Larval paralysis factor of sheep

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AYE KYAWT SOE

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Dedicated to the memory of Mya

MASSEY UNIVERSITY

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Abstract

This study aimed to identify the origin and molecular nature of larval paralysis factor (LPF), an uncharacterised natural anthelmintic agent(s) known to be secreted by cells in the small intestine of nematode-parasite-immune sheep. The study first confirmed previous findings that gut mucus and small intestinal mucosal cell culture supernatants (CCSs) from parasite-immune sheep contain LPF. An *in vitro* larval migration inhibition (LMI) assay showed that LPF is active against numerous nematode parasite larvae of ruminants and adults of *Trichostrongylus colubriformis*. Lamina propria cells (LPCs) were a richer source of LPF than epithelial cells, and release of the factor was specifically triggered by parasite larval antigens.

A series of trials was performed to optimise LPF production *in vitro*. LPF production was highest when LPCs were selectively extracted from the first three metres of small intestine of resistant-line or hyperimmune parasitized sheep three days after oral challenge with *T. colubriformis* larvae. LPF release *in vitro* appeared to be related to the percentage of eosinophils present in cell cultures, but not with mucosal mast cells or globule leucocytes.

Subsequent *in vitro* experiments showed that mucus glycoproteins released from goblet cells enhanced and sustained the activity of LPF, which may point to a previously unrecognised effector link between goblet cell hyperplasia and mucosal immune responses to gut nematode parasites.

Sequential purification and molecular analysis of LPF showed that the active agent has a molecular mass less than 1kDa, it is polar and heat and enzyme resistant. Further purification using solid phase extraction and HPLC established that LPF did not bind either C18 or ion exchangers, but LPF activity in the flow through from these sorbents was retained on aminopropyl HPLC columns and eluted as a single active peak after flushing with acetonitrile/water mixture (70:30). This peak, analysed by LC-MS, comprised three main compounds of interest: a UV absorbing compound at 254nm with no MS data, and two non UV absorbing compounds at 254nm: one was a component of m/z 104 (ESI+ve) and the other was detected at m/z113 (ESI-ve).

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List of Abbreviations

AD	Arrested development
Ag	Antigen
ANOVA	Analysis of variance
*auto*MACS	Automated magnetic cell sorting
CCS	Cell culture supernatants
CD	Cluster of differentiation
COX_1	Cyclo-oxygenase 1
COX_2	Cyclo-oxygenase 2
CTMC	Connective tissue mast cells
DAD	Diode array detector
DMSO	Dimethylsulphoxide
EDTA	ethylenediaminetetraacetic acid
eEP/GO	enriched epithelial/goblet cell
EOF	Eosinophil factor
EP/GO	epithelial and goblet cell
EPA	Eosinophil potentiating activity
EPG	Eggs per gram
EPO	Eosinophil peroxidase
ES	Excretory secretory
ESI-MS	Electrospray ionisation-mass spectrometry
FcR	Fc receptor
FcRn	Fc-receptor of neonate
FCS	Foetal calf serum
FEC	Faecal egg count

FITC	Fluorescein Isothiocyanate
FMLP	N-formyl-methionyl-leucyl-phenylalanine
GI	Gastrointestinal
GL	Globule leucocyte
GLF	Globule leucocyte factor
GM-CSF	Granulocyte macrophage colony stimulation factor
GO	Goblet cell
HID	High iron diamine
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N' -2-ethane
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IEL	Intra-epithelial lymphocyte
IFNG	Interferon gamma gene
IFN-γ	Interferon gamma
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgG1	Immunoglobulin G1
IgG2	Immunoglobulin G2
IgM	Immunoglobulin M
Igs	Immunoglobulins
IL-1 – IL-13	Interleukin-1 - Interleukin - 13
L1	Larval stage 1
L3	Infective larval stage 3
L4	Larval stage 4

LCA	Leucocyte common antigen
LC-MS	Liquid chromatography- mass spectrometry
LMI	Larval migration inhibition
IFNG	interferon gamma gene
LP	Lamina propria
LPC	Lamina propria cell
LPA	Lymphocyte proliferation assay
LPF	Larval paralysis factor
LPL	Lamina propria lymphocyte
LR	Lactation rise
LT	Leukotriene
LTB_4	Leukotriene B4
LTC_4	Leukotriene C4
LTD_4	Leukotriene D4
LTE_4	Leukotriene E4
m/z	Mass/z (z = charge of the ion in the mass spectrometer)
MHC I	Major Histocompatibility Complex I
MHC II	Major Histocompatibility Complex II
MMC	Mucosal mast cell
MMCF	Mucosal mast cell factor
MMD	Monocyte-macrophage-dendritic cell
MMDF	Monocyte-macrophage-dendritic cell factor
MQ	Milli-Q
MS-MS	Tandem mass spectrometry
MWD	Molecular weight distribution
P1	Population 1

P2	Population 2
PAF	Platelet activating factor
PAMPs	Pathogen-associated-molecular-patterns
PAS	Periodic acid-Schiff
PBS	Phosphate buffered saline
PG	Prostaglandin
PGD2	Prostaglandin D2
PGE1	Prostaglandin E1
PGE2	Prostaglandin E2
PGF2	Prostaglandin F2
PGI2	Prostaglandin I2
Pk1	Peak 1
PPR	Periparturient rise
QTL	Quantitative trait locus
RE	Rapid expulsion
rIL	Recombinant interleukin
RMCP I	Rat mast cell proteinase I
RMCP II	Rat mast cell proteinase II
S.D.	Standard deviation
SAX	Strong anion exchange
SCF	Stem cell factor
SCX	Strong cation exchange
SMCP	Sheep mast cell proteinase
SPE	Solid phase extraction
SRS-A	Slow releasing substances of anaphylaxis
TcL3	*Trichostrongylus colubriformis* larval stage 3

TCR	T cell receptor
TGF-β	Transforming growth factor-β
Th1	T helper cell 1
Th2	T helper cell 2
TLC	Thin layer chromatography
TLR	Toll-like-receptor
TMB	3,3,5,5-Tetramethylbenzadine
TNF- α	Tumour necrosis factor-α
UV	Ultraviolet
v/v	Volume by volume
VIP	Vasoactive intestinal peptide

Location	Nematodes
Abomasum	*Haemonchus contortus* *
	Teladorsagia circumcincta; Ostertagia trifurcate*;
	Trichostrongylus axei *
Small intestine	*Cooperia curticei**
	*Nematodirus filicollis**
	*Nematodirus spathiger**
	*Trichostrongylus colubriformis**
	*Trichostrongylus vitrinus**
Large intestine	*Chabertia ovina*
	Oesophagostomum venulosum; Oesophagostomum columbianum;
	Trichuris ovis.

* Considered as major important species.

Table 1.1 Economically important Trichostrongylid nematodes and their location in the gastrointestinal tract of sheep (Vlassoff & McKenna, 1994).

Chapter 1
Literature review

This chapter reviews literature concerned with the immunity of sheep to gut nematodes which are of economic importance because this study is about a natural anthelmintic - larval paralysis factor - originating in the gut mucosa of sheep, and their release into the mucosa is associated with mucosal immune response to gut nematodes. Also the chapter concentrates principally on the *Trichostrongylus colubriformis* species which has received the most attention in published studies among the other parasites living in the small intestine.

1.1 Introduction

From a human perspective, sheep consume low value food and produce wool and meat of high value. Economically, they are concentrated in the temperate areas of the world where moist and warm conditions are most favourable for nematode larvae to survive and grow. In this 'wormy world' the majority of grazing sheep become infected with a variety of parasites during their lives. The impact of nematode parasitism is detrimental, occurring in most districts subject to intensive livestock farming. The impact can be affected by age, nutrition, sex and genetic factors of the host, and type, frequency and dose of infection. For their survival, sheep naturally develop an immunity to gut nematodes for regulating the worm burden in the gut. Besides, lambs seem to have an innate immunity which is important to initiate the development of an acquired immunity in the later part of their lives, in response to the multi-stage life cycle of nematodes in the gut. Naturally the immune mechanisms involved in the elimination of worms from the gut are very complex; but they are important especially for sheep living in temperate regions, such as New Zealand, where ingestion of infective larvae occurs more or less through out the year.

1.2 Species of economic significance and their life cycle

Domesticated farmed sheep are commonly infected with most of the economically important GI trichostrongylid nematode parasites listed in Table 1.1. Generally, the life cycles of the gut

nematodes are direct and similar. Fertilised eggs released by inseminated female nematodes in the GI tract are passed with faeces. Under optimal conditions, eggs hatch, and stage 1 larvae (L1) to stage 3 larvae (L3) develop in the environment. Infection is by ingestion of the L3 – infective stage.

Following ingestion of L3 larvae, the stomach worms are exsheathed in the rumen and the intestinal worms in the stomach (Sommerville, 1957). The larvae of different species inhabit different niches of the GI tract. *H. contortus* is usually closely allied to the mucosal surface of the abomasum (Sommerville, 1963), while *O. circumcincta* enters the gastric glands (Armour *et al.*, 1966), and *T. colubriformis* tunnels through the epithelium of the small intestine (Barker, 1973a). L4 develop about the 7th day; young adult L5 as early as the 12th day; and although eggs are usually present in faeces of infected sheep on the 21st day, (Armour *et al.*, 1966; Sommerville, 1957) they could be present as early as the 17th day after infection. According to the nature of the nematode habitat in the gut, the establishment of an excessive number of worms could easily alter the normal gut physiology into a pathological state in a short time after infection.

1.3 Pathogenesis of nematode infections

Nematodes damage the GI mucosa in various ways. *Haemonchus* and *Bunostomum* spp. suck blood and others browse on the mucosa. In haemonchosis, both the fourth larval stage and adults of *H. contortus* suck blood and leave haemorrhages in the abomasum, which cause blood loss of about 50 µl / worm / day (Clack, 1962). As a consequence, the affected sheep show low serum iron and low bone marrow reserves of iron, followed by a rapid drop of packed cell volume, and severe anaemia which may be fatal to susceptible animals (Dargie & Allonby, 1975).

The gut browsers, *T. colubriformis* and *T. vitrinus,* which live in the anterior part of the small intestine, irritate the mucosa surface by tunnelling between the epithelial cells and the basement membrane. Sheep with continuous exposure to infection have flattened mucosa, and extensive changes in epithelial cell morphology (Coop *et al.*, 1979; Coop *et al.*, 1985) . Varying degrees of epithelial erosion from shortening of villi to total villous atrophy were observed in lambs with a single heavy infection of *T. colubriformis* (Barker, 1973b; Jackson *et al.*, 1983). The actual cause of flat mucosa is still unknown. However, the worms' physical activity and host factors, especially in heavy infections, may cause sloughing of epithelial cells, leading to the formation of flat mucosae. Moreover, increased plasma loss into the gut lumen was also

observed after 10-12 days of heavy infection (85,000 larvae) in Merino lambs, which coincided with the onset of anorexia, hypoproteinaemia and weight loss (Barker, 1973b; Prichard *et al.*, 1974b).

With the other pathogenic nematode, *Tel. circumcincta,* infective larvae penetrate the gastric glands of the abomasum, and develop into adults. In sheep parasitized with a single heavy infection, the glands show replacement of normal gastric epithelial cells such as mucous cells, zymogenic cells (pepsinogen secreting) and parietal cells (acid secreting) with the stretched, dilated, flattened and non-functional undifferentiated cells (Armour *et al.*, 1966). The infected glands can malfunction, not producing an acidic proteolytic gastric juice, and in consequence there may be an increased pH of the abomasal contents, reduced pepsin formation, and heightened plasma pepsinogen level (Armour *et al.*, 1966; Mulligan, 1972; Wiggin & Gibbs, 1987). The regression of most lesions occurs, and normal gastric function resumes, soon after the loss of the adult worm population at 2-3 weeks (Angus *et al.*, 1979) but complete recovery is impossible if the infection is continuous and drench use is irregular.

The damage caused by any of these GI nematodes has a profound effect on metabolism and performance of growing lambs (Barker, 1973b; Coop *et al.*, 1986; Steel *et al.*, 1980; Sykes *et al.*, 1975) and reductions of weight gain and fleece production are quite common in animals with higher worm burdens (Gibson & Parfitt, 1972; Thompson *et al.*, 1982).

Generally the degree of pathology depends on worm burden (Steel *et al.*, 1980), nutritional status of animals (Gennari *et al.*, 1995) and genetic status of the host (Altaif & Dargie, 1978a).

1.4 Immunity to nematode infections

Host response to nematode infection can be expressed in three ways. These are *resistance* which is host ability to reduce incoming and established worm populations; *susceptibility* which is the reverse of resistance; and *resilience* which is host ability to withstand the effect of large worm burdens without losing productivity. Sheep can acquire immunity from natural or induced infection (Stewart, 1950b; Stoll, 1929) by one year of age. The investigations into how sheep develop immunity are extensive. Most experiments used worm-free lambs, and gave a single or trickle sensitisation infection with normal or irradiated larvae or allowed natural infection to occur. Stages of development of immunity were observed by daily faecal egg count and worm count at necropsy. These studies showed that the immunity was not absolute (Altaif & Dargie, 1978b); it could wane in stress related conditions such as weaning (Watson & Gill, 1991), low

nutrition (Whitlock *et al.*, 1943) and during the peri-parturient period (Gibbs & Barger, 1986). Development of immunity is affected by intrinsic and extrinsic factors.

1.4.1 Factors affecting host immunity

The development of host immunity to gastro-intestinal nematodes is influenced by several factors such as age (Manton *et al.*, 1962), hormones (Dobson, 1964; McMillen *et al.*, 1987), genetics (Douch, 1988), magnitude of infection and duration of exposure to infection (Windon *et al.*, 1984), nutrition (van Houtert & Sykes, 1996), and pregnancy and lactation (Coop *et al.*, 1990; Kahn *et al.*, 2003a).

1.4.1.1 Age

Age of the host is considered to be an important determinant for susceptibility to parasitic infection (Africa, 1931). Generally, lambs under 6 months of age are not responsive to nematode infections, either natural or experimental (Brunsdon, 1970; Douch & Morum, 1993) using normal larvae of *T. colubriformis* (Gibson & Parfitt, 1972) or *H. contortus* (Manton *et al.*, 1962), or *Tel. circumcincta* (Smith *et al.*, 1985b) and γ-irradiated larvae of *T. colubriformis* (Gregg *et al.*, 1978) or *H. contortus* (Smith & Angus, 1980; Urquhart *et al.*, 1966b). The same immunizing procedure produces a consistently high immunity in older sheep.

However, there are cases where lambs of 12-17 weeks of age have shown evidence of partial protection to induced *H. contortus* (Christie & Brambell, 1966; Emery *et al.*, 2000), *T. colubriformis* (Emery *et al.*, 1992a; McClure *et al.*, 1998) and *T. vitrinus* (Jackson *et al.*, 1983). Considerable protection (70-87%) was achieved when 3-5 month old lambs were given massive doses of infection followed by a drench (Christie & Brambell, 1966; Douch, 1989). The outcomes of various experiments on systemic antigenic stimulation of the foetus (Fahey & Morris, 1978; Silverstein *et al.*, 1964; Silverstein *et al.*, 1963) and postnatal lamb (Cameron, 1980; Cripps *et al.*, 1974; Duncan *et al.*, 1978; Emery *et al.*, 2000; Emery *et al.*, 1999; Husband & Lascelles, 1974) showed that immune responsiveness exists long before and until after birth. Despite being able to respond to antigens from an early age, the following researchers (Benitez Usher *et al.*, 1977; Duncan *et al.*, 1978; McClure *et al.*, 1998; Urquhart *et al.*, 1966a) have showed that the ability of lambs to combat GI nematode infections was very weak compared to the adults.

Several explanations have been proposed for the age phenomenon as follows:-

(1) *Immunologic immaturity*: From the view point of natural process of survival, the new born lamb's immune system is still in a stage of development which makes it more susceptible to infections than adult sheep (Hein, 1998). However, it is protected by the effective defence given by its mother's supply of antibodies mainly in colostrum and very little in milk (reviewed in Watson *et al.* 1994). It has been shown that colostrum-deprived lambs are likely to have serious complications in life (Campbell, 1974; Halliday, 1978). However, the maternal antibody has also been shown to interfere with the development of active immunity (Dineen *et al.*, 1978; Husband & Lascelles, 1975; Shubber *et al.*, 1984). Also the speed and number of effector cells (mucosal mast cells, eosinophils, lymphocytes and plasma cells) infiltrated into the lamina propria was much lower in vaccinated young lambs than vaccinated ewes (Salman & Duncan, 1985) and even in the worm-free stage, ewes had a much higher number of these cells than lambs (Salman & Duncan, 1984).

(2) *Stress factors*: Weaning can cause stress in all domestic animals due to change of diet (Blecha & Charley, 1990) and the breaking of the mother-offspring bond (Watson & Gill, 1991). It has been shown that there is an elevation of blood cortisol at weaning (Rhind *et al.*, 1998) and possibly this may cause immunosuppression (Collins & Suarez-Guemes, 1985; Gwazdauskas *et al.*, 1978; Shannon & Jones, 1974b) and lead to susceptibility to infections (Matthews *et al.*, 1979; Presson *et al.*, 1988).

(3) *Genetic factor*: It has been shown that this has a considerable effect on the development of protective immunity in lambs (Bisset *et al.*, 1996; Windon *et al.*, 1980).

(4) *Possible impairment in antigen-recognition or presentation process* may occur (Monsell *et al.*, 1984).

In order to make lambs under 6 months immune whether they are in the field or in the pen, it generally requires 12 weeks repeated dosing of low dose infection (Emery *et al.*, 2000; Emery *et al.*, 1999; Israf *et al.*, 1997) or a single massive dose of infection with a drug-abbreviation regime (Brambell, 1966; Douch, 1989; Douch *et al.*, 1995). However, in practice, the immunization regime, which requires repeated dosing is unrealistic, while a single, massive dose of immunization with drug-abbreviated regime (Brambell, 1966; Douch, 1989; Douch *et al.*, 1995) seemed to be effective and saves time, but the amount of native antigens required is massive. Still, the development of stronger immunity would be possible with a method which takes into account the above constraints (age, nutrition and genetic factors), selects potent immunomodulators such as an adjuvant (Watson *et al.*, 1994) and provides the best nutrition (Abbott *et al.*, 1985; van Houtert *et al.*, 1995; Wallace *et al.*, 1995).

1.4.1.2 Genetic factors

Many years ago, Stewart and colleagues were the first to demonstrate the responses of sheep, of different breeds, to GI nematode infection (Stewart *et al.*, 1937). Host genetic background influences the severity of GI nematode infection (Barger, 1989). However, little is known about the mechanisms which underlie such genetic resistance.

A variation in nematode resistance in sheep is widely recognised. The New Zealand Perendale breeds are more resistant than Romneys and Coopworths (McEwan *et al.*, 1994b; Watson *et al.*, 1992), and Texel cross Romneys are more resistant than pure Romneys (McEwan *et al.*, 1994a). Six-month old red Maasai lambs from Kenya had lower faecal egg count, higher numbers of blood eosinophils, higher PCV and serum protein than Dorper lambs in repeated infections with *H. contortus,* but the weight gain was not significantly different between the two groups (Mugambi *et al.*, 1996). St Croix lambs from the U.S shed fewer eggs than Dorset lambs in both pen and grazing trials with *H. contortus* infections, but the differences in immunological parameters were not significant (Gamble & Zajac, 1992). Similarly, in Glasgow, Dorset lambs were found to be more susceptible to re-infection with *H. contortus* than Scottish Blackface with haemoglobin A-type (Altaif & Dargie, 1978b).

Genetic variation within breeds allowed the selection of Romney lambs into "resistant" and "susceptible" groups using FEC and necropsy worm counts (Bisset *et al.*, 2001; Bisset *et al.*, 1992), and also segregation of lambs into "responders" and "non-responders" in response to immunization with irradiated *T. colubriformis* larvae and subsequent challenge with normal *T. colubriformis* larvae (Windon *et al.*, 1980). The resistant-line ewe lambs could also exhibit a lower peri-parturient rise in nematode egg count as adults, when compared with outbred or susceptible-line sheep (Woolaston, 1992).

In the search for reliable genetic markers for good breeding, the ovine lymphocyte antigen (SY1) appears to be the marker for responsiveness to vaccination against *T. colubriformis* in Merino lambs (Outteridge *et al.*, 1988) but it is not related to non-specific expulsion mechanisms for nematode parasites (Hohenhaus & Outteridge, 1995; Woodbury *et al.*, 1984). Studies in the field and indoor trials have indicated that FEC, anti-nematode IgA or IgG, mucosal mast cell and eosinophil hyperplasia were correlated with resistance (Bisset *et al.*, 1996; Buddle *et al.*, 1992; Gill, 1991). Therefore, the FEC, anti-nematode IgG and IgA (Gill *et al.*, 1993a; Stear *et al.*, 1997a), and eosinophil count (Stear *et al.*, 2002) have been considered as promising predictive markers for a nematode-resistant trait for breeding. Earlier information regarding quantitative trait locus (QTL) in Soay lambs and yearlings indicated that a parasite resistance gene was located in the first intron of the interferon gamma gene (IFNG) (Coltman *et*

al., 2001). However, the studies on QTL associated with natural nematode parasite infection in Scottish Blackface sheep indicated that QTL associated with specific IgA activity were identified on chromosomes 3 and 20, in region close to IFNG (chromosome 3) and MHC (chromosome 20); on chromosome 2, 3 and 14 associated with *Nematodirus* FEC and on chromosome 3 and 20 associated with non-*Nematodirus* Strongyl FEC (Davies *et al.*, 2006). Interestingly, a recent large study using the most extreme (resistant and susceptible) Romney lambs showed that a QTL associated with parasite resistance was identified in the telomeric end of chromosome 8, and a QTL for associated immune function traits (serum IgE and specific-anti-*T.* on chromosome 23 (Crawford *et al.*, 2006). This indicates parasite resistance is under genetic control, and the QTL-guided selection of nematode-resistant-line sheep may be an attractive alternative means to help control the world-wide drench-resistance problem.

1.4.1.3 Nutrition

Many years ago, Whitlock proposed that nutrition has a significant role in host immunity to nematodes. One of his trials revealed that sheep infected with *H. contortus* placed on a high plane of nutrition, reduced their worm burden significantly and recovered quickly (Whitlock *et al.*, 1943). The wide-spread appearance of drench-resistant nematode strains in ruminants (Besier & Love, 2003; Sargison *et al.*, 2005; Waller, 1997) has led to dietary protein supplements being considered as an alternative means to increase resistance and resilience of sheep to gut nematode infections (Knox *et al.*, 2003).

Many studies have shown a dietary protein enhanced immunity to GI nematode infection in lambs (Emery *et al.*, 1999; Israf *et al.*, 1996; Kahn *et al.*, 2000; Kambara & McFarlane, 1996; McClure *et al.*, 1999; Wallace *et al.*, 1995; Watson & Gill, 1991). Interestingly, a specific level of molybdenum (McClure *et al.*, 1999) also enhanced immunity to *T. colubriformis* infection in lambs; molybdenum seems to have a direct effect on the proteinase enzyme activity of the worms; enzyme secretion was inhibited when molybdate was added into the *Trichostrongylus vitrinus* culture (Knox & Jones, 1988), or in the retrieved worms from lambs given molybdenum (Suttle *et al.*, 1992) although the mechanistic effect of these supplements on the immune systems was not defined. Further research examining host diet influence on periparturient rise (PPR) in ewes suggested that PPR was more severe in low protein diet ewes (Houdijk *et al.*, 2006; Houdijk *et al.*, 2005; Huntley *et al.*, 2004).

In the face of nematode challenge, the host inflammatory response may cause damage to host tissues, resulting in excessive loss of proteins through damaged gut epithelium into the lumen (Barker, 1973b; Holmes & MacLean, 1971; Steel *et al.*, 1990). Even more proteins are needed

when the normal functions of the damaged tissues are repaired and restored in the 2-3 weeks following infection (Angus *et al.*, 1979). Hence, productivity is commonly compromised in highly nematode-resistant sheep (Bisset *et al.*, 2001; McEwan *et al.*, 1992).

Taken together, these findings support the view that dietary protein increases the resistance and resilience of sheep to nematode infections and keeps sheep healthy, especially in the critical periods (periparturient period, weaning and grazing on heavily nematode contaminated pasture) of their life.

1.4.1.4 Hormonal factors

The immune-endocrine connection in sheep is prominent during periparturition, and that creates a disadvantage for the sheep's immunity against nematode infection (Gibbs & Barger, 1986; Kahn *et al.*, 2003a). It is believed that these functional changes of the immune system are mediated by the release of stress hormones, especially adrenal corticosteroids (Prichard *et al.*, 1974a). Several cases of sex difference in host immunity to nematode infections have been reported in ruminants (Barger, 1994). There is a link between the immune, nervous and endocrine systems of the body, but as yet how they interact with each other is difficult to understand (Hohenhaus *et al.*, 1998).

1.4.1.4.1 Stress hormones

It is evident that adrenal hormones are released when sheep are under stressful conditions, such as suffereing from nematode infections (Prichard *et al.*, 1974b), weaning (Rhind *et al.*, 1998), periparturient period (Chamley *et al.*, 1973), extreme physiological conditions such as heat (Minton & Blecha, 1990), transport (Ali *et al.*, 2001; Ruiz de la Torre *et al.*, 2001), handling (Lester *et al.*, 1991), acute pain associated with dehorning and castration (Mellor & Murray, 1989; Molony *et al.*, 1997) and isolation (Cockram *et al.*, 1994). Evidence has shown that these hormones are destructive to the effector cells which are the key components of immunity to nematode infections in ruminants. They produce lymphocytolysis, eosinopenia, and inhibit antibody synthesis (Ambrose, 1970; Quinn, 1990; Schalm, 1965), antigen presentation (Snyder & Unanue, 1982) and even lymphocyte apoptosis (Brunetti *et al.*, 1995). It may be that they induce characteristic changes in populations of leucocytes and in cell-mediated immunological functions in sheep (Buddle *et al.*, 1992; Collins & Suarez-Guemes, 1985; Presson *et al.*, 1988; Shannon & Jones, 1974a). These hormones have been commonly used as an immunosuppressant to reduce host immunity against gut nematodes in sheep trials (Buddle *et al.*, 1992; Douch *et al.*, 1994; Matthews *et al.*, 1979; Prichard *et al.*, 1974a).

1.4.1.4.2 Sex hormones

Sex differences in response to parasites have been reported in vertebrates and this phenomenon is universal (Klein, 2004). The available reports from ovine studies on this topic have shown that ram lambs had markedly higher FEC than ewe lambs after vaccination and challenge with normal larvae *T. colubriformis* (Windon & Dineen, 1981), but Dawkin *et al.* observed no differences in FEC or eosinophil count between two sexes in line-lambs selected for high/low responsiveness to *T. colubriformis* infection, after vaccination and challenge with normal *T. colubriformis* larvae (Dawkins *et al.*, 1989). Eight month old sheep with *H. contortus* infection showed *self-cure*, and better immunity in ewes than rams (Luffau *et al.*, 1981) and a similar observation was made by Courtney that sex differences were significant only after puberty, when ewe lambs were more resistant to secondary infection with *H. contortus* than ram lambs (Courtney, 1983). Likewise, in lambs with *O. columbianum* infection, ewe lambs have lower FEC or more nodules than ram lambs or shorter worm length, but the gonadectomised lambs showed no differences (Dobson, 1964). Taken together, these data demonstrate that there is a sex difference in response to nematode infections. However, they raise the possibility that rams and ewes may differ in their response to a vaccine as well. However, whether sex differences in response to nematodes are mediated by endocrine-immuno interactions, has not been adequately explored and this represents an important area of future research.

1.4.1.5 Lactation and Pregnancy

The phenomenon termed the periparturient rise (PPR) or lactation rise (LR) denotes a transitory increase in faecal egg counts observed during late pregnancy and lactation of ewes. PPR is a well recognised phenomenon of ewes regardless of their nematode resistant genetic status (Kahn *et al.*, 2003b). The research data on the mechanisms of PPR is conflicting and still in question (Coop *et al.*, 1990; Fleming & Conrad, 1989; O' Sullivan & Donald, 1973). However, the available concepts are that hormonal changes during pregnancy and lactation somehow lead to suppression of the immune functions responsible for regulating worm burdens in the gut (Chamley *et al.*, 1973; McMillen *et al.*, 1987); and the other explanation is that during the periparturition period host nutrition is prioritised more for reproduction than immune functions, especially those associated with host protective acquired immunity (Coop & Kyriazakis, 1999). Nutrition supplementation has shown an immune-enhancement in periparturient ewes (Huntley *et al.*, 2004; Kahn *et al.*, 2003a) although it was ineffective in genetic-resistant line sheep. It is tempting to assume that PPR could be a typical innate adjustment by ewes to prioritise their resources at a critical moment for the survival of newborn lambs. Perhaps, in order to achieve this adjustment, the stress hormones are released to suppress the ewe's highly active immune system. In fact, as less activity uses less energy or resources, energy not used by the immune

system could be allocated to high priority functions, *i.e.* reproduction and maintenance of the ewe.

1.4.1.6 Host factor

1.4.1.6.1 Previous exposure to nematode infection

When sheep previously exposed with *Ostertagia* spp. or worm-free sheep were challenged, worm burden, rate of worm development and gut pathology of the former group were greatly reduced compared to the latter; and age of the host played no part in the differences (Elliott & Durham, 1976). Similarly, worm-free adult Merino ewes, which were previously vaccinated twice with 20,000 irradiated *T. colubriformis* L3 , showed a markedly lower FEC and necropsy worm burden than control sheep when they were exposed to contaminated pasture (Windon *et al.*, 1984).

1.4.1.7 Parasite factors

1.4.1.7.1 Infection dose

A worm population or antigenic threshold level is required to stimulate and maintain the host immune systems for the control of parasite population rather than complete removal of the population (Dineen, 1963). Studies with *T. colubriformis* and *H. contortus* have suggested that the threshold could be between 2,400-4,800 normal larvae (Barger *et al.*, 1985b; Dobson *et al.*, 1990a; Windon *et al.*, 1984). However, these estimates are not definitive and can vary according to age of animals, sensitization or challenge dose and period and species of nematode used.

In most cases, immunity developed in lambs given continuous infection with *T. colubriformis* and when worm population accumulated (Dobson *et al.*, 1990a; Dobson *et al.*, 1990c; Dobson *et al.*, 1990d). The initial expression of immunity is the inhibition of larval establishment after about 4 weeks of continuous infection with *T. colubriformis* (Seaton *et al.*, 1989b) or with *H. contortus*. The inhibition occurred earlier with a higher level of larval intake (Barger *et al.*, 1985b; Waller & Thomas, 1981) but the adult population persisted. Faecal egg count declined slowly over 6 to10 weeks (Barger *et al.*, 1985b; Dobson *et al.*, 1990c; Gibson & Parfitt, 1973). The developed immunity persisted for a very long time in the absence of worm burden (Gibson *et al.*, 1970) and even lasted for several months in highly immune sheep (Kimambo *et al.*, 1988), although the moderately immune sheep were susceptible again after 12 weeks.

1.4.1.7.2 Stage-specific antigens

Parasitic nematodes complete a major part of their life cycle in the host (L3, L4 and egg-producing adult), and thereby the host is naturally exposed to a variety of stage-specific-antigens (surface, somatic, excretory-secretory antigens and eggs) that are immunologically recognised (Charley-Poulain *et al.*, 1984; Emery *et al.*, 1991). Lambs previously exposed to only immature stages of *H. contortus* were able to demonstrate at least short-term resistance to re-infection (Christie *et al.*, 1964). Similarly, the humoral response to L3 and L3-L4 antigens was much higher than the response to adult and egg antigens in seven-month-old ewes with *H. contortus* infection (Charley-Poulain *et al.*, 1984). Experimental observations have shown that ewe lambs immunised with multiple normal infections of *T. colubriformis* L3 for 13 weeks, can reject L3 or adult challenge. However, when ewe lambs were immunised by specific stage of infection combined with drenching (4, 7 or 10-day infections with *T. colubriformis* L3) the 7 and 10-day groups rejected subsequent challenges, but the 4-day infection group did not. Ewe lambs given 5 x 7 day-infections were less effective against surgically transferred adult worm challenges (Emery *et al.*, 1992a). Furthermore, sheep immunised by surgical transfer of adult *T. colubriformis* prior to challenge infection with *T. colubriformis* L3 inoculated into the duodenum, did not reject a challenge until 7-10 days (Emery *et al.*, 1992b). Taken together, these findings demonstrate that stage-specific parasite antigens influence the worm rejection mechanisms of immune sheep in varying ways.

1.5 Expressions of immunity

Host immunity is expressed in several ways, *viz.* prevention of larval establishment, adult worm expulsion, arrested development of larvae, reduced worm fecundity, inhibition of worm growth, and reduced viability of free-living larvae.

1.5.1 Prevention of larval establishment

The most effective host responses to gut nematode infection in sheep is the ability of immunised sheep to expel incoming larvae within a day after oral (Elliot, 1981) and surgical challenge (Harrison *et al.*, 1999; Miller *et al.*, 1983a; Wagland *et al.*, 1996) with larvae, but in some cases it took several weeks (Gibson & Parfitt, 1972). In some cases it can be sustained without further sensitization for 42 days but it waned after 84 days or could be totally abrogated by corticosteroid treatment (Jackson *et al.*, 1988). It has been proposed that the expulsion process is initiated by specific antigens and the final expulsion of larvae is non-specific (Dineen *et al.*, 1977). It was suggested that local immediate hypersensitivity reactions were responsible for

rapid expulsion (RE) (Jones *et al.*, 1992; Miller, 1984). In fact, the development and sustainability of expulsion of incoming larvae may depend on several parameters such as dose of infection and length of exposure, timing of challenge, and breed and age of animals. This larval expulsion is non-lethal for worms (Elliot, 1981) and the most significant host immune response to incoming larvae in immune sheep.

1.5.2 Adult worm expulsion

A *self-cure* phenomenon is a classic example of host resistance to gut nematodes in ruminants and was the first evidence of immune expulsion of adult population of GI nematodes observed by Norman Stoll in sheep in 1929 (Stoll, 1929). In his experiment, Stoll used twin lambs and one twin was fed 45 infective larvae (stage 3 larvae) and the other was allowed to graze on the *H. contortus* infective pasture. He described *self-cure* as the sudden drop in FEC in the lambs following intake of infective larvae (Stoll, 1929). After 20 years, Stewart critically examined the *self-cure* reaction in penned and field sheep with *H. contortus* or *T. colubriformis* infection. A sudden drop in faecal egg count and increase in serum anti-nematode antibody were manifested after a dose of infective *H. contortus* larvae was superimposed upon the existing infection in sheep; although the *self-cure* occurred, sheep were not necessarily immune to that superimposed infection but the faecal egg count was suppressed until the serum antibody started to fall (Stewart, 1950a). It was demonstrated that *self-cure* was not site specific, because the abomasal induced *self-cure* can eliminate intestinal infection with *T. colubriformis* but the elimination of intestinal species did not result in loss of abomasal species (Stewart, 1950a). It was suggested that a downstream movement of larval antigenic materials from the abomasum to the small intestine triggered the host responses in the small intestine whereas the reverse was unlikely to occur (Stewart, 1955). Stewart's observation speculated that immunological reactions to *self-cure* were associated with local hypersensitivity (type I allergy) and increase in blood histamine level. Although anti-histamine drugs prevented the *self-cure* reaction there was no evidence that blood histamine was a direct cause of *self-cure* (Stewart, 1953). The mechanisms of adult worm expulsion remain poorly understood.

1.5.3 Arrested development of larvae

Arrested development (AD) or *hypobiosis* of larvae at the L4 stage is another phenomenon of host resistance to gut nematode parasites (Seaton *et al.*, 1989a; Seaton *et al.*, 1989b). However, it is different to the phenomenon that expels incoming L3 (Barger *et al.*, 1985b). It was found to be markedly slower in younger hosts (Dobson *et al.*, 1990b). It was suggested that in sheep infected with *Tel. circumcincta,* local IgA inhibited worm development. Initially, it was thought

that AD was induced only by host immune responses (Adams, 1983; Waller & Thomas, 1975). However, others have proposed that seasonal changes and nematode density (Eysker, 1997) or the genetic make-up of the worm (Frank *et al.*, 1986; Frank *et al.*, 1988) can also trigger the onset of AD. Perhaps, this phenomenon may be a parasite survival mechanism in immune hosts or in the external ecosystem during a period unsuitable for its further development (Soulsby, 1979).

1.5.4 Inhibition of worm growth

This manifestation of immunity appears as slow growth and development of nematodes. It was reported that the length of fourth stage larvae had a strong association with IgA responses in *Tel. circumcincta* infected sheep (Smith *et al.*, 1985a). Further research by Stear and colleagues has confirmed and extended this result. In their experiments, local IgA activity against L4 was associated with increased larval inhibition, and decreased adult worm length (Stear *et al.*, 1995; Strain *et al.*, 2002).

1.5.5 Reduction in fecundity of female worms

Reduction in fecundity (RF) has been reported in connection with immunity of sheep to *T. colubriformis* (Chiejina & Sewell, 1974; Dineen & Windon, 1980a; Gibson & Parfitt, 1972), *Tel. circumcincta* (Stear *et al.*, 1995), and *H. contortus* (Altaif & Dargie, 1978b), of cattle to *O. ostertagi* and of goats to *T. colubriformis* infections (Pomroy & Charleston, 1989). The anti-fecundity mechanism is different to that which expels incoming L3. No evidence was found to prove density-dependent RF in lambs given long exposure to infected pastures, similar dose of infection in the pen and challenge infection with 30, 000 *H. contortus* larvae (Coyne & Smith, 1992). Likewise, lambs given a single dose of 3000 infective larvae of *H. contortus* or 100 infective larvae per day for 30 consecutive days showed retardation of fourth larval stages but no evidence of RF of female worms (Dineen *et al.*, 1965). Lately, the finding of strong association between plasma anti-nematode IgA and anti-fecundity led researchers to speculate that the IgA response was the possible proximate mechanism of immunity to infection in lambs (Stear *et al.*, 1997b; Strain & Stear, 1999).

1.5.6 Reduced viability of free living stages

It is known that adult sheep are more immune to gut nematode parasites than young lambs (Gibson & Parfitt, 1972), and this immunity of adult sheep may have some influence on the development of nematode parasites outside the host (Jorgensen *et al.*, 1998). In the experiments of Jorgensen and co-workers, the results of *in vitro* larval development assay consistently

showed that nematode eggs from adult ewe faeces produced significantly fewer L3 than eggs excreted by lambs. The lower L3 developmental success was also demonstrated in sheep selected for resistance based on faecal egg counts. However, the possible immune mechanism behind these variations is unknown.

1.6 Nature of immunity

Host responses to gut nematodes in ruminants and laboratory animals at gut surfaces have been the subject of many reviews (Balic *et al.*, 2000b; Gasbarre *et al.*, 2001; Miller, 1984; Rothwell, 1989; Urban *et al.*, 1989; Wakelin, 1984). All of these focused mainly on the role of final part of acquired host immunity. In fact, host defence against pathogens is initiated by the innate immune system, which may provide early, rapid protection and then assist the induction and generation of an appropriate primary acquired immune response regulating the co-stimulatory activity of antigen-presenting cells and releasing effector cytokines (Medzhitov & Janeway, 1997a).

1.6.1 Innate immunity

Innate immunity is a natural host defence system mediated by non-specific immune factors, such as inflammatory cells and their secretions - vasoactive amines, proinflammatory cytokines, and chemokines. These elements likely play important roles in defence against incoming nematode larvae, however the nature of innate immunity to nematode infection is poorly understood (Wakelin *et al.*, 1993). Initially, the system has a crucial task to discriminate antigens on pathogens from self, commensals, and dietary products in the gut. Recent reports suggest that the innate system senses pathogens by recognizing pathogen-associated-molecular-patterns (PAMPs) via pattern recognition receptors, such as Toll-like receptors (TLRs) (Beutler, 2004; Medzhitov & Janeway, 1997b; Moncada *et al.*, 2003). To date, ten TLRs have been discovered in mammals (Beutler, 2004) including cattle and sheep (Menzies & Ingham, 2006). TLRs are expressed on antigen-presenting cells - dendritic cells, macrophages and B cells (Hornung *et al.*, 2002; Ochoa *et al.*, 2003), inflammatory effector cells - neutrophils, eosinophils and mast cells (Nagase *et al.*, 2003; Okumura *et al.*, 2003), and even in intestinal epithelial cells (Cario & Podolsky, 2000; Menzies & Ingham, 2006). TLRs are known to sense bacteria, viruses, fungi, and protozoa (Beutler, 2004), but their role in mucosal innate defence against nematode infection is poorly understood (Li *et al.*, 1998; Rosbottom *et al.*, 2002; Stadnyk & Kearsey, 1996).

When naïve or worm-free sheep were experimentally infected, in most cases, 60% of the primary infection (Barger & Le Jambre, 1988; Emery *et al.*, 1992a) was established. Of the remaining 40%, some might have been affected by the innate immune responses at the site-of-entry and then expelled or some might have attached and then die during their sojourn in the gut. When worm-free lambs were infected intra-duodenally with *T. colubriformis* larvae, histamine was detected in gut mucus within two hours, and the mucus showed anti-nematode activity *in vitro* (Harrison *et al.*, 1999). Histamine release into the tissues is a sign of acute inflammation and the initiation of innate immunity at the site of infection. Similar pictures of acute inflammation in naïve lambs were also reported by Barker (1973a & 1975c) and Salman & Duncan (1984).

GI nematode entry into their preferred gut niche is quite invasive and may cause extensive injury to the epithelial tissues (Barker, 1973a; Barker, 1975b; Salman & Duncan, 1984). Since capillary and nerve plexuses are closely associated with the base of the epithelium, injury could extend to these tissues and blood-bound inflammatory components such as platelets, complement and neurotransmitters such as vasoactive intestinal peptides (VIP) may be released from damaged capillaries into the surrounding tissues. Platelets release a variety of potent vasoactive mediators (histamines, thromboxane, serotonin, TGF-β, LTC_4 and platelet activating factor) that can immediately modulate chemotaxis, vasodilation and recruit inflammatory cells. Even if there is no physical damage to the mucosa, TLRs on the epithelial cells (Menzies & Ingham, 2006) could send signals for the induction of innate immune responses by secreting pro-inflammatory cytokines such as IL-6, IL-8, monocyte chemotactic protein-1, GM-CSF, and TNF-α (Jung *et al.*, 1995).

Histamine release into the gut mucus is probably the sign of acute inflammation and activation of blood and tissue-bound immune components. Histamine is the best-known chemical mediator in acute inflammation. Sources are polymorphs (Elsner *et al.*, 1999) and human platelets (Masini *et al.*, 1998; Vaczi *et al.*, 2001) although highest storage of histamine is in mast cells and basophils (Levy, 1974). Histamines can be released from activated platelets (Masini *et al.*, 1998) or from mast cells mediated by activating complement (Erdei & Pecht, 1996; Nilsson *et al.*, 1996) or by the neurotransmitters e.g. VIP (Stewart *et al.*, 1996). Perhaps, the damaged nerve plexus could release neurotransmitters such as VIP at the injury site. VIP has been found in the nerve endings of sheep small intestine (Bounjoua *et al.*, 1991) and they are capable of increasing mucosal mast cell sensitivity to parasite antigens in sheep *in vitro* (Stewart *et al.*, 1996).

Although, innate immune responses do not seem powerful enough to eliminate a nematode infection, the acute inflammation and its by-products can at least limit the worm burden to

reduce pathology in the gut and allow more antigen exposure to the lamina propria through the damaged gut wall. More importantly, it induces and generates the formation of potent antigen-specific T cell-dependent humoral and cellular effector responses (Gill *et al.*, 1993b; McClure *et al.*, 1995; McClure *et al.*, 1996) to eliminate the infection.

1.6.2 Acquired immunity

The acquired immune response has two broad categories: (1) antibody-mediated immunity; and (2) cellular-mediated immunity. However, it must be recognised that generally, a complete defence is mediated by both arms of the response.

1.6.2.1 Antibody-mediated immunity

The immunoglobulin (Ig) isotypes IgG_1, IgG_2, IgA, IgE and IgM occur in sheep body fluids (reviewed by Hein, 1998), and among these, complement-fixing IgG_1 predominates in serum and colostrum, and agglutinating IgA in intestinal secretion (Smith & Christie, 1978). Accumulating evidence has shown that anti-nematode antibody levels are elevated in the body fluids of immune sheep after challenge (Emery *et al.*, 1991; Kooyman *et al.*, 1997; Shaw *et al.*, 1998; Sinski *et al.*, 1995; Smith, 1977). Specific-antibody secreting cells have also been found in mucosa, lymph and lymph nodes of nematode infected sheep but not in control animals (Curtain & Anderson, 1971; Gill *et al.*, 1992a; Gill *et al.*, 1994; Pfeffer *et al.*, 1996).

1.6.2.1.1 Antibody transport within the host

IgA, IgM and IgG are transported through epithelial cells in a receptor-mediated process known as transcytosis; these three classes of Igs are secreted into the lumen to protect against pathogenic invasion and contribute to humoral immunity (Rojas & Apodaca, 2002). Human intestinal cell lines (Lachaux *et al.*, 1996; Tu & Perdue, 2006) and rodent intestines (Yu *et al.*, 2001) expressed the low affinity receptor for IgE (CD23), and these receptors mediate IgE transport via a bi-directional transcytosis pathway (Tu *et al.*, 2005). However, there is currently no evidence for CD23 expression on ruminant epithelial cells, and the CD23-mediated IgE transport mechanism may not occur in these species.

Transport of IgA or IgM is mediated by a polymeric Ig receptor (pIgR) into the lumen in humans, ruminants and rodents (Brandtzaeg, 1981; Mostov & Deitcher, 1986; Solari *et al.*, 1986; Tomasi & Bienenstock, 1968) and the PIgR mediated transport is uni-directional, occurring only in the basolateral to apical direction (Woodard *et al.*, 1984). In ruminants, passive transfer of immunity from mother to offspring via colostrum is of paramount

Type of sample	Ig Iso-type	Infection*	Antigen	Reference
Serum	IgG_1	T. c	L3-ES	Douch *et al.* (1994)
	IgE	H. c	T	Kooyman *et al.* (1997)
	IgE	T. c	NS	Shaw *et al.* (1999)
Plasma	IgA	H. c	L4	Strain *et al.* (2002)
Mucus	IgG_2, IgA	T. c	L3	McClure *et al.* (1992)
	IgA	Tel. c	L4	Stear *et al.* (1995)
	IgG_1, IgA	T. c	L3-ES	Harrison *et al.* (1999)
Lymph	IgA	H. c	T	Smith *et al.* (1983)
ACC	IgA	H. c	T	Gill *et al.* (1994)

*T. c – *T. colubriformis*; H. c – *H. contortus*; Tel. c – *Tel. circumcinta*; T = total antibody; NS = not stated; L3-ES = larval stage three excretory secretory antigens; L4 = larval stage four antigens; antigen = type of antigen to which antibody was directed; ACC = antibody containing cell.

Table 1.2 Responses of immunoglobulin isotypes in the body fluids and gut tissues positively correlated with nematode resistance in sheep.

importance to neonate survival, since the ruminant placental structure does not permit transfer of antibodies during pregnancy. The transfer of antibodies from maternal blood to colostrum in the mammary gland is active and highly selective in favour of the IgG_1 isotype (Richards & Marrack, 1963) rather than other antibody iso-types. However, the mechanism by which IgG is transported across the epithelial cells for mucosal defence is still unknown.

Nevertheless, the special MHC class I-related Fc-receptor of neonate – FcRn, (Simister & Mostov, 1989) is a good candidate. In rodents, FcRn expression is limited to neonatal life (Rodewald, 1976; Rodewald, 1980), where it transports IgG into the gut (Ghetie *et al.*, 1996; Martin *et al.*, 1997). Recently, it has become obvious that FcRn continues to be expressed in epithelial cells of adult tissues such as human intestine, liver and kidney, rodent liver and mammary gland (Ghetie & Ward, 2000), ovine mammary gland (Mayer *et al.*, 2002b), bovine lungs (Mayer *et al.*, 2004) and intestines (Kacskovics, I *pers. com.*). Based on the human and rodent *in vitro* experiments, FcRn differs significantly from the pIgR in that its ability to transport IgG is bidirectional (Dickinson *et al.*, 1999; McCarthy *et al.*, 2000). Significant changes observed, in subcellular localization of the receptor in the acinar and ductal epithelial cells of the mammary gland of the ewe at the time of parturition, have prompted the hypothesis that FcRn is involved in IgG_1 secretion in ruminant epithelial cells (Mayer *et al.*, 2002a; Mayer *et al.*, 2002b). Real-time RT-PCR studies showed that FcRn expression occurred not only in the mammary gland but also was highly abundant in the liver, kidney, and the gastro-intestinal tract (jejunum & ileum) of adult sheep (Dzidic *et al.*, 2004). This suggests that the level of FcRn in the adult tissues could be useful for understanding serum Ig levels and for the development of new strategies for passive immunization.

1.6.2.1.2 Role of antibody in nematode immunity

Several studies have shown that anti-nematode antibodies - (IgG1, IgA, IgE) were positively correlated (Table 1.2) with host immunity to nematode infection. Also, the IgA and IgG_1-antibody-secreting cells in the gut mucosa and lymph (Adams *et al.*, 1980; Cripps & Rothwell, 1978; Gill *et al.*, 1994; Smith *et al.*, 1981) and IgE^+ cells in the mucosa and in mucus, of immune sheep were significantly higher than in controls (Harrison *et al.*, 1999; Pfeffer *et al.*, 1996). This implies that the anti-nematode antibodies were produced in the gut mucosa, and may have participated directly or indirectly in immunity to gut nematode infections.

1.6.2.1.2.1 Immunoglobulin A

IgA is predominant in mucosal secretions and protects mucosal surfaces in several ways. It is capable of inhibiting viruses (Mazanec *et al.*, 1992), agglutinating bacterial surfaces (Williams

& Gibbons, 1972), neutralizing toxins (Williams & Gibbons, 1972) and removing excessive antigen challenge (Lycke *et al.*, 1987) but it has no precipitation and complement-fixation capabilities (Tizard, 2000). Little is known about the role of IgA in immunity to gut nematode infection, although the magnitude of IgA response was highly significantly associated with host immunity to gut nematodes in several experiments. For instance, local IgA concentrations rose 7 times and IgA-containing cells rose 9 times in sheep given continuous *O. circumcincta* infection in sheep than in controls whereas IgG and IgM did not change (Smith *et al.*, 1981). Local anti-nematode IgA rose during haemonchosis, and fell very rapidly after self-cure, and it was concluded that IgA concentration was a reflection of local antigens (Charley-Poulain *et al.*, 1984; Smith, 1977). In addition, studies showed that reduction of adult worm growth and fecundity in adult female worms in teladorsagiasis and haemonchosis were strongly associated with local anti-nematode IgA (Strain *et al.*, 2002; Strain & Stear, 2001).

Furthermore, IgA may be able to form a particular association with mucin to render bacteria mucophilic (Magnusson & Stjernstrom, 1982). It could be that anti-nematode IgA in the gut secretions has a similar capability. Recent observations that mucus anti-larval IgA and IgG, which were specifically directed against a 35-kDa L3-specific cuticular antigen, reduced larval challenge in naïve sheep (Harrison *et al.*, 2003a) support this possibility. On the face of it, anti-nematode IgA may be a possible mechanism (Fig.1.1) of trapping nematodes in immune sheep where high levels of anti-nematode IgA antibodies occur in the mucosa and in the lumen. This 'mucus trapping' was considered as a popular mechanism in the 1980's for rapid expulsion of nematode larvae in immune animals (Bell *et al.*, 1984; Miller *et al.*, 1981).

1.6.2.1.2.2 Immunoglobulin G

Most of the IgG_1 and all IgG_2 in luminal secretions originates from blood plasma (Cripps *et al.*, 1974) and both fix homologous complement (Feinstein & Hobart, 1969). It is well documented that the level of IgG_1 in mucus and the number of secreting cells in mucosa of nematode immune sheep increase significantly after challenge (Gill *et al.*, 1992a; Harrison *et al.*, 1999). It seems IgG responses also reflect local infection, as for isotype IgA. By knowing host immunoglobulin access to the inside of the nematodes (Murray & Smith, 1994) one can expect that these antibodies may harm (possibly by means of neutralisation or blocking) vital components of nematodes required for their survival and growth in the host. This notion was strengthened by the findings that immune serum from a *T. colubriformis* infected goat inhibited nematode feeding more than normal serum, but the heat-inactivated immune serum did not affect its suppressive activity, and the suppression of nematode feeding was associated with IgG_1 in the serum (Bottjer *et al.*, 1985).

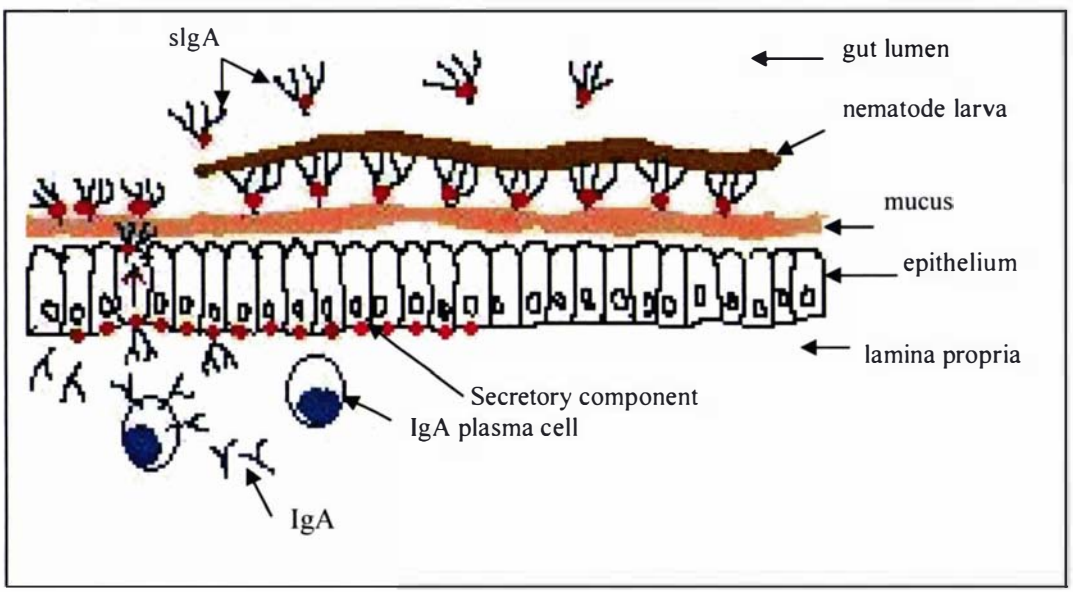

Fig. 1.1 Possible role of local IgA in gut nematode expulsion mechanism. The plasma cells residing within the lamina propria produce dimeric IgA and are transported through secretory component-expressing epithelial cells by endocytosis mechanism mediated by a polymeric Ig receptor (pIgR) into the lumen as secretory IgA (sIgA). The superficial mucus in the gut lumen is not only a mechanical protector for epithelium but also it may facilitate sIgA to trap microorganisms including large microbes like nematode larvae for preventing invasion and their establishment in the host in a process known as 'immune exclusion'.

Property	FcαRI	FcεRI	FcεRII	FcRn	pIgR
CD designation	CD89	NS	CD23	NS	NS
Cells (human)	DCs monocytes macrophages neutrophils kupffer cells	mast cell neutrophils activated eosinophils	B cells activated T cells eosinophils platelets follicular DCs thymic cells	bronchial epithelial mammary gland liver and intestine	intestinal respiratory urinary tract skin mammary gland
Affinity (high)		IgE		IgG	IgA
Affinity (low)	IgA		IgE		
Function		mediator release Anaphylaxis	enhance antibody response	IgG transport	IgA transport
Cells (ruminants)	NS	mast cells	NS	neonate duodenal crypt cells ewe mammary gland cells	intestinal liver lungs kidney mammary gland
Affinity (high)	**NS**	IgE		IgG	IgA
Affinity (low)			IgE		
Function		anaphylaxis recurrent urticaria mediator release	NS	IgG transport	IgA transport

Adapted from (Kacskovics, 2004); NS = not stated;

Table 1.4 Other Fc receptors

Property	FcγRI	FcγRIIA	FcγRIIB	FcγRIIIA	FcγRIIIB	Fcγ2R
CD designation	CD64	CD32	CD32	CD16	CD16	NS
Cells (human)	DCs monocytes macrophages neutrophils	macrophages neutrophils platelets Langherhan cells	B cells macrophages neutrophils eosinophils	NK cells macrophages neutrophils eosinophils	neutrophils	NS
Affinity (high)	IgG1 and IgG3					
Affinity (low)	IgG2, IgG4	IgG	IgG	IgG	IgG	NS
Function	phagocytosis ADCC	phagocytosis	inhibition	ADCC	NS	NS
Cells (ruminants)	IgG1 on monocytes macrophages IgG2 on Neutrophils Monocytes Macrophages		macrophages	NK cells polymorphs monocytes macrophages	NS	myeloid cells?
Affinity (high)	IgG1 & IgG2					IgG2
Affinity (low)		IgG1	NS	NS		
Function	phagocytosis	phagocytosis	NS	phagocytosis	NS	NS

Adapted from(Kacskovics, 2004) ADCC = antibody-dependent cell-mediated cytotoxicity ; NS = not stated

Table 1.3 Fcγ receptors

1.6.2.1.2.3 Immunoglobulin E

IgE is the immunoglobulin iso-type most commonly associated with immediate hypersensitivity and skin-sensitizing reactions, although other antibody isotypes like IgG_1 in mice and guinea pig, IgG_2 in rats and IgG_{1a} in sheep also mediate the reaction (Arkenase, 1977). However, a recent report in sheep demonstrated that only mAbs to IgE produced the skin hypersensitivity reaction within 1 minute of injection of mAbs, which was similar to histamine control but that mAbs to IgG_1, IgG_2 and IgM did not respond (Shaw *et al.*, 1996).

As with other Igs, it has been demonstrated that IgE binds to Fc receptors – strongly to FcεRI and weakly to FcεRII. In sheep, IgE was detected on gut mucosal mast cells, connective tissue mast cells, globule leucocytes and blood basophils from adult Romney sheep hyper-immunised with *T. colubriformis* L3 (Shaw *et al.*, 1996). The production of anti-nematode IgE against worm allergens in sheep is well documented (Huntley *et al.*, 1998a; Huntley *et al.*, 1998b; Kooyman *et al.*, 1997; Shaw *et al.*, 1998; Shaw *et al.*, 1999) and serum IgE levels were correlated to responsiveness (Shaw *et al.*, 1999). The presence of anti-nematode IgE in gut secretions (Harrison *et al.*, 1999) and IgE^+ cells in the small intestinal mucosa suggest local IgE synthesis occurs, though its concentration is much lower than other Ig iso-types.

The role of IgE in mucosal defence in ruminants is not clear, however it binds to mast cells, and upon interaction with specific nematode antigens is responsible for triggering the release of inflammatory mediators of immediate hypersensitivity. These mediators have a prominent anti-parasitic role in immunity to gut nematode infection (Bendixsen *et al.*, 1995; Douch *et al.*, 1996b; Murray *et al.*, 1971b; Rothwell *et al.*, 1971; Stewart, 1953)

1.6.2.1.3 Immunoglobulin receptors – FcRs

Interaction of antibody-antigen complexes with FcRs on haematopoietic cells triggers a wide variety of effector responses such as antibody-dependent cytotoxicity, mediator release, phagocytosis and antibody secretion (Ravetch & Kinet, 1991). As already outlined, FcRn mediates selective transport of IgG_1 from mother's circulation into the colostrum at the time of parturition (Mayer *et al.*, 2002a; Mayer *et al.*, 2002b). Other sheep cells such as macrophages have a FcγRI for IgG_1 (Yasmeen, 1981) and neutrophils have Fc receptor specific for IgG_2 (Watson, 1975). Fc receptors expressed on intact cells isolated from humans and ruminants are tabulated in Table 1.3 & 1.4.

1.6.2.2 Cell-mediated immunity

It is well documented that nematode infections trigger local and systemic inflammatory effector cell responses, especially in immune sheep (Buddle *et al.*, 1992; Douch *et al.*, 1986; Winter *et*

al., 1997). In response to nematode infection, gut tissues showed the presence of inflammatory mediators like histamine, prostaglandins, leukotrienes, thromboxane (Douch *et al.*, 1984; Jones & Emery, 1991), expression of pro-inflammatory cytokines IL-6 (Shen *et al.*, 2000), and variable expression of TNF-α and IL-1β (Paalangara *et al.*, 2003). Interestingly, IL-6 is the only cytokine which has a strong action on nerve tissues, especially on microglia (Galiano *et al.*, 2001), perhaps it is one of the important proteins that mediate neuro-immunological pathways during inflammatory responses to nematode infection.

These inflammatory mediators are well known, potent, chemo-attractants for inflammatory cells (Colditz, 1991; Colditz *et al.*, 1992) which accelerate histopathological changes (Rothwell, 1989) and promote the further influx of inflammatory cells - eosinophils, monocyte-macrophage-dendritic cells, globule leucocytes, lymphocytes (T and B cells) basophils and neutrophils - into the lamina propria. They are also important in the activation and proliferation of epithelial-goblet cells (Barker, 1975a; Charleston, 1965; Douch *et al.*, 1986; Salman & Duncan, 1984), which serve as an effector in the process of worm expulsion (Rothwell, 1989), independently or in concert with other cells.

There appears to be two types of response against nematode infection in sheep, viz. rapid and delayed type (Rothwell, 1989), which vary in speed of onset and are directed either against incoming larvae or existing adult populations. In immune sheep the rapid response can expel an entire larval challenge population in less than half a day (Harrison *et al.*, 1999). In some cases it takes 24-48 hours (McClure *et al.*, 1992; Miller *et al.*, 1983a), and is likely to be an acute allergic inflammatory response featuring infiltration of inflammatory cells and their mediators (Douch *et al.*, 1996b; Jones *et al.*, 1994). The delayed type of response usually occurs in 3-14 days after challenge (Emery *et al.*, 1992b; McClure *et al.*, 1992) and is also comprised of a high influx of inflammatory cells and their secretions (anti-nematode antibodies, mediators, cytokines and chemokines), together with additional T cell and B cell help (Gill *et al.*, 1993b; McClure *et al.*, 1992). These combine to deploy a more powerful and amplified response, to eliminate the population of worms which may have escaped the rapid expulsion process.

1.6.2.2.1 Mucosal mast cells

Mast cells and basophils were originally identified by Ehrlich in 1878, on the basis of their prominent meta-chromatic stained cytoplasmic granules. Both are bone marrow-derived cells; they have serine proteinase and histamine; they have high affinity Fc receptor to IgE molecules and through these molecules are involved in allergic reactions; they both produce arachidonic acid metabolites (Huntley, 1992; Kitamura *et al.*, 1993). Despite these similarities, there are

some differences between mast cells and basophils. Basophils are differentiated in bone marrow and after appropriate stimulus they migrate into the blood (Askenase, 1977), whereas the mast cell precursors are derived from bone marrow (Huntley *et al.*, 1992a; Oku *et al.*, 1984) and are differentiated in mucosa. Rat basophils live for 12 hours and mast cells for 40 days to 6months (Huntley, 1992). The basophil nucleus has 3-5 lobes and is more rectangular in shape whereas mast cells contain a single nucleus, and both the cell and its granules are bigger than in basophils (Rothwell *et al.*, 1994).

Many biologists have described mast cell heterogeneity in different tissues (Barrett & Metcalfe, 1984; Bienenstock, 1988; Enerback, 1981; Enerback, 1986; Jarrett, 1984; Kitamura, 1989) and their origin (Galli, 1990; Kitamura, 1989). Mast cells are classified into two basic types, viz. mucosal mast cell (MMC) and connective tissue mast cell (CTMC). The former are found abundantly in the lamina propria of gut mucosa. They can be identified by fixation and staining methods (Enerback, 1966a; Enerback, 1966b; Enerback, 1986). It was demonstrated that MMC staining with toluidine was blocked by 4% formaldehyde fixative, but this did not happen to CTMC. However, a lower concentration of 0.4% formaldehyde preserved the staining efficiency of MMC (Enerback, 1966b). In addition, MMC stain blue and CTMC stain red in Alcian blue/saffranin stain (Dimitriadou *et al.*, 1990).

IL3 was found to be critical for differentiation and maturation of bone marrow mast cells in sheep (McInnes *et al.*, 1993), mice and rats (Takao *et al.*, 2003). Lately, stem cell factor (SCF), the ligand for the membrane tyrosine kinase *c-kit*, was shown to be abundantly expressed on the mast cell surface (Galli *et al.*, 1994), and found to play an important role in the mast cell hyperplasia in normal rats (Newlands *et al.*, 1995). It was reported that antibodies to *c-kit* can abrogate the host response and deplete the intestinal mast cell population (Grencis *et al.*, 1993; Takao *et al.*, 2003). Furthermore, the mucosal mast cell response to nematode infection, in genetically resistant lambs, was defective when $CD4^+$ T cells were depleted after initial sensitization with *T. colubriformis* larvae (Gill *et al.*, 1993b).

