STUDIES ON ACQUIRED IMMUNITY TO *NEMATODIRUS BATTUS* INFECTION IN LAMBS

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ABSTRACT

Several experiments were conducted using housed sheep with the aim of obtaining information regarding the immune response of young lambs to *Nematodirus battus* infection, the influence of protein supplementation and the persistence of the response. Field observations were conducted also over a two year grazing period to determine the seroepidemiology of natural infections using an ELISA developed against worm antigens.

Prior to commencement of the main housed experiments, the results of a preliminary trial conducted by Dr.W.D.Smith prior to commencement of this PhD research project were evaluated to assess the kinetics of worm loss following a single infection of *N.battus*, essential information before 'trickle infection-challenge' experiments can commence. Four groups of young lambs were infected with a single dose of 30,000 L_3 and each group was killed after 7, 14, 21 or 28 days post-infection (PI). The worm burdens were significantly reduced in number after 28 days PI. Individual variation in burdens was evident after 21 days PI and therefore, in view of these findings, lambs in future experiments were killed at 9 or 10 days post-challenge (PC) to minimize between animal variation.

The housed 'trickle infection-challenge' trials followed a standard infection regime. Lambs were infected with escalating doses of L_3 over several weeks, treated with anthelmintic and challenged with a single dose of 30,000 L_3 a week later. Challenge controls did not receive a primary trickle infection. All groups were killed on either day 9 or 10 PC to obtain worms, blood and intestinal tissue. Blood samples were taken frequently throughout the primary infection period for peripheral blood eosinophil counts and antibody level determination. Two trials were conducted to assess the influence of supplemention of an adequate basal diet with a rumen bypass protein (fish meal). Both had a similar design except for differences in the levels of dietary protein, the age of lambs and the duration of infection. Previously infected and challenge control groups were offered either a basal diet (trial 1, 132 g CP kg^{-1} DM; trial 2, 125 g CP kg^{-1} DM) or a supplemented one (trial 1, 183 g CP kg^{-1} DM; trial 2, 178 g CP kg^{-1} DM CP). The results of both trials showed trends for enhancement of responsiveness without significant effects. Previous infection significantly enhanced immunity as characterized by reduced worm size and burdens,

elevated antibody levels, increased numbers of tissue mast cells and eosinophils. Supplementation significantly enhanced antibody but not inflammatory responses. Re-analysis of the results showed that lambs could be segregated into high- and low-responders based on their worm burden. However, the degree of responsiveness was not reflected in the inflammatory and antibody responses studied.

A trial was conducted to determine the persistence of the immune response described in the dietary trials. Six groups of lambs (3 infected, 3 challenge controls) were subjected to the standard design outlined above. An infected and control group was challenged either one, 6 or 12 weeks post-treatment and killed 10 days PC. The results showed an ability to respond without antigenic stimulus for up to 12 weeks which was expressed by retardation in development of the worm populations and also by worm expulsion.

Field trials were conducted over a two year grazing period to determine any difference in serum anti-worm antibodies of lambs and ewes and whether this could be correlated to developing immunity. The use of serum fructosamine concentration as a general index of gastrointestinal damage was also investigated. The results showed that antibody responses of ewes were maintained at high levels throughout the grazing period with no indication of a periparturient relaxation in immunity. Lambs developed increasing antibody levels over time and the FEC declined when the peak levels were reached. However, lamb antibodies were significantly lower in comparison to ewes. Serum fructosamine was not altered despite periods of clinical nematodirosis.

DECLARATION

The work reported in this thesis was conducted at the Parasitology Division of Moredun Research Institute, Edinburgh and this thesis was composed by myself. Some of the eosinophil and mast cell data reported was obtained in collaboration with colleagues at the institute. The field studies were part of a larger project concerned with the investigation of anthelmintic resistance in ovine gastrointestinal nematodes and the data presented in chapter 3 was generated by Dr.W.D.Smith. Nevertheless, most of the work presented in this thesis was carried out by myself and where conjoint experiments were undertaken, a full role was played in the design of the experiments and interpretation of the results.

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Table of Contents

Chapter 2 General materials & methods

Chapter 3 Kinetics of worm loss

List of Tables

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of Figures

Chapter 4

Chapter 5

Chapter 6

Chapter 7

CHAPTER 1
Introduction

1.1 Introduction

Gastrointestinal parasitism in ruminants is frequently associated with high stocking density and intensive production systems of husbandry and is responsible for considerable economic loss (Holmes, 1985). The common helminths of sheep, the nematodes of the trichostrongyle group, are acquired through ingestion of infective larval stages which are present on herbage. Established infections cause various pathological changes which can result in weight loss, anaemia, diarrhoea, dehydration and in severe cases death.

Currently, strategic control programmes are practiced which incorporate anthelmintic dosing combined where practicable with pasture spelling or incorporation of less susceptible classes of livestock. However, the emergence of resistant strains of parasites, growing consumer concern over the widespread application of chemotherapeutic drugs coupled with the fact that methods of pasture spelling alone are not a practical means of control has provoked a search for alternative approaches to the control of gastrointestinal parasitism.

Due to the rapid growth in the field of molecular immunology various aspects of immunity towards parasitic infections are being sought and the potential for manipulation of the host immune response in order to control infections is of great interest. In *Trichostrongylus colubriformis* infections Dineen, Gregg and Lascelles (1978) has shown that sheep can be segregated into 'responders' and 'non-responders'

based on their ability to regulate worm populations. These findings have not only stimulated work on selection of genetically resistant animals as a method of control of gastrointestinal nematodes but also allow comparisons to be made between the mechanisms of immunity of responsive and susceptible hosts. Hence the ability to detect markers of immune responsiveness is crucial in such a selection process. The potential for vaccination against nematodes is large and the bovine lungworm, *Dictyocaulus viviparus* has been controlled successfully with a commercially available attenuated vaccine. However, attempts to immunize sheep against their common gastrointestinal nematodes unfortunately have been only partially successful and in general have not been effective in the young growing lamb. Current research is directed to identifying protective antigens of common nematodes as a long-term solution to the problems of control strategies outlined above.

Despite the evidence that sheep can acquire immunity to gastrointestinal nematodes, there are still no reliable vaccines for controlling these infections. This is due partly to a lack of basic knowledge concerning the mechanisms involved in the development of naturally acquired immunity (Smith, 1988). Our knowledge to date is fragmentary. This is due to the complexity both of the parasite and the host immune response. In addition there is variation in host responses towards different parasite phyla and developmental stages. Hence, there is an urgent need to characterize the development of immunity towards nematodes of sheep. Such an approach would be to study host-parasite relationships based on an ovine model since extrapolation of findings from laboratory animal studies to infections in sheep may not be possible considering differences in the immune system of diverse species (Gamble and Zarlenga, 1986).

Among the various genera of nematodes that infect sheep, *Nematodirus battus* infection in lambs is of particular relevance in the sense that lambs respond rapidly parasitologically and acquire a solid immunity throughout the rest of their lives. Despite the knowledge of this established fact very little work has focused on the acquisition of immunity and the mechanisms involved. This may be because *N.battus* is not of wide geographical significance and that due to the immunity acquired and the seasonal prevalence, the disease is easily controlled by grazing management strategies and/or strategic anthelmintic treatments.

In view of the current trends in immunoparasitological research and given the failure of vaccination in young lambs (Murray, 1987; Emery and Wagland, 1991), the use of *N.battus* infection in lambs as a model for studies on immune responsiveness is considered of practical interest (Taylor and Thomas, 1986). As with *T.colubriformis* infections in sheep, studies with *N.battus* have shown also that lambs can be segregated based on their responsiveness to infection (Taylor and Thomas, 1986). Therefore, it is envisaged that the study of naturally acquired immunity to *N.battus* infections in lambs would yield important if not invaluable data that may be also relevant to other nematode infections.

The objectives set forth for this Ph.D. research project aim to :

1. develop a model infection which would provide baseline data concerning the immune response towards and regulation of *N.battus* populations.
2. enhance naturally acquired immunity of lambs towards *N.battus* by supplementation of feed with a by-pass protein.
3. determine whether the model used can maintain immunological memory as shown in the field.

4. study natural infections of grazing ewes and their lambs with *N.battus.*

Very little information on the development of immunity to *N.battus* is available in the literature. Therefore, reference to studies conducted with other common ovine gastrointestinal nematode infections has been used extensively.

1.2 Nematodirosis

1.2.1 General

Nematodirosis is a common roundworm infection caused by the small intestinal nematode, *Nematodirus battus* which generally affects young lambs of about 4 to 8 weeks of age (Coop, 1989). The disease is prevalent typically during the late spring to early summer months and rarely affects sheep over 4 months of age (Boag and Thomas, 1975). The disease condition was first described by Crofton and Thomas (1951) in North-east England. The origin of the species remains obscure. It has been suggested that it may have been present in Britain in small numbers for a long time and that changes in animal husbandry in the early 1950's were responsible for an increase in *Nematodirus* burdens. However, Jansen (1973) has argued with established historical and morphological facts that this species could have originally belonged to the helminth fauna of some species of deer. *N.battus* has some striking features in common with *Nematodirus roscidus,* Railliet, 1911 which has been found in many parts of Europe in roe deer, fallow deer and mouflon. Putting all his arguments together Jansen concluded that *N.battus* was imported into Britain somewhere in the 1920's or 1930's with foreign species of deer.

N.battus worms live in the small intestine causing local damage to the bowel lining, resulting in watery scour and dehydration (Coop, 1989). Death due to severe

infection can occur within a few days of scouring and losses may be as high as 30% of the lamb crop (Gibson, 1974). While mortalities, when they arise, are obvious and often disastrous, the more insidious aspect of this condition is that of the non-fatal clinical and sub-clinical disease. The major feature is the failure of parasitized lambs to achieve their potential rate of live weight gain. Such retardation in productivity is due to a combination of reduced voluntary feed intake and impaired feed utilization (Rowlands and Probert, 1972).

1.2.2 Life history and epidemiology

The parasitic life history of *N.battus* was first described by Thomas (1959). The direct life-cycle differs to that of other strongyles in that eggs voided in the host faeces do not hatch to produce first-stage free-living larvae (L_1). In contrast two successive non-parasitic larval stages (L_1 and L_2) followed by the third (L_3) or infective larval stage develop within the egg. The L_3 hatch from the egg following an increase in temperature (spring and summer months) after a prolonged period of cold exposure (autumn and winter months). The stereotyped feature of this seasonal pattern of infection is the reason why the disease is readily forecasted (Ollerenshaw and Smith, 1966; Smith and Thomas, 1972).

Studies on the development of *N.battus* in single infections (Mapes and Coop, 1972) showed that upon ingestion the L_3 exsheath and the third moult occurs between days 2 and 4 post-infection within the mucosa with day 4 being the time of maximum mucosal penetration. By day 6, during the mid-fourth larval stage (L_4) most larvae would have returned to the mucosal surface. However, some larvae may remain in the mucosa and moult to their fifth stage (L_5). Adult worms were able to be

distinguished as early as day 10 post-infection. The majority of worms tend to be found in the first 3 metres of the proximal duodenum (Thomas, 1959; Rowlands and Probert, 1972). Larval inhibition (arrest) in *Nematodirus* species has been demonstrated in penned sheep (Donald, Dineen, Turner and Wagland, 1964; Dineen, Donald, Wagland and Turner, 1965) and under natural grazing conditions (Waller and Thomas, 1983). It has been suggested that the size of the infecting dose or the duration of exposure might be the cause of inhibition under experimental infection (Donald *et al.*, 1964; Dineen *et al.*, 1965). In contrast, the results of Waller and Thomas (1983) suggest that both seasonal factors and age of the host may play an important role in larval inhibition for *Nematodirus* species. They also suggested that this phenomenon was not density-dependent nor associated with host immunity. However, Taylor and Thomas (1986) have shown that responder lambs had increased numbers of L_4s and attributed this to the immunity acquired by the lambs.

The epidemiology of nematodirosis is predictable (Ollerenshaw and Smith, 1966; Smith and Thomas, 1972) and after three successive annual grazings the degree of pasture contamination is frequently sufficient to cause clinical disease (Boag and Thomas, 1975). Eggs passed by infected lambs overwinter on the pasture and hatch to produce L_3 the following spring which are the source of infection for the following year's lamb crop (Boag and Thomas, 1975). Since adult sheep are immune to *N.battus* they do not play any major role in the epidemiology of nematodirosis (Bairden and Armour, 1987). Recently, the involvement of calves in perpetuating rather than reducing the degree of pasture contamination has been documented (Bairden and Armour, 1987; Coop, Jackson, Jackson, Fitzsimons and Lowman, 1988 and Coop, Jackson and Jackson, 1991). Hence the practice of alternate grazing

between sheep and cattle as a means of control has proven detrimental since young susceptible calves may transmit infection to the following years lamb crop by providing a suitable niche for growth and reproduction of *N.battus*.

The possibility of an alteration in the epidemiology of nematodirosis has been proposed (Gibson and Everett, 1981; McKellar, Bairden, Duncan and Armour, 1983; Hollands, 1984; Thomas 1991). This is due to findings that show an increased level of pasture contamination during the autumn months and incidences of patent infections during this period. These findings have been correlated to changes in the environment (climate) (Thomas, 1991), in particular microclimatic effects upon eggs. When the eggs are released from the faecal pellet into the surrounding soil due to harsh conditions, favourable weather conditions such as more constant moisture and even temperature (Gibson and Everett, 1981), may favour the transmission of infection due to increased hatching and larval activity upon pasture leys.

1.2.3 Clinical signs and pathology

The clinical signs observed in cases of nematodirosis are generally similar to those observed in trichostrongylosis, ostertagiosis and cooperiosis which are manifested by illthrift, weight loss and depressed voluntary feed intake (Blood and Radostits, 1989). In severe cases the lambs become diarrhoeic with dark soft faeces fouling the wool of the breech and dehydrated with sunken eyes (Gibson, 1974; Blood and Radostits, 1989). Deaths may occur within two days of the first observed illness (Blood and Radostits, 1989) and can continue for three weeks after which the survivors are solidly immune (Gibson, 1974).

Pathological changes observed in cases of nematodirosis vary from mild to acute inflammation of the intestine (Kingsbury, 1953; Thomas and Stevens, 1956; Baxter, 1957). Nematodirosis in young lambs can cause severe enteritis with marked hyperaemia, oedema and exudation which is normally cattarhal but in severe cases diptheritic (Reid and Murray, 1974). The general feature of nematodirosis is local villous atrophy (Thomas, 1959; Coop, Angus and Mapes, 1973; Martin and Lee, 1980) which is believed to be a sequel of pressure/damage upon the epithelial cells exerted by the parasites resulting in increased loss of cells into the lumen of the intestine. Hyperplasia of congested and oedematous intestinal mucosa is occasionally seen in sheep subjected to repeated challenge (Reid and Murray, 1974).

Nematodirus species are not likely to cause irreparable tissue damage since they do not penetrate deep into the mucosa. In fact the tissue phase is transient, superficial and limited to the larval stages (Rowlands and Probert, 1972). The actual number of worms associated with mortality is extremely variable. Deaths have been reported with 10,000 worms (Kingsbury, 1953; Thomas and Stevens, 1956). In contrast, lambs with burdens in excess of 30,000 worms have appeared healthy (R.L.Coop, personal communication). Thomas (1959) infected four 6 week old lambs with 50,000 L_3 and did not find any pathological changes apart from slight superficial flattening and erosion of villi which were in contact with the parasites.

The mechanism whereby *N.battus* damages the epithelium remains unknown. Direct contact resulting in pressure necrosis is one possibility (Coop *et al.*, 1973) and/or the effect of worm secretions/excretions (Coop *et al.*, 1973; Martin and Lee, 1980) may be another. In their study Coop *et al.* (1973) suggested that severe changes

to villous architecture led to a reduction in mucosal enzyme activity, in particular, alkaline phosphatase and disaccharidase, and was exhibited by marked scouring among lambs. It was suggested that in these lambs absorption may be impaired in the proximal region of the small intestine and this has been suggested also by Rowlands and Probert (1972). Thus unabsorbed residues may pass into and undergo fermentation in the distal bowel. It has been shown that parasitized lambs lacked leucine aminopeptidase activity in the first 3 metres of the small intestine (Rowlands and Probert, 1972). This enzyme is one of the terminal protein digestive enzymes and may well be a cause for reduced liveweight gain . Another common pathological change is mucus hypersecretion (Rowlands and Probert, 1972; Martin and Lee, 1980) and it has been suggested that this may be involved in the rejection mechanism (Martin and Lee, 1980).

Several workers (Thomas and Stevens, 1956; Munday, 1981; Mitchell, Mathieson and Fitzsimons, 1985) have noted nephrosis accompanying outbreaks of scour. The aetiology of the nephrotic lesions remains obscure. It has been suggested that it may be linked to an association between severe nematodirosis and production of *Clostridium welchii* type D toxin in the parasitized gut (Thomas and Stevens, 1959). The involvement of a nephrotic mycotoxin has also been suggested (Munday, 1981).

Under field conditions lambs are always faced with a multiple challenge with various parasites. Studies using rats (Bristol, Pinon and Mayberry, 1983) showed a synergistic effect of coccidial infections upon *Nippostrongylus brasiliensis* in terms of egg production and patency and this finding prompted Catchpole and Harris

(1989) to investigate the effects of a concurrent infection with coccidia and *N.battus*. Their results showed that concurrent infections produced severe clinical disease and death with increased worm fecundity. Clearly other parasitic and possibly bacterial infections may play an active role in the pathogenesis and epidemiology of nematodirosis.

1.2.4 Diagnosis, treatment and control

As with other nematode infections of ruminants nematodirosis is diagnosed by the presence of large coffee-coloured strongyle type eggs in the faeces. A tentative diagnosis can be made based on clinical appearance. Usually lambs scouring profusely in the late spring/early summer months are infected with *N.battus*. Despite the advances in diagnostic techniques, the diagnosis of ruminant nematodoses is still dependent on the visual inspection of faecal flotations. Apart from faecal egg counts, total worm burdens should be counted whenever possible and the results considered together with the clinical signs, the animals age and the season of the year (Blood and Radostits, 1989). A lack of relationship between larval intake and faecal egg output above a threshold level has been noted for *Nematodirus* infections (F.Jackson and R.L.Coop, unpublished data; Coop *et al.*, 1988). Thus the parasite is able to regulate egg production, and faecal egg counts may underestimate the actual worm burdens in heavily infected sheep.

There has not been any work conducted to upgrade the present method of diagnosing nematodirosis essentially because the prevailing method has proven reliable. However, recently serum fructosamine concentrations have been assayed (Heath and Connan, 1991) in lambs with single and concurrent infections with

Nematodirus spathiger and *Ostertagia circumcincta*. Falling levels of serum fructosamine may act as an indicator of parasitic protein-losing gastroenteropathy, therefore being of a more general application than serum pepsinogen levels in the diagnosis of parasitic gastroenteritis. From this study, in general, a correlation between fructosamine levels and parasitism was shown although it was weak in *N.spathiger*-infected lambs.

Anthelmintic treatment should not be left until clinical infection is evident since it is known that considerable damage will have been caused by juvenile worms (Coop, 1989) and therefore the prognosis, even after effective treatment will be poor. Most broad-spectrum anthelmintics such as benzimidazoles, levamisole and ivermectin can be used to remove worm burdens although one must be aware of the potential risk of the worms developing resistance to prolonged frequent use of one drug family. Indeed resistance of *N.spathiger* to benzimidazoles has been reported in Australia (Obendorf, Nicholls, Koan and Lacy, 1991).

The control of nematodirosis is based on the prevention of lambs grazing highly contaminated pastures particularly avoiding grazing young lambs on pastures which carried young lambs early in the previous season. Although peak levels generally coincide with lambing periods, this time may vary from year to year depending on climatic conditions and therefore may not coincide with lambing. In this case lambs may not be faced with pathogenic levels. The Ministry of Agriculture, Fisheries and Food (M.A.F.F) issue forecasts for nematodirosis and control can be achieved effectively by drenching lambs at 3 week intervals during the high risk period. Although cattle (calves) can transmit infection, alternate grazing with sheep

and cattle can still reduce contamination provided that calves are run with their dams and the third year of the rotation involves conservation and/or arable crops (Coop, 1989).

1.2.5 Features of Immunity

A limited amount of work has been conducted with the aim of understanding the mechanisms involved in the immune expulsion of *N.battus* from lambs. Most of the work conducted has focused upon population dynamics of the parasite during single (Mapes and Coop, 1972; Taylor and Thomas, 1986), multiple (Gibson and Everett, 1963; Mapes and Coop, 1973a) and concurrent (Mapes and Coop, 1970, 1973b, 1973c) infections without any emphasis placed upon host humoral and cellular responses. This fact is somewhat surprising since infections with *N.battus* are rapidly cleared and provide solid immunity throughout adulthood and therefore would make an interesting model for studies on immunity to ovine gastrointestinal nematodes.

One of the earliest experiments by Gibson (1959) suggested that resistance to *N.battus* was solely an age-dependent phenomenon and that previous exposure conveyed little or no additional resistance. However, subsequent studies (Gibson and Everett, 1963; Taylor and Thomas, 1986) have established that this is not true and acquired immunity plays an important role in the acquisition of immunity. However, age immunity also exists and has been described in lambs as early as 3 months of age in single (Taylor and Thomas, 1986) and multiple (Gibson and Everett, 1963) infections. Gallie (1973) described 3 types of resistance exhibited by rabbits to infection with *N.battus*, namely, natural, acquired and age resistance and claimed that

inhibition of larval development among older rabbits may be due to a rapid immune response or to morphological or physiological changes in the host which had developed with age.

Grazing lambs appear, on the basis of faecal egg counts (FEC), to develop a strong immunity to *N.battus* within 6 to 8 weeks of exposure to infective larvae. Apparently the magnitude and longevity of infection is related to larval intake. Maximum numbers of adult worms in the intestine and of eggs in faeces is reached around 16-18 days following administration of a relatively high dose of 60,000 *N.battus* L_3 to young lambs. These numbers begin to fall from days 20-22 until by day 28 there are very few nematodes left in the intestine (Mapes and Coop, 1972). This is associated with a well marked immunity to re-infection with this parasite (Gibson and Everett, 1963). A dose of 2000 *N.battus* L_3 given to young lambs has been shown to result in a relatively high worm burden which is maintained for a much longer period (at least 74 days) (Lee and Martin, 1976). A further example comes from the work by Taylor and Thomas (1986) who showed that a single primary infection with 50,000 L_3 resulted in only 10% establishment of a challenge dose whereas 3000 L_3 did not make any difference in terms of establishment compared to challenge controls. Taylor and Thomas (1986) were able to segregate lambs infected and challenged with *N.battus* L_3 on the basis of their worm burdens into 'responders' and 'non-responders'. This finding shows that as with *T.colubriformis* (Dineen *et al.*, 1978) sheep may vary in their ability to cope with nematodirosis therefore suggesting a genetically innate resistance.

It has been demonstrated that not only does immunization of sheep with *T.colubriformis* L_3 protect against homologous infection but also induces cross-

protection against infections with *N.spathiger* (Dineen, Gregg, Windon, Donald and Kelly, 1977). It was concluded that non-specific effector mechanisms generated towards *T.colubriformis* were effective in protection against *N.spathiger* infections. No significant protection occurred against a single species challenge with *N.spathiger* but 98-100% protection was attained against all 3 species in lambs challenged concurrently (*T.colubriformis, Trichostrongylus vitrinus and N.spathiger*) thus suggesting that protective antigens were not shared and that the effector mechanism was non-specific in nature. Field studies by Douch (1989) demonstrated a non-specific response against *N.spathiger* which was manifested by increased numbers of mucosal mast cells (MMC), eosinophils, globule leucocytes (GL) and enhanced larval migration inhibitory (LMI) activity. In contrast to the findings of Dineen *et al.* (1977), Douch found significantly higher numbers of immature *Nematodirus* species in *T.colubriformis*-resistant sheep and concluded that this was not arrested development, as suggested by Dineen *et al.* (1977), but retardation due to mediators from gut mucosal cells. Cross-protection between sheep nematodes is discussed in more detail later in this chapter.

Information on cellular and humoral responses in lambs towards *N.battus* or any *Nematodirus* species is extremely scarce. From what is known to date it seems that the effector mechanism is non-specific in nature. A local mast cell response (Buzmakova, 1980) and peripheral eosinophilia (Zurliiski, 1979; Usenov, 1978; Buzmakova, 1980) have been described in experimental infections with *Nematodirus* species. It is also evident that a local humoral response may be involved in the rejection of *N.battus* since numerous IgA-containing cells have been observed in dense aggregation in areas of the small intestine where *N.battus* predominated

(Sinclair, Wassall and Cawthorne, 1985). Other possible mechanisms have been suggested for expulsion of *N.battus* and include the effect of villus atrophy and gut mucus (Martin and Lee, 1980). According to these workers *N.battus* maintain their position in the host by bracing against intestinal villi. Therefore the villus atrophy which occurs during infection may increase the possibility of dislodgment. Using scanning electron microscopy they also noticed *N.battus* enclosed by mucus-like material at the time of worm rejection and when structural changes were beginning to occur. It was suggested that mucus may interefere with worm metabolism by restricting oxygen uptake or affecting locomotion or reducing the rate at which metabolic end products are lost from the worms.

So far this discussion has focussed on what is known of the host mechanisms involved in the acquisition of immunity. However, little has been described about the effects upon the worm. It has been mentioned earlier that worm expulsion is a major effect and a valid measurement of responsiveness. However, effects such as retardation in growth and reduced fecundity are equally important in assessing acquisition of immunity. Earlier work by Donald *et al.* (1964) with *N.spathiger* has shown that the mechanisms of resistance are mediated about threshold levels and are expressed by elimination of L_3 and adult stages, retardation of L_4 development, reduction in fecundity and discrimination against female worms in both the degree of L_4 retardation and extent of adult elimination. In contrast, Ballantyne, Sharpe and Lee (1978) suggested a discrimination against male worms. They demonstrated that the male *N.battus* energy charge falls more rapidly compared to females and suggested that this finding can be related to observations which indicate that males are being removed earlier and in greater numbers compared to females. A reduction in energy

charge which is essentially lack of ATP may result in impairment of physiological functions and also play a part in the eventual expulsion or retardation of worms. Other effects upon *N.battus* that may well be a consequence of immunity include changes in acetylcholinesterase activity, worm structure and the accumulation of crystals in the intestinal lumen of the worms (Lee and Martin, 1976).

1.3 Mechanisms of Immunity to Ovine Gastrointestinal Parasitism

Only recently have studies upon aspects of immunity towards nematode infections become of paramount interest. This may well be due to the fact that the current methods for containment of disease, which rely heavily upon anthelmintics, have been undermined due to the emergence of resistant strains of parasites. Therefore, a logical step in the control of these infections would be the use of immuno-prophylactic measures which essentially rely upon vaccines. However, a lack of in-depth knowledge concerning the mechanisms of immunity pose vaccinologists with a difficult task. Hence the current interest in mechanisms of immunity aim to provide information that will lead ultimately to the development of a reliable vaccine. It is worthy to note here that despite the lack of knowledge to date, research aimed at identifying protective antigens have yielded promising results. Artificially acquired immunity, with more than 90% protection, has been obtained with *H.contortus* gut membrane antigens (Munn, Smith, Graham, Greenwood, Tavernor and Coetzee, 1993; Smith, Smith and Murray, 1994). Protection has been obtained towards *T.colubriformis* with ES products (Savin, Dopheide, Frenkel, Wagland, Grant and Ward, 1990; Dopheide, Tachedjian, Phillips, Frenkel, Wagland and Ward, 1991).

The capacity of mammalian hosts to respond to gastrointestinal nematodosis is a function of age, nutritional and reproductive status, and genotype of the host and the ability of the parasite to evade, suppress or modify, the host response (Miller, 1984). Despite the gradually acquired immunity by ruminants to continuous or seasonal stimulation by ingestion of L_3, the immunity acquired is not usually solid. Generally the acquisition of immunity is delayed until or around about the age of 6 months providing lambs are continuously exposed to the parasite. It is suggested that the unresponsiveness of young lambs is partly due to immaturity of gut effector mechanisms rather than the failure to produce parasite-specific antibodies (Gregg, Dineen, Rothwell and Kelly, 1978). However, the cause of this age-related susceptibility has yet to be clearly identified (Miller, 1984).

Once immunity is acquired it is rapid since rejection of challenge infections of *H.contortus* or *T.colubriformis* by immune sheep occurs rapidly with the majority of L_3 being expelled within 24 hours (McClure, Emery, Wagland and Jones, 1992) and even as quickly as 4 hours (Jackson, Miller, Newlands, Wright and Hay, 1988). The precise mechanism of rejection is incompletely understood but involves collaboration between an immunologically-specific encounter with worm antigen and a non-specific episode with direct effects on the parasite or the local gastrointestinal environment which leads to expulsion (Dineen *et al.*, 1977). This immunity may regulate worm burdens to the extent of keeping worm numbers below pathogenic levels but seldom removes all the worms (Emery and Wagland, 1991).

Expression of resistance towards nematodes and the effects upon immune targets varies between host and parasite. Innate immunity is a pre-existing immunity

which prevents L_3 from establishing in hosts not previously exposed to infection. Alternatively hosts may not expel their worm burdens but are able to alleviate the deleterious effects of infection. Such resistance is termed resilience. Acquired immunity is a form of resistance acquired by hosts from exposure to infection (Emery and Wagland, 1991).

Acquired immunity exerts its effects through effector mechanisms which may prevent establishment of incoming L_3, probably by a reversable paralysis or dis-orientation as recovered L_3 can successfully infect naive hosts (Rothwell, 1989). This phenomenon has been described in a number of host-parasite interactions and is referred to as rapid expulsion (RE) (Bell and McGregor, 1980; Miller, 1984). In some instances the hosts effector mechanisms may cause a retardation in nematode development or fecundity (Miller, 1984; Rothwell, 1989). Another form of responsiveness is the expulsion of established adult worms due to sensitization with large numbers of incoming L_3 (Rothwell, 1989). This expulsion of patent infections may occur over several months in ruminants (Miller, 1984). Elimination of nematodes after they have established in the gut, but before achieving patency also occurs (Dineen and Wagland, 1966).

Information to date suggests that immunity is a combination of specific (antibody, T-cell response) recognition of worm antigens which leads to an inflammatory reponse of a non-specific nature. These mechanisms are discussed below.

1.3.1.Antibody responses

The role of antibodies in resistance to ovine gastrointestinal nematodes still remains speculative despite the fact of existing evidence of systemic and local responses. Earlier studies on the development of immunity towards *H.contortus* described anti-larval antibodies in serum and mucus of hyper-infected lambs (Smith, 1977a,b). The response developed slowly after 4 weeks of exposure and waned without further stimulation. This waning has later been further established with studies on responses of abomasal lymph in *O.circumcincta* infected and challenged sheep, where temporary interruption of larval challenge caused a reduction in IgA levels (Smith, Jackson, Graham, Jackson and Williams, 1987).

Generally antibody responses towards sheep nematode infections are characterized by elevated levels of serum IgG and IgM (Smith, 1977a,b; Charley-Poulain, Luffau and Pery, 1984, Schallig, Van Leeuwen, Bernadina and Hendrikx, 1994) and mucus/lymph IgA (Smith 1977a,b; Adams, Merritt and Cripps, 1980; Smith, Jackson, Jackson, Dawson and Burrells, 1981; Smith, Jackson, Jackson and Williams, 1983c; Smith, Jackson, Jackson, Williams and Miller, 1984a; Smith, Jackson, Jackson, Williams, Willadsen and Fehilly, 1984b; Smith, Jackson, Jackson, Graham, Williams, Willadsen and Fehilly 1986; Smith *et al.*, 1987). Schallig *et al* (1994) have investigated serum IgA responses towards *H.contortus* infections and suggested that they provide a poor reflection of the local IgA response and therefore are probably unsatisfactory for monitoring local responses. It has been shown that serum IgG and IgM, and mucosal IgA are elevated and directed against all of the developmental stages of the parasite. For instance, Charley-Poulain *et al.* (1984) described elevated levels of the above isotypes against adult, L_3 and egg antigens of

H.contortus in immune sheep after a challenge infection. Recently Schallig *et al.* (1994) have described elevated levels of serum IgG1, IgG2 and to a lesser extent IgA towards adult and L_3 antigens during primary and secondary infections of Texel sheep with *H.contortus.*

Probably the most important if not most studied humoral response is the local IgA response. Resistance in hyperinfected sheep with *O.circumcincta* was associated with a secondary response in gastric lymph which consisted of a cellular reaction 2 to 4 days post-challenge followed a few days later by an IgA response (Smith *et al.*, 1983c, 1984a). A temporal association between local and/or systemic IgA and IgG anti-parasite antibodies and resistance (reduced worm burdens and FEC) to haemonchosis is well documented (Smith and Christie, 1978; Charley-Poulain *et al.*, 1984; Duncan, Smith and Dargie, 1978; Gill, Gray, Watson and Husband, 1993b; Gill, Husband, Watson and Gray, 1994; Schallig *et al.*, 1994). Inverse correlations between worm length and the size of an IgA response has pointed towards a possible role of IgA in parasite stunting (Smith, Jackson, Jackson and Williams, 1985).

Cell transfer experiments demonstrated that lymphocytes from the gastric lymph of sheep made immune to *O.circumcincta* (Smith *et al.*, 1986) and *H.contortus* (Smith *et al.*, 1984b) were able to transfer immunity to naive recipients. This transfer of immunity is suggested to be the result of an indirect mechanism triggered by donor cells (Miller, 1984) and was associated with a local IgA response and mastocytosis which was reflected by worm loss and stunting (Smith *et al.*, 1985). Earlier cell transfer work by Adams *et al.* (1980) using *T.colubriformis*-immune sheep also established the importance of IgA by demonstrating increased numbers of

cells containing this isotype in intestinal lymph and in the lamina propria. However, they failed to transfer passive immunity to naive recipients. This may be due to the fact that the recipients were not histocompatible whereas identical twins were used in the studies of Smith *et al.* (1984, 1986).

Recent studies using *in situ* methods of labelling plasma cells of various isotypes have further strengthened the conclusions that IgA is elevated in immune sheep. Increased numbers of IgA-containing cells have been observed in the proximal third of the small intestine of lambs responding to *N.battus* (Sinclair *et al.*, 1985). Similarly Gill, Husband and Watson (1992) showed increased numbers of immunoglobulin-containing cells (ICC) in the abomasum of sheep immune to *H.contortus* and later demonstrated that only 2% of the ICC comprised specific anti-worm antibody-containing cells which increased in numbers from day seven post-infection to peak by day 21 (Gill *et al.*, 1994). These authors suggested that the response generated covered a range of antigen specificites which may be due to polyclonal activation of precursor cells (Cross, Klesius and Haynes, 1986; Gill, Pomroy, Charleston and Moriarty, 1991) or alternatively due to bacterial and food antigens which may more readily be exposed to plasma cells in the lamina propria due to mucosal tissue damage.

Smith (1977a) suggested IgA was produced locally and this suggestion has been confirmed by other workers. Radioisotope studies in sheep showed that intestinal but not mammary IgA is produced locally and not derived from serum (Sheldrake, Husband, Watson and Cripps, 1984). Husband, Beh and Lascelles (1979) demonstrated that IgA precursor cells originate from Peyer's patches in response to

local antigen challenge and selectively home to the gut via the intestinal lymph. Furthermore, lymph studies in *O.circumcincta* infected sheep (Smith *et al.*, 1985) suggest an influx of antibody-containing cell precursors generated in gut associated lymphoid tissue (GALT) in response to infection.

Various mechanisms by which antibodies exert their protective effects have been proposed. Stunting, reduced fecundity and loss of worms is frequently associated with resistance and, as suggested earlier, may possibly be an effect of antibodies (Smith *et al.*, 1985). This effect may be brought about by the ability of antibodies to block or neutralize vital parasite enzymes (Gill *et al.*, 1994) or inhibit essential metabolic processes which are vital for parasite establishment and maintenance (Carlisle, McGregor and Appleton, 1990). *In vitro* experiments have demonstrated that worm specific IgG1 can suppress *T.colubriformis* feeding (Bottjer, Klesius and Bone, 1985).

It is worthy to note that studies comparing the antibody response of resistant and random-bred strains of sheep have further emphasized the importance of a local IgA and IgG1 response towards *H.contortus* infections (Gill *et al.*, 1993b, 1994). It is also becoming more evident that IgG1 is also being produced locally and of equal importance in the immune response directed at invading gastrointestinal nematodes (Gill *et al.*, 1992, 1993b, 1994).

1.3.2 T-cell responses

An essential component of the immune response to a number of gut nematodes (*T.colubriformis, T.spiralis, N.brasiliensis*) of laboratory animals is the involvement of T cells (Wagland and Dineen, 1965; Ogilvie and Parrott, 1977). As well as

participation in antibody production, changes in T cell responses (migration, activation and effector function) have been observed in *T.colubriformis* challenged sheep (McClure *et al.*, 1992).

Studies in laboratory rodents have shown that protective immune responses induced by helminths are T-cell dependent (Mitchell, 1980) and that the $CD4^+$ subset plays a major role in mediating protection against challenge infections with *T.spiralis*, *H.polygyrus* (Grencis, Riedlinger and Wakelin, 1985; Urban, Katona and Finkelman, 1991) and spontaneous expulsion of *N.brasiliensis* during a primary infection (Katona, Urban and Finkelman, 1988). It was recently established that $CD4^+$ cells play a regulatory role in mediating genetic resistance to *H.contortus* in sheep (Gill, Watson and Brandon, 1993a) and McClure *et al.* (1992) described increased numbers of this subset in the lamina propria of *T.colubriformis*-immune sheep.

Over the last decade athymic mice and inbred rodents have been used extensively in cell-transfer studies to define lymphocyte populations involved in the regulation of protective immune responses and their possible mode of action. However, in sheep, similar studies have not been possible due to the unavailability of inbred lines or athymic strains. The studies by Smith *et al.* (1984, 1986) using genetically identical lambs produced by embryo micro-manipulation showed that lymphocytes could transfer immunity to *H.contortus* and *O.circumcincta* which was manifested in the form of a mastocytosis and local IgA response. However, the lymphocyte subsets that were crucial for transfer of immunity were not determined probably due to the unavailability of specific markers. Furthermore, cell transfer

studies like these are extremely laborious and time consuming with only a limited number of sheep per experiment. The development of monoclonal antibodies to lymphocyte surface molecules in sheep (Mackay, Maddox, Gogolin-Ewens and Brandon, 1985; Maddox, Mackay and Brandon, 1985) has provided new opportunities to define lymphocyte populations in sheep either *in vitro* or *in vivo*.

The major sub-populations of T-lymphocytes identified in sheep are SBU $T4^+$ and SBU $T8^+$ cells, the homologues of the human $CD4^+$ and $CD8^+$ cells respectively (Maddox *et al.*, 1985). $CD4^+$ cells comprise approximately 20% of peripheral blood mononuclear cells (PBMC), 80% of thymocytes and 50% of lymphocytes in efferent lymph. $CD4^+$ thymocytes co-express CD8 antigen, whereas $CD4^+$ PBMC do not express CD8 antigen. $CD8^+$ cells comprise 12% of PBMC, 80% of thymocytes and 15% of lymphocytes in efferent lymph (Miyasaka, McClure, Hein, and Trnka, 1988). Cells expressing CD4 antigen recognize specific antigen in association with MHC class II molecules and function as helper cells, whereas cells expressing CD8 antigen recognize antigen in the context of major histocompatibility complex (MHC) class I molecules and function as suppressor/cytotoxic cells (Swain, 1983; Miyasaka *et al.*, 1988).

