IMMUNE RESPONSES TO ANTIGENS OF *Haemonchus contortus*

by

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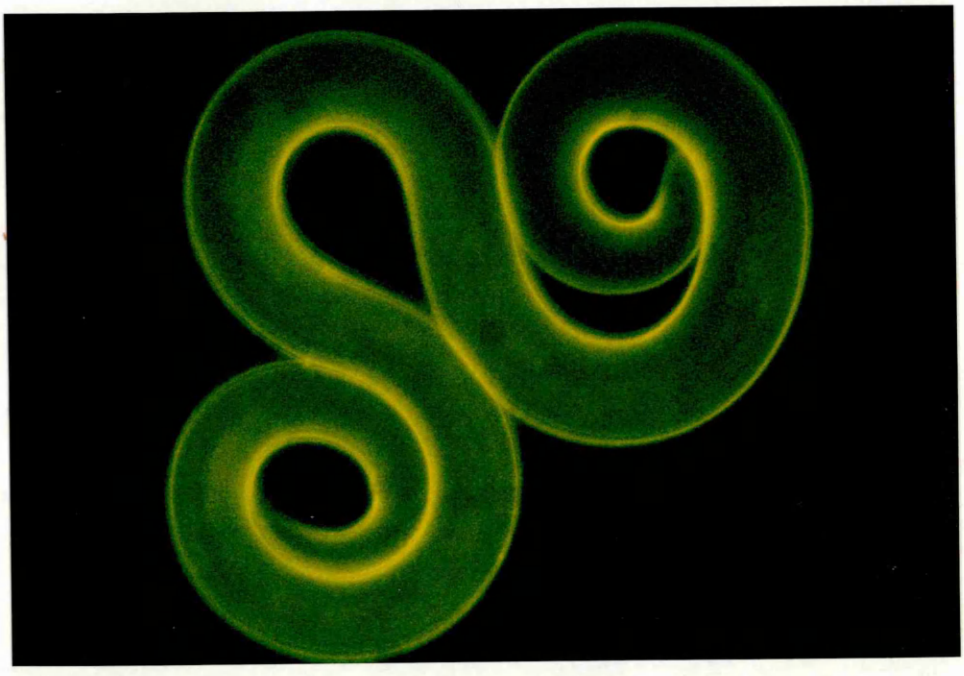
I CAN DO ALL THINGS THROUGH CHRIST

WHICH STRENGTHENETH ME:

PHILLIPIANS 4:13



DEDICATION

I dedicate this thesis to the memory of my mother to whom I owe so much and whom together with my father has always had every confidence in my abilities.

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DECLARATION

I declare that the work described in this thesis is my own carried out with the assistance of those I have acknowledged.

ACKNOWLEDGEMENTS

First and foremost I give thanks to the Almighty, for without His guidance the work described in this thesis would never have come to pass.

To my supervisors Professor George Urquhart, Professor James Duncan and Professor Andrew Tait go my most sincere thanks and appreciation for the constant support, help and encouragement I received from them, each is his own way. The statement "whichever way things go, remember that you have learnt a lot of new techniques" will always be a source of encouragement thanks to Professor Urquhart.

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SUMMARY

The work undertaken in this thesis was designed to examine the antigenic nature of various stages of the abomasal parasite Haemonchus contortus. The parasitological and immunological responses of sheep "vaccinated" with surface and somatic proteins derived from third stage larvae and adult worms were also investigated.

Chapter One reviews the literature on H. contortus and describes the life cycle of the parasite together with the pathogenesis, clinical signs, treatment and control of infection. Discussion of the immunological aspects of ovine haemonchosis relating to natural immunity, acquired immunity and vaccination is followed by an introduction to the molecular techniques and approaches used in subsequent investigations. The materials and methods are presented in Chapter Two and include a description of the regimen of serial infections of adult and young sheep with infective third stage larvae which was used in an attempt to produce serum antibodies against Haemonchus. Hyperimmune sera was obtained only from the adult sheep, the younger sheep remaining susceptible to repeated challenge infections.

The results of the experimental work is presented in Chapters Three and Four. The findings of five experiments in which sheep were immunised/vaccinated with surface proteins derived from Haemonchus contortus larvae are described in Chapter Three. The first of these, a pilot experiment involving

a small number of animals suggested that a significant protective immunological response resulted from vaccination with L_3 and L_4 surface proteins. This was based on the low faecal egg outputs and high packed cell volumes of the vaccinated animals compared with the control lamb, after experimental challenge. Antibody levels resulting from vaccination were determined by the ELISA technique and these indicated a significant immune response to larval antigens in the vaccinated animals. These preliminary results led to a series of subsequent vaccination experiments. In these (Experiments Two - Five) post-vaccination challenge infections resulted in variable changes in packed cell volumes and faecal egg counts both between experiments and between vaccinated and control animals in the same experiment. The parasitological and haematological results of these experiments were not as encouraging as those obtained in the preliminary experiment, although up to 50% reductions in adult parasite establishment were recorded in some of the vaccinated groups compared with controls. These five experiments also showed varying levels of antibody response to larval antigens detectable by the ELISA but again the levels of response were less than those recorded after immunisation in the preliminary experiment.

The last experiment in Chapter Three described the results of a vaccination experiment using *H. contortus* L_3 or adult somatic antigens. Although a low antibody response to the proteins used in the vaccination was detected, there was no apparent protection against challenge with normal larvae.

Chapter Four is concerned with investigations of H. contortus at the molecular level. These included characterisation and comparison of surface and somatic protein profiles of various stages of the parasite by direct staining of proteins obtained by SDS-PAGE and by the use of biotin labelling techniques. The protein profiles of first and second stage larvae were found to be very similar. Although differences in protein profiles were demonstrated between third and fourth stage larvae and adult parasites these were especially marked between the third stage larvae and adults. Serum from animals in the experiments described in Chapter Three were also studied by immunofluorescence and immunoblotting techniques. Using these techniques serum antibodies from vaccinated lambs were found to vary both quantitatively and qualitatively.

The results of the experimental work carried out in this thesis are discussed in Chapter Five together with suggestions for possible avenues of future research.

CHAPTER ONE

GENERAL INTRODUCTION

INTRODUCTION

Haemonchosis is a parasitic disease of sheep, cattle and goats economically important worldwide, but especially so in tropical and subtropical areas. It is caused by the abomasal nematode Haemonchus which is classified as a member of the Phylum Nemathelminthes, Class Nematoda Superfamily Trichostrongyloidea, Genus Haemonchus (Soulsby, 1968). Three species are known - contortus, placei and similis.

Gibbons (1979) rejected the theory that H. placei was a valid species, regarding H. contortus and H. placei as synonyms. There are minor taxonomic differences such as the shape of the vulval flap, which in H. placei is button-shaped whilst in H. contortus, the flap is tongue-shaped. There are also differences in the Host-Parasite relationship e.g. prepatent period, the ability to "self-cure", which has not been proven to occur in cattle, and the development of immunity which occurs invariably in cattle, but not necessarily so in sheep and may be attributed to the host response. H. similis is regarded as a valid species, a major taxonomic difference being the placement of the vulva at the tip of the vulval flap.

H. placei and H. similis are primarily parasites of cattle whilst H. contortus is the parasite found in sheep and goats.

In this thesis, the host-parasite relationship discussed is that of Haemonchus contortus in sheep.

The worms are unsegmented, cylindrical and elongate, the sexes separate and the females are larger than the males. They are 10-30 mm long, possessing small buccal cavities armed with a

slender tooth or lancet. Cervical papillae are prominent and the bursa is large, especially so the lateral lobes, whereas the dorsal lobe is small and assymetrical. The vulva is anterior in the female and the spicules of the male are barbed.

Grossly the parasites are easily identified because of their specific location in the abomasum, relatively large size and the "barber's pole" appearance of the female parasites' white ovaries winding spirally around the blood filled intestines, clearly visible in fresh specimens.

Commonly known as the stomach worm or wireworm of sheep, it is one of their most pathogenic parasites.

LIFE CYCLE

The life cycle of H. contortus is direct and the stage that is infective to the host animal is the third stage larva (L_3). Normally four moults or ecdyses, in which the whole cuticle is shed and replaced by a new one, take place before the adult stage is reached. The L_3 becomes infective for the host animal after the second moult and the cuticle of this moult is retained as a protective sheath until the worm enters the host.

Following ingestion, exsheathment or the second moult is completed in the rumen, the actual stimulus for which is not known, but is thought to be the dissolved gaseous CO_2 and/or undissociated carbonic acid in the gut (Mapes, 1969). The now parasitic L_3 migrate to the abomasum and become closely associated with the mucosa, where the third moult takes place and the fourth stage larva (L_4) emerge.

The fourth-stage larva is able to feed once the third-stage sheath has been lost (Mapes, 1969), and just before the time of the fourth moult, the piercing lancet which enables the larva to penetrate the surface of the abomasal mucosa develops. The fourth stage larvae are also found in close apposition with the abomasal mucosal surface. Feeding commences and is soon followed by the fourth moult to the fifth or pre-adult stage. After further feeding these fifth stage larvae mature into adult females and males which are to be found moving freely on the surface of the mucosa. Differentiation into female and male begins around the time of the fourth moult.

Mating followed by the laying of many eggs by the prolific females takes place soon after and the eggs are passed out in the faeces of the infected animal. First stage larva begin to develop within the eggs as they pass along the gut and under optimal conditions the eggs hatch on the pasture within two days. Under favourable conditions, L_3 will be present on pasture within 4-6 days (Le Jambre, 1981). The first and second stage larva are free-living and are digested if ingested by a grazing animal.

The prepatent period for H. contortus is 18-21 days in sheep and goats and 23-28 days in cattle.

EPIDEMIOLOGY

Optimally, conditions for the survival and development of H. contortus are relatively high temperatures and high humidity. This has been summarised by Gordon (1948), as a monthly mean of 18°C and minimal monthly rainfall of 55 mm. Low temperatures

retard development whilst extreme heat causes desiccation. Under 9°C little or no development takes place although, pre-hatch eggs appear to be more resistant to adverse conditions and can survive freezing and desiccation more readily than other stages (Wharton, 1982).

The longevity of infective larvae depends primarily on the humidity of the microclimate on the pasture and in the Kenyan Highlands for example, at best is probably several months (Dinnik and Dinnik, 1958) under prevailing cool and dry conditions. Dinaburg (1944), in experiments carried out in the U.S.A. to determine the outdoor survival of infective larvae, found that the percentage of larvae recovered fell to about one percent of the inoculum after 14 to 28 days outdoors in the summer, between 27 to 41 days in the winter, 42 to 56 days in autumn and 56 to 70 days in the spring.

In an evasive strategy to survive adverse weather conditions i.e. cold or hot and dry, H. contortus larvae undergo hypobiosis in the host. This arrested development of early fourth stage larvae (EL_4) is seen in both the tropics and temperate areas. Because environmental conditions suitable for the development of infective larvae of H. contortus vary in different areas of the world, the epidemiology of the disease shows distinct variations. Broadly speaking these may be reviewed under the following headings.

(a) Cold temperate areas such as the United Kingdom and Holland.

(b) Warm temperate to subtropical areas such as Australia and the Highlands of Kenya.

(c) Tropical areas with sharply defined wet and dry seasons.

COLD TEMPERATE AREAS

The essential characteristic of the epidemiology of Haemonchus in such areas is a prolonged period of hypobiosis of the early L_4 stage. Thus in Southern Britain, the proportion of arrested larvae in sheep has been found to increase in August, reaching maximum numbers (100%) from October to March (Connon, 1971), while any larvae seen in April or May were found to be developing normally. A rather similar observation was made by Waller and Thomas (1975) in Northern Britain, except they recorded that arrested larvae were seen as early as July and maximum numbers (100%) were recorded by September.

The thrust of these observations as reported by Waller and Thomas (1975) indicates that, in Britain and possibly Northern Europe in general, the life cycle of H. contortus consists predominantly of one generation per year, the majority of time being spent within the host. Thus the bulk of the worm population ingested in the summer is carried forward as inhibited stages to mature and contaminate the pasture the following spring and early summer as part of the spring rise. The long intra-host period is presumably an evolutionary adaptation to overcome unfavourable conditions in the external environment.

This theory incidentally, is corroborated by earlier work

carried out by Connan (1971) who reported that haemonchosis had occurred in housed sheep at the same time as in sheep at pasture, presumably indicating that the worms in the adult housed sheep were not recently acquired but carried over from the previous year as inhibited larvae.

The environmental stimulus which "conditions" larvae to undergo hypobiosis in the host is unknown and experiments have given conflicting results. Connan (1975) concluded that the seasonal inhibition of H. contortus in East Anglia was brought about primarily by an environmental stimulus acting upon the pre-parasitic stages, but that unlike O. ostertagi this was not associated with falling temperatures.

In Canada H. contortus inhibition has been reported to follow a period of exposure of infective larvae to autumnal conditions (Blitz and Gibbs, 1972) and photoperiod apparently has an influence also (Gibbs, 1973). Similarly, in New Zealand (McKenna, 1973) and The Netherlands (Cremers and Eysker, 1975), inhibition has been shown to be induced by a period of cold conditioning.

In contrast Connan (1975) found that the stimulus for inhibition could be provided in a culture kept in the dark at 25°C for 12 days, while Eysker (1981), found that a very low tendency for inhibited development followed a seven or 12 day culture period at 25°C. Some of these differences may represent differences in the strains of H. contortus (Eysker, 1981).

The stimulus which induces resumption of development, which occurs at a time when environmental conditions are suitable for

the development of the free-living stages, is unknown. Possibly it may be fixed for the phenotype of the parasite or alternatively may depend on some physiological change in the host associated perhaps with lengthening daylight. It is assumed that resumed development is always correlated with a seasonal stimulus (Eysker, 1978; 1981). In temperate areas such as Britain the number of developing stages in sheep also increases with the onset of warmer weather in April (Connan, 1978).

WARM TEMPERATE OR SUBTROPICAL AREAS

In such regions which are not associated with any prolonged periods of drought, the necessity for hypobiosis as a factor in survival is minimal. Thus Allonby and Urquhart (1975) reported that although a small population of inhibited larvae were observed intermittently in a flock of sheep in East Africa, no obvious factor was found to account for them. Similarly, in summer rainfall regions and areas with mild winters in Australia, larval availability increases in late spring reaching maximum levels after mid-summer and declines through the winter. However, hypobiosis can occur in such areas and has also been found to occur on the Highveld and Karoo regions of South Africa from March onwards, presumably associated with the cold temperatures of autumn/winter (Reinecke, 1983).

TROPICAL AREAS WITH DEFINED DRY SEASONS

The epidemiology of H. contortus infection in these areas is more akin to that of cold temperate areas in as much as larval

hypobiosis plays a significant role, although the "trigger" is presumably different.

Thus in Nigeria in calves it has been observed that the numbers of arrested larvae increase from August onwards into the dry season starting in October. In this way the parasite is able to survive the hot dry season lasting from October until May (Ogunsusi and Eysker, 1979). In Senegal Vercruysse (1984; 1985) found in sheep, H. contortus survives the dry season i.e. November to April, largely as hypobiotic larvae and similar observations have been reported in the neighbouring Gambia by Kaupfmann and Pfister (1990), who found that in calves almost 100% of the Haemonchus burden is present as inhibited larvae from October to May i.e. the dry season.

The stimulus for resumption of development in these areas is again uncertain. In Northern Nigeria, developing stages start to reappear at the onset of the rainy season which lasts from May until October (Ogunsusi, 1979).

Despite its ubiquity and importance, it is surprising that so little precise information is available on the epidemiology of haemonchosis in sheep. Perhaps it is the case that its complexity was underestimated until approximately 20 years ago.

As far as the factors which precipitate actual clinical disease are concerned these are well known, at least in principle, and have been summarised in a review by Armour (1980), as follows. The first and most frequent is an increase in infecting mass which is often associated with seasonal change and occurs after at least one parasite generation. The second is

where the susceptibility of the existing stock is altered either to the effects of an existing infection or to the acquisition of new infections e.g. change in diet and stage of pregnancy and lactation due to the periparturient relaxation of immunity. The third occurs when susceptible stock is introduced to an infected area, especially worm-naive young animals, but may also occur in non-endemic areas due to the introduction of infected stock. All of these aspects are discussed in some detail later in this review.

PATHOGENESIS AND CLINICAL SIGNS

Classically the pathogenesis of haemonchosis is that of a moderate to severe acute haemorrhagic anaemia caused by the blood sucking stages of the parasite i.e. fourth stage and adult stages. The pathogenic effects are related to the intensity of infection and young animals and those in a poor state of nutrition are more likely to be severely affected than older animals or their counterparts (Parkins and Holmes, 1989). These effects include reduced productivity in terms of weight loss or reduced growth rates, agalactia and, in severe infections, death (Allonby and Dargie, 1973). Whilst anaemia is associated with many cases, diarrhoea is only a feature of the disease during the so-called "self-cure" reaction (Allonby and Urquhart, 1975).

The pathogenesis of the anaemia has been described by Fourie (1931), and Andrews (1942), who compared the development of the anaemia in infected sheep with that induced by phlebotomy. Essentially the results were similar, both approaches producing a

normochromic or hypochromic, macrocytic anaemia. The macrocytosis was associated with the release of immature red cells or reticulocytes from the hyperplastic bone marrow. However, in fatal cases, bone marrow failure occurred and was associated with the development of a hypochromic microcytic, anaemia.

All of these features indicate a haemorrhagic aetiology, although subsequently Charleston (1964), also comparing infected and phlebotomised sheep, noted the presence of macrocytic megaloblastic normoblasts in the marrow of infected sheep and concluded that there must also be impaired synthesis of red cells.

Attempts to measure the actual blood loss caused by H. contortus have been made by a number of workers (Martin and Clunies-Ross, 1934; Andrews, 1942; Clark, Kiesel and Goby, 1962). The last authors used red cells radio-tagged with ^{51}Cr and ^{59}Fe and estimated that the blood loss started between six and 12 days after infection and that each adult removed between 0.03 and 0.31 ml of whole blood per day. Since then the subject has been studied more extensively by Allonby (1973), Dargie (1973b) and Dargie and Allonby (1975). They showed that an initial rapid drop in the packed cell volume (PCV) was followed during the next six to 14 weeks by a period when the PCV was maintained at a steady but lower than normal level. This was followed by a rapid drop in PCV terminating in death. They explain these processes as occurring progressively due to a time lag between the initial

blood loss and a stimulation of the erythropoetic system, a period of continued compensation by erythropoesis, and dyshaemopoesis resulting from iron deficiency due to the rapid plasma iron turnover, the loss of iron in faeces (>10 mg daily) and the animals' limited ability to reabsorb iron from the intestine. If serum iron falls to levels of less than 40 ug%, erythropoesis cannot take place, the haematocrit falls and the animal dies.

Infected animals may lose large quantities of serum proteins into the gut due to the blood sucking habits of the parasites. Dargie (1975), reported that the mean daily faecal clearance of plasma can be up to 210 - 341 ml/day when estimated from the losses of ^{131}I-labelled polyvinylpyrolidone. Albers and Le Jambre (1983), reached similar conclusions, but also established that there was a good correlation between erythropoesis as measured by ^{59}Fe clearance from the blood and erythrocyte potassium concentration ($r=0.79$ $P<0.0010$).

Although there is a continual loss of albumin associated with haemonchosis, only a mild hypoalbuminaemia may develop. However, in severe cases the metabolism of albumin may increase four-fold and serum albumin levels fall to less than 1 g%.

In practice, the clinical signs of H. contortus infection may be divided into three syndromes i.e. hyperacute, acute and chronic (Allonby, 1973).

The hyperacute syndrome is uncommon and is associated with a sudden massive infection. It is characterised by sudden death in previously healthy sheep due to haemorrhagic gastritis. At

necropsy, 10,000 - 35,000 L_5 or immature adults may be found in the faeces since death commonly occurs during the prepatent period (Soulsby, 1968).

The acute syndrome is more common and is the result of susceptible sheep ingesting moderate numbers of infective larvae so that 1,000 - 10,000 adults mature in the abomasum. This disease is characterised by anaemia, variable degrees of oedema, especially the submandibular form and ascites, agalactia in ewes, lethargy, dark coloured faeces and falling wool. The oedema is a result of hypoproteinaemia and faecal egg counts are high and may reach 10,000 eggs per gram (e.p.g.) (Soulsby, 1968; Urquhart et al, 1987).

The chronic form of the disease, only first described about 20 years ago (Allonby, 1973), is almost certainly very common and probably is the form of most considerable economic importance. It is a result of repeated infection with fairly low numbers of infective larvae leading to the presence of 100 to 1,000 adult Haemonchus in the abomasum. Although morbidity is 100%, mortality is usually low and this syndrome usually leads to unthrifty, weak, emaciated animals in which neither severe anaemia nor gross oedema are present. It is commonly seen during a spell of prolonged dry weather when the pasture is of poor quality and has been postulated to be the result of an interaction between prolonged though small, blood loss during a lengthy period of poor nutrition (Allonby and Urquhart, 1975).

The role of nutrition in predisposing to haemonchosis has

been a subject of some controversy for the past half century. In 1933, Clunies-Ross and Gordon showed that acquired resistance to haemonchosis in aged sheep could be reduced by feeding a low protein diet.

The results of a subsequent experimental study by Kates and Wilson (1955), concluded that high protein diets altered the pathogenic effect of ovine haemonchosis. However, this conclusion was disputed by Abbott (1982), who calculated that the "high" and "low" protein diets in this earlier study were both "high protein" although the former had a higher energy content. She also thought that the small improvements in food conversion ratios observed between the infected and control sheep were unlikely to be significant.

In 1978, Preston and Allonby investigated the effect of high and low protein diets on haemonchosis in two groups of six sheep infected with a single dose of 350 H. contortus larvae/kilogramme body weight. While faecal output was similar in both groups, the one on the low protein diet had higher faecal egg counts and showed more severe clinical disease. Although they concluded that the clinical signs were due to a greater establishment of adult worms, no necropsies were carried out.

It has also been shown experimentally that in Scottish Blackface lambs dietary protein did not significantly influence the establishment of the parasite, while Finn-Dorset lambs on a low protein diet had significantly higher faecal egg outputs four weeks after infection and more severe clinical signs (anaemia, hypoproteinaemia and hypoalbuminaemia) than similarly infected

lambs on a high protein diet (Abbott, Parkins and Holmes, 1985).

Unlike many gastrointestinal nematode infections, haemonchosis does not normally seem to be associated with anorexia as an indirect cause of "poor nutrition". Thus Allonby and Dargie (1973) in a study of weight loss in sheep infected with H. contortus, concluded that the losses observed in the infected animals were not due to anorexia, since these sheep were consuming more food than the controls. Thus, the weekly nitrogen intake of the infected and control animals was 42.5 and 38.7 kg respectively. They suggested that "during the period of induced growth, the infected sheep entered a state of reduced N balance due to the increasing loss of urinary and faecal nitrogen. This indicated that, not only are such sheep unable to reabsorb all the nitrogen lost into the gut as a result of plasma leak, but that they actually mobilise tissue protein in order to supply the constituents necessary for the synthesis of physiologically more important molecules such as albumin and haemoglobin".

The most recent work is that of Abbott (1982), who investigated, under controlled experimental conditions, the influence of nutrition on the establishment and pathophysiological consequences of H. contortus infection in sheep using radioisotope tracers as well as conventional biochemical, haematological and parasitological techniques. She found that lambs aged four months on a low protein diet and repeatedly infected with 200 L_3/kg body weight, suffered from clinical haemonchosis and had higher worm burdens at slaughter

than lambs on a high protein diet. The principal finding was that many of the lambs fed a high protein diet developed resistance to continuing infection, and she suggested that the high worm burdens in lambs fed the low protein diet might indicate an imparied immune response in these lambs. She concluded that the lambs on the low protein diet were less able to develop an acquired resistance due to a delay in the onset of immunological competence. Abbott (1982), also concluded that the depressed productivity associated with ovine haemonchosis is probably the result of a combination of reduced feed intake and increased energy requirements for blood and tissue regeneration, rather than a failure to absorb the blood protein lost into the abomasum as suggested by Allonby and Dargie (1975).

Information on the effects of H. contortus on production in sheep is not very extensive. Under experimental conditions, Barger and Cox (1984), found that relatively mild effects on live weight gains and wool growth resulted from chronic infection and suggested that mortalities induced by acute infections may represent some of the most important economic effects attributable to the parasite. These results (Barger and Cox, 1984), were similar to those obtained in field studies by Allonby and Urquhart (1975), where low worm burdens, anaemia and progressive weight loss accompanied the chronic form of haemonchosis in Kenyan sheep. They found that a moderate infection of a few hundred worms persisting over a period of several months would produce chronic anaemia and ultimately, severe loss of body condition and death in ewes and lambs grazing

on poor quality pasture.

DIAGNOSIS

History and clinical signs are usually sufficient for the diagnosis of H. contortus infection in sheep especially when coupled with faecal examination to detect frequent high egg counts and to allow larval identification after culture. However, definitive diagnosis can only be made by necropsy of a representative clinical case.

The following description is adapted from that of Allonby (1973).

The hyperacute syndrome, which is relatively uncommon, usually presents as sudden deaths in a small proportion of the flock after a long spell of warm humid weather. Otherwise clinical signs if observed, are those of dullness, severe anaemia and dark coloured faeces. The faecal worm egg counts range from zero, if the worms have not reached patency, to 400,000 e.p.g. At necropsy, the abomasum contains 10,000 to 35,000 adult or immature worms and shows all the signs of a severe haemorrhagic gastritis. The mucosa is extremely petechiated due to multiple "bite" marks and may even be eroded. Section of the long bones usually shows a modest expansion of the red marrow.

The acute syndrome is characterised by dullness, anaemia, sub-mandibular oedema and possibly ascites. The faeces are dark coloured and ewes may become agalactic. Faecal worm egg counts range from 1,000 to 100,000 e.p.g. At necropsy, the typical petechiae are present, usually associated with oedema of the

mucosal folds and the presence of several thousand adult worms. Section of the long bones usually shows marked expansion of the red marrow into the medullary cavity.

The chronic syndrome is not necessarily associated with wet weather and indeed is more prevalent during a prolonged spell of dry weather when the nutrition becomes poor. It is mainly characterised by weight loss or poor growth rates and only a modest degree of anaemia. Faecal egg counts are low, under 2,000 e.p.g. Clinically, the syndrome is difficult to distinguish from one caused by poor nutrition per se. At necropsy, up to 1,000 adult worms may be present in the abomasum and the mucosal folds are thickened. In the long bones, the red marrow may be expanded, although if iron deficiency is present, the marrow may be gelatinous. Larval culture and identification may be performed if thought necessary, especially in cases where faecal egg counts are very low.

In general, the histopathological lesions seen in H. contortus infection in sheep include, infiltration of the mucosa by lymphocytes, eosinophils, mast cells and IgA plasma cells together with an apparent increase in the production of mucus (Salman and Duncan, 1984; 1985).

TREATMENT

Currently, a wide range of drugs are available for the treatment of haemonchosis. These include the benzimidazoles, the pro-benzimidazoles, levamisole and the avermectins e.g. ivermectin (Anon, 1990). All of these drugs are highly effective

against H. contortus, having efficacies of 95% to 99%.

Generally it is unwise to wait until an outbreak of haemonchosis has occurred before instituting drug treatments. However efficient and rapid an anthelmintic is at expelling worms, the attendant loss of productivity whether in terms of lamb growth, meat, milk or wool production is not immediately rectified and is financially costly. Also, by the time clinical signs are apparent, the pastures have often become heavily contaminated with developing eggs and larvae. For example, Le Jambre (1978b), pointed out that, at the time of treatment, the number of parasites within the host approximates only 3% of the total parasite population and thus the chances of reinfection are very high. The current trend therefore is to use anthelmintics, whenever possible, in a prophylactic fashion i.e. to institute treatment before overt clinical signs are apparent. The efficacy of treatment may be affected by the development of acquired resistance to anthelmintic drugs and this is discussed in the next section.

CONTROL

Attempts to control haemonchosis have depended on three strategies i.e. chemoprophylaxis, genetic selection for resistance and artificial immunisation. In this section only the first two are reviewed, the third being discussed in the following section on immunity.

CHEMOPROPHYLACTIC CONTROL

Chemoprophylaxis is currently the only practical approach to the control of the disease and depends essentially on repeated anthelmintic treatments during that part of the year when epidemiological conditions are suitable for the development of the infective larvae on pasture. Where the threat of haemonchosis is serious and prolonged, it has been advocated, at least until the last few years, that the drugs should be administered at intervals of three weeks or less, the prepatent period of Haemonchus, so that the pasture contamination by eggs and larvae is largely eliminated; in practice, it is more useful to depend on dosage at monthly intervals. Traditionally, in areas of intensive sheep rearing, broad spectrum drugs have been used which also control other pathogenic nematodes such as Ostertagia and Trichostrongylus.

This approach although expensive in terms of labour and cost of drugs, was used successfully until the early 60's when reports of reduced drug efficacy due to the acquisition of drug resistance by gastrointestinal nematodes, especially Haemonchus contortus, started to appear. Among the earliest reports, was one by Drudge, Szanto, Wyant and Elam (1964), who reported a resistant strain of H. contortus just three years after the commercial release of thiabendazole. Since then, drug resistant strains of H. contortus have been widely reported in the summer rainfall areas of Australia, in South Africa, Brazil and Uruquay and to a lesser extent in Europe and North America (Waller, 1986).

Resistance can be defined as a significant increase in the level of tolerance to a drug i.e. an increase in the number of individuals within a population able to withstand doses of a compound which would prove lethal to the majority of individuals in a normal population of the same species (Prichard, Hall, Kelly, Martin and Donald, 1980). This leads to a poor clinical response to anthelmintic treatment by which time selection for resistance has already occurred.

Resistance is largely confined to the benzimidazole group of anthelmintics and in Australia is estimated to occur in 68% of farms (Anderson, Martin and Jarrett, 1988). Levamisole resistance has also been reported to a lesser extent in Australia (Green, Forsyth, Rowan and Payne, 1981; Anderson et al, 1988), and more recently ivermectin resistance has been described in South Africa (van Wyk and Malan, 1988), and Brazil (Echevarria and Trindade, 1989).

It seems to be generally agreed that drug resistance is most likely to develop when a drug is used frequently and Donald (1983) has stated that selection pressure increases dramatically if the treatment intervals approach the prepatent period of Haemonchus i.e. the basis of the control schemes commonly practised in Australia (Johnson, Darvill, Bowen, Brown and Smart, 1976; Johnson, Darvill, Bowen, Butler, Smart and Pearson, 1979), and elsewhere. Under such regimens, only resistant strains of nematodes are likely to survive. Another factor which favours the development of drug resistance is the practice of dosing to

the average weight of the flock rather than to the heaviest animals in the flock (Edwards, Wroth, de Chaneet, Besier, Karlsson, Morcombe, Dalton-Morgan and Roberts, 1986), resulting in the use of sub-optimal dose rates in a number of animals.

In general the more effective a treatment programme is and the more its effectiveness depends on anthelmintic efficiency, the greater the potential for development of anthelmintic resistance.

The occasional administration of drugs to clinical cases of H. contortus infection is the form of treatment least likely to select for resistance as the adult (reproducing) population that may be resistant does not last long after treatment in the face of rapid reinfection and hence contributes little to pasture contamination (Prichard et al, 1980).

Strategies recently advocated in Australia to minimise the effect of anthelmintic resistance have included the use of a single drug until it fails followed by another (Le Jambre, Southcott and Dash, 1977), and a slow rotation at approximately yearly intervals between anthelmintics with different modes of action (Prichard et al, 1980).

Strategic control is based on the administration of drugs when parasites are at their lowest level on pasture or when the weather is against the survival of the free-living stages and results in a decrease in pasture contamination. Recently, two successful programmes have been introduced in Australia which rely for their effect on their strategic nature i.e. the "Worm Kill" and "Drench Plan" control programmes (Dash, Newman and

Hall, 1985; Waller, 1986). Both rely on the limited use of broad spectrum anthelmintics to control gastrointestinal nematodes such as Ostertagia and Trichostrongylus species, thus delaying the onset of resistance, and the use of the narrow spectrum drug closantel (Seponver(R)) which is specifically effective against the haematophagic H. contortus and is used exclusively when haemonchosis alone is a threat. Closantel has additional advantages in that it also has efficacy against liver fluke and nasal bots.

The basis of the Australian "Worm Kill" programme which is designed for August lambing is that three doses of Seponver(R) are administered; one on the 1st of August, the second on the 1st of November and the third on the 1st of February. The first dose is given to ewes as a pre-lambing dose and though the manufacturers claim that Seponver(R) has a 100% prophylactic effect on H. contortus for four weeks, in practice scientists associated with the Department of Agriculture, New South Wales, Australia found that it gives effective control for 12 weeks due to its residual effect (Davidson, 1985).

The second dose given in November, is administered to both ewes and lambs and the third dose given three months later in February is also administered to both ewes and lambs.

This technique almost completely prevents pasture contamination during the critical months of August to February without the need for repeated monthly drenching (Davidson, 1985). Broad spectrum anthelmintics to control Ostertagia and

Trichostrongylus species are administered in November and February and the inclusion of suitable grazing management i.e. two pasture changes to "low worm" pasture at the time of the second and third doses of Seponver(R) ensures the success of the programme. This programme may be classified as a form of "Integrated control" i.e. the use of anthelmintic treatment coupled with the relocation of stock to a "safe" pasture i.e. that with a relatively low helminth infectivity (Prichard et al, 1980).

As a result of the "Worm Kill" programme, H. contortus has virtually disappeared from farms that have been following the programme for two successive years and a maintenance programme "Worm Kill 2" has now been initiated. In this programme Seponver(R) is used only twice per year, whilst the broad spectrum drench treatments remain the same (Davidson, 1987).

However, there are two possible disadvantages of the "Worm Kill" programme. Firstly the likelihood of the development of resistance to closantel and secondly, whilst the programme is currently ideal for wool sheep, its use in meat producing lambs would be restricted due to drug residues in the tissues.

As a result of the evolution of parasites resistant to available drugs, more and more attention has been drawn towards alternate means of control.

GENETIC CONTROL

The control of H. contortus infections in sheep through genetic selection has been proposed by several authors. Most

publications concerned with haemonchosis in sheep are based on observations in wool producing Merino sheep and it is surprising that over the last few centuries, the breed has not developed a better resistance against H. contortus comparable to the immunity of adult sheep to Ostertagia and Trichostrongylus species. It may be that the constant availability of relatively efficient anthelmintics over the years, has resulted in little conscious selection of animals for resistance to the disease. The genetics of resistance will be covered under two headings namely Influence of haemoglobin (Hb) type and Breed susceptibility.

Influence of Haemoglobin Type

Some evidence that a small degree of selection may have occurred naturally, has been documented by Evans and Blunt (1961), who showed that the gene frequencies of the haemoglobin types of Romney Marsh and Southdown sheep in Australia were predominantly Hb A, whereas the original stock in the nature British environment was predominantly Hb B.

Evans, Blunt and Southcott (1963), suggested that Hb types were indicators of adaptation by sheep to environmental conditions e.g where high infection rates with H. contortus were a major factor, and demonstrated that adult worm burdens were smaller in sheep with Hb A than in those with Hb AB after infection with the parasite, although there were no significant differences in the haematological indices of the two haemoglobin types.

A relationship between Hb types and packed cell volumes

(seasonal maximum haematocrit) in grazing sheep was shown by Evans and Whitlock (1964), where mean packed cell volumes for sheep with Hb A were greater than in sheep with Hb B and sheep with Hb AB had intermediate values.

Haemoglobin types are genetically determined and once established the adult type in a sheep does not change (Jilek and Bradley, 1969). These authors and Radhakrishnan, Bradley and Loggins (1972), found that Florida Native ewes had a high frequency of Hb A and were adapted to the environment and therefore postulated that Hb A was found in sheep adapted to the adverse environment existing in Florida: they also reported that sheep with Hb A were more resistant to parasites than sheep with Hb B and that besides the higher frequency of Hb A sheep, those sheep had higher Hb concentrations and packed cell volumes and lower faecal egg counts than Rambouillet ewes.

Allonby and Urquhart (1976), found, in a study conducted on the performance of set-stocked Merino ewes and lambs in relation to their haemoglobin phenotypes, that those sheep with Hb A showed "self-cure" more frequently, had the highest haematological parameters and the heaviest body weights, whilst the Hb B type sheep were consistently lowest.

Breed Susceptibility

The influence of breed on the susceptibility of sheep to haemonchosis has been studied by several workers. Bradley, Radhakrishnan, Patil-Kulkarni and Loggins (1973), found that Florida Native lambs were more resistant than Rambouillet lambs,

as indicated by the higher packed cell volumes and greater weight gain, as well as the lower number of adult parasites recovered at necropsy in the Florida Native lambs.

Preston and Allonby (1978; 1979), compared the susceptibility to H. contortus infections of six sheep breeds in East Africa and found that the Red Maasai was the most resistant sheep breed, as judged by faecal egg counts and anaemia, and they observed that clinical cases of acute haemonchosis in this breed were rare. The increased resistance of this indigenous East African breed may be a result of natural genetic selection in an endemic area as the Red Maasai sheep were shown to achieve higher mean levels of anti-larval IgA antibodies than Merinos (Preston and Allonby, 1979).

Differences in susceptibility to H. contortus infection are greater between breeds than between Hb phenotypes within a breed. This was studied extensively by Altaif and Dargie (1978a; 1978b) using the Scottish Blackface and Finn-Dorset sheep. They found that, though sheep of both breeds homozygous for Hb A showed lower worm burdens and reduced effects of parasitic infection than sheep homozygous for Hb B, Scottish Blackface sheep of the same haemoglobin type as the Finn-Dorsets displayed less severe clinical and pathological effects and fewer worms and eggs were recovered from them as compared with the Finn-Dorsets. This they suggested, indicated that genetic factors operate in determining resistance and that the genetic resistance observed was directed against parasite establishment and was controlled by the immune response elicited.

A breed variation was also shown in Yugoslavia, where lambs and adults of three breeds of sheep (Cigaja, Merino Karkaz and Merino Prekos) were grazed together for several months on contaminated pasture. As measured by faecal egg counts, all lambs appeared susceptible. However the mean faecal egg counts of the Cigaja and Merino Prekos adults were almost persistently negative throughout the year, even after just one previous season of exposure to H. contortus (Cvetkovic, Lepojev and Vulic, 1973).

In more recent studies of four breeds of sheep, namely the Florida Native, the St. Croix, the Barbados Blackbelly and domestic sheep it was found that, based on faecal egg counts and packed cell volumes, the St. Croix lambs were the most resistant followed by the Florida Natives and the Barbados Blackbelly, whilst the domestic sheep were the least resistant (Courtney, Parker, McClure and Herd, 1985). These breed differences were found to be most pronounced in young lambs, with the domestic lambs being better able to resist secondary infections after sexual maturity.

Artificial selection and breeding for genetically resistant animals under natural infection, has been underway since 1978 (Gray, 1987). Australian workers have been heavily involved in the selection of genetically resistant Merino sheep based on individuals rather than breed and as such the progress has been slow.

Evidence of genetic resistance has been based on numbers of worms in sheep at necropsy, faecal egg output (e.p.g.) and

anaemia as measured by PCV. Under experimental H. contortus infection Gray (1987), reports that resistance has a moderate heritability i.e. to the order of 0.3 and resistance traits in a flock could increase by 6% per year given that variation of resistance with a flock is 40% and selection intensities for males and females are 5% and 30% respectively. Although a possibility, this increase in resistance would take some time to be achieved. The dependence on the selection of individual animals for the development of worm-resistant sheep is a major constraint and could largely be removed if there was "an entire breed which is resistant and has already evolved, at least to some degree, for meat production, such as the Red Maasai sheep of East Africa" (Urquhart, 1988).

IMMUNITY TO H. contortus

Since the experimental section of this thesis is concerned with the possibility of a protective immune response being stimulated by the surface antigens of H. contortus infective larvae, the literature relating to immunity is reviewed in rather greater detail.

Since the first attempt by Stoll (1929) to vaccinate sheep against H. contortus by the intraperitoneal injection of infective larvae, there have been intermittent reports of attempts to induce immunity, but for one reason or another none of these attempts have had any practical consequences. However, within the past ten to 15 years, stimulated partly by the possibilities opened up by recombinant technology and partly by

the increasing problem posed by the acquisition of anthelmintic drug resistance by H. contortus, there has been renewed interest in this area.

As noted earlier in this review, the knowledge of the immune responses of sheep to H. contortus is still rudimentary and the possibilities for immunisation are not very encouraging. In practical terms the situation is best summed up by Gordon's observations (1948; 1950), that Merino sheep in Australia acquire no immunity to H. contortus infection and are susceptible throughout their entire lives.

A similar observation was made by Allonby and Urquhart (1975) in Kenya, who observed that under natural conditions Merino ewes did not appear to develop a significant degree of immunity to the infection since on two occasions, over a period of 21 months, when it was known from tracer lamb worm burdens that large numbers of H. contortus larvae were available on pasture, heavy infestations in adults were rapidly established.

Despite these observations, which were confined to Merino sheep exposed to natural infection from birth, it was shown that Down Lambs (Manton, Peacock, Poynter, Silverman and Terry, 1962) and lambs of the Scottish Blackface breed (Urquhart, Jarrett and Mulligan, 1962) could be readily vaccinated against a single experimental challenge using either primary infections of normal or X-irradiated larvae so that challenge worm burdens were reduced by 98%. Subsequently, Smith and Christie (1978), found that the administration of two vaccinations of irradiated H. contortus larvae to 9-month old Scottish Blackface cross lambs

conferred protection against challenge with 10,000 normal larvae, whilst 7-month old Scottish Blackface lambs vaccinated with infective H. contortus larvae attenuated by irradiation at 60 kr acquired a striking resistance to challenge with 10,000 infective larvae. This resistance was accompanied by stunting of the challenge larvae recovered six days after challenge, thus indicating that at least part of the protective effect of the vaccination was directed against the larval stages of the challenge infection (Smith and Christie, 1979).

Unfortunately, this level of acquired resistance was only found in lambs aged over seven or eight months of age (Jarrett, Jennings, McIntyre, Mulligan and Sharp, 1959; Manton et al, 1962; Urquhart, Jarrett, Jennings, McIntyre, Mulligan and Sharp, 1966a; Benitez-Usher, Armour, Duncan, Urquhart and Gettinby, 1977; Neilson, 1975; Smith and Christie, 1979).

AGE RESISTANCE

Manton et al (1962) found that age was an important factor in the ability of sheep to resist reinfection with H. contortus. Under experimental conditions using two age groups of sheep (2-4 months and 10-12 months), it was seen that primary infections produced similar results in both groups namely reduced haematocrit values, haemoglobin levels and erythrocyte counts, but while subsequent infections resulted in consistently low haematological and high parasitological parameters in the young lambs, there was no marked drop in blood values and egg counts remained low in the older group of sheep. Abomasal worm counts

also showed an age variation with the young lambs producing consistently high worm burdens, whilst the older sheep showed very highly significant reductions in worm burden when compared with their respective controls.

Urquhart et al (1966a) found that the oral administration of 10,000 H. contortus larvae irradiated at 40kr, once or twice to three month old Blackface lambs, did not confer any protection against a challenge infection with 20,000 normal larvae one month after vaccination. Subsequent studies (Lopez and Urquhart, 1967) on worm-free Merino lambs aged six to seven months which were vaccinated with X-irradiated larvae on two occasions at monthly intervals also failed to demonstrate any evidence of protection against challenge. However, a group of seven Merino sheep reared worm-free until two years of age and subjected to the same regimen of vaccination were almost solidly protected against a challenge of 10,000 larvae compared to a group of control sheep (i.e. a mean of 61 $\pm$ 69 worms compared to 2,550 $\pm$ 1,629).

All of these results, taken together, have led to the hypothesis that lambs, due to immunological immaturity, are unresponsive to H. contortus infection until they are over seven months old (Duncan, Smith and Dargie, 1978). It has been suggested that if lambs are exposed to infection during this unresponsive period they fail, in subsequent life, to develop an adequate immunity due to a permanent suppression of their immune system to the protective antigens of H. contortus (Urquhart, 1980), although clear experimental proof of this hypothesis is

still lacking. The reason for this age related unresponsiveness is not known and is difficult to understand because neonatal and even foetal lambs are capable of mounting protective immune responses against various viral and bacterial infections (Smith, Jackson, Jackson and Williams, 1985). In a small laboratory animal model however, it has been shown that young rats up to the age of six weeks show a diminished response to infection with Nippostrongylus brasiliensis (Jarrett, 1971). Moreover, if they are repeatedly infected during this period and into adult life, they remain immunologically incompetent and fail to eliminate their challenge infection beyond a certain threshold level.

Sinclair (1970), queried whether this unresponsiveness in young animals was due to stimulation of the host by some helminth antigens and not others. More recently, vaccination experiments have been carried out using various somatic, metabolic or excretory-secretory antigens obtained from larval and adult Haemonchus, with varying degrees of success. These antigens can be referred to as "hidden" antigens and comprise those parasite antigens that the host animal is not normally exposed to, such as somatic antigens obtained by homogenisation of the parasite or those parasite antigens that are only transiently present such as the stage specific cuticular proteins.

Neilson (1975), showed that vaccination of 3-month old lambs with functional metabolic products released by H. contortus third and fourth stage larvae during in vitro culture, did not confer any protection against challenge with 3,000 L_3, though circulating antibodies which reacted with four of the larval

products were present in the serum. In contrast, vaccination of sheep with a high molecular weight fractionated extract of somatic antigens of H. contortus and concentrated excretory-secretory fluids obtained by the cultivation of third larval stages in vitro, produced partial protection in lambs aged under six months i.e. a 59% reduction in adult worm burden was observed in vacinates when compared with the controls (Neilson and Van De Walle, 1987).

A purified metabolite of exsheathed H. contortus L_3 isolated and concentrated from culture fluid during exsheathment was used as a vaccine in an attempt to protect young lambs aged less than four months against subsequent infection. Whilst the vaccine did not protect the lambs from succumbing to infection, there seemed to be an inhibition of worm egg production (Boisvenue, Galloway and Hendrix, 1987). Recently also, young sheep approximately six months and younger have been induced to gain some degree of protection against experimental infection with normal Haemonchus larvae after the administration of a protein fraction extracted from adult H. contortus (Munn, Greenwood and Coadwell, 1987). Local presentation of antigen is also important for potent expression of protective immunity against gastrointestinal nematodes (Rothwell and Griffiths, 1977).

Failure to successfully vaccinate sheep against H. contortus infection with secretions or tissue extracts of the parasite may be due to several factors. These include administration of insufficient or inappropriate material, insufficient time allowed

between vaccination and challenge for the development of acquired immunity and inappropriate route of injection (Adams, Beh and Davies, 1982).

Apart from these attempts to develop a form of practical immunisation against H. contortus infection, considerable effort has been devoted to the study of the immunological mechanisms which can enable sheep to develop immunity to this parasite.

One of the earliest reports is that of Stewart (1953) in Australia who studied the "self-cure" reaction of sheep to H. contortus. The term "self-cure" was first introduced by Stoll (1929), but was only described in detail by Stewart (1953), who defined the reaction as "the expulsion of an existing population of adult H. contortus a few days after challenge with relatively large numbers of infective larvae".

Stewart (1953), showed that the "self-cure" reaction was precipitated in an animal harbouring a population of adult worms by intake of a new dose of infective larvae and that "self-cure" did not occur in all infected sheep challenged with larvae, but was most likely to occur in animals which had had several previous infections with the parasite. He also showed that in some cases the adult worm population was largely eliminated, in others the "self-cure" reaction was partial and sometimes all that was produced was a transient suppression of egg production.

Stewart (1953) also suggested that "self-cure" was not completely specific e.g. when sheep infected with both H. contortus and Ostertagia spp were challenged with H. contortus larvae, adults of both species were expelled and when the "self-

cure" took place in the presence of T. colubriformis in the small intestine the adults of this species were also eliminated: this indicated that both parasite species need not be in the same locus however, intake of larvae of T. colubriformis, a small intestinal nematode, did not trigger "self-cure" of the abomasal parasites.

This phenomenon is of considerable value to the grazing animal as it is able to get rid of a substantial proportion of its adult parasite burden (Stewart, 1953). However, since the sheep usually offers no resistance to the development of a challenge infection, one wonders if this "immune" reaction is in fact an evolved adaption of benefit to both the host and parasite i.e. the host gets a few weeks respite from the haematophagic activities of the adult worms, but in return there is replacement of the old population of adults with young vigorous males and females (Urquhart, 1980).

The stimulus for the "self-cure" reaction is unclear but Soulsby, Sommerville and Stewart (1959), were able to induce the reaction experimentally and suggested that it is initiated by substances released when third stage larvae undergo the third ecdysis. That the reaction is dependant on antigens from living larvae acting locally is indicated by the fact that living larvae injected intraperitoneally or dead larvae given orally failed to induce it (Soulsby, 1968). "Self-cure" is accompanied by a transient rise in blood histamine, increase in complement fixing antibodies and intense mucosal oedema in the abomasum, suggesting

an anaphylactic reaction (Stewart, 1953). None of these reactions were shown in animals failing to show the "self-cure" reaction. This pioneering work of Stewart has been the basis of much subsequent study on the role of such agents as reaginic antibody, mucosal mast cells and goblet cells in helminth immunity in both sheep and laboratory animal models.

