsiveness in animals of this age was due to an inability to respond locally as mucosal IgA levels were significantly lower than those obtained from older vaccinates. Subsequently, however, no significant differences in local IgA or systemic IgG were obtained between young and adult vaccinates and it was suggested that the development of protective immunity was dependent upon several factors (Smith and Angus, 1980). Transfer of lymphoid cells from immune donor lambs to their monozygotic twin was observed to reduce the susceptibility of the recipients to challenge (Smith *et al.*, 1984) and parasite-specific Th cell responses have since been demonstrated in sheep infected with *H. contortus* (Haig *et al.*, 1989).

Similar results were obtained when sheep and lambs were vaccinated with a radiation-attenuated *T. colubriformis* larval vaccine. When worm burdens were compared with those of naive challenge controls, adult sheep were protected by more than 90% (Gregg and Dineen, 1978; Gregg *et al.*, 1978). In contrast, the degree of protection achieved in the lambs was only 37.7% (Gregg *et al.*, 1978). No significant differences in serum antibody levels or peripheral cellular responses were observed between resistant adult sheep and susceptible lambs. Local responses in mast cells, neutrophils and eosinophils were similar in both groups and only the levels of globule leucocytes in duodenal sections were significantly higher in immune sheep (Gregg *et al.*, 1978).

Successful vaccination against *Schistosoma* species has been achieved using irradiated cercariae in laboratory models (Simpson *et al.*, 1985). Moreover, Zebu cattle have been significantly protected against *S. bovis* using irradiated schistosomulae or cercariae of the homologous species (Bushara *et al.*, 1978).

1.9.5 Vaccination with dead parasite material

One of the first attempts to immunise animals with dead whole parasites (somatic antigen) was carried out by Chandler (1932) who obtained some degree of protection with heat-killed larvae of *N. muris* (*brasiliensis*) in rats. Immunised animals excreted fewer eggs but the number of established adult parasites did not differ significantly from challenge controls. Despite many similar studies, only a limited number have achieved successful immunisation using somatic parasite extracts (reviewed by Clegg and Smith, 1978).

Results obtained with *T. colubriformis* by Rothwell and Love (1974) demonstrated that somatic extracts of adult worm or L4 stages could protect guinea pigs against re-infection when compared with challenge controls. The protection induced was independent of adjuvants and appeared to be stage-specific as third stage larval homogenate did not confer a significant degree of protection.

Sonicates of adult *T. colubriformis* parasites, in the context of the adjuvant berylium hydroxide ($Be(OH)_2$) have been shown to induce 71 to 85% protection to re-infection in New Zealand rabbits (Wedrychowicz, Bezubik and Trojanczuk, 1989), however, *Corynebacterium parvum* and $Be(OH)_2$ alone also reduced worm burdens by over 50%. L3 somatic antigens were also observed to stimulate immunity against this parasite in rabbits and the immunity induced was higher than that following vaccination with adult excretory/secretory materials (Wedrychowicz, Doligalska, Bezubik and Graczyk, 1990). As outlined above, Rothwell and Love (1974) did not obtain immunity following vaccination with L3 extracts, but this may have been a result of the different adjuvants used in the two studies. The level of protection induced by the L3 somatic preparation was later observed to be adjuvant-dependent as significant reductions in worm burdens were only achieved with berylium hydroxide and not with FCA (Wedrychowicz and Bezubik, 1990).

Some success with somatic preparations has also been achieved with *H. contortus* in sheep. Whole sonicate of adult worms, but not of third stage larvae, resulted in 63% protection against challenge (Adams, 1989). In these studies, Freund's Complete adjuvant (FCA) appeared to increase the susceptibility of sheep immunised with the third stage preparation when compared with animals that received this antigen in the context of Freund's Incomplete adjuvant. These results implied that the killed mycobacteria in the FCA had a profound effect on the development of resistance in this system. Subsequently, a fraction extracted from *H. contortus* adult worms which was enriched for "fibrinogen-degrading products" was shown to induce 87% protection against challenge (Boisvenue *et al.*, 1990).

Using soluble extracts of *Oesophagostomum radiatum* L4 homogenates, East, Berrie and Fitzgerald (1988) obtained between 81 and 99% reductions in adult parasite worm burdens in vaccinates compared with challenge controls. The fact that soluble proteins could induce significant levels of protection would suggest that parasite excretory/secretory products may stimulate protective immune responses.

1.9.6 Vaccination with products released from live parasites

Due to the limited success achieved with somatic material, it was thought that antigens which were present at host/parasite interfaces could be the source of protective antigens but that these antigens might be insufficient in quantity in somatic extracts to stimulate protection.

The first indication that ES antigens might be involved in protection against parasites was demonstrated by Sarles and Taliaferro (1936), who observed immune precipitates at the mouth, excretory pore and anus of *N brasiliensis* adult parasites incubated with serum from immune rats. It was suggested that although the antigen-antibody complexes formed at the orifices of the parasite did not appear to cause direct damage, they might be responsible for blocking the activity of secretions essential to parasite survival. Many studies have since been performed to examine the efficacy of helminth ES materials in stimulating protective immunity, only a few of which are described below.

Trichinella spiralis

This is one of the few parasites which can be easily maintained in the laboratory and in which a strong immunity is induced post-infection. These factors make this parasite suitable for the investigation of nematode immunobiology.

Protective immunity against this parasite can be induced in mice using secretions (Campbell, 1955; Vernes ,1976) and soluble fractions (Despommier, 1981) from muscle stage larvae. Despommier and Muller (1976) demonstrated that the majority of the secretory products appeared to be released from secretory granules found within the stichosome oesophagus. Silberstein and Despommier (1984) subsequently obtained high levels of protection in mice using a single immunoaffinity-purified 48kDa molecule from muscle stage larvae. This molecule protected animals at biologically-relevant doses (0.1-1μg per mouse) and was located, by immunocytochemistry, to the ß-stichocytes, the gut lining and the surface of the cuticle, and comprised 12% of parasite secretions. These elegant experiments demonstrated that strong protective immunity could be induced by a single nematode secretory component.

Unfortunately, when experiments with L1 stichocyte antigens (Murrell and Despommier, 1984) or ES products (Gamble, Murrell and Marti, 1986) were attempted in pigs, only "moderate" (less than 55%) levels of protective immunity were induced

suggesting that different immune mechanisms were involved in the different hosts. These studies highlighted the danger of directly extrapolating experimental results from one host species to another.

T. colubriformis

ES products have also been shown to successfully immunise guinea pigs against infection with *T. colubriformis* (Rothwell and Love, 1974). When chambers containing live L4 were implanted into guinea pigs, protective immunity was induced. Chambers containing dead worms also induced a degree of protection, but this was not as high as that obtained using live parasites. To further establish the efficacy of ES materials, these workers immunised guinea pigs with *in vitro* -released products of fourth stage larvae and obtained significant protection in groups vaccinated in the presence or absence of adjuvant.

The major secreted glycoprotein of L4 and adult stage ES was subsequently gel-purified and tested in guinea pigs (Savin *et al.*, 1990). This 30kDa molecule induced a significant degree of host protection, even after denaturation in SDS and the reduction in worm burdens (average 59%) which it induced was similar to that achieved following immunisation with whole ES material treated in a similar manner. This protein was predominantly expressed in the L4 stage and its amino acid sequence had 28% homology to valosin, a protein from the small intestine of pigs which may regulate gastric secretion and duodenal motility.

Another gel-purified protein from *T. colubriformis* L4 ES was found to be highly protective in guinea pigs (Dopheide *et al.*, 1991). This 11kDa protein induced 100% protection in one third of the vaccinates and more than 50% protection in 89%. Antibodies against this protein were not observed in the serum of animals subjected to normal L3 infection or vaccination and it was suggested that cell-mediated immunity was responsible for the protection obtained. On amino acid sequencing, two possible T-cell binding epitopes were observed, as well as evidence of similarity to a human gamma-interferon -induced protein.

L3 ES products of *T. colubriformis* were also observed to mediate moderate protection against re-infection in guinea pigs (O'Donnell *et al.*, 1989)

Other parasite species

ES products of L3/L4, but not adult, stage *A. suum* were shown to induce protection in guinea pigs (Stromberg and Soulsby, 1977). It was thought that the protective antigens may be released during the moulting process as antigens obtained from cultured L3 stages prior to the moult did not induce protection. A fraction of the

L3/4 ES in which a 67kDa glycoprotein predominated was subsequently demonstrated to induce a significant degree of protection (Stromberg, 1979).

Immunisation with ES products of nematodes however, has not always been successful as demonstrated by Neilson (1975) who immunised lambs with ES products from L3 and L4 stage *H. contortus* but found no differences in faecal egg counts (FEC) or worm burdens between vaccinates and challenge controls. The animals used in this experiment were, however, less than 3 months old and may have been immunologically unresponsive (Urquhart *et al.*, 1966b; Duncan, Smith and Dargie, 1978).

1.9.7 Vaccination with helminth surface antigens

An additional host/parasite interface which may be of importance in the development of successful immune responses is the cuticular surface of parasites and surface extracts of various species have been tested with varying degrees of success.

Cetyltrimethylammonium bromide (CTAB) surface extracts of *T. spiralis* larvae induced a significant level of protection to re-infection in mice (Grencis *et al.*, 1986). In addition, in sheep, CTAB surface extracts of *O. circumcincta*, in the context of beryllium hydroxide $Be(OH)_2$, were also able to induce protective immunity and levels of serum and bile IgA correlated with the degree of immunity (Wedrychowicz, *et al.*, 1992). Similar products, in the context of FCA, did not induce a significant degree of protection and $Be(OH)_2$ was observed to induce a relatively high non-specific level of protection in these studies (A. Tait, personal communication).

In sheep immunised parenterally with surface extracts of *H. contortus* third-stage larvae, there was no difference in susceptibility between vaccinates and controls after primary challenge (Turnbull *et al.*, 1992). Also when sheep were immunised with cuticular collagens of *H. contortus*, no significant levels of protection were obtained in vaccinated animals (Boisvenue *et al.*, 1991) and the authors suggested that effector mechanisms against the collagens could perhaps not reach the target antigens due to the presence of a protective surface coat.

An alternative strategy in assessing the potential of surface antigens in protection is to passively immunise naive recipients with surface epitope-specific monoclonal antibodies. This has been successfully achieved in mice with monoclonal antibodies raised against the surface of *T. spiralis* larvae (Ortega-Pierres, MacKenzie and Parkhouse, 1984) and, in calves, with a monoclonal antibody specific for an antigen present on the surface of invasive onchospheres of *T. saginata* (Harrison and Parkhouse, 1986).

1.9.8 Recent strategies in the development of helminth vaccines

Despite the plethora of work on the characterisation of non-living parasite antigens there is, as yet, no helminth vaccine based on these antigen sources. This is probably the result of a number of problems,including:

1) the complex nature of antigens that are presented by metazoan parasites during the course of an infection;

2) the inability of hosts, particularly those that are immature, to respond effectively to these antigens;

3) the remarkable capacity of parasites to persist and proliferate in the face of host effector mechanisms;

4) a failure to determine the responses which are critical to the development of immunity and

5) the difficulty in obtaining sufficient quantities of antigenic material to undertake meaningful protection studies.

With recent advances in recombinant DNA technology and protein chemistry, the latter problem is now being tackled as polypeptide antigens can now be produced in large quantities for further examination.

The major histocompatibility complex and other, as yet, undefined genes within a population of hosts may influence the capability of individuals to recognise a specific antigen and this, in turn, will establish the success, or otherwise, of any vaccine (Kennedy, 1990). For example, variable responsiveness was encountered when synthetic peptides of the circumsporozoite protein of *Plasmodium falciparum* were used to vaccinate mice and it was observed that both antibody and T cell responses to this antigen were H-2b-restricted in immunised mice (Del Giudice *et al.*, 1986, Togna *et al.*, 1986).

In order to define specific antigens as potential vaccine candidates two main strategies may be attempted. The first involves systematic fractionation of parasite extracts, followed by the assessment of the efficacy of these fractions by vaccine/challenge experiments. The first potentially viable recombinant helminth vaccine for the control of *T. ovis* in sheep was developed in this manner (Rickard, 1989). An alternative strategy is to define components which appear to be essential to parasite survival within the host and to determine the protective value of such components. Several studies have been performed in which animals have been immunised against enzymes which may function in day-to-day parasite maintenance and which may also participate in parasite immune evasion. One of the first studies in this area involved the immunisation of guinea pigs with products enriched for *T. colubriformis* acetylcholinesterases (Rothwell and Love, 1974). The results of this study were rather

disappointing as the enriched fractions were not consistently protective and the results were rather inconclusive as neither purified product nor adjuvant effects were examined.

In both *S. mansoni* and *S. japonicum*, two species of glutathione S-transferase exist which are thought to function in detoxifying potentially-damaging electrophilic molecules released from phagocytes (reviewed by Mitchell, 1989). A significant level of protection has been obtained in mice and rats with both the native (Balloul *et al.*, 1987a) and recombinant forms (Balloul *et al.*, 1987b) of the 28kDa glutathione S-transferase of *S. mansoni* .

Antigens which are not normally exposed to the host during the course of natural infection have also been examined as vaccine candidates, examples of which are gut antigens of blood-sucking parasites. The philosophy behind this strategy is that when the parasite ingests blood in the immunised host , it will ingest antibodies directed against epitopes expressed in its gut . The effector mechanisms induced by these antigens are different to those induced by natural infection so that vaccine and natural immunity may be co-expressed in the same host (Willadsen and Kemp, 1988; Willadsen and McKenna, 1991). Using purified gut antigens from the tick *Boophilus microplus*, successful immunity has been stimulated (Johnson *et al.*, 1986) and recombinant forms of the gut antigen, Bm86, have subsequently been produced which induce significant levels of protection in immunised cattle (Willadsen and McKenna, 1991). Recently, successful vaccination of sheep has been achieved against blood-sucking *H. contortus* nematodes using a purified extract from the adult parasite's gut (Munn, Greenwood and Coadwell, 1987).

Another possible source of "hidden" protective antigens are the structural collagens of the nematode cuticle. Pritchard, McKean and Rogan (1988) have suggested that if a "first wave" immunological or chemotherapeutic attack could be directed against surface antigens, a second attack against the structurally-important, underlying collagen molecules could mediate parasite destruction.

Recent advances in molecular biology and protein chemistry have provided impetus for the generation of synthetic antigens for use in helminth sub-unit vaccines. In addition to bacterial cloning and peptide synthesis, the development of novel vectors and adjuvants have the potential to optimise vaccine efficacy. It must be stressed, however, that the definition of the important immune effector responses against helminths and the recognition of the antigens that stimulate these, are essential to the development of effective helminth vaccines.

Several recombinant polypeptides have been developed experimentally for immunisation against parasite infections. For example, a recombinant form of a *T. ovis* onchosphere antigen has been shown to induce over 94% protection in challenged sheep (Johnson *et al.*, 1989). Cloned onchosphere antigens have also mediated protective

immunity in rats to *T. taeniaeformis* (Ito *et al.*, 1991) and as mentioned previously, recombinant forms of *S. mansoni* glutathione transferases were also observed to induce a significant degree of protective immunity in laboratory hosts (Balloul *et al.*, 1987b).

Synthetic peptides can be derived from amino acid or gene sequences and must be self-polymerised or administered in combination with a carrier molecule in order to evoke an immune response. A synthetic peptide derived from the sequence of a glutathione transferase of *S.mansoni* has also elicited partial protection against challenge in rats (Wolowczuk *et al.*, 1991). This peptide was derived from a sequence which contained both T and B cell recognition sites and was synthesised as an octomeric or "octopus" construct. Sera from immunised rats mediated platelet, macrophage and, to a lesser extent, eosinophil adherence to schistosomulae and indicated the potential of this type of vaccine strategy.

1.9.9 Anti-idiotype vaccines

Molecular cloning and synthetic peptide strategies depend on protective antigens being polypeptides and if the major protective epitopes prove to be carbohydrate, these methods are of no value in the production of synthetic vaccines. With anti-idiotype vaccines, antibodies are used which mirror the original epitopes, irrespective of their biological or biochemical nature. Monoclonal antibodies were produced which recognised an immunogenic glycoprotein, gp38, present in adult *S. mansoni* and vaccination with the anti-idiotypes induced 50-80% resistance to challenge in rats (Gryzch *et al.*, 1985). Moreover, the antibodies stimulated in immunised rats were strongly cytotoxic for schistosomulae, in the presence of eosinophils, and were able to transfer immunity passively. This study demonstrated the potential for the development of vaccines against non-protein moieties.

1.9.10 T cell epitopes

A major drawback of purified antigens is their potential lack of effective stimulation of cell-mediated immunity, including Th responses. In order to overcome this, there have been many studies which have attempted to define the nature of T cell epitopes so that these they can be identified within or, when necessary, built into subunit vaccines. It was initially thought that T cell epitopes had to conform to an alpha helical structure or could be modelled into an amphipathic helix (DeLisi and Berzofsky,1985) but it now seems apparent that, in contrast to B cell epitopes, linear T cell epitopes are equally as effective as those with a native conformation and a predictive algorithm for Th epitopes has now been derived (Rothbard and Taylor, 1988). It is not yet clear, however, if the algorithms apply to all MHC types or if they can be generalised at all. In addition, the

positioning of T cell epitopes, with respect to the relevant B cell epitopes is also thought to be of importance (Zanetti, Sercarz and Salk, 1987).

1.9.11 Carriers and adjuvants

The rapid developments in recombinant DNA technology and protein chemistry will hopefully provide antigens in quantity for experimental immunisation studies but in isolated form the individual molecules may lose their immunogenicity. To regain potency it will be necessary to present the vaccine components to the immune system in a live attenuated carrier (vector) or in the context of a novel adjuvant preparation (Liew, 1985).

Live microorganisms can not only be exploited to produce polypeptides *en masse in vitro*, but also to produce antigens following injection into the host. Enterobacteria such as *Salmonella* and *E. coli* and viruses from the herpes and vaccinia groups are presently being developed for use as vectors for helminth vaccines (Hyde, 1990). Viruses have the advantage that the antigen is processed in host cells and, therefore, will be glycosylated and processed in the correct manner. Moreover, their persistence in virus-infected cells should stimulate a vigorous cell-mediated immune response (Bomford, 1989). Vaccinia virus has been successfully used as a vector for a cloned 28kDa surface antigen of *S. mansoni* (Simpson and Cioli, 1987). In the future, vectors may be designed so that they express more than one foreign gene and, therefore, act as multicomponent vaccines or, alternatively, genes encoding cytokines may be incorporated into the vector in order to enhance immune responses.

Adjuvants work by forming a depot of antigen which prolongs its release and improves its interaction with APCs. In addition, adjuvants may activate macrophages to release IL-1 which, if combined with the presence of the antigen on T cells, will stimulate their proliferation and helper activities via IL-2 (Gregoriadis, 1990). The only adjuvant currently licensed for use in man is aluminium hydroxide ($Al(OH)_3$) which acts mainly to stimulate humoral immune responses (Bomford, 1989). This adjuvant is used in many veterinary preparations, however, immune stimulating complexes (ISCOM's) which consist of saponin complexed to membrane protein antigens (Morein *et al.*, 1984), have recently been incorporated into a commercial vaccine against equine influenza virus. Freund's complete adjuvant stimulates cell-mediated immunity but its commercial use is prohibited by the associated severe granulomatous and toxic side effects. The development of non-toxic adjuvants which promote both cellular and humoral immunity would therefore appear to be a prerequisite for the further development of subunit helminth vaccines.

The adjuvant systems currently under development for anti-parasite vaccines include liposomes, ISCOM's and plurionic block co-polymers (Bomford, 1989). When antigens are incorporated into a liposome structure, T cell responses, in particular, are enhanced.

Plurionic block co-polymers such as non-ionic surfactant vesicles (NISV's) have properties similar to those of liposomes, in that they stimulate cell-mediated responses but have the added advantage that they are stable in the atmosphere and, therefore, do not require special handling or storage (Brewer and Alexander, 1992). As yet, there has been little work detailing the potential of helminth subunit vaccines in the context of any of the novel adjuvant preparations and most studies have utilised FCA, Al $(OH)_3$ and, for veterinary vaccines, saponin. In determining the efficacy of novel adjuvant preparations with helminth antigens, it is essential to ascertain that vaccines designed to induce CMI responses do not induce severe immunopathological reactions to the parasite.

1.10 Aims of the work presented in this thesis

The studies reported in this thesis were designed to determine potential areas of host/parasite interaction which may be important to the development of successful immunity to *D. viviparus*. The experiments undertaken, examined the main interfaces at which the parasite comes into contact with the host, i.e. the parasite surface or products that the parasites may release *in vivo*. With regard to the parasite surface, phenomena such as surface stage-specificity and surface shedding were examined in detail. Furthermore, the humoral responses directed against the parasite surface in vaccinated and infected calves were examined and compared with the state of immunity in these hosts. The materials which adult parasites release in culture (ES products) were then examined for their protective capacity in guinea pigs and the immunity they induced compared with that stimulated by somatic extracts of adult and third stage *D. viviparus*. The ES components were subsequently fractionated and further examined in immunisation experiments and were also assessed for acetylcholinesterase activity. A function for these enzymes in the stimulation of immune responses was then investigated.

It is anticipated that the results from this work might eventually lead to the clarification of more defined host-protective antigens in *D. viviparus* infection, as well as providing insights into the immunobiology of this interesting host/parasite relationship.

Chapter 2

Materials and methods

Materials and Methods

2.1 Parasites

2.1.1 Source of *D. viviparus* third stage larvae

Infective third stage larvae (L3) and 400Gy (40Kr) gamma-irradiated L3 were obtained from Pitman-Moore, Cambridge, England and were maintained in flasks in phosphate-buffered saline (PBS, pH 7.2, Appendix I) at 4°C. In the studies where 1000Gy (100Kr) L3 were required, the larvae were irradiated in a ^{60}Cobalt source (Nuclear Engineering, Berkshire, England).

2.1.2 Exsheathment of *D. viviparus* third stage larvae

Exsheathment with Milton

Milton solution (Richardson-Vicks Ltd., England), containing sodium hypochlorite (2% w/v), sodium chloride (16.5% w/v) was made to 5% in PBS, equivalent to a final concentration of 0.001% w/v sodium hypochlorite, and stored at 4°C. Generally, up to $1x10^6$ L3 were exsheathed at any one time. The larvae were added to 10ml of 5% Milton at room temperature and left to incubate for 10 minutes with occasional agitation. During this time, samples were taken at three minute intervals and the larvae examined microscopically for degree of exsheathment. If all the larvae had exsheathed by 10 minutes, they were given three, 5 minute spin-washes in PBS at 500g. If exsheathment had not occurred after the initial 10 minutes, the larvae were washed and then re-incubated with fresh Milton and examined every three minutes until exsheathment was observed to have occurred. The exsheathed larvae were either used immediately or were stored at 4°C in PBS.

Exsheathment with glutathione

Approximately 5,000 pre-washed, third stage larvae were incubated in 8.5ml nematode culture medium (Kennedy and Qureshi, 1986, Appendix I), including 50mM oxidised glutathione and were maintained in this for 24 hours in a humid, 5% CO_2 incubator at 37°C. The larvae were then harvested from culture and examined microscopically to ascertain the degree of exsheathment. If this was low, the larvae were re-incubated in fresh medium for a further 24-36 hours and subsequently re-examined.

2.1.3 Enrichment of viable third stage *D.viviparus* larvae by density gradient centrifugation

When excessive (>10%) dead larvae were present in stock solutions of infective larvae, they were enriched for live larvae by density gradient centrifugation in Percoll (Pharmacia, Milton Keynes, England, catalogue number 17089101). The Percoll was made up to 45% in PBS, warmed to 37°C and 5ml of this was added to a 10ml test tube. Next, 5ml of up to $1x10^6$L3, in PBS, was slowly trickled down the inside wall of each test tube, so that the larvae came to lie on the surface of the Percoll solution. The samples were then spun at 600g, for 20 minutes, and the resulting supernatant, above the zone of live larvae, aspirated. This supernatant was examined and if live larvae were absent, it was discarded. The live larvae were then resuspended in approximately 1ml of PBS, pooled and washed three times in PBS prior to use or storage at 4°C.

2.1.4 Source of *D. viviparus* adult parasites

These were obtained from the respiratory passages of experimentally-infected calves between Days 28 and 40 of infection. The adult nematodes were either manually removed from the bronchi after dissection or recovered by pulmonary perfusion following the technique of Inderbitzen (1976). Briefly, the pericardial sac was opened and a small incision made in the wall of the pulmonary artery. A piece of rubber tubing, connected to a mains water tap, was introduced into the pulmonary artery and maintained in position by double ligatures. The aorta, pulmonary veins and caudal vena cava were tied off and the lungs perfused with water. The water and contents of the pulmonary tree were subsequently collected from the trachea into a bucket and the parasites recovered using a 37μm aperture sieve. All adults obtained were immediately placed into warm PBS prior to further manipulation.

2.1.5 Source of *D. viviparus* first stage larvae

First stage larvae were obtained by one of two techniques. In the first, they were harvested from the faeces of calves harbouring patent infections. L1 were monitored in the faeces by a modified McMaster flotation technique (Gordon and Whitlock, 1939) or by the modified Baermann method of Henrikson (1965). An 18.5cm Whatman filter (grade 113,Whatman International, England) was smeared with 10 grams of faeces and on top of this was placed an 18.5cm Maxa Milk Filter (A. McCaskie Ltd., Stirling, Scotland). This sandwich was then inverted and positioned in a glass Baermann filter funnel filled with lukewarm water, so that the lower surface of the milk filter just touched the water surface. The apparatus was left overnight to enable the active L1 to migrate through the filter and subsequently sediment within the Baermann funnel: 10ml was

subsequently run off, spin washed in PBS and the pellet examined for L1 which were then stored at 4°C until required. Using this technique, the number of L1 recovered was often low and the larvae invariably died within a few days of harvesting, thus necessitating their use soon after collection.

In the second method, first stage larvae were obtained by the homogenisation of eggs collected from cultures of adult parasites (Section 2.2.3). The eggs were homogenised in PBS, on ice, for only a few minutes, to enable release of a proportion of the L1 and before the larvae were themselves homogenised. A disadvantage of this technique was that it proved difficult to separate the L1 from the remaining intact and broken eggs. First stage larvae obtained in this way were therefore only used for procedures where it was not essential to have a pure L1 population.

2.1.6 Source of fourth / fifth stage *D. viviparus* larvae

These were obtained using the perfusion technique (Inderbitzen, 1976) from the lungs of a calf infected 14 days previously with 20,000 L3. At this point in infection, there is a mixed population of L4 and L5 stages which are difficult to distinguish and separate.

2.2 Production of parasite antigens

2.2.1 *D. viviparus* third stage larval homogenate and sonicate

Prior to homogenisation, a viability count was made on the larvae by counting ten, 10µl samples. If there was a significant degree of mortality , that is, more than 10%, the larvae were separated on a 45% Percoll gradient (Section 2.1.3).

Approximately $5x10^5$ L3 were homogenised and then spin-washed three times in PBS at 1,500g for approximately 2 minutes, at 4°C. Depending upon the prospective use of the homogenate, the larvae were either homogenised in PBS or proteinase inhibitors (Tris poisons, Appendix I). The L3 were homogenised manually in a hand-held 0.5 - 1ml volume glass homogeniser (Jencons Scientific Ltd., Bedfordshire, England) on ice: 0.1ml of L3 solution in approximately 0.25ml of Tris poisons or PBS were used and progress was monitored microscopically at frequent intervals. Generally, the samples required 10-20 minutes of homogenisation before the majority of the larvae were fully disintegrated. The samples were then spun at 13,000g for 30 minutes, at 4°C and the supernatant of L3 homogenate (L3H) was removed. All samples were immediately snap frozen in liquid nitrogen and stored at -70°C until required.

If required, a detergent soluble extract was obtained by re-suspending the remaining pellet in 0.5ml of a 1% solution of the anionic detergent, sodium deoxycholate in Tris Poisons, and incubating on ice for 30 minutes. The samples were shaken occasionally during incubation, after which the supernatant was collected as detailed above.

Antigens were obtained by sonication when live L3 were present in abundance. Between $1x10^5$ and $1x10^6$ L3 were spin-washed three times in PBS, resuspended in 0.5ml and added to the same volume of proteinase inhibitors or PBS. The parasites were sonicated on ice for approximately 10 minutes in a MSE Soniprep 150 ultrasonic disintegrator (MSE, Scientific Instruments, Crawley, England), at 16 μm amplitude, with frequent breaks in the sonication cycle to maintain the sample at as low a temperature as possible. The samples were checked microscopically for degree of sonication.and when this was considered adequate, the sonicate was spun at 13,000g for 30 minutes, at 4°C, and the supernatant was then removed. This was snap frozen in liquid nitrogen and stored at -70°C until required.

2.2.2 Adult homogenate

Live adult worms, removed from nematode culture medium (see 2.2.3), were washed three times in cold PBS and subsequently homogenised in a glass homogeniser on ice. Approximately 100 adults per 1ml of PBS, or proteinase inhibitors, were used and the homogenate was subsequently spun at 13,000g for 30 minutes, at 4°C, the supernatant removed, snap frozen and maintained at -70°C until required.

Note: L1 and L4/5 homogenates were also prepared in PBS or proteinase inhibitors in a similar manner.

2.2.3 Adult excretory/secretory products

Adult worms, obtained from calf lungs as described in Section 2.1.4, were carefully washed in PBS at 37°C and subsequently transferred to plastic universal bottles containing 15ml Hank's Balanced Salt Solution (HBSS, Gibco, 07601200A) containing 12,000 units/ml penicillin, 1,200μg/ml streptomycin, 125μg/ml gentamycin and 25μg/ml amphoteracin B. This was termed Hank's washing solution. Approximately 100 live adults were transferred to each universal and maintained in Hank's washing solution for 5-10 minutes, at 37°C. The wash solution was then carefully aspirated and discarded and a similar volume, heated to 37°C, was added and the parasites further incubated for 5 minutes. This process was repeated three times. After this, the adults were carefully transferred, using a hooked Pasteur pipette tip, to a fresh universal containing nematode culture medium. Once again, the adults were incubated for approximately 5 minutes at 37°C and were then transferred to culture flasks containing the same culture medium at 37°C.

The parasites were cultured in flasks (Greiner GmBH, Germany) at a concentration of approximately two adults per ml of nematode culture medium. Throughout this procedure, care was taken to ensure that as many dead adults as possible were removed

from the cultures to minimise contamination with products from dead or dying parasites. The loosely-sealed culture flasks were then placed in a humid, 5% CO_2 incubator at 37°C. The parasites were maintained in these cultures for 24 hours, whereupon the culture medium was replaced and that harvested, used as a source of adult parasite excretory/secretory (ES) material. The parasites were maintained in culture for a total of 3 days.

The medium removed from adult cultures was spun at 800g for 5 minutes to separate out any eggs, L1 and adult debris present. The supernatant was subsequently passed through a 0.22μm sterile filter (Millex GV, Millipore SA, Molshiem, France) and the resulting filtrate either snap frozen and stored at -70°C or dialysed and concentrated through Amicon Centriprep 10 or Centricon 10 devices (Amicon Div., W.R. Grace and Co., Danvers, MA., U.S.A.) following the manufacturer's guidelines. The nominal molecular weight cut-off of these concentrators is 10,000 kiloDaltons (kDa). The ES was dialysed by successive rounds of centrifugation and dilution until all traces of the incubation dye from the culture fluid were no longer visible. The material was concentrated to approximately twenty times the initial volume and subsequently snap frozen and maintained at -70°C until required.

Note: L4/5 ES was produced in a similar manner. These stages of parasite were cultured at a level of approximately six parasites per ml of culture medium.

2.2.4 Protein determination

The Pierce Coomassie Blue-based Bradford protein assay (Pierce Chemical Co., Illinois, U.S.A) was used for all protein determinations and was performed according to manufacturer's instructions.

2.3 Animals

2.3.1 Calves

Male Fresian calves between 4 and 8 months of age (100-200kg) were used for the provision of both adult and L1 antigen material. It was ensured that none of these calves had been previously vaccinated or exposed to natural *D. viviparus* infection. All calves were maintained indoors on straw and fed on approximately 1kg hay and 3kg proprietary concentrate feed daily. The calves were examined each day or several times per day during the later stages of infection.

2.3.2 Guinea pigs

Male albino guinea pigs of the outbred Dunkin-Hartley strain were purchased from either Harlan Olac, Blackthorn, Bicester, Oxon, England or Interfauna, Cambridge, England. These animals weighed between 300-600 grams at the start of experimentation.

Female inbred guinea pigs of the Strains 2 and 13 were obtained from the National Institute of Medical Research, Mill Hill, London, for use in protection experiments. These animals weighed approximately 300 grams on arrival.

All guinea pigs were maintained in cages, in groups of one or two, and had access to water and a proprietary concentrate diet *ad libitum* . They also had access to lettuce leaves. All animals were examined daily. Dermal abscesses and ulceration were occasionally encountered in the vicinity of Freund's Complete Adjuvant injection sites. When this occurred, the nearby hair was clipped and Savlon (Beecham Animal Health, Middlesex, England) and Dermisol cream (Beecham Animal Health, Middlesex, England) applied. Guinea pigs with severe ulceration were separated from their cage mates and if the ulcers did not heal promptly, a serum sample was taken and the animal subsequently euthanased using diethyl ether.

All guinea pigs were bled by cardiac puncture whilst under diethyl ether anaesthesia.

2.3.3 Rabbits

A single New Zealand White rabbit weighing 3kg was obtained from Harlan Olac for the production of antisera to ES components. This animal was caged singly and maintained on water and a proprietary concentrate diet *ad libitum* . When required, it was bled from an ear vein.

2.3.4 Rats

Sprague-Dawley and Wistar rats weighing 300 grams were bred in-house at the Wellcome Laboratories for Experimental Parasitology and used to raise stage-specific antisera. They were maintained in groups of two with access to proprietary feed and water *ad libitum* . These animals were bled by cardiac puncture under diethyl ether anaesthetic.

2.4 Infection and immunisation of animals

2.4.1 Infection of calves and guinea pigs with *D. viviparus* L3

Stock larvae were examined microscopically for the degree of larval mortality prior to infection. Dead larvae were taken to be those that were neither curled tightly nor moved freely. If viability was satisfactory, i.e. < 10% mortality, the larvae were used for

infection. The larval concentration was assessed by counting 25, 10µl aliquots of the stock solution and the computed doses were subsequently made up for each animal.

Guinea pigs were infected orally using a syringe attached to an intravenous catheter (Red Luer, 5FG, Portex Ltd., Kent, England). Guinea pigs were usually infected with 5,000 or 6,000 L3 in 1ml PBS.

Calves were infected orally by pouring the computed dose in 10ml PBS from a glass universal bottle into their oral cavity, then holding the mouth closed to encourage swallowing. Calves were generally infected at a dose rate of 20 L3 per kg.

2.4.2 Rat infection with *D. viviparus* L1 and adults

Sprague-Dawley and Wistar adult male rats were infected intra-peritoneally with either L1 or adult male *D. viviparus*. Forty male adult worms were removed from culture and examined for viability under an inverting microscope. They were then washed three times in sterile PBS and 10 of these, in 1ml PBS, were injected intraperitoneally via an Intraflon 14 gauge catheter. To eliminate the possibility of immunising the rats with first stage larvae, female *D. viviparus* adult worms were not used.

L1, obtained from the faeces of infected calves as described earlier, were spin-washed five times in PBS and approximately 5,000 were injected intraperitoneally into each rat via a 14 gauge needle.

2.4.3 Guinea pig infection with *D. viviparus* L1 and adults

Dunkin-Hartley guinea pigs were infected in the same manner as rats to raise specific antisera against the adult and the L1 stages.

2.4.4 Guinea pig immunisations with parasite antigens

Both inbred and outbred strains of guinea pig were immunised with various antigen preparations in the context of Freund's Complete adjuvant (FCA). Depending upon the experimental protocol, the required amount of antigen for each animal was made up in sterile PBS to 1ml and, for the primary immunisation, was added to 1ml of FCA (Sigma, F5881). The samples were then thoroughly emulsified using an Ultra Turrax homogenisor (T25 homogeniser, R. and J. Wood, Paisley, Scotland). The degree of emulsification was determined by placing a small drop of the antigen/Freund's preparation in PBS and visually evaluating the degree of miscibility. When the emulsion was no longer observed to be miscible in the PBS, the sample was assumed to be ready for injection. The guinea pigs were injected subcutaneously via a glass syringe with Luer fitting and, to minimise post-immunisation abscessation and ulceration, the injections

were carefully distributed over three sites on the lateral abdomen and flank. The animals were examined daily and any ulcers that appeared, promptly treated as outlined above.

For the secondary immunisation, animals were injected with the same antigen in the context of Freund's Incomplete adjuvant (FIA, Sigma, F5506). The antigen was mixed and administered as described above, except that the animals were injected on the flank opposite to that used for primary immunisation.

Animals were challenged by infection 4 to 8 weeks after the secondary immunisation and killed on Day 6 post-challenge. All experiments included a challenge control group. In general, all animals were bled prior to immunisation, on Day 14 of secondary immunisation and, finally, at kill.

Guinea pigs were subjected to terminal anaesthesia in diethyl ether on Day 6 post-challenge and had their worm burdens determined by Baermannisation of lung material obtained using a stomacher (Stomacher 80, A.J. Steward, UAC House, London). Briefly, the entire lungs and thoracic trachea were carefully removed and rinsed in water and were then placed in a stomacher bag containing 20ml of PBS. The lungs were subsequently disrupted for 5 minutes, or until the they formed a fine suspension which was subsequently incubated in two layers of gauze within a Baermann funnel filled with PBS for 6 hours at 37°C. The samples were then aspirated to leave 20ml volume which was examined for larvae at x12 magnification.

Niosome adjuvant was prepared with ES products by Dr. J. Brewer of the Department of Immunology, Strathclyde University. Empty niosomes were also prepared for the immunisation of a control group. The niosome vesicles were stored at 4°C until required. For the guinea pig immunisations, they were injected subcutaneously into one site per animal using a regime similar to that followed for Freund's adjuvant.

Statistical analysis: as worm recoveries in challenged guinea pigs were not normally distributed, the results of all the immunisation experiments were analysed by the Mann-Whitney non-parametric test (5% significance level).

2.4.5 Preparation of serum

From all animals, whole blood was collected into plastic syringes or Vacutainers (calves). The blood was left to coagulate at room temperature for 1 hour and subsequently allowed to contract at 4°C, overnight. The serum was then carefully aspirated, centrifuged at 13,000g for 10 minutes and the resulting supernatant aliquoted and stored at -20°C.

2.5 Immunological procedures

2.5.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins

SDS-PAGE was carried out using the discontinuous buffer system of Laemmli (1970). Gels of 0.7 or 1.5mm thickness were used depending on the procedure under study. 5-25% gradient or 20% homogeneous resolving gels of 120mm long, with a 10mm stacking gel, were used for protein separation on a Pharmacia system, GE 2/4 (see Appendix I). Depending on the procedure, single and multi-well formers of 0.7mm or 1.5mm thickness were used. Cast gels were wrapped in moist paper and plastic wrap (Clingfilm) and stored at 4°C for up to 7 days.

The protein concentration run in each track ranged from 10-100µg, as indicated. The antigen samples to be analysed were made up to 20µl with PBS and each was run with 20µl of PAGE sample buffer (SB, Appendix I) under reducing or non-reducing conditions. For reducing conditions, 5% 2-mercaptoethanol was included in the SB and for non-reducing conditions, 1mg/ml iodoacetamide was added. All samples were placed in a boiling water bath for 10 minutes and subsequently loaded on to the gel.

Samples that were to be run for enzyme activity were not reduced, boiled or exposed to SDS but were electrophoresed under native conditions in order to preserve enzyme activity as much as possible. Low Molecular Weight Markers (Pharmacia, 17-0446-01), with nominal molecular weights (M_r) of 94, 67, 43, 30, 20.1 and 14.4 kDa, were used to enable estimation of the size of the separated parasite proteins.

Electrophoresis was carried out at 4°C, to minimise gel heating and distortion. Each gel was run at an initial voltage of 100V and a constant current of 40mA for approximately 3 hours in Tris/glycine running buffer, 25mM Tris, 0.18M glycine, 3.5mM SDS. Often, two gels were run together at 250V, 65mA. Electrophoresis was terminated when the bromophenol blue tracking dye reached 1cm from the bottom of the gel.

2.5.2 Gel staining for protein

For protein visualisation most gels were stained with 0.1% Coomassie Brilliant Blue R-250 (Sigma, B0149) in 25% methanol, 10% acetic acid and 1% glycerol for 1-2 hours. The gels were subsequently destained by rocking in the solvent until the background was clear. They were then dried on to pre-boiled cellophane (Bio-Rad, Herts.) or 18.5cm Whatman filter paper on a dual temperature slab drier (Bio-Rad) at 60°C. For increased sensitivity, gels were stained for protein using a silver stain kit (Quick Silver Kit, Amersham) following manufacturer's recommendations.

2.5.3 Electro-elution of material from polyacrylamide gels

For this procedure, 1.5mm thick, 20% homogeneous SDS-PAGE gels were run with antigen preparations under reducing or native conditions. The gels were lightly stained by subjecting them to three washes in deionised water, and then to filtered 0.1% Coomassie Brilliant Blue R-250 in 25% methanol, 10% acetic acid and 1% glycerol, for 10 minutes. They were then washed several times in deionised/distilled water over the next few hours. This permitted exact visualisation of the desired bands without fixing the proteins into the gel, and thus allowed accurate band excision for subsequent electro-elution. Alternatively, a narrow strip of stained gel was used as a template for locating the correct region of the unstained portion.

Once the desired piece of gel was isolated, it was cut into approximately 5mm x 5mm squares which were placed in a Bio-Rad Model 422 Electro-Eluter and electro-eluted in protein elution buffer containing 25mM Tris, 0.18M glycine, 3.5mM SDS. The samples were electroeluted for 5 hours at a constant current of 10mA per chamber. Following this, the eluate was removed from below each frit and concentrated in a Centricon 10 microconcentrator to one tenth of the original volume. After concentration, the eluates were snap frozen in liquid nitrogen and stored at -70°C until required. From each concentrated eluate batch, a sample was taken and run on a standard SDS-PAGE gradient gel to visualise protein content. A sample of the filtrate was also examined in a similar manner to determine if protein leakage had occurred during the concentration step.

2.5.4 Western Blotting

Electroblotting

This was carried out following the method of Towbin *et al.* (1979). After standard gel electrophoresis, gels were prepared for transfer by equilibrating in Tris-glycine transfer buffer containing 25mM Tris, 0.18M glycine, 3.5mM SDS and 20% methanol. The gels were equilibrated for 30 minutes at room temperature. The transfer membrane, Hibond C-Extra, 0.45μm (Amersham), was also immersed in the buffer for 30 minutes.

The proteins were transferred in the Tris-glycine buffer in a Bio-rad Trans-blot cell at either 60V, 200mA for 2 hours or 30V, 100mA, overnight. The apparatus was maintained in a 4°C cold lab throughout transfer. Upon completion, the cassettes were dismantled, the membranes allowed to dry and subsequently cut into strips ready for probing with the relevant sera. One of the strips was stained for proteins with 0.1% Amido Black (BDH, 44314) in 45% methanol, 10% acetic acid for 15 minutes, and then destained with the solvent. Alternatively, the strips were stained with Ponceau-S (Sigma, P3504) and subsequently rinsed in several changes of distilled water. The post-

transfer gel was stained with Coomassie Brilliant Blue, following the protocol outlined in Section 2.5.2, to confirm transfer from the gel.

Antigen detection with antisera

In order to reduce non-specific binding of antibodies to the nitrocellulose, each strip was incubated for 1 hour in 4ml of 20% soya milk/0.5% "Tween 20" (polyoxyethylene sorbitan monolaurate; Sigma, P5987) in blot wash buffer containing 10mM Tris-buffered saline, pH 7.2 and 0.5% Tween-20. The blots were then subjected to three, 5 minute washes in blot wash buffer and then incubated with the relevant serum sample diluted in wash buffer, for 2 hours at room temperature. The serum dilutions varied between 1:100 to 1:500, as indicated.

Following this step, the blots were washed and subsequently incubated with enzyme conjugate; anti guinea-pig IgG (Sigma, A7664) conjugated to alkaline phosphatase. Alternatively horseradish peroxidase (HRP, Sigma) conjugates were used which were raised against guinea pig IgG whole molecule (Sigma, A5545). The conjugates were diluted in PBS; 1: 500 for AP or 1: 200 for HRP, and 4ml added to each well and incubated with the strips for 60 minutes at room temperature. The strips were then washed and substrate solution added: for AP, 4ml of 100mM NaCl, 5mM $MgCl_2$, 100mM Tris buffer (pH 9.5) containing 0.06%, 50mg/ml nitrobluetetrazolium (NBT, Sigma, N6876)/70% dimethylformamide (DMF) and 0.033%, 50mg/ml bromochloroindolphosphate (BCIP, Sigma B6149) /100% DMF was added. For HRP, the chromogen, 4-chloro-1-naphthol (Sigma, C8890) containing H_2O_2 at 0.01% final concentration was added. The reaction was terminated after 5-20 minutes, depending on banding intensity. The AP reaction was stopped with 10mM ethylenediaminetetracetic acid (EDTA)/PBS and the HRP reaction stopped with distilled water. The blots were then dried and stored in aluminium foil.

2.5.5 Enzyme-Linked Immunosorbent Assay (ELISA)

The antigens under examination were Tris Poison extracts of adult and L3 homogenates or adult ES, prepared as described above. The optimum concentrations of antigen, serum and conjugate were ascertained using a standard chequerboard technique. All plates included negative and positive controls. The antigens were made up at between 0.125 and 5µg/ml in buffer containing 14mM Na_2CO_3, 34mM $NaHCO_3$ and 3mM NaN_3, and 60µl of this was used to coat each well of a 96-well microtitre plate (Immulon A2, Dynatech Laboratories Ltd., Billingshurst, England). Coating was carried out at 37°C, for 2 hours, or overnight at 4°C. The plates were then washed three times in ELISA wash buffer containing 7.5mM Na_2HPO_4, 3.2mM NaH_2PO_4, 145mM NaCl and 0.05% Tween 20, and subsequently dried.

To reduce non-specific binding of antibody, 60µl of 4% skimmed milk in ELISA wash buffer was added to each well before incubation for 60 minutes at room temperature. After incubation, the plates were re-washed and dried and 60µl of the relevant serum dilution (between 1:50 to 1:500 in 2% skimmed milk/ELISA wash buffer) added. Duplicate or triplicate samples of each serum were included. The plates were then incubated for 30 minutes at 37°C and subsequently washed and dried.

Next, 60µl of anti-guinea pig IgG (whole molecule) conjugated to horseradish peroxidase (Sigma, A5545), diluted in PBS at either 1:10,000 or 1:20,000, was added to each well. The plates were then incubated, washed and dried under conditions similar to those described above for the primary antibody step.

Following this, 60µl of substrate solution containing 25mM citric acid, 1mM Na_2HPO_4, 0.05% H_2O_2 and 5mg/ml orthophenylene-diamine (OPD, Sigma, P9029), pH 5, was added to each well. The plates were subsequently incubated at room temperature until a colour change was clearly evident and the time for this change noted. This incubation time was used for the subsequent related assays. The reaction was then terminated with 4M sulphuric acid. The plates were then read in a Titertek Multiskan Plate Reader at 492nm (Flow Laboratories Ltd., Uxbridge, U.K.) and the results were expressed as the mean optical density of the duplicate or triplicate samples.

2.5.6 Indirect fluorescent antibody test

In order to investigate various surface properties of *D. viviparus* stages, the fluorescent antibody test was applied to intact, live parasites using fluorescein isothiocyanate (FITC) conjugated to anti-immunoglobulin antibody.

For larval stages, approximately 4,000 parasites were tested per sample, while for the adults, one worm was used per sample. The larvae under study were given three spin washes in 0.1% NaN_3/PBS or, in the case of the adult stage, were simply immersed in 0.1% NaN_3/PBS and agitated for 5 minutes. In all incubations, the parasites were incubated in 250µl (larval stages) or 500µl (adult stage) of sample. After washing, antiserum, at a final dilution of 1:30 in 0.1% Na N_3/PBS, was incubated with the parasites for 30 minutes on ice. The samples were agitated frequently throughout the incubation period before the parasites were washed as described previously.

In some studies, specific bovine antibody isotype responses were measured by incubating after the primary antibody step with mouse monoclonal antibodies (mAb) raised against bovine IgG_1, IgG_2 or IgM (Centraal Diergeneeskundig Instituut). These reagents were added at a final dilution of 1:50, in 0.1% NaN_3/PBS, for 30 minutes on ice. The parasites were then washed and subsequently incubated with FITC-conjugated IgG (whole molecule) raised against the appropriate species, i.e rabbit (Sigma, F0382), bovine (Sigma, F7509), rat (Sigma, F6258), guinea pig (Sigma, F3887) or mouse

(Sigma, F2883), at a final dilution of 1:30 in NaN_3/PBS. The samples were again incubated on ice for 30 minutes and subsequently re-washed. The parasites were then taken up into 30µl of 0.1% NaN_3/PBS and mounted onto a slide upon which they were circled with a ring of Vaseline and placed under a coverslip ready for viewing.

Fluorescence was quantified by photon counting using a Lietz MPV Compact 2 microscope photometer connected to an Olivetti H100 Computer. For larval stages and eggs, the photometer was set to measure a rectangular field in the range of 230-555μm^2, using a x40 objective, while for the adult stage, 920-2220μm^2, with a x10 objective. For larvae and eggs, the measuring box was positioned centrally, while for adults, which displayed a high degree of internal fluorescence, the edge of the parasite was measured. For each experiment, the fluorescence of a predetermined specimen, usually the brightest, was set at an arbitrary value of 650, and the measurements of all other samples were taken relative to this. A background measurement was also taken and this was immediately subtracted from all readings. For each specimen, 20-25 measurements were recorded and the mean and standard deviation of these calculated.

2.5.7 Serum adsorbance

In some cases, it was necessary to determine the presence of surface stage-specificity by pre-adsorbing immune serum with various parasite stages prior to the immunofluorescence study. Approximately $7x10^4$ first stage larvae, $1x10^5$ third stage larvae or 50 adult male parasites were washed three times in PBS and subsequently incubated with immune serum, diluted 1:20 in PBS. These were maintained on ice for 2 hours with frequent agitation after which the adsorbed serum was removed by centrifugation of the larval samples or, in the case of the adult samples, the serum was simply aspirated. The adsorbed serum was then adjusted with the addition of PBS to give a final dilution of 1:30 and subsequently incubated with various parasite stages; the degree of surface recognition was investigated following the IFAT technique detailed in Section 2.5.6.

2.5.8 Surface shedding of bound antibody

To detect if *D. viviparus* parasites maintained under physiological conditions undergo shedding of their surface antigens, the following procedure was carried out. Approximately 3,000 L3 (sheathed or Milton-exsheathed), or 1 adult, per sample, were initially incubated in PBS or PBS containing the metabolic inhibitor, NaN_3 at 0.1%, at either 2°C or 37°C for 30 minutes. The samples were then incubated with 250µl (larval stages) or 500µl (adult stage) of normal or immune bovine serum at a final dilution of 1:30 and maintained under each of the conditions outlined above. The samples were then washed three times in the relevant medium, i.e. PBS with or without NaN_3, and

subsequently incubated with 250µl or 500µl of FITC conjugated anti-bovine IgG (whole molecule) at a final dilution of 1:30, again following the conditions outlined above. After 30 minutes, the washing step was repeated and the samples viewed under UV light and their relative fluorescence recorded as described earlier. After viewing, the remainder of each sample or, in the case of the adult stage, the adult was returned to its tube, and the samples maintained overnight, in darkness, at either 2°C or 37°C. The next day, all samples were viewed and examined for any changes in their degree of fluorescence.

2.5.9 Iodination of soluble proteins

All proteins were iodinated using the Iodogen technique (Markwell and Fox, 1978) which permits labelling of protein at tyrosine residues. Plastic microfuge tubes were coated with 200µg Iodogen (1,3,4,6- terachloro- 3 alpha, 6alpha-diphenylglycouril, Pierce Chemical Co., Illinois, U.S.A.) evaporated in 1mg/ml chloroform. This was carried out in batches and the tubes stored at room temperature in a desiccation chamber. To each pre-coated tube was added 15µg of adult homogenate, ES or L3 sonicate in 200µl PBS, pH 7.2. This was incubated for 10 minutes on ice with 9MBq $Na^{125}I$ (Amersham IMS 30, Amersham International plc). The sample was shaken occasionally and the reaction terminated by the addition of 10% volume, saturated tyrosine solution. The sample was further incubated for 5 minutes on ice, after which it was loaded on to a Sephadex G-25 column (PD-10., Pharmacia, 17-0851-01) which had previously been equilibrated with 0.5% Triton X-100/PBS. The detergent was used in order to reduce loss of labelled protein by adsorption on to the Sephadex column.

The radiolabelled macromolecules were subsequently collected in fractions taken at 30 second intervals and a rough estimate of the counts in all fractions measured using a hand held gamma counter (Mini-Instruments, Scintillation meter). The first three fractions after the initial peak, and the first from the second peak were retained, and subsequently measured for total radioactivity and the amount of radioactivity that was present in trichloroacetic acid (TCA)-precipitates. All other fractions were discarded.

In order to determine the amount of radioactivity that was attributable to ^{125}I associated with macromolecules, a TCA precipitation was carried out. In triplicate samples, 5µl of normal rabbit serum, which acted as a carrier, was incubated with 2µl of radiolabelled material and 50µl 0.5% Triton X-100/PBS for 10 minutes, at room temperature. These were vortexed occasionally and subsequently counted for total radioactive counts per minute (input cpm) in a gamma counter. To an identical set of triplicate samples, was added approximately 1ml of 10% TCA. These samples were also mixed and incubated for 10 minutes and then centrifuged at 1,500g for 10 minutes. After centrifugation, the supernatant was aspirated and the pellets counted (output cpm). The

amount of radioactivity attributed to association with macromolecules was designated as the % TCA precipitate. This was calculated from 100% x output cpm/input cpm.

2.5.10 Immunoprecipitation

To 50µl of wash buffer containing 0.1% SDS, 0.5% Triton X-100, 20mM LiCl, 50mM Tris and 0.5M NaCl, was added 2.5µl of test antisera and enough radiolabelled material to give approximately 2 x10^5cpm. The wash buffer containing LiCl was used to reduce non-specific binding of serum to the radiolabelled antigen. Each sample was set up in duplicate. These were vortexed, the input counts of each measured in a gamma counter and subsequently incubated overnight at 4°C.

The next day, 50µl of formalin-fixed *Staphylococcus aureus* cell suspension (Gibco) was added which acted as a solid phase adsorbent of antibody-antigen complexes (Kessler, 1975). This was incubated with the samples for 60 minutes at room temperature and subsequently for 60 minutes at 4°C. The unbound material was then removed by three washes in wash buffer, including one tube change. This was done in order to reduce background counts as much as possible. The radioactivity of the precipitated material was then measured in a gamma counter, and the duplicates pooled and stored at -20°C until analysis by SDS-PAGE.

All pellets were resuspended in 40µl of sample loading buffer containing 5% 2-mercaptoethanol and placed in a boiling water bath for 10 minutes. The samples were then centrifuged at 13,000g for 10 minutes and the supernatants run on 5-25% SDS polyacrylamide gels, as described in Section 2.5.1. On termination of electrophoresis, the gels were fixed in 25% methanol, 7.5% acetic acid and 1% glycerol for 30 minutes and subsequently dried on a slab drier at 60°C. They were then exposed to pre-flashed autoradiographic film (Fuji RX, Technical Photo Systems, Cumbernauld, Scotland) in combination with Du Pont Cronex Lighting-Plus Intensifying Screens (H.A. West, Edinburgh, Scotland) in Harmer X-ray film cassettes and maintained at -70°C until development in an automatic processor (Cronex CX 130, Dupont).

2.6 Electron Microscopy

2.6.1 Scanning electron Microscopy

Parasite specimens were double fixed in the following manner. The larvae to be scanned were initially fixed in 2.5% Grade I glutaraldehyde (Sigma) in 0.1M phosphate buffer, pH 7.2, and were subsequently incubated for 1 hour at room temperature. The samples were then given three, 5 minute spin washes in the same phosphate buffer. After the three buffer rinses, the specimens were incubated in 1ml of 1% osmium tetroxide fixative in the fume cupboard for 1 hour. Next, the specimens were subjected

to three, 10 minute washes in distilled water and stained in 1ml of 0.5% aqueous uranyl acetate in the dark for 1 hour. After incubation, the samples were rinsed in distilled water and each was placed in a ring specimen chamber on top of a 0.2μm Nucleopore filter. The specimens, within their chambers, were then dehydrated by serial immersions in acetone as water is not miscible with the transition fluid (CO_2) used in the critical point drier. The samples were immersed in 30%, 50%, 70%, 90%, 100% acetone (twice), and 100% anhydrous acetone, each for 10 minutes. The specimens were then placed in a critical point drier to dry in CO_2 for 100 minutes. The liquid level was adjusted frequently to prevent turbulence damaging the sample during the procedure and the drier was refilled four times with fresh CO_2 to remove the acetone. Hairdriers were used to increase the chamber temperature to approximately 40°C to give a chamber pressure of 1,400 pounds per square inch (psi). This increased the pressure above the critical point for CO_2 and thus reduced surface tension, and hence cell distortion, as the samples dried out. After 5 minutes at 1,400psi, the gaseous CO_2 was slowly removed to prevent condensation due to local cooling and the specimens were withdrawn from the chamber.

After drying, the specimens were stored overnight in a desiccation chamber, and the next day, removed from the filter chambers and mounted, intact with the filter paper, onto aluminium stubs. The edge of the filter paper was then cut and this area painted with colloidal silver paint to enable conduction of the electron beam. The mounted specimens were then inserted at the anode of a Modified Polaron SEM Coating Unit, E 5,000 in which the samples were coated with a 200-500 Angstrom thickness of gold in argon at a pressure of 0.2 torr. Coating the samples in this manner eliminated the chance of electron beam charge build up which can distort the image and damage the specimen. The samples were then viewed under the scanning electron microscope at magnifications up to $2x10^5$. The images were recorded on Ilford FP4 film.

