Immune responses of sheep to *Psoroptes ovis* – the sheep scab mite.

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Studies were conducted to investigate the immunopathogenesis of cutaneous lesions in sheep scab. The initial studies examined the nature and temporal development of the lesional infiltrate and growth during experimental primary and challenge infestations of sheep with Psoroptes ovis. These demonstrated that granulocyte infiltration was dominated by eosinophils, accompanied by mast cell hyperplasia and degranulation and dermal edema, features characteristic of IgE-mediated Type I hypersensitivity reactions. In primary infestations eosinophil counts and epidermal and dermal pathology were maximal 9 weeks after infestation while in challenge infestations eosinophil counts fell rapidly 3 weeks after challenge, an observation consistent with failure of the mites to become established. Importantly, within 24 hours of primary infestation P. ovis had provoked an intense eosinophil infiltrate and marked degenerative and proliferative epidermal pathology. Immunohistochemistry of primary infestations revealed a massive lesional infiltration of CD4+ and CD45RA+ cells that was accompanied by smaller numbers of γδ T cells and dendritic cells. In primary infestations, lesional growth was exponential during the first 7 weeks and then plateaued. In challenge infestations lesional growth was significantly reduced (p< 0.001), an observation indicative of the development of a substantial protective immunity.

Alterations in haematology and serology were investigated in parallel with the initial studies of lesional histopathology. In primary infestations eosinophilia developed in all sheep and basophilia in 6/14 sheep but after challenge infestation basophilia was not detected and eosinophilia was recorded in only one animal. Analysis of isotype-specific antibodies indicated that P. ovis elicited antigen-specific IgG, IgM and IgE but apparently not IgA responses and that challenge infestations provoked a significant (p<0.01-0.03) amnestic IgE but not IgG or IgM antibody response. Detection of P. ovis antigen-specific IgE provided further evidence of the involvement of immediate hypersensitivity in the immunopathogenesis of cutaneous lesions but a significant rise in antigen-specific IgE level was not detected until late (7 weeks) in the course of primary infestation. SDS-PAGE/Western blots demonstrated that P. ovis antigens/allergens >100kD were labelled most consistently by IgG/IgE antibodies and that the number of antigens/allergens recognised and the intensity of labelling increased with time after infestation.

Intradermal skin tests demonstrated the occurrence of immediate hypersensitivity, late phase (LPR) and delayed-type hypersensitivity (DTH) reactions to P. ovis whole-mite extract. Histopathology of the LPR and DTH indicated that both responses, but particularly the DTH, were dominated by eosinophils. Prausnitz-Kustner tests demonstrated the IgE dependence of the LPR and that the DTH was not transferred by a serum factor. Examination of the temporal development of these hypersensitivity responses indicated that they were not manifest until late in the course of infestation. This research has demonstrated that P. ovis provokes an intense eosinophil dominated innate response, an IgE-mediated Type I reaction and eosinophil dominated late phase and non-classical DTH reactions and that in addition, it generates a significant protective immune response.
DECLARATION

I hereby declare that:

(i) This thesis has been composed by myself.

(ii) The work has been carried out by myself or, where jointly, this fact has been acknowledged.

(iii) This thesis has not been submitted in any previous application.

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Lastly, I would like to thank my wife Sue for her patience and support and also record my gratitude to God for all that immunology, pathology and statistics have taught me about himself.

'These are but the outer fringe of his works, how faint is the whisper we hear of him!
Who then can understand the thunder of his power?
'From him and through him and to him are all things. To him be glory for ever'
LIST OF ABBREVIATIONS

AF Ascites fluid
APC Antigen presenting cell
Ca Calcium
CBH Cutaneous basophil hypersensitivity
CD Cluster designation
cDNA Copy deoxyribonucleic acid
C3 Complement fragment 3
C3a Complement fragment 3a
C5a Complement fragment 5a
DAB 3,3'-diaminobenzidine
Der p Dermatophagoides pteronyssinus allergen
DTH Delayed type hypersensitivity
ECP Eosinophil cationic protein
EDN Eosinophil-derived neurotoxin
ELISA Enzyme-linked immunosorbent assay
EPO Eosinophil peroxidase
ER-DTH Eosinophil-rich delayed hypersensitivity reaction
γδ T cell gamma delta T cell
GM-CSF Granulocyte-macrophage colony-stimulating factor
FceR1 High affinity immunoglobulin E receptor
FceRII Low affinity immunoglobulin E receptor
H & E haematoxylin and eosin
HCl Hydrochloric acid
H_{2}O_{2} Hydrogen peroxide
H_{2}SO_{4} Sulphuric acid
HTPS Heat-treated positive serum
IDST Intradermal skin test
IFNγ Interferon γ
Ig Immunoglobulin
IgA Immunoglobulin A
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>IH</td>
<td>Immediate hypersensitivity</td>
</tr>
<tr>
<td>IL-</td>
<td>Interleukin</td>
</tr>
<tr>
<td>kD</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>LPR</td>
<td>Late phase response</td>
</tr>
<tr>
<td>LTC4</td>
<td>Leukotriene C4</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>Mab</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MBP</td>
<td>Major basic protein</td>
</tr>
<tr>
<td>MCP-</td>
<td>Monocyte chemotactic protein</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>N</td>
<td>Normal</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NC</td>
<td>Nitrocellulose</td>
</tr>
<tr>
<td>NS</td>
<td>Negative (control) serum</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OP</td>
<td>Organophosphate</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
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<td>Prausnitz-Kustner test</td>
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<tr>
<td>PS</td>
<td>Positive serum</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation, normal T cells expressed and secreted</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerisation chain reaction</td>
</tr>
<tr>
<td>SALT</td>
<td>Skin associated lymphoid tissue</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>Stromal cell-derived factor 1 alpha</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide agarose gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>sIg</td>
<td>Secretory immunoglobulin</td>
</tr>
<tr>
<td>SIS</td>
<td>Skin immune system</td>
</tr>
<tr>
<td>SN</td>
<td>Supernatant (from tissue culture)</td>
</tr>
<tr>
<td>SP</td>
<td>Synthetic pyrethroid</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>Th cell</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-tetramethylbenzidine</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>T20</td>
<td>Tween 20 (Polyoxyethylenesorbitan monolaureate)</td>
</tr>
<tr>
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<tr>
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<td>Very late antigen-4</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
<tr>
<td>WC-1</td>
<td>Workshop cluster-1</td>
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<tr>
<td>WME</td>
<td>Whole mite extract</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
</tr>
<tr>
<td>Zn</td>
<td>Zinc</td>
</tr>
<tr>
<td>ZSF</td>
<td>zinc salts fixative</td>
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1. GENERAL INTRODUCTION

Ectoparasites are a major cause of economic loss globally and may serve as vectors of several pathogens to animals and man (Wikel 1984, Fox 1993, Wharton 1993, McLeod 1995, Melancon 1998). The skin constitutes the interface between the host immune system and ectoparasites and plays an integral role in the development and expression of the complex interactions between them. These interactions determine the clinical severity of disease and host resistance to subsequent infections. Analysis of these interactions is, therefore, essential in understanding the immunopathogenesis of cutaneous responses to Psoroptes ovis, the subject of this thesis, and potentially will facilitate manipulation of the immune response to promote host protection.

This introduction has four sections. The first will present a brief overview of the skin immune system and the second a more detailed review of host immune responses to ectoparasites. In the third section, host responses to Psoroptes spp infestation will be examined in the context of the epidemiology, clinical manifestations and measures currently employed to control sheep scab. The final section will provide the rationale for the studies described in this thesis.

1.1 THE SKIN IMMUNE SYSTEM

1.1.1 The components of the skin immune system

The skin is continuously exposed to a diverse array of pathogens such as parasites, fungi, bacteria and viruses and their products as well as numerous biologically active substances. As a defence against these it provides a physico-chemical barrier and a specialised, integrated immunological surveillance and response system. Two models of this immunological defence system have been developed. The first introduced the concept of the skin associated lymphoid tissue
(SALT) (Streilein 1978, 1983). This focussed on the role of epidermal cells (Langerhans’ cells, epidermotropic T lymphocytes and keratinocytes), vascular endothelial cells and skin draining lymph nodes in the cutaneous immune response. Later, an alternative model, the skin immune system (SIS), was proposed and developed (Bos and Kapsenberg 1986, 1993, Bos 1997). This system, in contrast to SALT, excluded the skin draining lymph nodes but encompassed all cells (Table 1.1) and humoral factors (Table 1.2) with recognised innate and adaptive immune functions that are normally present in the epidermis and dermis (Bos and Kapsenberg 1986, 1993, Salmon et al. 1994, Luger et al. 1996, Williams and Kupper 1996, Misery 1997).

Table 1.1 The Skin Immune System - Cells

<table>
<thead>
<tr>
<th>CELLS</th>
<th>Location</th>
<th>Mainly innate</th>
<th>Mainly adaptive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermis</td>
<td>Keratinocytes</td>
<td>Langerhans’ cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Langerhans’ cells</td>
<td>Epidermal T lymphocytes</td>
<td></td>
</tr>
<tr>
<td>Dermis</td>
<td>Mast cells</td>
<td>Dermal T lymphocytes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Monocytes/macrophages</td>
<td>Tissue dendritic cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Basophils</td>
<td>Vascular endothelial cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eosinophils</td>
<td>Lymphatic endothelial cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neutrophils</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2 The Skin Immune System – Humoral factors

<table>
<thead>
<tr>
<th>HUMORAL FACTORS</th>
<th>Mainly innate</th>
<th>Mainly adaptive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement</td>
<td>Pro-inflammatory cytokines e.g. IL-1β, IL-8, TNFα</td>
<td>Immunoglobulins</td>
</tr>
<tr>
<td>Eicosanoids</td>
<td>Antimicrobial peptides</td>
<td>Immunomodulatory cytokines e.g. IL-2, IL-4, IL-10, IL-12, IL-13, IFNγ</td>
</tr>
<tr>
<td>Neuropeptides</td>
<td>Fibrinolysins</td>
<td></td>
</tr>
</tbody>
</table>

Although omitted from the SIS on anatomical grounds, functionally the skin draining lymph nodes constitute an integral part of the skin-specific immune surveillance and response system and are included in this review.
1.1.2 Anatomical distribution of cells of the skin immune system

Anatomically the continuously renewed layers of protein-rich, cornified keratinocytes, embedded in a lipid-rich matrix, constitute the outermost cells of the epidermis. Scattered among the suprabasilar keratinocytes are epidermotropic T lymphocytes, melanocytes and Langerhans’ cells whose extensive, interlacing dendritic processes form an intricate immunological surveillance system (Salmon et al. 1994). Below the epidermis, the connective tissue of the dermis is traversed by a network of lymphatics and blood vessels that connect the SIS to the rest of the immune system. Unidirectional, afferent lymphatics convey lymphocytes, macrophages and dendritic antigen presenting cells (APCs) and cytokines to the skin draining lymph nodes. Blood vessels are concentrated in vascular plexi with the most superficial lying immediately below the epidermis in close proximity to the keratinocytes. In these plexi, endothelial cells of post-capillary venules express adhesion molecules that bind to counter-ligands on circulating cells and facilitate the normal steady passage of granulocytes, monocytes and lymphocytes into the dermis. In disease states, these venules are a major site of immunoinflammatory reactivity due to upregulated adhesion molecule expression (Bos and Kapsenberg 1986).

Many cells of the SIS such as mast cells, monocytes/macrophages, dermal dendritic cells and up to 90% of T cells are concentrated around vessels of the superficial plexus, particularly the post capillary venules, and in the periadnexal areas (Bos et al. 1987, Sontheimer 1989). The remaining cells and the infrequent, migrant neutrophils, eosinophils and basophils are dispersed throughout the connective tissue matrix.
1.1.3 Response of the skin immune system to pathogen challenge

Pathogen challenge, associated for example with ectoparasite infestations, will upregulate activity of the SIS simultaneously initiating antigen-independent innate and an antigen-dependent adaptive immune responses (Parrish and Breathnach 1996).

1.1.3.1 The innate immune response

Cell damage and stimulation caused by physical and biological activity of pathogens and their products initiates the release of a cascade of pro-inflammatory and immunomodulatory cytokines by resident epidermal and dermal cells (Nickoloff and Naidu 1994, Medzhitov and Janeway 1997, Galli et al. 1999). This pathogen driven activity establishes the early cytokine microenvironment that will regulate the initial recruitment of effector cells and also contributes significantly to the information programmed into APCs (Medzhitov and Janeway 1997, Kapsenberg et al. 1998).

1.1.3.2 The adaptive immune response

Langerhans' cells capture antigens penetrating the outer layers of the stratum corneum. Subsequently antigen is internalised, processed and transported within activated Langerhans' cells to the paracortical region of the skin draining lymph nodes and presented to naïve T cells (Kapsenberg et al. 1998, Kimber et al. 1998). Antigen recognition by naïve antigen-specific T cells stimulates clonal expansion and differentiation into memory or effector T helper (Th) cells. Studies of functional differences in Th cells from mice and man have categorised these cells as Th1 and Th2 cells (reviewed by Del Prete et al. 1994). Th1 cells characteristically secrete the type-1 cytokines, interferonγ (IFNγ), interleukin-2 (IL-2) and tumour necrosis factor beta (TNFβ) that promote cell-mediated immune responses while Th2
cells secrete the type-2 cytokines IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 and promote B cell responses and especially immunoglobulin E (IgE) synthesis (Romagnani 1997, Kapsenberg et al. 1998, Murray 1998). These responses are not mutually exclusive and evidence of sequential involvement of Th2 and Th1 responses in atopic dermatitis has been presented (Thepen et al. 1996, Grewe et al. 1998). The Th cytokine profiles of ruminants have not yet been segregated into these functional categories. However, the immune response of ruminants to helminth infection, which is characterised by mast cell hyperplasia, tissue eosinophilia and raised antigen specific IgE, is typical of the Th2 response (Miller 1996). Polarisation of Th cells towards a Th1 or Th2 cytokine profile may occur in lymphoid or lesional tissue and is the outcome of a complex interaction of signals transmitted to the activated T cell by APCs. These polarising signals are generated by interaction of information programmed into the APC by the type, dose, affinity of binding and biological activity of the antigen, the cytokine microenvironment in which the antigen was captured and host factors (Romagnani 1992, Kapsenberg et al. 1998, Murray 1998).

Adaptive immune responses are normally regulated by a series of immunomodulatory loops that minimise tissue damage (Shearer and Fleisher 1996). Failure of control mechanisms may result in excessive responses and immunopathology. Classically four hypersensitivity responses have been recognised, three (Types I, II and III) mediated by antibody and one (Type IV) cell mediated (Coombs and Gell, 1968). An additional reaction, cutaneous basophil hypersensitivity (CBH) has also been recorded (Dvorak 1976). Recently a further, T cell-dependent, delayed hypersensitivity response that recruits eosinophils, as opposed to macrophages, has been proposed (Meeusen 1999). As this eosinophil-
rich delayed-type response may be an important element in the cutaneous response to
P. ovis, it will be examined in detail in section 1.2.2.3.7.

1.2 RESPONSE OF THE SKIN IMMUNE SYSTEM TO ECTOPARASITES

Ectoparasites may traumatise cells and deposit irritant, cytotoxic and
pharmacologically active substances, and an array of antigens and allergens and
immunomodulatory compounds on or in the epidermis or intradermally (Burns 1996,
Wikel 1996a). Histopathology at tick attachment sites has elegantly demonstrated
that this diverse range of biologically active substances elicits a multifaceted immune
response that evolves in the course of a primary infestation and in successive
challenge infestations (Allen et al. 1977, Brown et al. 1984, Walker and Fletcher
1986, Abdul-Amir and Gray 1987). The nature of the response is determined by
previous exposure to the same or related species of ectoparasite and host capacity to
respond. In the succeeding sections the innate, humoral and hypersensitivity
responses to ectoparasites will be reviewed. Responses to Psoroptes spp are omitted
from this survey but are examined later. As mast cells, basophils, eosinophils and
IgE antibody are crucial in the development of several hypersensitivity reactions, this
review will include a section describing these.

1.2.1 The innate response

In vitro studies have demonstrated that cellular damage caused by mouthparts
and biological activity of ectoparasite products will provoke immediate, antigen-
independent release of cytokines from resident cells such as keratinocytes and mast
cells (Arlian et al. 1996, Machado et al. 1996). Dermatophagoides pteronyssinus, a
house dust mite (HDM) that like P. ovis is a member of the Order Astigmata,
produces Der p 3 and Der p 9. The ability of these to stimulate the expression of
eotaxin, a potent eosinophil chemoattractant, by alveolar epithelial cells and of HDM
extract to induce the production of IL-8 and TNFα, two potent neutrophil
chemoattractants, by cultured epithelial cells has also been demonstrated (Lau et al. 1999, Sun et al. 2001). In addition, the chemoattractant activity of HDM products, such as Der p 1, has been demonstrated (Fahy et al. 2000). Ectoparasite homologues of these HDM products may similarly induce cytokine and chemokine expression that will initiate and influence the recruitment of effector cells.

Observation, in tick-naïve cattle, sheep and rabbits, of significant cellular infiltration within 1 to 24 hours of infestation is consistent with the activation of an innate response (Tatchell and Moorhouse 1968, Allen et al. 1977, Walker and Fletcher 1986, Abdul-Amir and Gray 1987). In cattle, neutrophils dominated the infiltrate provoked by *Ixodes holocyclus* while eosinophils were the principal cells detected in infestations with *Boophilus microplus* (Tatchell and Moorhouse 1968, Allen et al. 1977). This preferential recruitment of cells may be indicative of the injection or induction of different chemoattractants.

1.2.2 The adaptive response

1.2.2.1 Antibody responses

Increased serum IgG and IgM antibody titres have been reported in response to infestations with ticks and *Sarcoptes scabiei* (Falk 1980, Nassef et al. 1991, Morsy et al. 1993, Thoday 1993, Wikel 1996b). Studies of dogs, infested with *S. scabiei* demonstrated that compared to infestations with low numbers of mites, high numbers of mites provoked a greater and more rapid antigen-specific serum IgG antibody response (Bornstein and Zakrisson 1993). These investigations also demonstrated that challenge infestations elicited prompt anamnestic antigen-specific serum IgG responses. Increased levels of antigen-specific serum IgG have also been reported

IgA antibody responses to ectoparasites other than *S. scabiei* have not been reported. Paradoxically, the majority of studies of human patients with scabies and one of dogs found a fall in total serum IgA antibody that was reversed after successful treatment (Falk 1980, Nassef *et al.* 1991, Morsy *et al.* 1993, Thoday 1993).

Antigen-specific IgE responses have been demonstrated in flea infestations of cats and dogs and in human patients with *S. scabiei* infestations (Rantanan *et al.* 1981, Dahl *et al.* 1985, Halliwell and Longino 1985, McKeon and Opdebeeck 1994). A skin-sensitising antibody with physical and chemical properties analogous to human IgE has been isolated from sera of horses sensitised to *Culicoides* spp flies (Matthews *et al.* 1983). Although direct evidence of antigen-specific IgE responses to ectoparasites has been demonstrated infrequently, positive wheal reactions to intradermal skin tests (IDSTs) and Prausnitz-Kustner (PK) tests, have provided putative evidence of IgE-mediated responses to several ectoparasites, including ticks, mites, sucking lice and biting lice (*B. ovis*), biting flies (mosquitoes, *Culicoides* spp, blackflies) and fleas (Wikel 1982, Matthews *et al.* 1983, Allen 1987, Nelson 1987, Allen 1994, James and Moon 1998). In addition, positive passive cutaneous anaphylaxis and basophil degranulation responses have been obtained with sera from sheep infested with *B. ovis* (Pfeffer *et al.* 1994, 1997)

### 1.2.2.2 Lymphocyte and dendritic cell responses

A limited number of studies have examined lymphocyte infiltration of the skin during ectoparasite infestations. *CD4*⁺ T cells, accompanied by smaller numbers of *CD8*⁺ T cells, dominated the cutaneous lymphocyte response of mice to
Ixodes ricinus, sheep to Lucilia cuprina and human patients to S. scabiei (Bowles et al. 1992, 1994, Cabrera et al. 1993, Elhay et al. 1994, Mbow et al. 1994). Increased numbers of γδ T cells and B cells were detected in ovine L. cuprina infestations but B cells were detected infrequently in canine S. scabiei infestations and small to moderate numbers were detected in human patients with scabies (Reunala et al. 1984, Bowles et al. 1992, 1994, Cabrera et al. 1993, Arlian et al. 1997).

Primary and challenge infestations of dogs with S. scabiei and sheep with L. cuprina elicited an increase in numbers of Langerhans' cells and dendritic cells, respectively (Bowles et al. 1992, Stemmer et al. 1996). In Guinea pigs primary infestations with Dermacentor andersoni caused a decrease, and challenge infestations an increase, in numbers of Langerhans' cells (Nithiuthai and Allen 1984).

1.2.2.3 Hypersensitivity responses

Clinical observation of wheal responses to ectoparasite bites or intradermal injection of ectoparasite antigens has demonstrated that host responses to arthropod ectoparasites commonly include immediate and delayed hypersensitivity responses (Wikel 1982, Allen, 1987, Nelson 1987, Allen 1994). The intermediate, late phase, CBH and, possibly, Arthus type responses are detected less frequently. A number of studies have revealed a distinct temporal pattern in the development of these responses in the course of exposure to ectoparasites (Mellanby 1946, Benjamini et al. 1961, Davis and Moon 1990). Lesional histopathology has provided supportive evidence of hypersensitivity reactions (Goldman et al. 1952, Allen 1987, Nelson 1987). However, the histopathology of skin biopsies taken in the course of an infestation represents a composite picture of the infiltrate associated with this multilayered immune response at only one point in its evolution. The infiltrate may
be further complicated by self-trauma and secondary bacterial infection. In view of this, histological evidence of the involvement of individual hypersensitivity responses may be obscured.

In this subsection, Type I (immediate hypersensitivity) and the late phase response and then cutaneous basophil hypersensitivity, Type III (Arthus) reactions and finally Type IV (delayed) hypersensitivity reactions will be reviewed.

1.2.2.3.1 IgE-mediated, Type I immediate hypersensitivity (IH)

In this response the primary effector cells in skin are mast cells sensitised by antigen-specific IgE antibody bound to high affinity, FcεR1, membrane receptors. Antigen bridges two IgE molecules initiating mast cell degranulation that culminates in the secretion of a rich variety of preformed and newly synthesised mediators (Turner and Kinet 1999, Schwartz 2001). These increase vascular permeability, upregulate expression of adhesion molecules by vascular endothelium and promote the recruitment of granulocytes that augment the inflammatory response (Solley et al 1976, Parrish and Breathnach 1996). Typically, immediate wheal reactions are maximal 20-30 minutes after intradermal injection of antigen and regress after 1 to 3 hours. Histologically they are characterised by a superficial perivascular infiltrate of eosinophils and neutrophils, accompanied by mast cell degranulation and dermal oedema (Barnes 1996, Parrish and Breathnach 1996, Weller 1997).

The histopathological pattern described above, plus increased numbers of mast cells, has provided evidence suggestive of IH in several ectoparasite infestations and has been observed in lesional skin from sheep infested with *S. scabiei, B. ovis, Melophagus ovinus, Culicoides spp* and fleas (Nelson and Bainborough 1963, Allen 1987, Ibrahim and Abu-Samra 1987, Nelson 1987, Connan and Lloyd 1988, Heath et al. 1995, Yeruham et al. 1997). Positive IDSTs
or PK tests have provided further evidence of the involvement of IH in a number of infestations but antigen-specific IgE has been detected less frequently (section 1.2.2.1.).

1.2.2.3.2 Late phase response (LPR)

In contrast to the immediate response, wheal formation and cellular infiltration associated with the LPR develop 3 to 4 hours after intradermal challenge, are maximal between 6 and 12 hours and then gradually decline (Solley et al. 1976, Frew and Kay 1988). The wheal usually resolves from 24 to 48 hours but the cellular infiltrate may decline more gradually (Tsicopoulos et al. 1994, Ying et al. 1997). Evidence of the occurrence of LPRs is usually derived from wheal reactions elicited by IDSTs, however, recent studies have demonstrated that cellular infiltration can occur in the absence of wheal formation (Zweiman et al. 1998).

Histological examination of LPRs in patients with atopic dermatitis has demonstrated that the cellular infiltrate comprised variable proportions of neutrophils, eosinophils, basophils and mononuclear cells (Solley et al. 1976, Frew and Kay 1988, Ying et al. 1997). In the early stages granulocytes and mononuclear cells were present in equal proportions but in the later stages, from 24 hours after challenge, mononuclear cells accompanied by neutrophils dominated the infiltrate.

The observation that this response could be elicited in man by intradermal injection of anti-IgE antibody and Prausnitz-Kustner testing and was attenuated in mast cell deficient mice has provided strong evidence for the involvement of an IgE-mediated, mast cell and/or basophil dependent mechanism (Dolovich et al. 1973, Nagai et al. 1995). In the case of mast cells this may operate through late-acting eosinophil and neutrophil chemoattractant cytokines, such as TNFα, released during the preceding IH reaction (Lemanske and Kaliner 1983, Wershil et al. 1991).
Analysis of the LPR in atopic patients demonstrated a strong correlation between numbers of CD4+ cells and eosinophils which may indicate that T cell activity augments the initial IgE-mediated recruitment of cells in atopic patients (Frew and Kay 1988, Gaga et al. 1991, Werfel et al. 1995). This is supported by the demonstration of a strong association between eosinophil infiltration, increased expression of mRNA for IL4 and IL5 and increased infiltration of T lymphocytes, in the analogous late alveolar response (Robinson et al. 1993, Sur et al. 1995, Virchow et al. 1995, Peters et al. 1996).

Responses to intradermal injection of ectoparasite antigens consistent with a LPR have been recorded in canine flea allergic dermatitis, in rabbits and guinea pigs repeatedly infested with ticks and in horses with recurrent seasonal dermatitis caused by Culicoides spp (Gill et al. 1986, Gordon and Allen 1987, Halliwell and Schemmer 1987, Foster et al. 1995).

1.2.2.3.3 Cutaneous basophil hypersensitivity (CBH)

CBH reactions comprise a heterogeneous group of delayed responses characterised by a significant though variable infiltrate of basophils (Askenase 1996, Parrish and Breathnach 1996). Temporal studies of the infiltrate elicited by intradermal challenge of atopic patients and animals sensitised by fleas and ticks have demonstrated that CBH can constitute a component of the LPR (Solley et al. 1976, Gill et al. 1986, Gordon and Allen 1987, Halliwell and Schemmer 1987). However, Dvorak (1976) reported that in CBH reactions induced in Guinea pigs by soluble proteins in Freund’s incomplete adjuvant, the influx of basophils was greatest from 24-48 hours after intradermal challenge. Although basophils were prominent, particularly in CBH reactions of Guinea pigs, mononuclear cells usually dominated the infiltrate (Askenase 1976, Haynes et al. 1978, Graziano et al. 1983). Numbers
of eosinophils were inconsistent but could equal or exceed basophil counts and neutrophils were detected less frequently.

In Guinea pigs, CBH reactions have been elicited following passive transfer of IgE and IgG1 antibody and also after passive transfer of T cells from sensitised to naïve Guinea pigs (Askenase 1976, Haynes et al. 1978, Mitchell and Askenase 1982, Graziano et al. 1983). These experiments have demonstrated the involvement of antibody and T cell-dependent mechanisms in the genesis of CBH reactions, the same mechanisms that are responsible for the LPR. It has been proposed that IgE, or in Guinea pigs IgE/IgG1-mediated mast cell degranulation, results in the release of late-acting chemotactic cytokines that recruit basophils (Moore and Dannenberg, 1993, Askenase 1996). Basophil recruitment and activation are considered in more detail in section 1.2.2.4.1.


1.2.2.3.4 Type II, antibody-mediated cytotoxic hypersensitivity

As this reaction has not been recognised as a feature of lesional pathology in responses to ectoparasite infestations it will be omitted.

1.2.2.3.5 Type III, immune complex-mediated hypersensitivity

As evidence for the involvement of this response in ectoparasite infestations is equivocal, it will be dealt with briefly. The wheal reaction is most intense 4 to
8 hours after intradermal injection of antigen and the major histological feature is vasculitis associated with a massive infiltration of neutrophils (Parrish and Breathnach 1996).

Immunopathology of lesional skin from human patients with scabies has demonstrated the deposition of IgA and IgM immune complexes in dermal blood vessels and of IgM and IgG immune complexes at the dermo-epidermal junction (reviewed Arlian 1996). Deposits of complement (C3) were also detected at these sites. However, it is uncertain whether these complexes were associated with mite or bacterial antigens.

1.2.2.3.6 Delayed hypersensitivity, Th-1 cytokine-mediated (DTH Type 1)

The classical tuberculin-induced DTH, involves antigen-presenting cells and sensitised CD4+ T cells that express a Th-1 cytokine profile (IFN-γ, IL2, TNFβ) and recruit and activate monocytes/macrophages as the principal effector cells. Infiltration of neutrophils may occur, particularly in severe reactions, and basophils and eosinophils may also be detected but B cells are rare. (Tsicopoulos et al. 1992, 1994). The latent period between intradermal injection and wheal reaction is variable and determined by host sensitivity, nature and dose of antigen and the animal species. In guinea pigs and rabbits it may be 10-18 hours while in cattle, sheep and human subjects it may be up to 48 hours (Pyrah and Watt 1995, Parrish and Breathnach 1996).

