ANTI-PROTEASE GENE THERAPY IN THE LUNG

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Submitted for the degree of Doctor of Philosophy,

University of Edinburgh, 2004

DECLARATION

I hereby declare that this thesis has been composed entirely by myself and that no part of this work has been submitted for any other degree or professional qualification. All work presented in this thesis was executed by myself except where otherwise acknowledged.

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ABSTRACT

Previous work has demonstrated the importance of neutrophil derived proteolytic enzymes in the pathogenesis of acute pulmonary inflammation. The protease/anti-protease balance theory of lung disease relies on the presence of substances within the lung which inhibit these enzymes. These include alpha-1-antiprotease, secretory leukoprotease inhibitor (SLPI) and elafin. Indeed these inhibitors have been used to modulate the inflammatory response both *in vitro* and *in vivo*. Whilst only limited data currently exists in relation to the ovine orthologs of these inhibitors the potential of this species as an intermediate model for lung-directed gene therapy has recently been highlighted and warrants further applied research. This thesis is directed towards this aim.

The first section details the cloning and characterisation of the ovine forms of SLPI and elafin. The ovine orthologs show many characteristics in common with the human forms of these proteins including distributions at mucosal sites and activity against neutrophil elastase *in vitro*. The identification of the sequences of the genes encoding ovine SLPI and elafin has expanded the current knowledge based on members of the whey acidic protein (WAP) and Trappin families of protein.

The ovine elafin sequence facilitated the construction of a replication-deficient adenovirus (Ad-o-elafin) for use as an efficient vector in lung-directed gene therapy in the context of lipopolysaccharide (LPS)-mediated direct lung injury. The data demonstrates that local lung administration of Ad-o-elafin up-regulates the innate immune response to intra-pulmonary LPS both within areas of the lung directly exposed to the virus and also in spatially disparate lung segments not receiving adenovirus. Additionally, the endobronchial administration of Ad-o-elafin abrogates the circulating leukocytosis seen as a result of intra-pulmonary LPS instillation when compared to control adenovirus. This suggests a role for local elafin up-regulation in the stimulation of the innate immune response with a concomitant decrease in the systemic response.

These data expand a relatively narrow knowledge base in relation to lung-directed adenoviral-mediated gene therapy in large animal models. In particular the extent of influence of a local gene delivery has been highlighted and is potentially of direct relevance to the design of applied protocols aimed at modulating the inflammatory response in the lung.

ACKNOWLEDGEMENTS

Throughout the time spent completing the work presented within this thesis I have received excellent supervision, training and advice from Dr Jean-Michel Sallenave and Dr D David Collie. The combined efforts of the above-mentioned supervisors have facilitated my progress from enthusiastic novice to hopefully a more able researcher.

Additional thanks are directed towards the other members of the Gene Transfer Group including Drs Jason King, Gerry McLachlan, Jonathan McMichael and Peter Henriksen. Further welcome advice and encouragement has been made available by others members of the Centre for Inflammation Research such as Drs Trevor Walker and Neil Henderson.

Special mention must go to Dr Rohit Mistry also, without whose preliminary work on ovine anti-proteases the identification of o-elafin and o-SLPI would have been far more difficult and time-consuming.

The technical assistance I have received during my time in Edinburgh has been enormous. This includes the assistance of Mark Marsden for the huge amount of help and advice on all the molecular genomics work completed during the first stage of the thesis work, and Alison Baker and Peter Tennant for their caring and efficient assistance in the ovine studies. This greatly facilitated completion of this *in vivo* experimentation in a manner which showed the respect and consideration due when using animal models. A great deal of thanks also needs to go to Nina Rzechorzek who gave up her valuable free-time to assist with this often monotonous and always time-consuming section of experimental work.

Thanks and apologies also go to my parents who are still to receive an introduction to pulmonary directed gene therapy and hence in all likelihood consider my work to be a bit of a mystery, as do many of my friends in the Veterinary Profession.

Finally, gratitude is directed to the Wellcome Trust who have supported me financially throughout the time-course of this thesis.

ABBREVIATIONS

AAV	Adeno-associated virus
Ad	Adenovirus
Ad-GFP	Adenovirus encoding Green Fluorescent Protein cDNA
Ad-lacZ	Adenovirus encoding β-galactosidase
Ad-o-elafin	Adenovirus encoding the ovine elafin (TOM-1) cDNA
AEC	Alveolar epithelial cell
AM	Alveolar macrophage
α_1-PI	Alpha-1-antiproteinase
APC	Antigen presenting cell
ARDS	Adult respiratory distress syndrome
BAC	Bacterial artificial chromosome
BALF	Bronchoalveolar lavage fluid
BALT	Bronchus associated lymphoid tissue
bp	Base pairs
BPI	Bactericidal/permeability increasing protein
BSA	Bovine serum albumin
CaP	Calcium phosphate
CAR	Coxsackie-adenovirus receptor
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CPE	Cytopathic effect
COPD	Chronic obstructive pulmonary disease
CR	Complement receptor
Da	Dalton

DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
ECL	Enhanced chemoluminescence
E. coli	*Escherichia coli*
EDTA	Ethylene-diamine-tetra-acetic acid
EGTA	Ethylene-glycol-bis-(beta-aminoethyl ether)-N,N,N',N'-tetra-acetic acid
EIA	Elastase inhibitory activity
ELF	Epithelial lining fluid
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's minimum essential medium
FCS	Foetal calf serum
GFP	Green fluorescent protein
HBSS	Hank's buffered salt solution
HCMV	Human cytomegalovirus
HDL	High density lipoprotein
HEPES	N-(2-hydroxyethyl)piperazine-N'-2-Fethanesulfonic acid
HIV	Human immunodeficiency virus
HMG-1	High mobility group 1
HNE	Human neutrophil elastase
HRP	Horseradish peroxidase
ICAM-1	Intercellular adhesion molecule 1
Ifn	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP	Intra-peritoneal(ly)
IT	Intra-tracheal(ly)
IV	Intravenous(ly)

kb	Kilobases
KDa	Kilodaltons
LBP	Lipopolysaccharide binding protein
LFA	Lymphocyte function associated antigen
LPS	Lipopolysaccharide
LT	Leukotriene
M	Molar
M. haemolytica	*Mannheimia haemolytica*
MCMV	Murine cytomegalovirus
MCP	Monocyte chemotactic protein
MHC	Major histocompatability protein
MIP	Macrophage inflammatory protein
MOI	Multiplicity of infection
MOPS	3-(N-Morpholino)propanesulphonic acid
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dineucleotide phosphate (reduced)
NF	Nuclear factor
OCT	Optimal cutting temperature
P. aeruginosa	*Pseudomonas aeruginosa*
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEEP	Positive end expiratory pressure
PEG	Polyethylene glycol
PEI	Polyethylenimine
pfu	Plaque forming units
PLL	Poly-L-lysine
PMN	Polymorphonuclear leukocyte
PPE	Porcine pancreatic elastase

PR-3	Proteinase-3
PVDF	Polyvinylidine fluoride
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
S. aureus	*Staphylococcus aureus*
SDS	Sodium dodecyl sulphate
SLEI	Sheep lung elastase inhibitor
SLPI	Secretory leukoprotease inhibitor
SP	Surfactant protein
TBS	Tris buffered saline
TCC	Total cell count
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
TPBS	Phosphate buffered saline plus 0.1% Tween 20
UV	Ultraviolet
WAP	Whey acidic protein
WBC	White blood cell

CHAPTER 1

INTRODUCTION

1.1. AIMS

This chapter will outline and discuss the literature which forms the background for the work presented within this thesis.

The main objectives identified at the start of this work were:

1. To investigate and characterise the ovine orthologs of the anti-proteases elafin and secretory leukoprotease inhibitor (SLPI), proteins which have been extensively researched in man and other mammals.
2. To construct, characterise and optimise an adenoviral vector encoding one of these anti-proteases.
3. To assess the effects of adenoviral up-regulation of the anti-protease shield in the sheep lung on the response to local inflammation.

Component aims within objective 1 included (a) the isolation and sequencing of the cDNAs for these proteins followed by the screening of an ovine bacterial artificial chromosome (BAC) library to allow the sequencing of the relevant genes, (b) an assessment of the distribution of these anti-proteases in the sheep at the RNA level, and (c) an appraisal of the functional activity of the ovine proteins by transfection of mammalian cells with expression plasmids containing the anti-protease cDNAs, and investigation of anti-protease activity in the culture supernatant.

Component aims within objective 2 included (a) the construction of an adenoviral vector encoding one of these anti-proteases, (b) the optimisation of transfection efficiency *in vitro* and *in vivo*, and (c) the characterisation of the immediate response to adenoviral vector instillation in the lung.

Finally, in objective 3 an ovine model of acute inflammation was developed in order to determine whether adenoviral up-regulation of the anti-protease shield in the sheep lung would modulate aspects of the local or systemic response to endotoxin.

Chapter 1
Introduction

This introductory chapter will review important topics with relevance to the experimental work detailed in chapters 3 to 7 of this thesis. A crucial concept within this thesis is that of the protease/anti-protease balance in the lung and this is explained and discussed in the context of the potential benefits of being able to artificially manipulate the balance.

The available evidence in relation to manipulating this balance through protein delivery or gene transfer is reviewed and discussed prior to a short justification of the choice of the sheep as an appropriate large animal model to explore the applied aspects of gene therapy.

1.2. The proteinase/anti-proteinase balance in the lung

1.2.1. The role of neutrophil derived enzymes in lung inflammation.

The identification that the congenital deficiency of α1-antitrypsin leads to premature development of pulmonary emphysema (Laurell and Eriksson, 1963; Eriksson, 1964) coincided with work done to model emphysematous changes by instilling the proteolytic enzyme papain into the lungs of rats (Gross et al., 1965). This work led to the protease/anti-protease balance hypothesis of pulmonary inflammation with regards chronic lung disease, specifically chronic bronchitis, emphysema and chronic obstructive pulmonary disease (Kueppers et al., 1974 and 1978; Lieberman, 1976; Morse, 1978A and B; Janoff, 1985). The sources of proteases in lung disease include mast cells, neutrophils and macrophages. The neutrophil derived enzymes neutrophil elastase, cathepsin G and proteinase 3 are of particular significance as a consequence of the large number of circulating neutrophils found within the lung's vasculature and the critical role this cell plays in the progression of acute inflammation. These proteases are contained within the neutrophilic azurophilic granules which also contain myeloperoxidase and defensins. Neutrophils also contain specific granules (which harbour collagenase, gelatinase, histaminase, lysozyme and lactoferrin) and other non-specific granules. The proteolytic enzymes mentioned above are now discussed in more detail.

Human neutrophil elastase (HNE) is the major proteolytic enzyme contained within the azurophilic granules and is a serine protease which degrades various substrates such as elastin (Reilly and Travis, 1980), proteoglycan and collagen (Starkey et al., 1977), matrix proteins found within the alveolar/capillary membrane. HNE has a positive feedback function in the promotion of inflammation and neutrophil influx as it stimulates the release of IL-8, which is a potent neutrophil chemokine (Adams and Lloyd, 1997; Mukaida, 2003). In addition to both direct and indirect anti-bacterial activities HNE also stimulates goblet and mucosal cell secretion, increases vascular permeability and stimulates broncho-constriction (Smallman et al., 1984; Sommerhoff et al., 1990; Nadel, 1991; Suzuki et al., 1996). Additional effects include cleavage of complement components on opsonised bacteria

(Tosi et al., 1990), of lymphocyte surface markers (Doring et al., 1995), and of CD14 on Monocytes (Le Barillec et al., 1999) and macrophages (Henriksen et al., 2004A). Exogenous HNE instillation into the lungs of experimental animals causes the development of emphysematous lesions (Janoff et al., 1977; Senior et al., 1977).

Proteinase 3 (PR-3) is a cationic serine proteinase related to HNE which is more active than HNE in acidic conditions (Kao et al., 1988). Like HNE, PR-3 can induce IL-8 (Berger et al., 1996) and can cleave the anti-protease secretory leukoprotease inhibitor (SLPI) (Rao et al., 1993). PR-3 degrades elastin, fibronectin, collagen type IV, laminin and vitronectin (Rao et al., 1991). PR-3 has been shown to occur at higher concentrations than HNE in sputum from patients with cystic fibrosis and may be more resistant to proteolytic degradation *in vivo* (Witko-Sarsat et al., 1999). Additionally, the observation that DNA does not bind to and inhibit PR-3 (whereas this does occur with HNE and cathepsin G) has prompted speculation that PR-3 could be the most important proteinase with activity in the diseased lung (Duranton et al., 2000).

Cathepsin G is also related to HNE at the amino acid level but seems to be a relatively weak proteolytic enzyme. It has stimulatory effects on the activity of HNE against elastin (Boudier et al., 1981; Lucey et al., 1985). In common with HNE and PR-3, cathepsin G stimulates submucosal gland secretion and increases vascular permeability (Somerhoff et al., 1990; Peterson et al., 1995).

1.2.2. The anti-elastase shield of the lung.

The balance hypothesis predicts the presence of mechanisms to control the proteolytic activity of the enzymes discussed above. The major constituents of this anti-proteinase screen with respect to the neutrophil-derived proteases are the proteins α1-antiproteinase, elafin and secretory leukoprotease inhibitor, and also in sheep the novel Kazal type anti-proteinase sheep lung elastase inhibitor (SLEI).

α1-Antiproteinase (α1-antiprotease, α1-antitrypsin) is a 52kDa glycoprotein (in man) synthesized and secreted by the liver before entering the lung passively by diffusion from the circulation. It is a major anti-proteinase in the lung with estimates of its contribution to the total anti-proteinase screen varying between 50 and 90% (Gadek et al., 1981; Boudier et al., 1983; Sallenave et al., 1999). α1-Antiproteinase shows characteristics of acute phase proteins and plasma levels increase during acute inflammation (Dickson et al., 1974), though increases in production are seen secondary to IL-6 which leads to a delayed increase compared to the situation seen with SLPI and elafin discussed below. Within the lung α1-antiproteinase is also secreted by monocytes, macrophages and epithelial cells (Perlmutter et al., 1985; Sallenave et al., 1997A; Cichy et al., 1997). Functional modification of α1-antiproteinase may be an important effect of cigarette smoking due to the presence of an oxidation-sensitive methionine residue in the active site (Carp et al., 1982). Equally oxidants from macrophages and neutrophils have also been identified as a source of α1-antiproteinase inactivation (Hubbard et al., 1987; Wallaert et al., 1991 and 1993A and B). More than 75 mutations in the α1-antiproteinase protein have been identified, however the most important clinically is the Z mutation (Lomas and Mahadeva, 2002). Mutations in the α1-antiproteinase protein are associated with a reduction in the functional capacity of this enzyme and the premature development of emphysema (Eriksson, 1996). Interestingly, α1-antiproteinase can protect against the effects of TNF and LPS possibly due to the induction of IL-1 receptor antagonist (Tilg et al., 1993; Libert et al., 1996).

Secretory leukoprotease inhibitor (SLPI) is a small (11.7kDa in human), cationic, secreted protein found throughout mucosal sites in the body. In the lung SLPI is secreted by the serous cells of the bronchial sub-mucosal glands, the non-ciliated epithelial cells of the airway walls and by alveolar macrophages (Stolk and Hiemstra, 1999), and is also an important anti-protease in neutrophils (Sallenave et al., 1997B). SLPI inhibits HNE, cathepsin G, trypsin, chymotrypsin and chymase (Fink et al., 1986; Boudier and Bieth, 1992) via an active site in the second of two whey acidic protein (WAP) domains found in the boomerang-shaped mature SLPI protein (Grutter et al., 1988; Eisenberg et al., 1990). It is the WAP domains, containing 4 disulphide bonds each, which indicate a close relationship with the very similar anti-proteinase elafin discussed below.

Elafin (elastase-specific inhibitor, skin-derived antileukoprotease) is also a small (9.9kDa) secreted cationic protein. SLPI and elafin can be regarded as members of the anti-leukoprotease family, though it is more accurate to regard elafin as a member of the trappin family (Schalkwijk et al., 1999) due to the presence of a sequence made up of repeats of the consensus sequence PVKGQD which constitute the 'transglutaminase substrate domain'. Transglutaminase enzymes cross-link the lysine and glutamic acid residues of these molecules to other matrix proteins (Steimert and Marekov, 1995; Nemes and Steinert, 1999). Elafin has a more specific spectrum of activity than SLPI with human elafin inhibiting HNE, PR-3 and porcine pancreatic elastase (PPE) (Schalkwijk et al., 1999).

Due to their similar characteristics SLPI and elafin can be regarded as a functionally discrete group of low molecular weight cationic anti-proteases. Both have been termed 'alarm antiproteases' due to their up-regulation by IL-1, TNF, HNE, defensins and bacterial LPS (Sallenave, 2000). In addition to the well-recognised anti-proteinase activity of elafin and SLPI, several additional functions for these proteins in the innate immune response have been postulated. SLPI has been shown to have bactericidal activity against both *Escherichia coli* and *Staphylococcus aureus* representing both Gram-negative and Gram-positive organisms respectively (Hiemstra et al., 1996), and elafin shows activity against *Pseudomonas aeruginosa* (Gram-negative) and *S. aureus* (Gram positive) (Simpson et al., 1999). In addition SLPI inhibits the replication of HIV-1 in monocytes (McNeely et al., 1995) and also has anti-fungal properties (Tomee et al., 1997). SLPI has been characterised as a protein induced by, and antagonistic to, bacterial LPS and can also be used in transfection experiments to render macrophages hypo-responsive to LPS (Jin et al., 1997). Elafin modulates the LPS response in endothelial cells and macrophages (Henriksen et al., 2004B; McMichael et al., submitted manuscript). SLPI also inhibits the production of metallo-proteases by monocytes and macrophages by the inhibition of prostaglandin synthetase-2 (Zhang et al., 1997). These emerging roles for both SLPI and elafin in the innate immune response represent an exciting and interesting field which is of great relevance to potential therapeutic applications.

A final serine proteinase inhibitor has been isolated and characterised from the bronchoalveolar lavage fluid of the sheep. This is a Kazal-type inhibitor (Mistry et al., 1997) and is known as Sheep Lung

Elastase inhibitor (SLEI) and is a glycosylated anionic protein with a molecular weight of 16.8-17.3kDa produced locally within the lung. SLEI inhibits human neutrophil elastase and porcine pancreatic elastase but not cathepsin G. The median molar ratio of SLEI to α1-antiproteinase in the sheep BALF (3.2 to 1) indicates its potential importance in the ovine lung.

Preliminary work in man has suggested the presence of an analogous inhibitor in the BALF (Bingle et al., 1995).

1.3. The modulation of the lungs inflammatory response

1.3.1. Therapeutic manipulation of the anti-elastase shield.

Prior literature in relation to the therapeutic effects of these anti-proteinases on the pathophysiology of the inflamed lung concern either the direct administration of protein to the lung or the use of gene transfer (or gene therapy) techniques to deliver the anti-proteinase cDNA to the lung.

Most studies involving protein delivery have focused on potential benefits with respect to emphysematous lung damage associated with deficiency of α1-antiproteinase. Protocols of protein delivery have varied from the use of fractionated human plasma (MacDonald and Johnson, 1995) through to recombinant protein produced by transgenic animals (Colman, 1999; Tebbutt, 2000). The results of studies in human patients have been equivocal. Evidence from North America and Scandinavia suggests that protein treatment using recombinant α1-antiproteinase does indeed slow the decline of FEV_1 (Eriksson, 1996; The Alpha-1-Antitrypsin Deficiency Registry Study Group, 1998) although other studies have disputed this (Dirksen et al., 1999). The seemingly disappointing response to α1-antiproteinase may relate to the protein's sensitivity to oxidation or an inability to access the interstitial site of proteolytic activity (Campbell and Campbell, 1988). In cystic fibrosis patients, nebulized α1-antiproteinase augments neutrophil-mediated killing of *Pseudomonas aeruginosa* by epithelial lining fluid (McElvaney et al., 1991). Beneficial effects have also been shown in the modulation of allergic lung inflammation in sheep whereby pre-treatment with aerosolised α1-

antiproteinase inhibited antigen-induced broncho-constriction, possibly as a result of a modulation of tissue kallikrein activity (Forteza et al., 1996).

Recombinant SLPI has also been delivered by aerosolisation to sheep with allergic lung inflammation as a consequence of sensitisation and challenge with *Ascaris suum*. Administration for 4 days prior to antigen challenge inhibited both early and late phase broncho-constriction, inhibited hyper-sensitivity to carbachol and also inhibited the antigen-induced decrease in tracheal mucus velocity (Wright et al., 1999). The same group also demonstrated that SLPI inhibited airway hyper-responsiveness induced by histamine in ovalbumin sensitised guinea pigs, and allergen-induced influx of leukocytes into the lung in a murine model (Wright et al., 1999). rSLPI has also been shown to modulate the effects of the neutrophil influx seen after immune complex deposition in the rat lung. In this latter case rSLPI significantly decreased the lung damage as assessed by haemorrhage and vascular permeability (Mulligan et al., 1993).

Studies involving the use of elafin protein in the lung are limited to the work by Tremblay et al. who have shown that the intra-tracheal administration of human pre-elafin (the 117 amino acid form including the signal sequence) to Golden Syrian hamsters inhibited HNE-induced lung haemorrhage (Tremblay et al., 2002). Further work by the same group demonstrated that pre-elafin instillation led to a dose-dependent inhibition of LPS-induced neutrophil influx in C57BL/6 mice and significantly decreased gelatinase activity, macrophage inflammatory protein-2 and KC levels in the bronchoalveolar lavage fluid (Vachon et al., 2002).

Work performed within this laboratory has so far focused on the *in vivo* administration of human elafin to murine models, using adenoviral vectors. Principally, Simpson et al., (2001A) investigated the effects of an adenovirus encoding human elafin (Ad-elafin) on the progression of pneumonia in mice induced by the intra-tracheal instillation of *Pseudomonas aeruginosa*. The same workers had previously shown that purified human elafin protein had anti-microbial effects against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Simpson et al., 1999). The instillation of Ad-elafin 5 days prior to the intra-tracheal administration of *P. aeruginosa* led to a significant decrease in recovered bacterial colonies isolated from the BALF 24 hours later, with a concomitant decrease in BALF total protein,

when compared to both a PBS control and the instillation of the control adenovirus Ad-lacZ, encoding for the beta-galactosidase gene (Simpson et al., 2001A).

Importantly, in preliminary experiments the same group could not show any detectable human elafin in the serum of animals that received Ad-elafin administered intra-tracheally indicating that transgene expression in an anatomically and physiologically discrete lung compartment led to an improved outcome in bacterial clearance.

1.3.2. Models to investigate the modulation of the lung's innate immune response.

The experimental work of Simpson et al. (2001A) referred to in section 1.3.1 was of great importance in providing *in vivo* experimental evidence to support the existence of a link between the anti-protease shield and the innate immune system within the lung. This potential link has led to the concept that elafin may have a role in the compartmentalisation of the immune response within the lung (Sallenave et al., 2003). In addition to the direct bacterial infection of the lung (Simpson et al., 2001A), acute lung injury may also occur secondary to systemic sepsis. In this context the adult respiratory distress syndrome (ARDS) may occur (Fein et al., 1983; Fowler at al., 1983; Montgomery et al., 1985; Niederman and Fein, 1990). During sepsis the pro-inflammatory cytokine TNF-α (TNF) is elevated and the levels are correlated with severity (van der Poll and Lowry, 1995; Calandra et al., 1990). Equally TNF administration can mimic some of the deleterious effects of sepsis (Natanson et al., 1989). Conversely, the blockade of TNF in patients leads to a worsened prognosis (Fisher et al., 1996). This paradox may reflect a role for TNF in the innate immune response and indeed TNF has been shown to activate the phagocytosis and microbicidal activity of alveolar macrophages and neutrophils *in vitro* (Le and Vilcek, 1987; Tan et al., 1995). This background information led Chen et al. (2000) to examine the influence of adenoviral transfer of murine TNF cDNA in a murine model of experimentally induced sepsis. The model involved caecal ligation and puncture (CLP) followed by instillation of *Pseudomonas aeruginosa* into the lungs. The study showed that mice benefited from receiving Ad-TNF intra-tracheally relative to those receiving control virus or vehicle alone.

Additionally the Ad-TNF treated animals yielded significantly fewer *P. aeruginosa* colonies 24 hours after bacterial instillation compared to control adenovirus. Alveolar macrophages (AMs) from Ad-TNF treated mice also showed a higher phagocytic index compared to AMs from control adenovirus treated animals when cultured *ex vivo* with bacteria. This compartmental activation of the innate immune system by local adenovirus-mediated expression of a pro-inflammatory cytokine has interesting and important implications for the delivery of other potentially immuno-stimulatory cDNAs to the lung.

Of particular interest is the use of Il-10 in models of localised inflammation which has shown differing effects dependent upon the site of administration. Il-10 has been demonstrated to inhibit the clearance of bacteria from the murine lung with a concomitant increase in mortality (van der Poll et al., 1996) and equally the neutralisation of Il-10 leads to an improved survival and increased bacterial clearance (Greenberger et al., 1995). In contrast, the systemic administration of Il-10 appears to be protective in models of respiratory tract infection and sepsis (Sawa at al., 1997; Kurahashi et al., 1999). Morrison et al. (2000) showed that the intra-tracheal delivery of an adenovirus encoding for IL-10 could ameliorate lung damage in a porcine model of pneumonia caused by *Actinobacillus pleuropneumoniae* although this work looked at a single time-point 24 hours post-bacterial infection. Additional data utilising gene transfer techniques in pneumonia models have demonstrated a beneficial effect with regards bacterial clearance by IL-12 (Greenberger et al., 1996) and IFN-γ (Lei et al., 1997).

The lung's innate immune response is orchestrated by a complex balance of 'pro' and 'anti' inflammatory cytokines. The investigation of techniques allowing the manipulation of the anti-protease shield within the inflamed lung may yield important information with respect to a link between this protective barrier and the stimulation or otherwise of the innate immune response.

1.3.3. Large animals as models for lung-directed gene therapy.

Large animals have played an important role in the development of comparative models of diseases affecting the lung and the investigation of therapeutic strategies therein.

The reasons that prompt research in larger species are various but are frequently related to either particular species-specific similarities to human subjects in relation to host responses in the context of either natural or experimentally induced disease or are related to issues surrounding the development of delivery protocols of relevance to human clinical medicine.

Predominant within the literature are studies using dogs in models of lung cancer both by the topical application of known carcinogens (Okita et al., 1977; Shors et al., 1980; Benfield et al., 1981) or by the transfer of tumour tissue (Ahrar et al., 2002). Equally dogs have been used to investigate therapies for lung tumours including chemotherapy, radiotherapy and radiofrequency ablation (Pass et al., 1987; Hershey et al., 1999; de Boer et al., 1999; Demmy et al., 2002; Ahrar et al., 2003). However, other mammalian species have been utilised in the majority of gene transfer techniques to the respiratory tract. For example the pig has been used in several studies using both plasmid DNA formulations (Nabel et al., 1992) and adenoviruses (Chapelier et al., 1996; Morrison et al., 2000). Interestingly, Hackett et al. (2000) showed that 90% of adenoviral vector injected intravenously in the pig localised to the lung which is in stark contrast to the situation seen in rodent studies where vector localises to the liver illustrating an important difference between rodent and larger animal models.

By far the majority of gene transfer studies have utilised primates. Several studies relevant to viral gene transfer to the respiratory tract have been performed by the group of Dr. James Wilson of The Gene Therapy Programme at the University of the Pennsylvania School of Medicine. These have investigated the adenoviral delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) cDNA to primate lungs (Engelhardt et al., 1993; Simon et al., 1993; Goldman et al., 1995; Chirmule et al., 1998) as a preface to human clinical trials, and also the blockade of CD-40 interactions with its ligand to abolish the mucosal IgA response to the vector (Chirmule et al., 2000). Brody et al. (1994) demonstrated a dose-dependent inflammatory response to adenovirus which could persist for up to 2 months, and Sene et al. (1995) and Lerondel et al. (2001) developed a novel aerosol

based delivery protocol for adenovirus. Finally, primates have also been used to optimize the *in vivo* delivery of the use of aerosolised DNA/lipid complexes (McDonald et al., 1998).

The sheep has gained popularity as a model for human lung disease for a variety of reasons not least of which being the broadly comparable lung volumes shared by these species (the average lung volume for a 50kg young adult female human is approximately 5.5 litres compared to approximately 4 litres for a sheep of equivalent weight). Sheep have become widely used in surgical science (Borrie and Mitchell, 1960; Hecker, 1974; Zrunek et al., 1988; Ingenito et al., 2001 and 2003) with their size and docile nature facilitating general ease of handling. They are also amenable to experimental interventions such as repeated venepuncture and don't represent any particular anaesthetic risk. With regards the respiratory system, sheep are a good model for the human system on account of a similar anatomy (McLaughlin et al., 1961; Plopper et al., 1983; Mariassy and Plopper, 1983). Indeed this similarity extends to the histochemical and cytochemical characteristics of the secretory cells within the respiratory tract (Mariassy et al., 1988). The ovine respiratory tract has also proved more relevant when compared to that of dogs in the study of gaseous exchange (Werlen et al., 1984).

Early physiological data indicating the utility of the sheep as a model for studying basic lung biology and pathophysiology (Halmagyi and Colebatch, 1961A and B; Colebatch and Halmagyi, 1961 and 1963; Halmagyi et al., 1963) led to further use of the sheep in numerous disease models, including asbestosis (Begin et al., 1983A and B).

Further studies illustrate the physiological similarities with man. Estimates of static compliance in the ovine lung vary from approximately 4.1 to 4.9ml/cm.H_20/kg (Wanner et al., 1978; Begin et al., 1981) which is closer to the situation in man which is estimated at between 2.4ml/cm.H_20/kg (Butler and Smith, 1956) and 3.9ml/cm.H_20/kg (Turner et al., 1968) than that seen in other primates (9.1 to 9.4ml/cm.H_20/kg - Kosch and Gillespie, 1977; Pare et al., 1978), and compares favourably to that seen in the dog (4.0 to 5.0ml/cm.H_20/kg – Mead and Collier, 1959; Robinson and Gillespie, 1973).

Equally, ovine lung resistance has been estimated at between 1.2 and 3.5cm.H_20/l/s (Wheeler et al., 1990; Kleeberger et al., 1985) compared to 2.19 to 5.1cm.H_20/l/s in man (Saunders et al., 1977; Dohi and Gold, 1979). These similarities have also led to a more recent interest in the development of an ovine model for cystic fibrosis research (Harris, 1997).

In summary, the ovine lung shares structural and functional similarities with the human lung.

A comprehensive and detailed expertise in ovine respiratory research has developed at the R(D)SVS over the last two decades. This work initially focused on the characterization of the clinical pathology of Maedi-Visna infection (Watt et al., 1992; Collie et al., 1993, 1994 and 1995; Begara et al., 1995 and 1996; Lujan et al., 1995) and led on to investigation of allergic responses in the lung (Sture et al., 1995; Pemberton et al., 2000; Collie et al., 2001; Collie, 2003). Workers have also used ovine models to study broncho-constrictor activity within the lung (Corcoran and Haigh, 1992, 1993 and 1995). Finally recent advances have been made with regards gene transfer to the pulmonary epithelium by segmental delivery of plasmid DNA to the airways (Emerson et al., 2003).

This background of knowledge combined with current local expertise in low molecular weight anti-proteases (discussed in section 1.2.2) and plasmid mediated transfer of genes to the ovine respiratory epithelium, led to our recognition of the potential utility of this model system in exploring the effects of adenoviral-mediated delivery of anti-proteases to the ovine lung.

CHAPTER 2

MATERIALS AND METHODS

2.1. EXPERIMENTAL PROCEDURES - CLONING AND SEQUENCING OF ONE ELAFIN AND SLPI GENES

2.1.1. Molecular biology reagents.

Primers were synthesised and supplied by MWG-Biotech AG (Ebersberg, Deuch.): Elafin gene-specific reverse primer (GSP1) 5'- CAT GGC GCA CCG GAT CA - 3'; Elafin gene-specific forward primer (GSP2) 5'- ATG AAG ACC AGA AGC TTC TTG GTC C - 3'; Full length reverse primer (r-el) 5'- CTG GGG ATC CAT ACA GGT CTT – 3'; Bgl II forward elafin primer (Bgl-f-el) 5'- GCC GGA GAT CT**G ACA AC**A TGA AGA CCA GAA GCT TCT TGG TC – 3' (Bgl II site underlined; Kozak sequence in bold); Sac I elafin reverse primer (Sac-r-el) 5'- TAC GTG AGC TCT CAT CAC TGG GGA TCC ATA CAG GTC TT – 3' (Sac I site underlined); Forward BAC primer (fBAC) 5'- TGA AAA GGG TCC AAT CAA CG – 3'; Reverse BAC primer (rBAC) 5'- GAG CCA CGC TTA GTG AGG AG – 3'; Reverse Inverse elafin primer (r-inv-el) 5'- GCC CCT AAC AAC CTC TCC TC – 3'; Forward elafin promoter primer (f-el(prom) 5'- ATT CCA CCT TCC CTA TT – 3'; Forward SLPI 5' primer (fSLPI) 5'- CTC ACC ATG AAG TTC AGT GGG CTC TTC C – 3'; Forward SLPI primer 2 (fSLPI2) 5'- CCC TCC TAG AAA ATC TGC CC – 3'; Reverse SLPI primer 1 (rSLPI1) 5'- TCA GGC TTT CAC AGG CGA AAG GGC – 3'; Reverse SLPI primer 2 (rSLPI2) 5'- CAC TGG ACA CGT CCC AGG CTT CT – 3'; Bgl II forward SLPI primer (Bgl-f-SLPI) 5'- GCC GGA GAT CT**C TCA CC**A TGA AGT TCA GTG GGC TCT TC – 3' (Bgl II site underlined; Kozak sequence in bold); Sac I SLPI reverse primer (Sac-r-SLPI) 5'- TAC GTG AGC TCT CAG GCT TTC ACA GGG GAA AGG CA – 3' (Sac I site underlined); Reverse FLAG SLPI primer (r-FLAG-SLPI) 5'- **TCA TCA CTT GTC ATC GTC GTC CTT GTA GTC** GGC TTT CAC AGG GGA AAG – 3' (FLAG epitope in bold); Sac I FLAG SLPI reverse primer (Sac-r-FLAG-SLPI) 5'- TA CGT GAG CTC **TCA TCA CTT GTC ATC GTC GTC CTT GTA GTC** GGC TTT CAC – 3' (Sac I site underlined; FLAG epitope in bold).

Taq DNA polymerase, magnesium chloride 25mM, DNA polymerase buffer, dNTPs, oligo (dT) primer, EcoR1, Buffer H for EcoR1, BglII, Buffer D for BglII, Sac I, Buffer J for SacI, M-MLV

reverse transcriptase, 5x M-MLV RT buffer were supplied by Promega UK (Southampton, UK). Shuttle vector pDC516 was obtained from Microbix Biosystems Inc. (Toronto, Canada). Shuttle vector pIRES-GFP#3 was kindly donated by Dr. Mary Hitt (Dept. of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada).

2.1.2. RNA preparation.

Ovine tissues were obtained from 5 month old crossbred lambs (Moredun Institute, Roslin, Edinburgh). The tissue samples obtained from sheep organs were treated as follows. Samples of each tissue were removed at autopsy and frozen at –80°C. Approximately one gram of each of these was then homogenised and the RNA extracted using Trizol (Life Technologies). RNA quality was assessed by running 10µl on a 1% agarose gel. Quantification of RNA in samples was by dual wavelength spectrophotometry.