2.6.2 Transmission electron Microscopy

The larvae to be processed were initially fixed for 1 hour in 1ml 2.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.2. Next, they were given three, 5 minute rinses in the phosphate buffer, and the specimens transferred into 1ml of 1% osmium tetroxide/0.1M phosphate buffer and incubated for 1 hour. On termination of this incubation, the specimens were subjected to three, 10 minute washes in distilled water and embedded in agar. The agar was melted into 1.5ml microfuge tubes and a few drops of the larval pellet carefully mixed into it using a Pasteur pipette. The specimens were then placed in a freezer for a few minutes to allow the agar to set. Next, using a Pasteur pipette, the agar pellets were removed from the microfuge tubes, placed in a Petri dish and the excess agar trimmed using a scalpel blade. The larvae were then stained in the dark for 60 minutes with 2ml of 0.5% aqueous uranyl acetate.

After staining, the specimens were washed in distilled water and, still in their respective vials, were dehydrated through a series of ethanol solutions: 30%, 50%, 70%, 90%, 100% alcohol (twice) and anhydrous 100% alcohol. The alcohol was removed, the specimens given three, 5 minute incubations in 3ml epoxypropane, which is miscible with both alcohols and the embedding resin, and then left overnight in 4ml of a 1:1 mix of the epoxyresins, Araldite (BDH, 38008) and epoxypropane. During this time, the specimens were slowly rotated.

The following day, each sample was embedded in fresh Araldite in a section of a small embedding tray. A small label was inserted into the Araldite of each sample and the tray placed in a 60°C oven for 48 hours. The specimens remained in the trays until 70nm sections were cut with a diamond knife in an Reichert Ultracut E ultramicrotome using a diamond knife. Sections were mounted on to gold 700 mesh grids and were then immersed quickly in 0.2M NaOH, followed by a 30 second incubation in 0.02M NaOH in a sealed Petri dish. The grids were then totally immersed in Reynold's lead citrate stain for 5 minutes, then transferred back through 0.2M NaOH and 0.02M NaOH and finally into distilled water, for at least 30 seconds. Care was taken to avoid contamination of the grids and to ensure thorough rinsing after each stain step. The specimens were then viewed under the transmission electron microscope at $2x10^5$ magnification, while negatives were at $5x10^5$ magnification.

2.7 Investigations of larval exsheathment and host gut penetration

2.7.1 Exsheathment of *D. viviparus* L3 under simulated host conditions

Approximately $1x10^5$ L3 were incubated in 10ml bovine ruminal fluid for 24 hours and then examined for exsheathment. Alternatively, a 10ml solution of up to $1x10^5$ L3, in deionised water, was added to 10ml of 2% hydrochloric acid (HCl). Next, 20mg of pepsin A powder (BDH, 159032) was added and the sample agitated until the pepsin was fully dissolved. The samples were then incubated in a water bath at 37°C for 3 hours with occasional agitation. The larvae were then spin-washed three times in PBS and 50% of the larvae resuspended in 20ml nematode culture medium, containing 20% foetal bovine serum (FBS) (Flow Laboratories,Scotland, 29-101-49) and 1% bovine bile. These larvae were then incubated for 24 hours at 37°C. The other 50% were maintained under the original conditions and examined for exsheathment after a further 24 hours.

After this time, all samples were washed three times in PBS and resuspended in nematode culture medium and FBS and examined for any indication of ongoing exsheathment. They were re-incubated over the next 24 hours, while being maintained at 37°C and were then spin-washed three times in PBS. Unfortunately it proved difficult to collect large quantities of intact L3 sheaths as these rapidly disintegrated.

2.7.2 Fluoresceination of sheathed and Milton-exsheathed *D. viviparus* L3

In order to attempt to determine the mechanism of *D. viviparus* exsheathment and to define the fate of cast sheaths, third stage larvae were fluoresceinated directly with fluorescein isothiocyanate, FITC (Sigma, F7250) following the method of Kumar and Pritchard (1991). Approximately $1x10^4$ sheathed or Milton-exsheathed L3 were spin-washed three times in PBS and subsequently incubated in 1ml of 0.001% FITC overnight at room temperature, in the dark. After this, the larvae were spin-washed and then examined under a x40 objective lens on a UV microscope, to determine the degree of FITC binding to the parasite surface. The sheathed larvae were subsequently exsheathed in Milton and again viewed under UV light to examine if there was any binding of FITC to the underlying L3 cuticle.

2.7.3 Penetration of small intestinal mucosa by third stage *D. viviparus*

Following the method outlined by Kumar and Pritchard (1991) for the penetration of the skin of mice by *Necator americanus* larvae, 10,000 FITC-labelled *D. viviparus* third stage larvae were incubated on the surface of the mucosa of a 2cmx2cm piece of bovine small intestine. The square of intestine was tied to the end of a piece of rubber tubing which was then immersed in nematode culture medium in a 25ml universal bottle (Figure 2.1). The samples were incubated for either 30, 60, 120 or 240 minutes. After each incubation, the mucosal surface of the intestine was carefully washed with PBS and any larvae present in the washings counted. The medium underneath the mucosa was also examined for larvae that may have penetrated the full depth of the mucosa. The area with which the L3 were in contact was then carefully excised and placed in phosphate-buffered formalin and maintained in the dark until mounted in paraffin and cut into 20µm sections. The sections were later examined under UV light for any fluoresceinated larvae which were in the process of penetration.

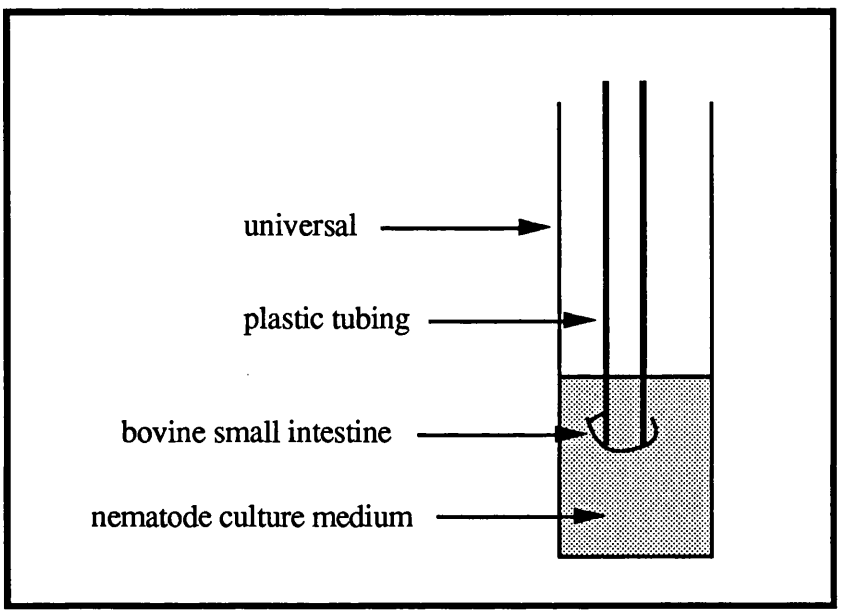


Figure 2.1
Apparatus used to examine the penetration of bovine small intestinal mucosa by *D. viviparus* sheathed L3 parasites.

2.8 Studies on Esterases and Acetylcholinesterases in *D.viviparus*

2.8.1 Ellman assay for AChE activity

To determine the esterase content of *D.viviparus* material, the colorimetric assay of Ellman *et al.* (1961) was performed in which acetylthiocholine iodide was used as a substrate which formed an intense yellow product in the presence of acetylcholinesterase (AChE). To 750µl of PBS, was added 5µl of 21.67mg/ml acetylthiocholine iodide (ATCI, Sigma, A5751)/0.1M phosphate buffer, pH 8, and 25µl of 39.6mg/ml 5'5'-Dithiobis-2-nitrobenzoic acid (DTNB, Sigma, D8130)/ 15mg/ml sodium bicarbonate/0.1M phosphate buffer, pH 7. These were warmed to 37°C, 12.5µl of test sample, in distilled water, subsequently added and the reaction immediately monitored for change in absorbance at 412nm using a spectrophotometer linked to a chart reader. The samples were referenced in relation to distilled water. To calculate the amount of AChE activity present, the following calculation was carried out:

IU/L = change absorbance /minute x 4662

In the initial studies, this assay was carried out at pH 4-8 in order to ascertain the pH optimum for the AChE present.

To determine if serum antibody or purified IgG had an effect on the activity of the esterases present in *D. viviparus*, the samples were pre-incubated with bovine or guinea pig serum or with purified bovine IgG (kindly provided by Dr. C.Britton, Department of Veterinary Parasitology, University of Glasgow) at a ratio of 2:1 and incubated at room temperature for 2 hours. The samples were then assayed as outlined above. In the case of guinea pig antiserum, which had high endogenous levels of esterase activity, the serum was pre-heated to 65°C for 5 minutes in order to inactivate this enzyme activity prior to incubation with the parasite material.

2.8.2 Polyacrylamide gel assays for the detection of esterase isoforms

10% polyacrylamide minigels (Mini Protean II Dual Slab Cell, Bio-rad) were cast according to manufacturer's instructions, except that SDS was excluded from all buffers because of its interference with esterase activity (D.P. Knox, personal communication). These gels were loaded with 10µl of test sample and 10µl of sample loading buffer, without SDS and proteinase inhibitors, and the samples run under native conditions at a constant current of 200mA for approximately 45 minutes in Tris/glycine running buffer, as detailed in Section 2.5.1 The electrophoresis tank was kept cool by immersion in ice.

The running buffer was replaced at frequent intervals with cold fresh buffer in order to preserve enzyme activity.

Following electrophoresis, each minigel was stained following the method of Grunder, Sartori and Stormont (1965). Briefly, 20ml of PBS containing 5mg/ml Fast Blue RR salt (Sigma, F0500) and 1ml of 2% naphthyl acetate (Sigma, N8505)/ acetone was incubated with the gels at room temperature until bands of enzyme activity were obvious. The reaction was then stopped by applying several rinses of distilled water and the gels photographed and dried. Samples analysed in this manner were also examined for the effect of serum and purified IgG on the esterases present, as outlined above. After pre-incubation, the samples were run on the gels and stained as before.

2.8.3 Eserine inhibition of AChE present in *D. viviparus* ES

In order to confirm that the esterase isoforms present were in fact acetylcholinesterases, and not pseudocholinesterases, eserine (Sigma, E8375), was used as a specific inhibitor (Pearse, 1972). The samples were electrophoresed on 10% polyacrylamide gels under native conditions, as described in the previous section. Prior to the staining step however, the gels were incubated in 20ml of 1mM eserine for 60 minutes at room temperature. The gels were subsequently stained in naphthyl acetate and Fast Blue RR salt as outlined above.

2.8.4. Staining polyacrylamide gels specifically for acetylcholinesterases

The basis of this method is that thiocholine, released by AChE by hydrolysis, is believed to reduce ferricyanide preferentially to ferrocyanide, and the latter combines with Cu++ to form an insoluble copper ferrocyanide precipitate. Following the method of Karnovsky and Roots (1964), polyacrylamide minigels run with parasite material were stained in the following manner. To 26ml of 0.77mg/ml acetylthiocholine iodide/0.1M phosphate buffer, were added 2ml 0.1M sodium citrate, 4ml 30mM copper sulphate, 4ml distilled water and 4ml 5mM potassium ferricyanide. The gels were incubated in this at 37°C until bands appeared, after which they were given multiple washes in distilled water. The gels were subsequently dried and photographed as before.

2.8.5 Purification of secreted acetylcholinesterases from adult ES

In order to further define the AChE present in *D.viviparus* ES, the enzymes were affinity-purified following the method of Hodgson and Chubb (1983). Epoxyactivated Sepharose 6B (Pharmacia) was hydrated and then made up with 0.2M edrophonium chloride (EdCl, Sigma, E3256) at 1 part gel: 2 parts EdCl. The pH was adjusted to 10

with 1M NaOH and the gel shaken for 24 hours at 50°C. The gel was then subjected to 10 volumes each of 0.1M sodium acetate (pH 4.5), 0.012M sodium borate (pH 10) and distilled water.

Approximately 0.7ml of the prepared gel was added to a 1ml syringe and washed five times with distilled water. Next, 200µl of ES was added to the top of the gel bed, followed by 10ml of 50mM $NaP0_4$ (pH 8), then 10ml 50mM Na PO_4/0.5M NaCl (pH 8) and finally with 8ml 12mM EdCl. Fractions of 1ml volume were collected and, those of potential interest, subsequently desalted twice through 1ml of swollen Sephadex G25 (Pharmacia,) constructed in 5ml syringes. At each wash, the columns were spun for 5 minutes at 200g. Finally, the samples were collected and assayed for AChE activity as outlined above.

2.8.6 Purification of esterase-enriched fractions from polyacrylamide gels

For immunisation studies, adult ES fractions enriched for esterases were isolated from polyacrylamide gels. This was carried out by running 200µl of adult ES, containing approximately 0.3mg/ml protein, on 120mm long, 0.7mm thick, 5-25% gradient polyacrylamide gels under native conditions. Following electrophoresis, 2cm strips were cut from each side of the gel and these portions stained for esterase activity following the method of Grunder, Sartori and Stormont (1965), outlined in Section 2.8.2. Once esterase bands were obvious, these portions were used as templates to enable the rest of the unstained gel to be cut in the correct region. The excised region was further cut into 1cm x 1cm squares which were subsequently loaded into a Bio-Rad Model 422 Electro-Eluter and eluted over the next 5 hours at a constant current of 60mA. SDS was, of course, omitted The resulting eluate was collected, pooled and subsequently concentrated in a Centriprep 10 microconcentrator. The concentrate was then assessed for esterase activity and a protein estimation made utilising the Pierce Bradford assay described in Section 2.2.4. All fractions were subsequently pooled and used for the immunisation of guinea pigs.

Chapter 3

Properties of the surface antigens of *D. viviparus*

3.1 Introduction

The surface and secretions of nematodes form the primary interfaces between the parasites and their external environment. The acellular nematode cuticle was initially thought to be an inert and non-immunogenic matrix (Lumsden, 1975) but subsequent studies have led to a re-assessment of this view and the cuticle is now regarded as a dynamic and immunogenic structure.

The overall composition of nematode cuticle remains to be fully elucidated but accumulated information was reviewed by Bird (1980) who proposed that the main areas of the cuticle be designated topographically as the outer epicuticle, the cortex, the median and the basal layers. The delineation of these four zones, especially in terms of their functional or biochemical properties, is far from clear cut (Rudin,1990). The nematode epicuticle varies in thickness between species and life cycle stages and has been proposed as a very highly modified cell membrane (Bird, 1980). It has, however, been shown to contain lipid components which display properties distinct from those of the classic lipid bilayer and which possibly exist in functional domains (Kennedy *et al.*, 1987a; Proudfoot *et al.*, 1990).

Associated with the epicuticle, are proteins and glycoproteins which are accessible to antibody binding on the surface of live, intact worms and which can be radiolabelled and extracted in mild detergents (Politz and Philipp, 1992). In addition to the soluble surface-associated proteins, the cuticle consists of two other major classes of proteinaceous components: the collagens and the cuticulins, neither of which appear to be surface-exposed. The collagens are cysteine-rich and require 2-mercaptoethanol for their extraction indicating that they are cross-linked by disulphide bonds, a feature which differentiates them from most known mammalian collagens (Selkirk *et al.*, 1989b). The biochemical disparities between vertebrate and nematode collagen have now been re-iterated at the gene level (Cox, 1990). The cuticular collagens of different nematode species appear to share similarities in their molecular weight and modes of cross-linking and studies on the gene families of *C. elegans* and *H. contortus* suggest that they fall into similar families (Cox, 1990). In some nematode species, for example *T. canis*, there exists an amorphous surface coat which consists mostly of carbohydrate and which lies approximately 20nm outside the epicuticle (Maizels and Page, 1990; Blaxter *et al.*, 1992).

The nematode cuticle was first considered to be an active structure when it was demonstrated that, in some species, it contains haemoglobin and enzymes (reviewed by Lee, 1966). More recent studies have indicated that the surface antigens of several nematode species are stage-specific. This was first reported by Mackenzie, Preston and Ogilvie (1978) who demonstrated, by adsorption studies, that sera taken from animals

infected with *T. spiralis* or *N. brasiliensis* contained antibodies which were specific for the surface of individual stages. These results were later confirmed by surface radio-iodination studies in *T. spiralis* by Philipp, Parkhouse and Ogilvie (1980) and *N. brasiliensis* (Maizels, Meghji and Ogilvie, 1983). Stage-specificity of surface antigens has since been demonstrated in several species including *O. circumcincta* and *O. ostertagi* (Keith *et al.*, 1990), *O.volvulus* (Lustigman *et al.*, 1990; Titanji, Mbacham and Sakwe, 1990) and *H. contortus* (Cox, Shamansky and Boisvenue, 1989; Rhoads and Fetterer, 1990). These studies demonstrated that the nematode surface was antigenic and further experiments indicated that it may also be a site of effective immune attack and parasite killing. The majority of this work was performed *in vitro* and showed that, under the mediation of specific antibodies, cytotoxic leucoytes can bind to the cuticle and, in some cases, result in death of the parasite. For example, newborn and infective *T. spiralis* larvae were killed when exposed to a combination of specific antibody and a peritoneal cell population enriched for eosinophils (Mackenzie *et al.*, 1980, Mackenzie *et al.*, 1981). Similarly, *N. brasiliensis* infective larvae were killed in the presence of a mononuclear suspension of cells in combination with complement (Mackenzie *et al.*, 1980, Mackenzie *et al.*, 1981). Such phenomena are not exclusive to nematode species. For example, *S. mansoni* schistosomulae were observed to be damaged by combinations of eosinophils or macrophages with antibodies or complement (Butterworth *et al.*, 1975, Capron *et al.*, 1976). A monoclonal antibody which bound a surface epitope of *T. spiralis* newborn larvae was demonstrated to promote parasite killing *in vitro* and also conferred protection against re-infection in mice (Ortega-Pierres, MacKenzie and Parkhouse, 1984). The importance of surface-exposed antigens in *T. spiralis* infection was further emphasised when monoclonal antibodies were used to immunoaffinity purify a 48kDa antigen which induced a significant level of protection in mice and which was confined to the surface and secretory apparatus of the muscle larvae (Silberstein and Despommier, 1984). Protection against re-infection with *T. spiralis* has also been induced in mice using antigens which have been stripped from the surface of muscle larvae using CTAB (Grencis *et al.*, 1986).

These studies have emphasised the importance of surface antigens in the stimulation of potential protective immune responses and have encouraged the identification and characterisation of these antigens in several clinically-important helminths. An example of this is the 30kDa surface-associated antigen present in adult *Brugia* species which is released from the surface of the parasite *in vitro* (Devaney, 1988, Selkirk, 1989a). This molecule is highly conserved across the filarial species (Devaney,Betschart and Rudin, 1990) and elicits an immune response early in the course of an infection (Maizels *et al.*, 1989).

In an effort to counteract immune effector mechanisms, it is thought that nematode parasites have co-evolved with hosts and devised their own mechanisms to assist them in avoiding or evading attack. The fact that some nematodes have the potential to change their surface-exposed antigens from one stage to the next, may provide the parasite with a means of evading host effector mechanisms. Theoretically, as the host attempts to mount an effective response to the surface-exposed molecules of one stage, the parasite has moulted to the next stage which expresses a different repertoire of surface epitopes. Some species of nematode even undergo antigenic adaptations in their surface without the intervention of a moult and this has been observed in *T. spiralis* (Mackenzie *et al.*, 1980), *N. brasiliensis* (Maizels, Meghji and Ogilvie, 1983), *T. canis* (Maizels *et al.*, 1987) and *T. cati* (Kennedy *et al.*, 1987b).

In several nematode species it has been noted that immunodominant epitopes included in detergent-soluble surface extracts have not been detected on the surface of live, intact parasites. Such cryptic epitopes may be hidden from potential host-protective immune responses by being buried in a sub-surface location within the cuticle. This phenomenon was epitomised when only two out of eight monoclonal antibodies, which precipitated radiolabelled surface extracts of *T. canis*, were observed to bind to the surface of intact parasites (Maizels *et al.*, 1987). Similar findings have also been obtained in studies with *T. spiralis* (Parkhouse, Phillip and Ogilvie, 1981; Ortega-Pierres *et al.*, 1984; Ortega-Pierres, Clarke and Parkhouse, 1986) and *T. cati* (Kennedy *et al.*, 1987b). The host may only be exposed to such occult antigens when they are shed or moulted from the parasite or when the parasite dies.

The nematode cuticle may also have the capacity for metabolic turnover with release of its surface-associated antigens. For example, infective larvae of *A. caninum* (Vetter and Klaver-Wesseling, 1978) and second stage larvae of *T. canis* (Smith *et al.*, 1981) were shown to shed bound antibody when maintained under mammalian tissue culture conditions. This phenomenon was further demonstrated for *T. canis* by Maizels, de Savigny and Ogilvie (1984) who observed that 25% of radio-iodinated surface antigens were lost from larvae within 1 hour of culture at 37°C. Surface shedding has since been demonstrated in radiolabelling studies with *D. immitis* (Ibrahim *et al.*, 1989) and *A. viteae* (Apfel and Meyer, 1990). Furthermore, *T. canis* parasites, to which eosinophils were adherent, were observed to slough their outer surface coat together with the associated effector cells (Rockey *et al.*, 1983). In addition to shedding surface-bound antibody and cells, the release of parasite antigens may also act to divert host effector mechanisms away from the surface of live parasites and, in the released products, there may exist enzymes which have the capacity to modify local immune responses.

In the investigations described in this Chapter, the indirect fluorescent antibody technique (IFAT) was used to examine some basic surface properties of *D. viviparus* .

This test enables the detection of epitopes regardless of their biochemical nature and will, therefore, potentially include carbohydrates and lipidic components in the analysis. This is in contrast to radiolabelling and immunoprecipitation techniques which will only detect responses directed against protein-containing molecules. Another disadvantage of surface radio-iodination is that sub-surface molecules may also be labelled and cuticular components, such as collagens, may be included in extracts, especially if they are isolated under harsh conditions. This problem was encountered when the surfaces of adult *H. contortus* (Cox, Shamansky and Boisvenue, 1989, Rhoads and Fetterer, 1990), *D. immitis* (Scott, Ibrahim and Tamashiro, 1990) and *D. viteae* (Betschart and Jenkins, 1987) were radiolabelled.

In the studies described here, *D. viviparus* was examined for stage-specificity in its surface-exposed antigens by the IFAT using sera from animals exposed to different stages of the parasite. Stage-specificity was further examined by adsorbing sera with different developmental stages of *D. viviparus* and then examining the specificity of the antibodies that remained after absorption. The outer surfaces of adult and third larval stage parasites were investigated for shedding of surface-bound antibody under mammalian tissue culture conditions. The possible location of L3 exsheathment was also examined, as this may have implications regarding the antigens to which the host is exposed during the course of *D. viviparus* infection.

3.2 Results

3.2.1 Stage-specificity of antigens exposed on the surface of *D. viviparus* eggs and L1

The surface of different stages of *D. viviparus* were examined for specificity in their surface-exposed antigens. This was performed by using antisera, in which parasite development was restricted to specific stages, and examining antibody binding by the IFAT. The types of infections and immunisations, and the stages of parasite which developed in each, are summarised in Tables 3.1 and 3.2.

Eggs and L1 were produced as described in Section 2.1.5 and incubated with the test sera in the IFAT as outlined in Section 2.5.6. The labelled parasites were viewed under UV light and their fluorescence quantified by photon counting. The results are presented as the mean and standard deviation of 20 readings per sample and are shown in Figures 3.1 and 3.2 and a photomicrograph of typical positive fluorescence, obtained with serum from calves infected with normal L3, is shown in Figure 3.3.

There was a relatively high degree of non-specific binding to the egg surface by the normal bovine serum, but the level of antibody binding in positive samples could be clearly delineated from the negative controls. Only in bovine serum samples where full patency was attained, i.e. normal L3 infection, was there any positive recognition of the egg surface. The normal guinea pig and rabbit sera did not display such high background levels of antibody binding to the egg surface and, interestingly, the only test serum that proved positive from these host species was that raised against adult ES products. All other samples, in which patency was not attained, were negative for the surface of the egg.

All sera, except those from normal L3-infected calves and adult homogenate-immunised guinea pigs, proved to be negative when used to probe the L1 surface. The anti-adult homogenate serum presumably contained antibodies to L1 present within the uterus of homogenised adult female worms. There was no recognition of the L1 surface by animals immunised with adult ES materials. When L1 parasites, obtained from the faeces of infected calves were examined, the results were similar to those obtained with newly-hatched larvae, suggesting that the latter expressed antigens representative of fully-developed L1 stages.

In summary, negative responses against the surface of eggs and L1 were obtained with all antisera where development terminated at the L4 or L5 stages. This suggested

that the surface-exposed antigens of the L1 and egg are antigenically different from those of the L3, L4 and L5 stages.

Abbreviation	Species raised in :	Raised against:	Route:
FBS	Foetal bovine	NA	NA
NBS	normal bovine	NA	NA
NBCS	neonatal bovine	NA	NA
BNL3	bovine	normal L3	oral
B40	bovine	400Gy irrad. L3	oral
B100	bovine	1000Gy irrad. L3	iv
NGPS	guinea pig	normal control	NA
GNL3	guinea pig	normal L3	oral
G40	guinea pig	400Gy irrad. L3	oral
G100	guinea pig	1000Gy irrad. L3	ip
GAH	guinea pig	adult homogenate*	sc
GL3S	guinea pig	L3 sonicate*	sc
GL1	guinea pig	live L1	ip
GAD	guinea pig	live male adult	ip
GES	guinea pig	ES products*	sc
GENZ	guinea pig	AChEenriched ES*	sc
mAb	mouse	L3 sheath antigen	monoclonal
NRS	rabbit	normal control	NA
RES	rabbit	ES products*	sc

Table 3.1 : Sera used for investigations of the surface properties of *D. viviparus*.

Note
iv = intravenous
ip = intraperitoneal
sc = subcutaneous
* = immunised in the context of Freund's Complete Adjuvant

NA = not applicable

Serum type	Stage of parasite exposed to L3	L4/5	adult	egg/L1	ES
NBS/NBCS	-	-	-	-	-
FBS	-	-	-	-	-
BNL3	+	+	+	+	+
B40	+	+	-[1]	-	-
B100	+	-	-	-	-
NGPS	-	-	-	-	-
GNL3	+	+	-	-	-
G40	+	+	-	-	-
G100	+	-	-	-	-
GAH	-	-	+	+	-
GL3S	+	-	-	-	-
GL1	-	-	-	+[2]	-
GAD	-	-	+[3]	-	-
GES	-	-	-	-	+
GENZ[4]	-	-	-	-	-
mAb[5]	+	-	-	-	-
NRS	-	-	-	-	-
RES	-	-	-	-	+

Table 3.2
Summary of the parasite stages that animals are exposed to during the course of infection or after immunisation.

1 Animals exposed to 400Gy larvae may harbour a few sterile adult worms
2 Animals infected with L1 in the absence of eggs
3 Animals immunised with male adult worms only
4 Animals immunised with an AChE-enriched fraction from adult ES
5 monoclonal antibody to specific L3 sheath antigen

Figure 3.1

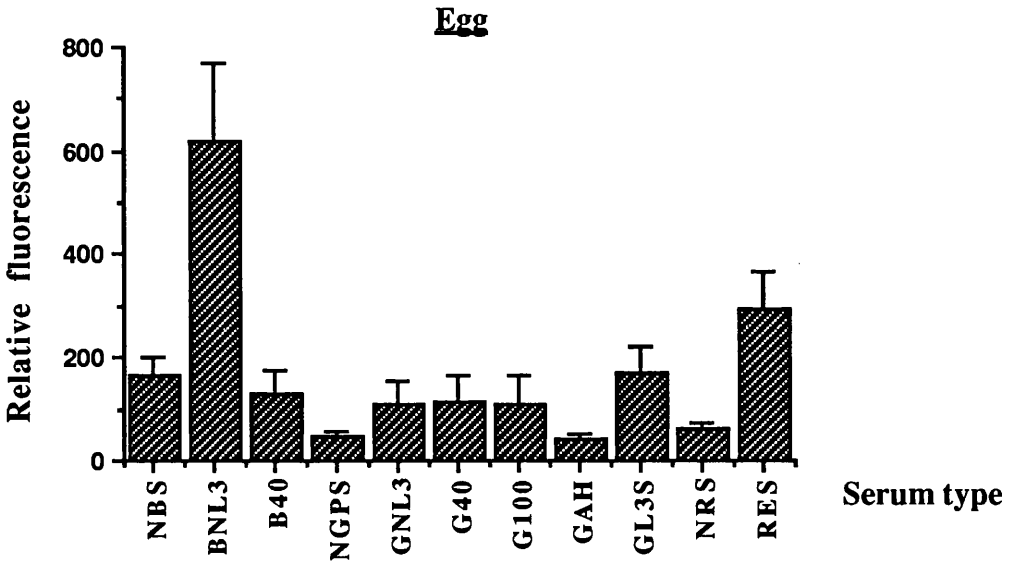
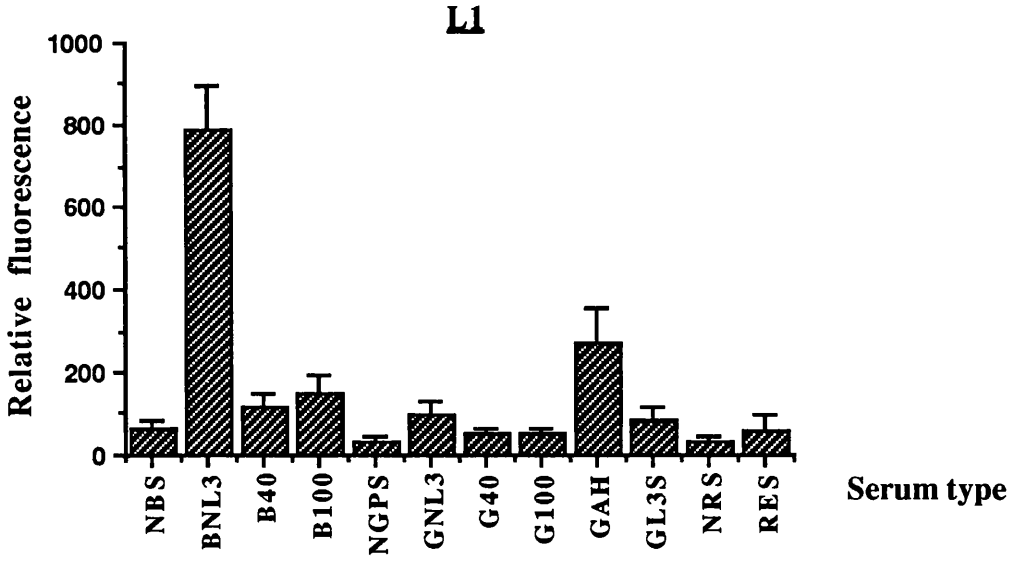


Figure 3.2



Figures 3.1 and 3.2

Stage-specificity of the surface-exposed antigens of *D. viviparus*.

Antibody binding to the surface of eggs (Figure 3.1) and first stage larvae (Figure 3.2) was examined by the IFAT (Section 2.5.6) using the bovine and guinea pig sera detailed in Tables 3.1 and 3.2. The calf sera were sampled on Day 21 of tertiary infection and the guinea pig and rabbit sera on Day 14 of secondary immunisation or infection.

Figure 3.3

Photomicrograph demonstrating typical positive fluorescence obtained with eggs and first stage larvae of *D. viviparus*.

The parasites were incubated with serum pooled from three calves infected on three occasions with 5,000 normal L3. The bovine antibody was detected using rabbit anti-bovine IgG (whole molecule) conjugated to FITC and the sampled examined under UV light.

3.2.2 Stage-specificity of the antigens exposed on the surface of third stage larvae of *D. viviparus*

Approximately 3,000 sheathed and Milton-exsheathed third stage larvae were probed by the IFAT with the above panel of antisera. The results are shown in Figures 3.4 and 3.5.

Pre-infection sera did not exhibit reactivity against the surface of the L3 sheath (Figure 3.4), but the surface of exsheathed third stage larvae showed an elevated degree of immunoglobulin binding by normal bovine sera (Figure 3.5).

All sera derived from animals exposed to third stage larvae manifested binding to both the L3 cuticle (i.e. exsheathed surface) and the L3 sheath. Following infection or vaccination, calves appeared to recognise the L3 sheath surface more strongly than they recognised the surface of the L3 cuticle. Animals not exposed to third stage larvae, did not recognise the L3 sheath or cuticle surface and the results indicated that antigens present in somatic and secretory extracts of the adult stages were not exposed on the surface of exsheathed or sheathed L3.

3.2.3 Stage-specificity of antigens exposed on the surface of fourth/fifth stage larvae of *D. viviparus*

Approximately 3,000 fourth and fifth stage larvae, obtained from calf lungs on Day 14 of infection, were probed for surface recognition by various sera using the IFAT. The L4 and L5 stages were difficult to discriminate so the results presented here were from a mixed population and are shown in Figure 3.6.

The pre-infection sera displayed no greater reactivity against the surface of these stages than the buffer controls but the standard deviations in each sample were relatively large due to the presence of strong internal autofluorescence. The wide variation observed in the degree of antibody binding to individual parasites may have been associated with the fact that two developmental stages were present. Only calves in which development proceeded beyond the third stage recognised the L4/5 surface. The results further suggested the presence of stage-specific surface antigens in *D. viviparus*. Guinea pigs that were immunised with live adult parasites also recognised the surface of the L4/5 stages. Presumably, the L5 present in this population represented young adults which expressed antigens similar to those present on mature stages.

Figure 3.4

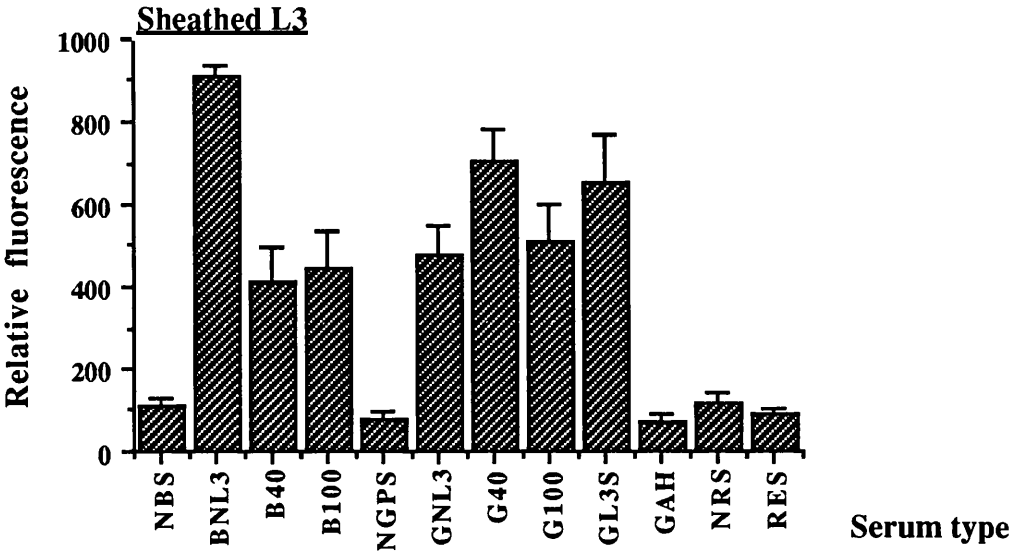
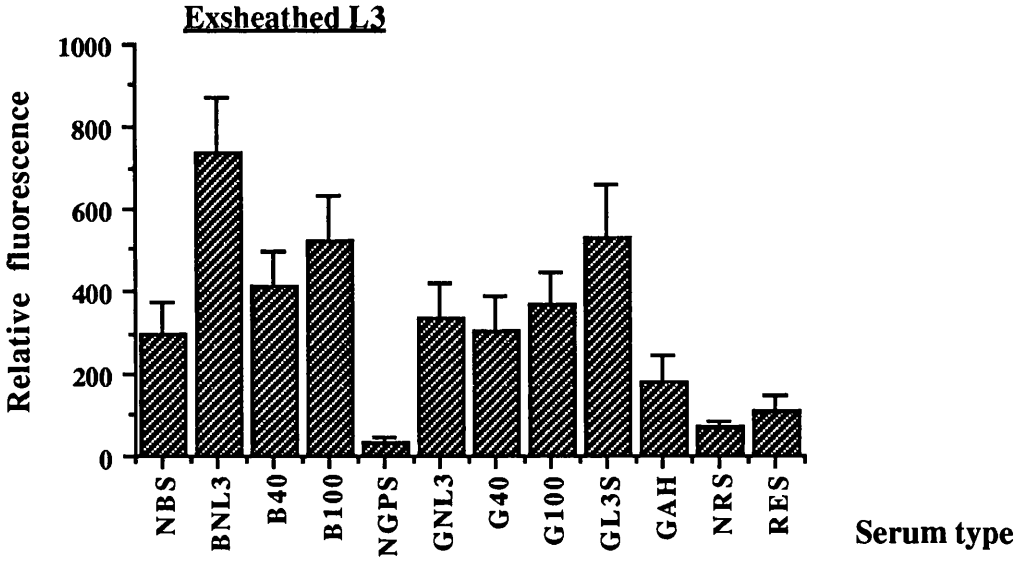


Figure 3.5



Figures 3.4 and 3.5

Stage-specificity of the surface-exposed antigens of *D. viviparus*.

Antibody binding to the surface of sheathed (Figure 3.4) and exsheathed (Figure 3.5) third stage larvae examined by the IFAT. The antisera were raised as outlined in Tables 3.1 and 3.2. The calf sera were sampled on Day 21 of tertiary infection and the guinea pig and rabbit sera on Day 14 of secondary immunisation or infection.

Figure 3.6

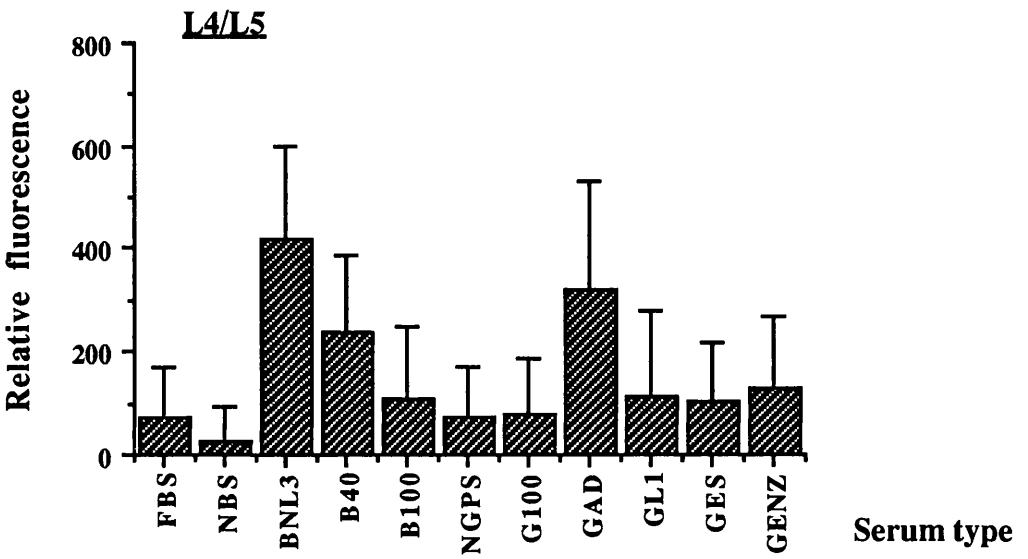


Figure 3.6

Stage-specificity of the surface-exposed antigens of *D. viviparus*.

Antibody binding to the surface of fourth and fifth stage larvae was examined by the IFAT. The antisera were raised as outlined in Tables 3.1 and 3.2. The normal L3 infection serum was pooled from three calves and was taken on Day 52 of primary infection, while the 400Gy and 1000Gy sera were sampled on Day 21 of secondary infection. All antisera from guinea pigs were sampled on Day 14 of secondary immunisation or infection.

3.2.4 Stage-specificity of antigens exposed on the surface of adult *D. viviparus*

Male or female adults, obtained from the lungs of infected calves, were probed for surface recognition by the same panel of sera. The results are presented in Figure 3.7.

These results reinforced those observed for the other stages of *D. viviparus* as it was only calves exposed to normal L3 infection and guinea pigs immunised with somatic extract of adult worms (not shown) which recognised the adult surface. In calves, the recognition measured at Day 52 of primary infection was confined to the IgM isotype, but by the time the second sample was taken (Day 21 of secondary infection), there had been a class switch to the IgG_1 isotype. Female and male adult worms did not differ in the pattern or level of antibody binding (not shown). Interestingly, the rabbit immunised with adult ES material did not recognise the adult surface.

3.2.5 Stage-specificity of *D. viviparus* demonstrated using antisera raised specifically against the L1 and adult stages

In the previous experiments it was impossible to determine whether the surfaces of L1 and adult parasites were different, because none of the sera used discriminated between the two stages. In order to examine this further, antisera were raised in Dunkin-Hartley guinea pigs against both living first stage and adult male parasites as outlined in Section 2.4.3. Male adult worms were used to avoid eggs and L1, present in the uteri of female parasites, being included. The responses these animals mounted to the L1 and adult surfaces were measured by IFAT and the results are shown in Figures 3.8a and b.

The levels of antibody to the adult and L1 parasite surfaces were relatively low in all sera. This was probably a result of the low antigen doses which were given in the absence of adjuvant. Immunisation with the adult parasites did not increase adult surface-specific binding above that observed with pre-infection serum. Animals immunised with L1 parasites recognised the surface of the homologous stage more strongly than those which received adult parasites.

To further investigate stage-specificity between these two stages, rat antisera, raised against live adult and L1 stages (Section 2.4.2), were immunoprecipitated with ^{125}I-labelled adult surface material (kindly provided by Dr C. Britton, Department of Veterinary Parasitology, University of Glasgow). The precipitates were analysed by SDS-PAGE under reducing conditions (Figure 3.9). There was one major component, of approximately 30kDa, which was recognised by all animals, including an uninfected rat. Only Sprague-Dawley rats which were immunised with adult parasites precipitated another adult surface component which had an M_r of approximately 45kDa. These two molecules appeared to be major components of radio-labelled adult surface material. The Wistar anti-adult serum and the anti-L1 sera from both strains did not precipitate the higher M_r component.

Figure 3.7

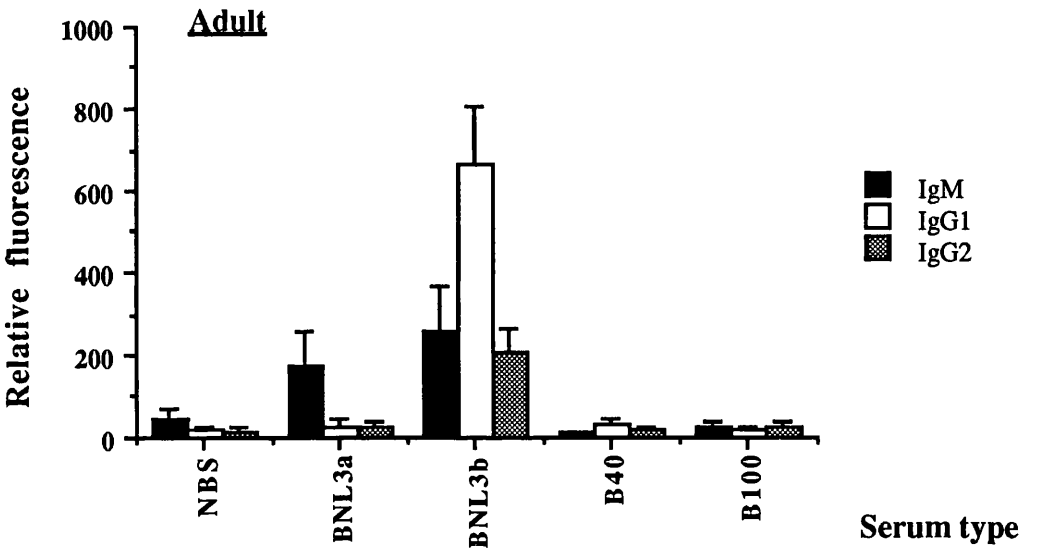


Figure 3.7

Stage-specificity of the surface-exposed antigens of *D. viviparus*.

Antibody binding to the surface of adult parasites was examined by the IFAT. The sera were raised as detailed in Tables 3.1 and 3.2. BNL3a was taken from a calf on Day 52 of primary infection and BNL3b sampled on Day 21 of secondary infection. The 400Gy and 1000Gy sera were sampled on Day 21 of the fifth infection.

Figure 3.8a

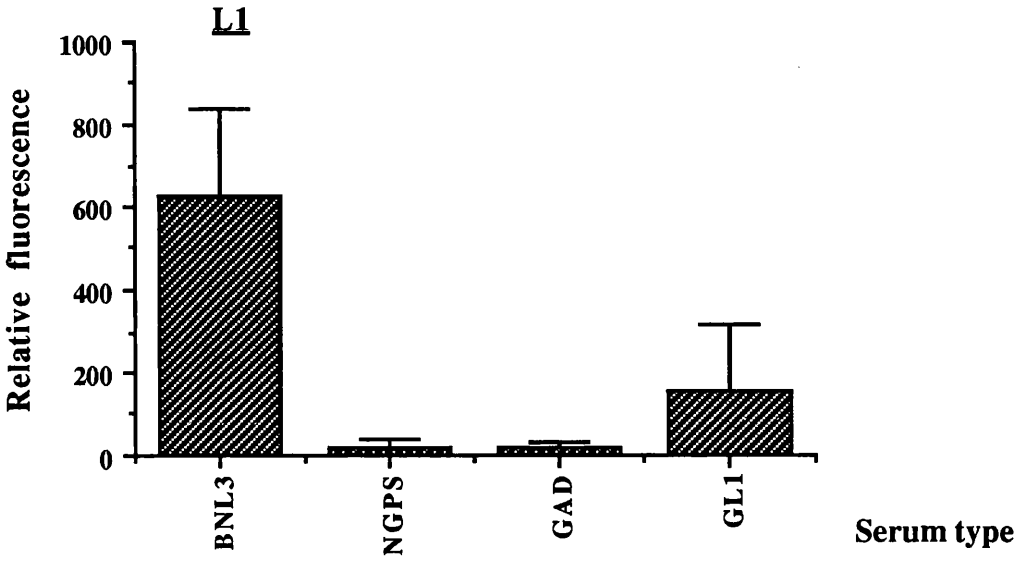
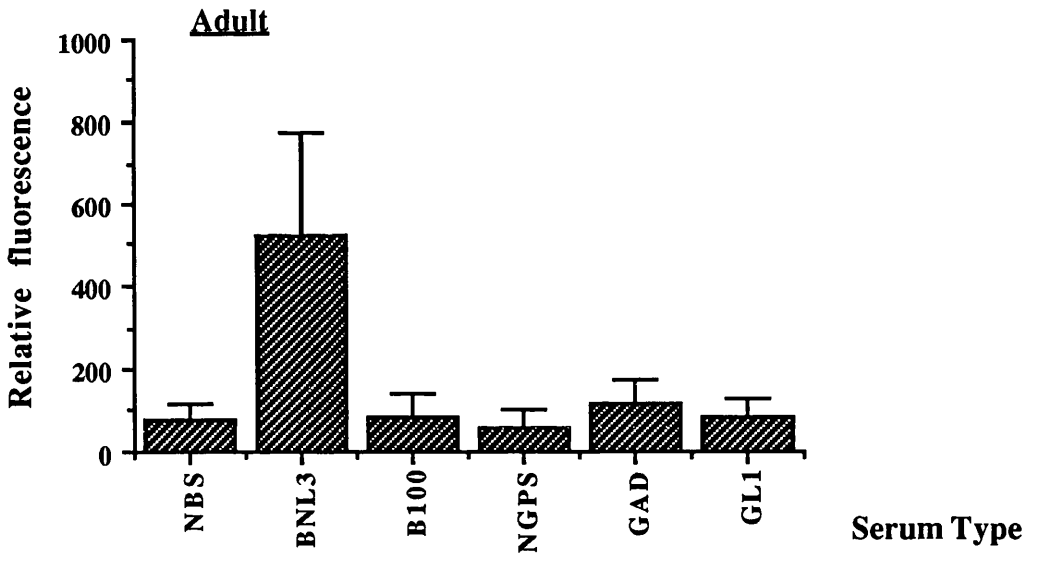


Figure 3.8b



Figures 3.8a and b

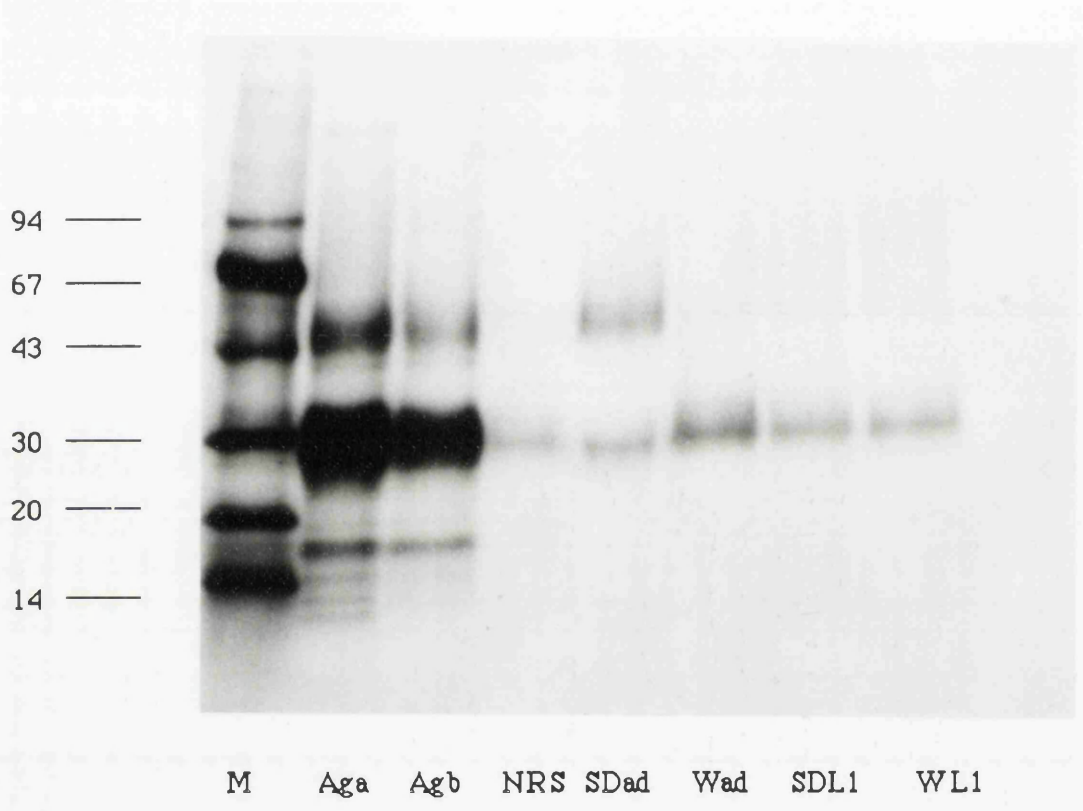
Stage-specificity of the surface-exposed antigens of *D. viviparus*.

Antisera raised against L1 (GL1) and adult male (GAD) stages were examined for surface recognition of the L1 (Figure 3.8a) and adult (Figure 3.8b) parasites using the IFAT. Bovine serum, sampled on Day 21 of tertiary infection (BNL3) was used as a positive control and normal bovine (NBS) and normal guinea pig (NGPS) sera were used as negative controls.

Figure 3.9

Autoradiograph of ^{125}I-labelled adult surface material immunoprecipitated with sera from rats infected intraperitoneally with either adult male (ad) or L1 (L1) parasites. The rats were bled on Day 14 following second immunisation. Aga was the original labelled material and Agb was antigen pre-cleared with Staph. A and was used for the immunoprecipitation. The serum used was pooled from two rats of Sprague-Dawley (SD) or Wistar (W) strains and was sampled on Day 14 of secondary immunisation. . The precipitates were analysed under reducing conditions on a 5-25% SDS-polyacrylamide gel.

Wad and WL1 are sera taken from Wistar rats which received live adult and L1 parasites respectively. SDad and SDL1 are sera taken from Sprague-Dawley rats which received live adult and L1 parasites respectively.


94
67
43
30
20
14
M
Aga
Agb
NRS
SDad
Wad
SDL1
WL1

3.2.6 Serum adsorption with L1, L3 and adult stages of *D.viviparus*

To further test whether the L3, L1 and adult surfaces were antigenically different, serum was pre-adsorbed with 70,000 L1, 100,000 L3 or 50 adult male worms before being used in the IFAT against the surface of these stages (see Section 2.5.7). Only the results obtained from the L1 and L3 stages are shown (Figure 3.10) as adsorption with adult worms did not reduce antibody binding to any stage. This may have been because too few adults were used in adsorption and parasite supply did not permit optimisation of the conditions.

The L3 parasites adsorbed out most of the reactivity against the surface of the homologous, but not the L1, stage. Similarly, the L1 parasites adsorbed out reactivity against the L1, but not the L3, surface. The results therefore confirmed the presence of stage-specific antigens between the L1 and L3 surfaces.

3.2.7 Serum adsorption with sheathed and exsheathed L3 stages of *D.viviparus*

In an attempt to compare stage-specificity between the antigens expressed on the surface of sheathed and exsheathed L3, $1x10^5$ of each were used to adsorb immune bovine serum. The results are shown in Figure 3.11 and indicated a degree of cross-reactivity between the sheath and cuticle of the L3 stage. The exsheathed L3 surface adsorbed most reactivity against the homologous surface and that of the sheath. Although the serum adsorbed with sheathed L3 totally ablated recognition of the sheath, it only partially reduced binding to the exsheathed L3 surface.

3.2.8 Surface shedding of bound antibody from adult *D. viviparus*

As previous experiments had demonstrated the ability of some parasites to shed their surface bound antibody (Vetter and Klaver-Wesseling, 1978; Smith *et al.*, 1981), the surfaces of adult and third stage *D. viviparus* were examined for this phenomenon. These stages of the parasite were incubated with post-infection and foetal bovine serum under various conditions (Section 2.5.8). The parasites were viewed under UV light immediately after incubation and after a further 24 hours of incubation. The results of this experiment are detailed in Figures 3.12a to c.

At both time points, under all incubation regimes, the FBS exhibited no binding to the surface of either stage of parasite. At the first time point, antibody from infected calves bound to the surface of adult parasites incubated at both 37°C and at 2°C. When the parasites were maintained at 37°C for 24 hours, in the absence of metabolic inhibitor, they lost 84% of their bound antibody and the fluorescence that remained appeared to occur in aggregates. The reduction in fluorescence was not due to a reduction in FITC reactivity as the adults maintained at 2°C, in the presence of metabolic inhibitor, maintained more than 91% of their original fluorescence.

Neither sheathed nor exsheathed L3 lost fluorescence between time zero and 24 hours, regardless of the conditions under which they were maintained. The relative fluorescence increased in some samples and was probably a result of slight differences in the arbitrary relative fluorescence settings taken from one day to the next.

Figure 3.10

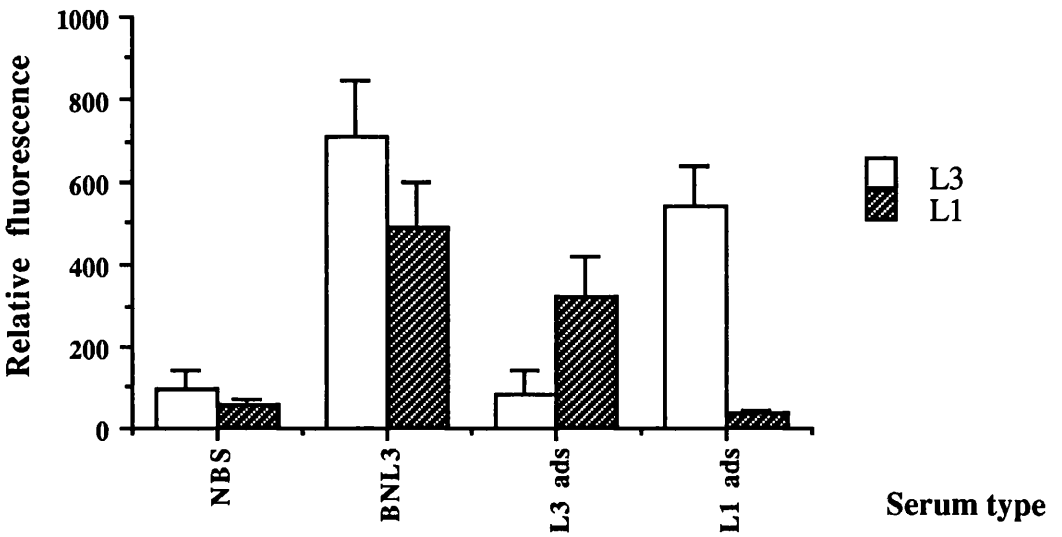


Figure 3.10

Stage-specificity of the surface-exposed antigens of *D. viviparus* L1 and L3.

Serum from calves infected on several occasions with normal L3 (BNL3) was adsorbed with sheathed L3 and L1 stages and the sera then examined for recognition of the surface of the two stages by the IFAT. "L3 ads" denotes normal L3 infection serum pre-adsorbed by the L3 stage and "L1 ads" normal L3 infection serum pre-adsorbed with L1 stage.

Figure 3.11

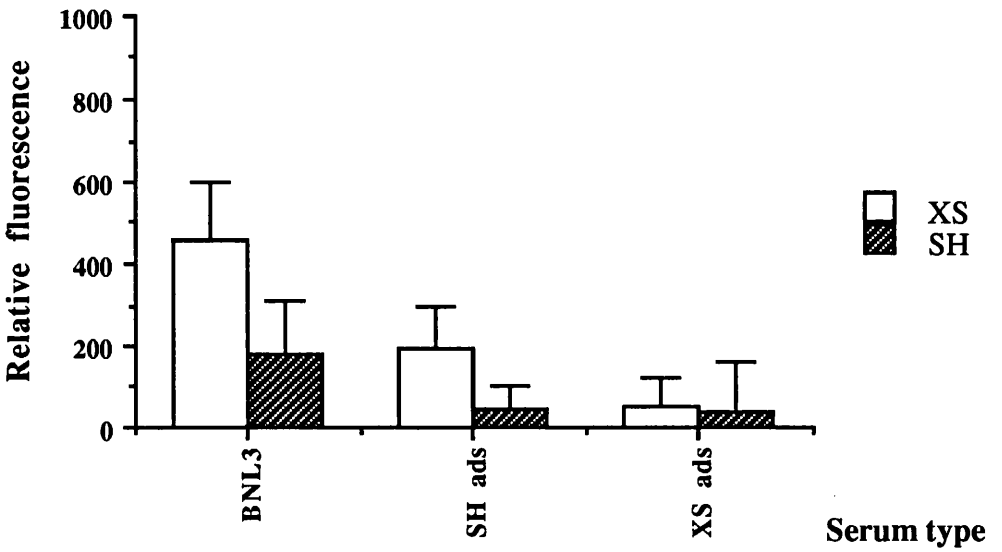


Figure 3.11

Cross-reactivity of the surface-exposed antigens of the *D. viviparus* L3 sheath and cuticle.