1.6.2.2.1.1 Mucosal mast cells in nematode infections

Evidence from sheep and rodent studies provide strong evidence that acute allergic inflammatory responses are key for immune expulsion of gut nematodes (Douch *et al.*, 1983; Jones & Emery, 1991; Miller, 1984). In sheep, mucosal mast cell hyperplasia was positively correlated with immunity to *T. colubriformis* and *H. contortus* infections in both outbred and genetically-resistant line sheep (Douch *et al.*, 1986; Gill *et al.*, 1993b). Mast cell-derived inflammatory mediators such as histamine, serotonin, thromboxane, leukotrienes, and serum

mast cell proteinase (Douch *et al.*, 1984; Huntley *et al.*, 1992b; Jones & Emery, 1991), were detected in the blood and intestinal secretions of immune sheep. Their frequency and the magnitude of secretion of preformed and synthesized mediators correlated well with nematode resistance (Douch *et al.*, 1983; Jones & Emery, 1991; Jones *et al.*, 1994; Stevenson *et al.*, 1994). These mediators have been proposed to account for increased mucosal permeability associated with nematode infection in sheep (Steel *et al.*, 1990) and rats (McDermott *et al.*, 2003). However, immune sheep, with no infection for 42 days and with very low mucosal mast cell counts (similar to control) were able to inhibit larval establishment (Huntley *et al.*, 1992b). Despite much circumstantial evidence, a direct anti-nematode role for mucosal mast cells remains to be convincingly demonstrated (Douch *et al.*, 1983; Douch *et al.*, 1996b; Emery & McClure, 1995).

1.6.2.2.1.2 Mast cell inflammatory mediators

Mast cells are the source of an array of inflammatory mediators, which upon activation, via cross linking of the IgE receptors or IgE molecules bound on their surface, initiate a speedy biochemical cascade, normally happening within 1-20 minutes (Meeusen, 1999; Shaw *et al.*, 1996), and that subsequently results in the release of preformed mediators (histamine, serotonin, serine protease, lysosomal enzymes, TNF, eosinophils-chemotactic factor) and de novo arachidonic acid derived lipid mediators, (see section 1.6.2.2.1.4), nitric oxide, PAF and cytokines of allergic reactions (Crews *et al.*, 1981; Hogaboam *et al.*, 1992; McGivney *et al.*, 1981). There is evidence that activated mast cells can release pro-inflammatory cytokines – IL6, TNF-α and INF-γ release was reported from human and rat (Bradding, 1996; Bradding *et al.*, 1994; Gupta *et al.*, 1996).

1.6.2.2.1.3 Preformed mediators of mast cells

Histamine and serotonin

These preformed vasoactive amines of mast cells are present in the blood and gut secretions associated with nematode infection (Douch *et al.*, 1984). They have similar biological activities to the leukotrienes, including modulating vascular permeability, chemotaxis, epithelial secretion and smooth muscle contraction; in addition they can induce, via H1 receptor-pathway, lymphocyte and monocyte proliferation leading to the production of cytokines and antibodies (Douch *et al.*, 1984; Krouwels *et al.*, 1998; Shea-Donohue *et al.*, 2001; Togias, 2003; Wasserman, 1979). Both histamine and serotonin are neurotransmitters (Dickson, 1977), but a direct involvement in establishing neuro-immunological links during nematode infection has not been investigated. Tests of the direct affect of both substances on *T. colubriformis* L3 larvae *in*

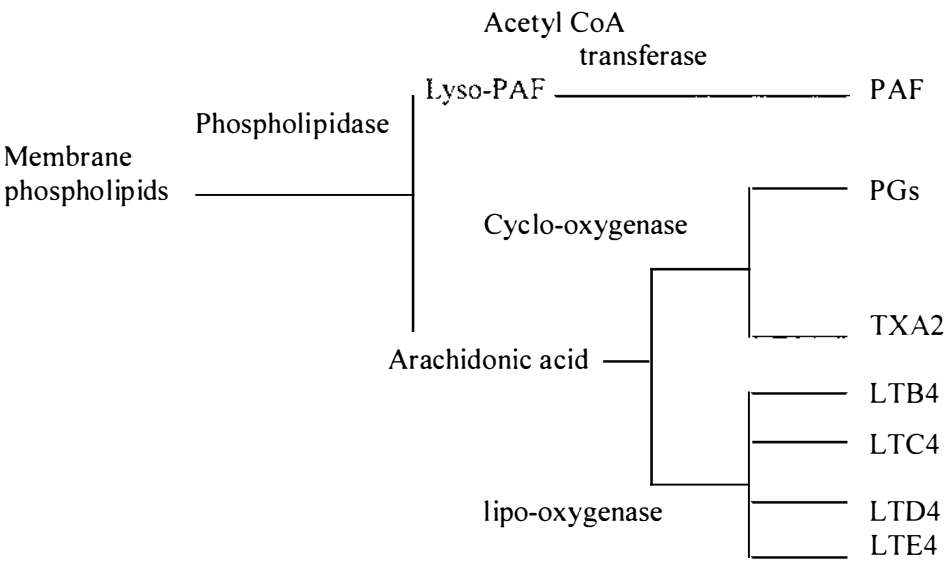

Fig. 1.2 Schematic diagram of the arachidonic acid cascade.

immunity to nematode infection is unclear. Data from rodent studies showed that the COX-blocker aspirin delayed expulsion of *Nippostrongylus brasiliensis* in rats (Kelly *et al.*, 1974), but indomethacin, another COX-blocker was found to accelerate mast cell sensitivity to antigens in *N. brasiliensis* infected rats (Befus *et al.*, 1982). In sheep, PGE1 and PGE2 had no inhibitory activity when tested against *T. colubriformis* L3 larvae in an *in vitro* assay (Douch *et al.*, 1983), neither did indomethacin treatment inhibit nematode rejection in immune sheep (Emery & McClure, 1995). Recent discoveries revealed two isoforms of COX, viz. COX-1 and COX-2. PGs which are synthesized under normal circumstances, and play crucial roles in modulating blood flow and mucus secretion, are derived via the isoform COX-1 pathway. On the other hand, the pro-inflammatory PGs are believed to be derived via the COX-2 pathway (Wallace & Chin, 1997). Interspecies variability in the synthesis and activity of PGs may account, at least partly for conflicting experimental results.

Leukotrienes (LTs)

LTs were described previously as slow-releasing substances of anaphylaxis. Like PGs, LTs are synthesized from arachidonic acid via 5-lipo-oxygynase enzyme pathway (Fig.1.2). There are two groups, viz. leukotrienes B_4 and peptido-leukotrienes (LTC_4, LTD_4, LTE_4). *Ascaris suum* antigen induced inflammation in lungs in sensitised sheep which suggested that leukotriene B4 (LTB4) is a potent chemotaxin for neutrophils, but that LTC4 and histamine contributed to bronchial contraction (Okayama *et al.*, 1989). However, data from human trials show that leukotriene B4 (LTB4) elicits several effects in inflammation, including polymorph recruitment, mucus secretion, and vascular permeability. LTB4 can up-regulate the expression of CD11 and CD18 on polymorphs; and elicit tissue damage by activating neutrophils to release reactive oxygen metabolites (Asako *et al.*, 1992; Ford-Hutchinson *et al.*, 1980; Hudson *et al.*, 1993). Larval paralysis activity was demonstrated in the LT-containing mucus (Douch *et al.*, 1983) and mast cell enriched culture supernatants (Douch *et al.*, 1996b) however the latter authors had no evidence that LTs or the other compounds in the samples elicited the activity (Douch *et al.*, 1996b). *In vivo* experiments showed that the LT-blocker piroxicam did not inhibit nematode expulsion ability of immune sheep when they were challenged (Emery & McClure, 1995).

Platelet activating factor (PAF)

PAF can be released from many inflammatory cells, such as mast cells, neutrophils, eosinophils, basophils, endothelial cells, macrophages and platelets (Barnes *et al.*, 1988). It is a particularly potent chemotactic agent for eosinophils (Coyle *et al.*, 1988) and causes degranulation in allergic patients (bHenocq & Vargaftig, 1986). In sheep, PAF was found to be a potent mediator, modulating vascular permeability within 10 minutes (Colditz, 1991). PAF was found with rodent mast cell proteinase II (RMCPII) in the gut contents of mice infected with

vitro were disappointing (Douch *et al.*, 1983). Antihistamine treatment in sheep (Emery & McClure, 1995) and rat (Bell *et al.*, 1982) did not downgrade the host immune response to nematode infection, although earlier work suggested that anaphylaxis was in part responsible for *self-cure* in sheep (Stewart, 1953 & 1955). However, histamine remains a useful biological marker for mast cell activity during gut nematode infection, especially in sheep (Douch *et al.*, 1984).

Sheep mast cell proteinase (SMCP)

This preformed neutral protease is a major component of secretory granules of mast cells and basophils. It is a chymotrypsin-like protease, termed sheep mast cell protease (SMCP) and has been purified from sheep mast cells (Huntley *et al.*, 1986). The rat has two types of protease, rat mast cell protease I (RMCP I) in CTMC and RMCP II in mucosal mast cells (Katunuma *et al.*, 1975). It provides another biological marker for mast cell hyperplasia in nematode infected animals, and has been widely used by a number of investigators to detect mast cell activity during infections with *H. contortus* and *Tel. circumcincta* (Huntley *et al.*, 1987) and *T. colubriformis* (Douch *et al.*, 1996a; Jones & Emery, 1991; Jones *et al.*, 1994) in sheep. In previously infected lambs, SMCP was detected in tissues and mucus 48 hours after challenge with abomasal worms, and it became inversely correlated with the worm burden on day of challenge (Stevenson *et al.*, 1994). The enzyme protease has been shown to be associated with intestinal permeability changes (King & Miller, 1984) and epithelial shedding (Miller *et al.*, 1983b) during anaphylaxis in rats.

In contrast, when Scudamore and colleagues injected larval antigens into the jejunal vasculature of primed rats, they did not see epithelial shedding during gut anaphylaxis, but observed a significant rapid increase of mast cell protease in the vascular perfusate which was not seen in naïve and immune rats challenged with an irrelevant antigen (Scudamore *et al.*, 1995).

1.6.2.2.1.4 Lipid mediators

Prostaglandins – DEGH & F

These are short-lived metabolites of membrane phospholipids, 20 carbon chain arachidonic acids, and are synthesized via cyclo-oxygenase (COX) enzyme pathway (Fig.1.2) by many cell types. PGE_1, PGE_2 and PGI_2 are generally considered to be anti-inflammatory and PGF_2 and PFD_2 are pro-inflammatory. They potentiate an increase in vascular permeability, epithelial secretion, peristalsis and smooth muscle contraction and also modulate mast cell histamine release (Moqbel & MacDonald, 1990; Shea-Donohue *et al.*, 2001). The function of PGs in host

Nippostrongylus brasiliensis (Hogaboam *et al.*, 1991; Moqbel *et al.*, 1989). Intra-mammary injection of PAF can induce tissue eosinophilia in parasitised sheep, although injection of parasite antigen itself attracts more cells than PAF (Topper *et al.*, 1992).

1.6.2.2.2 Eosinophils

Eosinophils are terminally differentiated and end stage bone marrow-derived polymorphonuclear leucocytes (Ogawa, 1993). In vitro studies showed their differentiation and maturation requires three cytokines – IL-5, IL-3 and GM-CSF (Clutterbuck *et al.*, 1989; Sonoda *et al.*, 1989). Murine rIL5 can initiate division and differentiation of eosinophil precursors in sheep (Stevenson & Jones, 1994), as can human rIL-5, which activates eosinophils much more potently and faster (15 minutes) than other stimulating factors like rIL-2, rIL-3 and rGM-CSF (Fujisawa *et al.*, 1990).

Like other bone marrow-derived inflammatory cells, eosinophils migrate from bone marrow via the circulation to tissues in response to chemotaxins - IL-8, PAF, IL-5, eotaxin, and RANTES-produced in the tissue and subsequently released into the circulation (Kameyoshi *et al.*, 1992; Palframan *et al.*, 1998; Seow *et al.*, 1994; Topper *et al.*, 1992). This migration is mediated by activated adhesion molecules (Moser *et al.*, 1992; Palframan *et al.*, 1998; Teixeira & Hellewell, 1998). The expression of integrins $\alpha_4\beta_1$ on sheep lung eosinophils was found to mediate ascaris antigen induced late bronchial reaction and persistence of hyper-responsiveness (Abraham *et al.*, 1994). In the mammary gland model of *T. colubriformis* infected sheep, in response to parasite antigen infusion, mammary gland eosinophils expressed CD11a and CD44, and the expression decreased at the late phase of the trickle infection (Stevenson *et al.*, 2001). A similar trend of CD11b and CD44 expression on mammary gland eosinophils was also observed on mammary eosinophils after mammary antigen challenge in *H. contortus* infected sheep (Bischof & Meeusen, 2002). In addition, sheep allergic to house dust mites showed an influx of eosinophils into bronchoalveolar lavage as early as 6 hours after antigen challenge, and this remained high for 48 hours, although the neutrophil infiltration was much more aggressive than that of the eosinophils (Bischof *et al.*, 2003). Likewise, nematode antigen can induce tissue eosinophilia in mammary glands at a rate 5-10 times greater than the platelet-activating-factor (PAF) in *H. contortus* infected sheep (Topper *et al.*, 1992). Nematode antigen has been demonstrated as a potent chemo-attractant for eosinophils (Dixon *et al.*, 2006; Wildblood *et al.*, 2005).

1.6.2.2.2.1 Eosinophils in nematode infections

Blood and tissue eosinophilia is a hallmark of helminth infection in many species, including humans (Butterworth & Richardson, 1985), although they are also present in non-parasitized animals (Huxtable & Rothwell, 1975) and in non-helminth related conditions such as allergic asthma (Gleich, 1986), myocytis eosinophilia (Trueb *et al.*, 1995; Trueb *et al.*, 1997) and acute inflammatory reaction to skin injection (Parish, 1972; Rothwell *et al.*, 1991). Blood eosinophilia was associated with the expression of resistance to *T. colubriformis* in both outbred and genetic line sheep (Buddle *et al.*, 1992; Gill *et al.*, 1993b; Rothwell *et al.*, 1993). An increase in blood eosinophils, tissue mast cell count, eosinophil potentiating activity (EPA) and SMCP level after secondary challenge coincided with adult worm rejection (Stevenson *et al.*, 1994; Winter *et al.*, 1997). In immune *H. contortus*-infected sheep, tissue eosinophilia peaked on day 3 after challenge and was sustained until killing on day 10, (Salman & Duncan, 1985). Even greater tissue eosinophilia (>100 times) was noted in challenged hyper-immune animals (Gorrell *et al.*, 1988a).

Douch *et al* (1986) observed a correlation between tissue eosinophilia and larval migration inhibition activity in mucus. Furthermore, breech soiling which is believed to be a sign of host hypersensitivity to larval challenge in the field was correlated with tissue eosinophilia but not with globule leucocyte and mucosal mast cell counts (Larsen *et al.*, 1994). Recent findings in gut mucosal (Bisset *et al.*, 1996; Buddle *et al.*, 1992; Dawkins *et al.*, 1989; Rothwell *et al.*, 1993) and mammary gland responses (Thamsborg *et al.*, 1999) to nematode infections indicated that systemic and local eosinophilia during nematode infection was under the genetic control of the host. However, there were cases where tissue eosinophilia did not correlate well with nematode resistance (Dineen & Windon, 1980b; Douch & Morum, 1993).

1.6.2.2.2.2 Anti-nematode roles of eosinophils

Many field and pen trials using line-bred and outbred immune sheep have shown that eosinophilia is characteristic of host immunity to nematodes. Based on rodent and ruminant studies, eosinophils have been regarded as anti-parasitic effector cells with the ability to kill helminths *in vitro* (Butterworth *et al.*, 1975; McLaren *et al.*, 1984). Eosinophils possess five types of helminthotoxic granules (Table 1.5). Despite these fascinating *in vitro* observations, a direct effector role for eosinophils in helminthiasis *in vivo* has been difficult to substantiate (Meeusen & Balic, 2000). However, recent *in vitro* and *in vivo* evidence (Meeusen & Balic, 2000; Rainbird *et al.*, 1998) of a direct anti-nematode effect of eosinophils on abomasal worms have re-validated their effector function in mucosal immunity of sheep. Rainbird *et al.* (1998) showed that IL5, which is an essential cytokine for eosinophil development and survival, also

Type of granule	Cell source	Molecular weight (Daltons)	Biochemical properties	Anti-microbial properties	References
Major basic protein (MBP)	Eosinophil*	9-13800	cation with sulfhydryl groups;	potent killer of schistosomula and *T. spiralis* larvae	(Gleich & Adolphson, 1986; Wasmoen *et al.*, 1988; Wassom & Gleich, 1979);
Eosinophil peroxidase (EPO)	same	70-75,000	cation, isoelectric point >11; glycosylated; provoke 5HT release from platelets	The combination of peroxide and halide makes the agent most potent among other granules; killer of schistosomula and *T. spiralis* larvae	(Carlson *et al.*, 1985; duduBass & Szejda, 1979; Jong *et al.*, 1981; Rohrbach *et al.*, 1990)
Eosinophil cationic protein (ECP)	same	16-22,000	cation; high affinity to zinc	more toxic than MBP and produces blebs on the schistosomula surface;	(Ackerman *et al.*, 1985; Ackerman *et al.*, 1983; Olsson *et al.*, 1977)
Eosinophil-derived neurotoxin (EDN)	same	18,400	less cationic than ECP	neurotoxic agent; cause paralysis in laboratory animals;	(Ackerman *et al.*, 1983; Durack *et al.*, 1981)
Charcot-leyden crystal protein	Both eosinophils and basophil	13,000	contain 1.2% carbohydrate; polypeptide with a single reactive sulphadryl group;	no record	(Ackerman *et al.*, 1980; Gleich *et al.*, 1976)

Table 1.5 Contents of eosinophil granules.

has the ability to promote the killing capacity of eosinophils. An influx of eosinophils, but not mucosal mast cells and globule leucocytes, in the gut tissues, was associated with diarrhoea in merino ewes (Larsen *et al.*, 1994). After challenge infection in immune sheep most $T19^+$ cells and eosinophils in gut lamina propria expressed IL5-mRNA (Bao *et al.*, 1996), and this suggested that paracrine and autocrine effector functions of T cells and eosinophils are operating in the mucosa during expulsion of nematode infection. IL5 is also particularly important for Peyer's patch-derived IgA-secreting plasma cell production in the mucosa (Beagley *et al.*, 1988).

1.6.2.2.3 B Lymphocytes

Considerable numbers of lymphocytes are normally present in the lamina propria of the small intestine and the number can be increased by the disease state of the gut, e.g. parasitic or bacterial infections. Comparatively, there are fewer B lymphocytes in lymph or blood than T lymphocytes (Mackay *et al.*, 1988).

The large number of B cells in lamina propria (LP) bearing surface Ig is of considerable interest. In immunised sheep, plasma cells appear in the lamina propria 4-5 days after nematode challenge (Gill *et al.*, 1994) and do not live long. In mice they live up to 4-5 days (Mattioli & Tomasi, 1973). Plasma cells were more abundant in nematode infected sheep than nematode-free sheep (Curtain *et al.*, 1971; Gill *et al.*, 1992a; Gill *et al.*, 1994). IgA is a major immunoglobulin in gut secretions and is derived from the IgA bearing B cells in the lamina propria but IgG1 and IgG2 are somewhat different and are derived from plasma (Cripps *et al.*, 1974). B cells function as effector cells during nematode infection in a number of ways, viz. by secreting antibodies which mediate damage in association with eosinophils (Rainbird *et al.*, 1998); assisting in eliminating nematodes from their preferred site (Harrison *et al.*, 2003a); secreting cytokines to activate other effector cells into the immune process leading to the elimination of the infection (Morimoto *et al.*, 2003; Pierrot *et al.*, 2001). Murine and human B cells are known to secrete pro-inflammatory cytokines - IL-1β and TNF-α, anti-inflammatory cytokines- IL-10, myeloid cell growth, differentiation and maturation factors-IL-3, IL-4, GM-CSF (O'Garra *et al.*, 1990) and IL-12 (Schultze *et al.*, 1999) upon receiving appropriate signals and stimulations through antigens and T cells. The effector role of antibodies in gut nematode immunity has been covered in Section 1.6.2.1.

1.6.2.2.4 T Lymphocytes

Thymus-derived lymphocytes (T-cells) are highly versatile and diverse cells of the immune

system. These cells develop from bone marrow progenitors, and mature through complex differentiation steps in the thymus to become the early T cells. Most peripheral T cells express CD4 and CD8, the majority use T cell receptor (TCR) αβ. $CD4^+$T cells are restricted to recognition of antigens via MHC II molecules on antigen presenting cells (macrophage-dendritic cells, B cells and others) and function as helper cells, whereas CD8+T cells recognise antigen in association with MHC I molecules and function as suppressor/cytotoxic cells (Crook, 1990; Swain, 1983).

Both $CD4^+$ and $CD8^+$ T cells, and MHCII positive lymphocytes occur constitutively in the mucosa of the gastrointestinal tract of sheep, and both $CD4^+$ and $CD8^+$ T cells expand variably within the lamina propria and epithelium in response to gut nematode infection in both immune and susceptible animals (Gorrell *et al.*, 1988a; Gorrell *et al.*, 1988b; Josefsen & Landsverk, 1996; McClure *et al.*, 1992). $CD4^+$ T cells in particular are more frequent in genetic-resistant-line sheep than either genetic-susceptible-line sheep or outbred immune sheep (Gorrell *et al.*, 1988b; Pernthaner *et al.*, 1995). Depletion experiments in adult sheep demonstrated that $CD4^+$ T cells are important for the generation of two effector arms, the recruitment of effector cells to the mucosa, and the production of specific antibodies in response to antigens (Gill *et al.*, 1992b; Gill *et al.*, 1993b; Howard *et al.*, 1989). $CD4^+$ T cells predominate in the T cell population of the blood and gut lamina propria. However, in the epithelium, intra-epithelial lymphocytes (IEL) are mostly $CD8^+$ (Gorrell *et al.*, 1988b; Lloyd *et al.*, 2000).

It has been discovered that $CD4^+$ T helper cells in humans and mice fall into two polarised T cell subsets - those that produce IL-2, INF-γ and TNF-β (Th1 cells) and those that produce IL-4, IL-5, IL-6, IL-10, and IL-13 (Th2 cells). Both cell types produce GM-CSF and IL-3. Th1 responses involving activated macrophages are more important to protect against intracellular pathogens while Th2 driven inflammatory responses (or the combination of Th1/Th2) predominate in anti-nematode and allergic inflammatory responses (Fiorentino *et al.*, 1989; Mosmann & Coffman, 1989; Punnonen *et al.*, 1993).

Compared to the rodent data (Else *et al.*, 1992; Grencis *et al.*, 1991; Ramaswamy *et al.*, 1994; Urban *et al.*, 1992), evidence for the Th_1/Th_2 paradigm has been slow to accumulate in ruminants. Continuous depletion of INF-γ and $CD8^+$T cells increased immunity against *T. colubriformis* in lambs (McClure *et al.*, 1995). Gut responses in immune sheep were associated with an increase in anti-nematode antibodies (*see:* Table 1.2) and the effector cells such as globule leucocytes (McClure *et al.*, 1992) mucosal mast cells (Douch *et al.*, 1986) and eosinophils (Buddle *et al.*, 1992; Dawkins *et al.*, 1989). IL-5 has increased immobilization of larvae *in vitro* with activated eosinophils (Rainbird *et al.*, 1998). *T. colubriformis* immunised

Mediators	Species	References
Histamine and Serotonin	Rabbit, guinea-pig; dog, human, rat, cat, ferret, goat	(Humphrey & Jaques, 1954; Masini *et al.*, 1998))
Thromboxane and lipoxin	Pig	(Stahl *et al.*, 1997)
LTC_4	Human	(Maclouf & Murphy, 1988)
Platelet activating factor (PAF)	Rabbit	(O'Neill *et al.*, 1991)
TGF-β	Human	(Iwasa *et al.*, 1993)
RANTES	Human	(Kameyoshi *et al.*, 1992)

Table 1.6 Inflammatory mediators released from the platelets of humans and animals

Receptors	Species	References
FcεRII	rat	(Joseph *et al.*, 1983)
FcγRIIA	human	(Thai le *et al.*, 2003)
Substance P	human	(Damonneville *et al.*, 1990)
INF-γ	human	(Pancre *et al.*, 1988)
PAF	human	(Valone, 1984)

Table 1.7 Surface receptors of platelets of human and animals

adult sheep have shown a five-fold increase in the number of eosinophils and IL-5 mRNA+ cells in lamina propria after 3 days of challenge (Bao *et al.*, 1996), and 10 times more IL-6 + cells were detected in the lamina propria of immune adult sheep than in controls (Shen *et al.*, 2000). Taken together, these findings suggest that protection in sheep against gut nematode infection is Th_2 biased and also the Th_1/Th_2 paradigm is operating in these circumstances in sheep. There is a smaller subset of T cells which are $CD4^-CD8^-$ TCR $\alpha\beta^-$ T cells, present especially in ruminants and termed as $T19^+$ (Mackay *et al.*, 1986). The majority of $T19^+$ are TCR $\gamma\delta^+$T cells, but not all the TCR $\gamma\delta^+$ T cells have T19 antigen, which usually appears late in $\gamma\delta^+$ T cell ontogeny. Thus the T19 antigen has been proposed as a 'maturation marker' for $\gamma\delta^+$ T cells (Mackay *et al.*, 1989; Mackay *et al.*, 1986). Most IEL are $CD8^+$ $\alpha\beta^+$ and 18% of them are $\gamma\delta^+$ T cells (Gyorffy *et al.*, 1992) . The function of $\gamma\delta^+$ T cells remains unknown.

However, it has been reported that $\gamma\delta^+$T cells occur more frequently in the gut mucosa than the lymph node in calves infected with *O. ostertagi* (Almeria *et al.*, 1997). Moreover, immunization and challenge infection in sheep increased the number of these cells 2-4 fold over that in controls in *T. colubriformis* infected sheep (McClure *et al.*, 1992; Shen *et al.*, 2000) and increased more in day 5 (McClure *et al.*, 1992). An important clue came from *in vivo* T cell depletion experiments of McClure *et al.* (1995), when the elimination of these cells enhanced natural immunity to nematode infection in sheep. Gorrell *et al.* (1988) suggested that $\gamma\delta^+$ T cells could be like murine $Lyt2^+$ IEL which lack Pan T antigens and are natural killer cells (Petit *et al.*, 1985; Schrader *et al.*, 1983). It is known that natural killer cells can produce IFN-γ upon appropriate stimulation; or in association with macrophages (Trinchieri *et al.*, 1984; Wherry *et al.*, 1991); and also 24% of $\gamma\delta^+$ T cells in the epithelium are found to be $CD8^+$ (Gyorffy *et al.*, 1992). Considered together, all these studies suggest that $\gamma\delta^+$ T cells modulate host protective responses. Perhaps, they take part in the 'damage control' process and tissue remodelling by sterilising the Th2 function - the inflammation which is known to be an important host protective mechanism (Larsh, 1975).

1.6.2.2.5 Platelets

Platelets are anucleate and 2 μm in diameter. Aside from their traditional haemostasis function, they play an inflammatory role and in acute phase reactions can release a variety of mediators (Table 1.6), and express adhesion molecules for fibronectin, laminin and collagen (George & Shattil, 1991; Parmentier *et al.*, 1990). Their classic activation is caused by adhesion after the rupture of endothelial cells and also via the activation of their membrane receptors (Table 1.7). Thromboxane is a major eicosanoid produced from activated platelets. Thromboxane was detected in the intestinal contents of immunised sheep in the first 6 days after challenge (Jones

& Emery, 1991). Interestingly, a cytotoxic effect of platelets on schistosomal and filarial parasites, possibly via IgE mediated mechanisms, has been demonstrated *in vitro* (Joseph *et al.*, 1983).

1.6.2.2.6 Intestinal Epithelium

The small intestinal tract is lined with a single layer of stem cell-derived columnar epithelial cells. Gut epithelium is quite heterogeneous, and features 4 epithelial cell types (goblet cells, absorptive cells, enterochromaffin cells and Paneth cells) and two other immunologically important non-epithelial immune cells, including the intra-epithelial-lymphocytes (IEL) and globule leucocytes.

1.6.2.2.6.1 Absorptive epithelial cells

Studies in humans and rats have shown that, aside from traditional roles in nutrient absorption, forming physical barriers (by secreting mucus), and aiding immunity (by producing defensin or specific sIgA), the epithelial cells act as 'early messenger' immune cells with intrinsic capabilities in host defence against luminal pathogen challenges (Podolsky, 1999). They can produce an array of messenger molecules (cytokines, prostaglandins) which can initiate inflammation for host protection and rapid epithelial restitution to reseal the protective physical barrier. Epithelial cells are the first cells to meet micro-organisms. For this, they should have a sensing system to discriminate pathogens as 'non-self' and to initiate host protective mechanisms.

In connection with this, it has been shown that freshly isolated ovine and bovine tracheal epithelial cells can synthesize a well known inflammatory mediator PGE2, similar to human and mouse intestinal epithelial cells (Holtzman, 1992). Human colonic epithelial cells are capable of producing pro-inflammatory cytokines, including IL-6, IL-8, IL-1β, monocyte chemotactic protein-1, GM-CSF, and TNF-α and epithelial neutrophil-activating peptide 78 in response to *Salmonella* infection (Jung *et al.*, 1995) and gut nematode parasites (Li *et al.*, 1998; Stadnyk & Kearsey, 1996). Furthermore, they can also express the receptors for IL-1β, IL-6, GM-CSF, and TNF-α (Panja *et al.*, 1998). In addition, stomach epithelial cells in parasitized sheep (Gorrell *et al.*, 1988a) and intestinal epithelial cells of human, guinea pig, rat and mice are known to express Major Histocompatibility Complex (MHC) II molecules (Bland, 1988; Scott *et al.*, 1980), and are capable of processing antigens when cultured with primed T cells (Bland & Whiting, 1989; Santos *et al.*, 1990), however, the intestinal epithelial cells of *T. colubriformis* infected sheep did not express these molecules (Gorrell *et al.*, 1988b; McClure *et al.*, 1992). Taken together, these findings suggest that epithelial cells are not only acting as traditional

mucus-secreting or IgA-bearing cells, but they are also immune effector cells designed to actively engage in mucosal defence network. In fact, the full role of epithelial cells in the mucosal innate defence is only beginning to be appreciated.

1.6.2.2.6.2 Paneth and enterochromaffin cells

Paneth cells migrate downwards from a stem cell precursor to the bottom of the crypt and are recognised by their distinctive zymosan granules. Human and animal Paneth cells produce antimicrobial peptides α-defensin (Ganz & Lehrer, 1994; Huttner *et al.*, 1998; Meyerholz *et al.*, 2004; Porter *et al.*, 1997). These peptides (also known as cryptdins) are also shown to induce Cl^- secretion from epithelial cells to facilitate washing out the pathogens from the intestinal lumen (Lencer *et al.*, 1997). Enterochromaffin cells are characterised by a wide basal region packed with dense granules and a very narrow apex, and produce the intestinal hormones secretin, cholecystokinin and serotonin (Ham & Cormack, 1979).

1.6.2.2.6.3 Goblet cells

Goblet cells are an exocrine type of intestinal epithelial cell specialising in compound exocytosis, and they contain high-molecular-weight mucus secretory granules. These granules are rich in glycoproteins, kallikrien protease, trefoil-type protein, vitamin-B_{12} binding protein and lactose-binding lectins (Phillips & Wilson, 1993). It is assumed that trefoil-type protein is protease-resistant and an important component of an intrinsic mechanism for defending mucosal integrity (Suemori *et al.*, 1991). These secretory granules fill the apical part of the cell like a balloon and push the nucleus along with the organelles such as mitochondria, endoplasmic reticulum and Golgi apparatus to the narrow basal part of the cell, and this makes the cell shaped like a goblet. Rapid release of mucus by the goblet cells can occur in response to different stimuli as follows:- (1) the secretogogues – VIP, prostaglandin E_1, carbachol and phorbol 12-myristate 13-acetate (PMA), cholera toxin, serotonin and neurotensin (Forstner *et al.*, 1981; McCool *et al.*, 1990; Moore *et al.*, 1996). (2) antigen-antibody complex (Carroll *et al.*, 1986). (3) pro-inflammatory cytokine IL-1β (Cohan *et al.*, 1991; Han *et al.*, 1987) and TNF-α (Arnold *et al.*, 1993). Studies in goblet cells from rat colon have shown that cholinergic stimulation evokes the discharge of these granules from the cells in the mid-crypt region within 5 minutes and the crypt was replenished with these cells in about 4 hours (Phillips & Wilson, 1993). In normal circumstances, both absorptive and goblet cells of the crypt-villus of the small intestine, migrate outward and it requires 3 days for their turn over (Karam, 1999).

Goblet cells in nematode infection and its anti-nematode role

Goblet cell hyperplasia accompanies many gut nematode infections in rodents and sheep (Miller, 1987; Newlands *et al.*, 1990). In rodents, changes in cell number and mucin chemistry were found associated with host immunity in *Nippostrongylus brasiliensis* infected rats and in *T. colubriformis* infected guinea pigs (Manjili *et al.*, 1998). It was suggested that cellular changes were immunologically mediated and T cell dependent, but that chemical changes in goblet cell mucin were associated with worms (Ishikawa *et al.*, 1994). However, somewhat different results occurred in mice where it was shown that CD4+ depletion reduced the quantity of mucus release, which interfered with worm expulsion in mice infected with *N. brasiliensis*, but that goblet cell hyperplasia and chemical changes in the mucin were not affected (Khan *et al.*, 1995).

Rodent studies revealed that pro-inflammatory cytokines like TNF-α and IL1-β, possibly produced by activated mast cells in the gut mucosa, were potent stimulators of mucus secretion (Arnold *et al.*, 1993; Cohan *et al.*, 1991), and so was the Th2-cytokine IL13, which was implicated in goblet cell hyperplasia and mucin production and secretion in immune rats with gut worms *Hymenolepis diminuta* (Webb *et al.*, 2007). The increased expression of this cytokine was found to be associated with resistance to *T. colubriformis* in sheep (Pernthaner *et al.*, 2005). Recent studies in rodents reported that manose-rich calcium-dependent galactose-binding lectins were expressed on *T. spiralis* infected small intestinal epithelium of rat (Pemberton et al, 2004b) and interestingly, the expression was higher in the genetically resistant strain than the susceptible strain. In a subsequent study, Paneth and goblet cells were detected as cellular sources of these intelectins (Pemberton *et al.*, 2004a) and mouse strains expressing these lectins rapidly expelled the worms whereas a strain without lectins, showed significant delay in expulsion (Pemberton et al. 2004a). These studies indicate the involvement of immune mediators in goblet cell hyperplasia and secretion, and also suggest an important effector role for goblet cells in immune responses to nematode infection.

1.6.2.2.6.4 Intra-epithelial lymphocytes (IELs)

IELs live in the paracellular space between two columnar cells of gut epithelium. Sheep, human and mouse IEL are reported to have distinctive cytoplasmic granules (Dobbins, 1986; Gyorffy *et al.*, 1992; Okayama *et al.*, 1989; Parrott *et al.*, 1983) and they were thought to be mast cells at one stage. In rodent studies, IEL exhibits cytotoxic activity in virus infected mice (Offit & Dudzik, 1989), and is shown to produce IFN-γ and IL-5 without cell proliferation (Taguchi *et al.*, 1991; Yamamoto *et al.*, 1993). IL5 is known to be an important cytokine for the Peyer's patch-derived B cells committed to becoming IgA secreting-plasma cells (Beagley *et al.*, 1988).

IELs seem to be important immunocytes which are exposed to a limitless variety of antigens derived from pathogens, commensals and ingested inert food ingredients where mucosal immunity, including effector T cells and antigen-specific-IgA, play critical roles for mucosal defence.

1.6.2.2.6.5 Globule leucocytes

Globule leucocytes, like IEL, are mostly found between columnar epithelial cells, but in sheep with heavy nematode challenge they may be present in the lamina propria as well. They are found in the mucosa of the intestinal, respiratory and urogenital tract of many species (Cantin & Veilleux, 1972; Douch *et al.*, 1986; Rahko, 1970; Tokashiki *et al.*, 1981). Their suggested origin has been granulated lymphocytes (Kent, 1966), plasma cells (Dobson, 1966), eosinophils (Casley-Smith, 1968), mesenchymal cells (Takeuchi *et al.*, 1969) and lately from degranulated mast cells (Huntley, 1992; Huntley *et al.*, 1984; Miller & Jarrett, 1971). They are easily characterised by their distinctive large globules stained pale eosinophilic with leishmann's stain in cytospot preparations (Huntley *et al.*, 1984). They contain sheep mast cell protease and have membrane-bound immunoglobulin E and G_1 (Douch *et al.*, 1981; Shaw *et al.*, 1996). So far, there are no reports of globule leucocytes being cultured. Several investigators reported that their numbers were directly proportional to anti-nematode activity of mucus (Douch *et al.*, 1986; Douch & Outteridge, 1989) and inversely proportional to worm burden (Dineen & Windon, 1980b; O' Sullivan & Donald, 1973) in sheep. Although they are abundant in parasitized sheep, their function is unknown (Douch *et al.*, 1986).

1.7 Summary of literature review

The foregoing points to a firm conviction that GI nematode parasitic infections of sheep remain a serious threat to intensive livestock farming in New Zealand and the rest of the world, while the emergence of multi-anthelmintic resistance in sheep and cattle nematodes has a great potential to damage the economy of the livestock farming industry. Consequently the suggestion has come forth that sustainable nematode control strategies less reliant upon anthelmintics should be used. The release of the first commercial lung worm vaccine in 1968 raised hopes that other helminth vaccines might be available soon, and by the 1990s, some progress had been made in the identification of promising vaccine antigens and in conducting protection trials; but still large scale production, selection of effective delivery systems, and induction of host immune memory responses remain major hurdles in nematode vaccine research.

Recently several options for nematode control in sheep have been offered even though the mechanisms underlying immunity to GI nematode infections are still not well defined. Significant progress has been made in breeding nematode-resistant sheep to lessen drench usage, and some genetic markers for resistance have been identified which help to improve breeding. Improved host nutrition has been put forward as an alternative means to increase resilience to nematode infection, especially at critical moments like periparturition and weaning, although it is not effective in genetically nematode resistant ewes. Nevertheless, despite the continuing search for lower drench usage for nematode control, the meat and wool industry world wide is still dependent on anthelmintics.

A better understanding of gut immune effector responses to nematode infection, particularly the origin, role and nature of larval paralysis factors in gut mucus, is important. Factors in mucus may be suitable for use as novel biological anthelmintics. Besides, the discovery of the origin and nature of the factor would enhance understanding of the host's protective immune response against nematode infection. However, information about the factor is very scarce, and it is obvious that more research is required to investigate its origin and biochemical identity.

1.8 Aim of the project

The project was to study the cellular origin of the larval paralysis factor and its biochemical identity, in a five step study. Firstly, the source of LPF was identified. Secondly, the optimal conditions required for maximal yields of the factor were ascertained. Thirdly, the cellular origin of the factor was defined. Fourthly, the role of gut mucus in LPF activity *in vitro* was examined. Finally, the biochemical identity of small intestinal LPF was examined.

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Chapter 2
General Methods and Materials

The methods for the studies are described in the section 2.1, and the materials which include reagents and recipes used in the studies are described in the section 2.2 on page 52.

All the animal experimentations in this Thesis were carried out with the approval of, and in accordance with requirements of the Wallaceville Animal Research Centre Animal Ethics Committee.

2.1 Methods

2.1.1 Immunization methods

2.1.1.1 Drug- abbreviated immunization

This method was used to immunise nematode-naive sheep raised in pens. It was similar to methods described by Stankiewicz *et al.* (1996), with modifications. Eight-month-old outbred-Romney sheep were infected with 30,000 *Trichostrongylus colubriformis* stage 3 larvae (TcL3) for two weeks and the infection was truncated by Nilverm™ (Levamisole HCl, Schering-Plough, Animal Health, Ltd) given orally with a dose rate of 8mg/kg. Animals were re-infected with a similar dose of TcL3 one week after drenching. This regime was repeated twice. The immune status of the animals was checked by re-infecting them with 10,000 TcL3 on three consecutive days two weeks after the last drenching, and then examining the FEC on day 21 and day 28 following the last re-infection.

2.1.1.2 "LPF regime" (Immunization method for producing larval paralysis factor)

This method was mostly used to immunise penned sheep for producing larval paralysis factor. In this method, sheep were brought into a pen and drenched; a week after drenching they were infected with 30,000 TcL3 three times a week for 3 weeks; and then challenged with 50,000 TcL3 two weeks after the last infection and killed 3 days after the challenge.

2.1.2 Preparation of *Trichostrongylus colubriformis* antigens

2.1.2.1 Larval preparation

TcL3 were kept at room temperature prior to ex-sheathing. Larvae were treated with sodium hypochlorite (*Clorax*: 5% sodium hypochlorite solution) at a concentration of 1% (v/v). The larval suspension was stirred with a magnetic bar for 20 min at room temperature. The ex-sheathing process was checked under a stereomicroscope prior to rinsing the larvae with tap water. Larvae were concentrated using a Nalgene concentrator having an 8 μm Sartorius cellulose acetate filter membrane. Cleaned larvae were retained on the filter membrane and retrieved from the membrane into a 20 μm nylon sieve by dropping phosphate buffered saline (PBS) or distilled water onto the membrane. Then the larvae were quickly passed through the sieve into a petri dish and in this way sheaths were removed from the larval suspension. This was an important step since the sheaths can interfere with the larval migration inhibition assay.

2.1.2.2 Preparation of Excretory secretory (ES) antigens of *T. colubriformis*

TcL3 were cultured from faeces of monospecifically infected Romney sheep. Larvae from the same batch were used for immunization, challenge infections and antigen preparation. Larvae were treated and exsheathed as described above. Exsheathed TcL3 were cultured in Hank's balanced salt solution (HBSS) supplemented with trehalose (1 g/litre) at a concentration of 100,000 larvae/2.5ml HBSS9 at 37°C for 48 hours. Antigen solution was collected, put through a 0.22 μm syringe-top filter (Sartorius, USA) and concentrated using a 'Stirred cell' (Amicon, USA) with a 3000 NMWL membrane. ES antigens on the membrane were collected and resuspended in HB9. The antigen solution was filtered through 0.22 μm syringe-top or bottle top filter (Sartorius, USA). Antigen concentration was determined by spectrophotometry at wave length 230/260 nm and the antigen aliquots were stored at -20°C.

2.1.2.3 Somatic antigens of TcL3

Ex-sheathed TcL3 in HBSS9 were frozen and thawed three times to disrupt the strong cuticle of larvae and then ground using a mortar and pestle. The resulting suspension was ultracentrifuged at 100,000 *g* for 1 h. Supernatants were filtered through a 0.22 μm syringe-top filter (Sartorius, USA), the antigen concentration was determined as above and aliquots were stored at -20°C.

2.1.2.4 Viable cell counting using Trypan Blue Exclusion technique

This method is based on the principle that live cells do not take up Trypan blue, whereas dead cells do. Ten micro-litres of cells were mixed with 90 µl Trypan blue solution (section: 2.2.2.14). The mixture was applied to a haemocytometer and left at room temperature for 3 minutes. The unstained (viable) and stained cells were counted separately in each of the four squares of the haemocytometer. The total viable cells in 1ml were calculated using the following formula:

Viable cell number ($x10^6$) = Total unstained cells in 4 squares X 0.025

2.1.3 Parasitological analysis

2.1.3.1 Faecal egg counts

The modified McMaster method[1] was used. Faeces (2 g) were added to 60 ml saturated NaCl in a 60 ml glass bottle. The contents were mixed by a motorised mixer for 45 seconds and the sample was quickly dispensed into a wetted McMaster egg counting chamber. All nematode eggs present in two chambers were counted. Since 2 g of faeces yielded 60 ml saturated NaCl suspension (1 g per 30 ml) and the volume of suspension examined was 0.3 ml (each square of counting chamber is 0.15 ml in volume) the eggs per gram (EPG) was calculated by multiplying the total number of eggs in the two chambers by 100.

2.1.3.2 Post mortem worm counts

The small intestine was stripped from the mesentery and cut into 1 metre pieces. Each gut piece was cut open over a bucket and the contents were caught. The gut mucosal surface was washed and rubbed thoroughly between two fingers to remove the contents under a jet of running tap water. A $^1/_{50}$ aliquot of contents was rinsed through 20 µm sieve and preserved in 10% phosphate buffered formalin until counted.

2.1.3.3 Cultivation of *T. colubriformis* adult worms *in vivo* and harvesting of the worms

A six-month old outbred Romney donor lamb was infected with 60,000 *T. colubriformis* L3, one week after the lamb had been drenched with 8mg/kg of Nilverm™. Faecal egg count was checked after 24 days to confirm infection. Then the sheep was killed by captive bolt and

[1] *In:* Technical Bulletin No. 18. Manual of Veterinary Parasitological Laboratory Techniques. Ministry of Agriculture, Fisheries and Food. Published by Her Majesty's Stationary Office.

exsanguination. The first 6 metres of small intestine was removed, and adult parasites recovered as described above (2.1.3.2) except that a 250 μm sieve was used. Adult worms were recovered manually under a stereomicroscope.

2.1.4 Lymphocyte transformation assay (LTA)

2.1.4.1 Isolation of peripheral blood lymphocytes

Jugular blood was collected into 15 ml heparinised tubes which were then centrifuged at 400g for 10 minutes at 20 °C and then at 1000g for 20 minutes. The buffy coat was collected into 2 ml of *cell rinsing medium* (*see* section: 2.2.2.8.) and layered gently over 3 ml Histopaque (Sigma-Aldrich, USA), in 15 ml centrifuge tubes. The tube was centrifuged at 750g for 30 minutes at 20°C. The mononuclear cell enriched population at the interface was collected and rinsed twice with the above media. The cell pellet was resuspended in *complete medium* and cell viability was determined by *Trypan blue exclusion*. The cells were adjusted to $2x10^6$/ml in complete medium.

2.1.4.2 Cell culture preparation

The above cell suspension (100 μl) was added in triplicate to wells of flat-bottomed 96-well plates (NUNC, USA). Either the TcL3 ES antigens (6 μg/ml in 100 μl) or Concanavalin A (Con A) at a final concentration of 5μg/ml in 100 μl as positive control or complete medium 100 μl alone as a negative control was added to triplicate wells. The plates were cultured for 52 hours at 37°C in a humidified 5% CO_2 incubator. Then, 0.5 μCi [methyl-^{3}H]thymidine (Amersham, The Netherlands) was added to each well and the cells were cultured for a further 18 hours.

2.1.4.3 Cell harvesting and Cell proliferation count

The above cells were harvested on glass fibre filters using an automated cell harvester (PhD, Cambridge Technology Inc). The unincorporated label was removed by washing and radioactivity was counted in a Beckman LS 6000C liquid scintillation counter. The results of LTA are expressed as stimulation indices (SI):

$$SI = \frac{\text{cpm (experiment)}}{\text{cpm (RPMI control)}}$$

2.1.5 Cytospot preparation and staining

2.1.5.1 Preparation of cytospots

Cells were suspended at a concentration of $1x10^6$ /ml in *cell rinsing medium.* Fifty microlitres of cell suspension and 200 ul of media was dispensed into a cytospin chamber and centrifuged at 500 *rpm* for 5 minutes in the *Cytospin 2* (Shandon, UK). Cytospots were air dried for 15 minutes.

2.1.5.2 Leishmann's staining and cell counting

Cytospots were stained with 1 ml Leishmann's stain for 6 minutes, when 2 ml *Leishmann's stain buffer* was added to the slide and staining continued for another 6 minutes. The slide was rinsed with a stream of buffer then left in buffer for 2 more minutes. Then the slide was air dried for 30minutes, cleared with xylol for 5 minutes, mounted with DPX mounting media, coverslipped and air dried overnight. Cells were examined under a Nikon CFI-60 Eclipse for differential cell type counts and for photomicrographic work, an Olympus BX50 was used. A total of 100 cells was counted as 20 cells/site.

2.1.6 Gut mucus preparation

Sheep were starved for 24 hours prior to slaughter. A 50 cm segment of small intestine was removed posterior to the bile duct entry immediately after killing. The removed segment was tied at one end, warm PBS (25 ml/metre) was put through, the other end tied and taken quickly to the lab. The segment was gently massaged three times, one end of the gut was opened and lumen contents were collected into a 15 ml centrifuge tube. In addition, the segment was pulled between two fingers to squeeze out all contents. The tube was vortexed for 3 minutes and then centrifuged at 4000G at 4°C for 15 minutes. The supernatants were collected and kept at –20°C.

2.1.7 Cell isolation and culture

2.1.7.1 Gut mucosal cell isolation

The cell isolation method was adapted from Stankiewicz *et al.* (1994). A 50 cm segment of small intestine was removed posterior to the bile duct entry immediately after killing. Both ends of a removed piece of gut were tied then placed in warm PBS and taken quickly to the lab. One end of the gut was opened and the lumen contents were removed, the mucosal surface was then everted and the ends tied again to protect against serosal cell contamination. The mucosal surface was rinsed 3 times with warm PBS, then incubated with 1mM Dithiothreitol (Sigma-

Aldrich, USA) in PBS for 20 minutes at 37°C and rinsed two times with PBS. Gut pieces were digested in complete medium (RPMI medium was supplemented with 10% FCS, 100 U/ml collagenase- Type I (Gibco, USA), 50 U/ml hyaluronidase (Sigma-Aldrich, USA), and DNase 20 U/ml (Boehringer Mannheim, Germany) at 37°C for 3 hours. The cell suspension was put through glass wool to remove cell clumps. Cells were washed twice with cell rinsing medium (Ca+Mg+free-Hank's balanced salt solution with 5% FCS and 2mM EDTA), once with HBSS9 (see section: 2.2.2.4.) and then resuspended in the latter medium.

The following cell isolation methods were adapted from previous investigators (Davies & Parrott, 1981; Nagi & Babiuk, 1987; Stankiewicz *et al.*, 1994).

2.1.7.1.1 Epithelial cell isolation

Two one-metre-long pieces of sheep small intestine were removed posterior to the bile duct entry immediately after slaughter. Both ends of the gut pieces were tied then placed in warm PBS and taken quickly to the lab. One end of the gut was opened and the lumen contents were removed and the mucosal surface was then everted. The mucosal surface was rinsed 3 times with warm PBS, then incubated with 1mM Dithiothreitol (Sigma-Aldrich, USA) in PBS for 20 minutes at 37°C and rinsed two times with PBS. Then the gut pieces were incubated in *epithelial cell dissociation medium* (Section 2.2.2.10) for 6 periods of 30 minutes at 37°C with fresh medium added each time. Cells from each successive batch were rinsed twice with cell rinsing medium. For counting and cytospot preparation, cells were resuspended in cell rinsing medium but for the cell culture, cells were rinsed, once with HBSS9 (see section 2.2.2.4.) and then resuspended in HBSS9 medium. The entire treatment took not more than 3 hours.

2.1.7.1.2 Lamina propria cell isolation

After EDTA treatment for 3 hours, gut pieces were rinsed for 3x with warm PBS and then treated with gut digest medium for 5 hours. Cells collected in the first hour were discarded to reduce epithelial cell contamination. Cells were then collected hourly, rinsed and resuspended in complete medium (RPMI with L-glutamine (Gibco, USA), N-2-hydroxyethylpiperazine-N' -2-ethane (HEPES) buffer 20 mM, $NaHCO_3$ 24mM, 2-mercaproethanol 50mM; penicillin 100 iu /ml, streptomycin 100 µg/ml, kanamycin 100 µg/ml, pH 7.2) and kept in a fridge. Cells were collected after five hours of digestion for culture. They were rinsed twice with *cell rinsing medium* (section: 2.2.2.8) and resuspended in a complete medium and kept in a fridge. Cytospots were prepared from cells isolated at each hour and stained with Leishmann's stain. The staining method is described in sections 2.1.5.1 & 2.1.5.2. Cell cultures were prepared as described in section 2.1.7.1.3.

2.1.7.1.3 Cell culture preparation

A 250 µl ($20x10^6$/ml) of epithelial cells or lamina propria cells or cells of each enriched cell type was first incubated in a CO_2 incubator at 37°C in a 24 well collagen-coated plate (as described below) for 15 min to equilibrate, 250 µl of HBSS9 with or without TcL3ES antigens (12µgm/million cells) was then added and re-incubated for 6-12 hrs in the same incubator. CCSs were collected in 1.5 ml microcentrifuge tubes, centrifuged (2000g, 10min) and stored at -20°C until assayed for larval migration inhibition activity *in vitro*.

Plates were collagen coated as follows: 0.5% human Type IV collagen (Sigma-Aldrich, USA), was dissolved in 0.1 M acetic acid by stirring 48 hours at 4°C. The collagen solution was filtered through 2-3 layers of sterile gauze and diluted with water to 1:20, and 200 µl was added to each well (25 $\mu g/cm^2$). The coated plates were air dried for 1-2 days in a laminar flow cabinet and then stored at 4°C.

2.1.8 Enriching specific cell types from the small intestinal mucosa

2.1.8.1 Discontinuous Percoll density gradient centrifugation

Discontinuous Percoll gradients (85%, 75%, 65%, 50%, 40% and 30%) were layered into 50 ml centrifuge tubes one day prior to use and were stored at 4°C. Cells ($200x10^6$ in 10 ml cell rinsing medium) were placed on top of the gradient and tubes were centrifuged at 1200g 10°C for 30 minutes. Cells at each interface were collected by pipette and rinsed twice with cell rinsing medium and once with HBSS9.

2.1.8.2 Immuno-magnetic cell separation

Note: All the media and cell incubation conditions were kept at 4°C to limit cell activation.

2.1.8.2.1 Enrichment of gut eosinophils

Strategy: Eosinophils were enriched by negative selection. In brief, epithelial cells and most globule leucocytes were firstly eliminated by the EDTA digestion method. Secondly, other unwanted cell types such as mucosal mast cells, remaining globule leucocytes, lymphocytes and monocytes-macrophages-dendritic cells from the digested lamina propria cell population were labelled with specific mAbs and depleted using an *auto*MACS method (Miltenyi Biotec GmbH, Gladbach, Germany).

Protocol: Epithelial cells were first eliminated (see section 2.1.7.1.1.), followed by enzymatic digestion to obtain lamina propria cells (see section 2.1.7.1.2). Cells were centrifuged at 300 g for 5 minutes at 10°C. Cells ($50x10^6$) were resuspended in 400 µl cell rinsing medium. The primary monoclonal antibodies: anti-ovine IgE antibodies (XB_6); anti-CD5; anti-CD21; anti-MHCII (section: 2.2.2.5: Table 2.1 on page 54) were added to the cell suspension at a dilution of 1:20 and incubated in the dark at 4°C for 15 minutes. Then the cells were washed twice with PBS (300 g for 5 minutes) and resuspended in 400 µl cold cell rinsing media. Fifty µl rabbit anti-mouse immunoglobulin –MicroBeads (10 µl MACS MicroBeads per $1x10^7$ total cells) was added to cell suspensions and incubated in the dark at 4°C for 15 minutes. The cells were washed, resuspended in buffer as above, and sorted with the *auto*MACS.

2.1.8.2.2 Enrichment of mucosal mast cells

Strategy: MMCs were enriched by positive selection. In brief, epithelial/goblet cells and globule leucocytes were firstly eliminated by the EDTA method. Secondly other unwanted cell types such as lymphocytes, eosinophils, MMDs and the remaining epithelial/goblet cells from the digested lamina propria cell population were depleted using the *auto*MACS.

Protocol: Epithelial cells were first eliminated (see section 2.1.7.1.1), followed by enzymatic digestion to obtain lamina propria cells (see section 2.1.7.1.2). Cells were centrifuged at 300 g for 5 minutes at 10°C. Cells ($50x10^6$) were resuspended in 400 µl cell rinsing medium. The primary monoclonal antibodies: anti-ovine IgE antibodies = XB_6 (see Table 2.1) were added to the cell suspension at a dilution of 1:20 and incubated in the dark at 4°C for 15 minutes. The rest of the procedure was similar to the method described in section 2.1.8.2.1.

2.1.8.2.3 Enrichment of goblet cells

Strategy: Goblet cells were first enriched using Percoll discontinuous density gradients. Cells collected from the interface of 85% Percoll gradient were labelled and depleted of all leucocytes using an *auto*MACS method, leaving an enriched population of goblet cells.

Protocol: Gut digest cells were prepared as per (see section 2.1.7.1.2.), layered on Percoll discontinuous gradients and centrifuged at 1200 g for 30 minutes at 10°C. Cells retained on the interface of 85% Percoll gradient were rinsed twice with cell rinsing medium and $50x10^6$ cells were resuspended in 400 µl cell rinsing media. Primary mAb to Leucocyte common antigens (CD45RA) (see Table 2.1) was added at a dilution of 1:20 and incubated in the dark at 4°C for 15 minutes. The rest of the procedure was similar to the method 2.1.8.2.1.

2.1.9 Larval migration inhibition (LMI) assay

The LMI assay was similar to that described by Rabel *et al.* (1994), with a modification which was to shorten the second incubation time to 2 hrs instead of 14-18 hrs. The micro-sieve was made out of 20mmx16mm OD (10mmID) polystyrene tube and 20 μm nylon mesh which was glued onto one end of the tube. A rubber 'O' ring was put on the tube for holding the sieve 1-2mm away from the bottom of the well. The TcL3 were ex-sheathed by the larval preparation method (*see* method: 2.1.2.1). The larvae were stirred using a magnetic stirrer and maintained at a concentration of approximately 1500-2000 L3/1ml.

Larval suspension (100 μl) and test sample (400 μl) were added into triplicate wells in alternate rows of flat bottomed 48-well-plates (Nunc, USA). A positive control (Levamisole 5 μgm/ml of 5% DMSO in distilled water) and negative control (PBS) were included in each assay. The plate was incubated at 40°C for 2 hr in a moist plastic container. After the first incubation, micro-sieves were wetted with buffer and placed in the empty wells, and all the contents from each well were transferred into a micro-sieve using a disposable transfer pipette. The sieves were tapped to release the air bubbles (if any) underneath the sieve. The plate was again incubated at room temperature in a moist plastic container for another 2 hours. The sieves were removed, 50 μl Lugol iodine solution was added into each well to kill the larvae for counting. The percentage larval migration inhibition (LMI)% was determined using the following equation:-

$$LMI\% = \frac{A - B}{A} \times 100$$

A = number of worms in negative control wells; B = number of worms in test sample wells;

2.1.10 *In vitro* larval paralysis (LP) assays

2.1.10.1.1 LP assay 1

The assay was developed to replace the conventional LMI assay of Rabel *et al.* (1994). The correlation between the two assays was 0.98. The new assay was reproducible, reliable and comparable to the conventional LMI assay and had the advantages that it needed very little sample (40 ul *versus* 400 μl) and was quicker (2 hours *versus* overnight or 14-18 hours). This assay adapted ideas for examining larvae for activity under a stereomicroscope from the work of Coles *et al.* (1975) who observed the action of anthelmintic and from the *in vitro* assay developed for detecting anthelmintic-resistance in ruminant gut nematode species (Martin & Le

Jambre, 1979) in the 1970s. Both assays observed the paralysed larvae whereas the new assay concentrated on moving larvae.

Protocol: *T. colubriformis* L3 were ex-sheathed by the method described in 2.1.2.1. Larval concentration was fixed to approximate 3000 L3/ml and stirred gently by magnetic stirrer. Larvae suspension (10 µl) and test sample (40 µl) were added to each well of flat-bottom 96-well plates. A positive control (Levamisole 5 µg/ml of 5% DMSO in distilled water) and negative control (PBS) were included in each assay. All samples were assayed in triplicate and plates were incubated at 37°C for 2 hr in a moisturised plastic container. After incubation, the contents of each well were agitated twice using a pipette, and all mobile larvae were counted stereomicroscopically after 1 min. Larval paralysis percentage was calculated using the following equation:-

$$LPF\ \% = \frac{A - B}{A} \times 100$$

A = number of moving larvae in negative control wells; B = number of moving larvae in test wells;

2.1.10.1.2 LP assay 2

This assay was developed to test samples which were normally available in very limited amounts, such as chromatographically purified samples (*i.e.* HPLC fractions in Chapter 7). The correlation with conventional LMI assay and LPF assay1 were $r = 0.97$ and 0.98 respectively.

Protocol: The larvae were maintained in suspension at a concentration of approximately 1500-2000 L3/ml and stirred gently by magnetic stirrer. Larvae suspension (10 µl) was added to each well of 72 well Terasaki mini plates (Nunc, USA) and excessive fluid was pipetted out when larvae settled at the bottom. The test or control sample (8 µl) was then added to each well. A positive control (Levamisole 5 µg/ml of 5% DMSO in distilled water) and negative control (PBS) were included in each assay. All samples were assayed in triplicate and plates were incubated at 40°C for 2 hr in a moisturised plastic container. The contents of each well were agitated twice using a pipette, and all mobile larvae were counted after 1 min. Larval paralysis percentage was calculated using the following formula:

$$LP\ \% = \frac{A - B}{A} \times 100$$

A = number of moving larvae in negative control wells; B = number of moving larvae in test sample wells;

2.1.11 Glycoprotein assay

This was a modified method of Mantle & Allen (1978). Mucus or cell culture supernatant samples were prepared at 20% dilution in distilled water. Sample (100µl) and 10 µl periodic acid solution (see section - 2.2.2.21.1) were dispensed into triplicate wells, and incubated at 37°C for 2 hours. After the incubation, 10 µl Schiff's reagent (see section 2.2.2.21.2) was added to each well, left on the bench at room temperature for 30 minutes, and read at 570nm absorbance on a *DynaTech* plate reader (Dynatech Laboratories, INC. Alexandria, Virginia, USA).

2.1.12 Histamine assay using glass micro-fibre method

Generating CCS for the assay

A minimum of 100 µl enriched mucosal mast cells/globule leucocytes ($20x10^6$/ml) was pulsed with 20 µl/ml of mAb to ovine IgE (XB_6) in a CO_2 incubator at 37°C in a 96-well plate (NUNC, USA) for 1 hr. The plate was spun (300g, 10min), and CCSs were collected in 0.6 ml microcentrifuge tubes.

Assay protocol

This is a modified method of Stahl Skov *et al.*(1984) using specialised glass-fibre coated 96-well plate. The plates were bought from RefLab, Denmark.

Binding step: CCS or mucus samples were boiled for 10 minutes and centrifuged at 2000g for 10 minutes. The supernatant was diluted (1:16) with distilled water. Diluted supernatant (50 µl) was added to triplicate wells and incubated at 37°C for 60 minutes in the incubator in order to let histamine bind to the solid phase glass-fibre surface at the bottom of the wells. The negative control was HBSS9 and the positive was histamine (Sigma-Aldrich, USA). Working standards of 1.6; 0.8; 0.4; 0.2 and 0.1µg/ml were prepared in HBSS9 by two-fold serial dilutions of the 3.2 mg/ml stock solution.

Washing Step: Plates were washed twice with double distilled water using an ELISA plate washer. SDS solution (0.4% in distilled water) (250 µl) was added into each well and incubated at 37°C for 30 minutes and followed by washing step as before.

OPT-Coupling Step: Coupling reagent was used fresh. It was prepared by dissolving 50 mg *o*-phthaldialdehyde (Sigma-Aldrich, USA) in 5 ml methanol (Reagent grade) in a 200 ml beaker, to which was added 100 ml 0.05 M NaOH. Coupling reagent (100 µl) was added to each well and incubated for 10 minutes. The reaction was stopped by adding 100 µl of 0.59% Perchloric acid (Merck, Germany). The histamine content was determined by Wallace Victor^2 1420 Multilabel counter (Turku, Finland) and read at 355/460 nm (excitation/ emission).

2.1.13 ELISA

2.1.13.1 IgA and IgG

Flat-bottom, 96-well immuno-micro-titre-plate (Costa, USA) wells were coated overnight at 4°C with 100 µl *carbonate coating buffer pH 9.6* (Sodium carbonate 0.795 g, sodium bicarbonate 1.465 g and 500 µl of 10% merthiolate in 500 ml distilled water), containing *T. colubriformis* larval ES antigen (5µg/ml). The antigen solution was flicked out into a sink and plates washed three times in *washing buffer* (0.05% Tween 20 in 0.2M PBS pH7.2). Plates were kept 3 minutes in a buffer between each wash and then blocked by adding 100 µl of *blocking buffer* (1% bovine serum albumin (Gibco, USA) and 0.1% Tween 20 in 0.2M PBS pH 7.2) for 1 hour in a 37°C incubator. The buffer in the wells was flicked out into the sink and 100 µl of control or test serum diluted 1:500 with *blocking buffer*, was added to triplicate wells, incubated for 1 hr at 37°C and plates were washed as above. For IgA, 100 µl mouse anti-bovine IgA (section 2.2.2.5: Table 2.2) diluted 1: 50 in blocking buffer was added to each well and incubated for 1 hr at 37°C and washed prior to incubation with goat IgG anti-mouse IgG conjugate to horse radish peroxidase (100 µl) (section 2.2.2.5: Table 2.3) diluted to 1:1000 in blocking buffer was added and again incubated in for 1 hr at 37°C. For IgG, 100 µl horse radish peroxidase-conjugated mouse anti-sheep IgG (see Table 2.3) diluted to 1:1000 was added to each well. Following a further incubation for 1 hr in a 37°C incubator, plates were washed as above and filled with 100 µl of *chromogen/substrate solution* (3'3'3'5'-tetramethylbenzidine 0.1mg (Boehringer Mannheim, Germany) was first dissolved in 100 µl DMSO (dimethylsulphoxide) and then added into 9.9 ml 0.1M sodium acetate pH6.0 buffer together with 10 µl 10% H_2O_2) to give a colour reaction within 30 minutes. The reaction was stopped by adding 50µl of 1 M sulphuric acid solution. The absorbance was read at 495nm using a *DynaTech* plate reader.