Although delayed (24 hour) reactions to intradermal injection of ectoparasite antigens have been recorded frequently, histopathology of these responses has been examined infrequently (Allen 1987, Nelson 1987, Allen 1994). However, delayed responses of pigs to S. scabiei antigen, of horses to Culicoides spp antigen and of dogs to flea antigen were dominated by lymphocytes accompanied by numerous
eosinophils while basophils were the predominant cell in 24 hour responses of sensitised guinea pigs to tick antigen (Sheahan 1975, Gross and Halliwell 1985, Gordon and Allen 1987, Halliwell and Schemmer 1987, Foster et al. 1995). The nature of the cellular infiltrate described in these responses is not typical of the classical delayed hypersensitivity response and may either represent an extension of the LPR or the delayed response described below.

1.2.2.3.7 Delayed hypersensitivity, Th2 cytokine-mediated (DTH Type 2)

It has been proposed that this reaction is analogous to the classical DTH response but is mediated by T cells with a Th2, as opposed to Th1, cytokine profile and is characterised by the preferential recruitment of eosinophils rather than mononuclear cells (Askenase 1996, Meeusen 1999). Evidence of this reaction has been derived principally from the observation of delayed eosinophil-rich responses to helminth infections and in particular, analysis of mammary gland responses of sensitised sheep to infusion of nematode larvae (Meeusen et al. 1990, Meeusen 1999). The latter study indicated that an early (10 hour) infiltrate of eosinophils and neutrophils was succeeded at 48 hours by an increasing influx of eosinophils accompanied by CD4+ T cells but not neutrophils. An eosinophil-dominated, delayed-type cutaneous response has also been observed in mice sensitised to Schistosoma mansoni cercariae and eggs (Ch’ang and Colley 1986, Teixeira et al. 2001). T cell-dependence of this response has been demonstrated by adoptive transfer of sensitised T cells and its abrogation by depletion of CD4+ lymphocytes (Ch’ang and Colley 1986, Teixeira et al. 2001). This response has parallels with the LPR but the recruitment of eosinophils, 18-72 hours after intradermal challenge, was maximal later and persisted longer. A similar delay in time recruitment of basophils has been reported in some CBH reactions (Dvorak 1976).
Pronounced eosinophil infiltration 24 hours after blackfly bites and detection of an eosinophil-rich infiltrate 24 hours after intradermal challenge of scabietic pigs with *S. scabiei* extract suggest that this type of DTH may be elicited in responses to arthropod ectoparasites (Sheahan 1975, Nelson 1987).

### 1.2.2.4 Mast cells, basophils, eosinophils and IgE.

The previous section reviewed the hypersensitivity responses that may participate in the immunopathogenesis of the sheep scab lesion. The involvement of inflammatory cells (mast cells, basophils and eosinophils) and IgE that characterise several of these reactions will now be described in more detail.

#### 1.2.2.4.1 Mast cells and basophils

Mast cells originate from pluripotential stem cells in bone marrow and are released into the peripheral circulation as committed but undifferentiated progenitor cells (Kitamura *et al.* 1981, Nakahata and Ogawa 1982). Studies in rodents and man suggest that recruitment and development of undifferentiated mast cells is promoted by upregulated expression of adhesion molecules, for example P selectin, on vascular endothelial cells and the activity of mast cell chemoattractants such as stem cell factor (SCF), IL-3, RANTES (regulated on activation, normal T cells expressed and secreted), monocyte chemotactic protein-1, (MCP-1), macrophage inflammatory protein 1α (MIP 1α), C3a and C5a (Matsuura and Zetter 1989, Nilsson *et al.* 1994, Taub *et al.* 1995, Costa *et al.* 1996, Sriramarao *et al.* 1996, Hartmann *et al.* 1997).

Recently, *in vitro* studies have demonstrated that eotaxin and stromal cell-derived factor 1 alpha (SDF-1α) are also potent chemoattractants for cultured human mast cells (Ochi *et al.* 1999, Romagnani *et al.* 1999, de Paulis *et al.* 2001). Observation of murine responses has indicated that differentiation of mast cells was also enhanced *in vitro* by IgE acting in concert with IL-3 and by IgE immune.
complexes (Ashman et al. 1991). The detection of sporadic mitosis by mast cells in lesional skin of patients with atopic dermatitis suggests that local proliferation may also make a minor contribution to increased mast cell numbers (Weber et al. 1995).

Basophils also originate from pluripotential bone marrow cells but, in contrast to mast cells, they enter the peripheral circulation as terminally differentiated cells (Galli 1990). Although morphologically similar to mast cells they are smaller and normally possess a lobulated, as opposed to the round to oval nucleus of mast cells. Normally they are detected infrequently in peripheral tissues. Although preferential recruitment occurs in the LPR and CBH, the mechanisms involved are unclear. Adhesion of basophils to endothelial cells may be promoted by IL-3 while, in man a number of cytokines and chemokines such as IL-3, eotaxin, RANTES, MCP-4, MIP 1α, SDF-1α and SCF have been shown to possess basophil chemoattractant properties (Bochner et al. 1990, Yamada et al. 1997, Dvorak et al. 1998, Bonville et al. 1999, Jinquan et al. 2000).

Mast cells and basophils play an integral role in responses to ectoparasites. Aggregation of FcεRI receptors on these cells by interaction of bivalent or multivalent antigens with receptor bound, antigen-specific IgE antibody constitutes the major pathway for activation by parasite antigens. Mediator release by this mechanism is significantly potentiated by upregulation of FcεRI receptor expression in response to increased concentrations of IgE antibody (Costa et al. 1997). In vitro studies have shown that priming with IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) also enhances basophil responses to IgE receptor stimulation and that priming with SCF potentiates mast cell responses (Amon et al. 1994, Parrish 1996). Degranulation of mast cells and basophils is also triggered by complement fragments C3a and C5a while chemokines such as eotaxin,
RANTES, MCP-3 and 4 and MIP-1α interact with the chemokine receptor-3 to stimulate histamine release by basophils but not mast cells (Costa et al. 1997, Schwartz 2001).

Mast cells are crucial and potent effector cells in the pathogenesis of acute inflammation associated with IgE-mediated Type 1 hypersensitivity. Activation of mast cells by this pathway results in the rapid release of preformed mediators such as histamine and neutral proteases. This is followed within minutes by secretion of newly synthesised lipid mediators which include prostaglandins, thromboxanes, leukotrienes and platelet activating factor (PAF) (Schwartz 1994, Schwartz 2001). These proinflammatory mediators generate an acute inflammatory response characterised by vasodilation, increased vascular permeability and leukocyte infiltration. Stimulation of IgE receptors also initiates the release of cytokines. These include proinflammatory (IL-1, IL-6, IL-8, TNFα) and haemopoietic (IL-3, IL-4, IL-5 and GM-CSF) cytokines (Schwartz 1994). The sustained release of haemopoietic cytokines and of TNFα and proteases plays a pivotal role in regulating the recruitment and activity of effector cells of the LPR, particularly of basophils and eosinophils. These augment and perpetuate the initial inflammatory response caused by histamine and lipid mediators. IgE-mediated activation of human basophils also provokes the release of several mediators including histamine, neutral proteases, leukotrienes and cytokines (IL-4, IL-13) (Costa et al. 1997).

Mast cell and basophil-derived IL-4 and IL-13 may transmit polarising signals to Langerhans' cells that will bias differentiation of naïve antigen-specific cells in lymphoid tissue towards a Th2 (allergic) cytokine profile and similarly influence signals relayed by APCs to Th memory and effector cells in lesional tissue (Schwartz 1994, Kapsenberg et al. 1998). Mast cells and basophils may also
promote B cell IgE isotype switching by production of IL-4 and IL-13 and theoretically by expression of the CD40 ligand (section 1.2.2.4.3).

1.2.2.4.2 Eosinophils

In normal tissue eosinophils occur in small numbers, principally below epithelial surfaces interfacing with the external environment (Kita et al. 1996). In man, tissue recruitment of eosinophils is promoted by the integrated activity of IL-5 and IL-13, by eosinophil chemoattractants such as PAF and by chemokines including, eotaxin, RANTES, MCP-3 and MCP-4 that interact with chemokine receptor-3 on eosinophils to induce site-directed migration (Lamkhioued et al. 1997, Dulkys et al. 2001, Pope et al. 2001, Terada et al. 2001). Although T cells may coordinate eosinophil recruitment, mast cells, basophils, eosinophils, macrophages, fibroblasts, and epithelial and endothelial cells are important sources of several of these chemoattractants (Gonzalo et al. 1996, Costa et al. 1997, Weller 1997, Teran et al. 1999, Ying et al. 1999a, 1999b, Lamkhioued et al. 2000). In vitro studies have indicated that eotaxin is the most potent of the eosinophil chemokines (Dulkys et al. 2001, Terada et al. 2001). Its integral role in the recruitment of eosinophils in murine models of allergic asthma has been demonstrated by a significant reduction of pulmonary eosinophilia in animals treated with rat anti-murine eotaxin monoclonal antibody 24 hours after provocative challenge (Kim et al. 2001).

Similarly in a murine model of schistosomiasis, pre-challenge administration of anti-eotaxin polyclonal antibody significantly reduced the infiltration of eosinophils in the normally eosinophil-rich delayed-type of cutaneous reaction (Teixeira et al. 2001). Eotaxin is produced by several cells including, dermal fibroblasts, epithelial and endothelial cells, T cells, macrophages, eosinophils and possibly mast cells (Hogabaum et al. 1998, Nakijima et al. 1998, Mochizuki et al. 1999, Ying et al.
1999a, 1999b). Fibroblasts constitute a major source of eotaxin and the synergistic activity of IL-4 and TNFα, derived from T cells and mast cells stimulate its expression (Mochizuki et al. 1999, Miyamasu et al. 2000). Basophils and eosinophils provide supplementary sources of IL-4.

Eosinophils are prominent in host responses to ectoparasites. Histological evidence indicates their involvement in the innate response (section 1.2.1). At sites of antigen/allergen challenge aggregation of immunoglobulin receptors by appropriate antibody (IgE, IgG, IgA, sIgA) bound to parasite antigen/allergen or engagement of complement receptors by complement fragments (C3a, C5a) bound to antibody-antigen complexes stimulates degranulation of eosinophils (reviewed Kita et al. 1996, Weller 1997). On activation, eosinophils release cytotoxic cationic proteins (MBP, ECP, EDN and EPO) and lysosomal hydrolases, elaborate several bioactive lipids (LTC4, PAF, lipoxin), and secrete proinflammatory cytokines IL-1, IL-6 and IL-8 (Desreumaux and Capron 1996, Weller 1997). Release of these mediators plays a major role in the immunopathogenesis of allergic inflammation and causes significant tissue damage and dysfunction (Gleich and Adolphson 1986).

Eosinophils are one of several sources of IL-4 that polarises the cytokine expression by Th cells towards a Th2 (allergic) profile and promotes IgE isotype switching by B cells (section 1.2.2.4.3)

1.2.2.4.3 IgE

Two distinct signals are essential to switch B cell isotype synthesis to immunoglobulin IgE (reviewed Bacharier and Geha 1999). The first is cytokine dependent and provided by interaction of IL-4 or alternatively the less potent IL-13 with their receptors on B cells. These cytokines are expressed principally by CD4+
Th2 cells. Supplementary secretion of IL-4 and IL-13 by mast cells and basophils and of IL-4 by eosinophils may upregulate IgE synthesis amplifying local IgE-mediated responses (reviewed Vericelli and Geha 1996). The second crucial signal is generated by ligation of the B cell CD40 receptor by CD40 ligand on T cells. In vitro studies have demonstrated that γδ T cells, mast cells and basophils cells also express the CD40 ligand and that γδ T cells and basophils are capable of inducing IgE isotype switching (Gauchat et al. 1993, Horner et al. 1995, Yanagihara et al. 1998). γδ T cells are relatively abundant in the ovine dermis and may, therefore, contribute significantly to the development of IgE-mediated hypersensitivity responses of sheep to ectoparasite infestations (Haig et al. 1999).

The interplay of several cytokines modulates IgE production, IL-4, IL-5, IL-6, and IL-9 upregulate IgE synthesis while IL-2, IL-12, and IFNγ inhibit IgE synthesis (reviewed Vercelli and Geha 1996, Bacharier and Geha 1999, Corry and Kheradmand 1999). It has also been suggested that binding of antigen-IgE complexes to the low affinity IgE receptor CD23 (FceRII) on B cells enhances IgE synthesis but at high IgE concentrations these receptors mediate an inhibitory influence (reviewed Corry and Kheradmand 1999). In addition, ectoparasite products with protease activity, such as Der p 1, may selectively cleave the CD23 receptor from B cells resulting in the loss of this inhibitory loop while the soluble fragments of CD23 may enhance IgE synthesis (Hewitt et al. 1998, Schulz et al. 1999)

IgE-mediated activation of FceRI receptors provokes degranulation of mast cells and basophils, a crucial effector mechanism in immediate hypersensitivity and cutaneous basophil hypersensitivity responses to ectoparasites. Moreover, this
response is augmented by IgE-induced upregulation of FcεRI receptor expression by mast cells (Costa et al. 1997). Human eosinophils, monocytes and neutrophils also possess FcεRI and RII receptors (Parrish and Breathnach 1996, Yamaoka et al. 1996, Weller 1997, Gounni et al. 2001, Novak et al. 2001). Aggregation of these receptors by antibody-antigen complexes will stimulate the release of mediators and contribute significantly to tissue damage in the LPR and possibly to host defence. In addition, capture of antigen by IgE bound to FcεRI and FcεRII receptors on Langerhans’ cells and monocytes and the FcεRII receptor on B cells enhances antigen presentation and T cell activation (Pirron et al. 1990, Gajewski et al. 1991, Heyman et al. 1993, Maurer et al. 1995)

1.3. PSOROPTES OVIS INFESTATION OF SHEEP

The previous section reviewed host responses and in particular cutaneous hypersensitivity responses to ectoparasites. In the present section host responses to Psoroptes spp mites are examined with an emphasis on those of sheep to P. ovis. In order to demonstrate the relevance of the investigations carried out in this thesis, this is done in the context of the epidemiology, clinical signs and measures currently employed to control sheep scab.

1.3.1 Classification of Psoroptes spp mites

The family Psoroptidae is a member of the Phylum Arthropoda and the Order Astigmata. It is closely related to mites in other families of the Order Astigmata such as the Sarcoptidae which includes Sarcoptes scabiei an obligate burrowing ectoparasite of mammals and the Pyroglyphidae which contains the house-dust mites Dermatophagoides farinae and D. pteronyssinus that cause atopic dermatitis and allergic rhinitis and asthma in man.
The family Psoroptidae comprises four genera of cosmopolitan, non-burrowing, ectoparasitic mites of mammals: *Psoroptes* spp, *Chorioptes* spp, *Otodectes* and *Caparinia* spp. Sweatman (1958) recognised five species of *Psoroptes*: *P. ovis*, *P. cuniculi*, *P. equi*, *P. natalensis* and *P. cervinus*. However, criteria used to differentiate species are equivocal (reviewed by Bates 1999a, Zahler *et al.* 1998) and analyses have demonstrated that genotypically isolates of different ‘species’ are highly homogeneous (Zahler *et al.* 1998). Within the species of *P. ovis*, differences in the pathogenicity or virulence of mite populations have been recognised and attributed to the existence of distinct ‘strains’ (Roberts and Meleney 1971, Bates 1999a, 1999b), though they may also reflect differences in the microflora of these mite populations (Mathieson and Lehane 1996).

### 1.3.2 Life cycle and Epidemiology

The life cycle of *P. ovis* comprises the egg and four developmental stages, namely the prelarva, larva, nymph (protonymph and tritonymph) and adult. Detailed analyses of the different phases have been provided by Downing (1936a) and Sweatman (1958). Each stage is completed on the host and under optimal conditions the life cycle, from egg to egg production by the adult female, takes 11 to 21 days. Adult males live for approximately one month and form a succession of attachment pairs with pubescent female tritonymphs or less frequently with female protonymphs. Each attachment continues until the female nymph becomes an adult. Adult females live for 11-42 days and if fertilised commence oviposition 1-2 days after emerging from the tritonymph stage and continue producing eggs for up to 29 days. They deposit 1-6 eggs a day and 15-40 eggs in total (Stockman and Berry 1913, Downing 1936a) and possibly 90 eggs per female under favourable conditions (Shilston 1915). Mites can survive off the host for 31-38 days though they are
infective for only 12-17 days (Babcock and Black 1933, Wilson et al. 1977, O'Brien et al. 1994).

Mathematical modelling has suggested that following infestation there is an initial period of instability (10 days), after which mite populations (larvae, nymphs and adults) increase exponentially, doubling every 6.3 days (Wall et al. 1999). However, population dynamics on the host will be determined by the interplay of host susceptibility, mite virulence, husbandry and environmental conditions (Roberts and Meleney 1971, Bates 1997b, O'Brien 1999). Bates (1997a) recognised six phases in the temporal development of mite populations on sheep, commencing with an extended, subclinical, lag phase that was characterised by low numbers of mites. In the succeeding rapid growth phase the mite population expanded rapidly, lesions spread and the accompanying clinical signs became evident. This was followed by the plateau phase when the mite population was static and then a phase of decline in the mite population that was accompanied by retardation and then cessation of lesional growth and the regression of clinical signs. In some animals the mite population appeared to die out completely (regressive phase), in others, although clinically recovered and apparently normal, mites which dispersed at random over the body during the phase of rapid lesional growth survived in cryptic sites (cryptic phase).

*P. ovis* is highly contagious and it requires successful transfer of only one ovigerous female from an infested to susceptible sheep to establish an infestation. Infestations can be transmitted by direct contact between susceptible sheep and animals with subclinical as well as those with clinical disease. Cryptic populations of mites may survive for two years (Babcock and Black 1933) and also provide a source of mites that may be transmitted to susceptible sheep. Husbandry practices
that concentrate sheep together, such as high stocking density and trough feeding predispose to rapid spread of the disease from these sources through a flock (Spence 1951). Survival of mites off the host facilitates indirect transfer of mites by tags of wool and scab caught on scrub and fences and through contaminated sheep pens and transporters. Contaminated clothing and shearing combs may provide additional sources of infestation.

In an extensive survey of the disease no evidence of any breed, sex or age predisposition was found (O’Brien 1992). However, other observations have suggested that lowland breeds with a high density of wool follicles and yearling sheep are more susceptible to infestation (Bates 1997b).

Although infestations can occur throughout the year there is typically a significantly increased incidence of disease in winter (Kirkwood 1986, O’Brien 1999, Bates 1997b, French et al. 1999). This seasonality has been attributed to a combination of management and climatic factors (reviewed by French et al. 1999). The reduced incidence of disease over the summer and autumn months may be attributable to shearing which adversely affects mite survival, the use of acaricides to control summer ectoparasites such as *Lucilia sericata* and autumn dips to control *P. ovis*. The risk of introduction and spread of infestation through a flock in the winter months is increased by the arrival of potentially infested sheep from autumn sales coincidentally with the influx of susceptible lambs and the concentration of sheep for overwintering and lambing.

1.3.3 Clinical signs

The rate of progress of the disease in individuals is variable. The initial subclinical phase persists for a variable period of 2-8 months in naturally occurring infestations and 10-35 days in experimental infestations (Bates 1997a, O’Brien
Lesions are generally first detected on the dorsum (Sargison 1995, Bates 1997a) and can rapidly extend over the flanks to the limbs and head while sparing the glabrous skin of the ventrum. Clinical observations of the cutaneous response indicate that mites provoke papular inflammation and serous exudation within hours of experimental infestation (Spence 1949, Bates 1999b). Subsequently vesicles and greenish yellow pustules develop, increase in size and rupture discharging their contents onto the skin surface (Downing 1936a). Here the serocellular discharges and exudate stain the base of wool fibres yellow-orange and as they coagulate and dry they produce an area of yellowish scale and crust and matted fleece (Sargison 1995, Bates 1997a). Typically, the scab is surrounded by a moist, faintly green zone of exudation with an outer ring of inflammation that merges into normal skin. Mites appear to congregate, feed and deposit eggs at this interface of lesional and non-lesional skin (Kirkwood 1986, Sargison 1995, Bates 1997a). As they advance outwards they provoke an intense inflammatory response and individual lesions expand, coalesce and eventually may extend to involve most of the body.

Initially lesions generate a mild to moderate pruritus manifested by head tossing and scratching, rubbing and biting at affected sites (Sargison 1995, Bates 1997a). As the disease progresses pruritus becomes more intense and the fleece overlying lesional skin becomes increasingly stained, moist, soiled and matted. Tufts and clumps of fleece may be shed or pulled out and self-trauma of exposed areas results in abrasions, ulcers, excoriation and secondary bacterial infection (Sargison 1995). Animals may be hyperaesthetic and when stimulated by rubbing, affected animals often exhibit a pronounced nibble reflex with protrusion of the tongue and lip smacking. Occasionally stimulation precipitates epileptiform convulsions and death (Bygrave et al. 1993).
As lesions become more extensive productivity falls. Reduced food intake due to anorexia and excessive protein loss through lesions result in reduced weight gain in growing animals and weight loss in adults (Kirkwood 1980). In pregnant ewes loss of protein causes low birth weight and high perinatal mortality while in lactating ewes milk yield is depressed (Sargison et al. 1995). In severe cases loss of condition may be extreme and result in depression, emaciation and death.

Most animals recover and lesions resolve (Bates 1997a). As this occurs new wool growth lifts scab away from the skin which may appear hyperkeratotic and lichenified. In some animals the mite population may die out completely while in others, mites may persist in cryptic sites such as the inguinal and infraorbital fossa and external auditory meatus (Downing 1936b, Spence 1949, Bates 1997a). Occasionally exuberant crusts and scales persist and may be peeled back to expose an abundance of mites. These cases, described as ‘flakers’ (Bates 1997a), appear to be analogous to Norwegian scabies in man, pigs and dogs and may be associated with immunodepression/deficiency (Espy and Jolly 1976).

Infestations may sometimes be limited to the external auditory meatus. These may cause head shaking and rubbing and scratching of the affected ear which result in aural haematomata, inflammation and excoriation of the pinna and base of the ear (Morgan 1992, Bates 1996).

At present diagnosis of sheep scab is suggested by history and clinical signs and confirmed by identification of *P. ovis* mites in scrapings from lesional skin (Sargison 1995, O’Brien 1999). Inevitably, animals with sub-clinical disease or minimal lesions may escape detection and it has been suggested that an enzyme linked immunosorbent assay (ELISA) to detect *P. ovis* antigen-specific antibody may provide an alternative method for screening a flock/herd (Lonneux et al. 1996, Ochs
et al. 2001). However, a recent study of field cases has suggested that positive evidence of *P. ovis* antigen-specific IgG binding was detected earlier with sodium dodecyl sulphate-polyacrylamide agarose gel electrophoresis (SDS-PAGE)/western blot techniques than ELISA tests (Grogono-Thomas et al. 1999) and that immunoblots may, therefore, provide a sensitive alternative immunodiagnostic test.

### 1.3.4 Current control measures for sheep scab

Recognition of the profound economic and welfare importance of *P. ovis*, the subject of this present study, resulted in its successful eradication in several countries including Australia (1884), New Zealand (1985), Canada (1924), the USA (1973) and Great Britain in 1952 (Kirkwood 1986, O’Brien 1999). However, in the U.K. it was reintroduced in 1973. Reinstatement of compulsory dipping in 1976 failed to eliminate the disease and a government working party concluded that an eradication policy was untenable. Consequently, in 1992, the disease was deregulated and responsibility for its control was placed on sheep farmers.

Since then, despite plunge dipping in organophosphate (OP) and synthetic pyrethroid (SP) compounds there has been an inexorable spread of the disease (O’Brien 1999). Moreover, OP dips have been incriminated in post-dipping sickness of stockmen and dipping contractors while disposal of large volumes of dip wash containing OP and SP compounds have raised ecological concerns (Littlejohn and Melvin 1991, Murray et al. 1992, Stephens et al. 1995, Beach et al. 1996, Gasser et al. 1997, Belfroid et al. 1998). Furthermore, analysis of OP and SP residues in the fleece samples have indicated that 26-37% of sheep were inadequately dipped (Anon 1986). In addition, plunge dipping is labour intensive and stressful to sheep (Bates 1993).
Resurgence of the disease, concerns for human health and the environment, the unpopularity of plunge dipping, and the emergence of strains of *P. ovis* resistant to propetamphos and to flumethrin, have created an urgent need for alternative acceptable methods of control (Synge *et al.* 1995, Clark *et al.* 1996). Although, at present, the avermectins may provide an acceptable form of control, those currently licensed fail to confer long-term protection and there is always the possibility that misuse by farmers will result in the emergence of resistant strains. Therefore, the need for research into other control measures remains. Various possibilities have been proposed. These include the development of novel insecticides and naturally-occurring substances and also generation of a vaccination strategy similar to those already employed successfully to control tick infestations and flies (Allen 1994, Casu *et al.*, 1997, O’Brien 1999). Understanding the nature of host immune responses to *P. ovis* will, potentially, contribute the development of an effective vaccination strategy.

**1.3.5 Host responses to *P. ovis* infestation**

*P. ovis* is a surface living mite. Its mouthparts do not penetrate beyond the outermost layers of loose keratin and electron microscopy has indicated that they are designed to abrade rather than pierce the epidermis and to siphon surface lipid emulsion and inflammatory fluid into a preoral cavity (Blake *et al.* 1978, Sinclair and Kirkwood 1983, Rafferty and Gray 1987, Sinclair and Filan 1989, Mathieson 1995). Mite products are, therefore, deposited on intact or abraded epidermis. These include exuviae, moulting enzymes, faecal pellets containing waste products such as guanine, enzymes and numerous bacteria enveloped in a peritrophic matrix, and substances released by degenerating dead mites (Bates 1997a, Mathieson 1995, Mathieson and Lehane 1996). By analogy with cysteine proteases produced by
house-dust mites, epidermal penetration of biologically active substances may be facilitated by proteases in faecal pellets (Herbert et al. 1995, Wan et al. 1999). It is clear from the clinical signs provoked by *P. ovis* infestation that following penetration of the epidermis mite products elicit a profound immunoinflammatory response.

### 1.3.5.1 Cutaneous histopathology.

Lesional histopathology of experimental and naturally occurring infestation of sheep with *P. ovis* has been reported (Rapeanu 1972, Rovere and Nunez 1977, Abu-Samra et al. 1981, Rosa and Moschini 1988). The experimental study of Rapeanu (1972) focussed on structural changes and largely ignored the cellular infiltrate while Rovere and Nunez (1977) biopsied lesions only once during the acute and chronic phases of the disease, respectively, at 23 and 92 days after infestation. In a later study, only 3 sheep were infested and lesions were biopsied at irregular intervals from 13 to 67 days after infestation (Rosa and Moschini 1988). None of these studies commented on lesional mast cells or investigated the dynamics of cellular infiltration into lesional skin. Lesional histopathology described in these studies, particularly the cellular infiltrate, was inconsistent but included the occurrence of subcorneal eosinophilic pustules and a dermal infiltrate of composed of variable proportions of eosinophils, neutrophils, macrophages and lymphocytes. There was also pronounced dermal oedema and proliferative and degenerative epidermal pathology (Rapeanu 1972). In experimental infestations of *P. ovis*-naive calves, eosinophils dominated the infiltrate and increased mast cell numbers were observed (Stromberg and Fisher 1986, Stromberg et al. 1986). However, in these studies calves were infested twice at an interval of 17-21 days. Stromberg and Fisher (1986) also found that this characteristic inflammatory response was provoked
more rapidly by challenge infestations of previously infested calves than in *P. ovis*-naive animals. These observations are consistent with the involvement of IH in the pathogenesis of cutaneous lesions (Romagnani 1994, Barnes 1996, Weller 1997).

### 1.3.5.2. Haematology

O’Brien *et al.* (1995) recorded the development of eosinophilia, neutrophilia, lymphopenia and a significant fall in haemoglobin concentration in the course of experimental primary infestations of sheep with *P. ovis*. An initial transient decrease in eosinophil and neutrophil counts and a more sustained decline in lymphocyte numbers was also seen in uninfested, control sheep and may have been attributable to the stress of housing and handling.

Significant increases in mean eosinophil counts compared to controls have also been reported in primary infestations of cattle but individual animals differed considerably in their response (Stromberg *et al.* 1986, Stromberg and Guillot 1987a, 1987b, Losson *et al.* 1988). Challenge infestations of cattle elicited a significant eosinophilia after only 10 days compared to 6 weeks after infestation in naive cattle (Losson *et al.* 1988). In contrast to sheep, stanchioned calves experimentally infested with *P. ovis* developed a significant neutropenia that was correlated with lesional area and attributed to a transepidermal efflux of cells (Stromberg *et al.* 1986, Stromberg and Guillot, 1987a). These studies also recorded lymphopenia in infested animals. However, lymphocytosis in the absence of neutropenia has also been documented (Losson *et al.* 1988). In this latter study the absence of a neutropenia may be attributable to the minimal extent of the lesions, while the observation of a lymphocytosis rather than lymphopenia may reflect the absence of stress induced by stanchioning.
Treatment of infested sheep (O’Brien et al. 1995) and cattle (Stromberg and Guillot 1987b, Stromberg and Guillot, 1989) resulted in a rapid normalisation of circulating neutrophil, lymphocyte and eosinophil counts though in cattle, particularly in animals with an extensive dermatitis, the decline in eosinophils was preceded by a transient but significant rise in numbers.