2.1.3. Elafin RACE-PCR.

RACE-PCR for ovine elafin was performed using ovine tracheal mucosal RNA. Firstly 5' RACE was performed by using the primer GSP1 (derived from partial sequence already obtained from preliminary experiments) and the 5' forward Generace Primer (GRP1). This was followed by a nested PCR using GSP1 with nested 5' forward Generace Primer (nGRP1). PCRs were carried out using a PTC-200 DNA engine (MJ Research) in 50µl mixtures. The following conditions were used: 10% *Taq* DNA polymerase 10x buffer, 1.1mM magnesium sulphate, 0.2mM for each dNTP, 2.5 units *Taq* DNA polymerase, and approximately 20pmol of each primer.

Cycling parameters were as follows: initial incubation of 92°C for 5 minutes, followed by 92°C 1 minute, 55°C 1 minute, and 72°C for 1 minute (30 cycles followed by a final amplification step of

72°C). Products were run out on a 1% agarose gel. These conditions were used for both the original and nested PCRs.

To perform the 3'RACE a slightly different strategy was used. Reverse transcription of the original RNA sample was performed using the Generace oligo-dT supplied in the Generacer™ kit and M-MLV RT (Promega). PCR was then performed using the forward primer GSP2 and the reverse Generace™ primer (GRP2) in order to obtain the full-length cDNA sequence. GSP2 was designed from the sequence derived from the 5'RACE procedure. PCR conditions were similar to those above except for a magnesium chloride concentration of 2.2mM. This was followed by a nested PCR using GSP2 and nested reverse Generace™ primer (nGRP2).

2.1.4. SLPI RACE-PCR.

Ovine SLPI full-length cDNA sequence was obtained using the following technique. Rapid amplification of cDNA ends (RACE) was performed using the Invitrogen™ Generacer™ kit as recommended by the manufacter. Using ovine kidney RNA, 5'RACE was performed using forward Generace Primer (GRP1) and the reverse primer rSLPI1 (derived from preliminary sequence – not shown) followed by nested PCR using nGRP1 and rSLPI2. This was followed by a full length PCR of cDNA using fSLPI and rSLPI. Cycling parameters were as follows: initial incubation of 92°C for 5 minutes, followed by 92°C 1 minute, 60°C 1 minute, and 72°C for 1 minute (35 cycles followed by a final amplification step of 72°C). Products were run out on a 1% agarose gel.

No 3'RACE was performed (sequence already available in house – Mistry, personal communication).

2.1.5 Extraction of PCR products and cloning reactions.

All PCR products were run on 1% agarose gels and bands of interest were cut out and purified using the QIAQuick Gel Extraction Kit (Qiagen, Crawley, W. Sussex).

Cloning of the extracted RACE cDNA fragments was achieved by using the TOPO TA Cloning^{R} Kit for Sequencing included with the GeneRacer^{TM} kit. The cDNAs were ligated into pCR^{R}4-TOPO^{R} plasmid which was transformed into TOP10 *E.coli* cells. Plasmid DNA was purified with the Promega SV Miniprep system.

2.1.6. Sequence analysis.

Sequence analysis was done both in house and by MWG-Biotech AG, and the sequence data analysed with the on-line computer software packages NCBI Open Reading frame finder (NCBI), NCBI Blast (NCBI) and BCM Multiple Sequence Alignment tools (BCM).

2.1.7. Bacterial artificial chromosome (BAC) library screening and subcloning.

An ovine BAC library was screened using primers fSLPI2 and rSLPI2 (for SLPI) and fBAC and rBAC (for elafin) by Dr. F. Piumi at Institut National de la Recherche Agronomique (INRA), Laboratoire mixte INRA-CEA de Radiobiologie et d'Etude du Genome (LREG), Jouy-en-Josas, France. Positive clones (one for elafin and one for SLPI) were isolated and DNA prepared with Qiagen Plasmid Maxi Kit (Qiagen, Crawley, UK). 1μg of each BAC positive clone was digested with EcoR1, run out on a 1% agarose gel, and blotted onto GeneScreen^{TM} membranes (PerkinElmer Biosciences, Inc.). These were probed for one hour with [α-^{32}P]dCTP labelled full length cDNAs coding for ovine elafin or ovine SLPI, using Express-Hyb (Clontech) following the manufacturers

protocol. Positive bands were identified with a Storm Phosphoimager system (Amersham) and further subcloned into plasmid PDC516 for sequencing using various internal primers.

2.1.8. Tissue distribution of elafin and SLPI by Northern Blot analysis and RT-PCR.

Twenty μg of each tissue RNA sample were denatured in 50% formamide, 16% formaldehyde, 20mM MOPS, pH 7.0, 5mM sodium acetate, and 1mM EDTA, for 15 mins at 65 , electrophoresed in a 1% agarose gel containing 2.2M formaldehyde and transferred to GeneScreen membrane. The membrane was hybridised with [α-^{32}P]dCTP labelled full length cDNA coding for ovine elafin in ExpressHyb™ (Clontech) following the manufacturer's recommendations. The membrane was then visualised on a Storm™ Phosphoimager system (Amersham).

One μg of each tissue RNA sample was used in a reverse transcriptase reaction using M-MLV RT (Promega). Product from this reaction was split into two PCRs, using either forward and reverse ß-actin primers or the full length elafin primers GSP-2 and r-el. The process was repeated for SLPI using the ß-actin primers and the partial length primers fSLPI and rSLPI2. Conditions were as for the RACE elafin PCR except for there being 35 cycles for elafin and SLPI, and 28 cycles for the ß-actin.

2.1.9. Genomic DNA preparation.

DNA was extracted from lung and liver samples by proteinase K digestion in NTES (50mM Tris, 50mM EDTA, 100mM NaCl, 1% SDS). Following phenol/chloroform extraction, washing with 100% isopropanol and then 70% ethanol, the DNA was re-suspended in water and stored at -20 °C.

2.1.10. Comparison of ovine elafin products synthesised by PCR from the positive BAC clone, the genomic DNA and RT-PCR synthesised cDNA.

In order to compare the elafin cDNA, identified by the RACE procedure, with the gene sequence identified by BAC screening (see above) and with genomic ovine DNA, PCR was performed using as template purified elafin BAC DNA and ovine liver DNA alongside cDNA synthesised from ovine skin and pIRES-GFP-ovine elafin, using the non intron-spanning primers fBAC and rBAC. PCR parameters were as for the RACE elafin PCR. Products from the genomic DNA PCR were ligated into pCR^{R}4-TOPO^R plasmid which was transformed into TOP10 *E.coli* cells. Plasmid was extracted with Promega SV Miniprep system and sequenced using internal primers.

An additional PCR was performed on ovine genomic DNA using the primers f-el and GSP2. Products from this PCR were also cloned into pCR^{R}4-TOPO^R as above and sequenced.

2.1.11. Construction of ovine elafin and ovine SLPI expression constructs.

Full-length elafin cDNA coding for a protein of 220 amino acids in pCR^{R}4-TOPO^R was used as a template for PCR with Bgl-f-el and Sac-r-el (to give BglII-elafin-SacI). PCR conditions were as for the RACE PCR for elafin.

Full-length SLPI cDNA coding for a protein of 132 amino acids in pCR^{R}4-TOPO^R was used as a template for PCR with Bgl-f-SLPI and Sac-r-SLPI (to give BglII-SLPI-SacI). The same template was used for PCR with Bgl-f-SLPI and r-FLAG-SLPI, followed by a nested PCR of this product with Bgl-f-SLPI and Sac-r-FLAG-SLPI (to give BglII-SLPI-FLAG-SacI). PCR conditions were as for the RACE PCR for SLPI.

These PCR products were ligated into the expression vector pIRES-GFP#3 using standard ligation techniques producing pIRES-GFP-ovine elafin, pIRES-GFP-ovine SLPI and pIRES-GFP-ovine SLPI-

FLAG. These plasmids were used to transform XL1-Blue subcloning grade E. coli (Stratagene). Plasmid was purified with the Qiagen Plasmid Maxi Kit (Qiagen, Crawley, UK).

2.1.12. Transfection of 293 cells with ovine elafin and ovine SLPI expression constructs.

293 cells (Microbix Biosystems Inc.) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal calf serum (FCS), penicillin G (final concentration 100U/ml), streptomycin sulphate (final concentration 100μg/ml) and L-glutamine (final concentration 2μM) in 6cm plastic tissue culture dishes and incubated at 37°C in a humidified incubator containing 5% $C0_2$ until fully confluent. 6μg plasmid DNA was suspended in 15μl 3M $CaCl_2$ and made up to 225μl with distilled water. This was then added to an equivalent volume of 2xHBSS (1.5mN $Na_2HPO_4(H_20)_2$, 10mM KCl, 280mM NaCl, 12mM D-Glucose, 50mM HEPES, pH 7.05) and mixed for 1 minute. This was combined with 4ml of DMEM containing 10% FCS, 100U/ml penicillin G, 100μg/ml streptomycin sulphate and 2μM L-glutamine and added to 293 cells which had been washed with PBS twice and the cells incubated at 37°C overnight. After 24hrs the medium was removed, the cells washed twice with PBS and medium replaced with fresh DMEM as above but containing no FCS. Medium was removed after a further 3 days, cells spun out at 1000G for 4 minutes and the conditioned medium stored at -20°C.

2.1.13. Anti-elastase activity of ovine elafin and ovine SLPI transfectants.

Anti-elastase assays were performed in 96 well microtitre plates. 10ng human neutrophil elastase (HNE, diluted in 50mM Tris, 0.1% Triton, 0.5M NaCl, pH 8.0) was incubated, at 37°C for 30 minutes, alone or with increasing volumes of conditioned medium from untransfected cells or from cells transfected with pIRES-GFP-ovine elafin or pIRES-GFP-ovine SLPI (final volumes 50ul).

Residual HNE activity was measured by change in absorbance at 405nm of the chromogenic substrate N-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide over 10 minutes at room temperature. Rate of change in absorbance was directly correlated with HNE activity. The percentage of residual HNE activity (y-axis) was plotted against the volume of conditioned medium used (x-axis) and the point where the linear part of the curve crossed the x-axis was used to calculate elastase inhibitory capacity (EIC) of the medium (Sallenave et al., 1991).

2.1.14. Western blot analysis of ovine elafin and ovine SLPI transfectants.

Supernatants from untransfected and plasmid transfected 293 cells were analysed by SDS-PAGE (reducing conditions, 15% acrylamide gel). Pre-stained molecular weight markers were included. Proteins were transferred to PVDF membrane in 25mM Tris, 192mM glycine, 10% methanol, at 100V for 60 minutes at 4°C. Membranes were blocked overnight at 4°C in TPBS (0.01% Tween 20 in PBS) with 5% dried skimmed milk then incubated in a 1:1000 dilution of primary antibody (anti-elafin Trab-20 monoclonal antibody directed against the PVKGQD repeats (Hycult, Nl), or anti-FLAG monoclonal antibody (Sigma-Aldrich) or polyclonal anti-human SLPI antibody (Sallenave et al., 1992) with 5% skimmed milk powder for 1 hour at room temperature. Membranes were then washed with TPBS and incubated in a 1:2000 dilution of either anti-mouse IgG conjugated to peroxidase (for Trab-2O and anti-FLAG monoclonal primary antibodies) or anti-rabbit IgG conjugated to peroxidase (for anti-human SLPI polyclonal primary) (both antibodies supplied by DakoCytomation, CA) for 45 minutes at room temperature. After washing in TPBS, membranes were developed using Western Lightning™ Chemiluminescent Reagent Plus (PerkinElmer Life Sciences, Inc.) and exposed on X-omat radiograph-quality film (Kodak).

2.1.15. Phylogenetic Tree analysis of the WAP domains of elafin and SLPI family members.

To investigate the phylogenetic relationships of the ovine forms of elafin a phylogenetic tree was constructed by the neighbour-joining method (Saitou et al., 1987) using data from multiple alignment of the amino acid sequences of the whey acidic protein (WAP) domains of members of the trappin family, the known SLPI sequences (human, mouse, rat, pig and sheep), the murine elafin-like proteins 1 and 2 (Hagiwara et al., 2003), and the human vasopressin molecule which contains a rudimentary WAP motif. The latter can be assumed to be evolutionarily related to the elafin and SLPI families of proteins.

2.2. EXPERIMENTAL PROCEDURES – THE CONSTRUCTION OF AN ADENOVIRUS EXPRESSING OVINE ELAFIN AND INVESTIGATION OF INFECTION OPTIMISATION PROTOCOLS *IN VITRO*

2.2.1. Insertion of the ovine elafin cDNA into the shuttle vector pDC516.

The cDNA coding for the short 220 amino acid form on ovine elafin (TOM-1) contained in the expression vector pCR^{R}4-TOPO^R was used as template for two consecutive PCRs with firstly Bgl-f-el and FLAG-r-el, followed by Bgl-f-el and Sac-r-FLAG-el (to yield BglII-elafin-FLAG-SacI). PCR conditions were as for the RACE PCR for ovine elafin. Final product from these PCRs was ligated into shuttle vector pDC516 (which contains the murine cytomegalovirus (MCMV) promoter) using standard ligation techniques to yield pDC516-o-elafin. Product from this reaction was used to transform XL1-Blue subcloning grade E. coli and a positive colony identified by restriction analysis. Plasmid was isolated with the Qiagen Maxi-Prep kit. This plasmid is referred to as pDC516-o-elafin.

2.2.2. Co-transfection of 293 cells with shuttle plasmid and adenoviral vector plasmid.

Human embryonic kidney cells (HEK 293 cells) were grown in 6cm dishes until 80-90% confluence. These cells were then co-transfected with pDC516-o-elafin and pBHGfrt(del)E1,3FLP (a kind gift from Dr. M. Hitt, McMaster University) at various ratios or with the single control plasmids pFG140 (as a positive control) and pDC516-o-elafin or pBHGfrt(del)E1,3FLP (as negative controls) in the following way. Plasmid DNA was added to 15µl 3M $CaCl_2$ and the volume made up to 225µl with sterile water. To this, 225µl 2xHBSS (8g NaCl, 5g HEPES, 0.27g KCl, 0.1g Na_2HPO_4, 1g D-glucose, pH 7.1, made up to 500ml with sterile water) was added and vortexed before adding to 5ml pre-warmed 293 medium (MEM Eagles, 10% FCS, 1% Penicillin/Streptomycin, 1% L-Glutamine, 3%

sodium bicarbonate). The resulting mixture was added to the 293 cells and incubated overnight before replacing the medium. The cell monolayers were monitored daily for signs of cytolysis. When monolayers showed 90-100% cytolysis (including the positive control dish transfected with pFG140) the contents of each dish were transferred to 14ml Falcon tubes and centrifuged at 1200rpm for 10 minutes at 4°C. The supernatant was then transferred to a fresh Falcon tube with 10% glycerol and stored at -80°C. DNA was extracted from the cell pellet for Southern blot analysis as described below.

2.2.3. DNA extraction from CPE positive dishes and Southern blot analysis.

The cell pellet from the co-transfections which resulted in cytolysis (see above) were incubated overnight at 37°C with 600µl 1xPronase-SDS solution (200µl 5mg/ml Pronase, 40µl 500mM EDTA, 100µl 10% SDS, 1.66ml 10mM Tris-HCl, pH 7.5). The resultant lysate was extracted twice with 500µl buffer-saturated phenol followed by centrifugation at 13,000rpm for 10 minutes at room temperature.

The aqueous layer was then added to 50µl of 3M sodium acetate and 1ml absolute ethanol and mixed several times by inversion. This was centrifuged at 13,000rpm for 10 minutes at room temperature to pellet the DNA and the supernatant discarded. The pellet was washed in 95% ethanol then air dried before being dissolved in 100µl 0.1xSSC (17.53g NaCl, 8.82g sodium citrate made up to 100ml with water, pH 7.0 then dissolved 1:200 in water).

10µl of this DNA solution was digested with Hind III overnight at 37°C and run out on a 1% agarose gel after which the gel was photographed to examine possible banding patterns. This gel was then transferred to Genescreen^{TM} membrane and probed using ovine elafin cDNA in a Southern blot analysis as described in 2.1.7.

2.2.4. Re-plaquing of Ad-o-elafin.

Stored supernatant (Section 2.2.2) was diluted 1 in 10^6 and 1 in 10^7 in PBS ++ (PBS with 0.01% $CaCl_2$ and 0.01% $MgCl_2$) and added to fresh 293 cells in 6cm dishes. The dishes were then incubated at 37°C for 45 minutes after which 10ml of an overlay solution (1% agarose in water, melted and maintained at 42°C, added to 1.6xMEM Eagles, 10%FCS, 0.1% yeast extract, 2% Penicillin/streptomycin, 2% L-Glutamine, 0.45% sodium bicarbonate, 1% fungizone) was added to the monolayer. The overlay was left to solidify for 10 minutes before being returned to the incubator. Plaques appeared within 5-7 days. Several well isolated plaques (>3mm diameter) were selected by removing an agar plug core with the broad end of a sterile P1000 pipette tip. The cores were placed into 1ml PBS ++ and 10% glycerol and stored at -80°C.

2.2.5. Re-infection of 293 cells with plaque lysate.

The plaques isolated in section 2.2.4 were subjected to 3 rounds of freeze-thaw lysis after which 200µl of the lysate was used to infect fresh 293 cells in 6cm dishes. After 100% cytolysis the dishes were treated as in section 2.2.3 above to isolate the DNA. This DNA was again digested with Hind III and the resultant DNA run on a 1% agarose gel to examine the banding pattern for possible transgene insertion. The supernatants from this experiment were used to screen for secreted ovine elafin by Western blot analysis in the same manner as described in 2.1.14 above and probed with Trab-20 monoclonal antibody (Hycult, Nl), or anti-FLAG monoclonal antibody (Sigma-Aldrich) diluted 1 in 1000 in PBS/0.1% Tween and 5% skimmed milk powder. The secondary antibody and detection by chemi-luminescence were as desribed in 2.1.14.

Further amplification and titration of one positive plaque was performed as described elsewhere (Graham and Prevec, 1991).

2.2.6. Optimising adenoviral infection efficiency *in vitro*.

A549 cells were plated out in 48 well plates at 80,000 cells per well. Ad-GFP at MOI 1 and 10 was applied to the monolayers in fresh medium (DMEM plus 1% pencillin/streptomycin, 1% L-Glutamine) for one hour. Additionally, Ad-GFP that had been complexed with polyethylenimine (PEI) at a ratio of 500 molecules of PEI to one viral particle in fresh medium and Ad-GFP which had been complexed with calcium phosphate co-precipitates (formed by adding 2.14µl 2.5M $CaCl_2$ per ml EMEM medium and vortexed well before incubating with adenovirus for 20 minutes) was added to the cells. After one hour the medium containing the Ad-GFP was removed and the monolayers were washed twice with sterile PBS. Fresh medium (DMEM plus 10% FCS, 1% pencillin/streptomycin, 1% L-Glutamine) was then added to the wells. The wells were examined for the presence of GFP 24 hours later by fluorescent microscopy and images captured using Openlab 3.0.0 image capturing software (Improvision Ltd., Coventry, UK). GFP positive pixel counts were measured using ImageJ software (http://rsb.info.nih.gov/ij/).

In a second experiment Ad-o-elafin was used to infect A549 cells in the same plate format as above at MOI 10 either alone or after pre-complexing with PEI (500 molecules PEI per viral particle) or calcium phosphate. Additionally, cells were infected with virus alone in hypotonic EGTA (100mM EGTA in 37.5mosm/l PBS). After one hour the medium was replaced with fresh DMEM plus 10% FCS, 1% pencillin/streptomycin, 1% L-Glutamine. Bacterial lipopolysaccharide (LPS) (10µg/ml of LPS purified from *E. coli* L28 B8) was added to the culture supernatant in one set of wells on day 3. On day 4 the medium was removed from all wells, cellular debris removed by centrifugation at 13,000rpm for 10 minutes at 4°C and the supernatant assayed for secreted elafin by direct ELISA as detailed below in 2.2.7.

Further experiments examined the effects on infection efficiency and cell death of varying the contact times between the adenovirus/calcium phosphate co-precipitates and the A549 cells. This experiment was performed using the same cells and well format as above and using Ad-GFP at an MOI of 10 with or without calcium phosphate co-precipitation (as detailed in the above section) with contact times

with the cells varying from 1 to 60 minutes before medium was replaced. Image J analysis of photomicrographs taken at 24 hours post-infection allowed GFP positive pixels to be quantified. The cells which were in contact with the virus alone or virus and calcium phosphate for 1 hour were also stained with trypan blue to assess viability after 24 hours.

2.2.7. Direct ELISA for secreted ovine elafin.

Secreted elafin in the culture supernatant was detected by a direct ELISA protocol in the following manner. A 96 well assay plate (Costar^{R}) was used to bind the different supernatants from the above experiments (neat and 1 in 10 dilutions) over night at 4°C. Test supernatants were compared against supernatants from cells transfected with plasmid containing the ovine elafin cDNA. The latter had been accurately titrated for elafin content by anti-elastase activity and were used as standards. Supernatants were removed on the following day and the wells rinsed 3 times with PBS-T (PBS plus 0.1% Tween 20) before adding Trab-2O monoclonal antibody (Hycult Biotechnology, Nl.) at a dilution of 1 in 1000 in PBS-T. After 2 hours the antibody solution was removed and the wells washed 3 times with PBS-T. Goat anti mouse monoclonal antibody conjugated to peroxidase was added at a dilution of 1 in 2000 in PBS-T for 1 hour. The wells were washed 3 times with PBS-T. Substrate was added (sodium acetate citrate pH 4.9 with 1% 3,3',5,5'-tetramethylbenzidine and 0.05% H_20_2) and a blue colour allowed to develop before the reaction was 'stopped' with 1M H_2SO_4. The colour was read at 490nm with reference readings taken at 560nm. The absorbances for each sample were cross-referenced with those from the known standards to allow the amount of secreted elafin in each sample to be calculated.

2.2.8. The use of calcium phosphate co-precipitation to improve the infection efficiency of alveolar macrophages *in vitro*.

Ovine alveolar macrophages (and other mobile cells) collected at routine bronchoalveolar lavage (see section 2.3.2.) were cultured in 24 well plates at a seeding density of 250,000 per well in RPMI containing 10% foetal calf serum (FCS), penicillin G (final concentration 100U/ml), streptomycin sulphate (final concentration 100μg/ml), L-glutamine (final concentration 2μM) and amphotericin B (0.5%). After 6 hours non-adherent cells were washed off the wells and fresh medium was added to each well. Ad-GFP MOI 100 with or without calcium phosphate co-precipitation was applied to the cells for 20 minutes before removal and replacement of medium. 24 hours later the monolayers were photographed and infection efficiency assessed by visualisation of GFP. The cells in contact with virus alone or virus and calcium phosphate for 20 minutes at an MOI of 100 were also stained with trypan blue to assess viability after 24 hours. In this experiment alveolar macrophages from 3 different donor sheep were used.

Additionally, Ad-o-elafin was used at MOIs of 100 and 200 both with and without co-precipitation with calcium phosphate in the same way as above. This time the culture medium was collected after 4 days and used in a Western blot as described in 2.1.14.

2.3. EXPERIMENTAL PROCEDURES – *IN VIVO* EXPERIMENTATION.

2.3.1. Anaesthesia and ventilation of sheep for experimental procedures.

Food was withheld for 12 hours prior to anaesthesia which was achieved by intravenous administration of a single bolus of thiopentone sodium (Intraval sodium; Rhône Mérieux Ltd.) at a dose rate of 20 mg/kg bodyweight. Following this the sheep was intubated and anaesthesia maintained with gaseous halothane (2-3 per cent) in oxygen and nitrous oxide. The sheep was placed in a large plexiglass whole body respirator (internal volume: 388 litres) in sternal recumbency. The proximal end of the endotracheal tube was connected to the anaesthetic circuit through a connector in the wall of the box. Negative pressure ventilation was achieved by connection to a large bellows pump (Cuirass; Cape Warwick, Warwick, U.K.) which induced a sinusoidal tidal respiratory pattern, the rate and magnitude of which could be controlled by adjustment of the pump itself. A tidal volume of 10ml/kg bodyweight was maintained. Respiratory rate was adjusted to maintain end-tidal CO_2 measurements in the range 4.5-5.5% (Oxicap Monitor Model 4700; Ohmeda, Louisville, CO, USA).

2.3.2. Bronchoalveolar lavage.

Two 20ml aliquots of normal saline (0.9% NaCl solution) were used to lavage lung segments after wedging a flexible fibre-optic bronchoscope (5.3mm OD) (Model FG-16X; Pentax U.K. Ltd.) in selected segmental bronchi.

Bronchoalveolar lavage (BALF) was passed through sterile gauze into a sterile Falcon tube and immediately placed on ice until subsequent analysis.

2.3.3. BALF handling.

BALF was spun at 400g for seven minutes to separate out the cellular fraction. The resultant pellet was re-suspended in sterile phosphate buffered saline and the total cell number counted before subsequent preparation of cytospins for differential cytology. Supernatants were re-centrifuged at 1,000 x g at 4°C for 20 min. The samples were stored at –70°C.

2.3.4. Differential cytology.

Cells were counted using a Neubauer haemocytometer and values expressed per millilitre BALF. Cytocentrifuge slides were prepared and stained using Diff Quick stain for differential counts on 500 cells. Cells were classified as neutrophils, macrophages, eosinophils, lymphocytes, mast cells or 'other' cells according to standard morphological criteria.

2.3.5. LPS-induced up-regulation of ovine elafin and SLPI in bronchoalveolar lavage fluid.

Bronchoalveolar lavage fluid collected by colleagues as part of a related protocol (Tate et al., 2004; submitted manuscript) provided the means to assess the regulation of ovine elafin and SLPI following local lung challenge with LPS. Briefly, in this protocol twelve cross-bred sheep (10 female and 2 male) were anaesthetised with 20mg/kg intravenous thipoentone (Intraval sodium, Merial Animal Health Ltd., Harlow, Essex, UK), intubated and maintained on 2-3% halothane in oxygen and nitrous oxide. 1mg lipopolysaccharide (LPS) from E. coli O26:B6 diluted in 5ml distilled water was instilled via bronchoscope into random segments. Bronchoalveolar lavages from these sheep were collected a week prior to the experiment and 6, 24 and 168 hours after the administration of LPS. The lavages were spun at 400g for 7 minutes and the supernatant stored.

The stored supernatant (200µl) was used in a dot blot onto GeneScreen™ membrane and blocked with 5% milk powder in TPBS. The membrane was then probed with either polyclonal anti-human SLPI antibody (Sallenave et al., 1992) or monoclonal Trab-2O anti-elafin antibody (Hycult, Nl). After exposure to X-omat radiograph-quality film, resultant films were scanned and densitometry measurements taken using a UVP white light Transilluminator (UVP, Inc. Upland, CA USA). Control membranes probed with secondary antibody alone showed no detectable protein (not shown).

2.3.6. Instillation of Adenovirus or Adenovirus/calcium phosphate co-precipitates into the ovine lung.

The anaesthetised and intubated animal was placed in sternal recumbency in the whole body respirator as above and maintained with 2-3% halothane in oxygen and nitrous oxide. The fibreoptic endoscope (5.3mm OD) (Model FG-16X; Pentax U.K. Ltd.) was advanced and wedged in selected segmental bronchi. The adenovirus was diluted from stock solution into 5ml sterile PBS in order to achieve the required number of active particles (plaque forming units or pfu). This 5ml volume was then instilled into the segment, through a polyethylene catheter passed through the giving port of the endoscope and passed as far into the chosen segment as possible without damaging the lung. Instillation was performed in a controlled manner in order to avoid flooding proximal to the tip of the endoscope and to facilitate dispersion of the adenovirus/PBS into the subtended segment. This was usually accomplished within approximately 30-60 seconds. At the end of the instillation air was allowed to enter the giving port of the endoscope to allow equilibration of pressures in the segment and the endoscope carefully withdrawn while monitoring and managing any reflux of the adenovirus/PBS.

Adenovirus/calcium phosphate co-precipitates were formed by incubating, with gentle intermittent agitation, the specific amount of stock adenovirus solution (calculated to yield the requisite pfu) with 1ml of the freshly made calcium phosphate solution (2.14µl 2.5M $CaCl_2$ added per ml EMEM medium and vortexed well) at room temperature for 20 minutes before dilution to 5ml with sterile PBS. This adenovirus/calcium phosphate co-precipitate was instilled in the same way as virus alone.

2.3.7. Culture of harvested alveolar macrophages.

After counting the total cells and making the cytospins for differential cytology, mobile cells were plated out in 48 well tissue culture plates at 50,000 cells per well in RPMI containing 10% foetal calf serum (FCS), penicillin G (final concentration 100U/ml), streptomycin sulphate (final concentration 100µg/ml), L-glutamine (final concentration 2µM) and amphotericin B (0.5%). After 6 hours non-adherent cells were washed off the wells and the adherent cells cultured for a further 7 days in fresh medium. Supernatants were then collected, spun at 13,000rpm for 1 minute to remove cellular contamination, and stored at -30°C. After 24 hours in fresh medium the number of GFP+ve cells was assayed by direct visualization of the wells using UV-microscopy.

2.3.8. Western blot analysis of cell culture supernatants.

25µl of supernatant along with known amounts of purified ovine elafin protein were reduced with 1% dithiothreitol and loaded on 4-12% gradient polyacrylamide gel using the Invitrogen NuPage gel system as recommended by the manufacturer. After running the gel proteins were transferred to Hybond ECL Nitrocellulose membrane (Amersham Pharmacia). Resultant membranes were blocked in 5% skimmed milk powder in TPBS (Phosphate buffered saline and 0.1% Tween 20). Membranes were probed overnight at 4°C with Trab-2O diluted 1 in 500-1000 in TPBS or anti-FLAG monoclonal antibody diluted 1 in 1000 in TPBS. Membranes were then washed in PBS-T before the secondary antibody was applied (Goat anti mouse IgG conjugated to HRP). This was followed by final washing, addition of Western Lightning™ Chemiluminescent Reagent Plus (PerkinElmer Life Sciences, Inc.), and exposure to X-omat radiograph-quality film (Kodak). Densitometry measurements were made on UVP white light transilluminated membranes (UVP, Inc. Upland, CA USA).

2.3.9. Post-mortem visualisation of GFP+ve cells in the ovine lung.

48 hours after lung segmental instillation of Ad-GFP the animals were euthenased and exsanguinated by severing both carotid arteries. The lungs were removed and inflated for 2 hours with 4% paraformaldehyde at room temperature. The lungs were then rinsed twice with sterile PBS and inflated with 30% sucrose overnight at 4°C. Small representative portions of lung were carefully dissected and stored at 4°C in 30% sucrose. Small pieces of these portions were then mounted in optimal cutting temperature medium (OCT) and 10μm sections cut and mounted on lysine-coated slides. The residual OCT was rinsed off with sterile PBS and then the slides were dipped in absolute alcohol to remove salts. The slides were subsequently dipped into 1μg/ml DAPI (4',6-diamidino-2-phenylindole) in methanol for 2 seconds and then immediately dipped firstly into PBS and then into absolute ethanol before being air-dried. Cover slips were then affixed with DPX mounting fluid and GFP+ve cells counted by direct visualisation with UV-microscopy.

2.3.10. Total protein content of bronchoalveolar lavage fluid.

The total protein content of the BALF supernatant was assayed using the bicinchoninic acid method (Pierce) using purified albumin as standards.

2.3.11. Serum neutralising anti-adenovirus titres.

Whole blood was collected into plain vacutainers by jugular venepuncture.and left to clot at room temperature for 2 hours before separation of the serum by centrifugation at 13,000rpm for ten minutes. 10µl of undiluted serum, or of a 1 in 10 or 1 in 100 dilution, was added to 90µl DMEM medium which in turn was added to 85µl of DMEM containing a specific number of adenovirus particles encoding for beta galactosidase (Ad-lacZ). The number of Ad-lacZ particles was calculated to give a multiplicity of infection (MOI 100) when previously cultured A549 cells were infected in 48 well plates. The virus/serum/medium mixtures were incubated at 37°C for 90 minutes and then applied to the washed A549 cells in 48 well plates for 20 hours at 37°C. The monolayers were washed and then lysed by applying 200µl sterile distilled water and passing the cells through 2 freeze-thaw cycles. Finally all the cells were disrupted by repeated passage through a P200 tip along with scraping of the final residual cells from the well. This crude protein solution was transferred into a 1.5ml eppendorf and vortexed for 30 seconds. Finally the insoluble debris was centrifuged out at 13,000rpm for 5 minutes. The resultant clear cell lysate was removed from the isoluble matter for further analysis. This lysate was assayed for beta galactosidase activity by using the β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer (Promega). Beta galactosidase activity was compared to control infections using MOI 100 but no incubation with ovine serum and also a negative control using no Ad-lacZ infection. At each dilution the residual beta galactosidase activity was calculated as a percentage of the positive control cells activity and hence the percentage inhibition calculated. The titre for each time-point was calculated by graphing the inhibition of beta galactosidase activity (y axis) against volume of serum used (x axis). The point where the linear portion of the resultant curve crossed the x axis was used to calculate the volume of serum needed to inhibit 100% of the Ad-lacZ particles. The titre was hence calculated by dividing the volume of the total virus containing solution (185µl) with the volume of serum calculated to be necessary to inhibit the Ad-lacZ 100%.

2.3.12. Modulation of the local and systemic responses to bacterial LPS by the segmental administration of recombinant adenovirus.

A flow diagram outlining the experimental protocol is depicted in figure 1. Virus was administered either once only (at day 0) or twice (at day 0 and then again at day 14). $1x10^8$ plaque forming units (pfu) were co-precipitated and instilled into 3 discrete segments in the right lung. Animals received either Ad-o-elafin or Ad-GFP, no animals received both. At 3, 7 and 10 days after instillation bronchoalveolar lavage fluid (BALF) was collected from one of the administered segments and one 'naïve' segment in the left lung.

4 animals in each group (Ad-o-elafin and Ad-GFP treated) received instillations at both day 0 and day 14 (fig. 1, left branch). BALF was therefore obtained from these animals on days 3, 7, 10, 14 (naïve segment only), 17, 21 and 24. Each of these BALF samples was used for analysis of total cell count numbers, differential cytology and total protein content.

A second group of animals was used to assess the response to bacterial LPS after a single dose of recombinant adenovirus (fig. 1, right branch). Virus was instilled into three segments in the right lung and BALF collected from both the right lung (virus treated segments) and the left lung (naïve segments) at day 3, 7 and 10. These BALF samples were used for assessment of total cell counts, differential cell counts and total protein content. At day 10 after virus instillation *M. haemolytica* LPS (3ml of a sterile solution in water at 150μg/ml) was instilled into both a virus treated segment in the right lung and a naïve segment in the left lung. The sheep were euthenased on day 14 and BALF samples obtained from the LPS instilled segments along with a further 'naïve' segment. This BALF was assessed as above for total cell counts, differential cell counts and total protein content.

The BALF samples from this second group were also used to isolate and culture alveolar macrophages as previously described and culture supernatants used in Western blot analysis after 1 week. The alveolar macrophages were also checked for GFP content after 24 hours as described above.

A more detailed outline of the experimental protocols is shown in fig. 2.

