A study on variation in immune responses and protective mechanisms to *Ostertagia circumcincta* and their relationship with parasitological parameters in Scottish blackface sheep

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ABSTRACT

The aim of this study was to examine the variation in immunological control of *Ostertagia circumcincta*, following natural exposure of sheep by grazing contaminated pasture, and relate this variation to that in parasitological parameters. Selection of sheep by faecal egg count resulted in different results in two consecutive years, in the first year those sheep with higher faecal egg counts had more adult female worms, in the second those sheep with higher faecal egg counts had longer, more fecund worms. These observations are consistent with earlier studies showing that faecal egg count is a function of worm burden and worm fecundity.

Immunophenotyping of peripheral blood revealed that lymphocyte subset percentages are repeatable and that lymphocyte numbers were greater in grazing lambs. A negative association between worm burden and B cell percentage representation of peripheral blood lymphocytes was observed, possibly as a result of immune modulation, whilst immunophenotyping of cells from lambs given a deliberate challenge provided further evidence of immune-modulation by *O. circumcincta*. Following natural infection those sheep with a larger abomasal nodes had shorter worms, suggesting that the magnitude of the immune response was important.

Proliferation assays emphasised the importance of the local immune response by showing that responses to antigen were greater in lymphocytes from local tissues. Lymphocyte responses were dependent on previous exposure. Negative associations were observed between worm burden and proliferative responses, and because there was no evidence of active control of worm burden the results also provided further evidence of immune modulation.

Enumeration of mast cells and globule leucocytes showed that there was a marked increase in the density of these cells following exposure to *O. circumcincta*, and

although there was no evidence that these cells were involved in the control of worm burden there was a relationship between the globule leucocyte density and worm length that suggest this cell type may have a direct role in restricting or controlling worm length. The observation that lambs were able to mount a vigorous mast cell and globule leucocyte response although there was no evidence that they were able to control worm burden raises the possibility that these cell types may have a direct role in the control of the *O. circumcincta* burden.

Parasite specific IgA responses were investigated and shown to increase with exposure and a positive association was observed between worm burden and local IgA activity following deliberate infection. The study provided further evidence that parasite-specific IgA activity has a role in regulating worm length.

In conclusion the work in this thesis demonstrated considerable individual variation in the mechanisms of immunological control and host protection. The results provided further evidence of the role of limiting worm length and fecundity in the genetic control of *O. circumcincta.* Many of the findings could best be explained by suggesting immune modulation of the host by the parasite. It is likely that further studies on how *O. circumcincta* attempts to modulate the immune response, and whether or not the host can resist these attempts, will greatly increase our understanding of the host-parasite interface.

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

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ABBREVIATIONS

A/Aug	August
AC	after challenge
ANC	abomasal node cells
AP I/II	Adult preparation I/II
αβ T cell receptor	alpha-beta T cell receptor
BC	before challenge
Bq	Bequerel
BSA	bovine serum albumin
°C	degrees Celsius
cm	centimetre
Con A	concanavalin A
Cpm	mean counts per minute
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	dithio-L-thrietol
EDTA	ethyldiamine-tetraacetic acid
EL_4	early fourth stage larvae
ELISA	enzyme-linked immunosorbent assay
epg	eggs per gram (of faeces)
FITC	fluorescein isothiocyanate isomer-1 conjugate
FWEC	faecal worm egg count
g	gram
g	unit gravity (10^{-11}Newtons)
GBq	Gigabequerel
gd/γδ T cells	gamma-delta T cells
GI	gastrointestinal
GL	globule leucocyte
GM-CSF	granulocyte-macrophage colony-stimulating-factor
GUVS	Glasgow University Veterinary School
HCl	hydrochloric acid
H. contortus	*Haemonchus contortus*
HI-FCS	heat-inactivated foetal calf serum
HWS	Hank's washing solution
IFN-γ	interferon gamma
IgA/D/E/G/M	immunoglobulin subclasses A, D, E, G and M
IL-2,-3,-4,-5,-6,-9,-10	Interleukin-2,-3-4,-5,-6,-9,-10
J/Jun	June
J/Jul	July
kDa	kilodaltons
kg	kilogram
L_3	third stage larvae

L_3SE	third stage larval somatic extract
L_4	fourth stage larvae
L_5	fifth stage larvae
log	logarithm
M	molar
MC	mast cell
2-ME	2-mercaptoethanol
mg	milligram
MHC	major histocompatibility complex
ml	millilitre
mM	millimolar
mm	millimetre
MMC	mucosal mast cells
MSB	Martius scarlet blue
µg	microgram
µl	microlite
µm	micrometre
N/A	not available
NBF	10% neutral buffered formalin
N. brasiliensis	*Nippostrongylus brasiliensis*
N. dubius	*Nematospiroides dubius*
nm	nanometre
O/Oct	October
O. circumcincta	*Ostertagia circumcincta*
O. ostertagi	*Ostertagia ostertagi*
OD	optical density indices
p	probability
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PGE	parasitic gastroenteritis
pH	negative log of the hydrogen concentration
PM	post-mortem
PMSF	phenylmethylsulphonyl flouride
RPE	R-phycoerythrin
RT	room temperature
S/Sept	September
SI	stimulation index
SMCP	sheep mast cell protease
spp.	species
TCM	tissue culture medium
T. colubriformis	*Trichostrongylus colubriformis*
T. vitrinus	*Trichostrongylus vitrinus*
TGF-α	tumour necrosis factor-alpha
Th1/2	T helper cells, subtypes 1 and 2
T. muris	*Trichuris muris*
T. spiralis	*Trichinella spiralis*

u	unit
UK	United Kingdom
USA	United States of America
v/v	volume per volume
w/v	weight per volume
%	per cent

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CHAPTER 1: GENERAL INTRODUCTION

Metazoan parasites have the most complex structure of the pathogens of mammals and consequently, relative to other pathogens, have a long period of development from egg to fecund adult. In helminths much of this development takes place within the host and survival depends on evading or resisting the host's protective response. The hosts and their parasites have evolved together; the hosts developing an increasingly sophisticated challenge to the parasites and the parasites developing increasingly sophisticated mechanisms of evading, neutralising or surviving that challenge, and therefore hosts and parasite have developed a very close, though not harmonious, relationship. This view of an evolutionary battle without winners - but over the millenniams there will have been many losers - has been described as the 'Red Queen Hypothesis', after the character in Lewis Carroll's *Alice in Wonderland,* (1865), because one must keep running to stand still. The hypothesis applies to both hosts and parasites.

Sheep, *Ovis aries*, have a foraging strategy that favours grass over frond species, and consequently they are particularly vulnerable to gastrointestinal (GI) parasites that have a direct faecal-oral life cycle, a niche successfully occupied by nematodes. Recently, on the evolutionary time scale, man has interfered in the relationship between sheep and nematodes by increasing the stocking density and changing population structures - usually in favour of young susceptible animals - and replaced natural selection with his own selection criteria. Many of these changes have favoured the parasite but man, through the application of epidemiological knowledge and the use of efficacious anthelmintics, has limited parasitism of sheep. Anthelmintics put great selection pressure on nematodes: nematodes are rapidly evolving resistance to the anthelmintics and now threaten sheep production.

The close physical relationship and evolutionary battle between sheep and nematodes makes the study of their association of great interest. The opportunity to interfere in their relationship, by manipulating the sheep's protective response or by

accelerating the evolution of the sheep by artificially selecting for enhanced resistance, provides economic justification for this area of study.

1.1: NEMATODE PARASITES OF SHEEP IN THE BRITISH ISLES

Several studies have provided information on the species of nematodes occurring in the GI tract of sheep in the United Kingdom (UK) (Morgan *et al.*, 1951; Wilson *et al.*, 1953; Thomas and Boag, 1972; Reid and Armour, 1975). Table 1.1 provides a comprehensive guide to the species and their predilection sites.

In the UK *Ostertagia circumcincta* (*Teladorsagia circumcincta*) is the most prevalent nematode in grazing sheep (Morgan *et al.*, 1951; Boag and Thomas, 1971; Thomas and Boag, 1972; Reid and Armour, 1975), and is considered to be the most important (Urquhart *et al.*, 1987). In natural conditions *O. circumcincta* infection always occurs with concurrent small intestinal parasitism and this mixed parasitic infection causes the clinical syndrome of parasitic gastroenteritis (PGE) (Armour and Coop, 1991). *Trichostrongylus vitrinus* is the most numerous of the ovine small intestinal species in the UK (Reid and Armour, 1975). Occasionally *Haemonchus contortus* can occur in large numbers (Wilson *et al.*, 1953).

Site	Species
Abomasum	*Ostertagia circumcincta* *Ostertagia trifurcata* *Ostertagia pinnata* *Haemonchus contortus* *Trichostrongylus axei*
Small intestine	*Trichostrongylus vitrinus* *Trichostrongylus colubriformis* *Cooperia curticei* *Nematodirus battus* *Nematodirus filicollis* *Nematodirus spathiger* *Strongyloides papillosus* *Bunostomum trigoncephalum*
Large intestine	*Trichuris ovis* *Oesophagostomum venulosum* *Chaberta ovina*

Table 1.1: Predilection sites of nematode species in the GI tract of sheep in the UK.

1.2: LIFE CYCLE OF *O. CIRCUMCINCTA*

The life cycle of *O. circumcincta* is direct, following a typical trichostrongylid pattern. Unembryonated eggs are passed in the faeces and develop to infective third stage larvae (L_3) within the faecal pellet, where they stay until the arrival of moist conditions initiates migration onto the sward (Urquhart *et al.*, 1987). Given optimum conditions the development from egg to L_3 can occur within two weeks, but the larvae can survive on pasture for many months (Gibson and Everett, 1972). The ingested L_3 ex-sheathe in the rumen, and within 48 hours invade gastric glands of the abomasum and moult to fourth stage larvae (L_4) (Smith *et al.*, 1984). Thereafter larvae may either develop directly through fifth stage larvae (L_5) to adults, or development may arrest with the larvae remaining as the early fourth stage larvae (EL_4) (Sommerville, 1953). Early fourth stage larvae can remain in the

gastric pits for up to three months (Sommerville, 1953), surviving for longer than the adults (Armour *et al.*, 1966), and then resume development (Dunsmore, 1963). If larvae develop without arresting, immature adults are present by day 8 or 9, females containing eggs are present by day 12, all the worms are adult by day 20, eggs are first passed in the faeces during days day 14 to 16 and faecal egg counts peak about day 35 before falling to very low levels by day 60 (Threkeld, 1934 cited by Denham, 1969; Armour *et al.*, 1966; Denham, 1969; Stear *et al.,* 1995a).

1.3: EPIDEMIOLOGY

There is a seasonal occurrence of PGE and a seasonal pattern of worm burdens and faecal egg counts. In the ewe both faecal egg counts and worm burdens peak in the spring (Taylor, 1935; Hawkins and Cole, 1945; Morgan *et al.*, 1950; Morgan *et al.*, 1951; Wilson *et al.*, 1953; Reid and Armour, 1975) whereas in lambs faecal egg counts and worm burden peak in late summer (Hawkins and Cole, 1945; Morgan *et al.*, 1950; Crofton, 1955; Reid and Armour, 1975; Waller and Thomas, 1978a). In the ewe the 'spring rise', or more correctly the peri-parturient rise, which occurs around parturition and peaks in early lactation, is caused by a relaxation in the immune response of the ewe (Soulsby, 1957; O'Sullivan and Donald, 1970; O'Sullivan and Donald, 1973), and results in a peak in the L_3 numbers on pasture in mid-summer (Crofton, 1949; Heath and Michel, 1969). Residual contamination of pasture from larvae that have over-wintered can also contribute to the L_3 numbers (Salisbury and Arundel, 1970). If there is no residual contamination from the previous year's grazing, eggs passed by the ewe during the peri-parturient rise are the only source of L_3 and the lamb faecal egg counts peak in the late summer (Boag and Thomas, 1971). Some years there is a biphasic pattern of egg output by the lambs (Crofton, 1955), with an early peak in lamb egg counts, resulting from patency of larvae acquired from the over-wintered population, followed by a second peak in the late summer, resulting from contamination of pasture by eggs passed either by the ewe during the peri-parturient rise or by the lambs in their first peak

(Thomas and Boag, 1972). The results presented by Boag and Thomas (1971) and Thomas and Boag (1972) provide a useful model for the epidemiology of PGE, but there are many variables that influence the epidemiological pattern, and disease outbreaks can occur in sheep of all ages at any time of year (Ollerenshaw *et al.*, 1978). These variables include weather (Morgan *et al.*, 1950; Gibson and Everett, 1972; Gibson and Everett, 1973a; Anderson, 1973), previous years weather (Ollerenshaw *et al.*, 1978), breed (Stewart *et al.*, 1938), anthelmintic treatment (Barger, 1988) and grazing and management factors (Crofton, 1949; Crofton, 1955).

1.4: PATHOLOGY

A classic study on the pathological changes caused by *O. circumcincta* was a study by Armour *et al.* (1966) of an experimental, large, single challenge infection of naïve sheep. They showed that infected gastric glands became stretched by the presence of larvae and that the epithelium became hyperplastic and poorly differentiated; similar changes occurred in the epithelium of adjacent glands and resulted in raised circular nodules which were appreciable on gross examination. After the larvae had emerged from the glands there was cytolysis and cell sloughing of much of the epithelium and the glands collapsed, giving the nodules an umbilicated appearance. The surviving epithelium of the infected glands contained mucus producing cells, whilst the surrounding glands resolved to the normal. In heavy infections the lesions were confluent and gave the mucosa the gross appearance of 'Moroccan leather'. The early cell response was a lymphoreticular reaction in the basal lamina propria, especially prominent around and adjacent to the vascular beds. Neutrophils and eosinophils migrated into the infected glands. As the lesions progressed, the lymphoreticular response became more marked with a radial distribution of plasma cells. By day 21 there were globule leucocytes in the epithelium, especially in the upper zone. These changes were similar to, but occurred more rapidly than, those described in calves infected with *Ostertagia ostertagi* (Ritchie *et al.*, 1966).

1.5: PATHOPHYSIOLOGY

The pathophysiology has been reviewed by McKellar (1993). The loss of parietal cells in hyperplastic and dysplastic glands results in a functional impairment in acid production and a consequent increase in abomasal content pH from 2 up to 6.8 (Armour *et al.*, 1966; Jennings *et al.*, 1966). *Ostertagia circumcincta* actively suppresses acid production and this may contribute to the increase of pH and favour parasite survival (McLeay *et al.*, 1973; McKellar, 1993). In bovine ostertagiosis an increase in plasma pepsinogen is associated with structural changes in the mucosa and there may be leakage of macromolecules, including pepsinogen, from the lumen into the circulation (Armour *et al.*, 1966). A similar mechanism may occur in ovine ostertagiosis, although more recent evidence suggests that *Ostertagia* spp. in sheep and cattle increase pepsinogen production by stimulating the zymogen cells (McKellar, 1993) - indeed new work suggests that zymogen cells from previously exposed sheep produce more pepsinogen, possibly due to a hypersensitivity reaction (Scott and McKellar, 1998). There is a net loss of albumin into the GI tract and this is thought to be due to the macromolecular leak following structural changes resulting from emergence of larvae (Holmes and MacLean, 1971; Coop *et al.*, 1977). The rise in abomasal pH may partly be responsible for increased plasma levels of the peptide hormone gastrin (Anderson *et al.*, 1981) although *Ostertagia* spp. may directly stimulate the gastrin release (Anderson *et al.*, 1985); it has been hypothesised that high gastrin levels reduce GI motility and appetite (Fox *et al.*, 1989; McKellar 1993).

Ostertagiosis causes a reduction in feed intake, nitrogen digestibility, energy digestibility, the gross efficiency of metabolisable energy for growth, and in calcium and phosphorus deposition (Holmes and MacLean, 1971; Parkins *et al.*, 1973; Coop *et al.*, 1977; Sykes and Coop, 1977). All contribute to a reduction in live-weight gain and poor carcass conformation (Coop *et al.*, 1977). Wool growth is also impaired (Symons *et al.*, 1981).

The reduction in nitrogen digestibility is largely due to an increase in endogenous protein losses, and although there may also be a reduction in the digestion of exogenous proteins due to reduced pepsin activity, the small intestine probably compensates for any failure of protein digestion in the abomasum, unless the infection is severe (Parkins *et al.*, 1973; Holmes, 1985). Parasitic gastroenteritis is a mixed infection and there is always an abomasitis and enteritis. Concurrent infection with *O. circumcincta* and *Trichostrongylus colubriformis* exacerbated the reductions in nitrogen digestibility and live-weight gain that were observed in *O. circumcincta* infections (Steel *et al.*, 1982; Sykes *et al.*, 1988) but experiments with concurrent infections of *O. circumcincta* and *T. vitrinus* have failed to replicate this effect, possibly due to the *O. circumcincta* population lowering the *T. vitrinus* worm burden (Coop *et al.*, 1986; Coop *et al.*, 1988). With the exception of the work by Holmes and MacLean (1971), these conclusions stem from experiments in which diarrhoea was not a feature. In clinical disease diarrhoea is a feature but the mechanism responsible for diarrhoea remains obscure (Barker *et al.*, 1993).

Pathophysiological changes following challenge are not restricted to naïve individuals, and following a heavy larval challenge of immune ewes plasma pepsinogen concentrations are elevated and there is evidence of albumin catabolism and loss of plasma protein into the GI tract (Yakoob *et al.*, 1983).

1.6: CLINICAL SIGNS

In grazing sheep *O. circumcincta* infection always occurs with concurrent small intestinal parasitism and the mixed infection causes PGE, which commonly occurs in groups of lambs grazing contaminated pastures in late summer. The clinical signs are well described in text books (Urquhart *et al.*, 1987; Armour and Coop, 1991; Radostitis *et al.*, 1996). Infected lambs fail to thrive, are inappetant and soiling of the hindquarters is common, and although lambs may have dark green or black, loose faeces, profuse fluid diarrhoea is infrequently seen. Severely affected

animals may be dehydrated. Similar clinical signs occur in Type II ostertagiosis, a disease caused by the mass emergence of inhibited larvae from the abomasum in hoggs, gimmers and mature ewes during the winter months (Morgan *et al.*, 1950; Reid and Armour, 1973).

Diarrhoea is not a feature of most experimental infections (Armour *et al.* 1966; Sykes and Coop, 1977; Coop *et al*, 1986), but diarrhoea has been induced by infections with 1.2 x 10^5 larvae (Jayawickrama and Gibbs, 1967) or 9 x 10^5 larvae (Holmes and MacLean, 1971). Jayawickrama and Gibbs (1967) also reported clinical anaemia and a reduced haematocrit. Although haematological changes have been reported (Threlkeld and Downing, 1936), anaemia is not normally considered a feature of ostertagiosis or PGE and other workers have failed to find haematological changes after a heavy challenge (Holmes and MacLean, 1971).

Chronic diarrhoea has been recorded in Finnish Landrace lambs, but not in the less resistant Suffolk cross lambs, exposed to high levels of larval challenge and dosed frequently with anthelmintic. These lambs did not have high egg counts and the diarrhoea probably reflects an immunopathology caused by the lambs' response to the larval challenge (Suttle and Brebner, 1995).

1.7: DIAGNOSIS

Diagnosis is based on grazing and treatment history and clinical signs. Faecal egg counts are not always proportional to, and may be independent of, the size of larval challenge or worm burden (Coop *et al.*, 1977; Gibson and Parfitt, 1977; Gibson and Everett, 1978; Jackson and Christie, 1979; Callinan and Arundel, 1982). Negative or low faecal egg counts may be misleading because Type II ostertagiosis may present before patency, lambs on a suppressive anthelmintic regimen may be exposed to high levels of larval challenge and develop diarrhoea despite zero egg counts (Taylor and Kenny, 1995) and large reductions in performance have been

recorded despite relatively low faecal egg counts of 200-600 eggs per gram of faeces (Coop *et al.*, 1977). Therefore, faecal egg counts can demonstrate that infection is present but they are an insensitive measure of pathological change. Plasma pepsinogen levels are a valuable diagnostic aid and in clinical disease exceed 2.0 International Units (Coop *et al.*, 1977; Urquhart *et al.*, 1987). A definitive diagnosis depends upon post-mortem worm counts of cohorts, with total worm burdens of 10,000 to 50,000 being considered significant (Barker *et al.*, 1993).

1.8: IMMUNITY TO *O. CIRCUMCINCTA* AND OTHER GI NEMATODES

Miller (1996) described and discussed the major findings from rodent models, as well as ruminants, to review the literature on immunity to GI nematodes. The rodent models have been extremely valuable to our understanding of the host-parasite relationship, but they remain as models and if we extrapolate from one host-parasite relationship to another we must be cautious. The following discussion will focus mainly on the responses of sheep to gastrointestinal nematodes in general and to *O. circumcincta* in particular.

1.8.1: Evidence of acquired resistance to *O. circumcincta*

In 1929 Stoll, through his work on *H. contortus*, provided evidence of acquired resistance of sheep against nematodes, and Stoll and Nelson (1931) demonstrated an immune response to parasite antigen. There is much circumstantial evidence of acquired resistance. Prior exposure to *O. circumcincta* reduces the late summer peak (Thomas and Boag, 1972; Anderson, 1973). A slow rise in pasture larvae count prevents a high level of infection following subsequent high levels of exposure (Gibson and Everett, 1973b) and a low level of experimental trickle infection prevents high worm burdens from a subsequent and much greater

challenge (Gibson and Everett, 1977; Gibson and Everett, 1978). Worms from lambs exposed to a long infection exhibit morphological changes in the vulvar flap that are not seen in worms from naïve tracer lambs (Waller and Thomas, 1978b). Definitive proof that resistance to infection can be acquired was provided by giving an immunising challenge, then treating with anthelmintic to remove the immunising population, and then re-challenging and comparing the parasitology with that from a challenge of naïve animals (Elliot and Durnham, 1976; Smith *et al.*, 1983a; Smith *et al.*, 1983b). This resistance is due to an active rather than a passive process (Smith *et al.*, 1984).

1.8.1.1: Control of *O. circumcincta* worm burden

When lambs were exposed to a trickle infection for varying lengths of time the worm burden in the host peaked and then declined (Hong *et al.*, 1987; Seaton *et al.*, 1989), but even when the worm burden was increasing it represented a decreasing percentage of the total challenge (Coop *et al.*, 1977). In trickle infections there is a turnover in worm population, the adults have a short life and the decline in total population is caused by a greater loss of adults than the acquisition of new parasites (Waller and Thomas, 1978a; Hong *et al.*, 1987). In deliberate trickle infections there is development of resistance to the establishment of incoming larvae (Seaton *et al.*, 1989; Hong *et al.*, 1989; Dobson *et al.*, 1992), although experiments in grazing lambs of a similar age have failed to demonstrate this effect (Waller and Thomas, 1978b). When lambs were immunised by a trickle infection and subsequently challenged with a large single dose of infective larvae, adults acquired from development of the immunising infection were rejected (Hong *et al.*, 1989) - this 'self cure' phenomenon had previously been described for sheep infected with *H. contortus* (Stoll, 1929; Stewart, 1950a). As was stated above, previous exposure provides protective immunity against subsequent challenge (Elliot and Durnham, 1976; Smith *et al.*, 1983b; Smith *et al.*, 1984).

There is a rapid turnover in the worm population and immune lambs acquire mechanisms to prevent new larvae from establishing and to limit worm numbers; there are also mechanisms whereby adult worms can be rejected.

1.8.1.2: Inhibition of *O. circumcincta* EL_4

There is a seasonal increase in the proportion of EL_4 at the end of the grazing season (Anderson *et al.*, 1965; Armour *et al.*, 1969; Reid and Armour, 1972; Reid and Armour, 1975) and, because this phenomenon occurs in susceptible lambs (Connan, 1968; Reid and Armour, 1972; Waller and Thomas, 1978a), it is likely that larval development arrests due to environmental factors rather than being due to the acquisition of host immunity. Cold stressing *O. ostertagi* larvae for eight weeks induces arrested development in calves (Armour and Bruce, 1974) but experiments in which *O. circumcincta* larvae were cold stressed for six months failed to demonstrate the phenomenon in sheep (Connan, 1969). However, the reason for failure may have been that the cold stress period in this experiment was too long because Armour and Bruce (1974) showed that there is an optimum period of cold stress, and that normal development can occur after extended periods of cold stress. Dunsmore (1960) demonstrated that there can be population effects, with a greater percentage of the challenge dose arresting at higher levels of challenge.

Soulsby (1957) suggested that the host may play a role in inhibited larval development, and Dunsmore (1961) showed that host factors can be responsible for larval inhibition in naïve lambs. When lambs received trickle infections for different periods of time, those that received a longer dosing regimen had a lower worm burden, but because there was a similar number of inhibited larvae a greater proportion of their worm burden was EL_4 (Coop *et al.*, 1977). Similarly, Smith *et al.* (1983b) challenged previously exposed, but worm free, sheep and naïve sheep and showed that the previously exposed sheep had fewer worms than the naïve

lambs but a similar number of EL_4, therefore a greater proportion of their burden was EL_4. Two mechanisms, both due to acquired immunity, can account for these findings: either the more immune sheep prevent establishment of L_3 and actively inhibit those worms that do establish, or a similar number of L_3 establish but there is a preferential loss of adults, leaving a reduced worm burden of mainly EL_4. In one study involving sequential post-mortem examinations it was shown that by 48 hours there was a reduced worm burden and that larval development ceased before day 5; this work supports the first hypothesis and showed that larvae arrest as a consequence of acquired resistance (Smith *et al.*, 1984). Seaton *et al.* (1989) elegantly showed that as experimental trickle infections continued, increasing proportions of the larvae that established remained as EL_4. In naïve controls no larvae arrested, or were inhibited, demonstrating that in this experiment arrested development was not due to seasonal effects, also the proportion of incoming larvae that inhibited increased at a time when the worm burden was decreasing, demonstrating that arrested development was not due to population effects but to an acquired response.

In natural and deliberate infections a high proportion of the worm burden can be EL_4. Depending on a number of variables, this may be due to larvae becoming arrested due to climatic effects on the free-living stages or host responses inhibiting larval development or a combination of the two.

1.8.1.3: Reduction in fecundity in *O. circumcincta*

In experimental infections faecal egg counts reach a peak and then decline: this occurs in single challenge infections (Armour *et al.*, 1966; Gibson and Parfitt, 1977) and also in trickle infections (Coop *et al.*, 1977; Gibson and Everett, 1977; Jackson and Christie, 1979; Hong *et al.*, 1987). Because the worm burden also peaks and then declines (see above) a peak and decline in egg counts might be expected, but it has been observed that the decline in egg counts was more rapid

than the decline in adults, suggesting that anti-fecundity mechanisms were responsible (Hong *et al.*, 1987). The existence of anti-fecundity mechanisms had been discussed in the literature; Taylor (1935) discussed the unpublished observation that the fecundity of trichostrongyles from resistant lambs was a twelfth that of the fecundity of worms from susceptible lambs and Tetley (1941) recorded that in sheep infected with *Nematodirus* spp. variation in faecal egg count was correlated with worm fecundity rather than worm burden. There is also evidence of morphological changes that may be responsible for a reduced fecundity, because as trickle and natural *O. circumcincta* infections progressed the adult worms became shorter and the vulvar flap smaller, rudimentary or absent (Waller and Thomas, 1978b; Hong *et al.*, 1987; Seaton *et al.*, 1989). Shorter worms contain fewer eggs *in utero* (Coop *et al.*, 1977; Gibson and Everett, 1978; Stear *et al.*, 1995c), therefore one manifestation of the host acquired response is shorter worms with fewer eggs. There is also a change in the sex ratio towards greater proportions of males as the hosts become resistant and this is an anti-fecundity mechanism (Waller and Thomas, 1978b).

In summary, as resistance to infection develops fewer larvae establish, more of the larvae that do establish are inhibited and the surviving adults reach shorter adult length, develop morphological defects and contain fewer eggs. In addition adults worms can also be rejected. Combined these mechanisms reduce the worm burden and the faecal egg count and hence reduce pasture contamination.

1.8.2: Identification of protective host responses to GI nematodes in sheep

There is an immune response that limits infection with GI nematodes and there are a number of candidate mechanisms which might be responsible for protection.

1.8.2.1: The mast cell and globule leucocyte

Immunoglobulin E-mediated Type I hypersensitivity has long been considered to be a key component of the host response in many host-parasite relationships (Jarrett and Miller, 1982). Experiments with rodent models provided evidence of a functional role for the mast cell in protection against gut nematodes. Following cross-linking of bound IgE by specific antigen, mast cells release mediators which increase mucosal permeability, resulting in a macromolecular leak that allows direct contact between circulating antibodies and nematodes (Mulligan *et al.*, 1965; Barth *et al.*, 1966; Murray *et al.*, 1971; Scudamore *et al.*, 1995). Mediators can act non-specifically to create a hostile micro-environment (Lee *et al.*, 1986; Rothwell, 1989; Baird and O'Malley, 1993) and they can also have direct anti-parasite activity (Douch *et al.*, 1996). Mast cells are functionally active during expulsion of nematodes in rats (Woodbury *et al.*, 1984). Expulsion of *Trichinella spiralis* and *Strongyloides ratti* is delayed in mast cell-deficient mice but the reconstitution of mastocytosis by bone marrow grafting restores expulsion (Oku *et al.*, 1984; Nawa *et al.*, 1985). If mastocytosis is abrogated, by preventing the function of stem cell factor, then expulsion of *T. spiralis* fails (Grencis *et al.*, 1993).

It is now known that the mast cell is more than an effector cell and can express and release a number of cytokines, such as interleukin-3,-4,-6 (IL-3, IL-4, IL-6), tumour necrosis factor-α (TNF-α) and granulocyte-macrophage colony-stimulating-factor (GM-CSF); therefore it has been proposed that the mast cell plays a role in regulating the immune response (Gordon *et al.*, 1990).

The globule leucocyte is derived from mucosal mast cells (MMC) and is only observed in the presence of parasites (Murray *et al.*, 1968; MacDonald *et al.*, 1980). Following antigenic stimulation, sub-epithelial MMC discharge and move into an intra-epithelial site to become globule leucocytes (Huntley *et al.*, 1984).

There is now a large body of evidence supporting an active role for mast cells and globule leucocytes in controlling worm numbers of GI nematodes in sheep, much of it coming from the association of a greater tissue response in individuals with fewer worms. O'Sullivan and Donald (1973) investigated the peri-parturient rise in ewes exposed to *H. contortus* and *T. colubriformis*, and observed that non-reproductive ewes had a much lower worm burden, but more mast cells and many more globule leuccytes than pregnant and lactating ewes. In the non-lactating ewes there was a negative correlation between the worm burden and the globule leucocyte count. Christie *et al.* (1978) observed large numbers of globule leucocytes and MMC in sheep resistant to *H. contortus*. Lambs genetically resistant to *H. contortus* had higher mast cell and globule leucocyte counts than susceptible lambs of the same breed (Gill, 1991). Gamble and Zajac (1992) investigated variation in resistance to *H. contortus* between breeds, and although there were no differences in MMC counts, resistant breeds had a higher globule leucocyte count.

In sheep grazing *T. colubriformis* contaminated pasture there was a strong correlation between the globule leucocyte count and resistance (Douch *et al.*, 1986). Sheep genetically resistant to *T. colubriformis* had more tissue and lumenal globule leucocytes than susceptible sheep, and within the group of resistant sheep there was a negative correlation between globule leucocyte count and worm burden (Stankiewicz *et al.*, 1993). In several studies it has been shown that there was within-breed variation in the response to vaccination against *T. colubriformis*; some individuals were high responders and were protected from a challenge following vaccination, others were low responders and were not protected (Dineen *et al.*, 1978; Dineen and Windon, 1980). High responders had a higher globule leucocyte count and, moreover, within the high responder population there was a trend towards those lambs with more globule leucocytes having fewer worms (Dineen *et al.*, 1978; Dineen and Windon, 1980). Douch and Morum (1993) suggested that globule leucocytes may also limit worm fecundity, after observing that in a short primary *T. colubriformis* infection globule leucocytes were not correlated with worm burden but were with the faecal egg count.

There is also evidence of mast cell function in protective responses. In various studies it has been shown that there is a chronological association between mast cell activity and protection as well as increased level of mast cell mediators in resistant sheep. When immune sheep were challenged with *T. colubriformis*, larvae were rejected within 24 hours and this process was chronologically correlated with the appearance of globule leucocytes (McClure *et al.*, 1992). During a trickle infection of *T. colubriformis* mast cells became sensitised to parasite antigen at the same time as effective immunity against larval establishment developed (Bendixsen *et al.*, 1995). During the 'self cure' of *H. contortus* there was an increase in the peripheral blood concentration of histamine, a mast cell mediator (Stewart, 1953). Mast cell proteases were detected in both serum and gastric lymph when immune sheep were challenged with *H. contortus* and *O. circumcincta* (Huntley *et al.*, 1987) and isolated mast cells and globule leucocytes from parasitised sheep released mast cell proteases when incubated with parasite antigen (Jones *et al.*, 1992). High responders to vaccination against *T. colubriformis* had higher levels of mast cell mediators, such as histamine and leukotrienes, than low responders (Jones *et al.*, 1990). Small intestine mucus from resistant sheep inhibited *in vitro* migration of *T. colubriformis* larvae (Douch *et al.*, 1983), and this effect was greatest in individuals with more globule leucocytes (Douch *et al.*, 1984; Douch *et al.*, 1986). More recent work shows that globule leucocyte mediators directly inhibited larval migration (Douch *et al.*, 1996).

Mast cell deficient strains of sheep are not available, but mast cell and globule leucocyte responses of resistant sheep have been reduced, by either treatment with corticosteroids or depletion of CD4+ T cells, resulting in abrogated resistance; some caution should be used in interpreting these findings because it is likely that the treatments also altered other active immune responses (Douch *et al.*, 1986; Huntley *et al.*, 1992; Gill *et al.*, 1993b).

The specificity of protective mechanisms has been investigated. Sheep immune to one species of nematode have been challenged with either a heterologous nematode

or a mixed dose of heterologous and the immunising nematodes. Generally, results show that an immunologically specific encounter is needed to initiate a non-specific effect which protects against the immunising species and, as long as the heterologous parasite occupies the same or a more distal predilection site, the heterologous species (Dineen *et al.*, 1977; Emery *et al.*, 1993). A specific trigger followed by a non-specific effect would be compatible with parasite antigen being bound by IgE on mast cells and leading to release of mast cell mediators that result in non-specific damage to nematodes.

There is a considerable volume of evidence for a role of mast cells in controlling the *O. circumcincta* burden. It has long been known that sheep exposed to *O. circumcincta* have increased numbers of globule leucocytes in the abomasum (Whur, 1966; Armour *et al.*, 1966; Jackson and Christie, 1979). In a more recent study in which sheep were given a trickle infection there was a negative correlation between the number of globule leucocytes, though not mast cells, and the worm burden (Seaton *et al.*, 1989). Similar observations have been made in sheep that were naturally exposed and then deliberately challenged (Stear *et al.*, 1995c). Within the first 24 hours of immune sheep being challenged with *O. circumcincta*, larvae are prevented from establishing and there is a rise in the pepsinogen concentration of gastric lymph, probably due to a hypersensitivity reaction (Smith *et al.*, 1984). The rise in pepsinogen is associated with an increased mast cell proteinase concentration in the gastric lymph (Huntley *et al.*, 1987; Stevenson *et al.*, 1994).

From these studies it has been surmised that mast cells and globular leucocytes control worm burdens by larval exclusion. However, larval exclusion can occur without the presence of large numbers of mast cells and other mechanisms may also play a role (Huntley *et al.*, 1992).

1.8.2.2: Antibody responses

Raised serum antibody to a pathogen is a feature of immunological responses. Serum antibody may function in the hypersensitivity reaction resulting from mast cell mediator release (see above). Immunoglobulin G (IgG) may also have direct anti-parasite activity and associate with intestinal mucus to reduce larval establishment (Carlisle *et al.*, 1990). Immunoglobulin A (IgA) is produced at the mucosa (Quin *et al.*, 1975; Lascelles *et al.*, 1984), where it protects the gastrointestinal tract against mucosal bacteria and viruses (Gangulay and Waldman, 1980) and is a potential candidate for protective immune responses against GI nematodes (Miller *et al.*, 1983). Immunoglobulin A may act by limiting worm growth by blocking nematode feeding mechanisms (Smith *et al.*, 1985). In rodent studies it has been demonstrated that passive transfer of immunity can be achieved using monoclonal IgA (Roach *et al.*, 1991). IgE is necessary for the mucosal hypersensitivity reaction mediated by mast cells (see above).

Hawkins and Cole (1945) observed that serum from lambs could precipitate on the mouth parts, anus, excretory pore and cuticle of *O. circumcincta* L_3, and that those lambs that had serum which produced a greater degree of precipitation had lower egg counts. Stewart (1950b) showed an enhanced serological response to a second infection with *Trichostrongylus* spp., and that older more resistant sheep had higher titres. Various workers have demonstrated within-breed variation in antibody responses, and titres have been negatively correlated with parasitological parameters (Stewart, 1950a; Soulsby, 1957; Windon and Dineen, 1981; Gill, 1991; Douch *et al.*, 1995a). Serum IgG titres have also correlated with other immunological responses, such as mast cell responses, and therefore they may have no direct immunological role and their importance may be as a marker of resistance (Douch *et al.*, 1995a). However, McClure *et al.* (1992) showed that following challenge of immune sheep with *T. colubriformis* there was an initial rise in mucus IgG_1, possibly due to a 'leak lesion', and this observation provides a mechanism for the direct interaction between serum IgG and parasite, and suggests that IgG may

play a role in preventing larval establishment. A glycoprotein from the oesophageal glands of *O. circumcincta* L_3 - an excretory/secretory product - is recognised by serum from resistant lambs, but not susceptible lambs (McGillivery *et al.*, 1989; McGillivery *et al.*, 1990), and when purified and used as a vaccine it raised antibody levels and induced protection; although this provided evidence for a protective role for serum antibodies (McGillivery *et al.*, 1992) subsequent attempts to repeat this work have failed (Morton *et al.*, 1995).

Abomasal mucus from sheep immune to *H. contortus* contains high levels of parasite specific IgA which may be involved in protection (Smith, 1977; Smith and Christie, 1978; Duncan *et al.*, 1978). Sheep genetically resistant to *H. contortus* had a greater local IgA response, and there was a negative correlation between IgA and faecal egg count (Gill *et al.*, 1993b; Gill *et al.*, 1994).

Much of the revealing work on IgA has been in *O. circumcincta* infected sheep. Challenge of sheep immune to *O. circumcincta* has been associated with a marked increase in parasite-specific and total IgA in the gastric lymph (Smith *et al.*, 1983b). In a single challenge infection this response occurred too late to be involved in preventing larval establishment (Smith *et al.*, 1984), but in trickle infections of immune sheep, larvae ingested after the first few days would encounter an existing IgA response that may play a role in preventing establishment (Smith *et al.*, 1987). The total IgA response in gastric lymph was correlated with worm stunting (Smith *et al.*, 1985). Partial immunity and an IgA response were transferred to a lamb from an immune identical twin by donating lymphoblasts, but a mast cell response was also transferred so the IgA may not have been directly responsible for protection (Smith *et al.*, 1986). Vaccination schedules against *O. circumcincta* that produced high serum and bile IgA responses, as well as high serum IgG responses, gave much greater protection than those that gave only high IgG responses (Wedrychowicz *et al.*, 1992).

Comparisons of antibody responses between lines of sheep resistant to *O. circumcincta* and lines of susceptible sheep failed to show any major differences in serum antibody levels but did show that resistant sheep had significantly greater mucosal IgA and IgG_2 responses (Yong *et al.*, 1991). In randomly selected sheep challenged with a deliberate infection following natural exposure, parasite-specific mucosal IgA responses were correlated with worm lengths and numbers of eggs per worm but not with worm burdens (Stear *et al.*, 1995c). Some sheep with strong mucosal IgA had long, fecund worms and this may reflect failure of these sheep to have recognised key antigens (Stear *et al.*, 1996b).

Summarising, plasma antibodies may contribute to mechanisms preventing larval establishment following a leak lesion. There is strong evidence to show that mucosal antibodies, and IgA in particular, are important in host resistance and they may act by limiting worm growth and fecundity. Tools to examine IgE responses in the sheep have only recently been available (Shaw *et al.*, 1996) and increases in total IgE and parasite-specific IgE (parasite homogenate and excretory-secretory products) have been reported in sheep parasitised with either *H. contortus* or *T. colubriformis* and have been temporally associated with protection or correlated with reduced faecal egg count (Kooyman *et al.*, 1997; Shaw *et al.*, 1998).

1.8.2.3: Eosinophils

Eosinophil responses are a feature of helminth infection that has been extensively documented in rodent models. Many species of helminths, including *H. contortus*, are susceptible to *in vitro* eosinophil mediated damage, and damage is accentuated when the eosinophils are sourced from primed hosts and in the presence of complement, cytokines and parasite-specific antibody but there is little direct evidence for an *in vivo* role of eosinophils against gut nematodes (Rothwell, 1989; Jones, 1996; Rainbird *et al.*, 1998). In mice, prevention of an eosinophilia by anti-IL-5 failed to reduce protection against GI nematodes (Coffman *et al.*, 1989; Urban

et al., 1991b). Eosinophils are strongly associated with gut nematodes but without evidence of a protective role. This may be due to the evolution of a stereotypical response against a group of pathogens - for example helminths - that induce a range of responses that will be effective against most members of that group, but with some responses in the range being redundant against certain sub-groups of pathogen (Finkleman *et al.*, 1995; Garside and Mowat, 1995).

In sheep, most of the studies on the role of the eosinophil have examined responses to *T. colubriformis*. Douch *et al.* (1986) showed that tissue eosinophilia was greater in sheep resistant to *T. colubriformis,* but the response quickly declined in the absence of further parasitic challenge. Dawkins *et al.* (1989) and Rothwell *et al.* (1993) showed that peripheral blood eosinophil responses were greater in sheep selected for increased responsiveness. Rothwell *et al.* (1993) also demonstrated a negative correlation between eosinophils and the faecal egg count but because the eosinophil counts were correlated to the globule leucocyte counts, the relationship between the eosinophils and egg count may have been a confounding effect. Buddle *et al.* (1992) showed that the peripheral eosinophil concentration increased when the faecal egg count decreased and suggested that eosinophils have a negative effect on fecundity. Pernthaner *et al.* (1995a) showed that resistant sheep had an earlier and greater peripheral eosinophil response; however, they failed to show a consistent association between eosinophil concentration and either worm burden or faecal egg counts. In contrast to the work showing an association between eosinophilia and resistance, earlier studies by Dineen *et al.* (1978) showed that tissue eosinophil counts were positively correlated with worm burden; that is sheep with more worms had more eosinophils, but subsequently Dineen and Windon (1980) failed to show a correlation between worm burden and the intensity of eosinophil responses.

Gill (1991), working with *H. contortus*, showed that following a secondary challenge, resistant sheep had fewer circulating eosinophils but more tissue

eosinophils. Gamble and Zajac (1992) identified breed differences in resistance to *H. contortus* but there were no between-breed differences in eosinophil responses.

In trickle infections of *O. circumcincta* a rise in peripheral eosinophil concentration was chronologically correlated with the development of host resistance to larval development (Dobson *et al.*, 1992). When naïve lambs and previously exposed lambs were challenged with *O. circumcincta* L_3, only the previously exposed lambs responded with a blood, bone marrow and tissue eosinophilia. In that study eosinophil potentiating activity (a bio-assay for IL-5-like activity) increased in the previously exposed lambs and was negatively correlated with worm burden (Stevenson *et al.*, 1994). Stear *et al.* (1995c) showed a negative association between tissue eosinophilia and worm length (which is a measure of worm fecundity) but not worm burden, but the association between eosinophilia and worm length may have been a confounding effect due to eosinophils being correlated to IgA responses.

When examined together the results from work on eosinophil responses in sheep are confusing. Eosinophil concentrations may reflect the responsiveness of the host. If this response is dose related, and wanes rapidly, then resistant animals may at times have a lower concentration of eosinophils due to a lower worm burden.

1.8.2.4: Mucus

Goblet cells, the mucus producing cells of the small intestine, may act as effector cells against nematodes. There is a chronological association between goblet cell hyperplasia and the expulsion of *Nippostrongylus brasiliensis* in the rat (Wells, 1963). Both worm expulsion and goblet cell hyperplasia are hastened by adoptive transfer of lymphocytes from immune animals (Miller and Nawa, 1979). There is also a qualitative change in the mucins at the time of expulsion (Ishikawa *et al.*, 1994). Recent work, reviewed by Nawa *et al.* (1994) shows that T cell-dependent

goblet cell hyperplasia damages *N. brasiliensis*, and that T cell-independent changes in mucin quality cause expulsion of the damaged worms.

Christie *et al.* (1978) observed that in sheep immune to *H. contortus* there was hypertrophy of the mucus secreting region of the gastric glands. When immune sheep were challenged with *H. contortus* L_3, the larvae failed to establish and were entrapped in mucus on the lumenal surface of the abomasum (Miller *et al.*, 1983). Nematode resistant sheep exhibit hyperplasia of the goblet cells (Douch *et al.*, 1986) and the mucus from nematode resistant sheep exerts a paralysing effect on larvae *in vitro* (Douch *et al.*, 1983; Douch *et al*, 1984), but because the paralysing effect is strongly correlated with the globule leucocyte count, but not to the numbers of goblet cells, the paralysing effect of mucus may be a result of globule leucocyte product rather than changes in mucus (Douch *et al.*, 1986). When an exteriorised section of jejunum from immune sheep was challenged with *T. colubriformis* L_3 there was an increase in mucus production and a change in mucus quality (Pernthaner *et al.*, 1996).

Mucous cell hyperplasia is a feature of the response to *O. circumcincta* infection (Armour *et al.*, 1966). Mucus is a physical interface between host and parasite, can interact with immunoglobulins and be a vector for inflammatory cell mediators. However, there remains a paucity of information on changes in mucus quality and quantity in parasitised sheep.

1.8.3: Control of immune responses to GI nematodes

1.8.3.1: The T helper 1 cell and T helper 2 cell dichotomy

Studies in rodent models have shown that protective immune responses to GI nematodes are T cell-dependent (Jacobson and Reed, 1975), and that T-helper cells play a major role in mediating the protective responses (Urban *et al.*, 1991a). It is widely accepted that the cytokine profile of T helper cells may, in certain environments, become polarised into T helper 1 (Th1) cells and T helper 2 (Th2) cells. T helper 1 cells secrete IL-2 and IFN-γ and Th2 cells secrete IL-4, IL-5, IL-6 and IL-10 (Mosmann and Coffman, 1989). T helper 2 cytokines activate and sustain responses typical of nematode infection, such as mastocytosis, eosinophilia and raised IgE, IgA and IgG_1 responses (Cox and Liew, 1992; Sherr and Coffman, 1992). When resistant and susceptible strains of inbred mice are challenged with *Trichuris muris* there is polarisation of the cytokine profiles from the mesenteric lymph cells. Cells from susceptible mice secrete Th1 cytokines whilst cells from resistant mice secrete Th2 cytokines (Else and Grencis, 1991; Else *et al.*, 1992). Interleukin 4 plays a crucial role in establishing and maintaining the Th2 response (Kopf *et al.*, 1993), and it is possible to abrogate protective responses by blocking IL-4 activity (Urban *et al.*, 1991b), and conversely administering IL-4 to susceptible strains results in the mice becoming resistant (Else *et al.*, 1994).

T helper 1 and Th2 phenotypes can be expressed in human T cells (Romagnani, 1991). T helper 1 and Th2 clones have been reported in ruminants, but care must be used when extrapolating between species because the immunoregulatory mechanisms differ (Brown *et al.*, 1995). There can be considerable overlap of Th1 and Th2 cytokine profiles in ruminants and cells other than CD4+ T cells, such as CD8+ or $\gamma\delta$ T cells, can produce Th1 or Th2 type cytokines; therefore cytokine responses can be described as Type I or Type II (Davis *et al.*, 1998; Brown *et al.*, 1998). If sheep are chronically infected with the enteric pathogen *Mycobacterium avium paratuberculosis* they may develop Johnes disease in which the

histopathological lesions may be classified as being either tuberculoid or lepromatous. Studies by Clark *et al.* (1996) showed that humoral responses and cytokine profiles from the two classification groups were polarised: tuberculoid lesions were associated with a Th1 response and lepromatous lesions with a Th2 response. Therefore Th2 responses can occur in sheep with natural disease, and investigations of cytokine responses to GI parasitism are likely to increase our understanding of immune responses to nematodes.

1.8.3.2: The role of lymphocytes in the control of GI nematodes of sheep

Immune responses are important in control of GI nematodes in sheep. Treatment of sheep with immunosuppressive doses of corticosteroids abrogates resistance to *H. contortus* (Jackson *et al.*, 1988; Presson *et al.*, 1988; Huntley *et al.*, 1992) and *T. colubriformis* (Douch *et al.*, 1986; Emery and McClure, 1995). In a more specific study Gill *et al.* (1993b) elegantly showed the pivotal role of T helper cells by depleting the $CD4^+$ T cell population and abrogating resistance to *H. contortus*.

In studies utilising lymphocyte proliferation assays to measure the responsiveness of lymphocytes to both non-specific mitogens and specific antigens, the proliferation depends upon T helper cells (Haig *et al.*, 1989). The measured responses in parasite specific proliferation assays were greater in previously exposed animals than naïve animals (Riffkin and Dobson, 1979; McClure *et al.*, 1992). Riffkin and Dobson (1979) suggested that pre-infection proliferation assays to *H. contortus* antigens were genetically controlled and negatively associated with worm burden following infection with *H. contortus*. Dineen and Windon (1980), working on the control of responses to vaccination against *T. colubriformis*, recorded greater post-vaccination proliferative responses to mitogen and parasite antigen in high responders than in non-responders. High responders did not have greater pre-infection responses (Windon and Dineen, 1981). Pernthaner *et al.* (1995a) compared proliferation of lymphocytes from lines of sheep that were either

resistant or susceptible to natural challenge by trichostrongyloid species and reported that lymphocytes from resistant sheep had a greater unstimulated proliferation, but a lesser response to T-cell mitogens. Gill *et al.* (1993a) showed that resistant sheep had greater responses to T cell-dependent but not to T cell-independent antigens. Yong *et al.* (1991) reported that lymphocytes from sheep resistant to *O. circumcincta* had greater proliferation responses to specific antigen, but because proliferation assays were one of the selection criteria to breed resistant sheep this observation may merely reflect that proliferation is a heritable trait. There were only minor differences in parasite specific antigen lymphocyte proliferation assays from breeds that have major differences in resistance status (Gamble and Zajac, 1992).

The numbers of lymphocytes in peripheral blood of parasitised sheep have been examined. Results from one research group show a greater lymphocyte concentration in the peripheral blood of susceptible sheep (Colditz *et al.*, 1996) whilst another research group apparently showed a greater concentration of CD4+ T cells in the peripheral blood of resistant sheep (Pernthaner *et al.*, 1995b). McClure *et al.* (1992) challenged immune sheep with *T. colubriformis* and showed that within the small intestinal mucosa there was an accumulation of the three main T cell subsets - CD4+, CD8+ and γδ T cells - and that in the peripheral blood the γδ T cell concentration increased. None of these changes occurred in susceptible sheep (McClure *et al.*, 1992). In *T. colubriformis* infection there is also an increase in γ/δ cells in draining lymph (McClure, *personal communication*). Although these results appear to show that the γδ T cell is important in the immune response, recent work shows that depletion of γδ T cells from peripheral blood improved protection against *T. colubriformis* (McClure *et al.*,1996).

A series of papers reporting work by Smith and colleagues provides much information about the site and timing of the immune response to *O. circumcincta* by examining changes in the flow and composition of gastric lymph. When previously exposed worm-free sheep and naïve sheep were challenged with a single dose there

was clear evidence of an immunological memory because the lymphoblast response was earlier and greater in the previously exposed sheep (Smith *et al.*, 1983b). There were age differences in lambs, with older lambs, which are more resistant than younger lambs, having a response that was similar in timing but greater in magnitude (Smith *et al.*, 1985). Smith *et al.* (1986) showed that the transfer of lymphoid cells from the gastric lymph of donor immune sheep can confer immunity to susceptible sheep, and that the lymphoid cells in the gastric lymph were not necessary for the efficacy of the donor sheep's response; the authors suggested that in the donor animals protection was mediated by primed lymphocytes resident within the lamina propria.

1.8.3.3: Antigen recognition and the MHC

If the host is to mount an effective immune response it is necessary to recognise pathogens as being foreign. The major histocompatibility complex (MHC) is intimately involved in antigen recognition, presentation and immune induction. Studies using congenic strains of mice infected with *T. spiralis* confirmed that MHC-linked genes do influence resistance to helminth challenge (Wassom *et al.*, 1979; Wassom *et al.*, 1984), but the influence of MHC-linked genes is influenced by background genes (Wassom *et al.*, 1984). Studies on *Nematospiroides dubius* produced similar results (Behnke and Robinson, 1985; Wassom *et al.*, 1987). In a recent review, Beh and Maddox (1996) discuss the associations between MHC class I antigens and resistance to ovine GI parasites, and suggest that early reports of significant associations may have been artefacts due to associations between background gene effects and the 'resistant' MHC antigens. However, work on MHC II polymorphisms has made allowances for such confounding associations and reports of associations with resistance are genuine (Schwaiger *et al.*, 1995; Stear *et al.*, 1996a). These more recent studies demonstrate that the effects were surprisingly strong, and lambs with the most common polymorphism (background and environmental effects being equal) had faecal egg counts 58-fold greater than

those with the most resistant polymorphism. The functions of the locus at which these polymorphisms occur have not been determined; they may have a role in antigen recognition or other immunoregulatory mechanisms.

Serum from sheep which are resistant to *O. circumcincta* recognises antigens that are not recognised by susceptible sheep (McGillivery *et al,* 1989) and work by McCririe *et al.* (1997), also on *O. circumcincta,* supports this observation indicating that variation in the recognition of specific parasite antigens may be partly responsible for variation in worm burden and worm length.

1.9: CONTROL OF GI NEMATODES OF SHEEP

1.9.1: Anthelmintic resistance

The development of resistance to anthemintics is a major threat to parasite control world-wide (Waller, 1994). In some areas of the world, such as Paraguay, the 'doomsday scenario' has arrived, with high levels of multiple resistance on nearly all farms and the sheep industry is facing imminent collapse (Waller *et al.*, 1995b). The problem is not yet so serious in the UK, but one survey in Scotland showed that over 24% of farms tested contained sheep nematodes, mainly *O. circumcincta*, that were resistant to benzimidazoles (Mitchell *et al.*, 1991). Strains of *O. circumcincta* that express multiple resistance to both the benzimidazoles and the macrocyclic lactone groups occur in Scotland (Jackson *et al.*, 1992) and imidazothiazole-resistant strains of *O. circumcincta* occur in the UK (Hong *et al.*, 1994).