2.1.13.2 IgE

Ammonium sulphate precipitation: All the sera were precipitated prior to ELISA to remove interference. This method is described in Shaw *et al.* (1998). A saturated ammonium sulphate solution (pH 7.2) was prepared in 0.85% saline. Serum samples (500 µl) were added to 500 µl of 80% of ammonium salt solution and vortex for 15 seconds twice. Samples were centrifuged

at 13,000g for 10 minutes at room temperature. Prior to the assay, all the ammonium sulphate treated samples were diluted with Tween 20 (0.1% Tween 20 in distilled water) to give a final serum dilution 1:4.

***Assay method*:** Plates were coated with TcL3ES antigen and blocked as per section 2.1.13.1. Diluted ammonium sulphate treated serum samples (100 µl/well) were added to triplicate wells and incubated for 1 hr at room temperature, then overnight at 4°C. The plates were washed six times with 150mM-NaCl 0.05% Tween 20 in 10 mM-phosphate buffer, pH 7.2 (PBST) using a semi-automate plate washer (NUNC, Denmark). A purified anti- ovine IgE (XB_6) (see Table 2.1) was diluted to 2.5 µg/ml with dilution buffer (0.5% BSA, 0.1% Tween 20, in PBS) was added (100 µl) to each well and incubated for 4 hrs at room temperature. Plates were washed as above and 100 µl horse radish peroxidase-conjugated rabbit anti-mouse IgG (see Table 2.3) diluted 1:1000 with dilution buffer was added to each well. Following a further incubation for 1 hr in a 37°C incubator, plates were washed as above and filled with 100 µl of *chromogen/substrate solution* (3'3'3'5'-tetramethylbenzidine 0.1mg (Boehringer Mannheim, Germany) was first dissolved in 100 µl DMSO (dimethylsulphoxide) and then added into 9.9 ml 0.1M sodium acetate pH6.0 buffer together with 10 µl 10% H_2O_2) to give a colour reaction within 30 minutes. The reaction was stopped by adding 50 µl of 1 M sulphuric acid solution. The absorbance was read at 495nm using a *DynaTech* plate reader.

2.1.14 Peroxidase micro-plate assay

Generating CCS for the assay

A minimum of 100 µl of enriched eosinophil population ($20x10^6$/ml) was incubated in CO_2 incubator at 37°C in a 96-well plate (NUNC, USA) for 15 min to equilibrate. Then a range of concentrations of human recombinant IL5 (0.625-10 ng/ml) prepared in HBSS9 (100 µl) was added and the cells were re-incubated for 1 hr. The plate was centrifuged (300g, 10min) and kept temporarily at 4°C, if the samples were assayed on the same day, otherwise they were stored at -20°C.

Assay protocol

The *substrate buffer* was prepared by dissolving 1 mg 3,3,5,5-Tetramethylbenzadine (TMB) (Boehringer Mannheim) in 100 µl DMSO and which was added into 10 ml 0.1 M Sodium Acetate pH 5.2 buffer solution (1.36gm Sodium Acetate in 100 ml Milli-Q water and acidity adjusted with HCl concentrate) together with 10 µl H_2O_2. Sample (50 µl) (5% cell culture supernatant in distilled water) was reacted with 50 µl *substrate buffer* for 30 minutes in a 96-

well plate, at room temperature and the reaction was stopped by adding 50 µl of 0.1M H_2SO_4. The plate was read at 490 nm using a DynaTech plate reader. The negative control was HBSS9 and the positive was peroxidase (Calbiochem, San Diego, California, USA); the peroxidase was diluted in two-fold dilutions at concentrations of 3.125-100ng/ml.

2.1.15 Methods of purification of larval paralysis factor (LPF)

2.1.15.1 Ultra-filtration method

Pooled cell culture supernatants (5 ml) were filtered through a series of Amicon ultrafiltration membranes (0.2 µm, 100KD, 10KD, 3KD & 1 KD) (Millipore, USA) using a 3 or 50 ml Stirred Cell filter unit (Amicon, USA). Materials retained on the membranes were resuspended in 5 ml HBSS9 buffer, and were assayed for larval paralysis activity along with pre-filtered CCS and controls (buffer and levamisole).

2.1.15.2 Solvent extraction method

CCS (5 ml) was lyophilised and resuspended in 5 ml 60% Methanol in milliQ-water. The mixture was centrifuged at 10,000 RPM at room temperature for 5 minutes and then the supernatant was lyophilised by Speed-Vac.

2.1.15.3 Solid phase extraction method (SPE)

2.1.15.3.1 SPE using C_{18} sorbent

The solvent extracted sample was resuspended in 0.5 ml Milli-Q water. A SPE column (C_{18} Mega Bond Elut 1 CC 500mg from Varian, USA) was conditioned with 2 ml methanol and rinsed twice with a similar volume of Milli-Q water. A sample of 250 µl was loaded into the column and rinsed with 2 column volumes of Milli-Q water, at a flow rate of 2 ml/min. Then the column was eluted with 300 µl of 60% methanol-water followed by 100% methanol. The eluent was lyophilised, resuspended in Milli-Q water (similar to starting volume) for bioassay.

2.1.15.3.2 SPE using ion exchange sorbent

A SAX cartridge (strong anion exchanger – quarternary amine, 100 mg sorbent, 1 ml volume) and a SCX cartridge (strong cation exchanger – Benzenesulfonic acid, 100mg sorbent, 1 ml volume) was conditioned with 1ml each of 1 M NaOH or 0.1M HCl respectively. Both cartridges were rinsed with Milli-Q water till the pH values were neutral. A control solution of amino acids was prepared by dissolving 1 mg each of four amino acids (Asparagine, Glutamic acid, Aspartic acid, Arginine) in 250 µl Milli- Q water. A 250 µl sample or control was applied first to the SAX cartridge, the column was eluted with 2 column volumes of 60% methanol-Milli-Q water (pH7) and the eluent was lyophilised. The sample was made up to 250 µl with

milli-Q water and made neutral before loading onto a SCX SPE cartridge. The cartridge was rinsed with 2 ml Milli-Q water, and the washings were lyophilised by Speed-Vac. Samples from each step were collected, reconstituted in a similar starting volume for bioassay. Sample pH was adjusted to pH7 prior to loading onto the exchanger or prior to bioassay using acetic acid and ammonium hydroxide.

2.1.15.4 Gel filtration using Bio-Gel P-2 method

2.1.15.4.1 Preparation of the Gel

Bio-Gel P2 (Bio-Rad, USA) 60g was soaked with 360 ml 0.01 M Phosphate buffer solution in a flask for 4 hours at room temperature. Typical hydrated bed volume was 3ml/ gm of dry gel. The column size was 1.5x70cm. After hydration was completed, half of the supernatant was removed and the suspension was degassed for 10 minutes. Then, 180 ml degassed buffer was added into the flask and swirled gently. After 90% of the gel had settled to the bottom, half of the supernatant was again removed. This procedure enabled the removal of the fines by repeating it up to 4 times.

2.1.15.4.2 Preparation of Gel Column

The column outlet was opened and two bed volumes of buffer were passed through the column with operating flow rate (1.0ml/min). The outlet was closed and the flow adaptor was adjusted down to the level of the gel bed. The void volume was determined by running the known substances MWD 500,000 dextran (5mg) and MWD 180 glucose. The chart flow was set to 3cm/min. The CCS (1-5mg) or controls (antigens or CCS without antigen or standards: dextran & glucose) was loaded onto the upper bed surface by pumping through the flow adaptor. All the eluted fractions were collected by a fraction collector (LKB 2000 Ultrorac, Sweden). The refractive index of the fraction was monitored with a refractive index detector (Dynamax – Rainin model RI-1, Germany) and chart speed recorded on a chart recorder. Fractions were collected according to the chromatogram profile, pooled, and lyophilised. The fractions were made up to the original volume and assayed for LPF activity.

2.1.15.5 Thin Layer Chromatography (TLC) method

2.1.15.5.1 Ninhydrin reaction

A 0.5 µl amount of seven biogel-P2 fractions were spotted on silica gel 60 F_{254} (Merck, Germany) metal plates. The plate was air dried, sprayed with 0.1% ninhydrin spray in a fume hood, and heated at 110°C until reddish-purple spots appeared.

2.1.15.6 High performance liquid chromatography (HPLC) method

2.1.15.6.1. Purification using C_{18} preparative HPLC column

A 250 µl purified CCS was fractionated on an Alltech Econosphere C_{18} 10µ 250x22mm column I.D stainless steel preparative HPLC column using a linear gradient from 5-80% CH_3CN in water at ambient temperature, at a flow rate of 2.5ml/min for 40 minutes. The solvent program was – 10 min. hold at 5%; 20 min. gradient from 5-80%; 5min. hold at 80%; 5min. gradient from 80-5%; 15 min. hold at 5%. Fractions were collected and pooled according to the corresponding peaks absorbed at 254nm, dried in a savant prior to bioassay.

2.1.15.6.2 Purification using C_{18} analytical HPLC column

Separation of Peak1 was performed on an Alltech Platinum C_{18} EPS 100 Å 5µ (250 mm x 4.6mm I.D) stainless steel analytical HPLC column with an isocratic mobile phase 5% acetonitrile in MQ water at ambient temperature at a flow rate of 0.5ml/min for 12 minutes. Fractions were collected and processed as above.

2.1.15.6.3 Purification using amino analytical HPLC column

HPLC separation was performed on an Econosphere amino 100 Å 5µ (250mm x 4.6mm I.D) stainless steel analytical HPLC column with isocratic mobile phase (75% acetonitrile-Milli-Q water) at ambient temperature at a flow rate of 0.8ml/min, and run for 30min. A total of seven fractions were collected in each run. All corresponding samples were pooled and kept at -20°C for bioassay.

2.1.15.6.4 Purification using amino micro-bore column

HPLC separation was performed on an Econosphere amino micro-bore 100 Å 3µ (250mm x 1m I.D) stainless steel HPLC column with mobile phase 75% acetonitrile-Milli-Q water isocratic mobile phase at ambient temperature at a flow rate of 0.1ml/min for 25 minutes. Two 254 UV reactive peaks were collected, pooled and kept at -20°C for bioassay.

2.1.16 Method of identification of LPF

2.1.16.1 Liquid Chromatography – Mass Spectrometry (LC-MS)

Chromatographic solvents were sparged with helium before use. Chromatographic separation was performed on a C18 Alltima column 150x2mmID with isocratic mobile phase (15% MeCN-water) at a flow rate of 0.2 ml/ml for 6 min. The column out let was connected to a 100

μ ID fused-silica capillary, which transferred the whole eluent into the ion source. Samples were analyzed on a Shimadzu QP-8000 alpha LC-MS system using both positive and negative electrospray ionization for ion production. The mass range monitored was 50-1000 m/z with a scan speed of 1000 m/z per second. The detector voltage was 1.7kV.

For the final analysis of the factor, LC-MS was also performed on a micro bore aminopropyl econosphere 250mm x 1mm I.D stainless steel column with mobile phase 75% acetonitrile-Milli-Q water isocratic mobile phase at ambient temperature at a flow rate of 0.1ml/min for 20min.

2.1.17 Solubility test for LPF

Cell culture supernatant aliquots (250 μl) were dried in a savant. Each aliquot was dissolved in 0.5 ml of milli-Q water or methanol or ethanol or ether. The mixture was thoroughly mixed by using a vortex mixer for 30 seconds. The supernatants were collected after centrifugation at 10,000 rpm for 5 minutes and the solvents were removed by Speed-Vac. The supernatants were re-dissolved in 250 μl Milli-Q water for bioassay.

2.1.18 Stability test

The aliquots (250 μl) of methanol (75%) extracted pooled active or pooled non-active cell culture supernatants were treated as follows: -

1) Sample was boiled for 10 minutes with or without 25 μl 1M HCl.
2) Sample pH 1 was adjusted to add 25 μl 1M HCl into the sample, incubated for 10 minutes at room temperature, then neutralised with 121 μl 0.2M NaOH, and lyophilised by Speed-Vac.
3) Sample pH 12 was adjusted to add 121 μl 0.2M NaOH into the sample, incubated for 10 minutes at room temperature, then neutralised with 25 μl 1M HCl, and lyophilised by Speed-Vac.
4) Two samples were frozen at -20°C for 1 hr and thawed in a 37°C water bath and the procedure was repeated 5 times for one sample and 10 times for another sample.
5) Sample was incubated with 1μg of Proteinase-K at room temperature for 1 hour.
6) Sample was incubated with 75 units of arylsulphatase in 0.2M sodium acetate buffer at 37°C for 1 hour.

All the lyophilised samples were made up to 250 μl with Milli-Q water, and assayed for LP activity.

2.1.19 Tissue fixation

A piece (25cm) of the small intestine from each sheep was cut open longitudinally and fixed in 4 % buffered formaldehyde solution for 6 hours for toluidine blue stain (pH0.5), and 48 hours for hematoxylin-eosin stain, Periodic Acid Schiff, and high-iron-diamine/Alcian blue (pH2.5). Tissues, for wax embedding, were washed with PBS, placed in a cassette and dehydrated in 70% ethanol. All the tissue slides were prepared by the histology laboratory in Wallaceville Animal Health Centre, Upper Hutt.

2.1.20 Cell counting technique for tissue slides

Cells were counted, from above the *musculais mucosae* to the tip of the villi, in villi which were well oriented and perpendicular to the mucosa. Counts were performed on a Leitz Wetzlar SM-LUX microscope (eyepiece 10X, objective 40X). Cells in 10 alternate villi were counted in each slide and the counts were expressed as mean ± S.D.

2.1.21 Statistical analysis

The significance of difference between sample means was determined using a student's t-test or analysis of variance (ANOVA). For the t-test the variances of each group were checked whether they were similar. For ANOVA the plot of residuals versus fitted values was checked that had no trends apparent, and also the similarity of group variances were checked. Normality of data was tested. Where there was doubt about homogeneity of variance or normality, the ANOVA or t- test results were verified with a non-parametric alternative test. Note that ANOVA and t-test are reasonably robust to non-normality, but less so to heterogeneity of variance. The level of rejection for the null hypothesis for all tests was set at $p \leq 0.05$. The correlations were assessed using Pearson's Linear Correlation Coefficient. Before calculation these coefficients we first checked that the relationships between the variables were approximately linear (see graphs in Appendices 1.3-1.5). In the cases of comparing two large samples using *t*-test (Appendices 24-26) because of the unequal variances of each group, we check the results of the *t*-test with a permutation test and a non-parametric test Mann-Whitney U (Wilcoxon rank-sum). All the analyses were performed using GenStat Statistical software (GenStat for Windows (10th Edition) Introduction. VSN International, Hemel Hempstead).

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2.2 Materials

2.2.1 Animals

The majority of the experimental sheep were from the Romney nematode-resistant selection line developed at the Wallaceville (Baker *et al.*, 1991) and of variable age (1-5 years old). Other types of sheep such as Wallaceville susceptible-line sheep (Baker *et al.*, 1991), outbred nematode-naïve Romney lambs and outbred Romney adults of variable age were also used occasionally in the experiments. The outbred nematode-naïve lambs were raised by separating lambs from ewes 3 days after birth and raising them worm-free in pens on a grating floor. They were made immune by drug abbreviated infection at 6 months of age. All were fed Lucerne chaff *ad lib*, and given concentrate sheep pellets 100gms/sheep/day.

2.2.2 Buffers, media, reagents and stains

2.2.2.1 PBS - 0.01 M phosphate buffered saline

The buffer was prepared as a sterile phosphate buffer containing NaCl 8.00g, KCl 0.20g, Na_2HPO_4 1.15g, NaH_2PO_4 0.20g in 1 litre of double distilled water.

2.2.2.2 PBS stock solution (10 x concentration)

NaCL 80 g, KCl 2.0 g, Na_2HPO_4 11.5 g, NaH_2PO_4 2.0 g were dissolved in 1 litre of double distilled water and filtered with bottle-top 0.22 μm membrane (Millipore, USA).

2.2.2.3 HBSS7 - Hank's Balanced Salt Solution, pH 7. 2

The medium was calcium/magnesium-free sterile Hank`s balanced salt medium and prepared by dissolving NaCl 8.0g, KCl 0.4g, $NaHCO_3$ 3.5g, Na_2HPO_4 0.9g, KH_2PO_4 0.06g, dextrose 1g, *N*-2-hydroxyethylpiperazine-*N*' -2-ethane (HEPES) buffer 20 mM, phenol red 2 gm, Penicillin 100 *iu*/ml (Sigma-Aldrich, USA), Streptomycin 100 μg/ml (Gibco, USA), Kanamycin 100 μg/ml (Gibco, USA) in 1 litre of double distilled water and filtered through 0.22 μm membrane (Millipore, USA).

2.2.2.4 HBSS9 - Hank's Balanced Salt Solution, pH 7.2

The medium was prepared as a sterile amino acid and antibiotic-free medium by dissolving NaCl 8.0g, KCl 0.4g, $CaCl_2$ 0.14g, MgSO4 7H2O 0.1g, MgCl2 6H2O 0.1g, NaHCO3 0.35g,

Na_2HPO_4 $2H_20$ 0.06g, KH_2PO_4 0.06g, glucose 1g, in 1 litre of double distilled water and filtered through 0.22 μm membrane (Millipore, USA). The growth factors such as insulin 10μg/ml (Sigma-Aldrich, USA), and transferrin 10μg/ml (Sigma-Aldrich, USA), were added only into the solution required for the cell culture.

2.2.2.5 Antibody reagents

Surface markers	Clone No.	Isotype	Specificity	Reference
IgE	XB6	IgG1	Ovine IgE	(Shaw *et al.*, 1996)
MHCII	28.1	IgG1	B cells, macrophages, dendrictic cells	(Puri & Brandon, 1987)
CD45RA (p220)	73B	IgG1	All leucocytes	(Puri *et al.*, 1985)
CD5	SBU T1	IgG1	T cells	(Mackay *et al.*, 1985)
CD21	DU14.24	IgG1	B cells	(Hein *et al.*, 2004)

Table 2.1 List of primary monoclonal antibodies.

Antibodies	Code number	Suppliers
Mouse anti-bovine IgA	MCA628	Serotec, UK

Table 2.2 List of primary antibodies.

Antibodies	Code number	Suppliers
Goat anti-mouse IgG MicroBeads	NA	Miltenyi Biotec, Germany
HPR conjugated rabbit anti-sheep IgG	213-4304	Rockland, USA
HPR conjugated goat anti-mouse IgG	A3673	Sigma, USA
HRP conjugated rabbit anti-mouse IgG	F 063	Dako, Denmark

Table 2.3 List of secondary antibodies.

2.2.2.6 HBSS11 - Hank's Balanced Salt Solution, pH 7. 2

The sterile medium was prepared similar to HBSS7 without adding phenol red. It was used for the preparation of Percoll density gradient medium.

2.2.2.7 Complete medium - RP6

RP6 was prepared by dissolving a packet of RPMI with L-glutamine (Gibco, USA), *N*-2-hydroxyethylpiperazine-*N'* -2-ethane (HEPES) buffer 20 mM, $NaHCO_3$ 24mM, 2-mercaproethanol 50mM; penicillin 100 U/ml, streptomycin 100 µg/ml, kanamycin 100 µg/ml and the pH was adjusted to 7.2.

2.2.2.8 Cell rinsing medium

This medium was HBSS7 with 5% FCS and 2mM EDTA.

2.2.2.9 Gut cell maintenance medium

Complete medium was supplemented with 5% FCS and 20 U/ml of DNase (Boehringer Mannheim, Germany).

2.2.2.10 Epithelial cell dissociation medium

This was HBSS7 supplemented with 5% FCS and 5 mM EDTA (Sigma-Aldrich, USA).

2.2.2.11 Gut digest medium

Complete medium RP6 was supplemented with 10% FCS, 100 U/ml collagenase- Type I (Gibco, USA), 50 U/ml hyaluronidase (Sigma-Aldrich, USA), and DNase 20 U/ml (Boehringer Mannheim, Germany).

2.2.2.12 Vasoactive amines for larval paralysis assay

Leukotriene B4, C4, D4, E4 (Cayman Chemical, USA) were shipped in 99% ethanol alcohol but were dried down using nitrogen gas prior to the assay. Serotonin (Sigma-Aldrich, USA), Dopamine (Sigma-Aldrich, USA), St. Louis, Missouri, USA), histamine (Sigma-Aldrich, USA),

and all the leukotrienes were prepared in 1.5 ml of 5% ethanol in water at a concentration of 100 µg/ml for the assay.

2.2.2.13 Fetal calf serum (FCS)

FCS was supplied as heat inactivated serum from Gibco, USA.

2.2.2.14 0.2% Trypan blue solution

Trypan blue 200mg was dissolved in 100 ml PBS and the solution was filtered and ready to use.

2.2.2.15 Leishmann's stain solution

Leishman's stain was prepared by dissolving stain powder 0.3 gm in 250 ml flask with 100 ml methanol. The solution was heated to 50°C and stirred for 30 minutes. Then the stain was gently stirred for 24 hours at room temperature, filtered and was ready to use.

2.2.2.16 Leishmann's stain buffer solution pH 6.8

This solution was prepared by dissolving a concentrate buffer tablet (BDH, UK) in a litre of double distilled water.

2.2.2.17 Acid alcohol solution

This was used for cleaning stains on microscope slides after staining. It consisted of 1% concentrate HCl in 70% ethyl alcohol.

2.2.2.18 Lugol iodine solution

The solution was prepared by dissolving 0.5 g Iodine crystals and Potassium Iodide 1 g in 500 ml distilled water.

2.2.2.19 Ninhydrin solution

Ninhydrin powder 0.1 g (BDH, UK) was dissolved in 100 ml *n*-butanol and mixed with 3 ml glacial acetic acid. The solution was heated to 60°C for about 30 minutes and kept at 4°C cold room. The solution was sprayed using a compressed gas sprayer (Alltech, USA).

2.2.2.20 Peroxidase assay substrate buffer

The substrate buffer was prepared by dissolving 1 mg 3,3,5,5-Tetramethylbenzadine-Boehringer Mannheim (TMB) in 100 µl DMSO and was added into 10 ml 0.1 M Sodium Acetate pH 5.2 buffer solution (1.36gm Sodium Acetate in 100 ml Milli-Q water and adjusted to pH5.2 with conc HCl) together with 10 µl H_2O_2.

2.2.2.21 Glycoprotein assay reagents

2.2.2.21.1 Periodic acid solution

The solution was prepared by adding 10 µl of 50% periodic acid (50 mg periodic acid dissolved in 100 µl distilled water) into 10 ml 7% acetic acid solution.

2.2.2.21.2 Schiff reagent

The reagent was made by adding 100 mg Sodium metabisulphide in 6 ml Schiff reagent solution (Sigma-Aldrich, USA), and incubated at 37°C for 90 minutes.

2.2.2.22 Histamine assay – coupling reagent

The reagent was used fresh. It was prepared by dissolving 50 mg *o*-phthaldialdehyde (Sigma-Aldrich, USA) in 5 ml methanol (Reagent grade) in a 200 ml beaker, to which was added 100 ml 0.05 M NaOH.

2.2.2.23 ELISA buffers

2.2.2.23.1 Antigen coating carbonate buffer 0.05 M, pH 9.6

Sodium carbonate 0.795 g, sodium bicarbonate 1.465 g and 500 µl of 10% merthiolate were made up to 500 ml with double distilled water and stored at room temperature.

2.2.2.23.2 0.2 M PBS pH 7.2

Di-sodium hydrogen phosphate 26.70 g was dissolved in 750 ml and sodium di-hydrogen phosphate in 300 ml double distilled water. The latter salt solution was added slowly into the former solution till the pH 7.2 was obtained.

2.2.2.23.3 Washing buffer

The buffer contained 0.05% Tween20 in 0.2M PBS pH7.2.

2.2.2.23.4 TMB substrate solution

This solution was made fresh every time. For 10 ml substrate buffer, 3'3'3'5'-tetramethylbenzidine 0.1mg (Boehringer Mannheim, Germany) was first dissolved in 100 µl DMSO (dimethylsulphoxide) and then added into 9.9 ml 0.1M sodium acetate pH6.0 buffer together with 10 µl 10% H_2O_2.

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Chapter 3
Mucosal source of larval paralysis factor

3.1 Introduction

Mucus from the abomasum and small intestine of nematode immune sheep and cattle has been shown *in vitro* to paralyse nematode larvae. This activity of mucus reflected the immune status of sheep, however, the levels varied and were strictly nematode-challenge dependent (Douch *et al.*, 1986; Douch *et al.*, 1983; Kimambo & MacRae, 1988). An *in vitro* assay has been developed for the presence of larval-paralysis factors (LPF) in intestinal mucus while investigating expulsion of *T. colubriformis* in immune sheep, and the acronym LMI (larval migration inhibition) was coined to describe both the factor in mucus and the effect which it exerted on nematodes (Douch *et al.*, 1983). When exsheathed larvae were treated with gut mucus from immune sheep they became paralysed temporarily and formed coils (Douch *et al.*, 1983); a similar anti-larval activity was demonstrated in the mucosal cell culture supernatant (CCS) derived from enriched mucosal mast cells and globule leucocytes from immune sheep (Douch *et al.*, 1996b). However, over the last 20 years very little progress has been made in identifying the active factor(s). Although Douch *et al.* (1996b) had demonstrated the larval paralysis activity was present in secretions from intestinal mucosal cells from immune sheep, the finding had not been repeated by other investigators. Thus, early research was planned first to confirm Douch's work, followed by experiments to determine a reliable mucosal source of LPF.

3.2 Materials and methods

All the materials and methods are described in Chapter 2.

3.2.1 Experiment 1: Confirmation of mucosal source of LPF

Aim: To confirm Douch's findings that substances having larval paralysis activity could be produced from isolated intestinal mucosal cells of immune sheep in response to *T. colubriformis* ES antigens.

Experimental approach

Five mixed age (1-5 years) nematode- resistant-line ewes from different farms were randomly selected from a culled group. Sheep were brought to the slaughter house one day prior to killing and food was also withheld for 24hr. They were killed (2 sheep a day on 3 consecutive days) by captive bolt and exsanguination. The first metre of small intestine after the bile duct entry was collected for isolation of mucosal cells and the second metre for the mucus. Small intestinal mucosal cells were isolated by the method described in Chapter 2: Methods & Materials: section: 2.1.7.1.

4.2.2.1 Results

3.2.1.1.1 Parasitology

The faecal egg counts at slaughter were all negative.

3.2.1.1.2 Lymphocyte transformation assay

The *T. colubriformis* larval ES antigens used in the experiments were assessed for their immunogenic property by lymphocyte proliferation assay, prior to the cell culture for LPF production. The antigens induced lymphocyte proliferation *in vitro* (data not shown).

3.2.1.1.3 Differential mucosal cell counts

Leishmann's stained cytospots of duodenal tissue digests showed a heterogeneous population of mucosal cells which contained eight cell types, *viz.* lymphocyte, eosinophil, neutrophil, macrophage, mucosal mast cell (MMC), globule leucocyte (GL), goblet cell (GO) and epithelial cell (Table 3.1; Fig. 3.1A & B). Although lymphocytes appeared in different forms (small and large lymphocyte, granular lymphocyte, lymphoblast and plasma cell), for convenience, all were counted as a lymphocyte (Fig. 3.1B, 8a, b, c). In the case of monocytes, macrophages and dendritic cells, the monocytes were easily identified by their horseshoe shaped nuclei, but the young macrophages and dendritic cells mostly were hard to differentiate and thus for convenience all of them were collectively named as monocyte-macrophage dendritic cells (MMDs). Eosinophils were easily identified by their characteristic densely packed orange-red granules and lobular nucleus but their size and granulation pattern varied. Epithelial cells were very heterogeneous in appearance, comprising different stages of three cell lineages *viz.*, absorptive columnar epithelial cells and GOs and Paneth cell lineage. Paneth cells were excluded from this study because these cells normally reside at the fundus of an intestinal gland (Dellmann, 1971) and the isolation method used in the present study did not digest the gut down to that level. For the convenience of cell counting, the non-differentiating stem cells (precursors of the three lineages), pre-absorptive columnar epithelial cell, differentiating absorptive

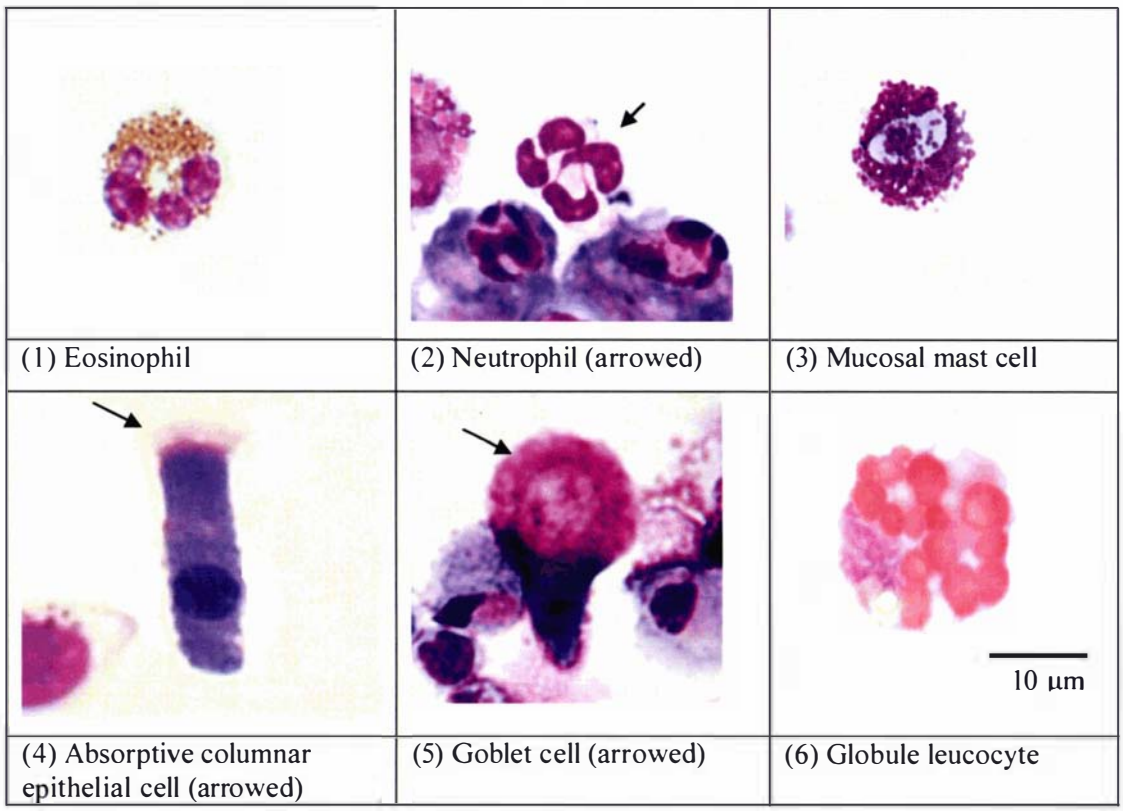
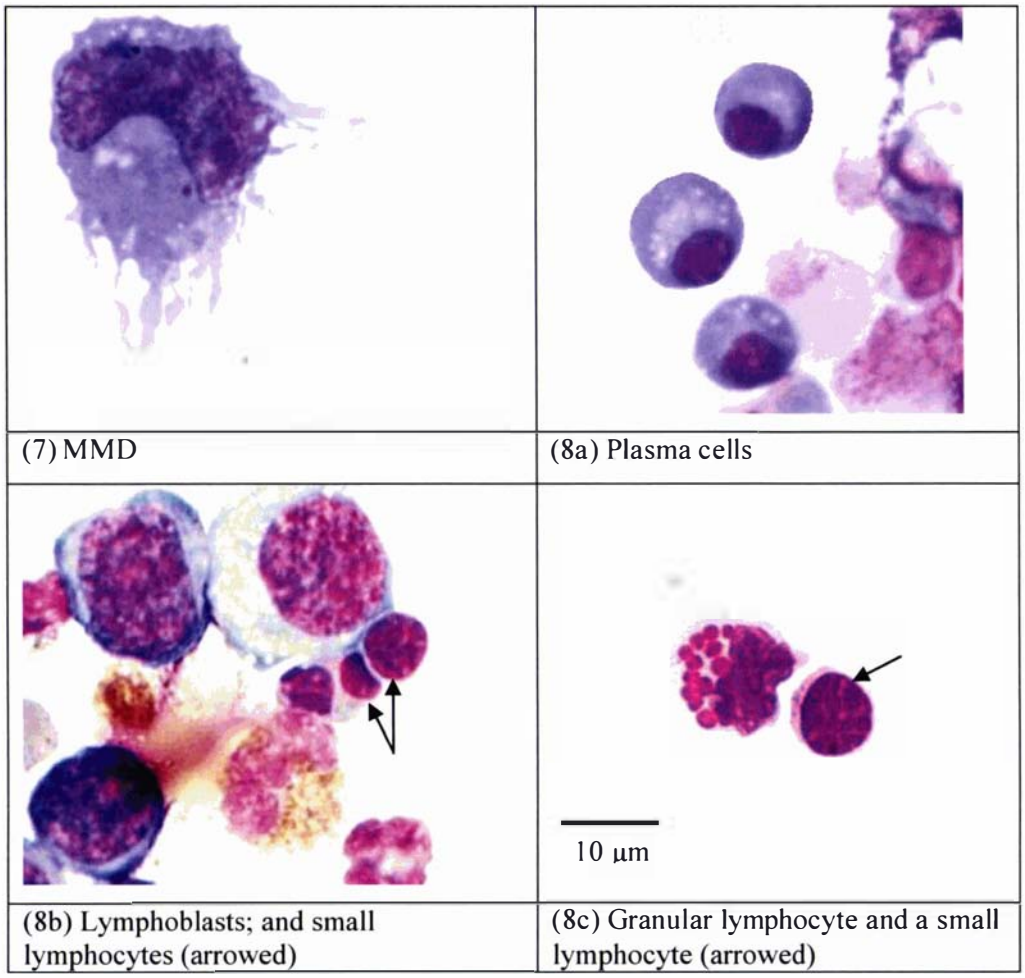


Fig.3.1, A Oil-immersion microphotographs of isolated small intestinal mucosal cells from Leishmann-stained cytospots. (1) Eosinophil: rounded, with large quatro-lobed purplish blue stained nucleus and oval shaped coarse orange red granules densely packed in the cytoplasm; (2) Neutrophil: rounded with typical multi-lobed purplish red nucleus; the lobes are pronounced and connected by a thin strand of chromatin; cytoplasm is granular and stains faintly pinkish. (3) Mucosal mast cell presents a variety of dark basophilic granules with typical oval shape lighter blue staining nucleus. (4) Absorptive epithelial cells are elongate, nucleus closer to basal part and characterised by lack of secretory granules and presence of a prominent apical brush border (where the arrow is pointed). (5) Goblet cell with characteristic goblet shape and its narrower end the basal part, the stem of the cell contains the nucleus and the bowl of the goblet is bulging sideways with membrane enclosed mucin globules. (6) Globule leucocyte is round or ovoid with characteristic large orange red eosinophilic globules and lateral displacement of the light purple staining nucleus.



(7) MMD | (8a) Plasma cells

(8b) Lymphoblasts; and small lymphocytes (arrowed) | (8c) Granular lymphocyte and a small lymphocyte (arrowed)

Fig 3.1, B Oil-immersion microphotographs of isolated small intestinal mucosal cells from Leishmann-stained cytospots. (7) MMDs are mostly oval shaped giant cells, with characteristic kidney shape nucleus in bluish coarse texture cytoplasm; and with distinctive numerous pseudopodia, surface folds and finger like processes projecting into various directions. (8a) Plasma cells are spherical or ellipsoid with rounded eccentric nucleus and cytoplasm is abundant and stains dark blue. (8b) Lymphoblasts are activated large lymphocytes, higher nuclear cytoplasmic ration with large rounded nucleus with fine chromatin in blue-gray agranular cytoplasm. Small lymphocytes (arrowed) are with round solid nucleus surrounded by a narrow rim of cytoplasm. (8c) Granular lymphocytes are large lymphocyte with distinct azurophilic spherical cytoplasmic granules which dispersed at one side of irregularly shaped nucleus.

Sheep	sLMI%	mLMI%	L	EO	MMD	N	MMC	GL	EP
5053	48	92	36	16	1	1	16	0	30
35	68	96	7	55	1	2	2	0	33
3022	32	90	54	18	3	2	15	4	4
36	51	81	37	21	3	1	6	0	32
3020	35	50	58	8	2	1	1	10	20
r		**0.53**	**-0.98**	**0.87**	**-0.57**	**0.20**	**-0.36**	**-0.70**	**0.82**

Keys: L – lymphocyte; ; N – neutrophil; EO – Eosinophil; MMD – monocyte-macrophage-dendritic cell; EP – epithelial cell; MMC- mucosal mast cell; GL-globule leucocyte; LMI – larval migration inhibition% - proportion of larvae whose migration is inhibited: mLMI% – LMI activity of mucus; sLMI% – LMI activity of CCS; r = correlation coefficient

Table 3.1 Proportion of recovered cell types in duodenal tissue digests and the correlations between LMI activity of CCS and the proportion of each cell type present in the cell culture. Crude data are tabulated in Appendix 1 & 2. The correlation coefficient (r) between sLMI% and other parameters at $p \leq 0.05$ level are tabulated and printed in bold.

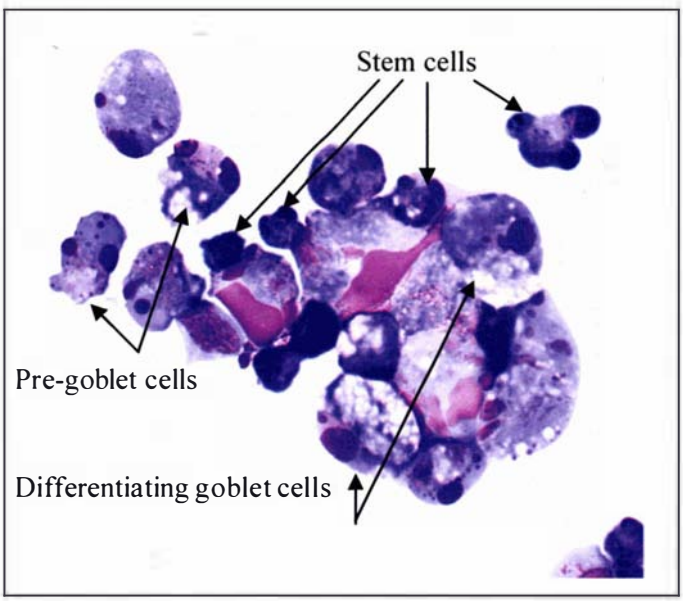


Fig. 3.1C Oil-immersion microphotograph of isolated small intestinal mucosal digest cells. Leishmann-stained cytospin preparation illustrating heterogeneity of epithelial cells. Magnification x 600.

columnar epithelial cells, absorptive columnar epithelial cells, pre- GOs (oligomucous cells), differentiating GOs and GOs (Fig. 3.1C) were collectively counted as epithelial cells.

According to Karam (1999), the absorptive cells from rat small intestine have no secretory granules like GOs, but they have a prominent apical brush border and tend to elongate (Fig 3.1A, 4). On the other hand, stem cells were the 'youngest' cells in the lineage, they are less differentiated, have a high nucleus to cytoplasm ratio and possess a high capacity to proliferate; whereas GOs are featured with a large group of mucous granules (Fig. 3.1C) but they do not always appear in a typical 'goblet' shape shown in Fig 3.1A. However, if a considerable number of typical GOs appeared in the cytospots, they were not included in epithelial cell count but counted as a separate cell type. MMDs, GLs and neutrophils were less frequent than other cell types (Table 3.1). GOs and MMCs were recovered less frequently than lymphocytes and eosinophils (~23%).

3.2.1.1.4 Larval migration inhibition activity of CCS and mucus

The larval migration inhibition (LMI) activity was determined using the improved *in vitro* LMI assay, which is similar to the method described by Rabel *et al.*, with minor modification (Rabel *et al.*, 1994). The mean LMI activity of mucus was 82% (±19% S.D.) and of CCSs was 47 % (±15% S.D.). Table 3.1 demonstrates that LMI activity of CCSs had a strong linear relationship with the proportion of eosinophils (r = 0.87; n = 5) and the proportion of epithelial cells (r = 0.82; n = 5) in the cell population, but only the correlation with the proportion of eosinophils was significantly different from zero (*see* Appendix 1.3), whereas the LMI activity was strongly negatively correlated with lymphocyte numbers (r=-0.98) (*see* Appendix 1.5). The intriguing result from this experiment was that the cell cultures without added nematode antigens had very little or no LMI activity (see Appendix 1.2).

Observation of larval motility in the wells under the microscope, after adding the CCS or mucus into the wells, revealed that some larvae stopped moving in 15 minutes and formed coils which were not seen in the negative control wells (medium only). A similar coiling of larvae was observed in the positive control wells (Levamisole 5µg/ml of 5%DMSO in distilled water); and there were some coiling larvae in the control wells which appeared in the late stage of the first incubation. The difference was that when the larvae were transferred by pipette and placed onto a sieve for the second incubation, the motion caused by pipetting could activate the resting larvae but not the paralysed larvae. Therefore, larvae in the test samples were still coiled whereas the larvae in the control media were active again, moving vigorously and wriggling through the sieve. Perhaps coiled larvae in control wells were resting.

3.2.2 Experiment 2: Determination of the mucosal source of LPF

Rationale and aim: Experiment 1 confirmed that *T. colubriformis* ES antigens stimulated low-level LPF release from intestinal mucosal cells of nematode-resistant sheep. In order to increase the LMI activity, further experiments were done to enrich for LPF-releasing cells. To address the question of whether LPF-releasing cells were situated in the epithelia or lamina propria compartment of the mucosa, two experiments (2.1 & 2.2) were conducted to devise a suitable method for isolating enriched epithelial or lamina propria cells (LPCs), and then to produce and compare LMI activity of CCS derived from these two compartments.

Experiment 2.1: Method development

Rationale and aim: Since the available methods for isolating gut cells do not achieve pure epithelial cells (contaminated with LPCs), the current research adapted and modified the methods of Nagi & Babiuk (1987) and Stankiewicz *et al.* (1994) to achieve this goal. Two experiments were conducted (2.1.1 & 2.1.2). The first experiment examined the effect of time after treatment with EDTA on the release of epithelial cells and their viability, and the recovery of LPCs in different enzymes at variable concentrations and their viability; and the second experiment optimised incubation time, and concentration of enzyme selected by the first experiment, for the maximum yield of LPCs.

Experimental approach

Experiment 2.1.1

One field-raised-outbred Romney ewe was brought to the slaughter house one day prior to killing for leaving with food. Sheep was slaughter by captive bold and exsanguination. Two one-metre segments of small intestine were removed after the bile duct entry; the first segment was incubated for 6 periods of 30 minutes at 37°C for the isolation of epithelial cells. The second segment was resected into 5 cm gut segments, digested in different enzymes (collagenase Type I, Type IV and with or without dispase) at their different concentrations for the recovery of LPCs; after 2 hour incubation cells were harvested and counted, and the gut segments were reincubated in the same media for a further 2 hours.

Experiment 2.1.2

Two field-raised-outbred Romney ewes were brought to the slaughter house one day prior to killing for starving. Sheep were killed, and a metre of duodenal tissue was removed after the bile duct entry. After epithelial cells were removed each intestinal tissue was resected into twelve 5 cm segments. Each segment was digested in 10 ml digestion medium at one of the four concentrations (50, 100, 200 & 400 U/ml) for one of three different times (2, 4 & 6 hours).

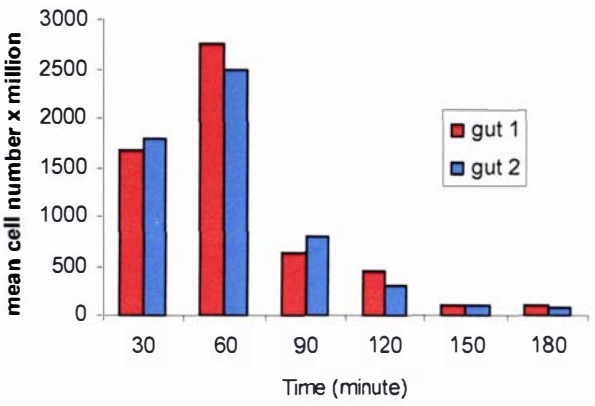


Fig. 3.2 Histogram of epithelial cell yield from a metre of small intestine after EDTA treatment at 30min intervals over a three hr period (n=2).

Time(min.)	L	N	EO	MMD	MMC	GL	EP/GO
30	18	0	1	0	0	5	78/0
60	23	1	4	0	0	11	60/2
90	29	3	4	1	1	13	52/0
120	22	0	2	1	1	7	62/6
150	19	0	3	10	1	7	57/4
180	29	0	7	6	0	3	53/3

Keys: L -lymphocyte; N – neutrophil: EO – Eosinophil; MMD – monocyte-macrophage-dendritic cells; MMC- mucosal mast cell; GL-globule leucocytes; EP/GO – epithelial/goblet cell;

Table 3.2 Mean percentage of cell types recovered after duodenal tissues were incubated with 0.5mM EDTA at 30 minute intervals over a 3 hour period (n = 2).

Enzyme concentration (U/ml)	Total cell number x 10^6		
	2 hr	4hr	Total cells No.
1. Collagenase Type1 250U	10.20	18.90	29.10
2. Collagenase Type1 50U + Hyaluronidase 50U	4.50	8.00	12.50
3. Collagenase Type1 250U + Hyaluronidase 50U	**12.80**	**24.80**	**37.60**
4. Collagenase Type1 500U + Hyaluronidase 50U	**19.40**	**29.70**	**49.10**
5. Collagenase Type1 50U + Hyaluronidase 50U*	9.15	11.50	20.65
6. Collagenase Type1 250U + Hyaluronidase 50U*	**24.00**	**19.40**	**43.40**
7. Collagenase Type1 500U + Hyaluronidase 50U*	**20.00**	**31.00**	**51.00**
8. Collagenase Type4 250U + Hyaluronidase 50U	10.00	11.20	21.20
9. Dispase 1U	1.95	1.25	2.75
10. Buffer only	1.50	1.25	2.75

* = Dispase 1 U/ml was included.

Table 3.3. Yield of viable cells recovered from duodenal tissue digests following treatment with different strengths and combinations of enzymes (n=1).

Experiment 2.2: Comparing LMI activity of epithelial and lamina propria cells

Aim: This experiment compared LMI activity of cell culture supernatants (CCS) of epithelial and LPCs.

Experimental approach

Four, two-year-old field-raised-resistant-line ewes were used. The first pair were brought into a pen, drenched, sensitised by 'LPF regime' as described in Chapter 2 (section 2.1.1.2) and left without food 24 hr prior to humane slaughter. The second pair received no treatment, they were brought from the field, not given food for 24 hr and slaughter like the other pair. In both cases, 50 cm of duodenal tissue was removed after the bile duct entry for isolating epithelial and LPCs for generating CCS. Epithelial cells recovered after 1 hour EDTA incubation, and LPCs recovered after 4-5 hours of enzymatic digestion, were incubated with *T. colubriformis* L3 ES antigens (TcL3ES) and the resultant CCSs were assayed for their LMI activity *in vitro.*

3.2.3 Results

3.2.3.1 Experiment 2.1.1 (EDTA treatment)

3.2.3.1.1 Yield and percentage recovered cell types

The highest number of epithelial cells was obtained at 60 minutes (Fig. 3.2) after the tissues were gently swirled in the EDTA containing cell culture medium. Thereafter the total cell yield declined. More dead cells were present in the 30-minute isolate but the viability of other isolates was 80-90%. Differential counts performed on cytospots, after Leishmann's staining (Table 3.2), revealed that over 50% of mucosal cells were epithelial cells, lymphocytes were second to most frequent (20%), and the remaining cells were neutrophils, eosinophils, MMDs and MMCs. GOs were present mainly in the late batches (120-180 min). GLs appeared in the first 30 min, peaked in the middle (60-90 min) and declined at the end of incubation.

3.2.3.2 Experiment 2.1.2 (Enzyme treatment)

3.2.3.2.1 Cell yield and viability

LPCs were effectively isolated by collagenase Type I but not by Type IV or by dispase (Table 3.3). Adding hyaluronidase to the cultures gave some enhancement in cell recovery. Dispase increased the cell yield up to double only with lower concentrations of collagenase (50 & 250 U) at 2 hours but not at 4 hours or with 500U of collagenase. Higher concentrations of the

enzyme tended to release a higher number of cells. A similar trend was shown in the second experiment (Table 3.4). Cell viability at all enzyme strengths was high and similar in the first 2 hours of digestion in both animals. At 6 hours, cell viability reduced markedly in the presence of higher enzyme concentrations (200 and 400 U/ml). Furthermore, at 6 hours, collagenase 100 U/ml yielded more viable cells than 200 U/ml.

3.2.3.3 Experiment 2.2

3.2.3.3.1 Enumeration of cell type

Differential counts performed on Leishmann's stained cytospots revealed that both epithelial and lamina propria isolates yielded high proportions of lymphocytes (29%-59%) but, as expected, epithelial isolates contained higher proportions of epithelial cells (38-54%) than lamina propria isolates (23%-34%). In addition, GLs (2%-9%) and GOs (7%-15%) were more frequent in the epithelial isolates whereas higher numbers of eosinophils (4%-16%), MMCs (5%-14%), MMDs (~3%) and very few neutrophils were recovered in the lamina propria isolates (Table 3.5). Granular lymphocytes (Fig. 3.1B, 8c) were easily identified by their distinctive red globules in the cytoplasm and appeared more in epithelial isolates than in lamina propria. In addition, there were some differences in the recovery of cell types between penned hyperimmunised and field sheep; the field sheep tended to have more GOs in the epithelial isolates whereas the penned sheep had higher yields of eosinophils in the lamina propria isolates (Table 3.5).

3.2.3.3.2 Larval migration inhibition activity

The LMI assay result showed that the LPCs consistently produced significantly higher LMI activity (56-99%) than the epithelial cells (0-28%). One sheep receiving hyper-immunisation in the pen produced a higher LMI activity than two sheep receiving only natural infection (Table 3.5). However, the sample number was so small that no meaningful conclusion could be made from that data set. The LMI activities were detected only in the cultures containing *T. colubriformis* L3 ES antigens and not in the control culture which contained only cells. This observation was similar to the results of Experiment 1.

Incubation time (hr)	Collagenase* strength U/ml	Sheep 1		Sheep 2	
		Cell x 10^6	viability (%)	Cell x 10^6	viability (%)
2	400	41	86	93	86
	200	45	89	96	90
	100	18	95	36	95
	50	16	96	49	92
4	400	53	72	43	70
	200	40	80	79	75
	100	20	91	49	87
	50	8	85	36	88
6	400	44	60	15	61
	200	17	69	30	56
	100	20	81	47	75
	50	28	70	12	78

*= 50u/ml hyaluronidase was added in all digest solutions

Table 3.4 Yield and percentage of recovered viable lamina propria cells from digested small intestinal tissues following incubation with different strengths of collagenase type I over a 6 hr period.

Sheep	Cell type	L	N	EO	MMD	MMC	GL	EP/GO	LMI%	
									Cell+ Ag	Cell only
7138P	EPC	38	0	1	0	0	5	49/7	5	0
7000P		29	0	6	0	0	2	54/9	12.2	0
2412F		34	0	0	0	4	2	47/13	28	0
5414F		37	0	1	0	0	9	38/15	0	0
mean		**35**	**0**	**2**	**0**	**1**	**5**	**47/11**	**11**	**0**
7138P	LPC	49	1	13	4	5	0	28/0	60	5
7000P		29	3	16	2	14	0	27/9	99	12
2412F		53	2	8	2	7	2	23/3	56	4
5414F		38	2	4	0	13	0	34/9	60	9
mean		**42**	**2**	**10**	**2**	**10**	**1**	**28/5**	**69**	**8**

Keys: P - field-raised-pen-boosted: F - field-raised no booster infection; L -lymphocyte; N – Neutrophil: EO – Eosinophil; MMD – monocyte-macrophage-dendritic cell; MMC- mucosal mast cell; GL-globule leucocyte; EP/GO – epithelial/goblet cell; Ag – *T. colubriformis* L3 ES antigen; EPC – epithelial cells; LPC-lamina propria cells;

Table 3.5 Percentage of cell types recovered from epithelial and lamina propria cell isolates and their LMI activity. Mean of cell types and LMI% are tabulated in bold print. Crude data are tabulated in Appendix 3.

3.3 Discussion

3.3.1 Confirmation of mucosal source of LPF

This chapter confirms Douch's work that demonstrated small intestinal mucosal cells isolated from nematode resistant-line sheep produced LPF in response to *in vitro* challenge with ES antigens (excretory secretory antigens) from the larvae of *T. colubriformis* (Douch *et al.*, 1996b). However, subsequent purification on Percoll discontinuous density gradients was not performed as described by Douch *et al.* because this was a preliminary investigation to establish the mucosal source of LPF. In addition, the *in vitro* cell culture system is shown to reliably produce factors which cause paralysis similar to that caused by immune mucus, indicating that the *in vitro* model can mimic this component of the *in vivo* mucosal response to nematodes.

The gut cell isolation technique using enzymatic digestion in the first experiment was adapted from the method of recovery for MMC and GL from the small intestine of parasitized sheep (Stankiewicz *et al.*, 1994). Table 3.1 illustrates that enzymatic digestion for 5 hrs recovered a heterogeneous population containing eight types of gut mucosal cells: lymphocytes, neutrophils, eosinophils, MMD, MMC, GL, GOs and epithelial cells. Epithelial cells were dynamically heterogeneous compared to other cell types, and ranged from immature stem cells to well differentiated elongated absorptive columnar cells, and from different stages of pre- GOs (oligomucous cells) to fully differentiated mature GOs. In the lymphocyte lineage, large and small lymphocytes, granular lymphocytes, lymphoblasts and plasma cells were collectively counted as lymphocytes. To date, this is the first published data on the differential small intestinal mucosal cell count of sheep. There are few publications concerning the staining pattern of single cell suspensions of heterogeneous populations of gut mucosal cells. Previous investigations have focused mostly on a particular cell type of the intestine; epithelial cells (Karam, 1999), lymphoid tissues, especially for T cell research (Bull & Bookman, 1977; Davies & Parrott, 1981; Gyorffy *et al.*, 1992; Nagi & Babiuk, 1987) and GL/MMC (Huntley *et al.*, 1984; Stankiewicz *et al.*, 1994). Although Nagi and Babiuk (1987) reported differential cell counts of Wright-Giemsa stained mucosal cells while working on isolating intra-epithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) from bovine jejunum, their results were not comparable with the present work. The reason being that the present work produced a mixture of cells from both compartments (*i.e.* epithelial & lamina propria) whereas Nagi and Babiuk (1987) and Davies and Parrott (1981) focused on IEL and LPL. Thus, they used EDTA treatment prior to the enzymatic digestion step for separating the two types of lymphocytes, and finally concentrated the cells with Percoll purification, and their differential cell count was on a Percoll purified population whereas the current work excluded both the Percoll gradient purification and the EDTA step in favour of the straight enzymatic digestion, and the

differential cell count was carried out on the crude mucosal tissue digest. Because of the differences cell preparation, their samples packed epithelial cells, and had more lymphocytes (77%-80%) than the current work which had 36%-58%; also it had much lower eosinophils (4%-7%) and MMCs (2%-5%) than the current study which had 38% and 8% respectively. Davis and Parrott (1981) isolated rodent lymphocytes from the two compartments and recovered even more lymphocytes (>90%), but eosinophils and MMCs were not counted separately, and were presented as granulocytes in low numbers (1%-4%). The high numbers of lymphocytes in both groups could result from the Percoll purification. Nevertheless, the cell viability in the present study and the relatively high numbers of granulocytes, especially MMCs and eosinophils, suggests that local stimulation was present in the gut.

Prior to cell culture for LPF production, the immunogenicity of ES antigens was assessed by using the lymphocyte proliferation assay (LPA). It was notable that the LMI activity produced in cell cultures without antigenic stimulation was profoundly less than that produced by cultures which were exposed to the *T. colubriformis* stage 3 larval antigens, suggesting that components released from larvae are potent and trigger gut mucosal cell activation leading to the release of LPF *in vitro*. This finding was in agreement with the report of Jackson *et al.* that anti-nematode immune responses to gut nematodes are nematode challenge-dependent (Jackson *et al.*, 1988).

In vitro rapid challenge studies, in which isolated small intestinal MMC/GL were exposed to *T. colubriformis* antigens or exsheathed L3 larvae for 30 min., have shown that the resultant CCS contained leukotrienes and substances having larval paralysis activity (Douch *et al.*, 1996). In the present study, CCS and immune mucus had LMI activity, although mucus had the higher activity. The reason for this difference is difficult to explain, but perhaps not surprising since the nature of the two samples is vastly different. CCS is relatively pure, containing cell secretions and parasite ES antigens, whereas mucus is a complex mixture of mucus glycoproteins attached to a milieu of microbes residing in the gut, antibodies, cell secretions, ions as well as dead cells and digesta. The LMI activity of CCS and mucus in the present study was higher than the activity reported from Douch *et al.* (1996). Perhaps the difference was due in part to cell concentration which was 10 times more, and in part by the immune status of the experimental sheep which were all nematode-resistant-line animals receiving natural infection, whereas Douch *et al.* used penned, immunised outbred sheep. It is well known that the magnitude of immunity reflects the genetic make up of the animal and the immunization regimes used for the animals (Balic *et al.*, 2006).

The present work shows eosinophils as the only cell type that correlated strongly (r =0.87; n= 5) with the LMI activity of CCS. In light of findings by other workers (Douch *et al.*, 1986; Douch

& Outteridge, 1989) anti-larval activity of mucus was always associated with high eosinophil numbers in the gut tissues of immunised sheep or naturally infected sheep. In addition, an elevated level of eosinophils was also associated with immunity to gut nematodes in sheep (Bao *et al.*, 1996; Douch *et al.*, 1986; Gorrell *et al.*, 1988b; Salman & Duncan, 1985). However, some other investigators have doubted the involvement of eosinophils in immunity to nematode infection in sheep (Douch *et al.*, 1988; Gill, 1991; Winter *et al.*, 1997) since eosinophil numbers did not correlate well with the parasitological data. MMCs, GLs and GOs are also known to be associated with immunity to gut nematode infection in sheep (Douch *et al.*, 1986; Newlands *et al.*, 1990). The current experiment did not conclusively establish the cell type responsible for the paralysis of larvae *in vitro* since many effector cells, *viz.* MMCs, GLs, lymphocytes, GOs and MMDs, were present in the cell cultures in addition to eosinophils. More work is needed before this can be fully elucidated.

3.3.2 Determination of mucosal source of LPF

3.3.2.1 Method development

The results reported in this chapter describe the optimal cell fractionation techniques which suited the isolation of epithelial (epithelial/GOs, intra-epithelial lymphocytes and GLs) and lamina propria-bound cells from the small intestinal mucosa of parasitized and normal sheep for the production of larval paralysis factor.

The aim of the present study was to compare LMI activity derived from epithelial and LPCs of the small intestine of parasitized and non-parasitized sheep. Therefore, this study required a technique to isolate cells from each compartment without contaminating cells between two compartments. Furthermore, in the process of developing the technique, since the amount of enzymes used by previous investigators for the enzymatic digestion technique were variable, the current study conducted a series of experiments to find the optimal strength of collagenase for isolating viable LPCs.

For the preparation of cells, gut tissues were removed immediately after slaughter and kept in warm PBS containing 2.5% foetal calf serum until the tissue was processed after 20 minutes. The cell isolation protocol, using the mucolytic agent dithiothreitol and cell dissociating agent diaminoethanetetraacetic acid (EDTA) in Ca+Mg+free medium, has been applied by others for the removal of epithelial-bound cells from the small intestine (Bull & Bookman, 1977; Davis & Parrot, 1981). This process helped to isolate or remove epithelial-bound cells without disrupting the lamina propria tissues.

EDTA removed most of the epithelial cells in the first 3 hrs of cell isolation work. To check whether there was any cross contamination of cells between two compartments, the EDTA treatment time of 3 hrs was divided into 6 periods of 30 minutes; and cells recovered at each 30 min period were carefully examined to check for the presence of lamina propria-bound cells. MMDs and MMCs can be use as indicator cells which are lamina propria-bound and have no history of trans-epithelial migration as eosinophils, neutrophils, GLs and lymphocytes do. But, one difficulty for MMCs is that sometimes, in the event of continuous nematode challenge, MMCs tend to move towards the epithelium as well as the epithelial border where they easily get damaged by the host inflammatory response to the nematodes; and thus they can be found in the epithelial isolates. In general, these two cells are good indicators for detecting LPC contamination in epithelial isolates; however, the best is MMDs, which do not populate like MMCs in the mucosa (the epithelial & lamina propria compartments) and always stay within the lamina propria compartment.

Gut tissues, soaked with 0.05mM EDTA in $Ca^{-}Mg^{+}$ free medium, released a maximum number of cells at 60 min, and 90 min was quite sufficient to remove most of the cells. This finding was in agreement with Davis & Parrot (1981), Bull and Bookman (1977) and Gyorffy *et al.* (1992). Cells, after isolation in each batch of EDTA treatment were immediately prepared for cytospots and stained with Leishmann's stain for differential counts. The highest yields of GL were recorded at 60 and 90 minutes which coincided with the time when the maximal yield of cells was recovered. MMDs (10%) appeared in the 150 and 180 min epithelial isolates (Table 3.2); and this indicates the connective tissues of basement membranes and lamina propria tissues were somehow damaged by EDTA treatment, or agitation by the orbital shaker may have released the LPCs into the epithelial isolates. This is an important observation, establishing that an incubation time of between 60-120 minutes is the best time to isolate more pure epithelial cells before contamination with lamina propria-bound cells (Table 3.2).

The enzyme technique used was a modification of a number of methods applied for the recovery of intestinal lymphoid cells from mice (Davies & Parrott, 1981), humans (Bull & Bookman, 1977) and cattle (Nagi & Babiuk, 1987). For the isolation of LPCs collagenase enzyme was selected as the enzyme of choice since it has been commonly used for isolating cells from different tissues by several investigators (Dirami *et al.*, 1991; Joshi, 1995; Laudes *et al.*, 2001; Nagi & Babiuk, 1987), and it is claimed to be gentle, and led to a greater recovery of cells (Fresheney, 1994). This quality of collagenase was attested when its concentration reached 200-400U/ml. The present experiment suggests that a high concentration of enzymes (200-400U/ml) recovered more cells than low concentrations (50-100U/ml), but there is a cost to pay

in that the cell viability could be affected. Taken together, the results from this study indicated that a 3 hr EDTA treatment to isolate/remove epithelial cells, and collagenase at a concentration of 100U/ml (in combination with hyaluronidase) was the most efficient method for the digestion of small intestines from all the experimental sheep, and this protocol was used for the isolation of epithelial or lamina propria cells. It is worth noting that the optimal concentration of collagenase type I (100U/ml) in this study was very close to that of Huntley *et al.* (1982). In addition, the technique developed in the study provided cells of sufficient purity from each compartment to assess their involvement in the production of LPF.

3.3.2.2 Comparing LMI activity of epithelial and lamina propria cells

Small intestinal LPCs produced significantly higher LMI activity than their counterpart epithelial cells. Interestingly, LPC cultures contained significantly higher yields of effector cells, notably, eosinophils and MMCs, but GLs were predominantly found in the epithelial cell fraction which produced no LMI activity (Table 3.5).

There are no previous reports ascribing the source of LMI activity to small intestinal LPCs, and the morphology and staining pattern of cells in the intestinal mucosa has not been reported as systematically as in the current study. The antigen-dependent release of LPF observed in this experiment was similar to the observation recorded in Experiment 1.

Among the experimental sheep, the two sheep hyper-immunised in the pen showed a tendency to produce higher yields of LPF than the two field resistant-line sheep with natural infection (Table 3.5). This finding supports the suggestion that the magnitude of anti-nematode response of sheep depends on both the intensity and the duration of infection to which sheep are exposed during immunization (Anderson, 1987; Barger *et al.*, 1985a; Barnes & Dobson, 1993).

This study also adapted a selective combined EDTA and enzymatic digestion technique which suited the needs of the current experiments. In most previous studies for detecting gut immune responses of sheep, researchers made differential counts of cells in situ in tissues rather than on cytospots after digestion into single-cell suspension. Information regarding the latter technique is scant, although some studies used single cell suspension for enrichment of MMC/GL (Douch *et al.*, 1996b; Huntley *et al.*, 1984; Stankiewicz *et al.*, 1994), but data on differential cell counts was not given. Even when it was, the results could not be compared since their techniques involved a straight digestion of whole gut tissues, whereas the present work isolated cells separately from each compartment of the mucosa.

Nagi and Babiuk (1987) recorded differential cell counts performed on cytospots of bovine epithelial and LPCs of jejunum after Wright-Giemsa staining. Although, their isolation procedures seemed similar to the present study, there was one important technical difference. Nagi & Babiuk opened up or diced the gut tissue and thus cell contamination between the two compartments was unavoidable. In the current study, the intestine was everted and both ends of it tied to stop contamination by serosal cells. This method of handling the gut allows cells from different layers to be obtained at different incubation times as described by Stankiewicz *et al.*(1994).