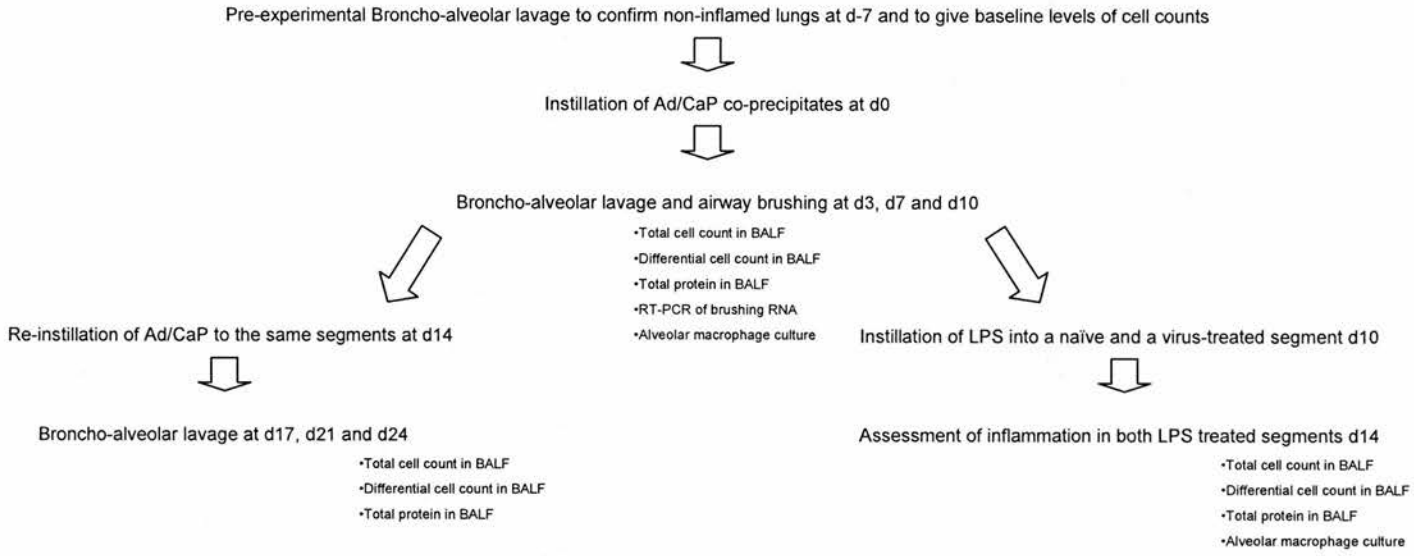


Fig. 1. Flow diagram of the administration of adenovirus/CaP co-precipitates to the ovine lung and the modulation of the response to locally administered LPS.

The diagram shows the time-course of the administration of either Ad-o-elafin or Ad-GFP to the ovine lung and subsequent sampling by broncho-alveolar lavage and airway brushing. The left hand branch of the diagram (A) represents the repeated administration of the viruses over a period of 24 days. The right hand branch (B) shows a single administration of each virus and the modulation of the response to LPS after 10 days.

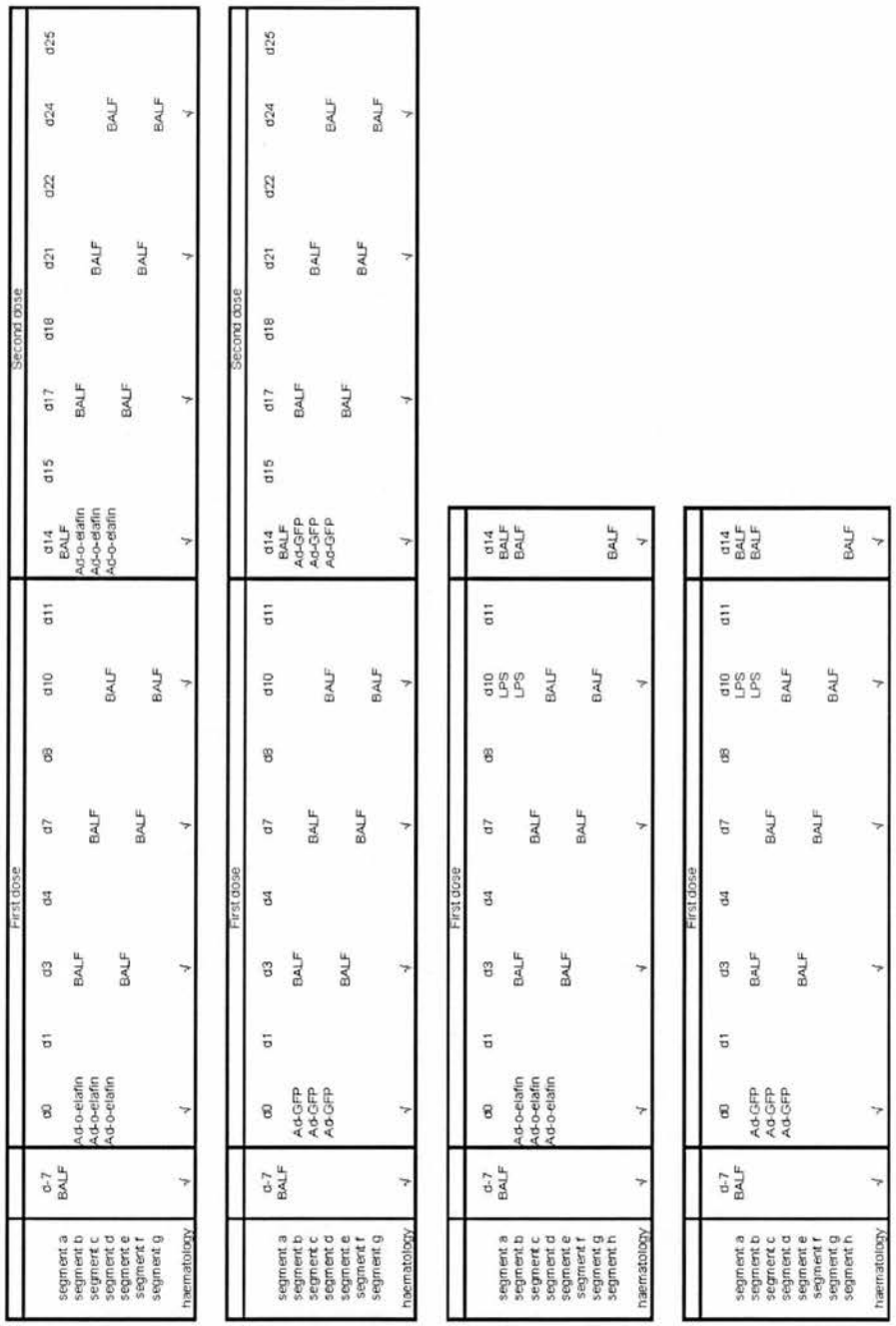

A
First dose
Second dose
segment a
segment b
segment c
segment d
segment e
segment f
segment g
haematology
d-7 BALF
d0
d1
d3
d4
d7
d8
d10
d11
d14 BALF
d15
d17
d18
d21
d22
d24
d25
Ad-o-elafin
BALF
B
First dose
Second dose
Ad-GFP
C
First dose
LPS
D
First dose

Fig. 2...

Fig. 2. Protocol for the repeated administration of adenovirus and also the modulation of the LPS response in the ovine lung.

The diagram shows the times of the administration of either Ad-o-elafin or Ad-GFP to the ovine lung and subsequent sampling by broncho-alveolar lavage and airway brushing. A represents the protocol for animals 188-192; B the protocol for animals 193-196; C the protocol for animals 453, 468, 809, 897 and 909; D the protocol for animals 472, 478, and 920.
(In protocols C and D, examining the effects of Ad-o-elafin and Ad-GFP on the response to bacterial LPS within the lung and also systemically, segment a represents Ad-/LPS+ segments, segment b represents Ad+/LPS+ segments and segment h represents Ad-/LPS- segments as discussed in section 7.3.5).

2.3.13. Production of *Mannheimia haemolytica* LPS.

A 0.5ml aliquot of *M. haemolytica* was inoculated into 50ml of Nutrient Broth and incubated at 37°C overnight. 30ml of this culture was then used to inoculate 3 litres of Nutrient Broth and incubated at 37°C for 18 hours in an orbital shaker at 100rpm. The bacteria were then pelleted at 5000g for 30 minutes and re-suspended in 50ml of sterile distilled water. This suspension was then warmed to 68°C and an equal volume of 90% aqueous phenol at 68°C was added. The resultant mixture was maintained at 68°C for 10 minutes after which it was placed on ice to allow phase separation. After centrifugation at 5000g for 30 minutes at 4°C the upper aqueous phase was collected and dialysed for 36 hours against several changes of distilled water using Spectra/Por^{R} dialysis tubing (3,500 molecular weight cut-off). The resultant solution was then lyophilized and diluted in sterile water to a final concentration of 150µg/ml.

CHAPTER 3

THE IDENTIFICATION AND CHARACTERISATION OF THE OVINE ORTHOLOG OF ELAFIN

3.1. INTRODUCTION

The human elastase-specific inhibitor elafin (Skin derived antileukoprotease/SKALP) has been studied extensively with regards gene/cDNA sequence (Sallenave and Silva, 1993), tissue distribution and antimicrobial activity (Simpson et al., 1999). Simian, bovine and porcine orthologs have also been characterized at the gene and cDNA level (Zeeuwen et al. 1997; Tamechika et al., 1996).

Historically, human elafin was first isolated from bronchial mucus (Hochstrasser et al., 1981; Kramps and Klasen, 1985; Sallenave and Ryle, 1991) and psoriatic skin (Wiedow et al., 1990; Molhuizen et al., 1993). Elafin messenger RNA has been demonstrated by Northern blot analysis in epiglottis, pharynx, vocal fold and psoriatic skin (Pfundt et al., 1996). This is similar to porcine elafin's distribution where elafin has been shown to be expressed in the trachea and large intestine by RNAse protection assay (Tamechika et al., 1996) and furthermore shown to be secreted by goblet cells in the tracheal mucosa and large intestinal crypts by in-situ hybridization (Suzuki et al., 2000). Human elafin has also been shown to be produced by alveolar macrophages (Mihaila and Tremblay, 2001) and Type II epithelial cells (Bingle et al., 2001). It is thought that this general distribution seen for currently known elafin orthologs, i.e. a distribution at mucosal sites and in inflamed epithelia, possibly reflects an immuno-regulatory function for elafin over and above its classically described anti-elastase activity (Simpson et al., 1999; Sallenave, 2000). The distribution of the known elafin orthologs will be looked at in more detail in chapter 5.

The human elafin gene has been studied in detail. It is found on chromosome 20 and spans approximately 2.2 kbps. The gene itself is made up of 3 exons and 2 introns and codes for a protein of 117 amino acids. This being a secreted protein, it has a signal peptide of 22 amino acids followed by two functional domains. The first is a transglutaminase substrate domain made up of five repeats of consensus sequence PVKGQD. This region has been termed 'cementoin' (Nara et al., 1994) in the trappin family of proteins (Schalkwijk et al., 1999) which the elafin members belong to. This glutamine and lysine rich region is thought to be involved in a transglutaminase reaction resulting in ε-(γ-glutamyl)lysine covalent bonds between the repeat region and matrix proteins such as loricrin and keratin-1, to promote the anchorage of the molecule to the interstitium, at least in the skin (Steinert

and Marekov, 1995; Nemes and Steinert., 1999). The second domain of o-elafin is composed of a whey acidic protein (WAP) domain which contains the anti-elastase site (Sallenave and Silva, 1993; Tsunemi et al., 1996).

Simpson et al. (2001A) showed the potential of human elafin cDNA delivery by an adenoviral vector (Ad-elafin) to the murine lung in a model of Pseudomonas induced acute lung injury. Here the delivery of Ad-elafin significantly improved survival rates compared to delivery of a control adenovirus encoding for beta galactosidase (Ad-lac Z). Additionally it was shown that human elafin protein had a chemo-attractant property for neutrophils both *in vitro* and *in vivo* in lungs stimulated with bacterial LPS. Overall the authors reported a beneficial outcome for animals pre-treated with Ad-elafin compared to pre-treatment with control virus or indeed with PBS.

An interest in ovine models of inflammatory lung disease has led to the current investigation into the ovine orthologs of elafin with regards the sequence of the ovine elafin gene and cDNA and the tissue distribution of the ovine elafin cDNA.

Once an ovine elafin ortholog had been shown to exist and dependent upon there being evidence for a similar function to human elafin, an expression plasmid expressing the ovine elafin protein could then be constructed. This would allow the investigation *in vitro* of the potential anti-protease activity of ovine elafin.

3.2. AIMS

The work presented in this chapter was aimed at:

1. Isolating cDNA species from ovine tissue that show homology with the cDNA for human elafin and hence represent an ovine ortholog of this protein.

2. Screening of an ovine genomic library (specifically a Bacterial Artificial Chromosome library) in order to obtain full gene sequence for an ovine elafin ortholog.

3. Screening a panel of ovine tissues to show ovine elafin expression at the RNA level by RT-PCR and Northern Blot techniques.

4. The construction of an expression plasmid containing the ovine elafin cDNA allowing transfection of lung derived epithelial cells *in vitro* to assess the functional activity of ovine elafin as an anti-elastase and also to investigate possible methods of detection of protein.

3.3. RESULTS

3.3.1. Isolation and sequence alignment of ovine elafin cDNA.

Using ovine tracheal mucosal RNA, and PCR primers GSP1 (indicated in fig. 1 and 2 and annealing to a portion of the ovine elafin sequence already available (Mistry, unpublished)) and forward primers annealing to sequences corresponding to the Generacer^{R} oligo ligated to the 5' terminus of the mRNA (GRP1 and nGRP1), 5' RACE PCR produced a product of approximately 610bps (fig. 2) which was cloned into pCR^{R}4-TOPO^{R} plasmid for sequencing. A selection of colonies was grown in LB broth and plasmid purified from these broths. Three sequences were obtained from positive colonies as shown in fig. 3. All of these products contained the sequence corresponding to the Generacer^{R} oligo ligated to the 5' terminus of the mRNA followed by 18bps of 5'UTR and then sequence corresponding to ovine elafin through to the priming site for GSP1. This information allowed 3' RACE to proceed using the forward primer GSP2 (indicated in fig. 3) and a reverse primer annealing to the Generacer^{R} oligo dT used in the reverse transcription step of the RACE procedure. This yielded a product of 887bps (fig. 4) which was also cloned into pCR^{R}4-TOPO^{R} plasmid for sequencing and was shown to contain sequence 3' of the stop codon corresponding to 3' UTR through to the polyadenylation signal. This product terminated with the sequence corresponding to the Generacer^{R} oligo dT sequence used in the reverse transcription step of the RACE procedure. Altogether, the 5' and 3' RACE procedure facilitated the identification of an open reading frame for a protein of 220 amino acids which showed homology with human, simian and porcine elafin (see fig. 5) and will be referred to as ovine elafin. The protein consists of a putative hydrophobic signal sequence of 23 amino acids at the N terminus followed by a distinct region of hexapeptide repeats of consensus sequence PVKGQD which corresponds to a potential transglutaminase substrate domain (Zeeuwen et al., 1997). The C terminal portion contains a presumed disulphide core region with eight cysteine residues in positions corresponding to those in the human elafin protein. This latter region corresponds to the ancestral whey acid protein domain (Schalkwijk et al., 1999).

```
          1           15 16          30 31          45 46          60 61          75 76          90
O-Elafin 2 --------------- --------------- --------------- --------------- --------------- ----AGTCAATGGAC     11
O-Elafin 3 AGGTCCAGTCAAAGG ACAAGATCCAGTCAA AGGTCAAGATCCAGT CAAAGGTCAAGATCC AGTCAAAGGGACAAG ATCCAGCCAAAGGTC     90
O-Elafin 4 --------------- --------------- --------------- --------------- --------------- ---------------      0
O-Elafin 1 --------------- --------------- --------------- --------------- --------------- ---------------      0

          91          105 106         120 121         135 136         150 151         165 166         180
O-Elafin 2 AAGATCCAGTCAAAG GACAAGATCCAGTCA AAGGTCAAGATCCAG TCAAAGGACAAGATC CAGTCAAAGGTCAAG A-TCCAGTCAAAGGT    100
O-Elafin 3 AAGATCCAGTGAAAG GTCAAGATCCAGTCA AAGGTCAAGATCCAG TCAAAGGACAAGATC CAGTCAAAGGTCAAG A-TCCAGTCAAAGGA    179
O-Elafin 4 --------------- --------------- --------------- --------------- --------------- ---------------      0
O-Elafin 1 --------------- --------------- --------------- --------------- CAGTCAAAGGACAAG GGTCCAGTCAAGGGA     30

          181         195 196         210 211         225 226         240 241         255 256         270
O-Elafin 2 CAAGGTCTAGTCAAG GGT-CAAGGTCCAGT CAAGGGACAAGATCC AGTCAAAGGTCAAGA TCCAGTCAAAGGTCA AGATCCAGTCAAAGG    189
O-Elafin 3 CAAGATCCAGTCAAA GGT-CAAGATCCAGT CAAAGGACAAGATCC AGTCAAAGGTCAAGA TCCAGTCAAAGGTCA AGATCCAGTCAAAGG    268
O-Elafin 4 --------------- -------GGTCCAGT CAAGGGACAAGATCC AGTCAAAGGTCAAGA TCCAGTCAAAGGTCA AGATCCAGTCAAAGG     68
O-Elafin 1 CAAGATCCAGTCAAA GGGACATGGTCCAGT CAAGGGACAAGATCC CGTTAAAGGACAAGT TTCAGTTAAAGGTCA AGATAAAGTCAAAGC    120

          271         285 286         300 301         315 316         330 331         345 346         360
O-Elafin 2 ACAAGAC-CGAGTCA GAAGTCCACTCCTCA CTAAGCGTGGCTCCT GCCCCAGGGTTCTGA TCCGGTGCGCCATGA TGAACCCCCCTAACC    278
O-Elafin 3 ACAAGAC-CGAGTCA GAAGTCCACTCCTCA CTAAGCGTGGCTCCT GCCCCAGGGTTCTGA TCCGGTGCGCCATGA TGAACCCCCCTAACC    357
O-Elafin 4 ACAAGAC-CGAGTCA GAAGTCCACTCCTCA CTAAGCGTGGCTCCT GCCCCAGGGTTCTGA TCCGGTGCGCCATGT TGAACCCCC-TAACC    156
O-Elafin 1 GCAAGAAGCCAGTCA AAGGTCCAGTCTCCA CTAAGCCTGGCTCCT GCCCCATTATCTTGA TCCGGTGCGCCATGT TGAATCCCCCTAACC    210
                                                                                  ←GSP1

          361         375 376         390 391         405 406
O-Elafin 2 GGTGTCTGAGAGATG CTCAGTGCCCAGGGG TCAGAAGTGCTGTGA AGGCT    328
O-Elafin 3 GGTGTCTGAGAGATG CTCAGTGCCCAGGG- TCAGAAATGCTGTTA AAGCT    406
O-Elafin 4 GCTGCTTGAAAGATA CTGACTGCCCAGGAA TCAGAAGTGCTGTGA AGGCT    206
O-Elafin 1 GCTGCTTGAAAGATA CTGACTGCCCAGGAA TCAGAAGTGCTGTGA AGGCT    260
```

Fig. 1. Multiple sequence alignment of possible ovine elafin cDNAs already known.

Four partial length ovine elafin cDNA clones (o-elafin 1 to 4) were aligned to choose an area of homology for use as an annealing site for a primer in 5'RACE-PCR. The annealing site for this primer (GSP1) is shown underlined.

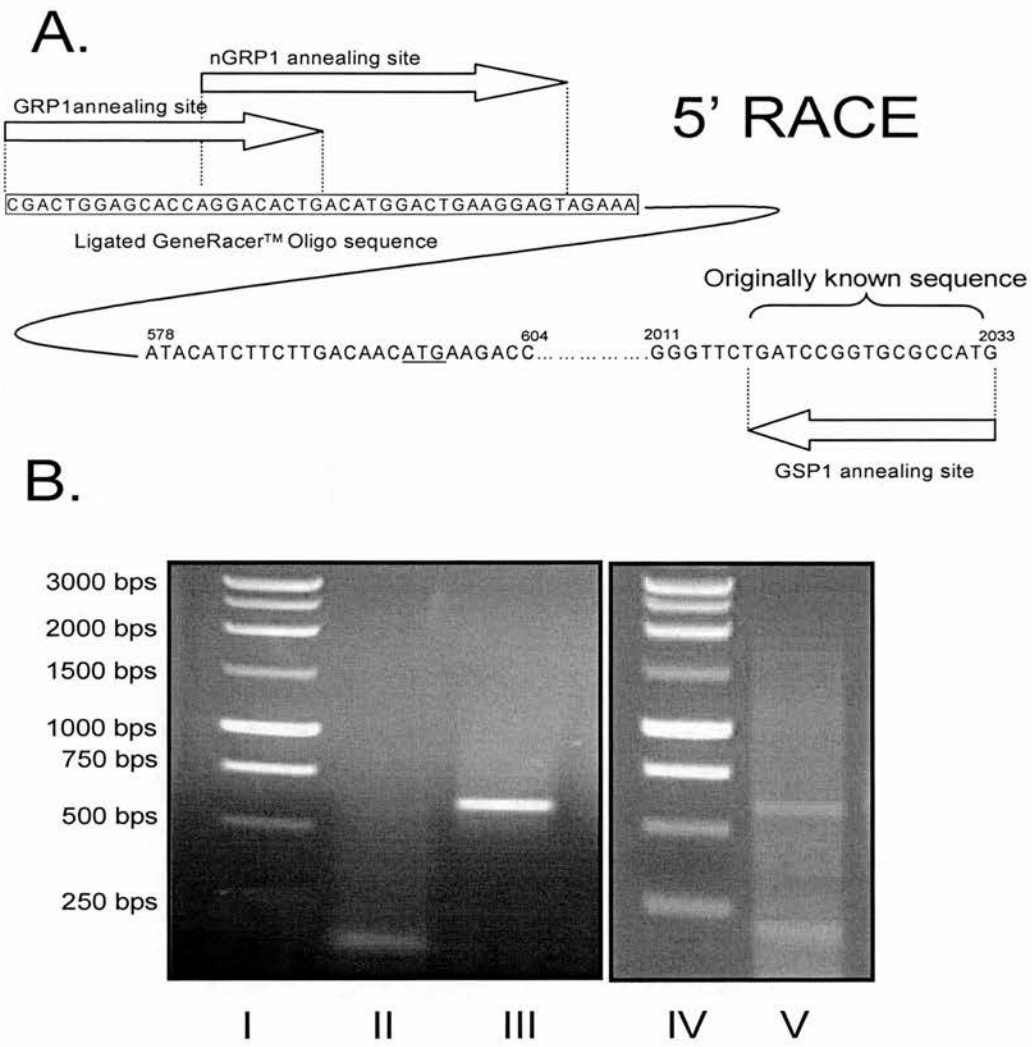


Fig. 2. 5'ovine elafin sequence was derived by 5'RACE-PCR using cDNA derived from tracheal mucosa.

A. Schematic representation of the 5' RACE procedure for ovine elafin using primers annealing to a known portion of the ovine elafin cDNA (GSP-1) and the ligated GeneRacer™ Oligo (GRP-1 and nGRP-1).
B. Agarose gel of the products from the 5' RACE PCRs.
I. and IV. 1kb ladder.
II. PCR performed on cDNA derived from ovine tracheal mucosa using β-actin primers β-actin 1 and 2.
III. PCR performed on cDNA derived from ovine tracheal mucosa using GSP-1 and GRP-1.
V. PCR performed on product in lane C using GSP-1 and nGRP-1.

```
         1           15 16          30 31          45 46          60 61          75 76          90
Clone 1  MKTRSFLVQVVVLLI LGTLVAEAAIIRGRP KGQGTKKTNVLVNEK GPINGQYPVKRPNPV KGQGPVKGQDPVKGQ DPVKGQDPVKGQDPV
Clone 2  MKTRSFLVQVVVLLI LGTLVAEAAIIRGRP KGQGTKKTNVLVNEK GPINGQYPVK----- -------GQDPVKGQ DPVKGQDPVKGQGPV
Clone 3  MKTRSFLVQVVVLL- LGTLVAEAAIIRGRP KGQGTKKTNVLVNEK GPINGQYPVKRPNPV KGQGPVKGQDPVKGQ DPVKGQDPVKGQDPV
         GSP2→

         91          105 106         120 121         135 136         150 151         165 166         180
Clone 1  KGQDPVKGQDPVKGQ DPVKGQDPVKG---- --------------- --------------- --------------- ---------------
Clone 2  KGQDPVKGQDPVKGQ DPVKGQGPVKGQDPV KGQ------------ --------------- ---DPVKGQDPVKGQ DRVRSPLLTKRGSCP
Clone 3  KGQDPVKGQDPVKGQ DPVKGQDPVKGQDPV KGQDPVKGQDPVKG- DPVKGQGPVKGQDPV KGQDPVKGQDPVKGQ DRVRSPLPH------

         181         195 196         210 211         225
Clone 1  --------------- --------------- ----------   116
Clone 2  RVLIRCAM------- --------------- ----------   146
Clone 3  --------------- --------------- ----------   172
           ←GSP1
```

Fig. 3. Amino acid sequence for 3 clones derived by 5RACE-PCR.

5' RACE PCR was performed using cDNA derived from ovine tracheal mucosa and primers GSP1 and primers annealing to the 5' GeneRacer™ oligo. Product was cloned into pCR⁴-TOPO™ which was used to transform TOP 10 *E. coli* cells. Positive colonies were grown up in broth and plasmid purified and sequenced. Three clones contained the 5' portion of an open reading frame showing homology with human and porcine elafin. The amino acid sequence of the open reading frames for these clones are shown aligned. Underlined are the priming sites for GSP1 (used in the 5'RACE-PCR) and GSP2 (to be used in the full-length PCR to follow).

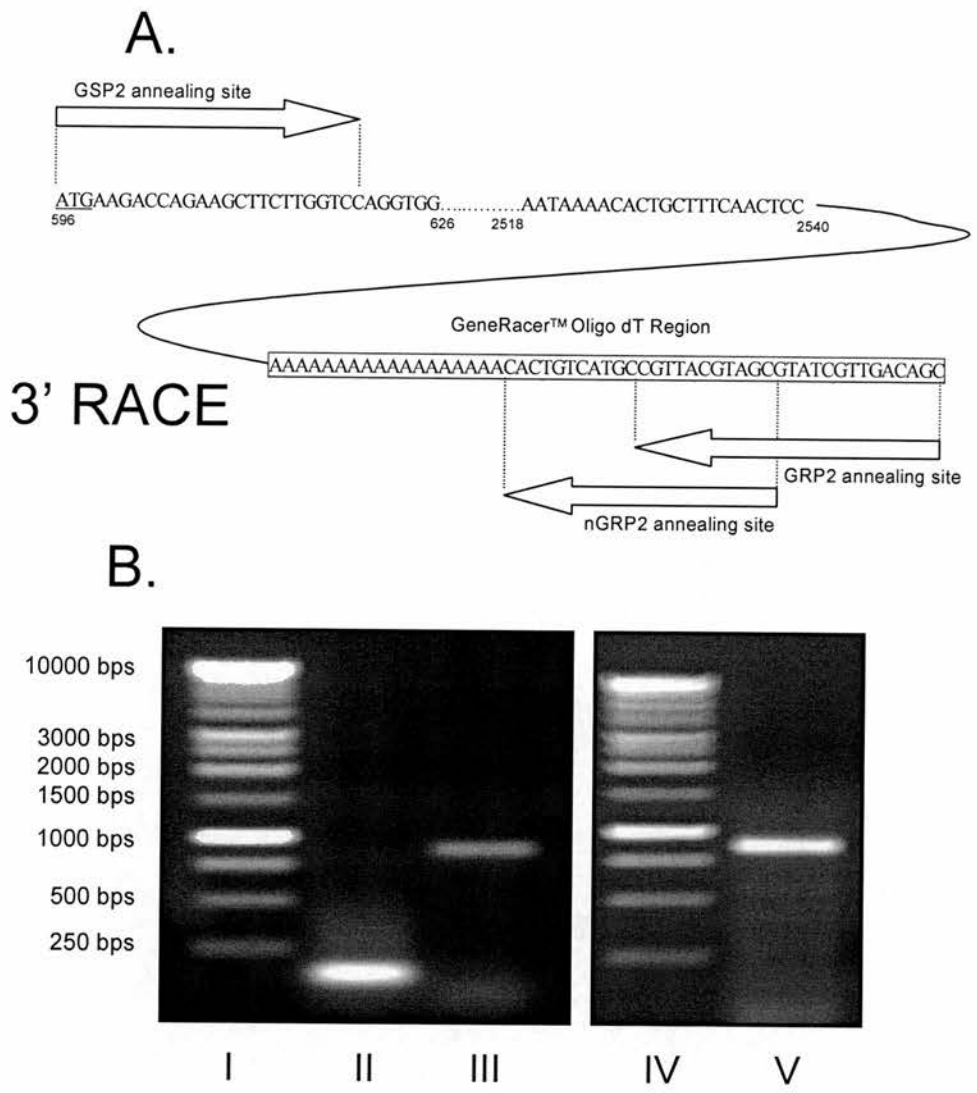


Fig. 4. PCR of full length ovine elafin using cDNA derived from tracheal mucosa.

A. Schematic representation of the full length RACE procedure for ovine elafin using primers annealing to a known portion of the 5' end of the ovine elafin cDNA (GSP-2) and the GeneRacer™ Oligo-dT region (GRP-2 and nGRP-2).
B. Agarose gel of the products from the full length RACE PCRs.
I. and IV. 1kb ladder.
II. PCR performed on cDNA derived from ovine tracheal mucosa using β-actin primers β-actin 1 and 2.
III. PCR performed on cDNA derived from ovine tracheal mucosa using GSP-2 and GRP-2.
V. PCR performed on product in lane III using GSP-2 and nGRP-2.

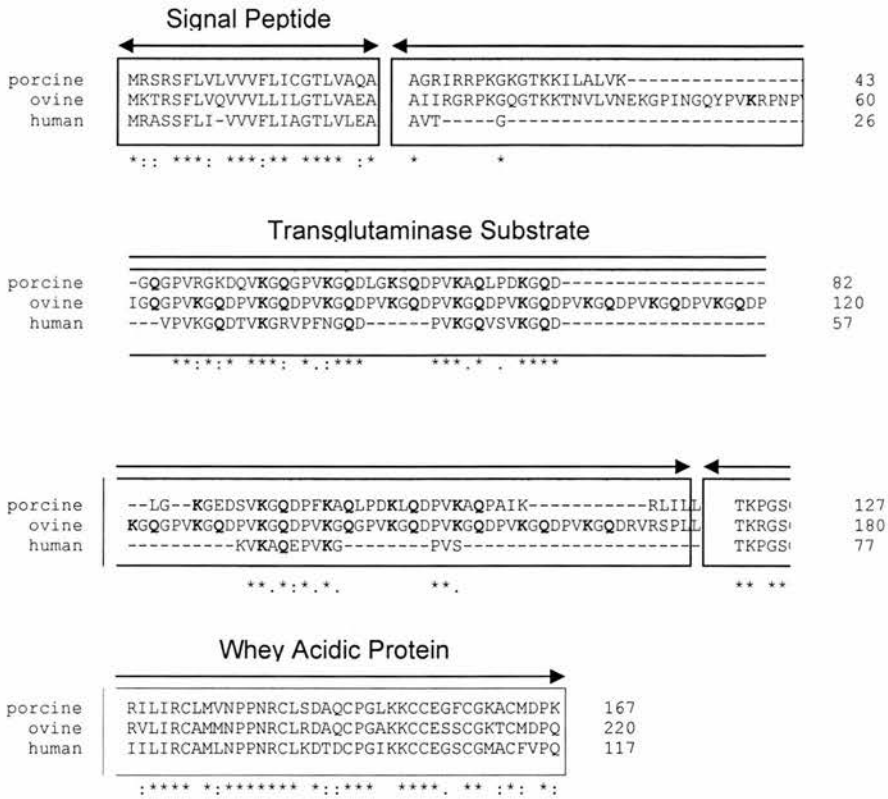


Fig. 5. The complete ovine elafin amino acid sequence shows high degree of homology with porcine and human orthologs.

PCR was performed on cDNA derived from ovine tracheal RNA using forward primer GSP2 and the reverse GeneRace™ primer GRP2. This was followed by a semi-nested PCR using GSP2 and the nested GeneRace™ primer nGRP2. PCR product was purified and ligated into pCRR4-TOPO™ which was used to transform TOP 10 *E. coli* cells. Positive colonies were grown up in broth and plasmid purified and sequenced. PCR product contained an open reading frame coding for a protein of 220 amino acids (ovine elafin). The amino acid sequence of the encoded protein is shown aligned with that for porcine and human elafin. The three domains are shown in separate boxed areas. The glutamic acid and lysine residues involved in the transglutaminase-mediated covalent bonding are indicated in bold type. '*' indicates a conserved residue, ':' indicates a conserved substitution and '.' indicates a semi-conserved substitution.

3.3.2. Sequence analysis of ovine elafin gene

Screening of an ovine BAC library was performed at the Institut National de la Recherche Agronomique (INRA) using probes generated by PCR (see materials and methods). This screening identified one clone for elafin. Southern blot analysis using 1μg of BAC clone DNA allowed identification of a positive EcoR1 band of approximately 15kb from the elafin clone (see fig. 6). This band was purified and subcloned into PDC516 (Microbix Biosystems Inc., Ca) for sequencing with sequential internal primers through to the polyadenylation signal at the 3' end and proximally through towards the promoter region. Unfortunately when this approach was applied to the 15kB EcoR1 fragment containing the elafin gene, we were only able to obtain full 3' sequence information (up to the polyA signal) and no 5' information. Because the BAC library was generated by partial Hind III digestion, and since the elafin signal peptide coding region contains a Hind III site (not shown), we were unable to obtain 5' information using this methodology and only obtained sequence from this restriction site forwards.

The promoter region sequence was obtained for the elafin gene by using PCR (with ovine genomic DNA as template obtained locally – see Chapter 2) with primer f-el(prom) annealing to the 5' end of the ovine elafin promoter (see fig. 7) along with a reverse primer annealing to a portion of the first intron of the ovine elafin gene (r-inv-el). This PCR product (fig. 8) was cloned into pCR^{R}4-TOPO^R and sequenced (fig. 9). This enabled us to obtain the rest of the elafin gene and we were able to show that the elafin gene spans approximately 2.5kb and is split into 3 exons and 2 introns. Positions of the exon/intron boundaries and other features of interest are shown in fig. 10.

3.3.3. Tissue distribution of ovine elafin.

Total RNA was extracted from multiple ovine tissues and analysed by Northern blot. A 750bp ovine elafin RNA species was detected in tongue, trachea, small intestine, large intestine (strongest signal) and skin (see fig. 11).