The occurrence of eosinophilia following infestation of naive animals with P. ovis, its increase following treatment which may have been associated with a sudden significant release of antigenic material, and its more immediate recurrence following secondary challenge are compatible with the involvement of hypersensitivity in the cutaneous response to P. ovis infestation. Rapid normalisation of haematology following treatment of animals with an endectocide despite the continued persistence of the scab suggests that live mites and not the bacterial microflora of the scab are principally responsible for the cutaneous lesions.

1.3.5.3 Immune responses

The immediate, innate immune response of P. ovis-naïve sheep to infestation has not been reported. However, different aspects of the adaptive response have been investigated and are reviewed in the following sections.

1.3.5.3.1 Antibody response

P. ovis antigen-specific, serum IgG levels but not serum IgE, IgM or IgA levels have been monitored by ELISA following experimental infestation of naive sheep (O’Brien 1992, Bates 1997a) and cattle (Fisher 1983, Pruett et al. 1986, Lonneux et al. 1996, 1998a). These studies recorded a rise in mean IgG level 3-4 and 1-5 weeks after primary infestation of sheep and cattle, respectively. Variation in immune responsiveness of different species and breeds, sensitivity of ELISA techniques and pathogenicity of the mites employed in these studies may have contributed to these
Serum IgG levels have also been monitored following challenge infestations 12 weeks after treatment of *P. ovis* infested cattle (Fisher 1983) but no anamnestic response in IgG antibody was noted. No comparable studies following challenge infestations sheep have been reported. Fisher (1983) suggested a positive relationship between *P. ovis* antigen-specific, serum IgG titres, mite numbers and lesional area in experimentally infested cattle. Subsequently a positive correlation between antibody titre and lesional area and between antibody titre and mite numbers was confirmed (Pruett *et al.* 1986, Lonneux *et al.* 1996, 1998b). It was also observed that the initial rise in antibody titre coincided with an increase in the mite population and the development of dermatitis (Fisher 1983, Pruett *et al.* 1986, Bates 1997a). This led to the proposal that mite products such as guanine, the peritrophic matrix and bacterial antigens (Eisemann and Binnington 1994, Bates 1997a, Mathieson and Lehane 1996) provoke an immediate hypersensitivity response that is responsible for the dermatitis and also produces a cutaneous microclimate that promotes survival and expansion of the mite population (Pruett *et al.* 1986, Stromberg and Fisher 1986).

SDS-PAGE/western blot techniques have been employed to explore the repertoire of *Psoroptes* spp antigens recognised by sera from infested animals. More than thirty IgG reactive antigens have been identified and although individual and temporal variation in the pattern of antigen recognition has been observed some antigens appear to be more consistently identified (Wikel 1989, Boyce and Brown 1991, Boyce *et al.* 1991a, Uhlir 1992, Matthes *et al.* 1996, Jayawardena *et al.* 1998). Immunoglobulin IgE reactive antigens have also been identified (Matthes *et al.* 1996) but their temporal development has not been studied.
Immunoblots have revealed almost complete homology in the antigen profile of *Psoroptes* spp mites (Boyce and Brown 1991) and have also demonstrated some cross-reactivity in the antigen repertoire of *Psoroptes* spp and related mites such as *Chorioptes bovis*, *S. suis* and *Notedres cati* (Matthes et al. 1996), *D. pteronyssinus* (Stewart and Fisher, 1986) and ticks (den Hollander and Allen 1986, Wozniak 1996). However, recognition of a particular profile of antigens may characterise *Psoroptes* spp infestations (Boyce et al. 1991a, Matthes et al. 1996, Jayawardena et al. 1998).

### 1.3.5.3.2 Hypersensitivity responses

There is limited, reliable evidence of the involvement of the different hypersensitivity reactions in the immunopathogenesis of cutaneous lesions. Histopathology and haematology (sections 1.3.1 and 1.3.2) may be indicative of the involvement of IgE-mediated Type 1 hypersensitivity.

In addition to these, wheal reactions to intradermal injection of *Psoroptes* spp extract have been examined in rabbits and cattle but no similar studies have been reported in sheep (Weisbroth et al. 1972, Wikel 1982, Losson et al. 1988, 1999, Revindran et al. 2000). Immediate wheal reactions were observed in infested and control rabbits and cattle and therefore provided equivocal evidence of Type 1, IgE-mediated hypersensitivity. However, Arthus or late phase (3-5 hour) reactions and delayed type hypersensitivity reactions were observed only in infested animals. The immediate responses detected in control animals were probably due to the high concentration of antigen used. For example, Losson et al. (1988, 1999) injected 0.1ml *P. cuniculi* whole mite extract (WME) with a concentration of 115µg protein/ml as opposed to 10µg protein/ml the standard concentration employed for IDSTs in dogs. There is also uncertainty as to the nature of the 3-5 hour and delayed responses reported since these reactions were not examined histologically.
Passive cutaneous anaphylaxis (Losson et al. 1999) has provided putative evidence for the involvement of a *P. ovis* antigen specific, homocytotropic antibody and suggests that this antibody is not present until fairly late in the course of infestation.

1.4 AIMS OF THIS THESIS

It is clear that the ectoparasite-host interaction generates a dynamic, multifaceted immune response (section 1.2). The early, innate response may influence the nature of the later adaptive immune response that encompasses the hypersensitivity reactions (sections 1.1.3.1 and 1.1.3.2). IDSTs have provided strong evidence of the occurrence of IH and DTH reactions in several ectoparasite infestations and of CBH responses to fleas and ticks (section 1.2.2.3). Although the LPR and DTH appear to be critical in the immunopathogenesis of cutaneous lesions, the histopathology and immunological nature of these reactions has generally not been defined.

Although descriptions of lesional histopathology in *P. ovis* infestations are inconsistent, it is clear that in cattle *P. ovis* elicits a pronounced infiltration of eosinophils (section 1.3.5.1). This recruitment of eosinophils may result from a combination of innate and hypersensitivity responses. The nature of the innate response to infestations with *P. ovis* has not yet been examined. Despite equivocal immediate wheal reactions after IDSTs, passive cutaneous anaphylaxis has provided putative evidence of an IgE-mediated response to *P. ovis* but the temporal development of antigen-specific IgE antibody levels has not been investigated and its involvement in the IH response has not been adequately demonstrated (section 1.3.5.3). More importantly, IDSTs have provided definite evidence of a delayed-type reaction and possibly a late phase response to *P. ovis* allergens (section
By analogy with the late phase response seen in atopic dermatitis and allergic asthma (section 1.2.2.3.2) and the eosinophil-rich delayed-type response seen in cutaneous reactions to *S. mansoni* (section 1.2.2.3.7), these reactions may be crucial in eosinophil recruitment in *P. ovis* infestations. In addition to lack of information on the immunopathology of sheep scab, there is currently no evidence that sheep generate a statistically significant protective response in to *P. ovis* infestation.

The purpose of the research presented in this thesis was to investigate the immunopathogenesis of cutaneous lesions caused by *P. ovis* infestation of sheep. In view of the observations made above, the primary aims of the research presented here were:

1. To establish a comprehensive picture of the nature and kinetics of the immunoinflammatory infiltrate in the course of primary infestations of *P. ovis*-naïve sheep, particularly the nature and kinetics of the early innate response and in addition, to establish whether or not primary infestations provoked a protective immune response.

2. To monitor the dynamics of isotype-specific antibody responses, particularly of IgE, elicited by primary and challenge infestations of *P. ovis* infestation and also to determine the profile of *P. ovis* antigens/allergens recognised by different isotypes and its temporal development.

3. The final aim, having established the nature of the cutaneous immunoinflammatory and antibody responses to *P. ovis*, was to investigate the histopathology and immunopathogenesis of the hypersensitivity responses, especially the delayed-type response, and to determine when they developed during the course of primary infestations.
2. LESIONAL GROWTH AND CUTANEOUS HISTOPATHOLOGY DURING PRIMARY AND CHALLENGE INFESTATIONS OF SHEEP WITH THE SHEEP SCAB MITE - *PSOROPTES OVIS*.

2.1 INTRODUCTION

In sheep *P. ovis* provokes a severe exudative dermatitis that is accompanied by intense pruritus and exuberant crust formation. Cutaneous inflammation is most intense at the advancing margin of lesions and it is here, at the interface of lesional and normal skin, that mites appear to be concentrated (Kirkwood 1986, Bates 1997a). Different aspects of lesional histopathology provoked by experimental primary infestations of naïve sheep have been described (Rapeanu 1972, Rovere and Nunez 1977, Rosa and Moschini 1988). However, these studies had limitations (section 1.3.5.1) and presented an inconsistent picture of the cellular infiltrate with variable proportions of eosinophils and neutrophils. Moreover, none reported the presence of lesional mast cells, investigated the dynamics of cellular infiltration into lesional skin or compared cellular infiltrates at different sites of the lesion.

Clinical observations (Spence 1949) have suggested that the dermatitis is more pronounced and lesional growth retarded following reinestation of sheep after their clinical recovery from sheep scab. Recently, shortly after the results of present study were reported (van den Broek *et al.* 2000), these observations were confirmed by measurements of lesional area in 3 sheep following primary and challenge infestations (Bates 2000). But neither Spence (1949) nor Bates (2000) examined cellular infiltration after primary and challenge infestations.

A more detailed study of calves compared both lesional growth and cellular infiltration after primary and challenge infestations with *P. ovis* (Stromberg and
Fisher 1986). This recorded a more rapid development of dermatitis and histopathological evidence of cellular infiltration, accompanied by a significant reduction in lesional growth following challenge infestations.

None of these studies have presented a comprehensive picture of the temporal pattern of histopathological events in the course of primary and challenge infestations, nor have they provided a detailed analysis of sequential changes in numbers of inflammatory cells, particularly mast cells and eosinophils, in lesional skin. Examination of these cellular responses in primary and challenge infestations may provide an indication of the mechanism(s) involved in generating cutaneous lesions and also in mediating the protective immune response.

In this chapter, in order to establish whether or not primary infestations generated a protective immune response, lesional expansion in primary and challenge infestations was recorded and compared. Temporal changes in lesion histopathology, particularly the kinetics of mast cell and eosinophil infiltration, in the course of primary and challenge infestations were also investigated. In addition, as mites appear to congregate at the advancing margin of the lesions histopathological events at this site were compared with those elsewhere in the lesion and related to the lesional distribution of mites.
2.2 EXPERIMENT 1: LESIONAL GROWTH AND CUTANEOUS HISTOPATHOLOGY IN PRIMARY AND CHALLENGE INFESTATIONS.

2.2.1 Materials and Methods

2.2.1.1 Animals

Primary infestation (Group 1)

Ten *P. ovis*-naïve Suffolk cross sheep aged one to two years were infested on the withers with a cluster of 25-50 ovigerous *P. ovis* mites which were removed from donor sheep with a mounted needle and selected by examination under a dissecting microscope with overhead lighting (American Optical, x40 magnification). Wool was plucked from a small area over the withers and the mites placed on the exposed skin. In each animal lesional area was measured at weekly intervals for 14 weeks. After injection of a local anaesthetic (Lignol, Arnolds Veterinary Products) 6mm punch biopsies were collected from normal skin prior to infestation and from the advancing border of the lesion at 3, 6, 9 and 12 weeks after infestation. In 5 sheep, in order to compare lesional pathology at different sites, biopsies were also collected from within a three centimetre radius of the original site of infestation at 3, 6, 9 and 12 weeks and from mid-way between this site and the advancing margin of the lesion, 6, 9 and 12 weeks after infestation (Figure 2.1). The infestation was terminated by injection of an endectocide (Ivomec, Merial Animal Health, 200μg/kg) at 12 and 13 weeks post-infestation. One animal collapsed with epileptiform convulsions 6 weeks post-infestation and was excluded from the trial.
Challenge (secondary) infestation (Group 2) and Controls (Group 3)

Five of the sheep from group 1 were maintained mite-free for 11 weeks after treatment and then were infested with 25-50 ovigerous *P. ovis* mites (group 2). At the same time, to ensure comparable pathogenicity of mites used in primary and challenge infestations, five *P. ovis*-naïve, control sheep (Suffolk cross and aged between one and two years), were infested with 25-50 ovigerous *P. ovis* mites from the same batch (group 3). Lesional area was monitored in both groups, as described above, for 8 weeks post-infestation. In both groups biopsies were collected from the advancing margin of the lesion, as described above, at 0, 3 and 6 weeks after infestation.
Figure 2.1 Location of biopsy sites 6 weeks after primary infestation

A = original area of infestation
B = mid-lesion area
C = advancing margin of the lesion
2.2.1.2 Lesional area

The method described by Guillot (1981) was modified and used to estimate lesional area. Briefly, the lesional shape and dimensions were mapped onto graph paper and the lesional area determined from this.

2.2.1.3. Histopathology

Biopsies were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.2) for 6 hours (Newlands et al. 1984), then stored in 70% ethanol at 4°C (Miller et al. 1983), processed and embedded in paraffin wax. Serial 5μm tissue sections were cut, stained and mounted under Shandon synthetic mountant (Shandon, Runcorn, Cheshire). General histopathology was examined and neutrophil counts determined in sections stained with Haematoxylin “Z” and Putts eosin (H & E) (CellPath, Newtown, Powys). Mast cells were stained with toluidine blue (0.5% in 0.5N HCl, pH 0.5) for 18 hours (Enerback 1966). Eosinophils were stained with Lendrums carbol chromotrope solution for 1 hour (see section 11.5) (Lendrum 1944) and counterstained for 10 seconds with haematoxylin. Dermal neutrophils, eosinophils and mast cells were counted in two semi-serial sections using an Olympus BX 50 microscope at 400x magnification. To ensure that cell counts were as objective as possible, cells were counted in an area covering two graticule fields to either side of the midline and extending from the dermoeidermal junction to the lower dermis, a depth 10 graticule fields - a total of 40 successive graticule fields (2.5mm²). Intravascular cells were excluded from the count. Basophils, identified as small, round cells with large granules were also detected but poor morphology frequently made differentiation from mast cells difficult and therefore they were included with mast cells as toluidine blue staining cells. Although for the purpose of
this study a precise count of mononuclear cells (lymphocytes, macrophages, plasma cells) was not undertaken, limited counts, in sections stained with H & E, were used to estimate the proportion of these cells relative to eosinophils.

2.2.1.4 **Statistical analysis**

The results are expressed graphically as mean and SEM. As statistical analysis required comparisons of repeated measures in different groups of animals and as some animals were present in two of the groups (Groups 1 and 2), differences between the groups were determined by analysis of co-variance using a Genstat 5 Release 4.1 programme (Lawes Agricultural Trust: Numerical Algorithms Group Ltd, Oxford, UK). Values of $p<0.05$ were considered significant.

2.2.1.5 **Image capture**

Images were acquired with a JVC TK-C1381 colour video camera attached to a Leica Laborlux S conventional compound microscope (Leica Microsystems UK Ltd., Milton Keynes, UK).

2.2.2 **Results**

2.2.2.1 **Lesional area**

Logarithmic transformation of area, $(\log_{10} \text{[area in cm}^2\text{]})$, revealed a rapid, exponential, increase in lesional area on sheep in group 1 and group 3 and restricted lesional growth in group 2 sheep (Figure 2.2). After one week the mean lesional area in challenged (group 2) sheep was 2cm$^2$ but no further significant increase in area occurred. The lesional area in challenged sheep was significantly less than in group 1 and group 3 sheep at 2 weeks ($p<0.01$) and from 3-8 weeks after infestation ($p<0.001$).
Clinical development of the lesion was more rapid following challenge than primary infestations. In *P. ovis*-naïve sheep the site of infestation was inflamed within 24 hours and serous exudation was detected in some animals. By 4 days several small (0.5-1 cm²) islands of scab in close proximity to each other were detected, subsequently these coalesced and the lesion progressed caudally and ventrally across the lateral thorax and flank. In contrast, in all challenged sheep there was a discrete area of pronounced inflammation and exudation at the site of infestation at 24 hours and exuberant crust formation by 48 hours after infestation.

**Figure 2.2** Lesional growth during primary and challenge infestations

Lesional area in challenge infestations (group 2) was significantly less than in primary infestations (groups 1 and 3) at 2 weeks (*p*<0.01) and from 3-8 weeks (*p*<0.001) after infestation.
2.2.2.2 Histopathology

2.2.2.2.1 Cellular infiltration in primary and challenge infestations

The pattern of infiltration in both primary and challenge infestations was diffuse, superficial and perivascular. Although concentrated in the upper dermis, inflammatory cells were distributed throughout the dermis and frequently patchy concentrations of cells, extending to the base of hair follicles, obscured the perivascular distribution except in the lower dermis where the infiltrate was relatively sparse (Figure 2.3). In all groups of sheep a substantial number of eosinophils and mast cells in the upper dermis were degranulated. At all time points and all sites, particularly at the advancing edge of the lesion, eosinophils dominated the infiltrate. Neutrophils were detected relatively infrequently though increased numbers occurred in sites of epidermal erosion.

Eosinophils: A massive infiltration of eosinophils was detected at the advancing margin of lesions (Figure 2.4) and many of these cells were degranulated. At 3 weeks the mean counts (Figure 2.5) were similar in all groups but by 6 weeks the count in group 2 sheep (challenge infestation) had fallen and was significantly lower than counts in group 1 (p<0.001) and group 3 (p<0.05) sheep (primary infestations) which had continued to rise. In group 1 (primary infestation) numbers of eosinophils were maximal at 9 weeks and fell rapidly from 9 to 12 weeks post-infestation. In primary infestations, eosinophil numbers in the different areas of the lesion (Figure 2.6) were not significantly different. However, at 9 weeks eosinophil counts tended to be less at the original site of infestation than at the advancing margin area and this difference approached significance (p<0.058).
Six weeks after infestation, there is a diffuse, perivascular infiltrate in the upper dermis with patchy concentrations of cells extending to the base of the hair follicles. (Original magnification x40, haematoxylin and eosin)
Figure 2.4  Lesional infiltration of eosinophils during primary infestation

Massive infiltration of eosinophils (cytoplasmic granules stained red) at the advancing margin of a lesion six weeks after infestation. (Original magnification x100, carbol chromotrope.)
Figure 2.5. Eosinophil counts at the advancing margin of primary and challenge infestations.

At 6 weeks eosinophil counts were significantly lower in challenge (group 2) than primary infestations (group 1 \(p<0.001\) and group 3 \(p<0.05\)).

Figure 2.6 Eosinophil counts at different lesional sites during primary infestation

No statistical difference was detected in eosinophil counts in different areas of the lesion.
Mast cells and basophils: A marked increase in dermal mast cell numbers occurred (Figures 2.7a and b) and followed a similar temporal pattern in all groups. Numbers were maximal at 3 weeks and fell from 3 to 6 weeks post-infestation (Figure 2.8). At six weeks, counts in group 2 (challenge) were significantly lower ($p=0.036$) than in group 3 (controls). Mast cell counts in different lesional areas (Figure 2.9) were not significantly different at 3 and 6 weeks. But at 9 and 12 weeks counts at the advancing margin were significantly lower ($p<0.03$ and $p<0.01$, respectively) than in the mid-lesion area and at 12 weeks were significantly lower ($p<0.01$) than at the original site of infestation. Basophils were only detected in lesional skin but poor morphology frequently made it difficult to differentiate them from mast cells.

Neutrophils: In groups 1 and 3 (primary infestations) counts were significantly higher at 3 ($p<0.05$) and 6 weeks ($p<0.05$ and $p<0.001$, respectively) than in group 2 (challenge infestation) (Figure 2.10). In group 1 (primary infestation) neutrophil counts at the advancing margin were maximal at 9 weeks after infestation and then fell dramatically (Figure 2.11). Although consistently higher at the advancing margin than at other sites, cell counts in different areas of the lesion were not significantly different except at 6 weeks when numbers in the mid-lesion area were significantly lower ($p<0.05$) than those at the advancing margin area.

2.2.2.2 Epidermal pathology

Three weeks after primary infestation the most consistently observed histopathology included multifocal to diffuse orthokeratotic and parakeratotic hyperkeratosis, hypergranulosis, epidermal hyperplasia with marked acanthosis and
irregular rete ridge formation (Figure 2.12a and b). Epidermal hyperplasia was accompanied by increased numbers of mitotic figures in the stratum basale.

**Figure 2.7a** Mast cells in normal skin

**Figure 2.7b** Mast cells in lesional skin

(Arrow indicates normal mast cell, original magnification x250, toluidine blue.)

Increased mast cell numbers and degranulation at the advancing margin of lesional skin 3 weeks after primary infestation (Arrow indicates degranulated mast cell, original magnification x250, toluidine blue.)
Figure 2.8  Mast cell and basophil counts at the advancing margin of primary and challenge infestations

Mast cell counts in primary (groups 1 and 3) and challenge (group 2) infestations were not significantly different.

Figure 2.9  Mast cell and basophil counts at different lesional sites during primary infestation

Mast cell counts at the advancing were significantly lower than in the mid-lesion area at 9 and 12 weeks (p<0.03 and p<0.01, respectively) and were significantly lower (p<0.01) than counts at the original site of infestation 12 weeks after infestation.
Figure 2.10  Neutrophil counts at the advancing margin of primary and challenge infestations

Neutrophil counts were significantly lower in challenge (group 2) than primary infestations (groups 1 and 3) at 3 weeks ($p<0.05$) and 6 weeks ($p<0.05$ and $p<0.001$, respectively)

Figure 2.11  Neutrophil counts at different lesional sites during primary infestation

Neutrophil counts were significantly lower ($p<0.05$) in the mid-lesion area than at the advancing margin 6 weeks after infestation.
Figure 2.12a  Epidermal pathology during primary infestation

Parakeratosis (solid arrow), acanthosis and rete ridge formation (dashed arrow) three weeks after primary infestation (Original magnification x100, haematoxylin and eosin)

Figure 2.12b  Pronounced disruption of epidermis during primary infestation

Oedema with disruption of intercellular bridges (solid arrow) and hydropic degeneration in epidermis and separation of collagen bundles (dashed arrows) in dermis, six weeks after primary infestation (Original magnification x100, haematoxylin and eosin)
Pronounced intercellular oedema with patchy disruption of intercellular bridges was often accompanied by hydropic degeneration and apoptosis of cells of the stratum basale and spinosum and less frequently by reticular degeneration. Despite disruption of the stratum basale dissolution of the basement membrane was rare. Parafollicular subcorneal pustules (Figure 2.13) were invariably present and intraspinous and intragranular pustules occurred less frequently. These contained numerous eosinophils with fewer neutrophils and mononuclear cells and rarely acanthocytes. Occasionally, vesicles filled with proteinaceous fluid and only a small number of cells were detected. There was transepidermal trafficking of numerous eosinophils and fewer neutrophils, mononuclear cells and mast cells. Occasionally erythrocytes were also seen in the epidermis. Focal to diffuse areas of exudate (eosinophilic proteinaceous material) coated the outermost layers of the stratum corneum and contained degenerating eosinophils, neutrophils and mononuclear cells. The proportion of neutrophils observed in the exudate was greater than in the dermal infiltrate.

Epidermal pathology developed and declined synchronously with the infiltrate of eosinophils and was most pronounced at 6 and 9 weeks after infestation (Figure 2.14). No differences were observed in the histopathology of the advancing margin and mid-lesional sites. However, compared to these areas, pathology at the original site of infestation was muted and no more severe at 6 and 9 weeks than at 3 weeks after infestation. By 12 weeks, resolution of lesions had commenced at all sites and hyperkeratosis was orthokeratotic and hypergranulosis limited to a narrow band of densely staining kerato-hyalin granules. Epidermal hyperplasia was reduced and mitotic figures were absent. Epidermal oedema was confined to focal areas
where intercellular bridges were more pronounced than normal. No epidermal pustules or vesicles were observed and exocytosis of eosinophils occurred rarely.

In challenge infestations, epidermal pathology was most obvious at 3 weeks and was resolving at 6 weeks post-infestation. At 3 weeks, diffuse hyperkeratosis was observed but parakeratosis and hypergranulosis were detected infrequently and epidermal hyperplasia was less pronounced than in primary infestations. Subcorneal eosinophilic pustules were observed in only 1 of 5 animals and were accompanied by intercellular oedema and epidermal exudate and crust formation.

2.2.2.2.3 Dermal pathology

In primary infestations, the profound infiltrate seen at 3 weeks was accompanied by a patchy to diffuse dermal oedema, which was particularly obvious at the dermo-epidermal interface. Collagen bundles were separated and disrupted and dilated lymphatics were detected in the upper dermis. There was follicular hyperkeratosis, hypergranulosis and spongiosis of the hair follicle infundibulum. Ducts of apocrine glands were dilated, sebaceous glands exhibited mild hyperplasia and occasionally arrector pili muscles were disrupted. Dermal pathology was most pronounced at 6 and 9 weeks post-infestation and was regressing by 12 weeks. At 12 weeks the number of hair follicles containing hair shafts appeared to be reduced and dermal fibrosis was evident. Dermal pathology after challenge infestation was relatively muted. Three weeks after challenge focal dermal edema was observed in three out five sheep but hair follicle pathology (spongiosis, hypergranulosis, hyperkeratosis) was detected in only one animal. Although dilatation of apocrine glands was detected sebaceous gland hyperplasia was not observed. At 6 weeks dermal fibrosis was evident.
Figure 2.13  Parafollicular subcorneal eosinophilic pustules

(Original magnification x100, carbol chromotrope.)
Multiple subcorneal pustules and vesicles and extensive disruption of epidermal architecture at the advancing margin of lesional skin nine weeks after infestation. (Original magnification x40, haematoxylin and eosin.)
2.3 EXPERIMENT 2. THE LESIONAL DISTRIBUTION OF MITES

2.3.1 Materials and Methods

Ten sheep were euthanased 8 weeks after infestation with 25-50 ovigerous *P. ovis* mites. Immediately after euthanasia the fleece was clipped to expose the lesion and a sample of skin (3 cm²) was excised from each of the following sites: the original area of infestation, the advancing margin of the lesion and mid-way between these sites. The skin samples were placed in Petri dishes and allowed to stand at room temperature. After 18 hours mites had migrated from the skin onto the lid of the Petri dish. Estimates of mite numbers were made as follows: numbers of mites on the lids from all Petri dishes were given a score from 0 to 4, subsequently the number of mites on a representative sample of scored lids was counted (section 11.6). No attempt was made to distinguish between larvae, nymphs and adults.

2.3.2 Results

Mites were found throughout the lesion but their distribution was not uniform (Table 2.1). Numbers were generally greatest at the advancing margin of lesions and lowest at the original area of infestation.

Table 2.1. Distribution of mites on lesional skin of *P. ovis* infested sheep

<table>
<thead>
<tr>
<th>Sheep No</th>
<th>Original infestation area</th>
<th>Mite score</th>
<th>Advancing margin area</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Original infestation area</td>
<td>Mid-lesion area</td>
<td>Advancing margin area</td>
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<tr>
<td>1</td>
<td>*</td>
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<tr>
<td>10</td>
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</table>

0 - no mites seen, * 5-50 mites, **50-200 mites, ***200-500 mites, ****> 500 mites
2.4 DISCUSSION

In the present investigation, the essential elements of lesional histopathology in primary and challenge infestations, included intense dermal infiltration dominated by eosinophils and accompanied by marked increase in mast cell numbers and degranulation and pronounced dermal oedema. These features are consistent with the occurrence of IH that is characterised by eosinophil infiltration and mast cell degranulation and accompanied by antigen-specific IgE (Romagnani 1994, Costa et al. 1997, Weller 1997). In addition, marked proliferative and degenerative epidermal pathology was observed in primary infestations.

Similar elements were observed in earlier studies of calves and sheep infested with *P. ovis* though the latter studies did not present such a consistent pattern of intense eosinophil infiltration or comment on the involvement of mast cells (Rapeanu 1972, Rovere and Nunez 1977, Abu-Samra et al. 1981, Stromberg and Fisher 1986, Rosa and Moschini 1988, Stromberg and Guillot 1989). Moreover, none of these studies quantified the numbers of different inflammatory cells nor examined the dynamics of cellular infiltration throughout the course of infestation. The present study has demonstrated clearly that eosinophils dominate the immunoinflammatory response to *P. ovis* and that neutrophils comprise a minor component of the infiltrate. It was also evident that eosinophil counts increased progressively from 3 to 9 weeks after infestation. This may indicate that eosinophil recruitment is dependent on number of distinct mechanisms, such as mite-derived eosinophil chemoattractants, immediate hypersensitivity, the late phase response and possibly the recently proposed Th2-like cytokine mediated delayed-type hypersensitivity response (Frew and Kay 1988, Parrish and Breathnach 1996, Meeusen 1999).
Eosinophil counts also paralleled the severity of lesional pathology. This was greatest from 6 to 9 weeks and it was also over this period that lesional growth plateaued. The release of mediators expressed by eosinophils and particularly the granule proteins has been cited as a cause of tissue damage in allergic and parasitic disease and may have contributed to lesional pathology (Frigas and Gleich 1986, Frigas et al. 1991). Human eosinophils express transforming growth factor-β1 (TGFβ1) which induces dermal fibrosis (Kadin et al. 1993). Expression of TGFβ1 by infiltrating eosinophils may contribute to the fibrosis seen observed in primary and challenge infestations with *P. ovis*. The possible involvement of eosinophils in resistance to parasites will be referred to later. Sequential mast cell counts indicated that after an initial increase, numbers of detectable cells fell significantly from 3 to 6 weeks. This was probably attributable to extensive mast cell degranulation which may have coincided with production of antigen-specific IgE though a significant rise in serum levels was not detected until 7 weeks after infestation (section 6.3.1.2).