1.9.2: Control of PGE

In the UK control of PGE is achieved by strategic dosing with anthelmintics (Mitchell and Fitzsimons, 1983) or 'clean' grazing (Rutter *et al.*, 1977) or a combination of both. Strategic dosing involves treatment of the ewes during the peri-parturient period, to limit pasture contamination, followed by dosing of the lambs during the grazing period. The frequency and timing of the strategic dosing of lambs depends upon the risks of nematodirosis and the stocking rate of the pasture. 'Clean' grazing is the practice of grazing sheep and lambs on pasture that has not carried young sheep or pre-parturient ewes during the previous 12 months. On many sheep farms 'clean' grazing is restricted and often reserved for the lambs after weaning and dosing. Even the Rutter system of 'clean' grazing involves an anthelmintic treatment of the ewes before they are moved onto 'clean' pasture. Therefore parasite control in the UK is dependent upon the use of anthelmintic.

Even on farms with benzimidazole resistance, efficacious benzimadizole treatments can be given if the pharmokinetics of delivery are improved (Sangster *et al.*, 1991; Ali and Hennessy, 1995) Successful parasite control can be still be maintained in regions with low levels of anthelmintic resistance (Waller *et al.*, 1995a) but steps must be taken to limit the development of resistance (Coles and Roush, 1992). There has been, and continues to be, much research on alternatives to chemotherapy for controlling nematodes. Novel approaches may not give complete parasitic control, but if integrated into a system in which anthelmintics are used more sparingly they may contribute to a sustainable control programme.

1.9.3: Novel approaches to the control of GI nematodes of sheep

1.9.3.1: Vaccines

Vaccination against worm parasites of animals has recently been reviewed, and much of the major work on the development of vaccines to GI nematodes has been discussed (Emery, 1996).

Perhaps the most promising reports are those on the development of a vaccine to *H. contortus* using the hidden antigen approach, where antibodies are raised to a gut antigen not normally exposed to the host immune system. The *H. contortus* vaccine gives protection of greater than 90% (Tavernor *et al.*, 1992; Smith, 1993). Recombinant glycoproteins have been produced (Munn *et al.*, 1993) and it is claimed that a commercial vaccine will be on the market in 'a few years' (Munn, 1995, *personal communication*). The hidden antigen approach works very well in protecting against haemonchosis because *H. contortus* is a blood feeder and delivery of parenterally raised antibody is direct. There is some optimism that a similar approach could be used to vaccinate against *O. circumcincta* or *T. colubriformis* because they ingest host immunoglobulin (Murray and Smith, 1994). However, which isotype they ingest, and whether or not it will be possible for vaccines to produce that isotype at the mucosal surface is not known.

Initially results from a vaccine based on an *O. circumcincta* L_3 excretory/secretory antigen that was recognised by resistant sheep were encouraging, but recent attempts have failed to replicate the success (Morton *et al.*, 1995). Trials, discussed by Emery (1996), using excretory/secretory antigens against *T. colubriformis* show promising results.

1.9.3.2: Biological control of the free-living stages of GI nematodes of sheep

Biological control of the free-living stages exploits species other than the host that affect parasite populations. These approaches are reviewed by Waller and Faedo (1996). Great interest lies with fungi that are nematophagous and delivery systems exist to administer spores of fungi which survive passage through the ruminant GI tract to the site of action in the faecal pellet (Larsen *et al.*, 1994). The species of grasses on pasture can impact on survival of free-living stages of *O. circumcincta* and can affect the establishment rate of challenge infections with *O. circumcincta*, and these findings support the proposal that it may be possible to exploit the constitution of the sward to limit parasite challenge (Niezen *et al.*, 1996; Niezen *et al.*, 1998a; Niezen *et al.*, 1998b)

1.9.3.3: Breeding for resistance to GI nematodes of sheep

Breeding for resistance is not a new idea and in the 1930s, before the introduction of the first generation anthelmintics, the possibilities of exploiting within-breed variation and between-breed variation were explored (Gregory, 1937; Stewart *et al.*, 1938). Substitution of susceptible breeds with resistant breeds is an approach that is practised in the developing world (Baker, 1995).

Phenotypic within-breed variation is well documented and nematodes in ruminants are known to have a negative binomial distribution (Barger, 1985) - the distribution is overdispersed with a small proportion of the hosts carrying a large proportion of the parasites. Stear *et al.* (1995b) showed that this effect is age-dependent and is stronger as animals mature, suggesting that the variation is due, at least in part, to acquired (probably immunological) responses. The variation is under genetic control (Scrivner, 1967) and there has been great success in breeding resistant lines of sheep (Christie *et al.*, 1978; Windon and Dineen, 1981; Albers *et al.*, 1987; Bisset *et al.*, 1992). Resistance is also associated with increased vigour in a range

of immune responses (Dineen *et al.*, 1978; Douch *et al.*, 1986; Gill, 1991; Douch *et al.*, 1995b). Polymorphisms for genes associated with the immune response have been shown to be associated with resistance (see above). Selection for resistance against one species of GI nematode confers resistance against heterologous species (Windon and Dineen, 1984; Woolaston *et al.*, 1990). Therefore it appears that resistance is associated with a more suitable response to GI nematodes rather than the ability to recognise key antigens, and it is not unreasonable to speculate that it may be due to an enhanced Th2 type response.

If breeding resistant animals is going to be a potential solution to nematode control it is important that the sheep are not only resistant but productive. The immune response to nematodes involves an immediate hypersensitivity reaction and this is likely to impair function and lead to a reduction in the nitrogen digestibility. Albers *et al.* (1987) demonstrated a strong association between resistance to *H. contortus* and resilience, which is a measure of productivity in the face of a parasite challenge. However, a decade later, with breeding for resistance being actively promoted on commercial farms, there has been no clear evidence of economic benefit (Woolaston and Baker, 1996). Conjecture and computer models predict that as the flock becomes increasingly resistant the pasture larval contamination will fall, the host response will lessen and productivity increase (Eady, S. *personal communication*).

Lines of sheep have been selected on the basis of low faecal egg count following natural exposure to a mixed infection of *Trichostrongylus* and *Ostertagia* spp., and although low egg count sheep had fewer worms there were unfavourable correlations between egg count and productivity - lambs with low faecal egg counts have a poorer live-weight gain and a lighter fleece (McEwan *et al.*, 1995). Programmes are now established to breed for resilience (measured by a reduction in necessity for treatments when exposed to challenge), and initial results show that although heritability is low, resilience is correlated with increased productivity (Bisset and Morris, 1996).

Workers on lines of *O. circumcincta* resistant sheep have not published details on productivity (Yong *et al.*, 1991). Resistant lines have not been established for sheep exposed to natural infections where *O. circumcincta* is the dominant species. However, work examining the faecal egg count in a flock of lambs, of known sire, exposed to natural infection with *O. circumcincta*, shows that the faecal egg count has a relatively low heritability but a very strong correlation with live-weight gain (Bishop *et al.*, 1996; Stear *et al.*, 1996b). Therefore breeding for resistance based on low faecal egg count should yield production benefits.

1:10 OBJECTIVES

There has already been a considerable number of studies on the immune responses of sheep to *O. circumcincta* but the interpretation of results from these studies has often been complicated by a large variation in both immunological responses and the parasitology in the experimental animals. Pilot studies by workers at Glasgow University Veterinary School (GUVS) on a flock of Scottish Blackface sheep, grazing *O. circumcincta* contaminated pasture, also revealed large variations in both immune responses and parasitological factors. The aim of the work described in this thesis was to exploit these variations by examining and attempting to make correlations between them, with the intention of differentiating between responses that were important in protection from those that were merely markers of infection. To increase the power of these analyses attempts were made to select animals from the extremes of a flock of 200 lambs. Three classes of parameter were studied: lymphocytes, candidate protective responses and parasitological factors.

- Lymphocytes are considered to play a pivotal role in the immune response to GI nematodes. The relative proportions and absolute numbers of CD4+, CD8+, $\gamma\delta$ T cells and B cells from both peripheral blood and gastric nodes were studied. Mononuclear cells were isolated from both peripheral blood and gastric

(abomasal) nodes and incubated with either non-specific mitogens or specific antigen to measure their proliferative responses.

- If variations in the number, proportion or behaviour of lymphocytes is responsible for variations in the parasitology of animals it is likely that this is mediated through variations in protective responses, therefore the correlations between lymphocyte parameters and protective responses may be stronger than those between lymphocyte responses and parasitological parameters. The mast cell and globule leucocyte responses in abomasal mucosa and parasite specific IgA responses in abomasal mucus and plasma were measured as candidate protective responses.

- Parasitological parameters were studied. Faecal egg counts were taken, worm burdens were estimated and adult female worm lengths were recorded as a measure of fecundity.

CHAPTER 2: MATERIALS AND METHODS

2.1: SOLUTIONS AND MEDIA

2.1.1: Phosphate buffered saline (PBS)

A sachet of PBS powder (Sigma-Aldrich Company Ltd, Poole, England) was made up to 1.0 litre in distilled water to produce 1.0 litre of 10 mM phosphate buffered saline, pH 7.4, with 138 mM sodium chloride and 2.7 mM potassium chloride. The PBS solution was sterilised in an autoclave.

2.1.1: Paraformaldehyde solutions

A mixture of 0.4 g of paraformaldehyde (Sigma-Aldrich) and 80 ml of PBS was placed in a water bath at 96^{O}C and given an occasional shake until the paraformaldehyde dissolved, normally in 10 minutes, when more PBS was added to give 100 ml of 0.4% (w/v) paraformaldehyde/PBS. Likewise, 1.0 g or 4.0 g of paraformaldehyde were added to PBS to make 100 ml of 1.0% or 4.0% paraformaldehyde/PBS solutions respectively.

2.1.3: Bovine serum albumin (BSA)

A mixture of 10 g of bovine albumin, fraction V (Sigma-Aldrich) and 1.0 g of sodium azide (Merck Ltd, Poole, England) was made up to 1.0 litre and stored at 4^{O}C in a bottle which was wrapped in aluminium foil to exclude light.

2.1.4: Iodine solution

A 45% (w/v) iodine solution was made up by adding 902 g of potassium iodide (Merck) to boiling tap water and when cool adding 510 g of iodine crystals (Merck) and then making the solution up to 1.0 litre with tap water.

2.1.5: Pepsin/hydrochloric acid

Pepsin/hydrochloric acid was made up by adding 100 g of Pepsin A (Merck) to 300 ml of 36% hydrochloric acid (Merck) and then making the solution up to 10 litres in tap water.

2.1.6: Lysis buffer

The buffer solution was made up by adding 8.0 g of ammonium chloride (Merck), 0.1 g of potassium dihydrogen orthophosphate (Merck) and 1.0 g of ethylenediamine-tetraacetic acid (EDTA) (Sigma-Aldrich) together and then dissolving in distilled water to 1.0 litre. The buffer was then autoclaved and stored at $4^{O}C$.

2.1.7: 0.6 M EDTA/PBS

This solution was made by adding 23.7 g of EDTA to 80 ml of distilled water and then placing on a heated magnetic stirrer until all the EDTA was dissolved, and then making up the volume to 100 ml. In the tissue culture hood the solution was passed through a 0.2 μm filter (Techmate Ltd, Milton Keynes, England) into a sterile bottle and stored at $4^{O}C$.

2.1.8: Heat-inactivated foetal calf serum (HI-FCS)

Bottles containing 500 ml of foetal calf serum (Sera-lab Ltd, Crawley Down, England) were placed in a water bath at 56^{O}C for 30 minutes to allow the temperatures to equilibrate and then a further 30 minutes to allow inactivation of the complement component. Aliquots were decanted in the tissue culture hood (Microflow Biological Safety Cabinet, Airflow, Andover, England) taking care to prevent contamination. Aliquots were stored at -20^{O}C and thawed in a water bath at 37^{O}C immediately prior to use. Serum from the same batch (201121) was used in all experiments.

2.1.9: Tissue culture medium (TCM)

Tissue culture medium was made up in the tissue culture hood using sterile reagents and stored at 4^{O}C until used. Glutamine has a limited half-life and TCM was not stored for longer than two weeks. The recipe below was used.

500 ml of RPMI 1640 (Dutch modification without L-glutamine) (Life Technologies Ltd., Paisley, Scotland),
2.5 ml of 1M Hepes buffer solution (Life Technologies),
12.5 ml of 200 mM L-glutamine (Sigma-Aldrich),
10.0 ml of 10,000 u (10 mg ml^{-1}) penicillin/streptomycin (Sigma-Aldrich),
5.0 ml of 250 mg ml^{-1} amphotericin B (Life Technologies).

2.1.10: Hank's washing solution (HWS)

The solution was made up in the tissue culture hood using sterile reagents, according to the recipe below, and stored at $4^{O}C$ until used.

500 ml Hanks balanced salt solution without calcium and magnesium (Life Technologies),
5.0 ml of 1M Hepes buffer,
5.0 ml of 10,000 u penicillin/streptomycin,
5.0 ml of 250 $mgml^{-1}$ amphotericin B,
500 µl of 500 µg ml^{-1} gentamicin (Life Technologies).

2.1.11: Cryopreservation medium

Two cryopreservation media were used. Cryopreservation Medium 1 was 60% TCM and 20% HI-FCS and 20% dimethyl sulphoxide (DMSO) (Hybrimax, Sigma-Aldrich) and Cryopreservation Medium 2 was 84% HI-FCS and 16% DMSO. The cell harvest was better with Cryopreservation Medium 2 (70% versus 50% at 1 x 10^7 cells ml^{-1}).

2.1.12: Carbonate buffer pH 9.6 0.06M

The buffer was made by adding 45.3 ml of 1M sodium hydrogen carbonate (84 g of sodium carbonate (Merck) in 1.0 litre) to 18.2 ml of 1M sodium carbonate (106 g of anhydrous sodium carbonate (Merck) in one litre) and making up the solution to 1.0 litre with distilled water. The pH was checked with a pH meter and when necessary adjusted by adding drops of the molar solutions until the pH was 9.6.

2.1.13: Percoll/PBS

Gradually, 45 g of Percoll (Sigma-Aldrich) was added to 40 ml of PBS on a heated magnetic stirrer, and when in solution this was made up to 100 ml with PBS.

2.1.14: Protease inhibitor buffer

Protease inhibitor buffer was a solution of 5 mM EDTA, 5 mM phenylmethylsulphonyl fluoride (PMSF), 5.0% (v/v) isopropanol, 0.01% (w/v) soya bean trypsin inhibitor in PBS. This was made up by dissolving 870 mg of PMSF (Sigma-Aldrich) in 50 ml of isopropanol and adding 50 ml of 0.1M EDTA in PBS and 100 mg of soya bean trypsin inhibitor (Sigma-Aldrich) and 800 ml of PBS to make a suspension, which was then stirred on a moderately heated plate for approximately 45 minutes until dissolved and made up to 1.0 litre with PBS.

2.1.15: Tris/deoxycholate proteinase inhibitors

This solution was made up by Dr Dawn Wallace using the recipe below.

10 mM Tris base (Boehringer Mannheim Biochemica, Lewes, England),
1% sodium deoxycholate (Merck),
1 mM PMSF in isopropanol,
2 mM EDTA,
5 μM pepstatin in methanol (Sigma-Aldrich),
2 mM 1,10 phenanthroline in ethanol (Sigma-Aldrich),
5 μM leupeptin (Sigma-Aldrich),
5 μM antipain (Sigma-Aldrich),

25 μg ml^{-1} N-p-tosyl-L-lysine chloromethyl ketone (Sigma-Aldrich),

50 μg ml^{-1} N-p-tosyl-L-phenyl alanine chloromethyl ketone (Sigma-Aldrich).

2.1.16: Metrizamide solution

A beaker containing 50 ml of distilled water was placed on a heated magnetic stirrer and 35.3 g of metrizamide (Metrizamide, analytical grade, Nyegaard, Oslo, Norway) was gradually added. The solution was made up to 100 ml and passed through a 0.2 μm filter.

2.2: ANIMALS

All the animals used in this study were Scottish Blackface sheep born in April on a commercial upland farm in Strathclyde, Scotland. The flock had approximately 800 breeding ewes and the policy on the farm was to graze ewes with twin lambs on improved pastures in three fields. Some years not enough ewes had surviving twins to fully utilise the improved pastures, and some ewes with single lambs were also grazed on the improved pasture. With the exception of sheep kept helminth-naïve, the lambs used in this study were grazed on the improved pasture. The ewes were removed in July and all the lambs were kept together in the largest field. Faecal samples were taken from 200 lambs in late May and subsequently at four-week intervals. With the exception of the sampling prior to the lambs being moved either to Glasgow University Veterinary School (GUVS) or to slaughter, the lambs were treated with a broad spectrum anthelmintic (albendazole sulphoxide; Rycoben, Young's Animal Health, Leyland, England) at the recommended dose of 5 mg kg^{-1} bodyweight, based on the bodyweight of the heaviest lamb.

Some sheep were reared in conditions designed to prevent helminth infection. These lambs were born outdoors and when a few days old moved indoors onto concrete covered straw yards with their dams, which were treated with albendazole immediately prior to housing. These lambs were weaned at eight weeks of age and fed on a complete ration.

2.3: PROTOCOLS

2.3.1: Sampling and necropsy protocols

2.3.1.1: Faecal samples

Faeces was taken directly from the rectum of sheep and stored at $4^{O}C$ until being processed within 72 hours, before the eggs embryonated.

2.3.1.2: Blood samples

Blood samples were collected by jugular venepuncture into either evacuated glass tubes that contained disodium EDTA as an anticoagulant (Becton Dickinson Ltd., Oxford, England) or into plastic syringes containing EDTA (Monovette, Sarstedt Ltd., Leicester, England). Assuming that 9 ml was drawn into the tube this would give a final concentration of 20 mM EDTA.

2.3.1.3: Necropsy

All sheep were killed by exsanguination following electrical stunning. The abomasum and small intestines were removed from the carcass. In sheep from which the dorsal abomasal lymph nodes were taken, the nodes were identified in

the fat adjacent to the lesser curvature of the abomasum and, taking care to limit contamination, were immediately transferred to 50 ml conical tubes (Falcon, Becton Dickinson) containing Hank's washing solution. Parasitological sampling was based on the methods of Armour *et al.* (1966). The abomasum and small intestine were carried to the laboratory and separated. The abomasum was opened along the greater curvature and the lumen was washed in cool tap water, taking care to collect all the contents and washings. The contents and washings were made up to 2.0 litres. The entire length of the small intestine was opened and washed and the contents and washings were also made up to 2.0 litres. Sub-samples, from the 2.0 litre volumes, of 200 ml were added to containers already containing 2.0 ml of 45% iodine. These were then stored for up to several months before counting. After washing the abomasum it was bisected along the longitudinal axis (from cardiac sphincter to pyloric sphincter). One half was digested with pepsin/hydrochloric acid for 6 hours at 42^{O}C, the digest was made up to 2.0 litres and 200 ml were stored after addition of 2.0 ml of iodine.

From the remaining half of the abomasum, sections were taken from the fundus leaf. In sheep from which three sections were taken for each fixative, sections were taken from three different leaves. Sections were fixed in either 10% neutral buffered formalin (NBF) or in 4% paraformaldehyde/PBS. Mucosal samples were collected by vigorous scraping of the abomasal surface with a glass microscope slide. The slide and samples were placed in a 50 ml centrifuge tube and frozen at -20^{O}C.

2.3.2: Parasitological protocols

2.3.2.1: Faecal worm egg counts

The concentration of nematode eggs in the faeces was estimated by modification of the McMaster technique (Wells, 1963; Miller and Nawa, 1979). A 3.0 g sample of faeces were added to 42 ml of tap water to make a final volume of approximately 45 ml, the mixture was then homogenised and passed through a 250 μm mesh screen (Endecotts Ltd, London, England) and the filtrate was collected in a 15 ml glass test tube and spun at 2,000 rpm for 2 minutes in a Centaur 2 centrifuge. The supernatant was discarded and the pellet was broken up by vortexing and resuspended in saturated sodium chloride solution. The tube was then gently inverted and a volume of suspension was withdrawn with a plastic Pasteur pipette and used to fill both chambers of a McMaster egg counting slide (Gelman Hawksley Ltd., Northampton, England). In the saturated salt solution the trichostrongyloid eggs floated to the underside of the chamber roof and the debris sank. By focusing a dissecting microscope just below the coverslip only eggs were in focus. The total number of eggs counted in the two squares of the McMaster slide represented the eggs present in 0.3 ml (2 x 0.15 cm x 1.0 cm x 1.0 cm) of the original 45 ml of homogenate that contained 3.0 g of faeces. Therefore each egg counted represented 50 eggs per g of faeces.

2.3.2.2: Estimation of worm burden

The technical staff of the Department of Veterinary Parasitology, GUVS, identified and counted the species, stage and sex of worms in the stored sub-samples. For each subsample, ten 4 ml aliquots were examined and each worm identified represented 50 worms present in the lumen or 100 worms present in the mucosa.

2.3.3.3: Measuring mean adult worm length

Stuart Mitchell measured all worms and calculated the mean adult worm length for each individual sheep. Twenty-five adult female worms were selected from each sheep at random from the stored samples of abomasal contents, and examined microscopically. Using a video camera an image of the worms was projected onto a monitor screen, and the length of each worm was estimated using an image analysis programme (PC-Image, Foster Findlay Associates Ltd, Newcastle-upon-Tyne, England). The mean was calculated.

2.3.3: IMMUNOLOGICAL PROTOCOLS

2.3.3.1: Protocol 1 for immunophenotyping of peripheral blood

A 4.0 ml sample of blood with EDTA as an anticoagulant was dispensed into a 50 ml conical polypropylene tube containing 4.0 ml of 0.4% paraformaldehyde/PBS at room temperature (RT) and mixed gently. This was incubated for 4 minutes. Lysis buffer, at 37^{O}C, was added to make the volume up to 50 ml and this was incubated for four minutes. Prolonged incubation in the hypotonic lysis buffer was avoided. The tube was centrifuged at 250 *g*, 1000 rpm in a Beckman GPR centrifuge (Beckman Instruments (UK) Ltd. High Wycombe, England), for 5 minutes.. The supernatant was discarded and the pellet of white blood cells resuspended, in BSA solution at 4^{O}C, to 50 ml and the cells washed by another spin at the same speed, but in a refrigerated centrifuge at 4^{O}C. All subsequent incubations and spins occurred at 4^{O}C. The wash was repeated once. The pellet was then resuspended in 4.0 ml of BSA solution and the suspension transferred to a Falcon 2054 tube (Falcon, Becton Dickinson) and this was centrifuged at 250 *g*. The supernatant was discarded and the pellet

resuspended. This wash was repeated once. After the second wash the supernatant was discarded and 100 µl of BSA solution was added and the pellet resuspended. Aliquots of 30 µl were transferred to 2054 tubes containing 50 µl of primary mouse monoclonal antibody. After discarding the supernatant there was some residual wash remaining with the cells, and on addition of 100 µl of BSA solution there was sufficient volume to allow at least eight 30 µl aliquots. The samples were vortexed and incubated at 4°C. After 20 minutes, 4.0 ml of BSA was added and the sample centrifuged and the supernatant discarded. This wash was repeated once. After the second wash 50 µl of either fluorescein isothiocyanate isomer 1 (FITC) conjugated $F(ab')_2$ fragment of rabbit anti-mouse IgG (Dako, High Wycombe, England) or R-phycoerythrin (RPE) conjugated $F(ab')_2$ fragment of goat anti-mouse IgG (Dako) diluted 1:20 in BSA solution with 5% heat inactivated normal sheep serum was added. The sample was incubated for 30 minutes before 4.0 ml of BSA solution was added and the sample centrifuged. Two further washes followed. The supernatant was discarded and 500 µl of 1.0% paraformaldehyde/PBS were added. The samples were stored in the dark at 4°C and analysed within a month using a laser fluorescent activated cell sorter (Coulter Elite, Coulter Corporation, Miami, USA). An electronic gate was set to include mononuclear cells but to exclude polymorphonuclear cells, red cells and debris according to their forward scatter and back scatter properties. The gate was set by a trained technician, Linda Andrews, and kept constant throughout each experiment. Ten thousand cells within the gated area were counted and the percentage of cells that had an intensity of fluorescence above a threshold level calculated. The percentage of cells stained by the primary antibody was then calculated by subtracting the percentage of cells that were stained by an irrelevant primary antibody.

The panel of monoclonal antibodies used was 17D, 7C2, 86D and VPM8. Monoclonal 17D binds ovine CD4, 7C2 binds CD8, and 86D binds the γδ T cell receptor (Mackay *et al.*, 1988; Mackay *et al.*, 1989; Innes *et al.*, 1995).

Monoclonal VPM8 binds to sheep immunoglobulin light chain (there is a massive predominance of λ over κ in sheep immunoglobulin and VPM8 is thought to have anti-λ specificity) and is a marker for B cells (Hopkins *et al.*, 1989; Bird *et al.*, 1995). The monoclonal antibodies 17D and 7C2 were kind gifts of Dr W. Hein (Basel Institute for Immunology, Basel, Switzerland) and 86D and VPM8 were kind gifts of Dr J. Hopkins (Department of Veterinary Pathology, University of Edinburgh, Edinburgh).

2.3.3.2: Protocol 2 for immunophenotyping of peripheral blood

This was essentially the same technique as Protocol 1 and was used when only two aliquots were needed. Instead of 4.0 ml of blood into 4.0 ml of 0.4% paraformaldehyde/PBS, 1.0 ml of blood was mixed with 1.0 ml of paraformaldehyde/PBS in a 15 ml Falcon tube. This modification used less reagent, and because more samples could be placed in a centrifuge, more samples could be processed at the same time.

2.3.3.3: Immunophenotyping of abomasal node cells

Abomasal node cells were prepared according to Protocol 2.3.3.12 (see below). The immunophenotyping was very similar to that for peripheral blood. A 1.0 ml of a suspension containing 2.0×10^7 node cells ml^{-1} in TCM was added to 1.0 ml of 0.4% paraformaldehyde/PBS and there were three washes in BSA solution before aliquoting into the 2054 tubes.

2.3.3.4: Lymphocyte counting

A 20 µl of cell suspension was mixed with an equal volume of 0.4% trypan blue (Sigma-Aldrich), transferred to an improved Neubauer haemocytometer (Weber, Teddington, England) and examined under the x 10 objective of a light microscope. Viable cells excluded the dye and a total cell count and the percentage of cells which were viable were calculated. Five squares within the central grid were counted and, if there were 200 or more cells, the figure was multiplied by 10^5 to give the number of cells in 1.0 ml of the original suspension. If there were fewer than 200 cells in five squares all 25 squares were counted and this figure was multiplied by 2 x 10^4 to give the concentration of cells in the original suspension. In suspensions from lymph nodes the sample was diluted before the addition of trypan blue.

2.3.3.5: Preparation of mucosal homogenate samples

Mucus and the abomasal mucosa were collected by vigorous scraping of the lumenal surface of the abomasum with a glass microscope slide. The samples were immediately placed in a 50 ml centrifuge tube and stored at -20^{O}C. The samples were removed from the freezer and, without allowing the samples to thaw, 0.3 g was cut away and transferred to a 15 ml centrifuge tube and 3 ml of cold protease inhibitor buffer was added. The protease inhibitor prevented autodigestion of antibody. The sample was homogenised for 4 minutes using an electric homogeniser (Janke & Kunkel, Stauffen, Germany) with the sample tube held over ice. The sample was incubated at 4^{O}C overnight and then transferred to a 6 ml ultracentrifuge tube and the volume was made up to 6 ml with cold protease inhibitor buffer and centrifuged at 20,000 *g* at 4^{O}C for 1 hour. The supernatant was removed and stored at -20^{O}C.

2.3.3.6: Preparation of plasma samples

Blood was collected into 10 ml EDTA tubes and centrifuged at 1200 *g* for 25 minutes at room temperature and a brake used to slow the centrifuge at the end of the spin. The plasma was transferred to glass vials and heat inactivated by placing the glass vials in a water bath at 56^{O}C for 45 minutes. To remove insoluble compounds the samples were centrifuged at 12,000 rpm (Eppendorf centrifuge) for 4 minutes at 4^{O}C. The supernatant was removed and stored at -20^{O}C.

2.3.3.7: Preparation of L_3 antigen for lymphocyte proliferation assays

A suspension containing 1x 10^6 *O. circumcincta* L_3 in 100 ml of cold PBS were provided by the Department of Veterinary Parasitology, GUVS, who obtained them from faecal culture of lambs infected with a laboratory strain obtained from the Moredun Research Institute, Edinburgh, Scotland. The suspension was divided into two 50 ml centrifuge tubes and centrifuged at 500 *g* for 5 minutes and the supernatant was discarded and made up to 5 ml in PBS at room temperature. Five ml of larval suspension were carefully layered over 5 ml of 45% Percoll /PBS at room temperature and centrifuged at 600 *g* for 20 minutes. The live larvae pelleted at the bottom of the tube, whilst the dead larvae remained at the Percoll/PBS interface. The pellet was resuspended in cold PBS and centrifuged at 500 *g*. There were two more washes in cold PBS. Following the washes 10 ml of PBS at room temperature were added to each tube and then these were combined and 200 µl of 2% sodium hypochlorite in 16.5% sodium chloride (Milton, Proctor and Gamble (Health and Beauty Care) Ltd, Egham, England) were added. After 10 minutes, microscopy revealed that the majority of the larvae had ex-sheathed. The larvae were washed twice in cold PBS by centrifuging at 500 *g* for 5 minutes and removing the supernatant. Approximately 5 x 10^5 parasites were added to 5 ml of cold PBS in glass universal tubes. The parasites were sonicated, with the universal tubes kept in a beaker of iced water, by a MSE Soniprep 150 ultrasonic disintegrator (MSE,

Scientific Instruments, Crawley, England) at an amplitude of 16 μm for approximately seven cycles of 30 seconds with 60 second rests between samples. Following sonication only broken sheaths were visible on microscopic examination. To remove low molecular weight insoluble material, which can act as mitogens resulting in non-specific proliferation of lymphocytes, the samples were ultracentrifuged. Both samples were made up to 6 ml in cold PBS in an ultracentrifuge tube and centrifuged at 1.0 x 10^5 *g* for 1 hour. The supernatant was removed and passed through a 0.2 μm filter. To ensure that the preparation contained no low molecular weight mitogens the ultracentrifuged antigen was pooled and dialysed using a membrane with a molecular cut-off of 15,000 kDa (Pierce & Warriner (UK) Ltd, Chester, England). The preparation was dialysed at 4^{O}C for 16 hours in three changes each of 1 litre of PBS, then filter sterilised by passing it through a 0.2 μm filter and stored at -20^{O}C.

2.3.3.8: Preparation of L_3 antigen for enzyme-linked immunosorbent assays

This was similar to the protocol used for preparation of antigen for lymphocyte proliferation. Once the larvae had been ex-sheathed with hypochlorite and washed twice in PBS then 5 ml of Tris/deoxycholate proteinase inhibitors were added to pellets of approximately 500,000 larvae. Aliquots (1.0 ml) of larval suspension were then homogenised in a pre-chilled hand held glass homogeniser (Jencons Scientific Ltd, Bedfordshire, England). The suspension was frozen and thawed ten times, transferred to Eppendorf tubes and then centrifuged at 14,000 *g* at 4^{O}C for 30 minutes. Aliquots were stored at -20^{O}C.

2.3.3.9: Protein assay

To measure the concentration of protein in the antigen preparations a bicinchoninic acid assay (BCA Protein Assay Kit, Pierce & Warriner (UK) Ltd.)

was used according to the manufacturer's directions. Standards were prepared with BSA and following incubation with bicinchoneic acid the optical densities were read using at 560 nm using an automatic plate reader (Dynatech MR5000, Dynatech Laboratories Ltd, Billinghurst, England). An optical density versus concentration linear curve was plotted, and the optical density of the antigen sample was fitted to the curve and the protein concentration calculated.

2.3.3.10: Enzyme-linked immunosorbent assay (ELISA)

A 96-well flat-bottomed microtitre plate (Costar UK Ltd, High Wycombe, England) was coated with antigen by adding 100 µl of 5µg ml^{-1} of antigen in carbonate buffer pH 9.6 per well and incubating overnight at $4^{O}C$. The plate was washed five times in ELISA buffer (50% PBS in distilled water with 0.05% polyoxylene-sorbitan monolaurate, Tween 20 (Sigma-Aldrich)). To reduce non-specific binding, 200 µl of blocking buffer (ELISA buffer with 4% skimmed milk powder (Premier Beverages, Adbastum, England)) was added to each well and incubated for 2 hours at $37^{O}C$, then washed as above. A 100 µl volume of either plasma or mucosal homogenate diluted in blocking buffer was added to each well and incubated at $37^{O}C$ for 30 minutes. After washing, 100 µl of rat IgG anti-sheep IgA diluted 1:50 in blocking buffer was added to each well and the plate was incubated for 30 minutes at 37 ^{O}C and the plate was then washed. A 100 µl volume of alkaline phosphatase-conjugated goat anti-rat IgG (Sigma-Aldrich) diluted 1:1000 in blocking buffer was added to each well and incubated for 30 minutes at $37^{O}C$. After washing 200 µl of substrate was added. The substrate was made up by adding two tablets (Sigma-Aldrich) to 20 ml of distilled water to make up 10 mg ml^{-1} p-nitophenyl phosphate in 0.2M Tris buffer. Phosphatase enzymes convert the p-nitrophenyl phosphate to yellow p-nitrophenol and free phosphate. The substrate was incubated at $37^{O}C$ until the known positive was a strong yellow to the naked eye, this always occurred in less than 15 minutes. Optical densities were read at 405 nm using an automatic plate

reader (Titertek Multiskan MC, Flow Laboratories Ltd., Irvine, Scotland). To minimise the variation between different days and different plates the optical densities were transformed into an optical density index (OD) for each animal by the following formula:

$$OD = \frac{\text{mean of test triplicates - mean of a weak positive standard triplicates}}{\text{mean of strong positive standard - mean of weak positive standard}}$$

2.3.3.11: Isolation of peripheral blood mononuclear cells (PBMC)

Blood in 10 ml EDTA tubes was centrifuged at 1200 *g* for 25 minutes at room temperature and a brake used to slow the centrifuge at the end of the spin. The buffy coat was carefully collected and made up to 4 ml with TCM at room temperature, then carefully layered over an equal volume of Histopaque 1083 (Sigma-Aldrich), at room temperature, in a 15 ml conical tube. Centrifuging at 600 *g* for 40 minutes with no brake resulted in a pellet of erythrocytes and neutrophils, a clear band of Histopaque, an opaque layer containing PBMC and a top layer of TCM. In some sheep there was erythrocyte contamination of the PBMC layer, therefore the PBMC were carefully aspirated and transferred to a new 15 ml tube and 10 ml of lysis buffer was added and incubated at 37^{O}C for 4 minutes to lyse contaminating red cells. After 4 minutes the suspension was immediately centrifuged at 300 *g* and the pellet resuspended in TCM. There were two further washes in TCM at 300 *g* to remove residual Histopaque, platelets and erythrocyte debris.

2.3.3.12: Isolation of abomasal node cells

The lymph nodes were dissected from the carcass and stored in HWS for between 10 minutes and 1 hour. They were then transferred to a plastic petri dish containing 20 ml of HWS at room temperature and any fat adhering to the nodes was carefully removed. In some experiments the nodes were then weighed. The nodes were washed twice in 50 ml of HWS at room temperature and then transferred to a plastic petri dish containing 20 ml of TCM with 10% HI-FCS at room temperature. The nodes were halved and forced through a sterile metal tea sieve using the plunger of a sterile 20 ml syringe. Cells from the parenchyma of the node passed through and much of the capsule and connective tissue remained. The cell rich TCM/FCS was then aspirated three times using a 20 ml syringe and a 19 gauge needle to obtain a single cell suspension. The suspension was transferred to a 15 ml centrifuge tube and allowed to settle for 5 minutes; 10 ml of suspension was then removed, leaving the debris, and centrifuged at 300 *g* for 5 minutes. The cells were resuspended in TCM and the wash repeated twice.

2.3.3.13: Isolation of abomasal intraepithelial and lamina propria lymphocytes

Within two hours of slaughter approximately 5 g of abomasal fundus leaf was gently washed in tap water and then had three washes of 5 minutes in 50 ml of HWS at room temperature before being cooled by a five minute incubation in HWS at 0^{O}C. To break down the tenacious abomasal mucus the tissues were then incubated in 50 ml of 1 mM dithio-L-thrietol (DTT) in HWS (770 µl of 1% DTT in 50 ml of HWS) at 0^{O}C for 15 minutes. The tissues were then washed twice for 5 minutes in HWS at room temperature before being cut into small pieces with a scalpel and transferred to a 50 ml container with either 2mM EDTA in HWS (830 µl of 0.6 M EDTA in 500 ml of HWS) or 2.4 U ml^{-1} of Dispase II (Boehringer Mannheim Biochemica) at 37^{O}C to remove the epithelial cells. The tissues were incubated in a shaking incubator for three 1 hour digests with the medium being changed after each hour. The digest solution from each incubation was passed through a 100 µm sieve (Falcon) and then through a

sterile nylon wool column to remove particulate matter. Cells were pelleted by centrifuging at 300 *g*. Cells were washed twice in TCM by centrifuging at 300 *g* before overlaying an equal volume of Histopaque 1083 and centrifuging at 650 *g* for 20 minutes at room temperature. The epithelial cells pelleted to the bottom and lymphocytes, if present, remained at the interface between Histopaque and TCM and were carefully removed and washed by centrifuging in TCM. After the third digest in EDTA the remaining tissues (lamina propria and sub-mucosa) were washed for 15 minutes in 5% HI-FCS/TCM and then digested by incubating in 5% HI-FCS/TCM with collagenase type XI (Sigma-Aldrich Company Ltd.) at 160 U ml^{-1}, hyaluronidase (Sigma-Aldrich Company Ltd.) at 300 U ml^{-1} and deoxyribonuclease I (Sigma-Aldrich Company Ltd.) at 0.25 mg ml^{-1}. The lamina propria was digested for 1 hour and then fresh digest medium was added. The digest medium was treated as for the EDTA digest medium.

2.3.3.14: Removal of dead cells from a lymphocyte suspension

If the percentage of non-viable cells in a cell suspension was greater than 10% then a protocol was used to deplete them. A 2.0 ml volume of 18% metrizamide (1.02 ml of 35.3% metrizamide, 0.94 ml of PBS and 40 µl of HI-FCS) was overlayed with 2.0 ml of cell suspension (not more than 1 x 10^7 cells ml^{-1}) in 15 ml centrifuge tubes. These were centrifuged at 500 *g* for 15 minutes with no brake. The dead cells pelleted to the bottom while the live cells banded at the interface between metrizamide and TCM. The live cells were gently aspirated; viability was greater than 95% and cell recovery was greater than 80%.

2.3.3.15: Cryopreservation

The cell suspensions were centrifuged at 300 *g* for 5 minutes and the supernatant discarded. The pellets were resuspended in HI-FCS to half the required volume,

an equal volume of cryopreservation medium was added and the suspension was immediately aliquoted into 1 ml Nunc cryopreservation tubes (Costar UK Ltd.). Cell concentrations of PBMC were no greater than 1 x 10^7 cells ml $^{-1}$ and of abomasal node cells concentrations were no greater than 1 x 10^8 cells ml^{-1}. The Nunc tubes were placed in a Nalgene Cryo 1^oC Freezing Container (Nalgene Company, Rochester, USA) which contained isopropyl alcohol at room temperature and the container was placed in the bottom of a -70^oC freezer for 24 hours. This process controls cooling at -1^oC $minute^{-1}$. The Nunc tubes were then placed in the liquid phase of liquid nitrogen. To recover cells the Nunc tubes were removed from liquid nitrogen and immediately plunged in a 37^oC water bath. As soon as the suspension had thawed it was transferred to a 15 ml centrifuge tube; 2.0 ml of warm TCM was added, drop at a time, the tube was shaken, and then another 7.0 ml of warm TCM were added. The suspension was then centrifuged at 300 *g* and the cells were washed twice by centrifuging at 300 *g* in TCM.

2.3.3.16: Lymphocyte proliferation assays

Peripheral blood mononuclear cells (PBMC) or abomasal node cells were incubated, in triplicate 200 µl volumes, in 96-well, flat-bottomed tissue culture plates (Costar UK Ltd) in TCM with FCS and 10^{-5}M 2-mercaptoethanol. The cells were cultured in the presence of various concentrations of Concanavalin A (Con A) (Sigma-Aldrich), a polyclonal T cell mitogen, parasite antigen or in medium alone at 37^oC with 5% CO_2. The cells in each well were pulsed with 46,200 Bq of 185 GBq $mmol^{-1}$ tritiated thymidine (Amersham International plc, Little Chalfont, England) six hours before harvesting so that proliferating cells incorporated tritiated thymidine into deoxyribonucleic acid (DNA). Harvesting was performed with an automated harvester (Filtermate 196, Packard, Pungbourne, England) onto a 96-well microplate with a bonded filter (Unifilter, Packard). A 25 µl volume of scintillation cocktail (MicroScint-O, Packard) was

added to each filter well before sealing with adhesive sheets (Topseal A, Packard). The amount of tritiated thymidine incorporated was measured by a scintillation counter (Topcount Microplate Scintillation Counter, Packard). Results were expressed either as the mean counts per minute (Cpm) of the triplicate wells or as a stimulation index (SI). The SI was calculated by dividing the mean Cpm of the triplicate of cells cultured with Con A or parasite antigen by the mean Cpm for cells cultured in medium alone.

2.3.4: Histology

Tissues were either fixed in NBF or 4% paraformaldehyde/PBS. Tissues fixed in paraformaldehyde/PBS were transferred to 70% ethanol (v/v) in tap water after 6 hours. Staff in the Department of Veterinary Pathology, GUVS, processed tissues using an automatic processing machine in which samples were passed through graded alcohols and chloroform before being vacuum embedded at 56^{O}C in paraffin wax. Tissue sections 4 μm thick were cut and mounted for staining. All fixed tissue sections were stained with a simplified astra blue (Blaies and Williams, 1981) and Martius scarlet blue (MSB) (Lendrum *et al.*, 1962). In sections stained with astra blue the mast cells stained blue against a pale pink background. In sections stained with MSB, globule leucocytes had large bright red granules stained against a blue background. In some MSB stained sections erythrocytes and chief (zymogen) cells also stained red, but this was a less intense red than that of globule leucocyte granules and because the granules in globule leucocytes are bigger the cell types were easily differentiated. In some sections plasma cells and erythrocytes also stained red but these cells were easily differentiated from globule leucocytes by morphology.

2.3.4.1: Cell counting

Mast cells and globule leucocytes were counted using an Olympus AHBT Research Microscope (Olympus, Tokyo, Japan). A graticule (Graticules Ltd, Tonbridge, England) was inserted into the right eyepiece. The graticule markings were one large square that contained 100 smaller squares and when viewed with the x 20 objective one side of the graticule square represented 0.49 mm and the large square enclosed 0.24 mm^2.

All sections were made through the fundus leaf and consisted of a projection into the lumen that was lined by mucosa on the 'dorsal', 'ventral' and 'distal' (polar) surfaces, see Figure 2.1. Twenty-one strips of mucosa were counted from each block. For each strip of mucosa the bottom of the large square was placed on the muscularis mucosa, and the cells contained within the large square or touching the north and west boundaries were counted. The small squares prevented the slide reader from missing some cells or counting others twice. If the height of the mucosa was greater than the height of the square (0.49 mm) the field of view was moved so that the bottom of the square was where the top had been previously; this ensured that a strip of mucosa 0.49 mm wide and the full height of the mucosa was examined. The number of cells and height of the mucosa was recorded.

The first strip counted was the most distal (or polar), with the bottom of the large square acting as a tangent at the most distal point of the muscularis mucosa. The second strip was on the 'dorsal' surface and separated from the first by 0.49 mm, at this position the bottom of the square was parallel to the muscularis mucosa. The third strip was separated from the second by the same distance. Ten strips were counted on the 'dorsal' surface. The twelfth strip was on the 'ventral' surface and separated from the first by 0.49 mm. Ten strips were counted on the 'ventral' mucosal surface.

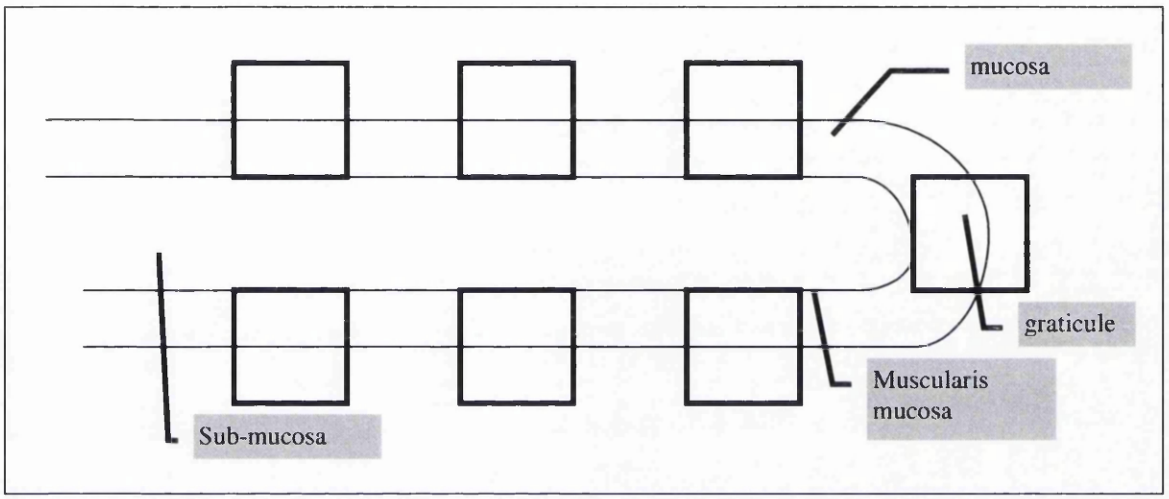


Figure: 2.1: Diagram of a section of fundus leaf with the location of mucosal strips counted.

2.4: STATISTICAL ANALYSIS

The univariate program on the SAS package (SAS Institute, Cary, NC, USA) was used to calculate the similarity of the observed distribution to the normal distribution. The Corr program on the SAS package was used to estimate product moment correlations and their significance. Unless otherwise stated in the relevant protocol, data sets that failed to approximate to the normal distribution were transformed to the logarithm base ten prior to running correlation analyses.

Comparisons between means for data sets were carried out using Microsoft Excel (Microsoft Corporation, Redmond, USA) to carry out the Student's *t*-test. Unless stated a two-tailed test was employed, Type 1 for paired samples and for non-paired samples a Type 3 test was used unless the variance was shown to be equal, when a Type 2 test would be used.

2.5: STUDY DESIGN

The experiments conducted in this study took place in three consecutive years and some of the results from one year were available before the next year's experiments were planned, allowing for modification of the study design. Some groups of lambs yielded tissues that were available for several studies, to examine different immune responses and effector mechanisms, thus gathering as much information as possible from each group of sheep. Many of the techniques were developed or adapted as the study developed and results from the earlier experiments prompted new lines of investigation for the later ones, thus more experiments were conducted in the second and third years.

The study is recorded in chapters according to the experimental techniques employed and the responses studied rather than according to groups of animals studied or the chronology of experiments. Since the course of a challenge is exposure to parasite, the immune response to the parasite and eliciting a protective effector response, therefore it is logical to describe experiments that study the parasitology, then those that investigate the lymphocyte response and finally those measuring the effector mechanisms - such an approach allows for a thorough focus on each response and the development of understanding of that response. Resultant disadvantages are a loss of chronological order, repetition of information, especially that referring to animals or parasitology, and the necessity for the reader to refer to findings in preceding or following chapters. To help the reader know which sheep were used for each experiment Table 2.1 was constructed, with the numerals indicating the sections where the experiments are described and discussed.

YEAR	SHEEP	PARASITOLOGY	EXPERIMENTS
1993	Sheep naturally exposed to *O. circumcincta* and selected according to faecal worm egg count.	3.2	Mast Cell Responses - 6.2
1994	Randomly selected sheep naturally exposed to *O. circumcincta*.		Immunophenotyping 4.2 IgA Responses 7.2, 7.3
1994	Sheep naturally exposed to *O. circumcincta* and selected according to faecal worm egg count.	3.3	Immunophenotyping 4.3, 4.4 Lymphocyte Proliferation Assays 5.3 Mast Cell Responses 6.3 IgA Responses 7.2, 7.3
1995	Sheep naturally exposed to *O. circumcincta* and selected according to the percentage of peripheral blood lymphocytes which were B cells. Some of the lambs were slaughtered when still carrying the burden acquired by natural exposure - the Group N lambs - and the rest were slaughtered following a deliberate challenge - Group D lambs.	3.4	Immunophenotyping 4.5, 4.6 Lymphocyte Proliferation Assays 5.4, 5.5, 5.6 Mast Cell Responses 6.4 IgA Responses 7.4, 7.6

Table 2.1: Study design.

CHAPTER 3: PARASITOLOGICAL FINDINGS

3.1: INTRODUCTION

It has long been known that there is between-breed and within-breed variation in resistance to GI nematodes (Stewart *et al.*, 1938) and that both the distribution of faecal worm egg count and worm burden fit a negative binomial distribution, in which a minority of the animals account for the majority of the worm burden or egg output (Donald, 1968; Barger, 1985). The variation is under genetic control and there has been success in breeding lines of sheep resistant to nematodes other than *O. circumcincta* (Windon and Dineen, 1981; Albers *et al.*, 1987; Bisset *et al.*, 1992). At the time of the design of the work in this thesis, pilot studies of lambs naturally exposed to *O. circumcincta* by grazing contaminated pasture showed considerable phenotypic variation in faecal worm egg count and worm burden (Stear, *personal communication*) and this work, now published, showed that faecal worm egg count in grazing lambs is under genetic control (Bishop *et al.*, 1996).

The objective of the experiments in this thesis was to identify which immunological parameters are important in controlling *O. circumcincta* infection in growing sheep. The approach taken was to compare findings in sheep that were able to mount an efficacious protective response to infection with those that failed to do so, and thus differentiate parameters that are protective from those that merely reflect exposure to the parasite. To facilitate this approach attempts were made to either select two groups of sheep drawn from the one population which differed significantly in parasitological parameters, or to select a sample which contained sufficiently wide variation to allow within-sample correlation or regression analysis.

3.2: SELECTION OF SHEEP INTO LOW AND HIGH FAECAL WORM EGG COUNT GROUPS. I

3.2.1: Introduction

All attempts at breeding parasite resistant sheep have been based on selecting individuals on the basis of faecal worm egg counts. There have been two different approaches, either assessing resistance using faecal worm egg counts after natural infection or following deliberate challenge with a known larval burden (Woolaston, 1990; Morris *et al.*, 1995). Selection of sheep following natural exposure, by grazing, results in a selection process that samples resistance to natural disease rather than the artificial constructs of a deliberate infection, which may not mimic all the processes involved in continuous exposure - however, deliberate challenge experiments exclude the variable of different larval intake due to different grazing behaviour. Selection following natural infection negates the necessity for keeping sheep in a worm free environment and harvesting infective larvae, thus making it easier to integrate with commercial sheep production and allowing a greater number of sheep to be tested, consequently increasing the power of selection.

The criterion for selecting sheep in this experiment was the mean of two monthly faecal worm egg counts following natural infection. Since work from previous years (Stear *et al.*, 1996b), had shown that there was an effect on faecal worm egg count by the birth type (single or twin) and sex of the sheep, animals were selected from a homogenous sex and birth type population.

3.2.2: Protocol

Faecal nematode egg counts were determined monthly from May to October for 108 male twin lambs using Protocol 2.3.2.1. All the lambs were April born Scottish Blackface, as described in Section 2.2. With the exception of the last sampling date in October the lambs were dosed with anthelmintic (albendazole; Rycoben) at the time of faecal sampling. The five lambs with the lowest sum of faecal worm egg counts in August and September were allocated to the low egg count group and the five with the highest egg count in the same months were allocated to the high egg count group. Both groups continued to graze the same pasture until the end of October and were transported to GUVS on 1 November 1993; they were killed two days later and the worm burden was calculated according to Protocol 2.3.1.3.

3.2.3: Results

The faecal worm egg counts for August, September and October are shown in Table 3.1. The mean faecal count in the high egg count group for October, not used in the selection process, was significantly higher than that in the low egg count group, 355 eggs per gram (epg) compared to 69 epg ($p<0.05$). No faecal worm egg count was available for sheep 36 because no faecal pellet could be obtained at the time of sampling.

The *O. circumcincta* burdens are shown in Table 3.2. There was no significant difference between high and low egg count groups in any of the data sets examined, L_4 ($p=0.08$), L_5 ($p=0.31$), adult worms ($p=0.07$), adult females ($p=0.05$) and total worm burden ($p=0.24$) but there was a trend that the high egg count group had more adult females, with a mean of 2350 compared to 920 in the low egg count group. The log transformed October faecal worm egg count was associated with the log transformed number of adult females, 0.72 ($p<0.05$, $n=9$).

LAMB	GROUP	AUGUST	SEPTEMBER	OCTOBER
6	LOW	0	0	50
35	LOW	50	0	0
36	LOW	50	0	N/A
62	LOW	25	25	125
69	LOW	25	25	100
2	HIGH	925	475	125
51	HIGH	1725	975	675
27	HIGH	600	550	275
43	HIGH	1025	575	225
60	HIGH	975	475	475

Table 3.1: Faecal worm egg counts (epg) for Low and High Faecal Worm Egg Count Groups. I.

LAMB	GROUP	L_4	L_5	ADULT	FEMALE	TOTAL
6	LOW	2700	0	1850	900	4550
35	LOW	3100	0	1500	700	4600
36	LOW	5800	0	850	500	6650
62	LOW	950	250	2650	1250	3850
69	LOW	0	0	2150	1250	2150
2	HIGH	1300	0	1850	1100	3150
51	HIGH	4400	100	5400	3500	9900
27	HIGH	1350	550	6450	3700	8350
43	HIGH	1200	100	2200	1350	3500
60	HIGH	2800	100	3800	2100	6700

Table 3.2: *O. circumcincta* burdens for Low and High Faecal Worm Egg Count Groups. I.

3.3: SELECTION OF SHEEP INTO LOW AND HIGH FAECAL WORM EGG COUNT GROUPS. II

3.3.1: Introduction

In the experiment described here all sheep grazed the same pasture from birth to housing prior to slaughter. Although there were faecal worm egg count records from 200 lambs the largest single group of lambs with identical sex, birth and grazing history was of 51 male singleton lambs grazed in Field One. Immunoglobulin A may have a role in limiting worm fecundity and plasma IgA may have a role as a criterion in selecting resistant sheep, but, in this group of 51 lambs, plasma IgA activity against L_3SE was shown to have no significant association with faecal worm egg count and was rejected as a selection criterion.

In the previous experiment the difference in numbers of adult females was not deemed significant, because at $p=0.05$ it was just too great for the conventional level of significance of $p<0.05$. If more sheep were used and group sizes were bigger the power of the statistical tests would be greater.

An examination of data from previous years showed that the closer to slaughter when the faecal worm egg counts were sampled, the greater the difference in worm burdens between selected groups would be, therefore emphasis was placed on the last sample, which was in October. Because the faecal worm egg count is repeatable from July onwards (Stear *et al.*, 1995b; Bishop *et al.*, 1996), suggesting that there is active control of faecal egg shedding from July onwards, the decision was taken to include July, August and September faecal worm egg counts in the selection criteria but to let the October count account for 50% of the weight of selection - the intention was that the inclusion of previous months' results would prevent the selection of an individual sheep which had an inconsistently high or low count due to some factor other than active control,

such factors may be measurement error, grazing behaviour or a change in faecal volume. To try to reduce variation due to measurement errors, four slides were read from one faecal sample from each lamb in October - the recorded October count was the mean of these four counts.