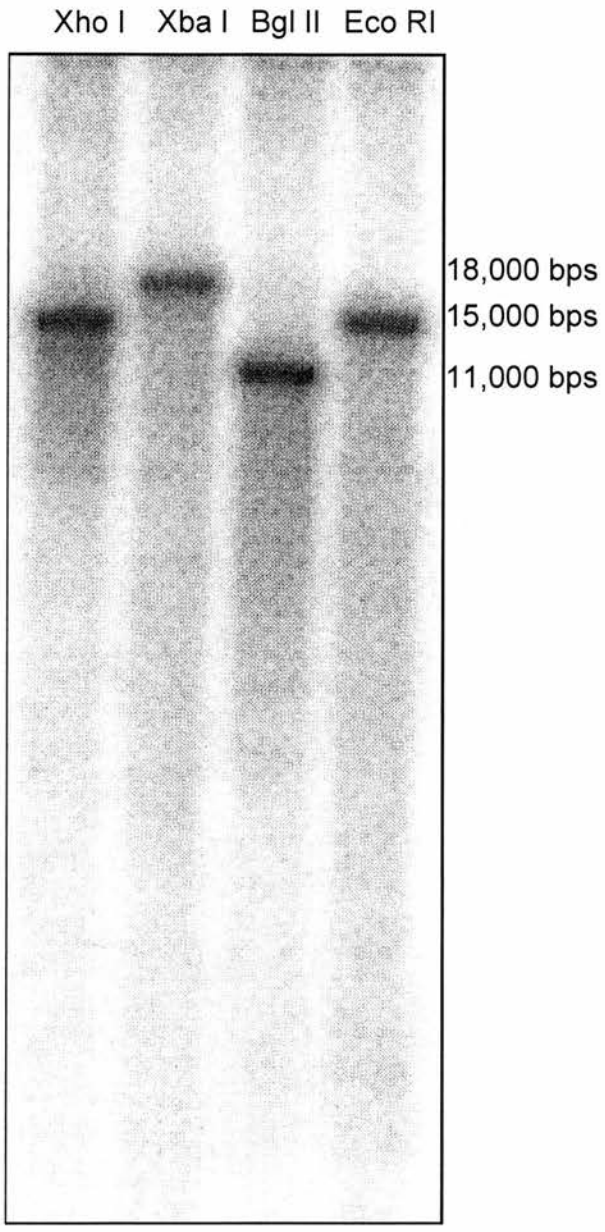


Fig. 6. Southern blot of ovine BAC DNA probed for ovine elafin.

1µg of BAC DNA was digested with restriction enzymes (Xho I, XBa I, Bgl II and EcoRI), fractionated on a 0.8% agarose gel, then blotted onto a nylon membrane. The membrane was probed with radiolabelled full length cDNA corresponding to the coding sequence of TOM-1. The size of the bands seen are indicated.

```
ovine      0 ------------------------------------------------------------
porcine    0 GAATTCCACCTTCCCTATTACTTTCTTGGTGCATTTCAGAGGGGATGACCCGTGAAGTGG
human      0 ------------------------------------------------------------

ovine      0 ------------------------------------------------------------
porcine   60 AAGAGCATGGTGATTAGTCCTTAGACATTCAAGGCAGGCTTGCCTTGGCGAGTTCTTCTT
human      0 ------------------------------------TTTGTCTTCAAGAGTTTTTC--

ovine      0 ------------------------------------------------------------
porcine  120 GGGGTCAGGGGAGAATACAAGGAAAGCCCAGCTGGAGGCTGGGAGGGGTCAACCGTTAG-
human     20 GAGACCAGGGAAGAAGGAAGGAAATGCCCAGTTTGATCGTGGGAGTGGTAAAATGATAAA

ovine      0 ------------------------------------------------------------
porcine  179 GTAGGTCTGGGTGGGGCTTGCAGGAAGAGGGAATAATTGGGAAACACCTGGGCTGTGAAA
human     80 GTAGATCTGGGTGGGGTTTGTAGCACCAGAGCATAATGGAGAAACACCTTGGTTTTGTAA

ovine      0 ------------------------------------------------------------
porcine  239 GCAGAACGCTGGACCGCCTCATGACTTTCTTGAATGACCTTGGGTGACTCTTCTCTCTTC
human    140 TCAAGA--CTGGATCTACCAGTGACTTGCT-GAAT-AACTTCGGTGATTCCTTTCTCTTC

ovine      0 ------------------------------------------------------------
porcine  299 TTTGGGCTTCATGTTCTTTCTAAGTAGGAAGAATGTGGTCGGGATGGTGCCTAAGG-GCC
human    196 TT-GGGTCTCACTGTATTTCAAAACATGAAGAATTTCATTGTAATGTTACCTAATAAGTG

ovine      0 ------------------------------------------------------------
porcine  358 AGCCAGCCCTTCT---CTGTGAGGACGTGAGATCTTCGGTCCTGGGGCTGTGAGGGGTGA
human    255 AGCCAGCACTTCTACTCTGTGAGAAAGTAGGAAAAC---TCTTGGGACAATCAGAGATGA

ovine      0 ------------------------------------------------------------
porcine  415 TGGAACAGGACCTGGCATA--TCTCCCTGTGAATAATCCTTGGGGAAAGGCCCAAGGGCC
human    312 TGTGATGTAATGTCCATTAGTTCTTCCTGTGAATAATCCTGAGGGAAAGCCCCCAGGTCC

ovine      0 ------------------------------------------------------------
porcine  473 CTCCCAGGATGGGGGTGGGTGTTTCCTGGCAGAATGAAGAAACCGTAGGTCTTGTCCCTC
human    372 CTCCCAGAATGGGG-TGGATATTTCCC---A------ATACAGCTAAGGAATTATCCCTT

ovine      0 ------------------------------------------------------------
porcine  533 GTGAA-ACCGCAGACCCACCCTTGGCGCCAGGCCAAGCTGGGGCTGCATAAAACTGAGCT
human    422 GTAAATACCACAGACCCGCCCT-GGAGCCAGGCCAAGCTGGA-CTGCATAAAGATTGGTA

ovine      0 ------------------ATACATCTTCTTGACAACATGAAGACCAGAAGC 33
porcine  592 TGGCCTCAGCTCTCAGCCATCCACCTTCCTGACAACATGAGGTCCAGAAGC 643
human    480 TGGCCTTAGCTCTTAGCCAAACACCTTCCTGACACCATGAGGGCCAGCAGC 531
```

Fig. 7. Design of primers for sequencing of ovine elafin promoter region.

The known promoter sequence for the human and porcine elafin genes were aligned with the 5' sequence for ovine elafin. A primer was designed based on the porcine sequence which anneals to the underlined portion of the porcine sequence. This primer (f-el)prom)) was used in PCR along with R Inv 1 primer. Template used was ovine genomic DNA. The start codons for the three sequences aligned here are shown in bold.

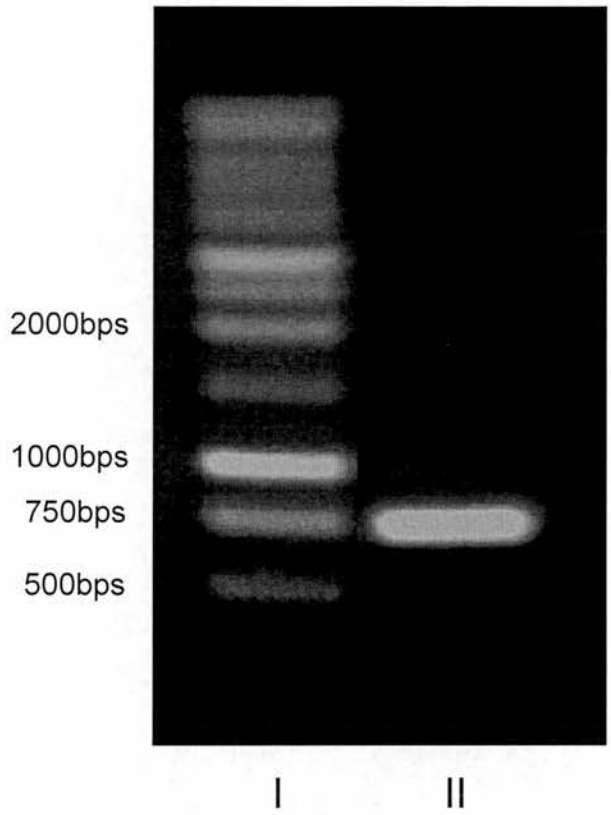


Fig. 8. PCR of ovine elafin promoter.

PCR was performed on ovine genomic DNA using the primers f-el(prom) and r-inv-el. Product was run out on 0.8% agarose gel.
I. 1kb ladder.
II. PCR performed on ovine genomic DNA using f-el(prom) and r-inv-el primers.

ACTTTCTTGGTGCATTTCAGAGGCTGCCTGTGAAATGGAAAAGTATAATG 50

ATTAGTCATTAAACACTCAGGGCAAATTTGCCTTTGAGAGTTTTTCTTGG 100

GATCAGGGGGAGAATGAAAGAAAAGCCTAGCATGATGCTGGGAAGAATAA 150

ATTGTTAGGGTAGGTCTGGGTGGGGCTTGCAGGAGCTGGGGAGAAATGGG 200

GGAACACCTTGGCTATACAAACAGTACTCCAGACCTACCACTGACTTGTT 250

GAATGACTGTGGGCGATTCCTCTCTCTCCTTTGGGCTTCATATTCTTTAA 300

GTATAAAGAATTTGGTCATGACGGTGCCTACAGACCAGCCAGCCCTACTT 350

CTTTGTGAGAATGTGAGATAGCTCTTGGGGCTATTAGAAATGATGTAACA 400

GCCATTTCTCCCTGTGGATAATCCTTGGGGAAGGGCCCAAGGTCCCTCCC 450

AGGATGAGGGTGTATATTTCCTGGTAAAACTAAGAAAATTTAAGTCTTAT 500

CCCTTGTGAAACCACAGACCCACCCTTGGCACCTGGCCAAGCTGAAGCTG 550

CATAAAACTAGGCTTCAGCTCTCAGTCATACATCTTCTTGACAAC**ATG**AA 600

GACCAGAAGC

Fig. 9. Ovine elafin promoter region sequence.

Sequence derived from the product of PCR reaction with 5' elafin and R Inv 1 primers using genomic ovine DNA as template. The 5' elafin primer annealed directly 5' to the 1st base shown. The start codon of the ovine elafin cDNA is shown in bold. The putative TATA box and Kozak sequence are underlined.

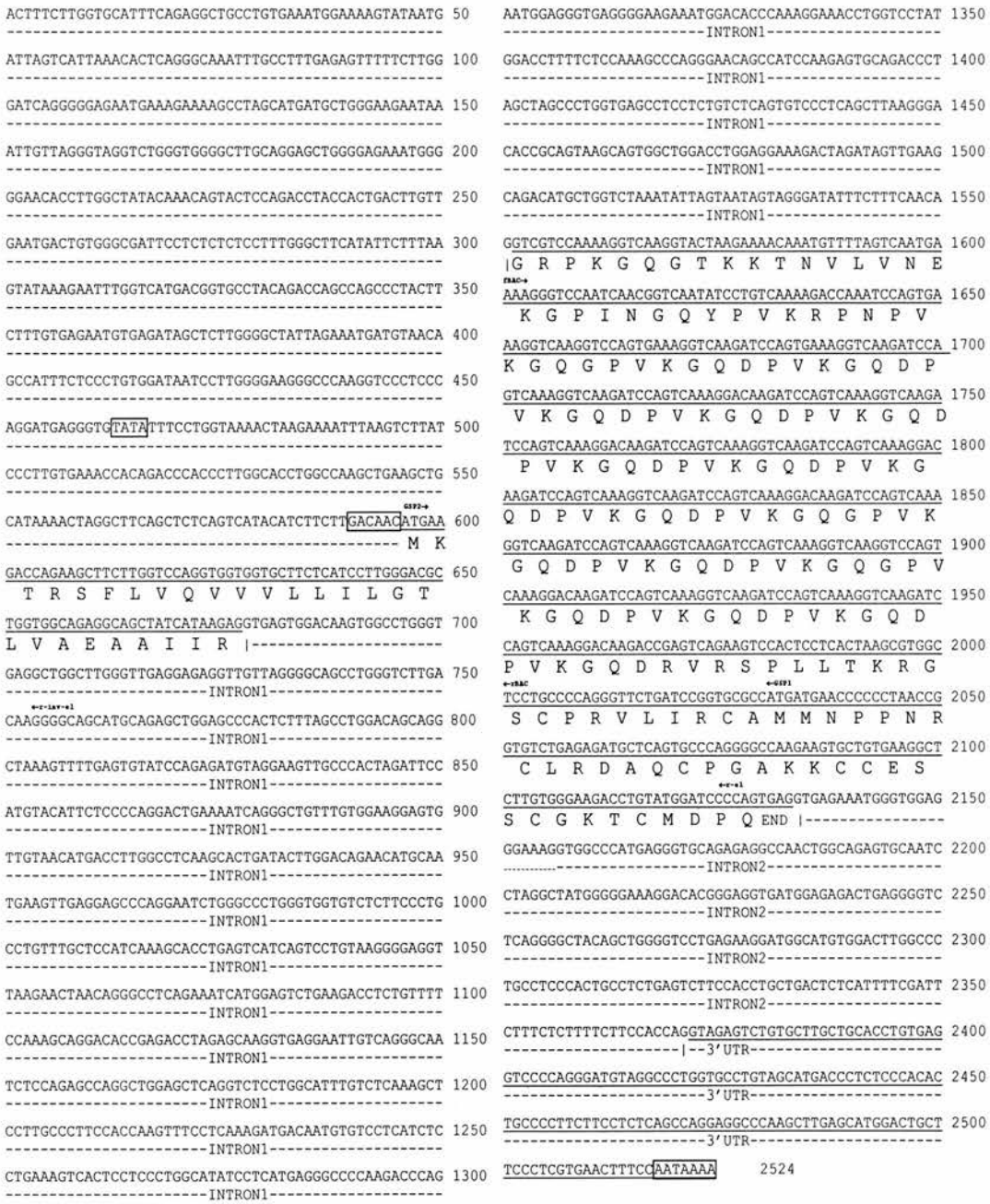


Fig. 10. Sequence for the gene coding for ovine elafin.

The 'tata' box, 5' Kozak sequence and 3' polyadenylation signal sequence are boxed. Exons are underlined. Predicted signal cleavage site is between amino acids 23 and 23 (between A and A). Annealing sites for the primers GSP1, GSP2, r-el (used in RACE-PCR), fBAC, rBAC (used in PCR screening of BAC library) and r-inv-el (used to amplify the promoter region) are indicated.

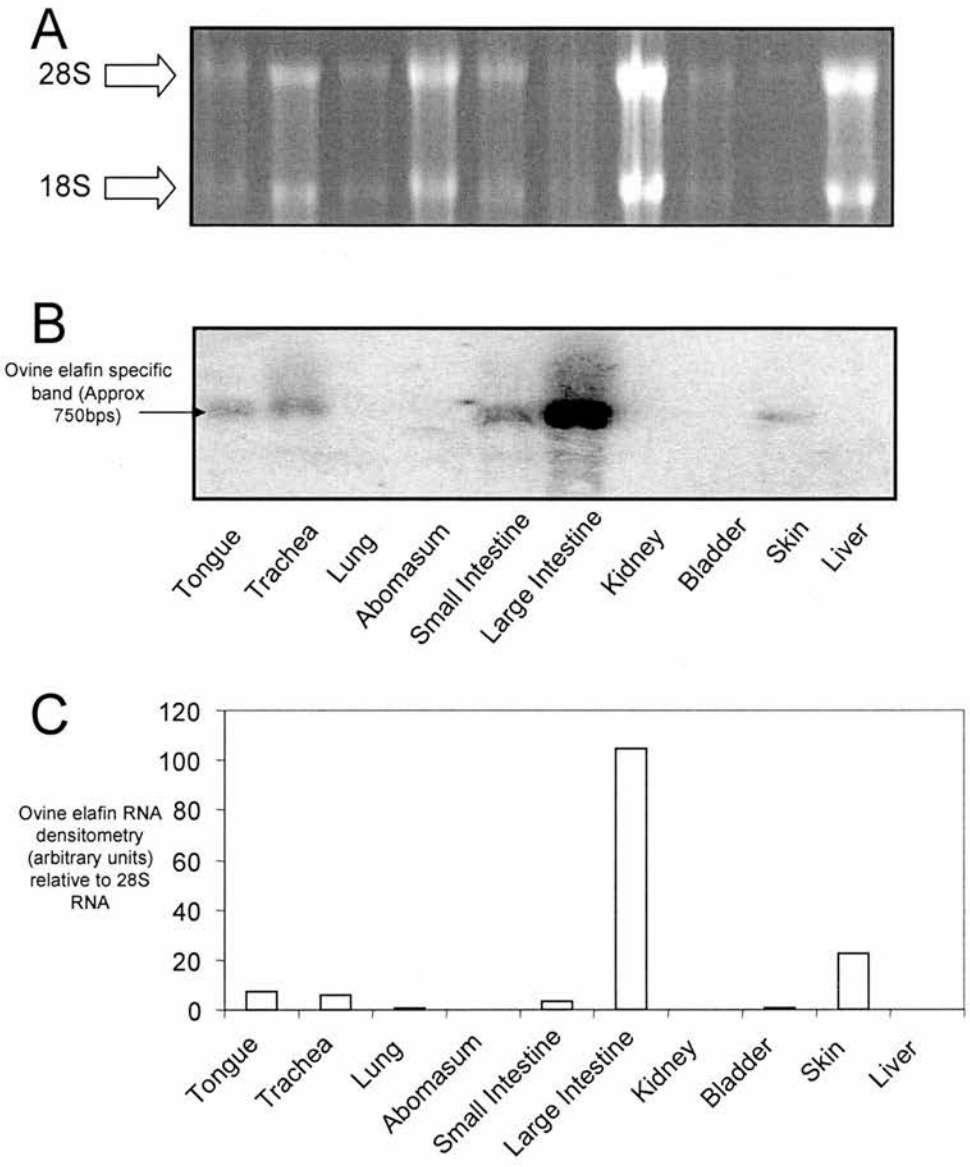


Fig.11. Tissue distribution of ovine elafin RNA as assessed by Northern Blot (loading of 20g of total RNA).

Ribosomal 18S and 28S RNA sub-units are indicated by arrows (A). The membrane was probed with the full length cDNA (660bps) corresponding to the coding portion of the gene for ovine elafin (B). Quantitative analysis of tissue expression of ovine elafin by densitometry of blot intensity relative to 28S RNA bands (C).

RT-PCR reaction using primers GSP2 and r-el (shown in fig. 10) for ovine elafin was performed using the same RNA samples (see fig. 12). Consistent with the Northern blot analysis, we found RNA to be present in tongue, trachea, small intestine, large intestine and skin. Interestingly, products of two different lengths were obtained, approximately 660bp (the expected size from fig. 10) and 800 bp. Two forms of ovine elafin are therefore being transcribed in the tissues analysed, the 660bp form shown and isolated by RACE-PCR and predicted by the BAC screening as shown in figs. 10, and an additional longer form of 800bp.

3.3.4. Genomic PCR for ovine elafin using a combination of non introns-spanning and introns-spanning primers.

On account of isolating two different ovine elafin mRNA products by RT-PCR (see fig. 12), although only one gene was isolated from the BAC library screening, we performed additional PCR reactions using non-intron spanning primers and a variety of templates to determine whether these RNA products are splice variants from a single gene or products of different/allelic genes. Fig.13A shows the PCR products when fBAC and rBAC primers are used with various templates: genomic ovine DNA (obtained locally), cDNA synthesised by RT-PCR from ovine skin RNA (shown to be positive for elafin expression), DNA extracted from the elafin positive BAC clone, and finally DNA extracted from the expression plasmid pIRES-GFP#3 containing the cDNA for ovine elafin isolated by RACE-PCR (see above). Two bands were seen with the first two templates (approximately 400bps and 525bps). The low molecular weight band corresponded to the ovine elafin cDNA product isolated by RACE-PCR (coding for a 220 amino acid protein) and present in the pIRES-GFP#3 plasmid containing the ovine elafin cDNA. The higher molecular weight band was of unknown origin. The two products obtained with the genomic DNA template were isolated and cloned into pCR^{R}4-TOPO^{R} and sequenced. As predicted, the low molecular weight product sequence matched exactly the

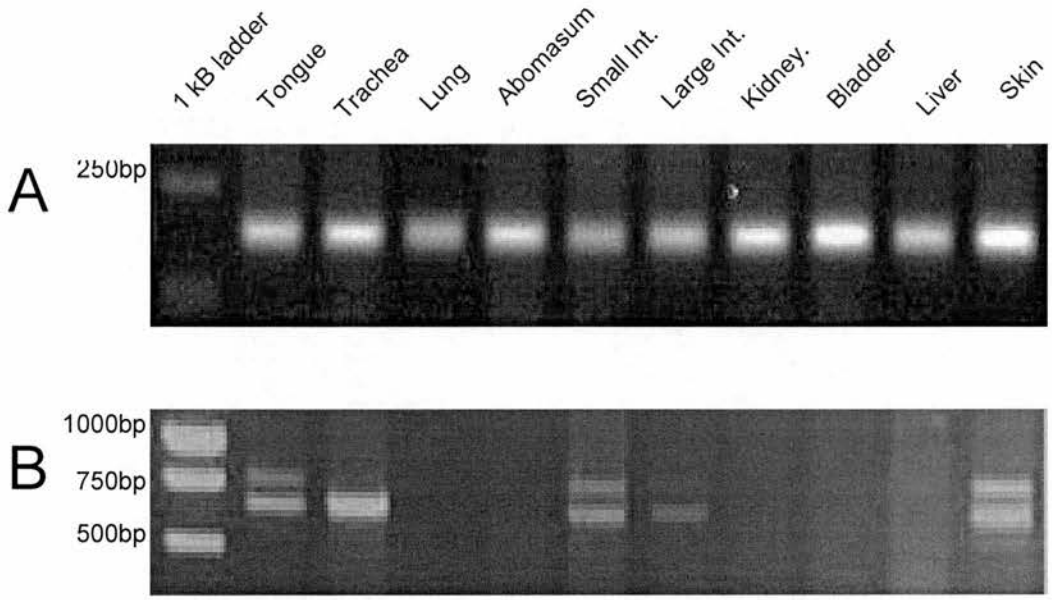


Fig. 12. Tissue distribution of ovine elafin expression by reverse transcription and PCR (RT-PCR).

RT-PCR for (A) ß-actin and (B) ovine elafin from RNA extracted from the various organs indicated. The position of molecular weight markers is indicated. Primers used for amplification of ovine elafin GSP2 and r-el yielding two products of approximately 660bps and approximately 800bps.

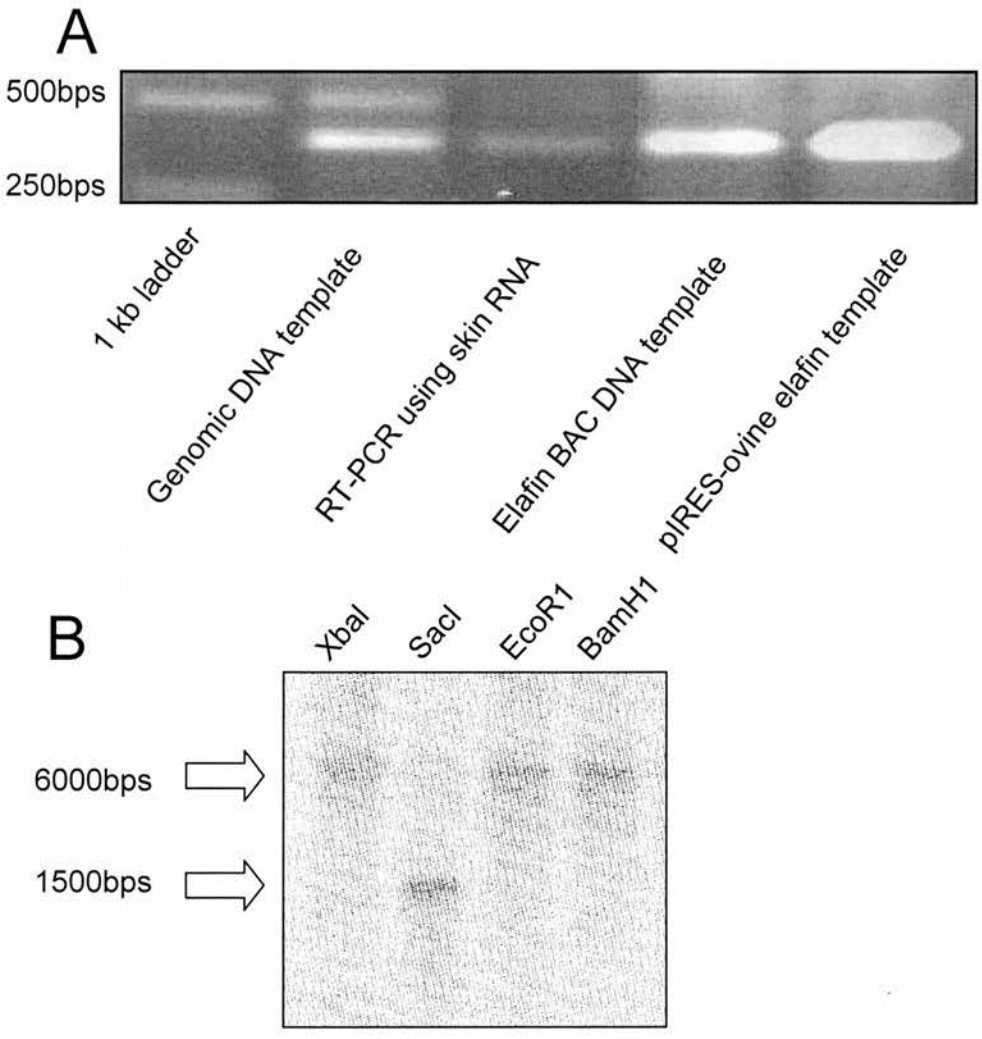


Fig 13. The Ovine Elafin gene exists as two alleles.

A. PCR was performed using the non intron-spanning primers fBAC and rBAC as detailed in the text. Templates used were ovine genomic DNA, cDNA synthesised by reverse transcription of RNA isolated from ovine skin, DNA isolated from an ovine BAC clone positive by PCR for the elafin gene, and the expression plasmid pIRES-GFP#3 containing the cDNA for ovine elafin (TOM-1). The lower molecular weight product of approximately 400bps is due to amplification of exon 2 of the ovine elafin gene or cDNA (and also inserted into pIRES-GFP#3); the longer product of approximately 525 bps corresponds to amplification of a longer form of ovine elafin.

B. 20μg of genomic ovine DNA was digested with restriction enzymes (XbaI, SacI, EcoR1 and BamH1), fractionated on a 0.8% agarose gel, then blotted onto a nylon membrane. The membrane was probed with radiolabelled full length cDNA corresponding to the coding sequence of TOM-1. The size of the bands seen are indicated.

sequence from the ovine elafin original sequence (fig. 10) while the high molecular weight product sequence showed an increase in number in PVKGQD repeats (26 instead of 19). The existence of two different genomic forms was further confirmed using a different set of PCR primers (GSP2 and f-el), yielding 2 distinct products of approximately 1500 and 1600bps (not shown). These products were cloned into pCR^{R}4-TOPO^{R} and sequenced and confirmed the presence of a second, heavier form of the ovine elafin gene coding for 26 PVKGQD repeats (instead of 19) but otherwise identical to the original gene isolated by BAC library screening and above. The original form of the gene described in Fig. 10 will here after be referred to as Trappin Ovine Molecule-1 (TOM-1) and the higher molecular weight form, coding for the longer form of ovine elafin of 262 amino acids, will be referred to as TOM-2. Alignment of TOM-1 and the known region of TOM-2 are shown in a diagrammatic representation in fig. 14.

Southern Blot analysis of ovine genomic DNA (from the same animal as the RNA used in the RT-PCR fig. 12) is shown in fig. 13B and indicates the presence of only one ovine elafin gene showing that indeed TOM-1 and TOM-2 are two allelic forms of the same gene.

3.3.5 Construction of expression plasmid encoding for ovine elafin.

The TOM-1 cDNA was successfully ligated into pIRES-GFP#3 (fig. 15) at three different insert to vector ratios as seen in Fig. 16. Product from the reaction between 7.5ng vector and 10ng insert was used to transform XL1-Blue subcloning grade E. coli. Positive clones were grown up in broth and plasmid isolated with the Qiagen Maxi-Prep kit. One such clone was chosen based on correct restriction enzyme analysis and sequencing. This plasmid will now be known as pIRES-GFP#3-ovine elafin.

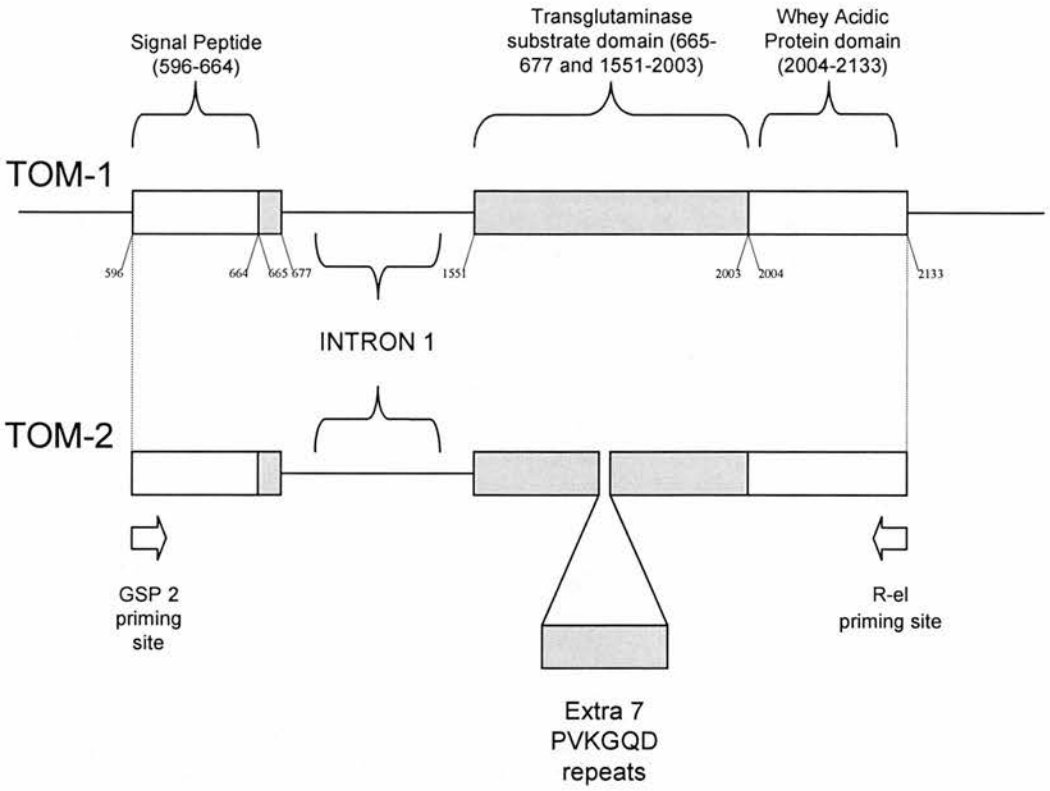


Fig.14. Figure showing the relationship between the two ovine elafin alleles (TOM-1 and TOM-2) coding for proteins of 220 amino acids and 262 amino acids respectively.

The promoter sequence and 3' sequence is known for the gene TOM-1 only (see Fig. 10). 100% homology exists between the known portion of TOM-2 and the corresponding portion of TOM-1, except for the fact that TOM-2 contains 7 extra repeats with the consensus PVKGQD as shown. Numbers below the representation of TOM-1 denote base-pairs from the start of the sequence shown in Fig. 10. TOM-2 has been sequenced after PCR of ovine genomic DNA using the primers GSP 2 and R-el as shown.

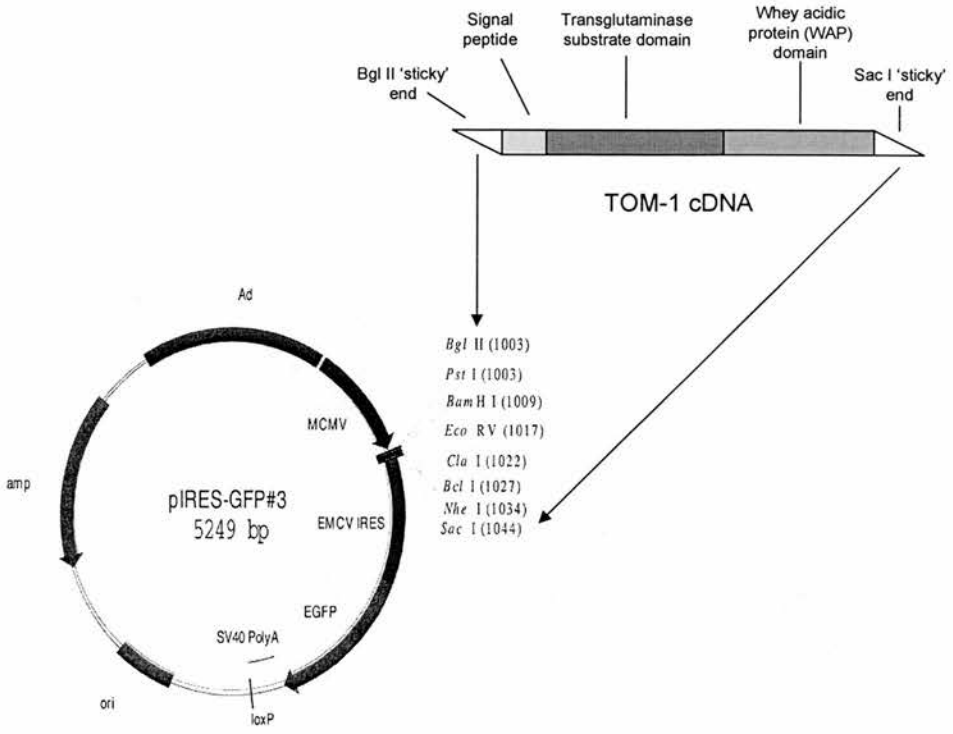


Fig. 15. Insertion of TOM-1 cDNA into pIRES-GFP#3.

PCR product consisting of the cDNA coding for TOM-1 with the addition of Bgl II and Sac I restriction sites (Bgl II-o-elafin-Sac I) was purified from agarose gel, digested with Bgl II and Sac I then re-purified and inserted into pIRES-GFP#3 expression vector which had also been purified post digestion with Bgl II and Sac I. This allowed the construction of pIRES-GFP-o-elafin.

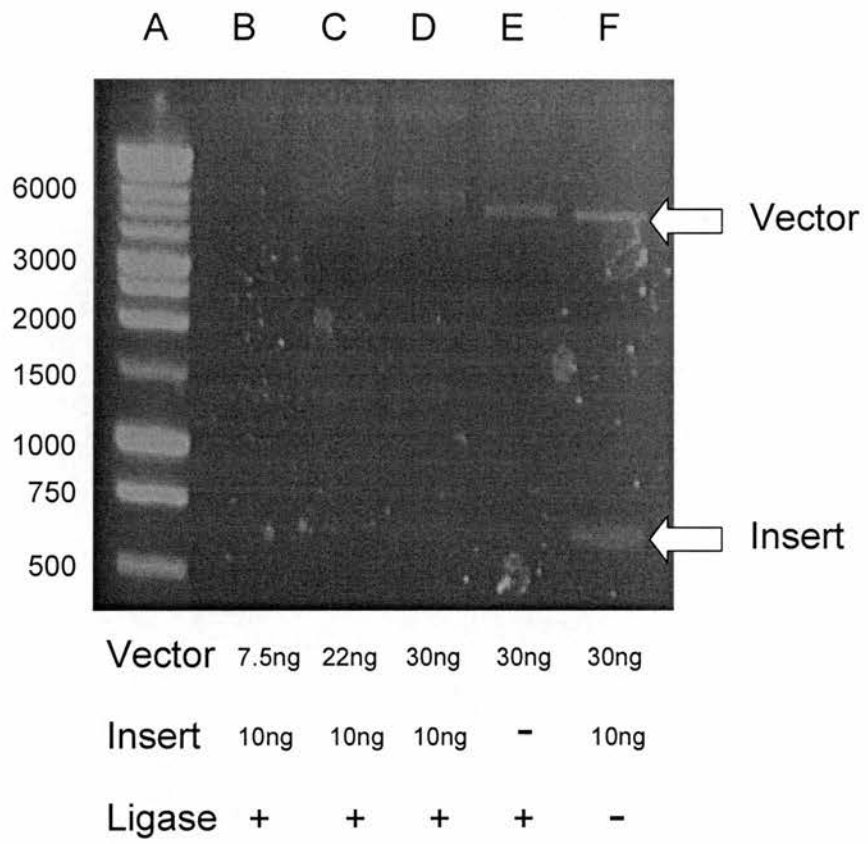


Fig. 16. Ligation of TOM-1 cDNA into pIRES-GFP#3.