Nagi & Babiuk (1987) illustrated monocytes (4%) and mast cells (4.5%) in their Wright-Geimsa and toluidine blue stained cytospot smears of Percoll purified epithelial isolates, although these cells are in fact strictly lamina propria-bound. This contamination was probably due to the dicing of jejunal tissue which opened up the lamina propria compartment, allowing the tissue to be exposed to EDTA. In contrast, the present study shows MMD were populated strictly in the lamina propria, and not in epithelia. In addition, Nagi and Babiuk combined plasma cells (which are lymphocytes), GOs, epithelial cells and dendritic cells together and all were tabulated as 'other' type of cells. Both of the studies recovered >70% lymphocytes in both compartments but the present work recovered 35% and 42% in epithelial and LPCs respectively.

The present study clearly demonstrates that lamina propria cells are a better *in vitro* mucosal source of LPF than epithelial cells, and judging from the differential cell count data, MMD were found only in lamina propria isolates and not in the epithelial isolates, indicating that the technique used in the present study is able to isolate cells from each compartment with less contamination. MMD are shown to be good indicators of lamina propria cell contamination in epithelial cell isolates during the cell isolation process. Furthermore, with regards to functional characteristics, isolated mucosal cells were shown to respond to TcL3ES antigens and produce larval paralysis activity which opens the way for further experimentation.

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Chapter 4
Optimisation of larval paralysis factor

4.1 Introduction

In Chapter 3 we reported a reliable mucosal source of larval paralysis factor (LPF), and for further research to identify LPF, a stock of quality LPF was needed. Of the two potential sources of LPF (*i.e.*cell culture supernatant & mucus) cell culture supernatant (CCS) seemed better suited to bioassay applications, purification, and chemical and structural elucidation of the factor. Nevertheless, the LMI activity of CCS reported in Chapter 3 was quite varied, and even when the mucosal source of LPF was limited to a specific anatomical compartment (*i.e.* small intestinal lamina propria), the activity was still not satisfactory. Another interesting finding was that LPF release *in vitro* was parasite-antigen dependent. To improve the quality and quantity of LPF, the relationship between host and parasite at the gut level was explored and three experiments were designed to optimise the best condition(s) required for *in vitro* LPF production.

4.2 Materials and methods

All the materials and methods are described in Chapter 2.

4.2.1 Experiment 1: Effect of immune status and husbandry practices on LPF production

Rationale and aim: It is known that anti-nematode immunity in sheep is influenced by the magnitude and duration of exposure to infection (Adams, 1983; Gill *et al.*, 2000; Jackson *et al.*, 1988; Smith *et al.*, 1985a) and also it is under genetic control (Bisset *et al.*, 1996), but it is not absolute (Altaif & Dargie, 1978b) . The variation of LMI activity in Chapter 3 may be due to either different levels of challenge received by the host or the immune capability of the individual host to respond to nematode parasites. To obtain the best animals for LPF production, this chapter compared the LMI activity of CCS from genetically-resistant sheep living in a pen, living in the field and out-bred sheep made immune in a pen using the truncated immunisation

method. The experiment used two other types of sheep, nematode naïve sheep living in a pen and genetically susceptible sheep living in the field, as experimental controls.

Experimental approach

Five groups of animals with different immune status or living conditions were used for comparing the LMI activity of CCS and mucus, as follows: - Group 1: four 6 month-old pen-raised out-bred lambs were used as nematode-naïve sheep; Group 2: four adult susceptible-line ewes raised in the field; Group 3: four resistant-line ewes raised in the field; Group 4: four resistant-line ewes raised in the field, and then sensitised and killed using the 'LPF regime' (Chapter 2.1.1.2) in a pen. Group 5: three 6 month-old nematode-naïve-outbred lambs reared in a pen were made immune using the truncated immunization, resensitized and killed using the 'LPF regime'. After food was withheld for 24 hrs and humane killing, two 50 cm gut pieces from the first metre of small intestine after the bile duct entry were collected, one for isolating lamina propria cells (LPCs) and another for preparing mucus. All the CCS and mucus samples were assayed for LMI activity *in vitro*.

4.2.1.1 Results

4.2.1.1.1 Parasitology

The faecal egg counts of the five groups were as follows: Group 1 (Naive control) - all zero; Group 2 (susceptible-line) – 1300, 3100, 3800, 1100; Group 3 (Field resistant-line) - 100, 100, 0, 0: Group 4 (field resistant-line ewes re-sensitised and killed using the LPF regime in pen) - all zero; Group 5 (nematode-naïve lambs made immune using the truncated immunization, re-sensitised and killed using the LPF regime) - all zero.

4.2.1.1.2 Enumeration of cell types

Table 4.1 demonstrates that lymphocytes formed the highest portion (20%-57%) of LPCs in all the groups, with the highest level (35%-57%) being in the non-immune groups (Group 1 and 2). Other cell types - MMDs, neutrophils, GLs and epithelial cells - appeared quite evenly in all the groups. Although the lowest levels (3%-8%) of MMCs were present in susceptible-line sheep, they were recovered more (10%-18%) in naïve lambs. Eosinophil numbers tended to be higher in nematode infected sheep rather than in nematode-naïve sheep. Interestingly, Group 4 (genetically resistant-line sheep) and Group 5 (outbred truncated immunised sheep) which received booster infections in the pen had higher percentages of goblet cells (GOs) than the other groups (Table 4.1).

4.2.1.1.3 Larval migration inhibition activity

Table 4.1 and Fig. 4.1 show that all sheep produced measurable LMI activity which varied

Group	Small intestinal lamina prpria cells in the cell culture							LMI%	
	L	EO	N	MMD	MMC	GL	EO/GO	Mucus	CCS
(1) A	39	11	1	12	10	0	27/0	16	2
B	35	19	2	15	11	0	15/3	29	8
C	55	8	1	4	14	3	14/1	9	12
D	57	5	1	2	18	3	10/4	19	15
(2) A	43	31	1	4	3	0	15/3	41	28
B	37	18	0	12	8	0	24/1	22	36
C	43	13	3	9	4	3	22/3	7	6
D	42	15	0	5	7	0	28/3	19	5
(3) A	29	38	0	2	7	2	19/3	60	71
B	23	55	0	8	8	0	4/2	75	63
C	25	18	1	7	23	0	22/4	56	60
D	47	13	2	6	11	1	18/2	50	58
(4)A	26	15	2	12	11	3	18/13	70	73
B	37	8	1	9	9	0	23/13	68	94
C	28	12	2	13	11	1	22/11	89	98
D	20	21	1	12	13	3	18/12	75	84
(5)A	25	21	0	4	15	2	21/12	92	89
B	35	28	0	6	9	0	14/8	74	66
C	32	22	3	4	11	1	16/11	86	95

Keys: L -lymphocyte; N – neutrophil: EO – Eosinophil; MMD – monocyte-macrophage-dendritic cells; MMC- mucosal mast cell; GL-globule leucocyte; EP/GO – epithelial/goblet cell; LMI% - proportion of larvae whose migration is inhibited. Group 1- nematode-naïve ewe lambs; Group 2 - field susceptible-line ewes; Group 3-field resistant-line ewes; Group 4 -field resistant-line ewes re-sensitised in pen; Group 5 - nematode-naïve outbred lambs which were made immune by using truncated immunisation regime and received similar re-sensitisation like group 4;

Table 4.1 LMI activity of CCS and mucus derived from groups of sheep with different immune status and rearing under different husbandry conditions. Small intestinal lamina propria cells in the cell cultures were differentiated and counted from their Leishmann stained cytospots. Crude data are tabulated in Appendix 4-5.1.

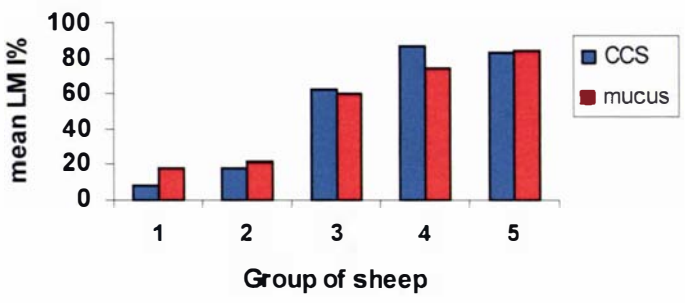

Fig. 4.1 Mean LMI activity from different types of sheep. 1 - Outbred nematode naive sheep; 2 - Field susceptible-line sheep; 3 - Field resistant-line sheep; 4 - Field resistant-line pen-sensitised; 5 - Outbred pen immunised and re-sensitised in pen. Each data point is the mean of four sheep; and the crude data are tabulated in Appendix 4-5.1.

Gut segments	L	EO	N	MMD	MMC	GL	EP/GO
1	43	8	0	5	12	3	19/10
3	26	13	1	9	13	6	25/8
6	26	20	1	8	11	2	23/9
12	25	46	0	6	7	0	11/4
18	30	56	0	2	4	0	6/2

Keys: L -lymphocyte; N – neutrophil: EO – Eosinophil; MMD – monocyte-macrophage-dendritic cell; MMC- mucosal mast cell; GL-globule leucocyte; EP/GO – epithelial/goblet cell;

Table 4.2 Mean percentage of each cell type recovered from the lamina propria cells of different sites of small intestine (n=5).

considerably according to the immune status of the group. The CCS and mucus data were analysed by one-way ANOVA with sheep as blocks (Appendix 5 & 6). There were significant differences between the groups. Mucus and CCS of all immune groups (Group 3, 4 and 5) had significantly higher levels of LMI activity ($p<0.01$) than the naïve (Group 1) and susceptible-line group (Group 2). The highest LMI activities (>75%) were obtained in the two immune groups receiving repeated sensitization in pens (Group 4 & 5).

4.2.2 Experiment 2: Effect of anatomical site on LPF production

Rationale and aim: In Chapter 3, the first metre of the small intestine was used to produce CCS and mucus. *T. colubriformis* populates preferentially in the anterior part of the small intestine (Wagland *et al.*, 1996), but whether this may affect LMI activity produced in other parts of the small intestine was unknown. The next experiment was designed to compare LMI activity of LPCs from different parts of the small intestine in resistant-line sheep.

Experimental approach
Five two year old field-raised resistant-line ewes were used. Sheep were drenched on arrival at the pen and a week later they were sensitised by the 'LPF regime'. Two weeks after the sensitisation period, 2 sheep per day on the first and second day, and one on the last day were slaughtered on 3 consecutive days. After food was withheld for 24hr and humane killing, a 50 cm small intestinal piece was removed at 1, 3, 6, 12 and 18 meter from the pylorus for the preparation of mucus and LPCs. The resultant CCS and mucus were stored at –20°C until assayed for LMI activity.

4.2.2.1 Results

4.2.2.1.1 Parasitology
Faecal egg counts were all negative.

4.2.2.1.2 Enumeration of cell types
Table 4.2 shows the differential composition of LPCs recovered from the different areas of the small intestine. Light microscopic examinations revealed that high proportions of lymphocytes (26%-43%), eosinophils (8%-56%) and epithelial cells (8%-33%), and low proportions of neutrophils, MMDs, MMCs and GLs, were commonly seen in the cytospots. Interestingly, GOs, MMCs and GLs appeared more frequently in the 1, 3 and 6 metre points whereas, eosinophils were more common in the distal part of small intestine (12^{th} & 18^{th} metre points).

Unexpectedly, the morphology of both eosinophils and MMCs varied between the different populations.

Three different types of eosinophil were observed. Marked distinctions in cell size, enlargement of the nucleus and the character of its segmentation, and in the distribution of cytoplasmic granules were observed in the populations. For simplicity and for discussion purposes, they were termed as Stage I – normal eosinophil or Stage II – activated eosinophil or Stage III - degranulated eosinophil. Stage I eosinophils were rounded and small (10.26 ± 0.78 μm), the nucleus was roundish and compact, and the cytoplasm was densely packed with coarse orange-red granules; these cells are presumed to be mature and at a pre-activation stage (Fig. 4.2,1). Stage II eosinophils were rounded and larger (13.66 ± s.d. 0.76 μm) than the former, they had a large multi-lobed nucleus, mostly with 3-4 lobes dispersed loosely in the cytoplasm and an increase in the amount of coarse orange-red granules which were densely packed in the cytoplasm (Fig. 4.2, 2). Stage III eosinophils were usually seen in the lamina propria cell population; they were also large with marked differences. These cells were elongated and irregular in outline, the nucleus was large with less defined segmentation, and the cytoplasm was lacking coarse deeply eosinophilic granules, but filled with fine loosely packed granules (Fig. 4.2,3).

Four different types of MMC were observed, based on a marked variation in the amount of large basophilic granules and the visibility of nuclei in the cytoplasm (Fig. 4.3). Stage I MMCs were granulated, filled with densely-packed characteristic basophilic granules which obscured the nucleus (Fig. 4.3,1); cell size was variable (15-25 μm) and most of the smaller ones were round and deeply basophilic. Stage II MMCs were partially degranulated in which some large granules had already escaped, revealing the nucleus (Fig 4.3, 2). In stage III, highly degranulated MMCs, only very fine granules were left scattered in the cytoplasm (Fig. 4.3, 3). In stage IV - completely degranulated MMCs, only a few fine granules were left in the globular-textured cytoplasm (Fig. 4.3, 4), and the cells revealed the true shape of the nucleus which was oval in outline and situated more or less centrally. In addition, very small perhaps immature MMCs were also recovered in the isolates. They had characteristic basophilic stained granules which totally obscured the nucleus, and some were even smaller than the size of small lymphocytes (Fig. 4.4).

Light microscopic observations revealed that the 1st and 3rd metre segments had higher numbers of Stage II – activated eosinophils than did the lower segments which were mainly populated with Stage I – normal eosinophils (see data in Appendix 10). Judging by one-way ANOVA

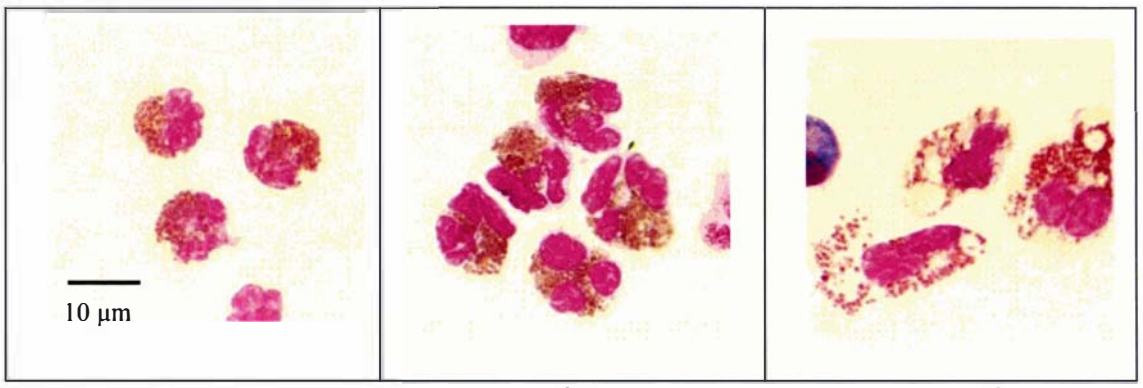

Fig. 4.2 Different morphologic profiles of eosinophil in the oil-immersion microphotograph of Leishmann-stained cytosmears of enriched lamina propria cells. (1) – Stage I – normal EO: rounded and small (10.26 ● s.d. 0.78 μm); the cytoplasm is densely packed with coarse orange-red granules; nucleus is bi-lobed and compact. (2) - Stage II - activated eosinophils are rounded but larger (13.66 ± s.d. 0.76 μm) than pre-activation stage, nuclei become bigger and subdivided into 3-4 lobes and they are dispersed in the cytoplasm; coarse orange-red granules are densely packed in the cytoplasm. (3) – Stage III - degranulated eosinophil: elongated and irregular in outline with large nucleus single or bi-lobed; cytoplasm is loosely packed with fine granules.

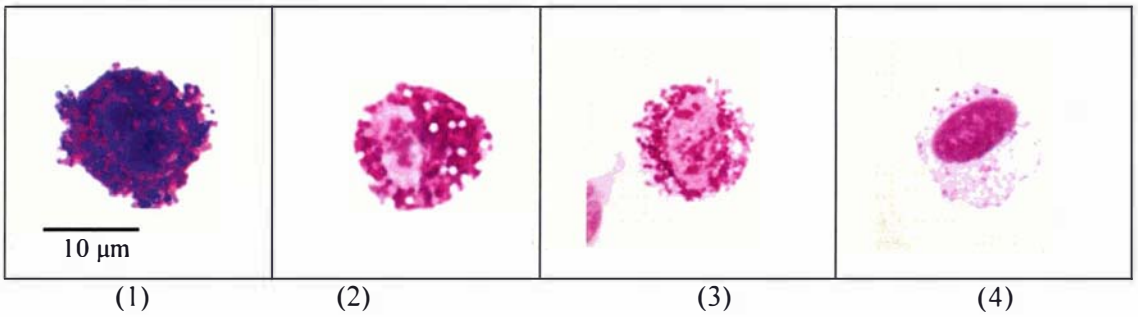

Fig. 4.3 Different morphologic profiles of mucosal mast cell in the oil-immersion microphotograph of Leishmann-stained cytosmears of enriched lamina propria cells. (1) Stage I – pre-degranulated MMC: it is in pre-degranulation stage with densely packed characteristic basophilic granules which obscure the nucleus. (2) Stage II - partially degranulated MMC: some large granules have escaped and that also reveals the nucleus. (3) Stage III - highly degranulated MMC: only very fine granules are left and scattered in the cytoplasm. (4) Stage IV - MMC in complete degraulation stage: it contains very few paler staining granules in the globular textured cytoplasm; it reveals the true shape of nucleus which is oval in outline and situated more or less centrally.

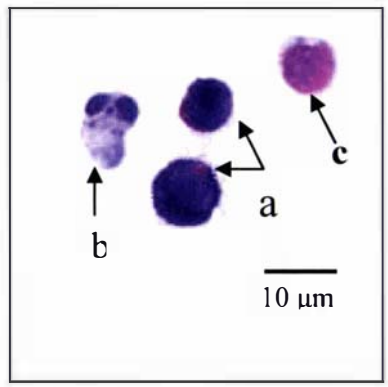

Fig. 4.4 (a) - Immature mucosal mast cells (7-10 µm); they are rounded with deep basophilic stained cytoplasm which obscure the intra-structure of the cell; (b) - Immature epithelial cell; (c) - Small lymphocyte.

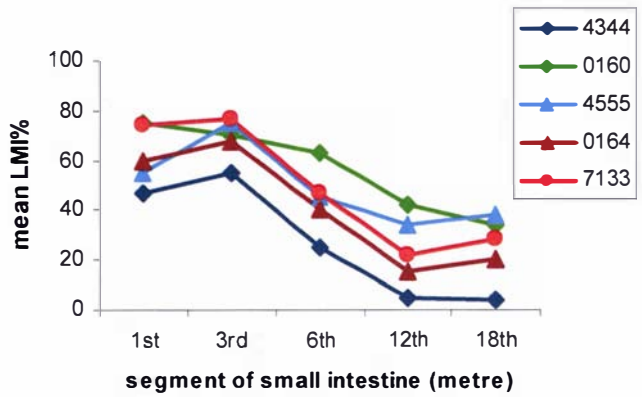

Fig. 4.5 LMI activity of CCS of enriched lamina propria cells derived from different segments of small intestines; each data point is the mean of triplicate sample. Data are tabulated in Appendix 6.

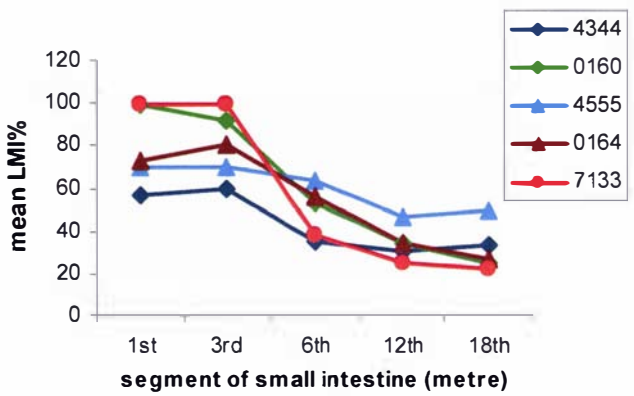

Fig. 4.6 LMI activity of mucus derived from different segments of small intestines; each data point is the mean of triplicate sample. Data are tabulated in Appendix 7.

DAC	Sheep ID	High anti-IgE	Sheep ID	Low anti-IgE
0	B008	0.31	0357	0.18
1	1208	0.52	3375	0.14
3	3802	1.04	1077	0.062
6	4029	0.17	3137	0.12
12	8135	0.44	3041	0.18

Key: DAC – day after challenge;

Table 4.3 Mean serum anti-*Trichostrongylus colubriformis* IgE level of experimental sheep after pen sensitization.

analysis, the mean percentages of stage I eosinophils were significantly different between segments (p level <0.001); and the 3rd segments obtained the highest numbers of stage II eosinophils (see data in Appendix 10 & 10.1). In contrast, there was no difference in the proportion of different MMC types between different gut segments (see Appendix 11.1-3). However, a trend of higher overall percentages of MMC or GL was found in the mucosal cell isolates of the anterior 6 metres than the posterior part of the small intestine (Table 4.2).

4.2.2.1.3 Larval migration inhibition activity

The LMI activity of CCS from the different segments was highly correlated with LMI activity of the mucus taken from corresponding gut segments (r = 0.88; n = 5) (*see* Appendix 8). According to the one-way ANOVA analysis (*see* Appendix 6.1 & 7.1), LMI activity of both CCS and mucus differed significantly between the segments. The LMI activities of the 1st and 3rd segments were not different but were significantly greater than the others. Also, the 6th was greater than the 12th and 18th segments, but the 12th and 18th were not different. However, regardless of the type of sample (CCS or mucus), the 3rd metre of the gut seemed to have the highest LMI activity (Fig. 4.5 & 4.6). Only the LMI activity of CCS of the 3rd segments showed some relationship with the proportion of eosinophils (r = 0.77; n = 5) and the proportion of MMCs (r = 0.66; n = 5) in the cell population, but the correlations were not significantly different from zero (Appendix 9).

4.2.3 Experiment 3: Effect of time after nematode challenge on LPF production

Rationale and aim: Evidence is accumulating that the immune sheep gut responds within a day to oral challenge (Elliot, 1981) or within 2 hr (Harrison *et al.*, 1999; Wagland *et al.*, 1996), 4 hr (Miller *et al.*, 1983a), 1-4 days (Chiejina & Sewell, 1974; Emery *et al.*, 1992b; McClure *et al.*, 1992) to a surgically inoculated nematode infection and the immunity can be sustained without further challenge for up to 6 weeks (Jackson *et al.*, 1988). This information could provide some indications as to when immune sheep should be killed after challenge to isolate reactive gut mucosal cells. Thus, the experiment was designed to investigate the best time after challenge to isolate mucosal cells to produce active CCS.

Experimental approach

Ten two year old resistant-line ewes (5-9 years old) were drenched on arrival in a pen and immunised as per 'LPF regime'. The ewes were grouped in pairs, so that one animal had low and another high levels of serum anti-*T. Colubriformis* IgE determined at the end of pen sensitisation (see Table 4.3). The reason for this was, at the time of the experiment, MMCs

were speculated as a source of LPF and it has been historically known that IgE mostly binds MMC in many species. Therefore, it was possible that the nematode-specific serum IgE level could be a good marker for MMC numbers in the gut as well as for LMI activity *in vivo* and *in vitro*. Two weeks after the sensitisation period, a pair of sheep was starved for 24 hr and killed at one of four different times (1, 3, 6, and 12 days) after receiving challenge infection, and one pair was killed without challenge infection (experiment control). Two 50 cm gut pieces were removed from the second metre of small intestine after the pylorus for the preparation of mucus, CCS and histology. All the mucus and CCS samples were assayed for LMI activity.

4.2.3.1 Results

4.2.3.1.1 Parasitology

The faecal egg counts at slaughter were all zero.

4.2.3.1.2 Differential cell counts

The cell types recovered in lamina propria isolates from each of the different days after challenge infection are tabulated in Table 4.4. It illustrates that lymphocytes comprised the highest proportion of cells in all samples.

Lymphoblasts (Fig. 3.1B, 8b) were recovered mostly in day 3 samples (~20%). Neutrophils were present in very low percentages (0-2%) in most of the samples and some were not included in the totals since they were less than 1%. GOs were recovered more on days 3 and 6. Interestingly, morphological gradation of eosinophils and MMCs (Table 4.5) was also observed in the cell isolates as described in Fig 4.2 & 4.3. Day 3 samples had the highest number of Stage II - activated eosinophils (Fig 4.2,2) in the groups, but day 6 had more of these cells than day 0 (control), 1 and 12 samples. Stage III - degranulated eosinophils appeared on day 6, but on day 12 the stage of eosinophils was similar to day 0 and 1 which had a higher number of Stage I - normal eosinophils. A similar morphological gradation of MMC as described in Experiment 2 was observed (Table 4.5). Immature MMC (Fig. 4.4) also appeared in a small proportion in day 0 samples, and the diameter was similar to lymphocytes (7-8 μm) and filled with dark basophilic stained cytoplasm (granules were not as distinctive as they appear in mature MMC). Higher numbers of mature granulated MMCs (Fig. 4.3,1) were observed on day 0, 1, 6 and 12 than on day 3. Marked distinctions in cell size of mature granulated MMCs between day 0 and day 1 pairs were displayed; in day 0 samples, cells were round, mean cell diameters were 11.90μm (sheep 0357) and 11.18μm (sheep B008) and had finer dark basophilic granules whereas MMCs on day 1 were irregular in shape and their diameters were larger (14.06 μm in sheep 3775 and 13.81μm in sheep 1208) and their granule content subsequently increased

DAC	Sheep ID	L	EO	N	MMD	MC	GL	EP/GO	LMI%
0	B008	50	7	2	6	21	0	10/-	48
0	0357	38	15	1	5	10	0	28/4	63
1	1208	68	7	1	3	8	3	7/3	50
1	3775	40	20	1	9	11	0	9/10	73
3	1077	42	19	0	2	12	5	13/7	98
3	3802	46	18	0	7	9	1	13/6	99
6	3137	58	6	0	2	23	0	6/5	73
6	4029	65	11	0	1	11	0	10/2	56
12	3041	50	14	1	5	18	3	10/0	69
12	8135	41	9	0	11	21	4	9/5	83
Correlation coefficient (*r*)		**-0.55**	**0.62**	**-0.5**	**0.24**	**0.02**	**0.22**	**0.02/0.62**	

Keys: L -lymphocyte; N – neutrophil: EO – Eosinophil; MMD – monocyte-macrophage-dendritic cell; MMC- mucosal mast cell; GL-globule leucocyte; EP/GO – epithelial/goblet cell; LMI% - larval migration inhibition %; Correlation coefficient (*r*) between LMI% and cell type present in CCS is tabulated and in bold print. DAC = day after challenge.

Table 4.4 Enriched lamina propria cells in the cell cultures from 5 groups of sheep and their relative LMI activity. LMI assay data are tabulated in the Appendix 12.

DAC	Sheep	(a) Type of eosinophils			(b)Type of mucosal mast cells			
		1	2	3	1(*)	2	3	4
0	**B008****	76	17	7	84(16)	0	0	0
0	0357	95	5	0	90(3)	7	0	0
1	**1208**	84	0	16	78	22	0	0
1	3775	66	34	0	84	16	0	0
3	**3802**	29	71	0	31	8	59	2
3	1077	39	61	0	21	9	65	5
6	**4729**	23	20	57	10	12	57	21
6	3137	59	25	16	8	15	64	13
12	**3041**	81	19	0	79	21	0	0
12	8135	88	12	0	58	38	0	4

DAC - day after challenge; * The numbers in brackets are immature MMC; ** High serum-IgE sheep are printed in bolt type.

Table 4.5 Morphological profiles of eosinophils and mucosal mast cells, recovered in different days after challenge, in Leishmann-stained cytosmears of lamina propria cells. One hundred cells of each cell type were counted. The morphologies of the variants were described in detail in Fig 3.5 & 3.6. (a) - Three types of eosinophils appeared in oil-immersion microphotograph of Leishmann stained cytosmears of lamina propria cells: 1- Mature eosinophils; 2 - Activated eosinophils; 3 - Degranulated eosinophil; (b) - Four stages of mucosal mast cell in oil-immersion microphotograph of Leishmann stained cytosmears of lamina propria cells. (1) Mature granulated MMC; (2) Partially degranulated MMC; (3) Highly degranulated MMC; (4) Completely degranulated MMC;

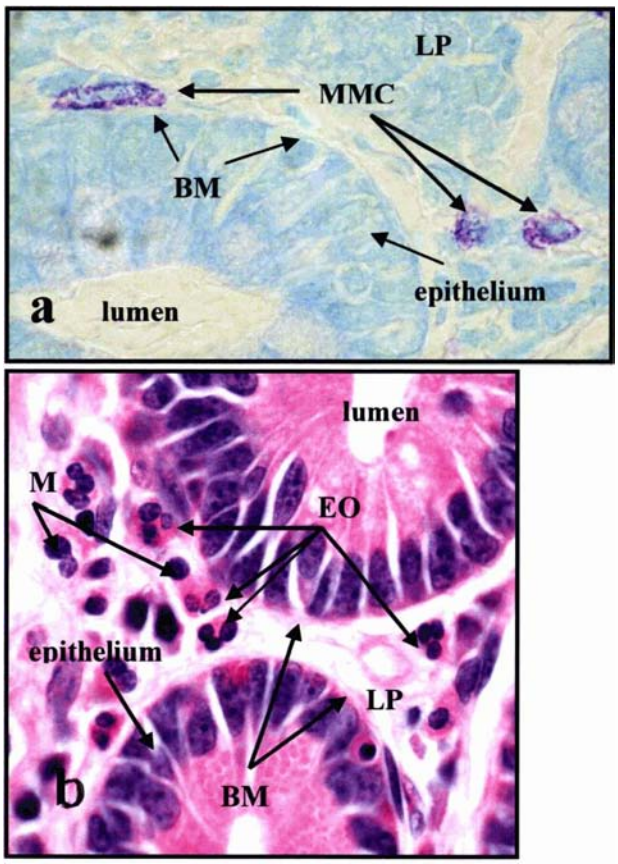

Fig. 4.9 Oil immersion microphotographs of small intestinal tissues of day 0. (a) Mucosal mast cells in gut mucosa of toluidine blue-stained tissue, (b) Eosinophils in gut mucosa of hematoxylin-eosin-stained tissue. It shows both cell types are positioned next to the basal membrane of epithelium. Magnification x 1000. Keys: BM - basal membrane of epithelium; EO – eosinophil: M – mononuclear cells; LP - lamina propria; MMC - mucosal mast cell. Magnification x1 000.

DAC	sheep	PAS stained goblet cell counts	s.d	HID stained goblet cell counts	s.d
0	B008	16	5	20	8
0	0357	25	8	24	8
1	1208	14	3	19	4
1	3775	27	7	28	13
3	1077	**32**	12	**37**	5
3	3802	**32**	11	**44**	14
6	3137	19	5	22	6
6	4029	27	11	25	11
12	3041	10	1	7	3
12	8135	10	4	11	4

Table 4.6 Changes of histochemical staining properties of goblet cell mucin in immune sheep after challenge infection. Both PAS and HID stained goblet cell numbers were peaked at 3DAC. Tissue cell counts are tabulated in Appendix 19 & 21.

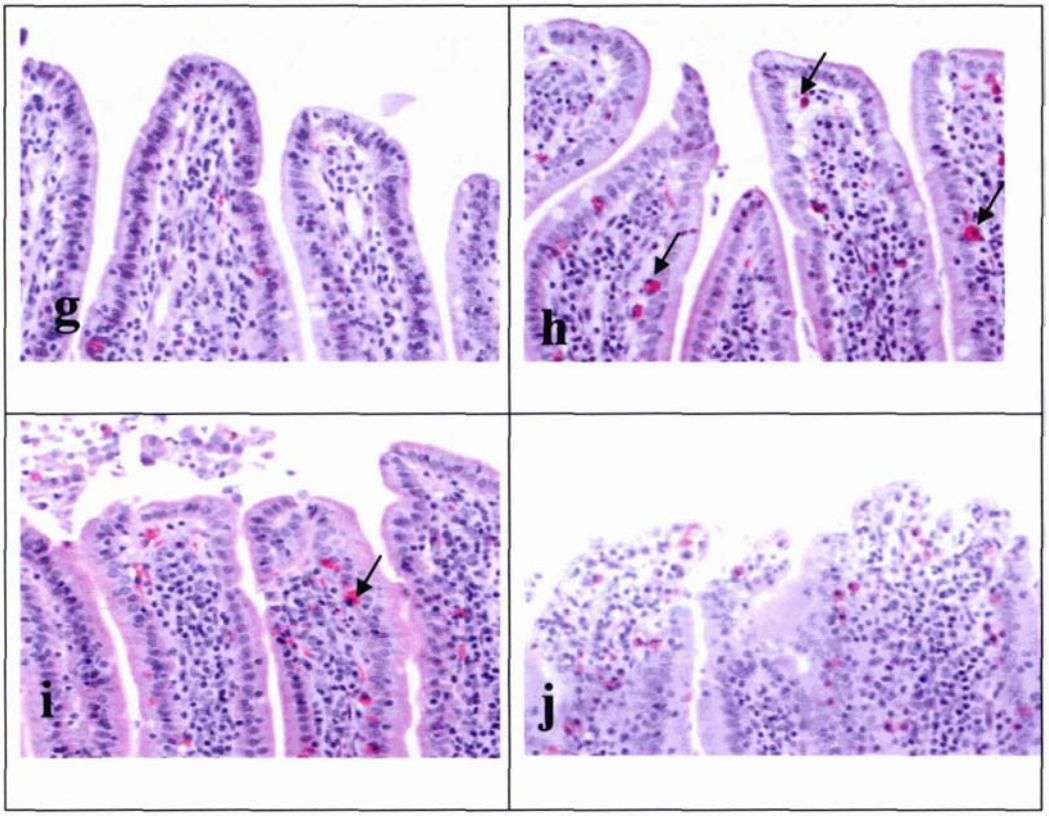

Fig. 4.7, B Oil-immersion microphotographs of hematoxylin-eosin stained small intestinal tissues. (g) & (h) – Day 6: gut tissues from sheep 4029 and 3137 showed the epithelium in most of the areas was nicely resealed. (i) & (j) – Day 12: sheep 8135 & 3041 showed epithelium was well sealed but the villus tips were flat and most were broad shoulders, but few areas in the latter sheep (high IgE) the villi were still open. Magnification x200.

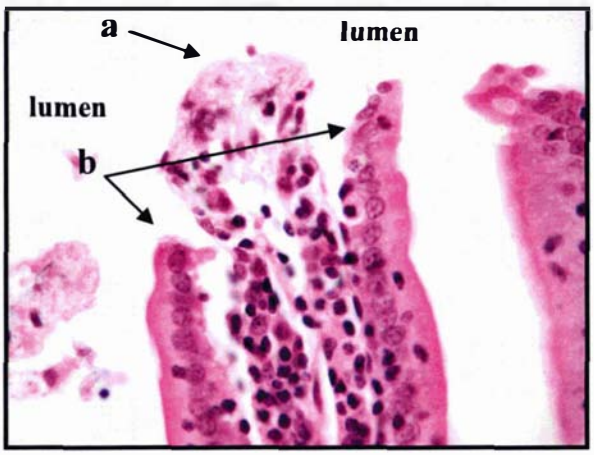

Fig. 4.8 Oil emersion microphotograph of a small intestinal villus on day 1 after challenge. (a) Lamina propria tissues exposed to the lumen. (b) Desquamative epithelium. Magnification: x500.

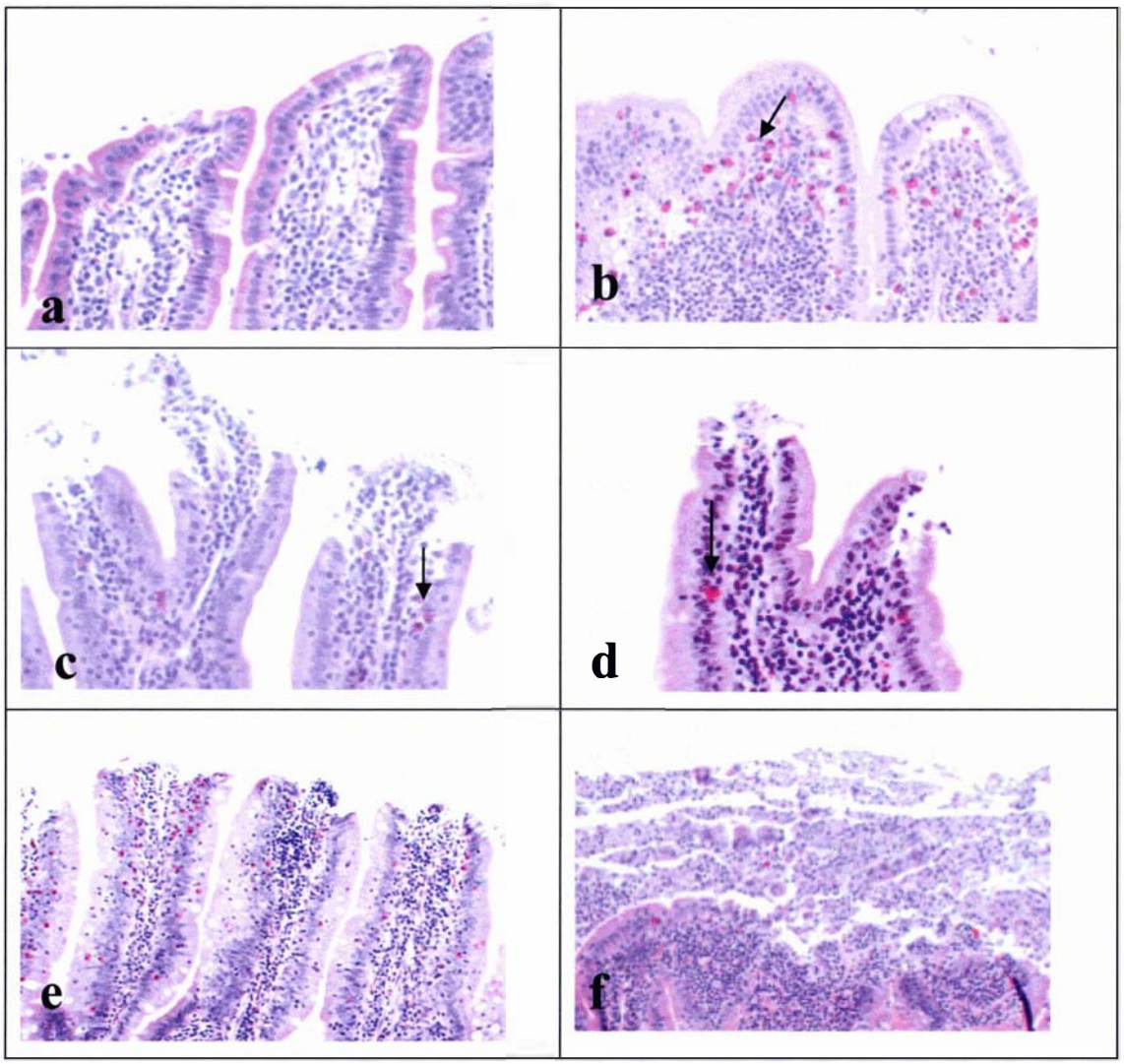

Fig. 4.7, A Oil-immersion microphotographs of hematoxylin-eosin stained small intestinal tissues. (a) & (b) - Control (sheep with no challenge): small intestinal villi from Sheep B008 and 0357 showed normal integrity and well sealed epithelium. (c) & (d) – Day 1: only the villus tips were damaged and opened, and some lamina propria tissues exposed to the lumen in both sheep. GLs with eosinophilic stained globules (arrowed) in sub-epithelial position. (e) & (f) - Extensive damage of villi on day 3 in both sheep; (e) - in most areas at least part of the remaining villi was gone; (f) - most areas were covered with a thick blanket of mucus. Magnification x200.

as well as the cell size and they had distinctive numerous dark basophilic granules. The size of cells between day 0 and day 1 was significantly different ($p=<0.001$) (crude data are tabulated in Appendix 24). The appearance of larger cells found on day 1 suggested they were undergoing activation. Furthermore, partially degranulated MMCs (~19%) started to appear by day 1 and the number quickly increased to approximately 60% by day 3 and 6, but they disappeared by day 12. LMI activities of CCSs were found to have some relationship with eosinophil yields ($r=0.62$; n=10) and with GOs ($r=0.62$; n=10) (Table 4.4). It was the second time eosinophil yields were correlated with the LMI% of CCSs, but the correlation was not significantly different from zero ($p \leq 0.5$).

4.2.3.1.3 Histological observations

The oil immersion microphotographs of small intestinal tissues at different days after challenge are displayed in Fig. 4.7, A & B. Not a single larva was seen in any of the tissues examined from challenged or unchallenged sheep. The tissue cell counts are tabulated in Appendices (13, 15, 17, 19 & 21).

On day 0, 6 and 12, epithelia were well sealed and had normal integrity (Fig. 4.7, A & B). However, intriguingly on day 1, the villous tips were damaged and showed marked epithelial desquamation with lamina propria tissues oozing out into the lumen and becoming slightly hyperaemic (Fig. 4.7, A, c & d and Fig. 4.8). On day 3 there were depressed surface lesions in the mucosa, with extensive villous atrophy and a thick blanket of mucus on most surfaces. On day 0, 6 and 12 the villi had epithelium with normal integrity but most of the villi were short with broad shoulders; villous atrophy was found most intensely on day 3, when some villi were even shorter than half the length of villus of other time points and some were almost flattened. The jejunum on day 0 (un-challenged but received the last immunisation infection 14 days prior to slaughter) and day 12 had less cellularity than day 1, 3 and 6. The villi in the latter group had a significant influx of eosinophils through out the villous lamina propria from sub-mucosa to epithelium (Appendix 13). In addition, day 3 had the highest GO counts (both PAS & HID stained GOs) among other days (Table 4.6) (Appendices 19 & 21).

According to one-way ANOVA analysis, tissue MMC counts differed significantly between days after challenge ($p<0.001$; n=5), but not between day 1 and day 3, and the MMC counts were lower on these two days than on other days (Appendix 18). MMCs were mostly dispersed in the lamina propria; but on day 1 most of them moved beneath the epithelium. Before challenge (day 0) MMCs as well as eosinophils and lymphocytes were usually located next to the basal membrane of the epithelium (Fig. 4.9, a & b). All the high serum IgE sheep had

higher tissue GL and MMC, and pathology (*i.e.* the shortening of villi and thicker mucus blankets) than did the low IgE group. However, the tissue MMC counts between the two groups were not statistically different at p level 0.05. In addition, the tissue MMC counts had medium relationship ($r = 0.58$; n = 10) with tissue GL counts. Perhaps, all the MMCs located in the mucosa might not be activated to transform into GL after degranulation as described by Huntley *et al* (1984) or all the degranulated MMCs might not become GL.

Notably, some patterns of MMC distribution were observed in toluidine blue stained tissue slides which (a) - corresponded to the duration of the challenge infection, and (b) - reflected the level of serum IgE. On day 0 in high serum-IgE sheep MMCs were present in both sub-mucosa and mucosa, whereas in low IgE sheep the cells were only present in the villi; however there were very few cells in both. On day 1, MMCs were only seen in the villi in both sheep, with more in high serum-IgE sheep, and the intensity of granules was higher than on day 0. On day 3, MMCs had proliferated in the sub-mucosa as well as in the mucosa, but the granule staining of villous cells was lighter than the sub-mucosa cells or the villous cells of day 1 and the high serum-IgE sheep had a higher number of cells. Interestingly, on day 6, the high serum-IgE sheep had more cells as well as deeply staining cells in both sub-mucosa and mucosa but the MMCs in low IgE sheep were localised mostly in the villi; and in comparison, the staining intensity was higher and the cells were bigger than other days. On day 12, both sheep had a few cells in the villi and none in the sub-mucosa but, as usual, high serum IgE sheep had more cells. GL counts were variable and the only noticeable result was that all the high IgE sheep had higher GL than the low IgE group.

Eosinophils were not plentiful in the mucosa on day 0 and day 12, but the number increased and the morphology profile changed significantly on day 1, 3 and 6. In practice, examining the detailed morphology of eosinophils in tissue sections was quite tedious compared with that of cells in cytospots. Nevertheless, cell shape and the intensity of granules in the cytoplasm, gave fair idea about their stages. Generally, on all the days except day 3, eosinophils in the villous lamina propria were mostly Stage III eosinophils with irregular shape and finely dispersed granules in the cytoplasm (Fig. 4.2, 3). The eosinophil profiles on day 0 and day 12 were similar, but day 12 had more Stage I and II eosinophils (Fig 4.2, 1 & 2) with densely staining reddish granules accumulating in the small intestinal sub-mucosa. On day 1, a mild influx of both Stage I and II eosinophils was observed in the sub-mucosa and few eosinophils were seen in the gut lumen (Fig 4.10 C). Interestingly, these eosinophils appeared to migrate out of the intestinal lamina propria via the basement membrane through the space of a denuded epithelial cell into the lumen (Fig. 4.10 A & B). On day 3, a significant high influx of Stage II activated eosinophils and more mononuclear cells were seen in the lamina propria of both villi and sub-

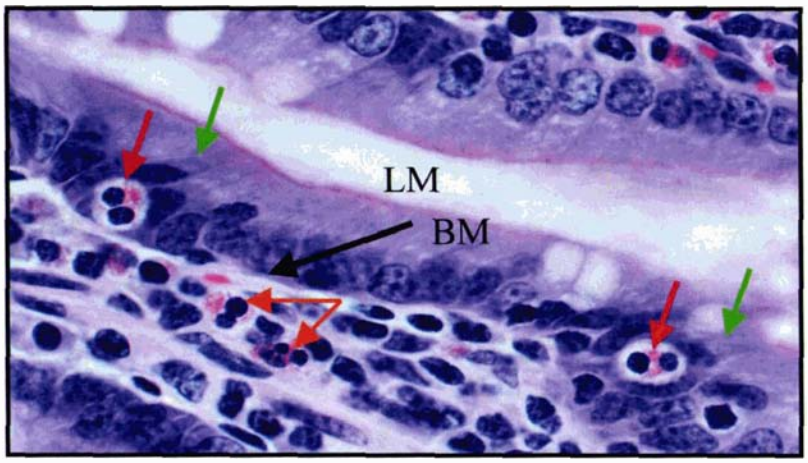

Fig 4.10 A

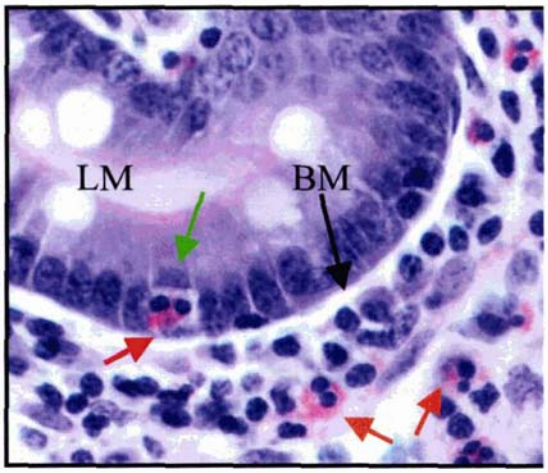

Fig 4.10 B

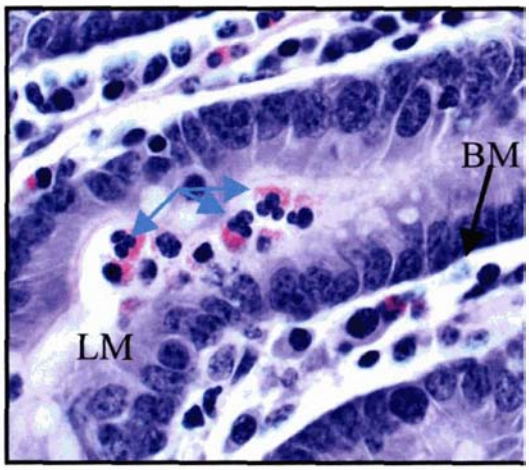

Fig 4.10 C

Fig. 4.10 Oil immersion microphotographs of the jejunum from challenged immune sheep on day 1 and showing eosinophil transepithelial migration. A & B - Eosinophils (orange arrows) are in lamina propria position. Eosinophils (red arrows) are observed within the epithelial cells of the villi. Eosinophils are located above the basal membrane (black arrows) and in the intra-epithelial space. The columnar epithelial cell (green arrows) is being lifted from the basal membrane and pushed out towards the lumen by the transepithelial migrating eosinophil. The displaced epithelial cells (green arrows) are above the eosinophil. C - Eosinophils (blue arrows) are in lumen position. The gut lumen (LM) and basal membrane (BM) are labelled for orientation. Microphotographs are hematoxylin-eosin-stained small intestinal tissue and the magnification is xl 000.

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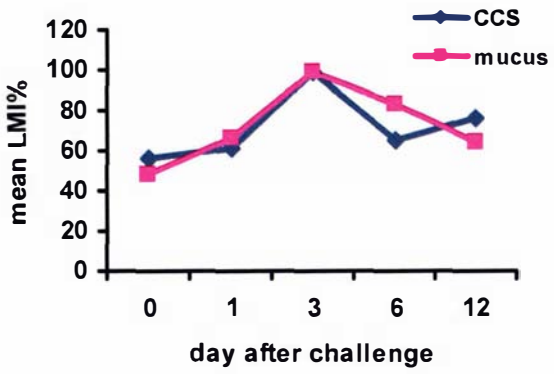

Fig. 4.11 LMI activity of CCS and mucus from sheep killed on different days after challenge. LMI = larval migration inhibition. Each data point is the mean of two sheep. Data for the graph is tabulated in Appendix 12.

Correlating parameters	Correlation (*r*)at p value 0.05
Mucus and CCS	0.53
Mucus LMI% and *T. colubriformis*- specific serum IgE	0.22
Mucus LMI% and tissue toluidine blue count	-0.31
Mucus LMI% and tissue EO count	**0.77**
Mucus LMI% and tissue GL count	-0.29
Mucus LMI% and PAS* stained GO count	-0.31
Mucus LMI% and HID** stained GO count	0.41

Keys: EO – eosinophil; GL – globule leucocyte; GO – goblet cell; *PAS – Peroxidase; ** HID - high iron diamine;

Table 4.7 Correlation of LMI activity of mucus with other important immunological parameters. All the immunological parameters for the calculations are tabulated in Appendix 23.

mucosa. The majority of the cell types gaining access to the lumen via the epithelium on day 3 were GLs, lymphocytes (*i.e.* the intra-epithelial lymphocytes), neutrophils and eosinophils. Notably, on day 3, higher numbers of both PAS and HID stained GOs were seen than on other days (Table 4.6). However, on day 6, the influx of Stage II activated eosinophils in mucosa was reduced, especially in the villi but they were still present in the sub-mucosa.

4.2.3.1.4 Larval migration inhibition activity

LMI activity of CCSs and mucus on different days after challenge are presented in Fig. 4.11 & Table 4.4. Table 4.4 demonstrates the correlation between LMI activity of CCS and the cell types present in the enriched LPCs. The correlations between LMI activities of mucus with other immunological parameters were also illustrated in Table 4.7. The results indicated that serum anti-nematode IgE titre of pre-challenge infection had no correlation with LMI activity of CCS ($r = 0.29$; $n = 10$) or mucus ($r = 0.34$; $n = 10$). LMI activity of CCSs and mucus produced on day 3 after challenge was the highest among other time points (Fig. 4.11). However, the activities on day 6 and day 12 were higher than on day 0 and day 1. In addition, LMI activity of CCSs had a medium positive correlation with the eosinophil counts ($r = 0.62$; $n = 10$) and the GO counts ($r = 0.62$; $n = 10$) among the cell types recovered in the tissue digests (Table 4.4). Interestingly, tissue eosinophils, but not other immunological parameters tabulated in Table 4.7, had a strong positive linear relationship with mucus LMI activity ($r = 0.77$; $n=10$) and its two-sided test correlation coefficient is significantly different from zero. All other correlations with mucus LMI activity were weak, or show evidence of non-linearity.

4.3 Discussion

4.3.1 Effect of immune status and husbandry practices on LPF production

These experiments were designed to test whether the husbandry practices imposed on sheep, and their immune status, influenced the LMI activity of mucus or CCS. The finding of significantly higher levels of LMI activity of mucus and CCS from immune sheep rather than susceptible or naïve sheep was in agreement with previous investigators (Douch *et al.*, 1984; Douch *et al.*, 1986; Douch *et al.*, 1996b). Interestingly, among immune sheep, the groups that received repeated infection in pens had a higher LMI activity than those only having natural infection. This indicates the magnitude of infection may play a role in activating the gut mucosa, because natural field infections are not as predictable as are experimental infections in terms of composition of nematode species in the challenge infection and size of the infection.

Interestingly, naïve sheep had remarkably similar levels of MMC population to the three immune groups (Gr.3-5), but they produced extremely low LMI activity. This finding may contradict the speculation of Douch *et al.*(1996b) that MMC enriched cell culture supernatant contains a soluble mediator with direct anti-parasitic activity. The other explanation could be that gut mucosa is bombarded with many microorganisms and luminal allergens derived from these organisms could possibly elicit MMC infiltration in the mucosa. In addition, the role of mast cells in mucosa becomes more complex, since they bear TLR-4 (Toll-like receptor-4) (Okumura *et al.*, 2003; Supajatura *et al.*, 2002a), and a non-allergic reaction of mast cells has been described recently, and they are considered to be an important component of the mucosal innate immune responses (Farhadi *et al.*, 2007). However, no data on this subject is available from ruminant studies.

Eosinophils appeared more frequently in nematode-infected sheep than in nematode-naïve sheep. Although eosinophilia in gut nematode infection is a hallmark of helminthiasis (Butterworth, 1984), its relevance is not well understood (Rothwell, 1989). Several factors contribute to eosinophilia in nematode infection. Eosinophils are attracted to the components of gut nematode parasites (Dixon *et al.*, 2006; Wildblood *et al.*, 2005). In addition, mucosal mast cell activation followed by nematode challenge in immune sheep (Jones *et al.*, 1994) could be a good source of many cytokines and mediators (Bradding *et al.*, 1994; Geotzl & Austen, 1977; Henz *et al.*, 2001) secreted into the gut mucosa. Among them preformed mediators (*i.e.* TNF-alpha, PAF, ECF-A) including IL5 are reported to be eosinophil chemoattractants (Colditz, 1991; Wang *et al.*, 1989; Yamaguchi *et al.*, 1988). IL5 is not only an eosinophil-selective chemoattractant (Wang *et al.*, 1989; Yamaguchi *et al.*, 1988), but also a potent selective cytokine to stimulate eosinophil production in bone marrow (Clutterbuck *et al.*, 1989). Eosinophils are also attracted to eotaxin, the eosinophil-selective chemoattractant released from the sub-epithelial tissue of the small intestine where it is constitutively expressed (Rothenberg, 1999).

The immune groups had higher proportions of GOs than other groups. Goblet cell hyperplasia in immune animals is believed to be under immunological control (Khan *et al.*, 1995; Miller & Nawa, 1979b). However, it may be an innate response to the physical irritation induced by the nematode infection (Specian & Oliver, 1991). Nevertheless, recent findings have reported that goblet cell hyperplasia and mucus secretion was found to be associated with IL13 released in the mucosa in response to nematode parasite challenge in rats (Webb *et al.*, 2007). Interestingly activated mast cells can generate IL13 (Burd *et al.*, 1995). Taken together, it is conceivable that eosinophilia together with goblet cell hyperplasia is the effector response of mast cell activation in response to nematode infection in the immune sheep.

Among the immune groups, Group 3 sheep (field living resistant-line) did not have as many inflammatory cells as the other two hyper-immunised groups. This may be due to differences in the amount and timing of larval challenge between the groups. In conclusion, the present study demonstrates that the genetic background is not the only important variable for the optimal level of LPF production in cell culture or in the mucus – the magnitude of parasite exposure to the gut is also important. These findings are in general agreement with Balic *et al.* (2006).

4.3.2 Effect of anatomical site on LPF production

The significant new findings in the study were the variation in LMI activity between different gut segments and the unexpected gradations in eosinophil or MMC morphology in the single-cell suspensions of lamina propria derived from different segments. Once again, there are no previous publications of ruminant studies regarding these findings.

Significant yields of lymphocytes, eosinophils, MMDs, MMCs and epithelial/goblet cells were obtained from the lamina propria cell populations of all gut segments. This indicates *T. colubriformis* nematodes evoke an influx of inflammatory effector cell populations in the intestinal mucosa. LMI activity was highest in the first 3 metres of small intestine and decreased downstream. In addition, the highest numbers of eosinophils (especially Stage II activated eosinophil), MMDs, MMCs and GLs were found in the 1st and 3rd segments, whereas the Stage I – normal eosinophils (pre-activation stage) were more frequent in the 12th and 18th segments. These findings indicate significant quantitative and/or qualitative differences in the anti-nematode mucosal immune response in the first 3 metres as compared to the posterior part of the gut. These differences may reflect more recent and higher exposure to challenge larvae at the sites of their establishment, because *T. colubriformis* populates preferentially in the first 3 metres of the small intestine (Barker, 1974; Steel *et al.*, 1990; Wagland *et al.*, 1996). Similar findings have been reported during infections with *T. colubriformis* and *H. contortus* in immune sheep, where increases in MMC and GL were more obvious at the predilection sites of the parasites, and were probably reflecting recent exposure to parasite challenge (Bendixsen *et al.*, 1995). The current study indicates that nematode exposure to the gut is critical for the mounting of an effective immune response against nematode infection. This concept is in line with previous findings: (1) the self-cure reaction in *H. contortus* infected immune sheep waned when the infection was absent for a month (Adams, 1983); (2) Jackson and colleagues observed a loss of immunity in immune sheep lacking antigenic exposure for nearly 3 months (Jackson *et al.*, 1988); (3) whether sheep were genetically-resistant-line or out-bred, challenged animals

showed significantly higher gut cell immune response than unchallenged sheep (Gill *et al.*, 2000; McClure *et al.*, 1992); (4) a marked secondary local immune response was only detected in the gut of immune sheep after challenge with 50,000 *Ostertagia* larvae but not after 1,000 larvae (Smith *et al.*, 1985a).

Interestingly, a positive relationship between LMI% of CCSs and eosinophil counts in the Experiment 3 (Table 4.4) is in agreement with the Chapter 3 result (Table 3.1).

Gradations of eosinophil and MMC morphology among LPCs isolated from different parts of the gut were unexpected results. It was first noticed when differential cell counts were performed on stained cytospots. Differences in cell size, the arrangement and numbers of nuclei in the cytoplasm, and the size and density of granules in both eosinophils and MMCs were clearly observed. Gradation of MMC and eosinophil morphology has not been commented on previously by other investigators. One reason could be that perhaps most previous studies used tissue cell counts to measure immune responses, and tissue preparations do not reveal the detailed morphology of the cells. The preparation of cytospots allows cells to reveal their morphology unhindered by stromal and other cells in tissues, and the features become more noticeable.

When viewed in the context of earlier results, LMI activity of LPCs derived from the 1st and 3rd metre points was higher than from the posterior part of the small intestine, even though these latter parts had higher numbers of eosinophils. It should also be recalled that some previous investigators (Amarante *et al.*, 2005; Amarante *et al.*, 1999; Dineen & Windon, 1980b; Douch, 1989; Gill, 1991; Winter *et al.*, 1997) did not find a positive correlation between the degree of immunity and tissue eosinophil counts. The present discovery of the morphological gradations of eosinophils, in relation to the treatments or to the locations in the infected immune gut, may provided some valuable clues to relate eosinophil numbers and the immune status of the gut in response to nematode challenge. It suggests that when assessing the role of eosinophils, cell morphology should be considered in order to make a meaningful interpretation of results. In contrast, although the morphological gradation of MMC was observed in all the gut segments, there was no location-based accumulation of particular stages of MMC in the segments as appeared with eosinophils. However, it was obvious that the stage II – activated eosinophils, GLs and the MMCs were localizing preferentially in the anterior 6 metres (where most *T. colubriformis* live) than in the posterior part of the gut. Perhaps this localization could be due to direct stimulation derived from nematode parasites which live there (Barker, 1974; Wagland *et al.*, 1996). This finding would accord with the observation of other investigators that local

immune responses are antigen dependent (Douch *et al.*, 1986; Douch *et al.*, 1983; Kimambo & MacRae, 1988).

4.3.3 Effect of time after nematode challenge on LPF production

The intestinal mucosa had been optimally primed 3 days after challenge to produce the highest LMI activity. Interestingly, differences in cell number and morphological gradations of eosinophils and MMCs between different days of challenge were also observed. Denudation of epithelium at the villous tips appeared as early as on day 1.

The study also revealed that the majority of resistant-line sheep with high anti-nematode-specific IgE produced higher tissue GL and MMC, and also more severe pathology than did the low IgE group. This result appeared to indicate that serum anti-nematode IgE, GL and MMC could not only be 'immune markers' but also an indication of pathology associated with nematode infection in resistant-line sheep. Interestingly, LMI activity did not correlated with any of the above parameters. It would appear that only a trigger level of MMC response may be needed to initiate the immune process for producing anti-nematode activity (LMI activity) *in vitro* or *in vivo*.

Comparing histological pictures of day 0 (no challenge) with other time points (challenge groups), clearly showed that nematode infection triggers gut inflammation which is associated with a high influx of inflammatory effector cells - eosinophils, MMDs, MMCs and epithelial cells - into the mucosa. These findings are similar to previous studies of mucosal immune responses to gut nematode infection (Gorrell *et al.*, 1988b; McClure *et al.*, 1992) which showed mobilization of high numbers of inflammatory cells into the gut mucosa of immune sheep after challenge with *T. colubriformis* nematode parasites.

An intriguing histological result was that epithelial shedding, particularly at the villous tips, was observed as early as on day 1 after challenge. McClure and her co-workers did not report this kind of pathology on day 1, although they did note a hyperaemic condition in the lamina propria which was broadly similar to the current observation (McClure *et al.*, 1992). This response could be part of the 'rapid type' immune response which causes an early rejection of challenge larvae and leads to the presence of inflammatory mediators in the gut contents (Jones *et al.*, 1992; Moqbel *et al.*, 1987). In general, the histological picture during the earliest stages of pathology have received less detailed study, although many investigations have focused their analysis on the mediators released into the gut contents immediately after challenge. Most

researchers (Balic *et al.*, 2003; Balic *et al.*, 2000a; Gill, 1991; Gorrell *et al.*, 1988a; McClure *et al.*, 1992; Salman & Duncan, 1985) have commented on the TH2-biased inflammatory effector responses developed on day 3-6, which featured the presence of inflammatory mediators and nematode-specific-antibodies in the gut mucus/contents, and increased mucus secretions, eosinophilia, mastocytosis, gut T cell expansions and goblet cell hyperplasia in the infected gut mucosa of immune sheep.

Careful examination of tissue sections in the present study suggested that this early damage may not be due to the physical invasion of 50, 000 challenge larvae into their niche, because none of the larvae were detected in any of the tissues or in the vicinity of the mucosa of day 1 to 12. Normally, *T. colubriformis* larvae enter through the lateral villous epithelium, and most localise above the basal membrane between the base and middle of the villi (Taylor & Pearson, 1979). The mucosa on day 0 showed normal integrity and had no signs of inflammation or epithelial breaks, but the gut condition changed abruptly within 24 hrs of challenge, with an inflammatory reaction which featured desquamation of epithelium, lamina propria tissues oozing out into the lumen at the villous tips, slight hyperaemia, changes in MMC morphology (increase intensity of granular staining and cell size) and mild influx of inflammatory cells, especially eosinophils, into the sub-mucosa. Since the infection was given orally, the larvae may not get to their niche all at once, but there was no evidence of clumped or trapped larvae in the superficial mucus or in the crypts of inflamed mucosa. This finding supports neither the 'leak lesion' hypothesis that luminal anti-nematode antibodies are implicated in the early rejection of larvae (Murray *et al.*, 1971a) nor the 'mucus entrapment' hypothesis that incoming larvae are physically trapped by mucus and excluded from the intestine of primed animals (Harrison *et al.*, 2003a; Lee & Oglivie, 1981; Miller *et al.*, 1981).

As a background to the current findings of pathology on day 1, mention should be made that over half a century ago, the immediate hypersensitive type of reaction in the gut after challenge with antigens was considered as being part of an immune expulsion mechanism in sheep with nematode parasite infection (Stewart, 1953; Stewart, 1955). This immune expulsion mechanism was initiated in an antigen-specific manner (Dineen *et al.*, 1977; Emery *et al.*, 1993) within 24 hrs (Elliot, 1981; Harrison *et al.*, 1999; McClure *et al.*, 1992) and was associated with the release of mast cell mediators such as protease, histamine and leukotriene in the gut contents (Harrison *et al.*, 1999; Huntley *et al.*, 1987; Jones *et al.*, 1994). It is generally accepted that immediate intestinal inflammatory responses are caused by biological mediators released from MMCs after antigen cross-linking of immunoglobulin (Ig) E bound to the cell surface. This reaction normally appears within 1-20 minutes after antigen challenge *in vivo* (Meeusen, 1999; Shaw *et al.*, 1996) and in *in vitro* experiments the release of histamine, mast cell protease and

leukotriene happened less than an hour after addition of specific nematode parasite antigens (Bendixsen *et al.*, 1995; Douch *et al.*, 1996b; Jones *et al.*, 1992). Taking these immediate inflammatory responses together, the dominant view at that time was that MMC inflammatory mediators were implicated in the rejection of nematode larvae in immune sheep (Douch *et al.*, 1983; Douch *et al.*, 1996b; Jones *et al.*, 1990). In this scenario, the rapid inflammatory immune response with mediator release in the mucosa seems to rest on the pillars of specific larval antigens and MMC population in the gut mucosa; and thus to evince the early pathology as seen in the current study, MMCs in the gut had to interact with luminal antigens.