There is however, evidence to suggest that the "self-cure" reaction is not always immunologically based. Allonby and Urquhart (1973) found that "self-cure" as judged by a dramatic fall in faecal egg count and loss of adult worm burdens, was found simultaneously in sheep on infected pasture and in sheep grazing on parasite-free pasture. They found that this phenomenon occurred after a period of significant rainfall and suggested that as a flock phenomenon, new pasture growth might play a role in initiating "self-cure" as this was the only common factor between the two flocks.

Protective immunity to H. contortus in sheep operates against various stages in the life of the parasite and perhaps the most important form of immunity is that acting against settlement in the host of incoming larvae (Adams, 1988).

In 1966 Bitakaramire showed that the immune response to Haemonchus was specifically directed against the third and fourth stage larvae, a result supported by Adams (1982), who, while working on the effects of corticosteroids on immunity, found that acquired immune responses were more important in controlling infection than innate mechanisms and that these responses appeared to be directed at the incoming third stage and

established fourth stage larvae. In an experiment involving vaccination of sheep with X-irradiated H. contortus third stage larvae followed by challenge with normal larvae, Bitakaramire (1966) recovered no worms beyond the fourth larval stage from 13 of the 14 vaccinated sheep allowed to survive for six or more days after challenge. Furthermore, the combined numbers of third and fourth stage larvae recovered on Days 3 and 6 were markedly reduced when compared with those from unvaccinated sheep. It was concluded that the "protective" antigens i.e. those that stimulated resistance to reinfection, were derived from the third or fourth stage larvae or from the third moult.

A similar result was reported by Miller, Jackson, Newlands and Appleyard (1983a), who termed the phenomenon "rapid expulsion" and reported that within 48 hours after challenge the vast majority of H. contortus larvae administered to hyperimmune sheep cannot be recovered from the surface of the abomasal mucosa or detected in sections of the mucosa i.e. they had been expelled from the abomasum.

Although the possibility that antibody may play a significant role in the immune response to gastrointestinal nematodes in small laboratory animal models has been demonstrated (Wakelin, 1978), passive protection by serum transfer has not been reported for such nematodes in ruminants. However increased levels of parasite specific antibodies, primarily IgA, have been described in the abomasum of H. contortus infected sheep (Smith, 1977), and its potential importance in immunity is suggested by

its relative absence from the mucosa of susceptible lambs when compared with resistant young adult sheep after both groups had been vaccinated with irradiated H. contortus larvae (Duncan et al, 1978).

Smith and Christie (1978), found that resistance to reinfection with H. contortus larvae was also associated with increasing concentrations of IgG anti-larval antibody in the serum as well as IgA and IgG antibodies in abomasal mucus. They suggested that the presence of these anti-larval antibodies demonstrated an association between the protection conferred by the irradiated vaccine and the antibodies. It was also noted that secondary antibody responses occurred after challenge and this was attributed to the vaccination priming the abomasal mucosal IgA system (Smith and Christie, 1978; 1979). In this connection Charley-Poulain, Luffau and Perry (1984), found that primary infections in seven month old female sheep did not result in an increase in anti-larval antibodies in the serum or abomasal mucus; however after a challenge infection, IgA, IgG and IgM serum antibody levels rose slightly. In contrast, the IgA antibody level in the abomasal mucus rose rapidly after the challenge infection and it was suggested that IgA anti-larval antibody in the mucus may be involved in the "self-cure" reaction by inducing adult worm expulsion, as its level diminished rapidly after worm expulsion.

A second antibody isotype, whose role has been extensively explored in immunity to gastrointestinal helminthiasis, at least in laboratory models, is the anaphylactic or reaginic antibody,

IgE, which has a high affinity for mast cells. It is well established that high levels of circulating IgE are invariably associated with helminth infections but to date attempts to determine their protective role in immune expulsion have been inconclusive (Jones, Edwards and Ogilvie, 1970; Miller, 1979).

Perhaps the most significant finding in recent years is that of Smith, Jackson, Jackson, Williams, Willadsen and Fehilly (1984), who showed that the transfer of whole lymph or washed lymph cells from three immune donor sheep to their homozygous co-twin recipients, reduced significantly the susceptibility of the recipients to challenge with 10,000 Haemonchus L_3 as measured by their faecal egg counts. In a further experiment, lymphocytes from an immune sheep transferred a secondary local IgA response to its homozygous recipient and also a marked reduction in worm count when compared with an infectivity control sheep.

The precise role of lymphocytes is still unclear and it is unlikely that they are active at the mucosal surface of the abomasum. However, in a review based on studies with H. contortus in sheep and supported by studies on laboratory models (Russell and Castro, 1979; Miller, Huntley and Wallace, 1981a), Miller (1984) has described an effector mechanism called "immune exclusion" by which larvae are excluded from entering the glandular tissue of immune sheep and are consequently rapidly lost from the host. The mechanisms of "immune exclusion" are still unknown but Miller (1984), has suggested a hypothetical system. In this, primed T cells of various sub-sets provide help

for B cells to differentiate into plasma cells, promote the differentiation of bone marrow precursors into mast cells, stimulate basophilopoesis and eosinophilopoesis and accelerate the differentiation of epithelial cells into goblet cells. Mast cells, eosinophils and basophils become sensitised by immunoglobulin and subsequently interact with worm antigen to promote the release of mediators such as histamine, 5HT, protaglandins and leucotrienes; these alter gut motility, permeability and secretion and may even have a direct effect on the parasite. In the gut lumen there is an increase of secretion and mucus and an influx of plasma proteins. These together with mucin polysaccarides interact with the worm cuticle and impede the penetration of worm secretions into the mucosa.

This hypothesis suggests an explanation for the results of work reprted by Adams (1982), who found that the corticosteroid dexamethasone abolished acquired immunity to H. contortus. He found that sheep, rendered immune after a primary infection, lost their immunity after dexamethasone treatment: treatment resulted in 68% establishment of infective larvae as determined by adult worm numbers, compared with 13% in untreated immune, and 65% in untreated and treated non-immune sheep. This dexamethasone effect was found to occur before Day 7 of the second infection, with many more adult worms being present in treated than untreated sheep at Days 21 and 24 after infection but no clear differences in the numbers of L_4.

Adams (1982), also found that acquired immune responses preventing the establishment of H. contortus infections, act

after Day 4 following infection and are complete by Day 7. Comparison of numbers of fourth stage larvae recovered from untreated and dexamethasone treated animals on Day 7 following challenge, showed that substantial numbers of infective larvae either failed to develop or were rejected before this time.

Some support for the mechanism of the "immune exclusion" hypothesis was subsequently produced by Jackson, Miller, Newlands, Wright and Hay (1988), who showed that the corticosteroid, dexamethasone, almost completely abolished the protective effect of "immune exclusion" in immune sheep; it is known that effector cells such as mast cells and globule leucocytes are particularly sensitive to corticosteroids.

One interesting and important feature of these experiments was that the immunity of the untreated sheep persisted, in the absence of antigenic stimulus, for six weeks but had waned by twelve weeks, perhaps indicating the necessity of a level of continuous challenge to maintain the cellular defences of the gastric mucosa. The relevance of this observation is the possibility that the regular use of anthelmintics may affect the development of immunity to H. contortus infection (Donald, Dineen and Adams, 1969), particularly with the development of new and highly effective anthelmintics.

This theory is supported by the work of Dineen and Wagland (1966) and Wagland and Dineen (1967) who found that sheep exposed to a series of infections with H. contortus terminated by anthelmintic treatment and followed by challenge were unable to

develop a significant degree of immunity. They thus concluded that a strong resistance was associated with prolonged uninterrupted infection and that anthelmintic treatment was more likely to interfere with, than assist the development of immunity. A more recent and unexpected development in this sphere was the observation of Benitez-Usher, Armour, Duncan, Urquhart and Gettinby (1977) who found that worm-free Scottish Blackface lambs vaccinated with irradiated larvae at nine and ten months of age, failed to develop any immunity to subsequent challenge, when anthelmintic (thiabendazole) treatment was administered three weeks after each immunising infection, whereas sheep of the same age undergoing similar vaccinations, but not treated with anthelmintic, developed a highly significant immunity to susequent challenge.

Barger (1988) found that young sheep were able to exhibit resistance to incoming H. contortus infection from as early as four months when exposed to a natural challenge, but this resistance was lost completely when they were treated with anthelmintic, suggesting that protective immunity in young lambs to H. contortus is most reliably achieved when moderate burdens of adult worms are allowed to persist in the host during challenge.

IMMUNOCHEMISTRY AND CULTIVATION OF H. contortus

THE NEMATODE SURFACE

There is no doubt that the surface of parasitic nematodes is recognised by the immune system in many infected hosts (Grencis,

Crawford, Pritchard, Behnke and Wakelin, 1986), and that antibodies are produced against the surface molecules and mediate antibody-dependent cell-mediated cytotoxic reactions. Considering the varying environments in which the different stages of a parasite live, it is not surprising that the surface associated proteins/molecules are in a state of constant turn-over (Maizels, Philipp and Ogilvie, 1982).

Biochemical studies of the adult stage cuticle of parasitic nematodes indicates that the cuticle consists of soluble components and collagen-like proteins which can be solubilised by detergents and 2-Mercaptoethanol (2ME) and non-solubilisable proteins. Characterisation of the soluble cuticular proteins by sodium dodecyl sulphate polyacrylamide gel electrophoreisis (SDS-PAGE) has revealed a large number of proteins covering a wide range of molecular weights (Fetterer, 1989).

The knowledge of the stage specificity of surface molecules was the stimulus for pioneering studies which showed that the surface of living nematodes can be radiolabelled without loss of viability and that the labelled molecules can be isolated in soluble form (Parkhouse, Philipp and Ogilvie, 1981). The surface proteins of several nematodes have been identified by radiolabelling followed by SDS-PAGE and autoradiography (Maizels et al, 1982; Philipp, Parkhouse and Ogilvie, 1980b).

The selective nature of the surface labelling technique amplifies those relatively few components exposed on the cuticle and allows a direct analysis of their antigenic nature by

conventional immunochemical techniques. The technique also allows for better molecular comparison between different stages of a given nematode as well as comparisons between species and facilitates studies on the nature of the nematode surface. The cuticle has, as a result of such studies, been found to be not only recognised by the host immune system, but also of a dynamic nature capable of alteration and development between moulting events. During their life cycle all nematodes undergo a series of moults in which they shed the external cuticle. Philipp et al (1980b) have shown that the cuticular surface of the parasite Trichinella spiralis expresses protein molecules which change qualitatively following the moulting process and quantitatively during the growth of the worms within one stage. Surface labelling studies have shown that the cuticle despite its dynamism has a relatively simple antigenic composition for each species examined. Philipp and Rumjanek (1984), found that the protein components of the cuticular surface are often strictly specific for a single stage of the parasitic life cycle. That cuticular surface proteins are a possible alternate source of potential protective antigen has been suggested by in vitro studies in which antibodies to surface antigens were shown to promote adherence and killing by granulocytes and macrophages (MacKenzie, Jungery, Taylor and Ogilvie, 1980).

Attempts to cultivate nematode parasitc stages in vitro have been made over the years. Silverman (1959) cited literature on the initial attempts to cultivate parasitic larval stages. He reported that Stoll (1940) had found that exsheathed and sterile

preparations of third stage H. contortus larvae underwent the first parasitic ecdysis when maintained in various balanced salt solutions, but apart from the development of a buccal cavity no growth or development followed, and death occurred soon after ecdysis. Silverman (1959) also reported on work done by Weinstein and Jones (1956) who managed to produce sexually mature adult Nippostrongylus muris in vitro, although the same media would not sustain Strongyloides spp. Silverman (1959), described his own success with cultivation of H. contortus and Ostertagia spp. The media used contained chicken embryo extract, autoclaved sheep liver extract, casein hydrolysate and sheep serum in a 2:2:2:1 ratio. In this medium using a roller tube technique, growth and metamorphosis of larval stages to the adult worm took 24 - 30 days but was accompanied by stunting of the worms. The media also required changing every 72 hours for the first 12 days.

Various advances in the in vitro cultivation of nematodes have been and are currently being made, though few published results are available. The media available vary from basic balanced salt solutions to very complex media. The conditions under which particular parasites will develop in vitro and the time scales relative to the course of development in vivo are all under investigation.

The advantages of in vitro cultivation are multifaceted and include the testing of anthelmintics and production of protective antigens with potential for control of helminthiasis (Leyland,

Ridley, Dick, Slonka and Zimmerman, 1971). One practical benefit of secreted antigens is that if they are released *in vivo* into the blood of parasitised hosts, they may serve as excellent diagnostic targets (Maizels and Selkirk, 1988).

A substantial proportion of research has been focussed on potentially protective antigenic fluids obtained by cultivation of exsheathed third stage (L_3) larvae to fourth stage (L_4) larvae *in vitro*. Substances released during the growth and development of parasitic stages of nematodes may also be important antigens for stimulating resistance to subsequent infection. The spectrum of molecules released, secreted or excreted by nematode parasites represents their major antigenic and functional challenge to the host. These functional molecules, some of which have been identified in excretory-secretory (ES) products of these nematodes, include invasive proteases, immunomodulatory components and enzymes such as acetylcholinesterase and superoxide dismutase (Maizels and Selkirk, 1988). Sinclair (1970) suggested that somatic antigens are not as important in stimulating immunity as ES products, which also include exsheathing fluids liberated by larvae as they moult. This thought has been supported by Maizels and Selkirk (1988), who reported that dead organisms or somatic extracts often fail to induce protection, whilst living irradiated parasites or soluble products secreted *in vitro* or released during moulting were efficacious.

The success of living vaccines implicates late L_3 and L_4 antigens as prime protective targets, but secreted molecules

particularly those performing essential functions may provide a more accessible part of the parasites life cycle (Maizels and Selkirk, 1988). There is some experimental evidence that ES antigens generate considerable protection and exsheathing fluids have been shown to contain several antigens (Sinclair, 1970).

Previous workers have stated that natural immunity to H. contortus is stimulated by antigens released during the fourth moult (Soulsby et al, 1959), and that the exsheathing fluids of infective H. contortus L_3 and fluid released during ecdysis of the fourth stage larvae are important in generating a protective immune response.

Soulsby, Sommerville and Stewart (1959) using the in vitro cultivation of H. contortus, suggested that exsheathing fluid played a role in the stimulation of the "self-cure" reaction. They found that larvae undergoing the second ecdysis in vitro secreted exsheathing fluid: when this fluid was tested against sera collected from animals undergoing the "self-cure", a reaction by the Ouchterlony agar diffusion precipitin technique was produced.

Soulsby et al (1959) also found serological evidence that, at the time of the "self-cure" reaction, antigens similar to those found in the second ecdysis exsheathing fluid were present. They concluded that, as third and fourth stage larvae as well as disintegrating adults were present at this time and although there was no evidence that exsheathing fluid is released at the time of the third ecdysis this suggested that exsheathing fluid

played a part in the "self-cure" reaction.

Antigens produced *in vitro* have been used for the vaccination of animal hosts against natural or experimental infection. Rickard and Bell (1971), reported successful vaccination of lambs against *Taenia ovis* with antigens produced from the *in vitro* cultivation of larval stages of *T. ovis* and *Taenia hydatigena*. Also Silverman, Poynter and Podger (1962) were able to obtain a high degree of protection in laboratory animals to *Dictyocaulus viviparus*, *T. colubriformis* and *Strongyloides papillosus* infections using L_3 antigens and antigens prepared from *in vitro* cultures of infective larvae which had reached the fourth stage of development (L_4).

In vitro cultivation coupled with surface labelling techniques confirmed that nematode parasites can change the composition of the surface in the absence of a moult (Philipp *et al*, 1980b). These workers found that the nematode *T. spiralis* expressed protein molecules on the cuticular surface that changed qualitatively following the moulting process and quantitatively during the growth of the parasite within one stage. By ^{125}I labelling of three stages of the parasite i.e. infective, new born (obtained by the *in vitro* culture of six-day old intestinal stages) and intestinal larvae and analysing the radiolabelled components by SDS-PAGE and autoradiography, they found that there were proteins specific for each stage. Analysis of the intestinal stage during its maturation (Days 1-6 after infection), indicated that modifications in the quantity of different proteins occurred over this period.

Further evidence for a dynamic cuticle was obtained by radiolabelling the surface of the infective and intestinal stages of T. spiralis with ^{125}I and culturing them in vitro. In this instance the surface labelling proteins were released into the culture fluids as detected by measurement of the released radioactivity expressed as a percentage of the total radioactivity present on the worms surface at time zero (Philipp et al, 1980b).

TECHNICAL APPROACHES

Detergents have been used widely in the solubilisation of nematode surface antigens. This has been possible primarily because of the polyanionic surface charge of the nematode and the fact that cationic detergents are available. One such detergent, cetytrimethylammonium bromide (CTAB) was found to be most efficient at stripping the surface of Nematospiroides dubius and the biochemical profile of the antigens removed was identical to that of surface antigens obtained by homogenisation (Pritchard, Crawford, Duce and Behnke, 1985). In addition, the detergent was shown to act in a non-invasive manner as electron microscopy failed to demonstrate any gross damage to the cuticle. Infection with CTAB treated muscle larvae of T. spiralis followed the normal course of development thus confirming that the detergent had no adverse effect on the parasite (Grencis et al, 1986).

This method of solubilising nematode surface proteins has its advantage in that sufficient quantities of relatively pure surface molecules can be produced for immunisation studies and

mice have been successfully immunised against T. spiralis by vaccination with CTAB stripped surface antigens. A substantial degree of protection against a challenge infection was shown in mice immunised with either total worm homogenates or CTAB antigen preparations (Grencis et al, 1986) as indicated by reduced adult worm burdens and the stunting and reduced fecundity of adult female worms.

An association between serum antibodies and parasitological parameters for resistance to infection has been found and both have been shown to be partially genetically determined for Cooperia spp (Keus, Kloosterman and van den Brink, 1981). Immunity to nematode infections is almost certainly not purely mediated by the humoral response and so cannot be solely measured by serum antibodies, but as serum antibody responses are easily assessed, it has been used as a parameter for large scale epidemiological work.

The Enzyme Linked Immunosorbent Assay (ELISA) technique has been used successfully to monitor the immune response to nematode infections by several workers. Keus et al (1981), found that the ELISA technique was useful for the detection of antibodies to trichostrongyloid worms in calves and that it could be applied using a variety of different soluble antigen preparations, required less labour than other techniques and had good reproducibility.

The use of fluorescent techniques (indirect immunofluorescence) has facilitated the study of serum antibodies

raised against various parasite antigens. For viable nematodes this labelling is only possible with those antigens that are surface related having limited use for internal antigens primarily because of the internal inaccessibility of the fluorescent label. However the internal structures of non-viable nematodes may be labelled by a method of freeze-fracture. As a technique it has proved to be useful for investigating surface labelling methods, solubilisation of surface proteins, post vaccination antibody levels and the titration of serum antibodies. It has also enabled the study of inter-stage surface profiles especially between sheathed/exsheathed third stage and fourth stage larvae and the response to the surface when exposed to various experimental sera.

The experimental work carried out in this thesis attempts to investigate the antigenic composition of H. contortus in its various stages with a view to preliminary studies on the potential of surface proteins from the L_3 parasitic stage as a source of antigen for vaccination of sheep against infection with the parasite.

CHAPTER TWO

MATERIALS AND METHODS

ANIMALS

In the work carried out in Glasgow, mature Scottish Blackface sheep and Suffok-Greyface and Finn-Dorset lambs were used. All were reared under worm-free conditions in an indoor environment which precluded acquisition of natural helminth infections. Their feed consisted of a ration of commercial sheep pellets and hay, and water was available ad lib. The Kenyan portion of the work involved the use of mature Dorper sheep which had been reared under a range management scheme prior to housing indoors under worm-free conditions.

STRAIN OF H. contortus

The strain of H. contortus used was one originally obtained from the Moredun Institute, Edinburgh and subsequently maintained in the Department of Veterinary Parasitology, Glasgow by passage in parasite-naive sheep.

PARASITOLOGICAL TECHNIQUES

FAECAL ANALYSIS

Faecal samples collected from the rectum were analysed by the method of Gordon and Whitlock (1939). In this, three grams of faeces are homogenised in 42 ml of water then passed through a coarse mesh sieve (Endecotts Ltd., London), with an aperture size of 250 microns, which allows only the passage of smaller size particles which includes nematode eggs (size range 70 - 165 microns). 15 ml of the filtrate is then poured into a test tube and spun at 2500 rpm for five minutes before the supernatant

is poured off and the remaining sediment mixed by rotary agitation (Whirlmixer, Scientific Industries Ltd.). The test tube is then filled with saturated sodium chloride, inverted six times to mix salt and faecal material before immediately withdrawing sufficient solution with a pipette to fill both chambers of a McMaster slide (Hawksley and Sons, London). The eggs floated by the salt solution are counted under a dissecting microscope and quantified by the method shown below:

3 g faeces in 42 ml of water = 1 g/15 ml

Volume under 1 square on slide = 0.15 ml

No. egg counted/square x 100 = No. eggs/g

Therefore the number of eggs counted in two squares multiplied by 50 equals the number of eggs per gram (epg) of faeces.

FAECAL LARVAL CULTURE

Four weeks post-infection a faecal collection bag lined with polythene was attached to a culture animal using a harness and the polythene bag lining changed daily. The total weight of faeces collected was determined together with the worm egg count of a random sample of faeces. The faeces were then evenly distributed into small plastic cartons which were loosely capped and incubated at 26°C. Cultures for first stage larvae (L_1) were incubated for two days, those for second stage (L_2) for three days and those for third stage larvae (L_3) for a minimum of ten days.

RECOVERY OF FIRST, SECOND AND THIRD STAGE LARVAE

On the appropriate day post-incubation for each larval stage, the culture cartons were removed from the incubator uncapped and filled with lukewarm water. After being left to stand for up to two hours, the faeces were sieved with a large household sieve and the filtrate collected in plastic beakers. The filtrate was vacuum filtered through a double layer of 8" milk filters (Maxa Filters, McCaskie, Stirling) using a Buchner apparatus. The milk filters were then placed on a Baermann apparatus which consisted of a large glass funnel filled with lukewarm water and closed at the stem by a length of rubber tubing and a clip. This was left in place for two to three hours for recovery of L_3 and overnight in the case of L_1 and L_2. This time interval ensured that the bulk of larvae present had either migrated (live larvae) or sunk (dead larvae) to the level of the rubber tubing and could then be drawn off into test tubes.

The larvae were spun for two minutes at 2500 rpm and the supernatant fluid poured off: they were then cleaned by flotation in 30% sucrose at 2500 rpm for five minutes with several subsequent washed in physiological saline.

The total numbers of larvae recovered was determined by a dilution technique whereby 10 ul of the larval concentrate was made up to 1000 ul in water and the number of larvae in 10 ul of this suspension determined. The total number of larvae was then calculated by first multiplying the number of larvae in 10 ul by ten and then multiplying by the volume (in ul) of larval concentrate.

RECOVERY OF FOURTH STAGE LARVAE

Sheep were orally infected with between 50,000 - 100,000 L_3 then killed four days later. The abomasum was removed, opened along its greater curvature, emptied of contents and gently washed under tap water. The abomasum was then cut into four long strips and suspended in physiological saline and incubated for one to two hours at 37°C. Larvae which migrated into the surrounding fluid were harvested every half hour and allowed to settle in collecting vessels at room temperature. After two hours incubation the abomasal strips were removed from the saline and discarded and the larvae were left to settle at room temperature overnight. The larvae were then pooled and counted, either individually if small numbers were present or, if numerous, by examination of diluted aliquots as described earlier.

RECOVERY OF ADULT PARASITES

For the recovery of adult parasites animals were infected orally with 10,000 L_3 and killed at least three weeks post infection. The abomasum was removed, opened along the greater curvature and the contents and mucosal washings pooled in a bucket to a total volume of either two or four litres. Two different methods of recovery were employed.

(a) Modified Baermann Technique

Abomasal contents were sieved through a double layer of gauze and the excess fluid squeezed out. The gauze was then attached to a plastic straw, suspended in lukewarm water and

incubated for two to three hours at 42°C. Viable worms moved out of the gauze into the water and were eventually collected from the bottom of the beaker.

(b) Manual Method

Small aliquots of the abomasal contents were poured onto a plastic tray and the adult parasites were then picked out individually.

NECROPSY PROCEDURE

Animals were killed using a captive bolt and immediately exsanguinated. On opening the carcass, the entire gut was removed from the body cavity and the pyloric sphincter - duodenal junction was immediately ligatured to prevent mixing or loss of contents. The abomasum and omasum were carefully separated from the rest of the gastrointestinal tract and removed. The omasum was detached from the abomasum which was then processed with or without the contents for the recovery of adult worms or L_4 respectively.

For the estimation of total adult worm burdens the abomasal contents plus the mucosal washings were made up to either two or four litres. From this total volume duplicate 200 ml well mixed samples were removed and placed into containers in which 2-3 ml of Iodine had been added. After thorough mixing 2 or 4 ml aliquots to a total of 40 ml were then removed from the 200 ml samples, placed on a petri dish and the total number of parasites counted. The total parasite burdens were determined by multiplying the number of parasites in 40 ml by either 50 or 100

for total contents of two litres or four litres respectively.

HAEMATOLOGICAL TECHNIQUES

COLLECTION AND STORAGE OF SAMPLES

Whole blood, and blood samples for serum and plasma were collected from the right jugular vein of infected animals into suitable vacutainer tubes (Becton - Dickinson, Rutherford, New Jersey, U.S.A.). Serum and plasma samples were stored at -20^{o}C until required. Whole blood samples in EDTA were processed immediately.

PACKED CELL VOLUMES (PCVs)

Whole blood was collected by jugular venepuncture into vacutainer tubes containing EDTA and PCVs determined by the microhaematocrit method. After thorough mixing by gentle inversion of the samples, blood was drawn up into duplicate capillary tubes (Gelman and Hawksley, England) and sealed at one end using "Crystaseal" (Hawksley and Sons, Lancing, Sussex). The samples were then centrifuged for six minutes in a microhaematocrit centrifuge (Gelman - Hawksley, England) and thereafter the percentage PCV was read using a microhaematocrit reader.

SERUM

Whole blood was drawn into additive-free silicone coated vacutainer tubes by jugular venepuncture. The caps were then removed from the tubes and the blood allowed to clot at room temperature overnight. The clotted blood was then removed from

the tubes and the raw sera spun at 2500 rpm for 20 minutes. The resultant clear serum samples were aliquoted into plastic tubes (Alpha Laboratories, Hampshire, England).

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

ANTIGENS

The antigens used in this technique were larval and adult somatic antigens and surface proteins extracted from third stage larave (see page 73). Somatic antigens were prepared from exsheathed third stage larvae and adult H. contortus by sonication in an MSE Soniprep 150 at 18 um amplitude. The sonication was carried out in ice cold PBS (pH 7.2) and the container in which the vessel containing the parasites was placed, cooled by ice to reduce the heating caused by sonication. Ten or 11 one minute sonicating bursts interspersed with one minute rest intervals were used and an aliquot of the larval suspension observed at intervals for degree of disintegration. After sonication the larval suspension was subjected to ultracentrifugation at 13,000 g at $4^{o}C$, after which the supernatant was stored at $-80^{o}C$ until needed. Protein estimation of the supernatant fluid was determined by the Bradford method (Bradford, 1976).

ELISA REAGENTS

1. Coating Buffer pH 9.6. Stock solutions of 0.2M sodium carbonate and sodium bicarbonate in distilled water.

   A. 21.2 g/litre Na_2CO_3

   B. 16.8 g/litre $NaHCO_3$

   Working solution

   8.0 ml Solution A

   17.0 ml Solution B

   75 ml Distilled water

2. Phosphate Buffered Saline (PBS pH 7.2).

   8.50 g NaCl

   0.32 g NaH_2PO_4

   1.10 g Na_2HPO_4

   Made up to 1 litre in distilled water

3. Washing Buffer.

   As PBS with the inclusion of 0.05% Tween 20

4. Blocking Buffer.

   As PBS with the inclusion of 4% skimmed milk

5. Diluting Buffer (Serum).

   As PBS with the inclusion of 2% skimmed milk

6. Diluting Buffer (Conjugate).

   As PBS with the inclusion of 0.05% skimmed milk

7. Substrate Solution.

Solution A. Chromogen Solution

Phosphate Citrate Buffer pH 5.0

5.11 g $Na_3C_6H_5O_7.H_2O$

9.15 g Na_2HPO_4

Made up to 1 litre in distilled water

Add 0.4 g Ortho-phenylene-diamine (OPD) and dissolve

Solution B. Substrate

30% H_2O_2

Working Solution

10.0 ml Solution A

0.004 ml Solution B

ELISA PROCEDURE

96-well flat-bottomed PVC microtitre plates (Titertek Flow Laboratories) were coated with 5 ug of the relevant antigen diluted in 1000 ul coating buffer, then incubated at 37°C for two hours or left overnight at 4°C. The plates were then washed three times in washing buffer using a Titertek (R) microplate washer 120 (Flow Laboratories) and then dried by shaking off excess buffer.

100 ul of the blocking buffer was then added to each well and the plates incubated at 37°C for 30 minutes. The plates were then washed three times, dried as before, then the diluting buffer containing the test sera samples at a 1:100 dilution added to the plates at 100 ul/well, but excluding the first column. All serum samples were prepared in duplicate. The plates were

then reincubated for a further 30 minutes at 37°C, washed three time in washing buffer and dried.

The Conjugate buffer was prepared using anti-Sheep IgG horseradish peroxidase (Sigma Laboratories) in PBS containing 0.05% skimmed milk at a 1:1000 dilution and 100 ul was placed in each well on the plates again excluding the first column. After incubation at 37°C for 30 minutes, the plate was washed three times in washing buffer then dried as before.

The Substrate buffer working solution was prepared and added at 100 ul/well to the plates which were then incubated at 37°C or at room temperature for ten to 15 minutes, after which the reaction was halted by the addition of 15% H_2SO_4 at 100 ul/well.

The optical densities of the colour reaction in each well was measured in a Titertek Multiscan ELISA reader at a wavelength of 492 nm. Four positive H. contortus antisera and four negative control sera were included in every plate. The first column on each plate acted as a blank.

ANALYSIS OF ELISA RESULTS

Means were determined for the absorbency values obtained from all sera examined by the ELISA technique. The means obtained for the four negative sera in each plate were used in the determination of the cut-off point in each experiment. This was calculated as being twice the value of the mean absorbency of the negative sera. Absorbency values above this confidence interval were interpreted as positive and those values falling below it as negative.

BIOCHEMICAL AND CULTURE TECHNIQUES

In Vitro CULTIVATION

Two different balanced salt solutions Mapes II and Phosphate buffered saline (PBS) to which 300 ug/ml Gentamycin Sulphate BP (50 mg/ml) and 5 ug/ml Fungizone-Amphotericin B (250 ug/ml - Flow Laboratories, Irvine, Scotland) were added, were used as culture media for exsheathed third stage H. contortus larvae as described by Mapes (1969) and Boisvenue, Emmick and Galloway (1977) respectively.

Larvae were exsheathed using [redacted] Milton in 0.85% saline prior to resuspension under sterile conditions into 4 ml of either Mapes II solution (pH 2.7) or PBS (pH 7.2) at a concentration of 5000 larvae/ml. The sterilely prepared culture tubes were then incubated on a rotator at 37°C for several days and larvae examined daily for any developmental changes.

Two other media were used for the cultivation of exsheathed larvae. They were high glucose Dulbecco Modified Eagle medium (Gibco BRL, UK) composed of L-glutamine and 4500 mg/l D-Glucose and Chicken embryo extract medium. Larvae for culture were prepared in the same manner as that described above.

Combinations of media were also used for culture. Larvae cultured in Mapes II solution for two days were gently spun down by centrifugation at 1000 rpm for two minutes and the Mapes II solution drawn off. The larvae were then resuspended under sterile conditions in 4 ml of either Dulbecco's medium or Chicken embryo extract medium then reincubated at 37°C for several days.

The cultures were observed daily for any development.

HOMOGENISATION OF PARASITES

Biotinylated and non-biotinylated intact larvae and adult parasites were washed in 0.85% saline then homogenised using either a Jencon's 0.1 ml glass homogeniser or a large Tri-Mix-homogeniser. The buffer (worm buffer) in which the parasites were homogenised comprised either 2.0 mM EDTA pH 8.0 containing the protease inhibitors N-alpha-p-tosyl-L-lysine chloromethyl ketone (TLCK) (50 ug/ml), N-I-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (25 ug/ml), phenyl methylsulphonylfluoride (PMSF) (1 mM), 1,10 phenanthroline (2 mM) and antipain (4.0 uM), or TRIS POISONS which in addition to the above reagents contained 2.0 mM Tris HCl pH 8.3. 1 ml of the worm homogenisation buffer was added to approximately one million larvae.

ELECTROPHORESIS AND ELECTROBLOTTING

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out as described by Laemmli (1970) on slab gels consisting of 10% running gel and 4% stacking gel. Homogenised samples were resuspended in sample buffer (0.5 M Tris, 3% SDS, 0.01 M EDTA pH 7.0, 10% glycerol), and then boiled for two minutes before being loaded on to the gel. Electrophoresis was carried out overnight at 50 volts, the voltage increased the next morning to 80 volts and the gel left to run until the tracking buffer reached the bottom of the gel plates when the current was switched off.

When mini-gel electrophoreses were carried out the voltage

used was 100 - 120 volts and electrophoresis normally took an average of two hours.

Electrophoretic transfer of the proteins from the polyacrylamide gel on to nitrocellulose acetate membrane (Amersham, Hybond C) was performed according to the method of Towbin, Stacklin and Gordon (1979), with a transfer time of three hours at 60 volts and at $4^{o}C$ (100 volts for one hour for mini-gels). The nitrocellulose membrane was placed over the polyacrylamide gel and the two sandwiched between layers of sponge lined with 3 MM paper. This gel "sandwich" was prepared under Tris glycine blotting buffer (0.028 M Tris, 0.19 M Glycine and 200 ml methanol made up to four litres with distilled water) in order that air bubbles be excluded. Both the gel and the nitrocellulose paper were cut at one corner to mark the side bearing the molecular weight markers.

In order to determine whether electrophoretic transfer had been accomplished successfully, the newly blotted nitrocellulose membranes were stained with a non-permanent stain comprising 0.3% Ponceau S in 5% TCA. The stain was removed by several washes in cold water.

Binding sites on blotted nitrocellulose membranes were then blocked by incubation at room temperature in 1% Tween 20 in Tris buffered saline (TBS - 10 mM Tris, 0.9% NaCl, pH 74) for at least two hours. Two methods were employed in the development of nitrocellulose blots for the detection of the protein bands separated by electrophoresis.

(a) Radioactive Iodine 125, (^{125}I) linked to Streptavidin (Amersham) was added to the blotted nitrocellulose membrane at 10 c.p.s./ul TBS and incubated on a rocking platform at room temperature for one hour. The blot was washed five times in the buffer to remove any unbound streptavidin, then exposed to radiographic film (Hyperfilm, Amersham, U.K.). The film was stored for up to one week at -80^{o}C before being developed.

(b) Blots were incubated in streptavidin linked peroxidase at a dilution of 1:200 for 90 - 120 minutes, after which the blots were developed. The developing solution contained 20 mg chloronaphthol in 20 ml methanol, 80 ml Tris saline and 100 ul 30% hydrogen peroxide. Protein bands after staining appeared bluish-purple. The developing reaction was stopped by placing the blots in cold distilled water; for permanent staining they were placed in 20 mM EDTA made up in PBS (pH 7.4).

STAINING OF GELS

After running overnight the polyacrylamide gels were stained in a 0.03% solution of Coomassie blue in 30% methanol and 10% acetic acid for one hour, then destained until the protein bands were visible in a solution of 30% methanol and 10% acetic acid. The destained gels were then dried down using a Biorad Model 483 Slab dryer (60^{o}C for two hours).

An alternative silver nitrate staining method was also used. In this polyacrylamide gels were incubated for 1 - 24 hours in 50% methanol then washed three times (ten minutes each time) in distilled water. The gels were incubated in silver stain (1 g

$AgNO_3$, 35 ml distilled H_2O, 3.5 ml aqueous NH_3, 25 ml 0.36% NaOH and 60 ml methanol prepared by adding the constituents in the given order for one to two hours, washed for 30 minutes in three changes of distilled water then developed for five to 30 minutes in developing solution (2.5 ml 1% sodium citrate, 250 ul 38% formaldehyde made up to 500 ml with distilled water). The reaction was stopped by placing the gels in 50% methanol.

BIOTINYLATION OF PARASITES

Larvae were prepared by flotation through 30% sucrose by centrifugation at 2500 rpm for ten minutes, followed by successive washes in 0.85% sodium chloride. Third stage larvae were then exsheathed by incubation in 1% sodium hypochlorite (Milton Solution, Richardson-Vicks Ltd., Egham, Surrey) for six minutes, then washed five times in 0.85% sodium chloride. The parasites were resuspended, after cleaning, in 0.85% saline at a concentration of 300 adults or 30,000 larvae per ml, then incubated at room temperature for one hour in D-biotin-N-hydroxy-succinamide ester (NHS-biotin) at a concentration of 0.3 mg/ml. Whole parasite biotinylation was terminated by washing extensively by centrifugation in 0.85% saline, whilst that of homogenates of the parasites was terminated by incubation in a final concentration of 10 mM ethanolamine and 0.1 M Tris (pH 7.2).

DETERGENT STRIPPING OF CUTICULAR PROTEINS

The cationic detergent cetyltrimethylammonium bromide - CTAB was used to strip the surface proteins from larval and adult parasites, by a method modified from Pritchard, McKean and Rogan (1988). 0.25% CTAB solution was added to 50 - 100,000 larvae or 100 adult parasites per ml 0.85% saline and incubated for six hours at 37°C. The CTAB extract was then separated from the parasites by centrifugation at 2500 rpm for ten minutes and the larvae washed several times in 0.85% saline. Alternatively the larvae/adults were separated from the CTAB solution by filtering through Whatman Filter paper. The CTAB preparation was stored at -20°C until required.

DETERMINATION OF SURFACE LABELLING AND REMOVAL OF LABELLED PROTEINS

Two methods were used in determining the effectiveness of the surface labelling process, one of which was also useful in determining the ability of the detergent CTAB to strip surface proteins.

(a) Detergent Action

CTAB was used to remove the cuticular proteins of exsheathed and biotinylated third stage larvae. After incubation in NHS-biotin (see page 68), larvae were incubated in CTAB as described above. The suspension was centrifuged at 13,000 g for five minutes to pellet the larvae and the CTAB stripped proteins were precipitated out of solution by the addition of 50% trichloroacetic acid (TCA) to a final concentration of 25%,

placed on ice for 30 minutes then separated from the supernatant by centrifugation at 13,000 g for five minutes. The CTAB treated parasites were then homogenised in worm buffer as described previously, centrifuged for five minutes at 13,000 g and the supernatant decanted and TCA precipitated on ice for 30 minutes. The pellet remaining after centrifugation was boiled for two minutes in 1% SDS, 0.125 M Tris pH 6.8 (ST buffer), centrifuged for five minutes at 13,000 g and the supernatant decanted and TCA precipitated as before.

The TCA precipitates from the CTAB, homogenised worm and ST buffered extracts were resuspended in 10 mM phosphate buffer pH 7.2 and SDS sample buffer with 2-mercaptoethanol (2-ME). Saturated Tris was added to increase the pH (a low pH was indicated by a yellow colour on addition of sample buffer). The final insoluble pellet was boiled for two minutes in 100 ul ST buffer and 10ul 2-ME and the resultant suspension prepared for loading as described for the three TCA precipitated supernatants. Prior to loading on to a 10% polyacrylamide gel, all four samples were boiled for two minutes. The prepared samples were run with high molecular weight markers, a surface biotinylated homogenate and an untreated somatic antigen preparation of the larvae. Table 1 shows a summary of the procedure.

Table 1

Summary of Determination of Surface Labelling

Exsheathed *H. contortus* L_3 resuspended in PBS pH 7.4 and incubated in NHS-biotin (0.3 mg/ml) one hour room temperature then washed five times in PBS pH 7.4.

Larvae resuspended in 0.25% CTAB, incubated for six hours 37°C then centrifuged at 13,000 g for five minutes - SN1.

Larvae washed five times in PBS pH 7.4 then homogenised in Worm buffer with proteinase inhibitors and the homogenate centrifuged at 13,000 g five minutes - SN2.

Pellet resuspended in ST buffer and boiled for two minutes then centrifuged for five minutes at 13,000 g - SN3. Insoluble pellet retained - P4.

TCA is added (to final concentration of 25%) to SN1, SN2 and SN3, incubated on ice for 30 minutes then SN precipitates centrifuged at 13,000 g for five minutes, the supernatant decanted and pellets retained - P1, P2, P3.

P1, P2, P3, P4 resuspended in 10 mM $NaPO_4$ buffer, run on 10% SDS gels, blotted, blocked and probed with ^{125}I-streptavidin or streptavidin-peroxidase.

SN - Supernatant P - Pellet

(b) Fluorescent Microscopy

Surface biotinylated third stage larvae were incubated at room temperature in 1:25 or 1:50 dilution of 200 ug/ml Streptavidin linked Fluorescein (FITC) (Sigma Laboratories) for 30 minutes, then washed several times in either 0.85% saline or PBS. After resuspenion in PBS a drop containing larvae was placed on a slide and dried over gentle heat. After immersion in Evans Blue dye for two minutes to diminish autofluorescence, the larvae were observed under ultra violet (UV) light.

An alternative procedure was also used whereby the larvae were kept on ice during the incubation period and the heat-drying and Evans Blue staining steps omitted. The purpose of the lower temperatures was to immobilise the larvae for photography.

COLLAGENASE DIGESTION

200 ul duplicate samples of a homogenate derived from 2000 biotinylated third stage larvae were resuspended in 200 ul ST buffer (0.125 M Tris pH 6.8, 1% SDS) with 10 ul mercaptoethanol/100 ul ST buffer and boiled for two minutes. The suspensions were then precipitated in a final volume of 25% TCA on ice for 30 minutes then centrifuged at 13,000 g for five minutes. The supernatants were discarded and the pellets resuspended in 600 ul of collagenase buffer (0.15 M NaCl, 50 mM Tris, 5 mM $CaCl_2$) containing 10 mM TPCK and 0.2 mM PMSF proteinase inhibitors. This was done to ensure that any digestion was specifically due to the presence of collagenase and not as a result of digestion by other proteases (Sage, Woodbury

and Bornstein, 1979). 150 units of collagenase (Sigma Type VII) were added (100 ug/ml) to one of the samples whilst an equivalent volume of collagenase buffer was added to the other and incubated for 16 hours at 37°C. The samples were then TCA precipitated on ice for 30 minutes, centrifuged and the pellet prepared for SDS-PAGE as described previously. Table 2 shows a summary of the procedure.

The samples were electrophoresed and electroblotted as described previously and after incubation in blocking buffer, incubated with ^{125}I-streptavidin for one hour. After several washes in Tris saline, the blot was autoradiographed on to Hyperfilm (Amersham, U.K.) at -80°C. Alternatively, the blotted nitrocellulose was incubated in a 1:200 dilution of 1 mg/ml streptavidin linked peroxidase (Sigma, U.K.) for two hours at room temperature after which the blots were stained as described previously (page 68) with chloronaphthol in methanol, Tris saline and H_2O_2.

CTAB EXTRACTION AND PROTEIN ESTIMATION

Exsheathed third stage larvae obtained from faecal culture and fourth stage larvae from Mapes II solution in vitro cultures were incubated in 0.25% CTAB detergent for six hours at 37°C then centrifuged at 2,500 rpm for five minutes to separate the larvae from the CTAB soluble proteins. The CTAB extract was then dialysed for three days at 4°C in phosphate buffered saline (PBS) pH 7.4 to remove the detergent. The dialysis was carried out using dialysis tubing (Visking size 3-20/32", Medicell

Table 2

Summary of Collagenase Digestion

200 ul ST buffer with 10 ul 2-mercaptoethanol/100 ul ST buffer added to 2 x 20/30 ul aliquots of 2,000 biotinylated L_3.

Suspension is boiled for two minutes.

TCA is added to final concentration of 25% and the suspension incubated on ice for 30 minutes.

Suspension is centrifuged at 13,000 g for five minutes, the supernatants discarded and the pellets resuspended in 600 ul collagenase buffer with proteinase inhibitors.

150 units collagenase added to one resuspended pellet and an equal volume of collagenase buffer to the other suspension.

Both suspensions incubated for 16 hours at $37^{o}C$.

TCA added to the suspensions to a final concentration of 25% incubated on ice for 30 minutes.

Suspensions centrifuged at 13,000 g for five minutes.

Samples run on 10% SDS gel, blotted, blocked and exposed to streptavidin-peroxidase or ^{125}-streptavidin.

International Ltd., London, England) and the PBS was changed every 24 hours.

At the end of the dialysis period the PBS containing the stripped surface molecules was subjected to protein estimation by the Bradford method (Bradford, 1976). This method involves the binding of Coomassie Brilliant Blue G-250 to protein. A standard graph of absorbence at 595 nm against a known concentration of Bovine Serum Albumin (BSA) standard was plotted after the addition of Coomassie reagent (Coomassie Protein Assay reagent, Pierce, Rockford, Illinois, U.S.A.). The absorbence of known volumes of the CTAB extracts was measured and then read off the standard curve. The total protein content of the unknown sample was then estimated by a multiplication factor determined by the actual volume of unknown sample used. All absorbencies were read off a Perkin-Elmer 550A UV-VIS spectrophotometer.

Pure CTAB detergent was also dialysed and the absorbence value obtained subtracted from that obtained for the surface extract. This was done because it was found that some CTAB detergent remained in solution even after dialysis and generated a colour reaction with the Bradford reagent, resulting in a higher protein estimation.

An alternate method of protein estimation was carried out in an attempt to eliminate the error caused by the presence of undialysed CTAB in surface protein extracts. This was the Lowry method (Lowry, 1951).

A standard curve was also plotted of known concentrations of BSA against their absorbencies at 750 nm, using sodium carbonate,

copper sulphate, sodium tartrate and Folin-Ciocalteau's reagent as the reagents for colour development.

PREPARATION OF CTAB EXTRACTED SURFACE PROTEINS FOR ANIMAL IMMUNISATION EXPERIMENTS

The CTAB extracted and dialysed proteins were prepared for injection into experimental sheep, emulsified with either Freunds Complete or Freunds Incomplete Adjuvants (Sigma Laboratories, U.K.) in a 6:4 or 1:1 V/V ration. The volume of extracted proteins containing the appropriate concentration of proteins required for vaccination was added to the appropriate volume of adjuvant and the two emulsified by vortex action until thick and milky white. The preparation was deemed ready for immediate use when a small amount of emulsion dropped into a beaker of cold water sank briefly as a discrete drop then floated without any dispersion.

IMMUNOFLUORESCENCE - INDIRECT FLUORESCENCE ASSAY (IFA)

Exsheathed third stage larvae were incubated in various sera i.e. from parasite-naive sheep (control sera) and sera obtained from the various animal experiments (test sera) for 30 minutes at room temperature. Serum dilutions used initially were 1:1, 1:2, 1:10, 1:25 and 1:50. After several washes in physiological saline or PBS pH 7.4, the larvae were then incubated in 1:25 or 1:50 dilutions of FITC antibody (Fluoroscein isothiocyanate (FITC) - anti-sheep/goat IgG (Scottish Antibody Production Unit, Lanarkshire, Scotland) for a further 30 minutes at room

temperature. The larvae were again washed several times in saline, then a drop of the larvae placed on multispot microscope slides (C.A. Hendley [Essex] Ltd., England) in order of increasing dilution.

The slides were dried over a bunsen flame then incubated in Evans Blue dye (in PBS) for two minutes to quench autofluorescence. The slides were then removed, placed on a microscope stage and the larvae observed through a 580 FITC filter under UV light. A summary of the procedure is shown in Table 3.

For photography, the IFA tests were carried out on ice and at $4^{o}C$ as this had the effect of reducing the mobility of the larvae. The heat treatment and Evans Blue staining steps were omitted as this tended to cause disruption of the parasites.

WESTERN BLOTTING

Electrophoresis and electroblotting of 50 - 70 ul aliquots of L_3 somatic antigen were carried out as described previously. Each polyacrylamide gel run carried ten such aliquots and an aliquot of high molecular weight markers. After the nitrocellulose membranes were blotted, they were checked for complete electrophoretic transfer of the proteins by staining with Ponceau S. The ten individual tracks of separated protein bands visible under the Ponceau S stain were then separated from one another by cutting using a sharp blade. The stained strips were then washed under warm water to remove the stain. The nitrocellulose strips were then incubated on a rocker in a

Table 3

Summary of Indirect Immunofluorescence Assay (IFA)

2,000 exsheathed L_3 incubated in test sera (1:25 dilution) 30 minutes on ice.

Larvae washed five times in PBS pH 7.4.

Larvae incubated in second antibody (FITC anti-sheep IgG 1:25 dilution) for 30 minutes on ice.

Larvae washed five times in PBS pH 7.4.

10 ul aliquots of larvae placed on microscope slide and observed under UV light.

blocking solution (3% skimmed milk in PBS, 1% Tween 20) for four hours at room temperature. The blots were then washed several times in TBS. The stained marker strip was retained for the determination of the molecular weights of antigens visualised by the colour reaction.

The test sera were prepared at a 1:50 dilution in a solution of 5% skimmed milk in PBS (pH 7.4) on the day of use and stored at $4^{o}C$.

