IMMUNITY TO *DICTYOCAULUS VIVIPARUS* INFECTION

by

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A thesis submitted for the degree of Doctor of Philosophy
in the Faculty of Veterinary Medicine of the University
of Glasgow

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July 1990

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TABLE OF CONTENTS

TABLE OF CONTENTS (Cont'd) Page no.

ACKNOWLEDGEMENTS

I would like to express my appreciation for the generous encouragement and help which I have received during the course of this work. Especially, I wish to thank most sincerely my supervisor, Professor George M. Urquhart, for the guidance and encouragement so readily and ably given during the past three years. Also Dr. M.W. Kennedy and Professor J.L. Duncan for their wise counsel and stimulating discussion.

For his expert assistance throughout, I wish to thank Mr. K. Bairden, the departmental superintendent.

I am also indebted to Miss C. Britton, Miss J. McKeand and Mr. J. Gilleard with whom I collaborated in some of the experiments described in Chapters five and six of this thesis.

I would also like to express my thanks to the staff of the Department of Veterinary Parasitology for their willing and able assistance. My thanks are also due to Mrs. J. Nybo for her skilled typing of the manuscript.

I am grateful to the Consejo Nacional de Ciencia y Tecnologia (CONACyT), de los Estados Unidos Mexicanos, for granting me a scholarship to carry out this work, and to the United Kingdom Committee of Vice-Chancellors and Principals from whom I received an ORS award.

Finally, this project was undertaken with the support of a research grant from The Wellcome Trust, London.

I would like to dedicate this thesis

to Maureen y a mis Padres

DECLARATION

The work described in this thesis is original and has not been submitted in any form to any other university. It was carried out by the author in the Department of Veterinary Parasitology, Veterinary School, University of Glasgow, under the joint supervision of Professor George M. Urquhart and Dr. M.W. Kennedy

LIST OF ABBREVIATIONS

APS	=	Ammonium persulphate
CF	=	Complement fixation
cm	=	Centimetre
cu	=	Cubic
ELISA	=	Enzyme-linked immunosorbent assay
FCA	=	Freunds complete adjuvant
FIA	=	Freunds incomplete adjuvant
E/S	=	Excretory/secretory
Fc	=	Fragment crystallizable
g	=	Gram
Gy	=	Gray
Ig	=	Immunoglobulin
IHA	=	Indirect haemagglutination
IP	=	Intraperitoneal
IV	=	Intravenous
kDa	=	Kilodalton
kg	=	Kilogram
Kr	=	Kilorad
lb	=	Pound
M	=	Molar
mA	=	Milliampere
mM	=	Millimolar
mAb	=	Monoclonal antibody
mg	=	Milligram
ml	=	Millilitre
mm	=	Millimetre
uCi	=	Microcurie
ug	=	Microgram
ul	=	Microlitre
um	=	Micrometre
uM	=	Micromolar
nm	=	Nanometre
OPD	=	Ortho-phenylene-diamine
PBS	=	Phosphate buffered solution
PAGE	=	Poly-acrylamide gel electrophoresis
RPM	=	Respirations per minute
SC	=	Subcutaneous
SDS	=	Sodium-dodecyl sulphate
TCA	=	Trichloroacetic acid
TEM	=	Triethylenemelamine
V	=	Volt
WBC	=	White blood cell

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SUMMARY

The studies reported in this thesis were conducted in order to obtain a better understanding of immunity to Dictyocaulus viviparus infection.

The first two chapters are concerned with a Literature Review and Materials and Methods, while the first experimental chapter, Chapter three, deals with the use of the guinea pig as a model for immunological studies of D. viviparus infection. The results showed that a high degree of protection could be achieved using soluble extracts of sonicated third stage larvae with FCA or liposomes as adjuvant which indicated that major protective antigens were present in this stage. This was confirmed when animals were vaccinated on two occasions with larvae irradiated at 1000 Gy, which do not develop beyond the third stage. Such guinea pigs, after challenge, showed a reduction of 94% in worm recovery.

Chapter four dealt with vaccination and passive transfer of immunity in calves. As found with the guinea pigs, it was shown that calves vaccinated with larvae irradiated at 1000 Gy were highly protected against challenge. A reduction of 76% in worm recovery after challenge was obtained after two doses of the irradiated L_3, while a regimen of seven infections gave 100% protection. The result of these experiments, albeit in a small number of calves, strongly suggest that the L_3 possess major antigens responsible for protection.

By means of the ELISA technique using mAb's against bovine immunoglobulins, a high non-specific IgM reaction to the L_3 soluble homogenates was detected in all negative and positive sera analysed. The presence of this heterophile antibody is discussed.

With regard to the passive transfer of immunity, it was confirmed that immune sera obtained from normal larval infections of calves is capable of conferring protection against challenge. A new observation was that the lungs of the serum recipients, after challenge, presented several hundred greenish-yellow lymphoid nodules, which, in the literature, have been related to protection and are typically found after challenge of calves immunised by natural infection or vaccination. On the other hand, the transfer of immune sera raised against 1000 Gy-irradiated L_3 did not protect at all and no nodules were observed. Possible explanations for this discrepancy are discussed.

In Chapter five, the results of experiments using quantitative immunofluorescence on the surface of L_3 and adult parasites are reported. It was shown that the surfaces of the third stage larvae and adult D. viviparus are antigenic and that the exposed antigens are both stage and species-specific. It was also observed that a non-specific IgM binding, similar to that encountered in the ELISA occurred when normal sera was used against the surface of exsheathed third stage larvae. This was not the case when adult parasites were used.

Chapter six is concerned with the electrophoretic analysis of soluble homogenates of adult and third stage larvae and E/S products of adult D. viviparus. It was found that most of the peptides detected with Coomassie blue staining were common between larval and adult stages, although some were specific to each stage. Immunoprecipitations and SDS-PAGE using E/S materials showed that bovine and guinea pig immune serum from third stage larval infections recognised most of the polypeptides present in the E/S, which indicated that these precipitated molecules are expressed by larval as well as adult parasites or are cross-reactive between stages. The possible potential of this observation in future vaccine strategies is discussed.

In the concluding experimental chapter, Chapter seven, the development of immunity to D. viviparus infection was studied over a period of two years in calves treated with a formulation of pour-on ivermectin during their first season of grazing on infected pasture. The results showed that treatment at three, eight and 13 weeks does not prevent calves from developing protective immunity against natural or artificial challenge with D. viviparus and that the immunity developed against the parasite is strong enough to prevent clinical signs during the second grazing season. It was also shown that the ELISA is a good method for detecting early infections in calves and that, as reported previously, the levels of antibody measured are not indicative of the degree of immunity, but of challenge.

CHAPTER ONE

GENERAL INTRODUCTION

The cattle disease known as Parasitic Bronchitis, caused by the nematode Dictyocaulus viviparus, has been recognised since the XVII century.

Nicholls in 1755 in a paper entitled "An account of worms in animal bodies", gives the first clear description of a disease known as Husk that "rarely affects animals older than one year producing a short dry cough", and that is caused by "small tape worms of about two inches long".

A review of the disease from its recognition until 1920 was presented by Daubney (1920). In his paper some historical facts are presented: Bloch (1782) gave the parasite its first name, Gordius viviparus, stating that Camper (1780) had found it to be the cause of a disease of cattle. Rudolphi (1809), redescribed the parasite and gave it the name of Strongylus vitulorum. Some years later, Gurlt (1831), gave another description of the lungworm under the name of Strongylus micrurus Mehlis. In 1907 the genus Dictyocaulus was created with the species viviparus affecting cattle and deer, filaria affecting sheep and goats and arnfieldi affecting horses and donkeys (Railliet and Henry, 1907).

Daubney (1920) also suggests, from his own observations, that the infection may occur by the ingestion of the final-stage larvae and the probability of a blood stream migration from the digestive tract to the lungs.

Bovine parasitic bronchitis has caused great economic losses in temperate climates for many years (Urquhart, Armour, Duncan, Dunn and Jennings, 1987). In response to this economic threat

many studies were instigated in the early 1950's with the purpose of finding a suitable way of controlling this disease. The work of a group of researchers at the University of Glasgow, in the course of a systematic study embracing epidemiology, pathology and immunology, achieved a most important advance in this area with the development of a vaccine which utilises the immunogenic properties of infective larvae attenuated by X-rays (Jarrett, Jennings, Martin, McIntyre, Mulligan, Sharp and Urquhart, 1958a).

As a result, in 1959, the commercial husk vaccine "Dictol" was launched (originally Allen and Hanburys Ltd., now Pitman Moore) and it, and a similar product (Huskvac, Intervet) are still widely used 30 years later. Paradoxically, although the vaccine has proved to be a success, it has been detrimental to some extent to the study of the disease, by limiting research funding in this field.

This thesis is concerned with studies on D. viviparus infection in calves and guinea pigs directed towards a better understanding of the identity of the source of protective antigens and the mechanisms underlying the immune response.

REVIEW OF LITERATURE

LIFE CYCLE

Dictyocaulus viviparus, often regarded as a member of the Superfamily Trichostrongyloidea, has a direct cycle, having a free-living phase followed by a parasitic phase within the host.

Dictyocaulus larvae are different from other trichostrongyloids e.g. Ostertagia and Cooperia in that they are slow moving and apparently do not feed in the preparasitic stages (Leuckhart, 1866; Maupas and Seurat, 1912).

The infective third stage larva is always surrounded by the cuticle of the second stage larva (L_2) and occasionally also by the cuticle of the first stage larvae (L_1) and they apparently rely for nutrition on their intestinal cells which are filled with dark brown food granules (Pfeiffer and Supperer, 1980). Infection of the host takes place by the ingestion of these third stage larvae (Daubney, 1920), after which they probably exsheath in the rumen or abomasum and penetrate into the wall of the small intestine (Smythe, 1937; Jarrett, McIntyre and Urquhart, 1957; Poynter, Jones, Nelson, Peacock, Robinson, Silverman and Terry, 1960), from which they travel via the lymphatic vessels to the mesenteric lymph nodes; there they are thought to undergo a moult to the fourth stage (L_4). After reaching the thoracic duct, the larvae migrate in the cranial vena cava to the right side of the heart, and via the pulmonary artery they reach the capillary bed of the lung. There, the larvae break through into the alveoli, and migrate to the bronchioles and small bronchi; after a final moult they mature to the adult stage in the upper bronchi.

The period of time from ingestion of the larvae until they can be found in the lungs has been a point of controversy for some time. Results from work undertaken in guinea pigs has shown that the L_3 can be found in the lungs of the animals in the first 24 hours after infection (Soliman, 1953; Poynter et al, 1960). Similarly, working with calves, Poynter et al (1960) were able to show that it was possible to find a very small number of L_3 in the lungs after 24 hours of infection with a dose of 200,000 L_3 and 36 hours later with a dose of 4,000 L_3. These results tend to support the theory that migration from the gut to the air passages occurs directly, where subsequent larval development occurs. However, the work of Jarrett, McIntyre and Urquhart (1957), infecting calves with doses of 5,000, 100,000 and 240,000 L_3, showed that microscopic lung lesions only started to appear five days after infection and that the fourth stage larvae was the earliest larval stage found in the lungs. This supports, at least in the natural host, the theory that the life cycle typically involves migration through the lymph nodes where the third moult occurs before entering the lymphatic vessels.

After maturing in the lung, the adult parasites mate in the larger air passages after which larvated eggs are laid. These hatch almost immediately and the first stage larvae pass up the trachea to the oesophagus where they are swallowed and voided with the faeces. Under optimal conditions, the L_1 develops to the infective L_3 in about one week.

PATHOGENESIS

The first detailed study of the pathogenesis of parasitic bronchitis was provided by the work of Jarrett, McIntyre and Urquhart (1957). They were able to reproduce all the lesions seen in field cases (Jarrett, McIntyre and Urquhart, 1954), by experimentally infecting young calves with different larval doses and slaughtering them at various times after infection. As a result of their work, Jarrett, Jennings, McIntyre, Mulligan, Sharp and Urquhart (1960a), subsequently divided the disease into four clinical phases.

1. Penetration phase

This phase covers the period from day 1 to day 7. It has no clinical importance as the larvae are presumably travelling through the lymphatic system to the lungs. However, Poynter et al (1960), showed that after 24 hours the lungs of calves infected with exceptionally large larval doses of around 200,000 L_3 presented numerous petechial haemorrhages associated with the penetration of the larvae from the capillaries into the alveoli.

2. Prepatent phase

This phase covers the period from day 8 to day 25. The presence of larvae in the alveoli provokes a host response which leads to a temporary blockage of bronchioles and small bronchi with a cellular infiltrate, principally of eosinophils, which prevents air from entering the alveoli; this causes foci of collapse after the air in the alveoli is absorbed into the blood.

In the third week, coughing and a marked increase in the respiratory rate (70-80 respirations per minute, (RPM)) occurs in typical cases. Death can occur in heavily infected animals as a consequence of complications such as pulmonary oedema and emphysema leading to heart failure.

If a necropsy is performed at the end of this period, many young adult worms are found in a frothy exudate in the bronchi.

3. Patent phase

This stage covers the period from around day 26 to day 55. The adult sexually mature lungworms are present in the bronchi causing much mucus exudate containing neutrophils, eosinophils and parasite eggs, which block the air passages. A marked consolidation of the lung lobules is produced when the eggs are aspirated into the lung tissue. This causes a primary parasitic pneumonia in which macrophages and giant cells surround these aspirated eggs and first stage larvae. In this phase a harsh bronchial breathing is evident on auscultation and the respiratory rate may rise to over 100 per minute with longer periods of coughing. Larvae are detected in the faeces from the beginning of this phase. If the animals do not die, regression of the clinical signs usually starts to occur after the sixth or seventh week.

4. Postpatent phase

This is the period in which the lungworm burden is eliminated and the majority of infected animals recover. It begins at day 56 and comes to an end around 80 to 90 days after

the initial infection. Respiratory rates and coughing decrease, but it is not until several months later that a complete recovery is achieved. Not all animals recover and some present a sudden increase of dyspnoea caused by an alveolar "epithelialisation". This is a result of the proliferation of Type II pneumocytes, which often involves whole lobes of the lung; the precise etiology of the lesion is unknown. This usually leads rapidly to the death of affected animals. At necropsy, large areas of consolidated red rubbery lung are present, despite the fact that almost all the worms have been eliminated.

Although vaccinated calves or recovered clinical cases develop a strong immunity against the disease, they may show clinical signs after reinfection such as coughing and tachypnoea, especially after a heavy challenge. This is due to ingested larvae which reach the lungs and are killed by the hosts' immune reaction in the small bronchioles, and is known as the "reinfection syndrome". A marked focal tissue reaction to the dead larvae in the form of lymphoid nodules, is produced. Macroscopically, these nodules are from 2.0 to 4.0 mm in diameter varying in colour from grey to greenish. They occur throughout the lung, although they are more frequently seen in the diaphragmatic lobes. Histological examination shows that the nodules are composed of lymphoreticular tissue surrounding a mass of degenerating eosinophils enclosing the remains of the parasite (Pirie, Doyle, McIntyre and Armour, 1971).

EPIDEMIOLOGY

I. Development and survival of infective larvae on pasture

First stage larvae passed in the faeces apparently do not feed and thus environment has no significance as a source of nutrition (Croll, 1973). However, it is influential in providing suitable conditions for the survival and development of the infective third stage larvae. As noted previously, the free living stages of the lungworm larvae live on reserve food substances which are generously stored in the form of granules in the intestinal cells.

The slow moving larvae moult twice to become the infective third stage larvae which can be found in large numbers on the surface of the faecal pat. The developmental time from the newly shed larvae to infective stage depends on climatic conditions such as humidity and temperature and has been shown to vary from three to 28 days (Taylor, 1942; Porter, 1942; Orlov, 1946).

Infective larvae do not withstand desiccation at any stage (Daubney, 1920; Rose, 1956); however they can survive sub-zero temperatures in the laboratory (Daubney, 1920) and out of doors (Rose, 1956). There are numerous reports that they can survive on pasture during temperate winters (Jarrett, McIntyre, Urquhart and Bell, 1955b; Michel and Shand, 1955; Rose, 1956; Allan and Baxter, 1957; Enigk and Duwel, 1962; Duncan, Armour, Bairden, Urquhart and Jorgensen, 1979). Also, Gupta and Gibbs (1970) in Canada and Jorgensen (1980) in Denmark, were able to demonstrate that even more severe winter conditions were not a limiting factor for lungworm survival. These overwintered larvae are

thought to be an important source of primary infection of young calves in the spring of the following year (Allan and Baxter, 1957; Oakley, 1977; Duncan et al, 1979). Such infected animals may carry a very light infection and present few, if any, clinical signs. However, they seed the pasture with sufficient numbers of larvae which are frequently responsible for outbreaks of the disease later in the year.

Circumstantial evidence that lungworm larvae may survive for several months in the soil was documented for the first time by Nelson (1977). Subsequently, Armour, Al Saqur, Bairden, Duncan and Urquhart (1980) were able to occasionally recover lungworm larvae from the soil of pasture ungrazed for a period of 12 months, from August to July. Therefore, it seems clear that under certain conditions lungworm larvae can survive in the environment for long periods of time during the winter and spring and that such larval population may even be high enough to sporadically give rise to outbreaks of clinical disease during the following grazing season.

In practice, this is often difficult to prove because of the ubiquity of the Pilobolus fungus and the possibility of pasture contamination by deer which may act as alternative hosts.

II. Larval migration

Lungworm larvae are relatively sluggish and only small numbers actively leave the cowpat (Michel and Rose, 1954). Because cattle are known to avoid grass around faecal pats (Michel, 1955b), external agents are therefore of great

importance for efficient transmission of the larvae to herbage. Thus, rain may wash larvae from the faeces, larvae may be carried from contaminated to clean grazing by tractor wheels, animals' hooves or the feet of men. Earthworms may also be involved as stated by Cobbold (1886), who by experimenting with eggs of D. viviparus in moist earth was able to demonstrate some larvae in the intestine of an earthworm; more recently, Oakley (1981b) isolated infective lungworm larvae in earthworms recovered from soil.

A special relationship has also been demonstrated between D. viviparus larvae and a fungus of the species Pilobolus (Robinson, 1962). This fungus, found throughout the British Isles, grows on the surface of the faecal pat from spores which have passed through the bovine alimentary tract. Infective lungworm larvae, from the surface of the faecal pat, climb the sporangiophore of the fungi on to the sporangia (Doncaster, 1981); these, in order to ensure dissemination of spores, explode violently propelling the spores and larvae up to a distance of ten feet from the faecal pat (Robinson, 1962); in windy conditions this distance could presumably be much greater. For these reasons no pasture in an endemic area can be thought of as clean.

III. The carrier animal

In order to understand the importance that the carrier animal plays in the epidemiology of D. viviparus, it may be useful to divide this phenomenon into two main categories namely: 1. Silent carriers and 2. Arrested larval development.

The term "silent carriers" was introduced by Wetzel (1948), to describe naturally infected animals, usually yearlings, which apart from excreting larvae, did not show signs of the disease. He attributed this to a less efficient host response in calves as compared with that of adult cattle.

Cunningham, Jarrett, McIntyre and Urquhart (1956), in a knackery and farm study were able to describe the phenomenon and interpreted it as follows "cases which have recovered from a severe attack of parasitic bronchitis are not only immune to reinfection but also usually throw off their entire primary infection. This may mean that most carriers are animals which have not had a heavy infection and have therefore never acquired a high degree of immunity. This hypothesis is supported by the fact that in the present survey the carriers (i.e. yearlings excreting larvae during the winter and spring) from eight of nine farms for which accurate individual histories were available had not been clinically affected the previous year, although animals of the same age which had been grazing with them had been markedly affected". Shedding of lungworm larvae during the winter and early spring has been observed in Germany (Wetzel, 1948), Scotland (Jarrett et al, 1955b), Canada (Gupta and Gibbs, 1970) and Austria (Supperer and Pfeiffer, 1971) and in all of these studies a low prevalence of silent carriers in young animals after their first grazing season was observed. As a result of this work, it can be concluded that silent carriers may play a part in the lungworm pasture contamination in the spring.

The occurrence of arrested D. viviparus larvae was reported for the first time by Taylor (1951) when he noted that many immature lungworms were observed in the lungs of calves which had been withheld from infection from periods longer than the prepatent period of infection. Since then this has been confirmed in natural or experimental infections by Taylor and Michel (1952); Michel (1955a); Supperer and Pfeiffer (1971); Eisenegger and Eckert (1975); Gupta and Gibbs (1975); Inderbitzin (1976) and Pfeiffer (1976). In contrast to the gastrointestinal nematodes in which arrestment occurs at the early fourth larval stage, in D. viviparus it occurs in the early fifth stage (Supperer and Pfeiffer, 1971; Inderbitzin, 1976), although a mixture of late fourth and early fifth stages was reported by Gupta and Gibbs (1975).

In order to explain the presence of arrested lungworm larvae, Michel (1955a) and Michel, MacKenzie, Bracewell, Cornwell, Elliot, Herbert, Holman and Sinclair (1965) suggested that it was connected with resistance of the host or depended on previous experience of infection. Jorgensen (1981) made a similar statement when he postulated that larvae which arrive later in the bronchial tree in resistant calves may be smaller and their development retarded.

An alternative theory to explain arrested development in D. viviparus infection was produced by the demonstration that infective larvae, subjected to a prolonged period of chilling before being administered to calves, undergo arrested development in the lungs (Inderbitzen, 1976; Pfeiffer, 1976; Oakley, 1979).

This brings the phenomenon into line with the hypobiosis of Ostertagia larvae which is generally attributed, at least in Europe, to the ingestion of infective larvae in the late autumn during a period of declining temperatures (Anderson, Armour, Jennings, Ritchie and Urquhart, 1969). This theory was summarised by Jorgensen (1981) "as an evolutionary adaptation towards the host living in a free state (i.e. without restrictions imposed by man). Then the phenomenon of arrested growth will have two implications. It will render the infection less harmful during the critical winter period and it will ensure that the infection is carried through the winter, not in order to survive winter climate but to ensure contamination of new grazing after the host's winter migration".

Resistant or partially resistant animals, or an evolutionary adaptation associated with the cooling of larvae during the colder months could each, or together, explain the presence of arrested lungworm larvae, and although these theories are somewhat different, both indicate that the arrested development suffered by D. viviparus during the winter period may be an important factor in carrying on the infection from one year to the next.

IMMUNITY

It is well known that a strong immunity to parasitic bronchitis develops in calves which survive an initial infection with D. viviparus (Porter and Cauthen, 1942; Jarrett, Jennings, McIntyre, Mulligan and Urquhart, 1955a; Rubin and Lucker, 1956; Weber, 1958; Jarrett, Jennings, McIntyre, Mulligan, Thomas and

Urquhart, 1959b).

Older animals, not previously exposed, remain relatively susceptible to infection (Selman and Urquhart, 1979). However, it has recently been shown that a degree of age immunity does exist, since yearlings without previous contact with the parasite had greater resistance to lungworm infection than calves as measured by worm burdens, size and percentage of worms established in the lungs (Taylor, Mallon, Green, McLoughlin and Bryson, 1988). Armour (1980) also postulated that age may influence the course of a helminth infection by increasing the patent period, reducing the pathogenic effect and possibly increasing the ability to acquire immunity.

The stage or stages of D. viviparus required to produce resistance to infection was studied by Kendall (1965). Using diethylcarbamazine to terminate the infection as early as possible, he concluded that young stages have a potent effect on the development of resistance, but also that they are not exclusively involved and that the latter stages are necessary for complete resistance. Cornwell (1963a), on the other hand, and in a similar experiment using diethylcarbamazine, concludes that "By the time the worms have developed to the stage where they are susceptible in large measure to the action of the drug (14-15 days), the host has been stimulated by antigenic materials capable of giving immunity".

Passive immunisation

An experiment to induce protection against D. viviparus by passive immunisation was reported by Jarrett, Jennings, McIntyre, Mulligan and Urquhart (1955a). Immune serum was obtained from six recovered field cases which received a further reinfection with doses ranging from 50,000 to 200,000 infective L_3 before being bled. After precipitation and dialysis, a final volume of 7.5 litres of globulin preparation with a protein concentration of 0.108 g/ml was recovered. Five calves received 500 ml of the immune serum daily for three days and were infected two days later with 4,000 infective lungworm larvae. The results based on worm recovery at necropsy 30 days after infection showed a 95% reduction when compared with a control group. Rubin and Weber (1955) using immune serum from an animal which had received 874,000 L_3 during a period of one year, immunised four calves intravenously at the time of infection at rates of 0.1, 1.0, 2.5 and 5.0 ml of the immune serum per lb of body weight, and one with 5.0 ml per lb, eight days after infection. After a challenge with 50,000 L_3, the two calves which received the highest dose of the immune serum survived and the others died, from which they concluded that a passive transfer of protective substances had occurred.

The value of these studies was that they showed the possibility of the eventual control of D. viviparus by immunological methods.

Active immunisation

Studies using adult homogenate (Jarrett, Jennings, McIntyre, Mulligan and Urquhart, 1960b) and adult worms plus third stage larval homogenate (Wade, Swanson, Fox, Simpson and Malewitz, 1962) showed a significant but minimal reduction in the number of worms recovered after a challenge infection when compared with the control groups. In addition, as stated by Jarrett et al (1960b) the method seemed of little practical value "since worms for the production of such a vaccine would have to be obtained from animals infected with the disease and the cost of this would be prohibitive".

Cornwell (1962b) showed in a study comprising eight calves, four treated and four controls, that a 70% worm reduction could be achieved after intraperitoneal administration of 2,500 fourth stage larvae followed by a second administration 21 days later of 1,000 L_4 subcutaneously and 2,500 L_4 intraperitoneally.

Vaccination

The possibility that an adequate degree of immunity could be obtained by the use of attenuated larvae capable of migrating through the gut and developing no further than the mesenteric nodes was presented by Jarrett, Jennings, McIntyre, Mulligan and Urquhart (1958b). The attenuation method adopted was X-irradiation. In a study designed to find the most suitable degree of X-irradiation in order to obtain the best immunogenic effect, Jarrett, Jennings, McIntyre, Mulligan and Urquhart (1960c), irradiated larvae at the levels of 20,000, 40,000 and 60,000 roentgens. Calves were immunised orally with one dose of

4,000 L_3 irradiated at those levels. The number of worms found in the animals of the various groups 35 days after challenge with 4,000 infective L_3, showed that the larvae irradiated at 40,000 roentgens produced a worm reduction of 98.5% and that the lung lesion score was minimal.

Calves immunised with larvae irradiated at 20,000 roentgens were not protected to the same extent, while those immunised with larvae irradiated to 60,000 roentgens were not protected at all presumably attributed to their failure to penetrate the intestine.

An experiment to assess the protection conferred by this form of vaccination under field conditions was reported by Jarrett et al (1958a). In this trial, the number of irradiated larvae was reduced to 1,000 to prevent fatal disease in case of a failure in the larval attenuation process. After a very high pasture challenge, ten out of 12 control calves died compared with three out of 15 vaccinates. It was therefore concluded that the 1,000 larvae used for vaccination were probably sufficient to protect the majority of animals at risk in the field. However, it was thought that two doses might provide a higher degree of immunity. Thus, Jarrett, Jennings, McIntyre, Mulligan, Sharp and Urquhart (1959a) performed an experiment in which animals were given an initial dose of 1,000 irradiated larvae and a second dose of 4,000, 2,000 or 1,000 irradiated L_3 after 42 days. The animals were challenged 50 days later with a dose of 10,000 infective lungworm larvae. Another group of animals were given 1,000 irradiated larvae at the start of the experiment and were

challenged at the same time and with the same number of L_3 as the double vaccinated groups. The results showed that in none of the groups that received irradiated larvae on two occasions were adult worms found at necropsy 33 days after challenge. In contrast, calves given a single dose of irradiated larvae harboured an average of 820 worms, similar to the unvaccinated control animals which had a mean of 958 worms.

Another small scale pasture trial was carried out using two immunising doses of vaccine, but this time, the dose interval was reduced to 30 days instead of the previous 42. The vaccinated animals together with the controls were turned out on a field in which 38 of 40 calves had died of parasitic bronchitis that year or the year before. During the trial, there was a marked difference in clinical signs, faecal larval output and growth rates in favour of the vaccinated animals. At necropsy, no adult worms were recovered from the vaccinated calves; in contrast, a mean of 440 mature worms was recovered from the controls (Jarrett, Jennings, McIntyre, Mulligan and Sharp, 1961).

These experiments showed that the X-irradiated vaccine protected against clinical lungworm disease and that it was of practical value under farming conditions.

Subsequent to this work, researchers in many parts of the world have confirmed the efficacy of the lungworm vaccine (e.g. in Britain, Nelson, Jones and Peacock, 1961; Poynter, Peacock and Menear, 1970; in the U.S.A., Engelbrecht, 1961; in Sweden, Olson, 1962; in France, Pierre, Euzeby, Malher and Jeannin, 1961; in Ireland, Downey, 1968).

Cornwell and Jones (1970b; c; d; and 1971) studied the possibility of attenuating D. viviparus larvae using the cytotoxic chemical agent Triethylenemelamine (TEM). After a double vaccination with 1,000 L_3 attenuated with 0.7% TEM, they were able to show a high degree of immunity to experimental challenge, similar to the one produced by the X-irradiated vaccine. However, this vaccine was never developed comercially.

Bonazzi, Grimshaw, Bairden, Armour and Gettinby (1983) in Scotland and Gennari and Duncan (1983) in Brazil, have used gamma-irradiated larvae from a ^{60}Co source instead of X-irradiation. In both cases, it was found that the gamma-irradiation larvae were as efficient in the immunisation of calves as the X-irradiated L_3. A vaccine was subsequently developed in the Netherlands using gamma-irradiation as a source of attenuation and is produced commercially in the U.K. (Huskvac, Intervet International, Cambridge).

Eosinophil leucocyte response

It has long been recognised that tissue and blood eosinophilia are characteristic features of helminth infections (McLaren, 1980). Histological studies have shown that eosinophils accumulate preferentially in close proximity to tissue invasive or tissue migratory stages (Conrad, 1971) and although the tissue reactions involve a number of different leucocytes, eosinophils predominate from the beginning to the end of the infection (Beeson and Bass, 1977).

Eosinophils have been shown to adhere and kill newborn and infective larvae of Trichinella spiralis via an antibody-dependent, complement independent mechanism (Bass and Szejda, 1979a; Mackenzie, Jungery, Taylor and Ogilvie, 1980). The exact mechanism has not been elucidated although the secretion granules and hydrogen peroxide generated from the oxidative metabolic burst have been shown to possess larvicidal properties (Bass and Szejda, 1979b; Wassom and Gleich, 1979).