Few experiments have been conducted on aspects of T-cell involvement in immunity towards gastrointestinal nematodes of sheep. PBMC and T-helper cell lines have been used to identify blastogenic responses to protein fractions from *H.contortus* (Haig, Windon, Blackie, Brown and Smith, 1989) and ES products of *T.colubriformis* (Emery, Bendixsen and McClure, 1991). Recently helper T-cell lines ($CD4^+$) recognizing antigen in the context of MHC class II molecules have been

shown to play a pivotal role in acquired immunity towards *H.contortus* in genetically resistant sheep (Gill *et al.*, 1993a). Cellular and humoral responses were abolished with increased faecal egg counts and worm burdens in sheep treated with a monoclonal antibody towards sheep $CD4^+$ subsets.

With the recently available T subset markers, interest on the involvement of intraepithelial lymphocytes (IEL) in parasitic infections has been able to progress into identifying lymphocyte subsets involved and their dynamics during infection. The $CD8^+$ IEL are described as the first line of contact between the immune system and gut contents (Gorrell, Willis, Brandon and Lascelles, 1988). In *T.colubriformis* challenged naive sheep, local accumulation of T cells following infection with L_3 was noted and accompanied by a transient decrease of peripheral $CD8^+$ cells. In immune sheep similarly challenged greater T cell accumulation was observed suggesting a superimposed antigen-specific T cell infiltration upon non-specific infiltration induced by local inflammation (McClure *et al.*, 1992). Infections of sheep with *T.colubriformis* and *H.contortus* did not cause any changes in tissue numbers of $CD4^+$, $CD8^+$, $CD5^+$ and SBU-$T19^+$ lymphocytes (Gorrell *et al.*, 1988). However, in immune sheep challenged with 50,000 *T.colubriformis* L_3 significant decreases in numbers of $CD4^+$ and $CD8^+$ lamina propria lymphocytes were demonstrated and this was suggested to be in response to the parasite (Gorrell *et al.*, 1988).

Further work on the role of T cells in acquired immunity towards gastrointestinal nematodes is envisaged due to recent advances in the cultivation of populations of $CD4^+$ parasite-specific T cells from immune and vaccinated sheep (Haig *et al.*, 1989; Emery *et al.*, 1991) and the development of monoclonal antibodies

to T cell antigens. Similarly the role of cytokines in the effector mechanism may well be understood with the advent of ovine cytokine assays (Emery, Rothel and Wood, 1990).

1.3.3 Mast cell responses

The development of resistance to nematode establishment and the elimination of adult burdens are associated with marked cellular infiltration of the intestinal mucosa with mucosal mast cells (MMC), basophils (Miller and Jarrett, 1971; Askenase, 1980) and globule leucocytes (GL) (Murray, Miller and Jarrett, 1968; Gregory, 1979). Goblet cell proliferation and increased mucus production have also been reported to be associated with nematode expulsion (Miller and Nawa, 1979; Lake, Bloch, Sinclair and Walker, 1980). The biogenic amine, histamine, released from mast cells has been implicated in the expulsion process (Rothwell, 1989) and retardation of worm fecundity (Jones, Windon, Steel and Outteridge, 1990).

The association between gastrointestinal nematodosis in ruminants and the appearance in the mucosa of cells with large acidophilic granules or globules, designated globule leucocytes, has been recorded by many workers (reviewed by Gregory, 1979). During nematode infections in rats, mice, sheep and some other, but by no means all species, MMC especially those entering the epithelia may undergo partial granule fusion and other changes and are then called GLs (Murray *et al.*, 1968). The derivation of these cells from MMCs has been demonstrated by histochemical and ultrastructural studies (Jarrett, Miller and Murray, 1967; Miller, Murray and Jarrett, 1967). Prolonged antigenic challenge has been shown to cause

alteration in the granule structure of the MMC in the intestine of sheep which lead to the formation of GLs (Huntley, Newlands and Miller, 1984).

A study of *Strongyloides ratti* infection in mast cell deficient W/W^v mice showed that only epithelial mast cells as opposed to lamina propria mast cells were effective in a normal expulsion (Abe and Nawa, 1987a,b). Similarly in sheep, GL (which are MMC that enter the epithelia) were more abundant in sheep with higher levels of resistance when responsiveness varied with periparturient phenomena (O'Sullivan and Donald, 1973), age (Gregg *et al.*, 1979) or genetically-determined factors (Dineen and Windon, 1980).

The release of mediators such as histamine and serotonin (5-hydroxytryptamine, 5-HT) from MMC during intestinal nematode infection in laboratory animals (Murray, Miller, Sanford and Jarrett, 1971; Jones, Rothwell, Dineen and Griffiths, 1974) and sheep (Steel, Jones and Wagland, 1990; Jones *et al.*, 1990; Jones and Emery, 1991) is a well documented phenomenon. Experiments with sheep infected with *T.colubriformis* have implicated MMC as the source of histamine released into gut contents of immune sheep during the first week of a challenge dose (Steel *et al.*, 1990; Jones *et al.*, 1990).

Studies using lambs selectively bred for high or low responsiveness to vaccination with irradiated *T.colubriformis* showed that tissue histamine levels were lower at 3 days compared to 28 days post-challenge (Jones *et al.*, 1990). This was suggested to be due to active secretion of this amine immediately following challenge, hence, increasing intestinal content levels but exhausting tissue levels. This assumption was strengthened by the demonstration of increased secretion of

histamine between days 2 and 6 post challenge into the duodenal contents of sheep challenged with a single large dose of L_3 after vaccination with irradiated *T.colubriformis*.

Apart from histamine, appropriately stimulated MMC release a variety of mediators during immune expulsion of intestinal nematodes (Miller, 1984; Rothwell, 1989). Those released from storage granules include histamine and 5-HT but a range of other mediators including various chemotactic factors, proteases, glycosidases and proteoglycans (Durham and Kay, 1985) are also found pre-formed in these granules. A particular protease which has been of considerable interest is sheep mast cell protease (SMCP). This protease has chymotrypsin-like esterase activity and has been isolated and purified from sheep MMC (Huntley, Gibson, Knox and Miller, 1986).

Detection of increased amounts of SMCP in blood would be a practical method of monitoring MMC activity *in vivo* and an ELISA has been developed for such purposes, however peripheral blood inhibitors made this test unreliable. Nevertheless, high concentrations of SMCP have been detected in the blood and gastric lymph of sheep undergoing a protective immune response to *H.contortus* and *O.circumcincta* infections respectively (Huntley, Gibson, Brown, Smith, Jackson and Miller, 1987). Similarly high levels of SMCP have been detected in intestinal contents of sheep challenged with *T.colubriformis* (Jones, Huntley and Emery, 1992), hence, suggesting a possible role for mast cells in protection.

In an attempt to culture MMC the generation of mast cells containing SMCP from bone-marrow precursors has proven successful and of great potential to facilitate mast cell biochemical and functional studies (Haig, Blackie, Huntley,

MacKeller and Smith, 1988). However bone-marrow-derived cultured mast cells (BMCMC) and MMC are probably not identical, thus caution must be applied when using *in vitro* derived MMC as models for mast cell populations *in vivo* (Gordon, Burd and Galli, 1990).

Other mediators released as a consequence of MMC stimulation are the newly formed, membrane-derived mediators. Arachidonic acid released from phospholipids as a result of membrane perturbation is metabolized by either the lipoxygenase pathway to form the leucotrienes (LT) and related compounds or by the cyclooxygenase pathway to form the prostaglandins (PG) and associated metabolites (Durham and Kay, 1985). Intestinal mediators from sheep resistant to *T.colubriformis* have been shown to contain substances with properties similar to that of slow reacting substances of anaphylaxis (SRS-A) (Douch, Harrison, Buchanan and Greer, 1983). The leucotrienes LTB_4, LTC_4, LTD_4 and LTE_4 constitute SRS-A and this finding has stimulated work on the effects of LT release upon gastrointestinal nematodes.

Release of leukotrienes after challenge in rats immunized against *N.brasiliensis* and *T.spiralis* has been documented (Moqbel, King, MacDonald, Miller, Cromwell, Shaw and Kay, 1986; Moqbel, Wakelin, MacDonald, King, Grencis and Kay, 1987). Jones *et al.* (1990) have demonstrated that LTB_4 and LTC_4 are secreted into mucus and are associated with larval rejection or exclusion. Levels of LTC_4 in intestinal contents of sheep immunized with *T.colubriformis* increased to maximum levels at day 6 post challenge (Jones and Emery, 1991).

Additionally other mediators such as prostaglandins (PG) and thromboxane which are also mast cell products have recently been reported to be released in *T.colubriformis* infections (Jones and Emery, 1991). Their consequences are still unknown but these mediators have potent pharmacological effects.

Despite the clear evidence shown for a functional relationship between MMC and parasite expulsion/exclusion one must bear in mind that there are other sources of biologically active mediators for which MMC are best known. For example basophils, platelets and enterochromaffin cells. An absence of MMC during expulsion/exclusion cannot be taken as evidence against a role for the mediators in the response (Rothwell, 1989). Indeed MMC and SMCP have been shown not to be a pre-requisite for parasite expulsion/exclusion (Huntley *et al.*, 1987).

1.3.4 Eosinophil responses

The importance of the eosinophil as a cellular component of the inflammatory response against helminth parasites has been described (Rothwell and Dineen, 1972). However, the role of this cell against these parasites is still uncertain (Butterworth, 1984). In laboratory animals, eosinophilia has been associated with expulsion of nematodes (Rothwell and Dineen, 1972; Dawkins, Carroll and Grove, 1982) while in sheep, blood eosinophilia has been shown to correlate well with the ability of lambs to respond to *T.colubriformis* infection and challenge (Dawkins, Windon and Eagleson, 1989; Buddle, Jowett, Green, Douch and Risdon, 1992). Furthermore, higher numbers of eosinophils have been found in the intestinal mucosa of sheep with enhanced resistance to nematodes (Douch, Harrison, Elliot, Buchanan and Greer, 1986) and sheep genetically resistant to *H.contortus* (Gill, 1991). In laboratory

animals there is evidence for an effector cell function which results in parasitic cell damage (Kazura and Grove, 1978; Olsen and Schiller, 1978; Handlinger and Rothwell, 1981).

Initially eosinophils were suggested to be attracted to mast cell products released during hypersensitivity reactions such as eosinophil chemotactic factor of anaphylaxis (ECF-A) and function by down-regulating the mast cell response by secretion of enzymes that degrade mast cell mediators such as histamine and SRS-A (Gleich and Loegering, 1984; Kay, 1985). However, in view of the demonstration that eosinophil major basic protein (MBP) can activate MMC and basophils for histamine release (O'Donnell, Ackerman, Gleich and Thomas, 1983) and that eosinophils themselves can generate LT (Jorg, Henderson, Murphy and Klebanoff, 1982; Shaw, Walsh, Cromwell, Moqbel, Spry and Kay, 1985), platelet activating factor (PAF) (Lee, Lenihan, Malone, Roddy and Wasserman, 1984) and reactive oxygen metabolites (Gleich and Loegering, 1984; Weiss, Test, Eckmann, Roos and Regiani, 1986), down-regulation appears an oversimplification of their role in hypersensitivities.

Regulation and production of eosinophils in bone-marrow is still not fully understood (Spry, 1988), however, cytokines play an important role (Stevenson and Jones (1992). Blood and tissue eosinophilia is enhanced in transgenic mice expressing the interleukin-5 (IL-5) gene (Dent, Strath, Mellor and Sanderson, 1990). However, there is no evidence to date that IL-5 transgenic mice or mice lacking the capacity to make IL-5 show a significantly altered response to parasitic infections (Spry, Kay and Gleich, 1992). Recently, Stevenson and Jones (1992) have described

the possible role for cytokines in the activation of eosinophils by developing an enzyme micro-assay to detect eosinophil potentiating activity (EPA). The assay was able to detect EPA in abomasal lymph of sheep collected 48 hours post-challenge with *O.circumcincta* (Stevenson, Huntley, Smith and Jones, 1994). IL-5, IL-3 and granulocyte macrophage-colony stimulating factor (GM-CSF) are potent sources of EPA (Stevenson and Jones, 1992) and could possibly account for the EPA observed.

The eosinophil effector function is essentially the degranulation of granule products toxic to the parasite (McLaren, Mackenzie, Ramalho-Pinto, 1977). However, not all target helminths are damaged during these processes (Rothwell, 1989). Earlier reports studying eosinophil degranulation employed systems in which helminths were used as targets. These reports demonstrated that IgG (Butterworth, Remold, Houba, David, Franks, David and Sturrock, 1977), IgE (Capron, Spiegelberg, Bennich, Butterworth, Pierce, Ouaissi and Capron, 1984), and C3b (Ramalho-Pinto, McLaren and Smithers, 1978; Metcalfe, Gadek, Raphael, Frank, Kaplan and Kaliner, 1977) are capable of mediating the eosinophil effector function. This function can be modulated by using a number of soluble cytokines (Silberstein and David, 1987). Recently, a novel finding that IgA was able to induce eosinophil degranulation (Abu-Ghazaleh, Fujisawa, Mestecky, Kyle and Gleich, 1989) encourages speculation that IgA and eosinophils could play an important role at mucosal surfaces especially in the case of gastrointestinal helminthiasis. Indeed, as discussed earlier, local levels of IgA are significantly elevated in ovine gastrointestinal nematode infections. Furthermore, IL-5, initially described as possessing eosinophil differentiation activity (Sanderson, Warren and Strath, 1985), has now been shown to exert its effects on both eosinophils and on lymphocytes

involved in IgA synthesis (Abu-Ghazaleh *et al.*, 1989). Thus, IL-5 may orchestrate the interaction of eosinophils and IgA and together they may play an important role in mucosal immunity.

Eosinophils are known to be in close contact with tissue parasites such as *N.brasiliensis* (Taliaferro and Sarles, 1939), *Bunostomum phlebotomum* (Sprent, 1946), *Oesophagostomum columbianum* (Shelton and Griffiths, 1967) and *S.ratti* (Moqbel, 1980). These findings suggest that some form of chemotactic factor may be attracting these cells. Intramammary infusion of an extract from *H.contortus* L_3 in sheep increased eosinophil accumulation as shown by increased numbers in the inflammatory exudate (Topper, Colditz and Windon, 1992). ECF has been demonstrated in soluble extracts (Washburn and Klesius, 1984; Klesius, Haynes and Cross, 1985), excretory/secretory (ES) products and intestinal cells and lateral hypodermal cords of *O.ostertagi* (Klesius, Haynes, Cross and Ciordia, 1986; Klesius, Snider, Horton and Crowder, 1989). Other studies have described soluble chemotaxins from helminths such as *Anisakis* (Tanaka and Torisu, 1978), *Ascaris* (Tanaka, Baka and Torisu, 1979) and *Metastrongylus apri* (Sasaki and Katsumo, 1983). Converesly, it is equally possible for other cell products involved in immunity to secrete chemotaxins. For instance, mast cells secrete ECF-A (Lewis and Austen, 1981) and PAF (Braquet and Rola-Pleszczynski, 1987) which stimulate eosinophil localization, and T-cells may mediate stimulation of eosinophil chemotaxis (Wakelin and Donachie, 1983). Topper *et al* (1992) demonstrated that PAF caused tissue accumulation of eosinophils in the sheep and suggested that it is likely that PAF is generated during the immune reaction of sheep to gastrointestinal parasites.

Genetic variation in the ability to mount eosinophil responses towards parasite infections is well documented (Vadas, 1982; Wakelin and Donachie, 1983). As described earlier, studies conducted with resistant sheep have shown some degree of correlation between eosinophils and responsiveness. Gill (1991) demonstrated that lambs bred for resistance to *H.contortus* had significantly increased numbers of tissue eosinophils than random-bred lambs after a challenge infection. Similarly, lambs genetically resistant to *T.colubriformis* had elevated local and peripheral eosinophil responses (Dawkins *et al.*, 1989; Buddle *et al.*, 1992; Rothwell, Windon, Horsburgh and Anderson, 1993)

Apparently among the few studies conducted on eosinophil responses to ovine nematode infections there seems to be a consistent feature of an association between high peripheral counts and suppression of FEC (Kimambo, MacRae and Dewey, 1988; Dawkins *et al.*, 1989; Buddle *et al.*, 1992). It has been suggested that a rapid peripheral eosinophilia may be the consequence of larval stages becoming embedded in the mucosa and stimulating a response (Kimambo *et al.*, 1988). Using Romney lambs Buddle *et al.* (1992) observed a much higher eosinophil response compared to Merino lambs (Dawkins *et al.*, 1989). It is possible that apart from breed differences this may be due to age and infection regime used (Buddle *et al.*, 1992).

Eosinophilia usually results from a local immune response which induces peripheral blood and tissue eosinophilia (Dawkins *et al.*, 1989). Previous studies in lambs where lamina propria eosinophils were counted were contrasting. While there was a positive correlation between eosinophils and worm burden in one study (Dineen *et al.*, 1978) there was no apparent correlation in another (Gregg *et al.*, 1978)

while a later study of high and low responder lambs showed a weak negative correlation (Dineen and Windon, 1980). Thus the role of lamina propria eosinophils in resistance to *T.colubriformis* is somewhat equivocal in lambs.

In general the eosinophil in ovine gastrointestinal parasitism shows great similarity in features to its response in laboratory animal models, which suggests an important role in the process of worm expulsion. However, the finding that mice lacking IL-5, the important cytokine involved in eosinophil proliferation, do not have an altered response to parasitic infection is somewhat surprising. It is possible that the mucosal role of eosinophils in worm expulsion has been underestimated in the past (Spry *et al.*, 1992). A reappraisal of the essential roles of eosinophils in parasitic infections has become necessary in view of these findings.

1.3.5 Role of mucus in immunity

Studies on various parasite/host interactions have described a role for mucus in the elimination of parasites (Lee and Ogilvie, 1981; Miller, Huntley and Wallace, 1981; Newlands, Miller and Jackson, 1990). Apart from acting as a lubricant (Tse and Chadee, 1991), mucus provides an excellent medium for trapping parasites (Miller, 1987). Mucus is secreted by goblet cells which are located between epithelial cells (Tse and Chadee, 1991). The viscosity and elasticity of mucus is attributed to mucus glycoproteins or mucins (Miller, 1987). The capacity of mucus to protect epithelial surfaces is highly dependent upon its gel-forming (visco-elastic) properties and highly heterogeneous oligosaccharide compositions (Tse and Chadee, 1991). It is these properties which provide intimate contact between effector cell mediators and antibodies with the parasite.

Miller (1984) described rapid expulsion of *H.contortus* from sheep and suggested that this response probably involved mucus and a hypersensitivity reaction. Indeed mucus has been observed to be in close adherence with *T.spiralis* (Lee and Ogilvie, 1981) and *N.brasiliensis* (Miller *et al.*, 1981) larvae. In *N.battus* (Martin and Lee, 1980) and *H.contortus* (Miller, Jackson, Newlands and Appleyard, 1983) infections in sheep, mucus was observed to be enveloping these parasites. Furthermore, in rats infected with *N.brasiliensis*, goblet cell hyperplasia accompanied by increased secretion of mucus was observed between 10 and 15 days post-infection and resulted in nematode trapping (Miller, 1987; Koninkx, Mirck, Hendriks, Mouwen and Duk, 1988).

Mucus from immune sheep contains larval-paralysing properties (Douch *et al.*, 1983, 1984, 1986; Kimambo and MacRae, 1988; Jones, Emery, McClure and Wagland, 1994) described as larval migration inhibitory (LMI) activity. This activity has been shown to be of a non-specific nature in the sense that not only were homologous larvae paralysed but heterologous larvae were equally inhibited in their ability to migrate through agar (Douch *et al.*, 1983). This paralysing activity has been ascribed to the leucotrienes C_4, D_4 and E_4 which are suggested to be secreted from mast cells into the surrounding mucus.

Amines and prostaglandins (PG) have been proposed as mediators of expulsion of adult nematodes from rodents (Rothwell, Jones and Love, 1974; Richards, Bryant, Kelly, Windon and Dineen, 1977). Douch *et al.* (1983) suggested that the detection of LMI activity in gastrointestinal mucus of sheep resistant to *T.colubriformis* was not attributed to amines and PG and therefore these substances may not be directly

involved in the resistance of sheep to nematodes. However, in a study comparing mucus mediator concentrations in sheep of high and low responsiveness to *T.colubriformis*, higher levels of histamine and leucotrienes were detected in mucus of high responders and it was suggested that these substances were secreted into the mucus by actively degranulating cells in response to infection (Jones *et al.*, 1990). Similar findings were demonstrated with selected Romney sheep after a mixed infection (Douch, Harrison, Buchanan and Brunsdon, 1984).

The role of mucus in worm expulsion is still not fully understood. Apart from the suggestion that it conveys inflammatory products to the worm, mucus may physically entrap larvae (Miller, 1987) and may also provide a medium for the retention of local antibodies which increases the chance of contact between antibodies and the worms (Miller, 1987; Jones *et al.*, 1990). Indeed, recently it has been demonstrated that the primary mechanism of rapid expulsion of *T.spiralis* from rats is an antibody-mediated inhibition whereby mucus and antibodies form an antigen-specific protective barrier. Essentially, mucus trapping is an IgG-mediated process that is associated with systemic sensitization by tissue phases of the parasite (Miller, 1987). Most of the common gastrointestinal nematodes in sheep do not have a prolonged tissue phase and therefore the interaction between trapping and delivery of inflammatory mediators in the lumen seems more likely in sheep.

1.4 Cross-Protection among Ovine Gastrointestinal Nematodes

Generally, the processes regulating population size in one species (host-mediated, parasite-mediated, density-dependent or otherwise) may cross regulate the population of another. More particularly, protective immunity against one species

may cross-protect against the other (Adams, Anderson and Windon, 1989). As discussed earlier, the precise mechanism of rejection is incompletely understood but involves collaboration between an immunologically-specific encounter with worm antigen and a non-specific episode with direct effects on the parasite or the local gastrointestinal environment which leads to expulsion (Dineen *et al.*, 1977).

Very few studies have investigated aspects of cross-protection during concurrent infections, even though multiple exposure is the norm under field conditions. Most studies using concurrent infections have concentrated on host productivity and pathophysiology (Steel, Jones and Symons, 1982; Coop, Field, Graham, Angus and Jackson, 1986; Sykes, Poppi and Elliot, 1988) rather than on parasite population dynamics and the development of host immunity.

Steel *et al.* (1982), using 900 or 3000 *T.colubriformis* L_3 per week and 38,000 *O.circumcincta* L_3 per week, found a 'synergistic' effect of concurrent infections on liveweight gain and wool growth, that is, the effect of both species concurrently exceeded the combined effects of the single infections. In contrast, Coop *et al.* (1986) concluded that there was no additive or synergistic effect on performance of lambs administered with concurrent infections of 5000 *T.vitrinus* and 12,500 *O.circumcincta* per week. However Sykes *et al.* (1988), using 7000, 14,000 and 21,000 *T.colubriformis* and 7000, 14,000 and 28,000 *O.circumcincta* per week, found a synergistic effect of mixed infection on feed intake and bodyweight gain in lambs fed fresh herbage. Their data showed that at the highest *T.colubriformis* infection rate *O.circumcincta* burdens increased dramatically, possibly indicating a

reduction in host resistance to the latter species caused by the large populations of *T.colubriformis*.

Earlier studies on mixed infections in sheep indicated that expulsive mechanisms worked down-stream but not upstream in the gut (Stewart, 1953; 1955). This suggestion has been supported not only by the findings of Stewart but also those of other subsequent studies (Gordon, 1968; Douch, 1989; Emery, Wagland and McClure, 1993). Such a hypothesis is worthy of further investigation since much evidence points to a non-specific final effector mechanism comprised of mediator release from MMC and eosinophils and LMI activity in mucus (Douch *et al.*, 1983) which may well be transported down-stream with the flow of digesta. The findings of Dineen *et al.* (1977) suggested that an early immunologically-specific encounter with worm antigens generated a response which, in its effector phase, was non-specific in nature. Their speculations were based on the ability of *T.colubriformis*-immune sheep to regulate a combined challenge with *T.colubriformis*, *N.spathiger* and *T.vitrinus* L_3. In single infections *T.colubriformis*-immune sheep were not able to expel *N.spathiger* L_3 and were only 34% protected against *T.vitrinus* L_3.

Similarly *T.colubriformis*-immune sheep did not reject abomasal parasites (*H.contortus* and *O.circumcincta*) when challenged singly or concurrently with *T.colubriformis*. However *H.contortus*- or *O.circumcincta*-immune sheep were able to reject all 3 challenge species (Gordon, 1968). Another example of the non-specific down-stream phenomenon is that of the results of Emery *et al.* (1993). They showed that sheep immunized by truncated *T.colubriformis* larval infections were more than 90% protected by day 4 post-challenge against L_3 given in a single or combined

infection, whereas no significant protection was exhibited against a single-species infection with unrelated nematodes *N.spathiger* and *O.circumcincta*. However, sheep given a combined infection with *T.colubriformis*, *N.spathiger* and *O.circumcincta* were equally protected against both intestinal species but not *O.circumcincta*.

Similar findings have also been recorded when *T.colubriformis*-immune sheep were subjected to natural challenge at pasture (Douch, 1989). Compared with naive controls, these sheep had reduced worm burdens of *T.colubriformis* (87%), *N. spathiger* (91%), *T.vitrinus* (52%), *T.axei* (67%) and *O.circumcincta* (42%) but similar numbers of *H.contortus* and *Cooperia curticei* to non-immune controls. Despite the demonstration of non-specific rejection of unrelated intestinal species in these results, there is evidence that the effector mechanism may work upstream against *T.axei* and *O.circumcincta* populations in the abomasum. Also evident is the failure of the effector mechanism to expel *C.curticei* populations down-stream. Stewart (1950) noted that cross-protection did not extend as far down as to influence *Oe.columbianum* populations. These findings may suggest that the final non-specific effector mechanism does not necessarily work down-stream. Instead, it may work either way but restricted to areas in close proximity to where it occurs in response to a secondary infection. This may well be true since, as discussed earlier, immunity to gastrointestinal nematodes is local in nature and, therefore, may well be restricted to sites of parasitism.

While on the subject of the spread of the effector mechanism it is important to note that physiological changes induced by one species have been shown to spread to adjacent regions and disturb establishment of a second species (Mapes and Coop,

1970; 1973b; 1973c; Blanchard and Wescott, 1985). Prior exposure to *O.circumcincta* has been suggested to cause alterations in gut pH and therefore interfere with establishment and fecundity of subsequent infection with *H.contortus* (Blanchard and Wescott, 1985). Similarly alterations in gut pH and sodium ion concentrations induced by *H.contortus* influence the establishment of *N.battus* populations further down-stream (Mapes and Coop, 1970; 1973c; 1973d). Changes in abomasal pH have been shown to depress establishment rates of *H.contortus* L_3 (Honde and Bueno, 1982).

Apart from directly causing changes in abomasal pH, *O.circumcincta* has been suggested to elaborate some substance that is antagonistic to *H.contortus* and that serum gastrin concentration is a measure of this substance indirectly (Blanchard and Wescott, 1985). Anderson, Hansky and Titchen (1981) suggested that *O.circumcincta* may produce a substance that acts directly on gastrin-producing cells to stimulate output. The same, or more likely another, substance produced by *O.circumcincta* might also cause reduction in acid output by blocking the acid-stimulating effects of gastrin on parietal cells (Blanchard and Wescott, 1985).

It is interesting to speculate on the effect that the non-specific effector mechanism exerts upon parasites. It is possible that the single mechanism affects the parasite in various ways. Under conditions of natural challenge not only did *T.colubriformis*-immune sheep reject infections with *O.circumcincta* and *N.spathiger* but also retarded their development as increased numbers of L_4s were counted in immune sheep (Douch, 1989). According to Dobson, Barnes and Windon (1992), the ability of hosts to block incoming larvae appears similar for *H.contortus*,

T.colubriformis and *O.circumcincta* infections. However, some aspects of immunity, such as inhibited development at pre-adult stages, are distinct for the species. In contrast to the findings of Douch (1989), Dineen *et al.* (1977) suggested that immunizing doses of *T.colubriformis* were not related to developmental arrest. The work by Douch (1989) is complicated by the fact that grazing lambs were faced with a variety of nematodes which may interact with each other and the host, and it is possible that if the duration of infection was prolonged the lambs would have become immune to all stages of one species and some cross-protection against the other species may have occurred (Dobson *et al.*, 1992).

Several investigators have described a degree of antigen-sharing between related parasites (see Lloyd, 1981). Excretory/secretory products from *H.contortus* L_3 contain an immunochemically-related protein to *T.colubriformis* (O'Donnell, Dineen, Wagland, Letho, Dopheide, Grant and Ward, 1989). Milner, Beall and Orwat (1987) have shown that there is considerable homology between antigens of *T.colubriformis* and *O.circumcincta*. The results of Dineen *et al.* (1977) suggested that, although not as effective as *T.colubriformis*, *T.vitrinus* antigens may have been responsible for 34% cross-protection and therefore may be generically-related antigens.

Adams *et al.* (1989) failed to show cross-protection between *H.contortus* and *T.colubriformis* based on FEC. However they only used single infections. According to serum antibody analyses they suggested that larval, but not adult, antigens may be shared. They suggested that one reason why, although sharing antigens, no cross-protection occurred, may be due to the effectiveness of these larval antigens in eliciting a protective response. However, it is well known that the classical self cure

reaction is evoked by larval antigens and recently Emery *et al.* (1993) showed that sheep immunized by exposure to *T.colubriformis* L_3 and L_4 (truncated infections) exhibited rapid clearance of not only *T.colubriformis* but also other intestinal worms within 4 days post challenge.

It is important to be aware that cross-protection involves extremely complex mechanisms which are possibly a combination of immunological, physiological, pathological and, no doubt, parasitological changes occurring simultaneously. Additionally one must note that the duration and rate of infection will affect the outcome of an infection and may well be a reason why conflicting results are frequently observed in studies on immunity to gastrointestinal nematodes.

1.5 Effects of Dietary Protein and Age upon Developing Immunity

It has long been established that age and plane of nutrition affect the host resistance to gastrointestinal nematodes (Smith *et al.*, 1985; Kambara, McFarlane, Abell, McAnulty and Sykes, 1993). Empirical studies on the effect of feed quality have emphasized the importance of the protein contents of the feed upon development of resistance towards gastrointestinal nematodes of sheep (Dobson and Bawden, 1974; Wagland, Steel, Windon and Dineen, 1984; Wedrychowicz, Abbott and Holmes, 1984; Abbott, Parkins and Holmes, 1985, 1986, 1988; Abbott and Holmes, 1990; Bown, Poppi and Sykes, 1991; Kambara *et al.*, 1993; van Houtert, Barger, Steel, Windon and Emery, 1995; Shaw, Nolan, Lynch, Coverdales and Gill, 1995).

Dietary protein markedly affects the pathogenic impact of parasitism on the host which could be of practical significance in field situations where sub-optimal

nutrition commonly occurs. It is not surprising that dietary protein affects the development of immunity, as many immune components such as antibodies and cytokines are largely proteinacous in nature (Kambara *et al.*, 1993). However, there are conflicting observations in the literature. Establishment of *Oe.columbianum* (Dobson and Bawden, 1974) and *Fasciola hepatica* (Berry and Dargie, 1976) was not affected by the level of dietary protein. Similarly, resistance to *H.contortus* and *T.colubriformis* in older lambs was not dependent upon dietary protein (Abbott and Holmes, 1990; Kambara *et al.*, 1993). Differences in acquisition of resistance between Scottish Blackface and Finn Dorset lambs on varying protein intakes were demonstrated by Abbott *et al.* (1985) in that different levels of protein did not affect worm establishment in Scottish Blackface lambs but did among Finn Dorset lambs. Despite these interesting findings there is little information about the effect of dietary protein on the development of immunity towards ovine gastrointestinal parasites.

Most of the work conducted to date has described significant increases in worm regulation and resilience among sheep offered diets with increased protein levels. Lambs fed a low protein diet below standard requirements (8.8% CP) and infected with *H.contortus* were more anorexic, lost weight and developed anaemia and oedema more frequently than lambs on a higher level of protein intake (16.9% CP), although, parasite establishment did not differ between the two groups (Abbott *et al.*, 1986). Recently, Kambara *et al.* (1993) infected young (8 weeks) and old (33 weeks) lambs with *T.colubriformis* and varied their dietary crude protein contents between 11% and 20%. After challenge the young lambs fed on a higher level of protein and previously infected had significantly lower FEC and worm burdens compared to the other groups. However, this significant dietary effect was not manifested by the older

lambs (although trends for enhanced responsiveness were evident). Similarly, 7 month old lambs fed either a high (16.9% CP) or low (8.8% CP) protein diet and infected and challenged with *H.contortus* did not differ in terms of worm regulation (Abbott and Holmes, 1990). Although the priming with 2 vaccinations of 10,000 irradiated *H.contortus* L_3 significantly enhanced their resistance by 90% or more, the additional dietary protein did not seem to boost the host response. The reason why this extra protein has no significant effects in older lambs remains unknown and speculative.

Abbott and Holmes (1990) ascribed the level and duration of priming with L_3 as being responsible for the differences in effects of dietary protein upon development of immunity between young and old lambs. Indeed this suggestion fits well with the findings of Kambara *et al.* (1993). They used identical infection regimes in both young and old lambs and although they claimed the extra protein had no significant effect upon the older lambs ability to regulate worm burdens, as mentioned earlier, trends for fewer worms in the high protein group were evident.

In laboratory animal studies expulsion of *N.brasiliensis* from protein-deficient rats was impaired and suggested to be related to an impairment in the MMC and GC response (Bolin, Davis, Cummins, Duncombe and Kelly, 1977; Cummins, Duncombe, Bolin, Davis and Yong, 1987). The importance of MMC in the response was suggested since syngeneic lymphocyte transfer did not correct impaired expulsion but transfer of bone marrow cells did (Duncombe, Bolin, Davis and Kelly, 1981). This would suggest that the protein deficiency is impairing precursor cell activity.

Studies on the effect of weaning upon responsiveness to *H.contortus* and *T.colubriformis* showed that weaned lambs not only gained less weight but also suffered a greater decline in packed cell volume (PCV), had mean FEC twice that of unweaned lambs and serum antibody responses towards both worm antigens were delayed and of a lesser magnitude (Watson and Gill, 1991). In contrast Shaw *et al.* (1995) showed that weaning did not affect the development of immunity to *H.contortus*. However, it is important to distinguish between the age at weaning in these studies. In the latter study, lambs were weaned at 4 months of age whereas those in the former study were weaned at 2 months of age. It is possible that weaning at a later age of 4 months would enable these lambs to respond more vigourously to infection. Differences in the relative proportion of total protein that by-pass the rumen may play a key role in accounting for the different rate and degree of response seen in these studies, since weaned lambs without a fully functional rumen will be forced to graze which will reduce metabolizable protein due to lower protein intake and ruminal losses whereas suckling lambs will benefit from the direct passage of milk proteins via the oesophageal groove to the abomasum. The studies by Bown *et al.* (1991) and Coop, Huntley and Smith (1995) complement this statement. Both groups showed that post-ruminal infusion of casein enhanced *T.colubriformis* and *O.circumcincta* regulation respectively. The adverse effects of *T.colubriformis* on nitrogen deposition were reduced through increasing duodenal protein supply (Bown *et al.*, 1991). Recently, van Houtert *et al.* (1995) have shown that supplementary feeding with a by-pass protein (fish meal) substantially reduced production losses attributable to infection with *T.colubriformis* and was associated with enhanced expulsion of the parasite burden.

The level of antibody produced and its correlation with resistance in infected animals appears to be affected by the level of dietary protein and age (Kambara *et al.*, 1993). Investigations into the influence of dietary protein on local immune responses in mice and rats have shown that protein deprivation can markedly reduce the gut localization of antigen-specific IgA plasmacytes (Pierce, Koster and Barry, 1981; McDermott, Mark, Befus, Baliga, Suskind and Bienenstock, 1982). Furthermore, reduced secretory IgA levels and low mucosal IgA responses to viral antigens have been previously observed in dietary protein deficiencies (Barry and Pierce, 1979).

In *T.colubriformis* infections, lambs receiving a lower plane of nutrition had lower serum titres of complement-fixing antibodies against worm antigen (Wagland *et al.*, 1984). Similarly lambs infected and challenged with *H.contortus* and maintained on different dietary protein levels differed in faecal antibody levels at 3 weeks post challenge with lambs on the high protein diet having higher titres (Wedrychowicz *et al.*, 1984). However worm burdens did not differ between dietary groups (Abbott, Parkins and Holmes, 1984). In contrast, Wagland *et al.* (1984) observed significant correlation between serum antibody titres and manifestations of resistance which include fecundity and worm burdens. Accordingly improved nutrition accounted for a 41% increase in protection. One possible reason why Wagland *et al.* (1984) obtained a considerable increase in protection is the fact that they used young lambs. The lambs used by Abbott *et al.* (1984) were older lambs and therefore, as discussed earlier, the extra protein ingested may have not had any significant effect.

The onset of immune responsiveness among lambs has been suggested to be a crucial factor in the development of immunity to gastrointestinal helminths (Abbott and Holmes, 1990). Generally immunity towards parasitic infections increases with age over the first 12 months of life in lambs (Watson and Gill, 1991). This is true for sheep infected with *H.contortus* (Manton, Peacock, Poynter, Silverman and Terry, 1962; Benitez-Usher, Armour, Duncan, Urquhart and Gettinby, 1977), *T.colubriformis* (Gibson and Parfitt, 1972; Dineen *et al.*, 1978) and *O.circumcincta* (Smith *et al.*, 1985). The factors involved in the onset are still unkown and have been linked to puberty, bodyweight and condition rather than chronological age (Abbott and Holmes, 1990). This seems a reasonable assumption since the plane of nutrition will affect bodyweight and condition and chronic deficiencies will no doubt delay puberty. This is frequently demonstrated in studies where protein deficiency in young lambs is associated with depressed resistance and immune responses.

Lambs infected and challenged with *T.colubriformis* varied in their lymphocyte responses depending on age irrespective of the level of dietary protein (Kambara *et al.*, 1993). Lymphocytes only responded among older sheep to T-cell mitogens and L_3 antigens. In contrast Togerson and Lloyd (1993) showed that adult immune sheep and naive lambs both responded (increased stimulation indices of lymphocyte blastogenesis tests) to similar fractions of *H.contortus* L_3 soluble antigens, although the magnitude of the response among the lambs was of a lesser degree. Studies with *O.circumcincta* using 4½ and 10 month old lambs showed that although the magnitude of the IgA and lymphoblast response was significantly depressed among young lambs, the timing of the local response was similar between age groups (Smith *et al.*, 1985). Resistance to a challenge infection with *H.contortus* among older lambs

was associated with elevated levels of local IgA and serum IgG (Smith and Christie, 1978; Duncan *et al.*, 1978).

However, this response was not observed among young lambs aged 3 months (Duncan *et al.*, 1978). It was suggested that the unresponsiveness of young lambs may in part be due to deficient antibody production due to a slow maturing secretory IgA system. Duncan *et al.* (1978) also suggested that young lambs are possibly unresponsive to complex nematode antigens or the abomasum has a slow capacity to produce IgA. In contrast Gregg *et al.* (1978) showed that there was no evidence that unresponsiveness of 3 month-old lambs towards *T.colubriformis* being due to a deficiency in antibody production. In fact, peripheral lymphocytes, monocytes, granulocytes and tissue mast cells, eosinophils and neutrophils did not differ in numbers between the two age groups in their study. The only cell that was associated with responsiveness was GL.