The present study also demonstrated that pathology at the mid-lesion site and advancing margin was not significantly different but was more severe than at the site of infestation. This variation in severity of histopathological changes may reflect the distribution of mites. Clinical observation of lesions in sheep has suggested that the mite population is concentrated at the interface of lesional and normal skin (Kirkwood 1986, Bates 1997a). However, a recent report has suggested that in sheep, as in cattle, *P. ovis* mites were distributed uniformly throughout the lesional area (Wall et al. 1999). The present investigation indicated that generally the highest concentration occurred at the advancing edge and the lowest at the site of infestation. This was compatible with lesional histopathology, as reduced density of
mites at the site of infestation was likely to result in a patchy and less intense inflammatory response while a higher concentration of mites at the other sites would elicit a more generalised and pronounced response. The reason for this differential distribution of mites is uncertain. However, the detection of a significantly higher proportion of abnormalities in sections of mites located at the margin of the scab lesion than in mites from adjacent normal skin suggests that the environment of the lesion is inimical to mite survival (Sinclair and Filan 1991).

Histopathology of *P. ovis* infestations suggests that the immediate hypersensitivity response is involved in the immunopathogenesis of cutaneous lesions. Earlier studies of experimental infestations in naive cattle and sheep have suggested that an initial ‘lag’ phase with minimal increase in mite population and lesional area, is succeeded by a phase of rapid growth in the mite population and lesional area that is promoted by the development of immediate hypersensitivity to mite antigens (Pruett *et al.* 1986, Stromberg *et al.* 1986, Bates 1997a). However, this suggestion was based on arithmetic as opposed to logarithmic data for mite numbers and lesional area. Logarithmic transformation of the values for lesional area obtained in Experiment 1 indicated that throughout the first 7 weeks of primary infestations the increase in lesional area was exponential with no evidence of either a ‘lag’ phase or a phase of accelerated lesional expansion attributable to the development of an immediate hypersensitivity response. This exponential increase in lesional area is consistent with the prediction, based on mathematical modelling, of an exponential growth of the mite population, following a brief period of instability, immediately after infesting sheep with 25 ovigerous mites (Wall *et al.* 1999).
The observation, in this study, of a statistically significant reduction in
lesional growth following challenge infestation has provided convincing evidence of
the development of a substantial protective immunity to *P. ovis* in sheep. This
confirms observation of restricted lesional growth after challenge infestation of cattle
and rabbits and more recently in 3 sheep (Stromberg and Fisher 1986, Uhlir 1991,
Bates 2000). Currently the mechanism mediating protection is unknown. The
present study failed to detect significant differences in numbers of eosinophils and
mast cells 3 weeks after primary and challenge infestations. However, clinical
observation indicated that challenge infestations elicited a more rapid and
pronounced inflammatory response and if biopsies had been collected earlier, for
example within one week of infestation, there may have been substantial differences
in cell counts.

The potential contribution of cells associated with IH and CBH to protection
has been suggested by studies of resistance to ticks. These have demonstrated
reduced resistance in Guinea pigs administered anti-basophil or anti-eosinophil
antibodies and the failure of mast cell-deficient mice to acquire resistance to
infestation with ticks (Brown *et al*. 1982, Matsuda *et al*. 1985). In addition,
eosinophils and basophils have been detected in the digestive system of ticks and
granules from both cells have been demonstrated in damaged midgut cells
(Voss-McCowan 1996). Eosinophils have also been observed in the midgut of
*P. ovis* mites obtained from infested sheep (Matthieson 1995) and may together with
ingested basophils cause similar damage in *P. ovis* mites. The significant increase in
histologically detectable mast cells observed 3 weeks post-infestation was followed
by a decline in detectable cells. But these alterations in mast cell numbers during the
first 6 weeks after primary infestation were not accompanied by any detectable change in the rate of lesional expansion. However, observations in calves have demonstrated that fecundity of mites placed on *P. ovis*-naive calves was initially high and then declined whereas in challenge infestations the fecundity of mites was consistently low (Stromberg and Fisher 1986, Guillot and Stromberg 1987). In primary infestations this would result in a delayed fall in mite numbers and would be compatible with the observation in Experiment 1 of a reduction in lesional expansion from 7 weeks after infestation. In challenge infestations, early impairment of mite fecundity, if severe enough, could result in failure of the challenge population to expand and may explain the apparent inability, noted in Experiment 1, of challenge infestations to become established. The contribution of IgE-mediated Type I allergic reactions to protective immunity would be consistent with this temporal reduction in mite fecundity. It has also been suggested that IH to mite allergens may increase pruritus and stimulate grooming which will contribute to the reduction in the mite population especially on challenged cattle (Pruett *et al.* 1998). However, in the present study, no evidence of increased grooming or self-trauma by challenged sheep was observed.

The investigations described in this chapter have provided evidence for the development of a significant protective immunity following primary infestations of sheep with *P. ovis*. They also suggest that mite products stimulate a multilayered immune response possibly involving an IgE-mediated Type-1 allergic reaction augmented by late phase and delayed-type hypersensitivity responses. In addition, the detection of basophils in the infiltrate, despite their poor morphology, may indicate the participation of CBH in the immune response but this requires further
investigation. The contribution of these hypersensitivity reactions to the protective immunity is unclear. Transepidermal trafficking of mast cells, eosinophils and basophils and efflux of antigen-specific immunoglobulins may influence mite survival and modulate mite fecundity but further investigation to elucidate their role is required. Determination of the mechanisms involved may contribute to the development of an effective vaccination strategy.

The next chapter will examine lesional pathology in the period immediately after infestation and subsequent chapters will examine evidence of the involvement of hypersensitivity reactions in the immune response to *P. ovis*.
3. CUTANEOUS HISTOPATHOLOGY IN THE PERIOD IMMEDIATELY AFTER PRIMARY INFESTATION OF SHEEP.

3.1 INTRODUCTION

Histopathology has demonstrated that ticks provoke an influx of inflammatory cells within 1 to 24 hours of attachment to tick-naïve hosts (Tatchell and Moorhouse 1968, Allen et al. 1977, Brown et al. 1984). Although, in contrast to ticks, there is no indication that the mouthparts of *P. ovis* penetrate the epidermis, clinical observation has indicated that within hours of experimental infestation of naïve sheep, *P. ovis* provoked a papular inflammation that was succeeded by the development of vesicles and pustules (Spence 1949, Sinclair and Filan 1989). The clinical observations reported in the previous chapter confirm this. However, the histopathology of events immediately following infestation does not appear to have been studied. These early inflammatory events may be of pivotal importance in the determining the nature of the subsequent adaptive immune response. Elucidation of this initial inflammatory response may, therefore, make a significant contribution to understanding the immunopathogenesis of cutaneous lesions and also to the development of an effective vaccination protocol for *P. ovis*.

In this chapter the temporal development of the cellular infiltrate and general lesional histopathology at 24 hours and 4, 8, 14 and 21 days after infestation of *P. ovis*-naïve sheep are described.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Five sheep Suffolk cross sheep aged between one and two years were infested, as described previously (section 2.2.1.1), with two doses of 25-50 ovigerous
*P. ovis* mites one to the right and the other to the left of the withers. After injection of a local anaesthetic (Lignol, Arnolds Veterinary Products), 6mm punch biopsies were collected from normal skin and from one of the two infestation sites 24 hours post-infestation. The latter biopsy obliterated this infestation site and further biopsies were collected from the advancing margin of the lesion at the contralateral site and from normal skin 4, 8, 14 and 21 days after infestation.

### 3.2.2 Histopathology

This was carried out as described previously (2.2.1.3).

### 3.2.3 Statistical analysis

For the reasons given previously (2.2.1.4), differences between cell counts in normal and lesional skin were determined by analysis of co-variance.

### 3.2.4 Image capture

Images were captured using the method employed previously (2.2.1.5).

### 3.3 RESULTS

#### 3.3.1 Cellular infiltration

**Eosinophils:** A significant (*p*<0.004) influx of eosinophils was detected at 24 hours (Figures 3.1 and 3.2a) and many of these cells were degranulated. Numbers of eosinophils increased from 24 hours to 3 weeks after infestation (Figure 3.2a).

**Mast cells and basophils:** A significant increase in mast cell counts (*p*<0.001) was evident by 4 days and numbers continued to increase until three weeks after infestation (Figure 3.2b). Basophils were not seen in normal skin but were detected in lesional tissue 24 hours post-infestation but as noted previously were frequently difficult to differentiate from mast cells.
Neutrophils: Compared to eosinophils, neutrophils comprised a minor component of the infiltrate. A significant ($p<0.001$) influx of cells was evident at 24 hours, numbers increased slightly and then declined after 8 days (Figure 3.2c).

3.3.2 Epidermal and dermal pathology

The essential features of epidermal and dermal pathology were no different from those described in detail in the previous chapter. However, the rapid development of cutaneous pathology following infestation was striking. Subcorneal eosinophilic pustules, transepidermal trafficking of eosinophils (Figure 3.1) and degenerative epidermal pathology and dermal oedema (Figure 3.3) were detected at 24 hours. By 4 days multifocal, proliferative epidermal pathology and disruption of dermal collagen bundles was evident.
Figure 3.1  Eosinophil infiltration 24 hours after primary infestation

Marked eosinophil infiltration and subcorneal pustule formation at the advancing margin of lesional skin 24 hours after infestation.
(Original magnification x250, carbol chromotrope.)
Figure 3.2  Cell counts in normal skin and at the advancing margin of lesional skin from 1 to 21 days after primary infestation

Eosinophil and neutrophil counts were significantly higher in lesional than normal skin 24 hours after infestation ($p<0.004$ and $p<0.001$, respectively) and mast cell counts were significantly higher ($p=0.001$) 4 days after infestation.
Cellular oedema accompanied by disruption of intercellular bridges and hydropic degeneration of cells in the stratum spinosum 24 hours after infestation. There is transepidermal trafficking of eosinophils and an acanthocyte is present (black arrow). (Original magnification x400, haematoxylin and eosin.)
3.4 DISCUSSION

The cutaneous immunoinflammatory response to arthropods is profoundly influenced by the cytotoxic and immunopharmacological properties of ectoparasite products and to a lesser extent by the site at which these are deposited (Burns 1996, Wikel 1996a). Electron microscopic examination has indicated that the mouthparts of *P. ovis* are adapted for siphoning surface fluids and may abrade the stratum corneum (Blake *et al.* 1978). However, there is no evidence of penetration beyond the inner layers of the stratum corneum (Sinclair and Filan, 1989). It appears, therefore, that mite products are deposited on intact or abraded stratum corneum with subsequent translocation through the epidermis to interact with cells of the skin immune system.

Spence (1949) noted clinical evidence of a papular inflammatory response within hours of infestation. Observations made in the previous study (section 2.2.2.1) confirmed this and lesional histopathology, recorded in this chapter, revealed that mite products provoked a prompt and intense inflammatory response, characterised by marked eosinophil and mast cell infiltration. In calves the response appears to be slower as a detailed study of animals infested with two doses of *P. ovis* mites, at an interval of 21 days, recorded only minimal histopathological changes one week after the final dose had been applied (Stromberg and Fisher 1986). This may reflect significant differences in transepidermal penetration of mite products and/or the cutaneous response of sheep and cattle to these substances.

Eosinophil and neutrophil chemoattractant properties of Der p 1, a significant constituent of faecal pellets voided by *D. pteronyssinus*, have been demonstrated (Fahy *et al.* 2000). House dust mite extract has also been shown to stimulate
expression of potent neutrophil chemoattractant cytokines, IL-8 and TNFα, by cultured epithelial cells while Der p 3 and Der p 9 stimulate expression of eotaxin, a potent eosinophil chemokine by lung epithelial cells (Lau et al. 1999, Sun et al. 2001). The rapid infiltration of eosinophils, observed in the present investigation, is consistent with the presence of potent eosinophil chemoattractants in the products of *P. ovis*. This chemoattractant activity may be augmented by the release of chemokines by epidermal and dermal cells (section 1.1.3.1). It is evident from the magnitude of eosinophil recruitment that the immediate, innate response contributes significantly to the influx of eosinophils seen in *P. ovis* infestations. However, the continued increase in lesional eosinophils seen in primary infestations from 3 to 9 weeks (section 2.2.2.2.1), suggests that during this period the early innate response is augmented by immediate hypersensitivity and possibly late phase and delayed-type hypersensitivity responses (Frew and Kay 1988, Meeusen 1999).

In the present investigation, epidermal pathology was also observed within 24 hours of infestation. This may be considered a sequel to the intense inflammatory response provoked by *P. ovis* products (Stromberg and Fisher 1986). However, these include an array of enzymes and other biologically active compounds which may have a direct effect on epidermal cells and architecture (Nisbet and Billingsley 1999). For example, Der p 1, a cysteine protease, has been shown to disrupt epithelial architecture and increase permeability of the bronchial mucosa (Herbert et al. 1995, Wan et al. 1999). A Der p 1 homologue has recently been identified in a *P. ovis*-cDNA expression library (Isaacs and Lee, personal communication). By analogy, this and other enzymes contained in faecal pellets of *P. ovis* may exert a direct effect on keratinocytes and provoke degenerative and
proliferative pathology and increase epidermal permeability, facilitating penetration of biologically active substances and allergens. Direct action of these substances may account for the degranulation of eosinophils and mast cells detected in the early stages of infestation. It may also contribute to the disruption of collagen bundles.

Cytokines, released by keratinocytes, mast cells and eosinophils may contribute significantly to polarisation of the developing adaptive immune response towards an IgE-mediated Type 1 allergic reaction. In this respect IL-4 and IL-13 are of particular importance (Fearon and Locksley 1996, Medzhitov and Janeway 1997, Corry and Kheradmand 1999, Finkelman et al. 1999). In vitro studies have demonstrated that parasite allergens with proteolytic activity, such as Der p 1, trigger release of IL-4 from mast cells and basophils and it is possible that the same mechanism may trigger the release of IL-13 (Machado et al. 1996). IL-4 is also expressed by human eosinophils (Weller 1997) and may therefore be released by degranulating ovine eosinophils that were invariably detected in lesional sites within hours of infestation.

The study carried out in this chapter has demonstrated that mite products stimulate a rapid and intense inflammatory response. Proteolytic activity of enzymes released from faecal pellets may play a pivotal role in the immunopathogenesis of cutaneous lesions by enhancing transepidermal penetration of antigens/allergens and other biologically active substances produced by *P. ovis* mites. Together with these, they may be responsible for the composition of the early inflammatory infiltrate and generate conditions promoting the development of IgE-mediated Type I allergic reactions.
4. IDENTIFICATION OF DENDRITIC CELLS AND LYMPHOCYTE INFILTRATES IN LESIONAL SKIN OF SHEEP WITH PRIMARY INFESTATIONS.

4.1 INTRODUCTION

Lesional histopathology has demonstrated that *P. ovis* infestation of cattle elicited a mixed cellular infiltrate that included substantial numbers of lymphocytes but in sheep only small numbers of lymphocytes were observed (Stromberg and Fisher 1986, Rosa and Moschini 1988). However, no analysis of the lymphocyte subpopulations infiltrating these lesions has been reported. Indeed, lymphocyte subpopulations involved in cutaneous responses to arthropod ectoparasites have been characterised in only a limited number of infestations, notably *L. cuprina, I. ricinus* and *S. scabiei* (Bowles et al. 1992, 1994, Elhay et al. 1994, Mbow et al. 1994, Stemmer et al. 1996, Arlian et al. 1997).

In view of the critical role played by dendritic and T cells in orchestration of the immune response, identification of the different cell subtypes infiltrating cutaneous lesions is essential in determining the immunopathogenesis of sheep scab and may be of benefit in devising a vaccination strategy.

The kinetics of eosinophil and neutrophil infiltration and mast cell hyperplasia in primary infestations with *P. ovis* were described in the last chapters. In the study described in this chapter, a panel of monoclonal antibodies (Mabs) was employed to determine sequential alterations in dendritic cells and lymphocyte subpopulations involved in the cutaneous immune response of sheep to primary infestations with *P. ovis.*
4.2 MATERIALS AND METHODS

4.2.1 Animals

Suffolk cross sheep, aged between one and two years and with no previous exposure to *P. ovis*, were used in this experiment. Five sheep were infested to the left of the withers with a cluster of 25-50 ovigerous *P. ovis* mites as indicated earlier (2.2.1.1). In one animal the infestation failed to be established, consequently it was omitted from the results. Six sheep were used as uninfested controls.

4.2.2 Histopathology

After subcutaneous injection of a local anaesthetic, 6mm punch biopsies were taken from the advancing margin of the lesion 3, 5, 7 and 9 weeks post-infestation. A biopsy was also collected from the withers area of the 6 uninfested, control sheep. Immediately after collection biopsies were bisected.

Half the biopsy was fixed in 4% paraformaldehyde, processed and later 5μm sections were stained with carbol chromotrope and eosinophil counts performed as described in section 2.2.1.3. For the purpose of this investigation it was decided to relate the lymphocyte responses to eosinophil counts as these had proved to be a sensitive indicator of the inflammatory cell response to infestation.

4.2.3 Immunohistochemistry

The other half of the biopsy was fixed in a non-aldehyde, zinc salts fixative (ZSF) as described by Gonzalez *et al.* (2001). Briefly, the biopsy was immersed in ZSF solution (0.1M Tris buffer with Ca acetate 0.05% [pH 7-7.4], containing Zn acetate 0.5% and Zn chloride 0.5%, section 11.2) for 72 hours at room temperature (RT). The biopsy was then placed in plastic cassettes and transferred to 78% ethanol 30 minutes before processing to paraffin wax. Sections 5μm thick
were cut, placed on treated glass slides (Superfrost Plus: Menzel-Glazer, Germany) and dried overnight at 37°C.

The EnVision Plus HRP System (Dako, Ely, UK) was used to amplify labelling with Mabs selected from those available at the Moredun Research Institute (Table 4.1). Sections were de-waxed and then submitted to two blocking procedures, quenching endogenous peroxidase activity with 0.03% hydrogen peroxide for 5 minutes at RT and then blocking of non-specific tissue antigens with 25% normal goat serum in Tris buffered saline (TBS, [0.05M Tris HCl, 0.015M NaCl, pH 7.2-7.6]) for 30 minutes at RT. Sections were then incubated overnight at 4°C with Mab in TBS at the dilutions detailed in Table 4.1. This was followed by incubation with the secondary antibody (peroxidase–labelled polymer conjugated to goat anti-mouse immunoglobulins) for 30 minutes at RT. Conjugate binding was detected by incubating with the substrate chromogen, 3,3'-diaminobenzidine (DAB) and hydrogen peroxide, for 7-8 minutes at RT. Sections were washed in TBS between each stage of the labelling procedure and after a final wash with distilled water, they were counter-stained with Haematoxylin “Z”, rinsed, dehydrated in graded alcohols, cleared and mounted under Shandons synthetic mountant. A negative control prepared by omission of the primary antibody was provided for each of the monoclonal antibodies used. Preliminary examination of sections revealed that the labelled cells were concentrated in the superficial dermis, therefore, the graticule was placed immediately below the stratum corneum and cells were counted in 5 successive fields, on each side of the midline (a minimum area of 0.2mm²).

4.2.4 Statistical analysis

As the number of animals was small and comparisons were made within a group of animals the significance of differences in cell counts in the course of the
infestation was determined by analysis of variance using a GenStat release 4.2 programme (5th edition), (Lawes Agricultural Trust; Numerical Algorithms Group Ltd, Oxford, UK). Values of \( p < 0.05 \) were considered significant.

**Table 4.1 Monoclonal antibodies used to characterise lymphocytes and dendritic cells in normal and lesional skin fixed in zinc salt fixative**

<table>
<thead>
<tr>
<th>Mab designation</th>
<th>Source</th>
<th>Dilution</th>
<th>Antigen specificity</th>
<th>Cellular expression</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>17D</td>
<td>Basel</td>
<td>1:50 SN</td>
<td>CD4</td>
<td>T-helper cells</td>
<td>Maddox et al. (1985)</td>
</tr>
<tr>
<td>SBU-T8*</td>
<td>Melbourne</td>
<td>1:50 SN</td>
<td>CD8</td>
<td>T-cytotoxic cells</td>
<td>Maddox et al. (1985)</td>
</tr>
<tr>
<td>CC15</td>
<td>Compton</td>
<td>1:10,000 AF</td>
<td>WC-1</td>
<td>( \gamma \delta ) T cells</td>
<td>Howard et al. (1989)</td>
</tr>
<tr>
<td>CC20</td>
<td>Compton</td>
<td>1:5,000 AF</td>
<td>CD1b</td>
<td>Dendritic cells</td>
<td>Howard et al. (1993)</td>
</tr>
<tr>
<td>73B</td>
<td>Basel</td>
<td>1:100 SN</td>
<td>CD45RA</td>
<td>B cells, subset of naive T cells</td>
<td>Mackay et al. (1990)</td>
</tr>
</tbody>
</table>

1 Basel: Basel Institute for Immunology, Basel, Switzerland. Melbourne: University of Melbourne, School of Veterinary Science, Parkville, Victoria, Australia. Compton: BBSRC Institute for Animal Health, Compton Laboratory, Newbury, Berks, UK.

2 AF, Mabs from ascites fluid
SN, Mabs from tissue culture supernatant

3 CD, cluster designation; WC-1, workshop cluster-1

* Clone 38-65

**4.3 RESULTS**

Cell counts in normal and lesional skin are expressed graphically in Figure 4.1 and photographs of the lymphocyte subpopulations in normal and lesional skin are shown in Figures 4.2-4.4. All the monoclonal antibodies employed labelled
cells in sections of normal and lesional skin but no labelling was detected in the negative controls.

Normal skin contained few lymphocytes and dendritic cells and no eosinophils (Figure 4.1). γδ T cells were the most abundant lymphocytes followed by CD45RA⁺ lymphocytes. Dendritic cells (CD1⁺) and CD4⁺ T lymphocytes were seen less frequently and were present in similar numbers. CD8⁺ T lymphocytes were detected occasionally.

After infestation the populations of lymphocytes and dendritic cells were maximal from 3 to 5 weeks after infestation and then declined. CD4⁺ T cells were distributed uniformly throughout superficial dermis with smaller numbers of cells in the deep dermis. There was a pronounced concentration of CD45RA⁺ lymphocytes in the superficial dermis with fewer cells, usually clustered perivascularly, in the deep dermis. Dendritic cells (CD1⁺) and γδ T cells were also concentrated in the superficial dermis with smaller numbers of cells located principally in perivascular tissue of the deep dermis. Some CD1⁺ cells, presumably Langerhans' cells were detected in the epidermis. The CD8⁺ lymphocytes appeared to be scattered randomly throughout the superficial and deep dermis. Eosinophil counts were raised from 3 to 9 weeks (Figure 4.1a) and although mean count fell at 7 weeks this fluctuation was not statistically significant.

At 3 weeks numbers of CD4⁺ and γδ T cells, CD45RA⁺ and CD1⁺ cells were significantly greater in lesional than normal skin (p<0.01, p<0.001, p<0.001, p<0.001, respectively). From 3 to 7 weeks after infestation CD4⁺ and CD45RA⁺ cells, accompanied by smaller numbers of CD1⁺ cells, dominated the mononuclear infiltrate. In normal skin the ratio of CD4⁺/CD8⁺ was 1.9/1 but at 3, 5 and 7 weeks after infestation this ratio had risen dramatically to 49.7/1, 15.2/1 and 70.5/1,
respectively. From 7 to 9 weeks numbers of CD4\(^+\), CD45RA\(^+\) and CD1\(^+\) cells in lesional skin declined significantly, \(p<0.01\), \(p<0.02\) and \(p<0.02\), respectively. By 9 weeks after infestation, numbers of CD4\(^+\) and CD1\(^+\) cells in normal and lesional skin were not significantly different, though numbers of \(\gamma\delta\) T cells and CD45RA\(^+\) remained significantly higher in lesional than normal skin, \(p<0.05\) and \(p<0.01\), respectively. However, numbers of CD8\(^+\) cells were significantly lower (\(p<0.05\)) in lesional than normal skin at 9 weeks.
Figure 4.1 Counts of eosinophils and subpopulations of lymphocytes and dendritic cells in normal skin and the advancing margin of lesional skin at different times during primary infestations.

a) Eosinophils

b) CD4+ T cells (17D)

c) CD8+ T cells (SBU-T8)

d) WC-1+ γδ T cells (CC15)

e) CD1+ dendritic cells (CC20)

f) CD45RA+ B cells/naive T cells (73B)

For each cell type, counts with the same letter are significantly different (a and c, p<0.01; b, f and k, p<0.001; d and h p<0.02; i, p<0.03; e, p<0.05)
Figure 4.2 Immunolabelling of CD4+ and CD8+ T cells (normal and lesional skin with monoclonal antibodies 17D and SBU-T8, respectively. (Original magnification x250, insets to show detail of a selected field are x500)

CD4+ T cells in normal skin

CD4+ T cells in lesional skin 5 weeks after infestation

CD8+ T cells in normal skin

CD8+ T cells in lesional skin 5 weeks after infestation
Figure 4.3 Immunolabelling of $\gamma^\delta$ T cells (WC-1') and dendritic cells (CD1') in normal and lesional skin with monoclonal antibodies CC15 and CC20, respectively. (Original magnification x250, insets to show detail of a selected field are x500)

$\gamma^\delta$ T cells in normal skin

$\gamma^\delta$ T cells in lesional skin 3 weeks after

Dendritic cells in normal skin

Dendritic cells in lesional skin 7 weeks after infestation
Figure 4.4 Immunolabelling of CD45RA⁺ B/naive T cells in normal and lesional skin with monoclonal antibody 73B. (Original magnification x250)

CD45RA⁺ cells in normal skin

CD45RA⁺ cells in lesional skin
3 weeks after infestation
4.4 DISCUSSION

In this and earlier investigations of the T cell population of normal ovine skin γδ cells were the most abundant subtype with smaller numbers of CD4\(^+\) and CD8\(^+\) cells (Bowles et al. 1992, McElroy et al. 1998, Haig et al. 1999). Previously, CD45R\(^+\)/CD45RA\(^+\) cells have been described as absent or infrequent in normal ovine skin and CD1\(^+\) cells were detected only occasionally (Bowles et al. 1992, Mwangi et al. 1990, Gorrell et al. 1995, McElroy et al. 1998, Anderson et al. 2001). However, in the present study, after γδ T cells, CD45RA\(^+\) cells were most prevalent cell population and CD1\(^+\) cells were as abundant as CD4\(^+\) cells. The use, in the present study, of a novel method of fixation (zinc salts fixation) may have contributed to the dissimilarities in numbers of cells detected. This new fixative was chosen as it provided excellent tissue morphology combined with good retention of antigenic reactivity of different ovine cell-surface epitopes to a number of monoclonal antibodies (Gonzalez et al. 2001, Stanley et al. 2001). The CD45R marker has been detected on the majority of ovine B cells and a subpopulation of naïve T cells (Mackay et al. 1990, Dutia et al. 1993). Studies in adult sheep demonstrated that naïve T cells selectively recirculate through peripheral lymph nodes rather than non-lymphoid tissue and that only cells with the memory phenotype (CD45R\(^+\)) homed to skin (Mackay et al. 1990, Mackay et al. 1992, Kimpton et al. 1995). These observations suggest that the CD45RA\(^+\) cells detected in the present investigation were B cells although further studies using double labelling for surface IgM and CD45RA would be required to confirm this.

The present investigation has shown that the lymphocyte infiltration elicited by P. ovis was dominated by helper/inducer T cells (CD4\(^+\)) and putative B cells (CD45RA\(^+\)), accompanied by a significant though less pronounced increase in
dendritic cells (CD1+). The dramatic increase observed in numbers of CD4+ and CD45RA+ cells relative to γδ T cells is consistent with the selective recruitment of these cells to lesional skin. CD4+ T cells also dominated the lymphocyte response to primary infestations of sheep with L. cuprina (blowfly) and of mice with I. ricinus nymphs and in the case of L. cuprina infections were accompanied by smaller increases in γδ and CD8+ T cells (Bowles et al. 1992, Elhay et al. 1994, Mbow et al. 1994). In the present investigation, the CD4+/CD8+ ratio, 15.2/1 to 70.5/1, was considerably greater than that noted in primary infections with L. cuprina and I. ricinus, 5/1 and 2.2/1, respectively. However, in these experiments the lymphocyte infiltrate was examined only for up to 72 hours after primary infestation, therefore a time-limited expression of the immune response to a discrete challenge was observed. This contrasts with the present investigation in which the extended period of primary infestation, up to 9 weeks, was equivalent to a continuous succession of challenges that may account for the heightened CD4+ response. The abundance of eosinophils in the inflammatory infiltrate (section 2.2.2.2.1) suggests that the CD4+ cells express a T helper 2-like cytokine profile but this needs to be confirmed by further investigations. At 9 weeks after infestation, despite a significant fall in numbers of CD4+ cells, there was an apparent increase in eosinophil numbers. Although T cell-derived cytokines play a key role in orchestrating eosinophil recruitment, mast cells, eosinophils, monocytes/macrophages and fibroblasts contribute several cytokines and chemoattractants, such as IL-4, IL-5, GM-CSF, PAF, MCP-3, eotaxin and RANTES, that are critical to eosinophil recruitment (Gonzalo et al. 1996, Costa et al. 1997, Weller 1997, Lamkhioued et al. 2000, Teran et al. 1999, Ying et al. 1999a, 1999b). Moreover, observation of a dramatic influx of eosinophils into the dermis within 24 hours of infestation (section 3.3.1) indicated
that mite products initiate eosinophil recruitment independently of antigen-specific T cell activity. The detection, in the present study, of a decline in CD4\textsuperscript{+} T cells but not eosinophils suggests that the contribution of a particular source of cytokines and chemoattractants to eosinophil recruitment varies in the course of disease.