However, an observation that seems inconsistent with this view is that, in practice, MMCs are not epithelial-dwelling cells like intraepithelial lymphocytes (IEL) and after thorough examination of histological samples in the current study, MMCs were absolutely lamina propria-bound (Fig. 4.9, a). Moreover, the transepithelial migration of MMC associated with nematode infection in sheep or in other species has not been reported by others, therefore, in order to manifest the rapid inflammatory immune responses, the luminal larval antigens must gain access through the epithelium to the lamina propria within 24 hrs to interact with the sub-epithelial MMCs. It is tempting to speculate that mast cell-independent innate immune mechanisms may operate at the mucosa immediately after the exposure to challenge by nematode larvae, to increase the epithelial permeability and allow some luminal nematode antigens to gain access to the lamina propria, for the triggering of a more potent mast cell-dependent immediate type of inflammation as seen on day 1.

Histological evidence on day 1 showed that a few activated eosinophils started to appear in the sub-mucosa but not noticeably in the villi, whereas many more MMCs with deep staining granules were found in the villi compared to the cells on day 0. It seemed that MMCs were more activated than eosinophils and they were localised close to the 'frontier' epithelial barrier. It is possible that the antigen-specific activation of sub-epithelial MMCs by cross-linking surface IgE or IgG, the MMC mediators (Peptido-leukotrienes, serum mast cell protease, thromboxane) could be released into the mucosa tissues and systemic circulation within 24 hrs, as reported in the work of Jones *et al.* (1994), Huntley *et al.* (1987) and Jones & Emery (1991). Rodent studies have proven that the function of mast cell protease *in vivo* is to elicit mucosal permeability but not epithelial shedding (McDermott *et al.*, 2003; Scudamore *et al.*, 1995). In fact, most inflammatory cells, not only MMCs are the source of this enzyme (DiScipio *et al.*, 2006; Jones *et al.*, 1992; Wahl *et al.*, 1974), also sheep gut nematode parasites secrete protease (Geldhof *et al.*, 2000; Knox & Jones, 1990; MacLennan *et al.*, 1997). Although the function of MMC enzymes in sheep remains an hypothesis, protease is a potent specialised enzyme effectively digesting the collagen tissues of the gastrointestinal tract, and thus it can disassociate

the epithelial cells by proteolysis of tight junction (TJ) proteins or type IV collagen of basal membrane or lamina propria tissues (Freshney, 1994; Sage *et al.*, 1979). However, interestingly, agents that have been described as mast cell stabilizers are capable of reducing gut tissue damage (Karmeli *et al.*, 1991; Takeuchi *et al.*, 1986) and agents that cause mast cell degranulation can increase the severity of gastrointestinal damage (Rees *et al.*, 1978) in various models. Especially, peptido-leucotrienes, TNF-α, PAF and thromboxane which are all MMC mediators, are capable of exacerbating gut tissue damage (Perdue *et al.*, 1989; Rioux & Wallace, 1994). In this context, it is possible that mast cell activation could significantly increase minor damage (*i.e.* the epithelial leakiness caused by protease) into high degrees of damage (*i.e.* epithelial shedding & lamina propria tissue opened up into gut lumen) such as that which appeared on day 1 through its mediator-dependent mechanism.

In the present study the most prominent intestinal mucosal pathology, consisting of villi denudation, oedema and an uneven thick layer of mucus covering the mucosal surface, and recruitment of multiple effector cells from the sub-mucosa to the sub-epithelial sites, was obvious on day 3. These findings are similar to the findings of other investigators who have reported the following histopathological changes, and increased cellular activities and secretions in immune sheep on day 3: (1) the pathology of intestinal mucosa was comparatively most prominent and characterised by the shorter, thicker and oedematous villi containing high influx of mononuclear cells, and increased local production of *T. colubriformis*-specific IgG1 and IgG2 (McClure *et al.*, 1992); (2) an increase in the output of lymphoblasts and IgA cells in gastric lymph (Smith *et al.*, 1985b); (3) vasoactive mediators (histamine and leukotriene) concentration in gut tissue and mucus was the highest (Jones *et al.*, 1990); (4) the number of eosinophils increased dramatically in *Teladorsagia circumcincta* immunised sheep (Balic *et al.*, 2003); (5) maximum eosinophil potentiating activity was detected between 2-3 days after challenge with abomasal worms in primed lambs (Stevenson *et al.*, 1994). These indicate the adaptive TH2-biased immune response to nematodes in sheep was well primed featuring the influx of inflammatory cells and specific-antibody secreting cells in the gut lamina propria. In fact, the adaptive immune response to nematodes is a high degree of inflammation and the result is significant tissue damage and mucus secretion, perhaps it may be the reason that the villous atrophy is greatest on day 3. However, the gut restitution took place on day 3, because epithelia of all sheep on day 6 were well sealed except the villi were wide and had broad shoulders.

Significant changes in MMC morphology (in terms of higher granulation and bigger cell size) were observed on day 1 and 6; changes on day 1 were quite mild compared to day 6, but the MMC granules were stained much deeper than the granules of day 0, 3 or 12. Perhaps MMC activation on day 1 could be associated with larval antigenic stimulation, and the evidence of

MMC activation and release of mediators into gut contents within 24 hrs of larval challenge has been reported (Jones *et al.* 1994; Harrison *et al.* 1996). The activation of MMCs could be a source of cytokines for proliferation, mobilization and activation of eosinophils and GOs appearing on day 3, since activated MMC can secrete a variety of cytokines (Burd *et al.*, 1995; Gordon *et al.*, 1990; Henz *et al.*, 2001). It is known that IL3, IL5, SCF and GM-CSF can activate eosinophils (Fujisawa *et al.*, 1990; Oliveira *et al.*, 2002); and also TNF-α (Arnold *et al.*, 1993), IL1- β (Cohan *et al.*, 1991; Han *et al.*, 1987) and IL-13 (Webb *et al.*, 2007) can induce goblet cell proliferation and secretion. However, the eosinophil mobilization on day 3, MMC activation on day 6 could be associated with a fully developed adaptive immune response followed by the release of TH2 cytokines (IL3, IL4, IL5 & IL13), in the lamina propria, since gut mucosa immune response to luminal pathogens via Peyer's patches normally takes 4-6 days (Mahida *et al.*, 1997). In the work of Salman & Duncan (1985) MMC numbers were increased nine fold in tissue sections from vaccinated ewes killed on day 5 which is close to the time of the present study.

Intriguingly, the most dominant and activated cells on day 3 were eosinophils and GOs rather than MMCs and they had a positive correlation with LMI activity of CCS. In fact, the finding of eosinophil activation and goblet cell hyperplasia on day 3 is a sign of Th2-biased immune responses (Gill *et al.*, 2000; Ishikawa *et al.*, 1997), and thus IL5 which is the most potent and fastest eosinophil activating factor (Stevenson & Jones, 1994) could be secreted in the gut mucosa tissue. Evidence is accumulating that leukotriene C4, which is a major mediator released from eosinophils (Shaw *et al.*, 1985) was released in the greatest quantity in the intestinal sample on day 4 after infection with *T. colubriformis* in immune sheep (Jones *et al.*, 1994); the highest eosinophil potentiating factors were produced in immune sheep 3 days after challenge with *Tel.circumcincta* (Stevenson *et al.*, 1994); the highest eosinophil count was recorded on day 3 in the vaccinated lambs with *H. contortus* infection (Salman & Duncan, 1985). These data lend considerable support to the findings of the current study that activation of eosinophils was the highest on day 3 and the LMI activity showed a positive correlation with the eosinophil proportions in the cell cultures. It seems likely that eosinophils are an important cellular source of LMI activity. Nevertheless, since the cell culture contained more than one type of cell, it does not exclude the other cells in the lamina propria from contributing to LPF production, directly or indirectly, by interacting with eosinophils. Thus, further controlled experiments are required to elucidate this issue.

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Chapter 5
Cellular source of larval paralysis factor

5.1 Introduction

Chapters 3 and 4 have shown that an *in vitro* cell culture system using small intestinal lamina propria cells (LPCs) of hyperimmunised sheep and *T. colubriformis* larval ES (TcL3ES) antigens was a reliable method to produce larval paralysis factor (LPF). Although they also demonstrated that eosinophils were a prominent cell type that had a positive correlation with LMI activity, there were other cell types present in the same cell population, *viz.* lymphocytes, monocyte-macrophage-dendritic cells (MMDs), mucosal mast cells (MMCs) and epithelial/goblet cells (EP/GOs). Thus, in order to better understand which cell type(s) release the nematode paralysis factor, a series of experiments was conducted to separate each cell type from the small intestinal tissue. The resultant enriched cell types were then stimulated with the antigens and assayed for their LMI activities *in vitro*.

5.2 Materials and methods

All the materials and methods are described in Chapter 2.

5.2.1 Experiment 1: Fractionation of small intestinal mucosal cells for LPF production

Rationale and aim: The results of Chapter 3 indicated that LPCs are the source of LPF. To establish a technique for quick fractionation of sufficient numbers of viable cells for further *in vitro* cell culture, the EDTA step was omitted, and following the enzymatic digestion of gut tissue, Percoll discontinuous density gradient separation was carried out. The distribution profile of mucosal cell types after Percoll separation was then determined. Cell fractions were then stimulated with *T. colubriformis* larval ES antigens and assayed for LMI activity *in vitro*.

Experimental approach

A total of the 11 field resistant-line ewes were used in the experiment. Sheep were brought into

a pen, drenched and sensitised by the LPF regime (Chapter 2: section 2.1.1.2). Two experiments (1.1 and 1.2) were performed: -

Experiment 1.1

Five sheep were killed, immediately after killing, two 50 cm pieces of small intestines from each sheep were removed at 2-3 metres from the pylorus for isolating mucosal cells using enzymatic digestion method. The digest cells were fractionated using Percoll density gradient centrifugation.

Experiment 1.2

Six sheep were used and the protocol for cell fractionation was similar to the above. The resultant Percoll fractions were stimulated with the ES antigens, and the CCSs were assayed for LMI activity *in vitro.*

5.2.1.1 Results

5.2.1.2 Experiment 1.1

5.2.1.2.1 Parasitology

Faecal egg counts of 11 sheep were between 0-100.

5.2.1.2.2 Differential mucosal cell counts

In the first experiment with 5 sheep, enzymatic digestion of small intestinal tissue for 3-5 hours yielded a heterogeneous population of cells, comprised of lymphocytes, neutrophils, eosinophils, MMDs, MMCs, GLs and EP/GOs. The recovered cell types are tabulated in Table 5.1. Cell viability with Trypan blue was 78% (n = 5; range 70%-88%) following enzymatic digestion.

5.2.1.2.3 Discontinuous Percoll density gradient separation

After the Percoll gradients were centrifuged, cell bands were formed at 30%, 40%, 50%, 65%, 75% and 85% Percoll bands. Trypan blue staining of cells recovered from each band showed that the mean cell viability was 74% (n = 5, range 68%-80%). Dead cells, epithelial cells and debris were found in the 30% Percoll band in all samples and few viable cells were trapped in the dead-cell aggregates. The majority of the viable cells were recovered above the 40%, 50% and 65% Percoll bands and very few in 75% and 85% bands (Table. 5.2).

Sheep	L	N	EO	MMD	MMC	GL	EP/GO
3578	47	2	9	9	3	5	25
213	48	2	12	10	4	4	20
1197	23	1	18	5	1	19	33
807	53	0	9	11	2	9	16
CD46	32	1	3	9	1	2	52
mean	**41**	**1**	**10**	**9**	**2**	**8**	**29**

Keys: L - lymphocyte; N – neutrophil; EO - eosinophil; MMD – monocyte-macrophage-dendritic cell; MMC - mucosal mast cell; GL - globule leucocyte; EP/GO - epithelial/goblet cell;

Table. 5.1 Proportion of recovered cell types in small intestinal tissue digests before Percoll separation (n=5). Mean percentage of each cell type recovered is tabulated and in bold print.

Percoll %	Total no. of viable cells x 10^6 (S.D.)
40	41.4 (26.5)
50	33.1 (18.7)
65	21.5 (10.0)
75	2.3 (1.0)
85	1.65 (0.9)

Table 5.2 Recovery of viable mucosal cells on each Percoll band after spinning 200x10^6 cells. (n=5).

Percoll%	Mean percentage of cell type					
	L	EO	MMD	MMC	GL	EP/GO
40	29	8	9	0	2	52
50	38	16	20	1	1	24
65	41	19	4	7	12	17
75	8	4	0	23	47	17
85	3	1	0	27	51	18
	Cell yield ($x10^6$)					
	L	EO	MMD	MMC	GL	EP/GO
40	13	2.9	3.4	0.1	0.7	21.3
50	12.9	6.2	6	0.4	0.3	7.4
65	9.3	4.2	1.1	1.3	2.1	3.5
75	0.2	0.1	0	0.4	1.3	0.4
85	0.1	0	0	0.5	0.9	0.2
Mean total cell no.	**35.4**	**13.3**	**10.4**	**2.7**	**5.3**	**32.8**

Keys: L - lymphocyte; EO - eosinophil; MMD - monocyte-macrophage-dendritic cell; MMC - mucosal mast cell; GL - globule leucocyte; EP/GO – epithelial/goblet cell;

Table 5.3 Distribution profile and total yield of each cell type in different Percoll density gradient bands after 200 crude digest cells were layered over Percoll density gradients and separated. Result expressed as mean of 5 experiments. Mean total cell yield of each cell type are tabulated and in bold print.

Sheep	L	N	EO	MMD	MMC	GL	EP/GO
7155	40	1	5	2	>1	>1	47/5
7389	42	2	12	2	6	>1	25/11
7635	29	1	40	1	>1	>1	18/11
2927	24	1	34	3	1	>1	35/2
8053	15	1	52	10	6	3	8/5
8051	22	2	34	21	2	5	12/2
Mean %	**29**	**1**	**30**	**7**	**3**	**1**	**25/5**

Keys: L - lymphocyte; N – neutrophil; EO - eosinophil; MMD - monocyte-macrophage-dendritic cell; MMC - mucosal mast cell; GL - Globule leucocyte; EP/GO – epithelial/goblet cell;

Table 5.4 Composition of crude enriched lamina propria cell population. Result expressed as mean percentage of six experiments. Mean percentage of each cell type in crude populations is tabulated and in bold print.

5.2.1.2.4 Distribution profile of each cell type on discontinuous Percoll density gradients

Table 5.3 shows that the majority of the lymphocytes (90%), eosinophils (80%) and MMDs (100%) recovered were predominantly localised between the 40% and 65% Percoll bands but few eosinophils were still present in high-density bands (75% & 85%). Approximately 50-80% of the MMDs accumulated on the 50% Percoll band and none of them passed through the 65% Percoll layer. A large proportion of MMCs and GLs was commonly recovered above the high-density bands (75% & 85%).

Similar to Chapter 3, since the non-differentiating stem cells (precursors of three epithelial lineages), pre- absorptive columnar epithelial cells, differentiating absorptive columnar epithelial cells, absorptive columnar epithelial cells, pre- GOs (oligomucous cells), and differentiating GOs were not easy to differentiate, for the convenience of counting, they were collectively counted as epithelial cells. However, GOs (Fig. 3.1, A,5) were counted separately for research purposes. Sixty-five percent and 22% of a mixture of epithelial and GOs were retained on 40% and 50% Percoll bands respectively. However, there were some GOs recovered in the 75% and 85% bands, and in comparison to the populations recovered in the 40%-65% Percoll bands, they were much purer. In addition to the GOs, a few eosinophils, and many GLs and MMCs were also recovered on the high density bands. The 85% band also contained some un-dissolved Percoll particles.

Based on these findings, two Percoll concentrations - 40% and 65 % - were selected to prepare two subpopulations of cells for further study. Hence, the next experiment was designed to assess the larval paralysis activity of the two selected populations – Population 1 (P1) which was recovered between 40% and 65% Percoll bands and contained mainly lymphocytes, eosinophils and MMDs, and Population 2 (P2) which was under the 65% Percoll band and contained mainly MMCs, GLs, and GOs.

5.2.1.3 Experiment 1.2

5.2.1.3.1 Cell composition of mucosal cells after digest

Enzymatic digestion of small intestinal mucosal tissues recovered a population of cells which consisted of lymphocytes, eosinophils and epithelial cells, neutrophils, MMDs, MMCs, GLs and EP/GOs in all sheep (Table 5.4). Among the cell types, the first 3 were the most frequent (approximately 28% each) and the rest were present at a lower percentage (1% - 7%) of the population, as determined by Leishmann's stain, which was consistent with previous results in Chapter 3. Cell viability was 76% (n=6, range=66%-82%).

5.2.1.3.2 Cell composition of P1 and P2 after Percoll density gradient centrifugation

Following Percoll density gradient centrifugation, the cell yield of P1 was 3 times greater than that of P2 (Table 5.5). The cell composition of P1 was quite similar to the unseparated LPC population tabulated in Table 5.4. Lymphocytes, eosinophils and epithelial cells were each recovered at 22%-34% (mean %) and the remaining cell types (neutrophils, GOs, GLs, MMCs and MMDs) comprised 1%-7% of cells. In contrast, GOs were the highest (50%) cell type recovered in P2 (Table. 5.6), and eosinophils (17%) were the second most common cell type. Very few neutrophils and MMDs were recovered in P2. MMCs formed a low proportion in all the samples. In addition, considerable numbers of immature mucosal mast cells were observed in P1, particularly in sample 7635 and 2927, although the latter had these cells in both populations (Table 5.7). GLs were present in all the samples at a low yield. Interestingly, the eosinophils found in P1 were mostly Stage I - pre-activated forms (Fig. 4.2, 1) whereas P2 (Table 5.7) was high in activated eosinophils (Fig. 4.2, 2). In addition, degranulated eosinophils appeared only in P2 but they were very few in number (Table 5.7).

5.2.1.3.3 LMI activity of P1 and P2

Table 5.8 demonstrates that CCS derived from different cell groups (unseparated LPCs or P1 or P2) without antigen stimulation had very low or no LMI activity. However, after antigen addition, the highest LMI activity was detected in the CCS from P2 whereas the CCS from P1 and unseparate LPCs were low. It also demonstrates clearly that the release of LPF in the cell cultures was mediated by the nematode larval ES antigens.

5.2.1.3.4 Mucus glycoprotein concentration in cell cultures

It was noticed that the consistency of CCS of P2 with or without antigens after 12 hours incubation varied greatly and CCS with antigens were more viscous than the CCS without. Mucus glycoprotein assay results (Table 5.9) showed that antigen-stimulated CCS had a higher concentration of mucus glycoprotein than its counterparts. The difference was statistically significant at p-value 0.05.

5.2.2 Experiment 2: In vitro stimulation by gut effector cell factors of glycoprotein release from enriched epithelial/goblet cell population, and determining LMI activity

Rationale and aims: The results of previous experiments indicated that P2 (under 65% Percoll band) produced higher LMI activity than P1 (between 40 and 65% Percoll band) and was

Sheep	Cell yield (x106)	Cell yield (x106)
	P1	P2
7155	480	100
7389	400	100
7635	350	128
2927	800	225
8053	625	150
8051	500	125
Mean	**526**	**138**
s.d.	**150**	**47**

s.d. – standard deviation; P1 – cell population between 40 and 65% Percoll bands; P2 - cell population between 65% and 85% Percoll bands.

Table 5.5 Total number of cells recovered in P1 and P2 after Percoll gradient separation. Mean cell yields and standard deviations of both populations are tabulated and in bold print.

Sheep	P	L	N	EO	MMD	MMC	GL	EP/GO
7155	P1	53	2	7	5	2	1	28/4
7389		21	1	10	5	4	9	22/28
7635		13	1	48	6	3	1	33/0
2927		17	2	43	1	4	1	26/8
8053		15	1	48	10	4	I	11/8
8051		10	1	47	9	1	4	26/2
Mean%		**22**	**1**	**34**	**6**	**3**	**3**	**24/8**
7155	P2	1	1	3	0	10	1	4/74
7389		2	0	2	0	13	3	14/64
7635		10	0	56	3	1	3	11/17
2927		1	1	3	1	5	6	42/42
8053		4	0	23	1	4	7	4/57
8051		1	1	14	0	3	1	29/42
Mean%		**3**	**1**	**17**	**1**	**6**	**4**	**19/50**

Keys: P – population; L - lymphocyte; N – neutrophil; EO - eosinophil; MMD - monocyte-macrophage-dendritic cells; MMC - mucosal mast cell; GL - globule leucocyte; EP/GO – epithelial/goblet cell; P1 – cell population between 40% and 65% Percoll bands; P2 - cell population between 65% and 85% Percoll bands.

Table 5.6 Proportion of each cell type recovered in P1 and P2. Mean percentage of each cell type in both populations is also tabulated and in bold print.

Cell population	Sheep	(a) Eosinophils			(b) Mucosal mast cell			
		1	2	3	1	2	3	4
P1	7635	88	12	0	8	21	71	0
	7389	85	15	0	2	28	70	0
	7155	90	10	0	0	27	69	3
	8051	93	7	0	0	10	66	14
	8053	88	12	0	0	20	74	6
	2927	84	16	0	76	16	8	0
P2	7635	27	65	8	12	68	17	3
	7389	2	98	0	58	39	3	0
	7155	11	89	0	48	40	12	0
	8051	6	62	32	12	26	62	0
	8053	35	52	13	63	37	0	0
	2927	20	80	0	98	2	0	0

Key: P – Cell population; P1 - cell population between 40% and 65% Percoll bands; P2 - cell population between 65% and 85% Percoll bands;

Table 5.7 Morphological profile of eosinophils and mucosal mast cells recovered in P1 and P2. One hundred cells of each cell type were counted. The morphologies of the variants were described in detail in Chapter 4, Fig. 4.2 & 4.3. (a) - Three stages of eosinophils appeared in oil-immersion microphotograph of Leishmann-stained cytosmears of lamina propria cells: 1- Normal eosinophils; 2 - Activated eosinophils; 3 - Degranulated eosinophils. (b) - Four degranulation stages of mature mucosal mast cell in oil-immersion microphotograph of Leishmann-stained cytosmears of lamina propria cells. (1) Pre-degranulated stage (2) Partially degranulation stage. (3) High degree degranulation stage. (4) Complete degraulation stage.

sheep	mucus	Crude cells only	Crude cells + Ag	P1	P1 + Ag	P2	P2 + Ag
7155	86±1.2	0	43±5.3	0	0	25±1.2	86±7.0
7389	50±5.4	0	42±2.3	0	0	26±5.0	100±0.0
7635	31±7.1	0	46±4.5	0	0	27±1.1	39±2.5
2729	65±5	<10	44±6	<10	60±4	18±1	95±3
8053	50±2	<10	46±8	<10	<10	<10	55±0
8051	53±2	<10	54±6	<10	21±4	27±5	91±0.4

Keys: crude cells - gut Lamina propria cells before Percoll separation; P1 – cell population between 40% and 65% Percoll bands; P2 - cell population between 65% and 85% Percoll bands; the unit is mean LMI% ±standard error of the mean.

Table 5.8 LMI activity of culture supernatants derived from different cell populations.

sheep	Mucus glycoprotein level in cell culture (µg/ml)					
	Crude cells	**Crude cells+Ag**	**P1**	**P1+Ag**	**P2**	**P2+Ag**
8051	1.53	2.2	5.53	6.2	7.98	11.76
8053	6.87	6.64	6.42	6.87	10.2	18.2
7635	4.2	5.31	0.42	5.31	5.76	11.76
7389	3.76	2.64	3.53	3.53	5.98	7.09
mean	**4.09**	**4.20**	**3.98**	**5.48**	**7.48**	**12.20**

Keys: crude cells - gut Lamina propria cells before Percoll separation; P1 – cell population between 40 and 65% Percoll bands; P2 - cell population between 65 and 85% Percoll bands;

Table 5.9 Concentration of mucus glycoprotein in cell culture supernatants before and after stimulation with *T. colubriformis* ES antigens for 6 hours. The negative control was buffer (HBSS9) only and the positive control was levamisole (5µg/ml) for *in vitro* LMI assay. The means are tabulated and in bold print. Cell culture supernatant samples were prepared at 20% dilution in distilled water. Sample (100µl) and 10 µl of 50% periodic acid solution were dispensed into triplicate wells, and incubated at 37°C for 2 hours. After the incubation, 10 µl Schiff's reagent (the reagent was made by adding 100 mg Sodium metabisulphide in 6 ml Schiff's reagent solution, and incubated at 37°C for 90 minutes) was added to each well, left on the bench at room temperature for 30 minutes, and read at 570nm absorbance on a *DynaTech* plate reader (Dynatech Laboratories, INC. Alexandria, Virginia, USA).

Cell type	Method	Purpose	Product
EO	1. EDTA	To remove epithelial bound cells	EOF
	2. Enzymatic digestion	To obtain EO in lamina propria	
	3. *auto*MACS	Further enriching EO by negative selection using mAb to ovine IgE antibodies, CD5; CD21, and MHCII antigens	
MMC	1. EDTA	To remove epithelial bound cells	MMCF
	2. Enzymatic digestion	To obtain MC in lamina propria	
	3. *auto*MACS	Further enriching MC by positive selection using XB6 mAb (anti-ovine IgE)	
GL	1. EDTA	To concentrate epithelial bound globule leucocytes	GLF
	2. 75% Percoll	To enrich GL and to remove unwanted cells	
EP/GO	1. EDTA	To remove epithelial bound cells	Enriched cells for further testing
	2. Enzymatic digestion	To obtain EP/GO along the pit of villi	
	3. Percoll density gradients	To concentrate EP/GO from other SLPCs	
	4. *auto*MACS	Further enriching EP/GO by negative selection using mAb to LCA (CD45RA)	

Keys: EO - eosinophil; MMD – monocyte-macrophage-dendritic cell; MMC - mucosal mast cell; GL - globule leucocyte; XB6 – mAb (anti-ovine IgE); LCA (CD45RA) –mAb (Leucocyte Common Antigen); EOF – eosinophil factor; MMCF – mucosal mast cell factor; GLF – globule leucocyte factor; EP/GO –epithelial/goblet cell.

Table 5.10 Strategy of cell type enrichment

composed of a significantly higher proportion of GOs and lower numbers of eosinophils, lymphocytes and MMDs. Interestingly, CCSs of the P2 were not only LMI-active, but were also high in mucus glycoproteins; possibly, the high numbers of GOs in the population was the reason, because GOs are the main source of mucus in the gut (Specian & Oliver, 1991). Whether the source of LMI activity was GOs, or another cell type in P2, and whether the mucus release reflected a direct response of GOs to nematode antigens or an indirect effector response of GOs to cell factors released by other cell types, remained unknown. Two further experiments (2.1 & 2.2) were carried out to explore these issues.

Experiment 2.1

The aim was to determine which specific cell type(s) contributed to the LMI activity. To do this, the first plan was to achieve further separation of each cell type from P2 using *auto* MACS (Magnetic cell sorter). However, recovery of P2 population was too low and did not provide sufficient cell numbers for further research, and thus, as an alternative, unseparated LPCs which are known to be a source of LPF (after Chapter 3) were used as a source of cells for *auto*MACS separation. Following immuno-magnetic enrichment, the enriched cell types were pulsed with *T. colubriformis* larval antigens, and the resultant supernatants were named as 'cell factors'; all the supernatants produced were assayed for LMI activity. A total of the 7 field resistant-line ewes were used in the experiment. Sheep were brought into a pen, drenched and sensitised by the LPF regime (Chapter 2: section 2.1.1.2).

Experiment 2.2

This work further enriched the EP/GOs from the LPCs, then co-cultured with each 'cell factor', and examined mucus glycoprotein or LPF release. Two field resistant-line ewes were used in the experiment. Sheep were brought into a pen, drenched and sensitised by the LPF regime (Chapter 2: section 2.1.1.2).

5.2.2.1 Results

5.2.2.2 Experiment 2.1

5.2.2.2.1 Parasitology

Faecal egg counts of seven sheep were between 0-200.

5.2.2.2.2 Immuno-magnetic enrichment of cell types

Using the strategies shown in Table 5.10, it was possible to enrich four cell types, *viz.* eosinophils, MMCs, GLs and EP/GOs. In *auto*MACS separation, cells stained with mouse monoclonal antibodies to sheep cell surface antigens (e.g. IgE antibodies, CD5, CD21 & MHCII) were incubated with iron beads coated with goat anti-mouse IgG, which recognised

primary monoclonal antibodies, and then passed through magnetic column to be sorted by either negative or positive selection.

5.2.2.2.2.1 Enrichment of eosinophils

Table 5.11 shows eosinophils could be purified to levels of 68%-94%, with the principal contaminants being small lymphocytes and EP/GOs (GOs were 20%-35%). The eosinophil-enriched population was incubated with recombinant human IL5 (rhIL5) and the supernatants were collected for measurement of eosinophil peroxidase (EPO) to confirm that *auto*MACS sorted eosinophils were functional. Degranulation of eosinophils through release of peroxidase enzymes was induced by concentrations of rhIL5 as low as 1.25 ng/ml (Fig. 5.1). Eosinophils recovered from all the methods had no obvious morphological changes. However, there were differences in the morphologic profiles of eosinophils (Table 5.12). Significantly, samples 1205 and 8151 contained a high proportion (61%-70%) of Stage II – activated eosinophils (Chapter 4; Fig. 4.2, 2), whereas sample 8421 and 6217 had a lower proportion of Stage II (10%-15%) and a higher proportion of Stage III – degranulated eosinophils (Chapter 4: Fig. 4.2, 3). Similarly, EPO production was much higher in the former pair than the latter (Fig. 5.1). Trypan blue exclusion tests showed that cell viability after enzymatic digestion was 77%±10% (n = 4; range 66%-89%) and after *auto*MACS was 62%±8% (n=4; range 52%-70%).

5.2.2.2.2.2 Enrichment of mucosal mast cells

A summary of the results of MMC enrichment is shown in Table 5.13. The purity of MMC increased significantly from 2%-5% to 83%-90% (n=2). Cell viability was 75%-82%, determined by Trypan blue exclusion test. Enrichment procedures had no obvious affect on MMC morphology and their ability to release histamine in response to agonists *in vitro* (Fig. 5.2). Partially degranulated mucosal mast cells (20%) appeared in sample 8151 after *auto*MACS, but in sample 4133 no difference was observed in the percentage of these cells before and after *auto*MACS (Table 5.14).

5.2.2.2.2.3 Enrichment of globule leucocytes

Highly enriched populations of globule leucocytes (90%-94%) were obtained in both samples (Table 5. 13). Most of the contaminating cells in the 75% Percoll band were small lymphocytes and young epithelial cells (stem cells) (Fig. 3.1, C). No significant difference in cell size or morphology was observed. Cell viability after Percoll was 80%-86%. A functionality test was not conducted on these cells.

Sheep	Method of enrichment	L	N	EO	MMD	MMC	GL	EP/GO
1205	After EDTA & enzymatic digestion	33	1	36	16	4	1	9
	After MACS	1	0	**81**	1	5	1	11
8151	"	29	2	16	31	5	1	16
		18	0	**68**	5	1	0	8
8421	"	31	0	61	4	0	0	4
		0	0	**93**	0	0	0	7
6217	"	26	2	57	11	1	1	2
		3	0	**94**	0	0	1	2

Keys: L - lymphocyte; N – neutrophil; MMD - monocyte-macrophage-dendritic cell; EO-eosinophil; MMC - mucosal mast cell; GL - globule leucocyte; EP/GO-epithelial/goblet cell;

Table 5.11 Purity of eosinophils after enrichment. EDTA cell dissociation method removed epithelial bound cells, dead cells and mucus on the mucosa. Enzymatic digestion isolated lamina propria cells to further enrich to high purity eosinophils using negative selection programs of *auto*MACS.

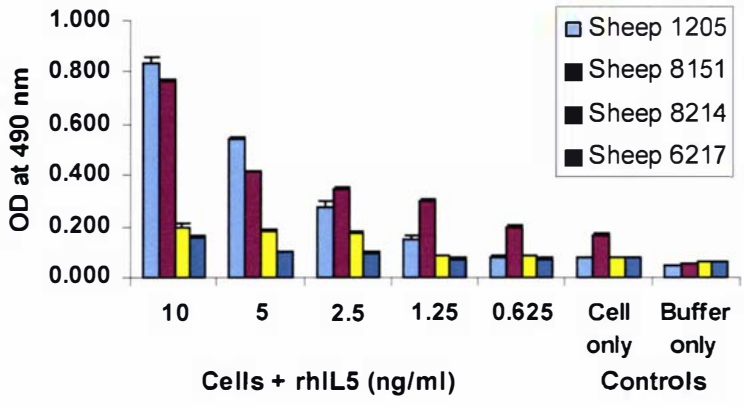

Fig. 5.1 Measurement of EPO release in the cell culture supernatants after stimulation with rhIL5 for 1 hr. For activation, approximately $1x10^6$ cells from eosinophil-rich population in 100 µl HBBS were incubated with a range of rhIL5 0.625-10ng/ml 100ul, or control medium (100 µl). Each treatment was carried out in triplicate. The error bar is S.D. Data were tabulated in Appendix 27.

	Stages of eosinophil (%)		
	1	2	3
8151	25	61	14
1205	20	70	9
8421	7	15	78
6271	15	10	75

Table 5.12 Morphological profiles of eosinophils in Leishmann-stained cytosmears of enriched eosinophil populations following *auto*MACS prior to cell culture. Twenty cells were counted at each of five different sites on each cytospot. The morphologies of the variants were described in detail in Fig 4.2. Three stages of eosinophils appeared in oil-immersion microphotograph of Leishmann-stained cytosmears of lamina propria cells: 1- Normal eosinophils; 2 - Activated eosinophils; 3 - Degranulated eosinophils.

Sheep	Cell type	Method	L	N	EO	MMD	MMC	GL	EP/GO
8151	MMC	EDTA, Enzymatic digestion	53	2	25	6	2	1	11
		*auto*MACS	4	0	0	2	**90**	0	4
4133		EDTA, Enzymatic digestion	29	1	17	31	5	1	16
		*auto*MACS	2	0	9	1	**83**	1	5
9146	GL	EDTA digestion	22	1	7	0	0	9	61
		75% Percoll	3	0	0	0	0	**94**	3
0375		EDTA digestion	45	2	30	1	0	18	4
		75% Percoll	4	0	2	0	0	**90**	4

Keys: L - lymphocyte; N – neutrophil; MMD - monocyte-macrophage-dendritic cell; EOeosinophil; MMC - mucosal mast cell; GL - globule leucocyte; EP/GO-epithelial/goblet cell;

Table 5.13 Purity of mucosal mast cells and globule leucocytes after enrichment. Enriched MMC and GL populations are in bold print.

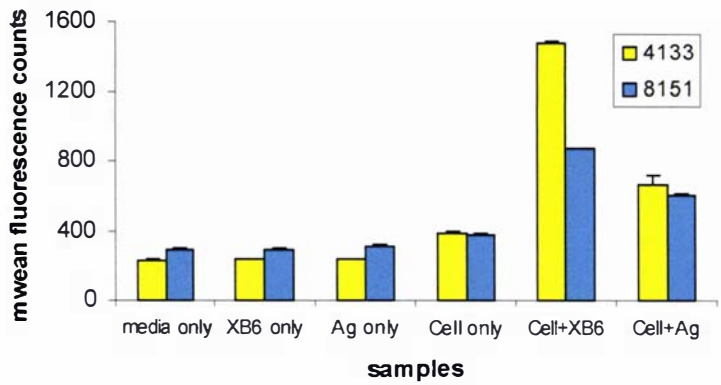

Fig. 5.2 Histamine release in the cell culture medium. Enriched mucosal mast cells (~1x10^6) in 100 µl HBBS were incubated with anti-ovine IgE XB6 (20 µg/ml) or *T. colubriformis* Larval stage 3 antigens (12.5 µg /ml) in 100ul, or control media – without agonists (100 µl). Each treatment was carried out in triplicate. Histamine content was determined spectro-fluorometrically in Wallace Victor^2 1420 Multilabel counter (Turku, Findland) and read at 355/460 nm (excitation/ emission). The error bar is S.D. Data were tabulated in Appendix 28.

Sheep	Status	Type of mucosal mast cells			
		1	2	3	4
4133	Before MACS	12	88	0	0
	After MACS	19	75	6	0
8151	Before MACS	94	0	2	4
	After MACS	80	20	0	0

Table 5.14 Morphological profiles of mucosal mast cells in Leishmann-stained cytosmears of enriched mucosal mast cell populations following autoMACS prior to cell culture. Twenty cells were counted at each of five different sites on each cytospot. Four degranulation stages of mucosal mast cell in oil-immersion microphotograph of Leishmann-stained cytosmears of lamina propria cells. (1) Pre-degranulation stage; (2) Partial degranulation stage; (3) High degree degranulation stage; (4) Complete degraulation stage. The morphology of the variants was described in Fig. 4.3.

Component	Mucus glycoproteins in supernatant (μg/ml)
eEP/GOs from sheep 7713 + Carbachol (100 μM)	6.37
eEP/GOs from sheep 5046 + Carbachol (100 μM)	3.76
Negative controls	
(1) - eEP/GOs only (from sheep 7713)	0.93
(2) - eEP/GOs only (from sheep 5046)	0.79
(4) - HBSS9 + Carbachol (100 μM)	0
(5) - HBSS9 only	0

Table 5.17 eEP/GOs response to secretogogue – carbachol (100 μM) *in vitro*. After *auto*MACS, the functional activities of eEP/GOs from 2 sheep were determined by mucus glycoprotein release *in vitro* in response to carbachol (100 μM). The CCS (20%) was assayed for mucus glycoprotein, and a double dilution of glycogen (2.5 – 80 μg/ml) was used as a standard. Crude data are tabulated in Appendix 30.

Source of cell factor (sheep)	Cell type	Mucus glycoproteins in eEP/GO supernatant after co-culture with a specific cell factor (μm/ml) *	
		sheep 7713	sheep 5046
8151	EO	4.21	2.03
1205	EO	9.01	2.23
8151	MMC	6.19	1.13
4133	MMC	4.05	0.45
9146	GL	9.01	4.02
0375	GL	0.42	0.47
eEP/GO + ES antigens			
7713		3.71	
5046		2.70	
eEP/GO only			
7713		0.93	
5046		0.73	

Keys: MMD-monocyte-macrophage-dendritic cell; EO - eosinophil; MMC - mucosal mast cell; GL - globule leucocyte; eEP/GO – enriched epithelial/goblet cells retained under 65%Percoll gradient. * after subtracting the value of negative control (eEP/GO only).

Table 5.18 Mucus glycoproteins in eEP/GO (n=2) after co-culture with cell factors derived from different cell types of small intestinal lamina propria; incubation time: 1h. Crude data for LMI% and mucus glycoprotein contents are tabulated in Appendix 31.1-3.

sheep	cell type	cell purity (%)	LMI%(S.D.)
8151	Eosinophil	61	84(9)
1205	"	68	60(9)
8421	"	93	17(5)
6271	"	94	23(5)
8151	Mucosal mast cell	90	31(7)
4133	"	83	13(7)
9146	Globule leucocyte	94	12(3)
0375	"	90	8(3)

S.D. - standard deviation.

Table 5.15 LMI activities of cell factors. Following the protocol of enrichment, a minimum of 250 µl cells ($20x10^6$/ml) was incubated in CO_2 incubator at 37°C with or without *T. colubriformis* ES antigens (3µgm) for 6 hrs. CCS are collected and assayed for LMI activity *in vitro*. Crude data are tabulated in Appendix 29.

Sheep	Method of enrichment	L	N	EO	MMD	MMC/GL	EP/GO
7713	EDTA & enzymatic digestion + 75%	6	2	17	2	17	56
	Percoll						
	*auto*MACS	3	0	7	1	2	**87**
5046	EDTA & enzymatic digestion + 75%	4	0	12	2	9	73
	Percoll						
	*auto*MACS	1	0	0	0	0	**99**

Keys: MMD - monocyte-macrophage-dendritic cell; L - lymphocyte; EO - eosinophil; MMC/GL - mucosal mast cell/globule leucocyte; EP/GO – epithelial/goblet cell;

Table 5.16 Purity of eEP/GOs after enrichment. After the epithelial bound cells were removed from gut tissues by EDTA cell dissociation method, the tissues were digested in enzymes to obtain lamina propria cells. Centrifugation of isolated cells on 75% Percoll density gradients was repeated till a sufficient number of eEP/GOs was obtained for further enrichment using *auto*MACS.

5.2.2.2.3 Larval migratory inhibition assay

The level of paralysis elicited by the cell factors is tabulated in Table 5.15. Eosinophil cell factors were the most active. However, high purity samples (6217 & 8421) did not yield higher LMI% as expected. In fact, the two samples giving the highest LMI activity (1205, 8151) contained a much higher proportion of activated eosinophils (Type II) than the samples with very pure eosinophils (6217 & 8421). Other cell factors derived from relatively pure mucosal mast cells and globule leucocytes demonstrated very poor LMI activity (Table 5.15).

5.2.2.3 Experiment 2.2

5.2.2.3.1 Parasitology

Faecal egg count of sheep of 7713 and 5046 at slaughter were 200 and 100 respectively.

5.2.2.3.2 Magnetic enrichment of epithelial/goblet cells

Enriched epithelial/GOs (eEP/GOs) prepared by density gradient centrifugation contained very low yields of lymphocyte, neutrophils and MMDs (less than 6%), and a considerable percentage of eosinophils (12%-17%), MMCs (9%-17%) and eEP/GOs (56%-73%) (Table 5.16). However, when these cells were enriched by *auto*MACS negative selection with a primary mAb to leucocyte common antigens (CD45RA), the purity increased to 87-99%. The ratios of GOs to epithelial cells were recorded as 58% and 25% in samples 7713 and 5046 respectively. The other portion of the epithelial cells was mostly undifferentiated GOs (Fig. 3.1C), and thus they were counted as epithelial cells. Viability with Trypan blue staining was 84% and 79% after Percoll, and 65% and 54% after *auto*MACS in samples 7133 and 5046 respectively.

5.2.2.3.3 Functionality of eEP/GOs

Table 5.17 tabulates the mucus glycoprotein release from eEP/GOs exposed to carbachol (100 μM). Sheep 7713 released more than the other sheep, perhaps, a higher proportion of GOs (58%) compared to sheep 5046 (25%) could be the reason. However, the release of mucus glycoproteins indicated that the eEP/GOs were still functionally active following *auto*MACS separation.

5.2.2.3.4 Effect of 'cell factors' on release of mucus glycoproteins from eEP/GOs

The amount of mucus glycoprotein released and the LMI activity of eEP/GO supernatants following exposure to different cell factors are tabulated in Table 5.18. The results show that all

of the cell factors, including TcL3ES antigens, were able to induce some level of mucus glycoprotein release by eEP/GOs.

5.2.2.3.5 Effect of 'cell factors' on the production of LPF by eEP/GOs

The LMI activity of eEP/GO supernatants after being co-cultured with cell factors derived from eosinophils, MMCs, and GLs is given in Table 5.19. It shows clearly that there was no increment in LMI activity during co-culture with eEP/GOs. LMI activity was virtually found only in supernatants from eEP/GO culture with eosinophil factors (EOF) from sheep 8151 and 1205. In fact, these two samples were themselves originally high in LMI activity (84% and 60% respectively). However, it was interesting that when LMI-active EOF was added to eEP/GO cultures (in both experiments), the LMI activity of the resultant supernatants were much higher than in samples that contained standard culture HBSS9 medium. This suggests that eEP/GO may secrete a substance(s) that preserves or is synergistic with LMI activity.

5.2.2.3.6 Effect of leucocyte vasoactive mediators and nematode parasite antigens on release of glycoproteins and LMI activity by eEP/GOs

Table 5.20 demonstrates a low level of mucus glycoproteins were produced by eEP/GO during co-culture with leucocyte vasoactive mediators. Mucus glycoprotein levels were similar to the levels induced previously by the mucosal mast cell and globule leucocyte cell factors (Table 5.18). This suggests that leucocyte vasoactive agents can trigger non-specific release of mucus glycoproteins from gut mucosal GOs. Antigen- induced release of mucus glycoproteins was also seen in P2 and eEP/GOs but the former released more mucus (Table 5.20). Perhaps, eEP/GOs had less effector cells than P2 to regulate the release of mucus glycoproteins since most effector cells of eEP/GOs were removed by *auto*MACS. Table 5.20 also shows the LMI activities elicited by P2 and eEP/GOs after being co-cultured with leucocyte vasoactive agents and ES antigens. Antigen-induced LMI activity was observed in P2 (68%) but not in eEP/GO population (12%). Vasoactive amines either alone (Table 5.21) or when co-cultured with eEP/GOs (Table 5.20), did not produce significant LMI activity, but the activity slightly increased when samples were pooled.

5.3 Discussion

The experiments reported in this chapter yielded new information about the cellular origin of LMI activity, and the seemingly preservative effect of epithelial/GO mucus.

Previous investigators used Percoll gradients to purify a specific cell type (mostly MMC/GL) from the rest of the gut mucosal cell population (Douch *et al.*, 1996b; Huntley *et al.*, 1984; Huntley *et al.*, 1982; Stankiewicz *et al.*, 1994). There was a lack of information about other cell types that could be recovered on different Percoll density gradients. The majority of epithelial cells were recovered above the 40% Percoll band, and most were columnar epithelial cells and differentiating GOs. Enrichment of the MMD population was partly achieved above the 50% Percoll gradient. The majority of lymphocytes, eosinophils and MMDs stayed above the 65% Percoll band, and the recovery and yields of MMC and GL (~70%) above the higher Percoll concentrations were similar to the findings of Stankiewicz *et al.* (1994) and Huntley *et al.* (1984). Based on the cell distribution profiles, two Percoll concentrations (40% and 65%) were selected to separate two Populations, *viz.* P1 which is between 40 and 65% Percoll bands, and P2 which is under the 65% Percoll band. P1 was comprised of lymphocytes, eosinophils, MMDs and EPI/GOs, and it showed less LMI activity than P2, which contained higher numbers of GOs and other minor cell types such as eosinophils, MMCs and GLs.

Interestingly, P2 had a higher yield of activated eosinophils and MMCs than P1. Perhaps the activated eosinophils are constituted with much larger granules and nuclei than other types, and similar to MMCs they are more granular, which may reduce their buoyancy and allow them to go through the 65% Percoll gradient and accumulate on the 75% Percoll cushion. P2 not only produced higher LMI activity, but also high levels of mucus. Perhaps this was due to the higher yields of GOs in P2. GOs are the chief mucus-producing cells in the gut, although columnar epithelial cells also produce some mucus (Cheng, 1974). This suggests the activated eosinophils and MMCs, together with EP/GOs were implicated in the release of LPF and mucus in P2. Therefore, further experiments were conducted to better understand the interaction between the effector cells in P2 that led to release of LPF and mucus.

The *auto*MACS separation allowed a sufficient number of viable and functional mucosal cells to be separated to generate supernatants, although cell viability after magnetic enrichment was lower (67%±9) than cell viability after enzymatic digestion (79%±7). Cell purity after *auto*MACS separation varied with different cell types, within a range of 68-94%. This is similar to that achieved by other investigators enriching cells from tissues. Human myoblasts have been purified to 90% level using the EDTA dissociation method followed by *auto*MACS separation (Lequerica *et al.*, 1999). Schmitz *et al.* isolated follicular dendritic cells from human tonsils using a three-step method similar to the current research (enzymatic digestion, and Percoll density gradient centrifugation followed by *auto*MACS), whereupon a purification of 79% was achieved (Schmitz *et al.*, 1993). As the former group yielded higher purity, it seems

that cell processing time, or the number of techniques involved in the enrichment, has some impact on cell viability and purity.

It is not an easy task to enrich viable eosinophils from gut tissues filled with a heterogeneous mixture of cells, and the absence of a specific antibody to sheep eosinophil surface antigens precludes a positive selection approach. In veterinary research many investigators (Duffus *et al.*, 1980; Rainbird *et al.*, 1998) used mammary gland eosinophils as a source, because they are relatively easy to obtain, in high purity ex *vivo* (80%) before further purification. However, the gut situation is different, a single cell suspension of gut mucosa digest showed the purity of eosinophils ranged between 15-50%, and they are among eight cell types. It was hoped that the purity would increase after discontinuous Percoll density gradient centrifugation, but it did not improve markedly, because the density range of eosinophils overlaps with other cell types, and they are not a predominant cell type in the population. Thus, the Percoll method was discontinued, and following enzymatic digestion, LPCs were prepared for a negative immuno-magnetic selection of eosinophils. This method increased the percentage of recovered eosinophils to 68%-90% with functionality intact. This result was similar to Hansel *et al.* and Bjerke *et al.* who enriched blood eosinophils and basophils using a similar technique respectively (Bjerke *et al.*, 1993; Hansel *et al.*, 1989).

Although several anti-GO antibodies have been produced for research purposes (Hibi *et al.*, 1994; Vecchi *et al.*, 1987), none of them are available commercially to facilitate GO isolation. Anatomically, GOs are most abundant in the colon. In the small intestine, they are scattered among absorptive columnar epithelial cells, and there are more absorptive columnar epithelial cells than GOs in the villi. According to Bennett, the GOs in the crypts are younger and tend to synthesize mucus more than secrete it, whereas in older cells the rate of secretion is faster than that of synthesis, causing mucus accumulation in the younger cells and depletion of mucus in the older cells (Bennett, 1976). This indicates that a longer enzymatic digestion is required to obtain younger GOs full of mucus from the crypts.

Interestingly, none of the factors secreted by MMCs and GLs into supernatants demonstrated significant LMI activity. In contrast, EOF had significant LMI activity. Surprisingly, the activity of high purity samples (sheep 8421 & 6271) was lower than that from low purity samples (sheep 8151 & 1205). Examination of Leishmann stained cytospots suggested that morphological gradations of eosinophils in the populations may partly account for this discrepancy. The higher purity samples contained a significantly lower number of stage II activated eosinophils (10%-15%) than did the lower purity samples (61%-70%), suggesting that activated eosinophils might be implicated in the release of LMI activity. It seems active cells are

more responsive to stimuli (*i.e.*ES antigens) than non-active ones. It may be the reason that in the functionality test for eosinophils (Fig. 5.1) that supernatants with high numbers of activated eosinophils contained higher EPO than supernatants with lower numbers of activated cells (Fig. 5.1). These findings were in line with the results reported in Chapter 4: Experiment 4.2.2, in that high numbers of activated eosinophils in the first six metres were also associated with high LMI activity in that location.

Table 5.11 demonstrates that other cell types such as MMCs and GOs, which did not produce LMI activity, were present in the low purity eosinophil enriched populations. Activated MMCs can rapidly secrete a variety of cytokines (Burd *et al.*, 1995; Gordon *et al.*, 1990; Henz *et al.*, 2001) and mediators (Jones & Emery, 1991; Jones *et al.*, 1994) which have a potent influence on eosinophil degranulation (Fujisawa *et al.*, 1990; Stevenson & Jones, 1994; Takafuji *et al.*, 1998). In addition, IL5 or nematode parasite antigen-induced activation of eosinophils have been shown to produce anti-larval substances *in vitro* (Jonas *et al.*, 1995; Rainbird *et al.*, 1998). Based on these findings, one could speculate that the mediators and cytokines released from activated MMCs were implicated in the activation of eosinophils leading to the release of LMI activity. It also indicates that the number of activated cells in the culture is perhaps equally important to the release of LPF as is the purity of the cell type of interest. Cell activation induced by other effector cells could further enhance LMI activity. In addition, the current work demonstrated that all commercially available vasoactive mediators contained no anti-nematode substances, and this result is consistent with Douch (1990).

The current work confirmed again that LMI activity was associated with enriched EOF. Supernatants from enriched epithelial/GOs (eEP/GOs) alone, or eEP/GOs with TcL3ES antigens, did not contain LMI activity, however the latter contained more mucus glycoproteins than the former. Mucus glycoprotein release was also observed in supernatants from eEP/GOs with EOF. The reasons could be several. All cell factors were induced by nematode antigens and were likely to contain a mixture of antigens, leucocyte vasoactive mediators, and immune complexes. It has been shown that antigen-stimulated gut MMCs can rapidly release the leucocyte vasoactive mediators *in vitro* (Jones *et al.*, 1992). Since the gut was well primed, it may have contained plasma cells secreting nematode-specific antibodies in the lamina propria isolates, and the formation of immune complex in the cell factors could be possible. Either immune complexes (Walker *et al.*, 1977) or vasoactive mediators (Hoffstein *et al.*, 1990; Shelhamer *et al.*, 1980) can stimulate non-specific release of mucus glycoproteins from GOs. Mucus release from GOs by leucocyte vasoactive mediators was also demonstrated in the

present study (Table 5.18). Thus, it is likely that these components in the cell factors could mediate the release of mucus from eEP/GOs. In comparison, antigen-induced release of mucus glycoprotein was seen to be much higher in P2 than in eEP/GO population (Table 5.20). Perhaps, eEP/GO populations had less cells to regulate the release of mucus glycoproteins than P2, since most effector cells were removed from eEP/GO population by *auto*MACS separation. Clearly, there was no increment in the LMI activity of any of the cell factors tested during co-culture with eEP/GOs. However, Table 5.19 demonstrates the intriguing result that when added to eEP/GO cultures (at 20%), the LMI activity of EOF was much higher than when the factor was added at 20% to buffered medium. This indicates that the LMI activity of EOF was in some way sustained or preserved by components released from eEP/GOs, possibly mucus glycoproteins. Interestingly, a similar comment was made by Jones that gut mucus could stabilise and prolong the biological activity of inflammatory mediators [cited as *pers. com.* in the review of (McClure, 2000)].

In conclusion, eosinophils appear to be the major cellular source of LMI activity, provided that they are in an activated stage to enable them to release LPF. As we know, eosinophil activation can be mediated by molecules released from other effector cell types in the gut mucosa, so other cell types may contribute to LPF production, directly or indirectly, by interacting with eosinophils. It will be important to investigate the cell type(s) which interact with eosinophils in LPF production *in vitro*. The experiments in this Chapter clearly demonstrated that EP/GOs were not a source of LPF, and that factors from eosinophils, MMCs, GLs, as well as nematode parasite antigens and vasoactive mediators, could mediate the release of mucus glycoproteins *in vitro*. In addition, the current studies show the use of various techniques to enrich each cell type in gut mucosa, while maintaining their functionality, viability and morphology. Lastly, this study points towards a potential missing link between GOs and other cells in the effector arm of mucosal anti-nematode responses.

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Chapter 6
Demonstration of additive effect of gut mucus on LMI activity

6.1 Introduction

Chapter 5 has shown that an enriched epithelial/goblet cells (eEP/GOs) did not produce LMI activity, but it could release mucus glycoproteins in response to gut effector cell secretions (cell factors), nematode parasite antigens and leucocyte vasoactive mediators. The most interesting result was that a secretion from the eEP/GOs (presumably mucus) appeared to sustain the LMI activity of eosinophil factor (EOF). The purpose of this experiment was to confirm the additive effect of gut mucus on LMI activity of test substances, *i.e.* LPF-active EOF and the anthelmintic levamisole.

6.2 Materials & methods

Experimental approach

One field-resistant-line sheep was immunised using the LPF regime; food was withheld for 24 hr and killed; and the eEP/GOs were was isolated as in the methods described in Chapter 5. Firstly, eEP/GOs were co-cultured with a range of concentrations (1.25-20%) of LPF active eosinophil cell factors (from sheep 8151) for 1 hr, and the resultant CCSs were assayed together with the two controls (*i. e.* cell factors diluted with HBSS9 or naïve mucus) for LMI activity. Secondly, a doubling-dilution titration of levamisole was prepared, using either gut mucus from a naïve lamb or buffered medium (HBSS9) as diluent, and the resultant levamisole-containing samples were assayed for LMI activity. All samples were assayed in triplicate.

Strategy of generating eEP/GOs

Method for generating eEP/GOs was identical to Chapter 5. Cells, collected after enrichment, were immediately co-cultured with EOF and used for other purposes: differential cell counts and functionality test. Cell viability was evaluated by Trypan blue exclusion following Percoll density centrifugation and *auto*MACS separation.

Functionality of eEP/GOs

Functional activity test was also identical to the test described in Chapter 5.

Co-culturing with EOF

eEP/GOs were adjusted to 20 x 10^6/ml in HBSS9 and plated out in 250 µl aliquots in a collagen coated 24-well culture plate (NUNC, USA). Cells were incubated at 37°C in CO_2 for 15 minutes to equilibrate the culture conditions. Two hundred and fifty micro-litres of HBSS9, with or without *T. colubriformis* ES antigens (3µgm) or with cell factor (final concentration – 20%), was added to each well and re-incubated for 6 hrs. The CCS were collected and spun (as above) and assayed for LPF activity and mucus glycoprotein *in vitro.*

6.3 Results

6.3.1 Faecal egg count

Faecal egg count of sheep at slaughter was zero.

6.3.2 Differential cell counts

Normally, eEP/GOs were in low yield, and thus, several density gradient fractionations were carried out and pooled to obtain a sufficient number of EP/GOs. Differential counts performed on cytospots, after Leishmann's staining, revealed that eEP/GOs (cell bands on 75% Percoll gradient) contained 1% each of lymphocytes and neutrophils, 14% eosinophils, 3%, MMDs, 11% MMCs/GLs and 70% EP/GOs (Table 6.1). However, when EP/GOs were enriched as a negative population by autoMACS separation using a mAb to leucocyte common antigens (CD45RA), the purity increased to 97% (Table 6.1; Fig. 6.1). In eEP/GOs, the percentage GOs to epithelial cells was recorded as 29%. Viability with Trypan blue staining was 71% after Percoll centrifugation and reduced to 64% following *auto*MACS separation. No significant difference in cell size or morphology was observed.

Method of enrichment	L	N	EO	MMD	MMC/GL	EP/GO
EDTA & enzymatic digestion + 75%	1	1	14	3	11	70
Percoll						
*auto*MACS	2	0	1	0	0	97

Keys: MMD - monocyte-macrophage-dendritic cell; L - lymphocyte; EO - eosinophil; MMC/GL - mucosal mast cell/globule leucocyte; EP/GO– epithelial/goblet cell;

Table 6.1 Purity of eEP/GO after enrichment. After the epithelial bound cells were removed from gut tissues by EDTA cell dissociation method, the tissues were digested in enzymes to obtain lamina propria cells. Centrifugation of isolated cells on 75% Percoll density gradients was repeated till a sufficient number of EP/GOs was obtained for further enrichment using *auto*MACS separation.

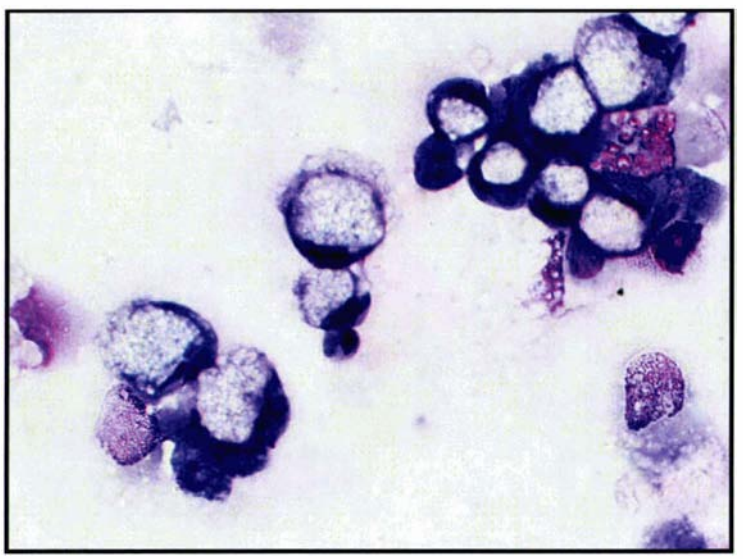

Fig. 6.1 Oil-immersion microphotograph of a Leishmann-stained cytospin preparation illustrating enriched epithelial/goblet cells (eEP/GOs) . Magnification x 600

Component	Mucus glycoprotein in CCS (μg/ml ± S.D.)
eEP/GO from sheep 6070 + Carbachol (100 μM)	3.04(1)
Negative controls	
eEP/GO only (from sheep 6070)	0.65
HBSS9 + Carbachol (100 μM)	0
HBSS9 only	0

Table 6.2 eEP/GOs response to secretogogue – carbachol (100 μM) *in vitro*. After *auto*MACS, the functional activity of eEP/GOs was determined by mucus glycoprotein release *in* vitro in response to carbachol (100 μM). The CCS (20%) was assayed for mucus glycoprotein, and a double dilution of glycogen (2.5 – 80 μg/ml) was used as a standard. Crude data are tabulated in Appendix 35.

Component	Mucus glycoprotein in supernatant (μg/ml)
eEP/GOs from sheep 6070 + EOF (8151)	5.37
eEP/GOs from sheep 6070 + ES antigens	3.83
Negative controls	
eEP/GOs only	0.91
HBSS9 only	0.00

Table 6.3 eEP/GOs response to eosinophil cell factor and ES antigens of *T. colubriformis* stage 3 larvae. After *auto*MACS, eEP/GOs were determined by mucus glycoprotein release *in vitro* in response to EOF (final concentration 20%) and ES antigens (3 μg). The CCS (20%) was assayed for mucus glycoprotein, and a double dilution of glycogen (2.5 – 80 μg/ml) was used as a standard. Crude data are tabulated in Appendix 35.1.

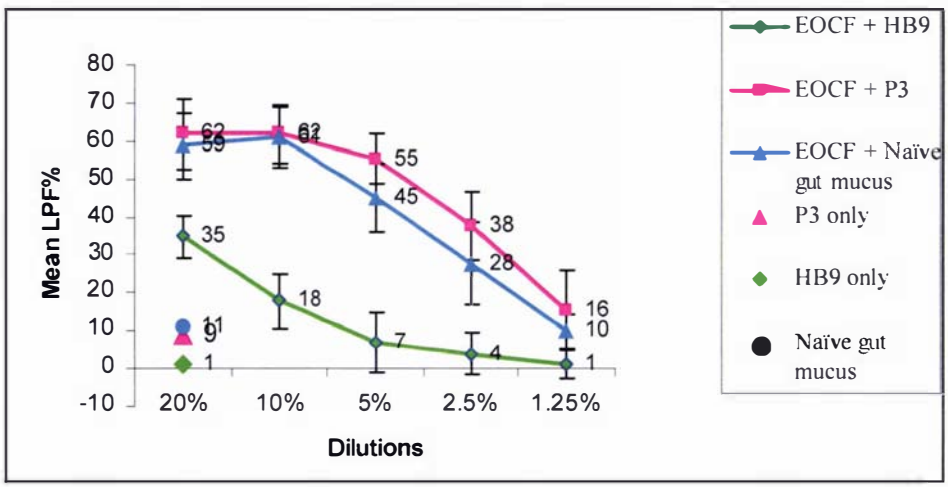

Fig. 6.2 Preservative effect of mucus glycoprotein on LMI activity of cell fatctors *in vitro*. P3 isolated from sheep 7133 was cultured with a range of concentrations (1.25-20%) of LPF active eosinophil cell factors (from sheep 8151), and the resultant CCSs were assayed together with control samples (Cell factors diluted with HBSS9 or naïve mucus) for LPF activity. The samples were in triplicate. The error bars are S.D. Crude data are tabulated in Appendix 36.

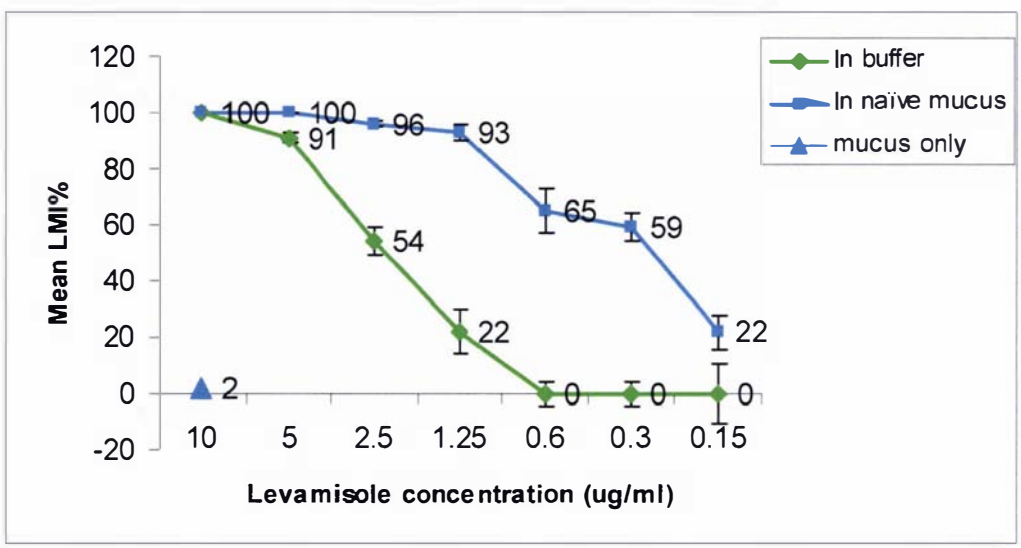

Fig. 6.3 Additive effect of mucus glycoprotein on LMI activity of levamisole *in vitro*. Levamisole was diluted with gut mucus from naïve lamb or buffered medium (HBSS9) and assayed for LPF activity. The samples were in triplicate. The error bars are S.D. Crude data are tabulated in Appendix 37.