3.3.2: Protocol

The protocol was similar to that for Experiment 3.2. The selection criteria differed, being the sum of the October count and mean of the July, August and September counts, therefore the October faecal worm egg count accounted for 50% of the selection weight. The animals were selected from the 51 male singleton lambs which had grazed Field 1 from birth. Eight sheep were selected with low faecal worm egg counts and eight with high counts. Four days after the last faecal worm egg count in October the sheep were transported to GUVS where they were housed and fed on hay. Four days after housing, 26th October 1994, the first four lambs were slaughtered, with four lambs being slaughtered each day for four consecutive days. A faecal worm egg count was taken post mortem. Parasite burdens were calculated as for Experiment 3.2. The mean length of adult female worms was also calculated according to Protocol 2.3.2.3.

3.3.3: Results

The results are shown in Tables 3.3 and 3.4. There were no significant differences between the high and low egg count groups for faecal worm egg count at slaughter, L_4 burden, L_5 burden, adult male, adult female, total adults or total worm burden (respective p values were 0.31, 0.14, 0.31, 0.30, 0.25, 0.39 and 0.49) but the low egg count group had a significantly shorter mean worm length, 0.83 cm compared to 0.90 cm ($p<0.05$). There was no association between the transformed faecal worm egg count at slaughter and worm length,

0.04 ($p=0.89$), but there was a strong and significant correlation between the transformed faecal worm egg count at slaughter and the transformed adult female worm burden, 0.65 ($p<0.01$).

Distribution curves were drawn for worm length and adult female burden, these are illustrated in Figures 3.1 and 3.2. The former approximated a bimodal curve, which suggests that the sample was from the extremes of the population.

LAMB	GROUP	JULY	AUG	SEPT	OCTOBER				PM
B23	LOW	600	0	0	50	50	0	50	200
B27	LOW	50	0	0	350	200	250	100	300
B37	LOW	50	0	0	150	150	100	50	200
Y165	LOW	0	0	0	100	100	0	0	1200
Y166	LOW	0	50	0	50	0	100	0	100
Y176	LOW	100	0	0	200	200	100	50	400
Y179	LOW	0	0	0	250	100	100	150	400
Y182	LOW	0	0	0	150	250	300	100	1200
B15	HIGH	100	550	300	350	450	800	600	350
B16	HIGH	450	100	200	250	200	350	450	650
B20	HIGH	250	100	350	350	0	200	50	250
B30	HIGH	600	250	200	700	600	450	500	700
B39	HIGH	400	200	550	350	150	300	150	200
B41	HIGH	300	450	50	300	250	200	150	550
B46	HIGH	1200	1000	400	550	400	600	800	1500
Y192	HIGH	400	150	250	450	350	300	450	650

Table 3.3: Monthly faecal worm egg counts (epg) and post-mortem (PM) faecal worm egg counts for Low and High Faecal Worm Egg Count Groups. II.

LAMB	GROUP	WORM LENGTH	L_4	L_5	ADULTS	ADULT FEMALES	TOTAL BURDEN
B23	LOW	0.93	0	0	1300	500	1300
B27	LOW	0.87	0	0	700	450	700
B37	LOW	0.79	3500	100	2150	1100	5750
Y165	LOW	0.78	4600	0	3500	2600	8100
Y166	LOW	0.79	900	0	800	500	1700
Y176	LOW	0.92	4600	0	3500	2100	8100
Y179	LOW	0.81	2900	50	1950	1200	4900
Y182	LOW	0.78	7600	100	3600	2000	11200
B15	HIGH	0.90	300	0	3050	1700	3350
B16	HIGH	0.84	2300	100	4900	2900	7300
B20	HIGH	0.84	1400	0	900	400	2300
B30	HIGH	0.96	0	0	3700	2150	3700
B39	HIGH	0.93	1500	0	1800	1100	3300
B41	HIGH	0.92	2800	0	1800	1300	4600
B46	HIGH	0.93	2300	0	3400	1800	5700
Y192	HIGH	0.86	700	0	2400	1300	3100

Table 3.4: *O. circumcincta* burdens and mean adult female worm length (cm) for Low and High Faecal Worm Egg Count Groups. II.

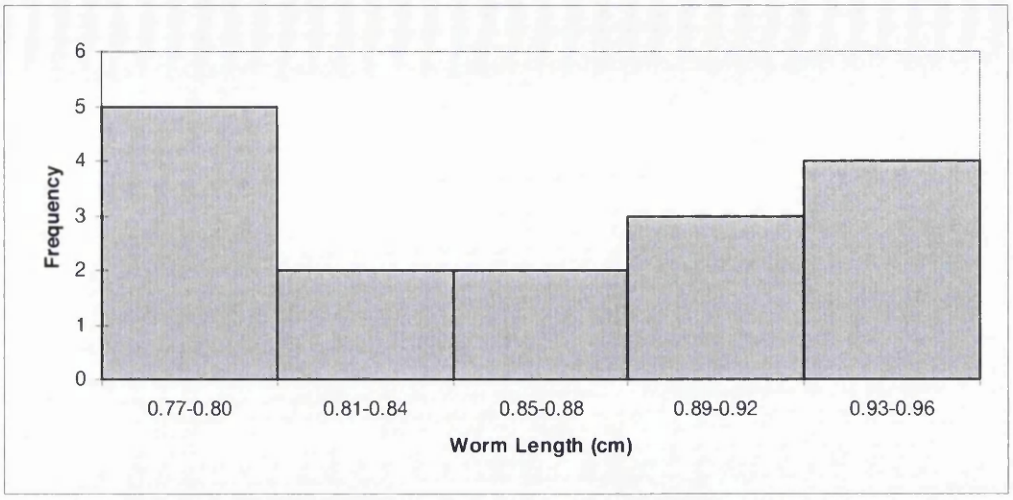


Figure 3.1: Frequency distribution of mean adult female *O. circumcincta* length from Low and High Faecal Worm Egg Count Groups. II.

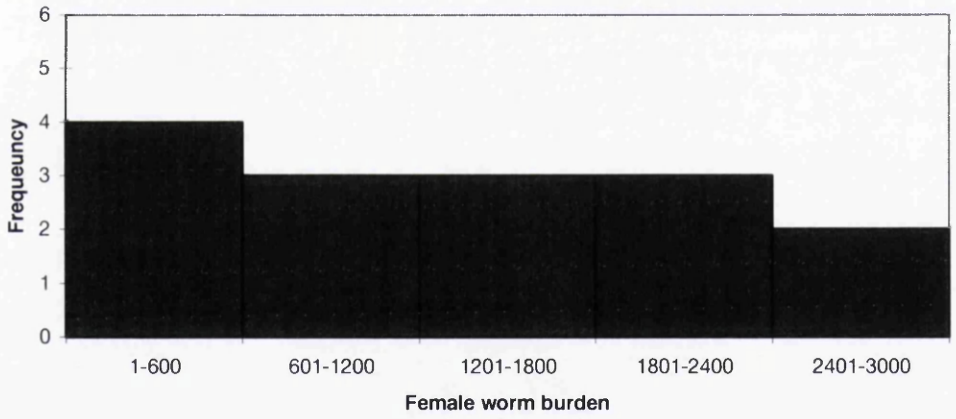


Figure 3.2: Frequency distribution of adult female *O. circumcincta* burden for Low and High Faecal Worm Egg Count Groups. II.

3.4: SELECTION OF SHEEP ACCORDING TO PERIPHERAL BLOOD B CELL PERCENTAGE REPRESENTATION

3.4.1: Introduction

The animals described in Experiment 3.3 were also used in Experiment 4.4 to investigate correlations between immunophenotyping results and parasitological parameters, and this work showed a negative association between B cell representation in peripheral blood and the total *O. circumcincta* burden. If this association was real it would be possible to select sheep with either high or low worm burdens by selecting those with low or high B cell representation in the peripheral blood. As well as investigating whether this selection criterion would be successful at identifying extremes of worm burden following natural infection, B cell percentage representation was used to investigate whether or not animals could be selected that would be either resistant or susceptible to a large single challenge deliberate infection with *O. circumcincta* L_3.

3.4.2: Protocol

The selection process is fully described in Section 4.5. In October 1995 peripheral blood B cell percentages were obtained for 54 female twin lambs which had been grazing Field 1 since birth. The 16 lambs with the highest and the 16 lambs with the lowest B cell percentages were selected and these sheep were assigned to Groups N and D, so that both Groups N and D had eight lambs with a high B cell percentage and eight lambs with a low B cell percentage, and both groups had equal means and equal variance in B cell percentage representation in the peripheral blood. The sheep were removed from pasture and transported to GUVS where they were housed and fed on hay. One week after being transported the first four lambs in Group N were slaughtered and four Group N lambs a day were slaughtered each day for the next three days. Worm

burdens, slaughter faecal worm egg counts and mean female worm lengths were calculated using the same methods as in Experiment 3.3. Each of the 16 lambs in Group D were dosed with an anthelmintic (ivermectin, Oramec Drench, MSD Agvet, Hoddeson, England) at the recommended dose of 200 $\mu g\ kg^{-1}$ bodyweight and, four weeks later, deliberately challenged with 50,000 infective *O. circumcincta* L_3. Ten days after challenge the lambs were slaughtered and the worm burdens calculated as in Protocol 2.3.2.2.

3.4.3: Results

The results of faecal worm egg counts, worm burdens and mean adults female worm lengths for Group N lambs are shown in Table 3.5. There were no significant differences in faecal worm egg count, L_4 burden, L_5 burden, adult worm burden, adult female worm burden, total *O. circumcincta* burden and mean adult female worm length between those sheep with high B cell percentages and those with low B cell percentages ($p=0.80$, 0.64, 0.37, 0.94, 0.90, 0.89 and 0.24 respectively). There was a significant correlation between adult female worm burden and faecal worm egg count, 0.54 ($p<0.05$) but not between mean adult female worm length and faecal worm egg count, -0.03 ($p=0.92$). Distribution histograms were drawn for mean worm length and adult female worm burden; the worm length approximated a normal distribution and adult female burden failed to approximate any recognised pattern (see Figures 3.3 and 3.4).

The results from the truncated challenge infection of Group D lambs are shown in Table 3.6. There was no significant difference in the worm burden between the high B cell lambs and the low B cell lambs ($p=0.35$).

The parasitology results from Experiment 3.3 were compared with those from Group N lambs in Experiment 3.4 and although the faecal worm egg count was greater for Group N lambs, 1381 epg compared to 553 epg ($p<0.05$), there was

no difference for worm length, L_4, L_5, adult female burden, adult burden or total *O. circumcincta* burden (p=0.90, 0.11, 0.26, 0.06, 0.07, 0.07).

LAMB	B CELL GROUP	PM FWEC (epg)	WORM LENGTH (cm)	L_4	L_5	ADULT	ADULT FEMALE	TOTAL ADULT
3	HIGH	500	0.76	1600	0	1350	700	2950
21	HIGH	400	0.87	900	0	1100	800	2000
100	HIGH	2100	0.94	2300	0	6050	3850	8350
31	HIGH	2400	0.80	10400	50	4850	3150	15300
47	HIGH	2500	0.82	7000	0	8500	4700	15500
199	HIGH	600	0.88	1250	0	2050	1000	3300
126	HIGH	200	0.87	5100	500	7850	5000	13450
58	HIGH	3000	0.81	4250	0	2950	1700	7200
92	LOW	1400	0.95	8900	3400	13700	7700	26000
198	LOW	2400	1.09	1750	0	2950	1500	4700
63	LOW	4250	0.84	6300	0	7350	4500	13650
15	LOW	700	0.82	1000	0	1900	1500	2900
35	LOW	350	0.82	1400	0	2800	1800	4200
1	LOW	500	0.83	2000	0	2900	1500	4900
149	LOW	50	0.96	1000	0	800	550	1800
11	LOW	750	0.84	4400	400	1150	750	5950

Table 3.5: Parasitology results for Group N lambs.

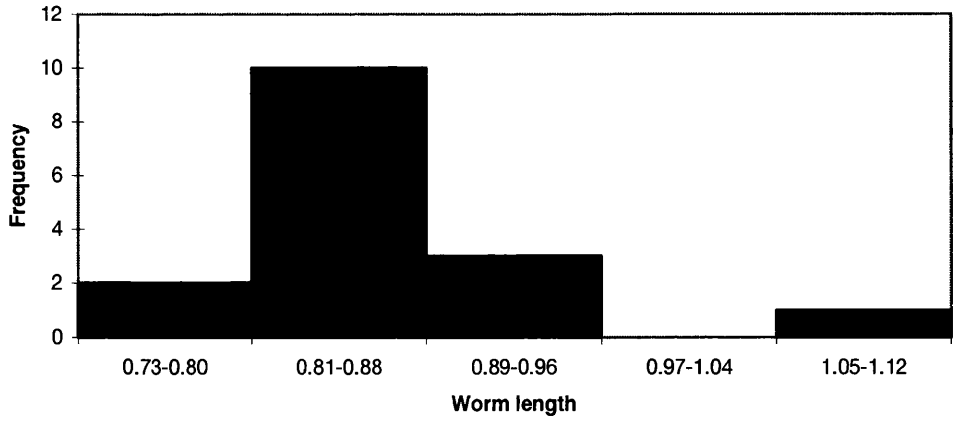


Figure 3.3: Frequency distribution of mean adult female *O. circumcincta* length from Group N lambs.

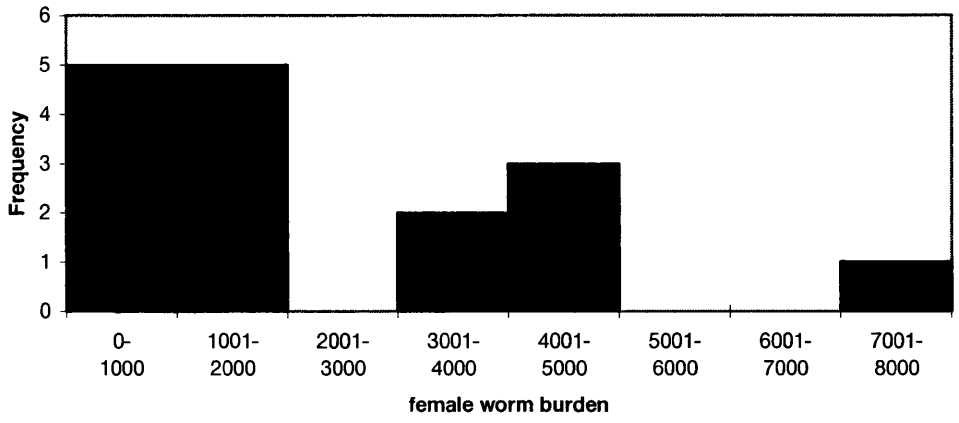


Figure 3.4: Frequency distribution of adult female *O. circumcincta* worm burden in Group N lambs.

LAMB	B CELL GROUP	WORM BURDEN
93	HIGH	14200
72	HIGH	17900
117	HIGH	23900
113	HIGH	24250
70	HIGH	10200
87	HIGH	12000
118	HIGH	20500
95	HIGH	19700
91	LOW	14900
25	LOW	15500
156	LOW	18500
26	LOW	10500
24	LOW	10800
68	LOW	17550
23	LOW	22800
147	LOW	13650

Table 3.6: Worm burdens for Group D lambs following a deliberate challenge with 50,000 infective L_3.

3.5: DISCUSSION

In these studies, selection of sheep according to faecal worm egg counts gave different results in two experiments run in consecutive grazing seasons. In the first year, selection based on the August and September counts succeeded in selecting sheep which differed significantly in their October faecal worm egg count and with a strong trend towards a significant difference in the adult female *O. circumcincta* burdens - that is those sheep selected for lower egg counts having fewer adult females. The faecal worm egg count in October was associated with the number of adult females. The following year a different selection process was used, still based on faecal worm egg counts, but including and emphasising the October count; this resulted in the low egg count group having a population of female worms that were significantly shorter, however there was no worm burden difference between the low and high egg count groups. The faecal worm egg count, at slaughter, was correlated with the adult female *O. circumcincta* burden, although not with the adult female worm length. Selection of sheep according to the percentage of peripheral blood lymphocytes which were B cells did not result in groups of sheep with significantly different worm populations.

The results from Experiment 3.2 clearly showed that it is possible to select sheep with low faecal worm egg count on the basis of previous faecal worm egg counts and that in one experiment sheep selected for low faecal worm egg count had fewer female worms, whilst in a second experiment the low egg count group had shorter worms. These results fit with the observations that faecal worm egg counts are repeatable and that there is a positive relationship between faecal worm egg count and the number of adult female worms (Stear *et al.*, 1995c; Stear *et al.*, 1996b). In Experiment 3.2 lambs grazed in one of two fields from April to July, prior to being moved to a pasture common to all lambs. All five of the lambs in the high egg count group were from the 77 which grazed in Field 1 prior to weaning, but only two of five in the low egg count group grazed Field 1,

the other three sheep were from the 28 which grazed Field 2. The egg counts for lambs grazed in Field 2 prior to weaning were significantly lower in July, August and October, than those in Field 1, 330 compared to 698 epg ($p<0.001$), 203 to 350 epg ($p<0.05$) and 151 to 246 epg ($p<0.01$) - the difference in September was not significant ($p=0.17$). Therefore the exposure to parasites may have been different, and consequently the apparent overrepresentation of low egg count sheep from the lambs which grazed Field 2 may have been due to environmental variables. The design of the thesis was to compare immunological responses between lambs that differed in their efficacy at controlling parasites despite having similar exposure to challenge, and therefore in subsequent experiments further attempts were made to control exposure variables by ensuring that all sheep selected had an identical grazing history.

Stear *et al.* (1996c) showed that in parasitology experiments with low group size and large variances in parasitological parameters, there is a high probability of getting a false negative, that is failing to identify a real difference between two groups. In Experiment 3.2 there was approximately a 2.5-fold difference in the burden of adult females, yet in a deliberate challenge experiment with a three-fold difference in worm burdens and a group size of seven, rather than five, the probability of getting a false negative would be 77% (Stear *et al.*, 1996c). An increase in statistical power can be achieved by increasing the group size, but because these animals were being selected for immunological experiments, which required harvesting of viable cells, there were practical limitations restricting group size. A second problem with increasing group size for selecting animals from the extremes, is that as group size gets large, then the animals approach the mean rather than the extreme, thus reducing the magnitude of the difference between the two groups - this is illustrated by looking at unpublished data from the same source farm in a previous year whereby five sheep with the lowest faecal worm egg count and five sheep with the highest faecal worm egg count gave groups with mean adult female burdens of 1066 and 2610 respectively, but when the twelve sheep with the lowest faecal worm egg counts

and the twelve sheep with the highest faecal worm egg counts were examined the respective means of adult female burden were 1433 compared to 2150, thus dropping from a difference which was almost a factor of 2.5 to one of 1.5.

The selection criteria for Experiment 3.3 included faecal worm egg counts over four months. A pilot study, in a previous year on the source farm, showed that the lesser the interval between faecal worm egg count and slaughter the greater the ability to differentiate groups according to worm burden, so considerable weight was given to the October sample. To increase the accuracy of the October count four McMaster slides were read from one sample from each sheep. Faecal worm egg counts are considered to be an imprecise measure of actual nematode egg count because Stear *et al.* (1996b) showed that as much as 28% of variation in faecal worm egg counts is due to measurement error, and Gasbarre *et al.* (1996) showed that repeatabilities for faecal worm egg counts taken from calves on consecutive days ranged from only 0.4 to 0.7. In Experiment 3.3, to try to prevent inappropriate selection due to misleading October values, the counts from previous months were utilised. The pilot study showed that the correlation of the September count to the October count was 0.43 but the correlation of the mean of the July, August and September counts to the October count was 0.51, and therefore the latter was chosen to account for 50% of the weight of selection.

Although Experiment 3.2 selected sheep which differed in the number of adult female *O. circumcincta*, this was not the case for Experiment 3.3, and although the group sizes were smaller in the first experiment, the population from which they were selected was larger and may have been more diverse because there was some difference in grazing history, thus resulting in a greater difference between extremes. Faecal worm egg count is a function of both worm fecundity and number of adult females (Stear *et al.*, 1996b) and although it is possible that the relative influence of these factors differed greatly between the two years, it is

unlikely because the correlation co-efficients between worm burden and faecal worm egg count were similar, 0.70 and 0.65.

In Experiment 3.3 there was a considerable difference in the mean faecal worm egg counts between the October sample, 251 epg, and the post-mortem sample, 553 epg ($p<0.01$), despite a short interval between these samples. This difference can be explained by the fact that at the time of the October sample, which was taken only 28 days after the sheep received an anthelmintic treatment, only a minority of the larvae ingested which could become patent would have done so, but at the slaughter sample a much greater proportion would be patent. The prepatent period of *O. circumcincta* is 14 to 16 days and egg output per worm does not peak until five to six weeks post-infection (Threlkeld, 1934; Armour *et al.*, 1966), therefore if we assume the grazing sheep would have had a consistent daily intake of larvae, then by day 28, the October sample, only a minority of the larvae that had the potential to reach patency would have done so, whilst at the time of slaughter a greater proportion would have done so, and those worms having reached patency earlier would be more fecund - experimental trickle infections show that worm numbers and faecal worm egg counts rise steeply in the initial weeks before reaching a peak or plateau. Thus the results observed here are compatible with our understanding of infection dynamics (Gibson and Everett 1978; Jackson and Christie, 1979; Hong *et al.*, 1987).

Because worm length is linearly associated with worm fecundity (Stear *et al.*, 1997b) and faecal worm egg count is a function of fecundity and worm burden, it is not surprising the selection of low egg count groups in Experiment 3.3 identified sheep with a shorter mean worm length. What is surprising is that there was no significant association between slaughter faecal worm egg count and mean worm length, even though there was an association between the mean of the October faecal worm egg count and mean worm length. As immunity develops local IgA regulates worm length (Stear *et al.*, 1995c) but another

possible role of IgA may be to retard worm development and delay patency, and therefore the local IgA response may account for a greater proportion of the variation of faecal worm egg count when the sampling date is close to the start of patency. The association between worm length and the October count may be partly due to the fact that those sheep with shorter worms had a greater local IgA response and were able to postpone patency.

There was no significant correlation between the October faecal worm egg count and the post-mortem faecal worm egg count, $r=0.40$ ($p=0.11$), despite the samples being only eleven days apart but there was a strong positive association between the September and the October faecal worm egg counts, $r=0.62$ ($p<0.05$). Interestingly adult female *O. circumcincta* burden was strongly associated with the post-mortem faecal worm egg count, 0.65 ($p<0.01$), but not the October one, 0.36 ($p=0.17$). Together these observations suggest that the factors which affect the faecal worm egg count 28 days after anthelmintic treatment differ from those 36 to 39 days after dosing, with worm burden becoming more significant as time progresses. These findings would be compatible with a delay in patency being an important factor contributing to low faecal worm egg count 28 days after anthelmintic treatment.

The results from Experiment 3.4 show that selecting animals according to B cell percentages failed to select groups which differed in any parasitological parameters. The failure to observe a difference may have been because host immunity was poorly developed due to the unusual weather during the grazing season, being unusually hot and dry for most of the year with a wet September and October, resulting in low exposure followed by heavy infections following mass emergence of larvae once the wet weather arrived (Hunt and Taylor, 1995; Veterinary Investigation Service, 1995). There was a strong trend towards higher worm burdens in Experiment 3.4, conducted in 1995, compared to Experiment 3.3, which was conducted in 1994, and it might have been that immunity was less well developed in 1995. If immunity was less well developed

it would be reasonable to predict that worm lengths would be longer, but this was not the case. However, the lack of difference in mean worm length may have been due to greater worm burdens in Experiment 3.4 restricting worm length due to population density effects (Bishop and Stear, 2000) rather than due to a functioning IgA response. There was an association between worm burden and faecal worm egg count in Experiment 3.4, but not between worm length and egg count.

The deliberate challenge in Experiment 3.4 revealed no significant difference between high and low B cell groups. In this experiment the mean worm burden was 16,678 from a 50,000 L_3 challenge, and compares with 17,299 reported by Stevenson *et al.* (1994) following an identical infection protocol of naïve lambs, also killed on day 10. Thus there is no evidence of the acquisition of an effective immunity that can regulate worm burden - this is consistent with the observations of Bishop *et al.* (1996) that following naturally acquired infections of six-month-old lambs there is no evidence that worm burden is under genetic control.

On the basis of experiments in two different grazing seasons whereby sheep were selected according to faecal worm egg counts there was sufficient success in identifying groups which differed in parasitological parameters to justify further attempts. The likelihood of selecting groups that differed significantly would be increased if the homogenous population was larger - the experiments described here used lambs from a commercial sheep farm and it is possible that population sizes could have been greater if attempts had been made to ensure that lambs of one type, such as male twins, all grazed the same field, although this may have caused further disruption to the farm management. In Experiment 3.2 the largest homogenous group was male twins, due to a successful lambing with a large number of twin lambs, and the success of lambing is a variable which is difficult to plan for unless another breed with a higher lambing percentage was substituted for the Scottish Blackface. Another variable which can affect results is the weather, leading to unusual levels of challenge, and no amount of planning

can predict the vagaries of a Scottish summer. A retrospective study of monthly faecal worm egg counts and slaughter worm burdens compared the results of selecting the five lambs with the highest faecal worm egg counts and five with the lowest counts in two successive grazing seasons and showed that in one year the low faecal worm egg count had a higher total worm burden but 54% were L_4 as opposed to only 17% in the high egg count group, whilst in the following year the percentage of L_4 was similar in both groups, but high faecal worm egg count group had many more worms - these results show that the findings from one year cannot be assumed to be true of all years.

One hazard of picking lambs with a low faecal worm egg count in a year when worm burdens are high is that it might result in lambs with very high worm burdens being selected, because recent work shows that the relationship between worm burden and faecal egg output is convex - this is because of density-dependent factors whereby in sheep with very high worm burdens, the mean worm length is very short and individual fecundity is very low, and at extremely high worm burdens faecal worm egg counts approach zero (Bishop and Stear, 2000).

CHAPTER 4: LYMPHOCYTE PHENOTYPING AND RELATIONSHIPS WITH PARASITOLOGICAL PARAMETERS

4.1: INTRODUCTION

Lymphocytes play a pivotal role in the immune response to GI nematodes (reviewed in Chapter 1). There are four main subsets of mature lymphocytes in the peripheral blood of sheep: CD4+ T cells, CD8+ T cells, γδ T cells and B lymphocytes. Mammalian T cells can be divided into two groups based on their T cell receptors, one group with αβ T cell receptors and the other with γδ T cell receptors (Lew *et al.*, 1986). Mammalian T cells which express αβ T cell receptors are either CD4+ or CD8+, the CD4 molecule marks a functional subset of T cells responsible for helper activity, whilst the CD8 molecule defines a population of cells which exhibit cytotoxic and suppressor function (Mason *et al.,* 1983; Mackay *et al.*, 1987). The γδ T cell subset has γδ T cell receptors and the function of these cells has not yet been elucidated.

In striking contrast to the lymphoid systems of humans and mice, lymphoid systems of ruminants contain large numbers of γδ T cells, which recirculate continuously between blood, solid lymphoid organs and tissues, and which can account for up to 50% of PBMC (Hein and Mackay, 1991). The predominance of γδ T cells in peripheral blood is particularly marked in the neonate and juvenile ruminant, the proportion of T cells that are γδ T cells is high in the neonate, at approximately 60%, and peaks at approximately 20 weeks, declines to 30% by one year and continues to decline with advancing age (McLure *et al.*, 1989; Hein *et al.*, 1990). In addition to differences in the numbers of γδ T cells there are also differences in the structure of the γδ T cell receptor between both

rodents and man and that in sheep, with the γδ T cell receptor of the latter showing hypervariability suggesting an expanded repertoire and a different function. Hein and Mackay (1991) suggested that ruminant γδ T cells may be an adaptive measure in response to a less reliable transmission of passive immunity (post-natal via colostrum in the ruminant compared to prenatal via the placenta in humans and rodents), or due to a functional immaturity of αβ T cells or possibly a response to a high mucosal pathogen load in young ruminants.

Although neonatal lambs have the ability to mount protective immune responses (Batty *et al.*, 1954), lambs are more susceptible than mature sheep to infectious disease, and GI nematode parasites in particular. Their immunological responses and disease resistance gradually increase during the first twelve months of life (Watson and Gill, 1991; Watson *et al.*, 1994). When helminth-naïve sheep of different ages were challenged with *O. circumcincta*, the younger sheep were measurably less resistant and exhibited poorer immune responses, thereby showing that the differences were due to age and not differences in previous exposure (Smith *et al.*, 1985).

There is substantial variation among individual sheep in virtually all immunological traits so far investigated. Given the importance of T cells in immune regulation, experiments on lambs were designed to quantify the variation in lymphocyte subset representation and investigate whether this variation has influence on resistance to GI nematode infection. The abomasal node receives lymphatic drainage from the abomasum and was chosen for investigation of cells involved in the local immune response (Schummer *et al.*, 1991). Two hypotheses relating to γδ T cells and immune responses to nematodes are plausible: one is that high numbers of γδ T cells are an adaptation to a high mucosal challenge by nematodes and contribute to protection, the other is that the overrepresentation of γδ T cells in juvenile ruminants contributes to the observed unresponsiveness to nematode infections. Both hypotheses merited investigation of γδ T cell numbers and associations with resistance. Although

the site of action of γδ T cells against *O. circumcincta* is likely to be the abomasal mucosa, lymphocytes re-circulate through the peripheral blood and the blood can be used as a diagnostic window on the lymphoid system (Westermann and Pabst, 1990).

4.2: AN INVESTIGATION OF THE REPEATABILITY OF LYMPHOCYTE NUMBERS AND SUBSET PERCENTAGES IN GRAZING LAMBS, AND A COMPARISON OF VALUES IN GRAZING LAMBS WITH THOSE IN HOUSED LAMBS

4.2.1: Introduction

Faecal nematode egg counts have frequently been used as a measure of resistance. Experiments on lambs, from the same flock as the lambs used in this study, have demonstrated variation in faecal worm egg count, and more importantly these experiments have shown that from the age of three months (July in April born lambs) the monthly faecal worm egg counts are repeatable, the variation is heritable, and both repeatability and heritability increase with age - this means that lambs with a low faecal worm egg count in one month would be expected to have a low count the next month (Stear *et al.*, 1995b; Bishop *et al.*, 1996). A heritable faecal worm egg count suggests that egg counts are actively influenced by the host as opposed to varying in a random manner. If any immunological parameter is involved in regulating faecal worm egg counts then it is expected that that parameter should show variation between individuals but be repeatable over time; the parameter may also show a response in the face of parasite challenge. The aim of this experiment was to ascertain whether peripheral blood lymphocyte subset numbers and percentages were repeatable

and whether grazing contaminated pasture influenced total numbers or subset percentages.

4.2.2: Protocol

Ten castrated male lambs, naturally exposed to *O. circumcincta* by grazing contaminated pasture, were blood sampled at intervals of four weeks from June to October. Five age, sex and breed matched control lambs were housed and kept helminth-naïve, these were blood sampled in July, August and October. The total number of leukocytes per ml of peripheral blood were counted by staff in the haematology laboratory using an automated cell counter and the number of lymphocytes was calculated following a differential count. Immunophenotyping was carried out using the protocol described in Chapter 2, Protocol 2.3.3.1 and the panel of monoclonal antibodies, the concentration used and the specificity are tabulated in Table 4.1.

In November the helminth-naïve lambs were slaughtered and immunophenotyping of abomasal node cells was carried out using Protocol 2.3.3.3., using the same panel of antibodies, at the same concentrations, as were used for the peripheral blood lymphocytes. The grazing lambs, which were part of a concurrent experiment at GUVS, were slaughtered at a licensed slaughterhouse and it was not possible to obtain abomasal nodes sufficiently fresh to carry out immunophenotyping. However, immunophenotyping was carried out on the abomasal nodes of grazing lambs in Experiment 4.3 - these were age, breed and sex matched - and the results from imunophenotyping their abomasal node cells were compared with those from the housed lambs.

To determine whether or not a parameter was repeatable the values from monthly samples were examined by analysis of variance to determine an overall repeatability value for the whole period of sampling. If a value was not

considered repeatable for the whole sampling period but there was a tendency for correlation co-efficents between adjacent months to become stronger and more significant as the season progressed, then that value was considered repeatable from the month when values were significantly associated with the following month.

MONOCLONAL	SPECIFICITY	CONCENTRATION
17D	CD4+ T cells	1:10
7C2	CD8+ T cells	1:100
86D	γδ T cells	1:50
VPM8	B cells	1:20

Table 4.1: Panel of monoclonal antibodies used for immunophenotyping.

4.2.3: Results

The results for the total lymphocyte counts from grazing and housed lambs are shown in Tables 4.2 and 4.3. Analysis of variance revealed a large difference in total lymphocyte counts between grazing and housed lambs (Figure 4.1), the mean values were 9.93 cells ml^{-1} and 5.34 cells ml^{-1} respectively ($p<0.01$). The repeatability of lymphocyte counts was 0.70 for grazing sheep ($p<0.05$) and 0.61 for housed sheep ($p=0.24$). There was a tendency for the correlations among counts for grazing sheep to increase as the season progressed (Table 4.4).

The percentage representation of lymphocyte subsets in monthly samples of peripheral blood is shown in Tables 4.5 and 4.6. When compared to housed lambs, grazing lambs have a greater percentage of B cells in July ($p<0.05$), August and October ($p<0.01$) but a lower percentage of CD4+ cells in July and August ($p<0.05$); these comparisons are illustrated in Figure 4.2. The other monthly comparisons were not significant.

The overall repeatabilities for each subset percentage in grazing lambs for monthly samples from June to October are tabulated (Table 4.7) but none are significant. The between month correlations for lymphocyte subset percentages in grazing lambs strengthened and became significant as the season progressed (Tables 4.8 to 4.11). Pearson correlation co-efficients demonstrated a positive and significant association between the percentage of CD4+ T cells in September with those in October, hence the percentage of CD4+ T cells was considered repeatable from September, although the August CD4+ percentages correlated with those in October, the co-efficient between August and September was not significant. By the same logic, the CD8+ T cell percentage was considered repeatable from August, the γδ T cell percentage from July and the B cell percentage also from July. As with the lymphocyte count the associations were stronger as the season progressed.

From the lymphocyte count and the lymphocyte subset percentages, the total number of lymphocytes for each subset per ml of peripheral blood was determined. Table 4.12 shows the overall repeatabilities for the total number of lymphocytes for each subset in the peripheral blood of grazing lambs; there was a strong trend, though not significant, towards overall repeatability for γδ T cells and B cells. Pearson correlation co-efficients for the total number of lymphocytes for each subset in samples collected for each month are recorded in Tables 4.13 to 4.16. The total number of CD4+ T cells was repeatable from September, CD8+ T cells from August, γδ T cells from July and B cells also from July. Again the repeatabilities increase as time progresses.

The percentage representation of lymphocyte subsets in the gastric nodes from helminth-naïve housed lambs are recorded in Table 4.17. When these were compared with the gastric node percentages of grazing lambs, from Experiment 4.3, Table 4.20, it was observed that housed lambs had a greater percentage of CD4+ T cells, 31.8 compared to 20.5 ($p<0.05$), and a greater percentage of

CD8+ T cells, 11.5 to 6.9 ($p<0.01$), but a lesser percentage of B cells, 23.2 compared to 37.9 ($p<0.01$). There was no significant difference in $\gamma\delta$ T cell percentages, $p=0.90$. These comparisons are illustrated in Figure 4.3.

LAMB	MAY	JUNE	JULY	AUGUST	SEPT	OCT
Y64	13.28	13.40	11.93	11.39	12.37	11.33
Y66	9.02	10.87	11.59	11.10	11.23	11.00
Y72	6.50	6.93	8.96	8.15	5.61	5.16
Y73	5.92	7.57	8.45	6.79	7.25	6.08
Y92	8.66	5.78	10.77	10.22	10.20	7.53
Y94	10.19	14.03	19.38	17.90	18.10	16.03
Y114	4.40	11.48	7.97	6.90	9.02	7.78
Y119	11.43	13.21	10.31	12.70	11.22	12.26
P78	6.52	9.26	10.29	7.42	7.26	8.08
B94	6.32	11.40	8.97	9.92	11.68	9.31

Table 4.2: Total lymphocyte counts (x 10^6 cells ml^{-1}) in monthly samples of peripheral blood from grazing lambs.

LAMB	JULY	AUGUST	OCTOBER
P17	5.29	5.88	3.90
P38	5.71	5.83	5.55
P46	6.88	5.60	7.33
P48	5.68	6.16	4.64
P53	4.16	3.88	3.65

Table 4.3: Total lymphocyte counts (x 10^6 cells ml^{-1}) in samples of peripheral blood from helminth-naïve lambs.

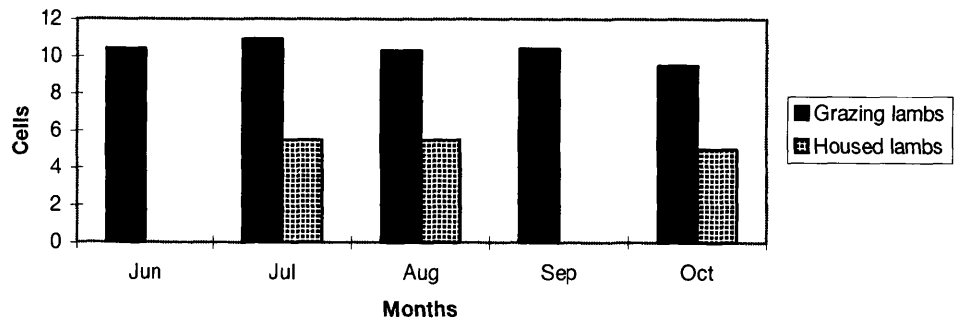


Figure 4.1: The means of total lymphocyte counts (x10^6 ml^{-1}) in the peripheral blood from grazing and housed lambs.

MAY	-					
JUNE	0.68	-				
JULY	0.70	0.75	-			
AUGUST	0.70	0.63	0.91	-		
SEPT	0.59	0.73	0.85	0.92	-	
OCTOBER	0.69	0.84	0.84	0.92	0.93	-
	MAY	**JUNE**	**JULY**	**AUGUST**	**SEPT**	**OCTOBER**

Table 4.4: The correlation matrix for the months from May to October for the total lymphocyte count per ml of peripheral blood in grazing lambs.

LAMB	CD4+ T CELLS J J A S O	CD8+ T CELLS J J A S O	γδ T CELLS J J A S O	B CELLS J J A S O
Y64	10 16 12 16 17	1 10 4 1 3	18 28 27 23 24	52 42 46 55 53
Y66	16 15 6 15 N/A	14 7 6 5 N/A	11 12 14 16 N/A	9 58 61 69 N/A
Y72	16 NA 17 19 19	4 7 9 8 10	13 23 15 19 14	44 50 44 63 49
Y73	13 16 14 14 17	14 8 6 10 8	9 23 29 19 18	13 48 50 58 45
Y92	11 14 10 11 14	7 8 5 7 5	3 11 16 12 15	60 60 62 67 51
Y94	11 16 11 10 7	2 2 4 2 2	12 18 12 12 12	53 64 69 75 71
Y114	13 12 14 13 14	6 7 4 5 6	23 30 28 33 24	41 50 40 53 49
Y119	15 9 15 13 15	3 3 5 4 3	8 15 13 12 12	55 65 53 73 59
P78	10 17 7 12 11	3 9 3 4 4	16 36 41 36 38	56 35 42 50 39
B94	13 18 17 18 20	4 7 8 6 6	8 33 29 26 27	8 34 47 53 35

Table 4.5: The percentage representation of lymphocyte subsets in monthly samples of peripheral blood from grazing lambs.

LAMB	CD4+ T CELLS J A O	CD8+ T CELLS J A O	γδ T CELLS J A O	B CELLS J A O
P17	31 N/A 17	6 6 5	20 23 17	23 32 27
P38	17 22 10	7 7 9	40 28 20	15 31 27
P46	24 24 20	7 4 4	16 20 18	38 32 27
P48	27 22 24	12 9 8	21 27 20	22 28 25
P53	23 27 24	10 11 22	20 16 14	23 30 29

Table 4.6: The percentage representation of lymphocyte subsets in samples of peripheral blood from helminth-naïve lambs in July, August and October.

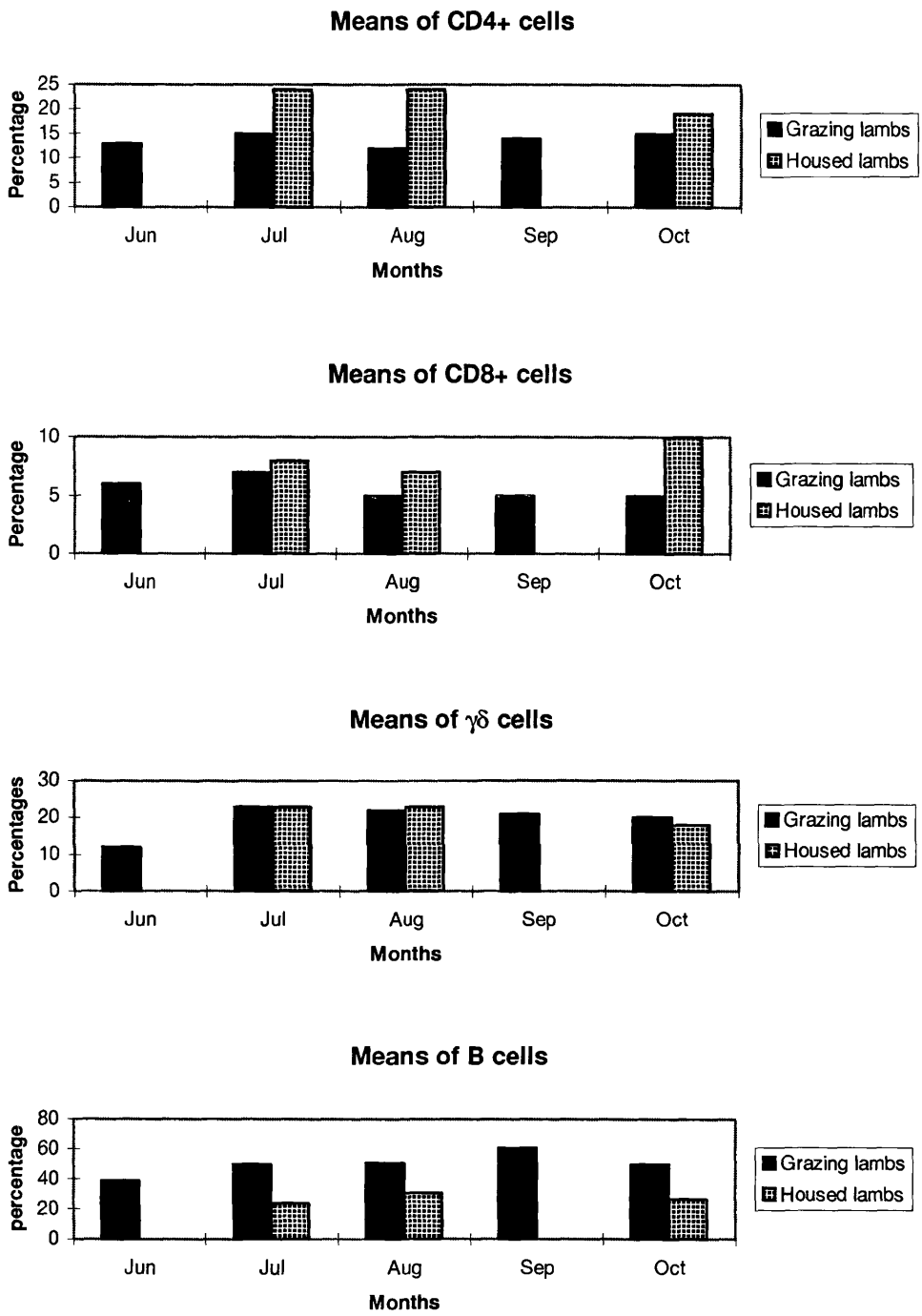


Figure 4.2: The means of the percentage representation of lymphocyte subsets from the peripheral blood of grazing and housed lambs.

LYMPHOCYTE SUBSET	REPEATABILITY
CD4+ T CELLS	0.29 p=0.17
CD8+ T CELLS	0.33 p=0.14
γδ T CELLS	0.57 p=0.07
B CELLS	0.4 p=0.11

The first figure is the strength of the association the second the probability of this occurring by chance.

Table 4.7: The overall repeatabilities for lymphocyte subset percentages in the peripheral blood of grazing lambs.

JUNE	-				
JULY	-0.47	-			
AUGUST	0.31	-0.20	-		
SEPT	0.50	0.31	0.53	-	
OCTOBER	0.50	0.07	0.73*	0.87**	-
	JUNE	**JULY**	**AUGUST**	**SEPT**	**OCTOBER**

For those correlations marked * then p<0.05, for those marked ** then p<0.01.

Table 4.8: The correlation matrix for the months from June to October for the percentage of lymphocytes that were CD4+ in the peripheral blood of grazing lambs.

JUNE	-				
JULY	0.19	-			
AUGUST	0.22	-0.00	-		
SEPT	0.64*	0.21	0.71*	-	
OCTOBER	0.56	0.34	0.79*	0.80**	-
	JUNE	**JULY**	**AUGUST**	**SEPT**	**OCTOBER**

For those correlations marked * then p<0.05, for those marked ** then p<0.01.

Table 4.9: The correlation matrix for the months from June to October for the percentage of lymphocytes that were CD8+ in the peripheral blood of grazing lambs.

JUNE	-				
JULY	0.59	-			
AUGUST	0.43	0.87**	-		
SEPT	0.69*	0.90**	0.87**	-	
OCTOBER	0.49	0.88**	0.88*	0.89**	-
	JUNE	**JULY**	**AUGUST**	**SEPT**	**OCTOBER**

For those correlations marked * then $p<0.05$, for those marked ** then $p<0.01$.

Table 4.10: The correlation matrix for the months from June to October for the percentage of lymphocytes that were γδ T cells in the peripheral blood of grazing lambs.

JUNE	-				
JULY	0.28	-			
AUGUST	0.05	0.73*	-		
SEPT	0.19	0.93**	0.84**	-	
OCTOBER	0.55	0.84**	0.70*	0.84**	-
	JUNE	**JULY**	**AUGUST**	**SEPT**	**OCTOBER**

For those correlations marked * then $p<0.05$, for those marked ** then $p<0.01$.

Table 4.11: The correlation matrix for the months from June to October for the percentage of lymphocytes that were B cells in the peripheral blood of grazing lambs.

LYMPHOCYTE SUBSET	REPEATABILITY
CD4+ T CELLS	0.29 p=0.17
CD8+ T CELLS	0.33 p=0.14
γδ T CELLS	0.53 p=0.07
B CELLS	0.70 p=0.05

The first figure is the strength of the association the second the probability of this occurring by chance.

Table 4.12: The overall repeatabilities for the total number of lymphocytes for each subset per ml of peripheral blood from grazing lambs.

JUNE	-				
JULY	-0.47	-			
AUGUST	0.31	-0.20	-		
SEPT	0.41	0.36	0.62	-	
OCTOBER	0.51	0.07	0.73*	0.88**	-
	JUNE	**JULY**	**AUGUST**	**SEPT**	**OCTOBER**

For those correlations marked * then $p<0.05$, for those marked ** then $p<0.01$.

Table 4.13: The correlation matrix for the months from June to October for the total number of CD4+ cells per ml of peripheral blood in grazing lambs.

JUNE	-				
JULY	0.19	-			
AUGUST	0.22	-0.04	-		
SEPT	0.42	0.01	0.72*	-	
OCTOBER	0.56	0.34	0.76*	0.80**	-
	JUNE	**JULY**	**AUGUST**	**SEPT**	**OCTOBER**

For those correlations marked * then $p<0.05$, for those marked ** then $p<0.01$.

Table 4.14: The correlation matrix for the months from June to October for the total number of CD8+ cells per ml of peripheral blood in grazing lambs.

JUNE	-				
JULY	0.56	-			
AUGUST	0.41	0.82**	-		
SEPT	0.68*	0.71*	0.80**	-	
OCTOBER	0.54	0.83**	0.95**	0.85**	-
	JUNE	**JULY**	**AUGUST**	**SEPT**	**OCTOBER**

For those correlations marked * then $p<0.05$, for those marked ** then $p<0.01$.

Table 4.15: The correlation matrix for the months from June to October for the total number of $\gamma\delta$ cells per ml of peripheral blood in grazing lambs.

JUNE	-				
JULY	0.49	-			
AUGUST	0.42	0.96**	-		
SEPT	0.48	0.93**	0.98**	-	
OCTOBER	0.77*	0.92**	0.92**	0.96**	-
	JUNE	**JULY**	**AUGUST**	**SEPT**	**OCTOBER**

For those correlations marked * then $p<0.05$, for those marked ** then $p<0.01$.

Table 4.16: The correlation matrix for the months from June to October for the total number of B cells per ml of peripheral blood in grazing lambs.

LAMB	CD4+ T CELLS	CD8+ T CELLS	γδ T CELLS	B CELLS
P17	37.4	10.2	4.1	34.7
P38	38.2	14.5	7.2	27.4
P46	19.0	12.5	2.4	18.7
P48	27.7	8.8	4.9	19.7
P53	36.9	11.3	1.3	15.0

Table 4.17: The percentage representation of lymphocyte subsets in the abomasal node of helminth-naïve lambs.

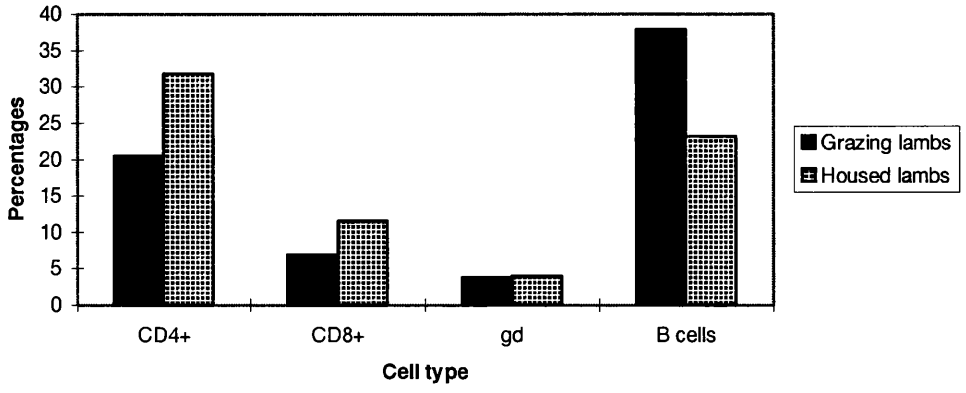


Figure 4.3: The means of the percentage representation of lymphocyte subsets from the abomasal node of grazing and housed lambs.

4.3: A COMPARISON OF LYMPHOCYTE NUMBERS AND SUBSET PERCENTAGES BETWEEN LAMBS WITH A LOW FAECAL WORM EGG COUNT AND THOSE WITH A HIGH FAECAL WORM EGG COUNT

4.3.1: Introduction

The results from the investigation described in Section 4.2 revealed that total lymphocyte counts, lymphocyte subsets and lymphocyte subset counts were repeatable and also showed that these parameters differed between grazing lambs exposed to infection and housed helminth-naïve lambs, thus meeting some of the criteria expected from a parameter involved in regulating faecal worm egg count. The aim of this experiment was to investigate whether or not there were any significant differences between lymphocyte numbers and percentages of subsets between two groups of lambs selected to represent the extremes of faecal counts present within the flock.

4.3.2: Protocol

From the 51 male singleton lambs that were grazed in Field 1 since birth, two groups of eight lambs were selected on the basis of their faecal worm egg counts in July, August, September and October, so that one group was selected for low faecal worm egg count and one group was selected for high faecal worm egg count (Experiment 3.3). Four days after the October faecal sample the selected lambs were removed from pasture and transported to GUVS where they were housed and fed on hay. Two days after they were transported the lambs were bled to enable a total lymphocyte count in the peripheral blood and immunophenotyping of peripheral blood using Protocol 2.3.3.1. Two days after the lambs were bled the first four lambs were slaughtered, with four lambs being slaughtered each day for four consecutive days. Immunophenotyping of

abomasal node cells was carried out using Protocol 2.3.3.3, and the panel and concentration of antibodies used were the same as those used in Experiment 4.2. On the day of slaughter faecal samples were taken for a faecal worm egg count and the abomasum was removed and worm burden calculated. The mean length of adult female worms was also calculated.

4.3.3: Results

The results for the total lymphocyte counts per ml of peripheral blood are shown in Table 4.18. The mean of 7.26 x 10^6 cells ml^{-1} for the low egg count group was not significantly different from the mean of 8.11 x 10^6 cells ml^{-1} for the high egg count group (p=0.57).

The percentage of the total lymphocyte populations represented by each subset in samples of peripheral blood and abomasal node cells from both high and low faecal worm egg count groups is shown in Tables 4.19 and 4.20. For both peripheral blood lymphocytes and abomasal node cells there was no significant difference in the means of CD4+ T cells, CD8+ T cells, γδ T cells, and B cells between the low and high egg count groups (p=0.91, 0.52, 0.76, 0.45 for peripheral blood lymphocytes and p=0.87, 0.37, 0.40, 0.71 for abomasal node cells). The total number of lymphocytes for each subset in 1.0 ml of peripheral blood from high egg count lambs was compared with low egg count lambs (Table 4.21) and no significant difference was identified (p=0.54, 0.24, 0.82, 0.59 for CD4+ T cells , CD8+ T cells, γδ T cells and B cells).

The means of lymphocyte subsets from peripheral blood of all 16 lambs were compared with the means of lymphocyte subsets from the abomasal node of all lambs, revealing no significant differences between the percentage representation in CD8+ T cells (p=0.96) and B cells (p=0.44), but the mean percentage of CD4+ T cells in peripheral blood was less than that in the abomasal node, 17.0

compared to 20.6 ($p<0.05$) and the γδ T cell percentage in peripheral blood was 22.4 compared with 3.8 in the abomasal node ($p <0.01$).

The means of total *O. circumcincta* burden, L_4 *O. circumcincta*, L_5 *O. circumcincta*, adult female, adult male *O. circumcincta* and faecal worm egg count immediately prior to slaughter were not significantly different between the high and low worm egg count groups, although the high egg count group had worms with a greater mean length ($p<0.05$) - these results were described in Experiment 3.3.

LAMB	FWEC GROUP	LYMPHOCYTE COUNT
B23	LOW	6.02
B27	LOW	5.67
B37	LOW	8.31
Y165	LOW	9.98
Y166	LOW	8.11
Y176	LOW	8.39
Y179	LOW	2.32
Y182	LOW	9.29
B15	HIGH	7.50
B16	HIGH	5.73
B20	HIGH	13.88
B30	HIGH	4.80
B39	HIGH	8.19
B41	HIGH	11.32
B46	HIGH	4.43
Y192	HIGH	9.01

Table 4.18: Total lymphocyte counts (x 10^6 cells ml^{-1}) in the peripheral blood of lambs selected as having either a low or high faecal worm egg count.