There was also a significant increase in numbers of γδ T cells. Increased numbers of γδ lymphocytes have also been reported in primary and secondary infections with \textit{L. cuprina} (Bowles \textit{et al.} 1992). The reason for this response is not clear. γδ cells are activated by bacterial lipoproteins and, therefore, it may be argued that they are responding to secondary bacterial challenge. However, it is notable that infections with \textit{L. cuprina} are accompanied by a marked infiltrate of neutrophils, an observation compatible with the presence bacterial infection, whereas in \textit{P. ovis} infections the number of neutrophils detected is relatively small. Alternative possibilities are that mite products contain significant amounts of lipoproteins or other substances that activate γδ lymphocytes or, as suggested recently, that γδ together with CD4\textsuperscript{+} cells are involved in the elicitation phase of delayed-type hypersensitivity (Jorundsson \textit{et al.} 1999).

In the present investigation numerous putative B cells (CD45RA\textsuperscript{+}) were detected in lesional skin. Although only small numbers of B cells (CD45R\textsuperscript{+}, sIg\textsuperscript{+}) were observed 48 hours after infestation with \textit{L. cuprina}, numbers had increased substantially at 72 hours but no increase in B cells (CD45R\textsuperscript{+}) was detected in primary infestations of mice with \textit{I. ricinus} (Bowles \textit{et al.} 1994, Mbow \textit{et al.} 1994). The latter observation may again reflect the time-limited observation of the immune response. In this context, it is notable that 5 days after experimental infection of sheep with \textit{Trypanosoma congolense} the lesional lymphocyte infiltrate was dominated by CD4\textsuperscript{+} and B cells (CD45R\textsuperscript{+}) (Mwangi \textit{et al.} 1990). B cells have also
been detected in *S. scabiei* infestations of man and pigs (Reunala *et al.* 1984, Morsy and Gaafar 1989) but, intriguingly, immunohistochemical analysis of the lymphocyte infiltrate in lesional skin from infested dogs revealed only small numbers of B cells (Arlian *et al.* 1997). In *P. ovis* infestation, antibody synthesis by lesional B cells will augment that produced in the regional lymph nodes and may contribute to host defence. In addition, lesional B cells may act as antigen presenting cells and increase T cell activation (Pirron *et al.* 1990, Gajewski *et al.* 1991).

A significant increase in the population of dendritic cells (CD1+) was observed in the present investigation. Similarly, increased numbers of dendritic cells were detected in lesional skin of dogs infested with *S. scabiei* (Stemmer *et al.* 1996). However, primary infestations of *L. cuprina* and *I. ricinus* did not result in a significant increase in numbers of dendritic cells detected (Bowles *et al.* 1992, Mbow *et al.* 1994). This may again be attributable to the time-limited nature of these experiments. Expansion of the population of dendritic cells, seen in the present study, is consistent with increased presentation of antigens to naïve T cells in drainage lymph nodes and amplification of the immune response. Dendritic cells may also augment the local immune response by presentation of antigen to effector T cells in lesional tissue (Mudde *et al.* 1990).

It is clear from the present experiment that CD4+, CD45RA+ and CD1+ cells dominated the profound cutaneous immune response elicited by *P. ovis* infestations. Different aspects of this acquired immune response will be examined in later chapters.
5. HAEMATOLOGY IN THE COURSE OF PRIMARY AND CHALLENGE INFESTATIONS.

5.1 INTRODUCTION

Studies in calves have demonstrated that primary infestations with *P. ovis* can exert a profound influence on circulating red and white blood cell counts and bone marrow activity (Stromberg *et al.* 1986, Stromberg and Guillot 1987a, 1987b). In cattle, secondary (challenge) infestations elicited less pronounced white blood cell responses than primary infestations (Losson *et al.* 1988). Although haematology has been monitored in the course of primary infestations of sheep with *P. ovis* it has not be examined in challenge infestations (O’Brien *et al.* 1995). The investigations in cattle and sheep have also demonstrated that treatment of infested calves and sheep with an acaricide resulted in rapid normalisation of red and white blood cell counts (Stromberg and Guillot 1989, O’Brien *et al.* 1995).

In chapter 2 lesional infiltration of mast cells and granular leukocytes in the course of primary and challenge infestations of sheep with *P. ovis* was examined. The alterations in circulating red and white blood cell cells, particularly those of eosinophils, basophils and neutrophils that accompanied these infestations and then followed endectocide treatment are described in the present chapter.

5.2 MATERIALS AND METHODS

5.2.1 Animals

Blood samples were collected from the three groups of sheep described in section 2.2.1.1.

*Primary infestation (Group 1)*

Blood samples were collected into heparinised vacutainers (Becton Dickinson, Franklin Lakes, NJ, USA) by jugular venipuncture at weekly intervals
until 14 weeks and then at 16 weeks after infestation. The sheep were treated with an endectocide (see section 2.2.1.1) at 12 and 13 weeks after infestation. Total red and white blood cell count was determined with a Baker 9110 Haematology Analyser (Biochem. Pharma. Inc., Allentown, PA, USA). The differential count was estimated manually after staining a blood smear with a modified Wright’s stain.

One hundred cells of each cell type were counted and the differential count per unit volume determined from this. The different cells were identified on the basis of characteristic features: neutrophils, band or multilobulated nucleus and pink/red cytoplasm; eosinophils, bilobed nucleus and red cytoplasmic granules; basophils, blue/purple densely packed granules often obscuring the multilobulated nucleus; lymphocytes, mononuclear cells with a sparse but variable amount of bluish cytoplasm; monocytes, mononuclear cells with an indented nucleus and abundant greyish cytoplasm containing variable numbers of vacuoles.

Challenge (secondary) infestation (Group 2) and Controls (Group 3)

Blood samples from these sheep were collected and examined at weekly intervals until 8 weeks after infestation.

5.2.2 Statistical analysis

Differences between red and white blood cell counts in the different groups of sheep were determined by analysis of co-variance as described earlier (2.2.1.4).

5.3 RESULTS

5.3.1 Red blood cell counts

Although in primary infestations (groups 1 and 3) mean counts gradually declined from pre-infestation numbers in the first 7 to 8 weeks after infestation, they remained at or above the upper limit of the normal range (Figure 5.1). After challenge infestation (group 2) mean counts initially increased and then stabilised,
also at the upper end of the normal range (Figure 5.1). No consistent statistical difference was found in counts recorded in challenge and primary infestations.

**Figure 5.1 Red blood cell counts in the course of primary and challenge infestations**

![Graph](image)

Dashed lines indicate lower and upper limits of the normal range
Black arrows indicate treatment with endectocide
Differences between primary and challenge infestations were not significant

### 5.3.2 White blood cell counts

The mean counts of the different white blood cells fluctuated in the course of infestation and individual animals, particularly those of group 3, differed considerably in their responses.

**Neutrophils:** After an initial fall, mean counts of group 1 (primary infestation) sheep increased until 8 weeks after infestation but no particular trend was apparent in counts of sheep from groups 2 and 3 (Figure 5.2). No consistent significant difference was detected in the neutrophil responses to primary and challenge infestations.
Figure 5.2 Neutrophil counts in the course of primary and challenge infestations

Caption as for Figure 5.1
Differences between primary and challenge infestations were not significant

Lymphocytes: In all groups mean counts initially developed a downward trend but remained within the normal range (Figure 5.3). In group 2 this trend was reversed at 7 weeks when numbers increased rapidly while in group 1 counts rose more gradually from 8 weeks after primary infestation. Counts in challenge (group 2) and primary infestations (groups 1 and 3) were not significantly different except at 8 weeks when lymphocyte numbers of group 2 animals were significantly higher ($p<0.001$) than those of group 1 and the mean count of group 2 sheep was higher than that of group 3.

Monocytes: Mean monocyte counts were erratic in all groups of sheep and no distinct trend in numbers was apparent (Figure 5.4). A marked increase in counts of group 1 animals occurred immediately after endectocide treatment at 12 weeks. Counts in sheep receiving challenge (group 2) and primary infestations (groups 1
and 3) were not significantly different except 1 week after infestation when counts in group 2 were significantly higher ($p<0.03$) than those in group 1. However, at this time the mean count of group 2 was lower than that of group 3.

**Figure 5.3** Lymphocyte counts in the course of primary and challenge infestations

![Graph showing lymphocyte counts over time for groups 1, 2, and 3.](image)

Caption as for Figure 5.1
Counts with the same letter are significantly different (a, $p<0.001$)

**Figure 5.4** Monocyte counts in the course of primary and challenge infestations

![Graph showing monocyte counts over time for groups 1, 2, and 3.](image)

Caption as for Figure 5.1
Counts with the same letter are significantly different (a, $p<0.03$)
Eosinophils: Primary infestation elicited progressive increases in mean counts in group 1 but appeared to provoke a biphasic eosinophil response in group 3 (Figure 5.5). All the sheep in these two groups developed eosinophilia (>1 x 10^9/l). In group 1 the count peaked at 11 weeks, one week before endectocide treatment, and then fell rapidly. After challenge infestation (group 2) there was a small and transient increase in eosinophil counts at 1 week but, in contrast to primary infestations, only one animal developed eosinophilia. Clinically, lesional development in this animal was no different from that of other sheep. Eosinophil counts in challenge infestations were significantly higher (p<0.001) than those of group 1 sheep at 1 week and were significantly lower (p<0.05) 8 weeks after infestation (Figure 5.5). At 5 and 8 weeks after infestation, they were also significantly lower (p<0.004) than those of group 3 sheep.

**Figure 5.5 Eosinophil counts in the course of primary and challenge infestations**

Caption as for Figure 5.1
Counts with the same letter are significantly different (a, p<0.001; b and e, p=0.004; c, p<0.03; d, p<0.05)
**Basophils:** Basophilia ($>0.3 \times 10^9/l$) was recorded in 6 of the 14 sheep that received a primary infestation (4 sheep in group 1 and 2 in group 3) and in one of the 5 sheep receiving challenge infestations (Figure 5.6). In these animals, a transient rise in basophil numbers, similar to that seen in the eosinophil counts, was detected one week after challenge. Differences between challenge (group 2) and primary infestations (groups 1 and 3) were inconsistent and not statistically significant.

**Figure 5.6 Basophil counts in the course of primary and challenge infestations**

![Graph showing basophil counts](image)

Caption as for Figure 5.1
Differences between primary and challenge infestations were not significant

5.4 **DISCUSSION**

In a previous study, experimental primary infestations of sheep provoked cutaneous lesions that were accompanied by significant decreases in haemoglobin concentration and lymphocyte counts and significant increases in numbers of neutrophils and eosinophils (O'Brien *et al.* 1995). In the present investigation, the principal features of primary infestations were a decline in mean lymphocyte counts and the occurrence of eosinophilia and basophilia. Arguably, stress of housing and handling contributed significantly to the fall in lymphocyte counts recorded in sheep
as a similar decline was recorded in uninfested control sheep (O’Brien et al. 1995).

Lymphopenia, neutropenia and eosinophilia have been reported in primary infestations of calves (Stromberg et al. 1986, Stromberg and Guillot 1987b).

In the present study, all sheep developed eosinophilia after primary infestation with *P. ovis*. In contrast, there was marked individual variation in response of calves to primary infestations, though when compared with uninfested controls significant increases in the mean eosinophil count have been recorded (Stromberg et al. 1986, Stromberg and Guillot 1987a, 1997b, Losson et al. 1988). This may reflect a species difference in the cellular response to ectoparasite infestation. A biphasic increase in eosinophils has been reported in some parasitic diseases such as, *Teladorsagia circumcincta* infection of sheep, and may be attributable to early mast cell-mediated and late T cell-mediated pathways (Haig et al. 1995, Kusama et al. 1995, Ushio et al. 1995). Although there was the suggestion of a biphasic response in group 3 sheep there was no evidence of this in group 1 sheep or in an earlier investigation (O’Brien et al. 1995). The delayed development of eosinophilia, observed in both the present and an earlier study (O’Brien et al. 1995), suggests that during the first 6 to 8 weeks after primary infestation, the generation and release of eosinophils from the bone marrow pool is matched by the efflux of cells from the blood into lesional tissue. Subsequently, the circulating eosinophil count continued to rise until 11 weeks, despite the rapid reduction in lesional eosinophils that was observed from 9 to 12 weeks after infestation. This divergence may be explained by reduced transepidermal loss of cells combined with a delay in normalisation of progenitor cell activity. In addition to eosinophilia, a sustained increase in mean basophil counts and, in some sheep, basophilia
counts > 0.3 x 10^9/l) were recorded in the present investigation. This observation has not been reported in earlier studies of *P. ovis* infestation in calves or sheep.

Calves infested with *P. ovis* developed a marked neutropenia that was inversely correlated with lesional area (Stromberg et al. 1986). However, an increase in mean neutrophil numbers was recorded in cattle that manifested minimal cutaneous lesions after primary infestations (Losson et al. 1988). Examination of bone marrow from infested calves revealed myeloid hyperplasia with a significant decrease in mature and an increase in immature neutrophils (Stromberg and Guillot 1987a). These observations suggest that the neutropenia detected in calves resulted from an inadequately compensated transepidermal loss of cells. In contrast to calves, neutropenia was not detected in this or an earlier study of infested sheep (O'Brien et al. 1995). This observation suggests that in sheep the transepidermal efflux of neutrophils is not as great as in cattle.

Challenge infestations of cattle elicited only a significant eosinophil response (Losson et al. 1988). This was more rapid but less pronounced than in primary infestations. In the present investigation, although a rapid eosinophil response occurred it was transient and the cell counts were relatively low. Later, at 6 and 8 weeks after primary infestation eosinophil counts were significantly lower in challenge than primary infestations, an observation consistent with the failure of the challenge infestation to become established. Apart from this eosinophil response, no substantial differences were detected in red and white cell responses to primary and challenge infestations.

In the present study, apart from an increase in monocytes, endectocide treatment 12 weeks after primary infestation did not have a significant effect on red or white blood cell counts recorded in the present study. The sharp rise in
monocytes was not accompanied by a corresponding increase of these cells in cutaneous lesions and the explanation for the increase is not clear. The absence of any other significant change in haematology was consistent with the observation that by 12 weeks after primary infestation lesional pathology was resolving (see section 2.2.2.2.2). Other studies, however, have reported a rapid normalisation of lymphocyte and neutrophil counts following treatment of infested sheep and calves (Stromberg and Guillot 1987b, O’Brien et al. 1995). In calves, particularly those with extensive dermatitis, treatment was followed by a transient increase in circulating eosinophils (Stromberg and Guillot 1987b, 1989). This increase may have been associated with the synchronous death of mites and subsequent release of mite products that resulted in increased antigenic challenge.

Although recorded in a number of endoparasite infections, eosinophilia and basophilia are not consistently associated with parasitic diseases of sheep (Greenwood 1977, Rothwell et al. 1994, Haig et al. 1995). These cells can release potent proinflammatory mediators and may contribute significantly to both tissue damage and host defence (Frigas and Gleich 1986, Frigas et al. 1991).

This investigation has demonstrated the occurrence of a marked eosinophilia accompanied by a less pronounced basophilia in P. ovis infestations of sheep, features compatible with the involvement of allergic immune responses in the pathogenesis of sheep scab.
6. TEMPORAL PATTERN OF ISOTYPE-SPECIFIC ANTIBODY RESPONSES IN PRIMARY AND CHALLENGE INFESTATIONS.

6.1 INTRODUCTION

The histopathology associated with *P. ovis* infestation, described in chapter 2, implicates IgE-mediated Type I hypersensitivity as a major contributor to cutaneous lesions. Host antibody responses may also be involved in the resistance to challenge infestations of *P. ovis* that was observed in the chapter 2. Analysis of antibody responses elicited by primary and challenge infestations and identification of antigens/allergens dominating these responses may, therefore, give an indication of their role in the development of pathology and/or resistance.

*P. ovis* antigen-specific IgG antibody responses have been monitored following experimental infestation of naïve sheep (O'Brien 1992, Bates 1997a) and cattle (Fisher 1983, Pruett et al. 1986, Lonneux et al. 1996, 1998a). However, despite the suggestion that IgE-mediated Type 1 hypersensitivity plays a major role in the generation of cutaneous pathology (Pruett et al. 1986, Stromberg and Fisher 1986) and the detection, by IDSTs and PK tests, of homocytotropic antibody in sera of infested animals (Weisbroth et al. 1972, Losson et al. 1988, 1999), the IgE antibody response has not been investigated nor have those of the IgM and IgA isotypes. Immunoglobulin IgG levels have also been tracked after challenge infestations of previously infested cattle (Fisher 1983, Pruett et al. 1986) but no comparable study of antibody responses following challenge infestations of sheep has been reported.

SDS-PAGE/western blot techniques have been employed to explore the repertoire of *Psoroptes* spp antigens recognised by sera from infested rabbits, cattle
and sheep (Wikela 1989, Boyce et al. 1991, Uhlir 1992, Matthes et al. 1996, Jayawardena et al. 1998). Several IgG and IgE-reactive P. ovis antigens/allergens have been detected but antigen binding by IgM and IgA antibodies and differences in the profile of antigen binding in primary and challenge infestations have not been reported.

The investigation described in this chapter assessed the isotype-specific IgE, IgM and IgA as well as IgG antibody responses to P. ovis that accompanied the primary and challenge infestations of the sheep studied in chapter 2. In addition, SDS-PAGE/western blot techniques were employed to identify immunodominant P. ovis antigens/allergens and the profile of antigen-antibody binding at different stages of primary and challenge infestations.

6.2 MATERIALS AND METHODS

6.2.1 Animals

Blood samples, from the three groups of sheep described in section 2.2.1, were collected into vacutainer tubes containing no anticoagulant (Becton Dickinson, Franklin Lakes, NJ, USA) by jugular venipuncture.

6.2.2 Antigens

P. ovis and P. cuniculi WME were prepared as follows. P. ovis and cuniculi mites (adults, nymphs and larvae) were collected from infestations in sheep and rabbits, respectively. The live mites were washed by vortexing for 5 minutes in ice-cold 0.015M PBS (pH 7.2), followed by 1% sodium dodecyl sulphate (SDS) at RT and finally 10 washes in ice-cold PBS. Mites were then homogenised in a ribolyser (FastPrep®Fp120, Qbiogene, Inc Carlsbad, USA) (4x30sec blasts [setting 6] separated by 2min incubations on ice) and sonicated (Polaron, Watford) in ice-cold
PBS with five, one minute, bursts at the maximum setting. The final homogenate was centrifuged at 10,000g for 10 minutes and the supernatant (whole-mite extract) harvested, aliquoted and stored at -70°C. Protein content of the supernatant was determined, in accordance with the manufacturer’s instructions, with a BCA™ Protein Assay Kit (Pierce, Illinois).

Preliminary analysis of SDS-PAGE/western blots confirmed the earlier observation of almost complete homology between in the profile of P. ovis and P. cuniculi antigens revealed by antibodies in sera from infested sheep (Boyce and Brown 1991). These preliminary studies also indicated that contamination of mite antigens with ovine immunoglobulin, ingested by mites harvested from sheep, did not produce ‘false’ antigens on western blots. In view of this and the difficulty in obtaining an adequate supply of P. cuniculi mites, it was decided to employ P. ovis antigen in the SDS-PAGE/western blot studies. However, antigen derived from P. cuniculi was used in studies employing the sensitive enzyme-linked immunosorbent assay in order to avoid any possible inaccuracy due contamination of P. ovis antigen by ovine immunoglobulin (Wassall et al. 1987, Beetham 1997, Pettit et al. 2000).

### 6.2.3 Enzyme-linked immunosorbent assay studies

Details of the buffers employed in this section are given section 11.1 and 11.4. Single point assays of the level of isotype-specific antibody were made as follows. Micro-ELISA plates (M129B, Dynex Technologies, Ashford, Middlesex) were incubated for 1 hour at RT with (50μl/well) P. cuniculi WME (10μg/ml) in 0.2M carbonate/bicarbonate buffer (pH 9.6) and then washed twice with PBS/05%V/V Tween 20 (PBS/T20). On the basis of preliminary titrations, sera were
diluted, 1/800 for IgG/IgM and 1/5 for IgA assays, in PBS/0.5% Tween 80/0.5M NaCl (PBS/T80/NaCl) and 50µl of the test serum added, in duplicate, to the wells.

In order to minimise the potential blocking of the binding of IgE to P. ovis allergens by IgG, serum samples (100µl) were heated at 56°C for 30 minutes and then precipitated in 30% and 50% saturated ammonium sulphate (Kooymen et al. 1997, see section 11.4). The final precipitate, containing IgE and residual IgG, IgA and IgM, was dissolved in 200µl PBS/T80/NaCl and 50µl of this treated serum added to the wells. After incubation for 1 hour at RT, the plates were washed six times with PBS/T20. Plates were then incubated with 50µl of the appropriately diluted mouse anti-ovine monoclonal antibody, IgG ([VPM6-supernatant], 1/10 in PBS/T80/NaCl), IgM ([VPM13-ascites], 1/1000 in PBS/T80/NaCl), IgA ([Serotec-purified monoclonal antibody], 1/2000 in PBS/T80/NaCl) or IgE ([IE7-supernatant, Kooymen et al. 1997], 1/10 in PBS/T80/NaCl). This incubation was followed by one with 50µl biotinylated goat-anti-mouse Ig conjugate ([Dako, Ely Cambs, UK], 1/1000 dilution in PBS/T80/NaCl) and streptavidin horseradish peroxidase (HRP) (Dako), 1/2000 dilution in PBS/T80/NaCl). Each incubation was for 1 hour at RT and plates were then washed six times in PBS/T20. Colour reaction was developed by incubating with 50µl 3,3'5,5'-tetramethylbenzidine (TMB) substrate (Dynex Technologies), IgG, IgM and IgA assays were incubated for 10 minutes and IgE for 30 minutes. Incubations were at RT and stopped by the addition of 50µl 0.18M H2SO4 (Voller et al. 1979). The colour reaction at OD (optical density) 450nm was measured in a Labsystems iEMF Reader (Labsystems Life Sciences International, Hampshire, UK) and the mean of the duplicate samples determined. In order to compare results between assays, aliquots from a pool of positive sera were stored at -20°C and serum
from a fresh aliquot was included on each plate. If necessary, the OD 450 of a positive serum control was corrected so that it gave the same reading as the other positive serum controls and the values of unknown samples were then adjusted relative to this. In practice, between test variability was minimal.

6.2.4 SDS-PAGE and western blots

Electrophoretic profiles of *P. ovis* WME were obtained by discontinuous SDS-PAGE (details of buffers, gels and solutions are given in 11.1, 11.3 and 11.4). Mite extracts were reduced and denatured by boiling for 5 minutes in sample buffer containing mercaptoethanol and then loaded (15-20μg/lane) onto 0.75mm thick 12% minigels with a 4% stacking gel. Protein standards were loaded into an additional well and each gel run at 200V for 35-45 minutes. The separated proteins were transferred onto nitrocellulose (NC) membrane using a semi-dry transfer apparatus at 70 A/gel for 60 minutes at RT. After transfer, the NC membranes were stained with 0.1% Ponceau S (w/v) in 5% acetic acid (v/v) (Sigma, Gillingham, Dorset) and cut into strips. The strip with molecular weight markers (MultiMark™, Novex, San Diego, USA) was dried and stain was removed from strips containing separated mite proteins by three washes in 5% acetic acid followed by a rinse in distilled water and a final wash in PBS/T80/NaCl. These were then incubated in PBS/T80/NaCl for 30 minutes at RT on an orbital shaker (100cycles/min) to block non-specific protein adsorption. Subsequently, to detect reactivity with *P. ovis* antigen-specific antibodies, the blotted proteins were probed with test sera. Antigens binding IgG and IgM antibody were identified with sera prepared at a dilution of 1/100 in PBS/T80/NaCl and IgA reactivity was probed with sera prepared at a dilution of 1/10 in PBS/T80/NaCl. Reactivity with IgE antibodies was determined by incubation
with test sera pre-treated as described earlier. After incubation for one hour at RT with constant rocking the blots were washed three times in PBS/T80/NaCl. They were then incubated with mouse anti-ovine monoclonal antibody (IgG [VPM6, 1/10 in PBS/T80/NaCl], IgM [VPM13, 1/1000 in PBS/T80/NaCl], IgA [Serotec, 1/200 in PBS/T80/NaCl], IgE [IE7, 1/10 in PBS/T80/NaCl]) for one hour. This was followed by incubation with biotinylated goat anti-mouse Ig ([Dako], 1/1000 in BS/T80/NaCl) for one hour and streptavidin-HRP ([Dako], 1/2000 in PBS/T80/NaCl) for 30-60 minutes. Each incubation was at RT on an orbital shaker (100 cycles/min) and followed by three washes in PBS/T80/NaCl. Finally peroxidase activity in blots incubated with anti-ovine IgG, IgM or IgA was revealed with DAB/urea/H₂O₂ substrate (Sigma Fast™ DAB tablet, Sigma). Peroxidase activity in blots incubated with anti-ovine IgE was detected by chemiluminescence as described by the manufacturer (ECL™ Western blotting, Amersham). In control blots serum or monoclonal antibody was replaced by wash diluent.

6.2.5 Statistical analysis

For the reasons given previously (2.2.1.4), differences between the groups in the isotype-specific antibody responses were evaluated by analysis of co-variance. As the estimation of differences in antibody levels within each group involved comparisons of paired samples, the two-tailed student’s t test was employed for this.

6.3 RESULTS

6.3.1 ELISA studies

Sera were collected weekly from 0-14 weeks and at 16 weeks after primary infestation (group 1) and antigen-specific ELISAs were used to estimate IgG, IgE,
IgM and IgA antibody levels to *P. ovis* in sera from the five sheep which later constituted group 2. These sheep were treated with an endectocide at 12 and 13 weeks after infestation (section 2.2.1.1). IgG, IgE, IgM and IgA antibody levels to *P. ovis* were also determined in sera collected weekly from these 5 sheep from 0-8 weeks after challenge infestation (group 2) and the accompanying control (primary) infestations (group 3).

### 6.3.1.1 IgG antibody

A significant (*p*=0.046) increase in mean IgG antibody level (Figure 6.1) was first seen 2 weeks after primary infestation (group 1). Antibody levels of group 1 sheep rose more rapidly than those of group 3 and were significantly greater (*p*<0.02) than those of group 3 at 7 and 8 weeks. Peak antibody levels (group 1) were achieved from 8-12 weeks after infestation. In challenge infestations (group 2) there was a prompt but transient IgG antibody response (Figure 6.1) that was not significantly different from the baseline value. No consistent significant differences were detected in antibody levels of sheep with primary (groups 1 and 3) and challenge (group 2) infestations.

### 6.3.1.2 IgE antibody

After primary infestation the increase in IgE antibody level (Figure 6.2) observed in group 3 paralleled that in group 1 with a significant (*p*=0.024) increase in antibody level first detected 7 weeks after infestation (group 1). In group 1, the peak antibody level occurred at 13 weeks, one week after the first dose of endectocide, and then declined. Challenge infestation (group 2) elicited a rapid and significant (*p*=0.015), anamnestic response (Figure 6.2) by 1 week and, except at 5 weeks, from
1-8 weeks IgE antibody levels were significantly higher ($p<0.01-p<0.03$) in challenge than primary infestations.

**Figure 6.1** *P. ovis* antigen-specific IgG levels in the course of primary and challenge infestations

The grey arrow indicates first occurrence of a significant increase in antibody level during primary infestations and the black arrows indicate treatment with endectocide. At 7 weeks the IgG level in group 1 was significantly greater ($p<0.003$) than in group 3 but no significant differences were detected between IgG levels of sheep with primary (groups 1 and 3) and challenge (group 2) infestations.

**6.3.1.3 IgM antibody**

A significant ($p=0.04$) rise in mean IgM antibody level (Figure 6.3) was first detected 7 weeks after primary infestation (group 1). There was no statistical difference between IgM antibody levels of sheep with primary and challenge infestations.

**6.3.1.4 IgA antibody**

*P. ovis* antigen-specific IgA levels in primary and challenge infestations were no different from background levels.
Figure 6.2 *P. ovis* antigen-specific IgE levels in the course of primary and challenge infestations

![Graph showing IgE levels over time for different groups.](image)

Caption as for Figure 6.1
Except at 5 weeks after infestation, from 1 to 8 weeks IgE antibody levels were significantly greater in challenge (group 2) than primary (groups 1 and 3) infestations (*p*<0.01- *p*<0.03)

Figure 6.3 *P. ovis* antigen-specific IgM levels in the course of primary and challenge infestations

![Graph showing IgM levels over time for different groups.](image)

Caption as for Figure 6.1
No significant differences were detected between IgM levels of sheep with primary (groups 1 and 3) and challenge (group 2) infestations
6.3.2 SDS-PAGE/western blots

In primary infestations, binding of IgG/IgE antibodies to reduced antigens/allergens was evaluated in blots of *P. ovis* proteins probed with sera collected from 6 sheep in group 1 before infestation; at 4-6 weeks and 11-13 weeks after infestation. Clinically, the 4-6 week period represented the mid-stage of lesional development and by 11-13 weeks lesions were resolving. In challenge infestations, binding of IgG and IgE antibody was determined in blots probed with sera collected from 3 sheep in group 2 before challenge and 2 and 6 weeks after challenge.