Serum from calves infected several times with normal L3 (BNL3) was adsorbed with sheathed and exsheathed L3 and the sera were then examined for recognition of the surface of the two stages by the IFAT. "SH ads" denotes serum pre-adsorbed by the sheathed L3 stage and "XS ads" serum pre-adsorbed with exsheathed L3.

Figure 3.12a

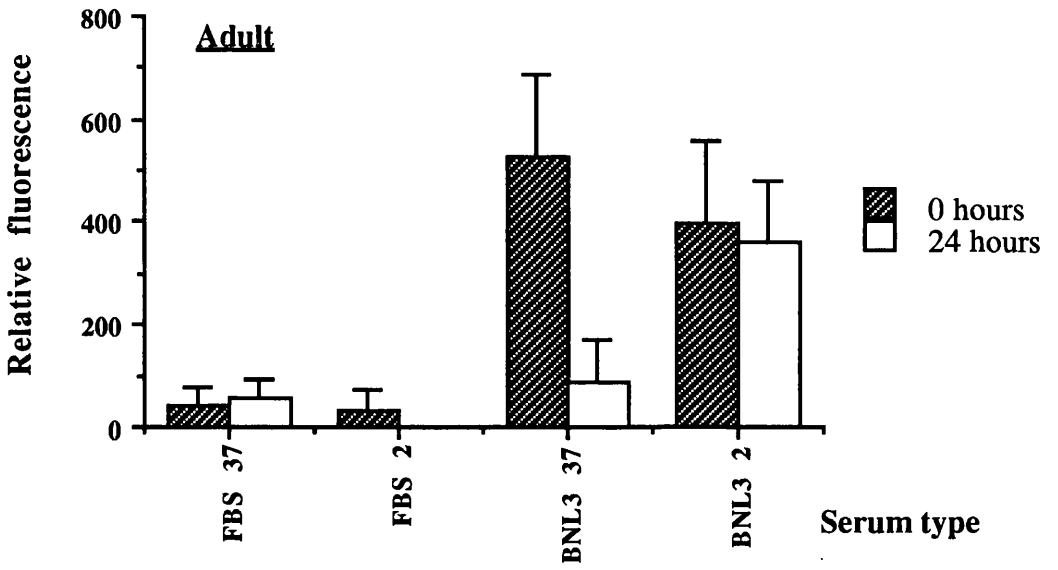


Figure 3.12a

The shedding of FITC-labelled antibody from the surface of adult *D. viviparus*.

Adult *D. viviparus* parasites were incubated with FBS or pooled sera from calves that had been exposed to multiple infections of normal L3 in PBS, at 37°C, (FBS 37 and BNL3 37) or incubated in 0.1% NaN3/PBS, at 2°C, (FBS 2 and BNL3 2) as detailed in Section 2.5.8. Fluorescence was quantified by photon counting immediately after labelling and after a further 24 hours.

Figure 3.12b

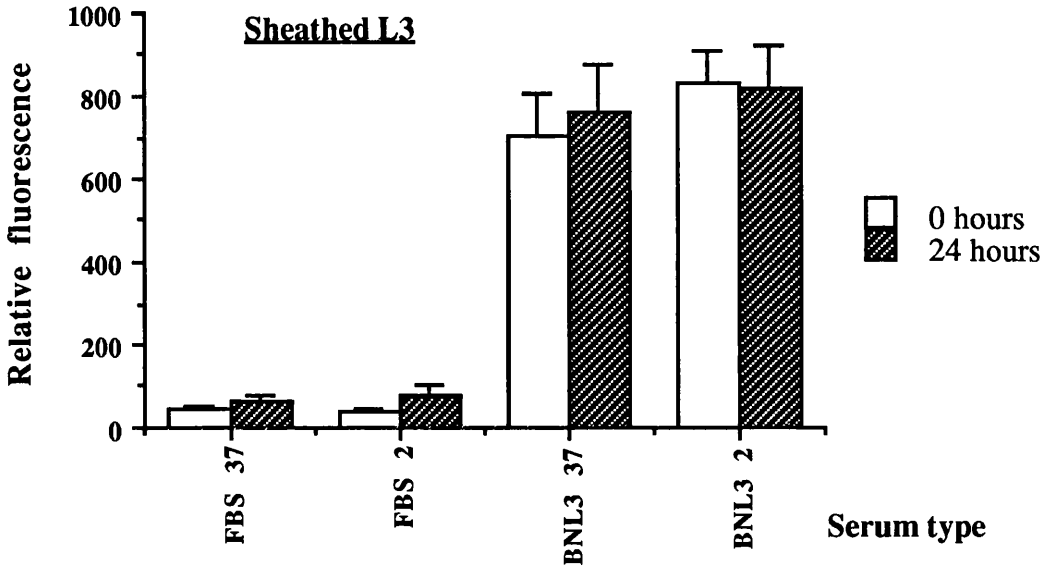
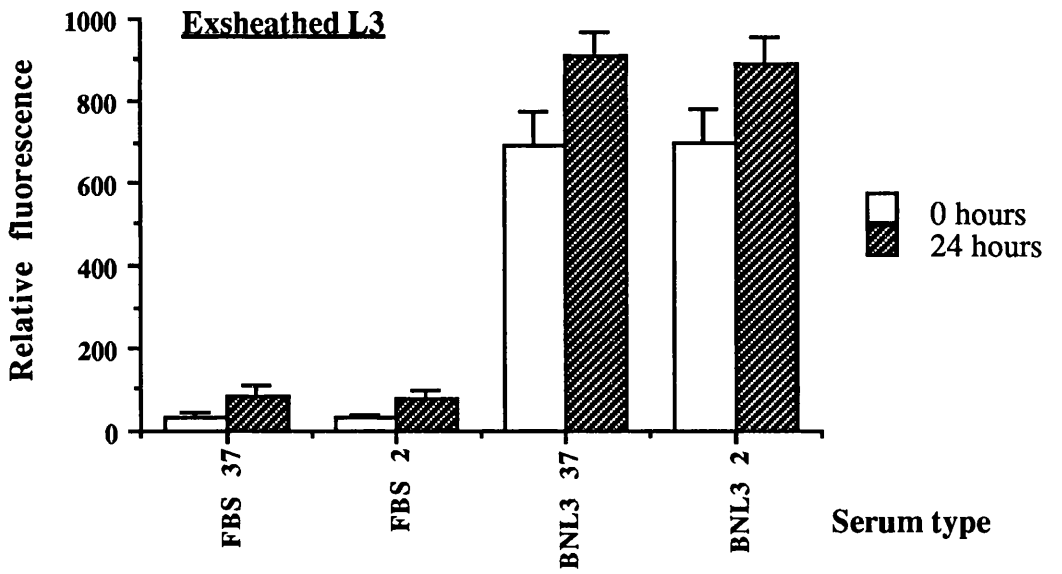


Figure 3.12c



Figures 3.12 a and b

The stability of FITC-labelled antibody bound to the surface of sheathed and exsheathed *D. viviparus* L3.

Sheathed (Figure 3.12b) and exsheathed (Figure 3.12c) *D. viviparus* L3 were maintained under physiological conditions (FBS 37 and BNL3 37) or at 2°C with 0.1% NaN3/PBS (FBS 2 and BNL3 2) as detailed in Section 2.5.8 and their relative fluorescence quantified immediately and after a further 24 hours.

3.2.9 Heterogeneity of surface antigens within two populations of *D. viviparus* larvae

In order to investigate whether the surface-exposed antigens within a population of larvae were heterogeneous, sera from three calves (B7, B8 and B9), infected on multiple occasions with normal L3, were used to probe the surface of two batches of *D. viviparus* L3 (A and B). Population A had been obtained from Pitman Moore 1 year prior to the experiment and had been maintained in PBS at 4°C during this interval. Sheathed and Milton-exsheathed L3 were examined and the fluorescence of 50 individual L3 per population quantified. The results are presented as the frequency of observation of the measurements in each population and are shown in Figures 3.13a to 3.13f.

Although the individuals differed in their responses quantitatively, the calves displayed a normal distribution of antibody binding against both populations of sheathed and exsheathed larvae. When the profiles of recognition of the two larval populations were compared for calves B7 and B9, the responses to population A mirrored those against population B. However, calf B8 appeared to respond differently to the two populations in that it recognised the sheath antigens in the older larval population (A) more markedly. Nevertheless, the recognition pattern still had a normal distribution.

3.2.10 Exsheathment of third stage *D. viviparus* larvae.

From the experiments outlined above, it was observed that infected calves displayed a stronger response to the surface of sheathed L3 than that against the surface of exsheathed L3. This was surprising in view of the previous assumption that this stage exsheathed in the rumen. The recognition of the sheath antigens may have been due to the presence of epitopes cross-reactive with those of later stages. Alternatively, it may be that the L3 invades the intestinal mucosa while retaining the sheath which is then deposited in host tissue and presented to the immune system. In light of the fact that third stage larvae can induce protective immunity in calves and guinea pigs (Canto, 1990), it was thought to be of relevance to investigate further the possible site of L3 exsheathment.

Various host sites were simulated so that the L3 were incubated in conditions equivalent to either the rumen, abomasum or small intestine with subsequent exposure to foetal calf serum (Section 2.7.1). Only when exposed sequentially to all of these conditions, did L3 exsheath to any significant degree and it was noted that, following exsheathment, larvae converted their motility pattern from tight coiling to sinusoidal motion. Thus it appeared that L3 require to be exposed to pepsin and HCl, followed by bile and serum, before they exsheath and continue their development *in vitro*.

To investigate if the L3 could penetrate the intestinal mucosa with their sheaths intact, $5x10^4$ L3 were fluoresceinated directly and $1x10^4$ of these L3 set up with bovine mucosa

and sections taken for subsequent examination. A summary of the results is presented in Table 3.3.

Within one hour of incubation, most larvae obtained from the upper side of the mucosa were in close association with the superficial epithelium and few remained in the PBS alone. Sheathed L3 were found below the mucosa which suggested that full penetration may have occurred. The possibility that these larvae passed through a disrupted piece of intestine must also be considered. The fact that these larvae were not observed until the last incubation suggested that they might have migrated there by active penetration.

Only one larva was observed in the 40 sections taken and this was seen in the connective tissue of a section taken after 60 minutes of incubation. This larva had retained its fluoresceination. These results suggested that the L3 observed in the tissue section had not lost its sheath, as fluoresceinated larvae, which were subsequently exsheathed, were observed to lose their fluorescence (not shown). Also, there may have been more larvae in the process of penetration, as these sections constituted a very small proportion of total intestine used and as the host tissue autofluoresced to a high degree, visualisation of fluoresceinated larvae was difficult.

Although rather tenuous, these results supported the findings of the stage-specificity work which implied that L3 may penetrate the host intestinal mucosa with their sheaths intact.

Figure 3.13a

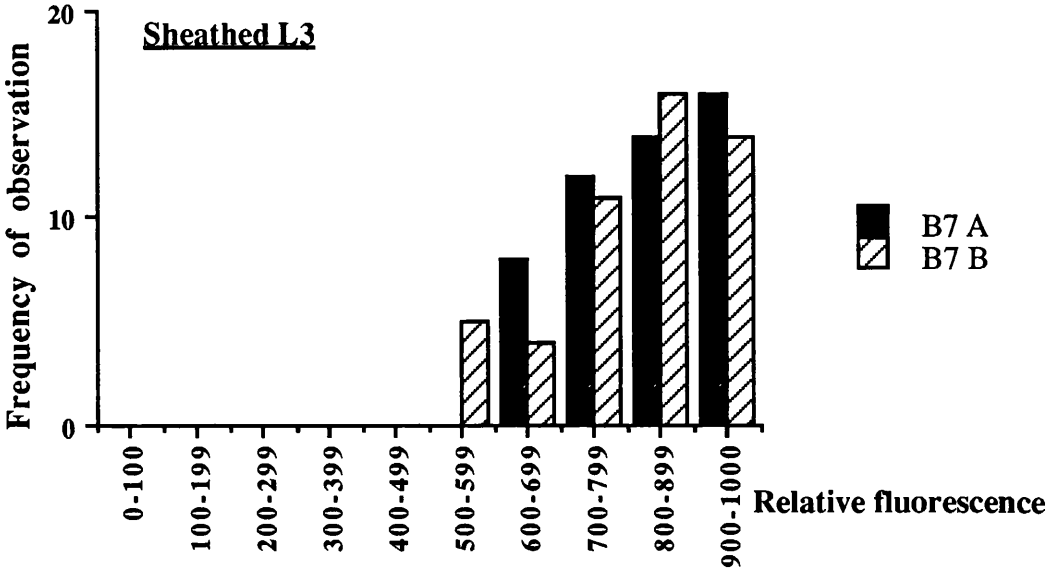
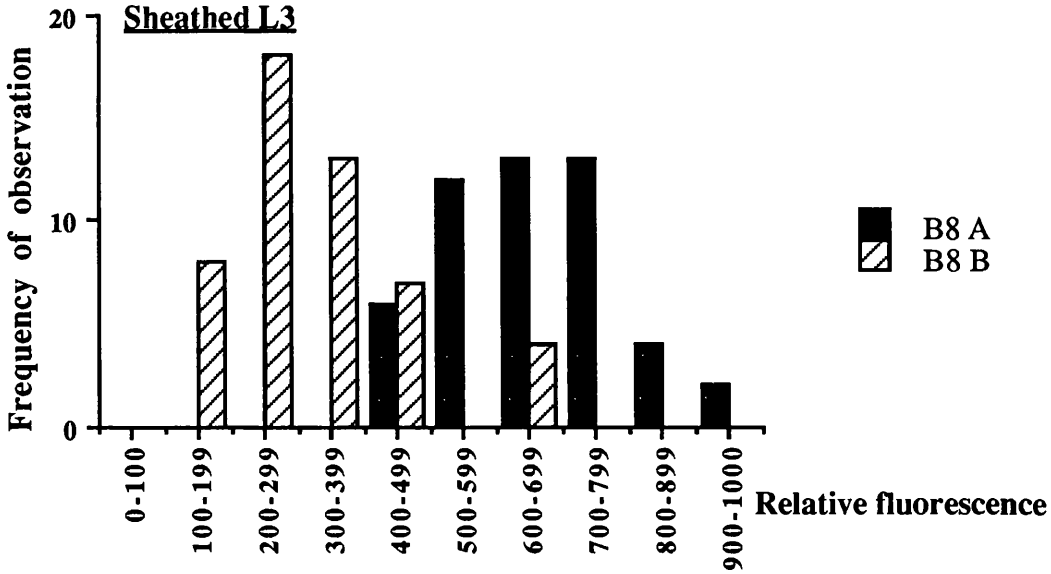


Figure 3.13b



Figures 3.13a and b

Lack of heterogeneity in the surface epitopes presented by *D. viviparus* sheathed L3.

Approximately 3,000 sheathed L3 from two batches, A and B, were incubated with individual hyperimmune calf sera, B7 and B8 (sampled on Day 21 of fifth infection). These calves had received 44,000 normal L3 by this stage. Following the IFAT, 50 L3 per sample were measured and the frequency of observation of the relative fluorescence of these plotted for each population. Antibody from calf B7 bound very strongly and a proportion of the samples were above the maximum of 1000 on arbitrary measurement scale, these were included in the 900-1000 group in Figure 3.13a.

Figure 3.13c

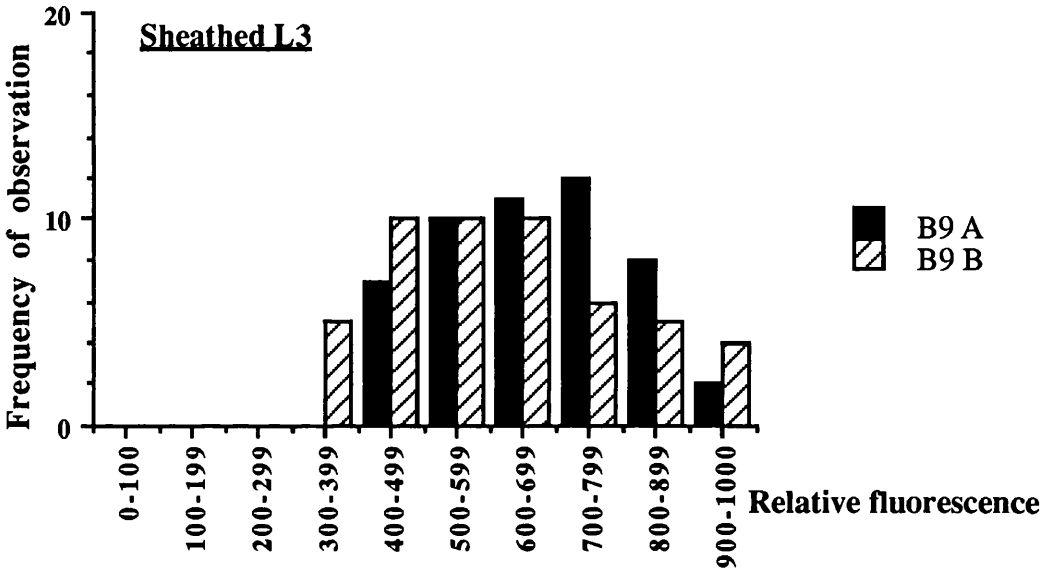


Figure 3.13c

Lack of heterogeneity in the surface epitopes presented by *D. viviparus* sheathed L3.

Approximately 3,000 sheathed L3 from two batches, A and B, were incubated with individual hyperimmune calf serum, B9, sampled at the same time as the calf in Figure 3.13a. Following the IFAT, 50 L3 per sample were measured and the frequency of observation of the relative fluorescence of these plotted for each population.

Figure 3.13d

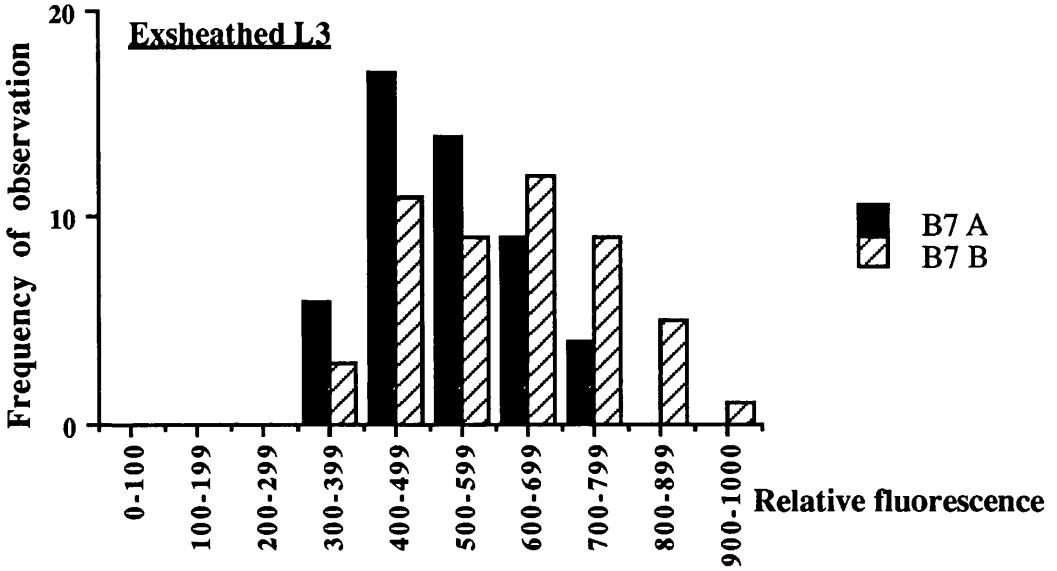
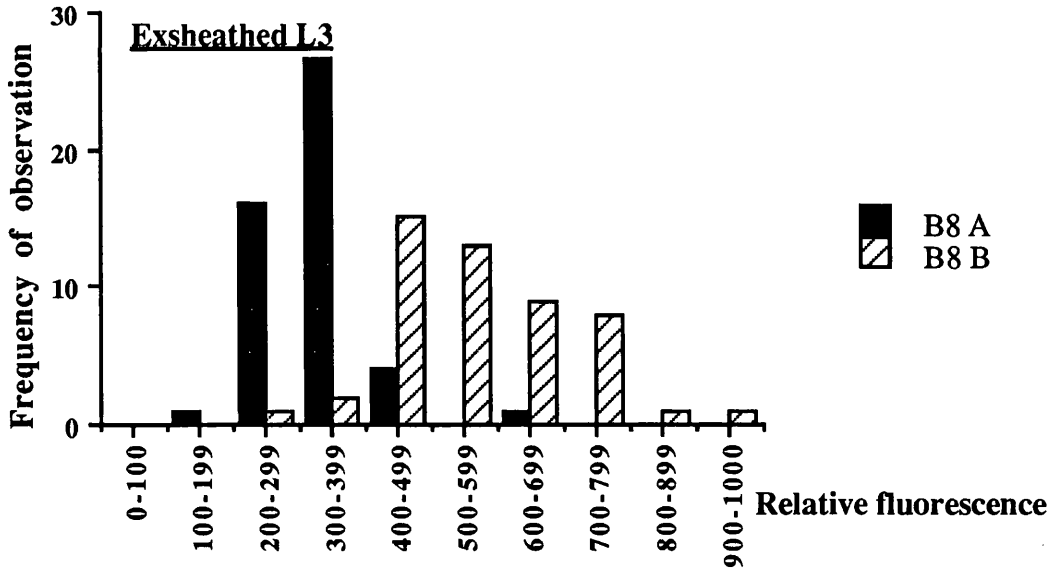


Figure 3.13e



Figures 3.13d and e

Lack of heterogeneity in the surface epitopes presented by *D. viviparus* exsheathed L3.

Approximately 3,000 exsheathed L3 from two batches, A and B, were incubated with individual hyperimmune calf sera, B7 and B8 (sampled on Day 21 of fifth infection). These calves had received 44,000 normal L3 by this stage. Following the IFAT, 50 exsheathed L3 were measured and the frequency of observation of the relative fluorescence of these plotted for each population.

Figure 3.13f

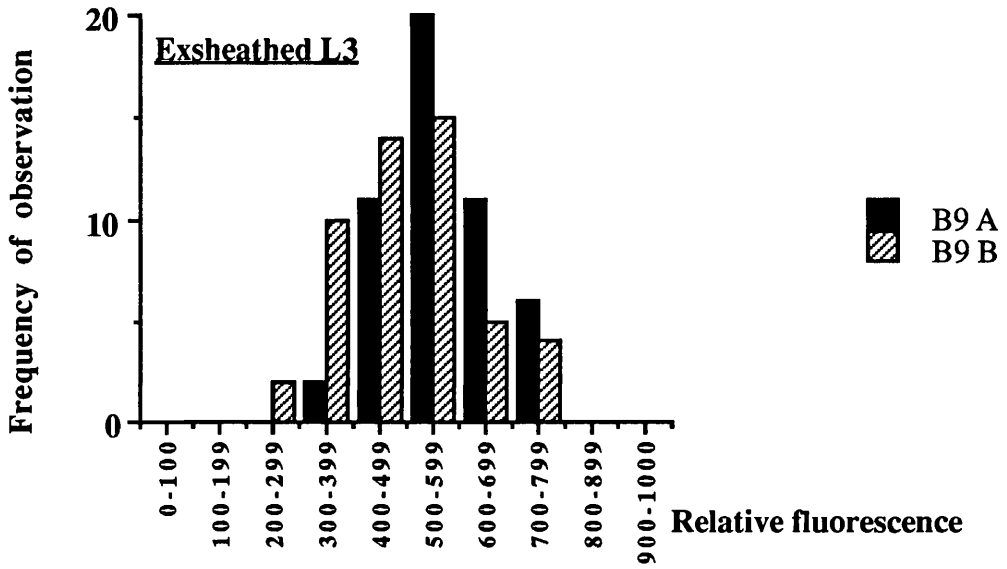


Figure 3.13f

Lack of heterogeneity in the surface epitopes presented by *D. viviparus* exsheathed L3.

Approximately 3,000 exsheathed L3 from two batches, A and B, were incubated with calf serum, B9, and the responses quantified as outlined in Figure 3.13a.

Incubation time (hours)	L3 above mucosa	L3 below mucosa	L3 within mucosa (20μm sections)
1	5000+	0	1
3	5000+	0	0
6	5000+	0	0
22	5000+	40	0

Table 3.3 Penetration of the bovine small intestinal mucosa with sheathed L3

Approximately 40,000 sheathed L3 were fluoresceinated directly, Section 2.7.2, and subsequently divided into four groups which were incubated under the conditions described in Section 2.7.3 for the time intervals indicated above. The top of the mucosa was rinsed at the end of each incubation and three, 50μl sub-samples of the washings counted. Three 50μl sub-samples of the medium below the mucosa were also examined for L3. The total number of larvae present in each was calculated by taking a mean of the sub-samples and then estimating the approximate number present in the total volume.

The mucosa samples were placed into phosphate-buffered formalin at the end of each incubation and ten 20μm sections taken from each. These were examined under UV light for the presence of fluoresceinated L3.

3.3 Discussion

The results described in this Chapter indicated that the surface of *D.viviparus* is no exception to the rule that the nematode cuticle is a dynamic structure which is potentially capable of having a complex and intimate interaction with the host.

The initial set of data demonstrated stage-specificity of the surface-exposed antigens in the stages of the life cycle examined. The unfortunate exception was the L2 stage, which could not be obtained in sufficient quantity for study. Some deductions can be made regarding the L2 surface, as the sheath of the L3 stage may be considered, in the majority of cases, to be the retained L2 cuticle. It could also be argued that, as a free-living stage, the L2 is not vital to the host/parasite interaction. The results presented here, however, indicated the possibility that the third stage larva may penetrate the host intestine with the retained L2 cuticle (L3 sheath) intact.

It was impossible, using the initial panel of sera, to differentiate between responses to the surface of adult and L1 stages and so sera, specific to these stages, were produced. Unfortunately, the results obtained by IFAT and immunoprecipitation studies were rather inconclusive but the latter results did indicate that there might be a component present on the adult surface which was not present on the L1 surface. Immunoprecipitations using this antisera and radio-labelled L1 surface extracts could be performed to examine this further.

The responses to the egg and L1 surfaces were similar, except that there was strong recognition of the egg surface by anti-ES serum. This was the only stage which was recognised by animals immunised with adult ES materials.

The results obtained with the L3 stage demonstrated immunogenicity of the retained sheath which implied that the surface of the sheath shared cross-reactive epitopes with a later tissue-penetrating stage. Alternatively, the L3 stage might penetrate the host while retaining its sheath and components of the sheath may subsequently be presented to the host immune system. The binding of antibody to the sheathed L3 appeared to mirror the responses directed against the exsheathed L3 and this was assumed to be due to the fact that there were no infections where the host was exposed to one surface without the other. However, when specific bovine isotype responses were examined, a high level of antibody binding to the exsheathed surface was observed in serum taken from uninfected calves. There was no equivalent recognition of the sheathed L3 surface. Moreover, following infection, there was a strong response to the L3 sheath but there were only small increases in recognition of the exsheathed L3 surface. Despite these differences, further adsorption studies indicated a partial cross-reactivity between the L3 sheath and the underlying cuticle and suggested that the L3 sheath surface had some, but not all, epitopes available on the L3 cuticle.

The surface-exposed antigens of the L4/5 stages were clearly different from those of the L3 surface; serum from animals immunised with L3 irradiated to 1000Gy exhibited no antibody binding to the L4/5 surface. The L4/5 population appeared to express a repertoire of antigens on their surface similar to that of the adult stage, as antibodies raised against adult worms bound strongly to the surface of L4/5 parasites. These results indicated that the host may be exposed to adult surface antigens as early as Day 14 of infection. The surface-exposed antigens of the adult stage were also demonstrated as being immunogenic and were only recognised by antibodies from animals in which full patency had been attained or by antibodies from animals immunised specifically with the adult stage.

Stage-specific surface antigens may provide *D. viviparus* parasites with a strategy for avoiding host humoral responses during a primary infection: by the time the animal mounts a primary anti-L3 response, the parasite may already have moulted on to the next stage which expresses a different repertoire of surface epitopes. In doing this, the parasite may be avoiding potentially lethal antibody-mediated responses which have been demonstrated *in vitro* to mediate killing of *N. brasiliensis* and *T. spiralis* larvae (Mackenzie *et al.*, 1980). That humoral responses may be of relevance in *D. viviparus* infection was inferred by the successful passive transfer of post-infection serum to naive recipient calves (Jarrett *et al.*, 1955a). Furthermore, antibody-mediated binding of eosinophils to *D. viviparus* L3 has been demonstrated *in vitro*, but the parasites were not killed by the cells (Knapp and Oakley, 1981). Third stage larvae, irradiated to 1000Gy, are also capable of inducing protective immunity in calves and guinea pigs (Canto, 1990) suggesting this stage as a source of protective antigens. Therefore, in providing a different epitope repertoire on the surface of later stages, the parasite may be avoiding potentially-successful host effector responses directed against the L3 surface. This might enable patency to be reached in primary infection but this strategy would be less effective on re-infection.

It was noted that antibodies in serum raised against ES products did not bind to the surface of L4/5 and adult stages. This implied a sub-surface location for the immunogenic ES epitopes or suggested that they were released from parasite secretory organs. The lack of reactivity of the anti-ES serum with the adult surface was rather confusing in light of the fact that the adult was demonstrated to shed surface-bound antibody when maintained under physiological conditions. This shedding was abrogated in the presence of the metabolic inhibitor, NaN_3, suggesting that it was an active process. It is possible that the antibody itself was being lost as a result of proteolytic cleavage. As adult *D. viviparus* have been demonstrated to release proteinases (Britton, 1991), it may be that these enzymes degraded the surface-bound antibody. Degradation of secretory IgA by the products of several bacteria species has been described (Gotschlich, 1983).

Furthermore, *S. mansoni* schistosomulae (Auriault *et al.*, 1981) and adult stage *F. gigantica* (Fagbemi and Hillyer, 1991) have been observed to release hydrolases capable of breaking down surface-bound antibody. In preliminary studies, *D. viviparus* adult ES products were incubated with bovine IgG and human IgA (not shown) and the incubation products analysed by SDS-PAGE. The results were inconclusive, however, as it was difficult to discriminate the light and heavy immunoglobulin chains from the ES components. Alternatively, adult parasites might only shed surface antigens when bound by antibody. To examine this hypothesis, ^{125}I surface-labelled adult worms could be cultured in the presence of serum, or purified immunoglobulin from infected calves, and SDS-PAGE analysis of the resulting culture supernatant might indicate whether loss of radiolabelled surface molecules was enhanced by the presence of antibody.

The work presented in this Chapter suggested that the source of adult *D. viviparus* ES material was not the surface and it was seen that the strongest degree of binding by anti-ES antibodies was against the surface of eggs. Subsequent immunocytochemistry studies have indicated that this antisera binds strongly to the female worm uterus (not presented). Sections of the cephalic region of the parasite were not obtained so excretory glands in this region might still be considered as a source of the secretory products. It is of interest that studies on adult *Trichostrongylus* worms have indicated that the reproductive tract of adult male parasites, along with the subventral excretory glands, contained high levels of esterases which are known to be secreted by this nematode species (Rothwell, Ogilvie and Love, 1973). The possibility must not be overlooked, however, that ES molecules released from other organs of *D. viviparus* may be sticking to the egg surface following release into culture.

In contrast to adult parasites, sheathed and exsheathed third stage larvae did not appear to release surface-bound antibody. This was not surprising in the case of the L3 sheath which is merely a metabolically-inactive structure left behind by the previous stage. That the L3 cuticle itself was also inert in terms of surface turnover was suggested by previous work which indicated that this stage released negligible ES products when maintained in culture (Britton, 1991). The L3 surface examined in the surface shedding experiment had not been exposed to the host environment, however, and the possibility of *in vivo* surface shedding should not be excluded. In contrast to L3 stages, which moult to the L4 within days of infection (Poynter *et al.*, 1960), adult parasites maintain their position in the bovine respiratory tract for up to 60 days. If *D. viviparus* -specific antibody in the respiratory tract reflects circulatory levels during infection, then surface-shedding of bound antibody may be of relevance to survival of the adult parasite.

The L3 cuticle surface appeared to be different from the other developmental stages in terms of lipid organisation (not shown). When various stages of the parasite were examined for surface insertion of the fluorescent lipid analogue, 5-(*N*-octadecanoyl)

aminofluorescein (AF18), only the exsheathed L3 parasites did not permit incorporation of this probe into the surface. This indicated a difference in the surface-associated lipid components of this developmental stage. Moreover, the L1 stage also had a reduced level of lipid probe insertion. Both these stages must undergo extreme changes in their environmental conditions. This characteristic difference in lipophilicity has been also observed with L3 stages of *O. ostertagi* (Duncan, 1990) and *H. contortus* , *T. spiralis* and *A. viteae* (Proudfoot *et al.*, 1990).

Not only may parasites differ between developmental stages in the structure and composition of their surface, but it is possible that individual parasites may exhibit heterogeneity in their surface antigen expression. This would be advantageous to parasite populations which encounter genetically diverse communities of hosts because selection pressure would favour parasites that were heterogeneous in their antigenic surface molecules. Heterogeneity in parasite surface antigen expression has already been described in *Ascaris lumbricoides* infection (Fraser and Kennedy, 1991). In contrast to *Ascaris*, *D. viviparus* L3 parasites appeared to present a comparatively homogeneous set of surface antigens to their host, as suggested by the normal distribution of antibody binding to both larval populations examined. This might have been due to the fact that the parasites used in these experiments comprised a population derived from a single strain, i.e. larvae which were cultured for the lungworm vaccine. It would be of interest to examine surface antigen expression from parasites derived from field cases of lungworm infection. Nevertheless, it could be that *D. viviparus* does not express much heterogeneity in its surface antigen repertoire and this may account for the success of naturally-acquired and vaccine-induced immunity. This situation is in complete contrast to *Ascaris* infection, where there is no evidence that acquired resistance mechanisms influence the proportion of immature parasites which become established in previously-infected hosts (Elkins, 1987).

It was suggested from the results presented in this Chapter that the L3 stage of *D. viviparus* may penetrate the host mucosa while retaining its sheath. The sheath antigens were recognised following infection and, if the sheath were to be left behind in host tissue, it might serve as a nidus of antigen against which the host may mount irrelevant effector responses. This may be an opportunistic strategy evolved by the parasite in order to divert host effector mechanisms away from the surface of migrating third or fourth larval stages.

In conclusion, it can be proposed that the surfaces of all developmental stages of *D. viviparus* are antigenic and the associated epitopes stage-specific. The results also indicate that adult parasites may be able to withstand host humoral responses by loss of bound antibody and by the release of antigenic ES products, the epitopes of which do not appear to be exposed on the surface of intact parasites.

Chapter 4

Antibody responses to the surface of *D. viviparus* in infected and vaccinated calves

4.1 Introduction

The antibody response in *D. viviparus* infection

In 1955 Jarrett *et al.* stated that, "In parasitic bronchitis there are humoral antibodies which are detectable and measurable by a complement fixation test and the serum containing these antibodies confers a considerable degree of protection against a single challenging infection of infective larvae of *D. viviparus* in calves which have not previously been infected".

This statement was based on experiments where antisera from naturally-infected animals, injected intraperitoneally at a dose rate of 500ml per calf, reduced the mean worm burden of recipients by 95% compared with challenge controls. This established the potential importance of antibodies in protective immunity against *D. viviparus*. Similar results were reported by Canto (1990) who obtained a 78.6% reduction in worm burdens in recipients of immune serum. In both passive transfer studies, the recipients of the immune sera were observed to have "nodules" in their lungs and, because of their characteristic histopathology, it was suggested that these nodules were sites of antibody production. Passive transfer against *D. viviparus* infection has also been successful in guinea pigs: when compared to challenge controls, recipients of normal L3 infection serum had 88-89% reductions in worm burdens (Wilson, 1966; Canto, 1990). The antibodies inducing the protective response were considered to be of the gamma anaphylactic type (Wilson, 1966).

Many studies have been performed in an attempt to elucidate the components of the humoral response which are relevant to protective immunity in parasitic bronchitis. Initially, the complement fixation (CF) test was used to examine antibody levels during experimental and natural *D. viviparus* infections (Jarrett *et al.*, 1959; Weber, 1958; Cornwell, 1959, 1960a, 1960b and Cornwell and Michel, 1960). Using heat-inactivated whole adult worm as antigen, it was observed that antibody levels increased from Day 30 after experimental infection to peak on Day 50, after which they gradually declined (Jarrett *et al.*, 1959). When responses were measured after secondary infection, antibody levels rose within 7 days of re-infection and peaked 14 to 21 days later (Jarrett *et al.*, 1959). Similar results were obtained by Cornwell and Michel (1960) who examined antibody responses during natural infections and demonstrated that the kinetics of the antibody response were similar in natural and experimental infections. In contrast, Weber (1958) observed a more rapid and sustained response in CF titres following primary infection. The antigen used in this case had not been subjected to heat-inactivation but, when the two antigen preparations were subsequently examined in the same experiment, differences in antibody directed at each were not observed

(Cornwell; 1963). The results, therefore, suggested that the calves in Weber's study (1958) had previous exposure to *D. viviparus* and were probably undergoing an anamnestic response when sampled.

It has been shown that CF-antibody titres to *D. viviparus* are not directly related to immune status (Michel and Cornwell; 1959) and that calves could be heavily infected before exhibiting significant rises in CF-antibody titres (Jarrett *et al.*, 1961a). It was also the case that calves, vaccinated with 40Kr (400Gy)-irradiated L3, did not necessarily display CF-antibody titres against *D. viviparus*, despite being resistant to challenge (Jarrett *et al.*, 1961a; Cornwell, 1960b). Contrary to this, calves immunised with dead adult worm material, were not resistant despite having high levels of *D. viviparus* -specific antibody (Jarrett *et al.*, 1960b).

Subsequently, Bokhout, Boon and Hendriks (1979) used soluble adult antigens in the indirect haemagglutination test (IHA) and obtained a good correlation (more than 80%) between positive IHA and clinical diagnosis in the field. They observed that antibody levels started to increase within a fortnight of infection and peaked at Day 45 to remain positive for several months. The earlier rises in antibody titres compared with those observed in the CF tests, were probably a reflection of the higher sensitivity of the IHA test. There have been a number of recent seroepidemiological surveys of lungworm infection in the Netherlands using the ELISA (Boon, Kloosterman and van den Brink, 1982; Boon, Kloosterman and van der Lende, 1984 and Bos and Beekman, 1985). These surveys indicated a high (more than 90%) prevalence of infection (Boon, Kloosterman and van der Lende, 1984) and emphasised that, although positive ELISA titres were a satisfactory indicator of recent herd exposure, they were inaccurate in determining the immune status of individual animals (Bos and Beekman, 1985).

Although humoral immunity is known to play some part in the development of immunity to *D. viviparus*, both the target antigens and the effective immunoglobulin isotypes involved remain to be defined. Additionally, the developmental stage(s) of parasite against which these antibodies exert their effect is unknown. The fact that 400Gy-irradiated larvae can induce resistance indicates that protective antigens must be present in the third, fourth or fifth larval stages. Third stage *D. viviparus* larvae, irradiated to 1000Gy, were also observed to stimulate a significant level of immunity in both calves and guinea pigs and as L3 irradiated at this level develop no further, this stage was assumed to contain protective antigens (Canto, 1990). Coincidentally, the immunity induced by these L3 could not be passively transferred in either calves or guinea pigs suggesting that non-humoral effector mechanisms were involved or that the quantity of antibody transferred was a limiting factor.

This Chapter describes a preliminary examination of bovine antibody subclass binding to the surface of live *D. viviparus* parasites. The responses induced in infected

and vaccinated calves to the surface of L3 and adult stage parasites were examined using the IFAT. In performing these experiments, it was hoped that some information would be obtained regarding the effector mechanisms relevant to the development of protective immunity in parasitic bronchitis.

4.2 Results

4.2.1 Bovine immunoglobulin responses to the *D. viviparus* L3 sheath.

Sheathed L3 were incubated with serum from calves exposed to various infection regimens with *D. viviparus* and their responses examined using the IFAT (Section 2.5.6). All animals used in these studies were found to be immune to re-infection when challenged with normal larvae (Canto, 1990). Antibody isotype responses were measured by incubation with mouse monoclonal antibodies against bovine IgM, IgG_1 and IgG_2 and the mouse antibody detected with FITC-conjugated anti-mouse immunoglobulin. The samples were quantified by photon counting and the results, presented as the mean and standard deviation of 25 samples, are shown in Figure 4.1a.

There was no reactivity in pre-infection serum against the sheath of third stage larvae. After two infections with normal L3 there was a strong IgG_1 response, with only slight increases in the IgM and IgG_2 . With further infections surface-specific IgG_1 actually decreased, so that by Day 21 of the sixth infection, the levels of this antibody were almost half that observed following secondary infection.

The level of anti-L3 sheath antibody in animals vaccinated twice with 400Gy or 1000Gy-irradiated L3 was only slightly increased above pre-infection levels. Following challenge with normal larvae, there was a rapid rise in IgG_1 with a small increase in the IgM and IgG_2 isotypes. The results implied that during these immunisation regimes, the induction of protective immunity was accompanied by only weak antibody responses to surface antigens. The strong secondary IgG_1 response observed following normal L3 challenge of 1000Gy vaccinates may have been a consequence of a more efficient priming in these calves which had been infected intravenously. The calves were infected by this route as larvae irradiated to this level do not appear to penetrate the intestinal wall after oral infection (Canto, 1990).

When the IgG_1 and IgM profiles of calves subjected to additional intravenous immunisations with 1000Gy-irradiated larvae were measured (Figure 4.1b), the IgG_1 levels were observed to increase substantially following tertiary infection. Thereafter, the levels of this antibody gradually decreased, despite further infections. The degree of binding did not, however, decrease to pre-infection levels. The IgM levels increased following primary immunisation but subsequently decreased gradually to almost pre-infection levels.

Figure 4.1a

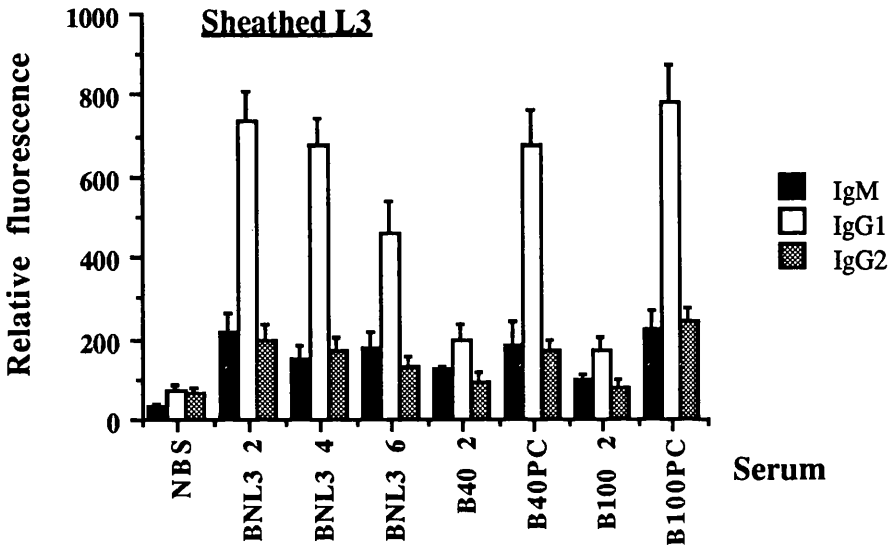
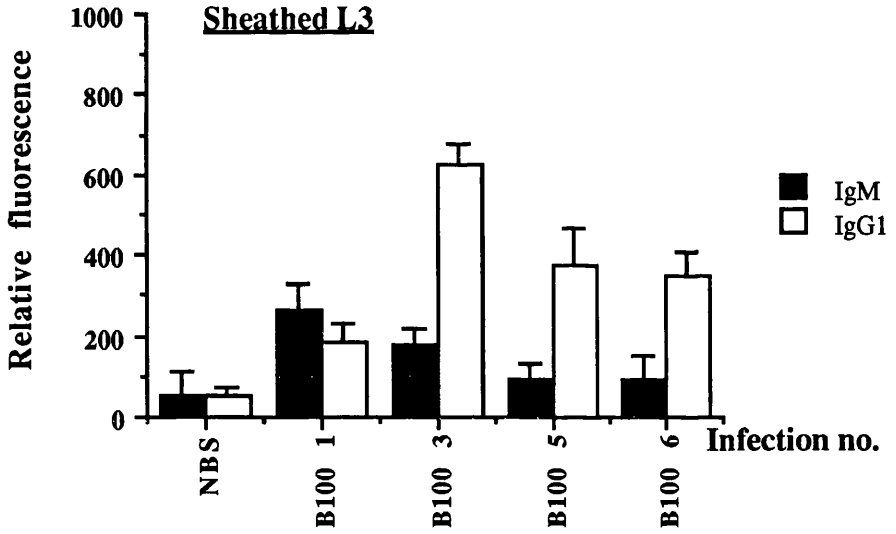


Figure 4.1b



Figures 4.1a and b

Bovine antibody responses to the surface of sheathed *D. viviparus* L3.

In Figure 4.1a,the sera were sampled from calves exposed to either normal L3 (BNL3), 400Gy (B40) or 1000Gy (B100) L3 infections. Normal L3-infected calves received two larval doses of 2,000 L3, then two of 10,000 L3 and two of 20,000 L3 (the number of infections are indicated). The 400Gy L3-infected calves received two doses of 1,000 L3 (B40 2) and the 1000Gy L3 calves (B100 2) 2,000L3, before challenge with 5,000 normal L3 (B40 PC, B100 PC).

In Figure 4.1b sera were from 1000Gy L3-infected calves (B100) given the number of infections indicated. Animals received 5,000 L3, 10,000 L3 (x3), 20,000 L3 (x2) intravenously. Sera were pooled from three calves and sampled 28 days after infection.

4.2.2 Bovine immunoglobulin responses to the surface of *D. viviparus* exsheathed L3.

In order to examine responses to the L3 cuticle itself, *D. viviparus* infective larvae were exsheathed in Milton and the cuticular surface subsequently examined for binding to antibody from animals subjected to normal L3 infection. The results of the IFAT are shown in Figure 4.2a.

The exsheathed L3 surface was recognised by the pre-infection sera of all calves and this binding was observed to be exclusive to the IgM isotype. This binding was not observed using foetal bovine serum. Following two infections, the L3 cuticle-specific IgM increased above pre-infection levels and decreased gradually thereafter, despite further infections. Interestingly, the level of IgG isotypes, directed against the exsheathed L3 surface, showed little change, with only a marginal increase in IgG_1 levels after four infections with large numbers of L3. The L3 cuticle, in contrast to the L3 sheath surface, did not appear to induce strong antibody responses during the course of normal L3 infection.

Anti-L3 cuticle antibodies induced in calves infected with 400Gy and 1000Gy irradiated L3 were also examined and the results are presented in Figure 4.2b.

IgM antibodies directed against the L3 cuticle were observed to increase above pre-infection levels following vaccination with 400Gy L3. The levels of IgM increased again when the vaccinates were challenged with normal L3. As with sheathed L3, minimal IgG increases were induced by 400Gy L3, however, the levels rose substantially following challenge. Intravenous vaccination with 1000Gy-irradiated larvae provoked little recognition of the L3 cuticle by any of the antibody subclasses but after challenge the animals manifested an antibody profile typical of an anamnestic response in that there was a rapid and considerable rise in IgG_1, in the absence of increased IgM. The IgG_1 levels stimulated were high indicating that these animals had been highly primed against the surface antigens of the L3 cuticle. This was in contrast to the results obtained with normal L3-infected animals and may have been related to the administration of the 1000Gy L3 intravenously.

In a separate group of calves, exposed to six infections of increasing doses of 1000Gy larvae, increased levels of IgG_1, directed against the exsheathed L3 surface were observed after the third infection (Figure 4.2c). On subsequent infections with 1000Gy L3, there were no further increases in antibodies directed against the L3 cuticular surface and it may have been that more avid antibodies were being produced which acted more effectively against the incoming L3.

Figure 4.2a

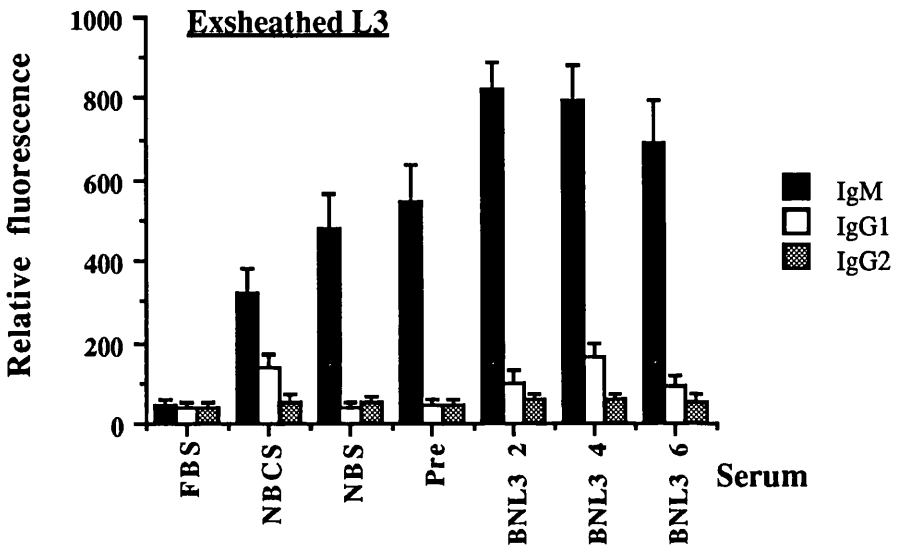
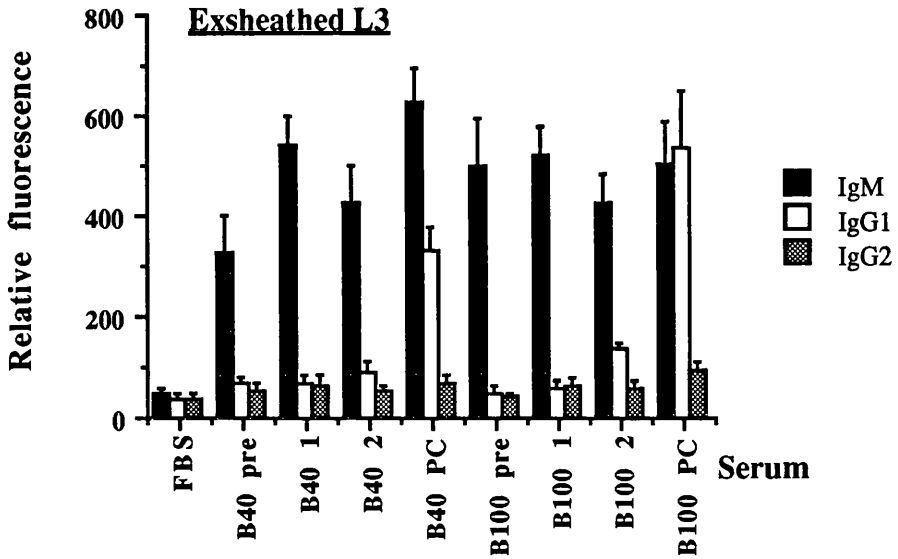


Figure 4.2b



Figures 4.2a and b

Bovine antibody responses to the surface of *D. viviparus* exsheathed L3.

In Figure 4.2a, the normal L3 infection sera (BNL3) used were the same as those referred to in Figure 4.1a and were compared to the antibody levels present in foetal bovine (FBS), newborn calf (NCBS) and normal bovine (NBS) sera.

In Figure 4.2b, the 400Gy-irradiated L3 vaccinates received two doses of 1,000 L3 (B40 1 and B40 2), followed by a challenge dose of 5000 normal L3 (B40 PC). The calves receiving 1000Gy-irradiated larvae were administered with two doses of 2,000 L3 per dose (B100 1 and B100 2), followed by a challenge with 5,000 normal L3. The sera were taken 28 days after each infection and compared to pre-infection sera (pre)..

Figure 4.2c

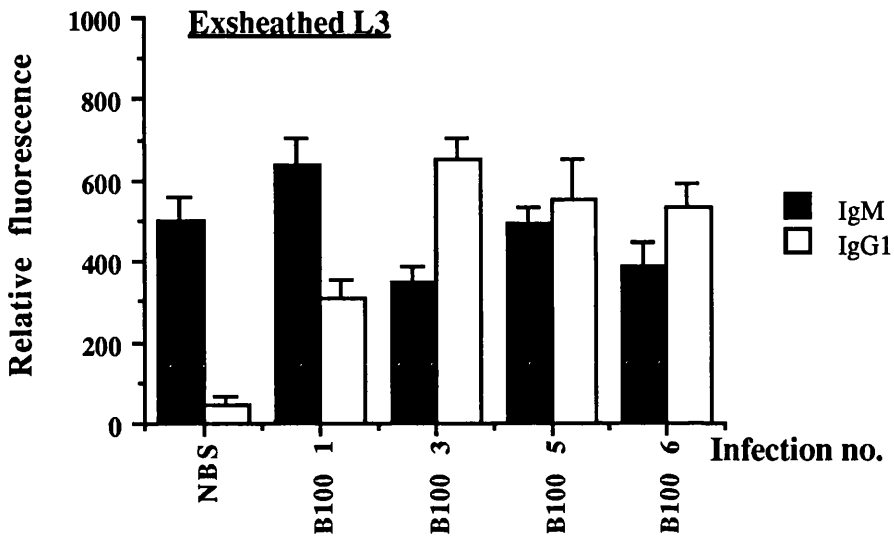
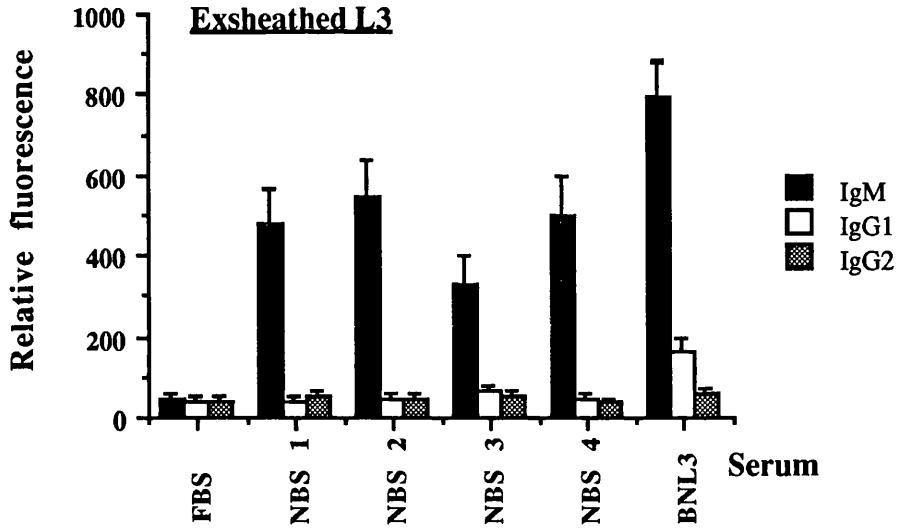


Figure 4.2d



Figures 4.2c and d

Bovine antibody binding to the surface of *D. viviparus* exsheathed L3.

Figure 4.2c demonstrates the responses of 1000Gy L3-infected calves (B100) to the exsheathed L3 surface after the number of infections indicated. The animals received 5000 L3, 10,000 L3 (three times) and 20,000 L3 (twice) intravenously, 28 days apart. All sera were pooled from three calves and were sampled 28 days after each infection.

Figure 4.2d compares the level of IgM binding to the surface of Milton-exsheathed L3 in individual uninfected calves (NBS 1-4), foetal bovine serum (FBS) and serum taken from a calf on Day 21 of a secondary infection with normal L3 (BNL3).

4.2.3 Non-specific binding of IgM to the L3 cuticle of *D. viviparus.*

In some the foregoing experiments with exsheathed L3, there was an apparent recognition of the L3 surface by normal bovine serum. This was also observed with new born calf serum but was absent when foetal calf serum was used (Figure 4.2a). When sera from other calves were examined, all showed increased levels of L3 cuticle-specific IgM prior to infection (Figure 4.2d). This "non-specific" recognition of the exsheathed L3 surface was mediated in NBS and NBCS by IgM antibody. There was negligible binding by IgG_1 or IgG_2, except in the newborn calf serum where colostrum-derived antibody may have been involved. As the IgM bound Milton-exsheathed larvae, it was thought necessary to examine the cuticle of such parasites more closely in case the Milton itself was inducing damage which mediated non-specific binding. The surfaces of sheathed and Milton-exsheathed larvae were therefore examined under scanning (SEM) and transmission (TEM) electron microscopy and the photomicrographs of most interest are presented in Figures 4.3a to 4.3g.

Figure 4.3a is an SEM of a sheathed L3 (x6,400). The surface was observed to be smooth with the underlying L3 cuticular annulations and lateral groove obvious. When the surface of Milton-exsheathed larvae were similarly examined at x6,400 and x19,200 (Figures 4.3b and 4.3c, respectively), it was clear that the L3 sheath had been completely removed and that the underlying L3 cuticle was smooth and appeared to be undamaged. These features were observed in all larvae examined in these preparations. The results are in contrast to the situation observed with Milton-exsheathed *O. ostertagi,* in which cuticular damage was obvious under SEM (Duncan, 1990).

The apparent lack of damage due to Milton treatment was confirmed by TEM. Figure 4.3d shows a sheathed L3 in cross-section and demonstrated the presence of only one retained cuticle, presumably that of the L2. The tri-radiate pharynx at the centre of the parasite was obvious. Figure 4.3e shows an exsheathed L3 viewed in cross-section (x 11,000): the surface of the parasite appeared to be intact and all other L3 in the preparation were of a similar appearance. When the surface of the exsheathed L3 was viewed at higher magnification, no obvious disruption was observed on the surface (Figure 4.3f) which appeared similar to that of the sheathed L3 (not shown). A surface coat outside the epicuticle was distinguishable at higher magnification (Figure 4.3g).