LAMB	FWEC GROUP	CD4+ CELLS	CD8+ CELLS	γδ T CELLS	B CELLS
B23	LOW	14.8	4.5	16.3	41.1
B27	LOW	25.9	10.2	37.0	59.0
B37	LOW	13.6	5.2	18.6	42.7
Y165	LOW	17.6	3.4	24.2	33.1
Y166	LOW	17.8	8.8	18.6	38.6
Y176	LOW	14.8	5.9	31.5	37.4
Y179	LOW	18.2	7.9	13.8	42.8
Y182	LOW	14.3	5.7	24.3	40.8
B15	HIGH	13.9	11.5	12.6	39.7
B16	HIGH	18.1	6.3	22.7	35.1
B20	HIGH	15.6	5.3	19.6	44.1
B30	HIGH	17.9	6.4	16.5	33.4
B39	HIGH	15.0	6.1	23.2	54.0
B41	HIGH	19.0	5.6	38.8	27.1
B46	HIGH	18.9	8.9	22.5	22.8
Y192	HIGH	17.1	7.3	18.7	50.0

Table 4.19: The percentage representation of lymphocyte subsets in the peripheral blood of lambs selected according to faecal worm egg count.

LAMB	F.W.E.C. GROUP	CD4+ CELLS	CD8+ CELLS	γδ T CELLS	B CELLS
B23	LOW	24.2	8.7	3.7	42.4
B27	LOW	25.9	10.5	1.9	25.6
B37	LOW	21.1	5.3	2.7	45.2
Y165	LOW	23.0	5.6	3.0	35.2
Y166	LOW	18.2	4.6	3.2	42.8
Y176	LOW	12.5	2.7	3.2	30.1
Y179	LOW	17.8	6.4	6.6	39.7
Y182	LOW	21.0	4.5	3.1	36.5
B15	HIGH	26.9	15.0	4.3	24.2
B16	HIGH	22.9	12.9	5.0	41.9
B20	HIGH	24.2	6.0	2.3	36.1
B30	HIGH	19.1	8.8	2.3	38.9
B39	HIGH	22.1	5.7	1.7	43.9
B41	HIGH	18.2	6.0	7.9	40.8
B46	HIGH	13.1	2.4	6.8	35.4
Y192	HIGH	20.0	4.6	3.6	46.5

Table 4.20: The percentage representation of lymphocyte subsets in the abomasal node of lambs selected according to faecal worm egg count.

LAMB	FWEC GROUP	CD4+ CELLS	CD8+ CELLS	γδ T CELLS	B CELLS
B23	LOW	0.89	0.28	0.98	2.47
B27	LOW	1.47	0.58	2.09	3.35
B37	LOW	1.13	0.43	1.55	3.55
Y165	LOW	1.76	0.34	2.42	3.30
Y166	LOW	1.44	0.71	1.51	3.13
Y176	LOW	1.24	0.50	2.64	3.14
Y179	LOW	0.42	0.18	0.32	0.99
Y182	LOW	1.33	0.53	2.26	3.79
B15	HIGH	1.04	0.86	0.94	2.98
B16	HIGH	1.04	0.36	1.30	2.01
B20	HIGH	2.17	0.74	2.72	6.12
B30	HIGH	0.86	0.31	0.79	1.60
B39	HIGH	1.23	0.50	1.90	4.42
B41	HIGH	2.15	0.63	4.39	3.07
B46	HIGH	0.84	0.39	1.00	1.01
Y192	HIGH	154	0.66	1.68	4.50

Table 4.21: The total number of lymphocytes (x 10^6 cells ml^{-1}) of each subset in the peripheral blood of lambs selected as having either a low or faecal worm egg count.

4.4: CORRELATIONS BETWEEN IMMUNOPHENOTYPING RESULTS AND PARASITOLOGICAL PARAMETERS IN LAMBS SELECTED FOR EITHER HAVING A LOW FAECAL WORM EGG COUNT OR HAVING A HIGH FAECAL WORM EGG COUNT

4.4.1: Introduction

The results from Experiment 4.3 revealed no difference in lymphocyte numbers or subset representation between the high and low faecal nematode egg groups, but the results from Chapter 3 also showed that, with the exception of mean worm length, there was no difference in parasitological parameters between the two groups.

4.4.2: Protocol

The phenotyping data was that used in Experiment 4.3, but rather than comparing between groups, quantitative analysis was used to compare trends within the complete group of 16 lambs. Phenotyping data consisted of the total lymphocyte count, the percentage representation of CD4+ T cells, CD8+ T cells, γδ T cells, and B cells in peripheral blood, the total number of CD4+ T cells, CD8+ T cells, γδ T cells, and B cells in the peripheral blood and the percentage representation of CD4+ T cells, CD8+ T cells, γδ T cells, and B cells in the abomasal node. Parasitological parameters examined were the slaughter faecal worm egg count, the number of L_4 *O. circumcincta,* the number of L_5 *O. circumcincta*, the number of female adult *O. circumcincta*, the number of male adult *O. circumcincta*, the total *O. circumcincta* burden, and the mean of adult female worm length. The data was transformed to prevent outlying results skewing the analysis. The log of faecal worm egg counts and worm burdens were calculated and because the lymphocyte subsets were expressed as percentages, arcsine values were calculated. The worm lengths were not transformed because they were normally distributed.

4.4.3: Results

The log of total number of lymphocytes per ml of peripheral blood was not significantly associated with any parasitological parameter ($p>0.05$).

The faecal worm egg count at slaughter was not associated with any lymphocyte parameter examined but there was a trend, not statistically significant, towards a moderately strong negative association between the arcsine of B cell percentages in peripheral blood and faecal worm egg count, as shown in Table 4.22.

The associations between the arcsine of lymphocyte subset percentages and the logs of worm burdens were examined. There was a significant association

between the percentage of CD4+ T cells and adult male *O. circumcincta;* this was negative and moderately strong, -0.52 ($p<0.05$), but there was no association with any other worm burden parameters. There were no significant associations between either the percentages of CD8+ T cells or γδ T cells and worm burden parameters. There was no significant association between the arcsine of B cell percentages and the numbers of L_4, L_5 and adult male *O. circumcincta*, but there were significant and moderately strong negative associations between the arcsine of B cell percentages and adult female *O. circumcincta*, -0.54 ($p<0.05$), and the total *O. circumcincta* burden, -0.56 ($p<0.05$). The relationship between worm burden and B cell percentage is shown in Figure 4.4. There was no association between subset percentages and worm length (Table 4.24).

There were no associations between the log of total number of cells of a subset per ml of peripheral blood and worm burden parameters, faecal worm egg count or worm length (Tables 4.22 to 4.24). There were also no associations between the arcsine of percentage representation of abomasal node cells and worm burden parameters, faecal worm egg count or mean worm length (Table 4.25).

CELLS	ARCSINE OF PERCENTAGE REPRESENTATION	LOG OF TOTAL NUMBER OF CELLS IN 1.0 ml
CD4+ CELLS	0.10 p=0.72	-0.09 p=0.75
CD8+ CELLS	-0.20 p=0.45	-0.36 p=0.17
γδ T CELLS	0.12 p=0.67	0.02 p=0.94
B CELLS	-0.49 p=0.06	-0.33 p=0.22

The first figure is the strength of the association, the second the probability of this occurring by chance.

Table 4.22: The associations between the log of faecal worm egg counts at time of slaughter and the arcsine of percentage representation of lymphocyte subsets in peripheral blood at time of slaughter and the log of the total number of lymphocytes of each subset per ml of peripheral blood.

CELLS	ARCSINE OF PERCENTAGE REPRESENTATION	LOG OF TOTAL NUMBER OF CELLS IN 1.0 ml
CD4+ T CELLS	-0.46 p=0.08	-0.11 p=0.70
CD8+ T CELLS	-0.39 p=0.14	-0.27 p=0.32
γδ T CELLS	-0.02 p=0.93	0.12 p=0.67
B CELLS	-0.56 p<0.05	-0.21 p=0.43

The first figure is the strength of the association, the second the probability of this occurring by chance.

Table 4.23: The associations between the log of *O. circumcincta* burden and both the arcsine of percentage representation of lymphocyte subsets in peripheral blood at time of slaughter and the log of the total number of lymphocytes of each subset per ml of peripheral blood.

CELLS	ARCSINE OF PERCENTAGE REPRESENTATION	LOG OF TOTAL NUMBER OF CELLS IN 1.0 ml
CD4+ T CELLS	0.06 p=0.80	-0.16 p=0.58
CD8+ T CELLS	0.15 p=0.60	-0.03 p=0.89
γδ T CELLS	0.15 p=0.60	-0.02 p=0.93
B CELLS	-0.15 p=0.60	-0.23 p=0.40

The first figure is the strength of the association, the second the probability of this occurring by chance.

Table 4.24: The associations between the mean of adult worm length and both the arcsine of percentage representation of lymphocyte subsets in peripheral blood at time of slaughter and the log of the total number of lymphocytes of each subset per ml of peripheral blood.

CELLS	LOG OF WORM EGG COUNT	LOG OF WORM BURDEN	WORM LENGTH
CD4+ T CELLS	-0.32 p=0.18	-0.35 p=0.16	-0.18 p=0.43
CD8+ T CELLS	-0.28 p=0.29	-0.29 p=0.26	0.14 p=0.62
γδ T CELLS	0.31 p=0.20	0.17 p=0.55	0.13 p=0.64
B CELLS	-0.02 p=0.93	0.25 p=0.33	0.11 p=0.70

The first figure is the strength of the association, the second the probability of this occurring by chance.

Table 4.25: The associations between the arcsine of percentage representation of lymphocyte subsets in the abomasal node and log of faecal worm egg counts at slaughter, log of *O. circumcincta* burden, and the mean adult female worm length.

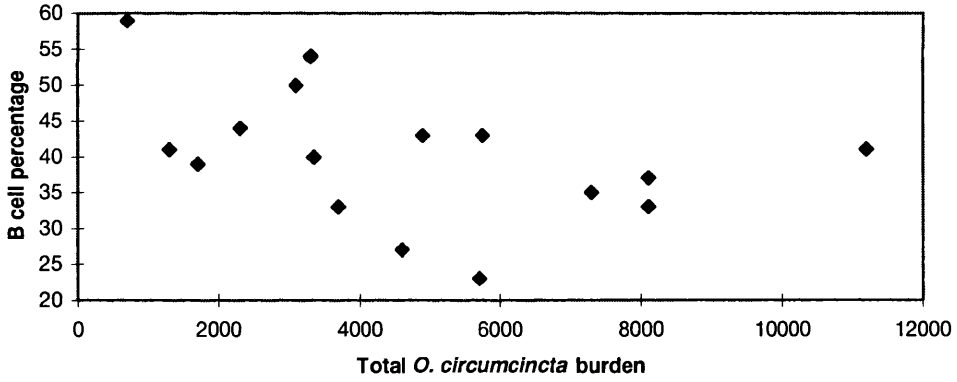


Figure 4.4: The relationship between the *O. circumcincta* burden and the percentage of peripheral blood lymphocytes which are B cells.

4.5: AN INVESTIGATION OF LYMPHOCYTE NUMBERS AND SUBSET PERCENTAGES IN LAMBS SELECTED AS EITHER HAVING A HIGH OR A LOW PERCENTAGE OF B CELLS IN PERIPHERAL BLOOD

4.5.1: Introduction

The results from Experiment 4.4 showed a negative association between the B cell percentages of lymphocytes in the peripheral blood and the total worm burden. The following year, in order to test this association, lambs with high and low B cell percentages in peripheral blood were selected. To test whether this association would also be true for lambs given a deliberate infection, half of these animals were killed following an experimental challenge.

This experiment necessitated immunophenotyping of the experimental lambs, providing the opportunity to investigate relationships between different lymphocyte subsets, in the peripheral blood and the abomasal node, from lambs selected from the extremes of B cell percentages, as well as investigating the responses of peripheral blood lymphocytes following deliberate infection, and this work is described here.

To maximise power of analyses lambs were selected from the largest possible single homogenous group. Both sex and type of birth (singleton or twin) contribute to variation in faecal worm egg count of grazing lambs (Stear *et al.*, 1996b). There were 197 lambs available but these lambs had been reared in three different fields prior to weaning in July. Therefore, using records from the three previous years, a general linear model was established to investigate whether or not the field of grazing prior to weaning was a factor that contributed significantly to variation in faecal worm egg count. A field effect was observed, and so lambs were selected which had a common grazing. From the 197 lambs the largest homogenous groups were 58 male singletons and 58 female twins. A

variance ratio test showed that variance was greater in females and that the variance increased as the grazing season progressed, and therefore lambs were selected from the female twins.

4.5.3: Protocol

A few days after all the lambs were sampled for faeces in October, all the female twins from Fields 1 and 2 were gathered and blood was collected and processed according to Protocols 2.3.1.2 and 2.3.3.2. Immunophenotyping was for B cell percentages using VPM8 at 1:20. The 16 lambs with the highest B cell percentages and the 16 lambs with the lowest B cell percentages were selected. From these 32 lambs, two groups were selected by ranking the lambs according to their B cell percentages and assigning lambs with odd numbers of ranking (1^{st}, 3^{rd}, 5^{th} ...) to Group N and those with an even number (2^{nd}, 4^{th}, 6^{th} ...) to Group D, thus giving two groups of 16 lambs each, with equal means and equal variance in B cell percentages but drawn from the extremes of B cell percentages so that both groups had high and low B cell subsets. The selected lambs were removed from pasture and transported to GUVS where they were separated into Groups N and D, housed separately and fed on hay.

One week after they were transported the first four lambs in Group N were bled and slaughtered, and four lambs a day were slaughtered each day for the next three days. Immunophenotyping of peripheral blood was done using the protocol described in Chapter 2, Protocol 2.3.3.1, immunophenotyping of abomasal node cells was carried out using Protocol 2.3.3.3, and the panel and concentration of antibodies used were the same as those used in Experiment 4.2.

Each of the 16 lambs in Group D were dosed with an anthelmintic (ivermectin, Oramec Drench) at the recommended dose of 200 $\mu g\ kg^{-1}$of bodyweight and, four weeks later, deliberately challenged with 50,000 infective *O. circumcincta*

L_3. Immunophenotyping was carried out on peripheral blood removed three days before and three days after challenge using Protocol 2.3.3.3 and the panel of monclonals in Experiment 4.2. Ten days after challenge the lambs were slaughtered and immunophenotyping of abomasal node cells was carried out using Protocol 2.3.3.3 and the panel and concentration of antibodies used were the same as those used in Experiment 4.2. The abomasal nodes from all lambs were weighed and, working on the assumption that the proportion of lymphocytes is uniform across the node, this measure was used to arrive at a value for the total number of lymphocytes of each subset in the abomasal nodes; the number of lymphocytes per gram of node was not calculated and the value was the product of the mass of nodes by the percentage representation of the subset and was expressed in units.

4.5.3: Results

From the 58 lambs selected, B cell percentages in the peripheral blood were determined from only 54 lambs because one lamb was not found and there were technical problems with three samples, two controls had high background fluorescence and one sample had no fluorescence. The mean B cell percentage was 40.1, the highest was 59.7 and the lowest was 27.7. The mean B cell percentage for Group N was 40. 9 and the mean B cell percentage for Group D was 40.2, but the mean for the high B cell subsets was 49.5 in Group N and 48.5 in Group D and the mean for the low B cell subsets was 32.4 in Group N and 31.9 in Group D. Tables 4.26 and 4.27 show the B cell percentages for Groups N and D at two different times.

When the B cell percentages from blood samples taken at time of selection were compared with those obtained from Group N lambs at the time of slaughter there was a strong correlation, 0.88 ($p<0.01$), but the pre-housing mean was greater, 40.9, than the post-housing mean, 32.1 ($p<0.01$). The B cell percentages from

Group D lambs whilst grazing and after housing and dosing with anthelmintic were compared, revealing a moderately strong association, 0.59 ($p<0.05$), and that the pre-housing mean was greater, 40.2 compared to 33.3, ($p<0.05$), confirming that grazing produces a B cell lymphocytosis.

Table 4.28 shows the representation of lymphocyte subsets in the peripheral blood of Group N lambs prior to slaughter, and Table 4.29 shows the total lymphocyte counts and the concentrations of each lymphocyte subset.

The percentage representation of all four subsets in peripheral blood of Group D lambs were compared before and after challenge and recorded in Table 4.30. The mean of CD4+ T cells decreased from 18.9 % to 15.1 % ($p<0.01$), the mean of CD8+ T cells from 8.5 % to 4.8 % ($p<0.01$), the mean of γδ T cells increased from 17.5 % to 24.6 % ($p<0.01$), but there was no significant difference between the mean B cell percentages ($p=0.37$); these results are represented in Figure 4.5.

Table 4.31 shows the total lymphocyte count per ml of peripheral blood from Group D lambs before and after challenge; the before challenge mean was 7.5 x 10^6 cells ml^{-1}, which is significantly greater than the after challenge mean of 6.6 x 10^6 cells ml^{-1} ($p<0.05$). The total number of lymphocytes belonging to each subset per ml of peripheral blood from Group D lambs before and after challenge is shown in Table 4.32. Following challenge absolute numbers of CD4+ and CD8+ cells decreased - CD4+ cells from 1.36 x 10^6 cells ml^{-1} to 0.97 x 10^6 cells ml^{-1} ($p<0.01$), CD8+ cells from 0.67 x 10^6 cells ml^{-1} to 0.33 x 10^6 cells ml^{-1} ($p<0.01$) - but there was no significant change in the absolute numbers of γδ T cells ($p=0.18$) and B cells ($p=0.16$); these results are represented in Figure 4.6.

Table 4.33 shows the percentage representation of lymphocyte subsets from the abomasal nodes of Group N lambs. Those lambs that were selected as having a high B cell percentage in their peripheral blood had a higher B cell percentage in the abomasal node than those selected as having a low B cell percentage - 29%

compared to 23%, ($p<0.05$). Table 4.34 shows the percentage representation of lymphocyte subsets from the abomasal nodes of Group D lambs but there was no significant difference between B cell percentages in the two groups ($p=0.09$). The mass of the abomasal nodes and the estimate of the total number of lymphocytes of each subset within the nodes are given in Tables 4.35 and 4.36. In Group N the high B cell group had a greater total number of B cells in the abomasal node, 127 cell units to 77 cell units ($p<0.05$), but this was not true for Group D lambs ($p=0.45$).

When the abomasal node lymphocyte percentages in Group N lambs were compared with those in Group D lambs the Group N animals had a higher percentage of CD8+ T cells, 9.6 to 7.3 ($p<0.05$), but a lower percentage of B cells, 26% to 31% ($p<0.05$); there was no significant difference in the CD4+ and $\gamma\delta$ T cells percentages ($p=0.41$ and 0.65). When the means of the mass of the abomasal nodes between Group N and Group D lambs were compared there was no significant difference ($p=0.08$) but the deliberately infected group had a greater total number of B cells, 149 cell units to 102 cell units ($p<0.01$).

LAMB	AT GRAZING	HOUSED
3	60	48
21	56	44
100	51	42
31	48	30
47	47	39
199	46	41
126	45	39
58	43	29
92	36	23
198	35	29
63	34	30
15	33	19
35	32	25
1	31	29
149	30	22
11	28	25

Table 4.26: The percentage representation of B cells in peripheral blood from lambs in Group N whilst grazing and after housing, immediately prior to slaughter.

LAMB	AT GRAZING	HOUSED AND DRENCHED
93	58	57
72	53	38
117	50	42
113	48	42
70	47	23
87	46	36
118	43	37
95	43	36
91	36	31
25	35	26
156	34	40
26	33	40
24	30	22
68	30	40
23	29	26
147	28	26

Table 4.27: The percentage representation of B cells in peripheral blood from lambs in Group D whilst grazing and following housing and treatment with anthelmintic.

LAMB	B CELL STATUS	CD4+ T CELLS	CD8+ T CELLS	γδ T CELLS	B CELLS
3	HIGH	12	5	14	48
21	HIGH	20	9	10	44
100	HIGH	16	7	15	42
31	HIGH	15	6	20	30
47	HIGH	18	5	25	39
199	HIGH	17	11	14	41
126	HIGH	15	6	17	39
58	HIGH	24	8	11	29
92	LOW	16	4	29	23
198	LOW	17	9	27	29
63	LOW	18	7	20	30
15	LOW	29	11	22	19
35	LOW	24	8	22	25
1	LOW	23	6	13	29
149	LOW	20	10	17	22
11	LOW	14	13	25	25

Table 4.28: The percentage representation of lymphocyte subsets in the peripheral blood from Group N lambs immediately prior to slaughter.

LAMB	COUNT	CD4+ T CELLS	CD8+ T CELLS	γδ T CELLS	B CELLS
3	10.1	1.21	0.50	1.41	4.85
21	10.4	2.08	0.94	1.04	4.58
100	8.2	1.31	0.57	1.23	3.44
31	9.0	1.35	0.54	1.80	2.70
47	9.1	1.64	0.46	2.28	3.55
199	8.2	1.39	0.90	1.15	3.36
126	8.9	1.33	0.53	1.51	3.47
58	9.0	2.16	0.72	0.99	2.61
92	13.0	2.08	0.52	3.77	2.99
198	10.1	1.71	0.91	2.73	2.93
63	8.6	1.55	0.60	1.72	2.58
15	7.6	2.20	0.84	1.67	1.44
35	10.6	2.54	0.85	2.33	2.65
1	7.4	1.70	0.44	0.96	2.14
149	14.2	2.84	1.42	2.41	3.12
11	8.4	1.18	1.09	2.10	2.10

Table 4.29: The lymphocyte counts and the number of lymphocytes of each subset (x 10^6 cells ml^{-1}) in the peripheral blood of lambs from Group N immediately prior to slaughter.

LAMB	B CELL STATUS	CD4+ CELLS BC	CD4+ CELLS AC	CD8+ CELLS BC	CD8+ CELLS AC	γδ T CELLS BC	γδ T CELLS AC	B CELLS BC	B CELLS AC
93	HIGH	15	21	8	5	18	14	57	37
72	HIGH	20	5	8	2	12	14	38	60
117	HIGH	11	10	5	2	23	23	42	30
113	HIGH	17	15	8	6	6	17	42	40
70	HIGH	27	21	8	6	24	12	23	41
87	HIGH	22	25	8	7	6	22	36	31
118	HIGH	13	10	8	3	20	24	37	39
95	HIGH	16	12	7	3	25	42	36	23
91	LOW	17	11	13	4	27	34	31	28
25	LOW	24	18	9	8	17	13	26	29
156	LOW	18	8	15	8	25	25	40	29
26	LOW	20	22	5	8	16	24	40	29
24	LOW	20	18	8	7	22	44	22	18
68	LOW	21	16	11	5	7	27	40	37
23	LOW	24	17	11	4	15	34	26	22
147	LOW	18	14	14	8	18	25	26	29

Table 4.30: The percentage representation of lymphocyte subsets in the peripheral blood before challenge (BC) and three days after challenge (AC) of lambs from Group D with 50,000 *O. circumcincta* L_3.

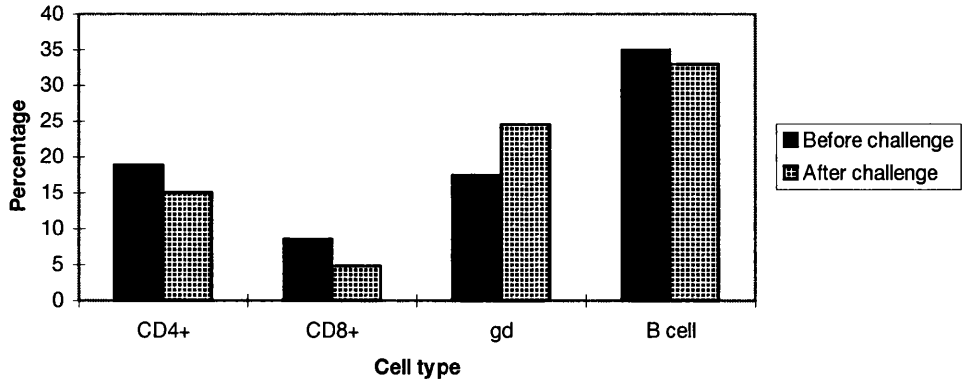


Figure 4.5: Changes in lymphocyte subset percentages following challenge of Group D lambs with 50,000 *O. circumcincta* L_3.

LAMB	B CELL STATUS	COUNT BEFORE CHALLENGE	COUNT AFTER CHALLENGE
93	HIGH	8.4	8.1
72	HIGH	7.0	6.1
117	HIGH	12.4	9.0
113	HIGH	6.4	6.5
70	HIGH	6.8	5.8
87	HIGH	6.0	4.6
118	HIGH	8.8	8.5
95	HIGH	8.1	7.0
91	LOW	10.9	7.8
25	LOW	5.2	6.0
156	LOW	5.0	5.6
26	LOW	7.0	4.6
24	LOW	5.9	4.1
68	LOW	6.8	8.1
23	LOW	7.0	6.9
147	LOW	7.9	6.8

Table 4.31: Total lymphocyte counts (x 10^6 cells ml^{-1}) in the peripheral blood of Goup D lambs before and three days after challenge.

LAMB	B CELL STATUS	CD4+ CELLS		CD8+ CELLS		γδ T CELLS		B CELLS	
		BC	AC	BC	AC	BC	AC	BC	AC
93	HIGH	1.26	1.70	0.67	0.40	1.51	1.13	4.79	3.00
72	HIGH	1.40	0.30	0.56	0.12	0.84	0.85	2.66	4.86
117	HIGH	1.36	0.90	0.62	0.18	2.85	2.07	5.21	2.70
113	HIGH	1.09	0.98	0.51	0.39	0.38	1.10	2.69	2.60
70	HIGH	1.84	1.22	0.54	0.35	1.63	0.70	1.56	2.38
87	HIGH	1.32	1.15	0.48	0.32	0.36	1.01	2.16	1.43
118	HIGH	1.14	0.85	0.70	0.26	1.76	2.04	3.26	3.32
95	HIGH	1.30	0.84	0.57	0.21	2.02	2.94	2.92	1.61
91	LOW	1.85	0.86	1.42	0.31	2.94	2.65	3.38	2.18
25	LOW	1.25	1.08	0.47	0.48	0.88	0.78	1.35	1.74
156	LOW	0.90	0.45	0.75	0.45	1.25	1.40	2.00	1.62
26	LOW	1.40	1.01	0.35	0.37	1.12	1.10	2.80	1 33
24	LOW	1.18	0.74	0.47	0.29	1.30	1.80	1.30	0.74
68	LOW	1.43	1.30	0.75	0.40	0.48	2.19	2.72	3.00
23	LOW	1.68	1.17	0.77	0.28	1.05	2.35	1.82	1.52
147	LOW	1.42	0.95	1.11	0.54	1.42	1.70	2.05	1.97

Table 4.32: The total number of lymphocytes (x 10^6 cells ml^{-1}) of each subset in the peripheral blood of Group D lambs before challenge (BC) and three days after challenge (AC) with 50,000 *O. circumcincta* L_3.

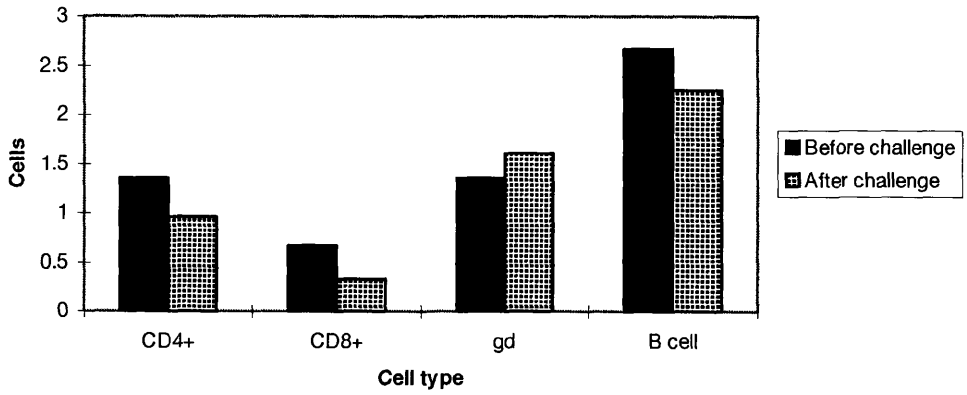


Figure 4.6: Changes in lymphocyte numbers in the peripheral blood (cells x 10^6 ml^{-1}) of Group D lambs three days after challenge with 50,000 *O. circumcincta* L_3.

LAMB	B CELL STATUS	CD4+ T CELLS	CD8+ T CELLS	γδ T CELLS	B CELLS
3	HIGH	32	8	3	28
21	HIGH	31	13	3	27
100	HIGH	27	10	5	37
31	HIGH	37	15	1	32
47	HIGH	35	5	4	26
199	HIGH	34	6	2	29
126	HIGH	31	8	3	33
58	HIGH	26	9	2	22
92	LOW	40	7	4	25
198	LOW	33	10	2	15
63	LOW	34	11	3	18
15	LOW	42	10	2	23
35	LOW	40	8	2	25
1	LOW	31	12	11	30
149	LOW	40	10	3	22
11	LOW	36	11	4	26

Table 4.33: The percentage representation of lymphocyte subsets in the abomasal nodes of Group N lambs.

LAMB	B CELL STATUS	CD4+ CELLS	CD8+ CELLS	γδ T CELLS	B CELLS
93	HIGH	41	6	1	16
72	HIGH	33	8	2	32
117	HIGH	27	4	5	39
113	HIGH	38	6	3	22
70	HIGH	35	7	4	32
87	HIGH	34	10	6	42
118	HIGH	32	7	5	38
95	HIGH	42	9	2	18
91	LOW	20	7	2	48
25	LOW	24	4	4	38
156	LOW	38	6	2	22
26	LOW	34	15	6	28
24	LOW	32	6	4	30
68	LOW	32	8	5	32
23	LOW	34	7	4	24
147	LOW	28	7	4	40

Table 4.34: The percentage representation of lymphocyte subsets in the abomasal nodes of Group D lambs.

LAMB	MASS	CD4+ CELLS	CD8+ CELLS	γδ T CELLS	B CELLS
3	6.8	218	54	20	190
21	2.5	78	32	8	68
100	3.6	97	36	18	133
31	6.2	229	93	6	198
47	5.4	189	27	22	140
199	2.4	82	14	5	70
126	3.9	121	31	12	129
58	4.3	112	39	9	95
92	2.5	100	18	10	62
198	1.6	53	16	3	24
63	3.1	105	34	9	56
15	3.6	151	36	7	83
35	4.2	168	34	8	105
1	3.0	93	36	33	90
149	2.7	108	27	8	59
11	5.3	191	58	21	138

Table 4.35: The mass of abomasal nodes in grams and the estimate of the total number of cells of each subset within the abomasal nodes of Group N lambs.

LAMB	MASS	CD4+ CELLS	CD8+ CELLS	γδ T CELLS	B CELLS
93	3.0	123	18	3	48
72	8.8	290	70	18	282
117	5.4	146	22	27	211
113	5.5	209	33	16	121
70	3.0	105	21	12	96
87	5.1	296	51	31	214
118	4.2	134	29	21	160
95	8.4	353	76	17	151
91	4.0	80	28	8	192
25	3.6	86	14	14	137
156	6.4	243	38	13	141
26	4.0	136	60	24	112
24	5.0	160	30	20	150
68	3.2	102	26	16	102
23	3.7	126	26	15	89
147	4.4	123	31	18	176

Table 4.36: The mass of abomasal nodes in grams and the estimate of the total number of cells of each subset within the abomasal nodes of Group D lambs.

4.6: CORRELATIONS BETWEEN IMMUNOPHENOTYPING RESULTS AND PARASITOLOGICAL PARAMETERS FOR LAMBS SELECTED AS EITHER HAVING A HIGH OR A LOW PERCENTAGE OF B CELLS IN PERIPHERAL BLOOD

4.6.1: Introduction

The results from Experiment 4.4 showed a strong negative association between the B cell percentages in peripheral blood and the total worm burden acquired by grazing and this association was significant at the $p<0.05$ level. However, because a large number of correlations were investigated, it is possible that some

correlations would have achieved this level of significance purely by chance. An experiment was designed to test this association and to investigate whether it extended to the worm burden of lambs given a deliberate infection. The design of the experiment was such that the lambs receiving a deliberate infection would be killed ten days after challenge; therefore if the peripheral blood B cell percentages were negatively associated with worm burden in these lambs then they would be associated with a mechanism preventing the establishment of *O. circumcincta* larvae.

4.6.2: Protocol

This experiment used the lambs and the immunophenotyping results from Experiment 4.5. On the day of slaughter, faecal samples were taken for faecal worm egg counts and samples taken from the abomasum to calculate worm burden and, for the naturally infected lambs, the mean length of adult female worms from each lamb. The parasitology results are recorded in Experiment 3.4.

The data was transformed to prevent outlying results skewing the analysis. The log of worm burden and faecal worm egg counts were calculated and because the lymphocyte subsets were expressed as percentages, arcsine values were calculated. In Group N lambs the parasitological parameters which were examined were the slaughter egg count, the number of L_4 *O. circumcincta,* the number of L_5 *O. circumcincta*, the number of female adult *O. circumcincta*, the number of male adult *O. circumcincta*, the total *O. circumcincta* burden and the mean adult female worm length. In Group D, the deliberate challenge group, the total *O. circumcincta* burden was calculated. The mean worm length was not transformed.

4.6.3: Results

In Group N lambs there was no association between the percentage representation of subsets within either the peripheral blood prior to slaughter or the abomasal node and the faecal worm egg count at the time of slaughter, any measures of *O. circumcincta* burden or the mean adult female worm length. These results are tabulated in Tables 4.37, 4.38 and 4.39.

The results from associations between the total number of lymphocytes in each subset from peripheral blood and abomasal node and parasitological parameters are shown in Tables 4.40, 4.41 and 4.42. There was no association between the total number of lymphocytes of each lymphocyte subset in peripheral blood and any parasitological parameter, nor was there an association between the total number of lymphocytes of any subset in the abomasal node and the faecal worm egg count at the time of slaughter or any measures of *O. circumcincta* burden. There were strong negative and highly significant correlations between the log of total number of CD4+ T cells, CD8+ T cells and B cells in the abomasal node and the mean adult female worm length, and the correlation between total number of γδ T cells in the abomasal node was strongly negative and trending towards significance. There was a very strong negative correlation between the log of the mass of abomasal nodes and the mean of worm length, -0.80 ($p<0.01$), shown in Figure 4.6.

Since the Group D lambs were slaughtered before the larvae from the infective dose matured, there is no measure of either adult female worm length or faecal worm egg count. The log of total lymphocyte count after challenge was positively associated with the log of worm burden, 0.64 ($p<0.01$), but this was not true for the before challenge count, 0.27 ($p=0.30$). There was a trend towards the CD4+ T cell percentage in peripheral blood sampled prior to the challenge to be negatively associated with worm burden, but the after challenge

percentage was strongly and highly significantly negatively associated; the post-challenge CD8+ T cell percentages were also negatively associated with worm burden. There were no other significant correlations in Group D lambs. These results are shown in Tables 4.43, 4.44 and 4.45 and Figures 4.7 and 4.8.

CELLS	PERIPHERAL BLOOD	ABOMASAL NODE
CD4+ T CELLS	-0.10 p=0.70	-0.30 p=0.25
CD8+ T CELLS	-0.28 p=0.29	0.05 p=0.86
γδ T CELLS	0.27 p=0.31	-0.10 p=0.72
B CELLS	0.05 p=0.86	-0.16 p=0.55

The first figure is the strength of the association, the second the probability of this occurring by chance.

Table 4.37: The associations between the log of faecal worm egg counts at time of slaughter and the arcsine of percentage representation of lymphocyte subsets in peripheral blood at time of slaughter and the abomasal node in Group N lambs.

CELLS	PERIPHERAL BLOOD	ABOMASAL NODE
CD4+ T CELLS	-0.38 p=0.15	-0.14 p=0.60
CD8+ T CELLS	-0.40 p=0.13	-0.32 p=0.22
γδ T CELLS	0.42 p=0.10	0.12 p=0.66
B CELLS	-0.01 p=0.99	-0.02 p=0.95

The first figure is the strength of the association, the second the probability of this occurring by chance.

Table 4.38: The associations between the log of total *O. circumcincta* burden and the arcsine of percentage representation of lymphocyte subsets in peripheral blood at time of slaughter and the abomasal node in Group N lambs.

CELLS	PERIPHERAL BLOOD	ABOMASAL NODE
CD4+ T CELLS	-0.14 p=0.61	0.02 p=0.96
CD8+ T CELLS	0.21 p=0.44	-0.05 p=0.84
γδ T CELLS	0.34 p=0.19	-0.04 p=0.88
B CELLS	-0.18 p=0.52	-0.30 p=0.25

The first figure is the strength of the association, the second the probability of this occurring by chance.

Table 4.39: The associations between the mean worm length and the arcsine of percentage representation of lymphocyte subsets in the peripheral blood at time of slaughter and the abomasal node in Group N lambs.

CELLS	PERIPHERAL BLOOD	ABOMASAL NODE
CD4+ T CELLS	-0.16 p=0.54	-0.01 p=0.96
CD8+ T CELLS	-0.35 p=0.19	0.08 p=0.77
γδ T CELLS	0.07 p=0.81	-0.05 p=0.84
B CELLS	-0.09 p=0.73	-0.02 p=0.94

The first figure is the strength of the association, the second the probability of this occurring by chance.

Table 4.40: The associations between the log of faecal worm egg count and the log of the total number of lymphocytes of each subset per ml of peripheral blood at time of slaughter and the total number of lymphocytes of each subset in the abomasal node of Group N lambs.

CELLS	PERIPHERAL BLOOD	ABOMASAL NODE
CD4+ T CELLS	-0.26 p=0.95	0.02 p=0.95
CD8+ T CELLS	-0.31 p=0.24	-0.16 p=0.56
γδ T CELLS	0.33 p=0.21	0.24 p=0.38
B CELLS	0.08 p=0.77	0.05 p=0.85

The first figure is the strength of the association, the second the probability of this occurring by chance.

Table 4.41: The associations between the log of *O. circumcincta* burden and the log of the total number of lymphocytes of each subset per ml of peripheral blood at time of slaughter and the total number of lymphocytes of each subset in the abomasal node in Group N lambs.

CELLS	PERIPHERAL BLOOD	ABOMASAL NODE
CD4+ CELLS	0.19 p=0.48	-0.75 p<0.01
CD8+ CELLS	0.41 p=0.11	-0.66 p<0.01
γδ T CELLS	0.42 p=0.10	-0.50 p=0.08
B CELLS	0.08 p=0.74	-0.75 p<0.01

The first figure is the strength of the association, the second the probability of this occurring by chance.

Table 4.42: The associations between the female worm length and the log of the total number of lymphocytes of each subset per ml of peripheral blood at time of slaughter and the total number of lymphocytes of each subset in the abomasal node of Group N lambs.

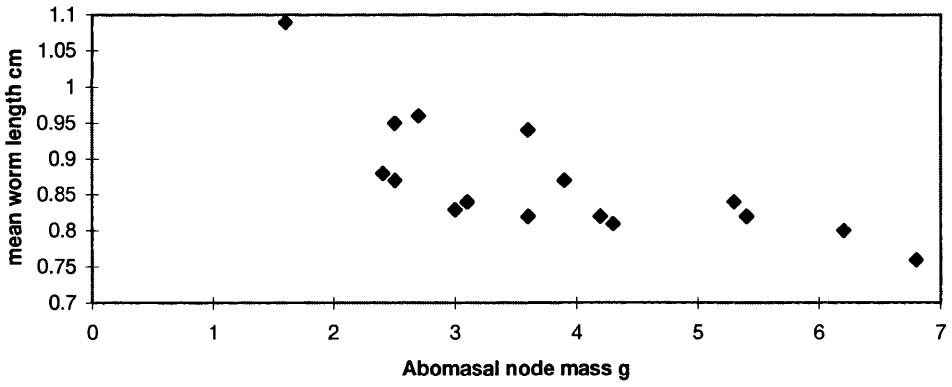


Figure 4.6: The relationship between the mass of the abomasal node and the mean of the adult female worm length for Group N lambs.

CELLS	ARCSINE OF PERCENTAGE	LOG OF CELL COUNT
CD4+ T CELLS	-0.48 p<0.06	-0.27 p=0.30
CD8+ T CELLS	-0.06 p=0.81	0.25 p=0.36
γδ T CELLS	-0.11 p=0.69	0.00 p=0.99
B CELLS	0.29 p=0.28	0.40 p=0.12

The first figure is the strength of the association, the second the probability of this occurring by chance.

Table 4.43: The associations between the log *O. circumcincta* burden and the arcsine of percentage representation of lymphocyte subsets and the log of the total number of lymphocytes of each subset per ml of peripheral blood in Group D lambs before challenge with 50,000 L_3.

CELLS	PERIPHERAL BLOOD	ABOMASAL NODE
CD4+ T CELLS	-0.63 p<0.01	0.08 p=0.75
CD8+ T CELLS	-0.58 p<0.05	-0.43 p=0.10
γδ T CELLS	0.04 p=0.87	-0.22 p=0.40
B CELLS	0.12 p=0.65	-0.20 p=0.46

The first figure is the strength of the association, the second the probability of this occurring by chance.

Table 4.44: The associations between the log *O. circumcincta* burden and the arcsine of percentage representation of lymphocyte subsets in peripheral blood after challenge and the abomasal node in Group D lambs.

CELLS	PERIPHERAL BLOOD	ABOMASAL NODE
CD4+ T CELLS	-0.20 p=0.46	0.31 p=0.23
CD8+ T CELLS	-0.33 p=0.23	-0.02 p=0.94
γδ T CELLS	0.43 p=0.10	0.07 p=0.80
B CELLS	0.44 p=0.09	0.12 p=0.65

The first figure is the strength of the association, the second the probability of this occurring by chance.

Table 4.45: The associations between the log of *O. circumcincta* and the log of the total number of lymphocytes of each subset per ml of peripheral blood at time of slaughter and the total number of lymphocytes of each subset in the abomasal node in Group D lambs.

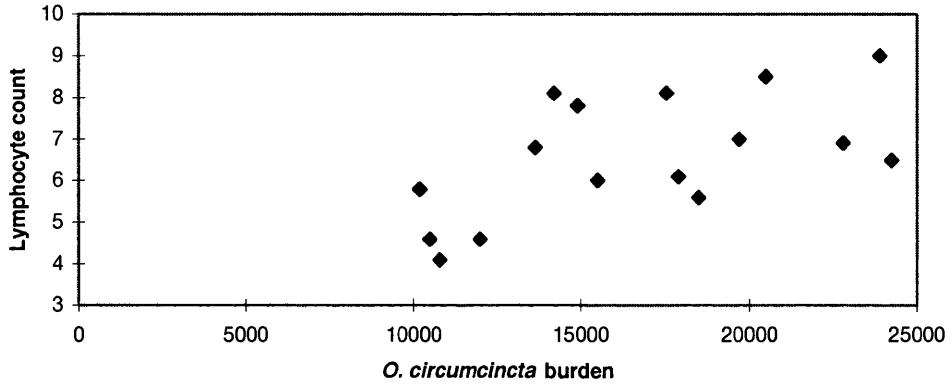


Figure 4.7: The relationship between the *O. circumcincta* burden and the lymphocyte count (x 10^6 cells ml^{-1}) in the peripheral blood of Group D lambs three days after challenge with 50,000 L_3.

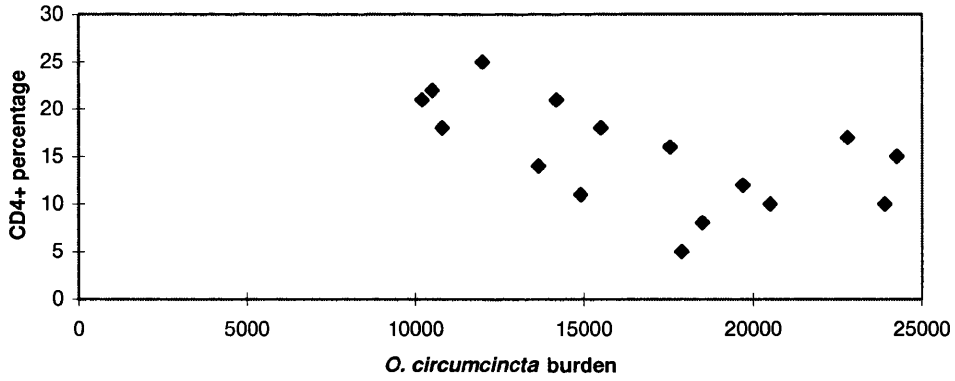


Figure 4.8: The relationship between the *O. circumcincta* burden and the percentage of lymphocytes which are CD4+ cells in the peripheral blood of Group D lambs three days after challenge with 50,000 L_3.

4.7: DISCUSSION

This study has shown that grazing lambs, compared to housed lambs, have an increased number of lymphocytes in the peripheral blood. The increase appears largely due to an increased number of B lymphocytes. Lambs in which a greater proportion of their peripheral blood lymphocytes were B cells had a smaller worm burden while an increased abomasal node weight was associated with a reduced worm length. Following a deliberate challenge with *O. circumcincta* L_3, sheep that had a greater worm burden had greater numbers of lymphocytes in the peripheral blood but a lower percentage of those lymphocytes were CD4+ T cells.

Compared to housed lambs, grazing lambs have a greater concentration of lymphocytes in peripheral blood and a greater percentage of these lymphocytes were B cells and, in some months, a lesser percentage were CD4+ T cells. Gorrell *et al.* (1988) compared sheep reared indoors in a worm free environment with sheep reared on pasture and found that pasture reared sheep had a much higher density of lymphocytes in the abomasal mucosa than indoor reared sheep, but when pasture reared sheep were immunised and challenged with *H. contortus* no increase in lymphocyte density was observed, possibly due to a high lymphocyte background. Therefore, the differences observed in the work described here may partly reflect environmental differences, especially an increased exposure to dietary antigens, rather than a specific response to nematode challenge. Indeed the observation that in Group N lambs the peripheral blood B cell percentages decreased after housing despite the fact that they were still carrying their worm burden, and the reduction was of a similar magnitude to that recorded in the Group D lambs which were wormed at housing, could imply that additional non-parasitic antigens act as the stimulus for the lymphocytosis. Alternatively, a continual challenge with incoming larvae may be necessary.

Another possible explanation for the observed differences in lymphocyte counts is that grazing lambs may have suffered more fear and excitement during handling and exhibited a physiological lymphocytosis - a well recognised phenomenon in other species (Bush, 1991).

Grazing lambs also had a greater percentage of B cells and a lesser percentage of CD4+ T cells in the abomasal node, and this finding is supported by work in cattle that demonstrated that abomasal nodes from pasture reared cattle had a greater percentage of B cells and a lower percentage of cattle CD4+ T cells than those from cattle reared indoors and helminth-naïve (Baker *et al.*, 1993). As in the peripheral blood, the changes in abomasal node may be a response to environmental factors but some of the increase in B cell percentages may be due to parasitism because if housed calves receive a primary *O. ostertagi* infection by deliberate challenge there is an increase in B cells and γδ T cells in both the abomasal mucosa and abomasal node (Almeria *et al.*, 1997, Canals *et al.*, 1997).

In Experiment 4.2 the overall mean of lymphocyte representation in peripheral blood of CD4+, CD8+ and γδ cells was 14%, 6% and 20% respectively, and 22%, 8% and 21% in the housed lambs. When these results are compared with 20% and 12% and 15% from mature Merino sheep sampled by Mackay *et al.* (1986), the CD4+ and CD8+ values for housed lambs are similar to those in the merino sheep but the lambs had a greater representation of γδ cells. Although some of the difference between Scottish Blackface lambs and Merino sheep may be due to technical differences, because Mackay *et al.* (1986) used a monoclonal 19-19 (T-19) that only identified 90% of γδ cells in peripheral blood, it is more likely that a combination of breed and age factors accounted for most of the difference. Indeed, in subsequent studies on different ages of sheep, the CD4+, CD8+ and γδ percentages were 15, 8 and 35 in lambs aged three to four months and 20, 11 and 22 in lambs aged eight to 12 months, indicating that the proportion of peripheral blood lymphocytes that are γδ cells decreases as sheep

mature (Mackay *et al.*, 1989). McClure *et al.* (1992), also using monoclonal 19-19 (T-19), immunophenotyped cells in the peripheral blood of two groups of mature Merino sheep, one group helminth naïve and the other having been exposed to *T. colubriformis*, and the CD4+, CD8+ and γδ cell percentages were 20, 14 and 12 for the helminth-naïve sheep, but in the other group the CD8+ and γδ cell percentages were 14 and 9 - exact CD4+ percentages were not given for the immunised group but they were greater than 15%. The values of T cell percentages recorded in the work described here concur with the values in the literature. The author is not aware of published values for B cell percentages in the peripheral blood of sheep but the strong correlations observed between the same animals sampled at different time points in Experiment 4.5 suggest that this is an accurate technique.

The immunophenotyping results from the monthly samples of peripheral blood from grazing lambs revealed that an individual's lymphocyte concentration and percentage of peripheral blood lymphocytes in each subset becomes more repeatable as the lamb matures and thus may play a role in regulation of faecal worm egg count.

Experiment 4.3 was designed to investigate differences in lymphocyte subset representation in the peripheral blood and in the abomasal node between two groups of lambs selected as having either a low or a high faecal worm egg count. Because both high and low egg count groups had been selected from the same population and received identical treatment it was appropriate to combine the two groups to investigate any associations between immunological and parasitological variables, and this was done in Experiment 4.4. The significant and moderately strong negative association between the B cell percentages in peripheral blood and the total *O. circumcincta* worm burden was of considerable interest. The initial interpretation of this result was that antibodies, produced by B cells, may play a role in regulating worm burdens. The association, if

confirmed, could be combined with faecal worm egg count and used as a selection criterion for resistant lambs for experimental or commercial purposes.

Although the association between B cell percentages and worm burden appeared significant a degree of caution is required because if a large number of possible associations are attempted it is probable that some may appear significant at the generally accepted significance level of $p<0.05$ merely by random events. Another association significant at the conventional $p<0.05$ level was the association between the CD4+ percentage of peripheral blood and the adult male *O. circumcincta* burden, and although it would be easy to speculate on a mechanism whereby hosts with a greater CD4+ population were better protected it is more difficult to suggest a hypothesis that restricts the effect to male worms, and the author considers that it is likely that this association is a result of chance. It was considered prudent to test the hypothesis that B cells are associated with worm burden, and to do this lambs from the extremes of the B cell percentage were chosen, and it was hoped that in so doing two samples of significantly divergent worm populations would be chosen.

The results from the work on lambs with a naturally acquired infection in Experiment 4.6 provide no evidence to support any significant association between B cell percentage representation in the peripheral blood and worm burden, nor was there any association between any of lymphocyte subset percentages in peripheral blood or abomasal node and any parasitological parameter. The B cell association observed in Experiment 4.4 may have been an artefact, or it may be that the animals were sampled from a very different population, because although lambs used in Experiments 4.4 and 4.6 were the same breed, age, sex and farm of origin, their exposure to parasites may well have been very different due to the fact that Experiment 4.4 was conducted in 1994, an unremarkable summer, whilst Experiment 4.6 was conducted in 1995, an unusually hot and dry summer which led to reduced parasite exposure and compromised the development of host immunity (Hunt and Taylor,1995) and led

to warnings in the veterinary press of disease outbreaks once wet weather returned (Veterinary Investigation Service, 1995).

It is now known that lambs of this age, in this system do not have genetic control of worm burden (Bishop *et al.,* 1996). One possible explanation for the association observed in the work described here, is that the parasite alters the immune response in an attempt to evade host immunity. A greater population of worms, occurring by a random process, may have inhibited B cell production. There is evidence that *O. ostertagi* can modulate immune function (Cross and Klesius, 1989), and parasites may be able to produce enough IFN-γ to subvert the host to a Type I response (Grencis and Entwistle, 1997), which may lead to a decreased percentage of B cells in peripheral blood in sheep with more worms. In contrast, other work suggests that evasion of the host immune response is through polyclonal B cell activation and lambs with more worms might then have a higher B cell percentage (Crandall *et al.*, 1978). The hypothesis that *O. circumcincta* modulates immune function to increase B cell percentage, could still be consistent with repeatable B cell percentages in a system whereby lambs are drenched and exposed to new infections at monthly intervals, because the effects of host evasion may facilitate the establishment of the next infection. Other non-genetic factors which may determine the host response and account for a causal association between B cell percentages and worm burden include non-genetic dam effects (quality and quantity of passive immunity may be related to dam's age, health and parasite exposure), age at which lambs were first exposed to significant challenge and any concurrent disease.

In Experiment 4.5, Group N lambs selected as having high B cell percentages in the peripheral blood had significantly higher B cell percentages and a greater number of B cells in the abomasal node, suggesting that the peripheral blood may reflect processes in the abomasal mucosa.

The deliberate challenge infection in Experiment 4.5 gave the opportunity to examine lymphocyte parameters before and after challenge. There was a

significant decrease in the lymphocyte concentration of peripheral blood after challenge, perhaps due to reduced migration into the peripheral blood from the tissues. There was a decrease in the percentage of CD4+ and CD8+ T cells and an increase in the γδ T cell percentages in peripheral blood three days after challenge; these changes are very similar in direction and magnitude to those reported by McClure *et al.* (1992) four days after immune sheep were challenged with *T. colubriformis*. The latter study also looked at changes in naïve sheep and did not observe any changes in either CD4+ or γδ T cell percentages, thus indicating that these were functions of an immune response rather than an inflammatory response. McClure (unpublished data) also recorded an increase in the absolute and relative numbers of γδ cells in the efferent lymph from the mesenteric node of previously exposed sheep three days after challenge with *T. colubriformis*, suggesting that a local response was the source of the increase in these cells observed in the peripheral blood.

In Experiment 4.6, the deliberate challenge, there were again associations between worm burden and lymphocyte parameters in the peripheral blood following challenge, and the association with CD4+ percentages was negative, strong and highly significant, -0.63 ($p<0.01$), the association with CD8+ percentages was negative, strong and significant, -0.58 ($p<0.05$), but the association with the total lymphocyte count was strong and positive, 0.64 ($p<0.01$). If the lymphocyte count increased after challenge then the relationship between count and burden could be attributed to a dose effect but because the challenge caused a reduced lymphocyte count an alternative explanation is required. Following a deliberate challenge with *T. colubriformis* immune sheep had a greater concentration of lymphocytes in the lamina propria than naïve sheep, suggesting that the accumulation of lymphocytes in the mucosa is immune mediated (McClure *et al.*, 1992). Lambs that are better able to regulate worm burden through a more efficacious immune response may have a greater accumulation of lymphocytes in the mucosa resulting in a lower lymphocyte count in the peripheral blood. The percentage of CD4+ and CD8+ T cells in

peripheral blood decreased after challenge and those lambs which have a greater worm burden appear to have had a lower percentage, therefore the effect may be a dose related immune response. It may be that those lambs with a more efficient immune response recruit more lymphocytes into the mucosa but worms that do establish trigger an immune mediated selective uptake of CD4+ and CD8+ T cells - therefore sheep with an efficient response would recruit many lymphocytes into the mucosal tissues, resulting in a lower worm burden and consequently limit the selective uptake of CD4+ and CD8+ T cells.