Major IgG and IgE-reactive antigens were defined as those binding IgG/IgE antibodies in at least 50% of the sera tested and demonstrating strong reactivity with at least 50% of these (Lowenstein 1978).

Blots demonstrating the development of antigen/allergen labelling by IgG and IgE antibodies in the course of primary and challenge infestations are shown in Figures 6.4 and 6.5, respectively. As western blots probed with IgM and IgA antibody revealed only faint labelling of antigens, which was difficult to interpret, these results are omitted.

6.3.2.1 Primary infestation

Individual animals exhibited considerable variation in the profile of antigens/allergens labelled by IgG/IgE antibodies. As the infestations progressed IgG/IgE antibodies reacted with a greater number of antigens/allergens and the intensity of labelling increased. At 11-13 weeks after primary infestation five major IgG-reactive antigens (Table 6.1) and three major and four minor IgE-reactive allergens (Table 6.2) were detected on western blots. Several other
antigens/allergens were labelled by immunoglobulin IgG (MWs 80kD, 65kD, 40kD, 20kD) and immunoglobulin IgE (MWs 90kD, 80kD) but these were weakly reactive and occurred less consistently. Antigens >100kD and <15kD and allergens >100kD were more consistent than others in stimulating substantial IgG and IgE antibody responses, respectively.

6.3.2.2 Challenge infestation

IgG antibodies in pre-challenge sera reacted weakly with up to 3 of the major antigens identified during primary infestation (Table 6.3) while IgE antibodies labelled one major and up to four minor allergens (Table 6.4). Two of these minor allergens (MWs 80kD and 90kD) were detected only inconsistently at 11-13 weeks after primary infestation. After challenge infestation, there was a marked increase in the intensity of allergen labelling and in the number of allergens identified. IgG antibody binding was also enhanced but not to the same extent as the IgE labelling.
Sheep 3: Development of *P. ovis* antigen recognition by IgG antibodies in the course of primary and secondary infestations

Primary infestation: Lane A - pre-infestation, Lane B - mid-infestation, Lane C - end-infestation
Challenge infestation: Lane D - pre-challenge, Lane E - 2 weeks post-challenge, Lane F - 6 weeks post-challenge

+ positive control, pooled sera collected from animals before primary infestation
- negative control, pooled sera collected from terminal bleed of animals at conclusion of challenge infestation
Figure 6.5 Sheep 3: Development of *P. ovis* allergen recognition by IgE antibodies in the course of primary and secondary infestations

![Image of gel electrophoresis](image)

Caption as for Figure 6.4

The evident differences in the intensity of IgG and IgE labelling seen in figures 6.4 and 6.5 are partly due to the use of chemiluminescence to detect peroxidase activity in blots incubated with anti-ovine IgE, this greatly enhanced detection of IgE binding.
### Table 6.1 Major IgG-reactive antigens detected in the course of primary infestations

<table>
<thead>
<tr>
<th>Molecular weight (kDa)</th>
<th>Control (pre-infestation)</th>
<th>Weeks 4-6</th>
<th>Weeks 11-13</th>
<th>pooled sera*</th>
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*sera collected from terminal bleed at conclusion of challenge infestation

Intensity of antibody labelling
- weak
- moderate
- strong
- very strong

### Table 6.2 Major IgE-reactive allergens detected in the course of primary infestations

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<tr>
<th>Molecular weight (kDa)</th>
<th>Control (pre-infestation)</th>
<th>Weeks 4-6</th>
<th>Weeks 11-13</th>
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Caption as for Table 6.1
Table 6.3  Major IgG-reactive antigens detected in the course of challenge infestations

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Caption as for Table 6.1.

Table 6.4  Major IgE-reactive allergens detected in the course of challenge infestations

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Caption as for Table 6.1.
6.4 DISCUSSION

The present ELISA studies have demonstrated that experimental infestations with *P. ovis* elicited IgE and IgM as well as the IgG isotype antibody response that has been reported previously (O’Brien 1992, Bates 1997a). They have also shown challenge infestation provoked a significant anamnestic IgE but not IgG or IgM antibody response. However, these ELISA studies failed to detect evidence of a *P. ovis* antigen-specific IgA antibody response, though detection of faint staining on SDS-PAGE/Western blots suggested that a weak response did occur. The IgA antibody response to ectoparasites other than *S. scabiei* has not been reported.

Several studies of human patients and one of dogs with sarcoptic mange recorded a fall in total serum IgA antibody levels that was reversed after successful treatment (Falk 1980, Morsy *et al.* 1993, Nassef *et al.* 1991, Thoday 1993). However, increased numbers of IgA-positive cells have been detected in lesional skin of pigs with sarcoptic mange (Morsy and Gaafar 1989). Studies in cattle, vaccinated intradermally with *Dermatophilus congolensis*, suggested that local production of antigen-specific IgA and its secretion onto the surface of the skin occurred in the absence of a rise in serum antigen-specific IgA (Lloyd and McEwan Jenkinson, 1981). It is possible, therefore, that IgA antibody may be involved in host defence even though it is not detected serologically.

The demonstration of *P. ovis* antigen-specific IgE antibody response, in addition to the lesional eosinophilia and mast cell infiltration described earlier (section 2.2.2.2.1), provides strong evidence of the involvement of IgE-mediated Type 1 hypersensitivity in the pathogenesis of cutaneous lesions. It has been hypothesised that development of IH initiates a phase of rapid lesional growth (Pruett
et al. 1986, Stromberg and Fisher 1986, Bates 1997a). In the present study, a significant increase in mean *P. ovis* antigen-specific IgE level was not detected until 7 weeks after primary infestation though its tissue activity may have preceded this. However, lesional growth, analysed in chapter 1, was exponential throughout the first 7 weeks of infestation and then plateaued (section 2.2.2.1). This study, therefore, provides no evidence that lesional growth is promoted by the development of an IgE-mediated Type 1 hypersensitivity response.

The role, if any, of the different isotypes responses in host defence is not clear. Ovine immunoglobulin has been detected in *P. ovis* mites harvested from infested sheep (Pettit et al. 2000). Host immunoglobulins could, therefore, have a direct, adverse effect on parasite survival. In a recent report, vaccination of calves with crude preparations of *P. ovis* allergens induced immediate and 24-48 hour hypersensitivity responses (Pruett et al. 1998). Although after challenge infestation no statistically significant difference was detected in mite numbers and lesional areas on vaccinated and control calves, there was a trend that suggested vaccination increased resistance to infestation. This was attributed to an augmented IH response that resulted in pruritus and increased self-grooming. In the present study, the group of challenged sheep were resistant to reinfestation. On challenge, these sheep mounted a prompt and significant IgE but not IgG or IgM antibody response. This observation is consistent with that of Pruett et al. (1998) and provides further evidence of the involvement of IgE-mediated mechanisms in host resistance.

The profile of *P. ovis* antigens/allergens on SDS-PAGE/western blots labelled by sera collected during primary infestation was similar to those reported previously in sheep and confirms the diversity of individual responses to *P. ovis*
antigens/allergens (Boyce et al. 1991a, Matthes et al. 1996, Jayawardena et al. 1998). However, contrary to a previous report (Boyce et al. 1991a) this study demonstrated that as the infestation progressed, intensity of antigen/allergen labelling increased with lesional area. In addition, it was found and that some immunodominant antigens, particularly those >100kD were recognised more consistently than others, an observation that may have implications for the serodiagnosis of sheep scab.

At present diagnosis of sheep scab is suggested by history and clinical signs and confirmed by identification of P. ovis mites in scrapings from lesional skin. Inevitably, animals with sub-clinical disease or minimal lesions may escape detection. Although studies of experimentally infested cattle have indicated that some cattle with minimal lesions remain seronegative on ELISA (Fisher et al. 1986, Lonneux et al. 1998a), employment of an immunodiagnostic test may provide a more reliable method for screening a flock/herd than clinical examination. Recently, Ochs et al. (2001) described a sensitive sandwich ELISA for the serodiagnosis of P. ovis infestation in sheep. Although this assay employed crude P. cuniculi extract its diagnostic sensitivity was cited as 93.7% and its specificity as 96.5%. However, the value of this latter figure is questionable, as 4 out of 4 sheep with chorioptic mange and 3 of 5 sheep with forage mites gave ELISA readings above the cut off value.

Earlier studies employing ELISA and/or SDS-PAGE/western blot techniques have revealed cross-reactivity in the antigen repertoire of Psoroptes spp mites and related mites such as Chorioptes bovis, S. suis and Notedres cati (Matthes et al. 1996) and D. pteronyssinus (Stewart and Fisher, 1986). Cross-reactivity with argasid (Ornithodorus spp) and ixodid (Dermacentor variabilis) ticks has also been reported
(den Hollander and Allen 1986, Wozniak 1996). This cross-reactivity may explain the observation in the present study of immunoglobulin IgE-reactive bands in a significant number of pre-infestation sera. More importantly, cross-reactivity of this nature will compromise specificity and reduce the reliability of serodiagnostic tests. However, the present study and earlier investigations suggested that a specific profile of antigen recognition may characterise P. ovis infestations (Boyce et al. 1991a, Matthes et al. 1996). The present study also indicated that some immunodominant antigens stimulate seroconversion early in the course of infestation. Identification and isolation of these Psoroptes spp-specific antigens and their expression as recombinant proteins might improve sensitivity and specificity of immunodiagnostic tests and importantly guarantee their reproducibility. These may provide a sensitive method for screening flocks and individual animals and facilitate the implementation of control programmes. They could also be used for epidemiological surveys (Lonneux et al 1996, Boyce et al. 1991b).

The SDS-PAGE/western blots also identified allergens dominating IgE antibody responses to primary and challenge infestations. Histopathology suggests that IgE-mediated responses contribute to cutaneous pathology while the present study indicates that they may also be involved in host defence. These allergens may, therefore, be suitable candidates for inclusion in vaccination studies aimed at modulating the host response to infestation. They would also be of value in more detailed studies of the immunopathogenesis of sheep scab.

The studies described in this chapter have demonstrated that P. ovis elicits an IgE as well as IgG and IgM antibody response and that IgG and IgE antibodies react
strongly with *P. ovis* antigens/allergens >100kD. In the next chapter the role of IgE in cutaneous responses to *P. ovis* will be investigated.
7. CUTANEOUS RESPONSES OF P. OVIS-NAIVE AND SENSITISED SHEEP TO INTRADERMAL INJECTION OF P. OVIS WHOLE-MITE EXTRACT.

7.1 INTRODUCTION

Ectoparasites elicit multiple humoral and cellular immune responses that can cause significant tissue damage (Wikl 1982, Allen 1994, Arlian 1996, Burns 1996). Observation of the wheal reactions of infested animals to intradermal injection of parasite extracts provides a convenient method for detecting the different hypersensitivity responses generated by specific infections, particularly when these reactions are also examined histologically (Parrish and Breathnach 1996). In naïve animals, histological examination of the cutaneous response to intradermal injection of parasite extract may provide valuable information about the early innate immune response. Identification of these responses and their contribution to host pathology and/or protection may facilitate the selective and advantageous manipulation of the immune response by vaccination.

Lesional histopathology (chapter 2) and the demonstration of a P. ovis antigen-specific IgE antibody response in infested sheep (chapter 6) suggested that IgE-mediated, Type 1 immediate hypersensitivity may play a significant role in the development of cutaneous lesions. However, wheal reactions of infested rabbits and cattle to intradermal injection of Psoroptes spp extract have provided equivocal evidence of IH (Weisbroth et al. 1972, Wikl 1982, Losson et al. 1988). But, these earlier studies and a more recent one (Ravindran et al. 2000) did reveal the occurrence of DTH and possibly an LPR or Arthus type reaction though neither of these was confirmed histologically.
In the series of studies described in this chapter, hypersensitivity responses of sheep previously infested with *P. ovis* were investigated by intradermal injection of *P. ovis* whole-mite extract (WME) and histological examination of the wheal reactions elicited. As antigenic cross-reactivity between *Psoroptes* spp mites and *D. pteronyssinus* has been demonstrated (Stewart and Fisher 1986), the cutaneous responses of these sheep to IDSTs with purified Der p 1, a major allergen derived from *D. pteronyssinus*, were also studied. The IgE dependence of immediate wheal reactions to *P. ovis* WME and Der p 1 was examined by the Prausnitz-Kustner test and histopathology of the responses obtained. In addition, IDSTs were performed in *P. ovis*-naïve sheep to investigate the innate, cutaneous response to *P. ovis* WME.

### 7.2 MATERIALS AND METHODS

#### 7.2.1 Animals

*P. ovis*-naive sheep (Suffolk cross, Texel cross, Dorset cross, Swaledale and Dorset) aged between one and two years were used in this study.

#### 7.2.2 Allergen extracts

*P. ovis* WME was prepared, the protein content determined and aliquots stored as described previously (6.2.2.).

Dr C Hewitt (University of Leicester, Medical Research Council) generously supplied immunoaffinity-purified Der p 1 that was obtained from cultured mites. Histamine (0.01%) was obtained from Artu Biologicals Europe B.V. Lelystad, Holland.

Fresh solutions of sterile PBS (pH 7.2) containing antigens (*P. ovis* WME and purified Der p 1) were prepared immediately before each skin test.
7.2.3 Performance of intradermal tests

All sheep were restrained in lateral recumbency and a grid with a minimum of 2.5 cm between each point marked on the clipped flank. Intradermal injections were made with a 25 gauge needle, bevelled edge rotated toward the epidermis, and an insulin syringe. Wheal reactions were calculated as the mean of two measurements of the wheal diameters made at right angles to each other.

7.2.4 Titration of antigen

Two crossbred, Texel sheep were used to determine the optimal concentration of *P. ovis* antigen for intradermal tests. Different concentrations, 2, 20 and 200μg protein/ml and 1mg protein/ml of *P. ovis* WME in PBS were prepared. 0.05ml of each antigen concentration and 0.05ml of PBS were injected intradermally and the wheal reactions were determined at 0.25, 0.5, 1, 2, 3, 6 and 24 hours.

7.2.5 Intradermal tests

7.2.5.1 Experiment 1. Time course of wheal reactions in *P. ovis*-sensitised sheep

Five Suffolk cross sheep were infested at the withers with a cluster of 25-50 ovigerous *P. ovis* mites as indicated earlier (2.2.1.1.). At 12 and 13 weeks post-infestation the sheep were treated with an endectocide (Ivomec, MSB Agvet, 200μg/kg).

When the sheep had been rested for 11 weeks, 0.05ml of *P. ovis* WME (10μg protein/ml), Der p 1 (10μg protein/ml) and the positive (histamine) and diluent (PBS) controls were injected intradermally at randomised sites on the flank. Wheal reactions were measured at 0.25, 0.5, 1, 3, 6, 24, 30, 48 and 72 hours after intradermal challenge.
7.2.5.2 Experiment 2. Wheal reactions and cell counts in 

*P. ovis*-naïve and sensitised sheep

The 5 sheep used in Experiment 1 to determine the time course of wheal reactions, were rested for one week and then employed in this study in which cell counts at reaction sites were analysed. On this occasion the IDSTs were performed on the contralateral flank to that used previously. Five Suffolk cross sheep were used as *P. ovis*-naïve, uninfested, controls.

Intradermal injections of 0.05ml of *P. ovis* WME (10μg protein/ml⁻¹), Der p 1 (10μg protein/ml⁻¹) and the positive (histamine) and diluent (PBS) controls were injected at two randomised sites on the flank of *P. ovis*-sensitised and naïve (control) sheep. Wheal reactions were measured at 0.25, 0.5, 1, 3, 6, 24 and 30 hours after challenge.

At 6 hours, after subcutaneous injection of a local anaesthetic (Lignol, Arnolds Veterinary Products), a 6mm punch biopsy was collected from one of the antigen and diluent control sites. The remaining antigen and diluent control sites were biopsied at 30 hours. Biopsies were fixed and processed and sections were stained and cell counts performed as described in section 2.2.1.3.

7.2.6 Prausnitz-Kustner tests

Three sera were used in the PK tests: negative (control) pooled serum (NS) collected from five *P. ovis*-naïve sheep prior to infestation; positive pooled serum (PS) obtained at the peak of the IgE antibody response in 5 sheep that had received two infestations of *P. ovis* and positive pooled serum that had been heat treated (56°C for 2 hours) to destroy IgE activity (HTPS) (Macaldowie 1997, Huntley personal communication).
A group of 5 sheep composed of 2 Suffolk cross, 2 Texel cross and 1 Dorset cross animal were employed as recipients for sera and subsequently challenged with *P. ovis* WME. A second group of 5 animals, comprising 3 Texel cross and 2 Dorset cross sheep, was used to test the response to challenge with Der p 1. A 5x2 grid was marked on the clipped flank and intradermal injections (0.05ml) of NS, PS and HTPS were made at two randomised sites. Twenty four hours later these sites were challenged by intradermal injection of 0.05ml of *P. ovis* WME (10µg protein/ml\(^{-1}\)) or Der p 1 (10µg protein/ml\(^{-1}\)) and two further sites were injected with 0.05ml *P. ovis* WME or Der p 1 and two with the diluent control (PBS). Wheal diameter was measured at 0.25, 0.5, 1, 3, 6 and 24 hours after intradermal challenge. One injection site was biopsied, as described above, at 6 hours and the other at 24 hours. Biopsies were processed and cell counts made as indicated earlier (2.2.1.3).

### 7.2.7 Innate response to intradermal injection of *P. ovis* WME.

Beck and Hiepe (1997) obtained 212µg protein from 1,000 *P. ovis* mites. Using this figure it was calculated that approximately 5µg protein would be derived from 25 mites, the number used to infest sheep in the earlier studies (chapter 2). It was decided, therefore, to determine the cutaneous response of *P. ovis* naïve sheep to the intradermal injection of 5µg *P. ovis* WME.

Twelve *P. ovis*- naïve sheep comprising 6 Swaledales and 6 polled Dorsets were used. These breeds have been reported to be relatively resistant and susceptible, respectively, to *P. ovis* infestation (Bates personal communication). A 3x2 grid was marked on the left flank and diluent (PBS) and *P. ovis* WME (5µg protein in 0.05ml PBS) were each injected into three randomised sites. After
subcutaneous injection of a local anaesthetic, biopsies were collected from one antigen and diluent site at 6, 24 and 48 hours. The biopsies were processed and cell counts made as on previous occasions (2.2.1.3.).

7.2.8 Statistical analysis

As comparisons between responses were made either between two groups of animals at the same time point or between two time points (6 and 30 hours) within a group of animals, the statistical significance of differences was determined by analysis of variance using a GenStat release 4.2 programme (5th edition), (Lawes Agricultural Trust, Numerical Alogrithms Group Ltd, Oxford, UK). Values of \( p<0.05 \) were considered significant.

7.3 RESULTS

7.3.1 Titration of antigen

At concentrations of \( 2\mu g \) and \( 20\mu g \) protein/ml\(^1\) the wheal reactions (Figure 7.1) elicited by 0.05 ml \( P.\ ovis \) WME and the diluent control in normal sheep were not significantly different. Higher concentrations of \( P.\ ovis \) WME provoked a significantly greater reaction than the diluent control from 2 hours (\( p<0.004 \)) to 6 hours (\( p<0.001 \)). A concentration of \( 10\mu g \) protein/ml\(^1\) was, therefore, selected for intradermal skin tests.

7.3.2 Intradermal tests

7.3.2.1 Experiment 1. Time course of wheal reactions in \( P.\ ovis \)-sensitised sheep

Intradermal injection of 0.05 ml \( P.\ ovis \) WME (10\( \mu g \) protein/ml\(^1\)) elicited an immediate wheal reaction which was maximal at 1 hour and then declined before merging imperceptibly, at 6 hours, with a late phase response that persisted until
24 hours (Figure 7.2). This was followed by a delayed response that peaked at 30 hours and persisted up to and beyond 72 hours after intradermal challenge. Der p 1 also provoked an immediate response but evidence of a delayed response was equivocal (Figure 7.2).

7.3.2.2 Experiment 2. Wheal reactions and cells counts in
P. ovis-naïve and sensitised sheep

7.3.2.2.1 Wheal reactions

Comparison of wheal reactions in P. ovis-naïve and sensitised sheep (Figures 7.3a and b) indicated that in sensitised animals 0.05ml P. ovis WME (10μg protein/ml⁻¹) elicited a marked immediate hypersensitivity response (p<0.01 at 0.25 hours and p<0.001 at 0.5 hours); a late phase response (p<0.001 at 6 hours) and a delayed hypersensitivity response (p< 0.01 at 24 hours and 30 hours). Compared to the 0.25-0.5 hour wheal, the 30 to 72 hour wheal in sensitised sheep was indurated suggesting a more cellular reaction. Wheal reactions to Der p 1 were less pronounced. However, the immediate and late phase responses to Der p 1 were significantly greater (p< 0.001 at 0.5 hours and p<0.001 at 6 hours) in sensitised than naïve sheep (Figures 7.3a and b). But, at 24 and 30 hours, the responses of naïve and sensitised sheep were not significantly different.
Figure 7.1  Titration of *P. ovis* WME in *P. ovis*-naïve sheep (n=2)

a) Titration of *P. ovis* WME in *P. ovis*-naïve sheep

*50μl of diluent and *P. ovis* WME injected intradermally.

200μg protein/ml-1 and 1 mg protein/ml-1 elicited significantly greater wheal reactions than the diluent control from 2 to 6 hours after challenge (a, p<0.004; b, p<0.001).

Figure 7.2  Time course of wheal reactions in *P. ovis*-sensitised sheep (n=5)

*50μl diluent, histamine and *P. ovis* WME 1 (10μg/1ml-1) and Der p 1 (10μg/1ml-1) injected intradermally.
Figure 7.3 Intradermal tests: Wheal reactions of *P. ovis*-naïve and sensitised sheep to challenge with *P. ovis* WME

a) Wheal reactions in *P. ovis*-naïve sheep (n=5)

b) Wheal reactions in *P. ovis*-sensitised sheep (n=5)

*50μl diluent, histamine and *P. ovis* WME and Der p 1 (10μg/1ml) injected intradermally. Wheal reactions to *P. ovis* WME were significantly greater in sensitised than naïve sheep from 0.25 to 30 hours after intradermal challenge (a, *p*<0.01; b, *p*<0.001).

Wheal reactions to Der p 1 were significantly greater in sensitised than naïve sheep from 0.5 to 6 hours after intradermal challenge (a, *p*<0.01; b, *p*<0.001).
7.3.2.2.2 Cell counts

**Eosinophils:** In sensitised sheep the infiltrate elicited by *P. ovis* WME was composed of eosinophils and fewer neutrophils. At 6 hours after challenge eosinophil counts (Figures 7.4a) were significantly greater (*p*<0.02) in sensitised than naïve sheep and by 30 hours this difference had increased (*p*<0.001) due to a dramatic influx of eosinophils in sensitised but not naïve sheep. Der p 1 also elicited an influx of eosinophils (Figure 7.4a) that was significantly greater (*p*<0.01) in sensitised than naïve sheep 6 hours after challenge but no increase in numbers was detected at 30 hours. At 6 and 30 hours eosinophils were distributed diffusely throughout the dermis with increased concentrations in the perivascular areas.

**Neutrophils:** Although at 6 hours *P. ovis* WME elicited a significantly greater (*p*<0.05) neutrophil infiltrate in sensitised sheep than the diluent control, this neutrophil response was not significantly different from that elicited by *P. ovis* WME and the diluent control in naïve sheep (Figure 7.4b). In contrast to *P. ovis* WME, Der p 1 provoked an infiltrate dominated by neutrophils. But this pronounced influx of neutrophils (Figure 7.4b) was not significantly different in sensitised and naïve sheep and declined from 6 to 30 hours after challenge. The distribution of neutrophils was similar to that of eosinophils.

**Mast cells and basophils:** Neither *P. ovis* WME nor Der p 1 provoked significant changes in mast cell and basophil counts (Figure 7.4c), although at 30 hours counts in sensitised sheep challenged with *P. ovis* WME were raised.
Figure 7.4  Intradermal tests: Cell counts in *P. ovis*-naïve and sensitised sheep, 6 and 30 hours after challenge with *P. ovis* WME and Der p 1

**a) Eosinophils (n=5)**

Status and challenge

- naïve, diluent*
- sensitised, diluent*
- naïve, *P. ovis* WME
diluent
- sensitised, *P. ovis* WME
diluent
- naïve, Der p 1*
- sensitised, Der p 1*

![Graph of eosinophils](image)

**b) Neutrophils (n=5)**

Status and challenge

- naïve, diluent*
- sensitised, diluent*
- naïve, *P. ovis* WME
diluent
- sensitised, *P. ovis* WME
diluent
- naïve, Der p 1*
- sensitised, Der p 1*

![Graph of neutrophils](image)

**c) Mast cells and basophils (n=5)**

Status and challenge

- naïve, diluent*
- sensitised, diluent*
- naïve, *P. ovis* WME
diluent
- sensitised, *P. ovis* WME
diluent
- naïve, Der p 1*
- sensitised, Der p 1*

![Graph of mast cells and basophils](image)

*50µl diluent, *P. ovis* WME (10µg/1ml⁻¹) and Der p 1 (10µg/1ml⁻¹) injected intradermally.

Responses of sensitised and naïve sheep marked with the same letter are significantly different. Eosinophil responses to *P. ovis* WME were significantly greater in sensitised than naïve sheep at 6 and 30 hours (a, p<0.02; b, p<0.001) while the response to Der p 1 was significantly greater 6 hours (c, p<0.01). Neither *P. ovis* WME nor Der p 1 provoked significantly different neutrophil or mast cell and basophil responses in sensitised and naïve sheep.
7.3.3 Prausnitz-Kustner tests

7.3.3.1 Wheal reactions

*P. ovis* WME elicited a positive PK reaction (Figure 7.5a) - the wheal diameter at the PS site was significantly greater than at the HTPS site from 0.25 hours (*p*<0.02) to 3 hours (*p*<0.03) after challenge. No wheal response was detected at 24 hours after challenge. Responses elicited by Der p 1 (Figure 7.5b) were less pronounced. But at 0.25 hours after challenge the wheal reaction at the PS site was significantly greater (*p*<0.001) than at the HTPS site. Although no significant differences were found in wheal responses recorded at 0.5 hours, at 1 hour the reaction at the PS site was significantly greater than at the HTPS (*p*<0.01) site.

7.3.3.2 Cell counts

**Eosinophils:** At 6 hours, the positive PK reaction elicited by *P. ovis* WME was accompanied by an influx of eosinophils that was significantly greater (*p*<0.001) than that detected at each of the other sites (Figure 7.6a). Although 6 hours after challenge with Der p 1 the number of eosinophils at the PS site was significantly greater (*p*<0.01) than at the diluent control site it was not significantly different from eosinophil counts at other sites challenged with Der p 1 (Figure 7.6b). Eosinophil numbers at the PS sites challenged with *P. ovis* WME or Der p 1 declined from 6 to 24 hours after challenge. Eosinophils were found throughout the dermis but were concentrated in the perivascular areas.

**Neutrophils:** Six hours after challenge neutrophil numbers were significantly higher (*p*<0.01) at the PS sites challenged by *P. ovis* WME (Figure 7.6c) than at the diluent control sites but were not significantly different from those at other sites.
Similarly, numbers of neutrophils at the PS site challenged with Der p 1 (Figure 7.6d) were significantly greater \((p<0.001)\) than at the diluent control site but were not significantly different from neutrophil counts at other sites similarly challenged. Neutrophil numbers at PS sites decreased from 6 to 24 hours after challenge. As with eosinophils, infiltrating neutrophils were more abundant in perivascular tissue. 

\textit{Mast cells and basophils}: Cell counts at PS sites challenged with \textit{P. ovis} WME (Figure 7.6e) and Der p1 (Figure 7.6f) were not significantly different from those at the other sites.

### 7.3.4 Innate response to intradermal injection of \textit{P. ovis} WME – cell counts

\textit{Eosinophils}: At 6 and 24 hours after challenge the influx of eosinophils elicited by 0.05ml containing 5µg \textit{P. ovis} WME (Figure 7.7a) was significantly greater (respectively, \(p=0.007\) and \(p=0.002\)) in Dorsets than Swaledales. In Dorsets, \textit{P. ovis} WME also induced significantly higher eosinophil counts than the diluent control at 6 and 24 hours (respectively, \(p=0.011\) and \(p=0.015\)). However, in Swaledales the eosinophil response to \textit{P. ovis} WME was significantly greater \((p=0.024)\) than to the diluent control only at 24 hours after challenge.