Knapp and Oakley (1981) observed in vitro that eosinophils became adherent to the surface of D. viviparus larvae earlier than other blood cells. Cell adherence was associated with an antibody-dependent, complement independent mechanism in hyperimmune sera and with a heat labile factor in normal serum presumed to be complement.

Observations of the blood eosinophil response in calves to normal infective and irradiated larvae of D. viviparus were reported by Weber and Rubin (1958), Cornwell (1962a) and Mackenzie and Michel (1964). Normal larvae elicited two peaks of blood eosinophilia, the first at nine to 15 days post infection and a higher one at about 40 days after infection. After reinfection, an even higher response was evident. Using irradiated larvae, the first dose stimulated a single response with a peak about 18 days, the second dose gave rise to a similar level at 14 days. Vaccinated animals after challenge with normal larvae developed an eosinophilia four times higher and with a double peak at 14 and 40 days.

Measurement of immunity

The responses to primary and secondary infections as well as the response to vaccination against D. viviparus have been followed using serological tests. Weber (1958), using the complement fixation (CF) test with a whole worm antigen prepared by a low temperature method, found an initial antibody response 12 days after infection which reached its peak four days later and remained at that level until the 63rd day. Such a response was seen both in calves exposed for the first time or in re-exposed animals.

Jarrett et al (1959b) and Michel and Cornwell (1959), using a heated adult whole worm antigen and the CF test were unable to confirm the results of Weber (1958). Both groups of researchers found that antibody titres required considerably longer to develop than did resistance to infection and no correlation between titres and resistance could be demonstrated.

Cornwell and Michel (1960) investigated the CF antibody response of calves exposed to natural and experimental infections. The results showed no essential difference in pattern of antibody response between grazing animals and indoor calves given a single dose of larvae.

Serological studies using the CF test on calves vaccinated with irradiated larvae were reported by Cornwell (1960a). His results showed that antibody response to the first dose of vaccine was usually very low, while the second vaccination "boosted" the titre in the low or middle range. Cornwell (1960b) followed the CF titre of calves vaccinated twice with irradiated

larvae and subsequently exposed to challenge. The majority of the challenged animals showed a titre increase after two weeks. Cornwell (1963b) also found cross-reactions between immune sera of D. viviparus infected animals and various other parasite antigens, especially those of Ascaris spp, Haemonchus contortus and Metastrongylus apri.

Bokhout, Boon and Hendriks (1979), suggested that more specific reactions could have been achieved if a non-denatured antigen had been used. Working with soluble antigens from adult worms in an indirect haemagglutination (IHA) test, they showed that the IHA findings agreed with 80% of the clinical field diagnoses. The authors found that titres were present 14 days after infection, reached a peak at day 45 and were still present several months later.

The enzyme-linked immunosorbent assay (ELISA) was applied for the first time to detect antibodies against lungworm by Marius, Bernard, Raynaud, Pery and Luffau (1979). They were able to show antibodies to adult D. viviparus antigen in serum and nasal secretions. Boon, Kloosterman and van den Brink (1982) using ELISA in artificially and naturally infected calves concluded that the ELISA titres correlate well with IHA titres and that the technique could be useful for herd diagnosis and survey work.

This was subsequently shown by Boon, Kloosterman and van der Lende (1984) in a study of the incidence of D. viviparus in the Netherlands.

TREATMENT

Many treatments have been used against D. viviparus infection; among them, diethylcarbamazine, tetramisole-levamisole, benzimidazoles and ivermectin have proved to be the most effective against one or more of the parasitic lungworm stages.

Diethylcarbamazine

Parker (1957) reported for the first time the potential value of diethylcarbamazine against D. viviparus. In a natural outbreak of parasitic bronchitis, an oral dose of 2.2 mg/kg of body weight prevented deaths in 37 calves, while 11 out of 37 control animals died or had to be slaughtered in extremis. In experiments with controlled infections, doses of 25 mg/kg daily for five days (14-18 days after infection) proved to be the best. Treatment at later stages of the infection, did not prevent death. Parker and Roberts (1958), concluded that doses of 22 mg/kg of diethylcarbamazine daily for five consecutive days controlled lungworm infection in the prepatent stage.

Later, Jarrett, McIntyre and Sharp (1962), using the same dose, found the drug to be 99.96% effective against 15-17 days old lungworms, but only 56.34% effective in reducing 31-33 day old infections. Cornwell (1963a), using 44 mg/kg of the drug for four days by the intramuscular route, found it to be very effective against 14-17 post infection lungworms, but it had only a moderate effect against 6-9 or 25-28 days old D. viviparus.

Tetramisole-Levamisole

Tetramisole proved to have a wider range of efficacy against the cattle lungworm (Ross, 1968). Forsyth (1968), states that the dose must be less than 12 mg/kg to prevent mild toxic changes and that the concentration must be less than 12% in order to avoid reactions in the injection site.

Tetramisole was separated into laevo and dextro isomers (Raymakers, Roevens and Janssen, 1967), known as levamisole and dexamisole. Levamisole proved to be as potent as dexamisole at half of the dose (Rohrbacher, Emro and Waletsky, 1967). Thus, using levamisole in place of tetramisole, the safety margin is doubled and the efficacy retained.

Levamisole has for a long time been considered to be one of the best treatments against parasitic bronchitis. Studies by Broome and Lewis (1974), Oakley (1980), Vazquez, Herrera, Najera, Gutierrez and Campos (1980) using doses of 7.5 mg/kg of body weight by the subcutaneous route, have shown an activity of over 95% against a nine day or older parasites and around 85% against younger larvae. Pouplard, Lekeux and Detry (1986), showed that levamisole at doses of 10 mg/kg was over 95% effective against fourth stage larvae of D. viviparus.

Levamisole has also been shown to be highly effective against inhibited D. viviparus larvae (Oakley, 1981a).

Benzimidazoles

The discovery of thiabendazole (Brown, Matzuk, Ilves, Peterson, Harris, Sarett, Egerton, Yakstis, Campbell and Cuckler,

1961), was followed by the development of many chemical derivatives with increased potency. Of these, fenbendazole (Baeder, Baehr, Christ, Duwel, Kellner, Kirsch, Loewe, Schultes, Schuers and Westen, 1974), oxfendazole (Averkin, Beard, Dvorak, Edwards, Fried, Kilian and Schiltz, 1975), albendazole (Theodorides, Gyurik, Kingsbury and Parish, 1976) and febantel (Wollweber, Koelling, Widdig, Thomas, Schulz and Muermann, 1978) have been useful against bovine lungworm infection.

Studies using fenbendazole (Saad and Rubin, 1977; Duwel and Kirsch, 1980), oxfendazole (Downey, 1976; Chalmers, 1979), albendazole (Benz and Ernst, 1978; Downey, 1978) and febantel (Grelck, Horchner and Woehrl, 1978), have shown these compounds to be virtually 100% effective against adults and developing larvae of D. viviparus.

Fenbendazole has also been shown to remove arrested or inhibited stages of the lungworm (Inderbitzin and Eckert, 1978; Pfeiffer, 1978).

Ivermectin

Avermectins are fermentation products of the actinomycete Streptomyces avermitilis (Burg, Miller, Baker, Birbaum, Currie, Hartman, Kong, Monaghan, Olsen, Putter, Tunac, Wallick, Stapley, Oiva and Omura, 1979). Ivermectin, a chemically modified derivative, has proved to be highly effective against adult worms and larval populations, including arrested and developing stages, of the most important cattle nematodes, including D. viviparus (Armour, Bairden and Preston, 1980).

Thus, many studies using ivermectin at doses of 200 ug/kg of body weight, have shown that almost 100% efficacy is achieved against all stages of lungworm (Alva-Valdes, Benz, Wallace, Egerton, Gross and Wooden, 1984; Benz, Ernst and Egerton, 1984; Pouplard et al, 1986).

One feature of particular interest is that ivermectin after injection persists at therapeutic concentrations against most gastrointestinal parasites for two weeks and in the case of D. viviparus for approximately 21 days (Armour, Bairden, Batty, Davison and Ross, 1985). This feature made it the drug of choice where treated calves continued to graze infected pasture. It has also led to the development of a chemoprophylactic regimen against pulmonary and gastrointestinal nematodes known as the "3, 8 and 13 week system" which is specially effective in the prevention of D. viviparus infection (Armour, Bairden, Pirie and Ryan, 1987; Taylor et al, 1988).

CHAPTER TWO

MATERIALS AND METHODS

Animals

Calves

In all experiments, weaned male Ayrshire or Friesian calves were used. These were obtained from local sources where calves were known to be reared under conditions in which infection with helminth larvae was unlikely. At the Veterinary School, the calves were bedded on clean straw which was replaced every two days. They received 1 kg of concentrate and 3 kg of hay daily. Water was available ad libitum. Before infection, their faeces were examined for nematode eggs to ensure that they were not infected.

Guinea pigs

Dunkin Hartley male albino guinea pigs, weighing 200 - 300 g initially, were used throughout the experiments. The animals were housed in plastic cages with wire mesh tops. They were fed on pelleted diet plus lettuce leaves and water ad libitum.

Dictyocaulus viviparus larvae

Infective and 40Kr X-irradiated third stage larvae were provided by Glaxovet, Ware, England and later by Intervet U.K., Cambridge, England. In this text we have used the current term Gray i.e. 100r. Thus, 40Kr equals 400Gy.

For some studies, 1,000Gy gamma-irradiated larvae were utilised. Normal infective larvae were irradiated in a ^{60}Cobalt source chamber (Nuclear Engineering, Berkshire, England) in the Department of Veterinary Physiology of the Veterinary School.

The number of larvae for infection was calculated from the mean number of larvae present in 40 aliquots of 0.025 ml of a

diluted stock, counted using a dissecting microscope.

Infection procedure

Calves

The required number of larvae were counted as described above and diluted with distilled water to a final volume of 20 ml. Calves were held, their mouths opened and each larval suspension poured down the animal's throat. The bottles were then rinsed with distilled water and the washings administered in the same fashion.

Guinea pigs

Guinea pigs were infected with an aliquot of 5,000 or 10,000 L_3 in 0.5 ml to 1.0 ml of water. The animals were lightly anaesthetised using trichloroethylene (Trilene, BDH Chemicals Ltd., Poole, Dorset). Oral infection was achieved by a shortened intravenous cannula (Red Luer, 5 FG, O.D. 1.65 mm, Portex Ltd., Hythe, Kent) connected to a plastic 1.0 mm syringe. In the case of an intraperitoneal infection, the same volumes were used and injected using a 1.0 in, 20 gauge needle.

Exsheathing process

When needed, larvae were exsheathed using 5% Milton's fluid (Milton 2, Richardson-Vicks Ltd., England) equivalent to a final concentration of 0.001 % w/v sodium hypochlorite. The larvae were suspended in this solution for 15 minutes then centrifuged three times at 100 g for five minutes, replacing the supernatant with distilled water each time.

Faecal examination of cattle

Faeces were collected from the rectum and examined for helminth eggs or larvae by a modified McMaster flotation technique (Gordon and Whitlock, 1939) or by a modified Baermann method (Henriksen, 1965).

Modified McMaster method

Three grams of faeces were homogenised with 42 ml of water and the suspension passed through a coarse mesh sieve of 250 microns aperture size (Endecotts Test Sieves Limited, Morden, London). After thorough mixing of the filtrate, 15 ml were withdrawn into a flat bottomed centrifuge tube (capacity 15 ml) and centrifuged at 400 g for two minutes. The supernatant was discarded and the remaining faecal pellet broken up by rotary agitation (Whirlimixer, Griffin and George, Wembley, Middlesex). The tube was then filled to its former level with saturated salt (NaCl) solution and after inverting six times, a volume of the suspension, sufficient to fill both chambers of a McMaster slide (Gelman Hawksley, Harrowden, Northampton) was quickly transferred. The number of larvae under the etched areas of the slide were counted and the result multiplied by 50 to give the number of larvae per gram of faeces.

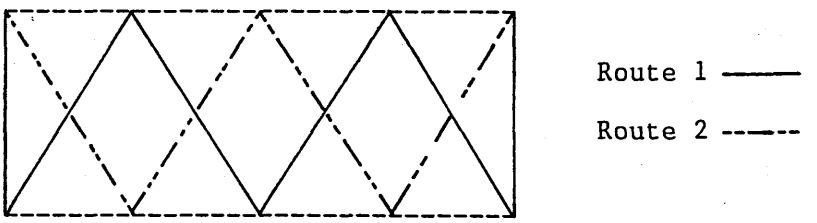
Modified Baermann technique

Ten grams of faeces were placed in gauze and suspended in warm water in a plastic 250 ml conical measure for a minimum of six hours after which both faeces and gauze were discarded. The suspension was allowed to sediment and the supernatant removed to give a final volume of 10 ml. This was well mixed and the larvae

present in a 1 ml aliquot counted and expressed as the number of larvae per gram of faeces.

Analysis of herbage for the presence of D. viviparus L_3

Pasture samples were collected by crossing the experimental plot as shown in the following diagram:



Fifty evenly spaced stops were made along routes 1 and 2 and at each stop four plucks of grass (the amount that could be grasped between thumb and forefinger) were taken, giving a total of 400 plucks per plot. The grass was processed by a method similar to that used by Parfitt (1955).

The samples were treated as follows: the bag containing the grass was weighed and then put into a small hand operated washing machine (Easy Pressure Washer, Classic Supplies Ltd., Leeds). Eight litres of lukewarm water were added, the bag was tied and the machine turned through one hundred revolutions. Before removing the bag and herbage from the machine, a small incision was made in the bag and the washings allowed to pass through a coarse mesh sieve (aperture size 2 mm) into a bucket. The remaining herbage, still in the bag, was then removed and as much fluid recovered from it as possible by squeezing. The grass was then spread on a tray and dried in an incubator at 70°C. When

thoroughly dried, the herbage was again weighed and this dried weight used in the final calculation of numbers of larvae per kilogram dried herbage (L_3/kdh). The washings contained in the bucket were filtered through a 38 micron sieve and the material retained transferred to a beaker. The resultant suspension was divided into two subsamples, one of which was processed using a Baermann apparatus and the other by the bile agar method of Jorgensen (1975). This latter method is used primarily for isolation of D. viviparus larvae from herbage and is based on the stimulating effect of bile on lungworm larvae and the ability of such larvae to migrate from agar gel.

In the first subsample, the traditional Baermann technique was employed as follows:

The larval suspension was drawn through a coarse filter paper (Whatmans Grade 113, 18.5 cm) using a Buchner funnel and vacuum pump, the larvae being retained in the paper. A single milk filter (Maxa Milk Filters, A. McCaskie Ltd., Stirling) was put on top, and the whole inverted and placed on the Baermann filter funnel. After standing for at least six hours, 10 ml of fluid were withdrawn and the larvae in 1 ml identified and counted.

The second subsample was made to a volume of 60 ml and preheated to 37°C in an incubator. To this was added 75 ml of the bile agar mixture (60 ml 3% Difco-Bacto agar plus 15 ml ox bile) at a temperature of 48°C and after gentle mixing, poured on to a wet 20 x 35 cm J-cloth (Johnson and Johnson, Slough) positioned on the base of a flat-bottomed plastic tray. After

setting for 15-30 minutes, the cloth was lifted, wound into a roll and immersed in warm water in a glass cylinder, the roll held in place by means of a plastic straw. Following incubation at 37°C for 12 hours, 10 ml of fluid were drawn off and the larvae in 1 ml differentiated and counted.

Haematological procedures and production of hyperimmune serum

Calves

Prior to the onset of the experimental period and at intervals of one week thereafter, blood was collected from the jugular vein in anticoagulant-free and EDTA vacutainer tubes (Becton and Dickinson, England). Blood smears were prepared and stained by 10% Giemsa in Phosphate Buffered Solution (PBS) pH 7.2 and an eosinophil count made using a X-100 objective and oil immersion. A minimum total of 200 white blood cells (WBC) were counted in each blood smear and the number of eosinophils present expressed as a percentage.

The blood in the anticoagulant-free vacutained tubes, was allowed to clot, the sera removed, centrifuged at 1220 g for 20 minutes and stored at -20°C for serological examination.

Guinea pigs

Blood was obtained by cardiac puncture before and/or seven to 21 days after challenge, allowed to clot at room temperature and then centrifuged at 1200 g for 20 minutes. The sera were stored at -20°C.

Guinea pig hyperimmune sera against normal, 400Gy and 1000Gy irradiated larvae was obtained from pooled serum samples of separate groups of animals. Each animal was infected with 5,000

L_3 21 days apart on three occasions.

Immunising antigens

The antigens used were extracts of adult worms or infective larvae and excretory/secretory (E/S) products of adult worms. The adult parasite extracts were prepared by disruption in ice-cold phosphate buffered saline (PBS) pH 7.2 in a glass tissue homogeniser, centrifugation of the homogenate at 13,000 g at 4°C for ten minutes, and the supernatant stored at -70°C until use.

Extracts of the third stage larvae were prepared by sonication in a MSE Soniprep 150 at 18 um amplitude. The container was cooled by ice and, to keep heating to a minimum, seven one minute burst were interspersed with one minute pauses. The sonication was carried out in ice-cold PBS pH 7.2 and the supernatant collected and stored as above.

Excretory/secretory material was obtained from the supernatant of cultures of adult parasites in Ascaris medium (Kennedy and Qureshi, 1986) for three days and collected daily. E/S was concentrated by centrifugal ultrafiltration using Centricon 10 (Amicon 4206), and the protein concentration estimated using a Coomassie blue-based assay (Pierce 23200). Despite several attempts no measurable protein was obtained from E/S products when L_3 were cultured.

Post-mortem techniques

Calves

Animal were stunned by a captive-bolt pistol and immediately pithed and exanguinated. The lungs were then removed together

with the trachea, oesophagus, heart and pericardial sac. Lungworms were recovered by one or other of the following procedures.

When live worms were required, the air passages were opened completely, starting from the trachea and cutting down to the small bronchioles with fine sharp pointed scissors. Worms were removed and placed in a petri dish containing warm saline for counting.

When live worms were not required, a modification of Inderbitzin's (1976) perfusion technique was employed. The pericardial sac was incised to expose the pulmonary artery in which a small incision was made. Rubber tubing was introduced into the artery and fixed in situ by double ligatures. The remaining great vessels were tied off and water from a mains supply allowed to enter the lungs via the pulmonary artery. Twenty litres were collected from the lungs via the trachea and the parasites concentrated by passing the washings through a 37 micron aperture sieve.

Guinea pigs

Infected guinea pigs were killed with trichoroethylene. The entire lungs and the thoracic portion of the trachea were removed, placed in a stomacher plastic bag containing 20 ml of saline soluion and homogenised in a Stomacher 80 (A.J. Seward UAC House, London, England) until very fine lung fragments were obtained. This suspension was passed through gauze into a flask, and the fluid examined immediately using a dissecting microscope and the parasites counted. The lung tissue remaining in the

gauze was Baermannised in 0.85% saline solution for six to 12 hours at 37°C. The sedimented worms were counted using a dissecting microscope.

Enzyme-Linked Immunosorbent Assay (ELISA)

Antigens

The antigens used were extracts of adult worms or infective larvae. The adult parasite and larval extracts were prepared in the same way as the immunising larval antigens but instead of PBS, Tris poisons pH 8.3 (see Appendix) was used.

Plate coating

PVC flat bottomed microtitre plates (Titertek, Flow Laboratories) were coated with 5 ug of protein (antigen) diluted in 50 ul of coating buffer (see Appendix). Coating was performed at 4°C overnight or for two hours at 37°C.

ELISA technique

The previously antigen coated plates were washed three times in washing buffer (see Appendix). The plates were filled with 50 ul of blocking buffer per well (see Appendix) for 30 minutes to reduce non-specific binding. After washing three times with washing buffer, 50 ul of serum dilutions in diluting buffer (see Appendix) per well were added to the plate wells, starting from a 1:20 dilution.

After a 30 minute incubation at 37°C, the plates were again washed three times with washing buffer and incubated again for 30 minutes at 37°C with a 1:1000 dilution of goat anti-guinea pig IgG-peroxidase conjugate (whole molecule) (SIGMA A-7289) or goat

anti-bovine IgG peroxidase conjugate (whole molecule) (SIGMA A-7414) in phosphate buffered saline solution. After incubation and a further triple wash, 50 ul of the chromogen suspension (see Appendix) containing hydrogen peroxide as substrate (see Appendix) were added per well. After 15 minutes at 37°C the colour reaction was terminated with 2M sulphuric acid (see Appendix) adding 100 ul per well.

The extinctions were measured in a Titertek Multiskan ELISA reader at a wavelength of 492 nm. All sera were analysed by duplicate. Two positive D. viviparus sera and four negative control were included at 1:20 dilution in every plate; in addition, two antisera, one against Ostertagia ostertagi and one against Cooperia oncophora were always included to detect any cross-specificity.

Interpretation

Means and standard deviations were determined for the absorbency values obtained from the six negative sera in each plate for the interpretation of the readings. A confidence interval was adopted extending to two standard deviations from the mean. Absorbency values above this confidence interval were interpreted as positive and values falling below as negatives.

In some experiments an "antibody titre" was used : an antibody titre of 1 corresponded to a serum dilution of 1:20, a titre of 2 to 1:40 and so on (Boon et al, 1982).

When monoclonal antibodies (mAb) were used, an extra step was performed. After the test serum incubation and washings, 100 ul of mAb to bovine IgM or IgG_1 or IgG_2 (Centraal

Diergeneeskundig Instituut, Lelystad, Netherlands) diluted 1:4000 was added to the wells and incubated for 30 minutes at 37°C. These reagents have been shown not to cross-react with other isotypes (van Zaane and Jzerman, 1984). The conjugate used was Mouse Polyvalent Immunoglobulins-Peroxidase conjugated (SIGMA A-0412). The results shown are the means of the optical density readings of two wells per sample.

Immunofluorescence technique

Parasites were washed three times with 0.1% sodium azide (NaN_3) and incubated with the bovine sera to be tested, diluted 1:25 in PBS, for 30 minutes on ice with occasional agitation. They were washed three times in PBS 0.1% NaN_3 and incubated for a further 30 minutes on ice with mouse monoclonal antibodies to bovine IgM, IgG_1 and IgG_2 diluted 1:50 in PBS. Following a further washing, they were incubated with FITC-conjugated antibody to mouse polyvalent immunoglobulins (IgM, IgG, IgA) (SIGMA F-1010) diluted 1:25 in PBS for 30 minutes. After further washing, parasites were mounted in microscope slides within a ring of vaseline to raise the coverslip from the surface of the parasites and viewed under ultraviolet light.

Quantitation

Fluorescence was quantified by photon counting using a Leitz MPV Compact 2 microscope photometer linked to an Olivetti H100 computer. The photometer diaphragm was set to measure a rectangular field in the range of 230-555 um^2 using a x 40 Fluotar air objective for larvae or 920-2220 um^2 with a x 10 objective for adult worms. For larvae, the area measured was in

the centre of the parasite, away from edge fluorescence. From adults, which all displayed internal autofluorescence centrally, the edge fluorescence was measured always keeping the edge line in the centre of the measurement field. This avoided most of the autofluorescence and, because the signal was stronger there, improved the signal to noise ratio. The fluorescence of one of the brightest specimens was set at the arbitrary value of 650. The results shown are the means of at least 15 estimations per each serum or pooled samples tested, and the backgrounds were subtracted automatically.

Poly-Acrylamide gel electrophoresis

Proteins were separated by poly-acrylamide gel electrophoresis in the presence of sodium-dodecyl sulphate (SDS-PAGE). When samples were stained with Coomassie blue, 20 to 30 ug of protein were used per track.

The samples were prepared by mixing 20 ul of the material with 20 ul of sample buffer (see Appendix). When non-reducing conditions were used, 50 ul of 2% iodoacetamide was added to 950 ul of the sample buffer, and when reducing conditions was employed, 50 ul of 2-mercaptoethanol was added to 950 ul of sample buffer. In each gel, molecular weight markers were added covering a range from 14.4 kDa to 94.0 kDa (Pharmacia 17-0446-01) and electrophoresed simultaneously.

For electrophoresis, the samples and the control markers were loaded into 3% acrylamide stacking gel (see Appendix) and electrophoresed in the presence of SDS on 5% to 25% acrylamide

gradient resolving gel (see Appendix) using a current of 60 mA and a voltage of 250 V for approximately three hours.

Following electrophoresis the gels were fixed and stained with Coomassie blue (0.1% Coomassie blue R-250 in methanol, 10% acetic acid, water [v/v/v]) for one or two hours and were destained by immersion in a solution containing 25% methanol, 10% acetic acid, 1% glycerol and water until a clear background, was obtained. Gels were vacuum dried onto filter paper or cellophane in a heated slab gel dryer at 80^{o}C (Bio-Rad 1125B).

Bolton Hunter labelling of E/S material of adult parasites

The E/S was diluted in 0.1M Borate buffer (see Appendix) and added to a glass tube in which an aliquot of 40 ul (200 uCi) of Bolton Hunter (Amersham IM-5861) had been added and allowed to dry overnight in a fumehood. The tube was agitated for ten minutes on ice and 0.1 volume of 2M glycine in borate buffer was added into the tube to bind any iodine excess. After a further agitation for five minutes at room temperature the labelled material was dispensed onto a G25 sephadex column (Pharmacia PD-10) and washed with PBS triton buffer (0.5% Triton X-100) (SIGMA T-6878). The fractions were collected in test tubes at 30 second intervals and the counts of each tube measured. Tubes around the peak containing the labelled antigen were pooled. High counts in later tubes were due to unreacted ^{125}I and were discarded.

TCA Precipitation

To estimate the amount of radioactivity which has bound to macromolecules, a trichloroacetic acid precipitation (TCA) was carried out. This eliminates the counts from free ^{125}I as only large molecules are precipitated by TCA. 50 ul of PBS triton buffer was dispensed into test tubes in triplicate, 5 ul of normal rabbit serum was added followed by 2 ul of labelled antigen obtained as described in the previous section. The tubes were counted in a gamma-counter and the input count was obtained; 10% TCA was added to this up to 1 cm from the tube neck. The tube was vortexed and centrifuged at 3000 g at $4^{o}C$ for ten minutes. The supernatant was removed and the pellet counted in the gamma-counter giving the output count. The percentage of TCA precipitated was calculated as follows:

%TCA precipitated = output counts/input counts X 100

Immunoprecipitation

50 ul of PBS triton buffer was dispensed to test tubes in duplicate followed by 2.5 ul of test serum. The activity of radiolabelled antigen preparation was counted in a gamma-counter and diluted with PBS triton buffer so as to add approximately 200,000 counts per minute to each immunoprecipitation tube in less than 10 ul. The tubes were incubated overnight at $4^{o}C$ and 50 ul of Staphyloccocus aureus protein A was added (this will bind to IgG). Each tube was vortexed, incubated for one hour at room temperature and one hour at $4^{o}C$. The tubes were centrifuged twice, PBS triton buffer added and the supernatant discarded.

For the third wash the precipitate was transferred to a new tube and centrifuged at 10,000 g for ten minutes. The supernatant was discarded and the pellets in duplicate pooled by resuspending one of the pellets in 500 ul of PBS triton buffer and transferring to the second tube. The resuspended pellet was centrifuged again and then used immediately or frozen at $-20^{o}C$.

To identify the antigens in the pellets, they were analysed by SDS-PAGE by resuspending them in 40 ul of SDS-PAGE sample buffer and boiling for ten minutes. The suspensions were loaded on to a 5%-25% gradient poly-acrylamide gel along with ^{125}I labelled molecular weight markers. After electrophoresis the gel was removed from its cassettes, fixed, dried onto filter paper and exposed to flashed Fuji film and the autoradiographs exposed at $-70^{o}C$. After exposure of approximately seven to 15 days the labelled antigens precipitated by antibodies appeared as bands on the autoradiograph.

CHAPTER THREE

THE GUINEA PIG AS AN EXPERIMENTAL HOST FOR THE BOVINE LUNGWORM *DICTYOCAULUS VIVIPARUS*

Studies on rabbits, guinea pigs, mice, rats and hamsters have shown that guinea pigs are the most susceptible of these small laboratory animals to infection with the bovine lungworm (Soliman, 1953; Douvres and Lucker, 1958). Subsequently they have been used on many occasions (Wade, Fox and Swanson, 1960a; Poynter et al, 1960; Wade, Fox and Swanson, 1960b; Wade, Swanson and Fox, 1961; Silverman, Poynter and Podger, 1962; Wilson, 1966; Cornwell and Jones, 1970a).

From these studies, it is apparent that worm recovery from the lungs of the infected guinea pigs has varied greatly from experiment to experiment, the best yield being around 5% when a dose of 5,000 L_3 was given orally (Poynter et al, 1960; Wilson, 1966). The worms were recovered by an adaptation of the Baermann technique in which the lungs and trachea were removed from the thoracic cavity, cut up by scissors and the tissue fragments suspended in gauze or nylon mesh in warm saline solution for up to 24 hours (Wade et al, 1960a; Cornwell and Jones, 1970a; Wilson, 1966).

The route followed by D. viviparus, after ingestion, to reach the lungs, was first described by Soliman (1953) and later confirmed by Poynter et al (1960). Both studies demonstrated that the migration was via the lymphatic and blood vessels and that no liver migration was involved.

The morphogenesis of the parasitic stages of the lungworm in the guinea pig was studied by Douvres and Lucker (1958). They were able to recover from the lungs of the infected animals the parasitic third stage just after 18 hours post-infection, and the

third moult, fourth stage, fourth moult and fifth stage, after 23, 43.5, 144 and 154 hours respectively. It has also been shown that these larvae remain in the lungs of the animals in constant numbers up to nine days after infection. On day 10, the worm burden shows a significant decrease and after 13 days only a few worms are found (Poynter et al, 1960; Wilson, 1966). Wade et al (1960a) showed that D. viviparus do not reach maturity in the guinea pig or any other laboratory animal, ceasing their development at the early fifth stage.

The use of irradiated larvae in the guinea pig proved that X-irradiation at the level of 400 Gy primarily affects D. viviparus at the transition from the fourth to the fifth stage (Poynter et al, 1960). They showed this by infecting guinea pigs with doses of 5,000 irradiated L_3 and killing the animals at 24 hour intervals. The results showed a marked reduction of worm recovery on day 6 and no irradiated larvae were found after eight days.

Active and passive immunity to D. viviparus has been demonstrated in the guinea pig. The fact that both infective and X-irradiated larvae are equally capable of evoking immunity in the animals was shown by Poynter et al (1960). They were able to demonstrate, after immunising guinea pigs twice with 5,000 L_3 of either type and then challenging them with 5,000 larvae that the animals were able to eliminate the challenge infection after just five days. Wilson (1966) also showed that second and subsequent challenge infections were eliminated more rapidly than the first infection.