The above mechanism would explain the observations from this study but would be dependent upon an immune response capable of limiting larval establishment. Lambs slaughtered at six months of age have no genetic control over worm burden but because the last anthelmintic was given a month prior to slaughter this reflects lack of genetic control in preventing establishment as early as five months of age (Stear *et al.*, 1997a), yet lambs of the same breed and farm of origin challenged at nine months of age can regulate worm burden through local immediate hypersensitivity reactions (Stear *et al.*, 1995c). The lambs in Experiment 4.7 were seven months old at time of challenge and it is possible they were regulating worm establishment, although the larval establishment rates were similar to those previously recorded for naïve sheep (Stevenson *et al.*, 1994).

Another explanation would be that sheep with a previous exposure to parasites respond with an infiltrate of lymphocytes into the abomasal mucosa resulting in a reduction in the peripheral blood lymphocyte count, but the parasite modulates and reduces this infiltration in a dose dependent manner, therefore sheep with more worms have a less intense response resulting in a positive association between worm burden and lymphocyte count. The parasite immune modulation may be cell specific, perhaps altering the quality of the immune response, and the influx of CD4+ cells and CD8+ cells may still be dose dependent.

The work described here reveals an association between increased mass of the abomasal node and reduced worm length. This observation is of particular interest because work by Stear *et al.* (1997b), on lambs of the same age, breed and farm of origin, shows faecal worm egg count is regulated by restricting worm length, which is in turn inhibited by a local IgA response to parasite-specific antigens (Stear *et al.*, 1995c). Therefore, it is reasonable to suggest that an increased mass of abomasal node may be associated with an increased local IgA response. Calves challenged with *O. ostertagi* have abomasal nodes of greater mass than those of control calves, but the mass of abomasal nodes was strongly and positively correlated with worm burden, suggesting that the size of the abomasal node was directly associated with the magnitude of the antigen challenge (Yang *et al*, 1993; Gasbarre, 1997). Taking into account the work on cattle and the observation that, due to population density effects, sheep with more worms have shorter worms (Stear *et al.*, 1996b), it may be argued that the association between abomasal node mass and reduced worm length is a confounding effect of sheep with more worms have bigger abomasal nodes and shorter worms, but this argument appears invalid because there was no direct correlation between worm burden and abomasal node weight in these lambs.

Studies on immunoglobulin production in the sheep intestine suggest that IgA antibody-containing cells in the lamina propria are responsible for local IgA production and that up-regulation of IgA in immune sheep occurs following local proliferation of antibody-containing cells (Beh, 1977; Husband *et al.*, 1979). If such a mechanism occurs in the abomasum, then an association between an increased mass of abomasal node and a reduced worm length may be due to the proliferation of plasma cell precursors in the abomasal node. Antibody-containing cells proliferate, with T cell help, in the lamina propria, but it may be that to produce sufficient antibody in the face of a chronic infection continued repopulation by newly induced antibody-containing cells is necessary and those lambs with a greater mass of node may be providing a greater level of repopulation. The inductive sites for IgA production in the small intestine are

the Peyer's patches, which are primary lymphoid organs in sheep (Craig and Cebra, 1975; Landsverk *et al.,* 1991), but the inductive site for IgA antibody containing cells in the abomasum has not been elucidated. Although lymphoid aggregates and follicles have been identified in the abomasum of parasitised sheep (Christie *et al.*, 1978; Hunter and MacKenzie, 1982; Gorrell *et al.*, 1988), these are most likely to be analogous to structures described in human gastric mucosa, following pathogen challenge, which lack the anatomical organisation of mucosally associated lymphoid tissue (Genta *et al.*, 1994), and may not be the sites of IgA induction. Other candidates for the site of induction for the IgA antibody-containing cells in the abomasal mucosa would include the abomasal node or distally in the Peyer's patches of the jejunum. If the abomasal node was the site of IgA antibody-containing cell induction, then those lambs that have a greater node mass may have had a greater magnitude of induction and consequently a greater IgA response.

Immunoglobulin A antibody-containing cell induction may not occur in the abomasal node, or even if it does so an enhanced induction may not contribute to enhanced IgA production in the lamina propria, but IgA production is T cell dependent and cognate T cell help is required in the inductive sites as well as T cell message at the effector site (Clough *et al.*, 1971; Crewther and Warner, 1972; Dunkley *et al.*, 1990; Dunkley and Husband, 1991; Boa *et al.*, 1993), therefore lambs with a greater mass of node may have enhanced recruitment of T cell populations required to provide that help.

A third possible scenario is that an increase in abomasal node weight has no direct mechanistic effect on local parasite-specific IgA production but may be a marker, albeit not a very useful one, for an appropriate or greater immunological response that regulates *O. circumcincta* worm length in grazing lambs.

The aim of the work in this chapter was to examine variation in lymphocyte subset numbers and relate these to variation in parasitological parameters, and try

to understand the role, if any, of these subsets in host protection against *O. circumcincta*. The observation of a significant association between B cell percentages and worm burden did not stand up to further investigation and remains an interesting observation which should be treated with caution. The negative association between CD4+ T cell percentages and worm burden in a controlled deliberate infection was more significant and merits further investigation. A different approach to understanding the role of different lymphocyte subsets has been taken by McClure *et al.* (1996), who examined immune responses of sheep to *T. colubriformis*, and using monoclonal antibodies to delete given subsets from the peripheral blood, though not tissues, they altered the host-parasite dynamic, demonstrating that deletion of CD8+ and γδ cells enhances protection - demonstrating that although a γδ cell response is a feature of the immune reaction, the cells retard protection in both primary and challenge infections. Interestingly McClure *et al.* (1996) also improved protection by using a monoclonal antibody to IFN-γ, demonstrating that IFN-γ hampers protection and work by Canals *et al.* (1997), using *in vitro* culture techniques, suggests that stimulated abomasal γδ cells express IFN-γ.

Further experiments could examine peripheral blood lymphocyte numbers and the lymphocyte subset percentages in housed animals given a trickle *O. circumcincta* challenge infection to confirm whether the lymphocytosis and relative over-representation of B cells and under-representation of CD4+ cells in grazing lambs was due to environmental factors rather than nematode challenge. Such an experiment, if continued for several months, would also allow further testing of the putative negative association of B cell percentages with worm numbers. The negative association between mass of abomasal node and worm length merits further study, especially links with local parasite-specific IgA production. The finding of a negative association between CD4+ cell percentages in the peripheral blood and worm burden following a truncated deliberate infection could also be further explored and an area of interest would be to examine the CD4+ population at the abomasal mucosa. It would be of

interest to compare lymphocyte subsets and investigate any parasite modulation of the immune response. Intra-epithelial lymphocytes and lamina propria lymphocytes have been isolated from the abomasum of cattle (Almeria *et al.*, 1997) and the author has isolated lymphocytes from the abomasal mucosa of sheep but the protocol was not systematic enough to dovetail with the other procedures. Indeed the author would agree with the comments of Gasbarre (1997) that procedures that normally result in recovery of large number of lymphocytes from the mucosa of the intestine yield few lymphocytes from the abomasum.

CHAPTER 5: LYMPHOCYTE PROLIFERATION ASSAYS AND THEIR RELATIONSHIP WITH PARASITOLOGICAL PARAMETERS

5.1: Introduction

The magnitude of a protective response may be dependent upon the number of activated effector lymphocytes producing the appropriate cytokine. Following exposure to pathogens, antigenic stimulation of lymphocytes results in clonal expansion, whereby cells proliferate and differentiate into clones of effector cells or memory cells - subsequent stimulation by identical antigen will result in clonal expansion of these memory cells, thus resulting in a secondary response and further increasing the size of both effector and memory cell pools (Roitt *et al.*, 1996a). Therefore a host's ability to mount a protective response partly depends upon clonal expansion of lymphocytes, and variation in the magnitude of protective responses may result from variation in the mechanisms of clonal expansion. The *in vivo* process of clonal expansion can be mimicked by the *in vitro* technique of lymphocyte proliferation assays using specific antigen to stimulate a secondary response, or by stimulation with mitogenic lectins which result in a non-specific clonal expansion of lymphocytes.

Work in cattle and swine has shown marked sire effects on *in vitro* lymphocyte proliferation responses to mitogens (Kehrli *et al.*, 1991; Edfors-Lilja *et al.*, 1994), and studies of between-strain differences in proliferative responses in mice have shown that resistant mice had greater stimulation responses (Robinson *et al.*, 1995). In sheep it is very likely that proliferative responses are under genetic control and it is possible that genetic control of lymphocyte responsiveness contributes to variation in parasite resistance.

Although several studies have investigated lymphocyte proliferative responses in sheep to various parasites, no clear pattern is emerging (reviewed in Chapter 1). The findings include primary responses to parasite antigen correlating with subsequent protection (Riffkin and Dobson, 1979), sheep from resistant lines having greater post-exposure responses to both mitogen and specific antigen (Dineen and Windon, 1980), sheep from susceptible lines having greater responses to mitogen after exposure to parasites (Pernthaner *et al.*, 1995a), resistant or immune sheep having greater spontaneous proliferation (McClure *et al.*, 1992; Pernthaner *et al.*, 1995a) and no significant difference between groups despite considerable differences in parasitology (Gamble and Zajac, 1992) - all these findings were from studies which used peripheral blood lymphocytes.

Sheep that were either resistant or susceptible to *O. circumcincta* have been bred, and following challenge the proliferative responses of peripheral blood lymphocytes to *O. circumcincta* antigen were greater in the resistant line (Yong *et al.*, 1991). However, as one of the selection criteria for resistant lines was the proliferation assay, this observation can be explained by genetic control of proliferative responses and does not show that variation in resistance is due to variation in the capabilities for clonal expansion.

Studies have shown increased numbers of lymphocytes and lymphoblasts in the efferent gastric lymph of sheep infected with *O. circumcincta*, that lymphocyte and lymphoblast numbers were greater in immune animals and that lymphocytes and lymphoblasts could be used to adoptively transfer immunity between siblings (Smith *et al.*, 1983a; Smith *et al.*, 1983b; Smith *et al.*, 1986). These studies emphasise the importance of the local response.

The work described in this chapter was designed to investigate proliferative responses following stimulation with both mitogen and specific antigen in lymphocytes isolated from the abomasal node as well as those isolated from

peripheral blood, and spontaneous proliferation of cells was also investigated. Relationships with parasitological parameters were analysed.

5.2: OPTIMISATION OF PROLIFERATION ASSAYS

5.2.1: Introduction

It was intended that proliferation assays would be used to investigate responses of individuals to mitogen and specific *O. circumcincta* antigen, and that investigation would involve examining within-group variation using analysis of variance. Many studies have used proliferation assays to compare responses between different groups (exposed versus naïve, or vaccinated versus non-vaccinated) where between-group differences can be expected to be substantial, but it was anticipated that variation may be more subtle and therefore attempts were made to optimise experimental conditions and gain an impression of the usefulness of this technique for such an approach.

5.2.2: Protocol

Titrations were set up to investigate the effect of different incubation conditions. The source of PBMC depended on the availability of sheep under licence at GUVS for PBMC, and sheep that had been killed at GUVS as part of earlier work in this thesis were used as the source of abomasal node cells. Isolation of PBMC and abomasal node cells was done according to Protocols 2.3.3.11 and 2.3.3.12 respectively. Cryopreservation was done according to Protocol 2.3.3.15. Cell counting was done according to Protocol 2.3.3.4 and if 10% or more of cells were non viable then dead cells were removed according to Protocol 2.3.3.14.

Unless otherwise stated, proliferation assays were set up according to Protocol 2.3.3.16, with the exception that those assays described prior to the one showing the benefit of 2-mercaptoethanol (2-ME) did not contain 2-ME in the TCM. All samples were set up in triplicate and the results are expressed as the mean count per minute (Cpm) or as stimulation index (SI), which was the ratio of the Cpm for the three stimulated wells and the Cpm for the three control wells.

5.2.3: Results

A series of three titrations to investigate the effect of abomasal cell concentration, incubation period and concentration of Con A indicated that the highest stimulation occurs with a cell concentration of 5×10^6 cells ml^{-1} and for a 48 hour incubation (Tables 5.1, 5.2 and 5.3). Table 5.3 shows that although Cpm were higher at a cell concentration of 5×10^6 cells ml^{-1}, so were the counts from wells without Con A, and a concentration of 1×10^6 cells ml^{-1} gave a higher SI.

The relationship between Con A concentration and proliferative responses was bimodal (Table 5.4 and Figure 5.1) and the first and highest peak was at approximately 10 $\mu g\ ml^{-1}$, whilst the second peak was close to, or plateauing at, 40 $\mu g\ ml^{-1}$ for all four animals examined. For the first peak, the concentration of Con A for which maximal proliferation occurred varied from 5 to 15 $\mu g\ ml^{-1}$, but a slight deviation from optimum concentration could lead to a greatly reduced response, and the optimum concentration of Con A for some sheep produced a response greatly less than optimal for others. The ranking of indices for the four animals at 10 $\mu g\ ml^{-1}$ differed from that at 40 $\mu g\ ml^{-1}$.

Table 5.5 and Figure 5.2 show the SI from four different samples from the same sheep, all derived from the same stock solution prior to cryopreservation, and the results show that overall pattern of response was similar although values varied

and the ranking of samples in the first peak was not the same as those in the second.

Four blood samples were removed at the same time from one sheep and processed by the same protocol simultaneously and two sets of triplicate cells were set up for each sample. The results give some qualitative insight into the repeatability of the technique, showing that one sample gave results considerably greater than the others and appeared to be due to greater proliferation in the stimulated cells rather than lack of activity in the unstimulated cells (Table 5.6). At the same time as the PBMC from the one sheep, F, were used for blastogenesis, PBMC were cryopreserved and kept frozen for two months before being used in proliferation assays which were set up at the same reaction conditions as for fresh PBMC. The SI for preserved cells were of the same order of magnitude, showing that cryopreservation did not greatly reduce subsequent proliferation; the mean of four sets of triplicates for cryopreserved cells was 33 (23, 38, 34, 37) which compared with the mean SI of 29 (results in Table 5.6) for fresh cells.

A proliferation assay was set up to investigate the effect on stimulation indices of 2-mercaptoethanol (2-ME) and to compare the use of HI-FCS with heat inactivated autologous lamb serum. The results showed that stimulation indices were greatest for culture medium containing foetal calf serum and 2-ME (Table 5.7). All subsequent proliferation assays used 2-ME.

The optimum concentration of HI-FCS in the final culture medium for three of four animals was 10% (Table 5.8), the fourth sheep PBMC gave a higher SI at a final concentration of 5 % HI-FCS.

Following observation of the benefits of the addition of 2-ME, the effect of a small variation in cell concentration was investigated (Table 5.9). A small variation in cell concentration could produce dramatic variation in stimulation

indices and affect the ranking of SI for a group of sheep. It was also noted that the optimum concentration differed amongst individuals (Figure 5.3).

The optimum incubation period was re-evaluated following addition of 2-ME to the cell culture medium and Table 5.10 shows that the maximum stimulation occurred with 72 hours incubation. The ranking of the sheep for a 48 hour incubation differed from that for a 72 hour incubation, but ranking of results for the three sheep common to the experiments recorded in Tables 5.8, 5.9 and 5.10 was the same for a cell concentration of 1 x 10^6 cells ml^{-1}, HI-FCS at 10 % and an incubation period of 48 hours, although there were large differences in SI for the same individuals between experiments.

A titration across time, *O. circumcincta* antigen type, and antigen concentration was set up to investigate lymphoproliferative responses to antigen. The results are shown in Table 5.11 and show that positive responses occurred with both PBMC and abomasal node cells, and that for the reaction conditions tested the greatest stimulation occurred with 20 μg ml^{-1} of the *O. circumcincta* L_3 somatic extract (L_3SE) preparation (prepared according to Protocol 2.3.3.7) following a culture period of 168 hours.

Con A	1 x 10^7	5 x 10^6	1 x 10^6	5 x 10^5
40 μg ml^{-1}	21	30	17	9
20 μg ml^{-1}	17	70	16	6
10 μg ml^{-1}	16	26	6	2
5 μg ml^{-1}	4	7	2	1
2.5 μg ml^{-1}	2	2	1	1
1.0 μg ml^{-1}	1	2	1	1
0.5 μg ml^{-1}	1	1	1	1

HI FCS at 5 % and cultured for 48 hours

Table 5.1: Stimulation indices (SI) following incubation of abomasal node cells at range of concentrations of cells with a range of Con A concentrations.

TIME (Hours)	10 μg ml $^{-1}$	15 μg ml $^{-1}$	20 μg ml $^{-1}$	25 μg ml $^{-1}$	30 μg ml $^{-1}$	40 μg ml $^{-1}$	50 μg ml $^{-1}$
36	25	11	11	16	18	16	8
48	83	21	23	37	45	61	55
72	42	12	8	12	18	24	21
96		5	4		10		
120		4	3		1		

HI-FCS at 5 % and 5 x 10^6 abomasal node cells ml^{-1}

Table 5.2: Stimulation indices (SI) following culture of abomasal node cells with a range of concentrations of Con A for a range of culture periods.

SHEEP	1 X 10^7	5 X 10^6	1 X 10^6	5 X 10^5	2.5 X 10^5
A	73,051 (10)	78,788 (16)	42,077 (59)	21,942 (37)	9,666 (19)
B	26,483 (6)	34,586 (11)	14,784 (12)	9,432 (10)	3,577 (6)

HI-FCS at 5 %, Con A at 10 μg ml^{-1} and cultured for 48 hours

Table 5.3: Mean counts per minute (Cpm) and SI (in brackets) for abomasal node cells from two sheep at various abomasal node cell concentrations.

Con A	A	B	C	D
50 μg ml $^{-1}$	23	18	46	13
40 μg ml $^{-1}$	22	18	48	12
30 μg ml $^{-1}$	19	12	41	12
20 μg ml $^{-1}$	13	5	15	12
15 μg ml $^{-1}$	10	28	12	6
10 μg ml $^{-1}$	39	9	52	15
5 μg ml $^{-1}$	12	4	34	26
2.5 μg ml $^{-1}$	6	2	4	9

HI-FCS at 5 %, 1 x 10^6 abomasal node cells ml^{-1} and cultured for 48 hours

Table 5.4: Stimulation indices (SI) following culture of abomasal node cells from four different sheep at a range of concentrations of Con A.

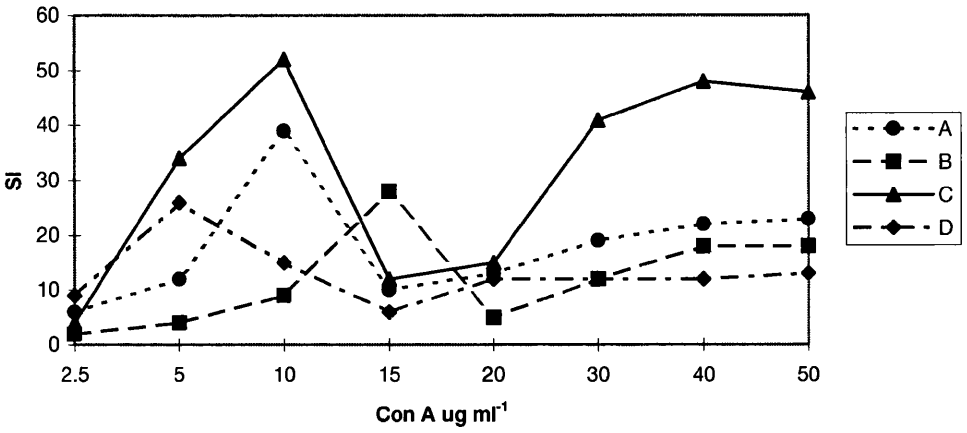


Figure 5.1: Optimisation of Con A concentration for proliferation assays, cultured for 48 hours, for 1 x 10^6 abomasal node cells ml^{-1} from four sheep.

Con A	E1	E2	E3	E4
80 μg ml^{-1}	20	14	37	18
40 μg ml^{-1}	22	16	34	24
20 μg ml^{-1}	20	10	25	18
10 μg ml^{-1}	33	22	39	22
5 μg ml^{-1}	41	34	52	28
2.5 μg ml^{-1}	14	6	36	12

HI-FCS at 5 %, 1 x 10^6 abomasal node cells ml^{-1} and cultured for 48 hours

Table 5.5: Stimulation indices (SI) from four different samples from the same sheep at a range of concentrations of Con A.

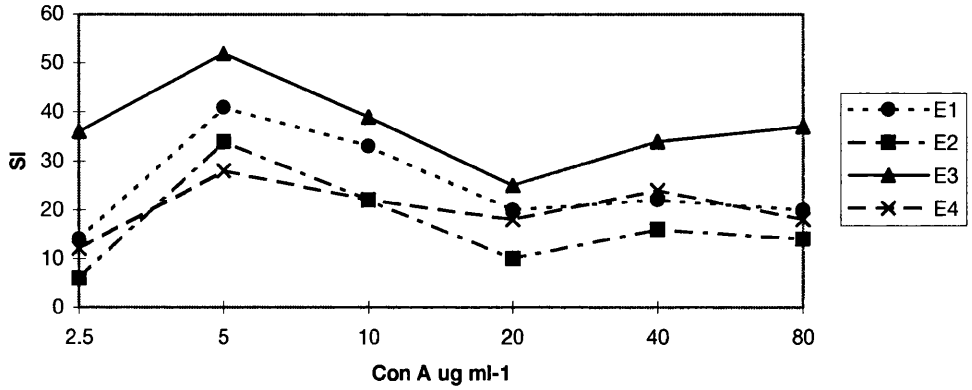


Figure 5.2: Optimisation of Con A concentration for proliferation assays for 1 x 10^6 abomasal node cells, cultured for 48 hours, from four different samples from the same sheep.

	F1	F2	F3	F4
I	134650 (16)	334195 (36)	96384 (17)	126355 (18)
II	159688 (21)	562676 (74)	87775 (15)	172675 (27)

HI-FCS at 5 %, Con A at 10 μg ml^{-1}, PBMC concentration 1 x 10^6 and cultured for 48 hours

Table 5.6: Mean counts per minute, and SI (in brackets), for results from two sets of triplicate wells, I and II, for four blood samples from the same sheep, sampled at the same time and processed by the same protocol simultaneously.

SHEEP	HI-FCS with 2-ME	HI-FCS	SHEEP SERUM 2-ME	SHEEP SERUM
G	59	21	13	8
H	84	26	11	9
I	33	8	7	2

1 x 10^6 PBMC ml^{-1}, Con A at 10 μg ml^{-1} and cultured for 48 hours

Table 5.7: Stimulation indices (SI) of PBMC from three sheep in culture medium that contained either heat inactivated sheep serum or HI-FCS at 5% and either with or without 2-ME (10^{-5}M).

SHEEP	20 %	15 %	10 %	5 %
J	21	18	50	28
K	9	21	25	22
L	4	10	35	10
M	3	86	97	160

1 x 10^6 PBMC ml^{-1}, Con A at 10 μg ml^{-1}, 2-ME at 10^{-5}M and cultured for 48 hours

Table 5.8: Stimulation indices (SI) following incubation of PBMC from four sheep in culture medium containing different concentrations of HI-FCS.

SHEEP	1.5 x 10^6 ml^{-1}	1.25 x 10^6 ml^{-1}	1.0 x 10^6 ml^{-1}	0.75 x 10^6 ml^{-1}	0.5 x 10^6 ml^{-1}
J	163	165	139	63	45
K	97	84	89	106	75
L	155	72	63	23	16
M	173	179	200	197	135

HI-FCS at 10 %, Con A at 10 μg ml^{-1}, 2-ME at 10^{-5}M and cultured for 48 hours

Table 5.9: Stimulation indices (SI) following incubation of PBMC at different cell concentrations.

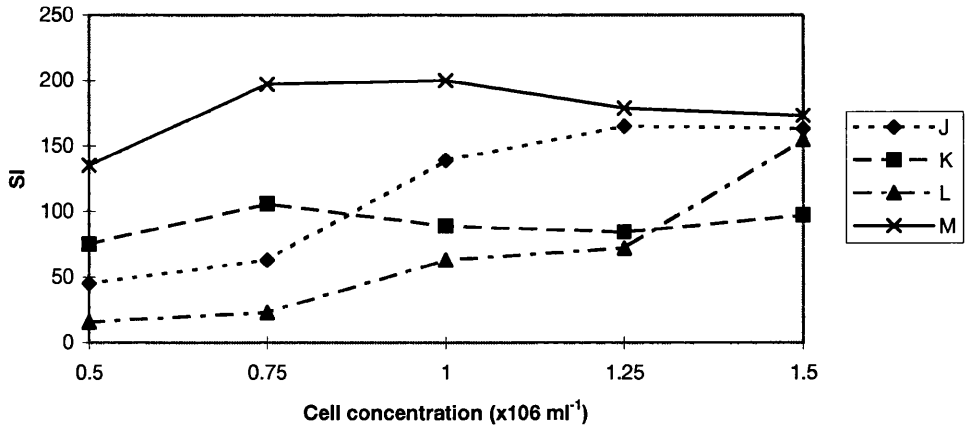


Figure 5.3: Stimulation indices (SI) for four different sheep over a range of PBMC cell concentration.

SHEEP	24 HOURS	48 HOURS	72 HOURS	96 HOURS
J	4	156	394	383
K	8	92	283	207
M	3	225	327	270

HI-FCS at 10 %, 1 x 10^6 PBMC ml^{-1}, Con A at 10 µg ml^{-1}, 2-ME at 10^{-5}M

Table 5.10: Stimulation indices (SI) following incubation of PBMC from three sheep in culture medium for different culture periods.

CELLS	ANTIGEN µg ml^{-1}	72 HOURS	96 HOURS	120 HOURS	144 HOURS	168 HOURS
PBMC	L_3SE 20	1.1	2.0	1.1	3.6	4.1
ANC	L_3SE 20	2.6	2.2	3.4	5.2	8.1
ANC	L_3SE 10	1.4	2.7	2.9	2.7	5.2
ANC	L_3SE 5	2.1	1.6	1.9	2.0	2.1
ANC	L_3SE 2.5	2.0	1.2	1.6	1.8	3.5
ANC	AI 20	2.0	0.7	0.9	1.1	0.8
ANC	AI 10	0.7	1.0	1.5	1.4	1.1
ANC	AI 5	0.6	1.1	1.6	2.6	1.7
ANC	AI 2.5	0.6	1.1	1.6	2.8	1.7
ANC	AII 20	1.8	3.2	3.2	3.0	2.3
ANC	AII 10	1.1	1.5	0.8	1.2	1.0
ANC	AII 5	1.3	1.0	0.8	0.7	1.3
ANC	AII 2.5	1.2	0.6	0.9	1.2	3.6

HI-FCS at 10 %, 1 x 10^6 cells ml^{-1}, 2-ME at 10^{-5}M

Table 5.11: Stimulation indices (SI) following incubation of PBMC and abomasal node cells (ANC), from one sheep, with different *O. circumcincta* antigens: L_3SE, Adult Preparation I (AI) and Adult Preparation II (AII), at different concentrations.

5:3: A COMPARISON OF PROLIFERATIVE RESPONSES OF ABOMASAL NODE CELLS FROM HELMINTH-NAÏVE LAMBS WITH THOSE FROM LAMBS EXPOSED TO A NATURAL *O. CIRCUMCINCTA* INFECTION

5.3.1: Introduction

This experiment was designed to test whether there was a difference between the proliferation of abomasal node cells from lambs which were helminth-naïve and those which had been exposed to *O. circumcincta* by grazing infected pasture.

5.3.2 Protocol

Five male castrated Scottish Blackface lambs were reared indoors from birth, and kept helminth-naïve. The lambs were slaughtered at six months of age and immediately after slaughter abomasal nodes were collected and abomasal node cells were isolated according to Protocol 2.3.3.12. Cell counting was done according to Protocol 2.3.3.4 and if 10% or more of cells were non viable dead cells were removed according to Protocol 2.3.3.14. Cells were cryopreserved and subsequently harvested according to Protocol 2.3.3.15.

Sixteen male castrate Scottish Blackface lambs were removed from grazing at six months old and kept housed for a few days prior to being slaughtered. The same protocols were used to collect and store abomasal cells as were used in the helminth-naïve lambs. Lymphocytes from 10 of these 16 lambs were recovered and used in this experiment. The reason for testing lymphocytes from only 10 was that there was a difference in storage procedure for the six not used.

Proliferation assays were set up according to Protocol 2.3.3.16, with a final HI-FCS concentration of 10%, 2-ME at 10^{-5}M and a cell concentration of 1 x 10^6 cells ml^{-1}. Those set up with Con A had a final Con A concentration of 10 μg ml^{-1} and were incubated for 72 hours, whilst those set up with specific antigen had a final L_3SE concentration of 20 μg ml^{-1} and were incubated for 168 hours.

Results are expressed as Cpm and SI from triplicate samples.

5.3.3 Results

The results from proliferation assays that included Con A and L_3SE are shown in Tables 5.12 and 5.13. Analysis of the responses to Con A showed no significant difference between the groups for Cpm in control wells, Cpm following stimulation and SI ($p=0.68$, 0.99 and 0.49). Table 5.14 shows that mean proliferative responses to L_3 antigen were much higher for abomasal cells from grazing lambs than for those from helminth-naïve lambs, Cpm were 10,230 compared to 704 and SI were 21 compared to 1.0 ($p<0.01$ for both comparisons). There was no significant difference for Cpm in unstimulated wells ($p=0.37$).

LAMB	GROUP	CONTROL Cpm	Con A Cpm	SI
P17	NAÏVE	1,496	29,471	20
P38	NAÏVE	961	42,476	44
P46	NAÏVE	266	9,204	35
P48	NAÏVE	905	56,562	62
P53	NAÏVE	441	12,128	28
B15	GRAZING	287	8,036	28
B16	GRAZING	752	62,491	83
B20	GRAZING	1,794	34,445	19
B27	GRAZING	610	10,797	18
B37	GRAZING	470	38,728	82
B41	GRAZING	315	14,207	45
B46	GRAZING	554	9,972	18
Y165	GRAZING	624	79,560	127
Y166	GRAZING	1,1O5	21,989	20
Y192	GRAZING	520	19,396	37

TABLE 5.12: Mean counts per minute (Cpm) and SI for abomasal node cells from helminth-naïve lambs and those from grazing lambs following culture with Con A.

LAMB	GROUP	CONTROL Cpm	L_3SE Cpm	SI
P17	NAÏVE	508	881	1.7
P38	NAÏVE	195	218	1.1
P46	NAÏVE	3,844	1,269	0.3
P48	NAÏVE	921	667	0.7
P53	NAÏVE	459	486	1.1
B15	GRAZING	535	13,592	25
B16	GRAZING	769	17,735	23
B20	GRAZING	322	23,477	73
B27	GRAZING	161	1,746	11
B37	GRAZING	1,202	25,250	21
B41	GRAZING	334	6,161	18
B46	GRAZING	553	4,126	7
Y165	GRAZING	472	2,362	5
Y166	GRAZING	320	3,857	12
Y192	GRAZING	328	3,995	12

TABLE 5.13: Mean counts per minute (Cpm) and SI for abomasal node cells from helminth-naïve lambs and those from grazing lambs following culture with L_3SE.

STIMULUS	GROUP	CONTROL Cpm	STIMULATED Cpm	SI
Con A	NAÏVE	814	29,968	38
Con A	GRAZING	703	29,962	48
L_3 SE	NAÏVE	1185	704	1.0
L_3 SE	GRAZING	500	10,230	21

TABLE 5.14: Mean counts per minute (Cpm) and SI for abomasal node cells, from helminth naïve lambs and those from grazing lambs, following culture with Con A or L_3SE.

5.4: PROLIFERATIVE RESPONSES OF PBMC AND ABOMASAL NODE CELLS FROM SHEEP WITH A NATURALLY ACQUIRED *O. CIRCUMCINCTA* INFECTION AND SELECTED ACCORDING TO B CELL PERCENTAGES

5.4.1: Introduction

The results from Experiment 4.4 showed an association between an increased percentage of peripheral blood lymphocytes which were B cells and a smaller number of *O. circumcincta* in the abomasum. A subsequent experiment, Experiment 4.5, selected lambs with the highest or lowest percentages of B cells in the peripheral blood, with the aim of selecting lambs with high and low worm burdens. Sixteen of these lambs were slaughtered when carrying the worm burden acquired by grazing, and eight of these lambs had a high B cell percentage and eight had a low B cell percentage - these were Group N lambs. Cells from these 16 were available for lymphocyte proliferation assays. Several lymphocyte parameters were examined - spontaneous proliferation in wells without either Con A or L_3SE and Cpm and SI following culture of cells with Con A or L_3SE.

5.4.2: Protocol

In October April born lambs were removed from pasture and transported to GUVS where they were housed and fed on hay. Four lambs a day were slaughtered on the 8^{th}, 9^{th}, 10^{th}, and 11^{th} days after housing. Immediately prior to slaughter peripheral blood was sampled and the abomasal nodes were recovered post mortem.

Isolation of PBMC and abomasal node cells was done according to Protocols 2.3.3.11 and 2.3.3.12 respectively. Cell counting was done according to Protocol 2.3.3.4 and if 10% or more of cells were non viable then dead cells were removed according to Protocol 2.3.3.14. Proliferation assays were set up as for those in Experiment 5.3.

5.4.3: Results

The results from proliferation assays following stimulation with Con A of PBMC and abomasal cells from Group N are shown in Tables 5.15 and 5.16. Tables 5.17 and 5.18 show the results following incubation with L_3SE for the same source of cells from the same sheep (no results were available for some samples due to contamination of the wells).

The mean counts per minute following stimulation with Con A of PBMC were greater in the low B cell group than in the high B cell group, 99,202 versus 52,585 ($p<0.05$), but there was no significant difference between Cpm in control wells and SI ($p=0.08$ and 0.15 respectively). Comparison of counts following culture of abomasal node cells with Con A showed no significant difference

between high and low B cell groups (p=0.74, 0.35 and 0.26 for Cpm in control wells, Cpm in stimulated wells and SI).

Following culture of cells with L_3SE there was no significant difference for any parameter between high and low B cell groups - the parameters examined were Cpm in control wells, Cpm in wells with antigen, and SI for PBMC (p=0.83, 0.90 and 0.93) and abomasal node cells (p=0.66, 0.82, 0.90). Because some results were not available only 11 sheep were involved in the analysis of abomasal cell responses.

When the results from PBMC were compared with those from abomasal node cells there were no significant differences in responses to Con A (p=0.73, 0.41 and 0.97 for control Cpm, stimulated Cpm and SI), and no significant differences for control wells at 168 hours and Cpm following culture with L_3SE (p=0.13 and 0.07) but the mean SI for abomasal node cells cultured with antigen was greater than that of PBMC cultured with antigen, 15.2 compared to 3.0 ($p<0.05$).

LAMB	B CELL GROUP	CONTROL Cpm	CON A Cpm	SI
3	HIGH	1,942	67,972	35
21	HIGH	1,301	29,625	23
100	HIGH	2,909	78,910	27
31	HIGH	417	29,402	71
47	HIGH	986	43,894	45
199	HIGH	806	98,657	122
126	HIGH	1,394	38,610	27
58	HIGH	2,134	33,749	16
92	LOW	2,169	147,569	68
198	LOW	775	91,021	117
63	LOW	2,226	116,367	52
15	LOW	756	43,031	57
35	LOW	1,300	54,405	42
1	LOW	1,841	133,366	72
149	LOW	1,195	73,890	62
11	LOW	1,692	133,187	79

TABLE 5.15: Mean counts per minute (Cpm) and SI following culture with Con A of PBMC from Group N lambs.

LAMB	B CELL GROUP	CONTROL Cpm	CON A Cpm	SI
3	HIGH	1,190	72,325	61
21	HIGH	1,885	46,136	24
100	HIGH	1,500	26,719	18
31	HIGH	417	84,271	202
47	HIGH	2,048	81,979	40
199	HIGH	1,038	96,309	93
126	HIGH	383	43,560	114
58	HIGH	5,602	134,363	24
92	LOW	1,250	85,611	68
198	LOW	1,478	73,882	50
63	LOW	4,704	109,834	23
15	LOW	1,268	80,561	64
35	LOW	1,479	20,195	14
1	LOW	426	16,694	39
149	LOW	707	42,889	61
11	LOW	700	20,470	29

TABLE 5.16: Mean counts per minute (Cpm) and SI following culture with Con A of abomasal node cells from Group N lambs.

LAMB	B CELL GROUP	CONTROL Cpm	L_3 SE Cpm	SI
3	HIGH	2,298	6,949	3.0
21	HIGH	864	4,387	5.1
100	HIGH	N/A	N/A	N/A
31	HIGH	812	2062	2.5
47	HIGH	1,447	2,126	1.5
199	HIGH	N/A	N/A	N/A
126	HIGH	N/A	N/A	N/A
58	HIGH	N/A	N/A	N/A
92	LOW	2,433	15,928	6.6
198	LOW	1,403	4,424	3.2
63	LOW	N/A	N/A	N/A
15	LOW	503	1038	2.1
35	LOW	487	1,650	3.4
1	LOW	1,826	2,288	1.3
149	LOW	724	1,665	2.3
11	LOW	1,414	2,236	1.6

TABLE 5.17: Mean counts per minute (Cpm) and SI following culture with L_3SE of PBMC from Group N lambs.

LAMB	B CELL GROUP	CONTROL Cpm	L_3 SE Cpm	SI
3	HIGH	953	8,073	8.5
21	HIGH	650	22,834	35
100	HIGH	N/A	N/A	N/A
31	HIGH	566	2,199	3.9
47	HIGH	1,078	10,955	10
199	HIGH	N/A	N/A	N/A
126	HIGH	N/A	N/A	N/A
58	HIGH	N/A	N/A	N/A
92	LOW	947	15,031	16
198	LOW	996	7,109	7.1
63	LOW	N/A	N/A	N/A
15	LOW	2,003	72,899	36
35	LOW	571	494	0.9
1	LOW	648	1,200	1.8
149	LOW	685	32,372	47
11	LOW	565	614	1.1

TABLE 5.18: Mean counts per minute (Cpm) and SI, following culture with L_3 SE of abomasal node cells from Group N lambs.

5.5: PROLIFERATION ASSAYS IN PBMC AND ABOMASAL NODE CELLS FROM LAMBS SELECTED ACCORDING TO B CELL PERCENTAGES AND SUBSEQUENTLY CHALLENGED WITH 50,000 INFECTIVE *O. CIRCUMCINCTA* L_3

5.5.1: Introduction

Following the observation of an association between B cell percentages in the peripheral blood and worm burden in grazing lambs a deliberate challenge experiment was conducted to test whether such an association would exist following a deliberate challenge with *O. circumcincta*. This experiment is described in Experiment 4.5 - eight lambs with high and eight lambs with low percentages of B cells (Group D lambs) were given a deliberate challenge, lambs, and cells from these 16 were available for lymphocyte proliferation assays. As in Experiment 5.4 the following parameters were examined: spontaneous proliferation in wells without either mitogen or specific antigen, and Cpm and SI following culture of cells with either mitogen or specific antigen. This work enabled comparison of responses in cells from a deliberate challenge with those from a natural challenge.

5.5.2: Protocol

In October April born lambs were removed from pasture and transported to GUVS where they were housed and fed on hay. The lambs were dosed with an anthelmintic at the recommended dose rate and four weeks later deliberately challenged with 50,000 infective *O. circumcincta* L_3. Peripheral blood mononuclear cells were isolated three days after challenge and abomasal node cells were isolated at slaughter, ten days after challenge.

Processing of cells and conditions for the proliferation assays were identical to those in Experiment 5.3.

5.5.3: Results

Tables 5.19 to 5.22 show the results from proliferation assays following stimulation with either Con A or L_3SE of PBMC and abomasal cells from Group D lambs.

Following culture with Con A, comparison of results between high and low B cell groups showed no significant differences for control Cpm, stimulated Cpm and SI for PBMC and abomasal node cells ($p=0.27$, 0.58, 0.40 and 0.50, 0.50 0.55). Similarly there were no significant differences between B cell groups following proliferation assays to antigen ($p=0.26$, 0.30, 0.58 and 0.39, 0.74 ,0.28 for control Cpm, stimulated Cpm and SI for PBMC and abomasal node cells respectively).

Responses of PBMC were compared with those of abomasal node cells. Following culture with Con A, Cpm were higher for PBMC than that of abomasal node cells, 94,828 compared to 71,129 Cpm ($p<0.05$), but there was no significant difference for Cpm in control wells and SI ($p=0.42$ and 0.94). For the antigen proliferation assay the control well mean Cpm and stimulated well mean Cpm were higher for abomasal node cells, 2,090 compared to 875 Cpm for control wells ($p<0.01$) and 51,495 compared to 17,159 Cpm for stimulated wells ($p<0.01$) - and although the mean SI was higher for abomasal node cells, 37 compared to 19, the difference was not significant ($p=0.11$).

Proliferative responses of cells from sheep exposed to a natural infection (Group N lambs) were compared with those from sheep which had been challenged with

50,000 infective *O. circumcincta* L_3 after being exposed to a natural infection (Group D lambs). Following culture with Con A, there was no significant difference for mean Cpm in control wells, mean Cpm in stimulated cells and mean SI for both PBMC and abomasal node cells (p=0.24, 0.24, 0.92 and 0.96, 0.56 0.94). The results from assays of PBMC assays to larval antigen showed no significant difference in control Cpm (0.11), a trend towards cells from Group D lambs having a higher count in wells cultured with antigen, a mean Cpm of 17,160 compared to 4,068 Cpm (p=0.06), and the mean SI was higher for cells from Group D lambs, 19 compared to 3.0 ($p<0.05$). When the responses of abomasal node cells following proliferation assays to specific antigen were compared, the mean Cpm in the control wells was higher for cells from Group D sheep, 2,090 compared to 878 ($p<0.05$), and mean SI were higher for cells from Group D lambs, 37 compared to 15 ($p<0.05$) - there was no significant difference for the mean Cpm from wells cultured with L_3SE (p=0.11).

LAMB	B CELL GROUP	CONTROL Cpm	CON A Cpm	SI
93	HIGH	1,714	128,389	75
72	HIGH	2,930	80,238	27
117	HIGH	5,943	154,684	26
113	HIGH	567	15,882	28
70	HIGH	1,515	126,652	84
87	HIGH	1,047	109,258	104
118	HIGH	1,992	61,396	31
95	HIGH	3,570	138,223	39
91	LOW	1,613	68,898	43
25	LOW	685	71,259	104
156	LOW	665	28,964	43
26	LOW	1,041	49,120	47
24	LOW	1,652	178,840	108
68	LOW	839	58,640	69
23	LOW	1,844	152,695	83
147	LOW	4,212	94,106	22

TABLE 5.19: Scintillation counts per minute (Cpm) and SI for PBMC from Group D lambs following culture with Con A.

LAMB	B CELL GROUP	CONTROL Cpm	CON A Cpm	SI
93	HIGH	803	42,793	53
72	HIGH	3,680	100,745	27
117	HIGH	1,081	100,611	93
113	HIGH	1,214	41,432	34
70	HIGH	1,290	88,078	68
87	HIGH	979	87,653	90
118	HIGH	4,855	88,096	18
95	HIGH	1,279	54,952	43
91	LOW	852	87,096	102
25	LOW	572	16,010	28
156	LOW	828	62,058	75
26	LOW	1,089	60,173	55
24	LOW	635	86,555	136
68	LOW	828	50,615	61
23	LOW	2,377	100,326	42
147	LOW	4,098	70,873	17

TABLE 5.20: Scintillation counts per minute (Cpm) and SI for abomasal node cells from Group D lambs following culture with Con A.

LAMB	B CELL GROUP	CONTROL Cpm	L_3SE Cpm	SI
93	HIGH	662	1368	2.1
72	HIGH	1,170	19,711	17
117	HIGH	572	52,348	92
113	HIGH	607	404	0.7
70	HIGH	1,143	2,805	2.5
87	HIGH	530	8,662	16
118	HIGH	886	8,587	9.7
95	HIGH	2,793	98,150	35
91	LOW	524	2,093	4.0
25	LOW	474	2,440	5.1
156	LOW	921	1,038	1.1
26	LOW	521	16,666	32
24	LOW	545	14,234	26
68	LOW	621	12,344	20
23	LOW	774	15,816	20
147	LOW	1,258	17,886	14

TABLE 5.21: Scintillation counts per minute (Cpm) and SI for PBMC from Group D lambs following culture with L_3SE.

LAMB	B CELL GROUP	CONTROL Cpm	L_3SE Cpm	SI
93	HIGH	1,703	21,082	12
72	HIGH	3,765	67,723	18
117	HIGH	990	16,706	17
113	HIGH	3,190	36,669	11
70	HIGH	1,478	76,540	52
87	HIGH	934	75,840	81
118	HIGH	5,742	74,926	13
95	HIGH	1,890	22,690	12
91	LOW	852	88,547	104
25	LOW	895	1,646	1.8
156	LOW	1,195	26,482	22
26	LOW	1,192	51,692	43
24	LOW	656	83,898	128
68	LOW	801	33,223	41
23	LOW	2,556	76,649	30
147	LOW	5,610	69,602	12

TABLE 5.22: Scintillation counts per minute (Cpm) and SI for abomasal node cells from Group D lambs following culture with L_3SE.

5.6: THE RELATIONSHIP BETWEEN LYMPHOCYTE PROLIFERATION ASSAY RESULTS AND PARASITOLOGICAL PARAMETERS

5.6.1: Introduction

The rationale for conducting proliferation assays was to identify whether or not there are associations between lymphoproliferative responses and parasitological parameters, with the intention of providing insight into the mechanisms that contribute to variation in protective responses to *O. circumcincta*. Proliferation assay and parasitological data sets from two groups of sheep were available, Group N lambs, which had been slaughtered shortly after being removed from pasture where they had acquired a natural *O. circumcincta* infection, and Group

D lambs, which were reared on pasture and housed, dosed with anthelmintic and subsequently given a challenge infection.

5.6.2: Protocol

The proliferation assay results were from Experiments 5.2 and 5.3, and the parasitology results were from the same sheep, recorded in Experiment 3.4. Quantitative analysis was used to investigate the strength and significance of correlations between data sets. For Group N lambs the parasitological parameters examined were the log of total *O. circumcincta* worm burden, the log of faecal worm egg count and worm length, but for the Group D lambs the only parameter available was log of worm burden. Proliferation assay parameters examined were Cpm in control wells, Cpm in stimulated wells and SI for assays to Con A and L_3SE for PBMC and abomasal node cells,

5.6.3: Results

The strength and association of correlations between results from proliferation assays to Con A and parasitological parameters for Group N lambs are shown in Table 5.23, whilst Table 5.24 shows results of similar correlations with results from proliferation assays to L_3SE. For Group N sheep there were no significant associations identified although there was a trend towards significance for a positive association between the log of faecal worm egg count and Cpm of abomasal cells in both control wells cultured for 72 hours and in wells with Con A ($p=0.08$ and $p=0.07$).

Group D lambs were given a deliberate challenge truncated infection and the only parasitological parameter available was the log of worm burden. A negative

association, -0.50 ($p<0.05$) was identified between the SI for PBMC stimulated with Con A and worm burden (Figure 5.4). There was a trend towards a negative association between the SI for abomasal node cells stimulated with L_3SE and worm burden ($p=0.06$) but no other correlations approached significance (Table 5.25).

	LOG OF *O. CIRCUMCINCTA* BURDEN	LOG OF FAECAL WORM EGG COUNT	MEAN WORM LENGTH
LOG OF PBMC CONTROL Cpm	0.30 p=0.27	0.20 p=0.45	-0.03 p=0.93
LOG OF PBMC CON A Cpm	0.12 p=0.65	0.04 p=0.89	0.35 p=0.18
LOG OF PBMC CON A SI	-0.06 p=0.84	-0.00 p=0.99	0.37 p=0.16
LOG OF ANC CONTROL Cpm	0.16 p=0.57	0.45 p=0.08	-0.19 p=0.48
LOG OF ANC CON A Cpm	0.12 p=0.67	0.46 p=0.07	-0.02 p=0.95
LOG OF ANC CON A SI	0.14 p=0.62	-0.08 p=0.77	0.11 p=0.69

The first figure is the strength of the correlation, the second the probability of it occurring by chance

Table 5.23: The associations between the log of Con A proliferation assay results, following culture of PBMC and abomasal node cells (ANC), and the log of *O. circumcincta* worm burden, log of faecal nematode egg count and the worm length from Group N lambs.

	LOG OF *O. CIRCUMCINCTA* BURDEN	LOG OF FAECAL WORM EGG COUNT	MEAN WORM LENGTH
LOG OF PBMC CONTROL Cpm	0.40 p=0.23	0.30 p=0.38	0.05 p=0.88
LOG OF PBMC L_3SE Cpm	0.31 p=0.35	0.26 p=0.45	0.30 p=0.37
LOG OF PBMC L_3SE SI	0.04 p=0.91	0.02 p=0.95	0.33 p=0.31
LOG OF ANC CONTROL Cpm	-0.26 p=0.44	0.20 p=0.56	-0.01 p=0.97
LOG OF ANC L_3SE Cpm	-0.35 p=0.29	-0.15 p=0.67	0.26 p=0.45
LOG OF ANC L_3SE SI	-0.40 p=0.22	-0.30 p=0.37	0.28 p=0.41

The first figure is the strength of the correlation, the second the probability of it occurring by chance

Table 5.24: The associations between the log of L_3SE proliferation assay results, following culture of PBMC and abomasal node cells (ANC), and the log of *O. circumcincta* worm burden, log of faecal nematode egg count and the worm length from Group N lambs.

	CON A	L_3SE
LOG OF PBMC CONTROL Cpm	0.28 p=0.29	0.16 p=0.54
LOG OF PBMC STIMULATED Cpm	-0.17 p=0.52	0.30 p=0.26
LOG OF PBMC SI	-0.50 p<0.05	0.03 p=0.90
LOG OF ANC CONTROL Cpm	0.24 p=0.38	0.30 p=0.27
LOG OF ANC STIMULATED Cpm	0.01 p=0.97	-0.22 p=0.41
LOG OF ANC SI	-0.32 p=0.22	-0.48 p=0.06

The first figure is the strength of the correlation, the second the probability of it occurring by chance

Table 5.25: The associations between the logs of the con A and L_3SE proliferation assays results, following culture of PBMC and abomasal node cells (ANC), and the log of *O. circumcincta* worm burden from Group D lambs.

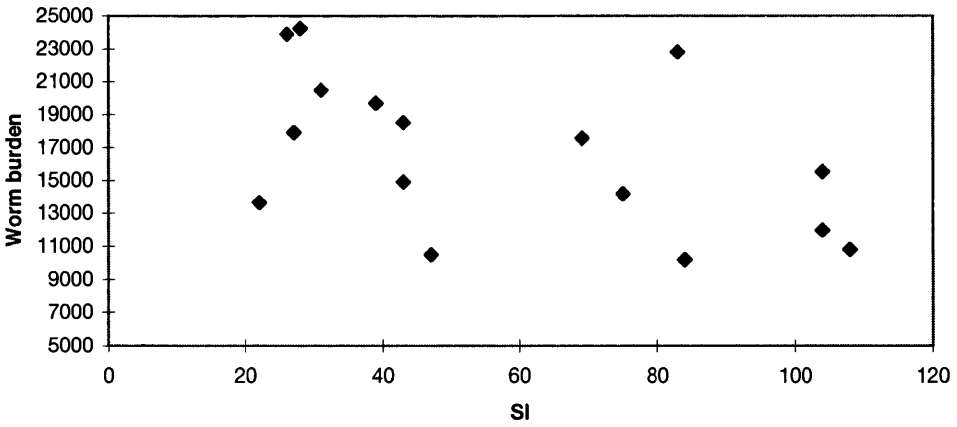


Figure 5.4: The relationship between the SI for PBMC, from Group D sheep, cultured with Con A and worm burden.

5.7: DISCUSSION

The work presented in this chapter shows that proliferative responses to L_3SE antigen are greater in sheep that have been previously exposed to *O. circumcincta*, are greater in abomasal node cells than in PBMC, and are greater in sheep which have recently received a large deliberate challenge infection than in those that have naturally acquired an *O. circumcincta* worm burden. Following the deliberate challenge there was a negative association between worm burden and proliferative responses of PBMC to the T cell mitogen Con A, and a trend towards those lambs from which abomasal node cells had a greater response to L_3SE having a lesser worm burden.

Results from preliminary experiments of attempts to optimise culture conditions have been recorded because of important observations that they highlight. Although this does not include sufficient samples to show statistically significant results and draw rigid conclusions, they suggest that the optimum culture conditions appear to vary between individuals, therefore making subtle comparisons of proliferative responses difficult. Also there was considerable variation in results from different samples from the one sheep sampled at the same time and processed using the same protocol, and this was despite the fact that the results expressed were means from triplicate wells. Problems due to variability in measured responses of monocytes to mitogens have long been recognised (Strong *et al.*, 1973) and previous reports have shown that culture of sheep lymphocytes is particularly difficult due to intra-animal variability being sufficient to mask other effects (Larsen, 1979; Staples *et al.*, 1981; Fiscus *et al.*, 1982).

Staples *et al.* (1981) attribute much of the variation to the recovery of critical subsets, but results described here illustrate large differences between samples sourced from the one preparation. Fiscus *et al.* (1982) partially restored counts in poorly responding cultures by addition of the reducing agent 2-ME, and

addition of this compound greatly enhanced responses in the experiment described here. Rai-El-Balhaa *et al.* (1987) showed that the hour of blood sampling can critically affect responses of PBMC and therefore all samples were collected between 09.00 and 11.00 hours. Small differences in cell concentration appeared to affect the ranking of SI from four sheep, and despite much effort to avoid errors in counting it is possible that variation in the final cell concentration of culture could significantly effect Cpm and SI. It could be argued that as the proliferation assay is an attempt to measure an individual's capability for clonal expansion the optimum conditions should be determined for each individual to allow for meaningful comparison, but such an approach would greatly increase the experimental workload and would be open to the criticism that the conditions that prevail *in vivo* at the site of clonal expansion, may not be the optimum ones for each individual.

The importance of representation of different subsets is shown by the observation in Experiment 5.4 in which PBMC from sheep with a greater B cell percentage but a lesser percentage of T cells in the peripheral blood have a poorer response to the T cell mitogen, Con A.

If the lymphocyte proliferation assay is viewed as an attempt at mimicry of the *in vivo* processes of clonal expansion then it is likely that the mimicry is crude. In the lymph node, the candidate site for clonal expansion, there is a sophisticated and complex architecture that will enable the development of a very specific micro-environment between antigen presenting cells, responding lymphocytes and their neighbours which is unlikely to be represented by the cell suspension within a tissue culture plate. The magnitude of response in a proliferation assay will depend upon the number of responding cells, the inherent responsiveness of those cells and any suppressing or activating effects produced by other cells in the suspension.

Gasbarre (1994) attempted to measure changes in the frequency of cells responding to specific *O. ostertagi* antigens by using limiting dilution assays of abomasal node cells from immunised and non-immunised cattle, and in so doing, identified a further problem of *in vitro* techniques. The nodes from previously exposed animals were much larger and although differences in the proportion of reacting cells were not great, there were large differences in the estimates of the total number of reactive lymphocytes (Gasbarre, 1994). It is possible that a lymphocyte proliferation assay would not identify this difference although it is likely that architecture of the node would enhance delivery of antigen to the appropriate population of sensitised cells producing a greater secondary response *in vivo*.