\textit{Neutrophils}: No significant difference was detected in the neutrophil responses of Dorsets and Swaledales to challenge with \textit{P. ovis} WME (Figure 7.7b). But \textit{P. ovis} WME provoked a significantly greater influx of neutrophils than the diluent control in both breeds at 6 (respectively, \(p=0.02\) and \(p=<0.001\)) and 24 (respectively, \(p=0.04\) and \(p=0.011\)) hours after challenge.
**Mast cells and basophils:** At 24 hours cell counts at sites challenged with *P. ovis* WME (Figure 7.7c) were significantly greater (*p*=0.021) in Dorsets than Swaledales but in both breeds cell counts at sites challenged with *P. ovis* WME and the diluent control were not significantly different.
Figure 7.5  PK test: Wheal reactions after intradermal challenge with *P. ovis* WME and Der p 1

**a) Wheal reactions to *P. ovis* WME (n=5)**

<table>
<thead>
<tr>
<th>Time after intradermal challenge (hours)</th>
<th>Diluent*</th>
<th>Histamine*</th>
<th><em>P. ovis</em></th>
<th>NS+<em>P. ovis</em> WME*</th>
<th>HTPS+<em>P. ovis</em> WME*</th>
<th>PS+<em>P. ovis</em> WME*</th>
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**b) Wheal reactions to Der p 1 (n=5)**

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<th>Time after intradermal challenge (hours)</th>
<th>Diluent*</th>
<th>Histamine*</th>
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*50μl negative serum (NS), heat-treated positive serum (HTPS) and positive serum (PS) were injected intradermally and challenged 24 hours later with 50μl *P. ovis* WME (10μg/ml*) or Der p 1 (10μg/ml*) additional sites were injected with 50μl diluent, histamine and *P. ovis* WME or Der p 1.

Wheal reactions to *P. ovis* WME were significantly greater at PS sites than at the corresponding HTPS sites from 0.25 to 3 hours after challenge (a, p<0.02; b, p<0.05; c, p<0.001; d, p<0.01; e, p<0.03).

Wheal reactions to Der p 1 were significantly greater at PS sites than at the corresponding HTPS sites at 0.25 and 1 hour after challenge (c, p<0.001; d, p<0.01).
Figure 7.6  PK test: Cell counts 6 and 24 hours after intradermal challenge
challenge with P. ovis WME and Der p 1

(a) Eosinophils (n=5)

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<tr>
<th></th>
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<th>24 hours post-challenge</th>
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<tr>
<td>HTTPS+P. ovis WME</td>
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(b) Eosinophils (n=5)

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(c) Neutrophils (n=5)

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<td>P. ovis WME</td>
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(d) Neutrophils (n=5)

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(e) Mast cells and basophils (n=5)

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(f) Mast cells and basophils (n=5)

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<td>Diluent</td>
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Figs a), c) and e) show response to P. ovis WME and Figs b), d) and f) show response to Der p 1
*Caption as for Figure 7.5
For each cell type, significant differences between responses at PS and other sites are marked with the same letter (a, b, c, d, p<0.001; e, p<0.01; f, g, h, p<0.05).
Figure 7.7 Responses of different breeds to intradermal challenge with *P. ovis* WME

<table>
<thead>
<tr>
<th>Breed and challenge*</th>
<th>Eosinophils (n=6)</th>
<th>Neutrophils (n=6)</th>
<th>Mast cells and basophils (n=6)</th>
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<tbody>
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<tr>
<td>Swaledale, diluent</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Dorset, <em>P. ovis</em> WME</td>
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</tr>
<tr>
<td>Swaledale, <em>P. ovis</em> WME</td>
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*Sites with challenged with 50µl diluent or *P. ovis* WME (100µg/ml−1).
For each cell type responses of Dorsets and Swaledales that are significantly differences are marked with the same letter (a, p=0.007; b, p=0.002; c, p=0.021).
7.4 DISCUSSION

Although the mouthparts of *P. ovis* may abrade the stratum corneum (Blake et al. 1978) there is no evidence of their penetration beyond the inner layers of the stratum corneum (Sinclair and Filan, 1989). This suggests that the principal cause of lesional pathology and pruritus is the host’s immunoinflammatory response to mite products. These substances include a diverse array of antigens which will stimulate a multifaceted humoral and cellular immune response and may include the classical Type I to IV allergic reactions (Coombs and Gell 1968).

In chapter 6 it was shown that *P. ovis* stimulated an allergen-specific IgE response in sheep. In the present investigation, the positive immediate wheal responses obtained after IDST and PK tests indicated that *P. ovis* allergens interacted with mast cell-bound IgE precipitating degranulation. This is consistent with the detection, by passive cutaneous anaphylaxis, of a heat-labile skin-fixing antibody in sera from cattle infested with *P. ovis* (Losson et al. 1999). It also confirms the participation of IgE-mediated Type I reaction in the generation of cutaneous pathology. The present investigation also demonstrated that above a certain concentration (>20μg/ml) *P. ovis* proteins provoked a non-immune, inflammatory reaction in naïve sheep. This may explain the equivocal evidence of IH obtained in some earlier studies of rabbits and cattle in which IDST elicited similar wheal reactions in infested and naive animals (Wikel 1982, Losson et al. 1988).

In contrast to reports of a distinct 3-5 hour wheal possibly associated with an Arthus reaction (Weisbroth et al. 1972, Wikel 1982), the histopathology observed 6 hours after challenge in this study was typical of a LPR (Solley et al. 1976, Frew and Kay 1988, Tsicopoulos et al. 1994) and the immediate wheal response was found
to merge imperceptibly with this response. However, the present study confirmed earlier reports of a delayed (24 hour) wheal reaction to *Psoroptes* spp allergens (Weisbroth *et al.* 1972, Wikel 1982, Losson *et al.* 1988, 1999, Ravindran *et al.* 2000). But the response observed was maximal at 30 hours and sustained until 72 hours. Typically LPR wheal reactions and the associated cellular infiltration peak between 6 and 12 hours then gradually subside (Dolovich *et al.* 1973, Solley *et al.* 1976, Frew and Kay 1988, Tsicopoulos *et al.* 1994). The kinetics of the response seen in the present investigation is similar to that described in some CBH reactions and the classical tuberculin-induced DTH reaction (Dvorak 1970, 1976, Parrish and Breathnach 1996). But, histopathology revealed that, at 30 hours, this response was dominated by eosinophils and therefore fundamentally different from both the CBH and the classical tuberculin-induced DTH reaction which in sheep (Pyrah and Watt 1995) as in other animals is characterised by an infiltrate of mononuclear cells and variable numbers of neutrophils. Delayed infiltrates of eosinophils have been observed in some parasite infections and in responses of sensitised ruminants to intradermal injection of helminth extracts (Snider *et al.* 1985, Meeusen *et al.* 1990, 1995, Starke *et al.* 1996). Analysis, in sensitised sheep, of mammary gland responses to infusion of nematode larvae has indicated that an early (10 hour), infiltrate of eosinophils and neutrophils was succeeded at 48 hours by an increasing influx of eosinophils accompanied by CD4+ T cells but not neutrophils (Meeusen 1999). This response appears to parallel the cutaneous reaction of sensitised sheep to *P. ovis* allergens.

The immune mechanism(s) involved in generating this response are unclear. Studies in man indicate that the influx of eosinophils associated with the LPR is
dependent on IgE-mediated mast cell degranulation, an effect that can be passively transferred by reaginic serum, and may be augmented by the selective recruitment and activity of CD4+ T cells (Dolovich et al. 1973, Frew and Kay 1988, Gaga et al. 1991, Tsicopoulos et al. 1994, Werfel et al. 1995). The decline in eosinophil numbers detected at 24 hours post-challenge in the positive PK test suggests that the delayed influx of eosinophils observed in the active cutaneous response (7.3.2.2.2) is not dependent on IgE antibody or another serum factor. T cell dependence of cutaneous, eosinophil-rich delayed (18-72 hours) reactions of mice to *S. mansoni* cercariae and eggs has been demonstrated by adoptive transfer of cells and abolition of the response by depletion of CD4+ T cells (Ch’ang and Colley 1986, Teixeira et al. 2001). Moreover, analysis of the tuberculin-induced DTH revealed a small but significant increase in eosinophils at 24 hours and provided evidence for the existence of small but significant population of T cells possessing a Th2 cytokine profile but with delayed in time activity (Tsicopoulos et al. 1992, 1994). These observations are consistent with the proposed existence of an allergen-induced, Th2 T cell-mediated, delayed hypersensitivity response that is responsible for the second wave of infiltrating eosinophils in some parasite infections (Askenase 1996, Meeusen 1999).

The magnitude of the DTH-mediated influx of eosinophils suggests that in sheep scab this response rather than IH may play a major role in the recruitment of eosinophils that dominate the cutaneous infiltrate and cause tissue damage (Frigas and Gleich 1986, Frigas et al. 1991). However, the relative contribution of the IH and DTH reactions to host defence against infestation with *P. ovis* is uncertain. In cattle vaccinated with partially purified antigens, induction of an IH response but not
of an IH plus LPR appeared to improve resistance to experimental infestations (Pruett et al. 1998). Furthermore, intradermal challenge of infested cattle with *Psoroptes* antigens elicited an IH reaction in a relatively resistant breed but both IH and DTH reactions in a susceptible breed (Losson et al. 1999). These observations may indicate that in cattle the IH response is associated with resistance or alternatively that the LPR and DTH reactions increase susceptibility to infestation. Further investigations need to be undertaken to determine the role of these responses in resistance of sheep to infestation.

Cross-reactivity between *D. pteronyssinus*, a house dust mite, and *P. cuniculi* antigens has been demonstrated (Stewart and Fisher 1986). In the present study, wheal reactions recorded in sensitised sheep after intradermal challenge indicated that Der p 1 cross-reacts with *P. ovis* allergens to elicit immediate and possibly delayed reactions. However, the infiltrate was dominated by a pronounced influx of neutrophils. The pathway(s) involved in this preferential recruitment of neutrophils is uncertain. Arguably, as no significant difference was detected in the neutrophil response of *P. ovis*-naïve and sensitised sheep to intradermal challenge with Der p 1, interaction of Der p 1 with mast cell-bound IgE played a minor role in neutrophil recruitment. However, the ability of proteases such as Der p 1 to provoke cytokine release from mast cells in the absence of IgE has been demonstrated (Machado et al. 1996). Der p 1 has also been shown to exert a direct chemoattractant effect on neutrophils and also induced the expression of potent neutrophil-chemotactic cytokines, IL-8 and TNFα, by peripheral blood mononuclear cells from allergic patients while housedust mite extract stimulated IL-8 and TNFα expression by
cultured epithelial cells (Lau et al. 1999, Fahy et al. 2000). A further possibility is the presence of endotoxin in the Der p 1 preparation that was injected.

Clinical observations have suggested breed differences in susceptibility of sheep to P. ovis infestation (Bates 1997b, Fourie et al. 1997). A recent study found that following experimental infestation lesional growth was significantly greater in fine woolled animals, the polled Dorset and Bluefaced Leicester cross Swaledale, than the coarse woolled Swaledale (Taylor 2002). The present investigation is the first to provide histopathological evidence of breed differences in cutaneous responses to intradermal injection of P. ovis antigens. The difference in eosinophil responses suggests that the initial, innate response to P. ovis may be of critical importance in determining breed differences in susceptibility to infestation. There was a striking, approximately tenfold, greater eosinophil count 24 hours after infestation with 25 live mites (section 3.3.1) than after intradermal injection of extract obtained from 25 mites. The reason for this is uncertain though it may be attributable to differences in the concentration and activity of the relevant antigens and the site at which they were deposited.

It is evident that P. ovis antigens/allergens induce a complex immune response. This study demonstrates that intradermal injection of antigens/allergens, particularly when accompanied by sequential histopathological examination of the cutaneous infiltrate can facilitate identification of the different hypersensitivity responses elicited. Potentially, this provides a potent tool not only for the analysis of immune responses such as the allergen-induced delayed Th2-mediated hypersensitivity but also for the identification of allergens eliciting IH and DTH
responses and the selection of suitable candidate allergens for manipulation of the immune response.
8. TEMPORAL DEVELOPMENT OF OVINE CUTANEOUS HYPERSENSITIVITY RESPONSES TO P. OVIS

8.1 INTRODUCTION

Host responses to ectoparasitic arthropods commonly include immediate and delayed cutaneous hypersensitivity responses (reviewed by Wikel 1982, Allen 1994). Clinical responses to bites of haematophagous arthropods typically progress through five distinct phases (Mellanby 1946, Benjamini et al. 1961, Larrivee et al. 1964, Feingold et al. 1968). These comprise; Phase I, a period of induction when no response occurs; Phase II, only delayed hypersensitivity responses are detected; Phase III, immediate and delayed reactions are manifested; Phase IV, only immediate responses are seen and Phase V, a period of desensitisation, when no hypersensitivity responses are elicited. However, histopathology accompanying these responses has been examined in only a limited number of studies (Larrivee et al. 1964, Gross and Halliwell 1985, Foster et al. 1995). Although a similar sequence of hypersensitivity reactions has been demonstrated in pigs infested with the burrowing mite, S. scabiei, the temporal development of responses to other ectoparasitic arthropods, such as P. ovis, that are not haematophagous has not been reported (Davis and Moon 1990).

The investigations carried out in chapter 7 demonstrated that in sheep P. ovis infestations provoke immediate, late phase and delayed-type cutaneous hypersensitivity responses. The study described here employed clinical and histological examination of responses elicited by intradermal injection of P. ovis WME to investigate the temporal development of hypersensitivity responses in P. ovis infestations. As the T cell dependence of the eosinophil-rich delayed-type response to S. mansoni cercariae and eggs has been demonstrated, the relationship between eosinophils and different lymphocyte subtypes and dendritic cells was
examined at a limited number of intradermal test sites (Ch’ang and Colley 1986, Teixeira et al. 2001).

8.2 MATERIALS AND METHODS

8.2.1 Animals

Suffolk cross sheep, aged between one and two years and with no previous exposure to P. ovis, were used in these experiments.

8.2.2 Allergen extracts

P. ovis WME was prepared, the protein content determined and aliquots stored as described previously (section 6.2.2). Fresh solutions of sterile PBS (pH 7.2) containing P. ovis WME were prepared immediately before each skin test.

8.2.3 Performance of intradermal tests

Intradermal skin tests (IDSTs) were carried out as indicated earlier (7.2.3).

8.2.4 Wheal reactions and eosinophil counts infested and uninfested sheep.

Five sheep were infested to the left of the withers with a cluster of 25-50 ovigerous P. ovis mites as indicated earlier (2.2.1.1.). Throughout the experiment the fleece over the right lateral thorax and abdomen was kept closely clipped, exposing the skin, and Petroleum jelly (Vaseline®, Leverfaberge, GmbH, 21614 Buxtehude, Germany) was applied daily at the perimeter of this area. This preserved a lesion free area for IDSTs. In one animal the infestation failed to become established, consequently it was omitted from the results. Five sheep were used as uninfested controls.

IDSTs were carried out 1, 3, 5, 7, and 9 weeks after infestation and at the same intervals in the case of the uninfested controls. Before each test a 3x2 grid was marked on the clipped flank and 0.05ml of P. ovis WME (10μg protein/ml⁻¹) and
0.05ml of the diluent (PBS) control were each injected intradermally at three randomised sites on the flank. Wheal reactions were measured at 0.5, 6, 24 and 48 hours after challenge.

Six hours after challenge, following subcutaneous injection of a local anaesthetic (Lignol, Arnolds Veterinary Products), a 6mm punch biopsy was collected from one of the antigen and diluent control sites. The remaining antigen and diluent control sites were biopsied at 24 and 48 hours. At 9 weeks two additional sites were injected one with *P. ovis* WME and the other with the diluent control. These sites were biopsied 72 hours later.

Immediately after collection biopsies were bisected. Half was fixed in 4% paraformaldehyde and processed and later 5μm sections were stained with carbol chromotrope and eosinophil counts performed as described in section 2.2.1.3. For the purpose of this investigation it was decided to evaluate the hypersensitivity responses on the basis of eosinophil counts as these proved to be the most sensitive marker for both the 6 hour (late phase response) and the 30 hour (delayed) responses (see section 7.3.2.2.2). The other half of each biopsy was fixed in a non-aldehyde, zinc salts fixative (ZSF) and processed as indicated previously (section 4.2.3).

### 8.2.5 Immunohistochemistry

Immunohistochemistry was carried out on biopsy samples fixed in ZSF as described in section 4.2.3. Lymphocyte subsets and dendritic cells were identified in biopsies collected from infested sheep 48 hours after intradermal injection of diluent and antigen at 1 and 9 weeks. The EnVision Plus HRP System (Dako, Ely, UK) was employed to detect immunohistochemical labelling (section 4.2.3) by the selection of monoclonal antibodies employed in chapter 4 (Table 4.1). Cells were counted in an area of 0.2mm² (4.2.3).
8.2.6 Statistical analysis

For the reasons given earlier (7.2.8) differences in wheal reactions and cell counts elicited by intradermal injection of *P. ovis* WME and the diluent control in *P. ovis* naïve and infested sheep were determined by analysis of variance (7.2.8) using a GenStat release 4.2 programme (5th edition), (Lawes Agricultural Trust; Numerical Algorithms Group Ltd, Oxford, UK). Values of *p*<0.05 were considered significant. The relationship between eosinophils and the different lymphocyte subtypes and dendritic cells 48 hours after intradermal challenge with *P. ovis* WME was investigated using a Minitab Statistical Software programme, Minitab Release 13.1.

8.3 RESULTS

8.3.1 Wheal reactions in infested and uninfested sheep.

Comparison of wheal reactions of infested and uninfested sheep to *P. ovis* WME (Figures 8.1a and b) demonstrated that at 7 weeks responses of infested sheep were significantly greater than those of uninfested sheep at 0.5 (*p*=0.003) and 6 hours (*p*=0.029) after challenge. Similar 0.5 and 6 hour wheal reactions were seen infested sheep at 9 weeks (Figure 8.1a) but statistically, due to the degree of variance, these were not significantly greater than those in the uninfested controls.
Figure 8.1 Temporal development of wheal reactions to intradermal injection of *P. ovis* WME in infested and uninfested sheep

a) Wheal reactions of infested sheep (n=4)

Week and challenge:

- wk 1, diluent
- wk 1, *P. ovis* WME
- wk 3, diluent
- wk 3, *P. ovis* WME
- wk 5, diluent
- wk 5, *P. ovis* WME
- wk 7, diluent
- wk 7, *P. ovis* WME
- wk 9, diluent
- wk 9, *P. ovis* WME

Wheal diameters marked with the same letter are significantly different (a, *p*=0.003; b, *p*=0.029)

b) Wheal reactions in uninfested sheep (n=5)

Week and challenge:

- wk 1, diluent
- wk 1, *P. ovis* WME
- wk 3, diluent
- wk 3, *P. ovis* WME
- wk 5, diluent
- wk 5, *P. ovis* WME
- wk 7, diluent
- wk 7, *P. ovis* WME
- wk 9, diluent
- wk 9, *P. ovis* WME

*50μl diluent and *P. ovis* WME (10μg/ml) injected intradermally.

Wheal diameters marked with the same letter are significantly different (a, *p*=0.003; b, *p*=0.029)
8.3.2 Eosinophil counts at sites of intradermal challenge

In both infested and uninfested sheep challenge with *P. ovis* WME elicited a greater eosinophil response than the diluent control at 6, 24 and 48 hours after injection (Figure 8.2a and b). At 7 and 9 weeks the eosinophil influx observed 6 hours after challenge with *P. ovis* WME was significantly greater, \( p<0.02 \) and \( p<0.01 \), respectively), in infested than uninfested sheep. The mean eosinophil count provoked by the diluent control was consistently greater in infested than uninfested sheep from 3 to 9 weeks (Figure 8.2a and b). This probably reflected the development of a circulating eosinophilia in infested (see section 5.3.2) but not uninfested sheep.

At 9 weeks in 3 of the 4 infested sheep there was a dramatic influx of eosinophils at 24 hours that persisted until 72 hours after challenge with *P. ovis* WME. Eosinophil counts at 24 and 48 hours were significantly greater \( p<0.01 \) in infested than uninfested sheep (Figure 8.2a and b). Numbers of eosinophils present 6 and 24 hours after challenge of infested sheep by *P. ovis* WME were also significantly greater \( p<0.02 \) at 9 than 7 weeks after infestation (Figure 8.2a). In addition, at 9 weeks but not before, eosinophil counts 24 and 48 hours after challenge of infested sheep with *P. ovis* WME were significantly greater, \( p<0.01 \) and \( p<0.03 \), respectively), than those at 6 hours (Figure 8.2a). In uninfested sheep eosinophil numbers at 6, 24 and 48 hours after challenge with *P. ovis* WME were significantly different only at 7 weeks when counts 48 hours after challenge were greater \( p=0.039 \) than those at 6 hours (Figure 8.2b). In uninfested sheep eosinophil responses to *P. ovis* WME were significantly greater \( p=0.01, p=0.01, p<0.02 \), respectively) than those to the diluent 6, 24 and 48 hours after
challenge at 7 weeks and at 24 and 48 hours (p<0.02 and p=0.01, respectively) after challenge at 9 weeks after infestation.

**Figure 8.2** Temporal development of eosinophil responses of infested and uninfested sheep to intradermal injection of *P. ovis* WME

a) Eosinophil responses in infested sheep (n=4) week and challenge*

wk1, diluent
wk1, *P. ovis* WME
wk3, diluent
wk3, *P. ovis* WME
wk5, diluent
wk5, *P. ovis* WME
wk7, diluent
wk7, *P. ovis* WME
wk9, diluent
wk9, *P. ovis* WME

b) Eosinophil responses in uninfested sheep (n=5) week and challenge*

wk1, diluent
wk1, *P. ovis* WME
wk3, diluent
wk3, *P. ovis* WME
wk5, diluent
wk5, *P. ovis* WME
wk7, diluent
wk7, *P. ovis* WME
wk9, diluent
wk9, *P. ovis* WME

*50μl diluent and *P. ovis* WME (10μg/ml) injected intradermally. Eosinophil responses marked with the same letter are significantly different (a, c, f, p<0.02; b, c, d, g, p=0.01; h, p<0.03)
8.3.3 Immunohistochemistry

Having first established that at 9 weeks after infestation intradermal challenge with *P. ovis* WME elicited a massive, delayed infiltration of eosinophils, counts of CD4+ cells and other subpopulations of lymphocytes present 48 hours after challenge at 1 and 9 weeks were determined. At 1 and 9 weeks after infestation mean counts of helper T cells (CD4+), cytotoxic T cells (CD8+), γδ T cells (WC-1+) and putative naïve T cells or B cells (CD45RA+) were greater at sites challenged with *P. ovis* WME than at sites injected with the diluent control (Figure 8.3a-e). However, at 1 week after infestation only CD4+ counts were significantly greater (*p*=0.035) at sites challenged with *P. ovis* WME than at sites injected with the diluent control. At 9 weeks CD4+, CD45RA+ and γδ T cell counts were significantly greater at sites injected with *P. ovis* WME than at the diluent control site, *p*=0.028 and *p*=0.022 and *p*=0.017, respectively. No statistically significant correlation was found between any of the different cell types and eosinophil numbers at diluent and antigen sites.

Comparison of mean cell counts elicited by challenge with *P. ovis* WME indicated that only CD4+ counts were substantially greater at 9 weeks than 1 week after infestation but this increase was not statistically significant. The distribution of all the infiltrating lymphocytes was similar. Cells were found throughout the dermis but concentrated in the superficial layer and particularly in perivascular sites. Although CD4+ cells were considerably more numerous and diffusely distributed at 9 than 1 week after infestation, the same pattern of infiltration was retained. Eosinophils also shared this distribution though at 9 weeks after infestation, perivascular concentrations of eosinophils were more pronounced in the deep dermis than those of the different lymphocytes.
Figure 8.3 Responses of lymphocyte subpopulations, dendritic cells and eosinophils, 48 hours after intradermal injection of *P. ovis* WME at 1 and 9 weeks after infestation (n=4)

a) CD4+ cells

b) CD8+ cells

c) WC1+ cells
d) CD1b+ cells

e) CD45RA cells

f) Eosinophils

*50μl diluent and *P. ovis* WME (10μg/ml) injected intradermally. Counts with the same letter are significantly different (a, p=0.035; b, p=0.028; c, p=0.017; d, p=0.022)
8.4 DISCUSSION

Jones and Mote (1934) observed successive phases in the development of responses of human patients to intradermal injection of foreign proteins; induction (no response); only a delayed response; both immediate and delayed responses; only an immediate response. Subsequently, clinical observations have indicated that this temporal pattern of cutaneous hypersensitivity reactions, plus a final phase of desensitisation, is typical of host responses to several haematophagous arthropods such as, mosquitoes (*Aedes aegypti*), fleas (*Ctenocephalides felis*), sandflies, sucking lice and bedbugs (Mellanby 1946, Benjamini *et al.* 1961, Larrivee *et al.* 1964, Feingold *et al.* 1968, Nelson 1987, Oka and Ohtaki 1989, Jones 1996, Ratzlaff and Wikel 1990). A similar response pattern to the burrowing ectoparasitic mite, *S. scabiei* has been demonstrated in pigs (Davis and Moon 1990).

In the majority of previous studies, the occurrence of hypersensitivity reactions was determined solely on the basis of clinical evidence of wheal reactions without reference to the accompanying histopathology. In the present study, although intradermal challenge with *P. ovis* WME elicited immediate wheal reactions at 7 and 9 weeks and delayed reactions at 9 weeks after infestation these reactions were not as pronounced as those recorded in chapter 7 (section 7.3.2). However, despite this, eosinophil counts at 6 hours and from 24 to 72 hours after intradermal challenge were as great as those recorded in the earlier study at 6 and 30 hours (chapter 7, Figure 7.3). This observation is compatible with the demonstration, in atopic patients, of similar inflammatory events in late phase responses whether or not they were accompanied by wheal reactions (Zweiman *et al.* 1998). In the present investigation, histopathology proved more reliable than wheal reactions in revealing the presence of the delayed response and also, as was noted in Chapter 7 (section
7.3.2.2.2), demonstrated the non-classical eosinophil-rich nature of this response. On the evidence provided by wheal reactions and histopathology it is clear that in contrast to the classical response pattern, *P. ovis* infestation of sheep first elicited an immediate response which was succeeded by a phase when both immediate and delayed hypersensitivity reactions occurred. As the experiment was terminated at this point it is uncertain whether this phase would have been followed by a stage when only immediate reactions were detected.

Deviation from the classical response has been recorded in a number of instances. Jones and Mote (1934) recorded immediate reactions before delayed reactions in 4% of patients and, contrary to classical response pattern elicited by fleas (*C. felis*) in Guinea pigs, no consistent sequence was found in the development of hypersensitivity reactions of dogs to *C. felis* (Gross and Halliwell 1985). Furthermore, in contrast to the response seen in pigs only immediate hypersensitivity reactions were elicited in the course of *S. scabiei* infestation of foxes (Little *et al.* 1998). Similarly, contrary to the observations in man, *A. aegypti* provoked only immediate and late phase hypersensitivity reactions in rabbits while immediate and 6 hour but not delayed reactions were observed in cats infested with *Otodectes cynotis* (Hudson *et al.* 1958, Weisbroth *et al.* 1974, Powell *et al.* 1980).

The typical sequence of hypersensitivity responses is associated with haematophagous arthropods that introduce antigens intradermally. However, as *S. scabiei* which deposits antigens intradermally and epicutaneously generated an apparently identical sequence of reactions in pigs it is unlikely that the atypical response of sheep to *P. ovis* was simply due to epicutaneous deposition of antigens (Davis and Moon 1990. Moreover, it is clear from the examples cited above that a given ectoparasite, such as *C. felis*, *A. aegypti* or *S. scabiei*, can deposit antigens at
the same site in different species and individuals and elicit dissimilar response patterns. Davis and Moon (1990) examined the relationship between antigen dose and the temporal development of hypersensitivity reactions and concluded that, although increased mite density and presumably exposure to mite antigen accelerated the onset of delayed hypersensitivity responses, the response pattern provoked by high and low levels of antigen exposure was identical. These observations and the diversity of response patterns exhibited by different individuals and species suggest that the host genotype plays a critical role in determining the pattern of hypersensitivity responses.

The present study has demonstrated that immediate and non-classical delayed hypersensitivity responses are not detected until relatively late, approximately 7 and 9 weeks, respectively, in the course of *P. ovis* infestation. This temporal occurrence of an immediate wheal reaction is consistent with the detection of a significant increase in the serum level of *P. ovis* antigen-specific IgE seven weeks after infestation (section 6.3.1.2). In protracted primary infestations and in challenge infestations that provoke an anamnestic response, immediate and, more importantly, the non-classical, delayed hypersensitivity response may have a critical role in recruitment of eosinophils to lesional sites. Both responses may participate in host defence mechanisms and may be associated with the failure of challenge infestations to become established (section 2.2.2.1).

Analysis of the influx of different lymphocyte subtypes at sites challenged intradermally with *P. ovis* WME revealed a similar pattern to that observed during the course of infestation (section 4.3). At 48 hours after intradermal challenge, in contrast to the classical ovine DTH response to purified protein derivative (Pyrah and
Watt 1995), there was a substantial increase in γδ T cells and putative B cells (CD45RA⁺). The CD4⁺: CD8⁺ ratio was also considerably higher, 12:1 and 27:1 at 1 and 9 weeks, respectively, after infestation as opposed to 2:1 in the classical DTH response. This distinct profile of lymphocyte infiltration provides further evidence of the non-classical nature of the delayed response to *P. ovis*.

In the last chapter (section 7.3.3.2) the results of PK tests demonstrated that IgE elicited a 6 hour (late phase) but not a 24 hour (delayed) influx of eosinophils. This suggested that different mechanisms were involved in the immunopathogenesis of these reactions. The observation in the present investigation, of a distinct temporal separation in the occurrence of IgE-mediated Type I hypersensitivity and delayed hypersensitivity reactions has provided further evidence of this. In murine models, T cell-dependence of the eosinophil-rich, delayed type hypersensitivity reaction to *S. mansoni* cercariae has been demonstrated by transfer of the response to naïve mice by T cells harvested from sensitised mice and by abolition of the same response to *S. mansoni* eggs by depletion of CD4⁺ cells with anti-CD4⁺ monoclonal antibody (Ch’ang and Colley 1986, Teixeira et al. 2001). In the present investigation numbers of CD4⁺ T cells at sites of *P. ovis* WME challenge were substantially greater at 9 weeks than one week after infestation. This may indicate the involvement of CD4⁺ cells in the genesis of the eosinophil-rich delayed hypersensitivity response to *P. ovis*. However, CD4⁺ counts at other times, particularly when the immediate response first occurs also need to be examined.