It is possible that hypo-gammaglobulinaemia may be part of a general immaturity of mechanisms of immunity towards worms in young lambs. However these observations suggest that young lambs are able to recognize worm antigens which contrasts the suggestion by Douch and Morum (1993) that unresponsiveness of lambs is, in part, associated with an inability to recognize worm antigen.

Results from work conducted with grazing lambs (Douch and Morum, 1993) of various ages showed that lambs aged 16 and 28 months had significantly lower FEC and elevated numbers of abomasal and small intestinal MMC and GL compared to that of 4 month old lambs and controls. However, tissue eosinophil numbers and worm burdens did not differ between groups. It was suggested that worm regulation

and suppression of fecundity are not affected by the same basic immunological mechanism. This is in contrast to the suggestion by Wagland *et al.* (1984) that both manifestations of resistance (worm burdens and fecundity) are affected by the same mechanism. Results from their experiment and from that of Dineen and Windon (1980) suggested that due to the high negative correlation between worm counts post-challenge in vaccinated sheep and counts of eggs *in utero* that both are affected by the same mechanism. These differences should be viewed with caution due to the nature of the infection in different experiments and the fact that Douch and Morum (1993) used grazing lambs which were faced with a multiple infection.

1.6 Conclusion

It is clear from this review of literature that a considerable amount of work has been undertaken in order to understand the mechanisms of immunity generated against common ovine gastrointestinal nematodes but very little has been directed to *N.battus* infections in lambs. The subsequent chapters will describe the approach taken to understand the host/parasite interaction and the role of nutritional status in the development of acquired immunity of young lambs to infection with *N.battus*.

CHAPTER 2
General materials & methods

2.1 Parasitological Techniques

2.1.1 Parasite strain and larval culture

The *N.battus* strain used for infection had been maintained at the Moredun Research Institute, Edinburgh in worm-free donor lambs after isolation from field material obtained from Firth Mains Farm (situated 12 km south-east of Edinburgh).

Faeces collected from donor lambs with patent infections was stored in 20 litre buckets and mixed regularly to avoid build up of anaerobic conditions. Half-full buckets were topped up with warm water (approximately 25°C), allowed to soak for 2 hours and thoroughly emulsified into a thick faecal suspension. The suspension was washed through a series of sieves with copious amounts of tap water. The sieves used were of 2 mm, 1 mm, 500 μm, 212 μm and 90 μm mesh and the sievings containing nematode eggs were retained by passing them over a 53 μm mesh sieve. The sievings were washed into a 2 litre measuring jug to allow for sedimentation.

Following overnight sedimentation the water supernatant was poured off and the faecal pellet (usually about 700 ml per 2 litre jug) suspended in saturated NaCl. This mixture was then poured through a chemical centrifuge via a large funnel and the eggs along with fine faecal debris which poured out from the outlet tubing was collected on a 53 μm mesh sieve. The eggs were then rinsed under tap water to remove remaining salt and collected in 2 litre measuring jugs.

The egg suspension was further cleaned of faecal debris by pouring it into a crystalizing dish and gently swirling the suspension. This allowed most eggs to sediment much quicker than the fine debris. While the debris was still in suspension dark brown masses of eggs were visible at the bottom of the dish and at this point the debris was gently poured off. This procedure was repeated several times until a relatively 'clean' suspension of *Nematodirus* eggs was obtained.

The eggs were cultured in 1 or 2 litre glass bottles that were connected to an aquarium pump via plastic tubing at approximately 20°C in a warm room. The tubing extended into the bottles and was connected to an aeration stone which enabled continuous pulsation of air through the suspension. Fungal growth was controlled weekly by addition of formalin to the egg suspension to make a final 2% solution which was left to bubble for 6 hours. The eggs took approximately 8 to 10 weeks to develop into L_3.

2.1.2 Artificial hatching and infection

The egg suspension was sedimented, the supernatant removed and the concentration of the remaining solid material was adjusted to approximately 1ml solid material (eggs) per 10 ml water. The eggs were then resuspended and 250 µl were dispensed in a thin line on a glass baseplate. Another glass plate (crushing plate) was placed over the eggs and pressed down firmly. Given the correct egg density, volume and pressure then almost all of the eggs should have split releasing the L_3 stages. The plates were separated ensuring that no shearing movement occured.

Both plates were washed by holding them over a large filter funnel and squirting tap water with a wash bottle so as to collect all the crushed eggs and emerged larvae into a 5 litre bottle. The crushing and washing process was repeated until sufficient larvae were obtained (allow for a 66% recovery rate i.e. crush 150 eggs for each 100 L_3 required). The L_3 collected in the bottle were cleaned of debris and egg shells by Baermannization using High Wet Strength Paper (Cleanaroll Ltd.). The bottle contents was poured through the paper which was held on a filter holder (plastic tube 50 mm in diameter and 100 mm long) by a rubber band. The paper was able to temporarily restrain the 'sluggish' L_3 (due to artificial hatching) while releasing all the water. The filter holder was then immersed in warm tapwater (22°C) which allowed the L_3 to migrate out and sediment to the bottom of the container. Baermannization was usually left overnight providing sufficient time for almost all the L_3 to migrate. The larvae were stored in tap water at 4°C prior to use. Eggs were only crushed a few days before infection so that the larvae had maximum chance of establishment.

For infecting lambs, the larval suspension was made up to the nearest volume in a volumetric flask and shaken to evenly disperse the L_3. A 1 ml aliquot was removed and made up to 10 ml. From this dilution 5 samples of 100 µl were streaked on to a glass slide and the L_3 counted under a stereo microscope. The total numbers of larvae were calculated and the concentration of the suspension adjusted for the required larval dose. In most circumstances a 10 ml dose was conveniently delivered *per os* via a glass McCartney vial.

2.1.3 Faecal egg count (flotation technique)

Rectal faecal samples were taken and placed into a polythene bag. The samples were weighed and for every gram of faeces 10 ml of tap water was added to the sample. The sample was emulsified using a stomacher. A 10 ml sub-sample was removed and passed through a tea strainer (1mm mesh) and washed through with an additional 5 ml of tap water. The retentate was squeezed and discarded. The filtrate obtained was poured into 20 ml cellulose acetate tubes and centrifuged at 100 *g* for 2 minutes.

The supernatant was removed using a vacuum line and the remaining faecal pellet resuspended with 12 ml saturated sodium chloride. The tubes were centrifuged again at the same speed and time as described earlier. Using artery forceps, the tube was clamped just below the meniscus of the supernatant and the contents of the upper chamber was poured into a cuvette. The upper chamber was rinsed with saturated NaCl and again poured into the cuvette which was filled totally and capped. The cuvette containing all the eggs in one gramme of faeces was placed on a counting slide mounted on the stage of a light microscope and examined under ×40 magnification.

The eyepiece contained a calibrated graticule (Kellner) which was used to count eggs in samples with high egg concentrations. For samples with few eggs all the eggs in the cuvette were counted. Whenever samples contained high numbers of eggs the graticule was used. Eggs that fell in the large square along two traverses of the cuvette were multiplied by 3 and those in the small square by 9 to obtain the total number of eggs per gram faeces.

2.1.4 Pasture larval counts

Pastures were sampled using the method of Taylor (1939). Herbage samples were plucked from sites 10 metres apart along a series of 'V' shaped traverses up and down the pasture. The sample (usually between 500 and 1000 grammes) was weighed and a small sub-sample removed for dry matter estimation (usually between 50-100 grammes) before being soaked overnight in 40 litres of warm tap water (25°C) which contained a small amount of detergent.

Following soaking, the herbage was removed in small handfulls which were squeezed to remove as much water as possi ble. The sample was then sedimented in a cold room (4°C) for 4 hours and its volume reduced to 2 litres. Following further sedimentation (500 ml) the sample was reduced to 100 ml which was poured into 6 cellulose acetate tubes. The larvae were recovered from each tube by centrifugation using saturated NaCl in the manner described for the faecal egg count method. The recovered larvae were pooled, washed twice centrifugally using water and the sample volume reduced to 0.3 ml.

The numbers of infective larvae were determined at ×100 magnification in saturated potassium iodide in a cuvette, the counting being done in the manner described in the faecal egg count method. The counts were converted to numbers of larvae per kg. of wet herbage (e.g. if the count was 200 L_3 recovered from 750 gm herbage then the count per kg. will be $200 \times (1000 \div 750) = 267$). The number of L_3 per kg. wet herbage were converted to numbers per kg. dry herbage according to this example:

$\Rightarrow$ if there were 267 L_3/kg. wet herbage and the dry weight was 25% then L_3/kg. dry matter = 267 × (100÷25) = 1068.

2.1.5 Removal and preparation of the small intestine for worm counts

Sheep were positioned on a cradle with all four limbs extended through the metal bars while an assistant restrained the sheep. A single captive bolt was delivered through the forehead and once stunned sheep were exsanguinated and the spinal cord severed. Each sheep was restrained until any remaining nervous stimuli diminished and then turned onto its back to open the abdomen along the ventral midline.

The entire gastrointestinal tract was removed and the proximal end of the small intestine located. The first two thirds of the entire small intestine were removed from the mesentry and the contents emptied into a 10 litre bucket. The intestine was split lengthwise and, along with the contents, soaked in 0.85% saline solution for 4-6 hours at 37°C. Following this saline digest lengths of intestine were pulled between finger and thumb to remove the superficial mucosa and the samples were made up to 5 litres and a 500 ml sub-sample (10% of total contents) removed and fixed with 20 ml formalin.

2.1.6 Worm counts

Samples were stained with a few mls of helminthological iodine (Appendix A). The sub-samples taken for worm counts varied and are described in the appropriate chapters. After a few minutes of staining the sample was passed and rinsed over a 38 μm sieve to remove excess iodine and fine debris and collected into a container. The stained sample was poured into 100 mm gridded contact plates (Sterilin) and searched for worms at ×100 magnification using a stereo microscope. Worms were

staged and sexed according to the descriptions given by Mapes and Coop (1972) during the count and preserved in 2% formalin in plastic bijou bottles.

2.1.7 Worm measurements

Where available 50 L_4 and/or L_5 male and female larvae per lamb were randomly chosen for length measurements. The stained worms were placed on a gridded counting slide (Sedgewick Rafter Cell) under a stereo microscope (WILD Heerbrugg) with attached television camera (Agar TV-LM) which was linked to an Archimedes 401/1 microcomputer with loaded Digit software (Watford Electronics). Worms were visible on a monitor screen and were digitized in order to trace the length. The tracings were measured automatically by the software which produced measurements in millimetres.

2.2 Tissue Histology & Eosinophil Counts

2.2.1 Blood and tissue samples

Blood samples were collected always at the same time of day for each time point by jugular venepunture into plain and ethylenediaminetetracetate (EDTA) vacutainer tubes for serum antibody ELISA and peripheral eosinophil counts respectively.

Sections of the small intestine (4-8 cm long), taken at approximately 2 metres from the proximal end, were fixed in 4% paraformaldehyde in PBS for 6 hours at room temperature. The tissues were then transferred into 70% alcohol at 4°C and dehydrated in ascending grades of alcohol (96%, 98%) after which they were cleared in toluene. The tissues were then embedded in hot wax and serially sectioned to 4 μm with a microtome and placed in a hot water bath prior to mounting. The sections

were mounted on glass slides and dewaxed with xylene. These were then passed through descending grades of alcohol into water and were then ready for staining.

2.2.2 Enumeration of tissue cells

For mast cell counts the sections were stained in 0.5% toluidine blue in 0.7N HCl (pH 0.3) at room temperature overnight (Enerback, 1966). For globule leucocyte and eosinophil counts the sections were stained for 30 seconds with haematoxylin (Appendix A) and washed in water followed by staining with carbol chromotrope solution (Appendix A) overnight (Lendrum, 1944).

All stained sections were washed briefly in water and dehydrated in ascending grades of alcohol. The sections were cleared in xylene and permanently mounted with DPX. Stained cells were enumerated using a ×10 eye piece containing a calibrated graticule and a ×40 objective lens. The villus-crypt unit method for counting cells (Miller and Jarrett, 1971) was used whereby cells were counted in 10 villi from each of 3 cut sections per lamb (a total of 30 villi).

2.2.3 Sheep bone marrow cell (SBMC) preparation

Sheep bone marrow cells (SBMCs) were isolated from sternal marrow obtained at post-mortem. Marrow preparations were filtered through double thickness sterile lens tissue (Whatman, Maidstone, U.K.) to remove fat and debris. Filtered cells were suspended in Hanks balanced salt solution (HBSS) containing 2% foetal calf serum (FCS; Gibco, Paisley, U.K.), 10 IU ml^{-1} penicillin, 50 μg ml^{-1} streptomycin (Sigma Chemical Co. Ltd., Poole, U.K.) and divided between six sterile plastic 20 ml universal bottles and centrifuged at 300 *g* for 10 minutes. After decanting the supernatant, pellets were resuspended as a single suspension, in a total volume of 24

ml in HBSS. Four ml of this suspension was then carefully layered onto 8 ml of Lymphoprep (d = 1.077 g ml^{-1}; Nycomed (UK) Ltd., Sheldon, U.K.) in sterile plastic universal bottles. The majority of the erythrocytes were separated from white cells by centrifugation of these gradients at 700 *g* for 30 minutes. The interface cells were removed using a pasteur pipette and washed twice in HBSS by centrifugation at 170 *g* for 10 minutes. These cells were then adjusted to 5×10^5 ml^{-1} viable nucleated cells ml^{-1} (assessed by 0.1% Nigrosin dye exclusion) in HBSS for preparation of cytospots.

2.2.4 Preparation of cytospots

Cytospots from bone marrow cell suspensions were prepared in a cytocentrifuge (Shandon Scientific, Runcorn, U.K.) using 0.1 ml of cell suspension containing 5×10^5 cells ml^{-1} and centrifuging for 5 minutes at 600 rpm. Cytospots were then air-dried for 5 minutes before staining. Percentage eosinophil numbers in bone marrow cytospots were determined from differential counts made of 600 cells on slides mounted with coverslips fitted with Coverbond mountant after they had been fixed and stained with Diff-Quik (Brownes, Reading, U.K.) according to the manufacturer's instructions.

2.2.5 Peripheral eosinophil counts

A 50 µl sample of blood was added to 450 µl Carpentiers eosinophil counting solution (2 ml 2% aqueous Eosin Y; 3 ml 40% formaldehyde saturated with $CaCO_3$ (BDH, Poole, U.K.); 95 ml distilled water prepared fresh each week) and the eosinophils counted in an improved Neubauer haemocytometer (Phillip Harris Scientific, Glasgow, U.K.). Eosinophils were expressed as $\times 10^4$ ml^{-1}.

2.3 Immunological & Biochemical Techniques

2.3.1 Soluble worm antigen preparation

Nematodirus (adults and L_4s) were collected from the proximal third of intestines of infected donor lambs (3-4 months old) and frozen at -20°C. The worms were placed on filter paper which was put on to a Buchner funnel attached to a vacuum pump and washed with PBS. The washed worms were ground up in a glass homogenizer as a 10% w/v suspension in homogenizing buffer (1mM EDTA, 1mM phenylmethylsulphonyl fluoride (PMSF) in PBS pH 7.4) and centrifuged (10,000 *g* for 20 min at 2°C). The supernatant collected after centrifugation was aliquoted and stored at -20°C prior to use. Protein concentration was estimated by the bicinchoninic protein assay reagent according to the manufacturer's instructions (Pierce).

2.3.2 Enzyme-linked immunosorbent assay for serum antiworm specific IgG

Optimal conditions were determined by checkerboard titration. For assaying IgG against L_4 antigens the optimal conditions which gave the largest difference between the optical density (O.D.) of reference sera and worm-free sera were an antigen concentration of 2μg protein/ml., sera dilution of 1/500 and conjugate dilution of 1/250. For assaying against adult worms the optimal conditions were 2 μg protein/ml of antigen, sera dilution of 1/1000 and conjugate 1/100 (Appendix B).

Flat bottomed microtitre plates (Dynatech Laboratories Ltd, Billinghurst, Sussex, U.K.) were coated overnight at 4°C with crude antigen diluted with 0.05M carbonate-bicarbonate buffer pH 9.6 (Appendix A), and subsequently washed twice with Tris-buffered saline comprising 10mM Tris and 0.5M sodium chloride pH 7.4 with 0.05% Tween 20 and 0.01% Thimerosal (TNTT). Non-specific binding was

blocked by applying 200µl/well 10% horse serum in PBS for 1 hour at 37°C and the plates were subsequently washed twice again with TNTT. Test serum (100µl/well) diluted with PBS was added to the wells in duplicate. The plates were incubated for an hour at 37°C and then washed three times with TNTT. Horseradish peroxidase-conjugated donkey anti-sheep IgG (Scottish Antibody Production Unit, Carluke, Lanarkshire) diluted with PBS was added to each well (50µl/well). The plates were incubated for 1 hour at 37°C then washed three times with TNTT followed by the addition of 3,3',5,5'-tetramethylbenzidine (150µl/well) (Appendix A) as the enzyme substrate. The enzyme reaction was stopped after 5 minutes by the addition of 2.5M sulphuric acid (30µl/well) and O.D. measured at 450 nm.

2.3.3 Enzyme-linked immunosorbent assay for sheep mast cell protease (SMCP)

A double antibody sandwich ELISA was used for the measurement of the concentrations of sheep mast cell protease (SMCP) in tissue homogenates as described by Huntley *et al.* (1987) using the German origin M129B flat bottomed plates (Dynatech Laboratories Ltd, Billinghurst, Sussex, U.K.).

Excised tissue samples were washed in PBS, weighed and stored at -20°C. The tissues were minced finely with scissors and suspended in 10 ml of emulsifying buffer (Appendix A). Homogenization of tissues was performed using a laboratory Mixer Emulsifier (Silverson Machines) at maximum speed for 60 seconds.

Flat-bottomed microtitre plates were incubated overnight (4°C) with rabbit anti-SMCP antibody in carbonate/bicarbonate buffer pH 9.6 (Appendix A) (1 µg/ml, 50 µl per well). The plates were washed 6 times with wash buffer (0.5% Tween 20 in PBS). Samples and standards were diluted in wash buffer. Samples were used at

1:10, 1:100 and 1:1000 fold dilutions and duplicated. The standards (known concentration of SMCP) were loaded at 0.5, 1, 2, 4, 6, 8, 10 and 12 ng/ml in duplicate. Controls consisted of samples incubated in uncoated wells.

After removing excess anti-SMCP antibody samples and standards were incubated in the wells (50 µl/well) for 1 hour at room temperature. This was followed by 6 washes and the addition of 50 µl horseradish peroxidase-conjugated rabbit anti-SMCP antibody (1:2000) for 1 hour at room temperature. After a further 6 washes the bound antibody was reacted with freshly prepared orthophenylenediamine (OPD) in H_2O_2 phosphate-citrate buffer pH 5.0 (150 µl/well) (Appendix A) for 5 minutes. The reaction was stopped by addition of 2.5 M sulphuric acid (25 µl/well) and O.D. determined on a Titertek multiscan ELISA reader (Titertek Flow Laboratories) at 492 nm. The concentration of SMCP in tissues was calculated from standard curves produced by plotting O.D. of standards against their concentration. The concentrations were expressed as µg SMCP/g wet weight of tissue.

2.4 Statistical analysis

All statistical analyses were performed using Minitab statistical software. Arithmetic means are given in the text, tables and figures with ± 1 standard error unless stated otherwise. Where data were skewed or had unequal variances they were transformed by $\log_{10}$ or $\log_{10}(x+1)$. The transformation for particular data sets and the tests used are explained in each chapter.

CHAPTER 3
Kinetics of worm loss

3.1 Introduction

This experiment was conducted by Dr.W.D.Smith prior to the commencement of the thesis but has been included as it contains important data for establishing the design of subsequent experiments in the thesis. The main purpose of this pilot experiment was to provide data on the kinetics of worm loss following a single infection of *N.battus*, essential information before 'trickle infection-challenge' experiments can commence. It is clear from the literature that worm counts of young lambs killed a few weeks after a single infection with *N.battus* are highly variable (Gibson and Everett, 1963; Mapes and Coop, 1972). This is probably due to expulsion of established worms from certain individuals rather than to an unequal establishment of infective larvae (L_3). The experiment's serial kill design will address this point.

3.2 Materials and Methods

The standard methods for recovery of worms have been described in detail in chapter 2.This section describes the animals used, their management and monitoring and also the experimental design.

3.2.1 Animals and feed

Twenty Blackface × Suffolk lambs aged approximately 3 months at the start of the experiment were used. The lambs had been maintained under worm-free

conditions from birth. Throughout the experiment the lambs were offered a restricted diet comprised of hay *ad libitum* and concentrates (Ruminant A; a pelleted complete basal diet {Dalgety Agriculture Ltd, Almondsbury, Bristol}) at 250-300 gms $head^{-1}$ day^{-1}. Water was offered *ad libitum.*

3.2.2 Experimental design

The lambs were balanced for sex and weight and allocated to 4 groups of 5 lambs. All groups were given a single infection with 30,000 *N.battus* L_3 and killed at various intervals post-infection (PI). Groups 1, 2, 3, and 4 were killed on day 7, 14, 21 and 28 PI respectively.

3.2.3 Sampling

At necropsy the proximal two thirds of the small intestine was taken to recover remaining worms as described in chapter 2. Sub-samples constituting 2.5% of the total wash and digest were examined for the presence of worms. Worms were counted and sexed.

3.2.4 Statistical analysis

Worm burdens were log_{10}-transformed prior to analysis. One-way analysis of variance (ANOVA) was used to test for differences between groups. Where significanct differences were noted differences between the means of groups was tested using a 2 sample t-test assuming equal variances.

3.3 Results

One lamb from group 2 died during the experiment and therefore, was excluded from the analyses. The worm burdens of each individual lamb are

Table 3.1. Individual worm burdens.

Group	Day PI	Lamb no	Males	Females	Non-sexed	Total
		R1	5920	8000	0	13,920
		R2	4240	6320	320	10,880
1	7	R3	5440	7920	0	13,360
		R4	3760	6240	0	10,000
		R5	5920	8320	0	14,240
		Mean	**5056**	**7360**	**64**	**12480 ± 856[a]**
		B1	5840	7600	0	13,440
		B2	4000	6880	0	10,880
2	14	B3	5600	6640	0	12,240
		B4	4320	9120	0	13,440
		Mean	**4940**	**7560**	**0**	**12500 ± 610[a]**
		Y1	8000	9120	0	17,120
		Y2	7520	8240	0	15,760
3	21	Y3	4880	4400	0	9280
		Y4	1680	1600	0	3280
		Y5	4800	5440	0	10,240
		Mean	**5376**	**5760**	**0**	**11136 ± 2481[a]**
		G1	320	240	0	560
		G2	3680	6240	0	9920
4	28	G3	400	1520	0	1920
		G4	1280	1200	0	2480
		G5	1520	2320	0	3840
		Mean	**1440**	**2304**	**0**	**3744 ± 1631[b]**

Mean total burdens with different superscripts were significantly different ($p<0.05$).

presented in Table 3.1. There was a significant decline in worm burdens over time ($p<0.01$). Worm burdens did not differ up to 21 days of infection. However, by day 21 PI individual variation in worm burdens became evident and by 28 days PI was more extensive with significantly lower worm burdens.

3.4 Discussion

The aim of this experiment was to determine the period after infection when worm regulation mechanisms began effective removal of *N.battus* populations. This preliminary investigation has fulfilled the objective in establishing an ideal period after a single infection with *N.battus* when lambs in the succeeding chapters could be killed.

The results suggest that within 2 weeks PI worm burdens do not decline and variation was minimal. Although worm burdens were not significantly reduced by 21 days PI there was evidence of individual variation. This suggests that some individuals may be responding at a more rapid rate, which could possibly be due to an innate ability to respond earlier. Ideally, lambs should be killed before or at 2 weeks PI or post-challenge in future experiments since individual variation may complicate the interpretation of treatment effects. This is relevant to designs where lambs given a trickle infection followed by a challenge infection are to be compared to challenge controls.

Previous experiments have described various effects of host immunity upon *Nematodirus* species (Dineen *et al.*, 1965; Taylor and Thomas, 1986). These include elimination of L_3 and adults, retardation in development of L_4 stages and discrimination against female worms resulting in reduced fecundity. The results of

this experiment do not encourage the prolongation of infection to more than 2 weeks since there is a possibility that variations in worm burdens may complicate interpretation of results. Additionally, since the majority of worms will be at the L_5 stage of larval development by day 10 PI (Mapes and Coop, 1972), and the fact that immunological mechanisms cause retardation of L_4, it is suggested that the lambs used in future experiments be terminated at around 10 days PI or PC. This will not only reduce individual variation to a minimal degree but also enable demonstration of retardation in the L_4 population. In conclusion it is suggested that future trickle infection and challenge experiments should terminate experimental animals at or very close to day 10 PI/PC.

CHAPTER 4

Influence of dietary protein upon development of immunity I

4.1 Introduction

Age and dietary protein are known to affect the host resistance to gastrointestinal nematodes. Apparently young lambs tend to be unresponsive towards gastrointestinal nematodes. This unresponsiveness has been correlated to impaired antibody production (Duncan *et al.*, 1978), depressed lymphocyte responsiveness (Rifkin and Dobson, 1979) and an inability to mount a MMC (Douch and Morum, 1993) and a GL response (Gregg *et al.*, 1978; Douch and Morum, 1993).

Grazing lambs appear, on the basis of FEC, to develop a strong immunity to *N.battus* within 6 to 8 weeks of exposure to infective larvae whereas immunity to other gastrointestinal nematode species may take 3 to 4 months. Although the mechanisms of resistance to gastrointestinal nematodes are as yet poorly understood, it has been suggested that they may in some way be different for this species. However, studies at Moredun Research Institute which have examined the development of resistance to *N.battus* in both grazing and housed lambs have not provided any conclusive evidence that the mechanisms associated with resistance to this species are different to those operating in other gastrointestinal nematodes (F.Jackson, unpublished data). The most notable difference between these two studies was that unweaned grazing lambs acquired immunity more rapidly than those that were weaned. Since the rate of development of resistance in sheep to gastrointestinal parasites may be influenced by protein status of the host (Abbott *et al.*, 1988; Bown *et al.*, 1991; Kambara *et al.*, 1993; van Houtert *et al.*, 1995),

differences in the relative proportion of total protein that by-pass the rumen may play a key role in accounting for the different rate and degree of response seen in these studies. Evidence supporting this view comes from an experiment in which lambs given a continuous infusion of casein direct into the abomasum were better able to regulate a challenge infection of *O.circumcincta* (Coop *et al.*, 1995). Bown *et al.* (1991) has also shown that post-ruminal infusion of protein reduced the worm burdens of lambs infected with *T.colubriformis* and also reduced the adverse effects of the parasite on nitrogen deposition through increasing duodenal protein supply.

The aim of the experiment described in this chapter was to acquire baseline data on the immune response of lambs towards *N.battus* and determine whether protein supplementation influenced the rate of development of immunity. If this proves to be the case then it clearly has important implications for studies on age related responsiveness.

4.2 Materials and Methods

Most standard methods have been described in detail in Chapter 2. This section will describe the animals used, their management and monitoring and also the experimental design used.

4.2.1 Animals and feed

Twenty-six Blackface × Suffolk lambs aged approximately 4 months at the start of the experiment were used. The lambs had been maintained under worm-free conditions from birth. Prior to the experiment the lambs were offered a restricted diet comprised of hay and concentrates and water was offered *ad libitum.* Approximately 4 weeks prior to experimental infection all lambs were fed their respective basal or

supplemented diets. The diets were either Ruminant A (132 g kg^{-1} of crude protein (CP) in the dry matter (DM)), a pelleted complete basal diet (Dalgety Agriculture Ltd, Almondsbury, Bristol), which supplied 66g of metabolizable protein (MP) kg^{-1} DM feed or this diet supplemented with 10% fish meal (183 g CP kg^{-1} DM) which supplied 95g MP kg^{-1} DM feed. Calculated metabolizable energy values for the basal and supplemented diets were 8.54 and 8.96 MJ kg^{-1} DM respectively. Feed offered varied individually during the experiment between 2 to 3 kg $head^{-1}$ depending upon appetite.

4.2.2 Experimental design

Two groups of 7 and two groups of 6 lambs were either continuously infected and challenged (groups 1 and 2) or challenged only (groups 3 and 4) with *N.battus* L_3 (Table 4.1). Groups 1 and 3 were offered supplemented Ruminant A and groups 2 and 4 basal Ruminant A. Due to constraints of accommodation, only continuously infected groups (1 and 2) were housed individually in metabolic cages. Group 3 and 4 lambs were group housed. The continuous infection regime was designed to mimic the field situation, using a rapidly increasing larval intake followed by decreasing intake over a 7 week period. All four groups were treated with levamisole (15 mg kg^{-1} bodyweight) and fenbendazole (5 mg kg^{-1} bodyweight) at week 8 to remove the primary infection and challenged one week later with a single dose of 30,000 *N.battus* L_3. All lambs were killed on day 9 post-challenge for worm population analysis and to obtain intestinal tissues for immunohistochemistry. The infection regime and timing of events are summarized in Table 4.1.

Three lambs maintained on Ruminant A were used to assess the efficacy of the anthelmintics used. These lambs were infected with 6000L_3 day^{-1} for 5 days and 3 weeks later drenched with the same anthelmintics and dose rate as mentioned above. These lambs were killed 6 days later and their small intestines removed for worm burden estimation (if any).

Table 4.1. *Experimental design.*

Group	Diet*	Infection+	Drench Week PI*	Challenge Week PI*	Kill Day PC*
1 (n=7)	RA+	10, 20, 40, 20, 10, 10,10	8	9	9
2 (n=7)	RA	as above	8	9	9
3 (n=6)	RA+	not infected	8	9	9
4 (n=6)	RA	not infected	8	9	9

\+ *lambs were infected with a 7 week trickle infection. The larvae were given daily, 7 days a week. The numbers given were those in the table multiplied by* 10^2.

* *RA - Ruminant A only, RA+ - Ruminant A + fish meal*
PI - post-infection, PC - post-challenge

4.2.3 Feed intake and liveweight gain

All lambs were weighed weekly prior to feeding. Daily food refusals were pooled on a weekly basis (individually for groups 1 and 2) and the dry matter intake (DMI) calculated weekly by drying a sample at 40°C for 36 hours.

4.2.4 Sampling

Blood samples, collected at the same time of day for each time point were taken weekly for serum and twice weekly for peripheral eosinophil counts by jugular

venepuncture. Faeces were collected weekly from the rectum from day 17 PI to obtain FEC.

When the lambs were killed a blood sample was taken in order to obtain serum, the proximal two thirds of the small intestine was taken for recovery of remaining worms and intestinal sections (4-8 cm long) were taken at approximately 2 metres from the proximal end of the small intestine for immunohistochemistry. The sternum was removed from selected individuals (based on FEC) for eosinophil counts.

4.2.5 Analysis based on responsiveness

Since there was no significant dietary effects upon responsiveness to a challenge infection, the lambs were re-grouped into high or low responders (HR or LR) for further analysis using an arbitrary cut-off point of 1000 worms as described by Taylor and Thomas (1986). Lambs in groups 1 and 2 which had total worm burdens below 1000 were grouped as high-responders and the remainder as low-responders. The challenge controls (groups 3 and 4) were pooled and referred to as controls.

4.2.6 Statistical analysis

Worm data, tissue mast cell and eosinophil counts, SMCP concentrations and antibody levels were $\log_{10}$-transformed prior to statistical analysis. FEC, GL and peripheral eosinophil counts were $\log_{10}(x+1)$-transformed prior to analysis. Data was subjected to either a one way, 2 way or 3 way analysis of variance (ANOVA) depending on the number of treatments and their interactions by fitting a general linear model (GLM). Where significant differences were noted, differences between

the mean of groups was tested using a 2 sample t-test. Correlation analysis was used to determine associations between cell counts and worm burdens.

4.3 Results

4.3.1 Growth and feed intake

All four groups did not differ in their growth rate throughout the experimental period (Figure 4.1) although weight gain was reduced in the infected lambs on the basal diet. Previous infection resulted in a significant reduction in DMI ($p<0.001$) (Figure 4.2). DMI did not differ significantly between previously infected groups (group 1 and 2) and between challenge controls (groups 3 and 4).

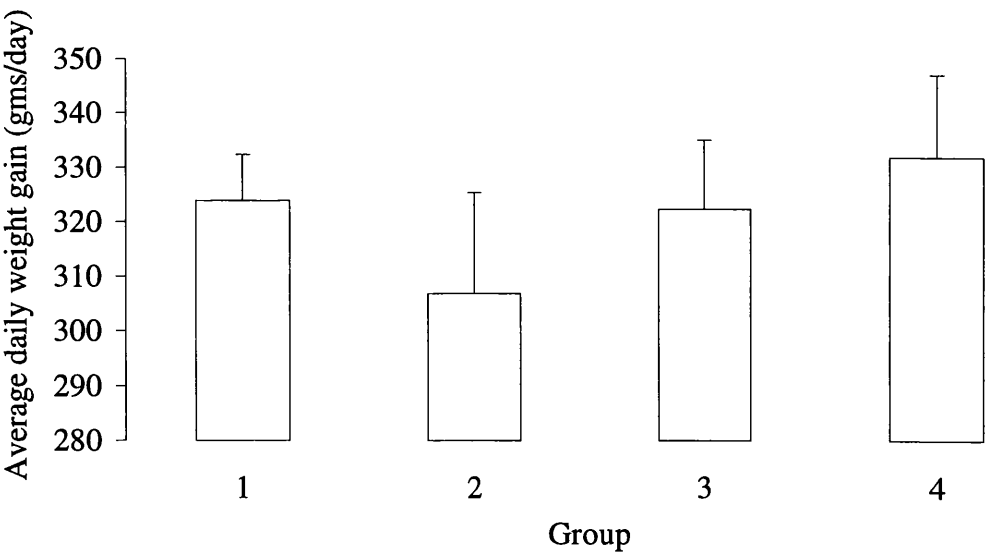

Figure 4.1. *Average daily weight gain ± S.E.M over the experimental period.*

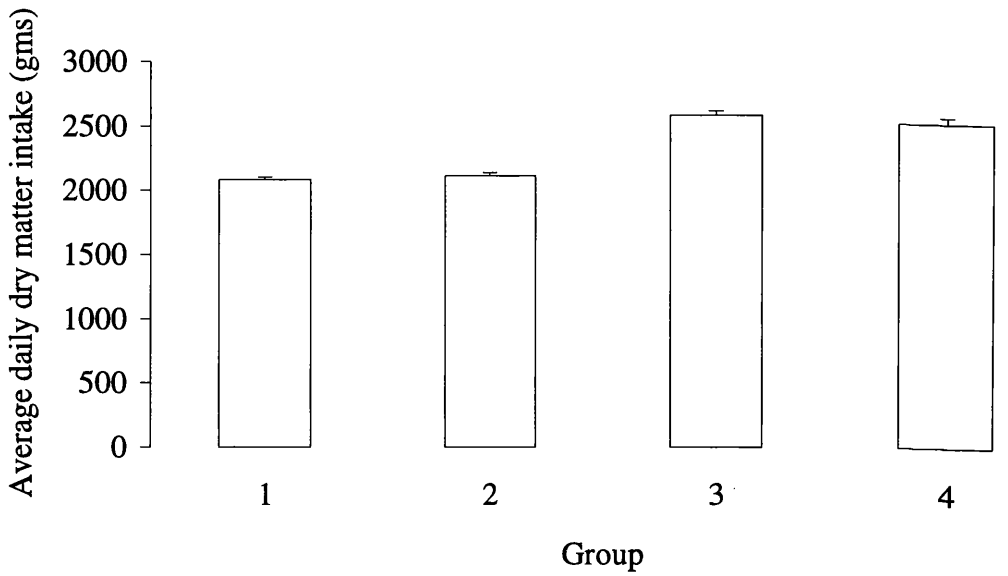

Figure 4.2. *Average daily DMI ± S.E.M over the experimental period.*

4.3.2 Faecal egg counts

The normal prepatent period of *N.battus* (14 to 18 days) was extended and FEC at day 31 post-infection were only 39 and 29 epg for groups 1 and 2 respectively (Figure 4.3). Egg output then increased steadily until treatment with anthelmintic at day 56. FEC were always low and never exceeded 100 epg and although group 1 showed a trend for higher FEC from day 31 onwards, these differences were not significant.

4.3.3 Worm burdens

The three lambs used to assess the efficacy of the anthelmintic treatment were completely free of worms. Therefore it was considered that any worms counted were derived from the challenge dose. The worms recovered 9 days after challenge were at the L_4 and L_5 stage of larval development. Mean percent establishments of the challenge infection were 6.6%, 9.2%, 28.5% and 16.8% for groups 1, 2, 3, and 4

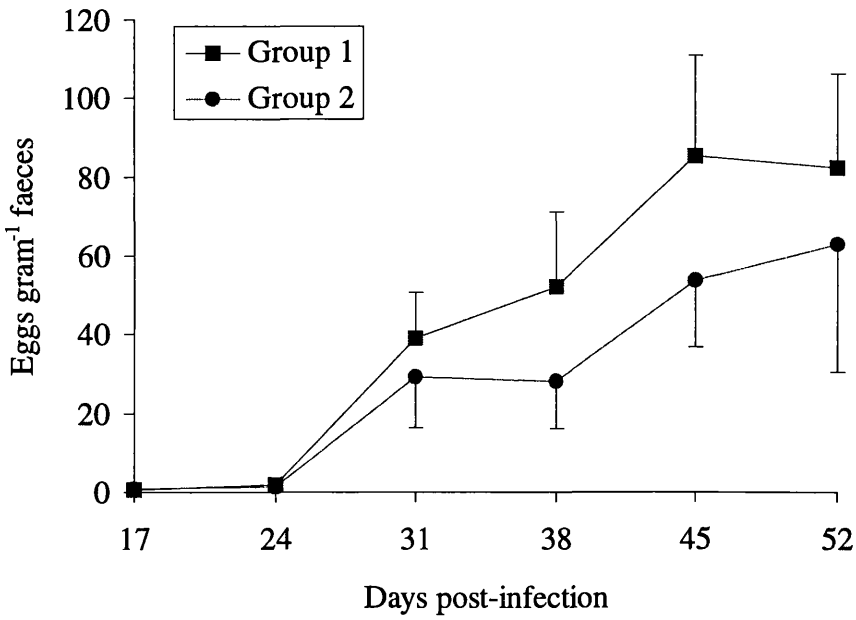

Figure 4.3. *Mean faecal egg counts ± S.E.M of the infected groups (1 & 2) over the continuous infection period.*

respectively. Analysis of individual worm burdens (Table 4.2) showed that more variation occurred within the previously infected groups (1 and 2) than in the challenge controls (groups 3 and 4). Previous infection caused a significant reduction in worm burdens ($p<0.001$) but supplementation failed to enhance the response significantly. There was no indication of an interaction between both treatments. Although supplementation had no significant effect upon the development of resistance, there was a trend for fewer worms in the supplemented previously infected lambs (group 1) which had 28% fewer worms than lambs in group 2.

