# An Immunochemical Study of *D.viviparus* Infective Larvae

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## Abstract

The bovine lungworm *Dictyocaulus viviparus* induces a highly effective immune response in infected cattle and a vaccine, consisting of radiation attenuated infective larvae, has been successfully used for over thirty years. In spite of this notable success, there is little understanding of natural or vaccine-induced immunity to this parasite. Since the infective larva is a potential source of important antigens and can be obtained in relatively large quantities, an immunochemical study of this stage formed the basis of the work presented in this thesis. An investigation of the mouse as a potential immunological model of *D.viviparus* infection revealed that larvae migrate to the lungs but are expelled without undergoing significant development. Although mice were capable of mounting an immune response to invading larvae, the results suggested the mouse was of limited value as an immunological model for this parasite. No polypeptides were detected by surface biotinylation of exsheathed $L_3$ but several molecules were revealed by labelling sheathed $L_3$. The generation of monoclonal antibodies and lectin binding studies on the $L_3$ cuticular surface demonstrated the presence of phosphorylcholine and carbohydrate epitopes respectively. Lectin binding studies suggested that carbohydrate was not exposed on the external surface of the $L_3$ sheath but was present on the internal surface. The generation of monoclonal antibodies revealed a 29-40kDa antigen on the external surface of the $L_3$ sheath which appeared to be highly immunogenic and responsible for the marked antibody response produced to this surface by immunised cattle. These monoclonal antibodies also bound to the surface of the $L_3$ sheath of numerous other nematodes from the order Strongylida, although the molecular weight of the detected antigen varied between some of the species. The antigen was located on a surface coat overlying the sheath epicuticle and was also found to be present in the somatic tissues of the $L_3$. The stage specificity and immunochemical properties of this antigen were examined. *In vitro* culture of $L_3$ revealed partial development to the $L_4$ with the production of several antigens which were detected by immune bovine serum. An $L_3$ cDNA expression library was produced but screening with immune bovine serum or the monoclonal antibodies failed to detect any positive recombinant clones.

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## Declaration

This thesis and the results presented in it are entirely my own work.

## Abbreviations.

| | |
|---|---|
| Ci | Curie. |
| cDNA | Complementary Deoxyribonucleic acid. |
| DNA | Deoxyribonucleic acid. |
| ELISA | Enzyme Linked Immunosorbant Assay. |
| E/S | Excretory/secretory. |
| G | guage. |
| g | gram. |
| IBS | Immune bovine serum. |
| IFA | Immunofluorescence Assay. |
| IPTG | Isopropylthiogalactoside. |
| kB | Kilobases. |
| kRad | Kilorad. |
| l | litre. |
| mab | Monoclonal antibody. |
| mg | milligram. |
| ml | Millilitre. |
| M | Molar. |
| mM | Millimolar. |
| MW | Molecular weight. |
| NBS | Naive bovine serum. |
| nm | Nanometre. |
| OD | Optical Density. |
| PBS | Phosphate buffered saline. |
| PC | Phosphorylcholine. |
| RNA | Ribonucleic acid. |
| SDS-PAGE | Sodium Dodecyl Sulphate Polyacrylamide Gel Electropheresis. |
| TCA | Trichloroacetic acid. |
| ug | microgram. |
| ul | microlitre. |
| X-Gal | 5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside. |

# Chapter 1

# CHAPTER 1

## GENERAL INTRODUCTION

The nematode *Dictyocaulus viviparus* is the cause of bovine parasitic bronchitis with the adult parasites occurring in the bronchi and trachea of cattle, deer, buffalo and reindeer (Urquhart 1985a). The parasite has been recognised as a cause of respiratory disease in cattle for over 200 years with numerous reports of the disease being published since the latter half of the eighteenth century (Nicholls 1755, Camper 1779, Bloch 1782, Goeze 1782, Mehlis 1831 and Daubney 1920).

Parasitic bronchitis has caused great economic loss in cattle in high rainfall temperate regions of Europe as well as parts of Canada, U.S.A., Australia and New Zealand (Urquhart 1985a). Since the introduction of a vaccine and modern anthelmintics, severe outbreaks of the disease have been less common, although the disease is still endemic in these regions. It also occurs on a sporadic basis in tropical areas such as Brazil, Venezuela and Jamaica. Severe outbreaks of parasitic bronchitis have also been reported in farmed red deer (Corrigall et al 1980).

The disease typically affects young cattle during their first grazing season, since on farms where the disease is endemic the older animals have usually developed a strong acquired immunity (Urquhart et al 1987). The effectiveness of the immune responses to this parasite is also reflected in the immune expulsion of adults between 60 and 90 days following infection of susceptible animals (Jarrett et al 1957a). Due to the economic importance of the disease and the observations regarding the highly effective immune response, work was performed which culminated in the development of a vaccine, based on radiation attenuated larvae (Jarrett et al 1959b). This was commercially introduced in 1959, since which time it has been widely and successfully used to control the disease throughout Europe (Urquhart 1985b).

Due to the remarkably effective natural immune response and the success of the vaccine, this parasite would seem to offer a opportunity, unique amongst parasitic nematodes, to examine immune responses which are highly successful at eliminating adult parasites and preventing re-infection. In spite of this, very little is known about the immune responses induced following infection or vaccination, or about the nature of the antigens responsible for the induction of these responses. Since the infective larvae ($L_3$) is considered to be a potentially important stage in the protective immune response of cattle against re-infection (Jarrett et al 1957b, Poynter et al 1960 and Jarrett & Sharp 1963) and since it can be obtained in relatively large amounts, the central objectives of this thesis were to identify and

characterise antigens of the *D.viviparus* infective larvae which may be relevant to the bovine immune response.

The introduction of this thesis is divided into two main sections. The first of these is a review of the literature concerning *D.viviparus* including its life cycle, epidemiology, pathogenesis, control and in particular the work which has been performed regarding immunity. The second section consists of a review of the current state of knowledge concerning antigens of parasitic nematodes which concentrates on those parasites about which there is most information available. Consideration of this information is important to the interpretation of the results obtained for *D.viviparus*.

## 1.1. The Bovine Lungworm *Dictyocaulus viviparus*.

### 1.1.1. Life cycle of *D.viviparus*.

Adult *D.viviparus* are slender nematodes of up to 8 cm in length which live in the bronchi and terminal bronchioles of the bovine lung. Females produce eggs containing fully developed larvae which hatch almost immediately. The $L_1$ then migrate up the respiratory tree, aided by the cough reflex, are swallowed and pass through the host's alimentary tract and out in the faeces. The $L_1$, $L_2$ and $L_3$ stages are free living and the time taken for development depends upon climatic conditions. At 25°C larvae reach the infective $L_3$ stage within 3 days but at 5°C this development can take up to 26 days (Rose 1956). The cuticle of the $L_2$ is not shed following the moult to the $L_3$ but is retained as a separated cuticle called the $L_3$ sheath and this phenomenon is common to many nematodes from the order Strongylida. The free living larval stages of *D.viviparus* are thought to be non-feeding and to rely on stored food reserves since they can develop to the $L_3$ in clean aerated water and the $L_3$ appears to be completely enclosed by the retained $L_2$ cuticle (Daubney 1920). It has been suggested that the granules associated with the larval alimentary tract serve as stored food material for the free living larval stages (Daubney 1920). The $L_3$ is the infective stage and when ingested by the bovine host is thought to exsheath in the alimentary tract prior to migration, however there is little experimental evidence to support this view.

Details of the parasite's migration have been investigated by the recovery of larvae and the study of histopathology following experimental infections (Soliman 1953, Jarrett et al 1957b, Poynter et al 1960). In all of these studies larvae were found in the mesenteric lymph nodes and lungs which was also the case for

similar studies in guinea pigs which, in addition, reported that larvae could not be recovered from the liver (Poynter et al 1960 and Wade et al 1960a). Therefore it has been concluded that, following penetration of the small intestinal mucosa, the larvae migrate to the lungs via the lymphatic/blood stream route. This would involve migration through the mesenteric lymph nodes and lymphatics to the thoracic duct which empties into the cranial vena cava. The larvae would then pass to the lungs in blood stream via the right side of the heart and pulmonary circulation. However the details of this migration, particularly the time taken for larvae to reach the lungs and the site at which the third larval moult occurs, has been the subject of some debate. Jarrett et al (1957a) reported that lesions attributable to the presence of larvae in the lungs were not seen until five days after infection with 5000 or 50,000 $L_3$, by which time the larvae were at the fourth stage. They went on to suggest that the third larval moult occurred in the mesenteric lymph nodes and that it was the fourth stage larvae which passed via the thoracic duct and blood stream to the lungs. However Poynter et al (1960) reported that third stage larvae were present in the lungs just 24 hours after infection with 200,000 $L_3$ and proposed that larvae "reach the lung where subsequent development occurs and that they do not dwell as fourth stage larvae in the lymph nodes". However Jarrett and Sharp (1963), in support of their previous work, reported that they were unable to recover larvae from the lungs of calves until the seventh day after infection with 1000 $L_3$ and that again these were at the fourth stage. They suggested that the results of Poynter et al (1960) were due to the extremely high doses of larvae (200,000) used and pointed out that the numbers of larvae recovered during the first few days after infection represented a very low percentage of the infective dose.

The situation has been further complicated by the results of experimental infections in the guinea-pig. Douvres & Lucker (1958) described finding third stage larvae in the lungs just 18 hours after infection and fourth stage larvae after 48 hours. Poynter et al (1960) found third stage larvae in the lungs after 24 hours and fourth stage larvae after 72 hours. Both of these groups suggested that the third larval moult had taken place within in the lungs. However Soliman (1953b) found third stage larvae in the mesenteric lymph nodes of guinea pigs 24 hours after infection but not in the lungs until three days later. In considering the validity of this data, Jarrett and Sharp (1963) proposed that such an experimental system is highly artificial and suggested that it was dangerous to extrapolate the behaviour of the parasite in the guinea pig to elucidate details of the life cycle in the natural host.

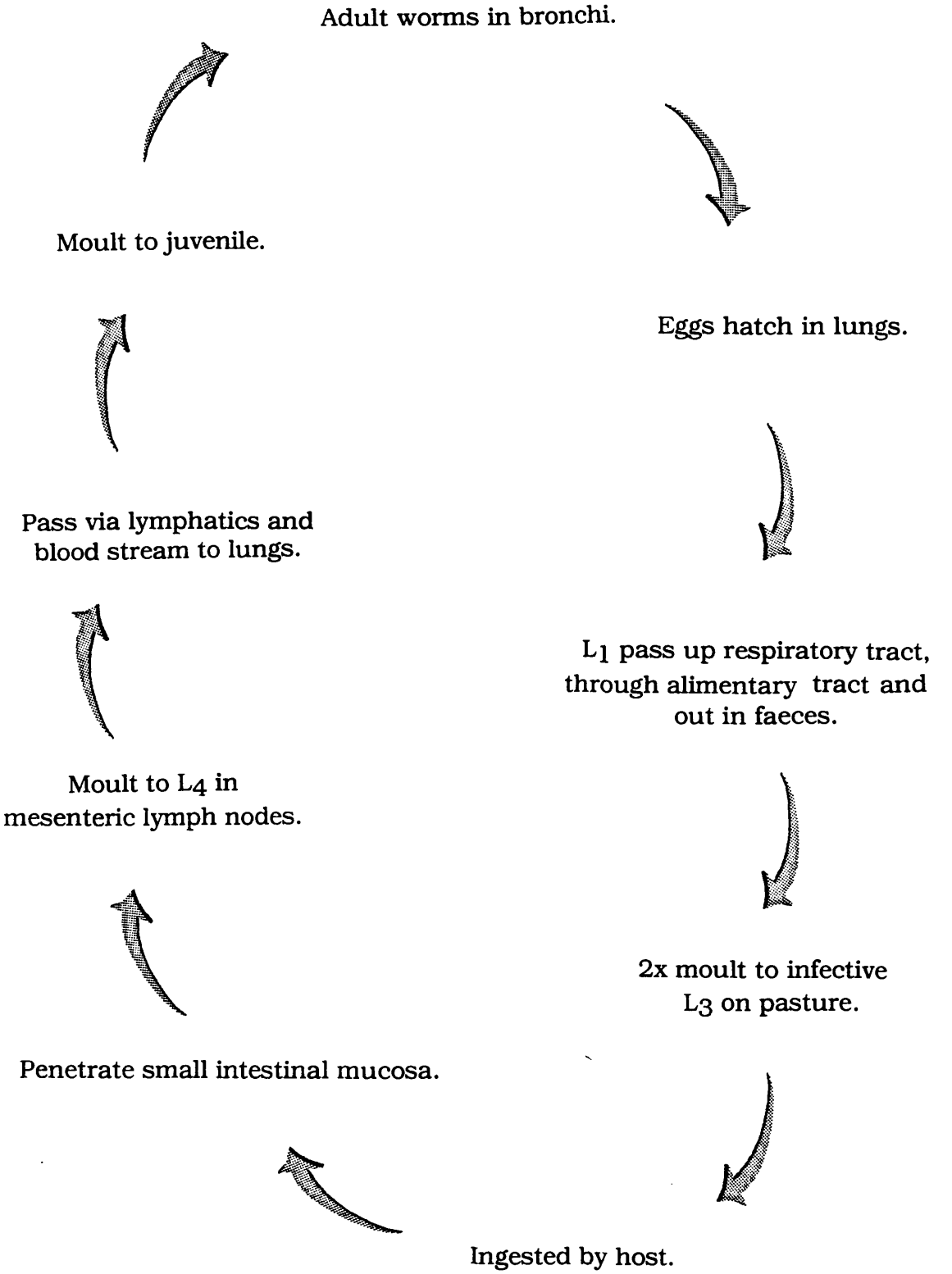
Therefore, in summary, the balance of evidence suggests that the migration and development of *D.viviparus* in the bovine host is as follows. Third stage larvae penetrate the small intestinal mucosa and enter the mesenteric lymph nodes

where they moult to the $L_4$, after which they migrate via the lymphatics and blood stream to the lungs. Larvae first reach the lungs, as the $L_4$, approximately seven days after infection, break through into the alveoli and commence their migration up the respiratory tree. The $L_5$ is present in the lungs by day 15 (Jarrett et al 1957b) and sexually mature adults are present by day 22 (Soliman 1953b and Jarrett & Sharp 1963). First stage larvae can be detected in the faeces by 21-24 days after infection (Jarrett et al 1957b). A summary of the life cycle is illustrated in figure 1.1.

There is little evidence to suggest that other routes of infection naturally occur. It is interesting to note that normal patent infections will develop following the subcutaneous administration of larvae to cattle (Wade & Swanson 1958) but no further evidence has been reported to support the authors suggestion that infections through skin wounds or abrasions may be possible. The only evidence of pre-natal infection of calves was a report by Kasparek (1900) of calves between 1.5 and 8 days of age being infected by lungworm, however no similar field cases have ever been subsequently reported. Porter and Cauthen (1942) attempted to infect a calf *in utero* by infecting the dam with 30,000 $L_3$ over a 1 month period ending two weeks before parturition but no evidence of infection was found in the calf over the first two months of its life. Soliman (1953) examined 26 foetuses and 2 bull calves from cows suffering from parasitic bronchitis but lungworms were not found in any of these progeny. It therefore seems unlikely that pre-natal infection occurs with this parasite.

The longevity of the adult parasites is not accurately known. Rubin & Lucker (1956) considered that most worms were expelled around 72 days after infection whereas Jarrett et al (1957) found that the presence of larvae in the faeces had reached low levels by 50 days after infection. Other studies have found that small numbers of adult parasites may persist for several months in some animals (Jarrett et al 1955b). This was confirmed by a survey of cattle on farms and in abattoirs which demonstrated that cattle could harbour lungworms for up to six months in the absence of reinfection (Cunningham et al 1956). However a complicating factor in the interpretation of this data is the ability of larvae to undergo inhibited development for several months (Michel 1955).

# Figure 1.1


Adult worms in bronchi.
Eggs hatch in lungs.
$L_1$ pass up respiratory tract, through alimentary tract and out in faeces.
2x moult to infective $L_3$ on pasture.
Ingested by host.
Penetrate small intestinal mucosa.
Moult to $L_4$ in mesenteric lymph nodes.
Pass via lymphatics and blood stream to lungs.
Moult to juvenile.

### 1.1.2. Epidemiology of *D.viviparus*.

The occurrence of clinical disease, transmission of infection and the persistence of infection from one year to the next depend upon the survival and behaviour of the free-living larval stages on the pasture and the persistence of infection within the host. Each of these factors will be considered in turn.

#### 1.1.2.1. Development and dissemination of the free living larval stages on the pasture.

Development from the $L_1$ to the infective $L_3$ stage is dependent upon the environmental temperature and humidity. At temperatures of 20-25°C the infective stage is reached within 3 days, at 5°C it can take up to 26 days and at 0°C development does continue but at a very slow rate (Rose 1956). The $L_3$ is the larval stage most resistant to cooling and desiccation and consequently is the most suited to survival on the pasture. It can survive freezing but is only partially resistant to desiccation (Daubney 1920 and Rose 1956).

Once the infective stage is reached it must be disseminated onto the pasture away from the faecal pat in which it developed. This is important to the transmission of infection as cattle have a marked aversion to grazing near to faecal matter (Michel 1955a). Unlike most trichstrongylid nematode larvae, *D.viviparus* $L_3$ are not very motile and remain coiled and motionless at environmental temperatures experienced in most temperate regions (Daubney 1920). This suggests that the migration of larvae onto the pasture from the faecal pats is unlikely to be a significant method of dissemination and this has been confirmed experimentally (Michel & Rose 1954). Robinson (1962) proposed a method of dissemination involving the fungus *Pilobolus*. This fungus grows on the surface of bovine faeces, indeed its spores must pass through the gut of a herbivore before further development occurs and so it is dependant on grazing animals for its own life cycle. A survey in the U.K. showed it to be present in 95% of bovine faecal samples examined (Robinson 1962). This author also observed that on increasing the illumination of faecal cultures the normally quiescent larvae became motile and on contact with a *Pilobolus* sporangiophore attached to it and migrated to the upper surface of the sporangium. When the sporangium ripens it undergoes a violent discharge to disperse its spores and any associated *D.viviparus* larvae are projected up to a distance of 3 metres. This effect could be further enhanced by the wind and so provide a very effective means of

dissemination. *D.viviparus* $L_3$ have also been observed inside the sporangia of *Pilobolus* (Doncaster 1981).

The consistency of the faeces is also important in the dissemination of larvae because this determines how easily faeces is spread over the pasture by mechanical means such as the movement of animals and vehicles etc. This is largely determined by climatic conditions in that when the rainfall is high the faecal pats break down and the faecal matter is spread much more quickly than during dry conditions. Therefore warm and wet conditions are the most favourable to both larval development and dissemination and so climatic conditions will have a major influence on the occurrence of disease in a particular year. The spreading of slurry has also been implicated in the contamination of pastures with *D.viviparus* larvae ( Jarrett et al 1955b).

### 1.1.2.2. Survival of infective larvae on the pasture.

The survival of larvae on the pasture is of great importance to the epidemiology of parasitic bronchitis but early work produced conflicting results. Porter (1942) reported that larvae survived for less than 5 weeks, Rose (1956) proposed a maximum survival of 13 weeks and Wetzel (1948) reported that larvae did not survive over the winter. However subsequent work has shown clear evidence of the ability of larvae to survive for prolonged periods and to overwinter on the pasture. Jarrett et al (1954) showed that in Scotland, larvae could survive for up to 13 months and overwinter. This has been subsequently confirmed in other temperate regions by the recovery of larvae in pasture samples and the demonstration of infection in susceptible calves grazing the following spring (Michel & Shand 1955, Allan & Baxter 1957, Downey 1973, Duncan et al 1979 and Armour et al 1980). Overwintering of *D.viviparus* $L_3$ has also been reported from areas with more severe winter conditions such as Canada (Gupta & Gibbs 1970) and Denmark (Jorgenson 1980). Overwintered $L_3$ are now generally considered to be an important source of infection for calves grazing the following spring (Oakley 1977 and Duncan et al 1979). Although the numbers of adult worms derived from such infections are generally too low to cause clinical disease, they continually seed the pasture with larvae which often leads to outbreaks of disease later in the year.

*D.viviparus* infective larvae have been found in the intestines of earthworms (Cobbold 1886 and Oakley 1981) and it is possible that these act as paratenic hosts and aid in the dissemination and survival of larvae.

### 1.1.2.3. Persistence of infection in the host.

*D.viviparus* infection can persist in animals for a longer period of time than the 2 to 3 month duration of a typical acute infection described by Jarrett et al (1957a). This can occur in two ways; firstly by the persistence of low numbers of adult parasites in animals which have been termed "silent carriers" (Wetzel 1948) and secondly by the ability of larvae to arrest their development inside the host (Taylor 1951).

**Carrier animals**

Wetzel (1948) first described the occurrence of naturally infected cattle which excreted larvae but showed no clinical signs of disease. He called these animals, which were usually yearlings, "silent carriers" and attributed the phenomenon to the less effective immune response of calves compared to that of adult cattle. Jarrett et al (1955) showed that adult worms could be present in the bronchi of cattle throughout the winter but could not conclude whether this was due to repeated reinfection or persistence of the same individual parasites. In an abattoir survey of cattle Cunningham et al (1956) found that 31% of lungs examined from yearling animals and 4% of older animals in the late winter and early spring contained adult lungworms. In a farm survey, also carried out by these authors, larvae were found in the faeces of animals on 11 out of 24 farms and many of these cattle had been tethered indoors throughout the winter and so had not been exposed to infection for several months. These authors proposed that the development of the carrier state was dependent upon the size of challenge to which cattle were exposed. Animals which had suffered from a relatively heavy infection became sufficiently immune to both expel the parasite burden and to resist reinfection. However animals only exposed to low levels of infection did not acquire such strong immunity and could harbour adult parasites for prolonged periods. The presence of lungworm larvae in the faeces of cattle in the winter and early spring has also been reported by Gupta and Gibbs (1970) and Supperer & Pfeiffer (1971).

Carrier animals may also arise following vaccination with the irradiated larval vaccine in two ways. Firstly, it has been shown that very low numbers of irradiated larvae can reach sexual maturity and survive in the lungs (Poynter et al 1960). Secondly, (and of much more significance in the field) because vaccination does not produce a sterile immunity, when vaccinates are exposed to field challenge a few adult parasites may develop and so "silent carriers" are produced (Cornwell & Berry 1960 and Menear & Swarbrich 1968). For this reason it is important that unvaccinated cattle are not grazed with vaccinates or follow them onto a pasture.

**Inhibited Larval Development**

The first description of inhibited larval development in *D.viviparus* infection was by Taylor (1951) when he reported that examinations of both natural and experimental infections showed that larvae could remain undeveloped in the lungs for at least 10 weeks. This has subsequently been confirmed by a number of authors who have described development being arrested at the early $L_5$ stage, ie. immature adults (Taylor & Michel 1952, Michel 1955b, Michel et al 1965, Supperer & Pfeiffer 1971, Eisenger & Eckert 1975 and Pfeiffer 1976), although Gupta & Gibbs (1975) reported a mixture of late $L_4$ and early $L_5$. This is in contrast to the situation with other trichostrongylid nematodes such as *Ostertagia ostertagi* (Anderson et al 1969) and *Haemonchus contortus* (Connan 1971) where inhibition occurs at the early $L_4$ stage.

It was originally suggested that inhibition of development was associated with the acquisition of immunity by the host (Michel 1955b). However more recent work has demonstrated that infective larvae which have undergone a period of prolonged chilling, prior to being administered to calves, are more likely to arrest their development at the early $L_5$ (Inderbitzen 1976, Pfeiffer 1976 and Oakley 1979). This is similar to arrested development in other trichostrongylid nematodes (Anderson et al 1969 and Jorgensen 1981) where it is viewed as a mechanism by which the parasite can survive inside the host when the external environment is hostile to development of the free-living stages. Therefore, in temperate climates, larvae ingested in the autumn arrest until the following spring when they complete their development to sexually mature adults.

### 1.1.2.4. Summary of Epidemiology.

In summary *D.viviparus* can overwinter in three ways; as infective larvae on the pasture, adult parasites in the host or inhibited early $L_5$ in the host. The contribution of each of these to the infection of cattle in the spring will vary with particular climatic conditions and husbandry practices. Clinical disease tends to occur later in the summer due to a build up of infection on the pasture. This is produced by the larval output of adults which have established infection in the spring, having overwintered by one of the three methods described above. However outbreaks of disease are less predictable than for the ruminant gastro-intestinal trichostrongylid nematodes and this may be partly due, to the smaller numbers of parasites required to produce clinical disease and to the relatively rapid immunity which develops to the

parasite. Nevertheless current understanding of the epidemiology of this parasite cannot fully explain sudden outbreaks of disease which can occur on farms which have been apparently free of infection for many years.

### 1.1.3. Pathogenesis of Parasitic Bronchitis.

The first description of the pathology of field cases of bovine parasitic bronchitis was by Jarrett et al (1954) and all the lesions observed were subsequently reproduced by experimental infection of calves (Jarrett et al 1957b). A more detailed account of the pathology was produced by Jarrett et al (1957a and 1960a) following experimental infection of calves with 5000 or 50,000 $L_3$. The lesions produced by these different infective doses varied only in a quantitative manner. On the basis of these experiments the pathogenesis of the disease was divided into four phases.

**The Penetration Phase (Days 1-7)**

This is the period during which the larvae enter the host and migrate to the lungs. There are no clinical signs associated with this phase unless the infecting dose is extremely large.

**The Prepatent Phase (Days 7-25)**

This is the period during which larvae enter the alveoli and migrate up the respiratory tree often causing coughing, tachypnoea and hyperpnoea, the severity of which depends on the size of the infecting dose. When larvae appear in the alveoli they are surrounded by a cellular infiltrate of polymorphs, macrophages and multinucleate giant cells. With time, these lesions progress further up into the bronchioles and blockage of the bronchiolar lumen causes collapse of dependant alveoli. Towards the end of this period parasitic bronchitis occurs with the bronchial mucosa heavily infiltrated with eosinophils, neutrophils, lymphocytes and plasma cells and a frothy mucous exudate is present in the bronchi. Immature adult worms are visible in the bronchi at this stage. Heavily infected animals may die from 15 days onwards from respiratory failure caused by the associated alveolar collapse, interstitial emphysema and pulmonary oedema.

**The Patent Phase (Days 25 -55)**

This phase is associated with bronchitis due to the presence of mature adults in the bronchi and pneumonia due to the aspiration of freshly laid eggs and larvae into the bronchioles and alveoli. The bronchiolar epithelium becomes

hyperplastic and there is further infiltration of the bronchiolar mucosa with large numbers of eosinophils and some neutrophils and plasma cells. There is a marked reaction around the aspirated eggs and first stage larvae in the alveoli, consisting of dense infiltrates of neutrophils, macrophages and multinucleate giant cells with dense masses of eosinophils often containing central areas of necrosis. Proteinaceous "hyaline membranes" can be seen covering the surface of many alveoli. There is widespread interstitial emphysema and pulmonary oedema and clinical signs include anorexia, coughing, hyperpneoa, tachypnoea, dyspnoea, dehydration, pyrexia (often exacerbated by secondary bacterial infection) and occasionally subcutaneous emphysema. Deaths are relatively common during this phase.

First stage larvae can usually be detected in the faeces during this period and counts are usually in the range of 50 to 1000 larvae per g (Urquhart 1985).

**The Post-patent Phase (Day 55 -70)**

This is the recovery period after which the adult worms have been expelled. Jarrett et al (1960a) found that most experimentally infected animals gradually recovered with a steady abatement of the clinical signs and complete recovery was apparent after several months. However "in approximately a quarter of those animals which were severely affected" there was a sudden exacerbation of dyspneoa which was often fatal. The pathology associated with these cases is described as alveolar epithelialisation involving proliferation of type II pneumocytes in large diffuse areas of the lung giving it a enlarged and rubbery appearance. The aetiology of this lesion is unknown but has been ascribed to the aspiration of material from dead parasites (Urquhart et al 1987).

**Re-infection syndrome.**

Cattle which are immune, either from previous infection or from vaccination, can show clinical signs if exposed to particularly heavy challenge. This is known as re-infection syndrome and is due to the migration and death of larvae in the lungs (Jarrett et al 1960a). The presence of lympho-reticular nodules in the walls of the bronchi and bronchioles of such animals has been described (Jarrett & Sharp 1962) and similar nodules had been previously reported in calves considered to be naturally resistant to infection (Simpson et al 1957 and Jarrett et al 1960a). These are thought to consist of lymphoid tissue surrounding the remains of dead larvae (Pirie et al 1971) and their relevance to immunity will be discussed in the next section.

In field outbreaks, the severity and duration of the pathology described above will depend numerous factors as has been reviewed by Urquhart (1985). These include the number of infective larvae ingested and the temporal pattern of infection. Also host factors such as age, immune status, plane of nutrition, environmental stress and the presence of intercurrent disease are also important.

## 1.1.4. Immunity and Vaccination.

### 1.1.4.1. Naturally acquired immunity to *D.viviparus*.

Most of the original published accounts of field cases of parasitic bronchitis reported that cattle which had recovered from the disease were resistant to re-infection (Wetzel 1948, Taylor 1951 and Jarrett et al 1954). This was supported by the demonstration that experimentally infected calves were also highly resistant to re-infection (Porter & Cauthen 1942 and Rubin & Lucker 1956). These authors also reported that previously uninfected calves of the same age were susceptible to infection which suggested that the resistance was due to acquired immunity and not simply related to age.

Selman and Urquhart (1979) have stated that older animals, not previously exposed to infection, remain susceptible to the disease. However it has been suggested that a degree of age resistance does occur because yearlings with no previous history of infection are less susceptible than calves (Wade et al 1962 and Taylor et al 1988). There also appears to be some variation in the degree of resistance produced following infection between individual calves of the same age and breed (Ruben and Lucker 1956 and Canto 1990). However this does not seem to be of practical significance to vaccination.

### 1.1.4.2. Experimental immunisation of cattle against *D.viviparus*.

Jarrett et al (1959b) demonstrated that a high degree of acquired immunity could result from a single sub-lethal dose or a series of small doses of infective larvae. The worms present in the lungs of such animals after challenge with 13,000 $L_3$ were greatly reduced in number, very stunted and underdeveloped. A rapid antibody response, measured by complement fixation using freeze dried adult worm antigen, was exhibited on challenge.

Jarrett et al (1955a) also showed that the globulin fraction of hyperimmune serum conferred a considerable degree of resistance to infection in passively immunised calves. The hyperimmune serum was produced by experimentally infecting recovered field cases and when the complement fixing antibodies were judged to be at a maximum, the animals were bled and the globulin fraction prepared. Five calves each received 500 ml of this preparation daily for 3 days by intraperitoneal injection and were then challenged with 4000 $L_3$. There was a 95% reduction in the number of worms in the lungs of passively immunised calves relative to challenge controls and also the associated lung damage was markedly reduced. Rubin & Weber (1955) intravenously injected four calves with differing doses of hyperimmune serum, from 0.1 to 5 ml/lb, and then challenged with 50,000 $L_3$. The two calves receiving the highest doses of serum survived whereas the two calves receiving the lower doses, and a control calf, died. More recently Canto (1990) reported a level of 75.6% protection in 2 passively immunised calves relative to a group of 4 control calves using the method described by Jarrett et al (1955a). It therefore appears that antibodies present in the serum of immunised cattle are capable of protecting animals against challenge.

Because of the solid immunity produced by natural infection and the success of passive immunisation, attempts were made to immunise cattle with dead whole worm material. Immunisation with freeze dried adult *D.viviparus* and Freund's adjuvant produced no protection against challenge with 4000 $L_3$ but a modest, although statistically significant, degree of protection against challenge with 2000 $L_3$ (Jarrett et al 1957a, Jarrett et al 1960b). However no visible reduction in the severity of associated lung pathology was detected in any of the immunised groups. Wade et al (1962) immunised calves with antigen prepared from a mixture of adult parasites and infective larvae and this produced a significant degree of protection in 8 month old calves but not in 2 month old calves. Therefore, although a degree of acquired immunity may be produced by immunisation with dead parasite material, Jarrett et al (1960c) suggested it was unlikely to be sufficient to enable calves to withstand infections of the magnitude common under field conditions. They also concluded that production of sufficient quantities of such material for use as a vaccine was impractical (Jarrett et al 1960b).

### 1.1.4.3. Development of a vaccine to *D.viviparus*.

The relatively moderate levels of protection afforded by immunisations with dead parasite material led Jarrett et al (1957a & 1960c) to speculate that stimulation of a protective immune response would be greatest when "actively metabolizing worms" were used as the antigenic stimulus. Particularly if such antigen was introduced at a site where "maximum production of immunity would be achieved and where maximum effect on the parasite would be exerted". Accordingly, a method of attenuating larvae to render them non-pathogenic but still capable of stimulating a protective immune response was sought. It had been shown by Tyzzer & Honeij (1916) that X-irradiation had a deleterious effect on *Trichinella spiralis* larvae and later work had shown that if such irradiated larvae were given to rats by mouth, an adult infection resulted but all the adults were sterile (Levin & Evans 1942 and Gould et al 1955). This prompted Jarrett et al (1960c) to perform a number of infection and immunisation experiments using larvae exposed to varying amounts of X-irradiation. It was found that irradiation at 20krad or 40krad inactivated the $L_3$ to the extent that a single dose of 4000 $L_3$ produced no significant disease but enabled the calves to successfully resist a challenge of 4000 normal larvae given 50 days later. Although infection with the irradiated larvae did not cause serious disease, mild clinical signs were noted and significant lung lesions could be seen on necropsy of animals killed 35 days after immunisation. Therefore vaccination with a smaller dose of irradiated larvae was considered desirable. Calves vaccinated with a single dose of 1000 $L_3$, irradiated at 40krad, were turned out onto a pasture which had been heavily contaminated (1300 larvae per sq. ft.) by the previous grazing of experimentally infected calves (Jarrett et al 1958a). Only 3 out of the 15 vaccinated calves died compared to 10 out of the 12 unvaccinated control calves with which they were grazed. There were also significant differences in the respiratory rates and faecal larval counts between the vaccinated and control calves. In view of the very heavy larval challenge and the fact that the immunising dose was only 1000 irradiated $L_3$, the degree of protection afforded was considered to be very good. A field trial was performed on 40 farms with a history of parasitic bronchitis in which half the calves were vaccinated with a single dose of 1000 $L_3$ irradiated at 40krad and half were left as controls (Jarrett et al 1958b). Outbreaks of parasitic bronchitis occurred on 6 farms where 62% of the controls died as opposed to only 6% of the vaccinates.

Although the single vaccination regime afforded significant levels of protection, it did not completely protect against disease in the face of high levels of challenge. Therefore further work was performed by Jarrett et al (1959a) on a double vaccination regime with irradiated larvae. Three groups of ten calves were all given a

first dose of 1000, 40krad irradiated $L_3$ and 42 days later each respective group was given a second dose of 4000, 2000 and 1000 40krad irradiated $L_3$. When challenged with 10,000 $L_3$, 51 days later, clinical signs were not evident in any of the calves from the immunised groups and no worms were recovered from the lungs of these calves compared to a mean of 879 worms per calf for the control group. A field trial using the same double vaccination regime was performed where the vaccinates and controls were turned out onto a field where 38 out of 40 calves had died from parasitic bronchitis the previous year (Jarrett et al 1961). There were significant differences in respiratory rates and clinical signs between vaccinated and control animals and at necropsy no worms were recovered from the lungs of the vaccinates whereas a mean of 440 were found in the controls.

This work led to the development of the commercial bovine lungworm vaccine "Dictol" which consisted of 1000 infective larvae irradiated at 40krad with two doses to be given at an interval of 4 weeks. Many workers have subsequently confirmed the efficacy of the *D.viviparus* irradiated larval vaccine in a variety of countries; Poynter (1960) and Nelson, Jones and Peacock (1961) in Britain, Englebrecht,H.J. (1961) and Edds,G.T. (1963) in U.S.A., Olson (1962) in Sweden, Vercruysse et al (1963) in Belgium, Enigk & Duwel (1963) and Blimdow (1966) in Germany, Pierre et al (1961) in France, Van Eck et al (1960) in the Netherlands and Downey (1968) in Ireland.

Alternative methods to attenuate larvae have been attempted. Larvae chemically attenuated with Triethylmelanine were found to give similar results to the X-irradiated larval vaccine but this system was never developed commercially (Cornwell & Jones 1970a and 1970b). More recently gamma irradiation from a $^{60}Co$ source has been used for the attenuation of larvae (Bonnazzi et al 1983 and Gennari & Duncan 1983) and this is now the method used to produce the two currently available commercial vaccines "Dictol" and "Huskvac" (Intervet, Cambridge, U.K).

### 1.1.5. Control of parasitic bronchitis

The *D.viviparus* irradiated larval vaccine has been in use for over 30 years and is still the only commercially successful helminth vaccine. Its role in the control of parasitic bronchitis and the relative merits of immunoprophylaxis versus chemoprophylaxis provide a interesting insight into the immunological control a helminth parasite.

### 1.1.5.1. Control by vaccination.

The irradiated larval vaccine was introduced in 1959 and has been highly effective in the control of parasitic bronchitis. Peacock and Poynter (1980) reported that in the first 21 years of its use only 266 incidents of confirmed or confidently diagnosed outbreaks of husk occurred in 205,000 "user years" which constitutes a prevalence of 0.13% on vaccinating farms. However there are a number of features of the vaccine which are important to its use under practical conditions.

Although vaccinated calves exhibit a high level of resistance to infection, the immunity is not absolute. This has two important consequences depending on the level of challenge. Firstly, vaccinated calves exposed to levels of challenge experienced under the majority of field conditions acquire small numbers of adult parasites and effectively become immune carriers (Cornwell & Berry 1960 and Menear & Swarbrich 1968). Secondly, under very high levels of challenge vaccinated calves can show clinical signs varying from mild coughing to severe respiratory signs although the latter situation is very rare (Urquhart 1985b).

There is no clear data on how long the immunity stimulated by vaccination lasts in the absence of boosting by field challenge. However it has been shown that vaccine-induced immunity is not as high or as persistent as naturally acquired immunity (Michell 1965) and it is generally accepted that maintenance of immunity is dependant upon boosting by natural field challenge (Peacock & Poynter 1980 and Urquhart 1985b).

These features of the vaccine have some important consequences with regard its use which provides an interesting insight into the practical control of a helminthic disease by vaccination.

(1) Calves are vaccinated with a double dose of vaccine (1000 irradiated $L_3$) at an interval of 4 weeks when they are over two months of age and should not be exposed to infection until at least 2 weeks after the second dose. Subsequent doses of vaccine are not usually required because immunity is boosted by natural field challenge.

(2) Vaccinated calves should not be suddenly exposed to heavily infected pastures. This is because resistance to infection is not absolute and gradually increases during exposure to field challenge.

(3) Vaccinates and non-vaccinates should not graze together or follow each other over the same areas of pasture. This is because vaccinated calves can

produce sufficient pasture contamination to produce disease in susceptible calves and examples of such outbreaks have been documented (Cornwell & Berry 1960). Also heavily infected susceptible calves can produce very high levels of pasture contamination which are capable of overcoming the immunity of vaccinates.

(4) Vaccination does not lead to lungworm free pastures and so it can never be considered safe to stop vaccinating new stock, irrespective of the length of time a farm has been free of disease.

(5) Control of intercurrent diseases, especially parasitic gastroenteritis, which reduce the level resistance to *D.viviparus* is important (Kloosterman et al 1989).

(6) Calves sometimes show transient episodes of coughing between 1 and 2 weeks following vaccination which is associated with the response to migration and death of larvae in the lungs (Urquhart 1985). Also, calves should not be vaccinated in the presence of other intercurrent respiratory disease as it may exacerbate the clinical severity of these conditions (Peacock & Poynter 1980).

### 1.1.5.2. Chemoprophylaxis as an alternative to vaccination

There are a number of anthelmintics available for treatment of parasitic bronchitis including the benzimidazoles (Inderbitzen & Eckert 1978 and Duwel & Kirsh 1980), levamisole (Oakley 1980 and Pouplard et al 1986) and ivermectin (Armour et al 1980 and Alva-valdes et al 1984). The traditional combination of strategic grazing and prophylactic use of anthelmintics, used to control the ruminant gastrointestinal nematodes, has generally been less effective for the control of parasitic bronchitis. This is because the occurrence of parasitic bronchitis is notoriously unpredictable and can occur at any time between June and October (Duncan et al 1979) and also because the numbers of parasites required to cause disease is relatively small. However, in recent years two new anthelmintic control stategies have been introduced which are more effective for the control of parasitic bronchitis. The oxfendazole pulse release bolus, when administered to calves, lodges in the rumen and releases a therapeutic dose at three weekly intervals for 5 doses. This does not allow any parasites to reach patency for the first 100 days of the grazing season and so minimises pasture contamination (Bogan et al 1987). This has been successfully used to control parasitic bronchitis (Armour et al 1988a) and it seems to allow sufficient exposure to infection for treated calves to develop immunity as

assessed by resistance to challenge in the following grazing season (Downey 1988). However other workers have reported that parasitic bronchitis can occur at the end of the grazing season following use of the bolus (Vercruysse 1987) and it is also possible that the prepatent stages could cause disease during the intervals between the pulse releases (Jacobs et al 1987). The other strategy which is being increasingly used for the control of parasitic gastroenteritis is the "3, 8 & 13" week regime of ivermectin treatment (Taylor et al 1985). This relies on ivermectin having a residual activity of 2 weeks against gastrointestinal nematodes and so a 5 week interval is sufficient to prevent any parasites reaching patency for the first 100 days of the grazing season as with the pulse release bolus. *D.viviparus* is particularly sensitive to ivermectin and there is a residual activity of at least 3 weeks for this parasite (Armour et al 1987). This means that there is less possibility of disease due to prepatent husk occuring between treatments, than there is for other anthelmintic control programmes. This regime has been effective in the control of lungworm infection (Taylor et al 1986 and Armour et al 1987) and it has been reported that calves treated in their first grazing season develop sufficient immunity to be resistant to challenge the following year (Taylor et al 1988 and Armour et al 1988b).

The fact that these recently introduced anthelmintic control strategies can be effective in the prevention of parasitic bronchitis leads to question of whether vaccination is still necessary. There are, in fact, still a number of dangers in a total reliance on anthelmintics for the control of parasitic bronchitis. Firstly the inherent unpredictability of *D.viviparus* epidemiology (Duncan et al 1979) is a potential problem for any control strategy which relies on minimising pasture contamination during the first part of the grazing season. Secondly, even after many years of use, the vaccine does not eradicate the parasite from a farm but allows sufficient challenge for the initial state of immunity to be continually boosted. In contrast, a major concern about the more modern methods of anthelmintic control is that because they prevent parasites reaching patency during the first part of the grazing season, very little seeding of the pasture occurs. Consequently, if used over a number of years, such regimes may reduce pasture contamination to such a degree that there is insufficient boosting of immunity and the whole herd may eventually become susceptible to the disease. This is a particularly hazardous state with *D.viviparus* as freedom from infection is very difficult to ensure due to the unpredictable nature of its epidemiology (Duncan et al 1979), the relatively small numbers of larvae required to cause disease and the ever present risk of introducing infection with bought-in stock. The economic impact of a significant outbreak of parasitic bronchitis in adult stock is very large and so reliance on chemoprophylactic control is not recommended.

Other disadvantages of strategic anthelmintic control include adverse environmental effects, presence of residues in meat from treated animals and the potential risk of anthelmintic resistance.

## 1.1.6. The immune response to *D.viviparus* infection.

The bovine immune response to *D.viviparus* is highly effective in both expelling established adult parasites and preventing re-infection. In spite of the remarkable success of the vaccine, very little work has been performed to identify the antigens which induce this immunity or to elucidate the effector mechanisms involved. The published work examining the immune response to *D.viviparus* infection and vaccination is reviewed in this section.

### 1.1.6.1. Stage of parasite and site of immune response.

Several parasite stages must be the targets of the bovine immune response to *D.viviparus*, since there is both destruction of established adult parasites and prevention of re-infection by the larval stages. However it is not known which stages are the most important in the induction of these responses or exactly which of the invading larval stages are destroyed by immune cattle. Also the site of the host responses to invading larvae has been the subject of some debate. Consideration of two situations may be useful in assessing the current state of knowledge; firstly the fate of irradiated larvae when used to immunise a susceptible host and secondly the fate of normal larvae when infecting an immune host.

**Infection of the susceptible host with irradiated larvae.**

Jarrett et al (1957b) speculated that the major site of relevant antibody production during *D.viviparus* infection was the mesenteric lymph nodes and the original intention was to attenuate larvae so that migration would be halted at this site. However in the initial studies of the irradiated larval vaccine mild, transient pulmonary signs were detected during the immunising period (Jarrett et al 1959a). Also Poynter (1960) found that irradiated larvae were present in the lungs as little as 24 hours after infection which was similar to experiments conducted with normal larvae and they concluded that there was "no evidence of a mesenteric lymph node hold up" of irradiated larvae. However Jarrett & Sharp (1963) commented that in these experiments the numbers of larvae recovered from the lungs and mesenteric lymph

nodes was very small, given that an infective dose of 200,000 larvae was used (eg. in 3 calves killed 48 hours after infection 1,480, 50 & 180 larvae were recovered from the lungs and 40, 5 & 0 from the mesenteric lymph nodes). They therefore suggested that such small numbers of larvae could not be assumed to be responsible for the immunising process, given that the fate of the vast majority of larvae was unknown. Jarrett & Sharp (1963) went on to show that significant numbers of larvae could be recovered from the lungs of calves from days 7 to 25 after infection with just 1000 40krad irradiated larvae, with none being recovered after day 45. Histological lesions in the lungs associated with the death of irradiated larvae, enlargement of the bronchial lymph nodes and the presence of dead larvae in the mesenteric lymph nodes were all described. The authors suggested that the immunogenic stages were probably the $L_3$ and $L_4$ and that immune responses in the mesenteric and bronchial lymph nodes as well as reactions in the lungs and bowel wall may all contribute to the protective immune response.

Experiments on guinea pigs have suggested that X-irradiation (at 40krad) affects the larvae at the moult from $L_4$ to $L_5$ (Poynter et al 1960). Cornwall & Jones (1971), also using guinea pigs, found that attenuation of larvae with 0.7% Triethylene Melamine also acted at, or shortly after, the moult from $L_4$ to $L_5$ and that larvae attenuated with greater concentrations failed to protectively immunise guinea pigs. They therefore concluded that the stimulation of an effective immune response depended on the host being exposed to larvae moulting from the fourth to the fifth stage. In contrast Canto (1990) found that gamma irradiation of larvae to 100krad from a $^{60}Co$ source did not allow development in the guinea pig beyond the $L_3$ but these larvae were capable of protectively immunising both guinea pigs and cattle. As has been pointed out by Jarrett & Sharp (1963) extrapolation of data from the guinea pig to the bovine may not always be valid. This is particularly the case when considering the effects of attenuation on the parasite since even normal *D.viviparus* larvae only develop to the early $L_5$ in the guinea pig and it is possible that similar differences in the development of irradiated larvae may occur between the two hosts.

**Infection of the immune host with normal larvae.**

The term "immune animals" used in this section refers to calves immunised either by vaccination or previous infection. Poynter (1960) found that larvae could be recovered from the lungs of immune calves from 24 hours after a challenge with 200,000 $L_3$ and concluded that invading larvae were not held up in the mesenteric lymph nodes of immune animals (similar to the conclusion for the behaviour of irradiated larvae in susceptible calves). Jarrett & Sharp (1963) also found that larvae reached the lungs of immune cattle following challenge with 1000 $L_3$ but

not until 7 days after infection (again similar to their results with irradiated larvae in susceptible calves). The differences reported by these separate workers in the time taken for the larvae to reach the lungs is again probably due to the different size of the infective dose. However both authors agreed that significant numbers of larvae do reach the lungs of immune animals.

A particularly interesting feature of infection in immune animals, which does not occur in susceptible animals, is the development of lympho-reticular nodules in the walls of the bronchi and bronchioles. These were first described in the lungs of calves which were found to be naturally resistant to infection when slaughtered after challenge (Simpson et al 1957 and Jarrett et al 1960a). Similar nodules had also been described in animals challenged after passive immunisation with serum (Jarrett et al 1955 and Canto 1992) and in infected calves treated with the anthelmintic drug diethylcarbamizine (Jarrett, McIntyre & Sharp 1962). They were found and described in detail by Jarrett & Sharp (1963) in the lungs of calves which were challenged following previous vaccination or infection but were absent from the lungs of calves infected or vaccinated for the first time. The nodules were 2 to 4 mm in diameter, clearly visible through the pleura and bulged through the surface of the lung when the parenchyma was excised. They tended to be grey/pink with greenish yellow centres and had the histological appearance of lymph nodal tissue, often with a centrally placed larvae surrounded by a mass of degenerating eosinophils. Germinal centres were present and some of the nodules had a well defined lobulated structure. The authors speculated that these might arise around dead larvae trapped in broncho-occlusive lesions and may be major sites of antibody production to "somatic" antigens released from the disintegrating parasites. However they also observed that many disintegrating larvae were found in the bronch-mediastinal lymph nodes which, along with the mesenteric lymph nodes, may also be a major site of antibody production. Similar results have subsequently been reported by Michel et al (1965) and Pirie et al (1971). The latter authors observed that animals which died following an experimental challenge had the lowest numbers of lympho-reticular nodules and the highest worm burdens and suggested that the nodules arose due to the immunological killing of larvae in the bronchioles. They further speculated that the presence of the nodules might be a good criterion of the host's immune status. However Poynter et al (1970) suggested that the presence of nodules did not always correlate with immunity and that the precise relationship between the two was unclear.

**Other evidence relevant to parasite stage and site of the immune response.**

Cornwell (1962) reported that immunisation of calves by intraperitoneal injection of fourth stage larvae produced a significant degree of protection (70% relative to controls) but not as great as that produced by vaccination or normal infection. He concluded that significant levels of protection could be produced without the $L_3$ stage passing through the mesenteric lymph nodes but it was not possible to determine whether the $L_3$ played a role in the development of immunity during normal infections. Bain & Urquhart (1988) reported that subcutaneous administration of the irradiated larval vaccine produced as high a degree of protection against challenge as that produced by oral administration. They concluded that the passage of larvae through the intestinal mucosa and mesenteric lymph nodes was not necessary for the the induction of a protective immune response. Canto (1990) showed that 100krad irradiated $L_3$, which do not develop beyond the $L_3$ stage in the guinea pig, could protectively immunise guinea pigs when administered orally or intraperitoneally and calves when administered intravenously. It therefore appears that although the $L_3$ is not essential in the production of protective immunity, it is capable of inducing such a response.

Jarrett et al (1955a) has shown that passive immunisation with the serum from hyperimmunised calves can confer protection to recipient animals and this has also been demonstrated in the guinea pig model (Wilson 1966 and Canto (1990). However Canto (1990) found that the serum from guinea pigs and calves protectively immunised with 100krad irradiated larvae could not confer protection to passively immunised recipients. These results can be used to hypothesize that antibody is important for that part of the immune response which is stimulated by the later parasite stages but not for that which is induced by the $L_3$. However caution should be exercised before this conclusion is reached for two reasons. Firstly, it is not certain that the 100krad irradiated larvae do not develop beyond the $L_3$ in cattle as this has only been examined in the guinea pig system and secondly, only two calves were passively immunised with the 100krad serum and so further experiments are necessary before any firm conclusions are drawn.

**Summary of parasite stage and site of immune response.**

It is clear that following vaccination, the irradiated larvae which comprise the vaccine develop to the $L_4$/early $L_5$ stage and reach the lungs in significant numbers. Similarly, normal larvae which invade immune hosts appear to be destroyed in the lungs, resulting in the formation of pulmonary lymphoid nodules but some are also destroyed in the mesenteric and bronchial lymph nodes. It seems

likely that immune effector mechanisms in all these sites, and also possibly in the intestinal mucosa, contribute to the immune response to re-infection. It is clear that the majority of invading parasites are destroyed by the host immune system in the early larval stages but their is no evidence to suggest whether this involves the $L_3$, $L_4$ or both of these stages. Similarly, there is no direct evidence to suggest which of the stages is the more important in the stimulation of immunity following vaccination or natural infection. However it appears that either stage on its own is capable of inducing a protective immune response.

### 1.1.6.2. The role of the eosinophil in immune response to *D.viviparus*.

A pronounced blood eosinophilia is characteristic of many helminth infections (Conrad 1971) and although a variety of myeloid cells are involved in inflammatory responses to helminths, the eosinophil often predominates (Beeson & Bass 1977). Eosinophils have been shown to adhere to and kill a number of helminth parasites *in vitro*, including *T.spiralis* newborn and infective larvae (Bass & Szedja 1979 and Mackenzie et al 1980), *Wuchereria bancrofti* infective larvae (Higashi & Chowdhury 1970) and *Schistosoma mansoni* schistomula (Capron et al 1983). However there is no direct evidence of parasite rejection or killing by eosinophils *in vivo*. In other helminth infections the eosinophil response may be evaded, for example it has been found that although eosinophils adhere to the surface of *Toxocara canis* $L_2$ and also degranulate, these larvae show little damage and even slough off the adherent cells (Fattah et al 1986). Bovine eosinophils have been shown to adhere to *D.viviparus* infective larvae *in vitro* with an associated reduction in motility (Knapp & Oakley 1981). This adherence was dependent upon a heat labile factor in normal bovine serum (possibly complement) and a heat stable factor in hyperimmune bovine serum (possibly antibody).

The previous description of the pathology associated with *D.viviparus* infection demonstrated that the eosinophil is very predominant in the various inflammatory lesions associated with the disease. The blood eosinophil response to *D.viviparus* infection has been described by several authors (Weber & Rubin 1958, Cornwell 1962a and MacKenzie & Michell 1964). There is a peak of eosinophilia at 9-15 days post infection which is followed by a second higher peak at around 40 days post infection. In contrast, just a single peak occurs 18 days after vaccination with irradiated larvae with a similar single peak following a second vaccine dose (Cornwell 1962a). It was suggested that the first peak of eosinophilia following normal infection was associated with migration of larvae through the lungs and the second peak with

the aspiration of eggs and first stage larvae following parasite patency. This would explain the single peak following vaccination as the irradiated larvae do not develop into mature adults. When vaccinated cattle were challenged with normal larvae the resulting eosinophilia was 4 times greater than that following vaccination and consisted of a double peak at 14 and 40 days post challenge, the second peak being the smaller. Presumably the second peak is smaller in this case because only a few parasites reach patency in the immune animals.

As with other helminths there is no direct evidence of eosinophils being important in the killing of parasites *in vivo* but Djafard et al (1960) reported a correlation between eosinophil response and survival in an outbreak of parasitic bronchitis in calves.

### 1.1.6.3. The antibody response to *D.viviparus* infection.

The antibody response to experimental *D.viviparus* infection was first measured by a complement fixation test using adult whole worm antigen prepared at low temperature (Weber 1958). An initial antibody response was detected as soon as 12 days after infection which reached a peak by day 16 and remained high until 63 days after infection. However subsequent use of the complement fixation test utilised heated adult whole worm antigen and produced somewhat different results (Jarrett et al 1959b and Cornwell & Michell 1960). A detectable complement fixing antibody response was not seen until 30-35 days after infection, reached a peak at 80-100 days and declined after 100-150 days. Peak levels of antibody production following reinfection occurred after about 21 days. Michel & Cornwell (1959) found that complement fixing antibody titres required considerably longer to develop than did resistance to reinfection and that there was no correlation between the degree of protection of individual calves and their antibody titre. Cornwell (1960a) investigated the complement fixing antibody response in calves vaccinated with irradiated larvae. The titres following both the first and second vaccination were significantly lower than those seen following normal infection and the response between individual calves varied widely. The majority of vaccinated calves showed a detectable increase in antibody titre 2 weeks after challenge but it was again concluded that the complement fixing antibody response did not necessarily reflect the immune status of the host.

Sera from lungworm infected calves cross-reacted with the complement fixation test when antigens from other parasites were used including *Ascaris*, *Moneizia* and *Haemonchus* species (Cornwell 1963b and Selman & Urquhart 1979). Also serum from calves infected with *O.ostertagi* cross-reacted with the complement fixation test

using *D.viviparus* heated whole adult worm antigen (Sinclair 1964). Bokhout et al (1979) reported that an indirect haemagglutination test (IHA) using non-denatured whole adult worm extract as antigen did not produce cross-reactions with sera from animals experimentally infected with *Fasciola hepatica*, *Cooperia oncophora*, *O.ostertagi* and *H.contortus*. They also reported that in an investigation of the presence of lungworm infection on 46 farms there was a positive correlation between IHA serology and clinical diagnosis for 80% of the herds and suggested that it may be of use as a diagnostic tool in the herd situation.

An enzyme linked immunosorbent assay (ELISA) has been used to demonstrate antibodies to adult whole worm extract in the serum and nasal secretions of *D.viviparus* infected calves (Marius et al 1979). Boon et al (1982) found a good correlation between the results of the IHA and the ELISA, using adult worm antigen, in both experimentally and naturally infected calves and suggested that the ELISA was a more convenient assay for survey work and herd diagnosis. Bos et al (1986) used the ELISA to measure the antibody response to $L_3$ and adult antigen preparations and following challenge of both vaccinated and non-vaccinated calves antibodies to $L_3$ antigens could be detected 1 week earlier than antibodies to adult antigens. They suggested that the anti-$L_3$ response may have reflected the number of larvae being ingested and the anti-adult response may have reflected the adult parasite burden. Similar results were reported by Taylor et al (1988) when performing the ELISA on calves treated with the "3, 8 & 13" ivermectin regime followed by housing and challenge the following year. They found that antibody to larval antigens tended to reflect exposure to infection but antibody against adult antigen was generally only high during patent infections.

Diagnosis of parasitic bronchitis is based on a combination of grazing (and vaccination) history, clinical signs and the identification of $L_1$ in the faeces. This is satisfactory in the majority of cases but the presence of larvae in the faeces is a relatively insensitive measure of adult parasite burden and does not detect prepatent, post patent and reinfection stages of the disease. Therefore a more precise and convenient diagnostic test would be useful both as a research tool and in routine diagnosis. Up to the present time serology has been mainly used for monitoring experimental infections and assessing exposure to infection in epidemiological studies. It has been of limited use for immunodiagnosis in the field because a knowledge of the immune status of individual animals (produced by previous exposure to infection or vaccination) is required to interpret the clinical significance of serological data. For example, a high antibody titre could reflect either the response of a solidly immune individual to field challenge or the response of a susceptible individual to a clinically significant challenge. Nevertheless, immunodiagnosis of

patent parasitic bronchitis (and the identification of carrier animals) would be a practical proposition if an assay which differentiated between larval and adult stages could be developed. The work of Bos et al (1986) and Taylor et al (1988) described above is interesting from this point of view. They suggested that the antibody levels measured by their adult homogenate ELISA were high only in patent infections as opposed to antibody measured by the larval homogenate ELISA which merely reflected exposure to infection. These results are encouraging but the inevitable presence of cross-reactive antigens between parasite stages means that such ELISAs cannot be used to reliably identify patent infections, particularly for individual animals. The identification and molecular cloning of stage specific antigens would allow the development of much more stage specific assays which could differentiate between larval challenge and infection with adult parasites. One possible candidate is the 17 kDa protein which has recently been identified and purified from *D.viviparus* adult somatic antigens (Leeuw & Cornelissen 1991). This appears to be stage specific and does not cross-react with sera from other common bovine helminthic infections. Other diagnostic strategies might include the detection of parasite products in the host's circulation by antigen capture ELISA or functional enzyme assays but no work of this nature has yet been published for *D.viviparus*. Such assays have the potential to allow a more direct assessment of parasite burden than tests which are based on the measurement of antibody titres.

In summary, the antibody response to *D.viviparus*, measured by complement fixation or ELISA, does not necessarily correlate with the immune status of the host but simply reflects exposure to infection. Serology has been used mainly for epidemiological surveys and research purposes and is of limited value for immunodiagnosis, particularly for individual animals, but the production of stage specific antigens may improve this situation.

## 1.2. Nematode Antigens and the Immune Response.

Helminths, unlike microbial or protozoan pathogens, are too large to be phagocytosed by cells of the host's immune and reticulo-endothelial system. Instead the immune response directs a whole spectrum of effector mechanisms which can lead to damage resulting in death and/or expulsion of the parasite (reviewed by Mitchell 1979). These include antibody-dependant-cell-mediated cytotoxicty (ADCC) reactions (involving numerous antibody isotypes and effector cells such as macrophages, neutrophils and eosinophils), immediate hypersensitivity reactions such as IgE mediated mast cell degranulation (particularly important in immunity at mucosal surfaces) and complement mediated damage following activation by both the classical and alternative pathways. T-lymphocytes also play a central role in the immune response to helminthic infections by producing cytokines which control the activities of antibody producing and other effector cells.

A comprehensive review of the immune response to helminths is beyond the scope of this introduction. Of primary relevance to this thesis are the molecules which constitute the nematode antigens and their relevance to acquired immunity.

### 1.2.1. Excretory/Secretory Antigens.

Molecules which are secreted/excreted by parasitic nematodes, along with surface antigens, represent the host-parasite interface and are consequently of particular interest from both a functional and immunological point of view.

#### 1.2.1.1. Nature of E/S antigens.

A wide range of functional molecules appear to be released during *in vitro* culture of parasitic nematodes.

Proteolytic enzymes have been reported in the secretions of many nematodes for which a variety of functions have been suggested. Examples include a role in host invasion in *Strongyloides stercoralis* (McKerrow et al 1990) and *Necator americanus* (Pritchard et al 1990b) and parasite feeding in *Ancylostoma spp.* (Hotez & Cerami 1983) and *H.contortus* (Knox & Jones 1991). Work on trematodes has also implicated secreted proteases in immune evasion by the cleavage of complement (Leid 1987) or immunoglobulin molecules (Auriault et al 1981).

Antioxidant enzymes have also been identified in nematode E/S products. Examples include superoxide dismutase in *Trichinella spiralis* (Rhoads 1983), catalase in *Nippostrongylus brasiliensis* and *Nematospiroides dubius* (Smith & Byrant 1986) and glutathione peroxidase in *T.spiralis* (Kazura & Meshnick 1984) and *N.brasiliensis* (Smith & Byrant 1986). These enzymes are thought to protect parasites from oxidant-mediated damage which can arise from normal cellular metabolism or the respiratory burst of activated host immune effector cells (Babior et al 1973).

High activities of acetylcholinesterase have been detected in the E/S products of many nematode parasites. Examples include *N.brasiliensis* (Edwards et al 1971), *Trichostrongylus colubriformis* and *Oesophagostomum radiatum* (Ogilvie et al 1973), *N.americanus* (McLaren et al 1974) and *Brugia malayi* (Rathaur et al 1987). The role of this secreted acetylchlolinesterase is not known but suggestions include it acting as a "biological holdfast" by inhibiting local gastrointestinal motility (Ogilvie & Jones 1971) or possibly having an immunomodulating effect in view of the fact that acetylcholine has been shown to stimulate cell mediated responses (Strom et al 1974 and Plaut,M. 1987).

Other functional molecules produced in nematode E/S products include an anticoagulant enzyme in *Ancylostoma caninum* (Hotez & Cerami 1983), an eosinophil attractant in *O.ostertagi* (Klesius et al 1986), protease inhibitors in *Ascaris suum* (Martzen & Peanasky 1985) and immunosuppressive agents from *T.spiralis* (Faubert 1976), *O.radiatum* (Gasbarre et al 1985) and *Onchocerca gibsoni* (Foo et al 1983).

E/S products are generally defined as material released from *in vitro* cultured parasites. Whether these reflect the physiological secretions of nematodes *in vivo* is open to question and probably depends on the particular parasite involved. For example, in the case of *T.canis* the infective larvae survive indefinitely in tissue culture and some of the *in vitro*-derived antigens have been detected in the circulation of infected rabbits and humans (Robertson et al 1988). Therefore, in this case, the *in vitro E/S* products are likely to be an accurate reflection of the molecules secreted *in vivo*. However for most other parasitic nematodes current *in vitro* culture systems are less satisfactory, for example the adult stages of ruminant trichostrongylid species die after several days in culture and so analysis of *in vitro* derived E/S products from these species should be treated with more caution.

The source of E/S antigens is not known in the majority of cases but immunocytochemical and immunogold electron microscopic techniques are being increasingly used to localise such antigens within parasites. Some E/S products have been clearly localised to internal secretory structures such as the stichocyte in *T.spiralis* (Despommier & Muller 1976) and *Trichuris muris* ( Jenkins & Wakelin 1977)

or to three sets of glands found in trichostrongylid nematodes (excretory, amphidial or oesophageal) such as *N.americanus* (McLaren 1974), *N.brasiliensis* (Edwards et al 1971) and *T.colubriformis* (Ogilvie et al 1973). Some components of the E/S material are shed from the nematode surface such as gp 29 of the filarial nematodes (Devaney 1988 and Selkirk 1990) and shedding of surface antigens has been reported for many nematode species (see below). The situation for *T.canis* $L_2$ is more complex as E/S products are thought to be produced in the oesophagus and, once exteriorized, are absorbed onto the cuticular surface and then subsequently shed from it (Maizels & Page 1990).

### 1.2.1.2. Immunological significance of E/S antigens.

The presence of specific antibody to E/S antigens has been well documented in many nematode infections. For example, infection sera has been shown to immunoprecipitate radiolabelled E/S antigens in many species such as *Ascaris* (Kennedy et al 1987d), *T.canis* (Kennedy et al 1987c and Maizels et al 1987b), *T.spiralis* (Ortega-Pierres 1984 & Kennedy 1991) and *D.viviparus* (Britton 1991). Also infection serum has been shown to specifically inhibit E/S enzyme activity of nematodes including proteases of *Ancylostoma caninum* (Thorson 1956), *A.suum* (Knox & Kennedy 1988) and *D.viviparus* (Britton 1991). Ogilvie & Hockley (1968) also demonstrated differences in the acetylcholinesterase isoenzyme profiles of *N.brasiliensis* harvested from immune mice (second and third infections) compared with those from susceptible mice (first infections).

Although E/S products are often highly antigenic, there is considerable qualitative and quantitative heterogeneity in the recognition of particular E/S antigens by infection serum between individual hosts (reviewed by Kennedy 1989). Also Bianco et al (1990) has shown evidence of size polymorphism in a secreted antigen of *O.lienalis*. Both heterogeneity in the host immune response and polymorphisms in parasite antigens have important implications for the potential use of such antigens in recombinant vaccines. In spite of this reservation, immunisation experiments have shown that E/S products are capable of eliciting protective immunity in a wide range of nematode species. Examples of particularly high levels of protection induced by immunisation with E/S antigens include 95% in mice against *N.brasiliensis* (Day et al 1979), 94% in cattle against *O.radiatum* (Keith & Bremner 1973) and 92% in guinea pigs against *T.colubriformis* (Rothwell & Love 1974). A fuller review of such experiments was presented by Maizels & Selkirk (1988).

Another reason for the current interest in E/S antigens is that they can sometimes be detected in the serum of infected individuals and so are of potential diagnostic use. Antibody capture assays using monoclonal antibodies have been developed to detect a high molecular weight proteoglycan present in the serum of animals and man infected with filarial parasites (Forsyth et al 1984b & 1985 and Maizels et al 1985 & 1987c) and a similar assay has been developed for *T.canis* infection (Maizels 1991). The measurement of functional activity of circulating parasite enzymes such as acetylcholinesterase has been suggested as another way in which E/S products could be used diagnostically (Knox & Jones 1991).

### 1.2.1.3. E/S products of *D.viviparus*.

Britton (1991) has performed preliminary investigations on the E/S products of *D.viviparus*. No proteins could be detected by Coomassie or Silver staining in the spent culture media of infective larvae but high levels of proteinase activity were detected using azocasein as a substrate. In contrast, a complex mixture of proteins were released from adult parasites during *in vitro* culture and many of these were recognised by bovine infection serum, although there was significant heterogeneity in the recognition profile produced by individual cattle. Interestingly, only one E/S molecule (approximately 30kDa) was strongly recognised by cattle immunised by 40 krad irradiated larvae and also a molecule of 30 kDa was the earliest to be seen by the infection serum. Lectin binding and $^{3}$H-glucosamine labelling suggested that most of the E/S products were not heavily glycosylated.

Significant protease activity was detected in adult E/S (as with $L_3$ E/S) and this was inhibited by bovine infection serum. Superoxide dismutase activity was also detected in both $L_3$ and adult E/S products. Recently, high levels of acetylcholinesterase activity have been detected in *D.viviparus* adult E/S products which is also inhibited by bovine infection serum (J.McKeand, unpublished data). Studies in other nematodes would suggest that these *in vitro* enzyme activities, ie. proteases, acetylcholinesterase and superoxide dismutase, may reflect the *in vivo* physiological activity of the parasites. However more caution is perhaps necessary when considering the *D.viviparus* E/S products, as the adult parasites only survive in tissue culture for a few days and the release of some products may be associated with dying or degenerating worms.

Cysteine protease activity has been detected in adult *D.viviparus* extracts and a proteinase of approximately 25 kDa has been partially purified (Rege et al 1989). These authors postulated that it may be involved in parasite nutrition

and/or lung pathology in infected cattle but no evidence of secretion of this molecule was presented.

Adult E/S material has been shown to produce significant, but variable, levels of protection against challenge in guinea pigs and so may be a potential source of protective antigens (Canto 1991 and McKeand unpublished data).

## 1.2.2. Antigens of the Nematode Cuticle.

The nematode cuticle is an obvious target for immune responses since its surface represents the host-parasite interface and should be accessible to the host immune system. However, as research has progressed, it has become apparent that the structure of the nematode cuticle and the associated immune response is far more complex than previously supposed. In order to appreciate the significance of particular cuticular antigens an understanding of the structure and composition of the nematode cuticle is required.

### 1.2.2.1. Structure of the Nematode Cuticle.

**Basic architecture of the cuticle.**

The body wall of a nematode is composed of the cuticle, an underlying cell layer called the hypodermis (or epidermis) and the longitudinally orientated somatic musculature (Wright 1987). The cuticle is a tough, but flexible, extracellular matrix which is secreted by the hypodermis (Bonner & Weinstein 1972, Martinez-Palomo 1978 and Bird 1980) and serves as an exoskeleton (Lumsden 1975). It also lines the invaginations of the nematode body wall such as the buccal cavity, pharynx, vulva, rectum and excretory pore. Most nematode cuticles have transverse grooves, called annulations, which confer a degree of flexibility and also their expansion in the larval stages can accommodate intermoult growth without an associated increase in cuticular surface area (Howells & Blaney 1983). Longitudinal expansions of the cuticle, known as alae, are a feature of some nematodes and are thought to assist in locomotion (Bird 1971). A number of other specialised cuticular structures exist including the male copulatory bursa which is used to grasp the female during copulation and papillae (usually at the anterior end) which are thought to have a sensory function (Bird 1971 and Lumsden 1975).

The basic structure and composition of the cuticle is highly conserved between nematodes but considerable variation of detail occurs between different

species or even different stages of the same species (Bird 1980). A schematic diagram of the cuticular architecture is shown in figure 1.2 (adapted from Wright 1987). The cuticle can be divided into three main zones on the basis of ultrastructural morphology; the basal zone, the median zone and the cortical zone. Bird (1984) suggested that zone was a more accurate term than layer as there are no distinct boundaries between these regions.

The **basal zone** lies immediately above the hypodermis (or epidermis) and has a striated appearance which Popham & Webster (1978) and Bird (1980) suggested might consist of blocks of protein separated by membranes. These striations are particularly prominent in the free-living infective larvae of many parasitic nematodes and the dauer larvae of *C.elegans*. These stages are all highly resistant to adverse environmental conditions and represent a suspension of development.

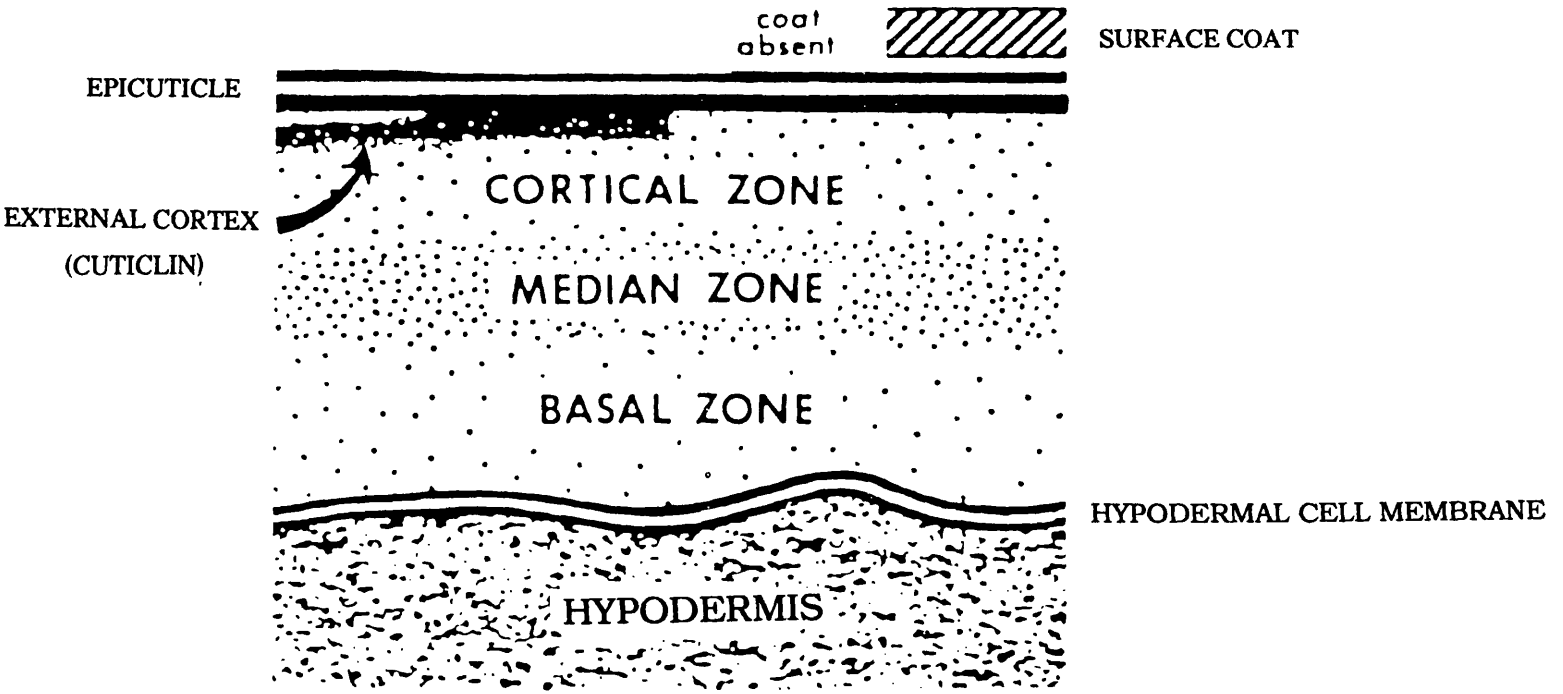
The **median zone** is homogenous and appears devoid of structure but is sometimes traversed by thin columns running between the cortical and basal layers. In adult *C.elegans* it has been shown to consist of fluid with "supporting struts" connecting the basal and cortical zones (Bird 1980). This zone is difficult to resolve in some nematodes and even appears to be absent in some cases such as the *C.elegans* dauer larvae (Bird 1980).

The **cortical zone** is often sub-divided into an outer and inner cortex. The outer cortex consists of the epicuticle, which will be discussed later, and an underlying area of structural proteins. At least some of this protein is linked with non-reducible covalent bonds making it a highly resistant layer (Fujimoto & Kanaya 1973 and Betschart & Jenkins 1987). The inner cortex is thought to be more complex and although it consists predominantly of structural proteins it appears to be metabolically active since both enzymes and RNA have been detected within it (Anya 1966).

**Biochemical composition and structure of the cuticle.**

The biochemical nature of the different zones of the nematode cuticle has been revealed by experiments involving extrinsic iodination of live parasites followed by solubilisation of the labelled molecules using a series of treatments. These have been conducted on numerous nematode species with broadly similar results which are well illustrated by the work performed on *B.malayi* (reviewed by Selkirk 1991). Homogenisation of adult worms in aqueous or detergent buffers, eg. 1% SDS, leads to the solubilisation of a limited number of proteins some of which have been subsequently localised to the epicuticle or outer cortical zone. The general architecture of the cuticle remains intact during this procedure.

# FIGURE 1.2


coat
absent
SURFACE COAT
EPICUTICLE
CORTICAL ZONE
EXTERNAL CORTEX
(CUTICLIN)
MEDIAN ZONE
BASAL ZONE
HYPODERMAL CELL MEMBRANE
HYPODERMIS

Homogenisation and boiling of these cuticles in the presence of 2-mercaptethanol results in the dissolution of the basal, median and inner cortical layers leaving the outer cortex and epicuticle intact. This treatment generally leads to the release of numerous proteins which are mainly over 50kDa and are hydrolysed by collagenase from *Clostridium histolyticum*. This suggests that the main structural components of the basal, median and inner cortical layers are collagenous proteins cross-linked by disulphide bonds. The outer cortex and epicuticle is highly insoluble and is not dissolved even by more stringent treatments, eg. 8M urea, 1%SDS, 88% formic acid and 0.1% sodium hydroxide. This insolubility of the outer cortex and epicuticle has been reported for numerous nematode species including *B.malayi* (Selkirk et al 1989), *Ascaris lubricoides* (McBride & Harrington 1967 and Fujimoto & Kanaya 1973), *C.elegans* (Cox et al 1981a), *Acanthocheilonema vitae* (Betschart et al 1985 and Betschart & Jenkins 1987), *N.americanus* (Pritchard et al 1988) and *H.contortus* (Fetterer 1989).

Therefore the major constituents of the nematode cuticle can be divided into broad three categories:

(1) The cuticular collagens which are the predominant components of the basal, median and inner cortical layers.

(2) The insoluble proteins of the outer cortex and epicuticle which are cross-linked by non-reducible bonds.

(3) The molecules of the nematode surface which may be located in the epicuticle or outer surface coat and may include soluble proteins, lipids and glycoconjugates.

### 1.2.2.2. The Cuticular Collagens.

Collagens are generally the most abundant component of the nematode cuticle and have been estimated to account for up to 80% of cuticular proteins (Kingston 1991). However work on *C. elegans* has shown that their abundance in the cuticle is stage specific with non-collagenous proteins contributing the larger component in the $L_1$ and dauer larval stages (Cox 1990). Dissolution of the basal, median and inner cortical zones in 2-mercaptethanol with the associated release of collagenase sensitive molecules suggests the cuticular collagens are predominantly located in these regions. Antibodies raised against 2ME soluble cuticular proteins do not bind to the surface of intact parasites on IFA or immunogold electron microscopic

studies (Pritchard et al 1988b). Also digestion of iodinated live worms with clostridial collagenase does not lead to the release of any cleavage products (Selkirk et al 1989). Maizels et al (1989) also found that tryptic digestion of live adult *B.malayi* did not digest collagenous proteins but did cleave the 29 kDa detergent soluble glycoprotein. These results all suggest that collagens are not exposed on the surface of the nematode cuticle.

The collagens and collagen genes of *C.elegans* are the best characterised of all the nematodes and these show a number of fundamental differences to their vertebrate counterparts. The total number of collagen genes in *C.elegans* is very large and has been estimated at between 50 and 200 (Cox et al 1984) compared with an estimated minimum of 20 for the vertebrate collagen gene family (Miller & Gay 1987). The reason for this large number of genes is not fully understood but may reflect the diversity of architectural features of the cuticle of the different developmental stages (Kingston 1991). Hybridization with specific probes indicates that their expression is developmentally regulated (Kramer et al 1985) and Cox and Hirsh (1985) suggested that the differences in cuticular morphology, ultrastructure and collagen composition between different nematode stages arise as a result of differential collagen gene expression. An alternative explanation for the large collagen gene number was proposed by Hirsh et al (1985) who suggested that it may reflect the need to produce large amounts of cuticle collagens in a short period of time, ie. at moulting.

Another surprising finding in *C.elegans* was that the mature cuticular collagens, which range in molecular weight from 60-110 kDa, are not primary translation products but are thought to be formed by covalent cross-linkage of numerous small precursors (Kingston 1991). The evidence for this includes the observation that the size range of collagenase sensitive *in vitro* translation products is much smaller than that of the mature collagens (Politz & Edgar 1984 and Cox 1990). Also antibody to 2ME soluble cuticular proteins bound to 38-52 kDa proteins in worms at the start of a lethargus but to molecules of 60-210 kDa (a size range analogous to mature collagens) at the end of the moult (Politz et al 1986). Work on *A.suum* (Kingston & Pettitt 1990) and *H.contortus* (Shamansky et al 1989) suggests that a similar process of collagen assembly occurs in these parasitic species, however this may not be universally true for all nematodes. Collagenase sensitive *in vitro* translation products of *Panagrellus silusiae* (Leushner et al 1979) and *B.malayi* and *Brugia pahangi* (Selkirk et al 1989) are of a similar size to the mature cuticular proteins.

The nucleic acid sequence of a number of collagen genes has been determined for *C.elegans* (Kramer et al 1982 and Cox 1989), *A.suum* (Kingston 1991)

and *H.contortus* (Shamansky 1989) and these show several common features which are quite distinct from the vertebrate collagen genes. They code for comparatively small molecules (30 - 40 kDa), have only 1 or 2 introns and have much shorter stretches of sequence coding for the Gly-X-Y repeating structure which are interrupted by stretches of non-repeating sequence coding for up to 18 amino acids. Another significant difference is the utilisation of disulphide bonds for cross-linking as opposed to vertebrate fibrillar collagens which are predominantly cross-linked by aldehyde formation from lysine or hydroxylysine side chains (Eyre et al 1984). Workers have repeatedly failed to find such cross-linking in nematode collagens (Bailey 1971, Fujimoto et al 1981 and Ouzana et al 1984). However the lack of dissociation of some of the larger *A.suum* collagen molecules into the 30-40 kDa monomers under reducing conditions and the isolation of isotrityrosine from *Ascaris* cuticle collagen has led to the suggestion that some non-reducible cross-links involving these residues may occur (Betschart & Wyss 1990).