Passive transfer of immunity was proven to be successful (Wilson, 1966) when pooled serum of guinea pigs, infected three times previously with 5,000 L_3, was administered intraperitoneally to naive animals in two 5.0 ml doses. The first dose of serum was given immediately prior to infection with 5,000 L_3 and the second dose on the following day. A worm recovery reduction of 89.2% was seen nine days later when the recovered worm burdens were compared with a control group receiving normal serum.

The immune status of the guinea pig against infection of D. viviparus has been measured by the complement-fixation technique (Wade et al, 1960a; b). Using a 5% suspension of lyophilised adult lungworm in NaCl solution containing 0.5% phenol as antigen, they were able to detect antibodies against the parasite ten days after a primary oral infection; however, they failed to detect any increase in complement-fixing antibody in sera of challenged guinea pigs (Wade et al, 1960b).

Currently, there are still many immunological aspects of D. viviparus infection that have not been elucidated such as the source of the protective antigens which generate immunity after infection or vaccination. It was considered that the guinea pig model could provide an appropriate and economic opportunity to study this and other aspects of immunity to D. viviparus.

EXPERIMENTS USING NORMAL, 400 Gy-IRRADIATED AND 1000 Gy-IRRADIATED LARVAE

EXPERIMENT ONE: THE RECOVERY AND MORPHOLOGY OF NORMAL AND 400 Gy-IRRADIATED LARVAE

The first experiment was designed to determine the recovery rate when the Stomacher-Baermann technique was used and to show the morphological changes undergone by normal and 400 Gy-irradiated larvae.

Experimental design

Twenty guinea pigs were divided at random into two groups of ten animals each. The ten guinea pigs of group 1 were each infected with 5,000 normal L_3, and each of the other ten in group 2 with 5,000 400 Gy-irradiated larvae. One animal of each group was killed daily for the first seven days and on days 9, 12 and 15. The lungs were removed, processed, the worms counted and the developmental stages recorded.

Results

The worm recovery numbers and their developmental stage are shown in Table 1. When the guinea pigs were infected with a dose of 5,000 L_3, normal or 400 Gy-irradiated, the parasites could be found in the lungs after 24 hours. Thereafter, the number of worms, ranging from over 100 to around 600, seemed to remain constant with the irradiated larvae up to day 6 and until day 9 with the normal larvae. In both groups the L_5 was the latest stage of development reached and no obvious differences between normal and irradiated larvae were seen under light microscopy.

No worms were found in the lungs of the animals killed on day 15.

Table 1

Recovery and morphology of normal and

400 Gy-irradiated larvae from guinea pig lungs

Day*	400 Gy-Irradiated Number of Larvae	Stage	Normal Number of Larvae	Stage
1	125	L_3	99	L_3
2	556	L_3	592	L_3
3	513	L_3, 3rdM	418	L_3, 3rdM
4	392	3rdM, L_4	280	3rdM, L_4
5	321	3rdM, L_4	136	L_4
6	422	L_4, 4thM	126	L_4, 4thM
7	46	L_5	140	L_5
9	37	L_5	349	L_5
12	1	L_5	11	L_5
15	0	-	0	-

* Days after infection

EXPERIMENT TWO: THE IMMUNISING ABILITY OF NORMAL AND 400 Gy-IRRADIATED LARVAE

This experiment was designed to test the degree of protection conferred by the double administration of normal or 400 Gy-irradiated larvae.

Experimental design

Fifteen guinea pigs were divided into three groups of five animals each. The animals in group 1 were each infected twice 21

days apart with 5,000 normal L_3. Those in group 2 were subjected to the same infection schedule but X-irradiated larvae were used instead. All animals including the naive control guinea pigs of group 3 were challenged with 5,000 normal L_3 21 days after the second immunising injection. One animal out of each group was killed from day 4 to day 8, the lungs were removed, processed and the worms counted.

Results

Normal and 400 Gy-irradiated larvae were capable, after two infections, of evoking immunity in the guinea pigs (Table 2). After five days no larvae were recovered from the normal L_3 infected guinea pigs and after day 7 from the X-irradiated L_3 infected animals.

Table 2

Normal larvae recovered from guinea pig lungs challenged with 5,000 infective L_3 after being infected twice with normal and 400 Gy-irradiated larvae

Days after Challenge	Normal	Irradiated	Control
4	20	61	177
5	5	6	133
6	0	4	171
7	0	1	193
8	0	0	405

EXPERIMENT THREE: THE ABILITY OF LARVAL AND ADULT WORM HOMOGENATES TO INDUCE IMMUNITY

This experiment, which was conducted in two parts because of the limited guinea pig accommodation, was designed to determine if antigen homogenates prepared from third stage larvae and from adult parasites respectively would induce resistance to lungworm infection in guinea pigs.

A. In the first part, the value of larval and adult homogenates administered IP in Freund's adjuvant was investigated.

Experimental design

Twenty animals were allotted to five groups of four each and by random selection each group was assigned a particular treatment as follows: group 1, adult worm homogenate; group 2, larval homogenate; group 3, 400 Gy-irradiated larvae; group 4, infective L_3 and group 5, challenge control.

The larval homogenate was prepared by sonication of third stage infective larvae in PBS and the adult homogenate was prepared by grinding adult worms also in PBS.

Just prior to injection the homogenates were emulsified with an equal volume of FCA adjuvant for the first injection and with FIA adjuvant for the second. The amount of protein given per injection per animal was adjusted to 50 ug in 1.0 ml.

400 Gy-irradiated larvae and infective L_3 were given to groups 3 and 4 at doses of 5,000 larvae per animal per infection.

Injections of the emulsified materials and live larvae were administered intraperitoneally on two occasions 21 days apart.

The challenge dose consisted of 5,000 infective L_3 administered orally after 21 days following the second injection of antigen or larvae. All animals were killed seven days after challenge.

Blood was obtained by cardiac puncture on the day of killing in order to measure antibody titres by the ELISA technique.

Results

The worm recovery numbers are shown in Table 3. The guinea pigs given adult homogenate harboured a similar number of worms as the challenge control group. Guinea pigs given larval homogenate had a 67.14% reduction in worm recovery when compared with the challenged controls ($P<0.01$).

In the groups in which live larvae, whether infective or irradiated, were administered protection was absolute, no worms being recovered.

The ELISA results are shown in Figure 1. Both groups of animals immunised with the homogenates in Freund's adjuvant presented titres of 1:20,000, while the guinea pigs immunised with live larvae had litres of 1:5120 for the normal infective L_3 and 1:2560 for the 400 Gy-irradiated larvae. This result shows a lack of correlation between antibody titres and protection.

Table 3

Larval recovery from a challenge of 5,000 L_3 after two IP injections of adult and larval homogenates in Freund's adjuvant

Animal No.	Adult Homogenate	Larvae	400 Gy Larvae	Infective Larvae	Challenge Control
1	397	135	0	0	361
2	316	129	0	0	253
3	294	59	0	0	251
4	238	42	0	-	243
Mean	311	91	0	0	277
% Reduction	0	67	100	100	

B. The second part of the experiment was concerned with the protection value of larval and adult homogenates and larval and adult E/S products given SC in Freund's adjuvant or liposomes.

Experimental design

Forty animals were divided into eight groups of five animals each and the treatments were administered as follows:-

Group 1 Larval homogenate in Freunds

Group 2 Saline in Freunds

Group 3 Larval homogenate in liposomes

Group 4 Adult homogenate in liposomes

Group 5 Larval E/S in liposomes

Group 6 Adult E/S in liposomes

Group 7 Saline in liposomes

Group 8 Challenge control

With the exception of group 5 in which no protein was detected, all antigens were given at doses of 50 ug/protein per injection following the regime of part A of the experiment.

Results

The worm recovery numbers obtained seven days after challenge, are shown in Table 4. Three of the animals which received the Freunds adjuvant presented skin ulcers after seven to ten days of the injection. No adverse effects were noticed when the liposomes were used. The inoculation of adult E/S and homogenate with liposomes as adjuvant achieved reductions in worm recovery of 76.6% and 75% respectively; this latter figure contrasts sharply with the zero protection found in part A of this experiment when adult homogenate was administered in Freund's adjuvant and is discussed later.

Similar and relatively high numbers of worms were recovered when L_3 E/S products were injected with liposomes and when the liposomes were administered with 0.85 saline solution. This was probably associated with the lack of measurable protein in the E/S products of the third larval stage.

The animals given the larval homogenates with either of the adjuvants gave good results. L_3 homogenate plus Freund's gave 85% protection and the same homogenate plus liposomes achieved a worm reduction of 79.44%. The protection obtained with the L_3 antigens was statistically significant when compared with the challenge control group ($P<.05$).

The use of Freunds alone or liposomes alone, injected SC, appeared to stimulate a modest degree of non-specific immunity against lungworm.

The ELISA results showed again no relationship between antibody levels and protection (Figure 3).

Table 4

Larvae recovery from a challenge of 5,000 L_3 after two SC injections of larval homogenate, adult homogenate, larval E/S or adult E/S using Freunds or liposomes as adjuvant

Animal No.	ADJUVANT							
	FREUNDS		LIPOSOMES					
	Homogenate		Homogenate		E/S			Challenge
	L_3	Saline	L_3	Adult	L_3	Adult	Saline	Control
1	3	36	24	28	71	12	72	87
2	16	41	26	39	76	26	83	148
3	24	73	29	45	129	42	90	196
4	28	104	46	54	152	51	123	211
5	66	126	62	58	161	81	145	258
Mean	27a*	76cbd	36ba	45ba	118dec	42ba	103dec	180e
% Reduction	85	58	79	75	34	77	42	

* Groups of animals with different subscript are statistically different $P<0.05$

The satisfactory results obtained with the three L_3 homogenates in experiments A and B suggest that this stage could be a major source of protective antigens.

In order to study this theory it was thought that a complete cessation of larval development at the third stage might be achieved if the larvae were irradiated at 1000 Gy. A series of experiments were designed to examine this and the antigenic properties of the third stage larvae.

EXPERIMENT FOUR: RECOVERY AND MORPHOLOGY OF THIRD STAGE 100 Gy-IRRADIATED LARVAE

This experiment was designed to test the ability of 1000 Gy-

irradiated larvae to reach the lungs and to study their subsequent development, if any. In order to obtain the 1000 Gy larvae, normal larvae were subjected to gamma-rays delivered from a cobalt source. Seventy-two animals were divided at random into three groups of 24 animals each. Subsequently, each group was divided in two subgroups of 12 animals each. Guinea pigs of group 1 were inoculated with 5,000 400 Gy-irradiated larvae, one subgroup orally and the other intraperitoneally. Animals in group 2 and group 3 were treated in the same way but 1,000 Gy-irradiated and normal larvae were used instead.

Three animals of each subgroup were killed on day 2, day 4, day 6 and day 8. The lungs were removed and processed by the Stomacher-Baermann technique, the worms were counted and their developmental stages recorded.

Results

The results are shown in Table 5. The number of worms recovered from the 400 Gy-irradiated and normal larvae remained similar throughout the experiment and generally more worms were recovered when the larvae were administered intraperitoneally.

A very low number of worms was recovered from the 1000 Gy-irradiated larvae group. On day 2 a mean of 48.3 L_3 was recovered and on day 8 a mean of only 0.83 worms was recovered from six animals. The reduction in worm recovery of the 1000 Gy-irradiated larvae relative to the 400 Gy-irradiated and normal larvae went from 79.35% on day 2 to 98.78% on day 8.

The morphological development of the 400 Gy-irradiated and normal larvae was as previously described. The 1000 Gy-

Table 5

Recovery and morphology of irradiated and normal D. viviparus larvae from guinea pig lungs

Day*	Group 1 400 Gy			Group 2 1000 Gy			Group 3 Normal		
	Oral	I.P.		Oral	I.P.		Oral	I.P.	
2	240	288		44	101		247	429	
	219	255	L_3	42	41	L_3	170	388	L_3
	185	220		42	20		158	202	
Mean	214	254		42	54		191	339	
4	370	294		38	61		211	502	
	358	288	3rdM	18	43	L_3	209	406	3rdM
	297	238	L_3	10	31		139	328	L_4
Mean	341	273		22	45		186	412	
6	257	285		4	10		187	204	
	181	182	L_4	3	6	L_3	169	200	L_4
	146	162		1	2		151	150	
			4thM						4thM
Mean	194	209		2	6		169	184	
8	15	48		0	3		152	21	
	10	41	L_5	0	2	L_3	114	9	L_5
	8	15		0	0		92	6	
Mean	11	34		0	1		119	12	

* Days after infection

irradiated larvae as judged by microscopic examination of fresh specimens, did not develop at all and remained as third stage larvae.

EXPERIMENT FIVE: THE ABILITY OF 1000 Gy-IRRADIATED LARVAE TO INDUCE PROTECTION

The results of the previous experiment which showed that L_3, irradiated at 1000 Gy, are unable to develop further indicated that this approach might be useful in studying the immunogenicity of the third stage larvae against challenge with normal L_3.

Experiment design

Thirty animals were divided into three groups of 12, 12 and six guinea pigs. Group 1 and group 2 were subsequently divided in two subgroups of six animals each. Animals in group 1, were infected on two occasions 21 days apart with 5000 400 Gy-irradiated L_3 each, one subgroup orally and the other intraperitoneally. Animals in group 2 received the same treatment except that the larvae were irradiated at 1000 Gy. All animals including those of the control group 3, were challenged with 5,000 normal L_3 21 days after the second infection and killed seven days after challenge. Just before killing, blood was obtained from all animals and processed as described in Materials and Methods.

The antibody response after challenge was measured by the ELISA technique using L_3 homogenate as the antigen.

In addition, immune sera from both immunised groups were immunoprecipitated using E/S of adult worms (the reason for the

use of this antigen is discussed in Materials and Methods), run in SDS-PAGE and the antigens visualised by autoradiography (these results are shown in Chapter Six).

Results

The results of Table 6 show that a very low number of worms were recovered from the immunised animals regardless of the type of larval irradiation or route of administration. Thus, the percentage reductions in worm recovery of the 1000 Gy and 400 Gy-irradiated larvae were 96.40% and 99.68% respectively when compared to the control group.

Both types of irradiated larvae were therefore capable of conferring protection against challenge.

The antibody titres of the groups after challenge are shown in Figure 2. As can be seen, both types of irradiated larvae were capable of eliciting a high antibody response, all of the sera being positive even when tested up to a 1280 dilution. The best antibody response was achieved when the 400 Gy-irradiated larvae were administered intraperitoneally. Otherwise the 1000 Gy-irradiated larvae given by both routes of administration and the 400 Gy-irradiated larvae administered orally produced a very similar antibody response.

Table 6

Larval recovery after challenge of guinea-pigs previously immunised with two doses of larvae irradiated with either 400 or 1000 Gy

Animal No.	400 Gy Oral	400 Gy I.P.	1000 Gy Oral	1000 Gy I.P.	Challenge Controls
1	0	0	4	0	110
2	1	0	6	0	160
3	1	1	11	2	252
4	1	1	11	3	258
5	1	1	17	3	263
6	2	2	38	10	417
Mean	0.83	0.66	14.5	3	243
% Reduction	99.6	99.7	94.3	98.7	

* Animals were killed seven days after challenge

EXPERIMENT SIX: PASSIVE TRANSFER OF IMMUNITY

This experiment was divided into three parts. The first part was designed to test the ability of bovine D. viviparus hyperimmune serum to confer protection against challenge of guinea pigs with normal larvae. If successful, this would eliminate the labour of accumulating small quantities of serum from a large number of guinea pigs. The second part had the purpose of comparing the effect of passively administered bovine and guinea pig D. viviparus hyperimmune serum (described in Materials and Methods) against challenge with normal L_3 and the third part was designed to test the ability of guinea pig serum raised against 1000 and 400 Gy-irradiated larvae to confer protection against lungworm infection.

Experimental design

For the first part of the experiment 24 guinea pigs were divided into three groups of nine, nine and six. Each animal in group 1 was given intraperitoneally 5.0 ml of bovine hyperimmune serum. Group 2 guinea pigs each received 5.0 ml of bovine normal serum by the same route. The serum was injected just prior to the oral infection. Animals in group 3 remained as a challenge control group. All the guinea pigs were infected with 5,000 normal L_3. Three animals from each of the first two groups and one from the control group were killed on days 5, 7 and 9 after infection. The worms were recovered by the Stomacher-Baermann technique and counted.

For the second part, 15 guinea pigs were divided into three groups of five animals. Each of group 1 received intraperitoneally 5.0 ml of bovine hyperimmune serum. Guinea pigs from group 2 each received 5.0 ml of guinea pig hyperimmune serum by the same route. In both cases the serum was injected just before the larvae were administered. The third group acted as challenge control. All animals were infected with 5,000 normal L_3 and killed eight days after infection when the lungs were removed, processed and the worms counted.

In the final part of the experiment, 26 guinea pigs were divided at random into four groups of five and one group of six animals. The animals of group 1 each received intraperitoneally 5.0 ml of the sera from a triple 400 Gy-irradiated larval infection just before a challenge oral infection with 5,000 normal L_3; groups 2 and 3 were treated similarly, except that

sera raised against 1000 Gy-irradiated and normal larval infections were used instead. Group 4 received normal guinea pig serum before the challenge and group 5 remained as a challenge control group. The 30 animals were killed eight days after infection, the lungs were removed, processed and the worms counted.

Results

In the first part of the experiment (Table 7), it was observed that the bovine hyperimmune serum was not capable of conferring any protection to the guinea pigs challenged with 5000 normal L_3.

Table 7

Larvae recovery from a challenge with 5000 normal L_3 after 5.0 ml injection of hyperimmune and normal bovine serum

Day*		Hyperimmune	Normal	Control
		104	178	
5		92	98	112
		90	92	104
	Mean	95.3	122.6	108
		198	475	
7		184	403	321
		182	91	219
	Mean	188	323	270
		169	140	
9		128	127	143
		60	62	195
	Mean	119	109.6	169

* Days after challenge

The second part of the experiment (Table 8) showed that the administration of guinea pig hyperimmune serum was capable of reducing the worm burden by 88.0% when compared to the control group. The use of bovine hyperimmune serum conferred no significant protection as observed previously.

Table 8

Larvae recovery from a challenge of 5000 infective L_3 after injection of 5.0 ml of bovine and guinea pig hyperimmune serum

Day*	Bovine	Guinea Pig	Control
	29	3	146
	114	4	147
8	157	21	173
	186	25	175
	229	49	213
Mean	143	20.4	170.8
% Reduction	15.8	88.0	

* Days after challenge

The results of the last part of the experiment are shown in Table 9. The use of hyperimmune serum against normal larvae produced similar results to those presented previously, i.e. 83.6% and 88.0% reduction in worm burdens respectively.

In comparison the protection conferred by the sera from the irradiated infections was relatively poor in that the 400 Gy-irradiated serum produced only a 44% reduction in worm recovery while the 1000 Gy produced no significant reduction in worm recovery. This latter finding, especially, was unexpected since guinea pigs actively immunised with such larvae are highly resistant.

Table 9

Worm recovery from passively immunised guinea pigs challenged with 5000 *D. viviparus* L_3*

Source of Serum				
400 Gy L_3	Infection X 3 1000 Gy L_3	Normal L_3	Uninfected Guinea Pig	Challenge Controls
43	82	4	80	139
56	107	23	89	140
90	151	31	114	169
124	157	36	203	185
151	175	41	224	196
-	181			
92b**	142bc	27a	142bc	165c
44.2	13.9	83.6	13.9	% Reduction

* All the animals were killed eight days after challenge
** Groups of animals with different subscript are statistically different ($P<0.05$).

Discussion

The first two experiments were carried out to confirm the results of previous studies using the guinea pig as a model for *D. viviparus* infection and to provide a base-line for subsequent work. They also served to evaluate the use of the Stomacher machine in homogenising the lungs of the infected animals.

With regard to the latter, the use of the Stomacher, which is faster and always consistent, proved to be as good as conventional chopping of the lungs with scissors in terms of worm recovery. The number of worms recovered and their stage of development after infection with normal or 400 Gy-irradiated L_3 were similar to the results reported by Poynter *et al* (1960).

Thus, L_3, whether normal or irradiated, were found in the lungs 24 hours after a primary oral infection with 5,000 L_3. Thereafter, the number of worms remained constant, with the irradiated larvae up to day 6 and until day 9 with the normal L_3. In both groups the L_5 was the latest stage of development. By day 15 all the worms in both groups had disappeared. These results generlly confirm those reported by Poynter et al (1960).

The immunising potential of normal and 400 Gy-irradiated larvae also reported by Poynter et al (1960) was confirmed in that guinea pigs immunised on two occasions with either type of larvae were subsequently highly resistant to challenge and by day 7 the immunity, in terms of worm reduction, was virtually 100%.

In Experiment 3A, the use of soluble homogenates of adult and larval parasites in Freund's adjuvant showed that a high degree of protection, of the order of 67%, was achieved when larval homogenates were used, which suggests that antigens important in the development of protective immunity are present in this stage.

When adult material was used, no protection was obtained, a result previously reported by Wilson (1966). This finding could be perhaps explained in two ways. First, the lack of protective antigens in this stage, which, as will be shown in Chapter Six, is improbable due to the multiple antigens in common between the third and adult stages. A second possibility is that the excellent properties of Freunds adjuvant in provoking a very strong host response against a very complex mix of antigens

(Figures 2 and 3) could militate against the successful stimulation of resistance due to antigenic competition.

The 75% reduction in worm recovery obtained in Experiment 3B when adult homogenate was used with liposomes, instead of Freunds, as adjuvant, together with the much lower antibody response in these animals (Figure 3), might tend to support the latter possibility. This result also suggests that antibody titres as measured in our system do not correlate with protection, a conclusion supported by the finding that the adult E/S, which was highly protective, provoked antibody levels little above zero.

Of some interest was the finding that the use of either FCA or liposomes alone produced a degree of protection i.e. 58% and 42% reduction in worm burden. The former was statistically significant and the latter just not. The same phenomenon has been shown to occur with a variety of immuno-stimulants against bacteria, fungi, protozoa and viruses (Warren and Chedid, 1988).

The high degree of protection, i.e. 67%, 85% and 89%, obtained with L_3 homogenates in three experiments indicated that potent antigens are present in this stage. Experiments 4 and 5 conducted with L_3 irradiated at 1000 Gy, which prevents larvae from developing beyond this stage, confirmed that third stage antigens are capable of producing a degree of protection, in this case, of the order of 94% against challenge. This might suggest that a search for protective antigens could be confined to this stage alone.

The last experiment was concerned with the prophylactic efficacy of hyperimmune serum when passively transferred to susceptible animals. It was shown on two occasions that bovine hyperimmune serum did not confer any protection to guinea pigs. In contrast, immune serum from guinea pigs produced a reduction in worm recovery of 84% and 88% in two separate experiments. A possible explanation for the absence of protection of the bovine immune serum could be that, if larval destruction is antibody-mediated, the Fc receptors present in the bovine immunoglobulins might not be able to interact with guinea pig complement or "K-cells" to induce larval damage.

An unexpected result was the failure of serum, obtained from guinea pigs immunised with L_3 irradiated at 400 Gy or 1000 Gy, to confer a significant degree of protection. This occurred despite the fact that guinea pigs immunised with either type of irradiated larvae are highly protected against challenge.

There are at least two explanations for this observation. First, that irradiated larvae do not stimulate the same quantity and range of antibodies as do normal larvae. Thus, while the antibody produced is sufficient to protect actively immunised animals, the passive transfer of several ml of serum is not enough to confer a significant degree of protection. The second is that protection is not solely produced by antibody and there is a cell-mediated component stimulated principally by the L_3. This is discussed further in the next Chapter.

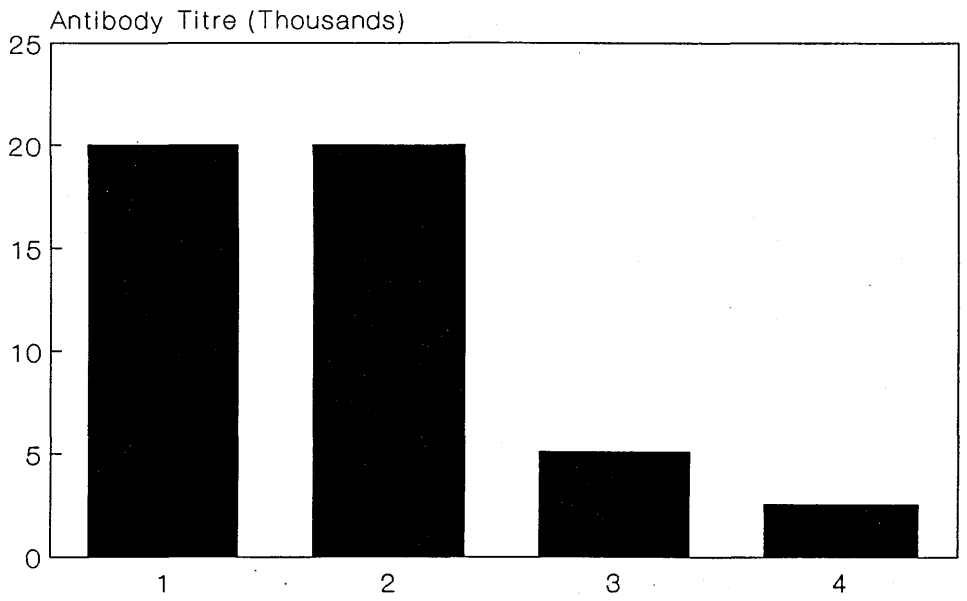


Figure 1. Mean antibody titres following challenge with 5000 infective L3 of groups of guinea pigs immunised twice with 1) soluble homogenates of L3 in Freunds adjuvant, or 2) soluble homogenates of adult worms in Freunds adjuvant, or 3) 5000 normal L3, or 4) 5000 L3 irradiated at 400Gy.

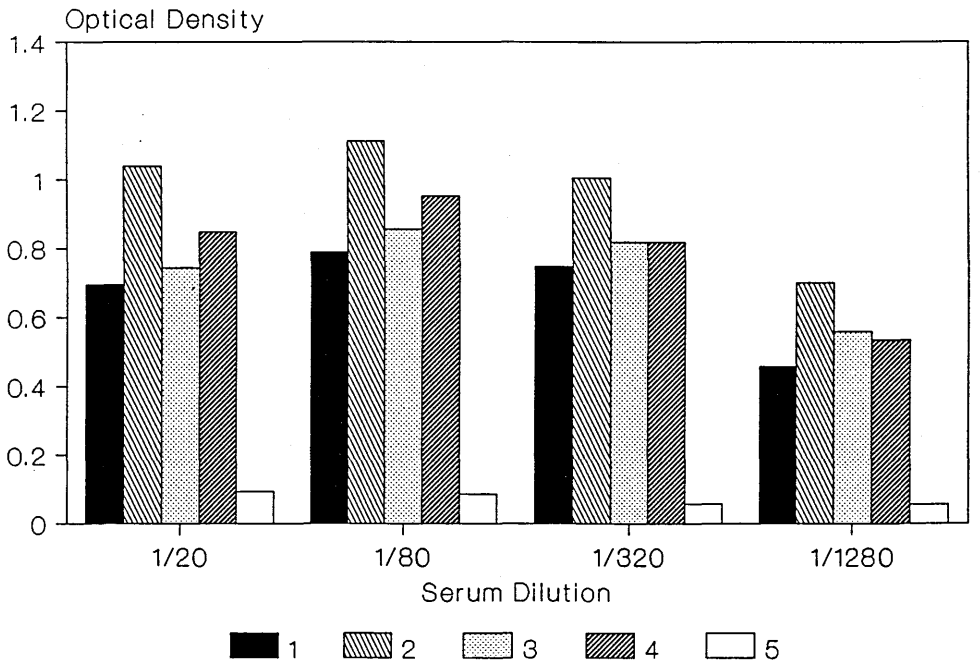


Figure 2. Mean optical densities of the serum antibody titres of guinea pigs after immunisation with 1) 5000 L3 irradiated at 400Gy orally, or 2) 5000 L3 irradiated at 400Gy intraperitoneally, or 3) 5000 L3 irradiated at 1000Gy orally, or 4) 5000 L3 irradiated at 1000Gy intraperitoneally and challenge with 5000 normal L3 compared with 5) challenge controls.

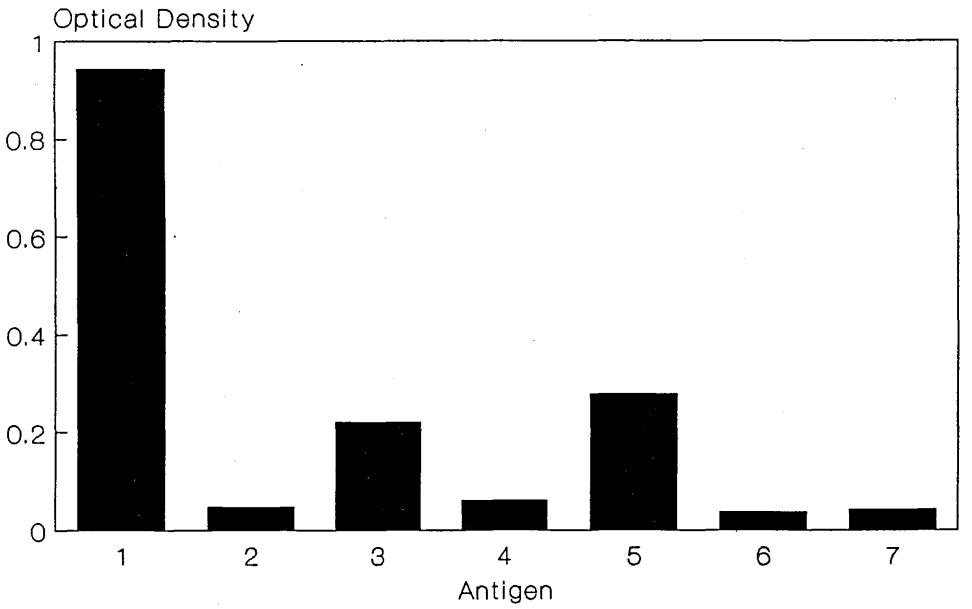


Figure 3. Mean optical densities of serum antibody from guinea pigs immunised twice with:
1=L3 homogenate in Freunds
2=L3 E/S in Liposomes
3=Adult homogenate in Liposomes
4=Adult E/S in Liposomes
5=L3 homogenate in Liposomes
6=0.85% Saline in Freunds
7=0.85% Saline in Liposomes
and challenged with 5000 infective L3.

CHAPTER FOUR

VACCINATION AND PASSIVE IMMUNISATION EXPERIMENTS IN CALVES

INTRODUCTION

In the previous chapter, concerned solely with the guinea pig as a model host, the new findings of most significance were: First, the fact that administration of L_3 irradiated to 1000 Gy produced as high degree of immunity as that observed with normal L_3. This occurred despite the fact that larvae irradiated to 1000 Gy do not develop beyond the third larval stage.