Despite reservations about lymphocyte proliferation assays the work described here shows some interesting results. Responses of abomasal node cells from grazing lambs to larval antigen were greater than those from helminth-naïve lambs. Although this observation may seem unsurprising, one study on responses of PBMC of cattle to *O. ostertagi*, has shown that proliferation of lymphocytes from previously exposed cattle can be suppressed by larval antigen (Klesius *et al.*, 1984) and a further study on *O. ostertagi* and one on *H. contortus* have demonstrated non-specific responses to larval antigen (Cross *et al.*, 1986; Gill *et al.*, 1994). The observation of a greater response in cells from the grazing sheep may have been helped by examining responses in the abomasal node, which receives afferent lymph from the site of infection. The difference in response between exposed and naïve sheep showed that the response was a secondary one; this is important because otherwise it could be argued that the long optimum culture period of seven days may have been associated with a primary response to antigen.

Abomasal cells from sheep exposed to *O. circumcincta* had greater proliferative responses to L_3SE than PBMC. In Group N sheep the abomasal cells had significantly greater SI and a trend towards higher Cpm following stimulation,

whilst in Group D lambs the Cpm in both the control and stimulated wells were significantly greater for abomasal cells, and although abomasal cells had a greater mean SI than PBMC, the difference was not significant. For responses to Con A there was no significant difference between abomasal node cells and PBMC from cells from Group N lambs, but responses to Con A were greater for PBMC for Group D lambs showing that their abomasal cells were not inherently more proliferative. Therefore, the greater responsiveness of abomasal node cells to L_3SE, compared to PBMC, could be due to there being a greater proportion of cells which recognise the specific antigen or alternatively a greater proportion of reactive cells may proliferate. The lack of a significant difference in the SI for the Group D sheep appeared to be due to the higher background count in control wells. The high Cpm in control wells of abomasal cells from Group D sheep may have been due to technical differences, but there may have been a large number of activated abomasal cells that were producing IL-2 or other T cell growth factors; IL-2 is hypothesised to function in an autocrine manner, promoting proliferation of T cells (Swain *et al.*, 1988; Powers *et al.*, 1988; Weinberg *et al.*, 1990). McClure *et al.* (1992) and Pernthaner *et al.* (1995a) have observed an increase in spontaneous proliferation of sheep PBMC following exposure to *T. colubriformis* and Pernather *et al.* (1995a) attributed this observation to *in vivo* activation of lymphocytes.

Responses of Group D lambs were compared with Group N lambs and were generally greater following antigen stimulation with the SI being greater for both PBMC and abomasal node cells. The PBMC from Group D sheep were harvested three days after challenge and Smith *et al.* (1984) showed that in a similar challenge infection of previously exposed sheep, the peak output of lymphocytes in the efferent lymph was three to four days after infection. As these cells will recirculate via the peripheral circulation, this sampling time should have been the optimum one for sampling a PBMC population enriched with cells derived from the abomasal node, a proportion of which would be expected to proliferate following exposure to antigen. The Group D sheep also

had greater proliferative responses in the abomasal nodes with higher SI, and it is likely that the deliberate challenge results in clonal expansion within the node producing a higher proportion of memory cells which recognise L_3SE.

Correlations between parasitological burdens and proliferative responses were examined following the natural infection in Group N lambs. No significant associations were identified although there was a trend towards animals with a high faecal worm egg count having a high Cpm following culture of abomasal node cells, both with or without Con A, for 72 hours. Egg count can be considered to be a function of the worm burden and worm fecundity, (Stear *et al.*, 1996b), and as neither of these show any association with Cpm in control and mitogen wells (fecundity was measured by worm length), then the trends between egg count and Cpm of abomasal node cells may be artefacts. However, it is possible that proliferation is associated with worm mass, and therefore may have a stronger association with faecal worm egg count than either worm burden or worm length alone.

A moderately strong negative association was observed between the SI for PBMC following culture with Con A and the worm burden of animals given a deliberate challenge infection, Group D. For the same group of sheep there had also been a negative association between the percentage of CD4+ T cells in the peripheral blood and the worm burden (Chapter 4), and it was considered possible that the increased response to Con A may be secondary to an increased proportion of CD4+ T cells, and indeed CD4+ T percentages and Con A responses were strongly correlated at 0.72 ($p<0.01$).

In Group D sheep the SI of abomasal node cells with L_3SE was negatively associated with worm burden, although the result was not quite significant ($p=0.06$). It would make sense if those sheep that could instigate a higher level of clonal expansion had a greater ability to control worm burden, but, as discussed in Chapter 3, there was no evidence that these sheep had acquired the

protective responses to control worm burden, because the mean worm burden compares with that previously reported following an identical experimental infection of naïve lambs (Stevenson *et al.*, 1994), and alternative explanations should be considered. Immune modulation of the host by parasites is a well recognised phenomenon (Raybourne *et al.*, 1983; Cross and Klesius, 1989) but there is strong evidence described in this chapter that exposure of the host to larval challenge leads to an enhanced response to antigen stimulation of isolated cells and has no effect on responses to Con A. The possibility that the poor response to antigen in those sheep with a high worm burden was due to immune modulation remains, and it is possible that as the surviving worms mature, they secrete immune modulating factors, and thus those sheep with a greater worm burden had a poorer response to antigen. The observation that the worm burden was not negatively associated with Con A responses might suggest that there was no general immune modulation but it might be that immune modulation occurs specifically, for example by interfering with presentation of parasite antigens.

The work in this chapter was carried out to investigate whether an individual's ability for clonal expansion contributed to protection against parasites, and the results are inconclusive. Partly this was due to the age of the experimental sheep, because at six months of age the mechanisms which regulate worm burden are not fully developed (Stear *et al.*, 1998), and experiments with older sheep may yield more conclusive results. In retrospect the use of proliferation assays to give an indication of *in vivo* clonal expansion results in difficulties in interpretation and may not be the most appropriate technique. Indeed it is technically possible to cannulate the gastric lymph duct and measure the flow and sample the lymph, and although this lymph may contain product draining from the mucosa as well as that of the abomasal node lymph, it may be a better and more useful measure of the processes of clonal expansion.

The *in vitro* methods may be useful to answer certain narrowly defined questions, such as are CD4+ cells from one group more responsive than CD4+

from another? Or do γδ T cells suppress or enhance responses? Magnetic separation could be used to enrich cell suspensions with a particular sub-population. The use of T cell lines has been used to investigate the responses of sheep to *H. contortus* antigens (Haig *et al.*, 1989), and this technique would be particularly useful in investigating responses to defined antigen fractions.

CHAPTER 6: MAST CELL AND GLOBULE LEUCOCYTE ENUMERATION AND THEIR RELATIONSHIP WITH PARASITOLOGICAL PARAMETERS

6.1: INTRODUCTION

The role of the mast cell and globule leucocyte in control of ovine GI parasites was reviewed in Chapter 1. It is now generally accepted that a local hypersensitivity reaction acts to control worm burden by worm expulsion or immune exclusion mediated by discharge of mast cells.

Mast cells are derived from common myeloid progenitor stem cells in the bone marrow and migrate to a variety of tissues, especially the skin, respiratory and GI tracts, and serosal surfaces (Galli, 1990). Not all mast cells are identical and there is considerable heterogeneity in both morphology and function between mast cells in different species, strains, individuals, different tissues within an individual and even the same tissue within an individual (Lee *et al.*, 1986). It is usual to classify mature mast cells as being either connective tissue mast cells, which are located in the skin, serosal surfaces and musculature, or as mucosal mast cells (MMC), which are found adjacent to mucosal surfaces (Enerback, 1966). Mast cells migrate from the peripheral blood into tissues where T cell dependent factors and type II cytokines such as IL-3, IL-4, IL-9, IL-10 are required for survival, differentiation and an optimal response (Finkleman *et al.*, 1991; Grencis *et al.*, 1991). Non T cell cytokines such as stem cell factor can also act as a growth and differentiation factor (Newlands *et al.*, 1995; Miller, 1996).

Mast cells share properties with both inflammatory effector cells and immune cells. Mast cells act as the critical effector cell in IgE-dependent protective responses and immediate hypersensitivity pathology, and classically mast cell

activation and release of mediators follows cross linking of the Fc_ε receptors by surface bound IgE binding antigen, but mast cell degranulation can also occur through anaphylotoxins following activation of complement by venom, lectins and neuropeptides (Roitt *et al.*, 1996b). The role of mast cells is not limited to being an end-stage effector cell and mast cells can produce a broad range of cytokines, thus playing an important role in immunoregulation (Gordon *et al.*, 1990). The distribution of mast cells in the mucosa is similar to that of lymphocytes and because mast cells can produce cytokines including IL-4, which is considered to be a type II response activator, it has been hypothesised that collaboration between mast cells and T cells in the mucosa drives the response phenotype of the host (Smith and Weis, 1996)

A series of histochemical and ultrastuctural studies in both ruminants and rats indicated that globule leucocytes were derived from mast cells (Jarrett *et al.*, 1967; Murray *et al.*, 1968; Miller and Walshaw, 1972) but some workers have suggested that the globule leucocyte is lymphoid in origin (reviewed by Gregory, 1979). Huntley *et al.* (1984) isolated cells from the abomasal mucosa and demonstrated a morphological, ultrastructural and histochemical graduation from mast cells, through transitional cells, to globule leucocytes. It is now generally accepted that globule leucocytes are mast cells activated in response to a parasite challenge.

The study by Huntley *et al.* (1984) showed that a previous exposure to parasites was necessary for mast cells to be isolated from the abomasum, but an active infection was necessary for mast cells to progress to globule leucocytes, and that those lambs which received a greater parasite burden not only had a greater number of mast cells and globule leucocyte but the ratio of globule leucocytes was greater. This evidence suggests that presence of globule leucocytes requires a two step process, the first being the recruitment of mast cells into the mucosa and second being the activation of mast cells to form globule leucocytes. Two studies have examined the number of mast cells and globule leucocytes in the

abomasal mucosa of sheep exposed to *O. circumcincta* and correlated worm burden with cell population density in the mucosa, and both studies recorded significant negative associations between globule leucocytes and worm burden but not mast cells and worm burden (Seaton *et al.*, 1989; Stear *et al.*, 1995c). Although the conclusions of both studies concur their approaches differed. Seaton *et al.* (1989) carried out analysis in three groups of lambs which were given a single challenge following trickle infections of four, eight and 12 weeks and the data shows that the ability to control worm burden was temporally associated with the development of the globule leucocyte response. Stear *et al.* (1995c) carried out analysis of one group in which all sheep had received identical treatment, a large single challenge following natural infection, and showed that individual variation in worm burden was strongly associated with globule leucocyte count.

The two studies described above showed that globule leucocytes were associated with protection in sheep at approximately nine months of age but younger lambs are more susceptible to *O. circumcincta* (Smith *et al.*, 1985). Therefore experiments described in this chapter were designed to test whether a similar mechanism was responsible for the control of worm burden in younger lambs. Attempts were made to select lambs from the extremes of variation in resistance within the flock in order to observe if there were differences in mast cell recruitment, through counting mast cells, and mast cell activation, through counting globule leucocytes.

6.2: MAST CELL AND GLOBULE LEUCOCYTE ENUMERATION IN LAMBS FROM LOW AND HIGH FAECAL WORM EGG COUNT GROUPS. I

6.2.1: Introduction

This experiment was designed in conjunction with Experiment 3.2 to investigate whether by selecting lambs from the extremes of faecal worm egg count within the flock it would be possible to select lambs with different worm burdens, and whether or not lambs with fewer worms would also have greater numbers of mast cells and globule leucocytes. If lambs with low faecal worm egg counts had a lesser worm burden but a greater mast cell and globule leucocyte population it would provide evidence that within-breed variation of worm burden in grazing lambs was a result of variation in the mast cell and globule leucocyte number.

6.2.2: Protocol

As described in Chapter 3, five lambs were selected for high nematode faecal worm egg count and five lambs were selected as having a low faecal worm egg count based on the mean of the August and September faecal worm egg counts. The lambs were removed from pasture on 1 November; five were killed two days later, and the remaining five the following day. One section of abomasal leaf was fixed in NBF and the samples were processed routinely for histology. Abomasal contents and a half abomasum were collected for parasitological examination. Slides were stained with a simplified astra blue for mast cells and MSB for globule leucocytes (see Section 2.3.4), and counting was done according to Protocol 2.3.4.1. The total number of graticule views needed to view 21 abomasal mucosal strips was recorded, thus giving the total area of the

count and allowing calculation of the population density for each cell type. The results from the two groups were compared by the Student's *t*-test.

6.2.3: Results

The total mast cell and globule leucocyte mean counts for 21 mucosal strips from one section per lamb, and the population density of cells are recorded in Tables 6.1 and 6.2. The section from Lamb 6 was too broken to allow enumeration of cells. The lambs in the low egg count group had a greater mean mast cell count and a higher mast cell population density, 653 cells and 149 cells mm^{-2}, than those in the high egg count group, 385 cells and 101 cells mm^{-2}, but these results were not significant (p=0.21 and 0.32). As with mast cells the low egg count group had a greater mean globule leucocyte count and higher mean globule leucocyte population density, 343 cells and 77 cells mm^{-2}, than those in the high egg count group, 212 cells and 51 cells mm^{-2}, but these results were not significant (p=0.21 and 0.2).

There was a strong and significant association between the mast cell and globule leucocyte count, 0.70 ($p<0.05$), but although there was a trend towards an association between the mast cell population density and globule leucocyte population density this was not significant, 0.61 (p=0.08) - perhaps because of the relatively small number of animals studied. The mean of the mast cell population density from all ten lambs was greater than the mean of globule leucocyte population densities, 270 cells mm^{-2} to 122 cells mm^{-2} ($p<0.05$).

LAMB	EGG COUNT GROUP	MAST CELL COUNT	MAST CELL mm^{-2}
6	LOW	N/A	N/A
35	LOW	653	134
36	LOW	943	230
62	LOW	734	163
69	LOW	280	69
2	HIGH	753	201
51	HIGH	258	65
27	HIGH	667	136
43	HIGH	120	32
60	HIGH	126	69

Table 6.1: Mast cell counts and mast cell population densities in lambs from Low and High Faecal Worm Egg Groups. I.

LAMB	EGG COUNT GROUP	GLOBULE LEUCOCYTE COUNT	GLOBULE LEUCOCYTE mm^{-2}
6	LOW	N/A	N/A
35	LOW	473	97
36	LOW	280	68
62	LOW	367	81
69	LOW	251	62
2	HIGH	301	80
51	HIGH	30	8
27	HIGH	471	96
43	HIGH	160	43
60	HIGH	99	29

Table 6.2: Globule leucocyte counts and cell population densities in lambs from Low and High Faecal Worm Egg Groups. I.

6.3: MAST CELL AND GLOBULE LEUCOCYTE ENUMERATION IN LAMBS FROM LOW AND HIGH FAECAL WORM EGG COUNT GROUPS. II

6.3.1: Introduction

The results from Section 6.2 showed a trend for animals with a low egg count to have a greater population density of mast cells and globule leucocytes in the

abomasal mucosa, but these differences were not significant. The results also showed considerable variation with some individuals having more than three times as many mast cells as others. To improve the power of the analysis the selection criteria included the faecal worm egg counts from October, the group size was increased to eight, and three sections from each lamb were examined.

6.3.2: Protocol

Two groups of eight lambs were selected on the basis of their faecal worm counts in July, August, September and October, so that one group was selected for low faecal worm egg counts and one group for high counts. Four days after the October faecal sample the selected lambs were removed from pasture and transported to GUVS where they were housed and fed on hay. Four days after they were transported the first four lambs were slaughtered, with four lambs being slaughtered each day for four consecutive days. On the day of slaughter faeces was taken for a faecal worm egg count, the abomasum was removed and four sections from different abomasal leaves were fixed in 4% paraformaldehyde/PBS and processed for histology. The worm burden and mean length of adult female worms was also calculated. Enumeration of cells was carried out on three sections from each lamb following the protocol in Experiment 6.2. The results from the two groups were compared, within-individual variation was examined and regression analysis across groups to examine any associations between parasitological parameters and mast cell or globule leucocyte counts.

6.3.3: Results

The total mast cell and globule leucocyte mean counts for 21 mucosal strips from each section of abomasal fold per lamb, the population density of cells for each

section, and the mean cell count and mean cell population density are recorded in Tables 6.3 and 6.4.

Analysis of the mast cell counts showed a very strong correlation between the mast cell counts in different slides from the same animal; the strength of the associations between the first slide read with the second and third slides read and between the second and third were 0.84, 0.89 and 0.97 respectively ($p<0.01$ for all three correlations). Strong correlations were also recorded for mast cell densities between slides from the same individuals, 0.66, 0.90 and 0.84 ($p<0.01$), revealing that between slide variation was small compared to between individual variation. There was also a strong correlation between the mean mast cell count and the mean mast cell density, 0.94 ($p<0.01$) and therefore the variation in mast cell count is largely dependent upon the mast cell density rather than the height of the mucosa and thus the total area counted. The mast cell counts and mast cell density for the first slide also correlated with the means from three slides, 0.95 and 0.91 ($p<0.01$), indicating that the result from the first slide is a good representation of the total population.

A comparison of the results between the high and low egg count groups reveals that those lambs in the low egg count group had a significantly higher mean mast cell count, 640 to 316 ($p<0.05$), and a significantly greater mean mast cell population density, 142 cells mm^{-2} compared to 73 cells mm^{-2} ($p<0.01$), these are illustrated in Figure 6.1.

Similarly, very strong correlations were observed for globule leucocyte counts and globule leucocyte population densities between the three slides read for each individual, 0.84, 0.95 and 0.84 for cell counts and 0.83, 0.95 and 0.84 for cell population densities (all $p<0.01$), and the correlation between the first slide read and the mean of three slides was also very high for both cell count and cell population density, 0.85 and 0.97 ($p<0.01$). The mean globule leucocyte count strongly correlated with the mean globule leucocyte population density, 0.93

($p<0.01$). When the globule leucocyte counts in the two groups were compared there was a trend towards the low egg count group having a greater number and density of globule leucocytes but this was not significant ($p=0.07$ and 0.07 respectively).

When the means of the mast cell population densities were correlated with the means of the globule leucocyte population densities there was a strong association, 0.76 ($p<0.01$), this association is represented in Figure 6.2. The mean of the mast cell population densities was greater than the mean of the globule leucocyte population densities, 109 compared to 74 cells mm^{-2} ($p<0.01$).

The variation between slides was small compared to the variation between individuals, thus the high values for correlation coefficients. Histograms for mast cell counts and globule leucocyte counts were prepared to record the distribution of counts within slides; these are recorded in Figures 6.3 and 6.4 and clearly show that whilst the distribution of mast cell counts approximates a normal distribution, that of the globule leucocytes is skewed and approximates a negative binomial distribution due to clustering.

The graphs confirm that the globule leucocytes were clustered and the clustering appeared to be greatest at the lumenal pole of the abomasal leaf. An analysis was carried out to test whether the density taken from the three most polar fields of view was greater than the average of the slide. The results show that the mean population density is greater in the lumenal pole area than in total area of the slide which was read, 148 mm^{-2} compared to 74 mm^{-2} ($p<0.01$). Photomicrographs of different regions of the abomasal fold from the same section are shown in Figures 6.15 and 6.16, illustrating the observation that the globule leucocyte density at the pole is greater than in tissue distant from the pole.

Associations between the transformed mast cell and globule leucocyte concentration and parasitological parameters were examined. There was a significant correlation between the log of globule leucocyte population density and the log of the *O. circumcincta* L_4 burden. Thus lambs with more globule leucocytes also had a greater number of L_4, the values are shown in Table 6.5 and represented graphically in Figure 6.5. There was no significant association between globule leucocyte counts and adult worms or total *O. circumcincta*. There was a significant negative association between the mean worm length and the globule leucocyte population density, -0.57 ($p<0.05$), this is illustrated in Figure 6.6. There was no association between globule leucocyte count or population density and faecal worm egg count. The only significant association between mast cell concentration and parasitological parameters was a negative association with worm length; the values were identical to the association between globule leucocytes and worm length -0.57 ($p<0.05$).

LAMB	GROUP	MAST CELL COUNTS	MAST CELL DENSITY mm^{-2}	MEAN COUNTS AND DENSITY
B23	LOW	670 476 445	133 95 101	530 111
B27	LOW	631 671 680	140 149 149	661 146
B37	LOW	1453 861 1253	289 125 265	1189 214
Y165	LOW	542 382 339	123 92 76	421 97
Y166	LOW	501 425 422	97 84 92	450 91
Y176	LOW	458 391 418	109 86 97	422 97
Y179	LOW	467 588 568	144 197 186	542 175
Y182	LOW	790 832 1094	174 184 250	905 203
B15	HIGH	271 367 338	73 103 93	325 89
B16	HIGH	323 283 284	69 57 53	297 59
B20	HIGH	215 383 421	58 91 100	340 84
B30	HIGH	343 350 388	76 74 81	360 77
B39	HIGH	226 172 159	50 42 42	186 45
B41	HIGH	343 355 309	79 88 72	336 80
B46	HIGH	120 106 86	29 25 22	104 25
Y192	HIGH	673 754 832	152 158 160	753 157

Table 6.3: Mast cell counts and mast cell population densities from each of three sections taken from lambs in Low and High Faecal Worm Egg Groups. II.

LAMB	GROUP	GLOBULE LEUCOCYTE COUNT	GLOBULE LEUCOCYTE DENSITY mm^{-2}	MEAN COUNTS AND DENSITY
B23	LOW	395 286 263	79 58 62	315 67
B27	LOW	82 68 67	18 14 14	72 15
B37	LOW	1142 1225 1200	217 174 263	1189 212
Y165	LOW	311 172 385	68 43 88	289 67
Y166	LOW	135 112 308	27 23 64	185 37
Y176	LOW	371 196 506	85 42 118	358 81
Y179	LOW	576 943 641	220 304 212	720 247
Y182	LOW	593 478 499	135 110 116	523 120
B15	HIGH	56 173 47	16 54 13	92 27
B16	HIGH	147 90 139	32 18 26	125 25
B20	HIGH	97 534 281	26 130 69	304 76
B30	HIGH	15 48 21	4 10 4	28 6
B39	HIGH	130 143 112	30 36 31	128 32
B41	HIGH	348 270 209	79 65 49	276 65
B46	HIGH	57 57 28	14 14 7	47 12
Y192	HIGH	488 223 607	95 43 136	439 89

Table 6.4: Globule leucocyte counts and cell population densities from each of three sections taken from lambs in Low and High Faecal Worm Egg Groups. II.

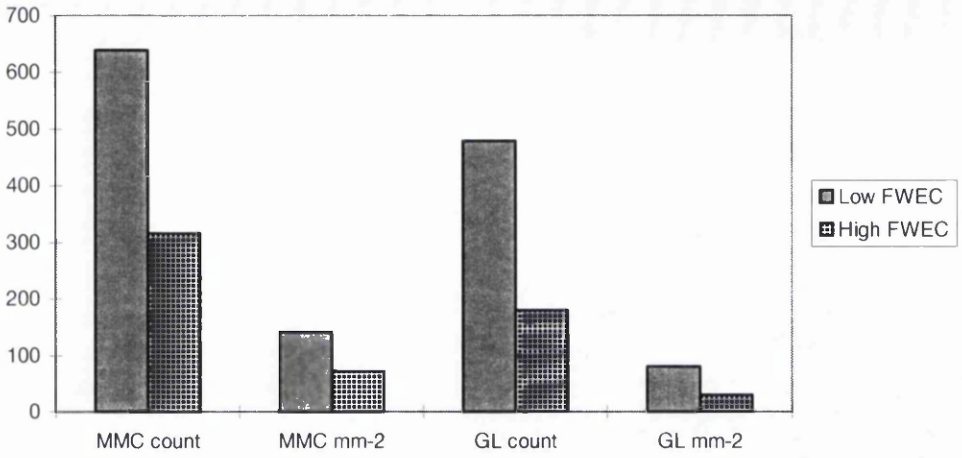
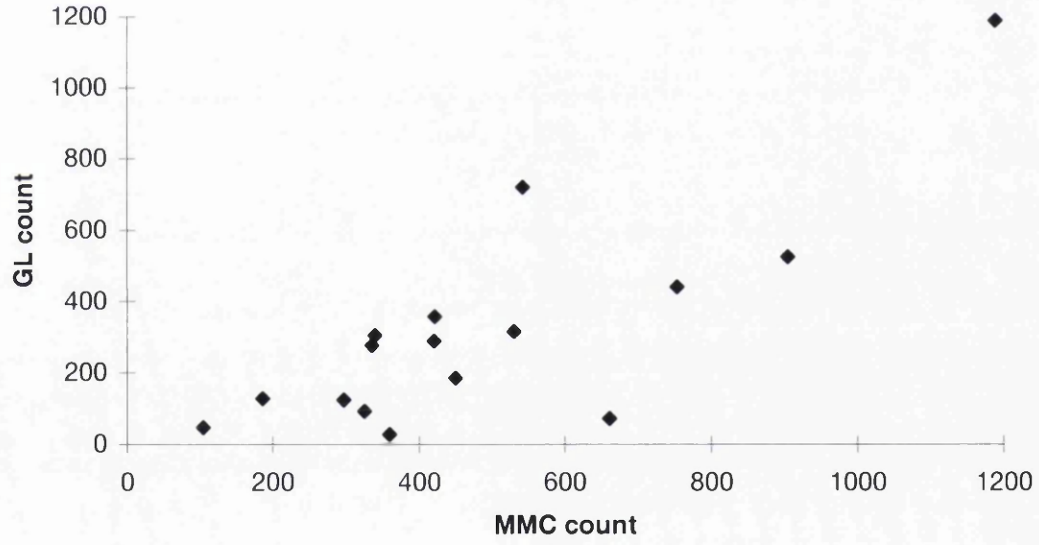


Figure 6.1: Mucosal mast cell (MMC) and globule leucocyte (GL) counts and population cell densities in Low and High Faecal Worm Egg Groups. II.



GL count is the mean globule leucocyte count for each lamb and MMC count is the mean mucosal mast cell count for each lamb.

Figure 6.2: A scatter graph of the mean of mast cell counts against the mean of globule leucocyte counts for three sections from each of 16 lambs in Low and High Faecal Worm Egg Groups. II.

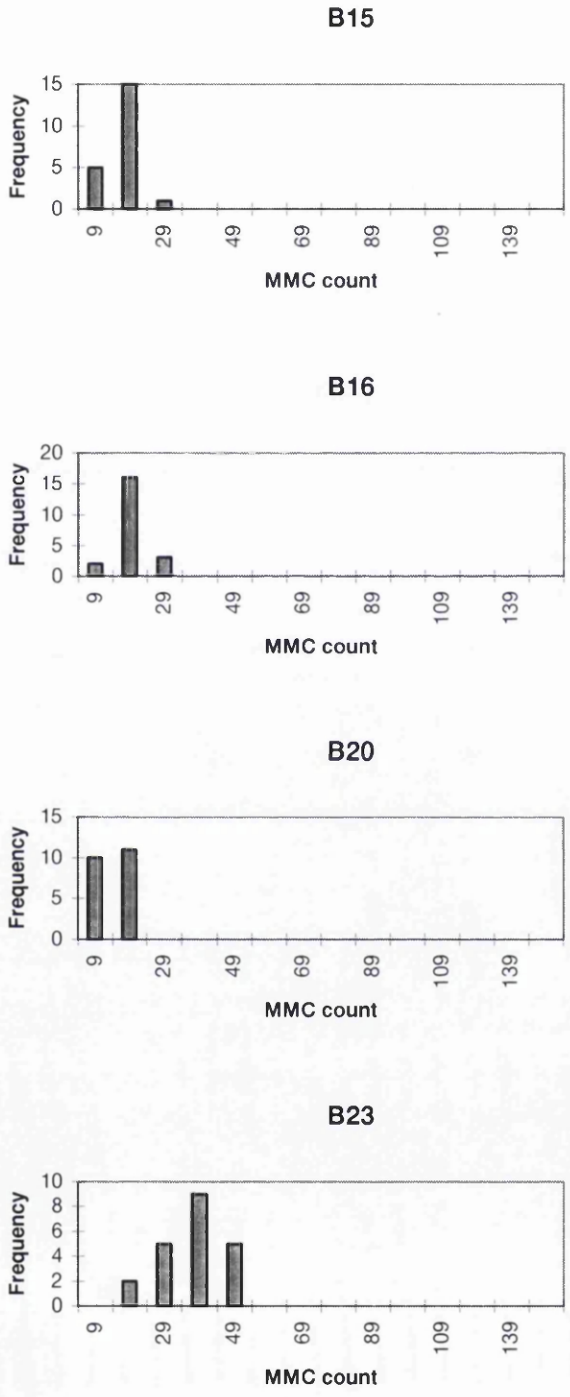


Figure 6.3a: Mucosal mast cell distribution in one section of abomasal mucosa from each of Lambs B15, B16, B20 and B23.

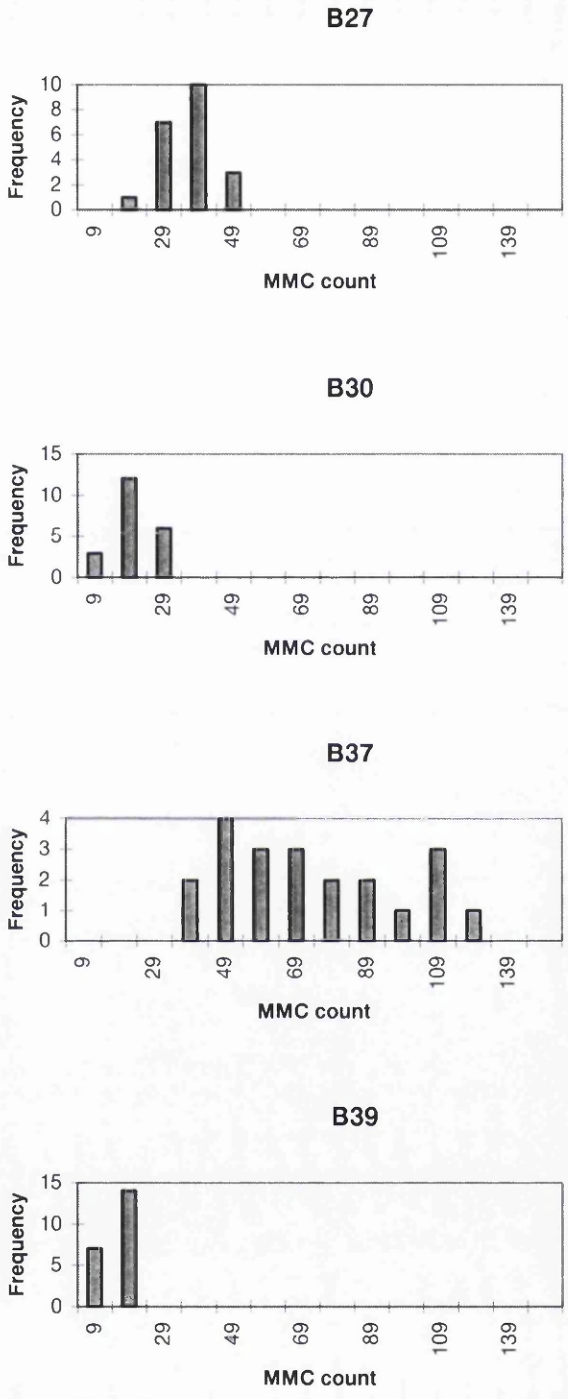


Figure 6.3b: Mucosal mast cell distribution in one section of abomasal mucosa from each of Lambs B27, B30, B37 and B39.

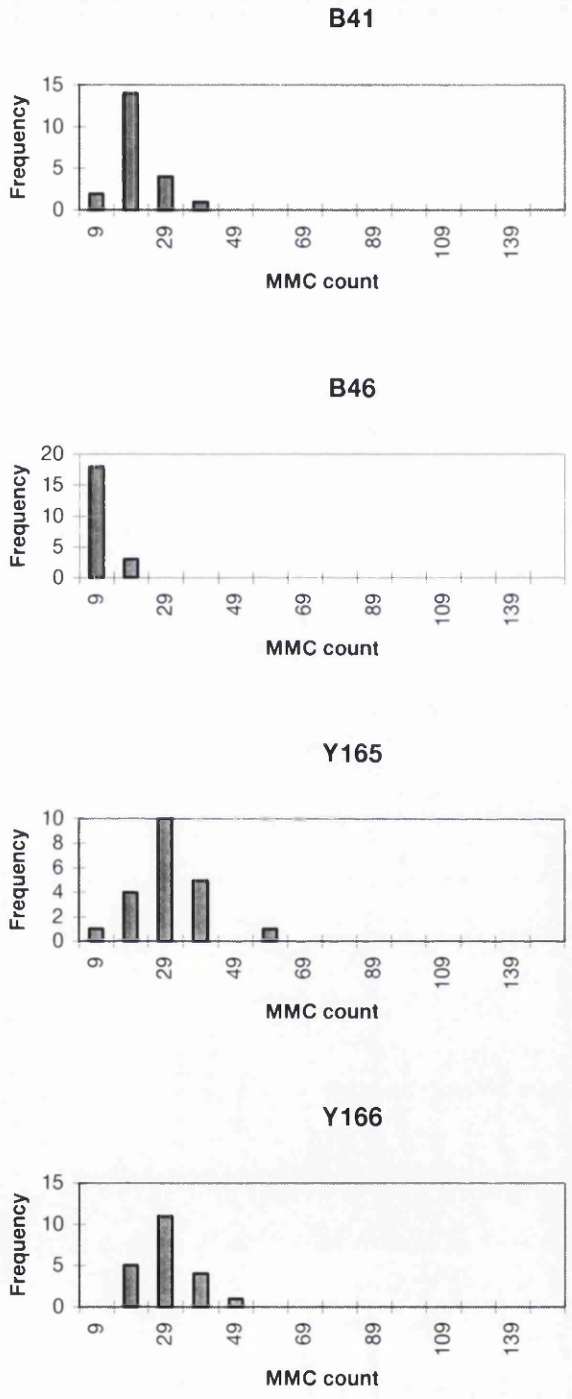


Figure 6.3c: Mucosal mast cell distribution in one section of abomasal mucosa from each of Lambs B41, B46, Y165 and Y166.

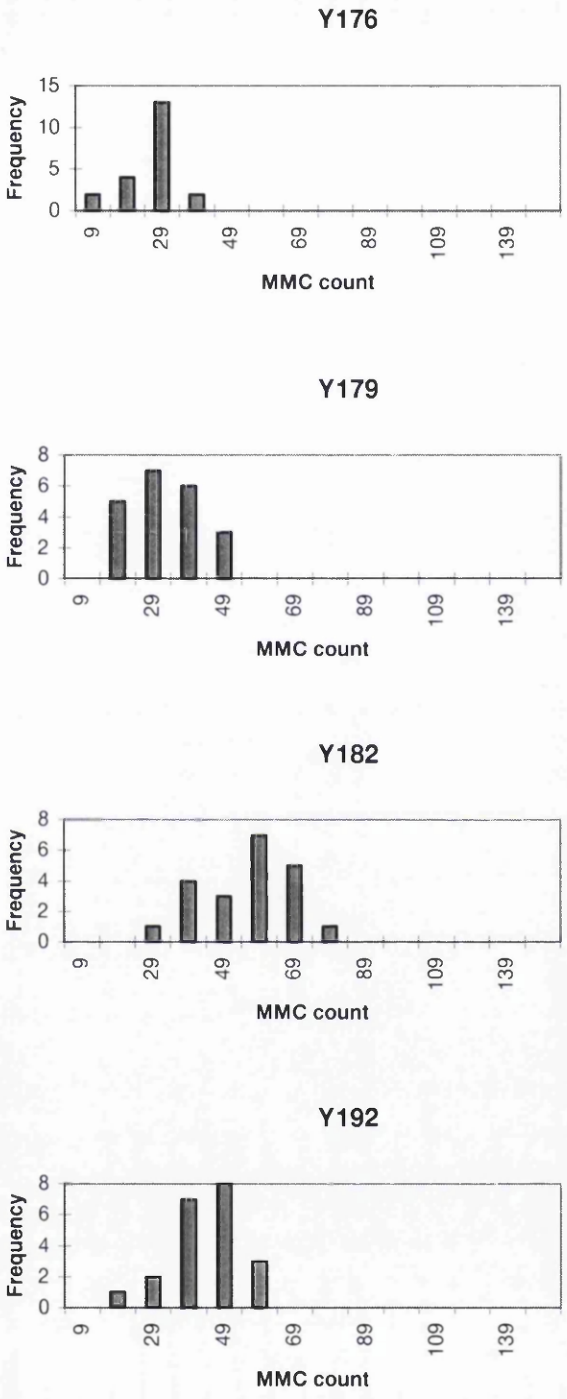


Figure 6.3d: Mucosal mast cell distribution in one section of abomasal mucosa from each of Lambs Y176, Y179, Y182 and Y192.

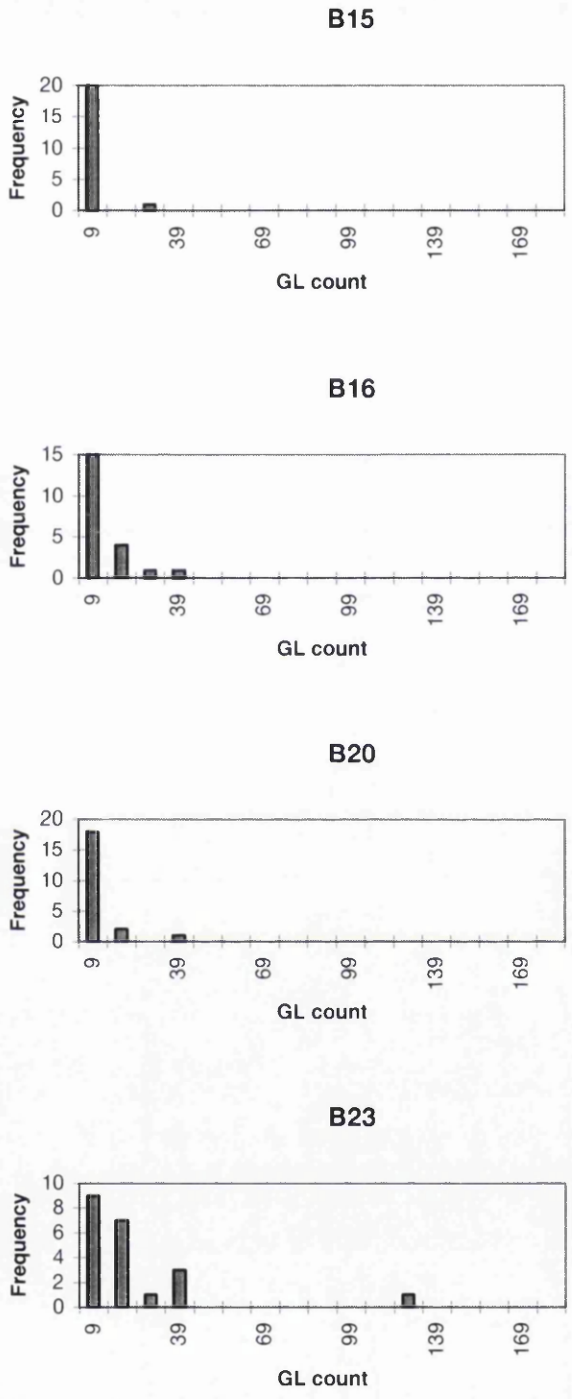


Figure 6.4a: Globule leucocyte (GL) distribution in one each section of abomasal mucosa from each of Lambs B15, B16, B20 and B23.

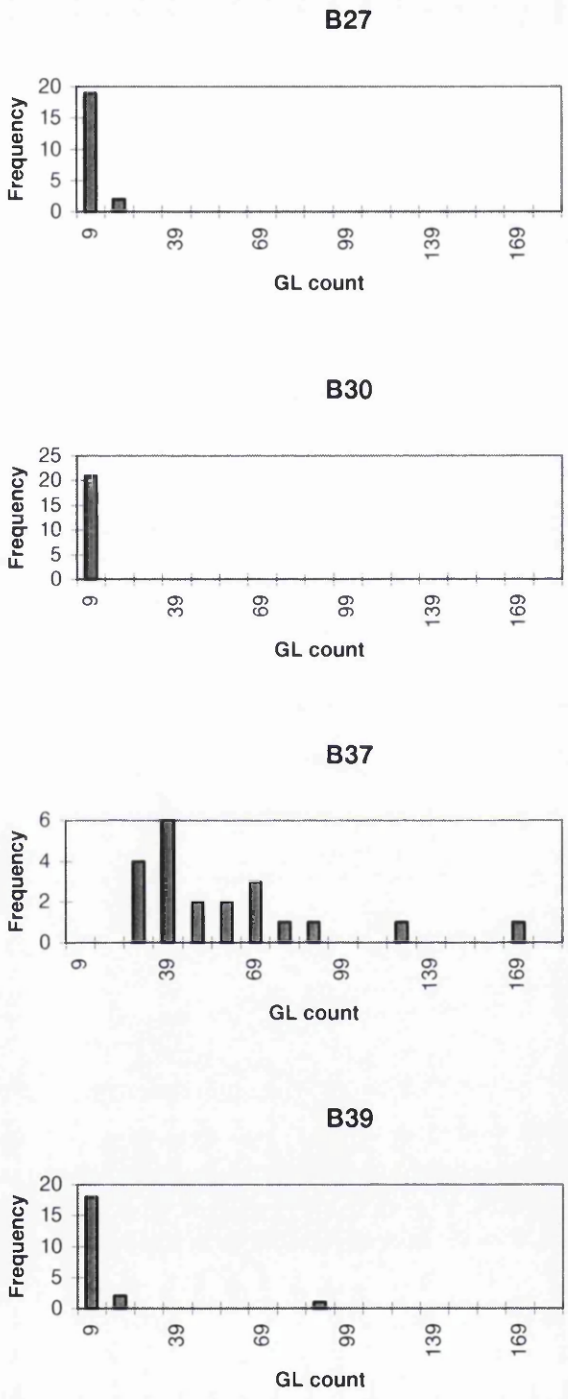


Figure 6.4b: Globule leucocyte (GL) distribution in one section of abomasal mucosa from each of Lambs B27, B30, B37 and B39.

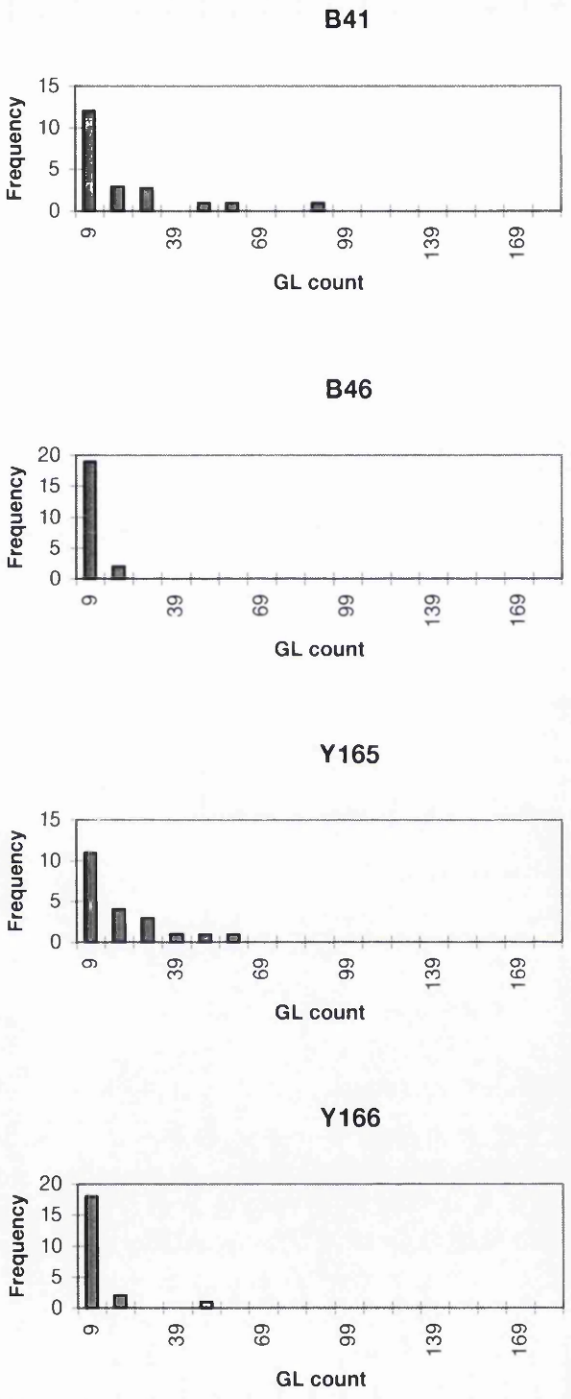


Figure 6.4c: Globule leucocyte (GL) distribution in one section of abomasal mucosa from each of Lambs B41, B46, Y165 and Y166.

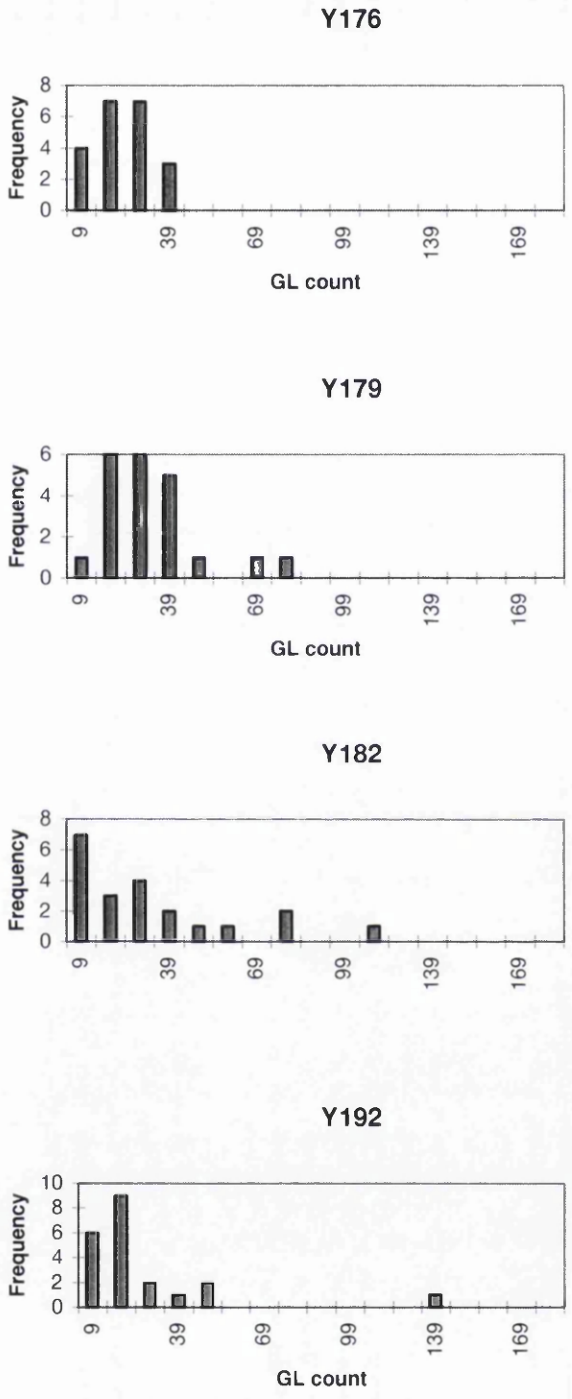


Figure 6.4d: Globule leucocyte (GL) distribution in one section of abomasal mucosa from each of Lambs Y176, Y179, Y182, Y192.

PARASITOLOGICAL PARAMETER	LOG MEAN MMC DENSITY	LOG MEAN GL DENSITY
Log L_4 *O. circumcincta*	-0.05 p=0.85	0.54 p<0.05
Log L_5 *O. circumcincta*	0.43 p=0.10	0.46 p=0.07
Log Adult *O. circumcincta*	-0.19 p=0.47	-0.08 p=0.78
Log total *O. circumcincta*	-0.05 p=0.85	0.30 p=0.26
Log of faecal worm egg count	-0.18 p=0.50	-0.16 p=0.56
worm length	-0.57 p<0.05	-0.57 p<0.05

The first figure is the strength of the correlation, the second the probability of it occurring by chance

Table 6.5: The correlations between the logs of the mean mucosal mast cell (MMC) density and mean of globule leucocyte (GL) density with the log of worm burden and faecal worm egg counts and worm length for lambs in Low and High Faecal Worm Egg Groups. II.

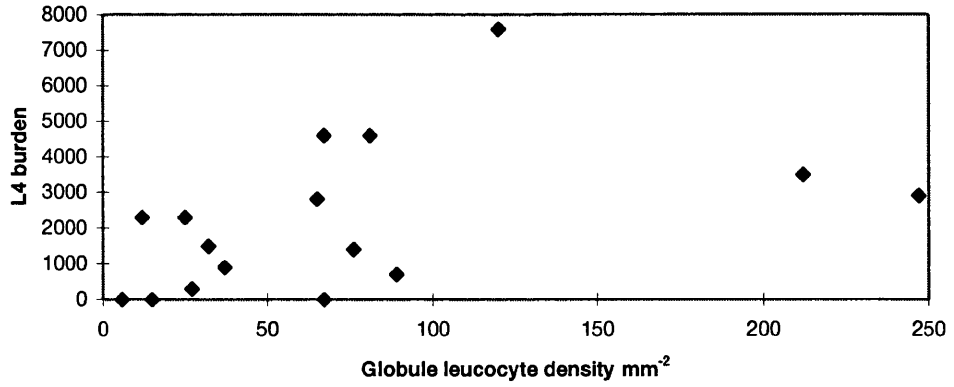


Figure 6.5 A scatter graph of the *O. circumcincta* L_4 burden against the mean of globule leucocyte population density for each of 16 lambs in Experiment 6.3.

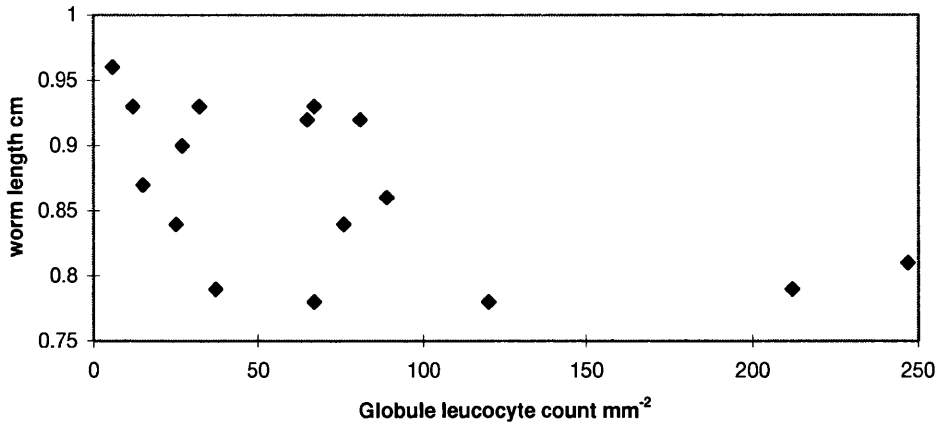


Figure 6.6 A scatter graph of the mean of worm length against the mean of globule leucocyte population density for each of 16 lambs in Low and High Faecal Worm Egg Groups, II.

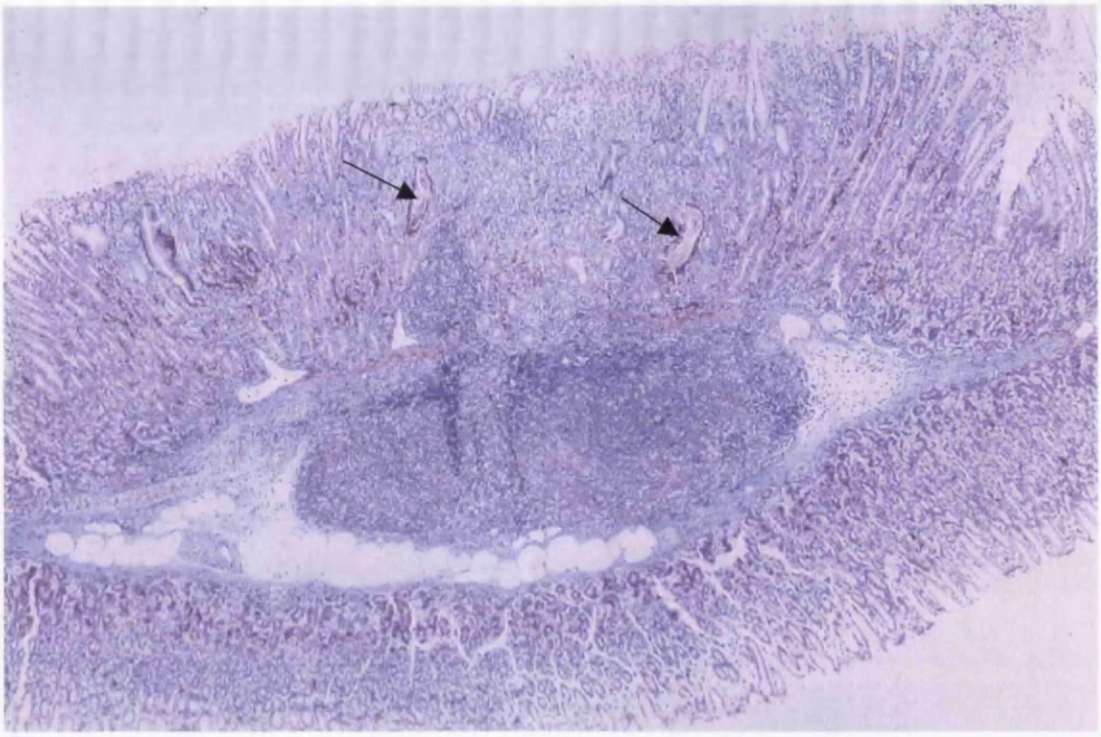


Figure 6.7: Photomicrograph of abomasal fold, revealing *Ostertagia circumcincta* larvae (arrows) present within abomasal gland tissue and lymphoid aggregates (MSB, original magnification was x 10).

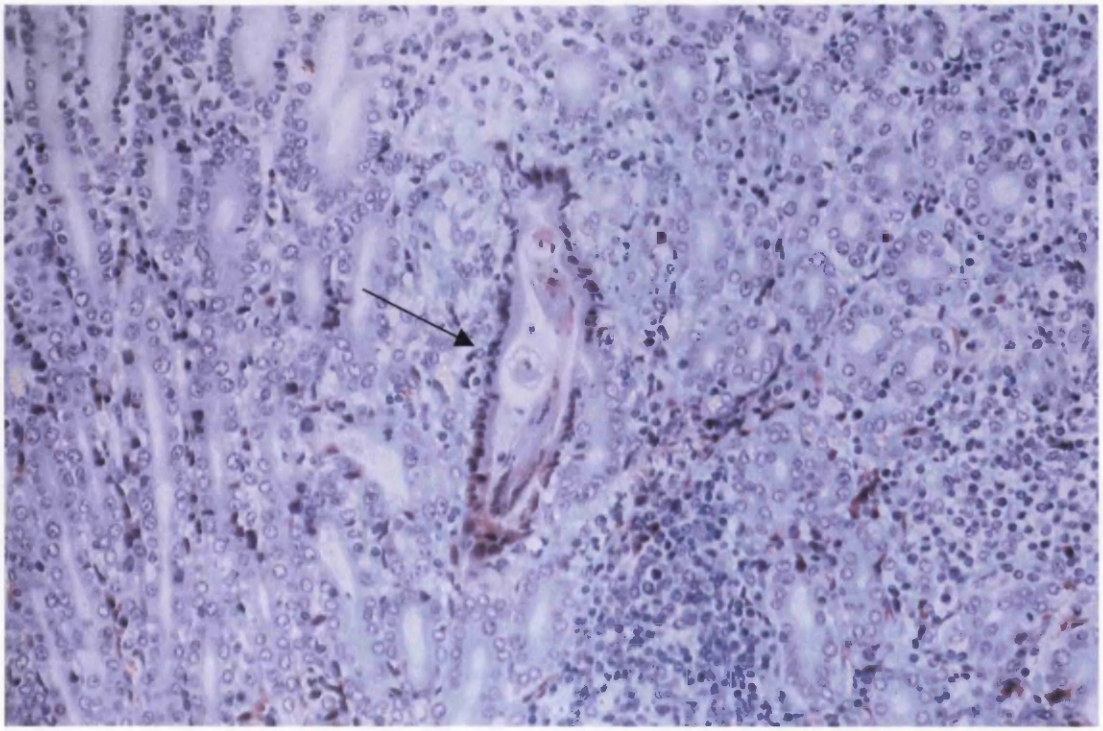


Figure 6.8: Photomicrograph of abomasal mucosa, revealing *Ostertagia circumcincta* larvae (arrow) present within an abomasal gland (MSB, original magnification was x 50).