Bgl II and Sac I digested TOM-1 cDNA was ligated into digested pIRES-GFP#3 with varying ratios of insert to vector as shown above and the products run on an 0.8% agarose gel.

A 1kb ladder.

B,C,D Vector and insert at varying ratios and T4 DNA ligase

E Vector and T4 DNA ligase showing minimal recircularisation of vector

F Vector and insert with no T4 DNA ligase

3.3.6. Transfection of 293 cells with an ovine-elafin plasmid construct.

In order to show potential anti-elastase activity attributable to the protein encoded for by the ovine elafin cDNA, supernatants were collected from 293 cells transfected with an expression plasmid encoding for ovine elafin (pIRES-GFP#3-ovine elafin containing the TOM-1 cDNA).

Supernatant from 293 cells either untransfected or transfected with pIRES-GFP-ovine elafin were collected and submitted to Western Blot analysis (see Fig. 17). The ovine elafin transfected cell supernatant contains a protein that reacts with the anti-elafin Trab-2O antibody and migrates at a molecular weight of approximately 38kDa. The untransfected cell supernatant does not contain any protein cross reacting to the Trab-2O antibody.

An anti-elastase assay was performed on this supernatant and on supernatant from untransfected 293 cells. Results are presented in fig. 18 as overall Elastase Inhibitory Capacity (EIC) i.e. the amount of pure human neutrophil elastase inhibited by the supernatant expressed as nmol/ml. The elafin plasmid transfected cell supernatant has a higher EIC compared to the untransfected cell supernatant.

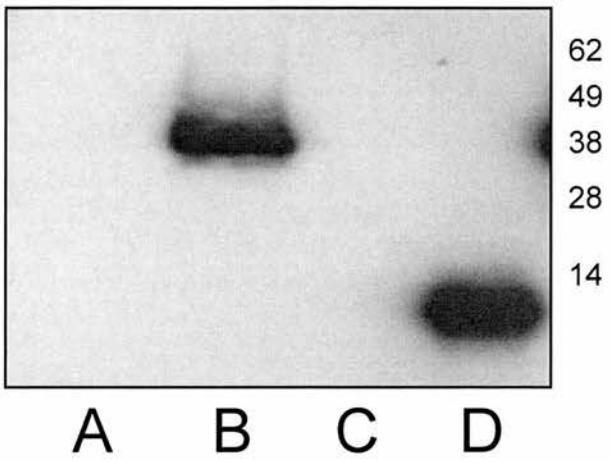


Fig.17. Western blot analysis of supernatants from plasmid-transfected 293 cells.

23µl of supernatant from A. untransfected 293 cells, B. pIRES-ovine elafin plasmid transfected 293 cells, or C. pIRES-ovine SLPI-FLAG transfected 293 cells was used in Western blot analysis. Membrane was probed with the anti-elafin Trab-2O monoclonal antibody directed against the repeat sequence PVKGQD. Recombinant human elafin used as a positive control runs at 10 kDa (lane D).

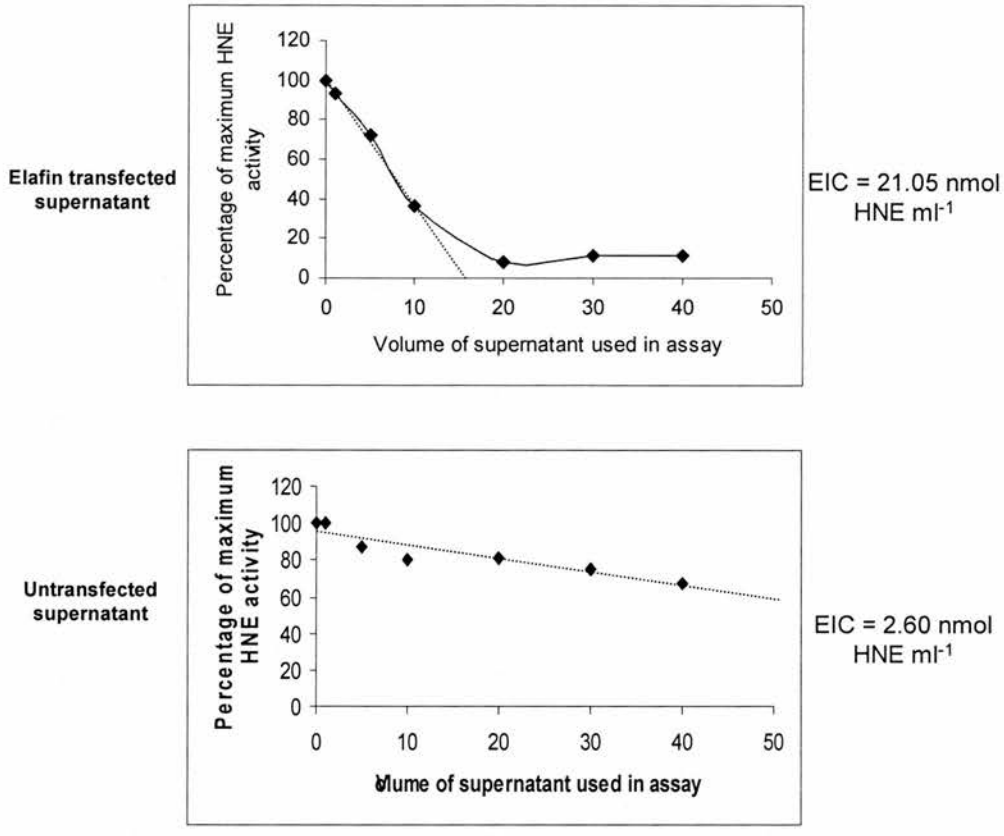


Fig. 18. Anti-human neutrophil elastase (HNE) activity of conditioned medium from 293 cells transfected with pIRES-ovine elafin plasmid.

10ng HNE was incubated for 30 minutes alone or with 40µl, 30µl, 20µl, 10µl, 5µl and 1µl of conditioned medium from untransfected 293 cells or from 293 cells transfected with pIRES-ovine elafin and resultant elastase activity assessed with MeO-succinyl-alanine(2)-proline-valine-p-nitroanilide as substrate. The rate of change of absorbance at 405nm was followed for ten minutes. Results shown as means of HNE activity over the ten minutes of the assay for two replicates against the volume of supernatant used. The dashed lines represent the line of best fit for the linear part of the curve from which we calculated the supernatant's Elastase Inhibitory Capacity (EIC) by the method described in Materials and Methods.

3.4. DISCUSSION

Neutrophil elastase inhibitors have been shown to be important endogenous molecules involved in limiting neutrophil-derived excess inflammation in humans, as well as in a variety of animal models (Laurell and Erikkson, 1963; Gross et al., 1965; Stolk et al., 1991; Rudolphus et al., 1993). Significant among these inhibitors is elafin, a member of the trappin multigene family (Wiedow et al., 1990; Sallenave et al., 1991). The 'trappin' name has been coined by Schalkwijk and collaborators (Zeeuwen et al., 1997; Schalkwijk et al., 1999) as an acronym for **TR**ansglutaminase substrate and w**AP** (whey acidic protein) domain containing **P**rote**IN**, to illustrate its property of being 'trapped' in a tissue and therefore potentially acting as an anchored protein. Trappins are constituted of an N-terminal transglutaminase substrate domain with hexapeptide repeats (PVKGQD), and a C-terminal four disulphide core, containing, in some members, the anti-elastase activity (Schalkwijk et al., 1999). The members of this family show similarities in their WAP domains with those seen in another elastase inhibitor, the secretory leukocyte proteinase inhibitor (SLPI), which has a broader spectrum of enzyme inhibition (Boudier and Bieth, 1992). Trappin members have been isolated from a number of animal species, including man, but no studies until now had identified the ovine orthologs of elafin and SLPI.

We have in the present study isolated both the genes and cDNA coding for the ovine orthologs of elafin.

Using RACE-PCR techniques, we have isolated from a tracheal sample a cDNA encoding for ovine elafin (o-elafin) which is composed of 220 amino acids, inclusive of a 23 amino acid signal peptide indicating that the protein is, like other species orthologs, a secreted protein. Following this signal peptide, we identified a region containing nineteen hexapeptide repeats of the consensus sequence PVKGQD. A similar region of repeats has been termed 'cementoin' (Nara et al., 1994) in the trappin family of proteins (Schalkwijk et al., 1999) which the elafin members belong to (see above). This glutamine and lysine rich region is thought to be involved in a transglutaminase reaction resulting in ε-(γ-glutamyl)lysine covalent bonds between the repeat region and matrix proteins such as loricrin and keratin-1, to promote the anchorage of the molecule to the interstitium, at least in the skin (Steinert

and Marekov, 1995; Nemes and Steinert, 1999). The second domain of o-elafin is composed of a 4 disulphide core region, named the WAP domain (see above), which, based on the sequence, contains the anti-elastase site (see below).

The predicted size for o-elafin after cleavage of the putative signal was 20.8 kDa. However a different migration profile on SDS PAGE was noted after over-expression of the o-elafin cDNA in 293 cells. It is anticipated that this is most likely due to the highly positive nature of the mature protein (16 net positive) as the protein will not be rendered anionic by the SDS to the same extent as a neutral protein of equivalent molecular weight. Hence the less anionic resultant protein will not migrate as quickly to the anode and hence will appear 'heavier' than it actually is. Alternatively the ovine elafin may be being cross-linked in the 293 cell supernatant to an un-identified protein, possibly by the action of a transglutaminase (Molhuizen et al., 1993; Schalkwijk et al., 1999).

When the biological activity of supernatant from 293 cells transfected with o-elafin was assessed (fig. 18), it was shown to have an elastase inhibitory capacity (EIC) for human neutrophil elastase (HNE) of 21.05nM. If we make the assumption that ovine elafin inhibits HNE with the same stoichiometry as human elafin i.e. one molecule of elafin interacts with one molecule of elastase (Sallenave and Ryle, 1991), then this equates to 437ng/ml of 'active o-elafin'. The protein sequence of the predicted active site is identical to bovine elafin (Zeeuwen et al., 1997). However, no spectrum of anti-protease activity is available for this protein.

Using both the ovine genomic BAC library described above and genomic DNA obtained locally, we also isolated the o-elafin gene (fig. 10), which sequence predicted exactly the cDNA sequence obtained from tracheal RNA. The gene structure is similar to that for human elafin (Saheki et al., 1992; Sallenave and Silva, 1993) in that the gene contains 3 exons and 2 introns with similar intron/exon boundaries.

The promoter region shown contains a 'tata' box at bp 463 but no sequence identifiable as a 'caat' box. An ATG initiation codon was observed, directly 3' of a consensus Kozak's sequence (Kozak, 1984). The o-elafin leader sequence coding for the signal peptide was found to be very similar to other species' orthologs.

RT-PCR and Northern blot analysis using RNA from various ovine tissues showed o-elafin to be present in tongue, trachea, small intestine, large intestine and skin. This is similar to porcine elafin's

distribution where elafin has been shown by in-situ hybridisation to be secreted by goblet cells in the tracheal mucosa and large intestinal crypts (Suzuki et al., 2000) and human elafin which was first isolated from bronchial mucus (Hochstrasser et al. 1981; Kramps and Klasen, 1985; Sallenave and Ryle, 1991) and psoriatic skin (Wiedow et al., 1990). Messenger RNA for human elafin has been demonstrated by Northern blot analysis in epiglottis, pharynx, vocal fold and in psoriatic skin (Pfundt et al., 1996). The mainly mucosal distribution of o-elafin is consistent with it being, like its human ortholog, an antimicrobial 'defensin-like' molecule (Simpson et al., 1999) with potential further functions in the regulation of the innate immune response (Sallenave, 2000; Sohn et al., 2003).

Interestingly, we obtained either one or two o-elafin products, by RT-PCR, depending on the tissues (fig. 12). The size of the smallest one (660bps) matched well with the size expected from the cDNA derived from the elafin gene isolated from the BAC library (hereafter named TOM-1). The origin of the longest (approximately 750bps) obtained from the tongue, small intestine and large intestine was investigated further, using locally obtained ovine genomic DNA. Southern blot and genomic PCR analysis allowed us to rule out the existence of splicing forms of the o-elafin gene as well as the existence of two genes and showed, instead, that the two cDNAs derived from allelic forms of the same gene (TOM-1 and TOM-2). When the two genomic alleles were sequenced, we found them to be identical but for the addition of 126bps coding for seven extra PVKGQD repeats in the longest form (fig. 14). The reason why we only obtained TOM-1 from the BAC library analysis while both TOM-1 and TOM-2 were obtained later probably lies with the origin of the samples: the INRA (France) BAC library used was derived from a different ovine breed (Romanov breed, Vaiman et al., 1999) from the one used for the RNA and subsequent analysis (Suffolk crosses from the UK). Our results suggest that the Romanov breed animal used to construct the BAC library only has TOM-1 while the Suffolk breed has both TOM-1 and TOM-2. The source of the RNA and DNA used in the RACE procedure and subsequent analysis was the UK commercial lowland flock (in this case Suffolk crosses as mentioned above) and as such were the result of much interbreeding between a variety of sheep breeds. It is possible that this will have an influence on the number of allelic forms of o-elafin and as such a 'screening' experiment involving PCR of a panel of individual animals from a variety of ovine breeds would be of undoubted interest.

Due to the high number of trappin family members identified so far, particularly in the hooved mammals compared to primates (Furutani et al., 1998), there has been interest in the evolution of the individual proteins and in the reasons that there are multiple trappins in one species but not in another. One *cis* acting element that has been implicated in the gene multiplication is the region of PVKGQD repeats in the transglutaminase substrate-coding region of the gene. Furutani et al. (1998) identify a correlation between number of repeats and number of trappin family members i.e. fewest repeats in human and peccary which have only one member each. When we apply this rationale to the ovine elafin gene we predict from the fact that we have at least 19 repeats in the ovine elafin gene first sequenced that there will be multiple trappin family members. This is the first time a trappin family member gene has been shown to be present in more than one allelic form.

The large transglutaminase substrate domain present within the ovine elafin molecule is of interest with regards interactions with other tissue components. As mentioned above this region is thought to be involved in cross-linking to extracellular matrix proteins. Human elafin has been shown to be cross-linked in this way to the cornified envelope of keratinocytes (Nakane et al., 2002; Steinert and Marekov, 1995). Human elafin contains 5 repeats loosely based on the consensus sequence PVKGQD. The ovine elafin equivalents discussed here contain 19 or 26 repeats with far higher conservation of the consensus sequence. This may mean there is a difference in the quantity and quality of transglutaminase-mediated cross-linking. Recent work has implicated transglutaminase repeats to be of importance in inhibiting tissue transglutaminase and in turn phospholipase-A2 hence leading to a down-regulation of pro-inflammatory mediators in a hypersensitivity model (Sohn et al., 2003). The extensive transglutaminase substrate region of the ovine elafin proteins may therefore have a beneficial effect in this regard also with an improved efficacy at down regulating the two enzymes compared to human elafin. This will be considered further in chapter 5.

It is important to note that the work identifying the o-elafin cDNA presented here has been based on products of PCR and as such may have preferentially isolated certain ovine trappins due to primer design. However, we also see only one DNA species by Southern blot analysis with full length o-elafin cDNA. If multiple trappin genes were present, we would expect to see multiple DNA species

annealing to this probe with varying degrees of homology with the probe. Further work needs to be performed to be certain that o-elafin is the only trappin gene present in ovines.

3.5. CONCLUSIONS

In summary, the genes encoding for the ovine ortholog of elafin have been successfully identified and cloned and its tissue expression has been determined at the RNA level. In addition, by over-expressing the o-elafin cDNA, it has been shown that the ovine elafin molecule is correctly processed and has anti-elastase activity. Ovine elafin is a member of the trappin family of genes that exists in two distinct allelic forms, each of which encodes a separate protein with different transglutaminase domain length. Although the significance of the existence of these 2 alleles is unclear, the particularly high number of repeats in either allele of o-elafin is interesting and may have physiological bearing.

CHAPTER 4

THE IDENTIFICATION AND CHARACTERISATION OF THE OVINE ORTHOLOG OF SECRETORY LEUKOCYTE PROTEASE INHIBITOR

4.1. INTRODUCTION

Secretory leukoprotease inhibitor (SLPI) is a non-glycosylated anti-protease first described in man (Stetler et al., 1986; Heinzel et al., 1986; Thompson and Ohlsson, 1986; Seemuller et al., 1986) which is expressed at multiple mucosal sites and is thought to be an important inhibitor of human neutrophil elastase but also shows activity against cathepsin G, chymotrypsin, trypsin and chymase. The pattern of secretion at mucosal sites reflects that already mentioned for elafin as discussed in chapter 3, and similarly prompts speculation that in addition to anti-protease functions SLPI may have a role in the innate immune response. Indeed human SLPI has been shown to have bactericidal activity against *E. coli* and *S. aureus* (Hiemstra et al., 1996). In addition recombinant human SLPI can kill *A. fumigatus* and *C. albicans* in a dose dependent manner providing the fungal cells are metabolically active (Tomee et al., 1997). In both these situations the anti-microbial activity was localised to the N-terminus of the protein. The combination of anti-microbial activity and an improved resistance to degradation by bacterial proteases (Sponer et al., 1991) has made SLPI a potentially attractive protein for use in inhalation therapy for lung inflammation (Bingle and Tetley, 1996).

The importance of SLPI in the human has led to the characterization of SLPI homologs in the mouse (Zitnik et al., 1997), rat (Gipson et al., 1999; Song et al., 1999) and pig (Farmer et al., 1990). In all the different species studied so far there is a high degree of homology at the protein level and the lengths of the proteins vary between 115 (porcine SLPI) and 132 amino acids (human SLPI). The porcine SLPI is shorter than others due to it lacking an obvious signal peptide (Farmer et al., 1990). The SLPI orthologs are all made up of two whey acidic protein (WAP) domains (Drenth et al., 1980). These domains consist of a conserved disulphide bonded region containing 8 cysteine residues. The second WAP domain contains the active anti-protease site.

SLPI has been shown to modulate the LPS response in the lung (Rudolphus et al., 1993; Stolk et al., 1993) and in macrophages (Jin et al., 1997). The effects that SLPI has on the LPS response may at least partially be explained by the inhibition of IκBα degradation (Taggart et al., 2002). My interest in the modulation of lung inflammation in a large animal model of lung inflammation has prompted this investigation of the ovine form of SLPI.

Prior to the work presented in this chapter, I already had access to the 3' cDNA sequence of a possible candidate for the ovine form of SLPI. This allowed the work presented to progress as outlined overleaf.

4.2. AIMS

The work presented in this chapter was aimed at:

1. The isolation of the full-length cDNA encoding for the ovine form of secretory leukoprotease inhibitor (SLPI).

2. The screening of an ovine genomic BAC library in order to obtain full gene sequence for an ovine SLPI ortholog.

3. The investigation of the distribution of the expression of ovine SLPI at the mRNA level by RT-PCR and Northern Blot techniques using a panel of ovine tissues.

4. The construction of an expression plasmid containing the ovine SLPI cDNA allowing transfection of cells *in vitro* to assess the functional activity of ovine SLPI as an anti-elastase and also to investigate possible methods of detection of this protein.

4.3. RESULTS

4.3.1. Isolation and sequence alignment of ovine SLPI cDNA.

Using ovine kidney RNA, and PCR primers rSLPI1and rSLPI2 (indicated in fig. 1 and 2 and annealing to a portion of the ovine SLPI sequence already available (Mistry, unpublished)) and forward primers annealing to sequences corresponding to the Generacer^{R} oligo ligated to the 5' terminus of the mRNA (GRP1 and nGRP1), 5' RACE PCR produced a major product of approximately 300bps (fig. 2) which was cloned into pCR^{R}4-TOPO^{R} plasmid for sequencing. A selection of colonies was grown in LB broth and plasmid purified from these broths. Plasmid was purified and contained sequence corresponding to the Generacer^{R} oligo ligated to the 5' terminus of the mRNA followed by 15bps of 5'UTR and then sequence corresponding to ovine elafin through to the priming site for rSLPI2. This information allowed full length PCR to be performed using the forward primer fSLPI (indicated in fig. 3) and reverse primer rSLPI annealing to the 3' end of the SLPI cDNA. This yielded a product of 405bps (fig. 3) which was also cloned into pCR^{R}4-TOPO^{R} plasmid for sequencing. Altogether, the 5' RACE and full length PCR procedures allowed us to identify an open reading frame for a protein of 132 amino acids which showed homology with human, murine, rat and porcine SLPI (see fig. 4) and will be referred to as ovine SLPI. This protein consists of a putative 24 amino acid hydrophobic signal sequence followed by two regions, each based on a whey acidic protein (WAP) domain with 8 conserved cysteine residues in each.

4.3.2. Sequence analysis of ovine SLPI gene

Screening of an ovine BAC library was performed at the Institut National de la Recherche Agronomique (INRA) using probes generated by PCR (see materials and methods). This screening identified one clone for SLPI. Southern blot analysis using 1μg of BAC clone DNA allowed

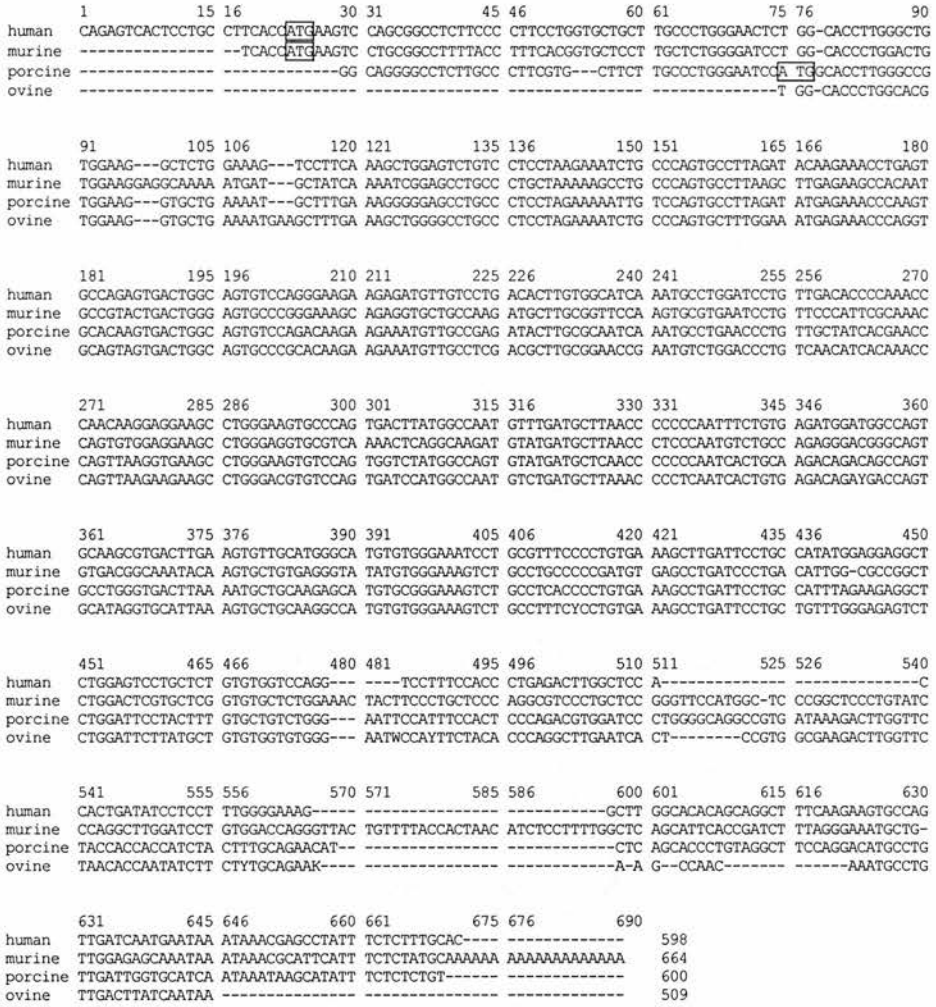


Fig. 1. Multiple sequence alignment of the known mammalian SLPI cDNAs.

The known nucleotide sequences for the human, murine and porcine forms of SLPI are shown aligned with the known portion of the ovine form of SLPI. Start codons for the known full-length cDNAs are boxed.

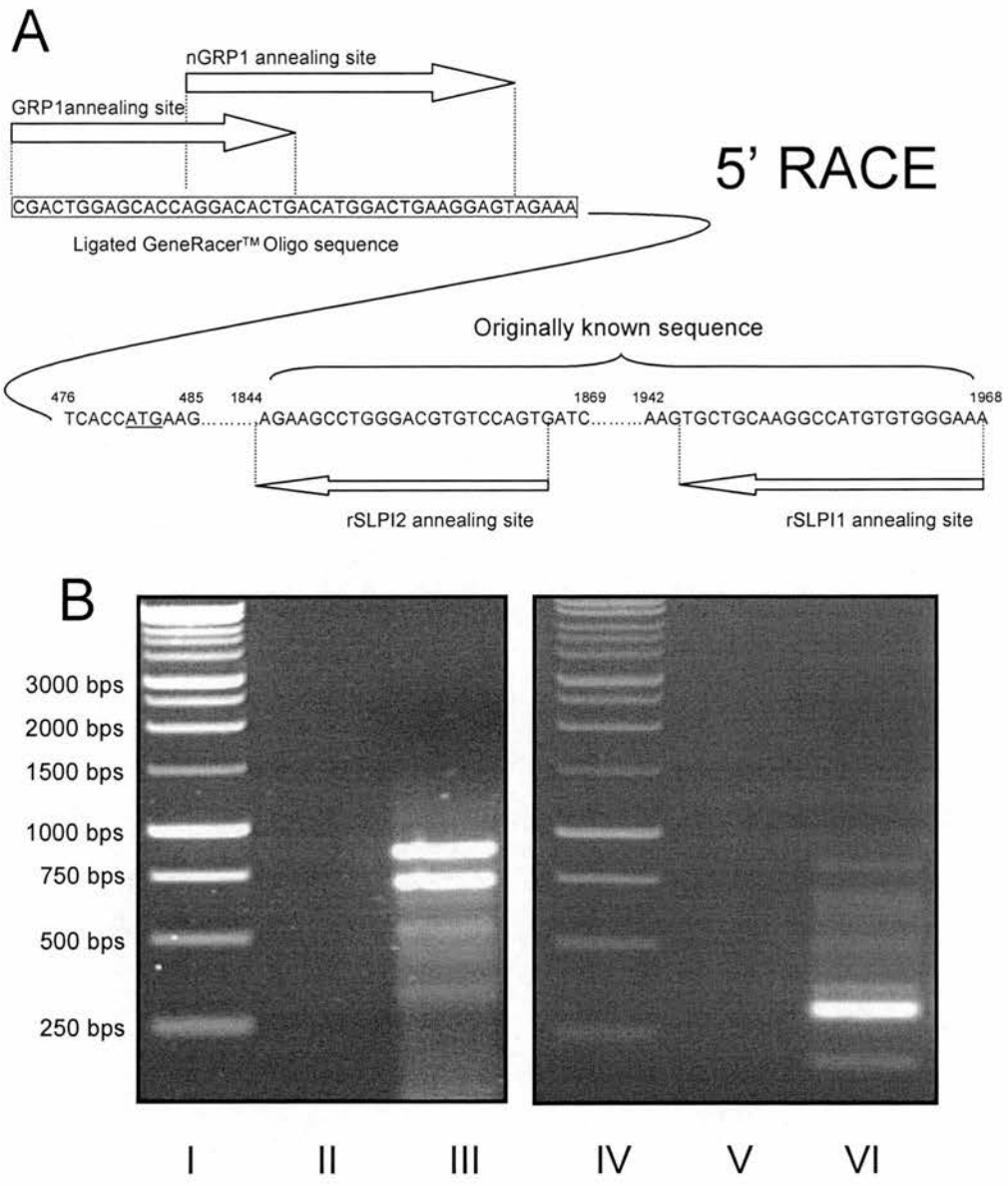


Fig. 2. 5RACE-PCR for ovine SLPI.

A. Schematic representation of the 5' RACE procedure for ovine SLPI using primers annealing to a known portion of the ovine elafin cDNA (rSLPI1 and 2) and the ligated GeneRacer™ Oligo (GRP-1 and nGRP-1).

B. Agarose gel of the products from the 5' RACE PCRs.
I. and IV. 1kb ladder.
II. PCR performed with no template using GRP-1 and rSLPI1.
III. PCR performed on cDNA derived from ovine kidney using GRP-1 and rSLPI1.
V. PCR performed with no template using nGRP-1 and rSLPI2.
VI. PCR performed on the product from lane III using nGRP-1 and rSLPI2.

A

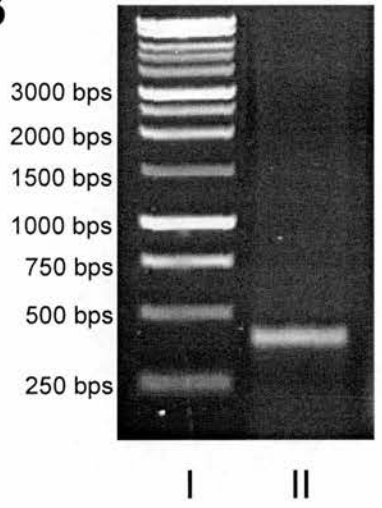
```
                1              15 16             30 31             45 46             60 61             75 76             90
5'OVINE SLPI    ACCCCTGCCCTCACC ATGAAGTTCAGTGGG CTCTTCCCCTTCCTG CTTCTTGCCCTGGGA ACCCTGGCACTTTGG GCTGTGGAAGGTGCT  87
                    →fSLPI

                91            105 106           120 121           135 136           150 151           165 166           180
5'OVINE SLPI    GAAAATGAAGCTTTG AAAGCTGGGGCCTGC CCTCCTAGAAAATCT GCCCAGTGCTTTGGA AATGAGAAACCCAGG TGCAGTAGTGACTGG  177

                181           195 196           210 211           225 226           240 241           255 256           270
5'OVINE SLPI    CAGTGCCCGCACAAG AAGAAATGTTGCCTC GACACTTGCGGAACC GAATGTCTGGACCCT GTCAACATCACAAAC CCAGTTAAGAAGAAG  267

                271           285 286           300
5'OVINE SLPI    CCTGGGACGTGTCCA GTG------------  292
                          ←rSLPI2
```

B

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                                                                                                                    fSLPI→
CTCACCATGAAGTTCAGTGGGCTCTTCCCCTTCCTGCTTCTTGCCCTGGGAACCCTGGCA 60
      M  K  F  S  G  L  F  P  F  L  L  L  A  L  G  T  L  A

                                                                                                                    fSLPI2→
CTTTGGGCTGTGGAAGGTGCTGAAAATGAAGCTTTGAAAGCTGGGGCCTGCCCTCCTAGA 120
L  W  A  V  E  G  A  E  N  E  A  L  K  A  G  A  C  P  P  R

AAATCTGCCCAGTGCTTTGGAAATGAGAAACCCAGGTGCAGTAGTGACTGGCAGTGCCCG 180
K  S  A  Q  C  F  G  N  E  K  P  R  C  S  S  D  W  Q  C  P

CACAAGAAGAAATGTTGCCTCGACACTTGCGGAACCGAATGTCTGGACCCTGTCAACATC 240
H  K  K  K  C  C  L  D  T  C  G  T  E  C  L  D  P  V  N  I

                                                                                                    ←rSLPI2
ACAAACCCAGTTAAGAAGAAGCCTGGGACGTGTCCAGTGATCCATGGCCAATGTCTGATG 300
T  N  P  V  K  K  K  P  G  T  C  P  V  I  H  G  Q  C  L  M

CTTAAACCCCTCAATCACTGTGAGACAGATGACCAGTGCATAGGTGCATTAAAGTGCTGC 360
L  K  P  L  N  H  C  E  T  D  D  Q  C  I  G  A  L  K  C  C