6.3.3 Functionality of eEP/GOs

Table 6.2 tabulates the mucus glycoprotein release from eEP/GOs exposed to carbachol (100 μM). The release of mucus glycoproteins in the CCS indicated that the eEP/GOs separated using the EDTA and enzymatic digestion, in combination with Percoll gradient separation and following *auto*MACS separation were still functionally active.

6.3.4 'Cell factors' induced mucus glycoprotein release by eEP/GOs

Table 6.3 shows that both EOF and TcL3ES antigens were able to induce some level of mucus glycoprotein release by eEP/GOs.

6.3.5 Effect of eEP/GOs on the LMI activity of EOF and levamisole

The experiment using a range of concentrations (1.25%-20%) of LPF-active EOF in eEP/GO cultures and the negative experiment controls (HBSS9 and LPF non-active naïve lamb gut mucus) demonstrated that the supernatants containing eEP/GOs or naïve mucus had a higher LMI activity than the supernatants containing only buffer. The other intriguing result was that the cell factor induced LMI activities in eEP/GOs or naïve mucus were somehow preserved in the range of 45%-62%; and even at the 5% level of concentration the activity was still 45%-55%. In contrast, in samples diluted with HBSS9, LMI activities dropped steadily as the dilution increased, and the activity was at low levels even at the 10% concentration (Fig. 6.2). The correlation coefficient (r) of LMI% between eEP/GOs with cell factors, and naïve mucus with cell factors was 0.98; and between the former and buffered medium HBSS9 with cell factors was 0.74 at p level 0.05.

The titration of levamisole (0.150 - 10 μg/ml) in HBSS9 and gut mucus from nematode-naïve sheep, demonstrated a similar effect to the above in that samples containing mucus sustained a higher level of LMI activity than did samples containing HBSS9 only (Fig. 6.3). High levels of LMI activity (93%-100%) were maintained in mucus samples containing levamisole concentrations as low as 1.25 μg/ml, whereas the samples without mucus have high activity at only 5 μg/ml and above. The results indicated that mucus-containing samples required only 0.3 ug/ml of levamisole to give rise to 60% LMI activity, whereas samples without mucus required as much as 2.5 μg/ml. This result indicates mucus-containing samples are 8 times more potent than non-mucus-containing samples.

6.4 Discussion

This study confirms the finding of Chapter 5 that mucus glycoproteins released from eEP/GOs enhanced and sustained LPF activity of eosinophil cell factor. This and the results for naïve mucus point to some probable missing links involved in the effector response of GO mucus in mucosal immune responses to gut nematodes in sheep.

The functionality test showed that isolated eEP/GOs were able to degranulate mucus glycoproteins in response to carbachol (Table 6.2), and a similar response was given when they were co-cultured with eosinophil cell factor or with nematode parasite antigen alone (Table 6.3). These responses were consistent with results in Chapter 5. GOs are in fact the chief cells to secrete mucins in the gut (Forstner *et al.*, 1973), and these mucins hydrate and gel, then interact with their aqueous environment in the lumen; therefore a variety of components secreted into the gut lumen such as water, electrolytes, arrays of cell secretions, immunoglobulins, and dead epithelial cells reside in this sticky gel to form a physical and chemical protective mucus coat overlying the epithelial surface (Specian & Oliver, 1991). GO hyperplasia and mucus secretion were reported to be one of several host cellular responses to gut irritations including gut nematode parasite infections (Manjili *et al.*, 1998; Neutra *et al.*, 1982). Traditionally, it has been proposed that the functional role of mucus is to form an epithelial protective barrier, as surface lubricants for smooth flow of particles on the mucosal surface (Florey, 1962). However, based on studies of immune animals, it has been suggested that another functional role of mucus is as an effector agent in worm expulsion from the gut. It has been shown that mucus contains a compound, similar to the slow reacting substance of anaphylaxis (SRS-A) whose incorporation into mucus paralyses worms (Douch *et al.*, 1983). This provided a physical barrier for immune-entrapment of nematode parasite larvae (Appleton *et al.*, 1988; Carlisle *et al.*, 1990) and of lumen bacteria by specific antibodies (Magnusson & Stjernstrom, 1982). Histochemical changes in GO mucins were found to be critical for worm expulsion (Ishikawa *et al.*, 1993; Oinuma *et al.*, 1995).

The experiments reported here identify a novel additional role for mucus derived from eEP/GOs, namely to augment LMI activity *in vitro*. As an alternative to the mucus of eEP/GOs, mucus from a naïve lamb was used to dilute a LPF-active EOF, similar results were obtained. A similar additive effect of gut mucus was reproduced in the experiment using levamisole as an anti-nematode agent. Therefore, this function of mucus is confirmed and can be added to the above list, *i.e.* to protect host anti-microbial molecules from rapid degradation.

However, the present results raise questions about the mechanics of the mucus-enhanced association between the surface of the worm and the anti-worm molecules. Although entirely speculative, it may involve lectin-like binding. Recent rodent studies have shown that a novel protein - intelectin - mediates anti-gut nematode response, and it was shown that Paneth cells and GOs were the cellular source of intelectins (Kuperman *et al.*, 2005; Pemberton *et al.*, 2004a; Pemberton *et al.*, 2004b). However, intelectins are predominantly expressed in immune animals, and in the current study it did not matter, whether the mucus was derived from immune or naïve animals. Perhaps, there is another kind of GO-specific mucus, released just to protect the functional integrity of gut epithelial surfaces that also serves a protective role in gut immune responses to nematode parasites.

Taken together, the results lend support to the concept (Miller & Nawa, 1979a) that mucus serves an important function in protection against small intestinal nematodes. This study also suggests that GOs themselves do not produce LPF, but do secrete substances which are synergistic with anti-nematode molecules released at the gut mucosa, and they may facilitate the expulsion mechanism by prolonging the existence of anti-nematode molecules. Further research is required to elucidate the proteins or other substances which enhance and sustain antimicrobial molecules at the gut mucosa.

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Chapter 7
Purification and characterization of larval paralysis factor

7.1 Introduction

The only information available regarding the molecular properties of larval paralysis factor (LPF) in mucus from nematode immune sheep was published in 1983 by Douch *et al.* Based on the arylsulphatase reaction, they concluded that the anti-larval activity in the mucus was associated with substances having some properties characteristic of slow reacting substances of anaphylaxis (SRS-A) (Douch *et al.*, 1983), and the activity was heat and base stable, though acid labile, and had a molecular weight less than 1000. This chapter reports further physicochemical characteristics and a purification process for LPF. The chapter also describes two *in vitro* larval paralysis assays which were developed, based on a previous larval paralysis assay used for detecting drench-resistant nematodes (Martin & Le Jambre, 1979), for assaying chromatographically purified samples which are normally produced in a very small amount.

7.2 Material and methods

7.2.1 Investigation design

Ten field resistant-line ewes aged between 2-4 years from a culled group were brought into a pen, drenched, and sensitised by the LPF regime (Chapter 2: section 2.1.1.2). The leftover pooled CCS from Chapters 3 and 4 was pooled with a new batch of CCS to conduct the biochemical analyses described in this chapter. Most of the materials and methods of this section are described in Chapter 2. The properties of LPF, such as solubility, were studied in water, ether, methanol, and ethanol; the stability was examined in acid, base, enzymes, and at different temperatures; and the LMI activity against L3 of other ruminant gut nematode

parasites such as *Haemonchus contortus*, *Teladorsagia circumcincta, Nematodirus spathiger,* and adults of *T. colubriformis* were also investigated. Several purification steps were undertaken to tailor the molecular characterization using liquid chromatography mass spectrometry (LC-MS).

7.3 Results and discussion

7.3.1 *In vitro* larval paralysis assay

The larval migration inhibition assay (LMI assay) developed by Rabel *et al.* (1994) was replaced by freshly developed paralysis assays which were based on the idea of Martin & Le Jambre (1979) for detecting drench-resistant worms. The main concern about the LMI assay was that it requires a 400 µl test sample which means it is not suitable for assaying chromatographically purified analytical samples which are mostly available in a few microlitres. The LMI assay had been used in earlier experiments when the sample size was not limited. Two alternative assays also detect larval paralysis (LP). LP assay 1 is performed in a 96-well microtitre plate and LP assay 2 is in 72-well Terasaki plates. Although the maximum well capacity of the former is 250µl, only a 40µl test sample is used, this assay was mostly applied to testing pre-purification samples with moderate supply. The Terasaki plate wells hold 10µl and were used for all chromatographically purified samples. Both assays take 2 hours, are reproducible and suitable for small sample analysis. LP assay 1 has been commonly used for testing anthelmintic resistance in ruminant nematodes, although there are some variations in the method (Kotze *et al.*, 2006; Lacey *et al.*, 1995; Varady & Corba, 1999).

The LP assay 2 in Terasaki plate format is the first larval paralysis assay performed in this type of plate. The assay performed on 10µl test samples is uniquely suitable for testing HPLC samples which are available in limited amounts. However, the drawback of LP assay 2 is that it is labour intensive, since it requires removing 8 µl of liquid from the larval suspension in a well after larvae have settled at the bottom, for accommodating the 8 µl test sample. Also, it is important to keep the assay plate in a moist container during incubation at 37°C to prevent evaporation.

Paralysis of larvae in the plate was determined by direct microscopic observation. Larvae were incubated with test samples or control media for 2hrs. Well contents were then agitated by

	Larval paralysis activity (%)			
CCS concentration (%)	80	40	20	10
Nematode species				
T. colubriformis	76	69	49	13
N. spathiger	87	67	31	0
H. contortus	92	88	56	56
Tel. circumcincta	74	49	22	0
O. Ostertagi	78	41	23	0
P. trichosuri	75	52	19	9
Levamisole (5ug/ml)	99			

Table 7.1 LP assay 2 of CCS against other nematode species. Larvae suspension (10µl) and 40µl test samples were added to each well of flat-bottom 96-well plates. A positive control (Levamisole 5µgm/ml of 5% DMSO in distilled water) and negative control (HB9) were included in each assay. All samples were assayed in triplicate and plates were incubated at 37°C for 2hr in a moisturised plastic container. After incubation, the content of each well was agitated twice using a pipette, and all mobile larvae were counted after 1 min. LP activity (%) was calculated from the formula described in Chapter 2: Methods and materials. Crude data are tabulated in Appendix 39.

Components	Number of immobile worms			
CCS concentration(%) in buffer	80	40	20	10
Adult *T. colubriformis*	5	5	5	1
Levamisole	5			
Buffer	0			

Table 7.2 Paralysis activity of CCS against adult *T. colubriformis*. Five adult worms were gently picked up and put into each well of a flat-bottom 24-well plate containing 500 µl CCS or control solutions. A positive control (Levamisole 5µgm/ml of 5% DMSO in distilled water) and negative control (PBS) were included in each assay. Plates were incubated at 37°C for 2hr in a moisturised plastic container. After incubation, the content of each well was agitated twice using a pipette, and all mobile worms were counted after 1min.

Duration of incubation in buffer	Mean percentage of larvae recovered			
	CCS 1	CCS 2	CCS 3	Mean %
2hr	44	58	34	**45**
6hr	53	40	34	**42**

Table 7.3 Mean percentage of larvae recovered from paralysis. After incubation with active CCSs from three sheep, the paralysed larvae in the wells were rinsed, re-incubated in fresh media at room temperature for 2-6hr and all mobile larvae were counted. Crude data are tabulated in Appendix 40.

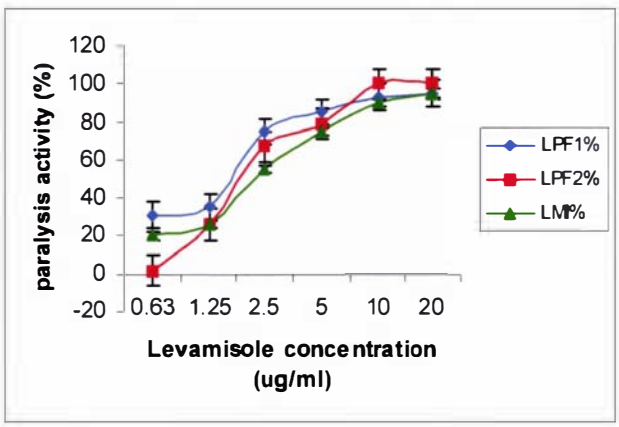


Fig. 7.1 Percentage of *T. colubriformis* larvae paralysed in the presence of levamisole in increasing concentrations in two types of LP assays and LMI assay. See Appendix 37 & 38 for crude data. Error bars are standard variations. LP assay 1 = 96-well microtitre plate assay; LP assay 2 = Terasaki microtitre plate assay; LMI assay = LMI assay of Rabel *et al.* (1994) with some modifications.

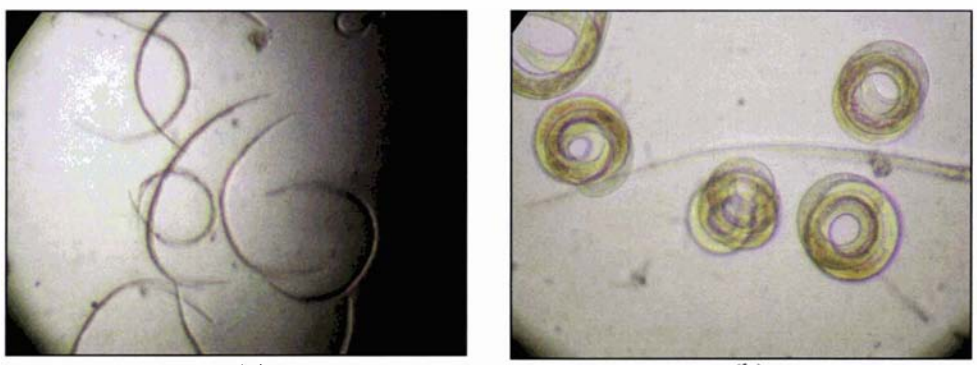


Fig. 7.2 Two types of larval paralysis produced by active CCS.
(a) - crescent; (b) - coiled;

pipetting and larval motility was assessed 1 minute later. Levamisole produced rapid paralysing contraction (coiling) of larvae immediately after larvae were exposed to the drug. Nevertheless, the most commonly used concentration (5 μg/ml) required at least 2 hrs to produce 100% paralysis (100-150 larvae). The contraction of larvae is believed to be caused by levamisole acting as a nerve ganglion stimulant (Coles *et al.*, 1975), and in the present bioassays levamisole gave a sigmoid dose-response (Fig. 7.1) similar to previous reports (Rabel *et al.*, 1994; Wagland *et al.*, 1992). LP assays depend on the subjective assessment of paralysis, whereas the LMI assay is based on the ability of larvae to migrate through a 20 μm sieve (Rabel *et al.*, 1994). Situations such as using unclean and blocked sieves in the LMI assay, clumping of larvae caused by other means, and the formation of air pockets underneath the sieve, when the larvae are transferred onto it for the second incubation, can create non-specific inhibition of larval migration. Therefore, the LMI assay cannot rule out that the inhibition of larval migration was caused by something other than paralysis. However comparing the three assays, in all cases the correlation coefficient (r) was significantly different from zero (Appendix 37).

All assays revealed the paralysis activity of CCS. The Terasaki microtitre plate assay made possible the development of a method for purification and characterization of LPF. In testing CCS samples against *T. colubriformis* larvae, observation revealed that there were two types of larval paralysis: some larvae became slightly curved like a crescent; but most were tightly coiled, as demonstrated by Douch *et al.* (1983) who reported that nematode larvae coiled when they were treated with levamisole or small intestinal mucus from immune sheep. The former type was like a crescent or slightly curved in shape, and the latter were coiled like a spring (Fig. 7.2 a & b). Both types of paralysis were observed in most active CCS, but more coiled than crescent type. However, a small number of CCSs produced only crescent type paralysis.

7.3.2 Determination of LMI activity against other nematode species

The rationale of this experiment was that the LMI activity had been primarily tested only against *T. colubriformis* L3 stage larvae. However, to assess the anthelmintic potential of LPF, its efficacy on other nematode parasites was investigated. Table 7.1 & 7.2 demonstrate that active CCS has the ability to paralyse a wide range of nematode species: larvae of *Trichostrongylus colubriformis*, *Haemonchus contortus*, *Teladorsagia circumcincta*, and *Nemtodirus spathiger* in sheep, *Ostertagia ostertagi* in cattle, *Parastrongyloides trichosuri* in possums, and adults of *T. colubriformis* in sheep. Compared to *T. colubriformis* larvae the

potency of CCS seemed to be higher in *T. colubriformis* adults and *H. contortus* larvae, and slightly lower in *Ostertagia* species. These results are consistent with the model of Dineen and co-workers that the expulsion of worms in immune sheep requires specific-antigen stimulation, but elimination is non-specific (Dineen *et al.*, 1977). Table 7.3 shows that only 42-45% of the larvae recovered from paralysis after rinsing twice and re-incubating in a fresh buffer for 2-6hr, indicating that the LP activity is partially reversible.

7.3.3 Physicochemical nature of LPF

Previous experimental evidence suggested that the LMI activity in immune sheep mucus was associated with a substance similar to slow releasing substances of anaphylaxis (SRS-A) (Douch *et al.* 1983). The basic components of SRS-A are leukotrienes (LTB4, LTC4, LTD4, LTE4) (Lewis & Austen, 1981); these lipid mediators are unstable and not easily soluble in water, but commonly soluble in ether and ethanol. The leukotriene-like substances in mucus were reported to be heat and acid labile, but stable in base. The results shown in Table 7.4 suggest that LPF was primarily soluble in water, to some extent in methanol, and not in ether, acetonitrile or ethanol. Solubility tests are commonly used to simplify the list of functional groups. If the compound is insoluble in ether, but soluble in water, then no more solubility tests are needed and they are in solubility class S_2; S2 are organic salts, amino acids, sugars, hydrophilic functional groups (Shriner *et al.*, 2004). The pH range of CCSs was 6-7. Besides, LPF was resistant to arylsulphatase which has been found to destroy leukotrienes (LTs) in the experiment of Douch *et al.* (1983). Moreover, CCSs were shown to be resistant to acid, boiling, and proteinase-K, although sensitive to base, repeated freeze and thaw, and boiling with acid (Table 7.5). Resistance to acid and enzyme is a unique property of LPF enabling survival to act as an anthelmintic in a hostile gut environment. Nevertheless, the loss of activity after 5 freeze and thaw cycles is one drawback of LPF to be aware of, especially during purification.

7.3.4 Purification of LPF

7.3.4.1 Centrifugal ultrafiltration

To estimate the molecular weight of LPF, 3ml of crude CCS was put through a series of ultra-filtration membranes (0.2 μm,100KD, 10KD, 3KD & 1KD), and the resultant fractions were assayed for LP activity. Table 7.6 shows that LP activity was accumulated under the 1KD ultrafiltration membrane, the mean LP activity (69%) was lower than the pre-filtration CCS

	Components	Mean LP Activity (%)
1	Untreated CCS	80
2	Milli-Q water (control)	76
3	Ether- S	0
4	Ether-I	65
5	Acetonitrile-S	0
6	Acetonitrile-I	76
7	Ethanol-S	21
8	Ethanol-I	59
9	Methanol-S	42
10	Methanol-I	67
11	Levamisole (5ug/ml)*	98

S= supernatant; I = insoluble materials ; * assay positive control

Table 7.4. Solubility of LPF. Each aliquot of 250µl lyophilised CCS (under MW3KD) was dissolved in 0.5ml each of water, ether, ethanol and methanol. Supernatants were separated by centrifugation; both supernatants and filtrates were dried, made up to the original volume with Milli-Q water and bioassayed. Crude data are tabulated in Appendix 41.

Treatments	Mean LP activity (%)
pH12, 10min, RT	8
pH1, 10min, RT	64
Boiling, 10min	51
Boiling with acid, 10min	1
Freeze/Thaw x 5	31
Freeze/Thaw x10	6
CCS + Proteinase-K, 1hr, 37^{0}C	61
Arylsulphatase (300U/ml)	64
Controls	
Proteinase-K only at 37^{0}C for 1hr	10
Untreated CCS	68
Levamisole (5ug/ml)*	100

* assay positive control

Table 7.5 The treatment which affects the LP activity is printed in bold type. Negative control is proteinase-K only in buffer; the positive controls are levamisole and untreated CCS. Crude data are tabulated in Appendix 42 & 43.

Components	Mean LP activity (%)	STD
crude CCS (pre-filtration)	89	5
0.2 um nylon membrane	9	12
100KD ultra-filtration membrane	14	9
10KD ultra-filtration membrane	11	11
3KD ultra-filtration membrane	12	14
1KD ultra-filtration membrane	13	8
*1KD ultra-filtration membrane	**69**	**5**
Levamisole (5 ug/ml)**	99	2

* *assay positive control

Table 7.6 LP activity of CCS fractions after high pressure filtration. Materials retained on each membrane were reconstituted in 3 ml HB9 the starting volume for bioassay.
* Materials went through 1KD membrane. The active fractions are in bold print. Crude data are tabulated in Appendix 44.

Gel fractions	Tube numbers	LPF%
1	90-98	24
2	99-103	10
3	104-112	24
4	113-116	45
5	117-120	3
6	121-124	14
7	**125-131**	**100**
levamisole		100

Table 7.8 Mean LP activity of fractions from a double bio-gel P2 filtration. The most active fraction 7 is in bold print. Crude data are tabulated in Appendix 46.

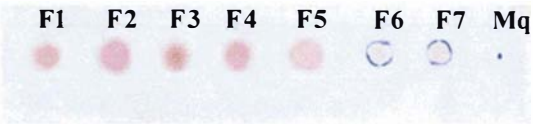


Fig. 7.5 Thin layer chromatogram of seven biogel-P2 fractions. Two microlitres of each fraction and control Milli-Q water were spotted on silica gel 60 F_{254} (Merck, Germany) plate and air dried. The plate was sprayed with 0.3% ninhydrin reagent in a spray box placed in a fume hood and then heated to 120°C in an oven for 10min until the colour spots appeared. Fractions 6 and 7 were circled with a pen before they faded out. Milli-Q water (control for the analysis).

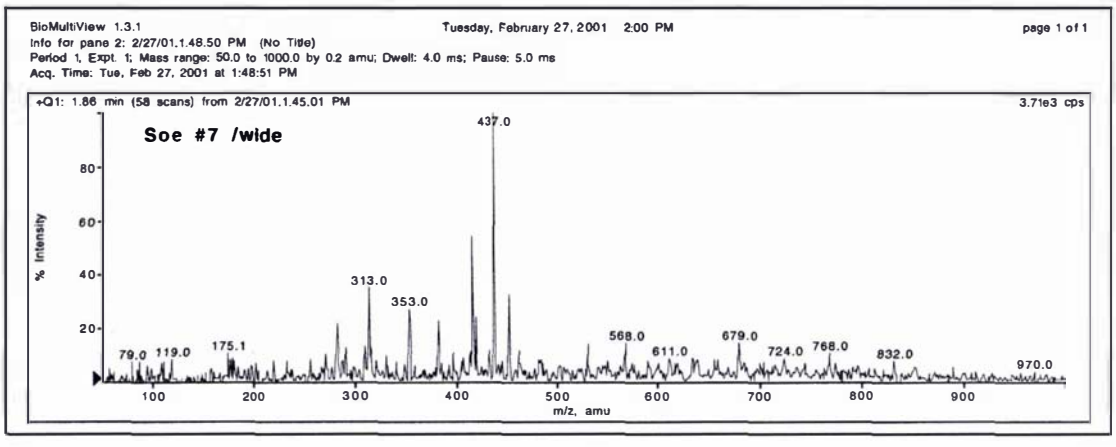
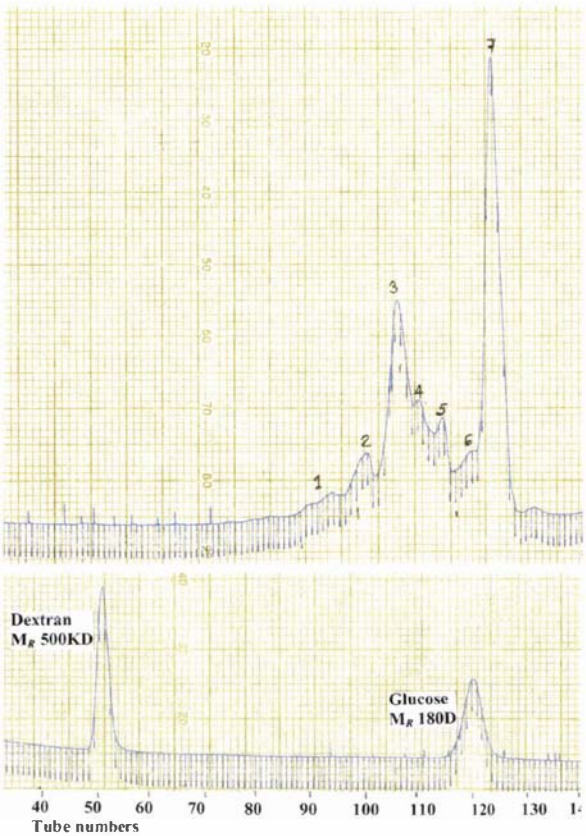


Fig. 7.6 Mass spectrum of the active gel fraction 7 of CCS (under 3KD). The sample was diluted to 10pmol/uL with 0.1%(v/v) acetic acid in 50% acetonitrile and injected by a syringe pump at 30uL/min into an ABI SciEx-300 ESI-MS with API-300 (atmospheric ionisation) module. Detector voltage is 1.8-2kV in positive mode. (The spectrum was recorded by Trevor Loo from Molecular Biosciences, Massey University.)

Gel fractions	Tube numbers	Mean LP activity (%)
1	1-20	0
2	21-35	30
3	36-45	18
4	46-52	9
5	53-56	10
6	57-61	14
7	62-65	21
8	**66-70**	**100**
9	**71-79**	**100**
10	**80-88**	**100**
11	89-92	38
12	93-100	3
levamisole		100

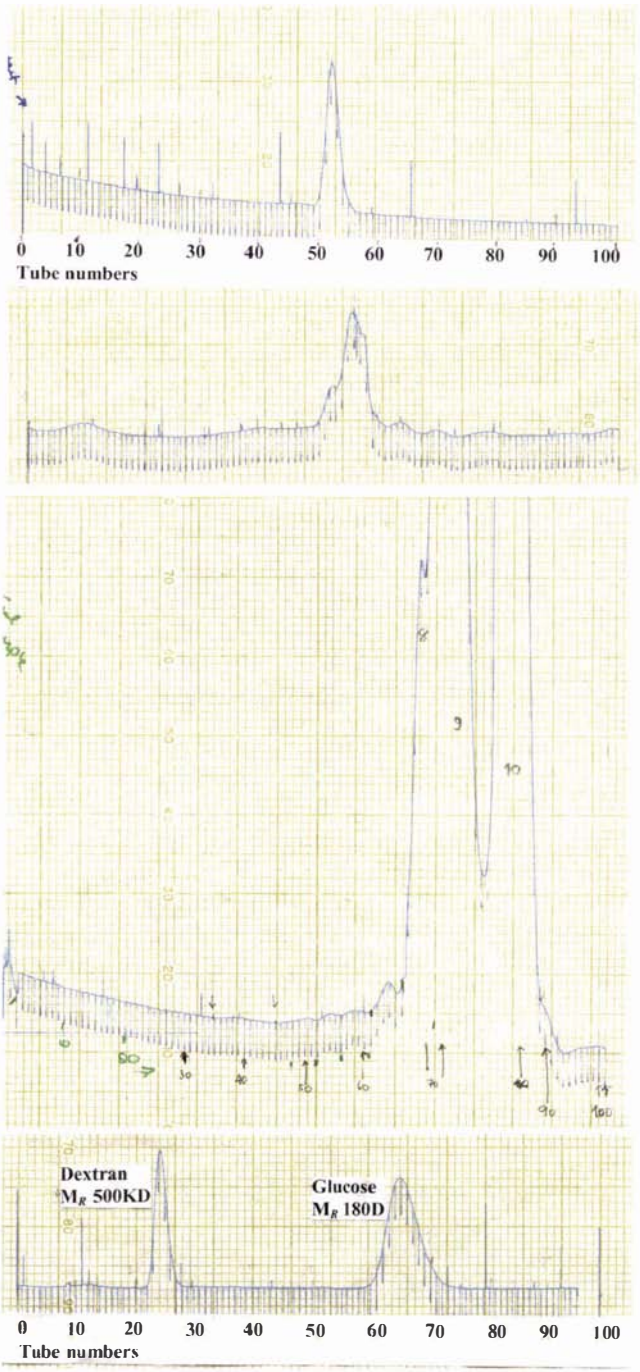
Table 7.7 LP activity of fractions from a single column biogel-P2 filtration. The active fractions are printed in bold type. Crude data are tabulated in Appendix 45.



(1)

(2)

Fig. 7.4 Chromatograms of samples run on a double column biogel-P2. (1) Active CCS under3KD ultrafiltration membrane; (2) Two standards.



(1)

(2)

(3)

(4)

Fig. 7.3 Chromatograms of samples run on a single column biogel P2.
(1) antigens; (2) CCS without antigen; (3) CCS with antigen; (4) Two standards;

(89%). The reduced activity may be due to the loss of LPFs during filtration, or the filtration may remove a co-factor like gut mucus which increase or stabilise LP activity *in vitro* (Ref: Chapters 5 & 6). The results in Table 7.6 indicate that the LPF was of low molecular weight, less than 1KD, and similar to the size of LMI substances in mucus demonstrated by Douch *et al.* (1983).

7.3.4.2 Size exclusion gel filtration

7.3.4.2.1 Single column Biogel-P2 filtration

Further, for the confirmation of molecular weight, the active CCS (under 3KD) was fractionated on a biogel-P2 (Elstow *et al.*, 1985; Vijayaraghavan & Hoskins, 1988). The fractionation resulted in 12 gel fractions (Fig. 7.3). Three large peaks and seven minor peaks were recorded. The large peaks appeared in the last third of the run and overlapped each other. The resolution of the chromatogram, especially for the 3 large peaks, was poor. LP activity was only detected in fractions 8, 9 and 10 (Table 7.7). Intriguingly, the active peaks were eluted close to the standard glucose (MW 180) on the chart. Clearly, this lends support to the results of the centrifugation filtration (Table 7.2) in which LPF was not retained on the 1KD ultra-filtration membrane. The controls (ES antigen alone and CCS without antigen stimulation) were re run on the gel, and both appeared as a single peak between tube number 50 and 60 (Fig. 7.3.1 & 2). The pooled fractions resulting from controls had no LP activities (data not shown). These results indicate that only the CCS stimulated with ES antigens produced LPF, and neither the CCS without antigen nor the antigen alone produced LPF. The result was in agreement with the findings in Chapter 3 that ES antigens trigger the release of LPF in the *in vitro* cell culture model system. To enable a better resolution of the chromatogram, the separation of same amount of CCS was separated on a double column biogel-P2 and produced seven gel fractions.

7.3.4.2.2 Double biogel-P2 filtration

This additional purification using a double column biogel-P2 resulted in seven fractions (Fig. 7.4). LP activity was confined to fraction 7 (Table 7.8) which responded very weakly to ninehydrin reaction (Fig. 7.5) and the spot was pencilled before it faded out. Fraction 7 appeared in the same region as the previous three active fractions of single column gel (Fig. 7.4). This confirmed that gel filtration purification of LPF is reproducible. Fraction 7 was subjected to electrospray-ionisation mass spectrometry (ESI-MS) analysis.

7.3.4.3 Electrospray-ionization mass spectrometry (ESI-MS)

Infusion ESI-MS analysis of fraction 7 showed that although it eluted close to the tube number of the standard (MW 180), it contained a complex mixture of compounds ranging in MW from 80-1000 Daltons (Fig. 7.6). However, the finding of compound 175 and 568 in both the sample and blank run indicated that the fraction 7 had some purities derived from the LC-MS system.

7.3.4.4 Sample preparation for HPLC using solid phase extraction (SPE)

For further sample purification, HPLC was recommended. Since the analytes were small unknown organic compounds, C18 sorbent was considered the best choice to begin isolating groups of compounds that are dissimilar in structure. The use of C18 reversed phase solid phase extraction (SPE) resulted in further purification of LPF with an increase in LP activity. Table 7.9 shows that LP activity of CCS before SPE was 79% and the activity was increased to 92% level after the C18 SPE. The reason was unknown, but the C18 SPE may remove some compounds that inhibit LP activity *in vitro*. For finer separation of the compound, the sample after SPE was subjected to HPLC purification.

7.3.4.5 Preparative C18HPLC purification

The CCS after SPE were separated by C_{18} reversed phase high performance liquid chromatography (C_{18}RP-HPLC). HPLC is an ideal technique for the analysis of small molecules less than 1000 Daltons (Lim, 1986). The logic for using C_{18}RP-HPLC purification was partly for sample clean-up, to remove apolar or less polar non-LPF compounds, although the results in Table 7.9 show LPF was not retained on the C18 sorbent. Following the SPE purification, the semi-purified CCS was put through a C18 preparative RP-HPLC column. A total of 5 fractions were collected and pooled according to the corresponding peaks absorbing at 254nm wavelength (Fig. 7.7), the fractions were lyophilised, and bioassayed. Table 7.10 indicates that only HPLC Fraction 1 (Pk1) was bioactive with reduced activity (68%) compared to pre-purification CCS (87%). The loss of activity may be due to the multi-step sample preparation which involved repeated HPLC fractionation and lyophilisation. It was eluted very close to the void volume (10min). Apparently, the LPF was very polar and not attracted to the apolar C_{18} bonded column saturated with the polar mobile phase (95% water- acetonitrile). This purification method concentrated polar analytes as well as removing unwanted apolar materials.

	LP activity (%)		
Sample dilution (%)	**80**	**40**	**20**
Type of sample			
Pre SPE CCS under 3KD	76	50	13
Washing- Milli-Q water	92	73	54
Eluent-60% methanol-water	17	13	0
Eluent-100% methanol	15	8	8
Levamisole (5ug/ml)	100		

Table 7.9 LP activity of CCS fractions after C_{18} reversed phase solid phase extraction (RP-SPE) purification. A C_{18} Mega Bond Elut 6 CC 1 gm from Varian, USA was conditioned with 2 column volumes each of methanol and milli-Q water. One millilitre CCS under 3K was loaded into the column and rinsed with 12ml Milli-Q water, the flow was 2 ml/min. Then the column was eluted with 3ml each of 60 or 100% methanol. The Milli-Q washing was freeze dried, and the eluents were lyophilised, resuspended in Milli-Q water (similar to starting volume) for bioassay along with the controls. Crude data are tabulated in Appendix 47.

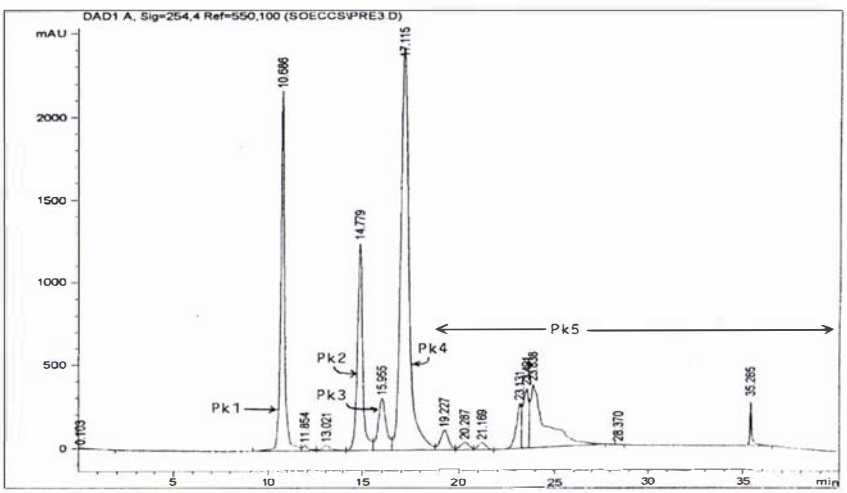


Fig. 7.7 Chromatograph of CCS under 3KD ultra membrane separated by C18 RP-HPLC. A 250 µl purified CCS was fractionated on an Alltech Econosphere C_{18} 10µ 250x22mm column I.D stainless steel preparative HPLC column using a linear gradient from 5-80% CH_3CN in water at ambient temperature, at a flow rate of 2.5ml/min for 40 minutes. The solvent program was – 10 min. hold at 5%; 20 min. gradient from 5-80%; 5min. hold at 80%; 5min. gradient from 80-5%; 15 min. hold at 5%. Fractions were collected and pooled according to the corresponding peaks absorbed at 254nm, dried in a savant prior to bioassay.

	Motile larvae			LP activity (%)	S.D.
Pre HPLC CCS	4	2	5	87	5
HPLC fractions					
F1	11	9	8	68	5
F2	28	33	31	0	9
F3	26	29	32	0	10
F4	30	30	30	0	0
F5	22	29	24	14	12
Controls					
Levamisole	1	0	0	99	2
Buffer	30	29	27		

Table 7.10 LP activity of preparative C_{18} RP-HPLC fractions. Crude data are tabulated in Appendix 48.

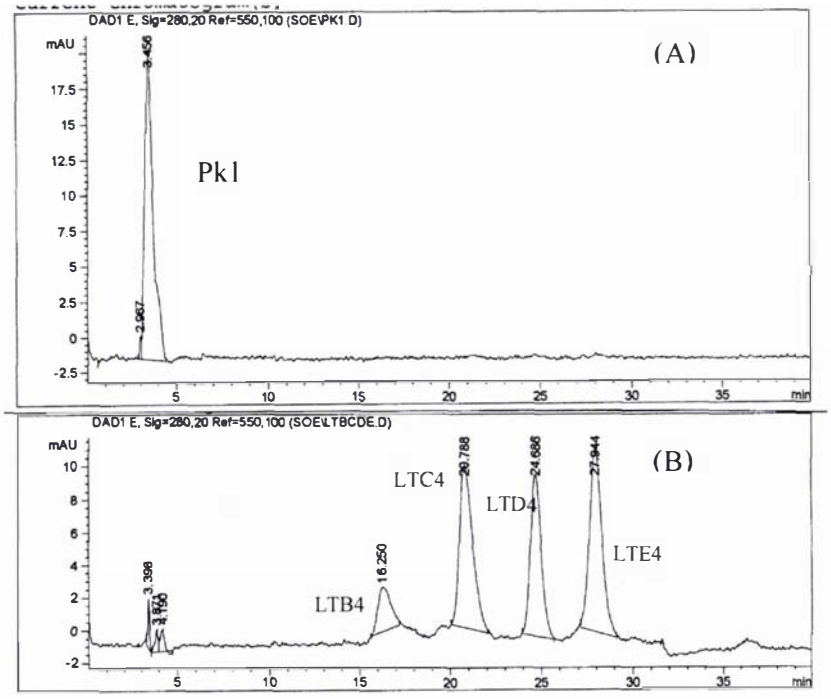


Fig. 7.8 HPLC profile of bioactive Pk1 and leukotrienes on C_{18} RP-HPLC. Separation of Pk1 (10µl) or four leukotrienes (1 ug each, Cayman, USA) was performed on an Altima Alltech analytical C_{18} 5µ stainless steel HPLC column (250mm x 4.6m I.D) with isocratic mobile phase (65% methanol-Milli-Q water plus 0.01 % acetic acid) at ambient temperature and a flow rate of 0.8ml/min, and run for 40 minutes. Detection was via DAD at UV280.

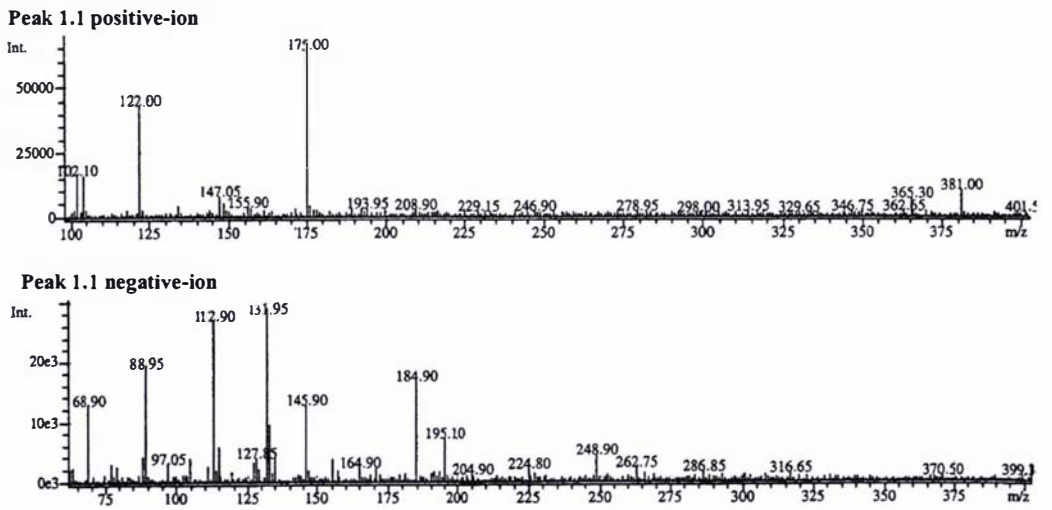


Fig. 7.10, a LC-MS chromatograms of PK1.1 in positive and negative mode.

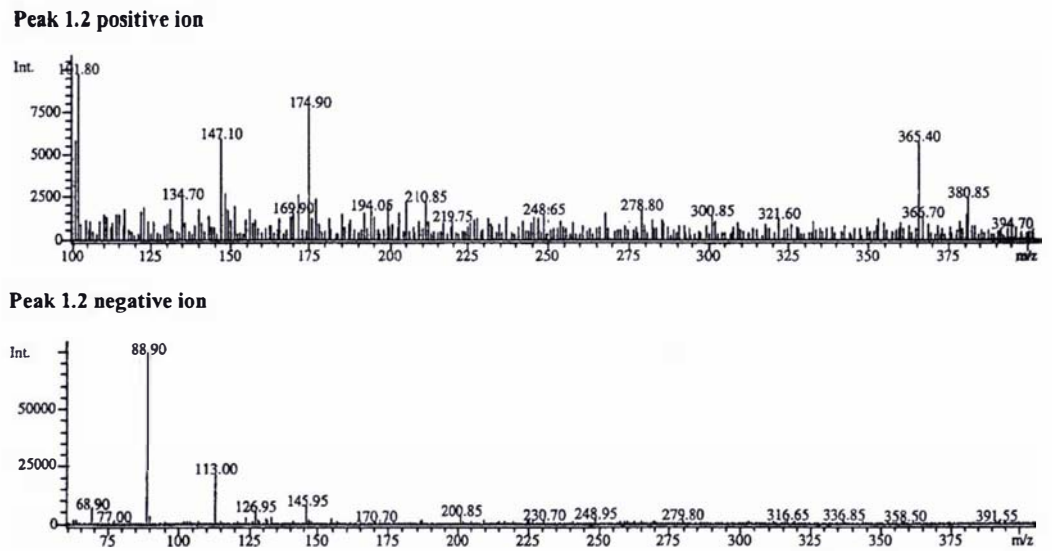


Fig. 7.10, b LC-MS chromatograms of PK1.2 in positive and negative mode.

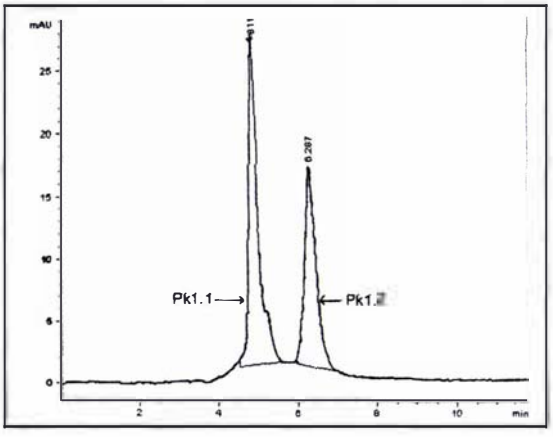


Fig. 7.9 Analytical HPLC profile of bioactive Pk1. Further separation of Pk1 was performed on an Alltech Platinum C_{18} EPS 100 Å 5µ stainless steel analytical HPLC column (250 mm x 4.6mm I.D) with isocratic mobile phase 5% acetonitrile in MQ water at ambient temperature at a flow rate of 0.5ml/min, and run for 12 minutes. Fractions were collected and pooled according to the corresponding absorbance at 254nm wavelength, evaporated to dryness and bioassayed. Detection was via DAD at UV254 nm.

	Motile larvae			LP activity (%)	S.D.
F1 (Pk1.1)	14	18	15	61	5
F2 (Pk1.2)	22	24	27	39	7
Controls					
Levamisole	1	2	0	98	4
Buffer	44	36	40		

Table 7.11 LP activity of the fractions of active Pk1. F1 - fraction 1 (Pk1.1); F2 – fraction 2 (Pk1.2). Crude data are tabulated in Appendix 49.

This result was in accord with the SPE results (Table 7.9) that most LPFs were eluted in the Milli-Q water wash.

Additional experiments were conducted to investigate whether Pk1 was similar to or contained slow-releasing substances of anaphylaxis (SRS-A) (leukotrienes). The reason was that more than 2 decades ago Douch *et al.* (1983) speculated, based on the arylsulphatase reaction of gut mucus of immune sheep, that leukotrienes (LTs) were the substances associated with mucus LMI activity. For the experiment, Pk1 or LTs (LTB4, LTC4, LTD4, LTE4) were fractionated using an Altima analytical C_{18} RP-HPLC column with an acidic methanol-water mobile phase. The resulting chromatograms (Fig. 7.8) demonstrate that Pk1 was eluted near the void volume at 3 mins whereas the different LTs were eluted from the column between 15-30 mins. This suggests that the LPF active PK1 does not contain substances like LTs, and that LP activity in CCS is not associated with LTs.

7.3.4.6 Analytical C_{18} HPLC purification

The subsequent further finer purification, of Pk1 on a smaller bore HPLC column with similar C18 bonded phase, resulted in two fractions, Pk1.1 and Pk1.2 (Fig. 7.9). The LP activity of fraction 1 (Pk1.1) and fraction 2 (Pk1.2) were 60% and 31% respectively (Table 7.11). The activity of fraction Pk1.1 was not significantly different from Pk1 activity (pre-HPLC), thus the assumption was that most of the LPF of Pk1 were eluted in Pk1.1. These two fractions were subjected to LC-MS.

7.3.4.6.1 L C-MS analysis of Peak 1.1 and 1.2

The LC-MS analysis of Pk1.1 and Pk1.2 (Figs. 7.10 a & b) established that the fractions remained mixtures of several small ion masses. The active Pk1.1 had more ion mass than the less active Pk1.2. The former fraction consisted of three strong (102, 122, 175) and two weak (147 & 156) positive-ion masses, and several negative-ion masses (69, 89, 113, 132, 146, 155, 185, 195). The latter fraction comprised of several weak positive-ion masses between 102 -400, but it had two strong (89 & 113) and three weak (69, 127 & 146) negative-ion masses. In addition, the two spectra suggest that there were ion masses similar to amino acids and di-peptides present in the active fraction Pk1. They were likely to be arginine (M_R-174+1H), cysteine (M_R-121+1H), and valine-glycine (M_R-174+1H) appearing as positive ion masses; aspartic acid (M_R-133-1H), glutamic acid (M_R-147-1H), and alanine-proline (M_R-186-1H),

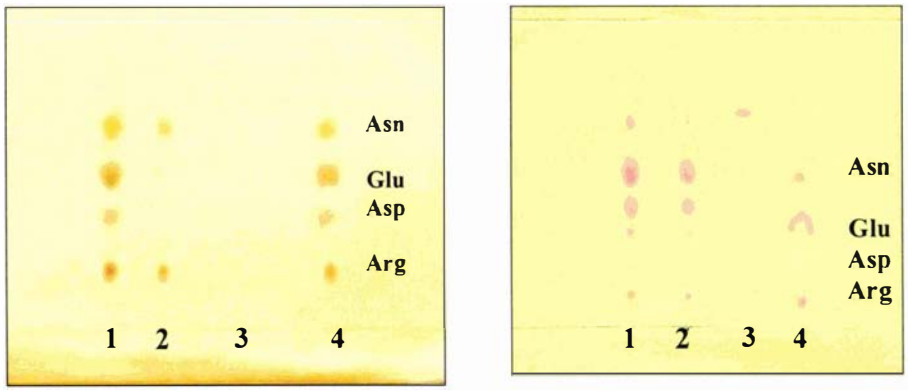
scanned as negative ion masses. Thus, the ion exchanger step was employed to clarify whether they were contaminants co-purified with LPF in the Pk1.

7.3.4.7 Removing contaminants from active fraction Pk1 by ion exchange SPE (solid phase extraction)

According to the previous LC-MS analysis of the HPLC fraction Pk1.1 & Pk1.2, the ion masses appeared to be glutamic acid, aspartic acid, asparagine, arginine and other di-peptide masses. Therefore, to establish the control system, the amino acid standards were spiked into the buffer and put through two SPE ion exchangers (SAX – strong anion & SCX-strong cation) consecutively. Comparison of TLC before and after ion exchangers (Figs. 7.11 a & b) showed that ion exchange SPE removed all the spiked amino acids in the buffer. In the case of Pk1, the fraction was put through the ion exchangers in a similar manner, the TLC profile (Figs. 7.11, b) shows the chromatogram did not change much after the anion exchanger, but it changed significantly after the cation exchanger. This indicates Pk1 contained more cation than anionic masses. Interestingly, the materials that survived, after two strong ion exchangers, produced LP activity (68%), only slightly lower than the pre-ion exchange sample (74%), and they appeared to react weakly to ninhydrin. This result was similar to the earlier active gel fraction 7 (Fig. 7.5). Comparison of LC-MS chromatograms before (Figs. 7.10 a & b) and after TLC (Fig. 7.12 a & b) demonstrate that the ion exchange system removed all the suspect ion masses. Furthermore, it also clarifies whether the ion masses were amino acids and peptides or not, clearly they were not contributing to the LP activity of Pk1. The results suggest that the LPF which survived going through two ion exchangers was likely to be a polar neutral compound lacking free carboxylic acid and a simple amino functional group.

7.3.4.8 Method development using SPE

Having had difficulties in retaining LPF on C18 bonded silica to improve purification and to obtain a better LC-MS chromatogram, the additional method developed using various SPE apolar sorbents (SDB, PH, CN) including ion exchangers (SAX, SCX, WCX, WAX) failed to resolve the problem. For these reasons, an alternative approach using polar sorbent aminopropyl was explored, and this allowed LPF to be retained on the sorbent, and it was finally eluted with 70% aqueous acetonitrile (Table 7.12). The possible mechanism behind this breakthrough could be that in an aminopropyl SPE cartridge saturated with a less polar mobile phase such as acetonitrile (compare to water), the polar compound like LPF adsorbed by the



(a) (b)

Fig. 7.11 Chromatograms of standard amino acids and active HPLC fraction Pk1. (a). Control (b). Fraction Pk1. 1. Pre ion exchange sample; 2. Sample after SAX SPE; 3. Sample after SAX SPE followed by SCX SPE. Amino acid standards: Asn - aspargine; Glu – glutamic acid; Asp – aspartic acid; Arg – arginine.

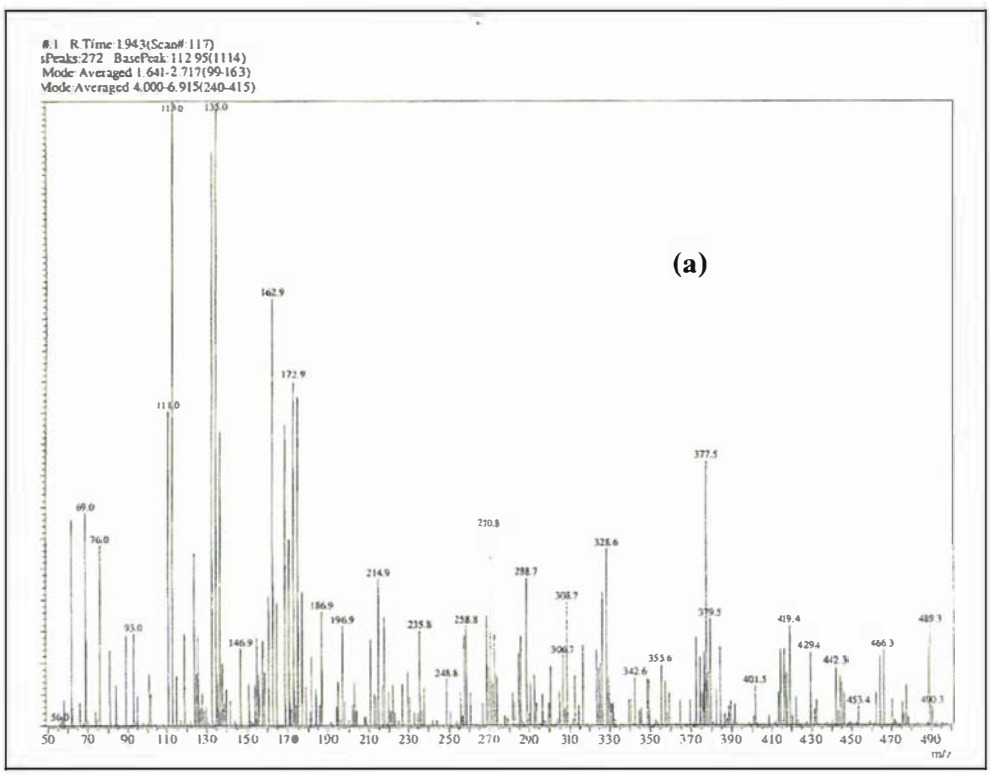


Fig. 7.12, a Negative ion electrospray ionization of LC-MS of Pk1 after ion exchangers. The spectra shows previously suspected amino acids and peptides masses had been removed.

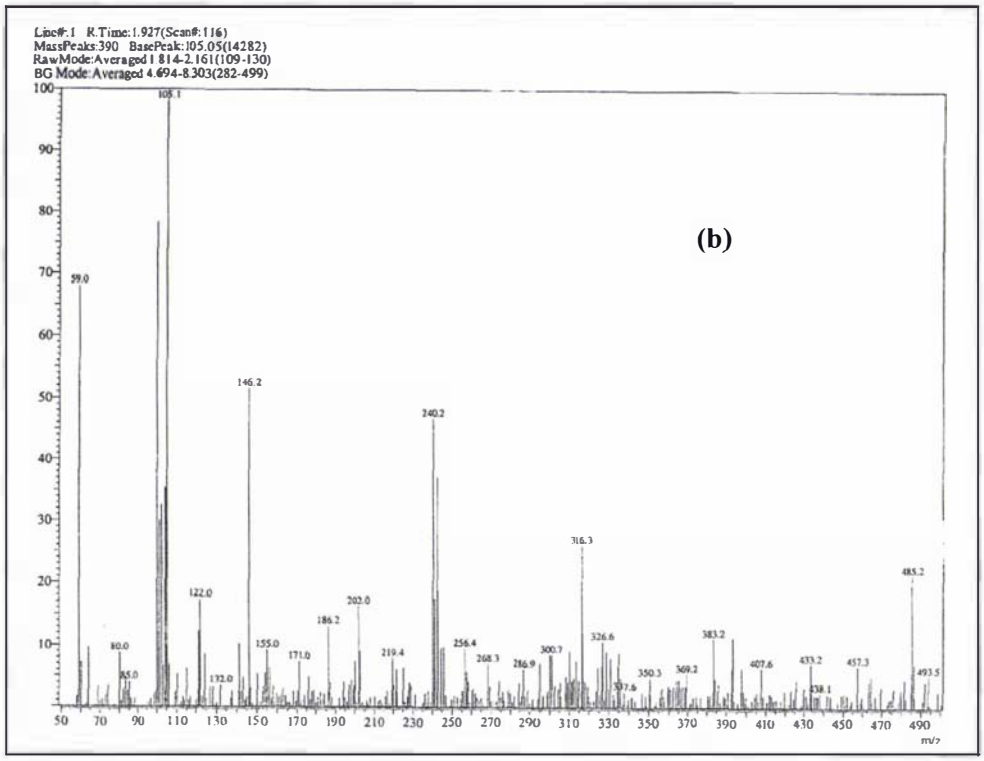
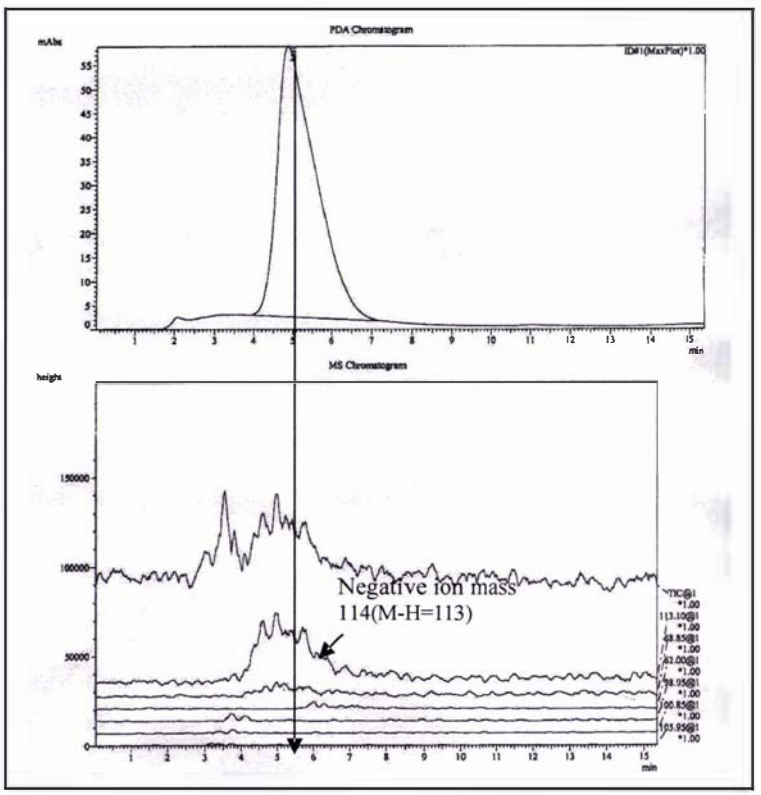


Fig. 7.12, b Positive ion electrospray ionization of LC-MS of Pk1 after ion exchangers. The spectra shows previously suspected amino acids and peptides masses had been removed.

	LP activity (%)		
Sample concentration (%)	80	40	20
Pre-SPE active Pk1	92		
Eluent			
Acetonitrile 100%	6	11	13
Acetonitrile 70%	87	54	14
Acetonitrile 50%	40	27	10
Acetonitrile 20%	11	10	11
Milli-Q water	8	6	10
Control			
Levamisole (5ug/ml)	97		

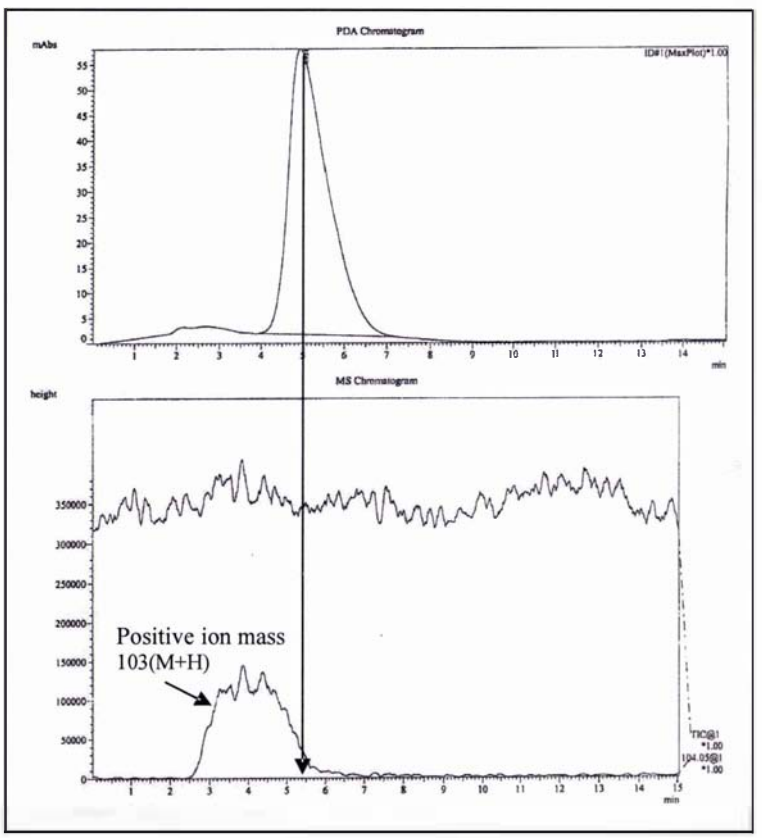
Table 7.12 SPE purification of active Pk1. An aminopropyl 3CC 500mg SPE was conditioned with 2 column volumes each of methanol and acetonitrile. A 500 µl active CCS (after C18SPE from Table 7.9) was loaded onto the cartridge, dried for 1 min., rinsed with 6 ml acetonitrile, followed by 2ml 90% or 70% or 50% or 20% acetonitrile-Milli-Q water or Milli-Q water. The flow was 2ml/min. Only the Milli-Q water was freeze dried, but the other eluents were lyophilised, resuspended in Milli-Q water (similar to starting volume) for bioassay along with the controls. Crude data are tabulated in Appendix 50.



(a)

(b)

Fig. 7.18 MS chromatogram of ion mass 114 with UV chromatogram of fraction Pk1.6.2.
(a) - UV chromatogram; (b) - Extracted ion MS chromatogram;



(a)

(b)

Fig. 7.19 MS chromatogram of ion mass 103 with UV chromatogram of fraction Pk1.6.2.
(a) - UV chromatogram; (b) - Extracted ion MS chromatograms.

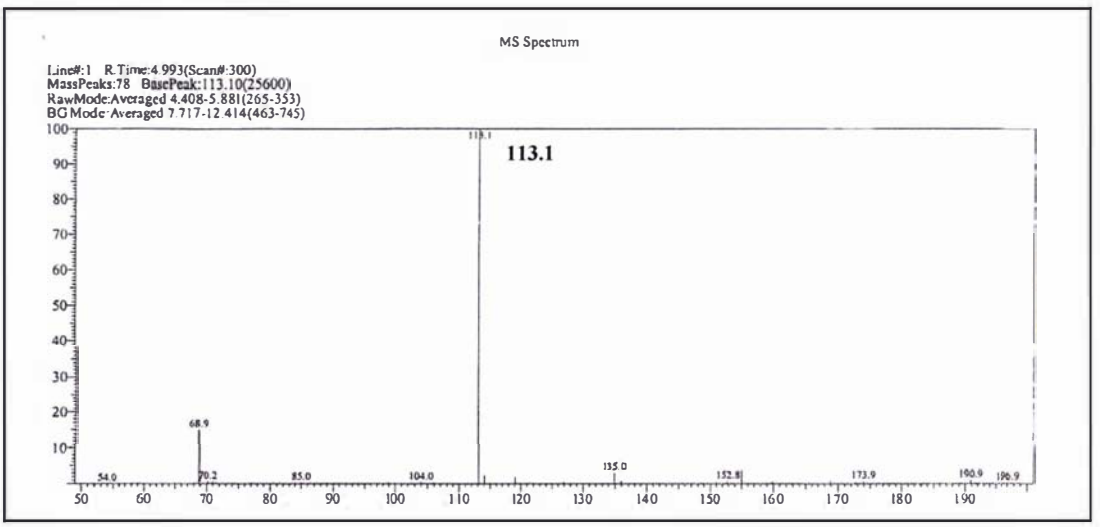


Fig. 7.16 Mass spectrum of negative ion mass 114 (M-H=113).

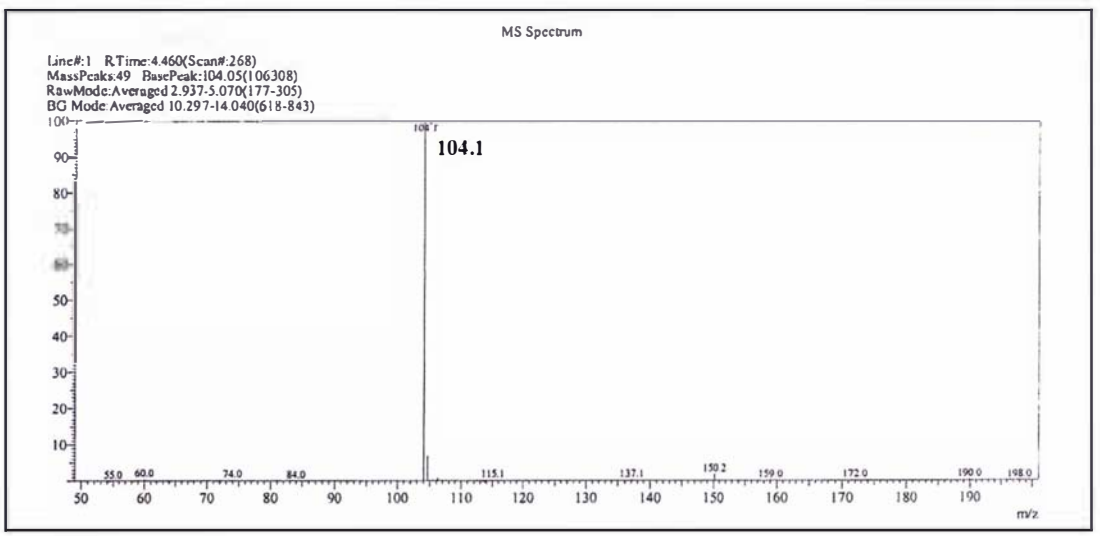


Fig 7.17 Mass spectrum of positive ion mass 103 (M+H=104).