The second was unexpected, in that passive immunisation with sera obtained from these 1000 Gy infections failed to confer any degree of protection against challenge. This contrasted with the high level of protection achieved by sera from normal infections (88.0 and 83.6%) and the modest protection (44%) obtained with the 400 Gy-irradiated larvae.

Since protection against D. viviparus infection in calves has been generally ascribed to humoral antibody, it seemed important to investigate in this species the transfer of serum derived from 1000 Gy infections. Before doing so, it was necessary to show that calves infected with 1000 Gy L_3 were also successfully protected against challenge. Secondly, it was also considered desirable to confirm the reports that, in our hands, calves could be successfully immunised with hyperimmune serum from animals infected with normal larvae.

VACCINATION IN CALVES USING 400 Gy and 1000 Gy IRRADIATED LARVAE

Two experiments were carried out. First, the relative immunising potential of 400 Gy and 1000 Gy-irradiated larvae administered intravenously at doses of 2,500 L_3 on two occasions 21 days apart, was investigated. The second experiment examined

resistance to challenge after multiple infections with normal, 400 Gy and 1000 Gy-irradiated L_3.

I. Administration of larvae on two occasions

Experimental design

Nine helminth-naive animals with a weight ranging between 137 to 172 kg were divided at random into three groups of three calves each. Animals in group A each received orally two doses of 1000 L_3, irradiated at 400 Gy, 21 days apart. Calves in group B received intravenously 2,500 1000 Gy-irradiated larvae at the same times as those in group A. The animals in group C remained as a challenge control group.

All animals were challenged 21 days after the second vaccination with 30 L_3/kg of bodyweight and were necropsied 25 days later.

During the course of the experiment, the animals were inspected daily, and their general condition and respiratory rates noted. In addition the calves were weighed at the start of the experiment, the day of challenge and one day prior to necropsy.

Faecal samples were collected and examined for the presence of Dictyocaulus larvae at weekly intervals during the prepatent period and subsequently daily.

At necropsy, the lungs and trachea were removed and the air passages opened completely, starting with the trachea, and the worms recovered and counted.

Total and differential white blood cells (WBC) were carried out weekly during the experiment.

At the same times serum samples were obtained and examined for the detection of specific antibodies against larval and adult antigens by the ELISA technique using anti-bovine IgG peroxidase-conjugate. Also, in order to show if any differences existed between the antibody isotypes elicited by the 400 Gy and 1000 Gy-irradiated larvae, monoclonal antibodies to bovine IgM or IgG_1 and IgG_2 were used.

Results

The mean respiratory rates of the three groups are shown in Figure 4. The range remain unaltered during both vaccination regimens.

Seven days after challenge the RPM of all three groups were increased and on the day of necropsy, the mean respiratory rates were 45, 54 and 76 for the 400 Gy, 1000 Gy and control groups respectively.

During the vaccination regimen, calves of all three groups gained weight steadily. Between the challenge and necropsy this slowed in the irradiated groups, the mean weight gains being six and 19 kg respectively for the 400 Gy and 1000 Gy groups. In contrast, the control group showed a mean weight loss of 17 kg during the same time period.

The results of McMaster and Baermann examinations from days 21 to 26 after challenge are shown in Table 11. In the 400 Gy group only one animal was positive by the Baermann examination on two occasions while in the 1000 Gy group, one

animal was positive by the McMaster and the other two by Baermann examination. In the control group two of the three calves were positive by the McMaster technique and the third by Baermann examination.

The lungworm burdens present at necropsy and the percentage reduction in the worm recovery of the vaccinated groups when compared with the control group are shown in Table 12. Although one of the control animals had a very low number of worms present in the lungs at necropsy, it was clear that a very high protection against infection had been achieved i.e. 91% and 77% reduction in worm numbers for the 400 Gy and 1000 Gy groups respectively.

The gross pathology of the controls was typical of calves infected with D. viviparus with extensive patches of consolidation primarily confined to the diaphragmatic lobes. Lesions were also present in the irradiated groups although much less extensive, specially in the group which received 400 Gy-irradiated larvae.

Using the larval antigen no antibodies against lungworm were detected in the animals of group A until a week after the second dose of vaccine. Afterwards, the mean titres increased markedly until the animals were killed. Calves of group B, showed a low titre after the first vaccination which continued to increase after the second dose. After challenge, the titres remained at the same level until the last week during which a sharp increase was observed. In the control animals no titres were detected until two weeks after challenge (Figure 5).

With the adult antigen no antibody response to the first vaccination was detected in any of the vaccinated animals. Titres started to appear one week after the second vaccination and showed a marked increase after challenge. The animals in the control group developed a strong antibody reaction three weeks after challenge (Figure 6).

When monoclonal antibodies were utilised to measure the isotype antibody response, a high non-specific IgM reaction was found in both groups of vaccinated animals prior to immunisation being at least five times higher than the background observed with IgG_1 or IgG_2 (Figures 7 and 8). This high IgM cross-reaction was also seen when sera from other parasite naive animals were used as controls.

After subtracting the readings of the backgrounds and non-specific reactions produced by the sera before the first vaccine administration (Figures 9 and 10), it is easier to appreciate that IgM is the first isotype being produced, followed by IgG_1. The fact that, during the vaccination period, an earlier increase of IgG_1 and IgG_2 levels was seen in the group immunised with the 1000 Gy L_3 is probably due to the intravenous route of administration which allowed most or all of the larvae to reach the lungs in a short time. However, by the day of challenge (21 days after the second vaccine) the IgG levels of the 400 Gy vaccinated animals were higher suggesting a better antigen recognition.

Two weeks after challenge, the levels of IgM in the 400 Gy vaccinated group started to decrease while the IgG_1 and IgG_2

showed a sharp increase possibly indicative of a secondary response. In contrast, the levels of IgM in the 1000 Gy vaccinated group remained the same suggesting the possibility that a previously poorly recognised set of antigens (derived from L_4 and adult parasites) were being presented and recognised by the host. The IgG_1 and IgG_2 in this group did not show a marked secondary response which also suggests that although the L_3 and adult parasites have antigens in common (see Chapter six) different antigens must be present.

The eosinophil count in the vaccinated animals rose slowly until one week after the second dose of vaccine was administered (Figure 11). A further more abrupt response was only seen at seven and eight weeks, i.e. two weeks after challenge, the mean count producing a peak of over 3,200 eosinophils per cu mm for group A and over 1,900 for the animals in group B. After that there was a rapid decline until the animals were killed. In the control calves a sharp increase of an order similar to the vaccinated calves was observed two weeks after challenge and subsequently started to decrease. These results are similar to those previously reported (Cornwell, 1962a).

The mean WBC showed an increment two and three weeks after the first vaccine administration (Figure 12). In group B they remained high until week 5 in which a decrease was noted. In group A, a decrease was seen after the second week post-vaccination. The WBC in all groups showed an increase over the normal values two weeks after challenge and from there started to decrease until the animals were killed.

Table 11

McMaster and Baermann results after vaccination with 400 Gy and 1000 Gy-irradiated larvae

Group	Days after infection							
	21		24		25		26	
A	M	B	M	B	M	B	M	B
400 Gy								
1	N	N	N	1	N	N	N	1
2	N	N	N	N	N	N	N	N
3	N	N	N	N	N	N	N	N
B								
1000 Gy								
1	N	N	N	1	N	N	N	1
2	N	N	N	1	N	2	100	-
3	N	N	N	N	N	1	N	1
C								
Control								
1	N	N	N	4	100	-	100	-
2	N	N	N	1	N	1	N	1
3	N	N	N	3	100	-	50	-

M = McMaster LPG
B = Baermann LPG

Table 12

Comparative protection conferred by double vaccination with larval lungworm vaccine irradiated at 400 Gy or 1000 Gy as measured by percentage reduction adult worm burdens after challenge 21 days later with 30 larvae/kg liveweight

Vaccine Protocol		Number of Lungworms in Calves
A	400 Gy	2, 12, 168 Mean 60.66 Reduction 91.29%
B	1000 Gy	97, 189, 200 Mean 162 Reduction 76.74%
C	Control	57, 729, 1304 Mean 696.66

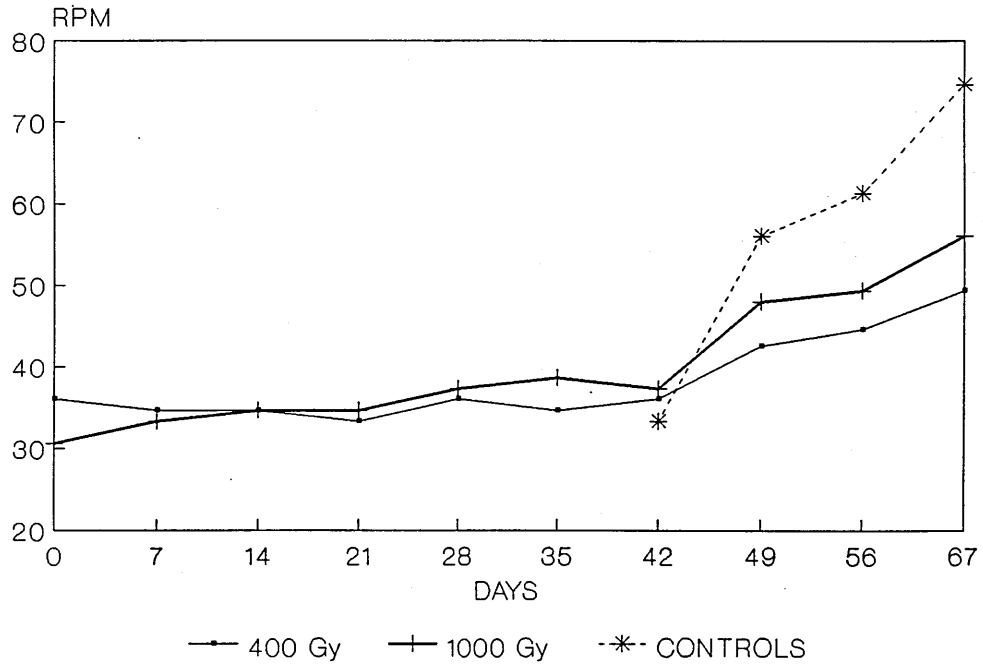


Figure 4. Mean respiratory rates of two groups of calves following vaccination with 1000 L3 irradiated at 400 Gy on days 0 and 21 orally, or 2500 L3 irradiated at 1000 Gy on days 0 and 21 intravenously. The calves were challenged with 50 L3/kg on day 42, and the results compared with a group of challenge controls.

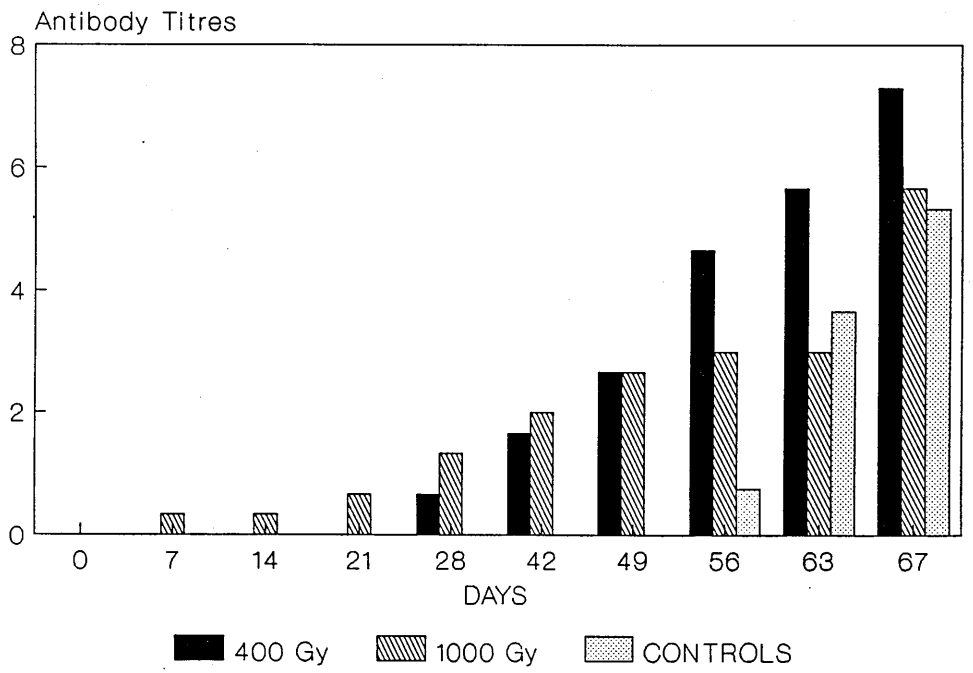


Figure 5. Mean antibody titres from pooled serum samples of 3 animals per group, detected by ELISA using L3 soluble antigens, following vaccination with 1000 L3 irradiated at 400 Gy on days 0 and 21 orally, or 2500 L3 irradiated at 1000 Gy on days 0 and 21 intravenously. The calves were challenged with 50 L3/kg on day 42, and the results compared with a group of challenge controls.

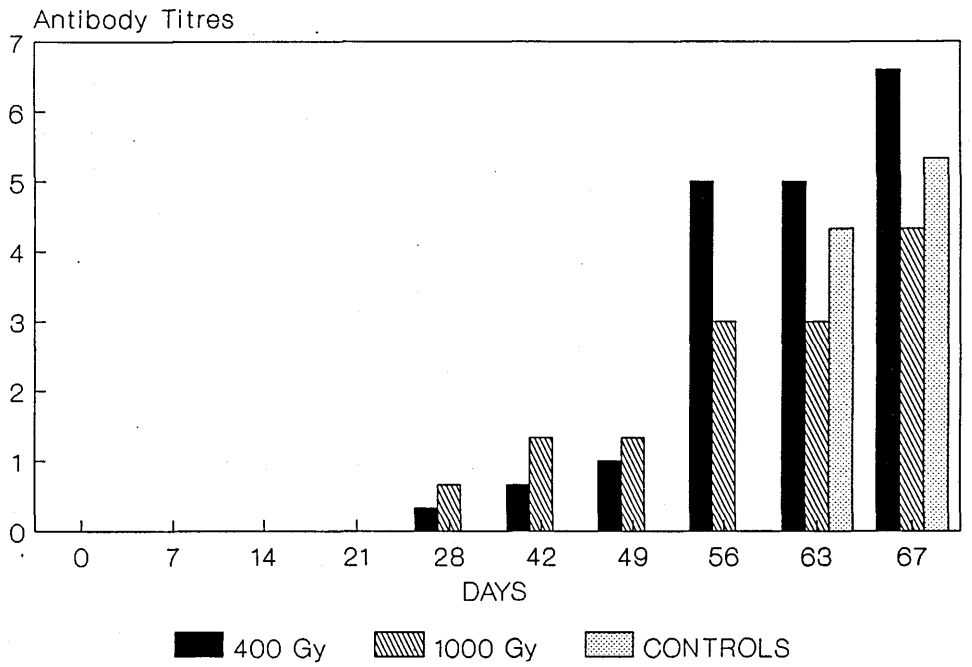


Figure 6. Mean antibody titres from pooled serum samples of 3 animals per group, detected by ELISA using adult soluble antigens, following vaccination with 1000 L3 irradiated at 400 Gy on days 0 and 21 orally, or 2500 L3 irradiated at 1000 Gy on days 0 and 21 intravenously. The calves were challenged with 50 L3/kg on day 42, and the results compared with a group of challenge controls.

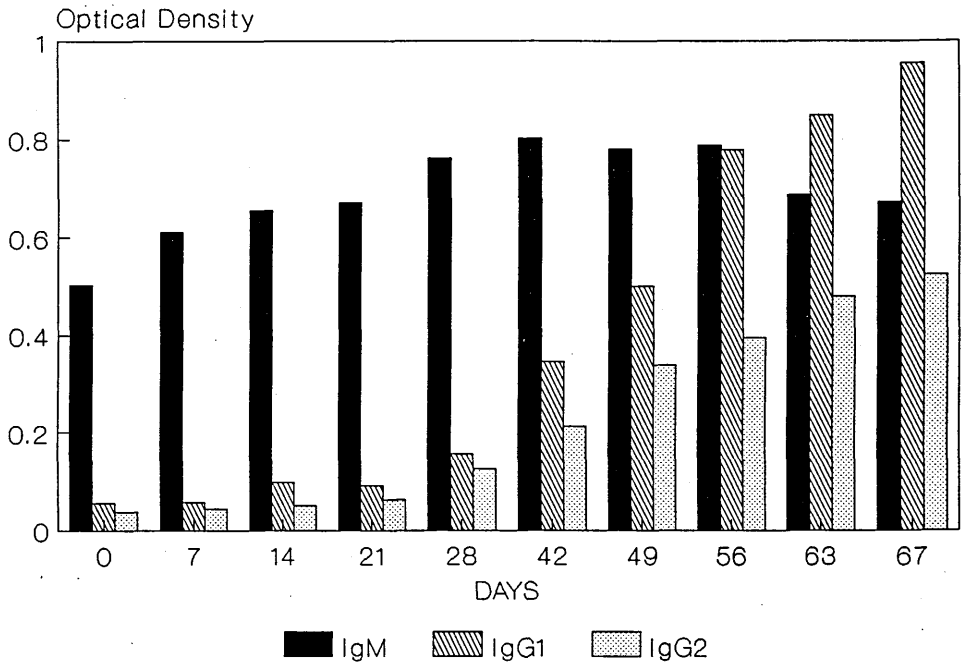


Figure 7. Mean IgM, IgG1 and IgG2 antibody levels from pooled serum samples of 3 animals, detected by ELISA using L3 soluble antigens, following oral vaccination with 1000 L3 irradiated at 400 Gy on days 0 and 21 and challenge with 50 L3/kg on day 42.

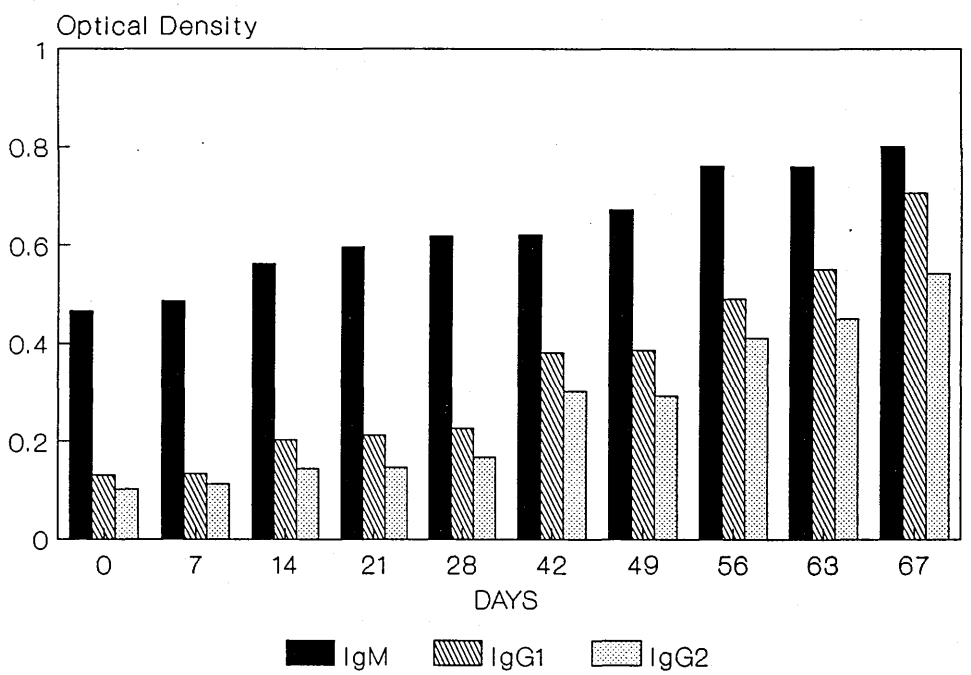


Figure 8. Mean IgM, IgG1 and IgG2 antibody levels from pooled serum samples of 3 animals, detected by ELISA using L3 soluble antigens, following intravenous vaccination with 2500 L3 irradiated at 1000 Gy on days 0 and 21 and challenge with 50 L3/kg on day 42.

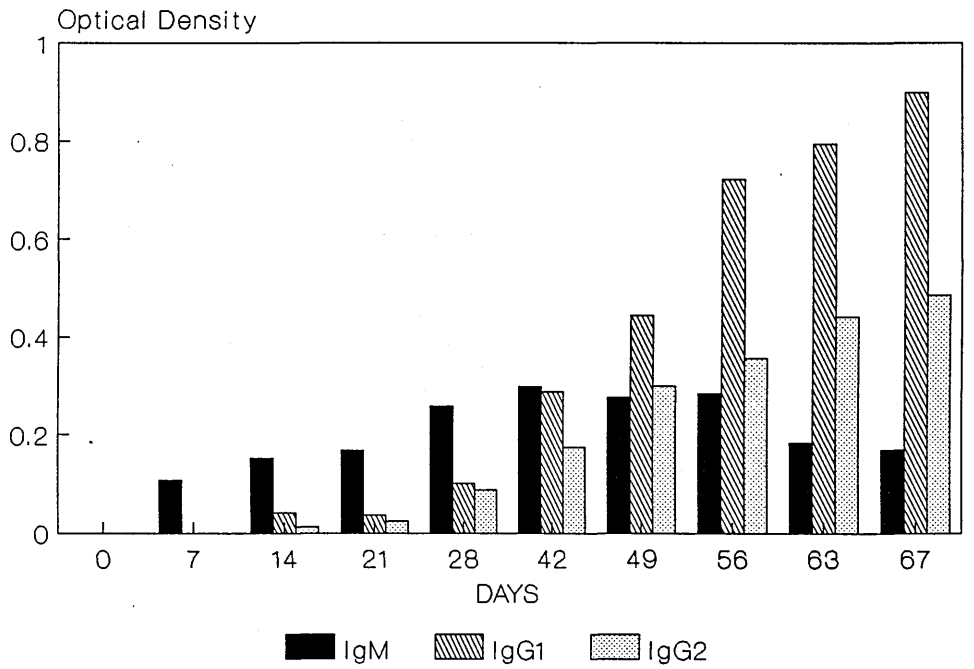


Figure 9. Mean IgM, IgG1 and IgG2 antibody levels from pooled serum samples of 3 animals, detected by ELISA using L3 soluble antigens, following oral vaccination with 1000 L3 irradiated at 400 Gy on days 0 and 21 and challenge with 50 L3/kg on day 42. The non-specific IgM reaction and the IgG backgrounds have been subtracted in this presentation.

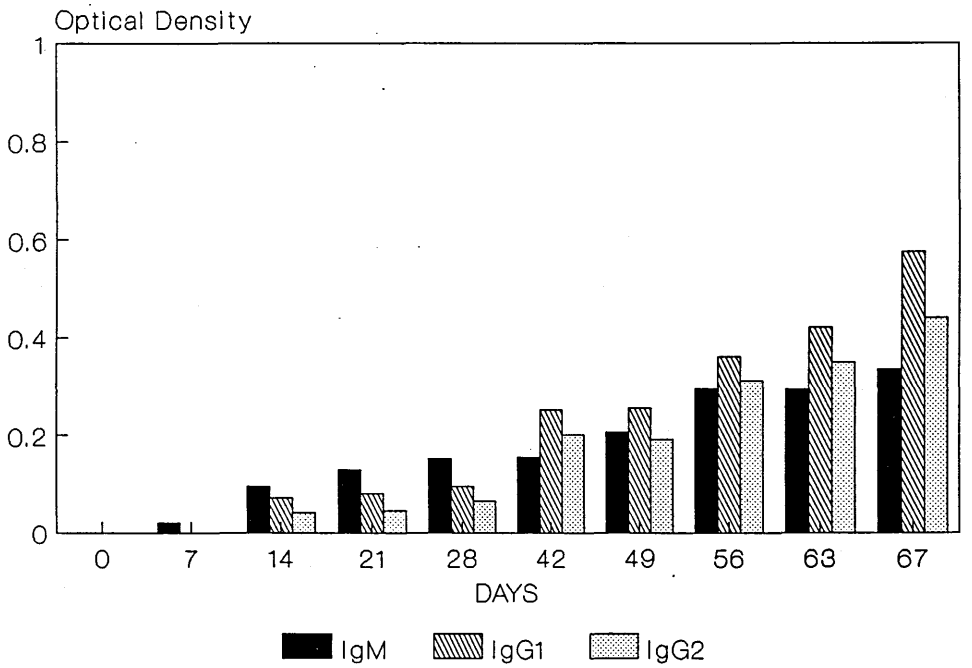


Figure 10. Mean IgM, IgG1 and IgG2 antibody levels from pooled serum samples of 3 animals, detected by ELISA using L3 soluble antigens, following intravenous vaccination with 2500 L3 irradiated at 1000 Gy on days 0 and 21 and challenge with 50 L3/kg on day 42. The non-specific IgM reaction and the IgG backgrounds have been subtracted in this presentation.

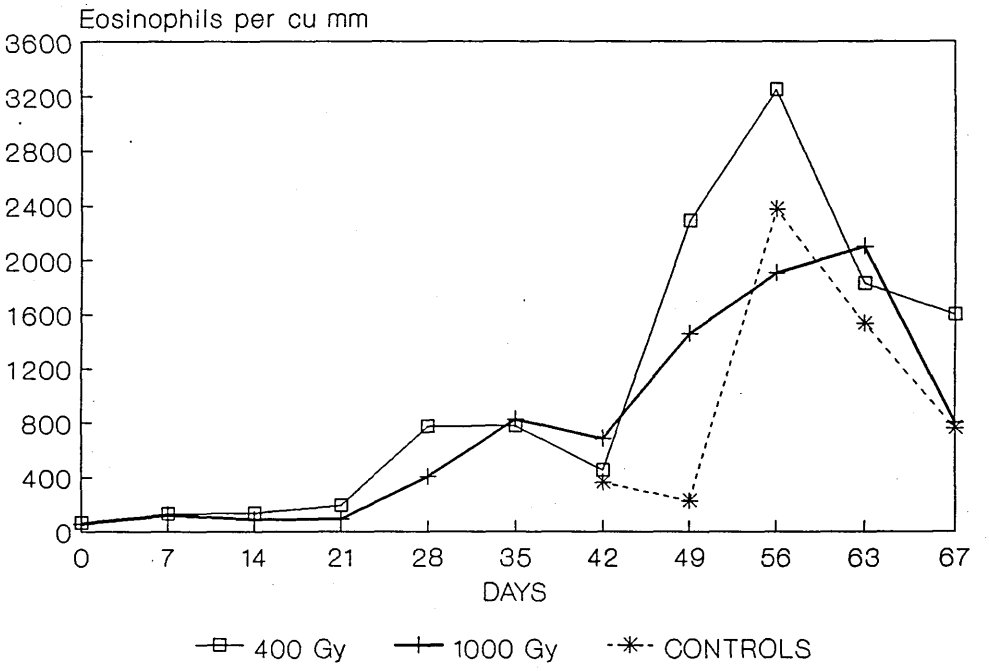


Figure 11. Mean eosinophil counts per cu mm of 3 animals per group, following vaccination with 1000 L3 irradiated at 400 Gy on days 0 and 21 orally, or 2500 L3 irradiated at 1000 Gy on days 0 and 21 intravenously and challenge with 50 L3/kg on day 42, compared with challenge controls.

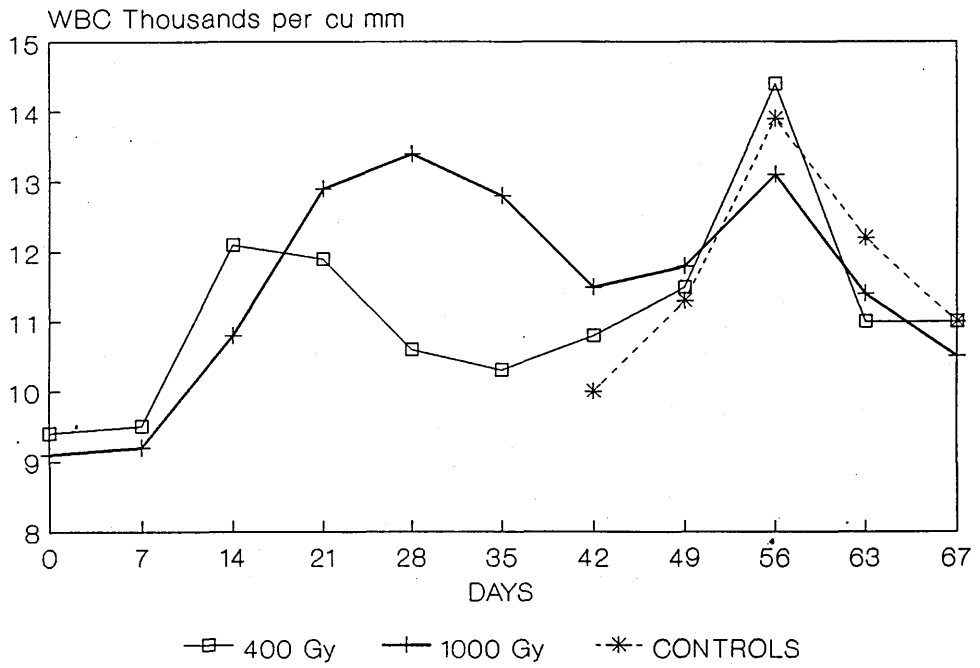


Figure 12. Mean white blood cells per cu mm of 3 animals per group, following vaccination with 1000 L3 irradiated at 400 Gy on days 0 and 21 orally, or 2500 L3 irradiated at 1000 Gy on days 0 and 21 intravenously and challenge with 50 L3/kg on day 42, compared with challenge controls.

II. Administration of larvae on multiple occasions

Experimental design

Six animals with weights ranging from 120 to 155 kg were divided into three groups of two animals each and infected on multiple occasions with normal or 400 Gy or 1000 Gy-irradiated L_3. The numbers of larvae administered, the routes and the dates of infection are shown in Table 13.

Faecal samples were obtained during the trial and the larvae administered only when the animals were negative by the Baermann technique. Serum was obtained prior to the start of the experiment and each time the animals were reinfected, to study the antibody development by the ELISA technique.

Table 13

Numbers of Larvae Administered and Route of Infection

Date	Normal L_3 Oral	400-Gy Oral	1000-Gy I.V.
09/02	2,000		
29/03	2,000		
06/04			5,000
26/04	10,000		10,000
23/05	20,000	5,000	10,000
16/06	20,000	10,000	10,000
10/07		10,000	20,000
28/07		20,000	
18/08	10,000	20,000	10,000
13/09	20,000	20,000	20,000
Total	84,000	85,000	85,000

Thirty-five days after the last infection the infected animals plus three parasite-naive control calves were challenged with 30 L_3 per kilogram of bodyweight and slaughtered 24 days

later for necropsy and estimation of worm burden.