4.3.4 Developmental stage

The worm populations in all groups were predominantly L_4. Considerably fewer worms in the previously infected groups were at the L_5 stage in comparison to the challenge controls. This is indicated by a significant effect of previous infection

Table 4.2. *Individual worm burdens on day 9 post-challenge.*

Group	Lamb no.	Male worms		Female worms		Total
		L_4	L_5	L_4	L_5	
	R1	1720	20	2060	420	4220
	R2	220	0	420	0	640
1	R3	820	0	1920	80	2820
	R4	1460	0	2340	120	3920
	R5	100	0	100	0	200
	R7	40	0	120	0	160
	Mean	**726**	**4**	**1160**	**103**	**1993±769[a]**
	B1	300	20	840	40	1200
	B2	100	0	120	0	220
	B3	3120	0	4360	140	7620
2	B4	100	0	220	0	320
	B5	2220	0	3160	60	5440
	B6	40	0	60	0	100
	B7	1740	40	1460	1200	4440
	Mean	**1088**	**10**	**1460**	**206**	**2764±1150[a]**
	R8	3450	750	2450	2200	8850
	R9	3500	800	1950	2850	9100
3	R10	3300	350	2750	1000	7400
	R11	3000	400	2600	2300	8300
	R12	3650	400	3700	2050	9800
	R13	3150	400	2100	2200	7850
	Mean	**3340**	**515**	**2590**	**2100**	**8545±357[c]**
	B8	2300	350	1900	1550	6100
	B9	1800	100	2250	650	4800
	B10	1400	100	1150	1200	3850
4	B11	1550	200	2550	100	4400
	B12	2600	300	1750	1900	6550
	B13	1500	500	850	1700	4550
	Mean	**1860**	**260**	**1740**	**1185**	**5045±429[b]**

Mean total burdens with different superscripts were significantly different ($p<0.05$).

in increasing both male ($p<0.001$) and female ($p<0.01$) L_4:L_5 ratios (Table 4.3). Among the male worm population, supplementation also significantly increased L_4:L_5 ratios and a significant interaction between previous exposure and supplementation was evident ($p<0.001$). Comparisons between the mean length of larvae (Table 4.3) showed that the previously infected groups (1 and 2) had significantly smaller worms than the challenge controls (3 and 4) ($p<0.001$). Supplementation did not affect worm length and there was no interaction between supplementation and previous infection. Previous infection significantly reduced sex ratios ($p<0.05$) (Table 4.3).

Table 4.3. *Mean worm lengths, L_4:L_5 and sex ratios.*

	Group 1	Group 2	Group 3	Group 4
Males				
L_4 length	3.12 ± 0.22^{a}	3.42 ± 0.33^{a}	4.78 ± 0.06^{b}	4.75 ± 0.05^{b}
L_4:L_5	86.0*	29.3 ± 14.3^{a}	7.15 ± 0.89^{b}	9.66 ± 2.21^{ab}
Females				
L_4 length	3.38 ± 0.17^{a}	3.77 ± 0.24^{a}	5.17 ± 0.06^{b}	5.14 ± 0.05^{b}
L_5 length	6.23 ± 0.1^{a}	6.21 ± 0.13^{a}	7.39 ± 0.13^{b}	7.15 ± 0.12^{b}
L_4:L_5	16.13 ± 5.76^{a}	26.5 ± 10.7^{a}	1.4 ± 0.31^{bc}	5.4 ± 4.04^{ac}
Sex ratio	0.59 ± 0.1^{a}	0.62 ± 0.06^{a}	0.83 ± 0.05^{b}	0.71 ± 0.03^{ab}

** Only one lamb from this group had male L_5*
Worm lengths are in millimetres. Sex ratios were derived by dividing the number of male worms by females. Figures with different superscripts were significantly different ($p<0.05$).

4.3.5 Peripheral eosinophil counts

The counts from one particular lamb (R5) were considerably elevated and atypical compared to the others in its group, therefore, this lamb was omitted from

the analyses. The peripheral blood eosinophil counts (Figure 4.4) were significantly elevated in previously infected groups ($p<0.001$). However, supplementation and time did not affect the counts. There were significant interactions between supplementation and previous infection ($p<0.001$) and between previous infection and time ($p<0.001$).

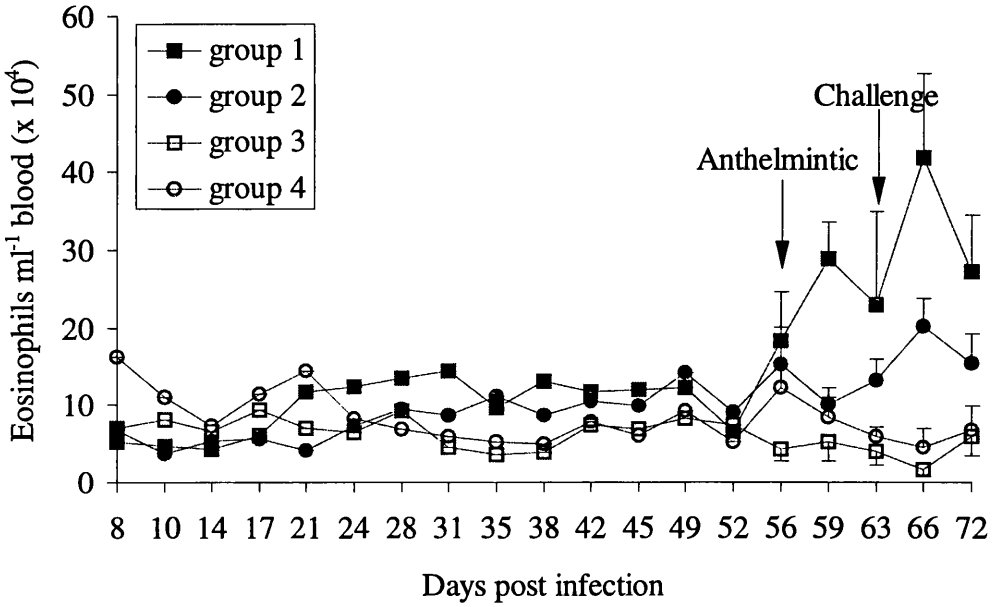

Figure 4.4. *Peripheral blood eosinophil counts throughout the experiment.*

4.3.6 Serological responses

The serum IgG antibody levels towards both L_4 and adult worm antigens are shown in Figures 4.5 and 4.6 respectively. Antibody levels were significantly elevated by supplementation ($p<0.05$), previous infection ($p<0.001$) and duration of time ($p<0.001$) with significant interactions between supplementation and previous infection ($p<0.001$) and between previous infection and time ($p<0.001$). Antibody levels of previously infected lambs (groups 1 and 2) towards both adult and L_4 antigens were generally higher than those of the naive challenge controls (groups 3

and 4) from day 38 PI. Challenge did not evoke an exaggerated anamnestic response towards adult antigens, antibody levels being maintained at pre-challenge levels.

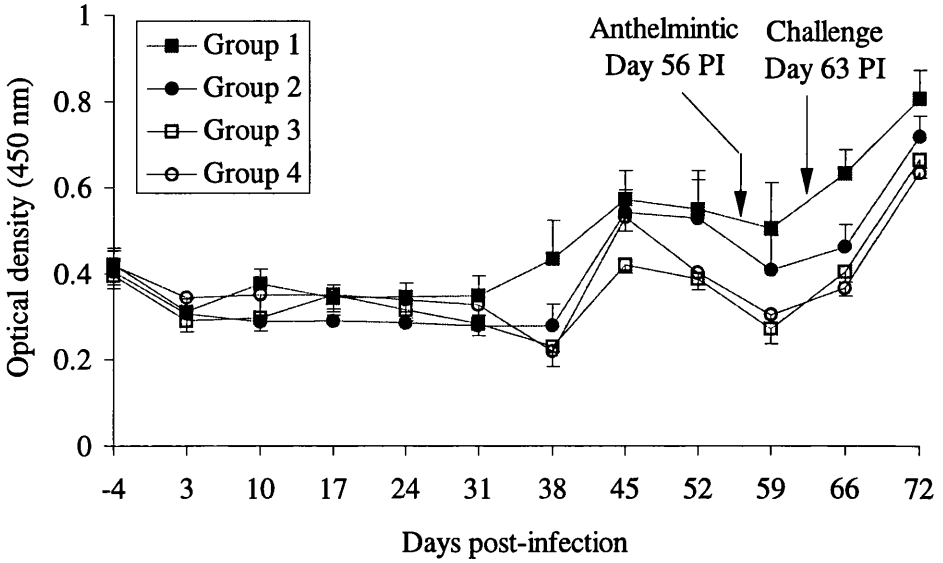

Figure 4.5. *Anti-L_4 IgG antibody responses ± S.E.M over the experimental period.*

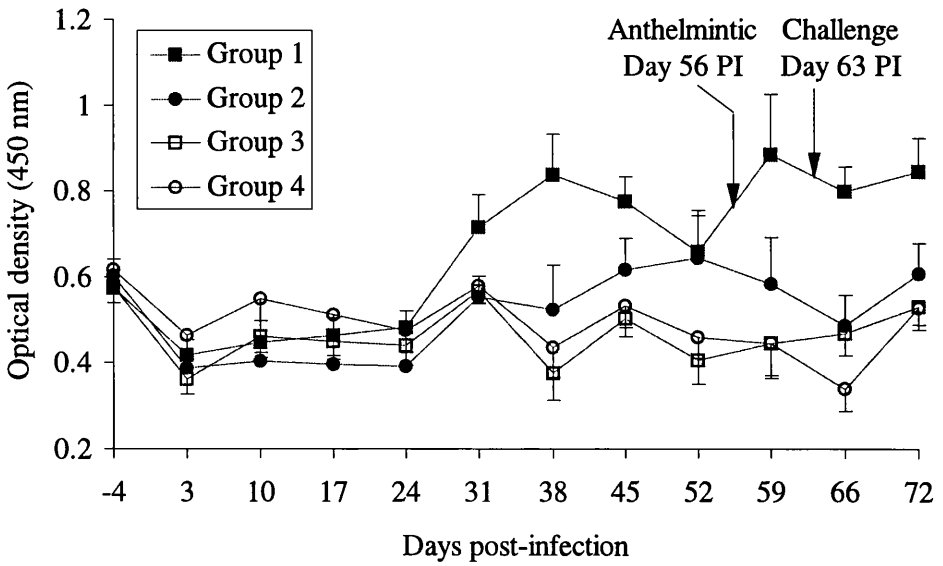

Figure 4.6. *Anti-adult worm IgG antibody responses ± S.E.M over the experimental period.*

4.3.7 Cell counts and SMCP concentrations.

The tissue cell counts and SMCP concentrations are shown in Table 4.4. Previous infection significantly enhanced eosinophil ($p<0.001$), GL ($p<0.01$) and MMC numbers ($p<0.001$). Supplementation enhanced GL numbers ($p<0.01$) while an interaction between supplementation and previous infection ($p<0.01$) was evident. Correlation analysis by group showed only a significant negative correlation between GL and worm burdens in group 1 ($r = -0.828$, $p<0.05$) and between SMCP and worm burdens in group 3 ($r= -0.805$, $p<0.05$).

Table 4.4. *Mucosal cell numbers and SMCP concentrations.*

Group	Lamb no.	Mast cells	SMCP	G.leucocytes	Eosinophils
	R1	50.4	10.6	0	88.1
	R2	57.1	12.8	0.8	62.3
1	R3	58.8	41.4	0.3	72.9
	R4	31.7	10.2	0.1	104.5
	R5	59.5	13.0	0.6	103.3
	R7	43.5	29.4	0.6	53.6
	Mean	**50.18 ± 4.45^{a}**	**19.57 ± 5.26^{a}**	**0.40 ± 0.13^{a}**	**80.76 ± 8.7^{a}**
	B1	36.27	11.85	0	43.63
	B2	30.90	3.71	0.1	49.43
	B3	58.63	5.92	0	44.17
2	B4	38.53	8.87	0	27.17
	B5	29.40	12.72	0	89.87
	B6	54.97	24.92	0	52.17
	B7	28.40	21.38	0	58.43
	Mean	**39.59 ± 4.67^{ac}**	**12.77 ± 2.96^{a}**	**0.003 ± 0.014^{b}**	**52.12 ± 7.3^{b}**
	R8	30.93	8.01	0	27.80
	R9	32.53	10.95	0	43.23
3	R10	30.23	11.90	0	43.73
	R11	17.90	12.27	0	22.60
	R12	26.97	6.30	0	49.0
	R13	12.57	13.28	0.03	21.30
	Mean	**25.19 ± 3.31^{bc}**	**10.45 ± 1.11^{a}**	**0.005 ± 0.005^{b}**	**34.61 ± 4.9^{b}**
	B8	54.50	4.88	0	47.30
	B9	18.47	21.22	0	37.93
4	B10	19.93	4.60	0	15.40
	B11	25.50	9.55	0	44.70
	B12	25.47	24.16	0	43.70
	B13	33.31	4.98	0	38.03
	Mean	**29.53 ± 5.43^{c}**	**11.57 ± 3.62^{a}**	**0^{b}**	**37.84 ± 4.7^{b}**

Figures with different superscript were significantly different ($p<0.05$). Cell count unit is numbers /villus crypt. SMCP unit is $\mu g\ g^{-1}$ wet weight tissue.

4.3.8 Parasitological results according to responsiveness

By re-grouping the lambs based on responsiveness (worm burdens) there was evidence of a high/low responder phenomenon. Six lambs were designated HR and 7 LR. Mean worm burdens of HR were significantly lower than LR ($p<0.001$) and pooled controls ($p<0.001$). Similarly LR had significantly fewer worms than the controls ($p<0.01$) (Figure 4.7).

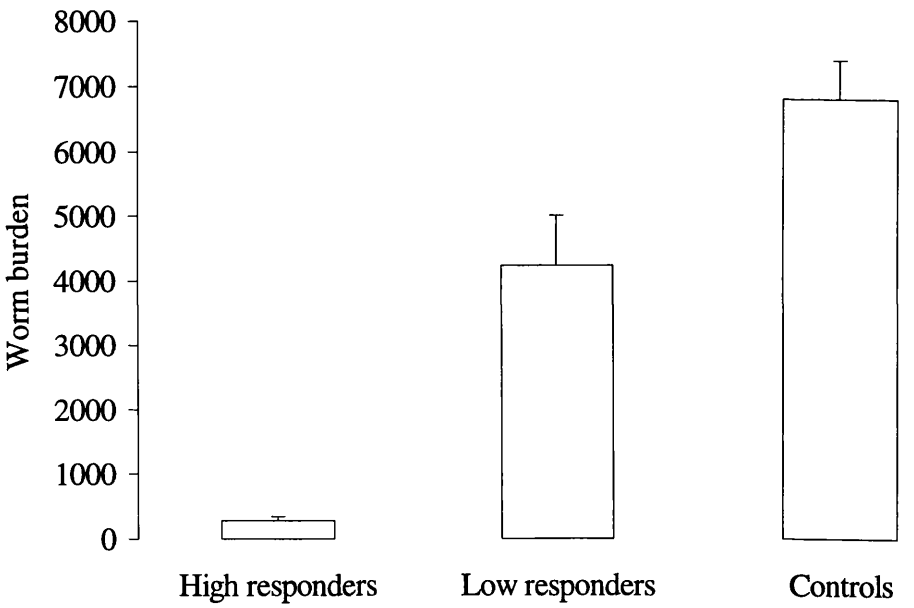

Figure 4.7. *Mean worm burdens ± S.E.M according to responsiveness.*

Since no L_5 were obtained from the HR, L_4:L_5 ratios could not be calculated. However, a significant decline in these ratios was observed between LR and controls (Table 4.5). Sex ratios were lower in responders in comparison to controls, although HR did not differ significantly. Worm lengths were significantly reduced in accord with responsiveness. Hence HR had the smallest worms followed by LR and controls (Table 4.5).

Table 4.5. *Mean L_4 lengths, L_4/L_5 and sex ratios according to responsiveness.*

	High responders	Low responders	Controls
Males			
L_4 length	2.77 ± 0.08^a	3.72 ± 0.28^b	4.76 ± 0.04^c
L_4:L_5 ratio	n.a.	48.2 ± 20.6^a	8.41 ± 1.2^b
Females			
L_4 length	3.12 ± 0.13^a	3.99 ± 0.14^b	5.16 ± 0.04^c
L_5 length	n.a.	6.22 ± 0.08^a	7.27 ± 0.09^b
L_4:L_5 ratio	n.a.	22.06 ± 6.47^a	3.42 ± 2.02^b
Sex ratio	0.63 ± 0.1^{ab}	0.59 ± 0.05^a	0.77 ± 0.03^b

Figures with different superscripts were significantly different at the 5% level.
n.a.- not available

4.3.9 Peripheral eosinophil counts and tissue histology according to responsiveness

Peripheral eosinophil counts rose significantly in responder groups ($p<0.01$) after anthelmintic treatment as they did when analysed according to dietary groupings, although there were no significant differences between HR and LR lambs (Figure 4.8). The duration of time had a significant influence ($p<0.05$) upon eosinophil counts. There was a significant interaction between responsiveness and time ($p<0.01$). Significant differences between LR and control lambs were evident from day 56 PI onwards. Similarly, differences between HR and controls were evident from day 63 onwards.

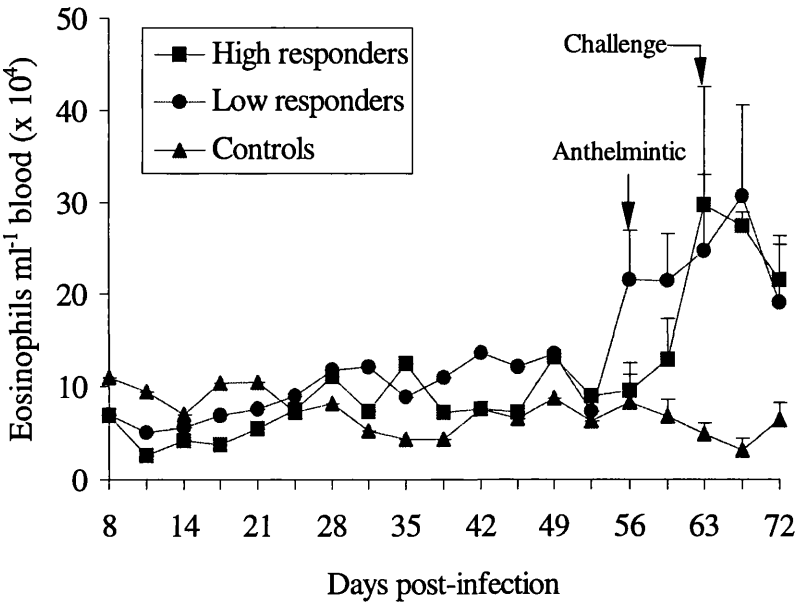

Figure 4.8. *Peripheral blood eosinophil counts S.E.M according to responsiveness.*

Cell counts and SMCP concentrations according to responsiveness are given in Table 4.6. Responders had significantly increased numbers of MMC, GL and eosinophils ($p<0.01$) in comparison to controls. There were no significant differences between HR and LR in terms of MMC, GL or eosinophil counts and SMCP concentrations. There was no correlation between cell counts or SMCP concentrations and worm burdens.

Table 4.6. *Mean ± S.E.M mucosal cell numbers and SMCP concentrations according to responsiveness.*

Group	Mast cells	Eosinophils	G.leucocytes	SMCP
HR	47.42 ± 4.71^{a}	57.98 ± 10.2^{a}	0.35 ± 0.15^{a}	15.45 ± 4.0^{a}
LR	41.95 ± 5.14^{a}	71.64 ± 8.99^{a}	0.05 ± 0.04^{ab}	16.3 ± 4.54^{a}
Controls	27.36 ± 3.10^{b}	36.23 ± 3.30^{b}	0.002 ± 0.002^{b}	11.01 ± 1.81^{a}

Comparisons between percentage of bone marrow eosinophil numbers of HR and LR (Table 4.7) were not significantly different. However, HR exhibited a trend for increased numbers.

Table 4.7. *Percentages of bone marrow eosinophils.*

Status	Lamb no.	Eosinophils (% of 600 bone marrow cells)
	R2	32.8
	R5	45.0
High-responders	B2	39.5
	B4	36.5
	Mean	**38.5 ± 2.6**
	R4	33.3
	B1	32.8
Low-responders	B5	37.0
	B7	15.8
	Mean	**29.7 ± 4.7**

4.3.10 Serum antibody levels according to responsiveness

Figures 4.9 and 4.10 show the anti-worm IgG antibody levels against L_4 and adult worm antigens for responder groups and challenge controls. Responders had significantly elevated levels ($p<0.05$) and duration of time had a significant influence ($p<0.05$) upon antibody responses. A significant interaction between both treatments was evident ($p<0.05$). Generally, the antibody levels in both responder groups were elevated from day 38 PI onwards. However, due to large individual variations, the antibody levels in the HR group did not differ significantly from those of the LR group.

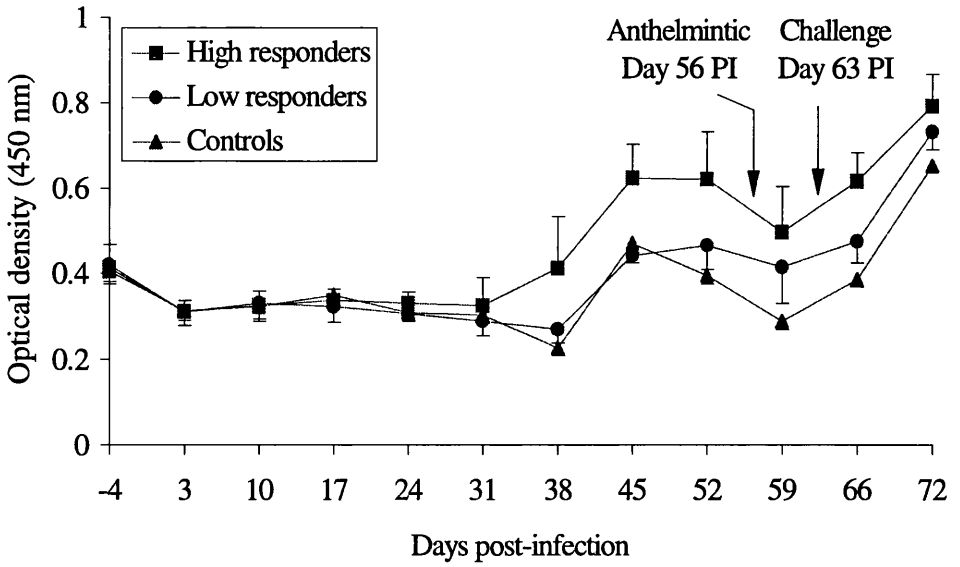

Figure 4.9. *Serum anti-L_4 IgG levels ± S.E.M according to responsiveness.*

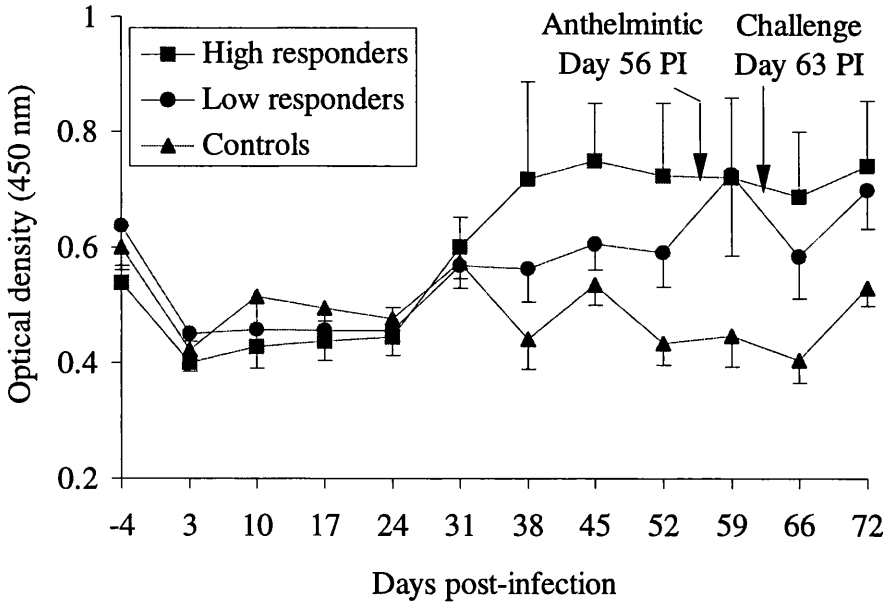

Figure 4.10. *Serum anti-adult worm IgG levels ± S.E.M according to responsiveness.*

4.4 Discussion

This experiment has provided data describing the immune response of lambs towards a model *N.battus* infection. The results described show also that by increasing protein intake from a basal dietary level of 13.2% to 18.3% CP that this was not sufficient to significantly enhance responsiveness either based on parasite elimination or in terms of the cellular and humoral responses. The results confirm previous findings and conclusions that immunity to this parasite is acquired due to previous infection.

The infection regimen was designed to induce a subclinical infection, without pathogenic effects which may compromise the development of immunity. Indeed none of the lambs showed any obvious clinical signs of nematodirosis or even a check in their growth rate. The rate of liveweight gain was high for lambs of 4 months of age but this was considered to be due to the early restriction on growth rate imposed in the pre-experimental period. The prepatent period was longer and the FEC low compared to other studies on *N.battus* infection (Thomas, 1959; Taylor and Thomas, 1986; Catchpole and Harris, 1989). One possible explanation would be the involvement of an innate age immunity. The lambs were 4 months old at the start of the experiment and age immunity has been described in lambs as early as 3 months in single (Taylor and Thomas, 1986) and multiple (Gibson and Everett, 1963) infections.

The worm burdens of previously infected lambs (groups 1 and 2) were much more variable in comparison with those of the challenge control groups (3 and 4). This finding is common among outbred populations. However, despite this finding,

worm burdens were significantly lower in previously infected lambs and therefore establish the fact that immunity was acquired through previous exposure. Similarly, worm lengths followed a similar pattern with the previously infected lambs having smaller worms which were delayed in their development. This suggests that not only did the response generated affect worm establishment, but also worm growth and development since worms recovered from challenge controls were predominantly at the fifth stage of larval development and those that were still at the fourth stage were comparatively larger than those recovered from the immune lambs. The previously infected lambs and HR had the lowest sex ratios, suggesting that male worms were more prone to expulsive effector mechanisms. Similar findings were described among *T.colubriformis* populations in lambs that were vaccinated and allowed to acquire a natural infection from grazing (Douch, 1989).

The failure to significantly enhance worm regulation among supplemented lambs may be due to the sufficient level of dietary protein in the basal diet. Previous studies on this subject have used groups of lambs offered low protein deficient diets and therefore were able to show significant differences (Abbott *et al.*, 1986; Kambara *et al.*, 1993). It is important to note that in this experiment the objective was to determine whether the inclusion of a rumen by-pass protein, by increasing duodenal protein supply, would be of any benefit in enhancing resistance to *N.battus* infection in weaned lambs. Previous studies on the interaction between parasitism and nutrition have used deficient diets in control groups and therefore, were likely to produce significant depression in immunity. Kambara *et al.* (1993) used diets with 11% and 20% CP contents and Abbott *et al.* (1988) used a deficient diet below basal requirements (8.8%CP). Furthermore, these workers used different parasites

(*T.colubriformis* and *H.contortus* respectively) and it is important to take into account the complexity of the inter-relationship between parasite, host and nutrition. This is exemplified by the findings of Abbott *et al.* (1986), in which lambs fed on a low protein diet (8.8% CP) were more susceptible to the pathogenic effects of *H.contortus* but did not differ in terms of parasite establishment compared to the better fed comparisons (16.9% CP).

Peripheral blood eosinophil kinetics were strikingly different in comparison to that seen in other ovine nematode infections. During the primary infection no obvious sign of peripheral eosinophilia was observed. This contrasts with the findings of other workers using *T.colubriformis* (Buddle *et al.*, 1992) and *O.circumcincta* (Stevenson *et al.*, 1994) whereby they generally observed an eosinophilic response during primary infection. Although counts started to rise in supplemented previously infected lambs after anthelmintic treatment they were still low in comparison to those of the above mentioned studies. Whether this is due to the parasite or its feeding habits or the site of infection is unknown. The eosinophil response was more significant at the tissue level with higher numbers in supplemented previously infected lambs. It is possible that additional protein aided the local recruitment of eosinophils to the site of infection indirectly by a general enhancement of the mechanisms involved in chemotaxis. It is also possible that this finding is a general feature of unresponsiveness in young lambs since previous studies on this aspect have used older lambs.

The involvement of MMC in rejection of parasitic nematodes is well established (see chapter 1). It has been suggested recently that in response to *H.contortus* and *T.colubriformis* infections, sheep MMC may degranulate to release

mediators which are accompanied by the release of SMCP (Huntley *et al.*, 1984; Jones *et al.*, 1990; Jones *et al.*, 1992). Degranulated MMC have been demonstrated to differentiate into GL (Huntley *et al.*, 1984). In this chapter, previous infection with *N.battus* significantly increased tissue MMC and GL numbers. However, supplementation failed to further increase MMC numbers significantly, although a trend existed. Supplementation increased GL numbers but these were extremely low. It is difficult to speculate on the mechanisms involved in these findings since the kinetics of this response was not followed. Therefore, conclusions based on one point in time would be misleading. It suffices to say that the development of immunity to *N.battus* is linked to an intestinal mastocytosis with the possibility of mediator involvement.

In contrast to previous work on the detection of SMCP in *H.contortus* infections (Huntley *et al.*, 1987), the results in this chapter do not show any correlation between SMCP concentrations and MMC numbers. Again, since the kinetics of this response was not followed a clear statement is not able to be put forward. It is possible that the parasite, its dose and duration, and the time when effective host responses are initiated may account for the differences in these findings.

The humoral responses observed in this model infection were influenced by the diet and previous exposure to the parasite. The trickle infection enhanced the antibody levels towards both adult and L_4 stages and generally a rise in antibody levels was observed after 38 days of continuous infection. It is important to note that the ELISA used crude worm preparations and therefore this may account for some

degree of cross reactivity with bacterial or food antigens which may explain similar trends in antibody levels of control groups.

Despite the increasing amount of literature on the subject of antibody responses to the common ovine gastrointestinal nematodes, the actual significance of these responses in the development of immunity remains questionable. Previous work has described elevated serum and local antibodies in sheep parasitized with *H.contortus*, *O.circumcincta* and *T.colubriformis* (see chapter 1). Recently, Gill *et al.* (1993b) have demonstrated that anti-parasite IgA and IgG1 antibodies may play an important role in genetically determined resistance of sheep to haemonchosis. The results in this chapter do not allow one to determine the importance of humoral responses in immunity to *N.battus*. However, these findings provide basic information on the kinetics of the response on which further work could be based.

It is quite common to obtain large variation in worm numbers in *N.battus* infections. Due to this and the fact that a significant enhancement of population regulation by supplementation failed, it was decided to determine whether there was some form of innate ability to respond to infection by segregating the lambs based on their worm burdens. This re-analysis of the data showed that not only did HR have significantly lower worm burdens but also the worms were significantly retarded. The identification of HR and LR confirms an earlier study (Taylor and Thomas, 1986) and essentially shows that when the protein supply is adequate (as it was in this experiment in the sheep on the basal diet) the predominant host effect influencing the pathogenicity of the parasites is the level of genetically-determined susceptibility of the host.

In conclusion, it is evident that immunity to *N.battus* is acquired and affects worm establishment and development. Protein supplementation of the diet failed to enhance responsiveness significantly. Lambs responded variably towards infections and, as with other sheep nematode infections, this may be genetically determined. The results from this experiment have provided baseline data concerning the immune response of young lambs towards *N.battus*. The results indicate that it would be profitable to repeat the experiment using younger lambs on different levels of protein intake.

CHAPTER 5
Influence of dietary protein upon development of immunity II

5.1 Introduction

The experiment described in the previous chapter demonstrated a trend for enhancement of immunity by supplementation of the basal diet with a non rumen degradable protein. Although supplementation did not cause significant enhancement of immunity, the fact that trends for enhancement existed justified repeating the experiment using a slightly lower plane of protein in the basal and supplemented diets.

5.2 Materials and Methods

Most standard methods have been described in detail in chapter 2. This section will describe the animals used, their management and monitoring and also the experimental design.

5.2.1 Animals and feed

Four groups of Blackface × Suffolk lambs were used. The lambs were approximately 2-3 months of age at the start of the experiment and had been maintained under worm-free conditions from birth. Groups 1 and 2 consisted of 14 and 12 lambs respectively and the challenge controls (groups 3 and 4) consisted of 6 lambs each. Prior to the experiment the lambs were offered a restricted diet comprised of hay and concentrates and water was offered *ad libitum.* One week prior to experimental infection all lambs were fed their respective basal or supplemented

diets. The basal diet contained 125 g CP kg^{-1} DM which supplied 86g MP kg^{-1} DM feed and the supplemented diet contained the addition of 10% fish meal (178.5 g CP kg^{-1} DM, which supplied 118g MP kg^{-1} DM feed). Calculated metabolizable energy values for the basal and supplemented diets were 8.5 and 8.96 MJ kg^{-1} DM respectively. Feed offered varied between individuals during the experiment between 2 to 3 kg $head^{-1}$ depending upon appetite.

5.2.2 Experimental design

Groups 1 (n=14) and 2 (n=12) were continuously infected and challenged and groups 3 and 4 were not infected but challenged only with *N.battus* L_3 (Table 5.1). Groups 1 and 3 were offered the supplemented diet and groups 2 and 4 the basal diet. Due to constraints of accommodation, only continuously infected groups (1 and 2) were housed individually in metabolic cages. Group 3 and 4 lambs were group housed. The continuous infection regime was designed to mimic the field situation, using a rapidly increasing larval intake followed by decreasing intake over a 12 week period. The infection period was initially planned to continue for a 7 week period but since there were insufficient numbers of L_3 larvae for the challenge infection this period was extended in order to allow time for culturing a new batch of L_3. All groups were treated with levamisole (15 mg kg^{-1} bodyweight) and fenbendazole (5 mg kg^{-1} bodyweight) at week 13 to remove the primary infection and challenged one week later with a single dose of 30,000 *N.battus* L_3. Half the number of lambs were chosen randomly from each group and killed on day 5 PC and the remaining lambs were killed on day 10 PC for worm population analysis and to obtain intestinal tissues for immunohistochemistry. The infection regime and timing of events are summarized in Table 5.1.

Table 5.1. Experimental design.

Group	Diet*	Infection+	Drench Week PI*	Challenge Week PI*	Kill Day PC*
1 (n=14)	S	10, 20, 40, 60, 40, 20, followed by three weeks of 500L_3 three times a week and the same number of L_3 twice a week for a further three weeks	13	14	5 & 10
2 (n=12)	B	as above	13	14	5 & 10
3 (n=7)	S	not infected	13	14	5 & 10
4 (n=7)	B	not infected	13	14	5 & 10

\+ *lambs were infected with a 12 week trickle infection. The larvae were given daily, 7 days a week until week 7 after which larvae were administered thrice and twice a week as described in this table. The numbers given were those in the table multiplied by 10^2.*

* *B - basal diet, S - supplemented diet*
PI - post-infection, PC - post-challenge

5.2.3 Feed intake and liveweight gain

All lambs were weighed weekly prior to feeding. Daily food refusals were pooled on a weekly basis (individually for groups 1 and 2 until week 8 PI afterwhich the lambs were too large to be housed in crates) and the DMI calculated weekly by drying a sample at 40°C for 36 hours.

5.2.4 Sampling

Blood samples, collected at the same time of day for each time point were taken weekly for serum and peripheral eosinophil counts by jugular venepuncture. Faeces were collected from the rectum weekly from day 21 PI to obtain FEC.

At necropsy, a blood sample was taken for serum, the proximal two thirds of the small intestine was taken to determine the remaining worm burden and intestinal sections (4-8 cm long) were taken at approximately 2 metres from the proximal end of the small intestine and sterna and mesenteric lymph nodes were removed for immunohistochemistry.

5.2.5 Analysis based on responsiveness

Since there was no significant dietary effects upon responsiveness to a challenge infection the lambs were re-grouped into HR or LR for further analysis using an arbitrary cut-off point of 1000 worms as described in the previous chapter. Lambs in groups 1 and 2 which had total worm burdens below 1000 were grouped as HR and the remainder as LR. The challenge controls (groups 3 and 4) were pooled and referred to as controls.

5.2.6 Statistical analysis

Worm burdens and GL counts were $\log_{10}(x+1)$ and other data $\log_{10}$ transformed prior to analysis. Data were subjected to either a one way, 2 way or 3 way analysis of variance (ANOVA) depending on the number of treatments and their interactions by fitting a general linear model (GLM). Where significant differences were noted, differences between the mean of groups was tested using a 2 sample t-test.

Correlation analysis was used to determine associations between cell counts and worm burdens.

5.3 Results

5.3.1 Growth and feed intake

Previous infection caused a significant increase in average daily weight gain (ADG) ($p<0.05$) but protein supplementation had no effect and there was no interaction between both treatments (Figure 5.1). Group comparisons did not show any significant differences between groups 1 and 2 and between groups 1 and 3. ADG of group 2 increased significantly ($p<0.01$) in comparison to group 4. Similarly group 3 had significantly higher ADG compared to group 4 ($p<0.05$). DMI did not differ significantly between any groups (Figure 5.2) although the intake of group 1 lambs was reduced compared to the respective previously uninfected challenge controls (group 3).

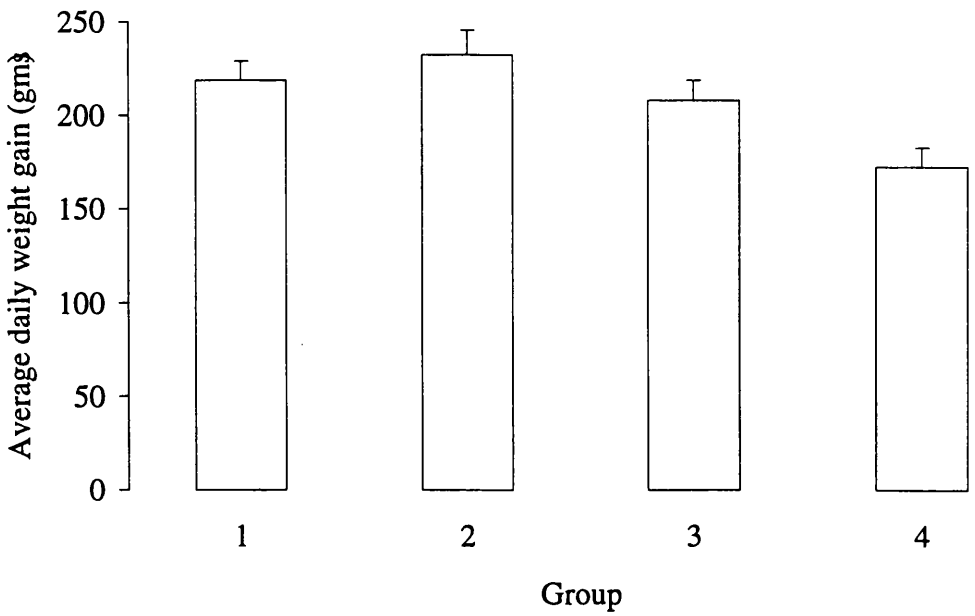

***Figure 5.1.** Average daily weight gain throughout the experiment.*

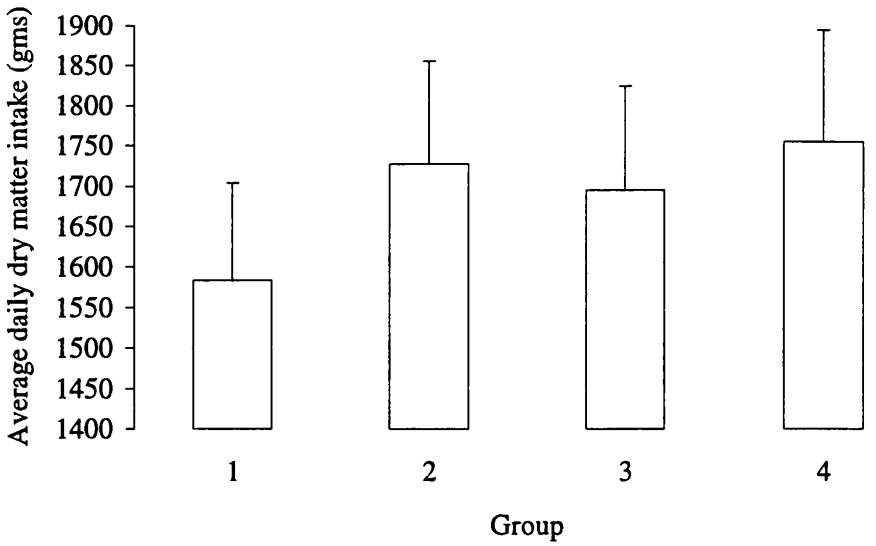

Figure 5.2. *Average daily DMI throughout the experiment.*

5.3.2 Faecal egg counts

There were no significant differences between FEC of previously infected groups throughout the infection period (Figure 5.3). FEC peaked between 35 to 42 days PI after which there was a steady decline.