                                ←rSLPI1                                                                 ←rSLPI
AAGGCCATGTGTGGGAAAGTCTGCCTTTCCCCTGTGAAAGCCTGA 405
K  A  M  C  G  K  V  C  L  S  P  V  K  A
```

Fig. 3. cDNA sequence of ovine SLPI.

A. Sequence derived by 5'RACE PCR. Underlined are the priming sites for rSLPI2 (used in 5'RACE PCR) and fSLPI (used in full length PCR shown in B.).

B. Full length cDNA of ovine SLPI.
The Gel shows in lane I 1kb ladder and lane II the PCR product using cDNA derived from ovine kidney and primers fSLPI and rSLPI. Nucleotide and amino acid sequence (predicted by sequence analysis software) is also shown. Underlined are the priming sites for fSLPI and rSLPI (used in full length PCR), fSLPI2 (used in screening the BAC library), rSLPI1 (used in 5'RACE PCR) and rSLPI2 (used in 5'RACE PCR and screening the BAC library. Start codon and stop codon are boxed.

colony identified by restriction analysis and plasmid isolated again with the Qiagen Maxi-Prep kit. This plasmid will be known as pIRES-GFP#3-ovine SLPI-FLAG.

4.3.5. Transfection of 293 cells with an ovine SLPI plasmid construct.

In order to show potential anti-elastase activity attributable to the protein encoded for by the ovine SLPI cDNA, supernatants were collected from 293 cells transfected with an expression plasmid encoding for ovine SLPI (pIRES-GFP#3-ovine SLPI-FLAG).

Supernatants from 293 cells either untransfected, or transfected with pIRES-GFP-ovine SLPI-FLAG were collected and submitted to Western Blot analysis (see Fig. 9). The ovine SLPI transfected cell supernatant contained 2 positive protein bands that reacted with the anti-FLAG antibody and migrated at molecular weights of approximately 12 and 20kDa. The untransfected cell supernatant does not contain any protein cross reacting to the anti-FLAG antibody.

An anti-elastase assay was performed on this supernatant and on supernatant from untransfected 293 cells. Results are presented in fig. 10 as overall Elastase Inhibitory Capacity (EIC). The SLPI plasmid transfected cell supernatant has a higher EIC compared to the untransfected cell supernatant.

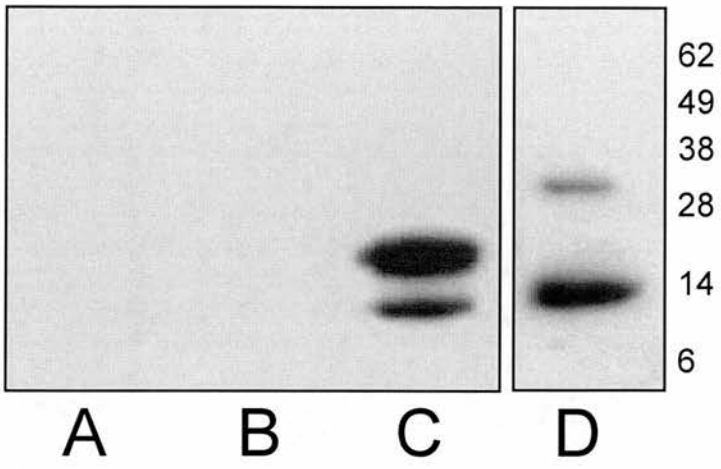

Fig.9. Western blot analysis of supernatants from plasmid-transfected 293 cells.

23µl of supernatant from A. untransfected 293 cells, B. pIRES-ovine elafin plasmid transfected 293 cells, or C. pIRES-ovine SLPI-FLAG transfected 293 cells was used in Western blot analysis. Membrane was probed in lanes A. to C. with the anti-FLAG monoclonal antibody M2. Lane D. was probed with polyclonal anti-human SLPI antibody. Human recombinant SLPI in lane D runs at 11.7kDa.

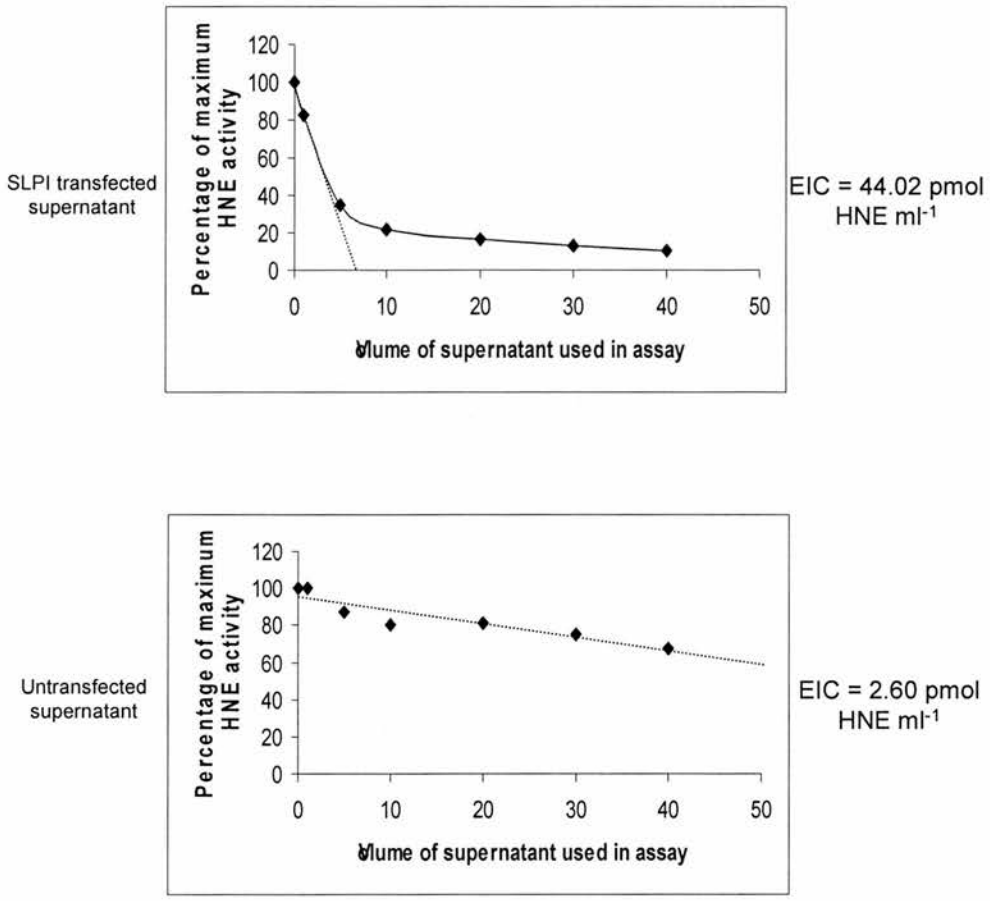

Fig.10. Anti-human neutrophil elastase (HNE) activity of conditioned medium from 293 cells transfected with pIRES-ovine SLPI plasmid.

10ng HNE was incubated for 30 minutes alone or with 40µl, 30µl, 20µl, 10µl, 5µl and 1µl of conditioned medium from untransfected 293 cells or from 293 cells transfected with pIRES-ovine SLPI plasmid and resultant elastase activity assessed with MeO-succinyl-alanine(2)-proline-valine-p-nitroanilide as substrate. The rate of change of absorbance at 405nm was followed for ten minutes. Results shown as means of HNE activity over the ten minutes of the assay for two replicates against the volume of supernatant used. The dashed lines represent the line of best fit for the linear part of the curve from which we calculated the supernatant's Elastase Inhibitory Capacity (EIC) by the method described in Materials and Methods.

4.4. DISCUSSION

I have isolated and cloned from ovine kidney a cDNA encoding for a protein of 132 amino acids with a putative signal peptide of 24 amino acids. This protein shows good homology with the already known orthologs of secretory leukoprotease inhibitor (SLPI) and shall be referred to as ovine SLPI. The alignment of the known orthologs shown in fig. 4 renders some information as to the possible structure and functional activity of the ovine SLPI protein. Firstly there is conservation of the 16 cysteine residues which take part in the intra-molecular disulphide bonding which has been shown in the human SLPI protein by X-Ray crystallography (Grutter et al., 1988). These bonds are thought to be important for the maintenance of the two discrete whey acidic protein (WAP) domains of the SLPI protein. Indeed the human SLPI protein has been shown to be in a 'boomerang' shape with the two WAP domains making up the globular ends of the protein. In addition to the cysteine residues, many of the residues taking part in the inter-main-chain hydrogen bonds in the human SLPI protein are conserved in ovine SLPI. This adds more credence to the prediction that ovine SLPI will also take on a 'boomerang' shape in the secreted form.

The human SLPI protein has its anti-protease site/primary binding segment exposed on the outer surface of a hairpin bend in the second WAP domain. The specific amino acid composition of this segment is important with regards the specificity of protease inhibition (Grutter et al., 1988; Eisenberg et al., 1990). Human SLPI inhibits human neutrophil elastase, trypsin, chymotrypsin, chymase and cathepsin G (Boudier and Bieth, 1992). The P_1 and P_1' residues identified for protease binding are Leu-72 and Met-73 (numbered after cleavage of the signal peptide hence in fig. 4 Leu-99 and Met-100) in the human SLPI protein. As shown these residues are conserved in the ovine SLPI protein and a similar spectrum of anti-protease activity can be predicted. Of interest is the P_1 residue being leucine in SLPI compared to alanine in the elafin protein. This difference in residue is thought to be the reason for the lack of an inhibition against chymotrypsin for human elafin, as the alanine residue is not large enough to fill the S_1 pocket of chymotrypsin (Tsunemi et al., 1996). This raises the interesting possibility that porcine elafin which has a P_1 leucine, may have a similar anti-protease activity to SLPI. Murine and porcine SLPI both have P_1 methionine residues and this may well have profound effects on their anti-protease activities. Indeed Zitnik at al. (1997) have shown that murine SLPI is less

effective at inhibiting human neutrophil elastase, bovine chymotrypsin and human cathepsin G compared to human SLPI. Also, the authors postulate that the P_1 methionine renders murine SLPI more susceptible to oxidative inactivation.

Interestingly, Junger et al. (1992) found that recombinant human SLPI had only a slightly reduced association rate constant (k_{on}) for ovine neutrophil elastase (ONE) when compared to human neutrophil elastase (HNE). This was in contrast to human recombinant eglin C which had a one hundred-fold decreased k_{on} for ONE compared to HNE. This highlights the importance of considering the source of protease inhibitor as well as the source of the target enzyme when describing enzyme inhibition studies.

The ovine SLPI cDNA isolated here has been shown to code for the secreted ovine SLPI protein which, on expression, is correctly processed and folded as shown by its detection in 293 cell supernatant after plasmid transfection. I have also shown functional data with a strong inhibition of human neutrophil elastase by the secreted protein.

I have also, by the use of a BAC library, identified and sequenced the gene encoding for ovine SLPI. The gene structure throughout the known mammalian SLPI orthologs seems to be well conserved when the exon/intron relationships are considered. In common with human and murine SLPI, the ovine gene is made up of 4 exons and 3 introns as shown in fig. 5. There is a signal peptide sequence as predicted by the use of the SignalP online sequence analysis tool (http://www.cbs.dtu.dk/services/SignalP/). This predicts cleavage of the signal peptide after Gly-24. This correlates precisely with the cleavage of the signal peptide in human SLPI after Gly-25. The putative regulatory 'caat' and 'tata' boxes were identified in the promoter region, at bp 192 and 435 respectively.

The distribution of ovine SLPI transcription was demonstrated by RT-PCR and RNA was found in the tongue, trachea, kidney, bladder and liver. This distribution shows similarities with that of human SLPI which has been shown by immunohistochemistry to be present in the lachrymal, respiratory, proximal digestive and genital tracts (Franken et al., 1989), and more recently in the kidney (Ohlsson et al., 2001). In addition, the mRNA for the murine ortholog of SLPI has been demonstrated in spleen and lung and, to a lesser degree, in liver, kidney and testis (Zitnik et al., 1997).

The expression of the ovine SLPI cDNA in 293 cells by the use of the plasmid pIRES-GFP#3-ovine SLPI-FLAG demonstrates that the ovine SLPI protein is secreted. Demonstration of the protein in the 293 cell supernatant was achieved by Western blot analysis. The predicted size for ovine SLPI after putative signal cleavage is 11.6 kDa. However, after expression of the o-SLPI cDNA in 293 cells, a different pattern of migration was apparent with two bands visible, a major one of approximately 20 kDa and a minor band of approximately 12 kDa (with the recombinant human SLPI positive control at approximately 14 kDa).

Since SLPI is, like elafin, heavily cationic (o-SLPI has an overall charge after putative signal cleavage of +9), and since cationic proteins are known to sometimes appear larger than their actual size, when analysed by SDS-PAGE, the higher 20 kDa form may reflect an artifact of migration, and the 12 kDa be a minor degradation product.

Alternatively, the native o-SLPI may form aggregates or be bound to an unknown culture-medium-derived protein, through SDS and β-mercaptoethanol-resistant bonds. This has indeed already been observed for both SLPI and elafin, when these proteins were derived from complex biological fluids (Nara et al., 1994; Tremblay et al., 1996; Sallenave et al., 1999).

When the biological activity of supernatant from 293 cells transfected with o-SLPI was assessed it was shown to have an elastase inhibitory capacity (EIC) of 44nM, well in excess of that of untransfected cells (2.6nM). This value (44nM = 510ng/ml of 'active o-SLPI') is in agreement with the antigenic levels measured by dot blot analysis (results not shown).

The mainly mucosal tissue distribution of o-SLPI is consistent with it being, like its human ortholog, an antimicrobial 'defensin-like' molecule (Hiemstra et al., 1996). Indeed, evidence is forthcoming that, like elafin (Simpson et al., 1999) SLPI may have important functions in innate immune responses (Hiemstra et al., 1996).

4.5. CONCLUSIONS

The data presented in this chapter has demonstrated the successful identification and cloning of the gene encoding for the ovine ortholog of SLPI and the determination of its tissue expression at the RNA level. In addition, by over-expressing the o-SLPI cDNA, I have demonstrated that the protein is correctly processed and has anti-neutrophil elastase activity.

CHAPTER 5

THE OVINE ORTHOLOGS OF ELAFIN AND SLPI ARE POTENTIAL ANTI-INFLAMMATORY PROTEINS IN THE LUNG

5.1. INTRODUCTION

The previous chapters have identified the ovine forms of the proteins elafin and SLPI and confirmed that they share certain characteristics with these proteins in other species. These characteristics, at the level of the gene, cDNA and protein, along with the respective tissue distributions, have led to the supposition that these anti-proteases may play important roles in the inflammatory response, specifically in the innate immune response in a similar fashion to the human orthologs (Sallenave, 2000).

Additionally, the identification of the ovine orthologs of these proteins will add to the currently available information with regards the trappin family (Schalkwijk et al., 1999) and the whey acidic protein family (Drenth et al., 1980; Seemuller et al., 1986) to which elafin and SLPI members belong respectively. Many members of these families have also been characterised in other species and this has enabled the identification of a shared evolutionary development between these two families of proteins (Furutani et al., 1998; Schalkwijk et al., 1999).

The specific characteristics of the elafin and SLPI family members with regards their suitability for use as potentially modulatory proteins in the field of pulmonary inflammation is of great interest and this chapter will address these issues in turn.

5.2. AIMS

1. This chapter will demonstrate how ovine elafin and SLPI relate to other members of the trappin and whey acidic protein (WAP) families both in terms of distribution and also at a phylogenetic level.

2. Data will be presented which show how both proteins are influenced by inflammation in the ovine lung.

3. The ovine elafin and SLPI orthologs, and their specific characteristics, will then be considered with regards their potential as anti-inflammatory proteins for vector based delivery to the lung in models of inflammation.

5.3. RESULTS

5.3.1. Phylogenetic Tree analysis of elafin and SLPI family members.

The whey acidic protein domains of the two ovine elafin proteins and ovine SLPI (with elafin containing one and SLPI two) are aligned with all currently known members of the WAP and trappin families in addition to the murine elafin-like proteins 1 and 2 and also human vasopressin in fig. 1A. The alignment is 'controlled' around the highly conserved cysteine residues shown by asterisks. The boxed region signifies the region conveying anti-elastase activity in those family members with this property.

The phylogenetic tree analysis performed on the WAP/trappin domain alignment is shown in fig. 1B. This shows that the two forms of ovine elafin are closely related to the bovine form of elafin and all of which appear to be evolutionarily older than the bovine trappins 4 and 5. Bovine trappin 6, however, seems to be more closely related to the larger group of porcine trappins included in which are trappins isolated from the peccary and warthog.

The tree also demonstrates the obvious divergence between the WAP domains one and two of the SLPI family members. Also we can see the close relationship between the second WAP domain in the SLPI members and the trappin WAP domains. Indeed as mentioned above it is this second domain in the SLPI family and the WAP domain in trappin members which confer the anti-elastase activity in those members which act in this way.

5.3.2. Comparison of tissue distribution between trappin family members.

Table 1 shows the distributions of the currently known elafin orthologs. There are wide differences between the mammals for which the elafin orthologs have been described i.e. man, pig, cow and now, sheep. As evidenced by this panel of mammals, no elafin orthologs have been described in rodent

A

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SLPI first WAP domain
  human SLPI WAP 1                 CPPK-KS-AQ C--LRYKKPE -CQSDWQCPG KKR-CCPD-T CG--IKC
  ovine SLPI WAP 1                 CPPR-KS-AQ C--FGNEKPR -CSSDWQCPH KKK-CCLD-T CG--TEC
  rat SLPI WAP 1                   CPAR-KP-AQ C--LKREKPE -CSTDWGCPG KQR-CCQD-T CG--FKC
  murine SLPI WAP 1                CPAK-KP-AQ C--LKLEKPQ -CRTDWECPG KQR-CCQD-A CG--SKC
  porcine SLPI WAP 1               CPPR-KI-VQ C--LRYEKPK -CTSDWQCPD KKK-CCRD-T CA--IKC

SLPI second WAP domain
  human SLPI WAP 2                 CPVT-Y--GQ C--LMLNPPN FCEMDGQCKR DLK-CCMG-M CG--KSC
  ovine SLPI WAP 2                 CPVI-H--GQ C--LMLKPLN HCETXDQCIG ALK-CCKA-M CG--KVC
  rat SLPI WAP 2                   C-LK-FQ-GK C--LMLNPPN KCQNDGQCDG KYK-CCEG-M CG--KVC
  murine SLPI WAP 2                C-VK-TQ-AR C--MMLNPPN VCQRDGQCDG KYK-CCEG-I CG--KVC
  porcine SLPI WAP 2               CPVV-Y--GQ C--MMLNPPN HCKTDSQCLG DLK-CCKS-M CG--KVC

Trappin family WAP domains
  TOM-1 WAP                        CP-R-VL-IR C--AMMNPPN RCLRDAQCPG AKK-CCES-S CG--KTC
  TOM-2 WAP                        CP-R-VL-IR C--AMMNPPN RCLRDAQCPG AKK-CCES-S CG--KTC
  simian elafin WAP                CP-N-IL-IR C--AMLNPPN RCLKDTD--- ---------- -------
  human elafin WAP                 CP-I-IL-IR C--AMLNPPN RCLKDTDCPG IKK-CCEG-S CG--MAC
  porcine elafin WAP               CP-R-IL-IR C--LMVNPPN RCLSDAQCPG LKK-CCEG-F CG--KAC
  warthog elafin WAP               CP-R-IL-IR C--MMVNPPN RCLSDAQCPG VKK-CCEG-F CG--KEC
  bovine elafin WAP                CP-R-VL-IR C--AMMNPPN RCLRDAQCPG VKK-CCEG-S CG--KTC
  porcine trappin 1 WAP            CP-R-IL-FR C--PLSNPSN KCWRDYDCPG VKK-CCEG-F CG--KDC
  warthog trappin 3 WAP            CP-W-IL-LR C--PLANPSN KCWRDYDCPG VKK-CCEG-F CG--KDC
  bovine trappin 4 WAP             CP-R-VL-FK C--LVMNPPN RCLRDAQ--- ---------- -------
  bovine trappin 5 WAP             CP-R-VL-FK C--LVMNPRN RCLRDVQC-- ---------- -------
  bovine trappin 6 WAP             CP-R-VR-IH C--NLWNPPN QCWRDAHCPG AKK-CCEG-F CG--KTC
  porcine trappin 7 WAP            CP-W-IF-LR C--PLPKPPN KCWRDSHCPG VMK-CCEG-F CG--NEC
  porcine trappin 8 WAP            CP-R-IL-IR C--LMVNPPN RCLSDAQCPG VKK-CCEG-F CG--KDC
  porcine trappin 9 WAP            CP-R-IE-IR C--RLLNP-N RCLIDAQCPG FQK-CCR--V CGV-KSC
  peccary trappin 10 WAP           CP-M-IK-IR C--ALFNPPN RCLTDAGCPG ARK-CCIG-S CG--KAC
  hippopotamus trappin 11 WAP      CP-K-IW-IE C--STLNPPK RCLRDAQCPR NKN-CCPA-S CG--KIC

Elafin-like proteins of mouse WAP domains WAP domain
  elafin-like protein 1 WAP        CP-E-YR-VP CPFVLI--PK -CRRDKGCKD ALK-CCFF-Y CQM-R-C
  elafin-like protein 2 WAP 1      CP-E-FL-LD CPFVLL--PV -CSRDKGCKG TKK-CCFY-Y CQM-RIL
  elafin-like protein 2 WAP 2      -P-D-YV-R- C--IRQDDPQ -CYSDNDC-G DQEICCFW-Q CGF-K-C

  human vasopressin                CQEENYLPSP C---QSGQKA -CGSGGRCAA FGV-CCNDES CVTEPEC
                                   *          *          *     *       **     *     *
```

B

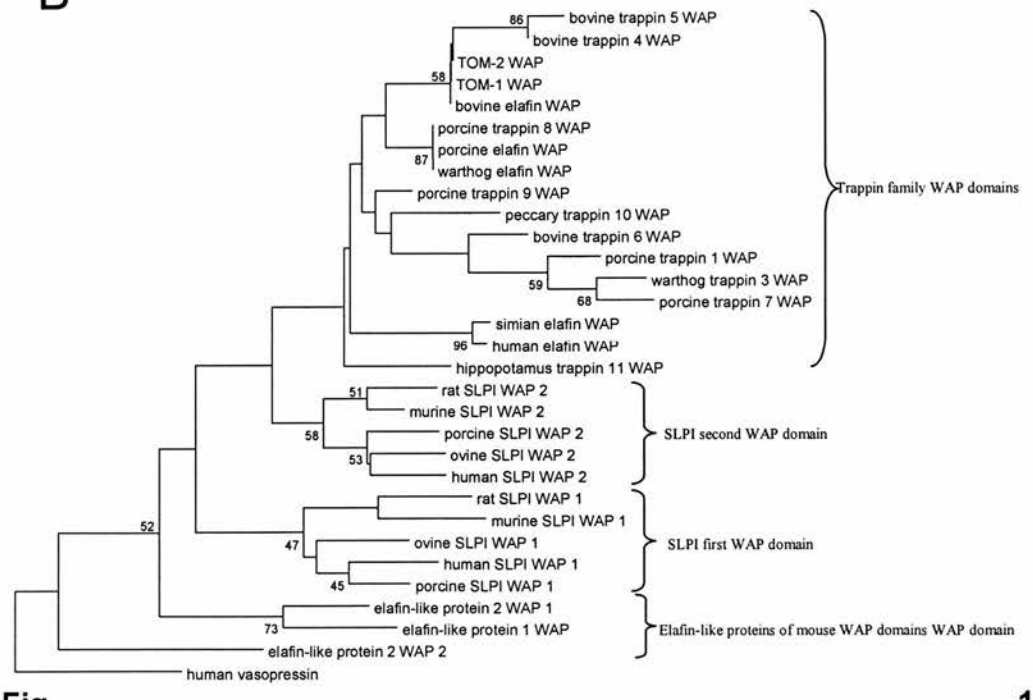

Fig. 1...

Fig. 1. Comparison of the Whey acid protein (WAP) domains for the proteins of the trappin family, SLPI family and the recently discovered elafin-like proteins in the mouse, and the WAP motif of the human vasopressin molecule.

A. Alignment of the 1st and 2nd WAP domains of the currently known SLPI proteins, along with the WAP domains for a variety of trappins including the ovine forms of elafin derived from the alleles TOM-1 and TOM-2, and including the WAP domains of the murine elafin-like proteins 1 and 2 (Hagiwara et al., 2003) and human vasopressin. The WAP motifs for all the proteins were aligned by using ClustalW software available online. Conserved cysteine residues are indicated by asterisks. The boxed region indicates the putative active anti-protease active site in the second WAP domains of the SLPI family and in the WAP domain of the trappin family. B. Phylogenetic tree constructed using a neighbour-joining technique with Poisson corrected data from the above alignment. Bootstrap values above 40 are indicated. Branch length is proportional to evolutionary distance.

Ovine elafin	Tongue, trachea, small intestine, large intestine, skin - present study
Human elafin	Sputum (Hochstrasser et al., 1981; Kramps and Klasen, 1985; Sallenave et al., 1991 and 1992) Skin (Schalkwijk et al., 1990; Wiedow et al., 1990; Molhuizen et al., 1993) Ocular secretions (Sathe et al., 1998) Endometrium (King et al., 2003) Oral cavity (Pfundt et al., 1996)
Porcine elafin	Trachea, large intestine (Tamechika et al., 1996; Suzuki et al., 2000)
Bovine elafin	Snout epidermis, tongue (Zeeuwen et al., 1997)

Table 1. The distribution of the members of the elafin family to date.

species. Hagiwara et al. (2003) have described two murine elafin-like proteins, SWAM-1 and 2. These have a whey acidic protein (WAP) domain with a conserved core containing 8 cysteine residues which are involved in 4 disulphide bonds. However, neither SWAM-1 nor 2 contain a discernible anti-elastase site (as shown by the boxed region in the alignment of the WAP motifs of all the known trappins (fig. 1 A)) and so cannot be regarded as murine elafin equivalents. The mammalian elafin orthologs investigated to date have been found either at the RNA level by RT-PCR or Northern blot analysis, or by direct demonstration of protein by ELISA, immuno-histochemistry or Western Blotting. These methods demonstrate a general distribution of elafin orthologs at mucosal surfaces like the buccal cavity, upper respiratory tract and intestinal tract. In addition, in the present study ovine elafin was found in the skin. This has also been shown in humans (Schalkwijk et al., 1990; Wiedow et al., 1990). Also human elafin has been shown to be upregulated in skin disease involving neutrophil infiltration (Pfundt et al., 1996). Overall a distribution of elafin orthologs where the body is exposed to the external environment is seen.

The distributions (where known) of other members of the trappin family as described in members of the suidae, tayassuidae, bovidae and hippopotamidae (Tamechika et al., 1996; Furutani et al., 1998) show similarities to that for elafin. For example, the bovine trappins 4 and 5 are expressed in trachea, ileum and tongue, and trachea respectively (Zeeuwen et al., 1997).

5.3.3. Comparison of tissue distribution between SLPI family members.

The present work using RT-PCR analysis showed the presence of o-SLPI RNA in tongue, trachea, kidney, bladder, liver and skin. Work in other species, namely the mouse, rat, pig and man, has shown a variation in tissue distribution as shown in table 2. Again we can see commonalities in the general distribution but with some specific differences. SLPI is thought to be secreted at sites exposed to the external environment in the same way as elafin. Hence we see ovine SLPI in the tongue, trachea, urinary tract and skin, as well as the liver. The murine, human and porcine orthologs show overlap

Ovine SLPI	Tongue, trachea, kidney, bladder, liver, skin – present study
Human SLPI	Uterus (Casslen et al., 1981) Serous glands maxillary sinus and trachea (Fryksmark et al., 1982) Middle ear mucosa (Carlsson et al., 1983) Synovial fluid (Ekerot et al., 1983) Salivary glands (Ohlsson et al., 1984) Nasal secretions/mucosa (Fryksmark et al., 1989; Westin et al., 1994) Male reproductive tract (Ohlsson et al., 1995) Cervical mucus and foetal membranes (Helmig et al., 1995) Intestinal mucosa (Bergenfeldt et al., 1996; Nystrom et al., 2001) Articular cartilage/Intervertebral disc (Bohm et al., 1991; Jacoby et al., 1993; Ohlsson et al., 1997) Mast cells (Westin et al., 1999) Neutrophils (Bohm et al., 1992) Pancreatic beta cells (Nystrom et al., 1999) Kidney (Ohlsson et al., 2001) Squamous cell carcinoma (Westin et al., 2002) Lacrimal secretions (Sathe et al., 1998) Alveolar macrophages (Mihaila et al., 2001) Clara/Type II alveolar cells (Sallenave et al., 1993) Endometriosis Endometrium (Suzumori et al., 1999) Psoriatic epidermis (Wiedow et al., 1993)
Porcine SLPI	Endometrium (Farmer et al., 1990) Myometrium (Simmen et al., 1991) Placenta/allantoic fluid/foetal cord blood/foetal liver (Simmen et al., 1992)
Murine SLPI	Spleen/lung/liver/skeletal muscle/testis (Zitnik et al., 1997) Tumour cell lines (Morita et al., 1999) Skin tumours (Schlingemann et al., 2003) Macrophages, Neutrophils, spleen (Jin et al., 1997;Odaka et al., 2003)
Rat SLPI	Lung (Gipson et al., 1999) Articular cartilage (Song et al., 1999)

Table 2. The distribution of the members of the SLPI family to date.

with their distributions. For example human SLPI is seen in lung, nasal secretions, salivary glands, intestinal mucosa and inflamed skin (table 2).

5.3.5 Up-regulation of ovine elafin and SLPI protein in bronchoalveolar lavage fluid after lipopolysaccharide administration *in vivo.*

Fig. 2. shows the dot blot analysis of ovine bronchoalveolar lavage fluid (BALF) after segmental administration of *Mannheimia haemolytica* LPS. The densitometry analysis demonstrates that both elafin and SLPI are upregulated in the lung after LPS administration. Ovine elafin levels increase slightly at 6 hours, are significantly raised by 24 hours ($P<0.005$) and then return to baseline levels by 1 week. SLPI, on the other hand, is significantly increased at both 6 hours and 24 hours ($P<0.0005$ and $P<0.005$ respectively).

Control dot blots probed with secondary antibody only showed no absorbance after exposure to X-Ray film.

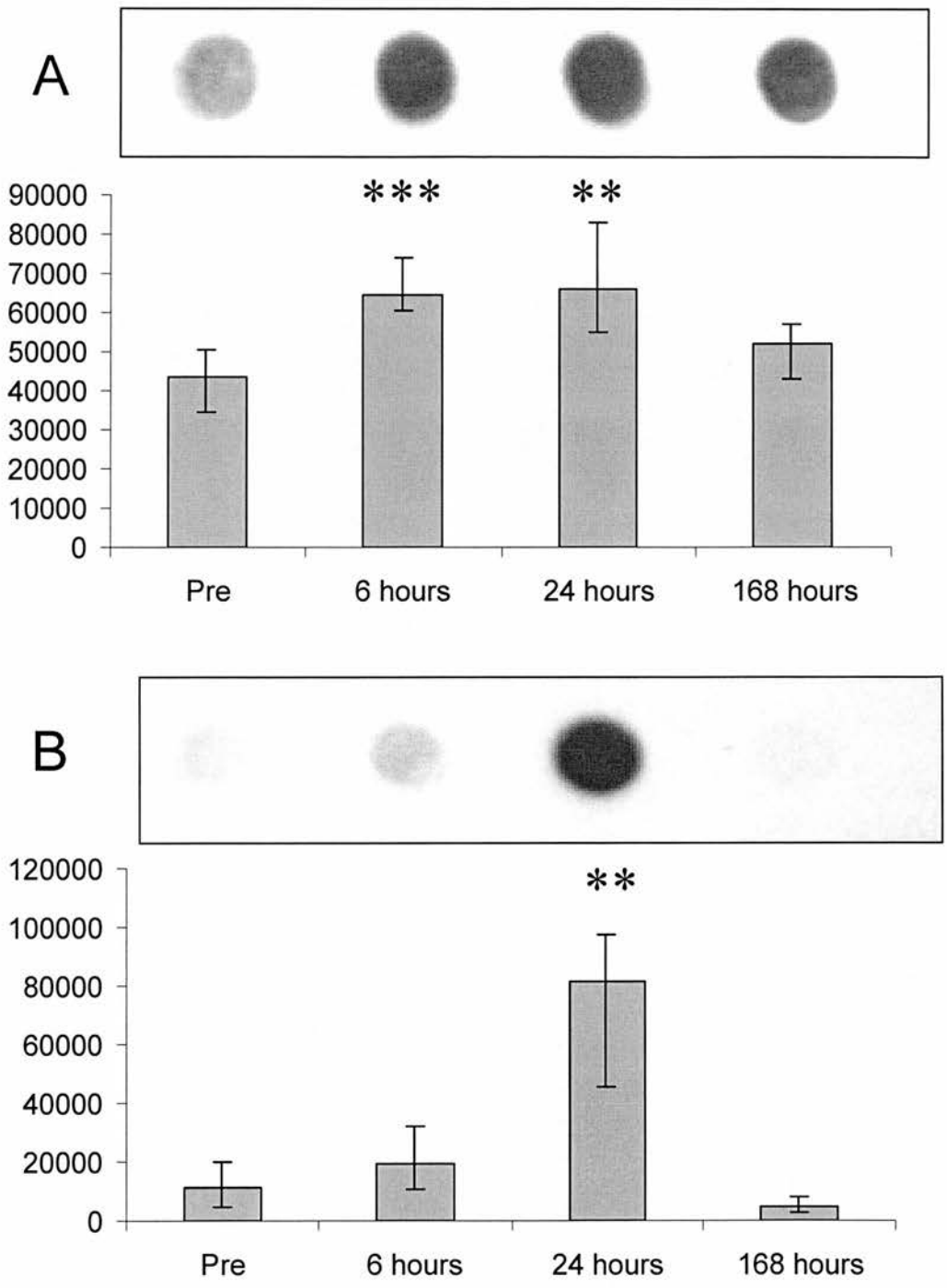
A
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Fig. 2...

Fig. 2. Secretion of SLPI and elafin in bronchoalveolar lavage collected from sheep lung segments exposed to lipopolysaccharide.

450μg Mannheimia haemolytica LPS was administered to discrete lung segments of twelve sheep. Bronchoalveolar lavage was collected a week prior to and 6, 24 and 168 hours after administration of the LPS. 200μl of broncholaveolar lavage from each animal at each time point was used in a dot blot and probed with either (A) human anti-SLPI polyclonal antibody, or (B) Trab 2-O monoclonal antibody detecting ovine elafin. Results are shown as median arbitrary densitometry units against time after dosage of LPS with error bars showing inter-quartile ranges. Also shown are representative dot blot results for each protein. ** signifies a P value of <0.005 and *** signifies a P value of <0.0005 compared to pre experimental values when Mann Whitney analysis was performed.

5.4. DISCUSSION

Over the last several decades there has been considerable interest in developing and refining laboratory animal models of inflammatory lung disease and in parallel with this work, interest has focused on modulating the inflammatory response through targeting a variety of cellular and secreted proteins, either by up-regulation or by down-regulation. This approach forms the basis of many lung-directed gene therapy applications and the possible targets which can be modulated *in vivo* by the use of viral and non-viral vectors have been discussed in depth elsewhere.

The orthologs of elafin and SLPI have multiple potential anti-inflammatory functions as discussed in chapter 1 but briefly these functions are summarised here with additional information derived from the newly described ovine orthologs of these proteins. Firstly the proteins' main function has been thought to be as locally produced anti-proteases with a slight difference of spectrum of inhibition for elafin compared to SLPI (Sallenave, 2000). Thus both proteins are of importance in maintaining the integrity of mucosal surfaces against the deleterious effects of neutrophil derived proteolytic enzymes. The kinetics of inhibition imply that neutrophil derived elastase is the major target for inhibition particularly with regards SLPI (Vogelmeier et al., 1996). Observations in relation to the distributions of elafin and SLPI orthologs have fuelled predictions of a protective role for these proteins in the innate immune system. Indeed, both human elafin and SLPI have been shown to have potent anti-microbial functions *in vitro* (against bacteria, fungi and a potential anti-HIV activity (McNeely et al., 1995 and 1997; Hiemstra et al., 1996; Schalkwijk et al., 1999; Simpson et al., 1999)). Interestingly, the trappin family members show a high degree of variation at the active anti-elastase site (Zeeuwen et al., 1997) which raises the possibility that these proteins have a role in inhibiting exogenous bacterial proteases in the same way that elafin (Trappin-2) has a primary role against endogenous neutrophil derived proteases. In addition to a direct protective function against enzymatic damage to mucosal surfaces, the inhibition of human neutrophil elastase (HNE) can have indirect anti-inflammatory effects due to the inhibition of HNE-stimulated up-regulation of the release of chemo-attractant cytokines such as Il-8 and MCP-1 from both epithelial and non-epithelial cells (Van Wetering et al., 1997; Ishihara et al., 1999; Devaney et al., 2003; Uehara et al., 2003). This function of HNE (and

other serine proteases such as proteinase 3 and cathepsin G) is due in part to activation of protease activated receptors.

The anti-proteases considered here have also been shown to have direct effects on the stimulation of macrophages by LPS and analogous substances. Jin at al. (1997) showed that murine SLPI was a major factor influencing the responsiveness of macrophages to LPS stimulation and that the stable transfection with mSLPI of cells normally responsive to LPS attenuated the LPS-stimulated release of nitric oxide and TNFα. This was due to mSLPI suppressing the LPS-induced activation of NF-κB. Indeed mice deficient in SLPI have been shown to have significantly greater susceptibility to LPS-induced shock than normal mice (Nakamura et al., 2003) which is at least in part due to an increase in the secretion of Il-6 and HMG-1, and NF-κB activation in the SLPI -/- animals' macrophages compared to SLPI +/+. Additionally this group found that SLPI attenuated B cell proliferation and IgM production after LPS stimulation. Further work (Jin et al., 1998) has shown a similar effect for SLPI in down-regulating macrophage responses to lipotechoic acid, a Gram +ve bacterial wall product analogous to LPS. In addition, Zhu et al. (1999) confirmed that this inhibitory activity can be repeated by the transfection of a non-secreted form of mSLPI into a murine macrophage cell line. Interestingly, recombinant human SLPI inhibits human monocyte production of cyclooxygenase 2 (and hence decreased PgE_2 and matrix metallo-proteases) after LPS stimulation (Zhang et al., 1997). This function of SLPI was shown to be largely independent of anti-protease activity.