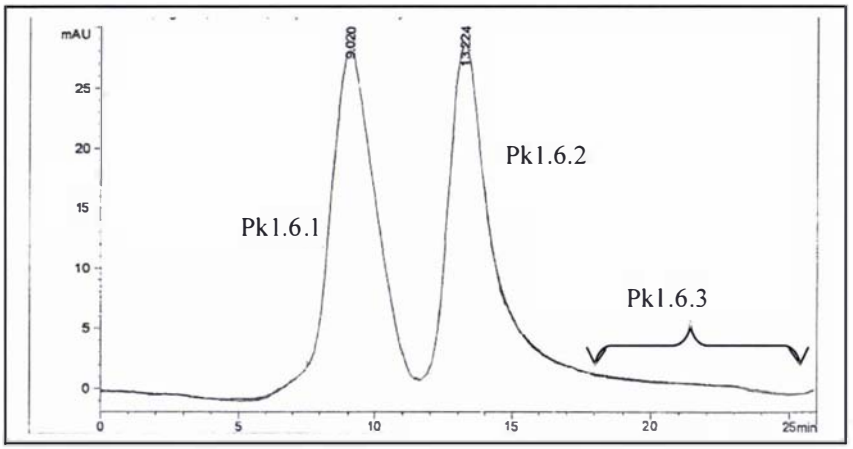


Fig. 7.14 HPLC profile of three fractions of Pk1.6 re-separated on an analytical amino HPLC column with isocratic 70% acetonitrile-water mobile phase, run for 30min. Detection was via DAD at UV254 nm.

Fractions	Motile larvae			mean LP%
F1(Pk1.6.1)	21	18	16	20
F2(Pk1.6.2)	**9**	**6**	**10**	**64**
F3(Pk1.6.3)	21	19	23	9
Levamisole(5ug/ml)	0	1	0	99
Buffer	20	19	22	

Table 7.14 LP activity of fractions of Pk1.6 re-separated on an aminopropyl analytical column. Active fractions are in bold print. Crude data are in Appendix 52.

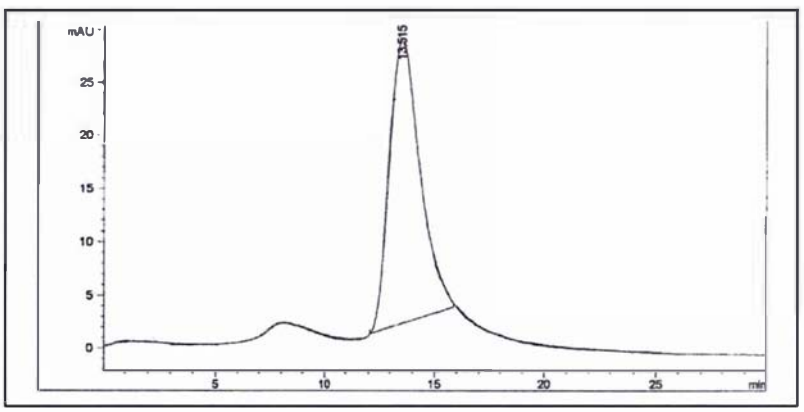


Fig. 7.15 HPLC profile of Pk 1.6.2 separated on a microbore (1mmx250mm) amino HPLC column with isocratic 70% acetonitrile-water mobile phase, run for 30min. Detect was via DAD at UV254 nm.

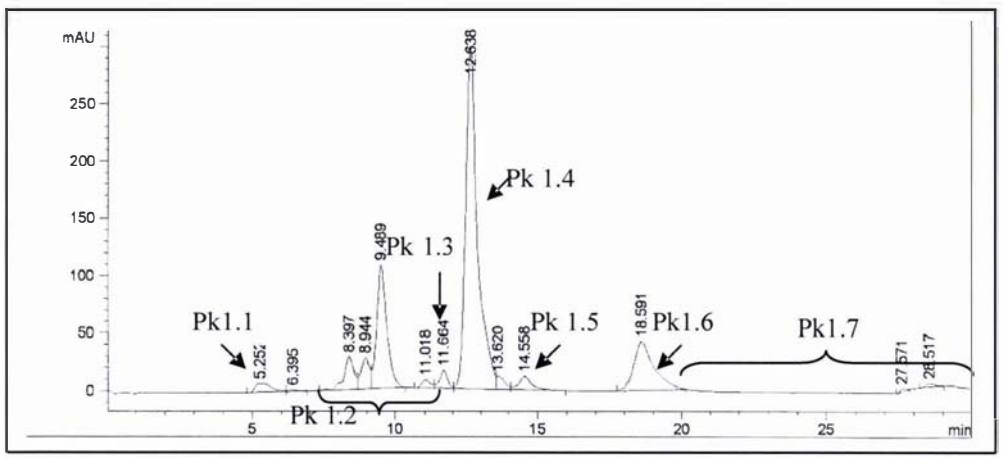


Fig. 7.13 HPLC profile of the fractions of Pk1 (after purification on ion exchangers) separated on an Econosphere amino 100 Å 5µ (250mm x 4.6mm I.D) stainless steel analytical HPLC column with isocratic mobile phase (75% acetonitrile-Milli-Q water) at ambient temperature and a flow rate of 0.8ml/min, and run for 30 min. Detection was via DAD at UV254 nm.

Fractions	Motile larvae			LP%
F1	24	20	20	7
F2	25	20	24	0
F3	21	19	23	9
F4	20	22	24	4
F5	**15**	**16**	**14**	**35**
F6	**10**	**7**	**5**	**68**
F7	22	25	21	1
Levamisole(ug/ml)	1	1	0	97
Buffer	24	25	21	

Table 7.13 LP activity of fractions of Pk1 (after ion exchanger purification) separated on an aminopropyl analytical column. Active fractions are in bold print. Crude data are in Appendix 51.

polar aminopropyl bonded silica surfaces would elute selectively as polarity was increased by adding water into the mobile phase. Therefore, washing under apolar solvents (100% acetonitrile) could not remove polar analytes like LPF from the amino polar sorbent. Thus, no LP activity was detected in the eluent of 100% acetonitrile (Table 7.12). This result was in accord with the previous results describing solubility of LPF in Table 7.4 that LPF was not soluble in acetonitrile. When the polarity of the mobile phase was increased by adding 10% water, apparently the polarity was still not strong enough to break the bond between the polar bonded surface and the LPF. Total elution was achieved by 30% water in the mobile phase solvent. The LP activity pre and post-SPE was similar.

7.3.4.9 Purification of Pk1 (after ion exchange SPE) using aminopropyl HPLC

The method development in the previous section led to the use of aminopropyl HPLC to purify the fraction further. HPLC separation of Pk1 was performed on an analytical aminopropyl HPLC column with isocratic mobile phase (70% acetonitrile-milli-Q water), a flow rate of 0.8ml/min and run for 30min. Fig. 7.13 shows a total of 7 fractions collected according to the chromatogram which absorbed at UV254 nm wavelength, samples from multiple runs were pooled and assayed. Table 7.13 indicates only fraction 6 (Pk1.6) produced LP activity and fraction 5 (Pk1.5) had low level activity. Further, the most active HPLC fraction (Pk1.6) was purified again on the analytical amino HPLC resulting in 2 fractions (Fig. 7.14). The result in Table 7.14 shows the second fraction (Pk1.6.2) produced higher LP activity than did fraction 1 and 3.

7.3.4.10 LC-MS analysis of active fraction Pk1.6.2

The active fraction 1.6.2 was run again on the 1mm microbore aminopropyl HPLC column (Fig. 7.15) to double-check the fraction was in the sample subjected to LC-MS analysis. Intriguingly, the LC-MS illustrated that there were three possible ion masses corresponding to the chromatogram detected at DAD UV254nm; one was 104 in positive ESI-MS mode (Fig. 7.17), and the other two appeared as 69 and 113 in negative ESI-MS mode (Fig. 7.16). However, in these three masses, the MS chromatogram of negative ion masses 69 and 113 aligned with the active peak 1.6.2 absorbed at UV254nm, but the positive ion mass104 did not (Fig.7.18 & 19). This finding suggests that the negative ion mass 69 and 113 were the ion masses of active

fraction 1.6.2. However, the ion detected at *m/z* 69 is probably a source fragment (Fig. 7.16) generated by the loss of 44 (e.g. CO_2) from the *m/z* 113 ion rather than a separate compound (*i.e.*113 - 44 = 69).

However, when this sample was re-analysed after two weeks, the aminopropyl column behaviour had changed and produced different UV chromatograms. The fraction absorbed at UV254 was eluted at 2.2min (Fig. 7.20) compared to the 4.921mins of the previous chromatogram (Fig. 7.18). And Fig. 7.20 shows the two negative ion masses were present and the mass appeared at the same position (4-7 min) as in the previous MS chromatogram (Fig 7.18). The obvious difference was that although the negative ion mass 113 was present, it did not correspond to any UV spectrum. The reasons for the change could be: firstly LPF is by nature sensitive to freeze and thaw (see Table 7.5), and that sample had been subjected to freeze and thaw several times during purification and several analyses; secondly the aminopropyl bonded silica is naturally not compatible with the aqueous mobile phase and the use of it may lead to early degeneration of the column (Ali & Aboul-Enein, 2006). Unfortunately, the amino column was the method of choice in this current research. The change was noticeable when studying a series of chromatograms produced by aminopropyl column during the LC-MS analysis. To begin with the UV-absorbing peak and *m/z* 113 ion species eluted on the 1 mm microbore column around 14 min as in Fig. 7.15. Subsequently on the same column they eluted around 5.5 min (Fig. 7.18). However in the final chromatogram (Fig 7.20) the UV-absorbing peak eluted at 2.2 min, while the *m/z* 113 peak eluted around 7 min. This indicates instability of the aminopropyl column under aqueous conditions, but also instability of the UV-absorbing compound.

This was 'a blessing in disguise' as this condition provided more information about LPF. Thus fraction 1.6.2 (Fig. 7.14) would appear to contain at least three compounds: a component of *m/z* 104 (ESI+ve) lacking significant UV absorption at 254 nm (Fig. 7.19), one of *m/z* 113 (ESI -ve; fragment *m/z* 69) also lacking significant UV absorption, and a separate UV absorbing component for which no MS data was obtained (Fig. 7.20). This latter compound appeared to degrade in the final stages, and was clearly distinct from the compound of *m/z* 113. However the sensitivity of the single quadrupole LC-MS used in this study is limited, and the presence of other undetected components in fraction 1.6.2 cannot be ruled out. Unfortunately there was inadequate material remaining to test the activity of the peaks in Fig 7.20, and to further refine the LPF. However, the information obtained in this study provides a good lead for future LPF researchers.

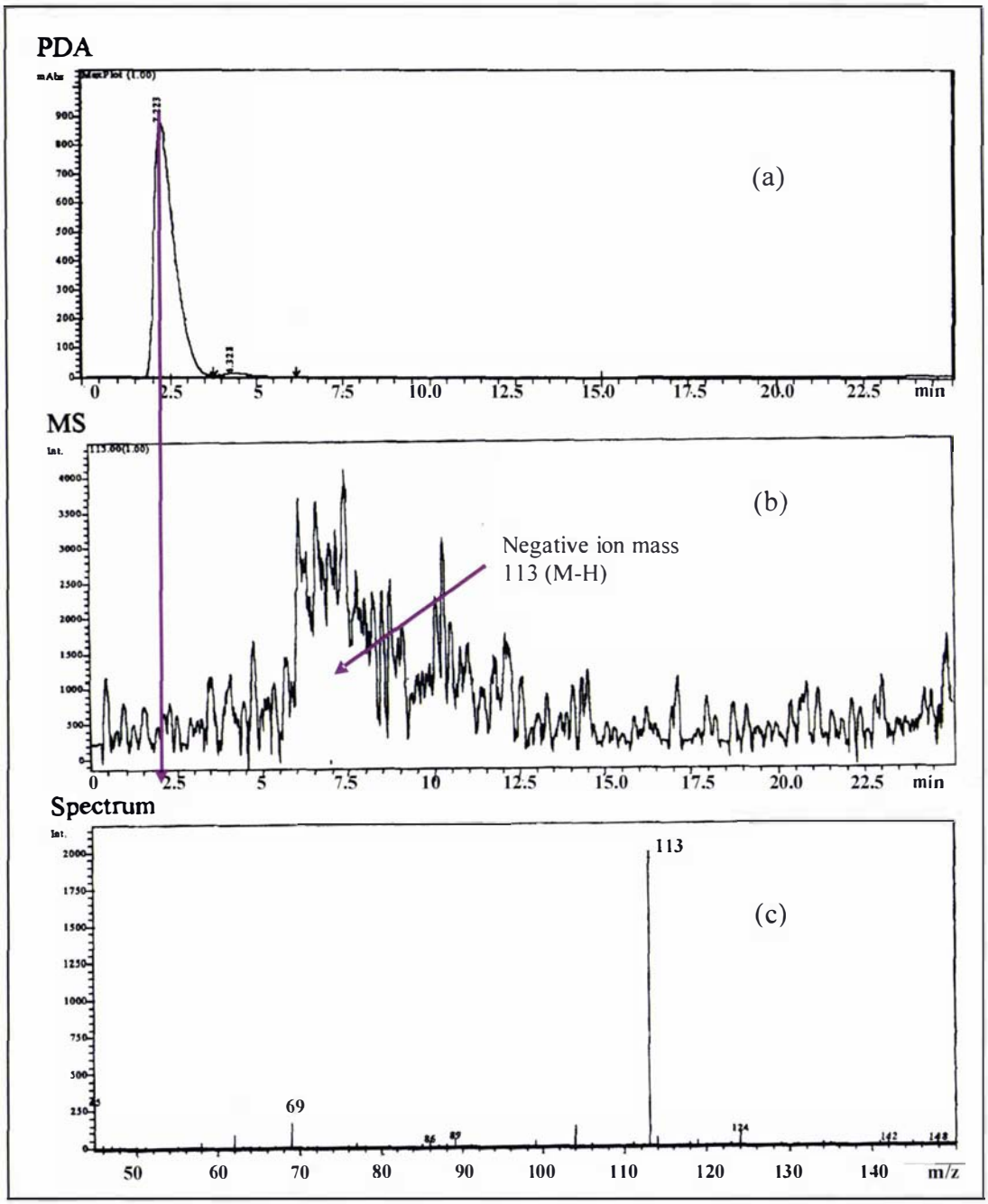


Fig. 7.20 MS chromatogram of ion mass 103 with UV chromatogram of raction Pk1.6.2. separated on 1mm microbore aminopropyl HPLC column 2 weeks after the first analysis. (a) UV chromatogram; (b) Extracted ion MS chromatogram; (c) Mass spectrum

In conclusion, the multi-step purification methods used suggest that LPF is a very small polar compound exhibiting non-specific activity - the ability to paralyse a wide variety of larval and adult stages. The addition of biogel-P2 and ultramembrane filtration steps resulted in an estimation of the molecular weight of the factor as being close to glucose (M_R 180Daltons). However, a preparative high performance liquid chromatography (HPLC) took the purification further and made possible the separation of the highly polar LPF from the non polar or less polar contaminants in the CCS. The present study used a powerful LC-MS detection system to determine contaminants in order to improve the purification for molecular characterisation. The addition of the solid phase extraction application resulted in two significant improvements in the purification, *viz.* the elimination of several amino acids and peptides, which were believed to be a contaminant in the active semi-purified HPLC fractions; and retaining the LPF longer on a HPLC column for a better resolution on the mass spectrometry chromatogram. Finally, these developments enabled the purification process to yield a highly purified fraction containing three compounds, *viz.* a UV absorbing compound λmax 250nm (for which no MS data was obtained), and two non UV absorbing compounds at 254 nm: one was a component of *m/z* 104 (ESI+ve) and the other was detected at *m/z* 113 (ESI-ve).

In addition, this study clearly demonstrates by comparison of molecular size (smaller than sugar molecule), physico-chemical characteristics such as resistance to arylsulphatase and acid, polarity, and retentive activity on C18 reverse phase HPLC, that LPF purified from active CCS of LPCs, derived from the small intestine of nematode resistant-line sheep, is different to the mucus-derived anti-parasitic substances reported previously (Douch et al. 1983) having some properties characteristic of leukotrienes (LTs). The two greatest differences between LTs and LPF are: firstly, LTs are lipid species, therefore they are readily soluble in solvents that are not suitable for highly polar species CCS, secondly, CCSs are resistant to arylsulphatase which destroys LTs. Furthermore, Chapter 5 of the present study tested anti-nematode activity of commercially available LTs, and also Emery & McClure used specific antagonists of leukotrienes to examine their ability to suppress the rejection of *T. colubriformis* infective larvae from immune sheep (Emery & McClure, 1995), neither in vitro nor in vivo work supports Douch's speculation. Nevertheless, the results from present study indicate that it has developed a reproducible method of preparative separation, which narrowed a complex mixture of organic compounds in the CCS to a highly purified fraction.

Hence, in the interest of discovering gut-originated natural anthelmintic, it is hoped that the method developed in the current study plus more sensitive molecular structure analysis (e.g. MS-MS) would provide the identity of the 3 putative LPFs in the very near future.

❁ ❁ ❁

Chapter 8
General discussion and conclusions

The goal of this thesis was to gain a better understanding of the nature of the intestinal larval paralysis factor (LPF), its cellular source, and its role in the immune response to *Trichostrongylus colubriformis*. At the beginning of this study, very little information existed about the factor, and only two publications were available. Initially, the present study identified and confirmed the mucosal source, followed by a series of experiments designed to optimize intestinal conditions for the best LPF production *in vitro*. Following investigations into the cellular origin of the factor, the study was extended to examine the role of gut mucus in LPF activity *in vitro*, and finally into examination of the biochemical identity of the small intestinal LPF.

Supernatants prepared from mucosal cells of resistant-line sheep showed a larval migration inhibition (LMI) activity in response to *T. colubriformis* excretory secretory antigens (TcES), thus confirming the earlier finding of Douch *et al.* (1996b). However, no LMI activity could be detected in cell culture supernatants (CCSs), from nematode-resistant-line sheep without exposure to antigens, or in CCSs prepared from nematode-naïve sheep, with or without antigens. This suggests LPF production *in vitro* is antigen dependent, but it is not clear whether it is antigen specific, since the entire study used only antigens from *T. colubriformis*. However, Douch *et al.* have demonstrated that no significant LMI activity was detected in CCSs in which mucosal cells were incubated with heterologous antigens (Douch *et al.*, 1996b). Further experiments determined that the cellular source of LPF lay in the lamina propria cells (LPCs), but not in the epithelial cells, which contained mostly lymphocytes, globule leucocytes (GLs) and epithelial/goblet cells (EP/GOs). There is some support for the findings that the small intestinal lamina propria isolates are enriched with antibody-bearing effector cells, that is eosinophils, mucosal mast cells (MMCs) and monocyte-macrophage-dendritic cells (MMDs), which are:- reported to possess anti-microbial activity (Butterworth, 1984; Feger *et al.*, 2002;

Jonas *et al.*, 1995; Murray *et al.*, 1980); considered to release substances which are anti-nematode by the cross-linking of surface-bound antigen-specific immunoglobulins (Jonas *et al.*, 1995; Jones *et al.*, 1992); thus they appeared to be implicated in the LMI activity of CCS. In the studies designed to optimise the conditions required for LPF production *in vitro*, the results were in agreement with previous findings that irrespective of whether the sheep were from selection lines or outbred, when they are immune they produce gastrointestinal mucus and mucosal cell culture supernatants with significant LMI activity (Douch, 1989; Douch *et al.*, 1986; Douch *et al.*, 1996b; Kimambo & MacRae, 1988; Rabel *et al.*, 1994). The substances secreted into the mucus or into the cell culture could contribute directly to the removal of worms from the immune sheep. In the study, immune sheep produced higher LMI activity (both mucus & CCS) than other groups.

Although the LMI activity of field-resistant sheep was consistently higher than the susceptible or naïve sheep, the study selected the indoor immune groups (resistant-line and outbred sheep), due to their superior LMI activity. Then, among the immune groups, the line sheep were chosen for their genetic assurance of resistance and the consequent saving of the time required for immunization, because it took more than 2 months to make outbred sheep immune. Even when age and immune status were similar, field resistant-line sheep produced much less LMI activity than did indoor line sheep. There may be at least two reasons for this low LMI activity in outdoor line sheep. First, field sheep are constantly exposed to nematodes from pasture, and exposure is strongly correlated with availability of pasture larvae. LMI activity can be affected by pasture contamination which is associated with several factors such as stock density, type of grazing stock (large or small ruminants or lambs or adults), growth of pasture, season of the year, climatic conditions, etc. (Brunsdon, 1980; Douch et al., 1984; Familton & McAnulty, 1997; Nicol & Everest, 1997; Vlassoff et al., 2001). Consequently, the variation in gut exposure to larvae could produce inconsistent levels of immunity, since the time of onset and the efficacy of immune mechanisms vary with the magnitude and duration of infection (Chiejina & Sewell, 1974).

Secondly, stress of living in the field is another possible reason for low LMI activity. For instance, outdoor sheep were not protected from extremes of climate, and had different types of food, compared to the regularity and *ad lib* basis of indoor animals. It is evident that adrenal hormone release is associated with sheep under stressful conditions, such as weaning (Rhind et al., 1998), periparturition (Chamley et al., 1973), extreme physiological conditions such as heat (Minton & Blecha, 1990), and isolation (Cockram et al., 1994).

It is known that stress hormones can damage effector cells (i.e. lymphocytes, eosinophils) (Ambrose, 1970; Brunetti et al., 1995; Quinn, 1990; Schalm, 1965) and antigen presentation (Snyder & Unanue, 1982), and they are sometimes used as an immunosuppressant to reduce host immunity against gut nematodes in sheep trials (Buddle et al., 1992; Douch et al., 1994; Matthews et al., 1979; Prichard et al., 1974). The present results indicate the optimal animals are both resistant-line sheep and outbred immune sheep living indoors and having controlled infections, however the latter are of less advantage than the former, because they require immunization which is a time consuming procedure and also the animals are not guaranteed to produce quality CCS unconditionally, since their genetic merit is not known. Therefore, through out the present study, use was made of the progeny of a line of elite nematode resistant sheep, which has been established at Wallaceville Animal Research Centre for research purposes since 1979 (Baker *et al.*, 1991).

An interesting observation emerged during this study concerning breech soiling (dags) and diarrhoea in resistant-line sheep maintained under different husbandry practices. Factors contributing to dags are complex. The prevailing dogma is that dags and diarrhoea formation occur due to acute inflammatory response to gut nematode infection associated with MMCs, GLs and eosinophils, and their mediators include serotonin and platelet activating factor (Bisset *et al.*, 1996; Brandt *et al.*, 2003; Larsen *et al.*, 1999; Larsen *et al.*, 1994). However, Larsen *et al.* did not find a relationship between parasitology data and dag scores (Larsen *et al.*, 1994). Dags on sheep are a major and costly problem for the meat and wool industries (Bisset *et al.*, 1992). The cost comes from extra crutching to remove soiled wool, which is then sold at a reduced price (Larsen *et al.*, 1994), and the increased likelihood of flystrike must also be combated (Morley *et al.*, 1976).

In the present study, resistant-line sheep living in pens (Group 4) and receiving repeated high doses of infection (30-50,000 *T. colubriformis* larvae per inoculation 3 times a week) showed no sign of dags or diarrhoea when compared with their counterparts living in the field (Group 3). Under New Zealand pasture conditions, an adult field sheep can acquire an average of 16,000 nematode larvae a day (Familton & McAnulty, 1997), and thus both groups may have experienced similar levels of antigenic challenge in a week. However, the former group always had normal faecal pellets while the latter had significant dags and mushy faeces. Thus it would appear that these problems may also be associated with environmental (dietary and living) conditions rather than immunological factors alone. Further research taking all these factors into account needs to be pursued to get a better understanding of the root causes of dag and diarrhoea formation in grazing sheep.

The study showed that only GO numbers increased significantly in indoor immune groups with repeated infection and whose LMI activities were higher than in sheep with only pasture infection. The cause of the increment may be due in part to exposure to repeated high doses of nematodes, since the level of challenge infection has been shown to increase local immune responses (Gill *et al.*, 2000; Gorrell *et al.*, 1988; McClure *et al.*, 1992). Gut mucosal immune responses to nematodes are known to have a Type-2 cytokine-bias (Gill *et al.*, 2000; Pernthaner *et al.*, 2005), and the cytokine IL13 in particular is associated with GO hyperplasia (Webb *et al.*, 2007). In the current study, the enriched epithelial/GO (eEP/GO) population did not produce LPF directly. However, these cells secreted mucus which either sustained the activity of LPF or impeded its degradation, *in vitro*. The results reported in Chapter 4, in which indoor immune sheep produced the highest LMI activity and also had the highest GO count, may have been due in part to mucus derived from GOs in cell cultures which may have improved the LMI activity of the indoor group. These appears to be no previously published experimental data on the effector capability of goblet cell, although W.O. Jones has speculated on the activity of gut mucus and was cited as *pers. comm.* by McClure (McClure, 2000).

Interestingly, this activity of mucus seems different to that of another novel effector molecule, the intelectins (Pemberton *et al.*, 2004), which are found at elevated levels in the abomasal mucosa of immune sheep infected with *T. circumcincta* (French *et al.*, 2007). The gut mucosa produces several anti-microbial molecules (α, β, & θ-defensins, cathelicidins, cecropins) (Dann & Eckmann, 2007; Mahida *et al.*, 1997), and effector molecules (resistin-like molecule- α, β & γ, arginase, chitinase, intelectins-1 & 2, intestinal trefoil factor) (Nair *et al.*, 2006; Podolsky, 1999) which help to maintain normal epithelial barrier function. To ensure that these molecules survive and carry out their function, there may be other molecules which in some way protect them from the hostile gut environment. Perhaps this is a basic and innate property of epithelia which has not been reported before.

The first 3 metres of the small intestine produced the highest level of LMI activity in mucus or in CCS, and as well contained the highest numbers of MMCs, GLs and activated eosinophils. This localised activation may reflect the higher concentration of challenge larvae available in that area, which is a predilection site for *T. colubriformis* (Wagland *et al.*, 1996). Furthermore, the high numbers of GL in the anterior part of the gut indicated increased MMC activation and degranulation in this area, since GLs are an index of MMC degranulation (Gregg et al., 1978). The evidence for MMC secretions being released into gut mucus immediately after larval challenge in immune sheep (Jones *et al.*, 1994) or into cell culture 5min after larval extracts were added (Jones *et al.*, 1992) has been reported before. Perhaps, the high numbers of

eosinophils in the area could be associated with MMC activation, because several cytokines released by activated mast cells were found to influence eosinophil survival and activation. These include IL5, IL3 and GM-CSF (Sanderson, 1992; Stevenson & Jones, 1994). It is conceivable that the higher concentration of larvae could generate higher larval antigen density at the site which in turn could sensitise mucosal cells bearing nematode-antigen-specific antibodies, followed by cellular activation and release of LPF into mucus to produce LMI activity. Similarly, the activated gut would contain activated cells, and thus LPF could be produced from isolated activated cells in response to larval antigens *in vitro.*

The observations about morphological gradations in eosinophils and MMCs, have not been reported previously in studies of ruminant immunity to gut nematode parasites. These findings gave new insights into the kinetics of mucosal cell activation in response to nematode challenge, relating local immune responses with associated cell type(s), thereby providing a more meaningful interpretation of the results. If conventional cell counting procedures had been used (*i.e.* without observing morphological changes), the activity of CCSs from the first 3 metres would have correlated negatively with eosinophil numbers in cell cultures, and LMI activity in the CCS derived from low eosinophil population would have been higher than in the CCS derived from high purity eosinophil populations. Then, like many previous investigators, the current study would have concluded that there was no correlation between eosinophilia and immunity to gut worms (Amarante *et al.*, 2005; Amarante *et al.*, 1999; Dineen & Windon, 1980; Douch, 1989; Gill, 1991; Winter *et al.*, 1997).

However, the study shows that when the morphology of eosinophils in the above samples was graded and counted carefully, the LMI activity correlated positively with type II cells (activated eosinophils). This suggests that type II eosinophils are associated with high LMI activity both in *vitro* and *in vivo.* A probable scenario for the morphological changes seen in both eosinophils and MMCs, is that both cells develop and mature in the bone marrow, and then migrate to the mucosa via the systemic circulation under the influence of IL3 and IL5 (Korenaga & Tada, 1994; McInnes *et al.*, 1993). In the case of eosinophils, they are end stage cells, but upon stimulation by nematode challenge as in the present study, eosinophils appear to undergo sequential activation stages as reflected by the different morphological states observed in single cell suspensions or in tissue samples. In the case of MMCs, activation and degranulation transforms MMCs into another phenotype – GLs (Huntley *et al.*, 1984; Jarrett *et al.*, 1967; Murray *et al.*, 1968), but whether all degranulated MMCs develop into GLs is uncertain, and to date all GLs are regarded as end cells. Nevertheless, in the current study MMCs also displayed several degranulation stages having different morphologies before they

transformed into a transitional stage cell (*i.e.* a stage before developing into a typical GL) (Huntley *et al.*, 1984). The GL appears to be an end stage cell, commonly populating the sub-epithelium of gut mucosa, but its function and fate is still an enigma. Thus, the important message from this discovery is that the cell count alone (especially eosinophils and MMCs) does not reflect the true picture of mucosal activation against nematodiasis, and providing the morphologic profile of these cells would be of significant value in future investigations.

The results of experiment 3 in Chapter 4 demonstrated that day 3 after challenge was the most active period, based on bioassays and gut histology results. This peak immune response occurring on day 3 after challenge is in line with the findings of other investigators (Balic *et al.*, 2003; Jones *et al.*, 1990; McClure *et al.*, 1992; Smith *et al.*, 1985). It is clear that it takes 3 days for TH-2- biased adaptive immune response against nematodes to develop in hyperimmune sheep, and for it to manifest in a full-scale inflammatory response, although interestingly, the earliest pathology and signs of inflammation were observed on day 1. Signs of gut inflammation immediately after larval challenge have been reported by other investigators (Huntley *et al.*, 1987; Jones & Emery, 1991). They postulated that allergic inflammation is caused by antigens cross-linking IgE bound to the MMC surface (Jones *et al.*, 1992). However, MMCs are not transepithelial migratory cells like neutrophils, GLs, intra-epithelial lymphocytes and eosinophils, so they cannot be available in the epithelia. Besides, in day 0 histology, the epithelia were healthy and well sealed, their barrier integrity seemed not easily compromised. Thus in this situation, to trigger MMC-dependent allergic inflammation within 24hrs, the antigens must go through epithelial tight junctions into lamina propria, to meet subepithelially-located MMCs or other inflammatory cells bearing parasite-specific antibodies. Therefore, to initiate this process, there should be a system which is MMC-independent for antigen transport into the lamina propria. There are four scenarios that might contribute to this outcome.

Scenario 1- Many previous experiments have used surgically challenged larvae that are inoculated directly into the gut. This means the gut mucosa was exposed to all inoculated larvae at once, which is unnatural, and would concentrate the antigenic stimulus compared to an oral challenge and may also exacerbate the gut conditions that developed normally after further oral dosing. The current experiment used the natural route (*i.e.* oral challenge). Thus, it has to be considered whether larvae would be available in the small intestine to provoke the host immune system for the response as seen on day 1. Earlier work has confirmed that in sheep, *T. colubriformis* larvae can reach the first 6-8 metres of small intestine within a day after oral infection (Crofton, 1963; Elliot, 1981). They could be in the stomach earlier than 24 hours. *T. colubriformis,* larvae normally exsheath in the stomach (Crofton, 1963) and the fluids that are

released during the exsheathing process are known to be antigenic (Knox & Jones, 1988). Besides, sheep stomach epithelial cells are MHCII positive (Gorrell *et al.*, 1988a), but it is not certain whether they actively process and present antigens to naïve T cells nearby without co-stimulatory molecules. However, both the excretions released by the larvae (Emery *et al.*, 1991) and the carbohydrate-rich larval surface (Harrison *et al.*, 2003b) are also antigenic. Thus, the down stream exsheathing fluids together with other larval antigens available at their predilection sites could be potent stimuli for the mucosal immune system. These soluble larval antigens plus the 'early bird' larvae could be exposed to the mucosa of the small intestine even in the early part of 24 hrs after challenge, before the arrival of the majority of the population.

Scenario 2- In connection with the above scenario, the gut mucosa is constantly bombarded by pathogens and the epithelia restrict their passage into the underlying tissue. Nevertheless, in an inflammatory condition such as nematode infection, neutrophils and eosinophils have been observed in both mucosa and lumen in experiments with nematode infected naïve lambs (Baker, 1975; Salman & Duncan, 1985) or immune sheep (Balic *et al.*, 2006) or in experiments using model epithelia (Nash *et al.*, 1987; Resnick *et al.*, 1995). This implies the epithelial tight junction can be compromised in certain conditions. In light of finding eosinophils in the gut lumen in the current study on day 1 and day 3, although the mechanism involved in the transepithelial migration of leucocytes is poorly defined, the evidence suggested that eosinophils do not use their usual diapedesis movement such as when they go through the tight junction of the vascular endothelium, but instead they seemed to use some kind of mechanical force to push the entire epithelial cell out of the basal membrane towards the lumen. Similar observations and comments have been made previously by Nash *et al.* that leucocytes may generate the force required to separate the tight junction (Nash *et al.*, 1988). Neutrophils and eosinophils also secrete proteases (DiScipio *et al.*, 2006) which can effectively digest the tight junction proteins of epithelium and the collagen support frame of lamina propria of the small intestine (Freshney, 1994). Possibly, this type of inflammatory cell migration can elicit extensive leaks in the epithelium, which could allow luminal antigens to gain access to the lamina propria. Nevertheless, in the current study eosinophil transmigration was not a widespread phenomenon, occurring occasionally in the crypts but not at the villous tips. Thus, it is difficult to conclude that eosinophil transepithelial migration induced disruption of epithelial barriers for the convenience of luminal larval antigen transport into the lamina propria.

Scenario 3- It has been reported that IgA and IgG1 are dominant Igs in mucosal humoral responses immediately after nematode challenge in immune sheep (Harrison *et al.*, 1999;

McClure *et al.*, 1992). Recent *in vitro* studies, of receptor associated Ig transport in mucosal surfaces, have suggested that in neonates and adults the pIgR (polymeric immunoglobulin receptor) mediates unidirectional transport of dimeric IgA and IgM (basolateral to apical direction), whereas transport of IgG in the mucosa is mediated by FcRn (Fc receptor of neonate) and is bidirectional (Dickinson *et al.*, 1999; McCarthy *et al.*, 2000; Rojas & Apodaca, 2002). Recent findings of high FcRn expression levels in small intestinal tissues of adult sheep (Dzidic *et al.*, 2004) suggest that FcRn is used for IgG transport across mucosal epithelial barriers at sites other than the liver in adult as well as neonatal animals (Mayer *et al.*, 2002b). Besides, this novel bidirectional IgG transport ability of FcRn has been proven (Yoshida *et al.*, 2004) to be a vehicle for transporting IgG across epithelial barriers into the lumen to capture luminal antigens (Abrahamson *et al.*, 1979) to transport back into the lamina propria for antigen processing by dentritic cells and for CD4 activation. It is therefore tempting to draw a scenario where the FcRn could transcytose nematode-specific IgG into the lumen to capture luminal larval antigens, then transport them back across the epithelial barrier to sub-epithelially located anti-nematode-antibody-bearing MMCs and other inflammatory cells in the absence of 'leaky' epithelial barriers; and this could trigger a cascade of inflammatory processes and gut tissue damage as seen on day 1 after challenge.

Scenario 4- When considering antigen transport into the lamina propria in nematode sensitised animals, another important point to take into account is that the first host cells which 'meet' larvae or luminal larval antigens are in fact the single layer of epithelial cells: enterocytes & intraepithelial lymphocytes (IEL). These cells are an important barrier between the immune compartment of mucosa and luminal antigens derived from pathogens, food and commensals. Recent studies have shown that these cells signal the presence of pathogens via Toll-like receptors (TLRs) (Aderem & Ulevitch, 2000) and they appear to regulate inflammatory and immune responses (Akira *et al.*, 2001) by inducing the expression of proinflammatory cytokines that recruit circulating leucocytes. It has been shown lately that sheep gut epithelial cells bear TLR receptors 1-10 which are homologues of human TLRs (Menzies & Ingham, 2006); also human and rodent studies indicate that intestinal epithelial cells can express and produce proinflammatory cytokines, chemotaxins (IL1-β, SCF, TGF-β1 & IL-8, TNF-α, GM-CSF, IL6), and chemokines (*monocyte-chemo-attractant protein*-1, RANTES, epithelial neutrophil-activating peptide 78,) in response to intestinal nematode parasites (Li *et al.*, 1998; Rosbottom *et al.*, 2002; Stadnyk & Kearsey, 1996) or to bacterial infection (Jung *et al.*, 1995).

Furthermore, IELs live at the gut frontier within a sea of epithelial cells, and although the IEL activation process remains poorly understood, it has been shown that IELs are capable of

proliferating in response to *T. colubriformis* larval challenge in immune sheep (McClure *et al.*, 1992). Since nematode antigens contain a mitogenic property (Cross *et al.*, 1986), they may be activated directly to produce IFN-γ, TNF-α and IL5 (Kohyama *et al.*, 1997; Taguchi *et al.*, 1991; Yamamoto *et al.*, 1993). Thus, a non-specific activation of IELs during larval challenge, leading to expression of innate and adaptive cytokines in mucosal cells, may be possible. Gut lymphocytes of both non-immune and immune sheep were found to express IFN-γ and TNF-α in response to nematode challenge (Pernthaner *et al.*, 2006).

Interestingly, the inflammatory cytokines IFN-γ and TNF-α can directly induce an increase in epithelial permeability (Grotjohann *et al.*, 2000; Madara & Stafford, 1989; Mullin & Snock, 1990; Schmitz *et al.*, 1999; Sutas *et al.*, 1997) by regulation of tight junction (TJ) proteins (Berkes *et al.*, 2003; Nusrat *et al.*, 2000), and contributed to the increased epithelial permeability to macromolecules in humans with bowel disease (Soderholm *et al.*, 2002) and rodents with gut nematode infection (Murray *et al.*, 1971a). These cytokines have been found to be expressed in IELs, mast cells and epithelial cells from humans and rodents (Kearsey & Stadnyk, 1996; Kohyama *et al.*, 1997; Supajatura *et al.*, 2002b; Yamamoto *et al.*, 1993). This information indicates that a non-specific mast cell-independent mechanism, which induces the release of inflammatory cytokines and chemokines from epithelia in response to larval challenge, could be implicated in the initial epithelial leakiness for antigen transport into lamina propria.

Taking these four scenarios together, based on the histological results of the current study, the epithelial barrier on day 0 did not show any defects at the tip of the villi, consistent with antigens gaining access into the gut lamina propria. The transepithelial migration of leucocytes in the crypts also did not appear to influence the delivery of antigens into the gut lamina propria, since this activity was not wide-spread on day 1. Therefore, it is likely that the larval antigen transport into the lamina propria involved innate TLR-mediated process, and IgG- or FcRn-mediated-transcytosis of antigens across the epithelial barrier. In addition, the rapid eosinophil influx on day 1 indicates the probable presence of eosinophil attractants such as eotaxins (Rothenberg, 1999), IL5 (Wang *et al.*, 1989; Yamaguchi *et al.*, 1988), and parasite antigens (Dixon *et al.*, 2006; Wildblood *et al.*, 2005) in the mucosa. The changes in MMC morphology on day 1 (increase of cell size & granulation) suggest that cytokines like SCF (stem cell factor), which is a potent mast cell attractant (Meininger *et al.*, 1992) as well as promoting mast cell growth and proliferation, could also be available in the mucosa by day 1.

Once the luminal larval antigens are in the lamina propria, they may be attracted to the sub-epithelially located anti-nematode antibody-bearing inflammatory cells, such as MMCs, MMDs and eosinophils. In fact, the experimental sheep in the current study were hyperimmunised and received their last immunization dose 2 weeks before they received a final challenge dose, meaning their gut mucosae were well primed. Furthermore, antibody bearing cells can reside in the gut mucosa of immune sheep for a month after the infection (Gill *et al.*, 1994) and thus, the lamina propria of these sheep could be filled with nematode specific-antibody bearing immunocytes 'living ' very close to the basal membrane.

On day 3 the intestinal mucosa was optimally primed, and the LPCs isolated from the small intestine produced the highest level of LMI activity, which was positively correlated with Type II eosinophils and GOs, but not with MMCs. The histological results indicated that MMCs were highly activated on day 1, but not on day 3, that is during the acute stage of inflammation, and this may be the source of TH-2 type cytokines such as IL5 and IL13 (Bradding *et al.*, 1994; Henz *et al.*, 2001) which mediated respectively the eosinophil and GO activation on day 3. Although activated eosinophils can damage tissues physically by infiltration (Balic *et al.*, 2006), by direct cellular cytotoxicity (Fredens *et al.*, 1988), and possibly by other tissue damaging substances like proteinases (Okada *et al.*, 1997) and collagenases (Shlopov & Hasty, 1998), based on the impressive history of eosinophils on the pathogenesis of asthma (Frigas & Gleich, 1986; Frigas *et al.*, 1991; Gleich *et al.*, 1979) the release of eosinophil granule proteins and enzymes are a likely cause of gut tissue damage including villus atrophy and denudation of epithelia on day 3. The damage on day 3 seemed so extensive that if there had been worms residing in their sub-epithelial niche none would have survived in this situation, and most likely the whole worm colony had been wiped out because many of the villi were removed from their niche. This pathology resulting from inflammation seemed to be the most simple, effective, and natural mechanism for the removal of resident nematodes (adult worms) from their niche in immune sheep. In other words, this could be the *self-cure* reaction, named by Norman Stoll in 1929, in demonstrating that the worm burden was reduced immediately after a challenge larval dose coupled with a subsequent violent gut response in previously infected sheep (Stoll, 1929).

The results in Chapter 5 clearly demonstrate that using *auto*MACS (automated magnetic cell sorting) for enriching gut mucosal cells did not interfere with the functional activity and morphology of isolated cells, and this was in agreement with other researchers using *auto*MACS (Gangopadhyay *et al.*, 2004; Lequerica *et al.*, 1999; Park *et al.*, 2006). The isolation of functionally intact viable cells from multi-cellular tissues is difficult. However, the use of *auto*MACS gave a measure of success in obtaining sufficient numbers of highly purified

cells of the type of interest, which was not possible with the Percoll technique alone. Percoll solution is non-toxic to a wide variety of cultured cells (Pertoft & Laurent, 1977) which is consistent with the high levels of cell viability after Percoll purification seen in the present study (80%-95%). Percoll is useful to purify cells whose buoyancy does not overlap with many other cell types, e.g. monocyte-macrophage-dendritic cell (MMD). In the current study MMDs were recovered mostly on the 50% Percoll band, but the purity of other cell types [*i.e.* eosinophils, mucosal mast cells (MMCs), globule leucocytes (GLs) and epithelial/goblet cells (EP/GOs)] was poor after Percoll gradient centrifugation, similar to the results of Stankiewicz *et al.* (1994), who had little success separating eosinophils from the MMC/GL population of the small intestine of *T. colubriformis* immunised sheep. The biggest challenge is trying to separate MMCs and GLs, since their buoyancy and surface antigens (*i.e.* IgE and IgG_1) are similar. This may be the reason why previous investigators (Douch *et al.*, 1996; Huntley *et al.*, 1984; Huntley *et al.*, 1982; Stankiewicz *et al.*, 1994) when working with these cells from sheep, did not separate them and counted them under one description (MMC/GL). However, *in situ,* these two cells populate different areas of the mucosa, GLs are mostly in the epithelia location, whereas MMCs are in the lamina propria. Based on this observation, GLs were isolated from the epithelium using EDTA treatment to release intra-epithelial GLs from the epithelium, then they were concentrated by Percoll gradient centrifugation (Table 5.10) before being contaminated by lamina propria-bound MMCs, and the result was 90%-94% purity. Hopefully, the availability of more reliable cell isolation and cell culture systems will permit further characterization of these cells and should be of value in elucidating their role in immunity to nematodes.

Considering the origin of LPF, the experiments of Chapters 3-5 repeatedly demonstrated that LMI activity was associated with the number of activated eosinophils in the cell cultures or small intestinal tissues, but not with GLs, MMCs or EP/GOs. These results suggest that eosinophils have a direct effect on LMI activity, but it cannot be excluded that, other cells may also have a role in the production of LMI activity *in vitro* and *in vivo.* One possible scenario is that mast cells react to antigens *in vitro* within 5min (Jones *et al.*, 1992) or within 2hrs after inoculation of *T. colubriformis* larvae into the small intestine (Harrison *et al.*, 1999). Upon activation, they release mediators, proinflammatory and TH2-cytokines (Burd *et al.*, 1995; Jones *et al.*, 1992; Supajatura *et al.*, 2002), and these secretions then mediate eosinophil activation and product release (Stevenson & Jones, 1994), as well as GO hyperplasia and mucin secretion (Arnold *et al.*, 1993; Han *et al.*, 1987; Webb *et al.*, 2007). There is evidence from *in vitro* and *in vivo* studies to suggest that among the effector cells, only eosinophils and their products are cytotoxic (Gleich *et al.*, 1979) and anti-helminthic (Butterworth, 1984; Jonas *et al.*, 1995; Rainbird *et al.*, 1998). The products of GLs, MMCs and EP/GOs have never been

considered as anti-helminthic, except Douch's speculation that leukotrienes (LTs) and other substances with LMI activity could be present in immune mucus, or produced from enriched MMC/GLs in response to antigens (Douch *et al.*, 1983; Douch *et al.*, 1996). However, when all the commercially available mediators were tested in the current study and by Douch (1990), none of them produced LMI activity. Thus, taking all the evidence of current and previous studies together, one concludes that eosinophils are the cell type that best correlates with LMI activity. Nevertheless, further research is needed to show conclusively the presence of LPF in eosinophils or demonstrate their influence on other cell type(s) to form and release LPF.

It had become clear from the present study that GLs do not produce LPF. Most investigators have regarded GLs as an indicator of resistance to nematodes, since their numbers correlated negatively with worm burdens. There has been no report of them being phagocytic, or anti-helminthic, or to cause tissue damage. GLs are degranulated MMCs (Gregg *et al.*, 1978), commonly populating in the sub-epithelium, and they were found in the lamina propria of the small intestine when sheep received heavy doses of nematode parasites (Douch *et al.*, 1986). After degranulated MMCs have transformed into a typical GL (Huntley *et al.*, 1984), they travel across the lamina propria tissue to reach their destination in the sub-epithelium within 24hrs (McClure *et al.*, 1992). GL has been acknowledged as a silent partner with the epithelium in the host defence against nematodes for a long time, but all this evidence leads to questions of their migration pattern towards the epithelium, accumulation, of their purpose in being in the intra-epithelial location, and of their ultimate fate, which remain mysteries.

Leucocyte migration of the mucosa is complex and involves cell surface molecules (Teixeira & Hellewell, 1998) and found to be mediated by chemokines and cytokines (Moser *et al.*, 1992; Taub *et al.*, 1995). Although the mechanism involved in migration of GLs towards the epithelium is unknown, its precursor mast cells migrate under the influence of cytokines - TNF, TGF-β, IL8 and SCF. Interestingly, these cytokines are expressed and released by epithelial cells in response to gut nematode parasites (Li *et al.*, 1998; Rosbottom *et al.*, 2002; Stadnyk & Kearsey, 1996). It is conceivable that the epithelial cell activation and release of pro-inflammatory cytokines associated with nematode infection may attract the newly transformed GLs via pro-inflammatory cytokines to the epithelial site and position themselves into an intra-epithelial position where the epithelial cell is denuded. Perhaps, this migration may be part of the epithelial restitution process.

The study resulted in the development of a reliable *in vitro* cell culture system for separation of cells producing LPF. Together with *auto*MACS, cell culture helped in finding and enriching

source materials for the purification of LPF to relatively pure fractions. LPF activity was produced specifically in response to *T. colubriformis* larval antigens *in vitro* and *in vivo,* suggesting that it could be released into the mucosa during nematode challenge and is implicated in the expulsion of an infection in immune sheep. This suggests the *in vitro* model system is likely to portray the gut immune response to nematode infection. Therefore, the system could be used to investigate how LPF release is regulated, which could be useful in increasing LPF production *in vitro* and *in vivo.* This may also lead to the development of therapeutic approaches to enhance nematode control. In addition, it is a logical system to elucidate potent larval antigen fractions eliciting LPF release in the gut, and it may become a useful *in vitro* tool to screen potential antigens for nematode vaccines.

Microplate-bioassay-guided results, pertaining to the semi-purification of CCS (from small intestinal LPCs from immune sheep) using molecular size sieving through ultra-membranes and gel filtration chromatography, indicated that LPF is a small molecule, of a size close to that of glucose, having anthelmintic efficacy against a wide variety of nematode parasite larvae and adult stages. The molecular size was much smaller than the antimicrobial molecules known to be secreted by the gut epithelial cells such as defensins (2-6 kDa), and cecropins (3339 kDa) (Dann & Eckmann, 2007).

The purification and characterisation of LPF was a difficult process, due to the lack of research data on the subject. Nevertheless, the step-by-step analysis of biochemistry and HPLC chromatography suggests that LPF is a polar, heat and enzyme resistant compound which is very different in biochemical nature to the leukotrienes that Douch *et al.* postulated two decades ago (Douch *et al.*, 1983). The present study demonstrates that the LPF is polar and did not bind to a C18 sorbent whereas the leukotrienes are lipid compounds, less polar and C18-binding. In addition, all commercially available MMC mediators have been bioassayed in this study and by Douch (1990), and none were active against *T. colubriformis* larvae. The study used a LC-MS-guided analysis to remove amino acids and peptides from the active fraction and to determine a suitable HPLC method for the development of a purification procedure which provides a highly purified fraction comprising three main compounds. They are a UV absorbing compound at 254nm with no MS data, and two non UV absorbing compounds at 254nm: one was a component of *m/z*104 (ESI+ve) and the other was detected at *m/z*113 (ESI-ve).

In future research, with the leads and evidence obtained from the current study, the candidate compounds 104 (ESI+ve) and 113 (ESI-ve) could be checked and analysed with more sensitive LS-MS or MS-MS methods. An MS-guided analysis could continue to be applied to subsequent

purification steps, thus speeding up the concentration and isolation of the candidate compounds. In addition, when the active compound are identify, there are several applications have to be considered:

Conclusions

Several strategies are used to control nematodes (drenching, pasture management, biological control, vaccination, nutritional supplement, and genetic approach), with their own advantages and disadvantages. Without effective anthelmintics the sheep industry cannot continue to survive in its current form, therefore for the time being, anthelmintics are still needed for effective nematode control.

One strategy to discover new anthelmintics is to explore the gut-origin of natural anthelmintics from cells of immune sheep. The results reported in this thesis demonstrate that the release of that LPF is antigen-mediated, and LPF activity is associated with the activation of eosinophils, MMCs and GOs. The study supports concepts that the anti-nematode response is strongly genetically-biased and antigen-challenge dependent, and that it involves inflammation. In addition, with the help of *auto*MACS separation, the study provides the first evidence of a functional link between GOs and other inflammatory cells in host cellular effector mechanisms against nematode infection. Elucidation of the biochemical nature of the mucus component will provide more insight into the effector function of GOs, with a future goal of perhaps providing new therapy for treating not only nematode infection, but also other mucosal diseases.

Initial purification steps demonstrated that LPF is a very small, polar, broad-spectrum natural anthelmintic, with such biochemical properties as are required for it to withstand the hostile environment of the gut. In addition, the molecular size and retention behaviour in C_{18} RP-HPLC of LPF confirmed that they are not leukotrienes, which were postulated to be associated with larval migration inhibition activity in mucus from immune sheep. This study suggest the possibility that there is more than one mediator with anti-nematode activity, which is released into the gut mucosa in response to nematode parasite larval challenge in immune sheep, and which may also be associated with rapid larval expulsion in immune sheep. The use of bioassay-guided suitable HPLC method for the development of a purification procedure disclosed a highly pure analytical fraction, and the results suggest that it contained three molecular species.

This is the first report of the preparation of LPF at high purity. There is no doubt that this lead combined with a highly sensitive MS-monitoring system can speed up the search for a new class of anthelmintic agents based on natural mediators. The method developed for *in vitro* production and bioassay, identification of an association between LMI activity and gut mucosal cell types, and partial purification and analysis of LPF could definitely be a good lead to answer the cellular origin and chemical identity of LPF, which were not fully elucidated in the present study.

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Oral presentations at conferences based on work described in this Thesis

1. Abstract for the 29th Annual Conference of the Australasia Society for Immunology in Dunedin (5th-8th December 1999)

OPTIMISATION OF GUT MUCOSAL ANTI-PARASITIC ACTIVITY IN SHEEP BY USING *IN VITRO* LARVAL MIGRATORY INHIBITION ASSAY

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Several years ago small intestinal mucus of nematode-immune sheep was shown by *in vitro* larval migration inhibition assay (LMI), to contain anti-parasitic activities which affect the motility of larvae. The LMI activity is expressed as percentage inhibition of migration of larvae through a 20 μm sieve (LMI%). A similar activity was also detected in the cell culture supernatant (CCS) generated by culturing enriched small intestinal lamina propria cells from immune sheep in the presence of parasite antigens. The level of activity in mucus and or CCS was significantly greater in nematode resistant-line sheep than susceptible-line sheep in the field, but these two groups were not different when kept in pens. In the pen, the anti-parasitic activity depended on size of dose, frequency of infection and timing of killing after challenge infection. Localisation of anti-parasitic activity in the small intestine was also observed.

2. Abstract presented at the 29th Conference of the New Zealand Society for Parasitology, held jointly with the Australian Society for Parasitology, at Te Papa, Wellington, 24–28 September 2000

ANTI-PARASITIC MOLECULE ACTIVITY OF GUT MUCOSAL CELLS FROM NEMATODE-RESISTANT SHEEP

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The gut mucosa is bombarded with a variety of pathogens, including nematodes, and host animals are armed with different mechanisms to protect themselves against pathological damage. In response to nematodiasis, the ovine gut becomes conditioned to expel the incoming infective nematode larvae. The mechanism is which expelled larvae are still in question. How ever, one of the proposed mechanisms is that anti-parasitic molecule(s) [APM] released from the effector cells in the gut wall contribute to the rejection. Gut mucosal cells are composed of eight types – lymphocytes, eosinophils, mucosal mast cells/globule leucocytes, macrophages/dendritic cells, and goblet/epithelial cells. After enzyme-digestion, crude cell population was fractionated by MACS (Magnetic cell sorter) and density to enrich or deplete the selected cell type. Cell fractions were cultured with/without nematode antigens and resulting cell culture supernatants (CCS) were assayed for larval migration inhibition (LMI) and larval paralysis activities. Results indicated that among gradient fractions, APM activity was shown predominantly in the cell population which separated under 65%gradient. The fraction enriched in epithelial cells or macrophages/dendritic cells or lymphocytes had no acativity. In MACS separated fractions, activity was found predominantly in eosinophil-enriched populations, but the activity dropped when the purity of eosinophils increased to very high levels. Eosinophils or macrophages of mammary gland-origin had no APM activity. Panning, *auto*MACS and density separated mucosal mast cells had very low or no APM activity, even though they had undergone degranulation and histamine release.

3. Abstract presented at the New Zealand Society for Parasitology Annual Meeting No. 31, The Quality Hotel, Palmerston North, 22nd – 23rd October 2002

NATURALLY OCCURING ANTI-PARASITIC MOLECULE(S) IN SHEEP

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Gut nematode infection is a major problem in several countries including New Zealand and imposes an economic burden on agriculture. This problem escalates when anthelmintic resistant nematode strains spread widely. At present we are investigating some alternative means of overcoming the problem. One way is to identify natural anti-parasitic molecules (APM) expressed in the gut mucosa and develop technologies to use them as anthelmintics. Experiments performed *in vitro* indicate that gut mucosal cell culture supernatants (CCS) from nematode immune sheep contain an anti-parasitic agent against *Trichostrongylus colubriformis* 3rd stage larvae and a wide range of other parasitic gut nemtodes; also soil nematode *Caenorhabditis elegans*; and adult *T. colubriformis*. It was demonstrated that anti-parasitic activity is manisfested as temporary paralysis rather than clumping or killing. The release of this activity *in vitro* was associated with eosinophil-enriched gut mucosal cells and *T. colubriformis* larval antigens. Although the clinical nature and the molecule mass of the APM(s) are not fully understood, it is a very small heat stable molecular (~less than 1000daltons) and resistant acid and protease enzyme. However, the APM activity cannot be maintained in high alkaline medium. This work demonstrates the ability of gut mucosa to provide anti-parasitic substances. It supports the hypothesis that gut effector cells play an important role in the host resistance to gut nematodes.

Appendices

1. LMI assay

1.1. LMI activity of CCS with *T. colubriformis* ES antigens

Sheep	Larval count (Triplicate)			LMI%			Mean LMI%	S.D.
5053	60	49	70	47	57	39	48	9
35	38	31	42	67	73	63	68	5
3022	68	84	80	40	26	30	32	7
36	49	54	64	57	53	44	51	7
3020	74	68	79	35	40	31	35	5
Buffer control	121	107	115					
Levamisole	2	1	0				99	

1.2. LMI activity of CCS without *T. colubriformis* ES antigens

Sheep	Larval count (Triplicate)			LMI%			Mean LMI%	S.D.
5053	106	96	115	4	1	17	7	8
35	89	98	102	22	14	11	15	6
3022	97	103	115	15	10	-1	8	8
36	100	108	86	12	5	25	14	10
3020	102	110	112	11	4	2	5	5
Buffer control	121	107	115					
Levamisole	2	1	0				99	

1.3. Correlation between cell type and LMI% (Analysed by GENSTAT)

EO							
EP	0.4392						
GL	-0.5592	-0.5474					
LYM	-0.9259	-0.7102	0.7323				
MMC	-0.3363	-0.3859	-0.3372	0.2516			
MMD	-0.4395	-0.5526	0.2282	0.5961	0.1055		
N	*0.6470*	*-0.3961*	*-0.1667*	*-0.3582*	*0.0642*	0.0000	
sLMI	0.8718	0.8183	-0.7032	-0.9817	-0.3597	-0.5738	0.2032

EO	EP	GL	LYM	MMC	MMD	N

Keys: LYM – lymphocytes; EO – eosinophils; EP – epithelial cells; GO- goblet cells; MMC- mucosal mast cells; sLMI% – larval migration inhibition percentage of cell culture supernatants;

Correlations

EO			
LMI%	0.8718		
EP	0.4392	0.8183	
	EO	LMI%	EP

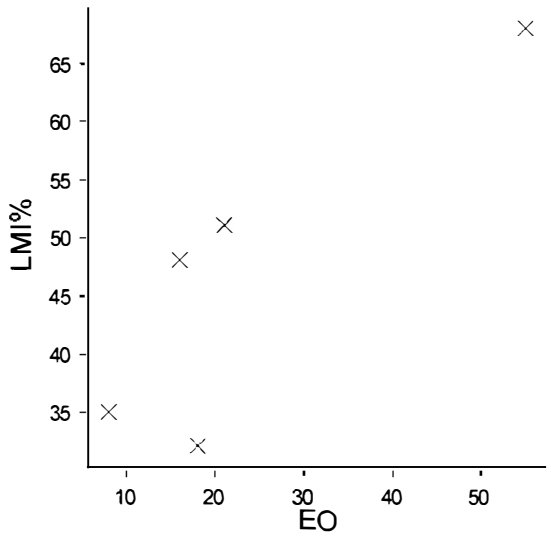
Number of observations: 5

Two-sided test of correlations different from zero probabilities

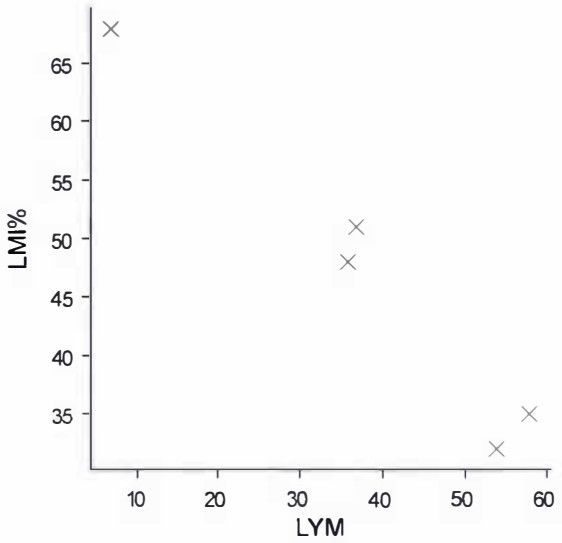
EO			
LMI%	0.0540		
EP	0.4593	0.0904	
	EO	LMI%	EP

Comments: *Data shows there is a positive relationship between LMI% of CCS and the proportion of eosinophils or epithelial cells, and there is high evidence (P~0.05) that the correlation of LMI% with the proportion of eosinophils is significantly different from zero, but not with the proportion of epithelial cells.*

1.4. Relationship between percentage of eosinophil and LMI% from CCS



1.5. Relationship between percentage of lymphocytes and LMI% from CCS



2. LMI activity of mucus (for Table 3.1)

Sheep	Larval count (Triplicate)			LMI%			Mean LMI%	S.D.
5053	9	4	15	92	96	87	92	5
35	2	9	4	98	92	96	96	3
3022	17	11	5	85	90	96	90	5
36	22	15	28	81	87	75	81	6
3020	49	70	53	57	39	54	50	10
Buffer control	121	107	115				114	
Levamisole	2	1	0				1	

3. LMI assay data (for Table 3.5)

Cell cultures with antigens						
cell type	Sheep	larval count (triplicate)			mean	LP%
EP	7138P	149	143	146	146	5
EP	7000P	133	138	135	135	12
EP	2412F	106	110	115	110	29
EP	5414F	145	150	160	152	1
LPC	7138P	60	69	55	61	60
LPC	7000P	2	2	1	2	99
LPC	2412F	62	66	74	67	56
LPC	5414F	63	71	52	62	60

Cell cultures with no antigens						
cell type	**sheep**	**larval count (triplicate)**			**mean**	**LP%**
EP	7138P	156	140	149	148	4
EP	7000P	151	148	154	151	2
EP	2412F	156	139	152	149	3
EP	5414F	144	158	143	148	4
LPC	7138P	147	141	152	147	5
LPC	7000P	137	131	138	135	12
LPC	2412F	145	152	147	148	4
LPC	5414F	131	150	139	140	9
Buffer		156	163	144	154	
Levamisole		0	0	1	1	

Keys: EP – epithelial cells; LPC – lamina propria cells

4. LMI activity of CCS from sheep with different immune status

Status	**Sheep**	**larval count (triplicate)**			**mean**	**LMI%**	**group mean**
out-bred nematode naïve	7065	154	160	144	153	2	9
	7022	141	150	139	143	8	
	8031	129	136	143	136	12	
	8032	142	134	120	132	15	
Field susceptible-line	6018	104	111	120	112	28	19
	6162	98	109	91	99	36	
	7362	138	151	146	145	6	
	7306	156	139	147	147	5	
Field Rresistant-line	6102	51	43	40	45	71	63
	6358	49	58	63	57	63	
	7054	55	62	68	62	60	
	7138	69	60	68	66	58	
	6521	38	48	40	42	73	87
Field resistant-line	7174	6	9	15	10	94	
pen-sensitised	6410	1	3	7	4	98	
	7640	29	25	19	24	84	
Outbred immunised and	8034	13	15	24	17	89	83
re-sensitised in pen	8035	42	67	48	52	66	
	6003	7	11	4	7	95	
Buffer control		138	159	168	155		
Levamisole		0	1	1	1		

Variances and degrees of freedom

For checking that the groups have homogeneous variance

Group	Var_	d_f
1	31.6	3
2	244.9	3
3	32.7	3
4	124.9	3
5	234.3	2

Bartlett's test for homogeneity of variances
Chi-square 4.40 on 4 degrees of freedom: probability 0.355

***Comments**: It indicates variances are not significantly different from one another*

Shapiro-Wilk test for Normality

For checking that the LMI activity is normally distributed

Data variate:	LMI%
Test statistic W:	0.8915
Probability:	0.034

***Comments**:It indicates data is not normally distributed. However, ANOVA is generally robust to non-normality so the analysis can be carried out.*

4.1. One-way ANOVA of LMI% of CCS derived from sheep with different immune status

Variate: LMI%

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Status	4	20099.2	5024.8	39.72	<.001
Residual	14	1770.9	126.5		
Total	18	21870.1			

Tables of means

Variate: LMI%

Grand mean 50.7

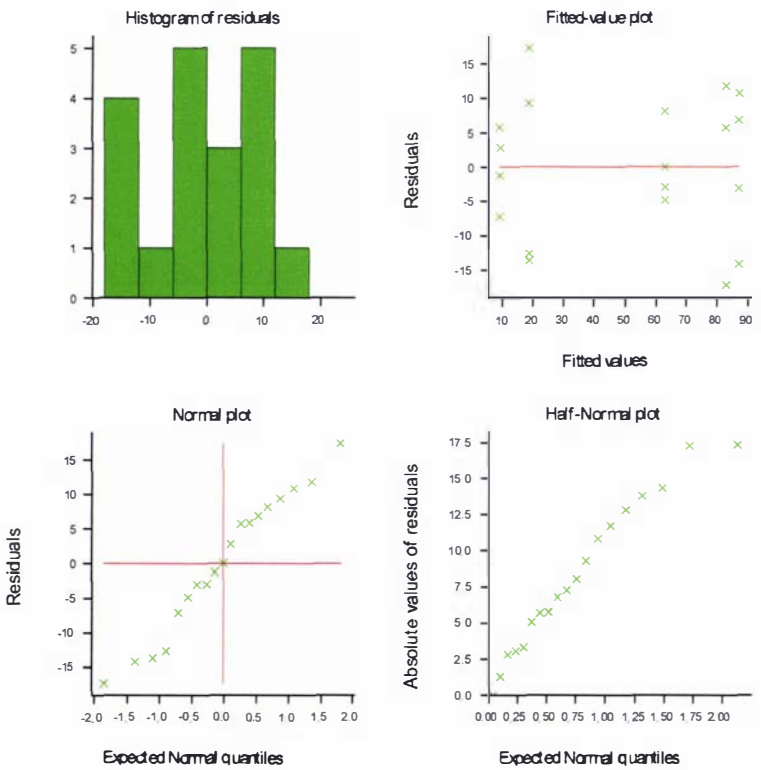
Status	OBN	FS	FR	FRPB	OBPl
	9.2	18.8	63.0	87.2	83.3
rep.	4	4	4	4	3

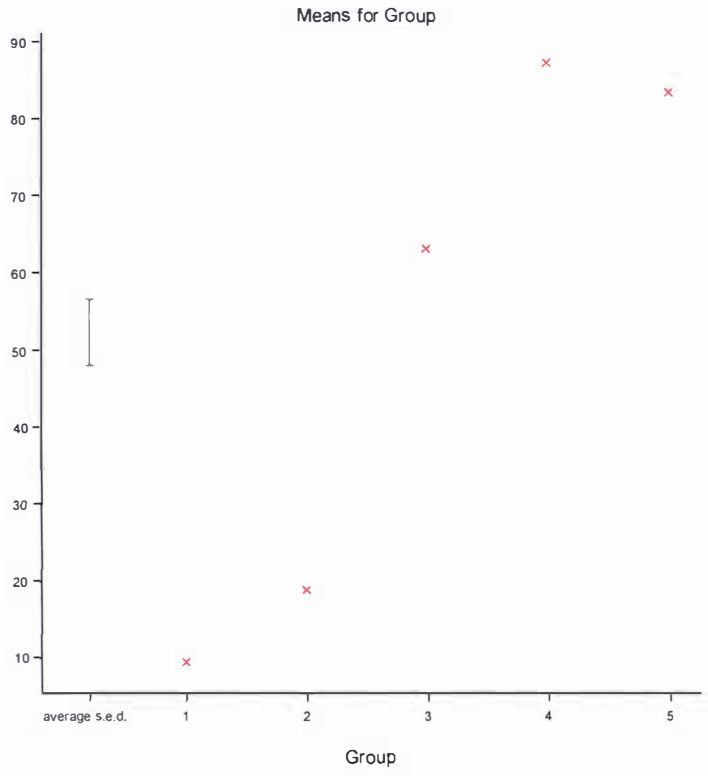
Least significant differences of means (5% level)

Table	Status	
rep.	unequal	
d.f.	14	
l.s.d.	19.70	min.rep
	18.42	max-min
	17.06	max.rep

Least significant differences (at 5%)

1	*				
2	17.057	*			
3	17.057	17.057	*		
4	17.057	17.057	17.057	*	
5	18.424	18.424	18.424	18.424	*
	1	2	3	4	5





***Comments**: Groups 1 and 2 (naïve and susceptible groups) have very much lower means than the other immune groups (3, 4 and 5), and the difference is much greater than the least significant difference (5% l.s.d.). The difference is so large that we believe that some degree of non-normality would not affect this conclusion. For ANOVA, the assumption of homogeneity of variance is more important to be satisfied than normality of the data.*

5. LMI activity of mucus from sheep with different immune status

Status	Sheep	larval count			mean	LMI%	groupmean
Out-bred nematode naïve	7065	127	130	133	130	16	18
	7022	112	98	119	110	29	
	8031	135	141	148	141	9	
	8032	119	124	134	126	19	
Field susceptible-line	6018	97	86	91	91	41	22
	7362	138	151	144	144	7	
	6162	116	120	125	120	22	
	7306	126	130	120	125	19	
Field resistant-line	6102	54	63	71	63	60	60
	6358	37	39	41	39	75	
	7054	77	70	56	68	56	
	7138	71	75	87	78	50	
Field resistant-line	6521	40	46	55	47	70	75

pen-sensitised	7174	56	50	45	50	68	
	6410	22	14	17	18	89	
	7640	47	37	33	39	75	
Out-bred pen-immunised	8034	10	16	11	12	92	84
and re-sensitised in pen	8035	34	41	46	40	74	
	6003	26	20	19	22	86	
Buffer control		138	159	168	155		
Levamisole		0	1	1	1		

5.1. One-way ANOVA of LMI% of mucus derived from different segments of gut

Analysis of variance

Variate: LMI%

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Status	4	13599.2	3399.8	30.14	<.001
Residual	14	1579.2	112.8		
Total	18	15178.4			

Tables of means
Variate: LMI%
Grand mean 50.4

Status	1	2	3	4	5
	18.2	22.2	60.2	75.5	84.0
rep.	4	4	4	4	3

Least significant differences of means (5% level)

Table	Status	
rep.	unequal	
d.f.	14	
l.s.d.	18.60X	min.rep
	17.40	max-min
	16.11	max.rep

6. Larval migration inhibition activity of CCS from different segments of gut

Sheep	Segment	larval counts			mean	LMI%
4344	1st	88	81	76	82	57
	3rd	76	82	70	76	60
	6th	131	121	118	123	35

	12th	135	142	122	133	30
	18th	128	120	134	127	33
	Buffer only	203	175	193	190	
	Levamisole	0	0	0	0	
0160	1st	2	1	1	1	99
	3rd	15	12	9	12	92
	6th	78	64	71	71	53
	12th	108	99	92	100	34
	18th	112	119	109	113	25
	Buffer only	159	145	148	151	
	Levamisole	0	0	0	0	
4555	1st	42	48	50	47	70
	3rd	45	52	44	47	70
	6th	59	65	49	58	63
	12th	84	89	79	84	46
	18th	78	87	72	79	49
	Buffer only	164	162	142	156	
	Levamisole	0	1	1	1	
0614	1st	55	47	41	48	73
	3rd	34	42	28	35	80
	6th	75	84	71	77	56
	12th	108	113	124	115	34
	18th	123	137	128	129	26
	Buffer only	163	185	176	175	
	Levamisole	1	0	0	0	
7133	1st	1	2	1	1	99
	3rd	0	2	2	1	99
	6th	91	76	86	84	38
	12th	102	92	112	102	25
	18th	108	113	97	106	22
	Buffer only	143	130	134	136	
	Levamisole	0	0	1	0	

Shapiro-Wilk test for Normality

Data variate: LMI%
Test statistic W: 0.9158
Probability: 0.041

Variances and degrees of freedom

GutSegment	Var_	d_f
1st	349.8	4
3rd	251.2	4
6th	144.5	4
12th	60.2	4
18th	117.5	4

Bartlett's test for homogeneity of variances

Chi-square 3.17 on 4 degrees of freedom: probability 0.530

Comment: The data again is not-normally distributed but does have homogeneity of variance.