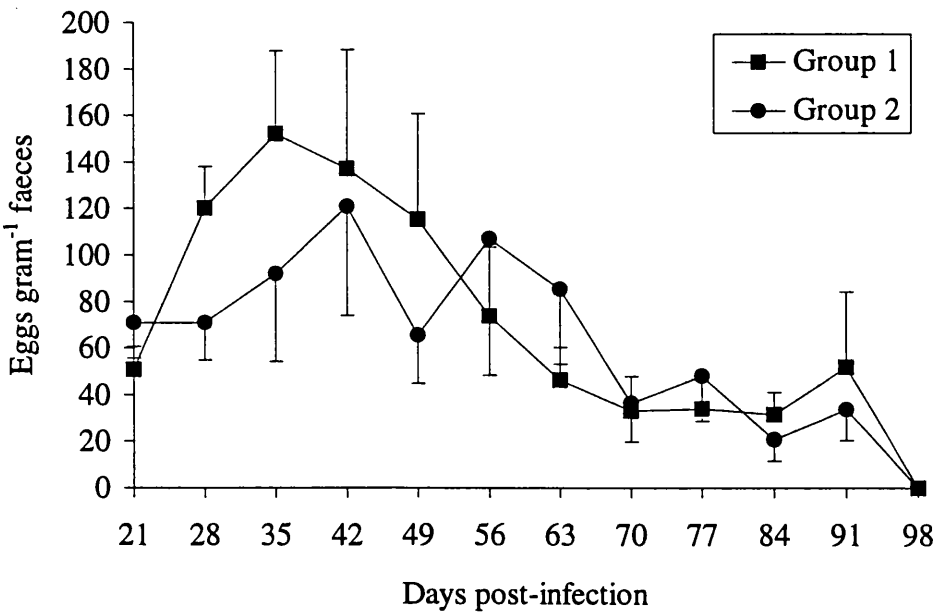

Figure 5.3. *Faecal egg counts of previously infected groups.*

5.3.3 Worm burdens

The worm burdens on both day 5 and 10 PC are shown in Tables 5.2 and 5.3 respectively. Previous infection significantly reduced worm burdens on both occasions ($p<0.05$). Supplementation did not affect worm burdens and no interaction between previous infection and supplementation was shown. Comparisons between worm burdens on day 5 and 10 PC showed a significant reduction in worm burdens of the challenge control groups on day 10 PC ($p<0.05$). Worm burdens of previously infected groups did not differ.

5.3.4 Developmental stage

The worm populations in all groups killed on day 5 PC were at the L_4 stage of development (Table 5.2). At day 10 PC the worm population of previously infected groups were predominantly L_4 but the challenge controls had a similar number of worms at the L_5 stage (Table 5.3). Considerably fewer worms in the previously infected groups were at the L_5 stage in comparison to the challenge controls. This is indicated by a significant effect of previous infection in increasing the female ($p<0.001$) L_4:L_5 ratios (Table 5.4). Comparison between the male larval ratios was unable to be conducted since group 1 had only one lamb with worms at the L_5 stage and group 2 did not have any.

Table 5.2. Individual worm burdens on day 5 PC.

Group	Lamb no	Unsexed L_4	L_4		Total
			Males	Females	
	R4	1080	280	360	1720
	R5	800	0	0	800
1	R8	0	0	0	0
	R10	100	0	0	100
	R11	440	0	0	440
	R13	1700	500	300	2500
	R14	0	0	0	0
	Mean	**589**	**111**	**94**	**794 ± 366[a]**
	B1	540	0	60	600
	B2	1160	540	460	2160
	B3	220	80	0	300
2	B5	980	120	100	1200
	B8	1200	300	240	1740
	B9	0	0	0	0
	Mean	**683**	**173**	**143**	**1000 ± 346[a]**
	R15	400	3900	3540	7840
3	R17	1200	3480	4800	9480
	R18	900	2020	1800	4720
	Mean	**1250**	**3133**	**3380**	**7346 ± 1396[b]**
	B15	400	6280	4540	11220
4	B18	500	6000	4000	10500
	Mean	**450**	**6140**	**4270**	**10860 ± 360[b]**

Mean total burdens with different superscripts were significantly different ($p<0.05$).

Table 5.3. Individual worm burdens on day 10 PC.

Group	Lamb no.	Male worms L_4	Male worms L_5	Female worms L_4	Female worms L_5	Total
	R1	0	0	0	0	0
	R2	0	0	0	0	0
1	R3	180	0	460	0	640
	R6	100	0	40	0	140
	R7	600	0	900	0	1500
	R9	320	40	1400	160	1920
	R12	460	0	800	0	1260
	Mean	**237**	**6**	**514**	**23**	**780 ± 297[a]**
	B4	240	0	200	0	440
	B6	780	0	800	40	1620
	B7	900	0	1840	100	2840
2	B10	300	0	300	0	600
	B11	260	0	0	0	260
	B12	460	0	1680	0	2140
	Mean	**490**	**0**	**803**	**23**	**1317 ± 428[a]**
	R16	600	100	700	580	1980
	R19	900	700	1100	1100	3800
3	R20	420	200	500	600	1720
	Mean	**640**	**333**	**767**	**760**	**2500 ± 654[ab]**
	B13	1240	1200	1700	1200	5340
4	B14	1100	1000	1500	2000	5600
	B17	1000	2240	1500	2500	7240
	Mean	**1113**	**1480**	**1567**	**1900**	**6060 ± 595[b]**

Mean total burdens with different superscripts were significantly different ($p<0.05$).

Comparisons between the mean length of larvae on day 10 PC showed that the previously infected groups (1 and 2) had significantly smaller worms than the challenge controls (3 and 4) ($p<0.001$) (Table 5.4). Supplementation did not affect worm length and there was no interaction between supplementation and previous infection. Sex ratios did not differ significantly on either day 5 or 10 PC (Table 5.4).

Table 5.4. Mean worm lengths, L_4:L_5 and sex ratios.

	Group 1	Group 2	Group 3	Group 4
Males				
L_4 length	3.21 ± 0.19^a	3.53 ± 0.36^a	4.62 ± 0.12^b	4.71 ± 0.05^b
L_4:L_5	8*	n.a.	3.1 ± 1.4^a	0.8 ± 0.2^a
Females				
L_4 length	3.42 ± 0.09^a	3.82 ± 0.31^a	5.18 ± 0.05^b	5.16 ± 0.06^b
L_5 length	6.26 ± 0.12^a	6.19 ± 0.14^a	7.25 ± 0.18^b	7.19 ± 0.13^b
L_4:L_5	8.7*	19.2 ± 0.8^a	1.0 ± 0.1^b	0.9 ± 0.2^b
Sex ratio day 5PC	1.2 ± 0.4^a	1.2 ± 0^a	1.0 ± 0.1^a	1.4 ± 0.05^a
Sex ratio day 10PC	0.9 ± 0.4^a	0.8 ± 0.2^a	0.6 ± 0.06^a	0.7 ± 0.07^a

** - only one lamb in this group had worms at the L_5 stage*
n.a - not available
Worm lengths are in millimetres. Sex ratios were derived by dividing the number of male worms by females. Figures with different superscripts were significantly different ($p<0.05$).

5.3.5 Peripheral eosinophil counts

The peripheral blood eosinophil counts are shown in Figure 5.4. Both supplementation ($p<0.05$) and previous infection ($p<0.001$) increased eosinophil counts significantly over time. There was no significant interaction between both treatments.

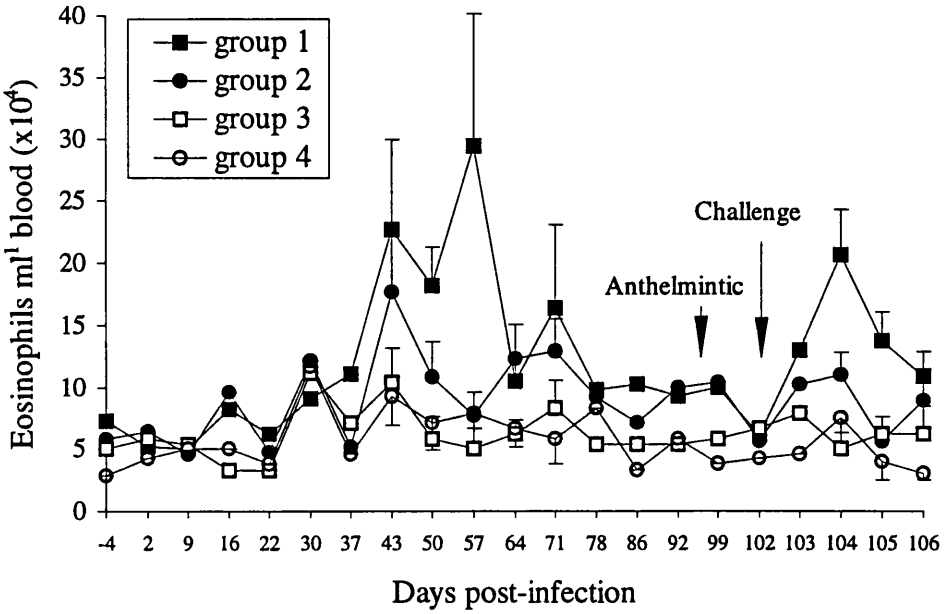

Figure 5.4. *Peripheral blood eosinophil counts throughout the experiment.*

5.3.6 Serological responses

The serum IgG levels towards both adult and L_4 worm antigens are shown in Figures 5.5 and 5.6 respectively. Antibody levels were significantly elevated by supplementation (L_4, $p<0.001$; adult, $p<0.01$), previous infection ($p<0.001$) and duration of time ($p<0.001$) with significant interactions between supplementation and previous infection (L_4, $p<0.05$; adult, $p<0.001$), supplementation and time (L_4 only, $p<0.05$) and between previous infection and time (L_4, $p<0.001$; adult, $p<0.01$). Antibody levels of previously infected lambs (groups 1 and 2) towards both adult and L_4 antigens were generally higher than those of the naive challenge controls (groups 3 and 4) from day 37 PI. Challenge did not evoke an exaggerated anamnestic response towards both L_4 and adult antigens, antibody levels being maintained at pre-challenge levels.

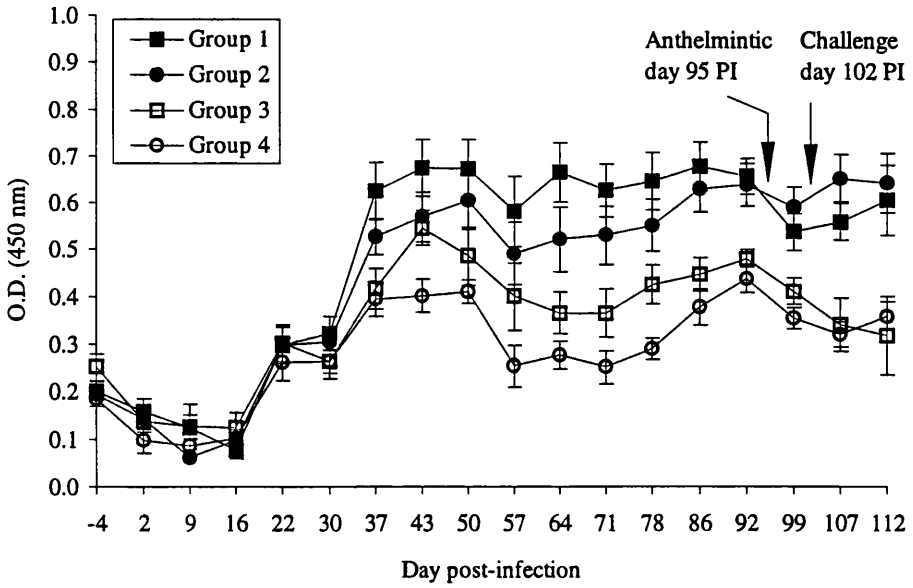

Figure 5.5. *Anti-L_4 IgG antibody responses ± S.E.M over the experimental period.*

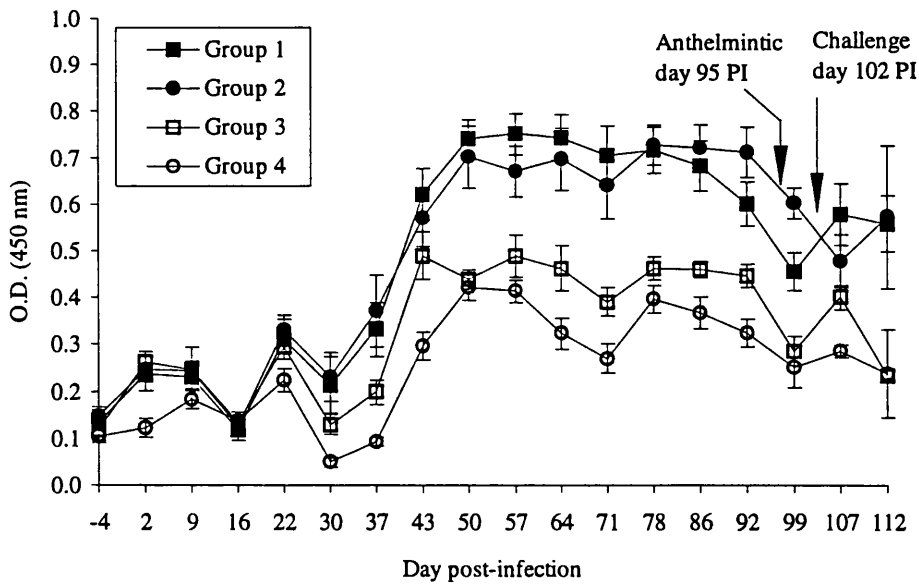

Figure 5.6. *Anti-adult worm IgG antibody responses ± S.E.M over the experimental period*

5.3.7 Cell counts and SMCP concentrations.

The tissue cell counts and SMCP concentrations on days 5 and 10 PC are shown in tables 5.5 and 5.6 respectively. The cell counts and tissue SMCP concentrations did not differ significantly between both occasions. Mast cell numbers were not influenced by supplementation, and previous infection only increased their numbers significantly on day 5 PC ($p<0.001$). SMCP concentrations were not elevated by supplementation on day 5 PC but were on day 10 PC ($p<0.05$). Previous infection caused a significant rise in SMCP concentrations on both day 5 ($p<0.001$) and day 10 ($p<0.05$) PC. Supplementation did not cause an increase in GL numbers however, previous infection caused a significant increase on day 10 PC only ($p<0.01$). Both supplementation and previous infection did not cause an increase in eosinophil numbers of the mesenteric lymph nodes and previous infection caused only a significant rise in mucosal eosinophil numbers on day 10 PC ($p<0.01$). There was no evidence of an influence upon cell counts and SMCP concentrations by interaction between both treatments on either day 5 or 10 PC. Correlation analysis by group did not show any correlations between cell counts and SMCP concentrations and worm burdens.

Table 5.5. Cell numbers and SMCP concentrations on day 5 PC.

Group	Lamb no.	MMC	SMCP	GL	Eosinophils	
					lymph node	mucosa
	R4	148	1098.4	22	28.8	41.6
	R5	35	284.6	0	0.9	3.0
	R8	76	233.8	2	5.9	22.1
1	R10	31	367.9	0	1.1	23.3
	R11	37	17.7	0	4.5	53.3
	R13	64	181.6	33	70.6	42.2
	R14	40	91.4	5	5.2	23.5
	Mean	**61.6 ± 15.7^{a}**	**325 ± 136^{a}**	**8.9 ± 5.0^{a}**	**16.7 ± 9.7^{a}**	**29.9 ± 6.4^{a}**
	B1	45	31.8	0	2.9	34.2
	B2	40	27.6	30	11.0	27.4
	B3	45	227.7	6	3.3	28.8
2	B5	66	154.7	7	3.3	22.0
	B8	56	111.2	0	2.5	14.3
	B9	23	34.8	0	9.1	18.5
	Mean	**45.8 ± 6.0^{ac}**	**98 ± 33.4^{ac}**	**7.2 ± 4.7^{a}**	**5.3 ± 1.5^{a}**	**24.2 ± 3.0^{a}**
	R15	5	0.5	0	1.3	9.7
3	R17	10	2.9	0	3.3	12.4
	R18	18	9.2	0	1.2	30.8
	Mean	**11.0 ± 3.8^{bc}**	**4.2 ± 2.6^{bc}**	**0^{b}**	**1.9 ± 0.7^{a}**	**17.6 ± 6.6^{a}**
4	B15	16	10.0	0	2.8	9.6
	B18	14	7.6	0	1.4	23.6
	Mean	**15.0 ± 1.0^{b}**	**8.8 ± 1.2^{b}**	**0^{b}**	**2.1 ± 0.7^{a}**	**16.6 ± 7.0^{a}**

Figures with different superscript were significantly different ($p<0.05$). Cell count unit is numbers villus^{-1} crypt. SMCP unit is μg gram^{-1} wet weight tissue.

Table 5.6. Cell numbers and SMCP concentrations on day 10 PC.

Group	Lamb no.	MMC	SMCP	GL	Eosinophils lymph	Eosinophils mucosa
	R1	77	75.7	7	13.6	18.7
	R2	23	31.2	5	41.6	25.8
	R3	126	402.6	29	0.5	50.8
1	R6	62	200.4	12	10.2	39.7
	R7	42	66.8	16	14.7	15.5
	R9	42	29.1	0	20.2	21.4
	R12	42	53.8	5	5.3	10.4
	Mean	**59.1 ± 12.9^{a}**	**122.8 ± 51.5^{a}**	**10.6 ± 3.6^{a}**	**15.2 ± 5.0^{a}**	**26.0 ± 5.4^{a}**
	B4	18	26.4	3	0.4	18.8
	B6	9	5.0	0	15.4	25.7
	B7	27	26.0	0	2.3	9.6
2	B10	36	37.4	0	5.8	16.6
	B11	26	29.0	9	4.4	19.2
	B12	63	43.4	2	1.3	20.9
	Mean	**29.8 ± 7.6^{a}**	**27.9 ± 5.4^{bc}**	**2.3 ± 1.4^{b}**	**4.9 ± 2.2^{ac}**	**18.5 ± 2.2^{ab}**
	R16	42	66.9	0	0.3	18.9
3	R19	15	7.4	0	2.5	10.0
	R20	25	21.0	0	4.0	13.2
	Mean	**27.3 ± 7.9^{a}**	**31.8 ± 18.0^{ac}**	**0^{b}**	**2.3 ± 1.1^{ac}**	**14.0 ± 2.6^{ab}**
	B13	13	2.1		2.0	9.8
4	B14	17	13.3	0	1.2	10.4
	B17	36	19.7	0	1.6	4.9
	Mean	**22.0 ± 7.1^{a}**	**11.7 ± 5.1^{ac}**	**0^{b}**	**1.6 ± 0.2^{bc}**	**8.4 ± 1.7^{b}**

Figures with different superscript were significantly different ($p<0.05$). Cell count unit is numbers villus^{-1} crypt. SMCP unit is μg gram^{-1} wet weight tissue.

5.3.8 Parasitological results according to responsiveness

By re-grouping the lambs killed on day 10 PC based on responsiveness (worm burdens) there was evidence of a high/low responder phenomenon. Seven lambs were designated HR and 6 LR. Mean worm burdens of HR were significantly lower than LR ($p<0.001$) and controls ($p<0.001$). Similarly LR had significantly fewer worms than the controls ($p<0.01$) (Table 5.7).

Since no male L_5 and only one lamb with female L_5 were obtained from the HR, L_4:L_5 ratios could not be calculated. However, a significant decline in female ratios was observed between LR and controls (Table 5.7). Sex ratios did not differ significantly, although a trend for a higher ratio was evident among HR. Worm lengths were significantly reduced in accord with responsiveness. Hence HR had the smallest worms followed by LR and the controls (Table 5.7).

Table 5.7. *Mean worm burdens, L_4 lengths, L_4/L_5 and sex ratios according to responsiveness.*

	High responders	Low responders	Controls
Worm burden	297 ± 101^a	1880 ± 230^b	4279 ± 889^c
Males			
L_4 length	2.68 ± 0.11^a	3.73 ± 0.3^b	4.68 ± 0.07^c
L_4:L_5 ratio	n.a	8.0*	2.0 ± 0.8
Females			
L_4 length	3.19 ± 0.16^a	4.05 ± 0.18^b	5.21 ± 0.03^c
L_4:L_5 ratio	18.4*	14.3 ± 5.6^a	0.9 ± 0.1^b
Sex ratio	1.3 ± 0.4^a	0.5 ± 0.1^a	0.7 ± 0.05^a

Figures with different superscripts were significantly different at the 5% level.
** - only one lamb in this group had worms at the L_5 stage*
n.a.- not available

5.3.9 Peripheral eosinophil counts and tissue histology according to responsiveness

Peripheral eosinophil counts rose significantly in responder groups ($p<0.01$) although there were no significant differences between HR and LR lambs (Figure 5.7). The duration of time had a significant influence ($p<0.001$) upon eosinophil counts. There was a significant interaction between responsiveness and time ($p<0.01$).

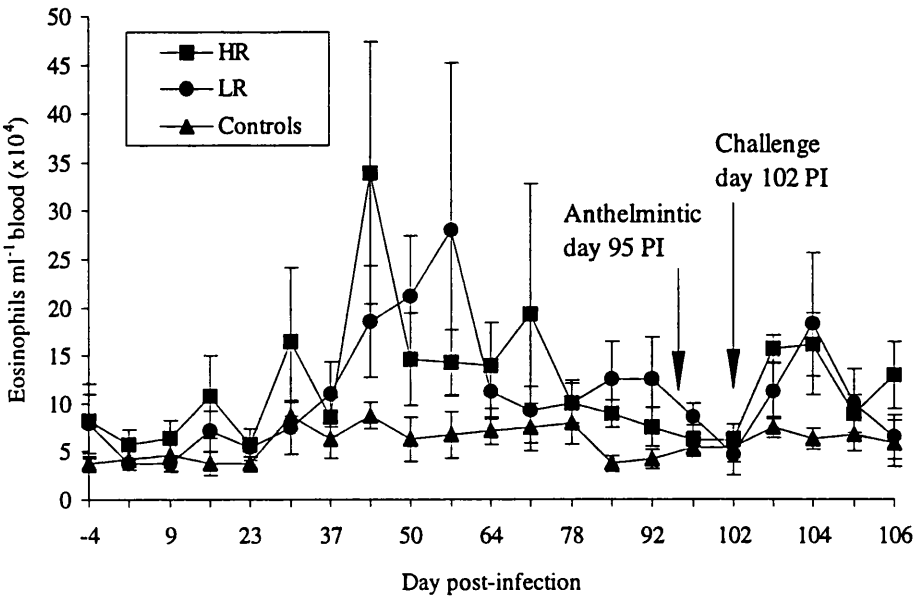

Figure 5.7. *Peripheral blood eosinophil counts ± S.E.M according to responsiveness*

Cell counts and SMCP concentrations according to responsiveness are given in Table 5.8. Responders had significantly increased numbers of GL and mucosal and mesenteric lymph node eosinophils ($p<0.01$) in comparison to controls. Mucosal tissue concentrations of SMCP were also significantly elevated according to responsiveness ($p<0.05$).There were no significant differences in cell counts and SMCP concentrations between HR and LR. There was no correlation between cell counts or SMCP concentrations and worm burdens. Bone marrow eosinophil

numbers of randomly selected individuals maintained on the basal diet did not differ significantly (Table 5.8).

Table 5.8. *Mean ± S.E.M cell numbers and SMCP concentrations according to responsiveness.*

Group	MMC	SMCP	GL	Eosinophils		
				lymph node	mucosa	sterna
						% of 600 marrow
HR	52.6 ± 14.7^{a}	114.7 ± 53.4^{a}	9.3 ± 3.6^{a}	10.9 ± 5.4^{a}	27.1 ± 5.0^{a}	13.3 ± 0.11^{a}
LR	37.5 ± 7.4^{a}	37.3 ± 9.0^{ab}	3.8 ± 2.6^{a}	9.9 ± 3.2^{a}	17.2 ± 2.6^{ab}	16.7 ± 4.9^{a}
Controls	24.7 ± 4.9^{a}	21.7 ± 9.5^{b}	0^{b}	1.9 ± 0.5^{b}	11.2 ± 1.9^{b}	11.4 ± 1.8^{a}

5.3.10 Serum antibody titres according to responsiveness

Figures 5.8 and 5.9 show the anti-worm IgG antibody levels against L_4 and adult worm antigens for responder groups and challenge controls. Responders had significantly elevated levels ($p<0.001$) and duration of time had a significant influence ($p<0.001$) upon antibody responses. A significant interaction between both treatments was evident ($p<0.001$). Generally, the antibody levels in both responder groups were elevated from day 37 PI onwards. However, due to large individual variations, the antibody levels in the HR group did not differ significantly from those of the LR group.

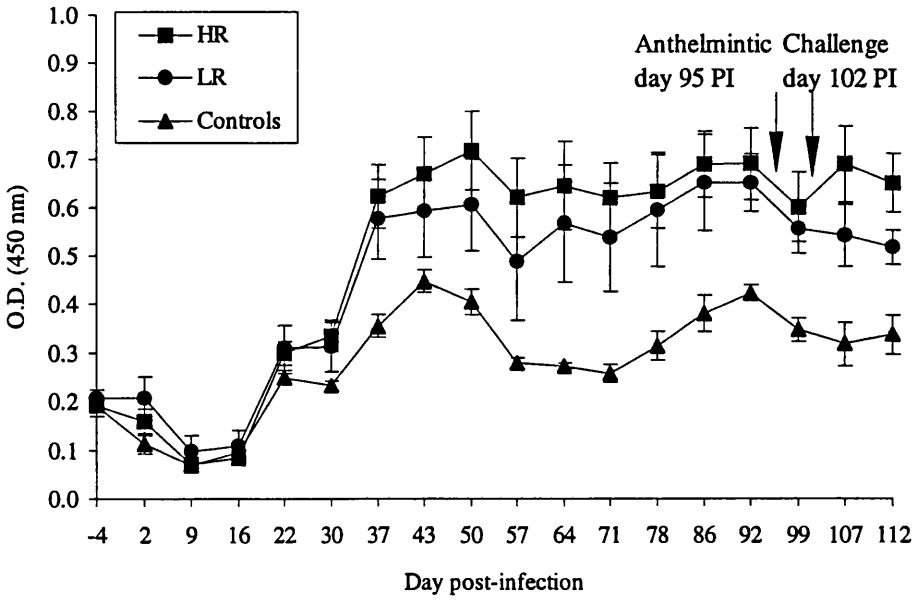

Figure 5.8. *Serum anti-L_4 IgG levels ± S.E.M according to responsiveness.*

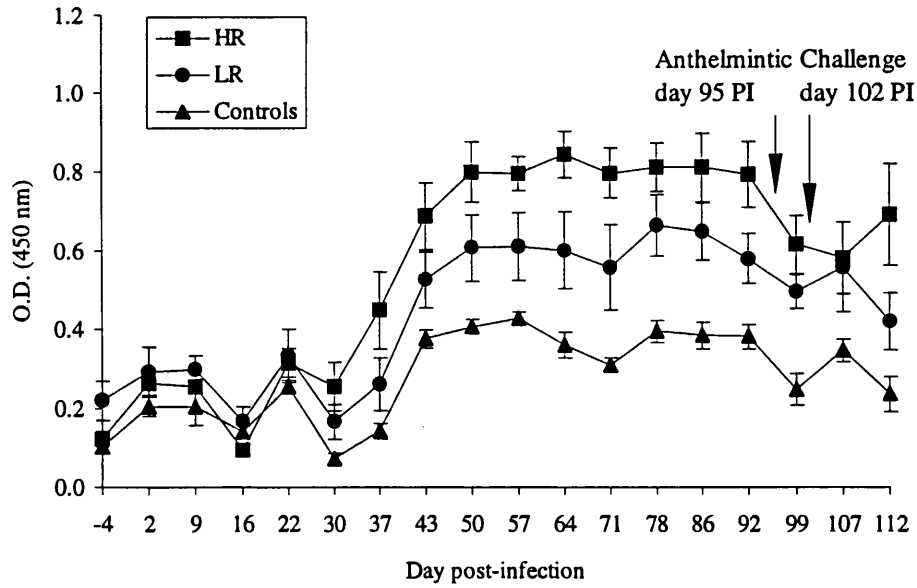

Figure 5.9. *Serum anti-adult worm IgG levels ± S.E.M according to responsiveness*

5.4 Discussion

The results of this experiment are very similar to those described in chapter 4. As shown previously, the principal manifestation of acquired immunity in this model is retardation of growth and development of larvae and expulsion of worms.

The interesting finding in this experiment is the data which shows that previous infection enhanced effector mechanisms as shown by similar worm burdens on day 5 and 10 PC as opposed to those of the control groups. Despite the further removal of worms from day 5 to day 10 PC among the challenge controls, the cell counts and SMCP concentrations did not increase. This could possibly be due to the existence of a threshold level at which worms can be effectively expelled and retarded. The delay in expulsion among these groups could be possibly linked to significantly lower inflammatory cell numbers.

The slight reduction in the level of crude protein (CP) of both diets compared to that used in the previous chapter did not produce significant effects in reducing worm burdens, retardation of larval development and enhancement of inflammatory responses. The levels of CP used were not formulated to be extremely far apart since this would be unpractical under field conditions and the non-rumen degradable nature of the fish meal would theoretically increase duodenal protein supply.

Surprisingly, previous infection significantly increased ADG. The results of this analysis were dominated by the differences between groups 2 and 4. This can only be explained by the assumption that the low level of infection used, was insignificant in affecting growth and presumably by chance, the lambs in group 4

were growing at a slower rate. Furthermore, it is worth considering the fact that both groups 3 and 4 were group housed.

The similarity in the worm burdens on day 5 and 10 PC in the previously infected lambs suggest an earlier response to challenge since among these groups the worm burdens did not differ. However, among the challenge controls there was a significant reduction in worm burdens by day 10 PC which suggests that the mechanisms involved in rejection were still operating against the challenge infection.

The peripheral eosinophil response was significantly enhanced by supplementation and infection. However, as shown previously, the counts were still low in comparison to those observed in other ovine nematodoses.

Once again it is evident that lambs were able to be segregated into HR and LR and this was reflected by reduced worm burdens, worm size and retardation in development. The cellular response remained similar between HR and LR and strengthens the suggestion that the inflammatory response may have a threshold level and that there is a possible involvement of other components of the immune system or direct effects of changes in gut physiology in the acquisition of resistance to *N.battus*.

CHAPTER 6

Persistence of immunity to experimental infection

6.1 Introduction

The preceeding chapters have described the effects of dietary protein supplementation upon the immune response of lambs towards *N.battus*. Data presented earlier showed that the response is acquired from a continuous trickle infection. Similarly, under field conditions, lambs that mount an effective immune response and survive early infections develop a persistent immunity to adulthood.

Barnes and Dobson (1993) claimed that there is lack of knowledge concerning the persistence of immunity to ovine gastrointestinal helminths after their removal. This knowledge would be important particularly under farming conditions where anthelmintic treatment may abruptly reduce the level of infection in the host. Such conditions may arise where sheep are drenched and moved to pastures of low infectivity or through seasonal changes in larval availability.

Studies on *T.colubriformis* infections showed that sheep given a trickle larval challenge develop an acquired immunity which persists after removal of the resident populations for varying intervals depending on the dose and duration of the primary infection. Gibson, Parfitt and Everett (1970) found that worms removed with anthelmintic after 12 or 24 weeks of a trickle infection with *T.colubriformis* were not replaced by incoming larvae (as judged by faecal egg counts). Furthermore, prolonged exposure (34 weeks) to *T.colubriformis* generated immunity which persisted for at least 24 weeks (Kimambo *et al.*, 1988). By contrast, immunity to

H.contortus appears to be fairly short-lived (Jackson *et al.*, 1988; Coyne and Smith, 1992) since removal of a primary infection rendered immune lambs as susceptible to challenge as naive lambs after 9 and 12 weeks without antigenic exposure.

The phenomenon of persistence of immunity (immunological memory) has not been studied in infections of lambs with *N.battus*. Based on previous parasitological observations one would expect lambs that had been previously infected to be relatively immune to further challenge. However, since the housed model infection described in previous chapters used a low level of infection in comparison to that which occurs in the field it is of interest to determine whether immunological memory due to prior exposure operates against *N.battus* using this model and if so, for how long. The results of this experiment will provide an understanding of the dynamics of the immune response and whether this model would be able to be used in describing events that occur in field situations.

6.2 Materials and Methods

6.2.1 Animals and feed

Fifty-four Blackface × Suffolk lambs aged approximately 3 months at the start of the experiment were used. The lambs had been maintained under worm-free conditions from birth. Throughout the experiment the lambs were offered a restricted diet comprised of hay *ad libitum* and concentrates (Ruminant A at 250-300 gms $head^{-1}$ day^{-1}). Water was offered *ad libitum.*

6.2.2 Experimental design

Lambs were allocated into 7 groups based on weight and sex to provide uniformity. Groups 1, 3, 5, and 7 were continuously infected with *N.battus* L_3 and challenged. Groups 2, 4, and 6 were not infected and served as challenge controls. Details of the infection regime and timing of events are summarized in Table 6.1. The continuous infection regime was designed to mimic the field situation, using a rapidly increasing larval intake followed by decreasing intake over a 7 week period. All groups were treated with levamisole (15 mg kg^{-1} bodyweight) and fenbendazole (5 mg kg^{-1} bodyweight) at week 8 to remove the primary infection. In order to determine the persistence of the response one infected and one non-infected group was challenged either one week post-treatment (PT) (groups 1 & 2), 6 weeks PT (groups 3 & 4) or 12 weeks PT (groups 5 & 6), thus enabling the measurement of immunological memory up to 12 weeks without exposure to further infection. Group 7 was further divided into four sub-groups of 3 lambs (group 7a, 7b, 7c, and 7d). Lambs in group 7a were killed immediately after anthelmintic treatment and groups 7b, 7c and 7d were killed on the same day that the lambs in groups 1 and 2, 3 and 4, and 5 and 6 were challenged respectively. This design enabled analysis of cellular responses prior to challenge which would aid in explaining any cellular responses noted post-challenge. The challenge infection was the standard single dose of 30,000 *N.battus* L_3. All lambs were killed on day 10 post-challenge (PC) (except group 7) for worm population analysis and to obtain intestinal tissues for immunohistochemistry.

6.2.3 Sampling

Details of the sampling techniques and analyses are explained in Chapter 2. During the primary infection blood samples (collected at the same time of day) were taken every third week for serum and weekly for peripheral eosinophil counts by jugular venepuncture. Faeces were collected weekly from day 14 post-infection (PI) for determination of faecal egg counts (FEC). At necropsy two thirds of the proximal small intestine was taken and processed for recovery of *N.battus* worms. Subsamples of 2% of the total intestinal digest and washings were counted. Intestinal sections (4-8 cm long) were taken at approximately 2 metres from the proximal end of the small intestine for immuno-histochemistry. The sternum was removed for bone-marrow eosinophil counts.

Table 6.1. *Experimental design.*

Group	Infection+	Drench Week PI*	Challenge Week PI*	Kill Day PC*
1 (n=7)	10, 20, 40, 80, 40, 20,10	8	9	10
2 (n=7)	not infected	8	9	10
3 (n=7)	as above	8	14	10
4 (n=7)	not infected	8	14	10
5 (n=7)	as above	8	20	10
6 (n=7)	not infected	8	20	10
7 (n=12)	as above	8	no challenge	killed in four groups of 3 as described in text

\+ *Lambs were infected with a 7 week trickle infection. The larvae were given daily, 5 days a week. The numbers given were those in the table multiplied by 10^2.*

* *PI - post-infection, PC - post-challenge*

6.2.4 Statistical analysis

Worm burdens and larval ratios, antibody titres, tissue mast cell and eosinophil counts and SMCP concentrations were $\log_{10}$-transformed and peripheral blood eosinophil counts $\log_{10}(x+1)$-transformed prior to analysis. Variables of balanced designs were compared using 2 way analysis of variance (ANOVA) and for unbalanced designs a general linear model (GLM) was fitted for a 2 way ANOVA. ANOVA was performed to establish the probability of significant effects of previous infection, time and the interaction of both factors. Where significant differences were noted differences between the means of groups was tested using a 2 sample t-test assuming equal variances. Correlation analysis was used to determine associations between cell counts and worm burdens.

6.3 Results

6.3.1 Faecal egg counts

Since groups 1, 3, 5, and 7 received similar infections, FEC during the primary infection were pooled. Very few eggs were detected on day 14 PI but by day 21 PI the egg count had peaked to a mean of 211 eggs per gram (epg) faeces. The counts rose to a mean of 227 epg by day 28 PI after which they gradually declined (Figure 6.1).

6.3.2 Worm burdens

The worms recovered on day 10 PC were at the L_4 and L_5 stage of larval development. Analysis of individual worm burdens (Table 6.2) showed a significant effect of previous infection ($p<0.05$) and time ($p<0.01$) upon worm burdens. There was no evidence of an interaction between both factors. Comparisons between means of groups showed no significant differences between previously infected and

challenge control groups although there was a trend for a lower establishment in the previously infected lambs (groups 1, 3 & 5). Worm burdens were significantly reduced in groups 5 and 6 compared to groups 1-4. Mean percent establishments of the challenge infection were 42.8%, 60.8%, 41.9%, 46.5%, 13.3%, and 19.6% for groups 1, 2, 3, 4, 5 and 6 respectively.

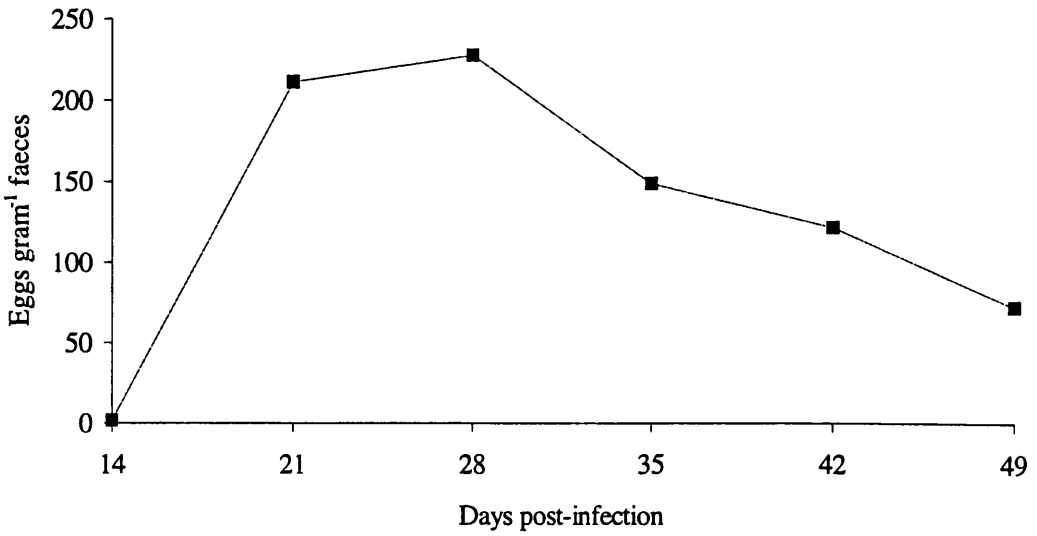

Figure 6.1. *Pooled FEC during primary infection (groups 1, 3, 5 & 7).*

6.3.3 Developmental stage

The worm populations in the previously infected groups (1, 3 & 5) were predominantly L_4 with considerably fewer worms at the L_5 stage in comparison to the challenge controls (2, 4 & 6). Significant increases in the percentage of L_4 ($p<0.001$) were demonstrated among previously infected groups. However, time had no significant effect and there was no interaction.

Comparisons of means between previously infected and challenge control groups were significant at week 9 (groups 1 & 2) and 20 PI (groups 5 & 6) but not at

Table 6.2. Individual worm burdens ± S.E.M on day 10 PC.

Group	Lamb no.	Male worms		Female worms		Total
		L_4	L_5	L_4	L_5	
	G1	200	0	350	50	600
	G2	5750	3150	6850	2100	17850
	G3	1900	5400	3750	5650	16700
1	G4	1150	6350	1900	6650	16050
	G5	300	0	850	50	1200
	G6	5700	1550	8450	1400	17100
	G7	4650	2000	6050	2100	14800
	Mean	**2807.1**	**2635.7**	**4028.6**	**2571.4**	**12042.9 ± 2900[a]**
	B1	1550	6600	2250	7850	18250
	B2	1400	8100	2050	9700	21250
	B3	1400	7200	1750	7950	18300
2	B4	1200	6450	1500	7000	16150
	B5	1200	5500	1850	5850	14400
	B6	1900	8850	1900	9400	22050
	B7	2600	6100	2750	5800	17250
	Mean	**1607.1**	**6971.4**	**2007.1**	**7650.0**	**18235.7 ± 1020[a]**
	G8	1300	4850	2450	5350	13950
	G9	6250	0	12350	150	18750
	G10	300	900	650	1150	3000
3	G11	5200	250	7700	600	13750
	G12	6750	50	7800	1050	15650
	G13	1900	5450	2400	6450	16200
	G14	1200	0	5400	0	6600
	Mean	**3271.4**	**1642.9**	**5535.7**	**2107.1**	**12557.1 ± 2135[a]**
	B8	2800	5050	5150	3900	16900
	B9	750	1250	1150	1400	4550
	B10	1850	6600	3450	6700	18600
4	B11	1050	7600	1900	7950	18500
	B12	1600	5050	2800	5100	14550
	B13	900	2900	1550	2500	7850
	B14	5050	2650	5950	3000	16650
	Mean	**2000**	**4442.9**	**3135.7**	**4364.3**	**13942.9 ± 2094[a]**
	G15	2050	250	2800	100	5200
	G16	750	1000	700	1300	3750
	G17	550	0	1250	0	1800
5	G18	1200	550	1950	1050	4750
	G19	950	2000	1450	1600	6000
	G20	150	0	500	0	650
	G21	700	1100	2000	2050	5850
	Mean	**907.1**	**700**	**1521.4**	**871.4**	**4000.0 ± 780[b]**
	B15	550	1550	1550	1400	5050
	B16	650	1200	1350	1350	4550
	B17	900	2100	1250	1950	6200
6	B18	650	1700	300	1750	4400
	B19	350	2200	800	2550	5900
	B20	700	3750	1200	2650	8300
	B21	850	2600	850	2500	6800
	Mean	**664.3**	**2157.1**	**1042.9**	**2021.4**	**5885.7 ± 522[b]**

Figures with different superscripts were significantly different ($p<0.05$).

week 14 PI (groups 3 & 4) although a trend for fewer L_4 in group 4 was evident (Table 6.3). The percentage of L_4 in groups 3 and 4 did not differ significantly due to

the extremely high numbers of L_4 in a few lambs from group 4. This also caused the male and female larval ratios of both groups not to differ significantly (Table 6.3). Analysis of treatment effects upon male and female larval ratios showed that for both variables previous infection significantly increased the ratios (male: $p<0.001$; female: $p<0.001$). However, time had no significant effect and there was no interaction. This finding is directly related to the increased numbers of L_4 in previously infected groups. Similarly the sex ratios were significantly influenced by previous infection ($p<0.01$) but not by time and there was no interaction. Means of previously infected and challenge control groups at week 9 and 14 PI did not differ significantly although a trend for lower ratios (fewer males) was noted. The sex ratio of group 6 was significantly elevated compared to group 5 at week 20 PI.

Table 6.3. *Percent L_4 of total population, L_4:L_5 and male:female sex ratios.*

	Group 1	Group 2	Group3	Group 4	Group 5	Group 6
% L_4	66.6 ± 11.1^{ad}	20.0 ± 2.0^{b}	67.3 ± 13.8^{ad}	37.2 ± 6.1^{ac}	69.2 ± 10.7^{d}	29.9 ± 3.8^{c}
Male L_4:L_5	1.7 ± 0.65^{acd}	0.2 ± 0.03^{b}	31.3 ± 26.2^{abc}	0.6 ± 0.24^{abc}	2.4 ± 1.47^{c}	0.3 ± 0.05^{bd}
Female L_4:L_5	5.3 ± 2.17^{a}	0.3 ± 0.04^{b}	17.3 ± 13.2^{ac}	0.9 ± 0.22^{c}	6.5 ± 5.39^{ac}	0.6 ± 0.14^{bc}
Sex ratio	0.7 ± 0.09^{ab}	0.9 ± 0.03^{a}	0.6 ± 0.08^{bc}	0.9 ± 0.02^{ac}	0.6 ± 0.10^{b}	0.9 ± 0.08^{ac}

Sex ratios were derived by dividing the number of male worms by females. Figures with different superscripts were significantly different ($p<0.05$).

6.3.4 Antibody titres

Serum titres were pooled for previously infected and also for the challenge control groups. The primary infection did not evoke an exaggerated and rapid response since there were no differences after 21 days of infection. Significant elevation of anti-adult worm ($p<0.05$) (Figure 6.2) and anti-L_4 ($p<0.01$) (Figure 6.3) serum IgG titres of previously infected lambs was evident by day 42 PI.

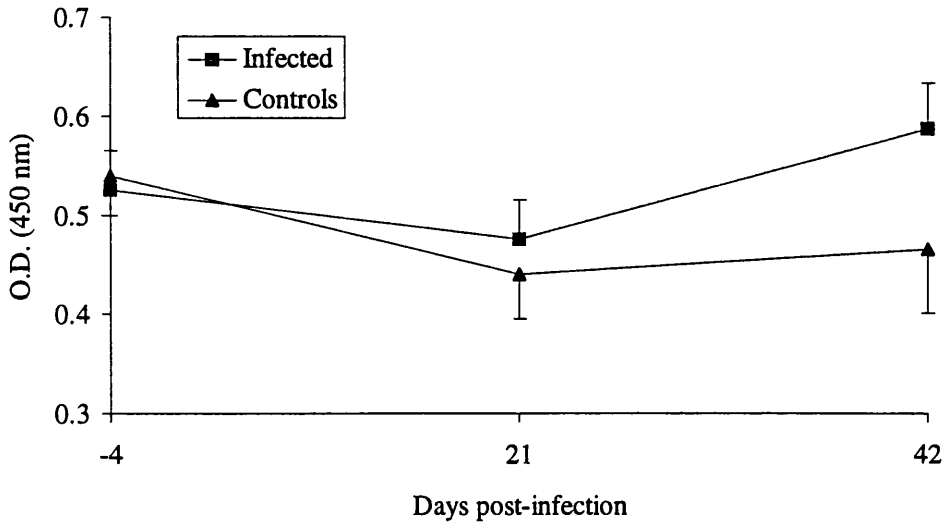

Figure 6.2. *Anti-adult worm IgG antibody response ± S.E.M during primary infection.*

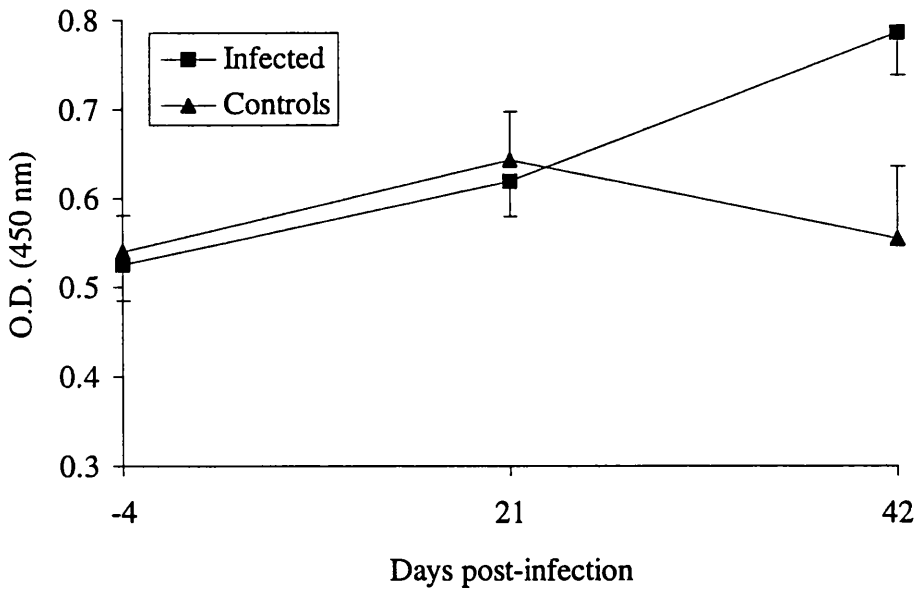

Figure 6.3. *Anti-L_4 IgG antibody responses ± S.E.M during primary infection.*

6.3.5 Peripheral blood eosinophil counts

The peripheral blood eosinophil counts did not differ significantly between previously infected and challenge control groups except on day 42 PI where infected

lambs had significantly ($p<0.001$) higher counts (Figure 6.4). However, the counts were still very low and considered to be in the normal range for lambs of this age.

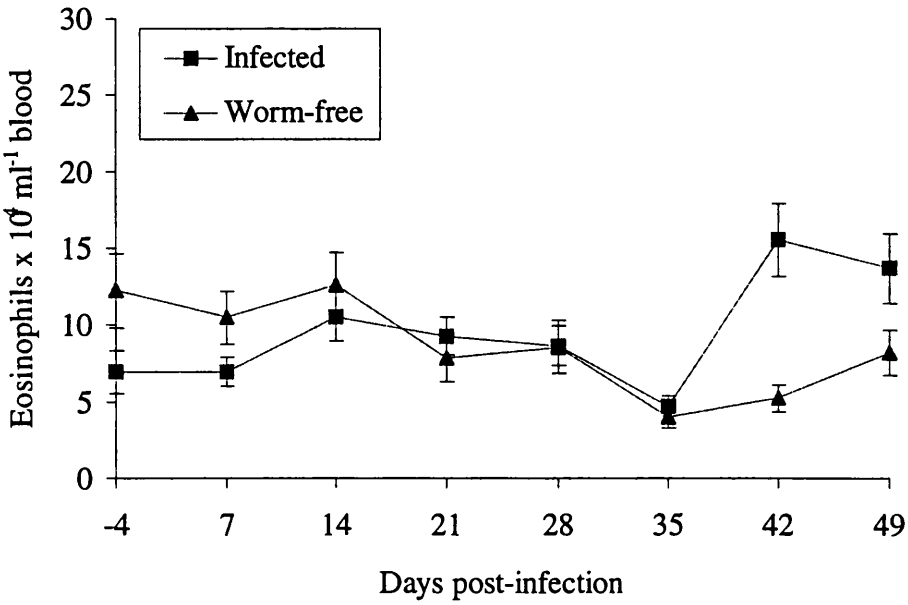

Figure 6.4. *Peripheral blood eosinophil counts ± SEM during primary infection.*

6.3.6 Tissue cell counts

All cell counts were extremely variable and the small group sizes further complicated the analysis (Table 6.4). Tissue eosinophil counts did not differ between any group and thus were not influenced by previous infection or time. In contrast, bone marrow eosinophil counts were significantly increased due to previous infection ($p<0.05$) and time ($p<0.001$) without any significant interaction between both treatments. There was a significant ($p<0.05$) negative correlation between bone marrow eosinophil counts in groups 5 ($r= -0.732$, $p<0.05$) and 6 ($r= -0.796$, $p<0.05$). Tissue controls (group 7) had similar pre-challenge eosinophil counts irrespective of time which did not differ from post-challenge counts.

Both mast cell counts ($p<0.05$) and tissue SMCP concentrations ($p<0.05$) were significantly enhanced by previous infection but not by time and no interaction was evident. Pre-challenge mast cell counts (group 7a & 7b) were significantly lower ($p<0.05$) at termination of the primary infection and after anthelmintic treatment in comparison to group 1. However, such differences did not occur between group 7c and groups 3 and 4. Group 7d did not differ significantly from group 5 but was significantly higher in comparison to the counts of group 6. Significant correlation between SMCP concentrations and worm burdens was detected in group 2 lambs ($r= -0.823$, $p<0.05$).

Table 6.4. *Mucosal cell counts, SMCP concentrations and bone marrow eosinophil numbers on day 10 PC.*

Group	Lamb	Mast cells (cells/VCU)	SMCP ($\mu g\ g^{-1}$ tissue)	Eosinophils (cells/VCU*)	Bone-marrow eosinophils (%)
	G1	32.2	87.72	65.6	16.6
	G2	19.0	13.50	38.7	21.1
	G3	3.7	1.31	12.0	4.8
1	G4	5.0	3.42	21.9	10.6
	G5	23.2	20.56	41.9	32.0
	G6	21.5	14.40	17.1	12.0
	G7	24.0	50.22	42.0	23.3
	Mean	**18.4 ± 3.9^{a}**	**27.3 ± 11.8^{a}**	**34.2 ± 7.0^{a}**	**17.2 ± 3.4^{a}**
	B1	12.2	3.18	52.2	10.6
	B2	3.2	2.11	11.9	5.1
	B3	14.2	4.81	10.4	7.0
2	B4	9.5	3.74	22.5	7.1
	B5	8.7	n.a.	16.3	14.6
	B6	5.5	2.00	52.0	5.8
	B7	21.2	5.47	16.1	5.3
	Mean	**10.6 ± 2.2^{ab}**	**3.55 ± 0.57^{b}**	**25.9 ± 6.9^{a}**	**7.9 ± 1.3^{b}**
	G8	9.7	3.49	5.8	33.4
	G9	17.5	24.55	34.4	26.1
	G10	4.2	3.47	49.8	17.6
3	G11	7.7	6.43	42.1	22.8
	G12	18.7	17.20	63.8	29.3
	G13	5.0	2.46	24.5	20.1
	G14	8.5	5.46	18.6	44.5
	Mean	**10.2 ± 2.2^{ab}**	**9.01 ± 3.21^{a}**	**34.1 ± 7.4^{a}**	**28.4 ± 3.7^{ac}**
	B8	5.0	3.38	25.1	25.1
	B9	5.2	7.02	38.3	35.3
	B10	6.5	8.09	42.4	19.3
4	B11	22.2	3.11	41.4	48.0
	B12	13.0	7.23	27.6	12.6
	B13	15.0	9.20	45.6	25.1
	B14	1.2	7.97	61.8	47.1
	Mean	**9.7 ± 2.8^{ab}**	**6.57 ± 0.90^{ab}**	**40.3 ± 4.6^{a}**	**30.3 ± 5.1^{ac}**
	G15	16.2	7.12	27.4	35.0
	G16	6.5	4.05	54.3	28.3
	G17	21.0	9.04	60.0	39.6
5	G18	13.2	1.18	26.3	27.8
	G19	14.0	8.91	30.6	30.5
	G20	26.7	24.09	48.3	42.3
	G21	9.0	10.81	30.7	20.1
	Mean	**15.2 ± 2.6^{a}**	**9.31 ± 2.76^{ab}**	**39.7 ± 5.3^{a}**	**31.9 ± 2.9^{c}**
	B15	11.5	12.30	56.5	30.8
	B16	4.2	3.57	37.6	27.6
	B17	2.0	2.03	48.1	26.6
6	B18	4.0	12.89	16.8	34.0
	B19	9.2	3.24	31.7	32.5
	B20	4.5	6.26	17.9	21.0
	B21	8.2	4.86	32.7	20.8
	Mean	**6.2 ± 1.3^{b}**	**6.45 ± 1.66^{ab}**	**34.5 ± 5.5^{a}**	**27.6 ± 2.0^{c}**

Figures with different superscripts are significantly different ($p<0.05$).

Table 6.5. Mucosal cell counts, SMCP concentrations and bone marrow eosinophil numbers of group 7.

Group	Lamb no.	Mast cells (cells/VCU*)	SMCP ($\mu g\ g^{-1}$ tissue)	Eosinophils (cells/VCU*)	Bone-marrow eosinophils (%)
	R1	5.2	0.65	18.8	17.1
7a	R2	5.7	5.82	12.9	14.3
	R3	2.7	0.80	42.9	33.5
	Mean	**4.5 ± 0.9^{a}**	**2.42 ± 1.70^{ab}**	**24.9 ± 9.2^{a}**	**21.6 ± 6.0^{a}**
	R4	8.0	2.91	26.5	15.0
7b	R5	7.2	2.88	24.5	10.8
	R6	5.2	1.13	22.5	5.0
	Mean	**6.8 ± 0.8^{a}**	**2.31 ± 0.59^{a}**	**24.5 ± 1.1^{a}**	**10.3 ± 2.9^{a}**
	R7	31.2	51.16	32.2	30.8
7c	R8	3.7	2.21	14.9	21.5
	R9	19.0	24.44	40.1	36.1
	Mean	**18.0 ± 8.0^{ab}**	**25.9 ± 14.2^{ab}**	**29.1 ± 7.4^{a}**	**29.5 ± 4.3^{a}**
	R10	16.0	8.54	27.8	19.8
7d	R11	24.0	14.37	36.6	32.5
	R12	14.0	4.18	20.3	26.0
	Mean	**18.0 ± 3.1^{b}**	**9.03 ± 2.95^{b}**	**28.2 ± 4.7^{a}**	**26.1 ± 3.7^{a}**

* *villus crypt unit*
Figures with different superscripts are significantly different ($p<0.05$).

6.4 Discussion

The results of this experiment suggest that acquired immunity towards *N.battus* is maintained for up to at least 12 weeks without further antigenic stimulus. The expression of immunity is principally manifested in the ability of lambs to retard larval growth and development and to a lesser extent by exclusion/expulsion of incoming larvae.

Analysis of variance showed that previous infection caused a significant reduction in worm burdens, although comparisons between group means did not differ significantly. However, the total worm burdens of previously infected groups

(1, 3 & 5) were 34%, 10% and 32% fewer than that of their respective controls. These differences, when viewed from a point of percentage establishment reveal hidden information which may be important in describing events over time. Although group 1 had 34% fewer worms than group 2 this difference was reduced after 5 weeks to just 10%. This finding may be a result of partial waning of immune effector mechanisms. However, after 6 weeks the reduction in establishment was more marked (32%). This could possibly be explained by the combination of previously acquired immunity and the so called 'age-resistance' phenomenon. Furthermore, a preferential rejection of male worms was evident, an occurrence which has been described among *T.colubriformis* populations in lambs that were vaccinated and allowed to acquire a natural infection from grazing (Douch, 1989). The reason for this preferential rejection remains obscure and may be of a complicated molecular nature. One may postulate that the hormonal and/or size differences between males and females render the males more prone to immunological mechanisms.

The significantly lower burdens of both groups 5 and 6 at week 12 PT could be due to two possibilities. The development of an innate resistance with age or a low viability of infective larvae. The larvae used for challenge were from the same batch and the longer period in culture may have affected viability. However, age-related resistance has been demonstrated as early as 3 months of age in *N.battus* infections (Gibson and Everrett, 1963; Taylor and Thomas, 1986). Although worm burdens were comparatively high in this experiment the prominent feature was the degree of retardation as a result of priming with the primary infection. This effect was evident after all challenges, although not significant between groups 3 and 4, a well marked trend existed and this form of expression of immunity was demonstrated in both

worm sexes. Again as with worm burdens, the differences in percentage of L_4 was not significant between group 3 and 4 which further strengthens the argument that partial waning of immunity existed after 6 weeks without antigenic stimulus.

The prominent feature of this model infection is the kinetics of the eosinophil response. In contrast to other economically important ovine gut nematodes there was no indication of a peripheral response, at least during primary infection. However, there is evidence that previous infection and time both had a significant effect on stimulating the production of eosinophils in the bone marrow. Unfortunately this was not reflected in the gut tissue. A further complication is the fact that pre-challenge bone marrow and tissue eosinophil numbers were similar irrespective of infection and time. It is possible that responses, whenever they occurred, were maintained since the termination of the primary infection.

The mast cell responses followed a similar pattern to that of the eosinophils. Both mast cell numbers and SMCP concentrations were significantly enhanced by previous infection but did not change with time. The large variation in the data complicates the inferences made but in general do suggest the possible involvement of these cells. As with the worm burdens, the mast cells also exhibited a trend for increased numbers in group 1 compared to group 2 but after 5 weeks this trend was not evident since numbers in groups 3 and 4 were very similar. However, towards the end of the experiment (week 12 PT) the differences became apparent again and were significant. These findings fit well with that of worm regulation and can possibly be explained by partial waning of immunity followed by the involvement of age resistance.

Antibodies towards both developmental stages were being produced after a prolonged period (42 days). However, the significance of these findings in terms of worm regulation remains to be established. The moderate responses observed in comparison to those described in infections with *H.contortus* (Gill *et al.*, 1993b; Schallig *et al.*, 1994) may depend on various factors which differ between experiments. The total worm burden, the worms' feeding habits and level of tissue penetration, and the parasitized organ involved all may account for these differences. *N.battus* does not penetrate deeply into mucosal tissue and as a result of this 'loose' contact the uptake of antigens is likely to be more restricted. This may not affect only the humoral response but also may be a cause for the less pronounced inflammatory response observed.

It is evident from this study that the inflammatory response, although in some cases significant, was not a salient feature of the developing immunity in this model. It is important to note that various factors such as the level and duration of primary and challenge infections may have a profound effect upon the outcome of the experiment. Indeed density dependent regulation has been described in *N.battus* infections (Coop *et al.*, 1988). Cellular responses cannot be compared directly to studies conducted on other ovine nematodoses since they may vary between parasite species and strains.

In general *N.battus* did not induce severe pathology at the level of infection used in this study. Studies by Thomas (1959) have shown that lambs can be infected with 50,000 L_3 without any apparent ill effects. The mucosal browsing nature of *N.battus* excludes close contact between cellular and humoral components which

may also be responsible for the very mild responses noted. Additionally, it is possible that the challenge infection was insufficient to cause an amplified response. Studies on lymph cell traffic of sheep infected with *O.circumcincta* have shown that a single challenge was insufficient to invoke a cellular or humoral response whereas continuous challenge with 2000 L_3 day^{-1} resulted in increased lymphocyte traffic and maintained raised antibody levels (Smith *et al.*, 1984a; 1987).

Based on these arguments it is tempting to suggest alternative effector cells/molecules of more direct involvement in the acquisition of immunity. It is well known that IgA cells proliferate and secrete antibody in increased amounts during infections with *H.contortus* (Gill *et al.*, 1994), *O.circumcincta* (Smith *et al.*, 1983c, 1984a), and *T.colubriformis* (Adams *et al.*, 1980). In fact IgA-containing cells have been demonstrated to be in preponderence where *N.battus* populations are abundant (Sinclair *et al.*, 1985). Similarly, IgG, especially IgG1, has been shown to be involved in the local mucosal response and may be involved in the regulation of *N.battus* since anti-worm specific IgG was shown to increase during primary infection (Figures 6.3 and 6.4). Furthermore, inflammatory mediators, cytokines, hormones and/or physiological changes in the gut may also play an important role in the complex mechanisms involved in developing immunity.

In conclusion, despite the small differences between previously infected and challenge control lambs, it is evident that acquired immunity does operate and is maintained up to 12 weeks PT in this model. The results have generated further questions on the regulation of this parasite which provoke further experimentation in order to determine factors more directly related to worm regulation.

CHAPTER 7
Sero-epidemiology of field infections

7.1 Introduction

Previous chapters have described aspects of immunity towards *N.battus* by adopting a model experimental infection. Although this model has generated data which has extended the understanding of the immune response to *N.battus*, this may vary under field situations, especially since lambs will be faced with a much larger infective dose of larvae and a more pathogenic infection. Lambs may well have to respond more vigorously in order to survive the high level of larval challenge.

Epidemiological studies on *N.battus* infections in sheep have been conducted by various workers (Thomas, 1959; Boag and Thomas, 1975; Mitchell *et al.*, 1985) and the general pattern of infection described involves a massive spring hatch of larvae derived from over-wintered eggs deposited by the previous years' lamb crop. Young naive lambs will be infected with these L_3 and suffer varying degrees of infection depending on the level of pasture contamination and their intake of herbage. The contamination is usually high since the hatch is synchronized due to sudden changes in ambient temperature. Under field conditions lambs show a characteristic rapid peak and fall in FEC over the first 6 to 8 weeks of grazing. The fall in FEC is the consequence of developing immunity which not only expels worms but also depresses female fecundity (Taylor and Thomas, 1986). It has been demonstrated also that ewes play a negligible role in the transmission of disease since they produce insignificant numbers of eggs even during the periparturient period. This is presumed

to be the consequence of the acquisition of a very effective and prolonged immune response among older pre-exposed ewes (Bairden and Armour, 1987).

With these facts in mind and since an ELISA for the measurement of serum antibodies had been developed, the purpose of the experiments in this chapter was to characterize the serological response of grazing ewes and their lambs over a 2 year grazing period with the aim of determining whether serological responses differed between ewes and lambs and whether this could be correlated to developing immunity. It was also of interest to determine whether the use of serum fructosamine concentration as a general index of gastrointestinal damage (Heath and Connan, 1991) would be applicable in monitoring infections with *N.battus* which, in contrast to some of the more common ovine nematodes, does not cause extensive tissue damage. The findings of these grazing experiments will provide a comparison for the model infection used in earlier chapters since the strain used in these indoor experiments was isolated from the farm on which these grazing trials were conducted.

7.2 Materials and Methods

7.2.1 Sheep and their management

The experiments were conducted on the institute farm (Firth Mains, 14 kilometres south east of Edinburgh, elevation about 230 m). The sheep sampled were Greyface × Suffolk ewes with twin lambs which grazed a permanent pasture (originally sown with a mixture containing 60% perennial rye grass, 10% timothy and cocksfoot and 5% red and white clover). This pasture had been grazed with

sheep for several years prior to the study and, therefore, contained infective stages of *N.battus*

7.2.2 Experimental designs

Experiment 1 (1992)

Twenty-one ewes with 36 lambs were grazed between five adjacent 0.9 ha paddocks (P6, P7, P8, P9 and P10) from April to July to ensure a uniform contamination with *N.battus* eggs. The flock was moved from paddock to paddock after 2 weeks of grazing. The lambs were born in February and were reared indoors with the ewes until turn out in April.

Experiment 2 (1993)

Sixty-two ewes with 119 lambs were divided into 5 groups based on sex and weight and each group was assigned to a 0.9 ha paddock (same paddocks used in 1992). The animals remained permanently on each paddock over the grazing season. The reason for grouping the animals was because each group was to be treated with a different anthelmintic throughout the grazing period. This was part of a separate ongoing research project on anthelmintic resistance in *Ostertagia* infections. However, the treatments given were equally effective against *N.battus* and therefore, for the purpose of this study, the data collected from the ewes and lambs were pooled. The lambs were born in February and had been reared indoors with the ewes from birth. At 2 months of age the lambs were allowed to graze alongside the ewes.

7.2.3 Sampling

During experiment 1 (1992) the sheep were sampled weekly commencing on the 5th May until 16th June. Samples taken included individual faeces for FEC and blood for determining antibody titres. Grass for pasture larval counts was collected fortnightly. Experiment 2 (1993) was conducted throughout the period from 5th May until 14th December. Faecal samples were collected weekly but are plotted in the results section at monthly intervals since anthelmintic treatment reduced the counts significantly. Blood was collected monthly and grass samples fortnightly. The results from the 5 groups were pooled. Blood samples were taken randomly from 30 lambs and their dams (20 ewes).

7.2.4 Serum fructosamine assay

The assay was performed according to the manufacturer's instructions (Raichem Fructosamine test kit; Bio-stat diagnostics). A mixture of 10 µl of sample pre-treatment reagent and 100 µl of serum or reconstituted calibrator was incubated at 22°C for 30 minutes. Absorbance was measured using a centrifugal analyser (Monarch). The analyser was zeroed at 500 nm using distilled water. All incubations and dilutions were carried out automatically using the analyser with code 635 for test assays and 636 for control assays. Absorbances were calculated by subtraction of the control values from the test values. The concentration of fructosamine was then calculated using the following formula:

$$\frac{\textit{sample absorbance}}{\textit{calibrator absorbance}} \times \textit{sample absorbance} = \textit{fructosamine concentration } (mmolL^{-1})$$

7.2.5 Statistical analysis

Data were $\log_{10}$-transformed prior to analysis. Differences between means were compared using a 2 sample t-test. One way ANOVA was used to compare serum fructosamine concentrations between weeks.

7.3 Results

7.3.1 Experiment 1 (1992)

Analysis of herbage samples from paddocks P6, P7 and P10 showed no detectable numbers of *N.battus* L_3. Paddocks P8 and P9 contained moderate to high numbers of L_3 (Figure 7.1). P8 had much higher numbers of L_3 (13,227 L_3) compared to P9 (6780 L_3) on the 16th April. Numbers of larvae decreased to very low levels (P8, 313; P9, 416) by the end of the sampling period in mid June.

Figure 7.2 shows the FEC of both lambs and ewes. Ewes produced negligible numbers of eggs in their faeces whereas the lambs had high FEC on the first day of sampling and the counts rose to a peak 3 weeks later followed by a rapid decline. The mean count at the end of the sampling period (16 June) was still significantly higher than that of the ewes ($p<0.001$).

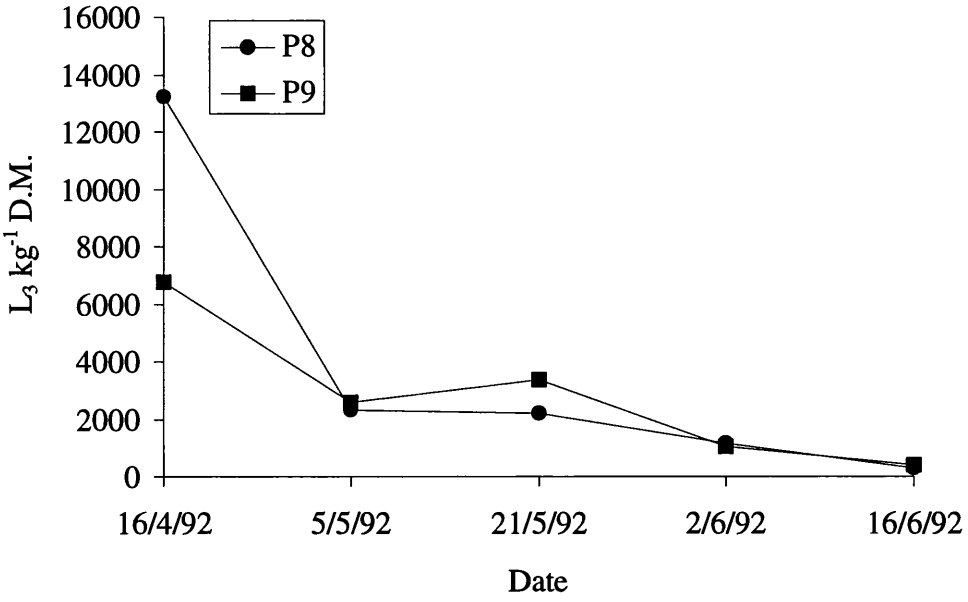

Figure 7.1. *Pasture larval counts for experiment 1 (1992).*

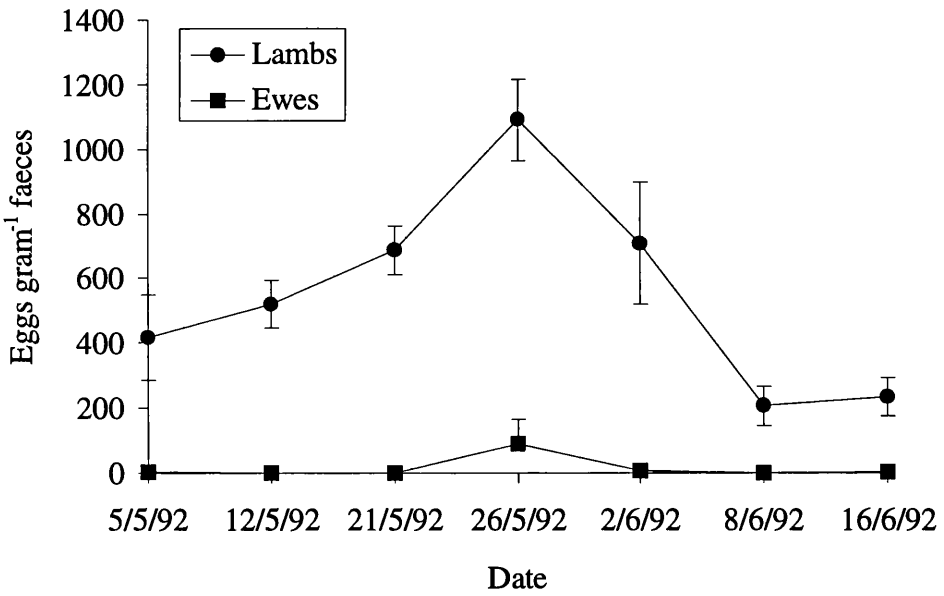

Figure 7.2. *Faecal egg counts of lambs and ewes during experiment 1 (1992).*

Figures 7.3 and 7.4 show the anti-L_4 and anti-adult worm IgG titres respectively. Throughout the grazing period lambs developed increasing antibody titres. However, these titres were still significantly lower in comparison to that of the

ewes. The antibody titres of the ewes remained fairly constant throughout the sampling period.

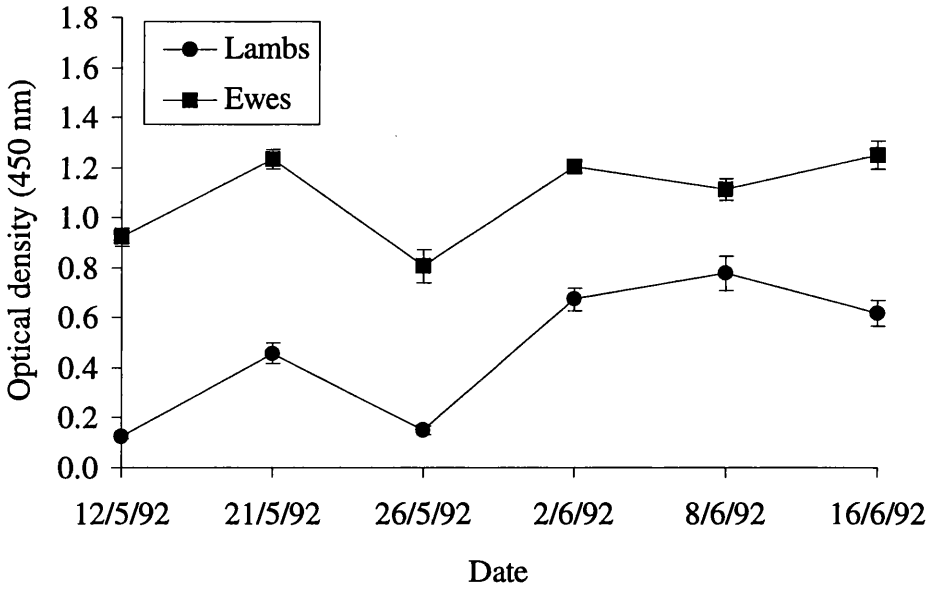

Figure 7.3. *Anti-L_4 IgG titres of lambs and ewes in 1992.*

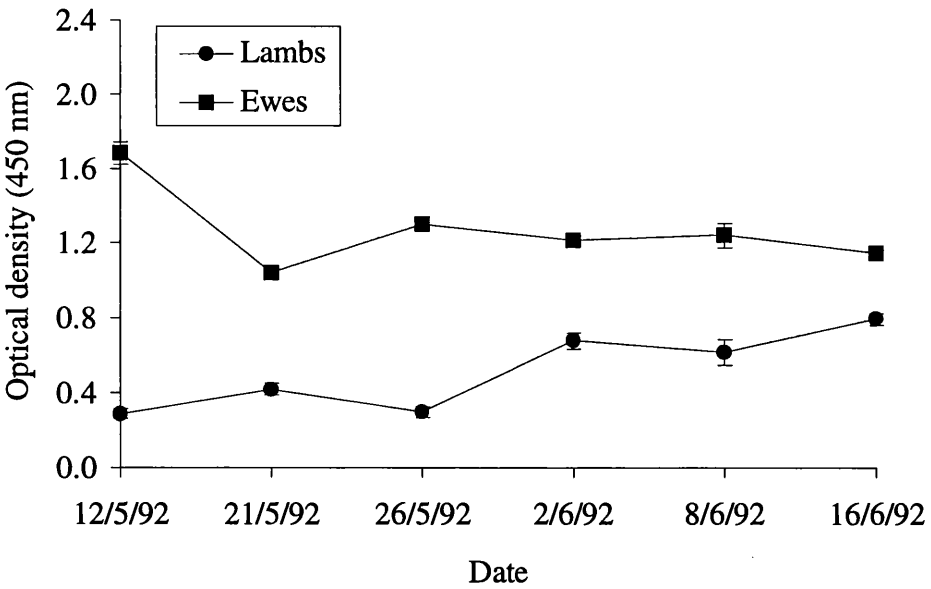

Figure 7.4. *Anti-adult worm IgG titres of lambs and ewes in 1992.*

7.3.2 Experiment 2 (1993)

Mean pasture larval counts from the five paddocks were pooled and are plotted in Figure 7.5. Considerable variation was evident on the first two sampling dates afterwhich larval counts declined abruptly with little variation between paddocks.

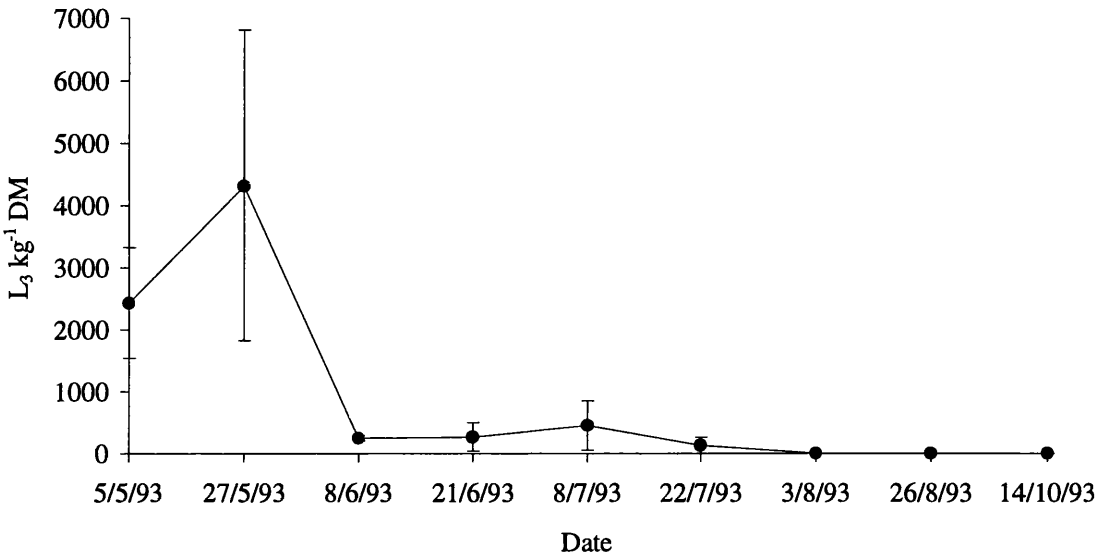

Figure 7.5. *Mean pasture larval counts for experiment 2 (1993).*

Faecal egg counts of the total population are shown in Figure 7.6. The mean counts on the 5th May were relatively high afterwhich they declined rapidly. This was the result of the anthelmintic treatment given on 19 May. A similar trend was observed when the FEC of randomly chosen lambs and ewes were plotted (Figure 7.7). The counts of the total and selected populations did not differ at any point in time. This enabled any pattern observed in the serological response to be considered as representative of the total population.

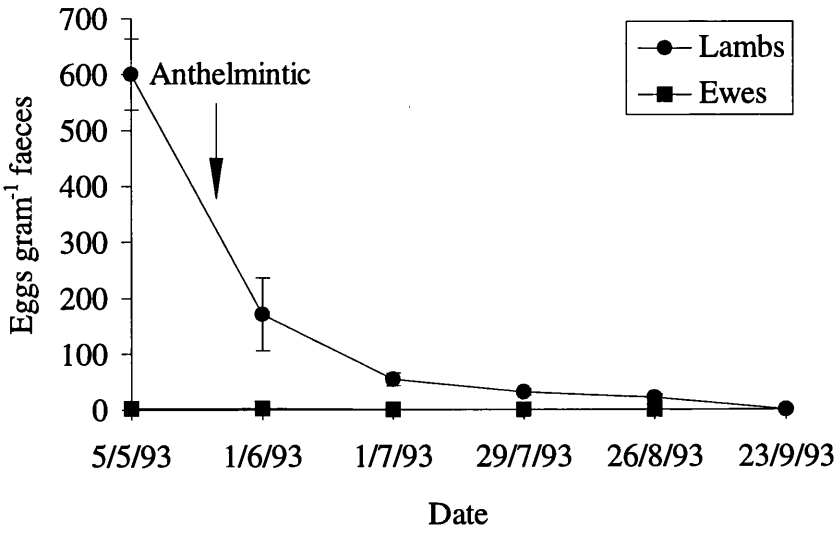

Figure 7.6. FEC of the total population (1993).

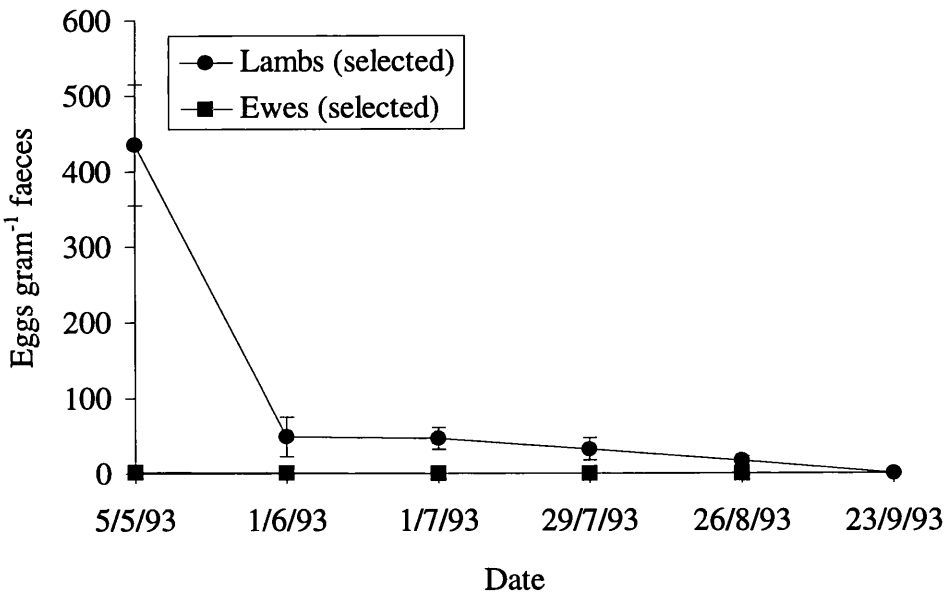

Figure 7.7. FEC of the selected population (1993).

Antibody titres of the selected sheep are shown in figures 7.8 and 7.9. The anti-L_4 titres were higher compared to that observed in the previous year. However, the ewes still had significantly higher titres than the lambs. Similarly, anti-adult worm

titres followed the same pattern with significant differences between ewes and lambs. Anti-adult worm antibody titres of the ewes were always significantly higher than those of the lambs.

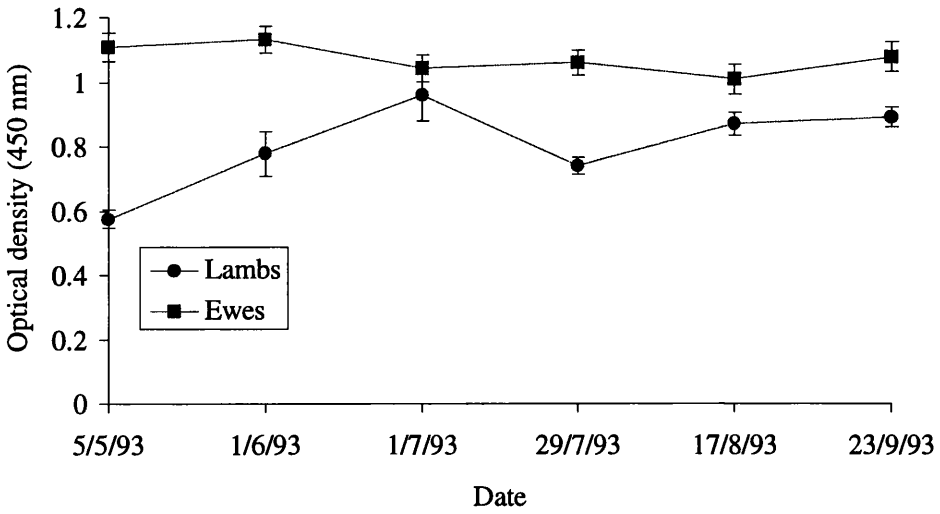

Figure 7.8. *Anti-L_4 IgG titres of lambs and ewes in 1993.*

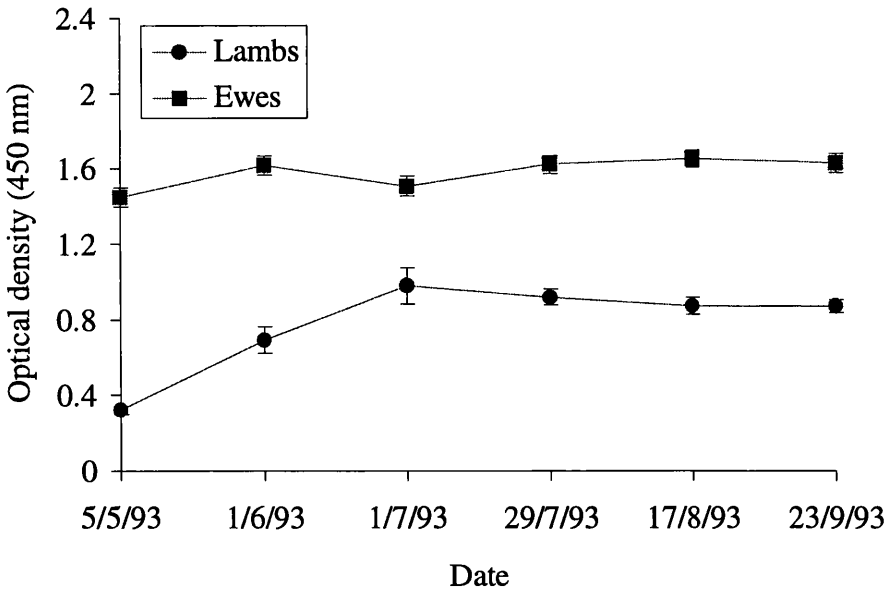

Figure 7.9. *Anti-adult worm IgG titres of lambs and ewes in 1993.*

Serum fructosamine concentrations in the selected lambs are plotted in Figure 7.10. There was no indication of a reduction in concentrations throughout the sampling period and comparisons between samples of adjacent weeks did not differ significantly.

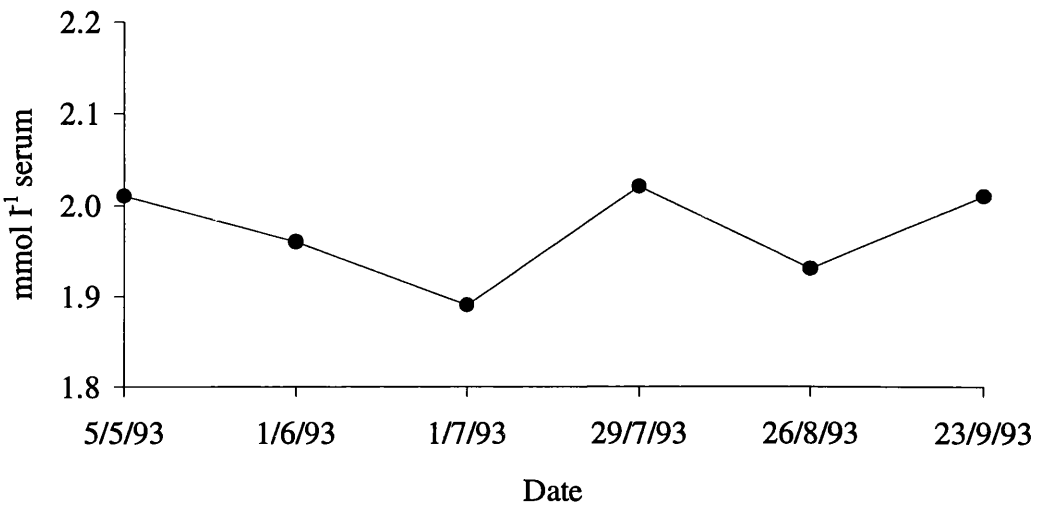

Figure 7.10. *Serum fructosamine concentrations of selected lambs.*

7.4 Discussion

The important findings in this study are a) the demonstration that an antibody response accompanies *N.battus* infection and is acquired through natural exposure and b) that the apparent immunity among ewes was manifested by higher antibody titres compared to lambs . It is also evident that a feature of developing immunity in lambs was their ability to mount a serological response which increased with the duration of exposure. However, this response was still significantly lower in comparison to that of the ewes. The importance of serum IgG in the regulation of *N.battus* is yet to be determined, however, the results from experiment 1 show that

the FEC started to decline in lambs once the antibody levels reached a peak. This observation was not able to be demonstrated in experiment 2 since the worms were removed by anthelmintic treatment before antibody levels had reached a peak.

The results from these observations strengthen the conclusion that the ewe plays a negligible role in the epidemiology of nematodirosis. This was explained not only by the negligible FEC during both years but also by the elevated antibody titres compared to those of the lambs. It is also clear that periparturient relaxation in immunity did not provide favourable conditions in the gut for *N.battus* larvae to establish and did not result in suppression of the antibody response. This suggestion is supported by the fact that the ewes in this study were grazing with their lambs which were still suckling. Interestingly, there was no indication of passive maternal transfer of antibodies since titres were very low in the young lambs at the start of the sampling period and only increased with time. A possible explanation could be due to the fact that the lambs were three months of age and had been grazing for one month before blood samples were obtained and therefore, catabolism of passively acquired maternal antibodies may have occured. Watson and Gill (1991) have shown that Merino lambs exhibited declining serum antibodies to *H.contortus* and *T.colubriformis* over the first 10 to 12 weeks of age and suggested that their findings were a feature of catabolism of maternal antibodies.

The negligible *N.battus* FEC of ewes is well documented and has been suggested to be the result of a solid immunity (Gibson, 1974). If this is the case then the immunogenicity of *N.battus* antigens could possibly be very effective since exposure to high levels of these antigens is restricted to a relatively short period of the grazing season. The maintenance of elevated antibody titres, despite zero pasture

larval counts from August onwards in experiment 2, can be explained by the fact that the pasture larval count technique is not a sensitive and accurate measure of larval abundance and may be underestimating the actual level of contamination. Therefore, it is possible that small numbers of L_3 persisted into the autumn and provided the antigenic stimulus required to stimulate a response and maintain elevated antibody titres. *Nematodirus* L_3 are very resistant to harsh conditions and have been shown to survive over prolonged periods of unfavourable weather conditions (Boag and Thomas, 1975). This characterisitc may enable sufficient numbers of *N.battus* to persist and stimulate a response in previously exposed animals.

An alternative explanation for the failure of *N.battus* to establish in ewes and the relatively short duration of infection in lambs could be a purely physiological effect. It is possible that a mature gut may lack vital elements pertinent for the successful establishment of *N.battus*. Physiological changes occur in the gut of young lambs as they start to consume solid food and it is possible that these changes are responsible for what is believed to be, developing immunity in lambs and solid immunity amongst ewes. Such a hypothesis can only be proven by exposing naive ewes to *N.battus*.

The finding that serum fructosamine concentrations were not reliable in monitoring clinical nematodiriasis has extended the work by Heath and Connan (1991) on the potential use of serum fructosamine concentrations in monitoring gastrointestinal parasitism. These workers showed that in experimental infections with *O.circumcincta* and *N.spathiger*, serum fructosamine was of value in monitoring sub-clinical concurrent infections and argued that in single species infections, lesions are less extensive and the scope for compensatory mechanisms for

the re-absorption of lost plasma proteins is greater. The results in experiment 2 show that under field conditions heavy clinical infections with *N.battus* in the months of May and June were accompanied by severe diarrhoea and lighter infections with other strongyles. One would expect under such conditions that lambs would have declining serum fructosamine concentrations due to the severity of infection. However, *Nematodirus* may reduce the absorptive capacity of intestinal villi with little tissue damage and little plasma protein loss and this may be why fructosamine concentrations were not altered.

CHAPTER 8
General discussion and conclusions

8.1 General discussion

Throughout the course of this research project the experiments conducted have produced valuable data which describes some elements of the humoral and cellular responses of young lambs to experimental and natural infections with *N.battus*. In general, the responses observed were similar to those described in other common ovine gastrointestinal nematode infections, although, the magnitude was of a lesser degree. It is clear that immunity to *N.battus* is a combination of acquired and age immunity and is expressed as both retardation and expulsion of worms. This discussion will attempt to link the findings in previous chapters and discuss the possible mechanisms involved.

The experiments described in previous chapters have concentrated on the local inflammatory responses, peripheral blood eosinophil and antibody kinetics. Since there is extremely limited availability of data concerning the immune response to *N.battus*, the first objective of this research project was to accumulate baseline data regarding this aspect. To date, the crucial effector phase in immunity to ovine gastrointestinal parasitism has been described to be the non-specific local inflammatory response in the gut (Dineen *et al.*, 1977). This is characterised by mastocytosis, SMCP secretion, degranulation and differentiation of MMC into GL and, local and peripheral eosinophilia. With this in mind a decision was taken to investigate these components of the immune response in *N.battus* infections.

In general, the results show that a similar response to that described in other ovine gastrointestinal nematode infections, is generated by infection with *N.battus*, albeit to a lesser degree. However, an interesting finding which was consistent throughout the series of experiments was the very low peripheral blood eosinophil counts in spite of elevated tissue numbers in previously infected and challenged lambs. Even though at some time during or after trickle infection there were peaks, these were relatively low in comparison to results obtained with other common ovine nematodoses (Buddle *et al.*, 1992, Stevenson *et al.*, 1994). Similar findings have been described by colleagues working with single *N.battus* infections in lambs (M.Winter and D.Lee, unpublished data).

This finding is surprising since there is evidence that 'messages' were being received at the bone marrow level as indicated by the increased numbers of bone marrow eosinophils, however these cells failed to enter the peripheral circulation. It was also evident that lambs were capable of mounting a local (mucosal) eosinophilia despite the absence of a peripheral response. One possible explanation for this finding is that increased tissue numbers were a consequence of local mobilisation of neighbouring eosinophils in the gut. It is possible that an eosinophil chemotactic factor (ECF) of either worm or mast cell origin was involved. ECF has been demonstrated in soluble extracts from helminths such as *O.ostertagi* (Klesius *et al.*, 1986, 1989), *Anisakis* (Tanaka and Torisu, 1978), *Ascaris* (Tanaka *et al.*, 1979) and *Metastrongylus apri* (Sasaki and Katsumo, 1983). Mast cells secrete ECF-A (Lewis and Austen, 1981) and PAF (Braquet and Rola-Pleszczynski, 1987) which stimulate eosinophil localisation, and T-cells may mediate stimulation of eosinophil chemotaxis (Wakelin and Donachie, 1983).

The absence of peripheral eosinophilia could be a feature of the lack of responsiveness frequently associated with young lambs. Additionally, it is possible that chronic infection with *N.battus* is needed in order to stimulate peripheral eosinophilia. It has been shown that significant increases in peripheral blood eosinophil counts (above baseline levels) in *T.colubriformis*-infected lambs only occurred after chronic infection over 70 days (van Houtert *et al.*, 1995).

Although mast cell responses were elevated as a consequence of previous exposure, the levels of SMCP did not correlate well with mast cell activity. This finding is in contrast to that found in *H.contortus* infections (Huntley *et al.*, 1987). However, direct comparisons are complicated by the fact that the parasite species, dose and duration of infection varied considerably between these studies. The GL response was extremely low in comparison to other studies using *T.colubriformis* (Douch *et al.*, 1986) and *H.contortus* (Huntley, Newlands, Jackson and Miller, 1992) or in concurrent field infections with several species of nematode (Douch, 1989) and in some experiments (chapter 6) were not observed at all.

The mast cell response is dynamic and would be ideally studied by serial kill experiments. However, since the aim of this research project was of a more general nature such an approach was not pursued. Additionally, such studies would require large numbers of animals. Nevertheless, the results described in this thesis may form a base on which future work can be conducted.

The studies have characterised the inflammatory response of the model adopted and in general suggest that the inflammatory response is an integral component in the

regulation of *N.battus* populations. However, whether this response is essential in the expulsion and retardation of *N.battus* remains unanswered.

The finding that lambs were capable of producing anti-worm IgG in experimental and field infections is not surprising since administration of most foreign bodies would evoke such a response. Whether this response is essential to worm expulsion/retardation is unclear, but from the field study it is clear that once antibody levels in lambs had increased, there was a drastic reduction in FEC. Studies on the involvement of serum antibodies in ovine nematode infections have been dominated by work on *H.contortus* with conflicting results. While increased antibody levels have been found by some workers in primary and hyperinfections (Smith, 1977ab; Gill, 1991; Schallig *et al.*, 1994) others failed to demonstrate any differences (Duncan *et al.*, 1978; Charley-Poulain *et al.*, 1984; Cuquerella, Gomez-Munoz and Alunda, 1991).

The ELISA developed for the quantification of serum IgG levels has shown acceptable levels of reactivity. It clearly demonstrated that young lambs do not respond serologically as strongly as ewes, either in housed experiments or at pasture. Other workers have described poor reactivity of ELISA using somatic antigens. Charley-Poulain *et al.* (1984) suggested that poor immunogenicity of worm components or complexing of antibodies with circulating antigens may be responsible for weak increases in anti-*H.contortus* antibodies of previously infected lambs. Cuquerella *et al.* (1991) suggested that low antigenicity of soluble proteins compared with surface and excretory/secretory products could be a possible reason why their ELISA reacted poorly. The difficulty in assaying the antibody response against parasite antigens lies in the fact that each antigen preparation is a complex cocktail with a large variety of molecules. The

titre measured by the ELISA test represents the combined activities of antibodies against different antigens. This problem could be resolved by purification of specific antigens. Nevertheless, in view of the differences in antibody levels between ewes and lambs in the field experiments, and the significant elevation in levels in housed experiments, the ELISA developed was successful in detection of changes in serum anti-*N.battus* IgG levels.

It was evident from the field experiments that even when lambs were immune to *N.battus* infections (as judged by FEC) their antibody levels were still significantly lower than the ewes. This finding and those from the dietary experiments suggest that the peripheral antibody response to infection with *N.battus* in young lambs is slow to develop and significantly lower in comparison to that of ewes. Thus this response is either not an essential component of the expulsive mechanisms or possesses a threshold level which, once reached, is equally effective in the retardation/expulsion of *N.battus*. Personally, the first option is more favourable since studies on *N.battus* (Sinclair *et al.*, 1985), *O.circumcincta* (Smith *et al.*, 1983c, 1984a; Smith *et al.*, 1985), *T.colubriformis* (Adams *et al.* 1980) and *H.contortus* (Gill *et al.* 1992; Gill *et al.*, 1994) infections have suggested an effector role for local as opposed to systemic antibodies. Indeed, an investigation into the role of local antibodies and mucus in the development of immunity to *N.battus* was planned but, due to the limited time available these experiments were unable to be conducted.

The dietary experiments described in chapters 4 and 5 showed that supplementation with fish meal as a source of by-pass protein was of little benefit in enhancing the acquisition of immunity. These findings are in direct contrast to similar

work with lambs of the same age group and breed that were infected and challenged with *O.circumcincta*. These lambs were offered the same diets as described in chapter 5 which resulted in significant enhancement of acquired immunity among previously infected supplemented lambs (R.L.Coop, unpublished results). In this experiment supplementation significantly enhanced immunity as assessed by a reduction of the size and length of a challenge infection. Similar studies investigating the effect of protein supplementation upon developing immunity to *T.colubriformis* in sheep have shown significant enhancement of worm regulation and immune responses (Bown *et al.*, 1991; Kambara *et al.*, 1993; van Houtert *et al.*, 1995). Kambara *et al.* (1993) and Abbott and Holmes (1990) described significant enhancement of immunity to *T.colubriformis* and *H.contortus* by supplementation of a deficient diet. A possible reason for the inability to enhance the responses to *N.battus* could be due to the fact that the non-supplemented groups had an adequate level of protein in the diet (132 and 125 g CP kg^{-1} DM) as opposed to the experiments described above in which supplemented diets were compared with deficient diets.

The reasoning for the use of a by-pass protein in the experiments described in chapters 4 and 5 stemmed from the findings of Bown *et al.* (1991) and Coop *et al.* (1995), whereby post-ruminal infusion of casein accelerated the development of immunity to *T.colubriformis* and *O.circumcincta* infections respectively. Bown *et al.* (1991) observed inferior nitrogen balances of sheep with pathological damage to the small intestine as a result of infection with *T.colubriformis* and suggested this to be a consequence of increased endogenous protein loss into the gastrointestinal tract. This effect was reversed when protein arriving at the small intestine was supplemented by an amount calculated to be equivalent to that of the irreversible endogenous loss. Since

endogenous protein loss was not calculated in the experiments described in chapters 4 and 5 it is difficult to comment on this aspect. However, it is known that the pathological consequences of infection with *N.battus* are relatively mild compared to that of *T.colubriformis*. It is possible that the mild infection regime imposed upon the lambs did not inflict severe intestinal damage and significant losses of endogenous protein. It is also possible that if there were endogenous losses, they may have been minimal and efficiently reabsorbed before the terminal ileum which was free of infection and presumably fully functional. This suggestion is based on the findings that liveweight gain did not differ either as a result of previous infection or by-pass supplementation.

Rumen protein losses are a common physiological process (McAllan, 1991) and where animals are fed poor quality forage crops, these processes may limit the potential availability of dietary proteins and incur production and disease penalties. Increasing protein supply at the duodenum of animals receiving low quality diets have resulted in improved production responses and occasionally improved intake and rapid acquisition of immunity towards gastrointestinal parasitism.

The use of fish meal as a by-pass protein is expensive and was used for its known capability of being protected from ruminal degradation. However, economic considerations would probably rule out supplying protein post-ruminally by means of by-pass proteins. A more realistic approach would be to increase the duodenal protein supply by dietary protein supplementation or the addition of a non-protein nitrogen source such as urea.

Recently, van Houtert *et al.* (1995) have used fish meal in similar studies on *T.colubriformis*-infected lambs and showed significant improvement in production and

immunity. It is difficult to compare results with this study, however, it is possible that the differences between supplemented and non-supplemented groups in chapters 4 and 5 were not sufficient to produce desirable effects. Although supplementation could have been increased to high levels, this would be impractical, hence the reason why this option was not investigated.

Although the investigation on the persistence of immunity to *N.battus* described in chapter 6 was not designed to examine the role of dietary components, the results suggest a possible effect of diet upon the responses which were quantified. This experiment followed the standard trickle infection and challenge regime using young lambs which was similar to that described in the dietary experiments. However, the major difference was that the animals used in this experiment were offered a diet which was sufficient only for maintenance purposes. The results clearly indicate that these lambs were less able to regulate their worm burdens since they were extremely high in comparison to those obtained in the dietary experiments. Similar findings are described in chapter 3 where a maintenance ration was used. Additionally the inflammatory response of lambs was of a lesser magnitude to that observed in chapters 4 and 5 and this finding may also be due to inadequate nutrition.

It is possible that a maintenance diet which allowed for only small increments in growth was inadequate to enable the lambs to respond more vigorously due to competition between developing immunity and production requirements. Experiments conducted with *T.colubriformis*-infected sheep have suggested that there is competition between developing immunity and production for limited physiological resources (Wagland *et al.*, 1984; Steel, Wagland and Dineen, 1985; van Houtert *et al.*, 1995). This

competition was also described by Kambara *et al.* (1993) who demonstrated significant depression of liveweight gain among young, protein-deficient *T.colubriformis*-infected lambs. However, despite the low level of dietary constituents offered to the lambs in chapter 6 these lambs were still able to show some degree of responsiveness in terms of retardation of larval growth. This persisted throughout the period of study.

The overall finding that young lambs were able to mount significant responses in the regulation of their worm burdens is in direct contrast to the general statement that lambs below 6 months of age are un-responsive to gastrointestinal parasitism. Could it be that *N.battus* possesses a highly immunogenic component? This suggestion is speculative but forms a relevant question in the approach to immunological control of gastrointestinal parasitism. If this is so, then it may be worth while identifying and purifying such a molecule which could be used to induce inflammatory responses towards other nematodes.

The fact that lambs encounter *N.battus* earlier in life and become rapidly immune whereas this is followed by infections with other nematode species, namely, *O.circumcincta* and *T.vitrinus* which do not seem to be cross-protected by *N.battus* immunity is of interest. One would expect that if the mechanisms eliminating *N.battus* are similar to those directed against other nematodes, that lambs would also be able to cope with other species, at least those infecting the intestine. The results in earlier chapters describe an antibody response which was always accompanied by an inflammatory response. Given the higher level of exposure to larval challenge in the field one would assume a similar, if not enhanced, inflammatory response to accompany the changes in antibody levels. The field experiments in chapter 7 show that elevated

antibody titres were maintained throughout the grazing season and it is reasonable to assume that the continuous infection sustaining this response would no doubt be accompanied by an inflammatory response. If this were the case, then, since it is well known that non-specific inflammatory responses are involved in the regulation of gastrointestinal parasites one would expect ensuing infection with other common ovine gastrointestinal nematodes in the same site or, at least down-stream to that of *N.battus* to be affected. This is not the case since lambs take several months to acquire immunity to these species such as *Ostertagia* and *Trichostrongylus* which therefore raises another question. Is there another component which is involved in the rapid acquisition of immunity to *N.battus*? Young grazing lambs will undergo physiological changes in the gut due to the developing rumen and associated flora and the change from a predominantly milk diet to one of roughage. One speculation is that developing immunity is in itself insufficient to cause the rapid expulsion of the worm populations but that physiological changes in the gut may be involved also. It is possible that *N.battus* has, over time, adapted very well to the under-developed gut of the young lamb and as a result has evolved a different life cycle in order to sustain its existence. This may be why *N.battus* eggs overwinter and develop to L_3 within the egg as opposed to other ovine nematodes.

8.2 Conclusions

In conclusion, it is evident that immunity to *N.battus* is acquired through previous exposure. There is also evidence of an innate age immunity which may vary among individuals. These experiments have shown that immunity is expressed as retardation of growth and development of L_4 larvae and as expulsion of resident populations. These

effects were linked to a local inflammatory and humoral response. The level of protein supplementation in the diet failed to enhance immunity significantly although trends for enhancement were evident. It is also clear that immunity in this model infection persists for at least 12 weeks without further antigenic exposure and this was demonstrated also in the field studies. The humoral responses correlated well with developing immunity (as judged by FEC) in the field and also in the housed experiments.

Nematodirus battus is a difficult parasite to work with. The seasonal availability of infective material coupled with the laborious cleaning and culture of *N.battus* eggs reduces the amount of animal research that can be conducted in comparison to work on other common ovine gastrointestinal nematodes. Even interpretation of results is often difficult since the degree of individual variation in worm burdens of *N.battus* is generally large. Despite these drawbacks it is evident from this research project that *N.battus* infections do evoke a response similar, albeit of lesser magnitude, to that described for other ovine gastrointestinal nematodoses. Therefore, there is considerable scope for future research on this parasite. It is essential to study infections in the natural host since the sheep is an ideal experimental animal and results obtained would not have to be extrapolated.

Other components of immunity need to be examined and investigations into the effect of changes in gut physiology of young lambs would yield invaluable data. It is possible that *N.battus* triggers a different effector pathway which, if known, could lead to manipulation of the immune response towards other gastrointestinal nematodes in an attempt to control gastrointestinal parasitism. Future work on this parasite should focus on other components of the immune response and an investigation into immunogenic

parasite molecules would no doubt extend the understanding of parasite/host interactions and may shed more light on the un-responsiveness of lambs to other common ovine nematodes.

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Appendix A

Recipes for buffers and solutions

Helminthological Iodine

250 g Potassium Iodide (Fisons)

50 g resublimed Iodine (BDH chemicals Ltd.)

500 g tap water

Haematoxylin

1.0 g Haematoxylin

0.2 g Sodium iodate

50 g Potassium alum (Aluminium potassium sulphate)

1000 ml distilled water

* the substances above are left overnight and the following day add:

1.0 g Citric acid

50 g Chloral hydrate

* bring to boil for 5 minutes, cool and filter.

Lendrums Carbol Chromotrope

1.0 g Phenol crystals

0.5 g Chromotrope 2R

100 ml distilled water

* melt Phenol crystals in flask by running hot water over the flask. Dissolve Chromotrope in phenol then add water and filter.

ELISA Coating Buffer

0.8 g Sodium carbonate

0.1 g Sodium azide

500 ml distilled water

* add water to Na_2CO_3 and pH to 9.6 with $NaHCO_2$ then add azide.

Tissue Emulsifying Buffer

Titrate 20mM TRIS with HCl until pH is 7.5. Then add 1.5M NaCl and 0.01% azide.

Phosphate Citrate Buffer

24.3 ml 0.1M Citric acid

25.7 ml 0.2M Na_2HPO_4

50 ml distilled water

* pH should be 5.0

Preparation of Tetramethylbenzidine

18 ml distilled water

2 ml 1M Sodium acetate pH 6.0

0.2 ml 10mg/ml Tetramethylbenzidine (TMB) in DMSO (dimethylsulphoxide)

20 µl Hydrogen peroxide

Preparation of Ortho-phenylenediamine

100 ml Phosphate/citrate buffer

40 µg Ortho-phenylenediamine (OPD)

40 µl 30% Hydrogen peroxide

Appendix B

Results from checkerboard titrations

The calculated differences between the O.D. of worm-free and infected sera are presented. The optimal conditions chosen are highlighted.

L_4 antigens

Antigen μg/ml	*Conjugate Dilution*	*Sera Dilution*					
		1/100	1/500	1/1000	1/2000	1/4000	1/8000
2	1/100	-0.055	0.934	0.785	0.594	0.216	0.232
	1/250	0.010	**0.929**	0.857	0.656	0.316	0.282
	1/500	0.015	0.736	0.663	0.464	0.263	0.250
5	1/100	0.036	0.647	0.550	0.491	0.239	0.221
	1/250	0.330	0.624	0.557	0.484	0.229	0.190
	1/500	0.218	0.510	0.363	0.269	0.166	0.108
10	1/100	0.103	0.860	0.796	0.767	0.367	0.224
	1/250	0.162	0.843	0.787	0.734	0.410	0.372
	1/500	-0.075	0.736	0.728	0.499	0.350	0.251

Adult antigens

Antigen μg/ml	*Conjugate Dilution*	*Sera Dilution*					
		1/100	1/500	1/1000	1/2000	1/4000	1/8000
2	1/100	-0.273	0.427	**0.549**	0.369	0.081	0.093
	1/250	-0.373	0.423	0.422	0.309	0.146	0.118
	1/500	-0.262	0.381	0.361	0.254	0.117	0.117
5	1/100	-0.346	0.298	0.271	0.238	0.086	0.033
	1/250	-0.325	0.268	0.258	0.147	0.101	0.127
	1/500	-0.302	0.132	0.210	0.147	0.071	0.073
10	1/100	-0.365	0.496	0.456	0.427	0.156	0.067
	1/250	-0.323	0.401	0.436	0.037	0.140	0.138
	1/500	-0.297	0.365	0.370	0.241	0.089	0.054

ABBREVIATIONS

ADG	Average daily weightgain
ANOVA	Analysis of variance
BMCMC	Bone marrow derived cultured mast cells
BSA	Bovine serum albumin
CP	Crude protein
DM	Dry matter
DMI	Dry matter intake
ECF-A	Eosinophil chemotactic factor of anaphylaxis
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme-linked immunosorbent assay
EPA	Eosinophil potentiating activity
EPG	Eggs per gram
ES	Excretory secretory
FEC	Faecal egg counts
GALT	Gut-associated lymphoid tissue
GL	Globule leucocyte
GM-CSF	Granulocyte macrophage colony stimulating factor
5HT	5-hydroxytryptamine
ICC	Immunoglobulin-containing cells
IEL	Intraepithelial lymphocyte
IL	Interleukin
L_1	First-stage larvae
L_3	Third-stage infective larvae
L_4	Fourth-stage larvae
L_5	Fifth-stage larvae
LMI	Larval migration inhibition
LT	Leukotriene
MBP	Major basic protein
MMC	Mucosal mast cell
MHC	Major histocompatibility complex
MP	Metabolizable protein
OD	Optical density
PAF	Platelet activating factor
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PC	Post-challenge
PCV	Packed cell volume
PG	Prostaglandin
PI	Post-infection
PMSF	Phenylmethylsulphonyl
PT	Post-treatment
RE	Rapid expulsion
SBMC	Sheep bone marrow cells
SEM	Standard error of the mean
SMCP	Sheep mast cell protease
SRS-A	Slow reacting substance of anaphylaxis
TNTT	Tris-sodium-tween-thimerosal

PUBLICATIONS ARISING FROM THIS THESIS

1. Israf, D.A., Coop, R.L, Stevenson, L.M., Jones, D.G., Jackson, F., Jackson, E., MacKellar, A. & Huntley, J.F. (1995). Dietary protein influences upon immunity to *Nematodirus battus* infection in lambs. Veterinary Parasitology (in press).

2. Israf, D.A., Coop, R.L., Jackson, F. & Jackson, E. (1995). Dietary protein influences upon population regulation of *Nematodirus battus* by lambs. Research in Veterinary Science (in press).

3. Israf, D.A., Coop, R.L, Stevenson, L.M., Jones, D.G., Jackson, F., Jackson, E., MacKellar, A. & Huntley, J.F. (1995). Persistence of immunity to *Nematodirus battus* infection in lambs. Veterinary Parasitology (submitted).

Dietary protein influences upon population regulation of *Nematodirus battus* by lambs

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Two groups of 7 and two groups of 6 lambs were offered either a complete basal ruminant diet (13.2% CP) or the diet supplemented with fish meal (18.3% CP). Groups 1 and 2 were infected daily for 7 weeks with *Nematodirus battus* larvae (L_3) and groups 3 and 4 served as uninfected challenge controls. All groups were treated with anthelmintic at week 8, challenged with a single dose of 30,000 *N.battus* L_3 one week later and killed 9 days post-challenge. Although supplementation showed trends for enhanced population regulation in lambs which had received continuous infection, these findings did not reach levels of significance. The worm burdens in both groups of previously infected lambs were significantly reduced both in number and size ($p<0.001$) and there was a preferential loss of male worms compared to that of the naive challenge controls. Further analysis of the results demonstrated that lambs were able to be segregated into high or low responders (HR & LR) based on their worm burden with significant reduction in worm burdens ($p<0.001$) and size ($p<0.01$) in line with responsiveness. The identification of HR and LR essentially shows that when the protein supply is adequate the predominant host effect influencing the pathogenicity of the parasites is the level of genetically-determined susceptibility of the host.

Previous studies have shown that unweaned grazing lambs acquire immunity to *N.battus* more rapidly than those that were weaned (F.Jackson, unpublished data). Since the rate of development of resistance in sheep to gastrointestinal parasites may be influenced by the protein status of the host (Holmes and Coop, 1994), differences in the relative proportion of total protein that by-pass the rumen may play a key role in accounting for the different rate and degree of response seen in these studies. Evidence supporting this view comes from experiments in which lambs given a post-ruminal infusion of protein were better able to regulate their worm burdens (Bown *et al.* 1991; Coop *et al.*, 1995). The aim of this experiment was to determine whether supplementation of an adequate basal diet with a rumen by-pass protein influenced the rate of development of immunity towards *N.battus* infections in weaned lambs.

Twenty-six worm-free Blackface x Suffolk lambs aged approximately 4 months at the start of the experiment were used. Two groups of 7 and two groups of 6 lambs were either continuously infected and challenged (groups 1 and 2) or challenged only (groups 3 and 4) with *N.battus* L_3. Groups 2 and 4 were offered a basal diet (Ruminant A; 132 g kg^{-1} of crude protein (CP) kg^{-1} DM which supplied 66g of metabolizable protein (MP) kg^{-1} DM feed) and groups 1 and 3 this diet supplemented with 10% fish meal (183 g CP kg^{-1} DM which supplied 95g MP kg^{-1} DM feed). Due to constraints of accommodation only continuously infected groups (1 and 2) were housed individually in metabolic cages. The continuous infection regime was designed to mimic the field situation, using a rapidly increasing larval intake followed by decreasing intake over a 7 week period (week 1: 1000 L_3 day^{-1}, wk 2: 2000 L_3 day^{-1}, wk 3: 4000 L_3 day^{-1}, wk 4: 2000 L_3 day^{-1}, wk 5 to 7: 1000 L_3 day^{-1}). All groups were treated with levamisole (15 mg kg^{-1} bodyweight) and fenbendazole (5 mg kg^{-1} bodyweight) at week 8 to remove the primary infection, challenged one week later with a single dose of 30,000 *N.battus* L_3 and killed on day 9 post-challenge. Feed intake and liveweight gain were monitored weekly and faecal egg counts (FEC) were determined weekly by a modification of the method of Christie and Jackson (1982) using flotation in saturated sodium chloride. The *N.battus* strain had been isolated recently from the field and cultured and extracted as described by Mapes and Coop (1970). At necropsy the first two thirds of the small intestine was removed and processed as described by Seaton *et al.* (1989). Worms were collected from 5% of the total intestinal contents of groups 1 and 2 and 2% of groups 3 and 4. Where available 50 fourth or fifth stage (L_4 or L_5) male and female worms were randomly removed from aliquots for measurement of length using a stereo

microscope fitted with a video camera, linked to an Archimedes Digit Image Analyzer.

Due to the high variability in worm burdens of previously infected lambs (groups 1 & 2) and since there was no significant dietary effects, the lambs were re-grouped into high- or low-responders (HR & LR) for further analysis using an arbitrary cut-off point of 1000 worms as described by Taylor and Thomas (1986). The challenge controls (groups 3 and 4) were pooled and referred to as controls. All data were $\log_{10}(x + 1)$-transformed prior to statistical analysis. Means of average daily weight gain, dry matter intake and FEC were compared using a 2 sample t-test and worm count and length data by 2 way analysis of variance using a Minitab statistical software. Means are given in the text and table with $\pm$ 1 standard error of the mean.

During the course of the experiment one lamb from group 1 died of suppurative pneumonia and was excluded from subsequent analyses. There were no significant differences in growth rate from week 0 to week 8 between groups 1 (323.8 $\pm$ 8.52 g day^{-1}) and 2 (306.8 $\pm$ 18.5 g day^{-1}). Similarly there were no differences in dry matter intake (group 1, 2.08 $\pm$ 0.02 kg DM day^{-1}; group 2, 2.11 $\pm$ 0.03 kg DM day^{-1}).

FEC did not differ significantly between previously infected groups (1 & 2) and were generally low. The mean counts for both groups increased steadily to peak on day 52 post infection (group 1: 82 epg; group 2: 63 epg). Analysis of individual worm burdens (Figure 1) showed that more variation occurred within the previously infected groups (1 and 2) than in the challenge controls. Previous infection caused a significant reduction in worm burdens ($p<0.001$) but supplementation failed to enhance the response significantly. There was no indication of an interaction between both treatments. Although supplementation had no significant effect upon the development of resistance, there was a trend for fewer worms in the supplemented previously infected lambs (group 1) which had 28% fewer worms than group 2. Previous infection resulted in significantly smaller worms ($p<0.001$). However, supplementation did not affect worm size although, a trend for smaller L_4s in group 1 was evident. The worm populations in all groups were predominantly L_4 and considerably fewer worms in the previously infected groups were at the L_5 stage in comparison to the challenge controls (Table 1). This is indicated by the higher L_4:L_5 ratio which was much more pronounced among the male population. The sex ratios were higher among the challenge controls indicating a larger male population.

Further analysis by segregating the lambs based on their response to challenge infection revealed a HR/LR phenomenon. Mean worm burdens of HR (273 $\pm$ 79) were significantly lower ($p<0.001$) than LR (4273 $\pm$ 760) and controls (6796 $\pm$ 592). Similarly, LR had significantly fewer worms than controls ($p<0.05$). A marked increase in the L_4:L_5 ratios was observed between LR (48.2) and controls (8.4) in the male population and to a lesser extent among the females (LR: 22.1; controls: 3.4). Larval ratios could not be calculated for HR since there were no L_5s in the population. Sex ratios were higher in controls (HR: 0.63, LR: 0.59, controls: 0.77). Worm length of both male and female L_4s followed a similar pattern, the HR having significantly smaller worms ($p<0.01$) followed by LR and controls.

The rate of liveweight gain was high for lambs of 4 months of age but this was due to the early restriction on growth rate imposed in the pre-experimental period. Supplementation did not increase liveweight gain and there was no significant enhancement of worm regulation and retardation as shown in Figure 1 and Table 1.

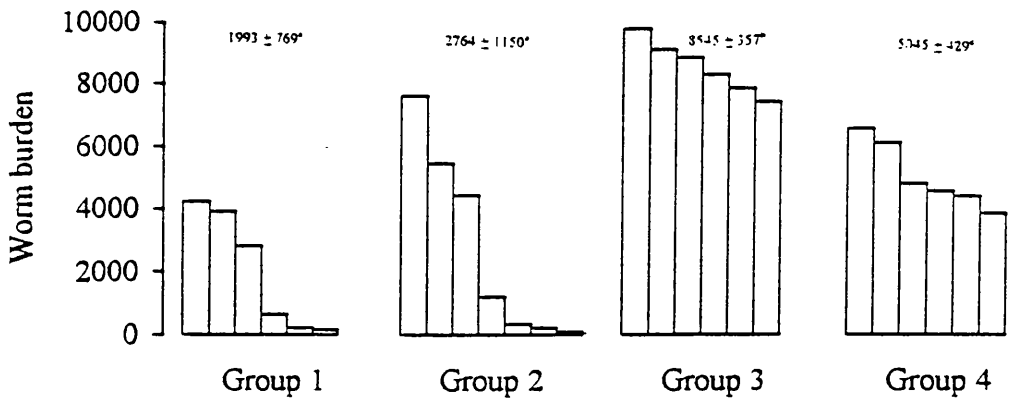

Figure 1. Mean ± S.E.M and individual *N.battus* worm burdens for lambs in groups 1–4.

However, a trend for fewer and smaller worms was observed in the supplemented previously infected group. This failure to significantly enhance worm regulation by the host may be due to the sufficient level of dietary protein in the basal diet. Previous studies on this subject have used groups of lambs offered low protein diets and therefore were able to show significant differences (Abbott *et al.*, 1986; Kambara *et al.*, 1993). It is important to note that in this experiment the objective was to determine whether the inclusion of a rumen by-pass protein, by increasing duodenal protein supply, would be of any benefit in enhancing resistance to *N.battus* infection in weaned lambs. Calculations of larval and sex ratios and worm measurements have shown that previous infection causes retardation in growth and development and protein supplementation showed a trend for enhancement of these effects.

It is quite common to obtain large variation in worm numbers in *N.battus* infections. In view of this and the fact that we were unable to demonstrate significant enhancement of population regulation by supplementation it was decided to determine whether there was some innate ability to respond to infection by segregating the lambs based on their worm burdens. This re-analysis of the data showed that not only did HR have significantly lower worm burdens but also that the worms were significantly retarded. The identification of HR and LR confirms an earlier study (Taylor and Thomas, 1986) and essentially shows that when the protein supply is adequate (as it was in this experiment in the sheep on the basal diet) the predominant host effect influencing the pathogenicity of the parasites is the level of genetically-determined susceptibility of the host.

Table 1. Mean larval length, L_4:L_5 and sex ratios.

	Group 1	Group 2	Group 3	Group 4
Males				
L_4 length	3.12ª	3.42ª	4.78ᵇ	4.75ᵇ
ratio L_4:L_5	86	29.3	7.1	9.7
Females				
L_4 length	3.38ª	3.77ª	5.17ᵇ	5.14ᵇ
L_5 length	6.23ª	6.21ª	7.39ᵇ	7.15ᵇ
ratio L_4:L_5	16.1	26.5	1.4	5.4
Sex ratio (male:female)	0.59	0.62	0.83	0.71

Figures with different superscripts were significantly different ($p<0.05$).

Acknowledgements

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Dietary protein influences upon immunity to *Nematodirus battus* infection in lambs

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Abstract

Several indices of the immune response to *Nematodirus battus* infection in lambs offered differing levels of dietary protein were quantified. Lambs were offered either a complete basal ruminant diet (13.2% crude protein {CP}) or the same diet supplemented with fish meal as a source of rumen bypass protein (18.3% CP). Lambs from each dietary treatment group were given either a 7 week continuous trickle infection with *N.battus* L_3 or remained uninfected. All lambs were drenched with anthelmintic at week 8 post-infection (PI), challenged with a single dose of 30,000 *N.battus* L_3 one week later, and killed 9 days post-challenge (PC). Previous infection induced a significant reduction in worm burdens ($p<0.001$) and enhancement of immune responses when compared to challenge controls. Among previously infected lambs, protein supplementation did not reduce worm burdens significantly, although there was a trend for fewer worms in the supplemented lambs. However, a significant increase in mucosal globule leucocyte ($p<0.05$) and eosinophil ($p<0.05$) numbers was evident. Supplementation ($p<0.05$) and previous infection ($p<0.001$) both enhanced serum anti-worm IgG titres over time. Peripheral blood eosinophil counts were not affected by supplementation but were significantly elevated over time as a result of previous infection ($p<0.001$). Since there were no significant differences in worm burdens of supplemented and unsupplemented previously infected lambs, it was of interest to determine whether lambs possessed an innate ability to regulate their parasite burden. Hence they were re-grouped based on an arbitrary cut-off burden of 1000 worms. High responders (HR) had burdens below 1000 worms while low responders (LR) had burdens above this value and challenge controls were pooled. The data was re-analysed based on these groupings and showed significant reduction in worm burdens between all three groups ($p<0.001$). Globule leucocytes were the only cell type that appeared to be significantly more abundant in the intestinal mucosa of HR ($p<0.001$). Serum antibody responses ($p<0.05$) and peripheral blood eosinophil counts ($p<0.01$) were significantly elevated over time in accord with the degree of responsiveness. The results of this study suggest that supplementation of protein upon an adequate basal diet of lambs previously exposed to *N.battus* does not significantly enhance worm regulation despite significant increases in cellular and antibody responses. The immunity acquired is characterized by reduction in worm burdens, elevated anti-worm antibodies and a cellular inflammatory response. The identification of HR and LR essentially shows that when the protein supply is adequate the predominant host effect influencing the pathogenicity of the parasites is the level of genetically-determined susceptibility of the host.

Key words: Nematodirus battus, dietary protein, immune responses

1. Introduction

Immuno-regulatory mechanisms involved in the development of immunity of sheep to gastrointestinal helminths still are not understood fully (Emery *et al.*, 1993). These mechanisms are complex and involve immediate hypersensitivity reactions, inflammatory mediators and quantitative and qualitative changes in mucus (Miller, 1984). Ovine mucosal mast cells (MMC) increase in numbers in infected gut mucosa and secrete mast cell protease (SMCP) in response to gastrointestinal nematode challenge (Huntley *et al.*, 1987; Huntley *et al.*, 1992). Resistance to challenge is often associated with the presence of globule leucocytes which are considered to be the end-stage of maturation of MMC (Huntley *et al.*, 1984). Eosinophils are also associated with helminth infection and increase peripherally and locally following infection (Buddle *et al.*, 1992; Rothwell *et al.*, 1993; Stevenson *et al.*, 1994). Antibodies of the IgA and IgG isotype increase in response to infections (Gill *et al.*, 1994; Schallig *et al.*, 1994) and are particularly important at the mucosal surface.

Under field conditions, young lambs rapidly develop a strong immunity following a moderate to heavy infection with *Nematodirus battus*. This immunity is characterized by a rapid decline in faecal egg counts and a rather slower fall in worm burdens to very low or zero levels (Thomas, 1959). It is therefore tempting to speculate that the immune mechanisms involved against *Nematodirus* species are either of a greater magnitude or somewhat different to those operating in other ovine trichstrongyle infections. A comparative study conducted at Moredun suggested that unweaned lambs were more refractory to infection with *N.battus* than weaned lambs (F.Jackson, unpublished data). These differences in susceptibility may be due partly to the fact that weaned lambs will be faced with a decline in protein intake due to cessation of suckling. Previous studies have provided evidence that supplementation of the basal diet with protein enhances the development of resistance to gastrointestinal nematode infection in sheep (reviewed by Holmes and Coop, 1994). The aim of this study was to evaluate the influence of dietary supplementation with a rumen bypass protein upon the acquisition of immunity in lambs experimentally infected with *N.battus*. A previous paper (Israf *et al.*, 1995) has described the parasitological findings and the cellular and humoral responses are presented here.

2. Materials and methods

Details of the animals and their management, experimental design and parasitological techniques have been described previously (Israf *et al.*, 1995) and are briefly summarized.

2.1. Animals and feed

Twenty-six 4-month old Greyface × Suffolk lambs which had been maintained indoors from birth were used. The lambs were fed a basal ruminant diet or the diet supplemented with 10% fish meal as a source of rumen bypass protein. The diets comprised a pelleted complete basal ration (Ruminant A; 132 g of crude protein (CP) kg^{-1} dry matter (DM), Dalgety Agriculture Ltd, Almondsbury, Bristol), which supplied 66g of metabolizable protein (MP) kg^{-1} DM feed or the basal diet

supplemented with 10% fish meal (183 g CP kg^{-1} DM) which supplied 95g MP kg^{-1} DM feed. Calculated metabolizable energy values for the basal and supplemented diets were 8.54 and 8.96 MJ kg^{-1} DM respectively.

2.2. Experimental design

Figure 1 shows the design of the experiment. Two groups (1 & 2) of lambs were infected daily for 7 weeks with *N.battus* L_3 (week 1: 1000 L_3 day^{-1}, wk 2: 2000 L_3 day^{-1}, wk 3: 4000 L_3 day^{-1}, wk 4: 2000 L_3 day^{-1}, wk 5 to 7: 1000 L_3 day^{-1}). and two others (3 & 4) served as challenge controls. Within each infection regime one group was offered the basal diet (groups 2 & 4) and the other the supplemented diet (groups 1 & 3). All four groups were drenched orally with anthelmintic (levamisole; 15 mg kg^{-1} bodyweight and fenbendazole; 5 mg kg^{-1} bodyweight) at week 8 and one week later challenged with a single dose of 30,000 *N.battus* L_3. All lambs were killed 9 days post-challenge (PC).

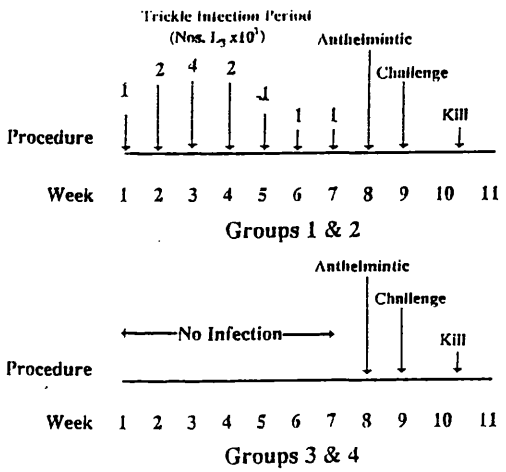


Figure 1. Experimental design. Lambs were infected with a 7 week trickle infection. Larvae were given daily, 7 days a week. Lambs in groups 1 (n=7) and 3 (n=6) were offered a supplemented diet. Lambs in group 2 (n=7) and 4 (n=6) were offered the basal diet.

2.3. Parasitological parameters, feed intake and liveweight gain

Parasitological techniques and monitoring of feed intake and liveweight gain have been described (Israf *et al.*, 1995).

2.4. Blood samples

Blood samples (collected at the same time of day) were taken weekly for serum and twice weekly for peripheral blood eosinophil counts by jugular venepuncture, the latter using ethylenediaminetetracetate (EDTA) vacutainer tubes.

2.5. Tissue samples

The gastrointestinal tract was removed immediately at necropsy and sections of the small intestine (4-8 cm long), taken at approximately 2 metres from the proximal end, were washed briefly in tap water and processed for enzyme extraction as described by Huntley *et al.* (1986). A further adjacent section was taken for histochemistry and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) pH 7.4 for 6 hours (Newlands *et al.*, 1984) and processed as described by Miller *et al.* (1983).

2.6. Enumeration of tissue cells

Sections of small intestine were stained with toluidine blue pH 0.5 (Enerback, 1966) for the histochemical enumeration of mast cells or with carbol chromotrope (Lendrum, 1944) for globule leucocytes and eosinophils. Stained cells were counted using a ×10 eyepiece, containing a calibrated graticule, and a ×40 objective lens. The villus-crypt unit method was used for counting cells (Miller and Jarrett, 1971), scanning a total of 30 villi from the sections cut from each lamb and the results were expressed as number of cells per villus-crypt unit.

2.7. Peripheral blood eosinophil counts

Percentage eosinophil numbers were determined from differential counts made of 600 leucocytes on blood smears fixed and stained with Diff-Quik (Brownes Ltd., Reading, U.K.) according to the manufacturer's instructions. Absolute eosinophil counts were estimated by relating the percentage count to the total leucocyte counts obtained on a Coulter counter (Coulter Electronics Ltd.) with a 3.617 μm diameter cut-off setting.

2.8. Enzyme-linked immunosorbent assay (ELISA) for sheep mast cell protease.

A double antibody sandwich ELISA was used for the measurement of the concentrations of sheep mast cell protease (SMCP) in tissue homogenates as described by Huntley *et al.* (1987) using the German origin M129B flat-bottomed plates (Dynatech Laboratories Ltd, Billinghurst, Sussex, U.K.).

2.9. Soluble worm antigen preparation

Nematodirus worms (adults and L_4s) were collected from the intestines of infected donor lambs as described by Smith (1993) and frozen at -20°C. Both L_4 and adult worm crude antigens were prepared separately in a similar manner. The worms were thawed, placed on filter paper and washed with PBS using a Buchner funnel attached to a vacuum pump. The washed worms were ground up using a glass homogenizer as a 10% w/v suspension in homogenizing buffer (1mM EDTA, 1mM phenylmethylsulphonyl fluoride (PMSF) in PBS pH 7.4) and centrifuged (10,000 *g* for 20 min at 2°C). The resulting supernate was aliquoted and stored at -20°C prior to use. Protein concentration was estimated using the bicinchoninic protein assay reagent (Pierce) according to the manufacturer's instructions.

2.10. ELISA for serum anti-worm IgG antibodies

Optimal conditions were determined by checkerboard titration. For assaying IgG against L_4 antigens the optimal conditions which gave the largest difference between the optical density (O.D.) of sera from infected and non-infected lambs were an antigen concentration of 2μg protein ml^{-1}, serum dilution of 1/500 and enzyme-conjugated antibody dilution of 1/250. For assaying against adult worms the optimal conditions were 2 μg protein ml^{-1} of antigen, serum dilution of 1/1000 and conjugate 1/100.

Flat-bottomed microtitre plates (Dynatech Laboratories Ltd, Billinghurst, Sussex, U.K.) were coated overnight at 4°C with crude antigen diluted with 0.05M carbonate-bicarbonate buffer pH 9.6, and subsequently washed twice with Tris-buffered saline comprising 10mM Tris and 0.5M sodium chloride pH 7.4 with 0.05% Tween 20 and

0.01% Thimerosal (TNTT). Non-specific binding was blocked by applying 200µl/well 10% horse serum in PBS for 1 hour at 37°C and the plates subsequently washed twice with TNTT. Test serum (100µl/well) diluted with PBS was added to the wells in duplicate. The plates were re-incubated for 1 hour at 37°C and then washed three times with TNTT. Horseradish peroxidase-conjugated donkey anti-sheep IgG (Scottish Antibody Production Unit, Carluke, Lanarkshire) diluted with PBS was added to each well (50µl/well). The plates were incubated for 1 hour at 37°C then washed three times with TNTT followed by the addition of 3,3',5,5'-tetramethylbenzidine (150µl/well) as the enzyme substrate. The enzyme reaction was stopped after 5 minutes by the addition of 2.5M sulphuric acid (30µl/well) and O.D. measured at 450 nm. The results were expressed as O.D. values.

2.11. Analysis based on responsiveness

After it was established that there was no significant dietary effect upon reduction in worm population the lambs were re-grouped according to total worm burdens for further analyses. Continuously infected lambs (groups 1 & 2) were re-grouped using an arbitrary threshold of 1000 worms (Taylor and Thomas, 1986). Lambs from these groups which had total worm burdens below 1000 were grouped as high-responders (HR) (n=6) and the remainder as low-responders (LR) (n=7). The challenge controls (groups 3 and 4) were pooled (n=12) and referred to as controls.

2.12. Statistical analysis

Antibody titres, tissue mast cell and eosinophil counts, SMCP concentrations and worm burdens were ^{10}log transformed and globule leucocyte counts and peripheral eosinophil counts ^{10}log(x+1) transformed prior to statistical analysis. Data was subjected to either a one way, 2 way or 3 way analysis of variance (ANOVA) depending on the number of treatments and their interactions by fitting a general linear model (GLM). Where significanct differences were noted, differences between the means of groups was tested using a 2 sample t-test. Correlation analysis was used to determine associations between cell counts and worm burdens.

3. Results

3.1. Worm burdens

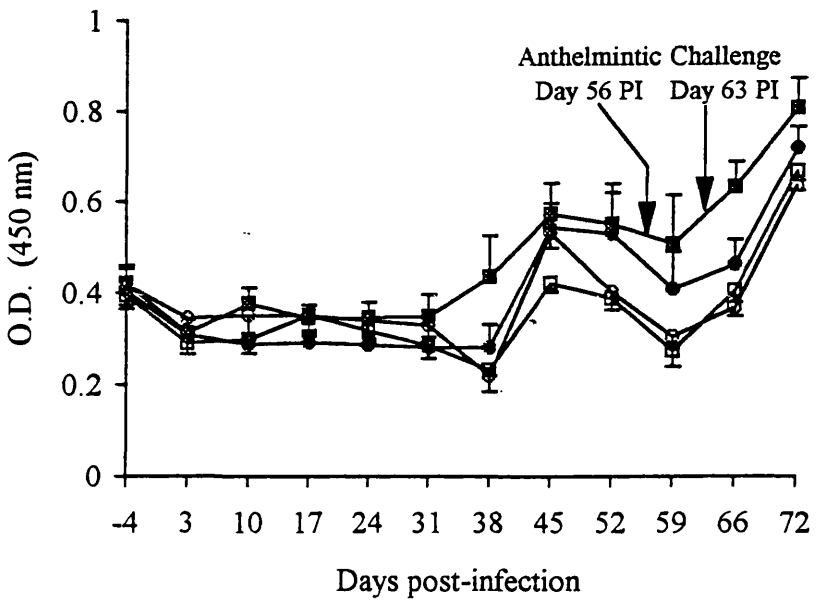
Table 1 shows the mean worm burden of the four groups of lambs. Previous infection caused a significant reduction in worm burdens ($p<0.001$) but supplementation failed to enhance the response significantly. There was no indication of an interaction between both treatments. Although supplementation had no significant effect upon the development of resistance there was a trend for fewer worms in the supplemented previously infected lambs (group 1) which had 28% fewer worms than group 2. Analysis of worm burdens based on responsiveness are shown in Table 2. There were significant differences ($p<0.001$)between all three groups (HR, LR and controls).

3.2. Antibody titres

The serum IgG antibody titres towards both L_4 and adult worm antigens are shown in Figure 2. Titres were significantly elevated by supplementation ($p<0.05$), previous infection ($p<0.001$) and duration of time ($p<0.001$) with significant interactions

between supplementation and previous infection ($p<0.001$) and between previous infection and time ($p<0.001$). Antibody titres of previously infected lambs (groups 1 and 2) towards both adult and L_4 antigens were generally higher than those of the naive challenge controls (groups 3 and 4) from day 38 PI. Challenge did not evoke an exaggerated anamnestic response, antibody titres being maintained at pre-challenge levels despite removal of the primary infection and a subsequent large challenge.

a)



b)

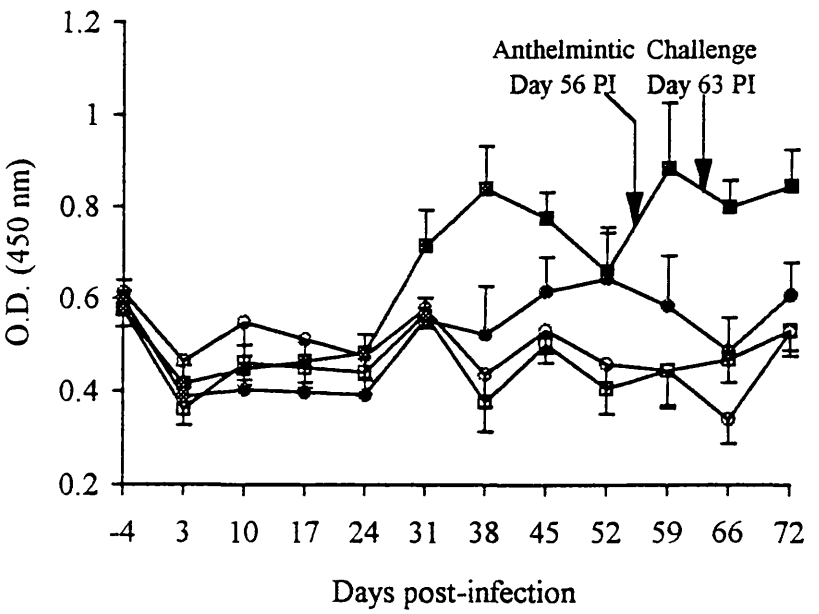


Figure 2. Anti-worm IgG antibody responses ± S.E.M over the experimental period. (a) response towards L_4 antigens, (b) response towards adult worm antigens.
—■— Group 1 (infected, supplemented), —●— group 2 (infected, basal diet), —□— group 3 (non-infected, supplemented) and —○— group 4 (non-infected, basal diet).

3.3. Peripheral eosinophil counts

The peripheral blood eosinophil counts (Figure 3) were significantly elevated in previously infected groups ($p<0.001$). However, supplementation and time did not affect the counts. There were significant interactions between supplementation and previous infection ($p<0.001$) and between previous infection and time ($p<0.001$).

3.4. Tissue cell counts and SMCP concentrations

The tissue cell counts and SMCP concentrations are shown in Table 1. Previous infection significantly enhanced eosinophil ($p<0.001$), globule leucocyte ($p<0.01$) and

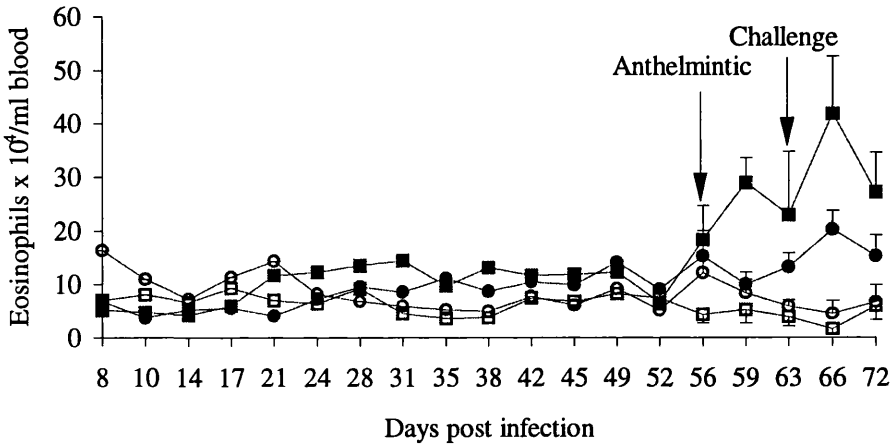


Figure 3. Peripheral blood eosinophil counts ± S.E.M over the experimental period. Symbols as described in Fig. 2

mast cell numbers ($p<0.001$). Supplementation enhanced globule leucocyte numbers ($p<0.01$) while an interaction between supplementation and previous infection ($p<0.01$) was evident. Correlation analysis by group showed only a significant negative correlation ($r= -0.828$, $p<0.05$) between globule leucocytes and worm burdens in group 1 and between SMCP and worm burdens in group 3 ($r=-0.805$, $p<0.05$).

Table 1. Mean ± S.E.M mucosal cell numbers, SMCP concentrations and total worm burdens.

Group	Mast cells*	SMCP[#]	Globule leucocytes*	Eosinophils*	Worm burden[+]
1	50.2 ± 4.4^{a}	19.6 ± 5.3^{a}	0.4 ± 0.1^{a}	80.8 ± 8.7^{a}	1993 ± 769^{a}
2	39.6 ± 4.7^{ab}	12.8 ± 3.0^{a}	0.01 ± 0.01^{b}	52.1 ± 7.3^{b}	2763 ± 1150^{a}
3	25.2 ± 3.3^{b}	10.4 ± 1.1^{a}	0.005 ± 0.005^{b}	34.6 ± 4.9^{b}	8550 ± 357^{b}
4	29.5 ± 5.4^{b}	11.6 ± 3.6^{a}	0^{b}	37.8 ± 4.7^{b}	5042 ± 429^{c}

Figures with different superscripts within each column are significantly different at the 5% level

* Cell numbers per villus crypt

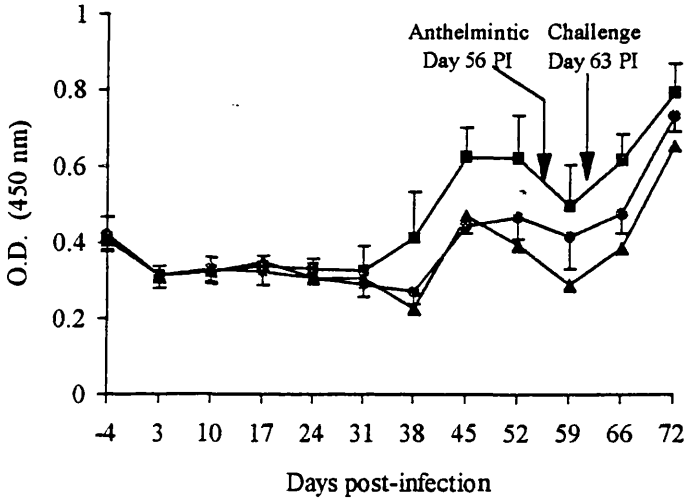
[#] µg sheep mast cell protease/gm wet weight of tissue

[+] data presented previously (Israf *et al.*, 1995)

3.5. Analysis based on responsiveness

Six lambs were designated HR and 7 lambs LR. Figure 4a and 4b shows the anti-worm IgG antibody titres against L_4 and adult worm antigens for responder groups and challenge controls. Responders had significantly elevated titres ($p<0.05$) and duration of time had a significant influence ($p<0.05$) upon antibody responses. A significant interaction between both treatments was evident ($p<0.05$). Generally, the antibody titres in both responder groups were elevated from day 38 PI onwards. However, due to large individual variations, the trend for elevated antibody titres in the HR group did not differ significantly from those of the LR group.

a)



b)

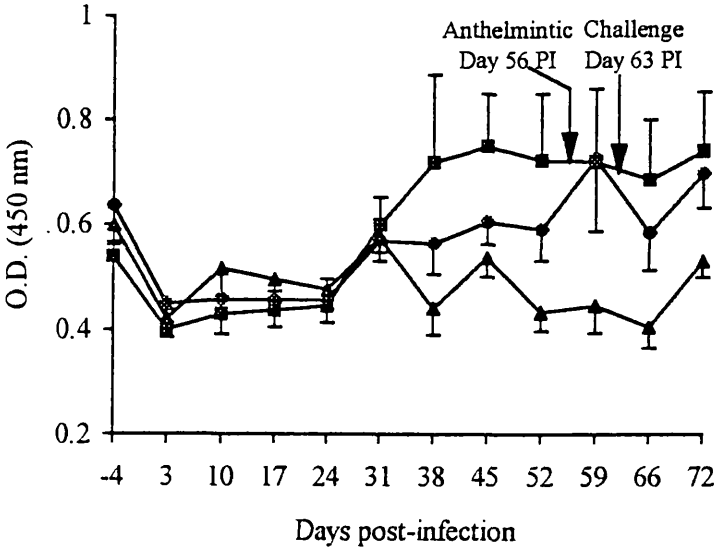


Figure 4. Anti-worm IgG antibody responses ± S.E.M according to responsiveness. (a) response towards L_4 antigens, (b) response towards adult worm antigens. —■— High-responders, —●— Low-responders, —▲— Controls.

Peripheral eosinophil counts rose significantly in responder groups ($p<0.01$) after anthelmintic treatment as they did when analysed according to dietary groupings, although there were no significant differences between HR and LR lambs (Figure 5). The duration of time had a significant influence ($p<0.05$) upon cell counts. There was a significant interaction between responsiveness and time ($p<0.01$). Significant differences between LR and control lambs were evident from day 56 PI onwards. Similarly, differences between HR and controls were evident from day 63 onwards.

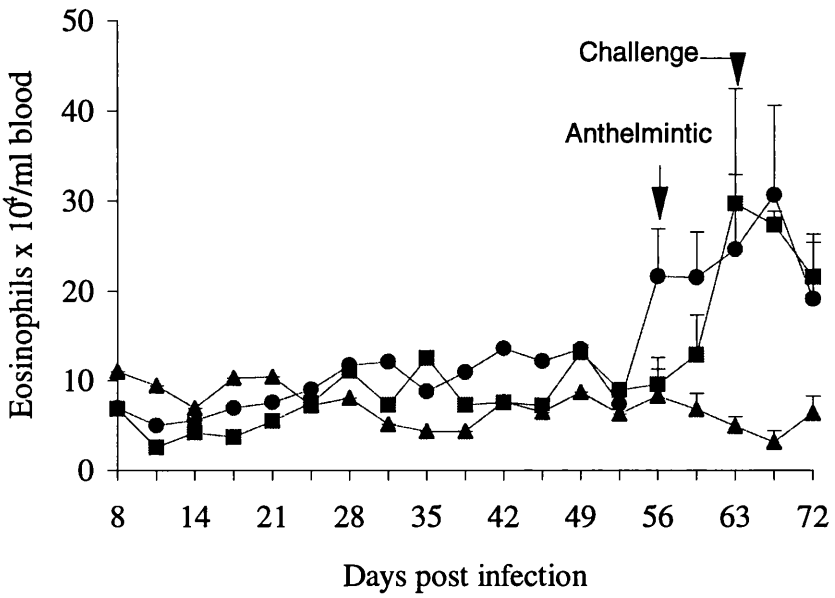


Figure 5. Peripheral blood eosinophil counts ± S.E.M according to responsiveness. Symbols as described in Fig. 4.

Cell counts and SMCP concentrations according to responsiveness are given in Table 2. Responders had significantly increased mast cells, globule leucocytes and eosinophils ($p<0.01$). There were no significant differences between HR and LR in terms of mast cell or eosinophil counts and SMCP concentrations. However, significant differences were noted for globule leucocyte numbers between HR and LR. No correlation existed between cell counts or SMCP concentrations and worm burdens.

4. Discussion

The results of this trial show that the immunity to *N.battus* is acquired through previous exposure. The immunity is manifested by a reduction in worm burdens accompanied by increased infilteration of the intestinal mucosa with MMC, GL and eosinophils, and increased levels of serum IgG. Exposure to 7 weeks of infection with *N.battus* resulted in only a moderate expression of immunity in both supplemented and non-supplemented lambs. Following challenge 77% fewer worms were found in the supplemented lambs compared to their naive controls and 45% in the non-supplemented lambs. Previous infection enhanced MMC, GL and tissue eosinophil responses, all of which are recognized as components of regulatory mechanisms in other gastrointestinal nematode infections (Miller, 1984). However, it is apparent that

Table 2. Mean ± S.E.M mucosal cell numbers, sheep mast cell protease (SMCP) concentrations and total worm burdens according to responsiveness.

Group	Number of lambs (n)	Mast cells*	SMCP#	Globule leucocytes*	Eosinophils*	Worm burden+
High-responders	6	47.4 ± 4.7^a	15.4 ± 4.0^a	0.3 ± 0.1^a	58.0 ± 10.2^a	273 ± 79^a
Low-responders	7	41.9 ± 5.1^a	16.3 ± 4.5^a	0.05 ± 0.04^b	71.6 ± 9.0^a	4237 ± 760^b
Challenge controls	12	27.4 ± 3.1^b	11.0 ± 1.8^a	0.002 ± 0.002^b	36.2 ± 3.3^b	6796 ± 592^c

Figures with different superscripts within each column are significantly different at the 5% level
* Cell numbers per villus crypt
# µg sheep mast cell protease/gm wet weight of tissue
+ data presented previously (Israf *et al.*, 1995)

these are not the sole factors governing regulation since there were no differences in MMC and tissue eosinophil numbers in HR and LR lambs.

Peripheral blood eosinophil kinetics were strikingly different to those reported in other ovine nematode infections. During the primary trickle infection there were no obvious signs of peripheral eosinophilia, a finding in direct contrast to those of other workers studying *T.colubriformis* (Buddle *et al.*, 1992) and *O.circumcincta* (Stevenson *et al.*, 1994) infections in sheep. Although other studies have correlated the degree of eosinophilia with responsiveness (Dawkins *et al.*, 1989; Buddle *et al.*, 1992) in the current trial no such correlations were apparent, peripheral eosinophil counts being similar in both HR and LR lambs. Furthermore, in guinea pigs, an anamnestic eosinophilia occurs during secondary nematode infection and peripheral and local eosinophilia is observed about the time of worm expulsion (Rothwell and Dineen, 1972). Handlinger and Rothwell (1981) demonstrated that guinea pigs of a high responder phenotype had higher numbers of peripheral and local eosinophils. However, one should be cautious when making extrapolations between host species.

The humoral responses observed in this model infection were influenced by the diet and previous exposure to the parasite. The trickle infection enhanced the antibody levels towards both adult and L_4 stages and generally a rise in antibody levels was observed after 38 days of continuous infection. It is important to note that the ELISA used crude worm preparations and therefore this may account for some degree of cross reactivity with bacterial or food antigens which may explain similar trends in antibody levels of control groups. Despite the increasing amount of literature on the subject of antibody responses to the common ovine gastrointestinal nematodes, the actual significance of these responses in the development of immunity remains questionable. Recently, Gill *et al.* (1993) have demonstrated that anti-parasite IgA and IgG1 antibodies may play an important role in genetically determined resistance of sheep to haemonchosis. The results in this trial are unable to determine the importance of humoral responses in immunity to *N.battus*. However, these findings provide basic information on the kinetics of the response on which further work could be based upon.

The protein-supplemented previously infected lambs had 28% fewer *N.battus* following challenge and it has been reported that these worms were retarded in their development compared to those recovered from unsupplemented lambs (Israf *et al.*, 1995). Dietary protein can markedly affect the pathogenic impact of parasitism in the host and thus, in those situations where suboptimal nutrition occurs, may have a practical significance (Sykes, 1994). In this study the 4-month old lambs were offered a basal diet adequate in protein and the selected infection regime was intended to minimise those pathogenic effects which might compromise the rate of development of immunity. These features of the experimental design served to minimise between group differences in this experiment and make it difficult to draw comparisons between this and previous studies on the impact of protein supplementation on gastrointestinal parasitism. Other studies using older lambs fed high and low protein diets have shown significant effects upon the pathogenicity of *H.contortus* (Abbott *et al.*, 1986) and *T.colubriformis* faecal egg counts/worm burdens (Kambara *et al.*, 1993). However, in both these studies the low protein diets would have markedly influenced both host resilience and resistance.

Within the constraints of the experimental design using 6 to 7 month old lambs it is apparent that protein supplementation did not significantly enhance immunoresponsiveness towards *N.battus*. However, it is clear that the mechanism involved is acquired from previous exposure and is manifested by a local inflammatory response and increased levels of anti-worm IgG. Differences in magnitude of the measured responses did not account for the ability of lambs to expel worms in this study. It is clear that further studies are required to determine the importance of other components of the immune response and to examine age effects upon host/*N.battus* interactions.

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