Figure 4.3a

Scanning electron micrograph of the surface of a sheathed third stage *D. viviparus*. (x12,800).

Scale bar: 0.5μm.

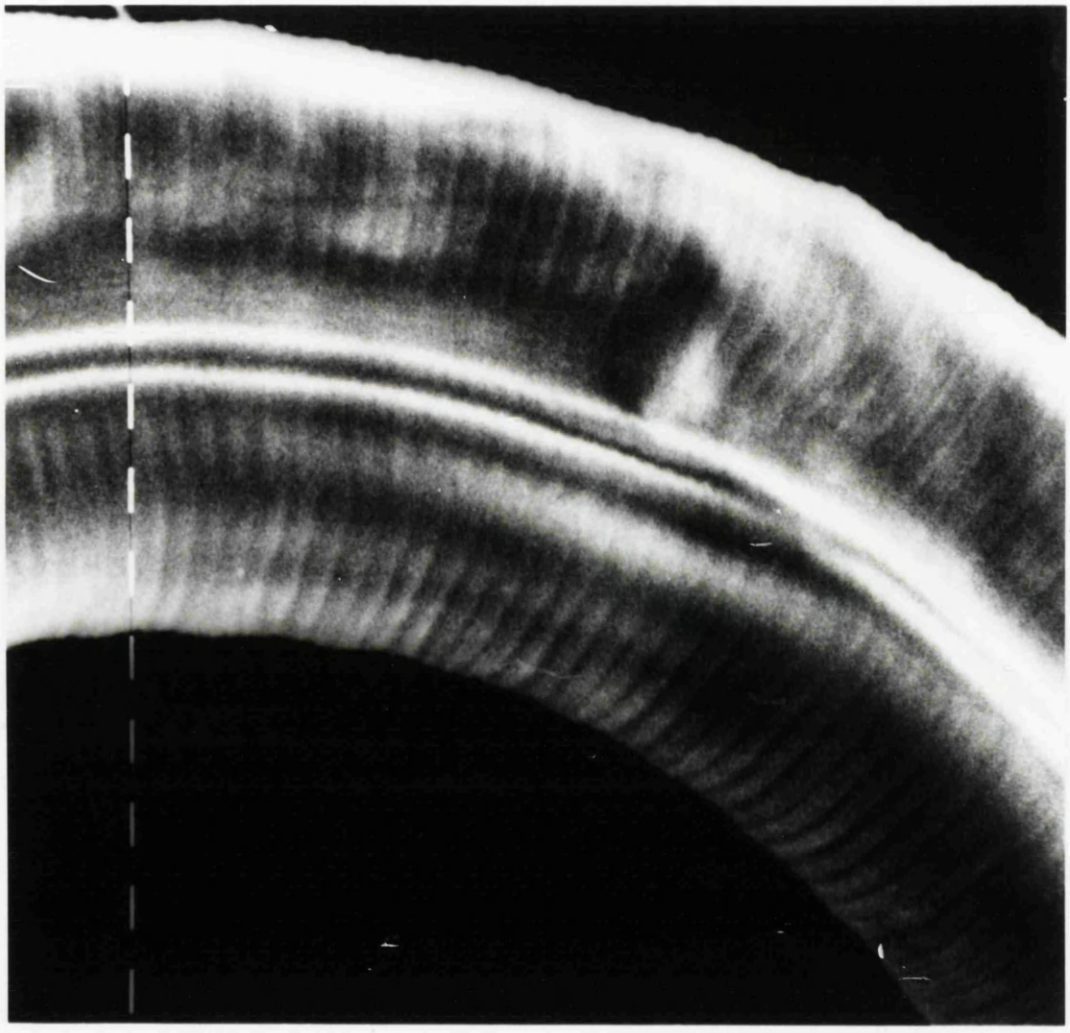


Figure 4.3b

Scanning electron micrograph of the surface of a Milton-exsheathed third stage *D. viviparus* larva (x6,400).

Scale bar: 1μm.

Figure 4.3c

Scanning electron micrograph of the surface of a Milton-exsheathed third stage *D. viviparus* larva (x19,200).

Scale bar: 0.1μm.

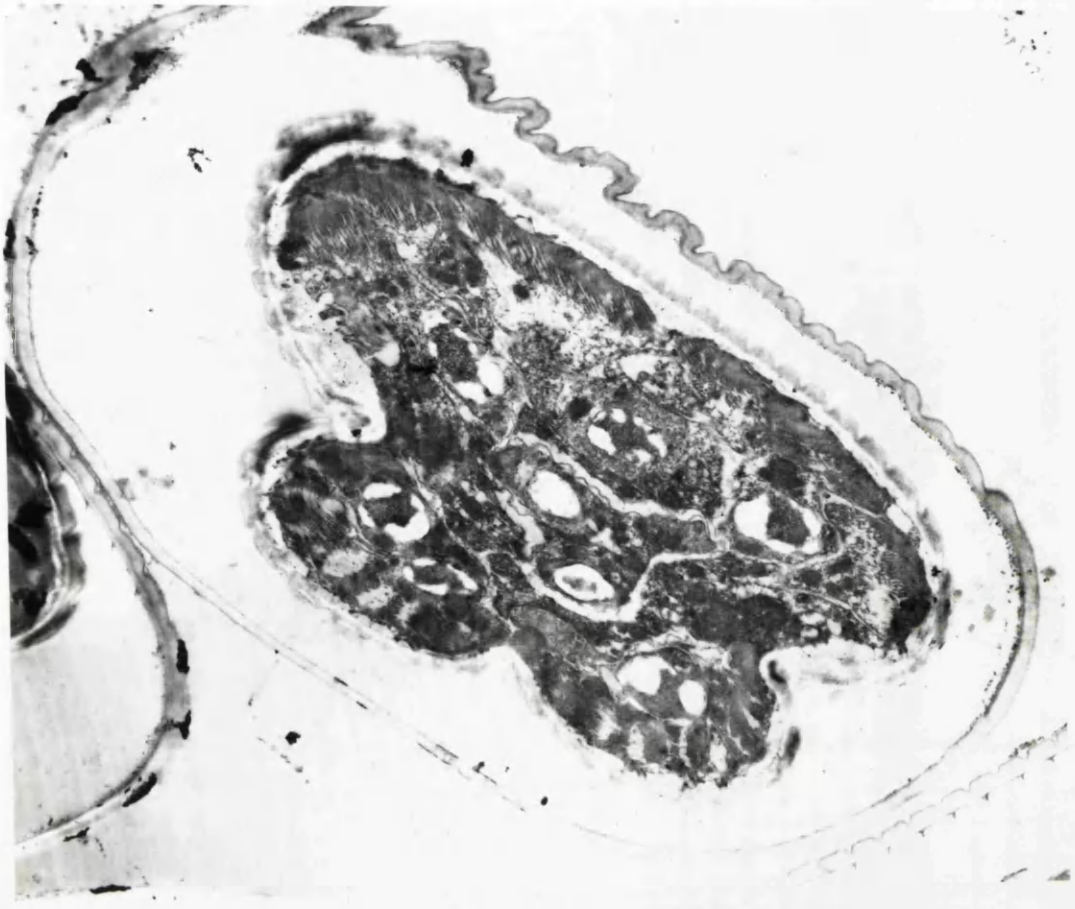
Figures 4.3d and e

Transmission electron micrographs of sheathed (Figure 4.3d) and Milton-exsheathed (Figure 4.3e) *D. viviparus* L3 in cross section.

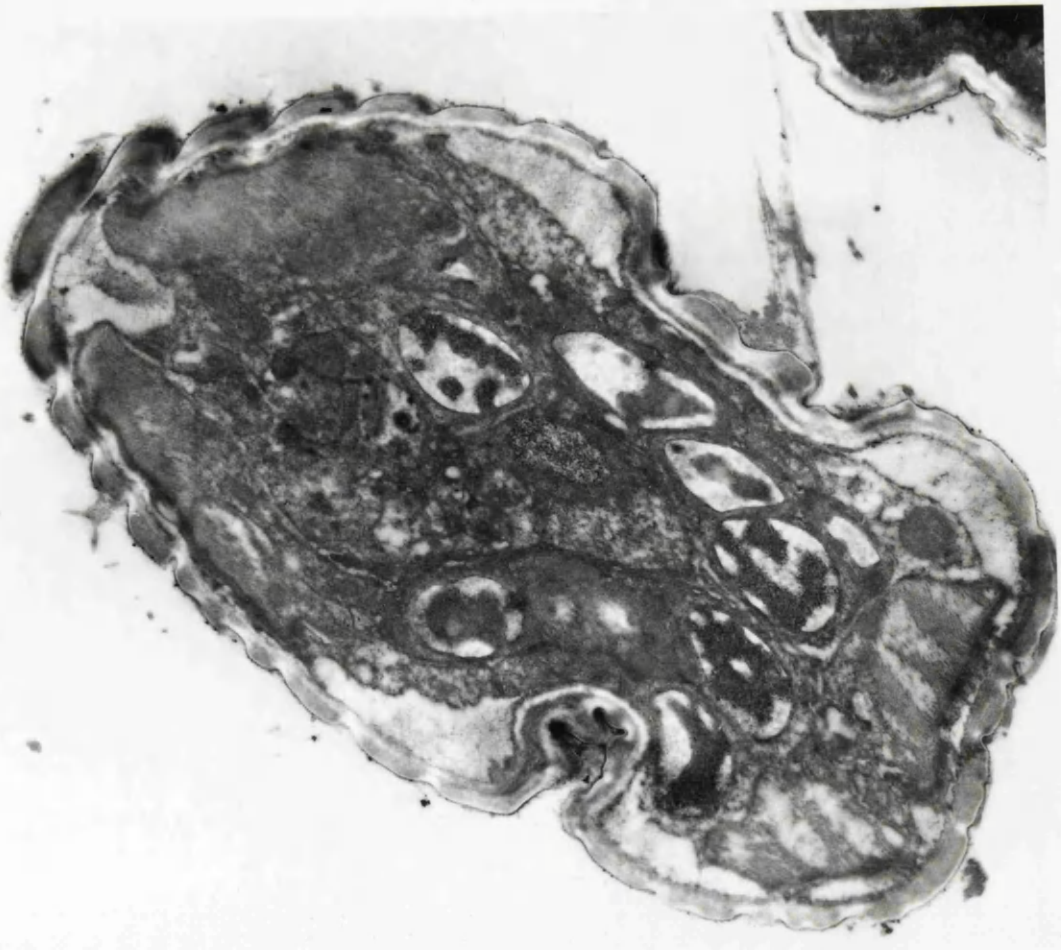
Magnification: Figure 4.3d: x7,500.
Figure 4.3e: x11,000.

Electron micrograph 4.3d was taken under negative contrast and 4.3e under positive contrast.

d



e



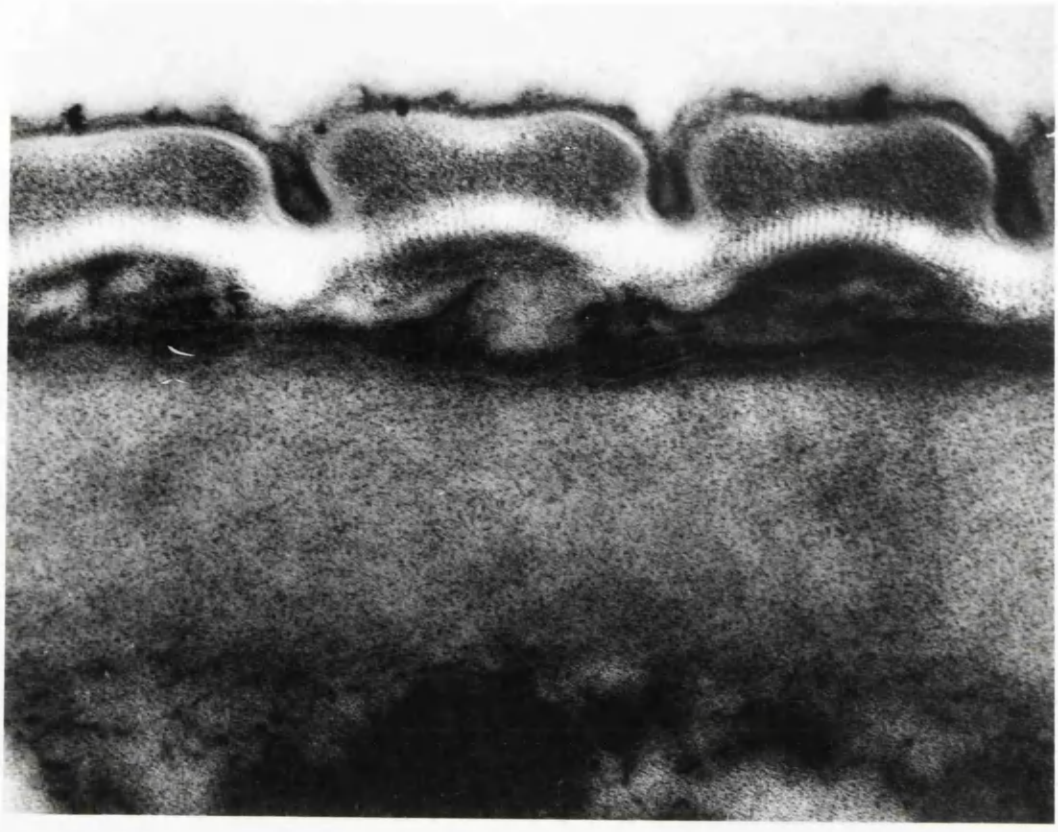
Figures 4.3f and g

Transmission electron micrographs of the cuticle of a Milton-exsheathed *D. viviparus* L3 in cross section.

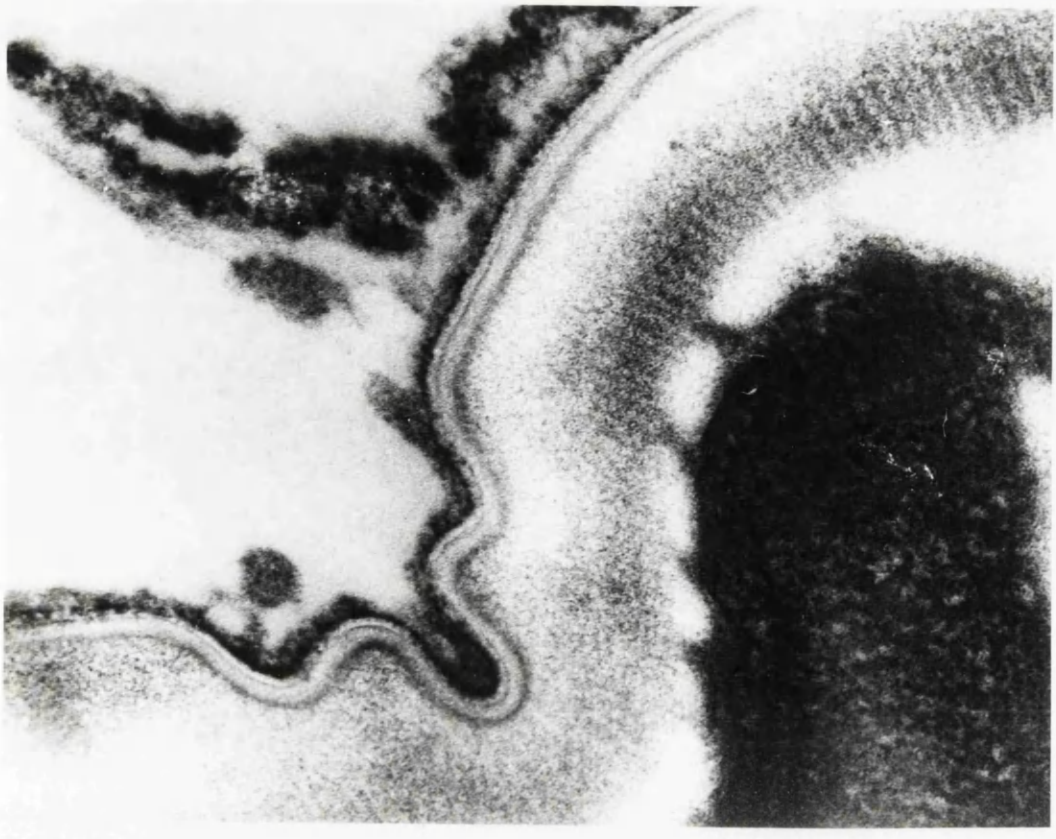
Magnification : Figure 4.3f: x75,000
Figure 4.3g: x125,000.

Electron micrograph 4.3f was taken under positive contrast and 4.3g under negative contrast.

f



g



Section 4.2.4 Bovine immunoglobulin responses to the surface of *D. viviparus* adult parasites.

As previous findings indicated that the surface antigens of *D. viviparus* are stage-specific, it was considered appropriate to examine bovine antibody responses to the surface of other stages of the parasite. Antibody responses to the surface of adult worms were therefore examined. Adult worms were incubated with individual sera from two calves exposed to normal L3 infection and the isotype responses determined as described above. The results are presented in Figures 4.4 a and 4.4b.

Apart from minor differences in the quantities of antibody produced, the isotype profiles of the two calves given normal L3 infections (Figure 4.4a) were similar and are therefore described together. There was no binding of antibody to the adult surface in serum taken prior to infection. By the time of the second infection on Day 50, there was a marked IgM response to the surface of the adult parasites. The responses against the adult worm surface followed classical primary and secondary antibody kinetics, in that by Day 80, there was an obvious class switch from IgM to IgG antibody. There was another increase in IgG antibody after the third infection. Later infections may have boosted adult-specific antibody because immunity stimulated by *D. viviparus* is not sterile (Porter and Cauthen, 1942; Jarrett *et al.*, 1959) and a small proportion parasites in each infection might have reached the adult stage. It was interesting to note that, for the first time, there appeared to be a substantial increase in surface-specific IgG_2 and this occurred around the time (Day 80) that adult worms are thought to be expelled from the lungs.

As found earlier (Chapter 3), calves which received 400Gy or 1000Gy-irradiated L3 did not produce antibody which reacted with the adult surface (Figure 4.4b).

Figure 4.4a

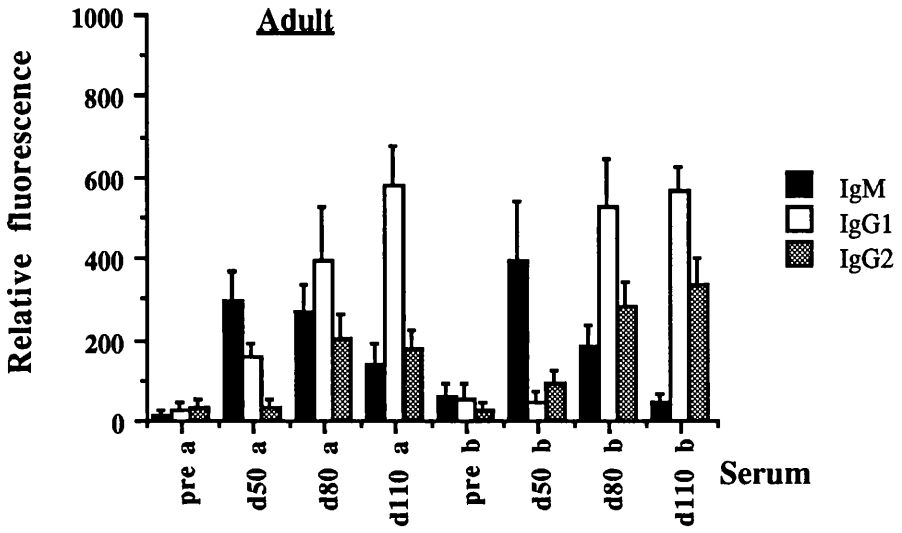
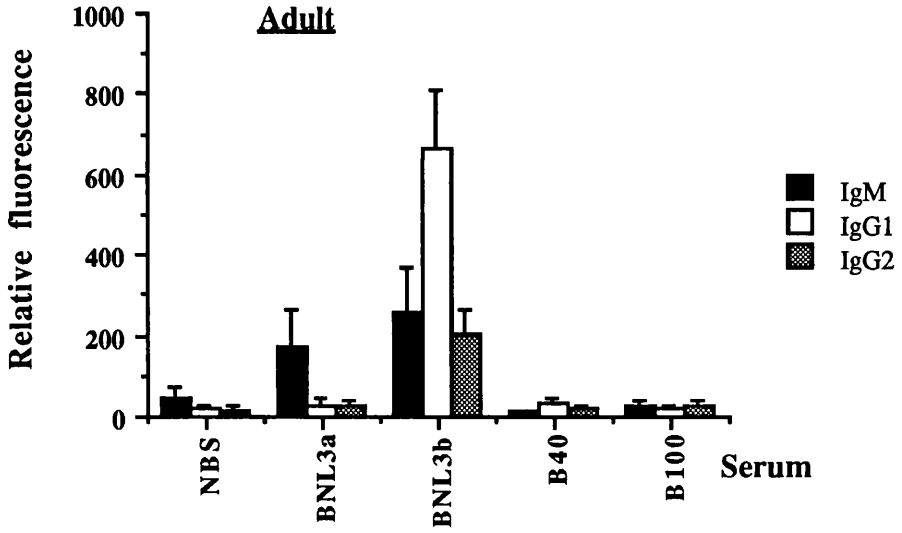


Figure 4.4b



Figures 4.4a and b

Bovine antibody responses to the surface of adult *D. viviparus*.

In Figure 4.4a sera from two individual calves infected with normal L3 were examined. The calves were infected on Days 0 and 50 with 2,000 normal L3, followed by 10,000 L3 on Day 80 and 20,000 L3 on Day 110. The sera were sampled on the days indicated in the Figure and the samples were compared to pre-infection sera (pre).

In Figure 4.4b, the responses of normal L3-infected calves (BNL3) are compared with calves vaccinated with 400Gy and 1000Gy L3. BNL3a was sampled on Day 52 of primary infection and BNL3b on Day 21 of secondary infection. Both infections were of 2000 L3. The 400Gy and 1000Gy sera were sampled on Day 21 of the fifth infection and the calves had each received 55,000 irradiated larvae by this time. All sera were pooled from three calves and compared to normal bovine serum (NBS).

4.2.5 Examination of bile and pulmonary mucus for *D. viviparus* surface-specific antibody.

Since local responses are considered important in immunity against luminal and mucosal-dwelling parasites (reviewed by Wakelin, 1978), the possibility of parasite interference by local immune mechanisms was investigated. Pulmonary mucus from a naturally-infected calf was measured for IgG_1, IgA and IgM antibodies reactive with the L3 surface. In addition, as circulating antibody levels were not boosted following several oral infections with *D. viviparus*, bile was examined for the presence of these antibody isotypes directed against the L3 surface.

Sheathed and Milton-exsheathed third stage larva of *D. viviparus*, were incubated with bile, pulmonary mucus and serum from a patent field case of parasitic bronchitis. Serum from a calf experimentally-infected on a number of occasions with normal L3 was used as a positive control and FBS as a negative control. Unfortunately, bile and pulmonary mucus from a calf known to have had no previous exposure were not available. The pulmonary mucus and bile were used at dilutions similar to those used for the serum and the IgM, IgA and IgG_1 isotype responses were determined as outlined above. The results are presented in Figures 4.5a and 4.5b.

Increased antibody was only detected in the sera from the infected calves and antibody levels higher than those of normal serum were not detected in either bile or mucus. Although bile and pulmonary mucus from a normal calf were not included, it appeared that there was no *D. viviparus* L3 surface-specific antibody present in the samples measured here. Together with the clinical and post mortem observations, the fact that this animal had a high IgG_1 anti-sheath response suggested that it had been under recent heavy challenge. It may be that this method was unsuitable for the detection of antibodies in pulmonary and biliary fluids. Furthermore, when the pulmonary mucus was examined for adult surface-specific IgG and IgM, negative results were obtained (not shown).

4.2.6 Species-specificity and cross-reactivity of surface epitopes present in *D. viviparus*, *O. ostertagi* and *C. oncophora* L3.

In order to examine if antibody stimulated by *D. viviparus* infection bound to the surface of related nematodes, sheathed third stage larvae of *O. ostertagi* and *C. oncophora* were incubated with antiserum raised in calves and guinea pigs against various stages of *D. viviparus*. The antibody responses were determined by IFAT and the results indicated that there were antigens present in the third stage of *D. viviparus* that cross-reacted with surface epitopes on the sheaths of third stage *O. ostertagi* and *C. oncophora* (Figures 4.6a and 4.6b). This was suggested by the fact that sera from guinea pigs and calves infected with 1000Gy L3 reacted with the L3 sheath of both

gastrointestinal parasites. Whether similar epitopes existed in other stages of *D. viviparus* was not examined. Reactivity using the guinea pig anti-1000Gy L3 serum was low but this was expected, in view of similar results obtained with this serum preparation in previous experiments (Chapter 3). Low antibody titres in this serum may have been due to the fact that the highly-irradiated L3 were injected intraperitoneally, and not intravenously, as they were in the calf infections.

When sheathed and Milton-exsheathed third stage *D. viviparus* larvae were incubated with antiserum raised in calves against *O. ostertagi* and *C. oncophora* L3 infections, evidence of cross reactive epitopes was absent (Figures 4.6c and 4.6d). This suggested one of two things. First, the cross-reactive epitope(s) which was located on the sheath of the two gastrointestinal parasites, was located internally in *D. viviparus* or, if it was a component of the sheath or cuticle, it was not surface-exposed. Alternatively, it could be that, as neither of the gastrointestinal parasites has a migratory life cycle, they consequently did not induce strong systemic responses in the host. In support of the latter hypothesis, it was observed that anti-*O. ostertagi* and anti-*C. oncophora* sera did not react with the sheath or L3 cuticle of the homologous species (not shown).

Figure 4.5a

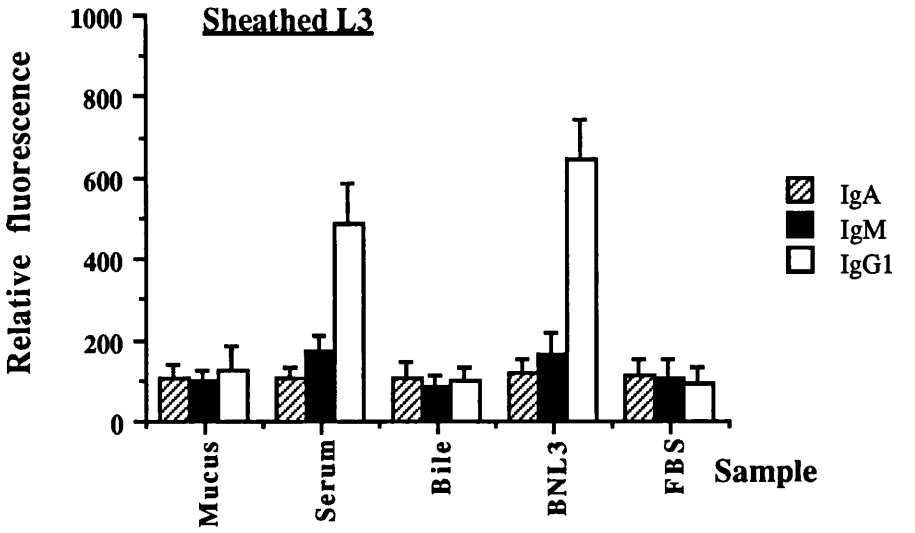
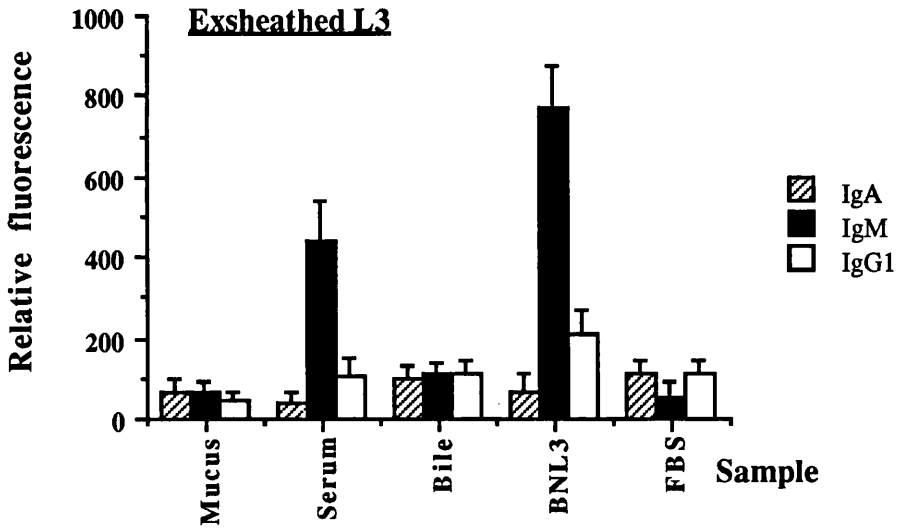


Figure 4.5b



Figures 4.5a and b

Examination of bovine bile and pulmonary mucus and bile for *D. viviparus* L3 surface specific antibody.

Sheathed (Figure 4.5a) and exsheathed (Figure 4.5b) *D. viviparus* L3 were incubated with pulmonary mucus, bile and serum obtained from a field case of parasitic bronchitis. Serum from a calf experimentally infected on three occasions with normal L3 (BNL3) was used as a positive control and foetal bovine serum (FBS), as a negative control.

Figure 4.6a

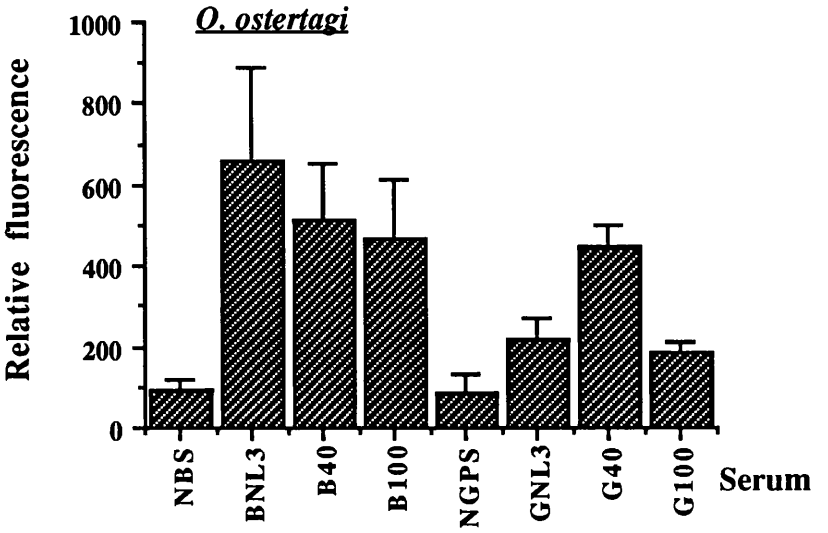
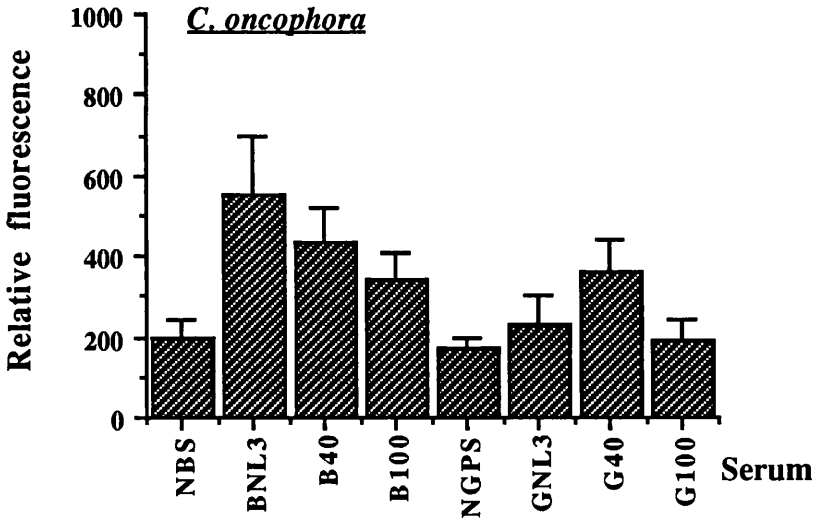


Figure 4.6b



Figures 4.6a and b

Animals exposed to various stages of *D. viviparus* produce antibody which binds to the L3 sheath of *O. ostertagi* and *C. oncophora.*

Antibody levels in the sera of uninfected calves (NBS) and guinea pigs (NGPS) were compared with those in pooled sera from calves or guinea pigs exposed to two infections of normal L3 (BNL3 and GNL3), or 400Gy L3 (B40 and G40) or 1000Gy L3 (B100 and G100). The serum samples were incubated with either *O. ostertagi* (Figure 4.6a) or *C. oncophora* (Figure 4.6b) sheathed L3.

Figure 4.6c

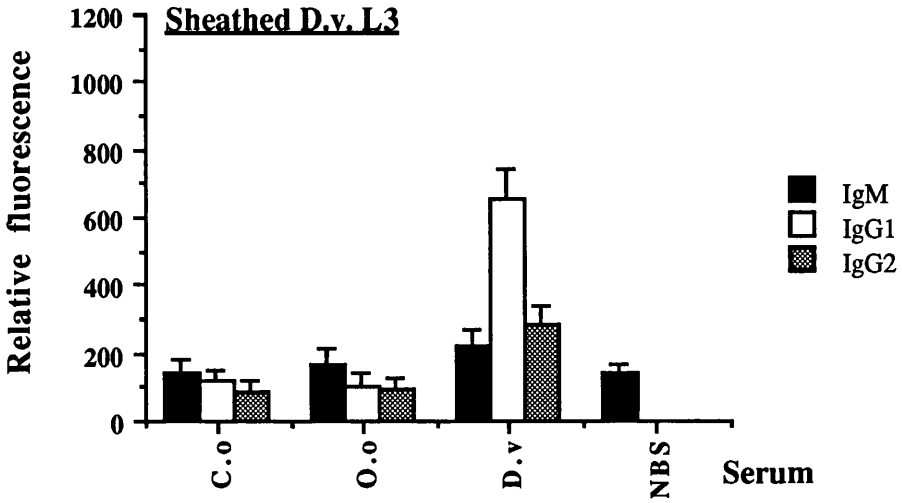
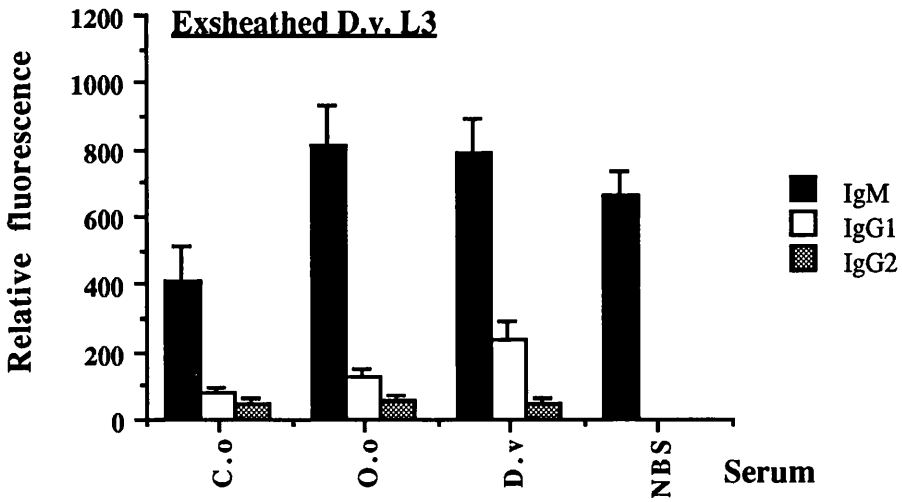


Figure 4.6d



Figures 4.6c and d

Calves infected with *O. ostertagi* (Figure 4.6c) or *C. oncophora* (Figure 4.6d) do not produce antibody specific for the surface of sheathed or exsheathed *D. viviparus* L3.

Antibody directed against antigens on the surface of sheathed L3 (Figure 4.6c) and exsheathed L3 (Figure 4.6d) were measured in the serum of calves exposed to three infections of *D. viviparus* (D.v), *O. ostertagi* (O.o) or *C. oncophora* (C.o). IgG antibody levels were not measured in the normal bovine serum (NBS).

4.3 Discussion

The measurement of antibodies during parasitic infection may not only assist in the diagnosis of some of the more covert parasitoses, e.g. human filariasis, but may also indicate profiles of antigen recognition which could be associated with protective immunity. Antibodies may also be used to isolate and/or locate potential immunogenic molecules from parasite material or, alternatively, from relevant clones within a cDNA expression library. Once the native or recombinant antigens have been purified in sufficient quantity they can be further defined in terms of their structural, biochemical and immunological properties. It is therefore of relevance in defining host:parasite interactions to examine antibody responses to live components of the parasite.

The first experiments presented here assayed the binding of bovine antibody to the surface of *D. viviparus* L3 and adult stages over the course of infection or vaccination. During the course of normal L3 infection in calves, there was a characteristic anamnestic response to the surface of the adult parasite as demonstrated by the initial burst of IgM antibodies which was succeeded by a class switch to IgG. These kinetics perhaps reflected the longevity of the antigens presented to the host immune system by the adult stages. Of interest here, was the fact that the IgG_1 and IgG_2 antibodies increased at the presumed time of adult worm expulsion.

Following infection with normal third stage larvae, a strong and persistent response, consisting predominantly of IgG_1, was observed against the surface of the L3 sheath. It could be envisaged that this response to the sheath might be to the parasite's benefit by diverting important effector functions away from the cuticle of migrating third larval stages.

The surface of the exsheathed L3 was bound strongly by IgM antibodies in all pre-infection calf sera examined. This binding was also present in newborn calf serum, but absent in foetal bovine serum, and suggested that the IgM antibody concerned could be transferred in the colostrum. It could be argued that the sodium hypochlorite, present in Milton used for exsheathing the L3 , may have damaged the L3 cuticle surface and exposed sub-surface epitopes which may then have been recognised by heterophile antibody. This possibility prompted the electron microscopy study which indicated that there was no apparent damage to the L3 cuticle following Milton exsheathment. That exsheathing larvae in this manner did not mediate cuticular damage was further suggested from studies which demonstrated that the surface of L3 parasites exsheathed in pepsin, HCl and bile was also bound by serum from normal calves (not shown). The results therefore suggested the presence of heterophile antibodies to the L3 cuticle surface and these may have been produced by a response to antigens present in the feed

or in the bacterial flora of the gastrointestinal tract of calves (Tizard, 1982; Tagliabue *et al.*, 1988). The presence of IgM antibodies on the L3 cuticle surface may have important repercussions for the recognition of this stage as these antibodies may act to block the binding of more effective immunoglobulin isotypes, for example IgG_1 or IgG_2 (Capron, 1987). This has been observed in *S. mansoni* infections in humans, rats and mice where both IgG_4 (Hagan *et al.*, 1991; Hagan, 1992) and IgM (Khaliffe *et al.*, 1986) have been implicated in blocking the effector functions of other antibody subclasses, for example IgE.

Following normal L3 infection, the levels of IgM directed against the L3 cuticle surface increased substantially while the IgG levels rose only marginally. This pattern was observed after several infections and the low IgG_1 anti-L3 responses may have been associated with the fact that , following exsheathment, the L3 soon moult to the next stage (Poynter *et al.*, 1960; Jarrett and Sharp, 1963). As the succeeding stages have been shown to express different surface antigens, animals would only be exposed to the L3 surface antigen repertoire for short periods of time and therefore might be unable to mount secondary responses against these antigens. Alternatively, it may be, that after the primary infection with normal L3, larvae are stopped at the level of the intestinal mucosa or mesenteric lymph nodes and systemic priming against these stages is therefore limited. This hypothesis, however, would not explain results obtained with the L3 sheath antigens, against which elevated IgG was observed following multiple infections. Furthermore, as indicated above, heterophile IgM may bind to the surface of the exsheathed L3 and this may have blocked the binding of IgG with a resultant reduction in clonal expansion and class switching to this immunoglobulin isotype.

The results obtained with the serum from calves vaccinated with 400Gy-irradiated larvae agreed with those of Jarrett *et al.*, (1961a), Cornwell (1960a) and Poynter *et al.* (1960), in that vaccination did not necessarily induce high circulating antibody levels. Only after normal L3 challenge, was there an obvious increase in the antibodies that bound to the surface of the sheathed and exsheathed L3. This increase was mostly in the IgG_1 subclass and was, therefore, indicative of a secondary response. In the case of 1000Gy L3 vaccination, it was observed that only after multiple vaccinations or after an ensuing normal L3 challenge, was there increased recognition of the sheathed and exsheathed L3 surfaces. The remarkably high levels of IgG_1 stimulated in these calves were probably a result of the high intravenous doses of 1000Gy L3 administered. The antigens of these L3 may have persisted longer than those of normal or 400Gy L3, as 1000Gy L3 develop no further. Furthermore, local intestinal effector mechanisms which may have been induced after oral infections might not have been stimulated following intravenous vaccination with 1000Gy L3 and perhaps on subsequent oral challenge, considerable numbers of parasites may have reached the systemic circulation

to stimulate high levels of IgG_1. The relevance of these antibodies to the development of protective immunity is questionable, however, as serum from calves infected repeatedly with 1000Gy L3 failed to passively confer immunity to naive recipients (Canto, 1990).

To further examine local effector mechanisms, bile and pulmonary mucus from a field case of parasitic bronchitis were examined for L3 surface-specific antibody. IgG_1 and IgA were examined as both these isotypes have been demonstrated in the external secretions of cattle and sheep, with the former isotype being the most predominant (Newby and Bourne, 1976; Lascelles *et al.*, 1986). Although IgA has been observed to be abundant in nasal secretions of cattle, this isotype was superseded by IgG_1 in bronchoalveolar lavage fluid (Mach and Pahud, 1971). Bile was used in the analysis due to its accessibility and also because it has been demonstrated as a source of intestinal IgA in both sheep (Husband, 1985) and rats (Jackson *et al.*, 1978; Hall *et al.*, 1979) and biliary IgG levels in sheep have been correlated with serum concentrations (Hall, 1986). It was therefore anticipated in these experiments that the high levels of *D. viviparus* -specific antibody present in the serum of the field-infected calf would be reflected in the bile. Neither *D. viviparus* -specific IgG_1 nor IgA, however, was observed in the bile or pulmonary secretions of this calf, despite there being high circulating levels of IgG_1. It is possible, however, that bile was not the ideal intestinal fluid to examine. It has been observed in *T. colubriformis*-infected sheep that locally-produced IgG_1 and IgG_2 diffuse through the intestinal mucosa and the presence of these antibodies was associated with early rejection of incoming larvae (McClure *et al.*, 1992).

D. viviparus, with its migratory life-cycle appeared to stimulate a strong antibody response to its own L3 sheath and to those of *O. ostertagi* and *C. oncophora*. In contrast, the latter two parasites did not appear to stimulate strong systemic antibody responses to either their own L3 surfaces or against that of *D. viviparus*. This was likely to be a consequence of their intra-luminal life-style where the stimulation of local antibody may be of more relevance to protective immunity. The cross-reactivity of epitopes on the L3 sheaths was in agreement with the results of Gilleard, Tait and Duncan (1992) who described a monoclonal antibody reactive against the L3 sheaths of these and other, related nematode species. The existence of such cross-reactive epitopes argues for a role in essential functions of the parasite and, although selective pressure may favour the presence of species-specific surface molecules, it must not be over-looked that these epitopes may represent common components in otherwise divergent molecules. In addition, cross-reactive epitopes may influence future vaccine design in that protection could be achieved against several nematode species using group-specific antigens. Cross-reactivity is well recognised in the surface epitopes of filarial species (Maizels, Philipp and Ogilvie, 1982), but in these species it has been suggested that the

conservation of specific molecules, for example those with tolerogenic epitopes, may be of relevance to immune avoidance (Maizels, Burke and Denham, 1987).

From the results obtained here it is difficult to interpret which humoral responses are of relevance to the acquisition of resistance to *D. viviparus*. Increases in IgG_1 or IgG_2 were observed at around the time of adult parasite expulsion and, in the case of the latter isotype, this was the only time that obvious rises were evident. IgG_1 also increased when animals were challenged after vaccination. However, it may have been coincidental that these immunoglobulins were increasing at the time of immune rejection and additional studies are warranted to further define their involvement.

It is assumed that death of adult *D. viviparus* is mediated by immune mechanisms 60-90 days after infection (Jarrett and Sharp, 1963) and the fact that corticosteroid-treated calves sustain their worm burdens beyond this time supports this theory. Bovine IgG_1 and IgG_2 antibody subclasses have been demonstrated to fix bovine complement and bind cultured monocytes and bovine IgG_2 has been shown to mediate phagocytosis by neutrophils and macrophages *in vitro* (McGuire, Musoke and Kurtii, 1979; Musoke, Rurangirwa and Nantulya, 1986). The involvement of these antibody subclasses in adult parasite damage by ADCC may, therefore, be proposed. Unfortunately, neither serum nor pulmonary mucus IgE levels could be measured in these studies. As IgE is thought to play a major role in the expulsion of other parasite species in various host locations (Jarrett and Miller, 1982), speculation regarding the precise role of antibody in *D viviparus* infection awaits the elucidation of the involvement of IgE.

Together with IgE, some IgG subclasses have been associated with the development of successful immune responses against several helminth species. For example, IgG_1, IgG_{2a}, IgG_{2c} and IgE were all demonstrated to stimulate rapid expulsion of *T. spiralis* following priming with *H polygyrus* infection or adoptive T- cell transfer (Bell *et al.*, 1992). In *T. muris* infections, elevated levels of IgG_1 were observed in resistant mouse strains, while IgG_{2a} was distinguished as the predominant isotype in susceptible mice (Else and Grencis, 1991a). Although these antibody levels were not absolute correlates of responder status, they were proposed as reflecting important responses at the intestinal level. The interstrain differences were thought to be related to which Th subpopulations were preferentially stimulated (Else and Grencis, 1991b). How relevant these rodent T-cell responses are to the development of immunity in the bovine is not clear. Similar Th cell subsets have, however, recently been demonstrated in human lymphocyte populations (Romagnani, 1991) and it is conceivable that they exist in cattle. It would be difficult to examine the relevance of Th subpopulation responses in *D. viviparus* infection as the cattle population is highly outbred and few inbred strains of guinea pigs are available to enable in depth inter-strain variation studies. Moreover, in mice, where inbred strains are readily available, this parasite terminates development by

Day 4 post-infection (J. Gilleard, personal communication). The examination of antibody subclass profiles following infection and vaccination with *D. viviparus* was therefore the first feasible step in the definition of immune responses to this parasite. The next step would perhaps be to attempt to define the role of T cell responses by harvesting, culturing and stimulating bovine T-cells from bronchial or mesenteric lymph nodes of infected or vaccinated calves and examining the cytokine profiles induced. This would verify the isotype patterns found in these studies and would enable the first examination of potential T cell responses to *D. viviparus* in calves.

Evidence obtained in other parasite/host systems indicates that the development of protective humoral or cellular immunity is rarely mutually exclusive, so it would be wrong to speculate on protective immune mechanisms without considering the role of cellular immunity. In addition, non-specific inflammatory components such as complement, myeloid cells and mast cell products, have also been implicated in the elimination of parasites (Wakelin, 1978). It was not within the scope of these studies to examine parameters relating to cell-mediated mechanisms but a strong multicellular response has been observed in the lungs of animals responding to *D. viviparus* challenge (Jarrett and Sharp, 1963). Also, in *S. mansoni* vaccination studies in mice using radiation-attenuated cercariae, lung-phase immunity was associated with pulmonary recruitment of mononuclear cells, including specific T cells (Wilson, Coulson and Dixon, 1986). The mononuclear cell population was capable of a potent secondary response which indicated the potential importance of T-cell memory at the level of the lungs (Ratcliffe and Wilson, 1992).

Chapter 5

Guinea pig immunisation experiments

5.1 Introduction

In any rational approach to the development of an anti-helminth subunit vaccine, a major aim is the definition of sources of protective parasite antigens. Candidate antigens may be distinguished in an empirical manner using various parasite preparations or fractions in successive vaccination and challenge experiments. This strategy of analysing responses to parasite components which stimulate different levels of immunity was used to define a fraction from onchospheres capable of protecting sheep against *Cysticercus ovis* infection (reviewed by Rickard, 1989). A polypeptide present in one of the protective fractions was subsequently observed to stimulate resistance in sheep when expressed as a fusion protein with *S. japonicum* glutathione S-transferase (Johnson *et al.*, 1989).

An alternative strategy is the definition of protective antigens by a rational approach in which components essential to parasite survival are identified. Immunisation against such components may interfere with the development of the parasite in the host. This scheme has been advocated for several human parasites, most notably *Schistosoma* spp where a glutathione S-transferase was isolated which was considered to act by scavenging potentially parasite-damaging oxygen radicals. When this enzyme was purified in native (Balloul *et al.*, 1987a) and recombinant (Balloul *et al.*, 1987b) forms it was able to confer protective immunity in mice.

Irrespective of the strategy, all protection studies must involve successive vaccination and challenge experiments in the definitive host species or an appropriate laboratory model. In this and the following Chapter, the preliminary steps in the characterisation of sources of host-protective antigens of *D. viviparus* are described. First, the empirical approach was applied in which three crude parasite preparations were examined for their protective capacity and one of these, adult ES products, further fractionated and assessed. Secondly, a parasite enzyme of potential immunomodulatory activity, acetylcholinesterase, was characterised and examined for potential interactions with host antibody.

Of the common laboratory animals, guinea pigs appear to be the most susceptible to infection with *D. viviparus* (Wade, Fox and Swanson, 1960a). Nevertheless, infection does not reach full patency in this species and the parasite lifespan is shortened (Poynter *et al.*, 1960). The migratory route taken by the parasite appears to be similar to that observed in the definitive bovine host with larvae reaching the lungs within 18 hours of infection (Soliman, 1953; Douvres and Lucker, 1958; Poynter *et al.*, 1960). Following two moults, the L5 remain in the lungs in constant numbers for approximately nine days before being expelled and very few parasites are present in the lungs by Day 14 of infection (Douvres and Lucker, 1958). Worm establishment varies greatly between

individual animals and also between experiments, the highest recorded recoveries being 5% (Poynter *et al.* , 1960). When guinea pigs were infected with 400Gy-irradiated L3, parasite development appeared to terminate at the transition from the fourth to fifth stage and it was suggested that these stages might be essential in the stimulation of protective immunity (Poynter *et al.*, 1960; Canto, 1990).

Guinea pigs have been used on several occasions in *D. viviparus* immunisation studies. Both normal L3 and 400Gy-irradiated L3 stimulated a high level of protection against re-infection in this species (Poynter *et al.*, 1960; Canto, 1990). An increase in *D. viviparus* -specific antibody following normal L3 infection in the guinea pig was first shown by Wade, Fox and Swanson (1960b) and was considered to be of relevance to the immune response following the successful passive transfer of immunity from infected guinea pigs to naive recipients (Wilson, 1966; Canto, 1990).

In an attempt to define possible sources of *D. viviparus* protective antigens, Canto (1990) immunised guinea pigs with somatic extracts of third stage larvae, in the context of FCA, and demonstrated a high degree of protection (67-85%). Moreover, both guinea pigs and calves, immunised with third stage larvae irradiated to 1000Gy, were significantly protected against challenge. As parasites irradiated at this level do not develop beyond the L3 stage, these experiments indicated that this stage contained antigens capable of stimulating protective immunity.

Similar studies using somatic extracts of adult *D. viviparus* have failed to produce a significant degree of protection in guinea pigs (Canto, 1990; Wilson, 1966) or calves (Jarrett *et al.*, 1960b), although high levels of antibody were observed in the immunised animals. Canto (1990) suggested that non-specific antibody may have interfered with relevant effector mechanisms and, interestingly, when somatic adult extracts were inoculated in the context of liposome adjuvant, significant levels of protection were obtained, despite the animals having low levels of circulating antibody (Canto, 1990). This adjuvant preparation injected on its own, however, reduced worm burdens by 42% and therefore probably had a significant effect on parasite development.

Products which adult *D. viviparus* release *in vivo* were first considered to be relevant in the development of host immunity by Wade, Swanson and Fox (1961) who vaccinated rabbits with lung exudate from infected calves. When the rabbits were subsequently challenged with normal L3, the parasites obtained from the lungs were stunted when compared with those recovered from challenge controls.

Several helminth species have been demonstrated to excrete or secrete substances which are likely to be essential to their survival and development within the host. For example, proteinases, which may assist in parasite nutrition and tissue penetration, have been identified in products released from adult *A. caninum* (Hotez *et al.*, 1985); larval *A. suum* (Knox and Kennedy, 1988); immature and mature *F. hepatica* (Dalton and

Heffernan, 1989); third stage *Strongyloides stercoralis* (McKerrow *et al.*, 1990); and third stage and adult *N. brasiliensis* (Healer, Ashall and Maizels, 1991). Moreover, adult *D. viviparus* have recently been shown to release several classes of proteinases which are recognised by antibody from infected and vaccinated calves (Britton, 1991). The possibility that ES products may be released in order to divert host effector mechanisms away from the surface of parasites has also been suggested (reviewed by Lightowlers and Rickard, 1988). Whatever their function, any search for potential vaccine candidates should include the examination of parasite ES products for their protective capacity and this Chapter describes a comparison of the protective capacity of *D. viviparus* ES products with that of somatic extracts. The humoral responses and their genetic control were examined and preliminary attempts were made to fractionate the adult ES for further definition of possible protective antigens.

5.2 Results

5.2.1 Optimising worm recoveries

In previous studies, the recovery of *D. viviparus* parasites from guinea pig lungs was observed to be highly variable (Poynter *et al.*, 1960; Canto, 1990). In an attempt to obtain more consistent results in the immunisation studies presented here, a preliminary experiment was carried out in which different methods of parasite infection and recovery were examined.

Two groups of six Dunkin-Hartley guinea pigs were infected with 5,000 *D. viviparus* L3. One group was infected directly into the oesophagus by stomach tube (Group A), the other *per os* via a 14g catheter (Group B). All animals were killed on Day 6 of infection and their lungs removed and processed for worm recoveries. Half of the disrupted lungs from each group were passed through fine gauze, the filtrate examined immediately for larvae and the remaining lung tissue Baermannised for 5 hours (Groups A1 and B1), while those of the remaining animals were immediately Baermannised for 5 hours (Groups A2 and B2). The results are shown in Table 5.1.

The average recovery rate was low, i.e. 2.6% overall. A high degree of variability in recovery was also observed among the individuals of each group, the most consistent level of parasite establishment being in those animals which were infected *per os*. Three out of the six guinea pigs infected by stomach tube had extremely low counts which were probably a result of regurgitation due to stress. All guinea pigs were subsequently infected *per os* and their disrupted lungs Baermannised without prior processing as these methods provided the most consistent worm recoveries.

Group:	A1	A2	B1	B2
Infected: Processed:	stom. tube filt.+Baer.	stom. tube Baer.	*per os* filt.+Baer.	*per os* Baer.
Individual recoveries	3 106 33	225 224 12	175 32 74	75 172 164
Mean recovery	47.3	157.3	93.7	137
Percentage Establishment	0.9	3.14	1.47	2.74

Table 5.1

Worm recoveries obtained from guinea pigs on Day 6 after a primary infection with 5,000 *D. viviparus* L3.

The guinea pigs in Group A were infected by stomach tube and those in Group B were infected *per os* via a 14 gauge catheter. At necropsy, the lungs from Groups A1 and B1 were disintegrated, passed through gauze, the filtrate examined for larvae and the remaining lung tissue Baermannised for 5 hours. The lungs of the animals in Groups A2 and B2 were disintegrated and Baermannised without further manipulation. The mean recoveries and establishment of larvae in each group are indicated.

5.2.2 Protection Experiment 1: which developmental stages of *D. viviparus* contain protective antigens?

Thirty Dunkin-Hartley outbred guinea pigs were randomly split into five groups of six. The animals in Group A acted as adjuvant controls and received two immunisations of PBS emulsified with an equal volume of FCA, on primary, and FIA, on secondary, immunisation. Those in Group B received two immunisations of a PBS-soluble extract of third stage larval homogenate (L3H), while those in Group C received a PBS-soluble extract of adult homogenate (AdH). The animals in Group D were immunised twice with adult ES products. All guinea pigs in Groups B to D were vaccinated twice and each received 60μg of antigen with FCA, on primary, and 100μg of antigen with FIA four weeks later. Four weeks after secondary immunisation, each animal in Groups A to D was challenged orally with 6,000 L3, together with six control animals (Group E). One animal in each group was bled prior to primary immunisation and all were bled fourteen days after secondary immunisation and at necropsy.

A sample of each antigen was examined by SDS-PAGE, under reducing conditions, and the protein profiles compared by Coomassie staining (Figure 5.1). In all three fractions, several components of similar mobility were observed but it could not be determined if these polypeptides were identical as two dimensional electrophoresis was not performed. As expected the somatic preparations were more heterogeneous than the adult ES products.

Four guinea pigs developed post-vaccinal ulcers, all of which healed, but there was no correlation between immunisation group and ulcer development. The worm recoveries are shown in Table 5.2.

The worm burdens of animals immunised with adult ES material were significantly different from the challenge controls (Mann-Whitney non-parametric test, 5% significance level). Although the mean reduction in worm burden for the animals immunised with L3 homogenate was 60.25%, the worm burdens obtained in this group were not significantly lower than those of the challenge controls The adult homogenate-immunised group also showed no significant reduction in worm burdens compared with the controls.

Total antibody levels to L3 and adult homogenate antigen extracts, measured by ELISA, were similar among individuals within each group and are not shown. The results obtained with pooled samples from each group taken following secondary vaccination and at necropsy are compared with pre-immunisation serum in Figures 5.2 a and b. Negligible increases in antibody levels were observed in the adjuvant and challenge control animals following immunisation. Adult homogenate-specific antibody levels increased by similar amounts in animals which received either L3 homogenate or

adult ES products (Figure 5.2a). The animals which were vaccinated with the homologous antigen (adult homogenate) had high specific antibody levels following secondary immunisation but were not protected, suggesting that much of the antibody response might have been directed against antigens irrelevant to the development of protection.

Animals vaccinated with all three antigen preparations displayed similar increases in antibody directed against the L3 homogenate preparation (Figure 5.2b) which suggested that there were antigens shared between the two developmental stages. A slight increase in antibody to the L3 stage antigens was seen in the control groups following challenge which indicated a response to migrating larvae.