Results

Clinical signs of mild parasitic bronchitis were only observed in the animals infected with normal larvae after the first and second infections. The repeated administration of large numbers of irradiated larvae and the final challenge of normal larvae did not produce any clinical abnormalities.

At slaughter the lungs of the reinfected animals presented no lesions at all. In contrast, large zones of consolidation were observed in the diaphragmatic lobes of the control animals. The numbers of worms recovered from the control animals were; 244, 943 and 1,466 while no worms were recovered from the lungs of the reinfected animals.

The results of the ELISA tests are shown in Figures 13, 14 and 15. The animals infected with normal larvae had a rapid increase of antibody levels reaching a peak after the third infection (Figure 13). After the fourth reinfection, the level of antibodies decreases slightly in one of the animals and remained almost at the same level in the other.

Calves infected with 400 Gy-irradiated L_3 showed a small increase of antibody levels during the first two infections which is characteristic of vaccination (Figure 14). In both animals a moderate increase was apparent up to the fifth infection in which the titres started to decrease.

The levels of antibody in the 1000 Gy-irradiated L_3 infected calves showed a marked increase after the first and second

infections, probably due to the intravenous route of infection, reaching a peak after four infections in one of the animals and after six in the other (Figure 15).

Discussion

Minor points of discussion have been dealt with in the results sections and it is proposed here to concentrate on the main findings.

Probably the most important result, subject to the constraints imposed by the small numbers of calves, was the observation that calves vaccinated with larvae irradiated at 1000 Gy were highly protected against challenge. Thus, two doses of such larvae conferred a protection of 76.7%, while a regime of seven infections gave 100% protection in terms of reduction in worm burdens.

Compared with the current double vaccination protocol, used commercially, the use of 1000 Gy-irradiated L_3 in the first experiment differed in that the larvae were administered IV and in larger numbers (i.e. 2,500 instead of 1,000). The reasons for this were that it was thought likely that the 1000 Gy-irradiated L_3 were incapable of penetrating the intestinal wall (Jarrett et al, 1960c) and the finding in guinea pigs that these larvae disappear very rapidly from the lungs.

The results of both experiments using 1000 Gy-irradiated larvae suggest that the L_3 present the major antigens responsible for protection. This does not exclude the possibility that these antigens could also be present in other stages of the parasite, but, being readily obtainable, make this stage a good source of

antigen for studies directed towards the development of a molecular vaccine.

An interesting finding arose with the use of mAb's to bovine immunoglobulins. A high non-specific IgM reaction was noticed in all negative sera analysed which was not detectable when IgG_1 and IgG_2 were used.

This reaction, presumably due to "heterophile" antibodies must have been produced, not as a response to previous immunisations with the parasite, but perhaps in response to antigens found in feed or in bacterial flora (Tizard, 1982). This subject is discussed further in Chapter five.

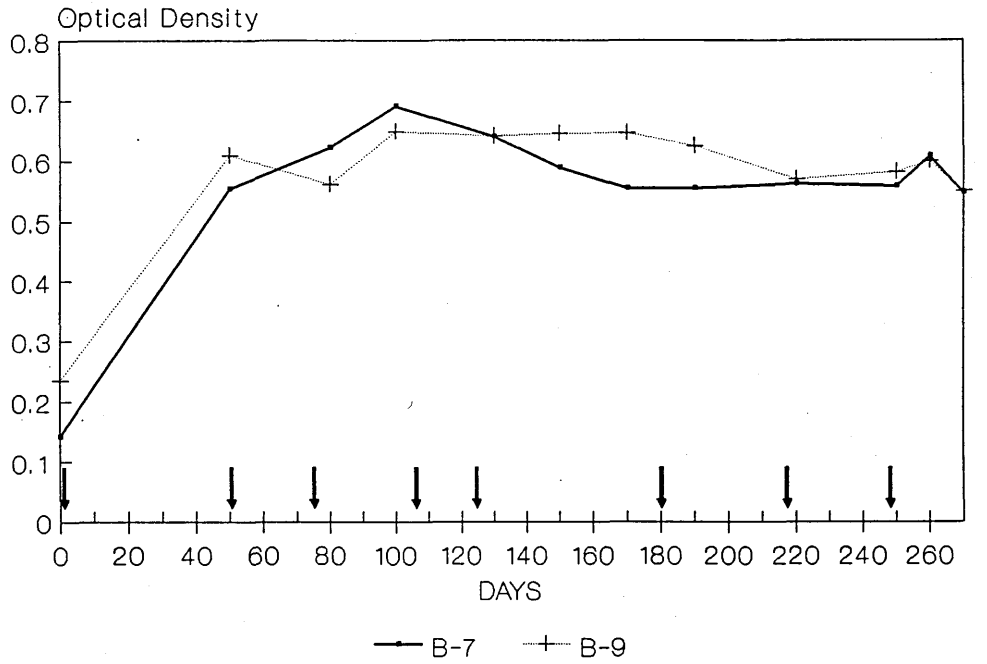


Figure 13. Mean antibody levels in calves, detected by ELISA using L3 soluble antigens, after multiple infections with normal L3 on days 0,48,76,103,127,188,216 and 250.

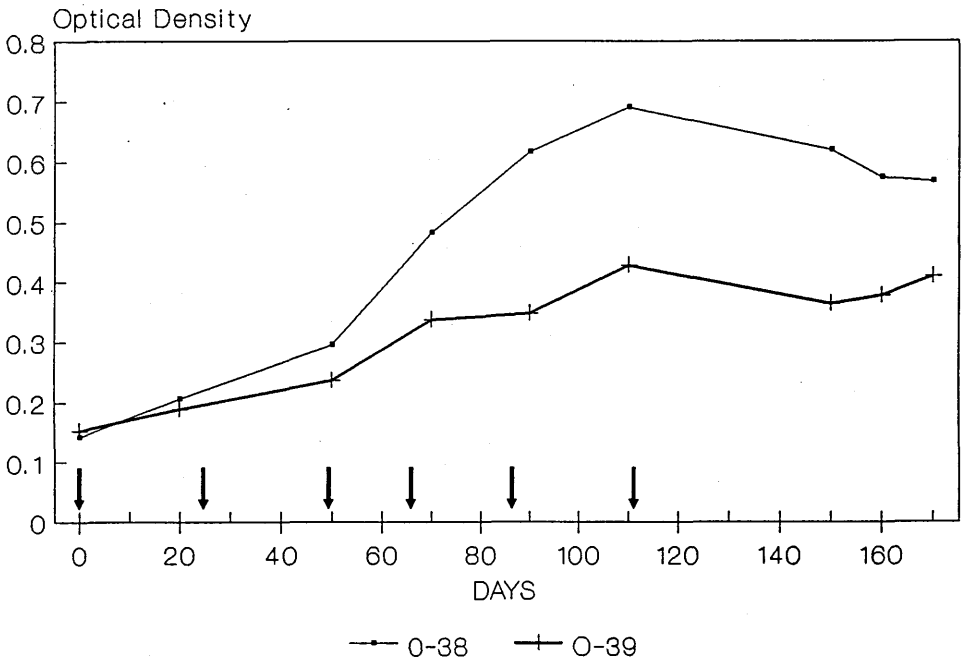


Figure 14. Mean antibody levels in calves, detected by ELISA using L3 soluble antigens, after multiple infections with L3 irradiated at 400 Gy on days 0, 24, 48, 66, 85, 113, and subsequent challenge with 30 normal L3/kg on day 147.

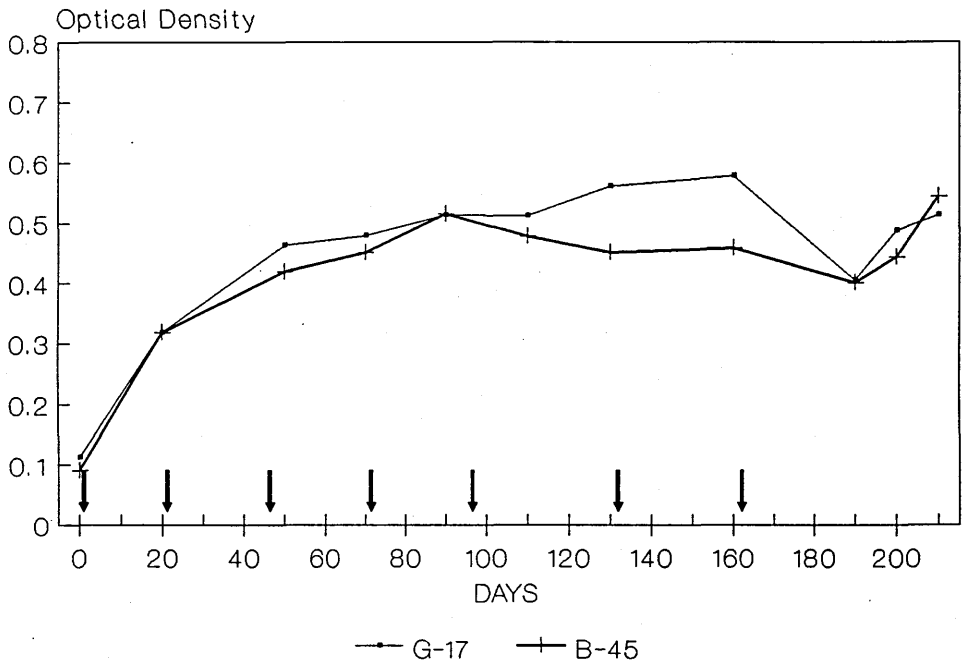


Figure 15. Mean antibody levels in calves, detected by ELISA using L3 soluble antigens, after multiple infections with L3 irradiated at 1000 Gy on days 0, 21, 47, 71, 95, 132, 160, and subsequent challenge with 30 normal L3/kg on day 194.

PASSIVE IMMUNISATION EXPERIMENTS IN CALVES

This had two purposes. The first was to confirm the results reported by Jarrett *et al* (1955a) in which sera, collected from six recovered field cases of parasitic bronchitis whose antibody levels had been boosted with experimental infections ranging from 50,000 to 200,000 infective larvae, was shown to confer protection against a single challenge of 4,000 infective *D. viviparus* L_3.

In the experiment reported by Jarrett *et al* (1955a), twenty-three litres of serum were obtained, and in order to reduce the volume to be administered in the passive transfer, a globulin preparation was obtained by adding Na_2SO_4 to a final concentration of 18 per cent. The globulin preparation was dialysed against running tap water overnight and then for 48 hours against isotonic NaCl. The final volume of this preparation was approximately one third of the original volume.

Five ten-week old calves were injected intraperitoneally with 500 ml of the immune globulin preparation daily on three consecutive days and two days after the last injection these five animals plus another five control calves were challenged with 4,000 lungworm larvae.

The results showed that all of the control animals became severely dyspnoeic. In contrast, and with the exception of one animal in which dyspnoea was apparent, the immunised calves did not develop any severe clinical signs during the experiment. At slaughter, the mean number of worms in the control animals was 786 as compared with 37.4 in the immunised calves, demonstrating

clearly that the serum had conferred a considerable degree of protection.

The second objective of the experiment looked at the possibility of transferring protection with sera raised against multiple infections of 1000 Gy-irradiated larvae.

Materials and Methods

Immune sera obtained from normal larval infections

Sera from six yearling cattle that had been grazing on endemic pastures from May until October, 1989, were used in the experiment. Each of the animals was subsequently infected in December on two occasions 14 days apart, the first time with 1,000 L_3 and the second with 10,000 larvae. None of the animals showed clinical signs of parasitic bronchitis all were killed 19 days after the second infection.

Immune sera from 1000 Gy-irradiated larvae infections

Two calves aged between three to four months were infected on four occasions IV with irradiated L_3; the first infection consisted of 5,000 larvae and on the three subsequent infections 15,000 irradiated larvae. Fifteen days after the last infection both animals were killed.

Immunoglobulin concentration procedure

At slaughter, blood was collected and allowed to clot, after which the serum was drawn off, centrifuged and pooled.

A crude immunoglobulin fraction, which contained all the immunoglobulins, was obtained by precipitation of the serum with

45% amonium sulphate saturation ($[NH_4]_2\ SO_4$) pH 7.0 (Heide and Schwick, 1973). The required amounts of $(NH_4)_2\ SO_4$ were added to both serum samples very slowly with stirring. The precipitation procedure was carried out at 4°C overnight. The globulin precipitate was freed from sulphate by 48 hours dialysis against running tap water and then for another 48 hours against a large volume of isotonic sodium chloride at 4°C. The final volume was reduced by approximately one half for the sera from the normal larval infections and by two thirds for the sera from the 1000 Gy L_3 infections.

Passive immunisation and challenge protocol

Two calves of 148 and 157 kg bodyweight and each injected intraperitoneally with 1.5 litres of the immunoglobulin preparation obtained from the normal larval infections. A second group of two calves of 128 and 159 kg bodyweight were each injected with 1.0 litre of the immunoglobulin preparation from the 1000 Gy infections.

Twenty-four hours later, these four calves and four controls of similar weights, were each infected with 30 L_3/kg. Blood samples were obtained prior to immunisation, the day of infection, and weekly afterwards until the animals were killed on day 24 post-challenge. Specific antibody levels were measured by the ELISA technique.

Clinical examination of the calves was carried out twice weekly for two weeks after infection and then repeated every day until the calves were killed.

ELISA interpretation

The optical density of the specific antibody using L_3 soluble homogenates was calculated by the formula: (V-N)/(P-N) X 100, V being the test serum reading, P the mean reading from the positive controls and N the reading from the mean of the negative controls (Taylor et al, 1988).

Results

No clinical difference between the immunised and the control animals was apparent during the trial. The RPM of both groups started to increase on day 15 and by day 24 was over 75 RPM in all animals. No larvae were detected in the faeces at any time during the experiment.

The numbers of worms present in the lungs of the four control calves were 1,174, 1,088, 973 and 405 i.e. a mean of 910 worms. The two calves passively immunised with serum from normal larval infections had 203 and 185 worms respectively, indicating a mean reduction of 78.6% in worm recovery. In contrast, the calves immunised with serum from 1000 Gy infections had worm burdens of 872 and 822 indicating a complete absence of protection.

The superficial lesions in the lungs of the control and "1000 Gy-serum" recipients were more extensive than those of the "400 Gy-serum" calves, mainly consisting of larger consolidated areas in the diaphragmatic lobes; also, the lungs of the two former groups of calves when compared to those of the last group were clearly augmented in size due to a well-marked interstitial emphysema.

A further finding of interest was that the pleural surfaces and parenchyma of the "400 Gy-serum" recipients were studded with multiple yellow or green nodules of around 1-4mm in diameter. These were completely absent in the other two groups.

The results of the ELISA tests on the immunised and control calves are shown in Figures 16 and 17. It is evident that the absorption of immunoglobulin into the circulation took more than 24 hours. Eight days after injection a high level of specific antibodies was detected in both immunised groups which increased until the calves were killed. However, a comparison of their antibody levels showed that the levels of the "400 Gy-serum" recipients were almost double of the "1000 Gy-serum" calves. In the control animals, a low level of antibodies was detected on day 14 after challenge which increased until the end of the experiment.

Discussion

Any interpretation of the results of this experiment must be undertaken with reservations in view of the small number of calves in the three groups. Nevertheless, the results were consistent and paralleled those found in Chapter three which were concerned with passive immunisation of guinea pigs.

With regard to the degree of protection conferred by the bovine serum from the normal larval infections, the result confirmed that reported for this species by Jarrett et al (1955a), namely that a high and reasonable similar degree of protection was achieved of the order of a 78% reduction in worm

burden. This result was also comparable with that reported in Chapter three for guinea pigs immunised with serum from normal larval infections.

Perhaps more interesting was the observation that the pleural surface and parenchyma of the lungs of both immunised calves were studded with several hundred greenish-yellow nodules up to a few millimetres in size. These nodules have been previously described as occurring after larval challenge of actively immunised calves (Jarrett and Sharp, 1963). Their presence supports the conclusion that the reduction in worm burdens in this experiment was due to immunological interference and also suggests that antibody plays a significant role in protection under conditions of natural challenge.

The calves immunised with serum from the 1000 Gy larval infections were not protected at all, a result also obtained from the experiment in guinea pigs. Furthermore their lungs were free of lymphoid nodules.

The failure of this type of serum to confer passive protection contrasts with the high degree of active immunity possessed by calves, similar to the donors, which were infected on two or more occasions with larvae irradiated at 1000 Gy.

There are, perhaps, three possible explanations for this finding. The first is that the level of protective antibodies stimulated by the 1000 Gy larval infections (and possibly restricted to the third larval antigens) is deficient in quantity or range. While this may be adequate to counter challenge infections of an actively immunised calf, the passive transfer of

a volume, albeit considerable, of such serum is insufficient to confer protection. In this contest it may be relevant that the titre of antibody transferred to the normal L_3 serum recipients, as measured by ELISA, was one third higher than the one transferred to the "1000 Gy-serum" recipients. However, as noted elsewhere (Chapter seven), the level of ELISA antibodies in this system cannot be used as an index of protection.

A second possibility is that immunity to D. viviparus is not due solely to circulating antibody and that a cell-mediated component operates against the invasive third larval stage. It is known that the production of mucosal mast cells, eosinophils and goblet cell hyperplasia show a marked T-cell dependency and the possible role of these elements as effector mechanisms of immunity against helminths has been reviewed extensively, most recently by Kassai (1989). Thus, while such a mechanism would protect a donor calf immunised on several occasions with 1000 Gy-irradiated L_3, it would not be transferred in the serum.

In line with the last theory there is perhaps a third possibility, namely, that local secretion of bronchial or mucosal IgA could play a role in protection as has been suggested by some workers (Duncan, Smith and Dargie, 1978; Smith, Jackson, Jackson, Williams, Willadsen and Fehilly, 1984).

These last two possibilities, if confirmed, would bring resistance mechanisms to D. viviparus more into line with those tentatively ascribed against gastrointestinal nematodes, with the proven antibody component acting as a "back-up" mechanism against larvae which successfully penetrate the mucosa.

However, at present, it must be concluded that the possible existence of immune mechanisms against D. viviparus, other than antibodies, remains a subject for speculation.

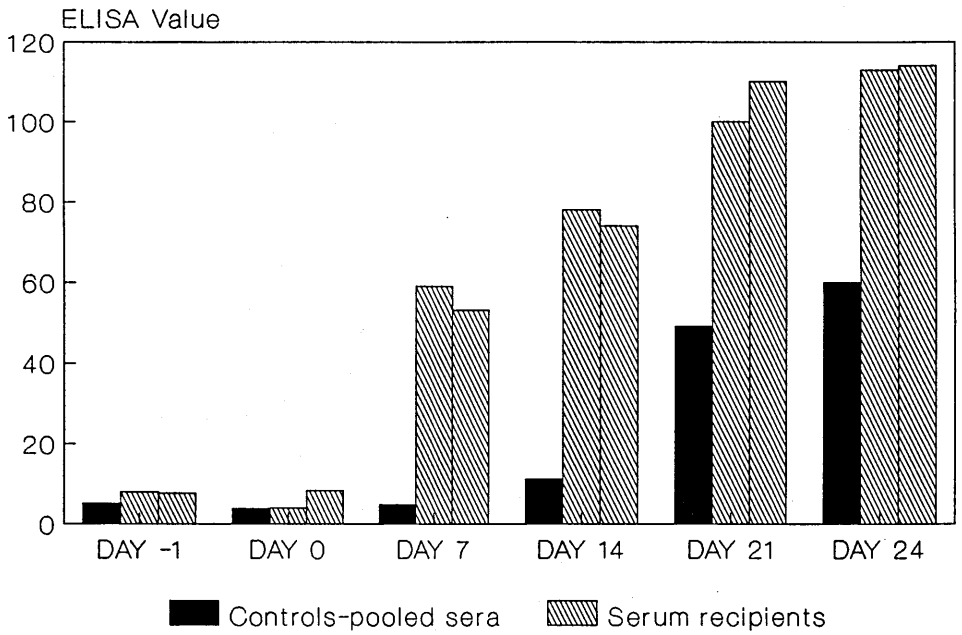


Figure 16. Mean antibody levels in calves after transfer of immune serum raised against normal L3, and subsequent challenge with 30 L3/kg on day 0, compared with pooled sera from challenge controls.

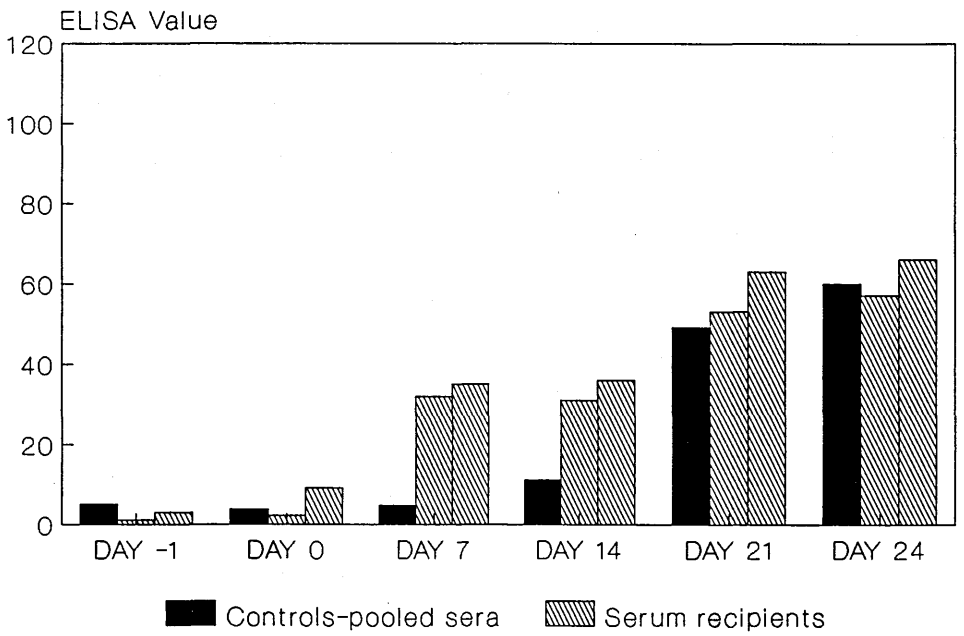


Figure 17. Mean antibody titres of calves after transfer of immune serum raised against L3 irradiated at 1000 Gy, and subsequent challenge with 30 infective L3/kg on day 0, compared with pooled sera from challenge controls.

CHAPTER FIVE

THE USE OF QUANTITATIVE FLUORESCENCE TO MEASURE THE ANTIBODY RESPONSE TO THE SURFACE-EXPOSED ANTIGENS OF THIRD STAGE LARVAE AND ADULT PARASITES

INTRODUCTION

Although it has been shown for several nematode infections that the surface proteins of the parasites are antigenic (Mackenzie, Preston and Ogilvie, 1978; Philipp, Parkhouse and Ogilvie, 1980; Maizels, Meghji and Ogilvie, 1983), there have been no equivalent studies on the bovine lungworm *D. viviparus*.

Most of the studies on surface of nematodes have used radio-iodination techniques and these have shown that the number of major proteins present is usually five or less (Maizels, Philipp and Ogilvie, 1982). However, this surprisingly small number may reflect a bias towards identifying only those polypeptides containing lysine and tyrosine residues, since only these are receptive to radio-iodination by the conventional techniques (Maizels *et al*, 1982). Other constituents such as glycolipids and polysaccharides have been excluded from the analysis. Nevertheless, it is possible that these non-protein cuticular antigens are important in the development of immunity to the parasite.

In the experiments presented in this Chapter, quantitative fluorescence, which is not dependent on the biochemical nature of the antigens, was used to study the antibody response to the surface-exposed materials of the infective third stage larvae and adult parasites.

The stage and species-specificity of *D. viviparus* was examined using sera from calves and guinea pigs that were infected with normal or irradiated larvae.

The materials and methods used in this section have been

described previously in Chapters two, three and four and only the results and discussion are presented here.

EXPERIMENT ONE

The specificity of the antibody response to larval and adult D. viviparus

Results

Using exsheathed L_3, which had spontaneously exsheathed or were exsheathed artificially, and which are presumably the invasive stage, the results were disappointing in that substantial levels of normal bovine immunoglobulins bound non-specifically to the surface of the larvae (Figure 18). While the level of binding of sera obtained from calves infected with normal or 400 Gy or 1000 Gy-irradiated larvae was substantially higher, the high background of non-specific binding made the interpretation of serum samples difficult.

Fortunately the use of sera from normal and infected guinea pigs, which did not exhibit this non-specific reaction, confirmed the specific binding of Ig to the surface of the parasite (Figure 18).

Using adult parasites as antigens (Figure 19), only serum from infection with normal larvae, leading to a patent infection, produced a response to the surface, which suggests stage-specificity of the surface of the adult parasites. In additon, serum from infected guinea pigs, in which the larvae do not mature beyond the early fifth stage, showed no response to adult surface antigens.

EXPERIMENT TWO

Attempts to define the basis of non-specific binding of normal serum to third stage larvae

The results reported above together with those presented in Chapter four on the ELISA isotype antibody response, suggested that the adsorption of bovine IgM might have been the cause of the non-specific fluorescence observed against the surface of L_3.

In order to examine this and to show the Ig-isotype response to the surface of third stage larvae and adult parasites, mouse monoclonal anti-bovine IgM, IgG_1 and IgG_2 antibodies were used in the immunofluorescence test.

Results

These showed that the high degree of non-specific fluorescence observed in the previous experiment was reproduced exclusively by the IgM (Figure 20). In contrast, very low binding was observed when IgG_1 or IgG_2 was used. When serum from newborn calves, i.e. serum collected from calves that were ten days old or less was used, a similar IgM cross-reaction to the one seen with normal serum was detected. This may indicate that the calves had contact with the cross-reacting antigen immediately after being born or, possibly, that some IgM antibodies were acquired via the colostrum. Certainly, using foetal calf serum the non-specific fluorescence was eliminated (Figure 20).

Serum from animals which had been infected on three occasions with 2,000 normal larvae the first two times and 5,000

on the third occasion, showed a marked increase in the IgM response but a low response of IgG_1 and IgG_2. This low increase in IgG_1 and IgG_2 is probably due to the rapid development of the L_3 into L_4, etc. which does not allow the host to mount a secondary response to the L_3 antigens.

Monoclonal antibodies were also used to analyse the adult parasite-bovine immune serum system. Only serum from animals infected with normal larvae elicited antibodies, primarily IgG_1, against surface antigens. Serum from animals infected on multiple occasions with 400 Gy or 1000 Gy-irradiated larvae did not produce fluorescence, nor did the sera from newborn or normal calves (Figure 21), again confirming the stage specificity of surface antigens of adult worms compared with L_3.

EXPERIMENT THREE

Species-specificity of the surface antigens of the third stage larvae and adult parasites

This was examined by submitting exsheathed L_3 and adult parasites to sera from cattle infected experimentally on two occasions with O. ostertagi and C. oncophora, two of the most common gastrointestinal parasites of cattle.

Results

As expected, a high non-specific IgM cross-reaction was observed with all the sera used against the exsheathed L_3 (Figure 22). No IgG_1 or IgG_2 reaction was detected using sera from C. oncophora or O. ostertagi infections, which indicates

species-specificity of the surface antigens of the L_3.

When adult D. viviparus worms were examined with immune sera from the three infections, only sera from patent D. viviparus infections evoked fluorescence, which suggests species-specificity also of the surface antigens of adult parasites (Figure 23).

EXPERIMENT FOUR

The kinetics of the isotype antibody response to the surface antigens of third stage larvae and adult parasites of D. viviparus

In order to study this, serum from two regimens of infection were used. First, vaccination with irradiated L_3 followed by challenge and secondly, multiple infections with normal larvae.

Results

In the first regimen, pooled sera from two groups of three animals which had been vaccinated on two occasions with either 400 or 1000 Gy-irradiated larvae before challenge three weeks later were tested (details in Chapter four).

As was seen with the ELISA technique using L_3 homogenate as antigen (Chapter four), serum from both of these vaccination regimens of irradiated larvae produced a very small increase in antibody levels. Thus, twenty-one days after first vaccination a small increase in IgM was detected while IgG_1 and IgG_2 remained at the same level. Three weeks after the second vaccination, i.e. the day of challenge, the IgM started to decrease and a small increase of IgG_1 was noted. Again, a high non-specific IgM

antibody response was detected prior to vaccination (Figure 24).

By day 67, (25 days after challenge), a new rise in the levels of IgM was detected in both groups presumably due to the number of larvae used in the challenge (30 L_3/kg). The IgG_1 response to the challenge was higher for the 1000 Gy vaccinated group, which suggests that a better recognition of the third stage antigens was produced, due probably to the lack of further development in this type of irradiated larvae.

The use of sera from two animals infected on several occasions with normal L_3 produced a specific but discrete increase in the IgM level for the first three infections after which a decrease in the relative fluorescence was observed (Figure 25). Very small increases in IgG_1 levels were observed up to the fifth infection.

When adult parasites were used as antigen, a switch in the levels of IgM to IgG_1 was observed after two infections. At the time of the fourth infection, the ratios of the levels of IgM to IgG_1 and IgG_2 were 1 to 5.8 and 1 to 2.4 respectively (Figure 26).

Discussion

The first observation that nematode surface antigens could be stage-specific was made on Trichinella spiralis using an eosinophil-binding assay (Mackenzie et al, 1978). Since then, it has been shown that stage-specificity of the pattern of labelled cuticular components is a general phenomenon (Philipp et al, 1980; Cox, Shamansky and Boisvenue, 1989).

The results observed in this Chapter using quantitative fluorescence showed that the surfaces of L_3 and adult D. viviparus are antigenic and that the exposed antigens are both stage and species-specific. Thus, it was found that immune sera from guinea pigs infected with normal L_3, and immune sera from calves infected with 400 Gy-irradiated L_3 (in both cases the host would have been exposed to L_3 and L_4 surfaces but little to fifth stages) did not bind to the surface of adult parasites.

It was interesting to observe that a non-specific IgM binding, similar to that observed previously against soluble antigens from L_3 homogenates (see Chapter four), was present when normal or neonatal calf sera was used against the surface of L_3 but not against adult parasites. This result confirmed the presence of "heterophile" antibodies on the L_3 surface. Also, when adult parasites were used, fluorescence only occurred with bovine serum from patent infections. This result strongly suggest antigenic specificity of the L_3 and adult surfaces.