6.1. One-way ANOVA of LMI% of CCS derived from different segments of gut

Variate: LMI%

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Segments	4	11657.0	2914.2	17.67	<.001
Residual	20	3298.4	164.9		
Total	24	14955.4			

Tables of means

Variate: LMI%

Grand mean 54.8

Segments	1st	3rd	6th	12th	18th
	80.2	80.2	49.0	33.8	31.0

Least significant differences of means (5% level)

Table	Segments
rep.	5
d.f.	20
l.s.d.	16.94

***Comments**: There is strong evidence ($p<0.05$) that segments 1 & 3 have higher LMI activity than the other segments. There is some evidence that the 6th segment has higher LMI activity on average than segments 12 and 18, but the difference was not significant at the 5% level. Because of the robustness of ANOVA to non-normality the comparison between the segments can be made.*

7. Larval migration inhibition activity of mucus from different segments of gut

Sheep	Segment	larval counts			mean	LMI%
4344	1st	93	99	112	101	47
	3rd	85	72	98	85	55
	6th	144	132	150	142	25
	12th	178	180	184	181	5
	18th	176	180	192	183	4
	Buffer only	203	175	193	190	
	Levamisole	0	0	0	0	
0160	1st	38	46	30	38	75
	3rd	45	51	38	45	70
	6th	63	55	48	55	63
	12th	80	95	86	87	42
	18th	97	108	94	100	34
	Buffer only	159	145	148	151	
	Levamisole	0	0	0	0	
4555	1st	75	66	70	70	55
	3rd	38	43	36	39	75
	6th	80	99	79	86	45
	12th	108	95	104	102	34
	18th	89	97	105	97	38
	Buffer only	164	162	142	156	
	Levamisole	0	1	1	1	
0164	1st	69	64	75	69	60
	3rd	57	60	49	55	68
	6th	110	99	105	105	40
	12th	145	153	147	148	15
	18th	135	144	140	140	20
	Buffer only	163	185	176	175	
	Levamisole	1	0	0	0	
7133	1st	36	30	42	36	74
	3rd	31	28	34	31	77
	6th	79	74	64	72	47
	12th	114	97	109	107	22
	18th	92	102	98	97	28
	Buffer only	143	130	134	136	
	Levamisole	0	0	1	0	

Shapiro-Wilk test for Normality

Data variate: LMI%
Test statistic W: 0.9542
Probability: 0.311

Variances and degrees of freedom

GutSegment	Var_	d_f
1st	147.7	4
3rd	74.5	4
6th	187.0	4
12th	217.3	4
18th	181.2	4

Bartlett's test for homogeneity of variances

Chi-square 1.11 on 4 degrees of freedom: probability 0.892

***Comment**: Indicates no issues with normality or homogeneity of variance so we can use ANOVA to analyse.*

7.1. One-way ANOVA of LMI% of mucus derived from different segments of gut

Variate: LMI%

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Segments	4	9164.2	2291.1	11.37	<.001
Residual	20	4030.8	201.5		
Total	24	13195.0			

Tables of means

Variate: LMI%

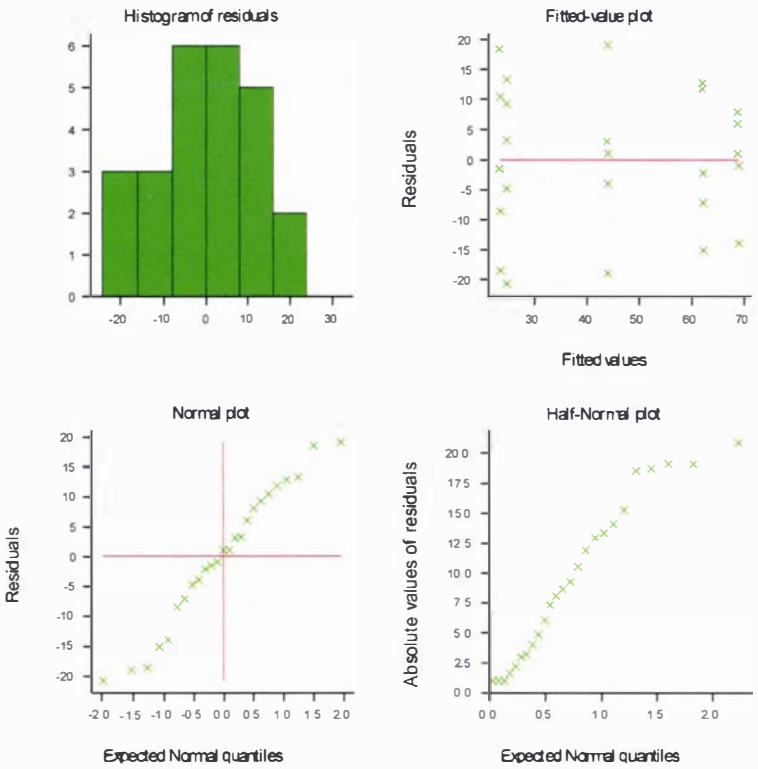
Grand mean 42.7

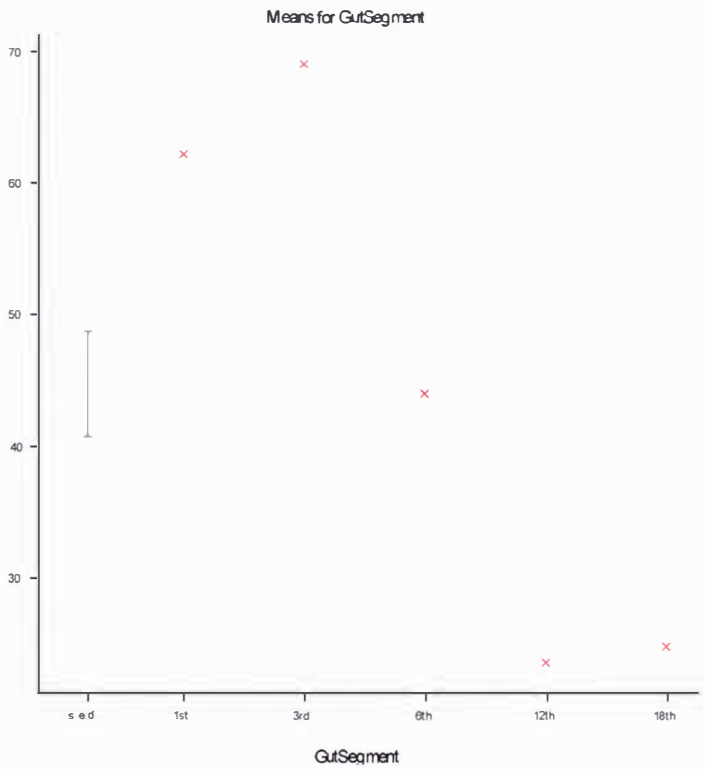
Segments	1st	3rd	6th	12th	18th
	62.2	69.0	34.0	23.6	24.8

Least significant differences of means (5% level)

Table	Segments
rep.	5
d.f.	20
l.s.d.	18.73

***Comment**: There is strong evidence ($p<0.05$) that segments 1 & 3 have higher LMI activity than the all other segments. There is also strong evidence that the 6^{th} segment has higher LMI activity on average than segments 12 and 18. The difference between segments 1 & 3 was not significant at the 5% level, and the same goes for the difference between segments 12 and 18.*


Histogram of residuals
Fitted-value plot
Residuals
Fitted values
Normal plot
Residuals
Expected Normal quantiles
Half-Normal plot
Absolute values of residuals
Expected Normal quantiles


Means for GutSegment
s e d
1st
3rd
6th
12th
18th
GutSegment

8. Correlation between LMI% of mucus and CCS (analysed by GENSTAT)

Correlation matrix

Mucus	1.000	
CCS	0.876	1.000
	Mucus	CCS

9. Relationship between LMI% and the proportion of cell types in the cell populations of third gut segments (for the experiment 3.3.4)

Sheep	L	EO	N	MMD	GO	MMC	GL	GO/EP	LMI%
4344	30	11	0	7	18	5	6	18/23	60
0160	31	12	1	15	6	21	0	6/14	92
4555	20	10	0	4	2	13	4	2/47	70
0164	31	13	2	10	6	14	5	6/19	80
7133	19	19	0	7	9	12	14	9/20	99
γ	-0.24	**0.77**	0.20	0.47	-0.38	**0.66**	0.31	-0.38/-0.50	

Keys: L -lymphocyte; N – neutrophil: EO – Eosinophil; MMD – monocyte-macrophage-dendritic cell; MMC- mucosal mast cell; GL-globule leucocyte; EP/GO – epithelial/goblet cell; *r* = correlation coefficient;

Correlations

EO			
MMC	0.0248		
LMI%	0.7674	0.6557	
	EO	MMC	LMI%

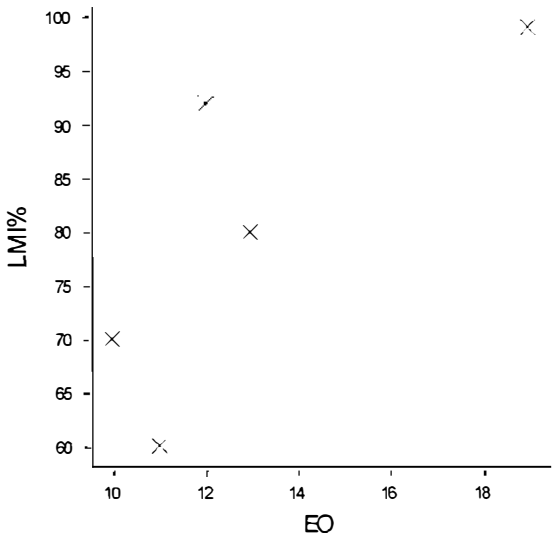
Number of observations: 5

Two-sided test of correlations different from zero probabilities

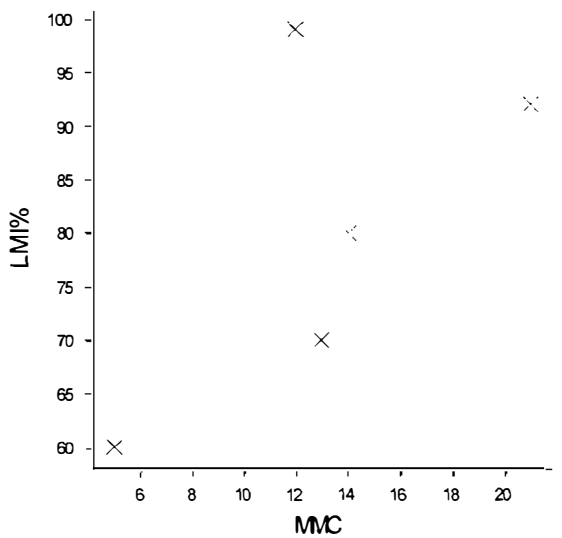
EO			
MMC	0.9684		
LMI%	0.1299	0.2295	
	EO	MMC	LMI%

***Comments**: Neither correlation coefficient is significantly different from zero. The lack of significance is because of the small sample size (5). This means that the data are insufficient to demonstrate a relationship between LMI and each of the two cell types.*

9.1. Relationship between percentage of eosinophils and LMI% from CCS



9.2. Relationship between percentage of mucosal mast cells and LMI% from CCS



10. Differential eosinophil stages in different gut segments

Sheep	Gut segment	Cell type		
		I	II	III
4344	1	22	55	23
7133	1	32	50	18
0160	1	45	40	15
4555	1	23	52	25
0164	1	36	48	16

mean		**31.6**	**49**	**19.4**
4344	3	21	74	5
7133	3	26	56	18
0160	3	13	71	16
4555	3	22	68	10
0164	3	20	63	17
mean		**20.4**	**66.4**	**13.2**
4344	6	57	32	11
7133	6	71	22	7
0160	6	65	30	5
4555	6	53	30	17
0164	6	70	26	4
mean		**63.2**	**28**	**8.8**
4344	12	90	9	1
7133	12	65	31	4
0160	12	24	41	35
4555	12	77	18	5
0164	12	69	21	10
mean		**65**	**24**	**11**
4344	18	94	4	2
7133	18	70	29	1
0160	18	86	10	4
4555	18	80	11	10
0164	18	79	13	8
mean		**81.8**	**13.4**	**5**

10.1. One-way ANOVA of Type II eosinophils derived from different segments of gut

Analysis of variance

Variate: TypeII

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Gut_segments	4	9058.96	2264.74	33.54	<.001
Residual	20	1350.40	67.52		
Total	24	10409.36			

Tables of means

Variate: Eosinophil Type II

Grand mean 36.2

Gut_segments	1st	3rd	6th	12th	18th
	49.0	66.4	28.0	24.0	13.4

Least significant differences of means (5% level)

Table	Gut_segments
rep.	5
d.f.	20
l.s.d.	10.84

11. Differential mucosal mast cell stages in different gut segments

Sheep	Gut segment	Stages of MMC			
		I	II	III	IV
4344	1	72	27	1	0
7133	1	62	37	1	0
0160	1	46	45	8	1
4555	1	31	38	29	2
0164	1	75	17	6	2
mean		**57**	**33**	**9**	**1**
4344	3	78	20	1	1
7133	3	69	31	0	0
0160	3	77	14	9	0
4555	3	1	58	41	0
0164	3	59	41	0	0
mean		**57**	**33**	**10**	**0**
4344	6	87	12	1	0
7133	6	36	64	0	0
0160	6	81	4	15	0
4555	6	42	16	31	11
0164	6	83	15	2	0
mean		**66**	**22**	**10**	**2**
4344	12	45	55	0	0
7133	12	2	36	62	0
0160	12	81	19	0	0
4555	12	2	93	5	0
0164	12	76	8	6	10
mean		**41**	**42**	**15**	**2**
4344	18	76	24	0	0
7133	18	86	14	0	0
0160	18	70	22	8	0
4555	18	5	3	89	3
0164	18	84	0	9	7
mean		**64**	**13**	**21**	**2**

11.1. One-way ANOVA of Stage I mucosal mast cells derived from different segments of gut

Analysis of variance

Variate: Stage_I

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Gut_segment	4	1895.0	473.7	0.52	0.725
Residual	20	18354.0	917.7		
Total	24	20249.0			

Tables of means

Variate: Stage_I

Grand mean 57.0

Gut_segment	1.	3.	6.	12.	18.	
		57.2	56.8	65.8	41.2	64.2

Least significant differences of means (5% level)

Table	Gut_segment
rep.	5
d.f.	20
l.s.d.	39.97

11.2. One-way ANOVA of Stage II mucosal mast cells derived from different segments of gut

Analysis of variance

Variate: Stage_II

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Gut_segment	4	2585.8	646.5	1.45	0.255
Residual	20	8934.4	446.7		
Total	24	11520.2			

Tables of means

Variate: Stage_II

Grand mean 28.5

Gut_segment	1.	3.	6.	12.	18.	
		32.8	32.8	22.2	42.2	12.6

Least significant differences of means (5% level)

Table	Gut_segment
rep.	5
d.f.	20
l.s.d. 27.88	

11.3. One-way ANOVA of Stage III mucosal mast cells derived from different segments of gut

Analysis of variance

Variate: Stage_III

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Gut_segment	4	519.4	129.8	0.23	0.917
Residual	20	11149.6	557.5		
Total	24	11669.0			

Tables of means

Variate: Stage_III

Grand mean 13.0

Gut_segment	1.	3.	6.	12.	18.	
		9.0	10.2	9.8	14.6	21.2

Least significant differences of means (5% level)

Table	Gut_segment
rep.	5
d.f.	20
l.s.d.	31.15

12. LMI activity of CCS and mucus from killed on different days after challenge

sample	Day after challenge	sheep	larval count (triplicate)			mean	LMI%	mean LMI%
CCS	0	B008	70	65	90	75	48	56
	0	357	65	55	38	53	63	
	1	1208	65	80	70	72	50	61
	1	3775	42	40	36	39	73	
	3	1077	1	3	4	3	98	99

	3	3802	0	2	1	1	99	
	6	3137	42	40	36	39	73	65
	6	4029	72	60	56	63	56	
	12	3041	37	45	51	44	69	76
	12	8135	26	20	27	24	83	
	Buffer		160	136	135	144		
	levamisole		0	0	0	0		
mucus	0	B008	76	70	67	71	35	32
	0	357	79	85	73	79	28	
	1	1208	38	29	42	36	67	56
	1	3775	60	45	76	60	45	
	3	1077	0	0	0	0	100	99
	3	3802	3	2	0	2	98	
	6	3137	22	30	24	25	77	83
	6	4029	10	14	12	12	89	
	12	3041	20	28	21	23	79	64
	12	8135	60	56	50	55	50	
	Buffer		106	104	120	110		
	levamisole		0	0	0	0		

12.1. Enriched lamina propria cells in the cell cultures from 5 groups of sheep and their relative LMI activity.

DAC	Sheep ID	L	EO	N	MMD	MC	GL	EP/GO	LMI%
0	B008	50	7	2	6	21	0	10/-	48
0	0357	38	15	1	5	10	0	28/4	63
1	1208	68	7	1	3	8	3	7/3	50
1	3775	40	20	1	9	11	0	9/10	73
3	1077	42	19	0	2	12	5	13/7	98
3	3802	46	18	0	7	9	1	13/6	99
6	3137	58	6	0	2	23	0	6/5	73
6	4029	65	11	0	1	11	0	10/2	56
12	3041	50	14	1	5	18	3	10/0	69
12	8135	41	9	0	11	21	4	9/5	83
Correlation coefficient (*r*)		**-0.55**	**0.62**	**-0.5**	**0.24**	**0.02**	**0.22**	**0.02/0.62**	

Correlations

EO			
LMI%	0.6151		
GO	0.6015	0.6193	
	EO	LMI%	GO

Number of observations: 10

Two-sided test of correlations different from zero
probabilities

EO			
LMI%	0.0584		
MMC	0.0658	0.0562	
	EO	LMI%	MMC

Comments: *Data shows there is a positive relationship between LMI% of CCS and the proportion of eosinophils or epithelial cells, and there is moderate evidence (P~0.06) that both of the correlations are significantly different from zero.*

13. Tissue eosinophil count

DAC	Sheep	villus										mean	SD
		1	2	3	4	5	6	7	8	9	10		
0	B008	34	28	27	20	32	27	13	33	36	25	27.5	7
0	357	18	26	25	20	28	20	18	19	31	28	23.3	5
1	1208	57	54	54	36	58	40	44	47	39	40	46.9	8
1	3775	29	47	34	40	27	36	36	45	37	23	35.4	8
3	1077	33	38	42	53	44	51	36	57	34	47	43.5	8
3	3802	51	42	35	57	54	60	54	56	59	41	50.9	9
6	3137	43	62	47	45	68	75	54	34	46	56	53	13
6	4029	30	47	42	47	52	43	36	42	26	48	41.3	8
12	3041	38	30	26	50	25	40	47	46	25	28	35.5	10
12	8135	30	37	38	40	45	31	25	44	55	51	39.6	9

14. One-way ANOVA of tissue eosinophil counts between different days after challenge

Analysis of variance

Variate: EO

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
DAC	4	6459.34	1614.84	17.90	<.001
Residual	95	8570.05	90.21		
Total	99	15029.39			

Tables of means

Variate: EO

Grand mean 39.69

DAC	0.	1.	3.	6.	12.
	25.40	41.15	47.20	47.15	37.55

Least significant differences of means (5% level)

Table	DAC
rep.	20
d.f.	95
l.s.d.	5.963

15. Tissue globule leucocyte counts

DAC	Sheep	villus										mean	SD
		1	2	3	4	5	6	7	8	9	10		
0	B008	50	34	57	49	70	53	48	68	40	47	51.6	11
0	357	22	12	16	8	16	17	14	15	16	11	14.7	4
1	1208	22	11	12	13	8	16	19	16	46	37	20	12
1	3775	9	9	14	7	13	18	10	11	16	20	12.7	4
3	1077	1	0	8	4	0	2	3	5	8	13	4.4	4
3	3802	46	32	37	34	23	35	29	19	26	19	30	9
6	3137	5	7	13	17	7	10	5	13	4	0	8.1	5
6	4029	23	15	21	14	22	29	35	21	14	20	21.4	7
12	3041	25	36	20	27	56	33	9	20	21	20	26.7	13
12	8135	28	13	22	18	21	41	34	24	18	8	22.7	10

16. ANOVA of tissue GL counts between different days after challenge

Analysis of variance

Variate: GL

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
DAC	4	4723.5	1180.9	6.29	<.001
Residual	95	17844.2	187.8		
Total	99	22567.7			

Tables of means

Variate: GL

Grand mean 21.2

DAC	0	1	3	6	12
	33.1	16.3	17.2	14.8	24.7

Least significant differences of means (5% level)

Table	DAC
rep.	20
d.f.	95
l.s.d.	8.60

17. Tissue mucosal mast cell counts

DAC	Sheep	villus										mean	SD
		1	2	3	4	5	6	7	8	9	10		
0	B008	97	91	82	82	94	74	90	68	73	66	81.7	11
0	357	53	49	48	36	66	39	54	51	51	46	49.3	8
1	1208	65	50	46	59	52	43	41	50	45	54	50.5	7
1	3775	24	61	38	39	26	37	36	42	41	39	38.3	10
3	1077	41	65	50	46	40	54	33	38	37	41	44.5	10
3	3802	44	42	29	41	41	35	46	30	27	30	36.5	7
6	3137	43	63	56	71	59	71	51	50	52	47	56.3	10
6	4029	71	81	84	63	59	42	64	92	65	61	68.2	14
12	3041	52	42	62	47	59	61	61	56	58	44	54.2	7
12	8135	53	62	65	69	36	75	51	51	75	75	61.2	13

18. ANOVA of mucosal mast cell counts between different days after challenge

Analysis of variance

Variate: MMC

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
DAC	4	9767.8	2441.9	14.13	<.001
Residual	95	16416.7	172.8		
Total	99	26184.5			

Tables of means

Variate: MMC

Grand mean 54.1

DAC	0	1	3	6	12
	65.5	44.4	40.5	62.2	57.7

Least significant differences of means (5% level)

Table	DAC
rep.	20
d.f.	95
l.s.d.	8.25

19. PAS-stained GO counts

DAC	Sheep	villus										mean	SD
		1	2	3	4	5	6	7	8	9	10		
0	B008	18	11	14	12	10	12	21	26	23	16	16	6
0	357	40	16	24	26	31	22	20	14	30	30	25	8
1	1208	10	16	17	14	11	16	17	14	10	14	14	3
1	3775	25	18	41	22	22	25	28	21	30	34	27	7
3	1077	34	34	37	61	33	24	32	15	35	19	32	12
3	3802	43	35	54	30	21	29	24	32	15	35	32	11
6	3137	21	14	15	15	29	25	16	18	19	20	19	5
6	4029	34	16	41	48	22	21	23	20	24	17	27	11
12	3041	11	11	8	10	10	12	9	9	10	12	10	1
12	8135	13	7	15	8	6	5	13	7	12	16	10	4

20. ANOVA of PAS stained GO counts between different days after challenge

Analysis of variance

Variate: PAS stained GOs

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
DAC	4	4875.00	1218.75	17.21	<.001
Residual	95	6727.75	70.82		
Total	99	11602.75			

Tables of means

Variate: PAS stained GOs

Grand mean 21.25

DAC	0.	1.	3.	6.	12.
	20.80	20.25	32.10	22.90	10.20

Least significant differences of means (5% level)

Table	DAC
rep.	20
d.f.	95
l.s.d.	5.283

21. HID-stained GO counts

DAC	Sheep	villus										mean	s.d.
		1	2	3	4	5	6	7	8	9	10		
0	B008	11	38	19	19	10	21	21	11	22	24	20	8
0	357	19	27	23	42	19	26	24	12	23	23	24	8
1	1208	7	14	12	9	11	14	18	18	14	13	13	3
1	3775	14	17	27	24	26	19	24	47	54	23	28	13
3	1077	39	35	29	32	42	39	45	33	36	38	37	5
3	3802	17	31	32	59	65	48	51	51	48	40	44	14
6	3137	19	27	13	16	26	31	22	18	28	21	22	6
6	4029	11	35	20	24	17	42	42	14	30	17	25	11
12	3041	7	4	3	6	10	10	9	10	6	4	7	3
12	8135	13	10	8	9	10	5	15	8	16	17	11	4

22. ANOVA of HID stained GO counts between different days after challenge

Analysis of variance

Variate: HID_GOs

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
DAC	4	4846.00	1211.50	17.44	<.001
Residual	95	6600.75	69.48		
Total	99	11446.75			

Tables of means

Variate: HID_GOs

Grand mean 21.15

DAC	0.	1.	3.	6.	12.
	20.80	20.25	32.10	22.40	10.20

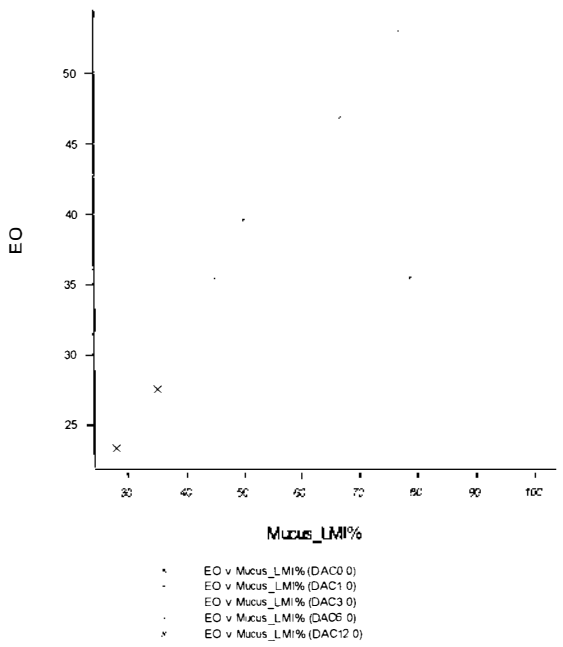
Least significant differences of means (5% level)

Table	DAC
rep.	20
d.f.	95
l.s.d.	5.233

23. Correlation of mucus LMI% with tissue cell counts or cell culture supernatant LMI% or serum anti-*T. colubriformis* IgE

DAC	Sheep	EO	GL	Tol.Blue	GO (PAS)	GO (HID)	CCS	SpIgE	Mucus LMI%
0	B008	27.5	51.6	81.7	16	20	48	0.451	35
0	357	23.3	14.7	49.3	25	24	63	0.261	28
1	1208	46.9	20	50.5	14	13	50	0.76	67
1	3775	35.4	12.7	38.3	27	28	73	0.205	45
3	1077	43.5	4.4	44.5	32	37	98	0.091	100
3	3802	50.9	30	36.5	32	44	99	1.524	98
6	3137	53	8.1	56.3	19	22	73	0.18	77
6	4029	41.3	21.4	68.2	27	25	56	0.255	89
12	3041	35.5	26.7	54.2	10	7	69	0.26	79
12	8135	39.6	22.7	61.2	10	11	83	0.632	50
r		0.77	-0.29	-0.31	-0.37	0.41	0.53	0.22	

Keys: *r* – correlation coefficient; EO – eosinophil; GL – globule leucocyte; GO (PAS) – PAS-satined goblet cell; GO (HID) – HID stained goblet cells; Tol. Blue – toluidine blue-stained mucosal mast cell; LMI% – larval migration inhibition %;



Correlations

GL	-0.3467				
Tol_blue	-0.3365	0.6053			
GO_HID	0.2775	-0.1973	-0.4911		
GO_PAS	0.1564	-0.3510	-0.4990	0.9445	
Mucus_LMI%	0.7655	-0.2878	-0.3098	0.4130	0.3736
	EO	GL	Tol_blue	GO_HID	GO_PAS

Number of observations: 10

Two-sided test of correlations different from zero
probabilities

EO					
GL	0.3264				
Tol_blue	0.3418	0.0637			
GO_HID	0.4377	0.5847	0.1495		
GO_PAS	0.6661	0.3200	0.1420	0.0000	
Mucus_LMI%	0.0099	0.4200	0.3837	0.2355	0.2876
	EO	GL	Tol_blue	GO_HID	GO_PAS

***Comments**: Data shows mucus LMI% had a relatively strong positive linear relationship with the tissue eosinophil numbers and its two-sided test correlation coefficient is significantly different from zero. All other correlations with mucus LMI were weak, or show evidence of non-linearity.*

24. The diameter (μm) of mucosal mast cells recovered in lamina propria cell isolates of day 0 and 1 after challenge infection

Sheep	0357	B008	3775	1208
	6.1	11.9	17.5	14.7
	11.7	10.9	13.6	14.6
	12	14.3	14.3	15.5
	11.6	15.1	16.41	14.3
	12	10.7	13.4	12.2
	7.2	11.9	13.7	15.36
	14.8	11.6	12.6	13.1
	15.4	11.9	13.5	14.6
	15.4	10.5	14.6	12.3
	10.8	10.7	13.5	11.7
	13.8	10.5	14.6	13.1
	13.1	9.8	13.7	13
	12.8	11	13.5	13.4
	10.5	13.4	13.1	13.8
	12.8	10.4	12	13.4
	13.5	9.6	14.6	14.6
	10.4	8.88	14	13.8
	11.6	9.6	15.06	13.8
	11.3	9.3	13.1	17.3
	11.14	11.6	14.5	11.6
mean	11.90	11.18	14.06	13.81
s.d.	2.34	1.62	1.25	1.39

Two-sample t-test

Variates: Day_0, Day_1.

Test for equality of sample variances

Test statistic F = 2.39 on 39 and 39 d.f.

Probability (under null hypothesis of equal variances) = 0.01

Note: strong evidence of unequal sample variances -
variances estimated separately for each group.

Summary

Sample	Size	Mean	Variance	Standard deviation	Standard error of mean
Day_0	40	11.54	4.087	2.022	0.3196

Day_1	40	13.94	1.712	1.308	0.2069

Difference of means: -2.398
Standard error of difference: 0.381

95% confidence interval for difference in means: (-3.158, -1.638)

Test of null hypothesis that mean of Day_0 is equal to mean of Day_1

Test statistic t = -6.30 on approximately 66.79 d.f.

Probability < 0.001

Permutation Test
Probability determined from 9999 random permutations < 0.001

Mann-Whitney U (Wilcoxon rank-sum) test
Variates: Day_0, Day_1.

Value of U: 251.0 (second sample has highest rank score).

Exact probability (adjusted for ties) < 0.001
(under null hypothesis that Day_0 is equal to Day_1).

Sample sizes: 40, 40.

***Comment**: Because of the unequal variances of each group, we check the results of the T-test with a permutation test and a non-parametric test. Both these are not affected by the fact that the two groups have unequally-sized variance. Both tests give the same conclusion as the T-test, namely that the means of the Day 0 and Day 1 groups are different (P<0.001).*

25. Two-sample t-test of tissue GL counts between low and high IgE sheep

Two-sample t-test

Variates: High IgE sheep, Low IgE sheep.

Test for equality of sample variances

Test statistic F = 2.24 on 49 and 49 d.f.

Probability (under null hypothesis of equal variances) = 0.01

Note: strong evidence of unequal sample variances -
variances estimated separately for each group.

Summary

Sample	Size	Mean	Variance	Standard deviation	Standard error of mean
High_IgE_gsheep	50	29.14	230.1	15.17	2.145
Low_IgE_sheep	50	13.30	102.6	10.13	1.432

Difference of means: 15.840
Standard error of difference: 2.579

95% confidence interval for difference in means: (10.71, 20.97)

Test of null hypothesis that mean of high IgE sheep is equal to mean of low IgE sheep

Test statistic t = 6.14 on approximately 85.45 d.f.

Probability < 0.001

Permutation Test

Probability determined from 9999 random permutations < 0.001

Mann-Whitney U (Wilcoxon rank-sum) test

Variates: Low_igE, high_IgE.

Value of U: 344.0 (second sample has highest rank score).

Exact probability (adjusted for ties) < 0.001
(under null hypothesis that Low_igE is equal to high_IgE).

Sample sizes: 50, 50.

***Comment**: Because of the unequal variances of each group, we check the results of the T-test with a permutation test and a non-parametric test. Both these are not affected by the fact that the two groups have unequally-sized variance. Both tests give the same conclusion as the T-test, namely that the means of the GL numbers in two groups are different (P<0.001).*

26. Two-sample t-test of tissue MMC counts of low and high IgE sheep

Two-sample t-test

Variates: Low_igE, high_IgE.

Test for equality of sample variances

Test statistic F = 2.77 on 49 and 626 d.f.

Probability (under null hypothesis of equal variances) < 0.001

Strong evidence of unequal sample variances - test with pooled variance estimate may be inappropriate; use separate estimates (VMETHOD=separate) instead.

Summary

Sample	Size	Mean	Variance	Standard deviation	Standard error of mean
Low_igE	627	18.07	84.7	9.20	0.368
high_IgE	50	29.94	234.8	15.32	2.167

Difference of means: -11.868
Standard error of difference: 2.198

95% confidence interval for difference in means: (-16.28, -7.457)

Test of null hypothesis that mean of Low_igE is equal to mean of high_IgE

Test statistic t = -5.40 on approximately 51.86 d.f.

Probability < 0.001

Permutation Test

Probability determined from 9999 random permutations < 0.001

Mann-Whitney U (Wilcoxon rank-sum) test

Variates: Low_igE, high_IgE.

Value of U: 344.0 (second sample has highest rank score).

Exact probability (adjusted for ties) < 0.001
(under null hypothesis that Low_igE is equal to high_IgE).

Sample sizes: 50, 50.

***Comment**: Because of the unequal variances of each group, we check the results of the T-test with a permutation test and a non-parametric test. Both these are not affected by the fact that the two groups have unequally-sized variance. Both tests give the same conclusion as the T-test, namely that the means of the GL numbers in two groups are different (P<0.001).*

27. Measurement of EPO release in the cell culture supernatants

Sheep	CCS+IL5 (ng/ml)					CCS -IL5	Buffer only
1205	10	5	2.5	1.25	0.625		
	0.809	0.532	0.242	0.164	0.084	0.075	0.048
	0.885	0.539	0.275	0.152	0.076	0.076	0.046
	0.853	0.549	0.301	0.129	0.075	0.074	0.047
mean	0.831	0.541	0.273	0.148	0.078	0.075	0.047
s.d.	0.031	0.012	0.030	0.018	0.005	0.001	0.001
8151	0.773	0.413	0.337	0.289	0.196	0.157	0.059
	0.756	0.416	0.348	0.299	0.196	0.177	0.053
	0.766	0.416	0.356	0.311	0.212	0.168	0.056
mean	0.765	0.415	0.347	0.300	0.201	0.167	0.056
SD	0.009	0.002	0.010	0.011	0.009	0.010	0.003
8214	0.190	0.178	0.178	0.089	0.078	0.075	0.060
	0.199	0.180	0.171	0.088	0.084	0.076	0.060
	0.210	0.188	0.173	0.087	0.088	0.074	0.065
mean	0.200	0.182	0.174	0.088	0.083	0.075	0.062
SD	0.010	0.005	0.004	0.001	0.005	0.001	0.003
6217	0.159	0.100	0.100	0.073	0.071	0.077	0.060
	0.165	0.101	0.098	0.071	0.076	0.074	0.060
	0.158	0.096	0.096	0.076	0.075	0.074	0.065
mean	0.161	0.099	0.098	0.073	0.074	0.075	0.062
SD	0.004	0.003	0.002	0.003	0.003	0.001	0.003

28. Histamine release in the cell culture medium

Sheep	media only	XB6 only	Ag only	Cell only	Cell+XB6	Cell+Ag
4133	237	238	238	404	1484	638
	220	237	235	398	1486	710
	223	232	231	412	1469	739
mean	226.67	235.67	234.67	404.67	1479.67	695.67
s.d.	9.07	3.21	3.51	7.02	9.29	52.00
8151	281	288	336	394	866	600
	306	308	313	368	879	624
	283	275	278	369	860	573
	290	290	309	377	868	599
mean	13.89	16.62	29.21	14.73	9.71	25.51
SD	8.02	9.60	16.86	8.51	5.61	14.73

29. LMI activities of cell factors

Sheep	Cell type	larval count			LMI%	SD
		Well 1	well2	well3		
8151	EO	40	16	17	84	9
1205	EO	71	47	65	60	8
8421	EO	128	121	136	17	5
6271	EO	122	110	125	23	5
8151	MMC	116	110	95	32	7
4133	MMC	143	122	139	14	7
6034	MMD	130	120	131	18	4
6070	MMD	132	117	104	27	9
9146	GL	136	142	133	11	3
375	GL	142	146	138	8	3
HBSS9 only		148	152	163	0	5
Levamisole		1	0	2	99	1

30. Glycoprotein level of enriched epithelial/goblet cells after exposure to carbachol (100 µM)

Component	Sheep	OD at 570nm	µg/ml	mean(µg/ml)
eEP/GOs + carbachol (100 µM)	7713	0.043	6.49	6.37
		0.045	6.85	
		0.039	5.76	
	5046	0.032	4.49	3.76
		0.029	3.95	
		0.023	2.85	
Negative controls				
eEP/GOs only	7713	0.013	1.04	0.93
		0.012	0.89	
		0.012	0.85	
	5046	0.012	0.76	0.79
		0.012	0.78	
		0.012	0.82	
		0.011	0.65	
HBSS9		0.002	-0.96	-0.90
		0.003	-0.78	
		0.002	-0.96	
HBSS9 + Carbachol		0.001	-1.15	-1.08

		0.001	-1.15	
		0.002	-0.96	

eEP/GOs – einriched epithelial/goblet cells.

31. Glycoprotein levels in the CCS of enriched epithelial/goblet cells after exposure to different cell factors

31.1. Mucosal mast cell factors

Source of eEP/GOs (sheep)	Source of cell factors	OD at 490nm	Glycoprotein level (µg/ml)			
			Total value	Total - Control	Mean	SD
7713	Sheep 8151	0.094	59.18	5.95	6.19	1.87
		0.092	57.69	4.46		
		0.097	61.41	8.18		
	Sheep 4133	0.086	53.23	0.00	0.45	0.77
		0.086	53.23	0.00		
		0.088	54.57	1.34		
5046	Sheep 8151	0.024	2.7	0.45	1.13	0.68
		0.025	3.38	1.13		
		0.026	4.05	1.8		
	Sheep 4133	0.028	5.4	3.15	4.06	1.03
		0.029	6.08	3.83		
		0.031	7.43	5.18		

Negative controls (eEP/GOs only): sheep 7713 and 5046 were 53.23 and 2.25 µg/ml.
eEP/GOs – enriched epithelial/goblet cells.

31.2. Globule leucocyte factors

Source of eEP/GOs (sheep)	Source of cell factors	OD at 490nm	Glycoprotein level (µg/ml)			
			Total value	Total - Control	Mean	SD
7713	9164	0.301	57.77	4.54	4.02	1.52
		0.291	55.54	2.31		
		0.304	58.43	5.20		
	0375	0.287	54.50	1.27	0.42	1.66
		0.288	54.72	1.49		
		0.275	51.75	-1.48		

5046	9146	0.108	12.84	10.59	9.01	1.70
		0.102	11.49	9.24		
		0.094	9.46	7.21		
	0375	0.061	1.45	-0.80	0.47	1.51
		0.065	2.33	0.08		
		0.073	4.39	2.14		

Negative controls (eEP/GOs only): sheep 7713 and 5046 were 53.23, and 2.25 μg/ml.
eEP/GOs – enriched epithelial/goblet cells.

31.3. Eosinophil factors

Source of eEP/GOs (sheep)	Source of cell factors	OD at 490nm	Glycoprotein level (μg/ml)			
			Total value	Total - Control	Mean	SD
7713	Sheep8151	0.304	58.43	5.20	4.21	1.14
		0.294	56.20	2.97		
		0.301	57.69	4.46		
	Sheep1205	0.298	56.95	3.72	2.23	1.49
		0.284	53.98	0.74		
		0.291	55.46	2.23		
5046	Sheep8151	0.074	4.73	2.48	2.03	0.78
		0.074	4.73	2.48		
		0.069	3.38	1.13		
	Sheep1205	0.094	9.46	7.21	9.01	2.06
		0.099	10.81	8.56		
		0.110	13.51	11.26		

Negative controls (eEP/GOs only): sheep 7713 and 5046 were 53.23, and 2.25 μg/ml.
eEP/GOs – enriched epithelial/goblet cells.

32. Glycoprotein content of CCS of enriched epithelia/goblet cells after co-cultured with parasite ES antigens and leucocyte vasoactive substances

Culture conditions	OD at 490nm	Glycoprotein (ug/ml)		
			Mean	**SD**
P2 + ES antigens	0.123	17.09	18.91	3.70
	0.115	16.54		
	0.113	16.18		
eEP/GOs + ES antigens	0.105	14.20	12.55	1.81
	0.090	10.61		
	0.100	12.85		
eEP/GOs + Leukotriene B4	0.073	6.39	6.18	2.28
	0.062	3.80		
	0.081	8.34		

eEP/GOs + Leukotriene C4	0.072	6.15	6.64	1.03
	0.079	7.83		
	0.071	5.95		
eEP/GOs + Leukotriene D4	0.069	5.41	5.50	2.32
	0.079	7.85		
	0.060	3.22		
eEP/GOs + Leukotriene E4	0.078	7.61	6.18	1.31
	0.071	5.90		
	0.067	5.02		
eEP/GOs + Prostaglandin D2	0.081	8.41	6.41	2.80
	0.078	7.61		
	0.060	3.22		
eEP/GOs + Prostaglandin E2	0.072	6.24	5.27	2.49
	0.076	7.12		
	0.057	2.44		
eEP/GOs + Serotonin	0.062	3.71	6.18	2.14
	0.077	7.32		
	0.078	7.51		
eEP/GOs + Dopamine	0.080	8.02	7.09	1.28
	0.078	7.61		
	0.070	5.63		
eEP/GOs + Histamine	0.079	7.83	7.09	1.52
	0.069	5.34		
	0.080	8.10		
eEP/GOs + Pooled mediators	0.088	10.00	8.00	1.84
	0.073	6.39		
	0.078	7.61		
P2 only	0.052	1.07	2.18	1.07
	0.055	3.22		
	0.060	2.24		
eEP/GOs only	0.065	4.44	4.00	1.89
	0.070	5.63		
	0.055	1.93		
Media + ES antigens	0.051	1.02	1.33	0.80
	0.056	2.24		
	0.050	0.73		
Media + pooled mediators	0.052	1.27	1.51	0.65
	0.051	1.02		
	0.056	2.24		
Media only	0.053	1.51	1.25	0.91
	0.048	0.24		
	0.055	2.00		

eEP/GOs – enriched epithelial/goblet cells.

33. LMI activity of CCS of enriched epithelial/goblet cells after co-cultured with parasite ES antigens and leucocyte vasoactive substances

No.	Component	larval counts			LMI%			Mean	SD
		well	well	well					
1	P2 + ES antigens	44	41	63	71	73	59	68	8
2	eEP/GOs + ES antigens	134	122	150	13	21	3	12	9
3	eEP/GOs + Leukotriene B4	110	120	129	29	22	16	22	6
4	eEP/GOs + Leukotriene C4	128	136	115	17	12	25	18	7
5	eEP/GOs + Leukotriene D4	117	144	135	24	6	12	14	9
6	eEP/GOs + Leukotriene E4	145	153	129	6	1	16	8	8
7	eEP/GOs + Prostaglandin D2	121	139	149	21	10	3	11	9
8	eEP/GOs + Prostaglandin E2	129	136	139	16	12	10	13	3
9	eEP/GOs + Serotonin	151	140	131	2	9	15	9	7
10	eEP/GOs + Dopamine	137	131	123	11	15	20	15	5
11	eEP/GOs + Histamine	142	132	134	8	14	13	12	3
12	eEP/GOs + pooled mediators	149	112	100	23	27	35	28	6
13	P2 only	145	128	132	6	17	14	12	6
14	eEP/GOs only	149	140	132	3	9	14	9	6
15	Media + ES antigens	164	159	147	-6	-3	5	-2	6
16	Media + pooled mediators	144	160	149	6	-4	3	2	5
17	Media only	148	158	155	154				

eEP/GOs - enriched epithelial/goblet cells.

34. LMI activity of selected leucocytes vasoactive amines

Component	larval counts			LMI%			Mean	SD
	well1	well2	well3	well1	well2	well3		
Leukotriene B4	145	160	149	15	6	13	12	5
Leukotriene C4	138	145	158	19	15	8	14	6
Leukotriene D4	117	144	135	32	16	21	23	8
Leukotriene E4	138	131	120	19	23	30	24	5
Prostaglandin D2	153	166	164	11	3	4	6	4
Prostaglandin E2	116	128	142	32	25	17	25	8
Serotonin	127	146	138	26	15	19	20	6
Dopamine	149	140	152	13	18	11	14	4
Histamine	153	167	141	11	2	18	10	8
Levamisole	7	6	4	96	96	98	97	1
HBSS9	161	149	168	159				

35. Glycoprotein level of enriched epithelial/goblet cells after exposure to carbachol (100 µM)

Component	Sheep	OD at 570nm	ug/ml	mean(µg/ml)
eEP/GOs + carbachol (100 µM)	6070	0.025	3.22	3.04
		0.027	3.58	
		0.02	2.31	

Negative controls		0.012	0.82	0.65
eEP/GOs only		0.010	0.49	
HBSS9		0.002	-0.96	-0.96
		0.002	-0.96	
		0.002	-0.96	
HBSS9 + Carbachol (100 μM)		0.001	-1.15	-1.15
		0.001	-1.15	
		0.001	-1.15	

eEP/GOs – enriched epithelial/goblet cells.

35.1. Glycoprotein level of enriched epithelial/goblet cells after exposure to eosinophil cell factor and ES antigens

Component	sheep	OD at 570nm	ug/ml	mean(μg/ml)
eEP/GOs + EOF	6070	0.161	5.13	5.37
		0.161	5.13	
		0.164	5.86	
eEP/GO + ES antigens		0.155	3.66	3.83
		0.156	3.91	
		0.156	3.91	
Controls				
eEP/GO only (cell control)		0.144	0.78	0.91
		0.143	0.98	
		0.143	0.98	
HBSS9 only (buffer control)		0.140	0	0
		0.139	0	
		0.140	0	

eEP/GOs – enriched epithelial/goblet cells; EOF eosinophil cell factor; HBSS9 – Hank's balanced salt solution 9.

36. The additive effect of mucus glycoprotein on LMI activity of enriched epithelial/goblet cells

Component	Dilution	larval count			LMI%			Mean	S.D.
		well1	well2	well3	well1	well2	well3		
EOF + eEP/GOs	20%	40	45	63	69	65	51	**62**	9
	10%	60	48	40	53	63	69	**62**	8
	5%	49	58	66	62	55	49	**55**	7
	2.5%	92	69	80	29	47	38	**38**	9
	1.25%	120	113	94	7	12	27	**16**	10

EOF + Naïve gut mucus	20%	56	63	41	57	51	68	**59**	9
	10%	40	61	50	69	53	61	**61**	8
	5%	58	75	80	55	42	38	**45**	9
	2.5%	107	79	94	17	39	27	**28**	11
	1.25%	109	118	120	16	9	7	**10**	5
EOF + HBSS9	20%	90	76	86	30	41	33	**35**	6
	10%	95	109	114	26	16	12	**18**	7
	5%	127	123	108	2	5	16	**7**	8
	2.5%	116	130	127	10	-1	2	**4**	6
	1.25%	123	126	133	5	2	-3	**1**	4
Negative controls									
eEP/GOs only		123	110	118	5	15	9	9	5
Naïve mucus		121	113	111	6	12	14	11	4
HBSS9 only		128	133	127					

Key: EOF – eosinophil cell factor; eEP/GOs –enriched epithelial/goblet cells.

37. Comparison of two larval paralysis assays and larval migration inhibition assay

LP assay 1	**Motile larvae**						**LP%**					
Levamisole (ug/ml)	20	10	5	2.5	1.25	0.625	20	10	5	2.5	1.25	0.625
well 1	0	0	2	6	14	19	100	100	89	68	26	0
well2	0	0	4	5	14	17	100	100	79	74	26	11
well3	0	0	4	4	12	20	100	100	79	79	37	-5
mean	0	0	3	5	13	19	100	100	82	74	30	2
Buffer control	20	17	20			STD	0.0	0.0	6.1	5.3	6.1	8.0
	18	20	17									
LP assay 2												
well 1	2	2	6	10	23	28	95	95	84	73	38	24
well2	2	3	6	10	23	23	95	92	84	73	38	38
well3	2	3	5	8	26	26	95	92	86	78	30	30
mean	2	3	6	9	24	26	95	93	85	75	35	31
Buffer control	37	37	36			STD	0.0	1.6	1.6	3.1	4.7	6.8
	37	36	40									
LMI assay												
well 1	13	22	58	114	179	189	95	91	76	52	24	20
well2	14	30	60	100	166	187	94	87	75	58	30	21
well3	12	22	62	107	180	196	95	91	74	55	24	17
mean	13	25	60	107	175	191	95	90	75	55	26	20
Buffer control	227	238	203			STD*	0.4	1.9	0.8	3.0	3.3	2.0
	261	277	214									

* = standard deviation; LP assay 1 = 96-well micro-plate assay; LP assay 2 = Terasaki plate assay; LMI assay = LMI assay of Rabel *et al.* (1994) with modifications.

38. Correlation of larval paralysis assays and larval migration inhibition assay (by Genstat)

Correlation matrix

LMI%	1.000	
LP * %	0.979	1.000
	LMI%	LP* %

* = LP assay 1 or 2

39. LP activity of CCS against different nematode species

	Larval paralysis (%)				Control
CCS concentration (%)	80%	40%	20%	10%	
	Number of motile larvae				
Nematode parasite species					
T. colubriformis	7, 9	12, 13	25, 29	38, 30	35, 40, 42
N. spathiger	6, 5	14, 11	25, 29	39, 42	39, 34, 45
H. contortus	1, 5	2, 7	17, 15	18, 15	30, 37, 42
Tel. circumcincta	10, 11	19, 21	29, 32	40, 41	40, 40, 37
O. ostertagi	10, 7	16, 22	26, 31	41, 43	40, 32, 40
P. trichosuri	11, 9	22, 16	36, 28	40, 32	37, 40, 42
Levamisole (5ug/ml)	0, 1, 0				
	Larval paralysis (%)				
T. colubriformis	76	69	49	13	
N. spathiger	87	67	31	0	
H. contortus	92	88	56	56	
Tel. circumcincta	74	49	22	0	
O. ostertagi	78	41	23	0	
P. trichosuri	75	52	19	9	
Levamisole (5ug/ml)	99				

40. Mean percentage of larvae recovered from paralysis

	Sheep	Prewashed			After washing			Percentage of recovery			Mean
2hrs	6217	32	34	37	14	13	19	44	38	51	44
	6034	36	32	30	21	18	18	58	56	60	58
	8151	31	32	34	9	11	13	29	34	38	34
6hrs	6217	35	33	35	18	16	21	51	48	60	53
	6034	32	30	29	13	11	13	41	37	45	41
	8151	28	28	34	10	8	11	36	29	32	32

41. Solubility nature of LPF

Components	motile larvae			LP%			mean	S.D.
Untreated CCS	5	6	9	85	82	73	80	6
Milli-Q water (control)	10	8	6	70	76	82	76	6
Ether- S	34	35	33	-3	-6	0	-3	3
Ether-F	9	16	10	73	52	70	65	11
Acetonitrile-S	33	31	31	0	6	6	4	3
Acetonitrile-F	5	8	11	85	76	67	76	9
Ethanol-S	25	31	22	24	6	33	21	14
Ethanol-F	12	16	13	64	52	61	59	6
Methanol-S	21	24	19	36	27	42	35	8
Methanol-F	8	11	14	76	67	58	67	9
Levamisole (5ug/ml) (control)	1	1	0	97	97	100	98	2
Buffer only	33							

42. Stability nature of LPF

	moving larvae							
Sample concentration (%)	80		40		20		10	
Treatment								
'pH12,	40	37	36	45	42	42	38	40
'pH1, 10min, RT	14	16	26	24	31	34	38	40
Boiling, 10min	23	18	19	22	30	27	43	40
Boiling with acid	45	38	35	40	42	35	39	36
Freeze/Thaw x 5	28	30	40	40	36	41	38	36
Freeze/Thaw x10	34	45	39	42	40	34	41	39
CCS + Proteinase	16	17	18	18	35	30	40	44
Arylsulphatase	13	17	19	22	31	28	42	42
Controls								
Proteinase-K	40	36	36	36	35	37	38	42
Untreated CCS	13	14	15	19	25	30	38	35
Levamisole	0	0						
Buffer								

43. Stability nature of LPF

	mean moving larvae				LP %			
Sample concentration(%)	80	40	20	10	80	40	20	10
Treatment								
'pH12,	39	41	42	39	8	4	0	7
'pH1, 10min, RT	15	25	33	39	64	40	23	7
Boiling, 10min	21	21	29	42	51	51	32	1
Boiling with acid	42	38	39	38	1	11	8	11
Freeze/Thaw x 5	29	40	39	37	31	5	8	12
Freeze/Thaw x10	40	41	37	40	6	4	12	5
CCS + Proteinase	17	18	33	42	61	57	23	0

Arysulphatase (300U/ml)	15	21	31	42	64	51	30	0
Controls								
Proteinase-K	38	36	36	40	10	14	14	5
Untreated CCS	14	17	28	37	68	60	35	13
Levamisole	0				100			
Buffer	42							

44. LP activity of CCS fractions after ultra-membrane centrifugal filtration

Components	Larval counts			LP%			Mean LP%	STD
Crude CCS	11	5	6	83	92	91	89	5
above 0.2um	53	68	56	18	-5	14	9	12
above 100KD	52	53	63	20	18	3	14	9
above 10KD	59	64	50	9	2	23	11	11
above 3KD	67	49	55	-3	25	15	12	14
above 1KD	57	61	51	12	6	22	13	8
under 1KD	17	24	20	74	63	69	69	5
Buffer	64	72	59					
Levamisole	2	1	0	97	98	100	98	2

45. Larval paralysis activity of fractions from a single Bio gel P-2 filtration

Fractions	Number of motile larvae			mean	LP activity (%)
1	36	42	31	36	0
2	22	31	20	24	30
3	34	30	22	29	18
4	42	29	24	32	9
5	21	38	35	31	10
6	27	33	30	30	14
7	30	24	29	28	21
8	0	0	0	0	100
9	0	0	0	0	100
10	0	0	0	0	100
11	22	19	24	22	38
12	38	27	37	34	3
Control	36	38	31	35	
levamisole	0	0	0	0	100

46. Larval paralysis activity of fractions from a double Bio gel P-2 filtration

Gel fractions	Motile worms	Mean	LP%

1	23	24	20	22	24
2	26	30	22	26	10
3	32	20	33	22	24
4	20	14	15	16	45
5	27	31	25	28	3
6	21	24	29	25	14
7	0	0	0	0	100
Buffer	34	23	31	29	
levamisole	0	0	0	0	100

47. Larval paralysis activity of fractions after C18 SPE

	Motile larvae									LP%		
Sample dilution (%)	80			40			20					
Eluent												
Washing- Milli-Q water	5	2	2	7	11	14	15	18	21	92	73	54
Eluent-60% methanol-water	35	30	32	39	30	33	40	39	36	17	13	1.7
Eluent-100% methanol	36	33	30	33	35	40	39	33	36	15	8	8
Levamisole (5ug/ml)	1	2	1							97		
Pre SPE CCS under 3KD	7	10	11	16	20	23	32	36	34	76	50	13
	36	42	44	41	35	38						

48. Larval paralysis activity of fractions after preparative HPLC C18 Platinum ESP column

	Motile larvae			LP%			mean LP%	S.D.
F1	11	9	8	62	69	72	68	5
F2	28	33	31	3	-14	-7	-6	9
F3	26	29	32	10	0	-10	0	10
F4	30	30	30	-3	-3	-3	-3	0
F5	22	29	24	24	0	17	14	12
Pre HPLC CCS	4	2	5	86	93	83	87	5
Levamisole	1	0	0	97	100	100	99	2
Buffer	30	29	27	29				

49. Larval paralysis activity of the fractions Pk1

	Motile larvae						LP%	S.D.
F1	14	18	15	65	55	63	61	5
F2	32	24	27	20	40	33	31	10
Levamisole	1	2	0		95	100	98	4
Buffer	44	36	40	40				

50. Larval paralysis activity of fractions after aminopropyl SPE

	Motile larvae									LP%		
Sample concentration (5)	80			40			20					
Eluent												
Acetonitrile 100%	19	20	20	17	18	21	16	18	21	6	11	13
Acetonitrile 70%	3	3	2	7	11	11	15	18	21	87	54	14
Acetonitrile 50%	12	15	11	15	17	14	19	17	21	40	27	10
Acetonitrile 20%	21	17	18	22	16	19	15	22	19	11	10	11
Milli-Q water	17	22	19	21	21	17	22	17	18	8	6	10
Levamisole (5ug/ml)	1	0	1							97		
Buffer only												
	21	20	22	20	20	22	21					

51. Larval paralysis activity of fractions after aminopropyl SPE

	Motile larvae			LP%			mean LP%	S.D.
F1	24	20	20	-4	13	13	7	10
F2	25	20	24	-9	13	-4	0	12
F3	21	19	23	9	17	0	9	9
F4	20	22	24	13	4	-4	4	9
F5	15	16	14	35	30	39	35	4
F6	10	7	5	57	70	78	68	11
F7	22	25	21	4	-9	9	1	9
Levamisole (ug/ml)	1	1	0	96	96	100	97	3
Buffer	24	25	21	23				

52. Larval paralysis activity of fractions after aminopropyl SPE

	Motile larvae			LP%			mean LP%	S.D.
F1	21	18	16	9	22	30	20	11
F2	9	6	10	61	74	57	64	9
F3	21	19	23	9	17	0	9	9
Levamisole(5ug/ml)	0	1	0	100	96	100	99	3
Buffer	20	19	22	20				

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