There is evidence that SLPI has a role in controlling inflammation in the lung by influencing the nuclear localization of the transcription factor NF-κB. Lentsch et al. (1999) showed that blocking endogenous SLPI in a rat model of immune complex mediated lung disease led to a significantly raised activation of NF-κB compared to immune complexes (ICs) alone. Administration of recombinant human SLPI intra-tracheally in addition to ICs led to significant decreases in lung vascular ICAM-1 expression, lung myeloperoxidase content and whole lung NF-κB activation compared to ICs alone. There was no effect of exogenous SLPI on the IC mediated activation of NF-κB in alveolar macrophages harvested from the rats post IC administration and also there were no changes in bronchoalveolar lavage levels of TNFα, MIP-2, CINC and C5a in SLPI treated rats compared to controls. Overall, the authors show that the down regulation of NF-κB activation by exogenous SLPI is due to an increase in IκBβ but not IκBα. Elafin has been shown to inhibit NF-κB

activation after adenoviral delivery to human umbilical vein endothelial cells in response to LPS and low density lipoprotein and also in peripheral blood derived macrophages in response to LPS (Henriksen et al., 2004B).

Evidence therefore exists to the effect that the elafin and SLPI orthologs both inhibit locally secreted proteolytic enzymes in addition to modulating the response of epithelial and non-epithelial cells to LPS type substances. The potential significance of the latter modulation is appreciated in the observation that manipulation of SLPI levels in the whole organism influences mortality following exposure to LPS (Nakamura et al., 1993). Considerable work has focused on the administration of these proteins in laboratory animal models of disease.

Simpson et al. (2001A) have shown the beneficial use of adenovirus delivery of the human elafin ortholog in a murine model of lung infection. This work has demonstrated the delivery of the human elafin protein improving the clearance of bacteria from the lungs of mice compared to that seen in virus control and uninfected mice. Human elafin has also been used as a recombinant protein administered directly to the lungs of hamsters that have received human neutrophil elastase or LPS endotracheally (Tremblay et al., 2002; Vachon et al., 2002). The administration of elafin led to a dose-dependent inhibition of HNE induced lung haemorrhage. Interestingly pre-elafin (complete with trappin-like repeats – see below) proved more effective than elafin at reducing HNE-induced haemoglobin release into the BALF. The importance of the trappin-like repeats is discussed in more depth below.

Wang et al. (2003) showed that in a rat model of ischemic stroke, the administration of Ad-rat SLPI attenuated ischemic damage 24 hours after middle cerebral artery occlusion as measured by lesion size and also by examining neurological deficits. The precise mechanism of action of SLPI in this model was not investigated.

Recombinant human SLPI has been used both by inhalation and by intravenous use in many models of lung disease as well as in human clinical trials (Mulligan et al., 1993; Wright et al., 1999). However the focus of this work has been on supplementing the anti-elastase activity as measured in bronchoalveolar lavage. As discussed above, such an approach may potentially ignore other possibly important functions which may be independent of anti-protease activity.

Multiple transglutaminase substrate repeats of consensus sequence PVKGQD (Schalkwijk et al., 1999) are present in the first domain of the trappins. This hexapeptide has been shown to take part in the transglutaminase-mediated cross-linking of elafin to matrix proteins (Molhuizen et al., 1993; Nara et al., 1994; Steinert and Marekov, 1995). An interesting consequence of this is that proteins containing these repeats can potentially competitively inhibit the transglutaminase itself. This enzyme is considered important in the direct formation of fibrosis in the lung (Griffin et al., 1979; Richards et al., 1991; Toida et al., 1991) as well as the activation of TGFβ (Kojima et al., 1993). Transglutaminase also has stimulatory effects on phospholipase A2 (Sohn et al., 2003), an enzyme which is also directly inhibited by proteins containing the PVKGQD repeats which serve as transglutaminase substrates. Indeed, peptides based upon the trappin PVKGQD repeat sequence have been shown direct clinical benefit in a guinea pig model of conjunctival hypersensitivity. In this model inhibition of these enzymes (Sohn et al., 2003), led to a decrease in both lipoxygenase and cyclooxygenase activities and hence controlled the arachidonate cascade. Another secreted protein which is a known substrate for transglutaminase is uteroglobin/Clara cell 10kDa protein (Manjunath et al., 1984) and this has also been shown to have immunomodulatory effects (Singh and Katyal, 2000) due, at least in part, to inhibition of phospholipase A2. The improved efficacy of pre-elafin compared to elafin in modulating HNE-induced lung damage (Tremblay et al., 2002) is possibly due to anti-inflammatory effects of the trappin-type region in the pre-elafin protein.

Ovine elafin is predicted to be a very good substrate for, and hence a competitive inhibitor of, transglutaminase and hence phospholipase A2. If the availability of the PVKGQD repeats has an influence on the competitive inhibition of transglutaminase (and hence phospholipase A2) then ovine elafin may have a significant advantage over human elafin in this regard due to the presence of 19 repeats compared to 5 in the human form.

5.5. CONCLUSIONS

Overall, both ovine SLPI and ovine elafin have characteristics (in common with other SLPI and elafin orthologs) that make them attractive for use as anti-inflammatory proteins in the field of inflammatory models. However, ovine elafin may have various advantages over ovine SLPI as a potential therapeutic, most significantly due to the presence of the large transglutaminase substrate domain. As discussed this may serve several useful functions in the inhibition of transglutaminase and hence phospholipase and so lead to a down-regulation of the arachidonic acid cascade locally which may convey an improved phenotype in an inflammatory model of lung disease.

It is this background which led to the construction of a replication-deficient adenoviral vector encoding for ovine elafin. This work is detailed in the next chapter.

CHAPTER 6

CONSTRUCTION AND OPTIMISIATION OF A REPLICATION-DEFICIENT ADENOVIRAL VECTOR ENCODING OVINE ELAFIN

Introduction

Chapter 5 highlighted the potential benefits that ovine elafin may have as a modulatory protein in the field of pulmonary inflammation. These can be summarised as shown below:

- Ovine elafin has intrinsic anti-protease activity
- Ovine elafin shows characteristic properties of other members of the elafin family and this has prompted speculation that the elafin members have 'defensin-like' functions (Sallenave, 2000)
- The presence of a large 'transglutaminase substrate domain' may confer additional beneficial characteristics to the secreted protein (Sohn et al., 2003)

These characteristics prompted the construction of a vector encoding for ovine elafin which would subsequently allow the investigation of the local up-regulation of this anti-protease in an ovine model of pulmonary inflammation. The data presented within this chapter requires as a background a brief review of the principles of gene therapy as a tool and therapeutic technique, and also the vectors available for this work with emphasis on the use of the recombinant adenoviruses.

The principles of gene therapy.

Gene therapy can broadly be defined as the use of genes to cure disease. This may involve the permanent correction of an incorrect gene for example in the treatment of severe combined immuno-deficiency (Cavazzana-Calvo et al., 2000), or the temporary up-regulation of a potentially therapeutic transgene as in the work in this laboratory using adenoviral vectors encoding for the human elafin protein (Simpson et al., 2001A). Each method of utilising a gene therapy or gene transfer technique may exploit different features of the chosen vector. The vectors in common usage today are discussed below along with a brief analysis of their advantages and disadvantages.

Vectors available for gene transfer to the respiratory tract.

These can be categorised as either non-viral or viral vectors. Non-viral methods of delivery have focused predominantly on the use of cationic liposomes which can be readily conjugated to anionic DNA in either plasmid or linear form. These complexes are generally cationic and interact with the negatively charged cell surface allowing the DNA to migrate into the cytoplasm of the 'transfected' cell (Schofield and Caskey, 1995). Other non-charged liposomes can also be used (neutral liposomes) and all liposomes can be made up of single or multiple lipid bilayers (unilamellar or multilamellar respectively) (Felgner et al., 1994). The DNA-liposome complexes are simple to produce and the liposomes can bind large amounts of DNA.

Other non-viral vectors include polycationic polymers such as polylysine and polyethylenimine (PEI) (Wagner et al., 1991; Boussif et al., 1995; Ferrari et al., 1997; Densmore et al., 2000; Wiseman et al., 2003). These have also been used conjugated to ligands to allow cellular targeting. Advantages of this system include ease of production, improved DNA packaging and the ability to target specific cells. However, the transfection efficiency remains lower than that seen with viral vectors.

Viral vectors for gene transfer to the respiratory tract include retroviruses, adeno-associated viruses (AAV), Sendai virus (SV) and adenoviruses. Adenoviruses will be discussed in more detail below. All the virus vectors have a limited capacity for 'foreign' DNA i.e. transgene. This varies from 4.7kb in AAV, through 7-8kb in retroviruses, to 37kb in adenoviruses.

Retroviruses can infect a broad range of cells, providing the cells are replicating, and integrate into the host cell's genome. This has been used to achieve stable infection of cells *ex vivo* followed by re-implantation of these cells. This has been widely reported recently in the treatment of severe combined immuno-deficiency in children (Cavazzana-Calvo et al., 2000). As illustrated by the potential sequelae to this type of intervention, the integration of the virus into the host genome can cause specific problems with regards activation of previously silent oncogenes with subsequent neoplastic potential (Marshall, 2003; McCormack and Rabbitts, 2004). Equally, the cells lining the respiratory tract are relatively differentiated and as such are not readily amenable to retroviral infection and hence this vector has limited applicability in the field of pulmonary gene therapy for inflammatory diseases.

Adeno-associated viruses (AAV), which typically remain epichromosomal but may integrate with high MOI, are members of the parvovirus family which as yet has not been shown to be pathogenic in

humans. This implies a good safety index for this virus as a possible vector. The main disadvantage of AAV as a vector is the limit on the size of inserted transgene (Tal, 2000). The clear advantage of AAV is a markedly reduced inflammatory response post –infection, which in turn allows the repeated administration of vector. Interestingly, Beck at al. (1999) showed that serum anti-AAV titres do not predict the neutralisation of AAV in the airways after instillation and indeed preliminary trials using repeated administration of aerosolised AAV type 2 vectors to deliver the CFTR cDNA to human patients have led to encouraging results (Moss et al., 2004).

Sendai virus, a relatively new viral vector, has been used to infect respiratory tissue *in vivo*. Its efficiency is several log orders higher than that seen with any other viral or non-viral vector (Yonemitsu et al., 2000).

The recombinant adenovirus as a vector for lung directed gene transfer.

Recombinant adenoviruses were chosen as the vector for gene delivery in this thesis. As such a more detailed appraisal of this vector is appropriate.

'Wild type' adenovirus has developed an exquisitely efficient biology related to the infection (in the mammalian context) of cells lining the respiratory tract. Such viruses have been subject to genetic modification to allow the insertion of foreign genes and hence these recombinant viruses allow the efficient delivery of these transgenes to the nuclei of infected cells. The genetic manipulation also removes the ability of the virus to replicate itself thereby serving as a control on infection. Recombinant adenoviruses have the additional advantages that they can infect both replicating and non-replicating cells equally efficiently, and also, unlike retroviruses, they do not integrate into the host cell genome and thereby avoid problematic up-regulation of oncogenes (Kremer and Perricaudet, 1995).

It is appropriate to examine the wild type virus structure as it has an important bearing on the mechanism of cell interaction and infection.

The wild type virus (and indeed recombinant adenovirus) is, grossly, icosahedral with a diameter of approximately 80nm. At the points where 5 of the faces meet are penton subunits to which are attached the fibres. These fibres terminate in a knob protein and it is this knob portion of the fibre

which interacts with the adenovirus receptor (the Coxsackie-adenovirus receptor or CAR) on the surface of permissive cells (Bergelson et al., 1997; Mayr et al., 1997). Following this interaction, internalisation of the virus depends upon an interaction between the penton base unit itself and α-β integrins on the cell surface (Wickham et al., 1993). The adenovirus particles are subsequently transported to the nucleus before disassembly occurs with release of the genetic material, allowing initiation of transcription and translation to begin. The genetic material is double stranded DNA and the wild type genome is approximately 36kb in size. The genome contains both early genes and late genes. The important genes with regards the manufacture of recombinant replication deficient adenoviruses are the E1 and E3 regions. The E1 region initiates viral transcription and does this without the need for trans-activating factors. E1 genes are transcribed rapidly after cell infection; indeed E1A mRNA/protein is detectable within 1 hour. The removal of the E1 region removes the possibility of virus replication.

The E3 region is not necessary for viral replication and has been deleted, either entirely or partially, from many of the currently used adenoviral vectors to allow larger transgenes to be inserted (Bett et al., 1994; Ng and Graham, 2003).The E3 region is potentially of interest as it contains genes which may decrease the immune response to the virus in the infected animal. For example, proteins encoded by this region interfere with the translocation of major histocompatability complex proteins to the surface of the cell, and also interfere with the TNF mediated cytolysis of infected cells (Feuerbach and Burgert, 1993; Wold, 1993; Beier et al., 1994; Lee et al., 1995; Wold et al., 1995). Indeed the co-expression of E3 proteins along with a transgene of choice has been shown to markedly reduce both the appearance of neutralising antibodies in sera and also the cyto-toxic lymphocyte (CTL) response of treated animals, when compared to expression of transgene alone (Ilan et al., 1997). The result of this attenuation of the immune response is that the repeated administration of the same virus led to a second wave of transgene-derived protein production whereas a second administration of the same virus minus the E3 genes was neutralised completely with no transgene product detected.

A significant disadvantage of adenoviruses relates to the fact that wild type adenoviruses are pathogenic and hence genetically modified viruses may still retain pathogenic features. Wild type virus can be associated with a fulminant pneumonia in man especially in immuno-compromised adults

(Klinger et al., 1998) although it can also act alongside other pathogens in respiratory disease with multiple aetiology. In the field of human gene therapy trials, two patients have been recorded as having respiratory pathology after receiving high doses of recombinant adenovirus and indeed one patient subsequently died (Crystal et al., 1995; Hollon et al., 2000). It is therefore of the utmost importance to carefully consider the dosage regime when any adenoviral vector is to be used and to take into account the route of administration in the particular situation under review. The pathology induced by the adenovirus, whether wild type or recombinant, is in part due to the host response towards a known foreign organism. This immune response is also of interest in that it is a major influence in the duration of transgene expression and also has an important influence in the possibility of repeated administration of the same vector.

The lung's immune response to adenovirus can be simplified if one considers as an example a single instillation of a recombinant virus into the air spaces of the lung. The majority of the instilled adenovirus is rapidly taken up by the resident alveolar macrophages present in large numbers in the air spaces themselves (Kuzmin et al., 1997; Worgall et al 1997). Additionally, infected cells are eliminated by a vigorous cytotoxic T cell response (Yang et al., 1994 and 1996; Wilson, 1996). Both these components make up the initial cell mediated response to the presence of virus. The humoral response comprises the formation of neutralising antibodies both locally within the bronchoalveolar fluid (BALF) and in the systemic circulation. It is this component of the immune response which is responsible for the difficulty in repeating administration of the same or a very similar vector. Both this humoral response and the cell mediated response may be directed not only towards the adenoviral components themselves (such as capsid proteins, proteins involved in replication and the genetic material) but also towards exogenous protein encoded by the inserted transgene. For example many marker genes used in gene transfer studies are derived from bacteria (beta galactosidase), jelly-fish (green fluorescent protein) or other organisms. In addition work involving rodent models of *in vivo* gene transfer may use viral (viral Il-10 – Lechman et al., 1999 and 2003; Whalen et al., 2001) or human (human elafin – Simpson et al., 2001A) cDNAs and hence human exogenous proteins will be produced following administration of virus. These proteins will be presented by antigen presenting cells in combination with MHC proteins and will elicit a further response. The obvious conclusion to draw from this data is that it is preferable to study gene transfer using a gene already present within

the organism of interest, such as up-regulation of a murine gene by adenoviral transfer in a mouse model and a human gene in human volunteers. In must be borne in mind, however, that adenoviral vector-induced inflammation can act as a 'danger signal' and as such play an important role in the breaking of tolerance to an endogenous protein (Camargo et al., 2000).

The incorporation of the ovine elafin cDNA into a replication deficient adenoviral vector would potentially be of great interest in the investigation of the roles of this anti-protease in the control of local pulmonary inflammation. Preliminary experimental work would also be of use in the optimisation of infection protocols using adenoviral vectors both *in vitro* and *in vivo*.

6.2. AIMS

As summarised in chapter 5 ovine elafin has potential interest in the modulation of the inflammatory response in the lung. To investigate the up-regulation of ovine elafin an efficient vector expressing this protein was constructed and its usage *in vitro* and *in vivo* investigated as summarised below:

1. This chapter details the construction of an adenoviral vector which codes for the shorter form of the ovine elafin allele (TOM-1).

2. Standard methods involving homologous recombination in human embryonic kidney (HEK-293) (Graham et al., 1977; Graham and Prevec, 1995) cells were employed.

3. Correct transgene insertion was confirmed by Southern blot analysis and restriction analysis, and functional activity assessed by infection of A549 cells and Western blot of the supernatants for secreted elafin.

4. Positive plaques were expanded to obtain a concentrated stock solution of active Ad-o-elafin and the concentration confirmed by titration on HELA cells.

5. The use of transfection reagents to up-regulate expression *in vitro* was explored.

6. Preliminary studies aimed at dose-finding *in vivo* were conducted.

6.3. RESULTS

6.3.1. Insertion of the ovine elafin cDNA into the shuttle vector pDC516.

Full length ovine elafin cDNA in pCR^{R}4-TOPO^R (see chapter 3) was used as a template for two consecutive PCRs with firstly Bgl-f-el and FLAG-r-el, followed by Bgl-f-el and Sac-r-FLAG-el (to yield BglII-elafin-FLAG-SacI). This product was ligated into shuttle vector pDC516 (which contains the murine cytomegalovirus (MCMV) promoter). Product from this reaction was used to transform XL1-Blue subcloning grade E. coli. A positive colony was identified by restriction analysis and plasmid isolated with the Qiagen Maxi-Prep kit. This plasmid will henceforth be known as pDC516-o-elafin.

6.3.2. Homologous recombination in 293 cells.

The techniques used in this chapter to produce a replication-deficient Ad have been developed by Professor Frank Graham's research group at McMaster University and the particular protocol used here utilising FLP/frt recombinase technology is discussed in one of his group's publications (Ng at al 2001). This protocol relies on the protein FLP catalysing a site-specific recombination event between the shuttle plasmid (here pDC516) and the adenoviral vector plasmid (pBHGfrt(del)E1,3FLP) so that a viral genome is produced lacking only the E1 and part of the E2 regions. The result is an Ad that can only replicate in the presence of E1 delivered in *trans* (in this case the human embryonic kidney cell line, HEK 293, are stably transfected with E1).

293 cells were co-transfected with pDC516-o-elafin and pBHGfrt(del)E1,3FLP (a kind gift from Dr. M. Hitt, McMaster University) at various ratios or with the single control plasmids pFG140 (as a positive control) and pDC516-o-elafin or pBHGfrt(del)E1,3FLP (as negative controls). This process is shown diagrammatically in fig. 1.

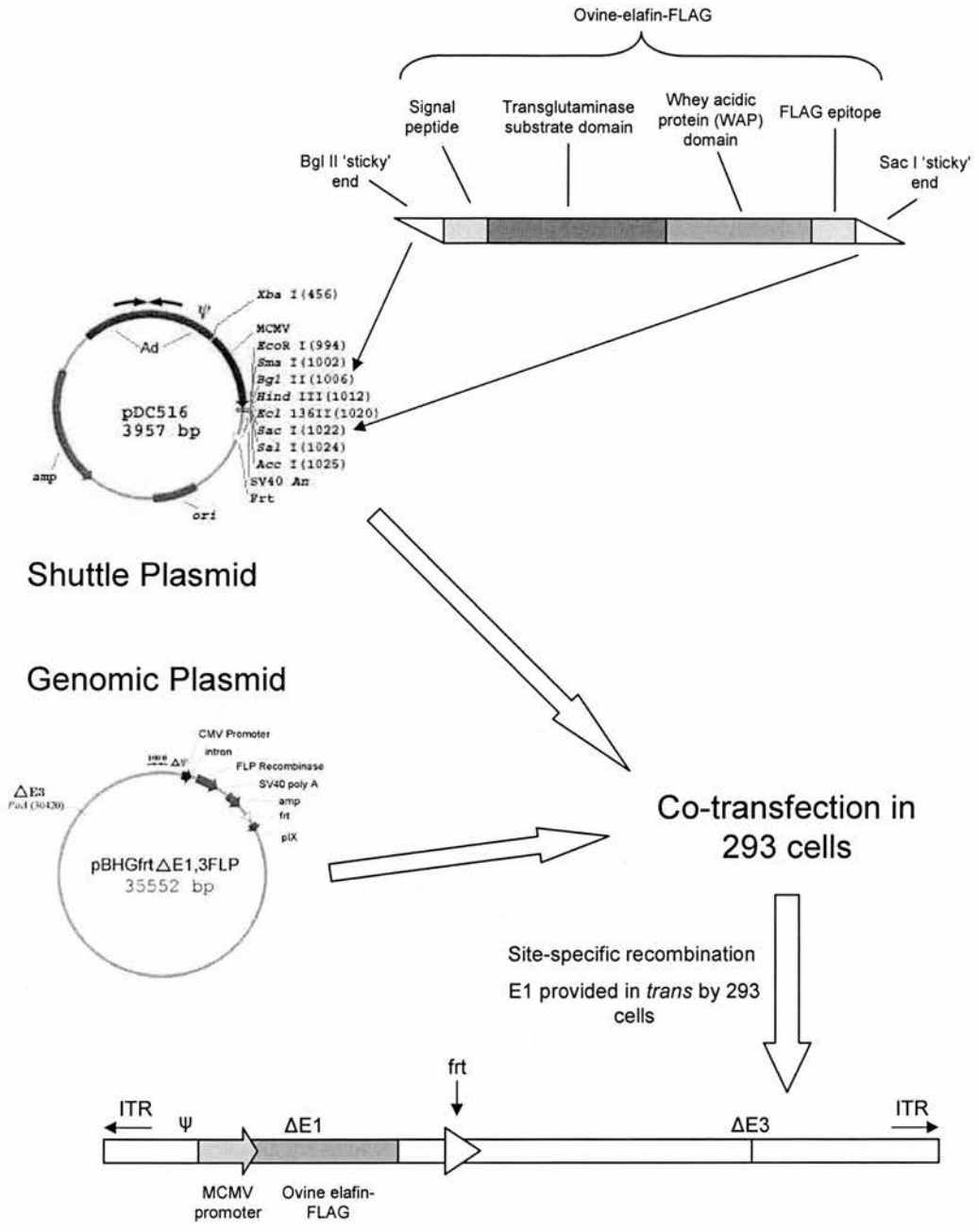
Ovine-elafin-FLAG
Signal peptide
Transglutaminase substrate domain
Whey acidic protein (WAP) domain
FLAG epitope
Bgl II 'sticky' end
Sac I 'sticky' end
Xba I (456)
Ψ
Ad
MCMV
EcoR I (994)
Sma I (1002)
Bgl II (1006)
Hind III (1012)
Xcl 136II (1020)
Sac I (1022)
Sal I (1024)
Acc I (1025)
SV40 An
Frt
pDC516
3957 bp
amp
ori
Shuttle Plasmid
Genomic Plasmid
CMV Promoter
intron
FLP Recombinase
SV40 poly A
amp
frt
pIX
pBHGfrtΔE1,3FLP
35552 bp
Co-transfection in 293 cells
Site-specific recombination
E1 provided in trans by 293 cells
frt
ITR
Ψ
ΔE1
ΔE3
ITR
MCMV promoter
Ovine elafin-FLAG
Replication-deficient adenovirus encoding ovine-elafin-FLAG

Fig. 1...

Fig. 1. Insertion of ovine elafin cDNA into shuttle vector followed by site-specific recombination in 293 cells to yield Ad-o-elafin.

The ovine elafin cDNA isolated in chapter 3 was used as a template in PCR reactions to produce BglII-ovine elafin-FLAG-SacI as described in chapter 2. This was ligated into the shuttle vector pDC516 as described. 293 cells were then co-transfected with this shuttle vector and also the adenovirus genome plasmid pBHGfrt(del)E1,3FLP and site-specific recombination events yielded the adenovirus genome as shown (Ad-o-elafin). This genome contains the ovine elafin cDNA driven by the MCMV promoter and the ψ packaging sequences derived from the shuttle vector. This genome is packaged in the 293 cells which deliver in *trans* the E1 proteins necessary for the initiation and continuation of viral replication.

Five dishes (referred to as 'a' to 'e') demonstrated complete cytopathic effect (CPE).

6.3.3. Southern blot analysis of CPE-positive cells for ovine elafin cDNA insert.

The cell pellets from dishes 'a' to 'e' were extracted for DNA. A 1% agarose gel of the products of Hind III digestion is shown in fig. 2A. Southern blot analysis, confirming two wells ('b' and 'e') as positive, is shown in fig. 2B. Positive bands were identified at approximately 3.5kbps and 4.5kbps.

6.3.4. Restriction digestion and Western blot analysis of six positive plaques.

Cell supernatant from dish 'b' was used to re-infect adherent 293 cells under agarose to isolate pure plaques. This led to the isolation of six positive plaques referred to as 1-6. These plaques were subject to freeze-thaw lysis and the resultant supernatant used on fresh 293 cells until 100% CPE to re-check the efficiency of infection, functional activity, and the DNA banding pattern after Hind III digestion.
The results of Hind III digestion are shown in fig. 3. The putative o-elafin positive band seen at 3.5kbps (see fig. 2B) was present in cell pellets from dishes 4, 5 and 6. All six dishes demonstrated a band at 4.5kbps which is the weight of the heavier band seen by Southern blot analysis (see above and fig. 2B).
The origin of the banding patterns seen in the Southern blot analysis and Hind III digestion (figs. 2 and 3) are discussed in section 6.4.1.
Western blot analysis on supernatants from these dishes is shown in fig. 4. Dishes 4, 5 and 6 were positive for secreted ovine elafin (detected by both the Trab-2O antibody and also the anti-FLAG antibody). Supernatant from dish 5 was used in further experiments to amplify the adenovirus stock

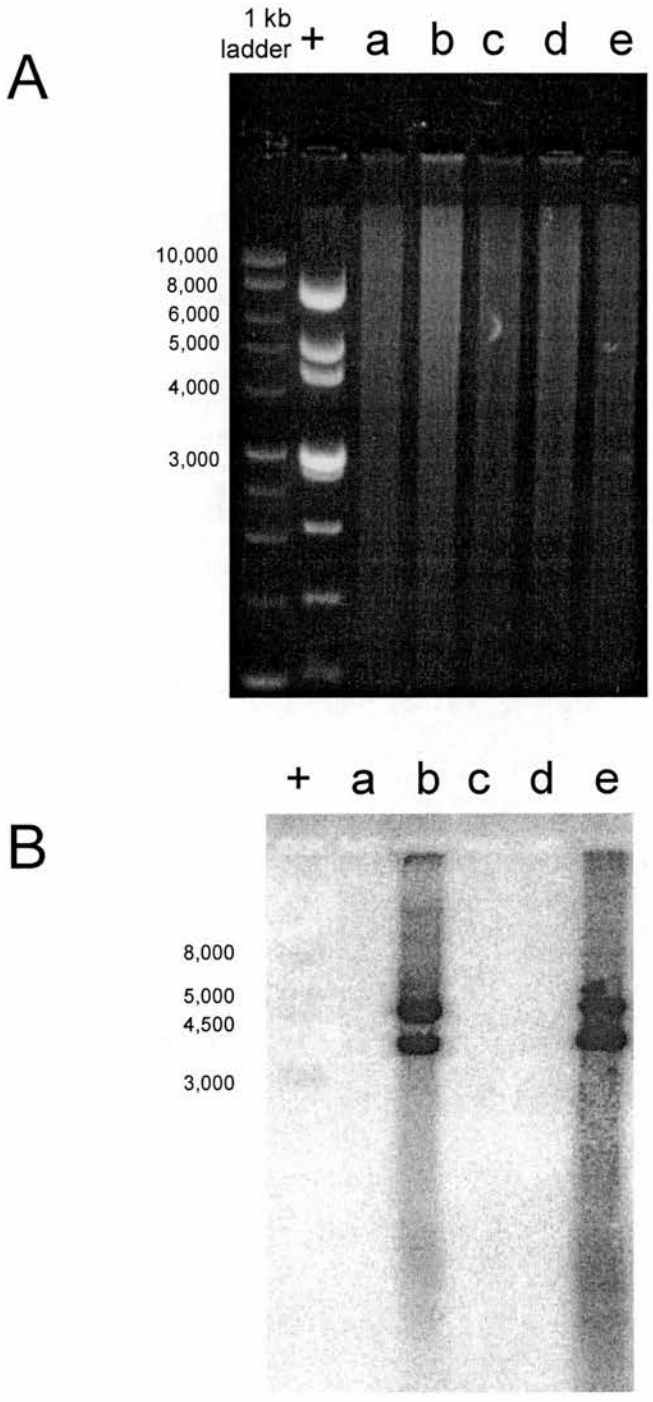
A
1 kb ladder
+
a
b
c
d
e
10,000
8,000
6,000
5,000
4,000
3,000
B
+
a
b
c
d
e
8,000
5,000
4,500
3,000

Fig. 2...

C

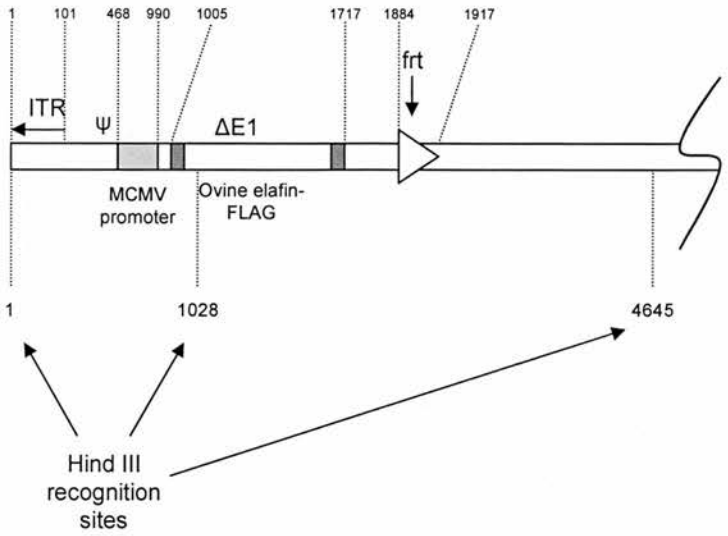

Fig. 2. Restriction digestion and Southern blot analysis of DNA post homologous recombination.

DNA from dishes positive for cytopathic effect after the homologous recombination stage of adenovirus production was digested with Hind III and run out on an agarose gel (A) before being transferred to PVDF membrane and used in Southern blot analysis (B) probed with the ovine elafin cDNA. The positive lane refers to DNA from the positive control dish transfected with pFG140 plasmid. a-e refer to the 5 dishes that showed full cytopathic effect. Dishes b and e are positive for the ovine elafin cDNA at approximately 3.5 and 4.5kb.
C shows the resultant map of the first portion of the resultant recombinant adenovirus genome after FLIP mediated recombination between pDC516 and pBHGfrt(del)E1,3FLP. The Hind III sites are indicated below. Figures denote the base pair number from the start of the Adenoviral genome.

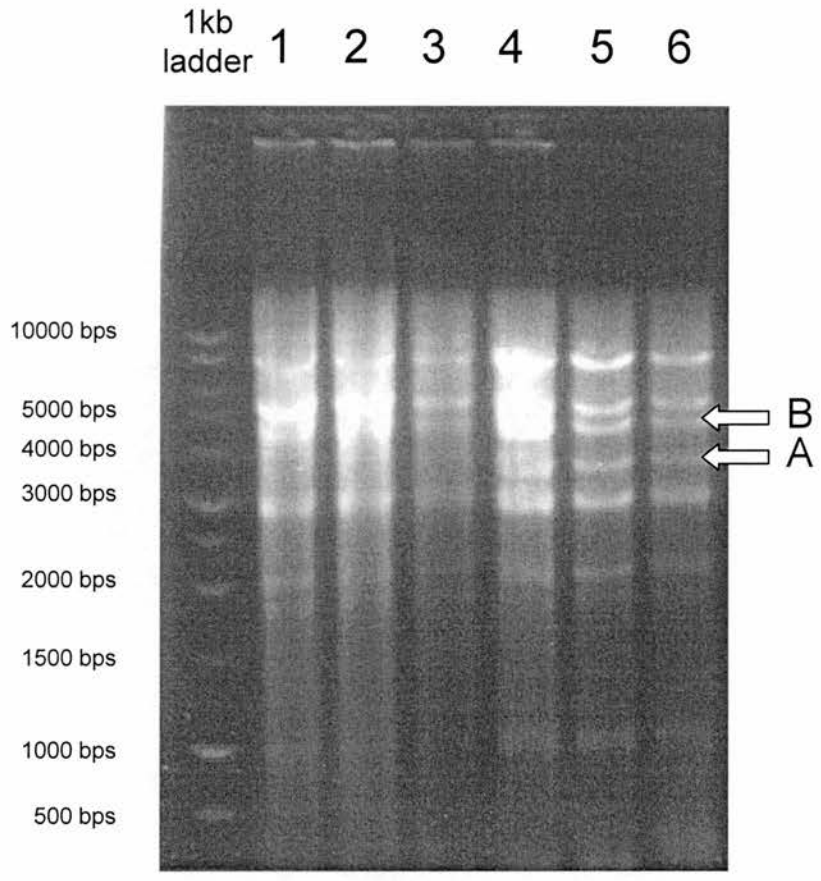

Fig. 3. Hind III digestion of DNA from 6 plaques isolated after 293 infection under agarose.

DNA isolated from the positive plaques isolated during adenovirus preparation was digested with Hind III as described in Chapter 2. The resultant digested DNA was run on an agarose gel and the photograph reproduced here. The lanes corresponding to plaques 1-3 have the same band pattern as each other and are different to the bands seen in the lanes corresponding to plaques 4-6 which again are the same as each other. Plaques 4-6 contain a band at the 3.5kb (A) seen by Southern blot in fig. 2B. A band is seen at approximately 4.5kb (B) which corresponds to the heavier band seen in Southern blot analysis but this band seems to be present in all 6 plaques.

by recognised methods (Graham and Prevec, 1995). This adenovirus will now be referred to as Ad-o-elafin.

6.3.5. The assessment of polyethylenimine, calcium phosphate, EGTA and LPS as transfection reagents for up-regulating adenoviral infection of A549 cells.

In order to investigate possible methods of improving the infection efficiency of adenoviral vectors *in vitro* (with potential subsequent use *in vivo*), a variety of infection protocols was used as described in chapter 2 (section 2.2.6). Fig, 5 shows representative photomicrographs taken 24 hours after infection of A549 cells with Ad-GFP under the following conditions. Virus was used either alone (multiplicity of infection (MOI) of either 1 or 10), or after co-precipitation with calcium phosphate (CaP), or pre-incubation with polyethylenimine (PEI, 500 molecules per virus particles) or in combination with hypotonic EGTA (100mM EGTA in 37.5mosm/l PBS). As seen in fig. 5 all three treatments caused an up-regulation in both the number of GFP +ve cells and their fluorescent intensity. The increase in GFP seen with the calcium phosphate co-precipitation was more marked than that seen with either the PEI complex or the pre-treatment with hypotonic EGTA.

Cell counts confirmed the visual impression (fig. 6) with calcium phosphate, PEI and EGTA significantly increasing the number of A549 cells infected ($P<0.005$, $P<0.05$ and $P<0.05$ respectively compared to virus alone).