The present study has confirmed the occurrence of immediate and eosinophil-rich delayed hypersensitivity responses in *P. ovis* infestations and demonstrated that their temporal development does not follow the classical response pattern to arthropod infections. These hypersensitivity responses developed relatively late in
the course of infestation and therefore their contribution to lesional pathology in primary infestations is of questionable significance. However, they may be more important in heavy infestations when responses appear to develop more rapidly and in protracted and challenge infestations. Distinct temporal separation of the first manifestation of immediate and delayed responses has provided further evidence that they are mediated by different mechanisms. Further investigations need to be carried out to determine whether or not the delayed response is mediated by CD4+ cells.
9. SUMMARY AND GENERAL DISCUSSION

This chapter is divided into three sections. The first section summarises the main findings of the research carried out in this thesis. In the second, these observations are integrated to formulate a hypothesis for the immunopathogenesis of sheep scab and in the third section some avenues for further research, arising from the present investigations, are proposed.

9.1. SUMMARY OF OBSERVATIONS MADE IN THIS THESIS.

The principal purpose of this research was to investigate the immunopathogenesis of the exudative, intensely pruritic cutaneous lesions that characterise sheep scab. It is clear from a review of the literature (section 1.2) that arthropod ectoparasites elicit a dynamic multifaceted immune response. IDSTs have demonstrated that hypersensitivity reactions play a critical role in this response (section 1.2.2.3). Immediate and delayed type hypersensitivity have been identified in most ectoparasite infestations but late phase responses and CBH have been recorded less frequently. Importantly, there is a lack of information on the histopathology and immunology of the delayed-type response. Although IDSTs in cattle and rabbits infested with *P. ovis* (section 1.3.5.3.2) provided questionable evidence of IH, they did indicate the occurrence of delayed-type reactions and possibly a late phase response to *Psoroptes* spp extract.

The research presented here demonstrated that lesional histopathology of sheep experimentally infested with *P. ovis* was characterised by an intense infiltrate of eosinophils, mast cell hyperplasia and degranulation and dermal edema (chapter 2). These features are typical of an IgE-mediated Type I hypersensitivity reaction. Investigation of isotype-specific antibody responses to *P. ovis* (chapter 6) confirmed the genesis of a *P. ovis* antigen-specific IgE antibody response as well as IgG and
IgM responses. This provided further evidence of an IH reaction while immediate wheal reactions after IDSTs and PK tests (chapter 7) confirmed the involvement of an IgE-mediated IH response to *P. ovis*. IDSTs also indicated the occurrence of late phase (6 hours) and delayed-type hypersensitivity responses (24-72 hours). Histopathology demonstrated that both these responses, particularly the delayed response, were dominated by eosinophils. Interestingly, IDSTs also revealed that these response developed late in the course of infestation (7 and 9 weeks, respectively) and that their temporal occurrence did not conform to the classical pattern of responses associated with ectoparasitic arthropods (chapter 8).

Although the IH, late phase response and non-classical, eosinophil-rich DTH (ER-DTH) play a crucial role in selective eosinophil recruitment is evident that the innate response provoked by *P. ovis* also makes a substantial contribution to the influx of eosinophils (chapter 3). This particular study also demonstrated that *P. ovis* caused the rapid development of degenerative and proliferative epidermal pathology.

Lastly comparison of lesional growth during primary and challenge infestations (chapter 2) demonstrated that primary infestations of *P. ovis* generate a substantial protective immunity.

9.2 THE IMMUNOPATHOGENESIS OF SHEEP SCAB – A HYPOTHESIS.

As the results have been fully discussed at the conclusion of each chapter, this section will seek to integrate the observations made in this thesis to formulate a hypothesis describing the immunopathogenesis of sheep scab. This is set out schematically in Figure 9.1 and may serve as a paradigm for the immunopathogenesis of other ectoparasite infestations.
One of the most striking observations made in this research is the multilayered nature of the immune response and its temporal development. It is evident from this study that histology may provide an adequate indication of the nature of the cellular infiltrate in the immediate innate response. However, subsequent adaptive responses are layered over this and histology, therefore, yields a composite picture of a multifaceted response at only one point of its development. Observation of wheal reactions, complemented by the staged histological examination of intradermal tests, carried out in the course of ectoparasite infestations, clearly facilitates discrimination of the different immune responses generated by an infestation and characterisation of the cellular infiltrates associated with them.

Clinical observations, confirmed by lesional histology, suggest that within hours of infestation *P. ovis* mites provoke an intense inflammatory response. As the mouthparts of mites abrade only the outermost layers of the stratum corneum it appears that the biologically active substances, contained in the faecal pellets deposited on the surface of the skin, elicit this response. These substances include diverse compounds such as immunogens including allergens, enzymes, chemoattractants, proinflammatory and possibly cytotoxic substances. Transepidermal penetration of these substances may be facilitated by proteases that, by analogy with Der p 1, disrupt tight junctions between epidermal cells and damage epidermal architecture. In addition to any direct proinflammatory and chemoattractant influence which they exert, mite products will interact with cells normally resident in the epidermis and dermis to initiate the release of a cascade of proinflammatory (IL-1, IL-6, TNFα) and immunomodulatory cytokines (IL-2, INFγ, IL-4, IL-5, IL-10, IL-12) and chemokines. The combined activity of these mite-
derived and mite-induced proinflammatory and chemoattractant compounds, particularly eosinophil chemoattractants, is probably responsible for the early inflammatory response that is characterised by infiltration of eosinophils and mast cells, for the degenerative and proliferative epidermal changes and for the development of pruritus.

At the same time mite-derived immunogens will interact with Langerhans’ cells and dermal dendritic cells initiating an adaptive response. The ability of APCs to polarise the resulting immune response along Th1 or Th2 pathways may be influenced by the characteristics of the immunogens, other mite-derived substances and the mite-induced release of immunomodulatory cytokines and other compounds such as histamine. Indeed, it is possible that, by analogy with Der p 14, some mite products act as proallergic adjuvants and promote a Th2 response (Epton et al. 2001).

In the course of infestation, the population density and relative numbers of larvae, nymphs and adults will alter. This would result in corresponding changes in the concentration and profile of antigen and allergen challenge – alterations which appear to be reflected in the western blots and will potentially influence that nature of the adaptive response. Although it is anticipated that the acquired immune response will involve both Th1 and Th2 CD4+ T cells, the detection of an IH, LPR and ER-DTH, suggest the response to P. ovis is dominated by the activity of CD4+ lymphocytes expressing a Th2-like cytokine profile. The contribution of Th2 response requires further investigation. Components of the immune response to P. ovis detected in this study have been identified in ovine responses to other arthropod ectoparasites (1.2). However, it remains to be seen whether the ER-DTH response to P. ovis is a feature of other ectoparasite infections and whether it is analogous to the CBH reaction seen in responses to ticks. The hypersensitivity reactions
generated by *P. ovis* will augment the inflammatory response, exacerbate tissue damage and increase pruritus. It is also possible that, by reducing mite fecundity, they make a significant contribution to the substantial protective response that was demonstrated in the reinfection study.

**Figure 9.1** Immunopathogenesis of sheep scab – a schematic representation.
9.3 POSSIBILITIES FOR FURTHER RESEARCH.

The observations made in the course of this research raise a number of questions and possibilities for further research.

1. **What mechanisms are involved in the generation of the innate, eosinophil-rich response to *P. ovis* and for the early degenerative and proliferative epidermal pathology?**

   *In vitro* research by my collaborators has indicated that *P. ovis* WME contains eosinophil chemoattractants and cytotoxic substances. The latter may contribute to the development of epidermal pathology. A major house dust mite allergen, Der p 1 exhibits neutrophil and eosinophil chemoattractant properties while Der p 1, 3, and 9 and crude house dust mite extract interact with cells to stimulate the release of cytokines such as IL-4, TNFα and IL-8 and the chemokines RANTES and eotaxin (Machado *et al.* 1996, Lau *et al.* 1999, Fahy *et al.* 2000, Sun *et al.* 2001). By analogy, *P. ovis*-derived homologues of these may play an important role in eosinophil chemotaxis and stimulation of the selective release of eosinophil chemokines that promote the preferential recruitment of eosinophils. Pilot studies conducted by myself in *P. ovis*-sensitised sheep have demonstrated that patch tests with *P. ovis* WME elicit an eosinophil-rich infiltrate. The same technique could be applied in *P. ovis*-naïve sheep to investigate the chemoattractant properties of fractions of *P. ovis* WME or homologues of house dust mite allergens identified in a *P. ovis* cDNA expression library. In addition, if combined with *in situ* hybridisation or the reverse transcriptase-polymerisation chain reaction (RT-PCR), patch tests could be employed to determine whether these substances stimulate mRNA expression for eosinophil chemokines. Indeed, as *P. ovis* products are deposited on an intact or abraded epidermis, patch tests may be of considerable value in
examining several aspects of the immunopathogenesis of sheep scab. If optimised, they could also be exploited to investigate early events in the genesis of the adaptive response by staged collection of biopsies that could be examined for cell and cytokine activity by histopathology, immunohistochemistry, in situ hybridisation and RT-PCR. Employed in conjunction with lymphatic cannulation they could also be used to determine the kinetics of cell and cytokine traffic in afferent lymphatics. This would give an insight into signals generating the adaptive response. Potentially this would not only improve our understanding of the immunopathogenesis of responses to P. ovis but also facilitate effective manipulation of the response to promote host protection. Furthermore, if developed successfully the ovine patch test model could be employed to investigate the immunopathogenesis of other allergic skin diseases. It may find particular application as a model for human atopic dermatitis which is frequently caused by products of house dust mites such as Der p 1 that have homologues in the products of P. ovis.

2. What is the role of eosinophils in polarisation of the adaptive response and mite survival?

The ability to selectively inhibit lesional recruitment of eosinophils would facilitate an insight into their function in these areas. In several animal species blockade of the very late antigen-4 (VLA-4), an α4 integrin expressed by eosinophils, with anti-VLA-4 antibody inhibits the influx of eosinophils to sites of allergen challenge (reviewed Kita et al. 1996, Weller 1997). Aerosol administration of a potent small-molecule inhibitor of VLA-4 significantly reduced the influx of eosinophils in an ovine model of allergic asthma and may be of value in inhibiting cutaneous recruitment of eosinophils (Abraham et al. 1997). Alternatives that could be explored include the use of monoclonal antibodies against ovine IL-5 (currently in
preparation, Jones and Huntley personal communication) or eotaxin (Kusama et al. 1995, Teixeira et al. 2001). Recently, administration of trifluoromethyl ketone, a selective inhibitor of cytosolic phospholipase A2, the enzyme responsible for initiating the conversion of membrane phospholipids to potent inflammatory mediators, and of a PAF antagonist (E6123) were shown to inhibit eosinophil recruitment in a guinea pig model of allergic asthma (Myou et al. 2001). It may be possible to use these compounds in sheep. Another approach would be to compare responses in breeds of sheep with significantly different eosinophil responses to *P. ovis* infestation.

A number of articles have suggested that the early innate response plays a critical role in polarisation of the later adaptive response towards an allergic Th2-cytokine profile (Fearon and Locksley 1996, Medzhitov and Janeway 1997, Kapsenberg et al. 1998). The pronounced, eosinophil-rich, innate response to *P. ovis* infestation presents an opportunity to examine this relationship. IDSTs carried out in *P. ovis*-naïve sheep of different breeds (chapter 7) demonstrated that within 24 hours of intradermal challenge a substantial infiltration of eosinophils occurred in the ‘susceptible’ Dorset breed but not in the ‘resistant’ Swaledale breed. Moreover, a parallel study found that in the 6 weeks after infestation of *P. ovis*-naïve animals lesions growth was significantly less in Swaledales than in Dorsets (Taylor 2002). Arguably, therefore, this marked eosinophil response may promote the establishment and expansion of *P. ovis* infestations. It is also possible that the early eosinophil response biased the adaptive response towards an allergic Type I IgE-mediated reaction. The nature of the adaptive response in Swaledales has not yet been investigated. Comparison of the temporal development of antibody and hypersensitivity responses in Dorsets and Swaledales may give an indication of the
role of eosinophils in polarisation of the adaptive response. In addition, immune responses of Dorsets in which the eosinophil response is abolished by administration of anti-IL-5 monoclonal antibody before infestation could be examined. In this investigation comparison of mite numbers in treated and untreated controls would also give an indication of the influence of eosinophils on mite survival. This could be explored further by treatment of cohorts of infested sheep at different times after infestation and determining its effect on the mite population and lesional expansion.

3. What immunological mechanisms mediate the non-classical, eosinophil-rich delayed-type hypersensitivity response (ER-DTH)?

In the research presented here, histopathology and immunohistochemistry demonstrated that this response was not typical of the classical DTH (chapters 7 and 8). A DTH reaction has frequently been identified in ectoparasite infestations (reviewed by Wikel 1982, Allen 1994). However, in the majority of instances its nature has not been defined histologically or immunologically. This is evidently an area that requires further investigation to determine whether the non-classical nature of the DTH observed in the studies presented here is typical of other ectoparasite infestations.

In addition, the immunology of the ER-DTH requires further investigation. PK tests (chapter 7) indicated that it was not transferred by a serum factor while the CD4+ infiltrate seen after intradermal challenge with P. ovis WME (chapter 8) suggests that CD4+ cells may be involved in generating the ER-DTH. Murine studies employing adoptive transfer of T cells and depletion of CD4+ lymphocytes have demonstrated the T cell dependence of eosinophil-rich delayed-type cutaneous responses to S. mansoni (Ch’ang and Colley 1986, Teixeira et al. 2001). Adoptive transfer of CD4+ cells from sensitised to naïve sheep followed by intradermal
challenge with *P. ovis* WME or prechallenge administration of anti-ovine CD4\(^+\) monoclonal antibody to sensitised sheep would clarify the role of T cells the ovine ER-DTH

4. *What mechanisms are involved in the genesis of the protective response?*

SDS-PAGE/Western blot studies (chapter 6) demonstrated that antigens/allergens >100kD consistently provoked an IgG/IgE antibody response while histopathology of IDSTs (chapter 7) and the pilot patch test studies conducted in *P. ovis*-sensitised sheep demonstrated that these tests are sensitive techniques for identifying *P. ovis* allergens. These techniques could be exploited in screening fractions of allergens identified on SDS-PAGE/Western blots for their ability to elicit immediate, late phase and delayed-type hypersensitivity reactions. Identification of allergens involved in the hypersensitivity reactions may facilitate their use in manipulation of the immune response. For example, prior immunisation with selected allergens in an appropriate adjuvant may induce a shift from the Th2-like to a Th0-like cytokine profile (van Neerven 1999).
10. BIBLIOGRAPHY


Mbow, M. L., Rutti, B., and Brossard, M. 1994. Infiltration of CD4+ CD8+ T cells, and expression of ICAM-1, Ia antigens, IL-1 alpha and TNF-alpha in the skin lesion of BALB/c mice undergoing repeated infestations with nymphal Ixodes ricinus ticks. Immunology 82:596-602.


Sheahan, B. J. 1975. Pathology of *Sarcoptes scabiei* infection in pigs. II. Histological, histochemical and ultrastructural changes at skin test sites. *J. Comp. Pathol.* **85**:97-110.


Shilston, A. 1915. Observations on the Life history of *Psoroptes communis* var *ovis* and some points connected with the epizootiology of the disease in South Africa. 3rd and 4th Reports to the Director of Veterinary Research, Union of South Africa, Department of Agriculture, Pretoria. 69-98.


11. APPENDIX

11.1 BUFFERS

Phosphate buffered saline (PBS)

8g NaCl
0.2g KCl
1.15g Na₂HPO₄
0.2g KH₂PO₄
1L Distilled water
Mix well and adjust the pH to 7.2-7.3

Tris buffered saline (TBS – 0.05M Tris HCl/0.15M NaCl)

1. **1M Tris HCl**
   121.1g Trizma® base (Sigma T 8524)
   800ml Distilled water
   Adjust the pH to 7.6 with concentrated HCl, autoclave at 121°C for 15 minutes

2. **5M NaCl**
   292.2g NaCl
   800ml Distilled water
   autoclave at 121°C for 15 minutes

3. **Tris buffered saline (TBS - 0.05M Tris HCl/0.15M NaCl)**
   125ml 1M Tris HCl
   75ml 5M NaCl
   2L Distilled water
   mix well and adjust the pH to 7.6 with concentrated HCl if necessary, add distilled water to give a final volume of 2.5L

Coating buffer for ELISAs

1. **Solution A. 0.2M Na₂CO₃**
   21.2g Na₂CO₃
   1L Distilled water
2. **Solution B. 0.2M NaHCO$_3$**

16.8g NaHCO$_3$

1L Distilled water

3. To prepare coating buffer, mix 80ml of solution A, 170ml Solution B and 750ml distilled water and adjust the pH to 9.6.

**Tank Buffer for ELISAs**

45g Tris

216g glycine

15g SDS

3L Distilled water

mix well

**Reducing sample buffer for Western blots**

2.5ml stacking buffer

2.3ml Distilled water

2.0ml Glycerol

2.0ml 20% Sodium dodecyl sulphate

1.0ml β-mercaptoethanol (Sigma M 6250)

0.2ml 0.1% bromophenol blue (Sigma B 7021)

**Resolving buffer for Western blot gels**

60.5g Tris

500ml Distilled water

Tris is allowed to dissolve and then concentrated HCl is added to bring the pH down to 8.8

**Stacking buffer for Western blot gels**

30.25g Tris

500ml Distilled water

Tris is allowed to dissolve and then concentrated HCl is added to bring the pH down to 6.8
Anode and cathode buffers for Western blots

1. **Anode 1 buffer (0.3M Tris/20% methanol pH 10.4)**
   
   - 18.5g Trizma® base (Sigma T 8524)
   - 100ml Methanol
   - 400ml Distilled water
   
   Mix the reagents well and adjust the pH to 10.4 if necessary

2. **Anode 2 buffer (25mM Tris/20% methanol pH 10.4)**
   
   - 1.5g Trizma® base (Sigma T 8524)
   - 100ml Methanol
   - 400ml Distilled water
   
   Mix the reagents well and adjust the pH to 10.4 if necessary

3. **Cathode buffer (25mM Tris/40mM 6-amino-n-hexanoic acid/20% methanol pH 9.4)**
   
   - 1.5g Trizma® base (Sigma T 8524)
   - 2.62g 6-amino-n-hexanoic acid
   - 100ml Methanol
   - 400ml Distilled water
   
   Mix the reagents well and adjust the pH to 9.4 if necessary

Transfer of electrophoretically resolved proteins onto nitrocellulose

2 sheets of filter paper pre-soaked in Anode 1 buffer are placed on the platinum anode and 2 sheets of filter paper pre-soaked in Anode buffer 2 placed on top of these. The pre-wetted nitrocellulose is put on top of the filter papers and the gel carefully layered on top of this. The 2 sheets of filter paper pre-soaked in the Cathode buffer are placed over the gel and a test tube rolled over the paper to exclude air bubbles. The cathode is placed on the stack and the current run for 60 minutes at 70 amps per gel.

11.2 FIXATIVES

4% Paraformaldehyde

- 8g Paraformaldehyde
- 200ml PBS
The solution is heated gently in a fume cupboard to dissolve the paraformaldehyde and allowed to cool before use.

**Zinc Salts Fixative**

- 0.1M Trizma® base (Tris) Sigma T 8524
- 0.05% calcium acetate
- 0.5% zinc chloride
- 0.55% zinc acetate

12.1g trizma base, 0.5g calcium acetate and 1000ml distilled water are mixed and then HCl added to give a pH between 7.0-7.4

5g zinc chloride and 5g zinc acetate are then added to the tris calcium acetate buffer

**11.3 GELS**

**12% Resolving gel for Western blots**

- 2.5ml Resolving buffer
- 0.5ml 20% Sodium dodecyl sulphate
- 10μl Temed (Sigma T 9281)
- 4.1ml 30% w/v Acrylamide/bis acrylamide stock solution *
- 2.8ml Distilled water

* Severn Biotech Ltd, Kidderminster, Worcs

**4% Stacking gel for Western blots**

- 1.4ml stacking buffer
- 0.14ml 20% Sodium dodecyl sulphate
- 10μl Temed (Sigma T 9281)
- 0.72ml 30% w/v Acrylamide/bis acrylamide stock solution
- 3.09ml Distilled water

100μl of 10% ammonium persulphate is added to the resolving and stacking gel solutions immediately before use

**11.4 SOLUTIONS**

**1% sodium dodecyl sulphate (SDS)**

- 2g Sodium dodecyl sulphate
- 200ml Distilled water
20% sodium dodecyl sulphate (SDS)

40g Sodium dodecyl sulphate
200ml Distilled water

PBS/0.05% Tween 20 (PBS/T20)

5L PBS
2.5ml Tween 20 (Sigma P 7949)

PBS/0.5% Tween 80 and 0.5M NaCl (PBS/T20/NaCl)

5L PBS
146.1g NaCl
25ml Tween 80 (Sigma P 8074)

Preparation of sera for IgE ELISA or Western blot

Heat inactivate (56°C for 30 minutes) 1ml of serum
Add 430μl of saturated ammonium sulphate and mix immediately
Centrifuge for 5 minutes at 8K
Pipette supernatant into new eppendorf and add 570μl saturated ammonium sulphate
Incubate overnight at 4°C or for a minimum of 30 minutes at 4°C
Centrifuge for 5 minutes at 8K
Discard the supernatant
Resuspend the pellet in 2ml of PBS/T80/NaCl

11.5 STAINS

Carbol chromotrope (0.5% Chromotrope in 1% Phenol) stain

5g Chromotrope*
10g Phenol crystals
1L Distilled water
Phenol is melted by gently heating in a fume cupboard then chromotrope is added and the mixture is dissolved in distilled water. After filtering the solution is ready for use.

*Chromotrope 2R, Gurr® microscopy materials, BDH, Poole, UK
11.6 DISTRIBUTION OF MITES ON LESIONAL SKIN OF P. OVIS

INFESTED SHEEP – MITE COUNTS

<table>
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<th>Original infestation area</th>
<th>Mid-lesion area</th>
<th>Advancing margin area</th>
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<td>6</td>
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<tr>
<td>10</td>
<td>10</td>
<td>60</td>
<td>200</td>
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</table>

Estimates of mite numbers, **50-200 mites, ***200-500 mites, ****> 500 mites

11.7 ANOVA RESULTS

11.7.1...Chapter 2

a) ANOVA results for section 2.2.2.1 - Differences in lesional area of group 1, 2 and 3 animals at different times in the course of infestation.

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b) ANOVA results for section 2.2.2.2.1 - Differences in eosinophil, mast cell and basophil and neutrophil responses of group 1, 2 and 3 animals at different times in the course of infestation.

Eosinophils

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Mast cells and basophils

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Neutrophils

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<td>Group 1 v Group 3</td>
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<td>P value</td>
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<td>Group 2 v Group 3</td>
<td>Degrees of freedom</td>
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c) ANOVA results for section 2.2.2.2.1 - Differences in eosinophil, mast cell and basophil and neutrophil responses in different sites of the lesion in the course of primary infestations.

Eosinophils

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<tr>
<td>Original site v mid-lesion</td>
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<td>Mid-lesion v advancing margin</td>
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Mast cells and basophils

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<td>Degrees of freedom</td>
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<td>F-statistic</td>
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<tr>
<td>Original site v mid-lesion</td>
<td>Degrees of freedom</td>
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<td></td>
<td>P value</td>
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<tr>
<td>Mid-lesion v advancing margin</td>
<td>Degrees of freedom</td>
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<td></td>
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11.7.2 Chapter 3

ANOVA results for section 3.3.1 - Comparison of eosinophil, mast cell and basophil and neutrophil counts in normal and infested skin.

Eosinophils

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<th>P value</th>
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<tbody>
<tr>
<td>Lesional v normal skin</td>
<td>1.8 1.8 1.8 1.8</td>
<td>1 4 8 14 21</td>
<td>16.65 20.33 20.36 27.4 71.08</td>
<td>&lt;.001 &lt;.001 &lt;.001 &lt;.001 &lt;.001</td>
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<tr>
<td>F-statistic</td>
<td>2.99 49.56 52.16</td>
<td>1.8 1.8 1.8 1.8 1.8</td>
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<tr>
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Mast cells and basophils

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<th>Time (days after infestation)</th>
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<th>P value</th>
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<tbody>
<tr>
<td>Lesional v normal skin</td>
<td>1.8 1.8 1.8 1.8</td>
<td>1 4 8 14 21</td>
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11.7.3 Chapter 4.

a) ANOVA results for section 4.3 - Differences in populations of lymphocytes and dendritic cells in normal and lesional skin at different times in the course of infestation

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<tr>
<td>CD4+</td>
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<td>Degrees of freedom</td>
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<td>F-statistic</td>
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<td>CD8+</td>
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<td>P value</td>
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<tr>
<td>WC1+</td>
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<td>Degrees of freedom</td>
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<tr>
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<td></td>
<td>P value</td>
</tr>
<tr>
<td>CD1+</td>
<td>Normal v lesional skin</td>
<td>Degrees of freedom</td>
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<td>CD45RA+</td>
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b) ANOVA results for section 4.3 - Differences in populations of lymphocytes and dendritic cells in lesional skin at different times in the course of infestation

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<tr>
<td></td>
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<td>3 v 5</td>
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<td>Degrees of freedom</td>
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### Chapter 5

**ANOVA results for sections 5.3.1 and 5.3.2 - Differences in red and white blood cell responses of group 1, 2 and 3 animals at different times in the course of infestation.**

#### Red blood cells

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<td>Grp 1 v Grp 2</td>
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<td>1,12</td>
<td>1,12</td>
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#### Neutrophils

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### Monocytes

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### Eosinophils

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### Basophils

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ANOVA results for sections 6.3.1.1, 6.3.3.2 and 6.3.3.3 - Differences in isotype-specific antibody responses of group 1, 2 and 3 animals at different times in the course of infestation.

IgG antibody

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IgE antibody

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IgM antibody

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11.7.6 Chapter 7

a) ANOVA results for section 7.3.2.2.2 - ID test, differences in eosinophil, neutrophil and mast cell and basophil responses of P. ovis-naive and sensitised sheep to intradermal challenge with P. ovis WME and Dp 1.

Eosinophils

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<td>Degrees of freedom</td>
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Neutrophils

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<td>Degrees of freedom</td>
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<td>Dp 1</td>
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<td>Degrees of freedom</td>
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Mast cell and basophils

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b) ANOVA results for section 7.3.3.2 - PK test: differences in eosinophil, neutrophil and mast cell and basophil responses to intradermal challenge at positive, heat-treated positive serum and normal serum sites, with P. ovis WME.

Eosinophils

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**Neutrophils**

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**Mast cells and basophils**

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<th>P value</th>
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**c) ANOVA results for section 7.3.3.2 - PK test: differences in eosinophil, neutrophil and mast cell and basophil responses to intradermal challenge at positive, heat-treated positive serum and normal serum sites, with Der p 1.**

**Eosinophils**

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### Neutrophils

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### Mast cells and basophils

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### d) ANOVA results for section 7.3.4 - Differences in eosinophil, neutrophil and mast cell and basophil responses of Dorsets and Swaledales to intradermal challenge with *P. ovis* WME.

### i) Differences between breeds

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(ii) Differences between *P. ovis* WME and diluent

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11.7.7 Chapter 8.

a) **ANOVA results for section 8.3.2 – Comparison of temporal development of eosinophil responses of infested and uninfested sheep to intradermal injection of *P. ovis* WME and diluent**

**Week 1**

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### Week 7

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### Week 9

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**b) ANOVA results for section 8.3.2 – Comparison of eosinophil responses 6 and 24 and 6 and 48 hours after intradermal injection of P. ovis WME in infested and uninfested sheep.**

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<th>Uninfested (24 hr v 6 hr)</th>
<th>Infested (48 hr v 6 hr)</th>
<th>Uninfested (48 hr v 6 hr)</th>
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**c) ANOVA results for section 8.3.2 – Comparison of eosinophil responses of infested sheep to intradermal challenge with P. ovis WME at 7 and 9 weeks.**

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**d) ANOVA results for section 8.3.2 – Comparison of eosinophil responses of infested sheep to intradermal challenge with diluent and P. ovis WME in the course of infestation.**

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<tr>
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<td>P. ovis WME v diluent</td>
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**e) ANOVA results for section 8.3.2 – Comparison of eosinophil responses of P. ovis-naive sheep to repeated intradermal challenge with diluent and P. ovis WME.**

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<table>
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<th>P value</th>
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<th>P value</th>
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<td>1,4</td>
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**f) ANOVA results for section 8.2.3 – Comparison of responses of lymphocyte subpopulations and of dendritic cells, 48 hours after intradermal challenge with P. ovis WME at 1 and 9 weeks after infestation.**

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<th>Diluent v CD8⁺</th>
<th>Diluent v WC1⁺</th>
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12. PAPERS ARISING FROM THIS THESIS

Published:

In press:
