THE ROLE OF INTERLEUKIN-8 AS A NEUTROPHIL CHEMOATTRACTANT IN BOVINE PNEUMONIC PASTEURELLOSIS

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SUMMARY OF DISSERTATION

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ABSTRACT

The role of interleukin-8 (IL-8) as a neutrophil chemoattractant in bovine pneumonic pasteurellosis was investigated by characterizing: (1) the expression of IL-8 in the lesions of pneumonic pasteurellosis, (2) the *in vitro* and *in vivo* effects of recombinant bovine IL-8 on neutrophil chemotaxis, and (3) the importance of IL-8 as a neutrophil chemoattractant in this disease.

The expression of IL-8 in bovine pneumonic pasteurellosis was assessed by Northern analysis, *in situ* hybridization and enzyme-linked immunosorbent assay. The expression of IL-8 mRNA was elevated dramatically in lesional lung compared to non-lesional lung, viral pneumonia, and normal lung. *In situ* hybridization revealed intense IL-8 mRNA expression in alveolar macrophages and neutrophils, and milder expression in several other cell types. Bronchoalveolar lavage (BAL) fluid from lesional lung contained 16.06 ± 4.00 ng/ml IL-8, but lower levels were present in non-lesional lung, viral pneumonia, and normal lung (mean ± SEM).

Recombinant bovine IL-8 (rbIL-8) was produced using a pGEX expression system. The rbIL-8 induced *in vitro* chemotaxis and shape change of bovine neutrophils at doses as low as 6.3 ng/ml. After injection of 1.0 ng to 3.3 μg of rbIL-8 into bovine skin, neutrophils marginated in vessels within 15 minutes and infiltrated the dermis by 1 hour after injection. These results demonstrate that bovine IL-8 is a neutrophil chemoattractant *in vitro* and *in vivo*, at levels equivalent to those present in pneumonic pasteurellosis.

An ovine model of pneumonic pasteurellosis was developed to ascertain the

effects of *in vivo* neutralization of IL-8 on the development of disease. At 0, 3, 8 and 20 hours after bacterial infection (p.i.), BAL fluid samples contained 6.5 ± 2.4, 110 ± 48, 554 ± 118, and 507 ± 70 ng/ml IL-8, respectively. Neutrophil infiltration was mild at 3 hours p.i. but progressively more intense at 8 and 20 hours p.i. Lambs were treated intravenously with up to 80 mg of an IL-8-neutralizing antibody prior to bacterial challenge. The antibody treatment failed to completely neutralize IL-8 activity, probably because the levels of IL-8 in the lung were unexpectedly high.

The contribution of IL-8 to the neutrophil chemotactic activity of BAL fluid from cattle and lambs with pneumonic pasteurellosis was assessed. Pre-treatment of pneumonic BAL fluid samples with a neutralizing antibody to IL-8, compared to pre-treatment with a control antibody, reduced the *in vitro* neutrophil chemotactic activity significantly, by 15-63% in 9/10 samples. An extract of pneumonic bovine lung induced vigorous neutrophil infiltration following injection into bovine skin, and depletion of IL-8 from the extract reduced this neutrophil influx by 60%. These data indicate that IL-8 is an important neutrophil chemoattractant in both early and well-developed lesions of pneumonic pasteurellosis, but that mediators with actions redundant to those of IL-8 must also be present at these times.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

aa, amino acid

BAL, bronchoalveolar lavage

BHI, brain-heart infusion (broth)

BHV-1, bovine herpesvirus-1

bIL-8, bovine interleukin-8

BRSV, bovine respiratory syncytial virus

BVD, bovine viral diarrhea

C5a, complement-derived anaphylatoxin 5a

cfu, colony-forming units

CXCR, CXC chemokine receptor

ELISA, enzyme-linked immunosorbent assay

ENA, epithelial cell-derived neutrophil attractant

FcγR, receptor for Fc component immunoglobulin G

Fig., figure

fMLP, formylated methionine leucine phenylalanine

GM-CSF, granulocyte monocyte colony stimulating factor

GCP, granulocyte chemotactic protein

GRO, growth-related oncogene

HBSS, Hank's balanced salt solution

IL, interleukin

IP-10, interferon-inducible protein-10

LT, leukotriene

LPS, lipopolysaccharide

M, molar

MIG, monokine-induced-by-interferon-γ

mRNA, messenger ribonucleic acid

P., Pasteurella

PAF, platelet activating factor

PBS, phosphate-buffered saline

PBST, phosphate-buffered saline with Tween-20

PG, prostaglandin

p.i., post-inoculation or post-infection

PI3, parainfluenza-3 virus

rbIL-8, recombinant bovine interleukin-8

roIL-8, recombinant ovine interleukin-8

RPMI-1640, Roswell Park Memorial Institute culture medium 1640

SDF, stromal-derived factor

SEM, standard error of the mean

TBST, Tris-buffered saline with Tween-20

TCID, tissue culture infective dose

TGF-β, transforming growth factor-β

TNF-α, tumour necrosis factor-α

WCVM, Western College of Veterinary Medicine

vs., versus

%, percent

1.0. INTRODUCTION AND LITERATURE REVIEW

1.1. Objectives

The importance of neutrophils in the defence against bacterial infection is irrefutable; however, in cases of overwhelming bacterial infection or non-infectious suppurative inflammation, neutrophil secretions may induce substantial tissue injury. In order to comprehend the general pathology of these disease processes and to devise effective adjunctive therapies to limit neutrophil-dependent tissue injury, it is desirable, therefore, to understand the mechanisms that recruit neutrophils to sites of bacterial infection. Pneumonic pasteurellosis is an economically important disease of cattle, and is the exemplar of neutrophil-dependent tissue injury in animals.

The objective of this research is to explore the role of interleukin-8 as a neutrophil chemoattractant in bovine pneumonic pasteurellosis, and to determine the importance of interleukin-8-dependent neutrophil recruitment in the development of clinical signs and lesions of this disease.

There were three approaches to this investigation of the role of interleukin-8 as a neutrophil chemoattractant in pneumonic pasteurellosis. Firstly, to evaluate the specific effects of IL-8 in cattle, bovine interleukin-8 was produced as a recombinant protein and the *in vitro* and *in vivo* effects of this chemokine were characterized. Secondly, to determine the potential for IL-8 to act as a neutrophil chemoattractant in

bovine pneumonic pasteurellosis, the expression of interleukin-8 was analysed in the lesions experimentally induced by *Pasteurella haemolytica*. Thirdly, two approaches were utilized to define the importance of interleukin-8 as a neutrophil chemoattractant and mediator of severe disease in pneumonic pasteurellosis: determining the effect of neutralization of IL-8 on the development of experimentally induced ovine pneumonic pasteurellosis, and measuring the contribution of interleukin-8 to the *in vitro* neutrophil chemoattractant activity of bronchoalveolar lavage fluid from calves and lambs with experimentally induced pneumonic pasteurellosis.

The remainder of this chapter is a review of the current literature that forms the framework for this project. Section 1.2 reviews the pathogenesis of bovine pneumonic pasteurellosis, with an emphasis on the molecular basis of lesion development and the contribution of neutrophils to the development of this disease. Section 1.3 is an overview of relevant aspects of neutrophil biology. The role of chemokines, particularly interleukin-8, as neutrophil chemoattractants is reviewed in Section 1.4. Section 1.5 is a detailed examination of the current understanding of chemokines as neutrophil chemoattractants in ruminants.

1.2. Bovine pneumonic pasteurellosis

1.2.1. Definition and importance

In this thesis, bovine pneumonic pasteurellosis denotes those cases of shipping fever pneumonia that are caused by *Pasteurella haemolytica*. Shipping fever is defined clinically by an acute onset of severe toxemic pneumonia in feedlot cattle, and pathologically by marked fibrinous and suppurative necrotizing lobar bronchopneumonia. This disease may be caused by *Pasteurella haemolytica*, *Haemophilus somnus*, *Pasteurella multocida*, or other pathogens, but the principal concern of this project is pneumonia caused by *P. haemolytica*. Shipping fever manifests clinically as fever of acute onset, depression, anorexia, rapid and shallow respiration, and mucopurulent nasal discharge. Coughing is a feature in many cases and dyspnea is present in later stages. Most cattle respond rapidly to appropriate antibiotic therapy if given early in the course of disease (Radostits et al., 1994).

Necropsy of acutely fatal cases reveals severe fibrinous lobar bronchopneumonia with characteristic foci of coagulative necrosis, and fibrinous pleuritis is often present. Histologically, alveoli are filled with fibrin, edema, and necrotic leukocytes. Current evidence suggests that most of these necrotic leukocytes are neutrophils, based on the predominance of neutrophils in early lesions and the lack of expression of markers of histiocytic differentiation (Ackermann et al., 1994). Irregularly shaped foci of coagulative necrosis are often present and are delimited by a band of leukocytes. There is usually prominent interlobular edema and thrombosis of interlobular lymphatics; in addition, thrombosis of intralobular or alveolar septal

capillaries is a common feature of the lesions (Dungworth, 1993).

In epidemiologic studies of disease in feedlot cattle, it is important to differentiate diagnoses of bovine respiratory disease (BRD), which includes disease of both the upper and lower respiratory tract, pneumonia of undetermined cause, pneumonic pasteurellosis that is not necessarily limited to feedlot cattle, and shipping fever. As stated above, bovine pneumonic pasteurellosis is used in this thesis to specify cases of shipping fever pneumonia that are caused by *P. haemolytica*. Shipping fever pneumonia frequently develops in calves soon after arrival in feedlots: many studies reveal an onset at 3 to 8 days and a peak incidence at 2 to 3 weeks after arrival. Most reports of shipping fever describe morbidity of 15% to 45%, mortality of 1% to 5%, and case fatalities of 5% to 10% (Radostits et al., 1994). In studies that examine multiple herds or feedlots, *P. haemolytica* is the most common bacterial pathogen isolated at necropsy from cattle with shipping fever (Martin et al., 1980; Radostits et al., 1994). In a recent review, 97 necropsy cases of pneumonia in cattle between 4 and 24 months of age were associated with the following pathogens or processes: 34% *P. haemolytica,* 16% interstitial pneumonia of unknown etiology, 11% bovine respiratory syncytial virus, 9% *Haemophilus somnus*, 9% bovine viral diarrhea virus, 5% *P. multocida*, 3% *Mycoplasma bovis*, 3% parainfluenza-3 virus, 2% embolic pneumonia, 1% infectious bovine rhinotracheitis virus, and 7% other diagnoses (Saskatchewan Veterinary Medical Association , 1998).

The effects of shipping fever on growth and performance are controversial, but most studies indicate that cattle treated for pneumonia have reduced performance

compared to their penmates. A large study of five feedlots in Ontario showed that calves that developed respiratory disease had significantly lower weight gains in the first 28 days in the feedlot compared to unaffected calves (0.59 vs. 0.73 kg/day, respectively). Average daily gains for the entire feeding period were also significantly lower in calves which had received treatment for BRD, compared to unaffected calves, but the magnitude of this difference was less than for the early feeding period (1.12 vs. 1.18 kg/day, respectively) (Bateman et al., 1990). A study of Alberta feedlots found that BRD had no effect on subsequent performance parameters, although the low incidence of severe pneumonia in these feedlots makes the applicability of these results questionable (Jim et al., 1993). Several studies describe 12% to 20% reductions in growth rates in the initial period following pneumonia. In one study, however, compensatory gains resulted in identical slaughter weights for previously pneumonic compared to healthy cattle (Cole et al., 1979). In contrast, others have shown that pneumonic cattle have 10% lower slaughter weights (Thomas et al., 1978), or 5.45 kg lower slaughter weights that cost $6.60 per animal (Hutcheson and Cole, 1986).

Pneumonic pasteurellosis is the disease of most economic importance to the North American beef industry and the most common cause of mortality in feedlot cattle (Radostits et al., 1994). The annual cost of pneumonia was estimated at $134,633 in one Colorado feedlot processing 57,727 cattle per year (Frank et al., 1988). In 1981, the cost of respiratory disease to Alberta feedlots was $7.20 for each animal in the feedlot. Based on data in this study, total disease costs to the Alberta beef industry were estimated at $15.6 million annually, of which $9.6 million was

attributable to respiratory disease (Church and Radostits, 1981). The costs of this disease include mortality, treatment costs including drugs, personnel and processing, vaccines, and effects on subsequent performance following treatment. Finally, shipping fever is a major reason for prophylactic antibiotic therapy of feedlot cattle, a practice which some criticize with increasing vigour as a cause of antibiotic-resistant infections of humans (Witte, 1998).

1.2.2. *Pasteurella haemolytica*

Pasteurella haemolytica is the most common causative agent of shipping fever pneumonia, although *Haemophilus somnus* and *P. multocida* induce diseases that are clinically and pathologically similar. There are 16 recognized serotypes of *P. haemolytica* in addition to many untypable isolates; these serotypes are defined by variations in capsular polysaccharide. Serotype 1 is the predominant isolate from pneumonic cattle (Frank, 1989), while serotype 2 is the most common isolate from pneumonia in sheep (Gilmour and Gilmour, 1989). The classification of *P. haemolytica* into biotypes is based on the ability to ferment arabinose or trehalose. In general, biotype A induces pneumonia in ruminants, septicemia in young lambs, and mastitis in ewes, while biotype T causes septicemia in weaned lambs or adult sheep (Adlam, 1989; Shewen and Rice-Conlon, 1993). It has recently been recommended that *P. haemolytica* biotype T be considered a separate species, *Pasteurella trehalosi* (Jaworski et al., 1998).

The virulence factors of *P. haemolytica* allow colonization of mucosal

surfaces, acquisition of nutrients, and avoidance of host defences. The factors permitting adherence of *P. haemolytica* to mucosal surfaces are poorly characterized, but include fimbriae and the capsular polysaccharide described below. A neuraminidase may promote mucosal colonization by hydrolysis of the sialoglycoproteins at the epithelial cell surface (Confer et al., 1990), while iron acquisition is aided by a transferrin-binding protein (Shewen and Rice-Conlon, 1993).

All serotypes of *Pasteurella haemolytica* produce a leukotoxin during the logarithmic phase of growth, with peak production *in vitro* at 6 hours of culture. Leukotoxin production is not detectable from bacteria in the stationary phase of growth, a fact that complicates the interpretation of older studies employing overnight cultures of bacteria (Adlam, 1989; Shewen and Rice-Conlon, 1993). Leukotoxin is a member of the RTX (repeats in toxin) family of bacterial toxins, and is encoded by a cluster of four genes, designated *leukotoxin* (*lkt*)A to D. The *lkt*A and *lkt*C genes encode 102 and 19 kDa proteins, respectively, which are both necessary for activity, whereas the *lkt*B and *lkt*D genes encode proteins that facilitate secretion of the toxin (Highlander et al., 1989; Shewen and Rice-Conlon, 1993). Leukotoxin damages the plasma membrane of ruminant leukocytes and platelets by forming transmembrane pores that lead to cell swelling and lysis (Shewen and Rice-Conlon, 1993; Clarke et al., 1998). Neutrophils are more sensitive than macrophages to the lytic effects of leukotoxin (Whiteley et al., 1992), and neutrophils from calves are more sensitive than those from adults (Adlam, 1989). Concentrations of leukotoxin which are 100- to 1000-fold lower than those which cause cell lysis

induce neutrophils to undergo a respiratory burst, to secrete the contents of their secondary granules (Czuprynski et al., 1991), and to augment neutrophil-mediated killing of endothelial cells (Maheswaran et al., 1993). Leukotoxin reportedly induces secretion of TNF-α and IL-1 by alveolar macrophages (Yoo et al., 1995a), but this may be an effect of contaminating endotoxin (Stevens and Czuprynski, 1995). The leukotoxin of *P. haemolytica* is specific for ruminant leukocytes and platelets: minimal or no effects have been demonstrated on bovine pulmonary parenchymal cells, or on leukocytes from pigs, rabbits, or humans (Shewen and Wilkie, 1982; Wilkie et al., 1990; Majury and Shewen, 1991).

Three lines of evidence indicate that leukotoxin is an important virulence factor in pneumonic pasteurellosis. Firstly, mutants of *P. haemolytica* that do not produce leukotoxin induce milder clinical signs and lesions than do wild type strains (Petras et al., 1995). Secondly, protection from natural and experimental disease is correlated with antibody titres to leukotoxin, as well as with titres to surface antigens detected by agglutination assays (Shewen and Wilkie, 1983). Thirdly, although injection of recombinant leukotoxin does not induce protective immunity, it does augment the protective effect of a vaccine prepared from culture supernatant (Conlon et al., 1991).

Lipopolysaccharide (LPS), or endotoxin, is a structural component of the cell wall of *P. haemolytica* and accounts for 12% to 25% of the dry weight of the bacterium (Adlam, 1989). The biologic effects of LPS are numerous and diverse, and mediated largely by the lipid A component. LPS isolated from *P. haemolytica* serotype A1 primes neutrophils for a respiratory burst, and increases the avidity of

surface integrins for the corresponding ligands on endothelial cells. High doses of LPS (over 10 μg/ml) are toxic to macrophages, while lower doses induce the production of the proinflammatory mediators tumour necrosis factor (TNF)-α, IL-1, chemokines, platelet activating factor (PAF) and leukotriene (LT) B4, and of procoagulants, oxygen radicals, nitric oxide, and proteases. Although LPS-induced activation of monocytes is dependent on serum LPS-binding protein and CD14 expression on the monocyte surface, other pathways exist for LPS-induced activation of macrophages (Jungi et al., 1997). Endothelial cells, following stimulation with LPS, secrete procoagulants and proinflammatory mediators, upregulate their surface adhesion molecules and, at higher doses, undergo necrosis due to injury of cell membranes. The humoral effects of LPS include activation of coagulation through Factor XII and complement activation by both the classical and alternate pathways (Davis et al., 1980; Confer and Simons, 1986; Confer et al., 1990; Whiteley et al., 1992; Cotran et al., 1994).

Administration of 96 μg of LPS from *P. haemolytica* to calves by intrabronchial bolus induces multifocal alveolar hemorrhage, edema, fibrin exudation, neutrophil infiltration, and platelet aggregation (Whiteley et al., 1991b). Intravenous administration of LPS from *P. haemolytica* induces dramatic cardiovascular changes in calves: an initial increase in pulmonary arterial pressure is followed by reduced cardiac output and pulmonary venous and systemic hypotension (Slocombe et al., 1990). In mice, the systemic effects of LPS may be due primarily to stimulation of TNFα and IL-1 secretion (Cotran et al., 1994). Although many of the sequelae of challenge with LPS mimic those of *P. haemolytica,* and although LPS

is an immunodominant antigen, the magnitude of the antibody response to LPS does not correlate with protection from disease (Confer et al., 1986).

The capsular polysaccharide of *P. haemolytica* prevents serum-induced bacterial agglutination, complement-mediated killing and phagocytosis by neutrophils (Chae et al., 1990). The latter effect is attributed partially to interference with opsonization, but a direct impairment of neutrophil function may also occur (Czuprinski et al., 1989). The capsular polysaccharide may permit binding of *P. haemolytica* to surfactant, as a mechanism of colonizing the alveolus (Brogden et al., 1989). Vaccination with purified capsular polysaccharide does not protect against subsequent challenge with *P. haemolytica* and does not augment the protective effect of other vaccines, but it is associated with the development of anaphylaxis in 36% of the vaccinates, suggesting that the immune response to the capsular polysaccharide is not protective (Conlon et al., 1993). Culture of *P. haemolytica* under iron-limiting conditions, mimicking that which occurs in animal tissues, results in growth of bacteria with smaller and less dense capsules than are present under iron-replete conditions (Clarke et al., 1998).

1.2.3. The pathogenesis of bovine pneumonic pasteurellosis

Although *P. haemolytica* is the etiologic agent of pneumonic pasteurellosis, factors that predispose to development of the disease are of critical importance in understanding its pathogenesis and in controlling the disease in the field. Cattle that are assembled from different sources are at highest risk of developing shipping fever,

probably because of the severe stresses commonly associated with this event and the exposure of naïve calves to a variety of respiratory pathogens. The stressors encountered by calves in this brief period often include weaning, transport, crowding, deprivation of feed and water, handling, vaccination, and pregnancy checking of heifers. Other risk factors for the development of shipping fever include changes in temperature and humidity, snow or rainfall, a dusty environment, and the month of purchase (Radostits et al., 1994; Ribble et al., 1995).

The pathogenesis of bovine pneumonic pasteurellosis is complex and not fully defined. A currently popular paradigm explaining the development of shipping fever may be summarized as follows: *P. haemolytica* bacteria are carried in the nasopharynx of many normal calves and, under the influence of the stressors described above, increase in number and are inhaled into the lung. Large numbers of inhaled bacteria overwhelm the pulmonary defences, particularly following viral infections or stress, and lead to the development of disease. Any model of the pathogenesis of shipping fever must attempt to explain the characteristic clinical and pathologic features of the disease: the clinical picture of severe depression and compromised pulmonary function, and the pathologic findings of exudation of proteinaceous fluid and fibrin into alveoli, neutrophil recruitment and lysis, coagulative necrosis of pulmonary tissue, and thrombosis of lymphatics and blood vessels.

1.2.3.1. The initial development of disease

Several anatomic and physiologic factors predispose cattle to the

development of severe pneumonia, and have been reviewed by Weekley and Veit (1995). Compared to other species, the bovine lung is small relative to body size, and the ratio of alveolar surface area to either body weight or basal oxygen consumption is less than 50% of the average for mammals. Poor collateral ventilation of bovine pulmonary lobules is a consequence of complete interlobular septation and few interalveolar pores. Although this tends to limit the direct spread of pathogens between alveoli, it also reduces the clearance of pathogens that reach the alveoli. Mast cells are numerous around airways in cattle relative to other species, and may enhance bronchospasm in response to aerogenous insults. Because of the large size of cattle, there is a greater tendency toward ventilation-perfusion mismatching: the ventral portions of the lung are overperfused by blood, while the dorsal aspects are underperfused but overventilated. The ability of pulmonary arterioles to constrict in response to hypoxia is particularly strong in cattle, but only partially overcomes this predisposition to ventilation-perfusion mismatch.

Finally, the bovine lung is replete with pulmonary intravascular macrophages, unlike that of humans and rodents. As a result, the lung is the target organ for endotoxemia in cattle, exacerbating the reductions in pulmonary function in pneumonia caused by Gram negative bacteria. Aerosol droplets that are 1 to 2 μm in diameter, such as bacteria, tend to be deposited in the terminal bronchioles, where respiratory defences are compromised by being beyond the limit of effective ciliary clearance yet proximal to the greatest concentration of alveolar macrophages (Dungworth, 1993).

Clinically normal calves carry low numbers of *P. haemolytica* in the nasopharynx, and inhalation of low numbers of these bacteria may be common and not of clinical significance (Frank et al., 1993). The development of disease probably depends on the additive effects of factors that enhance challenge of the lungs by bacteria and factors that impair respiratory defences.

Factors that increase the load of bacteria delivered to the lung include the following:

1. Stressful events and viral infections expand the populations of *P. haemolytica* colonizing the nasopharynx and tonsils (Frank, 1989) and result in delivery of aerosolized bacteria to the lower respiratory tract. As described above, these stresses include weaning, castration, dehorning, transport, mixing and altered social structure, crowding, deprivation of feed and water, handling, and vaccination.
2. Mixing of cattle serves to maximize exposure of naïve calves to those which are actively shedding *P. haemolytica.* The stresses described above compound this problem by increasing shedding of bacteria from carrier animals, and by reducing the innate resistance and immune responses of the naïve calves.

Factors that impair respiratory defences include the following:

1. Stressful events weaken innate and immunologic defences including the bactericidal activity of neutrophils (Shurin et al., 1994; Bartlett et al., 1997), by mechanisms which are not completely defined but include the production of immunomodulatory neuropeptides, adrenal corticosteroids and activation of the

sympathetic nervous system (Tizard, 1996).

2. The mucociliary clearance of bacteria is reduced by infection with BHV-1, PI3 or BVD viruses or *Mycoplasma sp.*, or exposure to irritant gases, such as ammonia. This effect may be induced either by inducing necrosis of tracheobronchial epithelium, or by sublethal cellular injury which reduces ciliary function (Rossi et al., 1977; Castleman et al., 1985).
3. Infection with BHV-1 or PI3 virus attenuates the phagocytic and bactericidal activities of alveolar macrophages (Forman and Babiuk, 1982; Bryson, 1990), and may reduce the recruitment of inflammatory cells to the site of infection (McGuire and Babiuk, 1984).
4. Pulmonary challenge with *P. haemolytica* induces dose-dependent exposure of alveolar macrophages and newly recruited neutrophils to leukotoxin, which impairs the bactericidal activity of these leukocytes directly or by inducing necrosis of these cells (Whiteley et al., 1992).

Despite this experimental evidence and supportive findings in some naturally occurring outbreaks, predisposing agents are often not identified in field cases of shipping fever. Additionally, the seroconversion of feedlot cattle to BHV-1, PI3, BRSV and BVDV does not correlate with the incidence of pneumonic pasteurellosis (Shewen and Rice-Conlon, 1993). Thus, since lesions typical of shipping fever pneumonia may be induced by challenge with *P. haemolytica* alone, it is likely that this bacterium may act as a primary pathogen in concert with the stressors outlined above (Radostits et al., 1994).

1.2.3.2. Molecular determinants of lesions

Pasteurella haemolytica induces pulmonary inflammation directly through the production of bacterial toxins, and indirectly by stimulating the secretion of inflammatory mediators from resident or recruited host cells. The virulence factors of *P. haemolytica* were described above, but toxins that directly modulate the inflammatory response are summarized here. Lipopolysaccharide induces macrophages to secrete a variety of pro-inflammatory mediators including TNF-α, IL-1, IL-6, IL-8, ENA, GRO-α, PAF and LTB4 (Lohmann-Matthes et al., 1994). Many of these mediators are chemotactic for neutrophils, but LPS has no direct chemotactic effect (Confer and Simons, 1986). Lipopolysaccharide activates complement by both the classical and alternate pathways, and induces dose-dependent sublethal injury or necrosis of endothelial cell membranes at high doses (Confer and Simons, 1986). Leukotoxin induces necrosis of alveolar macrophages; although it is controversial, sublethal concentrations probably do not induce cytokine secretion from macrophages (Stevens and Czuprynski, 1995). Leukotoxin also induces histamine release from bovine pulmonary mast cells (Adusu et al., 1994). Although no other toxins of *P. haemolytica* have been described to have direct injurious effects on resident pulmonary cells (Brogden et al., 1989; Whiteley et al., 1991b), bacterial toxins are frequently cited as a suspected cause of the coagulative necrosis that is characteristic of the disease (Dungworth, 1994). In contrast to the paucity of reported direct effects of *P. haemolytica* toxins, the inflammatory mediators produced in response to these toxins enhance vascular leakage, recruit

inflammatory cells, augment coagulation, and impair pulmonary function. The molecular and cellular bases for these processes are discussed in the following paragraphs.

Vascular leakage in inflammation may be caused by five general processes: direct endothelial injury, leukocyte-mediated endothelial injury, rapid transient endothelial cell contraction, delayed sustained endothelial cell retraction, and leakage from regenerating capillaries (Cotran et al., 1994). The first four of these are likely to contribute to the development of pulmonary edema in the acute phase of pneumonic pasteurellosis, while the first two may result in the fibrin deposition and hemorrhage into alveoli. Necrosis or sublethal injury of endothelial cells may be induced by LPS or oxygen radicals, or by enzymes released from neutrophils and macrophages, as described in Section 1.3.5. Histamine from leukotoxin-stimulated mast cells and leukotrienes C4, D4 and E4 secreted from LPS-exposed macrophages may cause rapid transient endothelial cell contraction. Finally, inflammatory cytokines produced by LPS-stimulated alveolar macrophages result in delayed sustained endothelial cell retraction (Cotran et al., 1994). The relative contributions of these causes of vascular leakage are unknown, but probably vary with the stage of disease.

The mechanisms of neutrophil recruitment in pneumonic pasteurellosis are not defined and their characterization is the major goal of this project. A number of potential neutrophil chemoattractants have been identified in cattle and are discussed

in detail in Section 1.5. The supernatant from *Pasteurella haemolytica* culture is chemotactic for bovine neutrophils, an effect which was attributed to more than one component of the culture fluid. At least one component had a molecular weight of over 30 kDa, was partially resistant to 60°C heat, and was present in both pathogenic serotype A1 and non-pathogenic serotype A11 (Brunner et al., 1989; Mdurvwa and Brunner, 1994). Endotoxin, leukotoxin, and capsular polysaccharide are not directly chemotactic for neutrophils (Gray et al., 1982; Confer and Simons, 1986; Brogden et al., 1989). Although the formylated bacterial peptide fMLP is a potent neutrophil chemoattractant in other species, it does not induce chemotaxis of bovine neutrophils (Gray et al., 1982).

In contrast to the apparently limited chemotactic effect of bacterial toxins, several mediators that are produced in response to *P. haemolytica* infection, including chemokines, leukotriene B4, platelet activating factor and C5a, are chemotactic for bovine neutrophils. These mediators will be reviewed in more detail in Section 1.5, but evidence for their expression in bovine pneumonic pasteurellosis is examined here. Interleukin-8 mRNA is expressed in alveolar macrophages exposed to LPS from *P. haemolytica*, and steady state levels progressively increase from 1 to 12 hours after inoculation of heat-killed *P. haemolytica* (Morsey et al., 1996). In the lesions of bovine pneumonic pasteurellosis, the chemokines GRO-α and ENA are expressed by type II pneumocytes and pleural mesothelial cells and by type II pneumocytes and alveolar leukocytes, respectively, and are secreted by alveolar macrophages stimulated *in vitro* with LPS from *P. haemolytica* (Allman-Iselin et al., 1994; Rogivue et al., 1995). Leukotriene B4, an eicosanoid neutrophil

chemoattractant derived from arachidonic acid by the lipoxygenase pathway, is synthesized and secreted by bovine neutrophils within five minutes of exposure to *P. haemolytica* leukotoxin, prior to the development of leukotoxin-induced cell lysis (Clinkenbeard et al., 1994). In a subcutaneous tissue chamber model of *P. haemolytica*-induced inflammation in calves, LTB4 concentrations in the chamber fluid increased from 265 pg/ml prior to inoculation to 816 and 812 pg/ml at 6 and 18 hours p.i., respectively, but declined to 581 pg/ml by 40 hours p.i. Neutrophil migration into these tissue chambers was insignificant at 2 hours p.i., but increased progressively between 6 and 40 hours p.i. (Clarke et al., 1994). The presence of IL-8 protein, LTB4, PAF, or C5a in the lesions of bovine pneumonic pasteurellosis has not been evaluated, nor has the relative importance of these mediators in inducing neutrophil chemotaxis in this disease. This topic will be considered further in Section 1.5.

The expression of TNF-α and IL-1 has been evaluated in pneumonic pasteurellosis in ruminants. Ovine alveolar macrophages exposed to *P. haemolytica* secrete TNF-α (Ellis et al., 1991). The expression of TNF-α and IL-1β mRNA is increased in bronchoalveolar lavage cells and in lesional tissue by 48 hours after infection with *P. haemolytica.* Bronchoalveolar lavage fluid harvested at necropsy of these calves contained on average 4.5 and 0.6 ng/ml TNF-α and IL-1, respectively, compared to 1.5 and 0 ng/ml, respectively in saline-inoculated controls (Yoo et al., 1995b). Following experimental infection with *P. haemolytica,* TNF-α levels in serum rose by 2 hours p.i., peaked at 8 hours p.i., and declined by 72 hours p.i. Peak TNF-α concentrations in serum varied from 0.12 to 5 ng/ml and did not correlate

with the extent of lesions at necropsy (Pace et al., 1993).

TNF-α and IL-1 have direct local effects on endothelial cells, including an increase in vascular permeability, increased expression of adhesion molecules, and a variety of procoagulant effects. These cytokines induce systemic effects including fever and hypotensive shock, and could contribute to the malaise or "toxemia" which is prominent in cattle with shipping fever. In addition, TNF-α and IL-1 stimulate a variety of cell types to synthesize and secrete IL-6, IL-8, eicosanoids and PAF, and these may in turn be important in mediating the hepatic production of acute phase proteins and recruitment of leukocytes to the site of infection (Cotran et al., 1994). Priming of leukocytes is another effect of TNF-α, IL-1 and the mediators induced by these cytokines, as described in Section 1.3.4.

Focal coagulative necrosis is a characteristic lesion of bovine pneumonic pasteurellosis, but the specific cause of this lesion is unknown. These foci are irregularly shaped and are typically sharply demarcated by a rim of leukocytes. Bacteria are most numerous at the margins, while the presence of neutrophils and fibrin in the central areas is presumably an effect of inflammation prior to the development of necrosis. Thrombosis and infarction may account for the lesion, but the presence of thrombosis is inconsistent (Dungworth, 1993). Other possible causes have not been adequately evaluated, but include bacterial toxins, inflammatory cytokines such as TNF-α or IL-1, or leukocyte-derived oxygen radicals or proteolytic enzymes. The necrosis of neutrophils with the formation of "oat cells" is probably an effect of leukotoxin, as discussed in Section 1.2.2.

Polymerization of fibrin is a central process in three lesions of bovine pneumonic pasteurellosis: intra-alveolar fibrinous exudate, capillary and venular thrombosis, and thrombosis of interlobular lymphatic vessels. Several mechanisms operating concurrently in this disease have an impact on coagulation, platelet reactivity, and fibrinolysis. Lipopolysaccharide is probably an important mediator of thrombosis by inducing tissue factor expression directly, or indirectly through induction of TNF and IL-1. *In vitro* treatment of alveolar macrophages, neutrophils, or endothelial cells with LPS, TNF or IL-1 induces procoagulant activity, which is a measure of the relative ability of a sample to induce coagulation of bovine platelet-poor plasma compared to tissue factor. In the case of macrophages, this procoagulant activity is attributable to tissue factor (Breider and Yang, 1994; Rashid et al., 1996).

Bronchoalveolar lavage (BAL) fluid from lesions of experimental bovine pneumonic pasteurellosis had increased procoagulant activity and reduced fibrinolytic activity compared to BAL cells from non-lesional or uninfected control lung. This increase in procoagulant activity was attributed to tissue factor expression by activated alveolar macrophages, because bovine neutrophils had no detectable procoagulant activity (Car et al., 1991).

A neutralizing antibody to tissue factor prevented the increase in the procoagulant activity of BAL fluid which followed *in vivo* pulmonary challenge with *P. haemolytica*, and reduced the deposition of fibrin in alveoli (Rashid et al., 1997). Pulmonary intravascular macrophages may also contribute to capillary thrombosis, based on: 1/ the close anatomic association of these cells with platelet aggregates and intravascular fibrin deposits in experimental pneumonic pasteurellosis, 2/ the ability

of these cells to produce tissue factor and activators of platelet aggregation, as described below, and 3/ the ability of LPS to diffuse across the alveolar wall and activate these macrophages (Whiteley et al., 1990, 1991a).

1.2.3.3. Alterations in pulmonary function

The alterations in pulmonary and cardiovascular function following intratracheal inoculation of *P. haemolytica* were described in six studies (Slocombe et al., 1984, 1989, 1990; Linden et al., 1995; Desmecht et al., 1996; Hare et al., 1996). Respiratory rates were consistently increased and, despite reduced tidal volume, resulted in an increased volume inspired per minute in most cases. In contrast, calves that developed severe disease had lower minute ventilation (Desmecht et al., 1996). Dynamic compliance was reduced in most cases, but total pulmonary resistance was often normal. Arterial oxygen partial pressures were reduced in all studies, by up to 65% (Linden et al., 1995). The arterial partial pressure of carbon dioxide, which is a measure of ventilation, was usually normal but was increased in severely affected calves (Desmecht et al., 1996). This may be caused by severe depression and reduced minute ventilation in terminal stages of disease. The gradient of alveolar to arterial oxygen tension (P_AO_2- P_aO_2), an indicator of the efficiency of gas diffusion across the air-blood barrier, was increased in all three studies in which it was evaluated. Systemic hypotension and pulmonary hypertension have been recorded but are inconsistent.

Lipopolysaccharide may cause some of these impairments of pulmonary function. Intratracheal inoculation of LPS derived from *P. haemolytica* induces

hypoxemia and increased P_AO_2- P_aO_2, but not hypercarbia or altered lung mechanics. Intravenous administration of LPS induces systemic hypotension, increased total pulmonary resistance and reduced dynamic compliance (Slocombe et al., 1990). Since LPS is readily absorbed across the alveolar wall (Whiteley et al., 1990), both of these routes of administration may be applicable in naturally occurring pasteurellosis.

1.2.3.4. The importance of neutrophils in development of the disease

Bovine pneumonic pasteurellosis is often presented as a paradigm of neutrophil-dependent tissue injury and of the important role of the inflammatory response in inducing severe disease. The mechanisms by which neutrophils injure tissue will be described in Section 1.3.5, while the three studies that provide evidence for a pathogenic role of neutrophils in pneumonic pasteurellosis are reviewed here.

Slocombe et al. (1985) examined the effect of hydroxyurea-induced neutrophil depletion on the development of experimental pneumonic pasteurellosis in calves. Neutrophil depletion prevented the development of tachypnea, bradycardia, hypoxemia and lymphopenia, but had no effect on rectal temperature or lymphocyte blastogenesis. At necropsy, calves with normal neutrophil status had acute severe necrohemorrhagic pneumonia, whereas grossly detectable lesions were not present in neutrophil-depleted calves. Microscopic lesions in infected calves with normal neutrophil numbers included filling of alveoli by edema fluid, neutrophils and hemorrhage, necrosis of alveolar septa, and interlobular edema, fibrin exudation and lymphatic thrombosis. In contrast, infected, neutrophil-depleted calves had mild lesions of alveolar hemorrhage, neutrophil aggregation and edema. Interlobular

lymphatics did not contain fibrin, although interlobular edema was present. A concern in this study was that although *P. haemolytica* bacteria were isolated from the pneumonic lungs of calves with normal neutrophil numbers, lungs from neutrophil-depleted calves were generally sterile. The authors addressed this concern by suggesting that neutrophil-induced lung injury might provide a milieu conducive to bacterial growth. Other studies, described below, suggest that neutrophil depletion has no effect on the numbers of bacteria present in these lesions.

Weiss et al. (1991) examined the effect of neutrophil depletion, using antiserum to bovine neutrophils, on the response to challenge with *P. haemolytica.* Neutrophil depletion reduced the percentage of lesional lung in the infected lobe from 41% to 7%, and abrogated the intra-alveolar exudation of neutrophils and fibrin. Neutrophil depletion did not prevent the hemorrhage and exudation of proteinaceous fluid into alveoli, and bacterial colonies were readily visible in lung sections from both groups.

Breider et al. (1988) also evaluated the response to intrabronchial challenge with *P. haemolytica* following neutrophil depletion induced by hydroxyurea. Infected, neutrophil-replete calves developed localized edema and consolidation at the inoculation site. Microscopic lesions included filling of alveoli with edema, fibrin, neutrophils, and erythrocytes; necrosis of alveolar walls; and edema and fibrinopurulent inflammation of interlobular septa. In contrast, microscopic lesions in infected, neutrophil-depleted calves were limited to severe interlobular and alveolar edema, hemorrhage, and fibrinous exudate. Neutrophil depletion did not affect the recovery of bacteria from the inoculation sites.

In summary, the effect of neutrophil depletion on the development of pneumonic

pasteurellosis has been somewhat variable. Neutrophil depletion prevented the development of severe gross pulmonary lesions, neutrophil exudation into alveoli, and necrosis of alveolar septa. Fibrin exudation may be partially dependent on neutrophil infiltration. Neutrophil depletion has little effect on the development of fever, alveolar edema and hemorrhage, and thrombosis, which probably represent direct effects of LPS or LPS-induced cytokines. These latter findings suggest that macrophages, in addition to neutrophils, may play a pathogenic role in pneumonic pasteurellosis by mediating the LPS-induced thrombosis that contributes to the characteristic foci of coagulation necrosis in this disease.

1.3. Mechanisms of neutrophil responses in acute inflammation

1.3.1. General aspects of neutrophil-dominated inflammation

Neutrophils are a common feature of acute inflammation. Suppurative and purulent are used synonymously in this review, and describe exudate that is dominated by neutrophils and is of a sufficient magnitude to be visible grossly. Further, suppurative exudate contains necrotic cellular debris, because of the lytic effect on host cells of neutrophil-derived lysosomal enzymes and oxygen radicals (Slauson and Cooper, 1990). Suppurative inflammation is almost always secondary to bacterial infection. In contrast, neutrophil-rich inflammation refers to microscopically identifiable infiltration of tissues by neutrophils. Although neutrophil-rich inflammation is often secondary to bacterial infection, neutrophils

may dominate the cellular infiltrate in inflammation incited by a wide variety of other stimuli. These include fungal infections such as aspergillosis, viral infections such as acute eastern equine encephalitis, type I hypersensitivity reactions as in some cases of chronic obstructive pulmonary disease in horses, type III hypersensitivity reactions induced by immune complex deposition in tissues, and certain autoimmune diseases such as pemphigus foliaceus.

Neutrophil responses are beneficial in most bacterial infections, because of phagocytosis and intracellular killing of pathogens and secretion of bacteriostatic molecules to the extracellular space. In addition to the beneficial effects of neutrophils in controlling bacterial infections, neutrophil-mediated tissue injury is an important component of overwhelming infections or infections with certain bacteria, as described in Sections 1.2.3.4 and 1.3.5. The important role of neutrophils in containing and eliminating bacteria is clearly shown by the fact that animals with neutropenia or impaired function predictably develop recurrent infections caused by bacteria of low pathogenicity (Gilbert et al., 1993). Functional abnormalities of neutrophils in animals include leukocyte adhesion deficiency syndromes in cattle and dogs, Chediak-Higashi syndrome in many species, and defects in production of reactive oxygen species in Doberman pinscher dogs. Neutropenia in animals may result from toxicity of chemotherapeutic agents, estrogens, or other xenobiotics, parvoviral infections, systemic lupus erythematosus, radiation injury and myelophthisis or neoplasia of the bone marrow (Breitschwerdt et al., 1987; Jain, 1993; Valli, 1993).

Neutrophil responses are initiated by soluble factors from bacteria, such as

the formylated peptide fMLP; by derivatives of plasma proteins, including complement-derived anaphylatoxins, fibrinopeptides, and fibrin degradation products; and by mediators, such as chemokines, leukotrienes and platelet activating factors, that are secreted from locally activated cells (Cotran et al., 1994). In responding to a localized insult to tissues, circulating neutrophils must adhere to the endothelium, traverse the vessel wall, and foray through tissues along a gradient of soluble or matrix-bound mediators. During this migration and upon arrival in the tissues, neutrophils are primed and activated to enhance their secretory and bactericidal functions. Finally, neutrophils secrete various compounds that impair bacterial growth or modulate the inflammatory response, and phagocytose bacteria to permit intracellular killing. These processes will be described in more detail in the following sections.

1.3.2. Transendothelial migration of neutrophils

The adhesion of neutrophils to endothelium at sites of inflammation occurs primarily in small venules. The molecules of importance in this process include selectins, integrins and members of the immunoglobulin superfamily, and their roles are summarized in the following model:

1. Local production of inflammatory mediators induces endothelial activation.
2. Selectins mediate the rolling adhesion of neutrophils along the endothelial surface.
3. Inflammatory mediators induce the rapid activation of neutrophils to

downregulate L-selectin expression and upregulate integrin expression and binding affinity.

4. Firm adhesion of neutrophils to endothelial cells is mediated by interactions between integrins on neutrophils and members of the immunoglobulin superfamily on endothelial cells.
5. Neutrophils migrate between endothelial cells and through the vessel wall (Carlos and Harlan, 1994; Cotran et al., 1994).

The selectins are a family of cell-surface carbohydrate-binding proteins and three have been identified: E-selectin, P-selectin, and L-selectin. Selectins bind to a variety of sialylated and fucosylated carbohydrate moieties of cell-surface glycoproteins and proteoglycans, which have been incompletely characterized but include the sialylated Lewis X and A tetrasaccharides, heparin-like molecules, and the specific glycoprotein receptors listed below. The importance of fucosylated carbohydrates is illustrated by human leukocyte adhesion deficiency type II, where defects in fucose metabolism impede the synthesis of sialyl Lewis X, resulting in ineffective neutrophil recruitment.

P-selectin (CD62P, PADGEM, GMP-140) is stored in Weibel-Palade bodies of endothelial cells and in α-granules of platelets. Thrombin, histamine, complement fragments, and oxygen radicals each induce rapid surface expression of P-selectin that peaks at 10 minutes and returns to baseline by 30 minutes after stimulation. In contrast, P-selectin surface expression is prolonged, up to 3 hours, following stimulation with inflammatory cytokines such as TNF-α. P-selectin ligands include

P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils, monocytes, and lymphocytes, and L-selectin (Carlos and Harlan, 1994).

E-selectin (CD62E, ELAM-1) is synthesized by endothelial cells in response to TNF-α, IL-1, IFN-γ, or lipopolysaccharide. E-selectin expression peaks at 4 hours and lasts up to 24 hours after stimulation. E-selectin ligands include E-selectin ligand-1 (ESL-1) on murine neutrophils, the cutaneous lymphocyte antigen (CLA) homing receptor for skin-seeking memory T lymphocytes, and L-selectin (Carlos and Harlan, 1994).

L-selectin (CD62L, MEL-14) is expressed on neutrophils, monocytes, eosinophils, B lymphocytes, naïve T lymphocytes, some memory T cells and natural killer cells, and erythroid progenitors. L-selectin expression on neutrophils is constitutive, but it is rapidly downregulated upon exposure to stimulants including IL-8, C5a and leukotriene B4. L-selectin ligands on endothelium include E- and P-selectin, which may play a role in neutrophil adhesion, and GlyCAM-1, CD34, and MAdCAM-1, which have putative roles in lymphocyte homing. Binding of L-selectin to these ligands is dependent on endothelial activation.

Integrins are transmembrane heterodimeric proteins that comprise α and β chains. The integrins of importance in neutrophil adhesion to endothelium are CD11a/CD18 (LFA-1, $\alpha_1\beta_2$), CD11b/CD18 (Mac-1, iC3b, $\alpha_2\beta_2$), and CD11c/CD18 (gp150,95, $\alpha_3\beta_2$). All three of these integrins are present on the cell surface of resting neutrophils, and the latter two are upregulated in response to GM-CSF, C5a, TNF-α, LTB4, PAF, IL-8 and formylated bacterial peptides. In addition, and

probably of more functional importance, these stimulants induce a conformational change in the surface integrins to increase the avidity for their ligands (Carlos and Harlan, 1994).

The endothelial ligands for the neutrophil integrins are members of the immunoglobulin superfamily. Intercellular adhesion molecule-1 (ICAM-1, CD54) is not constitutively expressed, but is present on endothelial cells that are stimulated by IL-1, TNF-α, IFN-γ, LPS, thrombin, hypoxia with re-oxygenation or oxygen radicals. ICAM-1 binds to the integrins CD11a/CD18 and CD11b/CD18. ICAM-2 (CD102) is expressed constitutively on endothelial cells, is not upregulated in response to proinflammatory cytokines, and binds to CD11a/CD18. ICAM-3 is also expressed constitutively, and binds CD11a/CD18 (Carlos and Harlan, 1994).

Local production of inflammatory mediators induces alterations in both marginated neutrophils and endothelial cells, and accounts for the selectivity and rapidity of cell recruitment by specific types of injurious agents. Thrombin, histamine, and C5a rapidly induce surface expression of P-selectin on endothelium. In contrast, TNF-α, IL-1β, IFN-γ, and LPS each result in delayed but more long-lasting activation of endothelium to upregulate surface expression of E-selectin and ICAM-1. Selectins mediate the initial rolling of leukocytes along the endothelium, which represents transient loose adhesion between the cell surfaces and allows exposure of leukocytes to local activating factors and other adhesion molecules at the endothelial surface. This rolling adhesion occurs within minutes after injury, peaks at about 30 minutes, and persists for at least 2 hours after the inflammatory stimulus

(Tedder et al., 1995). Evidence suggests that P-selectin mediates rolling adhesion of neutrophils to endothelium within minutes of stimulation. In contrast, L-selectin becomes important hours later, whereas the role of E-selectin is principally in adhesion of leukocytes to endothelium which has been stimulated by pro-inflammatory cytokines (Tedder et al., 1995).

The second phase in the neutrophil-endothelium adhesion cascade involves activation of neutrophils by locally produced inflammatory mediators, including IL-8, PAF, GM-CSF, C5a and formylated bacterial peptides. The effects of these mediators on neutrophils include reduced surface expression of L-selectin, a conformational change in surface integrins resulting in increased avidity for endothelial ligand, and increased surface expression of integrins. In contrast, nitric oxide or TGF-β inhibits these effects (Carlos and Harlan, 1994).

Firm adhesion, by necessity, occurs rapidly following selectin-mediated rolling; these events are separated by less than three seconds in the case of lymphocyte emigration (Carlos and Harlan, 1994). Firm adhesion is dependent primarily on interactions between ICAM-1 on endothelium, and either CD11a/CD18 or CD11b/CD18 on neutrophils. The importance of CD18 is reflected in leukocyte adhesion deficiency syndromes in cattle, humans and dogs, in which a genetic deficiency of CD18 results in severe impairment of neutrophil extravasation in most tissues, with concomitant persistent neutrophilia and recurrent bacterial infections. CD18 deficiency, however, does not impair neutrophil exudation into the bovine lung in response to *P. haemolytica*, indicating that CD18-independent mechanisms of neutrophil emigration probably exist in pulmonary endothelium (Ackermann et al.,

1996). A similar phenomenon has been described in rabbits and humans (Carlos and Harlan, 1994).

The molecular basis of diapedesis, the final step in neutrophil extravasation, is poorly defined. This process includes the squeezing of neutrophils through gaps between endothelial cells and their migration through the matrix components of the vessel wall. Recent work suggests that homophilic interactions between platelet endothelial cell adhesion molecule-1 (PECAM-1), a member of the immunoglobulin superfamily expressed on both neutrophils and endothelial cells, are important in neutrophil extravasation, but the specific mechanisms are unknown (Cotran et al., 1994). Other aspects of neutrophil diapedesis are probably similar to chemotaxis, as described in the following section.

1.3.3. Neutrophil chemotaxis

Chemotaxis refers to the directed migration of cells in response to a concentration gradient of a given mediator (Slauson and Cooper, 1990). Mediators which are chemotactic for neutrophils include the ELR-CXC chemokines (a subset of the α-chemokine family, described in Section 1.4), the complement-derived anaphylatoxin C5a and its stable derivative C5a-des-arg, leukotriene B4, platelet activating factor, bacterial products such as formylated oligopeptides (fMLP), and by-products of coagulation such as fibrinopeptides and fibrin degradation products (Slauson and Cooper, 1990; Cotran et al., 1994). Many of these mediators, including C5a, fMLP and LTB4, also recruit other leukocyte subsets, whereas the ELR-CXC

chemokines specifically attract neutrophils (see Section 1.4). These neutrophil chemoattractants also activate neutrophils to shed L-selectin and increase the avidity of their surface integrins to bind firmly to endothelium, as described above. Similarly, these mediators either activate neutrophils to undergo an oxidative burst and enhance phagocytosis and killing of bacteria, or prime neutrophils to improve these responses to other agonists (see Section 1.3.4). It is apparent, therefore, that chemoattractants have many other effects on neutrophil function in addition to their chemotactic consequences.

Instead of responding to gradients of soluble chemoattractants, neutrophils may migrate toward higher concentrations of matrix-bound molecules, a phenomenon termed haptotaxis (Rot, 1993). This theory is based on observations that some mediators, including IL-8, are able to bind to matrix glycoproteins, glycosaminoglycans such as heparan sulfate, endothelial cell surfaces, and the polycarbonate filters used in *in vitro* chemotaxis assays. In the case of *in vitro* neutrophil migration across perforated polycarbonate filters, IL-8-induced migration is primarily due to haptotaxis, C5a-induced chemotaxis is attributable to both soluble and membrane-bound effects of the mediator, and fMLP does not bind to the polycarbonate membrane (Rot, 1993). Although the *in vivo* role of tissue-bound chemattractants is uncertain, this phenomenon may affect the persistence of IL-8 at the endothelial surface or the way in which matrix-bound neutrophils recognize chemotactic gradients. Alternatively, matrix-bound chemokines may facilitate binding of leukocytes to matrix, or neutrophil-derived matrix-degrading enzymes may modulate ongoing neutrophil recruitment by cleaving chemokines from the

matrix (Gilat et al., 1996).

Neutrophils cannot swim through fluids, but are able to crawl through tissues. This fact has important ramifications in the mechanisms of chemotaxis in *in vitro* assays, and the failure of leukocytes to infiltrate and resolve pulmonary sequestra. The processes by which cells crawl have been recently reviewed (Gumbiner, 1996; Lauffenburger and Horwitz, 1996). In response to stimuli, neutrophils extend sheet-like lamellipodia or cylindrical filopodia, which are cytoplasmic protrusions that contain few organelles but abundant actin and actin-related proteins. These structures are extended in the direction of greatest concentration of the agonist, presumably due to localized additive effects of chemoattractant interactions with surface receptors. The force to generate these protrusions is probably generated by actin polymerization alone.

The lamellipodium must then attach to the matrix. This process may be mediated by interactions of β2 integrin or the β1 integrins VLA-5 and VLA-6 on the neutrophil cell surface with collagens, laminin, fibronectin, and proteoglycans in the basement membrane and tissue matrix (Gao et al., 1995). The final step in this process is release of the matrix attachments at the rear of the cell and forward movement of the rear and body of the cell. In the case of migrating fibroblasts, release from the matrix occurs by dissociation of β1 integrin from the cell surface, so that the integrins are left behind on the matrix. The mechanism of the force that pulls the cell forward is controversial, but probably involves myosin-dependent contraction of actin filaments. In this manner, neutrophils can progressively move through tissue toward the source of the chemotactic stimulus (Lauffenburger and

Horwitz, 1996).

Finally, secretion of proteases by neutrophils may be required for migration through the basement membrane. Alternatively, and more likely, neutrophils may simply squeeze through spaces between matrix proteins, because anti-proteinases have no effect on migration across endothelium and basement membrane (Cramer, 1992).

1.3.4. Neutrophil activation and effector mechanisms

The phases of neutrophil activation have been alluded to in previous sections: the resting or rolling phenotype, the primed or transitional phenotype, and the activated or motile-phagocytic phenotype (Ainsworth et al., 1996). Resting neutrophils are those that circulate in blood or undergo rolling adhesion to endothelium. This resting state is characterized by low intracellular calcium concentrations, high surface expression of L-selectin, and low surface levels and avidity of CD11b/CD18. Activation has been proposed to be a two-step process, requiring 1/ elevations in intracellular calcium, stimulated by G-protein-linked receptors such as the chemokine receptors, and 2/ signalling via tyrosine kinase, a component of receptors for TNF-α, IL-1, G-CSF, GM-CSF, and CD14 (Ainsworth et al., 1996). Priming, which may occur following stimulation of one of these pathways, results in reduction in L-selectin expression, increased expression and avidity of CD11b/CD18, assembly of NADPH oxidase components, and a heightened response to other agonists. In this model, triggering of the second

pathway, particularly by G-protein-linked receptors that induce elevations in intracellular calcium concentration, activates the neutrophil oxidative burst and secretory responses (Ainsworth et al., 1996). Phagocytosis of opsonized particles by neutrophils triggers intracellular signalling via Fcγ or complement receptors, and this also contributes to neutrophil activation by mechanisms that remain undefined (Unkeless et al., 1992; Wright, 1992). Details of the intracellular signalling pathways leading to neutrophil activation have been described (Snyderman and Uhing, 1992; Baggiolini et al., 1993; Hallet and Lloyds, 1995; Ainsworth et al., 1996) and will not be reviewed here.

The mechanisms by which neutrophil function is down-regulated are less well characterized. Firstly, several mediators have been described that counteract the stimulatory effects outlined above, including PGE2 and nitric oxide from alveolar macrophages, prostacyclin and adenosine from endothelial cells, lipoxin A4 from platelets, and histamine from mast cells (Sibille and Marchandise, 1993; Ainsworth et al., 1996). Secondly, elevations in intracellular calcium and generation of the superoxide burst are inherently transient, and are probably mediated concurrently by several intracellular regulatory pathways. Finally, receptor densensitization is a well-described phenomenon. Homologous densensitization involves internalization of ligand-receptor complexes, and possibly impaired interaction of receptors with G-proteins due to receptor phosphorylation. Heterologous desensitization, in which stimulation with one agonist reduces the response to a second agonist, may also occur, but the exact circumstances and mechanisms remain obscure (Snyderman and Uhing, 1992).

Phagocytosis is an important process in the bactericidal activity of neutrophils, and prevents tissue destruction by ensuring that the exposure of foreign material to injurious substances is isolated within the confines of the phagolysosome. Neutrophils, compared to macrophages, manage very little phagocytosis of inert particles, but readily phagocytose opsonized particles. Neutrophils constitutively express FcγRII (CD32) and FcγRIII (CD16), the low-affinity IgG receptors, while expression of the high-affinity receptor FcγRI (CD64) may be induced by treatment with GM-CSF (Daeron, 1997). Other immunoglobulin receptors on neutrophils include FcαR and the low-affinity IgE receptor S-lectin or Mac2/εBP. As described earlier, priming of neutrophils induces expression of complement receptor 3 (CD11b/CD18), the C3bi receptor (Unkeless et al., 1992; Wright, 1992; Sibille and Marchandise, 1993; Ainsworth et al., 1996). Particles which have been opsonized by IgG, IgA or C3b attach specifically to these receptors, triggering both engulfment of the particle by a pseudopod to form a membrane-bound phagosome, and activation of intracellular signalling pathways (Wright, 1992).

Bovine neutrophils contain three types of cytoplasmic granules: azurophil or primary granules that are rod-shaped, similar to lysosomes, and mediate intracellular killing of pathogens; specific or secondary granules that are larger, round, more numerous, and secrete their contents to the extracellular milieu; and large granules that are unique to ruminants, numerous, and electron-dense (Baggiolini et al., 1985; Cheville, 1994). The contents of these granules are listed in Table 1.1 (Bainton, 1992; Cotran et al., 1994; Ainsworth et al., 1996). Potentially important aspects of bovine neutrophils, compared to their human counterparts, include higher

bactericidal activity and higher content of lactoferrin, B12-binding protein, acid and alkaline phosphatases, glutathione peroxidase and glutathione reductase, but relatively low levels of lysozyme, myeloperoxidase, catalase, β-glucuronidase and β-galactosidase (Gennaro et al., 1978). The following have been specifically demonstrated in the different bovine neutrophil granules:

Table 1.1. A partial list of the content and function of neutrophil granules

Function	Azurophil granules	Specific granules	Ruminant large granules
Bactericidal effects	Myeloperoxidase BPI factor Defensins Azurocidin Lysozyme	Lysozyme	Bactenecin-5 and –7 Antibiotic dodecapeptide Indolicidin Lactoferrin
Nutrient-binding proteins		Lactoferrin B12-binding protein	
Proteolytic enzymes	Elastase Cathepsin G Collagenase		
Other		Alkaline phosphatase CD11b/CD18 Plasminogen activator Phospholipase A2 Histaminase Collagenase	

1/ Azurophil granules: myeloperoxidase I, II, and III (Cooray et al., 1993), bactericidal/ permeability-inducing factor (Elsbach and Weiss, 1992), defensins (Selsted et al., 1993), minimal amounts of lysozyme (Gennaro et al., 1978; Berenji, 1997), acid and alkaline phosphatase, β-glucuronidase and β-galactosidase (Gennaro et al., 1978);

2/ Specific granules: B12-binding protein (Watson et al., 1995) and lactoferrin (Gennaro et al., 1978); and

3/ Tertiary granules: lactoferrin (Baggiolini et al., 1985), bactenecins (Frank et al., 1990), antibiotic dodecapeptide (Storici et al., 1996), and indolicidin (Selsted et al., 1992).

Neutrophils kill phagocytosed bacteria both by non-oxidative mechanisms, attributed to the bactericidal factors listed in Table 1.1, and by the synthesis of reactive oxygen species including superoxide anion, hydrogen peroxide, and products of the peroxidase-H_2O_2-halide system. The synthesis of oxygen radicals is powered by the generation of NADPH via the hexose monophosphate shunt, a pathway that is particularly active in bovine neutrophils (Gennaro et al., 1978). NADPH oxidase, an inducible membrane-associated assembly of cytosolic and membrane-bound enzymes, transfers electrons from NADPH to oxygen. Activation of NADPH oxidase results in the synthesis of superoxide anion, which degrades spontaneously to form hydrogen peroxide under acidic conditions or in a reaction catalysed by superoxide dismutase. The direct effect of superoxide anion in inducing injury to bacteria is controversial; injury may also be a result of reaction with nitric oxide to

form peroxynitrite, or with hydrogen peroxide in the Haber-Weiss reaction to form the highly reactive hydroxyl radical (Klebanoff, 1992; Cotran et al., 1994).

Hydrogen peroxide is relatively less reactive than hydroxyl radical or hypohalous acids, but may react with ferrous iron in the Fenton reaction to form hydroxyl radical. The major mechanism of oxidative killing of bacteria by neutrophils likely involves a myeloperoxidase-catalysed reaction of peroxide with halide ions to form hypohalous acids within the azurophil granule. These hypohalous acids are highly toxic to microorganisms by halogenation of proteins or by oxidation of sulfhydryl groups, heme proteins, iron, or lipids in bacterial enzymes and membranes (Klebanoff, 1992). The role of neutrophil-derived oxygen radicals in injury to host tissues will be considered in the next section.

Secretory products of neutrophils include those that are stored in granules and others that are synthesized *de novo*. The contents of specific granules are actively secreted by viable cells, probably in response to elevations of intracellular calcium (Henson et al., 1992), and have a variety of actions. Several of these secreted products, such as lactoferrin, B12-binding protein, and lysozyme, are bactericidal or bacteriostatic. Others, such as collagenase, degrade extracellular matrix, while histaminase and C5a activator may modulate the inflammatory response. Finally, some contents of the secondary granules, including CD11b/CD18 and fMLP receptor, are not secreted; rather, they are transported to the plasma membrane (Ainsworth et al., 1996).

Although the contents of azurophil granules probably function primarily within the confines of the phagolysosome, they may be discharged to the exterior by

three mechanisms (Slauson and Cooper, 1990; Henson et al., 1992). "Regurgitation during feeding" results in leakage of granule contents when lysosomes fuse with phagosomes prior to their separation from the plasma membrane. A similar process has been termed "frustrated phagocytosis," in which attempts to internalize large masses of material, such as basement membranes or fibrin meshworks, result in leakage of granule contents to the exterior. Finally, lysis of neutrophils by bacterial toxins may result in spillage of the granule contents. Although these concepts are widely propagated in textbooks, they are perhaps simplistic and evidence for their existence is limited (Henson et al., 1992).

In addition to granule contents, activated neutrophils secrete several mediators that are synthesized *de novo*, including LTB4, prostaglandin E2, platelet activating factor, TNF-α, IL-1β, IL-1 receptor antagonist, IL-6, IL-8, IL-10, TGF-β, and GM-CSF (Casatella, 1995; Parsey et al., 1998). In contrast to the ability of human neutrophils to produce nitric acid, bovine neutrophils do not secrete nitric acid under the conditions tested (Goff et al., 1996). Because of the large number of neutrophils present in suppurative lesions, it is likely that secretion of mediators by activated neutrophils serves to augment the ongoing inflammatory response, recruit more neutrophils to the site of infection, and modulate the immune response to infectious agents (Lloyd and Oppenheim, 1992).

1.3.5. Neutrophil-mediated tissue injury

The role of neutrophils in inducing injury to host tissues is an established

theme in many infectious and inflammatory diseases. This section provides an overview of the evidence supporting this phenomenon, and outlines the mechanisms responsible for these injurious consequences. These mechanisms include damage induced by reactive oxygen species, lytic enzymes, inflammatory cytokines, and modulators of hemostasis. The production of these factors by neutrophils was described in the preceding section.

The direct toxicity of superoxide anion to tissues is controversial, because superoxide is relatively non-reactive except when in a hydrophobic environment, and it does not diffuse across membranes in most conditions (Klebanoff, 1992). However, approximately 15% of the superoxide anion produced by bovine neutrophils is released to the exterior of the cell (Gennaro et al., 1978). Furthermore, as superoxide dismutase inhibits superoxide-induced cellular toxicity in some *in vitro* systems but catalase does not, a direct role for this radical in causing *in vivo* injury should not be excluded (Klebanoff, 1992).

Hydrogen peroxide is produced in large amounts by activated neutrophils and readily diffuses across membranes. This, and the relative lack of extracellular inhibitors, may allow peroxide to be a major contributor to oxidative injury in exudate fluids (Klebanoff, 1992). As described in the previous section, superoxide anion and hydrogen peroxide may form the hydroxyl radical through the Fenton or Haber-Weiss reaction. The role of hydroxyl radicals in inducing tissue injury, however, is limited by at least two aspects: the lack of free ferrous iron in the extracellular space, and the limited diffusion of hydroxyl radical due to its extreme reactivity (Klebanoff, 1992).

The products of the hydrogen peroxide-myeloperoxidase-halide system include hypohalous acids such as HOCl, halogens such as Cl_2, and the chloramines RNHCl or $RNCl_2$, and it is likely that these species contribute substantially to neutrophil-induced oxidative tissue injury. Hypohalous acids are the most extensively studied of these, and induce injury by halogenation of proteins and oxidation of enzymes, heme proteins, and membrane lipids. In addition, hypohalous acids stimulate platelet and mast cell degranulation (Klebanoff, 1992). *In vivo* activation of this system in rat lungs induces acute pulmonary injury that manifests initially as increased vascular permeability with hemorrhage and fibrin exudation, and progresses to interstitial fibrosis (Johnson et al., 1981).

Other mechanisms which are probably less important in inducing neutrophil-induced oxidative tissue injury include the production of peroxynitrite by the reaction of superoxide anion with nitric oxide, and the synthesis of toxic halide radicals by reaction of hydroxyl radical, peroxide, and ferrous iron (Klebanoff, 1992).

In most situations, tissue injury by oxygen radicals is prevented by a series of protective mechanisms. Antioxidants, which scavenge free radicals but are consumed in the process, include vitamin E, glutathione, cysteine, albumin, ceruloplasmin, and transferrin. In addition, enzyme systems are active in tissue fluids to degrade reactive oxygen species. Superoxide dismutase converts superoxide anion to hydrogen peroxide, and catalase catalyses the transformation of hydrogen peroxide to water. Glutathione peroxidase converts reduced glutathione and either hydroxyl radical or hydrogen peroxide to oxidized glutathione and water; reduced gluathione is subsequently restored by the action of glutathione reductase.

These protective systems must be impaired or overwhelmed before the secretion of oxygen radicals by neutrophils will result in tissue injury (Cotran et al., 1994).

The role of neutrophil proteolytic enzymes in mediating tissue injury is dependent on the release of these products to the extracellular environment, as described in the previous section, and on the depletion of protease inhibitors from the interstitial fluid. The latter may occur through genetic deficiencies, as illustrated by the tendency of humans with α1-antitrypsin deficiency to develop neutrophil-induced pulmonary emphysema, or through impairment of anti-proteases by neutrophil-derived oxygen radicals (Cotran et al., 1994; Jaeschke and Smith, 1997). Alternatively, it has been postulated that adherence of neutrophils to target cells may activate the neutrophils to release proteases which locally deplete the anti-protease activity (Jaeschke and Smith, 1997).

Elastase and cathepsin G are important mediators of neutrophil-dependent lung injury in humans and in laboratory rodents. Human neutrophil elastase degrades not only elastin, but also numerous other matrix constituents, including collagen I, II, and IV, laminin, fibronectin, and proteoglycans (Jennings and Crystal, 1992). Elastase stimulates mucus production by tracheal submucosal glands (Schuster et al., 1992) and induces secretion by, and subsequent squamous metaplasia of, bronchial secretory epithelium. Neutrophil-rich sputum impairs ciliary function in tracheal explants and this effect is abrogated by α1-antitrypsin, an inhibitor of serine proteases including elastase (Schuster et al., 1992). In hamsters, elastase induces injury to alveolar septa and leads to emphysema (Snider et al., 1984) and it is a likely

contributor to the development of human emphysema associated with cigarette smoking (Cotran et al., 1994). Specific inhibitors of elastase reduce LPS- and TNF-α-induced neutrophil-dependent increases in pulmonary vascular permeability in rabbits (Sakamaki et al., 1996; Miyazaki et al., 1998). It should be noted, however, that the proteolytic enzyme content of neutrophil azurophil granules is considerably higher in humans than in cattle; in particular, the elastase content of human neutrophils is about 50 times greater than that of bovine neutrophils (Brown and Roth, 1991).

Activated bovine neutrophils induce injury to and ^{51}Cr-release from labelled endothelial cells, and these processes are dependent on both oxidative injury and proteolytic enzyme release (Maheswaran et al., 1993). The injurious effects of fMLP-stimulated human neutrophils on endothelial cells is dependent on proteases and oxygen radicals, whereas that induced by IFN-γ- or IL-1-stimulated human neutrophils is mediated partially by nitric oxide (Bratt and Palmblad, 1997). The relevance of this latter finding to bovine disease is unclear because, as described above, bovine neutrophils do not secrete nitric oxide.

The role of neutrophils in the pathogenesis of bovine pneumonic pasteurellosis has been detailed in Section 1.2.3.4. In humans, a role for neutrophil-dependent tissue injury has been established or suggested in several pulmonary disease processes: destruction of alveolar septa in the chronic bronchitis and emphysema induced by cigarette smoking, damage to alveolar septal endothelial cells in the acute respiratory distress syndrome associated with sepsis, bronchoconstriction and airway hyper-reactivity in asthma, and injury to endothelium and vessel walls in

Wegener's granulomatosis associated with circulating antibodies to neutrophil cytoplasmic constituents (Sibille and Marchandise, 1993). In rats, neutrophil depletion abrogates the increased pulmonary vascular permeability induced by intravenous LPS infusion (Tsuji et al., 1998).

In summary, the beneficial effects of neutrophil infiltration, activation, phagocytosis and secretion are obvious. However, in some circumstances, products of neutrophil activation contribute substantially to cellular injury and matrix degradation, and exacerbate the deleterious effects of the inciting agent.

1.4. Chemokine biology

The chemokines are a family of proteins that are defined by sequence homology and conserved cysteine residues, and probably arose by gene duplication and diversification. Most members of this family are 8 to 15 kilodaltons (kDa), consist of three β-pleated sheets and a C-terminal α-helix, and are mediators of leukocyte chemotaxis. The chemokine family is divided into four groups based on the number of amino acids separating the first two cysteine residues (reviewed in Rollins, 1997; Luster, 1998).

In the case of the C-X-C or α chemokines, a single amino acid separates these cysteines. The C-X-C chemokines are further subdivided into those in which the C-X-C sequence is immediately preceded by a glutamic acid-leucine-arginine (ELR) sequence and those that lack this motif. The former, termed ELR-CXC chemokines, are chemotactic for neutrophils and will be discussed in detail in the following

sections. The C-X-C chemokines that lack the ELR sequence include platelet factor 4, interferon-inducible protein (IP)-10, monokine-induced-by-interferon-γ (MIG), stromal-derived factor (SDF)-1α, and SDF-1β. Unlike the preferential effects of ELR-CXC chemokines on neutrophils, the non-ELR-CXC chemokines have diverse functions which have been recently reviewed (Rollins, 1997).

The second group of chemokines contains the C-C or β chemokines, in which the first two cysteine residues are adjacent. The C-X-C and C-C chemokines have similar primary, secondary and tertiary structures, but have differing quaternary structure and bind to distinct sets of receptors. The C-C chemokine group includes monocyte chemoattractant proteins (MCP) –1 to 5, macrophage inflammatory proteins (MIP) -1α, β, and γ, MIP-3α and β, RANTES (regulated upon activation, normal T cell expressed and secreted), eotaxin-1 and -2, and others. Most C-C chemokines have effects on leukocytes other than neutrophils. Their target cell specificity is due, at least in part, to the variable affinity of individual chemokines for specific C-C chemokine receptors, and to the dependence of receptor expression on leukocyte lineage and state of activation. Five receptors, termed CCR1 to CCR5, have been identified to date. The cellular specificity of these chemokines and the specific interaction of C-C chemokines with their respective receptors are complex and have been reviewed (Schall and Bacon, 1994; Epstein, 1998)

In the third group of chemokines, the C chemokines, the first and third cysteine residues are absent. Lymphotactin, the sole member of this group, is produced by a subset of T cells and is chemotactic for $CD8^+$ T lymphocytes (Tessier et al., 1997).

The CX_3C chemokines, in which the first two cysteine residues are separated by three amino acids, form the final group. Fractalkine is the only representative described to date, and is a transmembrane protein with an N-terminal chemokine domain on a mucin-like stalk (Schall, 1997).

1.4.1. Interleukin-8 (IL-8) and the ELR CXC chemokines

The ELR-CXC chemokines described to date are listed in Table 1.2 (Schall and Bacon, 1994; Rollins, 1997; Epstein, 1998). All of these chemokines are chemotactic for neutrophils, although PBP, CTAP-III, and β-thromboglobulin have weak activity without further processing to NAP-2 (Rollins, 1997). Interleukin-8 was initially described as a neutrophil chemoattractant secreted from LPS-stimulated macrophages, and current evidence indicates an important role for this chemokine in neutrophil recruitment in humans and rabbits. The remainder of this chapter summarizes the biology of interleukin-8, reviews its role in pulmonary disease, and examines the current understanding of interleukin-8 in ruminants.

The CXC chemokines form dimers in solution but, since the receptor affinity is much higher than the interchain dimer affinity, chemokines probably exist as monomers at concentrations of relevance in tissues (Rollins, 1997). The following are critical sites for functional integrity of IL-8: the ELR sequence, which is at the N-terminus of the 72 amino acid form of IL-8; the four cysteine residues, which form two disulfide bridges; and the glycine and proline residues in positions 31 and 32, which are critical for the "30-35 β turn" that is positioned adjacent to the ELR CXC motif in

the suggested quaternary structure (Clark-Lewis et al., 1994).

Table 1.2. ELR-CXC chemokines and their receptors

Name	Synonym and Comment	Receptor
interleukin-8	NAP-1	CXCR1, CXCR2
growth-related oncogenes (GRO)- α	melamona growth stimulatory activity (MGSA)	CXCR2
GRO-β and γ	MIP-2α and β	CXCR2
KC	rodent homologue of GRO- α	
epithelial derived neutrophil attractant (ENA)	ENA-78	CXCR2
granulocyte chemotactic protein-2 (GCP-2)		CXCR2
platelet basic protein (PBP)	CTAP-III, β-thromboglobulin, and neutrophil activating protein-2 (NAP-2) are proteolytic derivatives	CXCR2
lipopolysaccharide induced CXC chemokine (LIX)	Murine; AMCF-II is a related gene in pigs	

There are currently four described CXC chemokine receptors in humans, which are termed CXCR1 to CXCR4. Interleukin-8 is the only identified ligand with high affinity for CXCR1 (formerly IL-8R1), although GRO-α and NAP-2 bind this receptor with low affinity. Interleukin-8, GRO-α, -β, and -γ, ENA, and NAP-2 all bind to CXCR2 (formerly IL-8R2) (Ahuja and Murphy, 1996). The non-ELR-CXC chemokines IP-10 and MIG bind CXCR3. Finally, the ligand for SDF-1α is CXCR4, which has recently gained attention as being a co-receptor for human immunodeficiency virus (Oberlin et al., 1996). In contrast to the exclusivity of the CXC receptors 1-4 for CXC chemokines, the Duffy antigen binds both CXC and CC chemokines. The Duffy antigen is expressed on erythrocytes and, because no transmembrane signal is generated following ligand binding, it may bind and remove chemokines from the circulation (Neote et al., 1994). The CXC chemokine receptors are G-protein linked receptors with seven transmembrane domains, and effect intracellular signalling via protein kinase C, MAP kinases, and nuclear factor (NF) κB pathways (Murphy, 1997; Rollins, 1997). It has been postulated that CXCR2 may transmit important signals at sites distant from a lesion, where the chemokine concentration is lower, whereas high chemokine concentrations close to a source may saturate CXCR2 but allow effective signalling by CXCR1 (Murphy, 1997).

Although an IL-8 homologue has not been identified in rodents, there are several murine ELR-CXC chemokines that are homologous to GRO-α and are potent neutrophil chemoattractants. In mice, these chemokines are named KC (formerly designated N51) and macrophage inflammatory protein-2. Chemokines containing

the ELR-CXC sequence in rats include KC and cytokine-induced neutrophil chemoattractants- (CINC-) 1, 2α, 2β and 3. Rat CINC-3 was previously termed MIP-2 (Watanabe et al., 1993; Edamatsu et al., 1997).

1.4.2. Sources of IL-8 and stimuli for IL-8 secretion

All cell types that have been examined can, with the appropriate stimulation, secrete interleukin-8 (Furie and Randolph, 1995). The best characterized stimuli for IL-8 synthesis are lipopolysaccharide, IL-1, and TNF-α. Lipopolysaccharide induces IL-8 production by cultured human alveolar macrophages, with peak IL-8 mRNA expression induced by 1 μg/ml of LPS. Expression of IL-8 mRNA was detectable at 1 hour, peaked at 8 hours, and had declined by 24 hours after treatment with LPS. Cell-associated IL-8 protein was detected at 4 hours and persisted until at least 24 hours after treatment, while chemotactic activity in the culture supernatant peaked at 8 hours and persisted for 24 hours after exposure (Streiter et al., 1990). In another study, IL-8 was first detectable in supernatants of cultured human alveolar macrophages at 3 to 5 hours after stimulation with LPS, and peaked at 24 hours after exposure (Rankin et al., 1990). Cultured porcine alveolar macrophages expressed IL-8 mRNA within 30 minutes of LPS-stimulation, maximal expression occurred from 3 to 6 hours after exposure, and levels were detectable for at least 24 hours. Interleukin-8 protein was detected in the supernatant by 4 hours after LPS-treatment (Lin et al., 1994). The average peak IL-8 concentrations in supernatants of LPS-stimulated human alveolar macrophages, cultured at 2 x 10^6 cells/ml, ranged from

790 to 860 ng/ml (Rankin et al., 1990; Sylvester et al., 1990).

The timing of IL-8 secretion from LPS-stimulated macrophages relative to the release of other inflammatory mediators has been partially defined. Leukotriene B4 was detected in the supernatant of LPS-stimulated alveolar macrophages at less than one hour after stimulation, whereas IL-8 secretion was initially detected at 4-6 hours (Rankin et al., 1990). The IL-8-inducing effect of LPS on alveolar macrophages is probably direct and not mediated by secretion of IL-1 or TNF-α (Lin et al., 1994). The ability of LPS to induce IL-8 secretion by alveolar macrophages is not uniform across bacterial species; for example, compared to LPS from *Escherischia coli*, *P. haemolytica* LPS induces greater IL-8 mRNA expression in bovine alveolar macrophages (Morsey et al., 1996).

Lipopolysaccharide-induced IL-8 secretion can be modulated by various cytokines. The neutrophil chemoattractants C5a, LTB4 and fMLP augment LPS-induced IL-8 secretion by neutrophils (Kunkel et al., 1997). In contrast, IL-8 secretion by macrophages is impaired by pretreatment with IFN-γ (Schnyder-Candrian et al., 1995), prostaglandin E2 (Standiford et al., 1992), or interleukin-4 (Zhou et al., 1994). Similarly, IL-8 secretion by neutrophils is reduced by treatment with nitric oxide (Cuthbertson et al., 1997).

The proinflammatory cytokines IL-1 and TNF-α induce IL-8 secretion in all cell types that have been examined (Furie and Randolph, 1995). In contrast, the ability of LPS to induce biologically significant levels of IL-8 secretion in cells other than macrophages is more controversial (Kunkel et al., 1997). However, LPS-induced IL-8 secretion has been demonstrated in cultured bronchial epithelium

(Khair et al., 1994), endothelial cells (Baggiolini et al., 1992), neutrophils (Fujishima et al., 1993) and pleural mesothelial cells (Antony et al., 1995), albeit at much lower levels than for LPS-stimulated monocytes. For example, IL-8 secretion following LPS exposure was 70- to 200-fold lower in neutrophils compared to monocytes (Fujishima et al., 1993).

In addition to LPS, IL-1 and TNF-α, many other stimuli are capable of inducing IL-8 secretion in cells of marrow origin. Monocytes secrete IL-8 in response to a variety of bacterial cell wall components, phagocytosis of fungal conidia or inert silicate particles, adherence to plastic, or cross-linking of Fcγ receptors (Heinel et al., 1995; Marsh et al., 1995; Nessa et al., 1997). Neutrophil secretion of IL-8 is stimulated by zymosan (Au et al., 1994). Eosinophils from patients with atopy secrete IL-8 following dual stimulation with GM-CSF and either RANTES or PAF (Simon et al., 1995). Mast cells expressed IL-8 mRNA following aggregation of their immunoglobulin E receptors (Okayama et al., 1995). Platelets contained pre-formed stores of IL-8, which was rapidly secreted following activation with thrombin (Su et al., 1996).

As previously mentioned, cells which are not conventionally considered "inflammatory cells", such as epithelial cells, endothelial cells, fibroblasts, and smooth muscle cells, are able to secrete IL-8 following treatment with IL-1 or TNF-α. Interleukin-8 secretion from endothelial cells is also stimulated by fibrin (Qi and Kreutzer, 1995) or thrombin (Ueno et al., 1996). Epithelial cells produce IL-8 after infection with human respiratory syncytial virus (Mastronarde et al., 1995), maedi-visna virus (Legastelois et al., 1996), or *Yersinia enterocolitica* (Schulte et al., 1996).

In summary, a wide variety of cells can be induced to secrete IL-8. The importance of each of these cell types as a biologically important source of this chemokine depends on several factors and may vary with the type of stimulus, the tissue affected, and the stage of the disease process. These factors include the number of secreting cells present in the tissue at the time of interest, the presence of adequate concentrations of the stimulus, the timing and magnitude of IL-8 secretion by the given cell type, the presence of substances which potentiate or inhibit IL-8 secretion in response to the stimulus, local activators or inhibitors of IL-8 activity, and the presence of suitably responsive target cells. Although the data presented above provide insight into potential cellular sources of IL-8, the important *in vivo* sources of IL-8 following specific tissue insults have not been well defined in most cases.

1.4.3. Effects of IL-8 on target cells

The effect of IL-8 on neutrophil activation, the most extensively studied function of this chemokine, is described in this section. In addition, the ability of IL-8 to stimulate angiogenesis and its effects on eosinophils, lymphocytes, monocytes, and basophils are summarized.

Interleukin-8 was initially characterized as a macrophage-derived protein that induced chemotaxis and activation of neutrophils (Schroder et al., 1987; Peveri et al., 1988). *In vitro* neutrophil chemotaxis was elicited by IL-8 concentrations ranging from 0.1 to 10 nM (80-0.8 ng/ml), and the activity of naturally occurring,

recombinant and chemically-synthesized IL-8 were similar (Lindley et al., 1988; Clark-Lewis et al., 1991). Recombinant ovine IL-8 induced neutrophil chemotaxis of progressively increasing intensity from 1.0 to 32 nM (256-8 ng/ml) (the highest dose tested), which was of similar potency to recombinant human IL-8 (Seow et al., 1994).

Intradermal injection of naturally occurring or chemically synthesized human IL-8 induced infiltration of neutrophils (Leonard et al., 1991; Swensson et al., 1991). This effect was dose-dependent from 8 to 40 pmol (320-64 ng), and progressively more neutrophils were present from 0.5 to 1 and 3 hours after injection. The chemotactic effect was specific for neutrophils; neither eosinophil infiltration nor mast cell degranulation was present, but about 20% of the infiltrating cells at 3 hours after injection were monocytes. Intradermal injection of recombinant ovine IL-8 induced intense neutrophil infiltration at 6 and 24 hours, and less marked but significant infiltration of eosinophils and T lymphocytes (Seow et al., 1994).

Interleukin-8 induces the activation of neutrophils, in addition to the chemotactic effects described above. *In vitro* treatment of neutrophils with IL-8 induces an intracellular calcium flux within seconds, with a maximal effect elicited by 3 nM IL-8 (Lindley et al., 1988). Interleukin-8 reduced the surface expression of L-selectin but increased that of CD11b/CD18 and complement receptor 1 (Detmers et al., 1991). Interleukin-8 stimulated neutrophils to release vitamin B_{12}-binding protein from specific granules and β-glucuronidase from azurophilic granules (Lindley et al., 1988). The effect of IL-8 in inducing an oxidative burst in neutrophils has been controversial (Baggiolini et al., 1992). Most reports indicate that, like GM-CSF and TNF-α, IL-8 primes neutrophils for a greater response to

agonists such as fMLP but does not directly induce an oxidative burst (Elbim and Gougerot-Podicalo, 1996). Interleukin-8 augments the ability of neutrophils to phagocytose opsonized erythrocytes or fungal conidia, without altering the surface expression of Fcγ receptors (Detmers et al., 1991; Richardson and Patel, 1995). Finally, IL-8 improves the ability of neutrophils to kill *Staphylococcus aureus* and *Pseudomonas aeruginosa* but not *E. coli*, although this improvement is less pronounced than that induced by TNF-α or IL-1β (Ponglertnapagorn et al., 1996; Simms and D'Amico, 1997).

The N-terminus of secreted IL-8 is heterogeneous, because of differential processing of the 99 amino acid precursor to 77- or 72-aa variants. The 72-aa form is about 10-fold more potent than the 77-aa form in inducing *in vitro* neutrophil chemotaxis and activation. In contrast, these forms induce similar *in vivo* responses, perhaps due to *in vivo* extracellular proteolysis of the 77-aa form to the 72-aa molecule (Nourshargh et al., 1992).

Reports of IL-8-induced eosinophil chemotaxis have been controversial. Ovine IL-8 induced intradermal infiltration of very low numbers of eosinophils (Seow et al., 1994). *In vitro* studies indicate that unprimed eosinophils are not responsive to human IL-8 and do not express receptors for IL-8, but priming of eosinophils with IL-5 induces these cells to express the IL-8 receptor CXCR2 and undergo chemotaxis in response to IL-8 (Schweizer et al., 1994; Heath et al., 1997). Human basophils migrate toward IL-8, and release histamine and leukotrienes if these cells are previously primed (Baggiolini et al., 1992). Interleukin-8 is chemotactic for T lymphocytes (Larsen et al., 1989).

Angiogenesis may be stimulated by IL-8, thereby influencing the repair of inflamed tissue and the blood supply to growing tumours (Baggiolini et al., 1997). Interleukin-8 induces chemotaxis of endothelial cells *in vitro* and angiogenesis *in vivo*. Neutralization of IL-8 conferred a 34% reduction in the ability of supernatant fluid from LPS-stimulated monocytes to attract endothelial cells *in vitro*, and markedly suppressed the ability of this supernatant fluid to induce corneal angiogenesis (Koch et al., 1992). Interleukin-8 is expressed by a variety of tumour cells. The ability of *in vivo* neutralization of IL-8 to reduce by 40% the growth of tumour transplants in immunodeficient mice was attributed to an abrogation of tumour vascularity (Arenberg et al., 1996).

1.4.4. Expression of IL-8 in selected pulmonary diseases

Interleukin-8 expression has been documented in a wide variety of diseases, including infectious, immune-mediated, degenerative, toxic and neoplastic processes. This section summarizes the expression of IL-8 in selected diseases of the lung, with an emphasis on naturally occurring inflammatory processes in humans and rabbits. The role of IL-8 in diseases of ungulates will be discussed in Section 1.5. Because rodents lack an IL-8 homologue, models of pneumonia in mice and rats are not reviewed in this section.

Several studies have evaluated the expression of IL-8 in bacterial pneumonia in humans. Interleukin-8 levels in BAL fluid from patients with concurrent bacterial pneumonia and human immunodeficiency virus (HIV) infection ranged from 296 to

1161 pg/ml, whereas lower levels were present in HIV-positive patients with pneumonia caused by *Pneumocystis carinii* and in healthy controls (Krarup et al., 1997). Patients with community-acquired bacterial pneumonia had higher levels of IL-8 in BAL fluid, with a mean level of 214 ng per 10^6 alveolar macrophages, and enhanced IL-8 secretion from cultured alveolar macrophages compared to normal controls (Bohnet et al., 1997). Sputum samples from humans with chronic bronchitis or bronchiectasis contained, on average, 22 and 77 ng/ml IL-8, respectively (Richman-Eisenstat et al., 1993). In humans with unilateral bacterial pneumonia, IL-8 concentrations in BAL fluid from the lesional lung contained 18 to 1293 pg/ml IL-8 (mean of 395 pg/ml), whereas BAL fluid from the non-lesional lung contained IL-8 levels which were increased over normal but much less than the lesional BAL fluid (mean of 33 pg/ml) (Boutten et al., 1996). A comparison BAL fluid from patients with bacterial pneumonia revealed IL-8 concentrations of 569 ± 120 pg/ml and GRO-α concentrations of 1870 ± 314 pg/ml (Villard et al., 1995).

In BAL fluid from rabbits with experimentally induced *E. coli* pneumonia, the concentrations of IL-8, GRO-α and TNF-α were elevated within 4 hours of infection. At 24 hours after infection, these levels had declined in rabbits that cleared the bacteria but continued to rise in those with persistent infection (Fox-Dewhurst et al., 1997). The sequential generation of inflammatory mediators in LPS-induced pulmonary injury in rabbits has been evaluated. The levels of TNF-α were maximal at 30 minutes after challenge, whereas IL-1 receptor antagonist concentrations showed two peaks at 30 minutes and 2 hours. In contrast, IL-8 and IL-1 concentrations had a single peak at 2 hours, and were secreted from both

alveolar macrophages and infiltrating neutrophils at this time (Imamura et al., 1997).

Interleukin-8 levels in BAL fluid are elevated in a variety of other pulmonary diseases in humans; a selection of these are summarized in Table 1.3. A final study of interest, although it deals with cutaneous rather than pulmonary disease, involves analysis of IL-8 and GRO-α mRNA expression in the cutaneous lesions of psoriasis. Both of these chemokines were strongly expressed by neutrophils in the epidermis, with weaker expression by keratinocytes, whereas only GRO-α was expressed in vessels of the papillary dermis and its expression in this location was most prominent in early psoriatic lesions. These data suggest that GRO-α may be more important than IL-8 in the early recruitment of neutrophils in psoriasis, but both chemokines are produced by neutrophils in the more well-developed lesions and could contribute to ongoing neutrophil recruitment (Gillitzer et al., 1996).

1.4.5. Role of IL-8 as a neutrophil chemoattractant in inflammatory disease

The previous section summarized evidence for increased interleukin-8 expression in inflammatory diseases of the lung. The role that this chemokine plays in recruiting neutrophils to the sites of pulmonary inflammation depends not only on the levels expressed in BAL fluid, but also on the timing of IL-8 expression, the presence of local inhibitors of IL-8 function or counter-acting mediators, the presence of other mediators with effects that mimic those of IL-8, and the presence of suitably responsive neutrophils. There are several methods of assessing the importance of IL-8 as a neutrophil chemoattractant in pulmonary diseases, including:

Table 1.3. Human diseases associated with elevated interleukin-8 concentrations in bronchoalveolar lavage fluid.

Disease	IL-8 level in BAL fluid	Reference
Bacterial pneumonia	296 to 1161 pg/ml	Krarup et al., 1997
	18 to 1293 pg/ml	Boutten et al., 1996
	569 ± 120 pg/ml	Villard et al., 1995
	19.5 ± 3.3 ng/ml (ELF[1])	Goodman et al., 1996
Acute respiratory	507 ± 96 pg/ml	Villard et al., 1995
distress syndrome	87 ± 18 ng/ml (ELF)	Goodman et al., 1996
Pneumocystis carinii	123 ± 40 pg/ml	Villard et al., 1995
	42-254 pg/ml	Krarup et al., 1997
Hypersensitivity	N/A[2]	Denis, 1995
pneumonitis		
Cystic fibrosis	N/A	Schuster et al., 1995
		Bonfield et al., 1995
Asthma	244 pg/ml	Keatings et al., 1996
Idiopathic pulmonary	N/A	Car et al., 1994
fibrosis		
Normal lung	21.5 pg/ml	Keatings et al., 1996
	37-82 pg/ml	Krarup et al., 1997
	undetectable (ELF)	Goodman et al., 1996

[1] ELF- epithelial lining fluid
[2] N/A- not available

1. Correlating the concentration of IL-8 in BAL fluid, as measured by ELISA, with the magnitude of the neutrophil influx.
2. Comparing the neutrophil chemotactic activity of a BAL fluid sample of known IL-8 content, with that of recombinant IL-8.
3. Correlating the onset of IL-8 expression with the earliest influx of neutrophils.
4. Evaluating the effect of IL-8 neutralization on neutrophil chemotactic activity of BAL fluid.
5. Assessing the magnitude of neutrophil infiltration in animal models of inflammation or disease following *in vivo* neutralization of IL-8.
6. Studying models of inflammation or disease in chemokine- or chemokine-receptor-deficient animals.

Several studies have attempted to correlate the concentrations of IL-8 in BAL fluid with the magnitude of the neutrophil influx. In 27 HIV-positive patients with bacterial pneumonia, neutrophil counts in BAL fluid were correlated positively with IL-8 levels ($r = 0.6$) but not with LTB4 levels (Krarup et al., 1997). IL-8 concentrations correlated positively with several measures of neutrophil influx in unilateral bacterial pneumonia (Boutten et al., 1996). In pleural effusions associated with tuberculosis, empyema, pneumonia, and neoplasia, IL-8 levels and neutrophil numbers in pleural fluid were correlated positively ($r=0.46$) (Ceyhan et al., 1996). In contrast, others studying community-acquired pneumonia have found weak

correlations (r=0.24) between neutrophil numbers and IL-8 concentrations (Villard et al., 1995). In a study of IL-8 in community-acquired bacterial pneumonia, IL-8 release from cultured alveolar macrophages did not correlate with neutrophil counts in BAL fluid (Bohnet et al., 1997).

There are two obvious limitations to this method of analysis. Firstly, it does not account for the presence of other chemoattractants in the fluid sample. For example, BAL fluid from cases of bacterial pneumonia contain high levels of both IL-8 and GRO-α (Villard et al., 1995) and, because both of these chemokines are secreted by LPS-stimulated macrophages, their levels in BAL fluid are correlated (Villard et al., 1995). Therefore, this method of analysis could overestimate the role of IL-8 as a neutrophil chemoattractant in bacterial pneumonia. Secondly, a correlation of IL-8 levels with neutrophil numbers might simply reflect the fact that neutrophils are themselves a major source of IL-8 in the well-developed lesions of bacterial pneumonia (Streiter et al., 1992).

Fewer studies have evaluated the effect of IL-8 neutralization on neutrophil chemotactic activity of BAL fluid. In one report, alveolar macrophages were stimulated with LPS, TNFα, or IL-1β, and the *in vitro* chemotactic activity of the conditioned media was analyzed at 8 hours after stimulation, the time at which this activity was maximal. Neutralization of IL-8 in these LPS-, TNF-α- or IL-1β-conditioned media reduced the neutrophil chemotactic activity by 31%, 47%, and 44%, respectively, indicating that these percentages of the neutrophil chemotactic activity were attributable to IL-8 (Streiter et al., 1990). Similarly, stimulation of

human umbilical vein endothelial cells with IL-1α induces IL-8 secretion and neutrophil chemotactic activity, and co-incubation with an anti-IL-8 antibody reduced this bioactivity by 50% (Bittleman and Casale, 1995).

Three studies have evaluated the effect of IL-8 neutralization on the *in vitro* chemotactic activity of pleural fluids. In one description of patients with bacterial empyema, pleural exudates which contained 61.3 ± 21.0 ng/ml IL-8 induced strong *in vitro* chemotaxis of neutrophils, and neutralization of IL-8 in these fluids reduced the chemotactic activity by 65 ± 5% (Broaddus et al., 1992). Two comparable reports found similar but more variable results, with 20-90% (Miller and Idell, 1993) and 32% (Antony et al., 1993) of the neutrophil chemotactic activity of pleural fluid attributed to IL-8.

The method of most clinical relevance in assessing the importance of IL-8 as an *in vivo* neutrophil chemoattractant is to measure the magnitude of neutrophil infiltration in inflammation or disease following *in vivo* neutralization of IL-8. It is not yet practical to neutralize IL-8 *in vivo* in humans, and rodents lack an IL-8 homologue; therefore, these studies have been limited to disease models in rabbits and include injury induced by LPS, lyophilized bacteria, immune complexes, and reperfusion following ischemia.

A recent study examined a model in which rabbits were challenged intravenously with LPS, at 36 hours after pulmonary aerosol exposure to lyophilized *Streptococcus pyogenes*. This model was intended to mimic the acute respiratory distress syndrome by inducing mild pulmonary infiltration and activation of

macrophages, followed by LPS-induced neutrophil infiltration, pulmonary edema, hypoxemia and 70% mortality within 90 minutes of challenge. In this model, intravenous administration of an IL-8-specific antibody prior to priming and again before LPS challenge reduced neutrophil infiltration of the lungs by about 60%, lowered BAL fluid protein concentrations by 40%, lessened the ratio of wet to dry lung weights by 20%, and decreased the severity of hypoxemia by 30%. Treatment with anti-IL-8 antibody significantly reduced the mortality at 90 minutes after challenge from 70% to 30%, for ten rabbits per group (Yokoi et al., 1997).

In LPS-induced pleuritis in rabbits, intravenous and intrapleural administration of an IL-8-neutralizing antibody reduced neutrophil influx by 77% (Broaddus et al., 1994). The impact of intravenous anti-IL-8 antibody on neutrophil infiltration in LPS-induced dermatitis and arthritis is dependent on the stage of inflammation. Neutralization of IL-8 almost completely prevented neutrophil infiltration in the first 4 hours after LPS injection. However, this was less marked, conferring a 30% to 50% reduction in neutrophil numbers, from 6 to 24 hours, suggesting that IL-8 may be an important mediator of neutrophil infiltration in the early phases after stimulation but other mediators with redundant actions were active at the later stages (Harada et al., 1994).

Glomerulonephritis induced by immune complex deposition has traditionally been attributed to the effects of complement activation, but recent evidence suggests an important role for Fcγ receptor-mediated production of cytokines by macrophages or neutrophils in the development of this disease (Harada et al., 1994; Sylvestre et al., 1996). In a model of glomerulonephritis in rabbits induced by repeated injection of

bovine serum albumin, neutralization of IL-8 reduced glomerular neutrophil numbers by 40%, reduced fusion of epithelial foot processes, and completely prevented proteinuria (Wada et al., 1994).

Reperfusion injury following frostbite, hypovolemia, infarction or intestinal accident is an effect of oxidative tissue damage, and neutrophils are a major source of oxygen radicals in these lesions (Korthuis et al., 1988). In rabbits, experimental pulmonary ischemia followed by reperfusion induced neutrophil infiltration, edema and exudation of fibrin. Administration of an anti-IL-8 antibody reduced neutrophil infiltration by about 90%, and also partially prevented the exudation of fibrin (Sekido et al., 1993). These results imply that tissues undergoing oxidative damage in reperfusion injury secrete IL-8, creating a major stimulus for neutrophil influx in these lesions.

It should be noted that none of these studies examines infection with live bacteria; therefore, only the detrimental aspects of neutrophil responses are emphasized, and the beneficial role of neutrophils in eliminating infectious organisms is not considered. In contrast, an assessment of the utility of IL-8 neutralization as an adjunctive therapy in infectious disease must consider both of these aspects of neutrophil biology. No published studies have evaluated the effect of *in vivo* IL-8 neutralization in infectious diseases. In a study of intraperitoneal infection with *Klebsiella pneumoniae* in mice, administration of IL-8 at 1 hour prior to challenge conferred protection, whereas protection was not achieved by treatment at other times or in models of *Pseudomonas aeruginosa* infection (Vogels et al., 1993). In this experimental system, exogenous IL-8 probably stimulates receptors for

other ELR-CXC chemokines because, as indicated above, rodents lack an IL-8 homologue.

1.5. Neutrophil chemoattractants in ruminants

Despite the importance of bacterial infections and suppurative inflammation in ruminants, neutrophil chemoattractants in these animals have received comparatively little attention. The following neutrophil chemoattractants have been identified in cattle and/or sheep: the ELR-CXC chemokines IL-8, ENA, GRO, and GCP-2; the complement-derived anaphylatoxin C5a; leukotriene B4; and PAF. In contrast to the situation in most non-ruminant species, formylated bacterial peptides such as fMLP or supernatants from a variety of bacterial cultures do not induce chemotaxis or shape changes in bovine neutrophils (Gray et al., 1982; Forsell et al., 1985).

Bochsler et al. (1994) compared the ability of various neutrophil agonists to induce migration of bovine neutrophils across endothelial monolayers. Recombinant human IL-8 induced transendothelial chemotaxis *in vitro* at 75 ng/ml, and this effect was partially inhibited by an antibody to CD18. Neonatal bovine neutrophils were significantly more responsive to IL-8 than adult neutrophils. In contrast, transendothelial migration induced by either 10^{-7} M C5a or 10% zymosan-activated bovine serum was similar for adult and neonatal bovine neutrophils.

The *in vivo* and *in vitro* neutrophil chemoattractant activities of LPS, recombinant bovine IL-1β, recombinant human TNF-α, recombinant human IL-8,

C5a, LTB4, and PAF in cattle were compared by Persson et al. (1993). Recombinant human IL-8 and C5a induced *in vitro* chemotaxis of bovine neutrophils of comparable magnitude, whereas LTB4 was a weak attractant and LPS, IL-1, TNF, or PAF did not induce neutrophil migration. The chemotaxis induced by human IL-8 was significant from 0.05 to 500 ng/ml, and was maximal at 5 ng/ml. The ability of these agonists to induce neutrophil exudation *in vivo* was assessed by infusion into bovine teat cisterns. On a molar basis, LPS and IL-1 were the most potent inducers of neutrophil exudation and C5a, PAF and TNF were less effective, as assessed by administering the following range of each chemical: LPS, 1-1000 ng; IL-1, 100-1000 ng; TNF-α, 100-4000 ng; PAF, 1-10 μg; C5a, 65-650 ng; and LTB4, 34-340 ng. Surprisingly, neither 10 nor 1000 ng of human IL-8 induced neutrophil infiltration following infusion into bovine teat cisterns. The neutrophil influx in response to LPS, TNF and IL-1 were delayed compared to that induced by C5a or PAF. This suggests that the former do not attract neutrophils directly but probably induce synthesis of neutrophil chemottractants, as has been demonstrated in human and rodent systems (Faccioli et al., 1990; Dinarello, 1992).

1.5.1. ELR-CXC chemokines in ruminants

Bovine IL-8 was originally identified in media conditioned by bovine blood mononuclear cell cultures 3 to 24 hours after stimulation with LPS (Hassfurther et al., 1994). Bovine IL-8 has a molecular weight of 7.8 kDa and an isoelectric point of 8.65. Treatment at pH 3.0, pH 13.0 or 56°C reduced the *in vitro* chemotactic activity

of these supernatants of stimulated mononuclear cells by 72, 58, or 60 percent, respectively, but these treatments did not affect the IL-8 immunoreactivity detected in an ELISA. Affinity-purified bovine IL-8 induced *in vitro* chemotaxis of bovine neutrophils, which was dose-dependent from 5 to 650 pg/ml and was significantly but only partially inhibited by a monoclonal antibody to human IL-8. Receptors for IL-8 are expressed on bovine neutrophils, monocytes, and T and B lymphocytes (Canning, 1992).

The coding sequence of bovine IL-8 sequence is 643 base pairs (bp) in length, and has a single 303 bp open reading frame encoding a protein with a calculated molecular weight of 11.2 kDa (Morsey et al., 1996). The amino acid sequence shares 76%, 87% and 96% homology with human, porcine, and ovine IL-8, respectively (Seow et al., 1994; Morsey et al., 1996). Bovine alveolar macrophages express low levels of IL-8 mRNA and stimulation with heat-killed *P. haemolytica* or *P. haemolytica* LPS upregulate this expression within 1 hour (Morsey et al., 1996).

The coding sequence of ovine IL-8 is 1,434 bp, which encodes a single open reading frame similar to that described for bIL-8 (Seow et al., 1994). Recombinant ovine IL-8 (roIL-8) induces *in vitro* chemotaxis of ovine neutrophils in a dose-dependent manner from $10^{-9.5}$ to $10^{-7.5}$ M (about 2.5 to 250 ng/ml), similar to the potency of human IL-8. Intradermal injection of roIL-8 elicits infiltration of neutrophils, in addition to low numbers of eosinophils, $CD4^+$ lymphocytes and $T19^+$ $\gamma\delta$-T cells, but no $CD8^+$ cells.

Bovine GRO, also referred to as melanoma growth stimulatory activity, is the homologue of human GRO-α and was purified by high pressure liquid

chromatography (HPLC) (Rogivue et al., 1995). Bovine GRO is about 8 kDa, and shares 78%, 54%, 48%, and 39% amino acid homology with human GRO-α, ENA-78, NAP-2, and IL-8, respectively. Two forms of GRO were isolated which differed in their N-terminal sequences, presumably due to extracellular proteolysis. This chemokine elicited dose-dependent *in vitro* chemotaxis of bovine neutrophils in the range of 10^{-7} to 10^{-9} M (about 800 to 8 ng/ml). Neutrophils stimulated with bovine GRO underwent a shape change within 10 seconds and intracellular calcium concentrations increased. Bovine GRO was secreted by LPS-stimulated bovine monocytes. Using immunohistochemistry on lung tissue from cases of bovine pneumonic pasteurellosis, GRO was demonstrated in type II pneumocytes and pleural mesothelium, and to a lesser extent in alveolar macrophages. GRO was also expressed in mesothelium and type II pneumocytes in normal lung.

Bovine ENA was also purified by HPLC from LPS-stimulated bovine monocytes (Allmann-Iselin et al., 1994). Bovine ENA is about 8 kDa and has 74% homology with the amino acid sequence of human ENA-78. *In vitro* neutrophil chemotaxis was induced by 10^{-6} to 10^{-10} M (about 8,000 to 0.8 ng/ml) bovine ENA. In cases of bovine pneumonic pasteurellosis, ENA expression is extensive in type II pneumocytes and pleural mesothelium, patchy in capillary endothelium, and weaker in alveolar exudative leukocytes. No expression was noted in normal lung tissue.

Bovine GCP-2 was purified from Madin Darby bovine kidney cells which had been exposed to the supernatants of LPS- and concanavalin-stimulated human blood mononuclear cells (Proost et al., 1993). Bovine GCP-2 shares amino acid sequence homologies of 67%, 72%, 60%, and 39% with human GCP-2, ENA-78,

NAP-2, and IL-8, respectively. Bovine GCP-2 induced *in vitro* chemotaxis of human neutrophils in the dose range of 10^{-7} to 10^{-9} M (about 800 to 8 ng/ml); this effect was similar to that of human GCP-2 but about 10 times less potent than human IL-8.

The published amino acid sequences of bovine ENA and bovine GCP-2 differ by only one amino acid, apart from an extended N-terminus of up to five residues on the published sequence of GCP-2 (Proost et al., 1993; Allmann-Iselin et al., 1994). However, since the N-terminus of human GCP-2 is known to vary (Proost et al., 1993), it is possible that the molecule designated bovine GCP-2 is actually a variant of bovine ENA.

1.5.2. Relative importance of neutrophil chemoattractants in ruminants

The first portion of this section reviews the temporal expression of neutrophil chemoattractants in inflammatory diseases of ruminants, and compares the timing of IL-8 expression to the onset of neutrophil infiltration. The second portion of this section reviews studies that evaluate the impact of neutralization of these mediators on subsequent neutrophil responses.

The expression of inflammatory mediators was evaluated in bovine mastitis, which was experimentally induced by intramammary infusion of 30 colony forming units of *E.coli* (Shuster et al., 1997). In this model, increased vascular permeability was first detected at 12 hours after infection and peaked at 24 hours. Increases in the number of leukocytes in milk, apart from the normal diurnal variation, were first detected at 16 hours after infection and reached a plateau between 18 and 36 hours.

Neutrophil chemotactic activity in whey was detected at 14 hours after challenge and was maximal at 16 to 24 hours. TNF was first detected at 12 hours after challenge, whereas the initial increases in IL-1 and IL-6 concentrations were slightly later at 14 hours. IL-8 concentrations increased gradually, beginning perhaps between 12 and 16 hours p.i., and reached maximal levels of 0.1 ng/ml at 36 hours after infection. In contrast, C5a/C5a-des-arg concentrations increased rapidly between 12 and 16 hours, were maximal at 16 hours, and slowly declined over the next 56 hours.

A similar study evaluated concentrations of LTB4 in experimentally induced *Klebsiella pneumoniae* mastitis in cows (Rose et al., 1989). In this model, increases in milk somatic cell counts began within 4 hours of inoculation, whereas the increased LTB4 levels were not detected until 6 hours and were maximal at 8 to 12 hours after infection.

Changes in inflammatory cytokine concentrations were measured in experimental LPS-induced mastitis in sheep (Persson-Waller et al., 1997a). In this study, the initial increase of neutrophil numbers in milk occurred between 1 and 2 hours after challenge. The concentrations of IL-8 and TNF-α were elevated at 1 hour and peaked at 2-3 hours after challenge, but detection of IL-1β and IFN-γ was inconsistent. Similar changes were observed in a related study of cytokines in sheep milk following infection with *Staphylococcus aureus* or *E. coli*, although data were not available prior to 4 hours post-infection (Persson-Waller et al., 1997b).

These four investigations provide data to compare the timing of IL-8 expression and the onset of neutrophil infiltration. In general, these studies indicate that IL-8 and C5a are expressed slightly before or at the time of the initial influx of

neutrophils, whereas LTB4 is detected only in the later stages. However, the frequency of sampling in these experiments is not sufficient to prove definitively that the expression of IL-8 and C5a preceeds the onset of neutrophil infiltration.

Two studies have attempted to evaluate the importance of specific neutrophil chemoattractants in cattle by neutralizing these mediators in disease models. In bovine cutaneous tissue chambers inoculated with *P.haemolytica*, increases in LTB4 concentrations were related temporally to neutrophil influx, and both were inhibited by local dexamethasone treatment (Clarke et al., 1994). However, other neutrophil chemoattractants were not evaluated in this study and dexamethasone undoubtedly has anti-inflammatory effects other than LTB4 inhibition. For example, dexamethasone inhibits LPS-induced expression of IL-8 mRNA in porcine alveolar macrophages (Lin et al., 1994) and TNF-induced IL-8 expression in fibroblasts (Tobler et al., 1992).

The effect of specific neutralization of leukotrienes, PAF and IL-1β on neutrophil exudation in cows was studied using a teat infusion model (Persson-Waller, 1997). Treatment with the leukotriene biosynthesis inhibitor MK886 reduced LPS-induced neutrophil accumulation by 23% to 36% at 7 hours after challenge, despite the fact that LTB4 infusion did not induce neutrophil infiltration. MK886 had no effect on neutrophil accumulation at 3.5 hours after challenge. Treatment with the PAF receptor antagonist WEB 2086 reduced LPS-induced neutrophil accumulation by 23% to 30%, which was significant at both 3.5 and 7 hours after stimulation. Local treatment with anti-IL-1 antibody reduced neutrophil accumulation by up to 33%, but this effect was inconsistent and dependent on time

and LPS dose. The results presented in that paper indicate that the role of a specific mediator in recruiting neutrophils to sites of inflammation may vary with the severity of the inflammatory stimulus and the stage of the disease process. For example, PAF may recruit neutrophils to inflamed tissue at both early and late time-points after LPS challenge, whereas LTB4 may be secreted by activated inflammatory cells, including neutrophils, and augment recruitment of neutrophils late in the inflammatory process.

The results of these studies of mastitis in ruminants imply that many mediators of neutrophil chemotaxis are present after neutrophils have infiltrated the tissue, and that these could act in an additive or synergistic manner to induce the ongoing recruitment of these cells to the site of inflammation. In contrast, the importance of specific mediators in effecting the earliest influx of neutrophils is more difficult to assess. LTB4 is apparently not important in the initial recruitment of neutrophils to the infected mammary gland, because it is not detectable prior to neutrophil infiltration. The study of Shuster et al. (1997) described above suggests that C5a may be more important in inducing early neutrophil infiltration than is IL-8; however, a role for paracrine effects of relatively low local concentrations of IL-8 cannot be excluded because the increase in IL-8 concentrations were gradual and the standard deviation of these measurements was large. The studies of Persson-Waller et al. described above indicate that, for LPS-induced mastitis in sheep, IL-8 concentrations in milk increase prior to the influx of neutrophils and could be responsible for early recruitment of these cells. Thus, the specific role of IL-8 in ruminants has not yet been definitively assessed, but reports indicate that this chemokine could be an important mediator of the early mustering of neutrophils to

sites of bacterial infection, and that IL-8 is one of several products produced by neutrophils themselves that amplify recruitment of these cells in the later stages of disease.

Four concerns in these studies are: (1) cytokines could be present locally in biologically significant concentrations, yet their presence may not be sufficiently widespread to allow detection in milk, (2) the biologic activity of these mediators was not assessed, although this activity could be influenced by the presence of inhibitors, (3) in the late phases of disease, it cannot be determined whether the presence of these mediators is a cause or an effect of neutrophil infiltration and activation, because neutrophils themselves may secrete most of these chemicals, and (4) the role of the recently described chemokines ENA and GRO, and potentially other undiscovered neutrophil chemoattractants, was not assessed in these studies.

1.6. Conclusion

The studies reviewed in this chapter have demonstrated that, although the bactericidal effects of neutrophils are beneficial in bacterial infections, neutrophil-derived products contribute substantially to tissue injury and severe disease in bovine pneumonic pasteurellosis. Consequently, neutrophil recruitment to sites of pulmonary infection with *P. haemolytica* is critical in the development of this disease, and neutralization of the factors that mediate this neutrophil chemotaxis might be beneficial in preventing fatal or chronic relapsing disease. Interleukin-8 is an important neutrophil chemoattractant in humans and in rabbits, and preliminary

evidence suggests a similar role in ruminants. On this basis, the objective of this project was to evaluate the role of IL-8 as a neutrophil chemoattractant and mediator of severe disease in pneumonic pasteurellosis of ruminants.

This role of IL-8 was investigated using three approaches, which are presented in the following chapters. Chapter Two demonstrates that IL-8 is expressed in the lesions of bovine pneumonic pasteurellosis, illustrating a potential role for this chemokine in the pathogenesis of pneumonia. Chapter Three confirms the ability of bovine IL-8 to act as a neutrophil chemoattractant *in vitro* and *in vivo*. Chapters Four and Five investigate the importance of IL-8 as a neutrophil chemoattractant in pneumonic pasteurellosis of cattle and sheep, by measuring the effect of *in vivo* neutralization of IL-8 on the development of the disease, and by gauging the contribution of IL-8 to the *in vitro* neutrophil chemotactic activity of pneumonic lung fluid, respectively.

2.0. EXPRESSION OF THE NEUTROPHIL CHEMOATTRACTANT INTERLEUKIN-8 IN THE LESIONS OF BOVINE PNEUMONIC PASTEURELLOSIS.

2.1. Introduction

Pneumonia caused by *Pasteurella haemolytica* is a common and economically important disease of feedlot cattle. Following impairment of respiratory defences by viral infections and by the stresses of handling, shipping, and environmental changes, *P. haemolytica* can colonize the lower respiratory tract in large numbers and induce severe fibrinopurulent bronchopneumonia (Yates, 1982; Whitely et al., 1992).

Neutrophils have been implicated in the tissue injury observed in pneumonic pasteurellosis. Neutrophil-depleted calves develop comparatively mild clinical signs and lesions following challenge with *P. haemolytica* (Slocombe et al., 1985; Weiss et al., 1991). Bovine neutrophils are capable of phagocytosing and killing opsonized *P. haemolytica* (Berggren et al., 1981). However, during the logarithmic phase of growth, *P. haemolytica* secretes a leukotoxin which stimulates bovine neutrophils at low concentrations but induces functional impairment and has cytotoxic effects on these cells at higher concentrations (Czuprynski et al., 1991; Maheswaran et al., 1992). As a result, the lung is deprived of the beneficial effects of neutrophil

infiltration and activation, and also suffers the injurious effects of products secreted by activated neutrophils.

Several neutrophil chemoattractants have been described in cattle. The complement fragment C5a and leukotriene B4 are effective *in vitro* chemoattractants for bovine neutrophils (Persson et al., 1993), as are interleukin-8 (Hassfurther et al., 1994; Morsey et al., 1996) and several other α-chemokines including epithelial-derived neutrophil-activating protein (Allmann-Iselin et al., 1994), melanoma growth stimulatory activity (GROα) (Rogivue et al., 1995), and granulocyte chemotactic protein-2 (Proost et al., 1993). The relative importance of these neutrophil chemoattractants in cattle is not known. Interleukin-8 is an important neutrophil chemoattractant in pneumonia and empyema in humans (Broaddus et al., 1992; Standiford et al., 1996), and it has been shown in rabbits that neutralization of IL-8 abrogates neutrophil influx in lipopolysaccharide-induced pleuritis, endotoxic shock, and ischemic lung injury (Sekido et al., 1993; Broaddus et al., 1994; Matsumisha et al., 1997).

The objective of this study was to evaluate the expression of IL-8 messenger RNA (mRNA) and protein in pneumonia of cattle caused by *P. haemolytica* and bovine herpesvirus-1. Northern analysis and *in situ* hybridization were employed to assess mRNA expression and localization in tissues, and an enzyme-linked immunosorbent assay was used to measure IL-8 protein levels. Intradermal testing using lung extracts was performed to confirm the relevance of this IL-8 expression to neutrophil responses in cattle. In addition, IL-8 expression was compared in pneumonic pasteurellosis and in pneumonia caused by bovine respiratory syncytial

virus, because the latter disease characteristically lacks the intense neutrophil infiltration typical of pneumonic pasteurellosis.

2.2. Materials and Methods

2.2.1. Lung tissue

Lung tissue from these calves with pneumonic pasteurellosis was kindly provided by the Veterinary Infectious Disease Organization, Saskatoon, Saskatchewan, Canada (Godson et al., 1996). In brief, 6-10 month old beef calves with low antibody titres to *P. haemolytica* and bovine herpesvirus-1 (BHV-1) were infected by intranasal aerosol with 7.0×10^7 pfu bovine herpesvirus-1 strain 108, and challenged four days later with 10^9 cfu of aerosolized *P. haemolytica* A1. The calves became severely ill and were euthanized two to four days following bacterial challenge. These calves had severe cranioventral fibrinopurulent necrotizing lobar pneumonia and fibrinous pleuritis affecting 30-50% of the lung, typical of pneumonic pasteurellosis. Neutrophils predominated in the alveolar exudate. Grossly and histologically normal lung was obtained at necropsy from six market cattle at slaughter.

Lung tissue from eight calves infected with bovine respiratory syncytial virus (BRSV) was generously provided by Drs. Keith West and John Ellis, Department of Veterinary Microbiology, University of Saskatchewan. The calves were challenged by aerosol with bronchoalveolar lavage fluid obtained from a calf experimentally infected with a field isolate of BRSV, which contained 5×10^5 pfu BRSV. All calves became pyrexic and developed moderate to severe dyspnea. Three calves were

euthanized for humane reasons 5 days post-infection (p.i.), and five calves were euthanized on day 8 p.i. At necropsy, all calves had moderate to severe lesions including cranioventral atelectasis, generalized pulmonary edema, and dorsocaudal interlobular edema with bullous or alveolar emphysema. Histologically, at 5 days p.i., there was extensive necrotizing bronchointerstitial pneumonia with numerous syncytial cells and few intracytoplasmic inclusions. Bronchioles and alveoli contained low numbers of neutrophils. At day 8 p.i., hyperplasia of bronchiolar epithelium and type II pneumocytes was more prominent than bronchiolar necrosis. A full characterization of this technique of reproducing BRSV pneumonia will be published elsewhere by Drs. West and Ellis.

2.2.2. Bronchoalveolar lavage fluid and lung extracts

Bronchoalveolar lavage (BAL) fluid samples were obtained at necropsy from lesional cranioventral and non-lesional dorsocaudal lung in eight cattle with pneumonic pasteurellosis, from lesional cranioventral lung in eight cases of pneumonia caused by BRSV, and from normal cranioventral lung in six normal cattle. Sixty millilitres of PBS was infused into a large bronchus, and 20 to 35 ml of fluid was recovered by gentle suction. The amount of fluid recovered was usually greater from non-lesional than from lesional lung. Extracts of non-lavaged lung tissue from these same animals were prepared by homogenizing 1.2 g of lung tissue per ml PBS containing 0.05% Tween-20 (PBST), and recovering the supernatant following centrifugation for 10 minutes at 15000 g.

2.2.3. Northern blots

Steady state levels of IL-8 mRNA were assessed in lesional cranioventral and non-lesional dorsocaudal lung tissue from 11 cattle with pneumonic pasteurellosis, in lesional lung tissue from eight cattle with pneumonia caused by BRSV, and in lung tissue from two normal animals. All solutions were treated with 0.1% diethylpyrocarbonate (DEPC). Lung samples were homogenized in 5.5 M guanidinium isothiocyanate, 25 mM sodium citrate, and 0.5% sarcosine, and stored at -80°C. Particulate matter was cleared from the lysates by centrifugation at 42000 rpm in a Sorvall SW55Ti rotor for 30 minutes, and total cellular RNA was purified by ultracentrifugation of the supernatants over 5.5 M cesium chloride and 25 mM sodium acetate at 42000 rpm for 20 hours. The RNA pellets were resuspended in water and the RNA concentrations were determined by absorbance at 260 nm. For each sample, 20 μg of total cellular RNA was separated by electrophoresis in a gel containing 1.2% agarose, 6.5% formaldehyde, 40 mM MOPS (morpholinopropane sulfonic acid), 10 mM sodium acetate and 1 mM EDTA. RNA was blotted to nylon membranes using 10 × saline sodium citrate (SSC) and cross-linked using ultraviolet irradiation. The levels of RNA in each lane were confirmed by staining with methylene blue and by probing the blots for γ-actin (Gordon and Galli, 1990).

Bovine interleukin-8 (IL-8) cDNA was a generous gift of the Veterinary Infectious Disease Organization (Morsey et al., 1996). The IL-8/Bluescript KSII$^+$ construct was digested with HincII to generate a 640 base pair (bp) IL-8 cDNA

fragment, and the cDNA was purified by gel electrophoresis. Human γ-actin cDNA was generated from a plasmid provided by Drs. B. Murphy and V. Misra. ^{32}P-labelled cDNA probes specific for bovine IL-8 and γ-actin were prepared using a commercial kit (Oligolabelling kit, Pharmacia Biotech, Baie D'Urfé, PQ, Canada). The Northern blots were blocked with 0.1 × SSC / 0.5% sodium dodecyl sulfate (SDS) at 65°C for one hour. All subsequent procedures were performed at 42°C. The blots were prehybridized for 4 hours with 50% formamide, 12.5 µg/ml salmon sperm DNA, 0.1 M HEPES, 0.5 M NaCl, 5 mM EDTA pH 8.0, 5 × Denhardt's solution, 1% SDS and 0.5% sodium pyrophosphate. The ^{32}P-labelled cDNA probe was added at 1 x 10^6 cpm/ml, and allowed to hybridize overnight in the same solution. The post-hybridization treatments included two low stringency washes for 5 minutes in 2 × SSC / 0.1% SDS, and two high stringency washes for 30 minutes in 0.2 × SSC / 0.1% SDS. The membranes were exposed to Kodak X-omat AR autoradiography film using two Cronex Lighting plus intensifying screens for one to five days at -80°C (Gordon and Galli, 1990). Densitometry was performed using an image analyser (Image-1, Universal Imaging Co., W. Chester, PA), the signal densities as a percentage of background were calculated, and the ratios of IL-8 to γ-actin signal densities were calculated.

2.2.4. *In situ* hybridization

The right middle lung lobes from 5 calves with pneumonic pasteurellosis were perfused with fresh cold 4% paraformaldehyde in phosphate-buffered saline

(PBS) and fixed for 4 hours, and then post-fixed and/or held at -20°C in 70% ethanol until being embedded in paraffin using routine methods. Sense and anti-sense cRNA probes were transcribed (Riboprobe system, Promega, Madison, WI) from the appropriately linearized bovine IL-8/Bluescript KSII$^+$ construct in the presence of ^{35}S-UTP, and the cRNA probes were purified using scintered glass (RNAid, Bio101, Vista, CA). Five micron thick sections were deparaffinized and rehydrated, hydrolysed with 0.2 M HCl for 20 minutes, digested in 20 mg/ml proteinase K for 15 minutes, post-fixed in 4% paraformaldehyde at 4°C for 20 minutes, acetylated with 0.5% acetic anhydride in 0.1 M triethanolamine, and equilibrated to 2 × SSC. The sections were covered in 45 µl of hybridization buffer (50% formamide, 10% dextran sulfate, 0.3 M sodium chloride, 10 mM Tris hydrochloride pH 7.6, 5 mM EDTA, 10 mM dithiothreitol, and 0.1 mg/ml yeast tRNA) containing 11.25 ng cRNA probe and 1.25 µl non-radioactive thio-UTP and allowed to hybridize in a humidified chamber at 56°C overnight. Following hybridization, non-specifically bound probe was removed by washing twice for 15 minutes each at 56°C in 50% formamide/2 × SSC with 10 mM 2-mercaptoethanol (ME), followed by digestion at 37°C with 20 mg/ml ribonuclease A, and two final washes for 30 minutes each at 56°C in 50% formamide/2 × SSC with 10 mM 2-ME. The sections were overlaid with photographic emulsion (Kodak NBT-2), dried, and the slides were exposed for four to ten days, developed and fixed, and counterstained with toluidine blue (Gordon and Galli 1994).

2.2.5. Enzyme-linked immunosorbent assay (ELISA)

Mouse monoclonal antibody 8M6 and rabbit polyclonal antiserum specific for ovine IL-8, and recombinant ovine IL-8 were generously donated by Dr. Paul Wood, CSIRO-Division of Animal Health, Parkville, Victoria, Australia. Samples were assayed in triplicate at 10-fold dilutions from 1:10 to 1:10,000. The ELISA plates were washed four times with PBST after each step unless otherwise indicated, and all samples and antibodies were diluted in PBST with 1% sodium casein. Immulon-4 96-well plates (Dynatech, Chantilly, VA) were coated overnight with 0.2 μg/ml monoclonal anti-ovine IL-8 antibody in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, 3 mM sodium azide, without casein, pH 9.5). Non-specific protein binding sites were blocked for 4 hours with PBST containing 0.1% casein, then 100 μl of sample was applied without washing and incubated overnight. Polyclonal anti-ovine IL-8 serum was applied at a 1:8000 dilution, followed by horseradish peroxidase-labelled polyclonal anti-rabbit immunoglobulin (Zymed, South San Francisco, CA) at 1:2000, and detected with tetramethylbenzidine substrate (Sigma-Aldrich, Mississauga, ON, Canada). After 30 minutes, the reaction was stopped with 0.5 M sulfuric acid and absorbances were measured at 450 nm. A standard curve was constructed using threefold dilutions of ovine IL-8, and the dilution of each sample with absorbances in the linear portion of the standard curve was used to calculate the IL-8 concentration.

2.2.6. *In vivo* assessment of chemotactic activity

The *in vivo* neutrophil chemotactic activity of pneumonic lung extract was

evaluated by intradermal skin testing, as was the effect of depleting IL-8 from this extract. Lung extract, prepared as described above from lesional lung infected with BHV-1 and *P. haemolytica*, was depleted of IL-8 by the addition of 0.01 volumes of rabbit anti-IL-8 polyclonal serum. The sample was incubated for 60 minutes at 4°C, then 0.08 volumes of Sepharose CL4B beads coated with protein A (Sigma-Aldrich) were added to adsorb the IL-8/antibody complexes and, after a 30 minute incubation at 4°C, these complexes were removed by centrifugation. A parallel sample of lung extract was treated with normal rabbit serum in place of the IL-8 antiserum. The residual IL-8 concentrations in lesional lung extract treated with normal serum or with IL-8 antiserum were 123 or 13 ng/ml, respectively. Untreated non-lesional lung extract contained 8.7 ng/ml IL-8.

Two hundred microliters of lesional lung extract treated with IL-8 antiserum or normal rabbit serum, non-lesional lung extract, or PBST alone were injected intradermally into a normal calf, and the sites were biopsied 6 hours later. The samples were fixed in formalin, embedded in paraffin, and processed routinely for histopathology. Extravascular neutrophils were counted in 300 μm horizontal bands in each biopsy at three levels: the superficial dermis, immediately subjacent to the epidermis; the mid-dermis, immediately below and not including the apocrine glands or follicular vascular plexus; and the deep dermis, midway between the hair bulb and the panniculus. Counting was performed in duplicate by two people, without knowledge of the treatment status of the sample.

2.2.7. Data Analysis

Comparisons between lesional and non-lesional samples were performed using the paired t-test. Multiple groups were compared with a one-way analysis of variance (ANOVA) procedure, using logarithmically-transformed data when variances between groups were not equal. When significant differences between groups were detected by ANOVA, individual groups were compared with Tukey's multiple comparison test. Values were expressed as the mean ± standard error of the mean.

2.3. Results

2.3.1. Northern analysis of IL-8 expression

In six of seven cases of pneumonic pasteurellosis, there was high level expression of interleukin-8 (IL-8) mRNA in lesional cranioventral lung, but minimal expression in non-lesional dorsocaudal lung samples taken from the same calves (Figure 2.1). The Northern blots were analysed by densitometry, and the densities of IL-8 mRNA signal relative to that of γ-actin were calculated to control for variation in the amount of total RNA loaded in each lane. The overall ratio of IL-8 to γ-actin mRNA density was 2.37 ± 0.14 for lesional lung and 0.39 ± 0.06 for non-lesional lung. This difference was highly significant ($p<0.001$).

Steady-state levels of interleukin-8 mRNA were compared in the lesions of bacterial pneumonia, viral pneumonia, and in normal lung (Figure 2.2). In four other

calves with pneumonia caused by *P. haemolytica* and bovine herpesvirus-1 (BHV-1), the ratio of IL-8 to actin mRNA density was 5.46 ± 1.96. In comparison, the ratio of

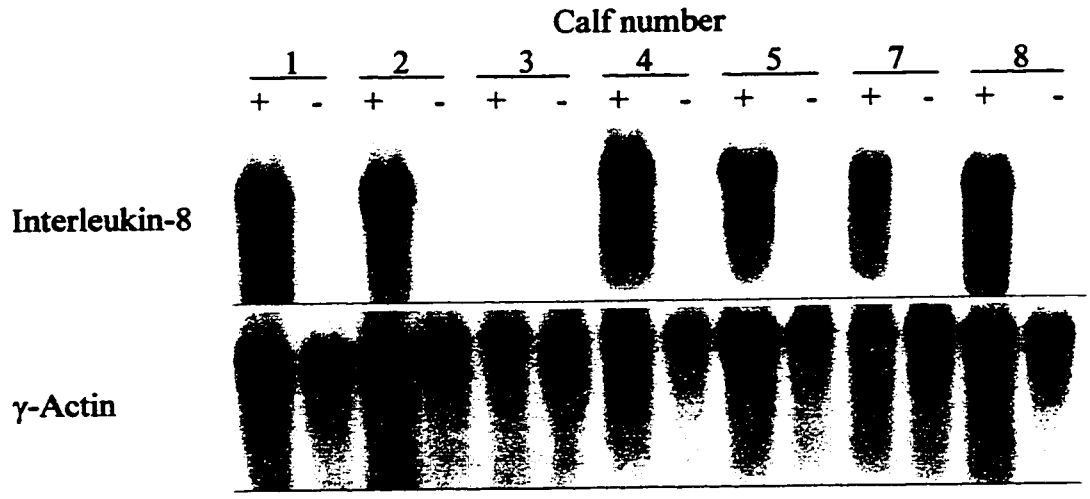

Figure 2.1. Expression of IL-8 mRNA in lungs from seven calves with pneumonic pasteurellosis. Northern blot comparing lesional (+) and non-lesional (-) lung from each calf when probed for IL-8 or γ-actin.

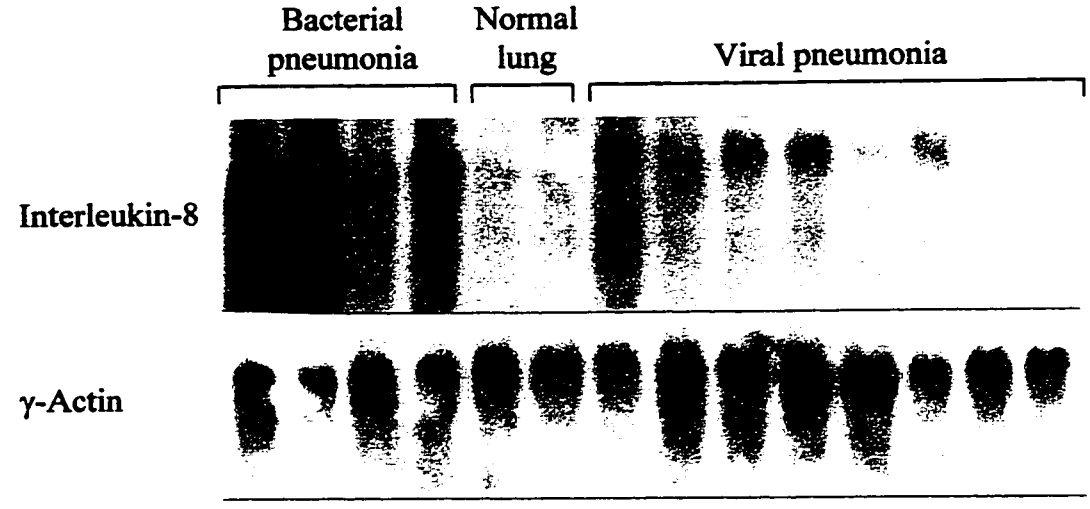

Figure 2.2. Comparison of IL-8 mRNA expression in the lesions of bacterial pneumonia caused by *Pasteurella haemolytica* and BHV-1, viral pneumonia caused by BRSV, and normal lung. Northern blot, probed for IL-8 or γ-actin.

IL-8 to actin was 0.95 ± 0.34 in calves with experimentally induced bovine respiratory syncytial virus (BRSV) pneumonia, and 0.94 ± 0.55 in normal lung.

2.3.2. *In situ* hybridization

The location of tissue IL-8 mRNA expression was examined using *in situ* hybridization in five cases of pneumonia caused by BHV-1 and *P. haemolytica.* Positive labelling was observed in three of the five cases evaluated. In these, IL-8 mRNA expression was most intense in the cellular exudate within alveoli (Figures 2.3A and 2.3B), and most areas with alveolar cellular exudate demonstrated IL-8 mRNA expression. The sense (negative control) IL-8 riboprobes did not hybridize significantly with any of the tissue sections (Figures 2.3C and 2.3D). While identification of the cell types expressing IL-8 mRNA was difficult in the *in situ* hybridization preparations, comparison of adjacent sections stained with hematoxylin & eosin confirmed that expression of IL-8 mRNA in the *in* situ hybridization sections corresponded to both neutrophils and macrophages. Positive staining was also prominent in "oat cells," the clustered leukocytes with streaming nuclear chromatin that are a characteristic histologic feature of pneumonia caused by *P. haemolytica.*

Interleukin-8 mRNA expression was detected at lower intensity in the epithelium of approximately 25% of bronchioles (Figure 2.4). In these areas, neutrophils often were present in the bronchiolar epithelium and in the bronchiolar lumens. Interleukin-8 mRNA was not detected in large bronchioles or bronchi.

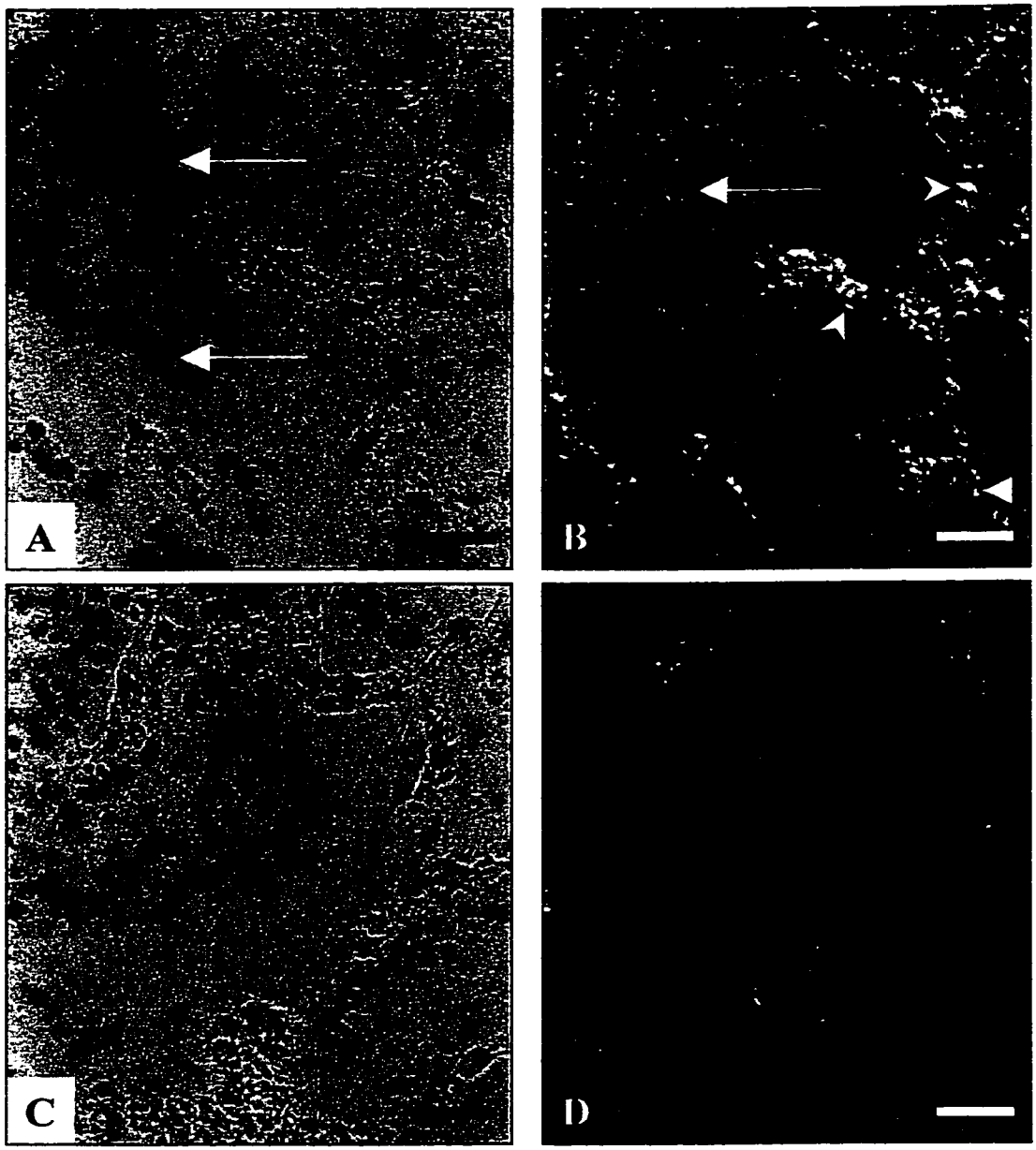

Figure 2.3. Lung; calf. Interleukin-8 (IL-8) mRNA is expressed by neutrophils and macrophages forming the alveolar exudate in the lesions of bovine pneumonic pasteurellosis. Serial sections, in situ hybridization, toluidine blue counterstain. Bar = 80 μm.**Fig. 2.3a.** Antisense probe, brightfield. Black silver grains over the alveolar cellular exudate (arrows) indicate expression of IL-8 mRNA. **Fig 2.3b.** Antisense probe, darkfield, same field as 2.3a. Illumination of the alveolar cellular exudate (arrow) indicates expression of IL-8 mRNA. Erythrocytes in the alveolar septa are nonspecifically illuminated (arrowheads). **Fig 2.3c.** Sense probe (negative control), brightfield. Note the absence of silver grains over the alveolar cellular exudate. **Fig 2.3d.** Sense probe (negative control), darkfield, same field as 2.3c. The alveolar cellular exudate is not illuminated.

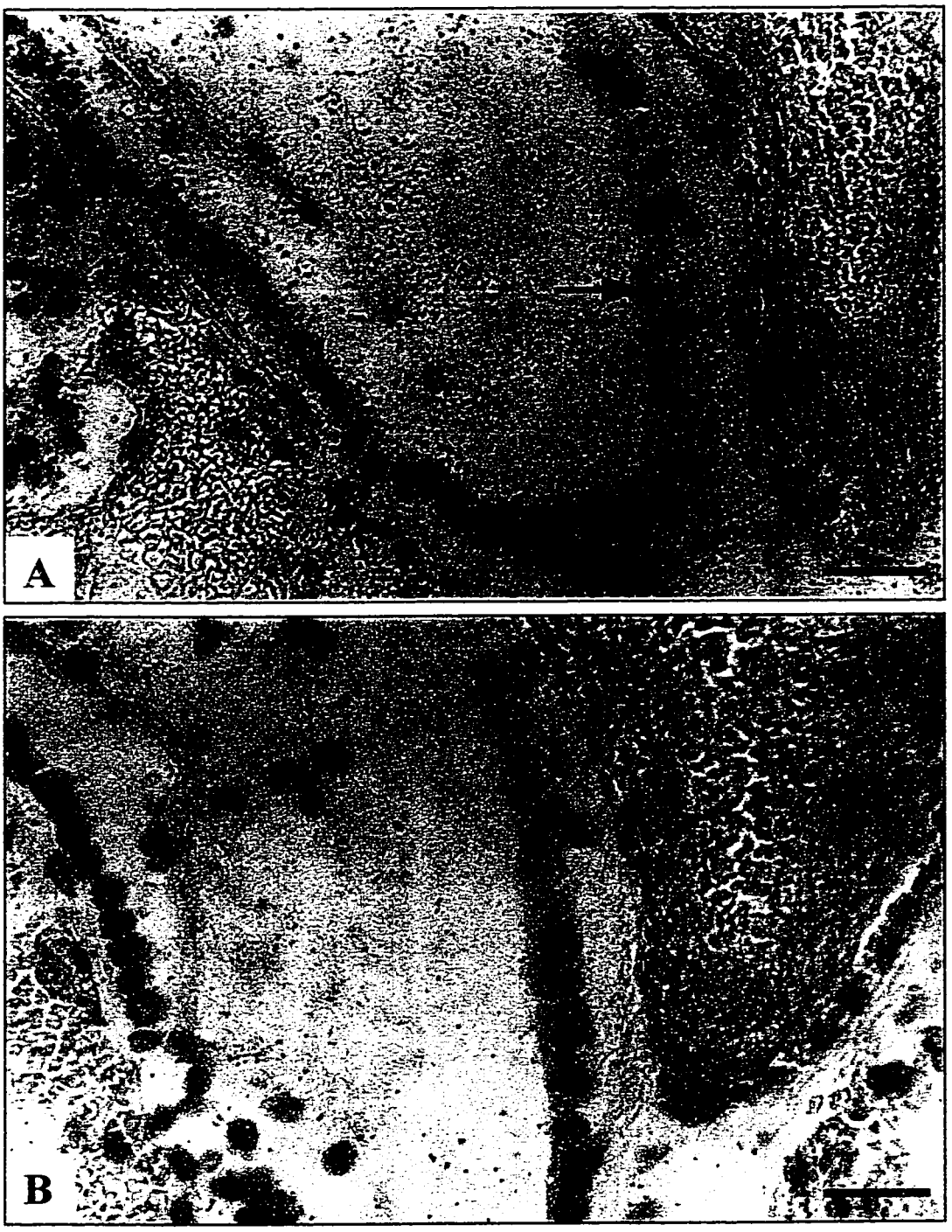

Figure 2.4. Lung, calf. Bronchiolar epithelial cells express IL-8 mRNA in the lesions of bovine pneumonic pasteurellosis. Serial sections, in situ hybridization, toluidine blue counterstain. Bar = 80μm. **Fig. 2.4a.** Antisense probe. The presence of black silver grains covering the bronchiolar epithelium (arrow) indicates expression of IL-8 mRNA. **Fig. 2.4b.** Sense probe (negative control), same field as 2.4a.

Alveolar epithelial cells expressed IL-8 mRNA, but this was unequivocal in fewer than 10% of alveoli and the signals were always less intense than in the alveolar exudate. Interleukin-8 mRNA expression was also prominent in scattered interstitial macrophages and fibroblasts of the interlobular septa and the pleura, and in pleural mesothelial cells.

2.3.3. ELISA

Interleukin-8 was measured in bronchoalveolar lavage fluid samples from 8 calves with pneumonia caused by *P. haemolytica* and BHV-1. Interleukin-8 concentrations in BAL fluid from lesional and non-lesional areas of lung were 16.06 $\pm$ 4.00 and 0.34 $\pm$ 0.11 ng/ml, respectively. In comparison, BAL fluid from the lesions of pneumonia caused by BRSV contained only 0.36 $\pm$ 0.09 ng/ml IL-8, while BAL fluid from normal cattle contained 0.01 $\pm$ 0.002 ng/ml IL-8 (Figure 2.5).

Interleukin-8 was also quantified in extracts of homogenized lung to measure the levels in tissue and to control for potential differences in the amount of epithelial lining fluid recovered by bronchoalveolar lavage. Lesional and non-lesional lung extracts from cases of pneumonia caused by *P. haemolytica* contained 76.91 $\pm$ 15.86 and 7.68 $\pm$ 1.15 ng IL-8 per gram of tissue, respectively, whereas lesional BRSV-infected and normal lung extracts contained 5.55 $\pm$ 1.72 and 0.19 $\pm$ 0.09 ng/g IL-8, respectively (Figure 2.6).

For both BAL fluid and lung extracts, IL-8 concentrations were significantly

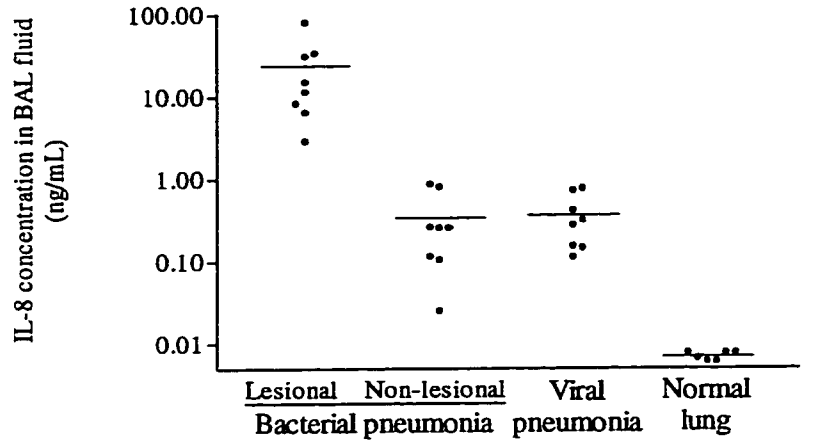

Figure 2.5. IL-8 concentrations in BAL fluid collected from lesional and non-lesional lung tissue from calves infected with *P. haemolytica* and BHV-1 (bacterial pneumonia), from the lesions of BRSV pneumonia (viral pneumonia), and from normal lung.

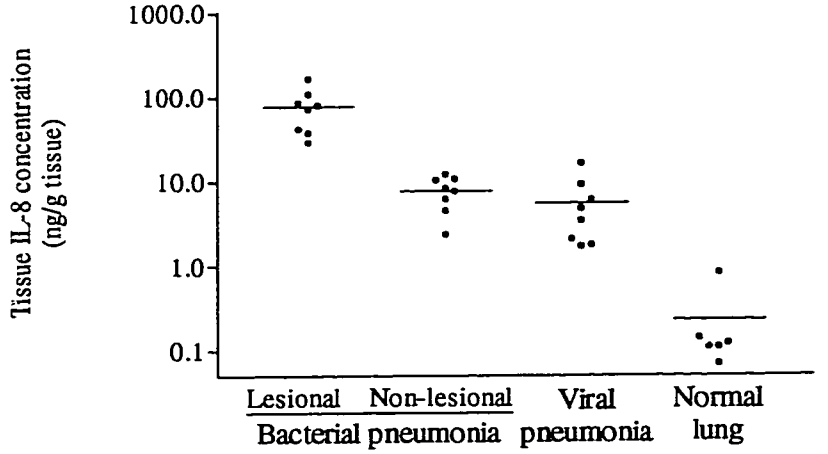

Figure 2.6. IL-8 concentrations in lung extracts prepared from lesional and non-lesional lung tissue from calves infected with P. haemolytica and BHV-1 (bacterial pneumonia), from the lesions of BRSV pneumonia (viral pneumonia), and from normal lung.

greater in lesions of pneumonic pasteurellosis than in other samples ($p<0.001$). IL-8 concentrations in BRSV-infected lung and in non-lesional lung from calves with pneumonic pasteurellosis were not significantly different, for either BAL fluid or lung extracts, but both were significantly greater than that in normal samples ($p<0.001$).

2.3.4. Neutralization of *in vivo* chemotactic activity

The ability of pneumonic lung extract to attract neutrophils *in vivo* was evaluated, and the contribution of IL-8 to this *in vivo* neutrophil chemotactic activity was estimated by depleting 89% of the IL-8 from the sample using IL-8 antiserum and Sepharose beads coated with protein A. This IL-8-depleted extract was injected intradermally into a normal calf, and extravascular dermal neutrophils were counted 6 hours later.

The IL-8-containing extract of pneumonic lung, which had been treated with normal rabbit serum and protein A-coated Sepharose beads, recruited on average 336.4 neutrophils/mm^2 to the site of intradermal injection (Figure 2.7). Adsorption of the lung extract with anti-IL-8 antibodies reduced the *in vivo* neutrophil chemotactic activity by 60.6 ± 4.8 percent, to 126.8 neutrophils/mm^2. This reduction was highly significant ($p<0.0001$) and was consistent at all sites in the dermis (Figure 2.7).

2.4. Discussion

Considerable recent research activity has demonstrated that interleukin-8 (IL-

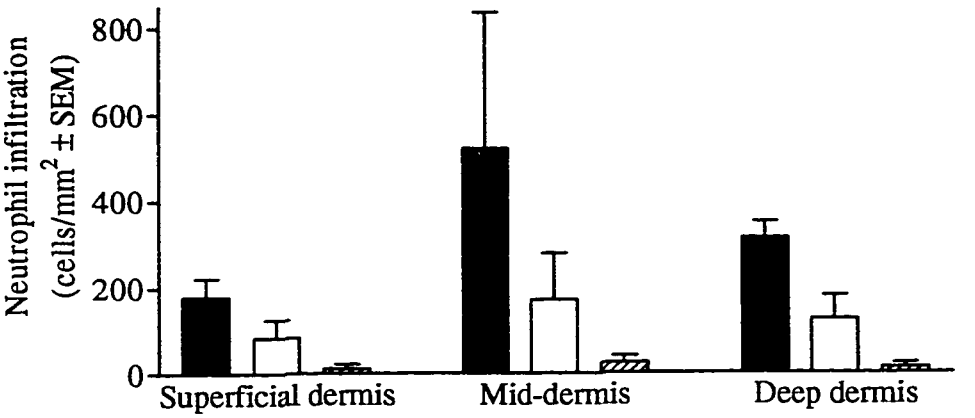

Figure 2.7. The effect of IL-8 depletion on the in vivo neutrophil chemotactic activity of pneumonic lung extracts. The neutrophil chemotactic activity was compared in pneumonic lung extract containing IL-8 (black bars), pneumonic lung extract depleted of IL-8 (white bars), and extract of nonlesional lung (cross-hatched bars). Depletion of IL-8 reduced the in vivo neutrophil chemotactic activity by 60.6 ± 4.8% (mean ± standard error).

8), a member of the α-chemokine family of small molecular weight chemotactic cytokines, is an essential mediator of neutrophil chemotaxis in inflammatory processes in rabbits and humans. In this study, we have demonstrated intense and consistent expression of IL-8 mRNA and protein in acute lesions of bovine pneumonia caused by bovine herpesvirus-1 and *P. haemolytica*. In contrast, IL-8 expression was minimal in non-lesional lung from the same calves, in lesions of BRSV pneumonia, and at background levels in normal lung. Furthermore, this IL-8 was biologically active in intradermal tests in cattle, suggesting that IL-8 would be an important neutrophil chemoattractant in the lungs of cattle with pneumonic pasteurellosis.

In all cases of pneumonic pasteurellosis, there were dramatic increases in IL-8 concentrations in lesional bronchoalveolar lavage (BAL) fluid. Interleukin-8 concentrations in BAL fluid from such lesions were, on average, 46 times higher than in non-lesional BAL fluid from the same calves and 1500 times higher than in BAL fluid from analogous sites in normal cattle. Differences in the amount of BAL fluid recovered from lesional and non-lesional lung were not great enough to account for this difference. To confirm this, IL-8 concentrations in standardized extracts of homogenized lung tissue were measured, and lesional lung contained 12-fold and 400-fold higher concentrations of IL-8 compared to non-lesional and normal lung, respectively.

Recombinant ovine IL-8 has been shown to induce significant neutrophil chemotaxis *in vitro* at concentrations of 10^{-9} M (approximately 8.6 ng/ml), and intradermal injection of 10^{-9} moles (approximately 8.6 μg) of recombinant ovine IL-8

effects dramatic neutrophil infiltration (Seow et al., 1994). In our study, the mean IL-8 concentration in BAL fluid from lesions of pneumonic pasteurellosis was 16 ng/ml. Because BAL fluid is diluted markedly compared to bronchoalveolar epithelial lining fluid (Rennard et al., 1986), this concentration of biologically active IL-8 should be more than adequate to induce vigorous neutrophil chemotaxis. Indeed, intradermal injection of extracts of pneumonic lung tissue containing about 25 ng IL-8 induced a dramatic neutrophil infiltrate. These data suggest that interleukin-8 recruits neutrophils to sites of pulmonary infection with *P. haemolytica.*

Because neutrophils are important mediators of tissue injury in pneumonic pasteurellosis (Slocombe et al., 1985; Weiss et al., 1991), these results imply that IL-8 may play an important role in the neutrophil-mediated tissue injury in this disease. We investigated the potential importance of IL-8 as a neutrophil chemoattractant in pneumonic pasteurellosis by quantifying the effect of depleting 89% of the IL-8 from lung extract on the subsequent *in vivo* neutrophil chemotactic activity of the sample. The results indicate that IL-8 was responsible for at least 60% of the *in vivo* neutrophil chemotactic activity of pneumonic lung extract. We are currently extending these preliminary experiments to fully evaluate the *in vitro* and *in vivo* effect of IL-8 neutralization on neutrophil chemotaxis.

Interleukin-8 mRNA expression was most prominent in intra-alveolar neutrophils and macrophages in cases of pneumonic pasteurellosis, but was also detected in bronchiolar and alveolar epithelium, pleural mesothelium, and interstitial macrophages and fibroblasts. Interleukin-8 mRNA was not detected in 2/5 cases by *in situ* hybridization and in 1/7 cases by Northern analysis. While we cannot rule out

the possibility of inconsistent IL-8 expression in these tissues, the dramatic elevation in IL-8 protein in all cases examined suggests that our failure to detect IL-8 mRNA by *in situ* hybridization was caused by low sensitivity or inadequate mRNA preservation rather than a true lack of expression.

The high-level expression of IL-8 mRNA in alveolar macrophages and neutrophils indicates that interleukin-8 may play a role in amplifying neutrophil recruitment to these established sites of inflammation. If IL-8 is an important amplifier of this suppurative response, then neutralization of this chemokine would be a potential method of down-regulating the injurious neutrophil influx during the clinically detectable phase of pneumonic pasteurellosis. In contrast to this putative role in the amplification of ongoing inflammatory responses, assessing the potential for IL-8 to induce the initial recruitment of inflammatory cells to *P. haemolytica*-infected alveoli will require an examination of IL-8 concentrations in the very early lung lesions of pneumonic pasteurellosis, prior to the infiltration of neutrophils. The finding of IL-8 mRNA expression in alveolar macrophages and bronchiolar and alveolar epithelium indicates a potential for IL-8 to mediate this early chemotaxis of inflammatory cells.

We compared IL-8 expression in pneumonic pasteurellosis and pneumonia caused by BRSV, because the latter lacks the severe neutrophil exudation that is typical of pneumonic pasteurellosis. Although dyspnea and pulmonary lesions were severe both in calves with pneumonic pasteurellosis and those with BRSV pneumonia, IL-8 expression was minimal in the calves with viral pneumonia. Thus, the IL-8 upregulation was a characteristic feature of pneumonic pasteurellosis, and

was not merely a non-specific consequence of injury to pulmonary tissue.

These investigations conclusively demonstrate that interleukin-8 mRNA and protein are expressed at high levels in the lesions of bovine pneumonic pasteurellosis, whereas expression is minimal in non-lesional and in normal lung. These results suggest that IL-8 amplifies the recruitment of neutrophils in the clinical stages of pneumonic pasteurellosis, and could attract neutrophils in the early stages after *P. haemolytica* infection. Because products released from activated neutrophils are important mediators of tissue injury in pneumonic pasteurellosis, upregulation of IL-8 may be a key event in the development of the severe clinical signs and lesions of this common and economically important disease.

3.0. PRODUCTION AND FUNCTIONAL CHARACTERIZATION OF RECOMBINANT BOVINE INTERLEUKIN-8 AS A SPECIFIC NEUTROPHIL ACTIVATOR AND CHEMOATTRACTANT.

3.1. Introduction

Neutrophils form a critical component of the host defence against infection, because of their ability to phagocytose and kill opsonized or non-opsonized bacteria, using an array of bactericidal enzymes and oxyradicals. However, these bactericidal agents may also damage host tissue, and neutrophil-dependent injury is an established mechanism in many infectious and non-infectious disease processes (Furie and Randolph, 1995). Interleukin-8 (IL-8), a member of the chemokine family of chemotactic cytokines, activates several steps in the neutrophil effector cascade in humans. These include augmentation of transendothelial migration of neutrophils through shedding of L-selectin from neutrophils, upregulation of neutrophil CD11/CD18 expression and avidity for ICAM-1 (Furie and Randolph, 1995), induction of potent and selective neutrophil chemotaxis, enhancment of the neutrophil oxidative burst induced by formylated bacterial peptides (Elbim et al., 1994), and improved phagocytosis and killing of some bacterial pathogens by neutrophils (Simms and D'Amico, 1997; Standiford et al., 1996).

Bovine IL-8 is a pH-sensitive, heat-labile, 7.8 kDa protein which shares 96,

87 and 76 percent amino acid homology with ovine, porcine and human IL-8, respectively. Bovine IL-8 cDNA is 643 base pairs (bp); the single open reading frame is 303 bp and contains a predicted signal sequence of 66 bp (Hassfurther et al., 1994; Morsey et al., 1996). Although human IL-8 induces chemotaxis of bovine neutrophils *in vitro* (Bochsler et al., 1994), infusion of human IL-8 into the bovine teat cistern reportedly does not induce measurable neutrophil exudation (Persson et al., 1993). Interleukin-8 is expressed at high levels in milk from cows with coliform mastitis (Shuster and Kehrli, 1992) and in the lesions of bovine pneumonic pasteurellosis, where a role for IL-8 as an important neutrophil chemoattractant has been proposed (Caswell et al., 1998).

In this study we describe the production and purification of recombinant bovine IL-8 (rbIL-8). The *in vitro* effects of this protein are characterized using neutrophil chemotaxis and shape change assays, and the dose-dependence and time course of IL-8-induced neutrophil infiltration are evaluated *in vivo*.

3.2. Methods

3.2.1. Materials

Rabbit antiserum to ovine IL-8 and monoclonal IgG2a antibody to ovine IL-8 (clone 8M6) were provided by Dr. Paul Wood, CSIRO Division of Animal Health, Parkville, Victoria, Australia. These antibodies have been used previously to identify bovine IL-8 in an enzyme-linked immunosorbent assay (ELISA) (Caswell et al., 1998). The hybridoma HB121, which secretes an IgG2a antibody to human IgE, was

obtained from the American Type Culture Collection and used to produce a control antibody which was purified from mouse ascites fluid using protein A affinity chromatography (Andrew and Titus, 1997).

3.2.2. Production and purification of bovine interleukin-8

Bovine IL-8 DNA was a generous gift of Dr. Mohammed Morsey and the Veterinary Infectious Disease Organization, Saskatoon, Saskatchewan. This DNA sequence had been amplified using the polymerase chain reaction to remove the signal sequence and introduce BamH1 restriction sites, and had been inserted into the vector pUEX-1 (Morsey et al., 1996). We subcloned the 240 bp bIL-8 DNA fragment into the expression vector pGEX-2T (Pharmacia Biotech, Uppsala, Sweden), so that the vector DNA sequence encoding glutathione-S-transferase (GST) was separated from the bIL-8 insert by a nucleotide sequence encoding a thrombin cleavage site. This bIL-8/pGEX-2T construct was used to transform competent HB101 *E. coli* cells using standard methods (Sambrook et al., 1989). The nucleotide sequence of the bIL-8 DNA insert and of the adjacent vector DNA was determined to confirm the correct orientation and reading frame (Sambrook et al., 1989).

One hundred ml of Luria broth containing 50 μg/ml ampicillin were inoculated with HB101 cells containing the bIL-8/pGEX-2T construct, and incubated for 10 hours at 37°C, then diluted in 1 L of fresh broth and incubated for a further 1 hour. Protein production was stimulated by the addition of 0.5 mM isopropyl-thiogalactopyranoside for 5 hours. The bacterial culture was centrifuged at 4000 g

for 15 minutes at 4°C, and the bacterial pellet was resuspended in 20 ml phosphate-buffered saline (PBS), pH 8.0, containing 0.1 mg/ml lysozyme, and then incubated on ice for 30 minutes. The bacteria were centrifuged as above, resuspended in PBS containing 1% Triton X100, 2 μg/ml aprotonin, 2 μg/ml leupeptin and 2 mM PMSF, and sonicated on ice using a Vibracell sonicator (Sonics and Materials Inc., Danbury, CT) for 5 minutes at a 10% duty cycle. The lysate was centrifuged at 27000 g for 15 minutes, and the supernatant containing the rbIL-8/GST fusion protein was passed through a 0.2 μm filter.

The fusion protein was purified using affinity chromatography by applying the lysis supernatant to a glutathione-Sepharose column (Sigma-Aldrich Canada, Oakville, ON, Canada), and washing the bound protein with 3 volumes each of PBS / 1% Triton-X100 and 150mM NaCl / 50 mM Tris-HCl / 5 mM $CaCl_2$ (NaCl/Tris/$CaCl_2$). Recombinant bovine IL-8 was then cleaved from the GST by incubation of the affinity matrix with NaCl/Tris/$CaCl_2$ containing 25 ug/ml thrombin for 10 hours at 37°C. A second eluate of rbIL-8 was collected by washing the column with one volume of NaCl/Tris/$CaCl_2$. Thrombin was removed from the eluates using benzamidine-Sepharose affinity chromatography (Pharmacia Biotech, Uppsala, Sweden), and the resulting rbIL-8 was dialyzed against PBS and stored at -80°C. Recombinant GST was eluted from the glutathione-Sepharose column by washing with 2 mg/ml glutathione.

3.2.3. Protein analysis

The concentration of protein in the rbIL-8 preparation was determined using the Bio-rad protein assay (Bio-Rad Laboratories, Mississauga, ON, Canada). Ten-fold dilutions of sample were diluted in PBS, mixed with 4 volumes of the diluted Bradford reagent, incubated for 20 minutes at 20°C, and the absorbance of each was read at 595 nm. A standard curve was generated using three-fold dilutions of bovine serum albumin, and the protein concentration in the sample was determined using the absorbance which fell within the linear portion of the standard curve.

The following samples were analysed by SDS-PAGE and Western analysis: rbIL-8/GST fusion protein bound to glutathione-Sepharose beads, the first and second eluates containing rbIL-8, and the recombinant GST following elution with glutathione. Samples were prepared by boiling with 0.2 M 2-mercaptoethanol and 45 mM sodium dodecyl sulfate, loaded on duplicate 15% polyacrylamide gels and separated by electrophoresis. One gel was stained with Coomassie brilliant blue (Sigma-Aldrich) and a Western blot was prepared by wet transfer from the second gel to a nitrocellulose membrane (Sambrook et al., 1989).

All subsequent procedures were carried out at room temperature. The Western blot was blocked for 4 hours in 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST), washed twice in PBS containing 0.05% Tween-20 (PBST), and incubated for 1 hour with a 1:2000 dilution of rabbit antiserum to ovine IL-8 in PBST containing 1% sodium casein. After washing the blot twice in PBST and once in PBST with 10% normal goat serum, a 1:2500 dilution of

biotinylated goat antiserum to rabbit immunoglobulins (Dako Corp., Carpinteria, CA) in PBST containing 1% casein was incubated with the blot for 1 hour. The Western blot was then washed three times in TBST, the streptavidin-alkaline phosphatase conjugate (Gibco-BRL Life Technologies, Burlington, ON, Canada) was applied at 1:5000 dilution in TBST for 1 hour, and after washing three times with water, the BCIP/NBT substrate was applied as directed (Gibco-BRL Life Technologies).

3.2.4. Neutrophil chemotaxis assay

Blood from clinically normal 2-week-old calves was collected into acid-citrate-dextrose Vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ). All procedures were performed at room temperature unless otherwise specified. Granulocytes were purified by centrifuging the blood at 900 g for 15 minutes and harvesting the lower 60% of the red cell layer. Erythrocytes were removed by hypotonic lysis, and the resulting granulocyte preparation was centrifuged at 500 g for 5 minutes and resuspended to 2 x 10^6 cells/ml in Hank's balanced salt solution containing 25 mM HEPES and 0% protein, pH 7.4 (buffered HBSS). The granulocyte preparation consisted of over 95% granulocytes, of which 74% to 98% were neutrophils depending on the number of eosinophils in the whole blood. Cell viability exceeded 95% based on trypan blue staining.

The chemotaxis assay was performed in a 48-well microchemotaxis chamber (Neuroprobe, Cabin John, ME). Three-fold dilutions of rbIL-8 that had been

dialysed against HBSS were analysed in triplicate. Buffered HBSS served as the negative control, and 10% zymosan-activated normal bovine serum was the positive control. To confirm that IL-8 was responsible for the chemotaxis, samples containing 10 μg/ml rbIL-8 were pre-incubated for 1 hour on ice with the neutralizing monoclonal antibody 8M6 (anti-ovine IL-8) or isotype-matched control antibody to human IgE, at a concentration of 5 or 50 μg/ml. Samples of chemotactic agonist were loaded in the lower chamber, and were separated from neutrophils in the upper chamber by a polyvinylpyrrolidone-free polycarbonate membrane with 5 μm diameter pores. The chamber was incubated in a humidified incubator with 5% carbon dioxide at 37°C for 30 minutes, then the apparatus was disassembled and neutrophils were removed from the upper surface of the membrane by washing with PBS and scraping the membrane three times. The membrane was air-dried, fixed with methanol, stained with May-Grunwald and Giemsa stains, and mounted on a glass slide. Cells that had migrated completely onto the lower surface of the membrane were enumerated in three fields per well with a 40X objective. As a control in initial assays, cells that had fallen into the lower wells were counted. These were insignificant in number and did not influence the results.

3.2.5. Neutrophil shape change assay

Neutrophils were isolated as described above from a calf with a low number of eosinophils in peripheral blood, and adjusted to a concentration of 2×10^7 cells/ml in buffered HBSS. Three-fold dilutions of rbIL-8 in buffered HBSS were prepared,

mixed with one volume of the neutrophil preparation, and incubated for 20 minutes in a 37°C water bath with gentle agitation every 5 minutes. The samples were fixed in 1% cold paraformaldehyde and held at 4°C until analysis. To confirm that IL-8 was inducing the shape change, samples containing 10 μg/ml rbIL-8 were pre-incubated with 1.0 μg/ml of monoclonal antibody 8M6 or an isotype-matched control antibody.

The fixed cells were analysed by flow cytometry. Neutrophils were gated based on characteristic forward and side scatter patterns, and the median forward light scatter within the neutrophil gate was recorded. As an additional method of analysis, wet mounts of one drop of the fixed neutrophil preparations were evaluated microscopically, and 100 cells were subjectively graded as follows: round with no projections from the cell surface, minor surface projections, major surface projections, or elongation and polarization of the cell.

3.2.6. Intradermal skin testing

The *in vivo* neutrophil chemotactic activity of the rbIL-8 preparation was assessed by skin testing in a 10-day-old Holstein calf which was clinically and hematologically normal, but had mild bronchopneumonia at the time of necropsy. For evaluation of the dose-dependence of the response, 3-fold dilutions of rbIL-8 in PBS were prepared in duplicate and 100 μl per site was injected intradermally on the lateral thorax 6 hours prior to euthanasia. For the time course evaluation, 3.3 μg (410 pmol) of rbIL-8 in 100 μl volumes were injected in duplicate at 15 and 30

minutes, and 1, 2, 4, 6, 8, 18 and 30 hours prior to euthanasia. Sites injected at the same time points with PBS alone served as negative controls. Blood leukocyte counts and differential leukocyte counts were performed at 15 minutes, 7 hours and 30 hours prior to euthanasia.

Six mm diameter samples of the injection sites were fixed in formalin immediately after euthanasia, and hematoxylin and eosin stained sections were routinely prepared. Sections were evaluated in a blinded manner in all cases. For evaluation of the dose-dependence, extravascular neutrophils were counted in 340-μm-wide bands parallel to the epidermis at three depths in the dermis: superficial dermis, immediately subjacent to the epidermis; mid-dermis, deep to but not including the follicular bulb; and deep dermis, close to but not including the panniculus. For evaluation of the time course of neutrophil infiltration, the same sites were evaluated, but neutrophil location was classified as intravascular or extravascular. Neutrophil numbers from these sites were pooled and expressed as the mean number of cells per mm^2 in each biopsy. Selected formalin-fixed biopsies were fixed in 5% glutaraldehyde, post-fixed in osmium tetroxide, and embedded in epon, and sections were examined by electron microscopy.

3.2.7. Statistical analysis

Results were analysed by two-way analysis of variance. If the results were significant at $p<0.05$, a Tukey multiple comparison test was used to compare individual groups. Differences were considered significant if $p<0.05$. Data on

graphs are expressed as the mean ± standard error of the mean.

3.3. Results

3.3.1. Sequence and protein analysis

The cDNA for bovine interleukin-8, from which the signal sequence had been removed (Morsey et al., 1996), was subcloned into the expression vector pGEX-2T, and the nucleotide sequence of the 240 bp bIL-8 insert and of the immediately adjacent pGEX-2T vector was determined. The 5' end of the bIL-8 insert is illustrated in Figure 3.1; the remainder of the bIL-8 nucleotide sequence was identical to that reported previously (Morsey et al., 1996). This nucleotide sequence predicts 8 additional amino acid residues of unknown origin on the N-terminus of the rbIL-8 protein. Bovine IL-8 was expressed as a glutathione-S-transferase (GST) fusion protein, purified by affinity chromatography, and cleaved from the GST using thrombin. The resulting rbIL-8 preparations contained 33 and 22 μg/ml of protein for the first and second eluates, respectively, for a total yield of 0.7 mg of protein from a 1 L culture.

Samples were analysed by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue (Figure 3.2). In the lane loaded with glutathione-Sepharose beads with bound fusion protein, bands were identifiable at 34, 26 and 8 kDa, the expected molecular weights of the bIL-8/GST fusion protein, GST, and rbIL-8, respectively. Lanes loaded with eluates 1 and 2 (above) contained a band at 8 kDa, as expected for rbIL-8, and were free of significant protein contaminants.

Western analysis using rabbit antiserum to ovine IL-8 resulted in strong

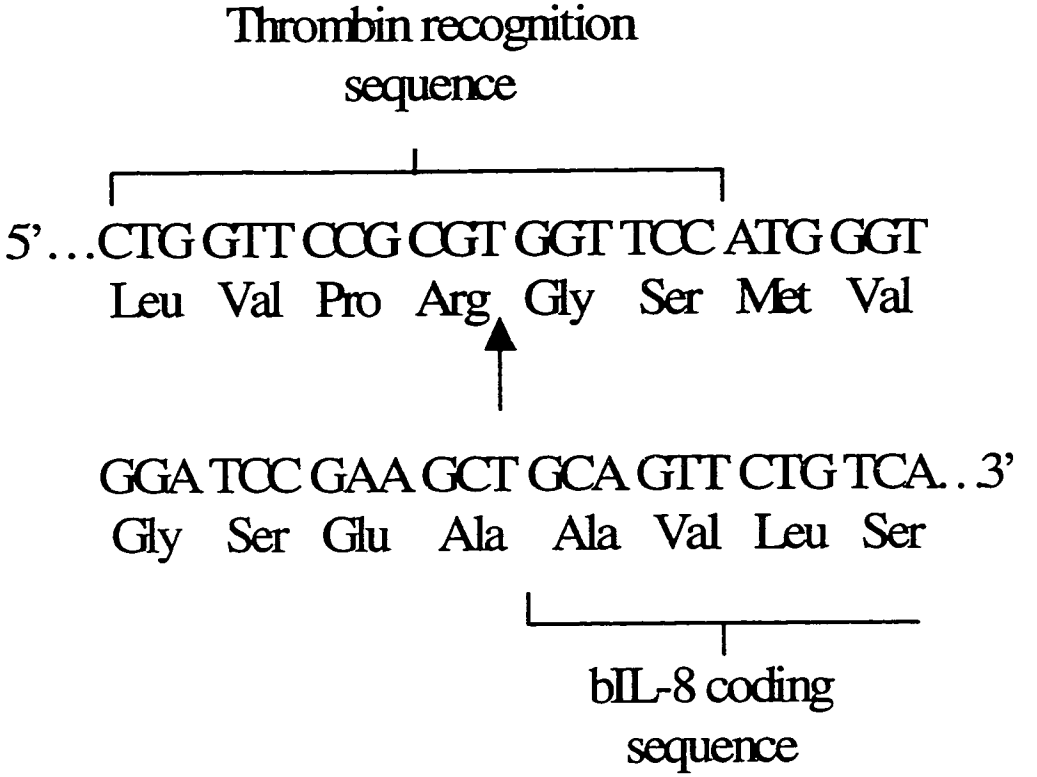

Figure 3.1. The nucleotide sequence of the 5' portion of the bovine interleukin-8 DNA insert, and adjacent pGEX-2T vector DNA. Note the thrombin recognition site encoded by the pGEX-2T vector DNA. The arrow indicates the thrombin cleavage site.

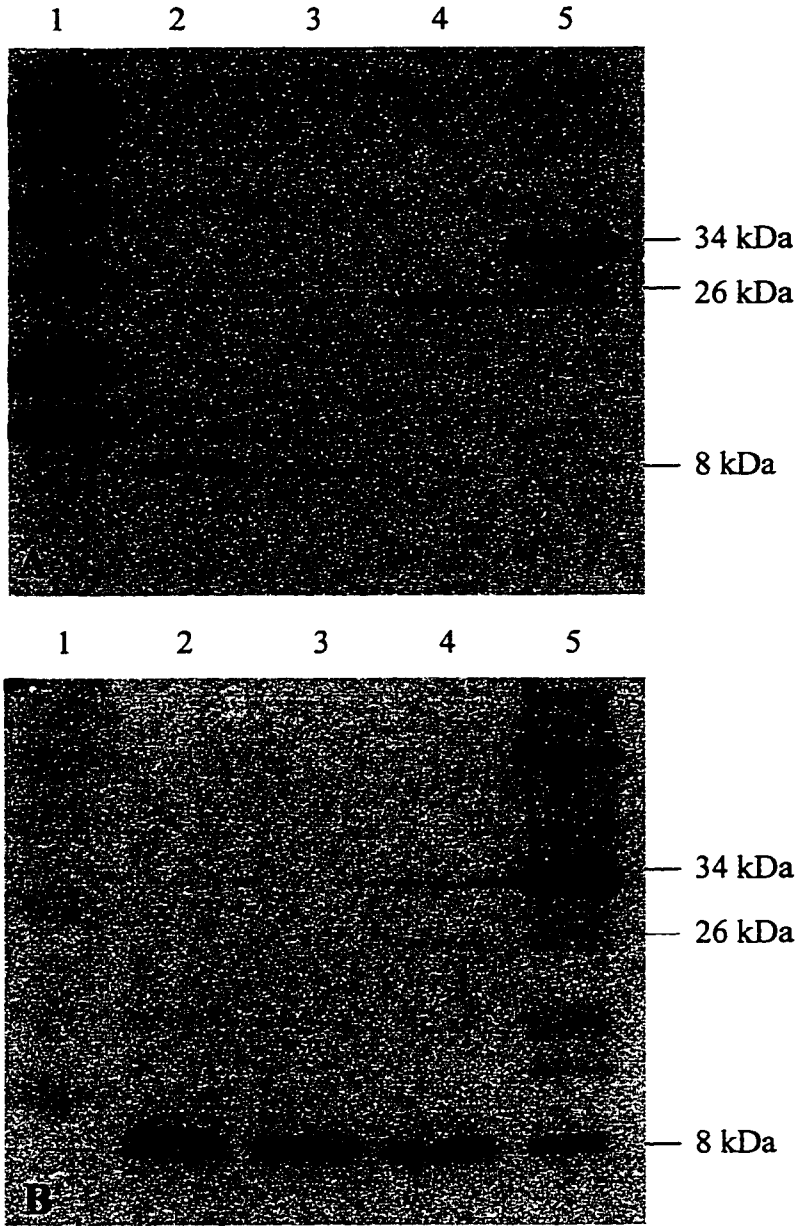

Figure 3.2. SDS-polyacrylamide gel electrophoresis (A) and Western blot using polyclonal rabbit antiserum to ovine interleukin-8 (B).

Lane 1: molecular size marker.
Lanes 2 & 3: first and second column eluates containing recombinant bovine IL-8 (rbIL-8) at 8 kDa.
Lane 4: recombinant glutathione-S-transferase (GST).
Lane 5: rbIL-8/GST fusion protein, containing bands at 8, 26, and 34 kDa.

labelling of the 8 kDa bands in all lanes and the 34 kDa band in the lane containing the rbIL-8/GST fusion protein, as expected for rbIL-8 and the rbIL-8/GST fusion proteins, respectively (Figure 3.2).

3.3.2. Neutrophil chemotaxis assay

Recombinant bIL-8 induced dose-dependent neutrophil chemotaxis when tested in microchemotaxis assays. This effect was significantly different from the negative control across a range of doses from 6.3 ng/ml to 7.9 μg/ml (Figure 3.3). To confirm the specificity of this neutrophil chemotaxis, IL-8 activity in some samples was neutralized using a monoclonal antibody to ovine IL-8 prior to the microchemotaxis assay. Treatment with 50 and 5 μg/ml of antibody reduced neutrophil chemotaxis by 93.4% and 46.0%, respectively.

The chemotactic activity of the rbIL-8 was specific for neutrophils. Eosinophil transmigration was rare (<0.5% of cells) in response to rbIL-8. In contrast, when granulocyte preparations containing 26% eosinophils were used, the granulocytes migrating toward 10% zymosan-activated bovine serum (containing C5a) were 80% neutrophils and 20% eosinophils.

3.3.3. Neutrophil shape change assay

Treatment of neutrophils with rbIL-8 induced shape changes that were detected microscopically and by flow cytometry. Neutrophils treated with HBSS alone were round or only rarely had surface projections. Treatment of these cells

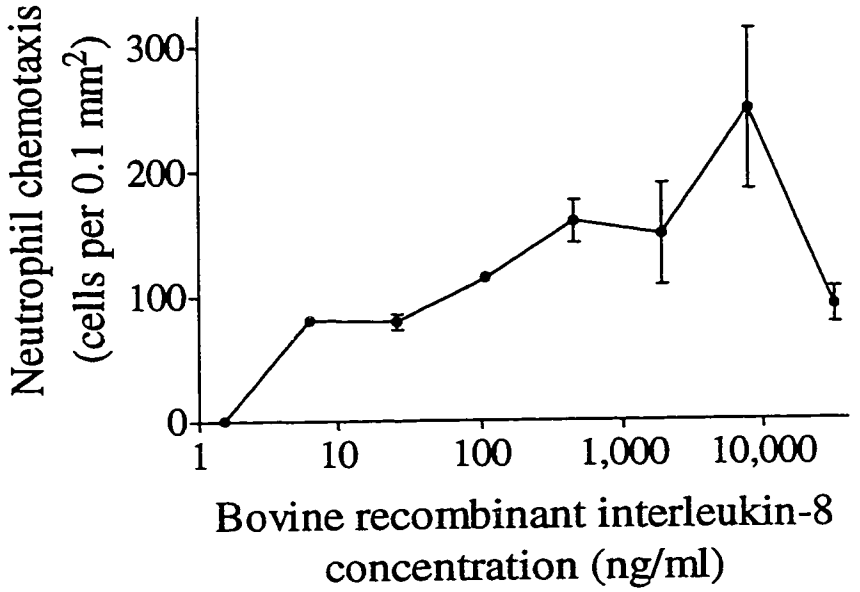

Figure 3.3. The dose effect of recombinant bovine interleukin-8 on *in vitro* chemotaxis of neutrophils. Interleukin-8 induces dose-related chemotaxis of bovine neutrophils in the concentration range of 6.3 ng/ml to 7.9 μg/ml. Mean ± standard error on the mean.

with 1.5 ng/ml rbIL-8 induced the formation of minor pseudopodia, while polarization and obvious distortion of the cell shape were evident at higher concentrations of rbIL-8. After incubation with 1.5 μg/ml rbIL-8, 34% of the neutrophils were polarized with elongation of the cell, 39% had major pseudopodia, 16% had minor surface projections, and 11% were round.

The formation of pseudopodia in response to rbIL-8 treatment was manifest as an increase in forward light scatter as determined by flow cytometry (Figure 3.4). This rbIL-8-induced shape change was dose-dependent from 1.5 ng/ml to 1.5 μg/ml, and pre-incubation of the rbIL-8 with a neutralizing antibody to IL-8 reduced this effect by 93%.

3.3.4. Intradermal skin testing

The efficacy of rbIL-8 as an *in vivo* neutrophil chemoattractant was evaluated by intradermal skin testing in a normal calf. A dose-dependent increase in neutrophil infiltration was elicited by injection of 1.0 ng to 3.3 μg (0.4 to 400 pmol) of rbIL-8 (Figure 3.5). In contrast, intradermal injection of PBS induced the accumulation of only 0.2 neutrophils/mm^2 at 6 hours post-injection (p.i.).

The time course of neutrophil infiltration in response to rbIL-8 was investigated by histologic examination of biopsies collected 15 minutes to 30 hours after administration of the cytokine (Figures 3.6-3.8). At 15 to 60 minutes p.i., neutrophils had accumulated and marginated in small dermal venules and infrequently

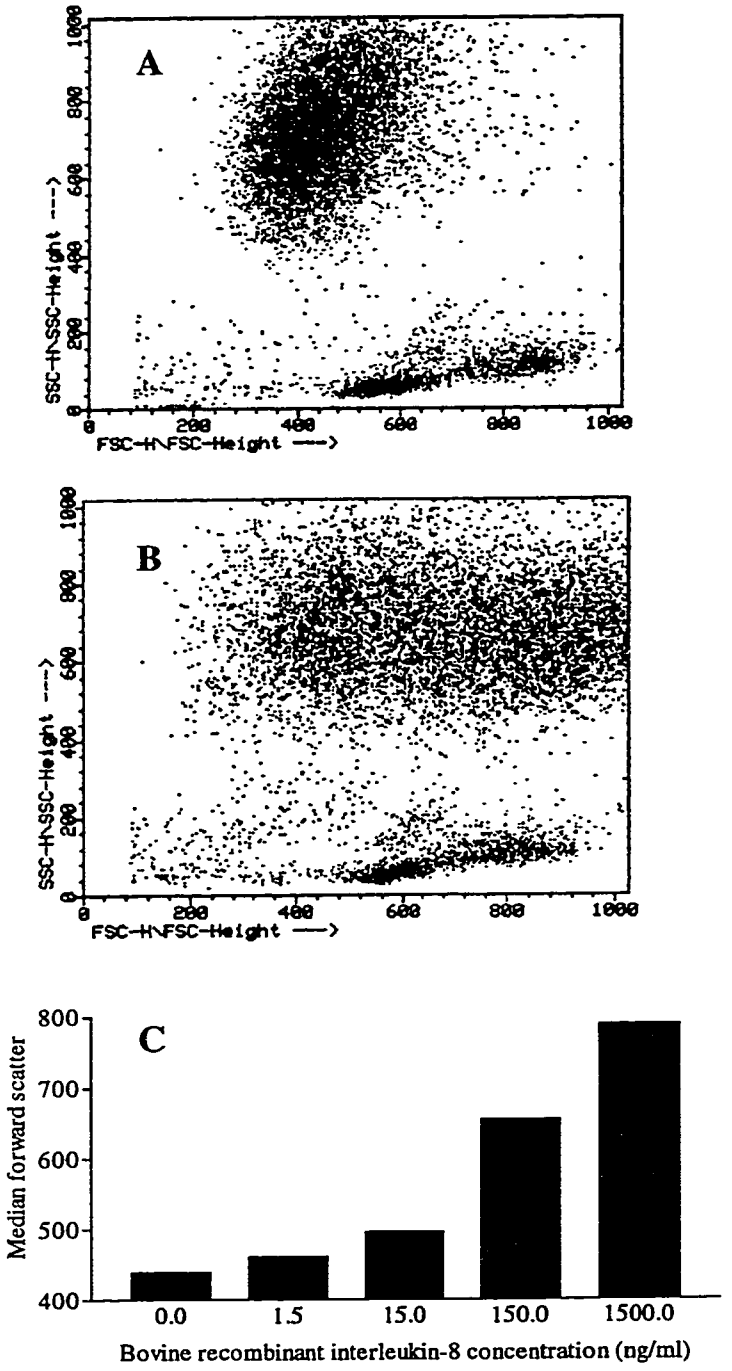

Figure 3.4. Flow cytometric analysis of bovine neutrophils, showing forward vs. side scatter of light. Compared to untreated neutrophils (**Fig. 3.4A**), treatment of neutrophils with 1.5 mg/ml recombinant bovine interleukin-8 (rbIL-8) (**Fig. 3.4B**) induces an increase in forward light scatter. **Fig. 3.4C**: The rbIL-8-induced increase in forward light scatter is dose dependent from 1.5 to 1500 ng/ml.

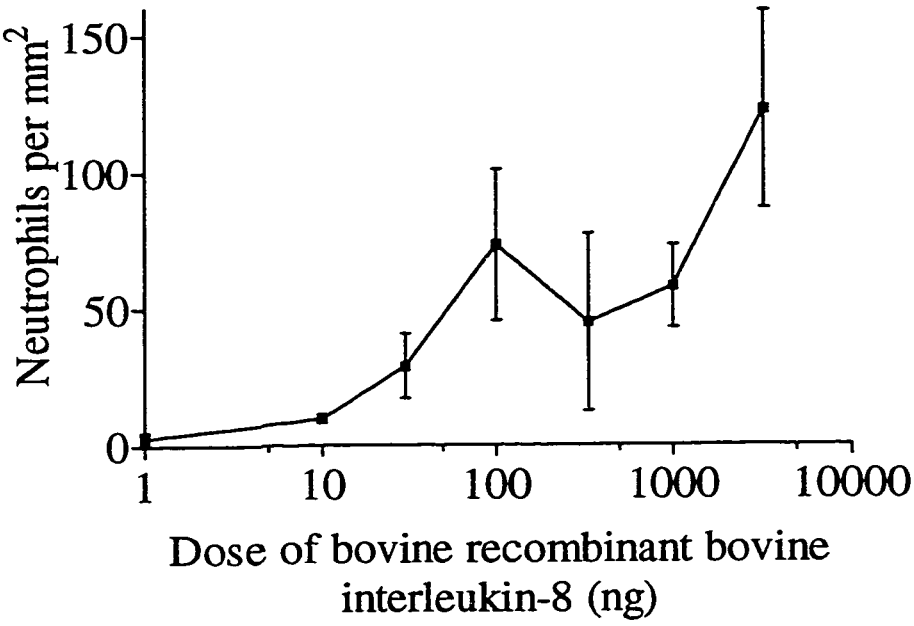

Figure 3.5. The dose response effect of recombinant bovine interleukin-8 *in vivo*. Intradermal injection of 1.0 ng to 3.3 μg rbIL-8 incited dose-related infiltration of neutrophils.

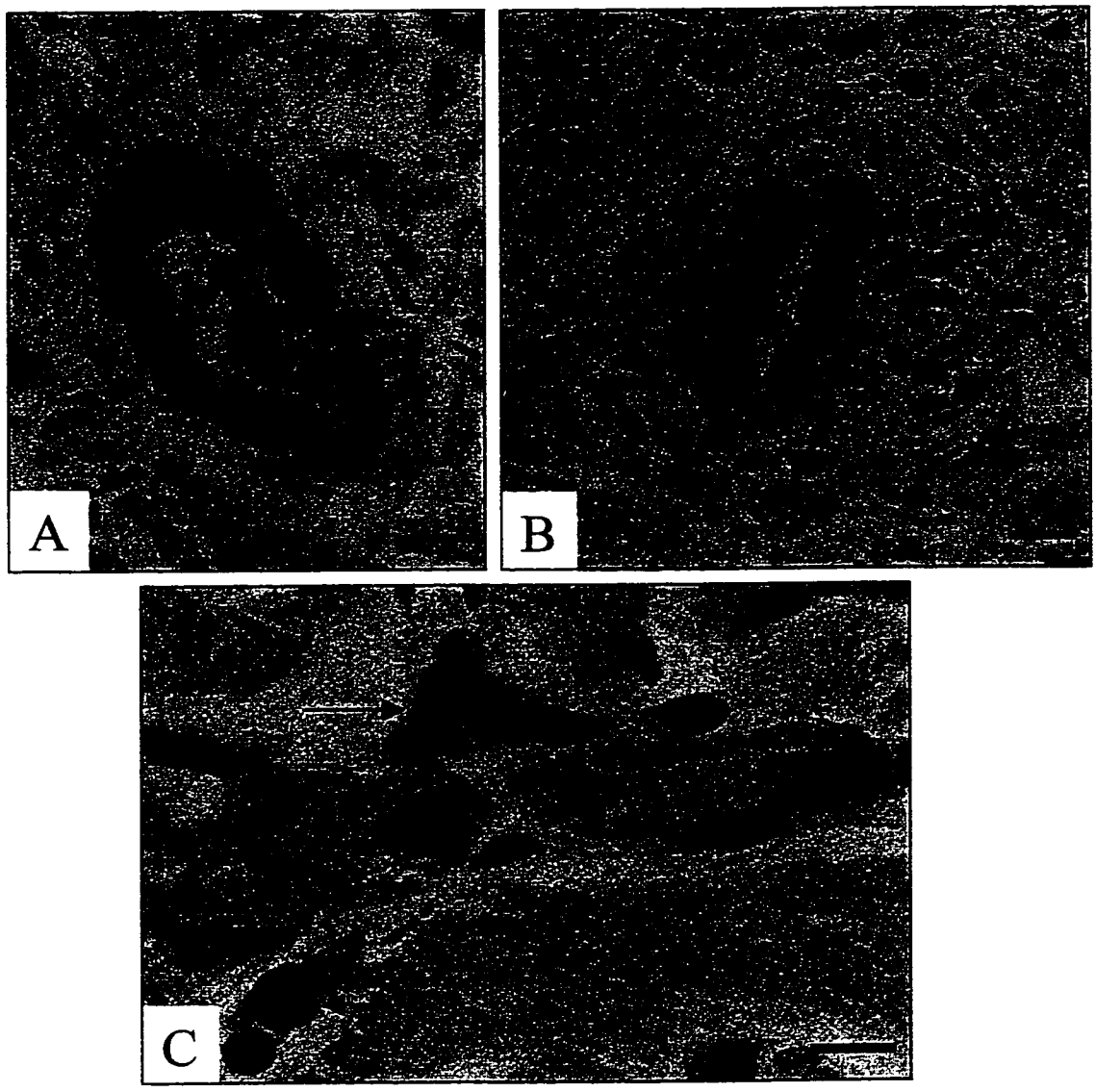

Figure 3.6. Hematoxylin- and eosin-stained sections of skin after injection of 3.3 μg recombinant bovine interleukin-8. **Fig. 3.6A.** Thirty minutes after injection. There is margination of neutrophils in a small dermal venule, but minimal infiltration into the dermis. Bar = 20 μm. **Fig 3.6B.** Two hours after injection. Neutrophils are present in the vessel wall and infiltrate the surrounding dermis (arrows). Bar = 15 μm. **Fig 3.6C.** Thirty hours after injection. Dermal neutrophils have morphologic evidence of apoptosis (arrows), including loss of nuclear segmentation, condensation of chromatin, and partial loss of cytoplasmic granules. Bar = 7 μm.

Figure 3.7. Electron micrograph of the dermis, 2 hours after injection of 3.3 μg of recombinant bovine interleukin-8. A venule lined by endothelial cells (arrowheads) contains several neutrophils (curved arrow) and erythrocytes. Neutrophils infiltrate the vessel wall and the adjacent dermis (straight arrow). Bar = 2.5 mm.

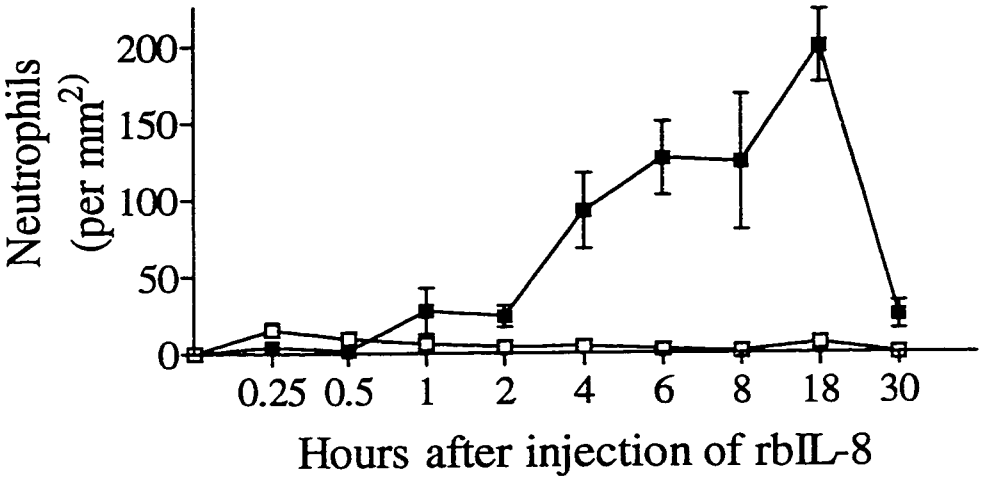

Figure 3.8. The time course of neutrophil responses to intradermal injection of recombinant bovine IL-8. Intravascular margination of neutrophils (open squares) was apparent from 15 minutes to 1 hour after injection. Extravascular neutrophils (closed squares) increased steadily in number from 1 to 18 hours after injection of rbIL-8, but declined by 30 hours.

in small arterioles. Neutrophil infiltration of vessel walls and perivascular interstitium was rare at 15 and 30 minutes p.i., but was obvious by 1 hour p.i. Neutrophil numbers in the perivascular and interstitial dermis increased gradually from 1 to 18 hours p.i., but declined by 30 hours p.i. At 18 hours p.i., and more prominently at 30 hours p.i., up to 50 % of perivascular cells had features suggestive of apoptotic neutrophils: condensed nuclear chromatin, rounding of nuclei with loss of nuclear segmentation, and hypereosinophilic cytoplasm with partial loss of cytoplasmic granules.

Eosinophils were present in low numbers in the superficial dermis at all times after 2 hours p.i., but always at fewer than 1 cell/mm^2. Infiltration of mononuclear cells was not noted, apart from the normal presence of these cells in the superficial dermis of ruminants (Scott, 1988). In negative control sites injected with PBS rather than rbIL-8, neutrophil infiltration was minor and usually associated with microscopic hemorrhage at the site of injection. Although neutrophil numbers were elevated in PBS-treated sites at 2 and 4 hours p.i., these were 6- and 8-fold lower, respectively, than in the corresponding rbIL-8-treated sites. Neutrophil counts in blood at 30 hours, 7 hours and 15 minutes prior to euthanasia were 2.8, 4.8, and 9.6 x 10^9/L, respectively. No macroscopic lesions were induced by intradermal injection of rbIL-8. In contrast, wheal formation was induced by intradermal injection of bronchoalveolar lavage fluid from a calf with pneumonic pasteurellosis (data not shown).

3.4. Discussion

In this study, recombinant bovine interleukin-8 (rbIL-8) was expressed as a GST fusion protein in a prokaryotic system and subsequently purified as a native protein. The protein expressed in this study differs from naturally occurring bovine IL-8, by the addition of eight N-terminal amino acids. The N-terminus of human IL-8 is known to be heterogeneous, probably due to post-translational proteolysis of N-terminal amino acids (aa), and the 72 aa form is more potent than the 77 aa form *in vitro*. Despite this, the 72 and 77 aa forms induce identical responses *in vivo*, perhaps because of extracellular proteolysis of the 77 aa variant (Nourshargh et al., 1992). In this study, the addition of eight N-terminal amino acids resulted in a protein with potent *in vivo* and *in vitro* effects, although these effects were not compared to naturally occurring bIL-8.

The rbIL-8 produced in this study was functionally active *in vitro*, as assessed by neutrophil shape change and microchemotaxis assays. Neutrophil shape change was quantified by measuring forward scatter of light using flow cytometry, and confirmed by microscopic observation of psuedopod formation and elongation or polarization of the cells. This shape change is dependent on the formation and asymetric distribution of filamentous actin (Coates et al., 1992) and thus may reflect a preliminary phase of neutrophil chemotaxis.

Recombinant bIL-8 was active *in vitro* at concentrations as low a 1.0 ng/ml (125 pM) and showed a dose-dependent increase in activity up to 10 μg/ml. Similarly, intradermal injection of rbIL-8 resulted in dose-dependent neutrophil infiltration across doses ranging from 3.3 ng to 3.3 μg (0.4 to 400 pmol). These

potencies are comparable to those reported for ovine and human IL-8 (Leonard et al., 1991; Seow et al., 1994), but lower than that described for the 72 aa form of human IL-8 when tested *in vitro* on bovine neutrophils (Hassfurther et al., 1994).

The molecular events that direct emigration of neutrophils from blood into tissue have been partially defined. Transendothelial migration of neutrophils requires transient selectin-dependent rolling adhesion of neutrophils to endothelium, and then firm adhesion mediated by interactions between neutrophil CD11/CD18 integrin and ICAM-1 on the endothelial cell surface. The events that induce movement of neutrophils through the vessel wall and tissue toward a chemotactic stimulus are less clearly defined, but probably involve local enzymatic degradation of matrix, and lamellipodium-dependent migration of the cell toward soluble or matrix-bound gradients of the chemotactic agent (Cotran et al., 1994). Intradermal injection of rbIL-8 induced neutrophil margination in small vessels by 15 minutes after injection. Infiltration into the dermis was present at 1 hour after injection, and increased steadily thereafter.

Neutrophils with morphologic features typical of apoptosis were present in the dermis 18 hours after administration of rbIL-8, and by 30 hours this feature was more prominent and dermal neutrophil numbers had declined. The survival time of neutrophils in tissue is not accurately known (Babior and Golde, 1995). Neutrophils that are purified from blood and cultured *in vitro* undergo apoptosis spontaneously, with a half-life of approximately 35 hours. Treatment with IL-1, TNF, GM-CSF, G-CSF, or IFN-γ prolongs neutrophil survival; whereas IL-8, C5a, or formylated bacterial peptides does not prevent apoptosis (Colotta et al., 1992). Our data suggest

that neutrophils have survival times in tissue similar to those reported *in vitro*, although accurate quantification of this would require more precise techniques of measuring neutrophil apoptosis.

In our study, IL-8 induced selective chemotaxis of neutrophils. Chemotaxis of mononuclear cells was not noted *in vivo*. Infiltration of eosinophils was present following intradermal administration of rbIL-8, but their number was low and unrelated to the dose of rbIL-8. In addition, when *in vitro* chemotaxis was evaluated using granulocyte preparations containing 26% eosinophils, eosinophil migration across the polycarbonate membrane in response to rbIL-8 was rare. Reports of IL-8-induced eosinophil chemotaxis have been controversial. Ovine IL-8 induced intradermal infiltration of very low numbers of eosinophils (Seow et al., 1994). *In vitro* studies indicate that unprimed eosinophils are not responsive to human IL-8 and do not express receptors for IL-8, but priming of eosinophils with IL-5 induces these cells to express the IL-8 receptor CXCR2 and undergo chemotaxis in response to IL-8 (Schweizer et al., 1994; Heath et al., 1997).

In this study, we have characterized the *in vitro* and *in vivo* effects of bovine IL-8. Interleukin-8 is expressed at a high level in bacterial diseases that are of tremendous economic importance to the beef and dairy industry. The importance of interleukin-8 as a neutrophil chemoattractant and as a mediator of neutrophil-dependent tissue injury in these diseases remains to be determined, and will require *in vivo* neutralization of this chemokine in experimentally induced or naturally occurring disease models.

4.0. THE EFFECT OF *IN VIVO* ADMINISTRATION OF A NEUTRALIZING ANTIBODY TO OVINE INTERLEUKIN-8 ON THE DEVELOPMENT OF PNEUMONIC PASTEURELLOSIS IN LAMBS.

4.1. Introduction

The previous two chapters showed conclusively that IL-8 is expressed in the lesions of pneumonic pasteurellosis, and that the levels of IL-8 in pneumonic lung extracts and in bronchoalveolar lavage (BAL) fluid are sufficient to induce vigorous neutrophil recruitment *in vivo* and *in vitro*. These results imply that IL-8 has the potential to act as a neutrophil chemoattractant in pneumonic pasteurellosis; however, the definitive role of IL-8 in this disease could be influenced by other factors, including the timing of IL-8 secretion, local inhibitors of IL-8 function, the presence of other redundant mediators of neutrophil chemotaxis, and the degree of neutrophil responsiveness.

The purpose of the experiments described in this chapter was to define the role of IL-8 as a neutrophil chemoattractant in pneumonic pasteurellosis. Since neutrophils may be important mediators of tissue injury and severe clinical signs in this disease, we anticipated that neutralization of IL-8 might abrogate the development of severe disease.

To evaluate the role of IL-8 in this disease, we studied experimentally induced pneumonic pasteurellosis in lambs, rather than calves, for the following reasons. Firstly, lambs commonly develop naturally occurring pneumonia caused by *P. haemolytica*. Although this often lacks the degree of fibrinous exudate typical of the bovine disease, it is clinically severe and is characterized by histologic lesions of fibrinopurulent bronchopneumonia containing foci of coagulative necrosis (Gilmour and Gilmour, 1989), as is typical of the disease in cattle. Secondly, although it is currently impractical for both financial and technical reasons to administer sufficient antibody to cattle to neutralize IL-8 *in vivo*, we believed that this approach would be feasible in young lambs. Finally, the studies that were reviewed in Section 1.5 suggest that mechanisms of neutrophil recruitment are similar in cattle and sheep, implying that conclusions derived from an ovine model are relevant to the bovine disease.

This chapter describes a) the development of the model of ovine pneumonic pasteurellosis, b) a preliminary experiment to determine the time course of IL-8 expression and neutrophil infiltration in this model and c) an experiment to assess the effect of *in vivo* neutralization of IL-8 on the development of disease.

4.2. Methods

4.2.1. Preparation of viral and bacterial stocks

The ovine parainfluenza-3 (PI3) virus isolate G2/425/8 was a generous gift of Dr. J.M. Sharp, Moredun Research Institute, Edinburgh, Scotland. An ovine isolate of *P. haemolytica* that had been passaged extensively in culture was obtained from

Dr. A. Potter, Veterinary Infectious Disease Organization, Saskatoon, SK. In a preliminary experiment, two Suffolk lambs were infected by intranasal aerosol administration of 2 x 10^8 $TCID_{50}$ of PI3 virus, and challenged four days later by intranasal aerosolization of 6 x 10^8 cfu of *P. haemolytica*, based on a previously described protocol (Sharp et al., 1978; Rushton et al., 1979). The lambs developed a transient fever 4 to 8 hours after challenge. Necropsies performed 17 and 24 hours p.i. revealed only mild lobular atelectasis, while histologic lesions were restricted to minimal neutrophil accumulation in terminal bronchioles (data not shown).

Because the disease induced by this method was too mild to assess the effect of IL-8 neutralization, three methods were used to increase the severity of the disease. Firstly, the virulence of PI3 virus was increased by four serial passages in Suffolk lambs that lacked serum antibodies to PI3 virus. The lambs were treated with 1 mg/kg dexamethasone twice daily, to enhance viral titres in BAL fluid and to minimize the development of an immune response. For the final passage, a lamb was treated with 200 mg enrofloxacin (Baytril™, Haver, Etobicoke, ON) once daily by intramuscular injection to prevent bacterial contamination of the BAL fluid, but did not receive dexamethasone. For the second passage of the virus, the lamb was infected by aerosol exposure to BAL fluid collected from a lamb in the preliminary experiment described above. For each subsequent passage, 1 litre of RPMI-1640 was used to lavage the lungs at necropsy four days after viral infection, and 10 ml of this BAL fluid was administered by intranasal aerosol to one lamb per passage for a total of four passages. Viral titres in BAL fluid were determined by Dr. Keith West, Department of Veterinary Microbiology, WCVM. The calculation of this viral titre

was based on the maximal dilution of BAL fluid that would induce a cytopathic effect in cultured fetal ovine pulmonary cells, in 50% of the cultures ($TCID_{50}$) (West et al., 1998). To confirm the presence of PI3 viral antigen, lung sections were processed for immunohistochemistry in the WCVM Diagnostic Immunology Laboratory.

Secondly, the severity of experimentally-induced bacterial pneumonia was augmented by using a minimally cultured isolate of *P. haemolytica.* The bacterial isolate was obtained from a lamb with fatal fibrinopurulent bronchopneumonia, and identified to species in the Diagnostic Microbiology Laboratory, WCVM. A swab of pneumonic lung was cultured once on Columbia blood agar, and a single colony was grown for 16 hours in 10 ml of brain-heart infusion (BHI) broth and was stored at –80°C. Thirdly, in an attempt to increase the severity of pneumonia, the bacterial culture was delivered by intratracheal challenge rather than by aerosol, as described in more detail below.

4.2.2. Infection of lambs

Two Dorset lambs and one Dorset-Arcott crossbred lamb, selected based on the lowest available serum antibody titres to *P. haemolytica* leukotoxin (≤1:25,600), were infected using the following technique. Fifteen ml of ovine BAL fluid, prepared as described above and containing 1.5×10^6 $TCID_{50}$ of PI3 virus, was administered by intranasal aerosol four days prior to bacterial challenge. A 16 hour culture of *P. haemolytica* was diluted 1:50 in BHI broth and incubated for a further 4

hours to obtain bacteria in the logarithmic phase of growth. The concentration of bacteria in the sample was estimated by comparing the absorbance of light at 600 nm to that of known concentrations of *P. haemolytica*. This estimate was confirmed later by inoculating Columbia blood agar plates with 10 µl of known dilutions of broth culture and counting the resulting colony forming units (cfu) after an overnight incubation. The lambs were challenged with 8.5×10^8 cfu of bacteria in BHI broth, delivered through a trans-tracheal catheter inserted to the level of the tracheal bifurcation. In this and subsequent sections, hours p.i. refers to the time after infection with *P. haemolytica*.

4.2.3. Assessment of pneumonic lambs

The lambs were monitored clinically before infection and at 1, 2, 3, 5, 8, and 20 hours p.i., by measuring rectal temperatures, respiratory rates, heart rates, and degrees of depression. Blood samples were collected in EDTA at these times, and neutrophil numbers were calculated based on total leukocyte counts assessed using a hemocytometer, and differential cell counts of stained blood smears. Lambs were selected randomly for euthanasia at 3, 8, and 20 hours p.i. At necropsy, lung lesions were noted and the percentage of lesional lung was estimated visually and by palpation. Representative sections from lesional and non-lesional areas were fixed by immersion in 4% paraformaldehyde at 4°C for 6 hours and stored in 70% ethanol overnight at 4°C , then histologic sections were prepared using routine methods. One cranial and one caudal portion of lung, chosen consistently to represent the most

severely affected areas, were lavaged with 40 ml and 20 ml of cold phosphate-buffered saline, respectively. The number of bacteria in the BAL fluid was determined by counting colony forming units as described above. The remaining BAL fluid was centrifuged for 10 minutes at 2000g and the leukocyte-free supernatant was frozen at –80°C. Interleukin-8 concentrations in the BAL fluid from these lambs and from the final passage of PI3 virus alone were measured as described in Chapter 2, using recombinant bovine IL-8 as the standard.

4.2.4. Effect of neutralization of IL-8 on the development of pneumonia

To define the role of IL-8 in the development of clinical signs and lesions of ovine pneumonic pasteurellosis, 4 lambs were immunized passively with differing doses of an antibody to IL-8 prior to infection with *P. haemolytica,* and 2 lambs served as non-immunized controls. The mouse monoclonal IgG2a anti-ovine-IL8 antibody 8M6, shown in Chapter 3 to neutralize the *in vitro* activity of bovine IL-8, was purchased from Dr. Paul Wood, CSIRO Division of Animal Health, Parkville, Victoria, Australia. The required doses of antibody were estimated based on the following information: (1) IL-8 levels in BAL fluid from calves euthanized because of severe experimental pneumonic pasteurellosis were 2.9 to 33.8 ng/ml, as described in Chapter 2; (2) the development of the ELISA system described in Chapter 2 indicated that 0.2 μg/ml of 8M6 antibody successfully captures recombinant ovine IL-8 in a linear fashion, up to a maximal level of 1.0 ng/ml IL-8; and (3) the volume of the normally distended lungs of a 15 kg sheep was estimated to be 1.5 L, based on

neutralized by 10 mg of the monoclonal antibody 8M6. To allow for calculation errors relating to imperfect delivery of antibody to the alveolar fluid, inefficient recovery of IL-8 from alveoli by lavage, and possible differences in the severity of pneumonia, the four lambs were each treated with 10, 20, 40 or 80 mg of antibody in 40 mL of phosphate-buffered saline.

Six clinically normal crossbred Dorset lambs were selected based on the lowest available serum antibody titres to *P. haemolytica* leukotoxin (≤1:25,600). Four days prior to bacterial challenge, the lambs were infected by intranasal aerosol with 0.9×10^6 $TCID_{50}$ of PI3 virus using the fourth *in vivo* passage BAL fluid described in Section 4.2.1. The monoclonal antibody was delivered intravenously 3 hours prior to bacterial challenge; the two control lambs received equal volumes of PBS. All lambs were challenged through an intratracheal catheter with 5×10^9 cfu of the low-passage ovine isolate of *P. haemolytica* in BHI broth, prepared as described above to&achieve a logarithmic phase of growth, with the lambs restrained in an upright sitting position.

All subsequent assessments were made in a double-blind manner. The lambs were monitored clinically twice daily prior to challenge, and at 2, 4, 8, 12, 18, 24 and 30 hours p.i. Clinical assessments included rectal temperatures, respiratory rates, and subjective scores of depression, dyspnea, appetite, strength, lung sounds on auscultation, and respiratory distress. An overall clinical score was determined subjectively using the following guidelines: 0- normal, 0.5- suspected illness, 1.0- definite but mild illness, 2.0- obviously sick, 2.5- very sick or moribund, 3.0- dead. Complete blood counts were performed before challenge and at 2, 4, 14 and 24 hours p.i. Aortic blood samples were collected from surviving lambs at 27 hours p.i. by

definite but mild illness, 2.0- obviously sick, 2.5- very sick or moribund, 3.0- dead. Complete blood counts were performed before challenge and at 2, 4, 14 and 24 hours p.i. Aortic blood samples were collected from surviving lambs at 27 hours p.i. by percutaneous puncture of the lumbar aorta, and blood gas analysis was performed in the WCVM Clinical Pathology Laboratory. To confirm that IL-8 was neutralized by treatment with the anti-IL-8 antibody, multiple intradermal test sites on each lamb were injected with either 1.65 μg of recombinant bovine IL-8 (rbIL-8) in 50 μl of PBS or an equivalent volume of PBS, and were biopsied 4 and 14 hours later. Histologic sections were prepared, and dermal extravascular neutrophils were counted as described in Chapter 3.

Each lamb was euthanized when its overall clinical score reached 2.5 or, in the less severely affected lamb, at 32 hours p.i. At necropsy, the percentage of consolidated lung was estimated and the ratio of lung weight to body weight was determined. The most severely affected portions of the right and left cranial and caudal lung were lavaged with PBS, using 40 ml for the caudal lung and 20 ml for the cranial lung. The BAL fluid samples were held on ice for use in subsequent assays. Analyses of BAL fluid samples included a) percentage of neutrophils, based on differential cell counts of stained cytocentrifuge preparations, b) IL-8 concentrations as assessed by ELISA, using the supernatants of BAL fluid samples that had been centrifuged for 10 minutes at 2000 x g and stored at –80°C, and c) *P. haemolytica* bacterial plate counts as described above. Representative lung samples from 7 standardized anatomic sites–the cranial and caudal parts of the right cranial lobe, the right middle lobe, the right caudal lobe, the cranial and caudal parts of the

left cranial lobe, and the left caudal lobe–were fixed in 10% buffered formalin, and histologic sections were prepared and stained with hematoxylin and eosin. The histologic lesions were graded without knowledge of their identity, using subjective 0-5 scoring systems for the extent of neutrophil accumulation and the intensity of fibrin exudation, hemorrhage, edema, bronchiolar exudate, and necrosis of bronchiolar epithelium. The percentage of the inflammatory lesion that was occupied by neutrophils was estimated.

4.3. Results

4.3.1. Serial *in vivo* passage of ovine parainfluenza-3 virus

Ovine PI3 virus, which was originally obtained from Dr. J.M. Sharp, Moredun Research Institute, Edinburgh, Scotland, was passaged *in vivo* in an attempt to increase virulence. Bronchoalveolar lavage fluid recovered after the third and fourth passages contained 10^4 and 10^5 $TCID_{50}$/ml, respectively. Lesions attributable to PI3 viral infection were similar in all lambs, and consisted of mild generalized lobular atelectasis affecting less than five percent of the lung. Microscopic lesions included mild bronchiolar epithelial necrosis or hyperplasia with infrequent eosinophilic cytoplasmic inclusions. Using immunohistochemistry, viral antigen was demonstrated in 1% to 10% of bronchiolar epithelial cells, and rarely in alveolar cells. In addition to these lesions, the lamb of the third passage developed cranioventral suppurative bronchopneumonia and a pure culture of *P. haemolytica* was isolated from the pulmonary lesions.

4.3.2. Evaluation of the time course of IL-8 expression and neutrophil infiltration

The time course of IL-8 expression and neutrophil infiltration was examined in three lambs infected experimentally with PI3 virus and *P. haemolytica*. All lambs became mildly to moderately depressed by two hours p.i. Very mild tachypnea was evident after 5 hours p.i., but respiratory rates never exceeded 60 breaths per minute. The overall clinical score of the lamb euthanized at 8 hours p.i. was consistently higher than that of the lamb euthanized at 20 hours p.i., as shown in Figure 4.1. Changes in rectal temperature and the numbers of blood neutrophils are illustrated in Figures 4.2 and 4.3. Neutropenia developed by 1 hour p.i. in all cases, and immature neutrophils were first detected in peripheral blood between 3 and 5 hours p.i.

The IL-8 concentrations in the two BAL fluid samples from the lamb infected with PI3 virus alone were 6.5 ± 2.4 ng/ml (mean ± SEM). In contrast, BAL fluid samples from the lambs euthanized at 3, 8 and 20 hours p.i. contained 110 ± 48, 554 ± 118, and 507 ± 70 ng/ml IL-8, respectively (mean ± standard error, two samples per group). The average concentrations of *P. haemolytica* in these BAL fluid samples were 11×10^7, 17×10^7 and 1.5×10^7 cfu/ml, respectively.

At necropsy of the lamb euthanized at 3 hours p.i., the lung contained mild cranioventral interlobular edema and a cranioventral distribution of indistinct, 2-5 mm diameter, red foci. Histologically, aggregates of 10 to 50 neutrophils were present in peribronchiolar alveoli and occasionally in terminal bronchioles, and these alveoli

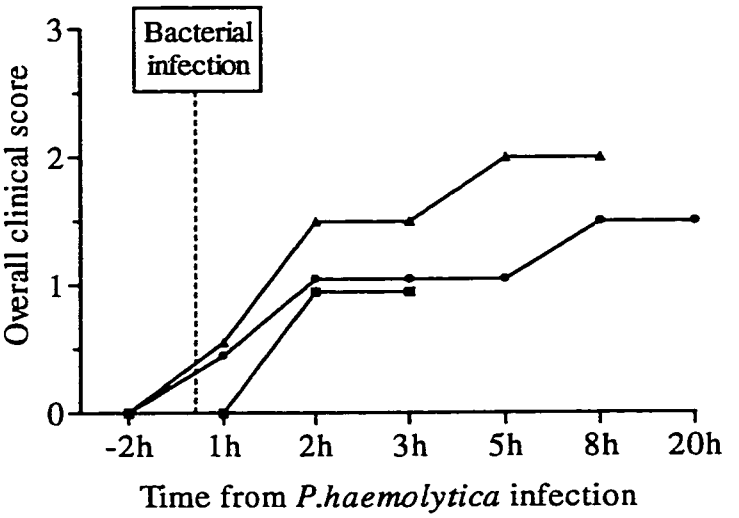

Figure 4.1. Changes in the overall clinical score in three lambs following infection with parainfluenza-3 virus and *Pasteurella haemolytica.* The clinical scores increased progressively after bacterial challenge. The clinical score of the lamb euthanized at 8 hours after bacterial challenge was consistently higher than that of the lamb euthanized at 20 hours.

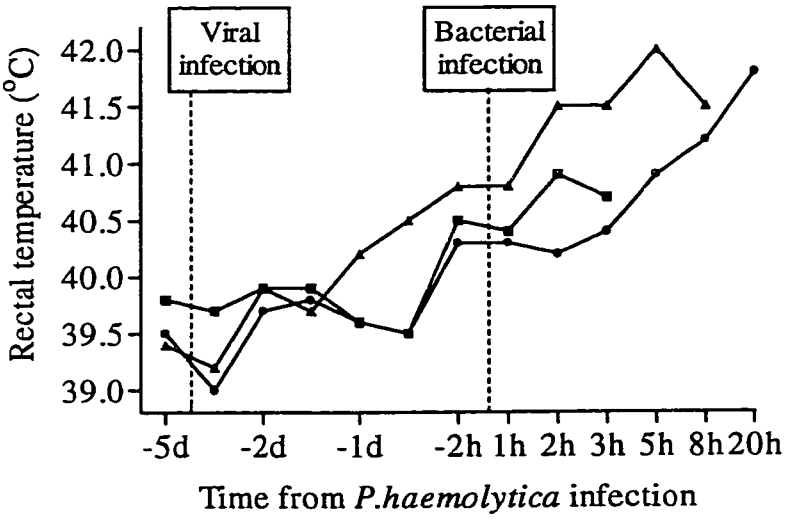

Figure 4.2. Changes in rectal temperature in three lambs following infection with parainfluenza-3 virus and *Pasteurella haemolytica.* The lambs developed a progressive febrile response after bacterial challenge.

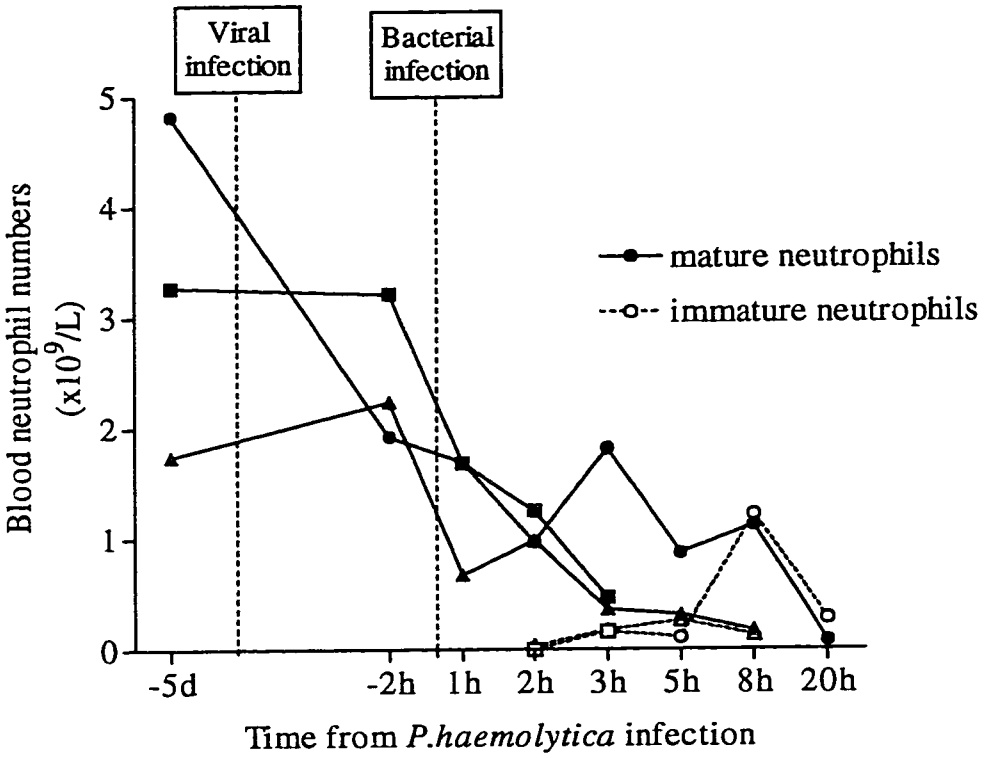

Figure 4.3. Changes in blood neutrophil numbers in three lambs following infection with parainfluenza-3 virus and *Pasteurella haemolytica.* Neutrophil numbers declined by 1 hour, and immature (band) neutrophils were first detected at 3-5 hours after infection. Reference range for ovine neutrophils, 0.6-4.0x10^9/L; and for ovine immature neutrophils 0-0.12x10^9/L.

were edematous. "Oat cells" typical of necrotic neutrophils were infrequent, and bacteria were present in low number (Figures 4.4 and 4.5).

Gross lung lesions in the lamb euthanized at 8 hours after infection were characterized by generalized, multifocal, 3 to 10 mm diameter areas of hemorrhage and slight firmness, and failure of the cranial lung lobes to collapse (Figure 4.6). In addition, the cranial part of the left cranial lobe was consolidated, but this was interpreted to be a pre-existing lesion based on the histologic lesions of fibrosis and lymphoplasmacytic peribronchiolitis. Microscopic examination of the hemorrhagic foci revealed extensive alveolar edema and moderate fibrin exudation, aggregates of moderate numbers of necrotic leukocytes with streaming chromatin, and numerous bacteria (Figures 4.4 & 4.5).

Lesions in the lung of the lamb euthanized at 20 hours p.i. were similar to those at 8 hours, but were firmer and bulged above the pleural or cut surfaces. Histologically, edema fluid and fibrin exudation were similar to that at 8 hours, but necrotic streaming leukocytes were more numerous and often filled alveoli (Figures 4.4 & 4.5). Bacteria were rare.

4.3.3. Effect of neutralization of IL-8 on the development of pneumonia

To assess the role of IL-8 in pneumonic pasteurellosis, the development of clinical signs and lesions following infection with PI3 virus and *P. haemolytica* were compared in untreated lambs and in lambs that received graded doses of a neutralizing antibody to ovine IL-8. The success of IL-8 neutralization was evaluated by two

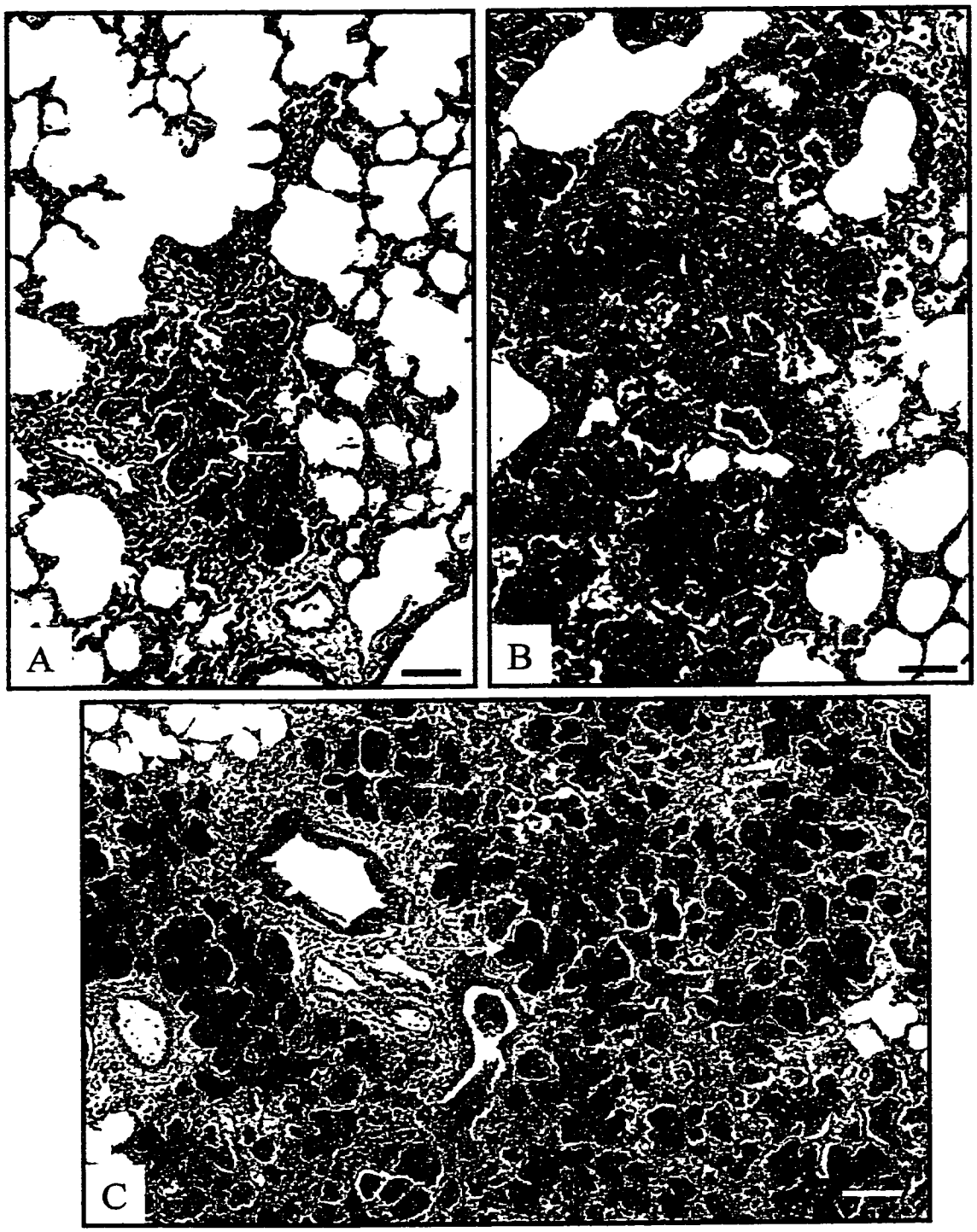

Figure 4.4. Low magnification of histologic pulmonary lesions in lambs infected experimentally with parainfluenza-3 virus and *Pasteurella haemolytica*. Bars = 100 μm. **Fig. 4.4A.** At 3 hours after bacterial infection (p.i.), there are scant aggregates of neutrophils in peribronchiolar alveoli (arrow). **Fig. 4.4B.** Neutrophils are more numerous at 8 hours p.i., and edema and fibrin are present. **Fig. 4.4C.** By 20 hours p.i., there is extensive filling of alveoli by neutrophils (arrow).

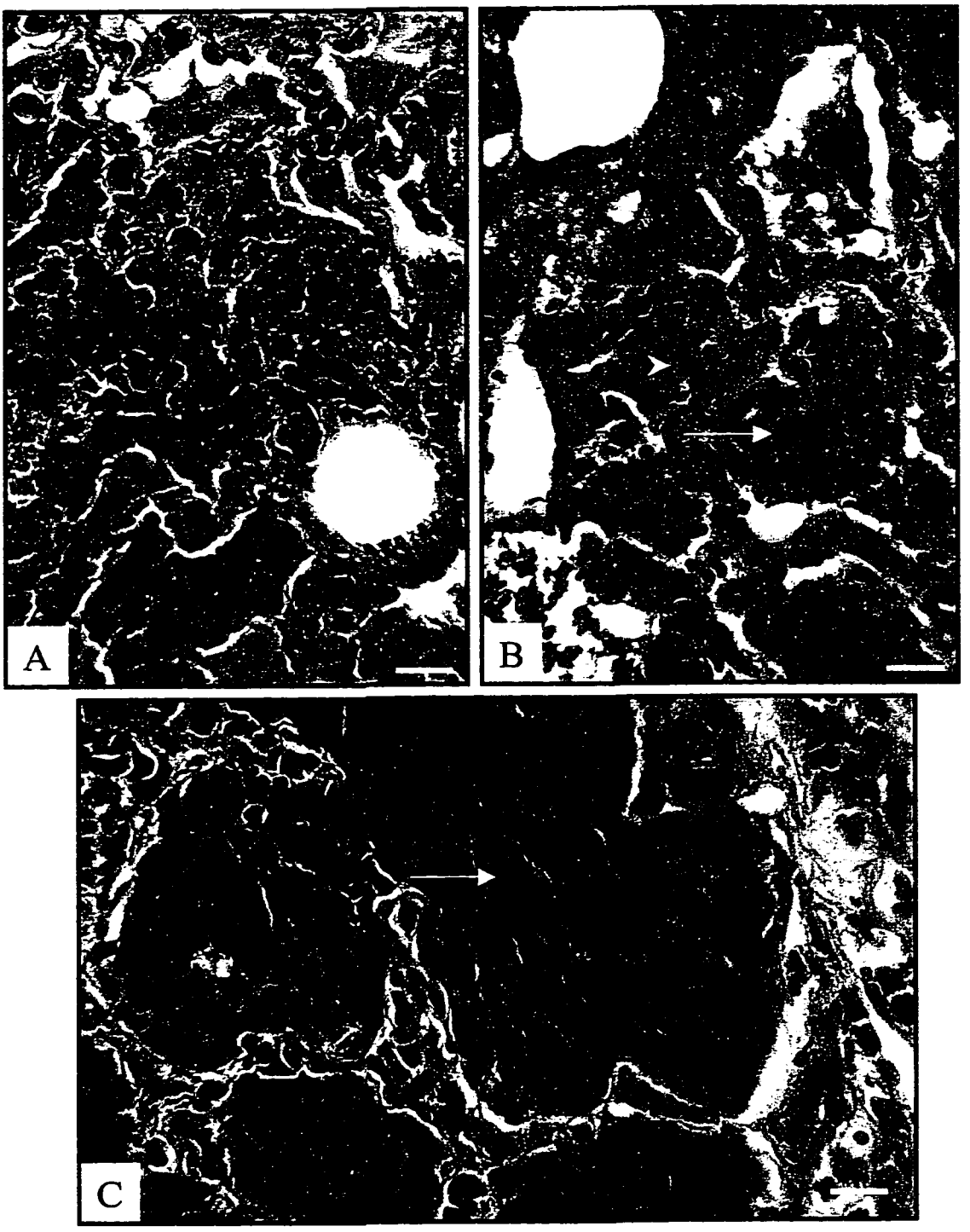

Figure 4.5. Higher magnification of histologic pulmonary lesions in lambs infected experimentally with parainfluenza-3 virus and *Pasteurella haemolytica*. Bars = 20 μm. **Fig. 4.5A.** At 3 hours after bacterial infection (p.i.), most neutrophils have multilobulated nuclei. **Fig. 4.5B.** At 8 hours p.i., neutrophils are often necrotic and have rounded nuclei (arrow), and edema and fibrin (arrowhead) are present. **Fig. 4.5C.** By 20 hours p.i., neutrophils are often necrotic and have streaming chromatin (arrow).

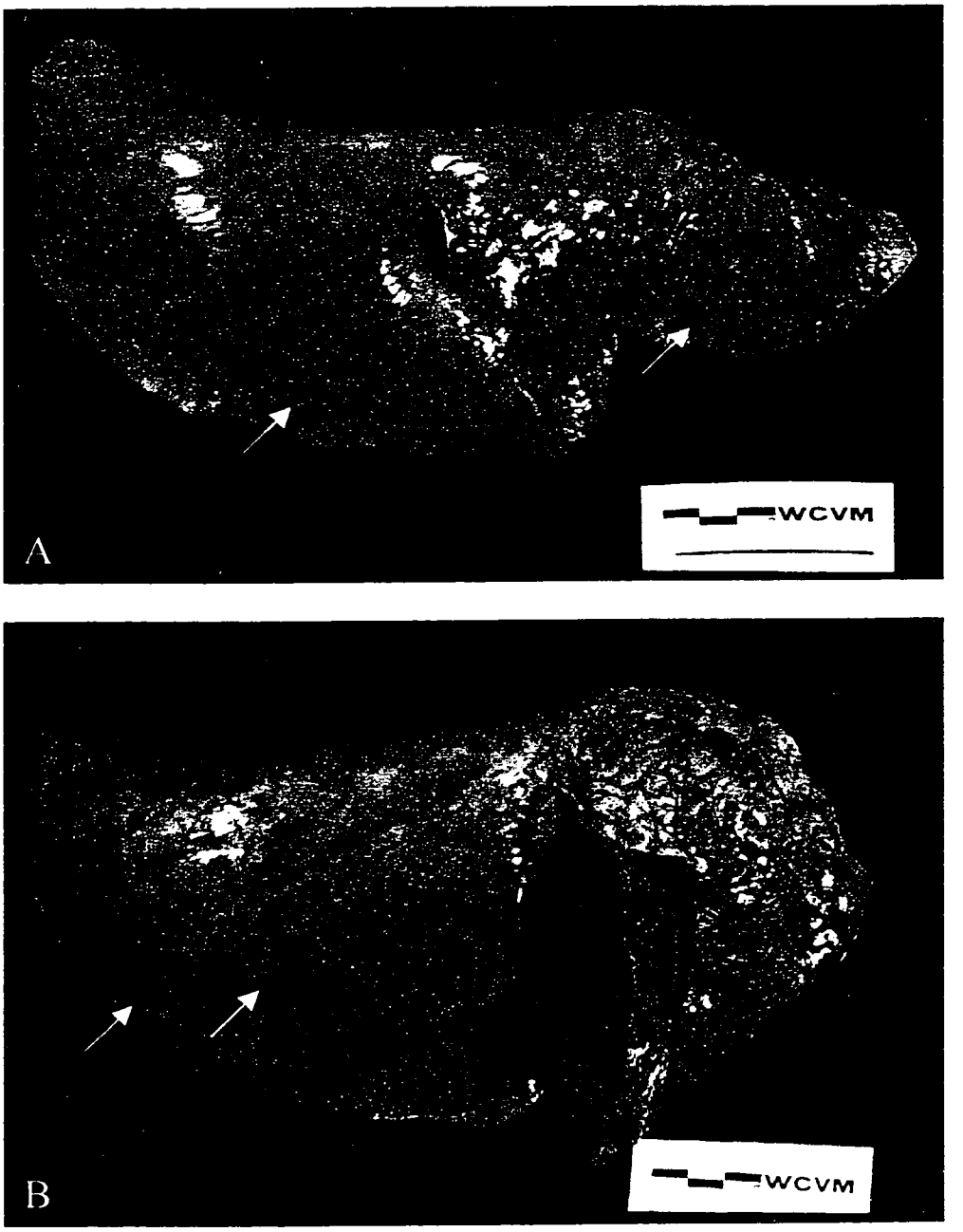

Figure 4.6. Gross pulmonary lesions in lambs infected experimentally with parainfluenza-3 virus and *Pasteurella haemolytica*. **Fig. 4.6A**. In the lamb from the time-course experiment euthanized at 20 hours after bacterial infection, gross lesions were restricted to mild multifocal areas of hemorrhagic inflammation in the cranial and ventral aspects of the lung (arrows). **Fig. 4.6B**. Lesions were more severe in the IL-8-neutralization experiment; the right caudal lung lobe from a lamb that received no antibody treatment had extensive consolidation, failure to collapse, and multifocal areas of hemorrhage (arrows). Bars= 1 cm.

methods: measurement of residual IL-8 in BAL fluid using an ELISA, and analysis of neutrophil infiltration at sites of intradermal injection of rbIL-8. The levels of IL-8 in BAL fluid were not different in antibody-treated lambs compared to control lambs. The mean IL-8 concentration in all BAL fluid samples was 910 ng/ml, and the maximal level in each lamb ranged from 1.0 to 2.6 μg/ml (Figure 4.7). Neutrophil infiltration of rbIL-8 injection sites was not eliminated in antibody-treated lambs; in fact, neutrophil infiltration in the three lambs given the most antibody was significantly more intense than in the controls ($p<0.05$, Figure 4.8).

All lambs developed severe depression, lethargy and weakness. In general, respiratory rates were lower after challenge, but the depth of respiration was greater. Abnormal lung sounds included increased bronchial tones, crackles and infrequent wheezes, but these abnormalities were inconsistent. The survival of lambs is shown in Table 4.1, and overall clinical scores are depicted in Figure 4.9.

Changes in blood neutrophil counts are summarized in Figure 4.10. The magnitude of these changes was not related to the dose of antibody. The results of aortic blood gas analysis for the four lambs that survived to 37 hours p.i. are presented in Table 4.2. All of these lambs were hypercapnic and hypoxemic. The lambs given 0 and 80 mg of antibody were acidotic and hypercapnic, typical of respiratory acidosis. In contrast, the lamb that received 20 mg of antibody was acidotic with low normal bicarbonate levels, suggesting the presence of both respiratory and metabolic acidosis.

At necropsy, consolidation was most severe in the caudal lung lobes, and was

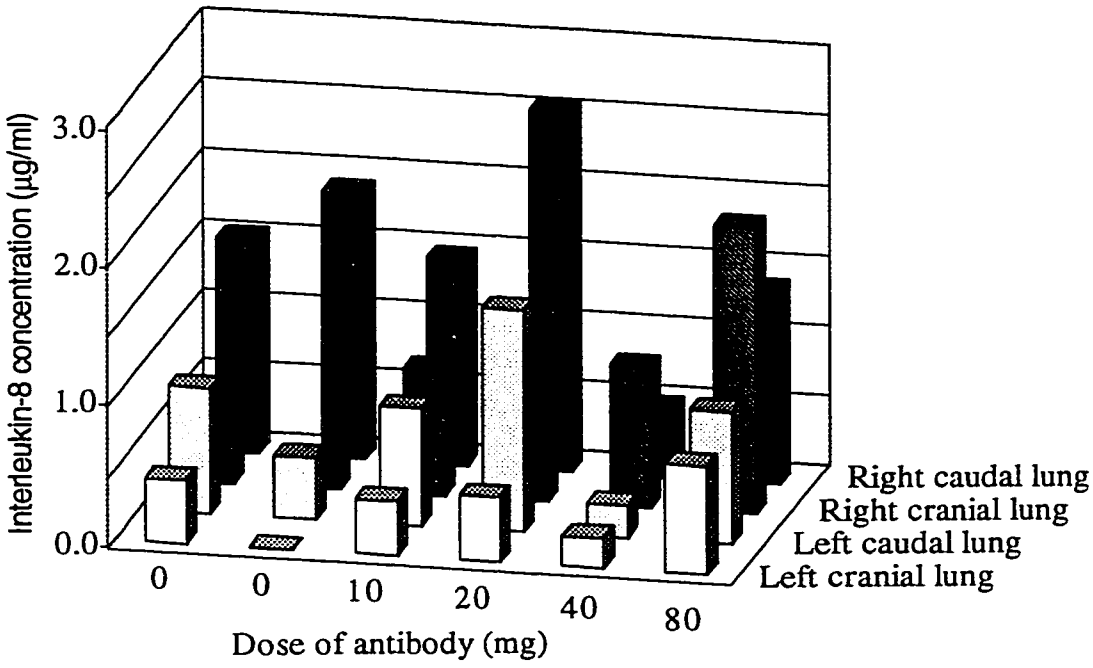

Figure 4.7. Interleukin-8 concentrations in bronchoalveolar lavage (BAL) fluid of lambs receiving various doses of IL-8-neutralizing antibody. IL-8 concentrations were similar in the antibody-treated lambs and in the controls.

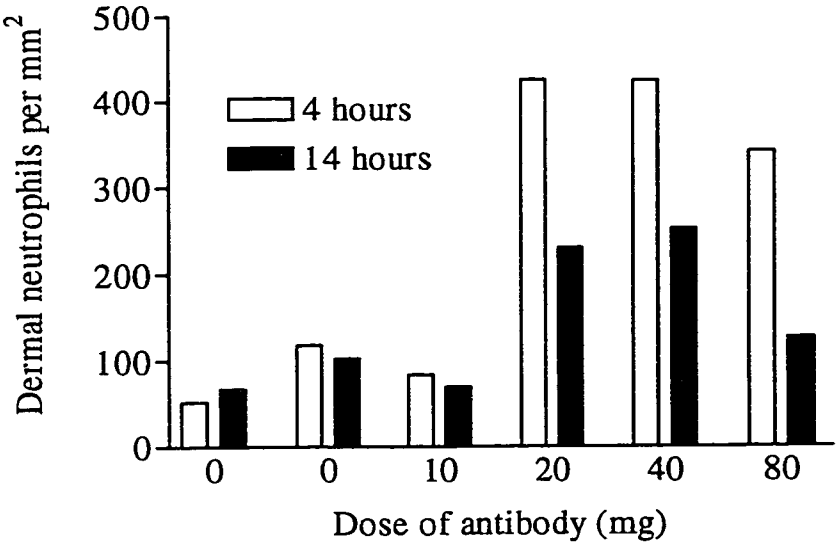

Figure 4.8. Neutrophil numbers in the dermis 4 and 14 hours after injection of IL-8, in lambs treated with differing doses of an IL-8-neutralizing antibody. IL-8-induced neutrophil responses were, suprisingly, higher in antibody-treated lambs than in controls.

Table 4.1. Survival of lambs infected with parainfluenza-3 virus and *Pasteurella haemolytica*, after administration of differing doses of a neutralizing antibody to ovine IL-8.

Antibody dose (mg)	Survival[1]	Outcome
0	32 hours	Moribund, euthanized
0	17 hours	Died
10	25 hours	Moribund, euthanized
20	27 hours	Died
40	32 hours	Depressed but not moribund, euthanized
80	30 hours	Moribund, euthanized

[1]Survival indicates the time between infection with *P. haemolytica* and death or euthanasia.

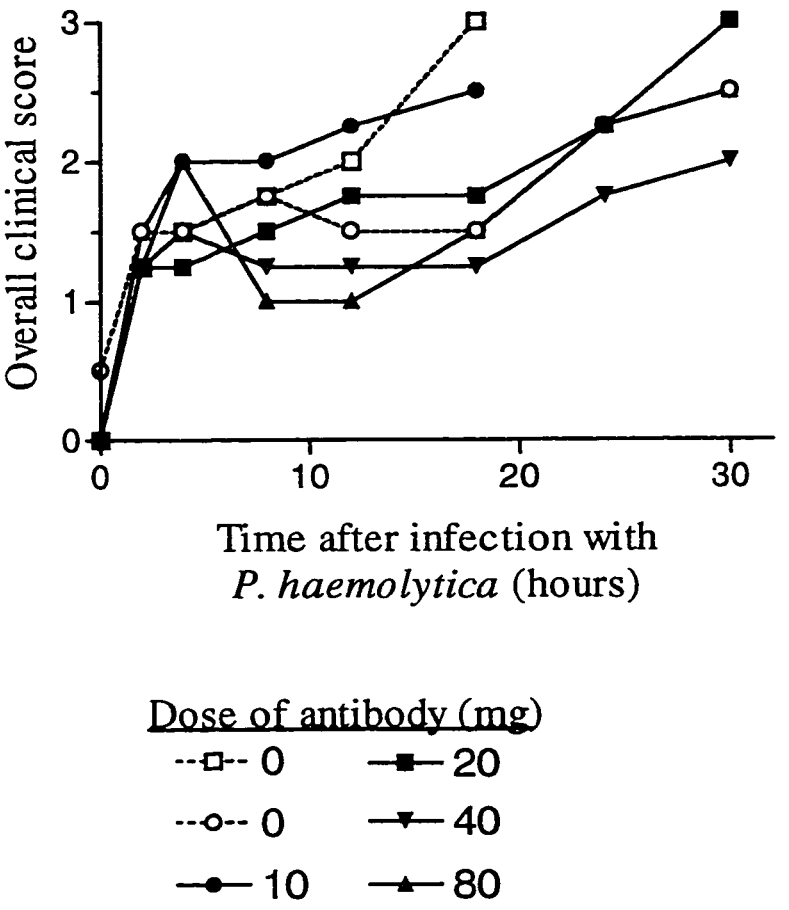

Figure 4.9. Changes in the overall clinical score following infection with *Pasteurella haemolytica*, in lambs given differing doses of anti-IL-8 antibody. Antibody treatment had no consistent effect on the clinical score.

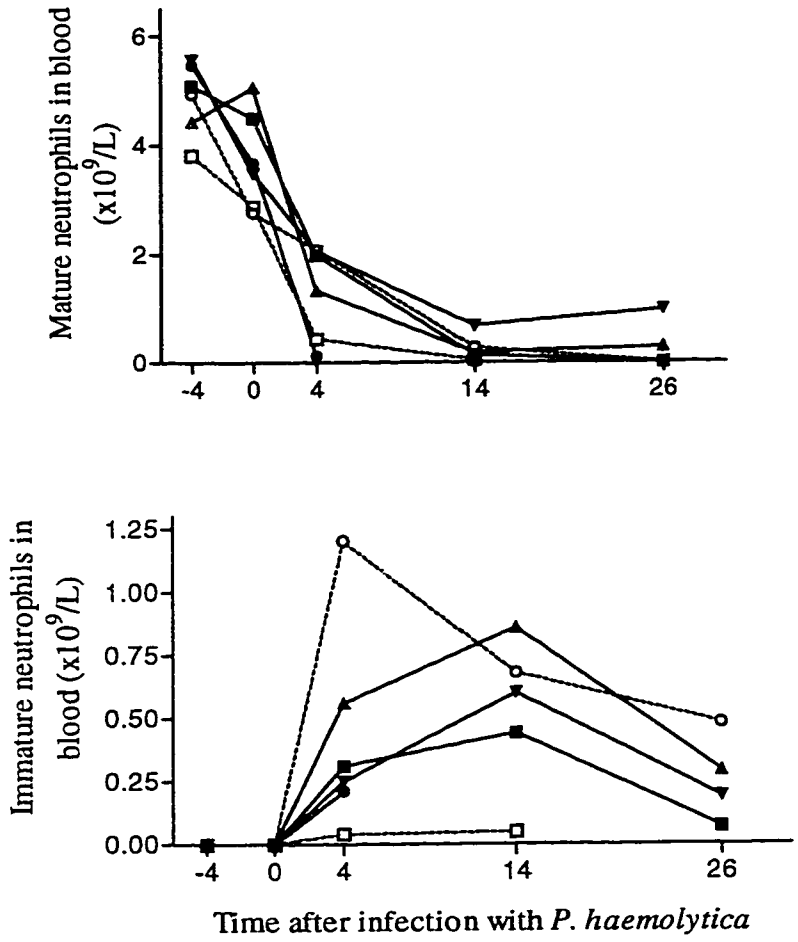

Figure 4.10. Changes in blood neutrophil numbers following infection with *Pasteurella haemolytica*, in lambs given differing doses of anti-IL-8 antibody. Antibody treatment did not significantly affect the changes in blood neutrophil numbers.

Table 4.2. Blood gas analysis after infection with *Pasteurella haemolytica,* from lambs given differing doses of a neutralizing antibody to ovine IL-8.

Antibody dose (mg)	pH	pCO_2 (mmHg)	pO_2 (mmHg)	HCO_3^- (mmol/L)	Base excess (mmol/L)
Normal[1]	7.32-7.50	30-40	80-110	20-28	-3 to 3
0	7.31	57.0	70.1	27.7	2.3
20	7.125	66.0	77.5	20.9	-8.2
40	7.356	56.6	70.3	30.4	5.7
80	7.278	76.2	47.8	34.8	6.3

[1] WCVM Clinical Pathology Laboratory reference values, ovine arterial blood

characterized by multifocal to locally extensive areas which were firm, hemorrhagic, and exuded fluid from the cut surface (Figure 4.6). The extent or the severity of the gross lesions did not correlate with antibody treatment (Table 4.3). Total lung weights, which ranged from 2.0% to 3.5% of body weight, were not related to the dose of antibody (Table 4.3).

Histologic lesions were qualitatively similar to those described in the time-course experiment (Section 4.3.2), but neutrophil infiltration and fibrin exudation were more severe. The antibody dose was not related to the extent of neutrophil accumulation, the intensity of neutrophil infiltration, or the amount of fibrin exudation, hemorrhage, edema, bronchiolar exudate, or bronchiolar necrosis (Table 4.4). The extent of neutrophil infiltration in the histologic lesions was graded subjectively using a blinded 0-5 scoring system, and the correlation of this score with the IL-8 concentration in BAL fluid was highly significant ($p< 0.0001$, $r=0.71$). Similarly, the correlation between the percentage of total leukocytes in BAL fluid that were neutrophils and the IL-8 concentration in this fluid was highly significant, although the strength of this correlation was not as great ($p<0.005$, $r=0.56$) (Figure

4.4. Discussion

The intent of these experiments was to establish a model of pneumonic pasteurellosis in lambs, and to use this model to evaluate the role of IL-8 as a neutrophil chemoattractant and mediator of severe disease. The latter objective was

4.11).

Table 4.3. Pulmonary lesions at necropsy after infection with parainfluenza-3 virus and *Pasteurella haemolytica*, in lambs given differing doses of antibody to IL-8.

Antibody dose (mg)	% Lesional lung	Lung weight: body weight (%)
0	40	2.6
0	60	3.2
10	60	3.4
20	30	2.0
40	15	2.4
80	30	3.5

Table 4.4. Pulmonary neutrophil responses in lambs infected with parainfluenza-3 virus and *P. haemolytica*, and given a neutralizing antibody to ovine IL-8.

Antibody dose (mg)	Neutrophils in BAL fluid(%)[1]	Extent of inflammation[2]	Neutrophil intensity (%)[3]	Fibrin and hemorrhage[4]
0	50	2.0	34	2.1
0	54	2.0	49	1.8
10	23	2.3	28	1.5
20	59	2.9	66	1.7
40	38	2.3	54	1.5
80	56	2.6	76	1.7

[1] The mean percentage of leukocytes in bronchoalveolar lavage fluid that were neutrophils for 4 standardized lavages per lamb.
[2] The mean score (0-5) of the extent of histologic inflammatory lesions for 7 standardized sections of lung per lamb.
[3] Estimated percentage of the inflammatory lesions occupied by neutrophils in 7 standardized histologic sections.
[4] The mean histologic score (0-5) of the intensity of fibrin exudation and hemorrhage for 7 standardized sections of lung per lamb.

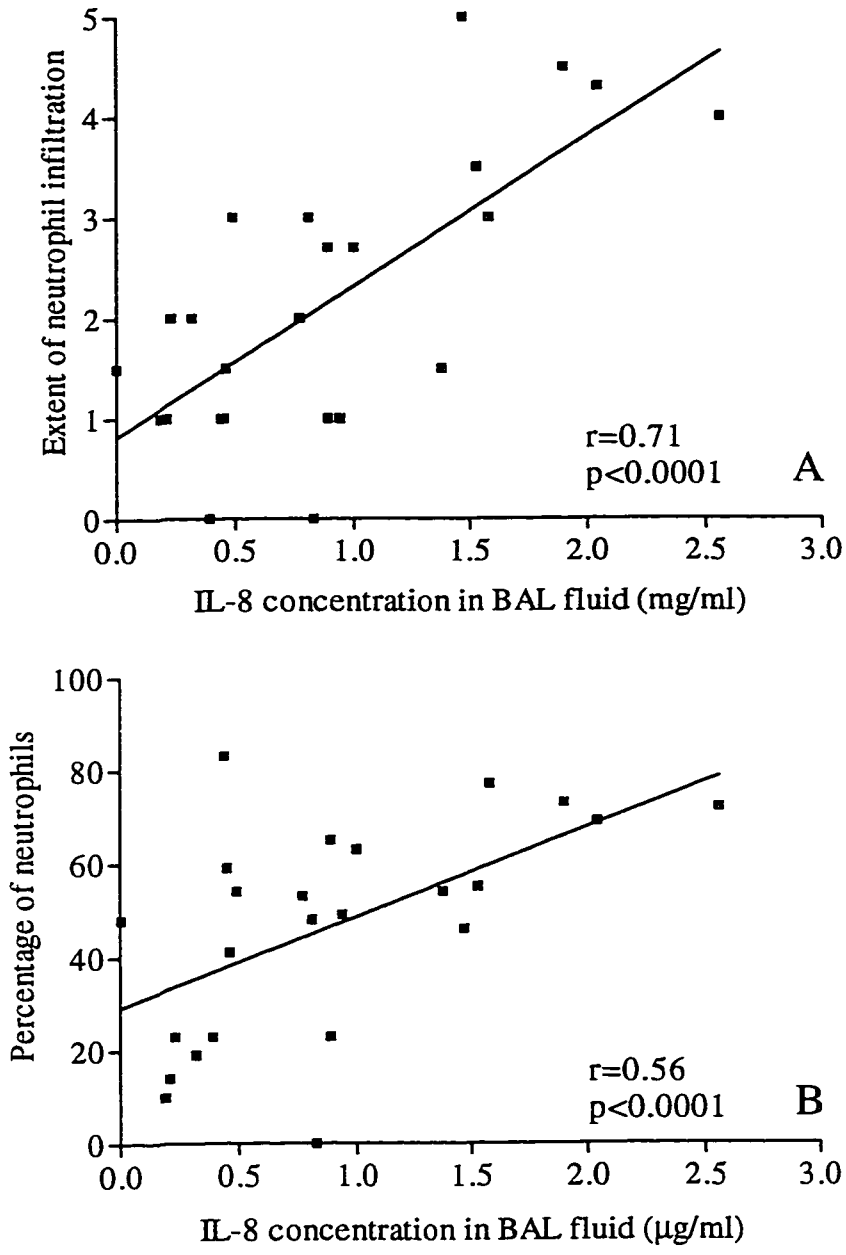

Figure 4.11. Correlation between the concentration of interleukin-8 (IL-8) in bronchoalveolar lavage (BAL) fluid, and either the histologic extent of neutrophil infiltration (Fig. 4.11A) or the percentage of neutrophils in BAL fluid (Fig. 4.11B). In both cases, there was a significant positive correlation between IL-8 concentrations in BAL fluid and neutrophil responses in the lung.

not achieved because the doses of antibody that were used failed to neutralize IL-8 in these lambs: antibody-treated lambs did not have lower IL-8 levels in BAL fluid than untreated controls, and neutrophil recruitment to sites of intradermal IL-8 injection was not impaired in the antibody-treated lambs.

The most probable cause for this failure was the high level of IL-8 expression in this disease. The calculated dose of antibody was based on IL-8 levels found in cattle with experimental pneumonic pasteurellosis. In contrast, the maximal IL-8 concentrations in BAL fluid from the six lambs in the experiment described in Section 4.3.3 were about 100-fold higher than in the lesional lung of the cattle described in Chapter 2, and this amount of IL-8 would greatly exceed the predicted neutralizing capacity of the highest dose of antibody. The causes of these apparent differences in IL-8 concentration were not determined in this study, but could include the following:

1. Bovine herpesvirus-1 (BHV-1) infection may impair IL-8 secretion, whereas ovine PI3 virus may not induce the same effect. Infection of calves with BHV-1 reportedly impairs the ability of alveolar macrophages to secrete mediators of neutrophil chemotactic activity (McGuire and Babiuk, 1984), but there are no published studies reporting a similar effect for PI3 virus.
2. The severity, extent or chronicity of lesions may differ between these specific groups of lambs and calves. It should be noted that the lambs described in Sections 4.3.3 died or were euthanized between 17 and 32 hours p.i., but the calves were euthanized because of severe disease at 2, 3 and 4 days p.i. The IL-8-generating capacity of 2-month-old lambs and 8-month-old calves may be

intrinsically different.

3. Differences in the response of the ELISA system could influence the measured IL-8 concentrations, because the ELISA system compared bovine samples and ovine standard in the experiments described in Chapter 2, but ovine samples and bovine standard in the experiments in this chapter. The importance of this difference as an explanation for the higher apparent IL-8 concentrations in ovine compared to bovine BAL fluid samples is probably minimal for two reasons. Firstly, as detailed in Appendix A, the greatest difference in reactivity of IL-8 standards was five-fold, at higher concentrations of IL-8 than were used as a standard in the ELISA, yet even this magnitude of difference would not explain the 100-fold higher measured IL-8 concentrations in ovine compared to bovine BAL fluid samples. Secondly, the absorbance values for ovine and bovine recombinant IL-8 were similar in the concentrations used as standards in the ELISA system (Appendix A). Thus, although the differing response of the ELISA system to ovine and bovine IL-8 standards could result in higher apparent IL-8 concentrations in ovine compared to bovine pneumonic BAL fluid samples, the magnitude of this effect is probably small and would not account fully for the measured differences.

Another potential cause for the failure of this experiment is that the interaction of the monoclonal antibody with IL-8 may not have completely abrogated the neutrophil chemotactic activity. This possibility is suggested by the significantly more vigorous neutrophil infiltration into sites of intradermal IL-8 injection in lambs receiving high

doses of the monoclonal antibody compared to those which received no antibody. As shown in Chapter 3, the monoclonal antibody 8M6 did neutralize 93% of the *in vitro* activity of this recombinant bovine IL-8. The activation of complement *in vivo* but not *in vitro,* with release of the neutrophil chemoattractant C5a, is one potential explanation for this effect, because murine and human complement are activated by murine immunoglobulin G2a (Neuberger and Rajewsky, 1981; Couderc et al., 1985). A second possibility involves the theory that some cytokine-antibody complexes may be resistant to degradation or excretion, and this may prolong the effective biological half-life of the cytokine (Finkelman et al., 1993; Martens et al., 1993).

Several approaches could be utilized to avoid these problems in future experiments. The simplest method would be to use a higher dose of antibody or an antibody with higher affinity for IL-8; however, the amounts of antibody that would be needed in this model make this a very expensive option, and other monoclonal antibodies specific for ruminant IL-8 are not yet available. A second option is to create a more localized lesion in the lung and neutralize IL-8 by local delivery of antibody. Preliminary calculations indicate that a large volume of antibody would still be required, even in a 200 cm^3 lesion, and there are several technical considerations that may preclude a successful outcome. A third option is to evaluate neutrophil infiltration very early in the course of disease, when IL-8 concentrations are dependent on resident cells rather than inflammatory cell sources. This option may be feasible, but would evaluate neither the role of IL-8 as a mediator of severe disease, nor the therapeutic potential of IL-8-neutralizing drugs in bovine pneumonic pasteurellosis.

Despite the failure to fulfil the primary objective of this experiment, the useful results of this study are a preliminary evaluation of the timing of IL-8 secretion and neutrophil infiltration in the lamb pneumonia model, and a correlation of IL-8 expression with the intensity of neutrophil infiltration during pneumonic pasteurellosis. Firstly, this study provides preliminary evidence for the timing of IL-8 secretion and neutrophil infiltration in pneumonic pasteurellosis. Interleukin-8 concentrations in BAL fluid and neutrophil infiltration of alveoli increased in parallel over time. Interleukin-8 levels and neutrophil numbers were very low in the lamb infected with PI3 virus alone, intermediate in the lamb euthanized 3 hours after challenge with *P. haemolytica*, and high by 8 and 20 hours p.i. These findings support the hypothesis that IL-8 is an important mediator of neutrophil recruitment in this disease, by demonstrating that neutrophil numbers increased in parallel with IL-8 concentrations at various times after challenge. An alternative explanation is that neutrophils themselves are a major source of IL-8 in this disease, so that IL-8 concentrations in BAL fluid do not rise substantially until numerous neutrophils are present. To determine whether either or both of these possibilities are correct, future experiments could analyze additional time points early in the disease and use more lambs per group to obtain data suitable for statistical analysis.

Secondly, in this study the concentration of IL-8 in BAL fluid was correlated with both the extent of neutrophil infiltration into alveoli and the percentage of neutrophils in BAL fluid. As described above and in Section 1.3.5, this supports the hypothesis that IL-8 is an important neutrophil chemoattractant in this disease, but alternatively may reflect the possibility that neutrophils themselves are a major

source of IL-8 in this disease.

In summary, the purpose of this experiment was to define the *in vivo* role of IL-8 as a neutrophil chemoattractant in ovine pneumonic pasteurellosis. This role could not be fully evaluated because of unexpectedly high levels of IL-8 in pneumonic lung, and these were not neutralized by the doses of monoclonal antibody used. The study does show that IL-8 expression and neutrophil infiltration are correlated both in time and in magnitude. It is not possible, however, to conclude whether this correlation is due to IL-8-induced neutrophil recruitment, secretion of IL-8 by neutrophils, or both.

5.0. THE IMPORTANCE OF INTERLEUKIN-8 AS A NEUTROPHIL CHEMOATTRACTANT IN THE LUNGS OF CATTLE AND SHEEP WITH PNEUMONIC PASTEURELLOSIS.

5.1. Introduction

Interleukin-8 (IL-8) is a potent neutrophil chemoattractant in humans and rabbits (Lindley et al., 1998; Beaubien et al., 1990) and has been identified in cattle and sheep (Hassfurther et al., 1994; Seow et al., 1994; Morsey et al., 1996). Interleukin-8 is expressed in the pulmonary lesions of cattle infected experimentally with bovine herpesvirus-1 (BHV-1) and *Pasteurella haemolytica* (Caswell et al., 1998) at levels that induce vigorous neutrophil chemotaxis *in vitro* and *in vivo* (see Chapter 3). These findings suggest that IL-8 may be an important mediator of neutrophil recruitment to the sites of infection with *P. haemolytica*; furthermore, because of the importance of neutrophils in the pathogenesis of this disease (Slocombe et al., 1985; Breider et al., 1988; Weiss et al., 1991), IL-8 could be a necessary factor in the development of severe clinical signs and lesions in pneumonic pasteurellosis.

In humans and in rabbits, interleukin-8 is an important mediator of neutrophil recruitment. In rabbits with lipopolysaccharide-induced pleuritis, neutralization of

IL-8 reduced neutrophil infiltration by 77% (Harada et al., 1994). Similarly, in a model of the acute respiratory distress syndrome induced by intravenous priming with lipopolysaccharide followed by pulmonary challenge with *Streptococcus pyogenes*, treatment of rabbits with an anti-IL-8 antibody resulted in lower mortality rates and less intense neutrophil and fluid exudation into the lung (Yokoi et al., 1997). Finally, neutralization of IL-8 in the pleural exudate from humans with empyema reduced the *in vitro* neutrophil chemotactic activity of these exudates by 65% (Broaddus et al., 1992).

The purpose of this study was to estimate the contribution of IL-8 to the *in vitro* neutrophil chemotactic activity of bronchoalveolar lavage (BAL) fluid from lambs and cattle with experimental pneumonic pasteurellosis. In a previous study, IL-8 depletion reduced the *in vivo* neutrophil chemotactic activity of an aqueous extract of pneumonic bovine lung by 60% (Caswell et al., 1998). This study extends this investigation by analyzing BAL fluid samples from multiple lambs and calves, and by comparing two experimental models of pneumonic pasteurellosis.

5.2. Methods

Bovine BAL fluid was obtained from the lesional portions of the lungs of five calves which had been infected experimentally with bovine herpesvirus-1 (BHV-1) and *P. haemolytica*, as described fully in Chapter 2 (Caswell et al., 1998). Five samples of ovine BAL fluid were harvested at necropsy 3, 8, or 20 hours after bacterial infection, from the cranial or caudal portions of the lungs of 3 lambs which

had been infected with ovine parainfluenza-3 virus and challenged with *P. haemolytica* as described in Sections 4.2.2 and 4.2.3. The levels of IL-8 in these bovine and ovine samples, as determined by ELISA, were reported in Sections 2.3.3 and 4.3.2, respectively.

The *in vitro* chemotactic activity in BAL fluid samples was measured using the chemotaxis assay described in Chapter 3. This procedure was modified to reduce mononuclear cell contamination of the neutrophil preparation to less than 1%, by overlaying the cells on Histopaque density gradient medium (Sigma-Aldrich, Mississauga, ON), centrifuging for 15 minutes at 2000g, and washing the cell pellet once in HBSS. Ovine neutrophils were used in assays evaluating ovine BAL fluid, and bovine neutrophils were used to assess bovine BAL fluid.

A pilot experiment was conducted to determine the optimal dilution of BAL fluid for further study. Serial two-fold dilutions, from undiluted to 1:256, of bovine samples 1 and 2 and ovine samples 1-cranial and 3-cranial were prepared in HBSS. The *in vitro* neutrophil chemotactic activity of these samples was analysed as described above.

A second pilot experiment determined the concentration of antibody required to neutralize IL-8 in the BAL fluid samples. Interleukin-8 was neutralized in the BAL fluid samples by pre-incubation with the monoclonal antibody 8M6, which is a purified mouse IgG2a antibody that neutralizes both ovine and bovine IL-8 (see Section 3.3.2; P.R. Wood, personal communication). The mouse monoclonal immunoglobulin-G2a anti-human-IgE antibody HB121, from the American Type Culture Collection, served as the isotype-matched control. Based on the results of

the previous pilot experiment, a two-fold final dilution of BAL fluid was used, to achieve a half-maximal *in vitro* neutrophil chemotaxis response. Replicates of the ovine BAL fluid samples 1-caudal and 2-caudal were prepared without antibody treatment, and with 15 or 150 μg/ml of the 8M6 and HB121 monoclonal antibodies, for a total of five treatment conditions per BAL fluid sample. All preparations were incubated on ice for 1 hour after the addition of the antibody, to allow neutralization of IL-8, and the samples were then tested in the chemotaxis assay as described above. Bovine BAL fluid samples 1 and 2 were prepared in a similar manner, using final antibody concentrations of 7.0 and 70 μg/ml.

To definitively assess the contribution of IL-8 to the *in vitro* neutrophil chemotactic activity of pneumonic BAL fluid, ovine samples were prepared as described above using two-fold final dilutions of sample and a final antibody concentration of 15 μg/ml. These specifications were based on the results of the two pilot experiments described above. After incubating the cocktail of sample and antibody on ice for 1 hour, the *in vitro* neutrophil chemotactic activity of the samples was analyzed in triplicate as described above, and the chemotaxis membranes were stained and mounted. Neutrophils that had migrated completely to the lower surface of the chemotaxis membrane were counted in a blinded fashion in three fields per replicate using a 40x objective. The three chosen fields were those with the highest concentrations of migrated neutrophils, and were separated by at least three field diameters. The effects of sample and antibody treatments were evaluated using a two-way analysis of variance, and values are expressed as mean ± SEM. All estimates of the importance of IL-8 were based on comparisons between anti-IL-8

antibody-treated samples and control antibody-treated samples, because it has been shown previously that the addition of protein, such as antibody, to the assay augments the migration of neutrophils across the chemotaxis membrane (Wilkinson, 1988).

5.3. Results

To determine the importance of IL-8 as a neutrophil chemoattractant in pneumonic pasteurellosis, the effect of neutralization of this chemokine on the *in vitro* neutrophil chemotactic activity of BAL fluid from calves and lambs with this disease was analyzed. The results of a pilot experiment to determine the optimal dilution of BAL fluid are presented in Figure 5.1. In all samples tested, the *in vitro* neutrophil chemotactic activity was maximal in the undiluted samples. A two-fold dilution of the BAL fluid induced an approximately half-maximal response, and this dilution was used in all subsequent assays. The chemotactic activity of the sample declined progressively with increasing dilutions, and no evidence of differential dilutional effects was present.

A second pilot experiment was conducted to determine the concentration of the IL-8-neutralizing antibody 8M6 required to reduce the *in vitro* neutrophil chemotactic activity of the BAL fluid. For the bovine samples tested, neutrophil chemotaxis was reduced in samples pre-incubated with 70 μg/ml of 8M6 antibody compared to those treated with the same amount of HB121 antibody, but this effect was not induced by 7 μg/ml of antibody (Figure 5.2A). In both ovine samples tested,

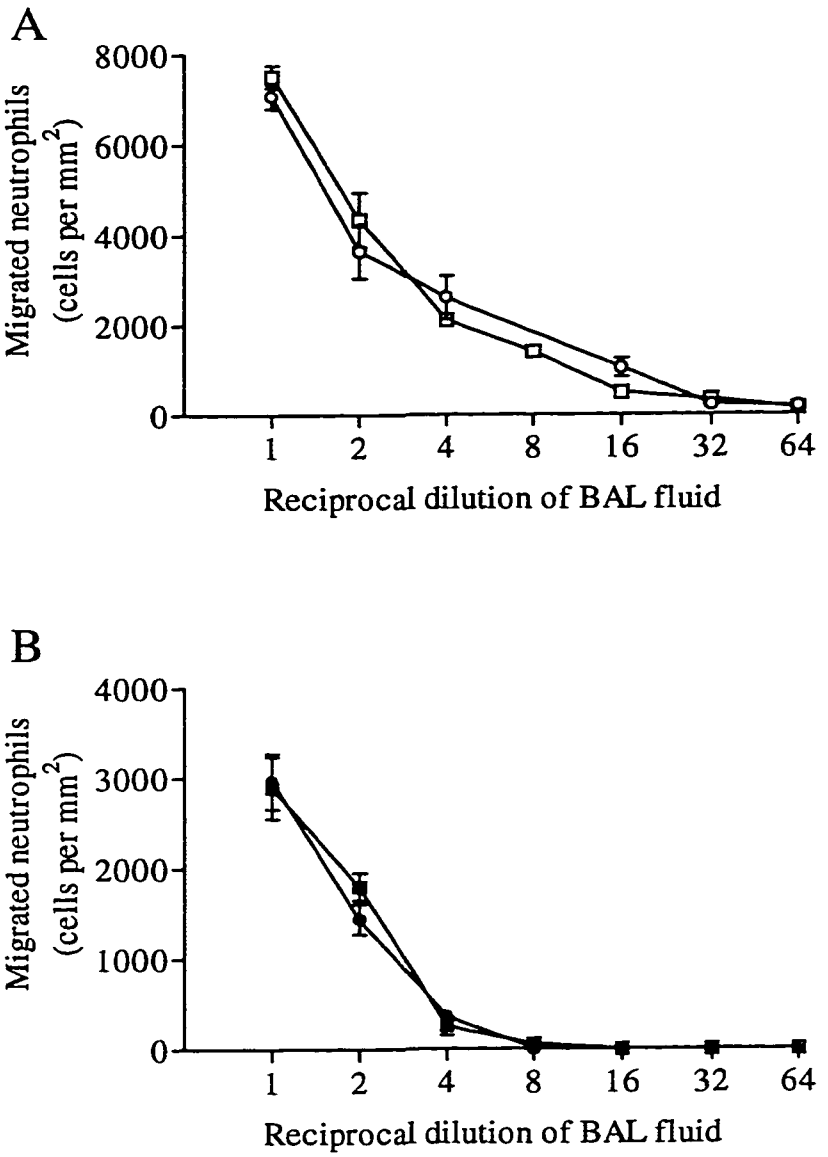

Figure 5.1. The *in vitro* neutrophil chemotactic activity of serial dilutions of bronchoalveolar lavage fluid samples, from calves (A) and lambs (B) with experimental pneumonic pasteurellosis. Bovine samples 1 (open circles) and 2 (open squares), and ovine samples 1-cranial (closed circles) and 2-cranial (closed squares), as identified in Table 5.1, were analyzed using species-matched neutrophils in separate assays. The neutrophil chemotactic activity was half-maximal activity at a two-fold dilution of BAL fluid, and declined progressively thereafter.

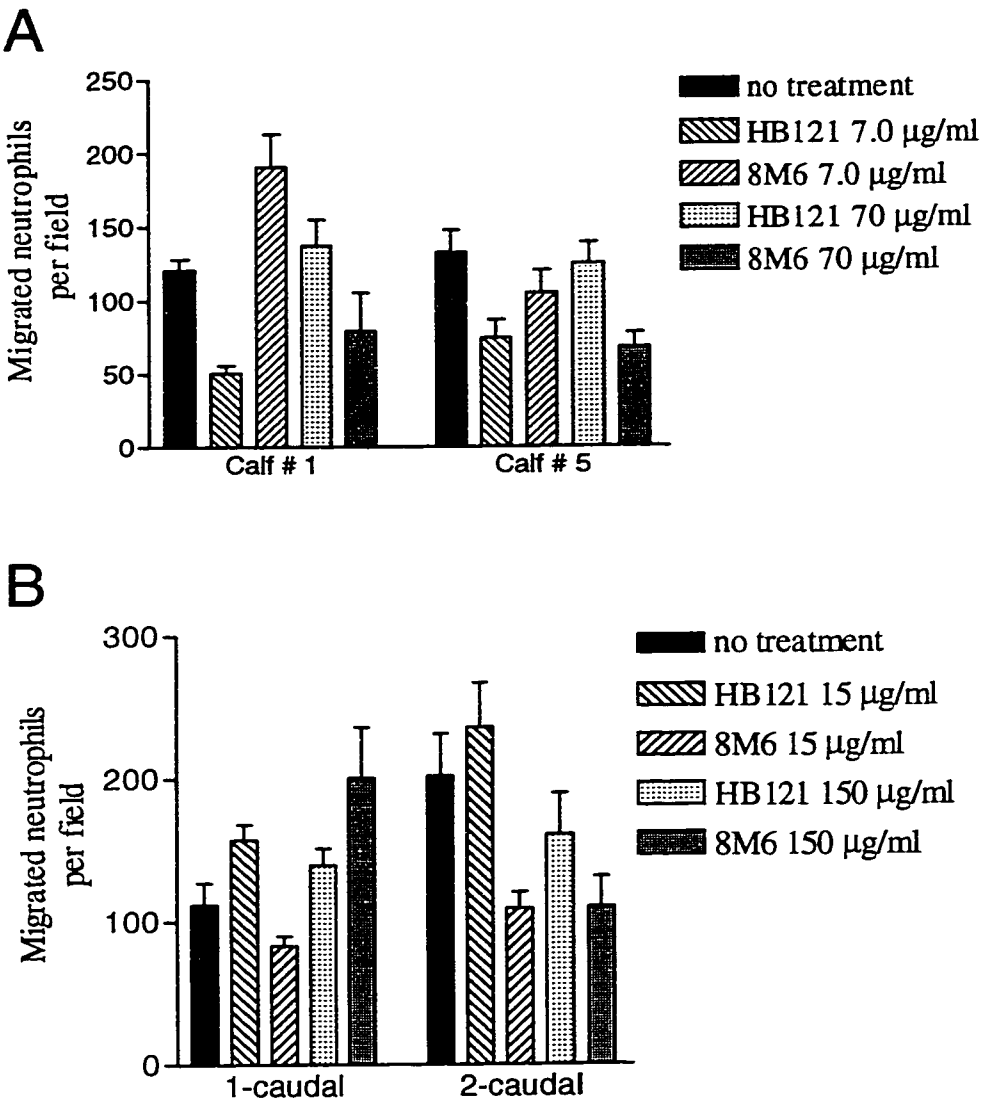

Figure 5.2. Pilot experiment to determine the concentration of antibody required to neutralize IL-8 in bronchoalveolar lavage (BAL) fluid samples from calves (A) and lambs (B) with experimental pneumonic pasteurellosis. **A**: In bovine samples 1 and 5, neutrophil chemotaxis was reduced by treatment with 70 µg/ml of the anti-IL-8 antibody 8M6, compared to treatment with the control antibody HB121. **B**: A similar effect was induced by 15 µg/ml antibody in ovine samples 1-caudal and 2-caudal.

an antibody concentration of 15 μg/ml of the IL-8-neutralizing antibody 8M6 reduced neutrophil chemotaxis, compared to the same concentration of isotype-matched control antibody HB121, while a reduction in chemotactic activity was observed with 150 μg/ml of antibody in one sample (Figure 5.2B). On this basis, antibody concentrations of 70 and 15 μg/ml were used in the next experiment for the bovine and ovine samples, respectively.

The final experiment was designed to measure the contribution of IL-8 to the *in vitro* neutrophil chemotactic activity of bovine and ovine pneumonic BAL fluid samples. In the bovine samples, neutrophil chemotactic activity was reduced, on average, by 35.8% in samples pre-incubated with the anti-IL-8 antibody 8M6 compared to those treated with the isotype-matched control antibody HB121 (Figure 5.3A). This reduction was demonstrated in all samples, from a minimum of 14.7% to a maximum of 59.5%, and was highly significant using two-way analysis of variance ($p<0.001$). The BAL fluid sample with the highest percent reduction in neutrophil chemotactic activity was from the same calf as the lung extract analysed previously, in Section 2.3.4.

Pre-treatment of ovine BAL fluid with the anti-IL-8 antibody 8M6 reduced the neutrophil chemotactic activity by 20.2%, on average, compared to that of the samples treated with the control antibody HB121 (Figure 5.3B). This reduction in neutrophil chemotactic activity was present in 4 of the 5 samples of BAL fluid (Table 5.1), and was highly significant using two-way analysis of variance ($p<0.001$).

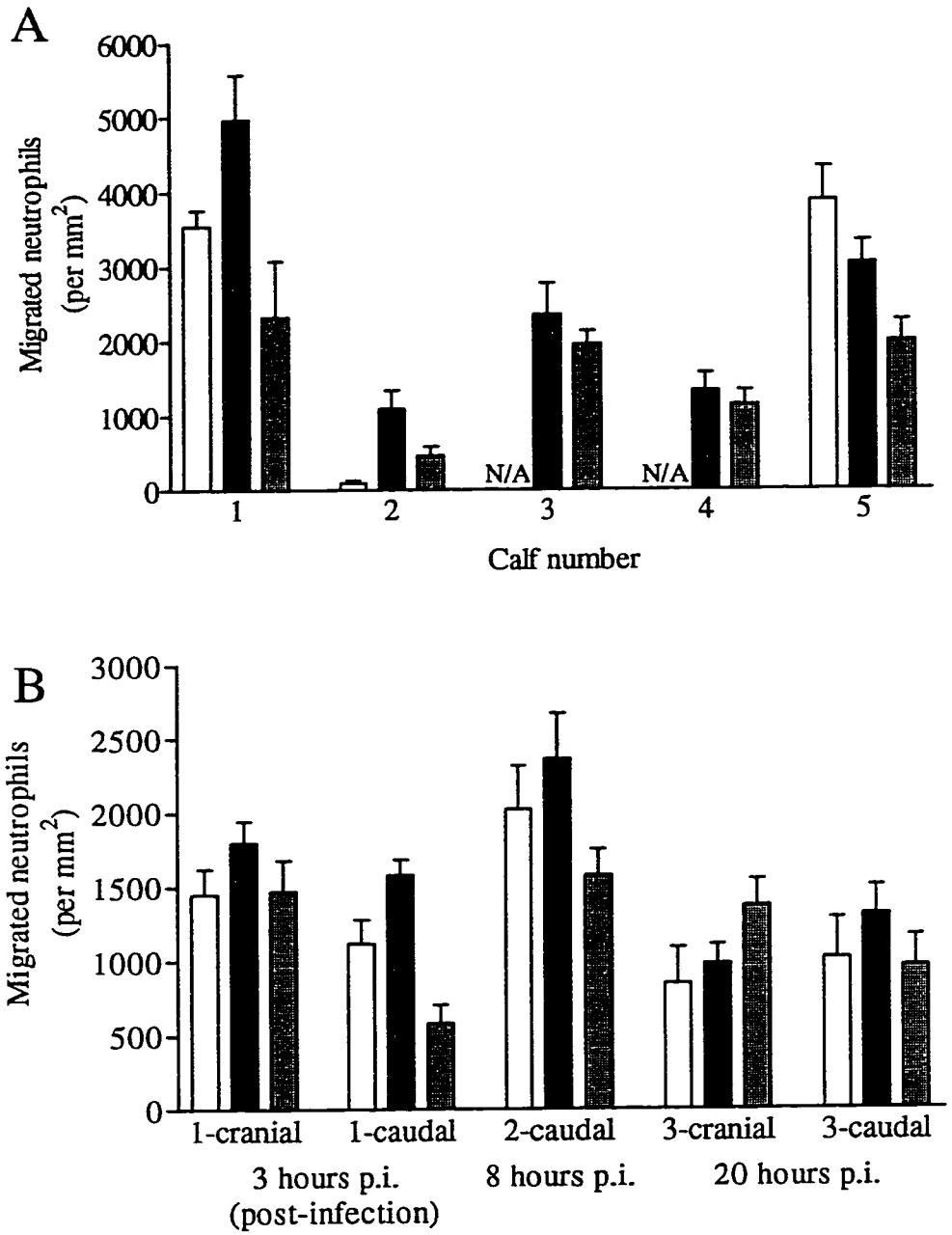

Figure 5.3. The effect of neutralization of IL-8 on the *in vitro* neutrophil chemotactic activity of bovine (A) and ovine (B) pneumonic bronchoalveolar lavage fluid. Samples that were not treated with antibody are indicated by white bars. Compared to samples treated with the control antibody HB121 (black bars), treatment with the IL-8-neutralizing antibody 8M6 (stippled bars) reduced neutrophil migration in 9 of 10 samples. N/A: data not available for bovine samples 3 and 4 without antibody treatment.

Table 5.1. The interleukin-8 (IL-8) concentrations in bronchoalveolar lavage fluid samples from calves and lambs with experimental pneumonic pasteurellosis, and percentage of neutrophil chemotactic activity in these samples attributed to IL-8.

Species	Sample	Time after infection[1]	Chemotaxis attributed to IL-8 (%)[2]	Concentration of IL-8 (ng/ml)[3]
Bovine	1	48 hours	53.2	33.8
Bovine	2	72 hours	59.5	6.5
Bovine	3	72 hours	17.0	11.5
Bovine	4	96 hours	14.7	15.0
Bovine	5	98 hours	34.4	31.0
Ovine	1-cranial	3 hours	18.2	158.1
Ovine	1-caudal	3 hours	63.3	62.2
Ovine	2-caudal	8 hours	33.4	672.5
Ovine	3-cranial	20 hours	-	437.7
Ovine	3-caudal	20 hours	26.3	577.8

[1] Time after infection with *Pasteurella haemolytica.*
[2] Percent reduction of *in vitro* neutrophil chemotactic activity of bronchoalveolar lavage fluid treated with anti-IL-8 antibody compared to control antibody.
[3] Interleukin-8 concentrations in the BAL fluid samples, determined in Chapters 2 and 4.

Overall, the concentration of IL-8 in BAL fluid samples was not significantly correlated with the percent reductions in neutrophil chemotactic activity attributable to IL-8 ($r=0.33$, $p=0.36$)(Table 5.1). Similarly, the magnitude of this difference did not correlate with the time interval between bacterial infection and collection of the BAL fluid (Table 5.1). There was no significant difference between the bovine and ovine samples in the mean percent reduction in chemotactic activity.

5.4. Discussion

This study evaluated the effect of a neutralizing antibody against IL-8 on the *in vitro* neutrophil chemotactic activity of bronchoalveolar lavage fluid from calves and lambs with experimental pneumonic pasteurellosis. The findings imply that IL-8 is a significant neutrophil chemoattractant in pneumonic pasteurellosis, and that 15 to 63% of the neutrophil chemotactic activity in pneumonic BAL fluid may be attributable to IL-8. This effect was highly significant for both bovine and ovine samples, and the difference between the bovine and ovine models was insignificant. In 7 of 8 samples in which it could be assessed, neutrophil chemotaxis was more vigorous in BAL fluid treated with the control antibody HB121 than in untreated samples. This phenomenon has been described as a non-specific effect of the addition of protein to the medium, which probably enhances the motility of leukocytes by reducing their attachment to the chemotaxis membrane (Wilkinson, 1988).

It is likely that pneumonic BAL fluid contains several mediators that are capable of attracting neutrophils to alveoli. Mediators of neutrophil chemotaxis in cattle include the ELR-CXC chemokines IL-8, ENA and GRO, the complement-derived anaphylatoxin C5a, leukotriene B4, and platelet activating factor (Persson et al., 1993; Allmann-Iselin et al., 1994; Bochsler et al., 1994; Hassfurther et al., 1994; Rogivue et al., 1995). Formylated bacterial peptides, which induce strong chemotaxis of neutrophils in humans and laboratory animals, do not attract bovine neutrophils (Gray et al., 1982; Forsell et al., 1985). It would be surprising if a single mediator was solely responsible for neutrophil chemotaxis, given this diversity and redundancy of potential agonists and the importance of neutrophil responses in the defence against bacterial infection. Nevertheless, the demonstration that 15% to 63% of the neutrophil chemotactic activity can be attributed to IL-8 indicates a potentially important role for this chemokine in the recruitment of neutrophils into diseased bovine and ovine lungs. The notion that IL-8 is unique among the neutrophil chemoattractants is supported by the high affinity of IL-8 for the CXC chemokine receptors (CXCR) 1 and 2 in humans, whereas the other ELR-CXC chemokines bind with high affinity to CXCR2, but not to CXCR1 (Ahuja and Murphy, 1996).

The overall importance of IL-8 estimated in this study is lower than predicted previously, where 60% of the neutrophil chemotactic activity in a single aqueous extract of lung from a calf with experimental pneumonic pasteurellosis was attributed to IL-8 (Caswell et al., 1998). However, because IL-8 was responsible for 59.5% of the neutrophil chemotactic activity in BAL fluid obtained from the same animal that provided this aqueous lung extract, the lower estimated importance of IL-8 suggested

by the current study may simply reflect differences among individual cases of pneumonic pasteurellosis. Compared to the earlier work, the current study analysed a larger number of samples, examined BAL fluid rather than lung extract, and evaluated *in vitro* rather than *in vivo* neutrophil chemotactic activity. An analysis of *in vitro* rather than *in vivo* neutrophil chemotactic activity may be more appropriate because the latter also measures the effect of indirect neutrophil chemoattractants such as lipopolysaccharide, TNF-α and IL-1. Finally, because the efficiency of IL-8 neutralization could not be measured, this study provides a conservative estimate of the percentage of neutrophil chemotactic activity that is attributable to IL-8.

The biologic significance of a 15% to 63% reduction in neutrophil chemotaxis cannot be predicted with certainty. It could be argued that such a reduction might result in no detectable effect on bacterial killing or neutrophil-mediated tissue injury. Conversely, a relatively minor reduction in neutrophil infiltration early in the course of disease could be of great significance, because it may prevent bacterial killing at a critical phase in the disease or, alternatively, quell the injurious inflammatory reaction before the stage of amplification by neutrophil-derived cytokines (Casatella, 1995).

Caution must be exercised in applying these *in vitro* findings to the study of disease *in vivo*. The estimated proportion of the neutrophil chemotactic activity that was attributed to IL-8 could be falsely high or low, if neutrophil chemoattractants were present that interacted with IL-8 in a synergistic or antagonistic manner, respectively. Additionally, it is probable that redundant mediators of neutrophil chemotaxis are present in these BAL fluid samples. Although IL-8 in the sample

might be sufficient to induce neutrophil chemotaxis, neutralization of IL-8 may not necessarily confer a reduction in chemotactic activity because of the presence of these redundant mediators.

To date, only one other published study has critically evaluated the importance of specific neutrophil chemoattractants in cattle (Persson-Waller, 1997). In that study, lipopolysaccharide, with or without a PAF antagonist or an inhibitor of LTB4 synthesis, was infused into the teat cisterns of cows and neutrophil accumulation was studied. Administration of the PAF antagonist or the inhibitor of LTB4 synthesis reduced neutrophil accumulation by up to 30% or 36%, respectively, although this effect was dependent on both the dose of LPS and the time after infusion. It is important to consider that these results are based on continuous neutralization of the inflammatory mediator during the response to LPS, whereas the current study evaluates the contribution of IL-8 at a specific time after infection. The findings in the lamb euthanized at only 3 hours after infection (ovine samples 1-cranial and 1-caudal), however, suggest that the contribution of IL-8 to total neutrophil chemotaxis may be similar in both the early and the well-developed stages of disease.

In summary, this study indicates that IL-8 is a significant neutrophil chemoattractant in pneumonic pasteurellosis, and is responsible for 15% to 63% of the neutrophil chemotactic activity in BAL fluid samples from calves and lambs with pneumonic pasteurellosis. This supports the concept that IL-8 is an effective neutrophil chemoattractant in this disease, but suggests that other mediators with redundant actions are also present.

6.0. GENERAL DISCUSSION.

The purpose of this research project was to investigate the role of interleukin-8 (IL-8) as a neutrophil chemoattractant in the lesions of bovine pneumonic pasteurellosis, and to evaluate the contribution of this chemokine to neutrophil-dependent tissue injury. The primary reason for undertaking these studies was to provide insight into mechanisms of neutrophil recruitment in cattle. Bacterial infections are common and of great economic importance in ruminants, and an understanding of the mechanisms underlying neutrophil responses are of intrinsic interest to those involved in the study of ruminant disease.

Secondly, it was anticipated that neutralization of IL-8 might reduce the clinical severity or chronic complications of this disease, because neutrophil-dependent injury to lung tissue is an important contributor to the lesions and clinical signs of bovine pneumonic pasteurellosis. The demonstration of this effect in an experimental setting could, therefore, provide the impetus for the development of pharmaceutical products that specifically neutralize IL-8 as an adjunctive treatment in overwhelming bacterial infections of humans and animals.

Finally, there is substantial interest in understanding mechanisms of neutrophil recruitment in humans; however, ethical concerns preclude many definitive experimental investigations of the importance of IL-8 as a human neutrophil chemoattractant. Comparative pathology provides an alternative but

complementary approach, whereby preliminary insights derived from human systems may be tested rigorously in more readily manipulable species. An important difficulty with this approach is that humans and rodents differ significantly in mechanisms of neutrophil recruitment; in particular, IL-8 is apparently an essential neutrophil chemoattractant in humans, yet rodents lack an IL-8 homologue. Because of this, studies that have investigated the *in vivo* role of IL-8 using methods amenable to experimental manipulation have been restricted mainly to rabbits. Therefore, an evaluation of the importance of this chemokine in a species other than rabbits is one method of providing reassurance that concepts derived from rabbit models are applicable to human pathophysiology.

There were three related components to this project: an investigation of the expression of IL-8 in the lesions of bovine pneumonic pasteurellosis, the production of recombinant bovine IL-8 and characterization of its effects *in vitro* and *in vivo*, and an evaluation of the importance of IL-8 as a neutrophil chemoattractant in pneumonic pasteurellosis of ruminants. The intent of these experiments was to prove that (1) bovine IL-8 is able to induce neutrophil recruitment, (2) IL-8 is expressed in the lesions of pneumonic pasteurellosis at levels sufficient to fulfil this function, and (3) IL-8 is an essential stimulus for neutrophil infiltration of these lesions.

The first step in evaluating the role of IL-8 as a neutrophil chemoattractant in bovine pneumonic pasteurellosis was to confirm that bovine IL-8 could, in fact, recruit neutrophils into tissues. Although Morsey et al (1996) had produced recombinant bovine IL-8 (rbIL-8) as a fusion protein with β-galactosidase, such

recombinant cytokines produced as fusion proteins often lack or have reduced biological effect, despite retaining their antigenic properties. For this reason, rbIL-8 was generated using the pGEX expression system, which allowed purification of the fusion protein and subsequent cleavage of the native chemokine from the carrier protein. The rbIL-8 had the expected physical and antigenic characteristics: it was about 8 kDa in size and it reacted with polyclonal antiserum to ovine IL-8 in a Western blot.

The rbIL-8 induced migration of neutrophils in an *in vitro* chemotaxis assay, whereas eosinophil transmigration was rare under the conditions tested. Neutrophil responses induced by IL-8 were dose-dependent in *in vitro* chemotaxis and shape change assays, from 6.3 ng/ml to 7.9 μg/ml and 1.5 ng/ml to 1.5 μg/ml, respectively.

Intradermal injection of rbIL-8 induced strong neutrophil infiltration, in contrast to a previous study where infusion of human IL-8 into the teat cisterns of cows did not elicit neutrophil exudation (Persson et al., 1993). This *in vivo* neutrophil recruitment was dose-dependent from 1.0 ng up to 3.3 μg, the highest dose tested. The changes induced by IL-8 were rapid: neutrophils marginated in dermal capillaries within 15 minutes of injection, and migrated across the vessel wall by 1 hour after injection.

The neutrophils summoned to the sites of rbIL-8 administration developed morphologic features of apoptosis between 18 and 30 hours after injection. As described in Section 1.3.4, neutrophil activation probably requires stimulation of two distinct signalling pathways. Interleukin-8 and other chemokines stimulate G-protein-linked receptors whereas TNF-α, IL-1 and GM-CSF trigger tyrosine kinases,

and these latter three cytokines are known to delay apoptosis and prolong survival of cultured neutrophils (Colotta et al., 1992). On this basis, it is likely that neutrophils responding to IL-8 alone have a limited life-span and undergo apoptosis; in contrast, the milieu of the infected lung would contain a deluge of other mediators, including IL-1, TNF-α and GM-CSF, that could prevent apoptosis and prolong neutrophil life-span.

The second step in evaluating the role of IL-8 as a neutrophil chemoattractant in bovine pneumonic pasteurellosis was to demonstrate that it was secreted in this disease, and that the magnitude of secretion and the anatomic and temporal patterns of expression were appropriate for this putative function. Chapter 2 provides definitive evidence that IL-8 mRNA is expressed and IL-8 protein is secreted in the well-developed lesions of acute bovine pneumonic pasteurellosis. Furthermore, the levels of IL-8 in the lesions of pneumonic pasteurellosis were much higher than in pneumonia caused by bovine respiratory syncytial virus, suggesting that selective expression of IL-8 occurs in bacterial pneumonia.

The magnitude of IL-8 secretion in pneumonic lung is probably sufficient to induce neutrophil recruitment. In calves experimentally infected with BHV-1 and *P. haemolytica*, BAL fluid and lung extact from lesional areas of lung contained, on average, 16 ± 4 ng/ml and 76 ± 15 ng/g, respectively. These levels should be adequate to recruit neutrophils *in vitro* and *in vivo*, based on the ability of 6.3 ng/ml and 1.0 ng of rbIL-8 to induce neutrophil chemotaxis *in vitro* and *in vivo*, respectively. Furthermore, because BAL fluid is highly diluted compared to the

alveolar lining fluid, these concentrations should incite robust neutrophil responses in inflamed lung tissue.

Interleukin-8 concentrations were markedly higher in the ovine than in the bovine model of pneumonic pasteurellosis. As with the bovine samples, the concentrations of IL-8 in pneumonic sheep lung should be adequate to induce neutrophil infiltration, based on the doses of recombinant ovine IL-8 required for *in vitro* and *in vivo* responses (Seow et al., 1994). The cause of the higher levels of IL-8 in sheep lung was not specifically determined, but could relate to differences in species, age, the viruses used in the respective models, the time interval between bacterial infection and sampling, and procedural changes in the ELISA.

The anatomic relationship between IL-8 expression and the lesions of pneumonia was examined using *in situ* hybridization. Expression of IL-8 mRNA was most intense in the neutrophils and macrophages that formed the alveolar exudate. This finding of expression of the neutrophil chemoattractant IL-8 by neutrophils themselves suggests that IL-8 may at least play a role in amplifying the recruitment of neutrophils to the sites of bacterial infection. It is reasonable to speculate that ongoing stimulation of macrophages and neutrophils by bacterial toxins or locally produced cytokines may incite further production of IL-8 in an attempt to recruit neutrophils to control the infection; in contrast, when this infection is controlled, the reduced presence of bacterial toxins and proinflammatory cytokines may not stimulate sufficient IL-8 production by these cells to promote ongoing neutrophil recruitment.

In contrast to this role in the mustering of neutrophils to sites of established inflammation, the initial influx of neutrophils may be regulated by a distinct set of chemoattractants that are secreted by resident cells. A role for IL-8 in this process is suggested by the finding that IL-8 was already present at substantial levels in the lamb that was euthanized 3 hours after challenge with *P. haemolytica*, despite the presence of only low numbers of neutrophils. In addition, IL-8 mRNA was expressed in resident cells such as alveolar macrophages and bronchiolar epithelium, and occasionally in alveolar epithelium, interstitial macrophages and fibroblasts, and pleural mesothelial cells, in the advanced lesions of bovine pneumonic pasteurellosis.

In summary, the concentrations of IL-8 that are present in the lesions of pneumonic pasteurellosis are adequate to induce neutrophil recruitment, at least into skin, and the anatomic and temporal aspects of IL-8 expression in this disease support such a role in the lung. In assessing the importance of IL-8 as a neutrophil chemoattractant in this disease, these findings do not take into account the effect of redundant mediators of neutrophil chemotaxis or the possibility of local inhibitors of IL-8; nevertheless, these findings are consistent with the following proposed model of neutrophil recruitment to the sites of infection with *P. haemolytica.*

1. Factors released from bacteria at the site of infection induce resident alveolar macrophages to secrete TNF-α and IL-1 as well as IL-8 and other neutrophil chemoattractants.
2. This early secretion of chemoattractants rapidly attracts neutrophils to the sites of bacterial infection.

3. The proinflammatory cytokines TNF-α and IL-1 induce IL-8 secretion from a wide variety of resident and newly recruited cells which, because of the number of these cells present relative to that of alveolar macrophages, may greatly augment the levels of IL-8 present.
4. As a result of these higher IL-8 concentrations, large numbers of neutrophils infiltrate the site of infection. These newly recruited neutrophils are stimulated by proinflammatory cytokines to produce even higher local concentrations of IL-8, which augment the ongoing recruitment of neutrophils to this active site of infection.
5. Down-regulation of this response may depend on clearance of bacteria and subsequent cessation of the original stimulus, reduced action of IL-8 because of a short half-life or the secretion of inhibitors, and reduced effect of IL-8 as a result of receptor desensitization or the secretion of mediators that have antagonistic actions to that of IL-8.

The third step in evaluating the role of IL-8 as a neutrophil chemoattractant in bovine pneumonic pasteurellosis was to demonstrate that IL-8 is an essential stimulus for neutrophil infiltration of these lesions. Three approaches were used in this evaluation. Firstly, a correlation was established between the concentrations of IL-8 in BAL fluid and the degree of neutrophil infiltration in the lesions. Secondly, IL-8 was neutralized in samples of pneumonic lung fluids, and the effect of this neutralization on the subsequent *in vitro* and *in vivo* neutrophil chemotactic activity of this fluid was measured. Finally, lambs were infected experimentally with *P.*

haemolytica, and the consequence of administering a neutralizing antibody to IL-8 on the development of disease was studied.

Chapter 4 was intended to be the first segment in a definitive study of the importance of IL-8 as a stimulus for neutrophil infiltration, and thus for tissue damage, in pneumonic pasteurellosis of ruminants. This first component was designed to establish an optimal dose of antibody, with the idea of repeating the study using that dose to obtain data suitable for statistical analysis. There are several reasons that this study was unsuccessful, but the strikingly high level of IL-8 expression in this model is probably the major factor. It is unlikely that modifications to this model of pneumonic pasteurellosis would reduce IL-8 concentrations enough to allow neutralization with the antibody 8M6, and still maintain clinical signs and lesions of sufficient severity to allow the extrapolation of findings to the naturally occurring disease. As a result, an alternative method of investigation was developed.

A positive outcome of this study was the demonstration of a relationship between IL-8 levels in pneumonic BAL fluid and the degree of neutrophil infiltration in these lesions. Firstly, in the 24 samples of BAL fluid from six pneumonic lambs described in Section 4.3.3, the concentration of IL-8 in BAL fluid was correlated with both the histologic extent of neutrophil infiltration and the percentage of neutrophils in BAL fluid. Secondly, IL-8 concentrations in BAL fluid ranged from 0.11 to 0.51 μg/ml in the 3 pneumonic lambs with relatively mild clinical signs and lesions that were described in Section 4.3.2; in contrast, 1.0 to 2.6 μg/ml of IL-8 was measured in BAL fluid from the most affected areas of lung in the 6 severely

pneumonic lambs discussed in Section 4.3.3. These results indicate that IL-8 concentrations are related to the severity of the disease and to the extent of neutrophil infiltration, but do not determine whether this is relationship is an effect of neutrophil recruitment in response to IL-8, IL-8 secretion from activated neutrophils or other cells, or a combination of these.

An alternative method of examining the importance of IL-8 as a neutrophil chemoattractant in pneumonic pasteurellosis was described in Section 2.3.4 and in Chapter 5. Here, the neutrophil chemotactic activity of pneumonic BAL fluid samples was measured using *in vitro* or *in vivo* assays, and the effect of neutralizing IL-8 in these samples was examined. Neutralization of IL-8 reduced the *in vitro* neutrophil chemotactic activity of pneumonic BAL fluid by 15% to 63%. In a single sample of lung extract, IL-8 neutralization conferred a 60% reduction of *in vivo* neutrophil chemotactic activity. These results imply that IL-8 is responsible for up to 63% of the neutrophil-recruiting ability of pneumonic lung fluids, and suggest that this chemokine is an important chemoattractant in both the early and well-developed lesions of this disease.

This analysis of the effect of IL-8 neutralization on chemotactic activity of pneumonic lung fluids must be interpreted with regard to the limitations of the method. Firstly, the effect of synergism or antagonism between mediators of neutrophil chemotaxis is not considered in this method, redundant effects among the various mediators may influence the interpretation of results, and the principle that cytokines have optimal concentrations for biological effect must be reconciled with the situation *in vivo*. Secondly, the biologic relevance of this magnitude of reduction

in neutrophil chemotaxis is uncertain, and probably depends on the impact of this reduction on the balance of bactericidal and pro-inflammatory effects of neutrophils in the early stages of disease. Finally, determining whether this abrogation of neutrophil responses improves or worsens the clinical severity of bacterial pneumonia will require an application of these findings to *in vivo* models of disease.

In summary, the major findings in this project with regard to the experimental models used are:

1. Recombinant bovine interleukin-8 induces selective recruitment of neutrophils *in vitro* and *in vivo*, at concentrations similar to those described in other species.
2. Interleukin-8 and IL-8 mRNA are expressed in the well-developed lesions of cattle experimentally infected with BHV-1 and *P. haemolytica*, at levels that are capable of inducing *in vitro* and *in vivo* neutrophil chemotaxis. Lower levels are detected in non-lesional lung in bacterial pneumonia and in the lesions of pneumonia caused by BRSV, and IL-8 mRNA is expressed at a low level in normal bovine lung.
3. In the well-developed lesions of bovine pneumonic pasteurellosis, alveolar neutrophils and macrophages express IL-8 mRNA at high levels, with more mild expression in bronchiolar and alveolar epithelium, interstitial macrophages and fibroblasts, and pleural mesothelial cells.
4. There is a highly significant correlation between the levels of IL-8 in pneumonic BAL fluid and measures of neutrophil infiltration, in lambs experimentally infected with parainfluenza-3 virus and *P. haemolytica*.

5. The contribution of interleukin-8 to the *in vitro* neutrophil chemotactic activity in pneumonic BAL fluid is variable between cases. On average, IL-8 accounts for approximately 36% and 20% of this *in vitro* activity in calves and lambs, respectively, and for 60% of the *in vivo* neutrophil chemotactic activity in a single lung extract using the bovine model.
6. Interleukin-8 concentrations in lambs infected with PI3 virus and *P. haemolytica* are apparently 10- to 100-fold higher than in calves experimentally infected with bovine herpesvirus-1 and *P. haemolytica.*

Future studies of neutrophil chemoattractants in cattle must confirm the importance of IL-8 in an *in vivo* setting. As previously stated, this may be impossible using the monoclonal antibody 8M6; therefore, these studies may need to develop alternative methods of neutralizing IL-8, such as an antibody with a greater neutralizing capacity, the development of an IL-8 receptor antagonist, or production of soluble IL-8 receptors. The latter two approaches have the potential advantage of neutralizing not only IL-8, but also the other ELR-CXC chemokines that may provide a redundant effect.

There is a need for similar studies to be performed concurrently on other neutrophil chemoattractants, including C5a, leukotriene B4, and the chemokines GRO-α and ENA. The initial phase of these evaluations should examine whether these mediators interact in an additive, synergistic or antagonistic manner *in vitro.* This has relevance in interpreting *in vivo* or *in vitro* experiments that evaluate the effect of neutralization of one mediator on subsequent neutrophil responses - the

estimate of the relative importance of this mediator as a neutrophil chemoattractant assumes that all such interactions are additive.

Finally, the role of specific chemoattractants should be evaluated in a variety of disease processes to determine general principles of neutrophil recruitment. It seems probable, however, that different neutrophil chemoattractants may serve differing roles depending on the type of insult, the specific anatomic location, and the phase of the inflammatory response. Elucidating these details of neutrophil recruitment will be challenging, but the prize will be insight into an important aspect of the inflammatory response and the host defence against infection, and the potential to modulate this response when it is no longer beneficial.

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APPENDIX A: RESPONSE OF THE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) TO OVINE AND BOVINE IL-8

The data presented in this appendix detail the differences in the sensitivity of the IL-8 ELISA system, which uses the monoclonal antibody 8M6 as the capture antibody and rabbit antiserum to ovine IL-8 as the detection antibody, for the measurement of ovine and bovine recombinant IL-8. This difference could be one reason for the higher apparent levels of IL-8 in pneumonic bronchoalveolar lavage (BAL) fluid from pneumonic lambs compared to calves, as discussed in Section 4.4.

Recombinant ovine interleukin-8 (roIL-8) was obtained from Dr. Paul Wood, CSIRO Division of Animal Health, Parkville, Victoria, Australia; the purity and protein concentration of this sample had been determined previously (Seow et al., 1994). The recombinant bovine IL-8 (rbIL-8) tested was that described fully in Chapter 3; the first and second eluate samples were tested separately. Serial 3.16-fold dilutions of these recombinant proteins were prepared in PBST containing 1% sodium casein, and tested using the ELISA system described in Chapter 2.

The reactivity of these recombinant proteins in the ELISA system is illustrated in Figure A1. As indicated in this graph, 0.69 ng/ml roIL-8 and 0.69 ng/ml rbIL-8 resulted in identical absorbance values of 0.08. In contrast, a five-fold higher concentration of roIL-8 compared to rbIL-8 was required for an absorbance value of 0.39.

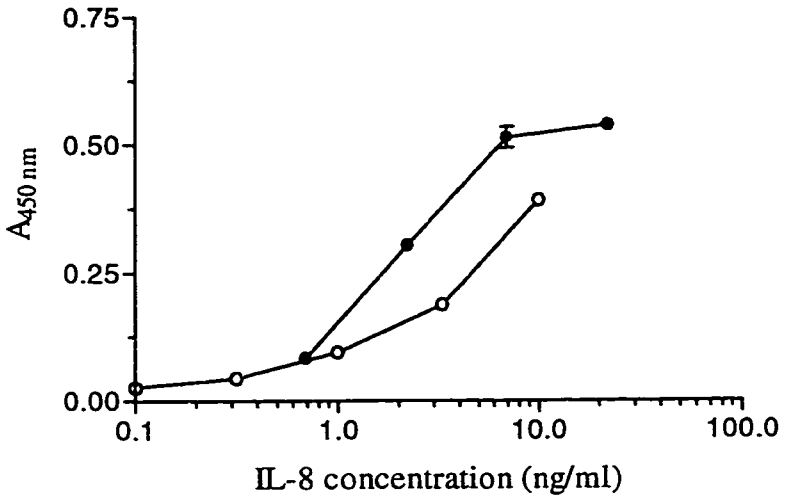

Figure A1. A comparison of the ability of the ELISA system to detect recombinant ovine (open circles) and bovine (closed circles) interleukin-8.

These data indicate that differences in the reactivity of rbIL-8 and roIL-8 in this ELISA system may contribute to the higher apparent IL-8 concentrations in BAL fluid from lambs compared to calves with experimental pneumonic pasteurellosis. In Chapter 2, interleukin-8 concentrations in bovine BAL fluid were measured using roIL-8 as a standard; in contrast, in Chapter 4, the detection of IL-8 in ovine BAL fluid was based on the rbIL-8 standard. Because up to five times more roIL-8 than rbIL-8 was required for the same absorbance value, it is possible that, in assays using roIL-8 as a standard, the calculated sample IL-8 concentration may be up to five-fold higher than that predicted by an assay using rbIL-8 as a standard.

The significance of this difference in reactivity is probably minimal for two reasons. Firstly, the actual magnitude of this difference is likely to be less than five-fold, because, (a) in the assays that measured IL-8 concentrations in pneumonic samples, the linear portion of the standard curve was typically from 0.03 to 1.0 ng/ml, and (b) in this range, rbIL-8 and roIL-8 gave similar absorbance readings in the ELISA shown in Figure A1.. Secondly, even a five-fold difference in reactivity of roIL-8 compared to rbIL-8 cannot explain the 100-fold higher IL-8 concentrations measured in BAL fluid from lambs compared to calves with pneumonic pasteurellosis. Other factors, as discussed in Section 4.4, may be the cause of this difference in IL-8 concentrations.