The species-specificity of the larval and adult surfaces was demonstrated in two ways. First, the use of sera from calves infected with 1000 Gy-irradiated L_3 against third stage larvae of C. oncophora and O. ostertagi showed that while a non-specific IgM response again occurred against the surface antigens of D. viviparus no fluorescence occurred with the IgG_1 and IgG_2 antibodies. Also sera from D. viviparus immune animals was tested against exsheathed third stage larvae of C. oncophora and O. ostertagi and no fluorescence was observed.

Secondly, when D. viviparus adult worms were used as antigen, only sera from patent lungworm infection evoked fluorescence.

Finally, a study of the kinetics of the antibody isotype response to the surface antigens of the third stage larvae and adult parasites confirmed that vaccination regimens of irradiated L_3 produce only a very small increase in IgM and IgG (see Chapter four).

After challenge the IgM increased in both groups of animals, and in the group vaccinated with 1000 Gy-irradiated larvae an IgG_1 response was also observed, due presumably to a better recognition of the third stage surface antigens.

Repeated infection with normal larvae is mainly characterised by an increase in the IgM level during the first three infections. Very low increases in IgG levels were observed which can probably be explained as a lack of recognition of surface antigens due to the short time that these normal larvae remain in the third stage.

When adult parasites were used as antigen, a switch from IgM to IgG_1 production was observed from the second infection onwards. This probably indicates that the long period of residence of adult parasites in the lungs of the infected animals allowed specific IgG_1 and IgG_2 antibody production to take place.

Table 14

List of the Sera used in the Immunofluorescence Studies

CALF

BNS	=	Bovine Normal Serum
NBS	=	Neonatal Bovine Serum
B400 Gy	=	Bovine serum after three infections with larvae irradiated to 400 Gy
B1000Gy	=	Bovine serum after three infections with larvae irradiated to 1000 Gy
BNL_3	=	Bovine serum after one infection with normal infective L_3
$BNL_3/3$	=	Bovine serum after three infections with normal infective L_3
BC.o.	=	Bovine serum after two infections with Cooperia oncophora L_3
BO.o.	=	Bovine serum after two infections with Ostertagia ostertagi L_3
BFS	=	Bovine Foetal Serum

GUINEA PIG

GPNS	=	Guinea Pig Normal Serum
$GPNL_3/3$	=	Guinea pig serum after three infections with normal L_3

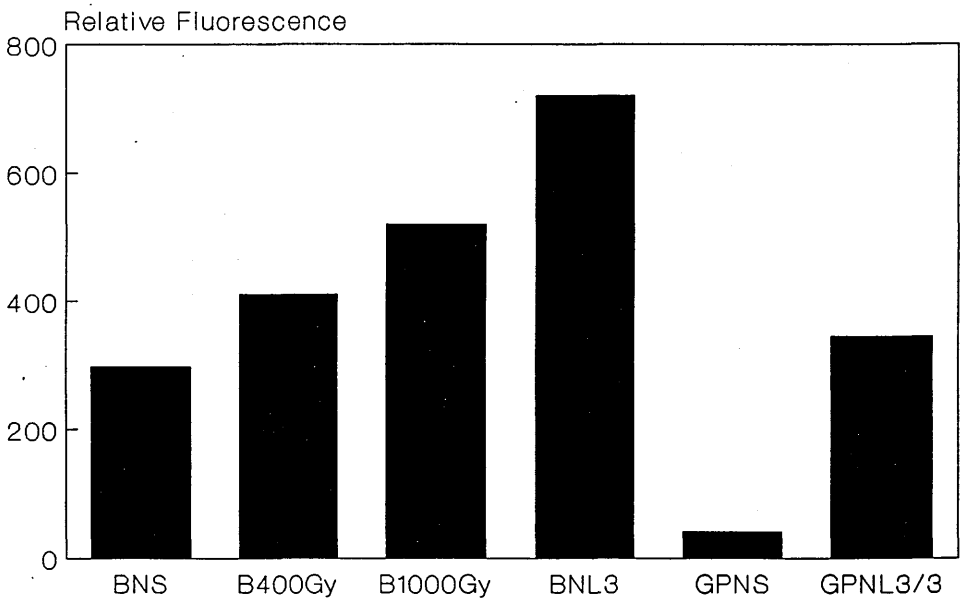


Figure 18. Antigenicity of the surface-exposed antigens on the exsheathed L3 as measured by fluorescence. Bovine sera raised against normal, 400 Gy and 1000 Gy irradiated L3 and guinea pig sera raised against normal larvae was compared with serum from normal calves and guinea pigs. See Table 14 for abbreviations used for each serum.

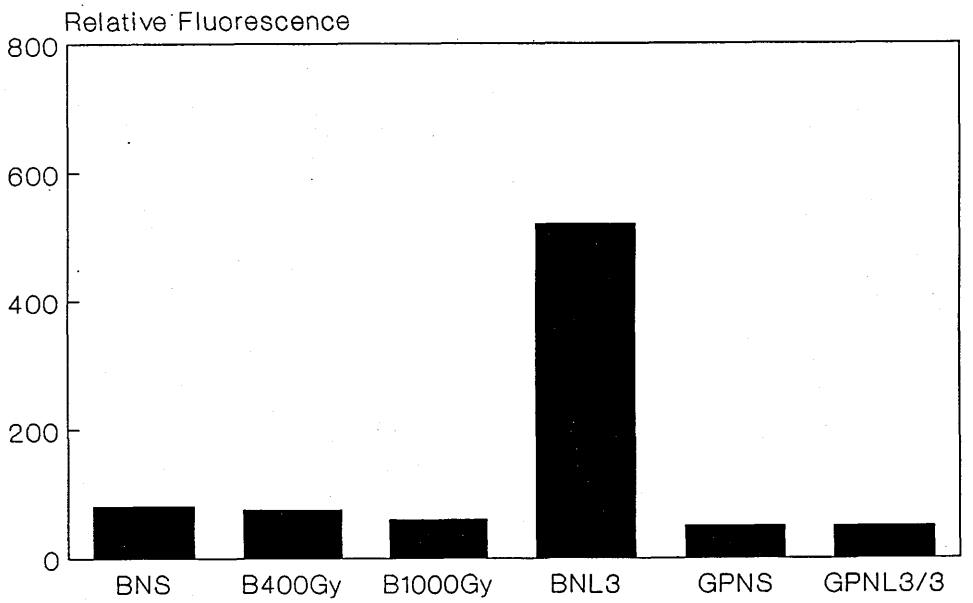


Figure 19. Stage-specificity of surface-exposed antigens of adult parasites as measured by fluorescence. Bovine sera raised against irradiated and normal larvae as well as guinea pig immune sera to normal L3 was compared with normal bovine and guinea pig sera. See Table 14 for abbreviations used for each serum.

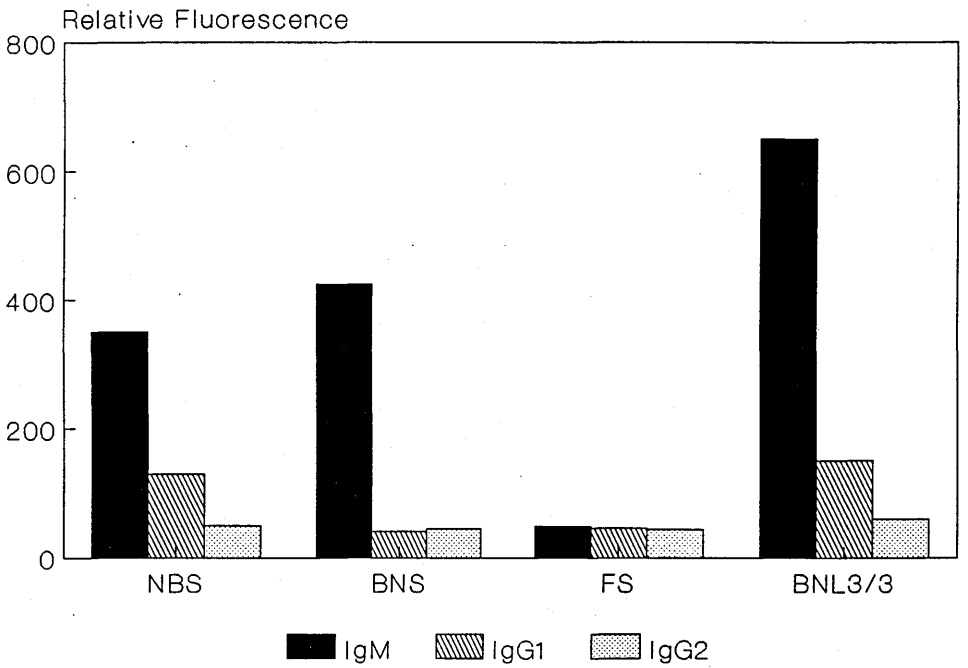


Figure 20. Isotype antibody response to the surface of exsheathed larvae as measured by fluorescence. Bovine sera raised against normal larvae was compared to bovine normal, neonatal and foetal serum using monoclonal antibodies to bovine IgM, IgG1 and IgG2. See Table 14 for abbreviations used for each serum.

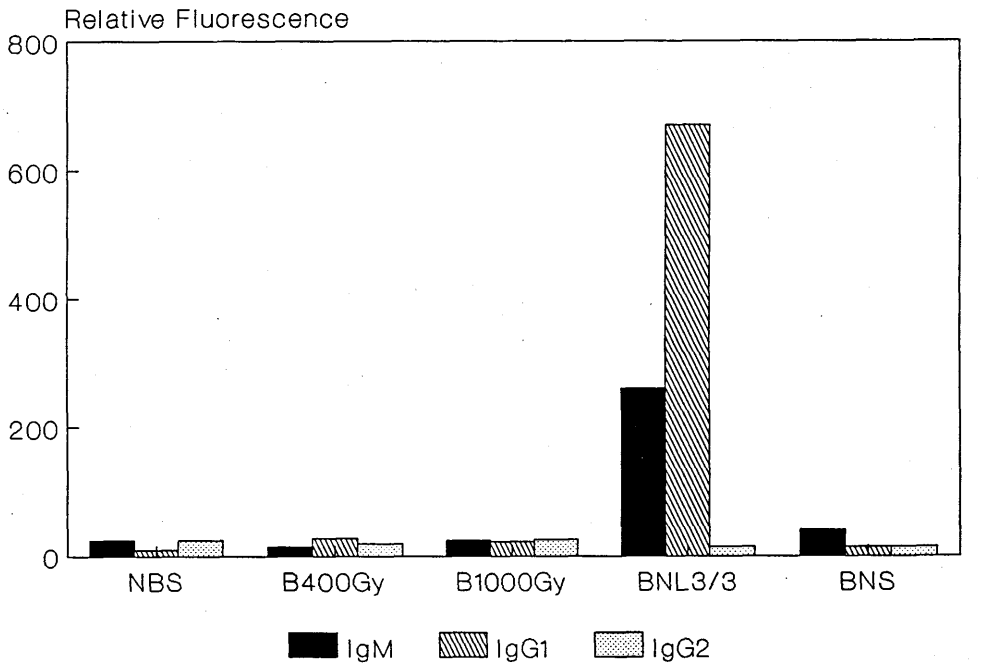


Figure 21. Isotype antibody response to the surface of adult parasites as measured by fluorescence. Bovine immune sera raised against normal or 400 Gy or 1000 Gy irradiated L3 was compared to normal and neonatal bovine serum. See Table 14 for abbreviations used for each antiserum.

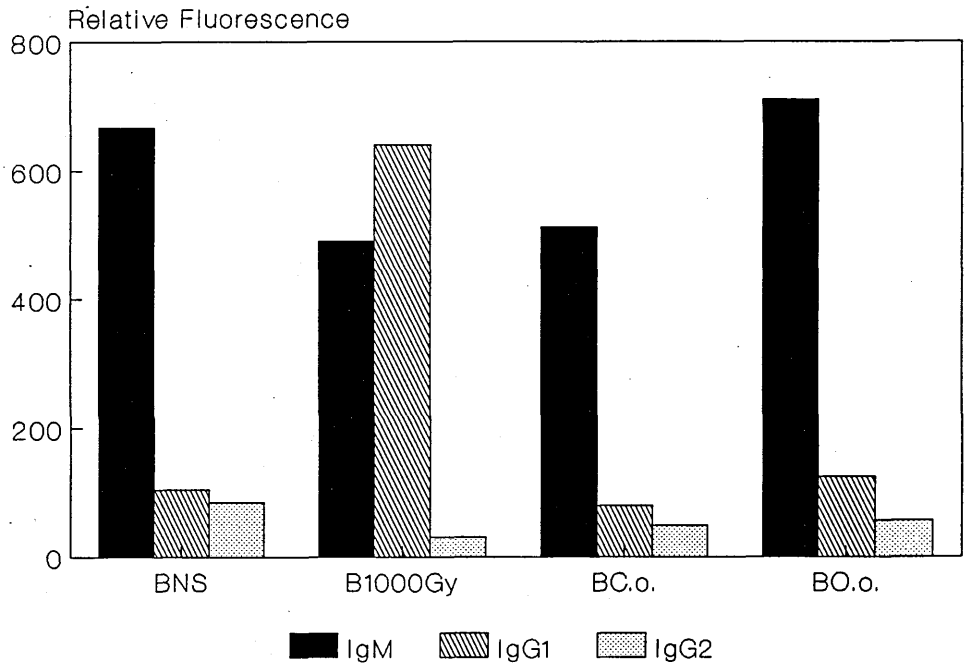


Figure 22. Species-specificity of the surface-exposed antigens of normal exsheathed L3 as measured by fluorescence. Bovine sera raised against C. oncophora or O. ostertagi were compared with normal sera and 1000 Gy irradiated L3 immune sera. See Table 14 for abbreviations used for each serum.

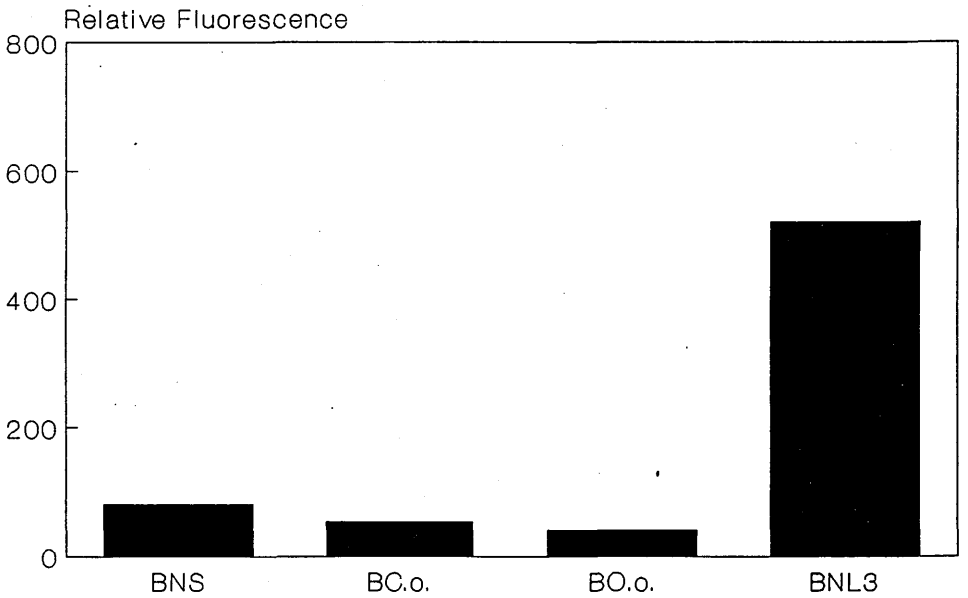


Figure 23. Species-specificity of surface antigens of adult parasites as measured by fluorescence. Immune bovine sera raised against C. oncophora or O. ostertagi were compared with normal bovine sera and sera raised against normal larvae. See Table 14 for abbreviations used for each antiserum.

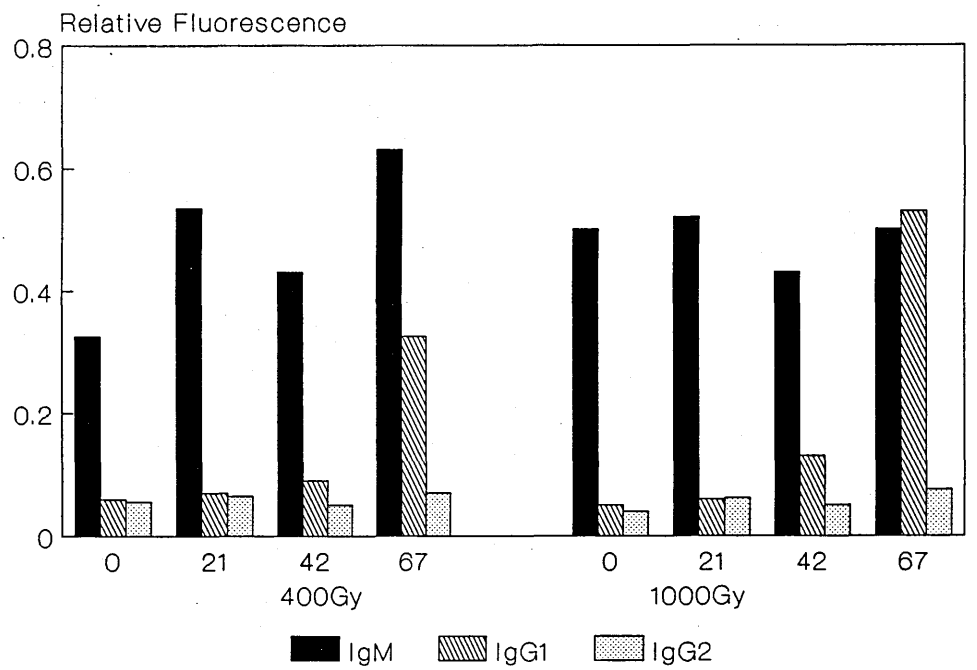


Figure 24. Kinetics of the isotype antibody response, as measured by fluorescence to the surface antigens of exsheathed L3, from pooled samples of 3 animals per group vaccinated with 400 Gy or 1000 Gy irradiated L3 on days 0 and 21 and challenged with 30 infective L3/kg on day 42.

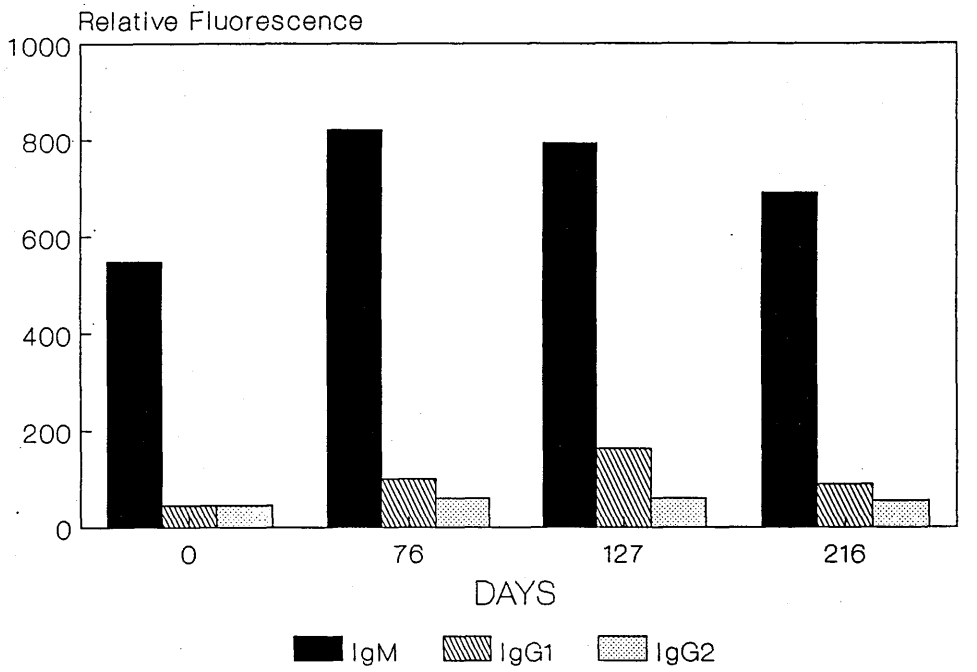


Figure 25. Kinetics of the isotype antibody response, as measured by fluorescence to the surface antigens of exsheathed L3, from pooled samples of 2 animals infected with normal L3 on days 0,48,76,103,127,188 and 216.

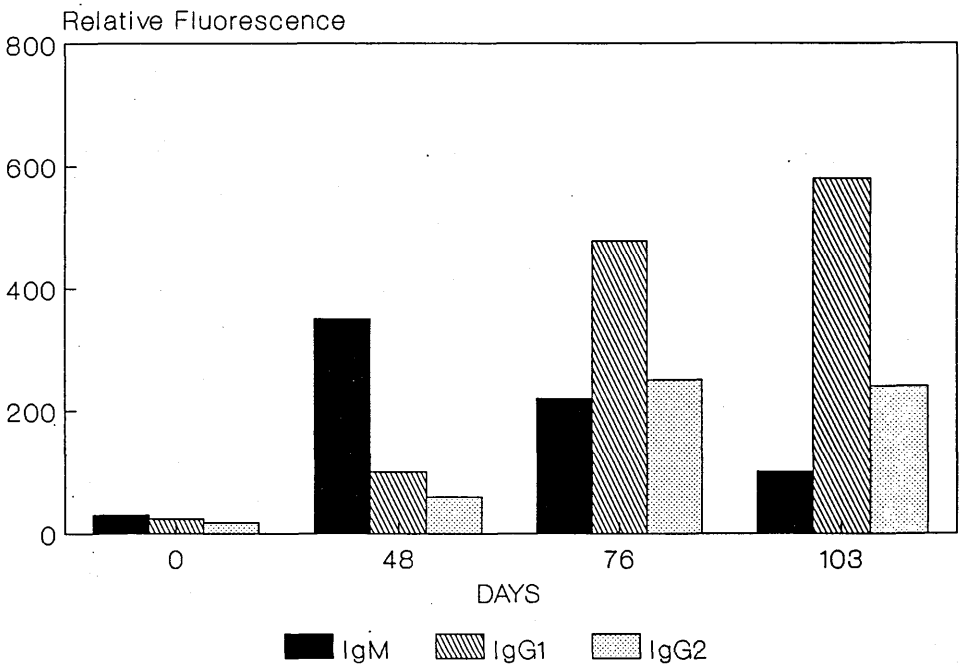


Figure 26. Kinetics of the isotype antibody response, as measured by fluorescence to the surface antigens of adult worms, from pooled samples of 2 animals infected with normal L3 on days 0,48,76 and 103.

CHAPTER SIX

PRELIMINARY STUDIES ON THE ANTIGENIC COMPOSITION OF THE THIRD STAGE LARVAE AND ADULT *DICTYOCAULUS VIVIPARUS*

INTRODUCTION

Nematodes are complex organisms containing many thousands of proteins with the potential to induce immune responses in the host; many of these responses, of course, will have no functional significance in terms of protection (O'Donnell, Dineen, Wagland, Letho, Werksmeister and Ward, 1989).

There are indications that E/S associated with parasites (Rickard, Boddington and McQuade, 1977) or purified somatic components (Despommier, Campbell and Blair, 1977) are more successful in stimulating host resistance when used as vaccines than are complex unfractionated preparations from parasites. A possible reason for this could be that antigenic competition among components diminishes the stimulation of specific immune responses (Neilson and Van De Walle, 1987).

One strategy for the development of a vaccine is to identify from the thousands of proteins present in a parasitic organism those which are associated with protective responses in the host (O'Donnell *et al*, 1989).

The SDS-PAGE technique has been widely used to obtain a better understanding of the antigenic composition of complex organisms, by separating the unknown proteins according to molecular size and estimating molecular mass by comparing their relative electrophoretic mobility to standard proteins.

Very little is currently known about lungworm antigens; thus, in this Chapter, the first stages of an antigenic analysis of *D. viviparus* using SDS-PAGE are described.

Two aspects were studied. First, a comparison of the

soluble extracts of sonicated third stage larvae and adult parasites and, secondly, the recognition by bovine and guinea pig immune sera of E/S products of adult worms. Only E/S from adult worms was examined here because, as pointed out in Chapter two, E/S products were not recovered from the third stage larvae.

Results

Coomassie blue staining of the SDS-PAGE patterns shown in Figure 27 indicated the complexity of aqueous somatic extracts of third stage larvae and adult parasites. More than 30 peptides with molecular weights ranging from 14.4kDa to greater than 94kDa were detected regardless of whether electrophoresis was carried out under reducing or non-reducing conditions. A comparison between the results of the two conditions can permit the identification of proteins or glycoproteins which have covalently linked subunits. Although a complete absence of such molecules is unlikely, none were detected in this analysis, among the major components at least. The presence of components common or specific to each stage was, however, clear, although similarity in apparent molecular mass does not necessarily infer identity.

Labelling of E/S products of adult parasites with ^{125}-I

Metabolic products of adult worms were successfully labelled using the Bolton-Hunter technique (described in Materials and Methods). After TCA precipitation, the percentage of radioactivity associated with the macromolecules was between 50% and 60%.

SDS-PAGE and autoradiographic analysis of adult E/S

Unlike somatic extracts, the metabolic products of adult parasites exhibited a much simpler pattern (lane 2 of Figures 28, 29, 30 and 31). Seven heavily labelled proteins with molecular weight between 14kDa and 43kDa were resolved as well as several less heavily labelled proteins with higher molecular weight.

Antigenicity of adult E/S labelled proteins as determined by immunoprecipitation and subsequent autoradiograph

To determine if metabolic products of adult parasites were antigenic, immunoprecipitates of the labelled E/S with guinea pig and calf sera were prepared.

Following SDS-PAGE procedure and autoradiography the results using guinea pig sera (Figures 28, 29, 30 and 31) showed that the E/S products were recognised by antibodies raised against larvae irradiated at 400 Gy or 1000 Gy and inoculated orally or intraperitoneally.

Recognition of adult E/S products was also observed in calves vaccinated on several occasions with large numbers of irradiated L_3 (Figure 32) (the numbers of larvae given are shown in Chapter four). In addition, it was also found that vaccinated calves before challenge recognise fewer E/S components than those given normal L_3 (Figure 33).

It is of interest that a variable antigen recognition pattern was seen within the experimental groups of guinea pigs and calves indicating that heterogenicity in the animals' capacity for antigen recognition was present. For example,

lanes 8 and 9 of Figure 29 show the presence of 1 and 2 low molecular bands respectively, that were not precipitated by serum from any of the other five infected animals. Similarly, in Figure 33, some low molecular weight bands precipitated by calf serum in lanes 4, 7 and 8 were not recognised by the sera of the animals represented in lanes 3, 5 and 6.

Discussion

A vaccine against D. viviparus, which is not dependent on attenuated larvae will probably only be possible by the application of recombinant DNA techniques. On the assumption that humoral immunity plays a major role in protection, the first step would be to analyse the protein components of the parasite. Hopefully it might be possible to recognise a specific antigen (or antigens), which is directly correlated with immunity. Failing that, it is possible to test for protection empirically by the excision of selected polypeptides from SDS-PAGE gels and, after their elution and concentration, to test for protective activity in an experimental host.

In the case of D. viviparus, as with other nematodes, the problems are formidable because of the complex composition of the different developmental stages and their excretory/secretory products.

In this Chapter a start was made on the analysis using homogenates of adult and third stage larvae and E/S products of adult worms. They were selected because all of them can be obtained in quantity.

The results of the SDS-PAGE analysis produced by the adult and larval homogenates yielded as anticipated, a complex pattern of bands. Most were common between both stages but some were specific to each stage. Since we know that protection can be achieved by third stage larvae alone, the next step of this work could be to determine which polypeptides are specific to the third stage larvae and which are shared with adult parasites. Perhaps, because a better immunity is likely to be obtained if all the stages are presented to the host, these shared antigens could first be isolated and tested.

Autoradiographs using E/S material showed that serum from guinea pigs immunised with larvae irradiated at 1000 Gy recognised most of the polypeptides present in the E/S which, since adult worms do not develop in guinea pigs, indicates that the precipitated molecules are expressed by larval as well as adult parasites or are cross-reactive between the stages. It is perhaps not unreasonable to consider, since a similar degree of protection was observed in guinea pigs vaccinated with either larval homogenate or adult E/S (i.e. 79% and 77% reduction in worm burden respectively pp. 55), that the major protective antigens are shared by both antigenic preparations. These E/S molecules could therefore represent a suitable starting point for further dissection of protective antigens, since, as noted in the results section there are relatively few polypeptide bands compared with those present in larval and adult homogenates.

One interesting feature was that the guinea pigs and calves showed a degree of heterogeneity in the specificity of their

antibody responses to E/S material. Thus, while all recognised certain components, each animal responded individually to others. More recently a study in guinea pigs, completed in collaboration with Miss Britton, has showed that this variability was essentially eliminated in inbred strains. This would infer a genetic basis for the heterogeneity and, as with other nematode infections (Kennedy, 1989), that the major histocompatibility complex (MHC) is probably in control of which parasite components elicit a response in a particular individual.

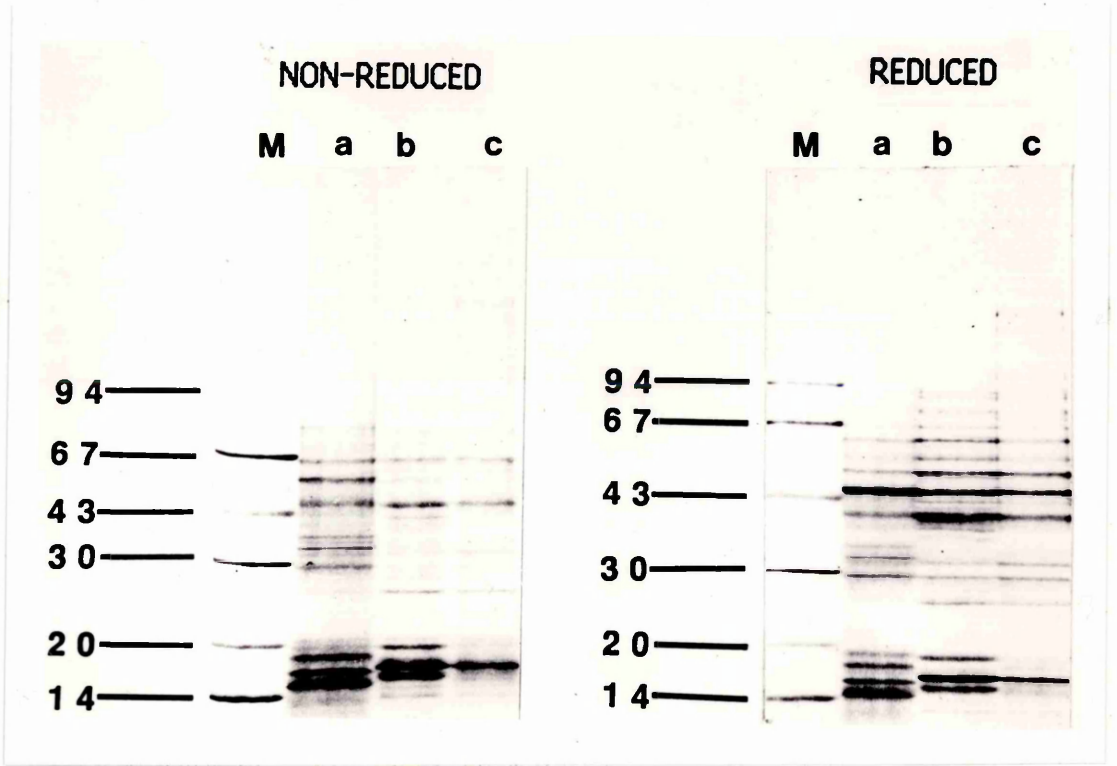


Figure 27. Coomassie blue staining of the SDS-PAGE patterns obtained from soluble homogenates of larvae (lane a), male (lane b) and female (lane c) adult parasites under non-reducing and reducing conditions. The relative molecular masses of standard markers (M) are indicated.