Cuticular collagens are inaccessible to the host immune system in intact worms as explained above. However collagen specific antibodies are produced in a number of chronic nematode infections eg. *B.malayi* infection in man (Selkirk et al 1989) and *H.contortus* infection in sheep (Boisvenue et al 1991). This is not unexpected since the host will be exposed to these proteins following moulting, immune mediated damage and natural death of parasites. However Selkirk (1991) reported that antibody binding to collagens in *B.malayi* infection serum is weak compared to that of non-collagenous cuticular proteins and Pritchard et al (1988a) reported that human post-infection sera did not recognise the cuticular collagens of *N.americanus*. It therefore seems unlikely that antibodies to cuticular collagens play an important role in the protective immune response to these nematode parasites. This has been supported by immunisation experiments of sheep with cuticular collagen proteins and a synthetic 18 amino acid cuticular collagen peptide of *H.contortus* which produced no significant resistance to challenge (Boisvenue et al (1991). However Pritchard et al (1988b) suggested that a successful nematode vaccine might include both surface antigens and cuticular collagens. The rationale for this being that the immune response to surface antigens might expose the underlying collagen molecules to a second wave of immune attack causing further, and perhaps more crucial, damage to the cuticle. The collagens would also be exposed to other host effector mechanisms such as mast cell proteases which have been shown to have collagenase activity (McKean & Pritchard 1989). The hypothesis that the stimulation of an immune response against cryptic cuticular molecules could contribute to parasite killing is given some credence if one considers the work of Gibson et al (1976). They suggested that the action of the anthelmintic drug diethylcarbamizine

(DEC) on *Onchocerca volvulus* microfilaria was due to the release of cuticular components rendering the parasites susceptible to immune attack. The role of the immune system in this phenomenon was confirmed by the demonstration that DEC fails to reduce microfilaraemia in *B.malayi* infections if the host lacks antibodies to the parasite (Piessens & Beldekas 1979). Similarly praziquantel increases the immunogenicity of schistosomes by exposing hidden tegumental epitopes (Harnett 1988). The validity of this principle is further supported by the ability of paramyosin to protectively immunise mice in spite of being located within the schistosome tegument and not being exposed on the parasite surface (Lanar et al 1986).

### 1.2.2.3. The Outer Cortex and Epicuticle.

**Insoluble protein of the outer cortex and epicuticle.**

This zone consists primarily of non-collagenous protein which does not dissolve following treatment with detergents and reducing agents. This insolubility has made the analysis of this material difficult and consequently relatively little structural or chemical information is available. Insolubility of the outer cortex and epicuticle has been reported in most of the nematodes which have been examined; *B.malayi* (Selkirk et al 1989), *A.lubricoides* (McBride & Harrington 1967 and Fujimoto & Kanaya 1973), *C.elegans* (Cox et al 1981), *Acanthocheilonema vitae* (Betschart et al 1985 and Betschart & Jenkins 1987), *N.americanus* (Pritchard et al 1988a) and *H.contortus* (Fetterer 1989).

Fujimoto & Kanaya (1973) isolated this insoluble material from the cuticle of *Ascaris lubricoides* and called it "cuticlin". Its amino acid content was broadly similar to the collagens but had a lower content of glycine (15% compared to 27%) and did not contain hydroxyproline. It was insensitive to bacterial collagenase treatment and had a X-ray diffraction pattern which was distinct from collagen. Fetterer et al (1990) reported stage specific differences in its amino acid composition in *A.suum*. Also it has been shown to be partially sensitive to a number of proteolytic enzymes such as elastase in a variety of nematodes including *A.suum* (Fetterer et al 1990), *C.elegans* (Cox et al 1981a) and *B.malayi* (Selkirk 1991) which may suggest that there are similarities in the biochemical composition of the outer cortex and epicuticle in a range of nematodes.

The current lack of knowledge about the nature of the insoluble proteins of the outer cortex and epicuticle means that the identity of the predominant superficial cuticular proteins of nematode parasites remains to be defined. These molecules are potentially of considerable importance to the host protective immune

response however little is known about their possible exposure on the surface. A number of workers have raised antiserum to the insoluble fraction of the cuticle and examined the cuticular binding by immunofluorescence and immuno-gold electron microscopy. Such antibodies have not been found to bind to the surface of intact worms in *A.viteae* (Betschart et al 1985 and Keifer et al 1986) and in *B.pahangi* (Devaney et al 1987). Betschart et al (1987 & 1990) reported that antibodies raised to the insoluble material from *Ascaris* cuticles bound to the epicuticle of *A.suum*, *A.viteae* and *B.pahangi* but not to the surface of living parasites.

Two distinct cuticlin genes, *cut-1* and *cut-2*, have recently been cloned from *C.elegans* (reviewed by Politz and Philipp 1992) and these may help to shed some light on the structure of the protein as well as enable the cloning of similar genes from parasitic nematodes.

**Soluble proteins of the outer cortex and epicuticle.**

As well as the insoluble protein matrix of the outer cortex and epicuticle there are also soluble proteins which, being more amenable to analysis, have been subjected to much greater investigation. These have been generally analysed by the labelling of live nematodes with $^{125}I$ followed by incubation or homogenisation in aqueous buffers or detergents which usually identifies a restricted set of polypeptides (Phillip & Rumjaneck 1984). The use of "surface-labelling" techniques to investigate the nematode surface will be discussed in greater detail elsewhere but it is important to emphasize here that none of these methods are truly surface specific. However, labelling is often confined to the cuticle with no labelling of underlying somatic structures and a number of molecules identified by these techniques have been localised to the superficial layers or surface of the cuticle by immunocytochemical techniques (Devaney et al 1990, Selkirk et al 1990).

The soluble "surface proteins" of many parasitic nematodes have been labelled and sized but few have been characterised in any great detail. The molecule for which there is most information is gp29 (sized at 30 kDa by some workers) which is the predominant "surface labelled" protein of the adult worms of *B.malayi* and *B.pahangi* and has been shown to be an homologous molecule in the two species by peptide mapping (Selkirk et al 1990). The size of the major labelled molecule in other adult filarial nematodes such as *Brugia timori*, *Wuchereria bancrofti* and *Loa loa* is also 29kDa and these molecules are immunologically cross reactive between the species (Maizels et al 1985 & 1987 and Selkirk et al 1990). Gp29 is expressed in the $L_4$ and adult stages but not the $L_3$ of *B.Malayi* (Selkirk 1990) and is expressed in the post-infection $L_3$, $L_4$ and adult but not the pre-infection $L_3$ of *B.pahangi* (Devaney 1991). Selkirk (1992) showed that in *B.malayi* the molecule was synthesized from a 32 kDa

precursor, secreted into culture medium within 5 hours of synthesis and on extended culture was converted to a 56 kDa product. *In vitro* secretion of the molecule has also been reported in *B.pahangi* but without any increase in molecular weight of the secreted molecule (Devaney 1988). Immunogold EM studies on both species suggest that the molecule is produced in the hypodermis and is present throughout the cuticle particularly in the outer cortical layer. However it does not seem to be present in the epicuticle or to be exposed on the cuticular surface (Selkirk 1991 and Devaney 1991). The molecule is glycosylated in both species by two N-linked carbohydrate side chains each of 1.5 to 2.0 kDa and there is no evidence of O-linked oligosaccharides (Selkirk et al 1990 and Maizels et al 1991). The anchorage of the molecule is not well understood but it is released into aqueous buffer by homogenisation of worms in the absence of detergents, it partitions entirely in Triton X-114 (therefore has only a hydrophilic and no hydrophobic form) and it cannot be released from live parasites by treatment with phospholipase C (Selkirk 1991). These results all suggest that the molecule is not anchored by a phospholipid membrane anchor which is perhaps not particularly surprising considering the immunogold EM localisation studies. The gp 29 molecule has been found to be highly immunogenic in most filarial infections although the significance of this response is not well understood and detailed B or T cell epitope mapping has not yet been performed (Devaney 1991).

The gene encoding gp29 has been cloned from *B.malayi* and the derived amino sequence suggests it is a homolog of the antioxidant enzyme glutathione peroxidase (Cookson et al 1992). The authors have suggested two possible roles for this enzyme which appears to be synthesized in the hypodermis, exported to the cuticle and released into the external environment. It could inhibit the oxidative burst of leukocytes and neutralize the secondary products of lipid peroxidation which would help to explain the marked resistance of these parasites to the host immune response. Alternatively, a peroxidase enzyme could catalyse the formation of non-reducible cross-linking tyrosine based residues which have been identified in both cuticular collagens and cuticlin.

**Lipids of the epicuticle.**

There are several pieces of evidence to support the presence of epicuticular lipids. Bird (1957) noted that Sudan-Black-stainable lipid could be extracted from *Ascaris* by their immersion in ether. Also Proudfoot et al (1991) reported that radio-iodination of live nematodes, using the Iodogen method, led to the iodination of double bonds in unsaturated lipids and Scott et al (1988) isolated a labelled glycolipid from the surface of adult *Dirofilaria immitis* following iodination of living parasites. Indirect evidence comes from the observation that fluorescent lipid

probes can be inserted into the epicuticle (Kennedy et al 1987a) which provides strong evidence for the lipophilic nature of the epicuticle, although the insertion of different probes was highly selective depending on their acyl chain length and physicochemical properties.

**Is the epicuticle a membrane ?**

The nature of the epicuticle is poorly understood and up to relatively recently it was considered to be a modified cell membrane. This was first suggested by Bird (1957) following the observation that Sudan-stainable lipid could be extracted from *Ascaris* by immersion in ether. This view was supported by electron microscopic studies which resolved the epicuticle as a trilaminate structure similar in appearance to the eukaryotic cell membrane (Lee 1965). Further support came from early ultrastructural studies on the moulting of nematodes which led to the view that cuticle synthesis was an intra-hypodermal cell phenomenon and the epicuticle was derived from the separated hypodermal membrane of the previous stage (Bonner 1970 & Lee 1970). However later work led Bonner & Wernstein (1972) to conclude that the hypodermal membrane remained intact during cuticle synthesis and that the cuticle (and therefore the epicuticle) was a truly extracellular product and this view has been supported by many subsequent studies (Lumsden 1975, Martinez-Palomo 1978 and Bird 1980). This concept was taken a step further by Locke (1982) who suggested that the epicuticle was really an envelope structure. He stated that "some cells in almost all groups from bacteria to vertebrates have the ability to secrete an envelope directly through or above their plasma membranes, and envelopes are not plasma membranes". He suggested that in this case the epicuticle was an envelope and formed a barrier to the environment that allowed the hypodermis to control the intervening space, ie.the cuticle. More recently, Proudfoot et al (1991) suggested that an analogy could be drawn between the epicuticle and the outer bacterial membrane of the *Enterobacteriaceae*, the major known functions of which are primarily physical whilst the bacterial cytoplasmic membrane is responsible for metabolic functions. This lack of clarity as to whether the epicuticle is a modified cell membrane or more analogous to a secreted envelope has led to a lack of consistency in the terminology used in the literature. This is important since to envisage the epicuticle as a cell membrane can lead to a range of invalid assumptions about its structure, composition and function as well to the inappropriate application and interpretation of analytical techniques.

It is clear from the above discussion that the epicuticle is extracellular and therefore not a true cell membrane, however this does not preclude it from being structurally and functionally analogous. There is not a clear consensus on what

precisely constitutes a cell membrane but the following simple definition is useful. "Membranes are lipid bilayers containing proteins and glycoproteins, forming a structure capable of controlling the internal cellular environment by selective interactions with the external environment" (Proudfoot et al 1991). Therefore an important question is whether the nematode epicuticle performs such selective interactions. This selectivity is achieved in eukaryotic cell membranes largely by integral membrane proteins and it is clear that both soluble proteins and glycoproteins are associated with the surface of many nematodes (reviewed by Maizels and Selkirk 1988). However the functions of these molecules are not known and their precise relationship to the epicuticle is not well understood. It is becoming increasingly apparent that many of these molecules actually reside outside, and separate from, the epicuticle in a "surface coat" (Blaxter et al 1992). Furthermore it has been shown by Devaney (1988) in *B.pahangi* and Scott et al (1988) in *Dirofilaria immitis* that the major surface associated proteins are extracted in buffer without the aid of detergents. This would suggest that their relationship to the surface of the nematode is very different to those of conventional integral membrane proteins. Also the detection of integral membrane proteins by freeze-fracture electron microscopy of nematodes has produced very inconsistent results. They have been reported as absent from the epicuticle of *T. spiralis* (Lee et al 1984 & 1986) and *O.volvulus* (Martinez-Palomo 1978) but present in *N.brasiliensis* (Lee & Bonner 1982). Howells et al (1983) suggested that the penetration of the nematode cuticle by many polar compounds was evidence that it was not an uninterrupted lipid bilayer, but was punctuated by components involved in nutrient translocation. However Howells (1987) later suggested that nutrient transport and homoeostatic control were functions of the hypodermal cell membrane and not the epicuticle.

Therefore it seems that the surface proteins of the nematode cuticle are not integral membrane proteins and there is little evidence of the epicuticle performing the transport and homoeostatic functions of a true cell membrane.

Recently biophysical studies have been performed on the nematode epicuticle using fluorescent lipid probes and these have revealed some unusual properties with regard to lateral mobility (Kennedy et al 1987a). The technique involves the measurement of the fluorescence recovery after photobleaching (FRAP) which has been used extensively to study the lateral diffusion of eukaryotic cell membranes (reviewed by Wolf 1988). A large non-diffusing fraction of lipid was detected on the surface of many adult parasitic nematodes including *B.pahangi*, *O.ostertagi*, *T.spiralis* and *A.viteae* using the anionic lipid probe AF18 (Kennedy 1987a). However for adult *L.carinii*, the inserted lipid probe AF18 showed small patches of mobilbilty which were distributed in an apparently random pattern

(Kennedy 1987b). In contrast to the results with parasitic nematodes, this probe showed unrestricted lateral diffusion over the whole surface of of the free-living nematode *C.elegans*. Whether the immobility of epicuticular lipid confers any survival advantage to parasitic nematodes within the host is as yet unclear. In spite of the immobility of the AF18 probe, a non-polar lipid probe NBD-chol detects mobile lipid in the same nematode species (Kennedy et al 1987b). This has led to the suggestion that the epicuticular lipid consists of discrete domains of differing composition and properties (Kennedy et al 1987b and Proudfoot et al 1991).

Therefore it is clear that the structure and behaviour of the nematode epicuticle is markedly different to that of a cell membrane.

### 1.2.2.4. The Nematode Surface

The original concept of the nematode cuticle simply being an inert protective covering (Sprent 1959) has radically changed in recent years. It has been shown that there is a rapid turnover of surface molecules in a variety of nematode species including *A.caninum* (Vetter & Klaver-Wesseling 1978), *T.canis* (Smith et al 1981a), *T.spiralis* (Phillip et al 1980), *S.ratti* (Murrell & Graham 1983a), *C.elegans* (Politz & Phillip 1992) and *D.immitis* (Ibrahim 1989). Also changes in the surface protein composition during the inter-moult period has been demonstrated in *T.spiralis* new born larvae (Jungary, Clark & Parkhouse 1983), *N.brasiliensis* infective larvae (Maizels et al 1983a) and *B.malayi* microfilaria (Furham et al 1987) and infective larvae (Carlow et al 1987). More recently Proudfoot et al (1990) described rapid changes in the epicuticular lipids of a number of species of infective larvae following entry into the definitive host. It can therefore be seen that the nematode surface is not merely an inert extracellular covering but a very dynamic structure.

The precise nature and organisation of molecules which are truly at the nematode surface is poorly understood. Lipids (Kennedy et al 1987a), carbohydrates (Kennedy et al 1987b) and proteins (Selkirk et al 1990) have all been associated with the cuticular surface and it has become clear that there is a large degree of diversity between different nematode species and even different stages of the same species.

The view of the epicuticle being the outermost layer of the cuticle is being revised for an increasing number of nematode species (Blaxter et al 1992). Electron microscopic studies have revealed an electron-dense layer outside the epicuticle in a variety of nematodes. These include free-living nematodes such as *C.elegans* (Politz and Philipp 1992) and *Caenorhabditis briggsae* (Himmelhoch and Zuckerman 1978), plant parasitic nematodes such as *Anguina funesta* and *Anguina*

*tritica* (McClure & Speigel 1991), parasitic nematodes of domesticated animals such as *T.canis* (Maizels & Page 1990), *D.immitis* (Cherian et al 1980), *T.spiralis* (Lee et al 1986) and *S.ratti* (Grove et al 1987) and human nematode parasites such as *N.americanus* (Pritchard et al 1988a) and *O.volvulus* (Lustigman et al 1990). It is likely that many of the putative surface antigens being investigated are associated with this structure rather than with the epicuticle itself. This is an attractive hypothesis since it is difficult to explain the dynamic properties of the nematode surface using the classical model of the epicuticle being an insoluble, highly cross-linked protein matrix bounded by a lipid "membrane".

The surface coat varies in thickness between 5 and 20 nm for different species and so can only be seen at the electron microscopic level, but its visualisation is very dependant upon the fixation and staining procedures used (Blaxter et al 1991). In many cases it can be easily seen by using osmium tetroxide staining prior to alcohol dehydration and embedding as with *O.volvulus* (Lustigman et al 1990) and *T.spiralis* (Lee et al 1986). In other cases it requires more specialised procedures in order to be clearly visible such as treatment with cationised ferritin as with *C.elegans* (Himmelhoch and Zuckermann 1983) or ruthidium red as with *D.immitis* (Cherian 1980). In *T.canis* the surface coat appears to be poorly preserved by ethanol based dehydration procedures used for immuno-gold electron microscopy fixation. This was clearly demonstrated by Maizels and Page (1990) who reported that two monoclonal antibodies which reacted strongly on surface IFA did not appear to bind to the cuticular surface using routine immunogold EM procedures employing ethanol dehydration. However when cryostat sections were used for immunogold EM studies these antibodies were clearly demonstrated to bind to the surface coat (Page et al 1992b). It can therefore be seen that detection of a surface coat is very dependant upon the methodology used and so it is possible that such a structure is present, but has escaped detection, in many more nematode species.

The nematodes are conventionally placed into two major classes: the Adenophorea and the Secernentea. Blaxter et al (1992) suggested that many of the properties of the surface coats of the secernetean nematodes (eg *Caenorhabditis* and *Toxocara*) are broadly similar whereas those of the adenophoran nematodes (eg.*Trichinella*) are somewhat different. In order to discuss the surface coat in more detail *T.canis* will be used as an example of the former type and *T.spiralis* as an example of the latter type since these are the nematodes for which most information is available. Similarities and differences to other nematodes will be highlighted during the discussion.

**The surface coat of *Toxocara canis*.**

Antigens on the surface of *T.canis* $L_2$ have been investigated both by "surface-specific" iodination and by generating monoclonal antibodies to surface epitopes. Radioiodination and SDS-PAGE analysis identified a restricted set of molecules with two components at 32 and 120 kDa being the most prominent and a further two molecules at 55 and 70 kDa being the most consistently labelled of the less abundant constituents (Maizels et al 1984). Radioiodination of $L_2$ E/S products labelled bands at exactly the same molecular weights as the surface molecules as well as a 400 kDa band (Maizels et al 1984). This commonality between the surface and E/S molecules of *T.canis* $L_2$ has been verified by studies in which monoclonal antibodies specific for E/S molecules have been shown to bind to the surface of live larvae and immunoprecipitate surface-iodinated antigens (Bowman et al 1987 and Maizels et al 1987b). It is interesting to note that the monoclonal antibodies do not bind to the surface of the $L_2$ until 24 hours of *in vitro* culture after hatching (Maizels et al 1987b). Two of the monoclonal antibodies (Tcn-2 and Tcn-8) which bind to the $L_2$ surface on IFA and to the surface coat on cryostat immuno-gold EM sections recognised the 55, 70 and 120 kDa surface molecules. The immunogold studies also showed that they bind to the mouth parts and oesophageal passage but not to the deeper layers of the cuticle (Maizels & Page 1990 and Page et al 1992a). Based on these results the authors suggested that molecules produced by the oesophageal glands are secreted and adhere over the surface of the larvae to form the surface coat. Interestingly, another monoclonal antibody (Tcn-3) recognised the 32 kDa molecule but did not bind to the surface of larvae on IFA. However it did bind to the surface of the epicuticle on immuno-gold EM indicating it is a true epicuticular molecule which is rendered cryptic by the presence of the surface coat (Maizels & Page 1990).

Another interesting feature of the *T.canis* $L_2$ surface coat is its rapid turnover. Smith et al (1981) noted that larvae maintained *in vitro* at 37°C did not bind antiserum raised against their E/S products. However if larvae were metabolically inhibited by being maintained at 2°C or preincubated at 37°C in the presence of metabolic inhibitors, such as 8mM sodium azide, the same sera produced intense fluorescence over the whole larval surface. This fluorescence was maintained whilst the larvae remained metabolically inhibited but if the temperature was returned to 37°C, or the sodium azide washed away, globules of fluorescence could be seen to lift away from the larval surface into the surrounding media. After 3 hours the larval surfaces were completely negative for fluorescence. Therefore it appears that *T.canis* $L_2$ shed their surface molecules and this is a metabolically active process. Maizels et al (1984) confirmed this active shedding of surface antigens by demonstrating that "surface" iodinated proteins are rapidly released into the surrounding medium in a

temperature dependant manner. Similar surface antigen shedding has also been demonstrated in a number of other nematodes including *A.caninum* (Vetter and Klaver-wesseling 1978), *T.spiralis* (Phillip et al 1980) and *D.immitis* (Ibrahim et al 1989). This phenomenon has clear implications for the evasion of host immune effector mechanisms which is discussed in detail below.

Some information is available on the biochemical nature of the surface coat of the *T.canis* $L_2$. The binding of cationised ferritin to the surface suggests a net negative charge and this is true of practically all the nematode surface coats (except for *T.spiralis*). Himmelhoch and Zuckerman (1983) observed that this binding of ferritin to the surface of *C.elegans* was independent of pH which, they suggested, indicated the presence of sulphated sugars. The binding of ruthidium red is again a feature common to most of the nematode surface coats and indicates the presence of acid mucopolysaccharides or substituted proteoglycan macromolecules (Luft 1971). The surface coat of *T.canis* $L_2$ along with a number of other species such as *N.americanus* (Pritchard et al 1988a) and *A.funesta* (McClure & Speigel 1991) is dissolved by cationic detergents such as CTAB but not by anionic detergents which may be due to the strong surface charge. However this is not true in all cases, eg.the surface coat of *S.ratti* is resistant to solubilisation with detergents, proteases, acids and organic solvents (Murrell and Graham 1983b) and so is very different to the labile surface coat of *T.canis* larvae.

Six of the eight monoclonal antibodies raised against *T.canis* $L_2$ E/S products (and which also immunoprecipitate surface iodinated proteins) (Maizels et al 1987b and Kennedy et al 1987c) recognise periodate sensitive carbohydrate epitopes. This evidence together with the staining of the E/S products with carbohydrate specific dyes and lectins demonstrates that the molecules common to the E/S and surface coat are heavily glycosylated (Menghi & Maizels 1986). Gas chromatographic analysis of the E/S products showed a 40% carbohydrate content with a predominance of carbohydrates such as N-acetylgalactosamine and galactose which are generally O-linked (Menghi & Maizels 1986 and Khoo et al 1991). These analyses also showed that the dominant structure in the E/S shared features with mucins which has led to the suggestion that this surface coat may be a lubricating and protective layer (Blaxter 1992).

**The accessory layer of *Trichinella spiralis*.**

The infective larvae ($L_1$) of *T.spiralis* has a surface coat outside the epicuticle which does not exist in the other stages (Lee et al 1986). This has been called the accessory layer and is markedly different to the various forms of surface coat described above. It was first described by Purkerson & Despommier (1974) as 2

dense lines on the surface of muscle larvae visible by electron microscopic examination of thin sections preserved with an osmium/dichromate fixative. In further studies Lee et al (1984) noted that glutaraldehyde/osmium fixation did not always uniformly preserve the structure and it most frequently appeared as a uniformly dense line of about 15nm thickness but could sometimes be resolved into a trilaminate pattern of 2 dense lines. Freeze fracture studies have shown the accessory layer to consist of an outer globular layer overlying a filamentous layer (Lee et al 1984 and 1986) suggesting it to be a more substantial and complex structure than the surface coats found in secernenean nematodes.

The work of Wright & Hong (1988) has shown that there are also substantial differences in the biochemical properties of the accessory layer compared to the surface coats of other nematode species. The surface of *T.spiralis* $L_1$ does not bind cationised ferritin and so may not be negatively charged. Carbohydrates could not be demonstrated in this layer by staining techniques and electron microscopy or by lectin binding studies (Ortega-Pierres 1984). The staining of the accessory layer by ruthidium red may be due to the presence of phospholipids rather than mucopolysaccharides (see below). Incubation of larvae in SDS (1 to 10%) or CTAB (0.25 to 10%) solubilised the outer globular layer but left the inner filamentous layer intact. Exposure of larvae to chloroform/methanol even for a few seconds led to solubilisation of both the globular and filamentous layers, and analysis of such solubilised material demonstrated the presence of a variety of lipids and phospholipids which were absent from the SDS and CTAB extracts. These results suggested that the accessory layer consists of an inner filamentous layer of lipid and the outer globular layer of protein. Wright & Hong (1987) went on to speculate on the possible details of such a structure. The predominant lipids extracted by the flash treatment with chloroform/methanol were ethanolamine phospholipids and these have been shown to form tubular micelles rather than the bilayers formed by choline phospholipids in cell membranes (DeKrujiff 1981). This would explain the filamentous appearance of the inner layer on freeze-fracture EM. Furthermore these micelles tend to form with the polar groups of the phospholipids inwards forming a hydrophilic core and a hydrophobic outer surface. Thus proteins could be anchored into these micelles by a hydrophobic portion of the molecule penetrating between the acyl chains of the phospholipids with a terminal hydrophilic moiety in the miceller core. A 47kDa surface protein has been demonstrated to have a lectin binding site, presumably carbohydrate, which is not accessible on the surface of intact larvae (Parkhouse et al 1981 and Ortega-Pierres 1984). It is possible that such a group is unavailable to lectins in intact larvae because it is buried in the hydrophilic core of the lipid micelles.

Obviously such a structure is highly speculative but it makes a useful model on which to base further investigation.

The function of the accessory layer is still unknown. It has been suggested that it may be primarily associated with the larvae's existence in the intracellular environment within the nurse cell (Stewart et al 1987), eg. involved in the transport of nutrients in or waste products out of the parasite. Alternatively it could simply be a protective barrier to enable the larvae to survive the environment of the host's stomach during infection (Wright & Hong 1989). The proteins on the surface of muscle larvae are resistant to proteases and the accessory layer is not disrupted by a variety of protease treatments including pepsin, chymoptrypsin and papain (Despommier 1983). However Stewart et al (1989) reported that it was degraded by *in vitro* trypsin and/or bile treatment of larvae which have been recovered from muscle with pepsin/HCL. This treatment also caused a change in motility from a coiling/uncoiling motion to a vigorous sinusoidal motion and also caused an increase in glucose uptake. These authors considered that exposure of larvae to conditions which mimic the host's enteric environment led to a loss of the accessory layer which might be a trigger to further development. However these changes have not been observed *in vivo* and Capo et al (1984) reported that following oral infection of mice, the surface of the $L_1$ remained positive for the surface specific monoclonal antibody 8A4 (Silberston & Despommier 1984) until after the second moult which occurred after penetration of the enteric epithelium. Also Wright & Hong (1989) found that 90% of $L_1$ had penetrated the enteric epithelium within 30 minutes of oral infection of mice and that the accessory layer was still intact. Therefore it may be that, in spite of the *in vitro* studies, the accessory layer is not degraded by exposure to the hosts enteric environment and is not a prerequisite for further development.

There has been little work on the synthesis of the accessory layer but the localisation of antigens recognised by monoclonal antibodies using immunocytological techniques has produced some interesting results. Silberstein & Despommier (1984) reported that a monoclonal antibody recognising a 48 kDa antigen (8A4.3.1.1) bound to the beta stichocytes, the gut lining and the cuticular surface and another antibody, recognising a 50/55 kDa antigen (7B2.3.1.1), bound to the alpha stichocytes, gut lining but only occasionally to the cuticular surface. Both these antigens represented significant components of parasite secretions (12% & 5% respectively). It has long been thought that the stichosome has a secretory role (Chitwood & Chitwood 1950 and Bruce 1970) and since the stichocytes are in direct communication with the oesophageal lumen through duct-like connections, secreted material could be passed to the exterior of the parasite via this route. Similar results were reported by McLaren et al (1987) who described that 2 monoclonal antibodies

which both bound to the surface of muscle larvae also bound to the stichosome and the gut lining of sectioned larvae. McLaren et al (1987) suggested it was conceivable that at least some of the components of the accessory layer associate with the nematode surface following secretion from the stichocytes via the oesophagus, particularly as secreted products may be relatively spatially confined around the larvae in the nurse cell. This has a striking similarity to the source of the *T.canis* surface coat proposed by Maizels & Page (1990). This hypothesis for *T. spiralis* may be supported by the report of Kehayov et al (1991) that a monoclonal antibody which recognised a 76 kDa excretory/secretory muscle larval antigen also bound to the stichosome and the cuticular surface. However the binding to the cuticular surface was not uniform but mainly located over the area of cuticle nearest the stichosome. These authors considered that it was more likely that the surface antigens were synthesized by the hypodermal cells and contained epitopes common to the stichosome and E/S antigens. More work to clarify the relationship between the molecules of the stichosome, excretory/secretory products and the accessory layer would help to resolve this debate. Interestingly Roach et al (1991) have described monoclonal antibodies which bind to the stichocytes, gut lining and the anterior cuticular surface of *Trichuris muris* adult worms. However there was also some cross-reaction with the egg lipid layer and pseudocoelemic fluid and so again these authors considered the result could either indicate that the stichocytes may be a source of surface antigens or that cross-reactive epitopes were present.

### 1.2.2.5. Immunological relevance of the cuticle.

Many early workers proposed that the nematode cuticle was an inert structure which was not immunogenic and believed that this was the reason many nematodes could survive for long periods in the host and so produce chronic infections (Sprent 1959). However the application of immunofluorescent techniques first demonstrated that antigenic determinants were present on the surface of nematode parasites (Sadun 1963). Subsequent work using immunofluorescence, immunoprecipitation of "surface labelled" molecules, immunocytochemical and immuno-gold electron microscopic techniques have confirmed that antibody responses to cuticular and surface antigens occur in many nematode infections (reviewed by Maizels & Selkirk 1988). The antigens involved encompass the full range of cuticular components described in the previous section, including cuticular collagens (Pritchard 1988a and Selkirk 1989), the insoluble protein matrix of the epicuticle (Betschart et al 1987, Selkirk 1991 and Devaney 1991b), the soluble

proteins identified by "surface labelling" techniques (Selkirk et al 1990, Pritchard et al 1986,Kennedy 1991) and molecules of the surface coat (Smith et al 1981 & 1982 and Blaxter et al 1992).

Many *in vitro* studies have now demonstrated the killing of nematodes in the presence of antibody, complement and leucocytes (Kazura & Grove 1978, Mackenzie et al 1978,1980 and 1981 and Subrahmanyam 1978). Mackenzie et al (1978) also showed that antibody which mediates cell adherence to the parasite surface could be removed by pre-absorption with living worms. This is strong evidence that specific antibody to surface antigens plays an important role in the *in vitro* killing of nematodes.

*In vivo* evidence which directly demonstrates the ability of nematode surface antigens to stimulate a protective immune response is limited. Grencis et al (1986) reported that a significant level of protection to *T. spiralis* infection was achieved by the immunisation of mice with antigens purified by a surface specific monoclonal antibody. Also, monoclonal antibodies which bind to the surface of *T.spiralis* infective larvae have been shown to produce high levels of protection by passive immunisation of mice (Silberstein & Despommier 1984,Ortega-Pierres et al 1984) and rats (Appleton et al 1988). Wedrychowicz et al (1992) has recently reported that sheep immunised with antigens extracted with detergent from live infective larvae of *Ostertagia circumcincta* showed significant immunity to challenge (71.7%) and that antibodies to the larval surface were present in serum and bile. However the nature and location of the antigens present in these detergent extracts is yet to be defined.

Although it has been suggested that molecules from the deeper layers of the cuticle such as collagen may be important in protective immune responses (Pritchard et al 1988b) there has been little experimental work to support this. Boisvenue et al (1991) reported that immunisation of sheep with cuticular collagens and a synthetic 18 amino acid cuticular collagen peptide of *H.contortus* produced no significant resistance to challenge.

Therefore, although it is now clear that the nematode cuticle is often highly immunogenic, the role of the anti-cuticular immune response in protective immunity is poorly understood. There are also a number of properties of the nematode surface which may enable nematode parasites to evade the immune response.

**Stage Specificity of Surface Antigens.**

MacKenzie et al (1978) first demonstrated that different antigens could be expressed on the surface of different nematode stages. Absorption of infection sera with *T.spiralis* adult worms removed the antibody specific to the surface of this stage but the binding of antibody to infective larvae was unaffected. This was confirmed by

Phillip et al (1980 & 1981) when they demonstrated that radio-iodination of live parasites labelled different proteins on the different stages of *T.spiralis*. Stage specific antigens have now been identified on the surface of many parasitic nematodes including *N.brasiliensis* (Maizels et al 1983a), *N.dubius* (Pritchard et al 1984), *N.americanus* (Pritchard et al 1984), *H.contortus* (Cox et al 1989), *C.elegans* (Politz et al 1987), *D.viviparus* (Britton 1991) and *O.circumcincta* and *O.ostertagi* (Keith et al 1990). In each of these examples there seems to be almost complete stage specificity, ie.antiserum raised to the antigens of one stage does not react with the surface of another. However this is not true of all nematode species as Maizels et al (1983b) showed that the surfaces of different stages of *B.malayi*, *B.timori* and *B.pahangi* are clearly cross reactive. Nevertheless some stage specific antigens do occur in the filarial nematodes (Abraham,Grieve & Mika-Grieve 1988, Lustigman et al 1990 and Selkirk et al 1990).

Stage specificity of surface antigens could help a nematode parasite to evade the host immune response since by the time antibodies have developed against one stage, the parasite may have moulted to reveal a new set of surface antigens. However the effectiveness of this strategy, although of some benefit to the parasite, may be limited by the non-synchronous nature of nematode infections which occur in the field. It is also interesting to note that stage specific differences occur in the cuticle of free-living nematodes such as *C.elegans* (Politz et al 1987). This suggests that the origins of stage specificity lie in the particular structural and environmental requirements which are imposed on the cuticle by different developmental stages rather than being a specific adaptation to the parasitic lifestyle.

Changes in surface antigen expression can also occur within a single stage of nematode. This has been reported in *T.spiralis* newborn larvae (Jungery et al 1983), *N.brasilienis* infective larvae (Maizels et al 1983a), hatching *T.canis* larvae (Maizels et al 1987a) and *B. malayi* microfilariae (Furham et al 1987) and infective larvae (Carlow et al 1987). These changes are often associated with a change of environment such as entry into the host and so may reflect important functional changes. Politz & Philipp (1992) suggested that exposure of new surface molecules in *C.elegans* may be associated with the loss of a surface coat as opposed to being directly due to changes in surface molecule gene expression. Proudfoot et al (1990) also described changes occurring in epicuticular lipid immediately after infection in a number of nematode species.

**Species specificity of surface antigens**

Many authors have described antigenic cross-reactivity between the most disparate of nematode species and this has made the use of crude parasite extracts of limited value for immunodiagnosis (Oliver-Gonzalez & Morales 1945 and Diesfield et al 1981). However parasite surface antigens are often more species or genus specific (Philipp & Rumjaneck 1984) and so the antigen profiles produced by "surface" labelling techniques can be characteristic for a given species of nematode and immune serum may only recognise surface antigens of the homologous species or genus. This specificity may be to the parasite's advantage from an immunological point of view and has also prompted some authors to speculate on the potential use of surface antigens as targets for immunodiagnosis (Parkhouse et al 1981). However there are a number of examples where surface molecules are well conserved between nematodes belonging to a different species or even genus. Maizels et al (1983b) noted that there was serological cross-reactivity between the surface antigens of *B.malayi*, *B.pahangi* and *B.timori* and Maizels et al (1985b) showed that there was also surface cross-reactivity between *Brugia* and other filariae such as *Wuchereria* and *Onchocerca*. "Surface" labelling experiments have identified 3-5 proteins on adult worms of *B.malayi* which are of identical molecular weight to those of *B.pahangi* (Selkirk et al 1986). Furthermore the major protein labelled by "surface" iodination of adult parasites is 29kDa (sized by some authors at 30kDa) in *B.malayi*, *B.pahangi*, *B.timori*, *W.bancrofti* and *Loa loa* and peptide maps confirm that this is an homologous protein in *B.malayi* and *B.pahangi* (Selkirk et al 1990).

Serological cross-reactivity has also been shown for the surface antigens of *T.canis* and *T.cati* with both infection serum and monoclonal antibodies although one of the monoclonal antibodies examined was species specific (Kennedy et al 1987c). This cross reactivity involved both carbohydrate and peptide epitopes.

**Shedding of surface antigens**

The active shedding of surface antigens by numerous species of parasitic nematode was discussed earlier and it has been suggested that this may constitute a mechanism for evading both antibody-dependant and antibody-mediated cellular cytotoxicity *in vivo* (Smith et al 1981 and Ghafoor et al 1984). Smith (1991) also suggested that antigen shed from the *T.canis* larval surface may be responsible for the granulomatous lesions observed in the absence of larvae in visceral and ocular toxocariasis and that prodigious shedding of antigens may divert immune responses away from the larval surface towards the antigen deposits. *In vitro* studies on the effects of immune serum with guinea pig (Rockey et al 1983 and Badley et al 1987 ) or human eosinophils (Fattah et al 1986) on *T.canis* larvae have shown that the

eosinophils rapidly adhere to the larval surface and degranulate. However the larvae show little sign of damage and remain viable in the culture, sloughing off the eosinophils together with the extracellular matrix. Scanning electron microscopic studies of this process have shown eosinophils binding to a partially shed surface coat (Badley et al 1987).

Serum taken from rabbits 28-35 days after infection with *T.canis* $L_2$ could significantly reduce the rate of surface shedding and larval motility *in vitro* (Smith 1991). However serum taken earlier during infection had no such effect suggesting that there is a maturation of the immune response during infection. Robertson et al (1988) demonstrated that the pattern of epitope recognition does change during long term *T.canis* infections in rabbits which may a reflection of this maturation.

Smith (1991) has suggested that the deposition of the secreted/surface antigens of *T.canis* may play an important role in the type of pathology produced by infection. In ocular toxocariasis it may be that retinal granulomata occur as a response to stationary (or dead) larvae whereas chronic endopthalmitis may occur in response to antigens deposited over a wide area by migrating larvae. This is supported by the ability of the appropriate monoclonal antibodies to detect antigen in many ocular inflammatory lesions in the absence of larvae (Smith 1991).

Detailed work on the consequences of larval shedding has not been performed on nematodes other than *T.canis* but the work on this parasite has shown that the shedding of surface antigens can have important implications for both protective immunity and immunopathology of nematode infections.

**Host mimicry by surface antigens.**

Acquisition of host molecules and synthesis of molecules cross-reactive with host antigens has often been described as a method of immune evasion by schistosomes (reviewed by McLaren 1984). However this phenomenon has received relatively little attention for the parasitic nematodes. "Surface" iodination of *Litomosoides carinii* and *Onchocerca gibsoni* labels a complex series of proteins on the surface of *in vitro* released microfilariae whilst only one molecule, shown to be host serum albumin, is labelled on the surface of host derived microfilariae (Philipp et al 1984 and Forsyth et al 1984). Human serum albumin has also been identified on the surface of blood-derived microfilariae of *W.bancrofti* (Maizels et al 1984b).

Living *T.canis* larvae microdissected from murine brains have been shown to bind a monoclonal antibody specific for Thy 1.2 antigen which is lost within 3 hours of *in vitro* culture by surface shedding (Smith 1991). There is very little inflammatory response to larvae in the CNS in contrast to that which occurs in other

parts of the body (Burren et al 1968 and Dunsmore et al 1983). Therefore it is possible that the passive acquisition of a Thy 1.2-like brain antigen could help the larvae to evade immune-mediated attack.

Proudfoot et al (1991) have shown that *in vitro* pre-incubation of infective larvae in host serum can reduce the binding of lipid probes to the nematode surface in a number of species and this effect is not seen with delipidated serum. It is possible that if such an acquisition of host lipids occurs *in vivo* it could play a role in masking the parasite surface from the host immune system.

The production of substances resembling the blood group antigens by nematodes has been known for a long time (Oliver-Gonzalez & Torregrosa 1944, Oliver-Gonzalez 1946). The presence of A and B blood group-like substances has been demonstrated on the cuticular surface of *A.suum* larvae (Soulsby & Coombs 1959). Also polyclonal human anti-A and anti-B typing sera bind to the surface of the metabolically arrested *T.canis* $L_2$ (Smith et al 1983) and two monoclonal antibodies which bind to the larval surface (Tcn 2 and Tcn 8) agglutinate human A group erythrocytes (Smith 1991). Furthermore the pretreatment of living larvae with human anti-A sera can block the binding of anti-E/S serum to the larval surface which prompted Smith (1991) to speculate that the binding of naturally occurring isohaemagglutinins *in vivo* could block the effects of infection derived antibody.

### 1.2.3. Covert Nematode Antigens.

Covert antigens are those which are not recognised by the host immune system during normal infections but are capable of eliciting an immune response when presented to the immune system in an appropriate manner. The best example of such antigens in nematodes are the gut membrane proteins of *H.contortus*. Munn & Greenwood (1984) reported that EM studies had shown that the gut microvillar membrane of the blood ingesting stages of *H.contortus* was coated with a material of a characteristic structure which they called Contortin. They produced a Contortin-enriched preparation (CEP) by differential centrifugation of adult homogenate which, when used to immunise sheep, gave 70% protection against experimental challenge (Munn et al 1987). Munn & Greenwood (1984) also reported that *H.contortus*, along with the other strongylid gastrointestinal nematodes, possessed an endotube which could be dissected from the intestine as a complex with the microvilli. A microvillar membrane protein (H11) of 110 kDa was isolated from the endotube (Munn et al 1986) and immunisation of sheep with H11 gave levels of protection of up to 93% (Munn et al 1989).

Neither contortin or H11 are recognised in normal infections and so are covert antigens. One potential advantage of covert antigens as vaccine candidates is that, unlike naturally immunogenic molecules, they have not been exposed to selection pressure by the immune system and so may be highly conserved between individual parasites. However a disadvantage is that host immune responses to covert antigens will not be boosted by natural challenge and so repeated vaccination may be required to maintain immunity. This is not necessarily a major disadvantage, for example in the case of *H.contortus* protection by vaccination may only be required for a relatively short period until the acquisition of natural immunity has occurred.

### 1.2.4. Phosphorylcholine.

Phosphorylcholine (PC) is a hapten which has been identified in many parasites by the binding of PC specific myeloma proteins and monoclonal antibodies. Strictly speaking, the binding of the such antibodies to parasite antigens does not necessarily prove that PC, and not some similar cross-reactive epitope, is present. This will be discussed in more detail in relation to results presented later in the thesis but the literature is reviewed here with this proviso in mind. PC is an epitope which deserves particular consideration since it may be responsible for much of the immunological cross-reactivity which occurs between antigens of different nematode species.

The PC epitope is extremely ubiquitous and has been found on molecules in a wide range of living organisms from human beta-lipoprotein (Leon & Young 1971) to the bacterial capsular polysaccharides of *Streptococcus pneumoniae* (Brundish & Baddiley 1968). It is also present in many protozoal and helminth parasites (Lal & Otteson 1989) and it seems to be particularly common and abundant in nematodes (Maizels & Selkirk 1988). These include *A.suum* (Gutman & Mitchell 1977), *T. spiralis* (Ubeira et al 1987), *N.brasiliensis* (Pery et al 1974), *T.canis* (Sugane & Oshima 1983), *B.malayi* and *B.pahangi* (Maizels et al 1987c), *O.gibsoni* (Forsyth et al 1985), *W.bancrofti* (Morgan et al 1986), *S.stercoralis* (Lal & Otteson 1989) and *H.contortus* (Pery et al 1974).

In some of the nematodes listed above, particularly the filarial nematodes, PC is present in the E/S products, eg. *B.malayi, B.pahangi* (Maizels et al 1987c) and *W.bancrofti* (Morgan et al 1986). However in other nematodes such as *T.canis* it is present only on somatic and not secreted antigens (Sugane & Oshima 1983). Immunocytochemical studies using PC specific myeloma proteins and monoclonal antibodies have been performed to localise PC in nematodes. In all these

studies the PC epitope was restricted to internal parasite structures and was not exposed on the nematode surface. Examples include *A.suum* (Gutman & Mitchell 1977) in which detection of the PC epitope was confined to internal membranous structures and the lining of the intestinal tract, *N.brasiliensis* (Pery et al 1979) and *Dipetalonema viteae* (Gualzata et al 1986) in which it was localised mainly to the lining of the intestinal tract, eggs and uterus but not the cuticular surface. An anti-PC monoclonal antibody did bind to the cuticle of *T.spiralis* muscle larvae but only to the inner layers close to the hypodermis and not to the cuticular surface (Choy et al 1991).

The immune response induced in mice to PC-bearing antigens is highly restricted and the antibodies produced are predominantly of the IgM isotype and are of a limited idiotype expression (Lee, Consenza and Kohler 1974). Mitchell & Lewers (1977) demonstrated that the anti-dinitrophenyl (DNP) response of mice was inhibited when a DNP-Ficoll-PC conjugate was used as the immunogen compared with a simple DNP-Ficoll conjugate. These authors suggested that this may be due to a cytotoxic effect of PC on the B cell precursors of high affinity antibody-secreting cells because of the cell membrane-active and lytic properties of PC reported by Weltzien (1973). They went on to suggest that "if parasite antigens contain PC, tolerance may be produced in many B cells with specificity for these parasite antigens ..... and the B-cells at risk would be those of relatively high antigen-binding capacity". Therefore the presence of PC on parasite antigens may have the effect of tolerising the host's immune system to otherwise highly immunogenic epitopes. The main weakness of this theory seems to be that PC is often found at sites in the parasite which are not exposed to the host immune system and not at more immunologically exposed sites such as the cuticular surface. Nevertheless PC present on secreted antigens is at the host-parasite interface and so could potentially play a role in masking epitopes on these secreted molecules. There is no evidence to suggest that antibodies directed at the PC epitope itself are involved in protective immunity.

Another interesting aspect of PC is that it binds to C-reactive protein. This is an acute phase protein released into the circulation by the liver in response to factors released from damaged tissue as a result of trauma or acute inflammation. Sugane & Oshima (1983) demonstrated the activation of complement in C-reactive protein positive sera by a PC-bearing antigen from a *T.canis* extract. The significance of this result to the parasite *in vivo* is not clear since PC is not present on the surface or in the ES products of this parasite, although it would be presented to the immune system if destruction of parasites occurs.

The various studies on the presence of PC in different nematode species would suggest that a wide variety of molecules can bear the PC epitope. In recent

years there has been particular interest in the PC-bearing ES molecules of the filarial parasites since these can be detected in the serum of both infected animals (Forsyth et al 1984b and Maizels et al 1985a) and human patients (Dissanayake 1985 and Selkirk et al 1986) and so may be of potential use as diagnostic targets. Multiple PC-bearing antigens are present in the ES products in each of the filarial species examined and many are thought to be proteoglycans. However, only some of these are detectable in infection serum with the dominant molecule being 90kDa in the *Brugia* species (Maizels et al 1987c and Wenger et al 1987), 200 kDa in *Wuchereria* (Lal et al 1987) and 56 and 62 kDa in *Onchocerca gibsoni* (Forsyth et al 1984).

The PC epitope is repetitive on the *Brugia* proteoglycans allowing antigen capture and detection by anti-PC antibodies (Maizels et al 1987c). However the ubiquitous nature of the PC epitope makes it unsuitable for specific diagnostic use and so non-PC, species specific epitopes on these molecules would be more suitable as diagnostic targets.

## 1.3. Aims and objectives of the thesis.

The immune response to the bovine lungworm *D.viviparus* is remarkably effective which has been reflected in the success of the commercial vaccine over the last thirty years. Although similar success has not been achieved for other helminth parasites, little is known about the nature of the immune responses to *D.viviparus* and an understanding this immunity might provide insights which are relevant to other parasitic nematodes for which immunoprophylaxis has been less successful.

In order to investigate host immune responses, a knowledge of the relevant parasite antigens is necessary. In spite of the rapid development of immunochemical and molecular techniques, which have been widely applied to other parasite species, little work of this nature has been performed on *D.viviparus*. Therefore, an important priotity of work on this parasite is the characterisation of antigens which are targets of the host immune response. The infective larvae of *D.viviparus* is considered to be a potentially important stage in the protective immune response of cattle (Jarrett et al 1957b, Poynter et al 1960 and Jarrett & Sharp 1963) and since it can be obtained in relatively large quantities, its immunochemical analysis is a practical proposition.

The work presented in this thesis is primarily aimed at identfying and characterising antigens of the *D.viviparus* $L_3$ which may be of importance to the

immune response. Unlike the adult parasite, the *D.viviparus* $L_3$ does not produce significant amounts of ES products during *in vitro* culture, although some protease activity has been detected (Britton 1991). Consequently, ES material does not constitute a good source of antigens from this stage. The surface of the $L_3$ cuticle is an obvious target for host immune responses and the surface of the $L_3$ sheath, although a less obvious target, is highly immunogenic in infected and vaccinated cattle (Britton 1991) and much of the work presented in this thesis involves the investigation of these surfaces.

# Chapter 2

# CHAPTER 2

# Materials and Methods.

## 2.1. Infection and immunisation of mice.

Male mice of three to six months old were used for all the immunisation experiments. Live infective *D.viviparus* larvae were supplied by Intervet U.K. (Cambridge, U.K.) and were stored in phosphate buffered saline, pH 7.2 (PBS, Appendix 1) at 4°C. Doses for immunisation and infection were suspended in a final volume of 0.5ml of distilled water. Oral immunisation was performed by administration of this suspension directly into the oesophagus by gavage. Subcutaneous or intraperitoneal immunisations were performed by injection of the 0.5ml suspension using a 23G needle. Parasite homogenates used for immunisation were prepared in 0.85% saline (see below) and 0.25ml was emulsified with an equal volume of adjuvent. Freund's Complete adjuvant (Sigma, F5881) was used for the first immunisation and Freund's Incomplete adjuvant (Sigma, F5506) for the second. These were administered by intraperitoneal injection using a 21G needle.

Mice were challenged by oral infection with *D.viviparus* $L_3$ and killed by terminal anaesthesia with trichloroethylene (Trilene, BDH Chemicals Ltd.). The lungs and thoracic trachea were removed, placed in a plastic bag containing 5ml of 0.85% saline solution and homogenised in a Stomacher 80 device (A.J. Seward, UAC House, London,U.K.). Larvae in the resulting suspension of small lung fragments were recovered by a modified Baermann technique (Baermann 1917). The suspension was filtered through gauze into a 250 ml conical measuring flask which was then filled with a luke warm solution of 0.85% saline. The gauze, which retained the homogenised lung tissue, was then suspended in the saline and the flask left at 37°C for 6 hours. After this the supernatant was removed to a volume of approximately 10 ml and the total number of larvae present in this remaining solution were counted using a dissecting microscope.

Blood samples were taken from the mice either by tail snip or intracardiac sampling under terminal anaesthesia.

## 2.2 Production of Parasite Material.

### Infective Larvae.

Infective third stage larvae ($L_3$) of *D.viviparus* were supplied by Intervet, U.K. (Cambridge, U.K.) and the infective larvae of other trichostrongylid nematodes were produced in the Department of Veterinary Parasitology, Glasgow by faecal culture of eggs harvested from experimental infections. Infective larvae were stored in PBS at 4°C.

Trichostrongylid infective larvae were exsheathed using a solution of 0.001% w/v sodium hypochlorite (Milton, 2 Richardson-Vicks Ltd., U.K.). Larvae were suspended in this solution for 15-20 minutes until they all appeared to have lost their sheaths when examined by light microscopy. They were then washed by centrifugation three times in PBS.

Following storage, live larvae were separated from the dead by centrifugation through a Percoll cushion. 5ml of a larval suspension was carefully pipetted as a separate layer onto 5ml of 45% percoll/PBS in a 10ml test tube which was then was centrifuged at 1000g for 20 minutes. This procedure pelleted the live larvae at the bottom of the tube whilst the dead larvae remained at the Percoll/PBS interface. The pellet of live larvae was washed several times and resuspended in an appropriate volume of PBS. This method was modified to separate $L_3$ sheaths from larvae of *H.contortus* following exsheathment in sodium hypochlorite solution. A pure preparation of sheaths was produced by centrifugation of exsheathed larvae through a 35% Percoll cushion at 1500g for 20 minutes and the sheaths removed from the Percoll/PBS interface with a pasteur pipette. Centrifugation of the purified sheaths through 35% Percoll was repeated several times until no contaminating larvae were visible. The method was also modified to produce a pure preparation of exsheathed larvae. These were prepared by centrifugation of exsheathed larvae through a 45% Percoll cushion at 1000g for 10 minutes and the exsheathed larvae collected as a pellet from the bottom of the tube. Centrifugation of exsheathed larvae through 45% Percoll was repeated as necessary until no contaminating sheaths were visible.

### Adult parasites.

Adult *D.viviparus* were collected by manual recovery from the bronchi and bronchioles of calves at necropsy, 21-35 days after oral infection with 20-40 infective larvae/kg. Indoor reared 3-6 month old Friesian calves were used which were unlikely to have encountered previous challenge or to be infected with other helminth parasites. Following removal from the lungs, the adult parasites were thoroughly washed in Hanks' Balanced Salt Solution (Gibco, 070-012 00A) containing 12,000/ml

units penicillin, 1,200ug/ml streptomycin, 125ug/ml gentamycin and 25ug/ml amphotericin B. They were maintained in *Ascaris* culture medium (Appendix 1) at 37°C, 5% $CO_2$ at a concentration of 2 worms/ml for 3-4 days which was the maximum time adult parasites remained viable during *in vitro* culture.

**Eggs.**

*D.viviparus* eggs, most of which were larvated, were collected from the spent culture medium of adult parasites by centrifugation at 1000g for 5 minutes. They were then washed five times by centrifugation in PBS to remove as much contaminating debris as possible.

**First stage larvae.**

*D.viviparus* first stage larvae ($L_1$) were recovered from the freshly collected faeces of patently infected calves. Faecal samples were diluted with an appropriate volume of water to form a paste which was then drawn through coarse filter paper (Whatmans Grade 113, 18.5cm) using a Buchner funnel and vacuum pump. A milk filter (Maxa Milk Filters, A.McCaskie Ltd., Stirling) was placed on top of the filter paper, on which the larvae and coarse faecal matter had been retained, and the resulting "sandwich" was inverted and placed on a Baermann apparatus (Baermann 1917) filled with luke warm water. After standing for at least six hours the 10 ml of the fluid in the neck of the funnel, which contained most of the larvae, was drawn off. First stage larvae were used within 24 hours of collection as storage at 4°C resulted in a high level of mortality.

## 2.3. Preparation of Parasite Extracts.

The $L_3$ somatic extracts were prepared either by the sonication or homogenisation of infective larvae. Sonication was used to prepare extracts for immunisation of mice, ELISA plate antigen and for the starting material for immunoaffinty purification with mab 2A6. However, Western blots of $L_3$ sonicate often produced poorly resolved bands and so extracts prepared from homogenised larvae were used for all the other immunochemical procedures presented in the thesis.

Sonication of larvae, at a concentration of $1x10^6$ larvae/ml, was performed with a MSE Soniprep 150 at 18um amplitude using a one minute sonication, one minute pause cycle. The process was continued until all of the larvae were completely disrupted and the larvae were kept on ice throughout the whole procedure. Larvae were homogenised, at concentrations of $5x10^4$ to $5x10^5$ larvae/ml

as the particular experiment required, using a 1ml glass tissue homogeniser (Jencons Scientific Ltd., Bedfordshire, U.K.). Complete disruption of larvae took approximately 15-20 minutes of homogenisation.

Extracts for immunisations were prepared by sonication of larvae in 0.85% saline and extracts for other purposes were prepared in 10mM Tris buffer, pH 8.3 containing a cocktail of proteinase inhibitors (Tris Homognisation Buffer, Appendix 1). Somatic extracts were not routinely prepared by homogenisation of parasites in detergents as larvae were very difficult to disrupt in this manner, presumably because of the lubricating effects of the detergent. Following disruption of larvae, by homogenisation or sonication, the resulting suspension was centrifuged at 7000 g, 4°C for 30 minutes and the supernatant recovered. This supernatant is referred to as the aqueous homogenate (or sonicate). In some experiments, as indicated in the results, homogenates were centrifuged at 100,000g for 30 minutes. The insoluble pellet was boiled for 10 minutes in a solution of 1% SDS (sodium dodecyl sulphate), 5% 2-mercaptethanol (2ME) and 5% urea. The supernatant resulting from centrifugation of this material at 7000g for 30 minutes is referred to as SDS/2ME/urea soluble extract.

Somatic extracts from adult parasites, eggs and $L_1$ were prepared by manual homogenisation as for infective larvae. Adults were homogenised at 2-5 parasites/ml whereas eggs and $L_1$ were homogenised at the same concentrations as for $L_3$.

Protein concentrations of the aqueous somatic extracts were estimated by the Bradford method (Bradford 1976). Briefly the somatic extract was diluted in PBS and added to an equal volume of Bradford reagent (Pierce, 23-200). The absorbance of this at 595nm was used to calculate the protein concentration using a standard curve constructed from a range of concentrations of bovine serum albumin (5-50 ug/ml).

## 2.4 Enzyme Linked Immunosorbant Assay (ELISA).

The details of all the buffers and solutions specified in the text are given in Appendix 1. *D.viviparus* $L_3$ aqueous sonicate or adult aqueous homogenate at a concentration of 10ug/ml was diluted in an equal volume of carbonate/bicarbonate buffer, pH 9.6. 96 well, flat-bottomed microtitre plates (Titertek, Flow Laboratories) were coated by incubation of 100ul/well of this diluted antigen overnight at 4°C. The coated plates were then washed three times with ELISA wash buffer and filled with 60ul/well of ELISA blocking buffer for 30 minutes at 37°C. After washing the plates

three times with ELISA wash buffer, 50ul/well of first antibody (serum diluted in antibody dilution buffer at 1:500 or neat hybridoma culture supernatant) was added and the plates incubated for a further 30 minutes at 37°C. After the plates had been washed three times with ELISA wash buffer, 50ul/well of second antibody, goat anti-mouse IgG (whole molecule)-peroxidase conjugate (Sigma, A5278) in antibody dilution buffer at a dilution of 1:300, was added and the plate incubated at 37°C for 30 minutes. After the plates were washed three times with ELISA wash buffer, 50ul/well of OPD/hydrogen peroxide solution (chromogen/substrate solution, Appendix 1) was added and the plates incubated for 30 minutes at 37°C. The reaction was terminated by the addition of 100ul/well of 4M sulphuric acid and the optical densities (OD) were measured at 492nm using a Titertek Multiscan MC plate reader (Flow laboratories, Ltd., Uxbridge, U.K.).

When serum from groups of mice were being assayed duplicate samples from each individual mouse were used and the result for the group expressed as the mean of the optical densities. When hybridoma supernatants were being examined the results were expressed as the mean of quadruplicate samples.

## 2.5 Immunofluorescence.

### 2.5.1. Indirect Immunofluorescence (IFA) on live parasites.

IFA was performed on live parasites to examine the binding of antibody to the cuticular surface. The assay was generally performed on 1000-5000 eggs or larvae or 4 adult parasites. All incubations were performed on ice and centrifugation was performed at 4°C.

Parasites were washed by microfugation three times in PBS/0.1% sodium azide and incubated in first antibody diluted in PBS/0.1% sodium azide for 30 minutes. Serum samples were diluted at 1:25, hybridoma supernatants were used neat and monoclonal antibody ascites were used at 1:50 dilution. The parasites were then washed three times in PBS/0.1% sodium azide and incubated in second antibody which was diluted 1:25 in PBS/0.1% sodium azide. The second antibody was either anti-bovine IgG-FITC (fluorescein isothiocyanate) conjugate (Sigma, F-7509) or anti-mouse IgG-FITC conjugate (Sigma, F-0257) depending on the species of first antibody used. Following a final three washes with PBS/0.1% sodium azide parasites were placed on a microscope slide in a drop of buffer within a ring of Vaseline. A coverslip was then placed on the Vaseline ring above the drop and the parasites viewed with a fluorescence microscope under ultraviolet light. IFA with FITC-

conjugated lectins (used at a 1:25 dilution) was performed by the same method except that only one step was involved (ie. no second antibody step). Parasites produced a degree of autofluorescence under UV light but this appeared yellow and so could be distinguished from the bright green fluorescence emitted by FITC.

The level of fluorescence was quantified to allow comparisons between samples or individual larvae to be made. This was undertaken using a Leitz MPV Compact 2 microscope photometer linked to an Olivetti H100 computer. The photometer diaphragm was set to measure the light emitted from a retangular field of 230-555um$^2$ using a x40 objective for larvae and eggs or 920-2220um$^2$ with a x10 objective for adult worms. For larvae and eggs a field was measured near the centre of the specimen but for adults edge fluorescence was measured since autofluorescence was particularly bright near the centre of this stage. The fluorescence of a bright specimen was set at the arbitrary fluorescence value of 650 and the values of fluorescence of other specimens were measured relative to this. At least 20 readings were taken for each sample and the mean value of relative fluorescence calculated from these. In the case of larvae and eggs, readings were taken from 20 separate individuals but for adult parasites multiple readings had to be taken from the same individual due to the limited number of adults available.

### 2.5.2 Indirect immunofluorescence on fixed parasites.

The method used for fixation of larvae on microscope slides was adapted from the method described by Sulston & Hodgkin (1988). Multiwell microscope slides were subbed by immersion in an aqueous solution containing 0.1% poly-L-lysine, 0.2% gelatin and then dried in an oven at 100$^o$C. A drop of distilled water, containing several hundred larvae, was pipetted onto each well on the slide and a coverslip placed over the top. The slide was then frozen by plunging into liquid nitrogen for approximately 15 seconds and after which the coverslip was flipped off with a scalpel blade. The slide was then immersed in methanol at -20$^o$C for 3 minutes and then in acetone at -20$^o$C for 5 minutes. Slides were stored at -20$^o$C until required.

IFA was conducted on the fixed specimens using the same antibody solutions as described for the IFA on live parasites. Single drops of antibody solutions were added to larvae on each well and the whole slide was washed between steps in PBS.

## 2.6. Monoclonal Antibody Production.

The generation of monoclonal antibodies was first described by Kohler and Milstein (1975) and the methods outlined below have been reviewed by Goding (1983).

**Immunisation Protocol.**

Mice were immunised for the first fusion by oral infection with *D.viviparus* $L_3$ and for the second fusion by intraperitoneal injection with $L_3$ aqueous homogenate with Freund's adjuvant. Details are given in the appropriate results section.

**Media for Cell Culture.**

The following tissue culture media were used for hybridoma production.

*Complete Medium.*

| | | |
|---|---|---|
| RPMI 1640 with Hepes | (Gibco 041-2400-M) | 100ml |
| 200mM Glutamine | (Gibco 043-5030-H) | 1ml |
| 7% sodium bicarbonate | | 1ml |
| Myoclone Foetal Calf Serum | (Gibco 011-6180-H) | 15ml |
| Penicillin-Streptomycin | (Gibco 043-5140-H) | 1ml |
| Gentamycin | (Gibco 043-5710-D) | 0.2ml |
| Amphotericin B | (Flow, 16-723-46) | 0.2ml |
| 0.1M mercaptethanol | | 0.01ml |

*Incomplete Medium* is the same as complete medium but without the foetal calf serum.

*HAT Medium*

| | | |
|---|---|---|
| Complete Medium | | 100ml |
| HAT (50x Concentrate) | (Sigma, H-0262) | 2ml |

*HT Medium*

| | | |
|---|---|---|
| Complete Medium | | 100ml |
| HT (50x Concentrate) | (Sigma, H-0137) | 2ml |

*Polyethylene glycol (PEG) solutions*

*41.6% PEG*

| | | |
|---|---|---|
| PEG | (Sigma, P-7777) | 5g |
| DMSO | (Sigma D-5879) | 1.8ml |
| Incomplete Medium | | 5.2ml |

*25% PEG*

| | | |
|---|---|---|
| PEG | | 5g |
| DMSO | (Sigma D-5879) | 1.8ml |
| Incomplete Medium | | 113ml |

**Myeloma Cell line.**

The myeloma cell line P3X63-Ag8.653 (Kearney et al 1979) was used as the fusion partner for the splenocytes harvested from the immunised mice. These were grown in complete media at 37°C, 5%$CO_2$ and maintained at a dilution of approximately $1x10^5$ cells/ml in a state of logarithmic growth until required for fusion.

**Preparation of feeder cells.**

Peritoneal macrophages from Balb/c mice were used as feeder cells and these were prepared as follows. A mouse was killed by cervical dislocation and the abdomen soaked in 70% ethanol. The skin was dissected from the abdomen and 5ml of chilled incomplete medium was injected into the peritoneal cavity using a 26G needle. The needle was withdrawn and the abdomen gently massaged for about 1 minute. A 21G needle was inserted into the abdomen and as much media as possible was aspirated into a 5ml syringe. The cells were washed by two centrifugations (400g for 7 minutes) and resuspended in 10ml of incomplete medium before being finally resuspended in 5ml of the appropriate media (HAT, HT or complete media). The cells were then incubated at 37°C, 5%$CO_2$ for 24 hours before use.

**Fusion of myeloma cells and splenocytes.**

The immunised mouse was killed by cervical dislocation and the spleen removed under sterile conditions. The spleen was then placed in a petri dish containing 5ml of warm incomplete medium and cut in half. The cells were squeezed into the medium from the cut ends of the spleen using tissue forceps. The cell suspension was then aspirated in and out of a 5ml syringe several times using a 23G

needle to disrupt any clumps of cells and centrifuged for 7minutes at 400g. The resulting pellet was washed once in 10ml of incomplete medium and the cells resuspended in a final volume of 5ml. An aliquot of these cells were counted in a haemocytometer in the presence of Trypan blue to determine the number of viable splenocytes harvested (10ul of cell suspension mixed with 90ul of 0.1% Trypan blue/PBS, viable cells exclude the dye).

The splenocytes were then added to myeloma cells at a 10:1 ratio in incomplete medium in a 50ml centrifuge tube, mixed and the cells pelleted by centrifugation at 400g for 7 minutes. The supernatant was discarded and 0.5ml of warm 41.6% PEG was added to the pellet in a dropwise fashion over a period of 1 minute, mixing the cells constantly. Next 0.5ml of the 25% PEG was added over a period of 1 minute, again with constant mixing of the cells. This was followed by the dropwise addition of 10ml of complete medium over a period of 3 minutes, with constant mixing of the sample. Finally, 50ml of HAT medium was added and the suspension of cells plated out into 96-well flat-bottomed microtitre plates at 100ul/well. The plates were incubated overnight at 37°C, 5%$CO_2$ and then 100ul of peritoneal macrophages, in HAT media, added to each well.

The plates were incubated at 37°C, 5%$CO_2$ for 7 days, after which time 100ul of media was removed and replaced with fresh HAT media. After approximately 14-18 days, when many of the hybridomas appeared to be confluent, 100ul of media was removed, replaced with fresh HAT media and retained for screening.

**Screening of hybridoma supernatants.**

Hybridoma supernatants were screened either by $L_3$ homogenate ELISA (section 2.4) or by IFA (section 2.5) on live $L_3$. Sheathed and exsheathed $L_3$ were mixed in equal proportions and used in the IFA so that antibody against both surfaces could be detected in a single sample.

**Cloning by limiting dilution.**

Cells were harvested from confluent wells, counted with Trypan blue and dilutions were made of 50, 20, 10, 5 and 2 cells/ml. 1ml of each dilution was plated out into 10 microtitre wells at 100ul/well into which 100ul of feeder cell suspension had already been added. The plates were incubated for 10-14 days and were examined by microscopy every few days to determine which wells contained single foci of growing cells. When the wells were confluent the supernatants were screened and selected positive wells were expanded. All such cell lines were cloned for a second time to ensure they were monoclonal.

**Cryopreservation of hybridoma cell lines.**

Cell lines were cryopreserved after they had been expanded into tissue culture flasks and were growing well. 10ml of a reasonably dense cell suspension was taken and the number of cells present counted ($10^6$-$10^7$ is a reasonable number of cells to aliquot into 2 cryotubes). The suspension was centrifuged at 400g for 7 minutes and the cell pellet resuspended in 3 ml of complete medium containing 20% DMSO. This volume was divided between two cryotubes and these were placed in a polystyrene box at -70°C for 12-24 hours. They were then transferred to liquid nitrogen.

To recover the cell lines from cryopreservation, the tubes were rapidly thawed by placing in a 37°C waterbath and the cells then suspended in 10ml of warm incomplete medium (37°C). The suspension was centrifuged, the pellet resuspended in 10ml of warm complete medium (37°C) and then transferred to a flask for culture.

**Isotyping of antibodies.**

Monoclonal antibody supernatants were isotyped using an erythrocyte haemagglutination kit (Serotec MMT RC1) or the mouse monoclonal antibody isotyping kit (Amersham, RPN 29).

## 2.7. SDS-Polyacrylamide Gel Electrophoresis.

### 2.7.1. Preparation and running of gels

SDS-PAGE was performed using the method of Laemmli (1970) using 5-25% acrylamide gradient slab gels with a 4% stacking gel (Appendix 1). The Biorad Protean II gel electrophoresis system was used and gels were run overnight at 80 Volts at a current of approximately 100mA.

Samples were made up to a volume of 30ul, mixed with 10ul of 4x sample buffer (Appendix 1) and boiled for approximately ten minutes prior to loading. If antigen bound to antibody-Protein A sepharose bead complexes were to be analysed 30-50ul of beads was added to 40ul of sample buffer and boiled for 10 minutes. The beads were then pelleted by centrifugation at 7000g for 10 minutes and the supernatant loaded onto the gel.

Molecular weights of parasite proteins were estimated by reference to molecular weight markers of either low weight range (14.2-66kDa, Sigma, MW-SDS-70L) or high molecular weight range (29-205kDa, Sigma, MW-SDS-200). If gels were to be analysed by fluorography $^{14}$C-labelled molecular weight markers were used (14.3-

205kDa, Sigma, M-5281). For each gel, the distance migrated by the molecular weight markers was plotted against the log of the their molecular weights and the molecular weight of parasite antigens was estimated from the distance migrated using this plot.

### 2.7.2. Staining of gels.

**Coomassie blue staining.**

In order to visualise polypeptides on gels following SDS-PAGE electrophoresis, gels were stained by a 2 hour incubation in 0.1% Coomassie Brilliant Blue R-250 (Sigma, B-0149) in 20% methanol, 10% acetic acid. Gels were then destained in 20% methanol, 10% acetic acid and 1% glycerol until the background was judged to be sufficiently clear.

**Silver staining.**

Silver staining was used as an alternative and more sensitive stain for polypeptides. Following electrophoresis, the gel was soaked for at least 12 hours in several changes of 50% methanol. It was then soaked for 1-2 hours in silver stain solution (Appendix 1) in the dark and then thoroughly rinsed with several changes of distilled water. The stain was developed for 5-10 minutes with citric acid/formaldehyde solution (Appendix 1) and then stopped with 50% methanol.

**Periodic Acid-Schiff (PAS) staining.**

PAS staining was used to detect glycoconjugates on SDS-PAGE gels. Following electrophoresis, gels were incubated for 30 minutes in 12.5% trichloroacetic acid, rinsed gently in distilled water, immersed for 60 minutes in 1% periodic acid (freshly prepared in 3% acetic acid) and then incubated in Schiffs reagent in the dark for 60 minutes. The gels were then fixed in freshly prepared 0.5% sodium disulphite.

**Stains All .**

Stains All has a strong affinity for acidic macromolecules including polypeptides and acid mucopolysaccharides (Green et al 1973).

Following electrophoresis, gels were incubated in 25% isopropanol, with agitation, at 50$^{o}$C for 30 minutes and then in 10% Triton X-100. The gels were then rinsed with distilled water and placed in the staining solution in the dark for at least 12 hours. The staining solution was prepared immediately before use from a 0.1% stock solution in formamide as follows; 10ml of this stock stain was added to 10ml formamide, 50ml isopropanol, 1ml 3M Tris-HCl, pH 8.8 and then made up to a

volume of 200ml with distilled water. The gels stained for 12 hours and then rinsed in distilled water (shielded from direct light) until the background was judged to be sufficiently reduced.

**Sudan Black staining.**

Sudan Black stains lipids and has been used to visualise human plasma lipoproteins on polyacrylamide following isoelectric focusing (Godolphin & Stinson 1974). The stain was prepared by dissolving 250mg of Sudan Black B in 10ml of acetone followed by the addition of 7.5ml glacial acetic acid and 40ml water. After mixing for 30 minutes, the solution was filtered to remove undissolved stain. The gel was then incubated in freshly prepared stain for at least 12 hours and then destained by several changes of acetone/acetic acid/water (20:15:54, v/v/v).

### 2.7.3. Fluorography of SDS-PAGE gels.

Fluorography was used to detect molecules labelled with $^{35}S$ or $^{3}H$ when extracts from metabolically labelled parasites were being examined. Following electrophoresis, gels were fixed for 30 minutes in 25% methanol, 7.5% acetic acid, 1% glycerol and incubated in scintillant (Amplify, NAME.100, Amersham) for 30 minutes. The gels were then dried onto Whatman 3MM filter paper using a slab gel drier (Bio-rad, 1125B) at 80°C for 3 hours. Gels were then exposed to flashed Fuji RX X-Ray film in cassettes with Ilford Fast Tungsten intensifying screens at -70°C.

## 2.8. Western blotting.

Immunoblotting of polyacrylamide gels (Western blotting) was performed using the method first described by Towbin et al (1979). The proteins separated on 5-25% polyacrylamide gels were transferred to nitrocellulose (Schleicer & Scheull, BA 85) in Tris/glycine/SDS transfer buffer (Appendix 1), using a Trans-blot cell (Bio-rad, 170-3910) at a constant current of 300mA for 3 hours.

Following transfer, the filter was stained by incubation in Ponceau-S (Sigma N3005, 0.2% in 3% trichloroacetic acid) for approximately 1 minute and destained with distilled water. This was performed to check the efficiency of transfer and check the approximate loading of the tracks. The molecular weight marker tracks were cut off and not destained. The filter was then incubated in blocking buffer (Appendix 1) overnight and then washed by three separate 10 minute washes in

washing buffer (Appendix 1). The filter was then incubated in first antibody solution for 1 hour; sera were diluted at 1:200 with antibody dilution buffer (Appendix 1) and hybridoma supernatants were used neat. After incubation the filter was washed three times in wash buffer and then incubated for 1 hour in second antibody diluted at 1:200 with antibody dilution buffer. The second antibodies used were anti-bovine IgG (whole molecule) peroxidase conjugate (Sigma A-7414), anti-rabbit IgG (whole molecule) peroxidase conjugate (Sigma A-6154) or anti-mouse IgG (whole molecule) peroxidase conjugate (Sigma A-4416) depending on the species of the first antibody used. Following three washes in wash buffer and a final wash in Tris saline (detergent may inhibit the peroxidase enzyme), the binding of the second antibody was visualised by incubating the membrane in the substrate/chromogen solution (hydrogen peroxide and 4-chloro-1-napthol, Appendix 1). The reaction was terminated by washing the filter in distilled water after sufficient development was judged to have occurred.

## 2.9. Surface Biotinylation of Parasites.

The biotin reagents used were D-biotin-N-hydroxy-succinamide-ester (NHS biotin, Boehringer Mannheim, GM1H), sulfosuccinimidobiotin (Sulfo-NHS-biotin, Pearce, 21217) or biotin hydrazide (Pearce, 21339). The NHS-biotin was made up as a stock solution of 10mg/ml in dimethylformamide and then diluted to a working solution of 0.5mg/ml in 0.85% saline. Sulfo-NHS-biotin and biotin hydrazide were dissolved directly to a working dilution of 0.5mg/ml in 0.85% saline.

The same method of labelling was used with NHS-biotin or sulfo-NHS-biotin. Live sheathed or exsheathed *D.viviparus* infective larvae were incubated at a concentration of 200,000 $L_3$/ml (50,000 $L_3$/ml for *H.contortus*) in 0.5 mg/ml of the biotin reagent in the dark for 1 hour at room temperature. Biotinylation was terminated by washing larvae extensively in 0.85% saline by repeated centrifugation (1000g for 2 minutes) and resuspension. The larvae were then homogenised in Tris homogenisation buffer (Appendix 1) to produce aqueous and SDS/2ME/urea homogenates as described in section 2.3. $L_3$ aqueous homogenate was labelled with 0.5 mg/ml of biotin reagent for 1 hour and the biotinylation was terminated by the addition of ethanolamine and Tris, pH 7.2 to final concentrations of 10mM and 100mM respectively.

The method for labelling with biotin hydrazide was the same as described above, except that the larvae were pretreated with sodium periodate in order to oxidise the hydroxyl groups on any exposed carbohydrates producing aldehyde groups with which the hydrazide moeity of the biotin reagent can react. This was

undertaken by incubating larvae in 40mM sodium periodate, 100mM sodium bicarbonate, 150mM sodium chloride, pH 9.5, in the dark for 30 minutes. The larvae were then washed with 0.85% saline and biotinylation performed as described above.

After labelling, the larvae were homogenised as described in section 2.3 and the polypeptides in the extracts separated by SDS-PAGE and electroblotted onto nitrocellulose as described in section 2.8. Molecules labelled with biotin were detected by probing the blot with streptavidin peroxidase conjugate. The filter was stained with Ponceau-S and then blocked for 1 hour by immersion in 5% skimmed milk, 1% Tween in Tris saline, pH 7.4 (Appendix 1). The filter was then washed in Western blotting wash buffer (Appendix 1) and incubated for 1 hour in streptavidin peroxidase (Sigma S-5512, stock solution of 0.5mg/ml diluted 1:200 in Tris saline). After a final washing the blot was developed using 4-chloro-1-naphthol and hydrogen peroxidase (Appendix 1).

Solubilisation of surface antigens was investigated by incubating live labelled larvae in a range of different detergents: 1% sodium dodecyl sulphate (SDS), 0.5% Sodium deoxycholate (NaDOC), 0.5% cetyl trimethylammonium bromide (CTAB) and 1%SDS/5% 2-mercaptethanol(2ME). Labelled sheathed and exsheathed $L_3$ were incubated in detergent at a concentration of 200,000 $L_3$/ml for varying periods of time and then removed by centrifugation. The presence of solubilised biotinylated protein in the resulting detergent supernatants was investigated by analysis of TCA precipitated material on Western blots probed with streptavidin peroxidase. The TCA precipitation was performed by addition of 10ul of foetal calf serum to 0.5 ml of detergent extract and then gradually adding ice cold TCA to a final concentration of 10% whilst vortexing the mixture. The solution was placed on ice for 30 minutes and then centrifuged at 10,000g for 10 minutes. The resulting pellet was dissolved in 30ul of SDS/PAGE sample buffer, separated by SDS-PAGE electrophoresis and analysed by Western blotting.

Biotinylation of antigen/antibody complexes on protein-A-sepharose beads was performed with NHS-biotin using essentially the same method as that used for live larvae. The antigen/antibody Protein-A-sepharose bead complexes were prepared using the standard immunoprecipitation technique (see section 2.11) using the following volumes: 300ul of *D.viviparus* $L_3$ aqueous homogenate (1.1mg/ml) was incubated with 2ml of monoclonal antibody supernatant and 50ul of beads. After washing (section 2.11) the beads were ready for incubation with the biotin reagent.

## 2.10. Metabolic labelling of parasites *in vitro*.

Metabolic labelling was performed using the following number of parasites of each stage in 5ml of culture medium (see below). 10 adults, 100,000 larvated eggs, 50,000 $L_1$ and 100,000 $L_3$.

### $^{35}$S-Methionine labelling.

The culture medium used for labelling parasites was prepared from RPMI 1640 made up from a kit (Gibco, Selectamine kit 062-07402 C) by adding all the individual amino acids except methionine. To this basic medium all the constituents of *Ascaris* culture medium (Appendix 1) were added. Parasites were washed several times in the *Ascaris* medium before being placed in tissue culture.

Parasites were placed in 5ml of medium with 20ul (0.2mCi) of $^{35}$S-methionine (Amersham, SJ 204, specific activity = 1000 Ci/mMol) and the flasks incubated at 37°C, 5% $CO_2$ for 4 days. After this time the parasites were recovered, washed several times in PBS and homogenised in 500ul of Tris homogenisation buffer (Appendix 1) to prepare aqueous and SDS/2ME/urea soluble homogenates as described in section 2.3.

### $^{3}$H-amino acid mixture labelling.

The same method was used as for the $^{35}$S-methionine labelling except that the Selectamine medium was made up without leucine, lysine, phenylalanine, proline and tyrosine as these are the amino acids present in the radiolabelled mixture. 200ul (0.2mCi) of high specific activity $^{3}$H-amino acid mixture (Amersham, TRK.550, Mean specific activity = 105 Ci/mM) was added to the 5ml of media containing the parasites.

### $^{3}$H-Glucosamine labelling.

The same method was used as for the $^{35}$S-methionine labelling except that the selectamine media was made up in full and 200ul (0.2mCi) of D-(6-$^{3}$H)Glucosamine (Amersham TRK.398, specific activity = 22 Ci/mM) was added to the 5ml of medium containing the parasites.

### Scintillation counting.

In order to allow approximate equal loading of radioactivity on SDS-PAGE gels for the homogenates prepared from different parasite stages, the labelled samples were assessed by scintillation counting. 5ul of sample was spotted onto a piece of glass fibre filter paper (FG/A Whatman) and this was dissolved in 1ml of

liquid scintillant (Optiscint "safe", LKB) and the radioactivity incorporated was measured by a liquid scintillation counter.

## 2.11. Immunoprecipitation.

Immunoprecipitations were performed using Protein-A-sepharose CL-4B beads (Pharmacia 17-0780-01) to immobilise antigen-antibody complexes. 100ul of aqueous homogenate was used for each sample unless otherwise specified in the results sections. The parasite extract was precleared by the addition of 30ul of a 50% protein-A-sepharose bead suspension and incubation for 1 hour. The beads were then removed by centrifugation at 10,000g for 2 minutes. The antibody (5ul of polyclonal antisera diluted in 200ul of PBS or 200ul of neat monoclonal antibody supernatant) was then added to the precleared antigen and the mixture incubated at 4°C overnight. 30ul of a 50% suspension of Protein-A-sepharose was then added to the samples which were incubated at 4°C for 1 hour and then centrifuged at 10,000g for 2 minutes. The beads were washed 5 times in NET buffer (Appendix 1) and 30ul of SDS-PAGE sample buffer was added. The samples were then boiled for 5 minutes and then centrifuged at 10,000g for 2 minutes. Negative controls using normal serum, complete medium or supernatant of the *Theileria annulata* mab 5E1 (Glascodine et al 1990) were performed where appropriate. The samples were subjected to SDS-PAGE; metabolically labelled samples were analysed by fluorography while unlabelled samples were analysed by Western blotting.

## 2.12. Immunochemical experiments.

### Chloroform/Methanol extraction of antigens.

This method was adapted from that described by Scott et al (1988). 200ul of *D.viviparus* $L_3$ homogenate (1.1mg/ml) was vortexed with 500ul of chloroform/methanol (1:1) for approximately 1 minute in an eppendorf tube and left to stand for 15 minutes. The aqueous and organic phases were separated by centrifugation at 10,000g for 5 minutes and the upper aqueous layer removed and the organic layer retained for analysis. The aqueous layer was vortexed with a further 500ul of chloroform/methanol, left to stand for 15 minutes, separated by centrifugation and both the aqueous and organic layers retained for analysis. The three resulting samples (1 aqueous and 2 chloroform/methanol) were lyophilised under vacuum and resuspended in 50 ul of PBS. These resuspended samples were

subjected to SDS-PAGE as described in section 2.7 and then probed with monoclonal antibody on Western blots.

**Periodate treatment of live larvae.**

Sodium periodate oxidises hydroxyl groups on the hexose ring of carbohydrates. This breaks the ring and disrupts any epitope comprising of this structure. Concentrations of periodate at 5mM are sufficient to achieve this whereas higher concentrations, above 100mM will oxidise other residues such as histidine on the peptide backbone and so may also disrupt peptide epitopes (Eylar & Jeanloz 1962). 1000 larvae were incubated in 500ul of 10mM sodium periodate (Sigma S-1878), in 5mM sodium acetate buffer, pH 4.7., at 4°C for 1 hour in the dark. They were then washed three times in PBS/0.1% sodium azide at 4°C and then subjected to IFA with the appropriate antibody.

**Digoxigenin hydrazide labelling.**

Mild oxidation of hydroxyl groups on carbohydrates by sodium periodate treatment results in the production of aldehyde groups. The hapten digoxigenin can then be covalently linked to these aldehyde groups via a hydrazide group. Such digoxigenin labelled glycoconjugates can be detected by immunoassay using a digoxigenin specific antibody conjugated with alkaline phosphatase. This system was used to examine whether antigens were glycosylated. The DIG Glycan Detection Kit (Boehringer Mannheim 1142 372) was used for this purpose.

Aqueous homogenates or suspensions of immune complexes on Protein-A-Sepharose beads were labelled (see below) and the results assessed by probing Western blots of this labelled material with a digoxigenin specific antibody-alkaline phosphatase conjugate. The protein A/antibody/antigen complexes were prepared by immunoprecipitating 500ul of *D.viviparus* $L_3$ homogenate with 1ml of 2A6 supernatant and 50ul of Protein-A beads. These were washed in NET buffer (Appendix 1) several times and were then ready for the labelling experiment. The labelling method was as follows;

Either 20ul of aqueous homogenate or 20ul of protein-A-sepharose bearing antigen/antibody complexes were added to 20ul of 10mM sodium acetate buffer, pH 5.5. and then 20ul of 15mM sodium metaperiodate was added. The mixture was incubated for 20 minutes in the dark at room temperature and the reaction terminated by the addition of 10ul of sodium disulfite solution (3.75mg/ml) followed by incubation at room temperature for 5 minutes. 5ul of the DIG-succinyl-amidocaproic acid hydrazide reagent (supplied in kit) was added to the sample and the mixture incubated at room temperature for 1 hour. After this period 25ul of 4x SDS-

PAGE sample buffer was added to the homogenate samples or in the case of the beads, these were collected by centrifugation and resuspended in 1x SDS-PAGE sample buffer.

The samples were then subjected to SDS-PAGE and Western blotting. Following staining with Ponceau-S, the nitrocellulose filters were incubated for 30 minutes in blocking solution (supplied in kit) and then washed 3 times in Tris buffered saline (Appendix 1). They were then incubated in anti-digoxigenin-alkaline phosphatase conjugate (1:1000) for 1 hour and again washed 3x in Tris buffered saline. The blots were developed using a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue terazolium (BCIP/NBT) substrate made up as directed in the kit.

**N-Glycosidase Treatment**

*D.viviparus* $L_3$ aqueous homogenate samples were treated with peptide N-Glycanase F (Boehringer-Mannheim, Glycopeptidase F, 913-782) to cleave N-linked oligosaccharides from polypeptide chains. This was performed to determine if the antigen contained any N-linked carbohydrate groups, in which case a reduction in molecular weight should be seen on SDS-PAGE following treatment. If the epitope recognised by a monoclonal antibody consisted of such a group the N-Glycosidase treated samples would not bind the antibody on Western blots.

50ul samples of *D.viviparus* $L_3$ homogenate were added to digestion buffer (0.5M sodium phosphate buffer,pH 7.5, 100mM 1,10 phenanthroline and 20mM 2ME) and PNGase was added to produce final concentrations of 1, 10, and 50 units/ml in a final volume of 100ul. The samples were then incubated at 37°C for 24 hours after which time SDS-PAGE sample buffer was added, the samples boiled for 5 minutes and subjected to SDS-PAGE. Western blots were probed with monoclonal antibody. A negative control using reaction buffer and no enzyme was included. To demonstrate the efficacy of the enzyme under the reaction conditions, a positive control of fetuin was digested at the same time. 40ul of a 2mg/ml solution of fetuin (Sigma 2379) was added to 40ul of digestion buffer and PNGase was added to a final concentration of 10 units/ml. An identical 40ul sample without enzyme was also prepared. These were incubated and analysed in parallel with the parasite samples except that the nitrocellulose filters were simply stained with Ponceau-S to detect the fetuin.

**Protease Treatments**

The proteases were prepared in the following reaction buffers: Pronase (Boehringer-Mannheim 165 921) was prepared as a stock solution of 10mg/ml in 0.01M tris pH 7.8, 0.01M EDTA, 0.5% SDS immediately before use. Trypsin

(Boehringer-Mannheim 109 827) was prepared as a stock solution of 10mg/ml in 1mM HCL, 20mM $CaCl_2$ immediately before use. Papain (Boehringer-Mannheim 108 014) was obtained as a suspension and immediately before use 2ME was added to a final concentration of 2M and the solution incubated at 37°C for 30 minutes.

Aqueous homogenates were prepared in 0.01M Tris, pH 8.3 (without protease inhibitors) for the protease digestion experiments. The protease stock solutions were added to 100 ul samples of *D.viviparus* $L_3$ homogenate (0.9mg/ml) to produce final concentrations of protease of 1, 2 and 3 mg/ml as well as a negative control sample to which reaction buffer alone was added. The samples were incubated at 37°C for 2 hours an equal volume of SDS-PAGE sample buffer added and were then subjected to SDS-PAGE and Western blotting. Blots were probed with antibody to assess the effect of the treatments on antigen.

## 2.13. Isoelectric focusing and capillary blotting.

Isoelectric focussing on agarose gels was performed by the method described by Ketch et al (1990). Agarose gels were prepared by dissolving 0.3g Pharmacia agarose IEF and 3.6g sorbitol in 27ml $H_2O$ at 100°C. After the agarose had dissolved and cooled, 0.38ml of ampholines, pH 3-10 (Pharmacia 80-1125-87) and 0.38ml of ampholines, pH 5-7 (Pharmacia 80-1125-91) were added. Gels (18.5 x 11.3 cm) were then immediately poured onto the hydrophilic side of Gel Bond (Pharmacia).

30ul samples of trichostrongylid $L_3$ aqueous homogenate were soaked into 3MM filter paper squares (approximately 3mm x 9mm). These sample papers were then placed along the length of the gel approximately 3cm from the cathode. The cathode solution was 1M NaOH and the anode solution was 0.05M $H_2SO_4$. The gel was run for 30 minutes at 1000V, 5W with the maximum current set at 20mA. After this time the sample papers were removed and the gel run for another 30 minutes. The gel was washed several times in 50mM Tris-HCl, pH 7.5 for 10 minutes to remove the ampholines. The proteins in the gel were then transferred to Immobilon PVDF paper (Millipore IPVH 151 5Q) by capillary blotting; the PVDF paper (cut to the same size as the gel, wetted with methanol and soaked in 50mM Tris-HCL, pH 7.5) was placed on the surface of the gel then overlaid with 3 pieces of 3MM paper soaked in 50mM Tris-HCl, pH 7.5, a block of paper towels and a weight. The apparatus was left overnight to allow the transfer to occur. The PVDF filter was then probed with monoclonal antibody using the same method as for Western blots.

Coloured pI markers (LKB, pI range 5.65-8.3, 1860-203 and pI range 2.4-5.65, 1860-202) were also run on the gels and capillary blotted so that an approximation of the pI of the detected antigens could be made.

## 2.14. Immuno-affinity Purification of the 2A6 antigen.

### Purification of mab 2A6 from tissue culture supernatant.

Protein A affinity chromatography was used to purify the monoclonal antibody 2A6 from tissue culture supernatant. A 1ml column of Protein-A Sepharose 4 Fast Flow beads (Pharmacia 17-0974-01) was prewashed with 5ml of 100mM glycine,pH 3 and then with 20ml of 100mM Tris, pH 8.0. 1 litre of tissue culture supernatant, buffered with 100ml of 1M Tris, pH 8 was passed through the column overnight using a peristaltic pump. The column was washed with 20ml of 100mM Tris,pH 8 followed by 20ml of 10mM Tris,pH 8 and the bound antibody was then eluted using 100mM glycine pH 3. 500ul fractions were collected in eppendorf tubes containing 50ul of 1M Tris, pH 8 and the tubes were gently mixed. The immunoglobulin-containing fractions were identified by measuring ultraviolet absorbance at 280nm and an aliquot of each fraction was run on SDS-PAGE to check that there were no major impurities. The purified antibody was also tested by immunofluorescence with live *D.viviparus* sheathed $L_3$ to ensure it still retained its antigen binding capacity.

### Production of a mab 2A6 immunoaffinty column.

The column was prepared by covalently coupling the monoclonal antibody to Protein-A beads using dimethylpimelimidate (DMP). This has the advantage that the antibody binds to Protein-A via the Fc domain and so the antigen binding site is orientated correctly for maximum interaction with the antigen. Once the antibody has bound to the protein A it is covalently coupled by the bifunctional coupling reagent DMP.

4.8 mg of purified 2A6 IgG was diluted in 5ml of 100mM Tris,pH 8 and mixed with 1ml of Protein-A Sepharose 4 Fast Flow beads. The mixture was incubated at room temperature for 1 hour with gentle rocking. The beads were then washed twice in 10ml of 0.2M sodium borate, pH 9 using centrifugation at 3000g for 5 minutes. The beads were resuspended in 10ml of sodium borate, pH 9 and a 10ul aliquot of beads were removed for analysis. Dimethylpimelimidate (DMP) was added to the remaining suspension to a final concentration to 20mM and the beads incubated at room temperature for 30 minutes with gentle rocking. After this time 10ul of

coupled beads were removed for analysis. The reaction was stopped by washing the beads once in 0.2M ethanolamine, pH 8 and then incubating for 2 hours at room temperature in 0.2M ethanolamine with gentle mixing. The beads were then washed several times in PBS and stored in PBS with 0.01% sodium azide at 4°C.

The efficiency of coupling was checked by boiling the 10ul samples of beads taken before and after coupling in 40ul of SDS-PAGE sample buffer. These samples were run on an SDS-PAGE gel which was stained with Coomassie blue. An efficient coupling was seen to have occurred as the immunoglobulin heavy chain was seen in the sample taken before coupling but not in the sample taken after coupling.

**Assessing the elution conditions for the immunoaffinity column.**

The column, consisting of 0.5 ml of the prepared beads, was pre-washed in 20ml of PBS and then 5ml of *D.viviparus* $L_3$ sonicate (0.5 mg/ml) was passed through the column three times ( 50 ul aliquots were taken for analysis each time). The column was washed with 20ml of PBS and then with 10ml of 10mM phosphate, pH 6.8 (pre-elution buffer). Acid elution conditions were tested by passing 5ml of 100mM glycine, pH 2.5 through the column and collecting 0.2ml fractions. After washing the column with 20ml PBS and then 10ml of 10mM phosphate buffer, pH 8 (pre-elution buffer), alkaline elution conditions were tested by passing 5ml of 100mM triethylamine pH 11.5 through the column and collecting 0.2ml fractions. The column was then washed and stored in PBS/0.05% sodium azide.

**Dot Blotting of fractions eluted from immunoaffinty column.**

10ul aliquots of each eluted fraction were spotted onto duplicate nitrocellulose filters and left to dry for 20 minutes. One filter was probed with mab 2A6 supernatant and the other with complete medium, as a negative control, using the method outlined for Western blots.

## 2.15. Immuno-Electron Microscopy.

### 2.15. 1. Pre-embedding labelling.

Pre-embedding labelling was performed to examine the binding of mab 2A6 to the surface of the *D.viviparus* $L_3$ sheath.

**Antibody/Protein-A labelling.**

50,000 *D.viviparus* sheathed $L_3$ were incubated in 1 ml of neat mab 2A6 supernatant for 30 minutes at 4°C and a duplicate sample was incubated with the anti-*Theileria annulata* merozoite mab 5E1 as a negative control. Both monoclonal antibodies are IgG2a and all the subsequent procedures were carried out in parallel for the two antibodies. After incubation in antibody, the samples were washed three times in PBS and then incubated in a 1:20 dilution of Protein-A-Gold (Sigma, 10nm Colloidal Gold P-1039) for 30 minutes at 4°C. The larvae were again washed 3 times in PBS.

**Fixation and dehydration.**

The labelled larvae were fixed in 0.5% glutaraldehyde and 2% paraformaldehyde in PBS for 30 minutes at 4°C, washed 3 times in PBS and then incubated in 1% osmium tetroxide for 1 hour. After washing 3 times in distilled water they were suspended a few drops of molten 5% agarose (40°C) which was allowed to set. This was performed to hold the larvae together as a pellet during the subsequent procedures. The pellet was incubated in 0.5% uranyl acetate for 1 hour in the dark, rinsed in distilled water and then dehydrated by sequential 15 minute immersions in 30%, 50%, 70%, 90%,100% and dried 100% ethanol.

**Resin infiltration and embedding.**

The specimens were given 3, five minute incubations in epoxypropane and then left overnight in Araldite:epoxypropane (1:1) with continual rotation. The following day the samples were embedded in fresh Araldite in a section of a small embedding tray and placed in an oven at 60°C for 48 hours.

**Sectioning and staining.**

Using a glass knife and ultramicrotome, ultrathin sections (approximately 80-140nm) were cut and picked up on copper grids using standard electron microscopy procedures. Each grid was submerged in a drop of 0.5% uranyl acetate for 5 minutes in the dark and then thoroughly washed in distilled water. After

this they were briefly immersed in 0.2M NaOH, placed in a drop of Reynold's lead citrate for 5 minutes, washed several times in 0.2M NaOH and then distilled water and left to dry. Stained sections were examined by transmission electron microscopy.

### 2.15.2. Post-embedding labelling.

Post-embedding labelling was performed to examine the binding of mab 2A6 to somatic larval tissues as well as to the sheath surface. Fixation and dehydration were the same as for the pre-embedding labelling except that the osmium tetroxide step was omitted. The samples were embedded in the acrylic resin Lowicryl, LR White (Agar Ltd. R-1281) as opposed to Araldite. Infiltration with this resin was performed at 4°C overnight and the resin polymerised at 40°C for 5 days as described by Aikawa & Atkinson (1990). After sectioning, ultrathin sections were picked up on Nickel grids and immuno-stained as follows: The grids were floated (section side down) on a drop of 1% bovine serum albumin/PBS at room temperature for 30 minutes. After washing in PBS they were floated on a drop of neat mab supernatant for 1 hour, washed in PBS, floated on a drop of Protein-A-gold (1:20 dilution in PBS) for 1 hour and again washed in PBS. The grids were then stained with uranyl acetate and Reynold's lead citrate as described above and examined by transmission electron microscopy.

## 2.16. Production and analysis of Parasite RNA.

**Isolation of total RNA.**

Larvae were taken up in 25 volumes of 4M guanadinium thiocyanate solution (Appendix 1) and were homogenised using a Tri-R, Stir-R homogeniser (Camlab) until all the larvae were disrupted. 0.75 volumes of ethanol were added and the sample left at -20°C overnight. The sample was then centrifuged in a siliconised tube (Appendix 1) at 10,000g for 30 minutes using a swing out rotor. The resulting pellet was resuspended in 10ml of 4M guanadinium thiocyanate solution and again thoroughly homogenised. 0.75 volumes of ethanol were added, the sample left at -20°C overnight and centrifuged the following day in a siliconised tube at 10,000g for 30 minutes. The pellet was resuspended in 10ml of phenol (equilibrated with 50mM EDTA), 10ml of 50mM EDTA, pH 8 (DEP treated, Appendix 1) and 10ml of chloroform. The sample was again homogenised and spun in a 50ml Falcon centrifuge tube at 3000g. The top layer was taken and again extracted with phenol after which it was

extracted with ether. The sample was then mixed with 3 volumes of 4M sodium acetate, pH 5.5 (DEP treated), left to stand overnight at 4°C and was finally centrifuged in a siliconised tube at 10,000g for 1 hour. The resulting pellet of precipitated RNA was resuspended in 100ul of DEP treated distilled water.

The yield and purity of RNA was assessed by ultraviolet spectrophotometry at 260 and 280 nm. The yield was calculated assuming that 1 OD unit corresponds to a concentration of RNA of 40ug/ml. The purity of the sample was assessed by the ratio of $OD_{260}/OD_{280}$ which should approach 2 for a very pure sample.

**Purification of poly (A)+ RNA**

Poly (A)+ RNA (messenger RNA) was purified from the samples of total RNA using a Poly(A) Quik mRNA purification kit (Stratagene 200348,200349). Each column of oligo (dT) cellulose could accommodate 500ug of total RNA and gave a 1-2% yield of poly (A)+ RNA.

***In vitro* translation of RNA**

*In vitro* translation of parasite RNA was performed using a standard rabbit reticulocyte lysate system. Either 0.5ug of poly(A)+ RNA or 5ug of total RNA was heated at 67°C for 10 minutes and then the reaction mixture was assembled in an eppendorf tube in the following order:

35ul nuclease treated lysate (Promega L4220)
7ul $dH_2O$ (DEP treated)
1ul ribonuclease inhibitor (Promega N2511)
1ul 1mM amino acid mixture minus methionine (Promega L9961)
2ul RNA substrate in $dH_2O$
4ul $^{35}S$ methionine (Amersham, SJ 204, specific activity 1000 Ci/mMol)

The reaction mixture was incubated at 30°C for 60 minutes after which time 10ul of reaction mixture was added to 30ul of 4x SDS-PAGE sample buffer and boiled for 5 minutes. The samples were run on SDS-PAGE gels and the translation products assessed by fluorography.

## 2.17. Agarose gel electrophoresis.

**Formaldehyde agarose gels for RNA.**

1.2% formaldehyde-agarose gels were prepared by adding 0.6g of agarose to 39ml of distilled water, microwaving until dissolved and then, when cool, adding 10ml of 5x MOPS buffer (Appendix 1) and 1ml of 37% formaldehyde. 20ml was then poured into the gel former (75mm x 50mm) and allowed to set at 4°C.

The samples were prepared by taking 10ug of total RNA and adding 4.5ul 5X MOPS buffer, 7.9ul of 37% formaldehyde solution, 22.5ul formamide and making up to a final volume of 45ul with $dH_2O$. The sample was heated at 55°C for 15 minutes to denature the RNA and immediately chilled on ice. Then 5ul of sample buffer (10mM EDTA/0.25% bromophenol blue in formamide) was added to each sample before loading onto the gel. The gels were run in 1x MOPS running buffer at 40V for several hours.

Gels were stained by immersion in 0.5ug/ml of ethidium bromide for 30 minutes, destained in distilled water and viewed on a transilluminator.

**Agarose gels for DNA.**

Agarose gels for analysis of DNA from recombinant phage (section 2.18.5) were prepared as above, except the agarose was dissolved in 1x TBE buffer (Appendix 1) and 110mm x 140mm gels were used. The DNA samples were diluted 1:1 in sample buffer consisting of 0.25% bromophenol blue, 0.25% Xylene cyanol and 15% Ficoll (type 400).

## 2.18. Construction, analysis and screening of a cDNA library.

### 2.18.1. Library construction.

A cDNA expression library was constructed using the Stratagene ZAP-cDNA synthesis and Uni-Zap$^{TM}$ XR vector system. The protocols of the kits were followed precisely and all the details of the cDNA synthesis, vector ligation and packaging of the library are given in the instruction manual of ZAP-cDNA Synthesis kit (Catalog number #200400, lot number #200400).

**Synthesis of cDNA.**

7.9ug of poly(A)+ RNA from sheathed *D.viviparus* $L_3$, which had been tested by *in vitro* translation, was used to synthesize the first strand. The total cDNA produced from the 7.9ug of poly(A)+ was not quantified but the finished cDNA was size fractionated with a Sephacryl S-400 spun column and the cpm detectable on a hand held monitor for the first three fractions was well within the range given in the protocol for optimal cDNA synthesis. These fractions were pooled, the cDNA ethanol precipitated and resuspended in 10ul of $dH_2O$. Also aliquots of first and second strand synthesis reactions were run on a thin alkaline agarose gel which was examined by autoradiography to check for successful synthesis and lack of hairpinning of the second strand. All these techniques were performed precisely as laid down in the kit protocol (Stratagene, 200400).

**Ligation of cDNA into vector and packaging**

The Uni-Zap$^{TM}$ XR vector was used and 2.5ul of the final cDNA solution was ligated into 1ug of vector arms as described in the instruction manual. The resulting ligated phage were packaged using four separate Gigapack II Gold packaging extracts (Stratagene 200214) which were pooled to give the final library.

Therefore in summary 25% of the cDNA synthesized from 7.9ug of poly(A)+ RNA was ligated into 1ug of vector arms and packaged to produce the final library.

### 2.18.2 Determining the size of the unamplified library.

A 50ml culture of host bacteria (PLK-F') were grown at 30°C in LB media (Appendix 1), supplemented with 0.2% maltose and 10mM $MgSO_4$, with vigorous shaking overnight. The cells were pelleted by centrifugation at 1000g for 10 minutes and resuspended in the appropriate volume of 10mM $MgSO_4$ to give a suspension with an $OD_{600}$ of 0.5. Serial dilutions of an aliquot of the packaging reaction (1:1, 1:10 and 1:100) were made in SM buffer (Appendix 1) and 1ul of each dilution was added to 200ul of the prepared host cell suspension and incubated at 37°C for 15 minutes. This was then mixed with 2ml of top agar (45°C) (Appendix 1) which was then poured onto a prewarmed (37°C) LB agar plate. The plates were incubated overnight at 37°C and the total number of plaque forming units (pfu) in the packaging extract calculated from the number of plaques at each dilution.

### 2.18.3. Amplification of the library.

PLK-F' cells were prepared as described above and aliquots of the packaging extract containing approximately $1x10^5$ recombinant phage were mixed with 600ul of host cell suspension ($OD_{600}$ in 10mM $MgSO_4$). After a 15 minute incubation at 37°C, 6.5ml of melted top agar (45°C) was added and this was poured onto a prewarmed 150mm plate of LB agar. Approximately 30 plates were required for the whole library. The plates were incubated at 37°C for approximately 6 hours and then 10ml of SM buffer was pipetted onto each plate. The plates were incubated overnight at 4°C with gentle rocking. The bacteriophage suspensions from each plate were then pooled into a sterile container, chloroform was added to a concentration of 5% and the mixture incubated at room temperature for 15 minutes. The cell debris was removed by centrifugation at 4000g for 15 minutes. The titre of the amplified library was determined using the same method as for the unamplified library.

### 2.18.4. IPTG/X-gal colour selection.

This assay was performed to determine the ratio of recombinants (white plaques) to non-recombinants (blue plaques) in the amplified library. XL1-Blue host cells were prepared, in the same manner as described for PLK-F' cells, to produce a suspension of $OD_{600}$=0.5 in 10mM $MgSO_4$. Serial dilutions of the amplified library were plated out with the cells by the same method used to determine the size of the library. The 2ml of top agar contained 15ul of 0.5M IPGT (Sigma 300127) and 50ul of 250mg/ml X-gal (Sigma 300201).

### 2.18.5. Determination of insert size for randomly picked plaques from the library.

Single plaques were picked from the library, plated out at a series of dilutions with XL1-Blue cells using 0.3% top agarose and incubated overnight at 37°C. Phage were harvested from the plates which produced confluent lysis by adding 2ml of SM buffer and scraping the agarose/buffer mixture into a 50ml centrifuge tube. A few drops of chloroform were added and the sample centrifuged at 6000rpm for 10 minutes to pellet the debris. 0.8 ml of supernatant was placed in an eppendorf tube and incubated with 0.5 ml of DEAE-cellulose (DE52, Appendix 1) by inverting the tube approximately 50 times. The sample was microfuged for 10 minutes and 0.8ml of

supernatant was transferred to a new eppendorf tube. 150ul of 2.5% SDS, 0.5M Tris-HCl, pH8, 0.25M EDTA was added and the sample heated at 67°C for 15 minutes. After cooling to room temperature, 200ul of 8M ammonium acetate was added and the sample left on ice for 15 minutes. The sample was microfuged for 10 minutes and 0.8ml of supernatant extracted twice with phenol/chloroform (1:1). The 0.8ml of supernatant was mixed with 480ul of isopropanol, left at room temperature for 10 minutes and then microfuged for 10 minutes. The pellet was washed with 70% ethanol and the dried pellet resuspended in 50ul of 10mM Tris, pH 7.5, 1mM EDTA.

10ul aliquots of these DNA preparations were double digested with 10 units EcoR 1 and Xho 1 restriction enzymes using 2ul of the 10x Eco RI incubation buffer supplied (Boehringer Manheim). The sample was made up to 20ul with $dH_2O$, incubated for 2 hours at 37°C after which time the enzymes were inactivated by heating at 65°C for 5 minutes. The samples, along with uncut vector DNA were analysed on a 1.2% agarose gel.

### 2.18.6. Immunoscreening of the library.

The library was plated out with XL1-Blue host cells on 150mm plates at a concentration of 20,000 pfu/plate. The method of plating was the same as that described for the amplification of the library. The plates were incubated at 42°C until small plaques were just visible (approximately 3.5 hours). During this time nitrocellulose filters, cut to the size of the plates, were immersed in 10mM IPTG and left to air dry for 30 minutes. The filters were then placed on the surface of the agar plates ensuring no air bubbles were trapped underneath. The position of the filter was recorded by making 3 asymmetrical stab marks through the filter into the agar. The plates were then incubated at 37°C for 3.5 hours and the filters were carefully removed and washed in Tris saline (Appendix 1) with 0.05% Tween-20. The filters were then probed with antibody using the same method as described for probing Western blots with the following modifications. The first antibody was used at a dilution of 1:100 (neat supernatant for mabs). The second antibodies were alkaline phosphatase conjugates, anti-mouse IgG (Sigma A-1902) or anti-bovine IgG (Sigma A-7914) used at a 1:200 dilution. The filters were developed using the BCIP/NBT substrate (Appendix 1).

Polyclonal antisera used for screening were preabsorbed by a mock immunoscreening procedure to remove any reactivity to bacterial products which might produce a high degree of background during screening. For this purpose a plaque from a test packaging reaction (using wild type DNA supplied with the

Gigapack II packaging extracts) was picked. This was plated out on XL1-blue cells to produce confluent 150mm plates and the screening procedure described above was performed up to the point of second antibody screening. The filters were then discarded and the first antibody solution kept to use for screening the library.

### 2.18.7. Antibody selection using recombinant peptides.

This is a rapid method for isolating the antibodies specific for a recombinant fusion protein of a bacteriophage lambda clone expressed in *E.coli* host cells. This antibody can then be used to probe Western blots of parasite antigens to identify the native protein for which the cloned gene codes. The method is adapted from that first described by Ozaki et al (1986).

The positive phage clone was plated out on XL1-Blue cells at a density of $10^4$ on a 90mm plate which was then incubated at 42°C for 3 hours until plaques were just visible. An IPTG impregnated nitrocellulose filter was overlaid, the plate incubated for two hours at 37°C after which time the filter was turned over and the plate incubated for a further 2 hours. The filter was removed from the plate, rinsed with Tris-saline (Appendix 1) and blocked in the normal manner. After washing the filter in Tris-saline the 10ml of antibody solution was added at a 1:50 dilution in the usual 1st antibody buffer and incubated overnight at room temperature. After washing the filter thoroughly in Tris-saline the bound antibody was eluted by a 5 minute incubation in 3ml of 0.2M glycine, pH 2.8. This solution was removed from the filter and the pH adjusted by the addition of 100ul of 2M Tris pH 7.4. Skimmed milk was added to this eluted antibody solution which was then used to probe a Western blot of *D.viviparus* $L_3$ homogenate using the standard method.

# Chapter 3

# CHAPTER 3

## Evaluation of the mouse as a laboratory animal model for the investigation of immunity to *D.viviparus*.

### 3.1. Introduction.

A small laboratory animal, susceptible to infection with *D.viviparus* and capable of mounting an effective immune response would be very useful for the study of immunity to *D.viviparus*. For a laboratory animal model to be of value in studying the details of host immune responses, the parasite life cycle and host-parasite interactions within the laboratory animal must accurately reflect those which occur in the natural definitive host. However a less than ideal model can be used in preliminary screens to search for candidate protective antigens which, in the case of *D.viviparus*, would otherwise require the use of cattle which would be expensive, even with small groups of animals, and would require relatively large amounts of material for immunisations.

The use of rabbits, guinea pigs, hamsters and mice as possible laboratory animal models for *D.viviparus* has been investigated by a number of workers (Soliman 1953, Douvres & Lucker 1958 and Wade et al 1960). The guinea pig was found to be the most susceptible host in all these studies and it has subsequently been used to investigate the migration route of the parasite and evaluate various methods of immunisation (Poynter et al 1960, Wade, Swanson and Fox 1961, Silverman, Poynter & Podger,1962 and Wilson 1966). However the mouse would have many inherent advantages over the guinea pig if it could be used as an immunological model for *D.viviparus* infection. These include the greater understanding of the murine immune system and the availability of a wide range of immunological reagents together with inbred and specialised mouse strains. For example SCID mice (a strain of mice lacking both B and T cells) can be repopulated with specific cell types, cytokines or antibodies to assess the role of these in the immune response. This can even include repopulation with cells from the natural host species of a particular parasite. Another advantage of the mouse is the ability to use large numbers of animals, relatively cheaply, in order to perform more statistically significant protection experiments. The small size of the mouse also means that smaller amounts of immunising material are required for each animal which is important when parasite material is in short supply. Also monoclonal antibodies can be relatively easily generated from mice to define parasite antigens and to evaluate their possible role in protection by passive immunisation of recipient mice of the same strain.

A brief discussion of the guinea pig model is useful, so that the relative merits of the mouse can be judged when the results are presented. Following experimental oral infection of guinea pigs with *D.viviparus*, larvae have been consistently recovered by baermannisation of tissue from mesenteric lymph nodes and lung but not from the liver (Soliman 1953 and Poynter 1960). Therefore, it seems likely that the parasite follows the lymphatic-blood stream migratory route to the lungs as is known to occur in cattle. The development of *D.viviparus* in the guinea pig has also been studied (Poynter et al 1960, Wilson 1966 and Wade et al 1960a). $L_3$ can be recovered from the lungs as early as 18 hours after oral infection, a mixture of $L_3$ and $L_4$ are present between days 3 and 6 and a mixture of $L_4$ and $L_5$ (immature adults) are present after day 6. The number of larvae recovered from the lungs reaches a maximum around day 8 and then decreases, with only very few larvae being recovered by day 13. The larvae do not develop to sexual maturity and so the life cycle is not complete. The numbers of larvae recovered from the lungs only reaches a maximum of approximately 5% of the infective dose, varying greatly between both different batches of larvae and different individuals infected with larvae from the same batch (Wilson 1966 and Canto 1990). This compares with recoveries from the lungs of cattle of between 37% and 60% of an infective dose of 1000 larvae on days 11 and 13 post infection, with 23% established as adults by day 35 (Jarrett & Sharp 1963).

Immunisation of guinea pigs with two oral infections of either normal or irradiated larvae produces a level of protection to subsequent challenge approaching 100% (Poynter et al 1960 and Wade et al 1960b). Passive immunisation using serum from infection immunised guinea pigs also confers a high degree of protection to recipients (Wilson 1966 and Canto 1990). These results are very similar to immunisation experiments performed on cattle (Jarrett et al 1955a and 1959b) and so the guinea pig has generally been considered to be an acceptable model for the study of immunity to *D.viviparus*. Its use has been mainly confined to experiments aimed at assessing the ability of crude antigen preparations to induce a protective immune response and such studies have produced a variety of different results. The degree of protection induced by immunisation with $L_3$ homogenate has been reported as 50% (Wade et al 1961) or 67% (Canto 1990) using Freund's adjuvant, whilst 97% protection has been reported using aluminium hydroxide as adjuvant (Silverman et al 1962). The protection induced by adult homogenate has been found to range from zero (Wilson 1966 and Canto 1990) to 39% with Freund's adjuvant (Wade et al 1961), 75% using liposomes (Canto 1990) and 94% using aluminium hydroxide as adjuvant (Silverman 1962). Immunisations of guinea pigs with adult E/S material with Freund's adjuvant has given levels of protection from 77% (Canto 1990) to over 90%

although there has been a large degree of variation between individual experiments (McKeand, personal communication).

The relative contributions of acquired immunity and non-specific resistance to the protection observed in these experiments is open to question. Silverman et al (1962) found that immunisation of guinea pigs with homogenates prepared from a number of different parasite species could induce a significant degree of protection against challenge with *D.viviparus*. This included 99% protection with *H.contortus* $L_3$ homogenate, 80% protection with *T.colubriformis* $L_3$ homogenate and even 63% protection with *Echinococcus granulosus* hydatid cyst material. Canto (1990) reported that groups of guinea pigs immunised with adjuvant alone showed significant degrees of protection (58% using Freund's and 42% using liposomes). This non-specific stimulation of resistance to challenge induced by adjuvants has also been reported for protozoal, viral and bacterial infections (Warren & Chedid 1988) and is likely to be a particular problem when the challenge is given only a few weeks after immunisation. Therefore, although the guinea pig is the best laboratory animal model presently available to study immunity to *D.viviparus* infection, it does have some inherent problems. Namely, inconsistent recoveries of larvae from the lungs of susceptible animals, limited development of larvae, a short duration of infection and problems with non-specific resistance to infection following immunisation with adjuvants.

The few experiments which have been performed to evaluate the mouse as a possible model for *D.viviparus* infection have shown that larvae can be recovered from the mesenteric lymph nodes (Soliman 1953) and lungs (Soliman 1953, Douvres & Lucker 1958 and Wade et al 1960) of mice following oral infection. All these workers found that the mouse was less susceptible to infection than the guinea pig and so did not pursue their investigations. However these were very superficial studies, simply involving single oral infections of small numbers of unspecified strains of mice. Therefore, given the potential advantages of the mouse over the guinea pig as an immunological model, a more serious attempt at assessing its possible use as a model for *D.viviparus* was considered worthwhile.

## 3.2. Results.

### 3.2.1. Infection of mice with *D.viviparus*.

In order to determine whether *D.viviparus* $L_3$ would migrate to the lungs of mice, five B10/HTT and five Balb/c mice were infected orally and another five mice of each strain infected intraperitoneally with *D.viviparus*. A dose of 2000 $L_3$ was used and one mouse from each group was killed daily from days 3-7. The numbers of larvae recovered from the lungs of each mouse is shown in table 3.1. Although the design of the experiment did not allow statistically significant conclusions to be drawn, it appeared that some larvae did reach the lungs and it was considered sufficiently encouraging to embark on a more detailed investigation.

Forty Balb/c mice were infected orally with 1000 *D.viviparus* $L_3$ and 4 mice were killed on each day post-infection from days 1 to 10. On days 2, 5 and 7 post-infection, ten of the larvae recovered from the lungs were picked at random and measured using a graduated eye piece along with ten infective larvae which were measured for comparison. A few larvae could be recovered from the lungs as early as 24 hours after infection (table 3.2). The numbers reached a maximum three days post-infection (4.6% of the administered dose) but declined rapidly after day 5 until none could be detected by day 9. These results were consistent with those of the previous experiment. All the larvae recovered appeared to be $L_3$ with no distinguishable signs of development, except that the retained $L_2$ cuticle ($L_3$ sheath) had been shed. There was no significant increase in the mean lengths of the larvae recovered on days 2, 5 and 7 compared to infective larvae which also suggests that there was no development within the murine host (table 3.3).

A further experiment was performed to determine whether the size of infective dose and the route of infection would affect the percentage of the administered dose reaching the lungs of Balb/c mice. Groups of five Balb/c mice were infected with doses of 250, 500 or 1000 larvae by oral, intraperitoneal or subcutaneous routes. All the mice were killed 3 days after infection and the numbers of larvae recovered from the lungs is shown in table 3.4. The percentage of the infective larvae which were present in the lungs three days after infection was independent of the size of the infective dose but a greater percentage of the dose was recovered from the lungs of mice infected by the intraperitoneal and subcutaneous routes compared with the oral route. The percentage of the infective dose reaching the lungs following oral infections seems to be quite consistent in both this and the previous experiments (between 3.4% and 4.7%). As in the previous experiments all the

larvae recovered appeared to be $L_3$ and showed no signs of development except for the loss of the retained $L_2$ cuticle ($L_3$ sheath).

### 3.2.2. Immunisation of mice by infection.

The experiments described above demonstrated that *D.viviparus* larvae migrated to the lungs of mice following infection by oral, subcutaneous or intraperitoneal infection. The proportion of the infective larvae administered which can be recovered from the lungs is very similar to that reported for guinea pigs (Poynter et al 1960, Wade et al 1960a and Canto 1990) and the numbers recovered seem to be quite consistent between experiments. Therefore in these respects the mouse is at least as good as the guinea pig model. However, unlike the situation in the guinea pig, there appears to be no larval development and the decline in larval numbers from 4 days after infection onwards, suggests that the larvae do not actually establish in the lungs. In spite of this limitation, if it is possible for mice to mount an immune response against migrating larvae, they may have some potential as a model to study immunity against reinfection with infective larvae. Also it may be possible to use the mouse to test ability of candidate antigens to stimulate a protective immune response against the $L_3$ stage. Therefore a number of experiments were performed to investigate whether mice could be immunised to prevent the migration of larvae to the lungs. Although the subcutaneous and intraperitoneal routes of infection resulted in larger numbers of larvae reaching the lungs, the oral route was chosen for these experiments in order to simulate the natural route of infection in cattle.

A group of six Balb/c mice were orally infected on two occasions with 1000 *D.viviparus* $L_3$ at an interval of three weeks. They were then challenged orally with 5000 $L_3$, three weeks after the second immunising dose, and a group of previously uninfected controls were challenged with the same dose. Two mice from each group were killed each day from days 2 to 4 post infection (table 3.5). Considering the total number of larvae recovered from the lungs of each group, the immunised group showed 74.1% protection against challenge relative to the control group. For the results taken from the two mice in each group killed on day 3 post-infection, a level of 89.8% protection is shown although the significance of this result cannot be assessed since only two mice are being considered. The morphology, size and viability of the larvae did not appear to differ between the two groups. Therefore this preliminary experiment suggested that fewer larvae reach the lungs of mice which have been immunised by previous infection.

A second experiment was performed with Balb/c, NIH and C57/BL10 mice in order to verify the previous result and to investigate whether a similar response is observed in different mouse strains. A group of six mice of each strain was immunised by a single oral infection of 1000 *D.viviparus* $L_3$ (Group 1) and a second group by a double infection at an interval of three weeks (Group 2). Both groups were challenged orally with 2000 $L_3$, along with a group of non-immunised controls (Group 3), three weeks after immunisation (table 3.6). The percentage of the infective dose recovered from the lungs of the challenge controls was slightly less than in previous experiments, but it was still sufficient for significant differences to be detected in some of the immunised groups. The groups of Balb/c and C57/BL10 mice immunised by the double infection showed 78.7% and 72.4% protection respectively, relative to challenge controls, and these were both statistically significant differences as assessed by the Student t-test at p=0.05. In contrast, the lungs of NIH mice immunised by double infection yielded more larvae than the challenge controls (although this was not a significant difference at p=0.05 by the Student t-test). Fewer larvae were recovered from the lungs of Balb/c and C57/BL10 mice immunised by a single infection than from the controls but these differences were not significantly different from the control groups (Student t-test at p=0.05).

In order that the antibody responses of the mice in each group could be examined, serum samples were taken from all the mice in each group 10 days after each immunisation and also when the mice were killed 3 days after challenge. Serum was also taken from six mice of each strain prior to immunisation to use as normal (pre-immune) serum. An ELISA using $L_3$ homogenate as antigen was used to measure antibodies in these different sera (figure 3.1). For the groups of Balb/c and C57/BL10 mice immunised by a single infection, significant levels of $L_3$ specific antibodies were not detected by the ELISA in sera taken 10 days after the single immunisation but a very low level of antibody was detected in sera taken 3 days after challenge (figure 3.1A). No antibodies were detected in either the post immunisation or post challenge sera of the NIH mice immunised by a single infection. For the groups of Balb/c and C57/BL10 mice immunised by a double infection, no $L_3$ specific antibodies were detected in the sera taken 10 days after the first immunisation but significant levels of antibody were detected in sera taken 10 days after the second immunisation (figure 3.1B). The levels of antibody were reduced in the post challenge sera which was probably due to the short interval of time between challenge and necropsy being insufficient for a full anamnestic response to develop. The same pattern of antibody production was seen in the doubly immunised NIH mice but the levels of antibody were much lower than for the other two strains (figure 3.1B). This is interesting since this strain, unlike the Balb/c and C57/BL10 mice, did not show significant levels of

protection against challenge following double immunisation. Therefore it appears there is a correlation between the level of antibody response and the outcome of challenge.

Immunofluorescence assays (IFA) were performed on viable sheathed and exsheathed $L_3$ to examine whether an antibody response had been generated to either the sheath or the cuticular surface. No such antibodies were detected with any of the Group 1 sera. Similarly there was no antibody produced to the surface of sheathed $L_3$ by post immunisation or post challenge sera from mice immunised by the double infection (Group 2). However the serum taken after the second immunisation of Balb/c and C57/BL10 mice produced a low level of fluorescence with exsheathed $L_3$, as did the post challenge sera of the Balb/c mice (figure 3.2). In contrast the NIH mice did not appear to produce significant levels of $L_3$ cuticular surface specific antibody.

Therefore, in summary, only the doubly immunised Balb/c and C57/BL10 mice showed significant protection against challenge. These mice produced greater levels of antibody to both $L_3$ somatic and surface antigens than the doubly immunised NIH mice which were not protected against challenge.

### 3.2.3. Immunisation of mice with $L_3$ and Adult Homogenates.

The previous experiment demonstrated that immunisation of mice by infection reduced the number of larvae reaching the lungs following a challenge infection. The main purpose of developing the mouse model was to produce a system of testing the ability of particular antigens to stimulate an effective immune response against migrating larvae. Therefore the following experiment was performed to determine whether such a response could be induced by immunisation of mice with crude preparations of parasite antigens. Balb/c mice were used in this experiment since the previous results suggested that C57/BL10 gave a similar response while NIH mice showed little or no response.

A group of six Balb/c mice were immunised twice with 0.2mg of $L_3$ homogenate at an interval of four weeks, using complete Freund's adjuvant for the first immunisation and incomplete for the second. Another group was immunised in the same manner with 0.2mg of adult homogenate and a third group with Freund's adjuvant alone. All three groups were challenged four weeks after the second immunisation with 2000 $L_3$, together with a group of non-immunised challenge controls. All the mice were killed 3 days post challenge and the numbers of larvae recovered from the lungs was determined (table 3.7). There was no significant

difference in the numbers of larvae recovered from the mice immunised with $L_3$ or adult homogenate compared to the Freund's or challenge control groups (Student t-test at $p = 0.05$).

Serum was taken from all the mice in each of the immunised groups before immunisation, 10 days after the second immunisation and also when the mice were killed 3 days after challenge. ELISAs, using both $L_3$ and adult homogenates as antigen, detected high levels of antibody in sera taken 10 days after the second immunisation of mice with either $L_3$ or adult homogenate (figure 3.3). The levels of antibody detected in the sera taken after challenge was less than in the post immunisation sera which may again reflect the lack of time for a full anamnestic response to develop following challenge. The $L_3$ homogenate ELISA detected higher levels of antibody in the sera from mice immunised with $L_3$ homogenate than from mice immunised with adult homogenate. Similarly, the adult homogenate ELISA detected higher levels of antibody in the sera of mice immunised with adult homogenate. Nevertheless, each of the two ELISAs detected significant levels of antibody in both mice immunised with $L_3$ and adult homogenate demonstrating the high degree of cross-reactivity between the antigens from the $L_3$ and adult stages. Significant levels of antibody were not detected in the sera from the Freund's control group.

These sera were also examined by IFA on live sheathed $L_3$, exsheathed $L_3$ and adult *D.viviparus* to determine the levels of surface specific antibody. None of the samples, including those from mice immunised with adult homogenate, produced significant levels of fluorescence with the surface of the adult stage (data not shown). The post immunisation and post challenge sera from mice immunised with $L_3$ homogenate produced only a low level of fluorescence with the exsheathed $L_3$ surface (figure 3.4A) but produced a much higher level of fluorescence with the surface of the $L_3$ sheath (figure 3.4B). In contrast, the sera from mice immunised with adult homogenate did not produce fluorescence with either of the $L_3$ surfaces indicating the stage specificity of the antibodies produced against the surface epitopes of the $L_3$ cuticle and sheath.

In summary, immunisation of mice with *D.viviparus* $L_3$ or adult homogenates using Freund's adjuvant stimulates a marked antibody response against somatic antigens but does not produce a significant level of protection against challenge. The $L_3$ homogenate immunisation stimulates a low level of antibody against the $L_3$ cuticular surface and a higher level of antibody against the $L_3$ sheath surface. Immunisation with adult homogenate does not induce antibody production against the surface of sheathed $L_3$, exsheathed $L_3$ or adult parasites. In spite of the

relatively high antibody produced by these immunisations the mice were not significantly resistant to challenge.

### 3.2.4. Passive Immunisation of mice against *D.viviparus*.

Protection can be transferred to recipient animals by passive immunisation of serum from immune cattle or guinea pigs, thus demonstrating the importance of antibody for protective immunity to *D.viviparus* in these species (Jarrett et al 1955a and Canto 1990). It was important to establish whether this was the case for the mouse, before considering its use as a model to examine immune responses to migrating infective larvae.

The following experiment was performed to produce hyperimmune serum from protectively immunised mice which could subsequently be used for passive immunisation. Two different immunisation regimes were used in an attempt to produce protectively immunised mice with as high a level of serum antibody as possible. Sixteen Balb/c mice were orally infected on four occasions with 5000 *D.viviparus* $L_3$ at three weekly intervals and a second group of sixteen mice were infected subcutaneously with 5000 $L_3$ at the same times. Ten days after the fourth infection, ten mice from each group were killed and bled out by cardiac puncture to produce hyperimmune serum. The remaining six mice in each group, together with 6 non-immunised controls, were challenged with an oral infection of 2000 $L_3$ and killed 3 days later in order to assess the degree of protection to challenge. The orally immunised mice showed 71.2% protection relative to the challenge controls which is significant as assessed by the Student t-test at $p = 0.05$ (table 3.8). However there was no significant difference between the subcutaneously immunised group and the challenge controls.

Serum was taken from six individuals from each of the immunised groups 10 days after each infection and the antibody level assessed by the $L_3$ homogenate ELISA (figure 3.5A). A similar level of antibody to $L_3$ somatic antigens was seen in both groups in spite of the fact that the mice immunised by subcutaneous infection did not show significant protection to challenge. IFA was also performed with the sera on viable sheathed and exsheathed $L_3$. Neither group produced a significant amount of fluorescence with sheathed $L_3$ but sera from both groups taken after the second and subsequent infections produced a low level of fluorescence with the exsheathed $L_3$ surface (data not shown).

Therefore, it appears that repeated oral and subcutaneous infection produced a very similar antibody response in mice as assessed by $L_3$ ELISA and IFA.

However only the oral infection induced a significant degree of protection. This suggests either that antibody is not important in the protective immune response of mice to *D.viviparus* or that there is a qualitative difference in the antibodies induced by subcutaneous infection compared to oral infection. In order to help resolve this, the hyperimmune serum collected from the orally immunised mice was used in a passive immunisation experiment.

Six Balb/c mice were each given 0.7ml of the hyperimmune serum by intraperitoneal injection and a second group of six mice were given the same volume of normal mouse serum. The mice were challenged by oral infection with 2000 *D.viviparus* $L_3$ 2 days after passive immunisation, together with a group of six non-immunised challenge controls, and then killed 3 days after challenge. Serum samples were taken from all the mice in each of the immunised groups before immunisation, immediately before challenge and also at the time of kill. The $L_3$ homogenate ELISA was used to determine whether any antibodies from the passively immunised serum could be detected in the circulation of the recipient mice (figure 3.5b). Low levels of *D.viviparus* specific antibody could be detected in these sera demonstrating that at least some of the passively immunised antibody was present in the circulation at the time of challenge. IFA was also performed but the sera did not produce detectable fluorescence with either sheathed or exsheathed $L_3$.

The numbers of larvae recovered from the passively immunised group were not significantly different from the numbers recovered from either the group immunised with normal serum or the challenge control group (Student t-test at $p = 0.05$) (table 3.9). Therefore, passive immunisation with hyperimmune serum from protectively immunised mice did not transfer a measurable degree of protection to recipient mice.

## 3.3. Discussion.

The purpose of these experiments was to investigate the potential of the mouse as a laboratory animal model to study immunity to *D.viviparus* infection. Such a model would be of particular value as a screen to find candidate protective antigens and as discussed earlier, the mouse would be a much more versatile and economic alternative to the currently used guinea pig system. A model can also be used to investigate mechanisms of host immunity but care must be taken to ensure that extrapolation of results is valid when drawing conclusions about immunity in the natural host. In order to be used for this purpose, a laboratory animal model must provide an accurate representation of the host-parasite interactions which occur in

the natural definitive host. Therefore during the discussion of these results it is useful to consider the potential of the mouse model in two separate ways. For it to be used as a model of bovine immunity to *D.viviparus* infection, the behaviour of the parasite and the resulting immune response would have to be similar in both the murine and bovine hosts. However, failing this, it may still be of use as a simple system to test the ability of particular antigens to stimulate a protective immune response. This is of similar validity as the use of *in vitro* killing assays to test the anti-parasitic properties of particular antibodies. For the mouse to be used for this purpose, the main requirement would simply be a statistically significant and repeatable difference in the larvae recovered from immunised and non-immunised animals.

The results presented have shown that the maximum number of larvae recovered from the lungs of mice following oral infection varies between approximately 2% and 5% of the infective dose. In this respect it is very similar to the results reported for the guinea pig, with a similar degree of variation in recoveries between individual animals (Poynter et al 1960 and Canto 1990). The maximum numbers of larvae were recovered 3 days after infection and after this time the numbers declined until none were present after day 8. Also there appeared to be no visible signs of development or any increase in size of larvae including those recovered as late as 7 days post infection. These results suggest that larvae migrate to the lungs but not establish themselves for very long once they have reached this site. It is possible that during the seven days following infection, larvae continually migrate to the lungs and are rapidly expelled resulting in a constant turnover of larvae in the lungs. In other words, those larvae recovered on day seven may have just reached the lungs rather than being the survivors of those which were present a few days after infection.

Similar results were reported by Wade et al (1960) and Douvres & Lucker (1958) with there being no visible development of larvae in the lungs and no larvae being recovered later than 6 days post infection. However Soliman (1953) reported that *D.viviparus* developed to immature adults in the lungs of mice but did not give any details as to the numbers of larvae found or the longevity of the infection. None of these authors specified the strain of mouse that was used and so it is difficult to make meaningful comparisons with the results presented here.

On the basis of the infection experiments reported here, it seems unlikely that the mouse could be used as a model of immune expulsion of *D.viviparus* from the lungs, since the parasite fails to establish itself and develop at that site in the mouse. However from these preliminary results it seemed possible that the mouse might have some potential as a model for the migration of infective larvae to the lungs and so allow protective immune responses directed at this part of the life cycle to be

examined. In order to investigate this possibility, it was first necessary to determine whether mice could be immunised to produce a measurable reduction in the number of larvae reaching the lungs. Infection with two oral doses of *D.viviparus* produced a level of 79.1% protection in Balb/c mice and 72.4% protection in C57/BL10 mice (the result for the Balb/c mice was supported by the level of 71.2% protection shown by the mice immunised by repeated oral infection to produce hyperimmune serum). This level of protection was statistically significant and suggests that mice can mount a reasonably effective immune response to migrating larvae. However it is somewhat less than the 90-100% protection seen in cattle and guinea pigs immunised by a similar regime (Jarrett et al 1959b , Poynter et al 1960 and Canto 1990). This may be due to the short period of time between challenge and kill which is insufficient to allow a fully effective immune response to develop. Unfortunately this period of time could not be extended due to the short lived nature of *D.viviparus* infection in mice.

Interestingly, the NIH mice immunised by a double oral infection regime did not show significant levels of protection to subsequent challenge. The sera from these mice contained much lower levels of antibody compared to the sera from the Balb/c and C57/BL10 mice. Also serum from the latter two strains produced a low level of fluorescence with the exsheathed larval surface whereas no such response could be detected in the NIH sera. This correlation between antibody levels and protection does not necessarily imply that this antibody is involved in the protective immune response. It may simply be a reflection of the generally poor immune response of NIH mice to *D.viviparus* infection and if other parameters had been measured, such as cellular immune responses, these too may have been relatively poor compared with those of the other strains.

Immunisation of Balb/c mice with $L_3$ or adult homogenate did not produce a significant degree of protection to subsequent challenge. This result is perhaps not very surprising given the variable results reported for immunisation of guinea pigs using these antigens (Wade et al 1961, Silverman et al 1962, Wilson 1966 and Canto 1990). However it does illustrate the difficulties which may be experienced in trying to induce protective immunity with non-living parasite material. An interesting feature of this experiment was the particularly marked antibody response against the surface of the $L_3$ sheath in the mice immunised by $L_3$ homogenate. These mice produced a significantly lower response against the $L_3$ cuticular surface. This is very similar to the antibody response of cattle, as measured by IFA, following infection or vaccination (Britton 1991). It is curious that immunisation of mice using $L_3$ homogenate produced an IFA antibody response more similar to that of infected or vaccinated cattle than that produced by immunisation of mice by infection.

In cattle, subcutaneous administration of larvae produces a high degree of protection, similar to that produced by oral immunisation (Bain & Urquhart 1988). Therefore the migration of larvae through the mesenteric lymph nodes is not essential for the stimulation of a protective immune response in the bovine host. However immunisation of mice by repeated subcutaneous infection did not produce a significant degree of protection against subsequent challenge. This difference is a good illustration of the nature and limitations of the mouse model. In cattle it is likely that immune mechanisms act on a number of different parasite stages in a number of different tissues. Therefore, even if the mesenteric lymph nodes are one of those tissues, the stimulation of immunity at other sites by subcutaneous immunisation is sufficient to produce an effective immune response. However in the mouse model, where animals were killed three days after challenge, it is likely that any protection seen is due to the effect of immunity on migrating larvae rather than the effect of immunity on the establishment of the parasite in the lung. Therefore it is quite possible that migration of larvae through the mesenteric lymph nodes is necessary to stimulate a protective immune response in the mouse.

In spite of the difference in protection between the mice immunised by subcutaneous or oral infection, the $L_3$ homogenate ELISA and the IFA detected similar levels of antibody in sera from these groups. This does not necessarily suggest that antibody is not important in the protective immunity of mice to *D.viviparus* since there could be qualitative differences in the antibody induced by the two different routes of administration. The passive immunisation experiment was performed to help resolve this issue. Recipients were not protected by passive immunisation with serum from mice protectively immunised by oral infection. This might suggest that the protective immunity seen in mice is not antibody mediated, however this experiment alone is insufficient to draw such a conclusion. Although the ELISA performed on the sera of recipient mice suggested that *D.viviparus* specific antibody had been successfully transferred, the levels of antibody were much lower than those present in the sera of the donor mice. Therefore the failure of this to protect the recipients may be a purely quantitative phenomenon.

In summary, the mouse is an unsatisfactory model to study immune-mediated expulsion of *D.viviparus* from the lungs since the parasite does not establish itself at this site in the mouse. Nevertheless, a significant degree of immunity to migrating larvae can be induced and so the mouse may have some potential as a model of immune responses to the early stages of infection with this parasite. However there are some serious reservations concerning its use in this manner. Firstly, the loss of larvae from the lungs after day three gives immune responses very little time to act and it may be over optimistic to expect significant immunity to be demonstrable

following immunisation with individual parasite antigens. Secondly, the IFA results suggest that there are some important differences in the antibodies produced to the $L_3$ cuticular and sheath surfaces following infection of mice compared with those seen following infection of cattle. For these reasons extrapolation of results from the murine to the bovine host may be invalid.

**Table 3.1**

Oral and intraperitoneal (I/P) infection of B10/HTT and Balb/c mice with 2000 *D.viviparus* $L_3$. One mouse from each group was killed daily from day 3 to day 7 post infection and the figures refer to the total numbers of larvae recovered from the lungs of each individual mouse.

**Table 3.2**

Forty Balb/c mice were orally infected with 1000 *D.viviparus* $L_3$. Four mice were killed on each day following infection from days 1 to 10 and the total numbers of larvae recovered from the lungs of individual mice are shown in the table.

**Table 3.3**

Ten larvae were randomly chosen from those recovered on days 2, 5 and 7 post infection and their lengths measured using a graduated eyepiece. The mean lengths are given in um and the standard deviations are given for measurements on the larvae recovered on each day. Day 0 refers to ten infective larvae which were measured for comparison.

**TABLE 3.1**

| Day | B10/HTT | | Bal b/c | |
|---|---|---|---|---|
| | Oral | I/P | Oral | I/P |
| 3 | 43 | 143 | 83 | 186 |
| 4 | 19 | 135 | 48 | 172 |
| 5 | 22 | 92 | 15 | 76 |
| 6 | 26 | 38 | 8 | 38 |
| 7 | 4 | 21 | 1 | 24 |
| **Total** | 114 | 429 | 155 | 496 |

**TABLE 3.2**

| Day | Number of larvae | | | | Mean |
|---|---|---|---|---|---|
| 1 | 12 | 9 | 8 | 6 | 8.75 |
| 2 | 35 | 26 | 16 | 10 | 21.75 |
| 3 | 58 | 49 | 42 | 28 | 46.75 |
| 4 | 40 | 29 | 23 | 15 | 26.75 |
| 5 | 46 | 30 | 24 | 5 | 26.25 |
| 6 | 5 | 3 | 3 | 1 | 3.00 |
| 7 | 7 | 4 | 4 | 2 | 4.25 |
| 8 | 2 | 2 | 1 | - | 1.67 |
| 9 | 0 | 0 | 0 | 0 | 0.00 |
| 10 | 0 | 0 | 0 | 0 | 0.00 |

**TABLE 3.3**

| Day | Mean Length | Standard Deviation |
|---|---|---|
| 0 | 328.4 | 16.6 |
| 2 | 354.1 | 22.5 |
| 5 | 321.9 | 25.6 |
| 7 | 343.8 | 20.2 |

**Table 3.4**

Groups of five Balb/c mice were infected with 250, 500 or 1000 *D.viviparus* $L_3$ by oral, intraperitoneal (I/P) or subcutaneous (S/C) infection. The figures in the table show the numbers of larvae recovered from the lungs of individual mice in each group. % dose = the mean number of larvae for the group expressed as a percentage of the infective dose.

**TABLE 3.4**

| | Infection with 250 larvae | | |
|---|---|---|---|
| | **Oral** | **I/P** | **S/C** |
| | 15 | 38 | 27 |
| | 9 | 14 | 24 |
| | 6 | 11 | 20 |
| | 4 | 9 | 20 |
| | - | 8 | 18 |
| **Mean** | 8.5 | 16.0 | 21.8 |
| **% dose** | 3.4 | 6.4 | 8.7 |

| | Infection with 500 larvae | | |
|---|---|---|---|
| | **Oral** | **I/P** | **S/C** |
| | 59 | 68 | 83 |
| | 19 | 46 | 82 |
| | 16 | 25 | 67 |
| | 12 | 10 | 61 |
| | 11 | 8 | 18 |
| **Mean** | 23.4 | 31.4 | 62.2 |
| **% dose** | 4.7 | 6.3 | 12.4 |

| | Infection with 1000 larvae | | |
|---|---|---|---|
| | **Oral** | **I/P** | **S/C** |
| | 65 | 107 | 98 |
| | 41 | 84 | 92 |
| | 38 | 79 | 81 |
| | 27 | 76 | 78 |
| | 23 | 48 | 52 |
| **Mean** | 38.8 | 78.8 | 80.2 |
| **% dose** | 3.8 | 7.9 | 8.0 |

**Table 3.5**

The immunised group were orally infected at an interval of three weeks, with 2 doses 1000 *D.vivíparus* $L_3$ and were challenged, together with the non-immunised control group, with 5000 $L_3$ three weeks after the second immunising infection. Two mice were killed from each group on days 2, 3 and 4 post challenge and the numbers of larvae recovered from the lungs of individual larvae are shown in the table.

**TABLE 3.5**

| Day | Immunised Group | Control Group |
|---|---|---|
| 2 | 122 | 317 |
| | 88 | 192 |
| 3 | 29 | 176 |
| | 16 | 265 |
| 4 | 19 | 138 |
| | 27 | 76 |
| **Total** | 301 | 1164 |

**Table 3.6**

Mice of three different strains were immunised by oral infection with *D.viviparus* $L_3$ and challenged with 2000 $L_3$ three weeks after immunisation. The figures shown are the total numbers of larvae recovered from individual mice killed 3 days after challenge.

Group 1 = Immunised once by oral infection with 1000 $L_3$.
Group 2 = Immunised twice by oral infection with 1000 $L_3$.
Group 3 = Non-immunised challenge controls.

$$\% \text{ protection} = \frac{\text{Control group mean - Immunised group mean}}{\text{Control group mean}}$$

**TABLE 3.6**

**Balb/c**

| | Group 1 | Group 2 | Group 3 |
|---|---|---|---|
| | 58 | 17 | 60 |
| | 39 | 13 | 59 |
| | 30 | 9 | 53 |
| | 21 | 9 | 41 |
| | 13 | 6 | 34 |
| | 9 | 3 | 18 |
| **Mean** | 28.3 | 9.5 | 45.5 |
| **% Protection** | 37.8% | 79.1% | |

**C57/BL10**

| | Group 1 | Group 2 | Group 3 |
|---|---|---|---|
| | 57 | 27 | 55 |
| | 45 | 19 | 46 |
| | 29 | 10 | 43 |
| | 12 | 6 | 40 |
| | 7 | 5 | 35 |
| | - | 3 | 33 |
| **Mean** | 30.0 | 11.6 | 42.0 |
| **% Protection** | 28.6% | 72.4% | |

**NIH**

| | Group 1 | Group 2 | Group 3 |
|---|---|---|---|
| | 58 | 69 | 60 |
| | 37 | 45 | 27 |
| | 29 | 36 | 26 |
| | 19 | 28 | 25 |
| | 15 | 19 | 24 |
| | 11 | 14 | 15 |
| **Mean** | 28.1 | 35.2 | 29.5 |
| **% Protection** | 4.7% | -19.2% | |

**Figure 3.1**

**A.** $L_3$ homogenate ELISA on sera from mice immunised by a single oral infection with *D.viviparus* $L_3$ (group 1). The optical densities are the means of duplicate samples from all six individual mice in each group.

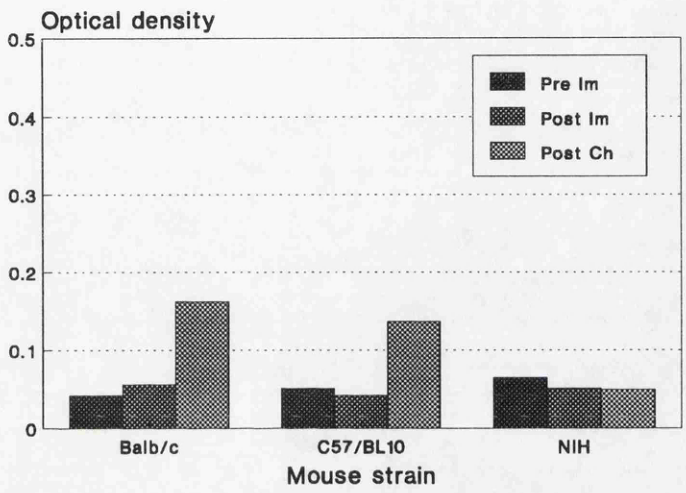
Pre Im = Serum taken from six mice prior to immunisation.
Post Im = Serum taken from mice 10 days after the single immunising dose of 1000 $L_3$.
Post Ch = Serum taken when mice were killed 3 days after challenge with 2000 $L_3$.

**B.** $L_3$ homogenate ELISA with sera from mice immunised by a double infection with *D.viviparus* $L_3$ (group 2). Optical densities are the means of duplicate samples from all six individual mice in each group.

Pre Im = Serum taken from six mice prior to immunisation.
Post Im 1 = Serum taken from mice 10 days after the first immunising dose of 1000 $L_3$.
Post Im 2 = Serum taken from mice 10 days after the second immunising dose of 1000 $L_3$.
Post Ch = Serum taken when mice were killed 3 days after challenge with 2000 $L_3$.

The ELISA was also conducted on pre and post challenge sera from the challenge control group (group 3) and no increase was detected in the optical densities following challenge.

A


Optical density
0.5
0.4
0.3
0.2
0.1
0
Pre Im
Post Im
Post Ch
Balb/c
C57/BL10
NIH
Mouse strain

B

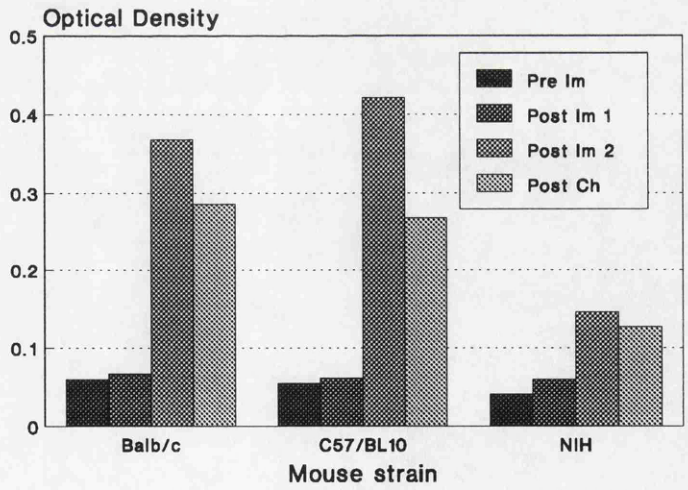

Optical Density
0.5
0.4
0.3
0.2
0.1
0
Pre Im
Post Im 1
Post Im 2
Post Ch
Balb/c
C57/BL10
NIH
Mouse strain

**Figure 3.2**

Quantitative IFA on exsheathed *D.viviparus* $L_3$ with serum from mice immunised by double infection (group 2). The relative fluorescence given for each sample is the mean of readings taken from 20 individual larvae using pooled sera from the six individual mice in each group.

Pre Im = Serum taken from six mice prior to immunisation.
Post Im 1 = Serum taken from mice 10 days after the first immunising dose of 1000 $L_3$.
Post Im 2 = Serum taken from mice 10 days after the second immunising dose of 1000 $L_3$.
Post Ch = Serum taken when mice were killed 3 days after challenge with 2000 $L_3$.

The levels of background fluorescence with the pre-immune sera are due to the autofluorescence of larvae which is a yellow colour and can be visually distinguished from the green fluorescence produced by the FITC conjugate.

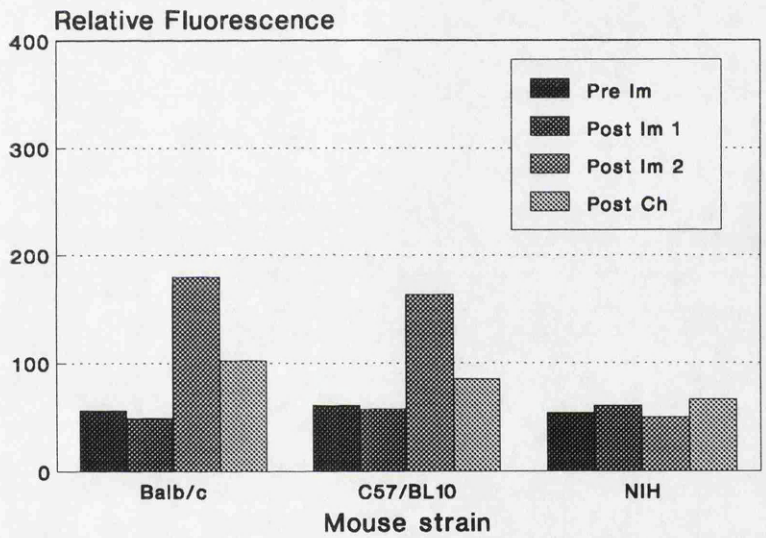

Relative Fluorescence
400
300
200
100
0
Pre Im
Post Im 1
Post Im 2
Post Ch
Balb/c
C57/BL10
NIH
Mouse strain

**Table 3.7**

The number of larvae recovered from the lungs of individual mice killed 3 days after challenge with 2000 *D.viviparus* $L_3$ following immunisation with parasite homogenates. Mice were immunised twice at an interval of four weeks by intraperitoneal injection of antigen with Freund's adjuvant and challenged 4 weeks after the second immunisation.

$L_3$ homogenate = Immunised twice with 0.2mg of $L_3$ homogenate.
Adult homogenate = Immunised twice with 0.2mg of adult homogenate.
Freund's = Immunised twice with Freund's adjuvant alone.
Control = Non-immunised challenge controls.

**TABLE 3.7**

| | L3 Homogenate | Adult Homogenate | Freunds | Control |
|---|---|---|---|---|
| | 113 | 142 | 123 | 94 |
| | 110 | 102 | 91 | 79 |
| | 67 | 69 | 84 | 62 |
| | 58 | 66 | 73 | 58 |
| | 55 | 47 | 39 | 54 |
| | 51 | Died | 19 | 39 |
| **Mean** | 75.7 | 85.2 | 71.5 | 64.3 |

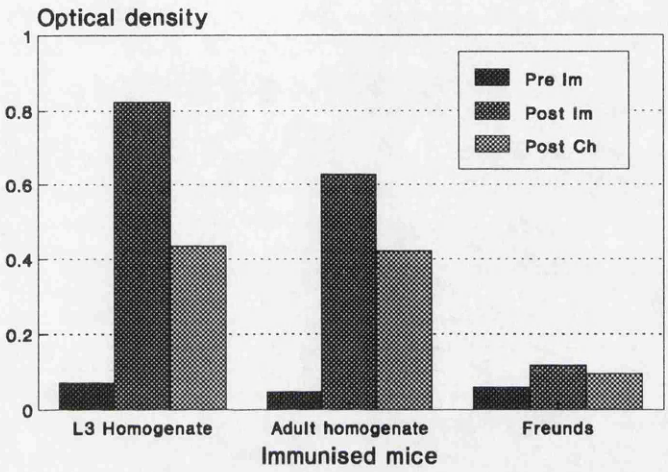
**Figure 3.3**

**A.** $L_3$ homogenate ELISA performed on sera from mice immunised with *D.viviparus* homogenates or Freund's adjuvant alone. The optical densities are the means of duplicate samples from all six individual mice in each group.

Pre Im = Sera taken prior to immunisation.
Post Im = Sera taken 10 days after the second immunisation.
Post Ch = Sera taken when mice were killed 3 days after challenge.

**B.** Adult homogenate ELISA performed on the same samples as in figure A.

A


Optical density
1
0.8
0.6
0.4
0.2
0
Pre Im
Post Im
Post Ch
L3 Homogenate
Adult homogenate
Freunds
Immunised mice

B

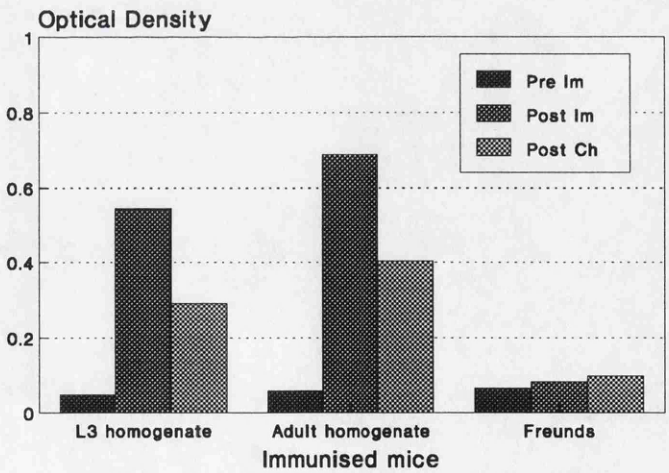

Optical Density
1
0.8
0.6
0.4
0.2
0
Pre Im
Post Im
Post Ch
L3 homogenate
Adult homogenate
Freunds
Immunised mice

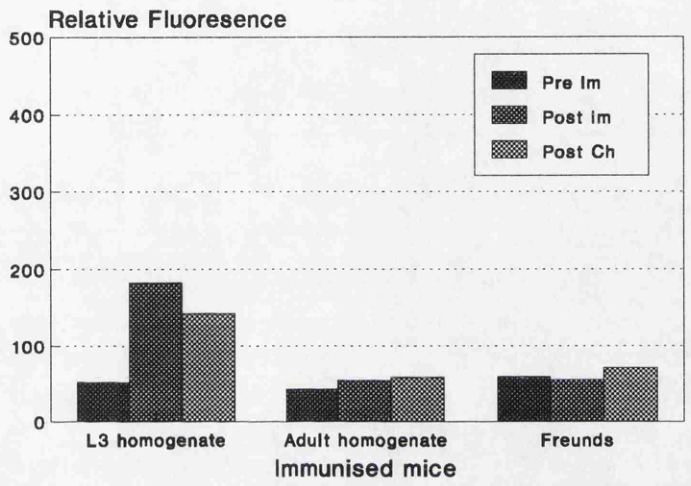
**Figure 3.4**

**A.** Quantitative IFA on exsheathed *D.viviparus* $L_3$ with serum from mice immunised with *D.viviparus* homogenates or Freund's adjuvant alone. The relative fluorescence given for each sample is the mean of readings taken from 20 individual larvae using pooled sera from the six individual mice in each group.

Pre Im = Sera taken prior to immunisation.
Post Im = Sera taken 10 days after the second immunisation.
Post Ch = Sera taken when mice were killed 3 days after challenge.

**B.** Quantitative IFA on sheathed *D.viviparus* $L_3$ using the same sera as in figure A.

A


Relative Fluoresence
500
400
300
200
100
0
Pre Im
Post Im
Post Ch
L3 homogenate
Adult homogenate
Freunds
Immunised mice

B

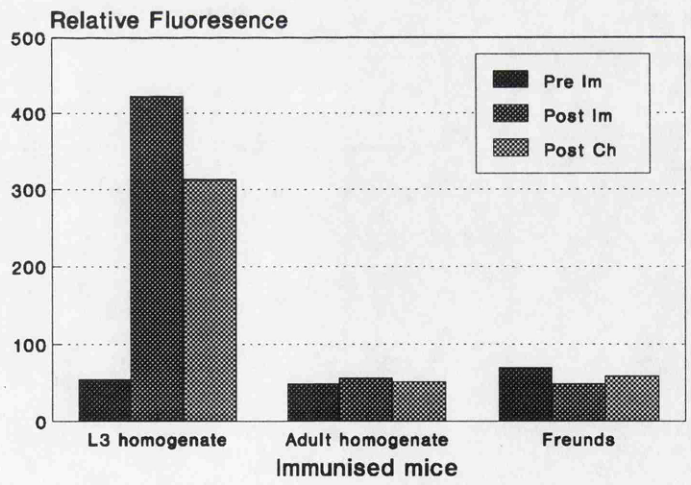

Relative Fluoresence
500
400
300
200
100
0
Pre Im
Post Im
Post Ch
L3 homogenate
Adult homogenate
Freunds
Immunised mice

**Table 3.8**

Numbers of larvae recovered from the lungs of individual Balb/c mice following challenge by oral infection with 2000 *D.viviparus* $L_3$. Mice were immunised by infection, either orally or subcutaneously, with 5000 $L_3$ on four occasions at an interval of 4 weeks. The challenge was given 10 days after the fourth immunising dose and the mice were killed 3 days after challenge.

**Table 3.9**

Number of larvae recovered from the lungs of individual Balb/c mice when killed 3 days after challenge by oral infection with 2000 *D.viviparus* $L_3$. Mice were passively immunised by intraperitoneal injection of 0.7ml of either hyperimmune or normal mouse serum and then challenged 2 days later.

**TABLE 3.8**

| | Oral Immunisation | Subcutaneous Immunisation | Controls |
|---|---|---|---|
| | 42 | 115 | 124 |
| | 28 | 72 | 106 |
| | 23 | 70 | 91 |
| | 17 | 69 | 74 |
| | 14 | 28 | 38 |
| | 8 | 22 | 25 |
| **Mean** | 22.0 | 62.7 | 76.3 |
| **% Protection** | 71.2 | 17.8 | |

**TABLE 3.9**

| | Hyperimmume Serum | Normal Serum | Challenge Controls |
|---|---|---|---|
| | 129 | 114 | 109 |
| | 115 | 91 | 75 |
| | 53 | 66 | 51 |
| | 49 | 53 | 43 |
| | 41 | 32 | 36 |
| | 15 | 26 | 14 |
| **Mean** | 67.0 | 63.6 | 54.7 |

**Figure 3.5**

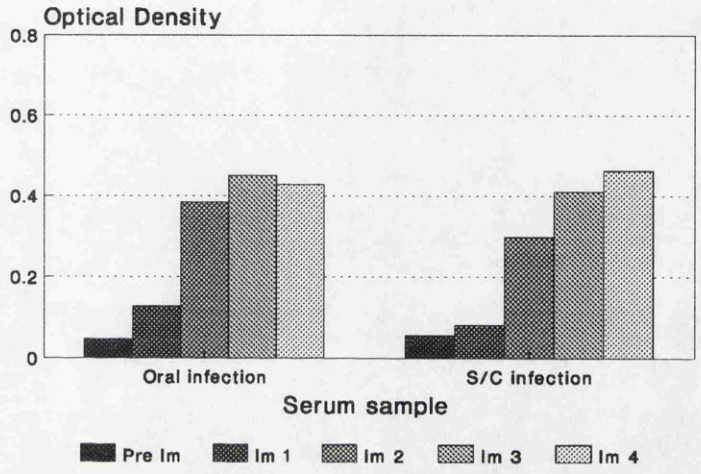
**A.** $L_3$ homogenate ELISA on serum taken 10 days after repeated oral or subcutaneous (S/C) infections of mice with 5000 *D.viviparus* $L_3$. The optical densities are the means of duplicate samples from six individual mice from each group.

Pre Im = serum taken prior to infection.
Im 1 to Im 4 = Immunisation 1 to immunisation 4.

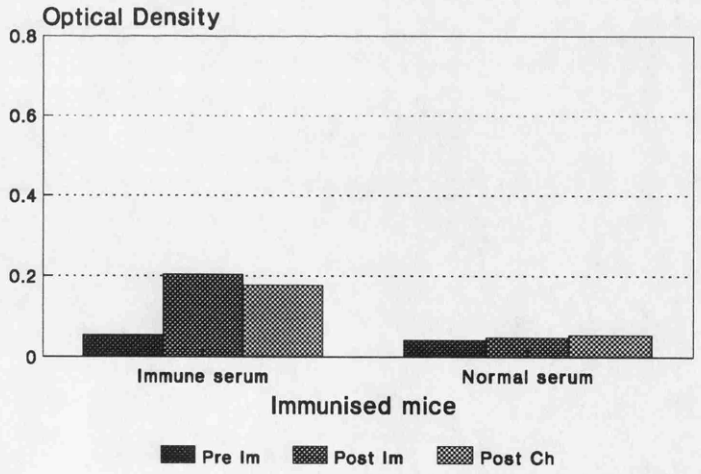
**B.** $L_3$ homogenate ELISA on serum taken from the recipient mice passively immunised either by intraperitoneal injection with 0.7ml hyperimmune donor serum (immune serum) or the same volume of serum from non-immunised mice (normal serum).
The optical densities are the means of duplicate samples from all six individual mice in each group.

Pre Im = Serum taken prior to passive immunisation.
Post Im = Serum taken 2 days after passive immunisation (immediately before challenge).
Post Ch = Serum taken when the mice were killed 3 days after challenge.

A


Optical Density
0.8
0.6
0.4
0.2
0
Oral infection
S/C infection
Serum sample
Pre Im
Im 1
Im 2
Im 3
Im 4

B


Optical Density
0.8
0.6
0.4
0.2
0
Immune serum
Normal serum
Immunised mice
Pre Im
Post Im
Post Ch

# Chapter 4

# CHAPTER 4

## Surface antigens of *D.viviparus* infective larvae.

### 4.1. Introduction.

The infective larvae of *D.viviparus* is considered to be a potentially important stage in the protective immune response of cattle against re-infection (Jarrett et al 1957b, Poynter et al 1960 and Jarrett & Sharp 1963). The surface of the $L_3$ cuticle is an obvious target for host immune responses and the surface of the $L_3$ sheath, although a less obvious target, is highly immunogenic in infected and vaccinated cattle (Britton 1991). Very little is known about the antigens present on these surfaces and so the characterisation of these antigens was considered to be an important priority in the investigation of bovine immunity to *D.viviparus*.

A number of different approaches can be taken to identify and analyse antigens on the cuticular surface of nematodes and a discussion of the relative merits of these is useful before considering the results obtained for *D.viviparus*. The three most commonly used approaches are, surface labelling techniques, monoclonal antibody production and the investigation of the binding of different lectins to the cuticular surface.

### 4.1.1. Surface labelling of the nematode cuticle.

This involves the labelling of living nematodes, usually with $^{125}$Iodine, followed by the release of labelled molecules either by physical disruption or by the use of detergents to selectively strip the surface of intact worms. The labelled molecules can then be analysed by SDS-PAGE and, in the case of $^{125}$Iodine labelling, autoradiography. This strategy was first used to label mammalian cell surfaces (Philipps & Morrison 1970) and was first applied to nematodes by Philipp et al (1980) in *Trichinella spiralis* and has subsequently become a standard technique to probe nematode surfaces. However a variety of different methods have been used and the assumption that the technique is "surface specific" can be misleading. Different catalysts often label different sets of molecules in the same nematode species (Baschong & Rudin 1982, Maizels et al 1983a and Marshall & Howells 1985) which is thought to be due to differences in the depth of penetration as well as the chemical specificity of the catalyst (Marshall & Howells 1985). The Bolton-Hunter reagent, for example, has been shown to penetrate to the somatic

structures of *Brugia pahangi* and so labels a larger subset of molecules than does Chloramine-T or lactoperoxidase which are restricted to the cuticle (Marshall & Howells 1985). The penetration of a particular catalyst varies with the species of nematode being considered, for example the Bolton-Hunter reagent labels the same molecules as Chloramine-T in *T.spiralis* and so appears to be more cuticle specific in this species (Parkhouse et al 1981).

Even though radiolabelling with Chloramine-T, lactoperoxidase and Iodogen is often confined to the cuticle (Parkhouse et al 1981, Baschong & Rudin 1982, Forsyth et al 1984a,Philipp et al 1984 and Marshall & Howells 1985) the labelling is not truly surface specific as is clearly shown by the labelling of collagens which invariably occurs (Philipp et al 1984, Selkirk 1991, Cox et al 1989, Rhoads & Fetterer 1990). A particularly pertinent example of this is the filarial "surface" glycoprotein gp29 which was first identified by radiolabelling and was thought for a long time to be a true surface molecule. However recent immunogold electron microscopic studies suggest that this molecule is not exposed on the surface or even present in the epicuticle but is predominantly located in the outer cortex and present in the deeper cuticular layers and hypodermis (Selkirk et al 1990 and Devaney 1991). Therefore the ability of radiolabelling techniques to selectively identify true surface molecules is very limited.

Another factor affecting which molecules are detected by radiolabelling methods is the chemical specificity of the catalyst used. Chloramine-T, Iodogen and lactoperoxidase mediate the covalent linking of $^{125}$Iodine principally to tyrosine residues (Hunter & Greenwood 1962), the Bolton-Hunter reagent to lysine residues (Bolton & Hunter 1973) and iodosulfanilic acid to lysine, tyrosine and histidine (Higgins & Harrington 1955). Iodogen and Lactoperoxidase will also label lipids (Hayunga & Murrell 1982 and Scott et al 1988) but carbohydrates will not be labelled by any of these methods.

Recently there has been some interest in the use of reactive biotin moeities to label cuticular molecules (Alvarez et al 1989, Keith et al 1990 and Hill et al 1990). This involves the labelling of living nematodes with biotin followed by the analysis of molecules released by physical disruption or detergent stripping as with radiolabelling. The analysis involves probing of Western blots with streptavidin conjugates (eg.peroxidase or alkaline phosphatase) after which the labelled molecules are visualised by development with the appropriate substrate. This system has some potentially important advantages over radiolabelling. Firstly, it is non-radioactive which means it is safer, labelled material can be stored indefinitely at -70$^{o}$C and there is no of the denaturation of labelled antigen which has been associated with some radiolabelling methods (Marshall & Howells 1985). Secondly, streptavidin can be used to localise labelled molecules within the parasite, for example with FITC conjugates on IFA,

peroxidase conjugates for histocytochemical examination or colliodal gold conjugates on electron microscopy. Thirdly, it should be possible to use streptavidin affinity columns to purify labelled molecules and fourthly different biotin moeities are available to allow selective labelling, eg. biotin-hydrazide which labels carbohydrate and NHS-biotin which labels lysine residues.

Alverez et al (1989) compared the cuticular proteins identified in *Brugia malayi* by labelling with Iodogen and sulfosuccinimidobiotin (sulpho-NHS-biotin) which label tyrosine and lysine residues respectively. A virtually identical series of molecules were labelled in each case and electron microscopic examination of sections probed with streptavidin revealed that the biotin was confined to the cuticle, although not specific to the surface. Furthermore labelled proteins could be separated from unlabelled molecules using avidin-agarose beads. Keith et al (1990) labelled polypeptides on the different developmental stages of *Ostertagia circumcincta* and *Ostertagia ostertagi* and demonstrated that at least some labelled molecules were exposed on the surface by probing live worms with a streptavidin-FITC conjugate followed by examination using fluorescent microscopy. Most of the fluorescence could be stripped away by incubation in 0.25% CTAB suggesting that most of the labelling was superficial, although electron microscopic studies were not performed to confirm this. Hill et al (1990) compared the cuticular molecules of different stages of *Ascaris suum* which were labelled by sulpho-NHS biotin (aqueous soluble) and NHS-biotin (organic soluble). Interestingly, in the $L_4$ and adult stages fewer proteins were labelled with sulpho-NHS-biotin than with NHS-biotin and electron microscopy demonstrated that the former label was confined to the outer cortex and epicuticle whereas the latter was present throughout the whole depth of the cuticle. The authors suggested that the organic solubility of NHS-biotin meant it could penetrate the lipid barrier of the epicuticle much more efficiently than the aqueously soluble NHS-biotin.

The selective stripping of surface layers by detergents, with and without reducing agents, has been applied in conjunction with radiolabelling to attempt to identify surface molecules more specifically. Examples include *Strongyloides ratti* (Murrell & Graham 1982), *Nematospiroides dubius* (Pritchard et al 1985), *B.pahangi* (Sutanto et al 1985), *Onchocerca volvulus* (Taylor et al 1986), *T.spiralis* (Grencis et al 1986) and *O.circumcincta* and *O.ostertagi* (Keith et al 1990). The ability of particular detergents to solubilise surface associated molecules differs between different nematode species. Murrell & Graham (1982) failed to solubilise any surface components from *S.ratti* with 1% NP40, 5% sodium deoxycholate or 1% SDS. Pritchard et al (1985) found that the cationic detergent CTAB solubilised most of the surface labelled components of *N.dubius* but a variety of anionic, zwitterionic and nonionic detergents had little effect and they suggested that this may reflect the net negative charge of the epicuticle. However 1%

SDS can solubilise molecules from the surface of intact *Dirofilaria immitis* $L_3$ (Mok et al 1988) and *Haemonchus contortus* $L_3$ (Cox et al 1990). Interestingly the *B.malayi* gp29 molecule cannot be solubilsed from intact parasites in the presence of detergent alone but incubation of parasites in 2-mercaptethanol causes its release (Maizels et al 1989 and Devaney et al 1991) which may suggest it is anchored by reducible bonds perhaps to the underlying collagen molecules (Devaney 1991). Therefore detergent solubilisation techniques are useful in producing enriched preparations containing surface antigens but do not provide direct evidence of surface location of a particular molecule.

The sensitivity of "surface" labelled molecules to incubation of live worms with proteases, eg. trypsin, has also been used as evidence of exposure at the cuticular surface (Maizels 1989). However little is known about the permeability of the epicuticle to proteins of this size (23.5 kDa) or whether such proteases are capable of progressively degrading the surface and releasing superficial as well as surface molecules. Alverez et al (1989) found that all of the Iodogen-labelled molecules in adult *B.malayi* were released by overnight incubation in trypsin and although Maizels et al (1989) found that gp29 could be released by incubations of as little as one hour no immunogold EM studies have yet demonstrated its presence on the cuticular surface.

In summary, although "surface" labelling and solubilisation techniques have played a central role in the study of nematode cuticular molecules, they are of limited value in defining which molecules are actually exposed on the nematode surface. Evidence for this must be obtained by complementary ultrastructural and immuno-electronmicroscopic studies.

### 4.1.2. Monoclonal antibodies as probes of the nematode surface.

The generation of monoclonal antibodies is another approach to identify molecules on the surface of the nematode cuticle and has been used in a variety of nematode species including *T.spiralis* (Oretega-Pierres 1984 and McClaren et al 1987), *B.malayi* (Carlow et al 1987), *B.pahangi* (Sutanto et al 1985), *Toxocara canis* ( Maizels et al 1987b and Kennedy et al 1987c) and *Trichostrongylus colubriformis* (Milner & Mack 1988). One inherent advantage it offers over surface labelling techniques is that any antibody binding to the surface of living parasites must recognise a true surface epitope. Other advantages of monoclonal antibodies are that they detect carbohydrate moeities as well as polypeptides (Maizels et al 1987b & Kennedy et al 1987c) and once produced they can be used for a range of purposes including immunoaffinity purification (Silberston & Despommier 1984), immunogold-EM localisation of antigens (Rudin 1990) and also for passive immunisation experiments (Appleton et al 1988 and Roach et al

1991). Disadvantages are that the molecules identified are biased towards those which are the most immunogenic in the animal used for the fusion and so may not be a true reflection of all the molecules exposed on the cuticular surface. Also antibodies are sometimes produced to cross-reactive epitopes and so are difficult to use as probes to examine a particular cuticular antigen (Sutanto et al 1985).

### 4.1.3. Lectins as probes of the nematode surface.

It is well documented that carbohydrates, conjugated to either protein or lipid, can be potent immunogens (Hughes 1976) and in recent years such antigens have been receiving increasing attention in both protozoal and helminth infections. Examples include the leishmania lipophosphoglycan which has been shown to suppress protective immune responses (Mitchell & Handman 1986) and an epitope on an oligosaccharide of a surface glycoprotein of *Schistosoma mansoni* which can induce a protective immune response (Grzych et al 1985). In nematodes both cuticular polypeptides (Maizels et al 1987a) and lipids (Scott et al 1988) have been shown to be glycosylated and many such glycoconjugates have been shown to be antigenic during infection (Kennedy et al 1987c, Smith et al 1983 and Furman & Ash 1983).

Lectins are high molecular weight substances, usually proteins, produced by plants and invertebrates which bind to specific carbohydrates. This specificity and large molecular size means that they can be used in the same way as antibodies to probe the nematode surface and also in techniques such as affinity purification, ultrastructural localisation and Western blotting. Fluorescein-labelled lectins have been used to characterise the carbohydrates on the cuticular surface of a number of nematode species including some filarial nematodes (Furnham & Ash 1983, Kaushal et al 1984 and Paulson et al 1984), *T.spiralis* (Ortega-Pierres 1984), *A.suum* (Hill et al 1991) and *T.colubriformis, H.contortus* and *Nippostrongylus brasiliensis* (Bone & Bottjer 1985). These studies have revealed stage specific expression of surface carbohydrate (Kaushal et al 1984, Paulson et al 1988 and Hill et al 1991) and also changes in surface carbohydrate during development of a particular stage (Furnham & Ash 1983).

A 47kDa glycoprotein has been shown to be exposed on the surface of *T.spiralis* muscle larvae by the binding of a monoclonal antibody to intact larvae (Ortega-Pierres et al 1984). Although this molecule binds to lentil lectin-Sepharose the lectin does not bind to the surface of the intact parasite (Parkhouse et al 1981 and Ortega-Pierres et al 1984). Therefore there must be carbohydrate residues on this surface glycoprotein which are buried and not exposed on the surface of the parasite.

The techniques discussed above have been applied to characterise the surface of both sheathed and exsheathed infective larvae of *D.viviparus*. In the data presented the surface of sheathed larvae is referred to as the $L_3$ sheath and the surface of exsheathed larvae as the $L_3$ cuticle.

## 4.2. Results.

### 4.2.1. Surface biotinylation of *D.viviparus* $L_3$.

Since few molecules have been identified on the surface of the *D.viviparus* $L_3$ cuticle and sheath using surface radioiodination (Britton 1991), the alternative surface labelling technique of biotinylation was investigated.

Live sheathed and exsheathed *D.viviparus* $L_3$ were labelled by incubation in 0.5 mg/ml NHS-biotin for 1 hour. *D.viviparus* $L_3$ were found to be particularly robust and were consequently very difficult to physically disrupt by homogenisation in detergent solutions. Therefore, the labelled parasites were homogenised in Tris buffer to produce aqueous and SDS/2ME/urea soluble homogenates as described in the materials and methods. Western blots of these were probed with streptavidin-peroxidase to detect biotin labelled molecules.

No labelled molecules were detected in either the aqueously soluble or SDS/2ME/urea soluble extracts of biotinylated exsheathed $L_3$ (figure 4.1.A.). As a positive control *D.viviparus* $L_3$ aqueous homogenate was labelled in solution with 0.5 mg/ml NHS-biotin at the same time and a complex mixture of polypeptides was detected which became very overdeveloped in the time allowed for development of the surface labelled tracks (figure 4.1.A.). This suggests that the lack of $L_3$ surface labelling was not due to limitations of the technique's sensitivity. Furthermore surface labelling of exsheathed *H.contortus* $L_3$ using precisely the same protocol labelled multiple bands in an SDS/2ME/urea extract (figure 4.1.A.).

Similarly no labelled molecules could be detected in the water soluble extracts of surface labelled sheathed *D.viviparus* $L_3$. However a number of molecules were labelled in the SDS/2ME/urea extracts; two major bands at approximately 70 and 140 kDa, a fainter high molecular weight band and two minor bands at 30kDa and 15kDa (Figures 4.1.A. and 4.1.B.). The 70 and 140 kDa bands were consistently labelled between different batches of larvae whereas the other minor bands were less consistent (compare tracks 5 and 7 of figure 4.1 and track 6 of 4.1B). In order to determine the solubility of these molecules in detergent in the absence of 2ME, surface biotinylated sheathed $L_3$ were homogenised in 1% SDS. In spite of the difficulty in disrupting the

larvae to solubilise reasonable amounts of protein, the 70 and 140 kDa molecules could be faintly seen in the detergent extract suggesting that the 2ME and urea were not necessary to solubilise these molecules (data not shown).

Exsheathed *D.viviparus* $L_3$ were also surface labelled with NHS-biotin, sulfo-NHS biotin and biotin hydrazide and again no labelled molecules were detected in any of these extracts (data not shown). These reagents were also used to label sheathed *D.viviparus* $L_3$ (figure 4.1.B). Labelling with sulpho-NHS biotin was similar to the labelling with the NHS-biotin but appeared to be less sensitive. The biotin hydrazide identified the same molecules in the SDS/2ME/urea extract but also labelled 2 bands at approximately 140 and 170 kDa in the water soluble homogenate (figure 4.1.B.). However the labelling of these two water soluble bands was not very repeatable.

Incubation of 2ME/SDS/urea soluble homogenates of surface biotinylated sheathed $L_3$ with 0.2mg/ml clostridial collagenase at 37°C for 60 minutes did not produce any visible digestion of the 70 and 150 kDa labelled molecules (figure 4.1.C.). The activity this enzyme was confirmed by its complete digestion of human placental collagen at 0.5mg/ml which was performed under the same conditions at the same time and assessed on a Coomassie stained SDS/PAGE gel (data not shown).

The surface biotinylated molecules could not be solubilised from the surface of living labelled larvae by a variety of detergents. Biotinylated sheathed $L_3$ were incubated in the detergent solution at 37°C for up to 5 hours at a concentration of 200,000 $L_3$/ml. TCA precipitation was performed on the detergent supernatant after removal of the larvae by centrifugation. The TCA precipitated material along with aliquots of the unprecipitated detergent extract and the aqueous and 2ME/SDS/urea homogenates prepared from the larvae were analysed on Western blots probed with streptavidin-peroxidase. In each case the labelled molecules were still present in the 2ME/SDS/urea larval extracts but none were detected in the TCA precipitated material or detergent supernatant.

### 4.2.2. Generation of Monoclonal Antibodies to the surface of the $L_3$ cuticle and sheath.

Surface labelling studies with biotin or $^{125}I$ has yielded no information about surface antigens of the $L_3$ cuticle and relatively few molecules were identified in the $L_3$ sheath of *D.viviparus*. Therefore, in an attempt to define surface antigens which may have escaped detection by surface labelling, fusions were undertaken to generate monoclonal antibodies against these surfaces.

**Fusion 1**

The ELISA and IFA results presented in the previous chapter demonstrated that although immunisation of mice by repeated infection produced only relatively low levels of antibody, there was some response to the $L_3$ cuticular surface and the mice did show a measurable degree of protection to challenge. Also antibodies induced by the migration of live infective larvae were considered more likely to reflect the natural immune response than those induced by antigens presented in parasite homogenates with adjuvant. The range of antibodies to $L_3$ antigens which is produced in mice following immunisation by multiple infection is illustrated by the Western blot probed with the donor serum used for passive immunisation in the previous chapter (figure 4.2.A.). For these reasons an immunisation protocol involving repeated oral infection with $L_3$ was used for the first fusion.

Three Balb/c mice were each immunised on three occasions at 21 day intervals by oral infection with 5000 $L_3$. Tail bleeds were taken from individual mice 10 days after the third dose and the sera analysed by IFA. All three sera produced a very low level of fluorescence with exsheathed $L_3$ and no detectable fluorescence with sheathed $L_3$. The mouse whose serum was judged to produce the greatest fluorescence was selected and three weeks later was orally immunised with 5000 $L_3$ for a fourth and final time. It was euthanased 3 days post infection and a Western blot of *D.viviparus* $L_3$ homogenate was probed with serum from the terminal bleed (figure 4.2.A.). Antibodies were present to a number of $L_3$ antigens although there were some differences to the antigens detected by the pooled donor serum.

The spleen did not appear particularly enlarged and only $4x10^7$ splenocytes were harvested. These were fused with $5x10^6$ myeloma cells and plated out in 250 microwells. Hybridomas developed in 43 out of the 250 wells and by 15 days post fusion most were sufficiently confluent to be screened. The first round of screening was performed by IFA in the presence of 10mM PC and none of the supernatants produced fluorescence with either sheathed or exsheathed $L_3$. The screening was repeated 2 days later in the absence of PC but still no positives were detected. Twenty nine of the hybridomas were successfully expanded into 24-well plates and were again screened by IFA without PC and found to be negative. In order to determine whether antibody to $L_3$ antigens could be detected from any of the hybridomas, supernatants were screened by ELISA with $L_3$ homogenate as plate antigen and in the presence of 10mM PC. 7 out of the 43 produced optical densities significantly above that produced by complete medium and rescreening in the absence of PC did not reveal any extra positive wells. The three hybridomas giving the highest optical densities were cloned and were still positive by ELISA following cloning. Only one of these antibodies, 3H2, detected antigen on Western blots which was a single band of approximately 100 kDa (figure 4.2.B.).

Therefore the fusion from a mouse immunised by repeated oral infection did not yield any hybridomas secreting antibody against $L_3$ surface antigens and yielded relatively few hybridomas producing detectable antibodies even against $L_3$ somatic antigens.

**Fusion 2**

Due to the lack of surface specific monoclonal antibodies produced by fusion 1, an immunisation regime using $L_3$ homogenate with Freund's adjuvant was used for the second fusion.

Three mice were immunised by intraperitoneal injection with 0.2mg of $L_3$ homogenate in complete Freund's adjuvant followed by a second similar dose 4 weeks later but using incomplete Freund's adjuvant. Serum samples were taken from each mouse 10 days after the second immunisation and analysed by IFA. The samples from 2 of the mice produced a high level of fluorescence against the surface of the $L_3$ sheath but only very faint fluorescence against the $L_3$ cuticular surface. The third mouse did not seem to produce significant levels of antibody against either surface. The individual whose serum was judged to produce the greatest fluorescence on IFA was selected and four weeks later was boosted simultaneously with 0.1mg of $L_3$ homogenate by intraperitoneal injection and 0.1mg by intravenous injection. The mouse was euthanased 24 hours later and serum from the terminal bleed was used to probe a Western blot of *D.viviparus* $L_3$ homogenate (figure 4.2.A.). The detection of antigen by this serum was much greater than that seen with any of the infection sera.

The spleen was grossly enlarged and $1x10^8$ splenocytes were harvested, fused with $1x10^7$ myeloma cells and plated out into 480 microwells. Hybridomas were visible in 163 out of 480 wells by 10 days post fusion and these were screened between 13 and 17 days post fusion by IFA in the presence of 10mM PC. Twenty one wells produced definite fluorescence against the surface of sheathed $L_3$ but none against the surface of exsheathed $L_3$. After several rounds of expansion and rescreening six hybridomas remained strongly positive for the surface of the $L_3$ sheath and were designated 2A6, 2F8, 1F2, 2D8, 2A3 and 2C6. Due to the lack of success of the primary screening with exsheathed $L_3$, 40 of the hybridomas which were negative on the primary IFA screen were rescreened by IFA in the absence of PC and the supernatants of five of these hybridomas produced fluorescence with exsheathed $L_3$. Three of these remained positive after expansion and rescreening and were designated 2C4, 1H1 and 1E4.

All of nine of the hybridomas successfully expanded were cloned by limiting dilution and quantitative IFA was performed on both sheathed and exsheathed $L_3$ with the supernatants from the nine clones. The antibodies fell into two discrete groups; firstly 2C4, 1H1 and 1E4 bound to the $L_3$ cuticular surface but not to the $L_3$

sheath surface (figure 4.3A) and secondly 2A6, 2F8, 1F2, 2D8, 2A3 and 2C6 bound to the $L_3$ sheath surface but not the $L_3$ cuticular surface (figure 4.3B). It can also be seen from the figure that the values of relative fluorescence produced by the six sheath specific monoclonal antibodies was greater than that produced by the three $L_3$ cuticle specific monoclonal antibodies. This difference was very marked and was always apparent when IFAs were performed with the two types of antibody.

## 4.2.3. Characterisation of the monoclonal antibodies specific for the $L_3$ cuticular surface.

**Pattern of binding of 2C4, 1H1 and 1E4 to the $L_3$ cuticle surface.**

Visual examination of the exsheathed $L_3$ subjected to IFA with 2C4 revealed that the fluorescence was very evenly distributed over the whole larval surface (figure 4.4A). If the values of relative fluorescence measured from individual larvae are plotted on a frequency histogram it can be seen that they approximate to a normal distribution (figure 4.4B). Therefore the antigen detected by 2C4 is evenly distributed over the larval surface and there is no significant variation in its presence between individual larvae. The pattern of binding of 1H1 and 1E4 was identical to that of 2C4 except the intensity of fluorescence was somewhat less (see figure 4.3A).

**2C4, 1H1 and 1E4 are anti-PC antibodies.**

It is common to find that some monoclonal antibodies generated against nematode antigens are directed at phosphorylcholine (PC) epitopes, particularly when crude parasite homogenates are used as immunogens. In the second fusion no $L_3$ surface specific monoclonal antibodies were detected by the primary screen which was conducted in the presence of 10 mM PC and a second round of screening without PC was required to identify hybridomas secreting the three $L_3$ surface specific monoclonal antibodies, 2C4, 1H1 & 1E4. Therefore it was considered possible that these antibodies were directed against the PC epitope.

The presence of PC in *D.viviparus* $L_3$ somatic antigens was investigated by measuring the binding of the monoclonal antibody Bp-1 to $L_3$ homogenate on an ELISA plate. This IgM monoclonal antibody was generated by Sutanto et al (1985) from mice immunised with living adult *Brugia pahangi* and boosted with a detergent solubilised surface fraction of adult worms and it has been shown to bind to the PC hapten (Maizels et al 1987). Ascites at used at a 1:1000 dilution produced a high optical density with the $L_3$ homogenate and this binding was completely inhibited in the presence of 1mM PC (figure 4.5A). Therefore, in common with many other nematodes, some of the somatic

antigens of *D.viviparus* larvae appears to contain the PC epitope. The ability of PC to inhibit the binding of monoclonal antibodies 2C4, 1H1 and 1E4 was also assessed using the ELISA and the binding of all three antibodies was completely abolished in the presence of 1mM PC (figure 4.5A). The monoclonal antibody 3H2 was also included in this assay and the binding of this was unaffected by PC indicating that the inhibition seen with the other antibodies was a specific effect and not simply due to PC interfering with the assay.

The binding of the monoclonal antibodies to the surface of exsheathed *D.viviparus* $L_3$ in the presence and absence of PC was also assessed by quantitative IFA (figure 4.5B). Relatively bright fluorescence was produced on the surface of the $L_3$ cuticle by Bp-1 ascites (1:50 dilution) and this was abolished in the presence of 10 mM PC. This specific binding of Bp-1 to the surface of live parasites indicates that PC, or PC-like, epitopes are exposed on the cuticular surface of *D.viviparus* infective larvae. The fluorescence produced by 2C4, 1H1 and 1E4 was also completely abolished in the presence of 10mM PC which again suggests that they too are anti-PC antibodies. IFAs with 2A6 and 2F8 on sheathed $L_3$ were unaffected by the presence of 10mM PC which demonstrates that PC does not interfere with the IFA in a non-specific manner (data not shown).

2C4, 1H1 and 1E4 were all isotyped as IgM using an erythrocyte haemagglutination kit (Serotec MMT RC1) which is generally the predominant isotype for anti-PC monoclonal antibodies produced from Balb/c mice.

Western blots of $L_3$ homogenate were probed with Bp-1 ascites (1:500 dilution) and the neat supernatants of 2C4, 1H1 and 1E4. The results with 1H1 and 1E4 were too faint for reproduction but the results for Bp-1 and 2C4 were more discernible (figure 4.2C). Both antibodies produce an identical series of bands which is further evidence that 2C4 is an anti-PC monoclonal antibody. The numerous bands recognised suggests that the PC epitope is present on a number of molecules in *D.viviparus* infective larvae. This is not unexpected but does mean that the PC-bearing molecule on the cuticular surface cannot be identified using this approach.

## 4.2.4. Investigation of the PC epitope on the *D.viviparus* $L_3$ surface.

**Anti-PC antibody does not bind to the cuticular surface of other trichostrongylid nematodes.**

In spite of the presence of PC epitopes in the somatic tissues and E/S products of many nematodes its exposure on the cuticular surface has not been previously reported. However most of the work on the localisation of PC epitopes has

been conducted on nematodes which are not very closely related to *D.viviparus* and a number of trichostrongylid nematodes were therefore examined to determine whether PC was exposed on the $L_3$ cuticular surface. The binding of Bp-1 and 2C4 to the surface of sheathed and exsheathed $L_3$ of six trichostrongylid nematode species was examined by IFA (figure 4.6). It can be seen that both antibodies bound only to the *D.viviparus* exsheathed $L_3$ surface and not to the $L_3$ surface of the other nematodes. *H.contortus, O.circumcincta, O.ostertagi, Cooperia oncophora* and *T.colubriformis* are all ruminant gastro-intestinal parasites and have no tissue migratory phase. Therefore the infective larvae of these species enter a very different host environment to that experienced by the *D.viviparus* $L_3$. However *Necator americanus* is a migratory parasite and so the infective larvae enter a host environment more similar to that experienced by the *D.viviparus* $L_3$. However neither Bp-1 or 2C4 bound to the exsheathed surface of *N.americanus* $L_3$ and so PC does not appear to be exposed on the infective larval surface. *T.canis* $L_2$, *T.spiralis* $L_1$ and a mixed stage culture of *Caenorhabditis elegans* were also examined by qualitative IFA with Bp-1 and 2C4 and did not show any observable fluorescence.

**The binding of anti-PC antibody to the surface of the *D.viviparus* cuticle is stage specific.**

Quantitative IFA was performed with both Bp-1 and 2C4 on a number of different stages of *D.viviparus* and both antibodies bind only to the exsheathed $L_3$ surface and not to the surface of eggs, $L_1$, sheathed $L_3$ or adult parasites (figure 4.7). The background values of relative fluorescence seen is due to the yellowish autofluorescence which is easily distinguished from the green fluorescence due to bound FITC. Therefore the exposure of the PC epitope on the $L_3$ cuticular surface seems to be entirely stage specific. The intact $L_2$ surface could not be examined by this technique since the $L_2$ is enclosed by the separated $L_1$ cuticle and the larvae only emerges from this as the $L_3$. Eggs, sheathed $L_3$, exsheathed $L_3$, $L_4$ and adult *H.contortus* and *O.circumcincta* were examined by qualitative IFA with Bp-1 and 2C4 and none of these stages fluoresced with either antibody (data not shown).

**Antibodies to the exsheathed $L_3$ surface present in *D.viviparus* naïve bovine sera are not of the anti-PC idiotype.**

Naive bovine serum (ie. serum from cattle previously uninfected with *D.viviparus*) and newborn calf serum produces fluorescence of the exsheathed surface of *D.viviparus* $L_3$ on IFA which appears to be due to IgM antibody (Britton 1992). Given that anti-PC monoclonal antibodies bind to the surface of the $L_3$ cuticle, it was considered that the presence of anti-PC antibodies in naive bovine serum could be responsible for this phenomenon. This possibility was investigated by performing an IFA on exsheathed

$L_3$ with normal bovine serum in the presence of increasing concentrations of PC. If the fluorescence was due to PC specific antibodies in normal bovine serum, the presence of free PC would be expected to compete for these antibodies and so reduce the signal. However there was no reduction in the fluorescence produced by this serum, even in the presence of 50mM PC (figure 4.8A). In contrast the binding of Bp-1, used as a positive control, was abolished even in the presence of 1mM PC. This result suggests that the fluorescence produced by naive bovine serum is not due to anti-PC antibodies. However it is worth noting that Maizels et al (1987a) have described monoclonal antibodies whose binding to parasite antigens is not inhibited by free PC but which do bind to PC in the absence of parasite material. This suggests they have a significantly lower affinity for free PC than for the parasite epitope to which they were raised and so are not of the classical anti-PC idiotype. Presumably the parasite epitope is either simply one which cross-reacts with PC or comprises of the PC-antigen junction. If such antibodies exist in bovine serum their binding to the larval surface would not be reduced in the presence of free PC.

As an alternative approach, an experiment was undertaken to assess the ability of Bp-1 to displace the $L_3$ surface specific antibodies in naive bovine serum. The normal anti-bovine (whole molecule) FITC conjugate (Sigma F 7509) used for IFA was found to cross react with Bp-1 which made it impossible to differentiate between the binding of bovine antibody and Bp-1 to the exsheathed $L_3$ surface using this conjugate. However an anti-bovine IgM (Fc) FITC conjugate (The Binding Site PF203 ) was found to be less cross reactive and so this conjugate was used to detect the binding of bovine antibodies to the surface of exsheathed $L_3$ in the presence of competing Bp-1 (figure 4.8B). Naive bovine serum at a 1:50 dilution produced marked fluorescence of the larvae but Bp-1 at a 1:10 dilution produced relatively little fluorescence, demonstrating that the binding of this bovine conjugate to Bp-1 was sufficiently poor that it can be used to assess the binding of bovine antibody even in the presence of competing Bp-1. There was no reduction in the fluorescence produced by the normal bovine serum (used at a 1:50 dilution) in the presence of increasing concentrations of Bp-1 (figure 4.8B). This suggests that the bovine antibodies are not displaced by Bp-1 and so are unlikely to bind to an epitope that includes or is closely associated with PC, unless of course such antibodies are of greater affinity than Bp-1. This experiment is not entirely satisfactory due to the slight, but measurable, binding of the anti-bovine antibody conjugate Bp-1. Species specific monoclonal antibodies to bovine IgM and IgG have recently become commercially available and so it would be useful to repeat this experiment with conjugates based on these antibodies.

In summary, the above experiments suggest that the antibodies present in normal bovine serum which bind to the surface of exsheathed *D.viviparus* $L_3$ do not bind to PC. However it is possible that the antibodies could be directed against a PC-like

epitope in which case the binding of these bovine antibodies to the $L_3$ surface might not be displaced by free PC. However given that the 2C4, 1H1 and 1E4 monoclonal antibodies are displaced by free PC there is no reason to suggest that such an epitope exists.

**Anti-PC antibodies are not predominant in *D.viviparus* infected cattle sera.**

The presence of PC epitopes on the surface of the *D.viviparus* $L_3$ cuticle makes the immune response of infected cattle to PC of particular interest. A simple experiment was performed to investigate whether cattle infected with *D.viviparus* produce significant amounts of antibody to PC. An ELISA was used to measure antibody to $L_3$ somatic antigens in the pooled sera from three calves (B7, B8 and B9) which had been repeatedly infected with *D.viviparus*. Serum samples had been taken several weeks after each infection (this sera was supplied by G.Canto - see Appendix 2 for details). Each sample was assayed in the presence of no PC, 10mM PC and 50mM PC (figure 4.9). The presence of PC does not cause a particularly marked reduction in the optical densities produced by the sera in the $L_3$ homogenate ELISA. This suggests that anti-PC antibody represents a relatively small proportion of the antibodies produced in cattle following infection by *D.viviparus*. A more detailed examination of the immune response to PC in cattle following vaccination and immunisation is required to help understand the true significance of the exposure of PC on the *D.viviparus* $L_3$ surface.

## 4.2.5. Preliminary characterisation of the monoclonal antibodies specific for the $L_3$ sheath surface.

**Pattern of binding of anti-sheath monoclonal antibodies to the sheathed $L_3$ surface.**

Examination of the sheathed $L_3$ after IFA with mab 2A6 revealed that the fluorescence was very evenly distributed over the whole larval surface (figure 4.10A). If the values of relative fluorescence measured from individual larvae are plotted on a frequency histogram it can be seen that they approximate to a normal distribution (figure 4.10B). Therefore the epitope detected by 2A6 appears to be evenly distributed over the sheath surface and there is no significant variation in the binding of the antibody between individual larvae. The pattern of binding of 2F8, 1F2, 2D8, 2A3 and 2C6 was identical to that of 2A6 (data not shown). The intensity of fluorescence produced by 2D8 and 2A3 was consistently less than that produced by the other anti-sheath antibodies (see figure 4.3B).

The quantitative IFAs presented in figure 4.3B demonstrate that all six anti-sheath monoclonal antibodies behave in a similar fashion with respect to their binding to the surface of exsheathed and sheathed $L_3$. This specificity of the monoclonal antibodies for the $L_3$ sheath is illustrated by typical examples of IFAs performed with these antibodies (figure 4.11). An IFA with 2A6 on a partially exsheathed *D.viviparus* $L_3$ (figure 4.11A) shows the lower half of the larvae still enclosed within the brightly fluorescent $L_3$ sheath with the upper half of the larvae exposing the non-fluorescing $L_3$ cuticle. An IFA performed with 2F8 on a mixed sample of sheathed and exsheathed *D.viviparus* $L_3$ produced bright fluorescence with the sheathed $L_3$ (figure 4.11B, larvae on the right) and no fluorescence of exsheathed $L_3$ (figure 4.11B, larvae on the left). IFAs with 1F2, 2D8, 2A3 and 2C6 all gave the same results.

**ELISA and Western blotting with the anti-sheath monoclonal antibodies.**

Only very low optical densities were produced with all six anti-sheath monoclonal antibodies with the *D.viviparus* $L_3$ homogenate ELISA relative to the 3H2, 2C4, 1H1 and 1E4 supernatants (data not shown). The optical densities produced with the *D.viviparus* adult homogenate ELISA were not significantly greater than the background values produced by complete medium alone. Therefore the ELISA was of limited value in the characterisation of these monoclonal antibodies.

Western blots of $L_3$ and adult homogenates were probed with the six anti-sheath monoclonal antibodies. All six supernatants produced a smear between 29 and 40 kDa with the $L_3$ homogenate blots (figure 4.12A). Each smear appears to consist of an ill defined banding pattern, but this is probably an artifact due to the superimposition of "ghost" bands of other polypeptides because on most subsequent blots, particularly with immunoprecipitated or immuno-affinity purified antigen, the pattern is much more homogenous. In contrast none of the antibodies recognised antigens on the adult homogenate blots (figure 4.12B). The positive control shown in the figure was $L_3$ homogenate probed by 2C6. The same amount of total protein was loaded on the gel for the $L_3$ and adult homogenates and the Western transfer was checked by Ponceau-S staining of the nitrocellulose filter. No antigen was detected by the monoclonal antibodies in the adult homogenate tracks even after prolonged development.

The six monoclonal antibodies were isotyped with an erythrocyte haemagglutination kit (Serotec MMT RC1) and all six reacted with both the $IgG2_A$ and the IgG3 isotypes. However the Amersham mouse Mab isotyping kit gave a clear cut result of $IgG2_A$ for 2A6, 2F8, 2D8, and 1F2.

### 4.2.6. Lectin binding to the $L_3$ sheath and cuticle.

The presence of glycoconjugates on the surface of the *D.viviparus* $L_3$ cuticle and sheath was investigated by examining the binding of a panel of fluorescent lectins to the surface of live sheathed and exsheathed larvae.

Sheathed and exsheathed *D.viviparus* $L_3$ were incubated in a number of FITC conjugated lectins, washed and examined by fluorescence microscopy. The lectins used, their abbreviations and their sugar specificities are given in table 4.1. The degree of fluorescence was scored from - (background autofluorescence only) to ++++ (very intense fluorescence due to bound FITC) and a summary of the results is presented in table 4.2. None of the seven lectins used bound to the surface of the $L_3$ sheath and only *Helix pomotia* agglutinin (HPA) bound convincingly to the exsheathed $L_3$ surface (Table 4.2 and figure 4.13A). This fluorescence produced by HPA was abolished in the presence of 200mM N-acetyl-D-galactosamine demonstrating the binding involved the lectins carbohydrate binding site. There was possibly some slight fluorescence produced by *Canavalia ensiformis* agglutinin (Con A), *Triticum vulgaris* agglutinin (WGA) and *Ricinus communis* agglutinin (RCA) but this was so faint that it was probably not significant (table 4.2).

Sodium hypochlorite is commonly used to exsheath trichostrongylid $L_3$ *in vitro*. *D.viviparus* $L_3$ need to be incubated in 0.01% sodium hypochlorite solution for approximately 15-20 minutes for complete exsheathment to occur. However if they are only incubated in the hypochlorite solution for about 5 minutes and then washed in PBS the exsheathment process is incomplete. Larvae which had been treated in such a manner were examined with 5 of the original panel of FITC conjugated lectins and the results are summarised in Table 4.2. HPA produced intense fluorescence with the partially intact sheaths and also caused fluorescence with the exposed $L_3$ cuticle (figure 4.13B). Con A and WGA also produced bright fluorescence with the partially cast sheaths but gave no fluorescence with the exposed $L_3$ cuticle (figures 4.13 B and C). *Tetragonolobus purpurea* and *Arachis hypogea* agglutinins both produced moderate fluorescence of the uncast sheaths but not the $L_3$ cuticle (see table 4.2). The fluorescence produced by Con A was abolished in the presence of 0.2M D-(+)-mannose or 0.2M D-(+)-glucose and that produced by HPA was abolished by N-acetyl-D-galactosamine demonstrating that the binding of the lectin involved its carbohydrate binding site.

Similar experiments also were performed on *O.ostertagi* and *T.colubriformis* $L_3$ for comparison. The results were the exactly the same for both these species and a summary of those for *O.ostertagi* is given in table 4.3. None of the lectins bound to the surface of sheathed or exsheathed $L_3$ of either species. When *O.ostertagi* or

*T.colubriformis* larvae were incubated for 5 minutes in 0.01% hypochlorite and then washed in PBS many of the larvae could be seen to be partially exsheathed. These were then examined with 5 of the FITC conjugated lectins (Table 4.3). Con A, HPA and WGA all bound to the cast or partially cast sheaths in the same very localised manner. In partially exsheathed larvae, ie. those which had lost the anterior "cap" but had not yet cast the rest of the sheath, there was bright fluorescence at the open end of the sheath and also in a well defined area towards the caudal end of the sheath (figures 4.14 and 4.15). In empty cast sheaths binding was localised in the same way, ie.fluorescence at the cranial and caudal ends (figure 4.15C). No binding to any part of the exposed $L_3$ cuticle was seen with any of the lectins. The pattern and intensity of binding of the lectins was exactly the same for *T.colubriformis*. The fluorescence produced by Con A was abolished in the presence of 0.2M D-(+)-mannose or 0.2M D-(+)-glucose and that produced by HPA was abolished by N-acetyl-D-galactosamine demonstrating the specificity of the binding.

In summary, no lectins bound to the surface of the intact *D.viviparus* $L_3$ sheath and only HPA bound to the $L_3$ cuticular surface. However five of the lectins were found to bind to the sheaths of partially exsheathed *D.viviparus* $L_3$. For *Ostertagia ostertagi* and *Trichostrongylus colubriformis* none of the lectins bound to the surface of the intact $L_3$ sheath or cuticle. However 5 lectins bound to cranial and caudal areas of sheaths which had lost their anterior cap. The binding of Con A and HPA appeared to be specific when examined by the appropriate sugar competition.

Western blots of *D.viviparus* $L_3$ homogenate were probed with five peroxidase conjugated lectins. No molecules were recognised in the aqueous fraction by any of the lectins but Con A recognised several bands very clearly in the 2ME/SDS/urea fraction at 29kDa, a doublet at 50 kDa, a band at 100 kda and several higher molecular weight bands (fig 4.16A). HPA and WGA also recognised these bands but only very faintly. The signal from Con.A on a Western blot was greatly diminished in the presence of 0.2M mannose or 0.2M glucose (figure 4.16B) suggesting that Con A binds to the parasite antigens in a specific manner. The specificity of the HPA and WGA binding on the blots could not be meaningfully assessed due to the very weak signal produced by these lectins.

The fact that only Con A seems to bind to the antigens *D.viviparus* $L_3$ antigens on Western blots does not correlate very well with the FITC-conjugated lectin studies in which HPA, Con A and WGA all seemed to produce a similar level of fluorescence. This suggests that the molecules detected by Con A on the blot are probably not those detected by fluorescent lectins binding to partially cast sheaths.

## 4.3. Discussion.

An attempt to identify the surface antigens of *D.viviparus* $L_3$ using surface radioiodination was made by Britton (1991). With exsheathed $L_3$ no molecules were identified by Bolton-Hunter labelling and labelling with Iodogen produced very faint and inconsistent results. Iodogen labelling of sheathed $L_3$ did detect some molecules which will be discussed in greater detail below. Nevertheless these surface radioiodination experiments yielded relatively little information about the cuticular antigens of *D.viviparus* infective larvae and for this reason the use of biotinylation as an alternative surface labelling technique has been investigated.

The results of these surface biotinylation experiments turned out to be very similar to the results of the radioiodination experiments performed by Britton (1991). No molecules were detected by labelling of live exsheathed larvae with NHS-biotin which leads to the following conclusions. Since this reagent reacts with lysine residues of polypeptide chains, there must be no such residues available on the cuticular surface. The result also suggests that the NHS-biotin cannot have penetrated to the deeper layers of the cuticle as one would have expected the cuticular collagens to be labelled. This is in contrast to previous reports of surface labelling with NHS-biotin performed in a number of nematode species such as *A.suum* (Hill et al 1990), *B.pahangi* (Alvarez et al 1989) and *O.circumcincta* and *O.ostertagi* (Keith et al 1990) in which numerous antigens were labelled, including cuticular collagens. In the first two examples penetration of the biotin into the deeper layers of the cuticle was confirmed by electron microscopic studies. As NHS-biotin is an organic soluble molecule surface labelling with the water soluble sulpho-NHS-biotin was investigated. This also failed to label any molecules on the exsheathed *D.viviparus* $L_3$ which suggests that the $L_3$ surface presents an impervious barrier to hydrophilic as well as hydrophobic molecules.

There are a number of other possible explanations for the lack of labelling with both NHS-biotin and sulpho-NHS biotin which should be considered. It does not seem likely that the results are due to the lack of sensitivity of the technique since the protocol is similar to that described for other nematodes (Hill et al 1990, Alverez et al 1989) and a number of molecules were clearly labelled using the same procedure on *H.contortus* $L_3$. Also the lack of molecules labelled by standard radioiodination procedures (Britton 1991) supports the results presented here. The possibility that the cuticular proteins of the *D.viviparus* $L_3$ cuticle contain very few lysine residues would also seem to be an unlikely explanation given the variety of proteins that exist in the cuticle. Furthermore, if this were the explanation one would also have to propose that

the cuticular proteins also lacked tyrosine residues in order to explain the radioiodination experiments.

Biotin hydrazide, which reacts with aldehyde groups generated by mild oxidation (periodate treatment) of carbohydrate residues, also failed to detect any glycoproteins on the $L_3$ cuticular surface. However it is possible that other glycoconjugates, such as proteoglycans, are present but not detected by this method since Western blotting is not a very effective technique for the analysis of non-protein (or predominantly non-protein ) molecules.

In conclusion, it seems that biotin labelling, like radioiodination, does not label molecules on or within the *D.viviparus* $L_3$ cuticle.

Surface biotinylation was somewhat more successful at identifying molecules of the $L_3$ sheath. The two most prominent molecules, at 70 and 140 kDa, were consistently labelled from batch to batch although the other minor bands were less reproducible. Again the results were very similar to the radioiodination experiments of Britton (1991) who reported that the two most prominent molecules identified by Iodogen labelling of sheathed larvae were sized at 70 & 155 kDa. These could easily be the same two predominant molecules which have been identified by the surface biotinylation experiments presented in this chapter, as some of their immunochemical properties are similar. Britton (1991) reported that the iodinated molecules were solubilised by homogenisation of larvae in NaDOC and were also present, along with a number of less prominent bands, in SDS/2ME solubilised material. In comparison, the two most prominent bands labelled in sheathed larvae by NHS-biotin, sulpho-NHS-biotin and biotin hydrazide were sized at 70 & 140 kDa and the discrepancy in the sizes between the molecules labelled with biotin and $^{125}I$ may be artifactual since the estimation of the high molecular weights on these gels is very approximate. These two biotin labelled molecules were always present in the 2ME/SDS/urea solubilised material but could also be extracted by homogenisation of larvae in 1% SDS (data not shown). Both the $^{125}I$ and biotin labelled molecules were also collagenase insensitive. However there was one difference between the iodinated molecules identified by Britton (1991) and the biotinylated molecules reported here which was that the iodinated molecules were released by incubation of live larvae 0.25% CTAB whereas the biotinylated molecules could not be solubilised by such treatment with a number of detergents including CTAB. However this may reflect differences in the sensitivity of the two techniques rather than differences in the nature of the molecules labelled.

Labelling of the sheathed larvae with biotin hydrazide identified two molecules at 140 and 160 kDa in the aqueous extracts. These were not detected by NHS-biotin or sulpho-NHS biotin and so could be glycoconjugate molecules. More work is

needed to confirm this since the result was not very repeatable, although this could be due to the poor binding of such molecules to nitrocellulose by Western blotting.

Therefore surface biotinylation and surface radioiodination give very similar results but appear to be of limited value in identifying cuticular molecules of the *D.viviparus* infective larvae. In general, when these techniques are used to label the nematode cuticle, a range of molecules are labelled in a reproducible manner. However there are a number of examples where the application of surface labelling techniques has been less successful. Maizels et al (1983a) reported that the surface of the infective larvae of *N.brasiliensis* could not be labelled in a reproducible manner using Chloramine-T and that detergent extraction of radiolabelled antigens was very inefficient. Also Rhoads and Fetterer (1990) reported similar problems of inefficient labelling and detergent solubilisation with the surface antigens of *H.contortus* $L_3$ when using the Iodogen method. Interestingly both of these examples are for $L_3$ infective larvae of strongylid nematodes and so could be refractory to these surface labelling techniques for similar reasons as *D.viviparus*. A possible explanations might be that surface consists predominantly glyconjugates (proteoglycans and glycolipids) or of an insoluble protein matrix such as cuticlin (with or without a lipid layer). However the surface would also have to be resistant to penetration by the labelling reagents otherwise there would be labelling of other molecules such as cuticular collagens.

Monoclonal antibodies were generated to the surface of the *D.viviparus* $L_3$ cuticle and sheath in order to characterise molecules which may have escaped detection by the surface labelling experiments. The only screening method available was an IFA using live larvae which was very time consuming due to separate tubes and multiple centrifugation steps being required for each sample. This limited both the numbers of wells which could be screened and the number of times each sample could be re-screened after expansion and cloning etc. More efficient screening methods based on an ELISA could not be used since detergents had not been found which would solubilise surface material from *D.viviparus* $L_3$ and so surface enriched material was not available for use as antigen. However, despite the disadvantages, one important feature of the live IFA is that it only identifies hybridomas secreting antibody to surface exposed epitopes.

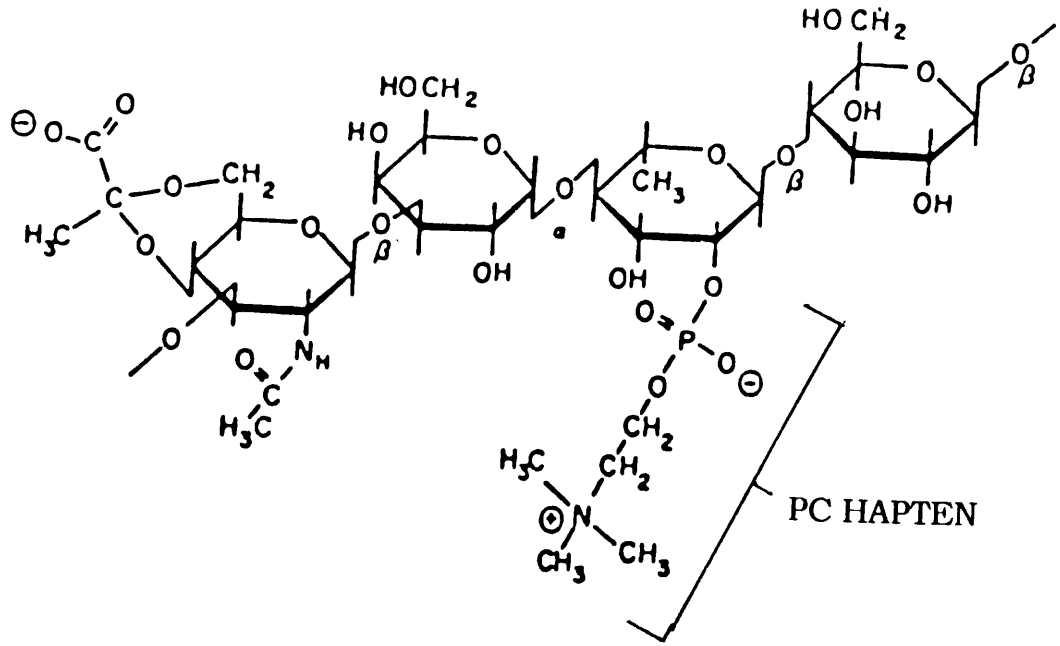
The inability to produce a surface enriched antigen fraction also limited the immunisation regimes available. A regime of repeated oral infection was chosen for the first fusion because although this only produced a low level of surface specific antibody it did induce a significant degree of protection to challenge. Therefore it was considered that monoclonal antibodies produced from such a fusion would be a more accurate reflection of the antibody repertoire to infection and may include antibodies with protective idiotypes. However the lack of splenomegaly, the relatively few splenocytes harvested and the low numbers of hybridomas produced by the fusion all

suggested that oral infection had stimulated a relatively poor B-cell response. No hybridomas secreting antibody to the surface of either the $L_3$ cuticle or sheath were ontained and only 7 hybridomas (out of 44) producing *D.viviparus* specific antibody were detected by an ELISA using aqueously soluble $L_3$ somatic antigens. This could be another reflection of the poor antibody response induced by the infections but it may also suggest that a proportion of B-cells were producing antibody to insoluble antigens. Interestingly, it appeared that no anti-PC antibodies were produced from this fusion as all 7 positive supernatants were unaffected by the presence of free PC in the ELISA. On the basis of these results it was decided that immunisation regimes based on infection were not likely to yield monoclonal antibodies to surface antigens.

Immunisation with $L_3$ homogenate and Freund's adjuvant was used for the second fusion and this induced a marked antibody response to the surface of the $L_3$ sheath and a much weaker response to the $L_3$ cuticular surface. Given the artificial nature of this regime, the antibody response is remarkably similar to the results of IFA with bovine infection and vaccination sera. The second fusion was much more successful, eventually producing 6 hybridomas against the surface of the $L_3$ sheath and three against the surface of the $L_3$ cuticle.

The hapten phosphorylcholine (PC) has been reported to be present in many nematode species based on the binding of PC-specific myeloma proteins or monoclonal antibodies and the literature has been reviewed in the introduction to this thesis. However it is important to understand the basis by which these antibodies are defined in order to fully appreciate their use as probes for PC. Myeloma proteins that precipitated pneumococcal C polysaccharide were found to be inhibited by choline, but only at relatively high concentrations (Cohn et al 1969). It was subsequently deduced, from biochemical analysis, that the choline was linked to the polysaccharide by a phoshodiester bond (see diagram 4.1) which suggested that PC might be the hapten detected by the myeloma proteins (Brundish & Badley 1967 and Leon & Young 1971). The zwitterion phosphorylcholine (PC) was found to be a much more potent inhibiter of binding than choline or a number of its other derivatives (Leon & Young 1971). The concentration required to produce 50% inhibition was between 430 and 870 times greater for choline than for PC, except for one myeloma protein for which their was only a ten fold difference. On this basis the myeloma proteins were defined as PC specific but it is important to realise that these antibodies will bind to other choline derivatives such as acetylcholine and phosphonocholine as demonstrated by the ability of these reagents to inhibit binding, albeit at higher concentrations than PC.

DIAGRAM 4.1



When considering the presence of PC in different parasites, it is important to clarify the nature of the data on which these conclusions are based. In the majority of parasite species the identification of PC is purely based on the binding of PC specific antibody probes. From the points made above, it can be seen that the binding of these antibodies does not necessarily distinguish between the presence of PC and other similar epitopes such other choline derivatives. Furthermore, the presence of free PC will inhibit the binding of PC specific antibodies to a cross-reactive epitope just as effectivley as it inhibits the binding to a true PC epitope. Therefore the use of PC competition is of limited use in determining whether a defined PC specific antibody is binding to PC or just a "PC-like" epitope on a parasite antigen.

The presence of PC on *B.pahangi* antigens is based on a more detailed immunochemical analysis. The monoclonal antibody Bp-1 was generated from mice immunised with living adult *Brugia pahangi* and boosted with a detergent solubilised surface fraction of adult worms (Sutanto et al 1985). Its PC specificity was subsequently defined using similar criteria to those used by Leon and Young (1971) for the myeloma binding proteins. Maizels et al (1987c) showed that 50% inhibition of antibody binding occured at concentrations of PC in the order of 0.01mM whereas 50% inhibition of binding required choline concentrations of above 1mM. Therefore Bp-1 appears to be highly specific for PC and, since it was generated to *B.pahangi* antigen, constitutes much firmer evidence for the presence of PC on this parasite's antigens.

The presence of PC epitopes on the surface of the *D.viviparus* $L_3$ cuticle was suggested by the binding of the anti-PC monoclonal antibody Bp-1 to live larvae and this has also been observed by Britton (1991). However from the preceding discussion it can be seen that this does not conclusively prove that PC as opposed to some other similar epitope is present on the $L_3$ surface. The experiments have shown that the binding of these antibodies on an $L_3$ homogenate ELISA was completely eliminated in the presence of 1mM PC. However this would occur whether Bp-1 was binding to PC or to some other PC-like epitope, such as another choline derivative, and so this again does not prove the presence of PC.

The inhibition of binding of the three surface specific monoclonal antibodies, raised to *D.viviparus* antigens, by 1mM PC demonstrates they have some specificity for PC. Furthermore, Western blots of *D.viviparus* $L_3$ antigens probed with 2C4 and Bp-1 produce the same pattern which is consistent with these two antibodies recognising the same epitope on a number of different molecules. However experiments comparing the relative abilities of low concentrations of PC and choline to inhibit binding are required to fulfil criteria used by Leon and Young (1971) for the myeloma proteins and Maizels et al (1987c) for Bp-1. If these three antibodies were shown to fulfil these

criteria, it would provide much more conclusive evidence that PC itself rather than some other, albeit very similar epitope, is present on the surface of the $L_3$.

In summary, the results have shown that PC, or a very similar epitope, is present on the surface of the *D.viviparus* $L_3$ cuticle. More detailed characterisation of the *D.viviparus* $L_3$ surface specific monoclonal antibodies might help to establish whether it is PC or some other closely related hapten which is present. Much of the literature concluding that PC is present in parasite antigens is based on the binding of PC specific antibody probes and little biochemical analysis appears to have been performed confirm this data. Therefore it seems sensible to use the term "PC epitope" rather than "PC" if, as in the case of *D.viviparus*, the evidence for the presence of this hapten is based on immunochemical rather than biochemical data.

Of particular interest was the exposure of the PC epitope on the *D.viviparus* $L_3$ surface. In all the nematodes which have been examined to date PC reactivity has been confined to somatic structures, the deeper layers of the cuticle and E/S products (see general introduction). However little work of this type has been performed on the trichostrongylid nematodes, which are the nearest relatives of *D.viviparus*, and so it was not clear whether the binding of PC specific antibodies to the *D.viviparus* $L_3$ surface was unique. The results presented in this chapter have shown that PC epitopes are not exposed on the cuticular surface of infective larvae of a number of nematodes from the order Strongylida including *N.americanus* in which the $L_3$ is a tissue migratory stage. Therefore it seems that the presence of PC epitopes on the surface of the *D.viviparus* infective larvae is very unusual and the results have also shown that this exposure on the cuticular surface is completely stage specific.

As discussed earlier in the general introduction, it has been proposed that PC may be relatively tolerogenic for specific host B cells other than the B-cell precursors of IgM-secreting cells with low antigen binding capacity (Mitchell & Lewers 1977 and Gutman & Mitchell 1977). It has been proposed that the host may be tolerised to certain otherwise immunogenic epitopes on PC-bearing parasite antigens (Gutman & Mitchell 1977). The presence of PC-bearing molecules in the ES products and intestinal tract of parasitic nematodes (Maizels & Selkirk 1989) could mean that PC plays such a role for antigens at these sites. However the observation that PC epitopes are not present on the surface of the nematode cuticle tends to militate against this being a particularly important immunomodulatory strategy in many cases. Therefore the binding of PC specific antibodies to the *D.viviparus* $L_3$ cuticular surface is very interesting from the immunological point of view. The presence of PC epitopes, together with the lack of other detectable antigens, would make the *D.viviparus* $L_3$ surface a particularly difficult structure for the host to mount an effective immune response against. This view is supported by the observation that the $L_3$ cuticular surface is relatively poorly

immunogenic in infected or vaccinated cattle, as assessed by IFA, in contrast to the $L_3$ sheath which is highly immunogenic (Canto 1990 and Britton 1991).

Since PC is a zwitterion (see diagram), another consequence of its exposure on the cuticle might be to produce a highly charged surface. It is tempting to speculate that this could affect the ability of biotin and other reagents to penetrate the surface which might explain the difficulty found in identifying $L_3$ cuticular molecules by surface labelling techniques. The surface charge of infective larvae could be investigated by using cytochemical stains such as ruthidium red and cationised ferritin in conjunction with electron microscopy (Himmelhoch & Zuckerman 1983 and Murrell et al 1983).

IFA performed with naive bovine serum from both adult cattle and new born calves (but not foetal calf serum) produces fluorescence with exsheathed but not sheathed *D.viviparus* $L_3$ (Canto 1990 and Britton 1992). This fluorescence was detected using specific anti-bovine IgM (or polyvalent) FITC conjugates but not with anti-bovine IgG conjugates suggesting that IgM specific for the surface of the $L_3$ cuticle is present in cattle serum soon after birth. Since it is thought that PC epitopes are responsible for much of the cross-reactivity between parasite antigens (Maizels et al 1987a) and given that these appear to be present on the *D.viviparus* $L_3$ surface, it seemed quite possible that the $L_3$ surface specific antibodies present in normal bovine serum were against the PC epitope. However the results of the PC and BP-1 competition experiments, performed to investigate this, suggested that antibodies to PC epitopes are not present in normal bovine serum. This implies that other, as yet unidentified, epitopes are present on the $L_3$ surface which are recognised by IgM present in naive bovine serum. The binding of the FITC-conjugated lectin HPA to the $L_3$ surface demonstrates the presence of glycoconjugates which raises the possiblity that the IgM may be binding to carbohydrate epitopes. Carbohydrates on the surface of *T.canis* have been shown to cross-react with antibodies to human A and B group erythrocyte antigens (Smith et al 1983 and Meghji & Maizels 1986) and it is possible that *D.viviparus* surface carbohydrates could be responsible for a similar phenomenon in cattle. The presence of carbohydrate on the *D.viviparus* $L_3$ surface is also consistent with the presence of PC as this is usually covalently linked to the oligosaccharide groups of glycoproteins or proteoglycans (Maizels et al 1987a and 1987c).

The immunological significance of $L_3$ surface specific IgM in normal bovine serum is not known but it is possible that the binding of IgM to the larval surface may prevent the binding of antibodies of other isotypes and so help the parasite to evade immune effector mechanisms. The presence of IgM "blocking antibodies" in the serum of young children infected with *Schistosoma mansoni* has been shown to prevent the *in vitro* binding of host effector cells to schistomula and to be associated with susceptibility to

re-infection (Butterworth et al 1987). Also because antibodies to both A and B human blood group antigens bind to the surface of *T.canis* $L_2$, it has been suggested that naturally occurring isohaemagglutinins in human serum could mask important epitopes on the larval surface (Smith et al 1983 and Smith 1991).

The immune response of *D.viviparus* infected or vaccinated cattle to PC was not examined in detail. However the optical densities produced on $L_3$ ELISA by sera taken from cattle after each of a series of infections with *D.viviparus*, was only slightly reduced in the presence of PC. This suggests that anti-PC antibodies make up a relatively small proportion of the antibodies directed against *D.viviparus* antigens.

The six monoclonal antibodies generated against the surface of the $L_3$ sheath all behaved in an identical fashion on IFA, ELISA and Western blots, except for the intensity of the IFA or Western blots which showed repeatable differences for each antibody. Therefore it would appear that all six antibodies probably recognise the same antigen, possibly with different affinities, although there is no evidence to suggest whether they recognise the same or different epitopes. Further use of these antibodies to investigate their target antigen and the nature of the sheath surface is presented in the next chapter.

The lack of $L_3$ cuticular molecules identified by surface labelling techniques and the presence of PC on the $L_3$ cuticular surface prompted the use of fluorescinated lectins to probe for surface glycoconjugates. A panel of seven FITC labelled lectins covering a range of sugar specificities (see table 4.1) were used to probe the exsheathed $L_3$ cuticular surface of *D.viviparus*. Only one of these, HPA, produced a relatively high degree of fluorescence and although Con A and WGA also gave very slight fluorescence, the significance of this was difficult to interpret. Nevertheless the binding of HPA demonstrates that carbohydrate is exposed on the surface of the *D.viviparus* $L_3$ cuticle and that N-acetyl-D-galactosamine is possibly a terminal residue. *O.ostertagi* and *T.colubriformis* were also examined for comparison with the same panel of lectins but no binding occurred to the $L_3$ cuticle in any of these species. This is supported by the results of Wharton & Murray (1990) who reported that Con A, WGA, SBA (soyabean agglutinin), PNA, HPA and UEA (Ulex europeans agglutinin) did not bind to the surface of exsheathed *T.colubriformis* $L_3$. Similarly Milner & Mack (1988) have also reported that Con-A, PNA, SBA and RCA did not bind to the surface of exsheathed *T.colubriformis* $L_3$. Therefore the presence of surface exposed carbohydrate residues, like the presence of PC epitopes, may be unusual to *D.viviparus* infective larvae amongst the trichostrongylid nematodes. Western blots of $L_3$ homogenate probed with peroxidase labelled HPA did not reveal any molecules in the aqueous fraction and only detected very faint bands in the SDS/2ME/urea soluble fraction. This suggests that the carbohydrate detected by IFA is not present on a soluble glycoprotein but may be present on another type of

glycoconjugate, such as proteoglycan or glycolipid, which may not bind to nitrocellulose filters. Alternatively the carbohydrate detected by the IFA might not be detected on Western blots because it is not solubilised by the methods used.

The same panel of lectins was used to probe the surface of the *D.viviparus* $L_3$ sheath and none of them bound to the surface of the intact sheath. However, five of these lectins were then used to probe partially exsheathed larvae and all of these bound to a greater or lesser extent. Con-A, WGA and HPA all produced bright fluorescence which was evenly distributed throughout the whole sheath. There are two possible explanations for this result. If carbohydrates are exposed on the inner surface of the sheath, they would only be accessible to the lectins after the integrity of the sheath had been breached, ie. during exsheathment. Secondly, if carbohydrates are present in the substance of the sheath, ie. not on the external or internal surfaces, then these might be exposed as the sheath is broken down during exsheathment, perhaps by the action of proteolytic enzymes. If this second explanation was correct then one might expect to see a more uneven fluorescence of the sheaths depending on the degree of degradation of particular areas of the sheath. However the effect seems to be "all or nothing" which is more consistent with the first explanation that once the integrity of the sheath is breached, the lectins can reach and bind to the internal surface. A similar effect has been reported for the binding of WGA on *N.americanus* sheaths by Pritchard et al (1991) who observed that this lectin did not bind to sheathed $L_3$ but did bind to the cast sheaths which they interpreted as evidence that N-acetyl-D-glucosamine is present on the internal sheath surface. Con-A was the only one of five peroxidase conjugated lectins which strongly detected antigen when used to probe Western blots of *D.viviparus* $L_3$ homogenate and this binding was abolished in the presence of competing sugars. HPA and WGA bound to these same molecules on Western blots but only very faintly and so this may well be due to non-specific binding. If any of these molecules were responsible for the binding of the lectins on IFA it seems likely that they would be strongly detected by all three lectins on Western blots. If these are not the antigens detected on IFA then the lectin binding sites on the sheath may be on predominantly non-proteinaceous or insoluble glycoconjugates which would not be detectable by Western blotting.

Sheathed and partially exsheathed *O.ostertagi* and *T.colubriformis* were also examined with the FITC-lectins. The result, which was the same for both species, was somewhat different to that found with *D.viviparus*. None of the lectins bound to the surface of the intact $L_3$ sheath. This result is supported by the work of Wharton & Murray (1990) who reported that Con-A, WGA, SBA, UEA, PNA and HPA did not bind to the surface of *T.colubriformis* sheathed $L_3$. However Bone & Bottjer (1985) did report that HPA bound to the intact sheaths of *N.brasiliensis* and *T.colubriformis* although there was no binding by 11 other lectins which were examined. All the lectins bound to partially or

fully cast sheaths in the same localised manner but producing differing intensities of fluorescence. Con A, HPA and WGA produced the brightest fluorescence (Table 4.3). They bound to the cranial end of sheaths which had lost their anterior cap (ie. the area of the refractile ring) and also to a well defined area towards the posterior end of the sheath. The presence of carbohydrates at these defined areas of the sheath may represent structural specialisation. The refractile ring is known to be structurally distinct from the rest of the sheath as this region is selectively degraded by exsheathing enzymes (Gamble et al 1989a and Gamble et al 1989b). The difference in lectin binding patterns between *D.viviparus* and the other trichostrongylid nematodes may reflect the difference in the mode of exsheathment which is discussed in Chapter 5.

In summary, it appears that the surface of the *D.viviparus* $L_3$ cuticle does not have exposed polypeptides amenable to surface labelling. The only surface epitope as yet identified is PC and the binding of the lectin HPA suggests that a carbohydrate with an N-acetyl-D-glucosamine terminal residue is also present. The infective larvae may evade host immune responses by a combination of exposing few immunogenic molecules on the surface, immunologically tolerising the host to exposed epitopes by the adjacent presence of PC and masking surface molecules by the binding of cross-reactive IgM. The $L_3$ sheath is much more immunogenic than the $L_3$ cuticle and six monoclonal antibodies generated to this surface all appear to recognise the same antigen. Lectins do not bind to the external surface of the *D.viviparus* $L_3$ sheath but do bind to the internal surface in a non-localised manner. This is in contrast to the binding of the same lectins to the $L_3$ sheaths of *O.ostertagi* and *T.colubriformis* which is localised to the refractile ring area and a region towards the posterior end of the sheath.

**Figure 4.1.**

**A.** Western blots of homogenates from surface biotinylated *D.viviparus* $L_3$ probed with streptavidin-peroxidase. Surface biotinylation was performed by incubating 200,000 larvae/ml (50,000 larvae/ml for *H.contortus)* in 0.85% saline with 0.5mg/ml NHS-biotin for 1 hour.After washing in PBS larvae were homogenised in Tris homogenisation buffer at concentration of 200,000 larvae/ml to produce aqueous and SDS/2ME/urea soluble homogenates in the normal manner.

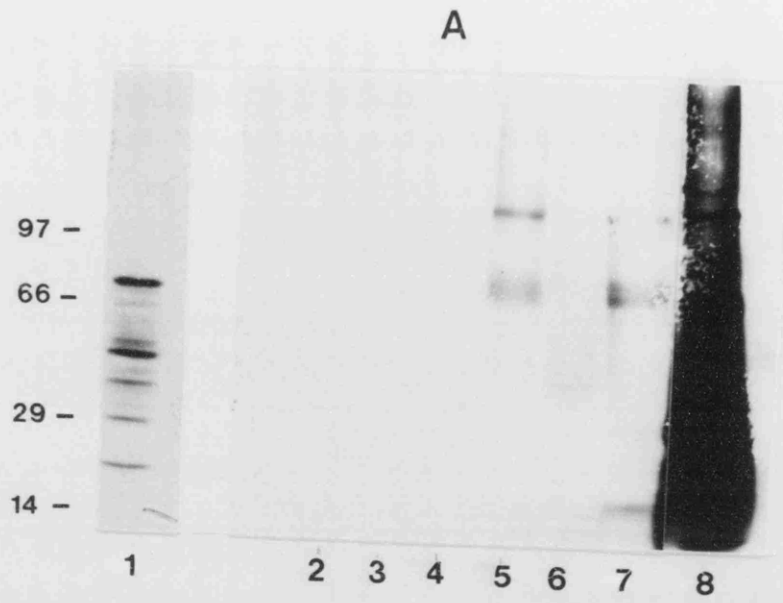
Track 1 = Exsheathed *H.contortus* $L_3$ water soluble fraction.
Track 2 = Exsheathed *D.viviparus* $L_3$ water soluble fraction.
Track 3 = Exsheathed *D.viviparus* $L_3$ SDS/2ME/urea fraction.
Track 4 & 6 = Sheathed *D.viviparus* $L_3$ water soluble fraction.
Track 5 & 7 = Sheathed *D.viviparus* $L_3$ SDS/2ME/urea fraction.
Track 8 = *D.viviparus* $L_3$ homogenate labelled with NHS-biotin.

Tracks 4,5 and 6,7 are identical except that they were performed on different batches of larvae.

**B.** Western blot, probed with streptavidin-peroxidase, of homogenates from sheathed *D.viviparus* $L_3$ which had been surface labelled with different biotin moieties. The same method as described in legend A was used.

Track 1 = sulpho-NHS biotin labelling, aqueous fraction.
Track 2 = sulpho-NHS biotin labelling, SDS/2ME/urea fraction.
Track 3 = biotin hydrazide labelling, aqueous fraction.
Track 4 = biotin hydrazide labelling, SDS/2ME/urea fraction.
Track 5 = NHS-biotin labelling, aqueous fraction.
Track 6 = NHS-biotin labelling, SDS/2ME/urea fraction.

**C.** Collagenase digestion of the surface biotinylated molecules of *D.viviparus* sheathed $L_3$. The SDS/2ME/urea fraction from labelled sheathed $L_3$ was incubated with clostridial collagenase at 37°C for 1 hour. The figure shows an extract treated with buffer alone (track 1) and an extract treated with collagenase (track 2) probed on a Western blot with streptavidin-peroxidase.


A
97 –
66 –
29 –
14 –
1
2
3
4
5
6
7
8

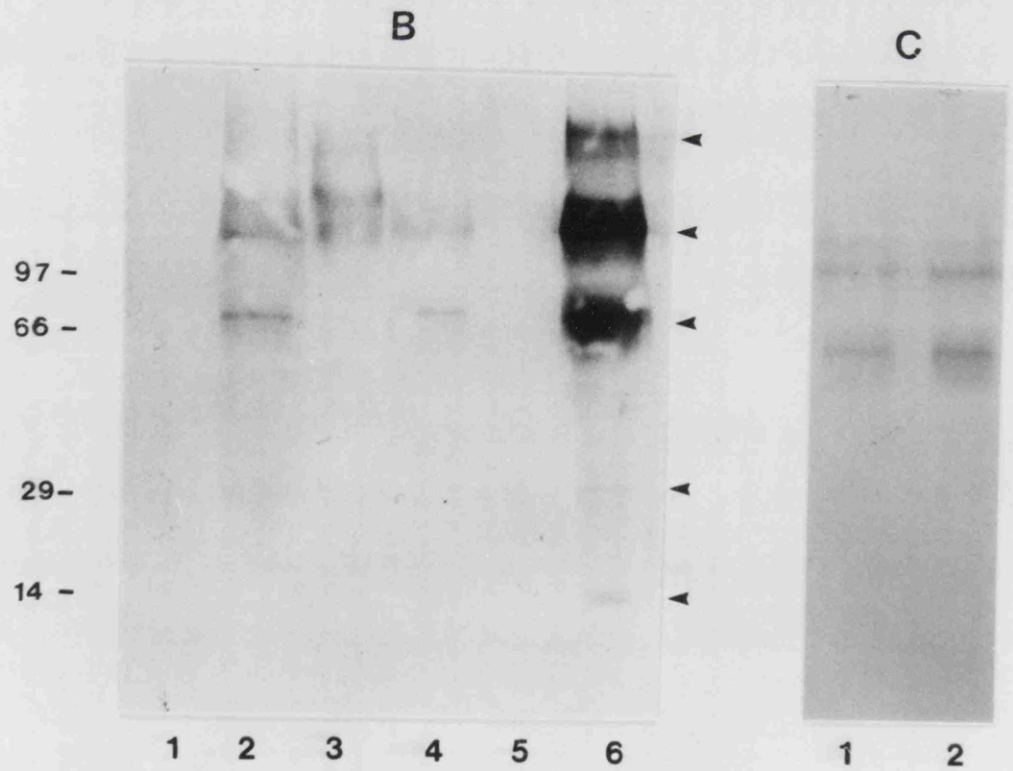
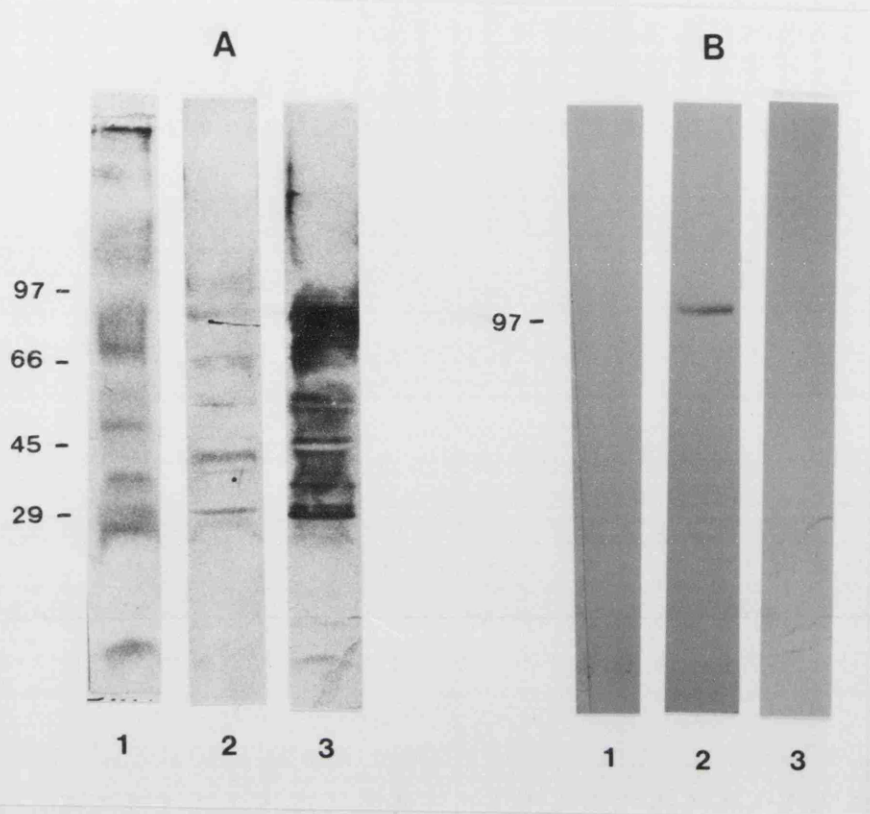

B
C
97 –
66 –
29–
14 –
1
2
3
4
5
6
1
2

**Figure 4.2.**

**A.** Western blots of *D.viviparus* $L_3$ homogenate probed with mouse sera (1:100 dilution). Track 1 is the pooled donor serum used in the passive immunisation experiment in the previous chapter. Tracks 2 and 3 are the sera taken at the time of euthanasia from the fusion 1 and fusion 2 mice respectively.

**B.** Western blots of *D.viviparus* $L_3$ homogenate (aqueous soluble) probed with hybridoma supernatants from fusion 1. Track 1; 1G3. Track 2; 3H2. Track 3; 1A5.

**C.** Western blots of *D.viviparus* $L_3$ homogenate (aqueous soluble) probed with the anti-PC monoclonal antibody Bp-1 ascites (1:100) (Track 1) and neat 2C4 supernatant (Track 2).


A
B
97 -
66 -
45 -
29 -
97 -
1
2
3
1
2
3

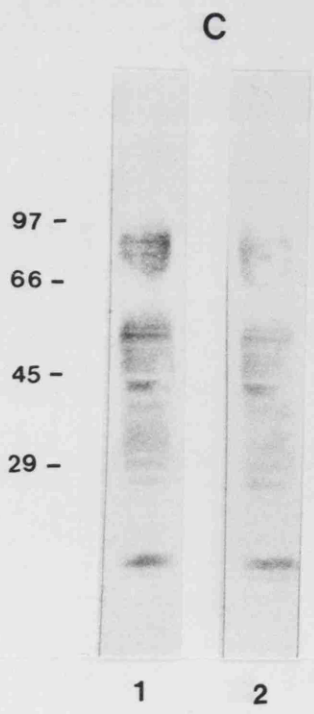
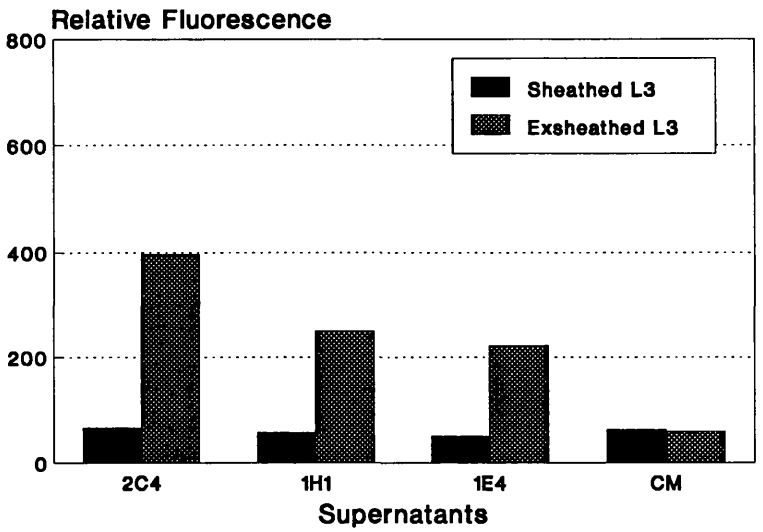

C
97 -
66 -
45 -
29 -
1
2

**Figure 4.3.**

**A.** Quantitative IFA for the three hybridoma supernatants 2C4, 1H1 and 1E4 on sheathed and exsheathed *D.viviparus* $L_3$. The values of relative fluorescence given for each antibody are the means of the readings from twenty individual larvae. CM is complete medium which was used as a negative control. The supernatant of 5E1, a *Theileria annulata* specific monoclonal antibody, also produced no fluorescence with sheathed or exsheathed $L_3$.

**B.** Quantitative IFA for the hybridoma supernatants 2A6, 2F8, 1F2, 2D8, 2A3 and 2C6 on sheathed and exsheathed $L_3$. The values of relative fluorescence given for each antibody are the means of the readings from twenty individual larvae.

A


Relative Fluorescence
800
600
400
200
0
Sheathed L3
Exsheathed L3
2C4
1H1
1E4
CM
Supernatants

B

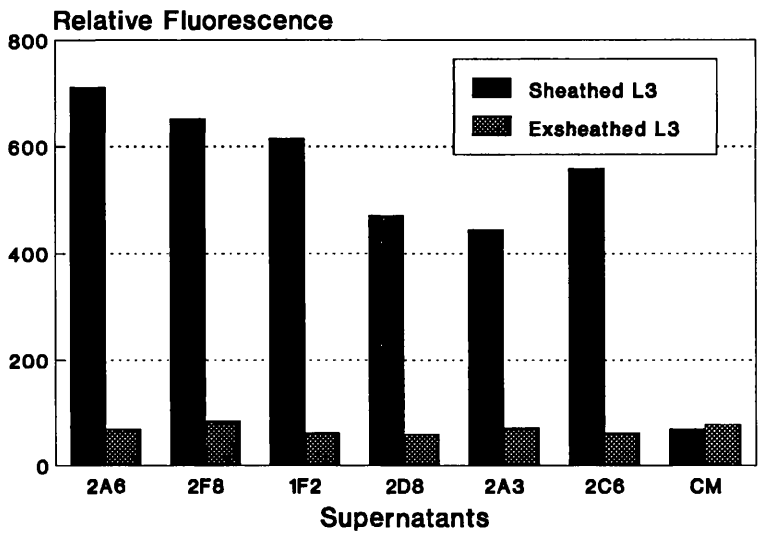
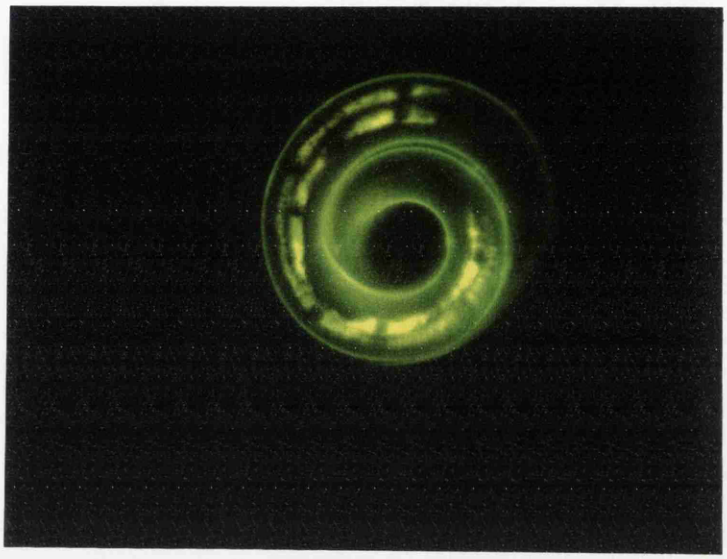

Relative Fluorescence
800
600
400
200
0
Sheathed L3
Exsheathed L3
2A6
2F8
1F2
2D8
2A3
2C6
CM
Supernatants

**Figure 4.4.**

**A.** Immunofluorescence of a *D.viviparus* exsheathed $L_3$ with neat 2C4 supernatant. Photomicrograph is taken at x400 magnification. All individual larvae appeared very similar on IFA with 2C4 as illustrated graphically in figure 4B. Exsheathed $L_3$ subjected to IFA with 1H1 and 1E4 appeared to be identical to this.

**B.** Frequency histogram displaying the readings of quantitative IFA from 25 individual exsheathed $L_3$ picked at random from several thousand larvae subjected to IFA with 2C4. A similar normal distribution was seen for the individual IFA readings of 1H1 and 1E4.

A



B

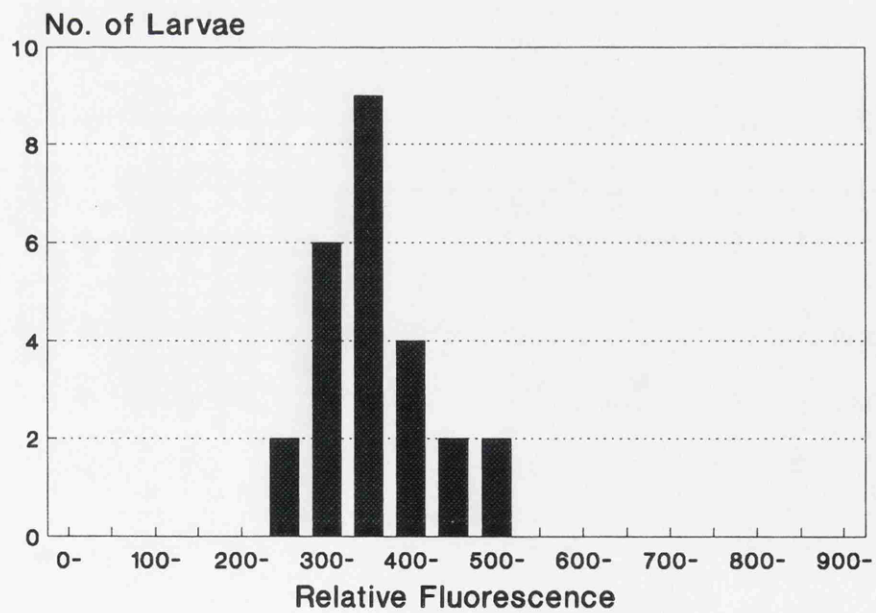
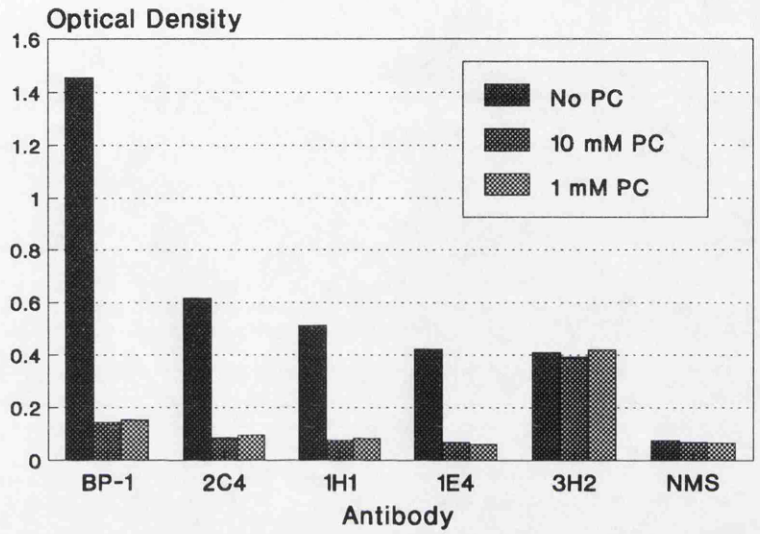

No. of Larvae
10
8
6
4
2
0
0-
100-
200-
300-
400-
500-
600-
700-
800-
900-
Relative Fluorescence

**Figure 4.5.**

**A.** Results of an $L_3$ homogenate ELISA used to measure the binding of antibodies to *D.viviparus* $L_3$ antigens in the presence and absence of PC. PC was added to the first antibody to produce final dilutions of 10mM or 1mM. The values given for each sample are the mean optical densities of quadruplicate wells. Bp-1 is ascites of an anti-PC monoclonal antibody used at a 1:500 dilution. 2C4, 1H1, 1E4, and 3H2 are undiluted tissue culture supernatants. NMS was normal mouse serum (1:100 dilution ) used as a negative control. Complete medium and the *T.annulata* monoclonal antibody supernatant 5E1 gave background optical densities similar to NMS (not shown in figure).

**B.** Quantitative IFA on *D.viviparus* exsheathed $L_3$ used to measure the binding of monoclonal antibodies to the surface of the $L_3$ cuticle in the presence and absence of 10 mM PC. The value of relative fluorescence given for each sample is the mean of 20 readings taken from individual larvae. Bp-1 was ascites used at 1:50 dilution and 2C4, 1H1 and 1E4 were all used as neat culture supernatants. The negative controls used were normal mouse serum (NMS) used at 1:50 and complete medium (CM) used neat as negative controls.

A


Optical Density
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
No PC
10 mM PC
1 mM PC
BP-1
2C4
1H1
1E4
3H2
NMS
Antibody

B

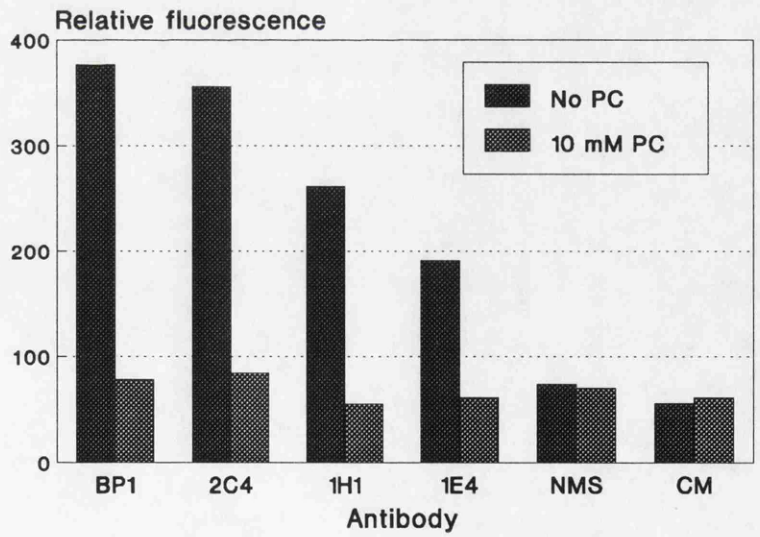
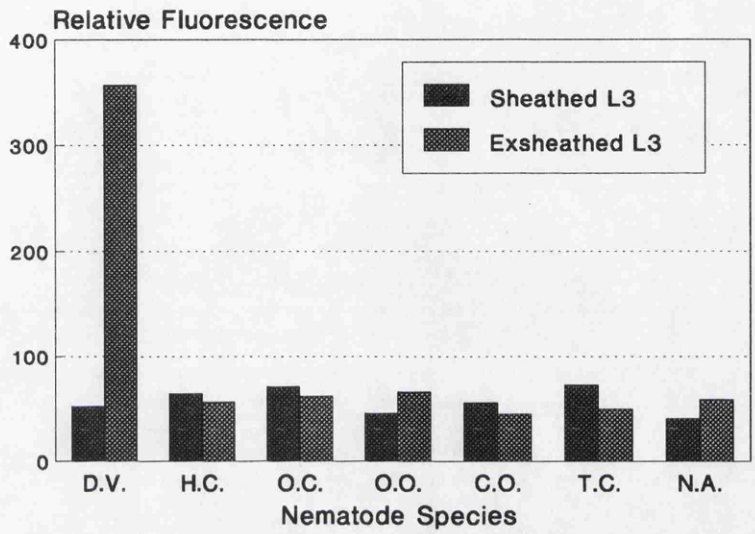

Relative fluorescence
400
300
200
100
0
No PC
10 mM PC
BP1
2C4
1H1
1E4
NMS
CM
Antibody

**Figure 4.6**

**A.** Quantitative IFA with BP-1 (ascites 1:50) on sheathed and exsheathed $L_3$ of different nematode species from the order Strongylida.

**B.** Quantitative IFA with undiluted 2C4 supernatant on sheathed and exsheathed $L_3$ of different nematode species from the order Strongylida.

A


Relative Fluorescence
400
300
200
100
0
Sheathed L3
Exsheathed L3
D.V.
H.C.
O.C.
O.O.
C.O.
T.C.
N.A.
Nematode Species

B

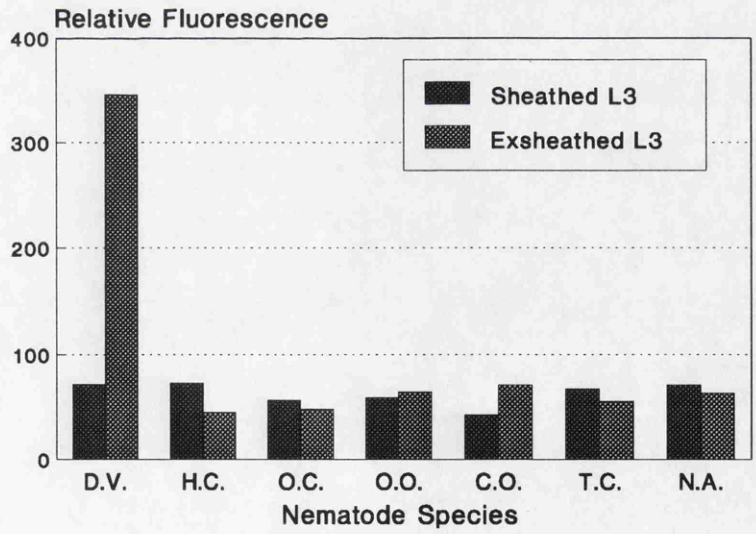

Relative Fluorescence
400
300
200
100
0
Sheathed L3
Exsheathed L3
D.V.
H.C.
O.C.
O.O.
C.O.
T.C.
N.A.
Nematode Species

**Figure 4.7.**

Quantitative IFA performed on different developmental stages of *D.viviparus* with Bp-1 ascites (1:50) and 2C4 undiluted supernatant.

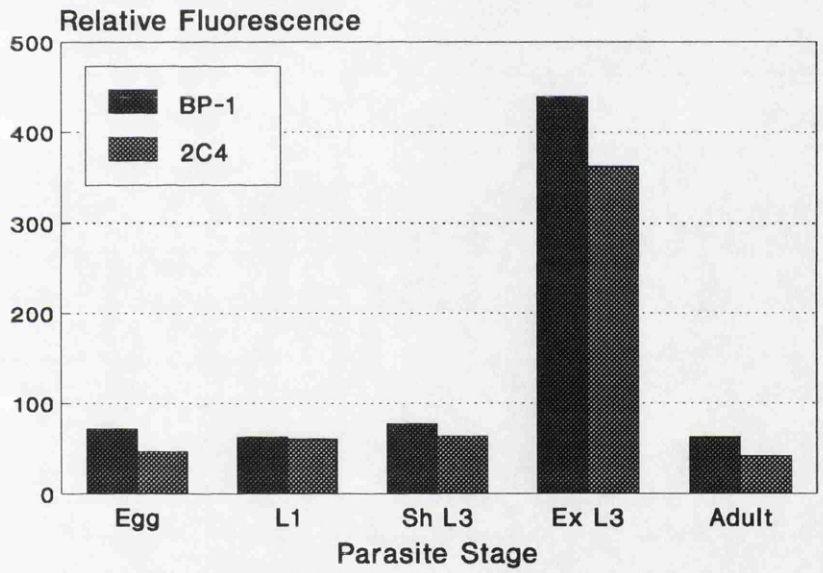

Relative Fluorescence
500
400
300
200
100
0
BP-1
2C4
Egg
L1
Sh L3
Ex L3
Adult
Parasite Stage

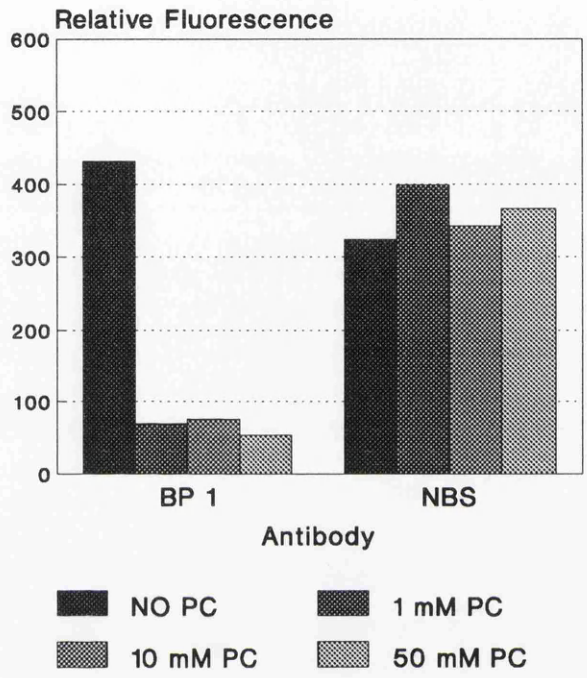
**Figure 4.8.**

**A.** Quantitative IFA on *D.viviparus* exsheathed $L_3$ with Bp-1 and normal bovine serum (NBS) in the presence of increasing concentrations of free PC. Bp-1 ascites was used at a 1:50 dilution and detected with an anti-mouse IgG (whole molecule) conjugate. Naive bovine serum (NBS) was used at a 1:50 dilution and detected with an anti-bovine IgG (whole molecule) conjugate.

**B.** Quantitative IFA on *D.viviparus* exsheathed $L_3$ with naive bovine serum (NBS) in the presence of increasing concentrations of competing Bp-1. An anti-bovine IgM (Fc) FITC conjugate was used at a dilution of 1:100 as the second antibody in all these samples in order to detect bovine antibody and not Bp-1. The first antibody used in each of the different samples are as follows;

NBS = Naive bovine serum at 1:50 dilution.
BP-1 = BP-1 ascites at a 1:10 dilution.
1 = NBS (1:50) and BP-1 (1:50).
2 = NBS (1:50) and BP-1 (1:20).
3 = NBS (1:50) and BP-1 (1:10).

A


Relative Fluorescence
600
500
400
300
200
100
0
BP 1
NBS
Antibody
NO PC
1 mM PC
10 mM PC
50 mM PC

B

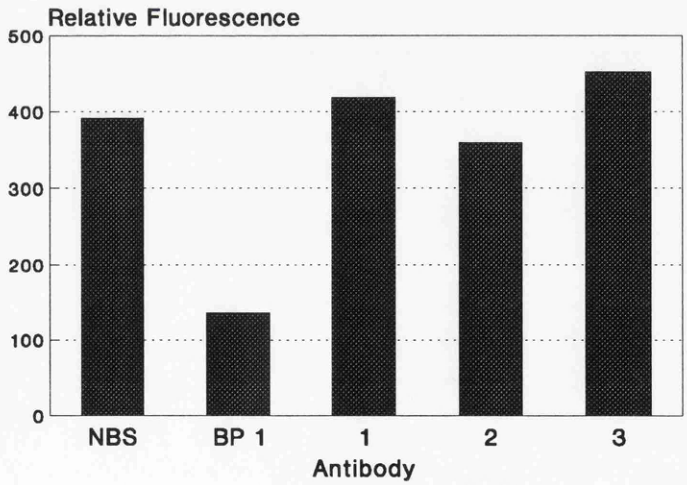

Relative Fluorescence
500
400
300
200
100
0
NBS
BP 1
1
2
3
Antibody

**Figure 4.9.**

$L_3$ homogenate ELISA with pooled serum from cattle following repeated oral infection with *D.viviparus* (B7,B8 & B9 sera -see Appendix 2).The points on the graph are the days on which the samples were taken and the infections were given on days 0, 48, 76, 103 & 127. Each point is the mean optical density for quadruplicate wells for each sample and the same samples assayed in the presence of 0, 10 and 50mM PC giving the three curves shown on the graph.

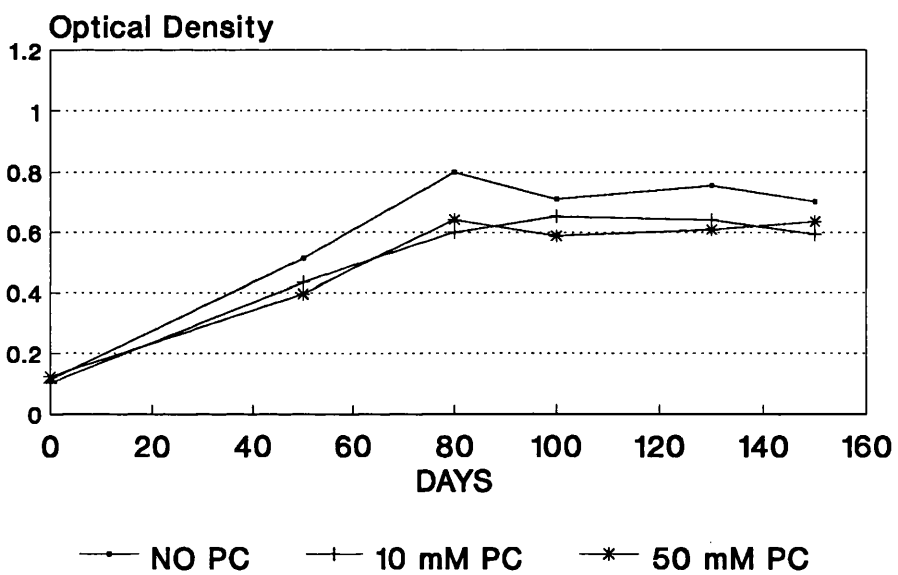
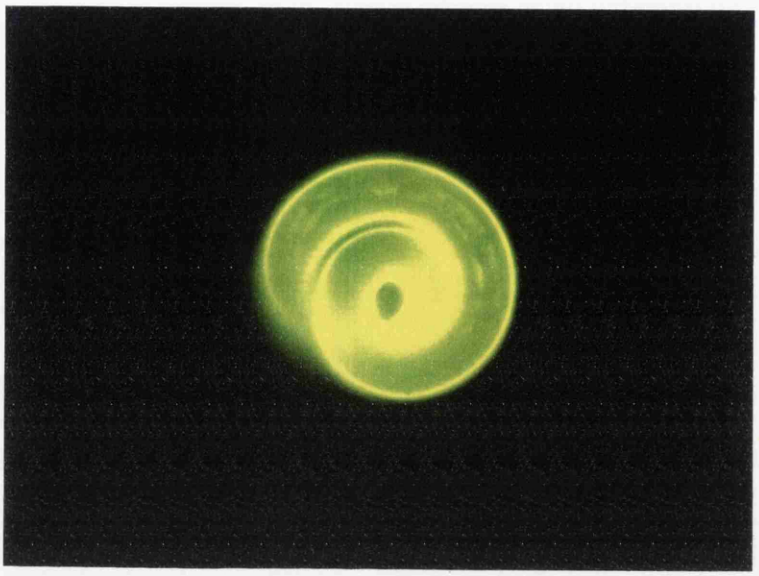

Optical Density
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
140
160
DAYS
NO PC
10 mM PC
50 mM PC

**Figure 4.10.**

**A.** Immunofluorescence of a *D.viviparus* sheathed $L_3$ with neat 2A6 supernatant. Photomicrograph was taken at x400 magnification. Sheathed $L_3$ subjected to IFA with 2F8, 1F2, 2D8, 2A3, and 2C6 had an identical appearance to this.

**B.** Frequency histogram displaying the readings of quantitative IFA from 25 individual sheathed $L_3$ picked at random from several thousand larvae subjected to IFA with 2A6. A similar distribution was seen for the individual IFA readings of 2F8, 1F2, 2D8, 2A3, and 2C6.

A



B

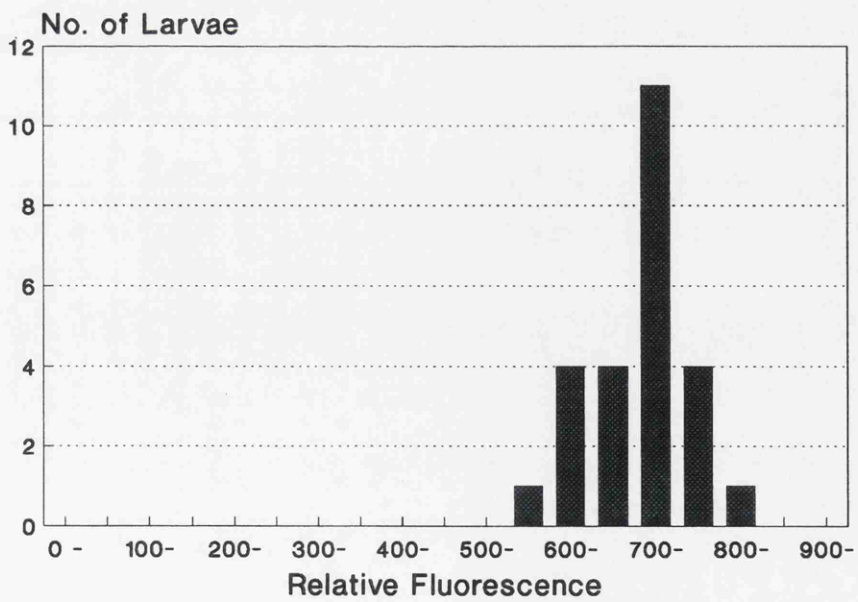

No. of Larvae
12
10
8
6
4
2
0
0 -
100-
200-
300-
400-
500-
600-
700-
800-
900-
Relative Fluorescence

**Figure 4.11**

**A.** IFA with 2A6 on a partially exsheathed *D.viviparus* $L_3$ at x400 magnification.

**B.** IFA with 2F8 on an exsheathed (left) and a sheathed (right) $L_3$ at x400 magnification in the same field of view.

**Figure 4.12.**

**A.** Western blots of *D.viviparus* $L_3$ homogenate probed with the anti-sheath monoclonal antibody supernatants. Track 1 = 2A6, Track 2 = 2C6, Track 3 = 2F8, Track 4 = 1F2, Track 5 = 1A3, Track 6 = 2D8, Track C = 5E1 (supernatant from a *Theileria annulata* specific monoclonal antibody used as a negative control).

**B.** Western blots of *D.viviparus* adult homogenate probed with the anti-sheath monoclonal antibody supernatants. The numbered tracks are for the same antibodies as figure A. In this case Track C is a positive control track of *D.viviparus* $L_3$ homogenate probed with 2C6.

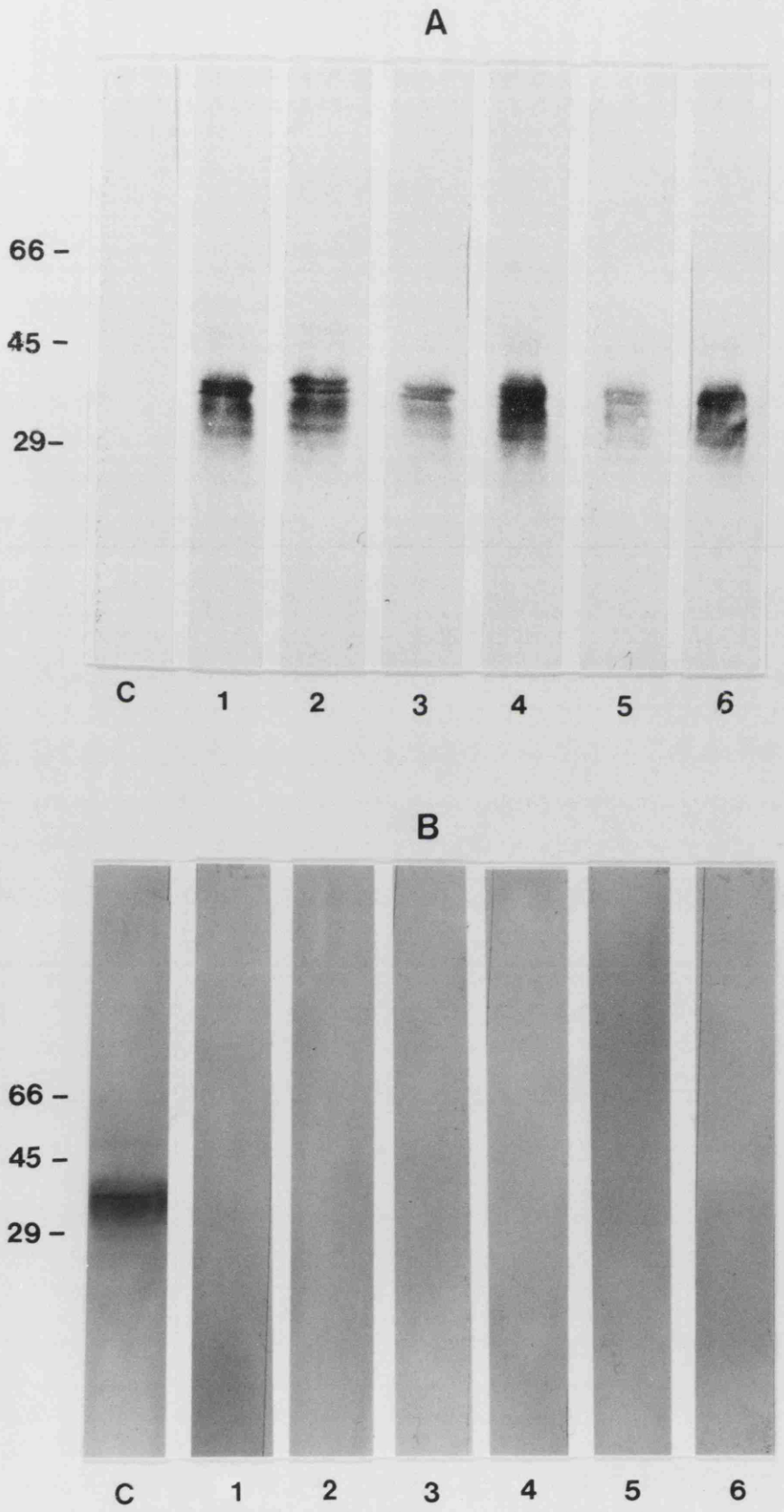

A
66 –
45 –
29–
C
1
2
3
4
5
6
B
66 –
45 –
29 –
C
1
2
3
4
5
6

**TABLE 4.1**

Sugar specificity of the FITC-conjugated lectins used to probe the surface of *D.viviparus*, *O.ostertagi* and *T.colubriformis* $L_3$.

| LECTIN | COMMON NAME | SUGAR SPECIFICITY |
|---|---|---|
| Canavalia ensiformis agglutinin (Con A) | Jack bean agglutinin or Concanavilin A | α-D-Mannose<br>α-D-Glucose |
| Helix pomotia agglutinin (HPA) | | N-acetyl-D-galactosamine |
| Triticum vulgaris agglutinin (WGA) | Wheat germ agglutinin | N-acetyl-D-glucosamine |
| Tetragonolobus purpureas agglutinin (TPA) | | α-L-Fucose |
| Arachis hypogea agglutinin (PNA) | Peanut agglutinin | D-galactose<br>N-acetyl-D-galactosamine |
| Ricinus communis agglutinin (RCA) | Castor bean agglutinin | β-D-galactose<br>N-acetyl-D-galactosamine |
| Lens culinaris (LCA) | Lentil agglutinin | α-D-Mannose<br>α-D-glucose |

**Table 4.2.**

Summary of qualitative IFA performed on *D.viviparus* $L_3$ with FITC-conjugated lectins. The fluorescence seen was generalised over the whole cuticle or sheath as is illustrated in figure 4.13. - = no fluorescence, ++++ = very bright fluorescence and NP = not performed.

**Table 4.3.**

Summary of qualitative IFA performed on *O.ostertagi* $L_3$ with FITC-conjugated lectins. The scores represent the intensity of fluorescence which was of a localised nature as illustrated in figures 4.14 and 4.15.

**TABLE 4.2**

| LECTIN | FLUORESCENCE | | |
|---|---|---|---|
| | SHEATHED L3 | EXSHEATHED L3 | PARTIALLY EXSHEATHED L3 |
| CON A | - | +? | ++++ |
| HPA | - | +++ | ++++ |
| WGA | - | +? | ++++ |
| TPA | - | - | ++ |
| PNA | - | - | ++ |
| RCA | - | +? | NP |
| LCA | - | - | NP |

**TABLE 4.3**

| LECTIN | FLUORESCENCE | | |
|---|---|---|---|
| | SHEATHED L3 | EXSHEATHED L3 | PARTIALLY EXSHEATHED L3 |
| CON A | - | +? | +++ |
| HPA | - | - | ++++ |
| WGA | - | - | ++++ |
| TPA | - | - | ++ |
| PNA | - | - | ++ |
| RCA | - | - | NP |
| LCA | - | - | NP |

**Figure 4.13.**

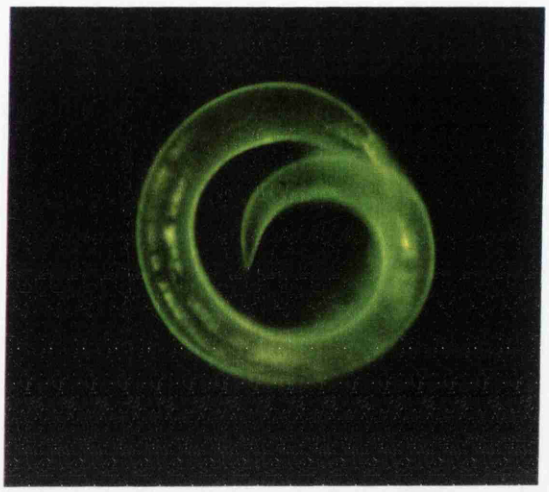
**A.** IFA on *D.viviparus* exsheathed $L_3$ with FITC-conjugated HPA. x400 magnification.

**B.** IFA on a partially exsheathed *D.viviparus* $L_3$ with FITC-conjugated HPA. x1000 magnification.

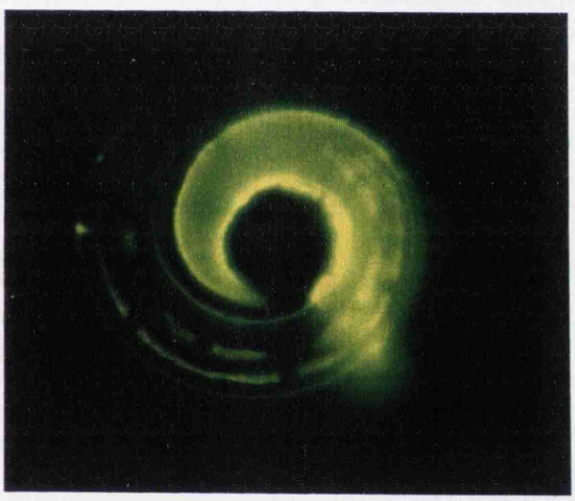
**C.** IFA on a partially exsheathed *D.viviparus* $L_3$ with FITC-conjugated Con A. x1000 magnification.

**D.** IFA on a partially exsheathed *D.viviparus* $L_3$ with FITC-conjugated WGA. x1000 magnification.

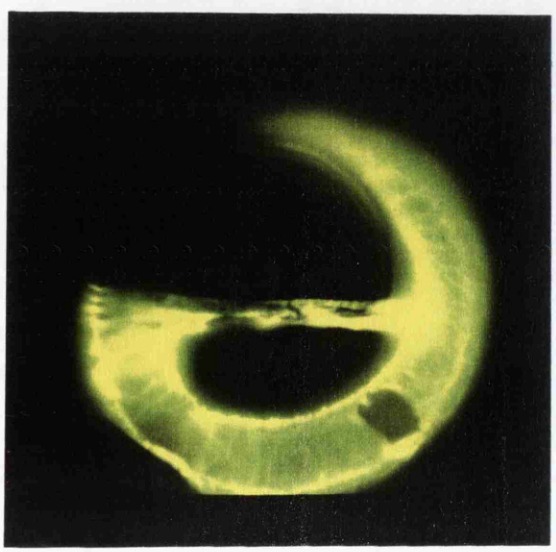
A



B



C



D

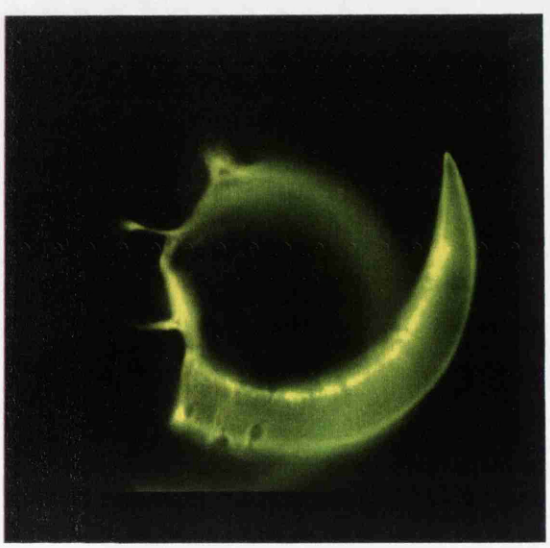


**Figure 4.14.**

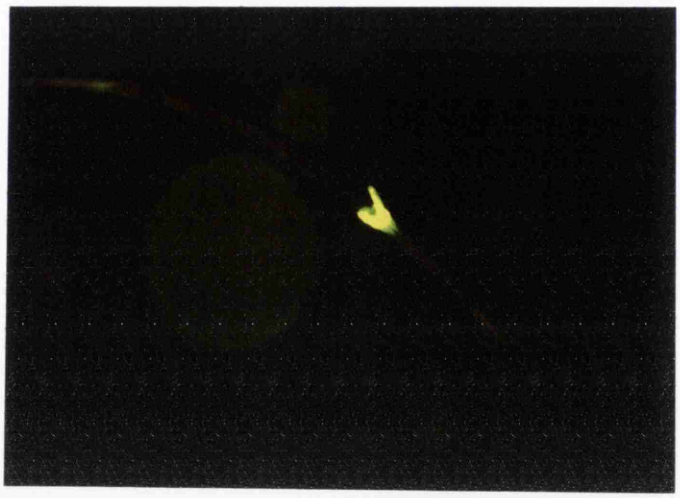
**A.** IFA on exsheathing *O.ostertagi* $L_3$ with FITC-conjugated HPA. x100 magnification.

**B.** Photomicrograph of the same larva in figure A. x100 magnification.

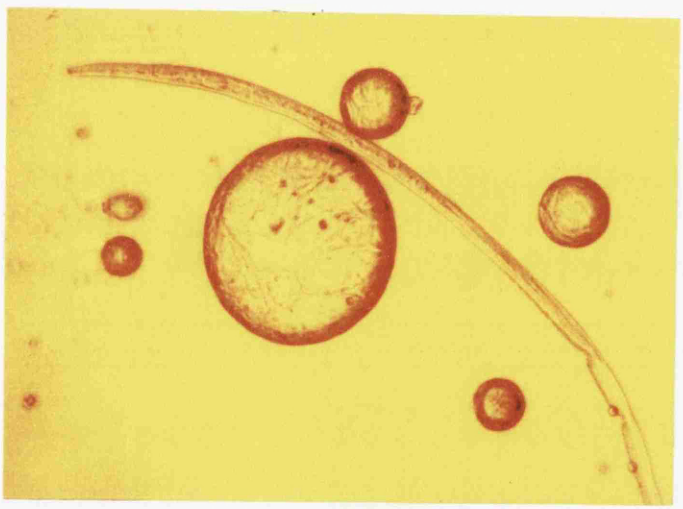
**C.** IFA on exsheathing *O.ostertagi* $L_3$ with FITC-conjugated Con A. x1000 magnification.

**D.** Photomicrograph of the same larva in figure C. x400 magnification.

A



B



C

D

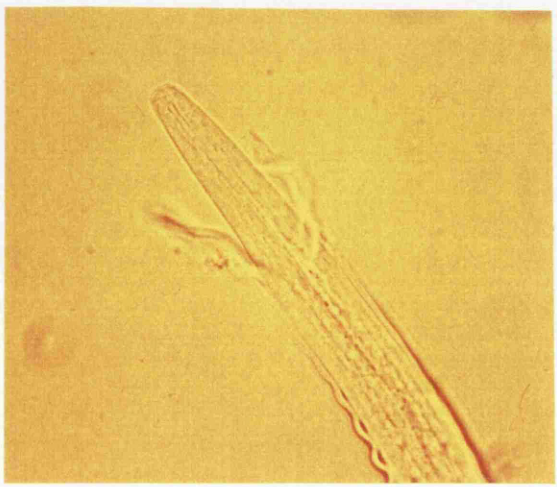


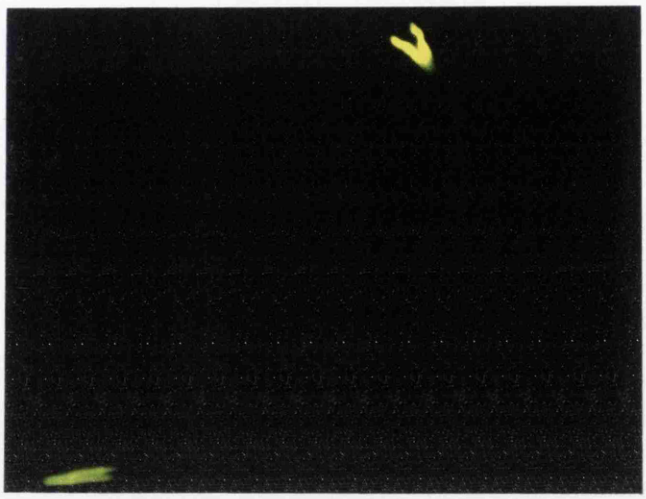
**Figure 4.15.**

**A.** IFA on exsheathing *O.ostertagi* $L_3$ with FITC-conjugated WGA. x100 magnification.

**B.** Photomicrograph of the same larva in figure A. x100 magnification.

**C.** IFA on a cast $L_3$ sheath of *O.ostertagi* with FITC-conjugated HPA. x100 magnification.

A



B

C

**Figure 4.16**

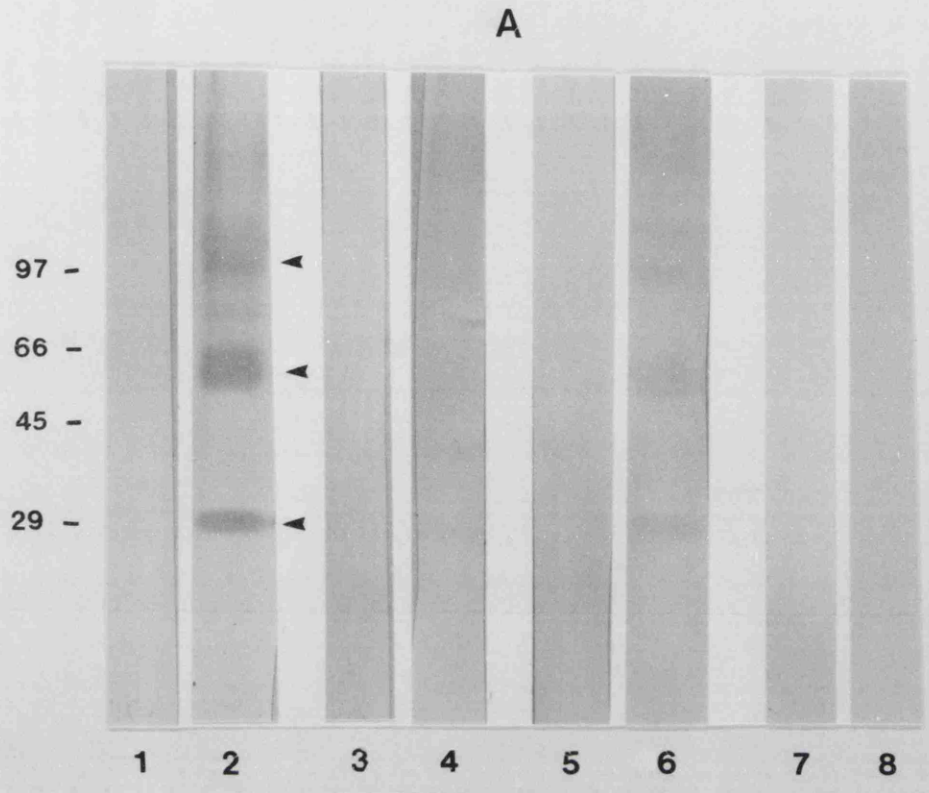
**A.** Western blots of *D.viviparus* $L_3$ homogenate probed with peroxidase conjugated lectins.

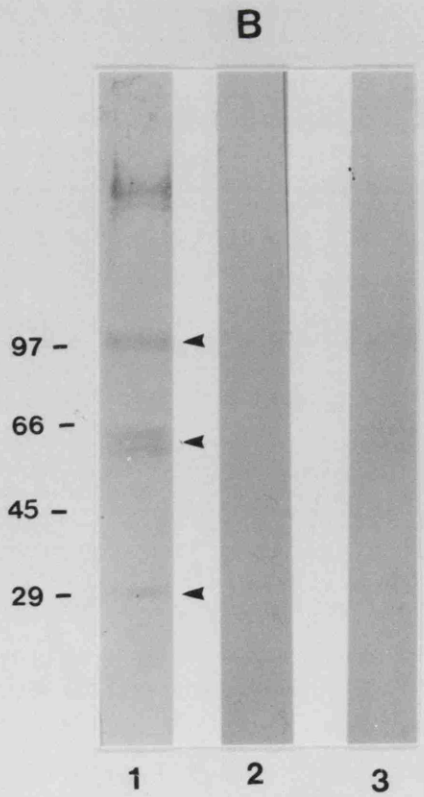
Tracks 1 and 2 = Con A.
Tracks 3 and 4 = HPA.
Tracks 5 and 6 = WGA.
Tracks 7 and 8 = PNA.

Tracks 1, 3, 5 and 7 are water soluble $L_3$ homogenate and tracks 2, 4, 6 and 8 are SDS/2ME/urea soluble homogenate.

**B.** Western blots of *D.viviparus* $L_3$ SDS/2ME/urea soluble homogenate probed with peroxidase-conjugated Con A (1:100 dilution) in the presence of competing sugars.

Track 1 = Con A alone
Track 2 = Con A and 0.2M mannose.
Track 3 = Con A and 0.2M glucose.


A
97 -
66 -
45 -
29 -
1
2
3
4
5
6
7
8


B
97 -
66 -
45 -
29 -
1
2
3

# Chapter 5

# CHAPTER 5.

## Characterisation of the *D.viviparus* $L_3$ sheath specific monoclonal antibodies.

### 5.1. Introduction.

The infective third stage larvae of trichostrongylid nematodes, which are free living and non-feeding, can survive on pasture for many months and are resistant to desiccation and sub-zero temperatures (Wharton 1982a and Wharton et al 1984). They are enclosed in the retained $L_2$ cuticle which has separated from the underlying $L_3$ cuticle but has not been shed during the moult from $L_2$ to $L_3$. This retained cuticle is often referred to as the $L_3$ sheath and is thought to be important in the ability of infective larvae to resist adverse environmental conditions (Ellenby 1968).

Relatively little work has been performed on the structure of the trichostrongylid $L_3$ sheath but from electron microscopic studies it was originally described as a relatively featureless structure surrounded by an outer and an inner electron dense layer (Smith 1970 and Eckert & Schwartz 1965). However, closer inspection of these published electron micrographs revealed bands of varying electron density in the basal area of the sheath (Wharton 1986). In a more detailed electron microscopic study of the *T.colubriformis* $L_3$, Wharton (1986) reported that the structure of the sheath followed the same basic pattern as the $L_3$ cuticle but was somewhat simpler. Four layers were described; an outer electron dense 11 nm thick epicuticle, an amorphous cortical zone, a fibrous basal zone and an inner electron dense layer. Freeze-fracture studies revealed that the inner basal zone consists of three layers of differently orientated fibres. Electron micrographs of *Haemonchus contortus, Necator americanus* and *Nippostrongylus brasiliensis* all show variation of electron densities throughout the basal zone (Smith 1970 and Inatomi et al 1963) and so the complex structure of the basal zone seen in *T.colubriformis* may be a structural feature common to other trichostrongylid $L_3$ sheaths. The sheath therefore possesses all the zones, except the median zone, of a living cuticle.

The sheath, like the cuticle, also shows some regional specialisation of structure. On the outer surface there are longitudinal expansions forming two lateral alae and on the inner surface of these there is a longitudinal projection which fits into a groove in the lateral alae of the underlying cuticle. This arrangement is thought to stop any rotation of the larvae within the sheath (Wharton 1982b and Eckert & Shwartz 1965).

Relatively little is known about the biochemical composition of the trichostrongylid $L_3$ sheath but Fetterer (1989) compared the proteins from isolated sheaths with those from the $L_3$ cuticle of *H.contortus*. The proteins were divided into two fractions, the 2ME soluble proteins many of which were collagenase sensitive and the 2ME insoluble proteins. The molecular weights of the most abundant 2ME soluble proteins differed between the sheath and the cuticle but their amino acid composition was very similar, with glycine, proline and hydroxyproline being the most abundant. The amino acid composition of the insoluble proteins was also similar for the sheath and the cuticle. Collagenase partially digested the basal layer of isolated $L_3$ cuticles but had no effect on the basal layer of the sheath. HPLC analysis has identified dityrosine and isotrityrosine in both 2ME soluble and insoluble sheath proteins (in greater amounts in the insoluble proteins) which suggests the presence of tyrosine derived cross-linking between both collagenous and none collagenous proteins of the *H.contortus* $L_3$ sheath. This is interesting since the insolubility of the *Ascaris* protein matrix cuticlin has been ascribed to this form of chemical cross-linking (Fujimoto 1975). Thus the protein composition of the *H.contortus* $L_3$ sheath seems to be very similar to that of the $L_3$ cuticle except that there may be more cross-linking of proteins by non-reducible covalent bonds.

The only other studies described have used FITC conjugated lectins to identify carbohydrates on the surface of the $L_3$ sheath. Bone & Bottjer (1985) reported that the lectin *Helix pomotia* agglutinin bound to the intact surface of *T.colubriformis* but this result could not be repeated by Wharton (1986) or in the lectin binding studies presented in earlier this thesis. Over a dozen other lectins have been tried by several authors on *T.colubriformis*, *H.contortus*, *N.brasiliensis* and *N.americanus* (Bone & Bottjer 1988, Wharton 1986 and Pritchard et al 1991) and none have been found to bind to the intact sheath surface.

Once the infective larvae enters the host exsheathment occurs and the parasite undergoes further development. The process of exsheathment for the ruminant gastro-intestinal trichostrongylids has been well characterised (Lapage 1935, Slocombe & Whitlock 1969, Gamble et al 1989a, 1989b and Wharton 1991). The first stage of exsheathment involves the formation of an indented ring, known as the "refractile ring", occurring at the 20th posterior annulus which is approximately 20um from the anterior end (Ozerol & Silvermann 1972b and Gamble et al 1989a). This ring is associated with degradation of the inner electron dense layer and basal layer leading to rupture of this region resulting in the release of the "apical cap" anterior to the ring. The $L_3$ then escapes from the sheath through the resultant opening. A number of workers have analysed the exsheathing fluids of *H.contortus* and several of the earlier studies reported the presence of leucine aminopeptidase activity (Rogers

1963, Rogers 1965 and Rogers & Brooks 1978) but this has been refuted by other workers (Slocombe 1974, Ozerrol & Silvermann 1972b and Gamble et al 1989). Gamble et al (1989) demonstrated that exsheathing fluids of *H.contortus* could induce refractile ring formation in mechanically prepared sheath heads of *H.contortus*, *Ostertagia ostertagi*, *Cooperia oncophora* and *T.colubriformis* and this fluid yielded a single sharp peak of protease activity at a molecular weight approximately 43 kDa. They then went on to purify a 44 kDa zinc-metalloprotease which could induce refractile ring formation in these nematode species. Further work identified a 160 kDa polypeptide which was unique to the refractile ring region of the sheath and was digested by the 44 kDa protease (Gamble & Fetterer 1990). Polypeptides of similar size were shown to occur in other trichstrongylid sheaths and so it seems that the process of exsheathment is well conserved amongst these nematode species. No work of this nature has been performed to investigate the exsheathment of the *D.viviparus* $L_3$.

The site of exsheathment in the host has been investigated by incubating larvae in fluid from various parts of the gastro-intestinal tract and also placing them directly into the rumen, abomasum, duodenum and small intestine in "cellophane" sacs (Sommerville 1954 & Bird 1955). These authors concluded that exsheathment occurs in the part of the alimentary tract that immediately precedes the region in which adults are normally found. Therefore *H.contortus*, *Trichostrongylus axei* and *Ostertagia circumcincta*, whose parasitic adult stages reside in the abomasum, were observed to exsheath in the rumen whereas the small intestinal parasites *T.colubriformis* and *Nematodirus* spp. exsheath in the abomasum. No experiments of this nature have been performed for *D.viviparus* and it has generally been assumed that these larvae exsheath in the abomasum prior to their penetration of the small intestinal wall and subsequent migration. This assumption is consistent with the observation by Silvermann & Podger (1964) that *D.viviparus*, unlike most other trichostrongylid larvae did not exsheath when exposed to $CO_2$ at the appropriate pH but did exsheath after exposure to pepsin and HCL. However Parker & Croll (1976) reported that *D.viviparus* could be made to exsheath in a variety of proteolytic enzymes including collagenase and concluded that the effect of pepsin was not specific. Therefore there is very little direct evidence to indicate the site of exsheathment of *D.viviparus* infective larvae. Interestingly Soliman (1953a) reported finding both dead ensheathed and live exsheathed larvae in the mesenteric lymph nodes of several cattle infected with *D.viviparus*. This suggests that at least some $L_3$ migrate to the mesenteric lymph nodes prior to exsheathment.

There is also similar debate about the site of exsheathment of the infective larvae of migratory hookworm species. Although the conventional view is that sheaths are lost during penetration of skin, several authors have suggested that this

might not be the case. Sheathed larvae of the feline hookworm *Ancylostoma tubaeforme* (Matthews 1972 and Matthews 1975) and the human hookworm *Ancylostoma duodenale* (Hotez et al 1990) have been shown to be capable of penetrating host skin tissue. Also Pritchard et al (1991) reported that following the application of fluoroscein labelled *N.americanus* infective larvae to intact skin, sheaths could be identified deep in the dermal tissues. Therefore it is possible that for migratory strongylid nematodes, such as *D.viviparus*, *Ancylostoma* spp and *N.americanus*, it is the $L_3$ sheath and not the $L_3$ cuticle which is the first parasite surface exposed to the host immune system.

Britton (1991) reported that IFAs with serum from both infected and vaccinated cattle on live *D.viviparus* larvae produced intense fluorescence with sheathed larvae. This was in contrast to the low levels of fluorescence produced by these sera with exsheathed larvae relative to normal bovine serum. This high antigenicity of the sheath surface is very surprising given the conventional belief that exsheathment occurs in the alimentary tract prior to larval migration. Interestingly, Pritchard et al (1991) have reported a similar phenomenon in *N.americanus* where human infection serum reacts strongly with the sheath in IFAs but shows little reactivity against the $L_3$ cuticular surface. The antigens responsible for this antigenicity of the $L_3$ sheath have not been identified for either *D.viviparus* or *N.americanus*.

In summary, molecules on the surface of $L_3$ sheaths of strongylid nematodes are of interest for a number of reasons. They form the interface between the free-living infective larvae and their environment and also constitute the first interface between the infective larvae and their host. The remarkably strong immune response to this surface which has been reported in migratory nematode species makes the associated antigens of particular interest.

## 5.2. Results.

The preliminary characterisation of the six sheath specific monoclonal antibodies presented in the previous chapter suggested that they are all directed against the same antigen, although they may recognise different epitopes. In this chapter the use of mabs 2A6 and 2F8 to investigate the target antigen and the nature of the $L_3$ sheath surface is described.

### 5.2.1. Examination of *D.viviparus* $L_3$ exsheathment using IFA with 2A6.

As discussed above the infective larvae of strongylid nematodes, upon infection of the host, escape from the $L_3$ sheath by proteolytic cleavage of a specialised area near the anterior end of the sheath. The rest of the sheath appears to be unaffected by this process and the discarded sheath can be easily seen by light microscopy (figure 5.1A). When observed by microscopy this process appears to be the same whichever *in vitro* method of exsheathment is used, eg. bubbling 100% $CO_2$ through the larval suspension or incubating the larvae in 0.01% sodium hypochlorite.

Attempts to exsheath *D.viviparus* $L_3$ by bubbling 100% $CO_2$ through the larval suspension were unsuccessful in spite of the effectiveness of this method with *H.contortus* and *T.colubriformis*. *D.viviparus* $L_3$ do exsheath when placed in a solution of 0.01% sodium hypochlorite for approximately 15-20 minutes. However, unlike the other strongylid nematodes, no refractile ring formation can be seen and only very occasionally was a *D.viviparus* larvae seen escaping from an intact sheath. For the vast majority of the larvae the sheath just gradually became less distinct and eventually disappeared with no discarded empty sheaths being visible.

IFA with mab 2A6 was performed on *D.viviparus* $L_3$ which had been washed in PBS after being incubated for varying lengths of time in 0.01% sodium hypochlorite. The first change apparent in the sheath was the appearance of transverse cracks throughout its length (figure 5.1B), followed by the appearance of holes due to the loss of sections of sheath (figure 5.28C). This continued in an apparently random manner until the whole sheath had disintegrated. Occasionally partially degraded cast sheaths could be seen by IFA but most of the sheath material appeared to be broken down into very small fragments (figures 5.1D and 5.1E).

### 5.2.2. 2A6 and 2F8 bind to the $L_3$ sheath of many strongylid nematodes.

There are certain common features to the life cycles of most parasitic nematodes belonging to the order Strongylida. The $L_1$ to $L_3$ stages are free-living and, as described above, the $L_2$ cuticle is retained as a sheath surrounding the infective $L_3$ stage. The binding of mab 2A6 to the $L_3$ sheath of a number of strongylid nematodes was investigated by quantitative IFA. Figure 5.2 shows the mean relative fluorescence produced by 2A6 (calculated from measurements on 20 individual larvae for each sample) on the sheathed and exsheathed $L_3$ of each species. The antibody produced a similarly high degree of fluorescence with the sheathed $L_3$ of all the species examined but did not bind to the surface of any of the exsheathed $L_3$. Qualitative IFA was also performed with 2F8 on the sheathed and exsheathed $L_3$ of the same nematode species and the results were the same as for 2A6. Further qualitative experiments showed that 2C6, 2D8, 2A3 and 1F2 all bound to the sheathed (but not exsheathed) $L_3$ of *H.contortus*, *T.colubriformis* and *O.circumcincta*.

The pattern of binding of 2A6 to the surface of all these different species of sheathed $L_3$ was similar to the binding seen with the *D.viviparus* $L_3$ as is illustrated for *H.contortus* (figure 5.3). The fluorescence was evenly distributed over the whole sheath surface (figure 5.3A) and the values of relative fluorescence measured from individual larvae approximate to a normal distribution when plotted on a frequency histogram (figure 5.3B). Therefore, as with *D.viviparus*, the antigen detected by 2A6 is evenly distributed over the *H.contortus* sheath surface and there is no significant variation in the binding of the antibody between individual larvae. The pattern of binding of 2A6 was similar for all the nematode species shown in figure 5.2.

Qualitative IFA was performed with both 2A6 and 2F8 on a number of nematodes which do not belong to the order Strongylida. *Ascaris suum* $L_2$, *Trichinella spiralis* $L_1$, *Toxocara canis* $L_2$ and a mixed stage culture of *Caenorhabditis elegans* were examined and no fluorescence was produced with any of these organisms. *Nippostrongylus brasiliensis* $L_3$ were also examined which are from the order Strongylida but the $L_2$ of laboratory strains shed the cuticle after moulting to the $L_3$ and consequently do not possess a sheath. The $L_3$ cuticular surface did not bind either 2A6 or 2F8 but cast $L_2$ cuticles could not be obtained for examination.

Western blots of $L_3$ homogenate (aqueous soluble) antigens from a number of nematode species from the order Strongylida were probed with 2A6 and 2F8 on Western blots and the two antibodies produced identical results. Figure 5.4A shows $L_3$ antigens of *D.viviparus*, *H.contortus*, *C.oncophora* and *Cyathostome* spp. probed with 2F8. Figure 5.4B shows Western blots of $L_3$ homogenate from *D.viviparus*, *H.contortus*, *T.colubriformis*, *C.oncophora*, *O.circumcincta*, *Cyathostomes* probed with

2A6. The antibodies bind to antigens of different molecular weights in some of the different nematode species. For *H.contortus*, *T.colubriformis*, *C.oncophora* and *O.circumcincta* an antigen of approximately 20 kDa is detected. There is some variation as to whether this resolves as a single band or doublet between individual blots and it has been observed as a triplet in *T.colubriformis* (Figure 5.4B). This variation is not dependant upon the batch of antigen or antibody used but is more related to the quality of resolution and the sensitivity of each particular blot. The antibodies detected 5 bands in blots of *Cyathostome* $L_3$ homogenates at 32, 30, 28, 16 and 18 kDa with little variation between repeated blots.

At this stage two different interpretations of the data are possible. Either an epitope is conserved between the different species but is present on different molecules or alternatively an antigenically similar, possibly homologous, molecule is conserved on the sheath of all these different nematode species. The similarity in the molecular weights of the antigens recognised in some of the species suggests that the latter explanation is the more likely and subsequent data to be presented supports this view.

*A.suum* $L_2$ (Figure 5.4A), *T.spiralis* $L_1$ (figure 5.4B) and *C.elegans* mixed stage culture homogenates (data not shown) were probed with both 2A6 and 2F8 on Western blots and no antigens were detected. Therefore it appears that 2A6 and 2F8 only bind to antigen from nematodes from the order Strongylida.

The results of the detection of antigen by the monoclonal antibodies 2A6 and 2F8 for the different species of nematode are summarised in figure 5.5.

### 5.2.3. The bovine immune response to the surface of the $L_3$ sheath is directed at the 2A6 antigen.

The antigens responsible for the surprisingly intense antibody response to the $L_3$ sheath have not been previously identified and the following experiments were performed to investigate whether the 2A6 antigen is involved in this response. The sera used in these experiments were produced by repeated oral infection of calves with normal non-irradiated or 40 Krad irradiated *D.viviparus* infective larve or intravenous injection with 100 Krad irradiated larvae (Canto Ph.d. Thesis and Appendix 2).

Western blots of *D.viviparus* $L_3$ homogenate were probed with *D.viviparus* hyperimmune bovine sera taken from 12 individual calves. The particular sera used were selected simply to examine the antibodies present in sera from cattle which had been protectively immunised by a variety of infection/immunisation

regimes. All 12 calves were solidly immune when eventually challenged and IFAs demonstrated that all 12 of these individual sera produced intense fluorescence with the *D.viviparus* sheathed $L_3$. The 12 samples fell into three categories: sera from cattle immunised by multiple infections of either non-irradiated, 40 krad irradiated or 100 krad irradiated larvae (the details of the immunisation regimes are given in figure 5.6 and Apendix 2).

A relatively complex pattern of bands was recognised by some of the 12 individual sera in blots of aqueous soluble $L_3$ homogenate which had only been cleared by low speed centrifugation at 10,000g for 5 minutes (figure 5.6A). This complexity may be deceptive since much of the immunoreactivity consisted of relatively poorly resolved material above 40kDa. The antigen most consistently recognised on these blots by the 12 sera is a broad band between 29 and 40 kDa which appears very similar to that recognised by 2A6 (see arrow on figure 5.6A). The other prominent antigens detected are a 28kDa antigen (appears as a curved band) and a low molecular weight antigen of less than 14 kDa which are seen by all the sera except O25 and O28. A somewhat simpler pattern of bands was obtained, with the 29-40 kDa antigen appearing even more prominent, when the same 12 sera were used to probe blots of a *D.viviparus* $L_3$ homogenate aqueous fraction which had been cleared by high speed centrifugation at 100,000g for 30 minutes (figure 5.6B). This suggests that the 29-40 kDa antigen is the most immunodominant of the genuinely aqueous soluble $L_3$ antigens. Nevertheless, the poorly resolved immunoreactivity above 40 kDa is still present in these blots as is the low molecular weight band although it is not easily seen in 5.6B (see figure 5.7B for comparison).

The antibody against the surface of the $L_3$ sheath in serum taken from cattle after successive infections with *D.viviparus* $L_3$ was measured by quantitative IFA. The level of fluorescence produced by serum taken three weeks after a single infection was relatively low compared to that produced by serum taken after subsequent infections (figure 5.7A). When the same serum samples were used to probe Western blots of *D.viviparus* $L_3$ homogenate, the 29-40 kDa antigen was only very faintly visible in the track probed with the serum taken after a single infection but was very clearly seen in the tracks probed with the sera taken after subsequent infections (figure 5.7B). A low molecular weight antigen of less than 14 kDa is also quite prominent in the blots probed with sera taken after all the infections.

Quantitative IFAs were performed on sheathed and exsheathed $L_3$ of *D.viviparus, H.contortus, T.colubriformis, O.circumcincta, O.ostertagi, C.oncophora* and *Cyathostomes* with hyperimmune *D.viviparus* bovine serum (pooled B7, B8 & B9 serum taken 3 weeks after three infections - see Appendix 2). This serum produced an equally intense degree of fluorescence with the $L_3$ sheaths of all the nematode species

examined but only reacted with the exsheathed $L_3$ surface of *D.viviparus* (Figure 5.8A). Naive bovine serum did not produce any fluorescence with the sheathed or exsheathed larvae of any of these species except for exsheathed *D.viviparus* (data not shown). Qualitative IFAs were also performed on larvae of these nematode species with sera taken from calves after three oral infections with 40krad irradiated larvae (pooled O38 and 039 serum) or three intravenous infections with 100 krad irradiated larvae (pooled G17 and B45 serum) and again there was strong recognition of the sheath surface for all the species. Therefore the three different types of *D.viviparus* immune bovine serum examined have a high level of antibody to the $L_3$ sheath surface of many different nematode species from the order Strongylida.

Western blots of $L_3$ homogenate prepared from a number of different nematodes were probed with hyperimmune *D.viviparus* bovine serum (the same pooled B7, B8 and B9 sera used for the IFA in figure 5.8A). The sera detected multiple bands on Western blots of *D.viviparus*, *H.contortus*, *C.oncophora* and *Cyathostome* $L_3$ homogenate demonstrating a high degree of cross-reactivity between the antigens of these parasite (figure 5.8B). However if this blot is compared to the $L_3$ homogenate blots probed with 2A6 and 2F8 in figure 5.4, it can be seen that the results are entirely consistent with, although do not prove, the hypothesis that the immunodominant antigen detected by immune bovine serum is the same as that detected by 2A6 and 2F8. The 29-40 kDa antigen is seen only in the *D.viviparus* track as was the case with 2A6 and 2F8. There is a 20 kDa antigen seen in the *H.contortus* and *C.oncophora* tracks (only faintly reproduced in the *C.oncophora* track of Figure 5.8) which is not present in the other two tracks and this could be the 20 kDa antigen seen by 2A6 and 2F8. There are also five bands at the appropriate molecular weights in the *Cyathostome* track which are not present in the other tracks (the bands which could correspond to the 32, 30 and 28 kDa antigens seen by 2A6 and 2F8 are marked by one arrow and the bands which could correspond to the 18 and 16 kDa antigens are marked with another).

All the experiments with immune bovine serum presented above suggest that the immunodominant 29-40 kDa antigen detected on Western blots by immune bovine serum is the same as that detected by 2A6 and 2F8. In order to prove this hypothesis the following experiment was performed. The principle of the experiment was to immunoprecipitate the antigen from *D.viviparus* $L_3$ homogenate using mab 2A6 and then to probe both the immunoprecipitated antigen and the remaining unprecipitated $L_3$ homogenate with hyperimmune bovine immune serum on a Western blot. 2A6 supernatant was added to a sample of $L_3$ homogenate and complete medium was added to an identical sample as a negative control. Immune complexes were adsorbed onto protein-A sepharose beads using the standard

immunoprecipitation protocol and both the protein A bead-immune complexes and the remaining supernatant were boiled with SDS-PAGE sample buffer. Details of the amounts of reagents used are given in Figure 5.9. Duplicate Western blots were performed with the resulting samples with one blot being probed with 2A6 and the other with *D.viviparus* hyperimmune bovine serum (pooled B7, B8 and B9 serum). Figure 5.9A shows the blot probed with 2A6 and it can be seen that the antigen is present in Track 1 (remaining $L_3$ homogenate supernatant after immunoprecipition with complete medium) but is absent from track 2 (remaining $L_3$ homogenate supernatant after immunoprecipitation with 2A6). It is also absent from track 3 (antigens immunoprecipitated by complete medium) but present in track 4 (antigens immunoprecipitated by 2A6). Therefore immunoprecipitation with 2A6 completely removed the target antigen from the $L_3$ homogenate. Figure 5.9B shows the duplicate blot probed with the pooled B7, B8 and B9 serum and it can be seen that the antigen immunoprecipitated by 2A6 is the same 29-40 kDa immunodominant antigen detected by immune bovine serum. Examination of this blot reveals that none of the other antigens detected by the immune bovine serum had been precipitated by 2A6 demonstrating the specificity of the immunoprecipitation. The heavy and light chains of the 2A6 immunoglobulin molecule can be seen in Track 4 of Figure 5.9B.

An identical experiment was also performed with *H.contortus* $L_3$ homogenate (figure 5.10) and this demonstrated that the 20kDa doublet is specifically immunoprecipitated by 2A6 (figure 5.10A) and that it is detected by the hyperimmune bovine serum (figure 5.10B).

Although complete medium was used as the negative control in these experiments, supernatant of the monoclonal antibody 5E1 (raised against *T.annulata* merozoites) has been used as the negative control in many similar immunoprecipitation experiments and this does not produce any non-specific precipitation of the 2A6 antigen.

This experiment proves that the antigen detected by 2A6 is the immunodominant antigen detected by *D.viviparus* hyperimmune bovine serum.

### 5.2.4. Sera from animals infected with gastro-intestinal trichostrongylid nematodes do not detect antigens on the surface of the $L_3$ sheath.

The previous experiments have shown that mab 2A6 detects an antigen in many nematodes from the order Strongylida and the antigen is immunodominant in cattle vaccinated or infected with *D.viviparus*. Therefore the possibility of this antigen

being immunogenic in other infections involving strongylid nematodes was investigated using IFA.

Quantitative IFAs were performed on sheathed *D.viviparus*, *H.contortus* and *O.ostertagi* $L_3$ with pooled serum taken from groups of cattle after two infections with *D.viviparus*, *C.oncophora* or *O.ostertagi* and also from sheep following two infections with *H.contortus*. As in previous experiments, the *D.viviparus* immune sera produced fluorescence with all the sheathed larvae but none of the sera from animals infected with the other nematode species produced fluorescence with the sheaths of any of the three larval species (figure 5.11). Western blots of *D.viviparus* $L_3$ homogenate were also probed with these sera and no antigens in the 29-40 kDa range were recognised (data not shown).

### 5.2.5. Binding of 2A6 to the $L_3$ sheath of *Necator americanus*.

The human hookworm *N.americanus* is a nematode from the order Strongylida in which the infective stage is the sheathed $L_3$. This parasite is of particular interest because, like *D.viviparus*, it is a migratory parasite and the $L_3$ sheath has been reported to be strongly recognised by serum taken from infected human patients (Pritchard et al 1991). The following experiments were performed to determine whether the 2A6 antigen was present on the surface *N.americanus* $L_3$ sheath and whether it was responsible for the immune recognition of this surface by infected humans.

Sheathed and exsheathed *N.americanus* $L_3$ were examined by IFA with 2A6. As with the other nematodes previously examined there was no binding to the exsheathed $L_3$ surface. However the IFA with sheathed $L_3$ produced very different results to those previously described for the other strongylid nematodes. The results of quantitative IFA on twenty individual sheathed $L_3$ are displayed as a histogram in figure 5.12B. It can be seen that three of the larvae exhibit a high degree of fluorescence and the rest just show background levels due to autofluorescence of the larvae. The fluorescence produced by 2A6 on the 3 positive larvae was of a similar intensity to that recorded for the nematodes from the order Strongylida previously examined. Figure 5.12A shows one of the larvae which was positive for the IFA and it can be seen that the fluorescence was evenly distributed over the whole sheath surface. This experiment was repeated twice by qualitative IFA; in the first case 4 out of 36 larvae fluoresced and in the second case 2 out of 23 larvae fluoresced.

Western blots of *N.americanus* $L_3$ homogenate probed with 2A6 detected two polypeptides in both the water soluble and SDS/2ME soluble

preparations (figure 5.13A), one very intense band at 17 kDa and a fainter one at 28 kDa. Interestingly the sensitivity of detection of the *N.americanus* antigens with 2A6 is similar to that of the *D.viviparus* and *H.contortus* antigen in these $L_3$ homogenate blots. This would be surprising if the antigen was only present in approximately 15% of the larvae and so it is possible that the antigen is present in all the larvae but only exposed on the sheath surface in a few individuals.

Following infection, *N.americanus* undergoes a tissue migration prior to reaching the site occupied by the adult parasites in the gastro-intestinal tract. As is the case for *D.viviparus* infection, IFA studies on human infection serum have detected a high level of antibody to the surface of the *N.americanus* $L_3$ sheath (Pritchard et al 1991). Therefore it is possible that *N.americanus* human infection serum might contain antibodies to the antigens recognised by 2A6. This could not be directly investigated using antigens from *N.americanus* $L_3$ due to shortage of material but a number of experiments were performed to determine whether human *N.americanus* infection serum contained antibodies which detect the 2A6 antigen of *D.viviparus*.

IFAs were performed on sheathed *D.viviparus* $L_3$ with sera from 15 humans from a *N.americanus* endemic area in Papua New Guinea (supplied by D.I. Pritchard). These sera had been demonstrated to have high levels of antibody to both the $L_3$ and adult stages of *N.americanus* by ELISA (Pritchard, personal communication). IFAs were performed several times on sheathed *D.viviparus* $L_3$ with these sera but none of the samples produced any significant fluorescence (data not shown). These sera were also used to probe Western blots of *D.viviparus* $L_3$ homogenate and although some of the sera detected a number of *D.viviparus* $L_3$ antigens none of them detected the 29-40 kDa antigen recognised by 2A6 (figure 5.11B). Antigen immunoprecipitated by 2A6 was Western blotted and probed with these sera and again the antigen was not detected (data not shown). Therefore although 2A6 binds to the sheath surface of a proportion of *N.americanus* $L_3$, no antibodies which cross-react with the 2A6 antigen of *D.viviparus* could be detected in human *N.americanus* infection sera .

### 5.2.6. Stage Specificity of the 2A6 antigen.

Quantitative IFA was performed on several of the developmental stages of *D.viviparus* with 2A6 and 2F8 and both antibodies only produced fluorescence with sheathed $L_3$ (Figure 5.14). Qualitative IFAs were performed on eggs, sheathed $L_3$, exsheathed $L_3$, $L_4$ and adults of *O. circumcincta* and *H.contortus* with both 2A6 and

2F8 and, as for *D.viviparus*, the sheathed $L_3$ was the only stage which produced significant fluorescence.

In order to examine the stage specificity of the antigen in the early larval stages in more detail $L_1$ were cultured to $L_3$ in calf faeces at room temperature, harvested daily by Baermannisation and examined by light microscopy and IFA with mab 2A6. After 24 hours all the larvae had the appearance of $L_1$ and IFA with 2A6 produced no fluorescence (figure 5.15A). After 48 hours there was no visible morphological change in most of the larvae but examination with IFA revealed a proportion (approximately 10-20%) of the larvae had a small "button" of fluorescence at the anterior end (figure 5.15B). After 72 hours the culture consisted of a mixture of stages; $L_1$ with and without the "cranial button" were still present but many of the larvae had moulted to the $L_2$ but still retained the $L_1$ cuticle which can be seen surrounding the larvae in loose corrugations (Figure 5.15C). 2A6 did not bind to this retained $L_1$ cuticle and no "cranial button" of fluorescence could be seen in any larvae of this type. Also at this time, some of the larvae had already developed to the $L_3$ and after 96 hours practically all the larvae had reached this stage. The $L_2$ cuticle, which was retained, enclosed the larvae in tight corrugations and fluoresced brightly with 2A6 (Figure 5.15D). At no point during the culture could $L_2$ be seen which were not enclosed by the retained $L_1$ cuticle (as in figure 5.15C) and so it seems likely that this is not cast until after the moult to $L_3$. This means that live IFA cannot be used to determine whether the 2A6 antigen is exposed on the surface of the $L_2$ cuticle prior to it becoming the $L_3$ sheath. The development of *D.viviparus* from embryo to $L_3$ is schematically represented in Figure 5.16. The appearence of a cranial button of fluorescence in the late $L_1$ is difficult to explain other than by the exposure of the newly formed $L_2$ cuticle at the oral orifice during apolysis of the $L_1$ cuticle.

The stage specific expression the 2A6 antigen was also examined by probing Western blots of parasite homogenates prepared from a number of developmental stages of *D.viviparus*. The antigen was detected by 2A6 in both the $L_1$ and the $L_3$ homogenate (figure 5.17A) and the detection of the antigen in both these stages was a very repeatable result. When two separate batches of adult homogenate were probed with 2A6, the antigen was faintly detected in one track but not in the other. (figure 5.17A). This experiment was subsequently repeated several times and the antigen could only be detected very faintly on one occasion. Homogenates were also prepared separately from male and female adults and probed with 2A6 on Western blots to determine if the antigen was present in larvated eggs *in utero* but again it was not detected (figure 5.17A). Homogenate was also prepared from larvated eggs which had been released from *in vitro* cultured adult worms. Two separate batches were probed with 2A6 and the antigen was detected in each case although

more faintly than in $L_3$ homogenate (figure 5.17B). Another interesting feature of the data presented in Figure 5.17 is that 2A6 detected the antigen equally well in homogenate prepared from either sheathed $L_3$ or exsheathed $L_3$. This was true even when exsheathed $L_3$ were checked by IFA with 2A6, prior to preparing the homogenate, to ensure no contamination with cast sheaths or fragments. This would suggest that the antigen is present elsewhere in the $L_3$ as well as on the sheath surface.

The results presented above suggest that the antigen first appears during the development of *D.viviparus* $L_3$ in the egg and is present in the subsequent larval stages up to and including the $L_3$.

If exsheathed *D.viviparus* $L_3$ are cultured at 37°C in RPMI they remain motile for up to 14 days and undergo partial development to the $L_4$ (see chapter 6). Exsheathed larvae cultured in this manner were harvested at day 4 and day 10 of culture and used to prepare homogenates. Equal amounts of total protein from these samples were Western blotted, along with homogenate from non-cultured exsheathed $L_3$ and Ponceau-s staining of the blot confirmed the equality of protein loading for each track. When the blot was then probed with 2A6 the intensity of antigen detection in the homogenate from the cultured larvae was significantly less than in homogenate from non-cultured larvae (figure 5.17C). This was also found to be the case when blots of cultured larvae were probed with immune bovine serum (see chapter 6). An aliquot of the spent culture medium was filtered and concentrated 10 fold by centrifugation in a centricon tube and probed on a Western blot with 2A6, but no antigen was detected (data not shown). Therefore it appears that the 2A6 antigen gradually disappears from *in vitro* cultured $L_3$ and cannot be detected in the culture medium. This would suggest that during *in vitro* culture of the $L_3$ the 2A6 antigen is metabolised at a greater rate than it is synthesised.

In order to examine the synthesis of the 2A6 antigen several developmental stages of *D.viviparus* were metabolically labelled and the resulting antigens immunoprecipitated with 2A6. Larvated eggs, $L_1$, $L_3$ (sheathed) and adult parasites were each metabolically labelled by three different methods. Firstly using $^{35}$S-Methionine, secondly using a high specific activity $^{3}$H-amino acid mixture (Leucine, Lysine, Phenylalanine, Proline and Tyrosine) and thirdly using $^{3}$H-glucosamine.

The radioactivity of aqueous and SDS/2ME/urea soluble homogenates prepared from the $^{35}$S-Methionine labelled parasites was assessed by scintillation counting. The counts for adult and $L_3$ antigens was higher than those for egg and $L_1$ antigens. These results were used to allow equal amounts of radioactivity for each sample to be loaded onto an SDS-PAGE gel so that the polypeptides labelled in each

stage could be compared following analysis of the gel by fluorography. However due to a lack of material, only half the amount of radioactivity was loaded onto the gel for $L_1$ homogenate compared to the other stages. A range of polypeptides appear to have been labelled in each stage, although the $L_1$ track is underloaded (figure 5.18A). Equal counts per minute of the water soluble homogenates of each stage were immunoprecipitated by 2A6 and protein-A sepharose beads (again half the amount for the $L_1$ stage due to the lack of material). The beads resulting from each sample were split into two equal samples and boiled in sample buffer and duplicate gels SDS-PAGE gels were run. One gel was electroblotted and the blot probed with 2A6 in order to determine in which samples the 2A6 antigen had been successfully precipitated (figure 5.18B) and the second gel was fluorographed to determine whether the precipitated antigen had incorporated any radiolabel (figure 5.18C). The results show that the 2A6 antigen has been successfully immunoprecipitated from the $L_1$ and $L_3$ stages but the fluorograph of the duplicate gel demonstrates that there was no detectable incorporation of the $^{35}$S-Methionone into the antigen. A further 2 months exposure of the same gel produced no specific signal.

Precisely the same experiment was performed with the parasites after labelling with the $^{3}$H amino acid mixture. Again, higher levels of radioactivity were measured in adult and $L_3$ homogenates and only half the counts per minute for $L_1$ antigens were used for the SDS-PAGE analysis and immunoprecipitation experiment compared to the other parasite stages. The antigen was successfully precipitated from $L_1$ and $L_3$ aqueous homogenates, although only faintly visible in the $L_1$ track (figure 5.19B) and the fluorograph of the duplicate gel again fails to detect incorporation of label into the precipitated antigen. The fluorograph still showed no specific signal even after a subsequent 2 month exposure.

The same experiment was also performed with $^{3}$H-glucosamine labelled parasites. Scintillation counting measured very little radioactivity in the egg and $L_1$ homogenates and this was reflected in the fluorograph of the SDS-PAGE gel of these homogenates (Figure 5.20A). Several glycoproteins were labelled in the $L_3$ and a low molecular weight antigen was labelled in both the adult and $L_3$ stages. This could be a glycolipid as such molecules often migrate with the dye front on SDS-PAGE. The 2A6 antigen was successfully precipitated from $L_3$ aqueous homogenate but not from the other stages (figure 5.20B). As with the previous experiments fluorography showed there was no incorporation of radiolabel into the precipitated antigen (figure 5.20C).

These experiments have failed to demonstrate the synthesis of the 2A6 antigen and the possible reasons for this will be discussed later in the chapter.

### 5.2.7. 2A6 detects antigen in $L_3$ somatic tissues.

The detection of the antigen by 2A6 in blots of exsheathed $L_3$ homogenate (figure 5.17A), coupled with the lack of binding of 2A6 to the $L_3$ surface, suggests that the antibody binds to antigen located internally in the $L_3$ as well as to the surface of the $L_3$ sheath. In order to investigate this in more detail, IFAs were performed with 2A6 on methanol/acetone fixed specimens of *D.viviparus* $L_3$. There was a marked difference in the appearance of the IFAs of fixed sheathed and fixed exsheathed $L_3$. With sheathed $L_3$ there was strong fluorescence of the sheath and faint internal fluorescence visible in a few of the larvae (figure 5.21A). In contrast, with the exsheathed $L_3$ there was no fluorescence of the $L_3$ cuticle but there was marked fluorescence unevenly distributed throughout the somatic tissues of the larvae (Figure 5.21B). Under high power this fluorescence had a very granular appearance (figures 5.21C and 5.21D). These IFAs were all performed with negative controls using the monoclonal antibody 5E1 (raised against *Theileria annulata* merozoites) which produced no fluorescence with any of the specimens described. The fluorescence produced by 2A6 could not be confused with autofluorescence, often seen in larvae, as this was relatively low in the fixed specimens and of a different colour to that produced by the FITC second antibody conjugate. The patchiness of the internal fluorescence seen could be due to the fixation method used which has led to uneven permeability of larvae to the antibody. This experiment was also performed on *H.contortus* $L_3$ and a similar patchy pattern of granular internal fluorescence was seen with IFA of 2A6 on exsheathed $L_3$ (figure 5.22A).

The binding of 2A6 to somatic tissues could be due to the antigen which is on the surface of the $L_3$ sheath also being present in larval somatic tissues or could be simply be due to a cross-reactive epitope at the two sites. The appearance of Western blots of sheathed and exsheathed *D.viviparus* $L_3$ homogenate probed with 2A6 are identical (figure 5.17A) which suggests that the same antigen is being detected at both sites. Ideally, to confirm this interpretation, the antigen from purified sheaths should be compared with the antigen from exsheathed larvae but a method for purifying *D.viviparus* sheaths has yet not been developed due to the fragmentation of sheaths during exsheathment. However this can be undertaken with *H.contortus* by centrifugation of exsheathed larvae through a Percoll cushion which allows separation of larvae and cast sheaths (see materials and methods). This was performed and the resulting larvae and sheaths were checked for respective cross contamination by light microscopy and IFA with 2A6 and then re-centrifuged through Percoll several times until each preparation was pure. The homogenates produced from these exsheathed larvae and sheaths were probed on Western blots with 2A6 (figure 5.22B). The antigen

recognised by 2A6 is 20 kDa in both preparations which suggests that the molecule present on the sheath surface is the same as that present in somatic tissues.

### 5.2.8. Immunogold EM studies with 2A6 on *D.viviparus* $L_3$.

In order to investigate the binding of 2A6 to the surface of the *D.viviparus* $L_3$ sheath surface in more detail, protein-A immunogold electron microscopy was performed. Sheathed $L_3$ were labelled firstly with 2A6, or 5E1 as a negative control, and then with protein-A gold prior to fixation, embedding and sectioning. No gold particles were observed on the surface of any of the sheathed $L_3$ following pre-embedding labelling with the negative control monoclonal antibody 5E1 (which is of the same isotype as 2A6) (figures 5.23A and 5.23B). These electron micrographs show the ultrastructural architecture of the sheath quite clearly and the main regions have been arrowed in Figure 5.23B. It appears to consist of an outer electron dense epicuticle (marked E), a cortical/basal zone which is mainly amorphous (marked am) except for one or two bands of varying electron density in the basal region (marked ed) and an inner electron dense layer (marked I). There is also a large amount of ill defined material between this inner electron dense layer of the sheath and the $L_3$ cuticular surface (marked D). Although not clearly shown in figures 5.20a and 5.20B, a layer outside the sheath epicuticle was seen in some larvae. This appeared to be well defined in some areas and less distinct in others. This ultrastructural appearance of the *D.viviparus* $L_3$ sheath is very similar to a previous description of the structure of the *T.colubriformis* $L_3$ sheath (Wharton 1986). The surface of sheathed $L_3$, which had been pre-embedding labelled with 2A6, were covered with densely packed gold particles (figure 5.23C). Closer inspection of these transmission electron micrographs reveals that the particles appear to have bound to a layer beyond the electron dense epicuticle of the sheath (figures 5.24A and 5.24B).

Post-embedding labelling was performed on sheathed *D.viviparus* $L_3$ with 2A6 and 5E1 using a fixation protocol without osmium. However no binding of 2A6 to the sheath or somatic structures could be detected other than a low level of, presumably non-specific, random binding of gold particles throughout both positive and negative control sections (data not shown). This lack of binding of antibody to the sheath surface and internal structures suggests that the epitope has not been preserved by the procedures used for post-embedding labelling. Other methods such as cryostat sectioning may be required to investigate the distribution of the antigen in somatic tissues.

### 5.2.9. The surface of the $L_3$ sheath is resistant to detergent solubilisation and protease treatment.

The nature of the $L_3$ sheath surface coat observed in the electron micrographs was investigated by incubating live sheathed $L_3$ in a variety of reagents and, after thorough washing, assessing its integrity by performing IFA with 2A6. The washing and IFA procedures were all performed at 4°C in the presence of 0.01% sodium azide. Incubation of sheathed $L_3$ in 0.02mg/ml, 0.1mg/ml and 1mg/ml of trypsin, papain or pronase at 37°C for up to 3 hours did not visibly damage the larvae or reduce the fluorescence subsequently produced by 2A6, relative to controls which had been treated with the protease buffers alone. The activity stock protease solutions used for this experiment was tested by digestion of $L_3$ homogenate and analysis by SDS-PAGE. Incubation with 0.5% CTAB, 2% sodium deoxycholate, 1.5% triton X-114 or 2% SDS with or without 5% 2ME for up to 3 hours also failed to reduce subsequent fluorescence with 2A6 relative to controls. Immersion of larvae in chloroform or chloroform/methanol (1:1) for periods of time from a few seconds up to 5 minutes had no effect on the subsequent binding of 2A6, but after fifteen minutes of incubation most of the larvae were killed, with visible disruption of the sheath surface and a marked reduction in the fluorescence produced by subsequent IFA with 2A6.

### 5.2.10. Biochemical characterisation of the 2A6 antigen.

**The 2A6 antigen is not detected by routine staining techniques.**

Examination of SDS-PAGE gels of *D.viviparus* $L_3$ homogenate stained with Coomasie blue, silver stain or Periodic Acid Schiff did not reveal any particularly abundant polypeptides or glycoproteins in the 29-40 kDa range (figure 5.25A). Staining of such gels with "stains all" or Sudan Black also failed to reveal any molecules of the appropriate molecular weight (data not shown). When immunoprecipitates of $L_3$ homogenate with 2A6 were Western blotted, the antigen was not visible by Ponceau-S staining and could only be detected by probing the blot with mab 2A6. This is illustrated in Figure 5.25B which shows the same track of a Western blot, first stained with Ponceau-S and then probed with mab 2A6. The heavy and light chains of the immunoglobulin molecule are clearly stained by the Ponceau-S but the immunoprecipitated antigen is not. Staining of such immunoprecipitates on SDS-PAGE gels with Coomassie or silver stain also failed to detect the antigen. Even when relatively large amounts of antigen and antibody were used in immunoprecipitations

the antigen could not be demonstrated by protein staining. For example if the resulting immunoprecipitate of 0.9mg of $L_3$ homogenate was loaded onto a single track of an SDS-PAGE gel the antigen was still not visualised by Coomassie blue or silver staining.

These results suggest that either the routine staining techniques are insufficiently sensitive to detect the amount of antigen present in the aqueous homogenates or that the biochemical properties of the antigen are responsible for the lack of staining.

**The 2A6 antigen partitions in the aqueous phase during chloroform/methanol extraction.**

*D.viviparus* $L_3$ homogenate was vortexed with chloroform/methanol (1:1) in an eppendorf tube and left to stand for 15 minutes. The aqueous and organic phases were separated by centrifugation, the aqueous layer removed and vortexed again with chloroform/methanol and separated as before. The three resulting samples (1 aqueous and 2 chloroform) were lyophilised under vacuum and resuspended in PBS. These resuspended samples were then probed with 2A6 on Western blots to determine into which phase the antigen had partitioned. Ponceau-S staining of the blot detected a 28 kDa molecule which had been extracted from the SDS/2ME/urea soluble proteins into the organic phase (figure 5.25C). However probing of the blot with 2A6 demonstrated that all of the 2A6 antigen had remained in the aqueous phase (figure 5.25D). The same experiment was performed with chloroform alone instead of the chloroform/methanol (1:1) mixture with a similar result except that the 28kDa molecule was not as efficiently extracted (data not shown).

**The epitope detected by 2A6 is periodate insensitive.**

Several thousand live sheathed *D.viviparus* $L_3$ were incubated in 10mM sodium periodate/sodium acetate buffer or sodium acetate buffer alone (as a negative control) in the dark at 4°C for 2 hours and then washed in PBS/0.01% sodium azide. The larvae were then examined by IFA with 2A6 in which all the steps were performed at 4°C in the presence of 0.01% sodium azide. In parallel to this, *Toxocara canis* $L_2$ were subjected to precisely the same periodate treatment and these were examined by IFA using the monoclonal antibody Tcn-2. This a monoclonal antibody known to recognise a periodate sensitive carbohydrate epitope on the surface of the *T.canis* $L_2$ (Maizels et al 1987b and Kennedy et al 1987c). The fluorescence produced by Tcn-2 on the *T.canis* $L_2$ was completely eliminated by the periodate treatment (figures 5.26A and 5.26B). However the fluorescence produced by 2A6 on the *D.viviparus* $L_3$ was

unaffected (figures 5.26C and 5.26D) suggesting that the 2A6 epitope is periodate insensitive.

**The 2A6 antigen could not be labelled with digoxigenin hydrazide.**

A digoxigenin hydrazide/anti-digoxigenin antibody system of glycan detection was used to probe blots of *D.viviparus* $L_3$ homogenate see materials and methods). There was a general diffuse binding above 60kDa and a few lower molecular weight bands were visible but no molecules in the 29-45 kDa range were detected (data not shown). This suggests that the 2A6 antigen is not heavily glycosylated and in order to investigate this in more detail the following experiment was performed. The principle was to label the antigen whilst it was bound to 2A6 antibody adsorbed onto protein-A sepharose beads, which would allow labelling of the immunoglobulin chains to act as an internal positive control for the labelling reaction. *D.viviparus* $L_3$ homogenate was immunoprecipitated with 2A6 supernatant and protein-A sepharose beads. After thorough washing in NET buffer, the beads, with the associated antigen/antibody complexes, were labelled in suspension with digoxigenin hydrazide. After further extensive washing the beads were split into duplicate samples, boiled in SDS-PAGE sample buffer and used to produce duplicate Western blots. One blot was probed with 2A6 to check the presence of the antigen and the other was probed with anti-digoxigenin antibody to determine if the antigen had been labelled. Two control samples were included in the Western blot to check the specificity of the glycan detection. The first control was to perform the procedure without $L_3$ homogenate resulting in digoxigenin labelled 2A6-protein A complexes and the second was to perform the procedure without $L_3$ homogenate and without labelling the complexes with digoxigenin hydrazide resulting in the presence of unlabelled immunoglobulin chains on the blot. Figure 5.27A shows that the controls worked in the appropriate manner in that the digoxigenin labelled immunoglobulin chains were clearly detected (tracks 3 & 4) whereas the unlabelled chains were not (track 5). The antigen was present in the appropriate track as revealed by the 2A6 detection (track 6) but was not clearly detected by the anti-digoxigenin antibody although there was possibly a very faint band present (track 3). The heavy and light immunoglobulin chains were clearly labelled in the same track acting as an internal positive control. This experiment was repeated several times without ever clearly labelling the antigen with digoxigenin hydrazide.

**The 2A6 antigen is unaffected by N-Glycosidase Treatment.**

Samples of *D.viviparus* $L_3$ homogenate were incubated with peptide N-Glycanase F (PNGase) at final concentrations of 1, 10, and 50 units/ml at 37$^{o}$C for 2

hours along with a negative control sample which was incubated in buffer alone. Fetuin was also incubated with PNGase at a final concentration of 10 units/ml as a positive control to ensure the activity of the enzyme. The resulting samples subjected to SDS-PAGE and Western blotting with the fetuin tracks being stained with Ponceau-S and the $L_3$ homogenate tracks being probed with 2A6. As expected there was a marked molecular weight shift in the fetuin demonstrating the activity of the enzyme. In contrast there was no visible reduction in the binding of 2A6 to the antigen or any change in the molecular weight of the antigen in the PNGase treated samples (figure 5.27B). In fact the PNGase treated samples produced a stronger signal when probed with 2A6 than the untreated control and this was seen again when the blot was repeated several times. This is difficult to explain except that the removal of carbohydrate residues may expose more epitopes to which 2A6 binds.

**The 2A6 antigen has a polypeptide component.**

Samples of *D.viviparus* $L_3$ homogenate were incubated with increasing concentrations (0, 1, 2 and 3 mg/ml) of pronase or trypsin at 37°C for 2 hours along with negative control samples which were incubated in the appropriate protease buffer alone. The resulting samples were then analysed by SDS-PAGE and probed on Western blots with 2A6. Pronase produced a ladder of bands between 40 and 15 kDa at each of the concentrations used (figure 5.28A). Trypsin treatment produced a new prominent band at approximately 23 kDa with a few fainter bands at a lower molecular weight and also possibly produced a slight reduction in the molecular weight of the antigen. Interestingly the antigen did not seem to be completely degraded even in the presence of 3mg/ml pronase or trypsin. Coomassie staining of these samples on SDS-PAGE gels reveals that practically all the stainable polypeptides have been degraded at this concentration. Also when these samples were probed with immune bovine serum (B7, B8 & B9) practically all the immunoreactivity had been removed by protease treatment except for the 2A6 antigen which was of similar appearance to that in figure 5.28A (data not shown). Treatment with papain (2 mg/ml) produced the same result as that produced by the trypsin treatment (figure 5.28B). It must be remembered that 2A6 is being used to visualise the antigen and so only fragments containing this epitope will be detected.

*H.contortus* $L_3$ homogenate was incubated with trypsin and pronase (2mg/ml) and a Western blot of the resulting samples was probed with 2A6. There was greatly reduced detection of the antigen by 2A6 in the protease treated samples but no digestion products were visible (figure 5.28C) suggesting that the antigen from this species may more susceptible to protease treatment.

**The 2A6 antigen could not be labelled with NHS-Biotin.**

An experiment, similar to that performed with digoxigenin hydrazide, was carried out using NHS-biotin to examine if the antigen could be labelled with this reagent. Antigen-2A6-protein A complexes were labelled in suspension with NHS-biotin and duplicate blots were produced one of which was probed with 2A6 to check for the presence of antigen and the other with streptavidin peroxidase to determine if it had been labelled with the biotin. The same controls as used in the digoxigenin hydrazide experiment were included to ensure the specificity of the detection system.

The antigen was not labelled by the NHS-biotin (figure 5.29A). The streptavidin detection appeared to be specific for the biotin since the biotinylated immunoglobulin chains were detected (track 3) but the chains in the unbiotinylated control sample were not (track 1). The 2A6 antigen was present in the appropriate track as shown by the probing with 2A6 (track 8) but was not detected with the streptavidin and so does not appear to have been labelled with the NHS-biotin (track 4). The immunoglobulin chains in the same track as the 2A6 antigen were clearly detected by the streptavidin which acted as an internal positive control for the labelling reaction.

**Examination of the 2A6 antigen by isoelectric focusing.**

Isoelectric focusing was performed on $L_3$ homogenates of *D.viviparus*, *H.contortus*, *C.oncophora* and *T.colubriformis*. The gels were capillary blotted onto PVDF paper and probed with 2A6. For the *D.viviparus* homogenate the antibody detected a smear which ran from the loading point to a pI of approximately 3.5 (figure 5.29B). For the *H.contortus* and *C.oncophora* a single band of pI 3.7 was detected and for *T.colubriformis* 2 bands at pI 3.7 and 3.8 were detected. The smear for *D.viviparus* was much more intense than the bands detected for the other species. Although these bands seem quite faint in the figure, these experiments were repeated several times with the same result in each case.

Therefore it appears that the antigen detected by 2A6 is an acidic molecule in each of the species examined. Interestingly isoelectric focussing resolves the antigen into a tight band(s) for *H.contortus*, *C.oncophora* and *T.colubriformis* whereas there is poor resolution for the antigen from *D.viviparus*. This is similar to the results of SDS-PAGE analysis.

### 5.2.11. Immunoaffinity purification of antigen with 2A6.

Although more information could be obtained from further immunochemical experiments of the type described above, much more rapid and detailed progress would be possible if the antigen could be purified. Immunoaffinty purification using 2A6, covalently bound to a protein-A sepharose column, was the method chosen to attempt this purification.

An immunoaffinty column was prepared using 4.8mg of 2A6 IgG with 1ml of Protein-A sepharose beads and the ability of the column to bind the antigen and the conditions required for elution were determined using the methods described in Chapter 2. The fractions eluted from the column by acid (100mM glycine pH 2.5) and alkaline (100mM triethylamine) elution conditions were analysed by dot blotting of aliquots onto duplicate nitrocellulose filters one of which was probed with 2A6 and the other with 5E1 as a negative control. The dot blots did not detect a significant reduction in the amount of antigen present in the $L_3$ sonicate after three passes through the column suggesting that not all the antigen present in the sonicate was bound by the column. The acid elution conditions did not remove detectable amounts of antigen from the column, but the antigen was detected in practically all the fractions of triethylamine, pH 11.5 (figure 5.30A). The elution conditions were refined by using 100mM triethylamine/0.5% sodium deoxycholate pH 11.5 as the elution buffer. This produced a sharper elution profile for the antigen when the eluted fractions were assessed by dot blotting (figure 5.30B). The fractions were also Western blotted and probed with 2A6 which confirmed the presence of the antigen in the appropriate fractions. Interestingly the antigen appeared to resolve more sharply as a single band when 2A6 was used to probe Western blots of the purified antigen as opposed to blots of $L_3$ homogenate (Figure 5.30C). There was no significant increased UV absorbance (280nm) of the eluted fractions containing the antigen (diluted 1:10 in PBS) and no antigen could be stained in these fractions on SDS-PAGE gels using Coomassie blue, silver or "stains-all" (data not shown). This could be due either to the biochemical nature of the antigen or the lack of sensitivity of such techniques.

In order provide a more sensitive method of detecting protein present in the eluates containing the antigen, the first 5 fractions from the elution shown in figure 5.30B were pooled by slot blotting them onto PVDF paper and having an amino acid compositional analysis performed on the strip. A negative control slot, through which an equal volume of elution buffer had been passed, was also analysed to determine the background amino acid contamination. The total amino acid levels were high on both the positive (2314 pmol) and negative (2196pmol) strips suggesting that there was a high level of background contamination with amino acids. This was

probably due to the method of concentrating the samples, which involved passing relatively large volumes of buffer through a small area of PVDF paper on the slot blot. The positive sample was not significantly greater than the negative sample but a small amount of protein could have been easily masked by the large background levels of amino acid. More analytical work on immunoaffinty purified material is required to help resolve the questions concerning the amount and biochemical nature of the antigen present.

## 5.3. Discussion.

The observation of *D.viviparus* $L_3$ exsheathment using IFA with 2A6 revealed that the process appears to be somewhat different to that which occurs in other trichostrongyloid nematodes. There was no degradation of any specific area of the sheath resulting in the formation of a refractile ring, but instead there was a generalised disintegration of the whole sheath. This could be due to the exsheathing protease(s) of *D.viviparus* being less specific than those of other trichostrongyloid nematodes or alternatively it may reflect fundamental differences in the structure of the sheath. It should be pointed out that these experiments involved the *in vitro* exsheathment of larvae using sodium hypochlorite treatment and consequently the observed disintegration of the sheath may not reflect the situation *in vivo*. The use of $CO_2$ to stimulate exsheathment *in vitro* is generally considered to a better mimic of the *in vivo* situation. However, unlike the other trichostrongyloid nematodes, it was found that *D.viviparus* $L_3$ did not exsheath when 100% $CO_2$ was bubbled through the larval suspension. Nevertheless, the unusual mode of exsheathment stimulated by sodium hypochlorite treatment of *D.viviparus* $L_3$ suggests the process is fundamentally different in this parasite since sodium hypochlorite treatment of other trichostrongyloid $L_3$ stimulates refractile ring formation and not a generalised disintegration of the sheath. Furthermore Davey & Rogers (1982) have shown that exsheathment of *H.contortus* with $CO_2$ is accompanied by a reduction in the volume of the oesophagus and excretory glands and they suggested that these might be the source of exsheathing fluids. They also observed a similar reduction in the volume of the oesophagus when larvae were exsheathed in sodium hypochlorite which implies that at least some of the same exsheathing fluids are released by this stimulus.

In summary, the exsheathment of *D.viviparus* $L_3$ appears to be different from that of other trichostrongyloid nematodes in two ways. It is not stimulated by exposure to 100% $CO_2$ *in vitro* and when stimulated by sodium hypochlorite, the process involves a generalised disintegration of the sheath and not the formation of a

refractile ring. Work previously performed on the exsheathment of *D.viviparus* has reported the ineffectiveness of $CO_2$ as a stimulus (Silverman & Podjer 1964) but has not reported the generalised disintegration of the sheath (Sommerville 1957 Silverman & Podger 1964 and Parker & Croll 1976).

This unusual mode of exsheathment is particularly interesting when the results of the lectin binding studies in the previous chapter are considered. The lectins bound specifically to the region of the refractile ring on the inner surface of the sheaths of *T.colubriformis*, *O.ostertagi*, and *H.contortus* infective larvae, a result similar to that reported for *T.colubriformis* by Wharton & Murray (1990). In contrast the same lectins bound throughout the whole internal surface of the *D.viviparus* sheath implying that this may lack any localised specialisation at the refractile ring region. Indeed the results suggest that molecules which are restricted to the refractile ring in many trichostrongyloid nematodes may be distributed throughout the whole of the *D.viviparus* $L_3$ sheath.

The binding of 2A6 and 2F8 to the surface of the $L_3$ sheath in all the nematodes examined from the order Strongylida was a surprising result. Antigenic cross-reactivity between the surface antigens of different nematode species has been previously reported by a number of authors (Maizels et al 1983b and Kennedy et al 1987c). However no antigen or epitope has been found on the surface of so many different nematodes, particularly between such widely divergent species (figure 5.5). It is also striking that the level of fluorescence produced in IFAs by 2A6 was very similar for each of the different nematode species which suggests that the target epitope is highly conserved. The nematodes containing the antigen are relatively distant taxonomically (from different superfamilies) and represent a wide range of parasitic life styles. For example, *N.americanus* parasitises the human small intestine, *D.viviparus* the bovine respiratory system and *Cyathostome spp.* the equine large intestine. However there a number of features which all the nematodes containing the 2A6 antigen have in common. They are all from the order Strongylida and the antigen was not detected on Western blots or IFAs of nematode species examined from other taxonomic orders (figure 5.5). Also the infective stage is a free-living $L_3$ which retains the $L_2$ cuticle as a protective sheath to which the monoclonal antibodies bind. Such a high degree of conservation of an epitope at a specific site in a defined group of nematodes suggests it may be of some functional significance. Furthermore the similarity in molecular weight and pI of the antigen in some of the species suggests that there is conservation of a molecule on the sheath and not simply an epitope. Interestingly, for those nematodes from the family Trichostrongylidae (figure 5.5) examined by Western blotting, mab 2A6 recognised a 20kDa antigen whereas different

sizes and numbers of antigens were detected in nematodes from different taxonomic families, ie. the Western blots appear similar in closely related species.

The binding of 2A6 to *N.americanus* was unusual in that only a minority of sheathed $L_3$ bound the antibody on IFA, but the sensitivity of antigen detection on Western blots was similar to that found with other nematodes. This might suggest that the antigen is present in all the nematodes but only present on the surface of the sheath in a few individuals. This result is in marked contrast to the other nematode species where the normal distribution of quantitative IFA readings from individual larvae suggests that there is only one population of larvae with respect to the binding of the 2A6 antibody.

The $L_3$ sheath, forming the interface between the external environment and the free-living infective larvae, is thought to be important in the ability of larvae to survive for prolonged periods and resist adverse environmental conditions such as freezing and desiccation (Ellenby 1968 and Wharton 1986). One particular threat to such larvae, in which the surface of the sheath must play a role, is predation by nematophagous fungi. These are a diverse group of fungi which feed on a large range of free-living, plant and animal parasitic nematodes (Shepherd 1955, Barron 1977 Norbring-Hertz & Mattiasson 1979 and Rosenweig et al 1985). Several fungal species feed on trichostrongylid nematodes (Hashimi & Connan 1989) and there have been a number of different accounts of the interaction of such fungi with the surface of the sheathed $L_3$. The most recent work was by Murray & Wharton (1990), on the predation of *T.colubriformis* by the nematophagous fungi *Arthrobotrys oligospora.* There appear to be two separate events which occur when a larvae becomes entangled in the fungal hyphae. Firstly there is capture of the larvae which is due to an adhesive substance associated with the surface of the hyphae and secondly there is growth of hyphae at the points of adhesion and penetration the nematode cuticle. This whole process takes approximately one hour. Interestingly, Murray & Wharton (1990) also reported that exsheathed $L_3$ were not captured by the fungus suggesting that the adhesive was either not produced or that it could not adhere to the surface of the $L_3$ cuticle. This would suggest that there is something specific about the sheath surface which allows capture to occur. The capture and invasion of nematodes by predacious fungi can be inhibited, in some species, by pre-incubating the fungi in various saccharides which has led to the hypothesis that lectins on the surface of hyphae interact with specific carbohydrates on the nematode cuticular surface (Nordbring-Hertz & Mattiasson 1979). However Wharton & Murray (1990) found no inhibition of trapping of *T.colubriformis* by *Arthrobotrys oligospora* by any of 12 different saccharides that were tested. Furthermore no lectins from a panel of 6 were found to bind to the surface of the *T.colubriformis* $L_3$ cuticle or sheath. In contrast hyphal

penetration of the nematode cuticle was completely inhibited by 2-deoxy-D-glucose and significantly reduced by fructose, melibiose, 2-deoxy-D-galactose and D-galactose. Exposure of the fungus to these saccharides resulted in larvae being trapped and left struggling in the trapping complexes, unpenetrated by hyphae, for more than 2 days. Therefore it appears that trapping (or adhesion) and penetration are two separate processes. Adhesion occurs to the $L_3$ sheath and not to the $L_3$ cuticle and is not inhibited by competition from free saccharides whereas penetration is inhibited by a number of saccharides and so may involve interaction with carbohydrates on the cuticular surface. The 2A6 antigen is the first molecule to be identified on the surface of any trichostrongyloid $L_3$ sheath and so it would be interesting to determine if it is involved in either of these interactions. This could be done by investigating the ability of the monoclonal antibodies to inhibit either of the processes in the same way as has been performed with saccharides.

The experiments presented in this chapter have shown that the 2A6 antigen of *D.viviparus* is highly immunogenic in both infected and vaccinated cattle. It was the most immunodominant antigen recognised on Western blots by all 12 of the hyperimmune cattle sera examined. This consistency of recognition by different individual cattle sera is in marked contrast to the heterogeneity seen in the recognition of adult E/S and somatic antigens (Britton 1991). The relevance of the antigen to the protective immune response cannot be determined from the results presented here, but the results of earlier passive immunisation experiments with some of the immune serum used to probe the Western blots are worth consideration. Serum from the cattle immunised by repeated infection (B7, B8 & B9) was highly effective when used to passively immunise recipient cattle against challenge which suggests that protective antibodies were present in this serum (Canto 1990). However serum from cattle immunised with 100krad irradiated larvae (G17 & B45) did not protect recipient cattle by passive immunisation, although the donors themselves were immune to challenge, suggesting that antibodies present in this serum was not protective. Since both of these types of sera strongly recognise the 2A6 antigen, these results would seem to suggest that antibodies recognising the antigen are not involved in the protective immune response. However care must be taken when drawing conclusions from these cattle experiments since they were conducted on groups of only 2 or 3 animals. Another point to consider is that IFA and Western blots showed that the 2A6 antigen is not strongly recognised after a single infection and since cattle expel adult worms approximately 60 days after a single experimental infection, this would suggest that antibodies to the 2A6 antigen are not involved in this process. This is consistent with the finding that the antigen does not appear to be present in the adult stage. However there is a strong antibody response to the antigen following a

second and subsequent infections and so it is possible that these antibodies could be involved in the protection of immune cattle against re-infection. In summary, the protective immune response to *D.viviparus* infection appears to be directed at $L_3$ and/or $L_4$ stages (Jarrett et al 1957b, Poynter et al 1960 and Jarrett & Sharp 1963) and whilst there is no direct evidence to suggest that the 2A6 antigen is involved in protective immunity, its immunodominance and consistency of recognition between individual cattle makes it a molecule worthy of further investigation.

Sera from cattle following 2 infections with *O.ostertagi* or *C.oncophora* or from sheep following 2 infections with *H.contortus* did not recognise the antigen on IFA or Western blots. This is not a particularly surprising result as these parasites are confined to the alimentary tract and so the antigen is less likely to be presented to the immune system than it is in a tissue migratory parasite such as *D.viviparus*. From this assertion and the report of Pritchard et al (1991) that the $L_3$ sheath of *N.americanus* was strongly recognised by sera from humans in endemic areas, it was suspected that the 2A6 antigen might be immunogenic in *N.americanus* infection. This was found not to be the case as none of the 20 individual human sera examined recognised the *D.viviparus* sheath or the 2A6 antigen on Western blots. Also the observation that mab 2A6 only binds to the sheath surface of minority of *N.americanus* $L_3$ does not fit with the report of Pritchard et al (1990), which stated that human infection sera recognised the sheath surface but did not suggest that there was any heterogeniety between individual larve.

In summary, the data presented in this chapter has proven that at least part of the marked immune response of cattle to the *D.viviparus* $L_3$ sheath is due to recognition of the 2A6 antigen. The immunodominance of the 2A6 antigen on Western blots suggests the possibility that this molecule could be entirely responsible for the bovine immune response to the $L_3$ sheath. It is of course possible that other major immunogens are present on the sheath surface but the balance of evidence would suggest that this is not the case. The fact that all the monoclonal antibodies raised to the sheath surface recognise the same antigen implies that it is antigenically homogeneous. Also a stronger piece of evidence is that the fluorescence produced by immune bovine serum was of equal intensity for all the different species of sheathed $L_3$ examined. This would be surprising if there were several different immunogenic molecules on the *D.viviparus* $L_3$ sheath surface as these would all have to be conserved in the different species of nematode which were examined from the order Strongylida.

The stage specificity of the antigen showed some interesting features. Its exposure on the cuticular surface was entirely stage specific in *D.viviparus*, *O.circumcincta* and *H.contortus* but it was present internally in larvated eggs, $L_1$ and

$L_3$ of *D.viviparus*. The 2A6 antigen was faintly detected in Western blots of adult homogenate on only two occasions which could have been due to the antigen being present in small amounts or alternatively could be an artifact caused, for example, by cross-contamination of samples between adjacent SDS-PAGE wells. The results suggest that the antigen first appears during late $L_1$ development within the egg. Attempts to demonstrate the synthesis of the antigen by biosynthetic labelling and immunoprecipitation were unsuccessful. This could be due to the synthesis of the molecule occurring only for a short period during a particular stage of development and being present in a stored form after this point. Alternatively, the radiolabelled amino acids used may not be particularly abundant in the antigen as is the case for the 400 kDa secreted molecule of *T.canis* $L_2$ which does not label with $^{35}S$-methionine (Menghi & Maizels 1986). The failure of $^{3}H$-glucosamine to label the antigen in these experiments was not conclusive as there was very poor incorporation of the label into larvated eggs and $L_1$. The hypothesis that the antigen is not synthesised in the $L_3$ is supported by the observation that it gradually disappears from larvae during *in vitro* culture which suggests that there is no active turnover of the molecule in this stage.

IFA on methanol/acetone fixed *D.viviparus* $L_3$ revealed that 2A6 and 2F8 did not bind to the $L_3$ cuticle but did bind to somatic tissues of the larvae. This binding appeared to be spread throughout the somatic tissues in an irregular fashion and had a granular appearance under high power. This granular appearance could be due, at least in part, to artifacts created by the fixation procedure and the irregular binding of the antibody could be due to uneven fixation of the larvae. The pattern of binding is unlike that reported for monoclonal antibodies to surface antigens of some other nematode species which bind to internal secretory structures such as the stichosomes of *T.spiralis* (McLaren et al 1987) and the oesophageal glands of *T.canis* (Maizels & Page 1990). In these cases the results can be explained by the production and secretion of material from these structures which is then exteriorised and subsequently becomes associated with the cuticular surface. The binding of 2A6 to the somatic tissues of *D.viviparus* $L_3$ is more similar to that reported other monoclonal antibodies such as DIM 229 against *D.immitis* $L_3$ and NEB-$D_1E_5$ against *B.malayi* $L_3$ (Rudin 1990). These antibodies bind to the epicuticular surface, but not to the deeper layers of the cuticle, and also bind to somatic structures such as the hypodermis, muscle and oesophagus. This pattern of binding is difficult to explain by the hypothesis that the internal binding represents detection of the antigen at its site of synthesis, since the binding is not restricted to secretory structures. In spite of this it seems that the internal binding of 2A6 is due to detection of the same antigen on the sheath and in the somatic tissues and not simply due to a cross-reactive epitope being

present at both these sites. This can be concluded because Western blots of homogenates prepared from sheathed and exsheathed *D.viviparus* $L_3$ are identical when probed with 2A6 and this is also true of homogenates prepared from exsheathed *H.contortus* $L_3$ compared to those prepared from purified *H.contortus* $L_3$ sheaths.

Immunogold EM studies were performed to investigate the internal distribution of the 2A6 antigen in more detail but the 2A6 epitope did not seem to be preserved by the procedures used for post-embedding labelling. More success might be obtained by post-embedding labelling of cryostat sections. The pre-embedding immunogold labelling of *D.viviparus* $L_3$ with 2A6 worked very well and revealed that the antibody bound not to the epicuticle but to a layer outside it. This surface layer appeared to be continuous and quite electron dense for most of the larvae examined on the electron micrographs. Interestingly, Wharton (1986) described the epicuticle of the *T.colubriformis* $L_3$ sheath as an 11 nm thick single electron dense layer "with regular strands of particulate material adhering to its outer surface" and in the electron micrographs reproduced in the paper, this surface material appears to form a relatively continuous layer. Therefore it appears that there is a surface coat on the $L_3$ sheath of *D.viviparus*, and possibly of other trichostrongyloid nematodes, which is similar in appearance to those described for a variety of other nematode species (reviewed by Blaxter et al 1992). It is to an antigen within this coat that the monoclonal antibody 2A6 binds. Unlike the surface coat of *T.canis* and many other nematodes (reviewed by Blaxter et al 1992 and in Chapter 1 of this thesis) the surface coat of the *D.viviparus* $L_3$ sheath does not seem to be shed or subject to any significant degree of turnover. Larvae which have been labelled with 2A6 and FITC conjugated second antibody can be left at room temperature for several days, in the absence of any metabolic inhibitors, without any significant loss of fluorescence. The surface coat also seems to be very resistant to solubilisation or degradation as the binding of 2A6 to the surface was unaffected by treatment of live larvae with detergents (with or without 2ME), proteases or flash treatment with chloroform/methanol. Treatment of live larvae with glycosidases may be useful to determine whether glycoconjugates are important in maintaining the integrity of the surface coat although no lectins from a panel of eight bound to the intact *D.viviparus* sheath surface (Chapter 4). Furthermore in spite of a number of studies no lectin has yet been found to bind to the external surface of other trichostrongylid sheaths (Bone & Bottjer 1985, Wharton & Murray 1990 and Duncan 1991). It has been generally assumed that the sheath, unlike the cuticle, is an inert structure as it has no direct contact with the hypodermis (Wharton 1985) and the results presented here are consistent with this view. One consequence of this is that the surface coat, which is just a few nanometres thick, must survive chemical and physical attrition without

repair for the lifetime of the larvae. It would be interesting to determine the manner in which this extreme resilience is achieved in a structure which can only be a few molecules in depth. The fact that the 2A6 molecule is solubilised by homogenisation of larvae in aqueous buffer would suggest that it is not the main structural component of the surface coat.

Immunochemical analysis of the 2A6 antigen proved to be a difficult task, particularly since the molecule could not be stained by routine protein or carbohydrate staining methods. Similar results to this have been reported for a number of parasite molecules such as the 400 kDa E/S proteoglycan of *T.canis* $L_2$ (Maizels & Robertson 1991) which cannot be stained with Coomassie or silver stains and does not incorporate $^{35}$S-methionine during metabolic labelling of parasites. The early work on the procyclic acidic repetitive protein (PARP) of *Trypanosoma brucei* (reviewed by Roditi & Pearson 1990) represents a particularly interesting parallel to the immunochemical problems encountered with the 2A6 antigen. It was first identified by raising monoclonal antibodies to the surface of the procyclic form of *T.brucei* and all ten of these monoclonal antibodies recognised the same molecule (Richardson et al 1986). Subsequent identification of this molecule turned out to be extremely problematical. It was not detectable by Coomassie blue staining (and only very faintly by silver staining) or UV absorption at 280nm. Also it could not be labelled with $^{125}$Iodine or metabolically labelled with $^{35}$S-methionine. The gene was eventually cloned in another laboratory by differential screening of cDNA libraries of bloodstream and procyclic forms and the gene sequence explained most of the original immunochemical problems encountered (Roditi et al 1987). It is a highly acidic protein which explains its poor staining characteristics and does not contain any aromatic amino acids which explains its lack of UV absorbance and failure to label with $^{125}$Iodine. The mature protein also lacks any methionine residues which explains the inability to metabolically label the molecule with $^{35}$S-methionine. Another unusual feature of PARP is that it does not resolve very well on SDS-PAGE gels appearing as a smear between 30 and 40 kDa and no simple explanation for this is apparent. One suggestion has been that there are numerous isomeric forms of the molecule, containing varying number of proline-glutamic acid repeats, which would have different migration properties on SDS-PAGE (Richardson et al 1988). In support of this, it is known that the procyclin genes exist as a polymorphic multigene family and that several genes are expressed simultaneously which differ only in the number of Glu-Pro repeats (Mowatt & Clayton 1988).

The above description of PARP bears some remarkable similarities to the properties of the 2A6 antigen presented in this thesis; all six monoclonal antibodies generated against the $L_3$ sheath surface recognised a single

immunodominant antigen which resolves as a smear or broad band on SDS-PAGE gels. It is not identified by surface labelling with $^{125}$Iodine (Britton 1991) or biotin, does not stain with Coomassie blue or silver staining techniques and has not been successfully labelled with $^{35}$S-methionine. It may be that some of the structural features identified in PARP may also apply to the 2A6 antigen. However there are several significant differences between the properties of the 2A6 antigen to those reported for PARP. Firstly PARP could be visualised by "Stains-all", a stain with a strong affinity for acidic macromolecules, and this did not detect the 2A6 antigen. Also PARP has been shown to be glycosylated whereas the experiments performed here have failed to demonstrate carbohydrate associated with the 2A6 antigen. The epitope to which 2A6 binds is periodate insensitive and no carbohydrate could be detected on the molecule by PAS staining or digoxigenin hydrazide labelling. These results cannot be taken as definitive proof that the molecule is not some form of glycoconjugate, as it has not been possible to quantify the amount of antigen present and so the negative results could be due to lack of sensitivity of the techniques. N-glycosidase treatment was performed to circumvent this problem but the antigen seemed to be resistant to this enzyme. Further experiments with other enzymes such as O-glycosidases may reveal some glycosylation of the molecule since Maizels & Page (1990) have reported that the major carbohydrates of the *T.canis* $L_2$ E/S products are O-linked. It is also worth noting that of the lectins used to probe Western blots of *D.viviparus* $L_2$, only Con A detected any molecules and this did not bind to the immunoprecipitated 2A6 molecule when it was used to probe such blots (data not shown).

Attempts to label the antigen with NHS-biotin were also unsuccessful but the presence of a polypeptide component was clearly demonstrated by the antigen's sensitivity to protease treatment. The results of these experiments were consistently repeatable with pronase digestion producing a ladder of bands and the trypsin and papain treatments producing a single major lower molecular weight product. The molecule could not be digested to completion even using high concentrations of protease and prolonged incubation times which completely digested most Coomassie stainable proteins. Therefore, although there must be a polypeptide component present, the molecule as a whole appears to be relatively resistant to protease treatment.

The purification of the antigen with a 2A6 immuno-affinity protein A column was relatively successful but although the antigen was easily detected in eluted fractions on Western blots probed with 2A6, significant amounts of protein could not be detected by U.V. absorbance or amino acid compositional analysis. Since Western blotting with 2A6 was the only detection method used for the antigen, it is possible that the amounts present in the eluted fractions were too small for detection

by U.V. absorbance and also too low to appear above the relatively high background amino acid contamination of the PVDF filters. The attempts of Richardson et al (1988) to immunoaffinty purify PARP using a monoclonal antibody were again remarkably similar. They could detect the molecule in fractions eluted off the column by ELISA but could not detect it by Coomassie blue, silver stains or UV absorbance. They managed to obtain an amino acid analysis of the protein but the yields they obtained from the column were extremely low (low microgram quantities from as many as $5x10^{10}$ organisms) and from this they concluded that PARP was not an abundant molecule. This conclusion was subsequently proved to be incorrect when much higher yields of PARP were produced from detergent extracts using a two step purification involving ion-exchange and Con A-Sepharose affinity columns (Clayton & Mowatt 1989). They estimated that PARP comprises of at least 0.7% of the total protein in procyclic trypanosomes which is about $6x10^6$ molecules/cell. They also suggested that the yields obtained by Richardson et al (1988) were much lower because of the use of aqueous supernatant from lysed parasites as the starting material, since most of the protein is attached to membranes and requires detergent for solubilisation.

These findings for PARP may be relevant to the attempts to purify the 2A6 antigen. It was surprising that, although the antigen could be clearly detected in fractions eluted from the column, it could not be detected by other methods and there was insufficient material present for compositional amino acid analysis. This was particularly puzzling when one considers the density of the immunogold labelling of the sheath surface with 2A6 and the fact that the molecule is present in somatic tissues since these findings would lead one to expect the antigen to be a relatively abundant molecule. However the Western blots show that although the antigen is present in the aqueous homogenates, it is also present in the SDS/2ME/urea soluble fraction and since the proportion of the antigen released into the water soluble fraction is not known, it is possible that the majority remains in the insoluble material. Thus an importanat priority in the analysis of this antigen, is the investigation of its solubilty in different detergents and determination of its abundance within the parasite.

In summary, the antigen responsible for the marked immunogenicity of the $L_3$ sheath has been identified and appears to be an extremely immunodominant $L_3$ antigen. Furthermore, the results suggest that an homologous molecule may exist on the surface of the $L_3$ sheaths of many other nematodes from the order Strongylida. Therefore the 2A6 molecule is of interest both in terms of *D.viviparus* immunology and as a conserved, and possibly functionally important, molecule of Strongylid nematodes.

**Figure 5.1**

**A.** Cast $L_3$ sheath of *H.contortus*. x100 magnification.

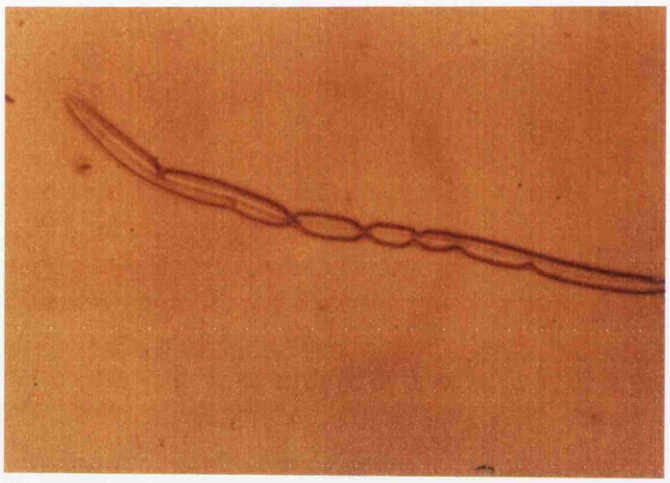
**B.** IFA with 2A6 on *D.viviparus* $L_3$ following 5 minutes incubation in 0.01% sodium hypochlorite. x400 magnification.

**C.** IFA with 2A6 on *D.viviparus* $L_3$ following 10 minutes incubation in 0.01% sodium hypochlorite solution. x400 magnification.

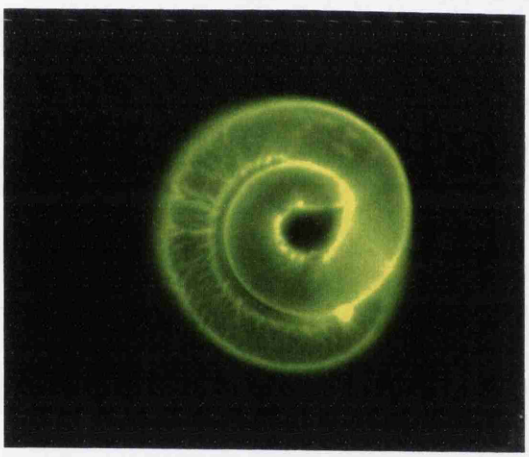
**D.** IFA with 2A6 on cast *D.viviparus* $L_3$ sheath following exsheathment in 0.01% sodium hypochlorite solution. x400 magnification.

**E.** IFA with 2A6 showing fragment of *D.viviparus* $L_3$ sheath following exsheathment in 0.01% sodium hypochlorite solution. x1000 magnification.

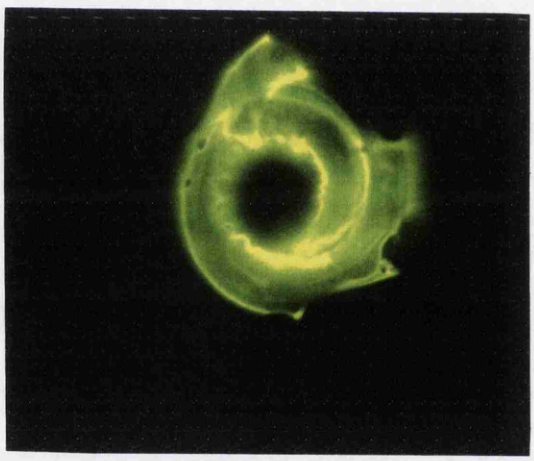
A



B



C



D

E

**Figure 5.2**

Quantitative IFA with undiluted 2A6 supernatant on sheathed and exsheathed $L_3$ of different nematode species from the order strongylida. Each value of relative fluorescence given in the figure is the mean of readings from 20 individual larvae.

D.V. = *D.viviparus*
H.C. = *H.contortus*
O.O. = *O.ostertagi*
O.C. = *O.circumcincta*
C.O. = *C.oncophora*
T.C. = *T.colubriformis*
T.V. = *T.vitrinus*
N.H. = *N.helvetianus*
CYATH = *Cyathostome spp.*

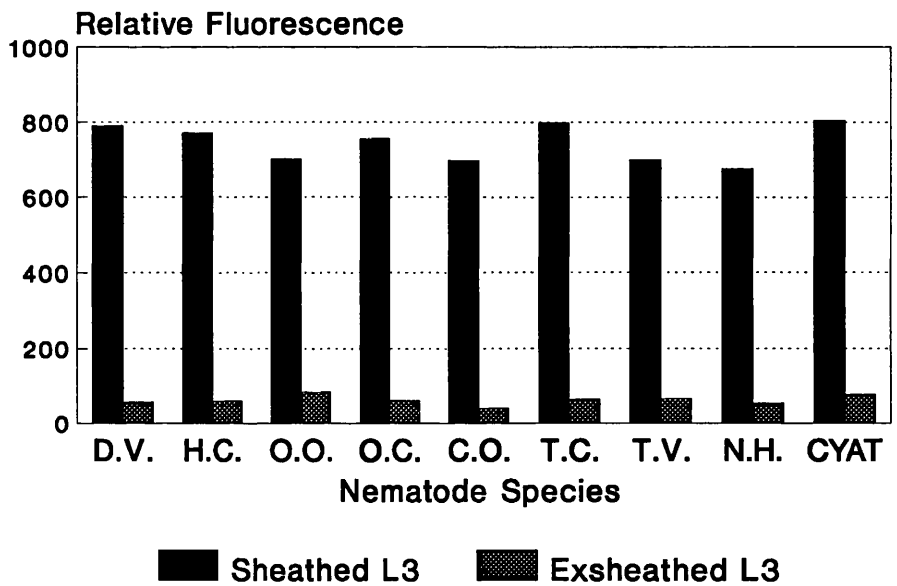
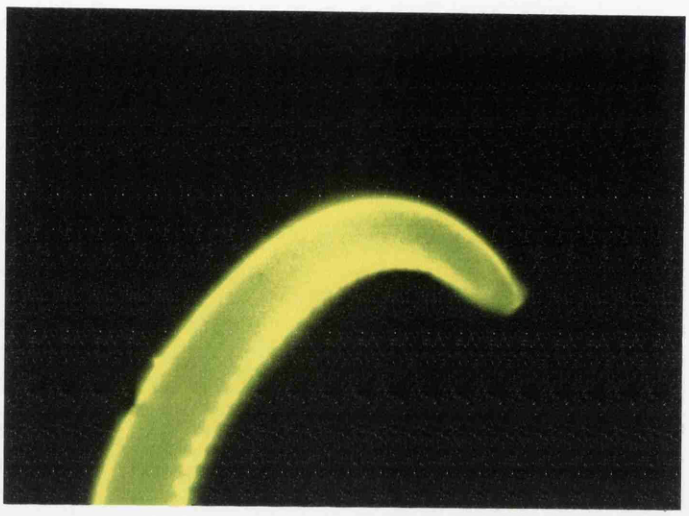

Relative Fluorescence
1000
800
600
400
200
0
D.V.
H.C.
O.O.
O.C.
C.O.
T.C.
T.V.
N.H.
CYAT
Nematode Species
Sheathed L3
Exsheathed L3

**Figure 5.3**

**A.** Immunofluorescence of an *H.contortus* sheathed $L_3$ with neat 2A6 supernatant. Photomicrograph is taken at x400 magnification. Sheathed $L_3$ of the other nematode species shown in figure 5.1 had an identical appearance to this.

**B.** Frequency histogram displaying the readings of quantitative IFA from 25 individual sheathed $L_3$ picked at random from several thousand larvae subjected to IFA with 2A6.

A



B

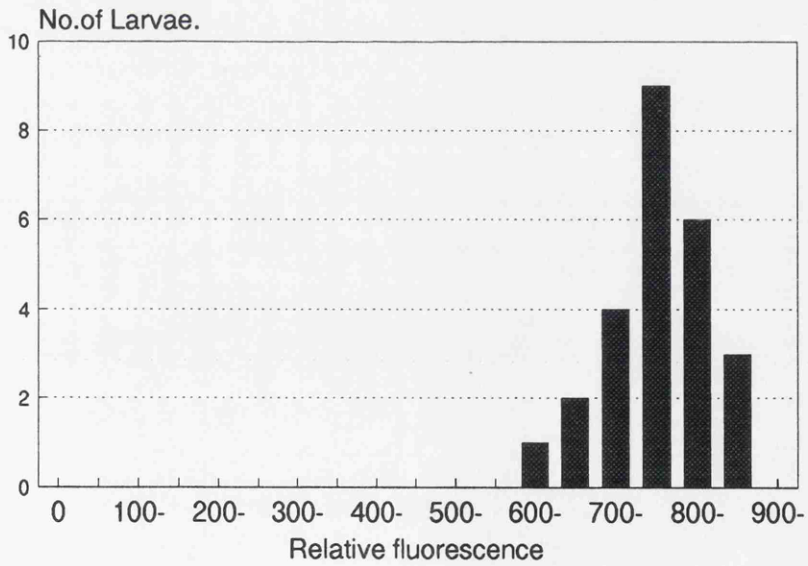

No.of Larvae.
10
8
6
4
2
0
0
100-
200-
300-
400-
500-
600-
700-
800-
900-
Relative fluorescence

**Figure 5.4**

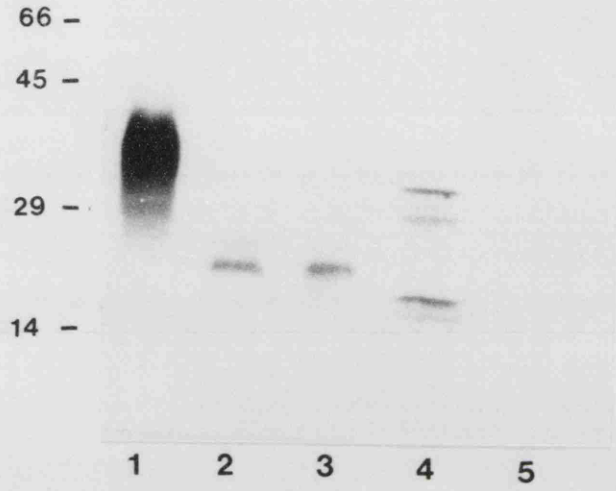
**A.** Western blot of $L_3$ aqueous homogenates prepared from a number of different nematode species probed with 2F8.

Track 1 = *D.viviparus*
Track 2 = *H.contortus*
Track 3 = *C.oncophora*
Track 4 = *Cyathostome spp.*
Track 5 = *Ascaris suum* $L_2$

**B.** Western blot of $L_3$ aqueous homogenates prepared from a number of different nematode species probed with 2A6.

Track 1 = *D.viviparus*
Track 2 = *H.contortus*
Track 3 = *T.colubriformis*
Track 4 = *C.oncophora*
Track 5 = *O.circumcincta*
Track 6 = *Cyathostome* spp.
Track 7 = *T.spiralis* $L_1$

A




B

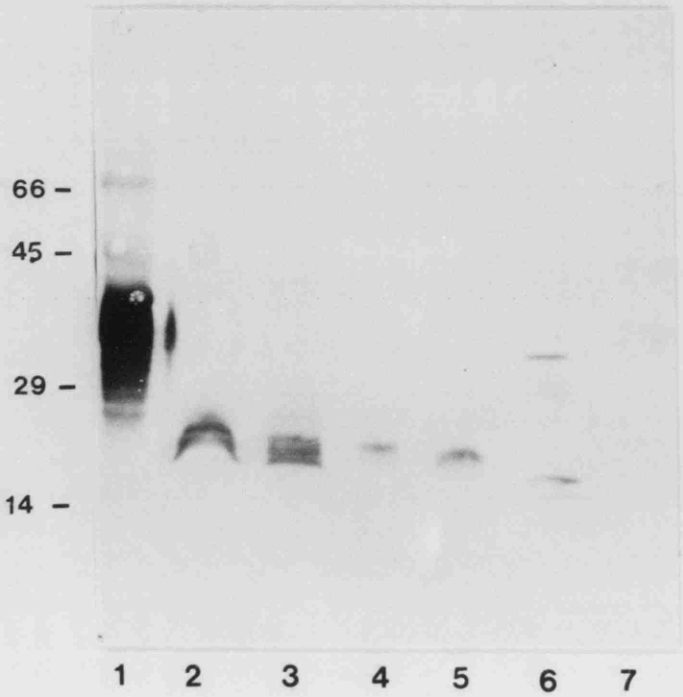


**Figure 5.5**

Summary chart showing the nematode species containing the 2A6 antigen and the taxonomic relationship between them. The taxonomy is adapted from Urquhart et al (1985) and Smith (1976).

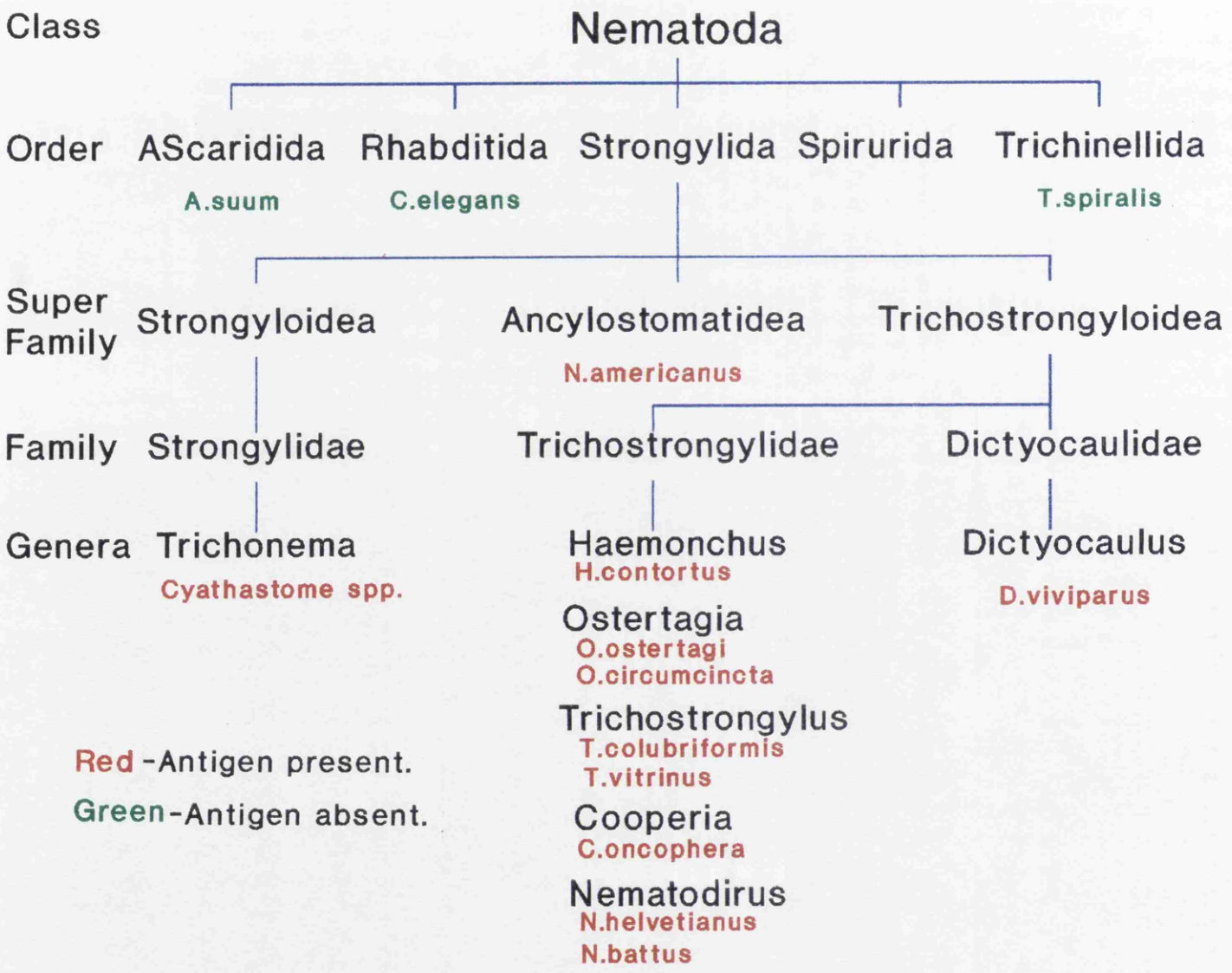

Class
Nematoda
Order
AScaridida
Rhabditida
Strongylida
Spirurida
Trichinellida
A.suum
C.elegans
T.spiralis
Super Family
Strongyloidea
Ancylostomatidea
Trichostrongyloidea
N.americanus
Family
Strongylidae
Trichostrongylidae
Dictyocaulidae
Genera
Trichonema
Cyathastome spp.
Haemonchus
H.contortus
Ostertagia
O.ostertagi
O.circumcincta
Trichostrongylus
T.colubriformis
T.vitrinus
Cooperia
C.oncophera
Nematodirus
N.helvetianus
N.battus
Dictyocaulus
D.viviparus
Red -Antigen present.
Green-Antigen absent.

**Figure 5.6**

**A.** Western blots of *D.viviparus* $L_3$ homogenate probed with 12 individual hyperimmune bovine sera. The $L_3$ homogenate is the aqueous soluble fraction cleared by centrifugation 5000 rpm for 5 minutes. The individual sera are as follows;

Tracks 2, 3 and 4 = B7, B8 and B9 sera respectively. Taken from cattle immunised three times by oral infection.

Tracks 5, 6 and 7 = 29, 31 and 30 respectively. Taken from cattle orally immunised twice with 40krad irradiated larvae and subsequently challenged.

Tracks 8 and 9 = O38 and O39 respectively. Taken from cattle immunised three times by oral infection with 40krad irradiated larvae.

Tracks 10 and 11 = G17 and B45 respectively. Taken from cattle immunised three times by intravenous administration of 100 krad irradiated larvae.

Tracks 12 and 13 = O25 and O28 respectively. Taken from cattle immunised twice by intravenous administration with 100krad larvae and subsequently challenged.

Track 14 = Naive bovine serum.

The arrow indicates the 29-40 kDa immunodominant antigen.

**B.** Western blots probed with the same serum as in figure A but using an aqueous fraction of *D.viviparus* $L_3$ homogenate cleared by centrifugation at 100,000 g for 30 minutes.

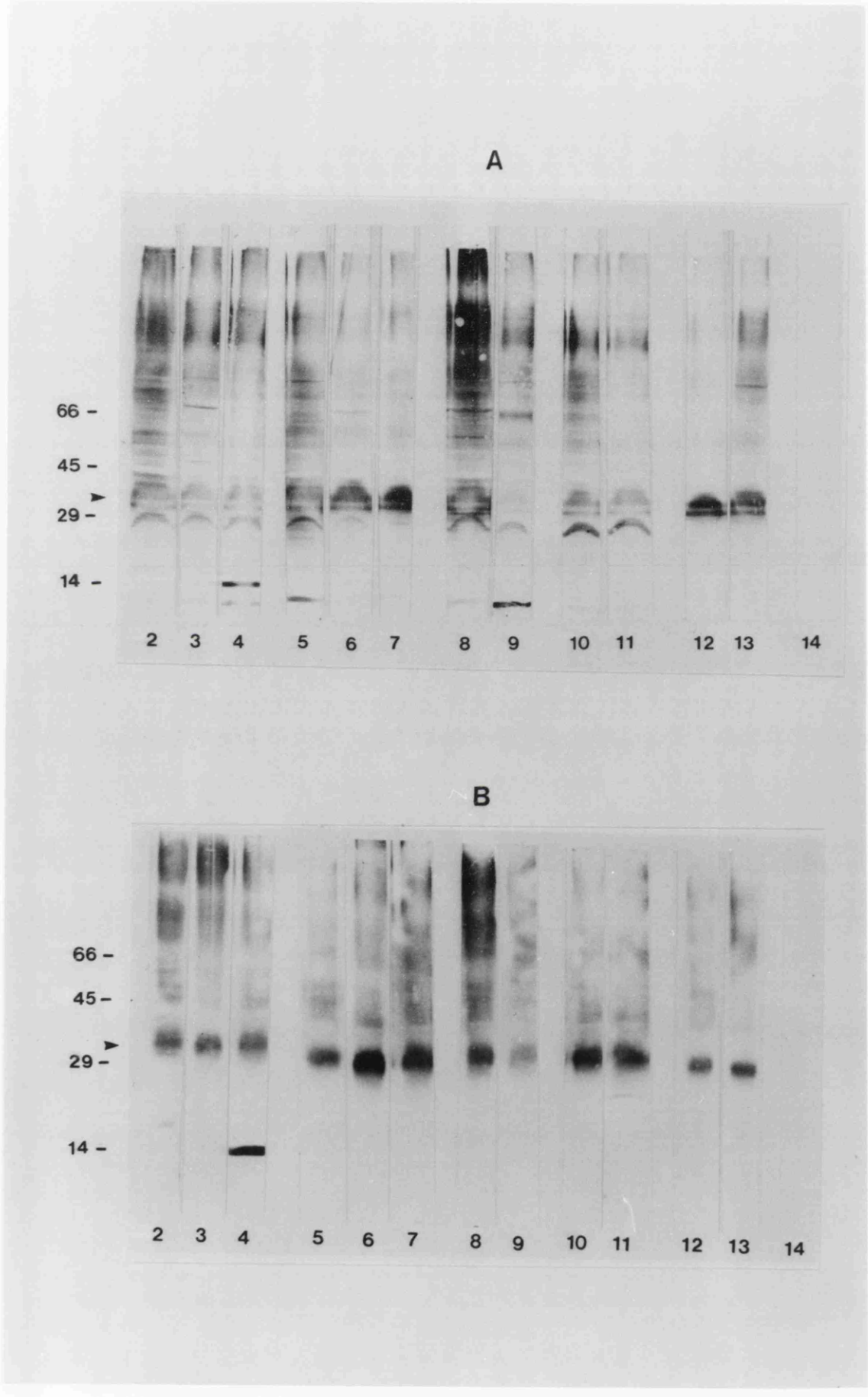
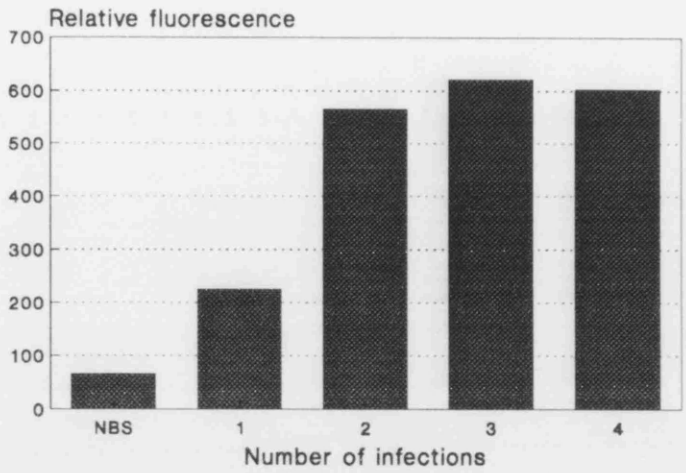

A
66 -
45 -
29 -
14 -
2 3 4 5 6 7 8 9 10 11 12 13 14
B
66 -
45 -
29 -
14 -
2 3 4 5 6 7 8 9 10 11 12 13 14

**Figure 5.7**

**A.** Quantitative IFA performed on *D.viviparus* sheathed $L_3$ with serum from taken, three weeks after each infection, from calves which had been orally infected with *D.viviparus* on successive occasions (pooled B7, B8 and B9 serum - see appendix 2). NBS = naive bovine serum.

**B.** Western blots of *D.viviparus* $L_3$ homogenate probed with the same pooled serum samples used in figure A. N = naive bovine sera. Tracks 1-4 are probed with the samples taken three weeks after infections 1 - 4 respectively.

A


Relative fluorescence
700
600
500
400
300
200
100
0
NBS
1
2
3
4
Number of infections

B

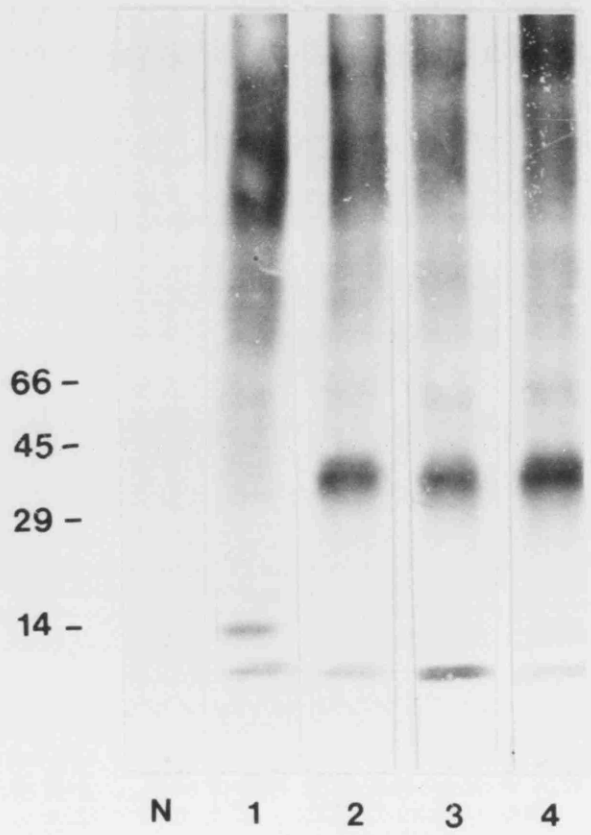

66 –
45 –
29 –
14 –
N
1
2
3
4

**Figure 5.8**

**A.** Quantitative IFA with pooled hyperimmune *D.viviparus* bovine serum (B7, B8 and B9 after third infection -see Appendix 2) on sheathed and exsheathed $L_3$ of different nematode species from the order Strongylida. Each value of relative fluorescence given in the figure is the mean of readings from 20 individual larvae.

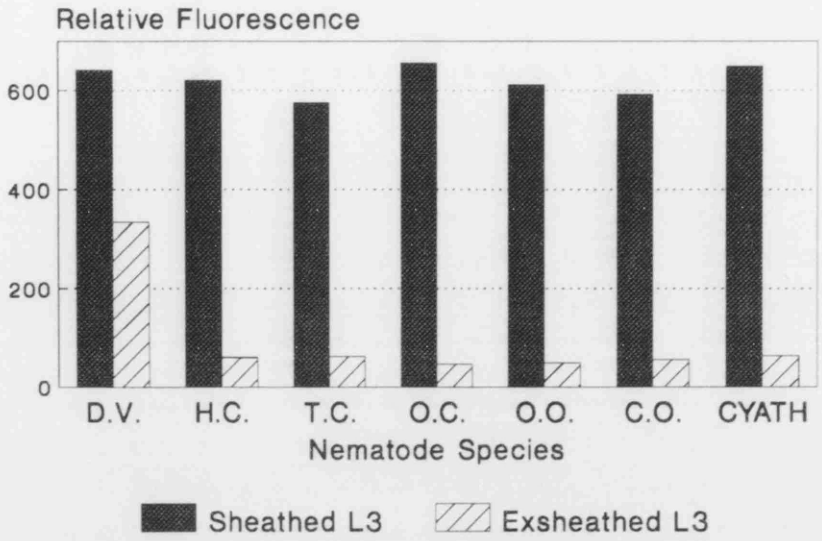
D.V. = *D.viviparus*
H.C. = *H.contortus*
T.C. = *T.colubriformis*
O.C. = *O.circumcincta*
O.O. = *O.ostertagi*
C.O. = *C.oncophora*
CYATH = *Cyathostome spp.*

**B.** Western blots of $L_3$ homogenate prepared from a number of different nematode species from the order Strongylida probed with the same *D.viviparus* hyperimmune bovine serum used in figure A.

Track 1 = *H.contortus*
Track 2 = *C.oncophora*
Track 3 = *Cyathostome* spp.
Track 4 = *D.viviparus.*

The arrows indicate the antigens of a similar molecular weight to those detected when mabs 2A6 and 2F8 were used to probe a similar blot. Compare to figure 5.4.

A


Relative Fluorescence
600
400
200
0
D.V.
H.C.
T.C.
O.C.
O.O.
C.O.
CYATH
Nematode Species
Sheathed L3
Exsheathed L3

B

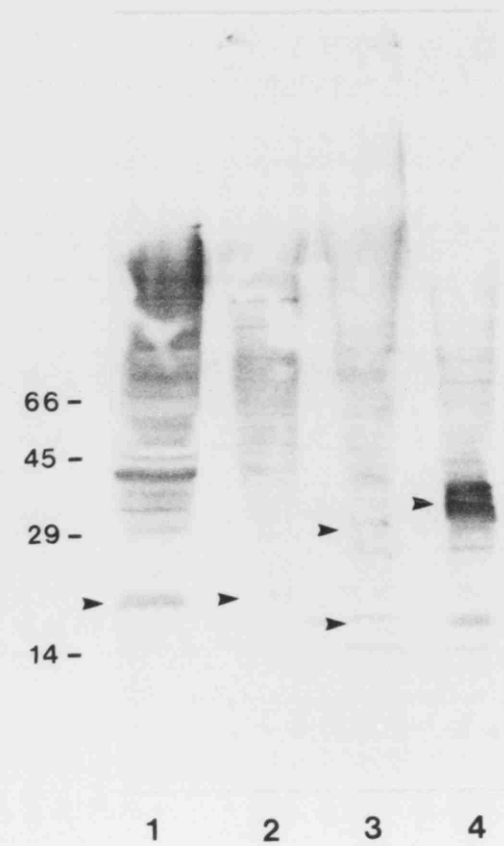

66 –
45 –
29 –
14 –
1
2
3
4

**Figure 5.9**

**A.** Western blot of supernatant and precipitated antigens resulting from immunoprecipitation of *D.viviparus* $L_3$ homogenate with 2A6 and protein-A beads and probed with mab 2A6.

Track 1 = $L_3$ homogenate supernatant following immunoprecipitation with complete medium and protein-A beads (negative control).
Track 2 = $L_3$ homogenate supernatant following immunoprecipitation with 2A6 supernatant and protein-A beads.
Track 3 = Antigens immunoprecipitated by complete medium and protein-A beads (negative control).
Track 4 = Antigens immunoprecipitated by 2A6 supernatant and protein-A beads.

**B.** Duplicate Western blot to figure A probed with *D.viviparus* hyperimmune bovine serum (pooled B7, B8 and B9 after third infection). Arrows H and L indicate the immunoglobulin heavy and light chains and arrow A indicates the 2A6 antigen.

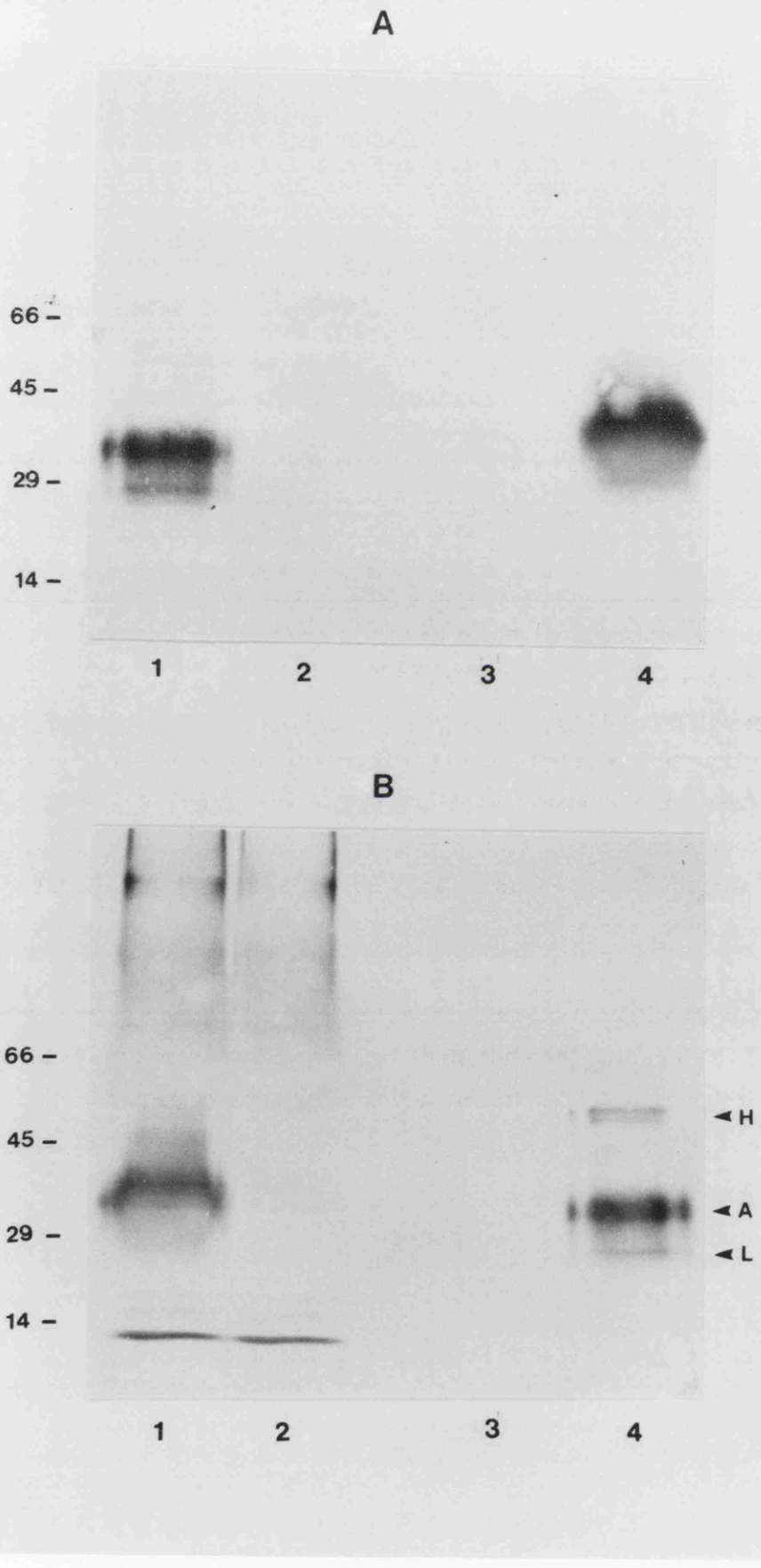

A
66 –
45 –
29 –
14 –
1
2
3
4
B
66 –
45 –
29 –
14 –
◄H
◄A
◄L
1
2
3
4

**Figure 5.10**

**A.** Western blot of supernatant and precipitated antigens resulting from immunoprecipitation of *H.contortus* $L_3$ homogenate with 2A6 and protein-A beads and probed with mab 2A6.

Track 1 = $L_3$ homogenate supernatant following immunoprecipitation with complete medium and protein-A beads (negative control).
Track 2 = $L_3$ homogenate supernatant following immunoprecipitation with 2A6 supernatant and protein-A beads.
Track 3 = Antigens immunoprecipitated by complete medium and protein-A beads (negative control).
Track 4 = Antigens immunoprecipitated by 2A6 supernatant and protein-A beads.

Arrow A indicates the 20kDa doublet detected by 2A6.

**B.** Duplicate Western blot to figure A probed with *D.viviparus* hyperimmune bovine serum (pooled B7, B8 and B9 after third infection). Arrow A indicates the 20kDa doublet detected by 2A6 and arrows H and L indicate the immunoglobulin heavy and light chains.

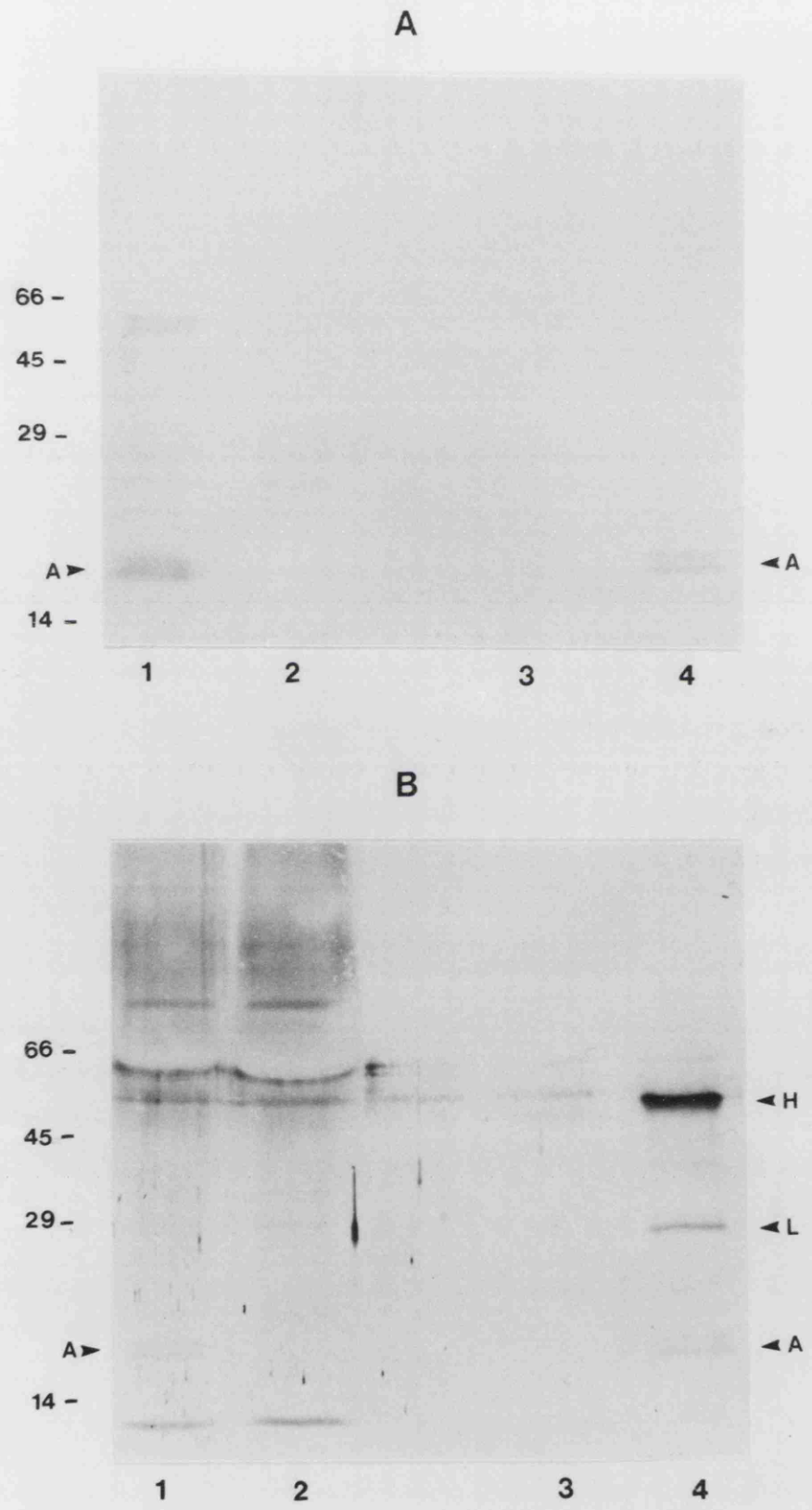

A
66 –
45 –
29 –
A ➤
14 –
◄ A
1
2
3
4
B
66 –
45 –
29 –
A ➤
14 –
◄ H
◄ L
◄ A
1
2
3
4

**Figure 5.11**

Quantitative IFA of *D.viviparus*, *H.contortus* and *O.ostertagi* sheathed $L_3$ probed with a number sera from animals following infection with different species of trichostrongyloid nematodes.

D.V. serum = Bovine serum after 2 experimental infections with *D.viviparus*.
H.C. serum = Ovine serum after 2 experimental infections with *H.contortus*.
O.O. serum = Bovine serum after 2 experimental infections with *O.ostertagi*.
C.O. serum = Bovine serum after 2 experimental infections with *C.oncophora*.

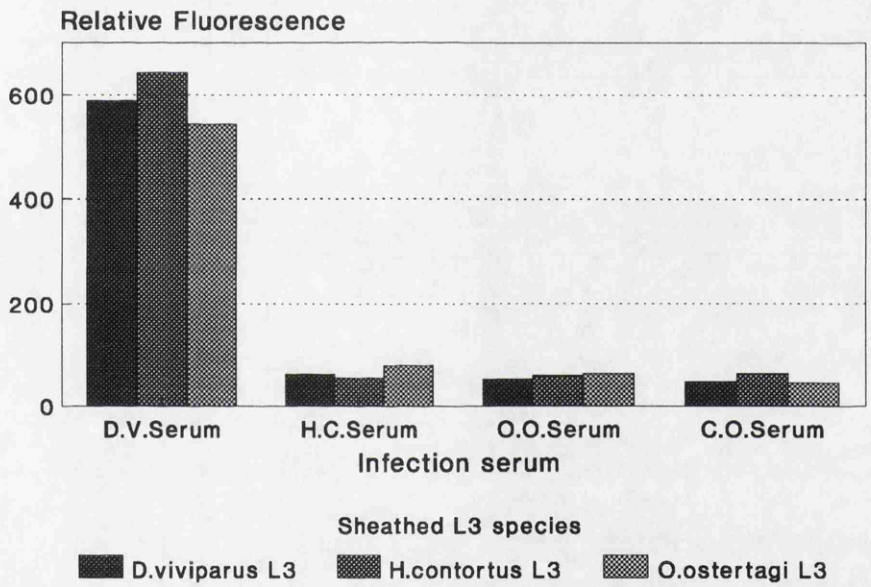

Relative Fluorescence
600
400
200
0
D.V.Serum
H.C.Serum
O.O.Serum
C.O.Serum
Infection serum
Sheathed L3 species
D.viviparus L3
H.contortus L3
O.ostertagi L3

**Figure 5.12**

**A.** An example of one of the *N.americanus* sheathed $L_3$ which produced significant fluorescence with 2A6 supernatant. x400 magnification.

**B.** Frequency histogram displaying the readings of quantitative IFA from 20 individual sheathed *N.americanus* $L_3$ subjected to IFA with 2A6 supernatant.

A

B

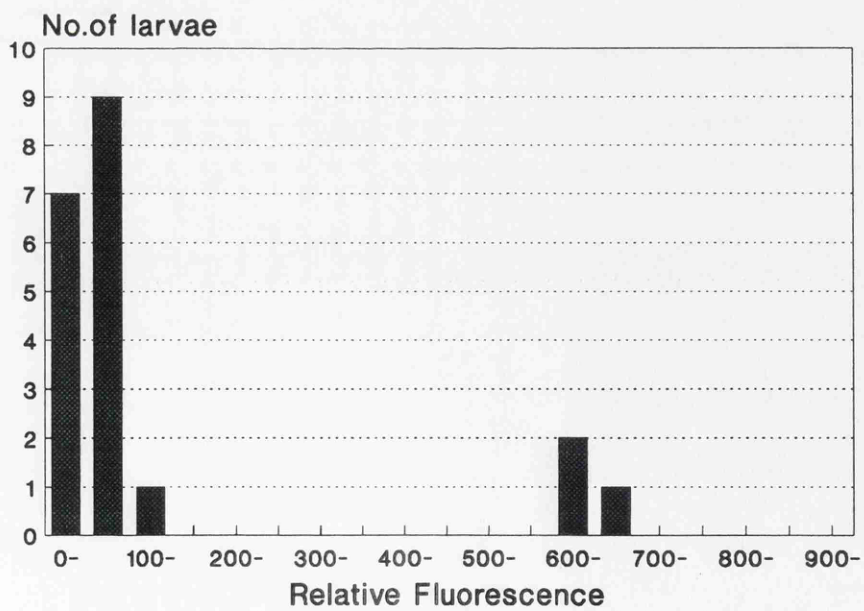

No.of larvae
10
9
8
7
6
5
4
3
2
1
0
0-
100-
200-
300-
400-
500-
600-
700-
800-
900-
Relative Fluorescence

**Figure 5.13**

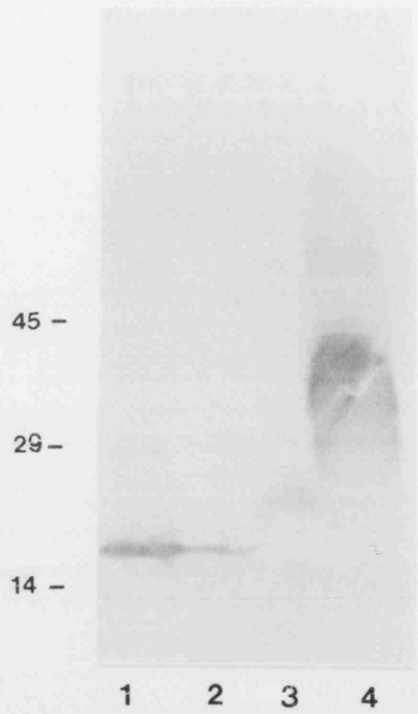
**A.** Western blot of a number of $L_3$ homogenates prepared from different nematode species and probed with 2A6 supernatant.

Track 1 = *N.americanus* $L_3$ homogenate SDS/2ME/urea fraction.
Track 2 = *N.americanus* $L_3$ homogenate aqueous fraction.
Track 3 = *H.contortus* $L_3$ homogenate aqueous fraction.
Track 4 = *D.viviparus* $L_3$ homogenate aqueous fraction.

**B.** Western blot of *D.viviparus* $L_3$ homogenate probed with *N.americanus* infection serum from 15 different human patients.

N = Naive human serum.

A




B

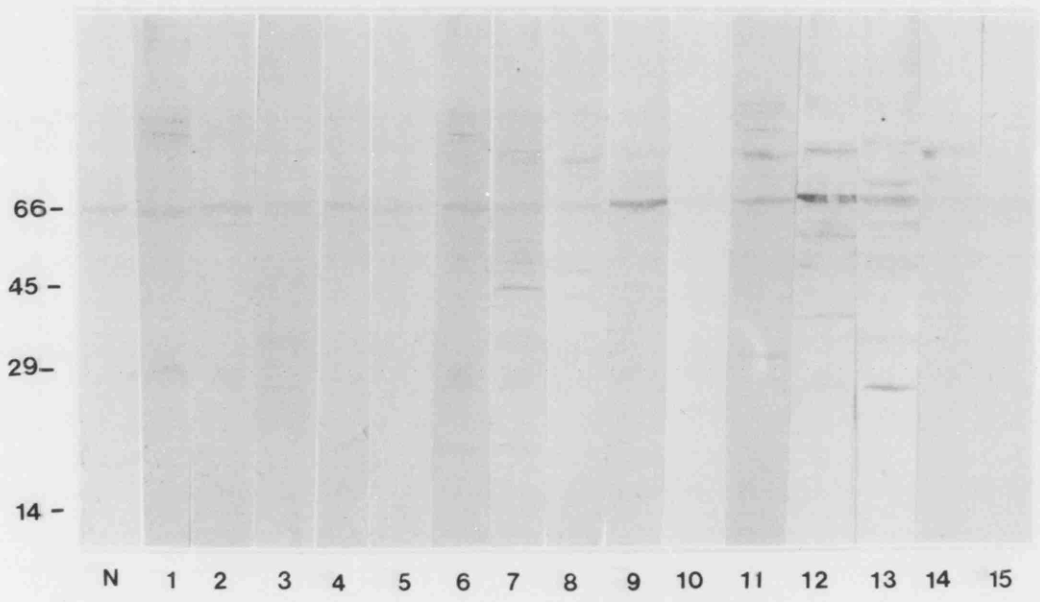


**Figure 5.14**

Quantitative IFA with 2A6 and 2F8 supernatants on several developmental stages of *D.viviparus*.

Egg = Larvated eggs.
L1 = $L_1$ harvested from bovine faeces.
SH L3 = Sheathed $L_3$.
ExSH L3 = Exsheathed $L_3$.
Adult = Adult parasites following *in vitro* culture for 24 hours.

All values are the mean of readings from 20 individual larvae/eggs except the values for the adults which are the mean from 6 individuals.

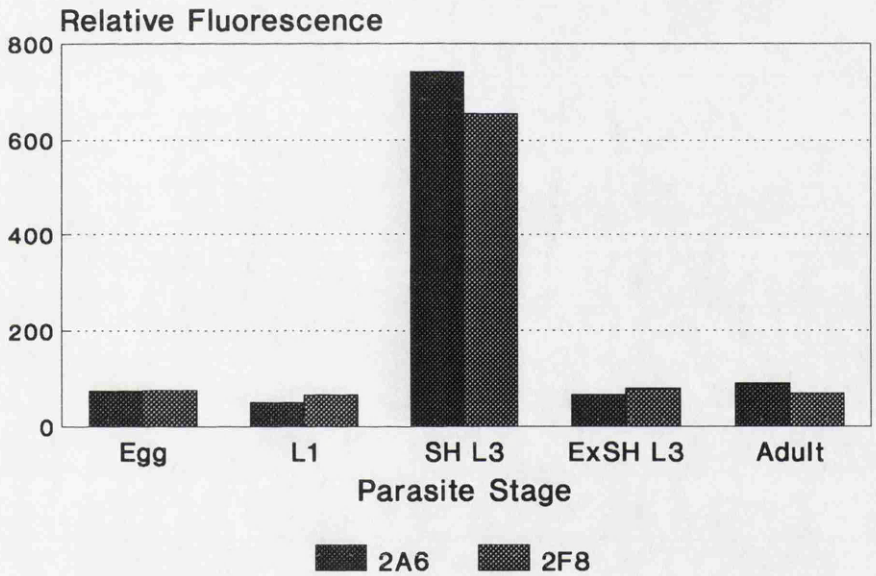

Relative Fluorescence
800
600
400
200
0
Egg
L1
SH L3
ExSH L3
Adult
Parasite Stage
2A6
2F8

**Figure 5.15.**

Examples of IFA performed with 2A6 supernatant on *D.viviparus* larvae cultured in faeces from $L_1$ to $L_3$.

**A.** Two larvae taken after 24 hours of culture. They appear to be $L_1$.

**B.** Two larvae taken after 48 hours of culture. The majority of larvae do not yet appear to have moulted to $L_2$ but IFA with 2A6 produces a "anterior button" of fluorescence in 10-20 % of larvae. This was not apparent in IFA performed with 5E1 as a negative control.

**C.** One larvae, typical of many examined after 72 hours of culture, examined under normal light and U.V. microscopy following 2A6 IFA. This is an $L_2$ which has retained the $L_1$ cuticle after moulting and this appears as a loosely corrugated cuticle relatively widely separated from the underlying $L_2$ cuticle. 2A6 produces no fluorescence with this retained $L_1$ cuticle.

**D.** One larvae, examined under light and U.V. microscopy after IFA with 2A6, which was typical of practically all those collected after 96 hours of culture. This is an $L_3$, in which the retained $L_2$ cuticle appears as a tightly corrugated cuticle relatively closely opposed to the underlying $L_3$ cuticle. This sheath appears to consist of a single retained cuticle and so the $L_1$ cuticle must have been shed by this stage.

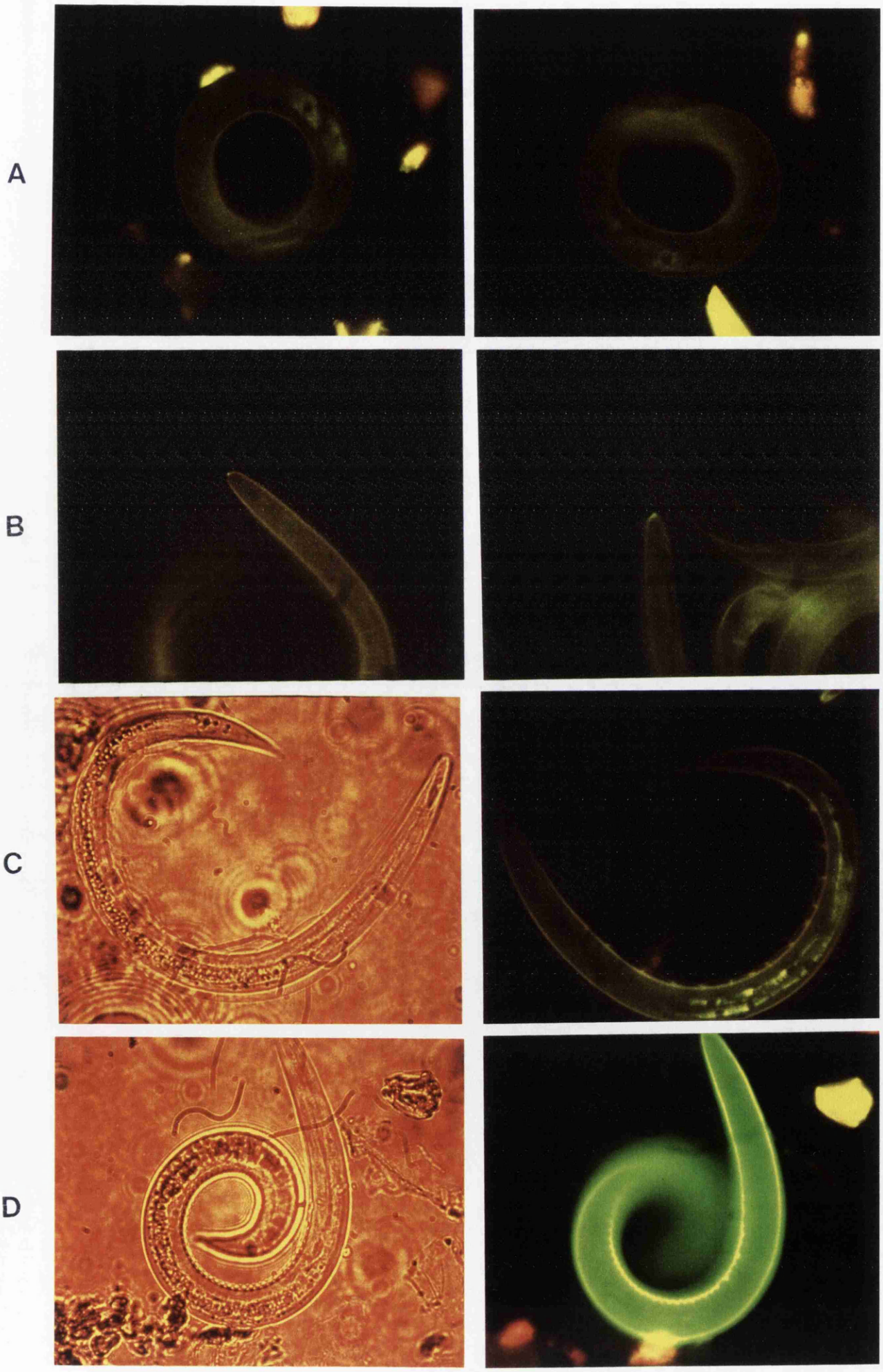

A
B
C
D

**Figure 5.16**

Diagram to illustrate the discrete stages which occur during development of *D.viviparus* from embryo to infective $L_3$. The exsheathment of the $L_3$ within the bovine alimentary tract is the traditional view although there is little experimental evidence to support this assumption. It can be seen that the $L_2$ cuticle is only ever exposed as the outer surface of the parasite following its separation to form the $L_3$ sheath. Consequently the intact, living $L_2$ cuticle cannot be probed with mab 2A6 on live IFA.

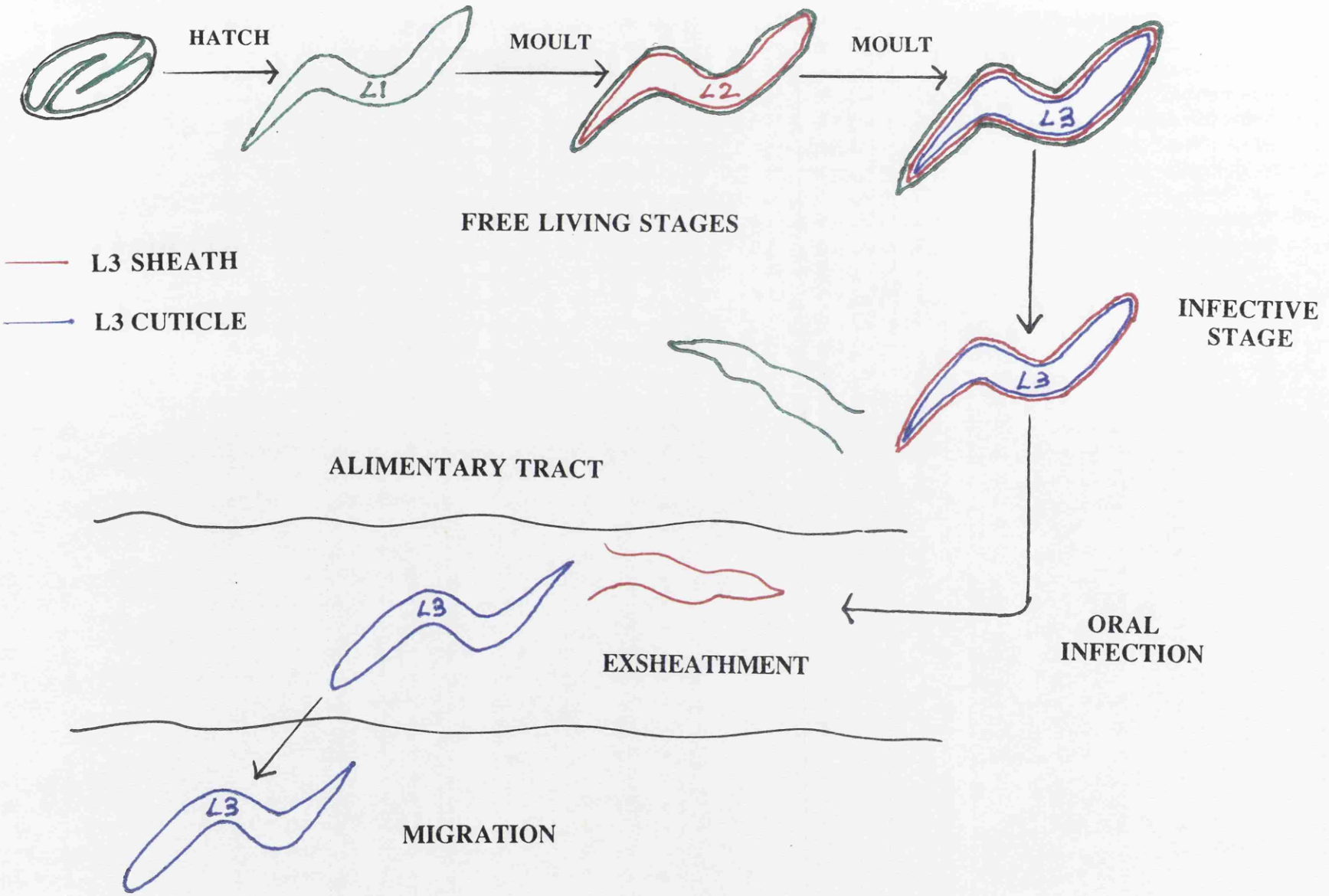

HATCH
L1
MOULT
L2
MOULT
L3
FREE LIVING STAGES
L3 SHEATH
L3 CUTICLE
INFECTIVE STAGE
L3
ALIMENTARY TRACT
L3
EXSHEATHMENT
ORAL INFECTION
L3
MIGRATION

**Figure 5.17**

**A.** Western blots of aqueous homogenate prepared from different developmental stages of *D.viviparus* probed with 2A6 supernatant.

Track 1 = $L_1$ harvested from faeces.
Track 2 = Sheathed $L_3$.
Track 3 = Exsheathed $L_3$.
Track 4 = Adult, batch 1.
Track 5 = Adult, batch 2.
Track 6 = Adult, male.
Track 7 = Adult, female.

**B.** Western blots of aqueous homogenate prepared from *D.viviparus* larvated eggs (released by *in vitro* cultured adults) probed with 2A6.
Track 1 = Larvated eggs, batch 1.
Track 2 = Larvated eggs, batch 2.
Track 3 = Sheathed $L_3$ homogenate.
Track 4 = Adult homogenate.

**C.** Western blots of homogenate, prepared from *D.viviparus* $L_3$ cultured in RPMI at 37°C in 5% $CO_2$, probed with 2A6 supernatant.

Track 1 = Exsheathed $L_3$ cultured for 10 days.
Track 2 = Exsheathed $L_3$ cultured for 5 days.
Track 3 = Non-cultured exsheathed $L_3$.

Homogenate was prepared in the same way for cultured and uncultured larvae and equal amounts of total protein loaded into each track. The Ponceau-s stain performed on the blot, prior to probing with 2A6, confirmed the equality of loading of each track.

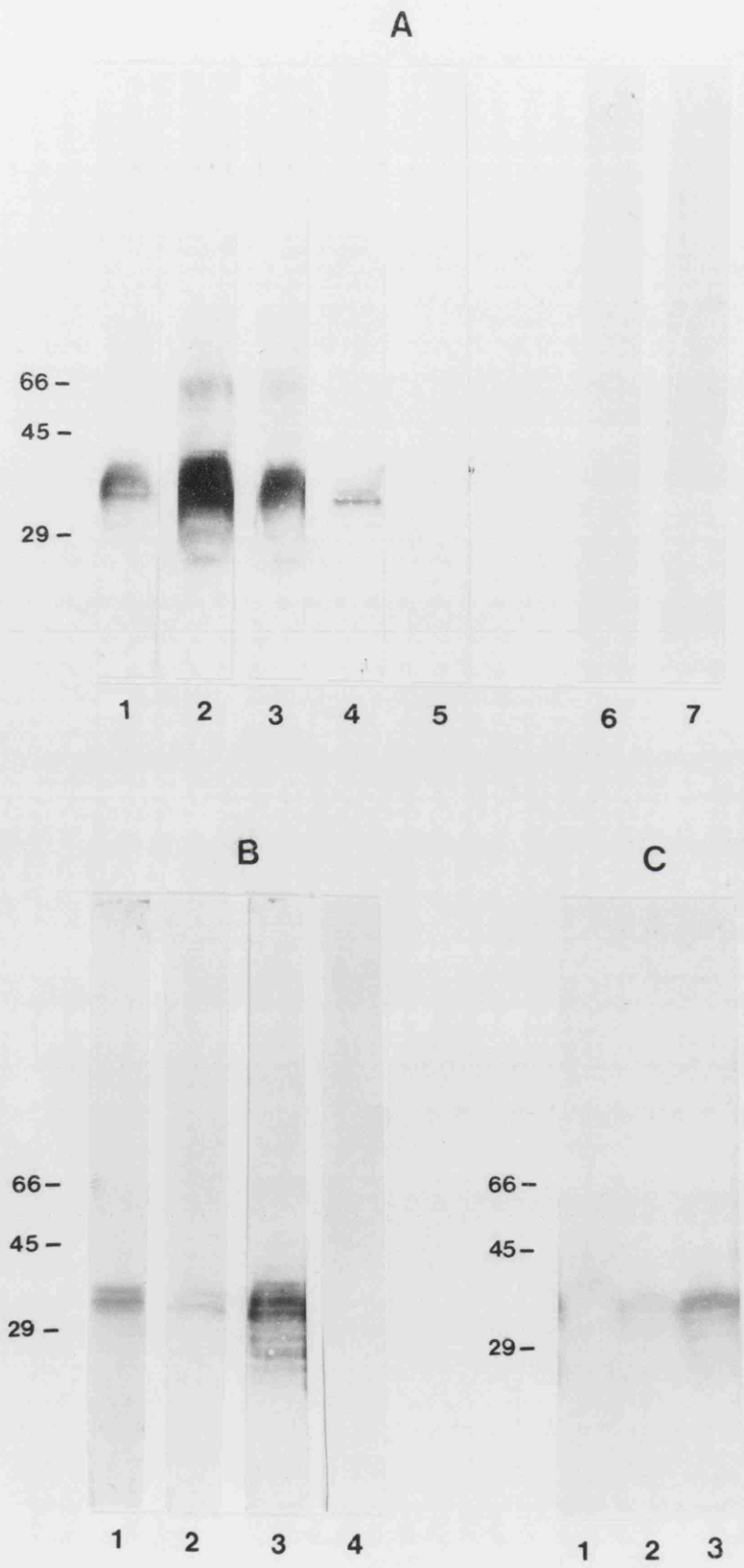

A
66 –
45 –
29 –
1
2
3
4
5
6
7
B
66 –
45 –
29 –
1
2
3
4
C
66 –
45 –
29 –
1
2
3

**Figure 5.18**

**A.** Fluorograph of SDS-PAGE gel of homogenates prepared from different stages of *D.viviparus* following metabolic labelled with $^{35}$S-methionine. The same number of counts per minute were loaded into each track except for $L_1$ for which half the counts per minute were loaded due to shortage of material. Tracks 1-4 contain aqueous soluble homogenate whereas Tracks 2-8 contain SDS/2ME/urea soluble homogenate.

Tracks 1 & 5 = Adult homogenate.
Tracks 2 & 6 = Larvated egg homogenate.
Tracks 3 & 7 = $L_1$ homogenate.
Tracks 4 & 8 = Sheathed $L_3$ homogenate.

**B.** Western blot of antigen, immunoprecipitated by 2A6/Protein-A sepharose beads from $^{35}$S-methionine labelled aqueous soluble antigens, probed with 2A6.

Track 1 = 2A6 immunoprecipitate from adult homogenate.
Track 2 = 2A6 immunoprecipitate from larvated egg homogenate.
Track 3 = 2A6 immunoprecipitate from $L_1$ homogenate.
Track 4 = 2A6 immunoprecipitate from $L_3$ homogenate.

Arrows H and L indicate heavy and light immunoglobulin chains and arrow A indicates immunoprecipitated antigen.

**C.** Duplicate gel, to that used to produce the blot shown in figure B, analysed by fluorography. 2 weeks exposure.

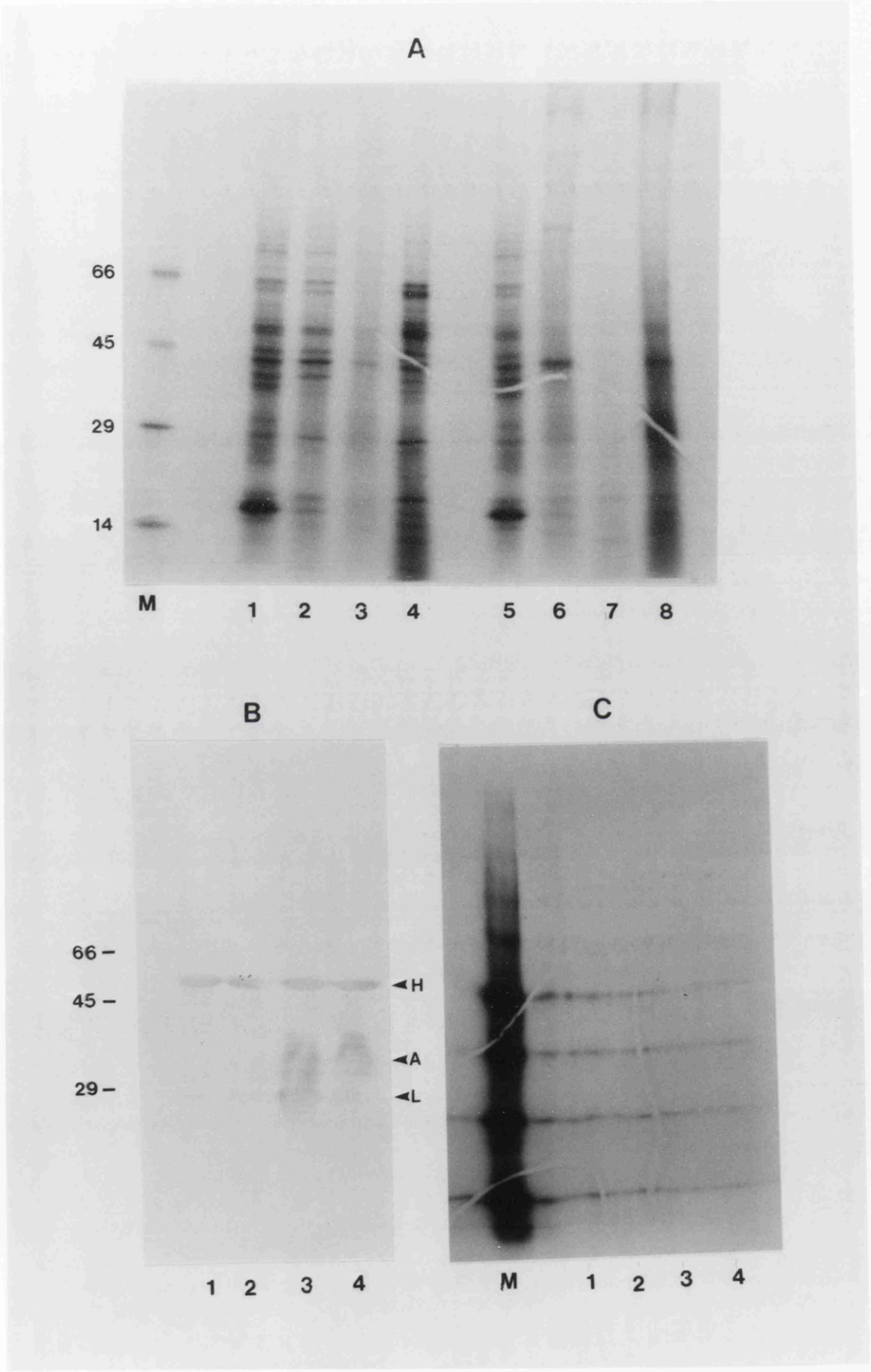

A
66
45
29
14
M
1
2
3
4
5
6
7
8
B
66 –
45 –
29 –
◄H
◄A
◄L
1
2
3
4
C
M
1
2
3
4

**Figure 5.19**

**A.** Fluorograph of SDS-PAGE gel of homogenates prepared from different developmental stages of *D.viviparus* following metabolic labelling with an $^{3}H$-amino acid mixture (Leucine, Lysine, Phenylalanine, Proline and Tyrosine). The same number of counts per minute were loaded into each track except for $L_1$ for which half the counts per minute were loaded due to shortage of material. Tracks 1-4 contain aqueous soluble homogenates whereas Tracks 2-8 contain SDS/2ME/urea soluble homogenates.

Tracks 1 & 5 = Adult homogenate.
Tracks 2 & 6 = Larvated egg homogenate.
Tracks 3 & 7 = $L_1$ homogenate.
Tracks 4 & 8 = Sheathed $L_3$ homogenate.

**B.** Western blot of antigen, immunoprecipitated by 2A6/Protein-A sepharose beads from $^{3}H$-amino acid mixture labelled aqueous soluble homogenate, probed with 2A6.

Track 1 = 2A6 immunoprecipitate from adult homogenate.
Track 2 = 2A6 immunoprecipitate from larvated egg homogenate.
Track 3 = 2A6 immunoprecipitate from $L_1$ homogenate.
Track 4 = 2A6 immunoprecipitate from $L_3$ homogenate.

Arrows H and L indicate heavy and light immunoglobulin chains and arrow A indicates immunoprecipitated antigen.

**C.** Duplicate gel, to that used to produce the blot shown in figure B, analysed by fluorography. 4 weeks exposure.

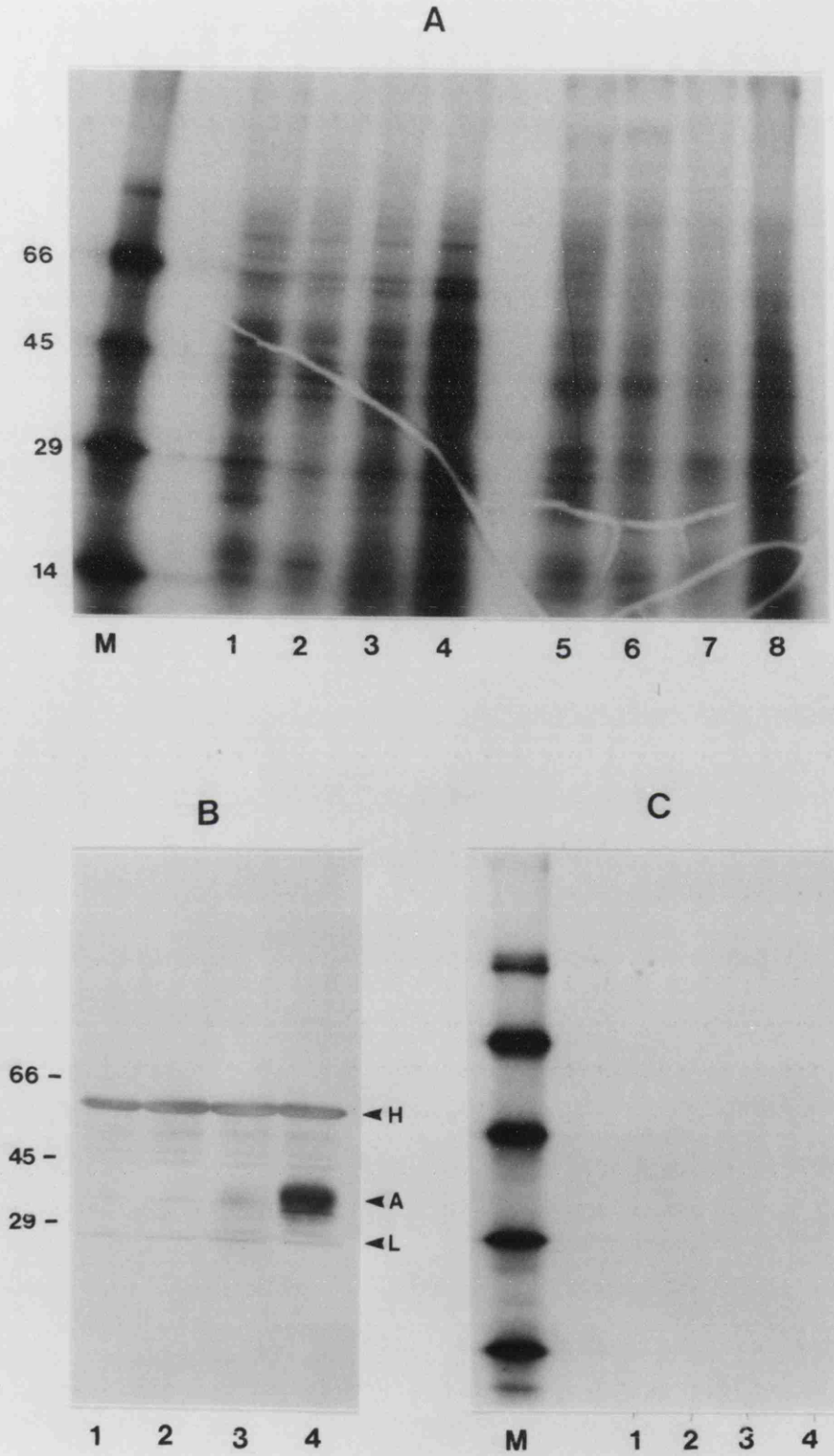

A
66
45
29
14
M
1
2
3
4
5
6
7
8
B
66 –
45 –
29 –
H
A
L
1
2
3
4
C
M
1
2
3
4

**Figure 5.20**

**A.** Fluorograph of SDS-PAGE gel of homogenates prepared from different stages of *D.viviparus* following metabolic labelling with $^{3}$H-glucosamine. The same number of counts per minute were loaded into each track except for $L_1$ for which half the counts per minute were loaded due to shortage of material. Tracks 1-4 contain aqueous soluble homogenates whereas Tracks 2-8 contain SDS/2ME/urea soluble homogenates.

Tracks 1 & 5 = Adult homogenate.
Tracks 2 & 6 = Larvated egg homogenate.
Tracks 3 & 7 = $L_1$ homogenate.
Tracks 4 & 8 = Sheathed $L_3$ homogenate.

**B.** Western blot of antigen, immunoprecipitated by 2A6/Protein-A sepharose beads from $^{3}$H-glucosamine labelled aqueous soluble homogenate, probed with 2A6.

Track 1 = 2A6 immunoprecipitate from adult homogenate.
Track 2 = 2A6 immunoprecipitate from larvated egg homogenate.
Track 3 = 2A6 immunoprecipitate from $L_1$ homogenate.
Track 4 = 2A6 immunoprecipitate from $L_3$ homogenate.

Arrows H and L indicate heavy and light immunoglobulin chains and arrow A indicates immunoprecipitated antigen.

**C.** Duplicate gel, to that used to produce the blot shown in figure B, analysed by fluorography. 3 weeks exposure.

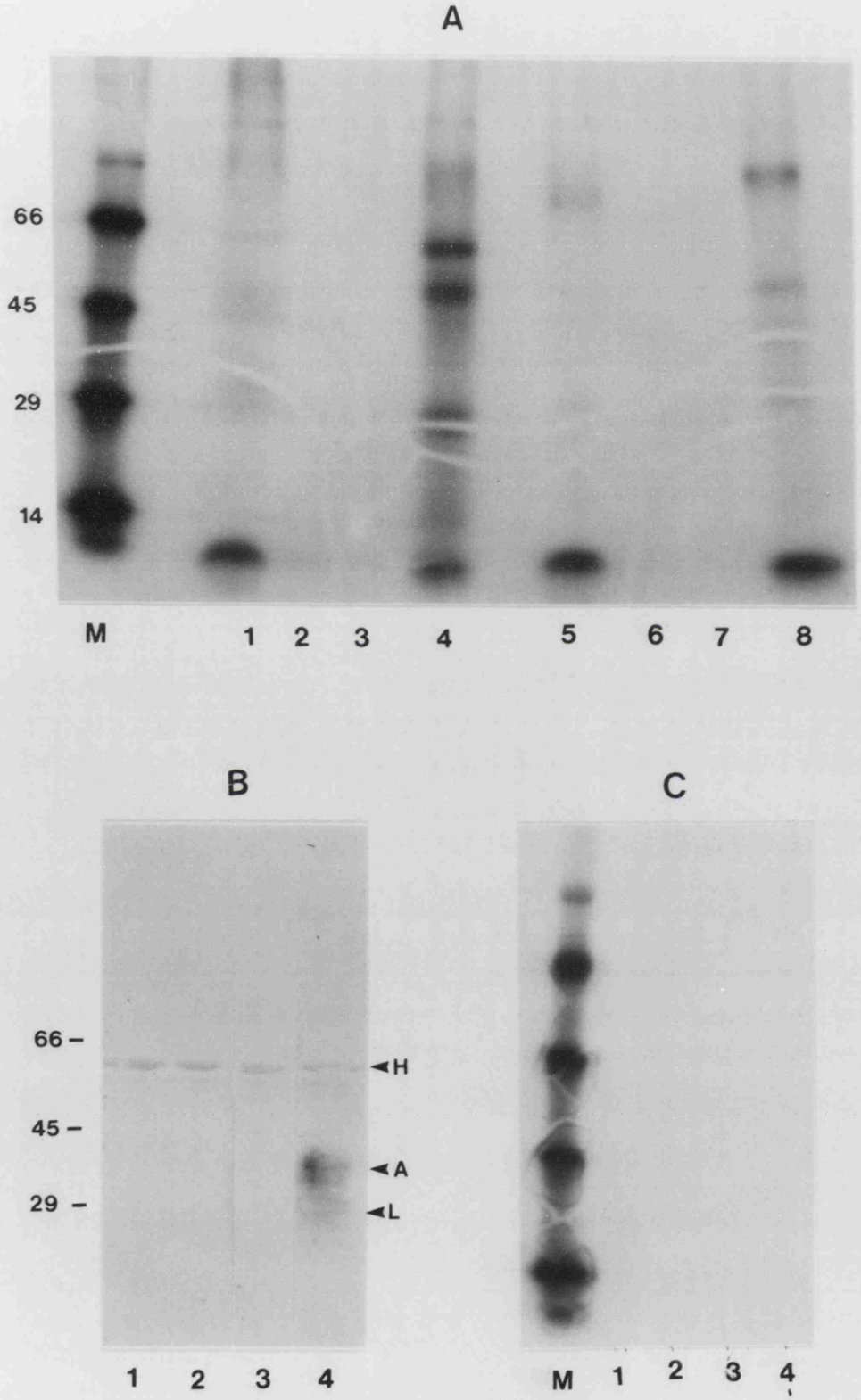

A
66
45
29
14
M
1
2
3
4
5
6
7
8
B
66 –
45 –
29 –
◄H
◄A
◄L
1
2
3
4
C
M
1
2
3
4

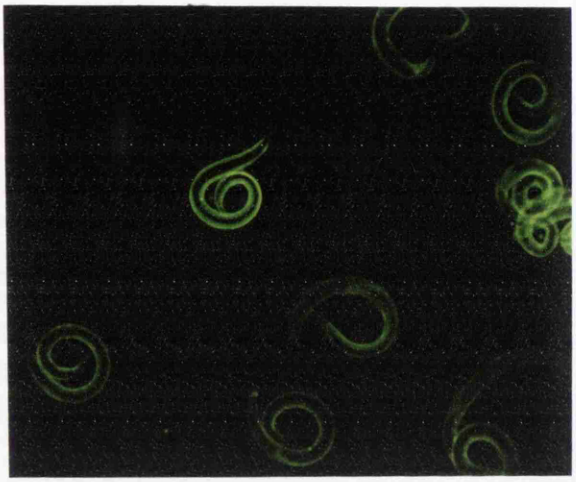
**Figure 5.21**

**A.** IFA of methanol/acetone fixed sheathed *D.viviparus* $L_3$ with 2A6 supernatant. x100 magnification.

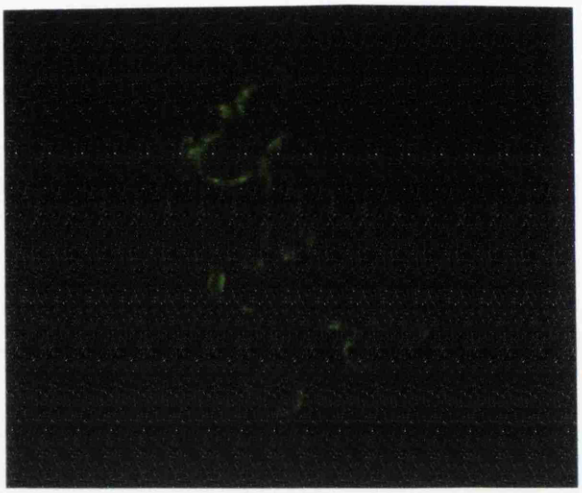
**B.** IFA of methanol/acetone fixed exsheathed *D.viviparus* $L_3$ with 2A6 supernatant. x100 magnification.

**C & D.** IFA of methanol/acetone fixed exsheathed *D.viviparus* $L_3$ with 2A6 supernatant. x1000 magnification.

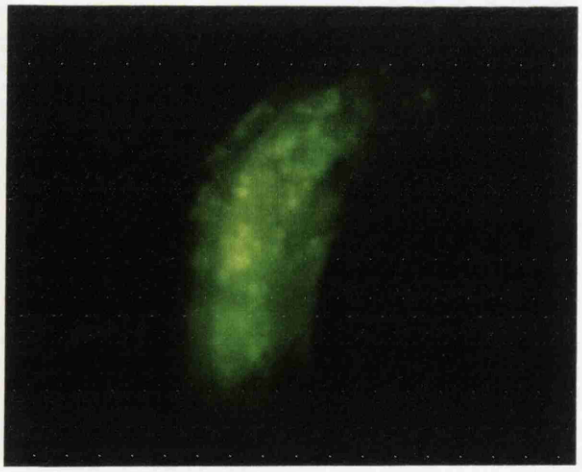
A



B



C



D

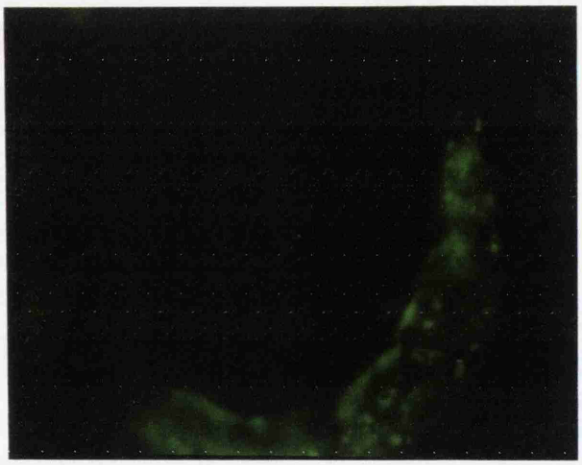


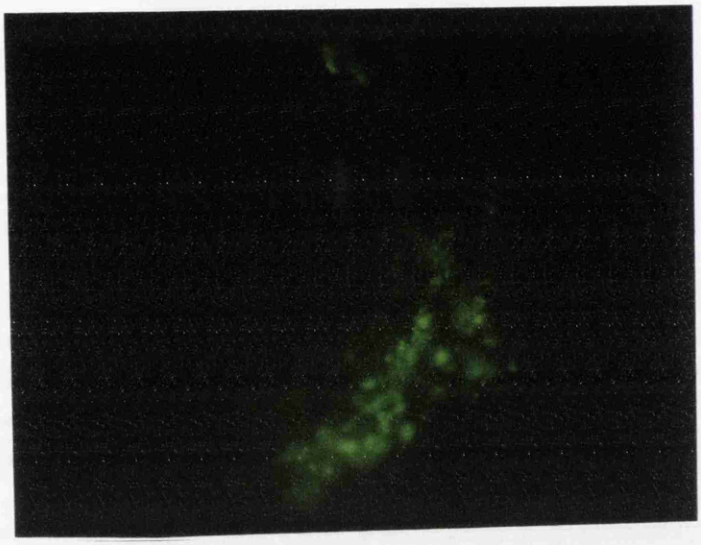
**Figure 5.22**

**A.** IFA of methanol/acetone fixed exsheathed *H.contortus* $L_3$ with 2A6 supernatant. x1000 magnification.

**B.** Western blot of homogenates of *H.contortus* infective larvae and sheaths probed with 2A6.

Track 1 = *D.viviparus* sheathed $L_3$ aqueous homogenate.
Track 2 = *H.contortus* sheathed $L_3$ aqueous homogenate.
Track 3 = *H.contortus* exsheathed $L_3$ aqueous homogenate.
Track 4 = *H.contortus* sheaths aqueous homogenate.
Track 5 = *H.contortus* sheaths SDS/2ME/urea homogenate.

A



B

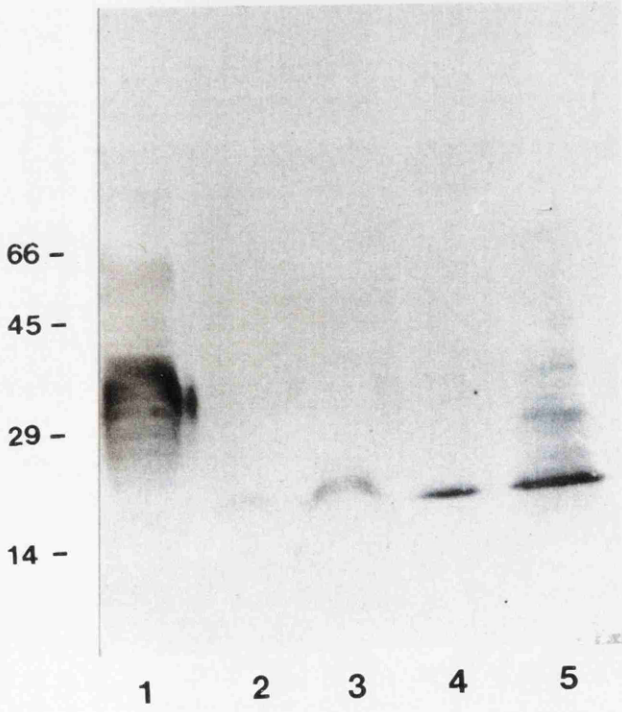


**Figure 5.23**

**A.** Transmission electron micrograph of a sheathed *D.viviparus* $L_3$ following pre-embedding protein-A immunogold labelling with 5E1 (negative control). x20,000 magnification.

S = $L_3$ sheath
C = $L_3$ cuticle
M = Somatic musculature

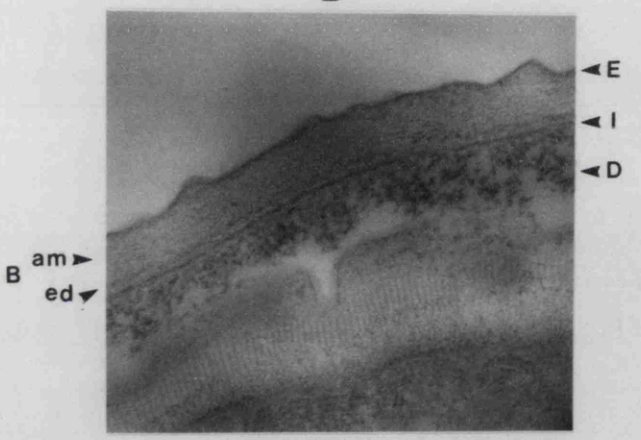
**B.** Transmission electron micrograph of a sheathed *D.viviparus* $L_3$ following pre-embedding protein-A immunogold labelling with 5E1 (negative control). x30,000 magnification.

E = Epicuticle
B = Basal layer: am = amorphous region
ed = electron dense region.
I = Inner electron dense layer
D = Debris between sheath and cuticle.

**C.** Transmission electron micrograph of a sheathed *D.viviparus* $L_3$ following pre-embedding protein-A immunogold labelling with 2A6. x30,000 magnification.

A


B




C

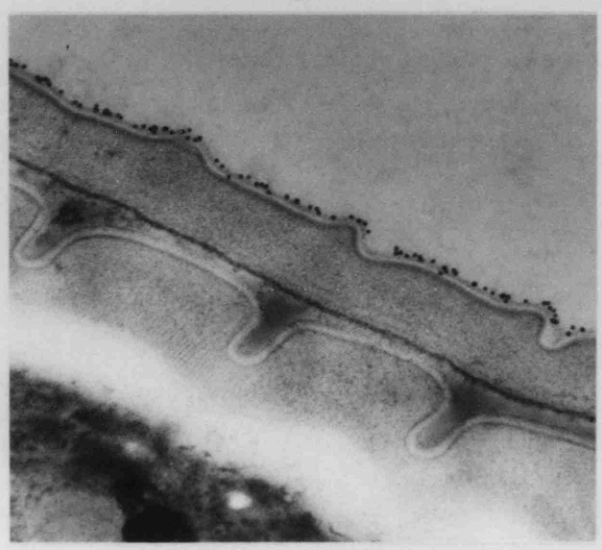
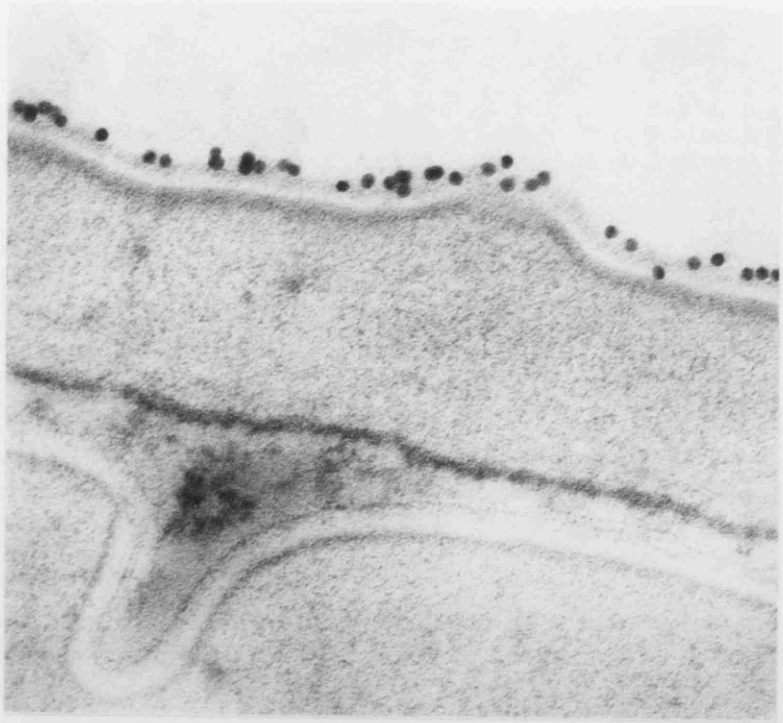


**Figure 5.24**

**A.** Transmission electron micrograph of a sheathed *D.viviparus* $L_3$ following pre-embedding protein-A immunogold labelling with 2A6. x85,000 magnification.

**B.** Transmission electron micrograph of a sheathed *D.viviparus* $L_3$ following pre-embedding protein-A immunogold labelling with 2A6. x140,000 magnification.

A



B

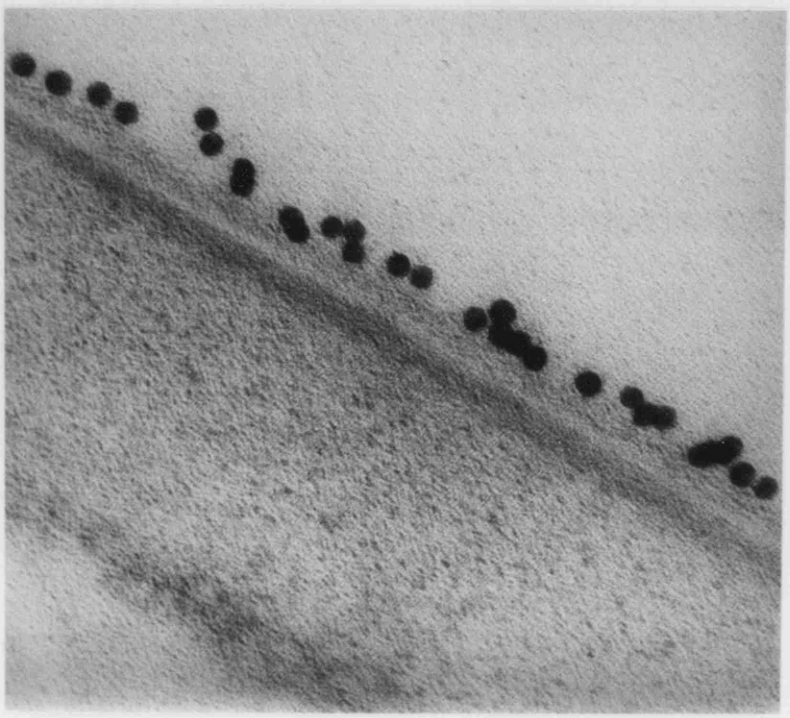


**Figure 5.25**

**A.** Staining of SDS-PAGE gels of *D.viviparus* $L_3$ homogenate. Tracks 1, 3 & 5 are SDS/2ME/urea homogenate and Tracks 2, 4 & 6 are aqueous homogenate.

Tracks 1 & 2 = Coomassie stained $L_3$ homogenate.
Tracks 3 & 4 = Silver stained $L_3$ homogenate.
Tracks 5 & 6 = PAS stained $L_3$ homogenate.

**B.** Western blots of 2A6 immunoprecipitated antigen. Both tracks on the figure are of the same lane on the blot but Track 1 shows the lane stained with Ponceau-S and Track 2 shows the lane probed with 2A6.

**C.** Western blot of *D.viviparus* $L_3$ homogenate following chloroform/methanol extraction stained with Ponceau-S.

Track 1 = Remaining aqueous soluble antigens following chloroform/methanol extraction.
Track 2 = First chloroform/methanol extract.
Track 3 = Second chloroform/methanol extract.
Track 4 = Remaining SDS/2ME/urea soluble antigens following chloroform/methanol extraction.
Track 5 = First chloroform/methanol extract.
Track 6 = Second chloroform/methanol extract.

**D.** The same Western blot as in figure C probed with 2A6.

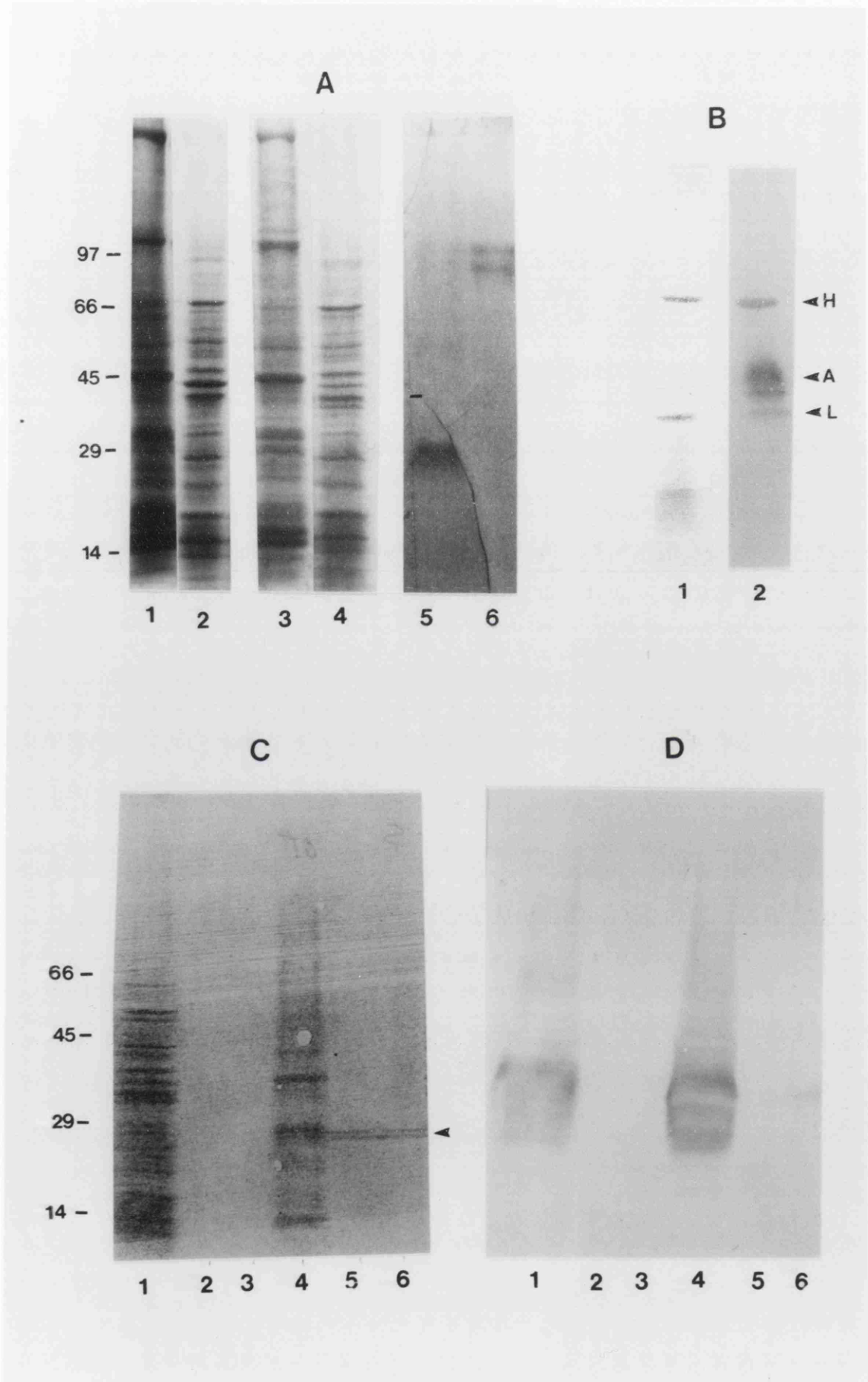

A
97 –
66 –
45 –
29 –
14 –
1 2 3 4 5 6
B
H
A
L
1 2
C
66 –
45 –
29 –
14 –
1 2 3 4 5 6
D
1 2 3 4 5 6

**Figure 5.26**

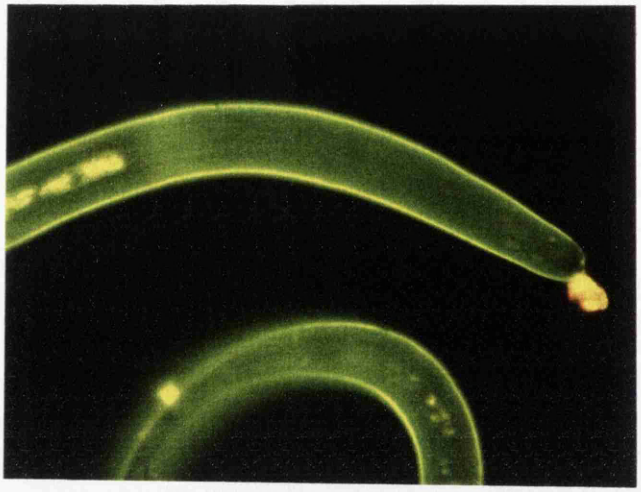
**A.** IFA performed on *T.canis* $L_2$ with monoclonal antibody Tcn-2 after incubation in sodium acetate buffer.

**B.** IFA performed on *T.canis* $L_2$ with Tcn-2 after incubation 10mM sodium periodate

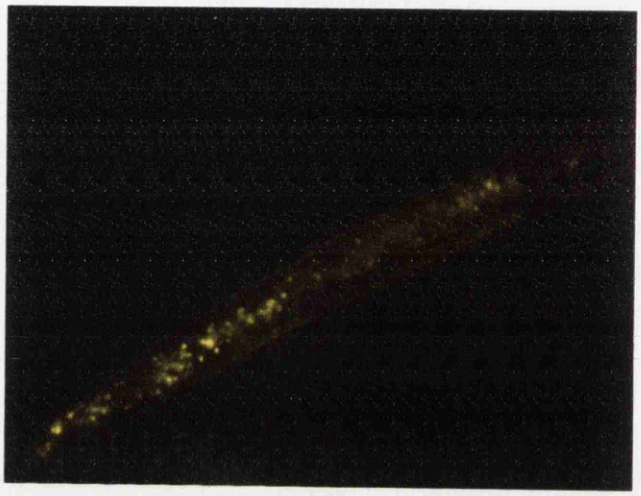
**C.** IFA performed on sheathed *D.viviparus* $L_3$ with 2A6 following incubation in sodium acetate buffer.

**B.** IFA performed on sheathed *D.viviparus* $L_3$ with 2A6 following incubation in 10mM sodium periodate.

A



B



C

D

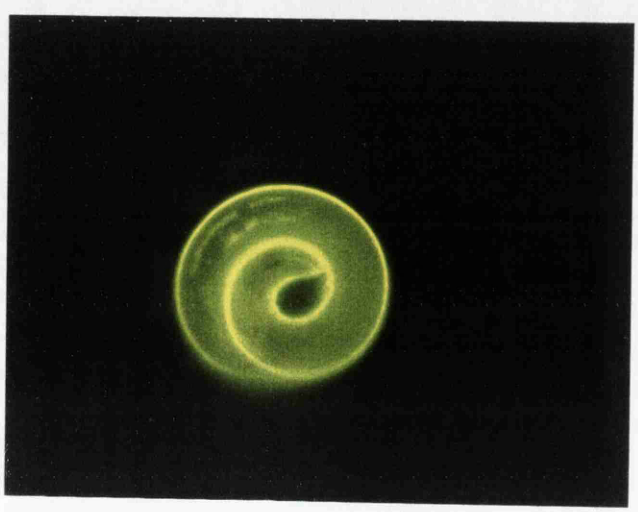


**Figure 5.27**

**A.** Western blot of digoxigenin hydrazide labelled antigen-2A6-protein A complexes probed with anti-digoxigenin antibody or 2A6. *D.viviparus* $L_3$ homogenate was immunoprecipitated with 2A6 and protein-A sepharose beads which were then labelled in suspension with digoxigenin hydrazide. All samples were split and analysed on duplicate Western blots, one of which was probed with 2A6 to demonstrate the presence of antigen (Tracks 6, 7 & 8) and the other with anti-digoxigenin antibody peroxidase conjugate to detect digoxigenin labelling (Tracks 3, 4 & 5).

Track 1 = Transferrin probed with anti-digoxigenin antibody (positive control).
Track 2 = Recombinant creatinase probed with anti-digoxigenin antibody (negative control).
Tracks 3 and 6 = Digoxigenin hydrazide labelled antigen-2A6-protein A complexes.
Tracks 4 and 7 = Digoxigenin hydrazide labelled 2A6-protein A complexes.
Tracks 5 and 8 = Unlabelled 2A6-protein A complexes.

Tracks 3-5 were probed with anti-digoxigenin peroxidase conjugate whereas Tracks 6-8 are the duplicate blot probed with 2A6.
The arrow marked "?" indicates a very faint band present in track 3 (antigen present) which is not in track 4 (antigen absent). The arrows marked H and L indicate the immunoglobulin heavy and light chains respectively.

**B.** Western blot of PNGase treated samples of *D.viviparus* $L_3$ homogenate probed with 2A6.

Tracks 1-4 probed with 2A6.
Track 1 = $L_3$ homogenate treated with 50 units/ml PNGase.
Track 2 = $L_3$ homogenate treated with 10 units/ml PNGase.
Track 3 = $L_3$ homogenate treated with 1 unit/ml PNGase.
Track 4 = $L_3$ homogenate treated with buffer alone.

Tracks 5 & 6 stained with Ponceau-s.
Track 5 = fetuin treated with 10 units/ml.
Track 6 = fetuin treated with buffer alone.

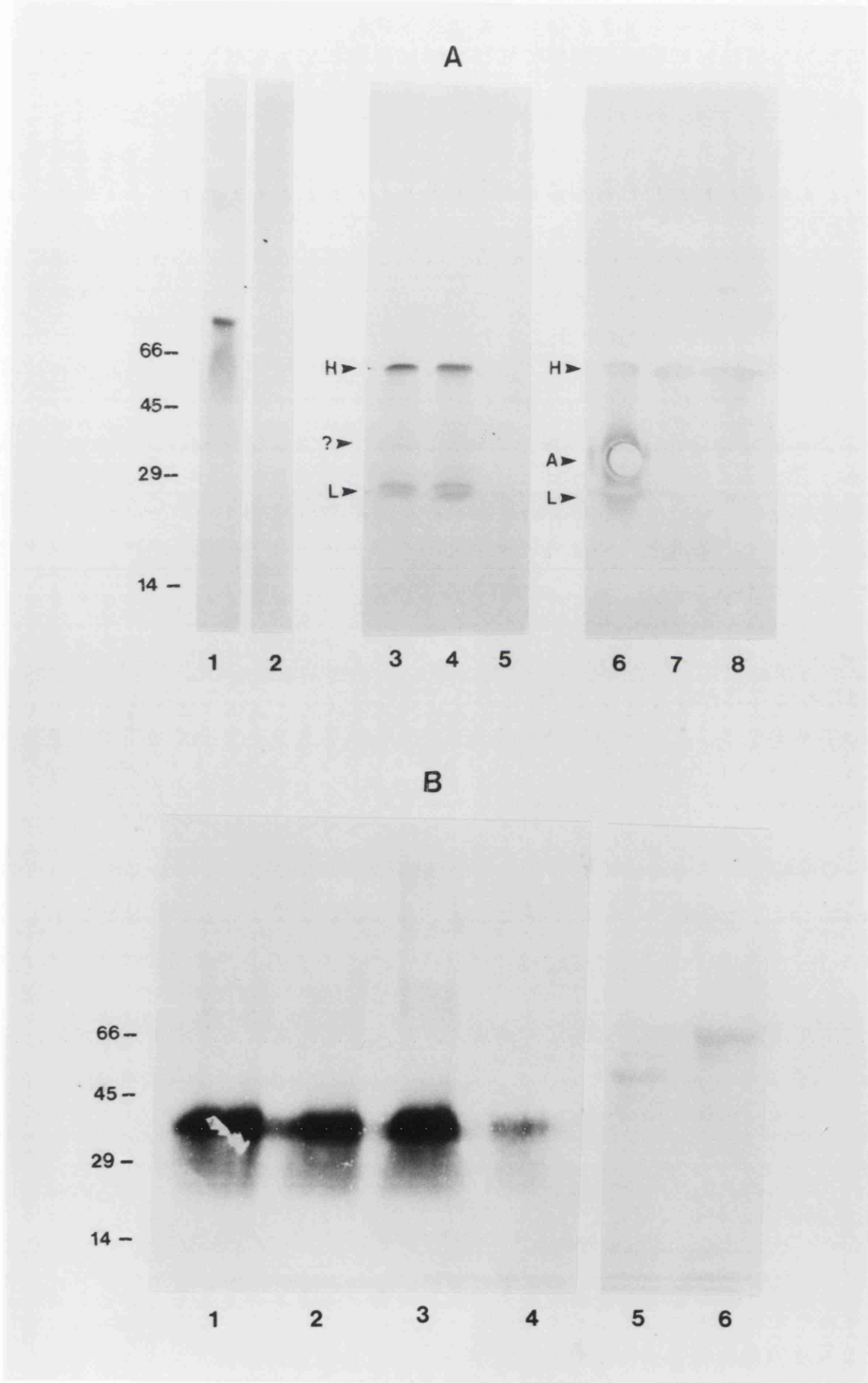

A
66–
45–
29–
14 –
H ►
? ►
L ►
H ►
A ►
L ►
1 2 3 4 5 6 7 8
B
66 –
45 –
29 –
14 –
1 2 3 4 5 6

**Figure 5.28**

**A.** Western blot of protease digested *D.viviparus* $L_3$ homogenate probed with 2A6 supernatant. *D.viviparus* $L_3$ homogenate was incubated with pronase or trypsin at varying concentrations at 37°C for 2 hours.

Track 1 = Incubated with protease buffer alone.
Track 2 = Incubated with 1mg/ml Pronase.
Track 3 = Incubated with 2mg/ml Pronase.
Track 4 = Incubated with 3mg/ml Pronase.
Track 5 = Incubated with protease buffer alone.
Track 6 = Incubated with 1mg/ml trypsin.
Track 7 = Incubated with 2mg/ml trypsin.
Track 8 = Incubated with 3mg/ml trypsin.

**B.** Western blot of trypsin and papain digested *D.viviparus* $L_3$ homogenate probed with 2A6.

Track 1 = Incubated with 2mg/ml trypsin.
Track 2 = Incubated in 2mg/ml papain.
Track 3 = Incubated in protease buffer alone.

**C.** Western blot of pronase and trypsin treated *H.contortus* $L_3$ homogenate probed with 2A6.

Track 1 = Incubated with 2mg/ml pronase.
Track 2 = Incubated with 2mg/ml trypsin.
Track 3 = Incubated with protease buffer alone.

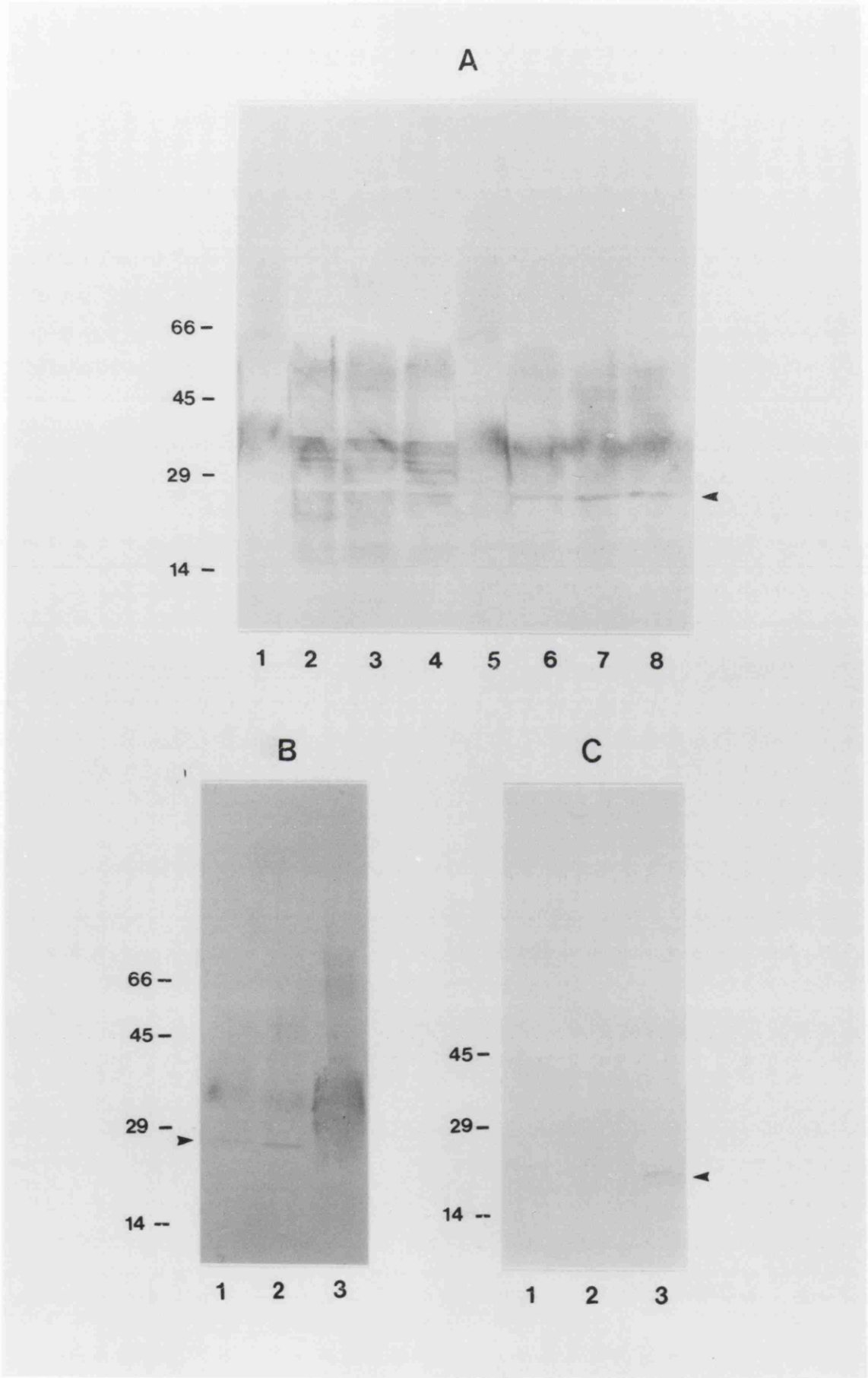

A
66 –
45 –
29 –
14 –
1 2 3 4 5 6 7 8
B
66 –
45 –
29 –
14 –
1 2 3
C
45 –
29 –
14 –
1 2 3

**Figure 5.29**

**A.** Western blot of NHS-biotin labelled antigen-2A6-protein A complexes probed with streptavidin peroxidase or 2A6. *D.viviparus* $L_3$ homogenate was immunoprecipitated with 2A6 and protein-A sepharose beads which were then labelled in suspension with NHS-biotin. All samples were split and analysed on duplicate Western blots, one of which was probed with 2A6 to demonstrate the presence of antigen (Tracks 5-8) and the other with streptavidin peroxidase conjugate to detect NHS-Biotin labelling (Tracks 1-4).

Tracks 1 & 5 = Unlabelled 2A6-protein A sepharose bead complexes.
Tracks 2 & 6 = Biotin labelled protein A sepharose beads.
Track 3 & 7 = Biotin labelled 2A6-protein A sepharose bead complexes.
Track 4 & 8 = Biotin labelled antigen-2A6-protein A sepharose bead complexes.

Tracks 1-4 are probed with streptavidin peroxidase to detect biotin labelling and tracks 5-8 are a duplicate blot probed with 2A6 to test for the presence of antigen. The arrow marked A indicates the immunoprecipitated 2A6 antigen and the arrows marked H and L indicate the immunoglobulin heavy and light chains respectively.

**B.** Capillary blots of isoelectric focussing gels probed with 2A6 and 5E1 supernatants.

Tracks 1-3 probed with 5E1 (negative control).
Track 1 = *T.colubriformis* $L_3$ homogenate.
Track 2 = *H.contortus* $L_3$ homogenate.
Track 3 = *D.viviparus* $L_3$ homogenate.

Tracks 4-8 probed with 2A6.
Track 4 = *T.colubriformis* $L_3$ homogenate.
Track 5 = *H.contortus* $L_3$ homogenate.
Track 6 = *D.viviparus* $L_3$ homogenate.
Track 7 = *C.oncophora* $L_3$ homogenate.
Track 8 = *D.viviparus* $L_3$ homogenate.

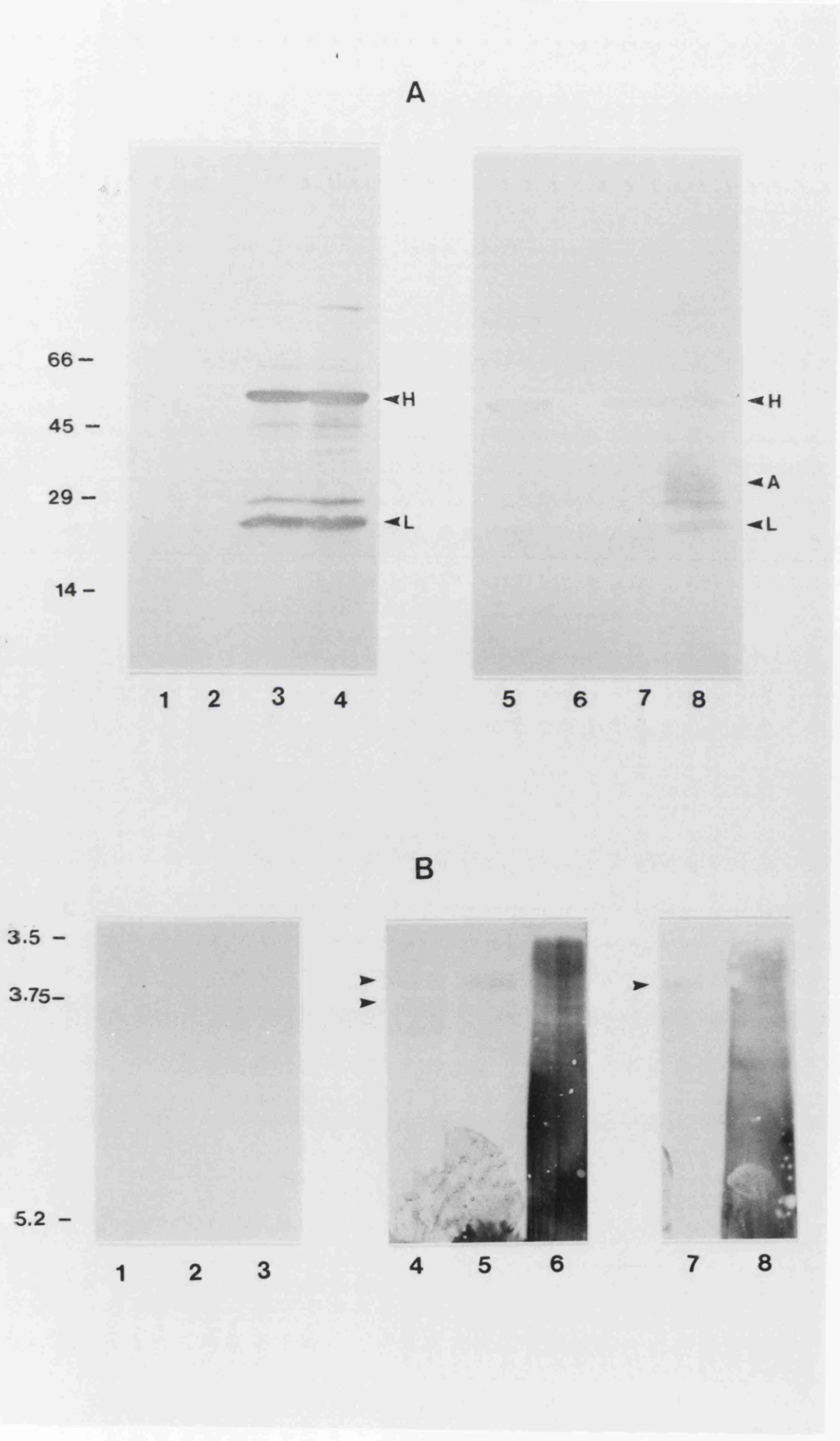

A
66 –
45 –
29 –
14 –
◄H
◄L
◄H
◄A
◄L
1 2 3 4
5 6 7 8
B
3.5 –
3.75–
5.2 –
1 2 3
4 5 6
7 8

**Figure 5.30**

**A.** Dot blot of 5ul aliquots of eluted fractions (running in sequence from left to right) from 2A6 immunoaffinity column probed with mab 2A6.

Rows 1-3 = Eluted with 100mM glycine, pH 2.5
Rows 4-5 = Eluted with 100mM triethylamine, pH 11.5

A duplicate dot blot was probed with complete medium as a negative control and no reaction was produced with any of the fractions.

**B.** Dot blot of 5ul aliquots of fractions (running in sequence from left to right) from 2A6 immunoaffinity column, eluted by triethylamine/0.5% sodium deoxycholate pH 11.5, probed with 2A6. A duplicate dot blot was probed with complete medium as a negative control and no reaction was produced with any of the fractions.

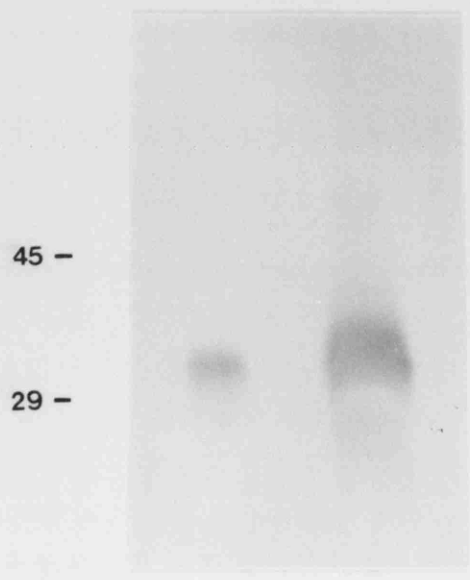
**C.** Western blot probed with mab 2A6.

Track 1 = Aliquot of pooled fractions 1-4 shown in figure B.
Track 2 = *D.viviparus* $L_3$ homogenate.

A


B


C



# Chapter 6

# CHAPTER 6

## Development of *D.viviparus* $L_3$ during *in vitro* culture and an attempt to clone the polypeptide component of the 2A6 antigen.

### 6.1. Introduction.

Both the $L_3$ and $L_4$ stages of *D.viviparus* may be important targets of the protective bovine immune response (Jarrett et al 1957b, Poynter et al 1960 and Jarrett & Sharp 1963). Therefore a thorough investigation of the antigens of both stages is desirable, however the production of sufficient numbers of $L_4$ for analysis is difficult. *In vivo* methods involve the infection of calves with large numbers of larvae followed, 14 days later, by slaughter and harvesting of $L_4$ from the lungs by Baermannisation (Baermann 1917) or the Inderbitzen technique (Inderbitzen 1976). This method of $L_4$ production is labour intensive and only relatively small numbers of larvae (usually several thousand per calf) can be produced in this manner. Therefore an attempt was made to culture *D.viviparus* $L_3$ *in vitro*, in order to determine whether any development would occur and to investigate whether any new antigens, recognised by immune bovine serum, would be produced. Since the $L_3$ can be obtained in large quantities, *in vitro* culture of $L_3$ might provide a source of immunogenic $L_4$ antigens.

The work presented in the previous chapter has revealed a number of fundamental problems in the immunochemical analysis of the 2A6 molecule, as well as problems in purifying sufficient amounts for analysis. Since the molecule is susceptible to degradation by proteolytic enzymes it must have a polypeptide component and if the gene encoding this polypeptide could be cloned, it might allow a number of aspects of the molecule to be studied which have proved to be refractory to immunochemical analysis. Sequence analysis of the gene would provide information about the structure of the molecule which may help to explain some of its immunochemical properties and may even suggest possible functions. Also the presence or absence of glycosylation sites might confirm or refute the immunochemical evidence for the lack of glycosylation of the molecule. Cloning of the gene would also allow its expression to be studied which is potentially interesting given the presence of the molecule in different sites and stages of the parasite.

The epitope detected by 2A6 is periodate insensitive and so is unlikely to consist of carbohydrate (Eylar & Jeanloz 1962 and Maizels et al 1987b) although it must be remembered that periodate insensitive carbohydrate epitopes do exist

(Woodward et al 1985). The epitope is also detected following Western blotting onto nitrocellulose filters and so is not a conformational epitope dependant on the tertiary structure of the polypeptide. Therefore it was considered that, if a cDNA expression library could be made from a parasite stage producing the 2A6 molecule, there was a good chance that mab 2A6 would detect the recombinant polypeptide. Unfortunately, experiments involving the metabolic labelling of *in vitro* cultured $L_3$ and immunoprecipitation of labelled antigens with mab 2A6 failed to detect synthesis of the molecule in this stage and it was also shown that the 2A6 molecule becomes less abundant during *in vitro* culture of $L_3$. However, the inability to demonstrate the synthesis of the molecule during *in vitro* culture does not necessarily mean that mRNA for the polypeptide component of the 2A6 molecule is not present in the $L_3$. Also it is possible that the radiolabelled amino acids used for metabolic labelling are absent or poorly represented in the polypeptide. Furthermore the labelling techniques may be insufficiently sensitive to detect the molecule if there is only a low level of synthesis during *in vitro* culture of $L_3$.

In spite of the disappointing metabolic labelling results, it was decided to construct a *D.viviparus* $L_3$ cDNA library in an attempt to clone the polypeptide component of the 2A6 antigen, since such a library should also provide a source of recombinant $L_3$ polypeptides which could be screened with appropriate antisera. A variety of sera from infected and vaccinated cattle is already available for this purpose (Canto 1990) and Britton (1991) has also produced an antiserum to adult E/S products which also detects molecules in the $L_3$ stage.

## 6.2. Results.

### 6.2.1. Comparison of sheathed and exsheathed $L_3$ polypeptides and antigens.

The polypeptide profiles of parasite homogenates prepared from sheathed $L_3$ or from exsheathed $L_3$ (immediately after exsheathment in 0.01% sodium hypochlorite or after storage of such larvae at 4°C for several days) were indistinguishable on Coomassie blue stained SDS-PAGE gels. These homogenates were also probed by Western blotting with 2A6 supernatant and pooled B7, B8 & B9 serum (cattle immunised by oral infection) and there were no discernible differences in the antigens detected between the sheathed and exsheathed $L_3$ (data not shown). Therefore, neither polypeptides or antigens exclusive to the $L_3$ sheath could be

detected by these methods and there appeared to be no new polypeptides or antigens expressed by larvae following exsheathment.

### 6.2.2. *In vitro* culture of sheathed and exsheathed $L_3$.

*D.viviparus* $L_3$ were exsheathed in 0.01% sodium hypochlorite and then incubated in RPMI + 10% foetal calf serum at 37°C and 5% $CO_2$. After approximately 2 hours larval motility changed from the coiled motionless appearance of infective larvae to a vigorous sinusoidal pattern. After 24 hours, the $L_3$ cuticle of some larvae appeared to have separated from the underlying surface and by 48 hours all the larvae had this appearance. No further development appeared to occur after this time and there was no apparent increase in larval size. Approximately 80% of the larvae were still alive after 4 days but only 60% after 10 days. All the surviving larvae remained highly motile but none had managed to shed the separated $L_3$ cuticle. However a few of the dead larvae did appear to have lost the separated $L_3$ cuticle and a small number of empty cuticles were visible in the culture medium from approximately four days onwards. Therefore although full development to the $L_4$ did not occur, the separation of the $L_3$ cuticle suggests that the $L_4$ cuticle had been produced and so some development had been induced by the culture conditions.

In order examine whether this development was dependant upon the prior exsheathment of larvae, sheathed $L_3$ were cultured under the same conditions. Precisely the same pattern of development appeared to occur and the $L_3$ sheath remained intact giving the cultured larvae a double sheathed appearance once the $L_3$ cuticle had separated from the underlying larvae. Again none of the living larvae appeared to have escaped from the two retained cuticles. This lack of visible difference between the development of sheathed and exsheathed $L_3$ suggests that exsheathment is not a prerequisite for the developmental changes to occur. The protein synthesis of the sheathed and exsheathed cultured larvae was compared by metabolic labelling with $^{35}S$-methionine and a $^{3}H$-amino acid mixture (Leucine, Lysine, Phenylalanine, Proline and Tyrosine) and there were no major differences in the synthesis of proteins detected by one dimensional SDS-PAGE and fluorography (figure 6.1). This supports the conclusion drawn from visual examination that the *in vitro* development of sheathed and exsheathed $L_3$ appears to be the same which implies that the stimulus for this development is not dependant upon exsheathment.

### 6.2.3. Polypeptides and antigens expressed during culture of the $L_3$.

Homogenates were prepared from $L_3$ immediately after exsheathment and from exsheathed $L_3$ cultured for 4 and 10 days in order to investigate whether any new proteins were produced by the cultured larvae. These were compared on SDS-PAGE gels by Coomassie blue staining and several changes were observed which were seen on a number of repeated gels using these preparations and also using homogenate from a separately prepared batch of cultured larvae. In the aqueous homogenate, a polypeptide at 65 kDa became more prominent and a polypeptide at 67kDa appeared to become less prominent in the cultured larvae (figure 6.2A). In the SDS/2ME/urea homogenate a band at 17 kDa become more prominent. Several other minor changes were apparent but these were not as reproducible.

These homogenates were also Western blotted and probed with a number of different types of *D.viviparus* hyperimmune bovine sera. Hyperimmune serum produced by repeated oral infection (pooled B7, B8 and B9 sera) detected three new antigens in the aqueous homogenates of cultured larvae; a very prominent band at approximately 65 kDa, a finer band at 50 kDa and a slightly curved band at 23kDa (figure 6.2B). It is possible that the new 65kDa antigen corresponds to the 65kDa polypeptide detected on the Coomassie stained gels of cultured larvae. There was also a new, and highly immunogenic, low molecular weight antigen in the SDS/2ME/urea homogenates of cultured larvae. The 29-40 kDa antigen (2A6 antigen) became much less prominent in the blots of the cultured larvae. There were no discernible differences between the antigens recognised in 4 day and 10 day cultured larvae. All the new antigens recognised by the hyperimmune bovine infection serum were also detected on Western blots by hyperimmune sera produced by repeated oral immunisation with 40krad irradiated larvae (pooled 038 and 039 sera) (figure 6.3A). However when similar blots were probed with hyperimmune serum produced by repeated intravenous immunisation with 100krad irradiated larvae (pooled G17 and B45 sera) the only new antigens detected in the cultured larvae were the low molecular weight 2ME soluble antigen and the 23kDa water soluble band (figure 6.4B). The disappearance of the 2A6 antigen in cultured larvae is again confirmed on the blots probed with both the 40 kRad and 100 kRad sera.

The blots of homogenates from exsheathed cultured larvae were also probed with rabbit antiserum raised to adult *D.viviparus* E/S products. There were no new antigens present in the cultured larvae which were detected by this serum. There were three clear bands recognised in all the $L_3$ aqueous homogenates, 30kDa, 23kDa and 17kDa, with a fainter doublet seen at approximately 14kDa (figure 6.4A). These were all present in the aqueous soluble $L_3$ antigens but absent from the

SDS/2ME/Urea soluble homogenates and were all detected on blots of adult homogenate probed with the same serum (figure 6.7). Since one would expect ES antigens to be soluble in aqueous solution, it is possible that these represent molecules present in the $L_3$ which are also present and secreted by adult parasites, although further experiments are required to investigate this possibilty. In contrast, the higher molecular weight molecules detected by this serum are present in both the SDS/2ME/urea soluble and aqueous soluble fractions which would not be expected for secreted molecules. Therefore it is more likely that the binding of the anti-adult E/S serum to these antigens represents cross-reactivity between epitopes in $L_3$ homogenate and adult E/S.

Pooled sera from mice immunised by infection with *D.viviparus* $L_3$ or by vaccination with $L_3$ homogenate did not recognise any new antigens when used to probe blots of cultured larvae (data not shown). If, as suggested in chapter 3, the $L_3$ does not appear to undergo any development in the mouse, sera from infected mice would only possess antibodies against $L_3$ antigens. This is also true of sera taken from mice immunised with $L_3$ homogenate. Therefore it is entirely understandable that these sera do not detect the new antigens and indeed these results support the conclusion that the $L_3$ undergoes no development following infection of the mouse.

### 6.2.4. Comparison of RNA extracted from sheathed, exsheathed and cultured $L_3$.

Preparation of large quantities of mRNA from the infective stage of trichostrongylid nematodes can be difficult and this has been ascribed to the quiescent nature of this stage (Dawkins & Spencer 1989 and Keith, personal communication). Therefore a number of experiments were performed to examine whether exsheathment or *in vitro* culture of larvae affected the yield and nature of the RNA which could be extracted.

*D.viviparus* $L_3$ were exsheathed and then incubated in PBS at 37°C in 5% $CO_2$ for 4 hours in order to allow time for any stimulus to transcription to occur. The RNA extracted from 2 million of these larvae was compared to that extracted from the same number of sheathed $L_3$. Approximately 47.5ug of total RNA ($OD_{260}/OD_{280}$ = 1.871) was obtained from the sheathed $L_3$ compared with only 29.5ug ($OD_{260}/OD_{280}$ = 1.915) for the exsheathed $L_3$. These samples were also examined by formaldehyde-agarose gel electrophoresis. The total RNA extracted from each sample of 2 million larvae was dissolved in the same volume of $dH_20$ and an aliquot of each sample run on a formaldehyde agarose gel. The two ribosomal RNA bands were clearly

visible in both samples suggesting the RNA was largely undegraded and the relative intensity of staining of each track is consistent with a greater yield of RNA being produced from the sheathed larvae (figure 6.4B). Therefore it appears that exsheathment and preincubation of larvae in PBS did not improve the yield of RNA. Attempts to *in vitro* translate both these samples of total RNA using rabbit reticulocyte lysate did not produce any visible products and it was found that the presence of these *D.viviparus* total RNA preparations inhibited the translation of the Brome mosaic virus mRNA used as a positive control. Therefore it is likely that there were impurities present in the total RNA that inhibit *in vitro* translation.

Five million exsheathed $L_3$ were cultured in RPMI + 10% foetal calf serum at 37°C in 5% $CO_2$ for 5 days and total RNA prepared. The yield was 459ug ($OD_{260}/OD_{280}$ = 1.963) and yield of poly (A)+ RNA was 9ug. The amount of total RNA obtained from 5 million uncultured sheathed $L_3$ processed at the same time was 774ug ($OD_{260}/OD_{280}$ = 2.021) and this yielded 14ug of poly (A)+ RNA. Therefore the yields of total RNA and poly (A)+ RNA were not increased by *in vitro* culture of the larvae.

*In vitro* translations were performed with aliquots of these poly (A)+ RNA samples from the sheathed (not cultured) and cultured larvae as well as with poly (A)+ RNA prepared from adult parasites and the translation products separated by SDS-PAGE and analysed by fluorography. There was a particularly prominent band at approximately 80 kDa in the sheathed $L_3$ translation products which was absent (or much less prominent) from the cultured $L_3$ translation products (figure 6.5A). Other than this the translation products of RNA from cultured and uncultured $L_3$ were broadly similar whereas there were a number of obvious differences between the adult and larval translation products. However two dimensional SDS-PAGE analysis of these samples would be necessary to accurately compare the *in vitro* translation products from cultured larvae with those from non-cultured larvae.

No antigens could be immunoprecipitated from the *in vitro* translation products of RNA from either cultured or non-cultured larvae with pooled B7, B8 and B9 sera, O38 and O39 sera or 2A6 supernatant. Western blots of the translation products probed with these antibodies also failed to detect any antigens (data not shown). However it was difficult to interpret these results due to a lack of a positive control antibody for both the immunoprecipitations and Western blots of *D.viviparus in vitro* translation products.

### 6.2.5. Construction of a cDNA library from *D.viviparus* $L_3$.

7.9ug of the mRNA extracted from uncultured sheathed larvae was used to synthesize cDNA using a ZAP-cDNA synthesis kit (Stratagene). The total cDNA synthesized was size fractionated using a Sephacryl S-400 spun column (see materials and methods), ligated into 1ug of Uni-ZAP$^{TM}$ XR vector arms and the resulting lambda library packaged using the Gigapack II Gold packaging extract. The primary library produced consisted of $3.08x10^6$ pfu (compared to the background titre of $1.8x10^4$ pfu/ug of unligated arms). This was amplified to produce a library with a final titre of $7x10^9$ pfu/ml. The presence of inserts was determined by IPTG/X-gal colour selection and 92% of plaques were white and therefore contained inserts. In order to assess the average insert size, ten plaques were picked at random and preparations of phage DNA were double digested with Eco R1 and Xho 1. Agarose gels showed that 9 out of the 10 phage contained inserts (figure 6.5B) and their sizes in kB are as follows; 2.0, 2.7, 0.7, 2.2, 1.9, 0.6, 0.6, 2.6 and 1.2 + 0.6. These would code for polypeptides ranging in size from of approximately 20kDa to 90kDa and so represent a reasonable size range for the inserts.

### 6.2.6. Immuno-screening of the cDNA library.

$2x10^5$ pfu were screened with 2A6 supernatant and no positives were detected. $3x10^5$ pfu were then screened with a pool of the monoclonal antibody supernatants 2A6, 2F8, 2C6, 2D8, 2A3 and 1F2 supernatants but again no positives were found.

Hyperimmune *D.viviparus* bovine infection serum (pooled B7, B8, and B9 sera) at a dilution of 1:100 was used to screen $2x10^5$ pfu but again no positives were detected. In order to ensure that the failure to detect positive plaques was not due to technical problems a further $1x10^5$ pfu were screened with the pooled B7, B8 and B9 sera and $7x10^3$ pfu were also screened at the same time with pooled sera from mice immunised with $L_3$ homogenate and Freund's adjuvant. The bovine immune serum did not identify any positive plaques but nine out of the $7x10^3$ plaques screened with the mouse serum appeared to be clearly positive. When these nine plaques were picked, replated and rescreened, eight appeared to contain positive plaques. Stocks were made from single plaques and each clone was replated; half of the nitrocellulose filter from each plate was screened with the mouse serum against $L_3$ homogenate and the other half with normal (naive) mouse serum. All eight produced negligible background with the normal serum (figure 6.6).

Further screening of the library with immune bovine serum was attempted and one of the positive mouse serum clones identified above was included each time as a positive control. $2x10^5$ pfu of the library were screened with 038 and 039 sera (cattle immunised with 40kRad irradiated $L_3$) and $1x10^5$ pfu with pooled G17 and B45 sera (cattle immunised with intravenous 100kRad irradiated $L_3$) but no positive clones were identified. A final screen of $2x10^5$ pfu with 2A6 again identified no positive clones. Therefore no recombinant antigens recognised by *D.viviparus* immune bovine serum could be indentified in the library.

Positive clones were identified by screening the library with rabbit antiserum against adult E/S products (C. Britton, unpublished data). An antibody selection experiment with the anti-ES serum was performed on two of these clones and the antibodies selected by both clones recognised a single band at approximately 14 kDa when used to probe Western blots of *D.viviparus* $L_3$ homogenate (figure 6.7). This corresponds to the faint 14kDa doublet detected on such blots by the original anti-ES serum.

The recognition of clones by the anti-homogenate mouse serum demonstrates that the library successfully expresses *D.viviparus* $L_3$ cDNAs. In spite of this, and the fact that the titre of the unamplified library was relatively high, none of the bovine immune sera or the monoclonal antibodies used for screening detected any recombinant polypeptides expressed by the library.

### 6.2.7. Production of antisera against *H.contortus* $L_3$ sheath antigens.

It is possible that the polypeptide component of the 2A6 molecule is expressed in the library but the epitope recognised by 2A6 is not. If this were the case, a polyclonal antiserum against the 2A6 molecule might detect the recombinant polypeptide. Unfortunately the molecule has not yet been purified in sufficient amounts to produce such a polyclonal antiserum. However since the 2A6 molecule appears to be so immunogenic, an antiserum raised against purified sheaths might be a suitable alternative for screening the library. As discussed in Chapter 5 pure preparations of *D.viviparus* $L_3$ sheaths could not be produced and so a preparation of sheaths from 500,000 *H.contortus* $L_3$ was purified by Percoll gradient centrifugation and homogenised in 0.85% NaCl. A Balb/c mouse was immunised, by intraperitoneal injection, with this aqueous soluble extract and another mouse was immunised with the insoluble pellet of homogenised sheaths. Freund's complete adjuvant was used for the first immunisation and the immunisations were repeated 4 weeks later with Freund's incomplete adjuvant. The mice were euthanased and bled 10 days after the

second immunisation. IFA was performed with the sera from the two mice on both *H.contortus* and *D.viviparus* sheathed $L_3$ but produced only very slight fluorescence. The sera against the aqueous soluble sheath material reacted only very faintly on Western blots of *D.viviparus* $L_3$ homogenate and the sera against the insoluble sheath material recognised numerous bands above 60 kDa which were probably cuticular collagens (data not shown). Since neither of these sera appeared to recognise the 2A6 antigen, they were not suitable for use in screening the library.

It is difficult to explain why this method of immunisation was so unsuccessful at inducing an immune response to the 2A6 antigen when immunisation with *D.viviparus* $L_3$ homogenate induces such a marked response. It could perhaps be due to the amount of immunogen present in the preparations or that the homologous molecule from *H.contortus* is much less immunogenic than that from *D.viviparus*.

## 6.3. Discussion.

Little work has been performed on the *in vitro* culture of *D.viviparus*. Bos & Panhuijzen (1982) reported that incubation of $L_3$ in RPMI 1640 at 38°C in 5% $CO_2$ allowed development to the early $L_4$ stage but larvae died soon after moulting. These authors did not examine whether any new polypeptides, or antigens recognised by immune cattle, were expressed by these cultured larvae. There has been more success with the trichostrongylid nematode *O.ostertagi* (Douvres & Malakatis 1977) in which development from infective larvae to egg laying adults has been achieved *in vitro*. However all the parasitic stages of this nematode develop within the bovine abomasum, unlike *D.viviparus* where the infective larvae are tissue migratory and the later larval stages develop within the respiratory tract.

An attempt was made to culture *D.viviparus* from $L_3$ to $L_4$ in RPMI and 10% foetal calf serum at 37°C in 5% $CO_2$ in order to investigate whether any new antigens appeared during development. Larvae were highly motile in culture and there was a separation (apolysis) of the $L_3$ cuticle from the underlying larval surface. A detailed ultrastructural study of the moulting of *O.volvulus* $L_3$ to $L_4$ during *in vitro* culture has shown that partial production of the $L_4$ cuticle occurs prior to apolysis of the $L_3$ cuticle (Lustigman et al 1990). Therefore it seems likely that some $L_4$ cuticular synthesis occurred in the *D.viviparus* cultured larvae suggesting that there had been at least partial development to the $L_4$. However ecdysis did not occur which suggests that the $L_4$ development was not complete or that the host factors necessary for ecdysis were not provided by the culture conditions.

It was interesting to observe that prior exsheathment of the infective larvae was not required for this partial development to the $L_4$ to occur. Also no differences were detected in the biosynthetically labelled proteins of sheathed and exsheathed larvae, although 2-dimensional SDS-PAGE analysis would be required to determine whether the profiles were truly identical. It therefore seems that exsheathment and the subsequent development to the $L_4$ are independent events. This conclusion has also been reached from studies on the ability of actinomycin-D to inhibit the *in vitro* development of infective larvae of several trichostrongylid species. At the appropriate concentration this antibiotic preferentially inhibits DNA-dependent synthesis of RNA (Hurwitz, Furth, Malamy & Alexander 1962 and Goldberg & Rabinowitz 1962) and it has been shown to block the *in vitro* development of $L_3$ to $L_4$ in *N.brasiliensis* (Bonner & Burrett 1976), *Neoaplectana glaseri* (Despommier & Jackson 1972) and *H.contortus* (Petronijevic & Rogers 1983). In the latter two examples it was also shown that the same concentration of actinomycin-D did not block exsheathment and the authors concluded that the mechanism controlling exsheathment does not involve DNA transcription.

Examination of Coomassie stained SDS-PAGE gels of homogenates of cultured larvae revealed that changes in the presence (or relative abundance) of several polypeptides does occur during *in vitro* culture. Of particular interest was the appearance of a number of new antigenic molecules in the cultured larvae. Firstly, the hyperimmune bovine infection serum (B7,B8 & B9) specifically recognised three new antigens in aqueous homogenates at 65, 50 and 23 kDa and a single low molecular weight antigen in the SDS/2ME/urea homogenates. The water soluble 65 kDa molecule and the SDS/2ME/urea solubilised low molecular weight antigen were particularly immunogenic. These antigens were not recognised on Western blots probed with normal bovine serum and so antibodies specific to these molecules must be produced in cattle following immunisation by infection with *D.viviparus*. This suggests that the molecules which are expressed by the *in vitro* cultured $L_3$ must also be produced during *in vivo* development of the parasite in the bovine host. This conclusion is consistent with the results of the Western blots probed with serum from cattle immunised by 40krad and 100krad irradiated larvae. All the new antigens were recognised by sera from cattle immunised with 40 krad irradiated larvae (038 & 039) which is to be expected since such larvae develop to late $L_4$/early $L_5$ in cattle and so should present these early $L_4$ antigens to the bovine immune system. In contrast, the serum from cattle immunised with 100krad irradiated $L_3$ did not recognise the new water soluble 65 kDa or 50 kDa antigens. This is consistent with the hypothesis that 100 krad irradiated $L_3$ do not undergo development to the $L_4$ in cattle (Canto 1990) and so would not present these early $L_4$ antigens to the bovine immune system.

Interestingly this serum did recognise the water soluble 23 kDa antigen and the low molecular weight 2ME solubilised antigen. This suggests that these antigens are produced very early in development to the $L_4$ (before the 65 & 50 kDa antigens) and that 100 krad irradiated larvae reach at least this point of development in the bovine host. Sera from mice infected with *D.viviparus* $L_3$ or immunised with $L_3$ homogenate did not recognise any of the new antigens produced by the cultured larvae. This is consistent with these antigens being expressed in the $L_4$ and there being no larval development following infection of the mouse which supports the results presented in Chapter 3. The probing of Western blots with the different types of bovine immune serum also confirmed the results of the previous experiments with monoclonal antibodies which suggested that the 2A6 antigen becomes less abundant in cultured larvae.

In summary, although larvae do not undergo full development to $L_4$ in *in vitro* culture, the above experiments have shown that some development does occur. Several antigens which appear early in the development to the $L_4$ have been identified (the 23 kDa and the low molecular weight antigen probably being produced earlier than the 65 & 50 kDa antigens) and the recognition of these by immune bovine serum suggests that they are produced during development *in vivo*. The experiment also supports the hypothesis that 100 krad irradiated larvae undergo very little development in the bovine host and that normal larvae do not develop in the mouse. This system of culture may therefore allow further investigation of these $L_4$ antigens which are of potential importance to the immune response. It could be used to produce relatively large amounts of antigen or provide an appropriate source of RNA to allow cloning of the cDNAs coding for these antigens.

Preparation of large amounts of mRNA from free-living trichostrongylid infective larvae has been reported as difficult and this has been ascribed to the quiescent nature of this stage (Dawkins & Spencer 1989 and Keith, personal communication). For the trichostrongylid nematodes *T.colubriformis* and *O.circumcincta*, Dawkins & Spencer (1989) found that a greater recovery of RNA could be obtained using exsheathed $L_3$ as opposed to sheathed $L_3$. This has not been found to be the case for *D.viviparus* in which a greater yield of RNA was obtained using sheathed $L_3$. Furthermore, exsheathed larvae which had been *in vitro* cultured for several days, still yielded less total and poly(A)+ RNA than the same number of unstimulated sheathed $L_3$. When the *in vitro* translation products of these RNA samples were compared by one dimensional SDS-PAGE, the profiles were broadly similar. Although 2-dimensional SDS-PAGE analysis would be required to provide an accurate comparison, the one dimensional profiles are remarkably similar given that quiescent and metabolically active larvae are being compared. It is generally assumed

that the general metabolic processes of trichostrongylid $L_3$ are suppressed since they are a non-feeding stage which can survive for many months or even years at 4°C in the laboratory or under appropriate environmental conditions in the field. However from the results presented here, it appears that *D.viviparus* $L_3$ possess a large amount of mRNA encoding a similar range of products as the mRNA found in metabolically active larvae. This would suggest that any down regulation of the expression of "house-keeping" genes in *D.viviparus* infective larvae must occur post-transcriptionally. Little appears to be known about the level of transcription which occurs in infective trichostrongylid larvae but the dauer larval stage of *C.elegans* is an analogous example of diapause in a nematode. This stage is thought to be relatively metabolically inactive since it shows reduced levels of metabolic enzyme activities and high energy phosphates (Riddle 1988). However it may not be particularly transcriptionally inactive since the level of run-on transcription by RNA polymerase II, in isolated dauer larvae nuclei, has been estimated as 40% of that observed for other developmental stages (Dalley & Golomb, personal communication).

The RNA from *in vitro* cultured larvae did not produce any new *in vitro* translation products of a similar molecular weight to the new polypeptides and antigens detected by SDS-PAGE and Western blotting of the homogenates from cultured larvae. This may simply reflect the poor resolution of the one dimensional SDS-PAGE analysis which is insufficient to investigate details of specific translation products. Alternatively it could be due to the fact that *in vitro* translation of nematode mRNA using a mammalian cell free translation system may not accurately reproduce the translational events which occur in the living nematode. This is particularly true for the simple rabbit reticulocyte lysate used in these experiments which produces translation products without any post-translational modifications such as signal peptide cleavage and glycosylation. Addition of commercially available pancreatic microsomal membranes to the reaction mixture would supply the enzymes necessary for some of these modifications to occur but these may again be different to the modifications produced in the living nematode.

Comparison of the antigens and RNA from unstimulated sheathed $L_3$ compared to cultured $L_3$ illustrate the relative merits of such larvae as a source of RNA for production of a cDNA library. The mRNA from cultured larvae should contain transcripts for the early $L_4$ antigens identified by Western blotting, however it would be unlikely to contain transcripts for the polypeptide component of the 2A6 antigen. On the other hand unstimulated sheathed larvae would not provide a source of message for the early $L_4$ antigens but might contain transcripts for the 2A6 antigen. On balance, given the abundance of RNA and the range of translation products produced, it was decided to use mRNA from sheathed unstimulated larvae as this

might enable cloning of the polypeptide component of the 2A6 antigen and also of other immunogenic $L_3$ antigens. The inability of 2A6 or immune bovine serum to immunoprecipitate *in vitro* translation products was of some concern but these experiments had to be interpreted with caution since none of the anti-sera used immunoprecipitated any antigens and so there was no positive control in these experiments. Also methionine might not be an abundant amino acid in the polypeptide component 2A6 antigen in which case the molecule would not be detected in this experiment.

The titre of the unamplified library was $3.08 \times 10^6$ pfu which is sufficiently high to allow screening for transcripts of relatively low copy number. The amplified library consisted of 92% recombinant phage with an estimated range of insert size from 0.6-2.7kB (coding for polypeptides of approximately 20-90kD). Mouse serum raised to $L_3$ homogenate (with Freund's adjuvant), which detected many $L_3$ antigens on Western blots (see chapter 4), recognised approximately 1 in 900 recombinant plaques. These were confirmed by picking and rescreening several times and did not react with normal mouse serum. This is a reasonable proportion of clones, in a cDNA expression library, for such an anti-serum to recognise. For example, four different adult *O.volvulus* cDNA libraries contained approximately 1 in 500 clones which reacted with a rabbit antiserum raised against adult *O.volvulus* homogenate (Donelson et al 1988). Therefore it seems likely that the library is reasonably representative of *D.viviparus* $L_3$ transcripts as shown by the range of mRNA *in vitro* translation products, the titre and range of insert size of the library and the proportion of recombinant phage expressing antigens specifically recognised by mouse antiserum raised to *D.viviparus* $L_3$ homogenate. Therefore it was surprising that none of the different types of immune bovine serum detected any positive clones in spite of repeated screening attempts and the concurrent use of a positive control (mouse antiserum to $L_3$ homogenate and a positive clone). Probing of Western blots of $L_3$ homogenate has shown that these bovine sera contain antibodies to antigens of this stage. However it is possible that many of these antibodies are directed at non-peptide epitopes and further work is required to investigate this possibility. Interestingly the use of these bovine sera to screen an adult *D.viviparus* cDNA expression library has also failed to detect any positive clones (Britton, unpublished data). This library also seems to be satisfactory in other respects, for example screening with anti-adult E/S serum has identified numerous positive clones.

The failure of mab 2A6, and the pooled anti-sheath monoclonal antibodies, to identify any recombinant molecules in the library could be due to a number of reasons. If the 2A6 molecule was not synthesized in the $L_3$, the appropriate cDNAs would not be present in the library and similarly, if the 2A6 molecule was

subject to a low rate of turnover in the $L_3$ the appropriate cDNAs would be very rare within the library. These two hypotheses are essentially the same, differing only in degree, and are supported by the failure of mab 2A6 to immunoprecipitate any labelled molecules from homogenates prepared from metabolically labelled $L_3$ or from $L_3$ *in vitro* translation products. Furthermore, since the antigen is present in larvated eggs and $L_1$, it could be synthesized in the earlier larval stages and be present in a stored form in the $L_3$ and this would be consistent with the reduction in abundance of the antigen observed during *in vitro* culture of the $L_3$. In order to explain the failure of mab 2A6 to immunoprecipitate labelled antigen from homogenates of metabolically labelled larvated eggs or $L_1$, it is possible to speculate that the molecule is only synthesized for a restricted period of time, perhaps during the early development of larvae within the egg. Of course it is also possible that the immunoprecipitation experiments failed to demonstrate synthesis of the molecule, in all the stages examined, because the amino acids used for labelling are poorly represented in the molecule. This has been reported for the 400 kDa secreted molecule of *T.canis* $L_2$ which does not label with $^{35}$S-methionine (Menghi & Maizels 1986). An attempt was made to minimise this possibility by using a mixture of several radiolabelled amino acids but these were labelled with $^{3}$H thus reducing the sensitivity of detection.

If, for the reasons outlined above, there was only a low level of message in the $L_3$ for the molecule recognised by 2A6, the number of cDNAs in the final library could be further reduced by the method of cDNA synthesis which was chosen. The nucleotide mixture used for the first strand synthesis contained 5-methyl dCTP which results in the cytosine residues in the first cDNA strand being methylated in order to protect the cDNA from restriction enzymes used in subsequent cloning steps. To avoid digestion by the mcrA, mcrB restriction enzymes present in many bacterial strains, recombinant phage containing hemi-methylated DNA must be first passed through a mcrA-, mcrB- host bacterial strain such as PLK-F'. After this the DNA is no longer hemi-methylated and can be grown in mcrA+, mcrB+ strains such as XL1-Blue. This has the important consequence that the library cannot be immunoscreened before amplification. Since some phage will grow preferentially to others during amplification, some cDNAs will inevitably be underrepresented in the final amplified library. Therefore cDNAs which are present in low number in the unamplified library can be lost altogether during amplification. Therefore even though $7x10^5$ plaques were screened with 2A6, if the mRNA was of low abundance in the $L_3$, positive clones could have been missed.

An alternative reason for the failure of 2A6 to detect positive clones is that, although recombinant phage expressing the polypeptide are present in the library, the 2A6 epitope itself is not expressed. If this was the case, the bovine immune

sera might be expected to detect some positive clones since they detect the molecule very strongly and, being polyclonal antisera, should recognise a number of different epitopes. However it is always possible that antibodies to the same immunodominant epitope recognised by mab 2A6 predominate in these antisera. A similar situation would occur if the epitope detected by 2A6 was carbohydrate and the antibody present in bovine immune serum was also predominantly directed against carbohydrates. Although the 2A6 epitope has been shown to be periodate insensitive, not all carbohydrates are susceptible to periodate oxidation (Woodward et al 1985). A good example of the importance of carbohydrates in this respect is the observation that the antibody produced to the major immunodominant molecule of *Theileria annulata* merozoites appears to be predominantly directed against carbohydrate epitopes (B.Shiels, personal communication). This is thought to be responsible for the failure of sera to detect the recombinant polypeptide in cDNA libraries. However the immunochemical experiments have suggested that the *D.viviparus* 2A6 molecule is not heavily glycosylated and so it seems less likely that the results presented here can be explained in this way.

In summary, the attempt to clone the gene encoding the polypeptide component of the 2A6 antigen from a *D.viviparus* $L_3$ cDNA library was unsuccessful. This library was also produced to enable other genes encoding immunogenic molecules of the $L_3$ to be cloned but, surprisingly, immune bovine sera also failed to recognise any recombinant clones. There are a number of questions which need to be resolved before a rational strategy for cloning the 2A6 gene can be developed. Most importantly the period of parasite development during which the molecule is produced needs to be identified. This could be approached by isolating RNA from different parasite stages and immunoprecipitating the *in vitro* translation products with 2A6 and immune bovine serum. The molecule has been identified in larvated eggs by Western blotting but appears to be absent (or of very low abundance) in adult parasites. Therefore the examination of RNA from both adult females and the eggs released from these during *in vitro* culture should encompass the time during which the molecule first appears and it should be relatively easy to produce sufficient amounts of this material by harvesting adult worms from infected calves. It may be also worthwhile examining RNA from mixed stage cultures of larvae developing from $L_1$ to $L_3$ so that the whole period of development from embryo to $L_3$ would be examined. Obtaining sufficient material for this might be more difficult and, if this was so, the same experiments could be performed on developing *H.contortus* larvae since these are easier to produce in large quantities. If these results appeared promising, it may be possible to use PCR amplification to produce a cDNA library from the appropriate stage of *D.viviparus* larvae to enable cloning of the gene. Also polyclonal

antisera to the 2A6 molecule should be raised to try and produce antibodies to other epitopes on the molecule. This is important since it is always possible that the 2A6 epitope (or the predominant epitopes recognised by immune bovine sera) may not be present on the *in vitro* translated product or recombinant polypeptide. One approach might be to try and purify sufficient antigen using a 2A6 immunoaffinty column to raise a polyclonal antiserum.

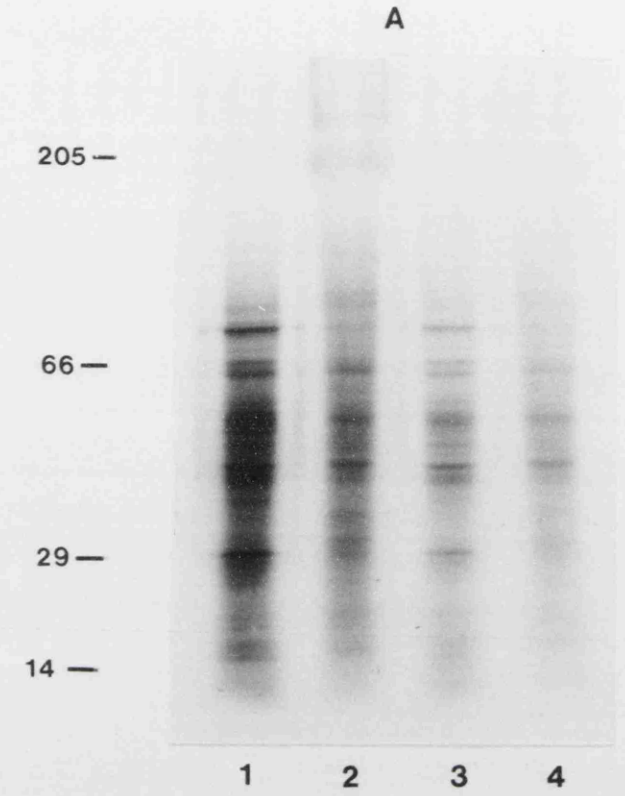
**Figure 6.1**

**A.** Sheathed and exsheathed *D.viviparus* $L_3$ metabolically labelled with $^{35}S$-Methionine.

Track 1 = Sheathed $L_3$ aqueous soluble homogenate.
Track 2 = Sheathed $L_3$ SDS/2ME/urea soluble homogenate.
Track 3 = Exsheathed $L_3$ aqueous soluble homogenate.
Track 4 = Exsheathed $L_3$ SDS/2ME/urea soluble homogenate.

**B.** Sheathed and exsheathed *D.viviparus* $L_3$ metabolically labelled with $^{3}H$-amino acid mixture (Leucine, Lysine, Phenylalanine, Proline and Tyrosine).

Track 1 = Sheathed $L_3$ aqueous soluble homogenate.
Track 2 = Sheathed $L_3$ SDS/2ME/urea soluble homogenate.
Track 3 = Exsheathed $L_3$ aqueous soluble homogenate.
Track 4 = Exsheathed $L_3$ SDS/2ME/urea soluble homogenate.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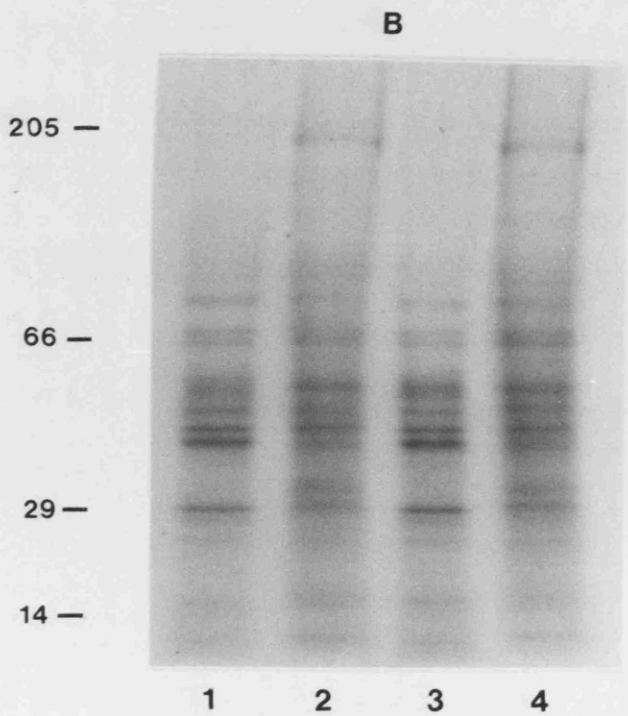
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**Figure 6.2**

**A.** Coomassie stained gel of homogenate prepared from cultured exsheathed *D.viviparus* $L_3$.

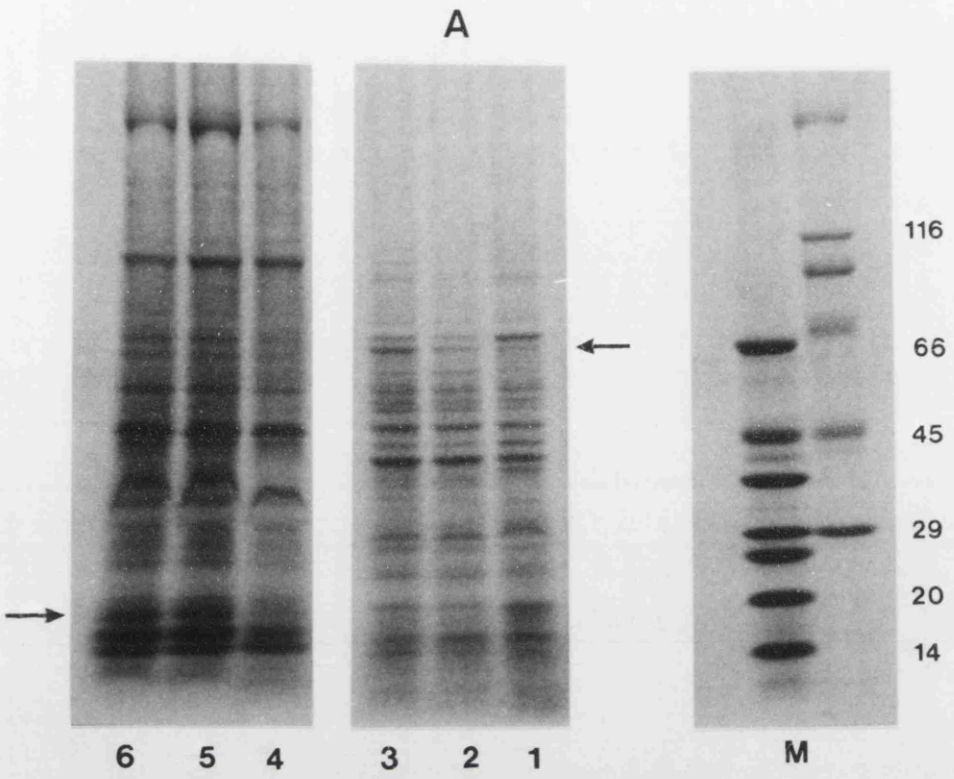
Track 1 and 4 = $L_3$ homogenate (larvae not cultured).
Track 2 and 5 = Homogenate from 4 day cultured $L_3$.
Track 3 and 6 = Homogenate from 10 day cultured $L_3$.
Tracks 1-3 = aqueous soluble antigens.
Tracks 4-6 = SDS/2ME/urea soluble antigens.
M = molecular weight markers.

Arrows indicate polypeptides which appear during culture.

**B.** Western blots of homogenate prepared from cultured exsheathed *D.viviparus* $L_3$ probed with hyperimmune bovine serum produced by repeated oral infection (pooled B7, B8 and B9 sera). A duplicate blot was negative when probed with normal bovine serum (data not shown).

Track 1 and 4 = $L_3$ homogenate (larvae not cultured).
Track 2 and 5 = Homogenate from 4 day cultured $L_3$.
Track 3 and 6 = Homogenate from 10 day cultured $L_3$.
Tracks 1-3 = aqueous soluble antigens.
Tracks 4-6 = SDS/2ME/urea soluble antigens.

Arrows indicate antigens which appear during culture.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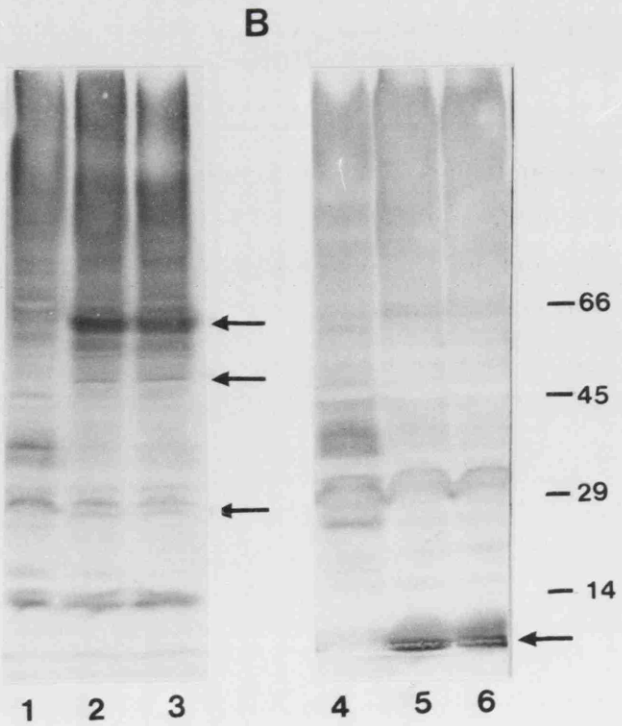
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**Figure 6.3**

**A.** Western blots of homogenate prepared from cultured exsheathed *D.viviparus* $L_3$ probed with hyperimmune bovine serum produced by repeated oral immunisation with 40 krad irradiated larvae (pooled O38 and O39 sera). A duplicate blot was negative when probed with normal bovine serum (data not shown).

Track 1 and 4 = $L_3$ homogenate (larvae not cultured).
Track 2 and 5 = Homogenate from 4 day cultured $L_3$.
Track 3 and 6 = Homogenate from 10 day cultured $L_3$.
Tracks 1-3 = Aqueous soluble antigens.
Tracks 4-6 = SDS/2ME/urea soluble antigens.
M = Molecular weight markers.

Arrows indicate which appear during culture.

**B.** Western blots of homogenate prepared from cultured exsheathed *D.viviparus* $L_3$ probed with hyperimmune bovine serum produced by repeated intravenous immunisation with 100 krad irradiated larvae (pooled G17 and B45 sera). A duplicate blot was negative when probed with complete medium (data not shown).

Track 1 and 4 = $L_3$ homogenate (larvae not cultured).
Track 2 and 5 = Homogenate from 4 day cultured $L_3$.
Track 3 and 6 = Homogenate from 10 day cultured $L_3$.
Tracks 1-3 = Aqueous soluble antigens.
Tracks 4-6 = SDS/2ME/urea soluble antigens.
M = Molecular weight markers.

Arrows indicate antigens which appear during culture.

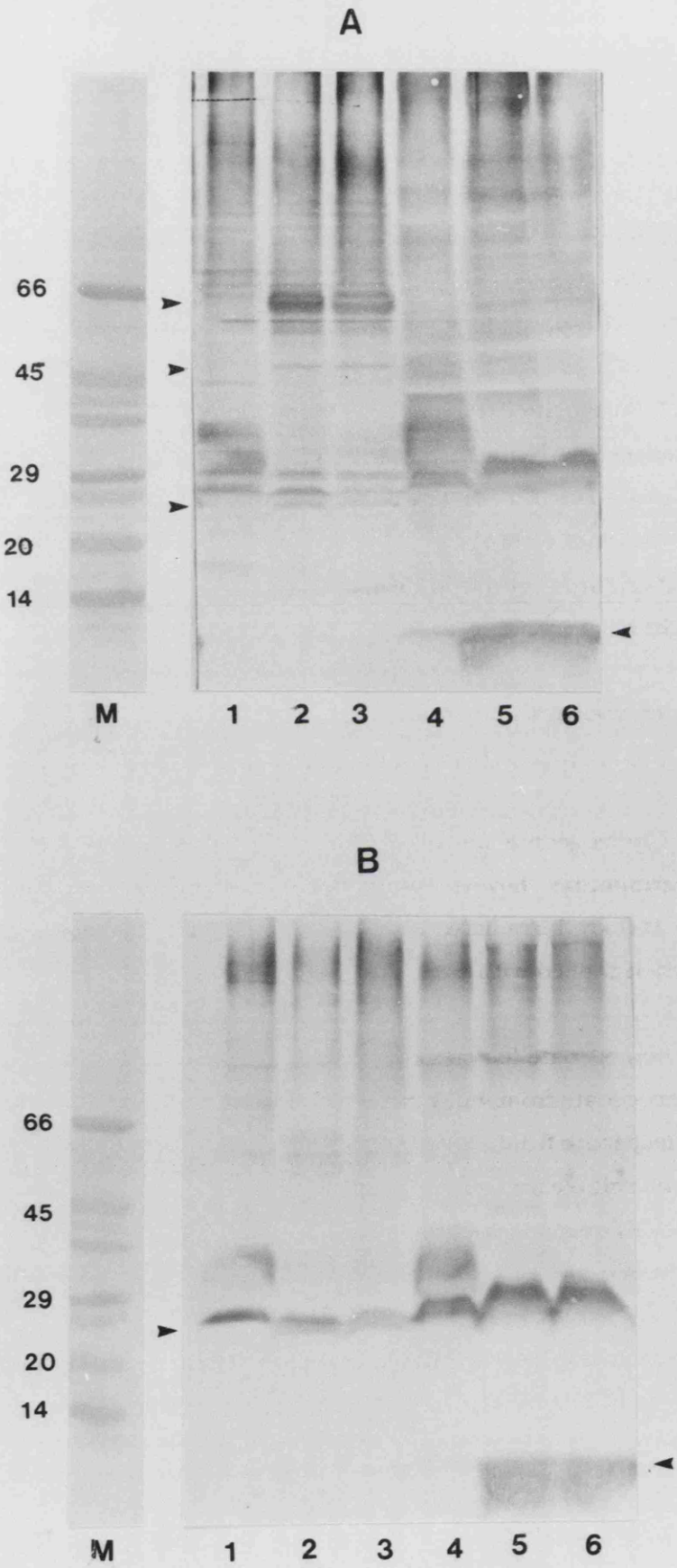
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**Figure 6.4**

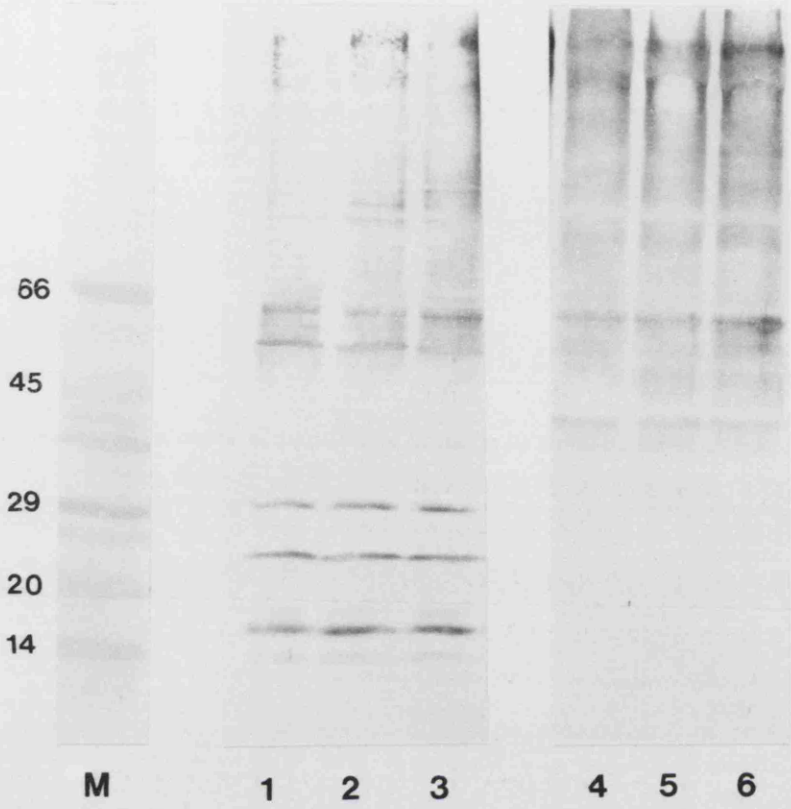
**A.** Western blots of homogenate prepared from cultured exsheathed *D.viviparus* $L_3$ probed with anti-adult ES rabbit serum. A duplicate blot was negative when probed with normal rabbit serum (data not shown).

Track 1 and 4 = $L_3$ homogenate (larvae not cultured).
Track 2 and 5 = Homogenate from 4 day cultured $L_3$.
Track 3 and 6 = Homogenate from 10 day cultured $L_3$.
Tracks 1-3 = Aqueous soluble antigens.
Tracks 4-6 = SDS/2ME/urea soluble antigens.
M = Molecular weight markers.

**B.** Ethidium bromide stained agarose gel of total RNA extracted from sheathed and exsheathed *D.viviparus* $L_3$. The total RNA extracted from $2x10^6$ larvae was dissolved in 60 ul of buffer and an aliquot of 10 ul was loaded onto the gel.

Track 1 = Ladder
Track 2 = Exsheathed $L_3$ incubated at 37°C and 5% $CO_2$ for 4 hours.
Track 3 = Sheathed $L_3$.
Tracks 4 and 5 = *T.annulata* merozoite RNA loaded as a control.

A

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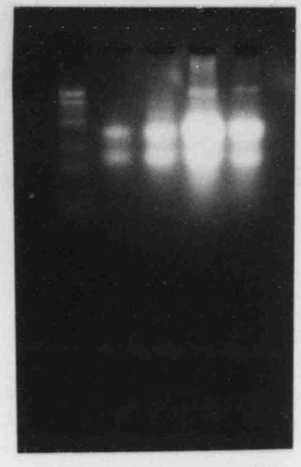


## Figure 6.5

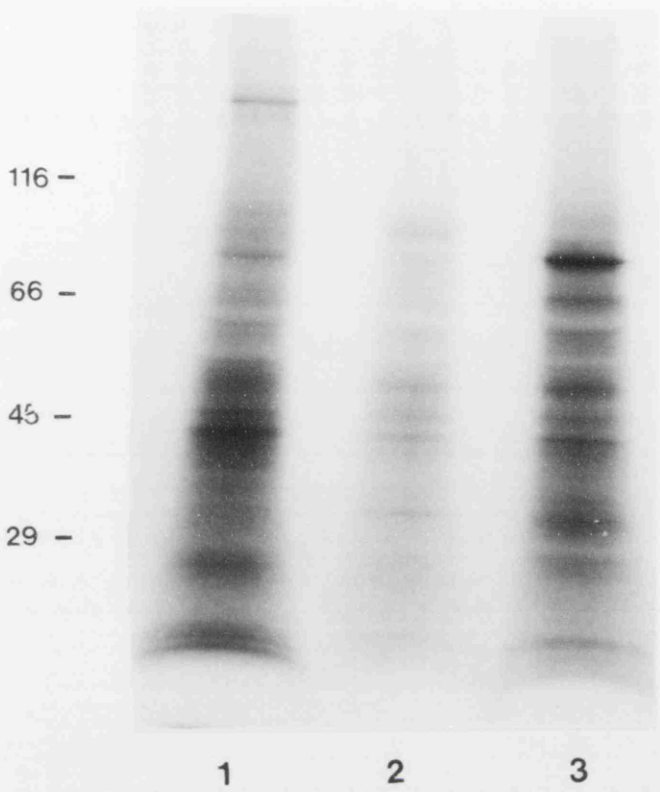
**A.** *In vitro* translation products of *D.viviparus* mRNA from larval and adult stages.

Track 1 = Adult translation products.
Track 2 = 4 day cultured exsheathed $L_3$ translation products.
Track 3 = Sheathed $L_3$ translation products.

**B.** Ethidium bromide stained agarose gel of phage DNA, double digested with Eco R1 and Xho 1, prepared from phage grown from 10 plaques picked at random from the library.

Tracks 1 and 2 = Undigested vector.
Tracks 2-12 = Digested recombinant phage containing inerts of size 2.0, 2.7, 0.7, 2.2, 1.9, 0.6, 0.6, 2.6 and 1.2 + 0.6.

A

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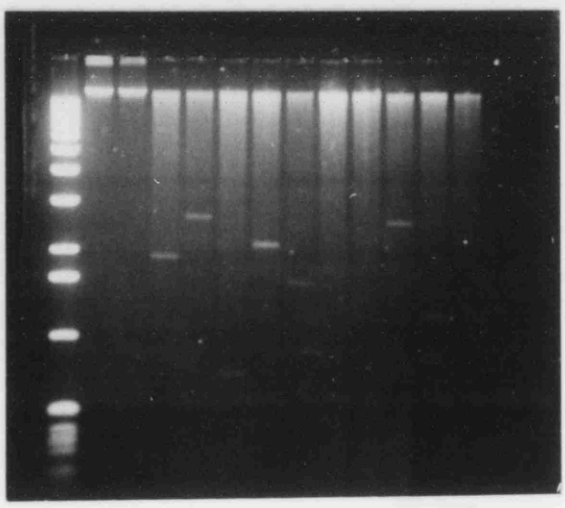


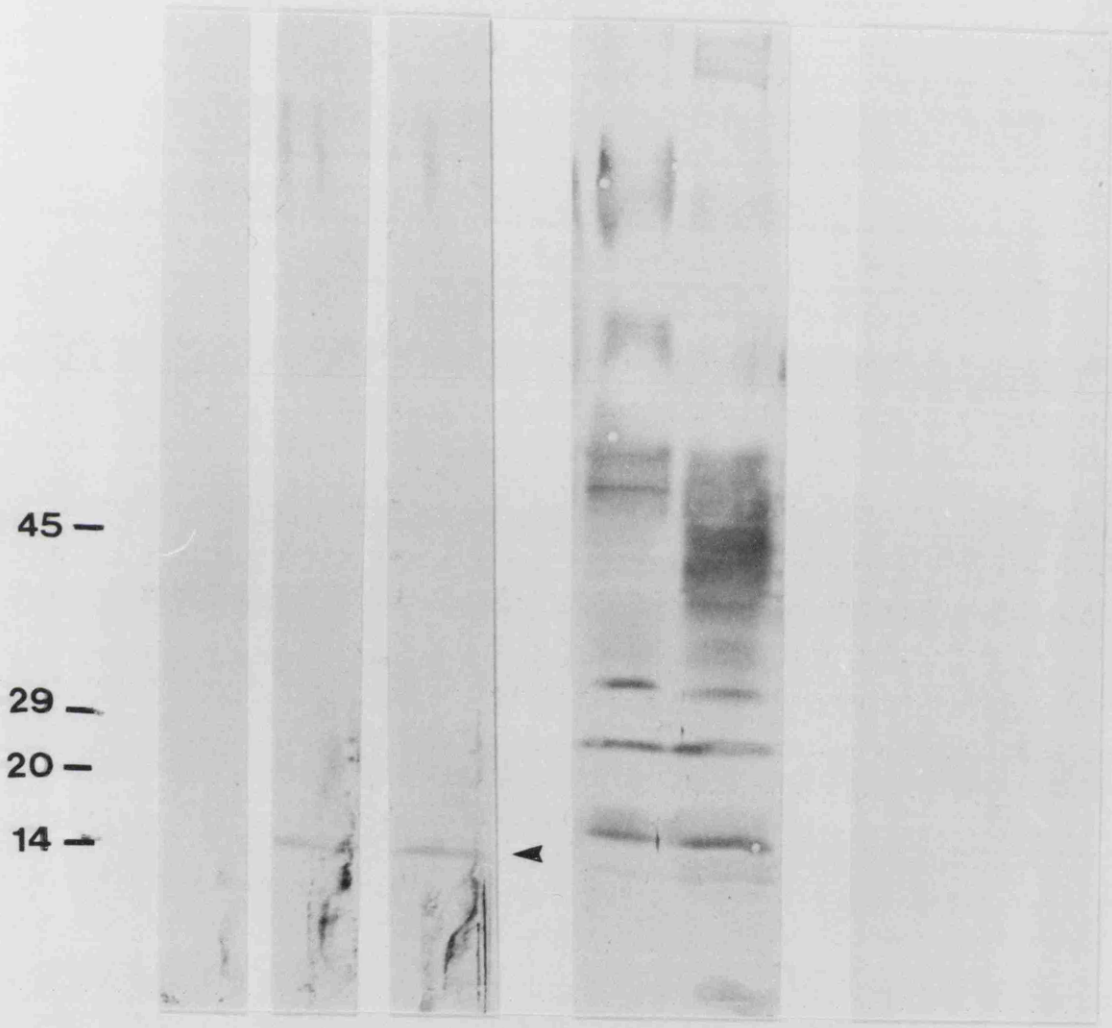
**Figure 6.6**

The eight positive clones detected by mouse serum against *D.vivlparus* $L_3$ homogenate. Half of each filter probed with the same mouse serum against *D.viviparus* $L_3$ homogenate (left) and the other half with normal mouse serum (right).

**Figure 6.7**

Western blot of *D.viviparus* homogenate probed with antibody selected by the two clones picked from the library by rabbit serum against adult ES.

Track 1 = $L_3$ homogenate probed with antibody selected by a clone randomly picked from the library (negative control).
Tracks 2 and 3 = $L_3$ homogenate probed with the antibody eluted from the two clones which were detected by the library screen with anti-adult ES serum.
Track 4 = $L_3$ homogenate probed with anti-adult ES serum.
Track 5 = Adult homogenate probed with anti-adult ES serum.
Tracks 6 and 7 = $L_3$ and adult homogenate probed with normal rabbit serum.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# Chapter 7

# CHAPTER 7

## Concluding Summary and Discussion.

The overall aim of the work presented in this thesis was to identify and characterise particular antigens of the *D.viviparus* $L_3$, which might contribute to the understanding of the highly effective immune response which is induced by normal infection or vaccination of cattle. The infective larva was chosen as the central subject of this thesis because it was considered to be a source of interesting and potentially important antigens. The radiation attenuated larvae which comprise the vaccine, develop only to the $L_4/L_5$ and once immunised, either by vaccination or previous infection, cattle appear to destroy the invading parasites during the early larval stages (Jarrett & Sharp 1963). Furthermore Canto (1990) has shown that immunisation of cattle with 100krad irradiated infective larvae, which are believed not to develop beyond the $L_3$, is capable of inducing a highly protective immune response. Additionally, it is relatively easy to obtain sufficient numbers of $L_3$ to allow detailed immunochemical analysis which is not the case for the other important larval stage, the $L_4$.

The identification of candidate protective antigens is difficult to achieve solely by studying the bovine immune response since, once immunised, all individuals appear equally resistant to challenge in spite of a high degree of heterogeneity in antigen recognition (Britton et al 1991). This means that it is not possible to correlate the state of immunity with the recognition of any particular antigens. Therefore an inexpensive and versatile laboratory animal model such as the mouse was considered to be potentially useful in the screening of antigens for their ability to induce protective immunity. The results showed that the mouse was a less permissive host than the guinea pig, the traditional laboratory animal model for *D.viviparus*, in that following infection the larvae did not appear to develop or establish themselves in the lungs. However, larvae did migrate to the lungs in numbers similar to those which occur in guinea pigs and a measurable degree of immunity to this migration could be induced by previous infection. This suggested that the mouse might provide a model for immunity to the early stages of *D.viviparus* infection, however the antibody response showed a number of fundamental differences to that seen in cattle. Infection did not induce a strong antibody response to the $L_3$ sheath, as occurs in cattle, and passive immunisation with serum from immune mice failed to transfer protection to recipients. The short lived nature of the infection allowed very little time for an effective anamnestic response to occur following challenge of immunised mice. Due to

these factors, the mouse was considered to be of limited value as a model for *D.viviparus* infection and immunity.

A characterisation of the surface antigens of the $L_3$ cuticular surface was undertaken since these represent the host-parasite interface and are consequently likely to be of immunological importance. Biotinylation of live exsheathed $L_3$ failed to identify any $L_3$ cuticular molecules which was also the case for the surface iodination experiments reported by Britton (1991). Generation of monoclonal antibodies to the $L_3$ cuticular surface revealed that PC, or a PC-like epitope, is exposed on the surface of the $L_3$ cuticle and this appears to be a completely stage specific phenomenon. Such an epitope, although present in many parasites, has not been reported to be exposed on the surface of any other nematode species. This is particularly interesting because it is often suggested that PC could have an immunomodulatory role since it is tolerogenic for high affinity host B cells (Mitchell & Lewers 1977 and Gutman and Mitchell 1977). It is also likely that the presence of PC, which is a zwitterion, produces a highly charged $L_3$ surface and it is interesting to speculate whether this could be responsible for the ineffectiveness of surface labelling techniques when applied to this stage. The presence of carbohydrate on the $L_3$ cuticular surface was demonstrated by the binding of the lectin *Helix pomotia* agglutinin and this is consistent with the presence of PC since it is usually linked to carbohydrate residues (Potter 1977). These findings suggest that the nature of the *D.viviparus* $L_3$ surface is particularly appropriate for the infective stage of a migratory parasite, since the presence of PC and the apparent lack of other exposed molecules would appear to represent a very poorly immunogenic surface. This helps to explain the relatively low antibody response to the $L_3$ surface following infection and immunisation of cattle (Britton 1991). The $L_3$ surface specific IgM present in naive bovine serum, reported by Britton (1991), does not appear to be directed at the PC epitope and this could act as "blocking antibody" for any non-PC epitopes which may be exposed on the surface.

In contrast to the $L_3$ cuticle, the surface of the $L_3$ sheath seems to be highly immunogenic following infection or immunisation which is surprising if one accepts the conventional view that the sheath is cast in the alimentary tract prior to the migration of the $L_3$. Biotinylation of live sheathed $L_3$ labelled a number of molecules of similar molecular weight to those identified by Britton (1991) using surface radioiodination. These could not be solubilised from the surface of live worms with detergents and were not susceptible to collagenase digestion. None of the lectins studied bound to the external surface of the sheath although a number did bind to the internal surface during exsheathment. The generalised pattern of binding of these lectins to the *D.viviparus* sheath was in contrast to their binding to *O.ostertagi* and

*T.colubriformis* sheaths which was restricted to the refractile ring region and a small area towards the posterior end of the sheath. These results seem to reflect the differences that were observed in the mode of exsheathment between these species in that the *D.viviparus* sheath completely disintegrated during exsheathment whereas the *O.ostertagi* and *T.colubriformis* sheaths were only degraded at the refractile ring region. This could be explained by the hypothesis that the lectins are detecting carbohydrates associated with the target molecules of the exsheathing enzymes responsible for the degradation of the sheath. It would also be interesting to know whether these differences in the mode of exsheathment reflect differences in the site of exsheathment within the host.

Six monoclonal antibodies were generated to the $L_3$ sheath surface and all recognised the same antigen which appeared as a poorly resolved band of approximately 29-36 kDa on Western blots. This was shown to be a very immunodominant antigen which was consistently detected on Western blots by serum from cattle immunised by protection or vaccination. The results presented have shown that this antigen is largely, and quite possibly solely, responsible for the surprisingly marked antibody response of immune cattle to the *D.viviparus* $L_3$ sheath surface. However the question remains as to how this antigen is presented so effectively to the host immune system, given its location on the sheath surface. The answer may lie in the observation that the antigen is also present in the somatic tissues of the $L_3$ and consequently any larvae which die during migration could release the antigen during their disintegration in host tissues. Alternatively, it may be that the larvae exsheath not in the host alimentary tract but at some point during migration, which would mean that the sheath itself presents the antigen to the host immune system. Soliman (1953a) reported the presence of both sheathed and exsheathed $L_3$ in the mesenteric lymph nodes of naturally infected cattle and that the sheathed $L_3$ were dead wheras the exsheathed $L_3$ were alive. It is tempting to speculate that larvae which failed to exsheath were killed as a result of the marked immune response to the sheath wheras larvae which successfully exsheathed survived due to the relatively poor immune response to the $L_3$ cuticle. As yet, it is not possible to draw any firm conclusions regarding the importance of the 2A6 antigen to the overall protective immune response of cattle to *D.viviparus* infection. Its location on the sheath surface might seem to militate against it being important in this respect, however more information concerning its presence elsewhere within the parasite and the reasons for its immunodominance in immune cattle is required before any firm conclusions can be drawn. Also its ability to protectively immunise animals could be tested by passive immunisation with the monoclonal antibodies or active immunisation with the purified antigen. However the results of such experiments must always be treated with

caution when relatively little is known about the structure, abundance, location and function of an antigen.

Of particular interest was the binding of 2A6, and the other sheath specific monoclonal antibodies, to the surface of the $L_3$ sheaths of many other nematode species from the order Strongylida. The molecule detected by this antibody had a similar molecular weight and isoelectric point in a number of the more closely related species, which suggests that there is an homologous molecule present on the surface of the $L_3$ sheath of these nematodes. It also seems more likely that the molecular weight differences of the antigens detected by 2A6 in the less closely related species is due to modifications of this homologous molecule rather than the presence of the same conserved epitope on completely different molecules. The conservation of a molecule at the same site in such a large range of parasitic nematodes would suggest that it is likely to be functionally important. For this reason, as well as its potential immunological importance in *D.viviparus* infection, the molecule was considered to be of particular interest and worthy of detailed investigation.

Immunochemical analysis of the molecule proved to be difficult, particularly as it could only be visualised by detection with antibody. Its partial degradation by protease treatment demonstrated it had a polypeptide component but a number of different approaches failed to demonstrate the presence of any carbohydrate. The possible glycosylation of the molecule requires further investigation since negative results of this nature should be interpreted with caution, in particular the possible presence of O-linked oligosaccharides should be investigated. Another question which remains unanswered concerns the abundance of the molecule in the parasite. The density of immunogold labelling with 2A6 on the sheath surface together with the presence of the molecule in somatic tissues would suggest that it is relatively abundant. However many of the results of the immunochemical analyses and the attempted immunoaffinty purification could be more easily explained if the molecule was of low abundance. One possible explanation of this could be that the situation is similar to that described for the PARP molecule of *T.brucei* (Roditi & Pearson 1990), where although the molecule is abundant in the parasite, it is relatively poorly extracted in aqueous buffers in the absence of detergents. More work is required to investigate this possibility.

The resolution of a number of other questions may also help to determine the function of the molecule in the parasite. The surface exposure of the 2A6 molecule is entirely stage specific whereas it is present within the parasite from the development of the $L_1$ inside the egg to the $L_3$ stage. The synthesis of the molecule could not be demonstrated and more work aimed at clarifying the precise point(s) of parasite development at which this occurs would be useful. This is also likely to be a

prerequisite to the successful cloning of the gene which encodes the molecule, since extensive screening of a *D.viviparus* $L_3$ cDNA expression library failed to detect any positive clones. Cloning of the gene would allow an alternative approach to the determination of the molecule's structure and possible function, as well as the control of its expression within the parasite. Similarly it would be interesting to investigate the location of the molecule in larval somatic tissues at different stages of development using immunogold-EM studies of cryostat sections. The immunogold studies already performed have shown that the molecule is present on a surface coat outside the epicuticle of the sheath. It appears that this is an inert and highly resilient structure unlike many of the surface coats described for other Secernentean nematodes (reviewed by Blaxter et al 1992).

*In vitro* culture of *D.viviparus* $L_3$ allowed partial development to the $L_4$ to occur and a number of new immunogenic molecules were expressed by these developing larvae. These antigens were detected by immune bovine serum suggesting that they were not simply an artifactual *in vitro* phenomenon. Thus the *in vitro* culture of *D.viviparus* $L_3$ could be used to produce these early $L_4$ antigens, or the RNA encoding them, in relatively large amounts. Also the fact that some of these antigens are not detected by serum from infection immunised mice and cattle immunised with 100krad irradiated larvae supports the view that the larvae do not develop beyond the $L_3$ in these situations.

Finally the screening of a *D.viviparus* $L_3$ cDNA library with a variety of different immune bovine sera failed to identify any positive clones and this has also been reported by Britton (unpublished data) for the screening of an adult cDNA library. In spite of this, the sera detected a range of antigens on Western blots and by immunoprecipitation which emphasises the need to investigate the nature of the epitopes recognised by these sera. If these were found to be predominantly non-polypeptide this would have profound implications for future approaches to the investigation of immunity to *D.viviparus*.

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# Appendix 1.

## Buffers and Standard Reagents.

**Phosphate Buffered Saline (PBS).**

| | |
|---|---|
| 8.5g | NaCl |
| 0.32g | $NaH_2PO_4$ |
| 1.1g | $Na_2HPO_4$ |

Made up to 1 litre with $dH_2O$.

***Ascaris* Culture Medium.**

| | |
|---|---|
| 100ml RPMI 1640 | (Gibco, 041-02409M) |
| 240ug L-glutamine | (Flow, 16-801-49) |
| 100mg D-glucose | (Formachem) |
| 40ug Tripeptide | (Sigma,G-1887) |
| 50ng glutathione | (Sigma, G-4251) |
| 10,000IU penicillin | (Flow, 16-700-49) |
| 10,000ug streptomycin | (Flow, 16-700-49) |
| 1mM sodium pyruvate | (Flow, 16-820-49) |
| 1mg sodium bicarbonate | (Flow, 16-883-49) |
| 2ml amphotericin B | (Flow, 16-723-46) |
| 0.25ml gentamycin | (Sigma, G-7632) |
| 1 tablet Cephalexin selectatab | (Mast Lab. Ltd., MS 10) |
| 1 tablet VCNT selecatab | (Mast Lab. Ltd., MS 6) |

**Tris Homogenisation Buffer.**

100ml 10mM Tris-HCl, pH, 8.3

2mM ethylenediaminetetra acetic acid (EDTA)

1mM phenylmethylsulphonyl fluoride in isopropanol (Pms-F, Sigma, P-7626)

5uM pepstatin in methanol (Sigma, P-4265)

2mM 1,10 phenanthroline in ethanol (Sigma, P-9375)

5uM leupeptin (Sigma, L-2884)

5uM antipain (Sigma, A-6271)

25ug/ml N-P-tosyl-L-lysine chloromethyl ketone (TLCK, Sigma, T-7254)

50ug/ml N-tosyl-L-phenyl alanine chloromethyl ketone (TPCK, Sigma, T-7254)

**ELISA Reagents.**

**Carbonate/bicarbonate plate coating buffer.**

| | | |
|---|---|---|
| 8ml | 0.2M | $Na_2CO_3$ |
| 17ml | 0.2M | $NaHCO_3$ |
| 75ml | $dH_2O$ | |

pH adjusted to 9.6.

**Wash Buffer.**

PBS with 0.05% Tween 20 (Sigma P-1379)

**Blocking Buffer.**

Wash buffer with skimmed milk (Marvel) at a final concentration of 4%.

**Antibody Dilution Buffer.**

Wash buffer with skimmed milk (Marvel) at a final concentration of 2%.

**Substrate/Chromogen solution.**

**OPD Buffer.**

| | |
|---|---|
| 5.11g | Citric acid |
| 0.15g | $NaH_2PO_4$ |

Made up to 1 litre with $dH_2O$

**OPD Substrate Solution.**

| | |
|---|---|
| 4mg | OPD (ortho-phenyl-diamine) (Sigma, P-1526) |
| 10ml | OPD buffer |
| 5ul | $H_2O_2$ (added immediately before use) (BDH, 45202) |

**SDS-PAGE Reagents.**

**Stock Solutions.**

**Gel Buffer.**

| | |
|---|---|
| 90.5g | Tris |
| 1.48g | EDTA |
| 2g | SDS (sodium dodocyl sulphate) |

Made up to 500ml with $dH_2O$ and adjusted to pH 8.8 with HCl.

**Stack Buffer.**

| | |
|---|---|
| 12.11g | Tris |
| 0.58g | EDTA |
| 0.8g | SDS |

Made up to 200ml with $dH_2O$ and adjusted to pH 6.8 with HCl.

**Acrylamide Solution.**

| | |
|---|---|
| 60g | Acrylamide |
| 1.6g | bis-Acrylamide |

Made up to 200ml with $dH_2O$ and filtered.

**5-20% Gradient Gels**

| | **5% Gel (Top)** | **20% Gel (Bottom)** | **Stacking Gel** |
|---|---|---|---|
| Acrylamide | 5ml | 20ml | 3.2ml |
| Gel Buffer | 7.5ml | 7.5ml | - |
| Stack Buffer | - | - | 6ml |
| Sucrose | - | 4.5g | - |
| H2O | 17.1ml | - | 14.4ml |

The above solutions are sufficient to produce 2 gels (178x138mm). Immediately before pouring the solutions, freshly prepared 10% ammonium persulphate (APS) and N,N,N',N'- Tetramethylethylenediamine (TEMED, Sigma T-8133) were added in the following volumes.

| | | | |
|---|---|---|---|
| 10% APS | 100ul | 100ul | 250ul |
| TEMED | 10ul | 10ul | 20ul |

**10% Minigels.**

| | **Separating gel** | **Stacking gel** |
|---|---|---|
| Acrylamide | 10.7ml | 1.6ml |
| Gel buffer | 8ml | - |
| Stack buffer | - | 3ml |
| $dH_2O$ | 13.3 | 7.4 |
| Immediately before use: | | |
| 10% APS | 250ul | 125ul |
| TEMED | 20ul | 10ul |

**SDS-PAGE Sample Buffer.**

| | |
|---|---|
| 25ml | 0.5M Tris pH, 7.5 |
| 4g | SDS |
| 20ml | glycerol |
| 10ml | 2-mercaptethanol |
| 44ml | $dH_2O$ |
| 1ml | 0.2% Bromophenol Blue |

**Electrophoresis Tank Buffer.**

| | |
|---|---|
| 120g | Tris |
| 576g | glycine |
| 20g | SDS |
| 14.9g | EDTA |

Made up to 4 litres produces a 5X stock solution.

**Silver Staining.**

**Staining Solution.**

| | |
|---|---|
| 2g | $AgNO_3$ |
| 70ml | $H_2O$ |
| 3.5ml | $NH_3$ |
| 50ml | 0.36% NaOH |
| 120ml | Methanol |

**Developing Solution.**

| | |
|---|---|
| 2.5ml | 1% Sodium Citrate |
| 250ul | 38% Formaldehyde |

Made up to 500ml with $dH_2O$ and used immediately.

**Western Blotting Reagents.**

**Transfer Buffer**

| | |
|---|---|
| 9g | Tris |
| 42g | glycine |
| 600ml | methanol |

Made up to 3 litres with $H_2O$

**Tris Saline.**

| | |
|---|---|
| 12.11g | Tris |
| 90g | NaCl |

Made up to 1 litre with $dH_2O$ and adjusted to pH 7.4 with HCl.

**Blocking Buffer.**

| | |
|---|---|
| 20% | Skimmed Milk (Marvel) |
| 10% | Horse serum |

Made up in Tris saline.

**Antibody Dilution Buffer.**

| | |
|---|---|
| 10% | Skimmed Milk (Marvel) |
| 5% | Horse serum |

Made up in Tris saline.

**Wash Buffer**

Tris saline with 0.05% Tween-20.

**Substrate/Chromogen solution.**

| | |
|---|---|
| 48mg | 4-chloro-1-napthol (Sigma C-8890) |
| 16ml | Methanol |
| 80ml | Tris saline |
| 32ul | $H_2O_2$ (hydrogen peroxide, BDH 45202) |

This was made up immediately before use.

**NET Buffer.**

(for washing Protein-A-sepharose during immunoprecipitations)

| | |
|---|---|
| 0.6g | Tris |
| 0.87g | NaCl |
| 370mg | EDTA |
| 100ul | NP-40 |
| 0.25g | gelatin |

Made up to 100ml with $dH_20$

**Reagents for the preparation of RNA.**

**DEP treatment.**

All solutions (except guanadinium thiocyanate) were treated with dimethyl pyrocarbonate (DEP, Sigma, D-5520) to destroy RNases by the following method. DEP was added to a final concentration of 0.1% and the solution incubated at 37°C for 2 hours. The solution was then autoclaved to destroy the DEP.

**Siliconisation of glassware.**

All glassware to be used for procedure involving the preparation and manipulation of RNA was siliconised to prevent loss of material due to adherence to the glass. Glassware was filled with dimethyldichlorosilene (Sigma, D-3879) for a few minutes,left to air dry and rinsed in $dH_2O$.It was theb baked at 180°C for eight hours to destroy RNases.

**4M Guanadinium thiocyanate.**

| | |
|---|---|
| 47.2g | GSCN (guanadinium thiocyanate) |
| 5ml | 1M Tris |
| 200ul | 0.5M EDTA |
| 1ml | mercaptethanol |

Made up to 100ml with $dH_2O$ and filtered.

**Reagents for agarose gels.**

**MOPS Buffer for formaldehyde gels (5X stock).**

| | |
|---|---|
| 41.8g | MOPS (morpholinopropane sulphonic acid) |
| 6.8g | sodium acetate |
| 10ml | 500mM EDTA |

Made up to 1 litre with $dH_2O$ and adjusted to pH 7 with 5M NaOH.

**TBE Buffer for DNA gels (10X Stock).**

| | |
|---|---|
| 540g | Tris |
| 275g | Boric acid |
| 200ml | 0.5M EDTA |

Made up to 5 litres and adjusted to pH 8.

**Media and Solutions for cDNA library analysis and screening.**

**LB Broth**

| | |
|---|---|
| 10g | NaCl |
| 10g | Bacto-Tryptone |
| 5g | Yeast Extract |

Made up to 1 litre with $dH_2O$.

**Bottom agar** = 15g/litre of Bacto-Agar in LB broth.

**Top agar** = 7g/litre of agarose in LB broth.

**SM Buffer.**

| | |
|---|---|
| 5.8g | NaCl |
| 2g | $MgSO_4.7H_2O$ |
| 50ml | 1M Tris, pH 7.5 |
| 5ml | 2% gelatin |

Made up to 1 litre with $dH_2O$

**Preparation of DE52.**

Add several volumes of 0.05M HCl to DE52 resin and chevk pH is below 4.5.

Add 4M NaOH, with constant stirring, until pH is 7.0.

Decant supernatant and add several volumes of LB broth,mix and allow to settle.

Repeat until pH is identical to that of the LB broth.

Resuspend to a final slurry of 75% resin, 25% LB broth.

Store at -20$^oC$.

**BCIP/NBT substrate.**

(for development of nitrocellulose filters from library screen).

**Diethanolamine buffer.**

| | |
|---|---|
| 1.05g | Diethanolamine |
| 0.1g | $MgSO_4$ hexahydrate |

adjust to pH 9.5 and then make up to 100ml with $dH_2O$

**Nitro Blue Tetrazolium (NBT).**

0.5g NBT (Sigma, N-6639) in 10ml of 70% Dimethylformamide

**Bromochloroindolyl phosphate (BCIP).**

0.5g BCIP (Sigma, B-9021) in 10ml of 100% Dimethylformamide

**Developing solution.**

| | |
|---|---|
| 10ml | Diethanolamine buffer |
| 66ul | NBT |
| 33ul | BCIP |

Made up immediately before use.

## Appendix 2.

### Bovine serum samples.

**B7, B8** and **B9** : Three calves orally infected with normal *D.viviparus* $L_3$ and blood sampled on the following days. Day 0 = 2000 $L_3$, Day 48 = 2000 $L_3$, Day 76 = 10,000 $L_3$, Day 103 = 20,000 $L_3$, Day 127 = 20,000 $L_3$, Day 190 = 20,000 $L_3$ and Day 219 = 20,000 $L_3$. The cattle were solidly immune when challenged 4 weeks after the final dose. Unless otherwise stated, the hyperimmune serum used was taken 3 weeks after the third infection.

**038** and **039** : Two calves orally infected with 40krad irradiated *D.viviparus* $L_3$ and blood sampled on the following days. Day 0 = 5000 $L_3$, Day 21 = 10,000 $L_3$, Day 45 = 10,000 $L_3$, Day 64 = 20,000 $L_3$, Day 84 = 20,000$L_3$ and Day 105 = 20,000 $L_3$. The cattle were solidly immune when challenged 4 weeks after the final dose. Unless otherwise stated the hyperimmune serum used was taken 3 weeks after the 3rd infection.

**G17** and **B45** : Two calves intravenously infected with with 100 krad irradiated *D.viviparus* $L_3$ and blood sampled on the following days. Day 0 = 5000 $L_3$, Day 21 = 10,000 $L_3$, Day 51 = 10,000 $L_3$, Day 86 = 10,000 $L_3$, Day 104 = 20,000$L_3$, Day 124 = 20,000 $L_3$ and Day 149 = 20,000 $L_3$. The cattle were solidly immune when challenged 4 weeks after the final dose. Unless otherwise stated, the hyperimmune serum used was taken 3 weeks after the 3rd infection.

**29, 31** and **30** : Three calves orally immunised by oral infection with 10,000 40krad irradiated *D.viviparus* $L_3$ on days 0 and 28. They were solidly immune when orally challenged 4 weeks after the second immunisation with 5000 normal $L_3$. Blood samples used for the blots were taken 3 weeks after the second immunisation.

**025** and **028** : Three calves intravenously infected with 40,000 100krad irradiated *D.viviparus* $L_3$ on days 0 and 24. They were solidly immune when orally challenged 4 weeks after the second immunisation with 5000 normal $L_3$. Blood samples used for the blots were taken 3 weeks after the second immunisation.