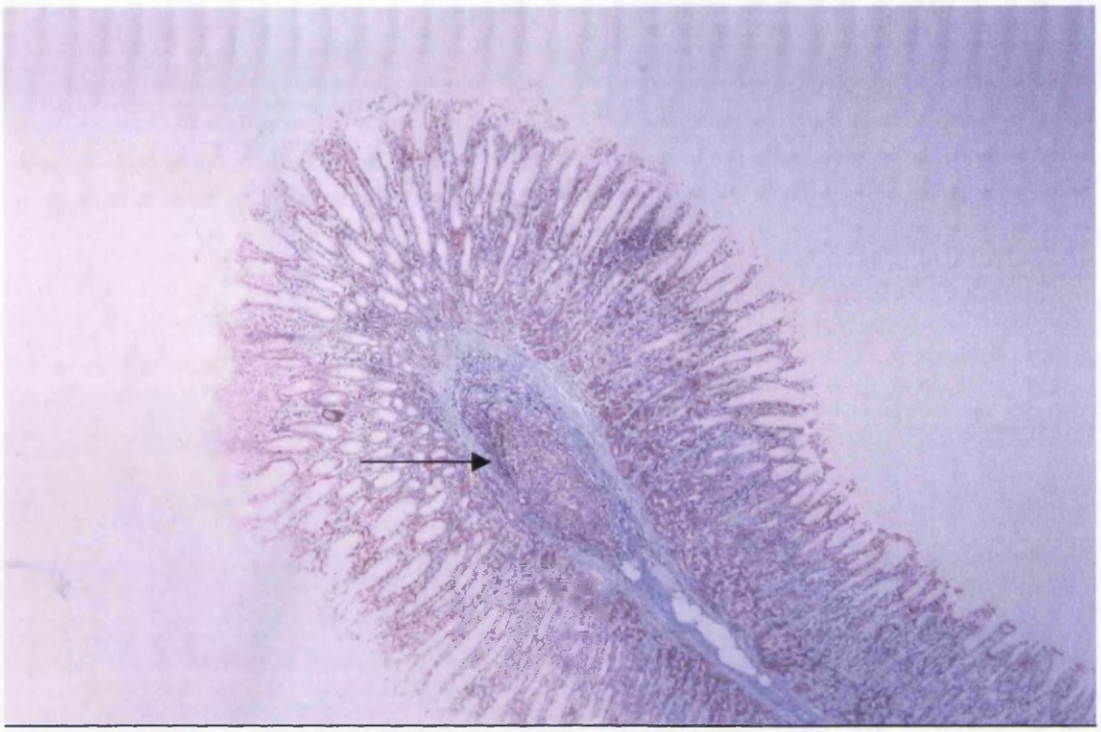


Figure 6.9: Photomicrograph of a leading edge of abomasal fold from lamb 182, which was exposed to *Ostertagia cirumcincta* by natural exposure, showing a large lymphoid aggregate (arrow) in the submucosal tissue (MSB, original magnification was x 10).

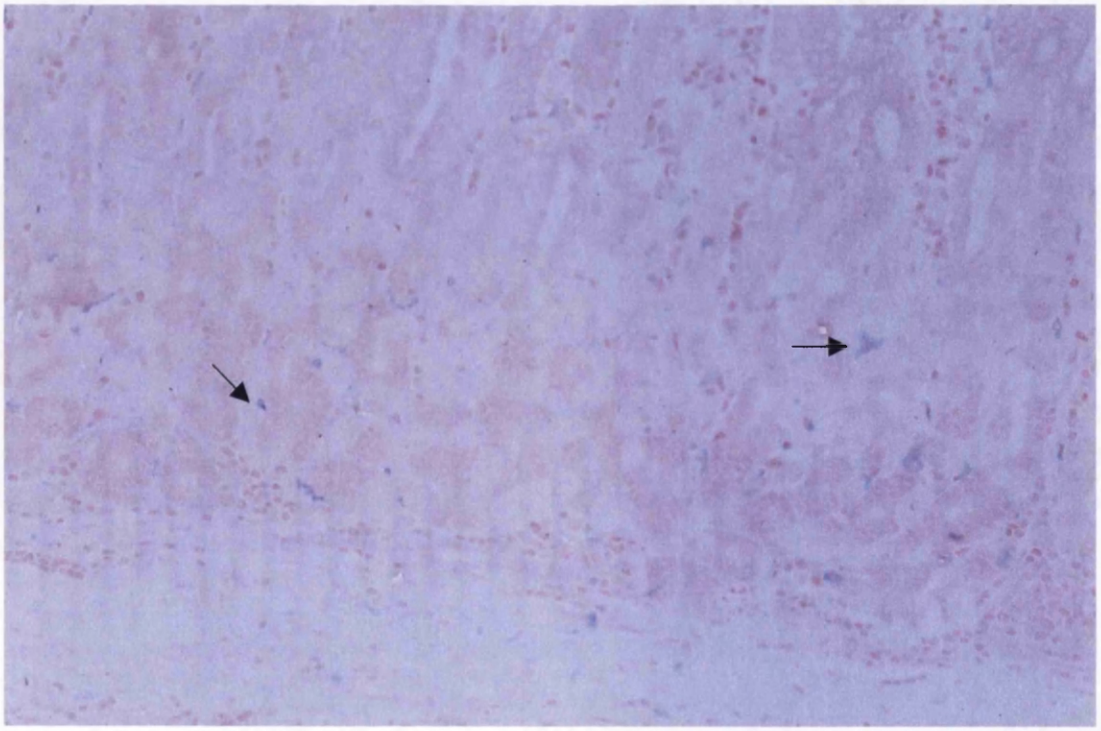


Figure 6.10: Photomicrograph of abomasal mucosa from lamb Y192 showing numerous mast cells (arrows) stained blue (modified astra blue, original magnification was x 50).

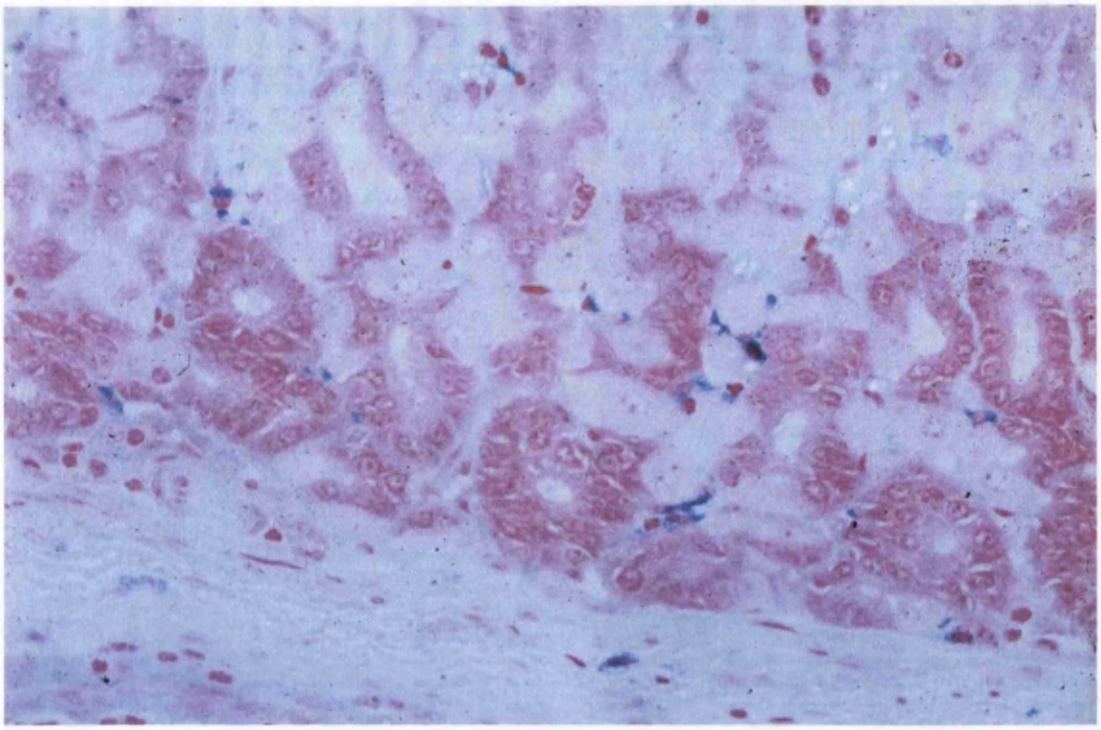


Figure 6.11: Photomicrograph of abomasal basal mucosa from lamb Y192 showing numerous mast cells in the basal region (modified astra blue, original magnification was x 100).

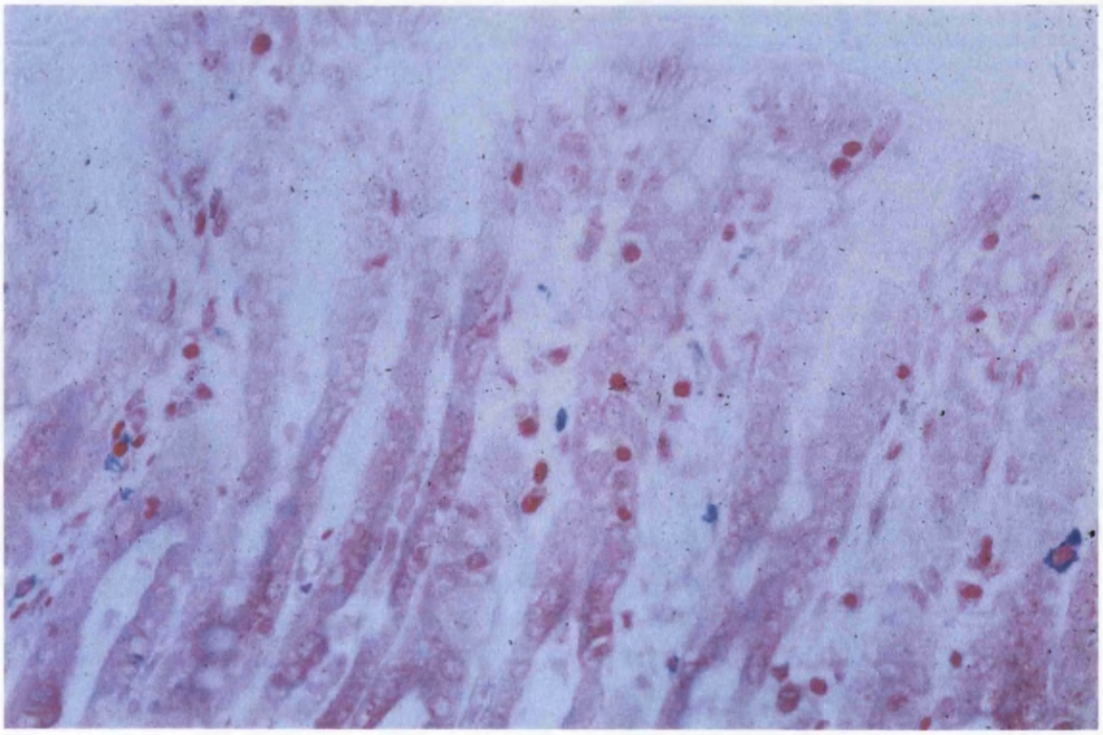


Figure 6.12: Photomicrograph of abomasal lumenal mucosa from lamb Y192 showing only a few cells in the lumenal region (modified astra blue, original magnification was x 100).

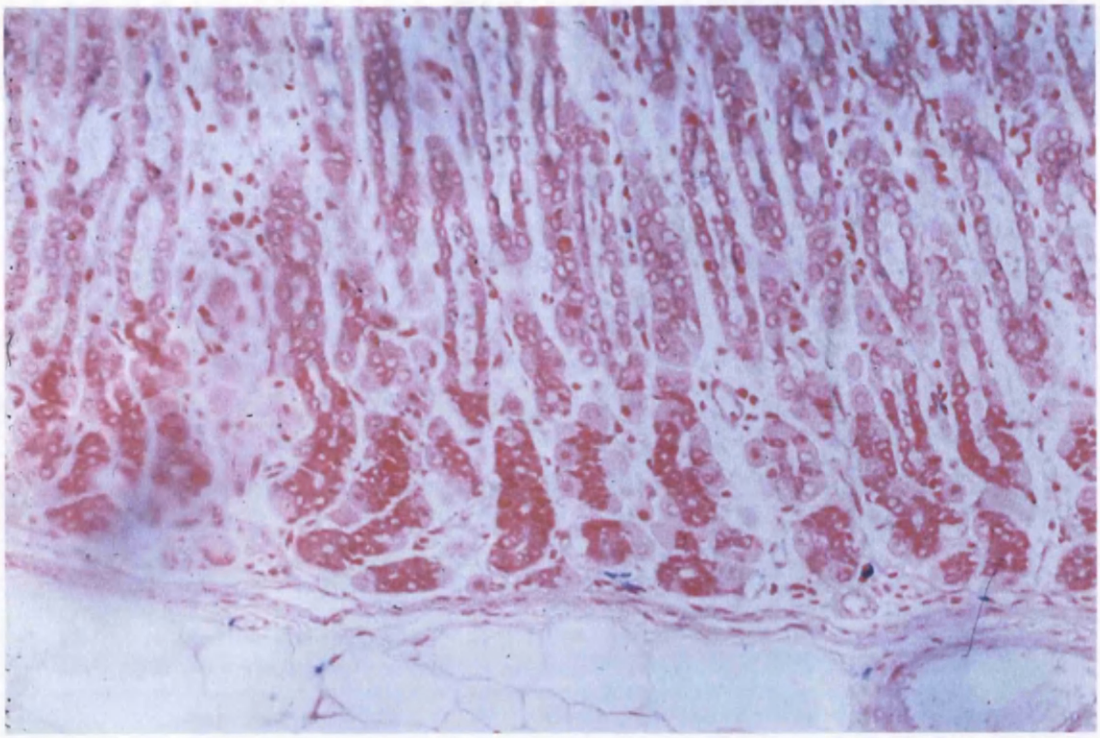


Figure 6.13: Photomicrograph of abomasal mucosa from lamb B46 showing only a few mast cells (modified astra blue, original magnification was x 50).

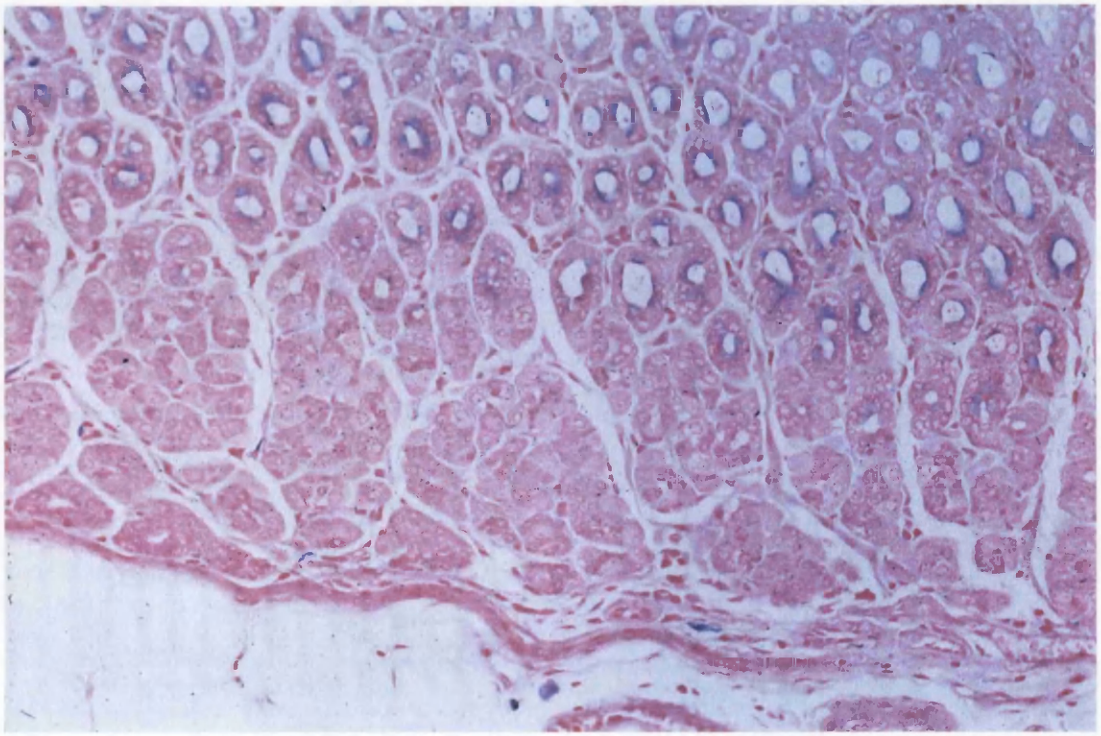


Figure 6.14: Photomicrograph of abomasal mucosa from a nematode naïve lamb showing only a few mast cells (modified astra blue, original magnification was x 50).

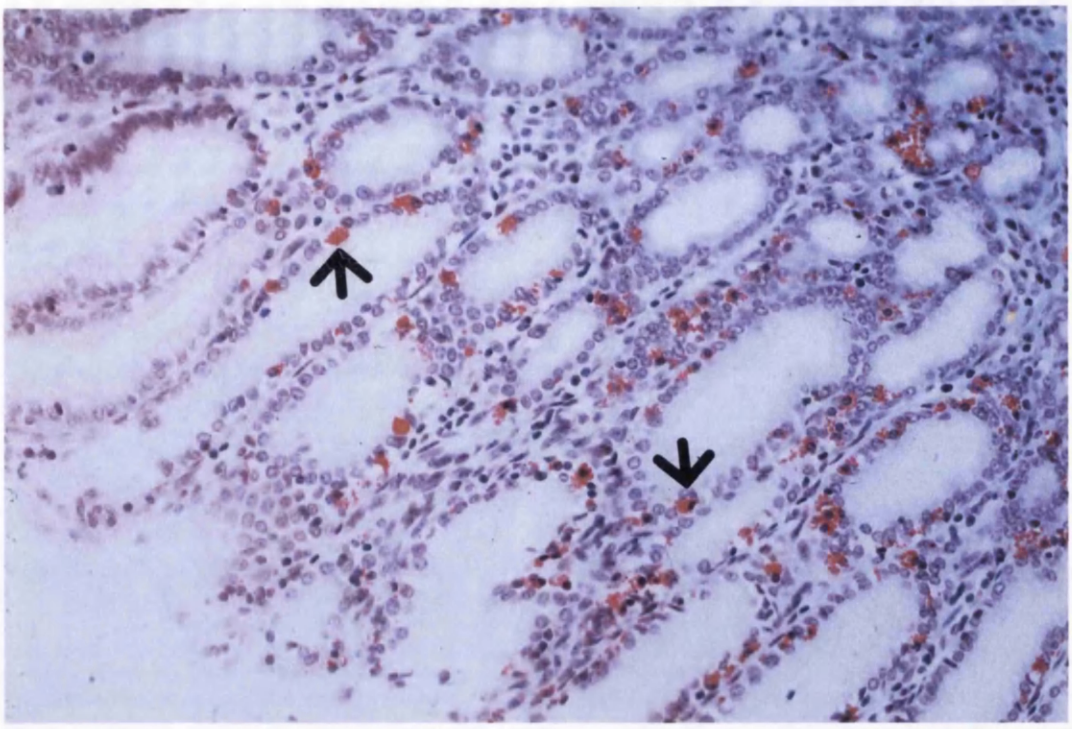


Figure 6.15: Photomicrograph of a leading edge of the abomasal fold from lamb Y182 showing numerous globule leucocytes (arrows) (MSB, original magnification was x 50).

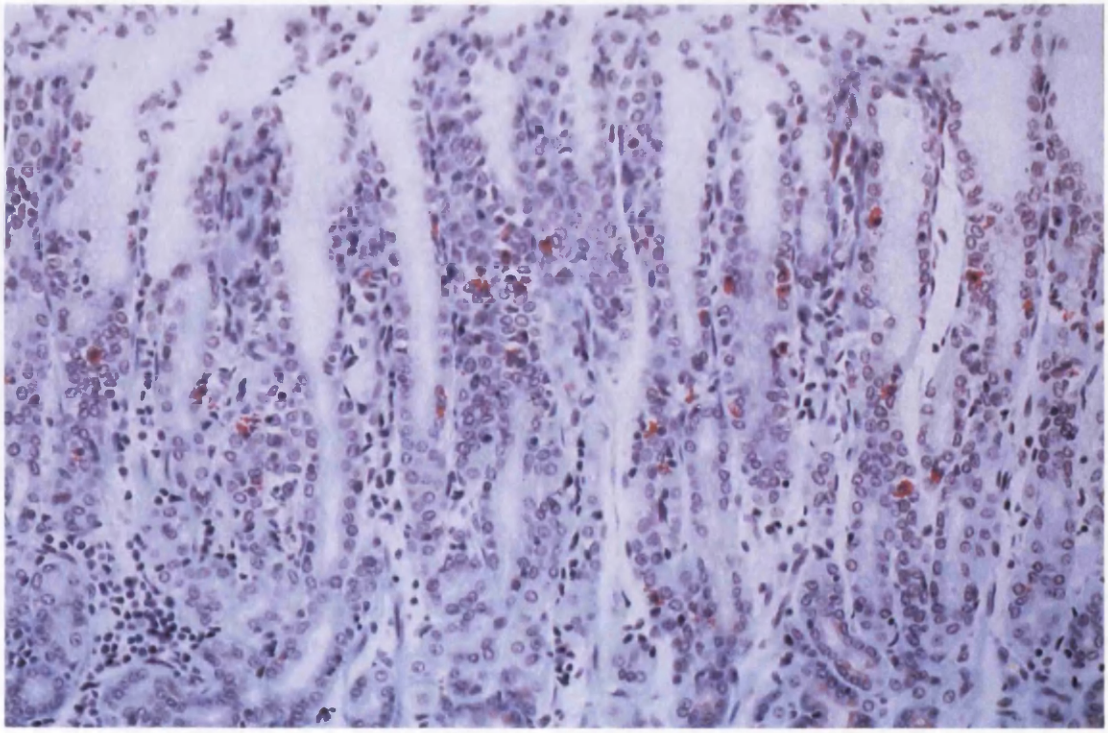


Figure 6.16: Photomicrograph of mucosa distant from the leading edge of the abomasal fold from lamb Y182 (MSB, original magnification was x 50).

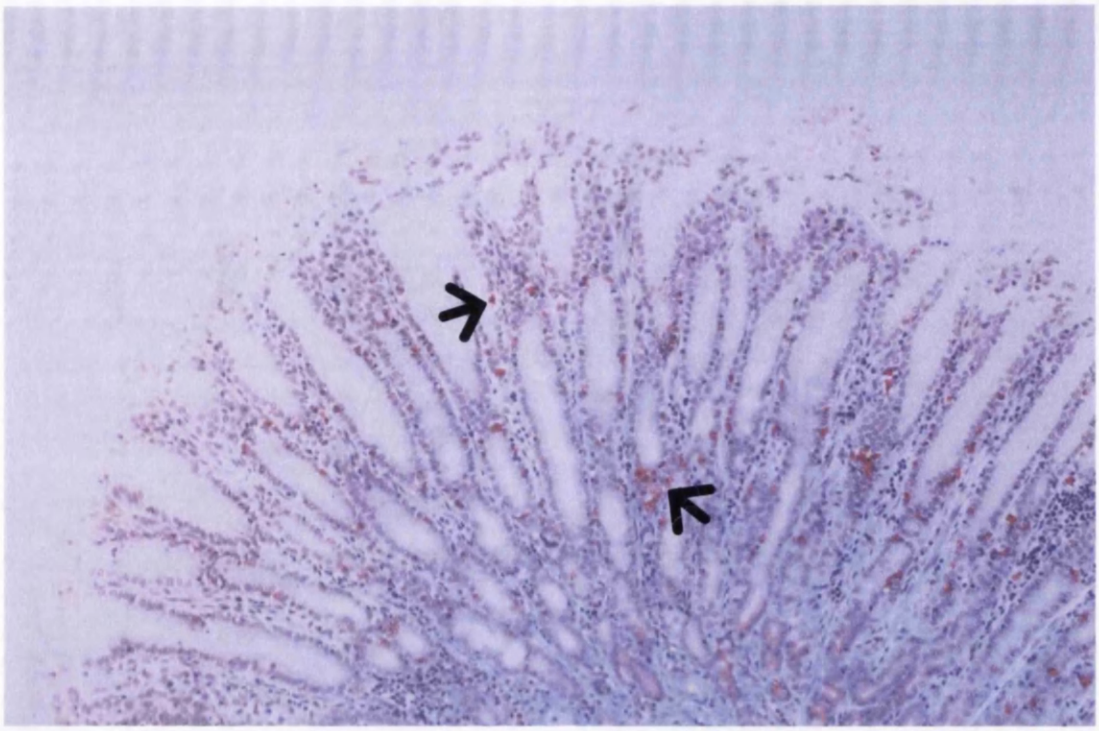


Figure 6.17: Photomicrograph of a leading edge of the abomasal fold from lamb Y182 showing numerous globule leucocytes (arrows) (MSB, original magnification was x 25).

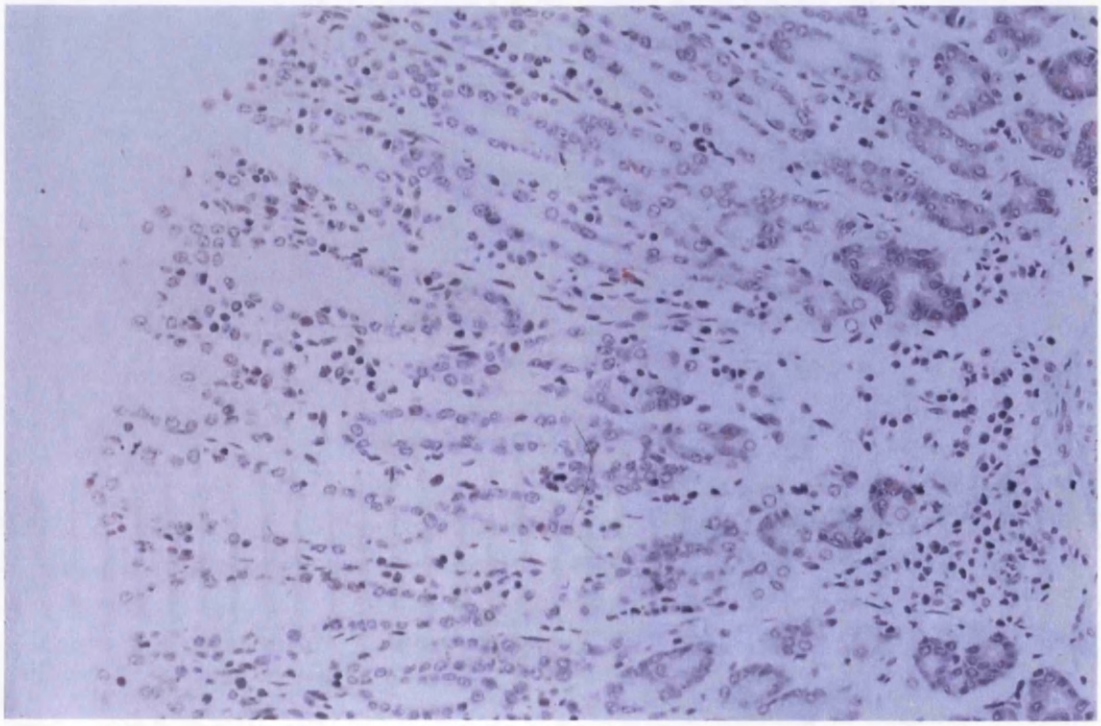


Figure 6.18: Photomicrograph of the leading edge of abomasal fold from lamb B30 showing a sparse population of globule leucocytes (MSB, original magnification was x 50).

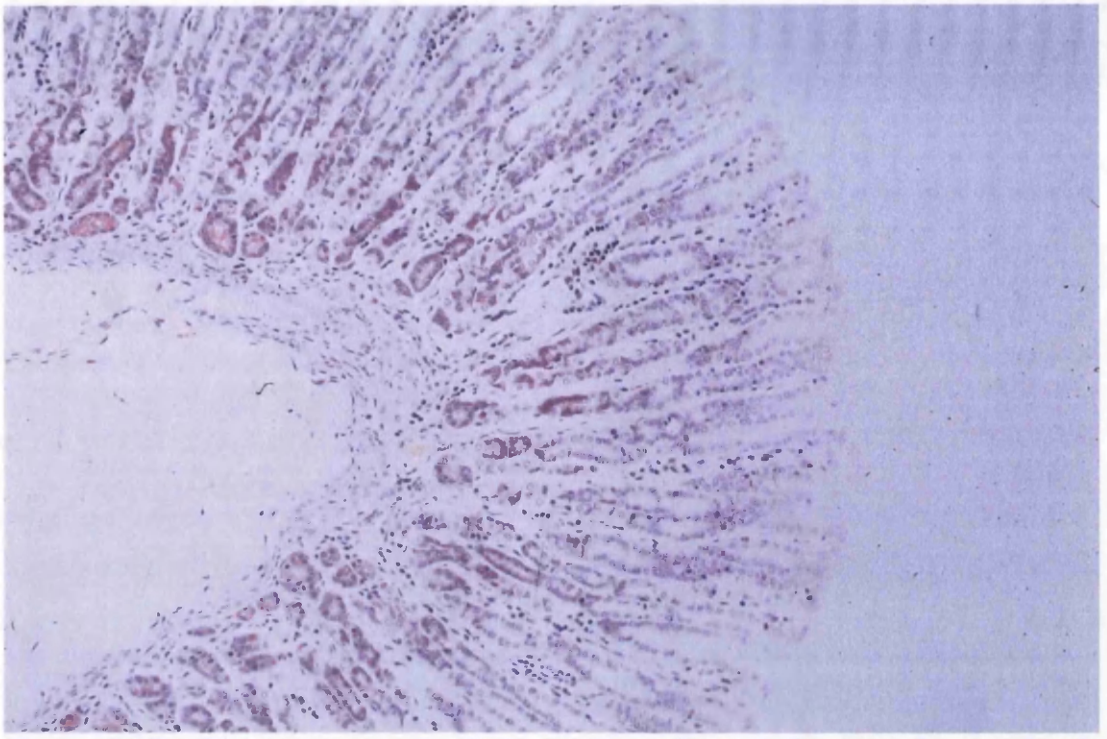


Figure 6.19: Photomicrograph of the leading edge of abomasal from a lamb, which was nematode naïve, revealing an absence of globule leucocytes and lymphoid aggregate (MSB, original magnification was x 25).

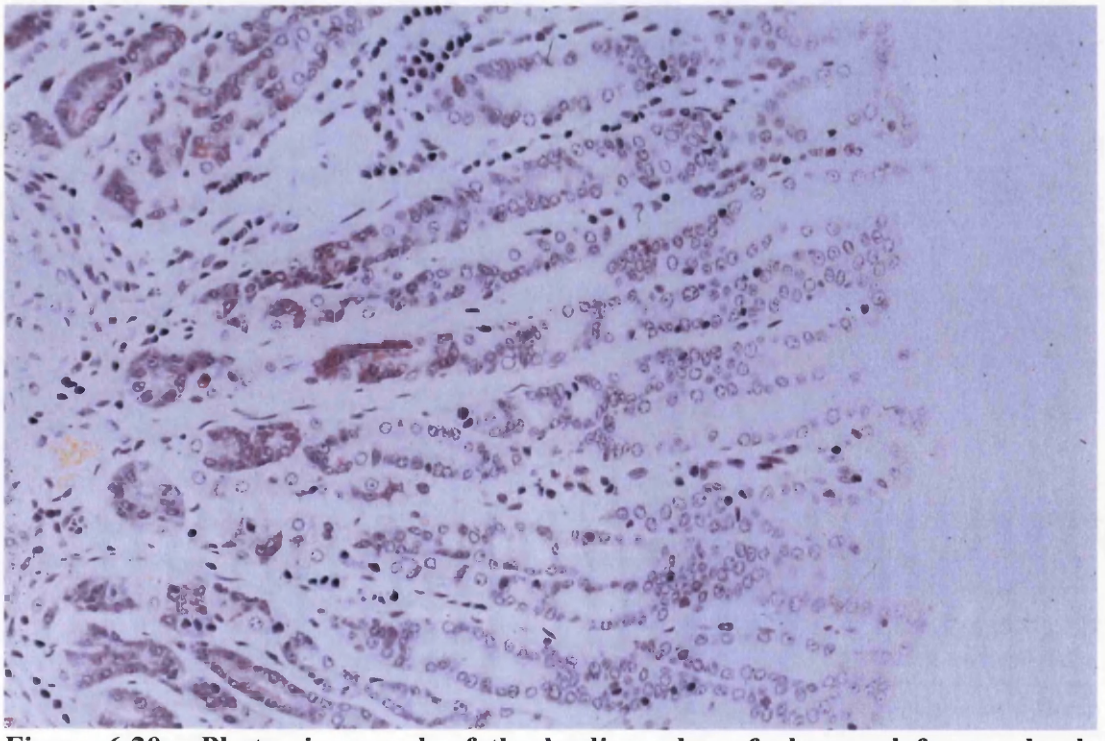


Figure 6.20: Photomicrograph of the leading edge of abomasal from a lamb, which was nematode naïve, revealing an absence of globule leucocytes (MSB, original magnification was x 50).

6.4: MAST CELL AND GLOBULE LEUCOCYTE ENUMERATION IN LAMBS SELECTED AS HAVING EITHER A HIGH OR A LOW PERCENTAGE OF B CELLS IN PERIPHERAL BLOOD

6.4.1: Introduction

Experiments 6.2 and 6.3 had failed to show that mast cells and globule leucocytes were involved in regulating worm burden. The failure to identify a relationship between these cell types and worm burden may have been due to the groups not being sufficiently different or the group sizes not being sufficiently large. The results from Experiment 4.4 showed a negative association between the percentage of peripheral blood lymphocytes which were B cells and the total *O. circumcincta* worm burden, and for the next experiment those lambs with the highest and lowest percentages of B cells in peripheral blood were selected. The intention was to select a sufficient number of lambs with high and low worm burdens to provide useful samples for understanding the mechanisms by which sheep control the worm burden. Enumeration of these cell types was again carried out in the hope that a suitable sample would be available to test the hypothesis that the mast cell-globule leucocyte axis is involved in regulating worm numbers. It also provided the opportunity to re-examine the association between mast cell responses and worm length.

The deliberate truncated infection experiment also provided the opportunity to study the mast cell and globule leucocyte response in a more rigorously controlled environment.

6.4.2: Protocol

Sixteen lambs with a high percentage of B cells in the peripheral blood and 16 lambs with a low percentage in peripheral blood were selected and assigned to Group N or D in such a manner that both groups had eight lambs with a high B cell percentage and eight lambs with a low B cell percentage and the means of B cell percentages for each group, and subgroup, were almost identical (Experiment 4.5).

In October the lambs were removed from pasture and transported to GUVS where they were separated into their respective groups and housed. After housing both groups were fed on hay. Four lambs a day from Group N were slaughtered on the 8 9 10 and 11 days after housing. Each of the Group D lambs was dosed with the anthelmintic ivermectin at the recommended dose rate and four weeks later deliberately challenged with 50,000 infective *O. circumcincta* L_3. Ten days after challenge the lambs were slaughtered.

Immediately after slaughter four sections of abomasal leaf were collected from each lamb and fixed in 4% paraformaldehyde/PBS and the sections were processed routinely and stained with simplified astra blue and MSB. Abomasal contents and a half abomasum were collected for parasitological investigation. Three sections of abomasum were read for globule leucocyte counts but only one for mast cell counts.

6.4.3: Results

The total mast cell counts for 21 mucosal strips from one section from each Group N lamb and the mast cell population densities are shown in Table 6.6. A

comparison of the results between the high and low egg B cell percentage groups reveals no significant difference in the mast cell population densities ($p=0.19$).

The total globule leucocyte mean counts for 21 mucosal strips from each section from Group N lambs, the population density of cells for each section, and the mean cell count and mean cell population density are recorded in Table 6.7. Very strong correlations were observed for globule leucocyte population densities between the three slides read for each individual, 0.93, 0.90 and 0.92 ($p<0.01$). As with the mast cells there was no significant difference between the two B cell percentage groups ($p=0.30$).

When the mast cell population densities were correlated with the globule leucocyte population densities (slide 1 and mean) there were also strong associations, 0.68 and 0.69 ($p<0.01$). The mean of mast cell concentration was greater than that of the mean of the globule leucocyte concentration, 138 cells mm^{-2} compared to 107 cells mm^{-2}, but the difference was not significant ($p=0.06$).

The results from the mast cell counts and globule leucocyte counts from Group D lambs are shown in Tables 6.8 and 6.9. Correlations between globule leucocyte population densities between slides gave mixed results, with some significant associations, but these were not as high as in earlier experiments, 0.3 ($p=0.25$), 0.68 ($p<0.01$) and 0.50 ($p<0.05$). The mast cell population densities were not associated with the globule leucocyte population densities for either slide 1 or the mean of three slides, 0.39 and 0.17 ($p=0.13$ and 0.54). As with Group N lambs there was no significant difference in mast cell and globule leucocyte population densities between high and low B cell percentage groups ($p=0.78$ and 0.52). The mast cell population densities were significantly greater than the mean globule leucocyte population densities, 90 cells mm^{-2} compared to 13 cells mm^{-2} ($p<0.01$).

Group N lambs had a greater mean mast cell population density than Group D lambs, 138 cells mm^{-2} to 90 cells mm^{-2} ($p<0.05$), and much greater mean globule leucocyte population density, 107 to 13 cells mm^{-2} ($p<0.01$), the comparisons are shown in Figure 6.20.

The total area from three slides that contributed to the globule leucocyte count was recorded (Tables 6.10 and 6.11), and mean area in the Group D lambs was greater, 15.6 mm^2 compared to 13.4 mm^2 ($p<0.01$) - as the same number of 0.49 mm mucosal strips were counted, this must mean that the mucosa of Group D lambs had a greater height.

Associations between globule leucocyte and mast cell concentrations and parasitological parameters were examined. There were no significant correlations between cell population densities with any of *O. circumcincta* burden results or with faecal worm egg counts (Table 6.12). As with experiment 6.3 there was a negative association between the log of globule leucocyte cell density and the mean adult female worm length, -0.63 ($p<0.01$); there was a slightly weaker negative association between mast cell density and worm length, -0.60 ($p<0.05$). The relationship between globule leucocyte density and worm length is shown in Figure 6.2. There was no association between globule leucocyte population density and faecal egg count. Following the deliberate challenge there was no significant association between either mast cell density or globule leucocyte density and worm burden, 0.00 and 0.21 ($p=0.99$ and 0.43).

LAMB	B CELL PERCENTAGE GROUP	TOTAL NUMBER OF MAST CELLS	MAST CELL mm^{-2}
3	HIGH	1066	245
21	HIGH	1091	216
100	HIGH	390	78
31	HIGH	317	61
47	HIGH	546	103
199	HIGH	557	120
126	HIGH	1504	286
58	HIGH	838	205
92	LOW	455	104
198	LOW	106	41
63	LOW	534	144
15	LOW	1132	244
35	LOW	716	142
1	LOW	342	79
149	LOW	76	19
11	LOW	516	118

Table 6.6: Mast cell counts and mast cell population densities in lambs selected for high and low B cell percentages in peripheral blood following natural infection.

LAMB	GROUP	GL COUNTS	GL DENSITY mm^{-2}	MEAN COUNTS AND DENSITY
3	HIGH	772 716 661	178 165 158	716 167
21	HIGH	606 715 457	120 140 102	592 122
100	HIGH	197 252 343	39 42 59	264 47
31	HIGH	784 616 650	150 126 128	683 135
47	HIGH	584 524 384	110 105 83	497 100
199	HIGH	266 171 158	57 35 35	198 42
126	HIGH	1749 1593 1276	333 322 251	1539 303
58	HIGH	360 380 316	88 96 82	352 89
92	LOW	718 438 456	164 115 110	537 131
198	LOW	16 98 27	6 32 9	47 16
63	LOW	302 311 217	82 67 53	277 67
15	LOW	747 591 775	161 141 168	704 157
35	LOW	258 175 336	51 36 75	256 53
1	LOW	514 380 523	122 78 106	472 106
149	LOW	180 418 251	46 94 64	283 69
11	LOW	339 529 615	77 123 150	494 116

Table 6.7: Globule leucocyte (GL) counts and cell population densities in lambs selected for high and low B cell percentages in peripheral blood following a natural infection.

LAMB	B CELL GROUP	TOTAL NUMBER OF MAST CELLS	MAST CELL mm^{-2}
93	HIGH	459	109
72	HIGH	551	106
117	HIGH	527	103
113	HIGH	643	97
70	HIGH	362	85
87	HIGH	346	52
118	HIGH	530	92
95	HIGH	536	91
91	LOW	512	100
25	LOW	167	38
156	LOW	496	88
26	LOW	686	125
24	LOW	419	98
68	LOW	451	88
23	LOW	340	69
147	LOW	511	104

Table 6.8: Mast cell counts and mast cell population densities in lambs selected for high and low B cell percentages in peripheral blood following a deliberate challenge infection.

LAMB	GROUP	GL COUNTS	GL DENSITY mm^{-2}	MEAN COUNTS AND DENSITY
93	HIGH	17 122 10	4 24 3	50 11
72	HIGH	24 11 106	5 2 19	47 9
117	HIGH	22 94 15	4 16 3	44 8
113	HIGH	46 170 32	7 27 6	83 13
70	HIGH	3 18 14	1 4 3	12 3
87	HIGH	2 7 18	0 1 3	9 1
118	HIGH	96 150 104	17 30 21	117 20
95	HIGH	131 181 165	22 32 30	159 28
91	LOW	46 41 29	11 8 6	39 8
25	LOW	30 236 79	7 48 15	115 23
156	LOW	78 60 26	14 12 5	55 11
26	LOW	134 75 111	24 14 24	107 21
24	LOW	41 116 55	10 25 13	71 16
68	LOW	59 37 8	12 7 2	35 7
23	LOW	38 35 20	8 8 5	31 5
147	LOW	57 159 138	12 32 27	118 24

Table 6.9: Globule leucocyte (GL) counts and cell population densities in lambs selected for high and low B cell percentages in peripheral blood following a deliberate challenge infection.

LAMB	GROUP	AREA mm^2
3	HIGH	12.88
21	HIGH	12.61
100	HIGH	12.34
31	HIGH	15.17
47	HIGH	14.48
199	HIGH	14.02
126	HIGH	16.85
58	HIGH	11.90
92	LOW	12.38
198	LOW	8.57
63	LOW	12.38
15	LOW	12.44
35	LOW	14.42
1	LOW	13.99
149	LOW	15.23
11	LOW	12.79

Table 6.10: The total area from three sections that was examined to give the globule leucocyte count for lambs exposed to a natural infection.

LAMB	GROUP	AREA mm^2
93	HIGH	13.32
72	HIGH	15.76
117	HIGH	16.08
113	HIGH	18.75
70	HIGH	13.41
87	HIGH	19.73
118	HIGH	17.51
95	HIGH	17.08
91	LOW	15.04
25	LOW	14.72
156	LOW	15.55
26	LOW	15.20
24	LOW	13.31
68	LOW	15.97
23	LOW	13.68
147	LOW	15.03

Table 6.11: The total area from three sections that was examined to give the globule leucocyte count for lambs challenged with a deliberate infection.

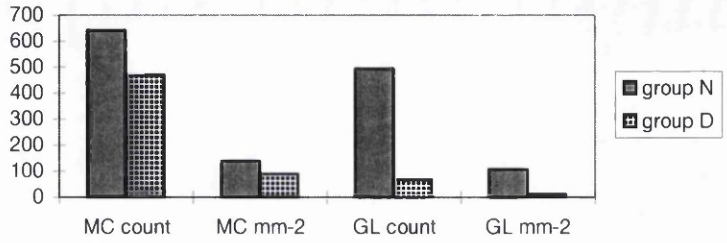


Figure 6.21: Mast cell (MC) and globule leucocyte (GL) counts and population cell densities in Group N and Group D lambs.

PARASITOLOGICAL PARAMETERS	LOG MEAN MC DENSITY	LOG MEAN GL DENSITY
Log L_4 *O. circumcincta*	0.19 p=0.48	0.21 p=0.43
Log L_5 *O. circumcincta*	0.10 p=0.72	0.49 p=0.06
Log Adult *O. circumcincta*	-0.10 p=0.72	-0.07 p=0.79
Log total *O. circumcincta*	0.13 p=0.62	0.17 p=0.52
Log of faecal worm egg count	0.12 p=0.66	-0.26 p=0.33
worm length	-0.60 p<0.05	-0.63 p<0.01

The first figure is the strength of the correlation, the second the probability of it occurring by chance

Table 6.12: The correlations between the logs of the mean mast cell (MC) density and mean of globule leucocyte (GL) density with the log of worm burden and faecal worm egg counts and worm length for Group N lambs.

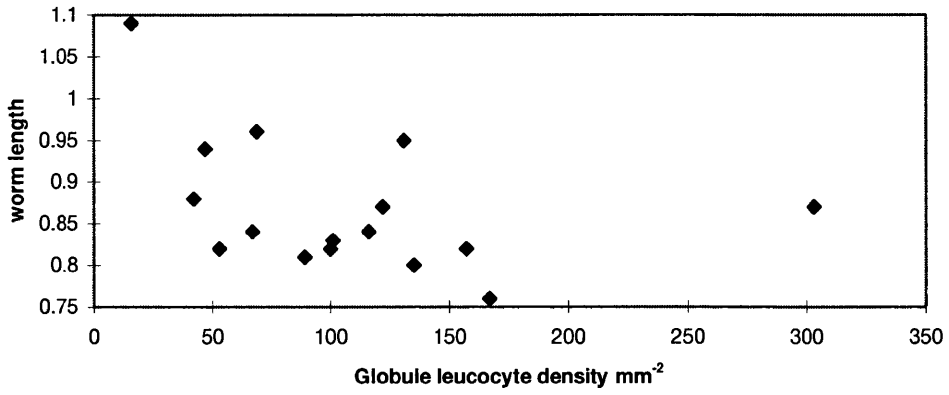


Figure 6.22: A scatter graph of the mean of worm length against the mean of globule leucocyte population density for each of 16 lambs in Group N.

6.5: MAST CELL AND GLOBULE LEUCOCYTE ENUMERATION IN HELMINTH-NAÏVE LAMBS

6.5.1: Introduction

This experiment was conducted to investigate the mast cell count and globule leucocyte count in lambs reared in an environment designed to be helminth-free.

6.5.2: Protocol

Five female Scottish Blackface lambs were reared indoors from birth, weaned at approximately two months of age and dosed with the anthelmintic albendazole sulphoxide at the recommended dose of 5 mg Kg^{-1}, based on the body weight of the heaviest lamb, before being transported to GUVS where they were housed on straw bedding and fed hay and concentrates. The lambs were slaughtered at six months of age and immediately after slaughter four sections of abomasal leaf

were collected from each lamb and fixed in 4% paraformaldehyde/PBS and the sections were processed routinely and stained with simplified astra blue and MSB. Abomasal contents and a half abomasum were collected for parasitological investigation. One section of abomasum was read for globule leucocyte counts and one for mast cell counts. The results were compared with those from lambs with a naturally acquired infection, the Group N lambs from Experiment 6.4.

6.5.3: Results

The mast cell and globule leucocyte counts are shown in Table 6.13. The mean mast cell count was 32.6 and the mean mast cell population was density was 9.4 cells mm^{-2}. No globule leucocytes were identified in all five sections examined. Parasitological examination identified no abomasal helminths. The mast cell and globule leucocyte counts were less than those in the Group N lambs ($p<0.01$). The mean area counted per slide was less for helminth-naïve lambs compared to Group N lambs, 3.52 mm^{-2} compared to 4.47 mm^{-2} ($p<0.05$).

LAMB	GL COUNTS	GL mm^{-2}	MAST CELL COUNTS	MAST CELL mm^{-2}	AREA mm^2
P1	0	0	31	8	3.91
P6	0	0	32	10	3.19
P13	0	0	25	7	3.43
P15	0	0	35	13	2.74
P21	0	0	40	9	4.32

Table 6.13: Globule leucocyte (GL) and mast cell counts, cell population densities and total area covered in helminth-naïve sheep.

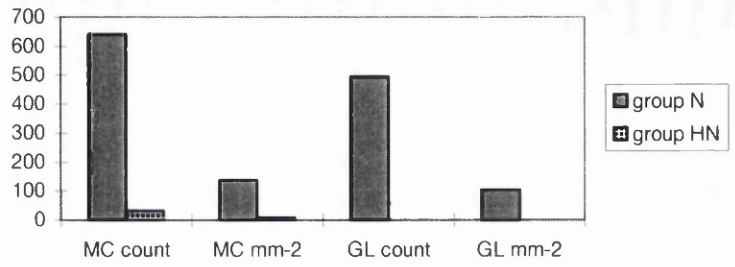


Figure: 6.23: Mast cell (MC) and globule leucocyte (GL) counts and population cell densities in Group N and helminth-naïve lambs (Group HN).

6.6: Discussion

This study has confirmed previous work by showing a strong relationship between the mast cell count and the globule leucocyte count and by showing that exposure to helminths increases the mast cell response and triggers the globule leucocyte response. Lambs selected for having low faecal nematode egg counts had more mast cells than those selected for having a high faecal worm egg counts. There was no evidence that either mast cells or globule leucocytes were involved in the control of the total worm burden, although an enhanced mast cell and globule leucocyte response was associated with a reduction in worm length and an increase in the number of *O. circumcincta* L_4. The globule leucocyte response to a truncated deliberate challenge infection was sub-maximal.

The results from Experiment 6.5, comparing counts in lambs exposed to a natural infection with counts in those maintained as helminth-naïve, concurs with the opinion that a helminth burden increases the mast cell concentration and triggers the globule leucocyte response (reviewed in Chapter 1). The mean cell population densities in the abomasal mucosa of the lambs exposed to *O. circumcincta* ranged from 90 to 138 cells mm^{-2} for mast cells and 13 to 107 mm^{-2} for globule leucocytes, but if the counts from the deliberate challenge infection

are excluded the ranges are from 109 to 138 cells mm^{-2} and 63 to 107 cells mm^{-2} for the two cell types. Compared to other studies with *O. circumcincta*, these concentrations are higher than those reported by some workers, 69 and 53 cells mm^{-2} (Stear *et al.*, 1995c), but less than that reported by others, over 750 cells mm^{-2} for the sum of mast cells and globule leucocytes (Smith *et al.*, 1984). Studies with *H. contortus*, in which the results were expressed as a sum of mast cells and globule leucocytes, had higher counts, ranging from 249 to 610 cells mm^{-2} (Huntley *et al.*, 1987; Gill *et al.*, 1991). Variation in results may be due to genetic differences between sheep, different infection and challenge regimes, the site of sampling the abomasum and techniques for counting cells (Seaton *et al.*, 1989; Gamble and Zajac, 1992; Huntley *et al.*, 1992).

The counting of cells and calculation of density required measuring the total area of lamina propria read in the mucosal strips and thus gave information on the height of the mucosal tissue. These results show that the area was greatest following deliberate challenge and least in naïve sheep, confirming the observation that ostertagiosis results in a hyperplastic gastritis (Murray *et al.*, 1970).

The associations between mast cell and globule leucocyte cell population densities in Experiments 6.3 and 6.4, at 0.76 and 0.69 are very strong and stronger than that previously reported, 0.51 (Stear *et al.*, 1995c). The failure to observe a significant association in Experiment 6.2 may be due to the small group size, or perhaps, because only one slide per lamb was read, the count may not have been an accurate estimate of the true number of cells in the abomasal mucosa. In subsequent experiments the group sizes were increased and in Experiment 6.3 three slides were read from each lamb. Mast cell density correlations between slides from the same individual lambs were very strong, ranging from 0.66 to 0.90. Globule leucocyte counts showed considerable within-individual variation between slides (sometimes differing by a factor of more than five), but because the between-lamb variation was so great, analysis

revealed associations even higher than those for mast cells (from 0.83 to 0.95). Therefore reading one slide per animal provides a reasonable estimate of the relative degree of the cell responses, though counting more slides provides even more accurate estimates.

The difference between the strength of the mast cell to globule leucocyte association in this study and that in the work by Stear *et al.* (1995c) may reflect biological or technical differences. A major biological difference was the infection regime, a natural infection with continuous exposure versus a large single challenge infection. Another was the site of the abomasum sampled, the fundus leaf compared to fundus and pylorus. The strength of the association of each cell type between slides indicates that the counting was an accurate estimate of the true level of mastocytosis and globule leucocyte activation in the abomasal folds. The use of the graticule ensured the areas of basal, central and lumenal mucosa which were counted were in proportion to the appropriate share of the tissue, which would have been more difficult to achieve using the circular field of view. Prevention of bias to a zone within the mucosa is particularly important as there is compartmentalisation of distribution of cell types with mast cells being more numerous in the basal mucosa adjacent to the muscularis mucosa and globule leucocytes being more numerous in the outer, lumenal mucosa (Christie *et al.*, 1978). The bias of mast cells to the basal area and globule leucocytes to the lumenal area is illustrated in Figures 6.10, 6.11 and 6.17. In Experiment 6.3 the distribution of globule leucocytes within the abomasal fold shows that there is a marked bias towards the lumenal projection or pole of the fold. Therefore the site of sampling and a systematic approach to reading the slide is important.

Results from all four experimental infections fail to show any association between either mast cell or globule leucocyte population density and worm burden, and therefore provide no evidence that these cell types are involved in control of worm burden. One possibility as to why no association was observed in the naturally acquired infections is that the variation in larval intake was so

great as to obscure an actual effect, although other studies on grazing sheep have shown negative associations between globule leucocytes and worm burdens, but in those infections *O. circumcincta* was not the most prevalent parasite (Douch *et al.*, 1986; Stankiewicz *et al.*, 1993). The deliberate challenge infection was tightly controlled with all animals receiving a similar dose and yet there was no negative association between either mast cells or globule leucocytes and worm burden. The observation that there appears to be no mast cell mediated control of the worm burden in six-month-old Scottish Blackface lambs exposed to a natural infection is supported by work showing no genetic control of worm burden in 500 lambs matched for age, breed, and farm of origin (Stear *et al.*, 1998).