Individual serum samples obtained after challenge were analysed by immunoprecipitation using ^{125}I-labelled adult homogenate or ES antigens (Section 2.5.11) and are shown in Figures 5.3 to 5.6. The larval homogenate preparation radiolabelled poorly and so analysis not was performed with this antigen preparation.

The challenge and adjuvant control animals demonstrated a very faint recognition of several polypeptides in the adult ES and homogenate extracts when the autorads were subjected to prolonged exposure (not shown). This may have been due to non-specific binding of antigen to the Protein A used in the assay. Sera from animals immunised with the L3 homogenate preparation precipitated most of the components labelled in both antigen preparations (not shown), but the banding patterns were unclear, particularly when the sera were precipitated against adult homogenate antigen. The recognition profiles of the individuals immunised with the L3 preparation were very similar, which was in contrast to the responses of animals immunised with the adult extracts.

Animals immunised with adult homogenate, although not protected to any degree, displayed a very strong recognition of both homologous and ES antigens (Figures 5.3 and 5.4). The radioactivity precipitated was higher than that obtained using serum from the adult ES-immunised animals but when the autorads were compared with those depicting the latter Group's responses (Figures 5.5 and 5.6), it was evident that a similar repertoire of molecules was precipitated. The degree of similarity was perhaps not surprising considering that both groups were vaccinated with adult-derived antigens in the context of a potent adjuvant. The upper component of the 38-42kDa doublet was only weakly recognised by animals which received adult homogenate but was strongly recognised by the adult ES-immunised guinea pigs. The adult ES components of M_r 14-18kDa did not appear to be present, or were not radiolabelled, in the adult homogenate preparation and this indicated that these molecules may be unique to adult ES products. Despite this, these components were recognised in the ES preparation by several animals which had been immunised with the adult homogenate antigen.

To summarise, no single antigen was exclusively recognised by the ES-immunised animals and so a specific recognition pattern could not be correlated with the development of protective immunity. Interestingly, the hyperimmune bovine serum did not recognise many antigens present in the adult homogenate preparation, in contrast to the strong recognition of the adult ES components (compare BNL3 tracks in Figures 5.4. and 5.5). This further implicated the importance of adult ES antigens in this host/parasite relationship and indicated that these products were being released during the course of a natural infection.

As the sera used in these immunoprecipitations were taken following challenge, it could be argued that some of the antibody detected may have been stimulated by the migrating third and fourth stage larvae but when post-secondary and post-challenge bleeds were compared (not shown), substantial differences in their recognition profiles were not observed.

The most striking aspect of the precipitation profiles was the high degree of heterogeneity in recognition displayed by the recipients of the adult parasite preparations. This occurred despite immunisation in the context of a potent adjuvant and the most obvious variability was evident in antibody responses against the lower molecular weight components.

Group	A	B	C	D*	E
Antigen	PBS/FCA	L3H/FCA	AdH/FCA	ES/FCA	chall. control
Individual recoveries:					
1	95	44	14	28	46
2	138	62	28	3	92
3	47	10	50	2	44
4	46	13	168	2	100
5	95	21	25	6	172
6	4	45	-	30	32
Mean	70.8	32.2	57.2	11.8	81.6
% reduction	13.2	60.2	29.9	85.5	-

Table 5.2

Protection Experiment 1 : worm recoveries of immunised animals six days after challenge with 6,000 L3 of *D. viviparus*.

Animals in Group B were immunised twice with L3 homogenate (L3H), while those in Group C received adult homogenate (AdH). Group D guinea pigs were immunised with adult ES products. Animals in these groups received antigen with an equal volume of FCA, on primary, and FIA, on secondary, immunisation. Animals which received adjuvant and PBS (Group A) acted as controls. The mean worm burden of each group was compared with that obtained from challenge controls (Group E) to give percentage reduction.

* indicates that the worm recoveries differed significantly from those of the challenge controls using the Mann-Whitney non-parametric test (5% significance level).

Figure 5.1

Coomassie-stained SDS-PAGE profile of antigen preparations used in Protection Experiment 1. The samples were run on a 5-25% SDS-polyacrylamide gradient gel under reducing conditions. The adult ES products were collected after 24 hours of parasite culture and were dialysed against PBS and concentrated to 20 times the original volume. They are compared to PBS extracts of adult and L3 homogenates. The relative molecular mass (M_r) of marker proteins (M) are indicated in kiloDaltons (kDa).

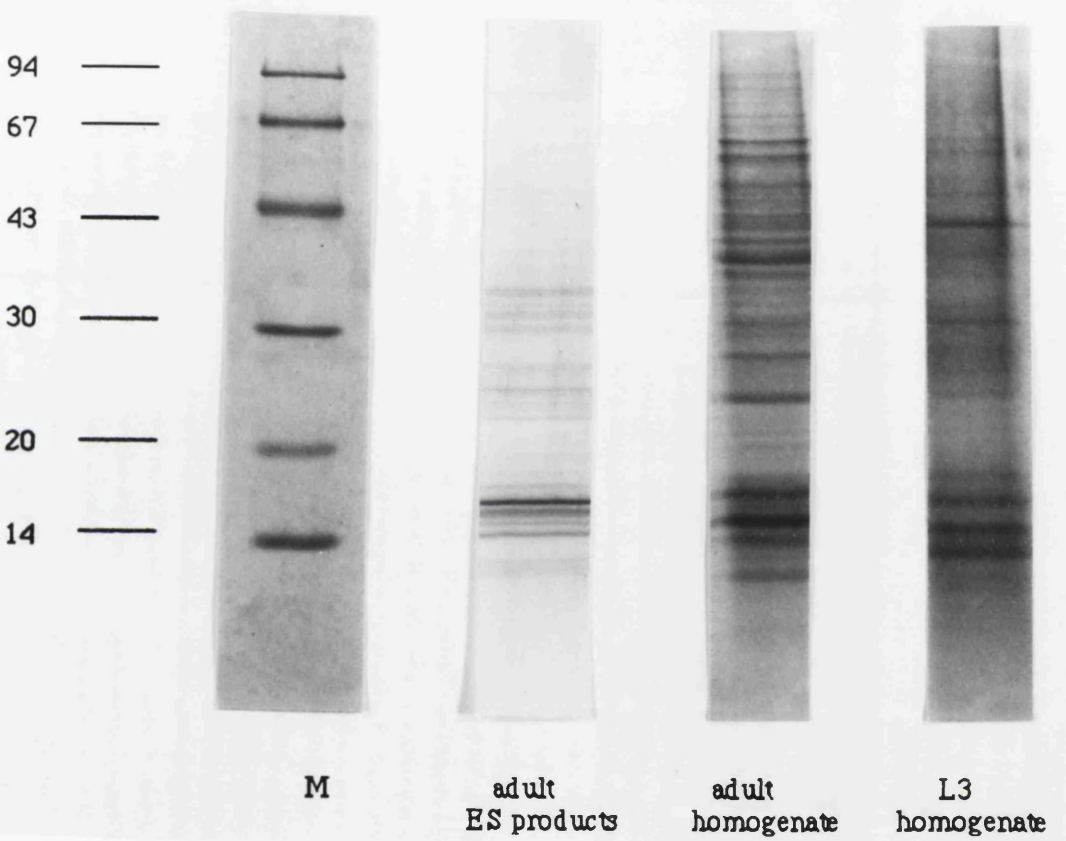

94
67
43
30
20
14
M
adult
ES products
adult
homogenate
L3
homogenate

Figure 5.2a

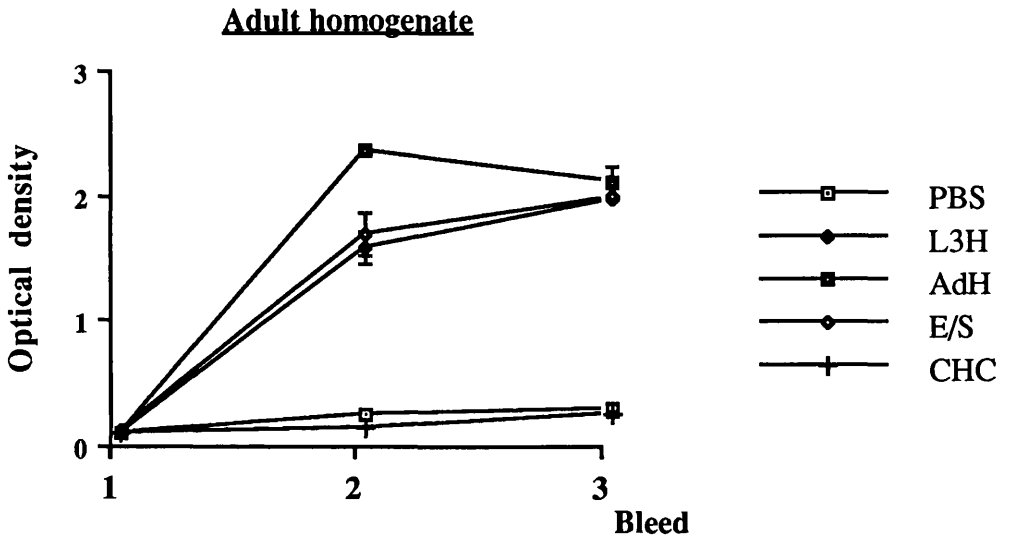
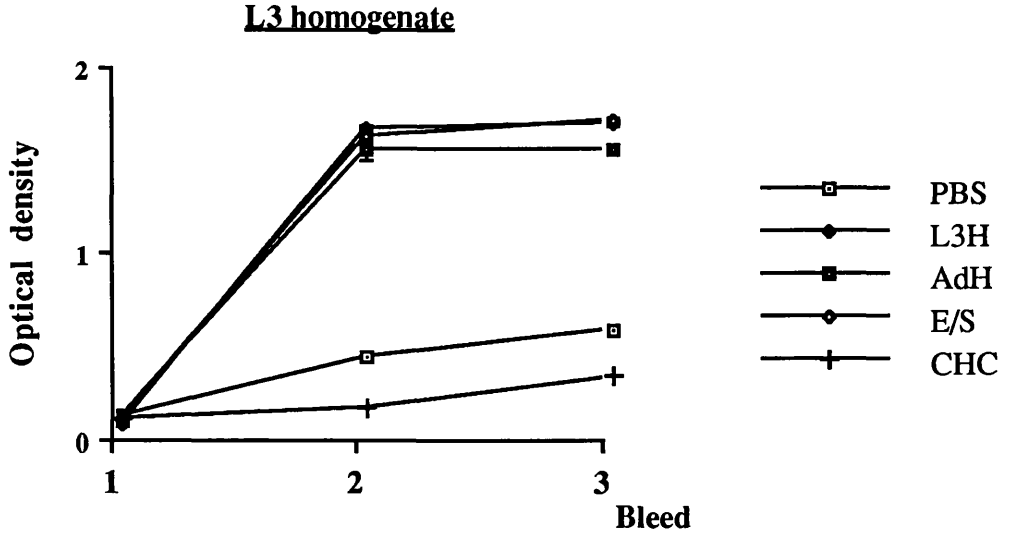


Figure 5.2b



Figures 5.2 a and b

Serum antibody levels present in pooled sera from guinea pig groups of Protection Experiment 1.

The group treatments are indicated in the legend. Pooled sera taken prior to immunisation (1), 14 days after secondary immunisation (2) and at kill (3) were measured against Tris/proteinase inhibitor extracts of adult homogenate (Figure 5.2a) and L3 homogenate (Figure 5.2b) by ELISA. All samples were carried out in duplicate and the means and standard deviations shown.

Figure 5.3 Protection Experiment 1: Recognition of adult homogenate antigens by antibodies in the serum of animals (C1-C5) immunised twice with adult homogenate PBS extract in the context of Freund's adjuvant. The serum was taken on Day 6 of challenge.

Radio-iodinated adult homogenate (Ag) was incubated with individual guinea pig serum and the immune complexes, precipitated by Staph A, were analysed by SDS-PAGE under reducing conditions. The antigen was also immunoprecipitated with bovine normal L3 infection serum (BNL3) for comparison and with normal guinea pig serum (pre) and foetal bovine serum (FBS) which acted as negative controls. The relative molecular mass (M_r) of marker proteins (M) are indicated in kiloDaltons (kDa).

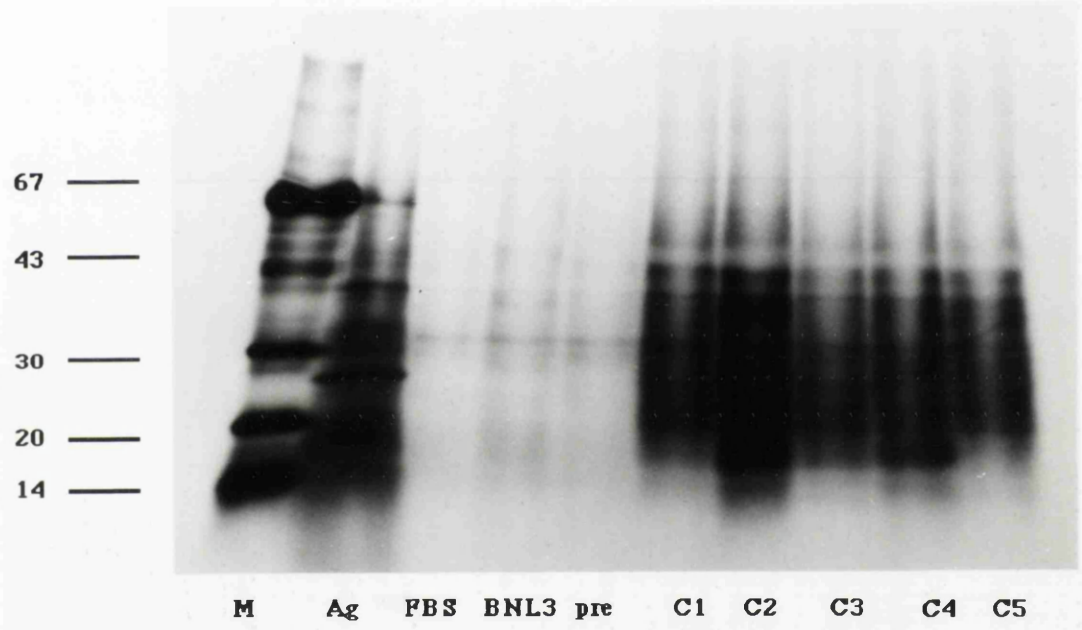

67
43
30
20
14
M
Ag
FBS
BNL3
pre
C1
C2
C3
C4
C5

Figure 5.4

Protection Experiment 1: Recognition of adult ES antigens by antibodies in the serum of animals (C1-C5) immunised twice with adult homogenate PBS extract in the context of Freund's adjuvant. The serum was taken on Day 6 of challenge.

Radio-iodinated adult ES products (Ag) were incubated with individual guinea pig serum and the immune complexes, precipitated by Staph A, were analysed by SDS-PAGE under reducing conditions. The antigen was also immunoprecipitated with bovine normal L3 infection serum (BNL3) for comparison and with normal guinea pig serum (pre) and foetal bovine serum (FBS) which acted as negative controls. The M_r of marker proteins (M) are indicated in kiloDaltons (kDa).

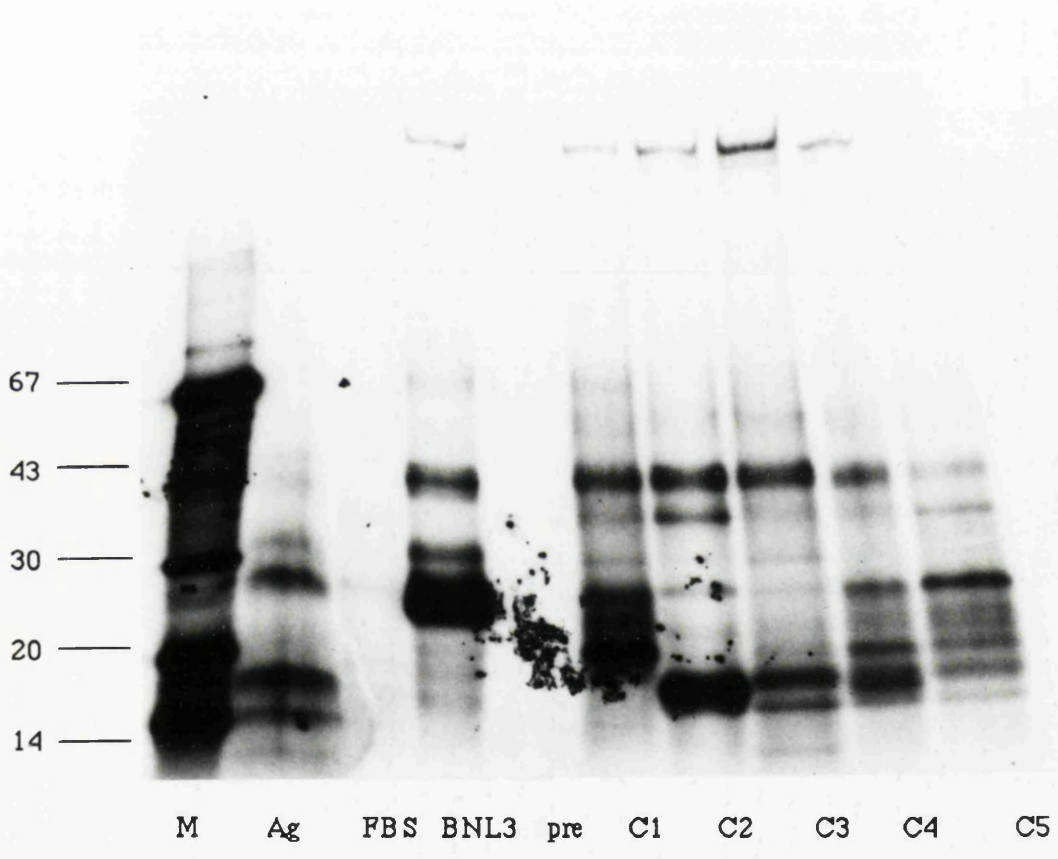

67
43
30
20
14
M
Ag
FBS
BNL3
pre
C1
C2
C3
C4
C5

Figure 5.5

Protection Experiment 1: Recognition of adult homogenate antigens by antibodies in the serum of animals (D1-D6) immunised twice with adult ES extract in the context of Freund's adjuvant. The serum was taken on Day 6 of challenge.

Radio-iodinated adult homogenate (Ag) was incubated with individual guinea pig serum and the immune complexes, precipitated by Staph A, were analysed by SDS-PAGE under reducing conditions. The antigen was also immunoprecipitated with bovine normal L3 infection serum (BNL3) for comparison and with normal guinea pig serum (pre) and foetal bovine serum (FBS) which acted as negative controls. The M_r of marker proteins (M) are indicated in kiloDaltons (kDa).

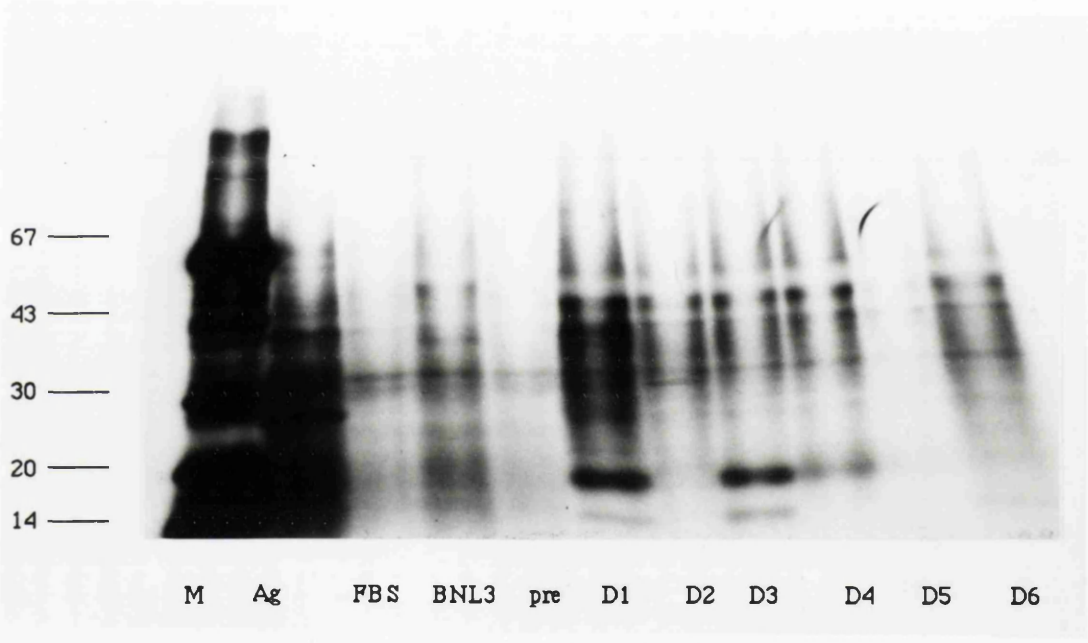
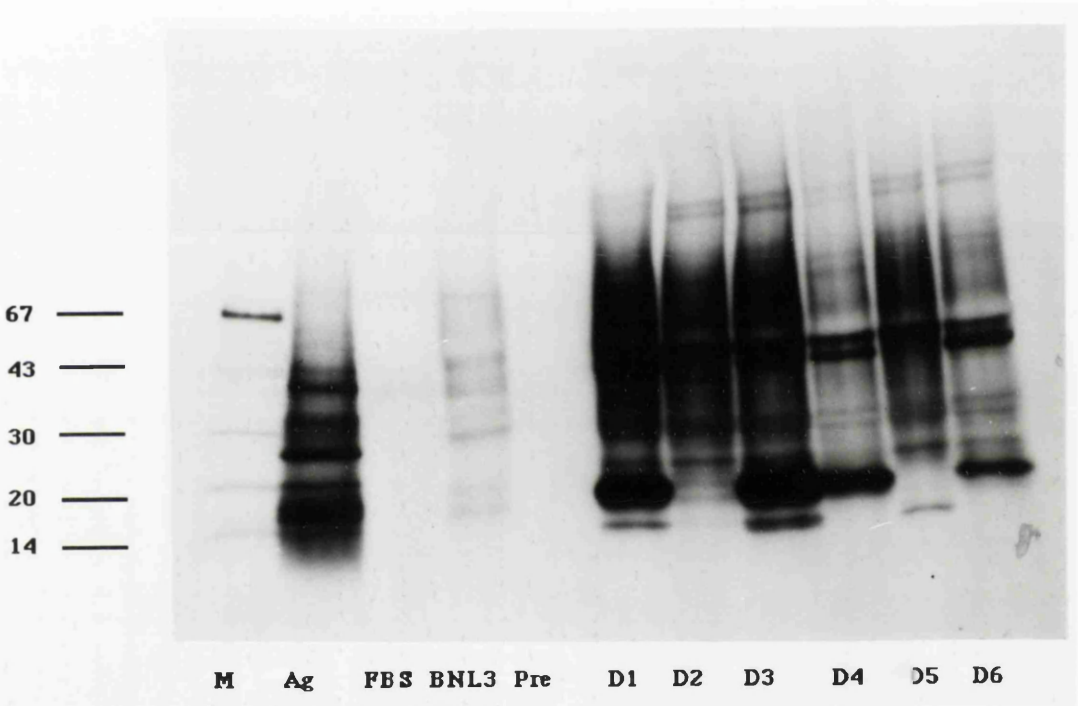

67
43
30
20
14
M
Ag
FBS
BNL3
pre
D1
D2
D3
D4
D5
D6

Figure 5.6

Protection Experiment 1: Recognition of adult ES antigens by antibodies in the serum of animals (D1-D6) immunised twice with adult ES extract in the context of Freund's adjuvant. The serum was taken on Day 6 of challenge.

Radio-iodinated adult ES products (Ag) were incubated with individual guinea pig serum and the immune complexes, precipitated by Staph A, were analysed by SDS-PAGE under reducing conditions. The antigen was also immunoprecipitated with bovine normal L3 infection serum (BNL3) for comparison and with normal guinea pig serum (pre) and foetal bovine serum (FBS) which acted as negative controls. The relative molecular mass (Mr) of marker proteins (M) are indicated in kiloDaltons (kDa).


67
43
30
20
14
M
Ag
FBS
BNL3
Pre
D1
D2
D3
D4
D5
D6

5.2.3 Protection Experiment 2: do adult ES antigens fractionated by SDS-PAGE retain their capacity to protect guinea pigs?

Protection Experiment 1 demonstrated that a high level of protective immunity could be induced by adult ES products, in contrast to somatic extracts of this stage, and an attempt was subsequently made to fractionate the ES antigens in order to further test their protective capacity.

A preliminary experiment was designed to compare the protective capacity of whole native ES products (Group B) with that of whole ES products either contained within (Group C), or electro-eluted from (Group E) SDS-polyacrylamide gels. The antigen preparation for Group C was produced by running whole ES products on SDS-PAGE under reducing conditions and the SDS was subsequently removed from the gel by washing it several times in Triton X-100. The gel was then frozen, ground in liquid nitrogen and emulsified with Freund's adjuvant. The ES preparation to be used for Group E was run on SDS-polyacrylamide gels under similar conditions and subsequently electro-eluted and concentrated (Section 2.5.3). As adjuvant controls, a group of guinea pigs were immunised with the adjuvant and PBS (Group A) and another group received Freund's and SDS-polyacrylamide gel without antigen (Group D).

Six male, Dunkin-Hartley guinea pigs in Groups A-E were vaccinated twice in the context of Freund's adjuvant following a protocol similar to that outlined for Protection Experiment 1. But in this case, the animals received 100μg of protein at both immunisations. The guinea pigs were challenged, together with 6 challenge controls (Group F), four weeks after secondary immunisation and killed on Day 6 post-challenge. In each group, one animal was bled prior to the start of the experiment and 14 days following primary immunisation. All animals were bled on Day 14 of secondary immunisation and at necropsy.

One animal in Group A and one in Group D were euthanased as severe dermal ulcers developed following primary immunisation. Less severe ulceration developed in single animals from Groups A, B, C and D.

The worm burdens are shown in Table 5.3. On statistical analysis there was no significant difference in the worm recoveries from any of the immunised groups when compared with the challenge and adjuvant controls. In contrast to Protection Experiment 1, the animals which received whole, native ES products had worm recoveries which did not differ significantly from those of the challenge control group.

The post-immunisation responses of each animal to adult ES antigen were measured by ELISA and the levels of total antibody of the individuals are depicted in Figures 5.7a to 5.7e. Animals in Groups A, D and F (controls) had negligible increases of antibody

levels which were therefore not examined further. The animals which received ES products within SDS-polyacrylamide gels (Figure 5.7c) had elevated levels of antibody following secondary immunisation, but these levels were substantially lower than those of animals which received either native ES materials (Figure 5.7b) or electro-eluted ES products (Figure 5.7e). It was difficult to accurately determine the amount of protein given to the guinea pigs which received ES products within gels, so these animals may have received less protein than those which were immunised with native or eluted adult ES materials. Individual antibody levels of the ES/SDS-polyacrylamide gel recipients were variable (Figure 5.7c) which possibly reflected the fact that animals received different parts of the gel containing different amounts of antigen.

In order to investigate further the responses of individuals which received native ES materials or ES materials within or eluted from gels, ES-specific IgM and IgG were measured. The IgG and IgM levels stimulated following secondary immunisation are demonstrated in Figures 5.8a to 5.8c.

In contrast to the results obtained with whole immunoglobulin, there was a considerable difference in IgG levels between the animals which received the native antigen and those which received the electro-eluted denatured ES products. In the former Group (B), IgG increased considerably following secondary immunisation (Figure 5.8a), while only two animals which received eluted adult ES (Group E) had elevated levels of this antibody subclass (Figure 5.8c). Although the animal in Group B which had the highest worm recovery (B4: 161 L5) had the lowest IgG levels, overall there was no correlation between IgG levels and individual worm burdens.

The ES-specific IgM levels in animals which received denatured ES products (Groups C and E) did not rise above pre-infection levels (Figures 5.8b and c), while the IgM levels of those which received the native antigen (Group B) were extremely variable (Figure 5.8a). Again, there was no correlation between antibody levels and individual worm burdens.

Sera obtained after secondary immunisation and challenge were immunoprecipitated with radiolabelled adult ES antigen. Interestingly, only the immunoprecipitates of secondary and post-challenge sera of the native adult ES recipients (Group B) were positive and the immunoprecipitation of the post-challenge bleed of this Group is shown (Figure 5.9). The counts and autorads of all the other groups were negative. As in Protection Experiment 1, heterogeneity in the antibody repertoire directed against adult ES antigens was obvious in the native ES-immunised Group. This was particularly so with regard to antibody responses directed against the lower M_r components and, as found with the ELISA, guinea pig B4 which had the highest worm recovery in this group, had low counts following immunoprecipitation. This guinea pig also exhibited a poor recognition pattern of the adult ES antigen.

Group	A	B	C	D	E	F
Antigen	PBS	ES	ES gel	gel	eluted ES	challenge control
Individual recoveries:						
1	4	101	25	99	22	4
2	48	12	12	died	33	103
3	70	13	26	67	86	28
4	114	161	194	460	117	46
5	78	24	237	157	160	133
6	died	24	48	70	69	139
Mean recovery	62.8	55.8	90.3	170.6	81.2	75.5
% reduction	16.8	26.1	none	none	none	-

Table 5.3

Protection Experiment 2 :worm recoveries of immunised animals six days after challenge with 6,000 L3 of *D. viviparus*.

Animals in Group B were immunised twice with whole native ES (ES). Those in Group C received ground SDS-polyacrylamide gel incorporating whole adult ES products (ES gel) and Group D animals were immunised with gel without antigen (gel). Group E guinea pigs were immunised with adult ES products electro-eluted from SDS-polyacrylamide gels. Animals in these groups received antigen with an equal volume of FCA on primary, and FIA on secondary, immunisation. The guinea pigs of Group A acted as Freund's adjuvant controls. The mean worm burden of each group was compared with that obtained in challenge controls (Group F) to give percentage reduction.

None of the group worm recoveries differed significantly from the challenge controls by the Mann-Whitney non-parametric test (5% significance level).

Figure 5.7a

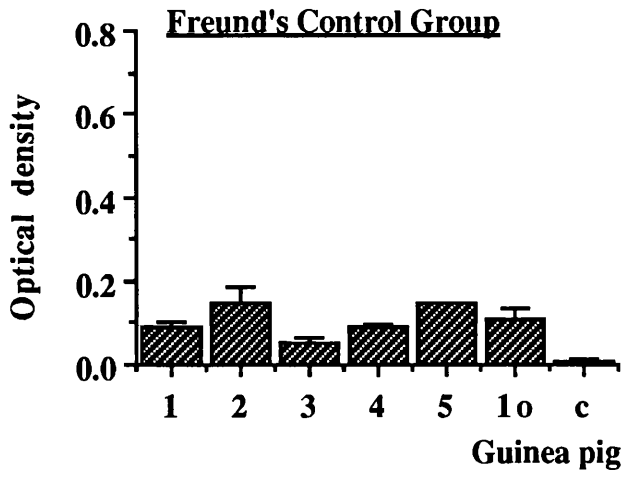
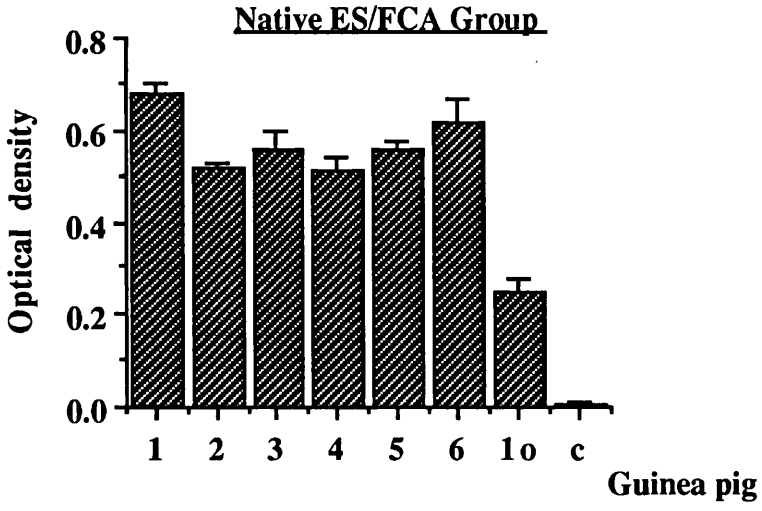


Figure 5.7b



Figures 5.7 a and b

Total antibody levels to adult *D. viviparus* ES products measured by ELISA.

Guinea pigs (1-5 or 1-6) received PBS (Figure 5.7a) or native whole ES (Figure 5.7b) in the context of Freund's adjuvant. Serum was taken on Day 14 of secondary immunisation. The samples were compared to pre-infection serum (c) and serum taken from one animal on Day 14 of primary immunisation (1^{o}). All sera were analysed in duplicate and the results are expressed as the mean optical density measured at 492nm.

Figure 5.7c

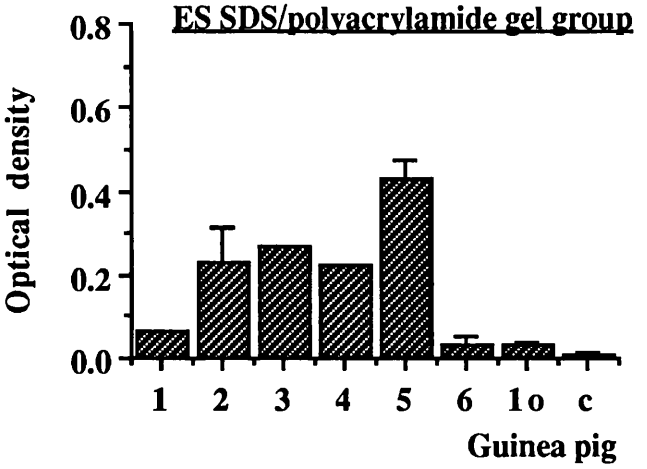
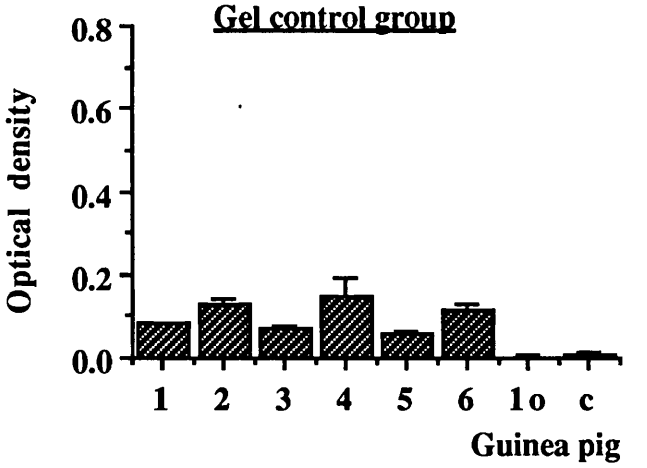


Figure 5.7d



Figures 5.7c and d

Total antibody levels to adult *D. viviparus* ES products measured by ELISA.

Guinea pigs (1-6) received whole ES products contained within ground SDS-polyacrylamide gel (Figure 5.7c) or were immunised with similarly treated gels in the absence of antigen (Figure 5.7d). Both groups were immunised in the context of Freund's adjuvant. Serum was taken on Day 14 of secondary immunisation. The samples were compared to pre-infection serum (c) and serum taken from one animal on Day 14 of primary immunisation (1^{o}). All sera were analysed in duplicate and the results are expressed as the mean optical density measured at 492nm.

Figure 5.7e

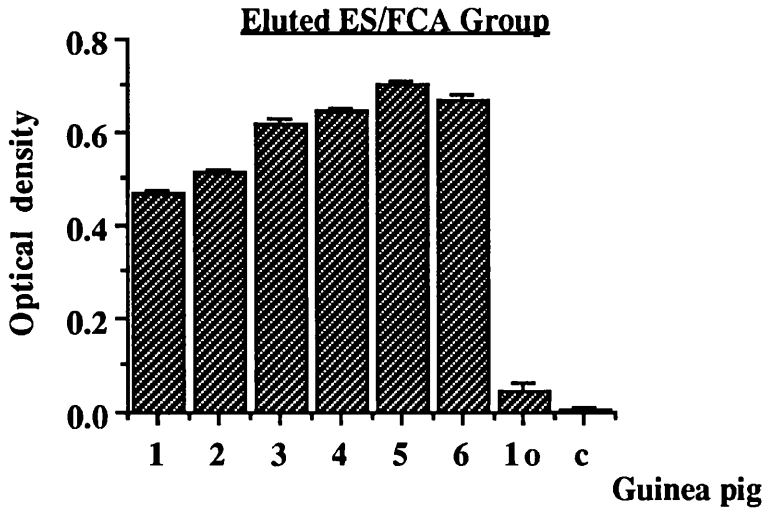
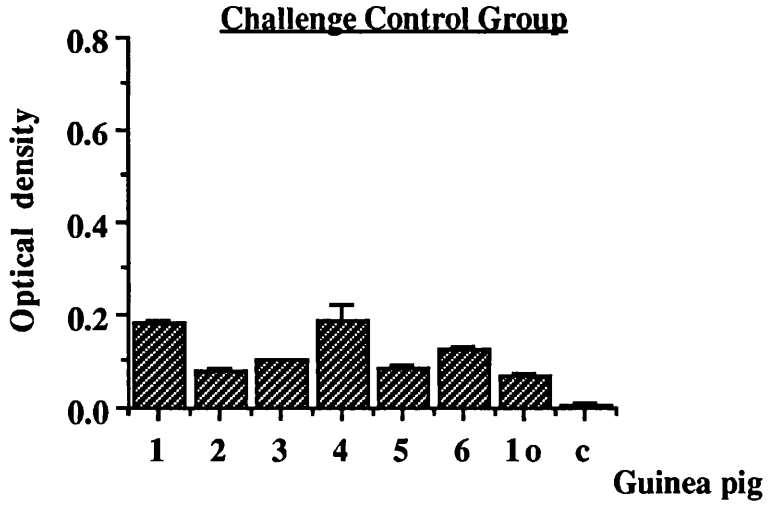


Figure 5.7f



Figures 5.7 e and f

Total antibody levels to adult *D. viviparus* ES products measured by ELISA.

Guinea pigs (1-6) received electro-eluted whole ES products (Figure 5.7e). Serum was taken on Day 14 of secondary immunisation. The antibody levels present in non-immunised controls are also shown (Figure 5.7f). The samples were compared to pre-infection serum (c) and serum taken from one animal on Day 14 of primary immunisation (1^{o}). All sera were analysed in duplicate and the results are expressed as the mean optical density measured at 492nm.

Figure 5.8a

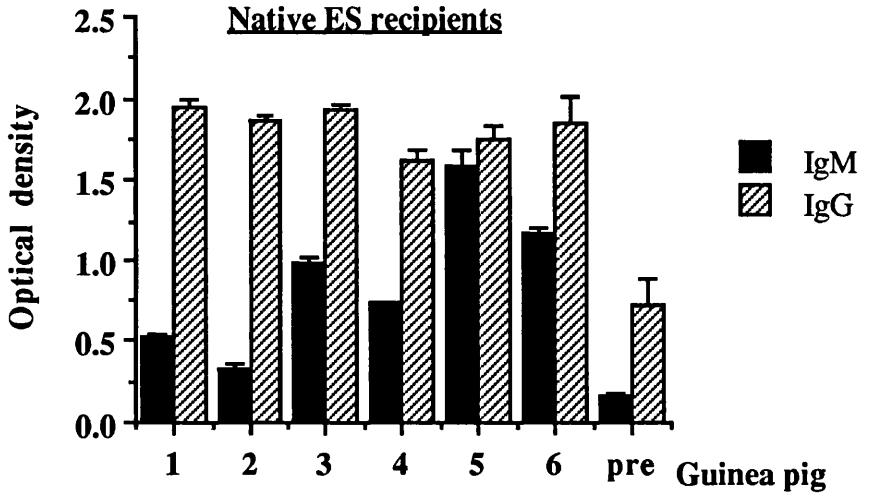
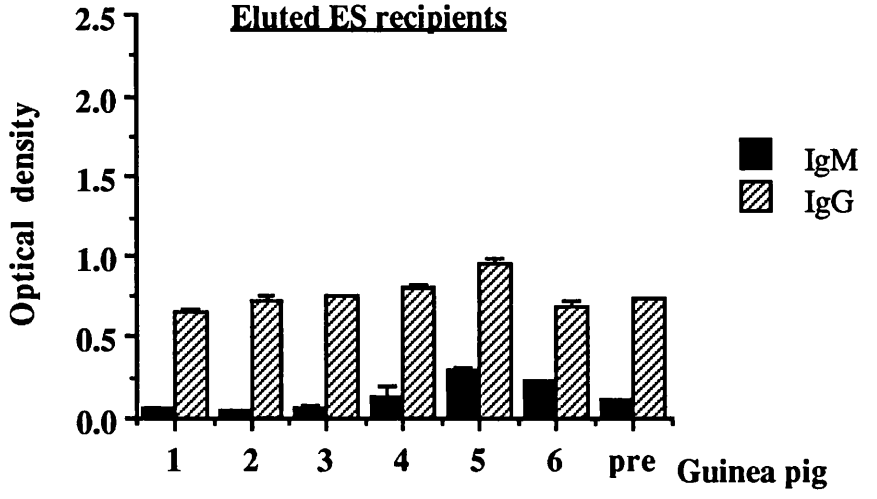


Figure 5.8b



Figures 5.8 a and b

IgM and IgG antibody levels to *D. viviparus* adult ES products measured by ELISA.

Serum IgM and IgG levels of individuals (1-6) which received native whole adult ES products (Figure 5.8a) or whole adult ES products electro-eluted from SDS-polyacrylamide gels (Figure 5.8b). The sera were sampled on Day 14 of secondary immunisation. The samples were compared to pre-infection serum (pre) and all were tested in duplicate. The results were expressed as the mean optical density measured at 492nm.

Figure 5.8c

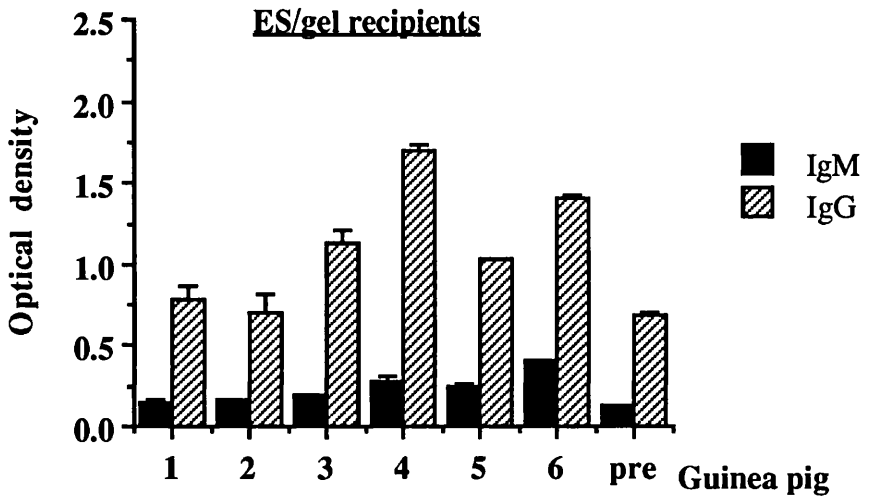


Figure 5.8 c

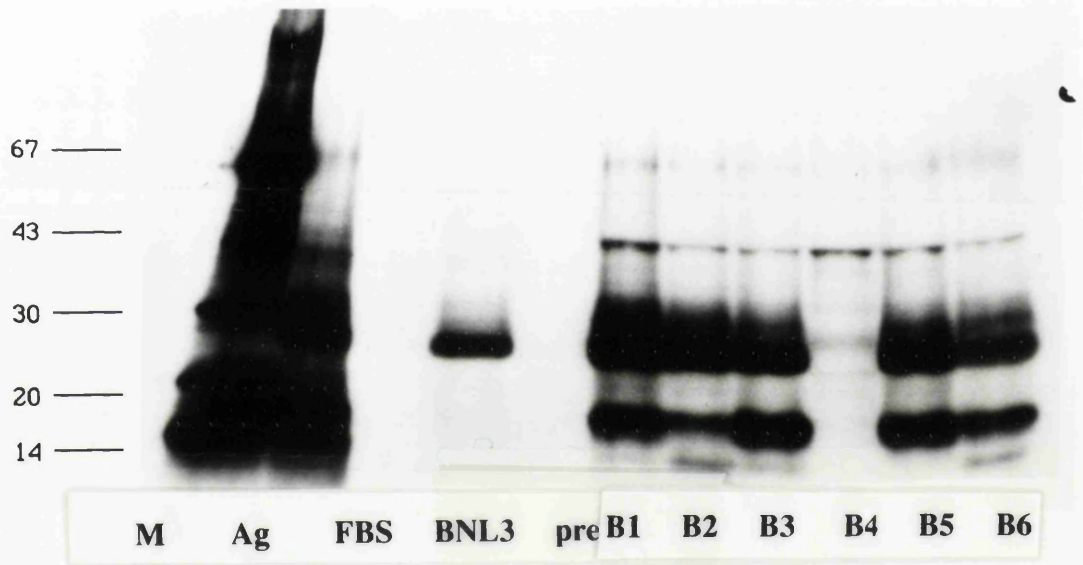
IgM and IgG antibody levels to *D. viviparus* adult ES products measured by ELISA.

Serum IgM and IgG levels of individuals (1-6) which received ground SDS-polyacrylamide gels containing whole adult ES products. The sera were sampled on Day 14 of secondary immunisation. The samples were compared to pre-infection serum (pre) and all were tested in duplicate. The results were expressed as the mean optical density measured at 492nm.

Figure 5.9

Protection Experiment 2: Recognition of adult ES antigens by antibodies in the serum of animals (B1-B6) immunised twice with adult ES extract in the context of Freund's adjuvant. The serum was taken on Day 6 of challenge.

Radio-iodinated adult ES products (Ag) were incubated with individual guinea pig serum and the immune complexes, precipitated by Staph A, were analysed by SDS-PAGE under reducing conditions. The antigen was also immunoprecipitated with bovine normal L3 infection serum (BNL3) for comparison and with normal guinea pig serum (pre) and foetal bovine serum (FBS) which acted as negative controls. The M_r of marker proteins (M) are indicated in kiloDaltons (kDa).


67
43
30
20
14
M
Ag
FBS
BNL3
pre
B1
B2
B3
B4
B5
B6

2.2.4 Protection Experiment 3: is there genetic control of the immune response to *D. viviparus* ES products?

A considerable degree of heterogeneity in antigen recognition was found to occur in outbred guinea pigs in their response to immunisation with various antigen preparations. This variability was unlikely to be due to variation in the antigens given to individual animals and was, therefore, likely to be host-related and genetically-determined. The genetic control of immune responses can be assessed using inbred strains of laboratory animals. Experiments using Major Histocompatibility Complex (MHC) congenic strains of mice and rats have indicated that genes mapping to this region may influence the antibody response to several nematode species (Kennedy, 1989). Unfortunately, congenic strains of guinea pig were not available for this study. However, strain 2 and 13 guinea pigs were available which are similar at their MHC class I loci, but disparate at their MHC class II loci (Klein, 1986). In order to investigate the control of the antibody repertoire in ES-immunisation, the two inbred strains of guinea pig were immunised with *D. viviparus* ES products and their immune responses compared.

Five female strain 2, six female strain 13 and six male Dunkin-Hartley guinea pigs were immunised twice with native ES products in the context of Freund's adjuvant following the same protocol described in Protection Experiment 2. Unfortunately, viable third stage larvae for challenge were not available until six weeks after secondary immunisation. Six male Dunkin-Hartley guinea pigs acted as challenge controls since suitable inbred guinea pigs were not available for inclusion in the experiment. The animals were bled as previously and all were killed on Day 6 after challenge.

Eight guinea pigs developed dermal ulcers following secondary immunisation but there was no correlation between guinea pig strain and ulcer development.

The worm burdens are detailed in Table 5.4. One of the challenge control animals was found to have regurgitated its gastric contents soon after infection and was therefore omitted from the analysis. Both the strain 13 and Dunkin-Hartley ES-immunised groups had worm burdens significantly lower than the challenge controls.

Individual serum samples taken after secondary immunisation and challenge were examined for *D. viviparus* adult ES-specific antibody by ELISA. The results are shown in Figures 5.10a to 5.10c.

After secondary immunisation, all animals displayed elevated immunoglobulin levels directed against the ES antigens. The antibody levels of the strain 2 guinea pigs were significantly lower than those of the other two groups. As expected, the outbred Dunkin-Hartley individuals exhibited a more variable pattern in antibody levels compared with the two inbred strains after secondary immunisation but not post-challenge. After challenge, the antibody levels stimulated may have been influenced by the number of

larvae migrating in each animal. As in the previous experiments, no correlation was observed when antibody levels were compared with individual worm recoveries.

Serum taken after secondary immunisation and after challenge was also precipitated against radiolabelled adult ES products. Autorads of the precipitates of the post-challenge sera from strain 2 (Figure 5.11) and Dunkin-Hartley (Figure 5.13) guinea pigs are presented, while the post-secondary immunisation sera of strain 13 guinea pigs are demonstrated in Figure 5.12.

Strain 2 guinea pigs had similar recognition patterns pre-challenge (not shown) and post-challenge, with the exception of an 18kDa molecule which was only precipitated by the animals following normal L3 challenge. This was also the case for five out the six strain 13 guinea pigs, but was not so obvious in the Dunkin-Hartley group. Compared to the strain 13 and Dunkin Hartley groups, the strain 2 animals displayed a weak recognition of the 28-32kDa doublet. Western Blots also identified this difference between the groups (Figure 5.14). Within each inbred strain, a small degree of heterogeneity in antigen recognition, was observed. An example of this being the unique recognition of the 18kDa component by strain 13 guinea pig B1. As with the ELISA results, the Dunkin-Hartley animals, appeared to exhibit the highest degree of heterogeneity in antigen recognition and the recognition profiles were similar to those observed for this outbred strain in Protection Experiment 2 (Figure 5.9). As in the previous experiments, no correlation between antigen recognition pattern and protection was observed.

Group	A	B	C*	D
Antigen	strain 2	strain 13	DH	chall. control
Individual recoveries:				
1	21	7	8	24
2	65	5	9	27
3	75	9	6	104
4	30	31	21	32
5	70	12	10	21
6	-	14	11	-
Mean recovery	52.2	13	10.8	41.6
% reduction	none	68.8	74.0	-

Table 5.4

Protection Experiment 3 : worm recoveries of immunised animals six days after challenge with 6,000 L3 of *D. viviparus*.

Group A consisted of strain 2 guinea pigs and Group B, strain 13 guinea pigs and those in Group C, Dunkin-Hartley. Animals in these groups received whole ES antigen with an equal volume of FCA, on primary, and FIA, on secondary immunisation. The mean worm burden of each group was compared with that obtained in Dunkin-Hartley challenge controls (Group D) to give percentage reduction.

* indicates that the group recoveries differed significantly from the challenge controls using the Mann-Whitney non-parametric test (5% significance level).

Figure 5.10a

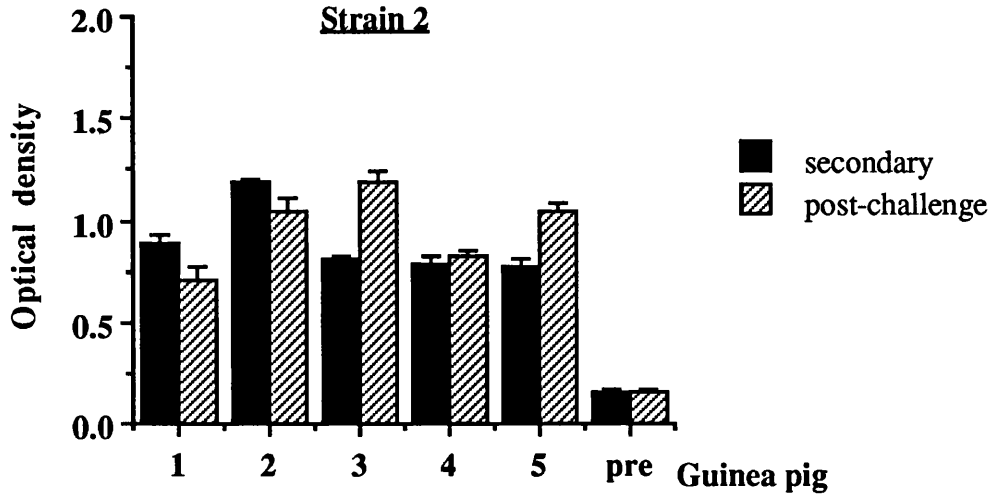
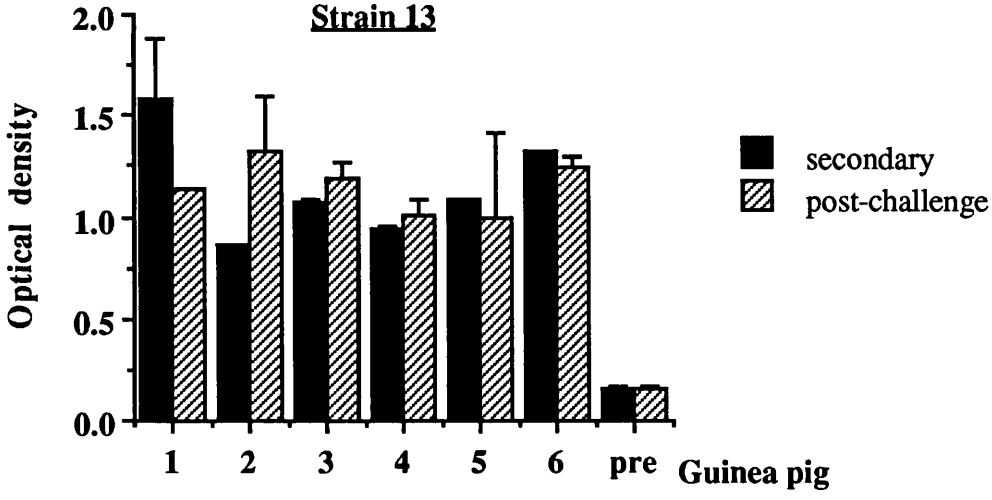


Figure 5.10b



Figures 5.10 a and b

Protection Experiment 3: Antibody levels directed against *D. viviparus* adult ES antigens in the serum of strain 2 and 13 guinea pigs.

The samples were taken from adult ES-immunised strain 2 (Figure 5.10a) and strain 13 (Figure 5.10b) guinea pigs two weeks after secondary immunisation and 6 days after normal L3 challenge. The samples were analysed by the ELISA in duplicate and the mean and standard deviation of each is indicated and compared to a pre-immunisation control (pre). The optical densities were read at 492nm.

Figure 5.10c

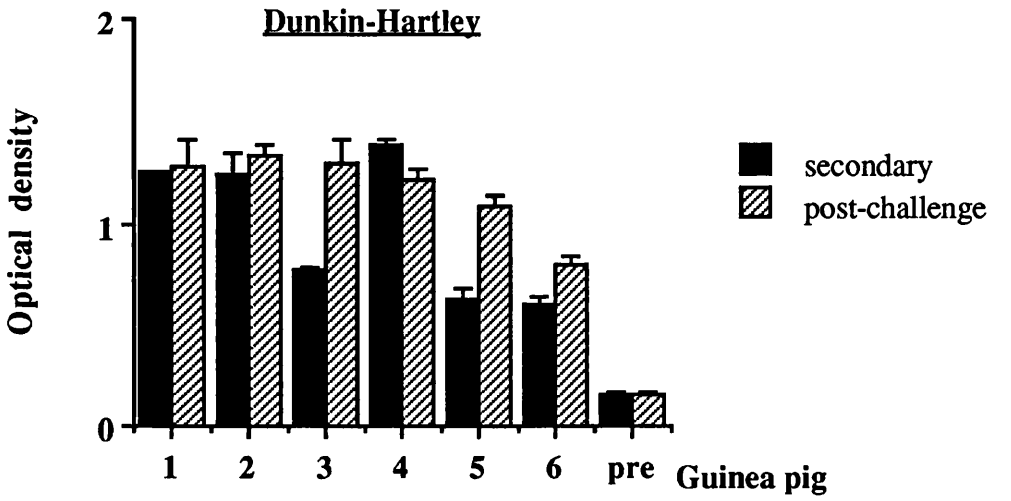


Figure 5.10c

Protection Experiment 3: Antibody levels directed against *D. viviparus* adult ES antigens in the serum of Dunkin-Hartley guinea pigs.

The samples were taken from adult ES-immunised Dunkin-Hartley guinea pigs two weeks after secondary immunisation and 6 days after normal L3 challenge. The samples were analysed by the ELISA in duplicate and the mean and standard deviation of each is indicated and compared to a pre-immunisation control (pre). The optical densities were read at 492nm.

Figure 5.11

Protection Experiment 3: Recognition of adult ES antigens by antibodies in the serum of strain 2 guinea pigs (A1-A5) on Day 6 of challenge. Prior to challenge, each animal had been immunised twice with adult ES products in the context of Freund's adjuvant.

Radio-iodinated adult ES products (Ag) were incubated with individual guinea pig serum and the immune complexes, precipitated by Staph A, were analysed by SDS-PAGE under reducing conditions. The antigen was also immunoprecipitated with pre-immunisation serum (pre). The M_r of marker proteins (M) are indicated in kiloDaltons (kDa).

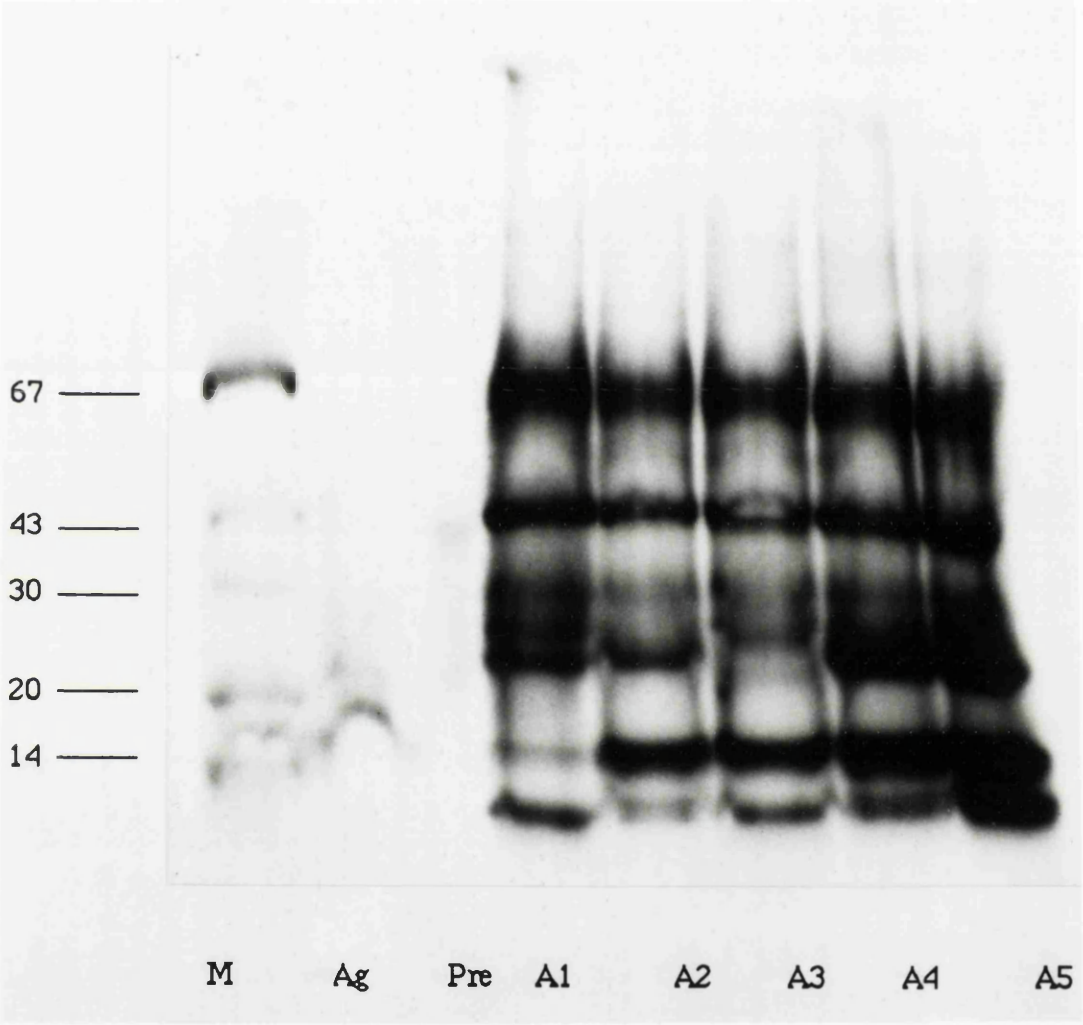

67
43
30
20
14
M
Ag
Pre
A1
A2
A3
A4
A5

Figure 5.12

Protection Experiment 3: Recognition of adult ES antigens by antibodies in the serum of strain 13 guinea pigs (B1-B6) 14 days after secondary immunisation with adult ES in the context of Freund's adjuvant.

Radio-iodinated adult ES products (Ag) were incubated with individual guinea pig serum and the immune complexes, precipitated by Staph A, were analysed by SDS-PAGE under reducing conditions. The antigen was also immunoprecipitated with pre-immunisation serum (pre). The M_r of marker proteins (M) are indicated in kiloDaltons (kDa).

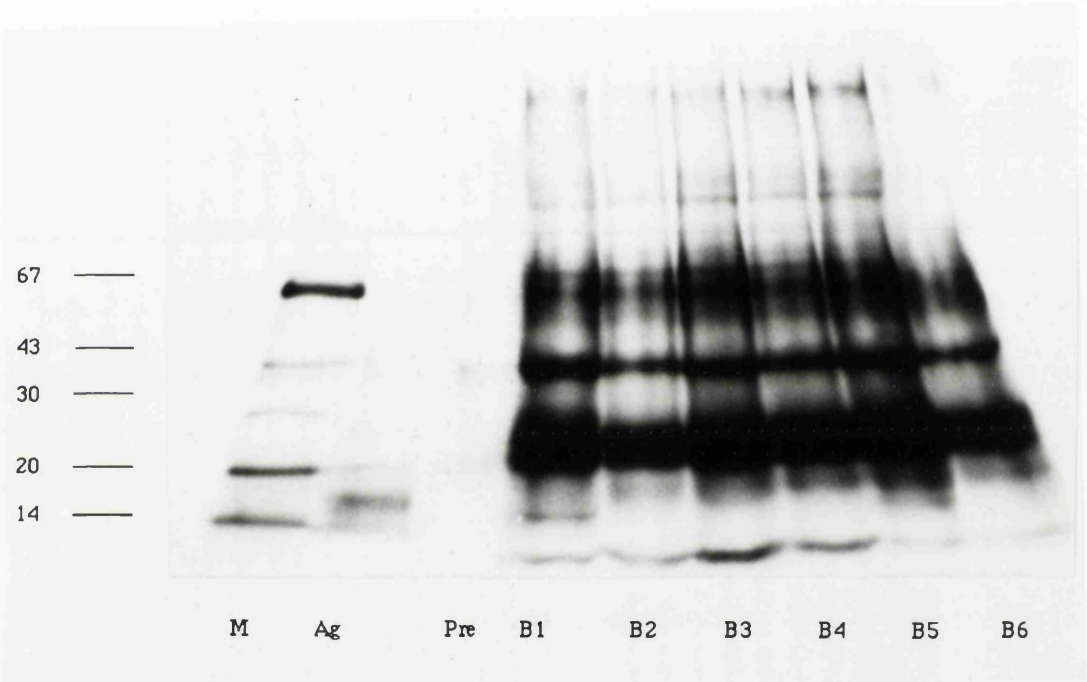

67
43
30
20
14
M
Ag
Pre
B1
B2
B3
B4
B5
B6

Figure 5.13

Protection Experiment 3: Recognition of adult ES antigens by antibodies in the serum of outbred Dunkin-Hartley guinea pigs (C1-C6) on Day 6 of challenge. Prior to challenge, each animal had been immunised twice with adult ES products in the context of Freund's adjuvant.

Radio-iodinated adult ES products (Ag) were incubated with individual guinea pig serum and the immune complexes, precipitated by Staph A, were analysed by SDS-PAGE under reducing conditions. The antigen was also immunoprecipitated with pre-immunisation serum (pre). The M_r of marker proteins (M) are indicated in kiloDaltons (kDa).

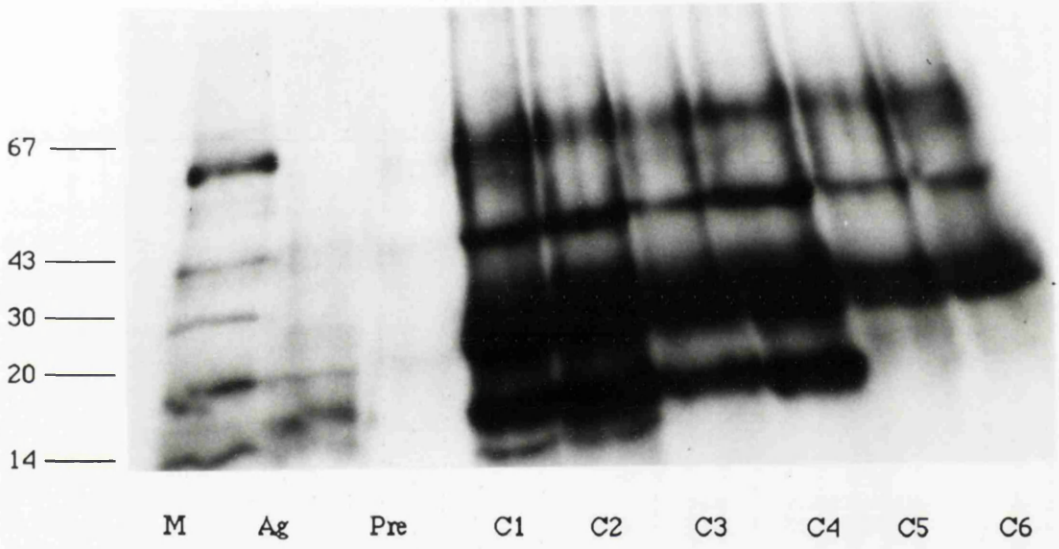

67
43
30
20
14
M
Ag
Pre
C1
C2
C3
C4
C5
C6

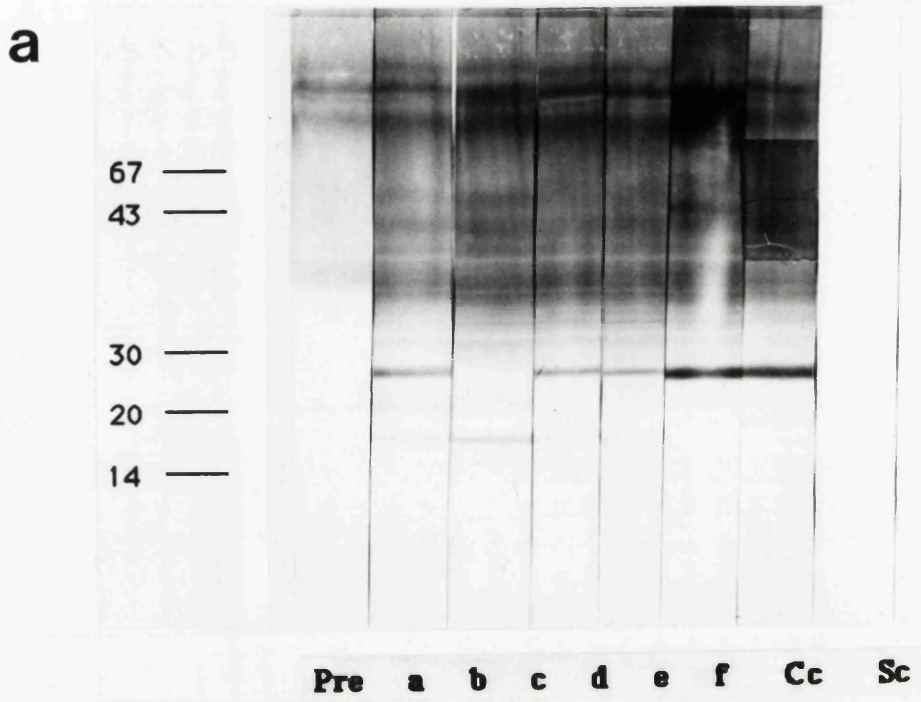
Figure 5.14

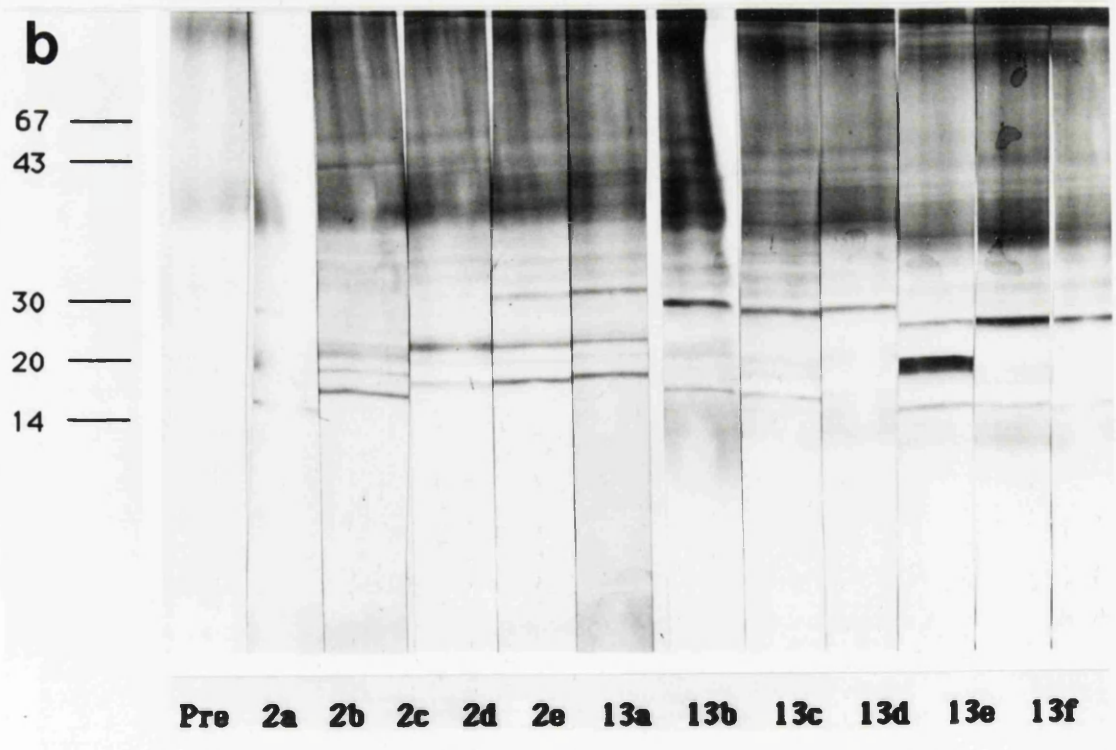
Protection Experiment 3: Western blot analysis of the post challenge responses to adult ES products of the different guinea pig strains used in Protection Experiment 3.

Figure 5.14a demonstrates the responses of individual animals (a to f) in the Dunkin-Hartley group. Pre-immunisation serum (pre) responses are also indicated, as are conjugate only (Cc) and substrate only (Sc) controls.

Figure 5.14b depicts the serum responses demonstrated by the two inbred strains. Pre-immunisation serum pooled from the all inbred animals is included as a negative control. The strain 2 responses are depicted in tracks labelled 2a to 2f and those of the strain 13 guinea pigs in tracks 13a to 13f.

Adult ES products were run on SDS polyacrylamide 5-25% gradient gels under reducing conditions and protein transfer and antigen detection were subsequently carried out as described in Materials and Methods. The relative molecular weights of marker proteins are indicated in kiloDaltons to the left of both panels of Blots.


a
67
43
30
20
14
Pre
a
b
c
d
e
f
Cc
Sc


b
67
43
30
20
14
Pre
2a
2b
2c
2d
2e
13a
13b
13c
13d
13e
13f

5.2.5 Passive Transfer: do antibodies stimulated by *D. viviparus* ES products confer immunity?

In order to define possible mechanisms underlying the immunity stimulated by adult ES products, serum from animals immunised on several occasions with adult ES was transferred to naive recipients.

Four male Dunkin-Hartley guinea guinea pigs were immunised once with 100μg protein of adult ES products, in the context of FCA, and twice, in the context of FIA. The injections were administered four weeks apart. A further six guinea pigs were infected at the same times with 5,000 *D. viviparus* L3. Seven days after the final immunisation or infection, the guinea pigs were exsanguinated under terminal anaesthesia and the serum collected.

Five ml of pooled serum from each group was then injected intraperitoneally into six guinea pigs, while six control guinea pigs received 5ml of normal guinea pig serum (NGPS). One hour later, each serum recipient and a further six guinea pigs, were infected orally with 6,000 L3. All serum recipients were bled at three and six days following transfer and the serum analysed by ELISA. All guinea pigs were killed on Day 6 and their lungs processed. The worm recoveries are shown in Table 5.5.

Animals which had received serum from either the normal L3 infection or adult ES immunised groups harboured worm burdens which were significantly lower than those of guinea pigs which received NGPS. Moreover, the animals which received serum from the normal L3 infected guinea pigs had significantly lower recoveries than the challenge controls. These results indicated a role for antibody in the immune mechanisms stimulated by normal L3 infection and adult ES immunisation in the guinea pig.

Antibody levels detected by ELISA three days after challenge are shown in Figures 5.15a to 5.15c. These indicated that all animals passively immunised with serum raised against normal L3 or adult ES products had received antibody specific for *D. viviparus* adult ES antigens. However, the levels of antibody detected by ELISA did not correlate with the degree of resistance in terms of individual reductions in worm burdens.

Group	A*†	B†	C	D
Serum	NL3	ES	NGPS	chall. control
Individual				
1	51	21	18	16
2	1	21	42	30
3	3	3	107	73
4	1	8	44	49
5	6	44	36	29
6	1	13	64	died
Mean recovery	10.5	18.3	51.8	39.4
% reduction				
- from C	79.7	64.6	-	23.9
- from D	72.5	53.5	0	-

Table 5.5

Passive Transfer: worm recoveries of passively immunised animals six days after challenge with 6,000 L3 of *D. viviparus*.

Group A guinea pigs received 5ml of serum from donor animals infected three times with 5,000 L3 (NL3). Animals in Group B received 5ml anti-adult ES hyperimmune serum (ES), while those in Group C received 5ml normal guinea pig serum (NGPS). The animals were challenged immediately following administration of the serum. The worm burdens of each group were compared with those of the control groups (Groups C and D) to give percentage reduction.

* indicates group recoveries differed significantly from the challenge controls by the Mann-Whitney non-parametric test (5% significance level).

† indicates group recoveries differed significantly from NGPS recipients by the Mann-Whitney non-parametric test (5% significance level).

Figure 5.15a

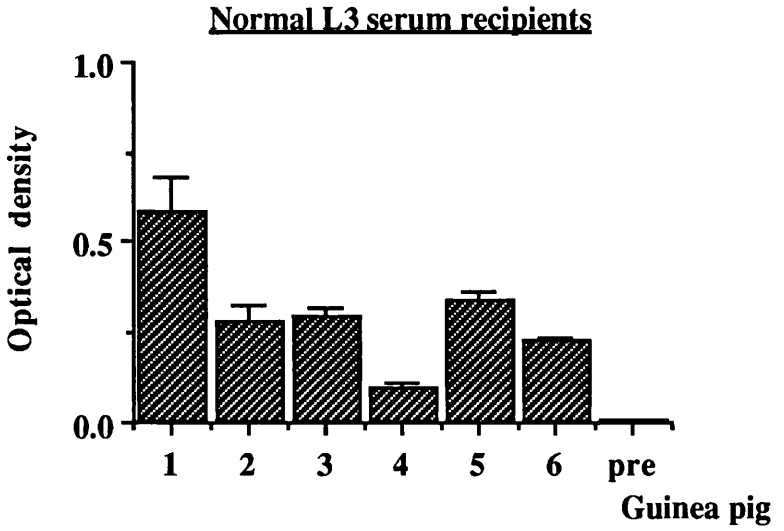
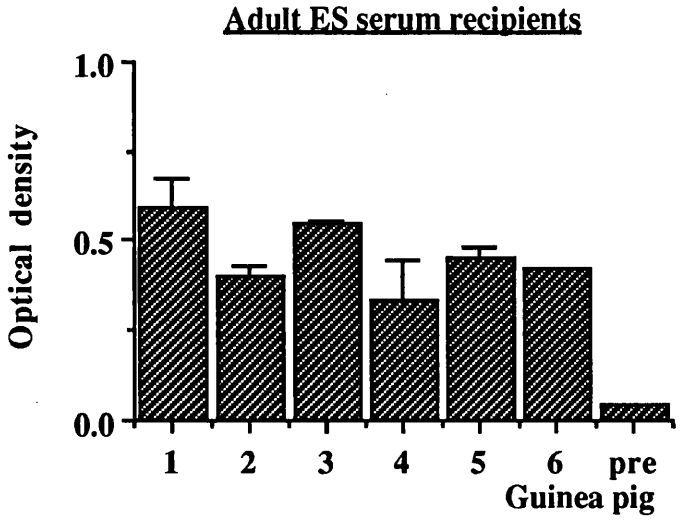


Figure 5.15b



Figures 5.15a and b

D. viviparus adult ES-specific antibody levels present in serum recipients three days following passive transfer.

Individual guinea pigs (1-6) were injected intraperitoneally with serum raised against normal L3 infection (Figure 5.15a) or adult ES products (Figure 5.15b). The sera were analysed in duplicate by ELISA and were compared to pre-transfer levels (pre). The mean and standard deviation of the samples are depicted.

Figure 5.15c

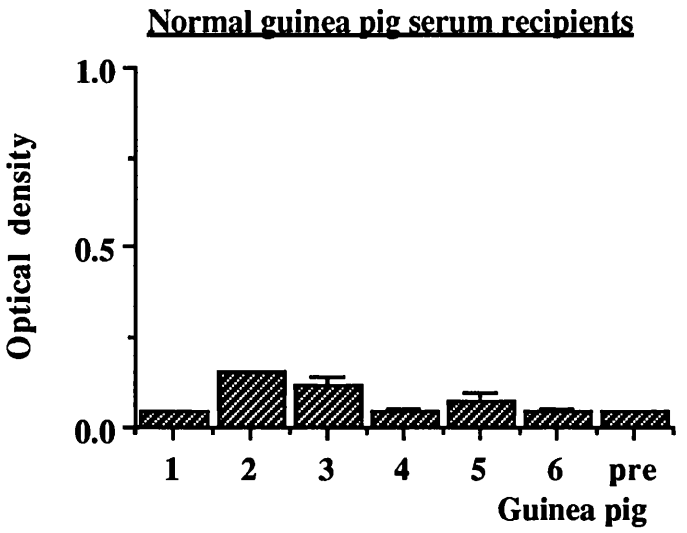


Figure 5.15c

D. viviparus adult ES-specific antibody levels present in serum recipients three days following passive transfer.

Individual guinea pigs (1-6) were injected intraperitoneally with normal guinea pig serum and their sera, analysed in duplicate by ELISA, were compared to pre-transfer antibody levels (pre). The mean and standard deviation of the samples are depicted.

5.3 Discussion

Many studies have demonstrated the efficacy of *in vitro*-released nematode products in the induction of protective immunity. The ES products of *T. spiralis* and *T colubriformis* have been the most characterised in this respect. Secretions released by *T. spiralis* muscle stage larvae proved to be highly protective in mice (Campbell, 1955; Vernes, 1976) and subsequently, Silberstein and Despommier (1984) obtained significant levels of protection in this host using a single immunoaffinity-purified 48kDa molecule from muscle stage larvae. This molecule protected animals at biologically-relevant doses and was shown to comprise 12% of the total parasite secretions. Excretory/secretory material released by *T. colubriformis* larval stages (Rothwell and Love, 1974) and purified molecules thereof (O'Donnell *et al.*, 1989; Savin *et al.*, 1990; Dopheide *et al.*, 1991) have successfully immunised guinea pigs against challenge.

In the studies described here, it appeared that ES products may be released by adult *D. viviparus in vivo* as both calves and guinea pigs which received normal L3 infections recognised most of the molecules present in the radiolabelled ES preparation. Moreover, in preliminary immunocytochemistry studies, rabbit anti-adult ES serum positively identified products in the lung tissue of a field case of parasitic bronchitis (J.B. McKeand, unpublished). The relative efficacy of adult ES products and somatic extracts of both adult and third stage larvae in the induction of protective immunity were compared and, as adult ES products had stimulated high levels of protection in a preliminary study, attempts were made to further fractionate this preparation using SDS-PAGE. The fractions obtained, however, failed to protect guinea pigs and, unexpectedly, the native ES products used in this second study exhibited a reduced protective capacity. In a preliminary attempt to identify effector mechanisms induced by ES vaccination, serum was successfully transferred from ES-immunised guinea pigs to naive recipients which indicated that antibody responses may play a part in the immunity stimulated by adult ES products. Since a considerable degree of heterogeneity in antigen recognition was found among individuals immunised with similar preparations, a further study examined the antibody repertoire induced in ES-immunised inbred guinea pigs. Differences were noted between two inbred strains which were reflected in their relative susceptibility to challenge following immunisation and in differences in their antibody repertoires.

The basis of these protection experiments was to compare the worm burdens of each treatment group with those of challenge controls. Unfortunately, the high degree of variability in worm recoveries from individual challenge control animals complicated statistical analysis of the results. Nevertheless, variability in parasite burdens is not uncommon in nematode infections in both laboratory and natural hosts and worm

burdens are often observed to have a negative binomial distribution (Barger, 1985; Adams, 1989). For this reason, the worm burdens in these immunisation studies were statistically analysed using a non-parametric test.

Although one criticism of the experiments performed here could be that too few animals were used per group, adult ES products were still shown to stimulate a highly significant level of protective immunity on two occasions. The causes of the variability in worm establishment in both immunised and control guinea pig groups were not determined but it may be assumed that both parasite and host factors were involved. The infectivity of larvae may vary from batch to batch and this was suggested by the considerable variation in percentage "takes" in the challenge controls between Protection Experiments 1 and 3. However, this should not influence worm burdens in animals of the same group given larvae from the same batch. Infection technique could influence takes and consequently special attention was given to aliquoting and administering the larvae. It was possible, however, that some animals could have regurgitated their challenge infection after being returned to their cage and this could account for some of the very low burdens observed in some of the challenge controls. Furthermore, individual guinea pigs, especially outbred animals, may vary in innate defence mechanisms, such as local gut inflammatory responses, which might affect larval migration and establishment. These differences are likely to be genetically-determined and are, therefore, unavoidable.

In the first immunisation study presented here, products released from adult stages were able to stimulate protective immunity in animals where parasite development was assumed to terminate at the early L5 stage. This may be attributable to the fact that the larval stages themselves may secrete similar products and, to examine this hypothesis, L4/L5 stages, recovered from the lungs of calves on Day 14 of infection, were cultured and their ES products examined using SDS-PAGE. It was noted that the products released from these stages had similar polypeptide profiles to those obtained from adult worms (not shown). Moreover, the ES material released by L4/L5 stages contained acetylcholinesterases similar to those released by adult parasites (see Chapter 6). In the guinea pig, immunisation with *D. viviparus* ES products may, therefore, prime host effector responses which act to impair nutrition and migration of lung stage parasites. These stages have previously been identified as being important in the development of immunity after either normal L3 infection or 400Gy L3 vaccination in guinea pigs (Cornwell and Jones, 1971).

The failure of adult somatic antigens may be attributable to their complexity (compare protein profile with adult ES products in Figure 5.1) which may have resulted in antigenic competition: the high antibody levels observed in the animals of this group suggested this. Alternatively, the antigens which were required for the stimulation of

protective immunity may have been in insufficient quantity in the adult somatic extract. The presence of cross-reactive antigens in the adult and L3 developmental stages, as indicated by ELISA and immunoprecipitation, was not unexpected as somatic preparations were used in which many components are likely to be similar. Of interest though, was the degree of complexity of the adult ES products and the number of cross-reactive epitopes shared with the two somatic preparations. This suggests that most of the adult ES products may be pre-formed and stored prior to secretion. Alternatively, it may indicate that the ES products obtained from culture were released by dead or dying parasites but this was unlikely as similar polypeptide profiles were obtained in ES products released from adult parasites incubated *in vitro* with ^{35}S-methionine (Britton, 1991).

Similarity was observed in the antigen recognition profiles of animals immunised with adult ES products (Group D) or adult homogenate (Group C). The ES recipients exhibited a clearer profile and only animals in this group recognised the upper molecule of the 38-42kDa doublet. The 42kDa component appears to be the only glycosylated species in adult *D. viviparus* ES products (Britton, 1991) and the results presented here suggest that it may be a unique secretory component. In addition, two low molecular weight immunodominant bands, present within the ES products, did not appear to be present within the radiolabelled adult homogenate preparation, despite being recognised by several of the adult homogenate-immunised guinea pigs. These antigens, which had approximate M_r of 14kDa and 18kDa, may be present in higher quantities within the ES products and this was implied by the stronger recognition of these by the ES-immunised group. Alternatively, it could be that these molecules, *per se* , were not present within the adult homogenate and were either breakdown products of higher M_r somatic components or shared epitopes cross-reactive with those present in higher molecular weight species. The existence of similar epitopes on different antigens has previously been observed in nematodes, for example *T. spiralis* (Robinson *et al.* , 1991).

The fundamental basis of the work presented in this Chapter relied upon the ability of whole adult ES material to induce a high level of protective immunity in guinea pigs. It was observed, however, that the immunity induced by this preparation varied considerably from experiment to experiment. This was most evident when one compared the immunity stimulated by adult ES products in Protection Experiments 1 and 2. In the former, significant levels of protection were obtained with a mean reduction in worm burden of more than 85%. This fell to 26.1% in the second immunisation study, where the worm burdens obtained from the ES-immunised guinea pigs did not differ significantly from the challenge controls. The reasons for these discrepancies are unclear but could be attributed to several factors.

First, the ES products, although removed from the second day of culture for use in both experiments, were obtained from different batches of adult parasites. The culture and extraction conditions applied to both batches were, nevertheless, identical and both adult ES samples exhibited similar profiles on Coomassie-stained SDS-polyacrylamide gels (not shown). This, however, does not imply that they were identical and two-dimensional electrophoresis would be required to further define their components. Of interest, was the fact that the adult ES material used for Protection Experiment 2 was obtained from adults harvested from calves on Day 23 of infection, while that used in the first experiment was obtained from a 28 day-old *D. viviparus* infection. This highlighted interesting possibilities regarding the influence of parasite maturity on the ES components released. Nevertheless, as mentioned previously, *D. viviparus* only develops as far as the L5 stage in the guinea pig host and, if ES products are to act against this stage, the age of the adult parasites which supplied the ES products should be immaterial.

Secondly, the antigen recognition profiles displayed by the ES-immunised animals in Protection Experiments 1 and 2 were slightly different. The guinea pigs of Protection Experiment 1 appeared to display a more complex pattern and there was stronger recognition of the 38-42kDa doublet. That these disparities were related to the level of immunity expressed was subsequently discounted, however, when the antibody responses of the ES-immunised Dunkin-Hartley guinea pigs of Protection Experiment 3 were examined. These animals were significantly protected, yet displayed antigen recognition profiles very similar to those of the ES-immunised group in Protection Experiment 2. It is possible that the differences observed in the recognition profiles between similarly-immunised groups reflected disparities in the proteins which were radiolabelled in each adult ES preparation. These results were similar to those observed in studies with other nematode species where attempts to correlate antibody recognition patterns with the development of immunity have failed (Wakelin *et al.*, 1986; Else and Wakelin 1989; Kennedy *et al.*, 1991). However, as nematode antigens may comprise several different epitopes (Robinson *et al.*, 1991), the relative importance of the antibody repertoire cannot be discounted until the epitopes themselves have been defined.

A third possible explanation for the variability in protective capacity of the adult ES preparation was that the larvae used in Protection Experiment 2 were more viable than those used in the other experiments. It could be inferred that more viable larvae might overcome the ES-induced immunity. This was unlikely, however, as similar takes were observed in the challenge controls of both Protection Experiments. Furthermore, it was noted in retrospect that the challenge larvae used in these experiments were from the same batch and that those used in Protection Experiment 2 had been stored for a longer period.

Although the animals in these experiments could have contributed to the observed variability in worm burdens, all were obtained from the same source and were the same strain, sex and age at the start of both experiments so that significant differences in responses between animals were unlikely. In Protection Experiment 2, two out of the six animals immunised with ES material, harboured considerably higher worm burdens compared with the other animals in the group. Interestingly, one of these guinea pigs displayed low antibody levels and the results could imply that this animal was a "low-responder", a phenomenon previously been observed in *T. colubriformis* infection in lambs (Dineen, Gregg and Lascelles, 1978; Windon, Dineen and Kelly, 1980) and guinea pigs (Rothwell *et al.*, 1978). The relative susceptibility of the lambs and guinea pigs in the latter experiments appeared to be associated with genetically-determined factors. An association between the expression of certain lymphocyte surface antigens and resistance to *T. colubriformis* has been observed in sheep which implicated a role for genes linked to the MHC in the development of immunity (Outteridge, Windon and Dineen, 1985).

In several helminth infections, the isotypes of antibody stimulated are thought to influence the outcome and may determine whether an infection is rejected, maintained or whether immunopathology ensues. For example, the development of an anti-cuticle IgM response has been associated with the development of protective immunity in mice to *D. viteae* (Philipp *et al.*, 1984). Moreover, the production of certain antibody isotypes may be related to increased susceptibility to some helminth infections, for example IgM (Khaliffe *et al.*, 1986) and IgG_4 (Hagan et *al.*, 1991) have been proposed to act as blocking antibodies in *S. mansoni* infection, while IgG_4 has been suggested to have this capacity in *Trichuris trichiura* infection (Bundy *et al.*, 1991). In an attempt to define the antibody isotypes stimulated by immunisation using various ES antigen preparations, IgG and IgM levels were measured in guinea pigs from Protection Experiments 1 (not shown) and 2. Interestingly, the levels of ES-specific IgG were significantly lower in the ES-immunised guinea pigs of Protection Experiment 2, compared with those in Protection Experiment 1. Also the "low-responder", B4, in Protection Experiment 2, had low levels of IgG which could suggest that the level of IgG antibody stimulated by ES immunisation may be of relevance to the development of protection to *D. viviparus.* in this host. Although elevated IgG levels to parasite ES materials have been associated with the development of protective immunity, for example in *T. muris* infection in some strains of mice (Else *et al.*, 1990), high levels of specific antibody do not necessarily indicate resistance to nematode infection. This was demonstrated with the adult homogenate-immunised group in Protection Experiment 1 and has also been observed with *T. spiralis* in mice (Else and Wakelin, 1989) and *N. americanus* in humans (Pritchard *et al.*, 1989). Unfortunately, antibody subclass levels could not be assessed

due to the lack of specific anti-guinea pig reagents. However, guinea pig IgG_1 and IgG_2 have been separated using Protein A (Lindmark, Thoren-Tolling and Sjoquist, 1983) and this technique may prove useful in future studies, as measurement of these subclasses could indicate which T helper subpopulations, if any, are involved in guinea pigs during *D. viviparus* infection or immunisation.

Another factor to emerge from the immunisation studies, was the heterogeneity in the antibody repertoire displayed by individuals immunised with similar preparations. This heterogeneity, which has been observed in calves and guinea pigs infected with normal or 400Gy-irradiated L3 (Britton *et al.*, 1992), was most pronounced against the lower molecular weight components of the adult ES products. This may have been related to the lower number of epitopes present in these components. Individual heterogeneity in antibody recognition of parasite antigens is well documented and appears to be under genetic control. Examples include, *T. muris* in mice (Else and Wakelin, 1989; Else *et al.*, 1990); *S. mansoni* in mice (Correa-Oliveira *et al.*, 1986), *N. brasiliensis* in rats (Kennedy *et al.*, 1990a); *T. spiralis* in mice (Kennedy *et al.*, 1991) and *A. suum* in mice (Kennedy *et al.*, 1990b; Christie, Fraser and Kennedy, 1992). During the course of natural parasite infection, the heterogeneity observed in antibody recognition patterns may be directly related to the parasite stages which are encountered. In these studies, however, heterogeneity in recognition patterns was observed after immunisation in which the animals had been exposed to the same preparation in the context of a powerful adjuvant. It was therefore unlikely that individual guinea pigs were exposed to different sets of antigens. As these animals were maintained under worm-free conditions, it was also unlikely that the different responses observed were related to exposure to cross-reactive antigens present in other parasite species. The disparities observed, were more likely a result of the genetically-determined capacity of individual animals to respond uniquely to a given set of epitopes. It has now been ascertained for many nematode species that the specific immune response of an individual is directed by genes encoding within and outside the MHC (reviewed by Wakelin, 1988; Kennedy, 1989).

In order to investigate the possible influence of genetic factors on the antibody repertoire in *D. viviparus* adult ES immunisation, two inbred strains of guinea pigs were immunised. The results showed that the two strains differed considerably in their antibody response and in their susceptibility to infection following ES vaccination. As these two strains have different sets of background genes, which can have a major influence on antigen recognition in nematode infection (Else and Wakelin, 1989), the observed difference cannot simply be attributed to their diversity at the MHC region. What can be stated from these studies, however, is that the differences observed between the inbred guinea pig strains were not due to genes which mapped to the MHC class I

locus as these strains are identical here (Klein, 1986) and, if the MHC were involved, then products encoded by the MHC class II locus mediated the differences observed.

The MHC class II region has been previously shown to influence antigen recognition in *A. suum* (Kennedy et *al.*, 1990b) and *T. spiralis* (Kennedy *et al.*, 1991) infections in mice. This region of the genome may direct specific T cell reactivity, as T cell receptors (TCR) can only recognise processed antigen when it is presented in association with polymorphic cell surface molecules encoded by the MHC (Kourilsky and Claverie, 1989). For example, responses to several T cell epitopes of a 19kDa antigen of *Mycobacterium tuberculosis* have recently been observed to be HLA-restricted (Faith *et al.*, 1991). If the particular MHC of an individual fails to bind peptides of a specific antigen, interaction of that antigen with T cells and the B cells which they help, will not occur (Schwartz, 1985; Germain, 1990). Thus the guinea pig strain differences observed here may have been directed by T or B cell responses, which themselves are MHC-restricted. A specific MHC haplotype may also restrict antigen recognition by coding for self-components which cross react with specific antigens present in the parasite. The T cells which would normally have reacted with such antigens are thought to be deleted during ontogeny in the thymus (Blanden, 1986; Vidovic and Matzinger, 1988). It was noted that, even within each strain, there was a degree of variation in the antigen recognition profiles and this may have been due to residual heterozygosity which occurs in background genes, for example, T cell receptor genes ,which lie outwith the MHC region (Schwartz, 1978; Kourilsky and Claverie, 1989). To further define the exact role of the MHC in the guinea pig response to adult *D. viviparus* ES immunisation, MHC congenic strains would be required.

Whether or not strain 13 guinea pigs were innately resistant to *D. viviparus.* challenge could not be ascertained, as challenge control groups of inbred strains were not included due to the limited availability. The immunological data suggested that the strain 13 guinea pigs responded better, both qualitatively and quantitatively, than the strain 2 animals. When the recognition profiles of the two groups were compared by immunoprecipitation and Western blotting, it appeared that strain 13 guinea pigs recognised the 28-32kDa doublet more strongly. This does not, however, indicate that the recognition of this molecule was related to the levels of immunity that were expressed since the relationship was purely correlative. To assess the protective capacity of such molecules, purification and further vaccination experiments would be required.

The failure of the gel-fractionated ES products to induce protective immunity was probably a result of epitope denaturation caused by the preparative procedures. The inactivation of protective peptides by SDS, 2-mercaptoethanol or heating to 100°C, has previously been observed in vaccination studies with *T. taeniaeformis* onchosphere antigens (Lightowlers *et al.*, 1984). When the onchosphere antigen preparation was

fractionated from gels in which the SDS had been replaced by sodium deoxycholate, the protective capacity was retained (Lightowlers, Rickard and Mitchell, 1986). This suggested that when antigens were run under less harsh conditions, their protective capacity could be retained. A *D. viviparus* extract was subsequently electrophoresed under native conditions and was able to protect guinea pigs against challenge (see Chapter 6).

The immunoprecipitation results from Protection Experiment 2 implied that antibody from the animals which received gel-fractionated adult ES products did not recognise native adult ES epitopes following immunisation. Despite this, however, elevated anti-ES immunoglobulin was detected by ELISA: either the epitopes which stimulated the increased antibody were not available in the radiolabelled preparation, or the immunoglobulin isotypes detected by ELISA could not be detected by the immunoprecipitation procedure. IgG_1 and IgG_2 are the only guinea pig immunoglobulin isotypes so far demonstrated to bind Protein A used in the immunoprecipitation assay (Lindmark, Thoren-Tolling and Sjoquist, 1983). In agreement with this, the ELISA studies indicated that the elevated antibody levels in the groups which received ES/gel or electroeluted ES fractions were not due to an increase in IgG or IgM. It may have been that IgE levels were elevated and these would not have been detected in the ELISA directed at the IgM and IgG antibody classes or by immunoprecipitation. It would be of interest to examine the IgE response in these guinea pigs, as adult *D. viviparus* cDNA clones expressing the immunodominant 14kDa ES component have recently been shown to exhibit a high level of similarity to the *Ascaris* ABA-1 allergen (C. Britton, personal communication).

The studies described here relied on antibody detection for assessment of immunity. The antibody repertoire was considered to reflect an individual's ability to respond to specific epitopes and, therefore, could determine the ability of an animal to respond to infection or immunisation. Of particular relevance, is the fact that the success of future subunit vaccines, which may comprise a few select polypeptides, will depend upon the ability of individuals in the vaccinated population to recognise vaccine components (Kennedy, 1990). Variability in responses to immunisation with sub-unit vaccines have already been encountered with a recombinant surface antigen vaccine developed for the control of Hepatitis B in man (Varla-Leftherioti *et al.*, 1990) and in the development of a circumsporozoite antigen vaccine against *P. falciparum* in mice (Del Giudice *et al.*; 1986, Togna *et al.*, 1986). In addition, by influencing recognition of parasite allergens, the antibody repertoire may also govern whether or not an individual will develop a detrimental immunopathological response.

The fact that specific antibody induced by *D. viviparus* adult ES products and Freund's adjuvant may contribute to the development of protective immunity was

suggested by the successful passive transfer experiment. The immunity transferred, however, was of a lower order than that transferred with serum raised against normal L3 infection which suggested that other immune effector mechanisms may have been involved, or that antibody induced by ES products was transferred in insufficient quantity.

For future studies, it would be of interest to examine the contribution that T cells make to the immunity induced by *D. viviparus* adult ES products: for example, is there a T cell dichotomy in the guinea pig which directs the immune mechanisms to success or failure? This would be of special interest with regard to the inbred strains which appeared to differ in their ability to respond to ES immunisation. Unfortunately, reagents, such as those developed for the detection of cytokines in mice, are not available for the guinea pig and, therefore, limited the present work to measurement of antibody recognition profiles and isotype responses. Also, *D. viviparus* cannot be used in the mouse as the parasite develops poorly in this host (Wade, Fox and Swanson, 1960a; J.S. Gilleard, personal communication). To investigate the possible influence of T cells on immunity to *D. viviparus* , T cell blots or proliferation assays could be attempted.

No attempts were made to define accessory cell involvement in immune expulsion of *D. viviparus*, although a considerable number of accessory cells, especially eosinophils, have been observed in the respiratory tract of infected calves (Jarrett and Sharp, 1963). It is of interest that, in some preliminary studies, a zymogen-stimulated respiratory burst of bovine neutrophils was reduced in the presence of *D. viviparus* adult ES products (data not shown) which indicated a possible anti-inflammatory role for these parasite derived-materials.

Because of the variability in worm burdens between experiments, future studies should perhaps include a comparison of the rate of adult expulsion in immunised animals and challenge controls. Wade, Fox and Swanson (1960b) found that animals immunised with normal L3, or 400Gy-irradiated L3, expelled their parasites at a faster rate than challenge controls and that the difference between the immunised animals and the controls was maximal 8 to 10 days after challenge. In the experiments presented here, however, all animals were killed on Day 6 of challenge and, therefore, differences in the rate of parasite expulsion could not be assessed. Although infected guinea pigs and calves display similar antigen recognition patterns of adult ES products following normal infection or 400Gy vaccination (Britton *et al*, 1992), it remains to be demonstrated whether *D. viviparus* adult ES products can immunise calves against re-infection. In this context, it has been shown that while *T. spiralis* larval ES antigens could significantly protect mice against challenge (Campbell, 1955; Vernes, 1976), only moderate protection was obtained in pigs (Gamble, Murrell and Marti, 1986). These

differences were thought to reflect disparities in the effector mechanisms stimulated in the two host species.

Chapter 6

Acetylcholinesterases of *D. viviparus* and their recognition by infected and immunised hosts

6.1 Introduction

Acetylcholinesterase (AChE, EC, 3.1.1.7) enzymes have been demonstrated to have an important role in the biology of several nematode species: in addition to their involvement in neuromuscular transmission, these enzymes are secreted by several nematodes. *N. brasiliensis* (Lee, 1970), *T. colubriformis* and *T. axei* (Ogilvie *et al.*, 1973; Rothwell, Ogilvie and Love, 1973), *O. radiatum* and *O. venulosum* (Bremner *et al.* 1973), *N. americanus* (Burt and Ogilvie, 1975), *Stephanurus dentatus* (Rhoads, 1981) and *B. malayi* (Rathaur *et al.*, 1987) all release AChE *in vitro* in quantities which exceed their initial body contents. Other nematode species, however, contain low quantities of AChE and do not appear to secrete these enzymes. In such species, the AChE enzymes have been primarily associated with nervous tissue, for example *A. suum* (Hutchinson and Probert, 1972), and were assumed to be of little relevance to the host/parasite relationship. Parasitic nematode cholinesterases so far characterised either exist as single proteins (Rothwell and Merritt, 1974) or in multiple molecular forms (Edwards, Burt and Ogilvie, 1971). They generally range from 60-200kDa, although that of *N. americanus* is 400kDa (Yeates and Ogilvie, 1976).

Evidence for the *in vivo* release of AChE by nematodes was provided by several studies in which specific AChE antibodies were demonstrated in the serum of infected hosts (Jones and Ogilvie, 1972; Bremner, *et al.*, 1973; Rothwell and Merritt, 1974; Rothwell *et al.*, 1976; Beaver and Dobson, 1978). In *T. colubriformis* (Rothwell and Merritt, 1974) and *N. brasiliensis* (Jones and Ogilvie, 1972) infections the anti-AChE antibodies were primarily of the IgG_1 subclass. The significance of anti-AChE antibody in protective responses has not been assessed in any detail, however, studies using purified *T. colubriformis* AChE enzymes failed to induce significant levels of immunity (Rothwell and Merritt, 1975).

The AChE isoenzymes of *N. brasiliensis* appear to form a unique relationship with the host in that the parasite AChE isoenzyme patterns were observed to alter as immunity developed in the infected rat (Edwards, Burt and Ogilvie, 1971; Jenkins, Ogilvie and McLaren, 1976). In view of the fact that the parasite AChE patterns also changed in infected naive rats which were passively immunised with serum, it was assumed that the enzyme changes could be induced by specific antibody. However, these alterations could also be induced in adult *N. brasiliensis* maintained in culture for several days and it was proposed that the enzyme changes were a feature of "damaged" parasites (Love, Ogilvie and McLaren, 1975). Furthermore, parasites which survived for longer periods in the face of host immunity were observed to have unique AChE

isoenzyme profiles (Jenkins, Ogilvie and McLaren, 1976) and these profiles reverted to the "normal" phenotypic pattern when the parasites were transferred to the intestines of non-immune rats (Sanderson, Jenkins and Phillipson, 1976).

The function of parasite AChE enzymes in the host is, at present, unknown but several roles have been envisaged (reviewed by Rhoads, 1984). It has been suggested that, by breaking down locally-released acetylcholine (ACh), AChE may act to block a number of host effector mechanisms directed against the parasite. Also, it has been suggested that AChE enzymes released by certain intestinal nematode species may function as a "biochemical hold fast"' whereby the enzymes act to reduce local peristalsis by breaking down ACh (Lee, 1969). AChE-specific antibody might interfere with these holdfast mechanisms and the resulting increase in peristalsis would then force worms to leave their predilection site close to the mucosa. Such evicted worms would then be forced to move to areas where environmental conditions would be less suitable for their continued survival (Lee, 1969).

AChE may also interfere with anti-parasite effector mechanisms by interfering with mucus secretion from intestinal goblet cells and thus reduce the effects of "mucus trapping" (Philipp, 1984). In the intestine, parasympathetic (cholinergic) control of mucus secretion by goblet cells has been demonstrated (Specia and Neutra, 1980) and this may be of relevance to *D. viviparus* infection in the lungs, where mucus secretion has also been observed to be under cholinergic control (Hafez, 1977).

A putative role has been suggested for nematode AChE in the deactivation of several cellular effector responses which may be stimulated by locally-released ACh (Rhoads, 1984). In these processes, the effects of ACh are thought to be mediated by intracellular cGMP levels. ACh and its analogues have been shown to stimulate lysosomal enzyme release (Ignarro and Colombo, 1973), as well as neutrophil-mediated ADCC (Gale and Zighelboim, 1974) and neutrophil chemotaxis (Hill *et al.*, 1975). Furthermore, it has been observed that histamine release in the lung can be enhanced by ACh (Kaliner, Orange and Austen, 1972; Tauber *et al.*, 1973; Kaliner and Austen, 1975). Deactivation of these responses by parasite AChE enzymes may be of particular relevance in helminth infections where they play an important role in parasite elimination. AChE may also influence the involvement of choline in phosphatidyl choline methylations at the level of cell membranes. These processes have been associated with immune and inflammatory cell functions such as mitogenesis (Hirata and Axelrod, 1980) and chemotaxis (Hirata *et al.*, 1979), as well as being involved in the release of histamine from mast cells and basophils (Hirata, Axelrod and Crews, 1979).

Although unrelated to the function of AChE in normal host/parasite relationships, it is of interest that their secretion can be inhibited by a number of benzimidazoles

(Rapson, Lee and Watts, 1981; Rapson, Chilwan and Jenkins, 1986) and benzimidazole resistance in some parasitic nematodes has been correlated with qualitative and quantitative changes in their AChE isoenzyme patterns (Sutherland, Lee and Lewis, 1988).

If the secretion of AChE is involved in immune modulation by parasites, this enzyme may be an important target of the host immune response. The work described in this Chapter was designed to define isoforms of AChE in *D. viviparus* and responses to these enzymes in infected or immunised hosts. Preliminary studies were also undertaken to characterise enzyme isoforms released by adult *D viviparus* and to investigate their potential as host-protective antigens.

6.2 Results

6.2.1 Adult *D viviparus* parasites secrete AChE

D. viviparus adult ES products were examined for AChE activity by the method described by Ellman *et al.* (1961; see Section 2.8.1) using acetylthiocholine iodide as a substrate. Adult ES materials were measured for AChE activity at a pH range of 4 to 11 in order to ascertain the optimum for activity. All samples were maintained at 37°C. The results are shown in Table 6.1.

A high level of AChE activity was detected in the adult ES products, and two peaks of optimum activity were apparent, one at approximately pH 7, the other at approximately pH 9.5. The two peaks of AChE activity may reflect the fact that there are several isoforms of AChE present in *D. viviparus* adult ES (see below) each of which may have different pH optima. When AChE levels present in adult ES material were compared with those in adult somatic extracts, the enzyme activity in the former was found to be considerably higher. As the adult ES products used in the analysis had been concentrated following culture, the levels in the two extracts could not be directly compared. Release of AChE by parasites was observed to decrease substantially by Day 3 of culture (not shown), by which time there was a considerable level of mortality.

In order to visualise the AChE isoforms present within *D. viviparus* adult ES, 10µl of ES products, diluted 1:10, was run on each track of a polyacrylamide minigel under native conditions. The gel was then sub-divided and the different tracks stained for protein, non-specific esterase or AChE activity (Figure 6.1). Two tracks were stained for protein using Coomassie Blue and a further two for esterase activity using naphthyl acetate/RR fast blue following the method of Grunder, Sartori and Stormont (1965; see Section 2.8.2). Using this staining technique, 5 separate isoforms of esterase were delineated which ran in close approximation near the lower margin of the gel. The five bands demonstrated by esterase-specific staining were also visualised in the tracks stained for AChE activity (Karnovsky and Roots, 1964; Section 2.8.4). The relative molecular weights of the AChE isoforms could not be assessed in these native polyacrylamide gels and when compared with the protein-stained tracks, there appeared to be little correlation between major protein components and esterase activity.

To confirm that the esterase isoforms present were AChE, and not pseudocholinesterases, eserine was used as a specific inhibitor (Pearse, 1972; see Section 2.8.3). The results, also shown in Figure 6.1, indicated that the esterases present in the adult ES products could be classified as AChE.

		Change in adsorbance(405nm)5-65 secs			
pH	buffer	ES A	ES B	Adh A	Adh B
4	acetate	0	0	0	0
5	(0.1M)	0.012	0.010	0	0
6		0.117	0.012	0.007	0.009
6	phosphate	0.112	0.110	0.003	0.004
6.5	(0.1M)	0.184	0.191	0.009	0.012
7		0.218	0.200	0.012	0.014
7.5		0.235	0.242	0.012	0.014
8		0.226	0.223	0.014	0.016
8.5		0.206	0.194	0.001	0.006
8.5	Tris	0.188	0.193	0.013	0.012
9	(0.1M)	0.180	0.197	0.014	0.012
9.5		0.238	0.222	0.009	0.010
9.5		0.309	0.302	0.058	0.059
10	carbonate/	0.242	0.222	0.006	0.004
11	bicarbonate	0.104	0.094	0	0

Table 6.1

pH optima of *D. viviparus* adult ES and homogenate extracts.

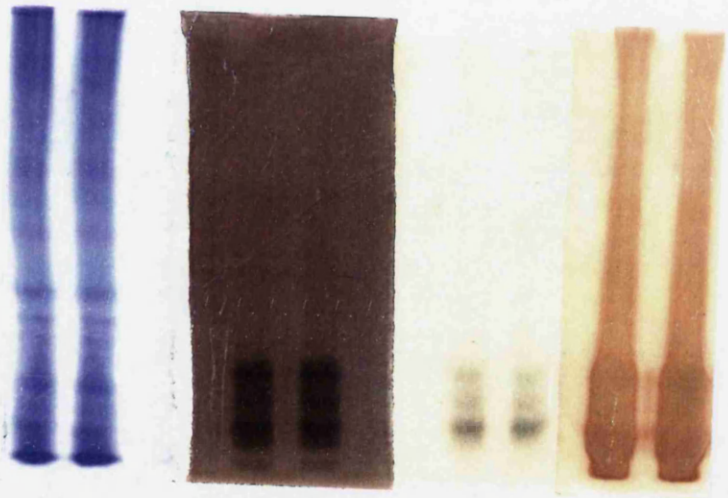
The pH optima activity of the AChE present in two batches of adult ES products (ES A and B) and adult homogenate extracts (AdH A and B) were assessed by the method of Ellman *et al*. (1961). The samples were examined at a pH range of 4 to 11 in the buffers indicated in the table.

The protein concentraions of the samples examined were ES A 0.30mg/ml; ES B 0.33mg/ml; AdH A 3.40mg/ml and AdH B 1.12mg/ml.

Figure 6.1

Examination of *D. viviparus* adult ES products for AChE activity.

Ten µl of adult ES material was run in each track of a 10% polyacrylamide minigel under native conditions. Two tracks were stained to visualise protein content using Coomassie Brilliant Blue R-250 and two were stained with naphthyl acetate/Fast RR Blue salt (Grunder, Sartori and Stormont (1965) which is specific for esterase activity. In order to examine if the esterases present were AChE, two tracks of the gel were pre-incubated with the specific inhibitor, eserine, prior to staining with naphthyl acetate/Fast RR Blue salt. A further two tracks were stained specifically for AChE following the method of Karnovsky and Roots (1964).



Coomassie Blue

Naphthyl acetate Fast Blue stain

Eserine inhibition

Karnowsky and Roots stain

6.2.2 A comparison of the AChE enzymes of *D. viviparus* with those of related nematodes.

The AChE isoforms in *D. viviparus* adult ES products were compared with PBS-soluble somatic extracts of adult worms and with similar extracts of L3 stages of *D. viviparus*, *O. ostertagi*, *C. oncophora* and *D. filaria*. These were assayed using polyacrylamide gels and the specific staining techniques outlined above and since the pattern of activity observed using both stains was similar, only the AChE-stained gel is shown (Figure 6.2).

No esterase activity was obtained in the tracks containing the L3 somatic extracts of *C. oncophora*, *O. ostertagi* and *D. filaria*. The dark areas apparent in the photograph in the *O. ostertagi* track, were not evident on the gel itself. The adult homogenate preparation of *D. viviparus* appeared to contain all the AChE isoforms present within the adult ES products, but the amount of activity varied considerably from batch to batch and only limited studies were performed using this preparation. In Figure 6.2, the esterase activity detected in the *D. viviparus* adult and L3 homogenate extracts had restricted migration on the gel. On most occasions, however, this band of activity was not present in either of these preparations and only AChE isoforms similar to those observed in adult ES material were observed. This band may have represented a complex of isoenzymes whose presence varied with experimental or storage conditions. A similar phenomenon has been observed with AChE of several other parasite species (Bremner *et al.*, 1973; Rothwell and Merritt, 1975; Wright *et al.*, 1983).

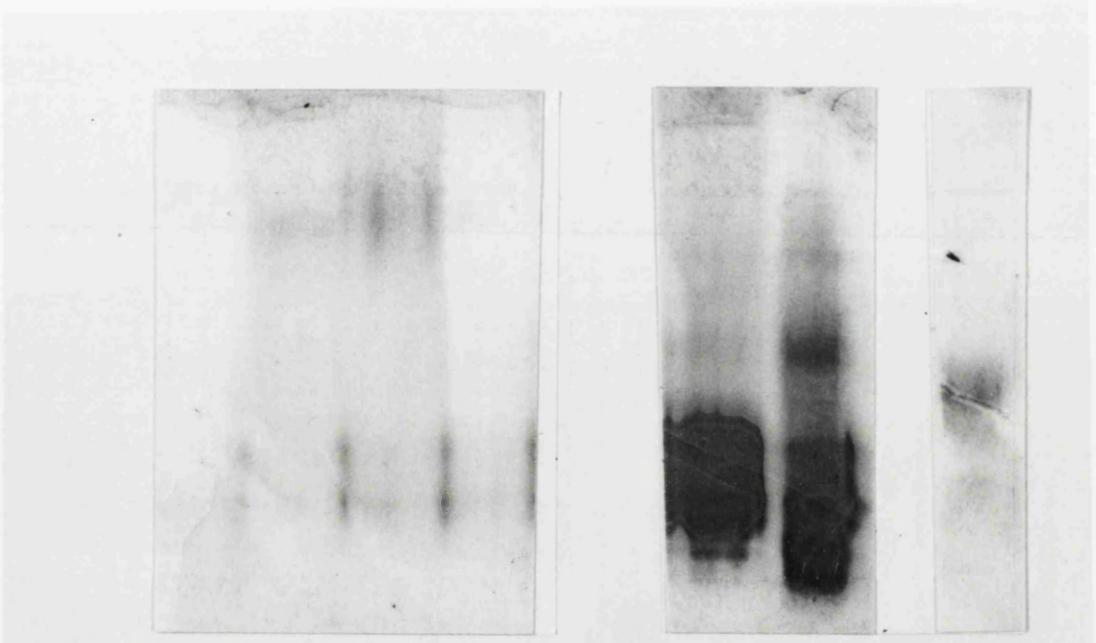
Somatic extracts of *D. viviparus* L3 always contained considerably less AChE activity than the adult stages, even when compared in terms of the protein concentration present in each extract (not shown).

The above experiments indicated that all esterase isoforms present in *D. viviparus* were AChE so all gels were subsequently stained by the method of Grunder, Sartori and Stormont (1965) which was simpler and quicker than the AChE-specific staining procedures.

Figure 6.2

Examination of different nematode species for AChE activity.

PBS-soluble somatic extracts of third stage *D. viviparus* (L3S), *D. filaria*, *O. ostertagi* and C. *oncophora* were examined for AChE activity following the method of Karnovsky and Roots (1964). The samples were compared to secretory (ES) and somatic (AdH) extracts of adult *D. viviparus* .



D.filaria *O. ostertagi* *C.oncophora* *D.viviparus*

ES AdH L3S

6.2.3 Recognition of *D. viviparus* AChE isoforms by infected calves and immunised guinea pigs.

To determine if specific antibody recognised the AChE present in *D. viviparus*, adult ES products and somatic extracts of the adult and L3 stages were incubated with serum from calves exposed to multiple infections. In addition, a sample of each was also incubated with serum pooled from six guinea pigs immunised twice with adult ES products in the context of Freund's adjuvant. Foetal bovine serum and normal guinea pig serum were also incubated with the antigen preparations as controls. Antigen preparations were incubated with the sera at a (volume) ratio of 2:1 for 2 hours at room temperature, the samples run on minigels under native conditions and stained for esterase activity. In this assay , the presence of specific antibody will result in complexing of the esterases so that they do not migrate into the resolving gel. Alternatively, if antibody binds to epitopes within the active site of the enzyme they will inhibit its activity. In order to examine if there was any endogenous AChE activity present in the sera, samples without antigen, were run and stained for esterase activity. The results are shown in Figures 6.3 a and b.

There appeared to be little activity due to endogenous AChE in the bovine serum which was in contrast to previous reports (Bremner *et al.*, 1973, Hodgson and Chubb, 1983). Two broad bands of esterase activity were present in the guinea pig sera, however, these migrated a shorter distance than the parasite AChE isoforms and therefore did not complicate the assay.

The results obtained with the antigen preparations run on their own were similar to those obtained previously, except that, in contrast to the previous experiment, the L3 somatic extract contained higher activity and the isoenzymes present appeared to be similar to those present within the adult stage.

Neither bovine pre-infection (not shown) nor guinea pig pre-immunisation sera had any qualitative effect upon the AChE isoenzymes present. Multiply-infected calves recognised all isoenzymes present in the adult homogenate preparation and no activity was detected in the resolving gel. A similar result was obtained when this antiserum was incubated with the somatic L3 extract. However, not all isoforms present in the adult ES products disappeared following incubation with serum from infected calves. This was likely to be a consequence of the very high levels of initial activity within the adult ES material.

Collectively, the guinea pigs immunised with adult ES products also demonstrated strong recognition of the esterase isoforms in the three *D. viviparus* extracts. This was particularly evident when the serum was incubated with the L3 somatic extract and all

but the upper two isoenzymes of the adult homogenate and ES preparations were recognised.

On closer inspection of the gels it was evident that in the samples in which there was antibody binding to parasite AChE, the enzymes were not inhibited but merely held back at the stack/resolving gel interface. This was especially obvious in the bovine serum samples, in which there was little endogenous esterase activity higher in the gel. These results indicated that the antibodies did not bind sufficiently close to the active site of the AChE isoenzymes to inhibit their activity. This was further emphasised by subsequent analysis in which adult ES products were pre-incubated with various types of bovine sera prior to being analysed by the test-tube assay (Ellman *et al.*, 1961). In order to perform this assay with guinea pig serum in which there were high levels of endogenous AChE activity, each serum sample was heated to 65°C for 5 minutes prior to incubation with the antigen preparation. This procedure eliminated endogenous esterase activity, whilst retaining the antibody activity present in the serum samples. The results are shown in Tables 6.2 a and b.

Both normal bovine and guinea pig sera appeared to have a non-specific effect upon the AChE isoforms present in the ES products and made it difficult to draw any comparisons with the post-infection or immunisation sera. The level of inhibition did not, however, appear to increase after incubation with sera from infected or vaccinated calves or sera from adult ES-immunised guinea pigs. As a high degree of enzyme recognition was obtained by gel analysis, without evidence of enzyme inhibition, the results would indicate that specific antibody was directed against epitopes outwith the active site of the enzyme.

6.2.4 Recognition of *D. viviparus* AChE by calves is mediated by antibodies of the IgG isotype.

In the experiments above, the endogenous AChE inhibitory activity in serum made it difficult to examine the effects of antibody on the enzyme. This problem was circumvented by incubating the samples with affinity-purified bovine IgG. These were obtained from normal and post-infection sera and were incubated with adult ES products and somatic extracts of the adult and L3 stages prior to gel assay. The results are shown in Figure 6.4.

In the tracks which contained antigen preparations which had been pre-incubated with IgG from normal L3-infected calves, there was some recognition of esterase isoforms present, as indicated by the band of activity which was retained at the stack/resolving gel interface. Equivalent bands were not evident in the samples which

had been pre-incubated with normal bovine serum or IgG affinity-purified from normal bovine serum.

Figures 6.3 a and b

Binding of antibody from infected calves and ES-immunised guinea pigs to *D. viviparus* AChE.

Figure 6.3a

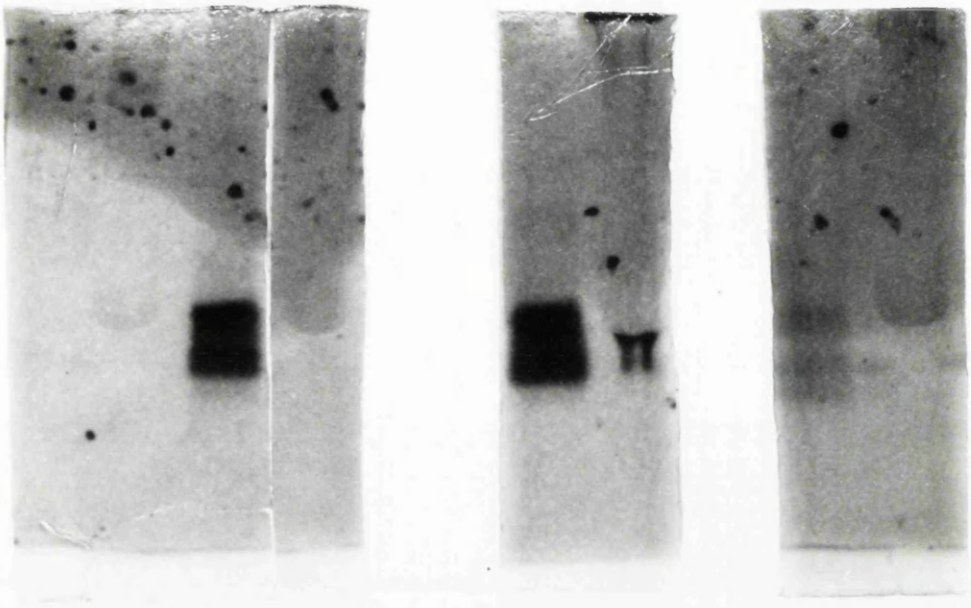
Serum pooled from three calves infected on three occasions with third stage larvae (BNL3) was incubated with either adult homogenate extract (AdH BNL3), adult ES products (ES BNL3) or L3 sonicate extract (L3S BNL3). The incubation products were then run on polyacrylamide gels and their esterase profiles compared to those in the original antigen preparations (AdH only, ES only and L3S only). Foetal bovine (FBS only) and post-infection (BNL3 only) serum were also run in the absence of antigen to examine for endogenous AChE activity.

Figure 6.3b

Normal guinea pig serum (AdH pre) and serum pooled from six guinea pigs immunised twice with adult ES products (AdH anti ES) were also incubated with AdH antigen and examined for recognition of parasite AChE. These sera were also incubated with adult ES (ES pre, ES anti ES) and L3S (L3S pre and L3S anti ES) extracts. The incubation products of each sample were assayed for esterase activity on polyacrylamide gels and their enzyme profiles compared to those of the serum samples run on their own (pre only, anti ES only).

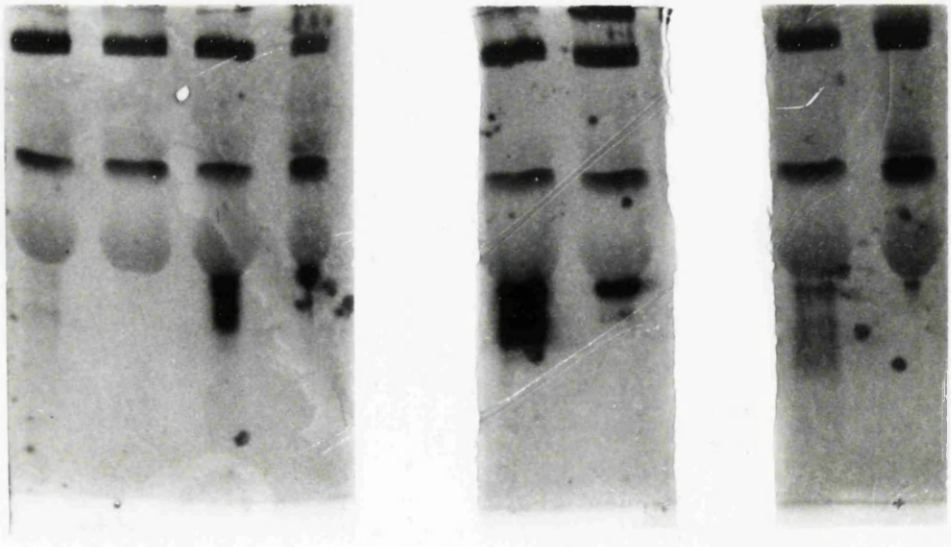
Both gels were stained for esterase activity following the method of Grunder, Sartori and Stormont (1965).

a)



FBS only | BNL3 only | AdH only | AdH BNL3 | ES only | ES BNL3 | L3S only | L3S BNL3

b)



pre only | anti ES only | AdH pre | AdH anti ES | ES pre | ES anti ES | L3S pre | L3S anti ES

Table 6.2a

Bovine sera	Percentage inhibition*
NBS	50
BNL3	75
400Gy	50
1000Gy	12.5

Table 6.2b

	% Inhibition by individual guinea pigs*					
Animal	NGPSa	NGPSb	DH	Str. 2	Str.13	anti AChE
1	40.6	37.5	12.5	68.7	56.2	37.5
2	53.1	53.1	12.5	37.3	40.6	56.1
3	56.0	50.0	75.0	43.7	56.2	46.9
4	56.0	56.0	50.0	40.6	75.0	59.4
5	43.7	54.7	68.7	81.2	96.2	46.9
6	48.4	45.3	-	-	90.6	34.4
7	53.1	-	-	-	-	-
8	53.1	-	-	-	-	-
Mean	50.5	49.4	43.7	54.3	69.1	41.0

Tables 6.2 a and b
Measurement of AChE inhibition by individual calves and guinea pigs.

Each serum sample was heated to 65°C for five minutes, to inhibit endogenous esterase activity, and 5µl incubated with 10µl of adult ES material. The incubation products were then assayed for AChE activity by the method of Ellman *et al.* (1961).

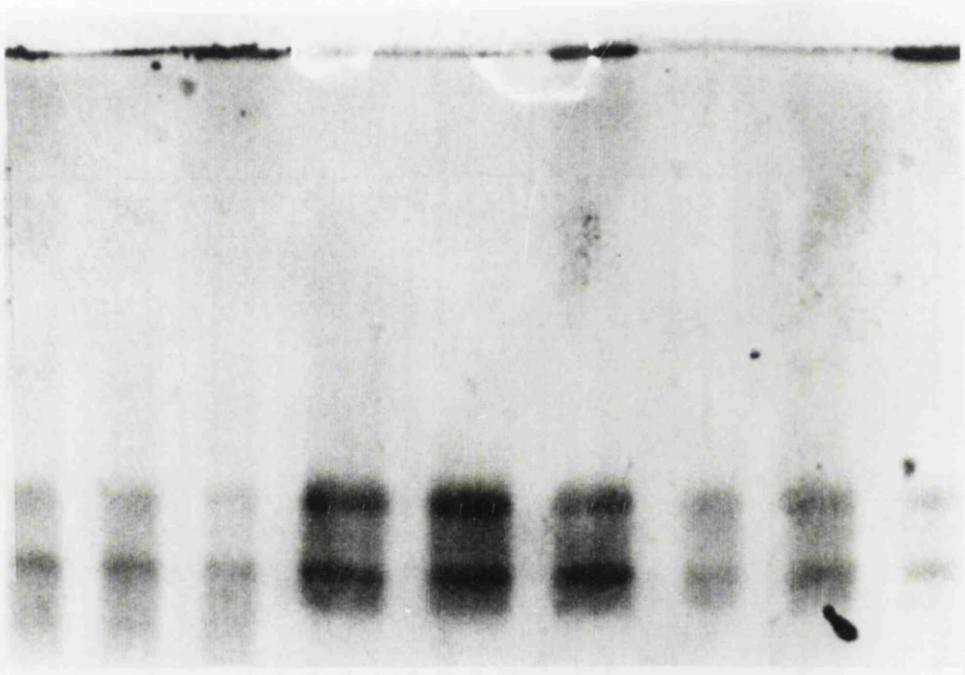
*Each sample is presented as a percentage inhibtion compared to the AChE activity present in adult ES products.

The bovine samples were pooled from three calves before infection (NBS) or after three infections with normal (BNL3), 400Gy or 1000Gy-irradiated L3. "NGPS a" and "b" were pre-infection bleeds from challenge control animals. Also examined, were serum samples taken on Day 14 of secondary ES-immunisation of Dunkin-Hartley (DH), strain 2 (Str. 2) and strain 13 (Str. 13) guinea pigs and a similar bleed from guinea pigs immunised with an AChE-enriched ES fraction (see Section 6.2.8).

Figure 6.4

IgG antibodies from infected calves bind *D. viviparus* AChE.

Normal bovine serum was incubated with adult homogenate extract (AdH1), adult ES products (ES1) and L3 homogenate extract (L3H1) for two hours at room temperature. IgG affinity-purified from either normal bovine serum (AdH2, ES2, L3H2) or post-infection serum (AdH3, ES3, L3H3) was also incubated with these antigen preparations. The incubation products of all the samples were run on a 10% polyacrylamide minigel under native conditions and their esterase profiles compared.



Adh1 Adh2 Adh3 ES1 ES2 ES3 L3h1 L3h2 L3h3

6.2.5 Recognition of *D. viviparus* AChE isoenzymes by individual calves

Individual calves were infected on several occasions with varying doses of normal L3 or L3 irradiated at 400Gy. Sera taken from the individual calves, prior to, and on Day 28 of each infection, were incubated with adult ES materials, the products run on native minigels and stained for esterase activity. The results are shown in Figures 6.5 and 6.6

The normal L3-infected calves (Figure 6.5) both recognised all AChE isoforms by Day 28 after primary infection. Calf "a" recognised all bands until the sixth infection, after which all isoforms re-appeared to some degree (track 4a). In contrast, calf "b" lost its recognition pattern after only the fourth infection (track 3b).

Calf "c", vaccinated with 400Gy-irradiated larvae (Figure 6.6), recognised all AChE isoforms by Day 28 of the fifth vaccination, while the other calf (d) did not recognise the enzymes until after the seventh dose of irradiated larvae. The fact that these calves, in which infection was likely to terminate at the early L5 stage (Jarrett and Sharp, 1963), recognised AChE released by the mature worms suggested that earlier stages may release isoforms of AChE similar to those from adults. It is possible, however, that a few mature adults may have developed in these calves and released AChE which stimulated the production of specific antibody.

In order to further investigate the appearance and longevity of anti-AChE antibodies during *D. viviparus* infection, sera, taken weekly after primary infection with 5,000 normal L3 or 400Gy-irradiated L3, were assessed for recognition of these enzymes. As before, adult ES materials were pre-incubated for two hours with the serum samples prior to being gel-assayed for AChE reactivity. The results are shown in Figures 6.7 a and b.

The calf infected with normal L3 (Figure 6.7a) recognised parasite AChE by Day 28 after infection and this was stronger by Day 49. In contrast, the calf infected with a single dose of irradiated larvae (Figure 6.7b) showed no recognition of any of the adult ES AChE enzymes by Day 49 after infection. Similar results were obtained with similarly treated calves and supported previous findings which indicated that calves infected with normal L3 appeared to be exposed to larger quantities of esterases compared with those infected with a similar number of 400Gy-irradiated L3.

Figure 6.5

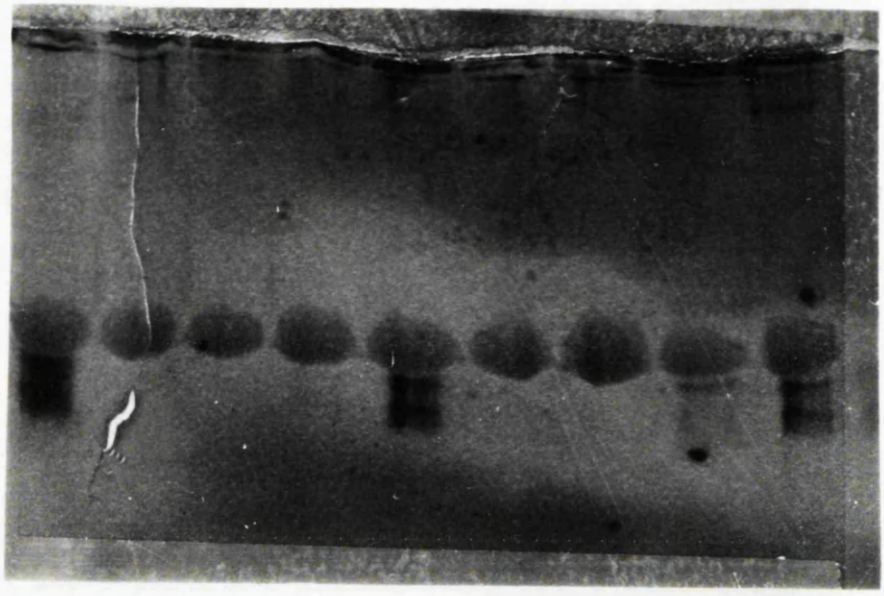
Individual calves exposed to natural *D. viviparus* infection recognise parasite AChE isoforms to different degrees.

Sera from two individual calves (a and b) taken on Day 28 of primary infection with 2,000 L3 (1a, 1b), secondary infection with a further 4,000 L3 (2a, 2b), quaternary infection with 20,000 L3 (3a, 3b) and on Day 28 of the sixth infection with 10,000 L3 (4a, 4b) were incubated with adult ES products as outlined previously. The incubation products of each sample were run on 10% polyacrylmide gels under native conditions and their esterase profiles compared to the level of recognition in a pre-infection serum pool (pre).

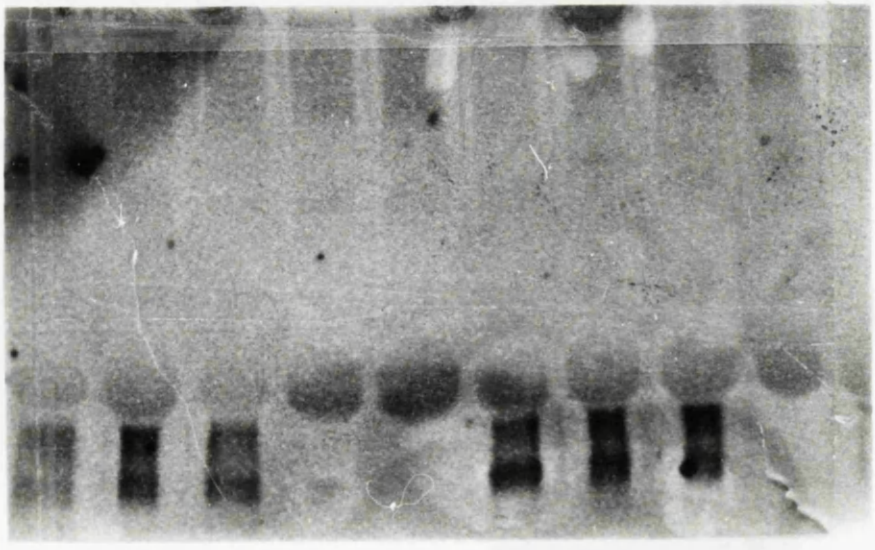
Figure 6.6

Individual calves vaccinated with 400Gy-irradiated *D. viviparus* L3 recognise parasite AChE isoforms to different degrees.

Sera from two individual calves (c and d) taken on Day 28 of primary infection with 5,000 irradiated L3 (1c, 1d), secondary infection with a further 10,000 irradiated L3 (2c, 2d), quinary infection with 20,000 irradiated L3 (3c, 3d) and on Day 28 of the seventh infection with 20,000 irradiated L3 (4c, 4d) were incubated with adult ES products as outlined previously. The incubation products of each sample were run on 10% polyacrylmide gels under native conditions and their esterase profiles compared to the level of recognition in a pre-infection serum pool (pre).



pre 1a 2a 3a 4a 1b 2b 3b 4b



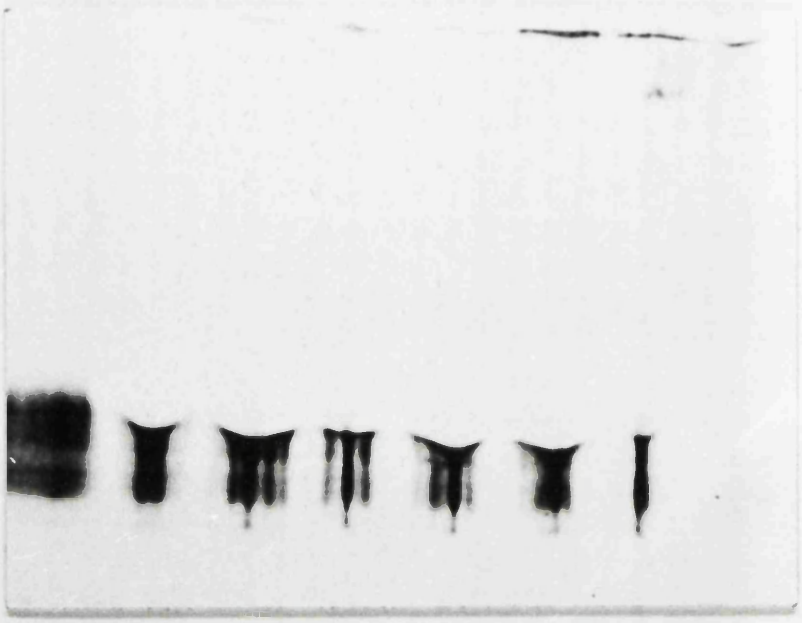
pre 1c 2c 3c 4c 1d 2d 3d 4d

Figure 6.7 a and b

The AChE recognition profiles of calves during the course of a primary infection with normal or 400Gy-irradiated *D. viviparus* L3.

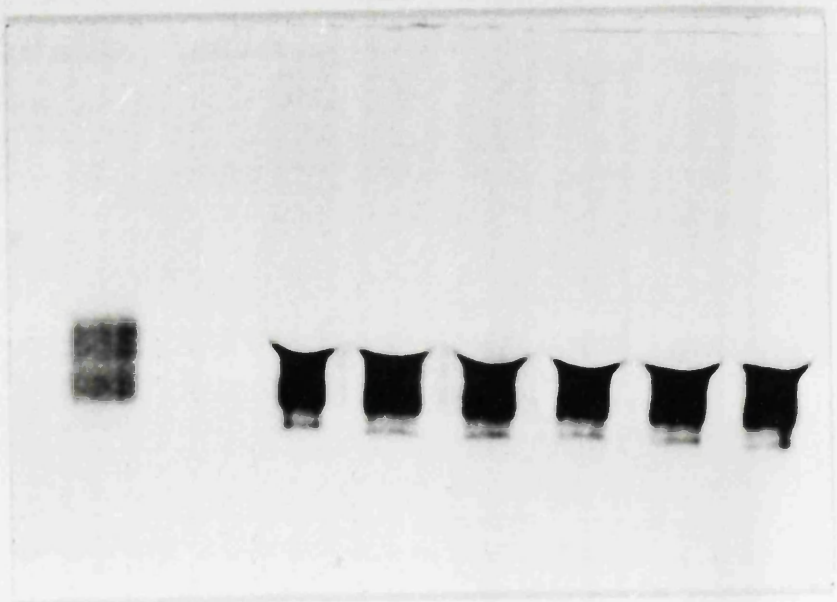
Serum samples taken on Days 0, 7, 14, 21, 28 and 49 of an infection with 5,000 normal L3 (Figure 6.7a) or with 5,000 400Gy-irradiated L3 (Figure 6.7b) were incubated with adult ES products as outlined previously. The incubation products were gel assayed for esterase activity and compared to adult ES materials (ES) or bovine serum (ser) run on their own.

a)



ES d0 d7 d14 d21 d28 d49

b)



ser ES d0 d7 d14 d21 d28 d49

The results obtained with the 400Gy L3 vaccinated calves indicated that immature stages of *D. viviparus* may release AChE. This was further suggested by the fact that AChE-specific antibodies were boosted in ES-immunised guinea pigs which were subsequently challenged with normal L3 (not shown) although challenge infections in guinea pigs do not produce mature adult stages (Poynter *et al.*, 1960). To find out if immature stages release AChE isoforms, a calf was infected with 20,000 normal L3 and necropsied on Day 14 of infection at which time the L4/5 stage parasites were recovered from the lungs . These parasites were subsequently maintained in culture for three days in a manner similar to that used for adult stages.

The ES material obtained from the L4/5 stages was gel-assayed for AChE and was observed to contain isoenzymes similar to those present within the adult ES products, with the exception of one esterase band of slower mobility (Figures 6.8 a and b) which was not recognised by any of the animals examined here. The other isoforms were recognised by guinea pigs immunised with adult ES products (in the context of FCA or in the context of niosomes) or an ES fraction enriched for AChE activity (Figure 6.8a). These esterases were also bound by purified IgG from normal L3-infected calves and by serum from calves vaccinated with 400Gy-irradiated L3 (Figure 6.8b). There was no obvious recognition of parasite AChE by calves which had been vaccinated with L3 irradiated at 1000Gy and similar findings were obtained when adult ES materials were incubated with sera from these calves (not shown).

6.2.7 Recognition of *D. viviparus* AChE isoenzymes by individual guinea pigs

To investigate if the heterogeneity in antibody response to parasite AChE exhibited by individual calves was also demonstrated by guinea pigs, sera from individuals, immunised twice with adult ES products, were examined for activity against *D. viviparus* AChE isoforms The samples were incubated with adult ES materials and gel assayed as outlined above. The results are shown in Figure 6.9.

As observed in the calf recognition patterns, antibody from individual guinea pigs bound adult ES AChE isoforms to different degrees. Animals B1, B5 and B6 recognised three out of the five isoenzymes, while guinea pigs B2 and B3 recognised all but one. The sera examined were from the ES-immunised guinea pigs of Protection Experiment 2 and it appeared that the animals which had the lowest worm burdens following challenge, recognised most of the parasite AChE isoenzymes. In contrast, guinea pig B4, which harboured a considerable number of L5 stages, demonstrated the most restricted enzyme recognition pattern. The inbred guinea pig strains immunised with whole ES material (Protection Experiment 3) were also assessed for parasite AChE recognition. The results are shown in Figures 6.10 a and b.

with whole ES material (Protection Experiment 3) were also assessed for parasite AChE recognition. The results are shown in Figures 6.10 a and b.

Strain 2 guinea pigs (Figure 6.10a) recognised few of the AChE isoforms present in *D. viviparus* adult ES products, in contrast to strain 13 guinea pigs which displayed a more extensive recognition pattern of the isoenzymes present (Figure 6.10b). This was of particular interest, as strain 13 animals showed significantly higher protection against challenge than animals of strain 2.

Figures 6.8 a and b

Antibodies from infected calves and immunised guinea pigs bind AChE released from *D. viviparus* L4 and L5 stages.

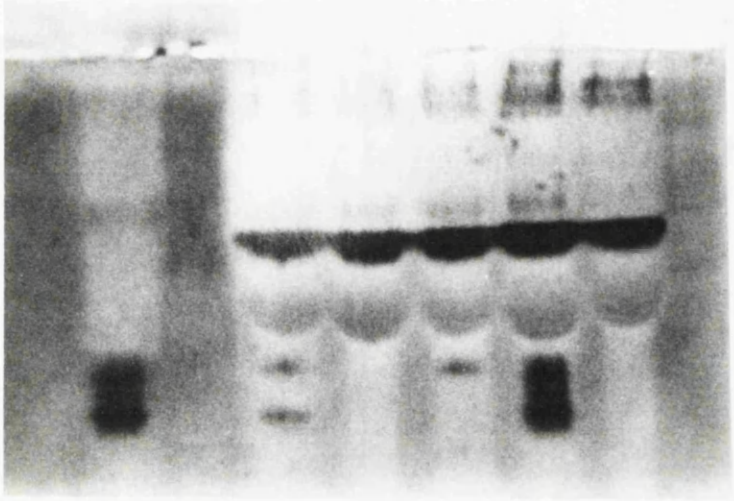
Figure 6.8a

L4/5 ES products were incubated with pooled sera from guinea pigs immunised twice with adult ES, in the context of niosome adjuvant (A) or Freund's adjuvant (C), or with serum from guinea pigs immunised with an AChE-enriched ES fraction (B). The incubation products were gel assayed for esterase activity and compared to L4/5 ES material (L4/5 ES only) and normal guinea pig serum (serum only) run on their own.

Figure 6.8b

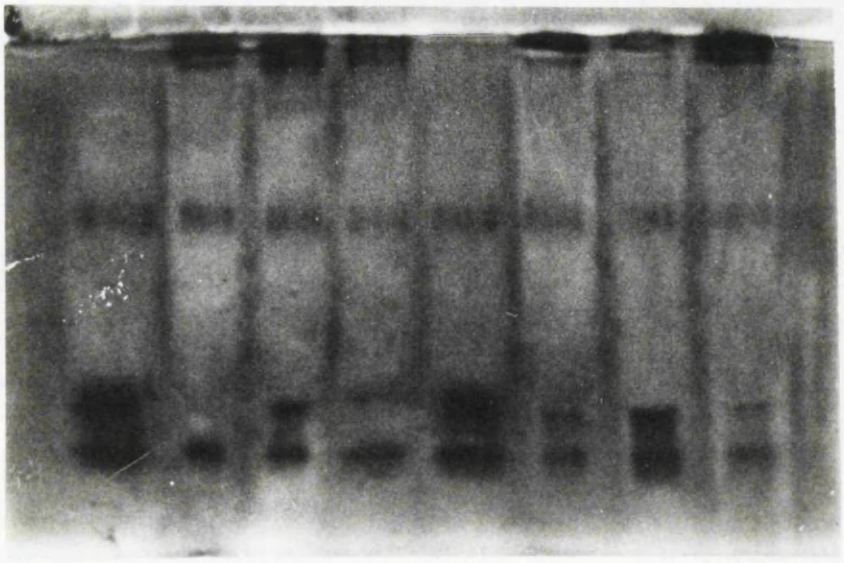
L4/5 ES products were incubated with pooled sera from calves prior to (NBS) and following multiple infections with normal L3 (BNL3), 400Gy- (400Gy) and 1000Gy- (1000Gy) irradiated L3. Purified IgG from normal bovine serum (NIg) and post-infection serum (ImIg), were also examined for reactivity against *D. viviparus* AChE. The esterase profiles of the incubation products were compared to the antigen preparation (L4/5 ES only) by gel assay.

a)



L4/5 ES only | A | B | C | pre serum | serum only

b)

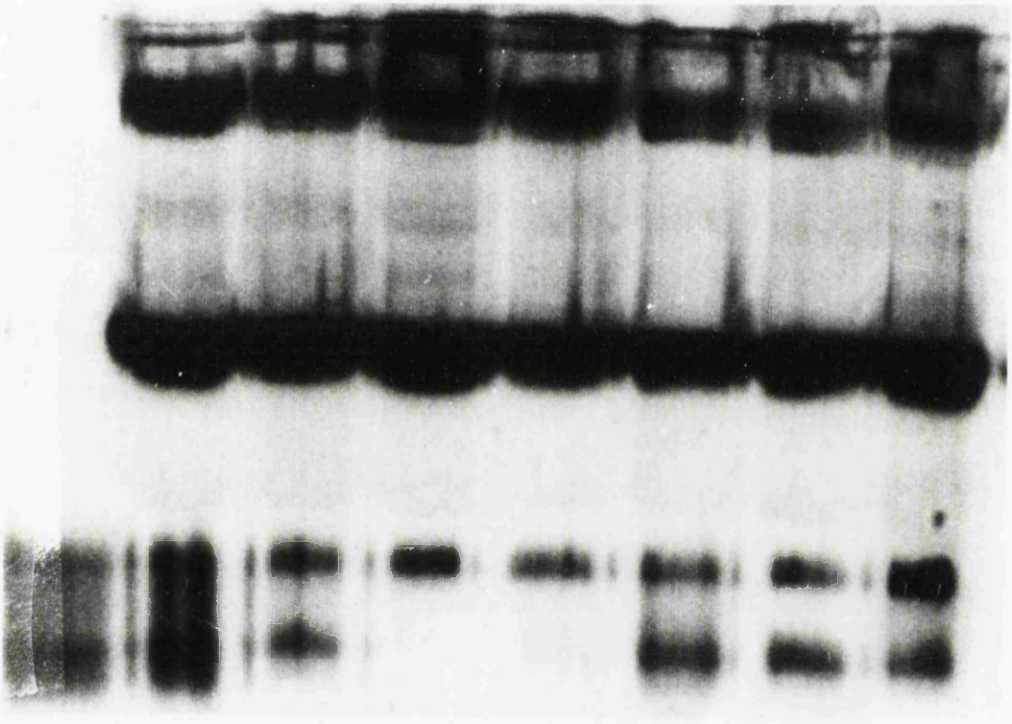


NBS | BNL3 | N Ig | Im. Ig | 1000 Gy | 400 Gy | L4/5 ES only

Figure 6.9

Dunkin-Hartley guinea pigs immunised twice with adult ES products display heterogeneous recognition profiles of the *D. viviparus*-secreted AChE isoforms.

Sera from the animals immunised with whole ES (from Protection Experiment 2) were used to examine individual guinea pig recognition patterns of *D. viviparus* AChE. Each serum sample, which was obtained on Day 14 of secondary immunisation, was incubated with adult ES products for two hours and the samples then analysed for esterase activity by gel assay. The individual worm burdens are depicted below the gel.



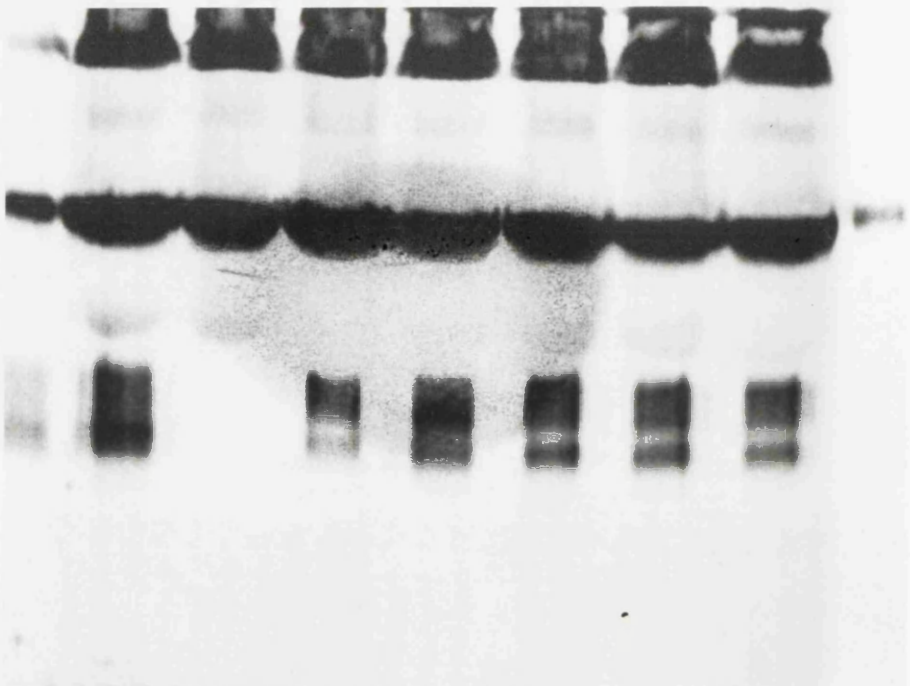
ES	Pre	B1	B2	B3	B4	B5	B6
-	-	101	12	13	161	24	24

Figures 6.10 a and b

Strain 2 and 13 guinea pigs immunised with adult ES products recognise the parasite AChE isoforms to different degrees.

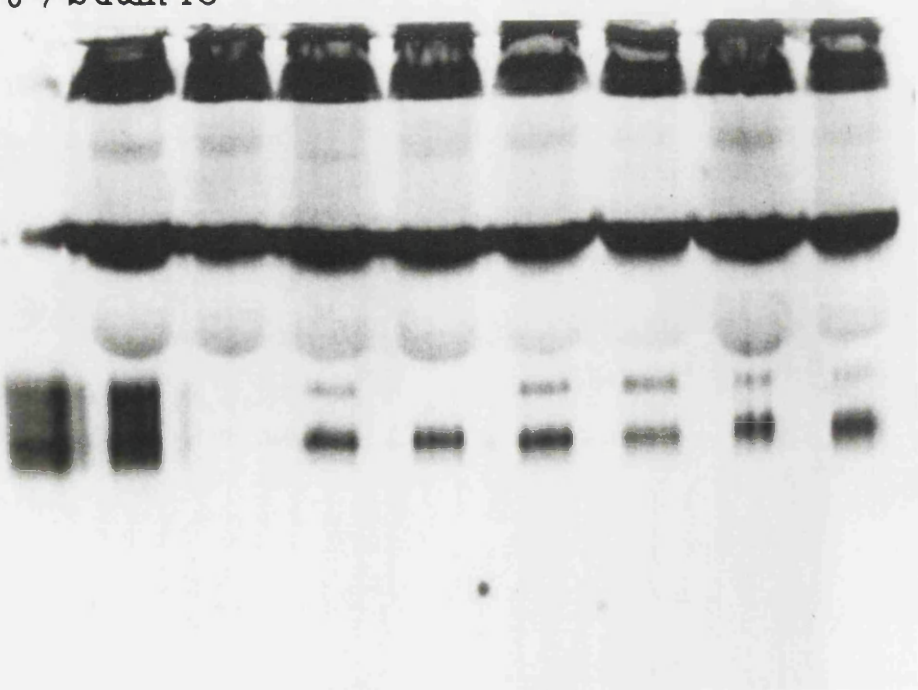
Individual sera taken from strain 2 (Figure 6.10a) and strain 13 (Figure 6.10b) guinea pigs on Day 14 of secondary immunisation were examined for AChE reactivity. Pooled pre-infection serum (pre) was also examined. Each serum sample was incubated with adult ES material as outlined previously and the products gel-assayed for esterase activity. The resulting esterase profiles were compared to those present in adult ES products (ES) and guinea pig serum (ser).

a) Strain 2



ES pre ser A1 A2 A3 A4 A5

b) Strain 13



ES pre ser B1 B2 B3 B4 B5 B6

6.2.8 Protection Experiment 4: immunisation of guinea pigs with AChE-enriched fraction of adult ES products and with whole ES products in the context of niosomes.

Since previous findings suggested a potential involvement of AChE in the host/parasite interaction in *D. viviparus* infection, an experiment was designed to find out if these enzymes have any functional role in the stimulation of protective immunity. *D. viviparus* AChEs were partially purified from whole ES products and administered, with Freund's adjuvant, to guinea pigs. In addition, a plurionic block co-polymer was assessed for adjuvant activity and, in this case, animals were given a niosome preparation in which whole adult ES products had been incorporated (courtesy of Dr. J. Brewer, Department of Immunology, University of Strathclyde).

Forty eight Dunkin-Hartley guinea pigs were randomly split into six groups of eight. The animals in Group A acted as Freund's adjuvant controls and received a primary immunisation of PBS emulsified with FCA and a secondary immunisation of PBS with FIA. Those in Group B acted as niosome adjuvant controls and received two immunisations of niosomes without antigen. Individuals in Group C were immunised twice with adult ES products, in the context of Freund's adjuvant, while those in Group D received the same antigen, incorporated into niosomes. Animals in Group E were immunised twice with an AChE-enriched adult ES fraction administered with Freund's adjuvant; this fraction was prepared by electroelution from polyacrylamide gels (Section 2.8.6) and was gel-assayed for AChE activity prior to use. The eight remaining animals acted as challenge controls (Group F). Details of the experimental design are shown in Table 6.3.

The esterase profile of the AChE-enriched fraction was compared with that of whole ES material (Figure 6.11) and it appeared that all the AChE isoforms were included in the enriched fraction.

Larvae were not available for challenge infection until 8 weeks after the second immunisation: all animals received 6,000 L3 *per os* and were killed on Day 6 after challenge for recovery of worms. One animal in each group was bled prior to primary immunisation and all were bled fourteen days after secondary immunisation and at necropsy.

The worm burdens are shown in Table 6.4. There was no statistical difference in the worm burdens of any of the immunised groups compared with the challenge controls. When the antigen recipient groups were compared with their relevant adjuvant controls, it was found that the animals which had received the AChE-enriched fraction in Freund's, harboured worm burdens which were significantly lower than those which received Freund's alone. The worm burdens of the animals which received whole ES materials in Freund's, however, were not significantly different from those of the appropriate adjuvant controls.

Serum samples taken after secondary immunisation and challenge of all groups were examined for adult ES-specific antibody by ELISA. The results are shown in Figures 6.12a to 6.12e. As in previous experiments, the adjuvant control groups (Groups A and B) did not show an increase in specific antibody following secondary immunisation. The animals which received ES products in the context of Freund's adjuvant had a variable increase in antibody following secondary immunisation and these levels were boosted considerably by Day 6 of challenge in all animals. In contrast, those animals which received ES antigen with niosome adjuvant had more uniform increases in antibody following secondary immunisation and these levels were not boosted following challenge. Following immunisation, the individuals which received the AChE-enriched fraction (Group E) had variable increases in ES-specific antibody which were not boosted after challenge. There was no correlation between the levels of antibody and the worm burdens obtained in individuals in any of the groups and, if anything, there appeared to be a negative correlation between antibody levels and worm burdens in the animals which received antigen in the context of Freund's adjuvant.

Serum samples from all animals were analysed by immunoprecipitation with ^{125}I-labelled adult ES products. The animals in the adjuvant control groups did not recognise any adult ES components following secondary immunisation or challenge.

The responses of the adult ES/Freund's-immunised animals (Group C) are shown in Figures 6.13 and 6.14. The results correlated well with those obtained by ELISA. Animals C2 and C3 had low levels of antibody on Day 14 of secondary immunisation but these increased considerably following challenge. In addition, these individuals also recognised more adult ES components following challenge. Overall, the antigen recognition profiles of the animals which received adult ES products with Freund's adjuvant were similar to those of animals immunised with the same preparation in the previous Protection Experiments (Chapter 5). All animals recognised both components of the 28-32kDa doublet but there was heterogeneous recognition of the 38-42kDa doublet and the 14kDa and 18kDa components. The increases in antibody following challenge were greater than those observed in the other Protection Experiments and this may have been related to the longer period between secondary immunisation and challenge.

Animals which received ES products in the context of niosomes (Figure 6.15) displayed a heterogeneous recognition pattern reminiscent of that observed in animals immunised with ES products and Freund's. The 28-32kDa doublet was strongly recognised by all individuals following secondary immunisation. The levels of specific antibody did not appear to be boosted following challenge and the animals did not recognise any additional adult ES components following challenge (not shown).

The immunoprecipitation results of the animals immunised with the AChE-enriched fraction and Freund's (Group E) correlated well with those obtained by ELISA; for example, guinea pig E3 displayed extremely low levels of ES-specific antibody by both procedures. Although these individuals received an ES fraction eluted from a small area of a polyacrylamide gel, most recognised several adult ES components which, when run under reducing conditions, varied considerably in molecular weight (Figure 6.16). These animals may have been immunised with many ES components, as the AChE isoforms were enriched from native gels in which components, sensitive to reduction on SDS-PAGE, might have run as one entity.

Sera from animals immunised with adult ES material and Freund's (Figure 6.17), adult ES material and niosomes (Figure 6.18) and AChE-enriched material and Freund's (Figure 6.19) were also immunoprecipitated with the AChE-enriched fraction radiolabelled with ^{125}I. A heterogeneous pattern of recognition was observed within each group and up to five components were recognised by some animals. The most immunodominant molecule had a relative molecular weight of approximately 67kDa and although recognised to different degrees by individual animals, there was no correlation between the recognition of this band and worm burdens. From these results, it was impossible to discriminate which molecules were associated with AChE activity. Pre-immunisation sera did not recognise any components present in this fraction (not shown).

All guinea pigs in Protection Experiment 4 were subsequently assessed for recognition of parasite AChE enzymes. By Ellman assay, the serum from immunised animals did not inhibit parasite esterases more than did normal guinea pig serum (not shown). When AChE reactivity was examined by gel assay, sera from individuals in the adjuvant and challenge control groups did not recognise the adult ES AChE isoforms (not shown). Secondary immunisation responses of animals which received adult ES material (Group C) or the AChE-enriched fraction (Group E) with Freund's are demonstrated in Figures 6.20a and b. In contrast to the responses of ES-immunised animals in the previous Protection Experiments, the animals which received this antigen in this study did not recognise any of the *D. viviparus* AChE isoforms (Figure 6.20a). Six of the eight animals which received the AChE-enriched fraction, recognised at least three of the isoenzymes (Figure 6.20b), while the individuals which received adult ES with niosomes did not recognise any of the AChE isoforms (not shown).

Following challenge, it was observed that the anti-AChE responses were boosted in animals which received adult ES (Group C) or the AChE-enriched fraction (Group E) with Freund's (Figures 6.21 a and c). Five of the animals which received whole ES products with Freund's adjuvant recognised the parasite AChE to variable degrees

(Figure 6.21a), in comparison to those which received this antigen in niosomes where recognition of the enzymes appeared limited (Figure 6.21b). Antibody, from the animals which received the AChE-enriched fraction bound very strongly to the enzymes and, in all but two individuals, all bands were retarded at the stack/resolving gel interface (Figure 6.21c).

Group	Antigen	Primary		Secondary	
		Protein (μg)	Vol. (ml)	Protein (μg)	Vol. (ml)
A	PBS/FA	NA	0.6	NA	1.0
B	PBS/nios.	NA	0.3	NA	0.3
C	ES/FA	81	0.6	80	0.8
D	ES/nios.	81	0.3	105	0.3
E	AChE/FA	21	0.8	34	0.4
F	ChC	NA	NA	NA	NA

Table 6.3
Protection experiment 4: total protein and volume of antigen given to immunised guinea pigs.

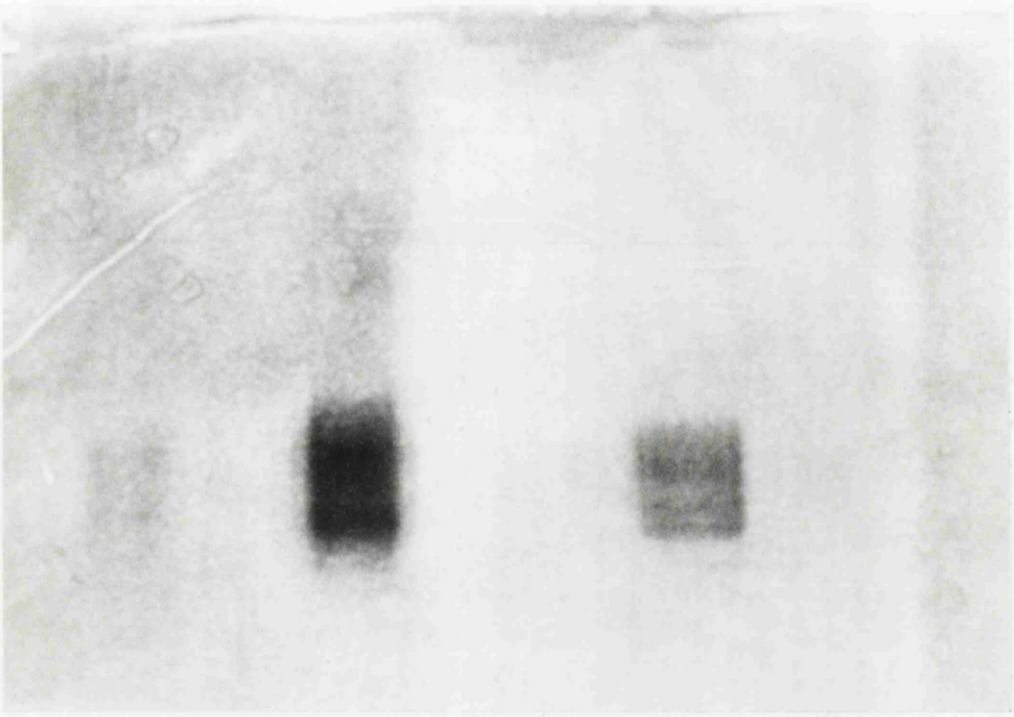
Animals in Groups A and B acted as adjuvant controls and received Freund's adjuvant (PBS/FA) or niosomes (PBS/nios.) with PBS. Those ir Groups C (ES/FA) and D (ES/nios.) received whole adult ES products in the context of either of these adjuvants. Those in Group E were immunised with an ES fraction enriched for AChE activity (AChE/FA) with Freund's adjuvant. The animals in Group F acted as challenge controls (ChC).

All animals recieved two immunisations, four weeks apart and were challenged with 6,000 L3 on Day 56 of secondary immunisation.

NA = not applicable.

Figure 6.11 AChE activity present in the enzyme-enriched adult ES fraction used in Protection Experiment 4.

A concentrated AChE-enriched fraction obtained by electroelution of gel cut-outs was run on 10% polyacrylamide gels and the esterase activity compared to that present in the adult ES batches (ES3 and ES2) used for the purification. The filtrate (filtrate of eluant), obtained following concentration of the fraction, was also examined for esterase activity. The gel was run under native conditions and stained for esterase activity.



ES3 ES2 concentrated AChE eluant filtrate of eluant

Group	A	B	C	D	E*	F
Antigen	PBS/ FA	PBS/ nios.	ES/ FA	ES/ nios.	AChE/ FA	ChC
Recoveries:						
1	15	16	28	5	13	45
2	92	17	4	1	15	35
3	47	19	9	55	21	43
4	49	20	28	33	43	140
5	31	13	78	29	3	40
6	37	38	47	18	13	5
7	124	12	31	10	56	12
8	80	15	44	34	3	21
Mean	59.4	18.8	33.6	23.1	20.9	42.6
% reduction	-	56.0	21.1	45.6	51.0	-

Table 6.4
Protection Experiment 4: worm recoveries of immunised guinea pigs on Day 6 after challenge with 6,000 *D. viviparus* L3
The Groups are as described in Table 6.3. The mean worm burden of each group was compared to that obtained from the challenge control group (F) to give percentage reduction.
* indicates that the worm recoveries differed significantly from those of the PBS/FA adjuvant control group by the Mann-Whitney non-parametric test (5% significance level).

Figure 6.12a

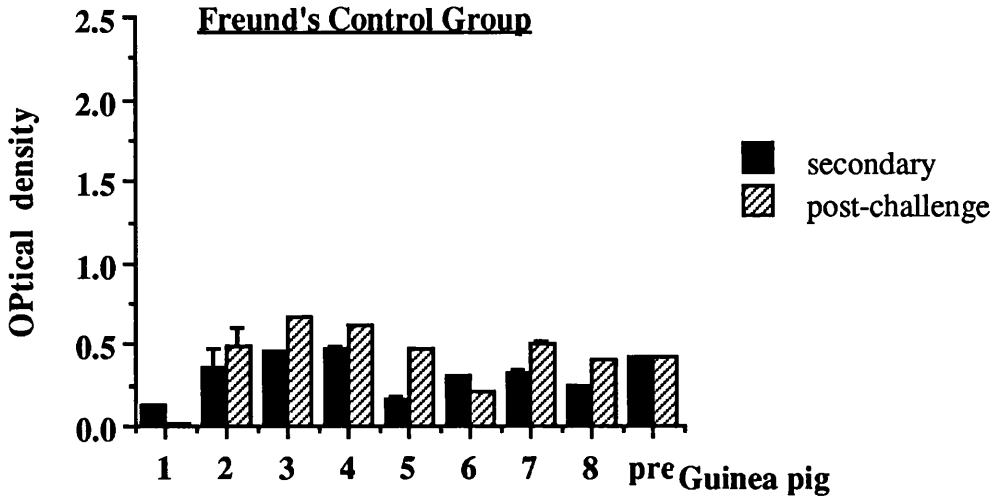
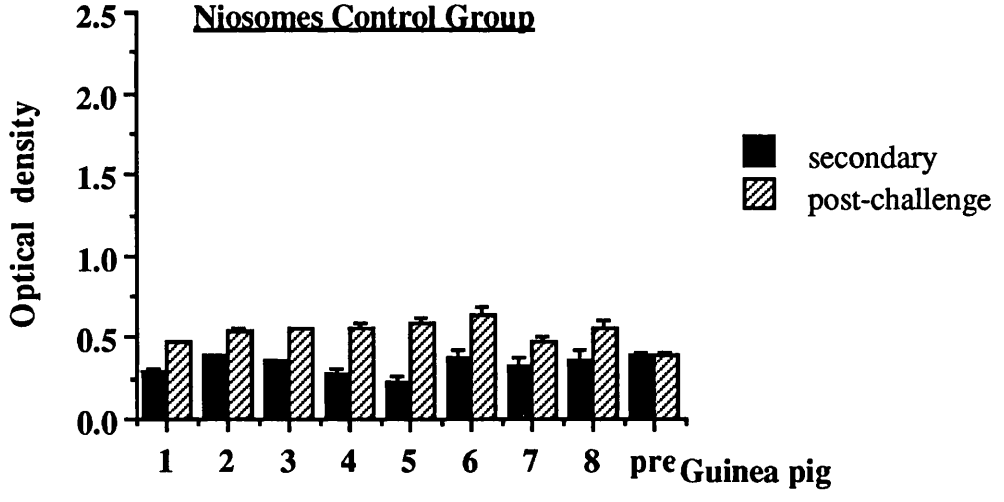


Figure 6.12b



Figures 6.12 a and b

Protection Experiment 4: Adult-ES specific antibody in adjuvant control guinea pigs measured by the ELISA.

The samples were taken from Freund's (6.12a) and niosomes (6.12b) adjuvant control guinea pigs on Day 14 of secondary immunisation and 6 days after challenge with 6,000 *D. viviparus* L3. The samples were tested in duplicate, the mean and standard deviation of each animal is indicated and compared with a pre-immunisation control (pre). The optical densities were read at 492nm.

Figure 6.12c

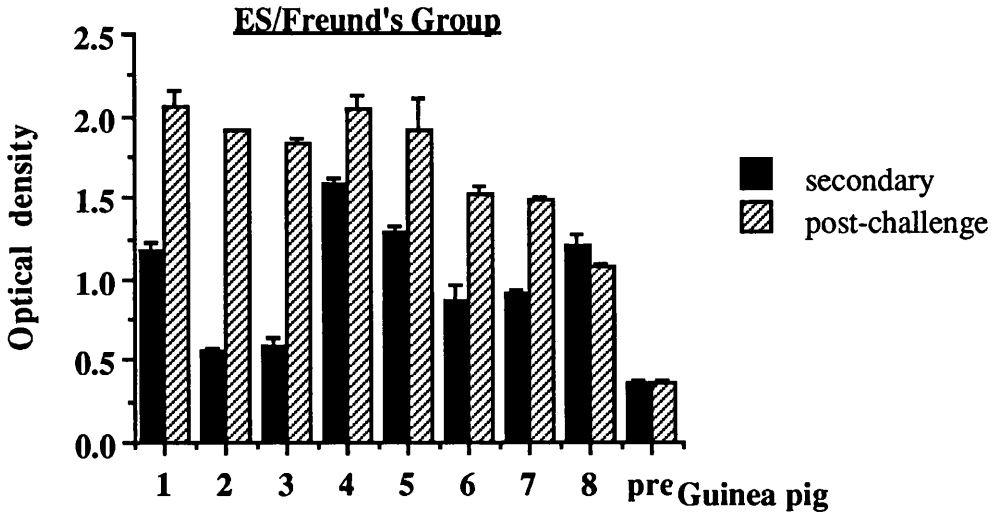
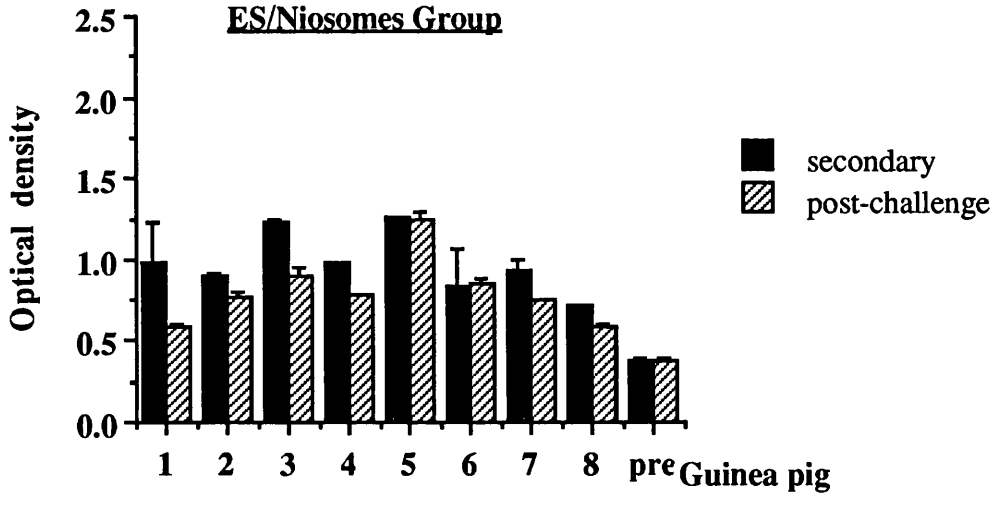


Figure 6.12d



Figures 6.12c and d

Protection Experiment 4: Adult-ES specific antibody in guinea pigs immunised with adult ES products in the context of Freund's or niosome adjuvants.

The samples were taken from guinea pigs immunised with adult ES in Freund's (6.12c) or niosome (6.12d) adjuvants on Day 14 of secondary immunisation and 6 days after challenge with 6,000 *D. viviparus* L3. The samples were tested in duplicate, the mean and standard deviation of each animal is indicated and compared with a pre-immunisation control (pre). The optical densities were read at 492nm.

Figure 6.12e

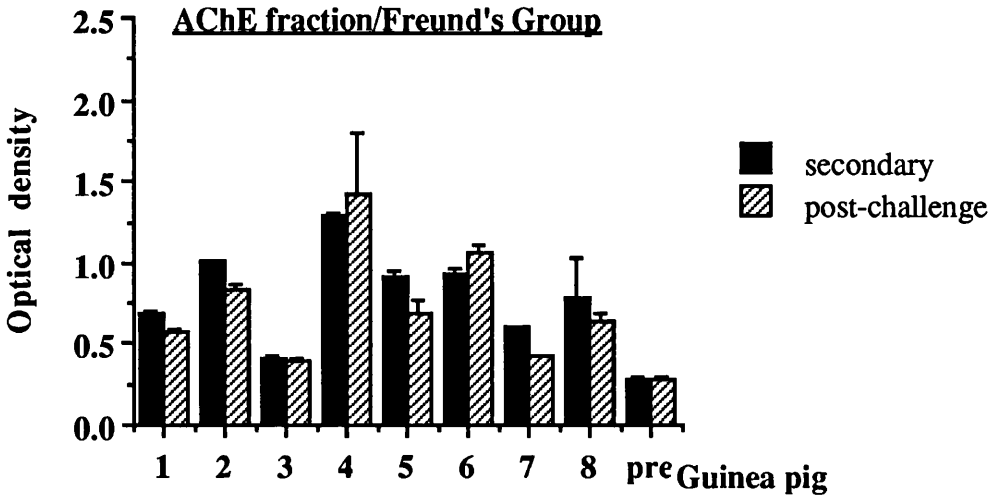


Figure 6.12e

Protection Experiment 4: Adult-ES specific antibody in guinea pigs immunised with AChE-enriched adult ES products in the context of Freund's adjuvant.

The samples, taken on Day 14 of secondary immunisation and 6 days after challenge with 6,000 *D. viviparus* L3. The samples were tested in duplicate, the mean and standard deviation of each animal is indicated and compared with a pre-immunisation control (pre). The optical densities were read at 492nm.

Figure 6.13

Protection Experiment 4: immunoprecipitation of *D. viviparus* adult ES antigens (Ag) by antibodies in the serum of individual animals (C1-C8) immunised twice with adult ES products in the context of Freund's adjuvant. The serum was taken on Day 14 of secondary immunisation.

Radio-iodinated adult ES material (Ag) was incubated overnight with individual guinea pig serum and immunoadsorbed with Staph A and the precipitates washed three times. The complexes were then analysed by SDS-PAGE under reducing conditions. For comparison, the antigen was also immunoprecipitated with normal guinea pig serum (pre) and with a pool of serum from three multiply-infected calves (BNL3). The relative molecular mass of marker proteins (M) are indicated in kiloDaltons.

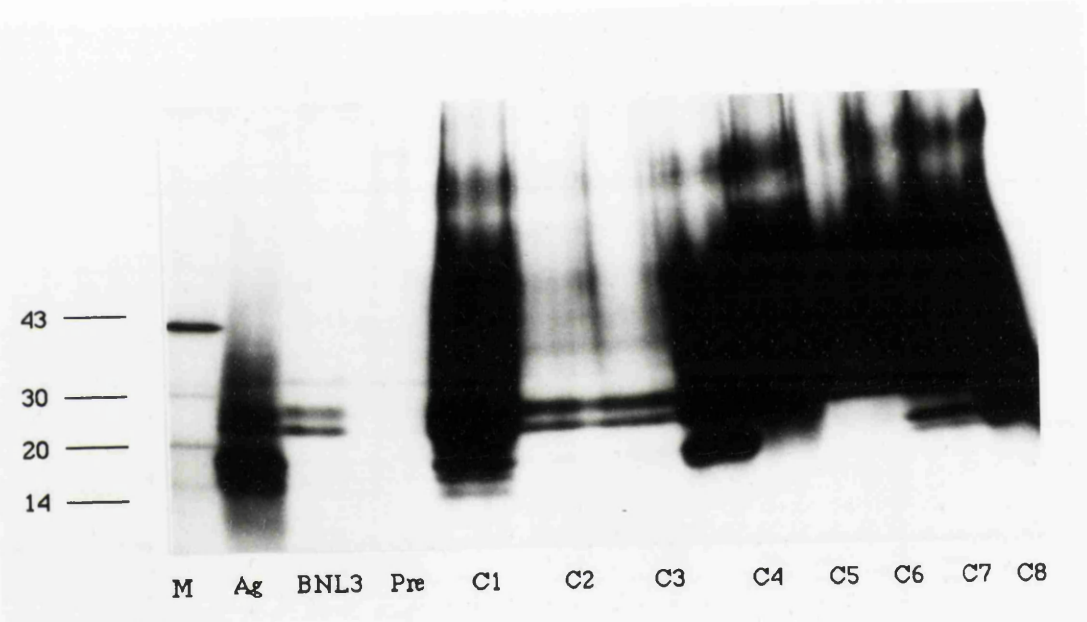

43
30
20
14
M
Ag
BNL3
Pre
C1
C2
C3
C4
C5
C6
C7
C8

Figure 6.14

Protection Experiment 4: immunoprecipitation of *D. viviparus* adult ES antigens (Ag) by antibodies in the serum of individual animals (C1-C8) immunised twice with adult ES products in the context of Freund's adjuvant. The serum was taken on Day 6 of challenge with 6,000 *D. viviparus* L3.

The immunoprecipitation was carried out as detailed in Figure 6.13. The relative molecular mass of marker proteins (M) are indicated in kiloDaltons.

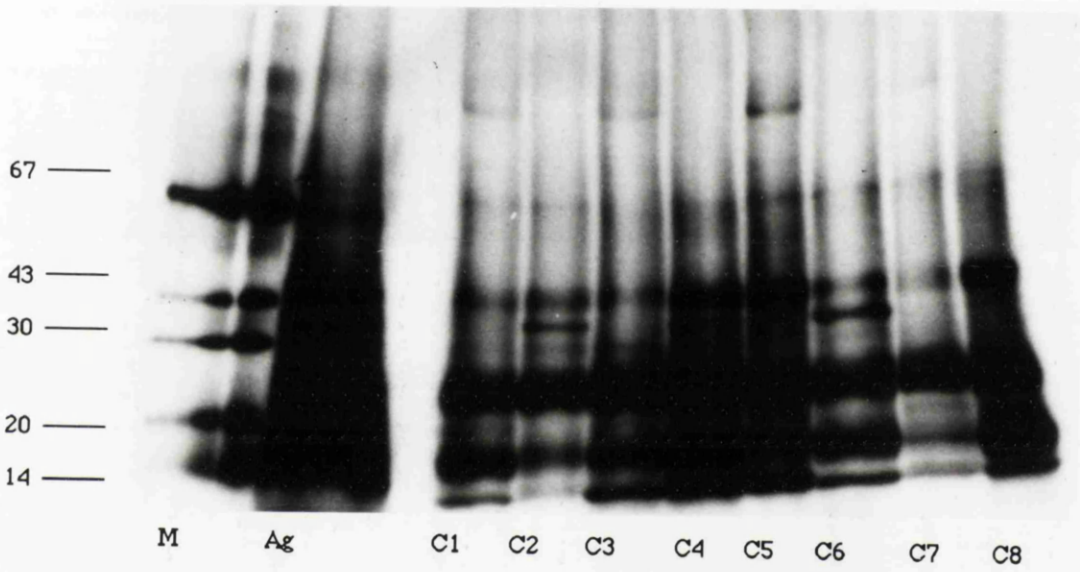

67
43
30
20
14
M
Ag
C1
C2
C3
C4
C5
C6
C7
C8

Figure 6.15

Protection Experiment 4: immunoprecipitation of *D. viviparus* adult ES antigens (Ag) by antibodies in the serum of individual animals (D1-D8) immunised twice with adult ES products in the context of niosome adjuvant. The serum was taken on Day 14 of secondary immunisation.

The immunoprecipitation was carried out as detailed in Figure 6.13. For comparison, the antigen was also immunoprecipitated with normal guinea pig serum (pre) and with a serum pool from three multiply-infected calves (BNL3). The relative molecular mass of marker proteins (M) are indicated in kiloDaltons.

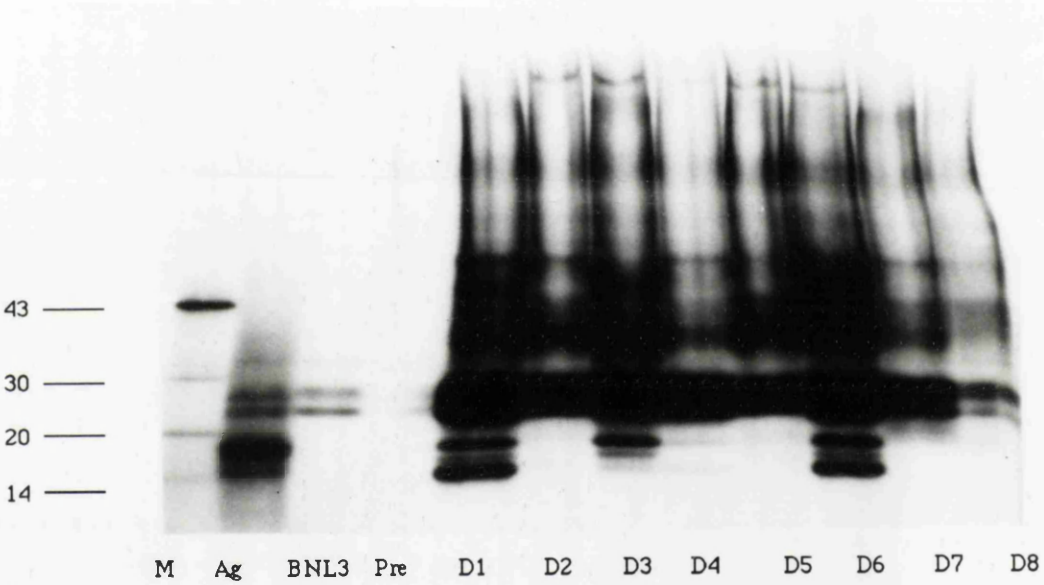

43
30
20
14
M
Ag
BNL3
Pre
D1
D2
D3
D4
D5
D6
D7
D8

Figure 6.16

Protection Experiment 4: immunoprecipitation of *D. viviparus* adult ES antigens (Ag) by antibodies in the serum of individual animals (E1-E8) immunised twice with an AChE-enriched adult ES fraction in the context of Freund's adjuvant. The serum was taken on Day 14 of secondary immunisation.

The immunoprecipitation was carried out as detailed in Figure 6.13. For comparison, the antigen was also immunoprecipitated with normal guinea pig serum (pre) and with a serum pool from three multiply-infected calves (BNL3). The relative molecular mass of marker proteins (M) are indicated in kiloDaltons.

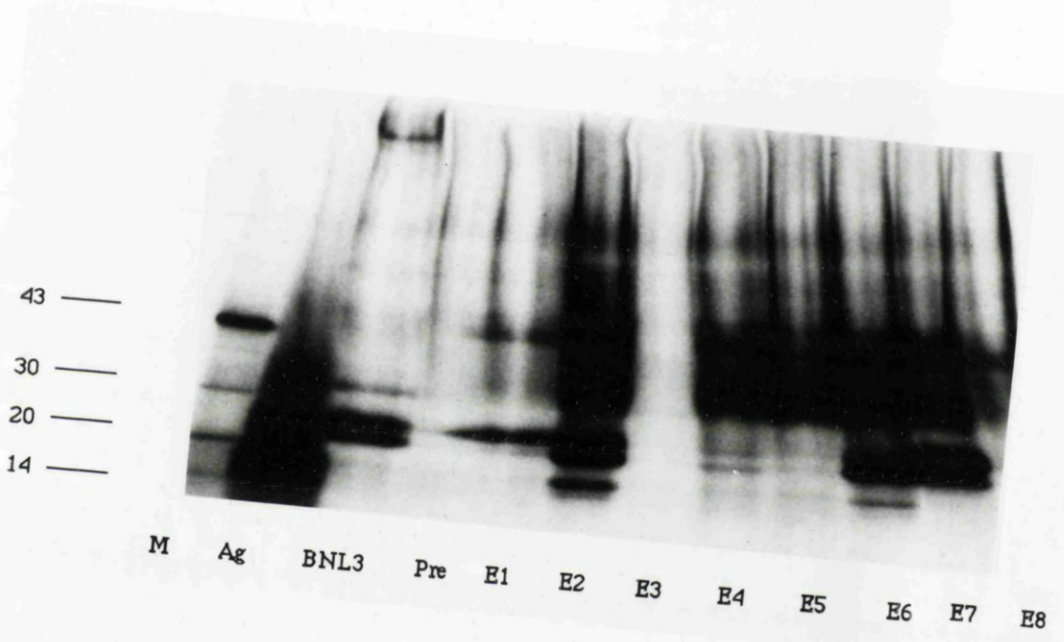

43
30
20
14
M
Ag
BNL3
Pre
E1
E2
E3
E4
E5
E6
E7
E8

Figure 6.17

Protection Experiment 4: immunoprecipitation of ^{125}I-labelled AChE-enriched fraction (Ag) by antibodies in the serum of individual animals (C1-C8) immunised twice with adult ES products in the context of Freund's adjuvant. The serum was taken on Day 14 of secondary immunisation.

The immunoprecipitation was carried out as detailed in Figure 6.13. For comparison, the antigen was also immunoprecipitated with a serum pool from three multiply-infected calves (BNL3). The relative molecular mass of marker proteins (M) are indicated in kiloDaltons.

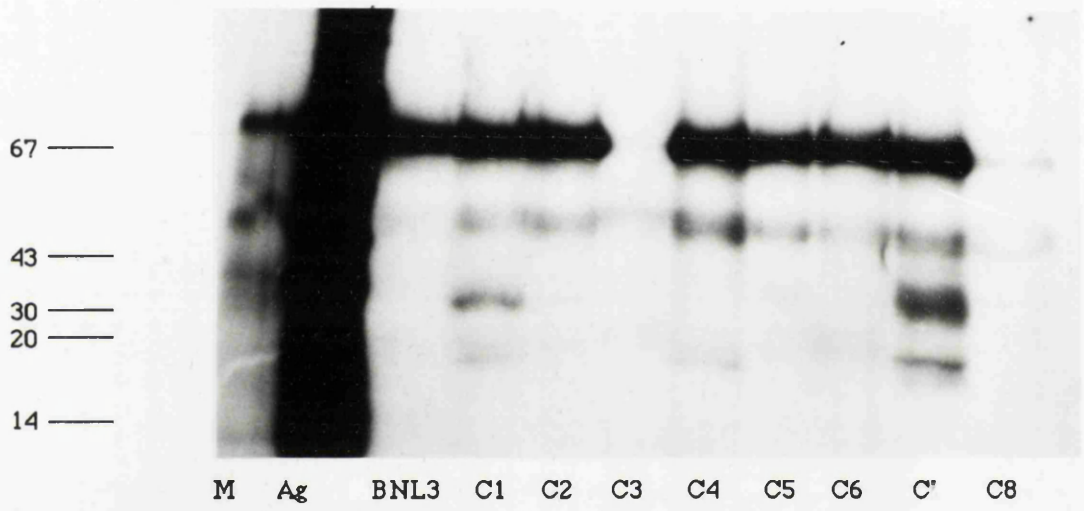

67
43
30
20
14
M
Ag
BNL3
C1
C2
C3
C4
C5
C6
C'
C8

Figure 6.18

Protection Experiment 4: immunoprecipitation of ^{125}I-labelled AChE-enriched fraction (Ag) by antibodies in the serum of individual animals (D1-D8) immunised twice with adult ES products in the context of niosome adjuvant. The serum was taken on Day 14 of secondary immunisation.

The immunoprecipitation was carried out as detailed in Figure 6.13. For comparison, the antigen was also immunoprecipitated with a serum pool from three multiply-infected calves (BNL3). The relative molecular mass of marker proteins (M) are indicated in kiloDaltons.

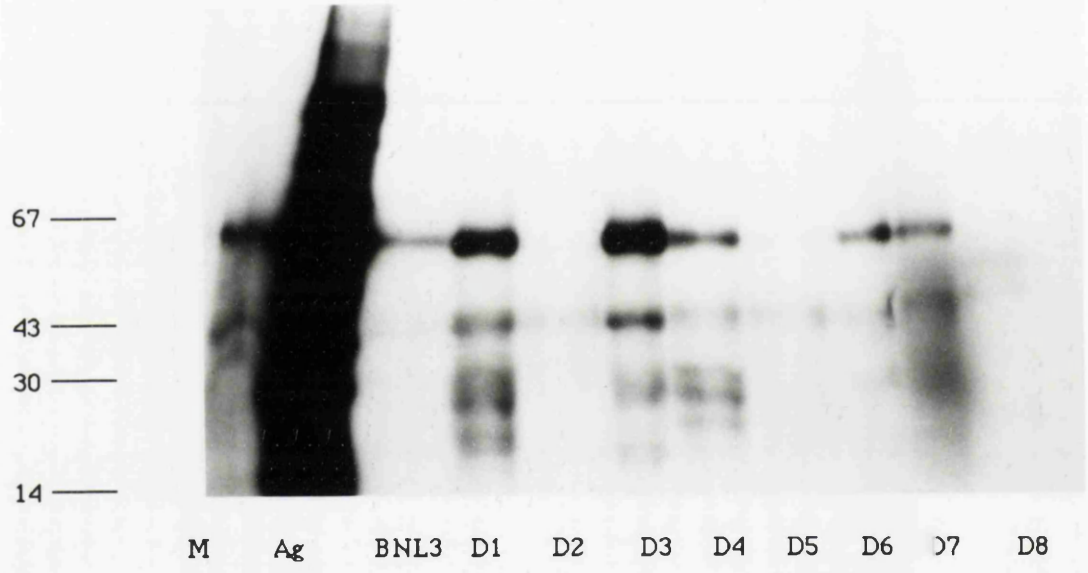
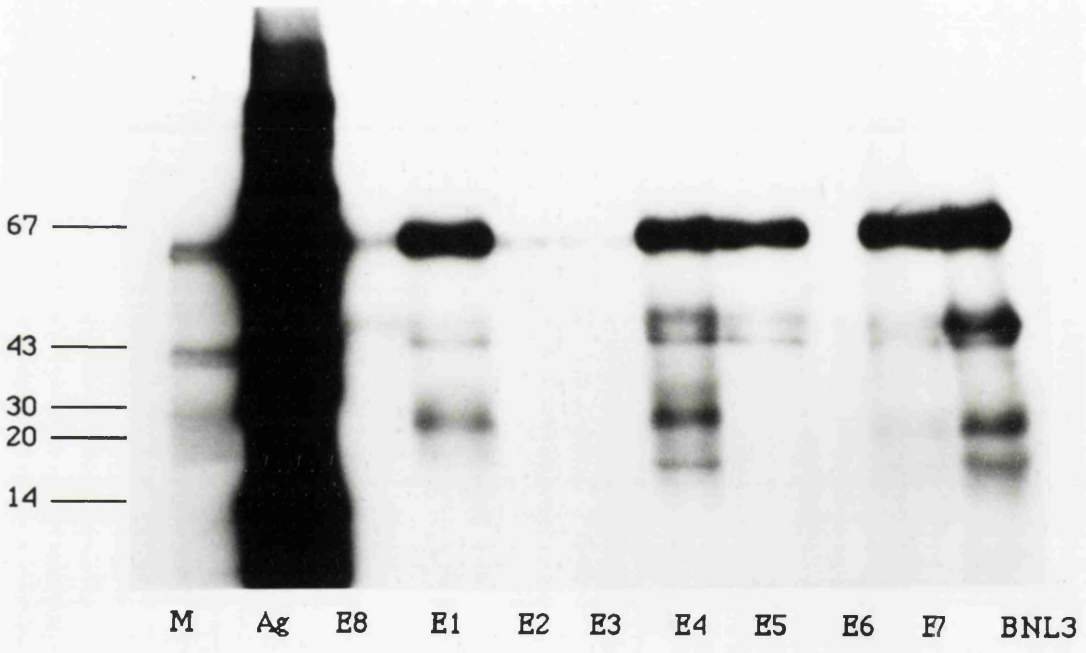

67
43
30
14
M
Ag
BNL3
D1
D2
D3
D4
D5
D6
D7
D8

Figure 6.19

Protection Experiment 4: immunoprecipitation of ^{125}I-labelled AChE-enriched fraction (Ag) by antibodies in the serum of individual animals (E1-E8) immunised twice with the homologous antigen preparation in the context of Freund's adjuvant. The serum was taken on Day 14 of secondary immunisation.

The immunoprecipitation was carried out as detailed in Figure 6.13. For comparison, the antigen was also immunoprecipitated with a serum pool from three multiply-infected calves (BNL3). The relative molecular mass of marker proteins (M) are indicated in kiloDaltons.

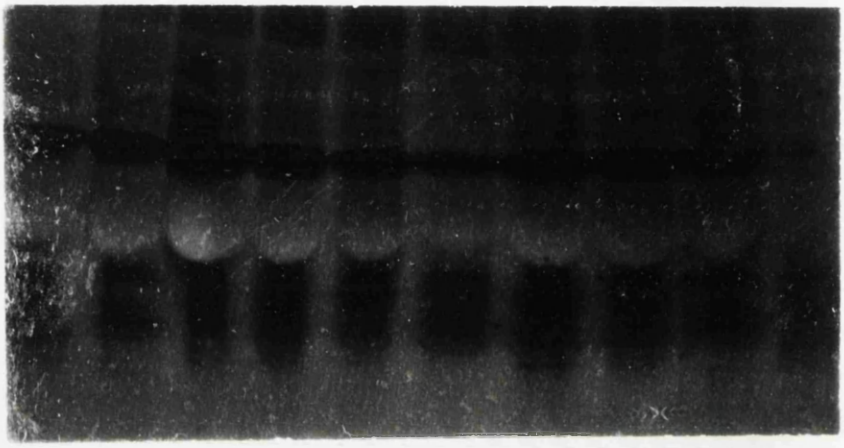

67
43
30
20
14
M
Ag
E8
E1
E2
E3
E4
E5
E6
E7
BNL3

Figures 6.20 a and b

AChE recognition profiles of animals immunised with ES or the AChE-enriched fraction in Protection Experiment 4.

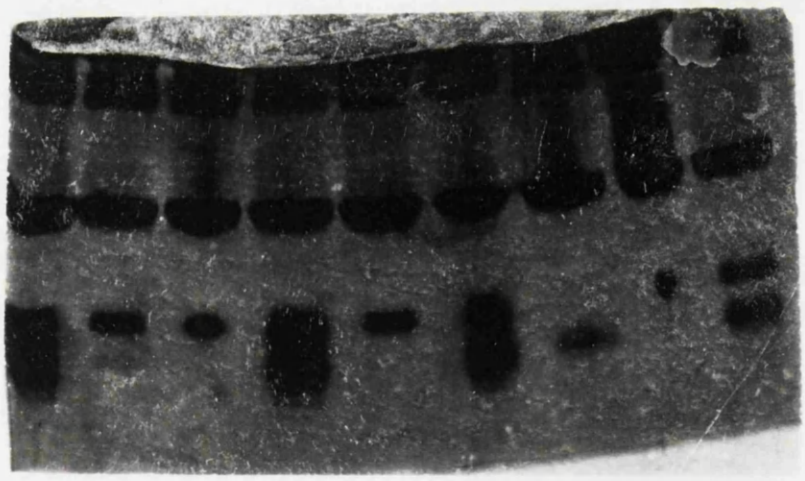
Individual sera from Day 14 of secondary immunisation were incubated 1:2 with adult ES products for two hours and the samples then analysed for esterase activity by gel assay. The animals in Group C (C1-C8) received two immunisations of adult ES (6.24a) and those in Group E (E1-E8) received two immunisations of the AChE-enriched ES fraction (6.24b). The animals in these groups received antigen in the context of Freund's adjuvant. The esterase profiles of the incubation products were compared with an adult ES sample incubated with pre-immunisation serum (pre) and also with adult ES antigen (ES only) run individually.

a



pre C1 C2 C3 C4 C5 C6 C7 C8 ES only

b



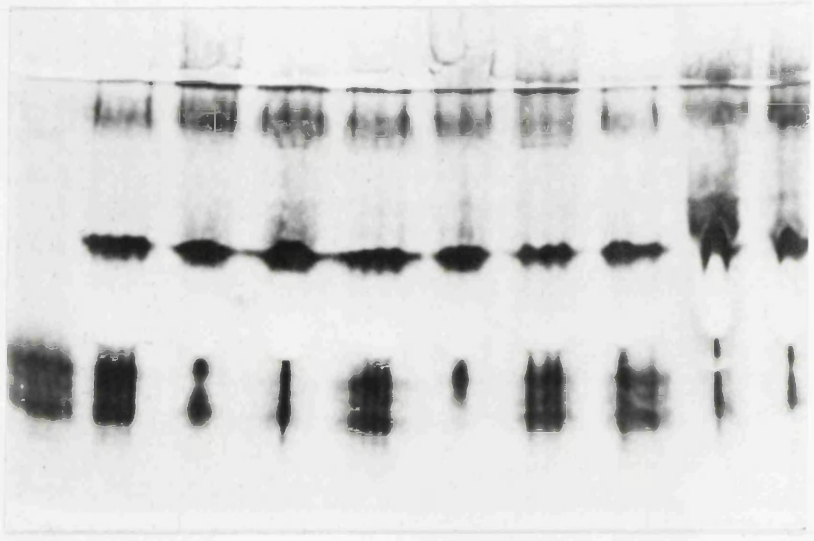
pre E1 E2 E3 E4 E5 E6 E7 E8

Figures 6.21 a, b and c

AChE recognition profiles of post-challenge sera of Protection Experiment 4 Groups C,D and E.

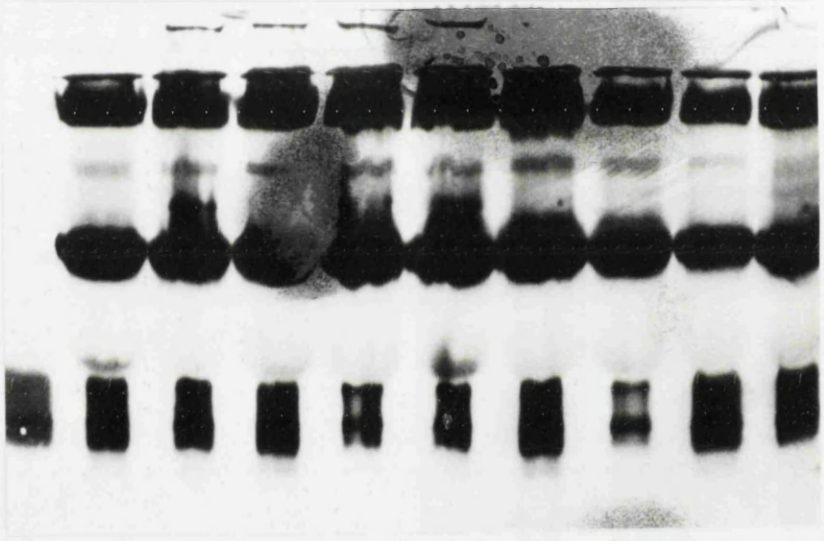
Individual serum samples taken on Day 6 of challenge with 6,000 L3 were incubated with adult ES antigen for two hours and the incubation products gel-assayed for esterase activity. The samples were taken from guinea pigs previously immunised with ES in the context of Freund's (Figure 6.21a) or niosome (Figure 6.21b) adjuvants. The reactivity in post-challenge sera of guinea pigs immunised with the AChE-enriched ES fraction (Figure 6.21c) was also examined. The recognition profiles were compared to pre-immunisation sera (pre) and also to the original adult ES activity (ES).

a)



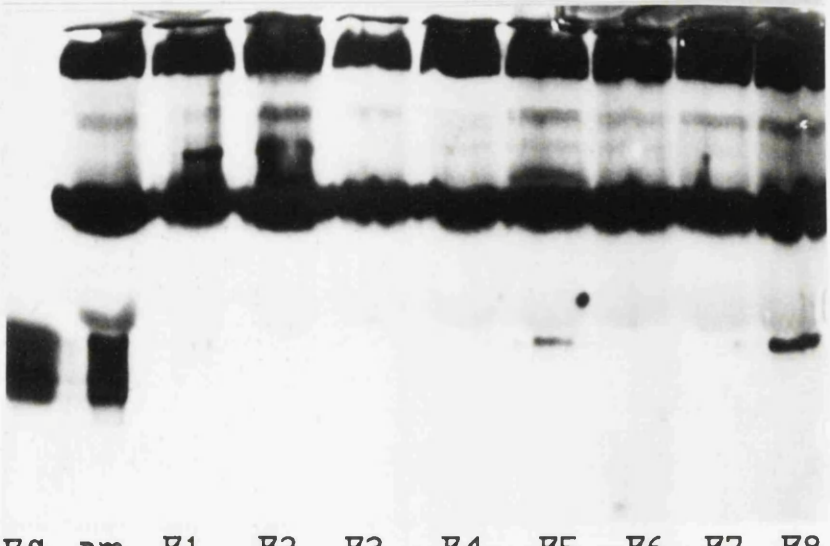
ES pre C1 C2 C3 C4 C5 C6 C7 C8

b)



ES pre D1 D2 D3 D4 D5 D6 D7 D8

c)



ES pre E1 E2 E3 E4 E5 E6 E7 E8

6.2.9 Purification of secretory AChE from *D. viviparus* adult ES products.

Since the above findings suggested a role for AChE enzymes in the stimulation of protective immunity against *D. viviparus* infection in guinea pigs, an attempt was made to purify the parasite secretory AChE to homogeneity following the method of Hodgson and Chubb (1983, Section 2.8.5). Briefly, 200µl of *D. viviparus* adult ES products was loaded onto an epoxy-activated Sepharose-B column coupled to edrophonium chloride (EdCl), the ES products eluted using 50mM $NaP0_4$, followed by 50mM Na PO_4/0.5M NaCl and finally with 12mM EdCl. The first five fractions (1ml) eluted by each buffer were analysed for AChE activity by test-tube and gel assay.

The Ellman assay results and efficacy of recovery are shown in Table 6.5. While 92% of the applied protein was recovered from the column, only 20.4% of the loaded AChE was apparently recovered. More than half (59%) of the recovered esterase activity was present in the first EdCl fraction which only contained 3.8% of the total priotein applied so some degree of purification had been achieved. As the activity obtained in the column fractions was lower than expected, it appeared that either the AChE had not been effectively recovered from the matrix or, that EdCl was still present in the EdCl fraction and thus inhibited the activity of the recovered AChE. The fractions were subsequently gel assayed for AChE activity (Figure 6.22).and it was observed that the activity present in the EdCl fraction contained fewer bands of AChE activity in comparison with the applied and earlier fractions.

The first EdCl fraction eluted from the column was radiolabelled with ^{125}I in an attempt to size the components obtained. The fraction was subsequently analysed by SDS-PAGE under reducing conditions, but did not appear to incorporate any radiolabelled components. The failure of the radiolabelling may have been the result of the low levels of protein present in the EdCl fraction used. Alternatively, low levels of tyrosine in the purified enzyme may have resulted in a lack of incorporation of the radioisotope. Further characterisation using this strategy was not attempted

Parameter	Total Applied:	Recovered: Fraction		
		Buffer: A	B	C
Protein (μg)	500	430	13	19
AChE (IU)	6.1	0.069	0.454	0.72
Specific activity (IU/μg protein)	0.0122	0.00016	0.035	0.038

Table 6.5

Affinity purification of secretory AChE from *D. viviparus* adult ES products.

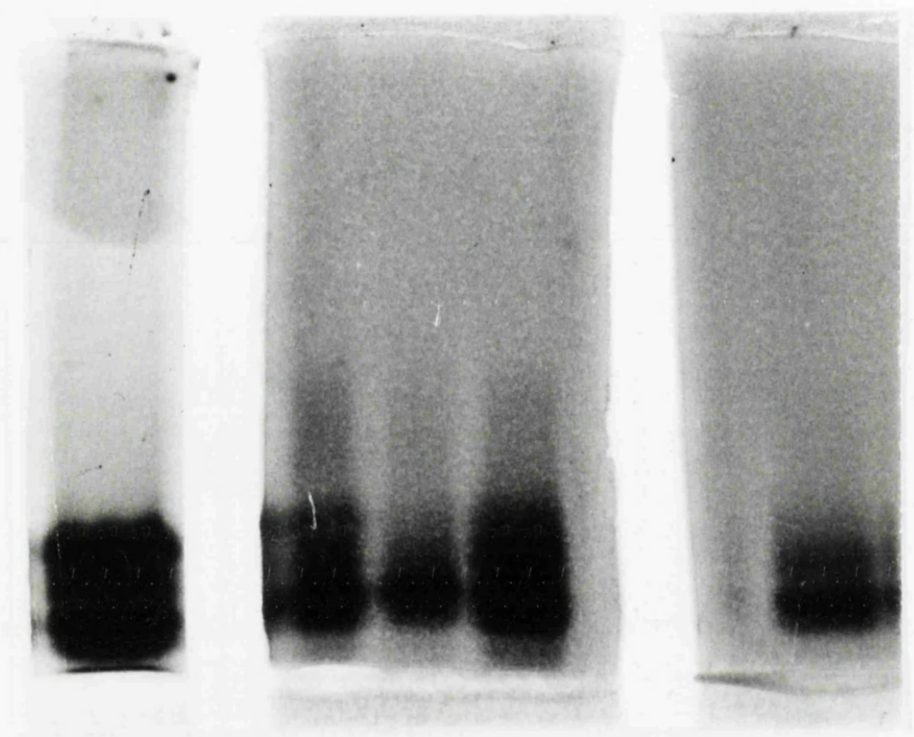
200μl of adult ES products, containing a total protein of 500μg and a total AChE activty of 6.1IU, were loaded onto an epoxy-activated Sepharose-B column bound with EdCl (Section 2.8.5). One ml fractions were collected following elution with 10ml 50mM $NaPO_4$ (buffer A), 10ml 50mM $NaPO_4$ /0.5M NaCl (buffer B) and 8ml 12mM EdCl (buffer C). All fractions were then analysed for AChE activity by the method of Ellman *et al.* (1961).

The fractions shown here represented a pool of the first three, 1ml volumes eluted from the column after each buffer change. All other fractions measured were negative for AChE activity.

Figure 6.22

AChE isoforms obtained by affinity purification.

Adult ES materials were subjected to affinity-purification using an epoxyactivated Sepharose-B column (Hodgson and Chubb, 1983, Section 2.8.5). The first three fractions eluted by 50mM $NaP0_4$ buffer (no salt fractions) were gel-assayed for esterase activity, along with the first eluted with edrophonium chloride (EdCl fraction 1). The samples were run on 10% polyacrylamide gels under native conditions and then stained for esterase activity. A sample from the adult ES batch (ES only) which was applied to the column was also examined for esterase activity.



ES only

no salt fractions
1 2 3

EdCl
fraction 1

6.3 Discussion

The possibility that *D. viviparus* adult ES components might be essential to host/parasite immunobiology, warranted a study of these materials for enzymes with potential host modulatory activity. AChE enzymes have been shown to be released by several nematode species and their potential roles in host/parasite relationships have been suggested by several authors (reviewed by Rhoads, 1984). Several developmental stages of *D. viviparus* were therefore examined for AChE activity and infected and immunised hosts tested for responses to this enzyme.

Five isoforms of AChE were demonstrated in both somatic and secretory extracts of adult and L4/5 developmental stages of *D. viviparus*. By gel electrophoresis, it was observed that the isoenzymes released from these stages migrated at a similar rate to those present in the somatic preparations. In contrast to the adult and L4/5 stages, the free-living L3 stage contained considerably less AChE activity, but the esterase isoforms present in all stages examined, displayed similar mobility on gel electrophoresis. These results are in accordance with those obtained for *N. brasiliensis* (Sanderson and Ogilvie, 1971), *N. americanus* (Burt and Ogilvie, 1975) and *Trichostrongylus* species (Rothwell, Ogilvie and Love, 1973) in which low levels of esterase activity were demonstrated in free-living compared with parasitic stages.

The capacity of infected or vaccinated calves to recognise the parasite AChE isoforms indicated that these enzymes were released by the parasite *in vivo*. Using immunocytochemistry techniques, additional attempts were made to prove that ES materials, and AChE in particular, were released in the lungs during the course of infection. Sections of lungs, removed at post mortem from *D. viviparus* -infected calves, were incubated with guinea pig sera raised against ES products or AChE-enriched ES fractions . Positive reactivity was obtained in the peribronchial tissues with both types of anti-serum when compared with the reactivity observed with normal guinea pig serum. Serum against purified *D. viviparus* AChE would have to be raised, however, in order to verify the release of this enzyme *in vivo*.

The other nematode species examined here did not appear to contain measurable levels of AChE activity in their L3 stages. This agrees with previous studies which demonstrated low levels of activity in the third larval stages of several nematode species (Ogilvie *et al.*, 1973).

AChE released by *D. viviparus* worms in the lungs might have several functional roles and, of particular relevance here, is their possible function in assisting the parasite to overcome host immune effector mechanisms. As outlined earlier, AChE may act by breaking down ACh and interfering with its local effects; for example, mucus secretion

(Hafez, 1977), lysosomal enzyme release (Ignarro and Colombo, 1973), neutrophil-mediated ADCC (Gale and Zighelboim, 1974), neutrophil chemotaxis (Hill *et al.*, 1975) and histamine release (Kaliner, Orange and Austen, 1972; Tauber *et al.*, 1973; Kaliner and Austen, 1975). It is possible that *D. viviparus* parasites release these enzymes in an attempt to reduce the increased mucus secretion which may be important in host defence mechanisms designed to reduce worm establishment and contribute to immunological "trapping". Although little is known of the immune responses directed against pulmonary nematodes, it is likely that chemical mediators released by mast cells may play a role in parasite expulsion but this remains to be defined. With regard to ADCC, results obtained with other helminth species have suggested that this may be an important arm of anti-parasite responses (Butterworth *et al.*, 1982; Mackenzie, Preston and Ogilvie, 1978). In preliminary experiments, it was observed that *D. viviparus* adult ES products inhibited the respiratory burst of zymogen-stimulated bovine neutrophils. Neutrophils are the most active cell type mediating ADCC in cattle (Wardley, Rouse and Babuik, 1976) and, as these cells are increased in the lungs of *D. viviparus* -infected calves (Simpson *et al.*, 1957), this mechanism may be of relevance in the development of immunity. By releasing AChE enzymes into its immediate environment, *D. viviparus* may be attempting to interfere with ACh-generated immunological processes, but antibodies directed against these enzymes may subsequently interfere with this immunomodulatory function. However, it is not yet known whether AChE-specific antibody is released locally in the lungs of *D. viviparus* -infected calves.

Vaccinated and infected calves responded heterogeneously to the AChE isoforms released by *D. viviparus* and this variability in recognition of parasite AChE has been observed in the responses of sheep to esterases present in *Trichostrongylus* , *Ostertagia* and *Oesophagostomum* species (Rothwell and Merritt, 1974; Rothwell *et al.*, 1976). The ability of individuals to respond to *D. viviparus* AChE may be under genetic control, as observed for other antigens of this parasite (see Chapter 5), and was suggested by the heterogeneity in AChE recognition which individual guinea pigs displayed following immunisation with the same antigen preparation in the context of a powerful adjuvant. Guinea pigs showing the most extensive AChE recognition patterns also had the lowest worm burdens; this was observed in individual Dunkin-Hartley guinea pigs and when animals of the two inbred strains were compared. This relationship could have been entirely coincidental, and may merely have reflected a general increase in antibody levels in these animals. However, no relationship was observed previously when attempts were made to correlate levels of parasite-specific antibody with individual worm burdens.

These results suggested a possible role for AChE in the stimulation of protective immunity against *D. viviparus* infection in guinea pigs and it would be interesting to carry out further immunisation studies with purified *D. viviparus* AChE. The likelihood of antigens, other than AChE, being involved in the stimulation of immunity in guinea pigs was indicated when serum from passive transfer studies were analysed for AChE reactivity (not shown). Serum from normal L3-infected guinea pigs, which transferred the highest level of immunity, did not exhibit reactivity against *D. viviparus* adult AChE enzymes.

AChE recognition was first observed in the serum of calves 28 days after infection with normal L3. This was similar to results obtained in *T. colubriformis* -infected sheep where anti-AChE antibodies were first detected on Day 21 of infection (Rothwell, Ogilvie and Love, 1973; Rothwell and Merritt, 1974). In general, calves infected with 400Gy-irradiated L3 of *D. viviparus* required several vaccinations before anti-AChE antibodies were detected. This was presumably because less AChE-secreting parasites were developing in vaccinates compared with calves infected with non-irradiated larvae (Jarrett and Sharp, 1963). However, these results suggested that earlier developmental stages also release AChE isoforms which may be antigenically related to those released by adult *D. viviparus*. This was confirmed when L4/5 stages were examined for esterase activity: all 5 isoenzymes present in adult ES products, were also evident in the somatic and secretory extracts of L4/5 stages. The fact that animals examined here did not appear to recognise the AChE after multiple infections may be a result of a more effective immune response by this time. For example, after several infections parasites may be restricted in their development within the host and, therefore, might not reach stages where AChE enzymes are released in large enough quantities to stimulate specific antibody. That *D. viviparus* L3 stages do not release AChE, was further suggested by the fact that calves vaccinated with 1000Gy-irradiated L3 did not recognise any parasite AChE (not shown).

Serum from infected calves and immunised guinea pigs did not inhibit the AChE activity present in *D. viviparus* any more than did pre-infection sera. This lack of inhibition by immune sera was also suggested in gel assays, when it was noted that recognised AChE enzymes were retained at the stack/resolving gel interface. This was particularly obvious when the antigens were incubated with bovine serum, in which there was little endogenous AChE activity in this region of the gels. Thus epitopes outwith the active site appeared to be recognised. Although *D. viviparus* AChE activity was not inhibited by sera from immune animals, specific antibodies might act to complex the enzymes and prevent their diffusion away from the parasite to their site of action. Complexing of parasite AChE enzymes, in the absence of inhibition of their activity, has been observed in *T. colubriformis* (Rothwell, Ogilvie and Love, 1973,

Rothwell and Merritt, 1974) and *Oesophagostomum* species (Bremner *et al.*, 1973) in sheep. Other nematode species, however, appear to stimulate antibodies which can inhibit parasite esterase activity, e.g *Ostertagia* species (Rothwell *et al.*, 1976).

In *D.viviparus* infection in calves, it has been observed that some parasites persist in immune hosts in an inhibited state of development (Taylor, 1951; Oakley, 1979, Supperer,1976). To find out if these parasites have altered AChE isoenzyme patterns analogous to those of "adapted" *N. brasiliensis* parasites (Edwards, Burt and Ogilvie, 1971), *D. viviparus* parasites, obtained from the lungs of immune field-infected calves, were cultured and their ES products analysed for AChE activity. Unfortunately, too few parasites were available to obtain sufficient levels of protein or AChE activity to examine for alterations in the released enzymes.

To investigate the capacity of an AChE-enriched fraction to stimulate protective immunity, an experiment was performed where the immunogenicity of this preparation was compared with whole adult ES products. Unfortunately, whole ES products and the AChE-enriched fraction failed to significantly protect animals when compared with the challenge controls. Previous vaccination studies using enriched or purified parasite AChE fractions, have also failed (Rothwell and Merritt, 1975; Wright *et al.*, 1983). The failure of adult ES materials to protect against challenge may have been due to the increased time between secondary immunisation and challenge (8 weeks), compared with the previous Protection Experiments.

When compared with the adjuvant control group, the enzyme-enriched fraction recipients (Group E) had significantly lower parasite burdens. The level of immunity induced by this preparation, however, may have been due to other, non-esterase, ES components. The heterogeneous nature of the AChE-enriched ES fraction was emphasised when sera from animals, immunised with this preparation, were observed to recognise several components of radiolabelled adult ES materials. It could be that epitopes present on the molecules included within the eluted gel region may have been shared with molecules not included in this fraction. However, when the AChE-enriched fraction itself was radiolabelled and immunoprecipitated with sera from animals which received adult ES materials or the AChE-enriched fraction, several components were noted to be recognised. A purified preparation of *D. viviparus* AChE isoforms would be required to further define the enzyme in terms of size and molecular forms. Of interest, is the fact that multiple molecular forms of AChE were affinity-purified from *N. americanus* excretory/secretory products using the EdCl column technique (Pritchard *et al.*, 1991). The immunodominant component of the AChE-enriched fraction of *D. viviparus* had a relative M_r of approximately 67kDa. Although it is not known if this molecule had AChE activity, it is of interest to note that gel filtration studies have estimated that the AChE enzymes of *N. brasiliensis* and *T.*

colubriformis have molecular weights corresponding to between 65 and 70kDa (Hogarth-Scott *et al.*, 1973).

The animals which were immunised with adult ES products in the context of niosomes developed specific antibody responses, but were not significantly protected against challenge. In fact, the niosome adjuvant control group had lower worm burdens than the group of animals which received antigen with this adjuvant. The niosome adjuvant controls, though not significantly protected when compared with challenge controls, had a mean reduction in worm burden of 56% and the worm burdens in the individuals of this group were very consistent. This suggested that the niosomes may have stimulated non-specific responses which affected the development of *D. viviparus* in the guinea pig. As with Freund's adjuvant immunisation, the niosomes were unable to circumvent the heterogeneity in antigen recognition patterns displayed by individual animals following immunisation with adult ES material.

To further characterise *D. viviparus* AChE isoforms, attempts were made to purify AChE using EdCl coupled to an epoxy-activated Sepharose-B column. The results were disappointing in that enzyme recovery was poor and a considerable proportion was obtained in fractions prior to the addition of the EdCl elution buffer. These results suggested that *D. viviparus* AChE may not have properties similar to the mammalian secretory AChE previously characterised by Hodgson and Chubb (1983). Further attempts to purify AChE will be made in the future using techniques such as those used for the purification of AChE from the root nematode, *Meloidogyne*. In this case, AChE enzymes were purified by using a combination of sucrose gradient centrifugation and ion-exchange chromatography (Chang and Opperman, 1991).

Chapter 7

Concluding discussion

In the introduction to a review by Emery and Wagland (1991) on recent advances in immunity and vaccine development in ruminant helminth infections, the authors refer to the;

"....dogged persistence and ingenuity of cadres of parasitologists who have done more than go through the motions".

It seems rather ironic, however, that the only commercially-available anti-helminth vaccine, against the bovine lungworm *D. viviparus*, was developed over 30 years ago in the absence of any detailed knowledge of the immunobiology of the host/parasite relationship. Also, the continued success of the vaccine has had an adverse effect on funding for investigations on the immune response to *D. viviparus* which has thus helped maintain this lack of knowledge. It seemed justifiable, therefore, to examine the immunobiology of this nematode in which both infection and vaccinaton induces a strong degree of protective immunity in the natural host. The main aims of the work presented here were:

a) to identify potential immune evasion strategies which *D. viviparus* may use in order to reach patency in its host;

b) to examine the humoral responses which may be involved in overcoming the infection in cattle and

c) to define a source of protective antigens in the guinea pig laboratory model with a view to characterising candidate antigens which could prove useful in the development of a subunit vaccine.

The experiments presented in Chapters 3 and 4 were designed to examine some of the properties of the surface of live parasites using antibodies from infected and immunised hosts. From these it seemed that *D. viviparus* may exhibit properties, hitherto postulated to occur with other helminths, which assist it in reaching patency during a primary infection. These include: the stage-specific expression of surface antigens; binding of the L3 cuticle by heterophile IgM which may hinder the binding of more effective antibodies; the retention of an inert but immunogenic L3 sheath which may divert effector responses away from the cuticle of migrating parasites and the shedding of surface-bound antibody by adult stages. In addition, in Chapter 6, the parasitic stages of *D. viviparus* were found to release an enzyme of possible immunomodulatory activity, namely acetylcholinesterase. With the exception of AChE release, the ramifications of the potential immune avoidance mechanisms are discussed in detail in Chapter 3 and are not elaborated on here. It should be emphasised, however, that all the evidence for immune avoidance was obtained *in vitro* and the

occurrence and importance of these mechanisms *in vivo* can only be surmised. Nevertheless, it was hoped that by using antibodies from immunised or vaccinated hosts and the surface of live parasites, a situation as near to that which occurs naturally would be achieved.

The importance of antibody in the development of immunity to *D. viviparus* infection was indicated by the success of passive immunisation experiments in both calves and guinea pigs (Jarrett *et al.*, 1955a; Wilson, 1966; Canto, 1990) and was confirmed here in the guinea pig model when passive immunity was obtained following the transfer of sera raised by immunisation with adult ES products or normal L3 (Chapter 5). From the results, however, it was difficult to interpret which, if any, humoral responses directed against the parasite surface were of relevance to the acquisition of protective immunity. Increases in adult surface-specific IgG_1 or IgG_2 were observed at around the time of adult parasite expulsion and, in the case of the latter isotype, this was the only time that obvious increased levels were evident. Since bovine IgG_1 and IgG_2 antibody subclasses have been shown to fix bovine complement, bind cultured monocytes and mediate phagocytosis by neutrophils and macrophages *in vitro* (McGuire, Musoke and Kurtii, 1979; Musoke, Rurangirwa and Nantulya, 1986), the involvement of these subclasses in ADCC-mediated damage of adult *D. viviparus* could be proposed. However, it remains to be shown that surface-specific antibodies are involved in immune elimination of adult *D. viviparus*, but *in vitro* studies might indicate whether these are capable of mediating parasite damage by the non-specific effector mechanisms mentioned above.

These studies measured circulating levels of antibody directed against *D. viviparus*, but gave no indication of immune responses which were occurring in the lungs. Preliminary attempts to examine such responses failed to detect *D. viviparus* surface-specific antibody in pulmonary secretions from an infected calf. The level of local pulmonary responses may be of particular relevance with regard to some of the pathological manifestations which are exhibited in the later stages of infection in some calves, namely alveolar epithelialisation. The characterisation of this response might have implications for allergic responses in the lungs in general and it is of interest to note that one of the immunodominant components of adult ES products was recently observed to have homology to ABA-1 allergen of *Ascaris* (C. Britton, personal communication). Unfortunately, neither serum nor pulmonary mucus IgE levels could be measured in these studies although IgE is thought to play a major role in the expulsion of other parasite species in various host locations (Jarrett and Miller, 1982).

Another fundamental question which remains to be answered is the involvement of T cells and accessory cells in the expression of host immunity in *D. viviparus* infection. For example, do Th subpopulations exist in the bovine and are these

preferentially stimulated by different types of pathogen? Th cell subsets, similar to those defined in the mouse, have been recently demonstrated in human lymphocyte populations (Romagnani, 1991) and it is conceivable that they also exist in cattle. Further definition of cell-mediated aspects of the immune response to *D. viviparus* await the development of reagents directed against bovine and guinea pig cytokines. Until these are available, it might be possible to examine T cell responses to *D. viviparus* infection using proliferation assays of T cells harvested from bronchial or mesenteric lymph nodes of infected or vaccinated calves and examining the cytokine profiles induced by stimulation with, for example, adult ES products. Alternatively, T cell Western blots could be attempted. Studies such as these would help to confirm the antibody isotype patterns reported in this thesis and would enable a preliminary examination of potential T cell responses to *D. viviparus* in calves.

It would also be of interest to investigate whether parasite-derived enzymes, such as superoxide dismutase and acetylcholinesterase, have an effect on bovine T cells or accessory cells. In some preliminary studies not reported here, a zymogen-stimulated respiratory burst of bovine neutrophils was reduced in the presence of *D. viviparus* adult ES products but as the cells were not incubated with purified parasite enzymes, the exact mechanisms behind this inhibition remain to be elucidated. Whether antibodies, specific to the parasite-derived enzymes, enable the immunomodulatory effect to be overcome and permit the effector cell population to eliminate the parasite should be examined further.

In the studies presented here, circulating levels of anti-AChE antibodies increased 28 days after infection in some calves, but it is not yet known whether similar increases occur in the pulmonary mucus. As IgG levels in intestinal mucus of sheep are thought to reflect those in the serum, it could be assumed that the anti-AChE antibodies might be present in the pulmonary mucus. However, it must be remembered that local stimulation of antibody, either in the gastrointestinal tract or in the lungs, does not appear to be essential to the development of protective immunity in *D. viviparus* infection as larvae irradiated to 1000Gy were capable of stimulating resistance in calves when given intravenously (Canto, 1990). These results would suggest that if protective antigens were defined, they could be effective if given parenterally.

For economic reasons the immunisation experiments presented here were performed in guinea pigs and it remains to be seen whether adult ES products or the AChE isoforms enriched from them, stimulate protective immunity in the bovine host. The level of immunity stimulated by adult ES products in guinea pigs varied between protection experiments; the groups immunised with this antigen in Protection Experiments 2 and 4 were not significantly protected against challenge. Especially discouraging was the fact that when the time between secondary immunisation and

challenge was increased to 8 weeks, the protective capacity of the adult ES products appeared to be lost. This could indicate that the immunity stimulated by these antigens in the guinea pig was of a short duration. Following challenge, however, high levels of antibody were stimulated in these animals which suggested that this was not the case. The reason for the variability in protective capacity of the adult ES materials remain unclear and perhaps the next step should be to examine the responses induced in the bovine host where more parameters relating to the development of immunity can be assessed. For example, the effects of adult ES-induced immunity on the fecundity of adult female worms and parasite development and length of patency can be more readily assessed in calves than guinea pigs where parasite development terminates within 15 days of infection. The effects of immunisation on clinical signs, following challenge, and the development of antibody responses could also be determined in more detail in the bovine host. Furthermore, worm establishment, although variable among individual calves, is likely to be more consistent and permit a better comparison between vaccinates and challenge controls than that provided by the guinea pig model.

To investigate whether or not AChE enzymes are protective antigens in their own right would require their purification to homogeneity, before their use in relevant immunisation experiments. It is unlikely that the amounts of antigen required to do this could be obtained from parasite material, but the purified enzymes could be used to raise AChE-specific antibodies for screening cDNA expression libraries, constructed from mRNA prepared from adult *D. viviparus*. Further protection studies could then be attempted with fusion proteins obtained from positive clones. Antibody against purified AChE might also be used to locate the source of AChE enzymes in parasites and for probing host tissue for evidence of their *in vivo* release. Furthermore, specific RNA probes, prepared from plasmids encoding conserved sites of the enzymes, could be used in *in situ* hybridisation for identification of the site of expression within the parasite and for examining for cross-reactivity of these enzymes between related nematode species.

The studies described here relied on antibody detection for the assessment of immunity as the antibody repertoire was considered to reflect an individual's ability to respond to specific epitopes and thus indicate the potential to respond to infection or immunisation. These studies highlighted the differences in antigen recognition patterns between animals, even within inbred strains of guinea pigs, and this heterogeneity was also evident when antibody binding to parasite AChE was examined. The results presented here indicated that the *D. viviparus* AChE isoenzymes were antigenically-different and this is of relevance because it increases the likelihood that all members of an outbred population will recognise at least one of the isoforms. Consequently, all vaccinates might develop a protective immune response. It must be remembered,

however, that individual animals which do not respond to all isoforms may still remain, for example, strain 2 guinea pigs remained susceptible to challenge following ES immunisation and showed poor recognition of the parasite-derived AChE.

One of the main conclusions of this work is that in the development of any subunit vaccine against *D. viviparus,* the complexity of the parasite antigens and the variability of the host responses to these will have to be taken into account. In addition, antigenic diversity within the parasite population itself will have to be considered, though this may be of less relevance in *D. viviparus* than in other parasite species. It is hoped that the results of the studies presented here have helped lay a foundation for the analysis of nematode antigens, which could be important in the studies of other disease-producing helminths against which no vaccine has yet been developed.

Appendix I

All chemicals obtained from Sigma Chemical Company unless otherwise stated.

(i) Phosphate-buffered saline (PBS), pH 7.2

10grams	NaCl	(BDH)
0.25grams	KCL	(Hopkin and William)
1.427grams	Na_2HPO4	(BDH)
0.25grams	KH_2PO4	(BDH)

deionised water to a final volume of 1L

(ii) Nematode Culture Medium

100ml	RPMI 1640	(Gibco)

supplemented with:

240µg	L-glutamine	(Flow)
100mg	D-glucose	(Formachem)
40µg	tripeptide (glycil-L-histidyl-L-lysine)	
50ng	glutathione	
10,000IU	penicillin	(Flow)
10,000µg	streptomycin	(Flow)
1mM	sodium pyruvate	(Flow)
1mg	sodium bicarbonate	(Flow)
2ml	amphoteracin B	(Flow)
0.25ml	gentamycin	
1 tablet	cephalexin Selectatab	(Mast Labs. Ltd.))
1 tablet	VCNT Selectatab	(Mast Labs. Ltd.)

(iii) Tris poisons

100ml	10mM Tris base (BoehringerMannheim)
2mM	EDTA
1mM	phenylmethyl sulponyl fluoride (Pms-F) (in isopropanol)
5μm	pepstatin (in methanol)
2mM	1,10 phenanthroline (in ethanol)
5μM	antipain
25μg/ml	N-p-tosyl-L-lysine chloromethyl ketone
50μg/ml	N-tosyl-L-phenyl alanine chloromethyl ketone

(iv) Reagents for SDS-PAGE

Stock solutions

1) Solution N

250grams acrylamide (BDH)

3.875grams N,N'-methylene bis acrylamide (BDH)

deionised water to a final volume of 625ml

Stored at 4°C with "Amberlite" (BDH)

2) Solution AA

480ml	1M HCl	(May and Baker)
336grams	Tris base, pH 8.9	
8grams	SDS	
2.3ml	N,N,N',N'-tetramethylethylinediamine (TEMED)	

deionised water to a final volume of 1L

Stored at room temperature

3) Solution S

121.1gram	Tris base/HCl, pH 6.8
8grams	SDS
4ml	TEMED

deionised water to a final volume of 1l

Stored at room temperature

4) Ammonium persulphate 100mg/ml (BDH)
Made fresh

Working solutions

1) Volumes for 2 x 0.7mm thick 5-25% gradient gels

Solution	5%	25%	Stacking gel
N	3ml	15ml	2.5ml
AA	3ml	3ml	-
dH_2O	18ml	6ml	15ml
S	-	-	2ml
AP	50µl	33.35µl	200µl

(v) PAGE sample buffer

5grams	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)	

deionised water to a final volume of 95ml.

Note: Pms-F, EDTA and SDS omitted in gels used for AChE detection.

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