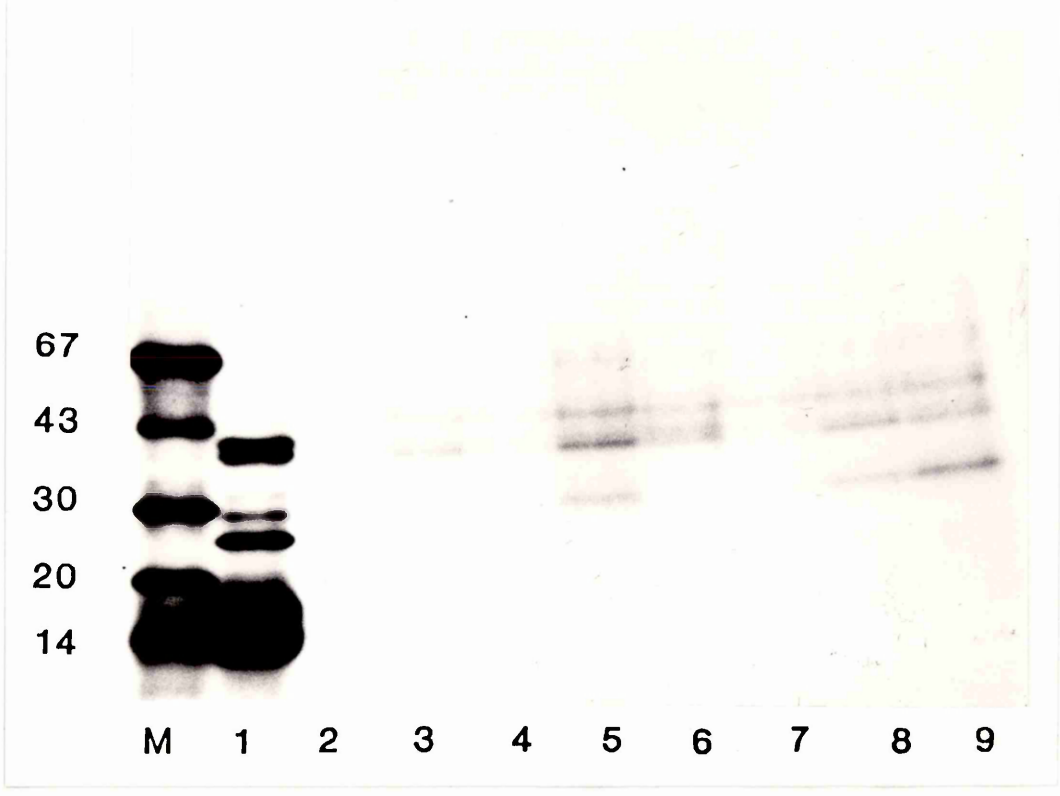


Figure 28. Autoradiographic patterns of 125-I labelled E/S products of adult D.viviparus (lane 1) before being immunoprecipitated with pooled normal guinea pig sera (lane 2) or with sera from 6 individual guinea pigs infected orally on 2 occasions with 5000 larvae irradiated to 400 Gy (lanes 3-8) or with pooled sera from guinea pigs infected twice with 5000 normal L3 (lane 9). The relative molecular masses of standard markers (M) are indicated.

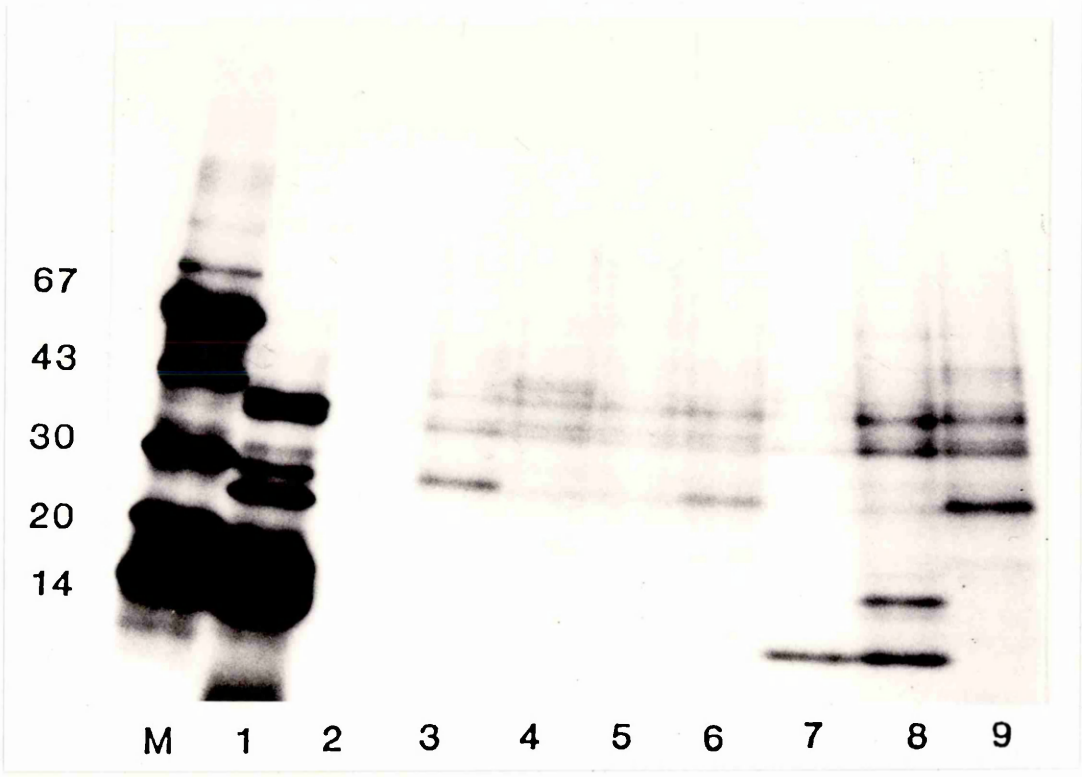


Figure 29. Autoradiographic patterns of 125-I labelled E/S products of adult D.viviparus (lane 1) before being immunoprecipitated with pooled normal guinea pig sera (lane 2) or with sera from 6 individual guinea pigs infected intraperitoneally on 2 occasions with 5000 larvae irradiated to 400 Gy (lanes 3-8) or with pooled sera from guinea pigs infected twice with 5000 normal L3 (lane 9). The relative molecular masses of standard markers (M) are shown.

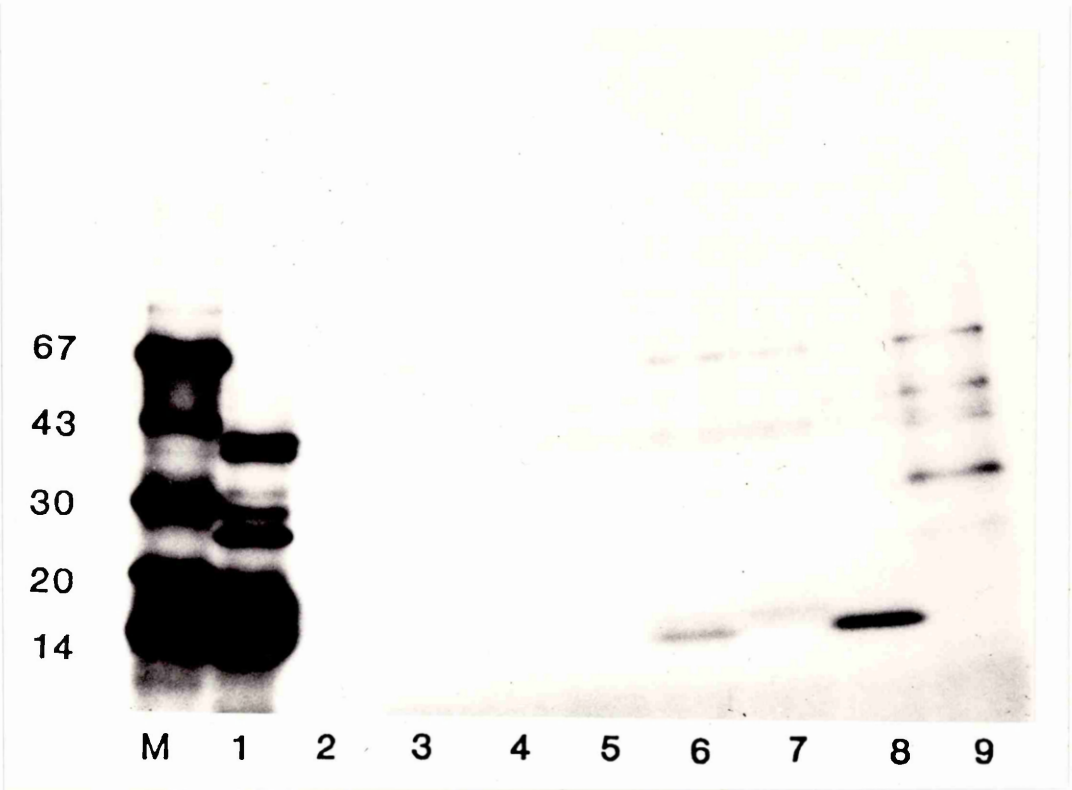


Figure 30. Autoradiographic patterns of 125-I labelled E/S products of adult D.viviparus (lane 1) before being immunoprecipitated with pooled normal guinea pig sera (lane 2) or with sera from 6 individual guinea pigs infected orally on 2 occasions with 5000 larvae irradiated to 1000 Gy (lanes 3-8) or with pooled sera from guinea pigs infected twice with 5000 normal L3 (lane 9). The relative molecular masses of standard markers (lane 1) are indicated.

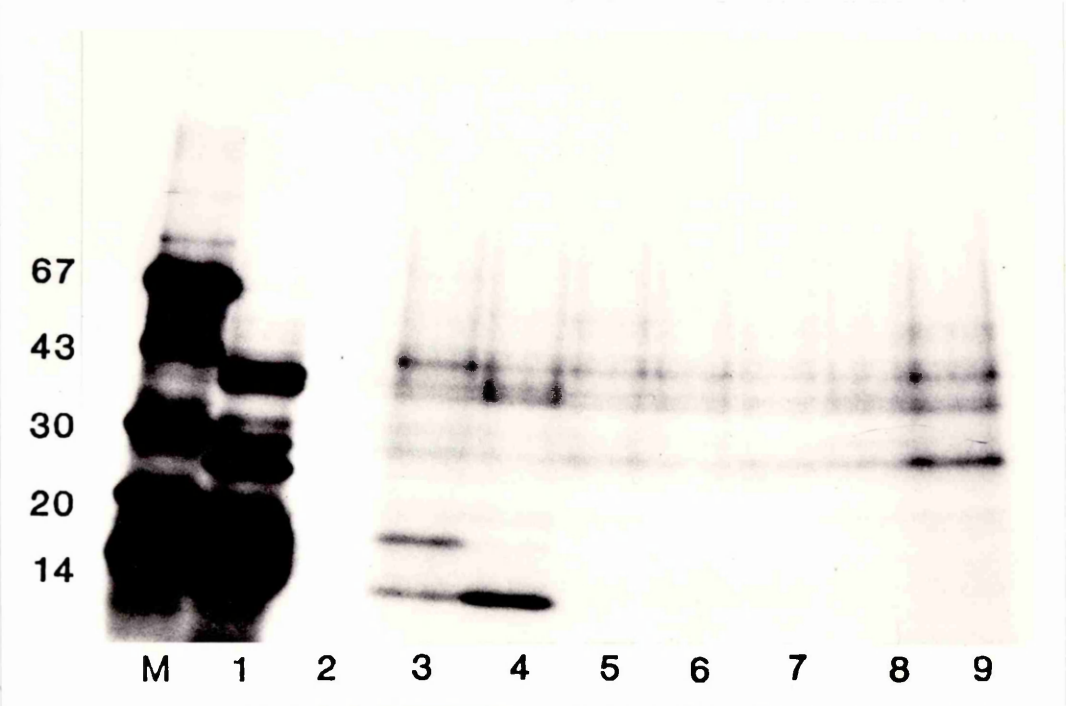


Figure 31. Autoradiographic patterns of 125-I labelled E/S products of adult D.viviparus (lane 1) before being immunoprecipitated with pooled normal guinea pig sera (lane 2) or with sera from 6 individual guinea pigs infected intraperitoneally on 2 occasions with 5000 larvae irradiated to 1000 Gy (lanes 3-8) or with pooled sera from guinea pigs infected twice with 5000 normal L3 (lane 9). The relative molecular masses of standard markers (M) are indicated.

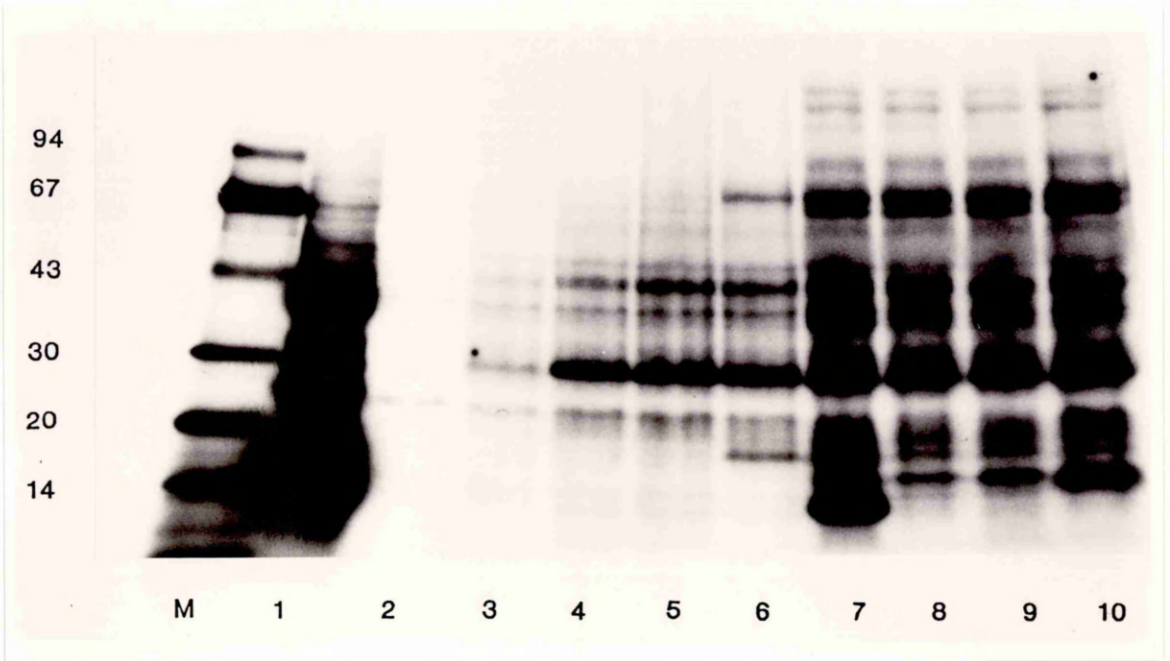


Figure 32. Autoradiographic patterns of 125-I labelled E/S products of adult D.viviparus (lane 1) before being immunoprecipitated with pooled sera from normal calves (lane 2) or with pooled sera from 2 calves infected on one occasion (lane 3), 3 occasions (lane 4), 4 occasions (lane 5), 5 occasions (lane 6) and 6 occasions (lane 7) with 1000 Gy irradiated L3 and with sera after 6 infections and challenge with normal larvae (lanes 8-10). The relative molecular masses of standard markers (M) are shown.

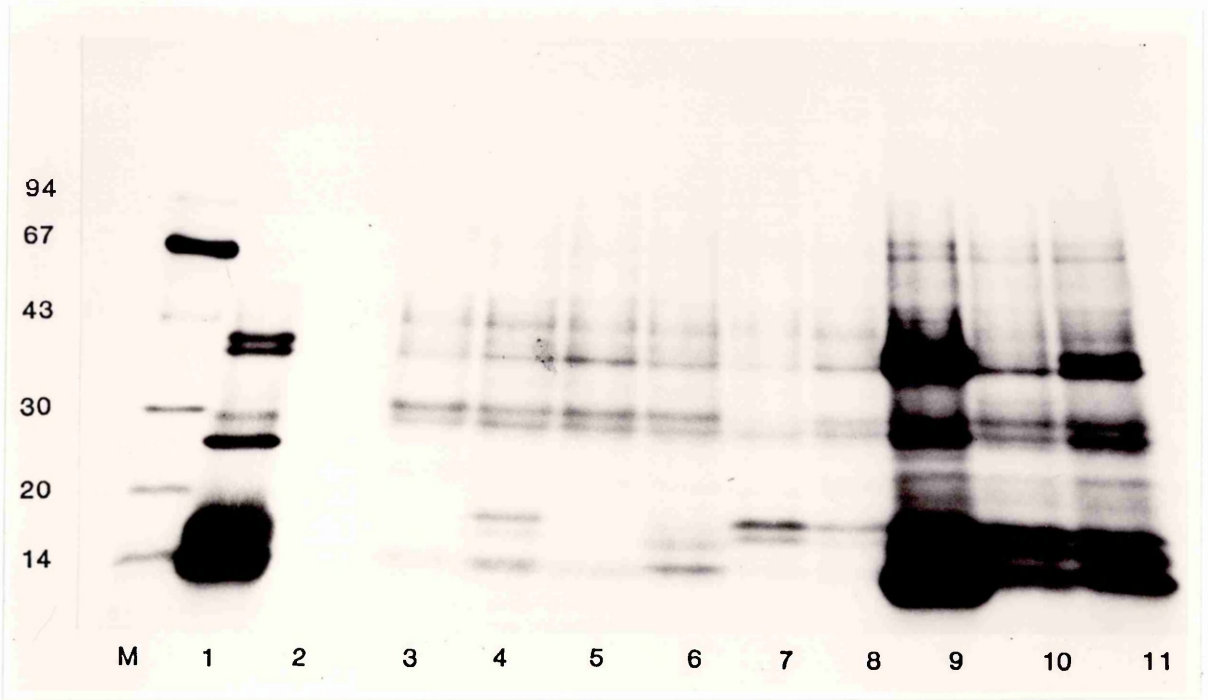


Figure 33. Autoradiographic patterns of 125-I labelled E/S products of adult D.viviparus (lane 1) before being immunoprecipitated with normal bovine serum (lane 2), or with sera from individual animals immunised twice with 400 Gy irradiated L3 and challenged with normal larvae (lanes 3-5), or with sera from individual calves immunised twice with 1000 Gy irradiated L3 and challenged with normal larvae (lanes 6-8) or with sera from individual animals infected twice with normal L3 (lanes 9-11). The relative molecular masses of standard markers (M) are shown.

CHAPTER SEVEN

A STUDY OF THE COMPARATIVE DEVELOPMENT OF IMMUNITY TO *D.VIVIPARUS* IN IVERMECTIN TREATED AND CONTROL CALVES DURING THEIR FIRST AND SECOND GRAZING SEASONS

INTRODUCTION

An understanding of the epidemiology of gastrointestinal helminthiasis of ruminants has led to the development of strategies for the control of these infections (Michel, 1969; Anderson et al, 1969). All of these are based on the knowledge that:-

(1) Overwintered L_3 are rarely acquired in sufficient numbers by young cattle grazed in spring to cause clinical disease.

(2) Following ingestion and subsequent development of such overwintered larvae, it takes until mid-July approximately for newly deposited eggs to develop into infective L_3 in sufficient numbers to precipitate clinical ostertagiasis.

An early strategy was to recommend, after a single dose of an effective anthelmintic, that the calves should be moved early in July to a pasture which had remained ungrazed by cattle since the previous autumn. This invariably resulted in a very low level of infection on the new pasture (Michel, 1969). If alternative grazing was not available, a second strategy of giving two or three anthelmintic treatments at intervals of three weeks during the early part of the season virtually eliminated contamination by preventing patency of ingested overwintered larvae (Pott, Jones and Cornwell, 1974; Armour, 1978). A more sophisticated strategy was the development of the morantel tartrate bolus which, given at turnout in spring, only allowed the acquisition of very low levels of infection which were not associated with clinical signs (Jones, 1981).

While these approaches worked well with gastrointestinal

helminthiasis they were unreliable in the prevention of parasitic bronchitis with outbreaks of the disease occurring in animals prior to and after being moved to aftermath grazing (Duncan et al, 1979; Oakley, 1979).

The reasons for this failure are unknown and have been variously ascribed to Dictyocaulus larvae emerging from the soil to the pasture at intermittent intervals in the year following deposition or to pasture contamination with wind-borne Pilobolus.

The development of ivermectin opened a new era in the control of parasitic bronchitis. Ivermectin apparently acts by potentiating the release and binding of gamma-aminobutyric acid (GABA) in some nerve synapses (Campbell, 1985). In nematodes, GABA acts as neurotransmitter sending signals between interneurones and motorneurones. The enhanced GABA binding results in an elimination of signal transmissions to the motor neurones resulting in paralysis of the nematodes.

In more recent years, the finding that ivermectin has a prophylactic effect which prevents the development of ingested D. viviparus for 21 days after administration (Armour et al, 1985) opened a new possibility for the therapeutic control of the disease. This was exemplified by the development of a prophylactic anthelmintic strategy of using the drug at three, eight and 13 weeks after turnout in set stocked calves, and has proved to be highly effective in the prevention of parasitic bronchitis (Taylor, Mallon and Kenny, 1985; Taylor, Mallon and Green, 1986; Armour et al, 1987).

Further investigations to explore the possibility that

calves subjected to this regimen and exposed to pasture contaminated with D. viviparus might develop protective immunity for the subsequent grazing season were conducted by Armour, Bairden and Ryan (1988). In order to assess the immunity of these yearlings, they were grazed on the same pasture as first season parasite naive calves. The results showed that all first season calves developed patent lungworm infections while no clinical signs of lungworm were detected in the yearling cattle at any stage of the trial.

In the study reported here, this work was repeated with two major differences: First, the ivermectin regimen was based on a pour-on preparation instead of the injectable drug, and secondly, the development of immunity was assessed by the use of the ELISA technique in consecutive serum samples.

MATERIALS AND METHODS

Animals

Thirty-six Friesian calves aged from four to six months which had been reared indoors and were thus considered to be helminth-free were used during the first year. Four groups of six calves were allocated to two treated and two control groups at the start of the grazing season. The remaining 12 calves were used as tracers to assess pasture larval contamination, one animal being grazed in each of the four paddocks in the spring, and two animals in each paddock in the autumn. In the course of the trial, two additional animals were added to take the place of two calves which had died in June. The principal calves in the

study weighed between 100 and 180 kg at the time of the first treatment on 28th May, 21 days after being turned out.

In the second year five yearlings which had been treated with topical ivermectin in their first grazing season and five yearlings which had received only salvage anthelmintic treatments with thiabendazole and a third group of six first year parasite naive calves, were separately grazed on three paddocks comprising part clean and part contaminated areas from the previous year.

Again, tracer calves (two in each paddock in the spring and autumn) were introduced. In addition a group of five parasite naive calves were used as controls for an experimental lungworm challenge at the end of the season.

Experimental design

In the first year the animals were turned out to graze on 7th May (Day 0). They were rotated daily through the four paddocks for the first 16 days after which a twice daily move took place until day 18 when they were weighed and allocated to the groups in which they remained throughout the 155 day grazing period. The calves in duplicate groups 1 and 3 were treated with ivermectin (0.5% v/w) solution topically at the rate of 1.0 ml per 10 kg of bodyweight on days 21, 56 and 91 after turnout and those of duplicate groups 2 and 4 kept as untreated controls. These latter were treated with therapeutic doses of thiabendazole as required. At the end of the grazing period five treated and five control calves were retained for a second year study. The remainder had died during the experiment or, if surviving, were necropsied 14 days after being housed.

The animals kept for a second grazing season were treated at housing with ivermectin subcutaneously at a dose rate of 200 ug/kg bodyweight.

One tracer calf was introduced to each paddock on day 0. These spring tracer calves were housed after two weeks and killed 16 days later. Two additional tracer calves were turned out in each paddock on day 175, housed after 13 days at pasture and killed eight to 12 days later.

In the second grazing season, the ten yearlings and six naive control calves aged between four and six months were turned out to pasture on the 4th May, 1988 (Day 0). These were allocated to one of the three paddocks as follows:-

Group A - The five yearlings which had been treated with ivermectin at three, eight and 13 weeks.

Group B - The five yearlings which had formed part of the control group, to which salvage anthelmintic treatments had been administered during the first grazing season.

Group C - Six parasite naive first year calves.

The animals were set stocked in their respective paddocks during the course of the 154 day grazing period and then housed. Two days after housing, the yearlings plus five parasite-naive control calves were challenged artificially with *D. viviparus* third stage larvae at a dose rate of 22 L_3/kg bodyweight. All the animals were necropsied 18 to 20 days after challenge in order to differentiate between natural and challenge infection.

The spring tracers of this second grazing season were assigned to the paddocks on day 0, housed after 14 days and

necropsied ten days later. The autumn tracer calves were grazed from day 154, housed after 14 days and also killed ten days after housing.

Respiratory rates and clinical observations

All calves were inspected daily and their respiratory rates were monitored weekly.

Parasitological data

Faecal and herbage samples were collected at turnout and at frequent intervals during the remainder of the trial. At necropsy total worm counts of D. viviparus were made using the Inderbitzin technique.

Serum samples

Blood samples were collected from the jugular vein on day 0 and then throughout the trial at frequent intervals and analysed for antibody levels.

Results

Clinical signs

In the first grazing season seven of the original 12 calves in the two control groups developed parasitic gastroenteritis or parasitic bronchitis and either died or had to be killed prior to the end of the grazing period. Following the death of the first control animal in June, which was attributed to nematodiriasis, the remaining controls were treated with thiabendazole as required. Thiabendazole was selected due to its poor efficacy against lungworm.

Throughout the second year no adverse clinical signs were observed in any of the yearling cattle. Mild and sporadic coughing was noted in the first year controls but no significant clinical signs of parasitic bronchitis were present.

Faecal D. viviparus larval counts

Group mean faecal larval counts throughout both grazing periods are presented in Figures 34 and 35. All surviving control calves showed patent lungworm infection in the first year during the late summer or autumn whilst no larvae were detected in the faeces of the ivermectin treated animals.

As can be seen from Figures 34 and 35 during the second year no lungworm larvae were detected in the faeces of the calves treated with ivermectin in the previous year. The faeces of the control group of the same age were also negative with the exception of two animals from which positive larval counts (1 LPG) were detected on day 140.

From late June, low number of L_1 were recovered from faeces of the young control calves (Figure 36).

Pasture larval counts

Lungworm larvae were recovered from the herbage in the two treatment paddocks throughout the first year in varying numbers (Figures 34 and 35) ranging from 0 to 555 larvae per kilogram of dried herbage (L_3/kdh) in the control paddocks and from 0 to 310 in the treatment paddocks. In both cases the highest recoveries were at the end of July or beginning of August.

In the second grazing season D. viviparus larvae were first recovered on day 21 from the pasture grazed by the control

yearling cattle, reappearing again in this paddock and for the first time in the areas grazed by the treated yearling cattle on day 49. Subsequently larvae were frequently detected until housing with a maximum recovery of 667 L_3/kdh from the paddock grazed by the control cattle compared with 333 L_3/kdh from the paddock of those previously given ivermectin (Figures 34 and 35). In the paddock where the naive control animals grazed, larvae were not detected until day 77 when 682 L_3/kdh were recovered (Figure 36).

Worm counts at necropsy

(1) Worm burdens of principal calves

Individual worm burdens of the first year control calves which died during the study plus those of the ivermectin treated animals killed 20 days after housing are given in Table 15. Although these results are difficult to compare due to the different necropsy dates, it can be seen that during the last six weeks of grazing the controls harboured about ten times as many worms than the treated animals.

Worm recoveries following experimental challenge of the two groups of yearlings after the second season and the naive challenge control group are presented in Table 16. There was a considerable variation in the worm burdens of the naive controls ranging from two to 266 parasites. In contrast, parasite recoveries fom the yearlings following challenge were very low with mean recoveries of 3.4 worms for the treated group and 2.0 worms for the controls indicating, even allowing for a degree of age resistance (Taylor et al, 1988), that the ivermectin treated

calves developed as high a degree of immunity as their untreated controls. The low mean burdens of 127 worms found in the naive calves which subsequently had grazed during the second year taken in conjunction with the mild clinical signs, suggested that both groups of yearlings had been exposed to a modest larval challenge during that year.

(2) Tracer calves

The D. viviparus worm burdens of the first year tracer calves are presented in Table 17. All of the spring tracers had very small numbers of adult worms present in the lungs compared with the autumn tracer calves in which there were marked differences in the number of worms recovered from thos grazed on the separate areas used by the control and the ivermectin treated calves. Thus, the latter harboured a mean of 49 worms compared with 290 from the tracers of the control paddock.

In the second grazing season (Table 18) small numbers of adult worms were recovered from the lungs of the spring tracers, ranging from 0 to 23. In the autumn tracers the lungworm burdens were unexpectedly low (five to 31) considering the numbers (56 to 264) found in the permanent controls of the same age (Table 16).

Antibody titres

First and second grazing seasons

Antibody titres against L_3 antigens started to appear in both groups of principal calves i.e. ivermectin treated and controls (Figures 34 and 35) after the animals had been grazing for 15 days. Anti-adult titres appeared between two and four weeks later which indicates that the antigens of the L_3 and the

adult stages differ in some respect from each other. This was shown previously in Chapters five and six. However, once the anti-adult titres were detected they followed a very similar pattern to those against L_3 suggesting the presence of common antigens. This was documented in Chapter six.

The reaction in the untreated group was greater than in the treated animals, reaching at the end of the grazing season titres of over 1:640 against titres of 1:160 in the treated animals. The titres of both groups decreased gradually after housing. In the untreated group, they reached their lowest point in the following year just before the start of the second grazing season (Figure 37), after which an almost immediate increase occurred, reaching levels of 1:160 by the end of the 154 day period. In the ivermectin treated group, the titres were at their lowest point three months before the start of the second grazing season (Figure 38) and remained at that level until 15 days after the animals were on pasture. This increase in titres was initially more modest than those of the control group but, by the end of the season were at the same level as those in the untreated group (Figures 39 and 40).

The first year control calves in the second grazing season showed a similar pattern of antibody reaction to those observed in the control group during the first year (Figures 39 and 40). The titres of anti-larval and anti-adult antigens appeared 15 and 30 days respectively after the animals were on pasture and continued to rise until the end of the season reaching titres of over 1:320.

Discussion

The objective of the trial was two fold: First, to study the efficacy of the pour-on ivermectin given to parasite naive calves during the first grazing season.

Secondly, to determine the immune status of these calves during the second year of grazing to D. viviparus infection.

In all the paddocks and in both years, infective lungworm larvae appeared sporadically. The treated animals were clinically normal and did not excrete any larvae during the first and second year of grazing. In comparison, the control calves suffer from parasitic bronchitis during the first year but were free of clinical signs during the second year; larval excretion was also negative apart from one occasion when a single larvae was observed in the faeces of two of the calves. This results showed that the ivermectin treated animals despite the absence of clinical signs during the first grazing season had acquired as high degree of immunity as the control calves.

This was confirmed by the experimental challenge at the end of the second year in which a high degree of immunity was apparent in both groups although this was not as absolute (Tables 15 and 16) as had been shown previously (Armour et al, 1988). This may be due to the fact that the level of exposure to lungworm larvae during the first grazing season and hence the subsequent acquired immunity was not as strong as in the experiments previously reported when "seeder" calves were used to contaminate the grazing plots.

With respect to the antibody titres, the results showed that the highest titres against larval or adult antigens were obtained in animals with patent infections (Figure 34) which may suggest that the mass of adult antigen is responsible for these high titres.

It has been shown by complement fixation (Cornwell, 1960a), by indirect haemogglutination (Bokhout et al, 1979) and by the ELISA technique (Bos, Beekman-Boneschanscher and Boon, 1986) that double vaccination with irradiated larvae (which do not develop to the adult stage) produces only a small to moderate rise in titres against the parasite although the animals are highly protected against challenge. This also suggests that developing parasites in the lungs are necessary for high titres.

The fall in the titres to their lowest level in both experimental groups during the housing period after the first grazing season was possibly a consequence of the treatment at housing and subsequent absence of challenge. Nevertheless, as judged by the second year on pasture, it is clear that they were strongly protected against the natural challenge of the second season suggesting that the moderate titres observed were possibly of antibodies with a better affinity for the parasite.

In the treated group it was observed that by the end of the first season the anti-adult titres were for the first time higher than the anti-larval titres (Figure 38). This change of pattern might be explained by the presence of adult worms in the lung of the animals after the anthelmintic effect of the ivermectin had disappeared.

During the second year a sharp increase of titres against larval and adult antigens was observed in the control group of yearlings (Figures 39 and 40), due probably to the immediate recognition of the larvae after ingestion. In contrast, the titres in the ivermectin treated group did not show a marked increase until August. This may be due to a less vigorous secondary response subsequent to the treatment regime of the first year.

From these observations it can be concluded that serology in the case of D. viviparus infection is a good method of detecting early infections in cattle and that the titres obtained do not indicate the degree of immunity but of challenge. Thus, it is important that the epidemiological and clinical data should be taken into consideration in assessing the immune status of the animals.

The results of this trial showed that a pour-on ivermectin treatment at three, eight and 13 weeks does not prevent calves from developing protective immunity against natural or artificial challenge with D. viviparus. A similar result was recently reported by Taylor, Mallon and Green (1990). The trial also showed that the immunity developed against the parasite is strong enough to prevent clinical signs during the second grazing season, a conclusion previously demonstrated with the injectable formulation (Armour et al, 1988). These results must however, be tempered by the fact that the calves were exposed to a significant challenge while under the ivermectin "umbrella". Calves exposed to a very modest challenge such as might be

anticipated after the repeated annual use of ivermectin, would not necessarily develop the same degree of immunity. The possible consequences of this i.e. the occurrence of parasitic bronchitis in older cattle, has been discussed in some detail by Taylor et al (1988).

A final point of considerable epidemiological interest was the detection of significant numbers of D. viviparus larvae on the herbage grazed by the treated calves during the first year, as late as September. Since the faeces of these calves were consistently negative for lungworm larvae, it can only be concluded that the larvae on herbage originated from one of two sources. First, they may have survived from the autumn of the previous year. While this is possible it is surprising that the numbers recovered were approximately similar to those recovered in May, July and August. This may indicate that lungworm larvae emerge in waves from the soil during the grazing season. A second possibility is that wind-borne Pilobolus spores had carried larvae from the two adjacent paddocks grazed by the untreated calves. A similar unresolved situation has been previously described by Duncan et al (1979).

Table 15

Individual *D. viviparus* worm burdens of first year ivermectin treated and control animals

Ivermectin treated		Controls	
No. of parasites	Day of killing	No. of parasites	Day of killing
17		5	04/06
12		2	19/07
8		5	22/07
8	29/10	0	24/08
5		88	04/09
5		102	10/09
0		53	23/09
		80	08/10

Table 16

Individual *D. viviparus* worm burdens

of yearling cattle and controls

	Yearlings		Permanent	Challenge
	Treated	Controls	Controls	Controls
	12	7	264	266
	4	2	127	207
	1	1	113	186
	0	0	104	38
	0	0	98	2
			56	
Total	17	10	762	699
Mean	3.4	2	127	139.8

Table 17

Individual *D. viviparus* worm burdens of spring and autumn tracers during the first year

Spring tracers		Autumn tracers	
Ivermectin paddock	Control paddock	Ivermectin paddock	Control paddock
0	2	97	461
0	1	84	378
		9	283
		5	38

Table 18

Individual *D. viviparus* worm burdens of spring and autumn tracers during the second year

Spring tracers			Autumn tracers		
Yearlings		Calves	Yearlings		Calves
Treated paddock	Control paddock	Control paddock	Treated paddock	Control paddock	Control paddock
12	16	23	11	14	31
8	2	0	9	5	18

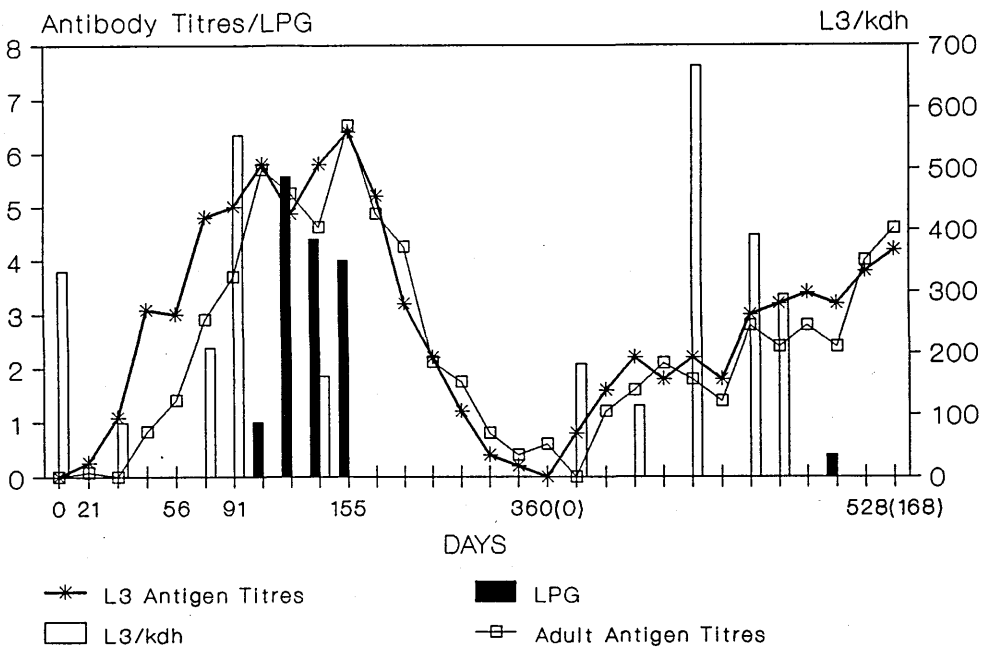


Figure 34. Mean ELISA titres with adult and larval D.viviparus antigens and larvae per gram of faeces from control calves during 2 grazing seasons. The animals were housed after 155 days on pasture starting the second grazing season on day 360 (0). The results of the analysis of herbage samples for the presence of larvae are also presented. The numbers 1-8 on the y-axis correspond to LPG in faeces (closed histogram) and ELISA antibody titres from 1:20 to 1:2560 (lines).

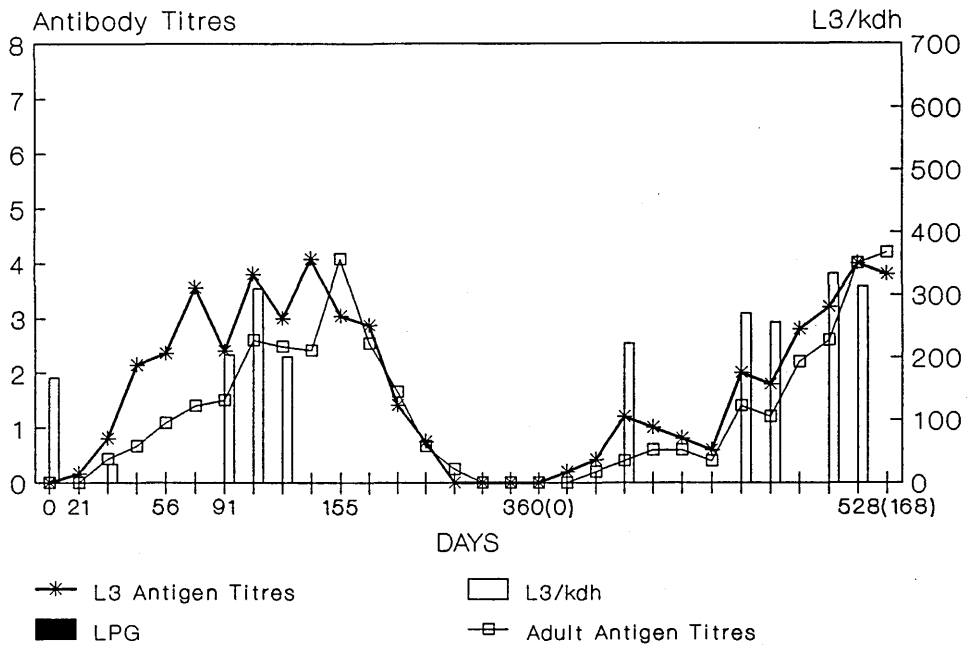


Figure 35. Mean ELISA titres with adult and larval D. viviparus antigens and larvae per gram of faeces of animals treated with ivermectin on days 21, 56 and 91 after being turned out on pasture. The animals were housed on day 155 starting the second grazing season on day 360 (0). The results of the analysis of herbage samples are also presented. The numbers 1-8 on the y-axis corrspond to LPG in faeces (closed histogram) and ELISA antibody titres from 1:20 to 1:2560 (lines).

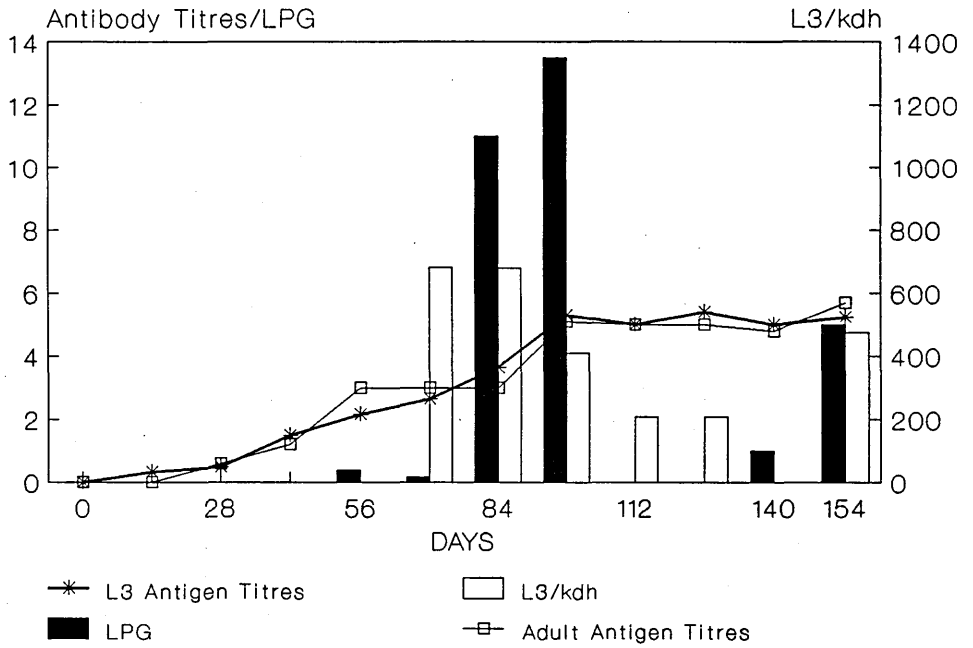


Figure 36. Mean ELISA titres with adult and larval D. viviparus antigens and larvae per gram of faeces of the second season control calves. Results of herbage analysis for the presence of larvae are also presented.

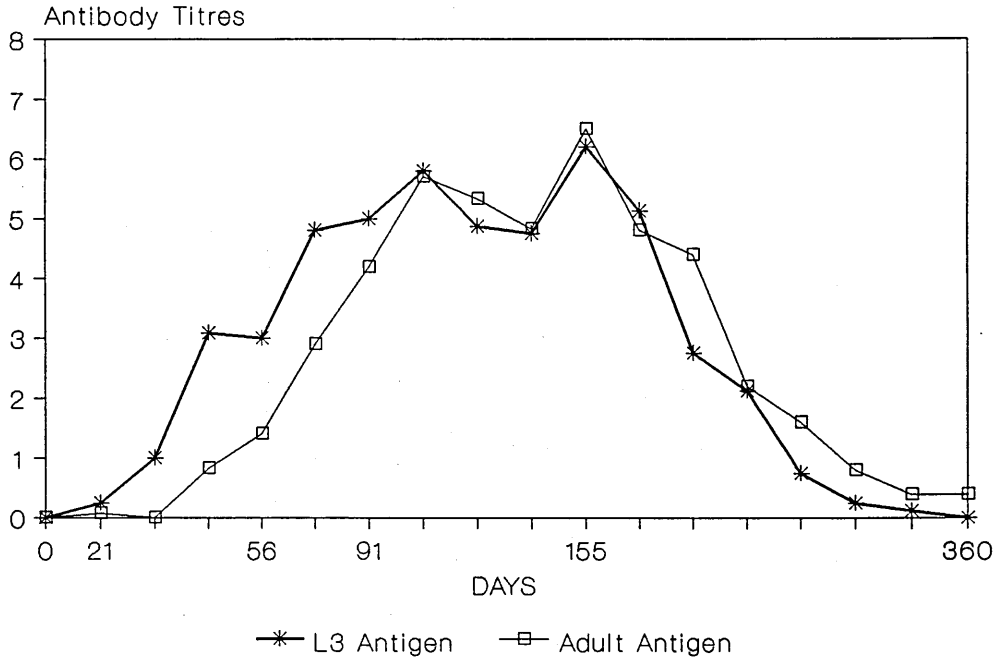


Figure 37. Mean antibody titres with adult and larval antigens from control animals during their first grazing season. The calves were housed on day 155.

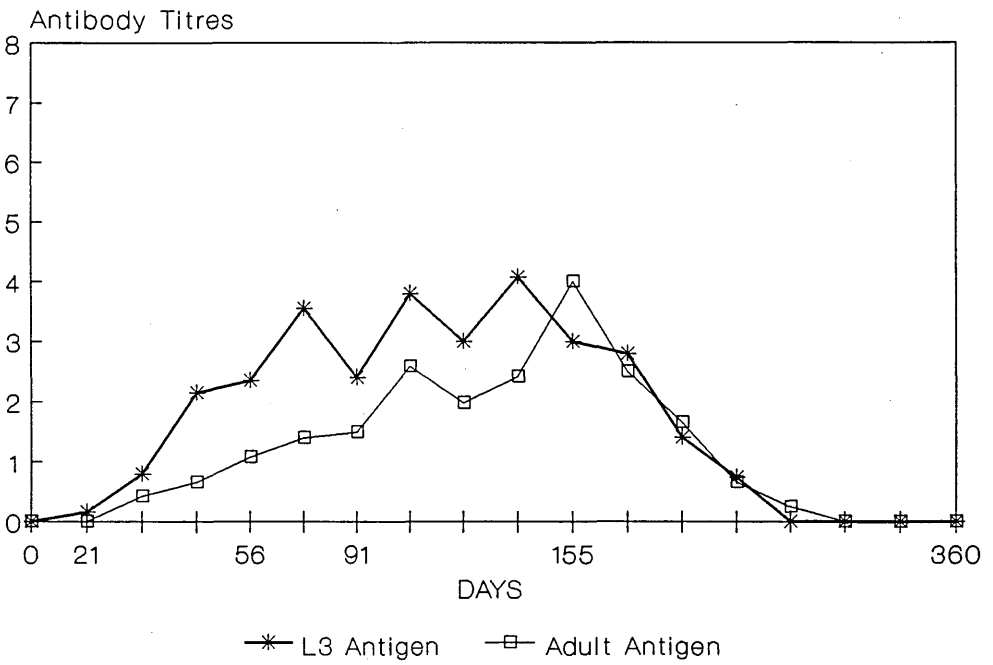


Figure 38. Mean antibody titres with adult and larval D. viviparus antigens of animals treated with ivermectin on days 21, 56 and 91 after being turned out on pasture. The calves were housed on day 155.

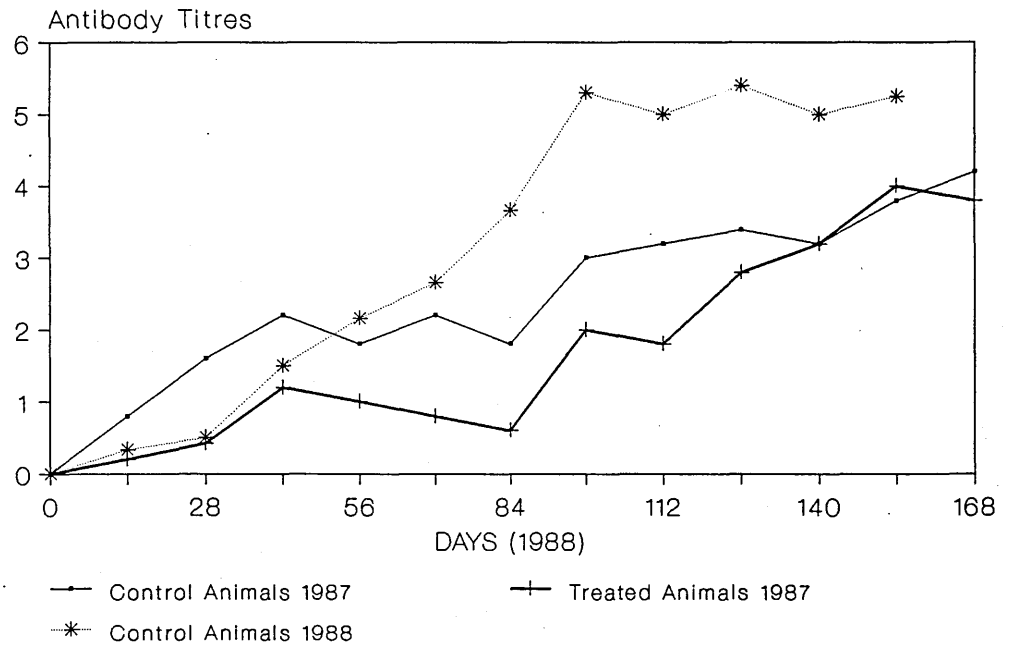


Figure 39. Mean antibody titres with larval D.viviparus antigens from control and ivermectin treated animals during their second grazing season and from first season control calves.

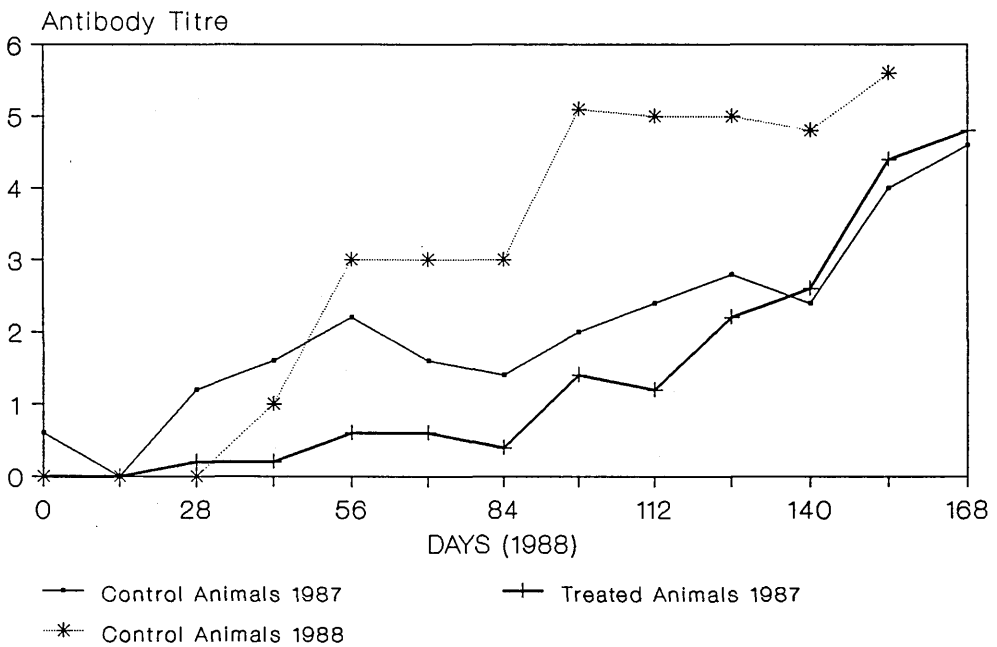


Figure 40. Mean antibody titres with adult D.viviparus antigens from control and ivermectin treated animals during their second grazing season and from first season control calves.

CHAPTER EIGHT

CONCLUDING DISCUSSION

While the mechanisms underlying immunity to gastrointestinal nematodes in ruminants and small laboratory animals have been the subject of quite extensive investigations over the past three decades, it is surprising that little or no attempt has been made to understand the well-documented immunity associated with infection of cattle with the bovine lungworm, Dictyocaulus viviparus.

The studies reported in this thesis were conducted in an attempt to redress, to some extent, this situation. For economic reasons the guinea pig was used as a model for much of this investigation and, whenever possible, corroboratory experiments were carried out in small groups of calves.

With the guinea pigs it was found that a high degree of protection ranging between 67% and 85% reduction in worm burdens could be achieved using soluble extracts of sonicated third stage larvae in FCA or liposomes as adjuvant which indicated that major protective antigens were present in this stage. This was confirmed when animals were vaccinated on two occasions with larvae irradiated at 1000 Gy which do not develop beyond the third stage. Such guinea pigs, after challenge, showed a reduction of 94% in worm recovery.

This latter observation was confirmed in cattle, in which it was shown that calves vaccinated with the same type of larvae i.e. irradiated at 1000 Gy, were highly protected against challenge. A reduction of 76% in worm recovery after challenge was obtained after two doses of the irradiated L_3, while a regimen of seven infections gave 100% protection. The result of

these experiments, albeit in a small number of calves, also strongly suggest that the L_3 possess major antigens responsible for protection.

In subsequent studies on the passive transfer of immunity, it was confirmed that immune sera obtained from normal larval infections of calves is capable of conferring protection against challenge. A new observation was that the lungs of the serum recipients after challenge, presented several hundred greenish-yellow lymphoid nodules, which, in the literature, have been related to protection and are typically found after challenge of calves immunised by natural infection or vaccination. This observation is interesting in that it strongly suggests that the development of these tiny nodular lesions is, initially at least, mediated entirely by antibody and that the subsequent cellular events are secondary.

Of equal interest was the finding that the transfer of immune sera raised against 1000 Gy-irradiated L_3 did not protect at all and no nodules were observed. This result paralleled that found in passive transfer of immune serum in guinea pigs i.e. serum from normal larval infections gave over 80% protection after challenge while serum from 1000 Gy infections gave none; the serum from 400 Gy infections gave a result intermediate between the two.

Three possible explanations are presented in an attempt to explain this finding. The first is that the level of protective antibodies stimulated by the 1000 Gy larval infections is deficient in quantity or range. While this may be adequate to

counter challenge infections of an actively immunised calf, the passive transfer of a volume, albeit considerable, of such serum is insufficient to confer protection.

A second possibility is that immunity to D. viviparus is not due solely to circulating antibody and that a cell mediated component operates against the invasive third larval stage. Thus, while such a mechanism would protect a donor calf, immunised on several occasions with 1000 Gy-irradiated L_3, it would not be transferred in the serum.

In line with the last theory there is perhaps a third possibility, namely, that local secretion of bronchial or mucosal IgA could play a role in protection as has been suggested by some workers.

A high non-specific binding of bovine immunoglobulin was encountered with the quantitative immunofluorescence assay when exsheathed third stage larvae were used. Using an ELISA technique which utilised mouse mAb's against bovine immunoglobulins it was shown that this was due to non-specific binding by bovine IgM. This was confirmed when the immunofluorescent assay was carried out using mAbs against IgM; in contrast, no non-specific binding occurred when mAbs against bovine IgG_1 or IgG_2 were used.

Some preliminary results were obtained with the electrophoretic analysis of soluble homogenate of adult and third stage larvae and E/S products of adult D. viviparus. It was found that most of the peptides detected with Coomassie blue staining were common between larval and adult stages, although

some were specific to each stage. Immunoprecipitations and SDS-PAGE using adult E/S materials showed that both bovine and guinea pig immune serum from third stage larval infections recognised most of the polypeptides present in the E/S. Since adult worms do not develop in the guinea pig this indicates that these precipitated molecules are expressed by larval as well as adult parasites or are cross-reactive between stages.

Since vaccination of guinea pigs with adult E/S products conferred as high a degree of protection as larval homogenates, i.e. 77% and 79% reduction in worm burdens respectively, it may be tentatively concluded that major protective antigens are common to both antigenic preparations. Since E/S contains relatively few polypeptide bands compared with larval homogenate it might therefore be a suitable starting point for the isolation of protective antigens.

In a final chapter, the development of immunity to D. viviparus infection was studied over a period of two years in calves treated with a formulation of pour-on ivermectin during their first season of grazing on infected pasture. The results showed that treatment at three, eight and 13 weeks does not prevent calves from developing protective immunity against natural or artificial challenge with D. viviparus and that the immunity developed against the parasite is strong enough to prevent clinical signs during the second grazing season. It was also shown that ELISA is a good method for detecting early infections in calves, titres appearing as soon as two weeks after going to pasture. However, as reported previously, the levels of

antibody measured are not indicative of the degree of immunity, but challenge.

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APPENDIX

REAGENTS LIST

ELISA

Tris poisons pH 8.3

2.0 M Tris HCl pH 8.3
2.0 mM ethylene diamine tetra acetic acid (EDTA)
1.0 mM phenyl methyl sulphonyl fluoride in isopropanol
5.0 uM pepstatin
2.0 mM 1, 10 phenanthroline
5.0 uM leupeptin
5.0 uM antipain
25 ug/ml N-p-tosyl-l-lysine chloromethyl ketone
50 ug/ml N-tosyl-l-phenyl alanine chloromethyl ketone

Coating Buffer pH 9.6

Stock solutions of 0.2M sodium carbonate and sodium bicarbonate

A. 21.2 g Na_2CO_3 Made in 1 litre with distilled water

B. 16.8 g $NaHCO_3$ Made in 1 litre with distilled water

Working solution

8.0 ml Solution A
17.0 ml Solution B
75.0 ml Distilled water

Phosphate Buffered Solution (PBS)

8.50 g NaCl

0.32 g NaH_2PO_4

1.10 g Na_2HPO_4

Made in 1 litre with distilled water

Washing Buffer

As PBS with inclusion of 0.05% of Tween 20

Blocking Buffer

As Washing Buffer with inclusion of 4.0% of skim milk

Diluting Buffer

As Washing Buffer with inclusion of 2.0% of skim milk

Substrate Solution

Solution A - Chromogen Solution

Phosphate Citrate Buffer pH 5.0

5.11 g Citric Acid

0.15 g Na_2HPO_4

Made in 1 litre with distilled water

Add 0.40 g OPD (ortho-phenylene-diamine) and dissolve

Solution B - Substrate

30% H_2O_2

Working Solution

10.0 ml Solution A
0.004 ml Solution B

POLY-ACRYLAMIDE GEL ELECTROPHORESIS

Sample Buffer

5.0 g	Sodium-dodecyl sulphate
5.0 ml	Tris pH 7.5
1.0 ml	100 mM EDTA
2.0 ml	100 mM Phenyl Methyl-Sulphonyl Fluoride in isopropanol
10.0 ml	Glycerol
2.0 ml	0.2% Bromophenol blue
	H_2O to final volume of 95 ml pH 6.8

Stock solutions for Poly-acrylamide gels

Solution		
AA	489 ml	1M HCL
	366 g	Tris base pH 8.9
	8 g	SDS
	2.3 ml	N,N,N',N'-Tetramethyl-ethylenediamine
		H_2O to final volume of 1000 ml
APS	100 mg/ml	Ammonium Persulphate
N	250 g	Acrylamide
	3.875 g	N,N'Methylene-bis-Acrylamide H_2O to final volume of 625 ml
S	121.1 g	Tris base
		HCL to pH 6.8
	8 g	SDS
	4.0 ml	N,N,N',N'-Tetramethyl-ethylenediamine
		H_2O to final volume of 1000 ml

Working solutions for Poly-acrylamide gels

Solution	5% gel	25% gel	Stacking gel
N	3 ml	15 ml	2.5 ml
AA	3 ml	3 ml	
dH_2O	18 ml	6 ml	15 ml
S			2.5 ml
APS (add last)	50 ul	33.35 ul	200 ul

BOLTON HUNTER LABELLING

Antigen

15 ug of protein in 0.1M Borate buffer to a final volume of 200 ul

Borate buffer

618 mg $H_3 BO_3$ in 100 ml of dH_2O pH 8.5 with NaOH