In the experiments reported in this chapter, it is possible that mast cells and globule leucocytes apparently failed to control the worm burden because they were present in insufficient number, but this is unlikely because the concentrations recorded in this study were greater than in one where a negative association was shown (Stear *et al.*, 1995c). It could also be argued that the mast cells in younger animals are immature and their response is sub-maximal, but the presence of globule leucocytes in the naturally acquired infections shows that mast cell activation occurred, although these globule leucocytes themselves may not have been fully functional. It may be that the mast cell globule leucocyte axis is dependent upon another mechanism to enable control of worm burden and that this factor was lacking in these sheep, or that the mast cell globule leucocyte axis does not control worm burden. Hosts have evolved a co-ordinated response to gastrointestinal nematodes, and even though some specific defence mechanisms may not protect against a particular parasite these mechanisms are still likely to be strongly associated with ones which are effective (Finkleman *et al.*, 1997). Thus it may be that mast cell responses are nearly always correlated with regulation of worm burden through association of the true effector responses, and the relationship between mast cells and worm burden may be a mathematical correlation rather than a causal association.

This speculation that the mast cell globule leucocyte axis may not regulate *O. circumcincta* burden would be extremely radical if it were not for the fact that effective protection against *H. contortus* has been recorded in immune sheep which did not exhibit an increase in mast cell or globule leucocyte cell population density (Huntley *et al.*, 1992), and that Gasbarre (1997) argued that in cattle *O. ostertagi*, relative to *Haemonchus* spp.. and *Trichostrongylus axei*, are refractory to gastric immunity and evade the initial host protective responses. If the mast cell axis were not responsible for controlling worm burden it would be necessary to consider which mechanisms might be. Smith *et al.* (1987) suggested that a local IgA response may be involved in worm exclusion mechanisms, but Stear *et al.* (1995c) showed no association between worm burden and local parasite-specific IgA response. One mechanism that is receiving increased interest in rodent models is the change in mucus character and quality (Nawa *et al.*, 1994). Indeed Miller *et al.* (1983), in their definitive study of the immune exclusion of *H. contortus*, discussed changes in the mucus and changes in biochemical structure of epithelial cells as being candidate protective mechanisms and Miller (1996) again suggested that mucus may be an important protective response.

The truncated deliberate challenge experiment reveals a very low globule leucocyte population despite there being an adequate population of mast cells, and at least three possible scenarios may explain this observation: one, that *O. circumcincta* larval stages are not the antigenic stimulus for mast cell degranulation and globule leucocyte formation; two, that the interval between anthelmintic treatment and challenge infection was so long that either the mast cells had become senescent or mucosal 'immunological memory' had been lost; or three, that the challenge infection was so overwhelming that there was almost total consumption of the mast cells and globule leucocytes yet repopulation of mast cells but not globule leucocytes was in process at the time of slaughter.

Sheep mast cell protease (SMCP) is a mast cell granule enzyme released during mast cell activation and a study has shown that the concentration of SMCP in gastric lymph had increased 24 hours after a deliberate challenge of immune sheep with *O. circumcincta* L_3, therefore showing that larval stages can precipitate mast cell activation (Huntley *et al.*, 1987; Jones *et al.*, 1992).

Work by Huntley *et al.* (1992) on the duration of immunity of sheep to *H. contortus* showed that the interval between an anthelmintic treatment, following exposure to parasite, and subsequent challenge has a marked effect on mast cell and globule leucocyte responses, and an interval of six weeks was sufficient to reduce the mast cell response to the level of naïve animals. In the deliberate infection described here the interval between dosing and challenge was four weeks, which may have been sufficiently long for the globule leucocyte response to diminish, although a previous study using this interval not only showed a globule leucocyte response, but also showed a negative association between that response and worm burden (Stear *et al.*, 1995c). There were major differences between the work by Stear *et al.* (1995c) and the work described in this chapter: one, the sheep were older and their previous exposure to parasites was greater, thus the cellular response may also have been greater and more persistent; and two, the animals were slaughtered eight weeks after challenge, thus allowing ample time for repopulation of mast cells and globule leucocytes in the abomasal mucosa.

A serial study of a large single challenge infection of previously exposed sheep with *T. colubriformis* showed that globule leucocytes can be recruited, consumed and then re-established, because there were few globule leucocytes prior to challenge, large numbers at day 1 (one day after challenge), low numbers at days 5 and 6 and very high numbers at days 8 and 14 (McClure *et al.,* 1992), and it is possible that a similar but more protracted dynamic process occurred in this experiment. Whether the globule leucocytes were consumed or the globule leucocyte response had waned, the lambs appear not to have acquired protective

mechanisms to control worm burden, as the mean worm burden from a 50,000 L_3 challenge was 16,678, which is approximately a third of the challenge dose, and compares with that reported by Stevenson *et al.* (1994) following an identical experimental infection of naïve lambs, which were also killed on day 10.

There is no evidence in the work described here that either mast cells or globule leucocytes control worm burden, but there is evidence to show that these cell types are associated with a reduced faecal worm egg count. Although there were no significant associations between these cell types and faecal worm egg counts at the time of slaughter, sheep selected for low faecal worm egg counts had a greater concentration of mast cells and globule leucocytes in their abomasal mucosa than sheep selected for high faecal worm egg counts; this difference was significant in Experiment 6.3 when the group sizes were larger. The failure to observe an association between mast cell count and faecal worm egg count at time of slaughter, although it was possible to select a high mast cell count on the basis of sequential low faecal worm egg count, may be due to the fact that faecal worm egg counts are of only low to moderate repeatability, 0.27 to 0.4 (Stear *et al.*, 1995b). Thus there remains considerable individual variability over time and consequently an average faecal worm egg count over several months may be more representative than one sample at the time of slaughter.

The faecal worm egg count is influenced both by the adult worm burden and by the fecundity of the adult worms (Stear *et al.*, 1996b), and because low egg count groups have more mast cells, but sheep with more mast cells do not have fewer worms, then we might expect that sheep with more mast cells and globule leucocytes have less fecund worms. Indeed, since worm length was negatively associated with globule leucocytes and mast cells in both experiments in which worm length was examined, and worm length is a measure of fecundity, sheep with more globule leucocytes did indeed have less fecund worms. Previous studies have reported negative associations between the globule leucocyte concentration and worm length, namely Stear *et al.* (1995c) with *O. circumcincta*

and Douch and Morum (1993) with a mixed infection which included *O. circumcincta*. Stear *et al.* (1995c) showed that the mucosal IgA was negatively associated with worm length and because globule leucocyte concentration was associated with IgA the negative association between globule leucocytes and worm length was considered to be a confounding effect, but this was not conclusive. Douch and Morum (1993) did not investigate antibody responses in their work and commented that as globule leucocytes appear to be the source of mediators which inhibit larval migration (Douch *et al.*, 1986) they might also be a source of substances affecting worm fecundity.

In Experiment 6.3 the globule leucocyte concentration was positively associated with the L_4 burden, which suggests that the globule leucocytes might have an additional protective mechanism by inhibiting larval development.

The distribution of mast cells and globule leucocytes was shown to be very different, with the mast cells distributed throughout the abomasal leaf and the globule leucocytes occurring in clusters with a very strong bias towards the pole of the abomasal leaf. These distributions would be consistent with the concept of a two stage activation process with a diffuse migration of mast cells into the mucosa as a response to parasitism followed by a process (reviewed by Huntley, 1992) in which mast cells are exposed to antigen and mature, migrate to an epithelial location and release their contents, thus forming globule leucocytes. Smith and Weis (1992) have proposed mechanisms that would explain this two stage migration, with immature mast cells expressing adhesion molecules recognised by endothelial ligand in the lamina propria allowing migration into the mucosa and then either cytokine stimulation or $Fc_{\varepsilon}R1$-mediated degranulation inducing expression of a different homing receptor, resulting in mast cells/globule leucocytes occupying an intra-epithelial position. Interestingly, mast cells and T cells share common adhesion receptors- and descriptions of changes in T cell distribution following challenge (McClure *et al.*, 1992) in some way mimic those of mast cells, with unchallenged immune

sheep having a greater lamina propria cellularity than naïve sheep and then following challenge a further increase in that lamina propria cellularity greater than that in the sheep with a primary exposure, and also a dramatic increase in the intra-epithelial lymphocytes.

It is generally recognised that the stimulus for mast cell degranulation is exposure to antigen and mechanisms exist whereby direct contact with an antigen can lead to degranulation through cross-linking of IgE bound to the mast cell membrane, and it has been shown that isolated and purified sensitised sheep mast cells are activated on contact with specific antigen (Jones *et al.,* 1992). On first examination the clustered distribution pattern of globule leucocytes would be consistent with direct local contact with antigen leading to degranulation, but rather than the clusters being random there appears to be a very precise anatomical localisation to the leading edge, or pole, of the fundus leaf. It is the author's opinion through observing the gross distribution of umbilicated nodules in parasitised abomasums that the leading edge is no more prone to parasitism than other parts of the abomasal leaf, and in the experiments described here the parasite challenge was not so great that practically every leading edge had been exposed. What is present at this leading edge of fundic leaves is a vascular bundle which, in sheep naturally exposed to *O. circumcincta,* is usually associated with a lymphoid aggregate - this lymphoid aggregate is illustrated in Figure 6.9. Local cytokine release from the polar lymphoid aggregate following exposure to soluble antigens may lead to mast cell degranulation and globule leucocyte activation - but other hypotheses would be valid: one, that the lymphoid aggregate provides a larger population of antigen presenting cells allowing more direct contact between antigen and lamina propria mast cells; or two, that direct contact may be responsible and that the adult worm's predeliction site for grazing is the leading edge of the fundus leaf.

Further studies could repeat the deliberate challenge experiment but have a shorter interval between dosing and challenge and also between challenge and slaughter, when a greater globule leucocyte response would be expected and the

lack of association between globule leucocytes and worm burden in a controlled challenge could be re-evaluated. Experiments on older, more immune sheep and sheep similar to the ones used in the experiments described could be used to examine other candidate mechanisms for controlling worm burden - if immune sheep had a similar mast cell response but a much greater mucous cell hyperplasia or qualitative changes in mucus biochemistry it would suggest that these were the effector mechanisms limiting worm burden.

Since most of the candidate protective responses are likely to be under the control of Th2 cytokines and therefore often linked with each other, attempts to identify causal associations by mathematical correlations are frustrated by confounding effects. It is now becoming possible to knock out or reduce some T cell subtypes or specified cytokines in sheep (Gill *et al.*, 1993b; McClure *et al.*, 1996) but, because these cell types or cytokines are upstream of the effector mechanisms, more than one response may be reduced and this may complicate interpretation. What would be very useful would be the ability to delete a specific effector response, such as can be done in mice in which mastocytosis can be blocked by treatment with a monoclonal antibody to *c-kit*, the ligand for stem cell factor, a mast cell growth factor (Grencis *et al.*, 1993).

Immunocytochemistry of abomasal sections could be used to investigate co-localization and collaboration of T cells and mast cells (Smith and Weis, 1996) and may be used to address the suggestion by Konno *et al.* (1995) that globule leucocytes are in fact a subset of γδ T cells rather than a derivative of mast cells.

CHAPTER 7: IMMUNOGLOBULIN A RESPONSES AND RELATIONSHIPS WITH PARASITOLOGICAL PARAMETERS

7.1: INTRODUCTION

There are five distinct classes of immunoglobulin molecule recognised in mammals, namely IgG, immunoglobulin M (IgM), immunoglobulin D (IgD), IgE and IgA. IgG is the major immunoglobulin in serum and extravascular tissue fluid and is considered to be the major antibody of secondary immune responses. IgM is largely confined to the intravascular pool and is an early antibody to complex antigens with levels peaking before those of IgG levels. IgD accounts for a small proportion of serum immunoglobulin and although it is present in large quantitites on the surface of many B cells its role has not been fully elucidated. IgE is a trace serum protein important in degranulation of mast cells, and IgA is present in serum but is abundant in mucosal structures.

In sheep IgA is dimeric in both plasma and mucosal secretions (Vaerman, 1970 cited by Quin *et al.,* 1975). IgA is the predominant class of immunoglobulin in intestinal secretions and mucosal IgA differs from that in plasma, being associated with a polypeptide, the secretory component, which facilitates transport into secretions as well as protecting against proteolysis (Chodirker and Tomasi, 1963; Newcomb *et al.*, 1968). The role of IgA on the mucosal surface is thought to be the binding of pathogen antigens and thus preventing colonisation, rather than the activation of inflammatory cascades or cytotoxic responses (reviewed by Kagnoff, 1993). Miller *et al.* (1983) postulated that IgA may play a role in the exclusion of nematodes and Smith *et al.* (1985) suggested that IgA present in abomasal mucus reduced the growth rate of *O. circumcincta* larvae in a dose dependent manner, perhaps by interfering with enzymes essential for normal feeding mechanisms.

In sheep, enteric mucosal IgA is produced locally rather than being derived from plasma IgA (Beh *et al.*, 1974; Quin *et al.*, 1975; Sheldrake *et al.*, 1984), and large numbers of IgA-specific cells in the lamina propria are considered to be responsible, with the contribution of regional nodes being trivial (Lee and Lascelles, 1970; Beh and Lascelles, 1974). It is reasonable to assume that mucosal IgA on the abomasal lumenal surface is derived from plasma cells in the lamina propria of the abomasum. Stear *et al.* (1995c) showed that IgA-positive plasma cells were present in the abomasal mucosa in significant numbers.

Increased antibody responses have frequently been associated with enhanced protection against parasites, reviewed in Chapter 1. There is an emerging hypothesis that in lambs exposed to *O. circumcincta*, faecal worm egg count is controlled by IgA regulating worm fecundity. In a flock of grazing lambs, from three months of age the genetically resistant sheep regulate faecal nematode egg count (Bishop *et al.*, 1996). The faecal worm egg count is a function of the number of adult female worms and mean fecundity of the worms (Stear *et al.*, 1996b), and although there is no genetic control of the number of adult worms in six-month-old lambs, there is genetic control of worm length, a measure of fecundity; thus regulation of faecal worm egg output is through restricting worm fecundity (Stear *et al.*, 1997b). Smith *et al.* (1985) observed a very close negative association between the mean length of *O. circumcincta* worms following a truncated deliberate challenge, and the total IgA concentration in the gastric lymph six days after infection, when the IgA concentration was at its greatest. Subsequently, Stear *et al.* (1995c) examined the relationship between the IgA activity against *O. circumcincta* L_4 antigens and mean adult female worm length following a deliberate challenge of twelve-month-old Scottish Blackface sheep, and showed a negative association between IgA activity and worm length.

The hypothesis that worm length and fecundity are regulated by antibody-mediated mechanisms was strengthened by the work by McCririe *et al.* (1997), which revealed heterogeneity in the recognition of *O. circumcincta* antigens by sheep, and

that recognition of one molecule from adult parasites was associated with shorter worms, thus suggesting that the specificity of antibody responses as well as the magnitude of antibody responses is important.

The mechanisms of host protection change as sheep mature, because the mechanisms that control worm length are functional before those that regulate worm burden (Seaton *et al.*, 1989; Stear *et al.*, 1996b). Although it is likely that IgA controls worm length in younger sheep as it is believed to do in older sheep, and even though the work by Smith *et al.* (1985) provides evidence of IgA controlling worm length in sheep that were only 4.5 months of age, it was considered prudent to investigate whether or not there was an observable association between IgA activity to *O. circumcincta* larval antigens and parasitological parameters following exposure of young sheep to parasites by grazing.

7.2: AN INVESTIGATION OF REPEATABILITY OF PARASITE-SPECIFIC IgA IN GRAZING LAMBS

7.2.1: Introduction

An experiment was designed to monitor the levels of parasite-specific IgA in plasma from grazing lambs, to test whether this parameter was repeatable and to test whether there was a tendency of the antibody levels to increase with increased exposure to parasite.

7.2.2: Protocol

Peripheral blood was collected in anticoagulant following venepuncture of ten castrated male lambs that had been exposed to *O. circumcincta* by grazing pasture. Plasma samples were prepared and stored by Protocol 2.3.3.6. Blood was collected at four-weekly intervals, in June, in July, in August and in September. The antigen used for quantifying parasite-specific antibody was a somatic extract of *O. circumcincta* L_3 (L_3SE) prepared according to Protocol 2.3.3.8. Serum was diluted 1:10 in blocking buffer and using Protocol 2.2.2.10 ELISA tests were used to determine IgA activity. The results of ELISA tests are expressed as optical density indices (OD).

7.2.3: Results

The OD for the ten lambs are shown in Table 7.1. Correlation coefficients between months were examined. There was a significant association between the OD in July and those in August, 0.75 ($p<0.05$), but there were no other significant associations - the strengths of the other correlation coefficients were: June and July -0.01 ($p=0.99$), June and August 0.22 ($p=0.55$), June and September 0.03 ($p=0.93$), July and September 0.43 ($p=0.21$) and August and September 0.27 ($p=0.45$).

The mean OD for the lambs in months from June to September were 0.02, 0.04, 0.09 and 0.35 (see Figure 7.1), showing an increase in IgA activity as the grazing season progresses. The differences between the June mean and both the August and September means were significant ($p<0.05$ and $p<0.01$), the differences between the July mean and August and September means were highly significant (both $p<0.01$), and the difference between the August and September means was significant ($p<0.05$). The difference between the June and July means was not significant ($p=0.41$).

LAMB	JUNE	JULY	AUGUST	SEPTEMBER
Y64	0.03	0.07	0.13	0.88
Y66	0.04	-0.02	0.02	0.11
Y72	-0.04	0.05	0.11	0.16
Y73	0.03	0.00	0.02	0.07
Y92	0.04	-0.02	0.12	0.09
Y94	0.01	0.03	0.08	0.81
Y114	0.01	-0.01	-0.01	0.41
Y119	0.02	0.10	0.09	0.36
P78	0.05	0.12	0.25	0.46
B94	0.02	0.04	0.09	0.16

Table 7.1: Optical density indices for ELISA tests to measure plasma IgA activity against L_3SE for grazing lambs at different months.

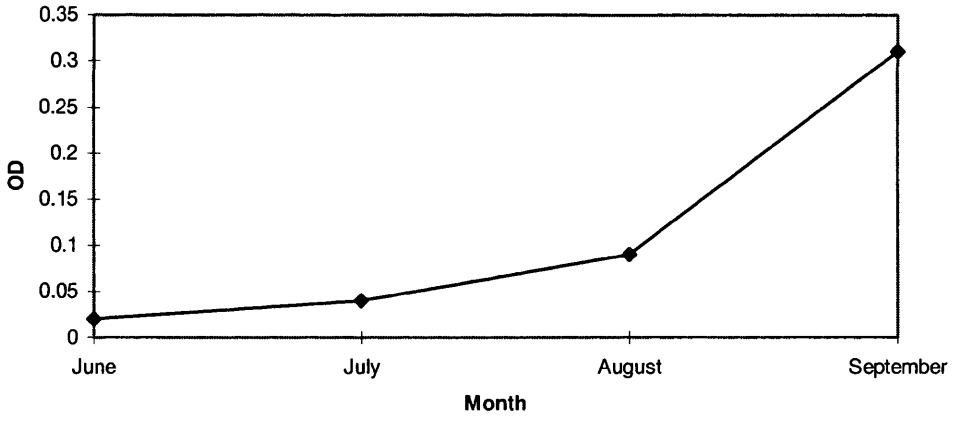


Figure 7.1: The mean optical density indices (OD) of ELISA tests for plasma IgA activity against L_3SE for grazing lambs.

7.3: A COMPARISON OF PLASMA IgA ACTIVITY AGAINST L_3SE IN GRAZING LAMBS WITH THAT IN HOUSED LAMBS

7.3.1: Introduction

The grazing lambs were exposed to *O. circumcincta* by grazing pasture, whilst the housed lambs were reared in conditions designed to prevent exposure to helminths. If the ELISA test measures reactivity to parasite then it is expected that the grazing lambs would have higher OD to L_3SE. This experiment was designed to demonstrate that difference.

7.3.2: Protocol

The values for the grazing lambs were those from the September blood sample for the ten lambs described in Experiment 7.2. The housed lambs were five age, sex and breed matched controls that had been housed since birth and kept helminth-naïve. Blood was taken in September and plasma removed and an ELISA test using the same protocol and antigen as for experiment 7.2 was carried out. The results were compared by a Student's *t*-test.

7.3.3: Results

The OD for the housed lambs are shown in Table 7.2. The mean OD for the grazing lambs was significantly greater than the mean OD for housed lambs, 0.35 compared to -0.08 ($p<0.01$). The negative OD occurred because the optical density in these samples was less than that of the low standard.

LAMB	OD
P17	-0.09
P38	-0.09
P46	-0.07
P48	-0.07
P53	-0.06

Table 7.2: Optical density indices (OD) for ELISA tests to measure plasma IgA activity against L_3SE for housed lambs in September.

7.4: A COMPARISON OF MUCOSAL HOMOGENATE IgA ACTIVITY AGAINST L_3SE IN GRAZING LAMBS WITH THAT IN HOUSED LAMBS

7.4.1: Introduction

This experiment was designed to show that the IgA activity against L_3SE in mucosal homogenate was greater in grazing lambs than in housed lambs reared in conditions so that they were helminth-naïve.

7.4.2: Protocol

The values for the mucosal activity in grazing lambs were those from the Group N lambs in Experiment 7.6. The housed lambs were five age, sex and breed matched controls that had been housed since birth, kept helminth-naïve and slaughtered when six months old. Abomasal mucosal homogenate samples were prepared according to Protocol 2.3.3.5. Mucosal homogenate IgA activity was assayed according to Protocol 2.3.3.10, with the dilution of mucosal homogenate in blocking buffer being 1:50.

7.4.3: Results

The results for ELISA tests to measure mucosal homogenate activity against L_3SE for five naïve lambs are shown in Table 7.3. The mean OD was significantly less than that for grazing lambs, -0.10 compared to 0.56 ($p<0.001$).

LAMB	MUCOSAL HOMOGENATE OD
N6	-0.08
N13	-0.09
N15	-0.09
N21	-0.11
N22	-0.12

Table 7.3: Optical density indices (OD) for ELISA tests to measure mucosal homogenate IgA activity against L_3SE for housed, helminth-naïve lambs.

7.5: A COMPARISON OF PLASMA IgA ACTIVITY AGAINST L_3SE IN LAMBS WITH A LOW FAECAL WORM EGG COUNT AND THOSE WITH A HIGH FAECAL WORM EGG COUNT

7.5.1: Introduction

The results from the investigations described in Experiments 7.2 and 7.3 revealed that the plasma IgA activity in grazing lambs increased as the animals matured and that by September grazing lambs had considerable antibody activity but there was no detectable activity in housed lambs. The aim of this experiment was to investigate whether or not there were significant differences in IgA activity between two groups of lambs selected to represent the extremes of the faecal worm egg

counts present within a flock, and whether or not there were any associations between IgA activity and parasitological parameters.

7.5.3: Protocol

Two groups of eight were selected on the basis of their faecal worm egg counts, so that one group was selected for low egg count and one group was selected for high egg count (Experiment 3.3). In October the lambs were removed from pasture and transported to GUVS where they were housed and fed on hay. Two days after transfer the lambs were blood sampled and plasma was removed and stored. An ELISA test using the same protocol and antigen as for Experiment 7.2 was carried out. The results from the two groups were compared and regression analysis across groups was used to examine any associations between IgA activity and parasitological parameters.

7.5.3: Results

The plasma IgA activity from sheep in the high and low faecal worm egg count groups is shown in Table 7.4. The mean OD for the low egg count group was 0.64 and the mean for the high egg count group was 0.47; these differences are not significant ($p=0.37$). The correlation coefficients for transformed IgA activity to parasitological parameters are shown in Table 7.5. There was a significant and strong negative association between plasma IgA activity and worm length, -0.60 ($p<0.05$), this relationship is shown in Figure 7.2.

LAMB	FWEC STATUS	PLASMA OD
B23	LOW	0.08
B27	LOW	1.07
B37	LOW	0.83
Y165	LOW	0.96
Y166	LOW	0.91
Y176	LOW	0.17
Y179	LOW	0.37
Y182	LOW	0.69
B15	HIGH	0.81
B16	HIGH	0.35
B20	HIGH	0.90
B30	HIGH	0.33
B39	HIGH	0.43
B41	HIGH	0.02
B46	HIGH	0.77
Y192	HIGH	0.15

Table 7.4: Optical density indices (OD) for ELISA tests to measure plasma IgA activity against L_3SE for lambs selected according to faecal worm egg count.

PARASITOLOGICAL PARAMETER	CORRELATION COEFFICIENT
Log of faecal worm egg count	-0.08 p=0.77
Log of *O. circumcincta* L_4 burden	0.06 p=0.82
Log of *O. circumcincta* L_5 burden	0.03 p=0.93
Log of *O. circumcincta* adult burden	-0.29 p=0.52
Log of total *O. circumcincta* burden	-0.17 p=0.52
Mean adult female worm length	-0.60 p<0.05

The first figure is the strength of the association, the second the probability of this occurring by chance

Table 7.5: The associations between plasma IgA activity against L_3SE and parasitological parameters for lambs selected according to faecal worm egg count.

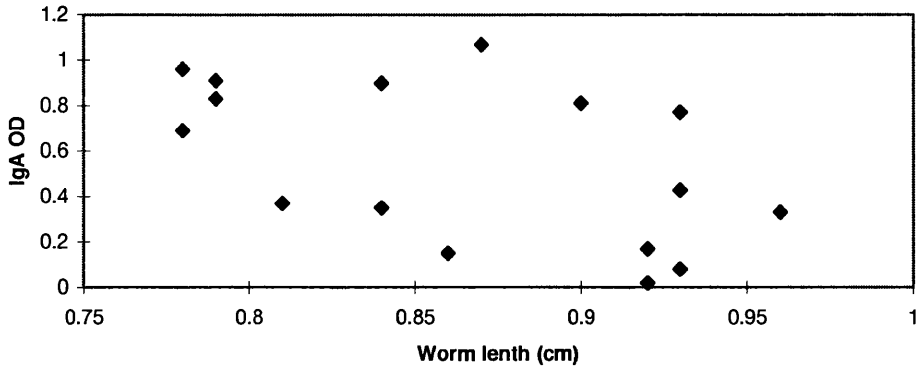


Figure 7.2: The relationship between female worm length and plasma IgA activity to L_3SE for lambs selected according to faecal worm egg count.

7.6: A COMPARISON OF IgA ACTIVITY AGAINST L_3SE IN LAMBS SELECTED FOR HIGH AND LOW B CELL PERCENTAGES

7.6.1: Introduction

The results from Experiment 4.4 showed an association between an increased percentage of peripheral blood B cells and fewer *O. circumcincta*. A subsequent experiment, Experiment 4.5, selected lambs with the highest or lowest percentages of B cells in the peripheral blood, with the aim of selecting high and low worm burdens. Sixteen of these lambs were slaughtered when carrying a worm burden acquired by grazing, and eight of these lambs had a high B cell percentage and eight had a low B cell percentage (Group N). Sixteen lambs, eight with a high B cell percentage and eight with a low B cell percentage, were given a deliberate challenge with *O. circumcincta* L_3 (Group D). Both plasma and abomasal mucus were sampled and used to investigate IgA activity against L_3SE. Comparisons of IgA activity were made between low and high B cell groups, and following natural

infection and deliberate infection, relationships between IgA activity and parasitological parameters were also investigated.

7.6.2: Protocol

In October all 32 lambs were removed from pasture and transported to GUVS where they were fed on hay. The Group N lambs were slaughtered shortly after transport with four lambs a day slaughtered on the 8^{th}, 9^{th}, 10^{th}, and 11^{th} days after housing. The lambs were blood sampled immediately prior to slaughter and plasma was removed and stored. Abomasal mucosal homogenate samples were prepared according to Protocol 2.3.3.5. Plasma IgA activity was assayed by the same protocol as used in Experiment 7.2 and mucosal homogenate IgA activity was assayed according to Protocol 2.3.3.10, with the dilution of mucosal homogenate in blocking buffer being 1:50.

Following arrival at GUVS the Group D lambs were dosed with an anthelmintic at the recommended dose rate and four weeks later deliberately challenged with 50,000 infective *O. circumcincta* L_3. Blood was sampled three days after challenge and plasma stored. Abomasal scrapings were taken at slaughter and mucosal homogenate was prepared. The protocols for estimating IgA activity in plasma and mucosal homogenate were identical to those for the Group N lambs.

The parasitological results are recorded in Experiment 3.4.

7.6.3: Results

The results of ELISA tests are shown in Tables 7.6 and 7.7. For both plasma and mucosal homogenate IgA activity in Group N lambs there was no difference between high B cell groups and low B cell groups ($p=0.50$ and 0.98 respectively).

The OD were not normally distributed and following transformation there was a significant association between IgA activity in plasma with that in mucosal homogenate, 0.50 ($p<0.05$).

The IgA activity in high and low B cell subgroups for Group D lambs was examined, and again there were no significant differences between groups, both for plasma and mucosal activity ($p=0.46$ and 0.29). There was no evidence of an association between plasma and mucosal homogenate activity for Group D lambs, 0.10 ($p=0.72$).

The mean OD for plasma IgA activity for Group N was greater than the mean for Group D, 0.56 compared to 0.34 ($p<0.05$), but the mean OD for mucosal homogenate was much less, 0.37 compared to 1.17 ($p<0.001$).

The correlation coefficients between the log of OD for plasma and mucosal samples and parasitological parameters were examined and the results for Group N are shown in Tables 7.8 and 7.9. There were no significant associations for Group N but there was a positive and significant correlation between log of worm burden and the log of mucosal IgA OD following the deliberate infection, 0.51 ($p<0.05$), whereas the correlation coefficient for plasma IgA activity to worm burden was not significant, 0.15 ($p=0.57$).

LAMB	B CELL STATUS	PLASMA OD	MUCOSAL OD
3	HIGH	0.38	0.67
21	HIGH	0.31	0.14
100	HIGH	0.60	0.23
31	HIGH	0.90	0.26
47	HIGH	0.80	0.30
199	HIGH	0.91	0.54
126	HIGH	1.01	0.78
58	HIGH	0.01	0.02
92	LOW	0.73	0.43
198	LOW	0.77	1.08
63	LOW	0.09	0.03
15	LOW	0.47	0.57
35	LOW	0.47	0.20
1	LOW	0.20	0.24
149	LOW	0.90	0.32
11	LOW	0.41	0.10

Table 7.6: Optical density indices (OD) for ELISA tests to measure IgA activity against L_3SE for Group N lambs.

LAMB	B CELL STATUS	PLASMA OD	MUCOSAL OD
93	HIGH	0.01	1.27
72	HIGH	0.58	1.12
117	HIGH	0.71	0.93
113	HIGH	0.22	1.54
70	HIGH	0.12	0.66
87	HIGH	0.59	0.79
118	HIGH	0.08	1.56
95	HIGH	0.76	1.30
91	LOW	0.46	1.19
25	LOW	0.30	1.45
156	LOW	0.33	1.20
26	LOW	0.61	1.54
24	LOW	0.09	0.60
68	LOW	0.13	0.74
23	LOW	0.23	1.81
147	LOW	0.15	1.05

Table 7.7: Optical density indices (OD) for ELISA tests to measure IgA activity against L_3SE for Group D lambs.

PARASITOLOGICAL PARAMETER	CORRELATION COEFFICIENT
Log of faecal worm egg count	-0.30 p=0.26
Log of *O. circumcincta* L_4 burden	0.09 p=0.72
Log of *O. circumcincta* L_5 burden	0.35 p=0.19
Log of *O. circumcincta* adult burden	0.19 p=0.49
Log of total *O. circumcincta* burden	0.16 p=0.56
Mean adult female worm length	0.41 p=0.12

The first figure is the strength of the association, the second the probability of this occurring by chance

Table 7.8: The associations between plasma IgA activity against L_3SE and parasitological parameters for Group N lambs.

PARASITOLOGICAL PARAMETER	CORRELATION COEFFICIENT
Log of faecal worm egg count	0.08 p=0.75
Log of *O. circumcincta* L_4 burden	0.24 p=0.38
Log of *O. circumcincta* L_5 burden	0.08 p=0.77
Log of *O. circumcincta* adult burden	0.36 p=0.17
Log of total *O. circumcincta* burden	0.29 p=0.28
Mean adult female worm length	0.15 p=0.58

The first figure is the strength of the association, the second the probability of this occurring by chance

Table 7.9: The associations between mucosal homogenate IgA activity against L_3SE and parasitological parameters for Group N lambs.

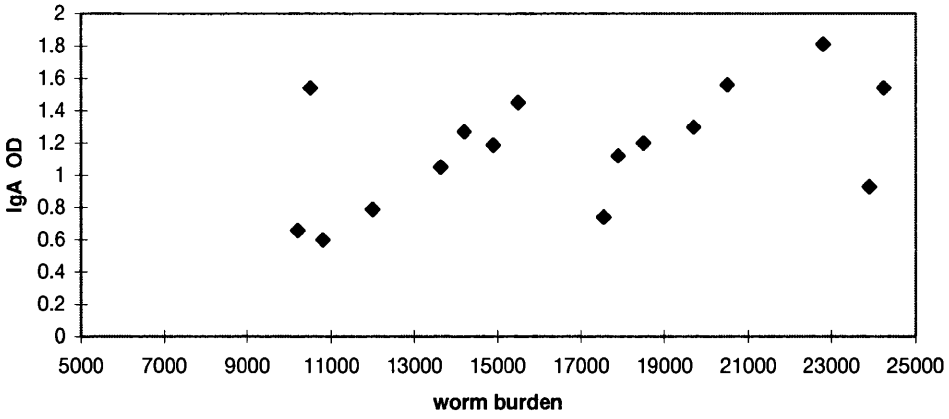


Figure 7.3: The relationship between worm burden and mucosal homogenate IgA activity against L_3SE for Group D lambs.

7.7: DISCUSSION

The work presented in this chapter shows that IgA activity to *O. circumcincta* antigen is greater in sheep exposed to infected pasture than those reared as helminth-naïve, and IgA activity increases with greater exposure to infected pasture. Following natural infection, a strong negative association between plasma IgA activity and worm length was found in lambs selected from the extremes of faecal worm egg count within the flock. Mucosal homogenate IgA activity against L_3SE was shown to be positively correlated with plasma IgA activity following natural infection but not following a truncated deliberate infection, however, following the deliberate infection mucosal homogenate IgA activity was much greater and positively associated with worm burden.

The work showing that both plasma and mucosal homogenate activity in sheep previously exposed to parasites by grazing is greater than those reared as helminth-naïve, and that the plasma activity increases during the grazing season, is evidence that the ELISA is detecting responses to specific parasite antigens. The assay does not appear to be measuring responses to cross-reacting environmental antigens, because the OD in the mucosal homogenate increases substantially following deliberate infection.

Following a deliberate infection of older sheep there is a strong negative association between parasite-specific IgA activity and worm length and that the specificity of antigen recognition is important in controlling worm length, whilst in younger sheep exposed to a natural infection worm length is under genetic control; therefore it has been assumed that the mechanism controlling worm length in lambs is the quantity and specificity of the IgA response (Stear *et al.*1995c; McCririe *et al.*, 1997; Stear *et al.*, 1997b). A strong negative association between plasma IgA activity and worm length was observed in sheep selected according to faecal worm egg count and this result supports the above hypothesis. However, no such association was observed for either plasma or mucosal homogenate IgA activity in sheep selected according to B cell percentages.

There may be technical reasons for this discrepancy because a different antigen, although prepared using the same protocol, was used for the two experiments. The same high pool sera and low pool sera were used in both experiments and both antigens reacted strongly with high pool sera, which was from animals with considerable exposure to *O. circumcincta*, and very weakly with low pool sera, which was from helminth-naïve sheep. The antigen preparation used in the earlier experiment may contained proteins for which activity against restricts worm length, whilst these proteins may have been missing from the second L_3SE preparation. In retrospect it could have been illuminating if gel electrophoresis preparations had been used to compare both.

One suggestion for the failure to observe a negative association may be that in the Group N sheep there were lambs that either had a good IgA response but failed to recognise the appropriate molecules necessary for restricting worm length or lambs that had a low quantity of IgA but a good specificity. There was a difference in selection criteria between the two experiments, extremes of faecal worm egg count selected a divergent worm length population for the earlier experiment, but extremes of B cell percentages selected a more homogenous population for the latter experiment making it less likely to observe any associations with worm length (the histograms in Figures 3.1 and 3.3 show the difference in the distribution of worm length). In the latter experiment the worm burden was greater, although not significantly ($p=0.06$), and because the number of worms present influences mean worm length through population effects (Stear *et al.*, 1996b), any effect of IgA may have been masked. Other explanations for the failure to observe an association may be the absence of a real effect or lack of sufficient statistical power.

In retrospect the choice of L_3SE antigen for examining a relationship between adult worm length and IgA activity was not the most appropriate. It is possible that an association might have been observed if adult somatic extract had been used, because if IgA does restrict worm length, then the target antigens may be proteins involved in feeding or metabolic processes specific to the adult.

Although no association was observed between IgA responses and worm length for Group N lambs, there were significant negative associations between two other immunological parameters, globule leucocyte density and abomasal node mass, and worm length, thus refuting the arguments that the population was not sufficiently diverse or too influenced by worm population to detect significant associations (neither parameter was significantly associated with worm burden). In Chapter 4 the negative association between node mass and worm length was discussed in detail and one suggestion was that sheep with a greater node mass produced a greater IgA response. However, since the strength of the association between the log of plasma IgA activity and log of node mass was only -0.11, and the strength of

the association between log of mucosal IgA activity and log of node mass was -0.20, therefore this data suggests that the effect of node mass on worm length is not mediated by IgA activity against L_3SE.

In Group N lambs the globule leucocyte count was negatively associated with worm length and this result was discussed in Chapter 6, where it was proposed that the association may be a confounding effect due to the association between IgA and globule leucocyte density, since IgA is the most likely candidate mechanism for controlling worm length. In the data presented here, there was no negative association between IgA responses and worm length, admittedly the most appropriate antigen was not used, but it raises the possibility that the relationship between globule leucocyte count and worm length may have been causal.

In the experiment on sheep selected according to faecal worm egg count, both globule leucocyte count and IgA responses were negatively associated with worm length. It remains possible that in this data set the relationship between globule leucocyte density was a confounding effect, but the strength of the association between globule leucocyte density and plasma IgA activity against L_3SE was only 0.03. One possible scenario regarding two variables which are both negatively associated with worm length, but are not associated with each other, is that both are acting independently to restrict worm length. However, in the data presented by Stear *et al.* (1995c) plasma IgA activity against L_3SE was significantly associated with plasma IgA activity against L_4 antigens, 0.53 ($p<0.01$), which was in turn significantly associated with globule leucocyte density, 0.43 ($p<0.05$), but the strength of the association between plasma IgA activity against L_3SE and globule leucocyte density was only 0.05. It remains possible that the mechanism responsible for restricting worm length is mucosal IgA activity against specific adult proteins and that the observation of negative associations between both plasma IgA activity against L_3SE and globule leucocyte density with worm length are simply confounding effects due to their association with IgA activity against adult antigens.

The results from the experiment on lambs selected according to extremes of faecal worm egg count support the hypothesis that IgA activity against parasite antigens restricts worm length but the data from Group N lambs does not support this hypothesis. Although it is not sufficient to reject the hypothesis it raises the possibility that globule leucocytes may also play a functional role in regulating worm length.

For Group N lambs the plasma IgA activity was positively correlated with mucosal IgA activity, and of a similar strength to that reported by Sinski *et al.* (1995) for activity against L_3SE in older lambs following a prolonged single challenge experiment. These observations are compatible with the view that plasma IgA is almost totally derived from the GI tract (Beh *et al.*, 1974; Quin *et al.*, 1975) but also show that for investigations of mucosal IgA the local source is more appropriate. For Group D lambs there was no significant association between plasma IgA and mucosal IgA. This apparent discrepancy may be due to plasma for IgA investigation being sampled three days after challenge whilst mucosal samples were taken ten days after challenge and it is likely that there was considerable excretory activity in the intervening seven days. Indeed Smith *et al.* (1984) showed that following a deliberate challenge of previously exposed sheep IgA in gastric lymph did not peak until day six or seven. Another possible explanation for the lack of association is that the Group D sheep had a high worm burden and Stear *et al.* (1995c) showed that the plasma activity was influenced by the interaction between mucosal activity and the worm burden.

Previous studies have suggested that mucosal IgA may play a role in regulating worm burden (Miller *et al.*, 1983; Smith *et al.*, 1987). In natural infections the worm burden is likely to be acquired by continuous low level infections; incoming larvae will continually stimulate local IgA production and therefore larvae would encounter an abomasal mucosa with a raised parasite-specific IgA concentration, and if IgA is protective then the IgA concentration should be negatively associated

with worm burden. No effect on worm burden due to IgA activity was observed in Group N lambs. This may be because IgA does not regulate worm burden or because individual variation in larval intake by grazing sheep was so great as to efface any IgA effect.

The sheep in Group D were given a large single challenge following a protracted period without exposure to parasite, and work by Smith *et al.* (1983b, 1984) showed that in a similar infection regime the IgA response occurred too slowly to be implicated in preventing larvae from establishing. The positive association between worm burden and mucosal IgA in Group D sheep was most likely a response to a greater concentration of antigen. The Group D sheep had greater mucosal IgA activity than Group N sheep, thus showing that there is a rapid response to challenge, and although all Group D sheep received an identical challenge, those in which the establishment was higher would have been exposed to a greater L_3 antigen load due to a larger proportion of larvae entering gastric glands. Also those with a greater worm burden will have been exposed to a greater level of L_4 antigens and it is likely that there is some sharing of antigens in the different larval stages.

More insight into the mechanisms of IgA control of parasites might have been gained if a different antigen had been used and future studies should use an adult antigen preparation because it is likely that worm length is controlled by restricting adult worm growth. Some studies have suggested that excretory/secretory products may be more appropriate antigens for investigating immune responses to nematodes but studies in *O. circumcincta* have shown that responses to somatic extract are very highly correlated with those to excretory/secretory products (Schallig *et al.*, 1994; Stear *et al.*, 1995c). If the antigens in adults on which local IgA mediates restriction on worm length could be identified then the strength of effective IgA responses could be more accurately determined. The results from the chapter described here and the chapter on globule leucocyte counts justify further research into the putative role of these cell types in restricting worm length. It is still not clear whether or not local IgA plays any role in restricting worm numbers, and the

best approach may be to measure local IgA responses prior to challenge - this would necessitate biopsy techniques.

CHAPTER 8: GENERAL DISCUSSION

Early studies on *O. circumcincta* defined the parasite and life cycle, with later studies investigating the epidemiology, pathophysiology and immunology of ovine ostertagiosis. The principal findings from these works are well described in text books and review articles (Michel, 1985; Holmes, 1985; Urquhart *et al.*,1987;McKellar, 1993; Stear *et al.,* 1997a). A standard approach to examining acquired responses of the host is to challenge parasite naïve and previously exposed sheep and compare differences. Studies using this approach contributed greatly to our understanding of ostertagiosis but were not able to differentiate between responses that were protective and those that were irrelevant or contributed to pathophysiology. These studies revealed large variation between individuals which at times made interpretation difficult (Smith *et al.*, 1983b; Smith *et al.*, 1987). Individual variation in host responses to nematode parasites has long been recognised. Many studies on rodent models have explored the Th1/Th2 paradigm and differentiated between protective and pathological responses, reviewed by Cox and Liew (1992), Finkelman *et al.* (1995) and Urban *et al.* (1996). Much of the research in sheep has been conducted with the intention of being able to exploit natural variation and consequently reduce dependence on anthelmintics (Woolaston and Baker, 1996; Bisset and Morris, 1996).

In order to distinguish between responses of exposure and responses of protection most experiments in this work were planned with the intention of having two groups of sheep with identical exposure but different levels of resistance. Because the sheep all had a common history it was possible to combine low and high egg count groups and use analysis of variance to investigate associations between immune parameters and parasitological parameters. This design allows a comparison of variation within a group, rather than a comparison of variation between groups. The within-group comparison is potentially more powerful and more likely to distinguish between protective and non-protective responses.

Selection according to faecal egg counts produced different results in different years. In the first year there was a strong trend towards low egg count sheep having fewer adult female worms whilst in the second year no such trend was seen but the low egg count group had shorter worms. Different results may be due to different selection criteria but may also be due to biological differences in different years. The aim of the project was to examine immune responses as they develop in sheep in a commercial production system, however there are many variables that can change from year to year leading to a different epidemiology that may affect the host-parasite relationship.

What was particularly noteworthy was that although selection by low faecal egg count resulted in selection of sheep with shorter worms, the association between worm length and faecal egg count weakened as the interval from anthelmintic dosing increased. This may have been because the faecal egg count in those sheep with short worms was lower because of two IgA mediated effects: the first effect being a reduction in adult female worm fecundity (Stear *et al.*, 1995c;Stear *et al.*, 1997a); and the second being delayed patency, and the effect of delay of patency would be expected to decrease as the interval between dosing and faecal sampling increases. It may be that a similar mechanism restricts worm length and retards development from L_4 to fecund adult, such as blocking feeding mechanisms or interfering with enzymes necessary for growth. Indeed, the plasma IgA work in Chapter 7 showed a negative association between plasma IgA and worm length in lambs selected on the basis of faecal egg counts, although the difference between groups was not significant. Although local IgA responses were investigated in later experiments, in which animals were selected according to B cell percentages, they were not available for lambs selected according to faecal egg count. It is recommended that future investigations should examine local IgA responses in more detail, concentrating on restriction of adult growth. Indeed, if the mechanisms of restricting adult worm growth were elucidated and the target proteins identified then future work on local responses may be very revealing.

Two other immunological parameters were also associated with reduced worm length - globule leucocyte density and abomasal node mass. A negative association between globule leucocyte density was observed in lambs selected on the basis of low faecal egg count and this association could be explained as being one of several mechanisms enhanced by increased Th2 responses. However in the lambs selected according to B cell percentages a similar negative association between globule leucocyte density and worm length was also recorded, but in this data set no association between IgA responses and worm length was observed. It would be wrong to use these results to reject the theory that it is local IgA responses which limit worm length (Stear *et al.*, 1995c; McCririe *et al.*, 1997) because the choice of antigen, being L_3SE rather than adult antigen, was not the most appropriate. However, it would be fair to suggest that, in addition to IgA responses, globule leucocyte activity may contribute to restriction of worm length and in different animals or infection patterns the relative effects may differ. Previous studies have shown that mediators released by globule leucocytes hamper larval migration (Douch *et al.*, 1986) and Douch and Morum (1993), and these studies have suggested a direct role for globule leucocytes in reducing worm fecundity.

The very strong negative association between abomasal node mass and mean worm length was not significantly associated with either IgA against L_3SE or globule leucocyte density. Abomasal node mass may reflect the degree of clonal expansion of lymphocytes recognising parasite antigens. Whilst much attention of late has focused on the quality of the host response in terms of Th1 and Th2 responses, the magnitude of the host response may also be an important factor. Animals with larger draining nodes would be expected to produce more helper lymphocytes and ultimately more effector cells than animals with smaller nodes. These animals would control worm growth more effectively.

Bishop *et al.* (1996) showed that worm burden in six-month-sheep exposed to natural infection with *O. circumcincta* is not under strong genetic control and this

observation is consistent with the findings in this thesis. The most important candidate mechanism for controlling worm burden involves the mast cell globule leucocyte axis (Seaton *et al.*, 1989; Stear *et al.*, 1995c), but no evidence was provided from this study to suggest that it regulated worm burden in the animals investigated. Furthermore results from the deliberate challenge infection in this study showed no evidence of acquisition of the ability to prevent larvae from establishing. Lambs selected for low faecal egg count had more mast cells than those selected for high faecal egg count but counts were not associated with worm burden. The observation that the mast cell response was well developed but apparently not efficacious at controlling worm burden was unexpected. The results are not sufficiently rigorous to disassociate globule leucocytes from control of worm burden as other plausible explanations are possible - such as worm burden was being controlled but the variation in uptake from natural exposure masked any effect of globule leucocytes, or that the globule leucocytes in these lambs were biochemically immature. However, the suggestion that the relationship between globule leucocyte or mast cell density and worm burden may merely be an association rather than causal merits further investigation.

The site of globule leucocyte activity may affect functional maturity due to local immune environment. It may be that in lambs the globule leucocytes within the abomasum are not effective, whilst those in the intestine are, because compared to the small intestine the abomasum has a much less well developed lymphoid anatomy and this may well have a functional cost resulting in poorer parasite control. Compared to the intestine, the abomasum could be considered as a hostile environment for any parasite due to low pH and simple mucosal architecture and few pathogens have the abomasum as their predilection site. *Ostertagia* species may parasitise the abomasum, despite the unfavourable conditions, because there are other advantages, such as less competition from other species and a less well developed immune response. Indeed, it is generally recognised that it takes longer to establish effective immunity to the nematodes of the abomasum, *Ostertagia* species and *H. contortus*, than those in the small intestine or respiratory system. It

is also possible that the adaptations made by *Ostertagia* species to live in the hostile abomasal environment result in greater resistance to mast cell mediators.

Immunophenotyping did reveal a negative association between the percentage of peripheral blood lymphocytes which were B cells and worm burden; this association was tested the following year by using B cell percentages as the selection criteria but the negative association with worm burden was not repeated. The initial association may have occurred by chance or there may have been a causal link which did not recur the following year because of a different pattern of exposure to parasite. Because there is no evidence that lambs of this age actively control worm burden, if the link was causal it may be that sheep with more worms have a lesser percentage of B cells because the worms down regulate B cells in an attempt at immune modulation (Cross and Klesius, 1989). The results comparing helminth-naïve lambs with those exposed to natural *O. circumcincta* infection by grazing indicated that exposure resulted in a B cell lymphocytosis. This could be further verified by comparing exposed and naïve lambs from identical environments. It is possible that there are two opposing forces, the host trying to mount a B cell response and the parasite trying to subvert one. There are a number of different approaches which could be used to investigate this hypothesis. One approach could be a variable dose infection in which there may be a threshold dose for B cell lymphocytosis, the magnitude of which may then decrease at higher levels of infection due to immune modulation. A second approach may be to look for significant expression of cytokines that could subvert the host response - IFN-γ has been shown to be produced by nematodes (Grencis and Entwistle, 1997). Experiments using exogenous cytokines or antibodies to cytokines to alter the response phenotype have done so by altering the cytokine early in the infection process (Urban *et al*, 1991b; Else *et al.*, 1994). Therefore, if immune modulations occurs, it may be that immature stages or early adults are responsible.

Immunophenotyping results from the deliberate challenge experiment showed that following a recent challenge the concentration of lymphocytes in peripheral blood

and the proportion of those lymphocytes which were CD4+ and CD8+ decreased, whilst the proportion of those which were γδ T cells increased. One explanation for these observations is that the challenge results in an inflammatory reaction within the abomasum, which led to less movement of lymphocytes from local tissue to peripheral blood and that CD4+ and CD8+ T cells are selectively retained - work by McClure (1992) suggests this reaction is immune-mediated. Sheep with a greater worm burden had a greater lymphocyte count than those with a lesser worm burden, which may have been due to those sheep that responded to the challenge infection by producing a greater local inflammatory reaction, with a consequently lower blood lymphocyte, managing to restrict larval establishment, or alternatively, it may be that those sheep with a greater worm burden had the local inflammatory reaction damped down by a greater parasite stimulus for immune modulation. Although the sheep with greater worm burdens had higher peripheral blood lymphocyte counts, a lower percentage of those lymphocytes were CD4+ and CD8+ cells. One explanation for this observation is that the retention of cells in the mucosa is more selective as parasite burden increases, resulting in a lymphocytic infiltrate of predominantly CD4+ and CD8+ cells. Some pilot work for this thesis (results not shown) used the T cell antibodies to stain cryostat sections of abomasal fundus with sufficient clarity to enable a quantitative count and further use of this technique could shed light on T cell changes within the mucosa. It would be interesting to compare T cell changes in animals that can control worm burden, such as animals over 12 months old, with those that cannot, and investigate whether associations with worm burden were due to efficacy of parasite control or immune modulation.

The results showing negative associations between worm burden and B cells in one experiment appear to contradict those showing a similar relationship between CD4+ and CD8+ T cells and worm burden in another, but the observations were made with different challenge regimes in different years. The results could be reconciled if they were due to γδ T cell percentages being positively associated with worm burden. One of the objectives of the thesis was to investigate whether or not variation in γδ T cell percentages were associated with variation in resistance, but

the work in this thesis provides no evidence that the γδ T cell percentages are associated with control of worm populations.

Results from the lymphocyte proliferation assay experiment showed that following the deliberate challenge those sheep with fewer worms had PBMC which produced a greater proliferative response to Con A than those with more worms. This may be explained by the observation that these sheep had a greater proportion of CD4+ cells and that Con A responses were strongly correlated to CD4+ percentages. The lymphocyte proliferation assays revealed another correlation between worm burden following deliberate challenge, this time the negative association between proliferative response of abomasal node cells to L_3SE and worm burden, and again immune modulation was suggested as the cause of this association.

Much of the interpretation of associations between immunological parameters and worm burden is based on the conclusion that active control was not occurring in these animals. That conclusion was drawn because Bishop *et al.* (1996) found no evidence of genetic control of worm burden and because the worm burdens following the deliberate challenge were of the same order of magnitude as those described in naïve sheep (Stevenson *et al.* 1994). The lambs observed by Bishop *et al.* (1996) were slaughtered at six months following a last anthelmintic dose at five months of age, and the conclusion must be that there was no evidence of control of worm burden acquired between five and six months of age. However, because there is evidence of control of worm burden at nine months of age (Stear *et al.*, 1995c), it is possible that control of worm burden may be occurring at seven months, which was the age of the sheep that were given the deliberate challenge. The high rate of larval establishment and the similarity of the worm burden results following challenge of naïve sheep (Stevenson *et al.*, 1994) suggests that worm burden was not regulated, but a control challenge of naïve age, breed and sex matched sheep would be needed to show this conclusively. If such a study did show evidence of reduced worm burden in previously exposed animals then it would necessitate re-interpretation some of the observations in this thesis.

The work in this thesis has produced many interesting observations but also raised many questions. Many associations were observed but more work is needed to show causal relationships. The approach of trying to examine responses in sheep following natural exposure has led to failure to replicate some findings and also difficulty in interpreting others because of the possibility of so many uncontrolled variables. However it also meant that the parasite-host interaction was studied in an environment as close as possible to that of the commercial sheep farm and although rigorously controlled deliberate challenge infections may yield very specific answers concerning those animals studied, those answers may not apply to the natural grazing animal.

In conclusion, this research has produced several interesting findings. It has shown that grazing sheep produce a B cell lymphocytosis, although further research is necessary to explore the findings as a marker or indicator trait of infection. An important finding of this research is that six-month-old sheep produce globule leucocytes in adequate numbers and of typical morphology, therefore the apparent inability of lambs to regulate worm numbers in the first grazing season is not a consequence of inadequate mast cell of globule leucocyte recruitment. While these results are not the final answer to the question of how lambs acquire resistance they do contribute to the development of this answer.

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