The nitrocellulose blots were incubated overnight in the test sera, each blotted strip in a different serum sample. The blots were then washed in TBS to remove any unbound serum antibody then reincubated collectively in a 1:100 dilution of second antibody (anti-sheep IgG peroxidase conjugate - Sigma) for two hours at room temperature. After thorough washing in TBS, the blots were incubated in a developer/staining solution (20 mg chloronaphthol, 20 ml methanol, 80 ml TBS) to which 100 ul hydrogen peroxide was added. The colour reaction was halted after approximately eight minutes by placing the blots in cold water.

PREPARATION OF HYPERIMMUNE SERA

Although it has been well documented that sheep can be rendered immune to infection with Haemonchus contortus by several exposures to the parasite, various attempts to achieve a protective immune response in animals under the age of seven months have met with little success. This has been attributed to immune unresponsiveness of young lambs.

The following method of multiple infection of sheep of different ages was applied in an attempt to produce serum for use in subsequent experiments. The methods used and the parameters monitored are described under two headings, the first dealing with mature sheep and the second with sheep aged 2 - 6 months.

MATURE SHEEP

One 9-month old female Scottish Blackface sheep which had been maintained worm-free was initially infected orally with 10,000 H. contortus third stage larvae (L_3); this was followed by a second infection with the same number of larvae eight weeks later. The sheep was then reinfected with 20,000 larvae six weeks later and a fourth infection with 50,000 larvae was given after a further six weeks. A final infection with 50,000 larvae was given 13 weeks later and the animal sacrificed 14 weeks after this final infection. Faecal and blood samples were taken weekly throughout the infection period.

The PCVs and faecal egg counts for each infection are shown graphically in Figure 1.

Packed Cell Volumes (PCVs)

The initial PCV values decreased in the period between the first and second infections but thereafter remained at a constant level. Values recorded after the fifth infection were higher than those recorded after the fourth. The lowest value obtained during the whole period was 28%.

FIGURE 1.

PCV's and faecal egg counts (e.p.g) in a 9-month old sheep after serial infections with normal H. contortus L3

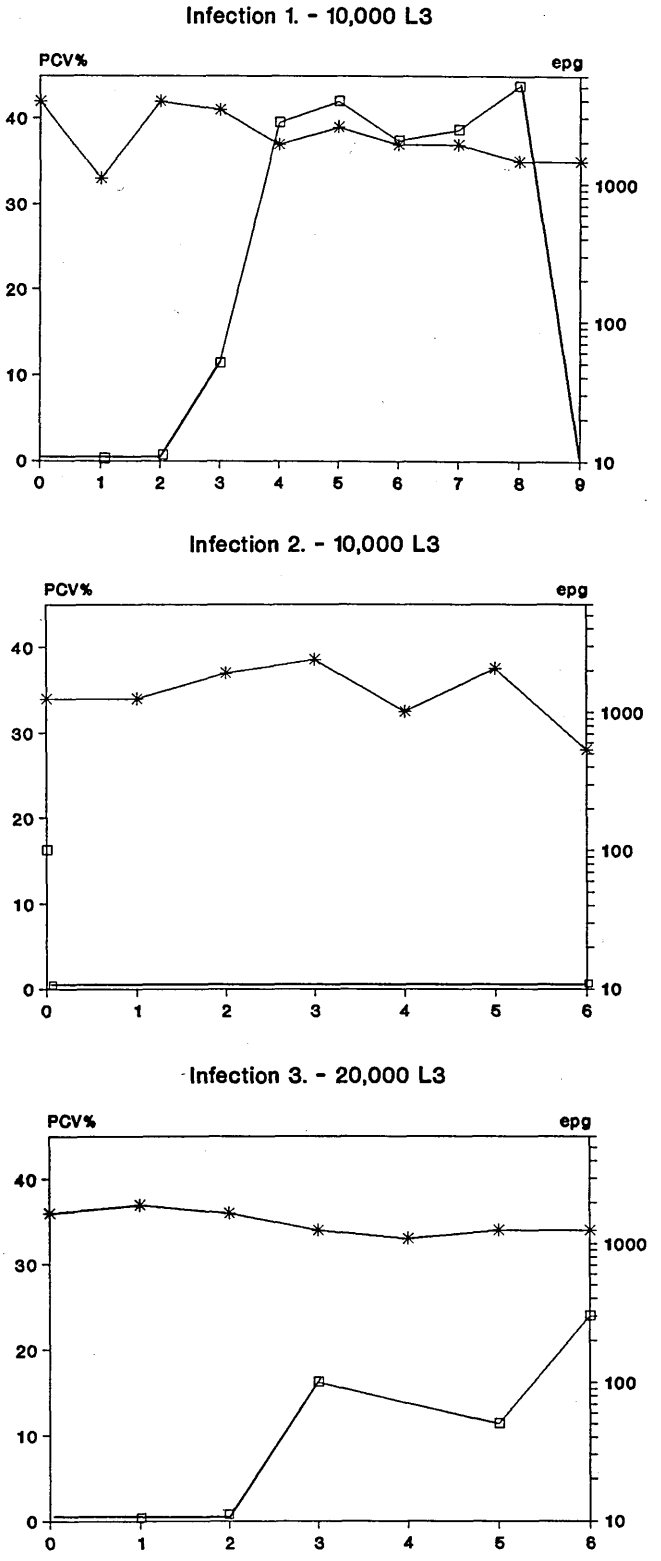
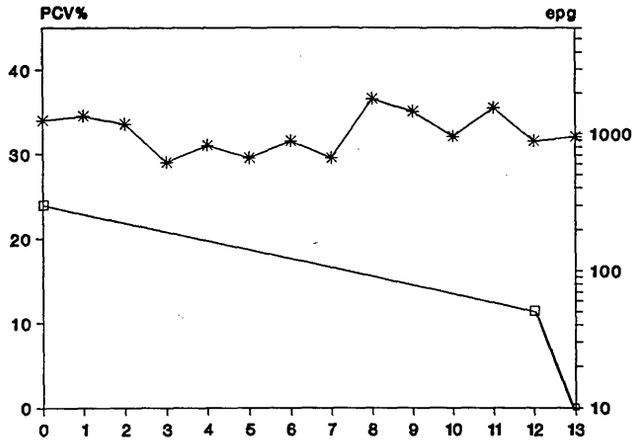
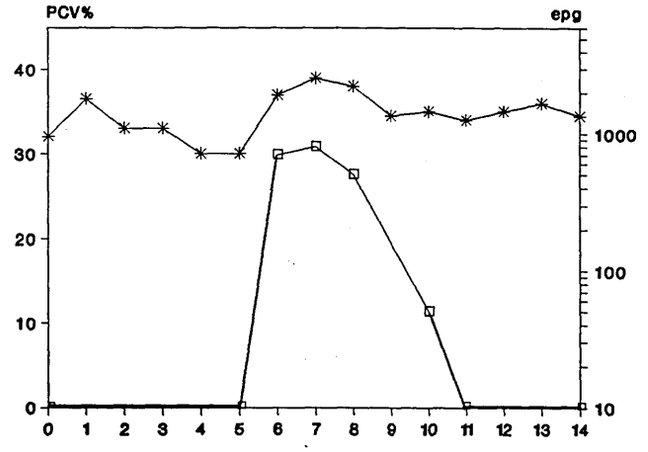


FIGURE 1. Cont'd

Infection 4. - 50,000 L3



Infection 5. - 50,000 L3



Weeks

Packed cell volumes

Faecal egg counts

Faecal Egg Counts

High positive counts were only obtained after the first infection. No eggs could be detected in faeces during the second infection and although there were positive faecal egg counts during the subsequent three infections these counts were below 1,000 e.p.g. By the termination of the experiment egg counts were negative.

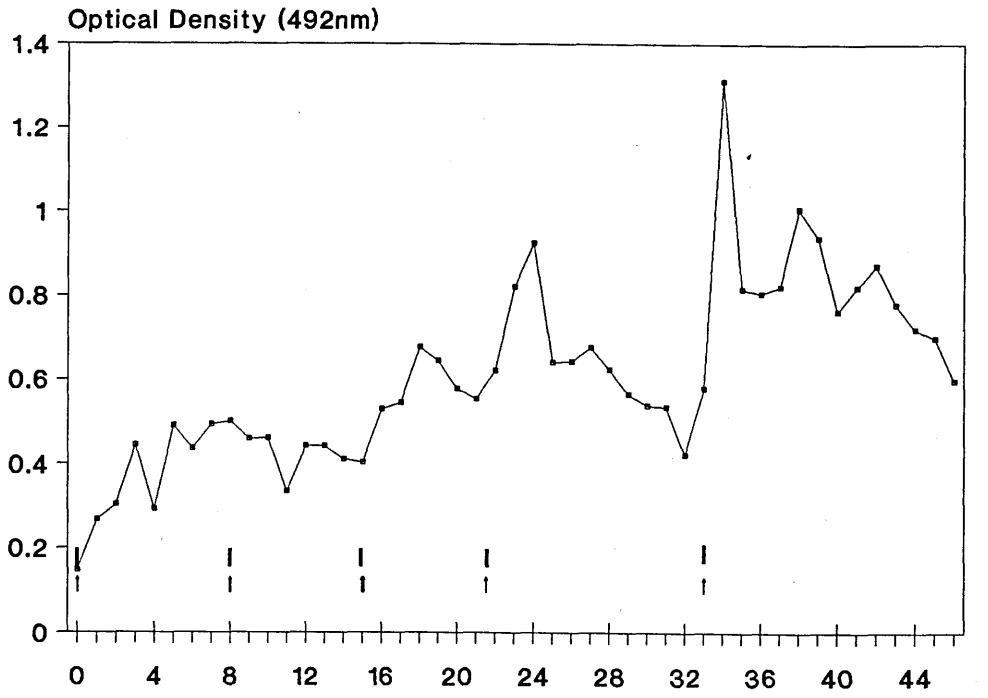
ELISA Results

An ELISA was performed using L_3 antigen and the optical densities (a measure of the serum antibody levels) of the serum samples taken weekly are shown in Figure 2. It can be seen that antibody levels were higher at the termination of the experimental period than at the outset and that following most infections there was a rise in antibody titre which was most marked after the fourth and fifth infections. Following the first, third and fourth infections peak antibody levels were reached within three weeks while after the fifth infection the peak was more marked and was reached within two weeks. In contrast after the second infection antibody titres showed little change. After peak values were achieved, antibody levels generally declined and fell to below the level recorded at the time of infection.

Based on these parasitological and antibody results it appeared that the animal had been successfully rendered immune to infection with *H. contortus* by the multiple infection regime employed.

FIGURE 2.

EILSA O.D's of sera after serial infections with normal H. contortus L3 in a 9-month old sheep using L3 somatic and surface antigens



I - Infection

Cut-off point - 0.394

SHEEP AGED 2 - 6 MONTHS

Two 6-month old male Suffolk-Greyface cross sheep and two 2-month old Dorset lambs (one male and one female) were used. Both groups of animals were worm-free at the start of the infection period. Faecal and blood samples were taken weekly during the course of infection.

All four sheep were infected initially and for all subsequent infections with oral doses of 10,000 infective L_3. Following the initial infection, the animals were each infected at different time intervals as follows.

The male Dorset lamb (Y13) was treated with the anthelmintic ivermectin (Oramec$^{(R)}$, MSD AGVET) at 0.2 mg/kg five weeks after the initial infection, then reinfected three weeks later. This infection was terminated by ivermectin treatment five weeks post infection and the animal rested before being infected for the third time two months later.

The female Dorset lamb (Y14) was reinfected in the absence of anthelmintic treatment 21 weeks after the first infection.

One Suffolk-Greyface sheep (Y9) was treated with ivermectin seven weeks after the first infection, then reinfected after one week. A third infection was superimposed 20 weeks later.

The second Suffolk-Greyface sheep (Y12) was reinfected in the absence of anthelmintic treatment after five weeks. This was followed by a third infection 12 weeks later, and a fourth after 17 weeks. This final infection was terminated after five weeks when the animal was slaughtered.

All animals were sacrificed at the end of the experimental period.

The PCVs and faecal egg counts for each animal during the experimental period are shown in Figures 3 - 6.

Packed Cell Volumes (PCVs)

Although the PCVs of all animals dropped very rapidly following the initial infection and lowest values were reached within 3 - 4 weeks, generally the PCVs did not fall below 25% after any infection. Exceptions were the PCVs of one animal from each age group which did fall to below 25%: this decline was transient in the 6-month old animal (Y12) but it was more marked and sustained in the 2-month old lamb (Y13).

After the initial decline in PCV, the values remained generally within the normal range averaging around 35%. Y13 (2-month old) showed the greatest variation from the other three animals in that PCV fell very quickly to below normal levels after every infection.

Faecal Egg Counts

All animals apart from Y12 (6-month old) achieved their highest counts after the first infection which reached over 25,000 e.p.g. in Y9 (6-month old) and Y13 (2-month old), within three and six weeks of infection. In contrast, Y12 (6-month old) achieved its highest egg count of 22,500 e.p.g. after the third infection.

In general the egg counts of all animals showed a decline within three weeks of achieving their peak values; an exception

FIGURE 3.
PCV's and faecal egg counts (e.p.g) after serial infections with 10,000 normal H. contortus L3 in a 6-month old lamb

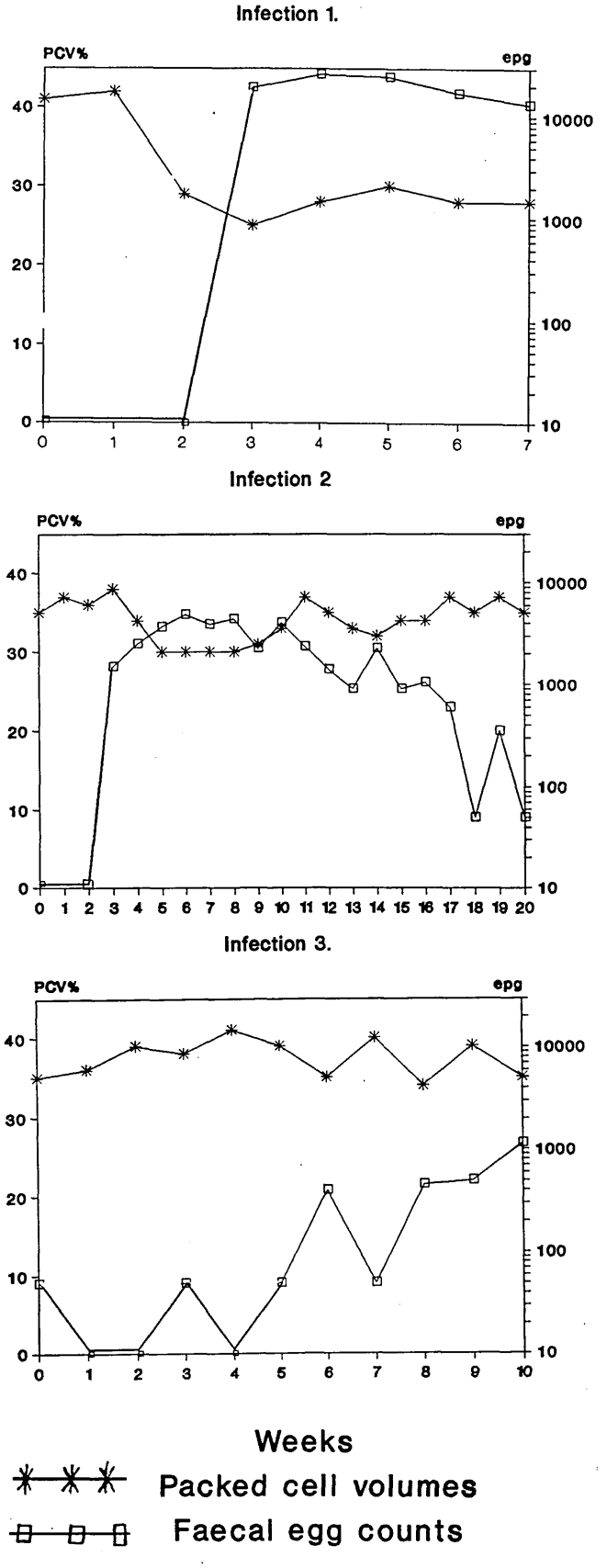


FIGURE 4.
PCV's and faecal egg counts (e.p.g) after serial infections with 10,000 normal H. contortus L3 in a 6-month old lamb

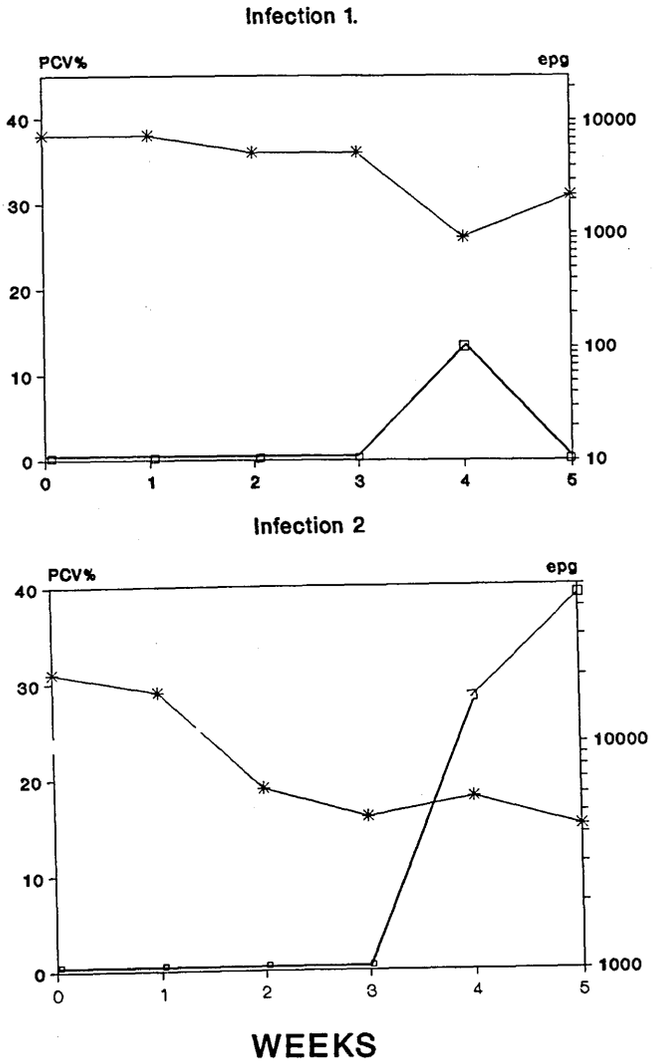
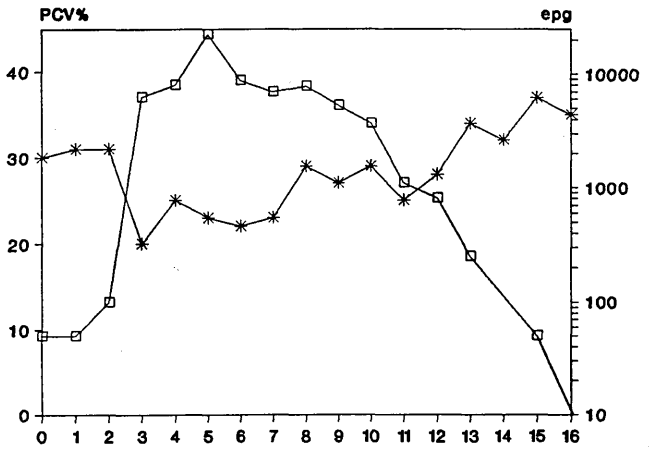
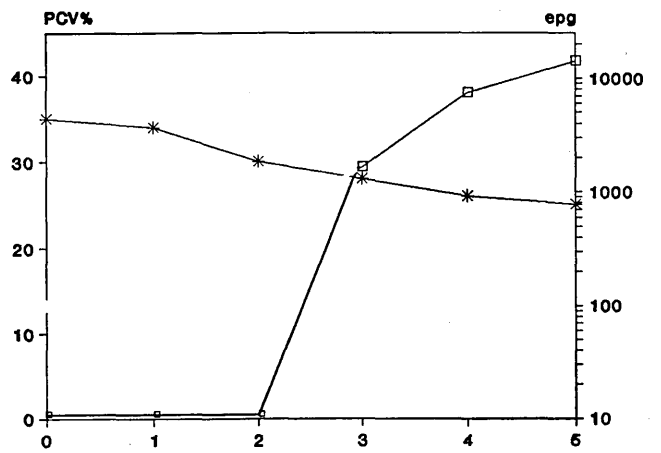


FIGURE 4. Cont'd

Infection 3.



Infection 4.



Weeks

Packed cell volumes

Faecal egg counts

FIGURE 5.

PCV's and faecal egg counts (e.p.g) after serial infections with 10,000 normal H. contortus L3 in a 2-month old lamb

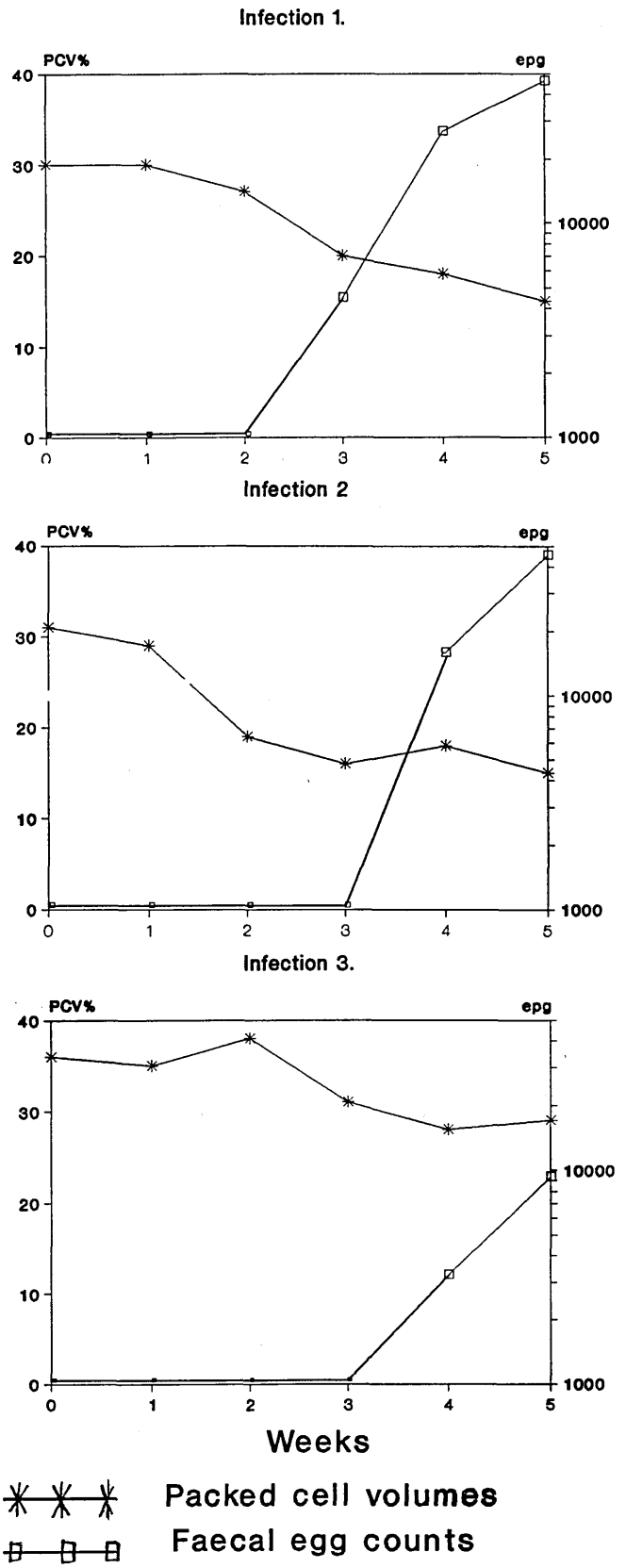
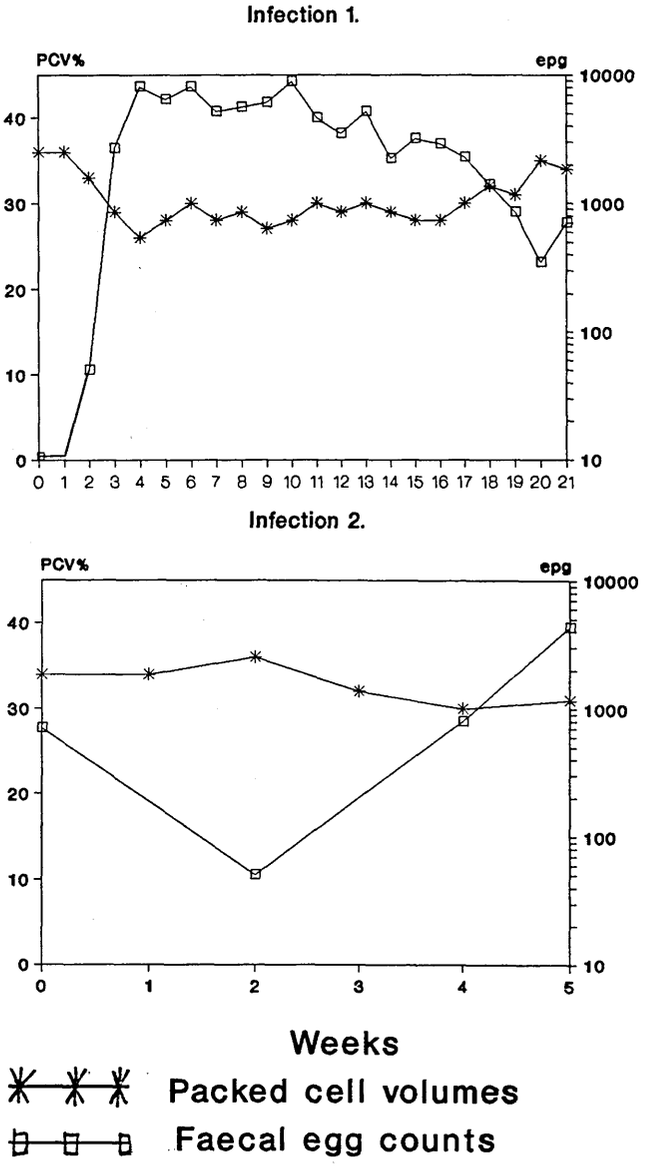


FIGURE 6.

PCV's and faecal egg counts (e.p.g) after serial infections with 10,000 normal H. contortus L3 in a 2-month old lamb



was Y13 (2-month old) where a sustained rise in faecal egg counts were seen after each infection.

Infections subsequent to the first resulted in lower ranges of egg counts and when allowed to continue beyond the tenth week these were found to increase and then steadily decline to a level where either negative or very low (<50) egg counts were recorded.

The results obtained indicated that despite individual variation in susceptibility to Haemonchus infection, animals under the age of six months do not appear able to develop any significant resistance to reinfection. Although subsequent infections resulted in lower faecal egg counts than the primary infection and PCV% values remained within the normal range, infective larvae were still able to establish and go on to produce mature males and egg laying females.

Both age groups of sheep appeared to be equally susceptible to primary infection, though the requirement for repeated treatment of Y13 (2-month old) might indicate that very young sheep may have a greater susceptibility.

The sera obtained from the hyperimmunisation of the mature sheep was subsequently used in the investigation of the antigenic nature of the surface proteins of exsheathed L_3. This is discussed in more detail in Chapter Four under the heading of Western Blotting (page 134).

Serum samples from the infection of the immature sheep were used in IFA and retained for later analysis of antibody

recognition of parasite antigens. It is hoped that it will be possible to carry out this analysis at some later date, but in the scope of this thesis this has not been included.

CHAPTER THREE

IMMUNISATION - VACCINATION EXPERIMENTS

INTRODUCTION

It has been known for some time that the surface of helminth parasites is antigenic (Philipp and Rumjanek, 1984) and currently there is a great deal of interest in the role of surface molecules in the immune response to nematodes. The first experiment described in this section was designed to induce antibody production against surface proteins from both third (L_3) and fourth (L_4) stage H. contortus larvae for use in some of the molecular biology studies described later in this thesis. Subsequent experiments reported in this chapter were carried out to determine whether surface proteins were capable of inducing a protective immune response in young sheep.

The antigens used in these experiments were surface proteins stripped from the surface of exsheathed L_3 and in vitro cultured L_4, by the cationic detergent cetyltrimethylammonium bromide (CTAB). The antigens were prepared for injection in Freund's Complete Adjuvant (FCA 6:4 v/v) for the primary immunising injection and in Freund's Incomplete Adjuvant (FIA 6:4 v/v) for the booster injection.

INDUCTION OF ANTIBODIES TO SURFACE PROTEINS OF THIRD (L_3) AND FOURTH (L_4) STAGE H. contortus LARVAE

Two worm-free Dorset lambs aged two months were used. They were maintained indoors on hay and a commercial sheep pellet diet in an environment which precluded the acquisition of helminth infection. One of the lambs (Y15) received L_3 surface antigens and the other (Y16) L_4 surface antigens. Initial injections

contained 2 mg of protein while the booster dose, containing 500 ug of the antigen, was given seven weeks later. The antigen preparations were divided and injected subcutaneously in two separate sites in the axilla to minimise any adverse effects of the adjuvant.

The lambs were monitored daily for any clinical signs which might have resulted from the immunising regimen and serum samples for use in an ELISA were collected weekly after the booster dose was administered.

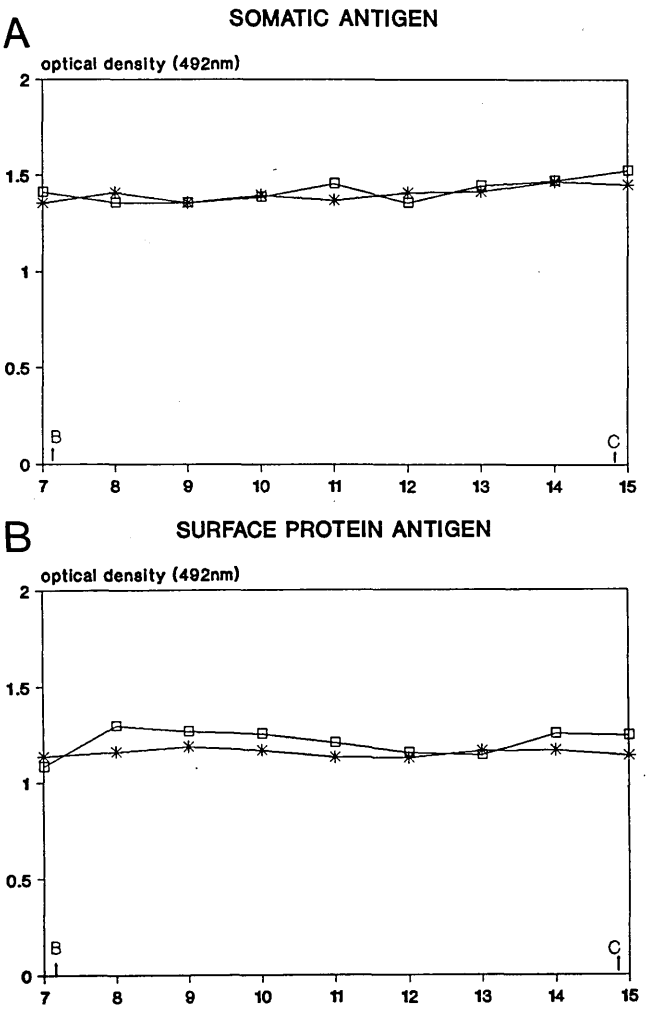
Determination of the serum antibody response to the injected surface antigens was based on the optical densities (O.D.s) obtained by the ELISA technique measured using both somatic and surface proteins as antigen. The results are shown in Figure 7.

The O.D. readings after booster immunisation revealed an increase in antibody level from negative (pre-infection serum) to very strong positive values. The sera from the lamb injected with the L_4 surface proteins (Y16) generally gave higher ELISA readings than the lamb injected with the L_3 surface proteins (Y15). In both cases, the O.D.s were higher when somatic antigens were used in the test, than when surface antigens were used. This suggested that the surface proteins used in the vaccination were a sub-set of the total proteins present in homogenates of the parasite.

Sera from the two animals was also analysed for antibody response by immunofluorescent microscopy. Exsheathed L_3 were incubated first in individual sera from each lamb followed by incubation in FITC-labelled second antibody before being observed

FIGURE 7.

ELISA O.D's of sera from two 2-month old lambs immunised with L3 (Y15) and L4 (Y16) surface antigens using H. contortus L3 somatic and surface antigens



Lamb Y15 Lamb Y16

B - Booster vaccination C - Challenge

Cut-off point - A:0.958 B:0.382

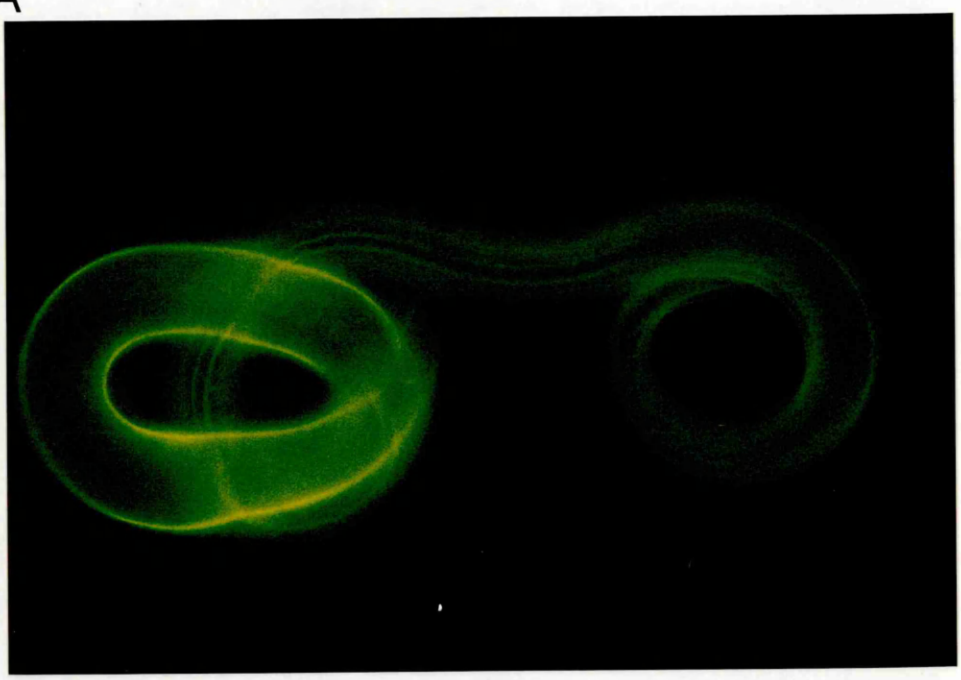
PLATE 1

H. contortus L_3 Surface Immunofluorescence with Post-Immunisation Sera from Two Vaccinated Lambs

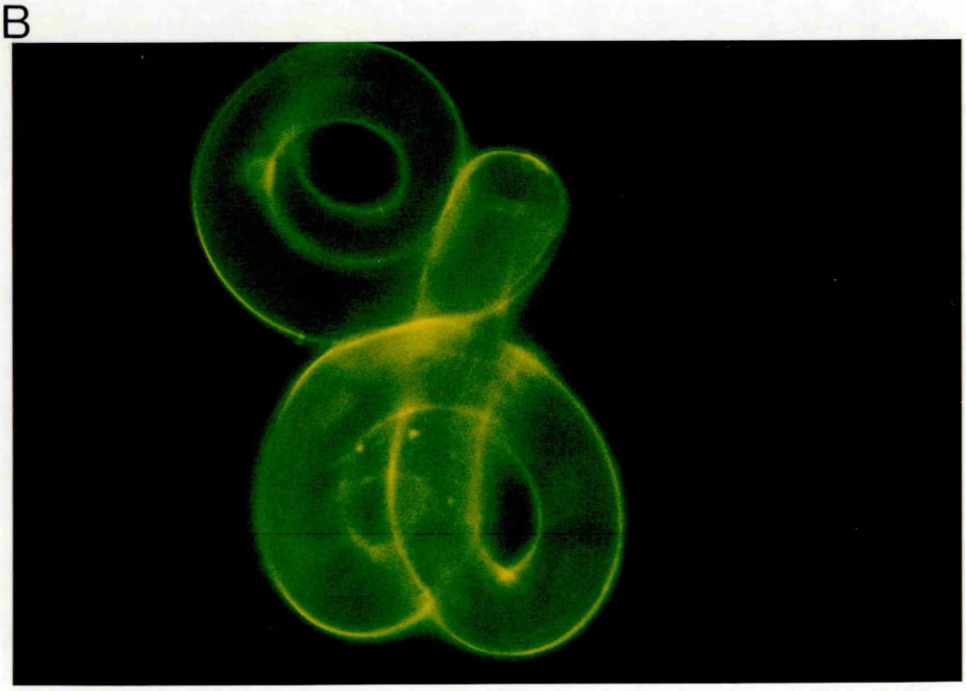
The two plates show surface fluorescence of exsheathed viable H. contortus L_3 after incubation in sera from L_3 vaccinated (Plate 1A) and L_4 vaccinated (Plate 1B) lambs on exposure to FITC-labelled second antibody.

There is a greater intensity of fluorescence visible on the larva in Plate 1B than in Plate 1A.

A



B



under UV light. The result of this procedure can be seen in Plate 1 which shows the strong surface fluorescence on the L_3 incubated in both sera.

CONCLUSION

The ELISA results showed that the injection of surface proteins into the lambs resulted in an antibody response. This response appeared much higher in the lamb injected with L_4 surface proteins. The result of the surface immunofluorescence also suggested a strong antibody response to the injected surface proteins, again apparently stronger in the animal injected with the L_4 antigens as shown by the greater intensity of fluorescence observed.

The sera obtained after immunisation in this experiment were used in investigations of the nature of L_3 antigens in subsequent vaccination experiments. More detail is given in Chapter Four under the heading Immunochemical Experiments.

EXPERIMENT ONE

IMMUNISATION: PROTECTIVE VALUE OF SURFACE PROTEINS AGAINST EXPERIMENTAL *H. contortus* INFECTION

INTRODUCTION

The fact that surface proteins are antigenic has been shown before, but antibodies to such antigens have not yet been shown to have significant protective properties. This experiment was aimed at determining whether *H. contortus* surface proteins could elicit a protective immune response.

EXPERIMENTAL DESIGN

The two Dorset lambs which were used for the induction of antibodies to L_3 and L_4 surface proteins of H. contortus were challenged with normal larvae to find out if there was any indication of a protective immune response.

Eight weeks after the booster dose of antigen in FIA, the two lambs together with a control lamb, were orally challenged with 10,000 normal H. contortus larvae. Blood for PCV estimation and faecal samples for worm egg counts were taken at the time of challenge and weekly thereafter until the end of the experiment. Serum was collected from each animal weekly for use in an ELISA against L_3 somatic and surface antigens.

The two immunised lambs were necropsied seven weeks post-challenge and their abomasa processed to determine the numbers of adult parasites present. Because it had a persistently high faecal egg count the control lamb was maintained as a source of eggs for culture of first, second and third stage larvae which were used in subsequent experiments.

RESULTS

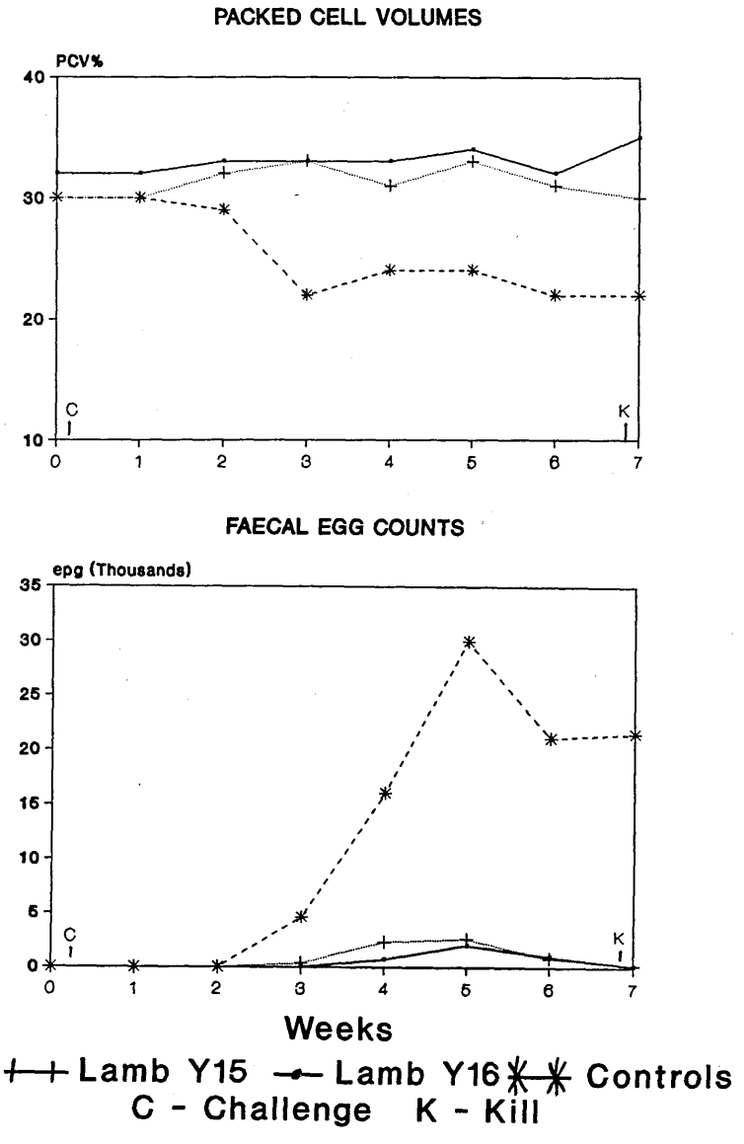
PCVs and faecal egg counts of the three lambs are shown in Figure 8.

Packed Cell Volumes (PCVs)

Between 14 and 21 days after challenge the PCV of the control lamb started to decline and by 21 days it had fallen to 22%; it subsequently remained below 25% until the end of the experiment. In contrast the PCVs of the two immunised animals

FIGURE 8.

Experiment 1: PCV's and faecal egg counts of two lambs immunised with L3 (Y15) and L4 (Y16) surface antigens and a control lamb after challenge with 10,000 normal H. contortus L3



remained at between 30-35% throughout the seven weeks post challenge period.

Faecal Egg Counts

By 21 days after challenge the control lamb had a faecal egg count of 4,550 e.p.g., which reached a peak of 30,000 e.p.g. at five weeks: the control lamb continued to have a high egg count averaging around 22,000 e.p.g. for the following two weeks. Both immunised lambs showed low egg counts following challenge with peak values of 2,300 e.p.g. (Y15) and 2,600 e.p.g. (Y16) being recorded four weeks post challenge; thereafter there was a decline in the egg counts, and by the end of the experiment few eggs could be detected in the faeces.

Worm Recoveries

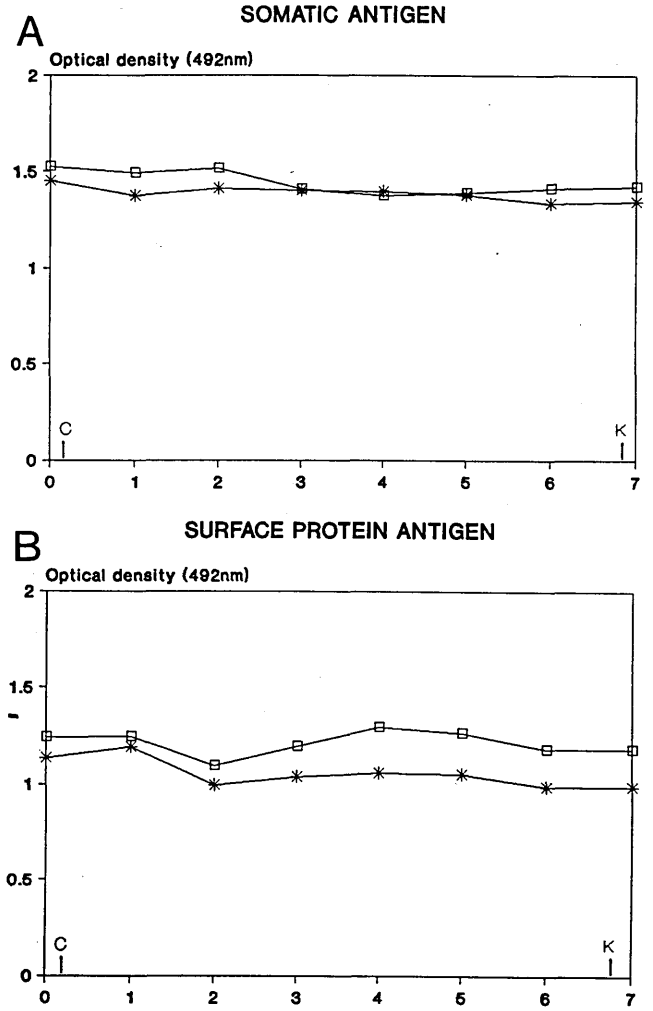
The total H. contortus worm recoveries from the abomasal contents were 220 and 380 for Y15 and Y16 respectively. There was little apparent damage in the abomasum and no parasites were seen attached to the mucosa.

ELISA Results

ELISA results are shown in Figure 9. In the two lambs which had received larval antigens, antibody levels detected by ELISA after challenge showed little change from those recorded during the immunisation period. Higher optical densities were obtained when the test was carried out using somatic antigens and the values using both antigens were higher in the lamb injected with L_4 surface proteins. The ELISA results using post challenge

FIGURE 9.

Experiment 1: ELISA O.D's of serum from two 2-month old lambs immunised with L3 (Y15) and L4 (Y16) surface antigens after challenge with 10,000 normal H. contortus L3: In the ELISA L3 somatic and surface antigens were used.



serum samples from the control lamb were below the cut-off point indicating a negative or extremely low antibody response.

CONCLUSIONS

These preliminary results indicated that injected surface proteins from H. contortus larvae induced an antibody response which appeared to be correlated on the basis of PCVs and egg counts with a poor establishment of a challenge dose of normal infective larvae.

There was little evidence of antibody production to larval antigens in the control lamb and in this animal parasite development seemed unimpaired. The ELISA results from the immunised animals suggest that despite little increase in antibody production after challenge, existing antibody levels had affected the establishment of the challenge dose.

EXPERIMENT TWO

VACCINATION: EFFECT OF IMMUNISATION WITH H. contortus L_3 SURFACE ANTIGENS ON CHALLENGE INFECTION WITH NORMAL LARVAE

INTRODUCTION

This experiment was aimed at investigating further the results obtained in the preliminary study using three animals. In this instance only the surface proteins from H. contortus L_3 were used.

EXPERIMENTAL DESIGN

Six 3-month old female Dorset lambs were used. All had been

reared worm-free and were maintained indoors for the duration of the experiment.

L_3 surface protein antigen was prepared from exsheathed H. contortus larvae as described previously using the cationic detergent CTAB.

Three lambs were designated as test animals and the remaining three as controls. The test lambs were each injected in the axillae with 2 mg of the L_3 surface protein extract emulsified with Freunds Complete Adjuvant (FCA) in a 1:1 v:v ratio. A booster dose of 500 ug surface protein in Freunds Incomplete Adjuvant (FIA) (1:1) was administered five weeks later. The three test lambs and the three control lambs were each given an oral challenge dose of 100,000 normal H. contortus larvae five weeks after the booster injection.

Prior to the onset of the experiment and weekly thereafter blood and faecal samples were collected for serum and PCV estimations and faecal egg counts respectively. The serum samples were examined by the ELISA using L_3 somatic and surface antigens.

The experiment was terminated 19 days after the lambs were challenged, and the abomasa from each animal processed in the laboratory for adult parasite recovery. The experimental design is summarised in Table 4.

Table 4

Experiment Two. Design

	Test Lambs	Control Lambs
1st Vaccination (Day 0)	2mg L_3 Ag	-
2nd Vaccination (Day 35)	500ug L_3 Ag	-
Challenge (Day 70)	100,000 L_3	100,000 L_3
Necropsy (Day 89)	+	+

RESULTS

Packed Cell Volumes (PCVs)

The mean PCVs for the two groups of animals are shown in Figure 10 with individual results in Appendix 1.

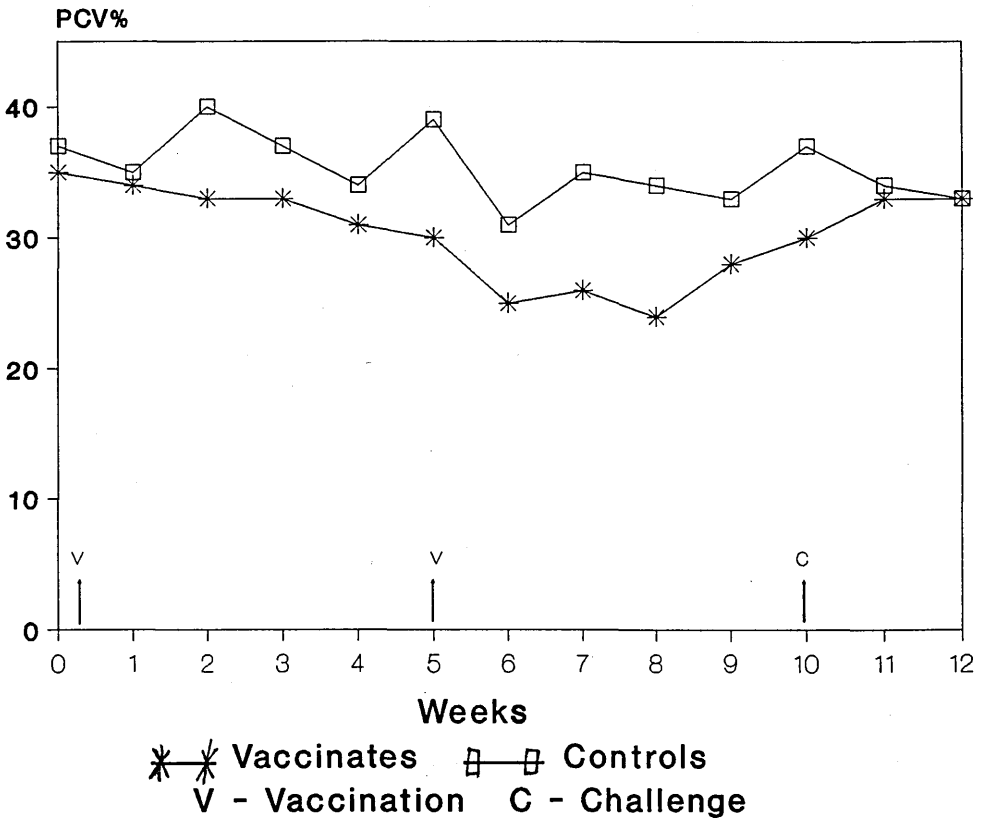
The mean PCVs of the test lambs showed a slight decline during the period prior to and following the primary vaccination but these had fallen to a level below the normal range by the time of the booster vaccination, reaching the lowest value of 18% in one lamb by the sixth week of the study. This trend was reversed between the eighth and ninth week and by the termination of the experiment the mean PCV of the test lambs had increased to a normal value of 35%.

The mean PCVs of the control lambs remained within the normal range throughout the experimental period with individual values ranging between 26 - 40%.

Faecal Egg Counts

The worm egg counts of both vaccinates and controls remained

FIGURE 10.
Experiment 2: PCV's of lambs vaccinated with H. contortus L3 surface antigens and non-vaccinated controls



negative for the duration of the experiment.

Necropsy Findings

The abomasa of the test lambs showed no gross signs of damage attributable to the presence of parasites, whereas those of the controls showed signs of damage in the form of pallor associated with numerous small round-edged regular depressions in the mucosa, presumed to the attachment sites of the blood sucking stages of the parasite.

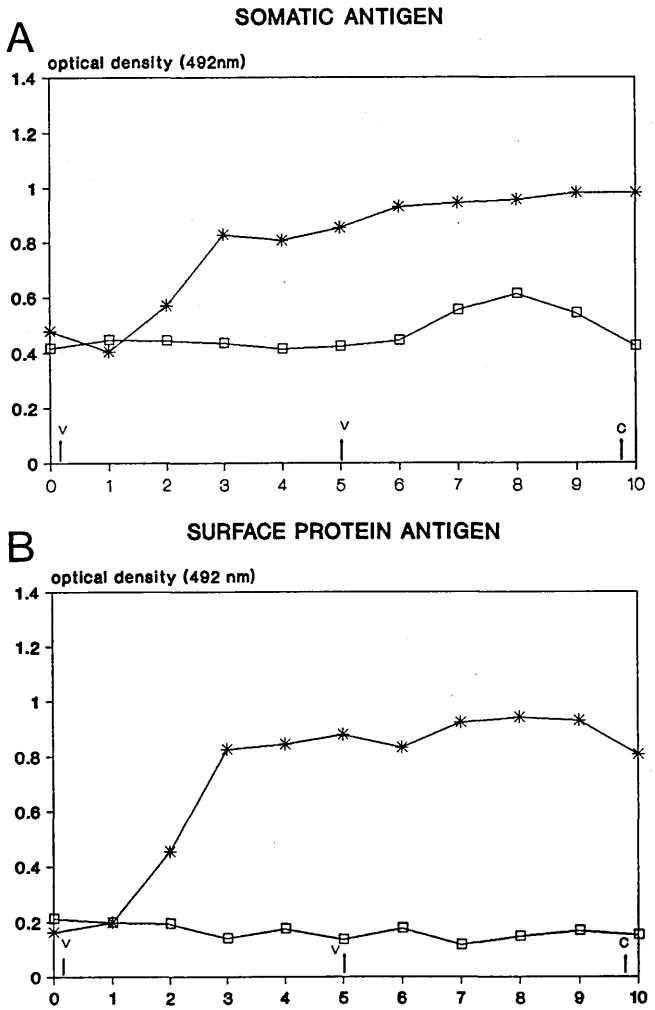
Worm establishment from the test lambs was between 15-36% of the challenge dose whilst those from the control lambs ranged from 20-78%. The mean numbers of adult parasites recovered from the test and control groups at necropsy 19 days post challenge were 28,000 and 51,520 respectively.

ELISA Results

As similar O.D.s were obtained within the two groups of lambs the ELISA results for one vaccinated and one control lamb are shown in Figure 11; individual results for the remaining animals are given in Appendix 2.

Weekly serum samples of all the lambs were subjected to ELISA using L_3 somatic and surface antigens. The results from both ELISAs (Figure 11) indicated that there was a marked antibody response to the injected surface proteins in the vaccinated animals which was significantly higher at the termination of the experiment than at its onset. The antibody titres obtained from the control animals remained below or just

FIGURE 11.
Experiment 2: ELISA O.D's of serum from lambs vaccinated with H. contortus L3 surface antigens and non-vaccinated controls: In the ELISA L3 somatic and surface antigens were used.



slightly above the cut-off point throughout the experiment.

CONCLUSIONS

The results of this experiment indicated that the immunisation with surface proteins of *H. contortus* L_3 induced a significant antibody response which appeared to be associated with an impairment in establishment and development of a challenge infection of normal larvae.

EXPERIMENT THREE

VACCINATION: EFFECT OF IMMUNISATION WITH *H. contortus* L_3 SURFACE ANTIGENS ON SUBSEQUENT CHALLENGE WITH A KENYAN STRAIN OF NORMAL LARVAE IN MATURE SHEEP

INTRODUCTION

In the two previous experiments in which parasite-naive lambs under the age of four months were used there were indications of a protective effect of immunisation with *H. contortus* L_3 surface proteins against challenge infection with normal larvae.

This experiment was carried out to determine the effect of a similar vaccination regimen on experimental challenge of mature Kenyan sheep.

EXPERIMENTAL DESIGN

Six yearling castrated male Dorper sheep were used in the experiment. These sheep had been reared outdoors under range management conditions and had thus been exposed to natural helminth infections: they were routinely dosed with anthelmintic

every month. After purchase the sheep were maintained indoors at the National Veterinary Research Centre, Muguga, on a diet of hay and sweet potato vines and water was supplied ad lib.

Three rams were allocated to the vaccination group and the remaining three acted as non-vaccinated controls. The vaccinated rams each received a primary injection of 2 mg of L_3 surface protein extract emulsified in FCA (6:4 v:v) in the axillae. A booster vaccination was administered five weeks later using a dose of 500 ug surface protein in FIA (6:4 v:v). The surface protein antigen used in this experiment was prepared from CTAB detergent treated third stage larvae of the Glasgow strain of H. contortus.

Five weeks after booster vaccination both test and control rams were orally challenged with 10,000 normal H. contortus infective larvae.

Due to unforeseen circumstances one of the original control rams was removed from the group. A substitute control animal was acquired immediately prior to challenge, but this ram was not worm-free and had a faecal egg count of 150 e.p.g. at time of challenge.

Blood samples for serum and PCVs and faeces for faecal egg counts were taken prior to the onset of the experiment and weekly thereafter. All rams were sacrificed 22 days after challenge. The experimental design is summarised in Table 5.

Table 5

Experiment Three. Design

	Test Animals	Control Animals
1st Vaccination (Day 0)	2mg L_3 Ag	-
2nd Vaccination (Day 35)	500mg L_3 Ag	-
Challenge (Day 70)	10,000 L_3	10,000 L_3
Necropsy (Day 92)	+	+

RESULTS

Packed Cell Volumes (PCVs)

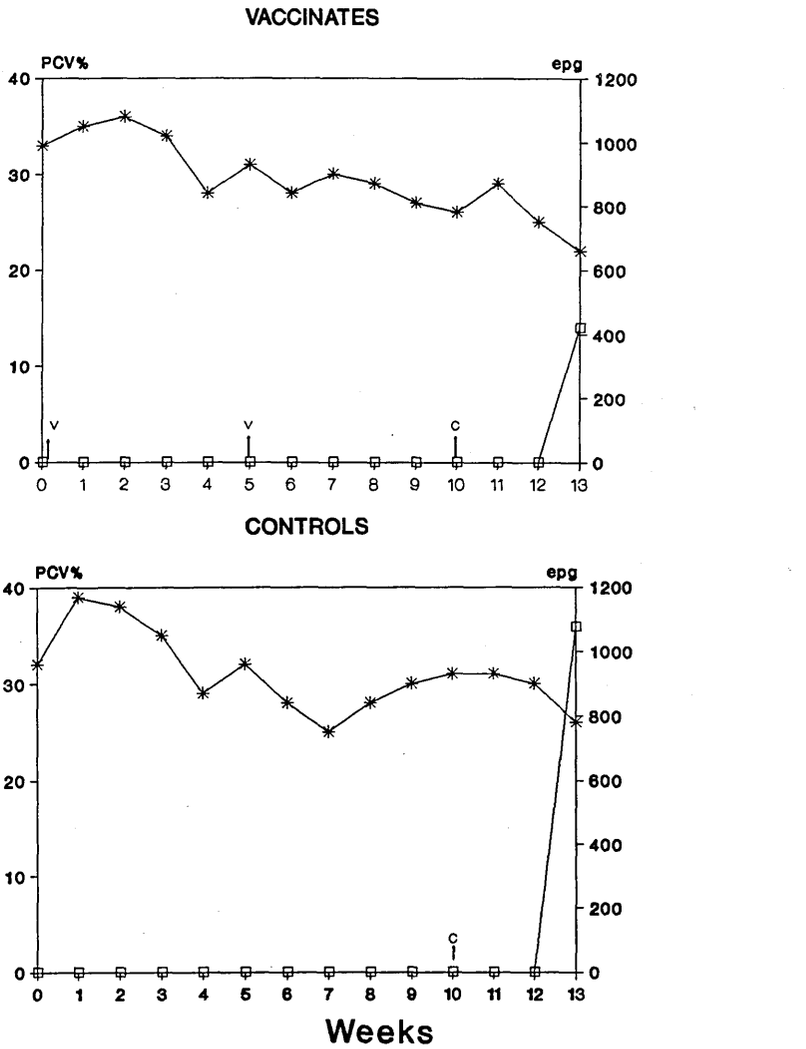
The mean PCVs and faecal egg counts for the vaccinated and control groups are shown in Figure 12 with individual PCV results detailed in Appendix 1.

Mean PCVs for both groups of animals showed a general decline and by the end of the experimental period values were 22% for the vaccinated group and 26% for the controls. Despite this there were no obvious signs of anaemia on physical examination of occular and oral mucous membranes.

Faecal Egg Counts

Worm egg counts of the six rams were negative for the first two weeks of the experiment. At three weeks however, positive egg counts of up to 500 e.p.g. were recorded and all six animals were dosed with Ivermectin (Oramec[R], MSD AGVET) at this point. Faecal egg counts two days later and the subsequent weekly faecal samples until three weeks post challenge were negative for

FIGURE 12.
Experiment 3: PCV's and faecal egg counts of adult sheep vaccinated with H. contortus L3 surface antigens and non-vaccinated controls



parasite eggs.

In the final week of the experiment the mean faecal egg counts of the vaccinated and control groups were 420 e.p.g. and 1,075 e.p.g. respectively.

Necropsy Results

At post-mortem examination the abomasa of the three vaccinated rams showed no gross signs of abomasal damage but adult parasites were easily visible on the mucosa during the washing process. The mean adult worm burden of the vaccinates was 1,630.

The abomasa from the two original control rams showed pallor of the mucous membrane and numerous pin-point depressions surrounded by raised pale areas. The mean adult parasite establishment from these two animals was 3,480. The necropsy resuts from the control ram which was substituted immediately prior to challenge were different in that there was no apparent damage to the abomasal mucosa and the number of adult parasites recovered was lower i.e. 700 worms. The mean adult parasite establishment when all three controls are included was 2,550.

CONCLUSIONS

Despite differences in age, breed and previous exposure of the animals used in this experiment compared with those in Experiment Two, the parasitological results were similar i.e. vaccination of adult Dorper sheep with L_3 surface proteins from a

Glasgow strain of H. contortus resulted in similar reductions in development of a challenge infection of normal Kenyan-derived H. contortus when compared with non-vaccinated controls. It was not possible to examine antibody responses in this experiment as serum samples remained in Kenya: it is planned to use these for further experimental work at a future date.

EXPERIMENT FOUR

VACCINATION: EFFECT OF FREUNDS ADJUVANT ON THE INDUCTION OF AN IMMUNE RESPONSE TO H. contortus

INTRODUCTION

The three experiments described previously in this chapter have indicated some potential value of H. contortus L_3 surface proteins as immunogens. This experiment was designed to determine whether the results obtained in these previous experiments were due to any non-specific effects of Freunds adjuvant.

EXPERIMENTAL DESIGN

Twelve Scottish Blackface lambs aged three months were used. All had been purchased as worm-free animals and were maintained indoors for the duration of the experiment. The animals were fed a hay and a commercial sheep pellet diet and water was available ad lib. The lambs were randomly allocated to one of three groups of four animals designated Group A, Group B and Controls.

Each lamb in Group A was vaccinated with 2 ml of L_3 surface protein extract (obtained from CTAB treatment of 3 million L_3)

emulsified in 2 ml FCA, those in Group B were given 2 ml PBS (pH 7.2) emulsified in 2 ml FCA and the Control group was left untreated.

Five weeks after the primary vaccination the animals in Groups A and B were given a second vaccination comprising 2 ml of a similar L_3 surface protein extract in 2 ml FIA (Group A) and 2 ml PBS in 2 ml FIA (Group B). Four weeks after this second vaccination, a further booster dose of 1.5 ml surface protein extract (obtained from 2.25 million L_3) and 1.5 ml PBS in FIA was administered to the Group A and Group B lambs respectively. All vaccinations were made in divided doses and injected in the axilla.

All three groups of animals were challenged with 10,000 normal H. contortus L_3 one week after the second booster vaccination and the experiment terminated four weeks after this challenge.

Prior to the onset of the experiment and weekly thereafter blood and faecal samples were taken for serum and PCV estimations and faecal egg counts respectively. The sera collected was subjected to analysis by ELISA using both somatic and surface antigens from H. contortus L_3. The experimental design is summarised in Table 6.

Table 6

Experiment Four. Design

	Group A	Group B	Control
1st Vaccination (Day 0)	2ml L_3 Ag	2ml PBS	-
2nd Vaccination (Day 35)	2ml L_3 Ag	2ml PBS	-
3rd Vaccination (Day 63)	1.5ml L_3 Ag	1.5ml PBS	-
Challenge (Day 70)	10,000 L_3	10,000 L_3	10,000 L_3
Necropsy (Day 98)	+	+	+

RESULTS

The mean PCVs and faecal egg counts of the three groups of animals are shown in Figure 13 with individual PCV results given in Appendix 1.

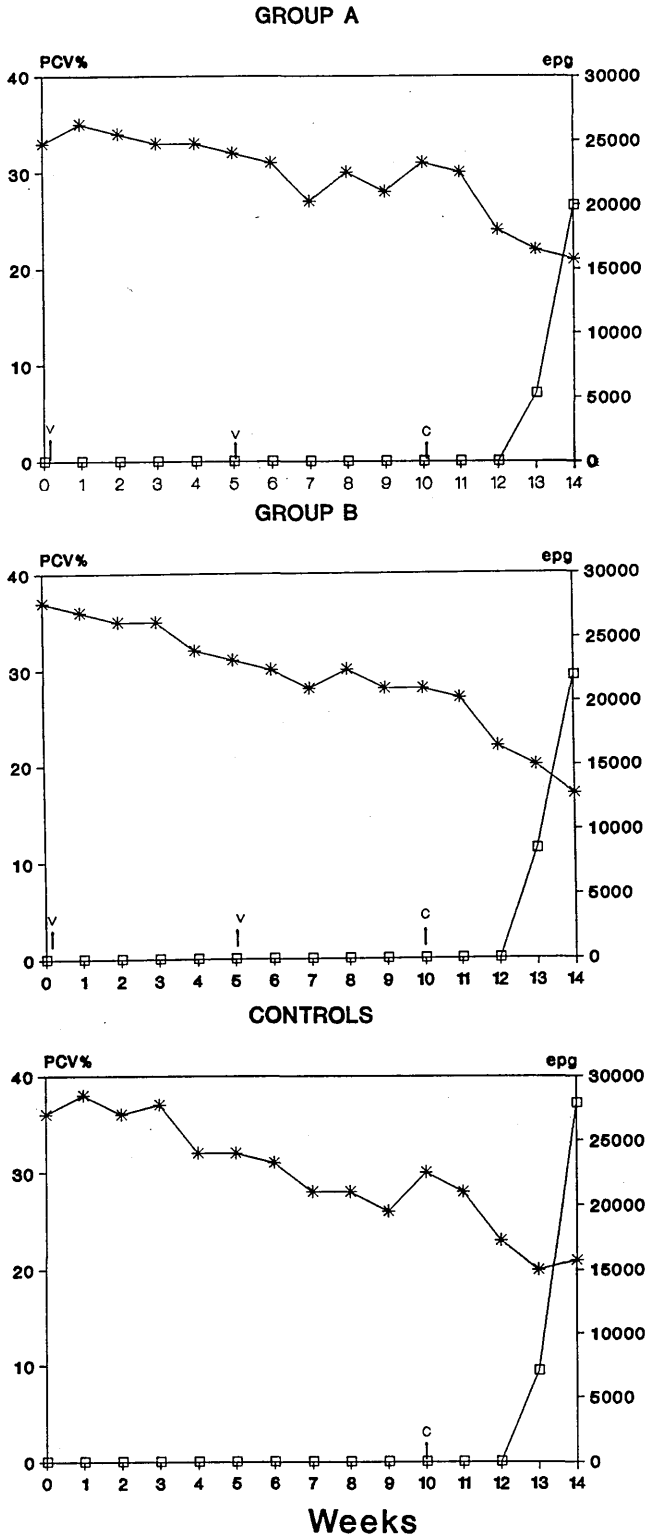
Packed Cell Volumes (PCVs)

The trend exhibited by all three groups of animals was a gradual decline in PCV up to the time of challenge. PCVs had fallen to below the normal range in all three groups by the second week after challenge and by the termination of the experiment they had reached approximately 20% in Group A and the Control group; Group B showed the lowest mean PCV of 16% at this time.

Visible signs of the developing anaemia were observed as a slowly progressive pallor of the occular mucous membranes in all animals: this was especially marked in the lambs of Group B.

FIGURE 13.

Experiment 4: PCV's and faecal egg counts of lambs vaccinated with H. contortus L3 surface antigens, Freunds controls and non-vaccinated controls



Faecal Egg Counts

Negative egg counts were recorded from all 12 lambs until the third week after challenge. At this time mean faecal egg counts of all three groups were between 5,000 and 8,500 e.p.g. which had risen to mean values of between 19,000 - 25,000 e.p.g. by the termination of the experiment.

There was a marked individual variation in the faecal egg counts especially within Group B and the Control group.

Necropsy Results

The abomasal mucosa of all animals showed varying degrees of pallor and one or two areas of ecchymotic haemorrhages. In addition, in one lamb in Group A, there was generalised petechiation and in one control lamb there was evidence of ulceration.

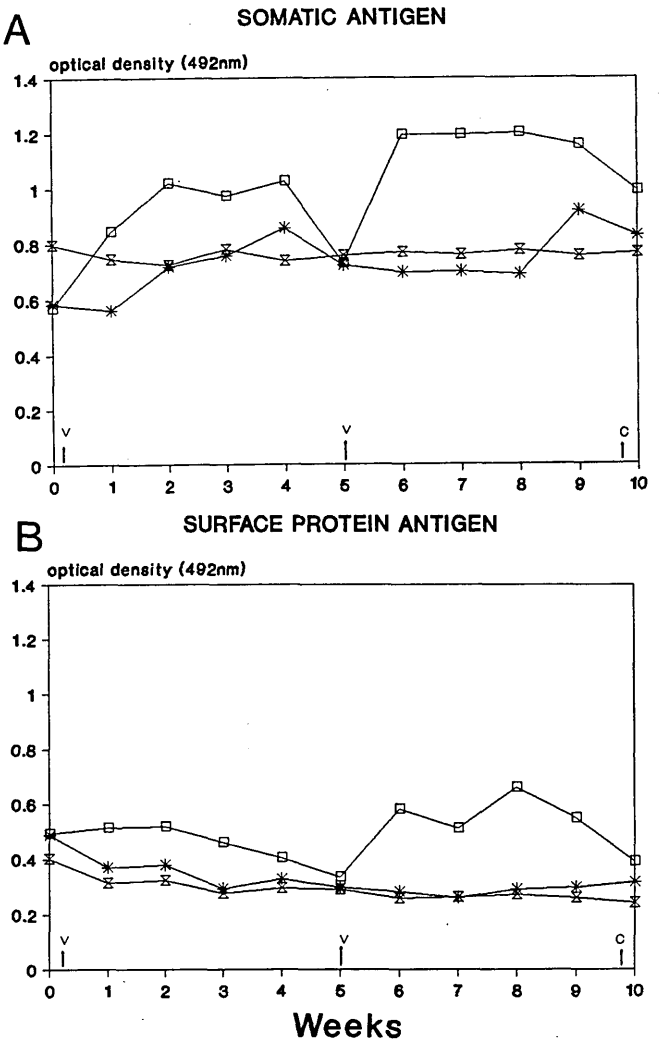
Adult parasite establishment was high in all groups with mean worm burdens of 7,363, 7,425 and 7,900 in Group A, Group B and the Controls respectively.

ELISA Results

The ODs obtained using serum samples from one animal from each group are shown in Figure 14; individual results for the remaining animals are given in Appendix 2.

The ELISA readings against L_3 somatic antigens in the Group A lambs indicated a slight increase in antibody response above the cut-off point from the second to fourth week after primary vaccination: after the second vaccination there was a more marked and sustained rise in ODs. The ELISA ODs from this

FIGURE 14.
Experiment 4: ELISA O.D's of serum from lambs vaccinated with H. contortus L3 surface antigens, Freunds controls and non-vaccinated controls: In the ELISA L3 somatic and surface antigens were used



Group A Group B Controls

V - Vaccination C - Challenge

Cut-off point - A:0.820 B:0.708

group using surface antigen only rose above the cut-off point on one occasion three weeks after second vaccination.

ELISA readings obtained for Group B against somatic antigens were varied with the values for one animal remaining below the cut-off point throughout the experimental period. The ODs for the other three lambs in Group B were consistently positive. When sera was analysed against surface antigens, the reverse occurred in that three of the animals produced results indicating a lack of antibody response while the results from the fourth animal were consistently positive.

The ELISA readings using somatic antigens, for two of the four control lambs, surprisingly indicated that there was antibody in their sera at different times during the experimental period; the sera from the remaining two lambs was consistently negative i.e. all readings were below the cut-off point: ELISA readings using surface antigens were all negative.

CONCLUSIONS

This experiment produced variable results which were difficult to interpret. From the trend in PCVs, faecal egg counts and total worm counts there was no indication that, as in the previous three experiments, the administration of the L_3 surface proteins was associated with any degree of protection against challenge. The administration of adjuvant alone also had no apparent effect on subsequent infection.

It appeared that the concentration of surface proteins administered to the animals in this experiment may have been very

low and that this may have played a part in the lack of success in demonstrating any significant immune response. The relatively high ELISA O.Ds obtained from pre-infection sera from some of these Blackface sheep suggested that the animals may have had non-specific cross-reacting antibodies or may have been previously exposed to H. contortus infection.

EXPERIMENT FIVE

VACCINATION: EFFECT OF IMMUNISATION WITH H. contortus L_3 WITH INCREASED DOSES OF SURFACE PROTEINS ON SUBSEQUENT CHALLENGE WITH NORMAL LARVAE

INTRODUCTION

The first three experiments described in this thesis were conducted with similar quantities of CTAB stripped L_3 surface protein extract, while in Experiment Four the antigen used had a lower protein concentration than that previously used. A further experiment was therefore carried out using large doses of the surface protein extract.

EXPERIMENTAL DESIGN

The procedure followed was in most respects similar to that of previous experiments, but in this case ten 4-month old Finn-Dorset lambs were allocated to two groups of five lambs each: one group was vaccinated, while the other remained as a non-vaccinated control group. The vaccinates were each injected with 3 mg L_3 surface protein extract emulsified in FCA (6:4 v/v) at the start of the experiment and a booster dose containing

650 ug of the extract in FIA (6:4 v/v) was given five weeks later; five weeks after this booster vaccination both vaccinated and control animals received challenge infections of 10,000 normal H. contortus L_3. Four weeks after challenge all of the animals were necropsied.

Prior to the onset of the experiment and weekly thereafter, all animals were blood sampled for serum and PCV estimations and faecal samples for worm egg counts were taken. Serum samples were examined by ELISA using L_3 somatic and surface antigens. The design of this experiment is summarised in Table 7.

Table 7

Experiment Five. Design

	Vaccinates	Controls
1st Vaccination (Day 0)	3mg L_3 Antigen	-
2nd Vaccination (Day 35)	650ug L_3 Antigen	-
Challenge (Day 70)	10,000 L_3	10,000 L_3
Kill (Day 98)	+	+

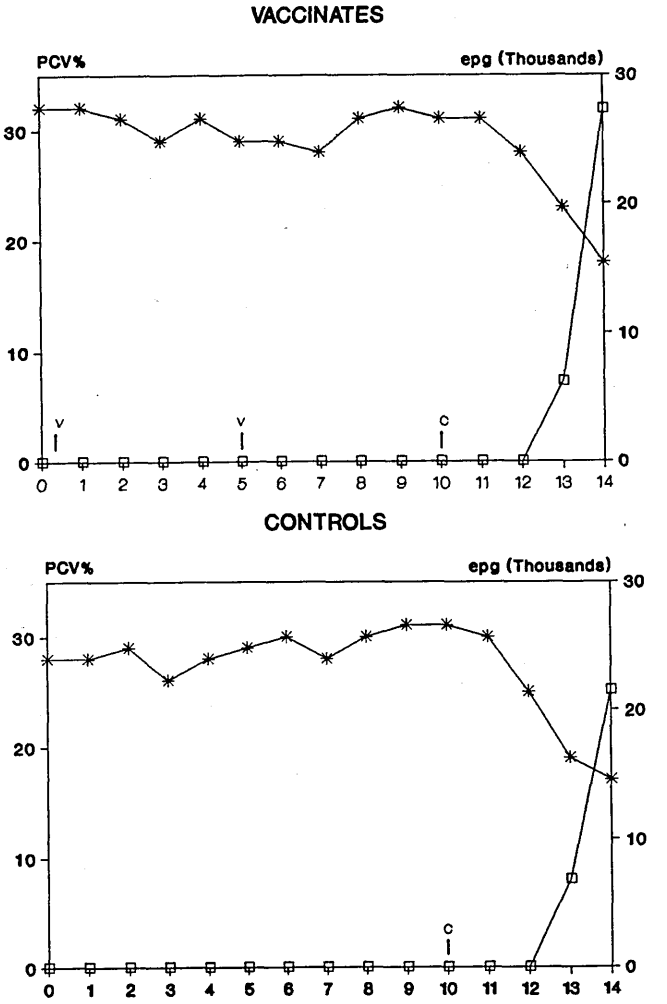
RESULTS

The mean PCVs and faecal egg counts for the two groups are shown in Figure 15; individual PCV results are given in Appendix 1.

Packed Cell Volumes (PCVs)

The mean PCVs of both groups of animals remained within the

FIGURE 15.
Experiment 5: PCV's and faecal egg counts of lambs vaccinated with H. contortus L3 surface antigens and non-vaccinated controls



normal range from the onset of the experiment until the time of challenge: after challenge the mean PCVs of both groups fell to below the normal range and by four weeks they had reached 17% in the controls and 18% in the vaccinates.

Faecal Egg Counts

The egg counts of both control and vaccinated lambs remained negative until the third week after challenge, when mean values of 6,000 - 7,000 e.p.g. were recorded for both the control and vaccinated groups. In both groups the egg counts showed a substained rise reaching values of over 20,000 e.p.g. by the termination of the experiment.

Necropsy Results

Abomasal contents and digests from each animal were examined for adult and immature parasite stages. The mean total parasite establishment in the vaccinates was 4,774 adults, whilst in the controls it was 6,684; the male to female ratio was approximately 1:1. No immature parasites were found in either group but it was noted in the vaccinated animals that there was an apparent stunting of the adult worms. One hundred randomly selected worms from each group were measured and their mean lengths were found to be 1.64 cm and 1.98 cm in the vaccinates and controls respectively. This variation in size was more apparent in female parasites whose mean length in the controls was 2.31 cm as opposed to 1.87 cm in the vaccinated group.

Grossly there was petechiation, ulceration and pallor of the abomasal mucosa in some lambs from each group although this was

not severe in any of these animals.

ELISA Results

Within group ELISA readings were similar and typical results from the sera of one vaccinate and one control lamb are shown in Figure 16: the O.D.s for the remaining animals are given in Appendix 2.

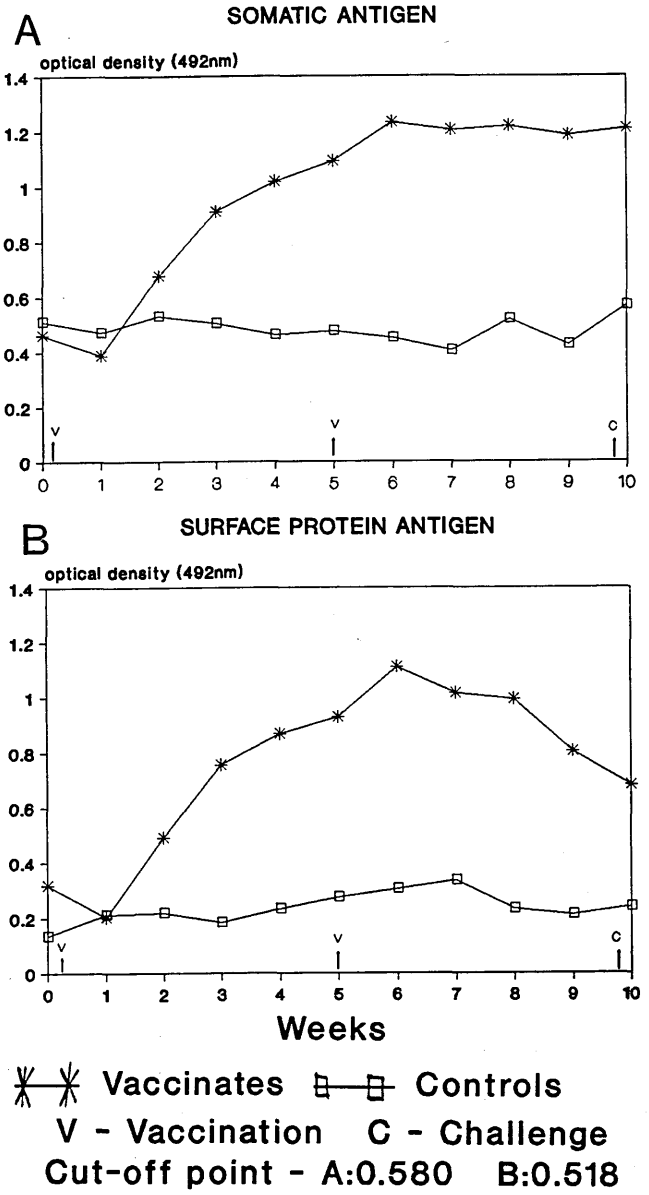
ELISA O.D.s against both L_3 somatic and surface antigens from the vaccinated group indicated a marked increase in antibody levels during the course of the experiment but especially following the first vaccination and the level of antibody response appeared greater when somatic antigens were used. In contrast, the ELISA results obtained with sera of the control group against both somatic and surface L_3 antigens were all below the cut-off points.

CONCLUSIONS

The results of this experiment indicated that despite a marked antibody response to the injected surface proteins in the sera from the vaccinated animals there was little evidence of any protective immune response. Both the vaccinated and control lambs appeared equally susceptible to the challenge infection, although there was a slightly lower adult worm establishment in the vaccinates. The presence of apparently stunted worms in the abomasa of the vaccinated animals might also indicate that vaccination was having some influence on the development of the challenge infection although this was not reflected in any group

FIGURE 16.

Experiment 5: ELISA O.D's of serum from lambs vaccinated with H. contortus L3 surface antigens and non-vaccinated controls: In the ELISA L3 somatic and surface antigens were used.



differences in faecal egg counts.

SUMMARY OF CONCLUSIONS FROM IMMUNISATION - VACCINATION STUDIES

In view of the small number of animals used in these experiments only general conclusions can be drawn which are not necessarily statistically significant.

The results obtained from the first five experiments indicated that surface proteins obtained from the surface of exsheathed H. contortus L_3, were generally able to induce the production of anti-L_3 antibodies when injected subcutaneously into experimental sheep of different breeds aged between two and 12 months. The results from the first three experiments suggested that the vaccination-induced antibodies may have played some role in protection as indicated by the lower egg counts and worm burdens in the vaccinated animals compared with controls. This was apparently unrelated to the level of the challenge dose, as similar results were obtained in Experiment Two when 100,000 L_3 instead of 10,000 L_3 were used. The results of Experiment Four are more difficult to interpret due to the low dose of antigen used for vaccination and varied intra-group results. This experiment did however confirm that Freund's adjuvant alone had little influence on protection; also it may not be unreasonable to conclude on the basis of the results of Experiment Four compared with Experiments One to Three, that the effects of the surface proteins are dose dependent and that there is a minimum effective dose. Unfortunately the results of the final experiment did not agree with the earlier findings in that

although the surface proteins again induced the production of antibodies detectable by ELISA, these appeared to be unrelated to any protection against H. contortus except for some evidence of a slight decrease in establishment and stunting of challenge worms in the vaccinated animals: this occurred despite using an increased dose of surface proteins for vaccination.

EXPERIMENT SIX

INVESTIGATION OF THE PROTECTIVE VALUE OF IMMUNISATION WITH H. contortus LARVAL AND ADULT SOMATIC ANTIGENS AGAINST CHALLENGE WITH NORMAL LARVAE

INTRODUCTION

This experiment was carried out to investigate the effects of vaccination of sheep with either larval or adult H. contortus somatic antigens. The responses in terms of type and quantity of antibody produced and any association with protection against challenge with normal larvae could then be compared with the results of previous experiments where L_3 surface proteins were used.

EXPERIMENTAL DESIGN

Third stage larvae (L_3) and adult parasites were obtained from the culture of infected faeces and recovery from infected sheep at necropsy respectively.

The parasites were homogenised as described under biochemical and culture techniques (page 65) and the homogenates ultracentrifuged at 14,000 rpm for two minutes using a L8-M

ultracentrifuge (Beckman, USA); after centrifugation the supernatants were decanted and their protein content estimated by the Lowry method (Lowry, 1951). These somatic extracts were stored at -20°C until required.

Six, 7-month old male Scottish Blackface sheep were used in the experiment; two animals received L_3 somatic antigens (Test 1), two received adult somatic antigens (Test 2) and the remaining two animals acted as challenge controls. The first immunisation consisted of 7 mg of either L_3 or adult somatic antigens in FCA (6:4 v:v) injected into the axillae. Five weeks later a booster dose of 1.8 mg of the appropriate antigen in FIA (6:4 v/v) was administered.

All animals were challenged orally with 10,000 normal H. contortus L_3 five weeks after the booster vaccination was administered and killed four weeks after challenge.

A week prior to the onset of the experiment and weekly thereafter, blood samples for serum and PCV estimations and faecal samples for worm egg counts were taken. Sera were examined for antibodies to either L_3 somatic antigens or adult somatic antigens using an ELISA. The experimental design is summarised in Table 8.

Table 8

Experiment Six: Design

	Test 1	Test 2	Controls
1st Vaccination (Day 0)	7mg L_3 Ag	7mg Ad Ag	-
2nd Vaccination (Day 35)	1.8mg L_3 Ag	1.8mg Ad Ag	-
Challenge (Day 70)	10,000 L_3	10,000 L_3	10,000 L_3
Kill (Day 98)	+	+	+

RESULTS

The mean PCVs and worm egg counts for each group are shown in Figure 17 while individual PCVs are given in Appendix 1.

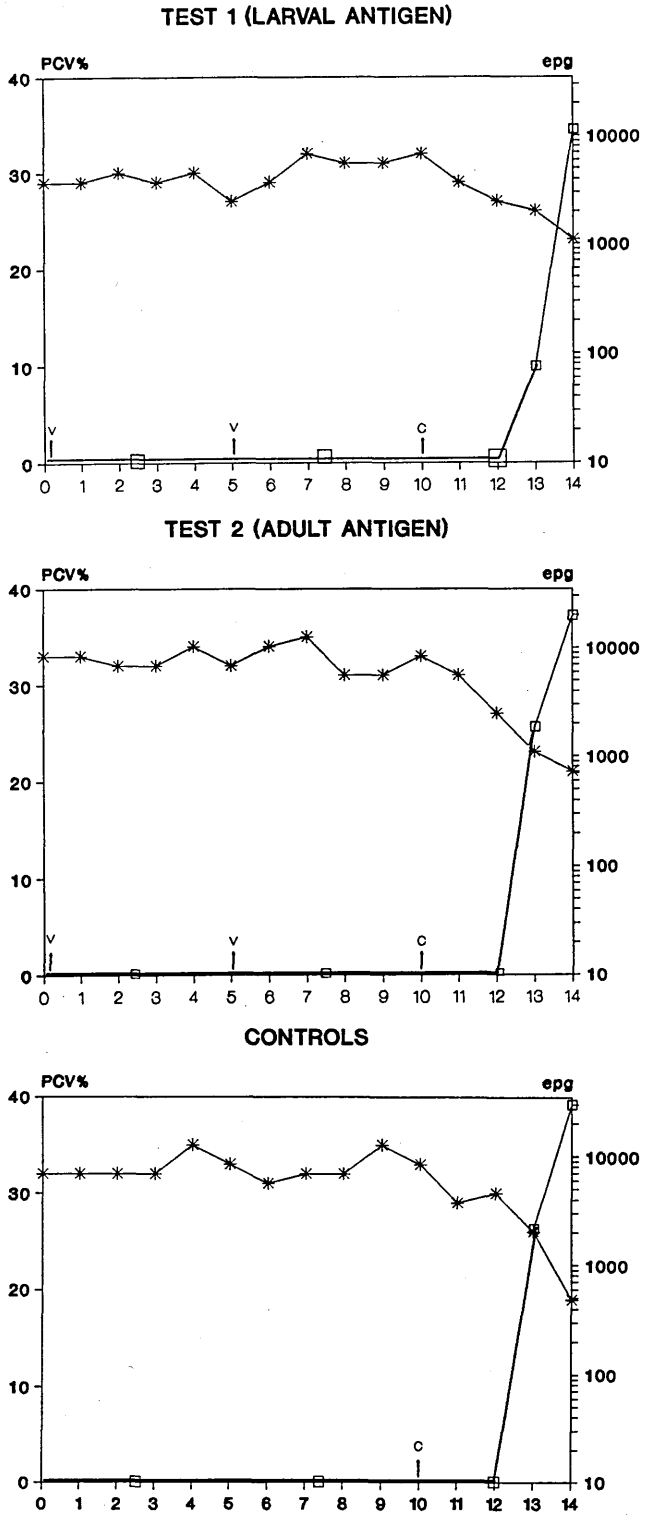
Packed Cell Volumes (PCVs)

The mean PCVs of the groups remained within the normal range until after challenge. Subsequently all three groups showed a decline until in the final week of the experiment mean values of 23%, 21% and 19% were recorded for Test 1, Test 2 and the Control groups respectively.

Faecal Egg Counts

The worm egg counts for all three groups remained negative until the third week after challenge when mean counts of 75, 1,850 and 2,125 e.p.g. were recorded for Test 1, Test 2 and Control groups respectively. By the termination of the experiment these values had increased to 11,350, 19,500 and 29,675 e.p.g. for Test 1, Test 2 and Control groups respectively.

FIGURE 17.
Experiment 6: PCV's and faecal egg counts of lambs vaccinated with H. contortus L3 (Test 1) and adult (Test 2) somatic antigens and non-vaccinated controls



Necropsy Results

Both abomasal contents and mucosal digests from each animal were examined for parasites. Mean adult parasite establishment was between 3,500 and 6,000 and the male to female ratio was approximately 1:1. Signs of abomasal damage were insignificant and seen as slight pallor of the mucous membranes with some petechiation.

ELISA Results

ELISA O.D.s of sera from both animals in each test group and the non-vaccinated controls are given in Appendix 2. The O.D.s from both test group and control animals showed little change and were well above the cut-off point at the beginning and for the duration of the experiment although the values recorded for the controls were consistently lower than the values recorded for both Test groups.

CONCLUSIONS

In view of the fact that the pre-vaccination sera from all animals produced ELISA results indicative of the presence of anti-H. contortus antibodies made interpretation of the results from this experiment difficult. The lack of any apparent increase in the level of antibody response despite vaccination with H. contortus somatic extracts suggests that these antigens may not be strongly immunogenic. The results obtained in this experiment also suggest that the animals may not have been

maintained parasite-free prior to the start of the experiment and that cross-reacting antibodies or antibodies against H. contortus may have been present.

CHAPTER FOUR

IMMUNOCHEMICAL EXPERIMENTS

EXPERIMENT SEVEN

In Vitro CULTIVATION AND SURFACE LABELLING OF *H. contortus* LARVAE

PART A. *In Vitro* CULTURE OF *H. contortus* LARVAE

INTRODUCTION

H. contortus has been cultivated to the fourth stage *in vitro* using various media (Mapes, 1969). This experiment was carried out to compare culture techniques using both simple balanced salt solutions, which had been used previously, and more complex media and to determine the effect of prolonged culture on larval development.

EXPERIMENTAL DESIGN

Two simple balanced salt solutions Mapes II and PBS were used to culture exsheathed third stage *H. contortus* larvae. In addition more complex media including Dulbecco's medium and Chicken embryo extract were also used. Cultures containing 5,000 *H. contortus* L_3 were prepared under as sterile conditions as possible and maintained at 37°C for several days on a rotator. The media were changed every four days and the larvae observed daily for any changes in morphology.

RESULTS

Larvae in both simple salt media appeared to develop at the same rate up to the fourth day in culture but thereafter the larvae in Mapes II solution developed faster. For example, 24 hours after being put into culture with Mapes II solution, larvae

were found to have less dense oesophageal and mouth regions compared with the exsheathed third stage larvae; at 48 hours after incubation this region was almost completely without visible structure. By the fourth day in culture however, the larvae were beginning to develop a darker region around the buccal area and there was structural development in the oesophageal area. In contrast, larvae at this stage in PBS still had clear anterior ends. Seven days post incubation, larvae in Mapes II solution had definite structures in the oesophageal region and the mouth area had a distinct shape. Sheaths with truncate tails were visible around most of the larvae which were moving much less vigorously than previously. The larvae in the PBS at seven days appeared to be at a similar stage of development as larvae in Mapes II solution at four days in culture with some structural development of the oesophageal area and a dark spot in the area of the buccal cavity.

By Day 8 of incubation, about 80% of the larvae in Mapes II solution had a fully reorganised oesophageal area with a clear distinction between this area and the rest of the gut. The tail region had also developed a slight kink in the last quarter and the gut region appeared cellular. Despite differences in early development, larvae in PBS culture were similar at this stage to those in Mapes II solution, although no kinking of the end of the tail was visible.

Very little further development in culture was seen after 8 - 9 days and dead larvae began to appear more frequently after

this time. Although some larvae maintained for up to four weeks in culture showed the presence of sheaths with truncated tails and "box-shaped" mouth parts characteristic of fourth stage larvae (L_4), in general the larvae appeared much the same as those seen at Day 9. No development of the genital areas could be seen.

Attempts to culture the exsheathed L_3 in the more complex media - Dulbecco's and the Chicken embryo extract - were unsuccessful when used either as primary media or as secondary media. Larvae survived for only up to 48 hours in culture in these more complex media and thereafter cultures were obscured by bacterial contamination despite observing careful aseptic handling and treatment of both cultures and culture vessels.

PART B: SURFACE LABELLING

Larvae that had been in culture for seven and 14 days were surface labelled with biotin, prepared for SDS-PAGE and electroblotted: the blots were blocked, incubated in ^{125}I-streptavidin and autoradiographed. The techniques used have been described previously in the Materials and Methods (Chapter Two).

RESULTS

The surface protein patterns from both the seven and 14 day cultured larvae were indistinguishable and similar to the surface patterns of *in vivo* derived fourth stage larvae but different from the surface patterns of exsheathed third stage larvae.

CONCLUSIONS

These experiments indicated that exsheathed third stage larvae cultured in either Mapes II solution or PBS were able to develop to the early fourth stage within seven days. It was also shown that Mapes II solution, which has a lower pH, allowed faster early development of larvae than PBS.

Prolonged culture in these media was not sufficient to allow further development, though some larvae seemed to have enough impetus to go on to the fourth moult as indicated by the presence of the fourth stage sheaths.

The failure of the larval cultures in either Dulbecco's medium or the Chicken embryo extract may indicate that these are inappropriate or that the conditions required for culture in these media are more exacting than those required for culture in media consisting of simple balanced salt solutions.

From the results obtained by surface labelling it appeared that the L_3 were able to change their coats without the third moult having occurred i.e. before L_4 were fully formed.

EXPERIMENT EIGHT

PROOF OF SURFACE LABELLING OF H. contortus LARVAE

INTRODUCTION

Previous work (Harley et al, 1985) has shown that the soluble vitamin biotin can be coupled to proteins and due to its high affinity for streptavidin can provide a sensitive method of identifying proteins separated by SDS-PAGE. As NHS-biotin was used in the study of surface and total parasite protein profiles,

it was necessary to confirm that it did indeed label only the surface proteins of intact worms and did not penetrate the cuticle. Two methods were used:-

METHOD A: USE OF THE DETERGENT CTAB

Since the cationic detergent CTAB has been used to strip the surface proteins of certain parasitic nematodes (Pritchard et al, 1985), it was assumed that if biotin was unable to penetrate the cuticle and therefore labelled only surface proteins, any biotinylated material would be stripped from the surface by the action of the detergent and would be present in the first supernatant; in contrast internal somatic proteins would only be released after the parasites were homogenised and therefore would not be labelled. Subsequent SDS treatment of CTAB stripped parasites would then allow the release of the remains of the body wall as well as any adhering muscle, whilst the final insoluble pellet might contain less soluble cuticular as well as somatic proteins.

EXPERIMENTAL DESIGN

First, second, third and fourth stage larvae as well as adult parasites were initially used in this investigation but later only third stage larvae, being the easiest to obtain, were used. A summary of the procedure is as shown previously in Table 1 (page 71).

RESULTS

Both autoradiographs and streptavidin peroxidase labelled blots of the supernatant extracts of surface proteins from larval and adult parasites produced similar results. In all cases the first supernatant (SN_1) containing the CTAB extracted proteins, showed polypeptides that corresponded in size to those detected in homogenates of biotinylated viable larvae or adults and which were a subset of the polypeptides detected by biotinylation of total homogenates. The second and third supernatants (SN_2 and SN_3) did not indicate the presence of any biotinylated proteins (clear strips on developing) whilst the insoluble pellet showed up several of these proteins when L_3 were examined but only faint or no polypeptide bands were seen when other H. contortus stages were used.

From this it was concluded that only the surface of the parasites was accessible to the biotin. Plate 2 shows the results obtained by autoradiography of surface biotinylated and CTAB stripped proteins from fourth stage larvae. Eight strong antigen bands of molecular weights 116, 109, 103, 97, 92, 71, 58 and 42 kDa appear in both tracks 1 and 2.

METHOD B: FLUORESCENT MICROSCOPY

Further proof that NHS-biotin was unable to penetrate the deeper layers of viable parasites was provided by fluorescent microscopy.

EXPERIMENTAL DESIGN

Only third stage larvae were examined. Viable exsheathed L_3

PLATE 2

Proof of Surface Labelling of *H. contortus* L_4 by Autoradiography

An autoradiograph of fractions from biotinylated whole fourth stage *H. contortus* larvae electroblotted and probed with ^{125}I-streptavidin is shown.

Lane 1: Surface labelled profile of whole larvae

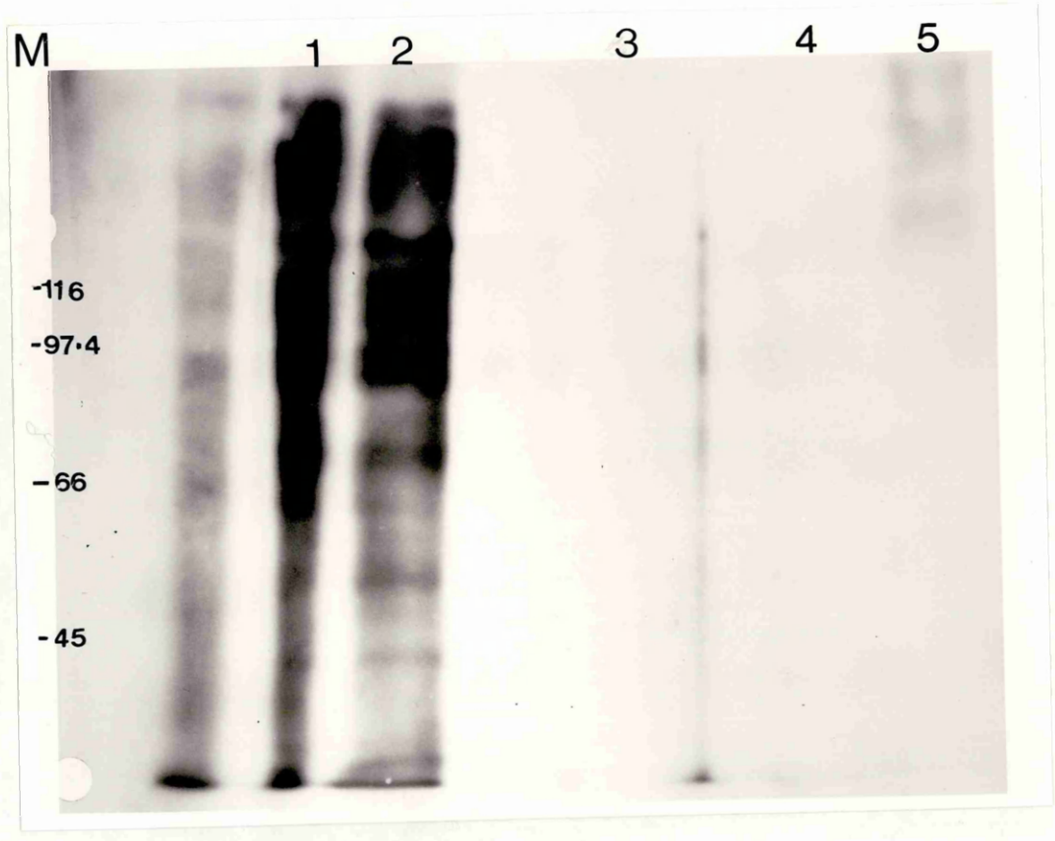
Lane 2: CTAB stripped proteins

Lane 3: Proteins released on homogenisation after CTAB treatment

Lane 4: Proteins removed from pellet by ST buffer

Lane 5: Proteins soluble in SDS and B-mercaptoethanol

M - Molecular weights markers in kDa.


M
1
2
3
4
5
-116
-97·4
-66
-45

exposed to NHS-biotin for 60 minutes, washed several times in PBS then incubated with Streptavidin-FITC for a further 30 minutes were examined under UV light. L_3 incubated in Streptavidin-FITC without prior exposure to NHS-biotin and L_3 incubated with NHS-biotin but incubated in CTAB before treatment with Streptavidin-FITC were also examined. All larval samples were prepared in duplicate and one sample of each was exposed to Evans Blue dye for 1 - 2 minutes before being viewed under UV light whilst the duplicate sample was observed in the absence of Evans Blue.

RESULTS

Plates 3 and 4 show the results of the fluorescence study. In the presence of Evans Blue dye, biotinylated L_3 treated with Streptavidin-FITC fluoresced a yellow-green colour whilst the non-biotinylated and CTAB treated larvae showed only a red fluorescence (Plate 3). In the absence of Evans Blue dye, autofluorescence was visible in all three samples of L_3 i.e. biotin-labelled, biotin-labelled/CTAB treated and untreated larvae (Plate 4).

CONCLUSIONS

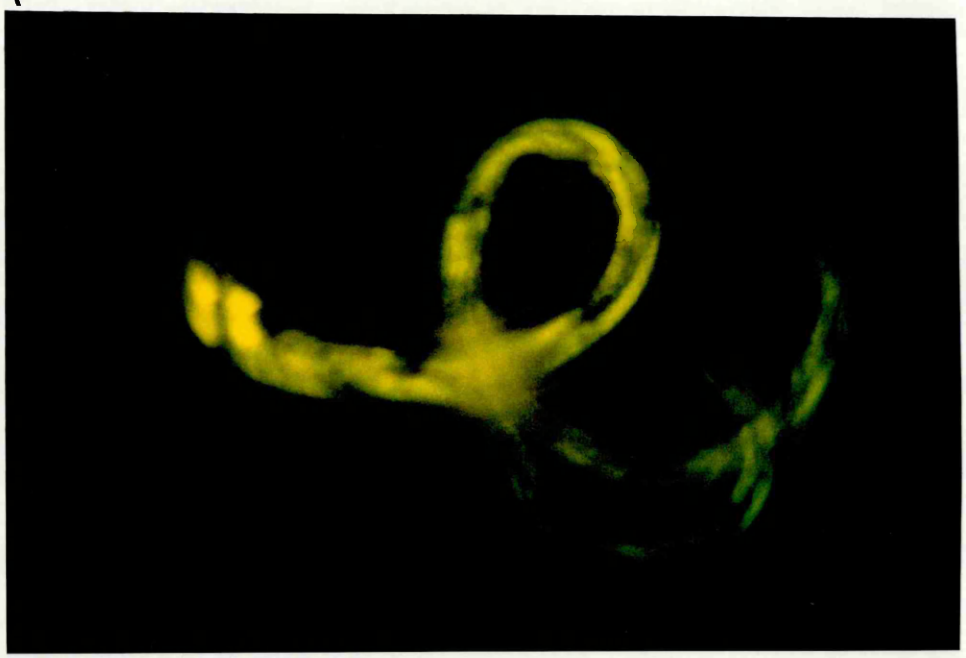
The findings of this experiment indicate that NHS-biotin is only able to label the surface of the cuticle of H. contortus L_3 and that the detergent CTAB is able to remove the labelled surface proteins from the parasites.

PLATE 3

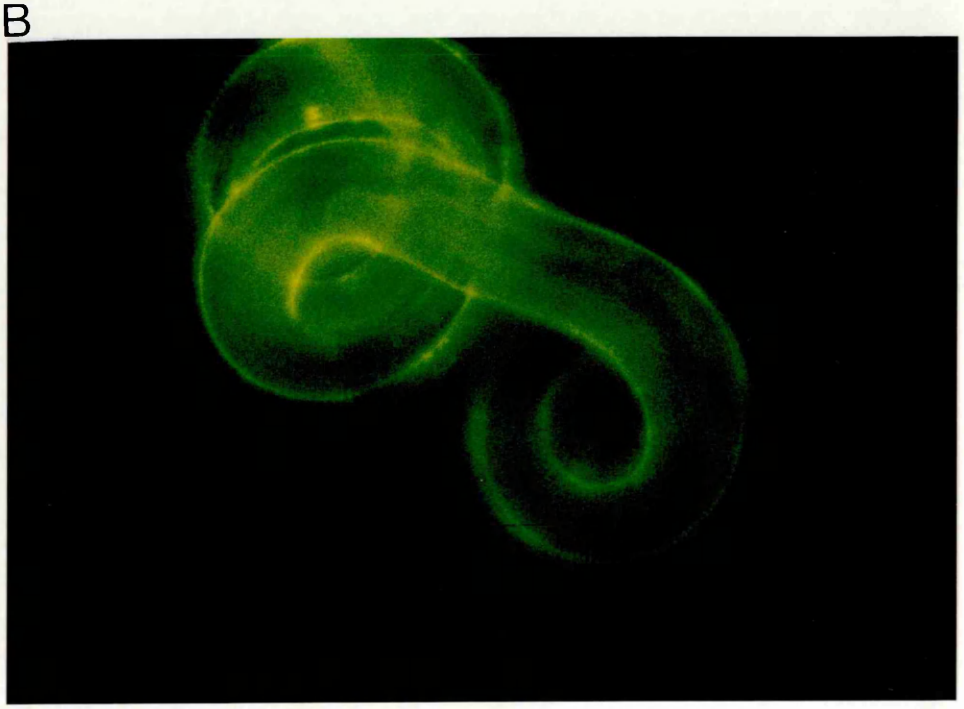
Proof of Surface Labelling of *H. contortus* L_3 by Fluorescence in the Absence of Evans Blue

This series of plates show the results of incubating viable exsheathed *H. contortus* L_3 in streptavidin - FITC without prior treatment (Plate 3A), after exposure to NHS-biotin (Plate 3B) and after exposure to NHS-biotin followed by exposure to the detergent action of CTAB (Plate 3C). Internal autofluorescence is visible in both Plates 3A and 3C.

A



B



C

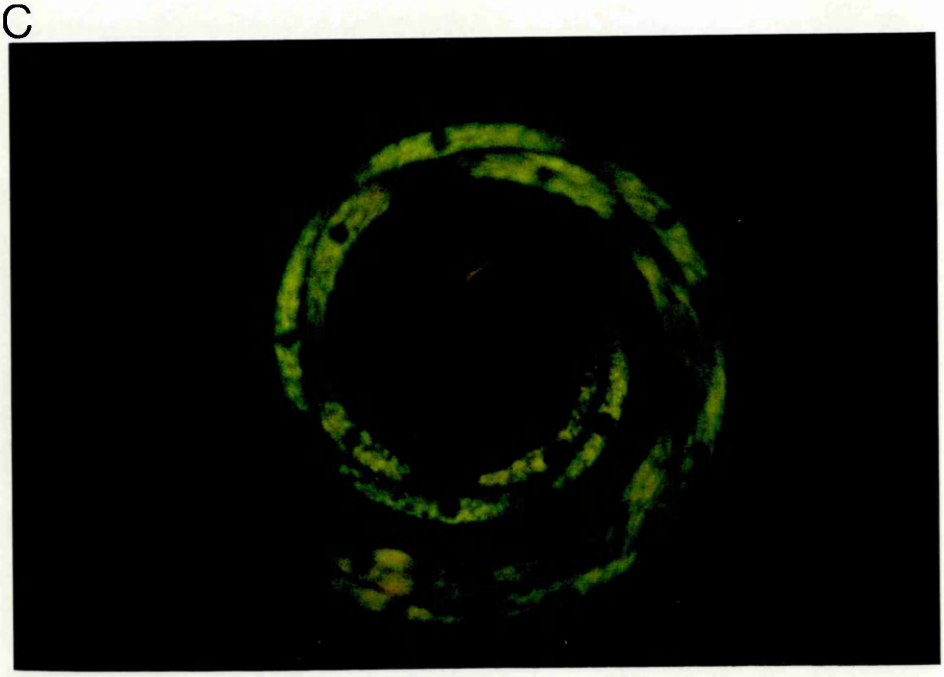


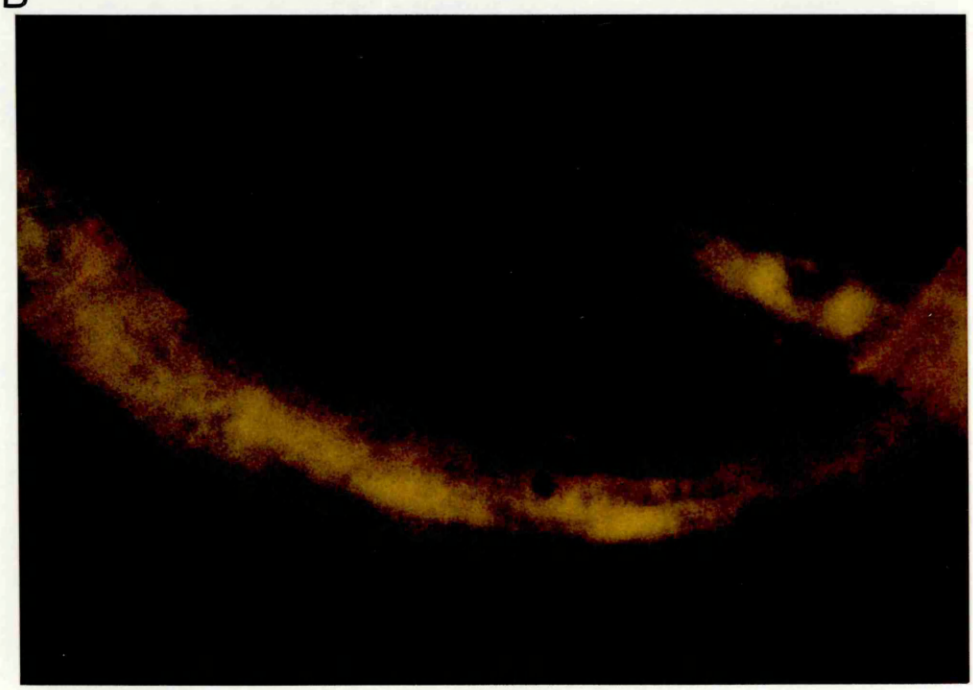
PLATE 4

Proof of Surface Labelling of *H. contortus* L_3 by Fluorescence in the Presence of Evans Blue

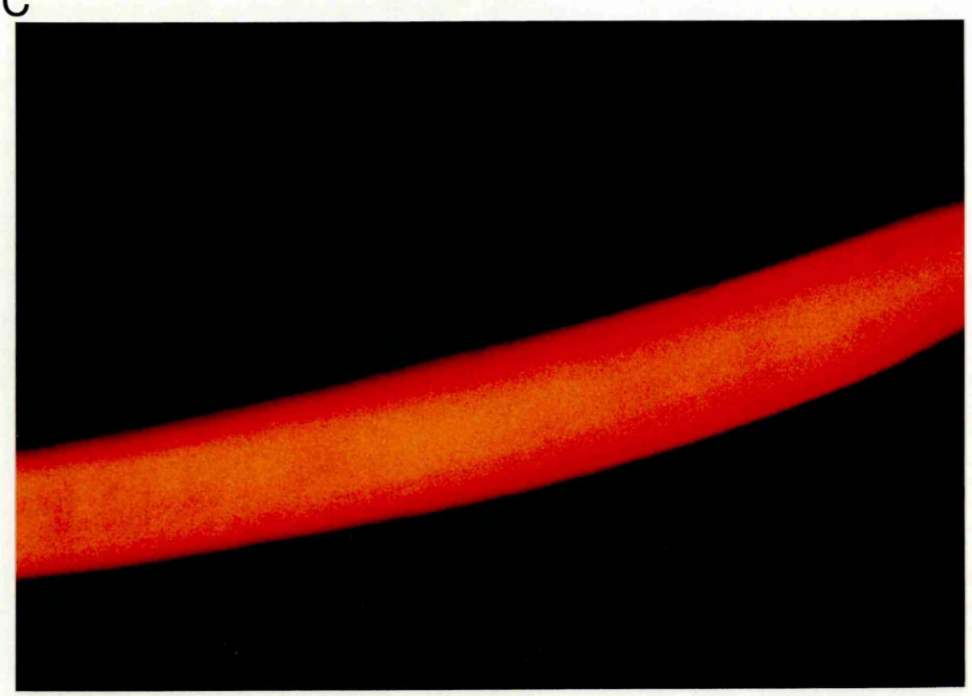
This series of plates show the results of incubating viable exsheathed *H. contortus* L_3 in streptavidin - FITC without prior treatment (Plate 4A), after exposure to NHS-biotin (Plate 4B) and after exposure to NHS-biotin followed by the exposure to the detergent action of CTAB (Plate 4C). In these plates incubation in streptavidin-FITC was followed by incubation of the larvae in Evans Blue dye for 1-2 minutes and this had the effect of quenching any autofluorescence and produced the red fluorescence visible in Plates 4A and 4C indicating negative surface fluorescence as opposed to the positive yellow-green fluorescence seen in Plate 4B.

A

B



C



EXPERIMENT NINE

H. contortus SURFACE AND TOTAL HOMOGENATE PROTEIN PROFILES

INTRODUCTION

The aim of this experiment was to compare the composition of both surface and total worm (somatic) protein profiles of the various stages of the parasite, and to characterise as far as possible, the surface proteins of third stage *H. contortus* larvae.

Both larval and adult stages of the parasite *H. contortus* were investigated and in the case of third stage larvae these were always exsheathed prior to any investigation.

EXPERIMENTAL DESIGN

First (L_1), second (L_2) and infective third (L_3) stage larvae were obtained from culture of infected faeces incubated at 26°C for varying lengths of time and subsequently harvested by the Baermann technique. Fourth larval (L_4) and adult stages were obtained by recovering these from the abomasal mucosa or contents of infected sheep.

All parasites were cleaned by flotation in 30% sucrose followed by several washes in 0.85% saline, after which L_3 parasites were exsheathed in a [redacted]1% sodium hypochlorite solution then washed several times in PBS (pH 7.4).

Biotinylation of surface proteins and homogenates of each stage, electrophoresis, electroblotting and the development of nitrocellulose blots were carried out as described earlier (page 67). An SDS gel of homogenised L_1,L_2, L_3 and adult

parasites was also run and stained with Coomassie blue for comparison.

RESULTS

The results of this experiment are shown in Plates 5 and 6. The Coomassie stained gel and the biotinylated homogenate blots (Plate 5) showed similar banding patterns for each stage of the parasite. The number of bands found using different stages is shown in Table 9.

Table 9

Number of Protein Bands Evident on a Coomassie Stained Gel and Nitrocellulose Blots of Biotinylated Homogenates

Stage	Coomassie Gel		Nitrocellulose Blot	
	Major	Minor	Major	Minor
L_1	9	11	7	-
L_2	8	7	8	-
L_3	4	12	9	-
Adult	5	4	6	-

For each of the four stages of parasite investigated the surface protein profile (Plate 6) appeared as a subset of the total worm profile with an average of 2 - 4 antigenic bands visible. Most of the antigens stained for L_3 surface occurred between 116 and 45 kDa with major antigens appearing at 90, 76 and 42 kDa. The L_1 and L_2 antigens which appeared were similar having molecular weights of approximately 76 and 60 kDa.

PLATE 5

Labelled Protein Profiles of *H. contortus* Homogenates

A Coomassie stained gel showing the homogenate (somatic) protein profiles of larval and adult stages of *H. contortus* is shown in Plate 5A. Plate 5B shows the homogenate biotinylated protein profiles of the same parasite stages but after electroblotting, probing with streptavidin-peroxidase and development of the nitrocellulose blot.

Lane 1: First stage larvae (L_1)

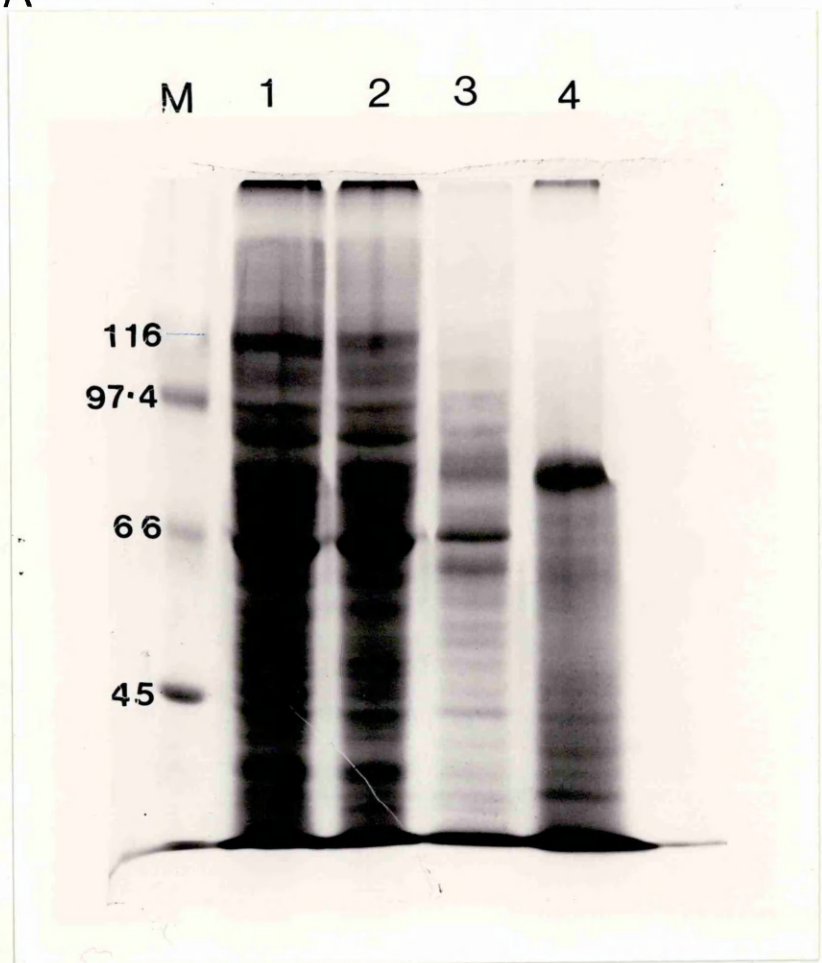
Lane 2: Second stage larvae (L_2)

Lane 3: Third stage larvae (L_3)

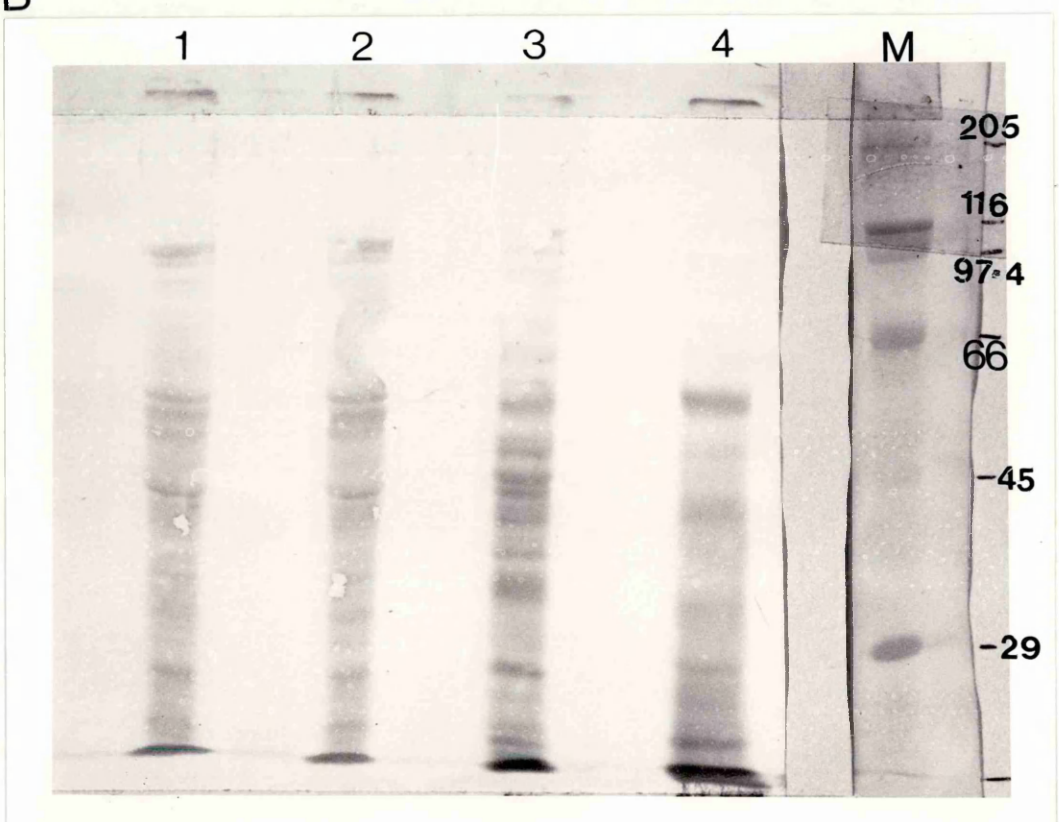
Lane 4: Adult parasites (Ad)

M - Molecular weight markers in kDa.

A



B



Many of the proteins were seen to be common to all stages although some distinct changes, both qualitative and quantitative, were visible. The complex banding pattern of the total protein profiles was more evident in the Coomassie stained gel than in the nitrocellulose blot where more bands were visible especially minor bands. The approximate molecular weights of antigens from each stage are shown in Table 10.

Table 10

Molecular Weights of Stained Antigen Bands

Stage	Molecular Weights (kDa)	
	Coomassie Gel	Nitrocellulose Blot
L_1 and L_2	101,92,86,80,70,59,47,38	101,80,78,66,38,
L_3	88,83,72,65,45	79,72,65,55,49,48
Adult	83,45,50,33	80,62,50,28

CONCLUSIONS

The results obtained from this experiment indicated that, whilst there are several proteins in common between the various stages of the parasite, there are some that are specific to individual stages. The results also showed a similarity in the proteins of the two free-living stages i.e. the first and second stage larvae as well as a large variation of proteins in the adult parasite.

PLATE 6

Surface Labelled Protein Profiles of H. contortus

Nitrocellulose blots showing the surface biotinylated protein profiles of larval and adult H. contortus after probing with streptavidin-peroxidase.

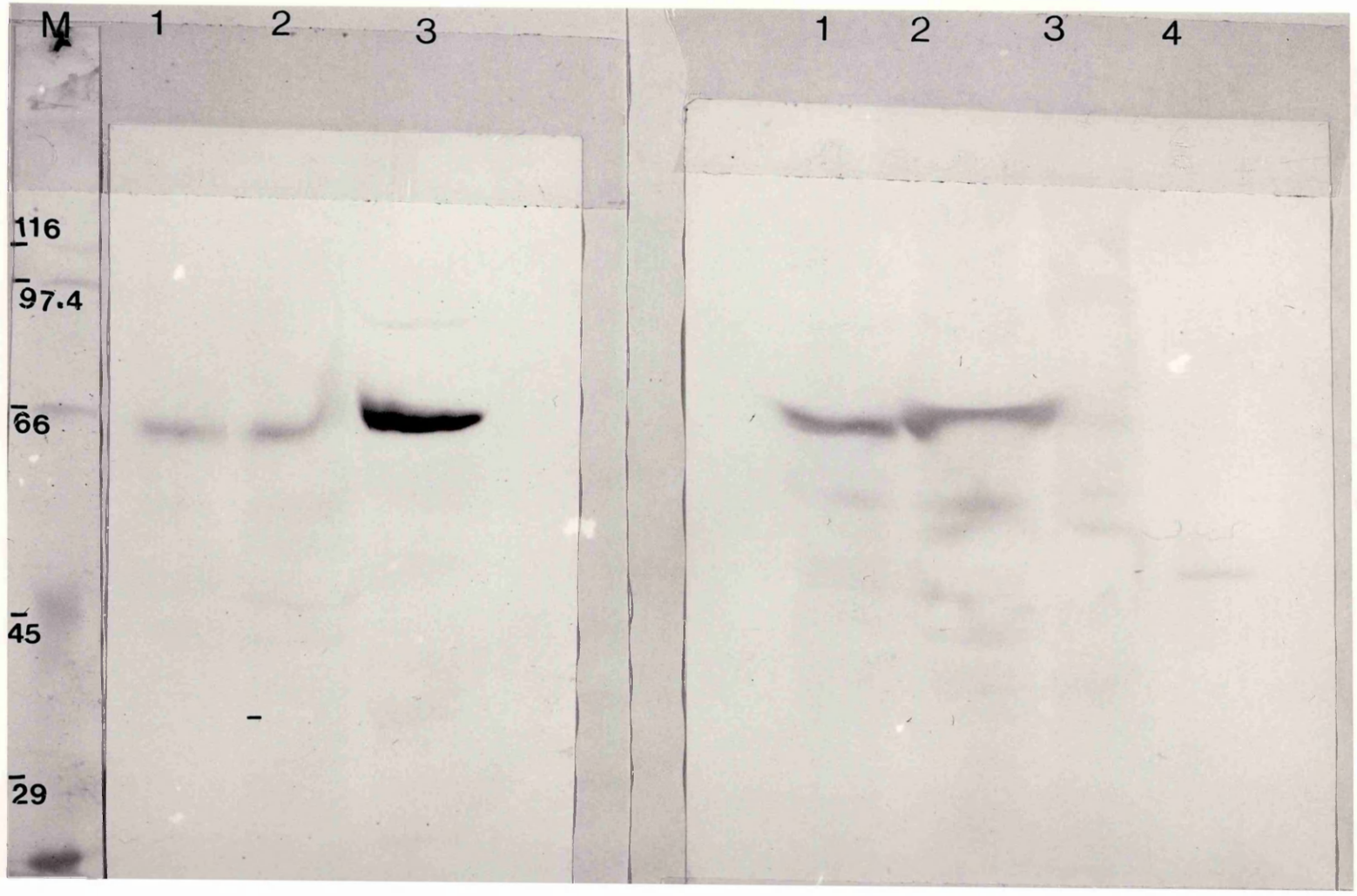
Lane 1: First stage larvae (L_1)

Lane 2: Second stage larvae (L_2)

Lane 3: Third stage larvae (L_3)

Lane 4: Adult parasite (Ad)

M - Molecular weight markers in kDa.


M
1
2
3
1
2
3
4
116
97.4
66
45
29

EXPERIMENT TEN

COLLAGENASE DIGESTION OF SURFACE PROTEINS

INTRODUCTION

Many of the molecules located on the surface of nematode parasites are proteins, however, below the cuticle surface collagens are a major structural component. This experiment was carried out to determine if any of the detergent extracted molecules of *H. contortus* L_3 were actually collagens and therefore indicating that the detergent extraction process had stripped proteins below the cuticular surface.

EXPERIMENTAL DESIGN

Collagenase was applied to several 200 ul aliquots of surface biotinylated exsheathed homogenates of third stage larvae. Non-collagenase treated samples were run in parallel as controls as described on page 74.

RESULTS

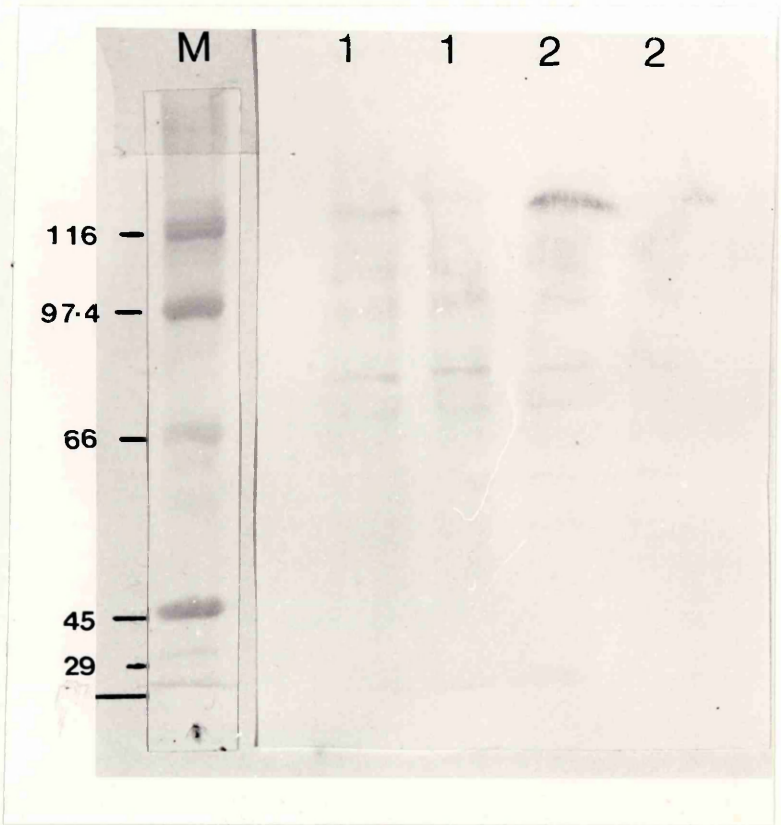
The results of this experiment are shown in Plate 7. Collagenase treatment was observed to have no effect on the biotin-labelled surface proteins as there was no difference in the banding patterns of the collagenase treated and collagenase free samples. In both samples five polypeptide bands were visible on staining the blots which had approximate molecular weights of 122, 110, 101, 86 and 78 kDa.

PLATE 7

Collagenase Digestion of *H. contortus* L_3 Surface Proteins

A nitrocellulose blot of third stage *H. contortus* larvae surface biotinylated, homogenised and treated with collagenase- Lane 1: untreated larvae - Lane 2:

M - Molecular weight markers in kDa.


M
1
1
2
2
116
97·4
66
45
29

CONCLUSIONS

The results of the collagenase experiment suggested that the molecules to which the biotin was able to bind were only those proteins located on the cuticular surface and that biotin was unable to penetrate below this superficial layer and bind to collagen.

EXPERIMENT ELEVEN

CHARACTERISATION OF SURFACE PROTEINS OF *H. contortus* L_3

INTRODUCTION

It has been known for some time that the surface proteins on the nematode cuticle are recognised by the immune system (Grencis *et al*, 1986) and that antibodies are produced against these proteins. Since the experiments described earlier in this thesis were designed to examine the protective nature of surface proteins, it was considered appropriate to examine the nature and composition of these antigens.

EXPERIMENTAL DESIGN

Exsheathed L_3 were incubated at $37^{o}C$ for six hours in the detergent CTAB to remove the surface proteins from the cuticle. The larvae were then sedimented by centrifugation at 2,500 rpm for ten minutes and the supernatant (CTAB and surface proteins) decanted. This was then dialysed for three days in PBS pH 7.4 at $4^{o}C$ to remove the detergent and 70 - 100 ul aliquots of the dailysed CTAB sample together with a 10 ul aliquot of L_3 homogenate were then analysed by SDS-PAGE. The resultant gel was

stained with silver nitrate to visualise the protein bands. High molecular weight markers were run on the gel adjacent to the two protein extracts to indicate the molecular weights of the separated proteins. A second gel was stained with Coomassie for comparison.

RESULTS

The results of this experiment are shown on Plate 8. Five polypeptide bands were visualised by the silver staining of CTAB extracted surface proteins with apparent molecular weights 137, 88, 74, 66 and 59 kDa. The bands were, however, fairly weak compared with the numerous bands visualised with the L_3 homogenate on silver staining. Some of the strongly staining antigen bands of the L_3 corresponded with the antigens from the CTAB preparation for example the antigens at 135, 88, 74 and 66 kDa.

No bands were visible on the Coomassie stained gel when CTAB stripped proteins were run in contrast to many polypeptide bands when the L_3 total homogenate was used.

CONCLUSIONS

The successful detergent extraction of surface proteins was confirmed by the presence of proteins on the gels. The quantity and number of proteins was however low indicated by the weak colour reaction and small number of bands. The fact that there are few proteins on the L_3 surface was also suggested by the absence of bands from the CTAB preparation on the Coomassie stained gel.

PLATE 8

Characterisation of Surface Proteins of *H. contortus* L_3

A silver stained mini-gel comparing the proteins separated by SDS-PAGE from a CTAB stripped L_3 extract and total protein profile of homogenised third stage *H. contortus* larvae is shown in Plate 8A.

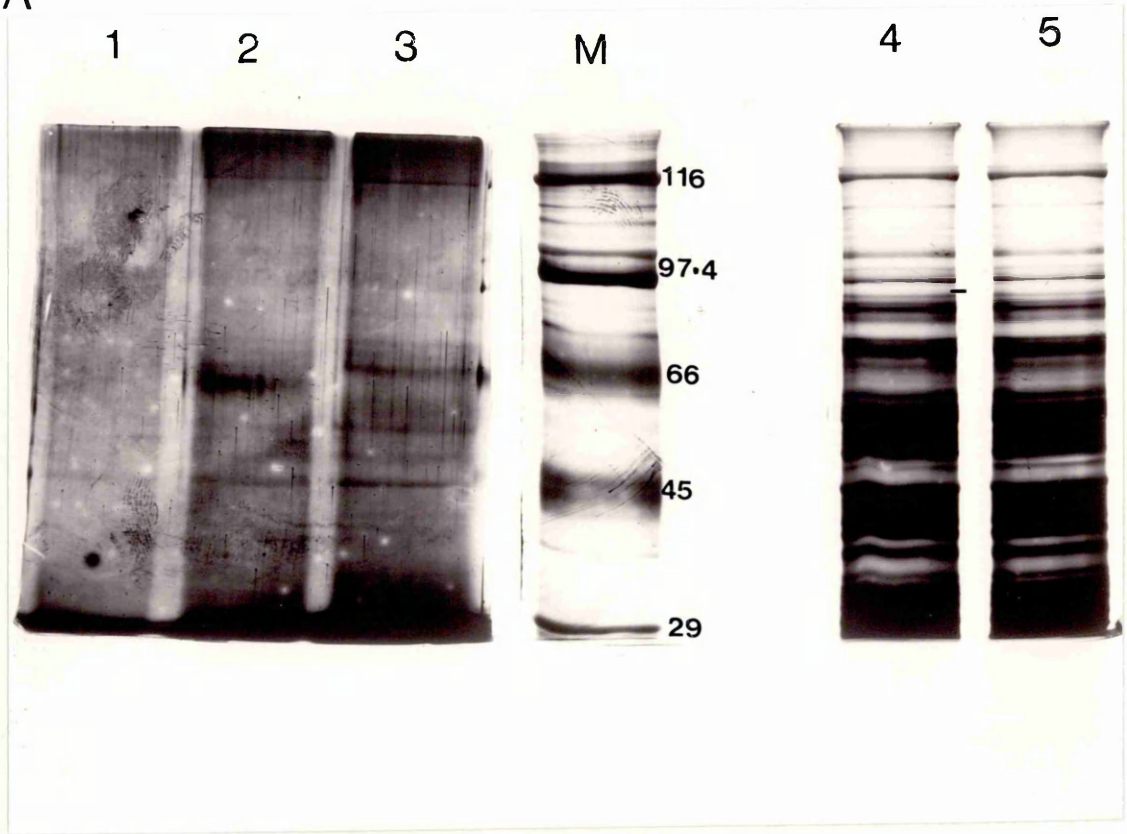
Lanes 1, 2 and 3: CTAB L_3 extract protein profile

Lanes 4 and 5: Third stage total protein profile

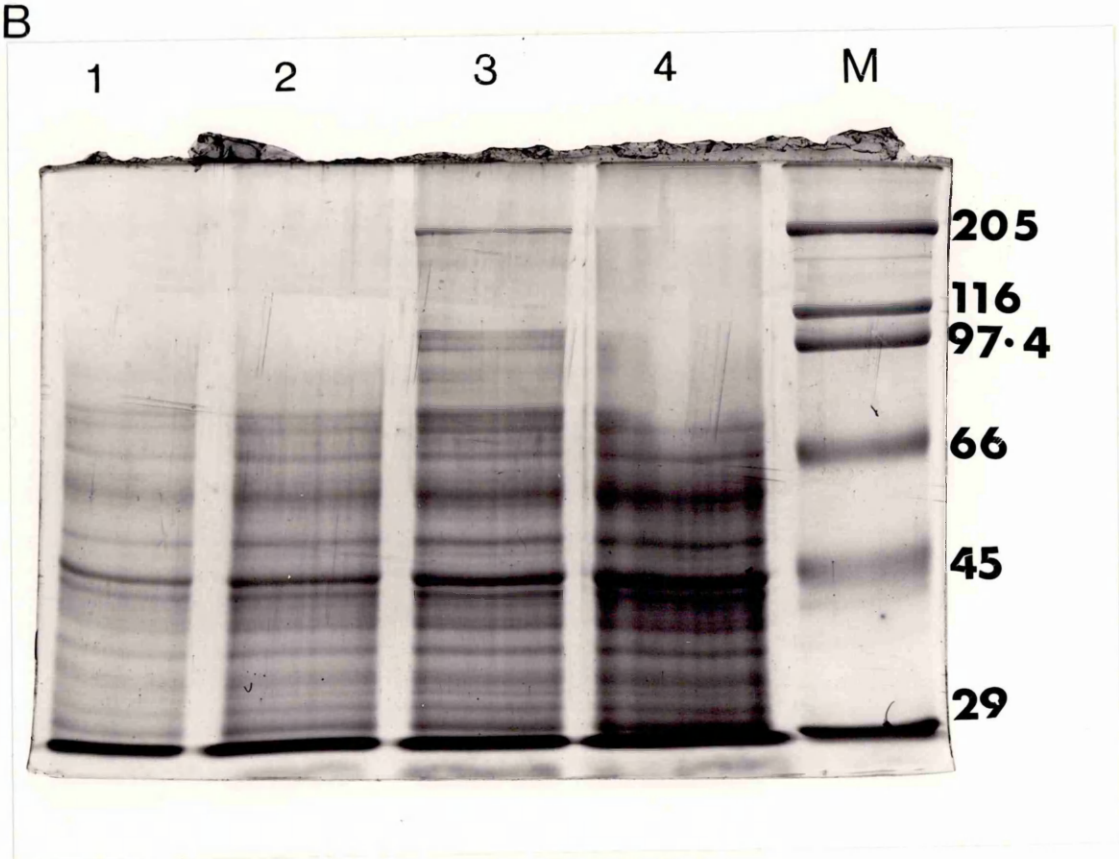
Plate 8B shows a Coomassie stained mini-gel of the total protein profile of homogenised L_3 in Lanes 1 - 4.

M - Molecular weight markers in kDa.

A



B



EXPERIMENT TWELVE

H. contortus SURFACE IMMUNOFLUORESCENCE

INTRODUCTION

Fluorescent microscopy can be used in the study of serum antibodies which recognise the surface of nematode parasites. In the immunofluorescence studies described here viable L_3 and adult H. contortus were used to investigate the presence and nature of antibodies in hyperimmune serum and in sera from animals used in the immunisation and vaccination experiments described previously.

PART A: HYPERIMMUNISATION

EXPERIMENTAL DESIGN

The immunofluorescence procedure has been described previously (page 76) and a flow chart was shown in Figure 3 (page 78).

Exsheathed third stage larvae were incubated in sera from the hyperimmune animal (Y8) for 30 minutes, incubated in FITC-second antibody for a further 30 minutes then observed under UV light. Both incubations were carried out on ice to reduce the movement of the larvae and thus facilitate observation. Sera from non-immune lambs was also examined.

RESULTS

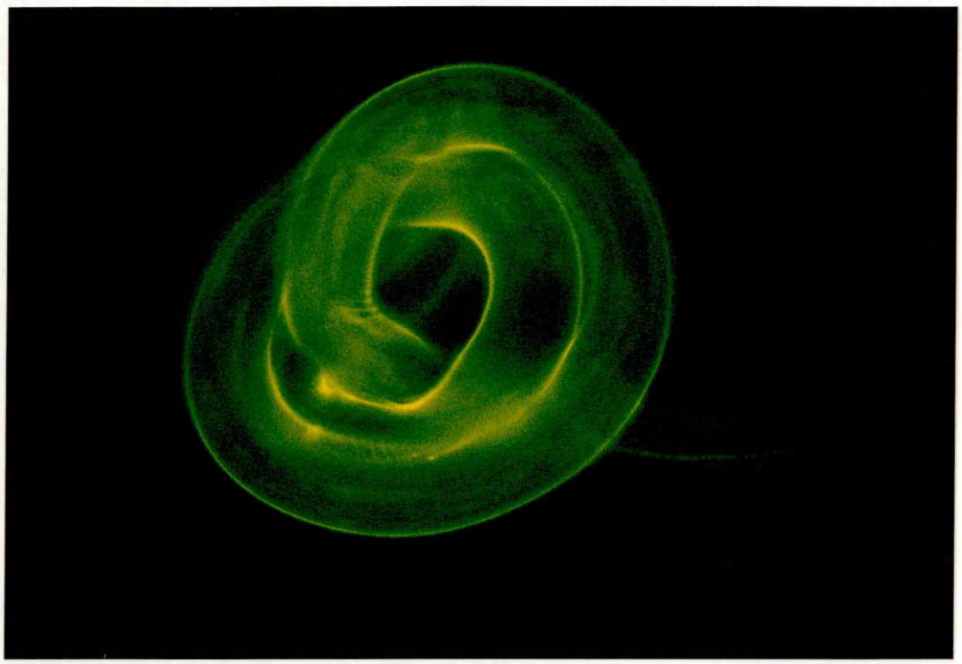
The result of this experiment showed positive surface fluorescence on larvae incubated in hyperimmune serum: when larvae were incubated in serum from non-immune young lambs no

PLATE 9

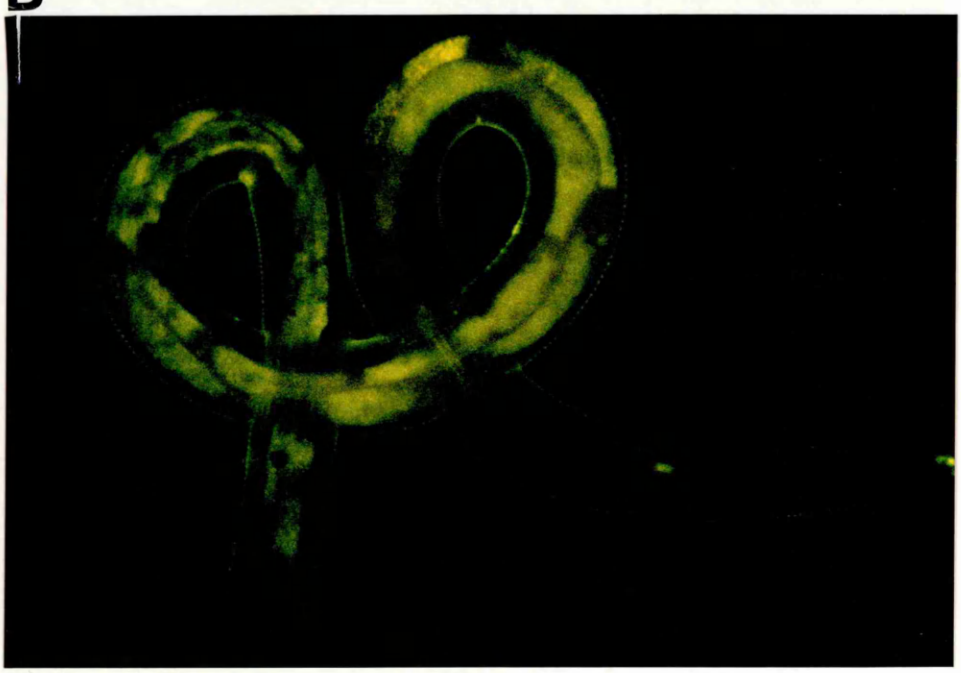
H. contortus L_3 Surface Immunofluorescence with Hyperimmune and Control Sera

Plate 9A shows the positive surface fluorescence obtained when exsheathed L_3 were incubated in sera from a hyperimmune adult sheep, whilst Plate 9B shows the negative surface fluorescence (with internal autofluorescence) of the L_3 after incubation in sera from non-immune young lambs.

A



B



surface fluorescence was observed, although internal autofluorescence was visible (Plate 9).

PART B: IMMUNISATION

EXPERIMENTAL DESIGN

Sera from the two immunised sheep from Experiment One were examined for the presence of antibody to surface antigens. Control serum was obtained from a parasite-naive sheep. The test serum samples were initially titrated at varying dilutions and subsequently a 1:50 dilution was selected.

Three sets of exsheathed third stage larvae were prepared. One set was incubated first in serum from the sheep immunised with L_3 surface proteins, the second in serum from the sheep immunised with L_4 surface proteins and the third set in normal serum.

RESULTS

The results from this part of the experiment can be seen in Plate 10.

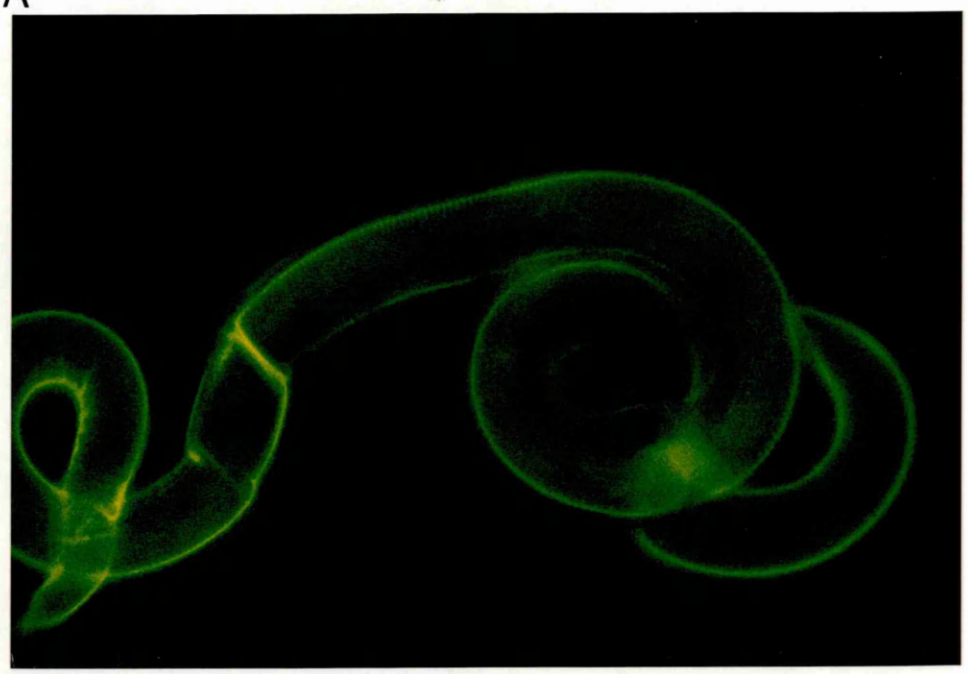
Fluorescence was observed on the surface of the third stage larvae incubated in both sets of sera from the vaccinated sheep (Plates 10A and 10B), but none with normal serum (Plate 10C). Fluorescence was more distinct with sera from the sheep vaccinated with the fourth stage larval antigens, and both vaccinated sheep sera produced clear fluorescence at the lower dilutions. This indicated that the antibody response to injected fourth stage proteins might consist of components shared between the L_3 and L_4 antigens with a higher titre to the infective third

PLATE 10

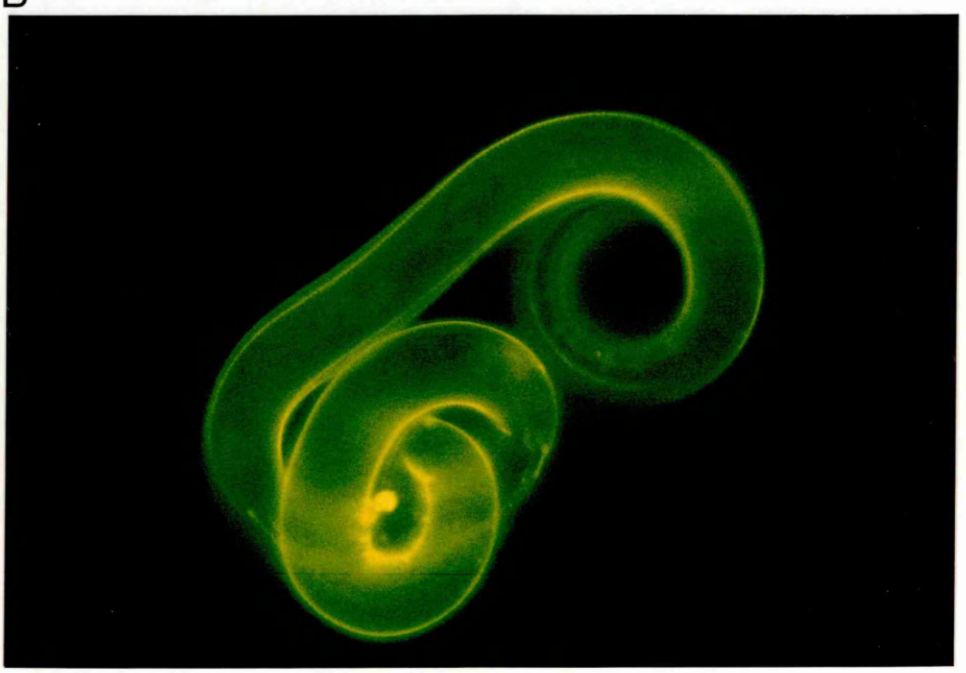
H. contortus L_3 Surface Immunofluorescence: Experiment One

Plates 10A and 10B show positive surface fluorescence resulting from the incubation of exsheathed viable L_3 in sera from surface protein immunised sheep: internal autofluorescence with little surface fluorescence was seen when larvae were incubated in serum from the non-immunised control animal (Plate 10C). Larvae in all three plates were exposed to FITC-second antibody.

A



B



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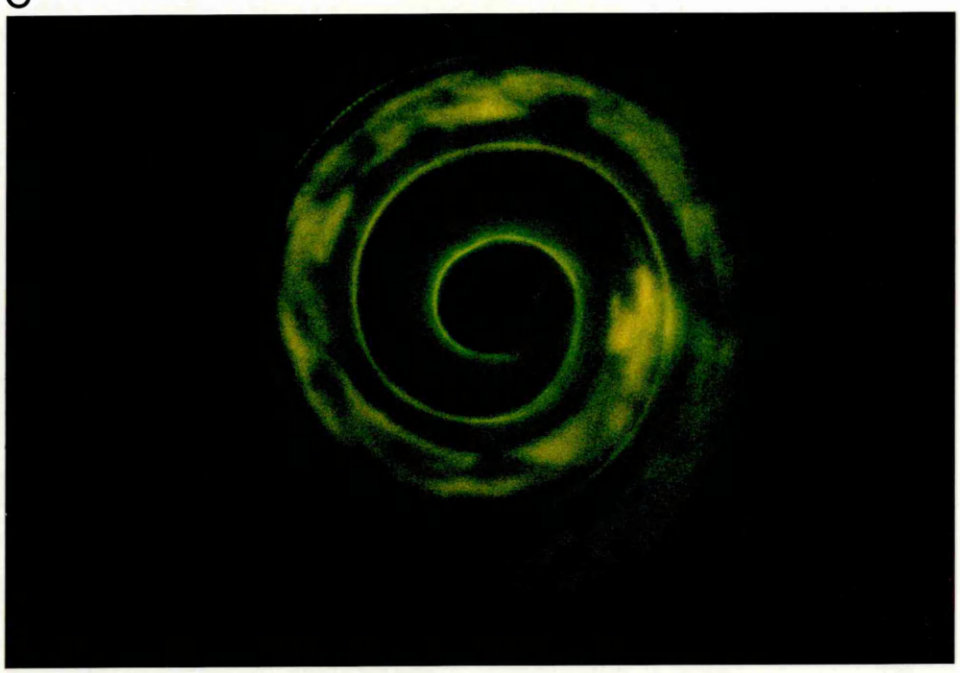


stage larvae as shown by the greater degree of fluorescence with this sera. This also suggests that the parasites used in the immunisation obtained by the in vitro culture may not all have been L_4.

PART C: VACCINATION

EXPERIMENTAL DESIGN

Pre-challenge sera from the CTAB surface protein vaccination Experiments Two, Four and Five were examined against viable L_3, while the sera from Experiment Six where somatic adult and larval antigens were used, were examined against either viable L_3 or adult parasites. In all cases a 1:50 dilution of both sera and the fluorescent label was used.

About 200 exsheathed L_3 were incubated in sera obtained in the week prior to challenge from both vaccinated and control animals from each experiment. Approximately ten adult parasites were incubated in the sera collected from the animals in Experiment Six which were vaccinated with adult somatic antigens: serum from the animals vaccinated with L_3 somatic antigens in this experiment was also examined to determine cross-reactivity. Known negative serum samples from parasite-naive animals were included as additional controls.

A titration of serum antibody levels for the hyperimmunised sheep, one surface protein immunisation lamb (Experiment One) and one animal from the vaccination Experiments Two, Four and Five, prior to challenge, was carried out with sera diluted at 1:25, 1:50, 1:100, 1:200, 1:400, 1:800 and 1:1000.

RESULTS

Results from the initial experiments are shown in Plates 11 - 14.

The sera of the vaccinated sheep from all experiments produced fluorescence on the surface of exsheathed L_3 although the intensity of fluorescence varied. Sera from Experiments Two and Five produced the strongest surface fluorescence (Plates 11 and 13 respectively), whereas serum from Experiment Four produced a weak fluorescence (Plate 12). The results from Experiment Six showed some surface fluorescence, although again this was very weak (Plate 14). There was some slight fluorescence on the surface of L_3 incubated in sera from sheep vaccinated with adult antigens, but adult parasites incubated in sera from animals vaccinated with L_3 antigens showed no surface fluorescence. L_3 incubated in serum from animals vaccinated with L_3 somatic antigens and adults incubated in sera from animals vaccinated with adult somatic antigens also produced a very slight fluorescence (Plate 14). All sera from non-vaccinated sheep i.e. the control groups produced no fluorescence.

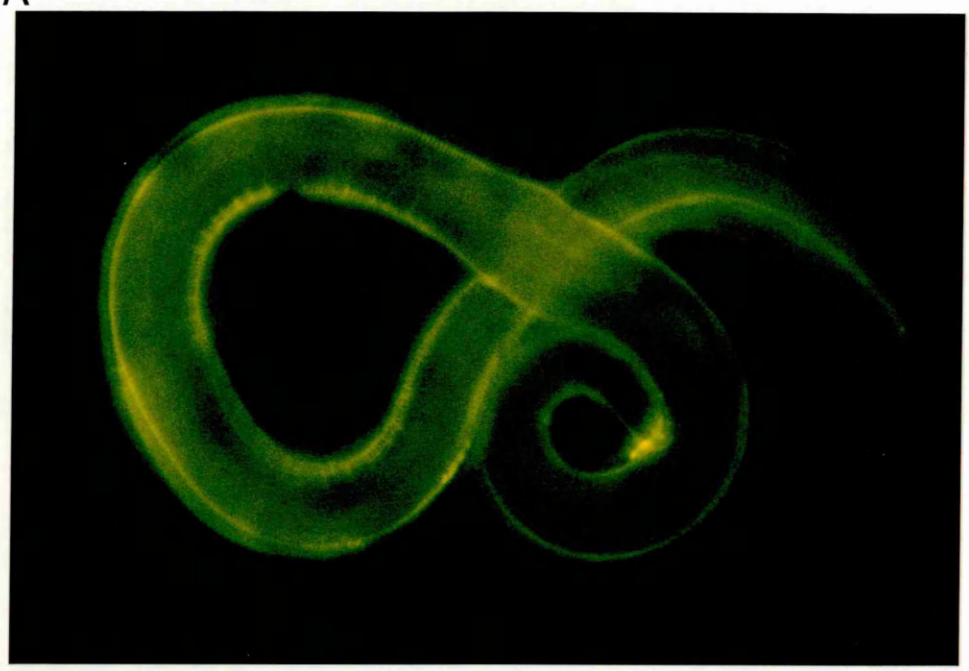
The results of the antibody titration experiment are shown in Plates 15 - 19 and scored subjectively in Table 11. The results for 1:25 and 1:50 dilutions were similar, therefore only the 1:50 results are shown.

PLATE 11

H. contortus L_3 Surface Immunofluorescence: Experiment Two

Plate 11A shows the positive fluorescence on the surface of exsheathed and viable *H. contortus* L_3 after incubation in sera from vaccinated animals followed by exposure to FITC-second antibody: Plate 11B shows the result with sera from non-vaccinated animals.

A



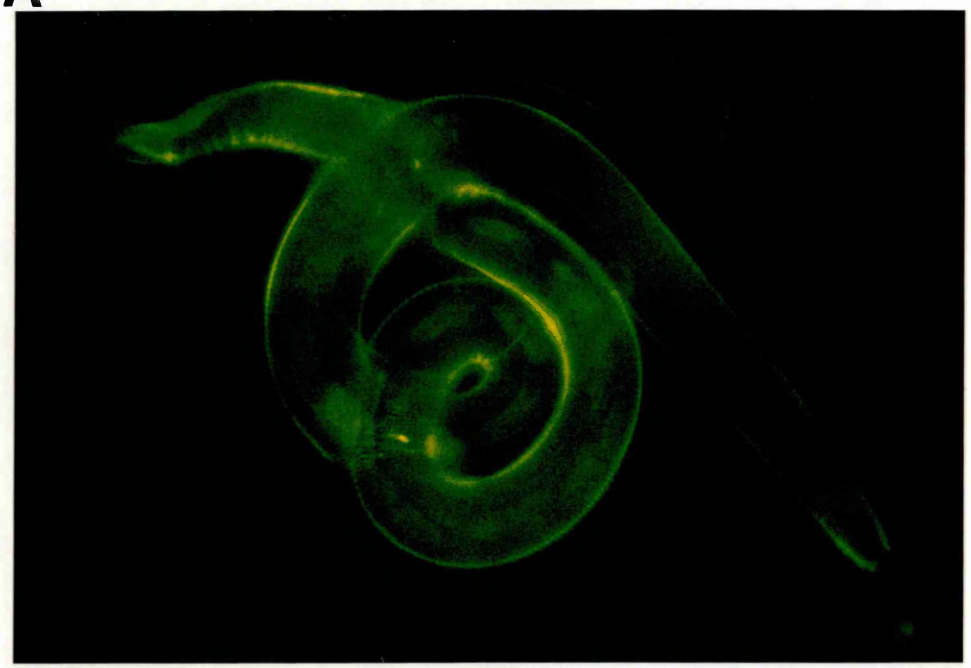
B

PLATE 12

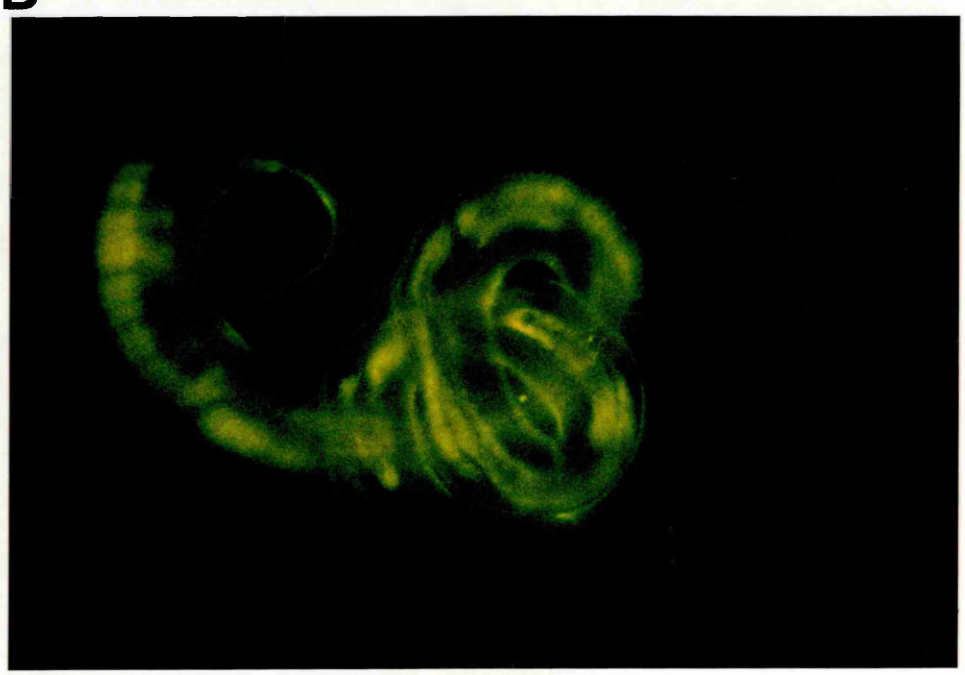
H. contortus L_3 Surface Immunofluorescence: Experiment Four

Plate 12A shows positive surface immunofluorescence of exsheathed and viable _H. contortus_ L_3 incubated in sera from vaccinated sheep: Plates 11B and 11C show the negative result (with internal autofluorescence) after L_3 were incubated in sera from animals 'immunised' with Freunds Adjuvant (Plate 12B) and non-immunised control sera (Plate 12C).

A



B



C

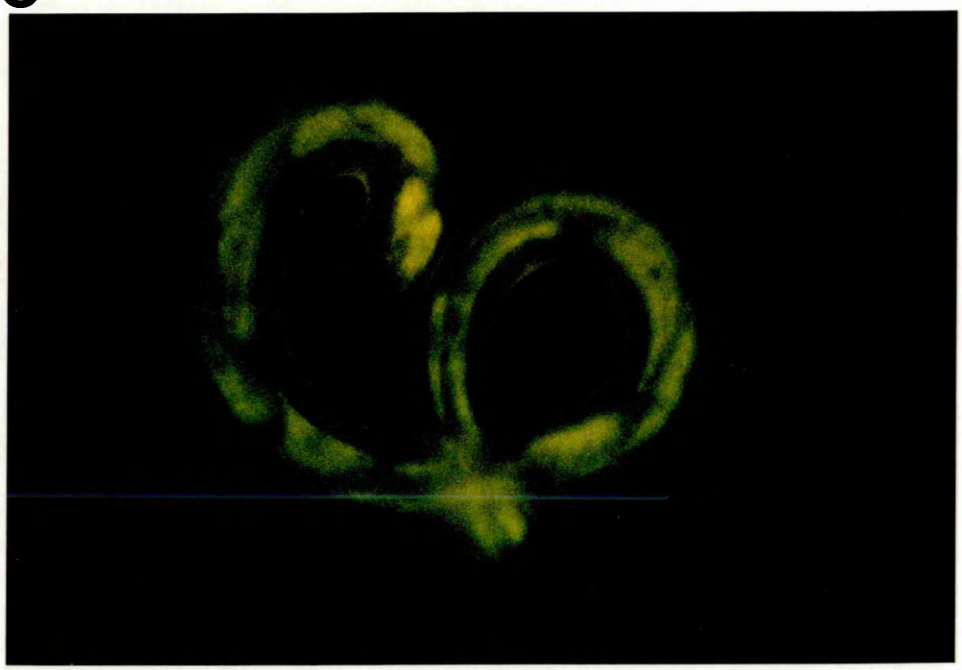
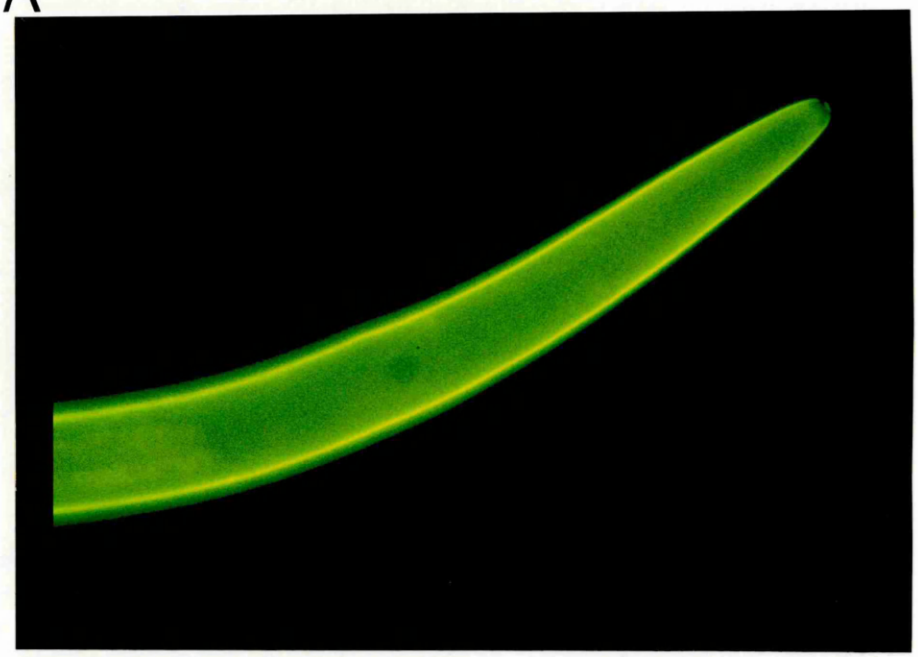


PLATE 13

H. contortus L_3 Surface Immunofluorescence: Experiment Five

Plate 13A shows strongly positive surface fluorescence of exsheathed and viable _H. contortus_ L_3 after incubation in sera from vaccinated sheep and Plate 13B the absence of surface fluorescence when larvae were incubated in sera from non-vaccinated animals: in each case larvae were exposed to FITC-second antibody.

A



B

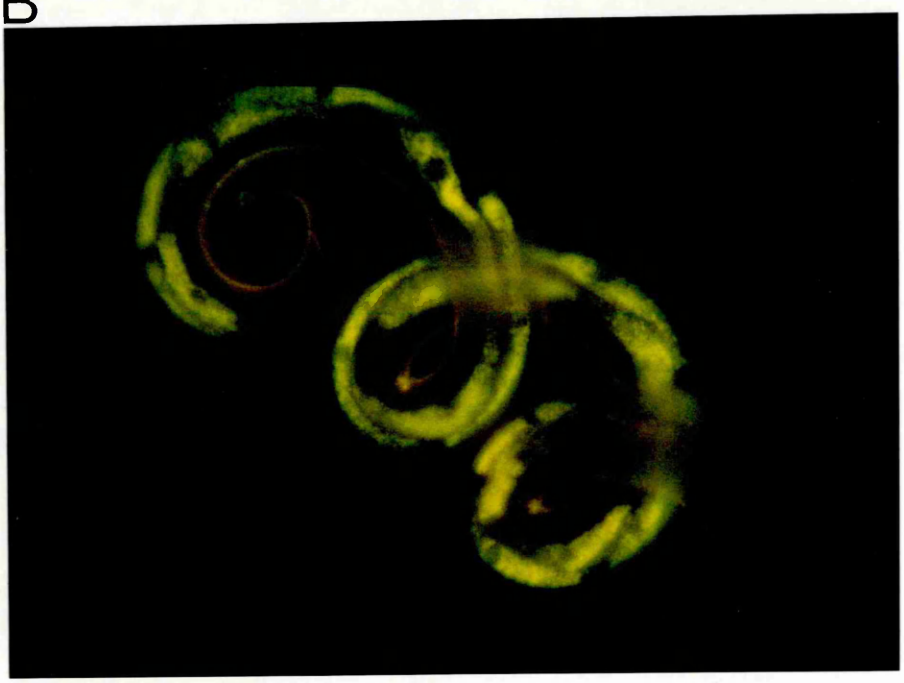


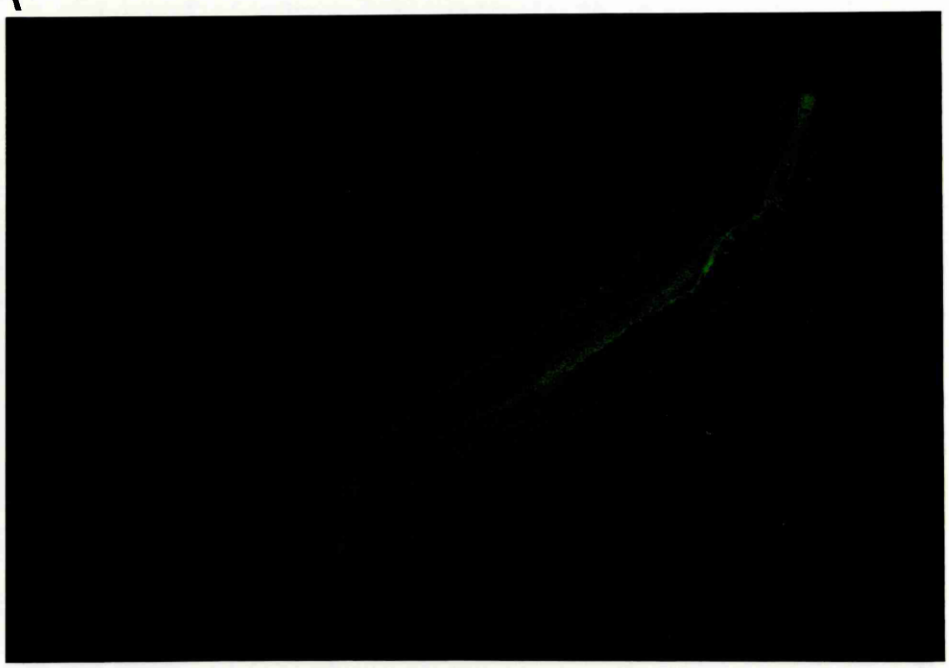
PLATE 14

H. contortus L_3 and Adult Surface Immunofluorescence: Experiment Six

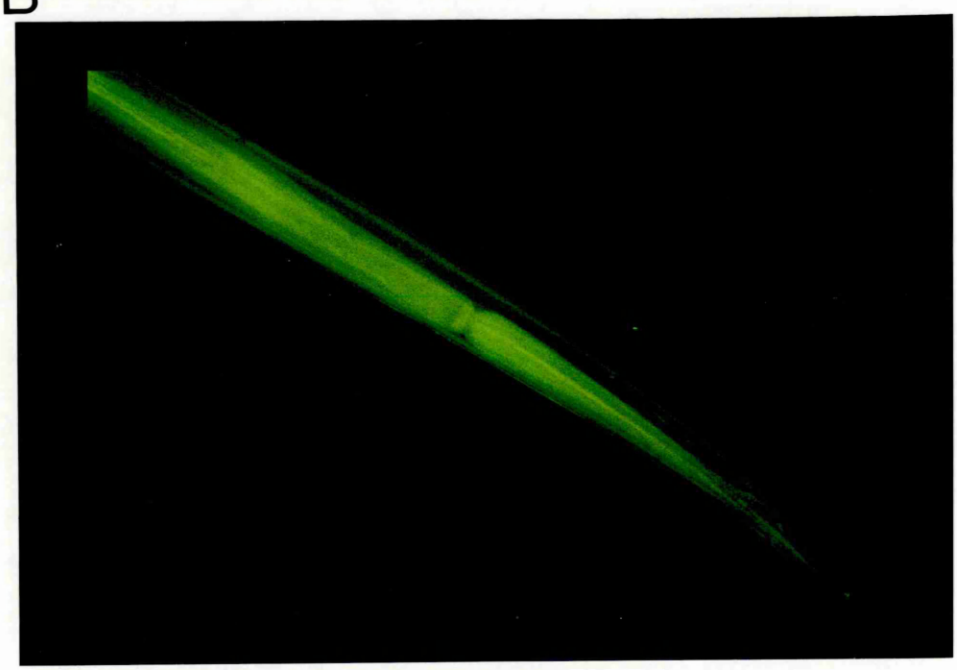
Plates 14A and 14B show the absence of immunofluorescence on the surface of adult *H. contortus* incubated in sera from sheep vaccinated with L_3 somatic antigens and adult somatic antigens respectively.

Plate 14C shows some light immunofluorescence on the surface of L_3 incubated in sera from sheep vaccinated with adult somatic antigens.

A



B



C

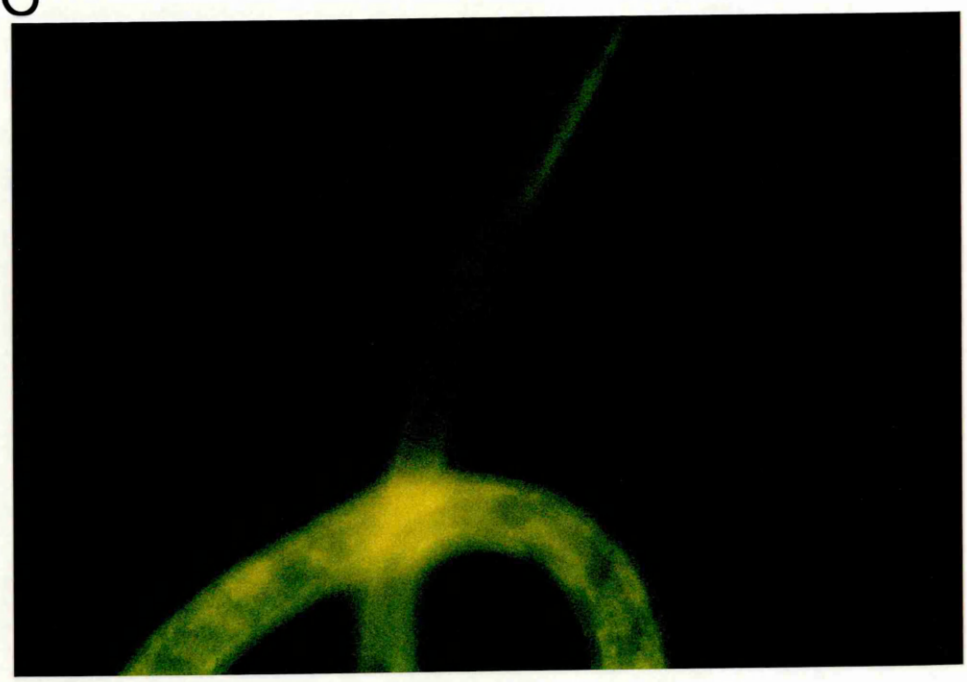


Table 11

Immunofluorescence Scores in a Serum Antibody Titration Against Viable Infective Larvae

	1:50	1:100	1:200	1:400	1:800	1:1000
HyI	+++	++	++	+	+	+
SP Imm	++++	++++	+++	+++	++	++
Expt 2	+++	+++	++	++	+	+
Expt 4	++	+	+	-	-	-
Expt 5	+++	++	++	+	+	-

Footnote:
++++ very strong fluorescence
+++ strong fluorescence
++ moderate fluorescence
+ minor fluorescence
- negative or vague fluorescence

key: HyI hyperimmunisation
SP Imm surface protein immunisation

CONCLUSIONS

The results obtained indicate that in the case of the surface protein vaccination experiments the antibodies generated were related to the L_3 surface antigens. In Experiment Four where low levels of antigen were administered, this was reflected by low or negative intensities of fluorescence.

The results from Experiment Six indicate that although there are surface proteins included in the somatic extracts, they have limited ability to generate antibodies to the surface and this was again reflected in the low intensity of fluorescence. The fact that adult homogenate-induced antibodies were able to

PLATE 15

H. contortus L_3 Immunofluorescence with Varying Dilutions of Hyperimmune Serum

This series of plates show the variation in intensity of surface immunofluorescence produced when exsheathed viable *H. contortus* L_3 are incubated in increasing dilutions of hyperimmune serum followed by incubation in FITC-second antibody.

Plate 15A: 1:50 dilution

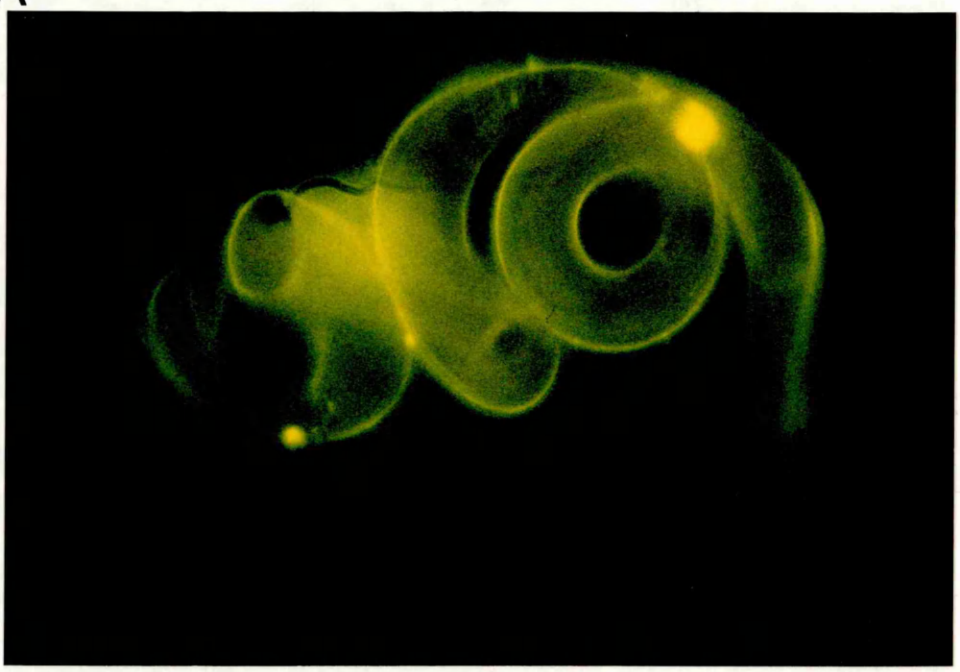
Plate 15B: 1:200 dilution

Plate 15C: 1:400 dilution

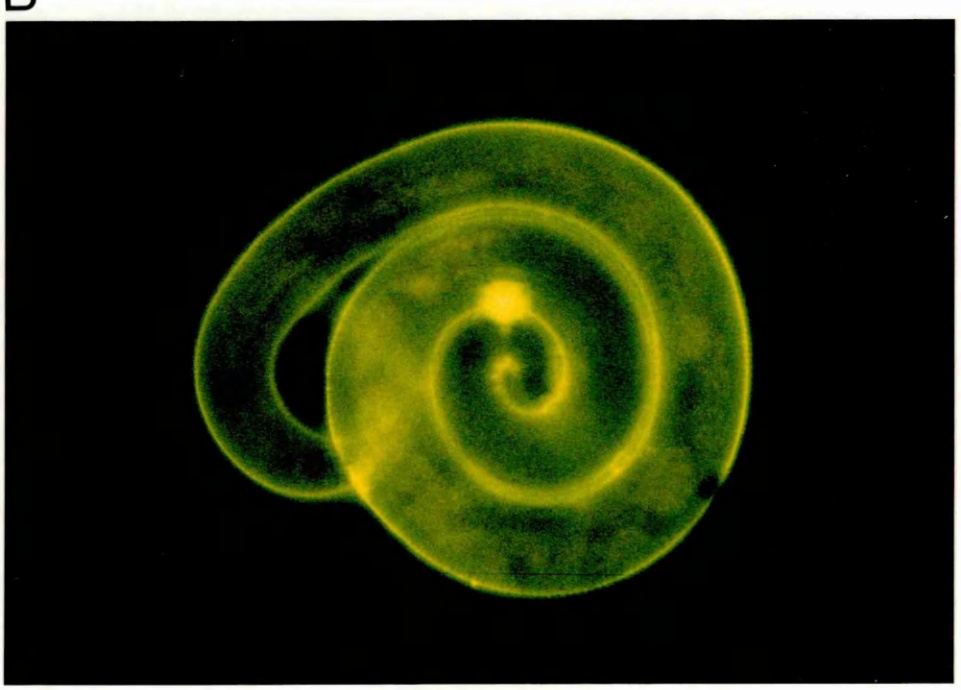
Plage 15D: 1:1000 dilution

With increasing dilutions internal autofluorescence becomes more evident and surface immunofluorescence diminishes.

A



B



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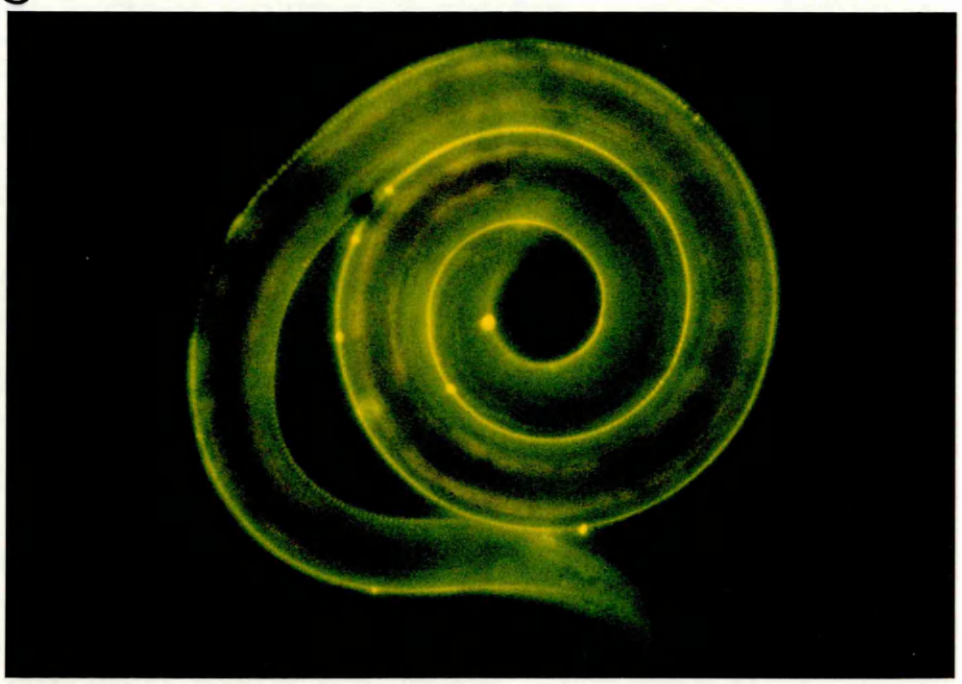


D

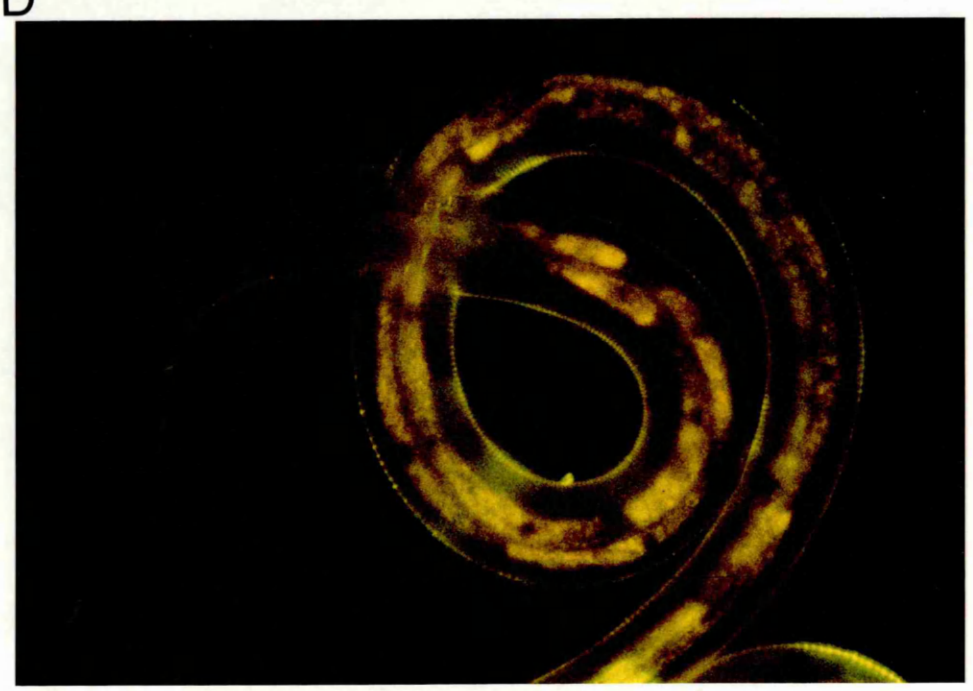


PLATE 16

H. contortus L_3 Immunofluorescence with Varying Dilutions of Serum from the Immunised Lambs in Experiment One

This series of plates show the variation in intensity of surface immunofluorescence produced when exsheathed viable H. contortus L_3 are incubated in increasing dilutions of sera from two immunised lambs, followed by incubation in FITC-second antibody.

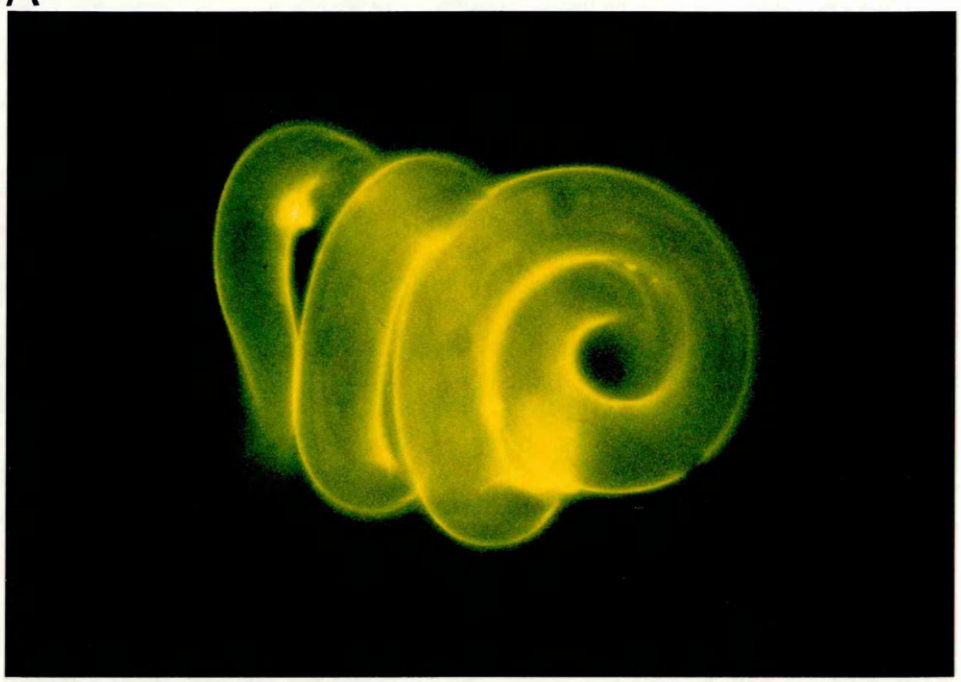
Plate 16A: 1:100 dilution

Plate 16B: 1:400 dilution

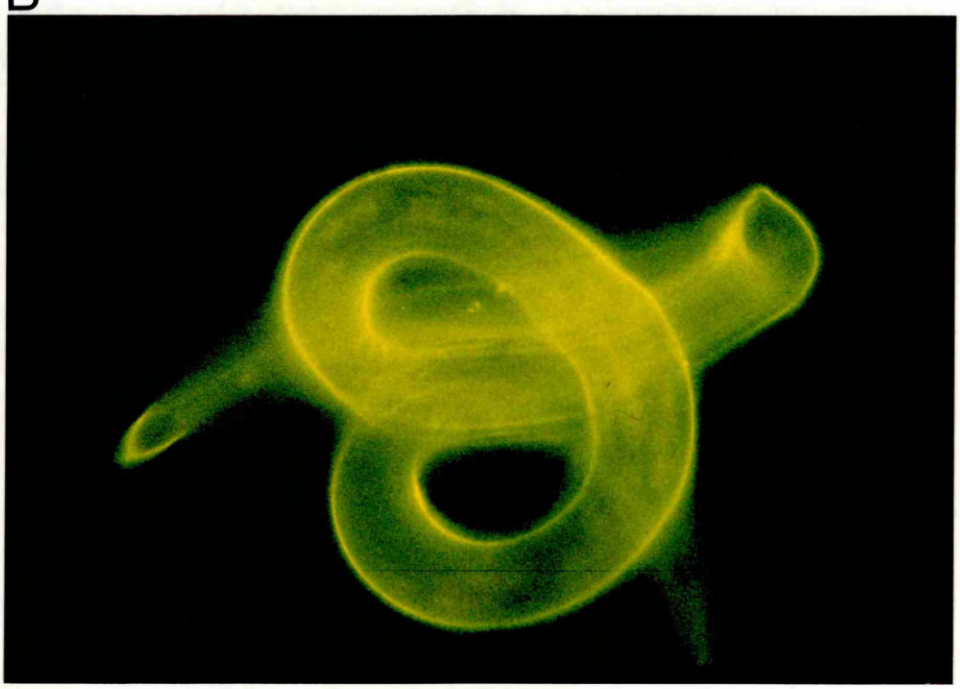
Plate 16C: 1:1000 dilution

With increasing dilution the surface immunofluorescence diminishes.

A



B



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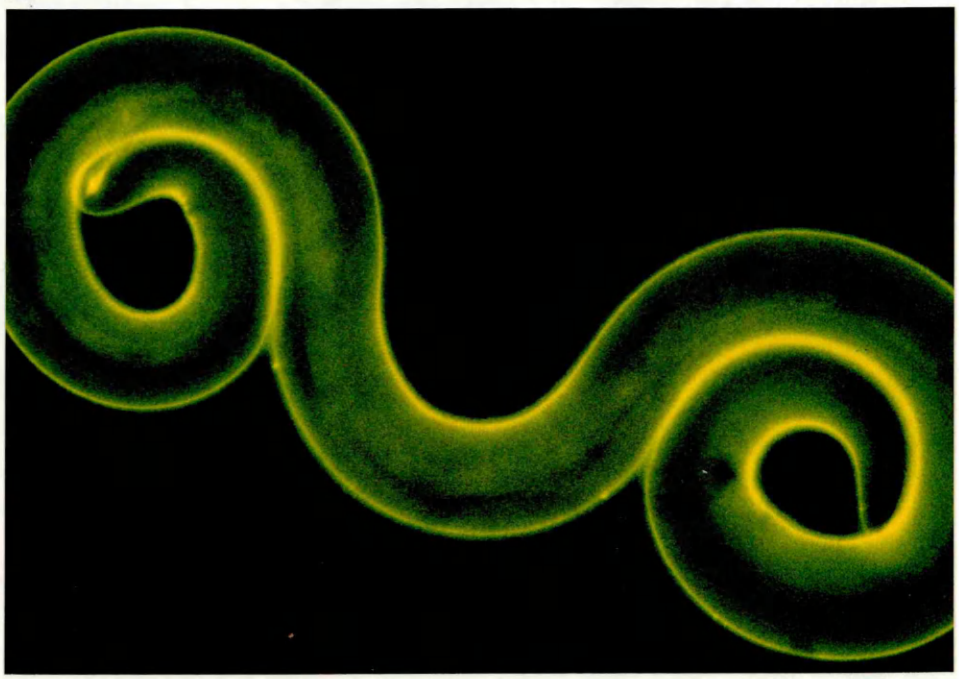


PLATE 17

H. contortus L_3 Immunofluorescence with Varying Dilutions of Serum from the Vaccinated Animals in Experiment Two

This series of plates show the variation in intensity of surface immunofluorescence produced when exsheathed viable H. contortus L_3 are incubated in increasing dilutions of sera from vaccinated animals followed by incubation in FITC-second antibody.

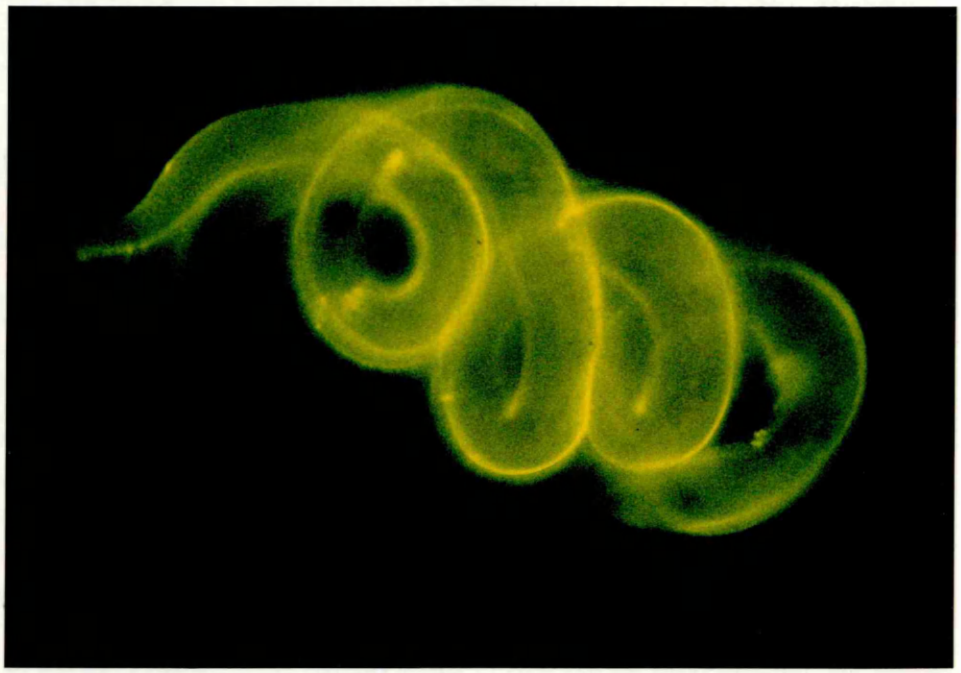
Plate 17A: 1:100 dilution

Plate 17B: 1:400 dilution

Plate 17C: 1:1000 dilution

With increasing dilution internal autofluorescence becomes more evident and surface immunofluorescence diminishes.

A



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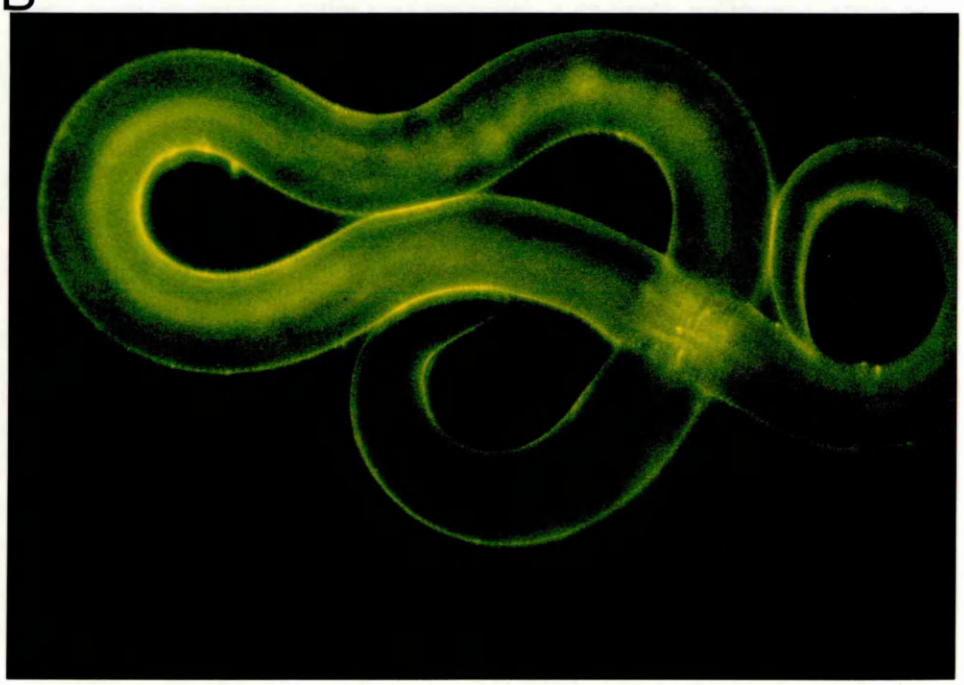


C

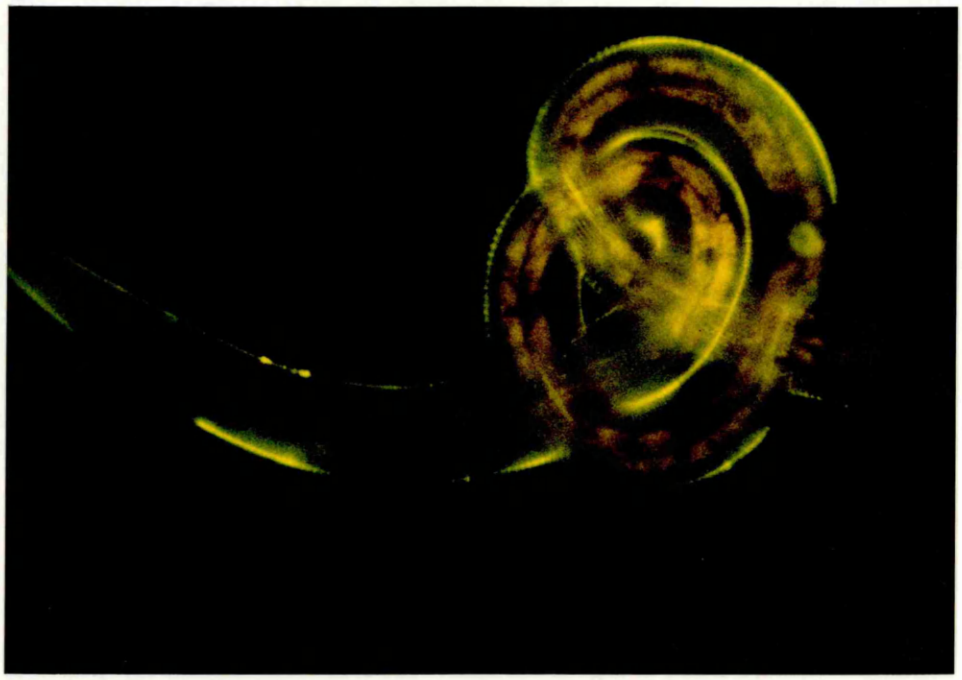


PLATE 18

H. contortus L_3 Immunofluorescence with Varying Dilutions of Serum from the Vaccinated Animals in Experiment Four

This series of plates show the variation in intensity of surface immunofluorescence produced when exsheathed viable _H. contortus_ L_3 are incubated in increasing dilutions of sera from vaccinated animals followed by incubation in FITC-second antibody.

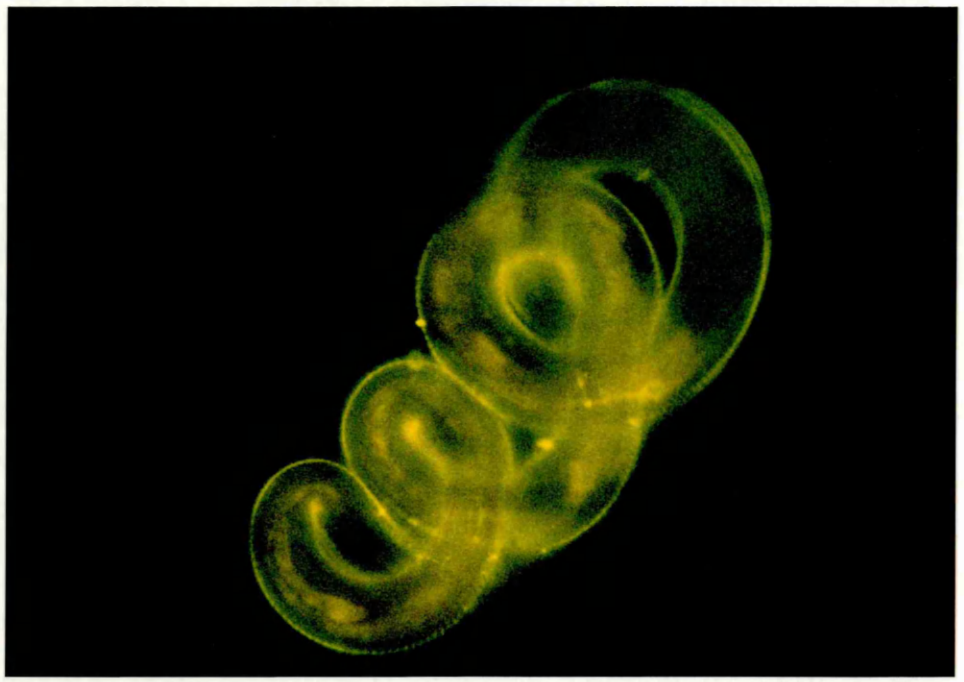
Plate 18A: 1:50 dilution

Plate 18B: 1:200 dilution

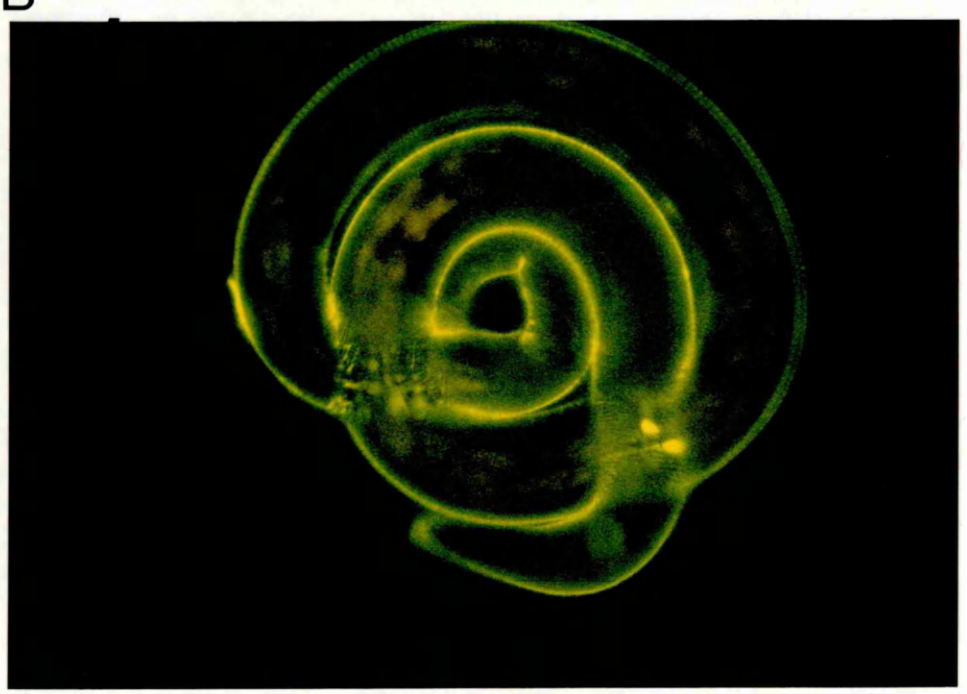
Plate 18C: 1:400 dilution

With increasing dilution internal autofluorescence becomes more evident and surface immunofluorescence diminishes.

A



B



C

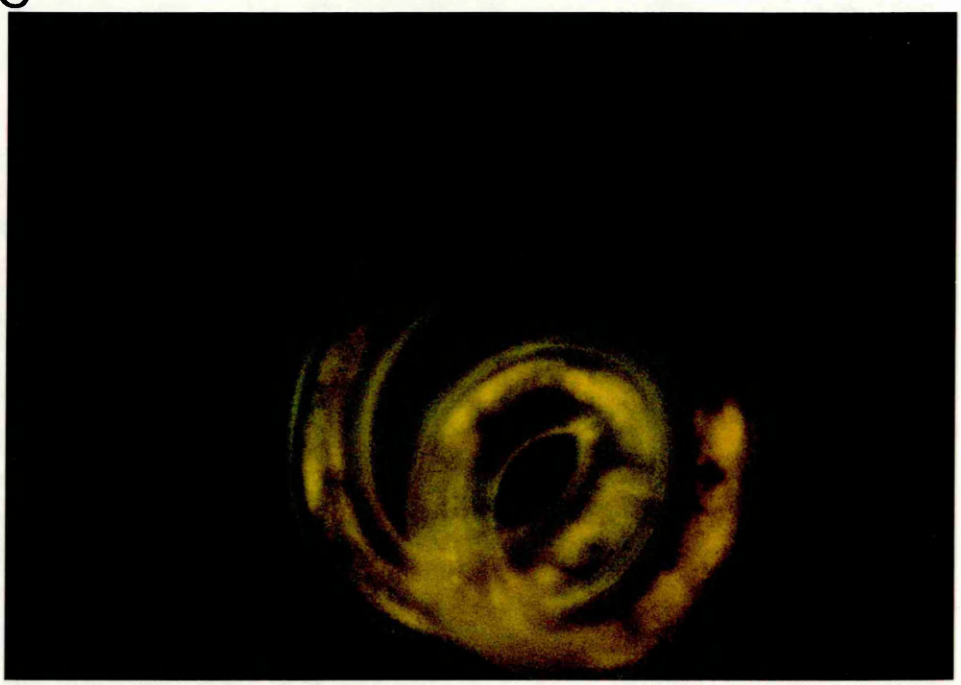


PLATE 19

H. contortus L_3 Immunofluorescence with Varying Dilutions of Serum from the Vaccinated Animals in Experiment Five

This series of plates show the variation in intensity of surface immunofluorescence produced when exsheathed viable H. contortus L_3 are incubated in increasing dilutions of sera from vaccinated animals followed by incubation in FITC-second antibody.

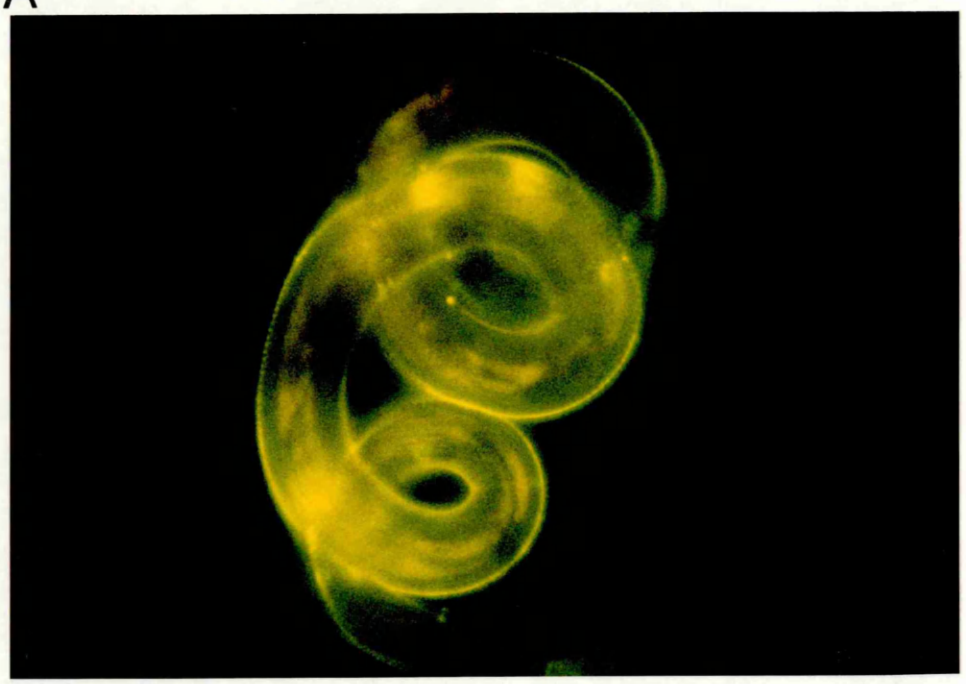
Plate 19A: 1:50 dilution

Plate 19B: 1:200 dilution

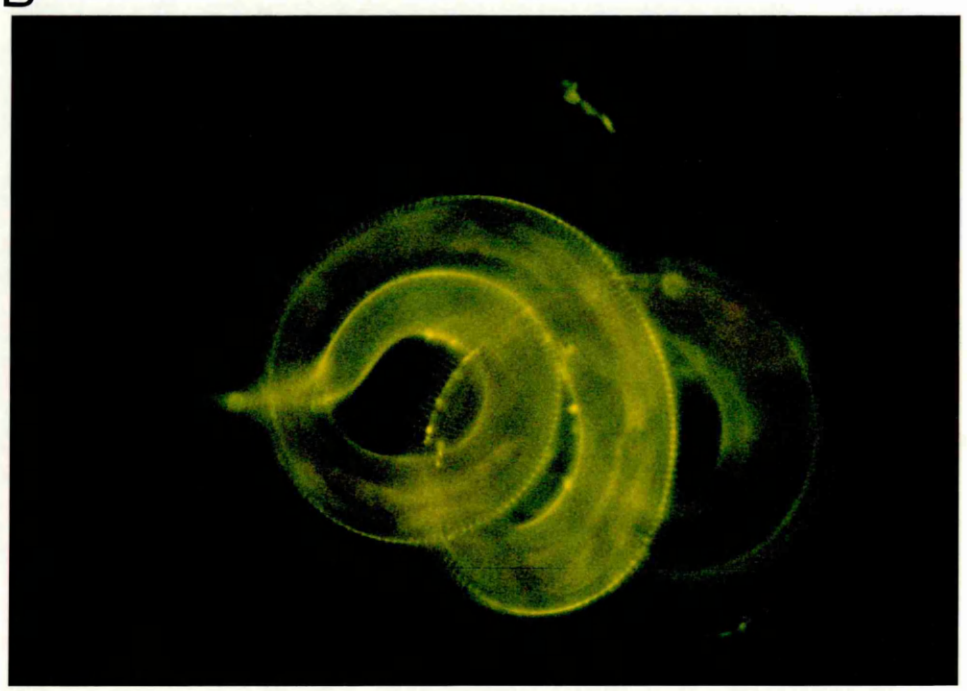
Plate 19C: 1:800 dilution

With increasing dilution internal autofluorescence becomes more evident.

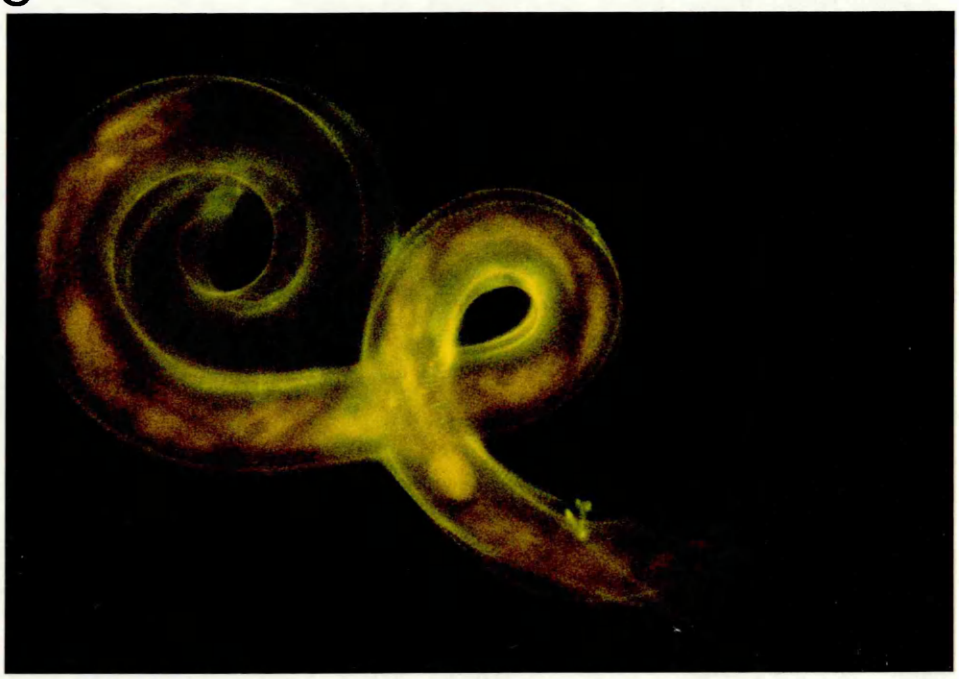
Å



B



C



recognise the surface of L_3 but no apparent recognition in the form of immunofluorescence was observed when adult parasites were incubated in L_3 sera, indicates that whilst there are shared antigens between the two stages of the parasite, those antigens that are surface related, although in short supply, are more abundant in the L_3 than the adult parasite.

The serum antibody titration experiment showed that where the antibody titres were high as determined by the ELISA technique, the corresponding immunofluorescence was also strong and in most cases remained strong up to a dilution of 1:800, suggesting that both methods of determining antibody levels in sera are equally sensitive.

EXPERIMENT THIRTEEN

WESTERN BLOTTING

INTRODUCTION

Western blotting may be used to assess the ability of serum antibodies to recognise parasitic antigens. The separation of the parasite antigens by polyacrylamide gel electrophoresis into polypeptides followed by blotting on to nitrocellulose and incubation with sera containing antibodies produces Antigen-Antibody (Ag-Ab) binding. The presence of this Ag-Ab complex enables a second enzyme conjugated antibody, with specificity against the serum antibodies, to form a three-way complex. By incubating the blot in a chromogenic substrate for the enzyme, any bound complexes will generate a colour reaction; this results

in visualisation of those antigens recognised by the test sera.

EXPERIMENTAL DESIGN

Nitrocellulose blots of L_3 somatic antigens were incubated in 1:50 dilutions of individual animal serum samples from the three surface protein vaccination experiments (Experiments Two, Four and Five) and the surface protein immunisation experiment (Experiment One): in addition nitrocellulose blots of L_3 somatic antigens and adult parasite antigens were incubated in sera from the somatic antigen experiment (Experiment Six). For each experiment ten or 11 serum samples (Week 0 to Week 9 or 10) were examined to investigate the time course of antibody response to the proteins used in vaccination. Pre-challenge sera from Experiment One was also examined. After washing in TBS - Tween 20 containing 3-5% skimmed milk, the blots were incubated in second antibody for two hours then washed again. Blots were then developed by placing them in a developing solution for up to eight minutes during which time the proteins recognised by antibodies in the sera appeared as distinct polypeptide bands. Molecular weight markers were run together with the L_3 somatic antigens and blots of these were stained with Ponceau S and retained. The stained markers were placed adjacent to the blots for determination of molecular weights of the visualised peptide bands.

A Western blot of pooled sera, on the day of challenge, from vaccination Experiments Two, Four and Five together with sera from the hyperimmunised sheep and sera from the two surface

protein immunisation lambs (Experiment One) was run to determine the antigens recognised as a result of the different immunisation/ vaccination regimens.

RESULTS

The results of the Western blots for each experiment are shown in Plates 20 - 22.

Plate 20 shows the results from two animals from Experiment Two. The polypeptides recognised varied between the two animals: Plate 20A indicates the appearance of antibodies to five polypeptides over the experimental period, whilst Plate 20B shows the appearance of a doublet and five minor polypeptides. Plate 21 shows the results from three animals from Group A, Experiment Four; in Plate 21B no polypeptide band development was seen using serum from one animal whereas Plates 21A and 21C show antigen bands visualised when sera from other lambs in the group were examined. Plate 22 shows the results from Experiment Five where two strongly recognised polypeptide bands and four more weakly recognised polypeptides appeared over the experimental period. The results from the pre-challenge sera from Experiment One are shown in Plate 23. The two immunised animals in this experiment recognised strongly a similar range of polypeptides.

The results obtained from sera from Experiment Six were poor. Polypeptides were recognised by pre-vaccination sera of both Test groups and apart from the recognition of two to three specific polypeptides by sera from the sheep vaccinated with L_3

PLATE 20

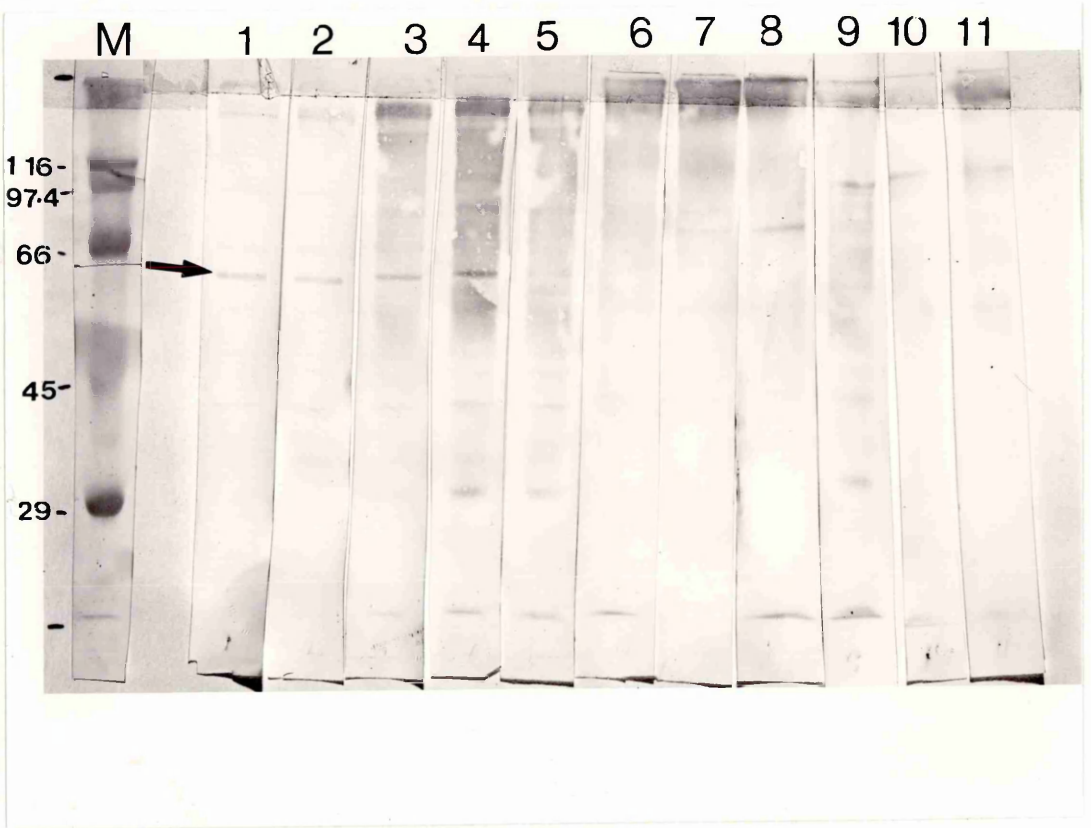
Western Blot Analysis: Experiment Two

These plates show the H. contortus third stage larval somatic antigens recognised by antibodies in weekly serum samples obtained from two vaccinated animals up to the week of challenge. An indication of the variability of individual response can be seen in the slightly different polypeptides recognised by the two animals. The arrows indicate polypeptides of similar molecular weight.

Lanes 1-11 (Plate 20A) and Lanes 1-9 (Plate 20B- Minigel) are the results for each week. Lane 1 is pre-vaccination, Lane 11 the week of challenge.

M - Molecular weight markers in kDa.

A



B

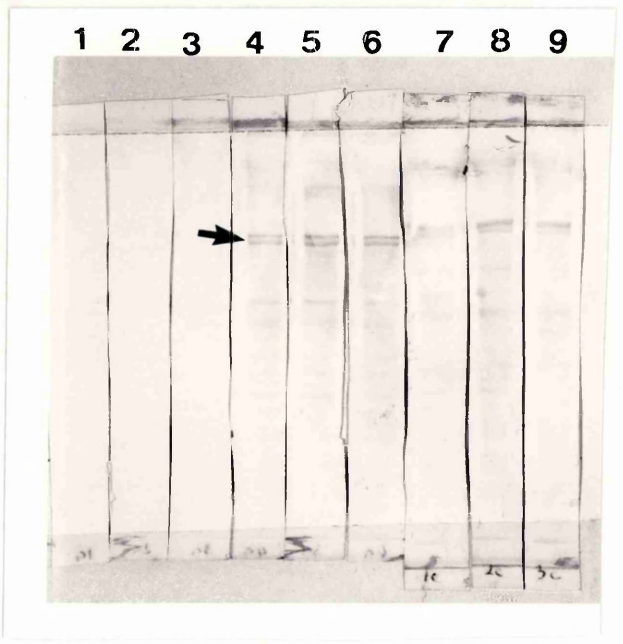


PLATE 21

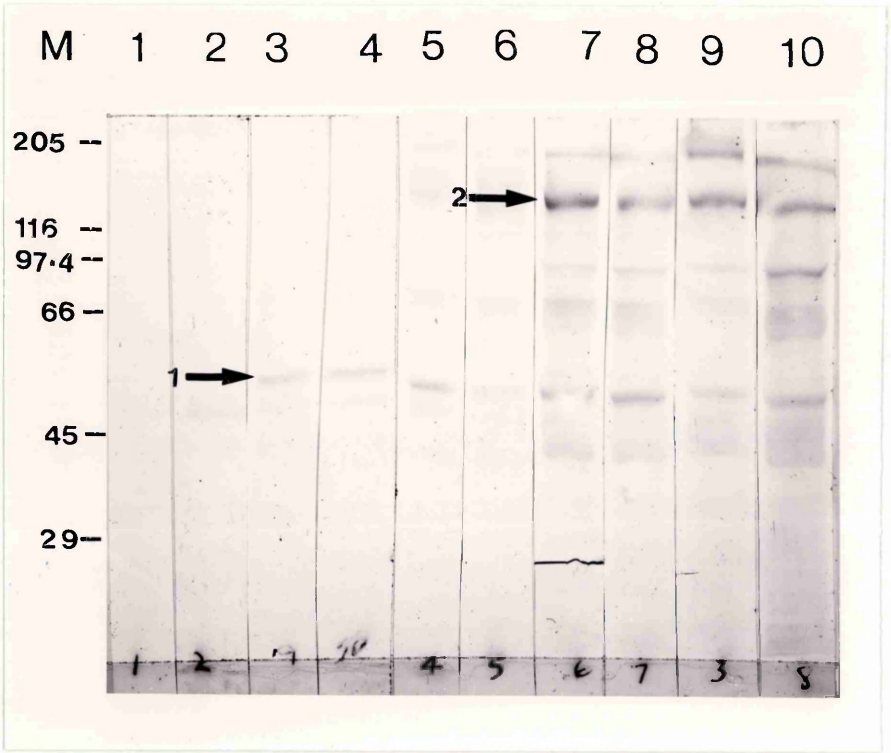
Western Blot Analysis: Experiment Four

These three plates show the H. contortus third stage larval somatic antigens recognised by antibodies in the sera obtained from three vaccinated animals from the time of first vaccination up to the week of challenge. An indication of the variability of individual response can be seen in that no polypeptides were recognised by one of these animals (Plate 21B) and antigen recognition patterns varied between the other two animals (Plates 21A and 21C). The arrows indicate polypeptides of equivalent molecular weight recognised by the two amimals.

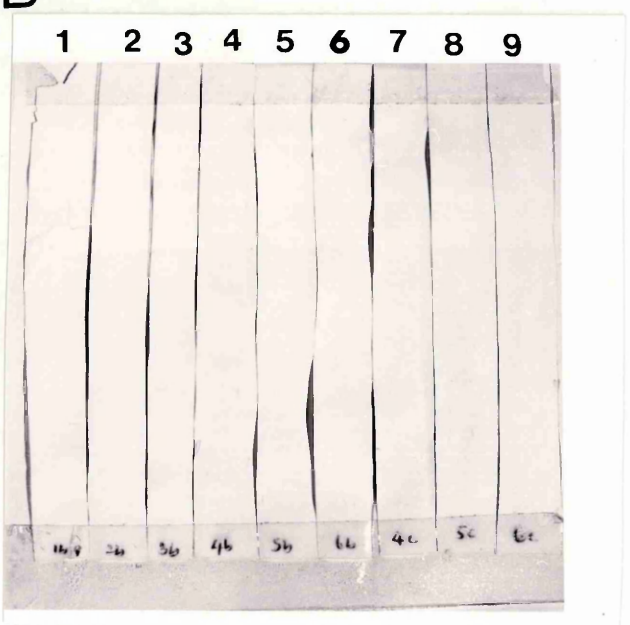
Lanes 1-10 (Plate 21A and 21C) and Lanes 1-9 (Plate 21B-Minigel) are the results for each week, Lane 1 is pre-vaccination, Lane 10 pre-challenge.

M - Molecular weight markers in kDA.

A



B



C

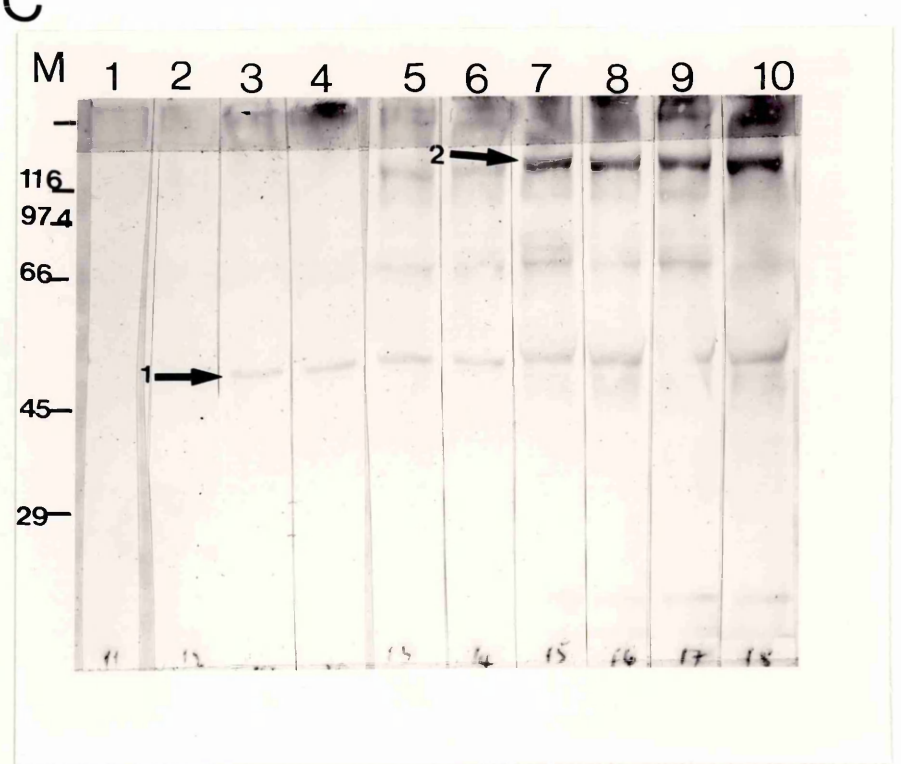


PLATE 22

Western Blot Analysis: Experiment Five

This plate shows the H. contortus third stage larval somatic antigens recognised by antibodies in the sera obtained from vaccinated animals from the time of first vaccination over the experimental period up to the week of challenge.

Lanes 1-11 are the results for each week, Lane 1 is pre-vaccination and Lane 11 the week of challenge.

M - Molecular weight markers in kDa.

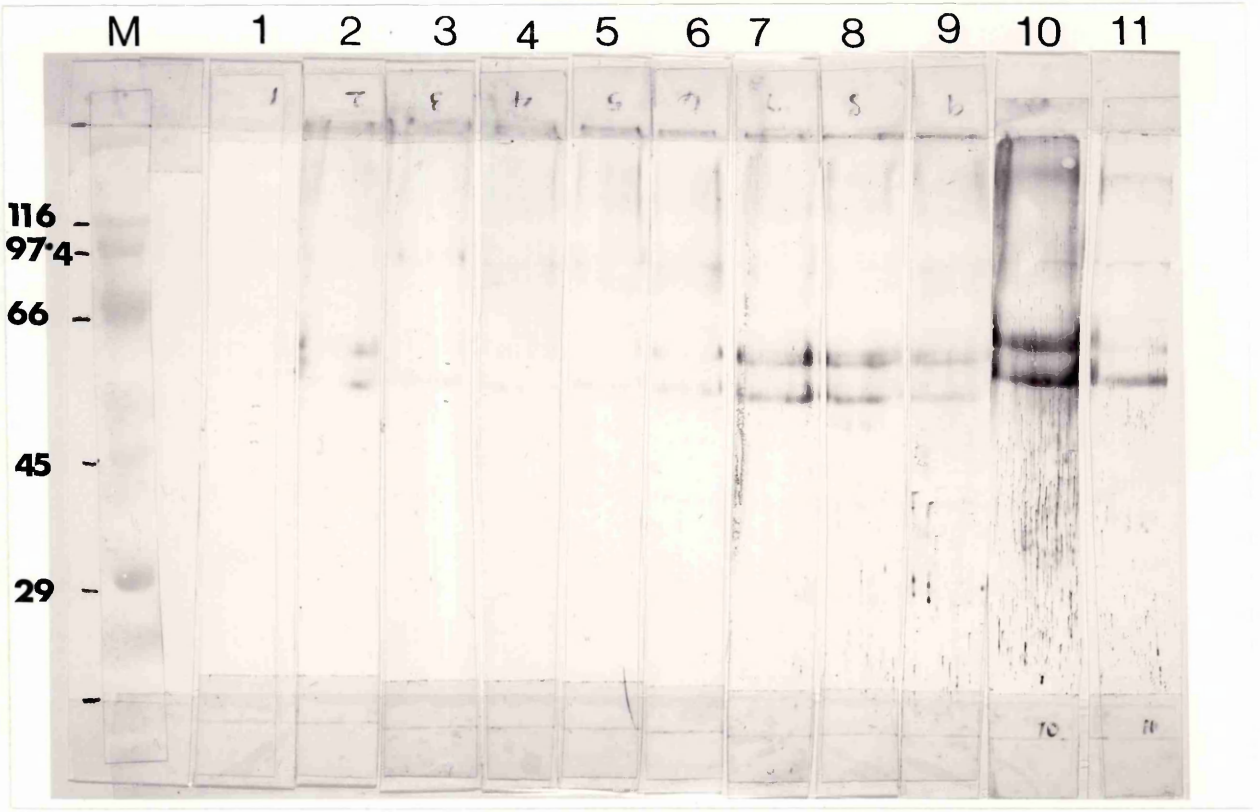

M
1
2
3
4
5
6
7
8
9
10
11
116
97·4
66
45
29

PLATE 23

Western Blot Analysis: Pre-Challenge from Experiment One

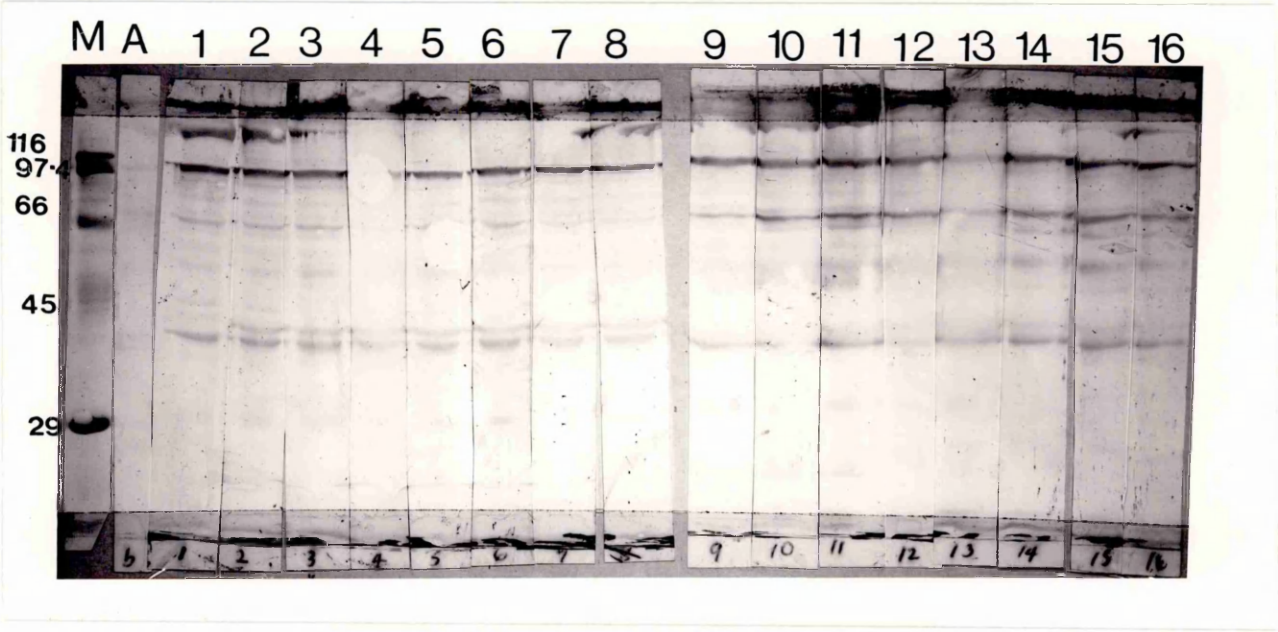
Western blots showing the H. contortus third stage larval somatic antigens recognised by antibodies in the sera obtained from the two larval surface protein immunised animals progressively from the week of booster immunisation up to the week of challenge. An indication of the variability of individual response can be seen in the slightly different polypeptides recognised by the two animals.

Lane A shows the results when sera from the hyperimmune animal was used.

Lanes 1-8 show the results for each week for sera from the animal immunised with L_3 surface proteins.

Lanes 9-16 show the results for each week for sera from the animal immunised with L_4 surface proteins.

M - Molecular weight markers in kDa.


M A 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
116
97·4
66
45
29

somatic antigens (Test 1) that appeared during the course of the experiment, there were no clear changes. Sera from the sheep vaccinated with adult somatic proteins (Test 2) recognised three polypeptides strongly after Week 7, but again this was not very clear.

The blot shown in Plate 24 using pooled sera from each individual experiment, shows that the sera from all three vaccination experiments (Lanes 4, 5 and 6) were able to recognise the same L_3 antigens, although the intensity of colour reaction indicating the level of antibody response, decreased with each successive experiment. In all three experiments four polypeptides were recognised. These polypeptides were also recognised by sera from the animal immunised with L_4 surface proteins (Y16) but only three of these were recognised by sera from the animal immunised with L_3 surface proteins (Y15). A faint polypeptide band recognised with Y15 sera was only visualised by pooled sera from Experiment Two. Sera from the hyperimmunised animal enabled the visualisation of three antigen bands albeit very faintly, and these corresponded to three of the four bands visualised by sera from the vaccination experiments and pre-challenge sera from Experiment One.

CONCLUSIONS

The results from these experiments indicated varying levels of antibody recognition of L_3 antigens and that there was some degree of individual animal variation, especially in vaccination Experiment Four. The results from the pooled sera blots showed

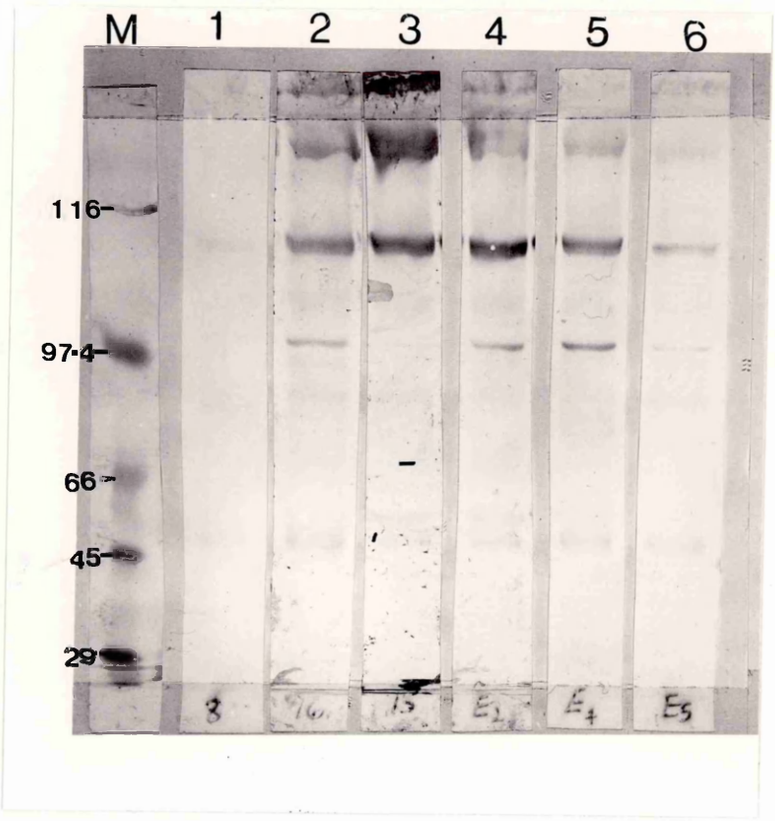
PLATE 24

Western Blot Analysis: Pooled Sera from Vaccination Experiments Two, Four and Five Compared with Hyperimmune Serum and Immunisation Sera from Experiment One

This plate shows the H. contortus third stage larval somatic antigens recognised by antibodies in the sera obtained from hyperimmunised, immunised and vaccinated animals. An indication of the variability of response can be seen in the different recognition patterns observed.

Lane 1 shows the result from serum obtained from a hyperimmune animal, Lanes 2 and 3 the results from pre-challenge sera obtained from lambs immunised with L_4 and L_3 surface antigens respectively, Lanes 4, 5 and 6 the results from pooled pre-challenge sera obtained from animals vaccinated with third stage larval surface antigens in Experiment Two, Four and Five respectively.

M - Molecular weight markers in kDa.


M
1
2
3
4
5
6
116
97·4
66
45
29

that individual animal variation could be masked by pooling the sera from each experiment. Western blotting also showed that the antigenic composition of the CTAB surface protein extract is simple comprising four to five major protein molecules. The Western blots run using sera from the hyperimmunised sheep indicated that although there is an antibody response to experimental challenge, the intensity of this response is low since only faint bands were visible on developing the blots.

CHAPTER FIVE

GENERAL DISCUSSION

The ubiquity and importance of Haemonchus contortus as a gastrointestinal parasite of sheep has led to numerous studies on the epidemiology (Gordon, 1948; Dinnik and Dinnik, 1958; Connan, 1971; 1975; 1978; Allonby and Urquhart, 1975; Ogunsusi and Eysker, 1979), pathogenesis (Allonby and Dargie, 1973; Allonby and Urquhart, 1975; Dargie and Allonby, 1975; Barger and Cox, 1984) and control (Le Jambre, Southcott and Dash, 1977; Dash, Newman and Hall, 1985; Waller, 1986) of infection. Although control relies almost entirely on the repeated use of anthelmintics, with an increasing number of reports of drug resistance over the last 20 - 30 years, this has become less effective despite the advent of newer more efficient drugs such as ivermectin. This has led to the development of parasite control programmes in countries such as Australia which include the strategic use of narrow spectrum compounds at certain times of the year when there is a greater risk of disease (Dash, Newman and Hall, 1985; Waller, 1986). These drugs while being highly efficient against H. contortus persist in the tissues resulting in problems of drug residues and prolonged withdrawal periods.

Experimentally, attempts to produce a vaccine against the parasite using irradiation attenuated third stage larvae have been shown to be successful against challenge with normal larvae in sheep over the age of seven months (Jarret et al, 1959; Benitez-Usher et al, 1977; Neilson, 1975), but similar vaccination schedules have been unsuccessful in lambs under this age which is presumed to be due to immunological unresponsiveness of young lambs (Urquhart et al, 1966a; Lopez and Urquhart, 1967).

In recent years there has been increasing interest in the study of nematode parasites at the molecular level. This has involved the investigation of specific parasite antigens including those released during the moulting process, excretory-secretory antigens and antigens on the cuticle. Much of this work in nematodes has centered around parasites which can be studied in rodents such as Nematospiroides dubius (Pritchard et al, 1985), Nippostrongylus brasiliensis (Maizels et al, 1983a), N. muris (Weinstein and Jones, 1956) and Trichinella spiralis (Philipp et al, 1980b) but there has been little work on H. contortus. In this thesis the results of the investigations on the antigenic nature of the different stages of H. contortus are presented together with the findings of preliminary studies on the immunochemical analysis of third stage larval surface proteins and their potential as a source of protective antigens.

As mentioned previously, the ability of sheep to mount a protective immune response under experimental conditions has been related to the age of the animals (Manton et al, 1962). In preliminary studies described in this thesis attempts were made to induce the production of hyperimmune serum in sheep both over and under the age of seven to eight months. The results of serial infections of a 9-month old animal and two groups of 2-month old and 6-month old lambs confirmed, in part, the findings of previous workers in that immunity to H. contortus infection based on faecal egg counts and PCV estimations, was only

demonstrated in the older animal. In contrast the younger lambs remained susceptible to repeated challenge which was attributed to immune unresponsiveness (Urquhart et al, 1966a; Lopez and Urquhart, 1967; Duncan et al, 1978). Two infections with 10,000 normal L_3 were sufficient to render the 9-month old animal immune to subsequent substantial experimental challenges. In contrast, the two groups of young lambs, aged two months and six months, continued to be susceptible to repeated challenge infections of 10,000 H. contortus L_3 as positive egg counts were obtained after each of these infections. The degree of susceptibility of these lambs however was variable between animals and also between infections. It has been reported previously that successive infections in sheep result in lower faecal egg counts and less markedly reduced PCVs but these are often variable (Manton et al, 1962). The degree of acquired resistance to reinfection has been studied most frequently in the situation where previous infections have been terminated by anthelmintic treatment (Donald and Wagland, 1966; Donald et al, 1969; Dargie and Allonby, 1975; Altaif and Dargie, 1978). The results obtained for one of the 2-month old lambs (Y13) in the work reported here fall into this category and in this animal faecal egg counts after the second and third infections were lower than those following the primary infection. In the other 2-month old lamb (Y14) and one 6-month old lamb (Y9) the reinfections were superimposed on existing infections and in these animals faecal egg counts were again lower after the second and third infections. Adams and Beh (1981) also found that primary infections of sheep conferred some

degree of immunity to reinfection with H. contortus in the absence of anthelmintic termination of the primary infection but this was not sufficient to prevent some establishment of the challenge larvae. The animals used by Adams and Beh (1981) were, however, 6 - 7 months old and at the age when immunological immaturity might be expected to have less influence on the suppression of the immune response. The remaining 6-month old lamb (Y12) produced results which fell into neither of the above categories in that lower egg counts were obtained after the first and second infections compared with those found following the two subsequent reinfections. This animal may fall into a category described by Urquhart (1980) who suggested that some sheep infected with H. contortus during the period of immune unresponsiveness might be subsequently unable to mount a protective immune response due to a permanent suppression of their immune system. However the primary objective of these preliminary studies in small numbers of animals was to provide reagents for subsequent immunochemical experiments; a large number of animals would have to be used to investigate more fully age-related immune responses to H. contortus.

Since the first attempt by Stoll (1929), to vaccinate sheep against H. contortus, a number of experimental studies have been carried out using irradiated H. contortus L_3 as potential "vaccines" with varying amounts of success (Jarrett et al, 1959; Neilson, 1975; Benitez-Usher et al, 1977). More recently vaccine materials derived from other sources have been examined including

somatic, metabolic and excretory-secretory products obtained from both adult and larval Haemonchus. Neilson and van de Walle (1987), successfully vaccinated Florida Native lambs with high molecular weight fractions of somatic and excretory-secretory antigens from larval H. contortus achieving a 59% reduction in adult worm numbers in vaccinates when compared with controls. Boisvenue et al (1987), used a purified metabolite of exsheathed H. contortus L_3 to vaccinate 4-month old lambs but no inhibition of challenge larval development was observed. When 48 - 150 day old lambs were vaccinated with a functional protein derived from adult H. contortus, the lambs were found to be less susceptible to challenge infections compared with control lambs and specific circulating antibodies were detected in the sera of these vaccinated lambs (Munn et al, 1987).

In this thesis the potential of H. contortus L_3 surface proteins in producing protection against challenge infections with normal larvae was investigated under experimental conditions. The material used for vaccination consisted of five polypeptides located on the surface of the exsheathed L_3 which were removed from the cuticle by detergent action.

A pilot experiment (page 87) produced encouraging results: the egg counts and PCVs of two lambs vaccinated with surface proteins were markedly different to those produced by a non-vaccinated control lamb. The egg counts after challenge peaked at 2 - 3,000 e.p.g. for the vaccinates before falling to <50 e.p.g. In contrast a maximum count of 30,000 e.p.g. was recorded in the control animal and the egg counts of this animal

subsequently were maintained at a high level. This suggested that the antigens in the vaccine generated an immune response which inhibited the development of a significant number of the challenge larvae. This was supported by the low adult worm recoveries from the vaccinated lambs at necropsy. The ELISA and immunofluorescence results using serum samples from these animals were also indicative of a significant antibody response to the injected surface proteins. The fact that the lambs were only two months old at the time of immunisation was also encouraging as it suggested that these young animals were in fact able to mount a protective immune response to these defined antigens at an early age. This might suggest that the immune unresponsiveness previously described in other studies (Duncan et al, 1978) is a result of immunosuppression following the use of large numbers of parasites or excessive doses of antigen.

In the following four experiments variable results were obtained. In Experiment Two for example, a 50% reduction in adult worm recovery was recorded for the vaccinates when compared with the controls, despite the high challenge dose of 100,000 H. contortus L_3. The animals in this experiment were killed at 19 days post-challenge at which time faecal egg counts were negative and the PCVs of both groups were still within the normal range. Sera from vaccinated animals in this experiment showed high antibody levels against L_3 antigens in the ELISA although these were not as high as those obtained in the preliminary experiment. This was confirmed in the immunofluorescence studies

when a weaker fluorescent signal was observed when larvae were incubated in sera from animals in this experiment compared with that obtained with sera from the animals used in the first experiment.

The parasitological results from the Kenyan experiment (Experiment Three) were similar to those obtained in Experiment Two, in that there was a 50% reduction in worm numbers in the vaccinates compared with controls. In this experiment however the animals were killed at 21 days after challenge and eggs were found in the faeces of all animals at this time. Unfortunately the serum samples collected from this experiment were unavailable for further study of any antibody responses generated by the surface proteins.

In Experiment Four low doses of antigen were used and although an antibody response to H. contortus L_3 surface proteins was detected by ELISA this was lower than that observed in previous experiments. The absence of any difference in the egg counts and PCVs between the vaccinates and controls suggested that a minimal dosage of proteins is necessary to induce a protective immune response capable of influencing the establishment and development of challenge larvae.

This experiment did however indicate that there are no non-specific effects from the administration of Freunds adjuvant alone. The use of different adjuvants has been shown to have some effect on the development of immunity to parasites. For example Wedrychowicz and Bezubik (1990), found that a much lower degree of protection was achieved when somatic antigens of

T. colubriformis L_3 emulsified in Freund's Complete Adjuvant (FCA) were administrated to rabbits compared with that obtained by the administration of the same antigen emulsified in beryllium hydroxide. Recently Bomford (1989) has suggested that "purified antigens are much less immunogenic than when they comprise part of a living parasite and the application of adjuvants is necessary".

Although the protective response to nematode antigens has been shown to depend in many cases on the type of adjuvant used (Monroy, Adams, Dobson and East, 1989) studies on the effects of different adjuvants were beyond the scope of the work presented in this thesis. Investigations into the potential protective ability of H. contortus L_3 surface proteins emulsified in a variety of different adjuvants would be a useful future areas of study.

The results from the last of the surface protein vaccination experiments (Experiment Five) were disappointing in view of the results of the initial experiments. In this experiment egg counts, PCVs and total worm burdens were similar in both vaccinated and control animals in spite of the increased dose of antigens used and the high antibody levels detected by the ELISA technique in the sera of the vaccinated lambs. The reason for this apparent lack of protection is not very clear, but it is possible that there is not only a minimum dosage of surface protein antigen required for protection but there may be a maximum dosage beyond which the administration of antigens

results either in immunosuppression or a less specific non-protective immune reaction.

The general conclusions from the five surface protein vaccination experiments are that although antibodies to these proteins can be induced by vaccination, their role in the protective immune response is variable. A major difference between the successful immunisation study and the subsequent vaccination experiments was the time interval between primary and booster vaccination, that between booster vaccination and challenge and the time interval between challenge and necropsy. In the preliminary experiment these were seven weeks, eight weeks and seven weeks respectively whereas in all other experiments a constant time interval of five weeks between vaccinations and subsequent challenge infections was adopted. Adams et al (1982), have suggested that sufficient time must be allowed between vaccination and challenge for the development of immunity, and it is possible that the interval of five weeks applied in the vaccination experiments described here might have been too short for the full protective potential of the vaccine antigens to be realised.

In Experiment Six, there was no evidence of any protective immunity conferred by administration of the L_3 or adult somatic antigens as vaccines. The egg counts, PCVs and total worm burdens from both vaccinated groups and the control group were similar and although the ELISA results indicated some antibody response, this was very weak. Perhaps this is not surprising since it has been previously reported that somatic antigens are

not very effective in stimulating immunity (Sinclair, 1970; Maizels and Selkirk, 1988).

Adams et al (1982) also suggested that quantity as well as quality of antigen administered was important in the stimulation of an immune response to H. contortus. In the experiments described in this thesis there were significant differences in the type and dose, in terms of protein concentration, of antigen used. The protein concentration of the somatic antigen used in Experiment Six was roughly 3.5 times greater than that administered in the surface protein Experiments Two, Three and Five. Despite the lower doses of surface proteins administered in these experiments antibody responses as determined by ELISA were very strong compared with those obtained with the higher doses of protein administered as somatic extracts in Experiment Six. This might suggest that the administration of large doses of somatic antigen instead of promoting the development of an immune response, resulted in immunosuppression of the host.

The results from the various experiments may have been influenced by host and breed variations as these have been shown to play a role in the development of immunity to parasite antigens both between and within breeds (Bradley et al, 1973; Altaif and Dargie, 1978a; 1978b). This phenomenon was observed to some degree within individual experiments as shown by the Western blot analysis of experimental sera where vaccinated animals within one group recognised different polypeptide

profiles, but also between experiments where variable results were shown by the same analysis.

The ELISA, immunofluorescence and Western blot studies produced some interesting results. First it appeared that the dose of antigen administered was related to the level of antibody response. For example in Experiments Two and Five the results of all three analyses were similar in that high antibody levels were detected by both ELISA and immunofluorescence and a number of polypeptide bands were revealed by Western blotting. In contrast in Experiment Four low to negative antibody levels were detected by ELISA and immunofluorescence and there appeared to be a variable detection of specific polypeptides by the Western blotting procedure. The major difference between these three experiments was the level of surface protein administered as vaccine with the sheep in Experiment Four receiving a much lower dose of antigen than the sheep in the other two experiments.

The strongest immunofluorescence on the surface of exsheathed L_3, the highest ELISA antibody levels and the best defined polypeptide band detection (high titre) by Western blotting were seen in Experiment One and these were associated with the most significant differences in PCVs and egg counts between control and vaccinated lambs after challenge. As stated previously, a longer time interval between vaccination, booster vaccination and challenge was allowed in this experiment compared with subsequent experiments and this is one possible explanation of the differences in the results obtained.

The brief attempt to cultivate third stage H. contortus larvae to the fourth stage and beyond was carried out in an attempt to provide an inexpensive and reliable method of acquiring fourth stage H. contortus larvae for studies on their surface proteins. This culture method could be considered partially successful in as far as alteration of certain internal structures of the larvae, which according to Mapes (1969) was related to development, were found. The area around the oesophageal region showed the greatest structural changes and the failure of the culture media to allow any further development of the parasites may have been related to insufficient nutrient materials within the media. However it did appear from this study that the development of infective larvae to the fourth stage requires only basic nutrients in the growth media. Previous in vitro studies on the cultivation of H. contortus have shown that the addition of heme to culture media facilitates development beyond the fourth stage (Stringfellow, 1984). In a later study Stringfellow (1986), found that the addition of ovine gastric contents to the medium API-1 and Fildes' reagent, which is a pepsin digest of defibrinated bovine blood, allowed the development of cultured L_3 to mature adult males in 28 days and to mature egg-laying females in 36 days. It was hoped to use these media for cultivation of H. contortus L_3 but these were unobtainable and further in vitro studies were not carried out.

The results of the culture methods carried out in this thesis did however suggest that the surface of the H. contortus were able to undergo changes before a moult actually occurred.

This was indicated by observed differences in the surface protein profiles of larvae which, after 4 - 7 days in culture, were similar to those of fourth stage larvae obtained at post-mortem of infected animals but different from those of L_3 obtained from standard faecal cultures. There is evidence that proteins on the surface of the cuticle of the parasitic nematode T. spiralis change both qualitatively and quantitatively following the moulting process and during the growth of the parasite within one stage (Philipp et al, 1980b). Although there are no previous reports of this occurring with H. contortus, the preliminary results obtained here suggest that such changes do in fact occur with Haemonchus. More extensive investigations are required to characterise stage-specific surface proteins of H. contortus.

In summary, the findings of the work carried out in this thesis have shown that:

1. Although the age of a sheep has an influence on the development of protective immunity to Haemonchus infection there are indications that animals under the age of seven months are capable of mounting an immune response to H. contortus L_3 surface proteins.

2. Somatic antigens from adult parasites and third stage H. contortus larvae are not effective in the stimulation of protection or in the induction of an immune response in terms of detection of significant antibody levels in sera.

3. Time intervals between vaccination and challenge and the dose of antigen administered as vaccine may be important determinants of the subsequent immune responses.

4. Antibody responses to vaccination with L_3 surface proteins can be detected between the 7th and 14th day after vaccination by both ELISA and Western blot analysis.

5. Freunds Complete and Incomplete Adjuvants alone have no effect on the development of an immune response to Haemonchus.

6. The use of simple balanced salt solutions in the culture of H. contortus larvae allows the development of exsheathed L_3 to the fourth stage, but more complex media and culture conditions are necessary for further development.

In conclusion the findings of the experiments described in this thesis have provided a basis for further studies on the immunochemistry of H. contortus especially in relation to the identification of specific surface protein molecules of third stage larvae and their potential as a source of vaccine material. In this respect future approaches would include hyperimmunisation and protection experiments using varying doses of L_3 surface antigens in larger groups of young and mature animals and the subsequent analysis of serum obtained from these experiments by ELISA, immunofluorescence, Western blotting and other antibody detection methods.

In addition purification and identification of the surface proteins of various stages of H. contortus might provide a range of stage-specific antigens which could be subsequently investigated as potential candidates for a vaccine against this important parasite of sheep.

APPENDICES

Appendix 1. Individual and Group Mean PCV's

Experiment 2.

Vaccinates	Week 0	1	2	3	4	5	6	7	8	9	10	11	12
G424	42	36	35	35	30	27	27	24	29	33	36	36	36
G545	35	35	32	32	31	22	23	23	24	27	27	31	30
G475	27	28	30	32	28	37	25	28	25	29	31	33	32
Mean	35	34	33	33	31	30	25	26	24	28	30	33	33
Controls													
G474	33	26	37	33	34	32	30	33	31	34	37	35	34
G551	37	40	42	40	34	41	31	35	35	37	39	30	30
G493	40	40	42	38	35	44	33	36	37	27	36	36	35
Mean	37	35	40	37	34	39	31	35	34	33	37	34	33

Experiment 3.

Vaccinates	Week 0	1	2	3	4	5	6	7	8	9	10	11	12	13
P19	30	35	34	32	25	25	25	25	30	20	16	20	17	20
P22	34	34	35	32	26	34	29	30	31	30	31	33	26	26
P27	34	37	40	37	34	34	30	36	35	31	30	34	32	19
Mean	33	35	36	34	28	31	28	30	29	27	26	29	25	22
Controls														
P20	32	42	39	33	29	31	27	25	25	26	27	28	26	24
P34	27	33	37	39	37	31	28	25	30	33	33	32	32	33
P35	37	41	38	33	32	35	28	-	-	-	34	33	32	21
Mean	32	39	38	35	29	32	28	25	28	30	31	31	30	26

Experiment 4.

Group A	Week 0	1	2	3	4	5	6	7	8	9	10	11	12	13
P3	35	37	33	34	34	33	31	31	33	29	31	32	26	24	15
P4	30	32	33	30	29	28	26	16	26	26	28	26	19	17	21
P5	35	35	33	34	31	32	30	29	29	26	31	32	22	23	22
P12	32	37	37	34	37	35	36	32	32	30	32	28	28	22	24
Mean	33	35	34	33	33	32	31	27	30	28	31	30	24	22	21
Group B															
P1	34	34	33	32	32	32	31	28	29	29	31	29	24	19	13
P2	38	40	34	37	34	32	31	28	30	26	24	26	17	19	17
P7	41	39	39	39	33	34	33	31	32	30	29	28	23	21	17
P9	33	32	34	33	28	27	25	25	27	25	29	26	24	21	19
Mean	37	36	35	35	32	31	30	28	30	28	28	27	22	20	17
Controls															
P6	33	36	35	35	29	29	30	27	26	27	29	29	28	21	20
P8	36	34	33	35	31	30	29	28	26	26	30	26	20	18	25
P10	34	36	35	35	29	29	30	27	26	27	29	28	23	21	20
P11	39	39	37	36	31	32	33	30	31	26	31	28	26	24	25
Mean	36	38	36	37	32	32	31	28	28	26	30	28	23	20	21

Experiment 5.

Vaccinates	Week 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
P27	30	30	32	30	33	27	29	28	28	29	28	29	25	18	17
P28	32	32	31	30	32	30	28	27	33	34	34	33	32	29	23
P29	30	30	27	26	28	28	28	28	31	33	29	31	27	22	11
P31	34	34	32	31	31	30	30	30	31	33	32	30	29	22	20
P32	32	32	31	29	31	29	29	28	31	32	31	31	28	23	18
Mean	32	32	31	29	31	29	29	28	31	32	31	31	28	23	18
Controls															
P23	31	31	29	22	26	27	34	30	29	32	30	30	29	24	23
P24	28	28	32	33	34	34	32	28	32	33	31	30	23	20	18
P25	25	25	24	22	24	24	25	26	28	27	32	28	24	21	17
P26	27	27	28	28	29	29	30	27	30	31	28	30	21	14	12
P30	30	30	32	27	26	33	31	31	31	33	32	32	26	18	17
Mean	28	28	29	26	28	29	30	28	30	31	31	30	25	19	17

Appendix 1. Cont'd

Experiment 6.

Test 1.	Weeks 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
B13	26	26	27	28	29	26	29	31	30	28	30	30	26	28	27
B16	31	31	32	30	30	28	29	32	31	33	34	28	28	24	18
Mean	29	29	30	29	30	27	29	32	31	31	32	29	27	26	23
Test 2.															
B14	31	31	29	29	32	30	33	36	32	30	31	28	23	22	23
B17	34	34	34	34	35	34	35	33	30	32	34	33	30	24	19
Mean	33	33	32	32	34	32	34	35	31	31	33	31	27	23	21
Controls															
B15	31	31	31	30	33	31	29	27	26	33	30	28	29	28	27
B18	33	33	33	34	37	34	33	36	37	37	36	30	30	23	11
Mean	32	32	32	32	35	33	31	32	32	35	33	29	30	26	19

Appendix 2. Individual ELISA O.D's

Experiment 2.

	Week 0	1	2	3	4	5	6	7	8	9	10
(S)											
G474	0.264	0.355	0.528	0.318	0.310	0.537	0.518	0.591	0.555	0.427	0.372
G475	0.418	0.541	0.826	0.969	1.036	0.984	1.021	1.103	1.078	1.074	0.899
G493	0.375	0.442	0.430	0.589	0.306	0.531	0.587	0.519	0.575	0.535	0.447
Cut-off point - 0.764											
(SP)											
G474	0.300	0.343	0.311	0.353	0.308	0.351	0.365	0.397	0.390	0.361	0.314
G475	0.398	0.538	0.598	0.651	0.751	0.851	0.928	0.949	0.954	1.009	1.074
G493	0.326	0.356	0.365	0.412	0.267	0.503	0.437	0.535	0.520	0.503	0.281
Cut-off point - 0.552											
(S)											
G545	0.256	0.422	0.665	0.702	0.806	1.009	1.074	1.037	0.973	0.872	0.880
Cut-off point - 0.670											
(SP)											
G545	0.201	0.367	0.610	0.647	0.751	0.954	1.019	0.982	0.918	0.817	0.708
Cut-off point - 0.560											

Experiment 4

	Week 0	1	2	3	4	5	6	7	8	9	10
(S)											
P2	0.706	0.781	0.815	0.767	0.763	0.881	0.885	0.102	1.341	0.972	0.991
P3	0.316	0.310	0.992	1.090	1.135	1.183	1.396	1.321	1.032	1.347	1.092
P6	0.348	0.338	0.481	0.489	0.548	0.501	0.493	0.421	0.501	0.490	0.493
Cut-off point - 0.668											
(SP)											
P2	0.579	0.545	0.458	0.420	0.528	0.442	0.587	0.571	0.596	0.449	0.840
P3	0.269	0.211	0.497	0.572	0.577	0.474	1.039	1.126	1.171	1.164	1.333
P6	0.329	0.319	0.223	0.225	0.254	0.183	0.277	0.228	0.220	0.287	0.448
Cut-off point - 0.443											
(S)											
P7	0.449	0.671	0.636	0.909	0.930	0.883	0.878	0.917	0.925	1.189	0.845
P4	0.503	0.824	1.149	1.162	1.187	1.220	1.210	1.240	1.263	1.468	1.204
P10	0.632	0.571	0.581	0.534	0.518	0.684	0.815	0.775	0.735	0.860	0.744
Cut-off point - 0.638											
(SP)											
P7	0.444	0.356	0.304	0.416	0.378	0.287	0.305	0.381	0.448	0.585	0.248
P4	0.414	0.448	0.646	0.570	0.795	0.763	1.146	1.136	1.147	1.092	0.742
P10	0.724	0.591	0.514	0.457	0.431	0.346	0.383	0.310	0.293	0.427	0.282
Cut-off point - 0.556											
(S)											
P9	0.861	0.559	0.905	0.903	0.723	0.700	0.796	0.712	0.746	0.705	0.579
P12	0.643	0.850	0.888	0.952	0.931	0.873	1.009	1.010	1.043	0.976	0.975
P11	0.845	0.741	0.766	0.709	0.617	0.628	0.687	0.520	0.531	0.479	0.597
Cut-off point - 0.670											
(SP)											
P9	0.313	0.416	0.324	0.421	0.386	0.340	0.336	0.467	0.353	0.318	0.330
P12	0.339	0.198	0.201	0.262	0.180	0.174	0.401	0.469	0.506	0.510	0.520
P11	0.361	0.302	0.367	0.264	0.248	0.269	0.285	0.216	0.193	0.209	0.238
Cut-off point - 0.512											

APPENDIX 2 cont'd

Experiment 5.

	Week										
	(S) 0	1	2	3	4	5	6	7	8	9	10
P24	0.609	0.531	0.777	0.434	0.591	0.548	0.612	0.540	0.638	0.551	0.524
P26	0.626	0.664	0.682	0.723	0.690	0.787	0.761	0.764	0.800	0.586	0.585
P32	0.674	0.595	0.920	1.139	1.215	1.217	0.125	1.287	1.363	1.208	1.200
Cut-off point - 0.816											
	(SP)										
P24	0.208	0.226	0.359	0.265	0.364	0.409	0.475	0.475	0.512	0.518	0.526
P25	0.320	0.262	0.288	0.327	0.312	0.357	0.347	0.000	0.417	0.640	0.416
P32	0.329	0.306	0.666	0.781	0.845	0.912	0.983	1.043	1.113	1.055	1.040
Cut-off point - 0.616											
	(S)										
P31	0.528	0.600	1.311	1.309	1.349	1.359	1.313	1.518	1.427	1.384	1.349
P29	0.379	0.553	1.063	1.259	1.283	1.267	1.286	1.337	1.289	1.427	1.235
P25	0.960	0.851	0.890	0.764	0.722	0.986	0.919	0.903	0.840	0.744	0.738
Cut-off point - 0.560											
	(S)										
P31	0.215	0.289	0.825	0.796	0.849	0.849	0.912	0.991	0.943	0.966	1.047
Cut-off point - 0.512											
	(S)										
P30	0.371	0.349	0.347	0.428	0.344	0.401	0.452	0.647	0.439	0.319	0.327
Cut-off point - 0.656											
	(S)										
P28	0.566	0.573	0.971	1.046	1.052	1.013	1.087	1.162	1.182	1.055	1.228
Cut-off point - 0.580											
	(SP)										
P28	0.163	0.198	0.453	0.825	0.843	0.878	0.832	0.922	0.942	0.930	0.805
Cut-off point - 0.518											
	(SP)										
P26	0.319	0.296	0.351	0.331	0.278	0.333	0.303	0.279	0.270	0.303	0.316
P30	0.345	0.394	0.356	0.401	0.325	0.448	0.383	0.268	0.368	0.418	0.424
P29	0.297	0.888	0.449	0.864	0.972	0.991	1.134	0.108	1.163	1.187	1.189
Cut-off point - 0.690											

Experiment 6.

	Week										
	(LS) 0	1	2	3	4	5	6	7	8	9	10
B13	0.905	0.980	1.031	1.088	0.987	1.057	1.136	1.126	1.124	1.031	0.911
B16	0.733	0.865	0.845	0.866	0.886	0.874	1.023	0.987	1.076	1.038	0.723
B15	0.749	0.717	1.687	0.675	0.631	0.629	0.486	0.589	0.606	0.649	0.458
Cut-off point - 0.374											
	(AdS)										
B14	0.601	1.039	1.061	1.127	1.276	1.240	1.312	1.314	1.233	1.194	1.261
B17	0.707	0.771	0.845	0.883	0.956	1.043	0.969	1.092	1.131	1.134	1.152
B18	0.643	0.710	0.649	0.667	0.672	0.651	0.730	0.773	0.985	1.021	1.003
Cut-off point - 0.394											

FOOTNOTE:

S --- Somatic antigens
SP -- Surface protein antigens
LS --- Larval somatic antigen
AdS - Adult somatic antigens

REFERENCES

REFERENCES

Abbott, E.M. (1982). Ph.D. Thesis. University of Glasgow.

Abbott, E.M., Parkins, J.J. and Holmes, P.H. (1985a). Influence of dietary protein on parasite establishment and pathogenesis in Finn-Dorset and Scottish Blackface lambs given a single moderate infection of Haemonchus contortus. Research in Veterinary Science 38, 6-13.

Adams, D.P. (1982). Time of onset and target of immune reactions in sheep with acquired immunity against Haemonchus contortus. International Journal for Parasitology 12 (5), 439-443.

Adams, D.B. (1988). The effect of Dexamethasone on a single and a superimposed infection with Haemonchus contortus in sheep. International Journal for Parasitology 18 (5), 575-579.

Adams, D.B. and Beh, K.J. (1981). Immunity acquired by sheep from an experimental infection with Haemonchus contortus. International Journal for Parasitology 11 (5), 381-386.

Adams, D.B., Beh, K.J. and Davies, H.I. (1982). The response of sheep to parenteral vaccination and immunising infections against the abomasal nematode Haemonchus contortus. International Journal for Parasitology 12 (5), 445-449.

Albers, G.A.A. and Le Jambre, L.F. (1983). Erythrocyte potassium concentration: A single parameter for erythropoesis in sheep infected with Haemonchus contortus. Research in Veterinary Science 35, 273-276.

Allonby, E.W. (1973). Ovine Haemonchosis; epidemiology, clinical signs and diagnosis. In: Helminth diseases of cattle, sheep and horses in Europe. Editors G.M. Urquhart and J. Armour, Glasgow, University Press pp. 59-63.

Allonby, E.W. and Dargie, J.D. (1973). Ovine Haemonchosis. In: Helminth diseases of cattle, sheep and horses in Europe. Editors G.M. Urquhart and J. Armour, Glasgow, University Press pp. 64-71.

Allonby, E.W. and Urquhart, G.M. (1973). "Self-cure" of Haemonchus contortus infections under field conditions. Parasitology 66, 43-53.

Allonby, E.W. and Urquhart, G.M. (1975). The epidemiology and pathogenic significance of haemonchosis in a Merino flock in East Africa. Veterinary Parasitology 1, 129-143.

Allonby, E.W. and Urquhart, G.M. (1976). A possible relationship between haemonchosis and haemoglobin polymorphism in Merino sheep in Kenya. Research in Veterinary Science 20, 212-214.

Altaif, K.I. and Dargie, J.D. (1978a). Genetic resistance to helminths: The influence of breed and haemoglobin type on the response of sheep to primary infections with Haemonchus contortus. Parasitology 77, 161-175.

Altaif, K.I. and Dargie, J.D. (1978b). Genetic resistance to helminths: The influence of breed and haemoglobin type on the response of sheep to reinfection with Haemonchus contortus. Parasitology 77, 177-187.

Anderson, N., Martin, P.J. and Jarrett, R.G. (1988). Mixtures of anthelmintics: A strategy against resistance. Australian Veterinary Journal 65 (2), 62-64.

Andrews, J.S. (1942). Stomach worm (Haemonchus contortus) infection in lambs and its relationship to gastric haemorrhage and general pathology. Journal of Agricultural Research 65, 1-18.

Annon (1990). National Office of Animal Health (N.O.A.H.). Compendium of data sheets for veterinary products. 1990-1991.

Armour, J. (1980). The epidemiology of helminth diseases in farm animals. Veterinary Parasitology 6, 7-46.

Barger, I.A. (1988). Resistance of young lambs to Haemonchus contortus infection and its loss following anthelmintic treatment. International Journal for Parasitology 18 (8), 1107-1109.

Barger, I.A. and Cox, H.W. (1984). Wool production of sheep chronically infected with Haemonchus contortus. Veterinary Parasitology 15, 169-175.

Benitez-Usher, C., Armour, J., Duncan, J.L., Urquhart, G.M. and Gettinby, G. (1977). A study of some factors influencing immunisation of sheep against Haemonchus contortus using attenuated larvae. Veterinary Parasitology 3, 327-342.

Bitakaramire, P.K. (1966). Studies on immunity to Haemonchus contortus infection. The elimination of a challenge infection by immune sheep. Parasitology 56, 619-622.

Blitz, N.M. and Gibbs, H.C. (1972). Studies on the arrested development of Haemonchus contortus in sheep. I. The induction of arrested development. International Journal for Parasitology 2, 5-12.

Boisvenue, R.J., Emmick, T.C. and Galloway, R.B. (1977). Haemonchus contortus: Effects of compounds with Juvenile Hormone activity on the in vitro development of infective larvae. Experimental Parasitology 42, 67-72.

Boisvenue, R.J., Galloway, R.B. and Hendrix, J.C. (1987). Protection of lambs with a purified metabolite of exsheathed third stage Haemonchus contortus larvae. American Journal of Veterinary Research 48 (8), 1236-1238.

Bomford, R. (1989). Adjuvants for anti-parasitic vaccines. Parasitology Today 5, 41-46.

Bradford, M.M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilising the principle of protein dye binding. Analytical Biochemistry 72, 248-254.

Bradley, R.E., Radhakrishnan, C.V., Patil-Kulkarni, V.G. and Loggins, P.E. (1973). Responses in Florida Native and Rambouillet lambs exposed to one and two oral doses of Haemonchus contortus. American Journal of Veterinary Research 34, 729-735.

Charleston, W.A.G. (1964). Comparison of blood and bone marrow changes in lambs with haemonchosis and in lambs repeatedly bled. Journal of Comparative Pathology and Therapeutics 74 (2), 223-240.

Charley-Poulain, J., Luffau, G. and Perry, P. (1984). Serum and abomasal antibody response of sheep to infections with Haemonchus contortus. Veterinary Parasitology 14, 129-141.

Clark, C.H., Kiesel, G.K. and Goby, C.H. (1962). Measurements of blood loss caused by Haemonchus contortus infection in sheep. American Journal of Veterinary Research 23, 977-980.

Clunies Ross, I. and Gordon, H. McL. (1933). Nutritional factors affecting resistance to haemonchosis. Australian Veterinary Journal 9, 100-107.

Connan, R.M. (1971). The seasonal incidence of inhibition of development in Haemonchus contortus. Research in Veterinary Science 12, 272-274.

Connan, R.M. (1975). Inhibited development in Haemonchus contortus. Parasitology 71, 239-246.

Connan, R.M. (1978). Arrested development in Haemonchus contortus. In: Arrested development of nematodes of sheep and cattle. Editors F.H.M. Borgsteede, J. Armour and J. Jansen, Central Veterinary Institute, Lelystad, pp. 53-62.

Courtney, C.H., Parker, C.F., McClure, K.E. and Herd, R.P. (1985). Resistance of exotic and domestic lambs to experimental infection with Haemonchus contortus. International Journal for Parasitology 15 (1), 101-109.

Cox, G.W., Shamansky, L.M. and Boisvenue, R.J. (1990). Haemonchus contortus: A simple procedure for purifying surface proteins from third and fourth stage larvae. Experimental Parasitology 70, 227-235.

Cremers, H.J.W.M. and Eysker, M. (1975). Experimental induction of inhibited development in a strain of Haemonchus contortus in The Netherlands. Tropical and Geographical Medicine 27, 229-230.

Cvetkovic, L.J., Lepojev, O., Vulic, I. (1973). Ispitivanje rasne otpornostic cigaje merino prekosa i merino kavkaz prema zeludacno-crevnim strongilidama u prirodnim uslovima infekcije. Veterinaski Glasnik 27,867-872.

Dargie, J.M. (1973b). Ovine haemonchosis. Pathogenesis. In: Helminth diseases of cattle, sheep and horses in Europe. Editors G.M. Urquhart and J. Armour, Glasgow, University Press, pp. 87-92.

Dargie, J.M. (1975). Application of radioisotope techniques to the study of red cell and plasma proteins and metabolites in helminth diseases of sheep. Symposium. British Society of Parasitologists 13, 1-26.

Dargie, J.M. and Allonby, E.W. (1975). Pathophysiology of single and challenge infections of Haemonchus contortus in Merino sheep. Studies on red cell kinetics and the "Self-Cure" phenomenon. International Journal of Veterinary Research 5, 147-157.

Dash, K.M., Newman, R.L. and Hall, E. (1985). Recommendations to minimise selection of anthelmintic resistance in nematode control programmes. In: Resistance in nematodes to anthelmintic drugs. Editors N. Anderson and P.J. Waller, CSIRO, Melbourne, pp. 161-170.

Davidson, S. (1985). "Wormkill": A strategic drenching programme. Rural Research 127, 17-20.

Davidson, S. (1987). Drenchplan: Research based worm control. Rural Research 137, 4-9.

Dinaburg, A.G. (1944). The survival of the infective larvae of the common ruminant stomach worm, Haemonchus contortus, on outdoor grass plots. American Journal of Veterinary Research 5, 32-37.

Dineen, J.K. and Wagland, B.M. (1966). The dynamics of the host-parasite relationship. IV. The response of sheep to graded and to repeated infection with Haemonchus contortus. Parasitology 56, 639-650.

Dinnik, J.A. and Dinnik, N.N. (1958). Observations on the development of Haemonchus contortus larvae under field conditions in the Kenyan Highlands. Bulletin Epizootic Diseases of Africa 6, 11-21.

Donald, A.D. (1983). Anthelmintic resistance in relation to helminth control and grazing systems. In: Facts and Reflections. IV. Resistance of parasites to anthelmintics. Editors F.H.M. Boorgsteede, Sv.Aa. Henriksen, and H.J. Over, Central Veterinary Institute, Lelystad, The Netherlands, pp. 187-198.

Donald, A.D., Dineen, J.K. and Adams, D.B. (1969). The dynamics of the host-parasite relationship. VII. The effect of discontinuity of infection on resistance to Haemonchus contortus in sheep. Parasitology 59, 497-503.

Drudge, J.H., Szanto, J.H., Wyant, Z.N. and Elam, G. (1964). Field studies on parasite control in sheep: Comparison of thiabendazole, ruelene and phenothiazine. Australian Journal of Veterinary Research 25, 1512-1518.

Duncan, J.L., Smith, W.D. and Dargie, J.D. (1978). Possible relationship of levels of mucosal IgA and serum IgG to immune unresponsiveness of lambs to Haemonchus contortus. Veterinary Parasitology 4, 21-27.

Echevarria, F.A.M. and Trindade, G.N.P. (1989). Anthelmintic resistance by Haemonchus contortus to ivermectin in Brazil: A preliminary report. The Veterinary Record 124,147-148.

Edwards, K., Jepson, R.P. and Wood, K.F. (1960). Value of plasma pepsinogen estimation. British Medical Journal 1, 30-32.

Edwards, J.R., Wroth, R., de Chaneet, G.C., Besier, R.B., Karlsson, J., Morcombe, P.W., Dalton-Morgan, G. and Roberts, D. (1986). Survey of anthelmintic resistance in Western Australian sheep flocks. 2. Relationship with sheep management and parasite control practices. Australian Veterinary Journal 63,139-144.

Evans, J.V. and Blunt, M.H. (1961). Variation in the gene frequencies of potassium and haemoglobin types in Romney Marsh and Southdown sheep established away from their natural environment. Australian Journal of Biological Science 14, 100-108.

Evans, J.V. and Whitlock, J.H. (1964). Genetic relationship between maximum haematocrit values and haemoglobin type in sheep. Science 45, 1318.

Evans, J.V., Blunt, M.H. and Southcott, W.H. (1963). The effects of infection with Haemonchus contortus on the sodium and potassium concentrations in the erythrocytes and plasma in sheep of different haemoglobin types. Australian Journal of Agricultural Research 14, 549-558.

Eysker, M. (1978). The significance of inhibited development on the epidemiology of Trichostrongylid infections in sheep in The Netherlands. Facts and Reflections III. Arrested development of nematodes in sheep and cattle. Editor F.H.M. Borgsteede, Central Veterinary Institute, Lelystad, The Netherlands.

Eysker, M. (1981). Experiments on inhibited development of Haemonchus contortus and Ostertagia circumcincta in sheep in The Netherlands. Research in Veterinary Science 30, 62-65.

Fetterer, R.H. (1989). The cuticular proteins from free-living and parasitic stages of Haemonchus contortus. I. Isolation and partial characterisation. Comparative Biochemistry and Physiology 94B (2), 383-388.

Fourie, P.J.J. (1931). The haematology and pathology of haemonchosis in sheep. 17th Report of the Director of Veterinary Services and Animal Industry, Union of South Africa, 495-572.

Gibbons, L.M. (1979). Revision of the genus Haemonchus (Cobb, 1898) (Nematoda: Trichostrongylidae). Systematic Parasitology 1, 3-24.

Gibbs, H.C. (1973). Transmission of parasites with reference to the strongyles of domestic sheep and cattle. Canadian Journal of Zoology 51, 281-289.

Gordon, H.McL. (1948). The epidemiology of parasitic diseases with special reference to studies with nematode parasites of sheep. Australian Veterinary Journal 24, 17-45.

Gordon, H.McL. (1950). Some aspects of parasitic gastroenteritis of sheep. Australian Veterinary Journal 26, 14-28, 46-52, 65-72, 93-98.

Gordon, H.McL. and Whitlock, H.V. (1939). A new technique for counting nematode eggs in sheep faeces. Proceedings of Helminthological Society, Washington 12, 50-52.

Gray, G.D. (1987). Genetic resistance to haemonchosis in sheep. Parasitology Today 3, 253-255.

Green, F.E., Forsyth, B.A., Rowan, K.J. and Payne, G. (1981). The isolation of a field strain of Haemonchus contortus in Queensland showing multiple anthelmintic resistance. Australian Veterinary Journal 57, 79-84.

Grencis, R.K., Crawford, C., Pritchard, D.I., Behnke, J.M. and Wakelin, D. (1986). Immunisation of mice with surface antigens from muscle larvae of Trichinella spiralis. Parasite Immunology 8, 587-596.

Jackson, F., Miller, H.R.P., Newlands, G.F.J., Wright, S.E. and Hay, L.A. (1988). Immune exclusion of Haemonchus contortus larvae in sheep: Dose dependency, steroid sensitivity and persistence of the response. Research in Veterinary Science 44, 320-323.

Jarrett, E.E.E. (1971). Diminished immunological responsiveness to helminth parasites. The effect of repeated reinfection of rats from an early age with Nippostrongylus brasiliensis. Clinical Experimental Parasitology 24, 9-25.

Jarrett, W.F.H., Jennings, F.W., McIntyre, W.I.M., Mulligan, W. and Sharp, N.C.C. (1959). Studies on immunity to Haemonchus contortus infection . Vaccination of sheep using a single dose of X-irradiated larvae. American Journal of Veterinary Research 20, 527-531.

Jilek, A.F. and Bradley, R.E. (1969). Haemoglobin types and resistance to Haemonchus contortus in sheep . American Journal of Veterinary Research 30 (10), 1773-1778.

Jones, V.E., Edwards, A.J. and Ogilvie, B.M. (1970). The circulating immunoglobulins involved in protective immunity to the intestinal stage of Nippostrongylus brasiliensis in the rat. Immunology 18, 621-633.

Johnson, I.L., Darvill, F.M., Bowen, F.L., Brown, P.B. and Smart, K.E. (1976). Optimum production from parasite control - gross margins in a Merino weaner sheep enterprise with different levels of parasite control. Proceedings of Australian Society of Animal Production 11, 369-372.

Johnson, I.L., Darvill, F.M., Bowen, F.L., Butler, R.W., Smart, K.E. and Pearson, I.G. (1979). The effect of four schemes of parasite control on production in Merino wether weaners in two environments. Australian Journal of Experimental Agriculture and Animal Husbandry 19, 303-311.

Kates, K.C. and Wilson, G.I. (1955). Effect of two rations differing primarily in protein, carbohydrate and crude fibre content on experimental haemonchosis in lambs. Journal of Parasitology 41 (6), Section 2, 43.

Kaupfmann, J. and Pfister, K. (1990). The seasonal epidemiology of gastrointestinal nematodes in N'dama cattle in The Gambia. Veterinary Parasitology 37, 45-54.

Keus, A., Kloosterman, A. and van den Brink, R. (1981). Detection of antibodies to Cooperia spp and Ostertagia spp in calves with the Enzyme Linked Immunosorbent Assay (ELISA). Veterinary Parasitology 8, 229-236.

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

Le Jambre, L.F. (1981). Hybridisation of Australian Haemonchs placei (Place, 1893). Haemonchus contortus cayugensis (Das and Whitlock, 1960) and Haemonchus contortus (Rudolphi, 1803) from Louisiana. International Journal of Parasitology 11, 323-330.

Le Jambre, L.F., Southcott, W.H. and Dash, K.M. (1977). Resistance of selected lines of Ostertagia circumcincta to thiabendazole, morantel tartrate and levamisole. International Journal of Parasitology 7, 473-479.

Leyland, S.E., Ridley, R.K., Dick, J.W., Slonka, G.F. and Zimmerman, G.L. (1971). Anthelmintic activity of Trichlorfon, Coumaphos and Naphthalos against the in vitro grown parasitic stages of Cooperia punctata. Journal of Parasitology 57, 1190-1197.

Lopez, V. and Urquhart, G.M. (1967). The immune response of Merino sheep to Haemonchus contortus infection. In: The reaction of the host to parasitism. Editor E.J.L. Soulsby, Lyons, France, pp. 153-159.

Lowry, O.H., Roseborough, N.J., Farr, A.L. and Randell, R.J. (1951). Protein measurement with Folin phenol reagent. Journal of Biological Chemistry 193, 136-275.

MacKenzie, C.D., Jungery, M., Taylor, P.M. and Ogilvie, B.M. (1980). Activation of complement, the induction of antibodies to the surface of nematodes and the effect of these factors and cells on worm survival in vitro. European Journal of Immunology 10, 594-601.

Maizels, R.M. and Selkirk, M.E. (1988). Biology and immunochemistry of nematode antigens. In: Biology of Parasitism. Editors P.T. Englund and F.A. Sher. Alan R. Liss Inc. New York.

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

Maizels, R.M., Philipp, M. and Ogilvie, B.M. (1982). Molecules on the surface of parasitic nematodes as probes of the immune response in infection. Immunological Reviews 61, 109-136.

Manton, V.J.A., Peacock, R., Poynter, D., Silverman, P.H. and Terry, R.J. (1962). The influence of age on naturally acquired resistance to Haemonchus contortus in lambs. Research in Veterinary Science 3, 308-314.

Mapes, C.J. (1969). The development of Haemonchus contortus in vitro. 1. The effect of pH and pCO_2 on the rate of development to the fourth stage larvae. Parasitology 59, 215-231.

Martin, C.J. and Clunies Ross, I. (1934). A minimal computation of the amount of blood removed daily by Haemonchus contortus. Journal of Helminthology 12, 137-142.

McKenna, P.B. (1973). The effect of storage on the infectivity and parasitic development of third stage Haemonchus contortus larvae in sheep. Research in Veterinary Science 14, 312-316.

Miller, H.R.P. (1979). Passive transfer of the mucosal mast cell response, its relationship to goblet cell differentiation: In: The mast cell, its role in health and disease. Editors J. Pepys and A.M. Edwards. Pitman, pp. 738-742.

Miller, H.R.P. (1984). The protective mucosal response against gastrointestinal nematodes in ruminants and laboratory animals. Veterinary Immunology and Immunopathology 6, 167-259.

Miller, H.R.P., Huntley, J.F. and Wallace, G.R. (1981a). Immune exclusion and mucus trapping during the rapid expulsion of Nippostrongylus brasiliensis from primed rats. Immunology 44, 419-429.

Miller, H.R.P., Jackson, F., Newlands, G. and Appleyard, W.T. (1983a). Immune exclusion, a mechanism of protection against the ovine nematode Haemonchus contortus. Research in Veterinary Science 35, 357-363.

Ministry of Agriculture, Fisheries and Food (1977). Manual of Veterinary Parasitological Laboratory Techniques. Technical Bulletin No. 18. Her Majesty's Stationary Office, London.

Monroy, F.G., Adams, J.H., Dobson, C. and East, I.J. (1989). Nematospiroides dubius: Influence of adjuvants on immunity in mice vaccinated with antigens isolated by affinity chromatography from adult worms. Experimental Parasitology 68, 67-73.

Munn, E.A., Greenwood, C.A. and Coadwell, W.J. (1987). Vaccination of young lambs by means of a protein fraction extracted from adult Haemonchus contortus. Parasitology 94, 385-397.

Neilson, J.T.M. (1975). Failure to vaccinate lambs against Haemonchus contortus with functional metabolic antigens identified by immunoelectrophoresis. International Journal for Parasitology 5, 427-430.

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

Ogunsusi, R.A. (1979). Termination of arrested development of trichostongyles of sheep in Northern Nigeria. Research in Veterinary Science 26, 189-192.

Ogunsusi, R.A. and Eysker, M. (1979). Inhibited development of trichostrongylids of sheep in Northern Nigeria. Research in Veterinary Science 26, 108-110.

Parkhouse, R.M.E., Philipp, M. and Ogilvie, B.M. (1981). Characterisation of surface antigens of Trichinella spiralis infective larvae. Parasite Immunology 3, 339-352.

Parkins, J.J. and Holmes, P.H. (1989). Effects of gastrointestinal helminth parasites on ruminant nutrition. Nutritional Research Reviews 2, 227-246.

Philipp, M. and Rumjanek, F.D. (1984). Antigenic and dynamic properties of helminth surface structures. Molecular and Biochemical Parasitology 10, 245-268.

Philipp, M., Parkhouse, R.M.E. and Ogilvie, B.M. (1980b). Changing proteins on the surface of a parasitic nematode. Nature 287, 538-540.

Preston, J.M. and Allonby, E.W. (1978). The influence of breed on the susceptibility of sheep and goats to a single experimental infection with Haemonchus contortus. Veterinary Record 103, 509-512.

Preston, J.M. and Allonby, E.W. (1979). The influence of breed on susceptibility of sheep to Haemonchus contortus infection. Research in Veterinary Science 26, 134-139.

Prichard, R.K., Hall, C.A., Kelly, J.D., Martin, I.C.A. and Donald, A.D. (1980). The problem of anthelmintic resistance in nematodes. Australian Veterinary Journal 56, 239-251.

Pritchard, D.I., McKean, P.G. and Rogan, M.T. (1988). Cuticle preparations from Necator americanus and their immunogenicity in the infected host. Molecular and Biochemical Parasitology 28, 275-284.

Pritchard, D.I., Crawford, C.R., Duce, I.R. and Behnke, J.M. (1985). Antigen stripping of the nematode cuticle using the cationic detergent cetyltrimethylammonium bromide (CTAB). Parasite Immunology 7, 575-585.

Radhakrishnan, C.V., Bradley, R.E. and Loggins, P.E. (1972). Host responses of worm-free Florida Native and Rambouillet lambs experimentally infected with Haemonchus contortus. American Journal of Veterinary Research 33, 817-823.

Reinecke, R.K. (1983). The ecology of free-living stages of Strongyloidea. In: Veterinary Helminthology. Butterworth Publishers.

Rickard, M.D. and Bell, K.J.C. (1971). Vaccination of lambs against infection with Taenia ovis using antigens produced during in vitro cultivation of larval stages of Taenia ovis and Taenia hydatigena. Research in Veterinary Science 12, 401-402

Rothwell, T.L.W. and Griffiths, D.A. (1977). Comparison of the kinetics of expulsion of Trichostrongylus colubriformis from previously infected, reinfected and vaccinated guinea pigs. Journal of Parasitology 63, 761-762.

Russell, D.A. and Castro, G.A. (1979). Physiological characterisation of a biphasic immune response to Trichinella spiralis in the rat. Journal of Infectious Diseases 139, 304-312.

Sage, H., Woodbury, R.G. and Bornstein, P. (1979). Structural studies on human type IV collagen. Journal of Biochemical Chemistry 254, 9893-9900.

Salman, S.K. and Duncan, J.L. (1984). The abomasal histology of worm-free sheep given primary and challenge infections of Haemonchus contortus. Veterinary Parasitology 16, 43-54.

Salman, S.K. and Duncan, J.L. (1985). Studies on the abomasal pathology of immunised and non-immunised sheep infected with Haemonchus contortus. Journal of Helminthology 59,351-359.

Silverman, P.H. (1959). In vitro cultivation of the histotrophic stages of Haemonchus contortus and Ostertagia species. Nature 183, 197.

Silverman, P.H., Poynter, D. and Podger, K.R. (1962). Studies on larval antigens derived by cultivation of some parasitic nematodes in simple media: Protection tests in laboratory animals. Journal of Parasitology 48, 562-571.

Sinclair, J. (1970). The relationship between circulating antibodies and immunity to helminthic infections. Advances in Parasitology 8, 97-138.

Smith, W.D. (1977). Anti-larval antibodies in the serum and abomasal mucus of sheep hyperinfected with Haemonchus contortus. Research in Veterinary Science 22, 334-338.

Smith, W.D. and Christie, M.G. (1978). Local and serum antibodies in sheep immunised with irradiated larvae. International Journal for Parasitology 8, 219-223.

Smith, W.D. and Christie, M.G. (1979). Haemonchus contortus: Some factors influencing the degree of resistance of sheep immunised with attenuated larvae. Journal of Comparative Pathology 89, 141-150.

Smith, W.D., Jackson, F., Jackson, E., Williams, J., Willadsen, S.M. and Fehilly, C.B. (1984). Resistance to Haemonchus contortus transferred between genetically histocompatible sheep by immune lymphocytes. Research in Veterinary Science 37, 199-204.

Smith, W.D., Jackson, F., Jackson, E. and Williams, J. (1985). Age immunity to Ostertagia circumcincta: Comparison of the local immune responses of 4.5 and 10 month old lambs. Journal of Comparative Pathology 95, 235-245.

Soulsby, E.J.L. (1968). Helminths, Arthropods and Protozoa of Domesticated Animals. 7th Edition. Bailliere Tindall, London.

Soulsby, E.J.L., Sommerville, R.I. and Stewart, D.F. (1959). Antigenic stimulus of exsheathing fluid in "self-cure" of sheep infested with Haemonchus contortus. Nature (London) 183, 553-554.

Stewart, D.F. (1953). Studies on resistance of sheep to infestation with Haemonchus contortus and Trichostrongylus species and on the immunological reactions of sheep exposed to infestation. Australian Journal of Agricultural Research 4, 100-117.

Stoll, N.R. (1929). Studies with the strongylid nematode Haemonchus contortus. 1. Acquired resistance of hosts under natural reinfection conditions out of doors. American Journal of Hygiene 10, 384.

Stoll, N.R. (1940). In vitro conditions favouring ecdysis at the end of the first parasitic stage of Haemonchus contortus (Nematoda). Growth 4, 383-406.

Stringfellow, F. (1984). Effects of bovine heme on development of Haemonchus contortus in vitro. Journal of Parasitology 70 (60),989-990.

Stringfellow, F. (1986). Cultivation of Haemonchus contortus (Nematoda: Trichostrongylidae) from infective larvae to the adult male and egg laying female. Journal of Parasitology 72 (2),339-345.

Towbin, H., Stacklin, T. and Gordon, J. (1979). Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proceedings of the National Academy of Science of the United States of America 76, 4350-4354.

Urquhart, G.M. (1980). Application of immunity in the control of parasitic disease. Veterinary Parasitology 6, 217-239.

Urquhart, G.M. (1988). The potential use of vaccines or genetically resistant animals in the control of helminthiasis. In: Increasing small ruminant productivity in semi-arid areas. Editors E.F. Thomson and F.S. Thomson, pp. 267-273.

Urquhart, G.M., Jarrett, W.F.H. and Mulligan, W. (1962). Helminth immunity. Advances in Veterinary Science 7, 87-129.

Urquhart, G.M., Armour, J., Duncan, J.L., Dunn, A.M. and Jennings, F.W. (1987). Ovine haemonchosis. In: Veterinary Parasitology. Longman, Scientific and Technical.

Urquhart, G.M., Jarrett, W.F.H., Jennings,J.F., McIntyre, W.I.M., Mulligan, W. and Sharp, N.C.C. (1966a). Immunity to Haemonchus contortus infection. Failure of X-irradiated larvae to immunise young sheep. American Journal of Veterinary Research 27, 1641-1643.

van Wyk, J.A. and Malan, F.A. (1988). Resistance of field strains of Haemonchus contortus to ivermectin, closantel, rafoxanide and the benzimidazoles in South Africa. Veterinary Record 123, 226-228.

Veglia, F. (1915). The anatomy and life history of Haemonchus contortus. Third and Fourth Reports of the Director of Veterinary Services, Onderstepoort S.A., pp. 347-500.

Vercruysse, J. (1984/5). The seasonal prevalence of inhibited development of Haemonchus contortus. in sheep in Senegal Veterinary Parasitology 17, 159-163.

Wagland, B.M. and Dineen, J.K. (1967). The dynamics of the host-parasite relationship. VI. Regeneration of the immune response in sheep infected with Haemonchus contortus. Parasitology 57, 59-65.

Waller, P.J. (1986). Anthelmintic resistance in nematode parasites of sheep. Agricultural Zoological Reviews 1, 333-373.

Waller, P.J. and Thomas, R.J. (1975). Field studies on inhibition of Haemonchus contortus in sheep. Parasitology 71, 285-291.

Wakelin, D. (1978). Genetic control of susceptibility and resistance to parasitic infection. Advances in Parasitology 16, 219-308.

Wedrychowicz, H. and Bezubik, B. (1990). Influence of adjuvants on immunity in rabbits vaccinated with infective larval somatic proteins of Trichostrongylus colubriformis. Veterinary Parasitology 37, 273-284.

Weinstein, P.P. and Jones, M.F. (1956). The in vitro cultivation of Nippostrongylus muris to the adult stage. Journal of Parasitology 42, 215-236.

Wharton, D.A. (1982). The survival of desiccation by the free-living stages of Trichostrongylus colubriformis. Parasitology 84, 455-462.