A second experiment assessed the effect, relative to the above conditions, of incubating the cells with bacterial lipopolysaccharide (LPS). This experiment was conducted in order to investigate the potential up-regulation of the activity of the MCMV promoter by bacterial LPS (Löser et al., 1998; Sallenave et al., 1998; Simpson et al., 2001B). After 1 hour incubation with the virus (MOI 10), virus+CaP, virus+PEI, or virus+EGTA the cell culture medium was changed. LPS (10μg/ml) was added to some of the virus alone wells on day 3. Supernatants were collected after 4 days and analysed by the use of a direct ELISA as described in Chapter 2 (Section 2.2.7).

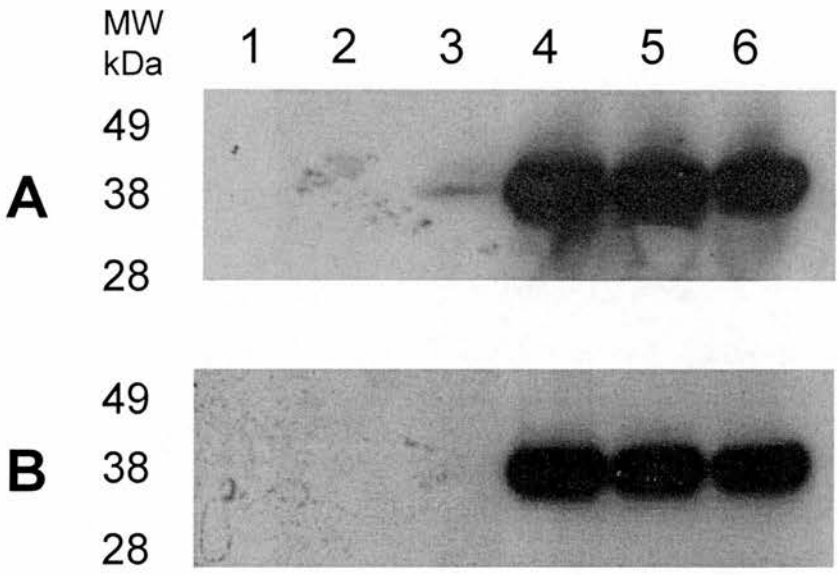

Fig. 4. Western blot analysis of supernatants from adenovirus positive plaques.

Supernatant from A549 cells infected with freeze/thaw lysate from the positive plaques (plaques 1 to 6) yielded after the 're-plaquing' stage of adenovirus production were used in Western blot analysis using either Trab-2O antibody (A) or anti-FLAG monoclonal antibody (B). Plaques 4-6 are positive for adenovirus producing elafin-FLAG protein.

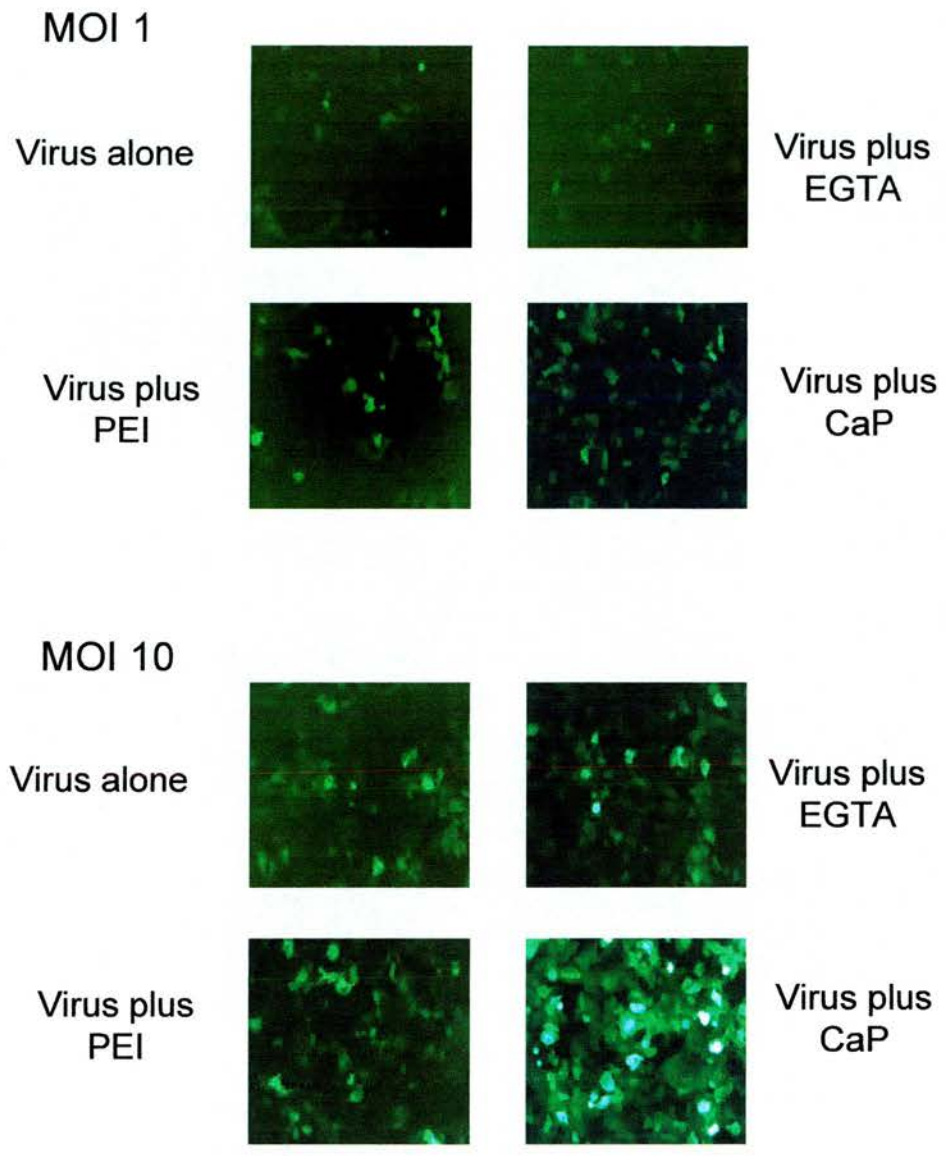

Fig. 5. The up-regulation of adenoviral infection of A549 cells by the use of polyethylenimine, calcium phosphate and EGTA.

A549 cells were plated out at 80,000 per well in 48 well format and infected with Ad-GFP MOI 1 (upper group of micrographs) or 10 (lower group) for 1 hour either alone or after pre-incubation with PEI or calcium phosphate, or with the addition of EGTA to the cells along with the virus as described in chapter 2 (section 2.2.6). GFP +ve cells were visualised by UV microscopy after 24 hours.

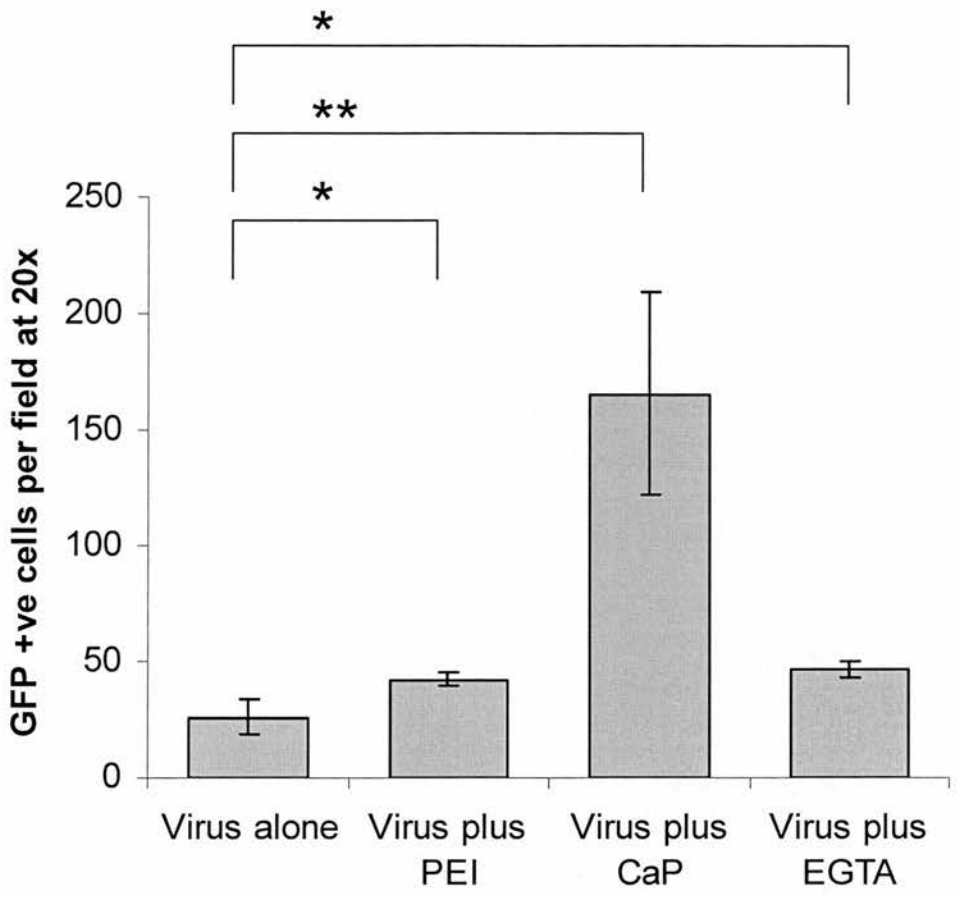

Fig. 6. The up-regulation of the number of A549 cells infected by adenovirus by the use of polyethylenamine, calcium phosphate and EGTA.

A549 cells were plated out at 80,000 per well in 48 well format and infected with Ad-GFP MOI 1 for 1 hour either alone or after pre-incubation with PEI or calcium phosphate, or with the addition of EGTA to the cells along with the virus. GFP +ve cells per high power field were counted after 24 hours. Results are displayed as the mean of 3 wells and error bars represent standard deviation. * Indicates $P<0.05$ compared to virus alone and ** indicated $P<0.005$ compared to virus alone when data analysed by T Test.

Both LPS and calcium phosphate co-precipitates significantly up-regulated the secretion of ovine elafin compared to that due to Ad-ovine elafin alone ($P<0.05$ and $P<0.005$ respectively) (fig. 7). and calcium phosphate was significantly more effective than LPS in this regard ($P<0.005$).

6.3.6. Optimisation of calcium phosphate as a transfection reagent.

6.3.6.1. Time of contact.

A549 cells were incubated with virus or virus+CaP (MOI 10) for 1 minute, 5 minutes, 20 minutes or 1 hour before washing and further incubation in fresh medium. Photomicrograph images were captured 24 hours post-infection and analysed by the use of Image J software to count GFP +ve pixels.

Calcium phosphate significantly increased the number of GFP+ve pixels relative to virus alone following all incubation times ($P<0.05$ for 1 minute, $P<0.0005$ for 5 minutes, 20 minutes and 1 hour) (fig. 8). The pixel count declined significantly between 20 minutes and 1 hour with virus+CaP ($P<0.05$).

6.3.6.2. Toxicity.

A549 cells were infected virus alone or virus+CaP (MOI 10) for 1 hour before washing and further incubation in fresh medium. After 24 hours cell viability was assessed by trypan blue exclusion.

The use of CaP led to a significant and marked increase in cell death relative to virus alone ($P<0.05$) (fig. 9).

6.3.6.3. Verification on primary cells.

Ovine alveolar macrophages, plated at 250,000 per well, were infected with Ad-GFP alone or Ad-GFP+CaP (MOI 100) for twenty minutes after which the medium was replaced and culture continued for 24 hours.

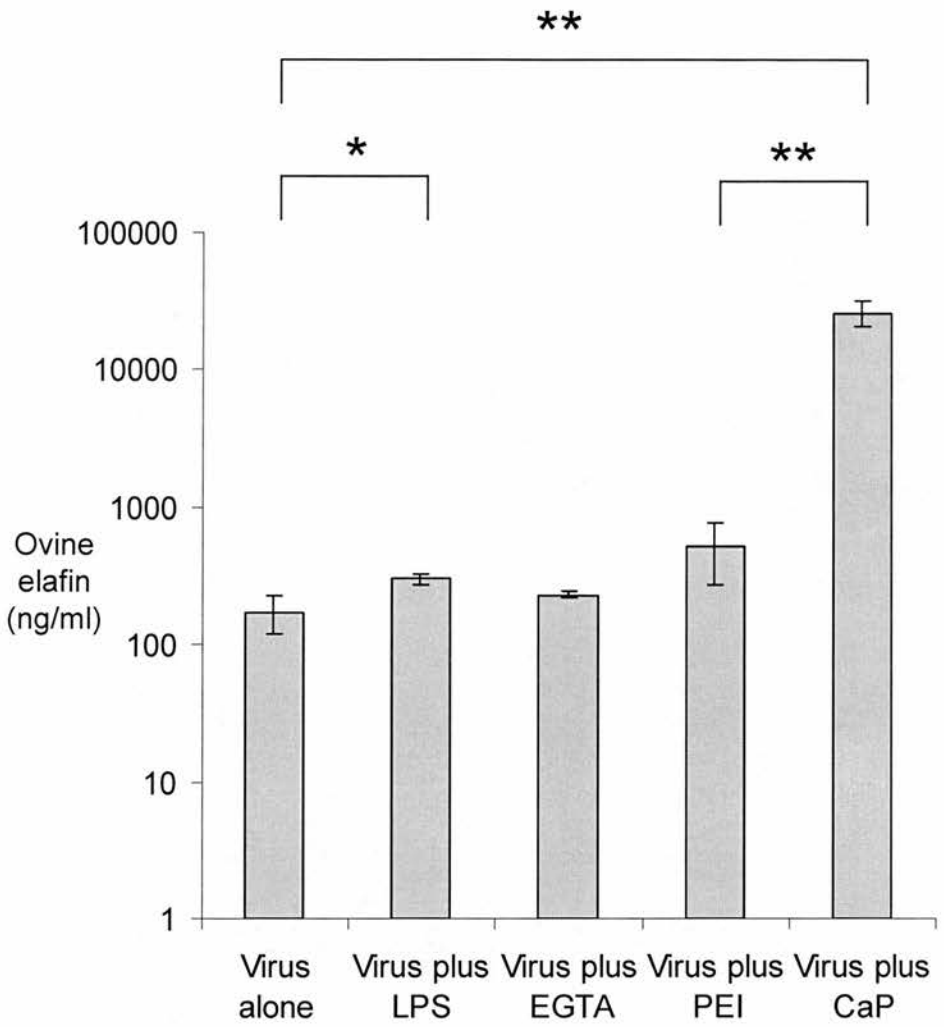

Fig. 7. The increase in the efficiency of Ad-o-elafin infection by the use of polyethylenimine, calcium phosphate, EGTA and LPS.

A549 cells were plated out at 80,000 per well in 48 well format and infected with Ad-o-elafin MOI 10 for 1 hour either alone or after pre-incubation with PEI or calcium phosphate, or with the addition of EGTA or LPS to the cells along with the virus. Secreted ovine elafin was assessed by direct ELISA after 4 days (See chapter 2 sections 2.2.6 and 2.2.7). Results are displayed as the mean of 3 wells and error bars represent standard deviation.
* Indicates $P<0.05$ and ** indicated $P<0.005$ when data analysed by T Test.

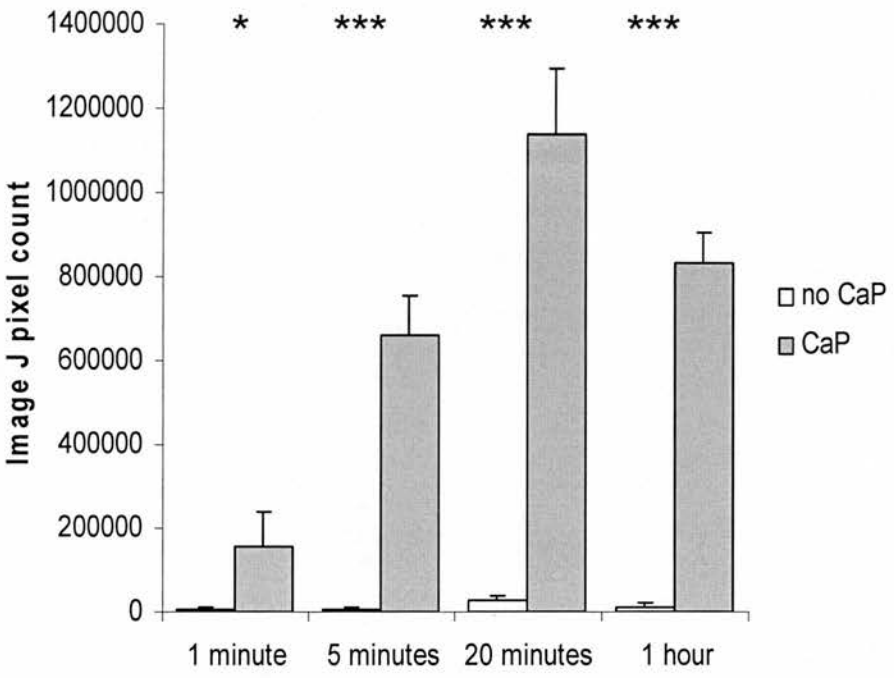

Fig. 8. The effect of contact time between vector and cells on the up-regulation of adenoviral infection of A549 cells with and without the use of calcium phosphate.

A549 cells were plated out at 80,000 per well in 48 well format and infected with Ad-GFP MOI 10 after pre-incubation with either calcium phosphate or control medium alone for 20 minutes. After 24 hours photomicrographs were taken and analysed by the use of Image J software. Pixel counts are shown as the means of 3 wells and error bars represent standard deviation.
* indicates $P<0.05$ and *** indicates $P<0.0005$ when CaP treatment is compared with no CaP for each contact time.

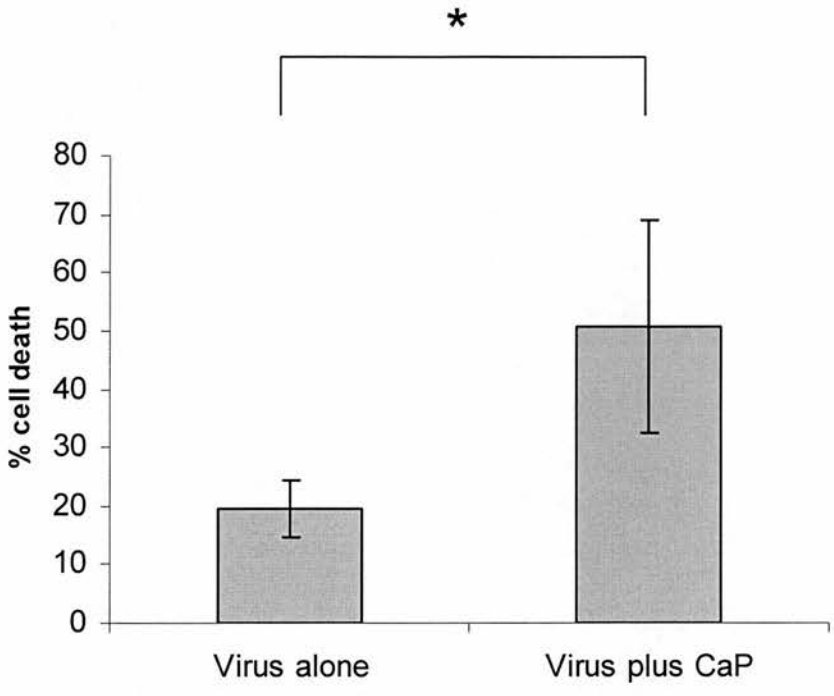

Fig. 9. The effect of adenovirus/CaP incubation on A549 viability.

A549 cells were plated out at 80,000 per well in 48 well format and infected with Ad-GFP MOI 10 for 1 hour after pre-incubation with either calcium phosphate or control medium alone for 20 minutes. After 24 hours cells were assessed for viability by trypan blue exclusion. Results are shown as mean of 3 wells and error bars indicate standard deviation.
* indicates $P<0.05$ when CaP treatment is compared with no CaP.

Twenty-four hours after infection, approximately 5% of the cells infected with Ad-GFP alone were GFP+ve (fig. 10A) whereas approximately 80% of the cells infected with Ad-GFP+CaP were GFP+ve (fig. 10B). The latter cells were more intensely fluorescent.

In a separate experiment, ovine alveolar macrophages, plated at 250,000 per well, were infected with Ad-o-elafin alone or Ad-o-elafin+CaP (MOI 100 and 200) for twenty minutes after which the medium was replaced and culture continued for 4 days. Western blot analysis of the supernatants 4 days after infection with o-elafin at both MOIs demonstrated an obvious up-regulation of secreted elafin with Ad-o-elafin+CaP relative to Ad-o-elafin alone (fig. 10C)

Cell viability at 24 hours was assessed by propidium iodide staining. The use of CaP led to a significant and marked increase in cell death relative to virus alone ($P<0.05$) (fig. 11).

6.3.6.4. Verification *in vivo*.

As discussed above the use of co-precipitation with calcium phosphate led to the most marked up-regulation of Ad infection efficiency and verification of this principle was sought *in vivo*. A one year old Suffolk cross sheep was instilled endo-bronchially in separate segments with $1x10^7$, $5x10^7$ and $1x10^8$ plaque forming units (pfu) of Ad-GFP either in PBS alone or after pre-incubation with CaP for 20 minutes (chapter 2 (section 2.3.6)). Other segments received PBS alone or PBS with CaP, or were not treated (naïve). The doses of Ad instilled in this experiment will be referred to as low, medium and high (relating to $1x10^7$, $5x10^7$ and $1x10^8$ plaque forming units (pfu) respectively) from this point. 48 hours after the instillation the sheep was killed and bronchoalveolar lavage (BALF) collected from each segment. The cellular content of the BALF samples was assessed for total and differential cell counts. The BALF supernatant was assayed for total protein content. Tissue sections were examined under UV microscopy for quantification of GFP +ve cells.

Ad-GFP alone (medium and high dose) and Ad-GFP+CaP (low, medium and high dose) increased the number of neutrophils in the BALF (Fig. 12A and B) relative to the naïve segment.

Treatment did not appear to influence the total protein content of BALF (fig. 13).

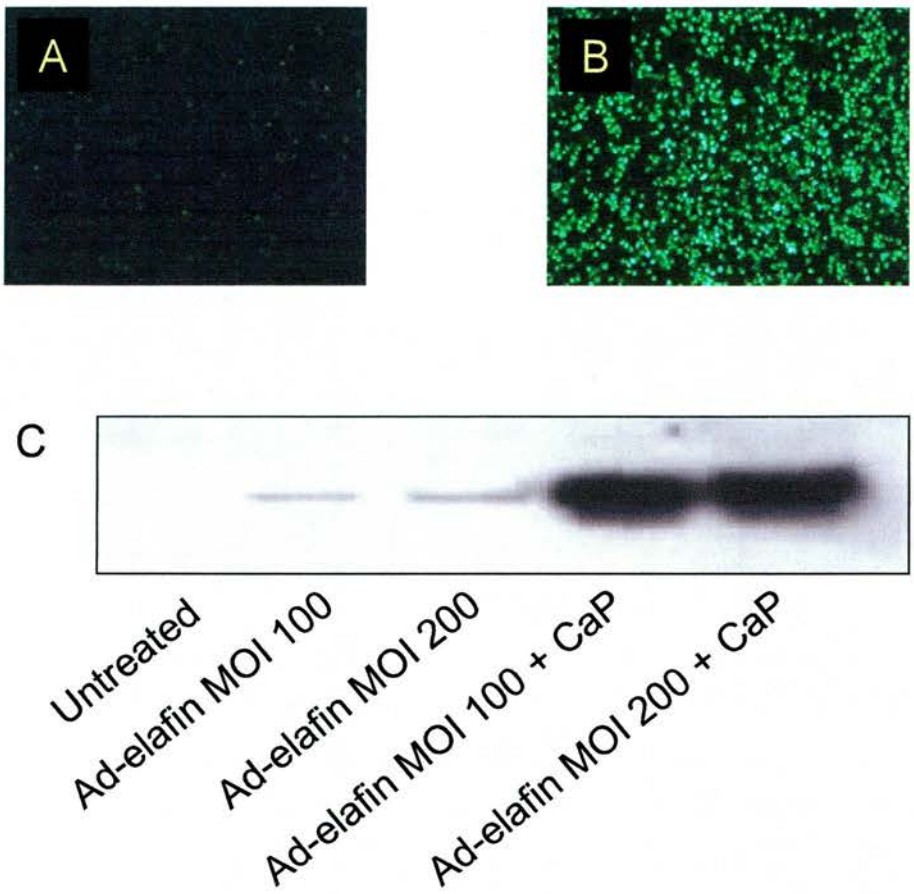

Fig. 10. Calcium phosphate increases the efficiency of adenoviral infection of ovine alveolar macrophages.

Ovine alveolar macrophages were plated out 250,000 per well in 24 well plates and were infected with Ad-GFP and Ad-ovine elafin at MOI 100 and 200 either pre-complexed with calcium phosphate or as virus alone.

A. Ovine alveolar macrophages 24 hours after infection with Ad-GFP MOI 100 alone.
B. Ovine alveolar macrophages 24 hours after infection with Ad-GFP MOI 100/calcium phosphate co-precipitate.
C. Western blot analysis of ovine alveolar macrophage supernatant using Trab-2O antibody 4 days after infection with Ad-ovine elafin at MOI 100 and 200 either with or without co-precipitation with calcium phosphate. Uninfected alveolar macrophage supernatant is included as a control.

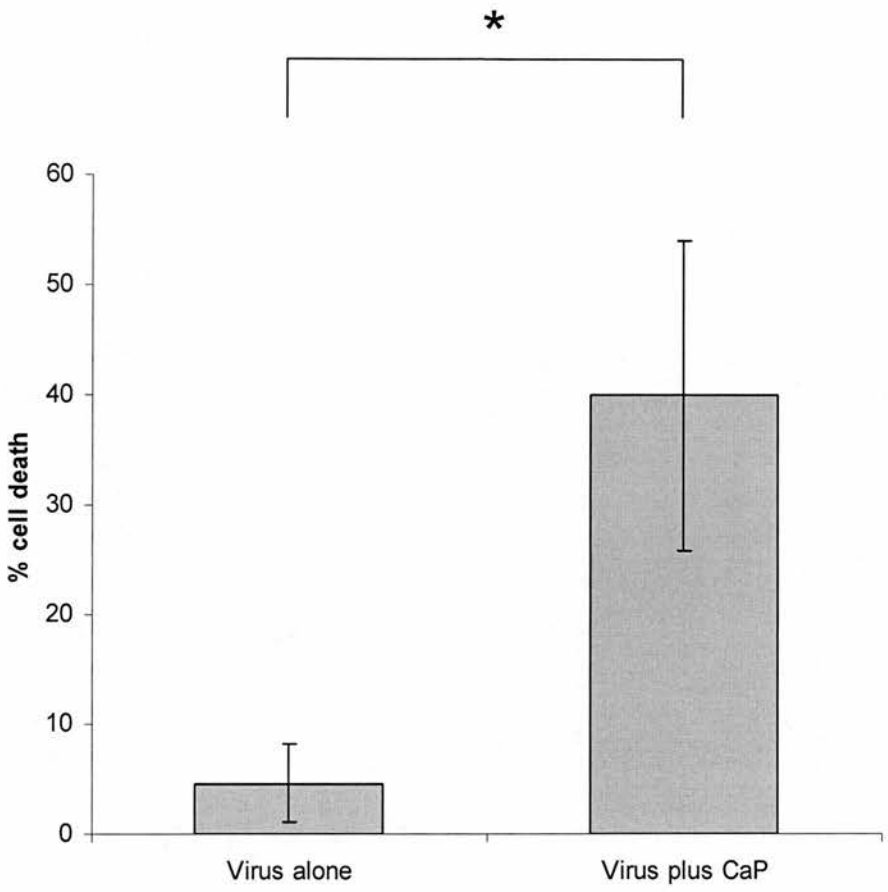

Fig. 11. The effect of adenovirus/CaP incubation on alveolar macrophage viability.

Alveolar macrophages were plated out at 250,000 per well in 24 well format and infected with Ad-GFP MOI 100 for 20 minutes after pre-incubation with either calcium phosphate or control medium alone for 20 minutes. After 24 hours cells were assessed for viability by propidium iodide staining. Results are shown as mean of 3 animals macrophages and error bars indicate standard deviation.
* indicates $P<0.05$ when CaP treatment is compared with no CaP.

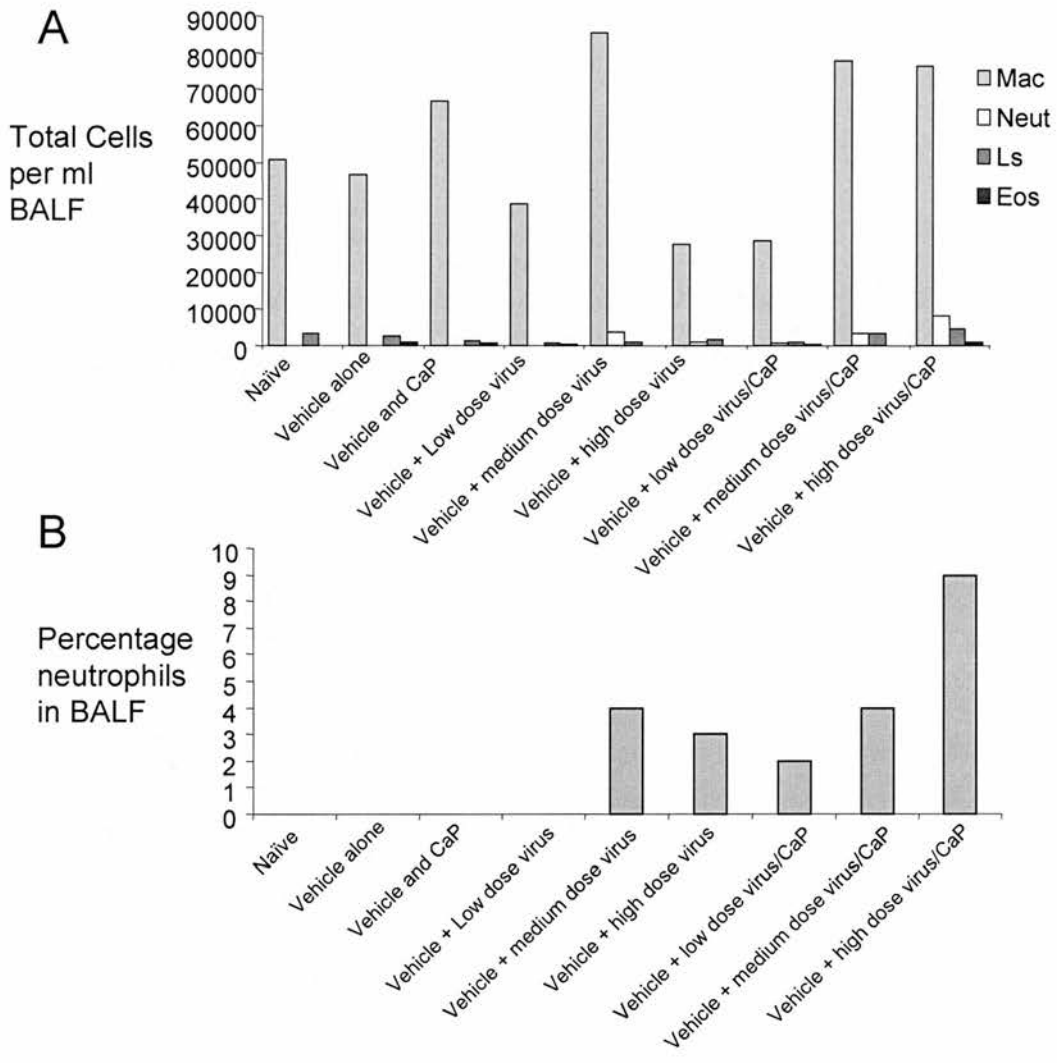

Fig. 12. The effects of adenovirus/CaP instillation on cell number and percentage neutrophils in bronchoalveolar lavage (BALF).

BALF was collected 48 hours after instillation into different segments of vehicle (PBS), vehicle and CaP, $1x10^7$, $5x10^7$ and $1x10^8$ pfu Ad-GFP either in PBS or after pre-incubation with calcium phosphate. Differential cell counts (A) and the percentage of neutrophils in the BALF (B) are shown.

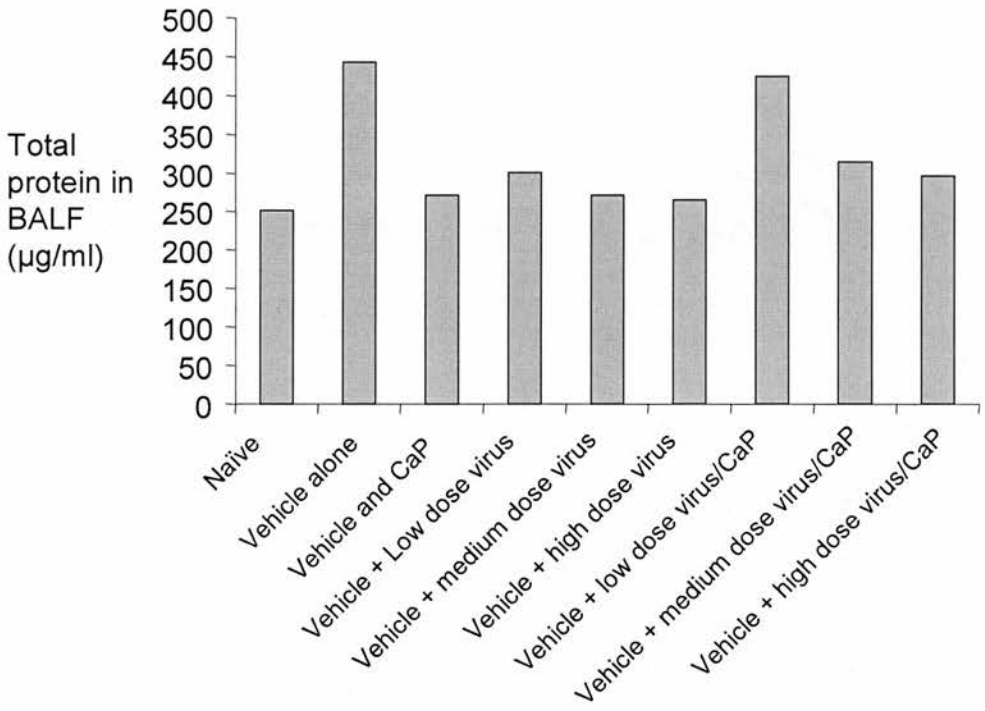

Fig. 13. The effects of adenovirus/CaP instillation on the total protein content of bronchoalveolar lavage (BALF).

BALF was collected 48 hours after instillation of vehicle (PBS), vehicle and CaP, $1x10^7$, $5x10^7$ and $1x10^8$ pfu Ad-GFP either in PBS or after pre-incubation with calcium phosphate, and the total protein content of the BALF shown.

High power fields in tissue sections from segments exposed to Ad-GFP alone (medium dose) and Ad-GFP+CaP (medium and high dose) had significantly more GFP+ve cells than fields in sections from the naïve segment. Co-precipitation with CaP significantly increased the number of GFP+ve cells in the segments exposed to medium and high dose Ad (fig. 14A).

Fig. 14B shows representative high power views from the high dose Ad-GFP+CaP treated segment. These photomicrographs show efficient infection of individual cells, mainly what appear to be Type II epithelial cells in the alveolar sacs (closed arrows) and alveolar macrophages (open arrow). Evidence of infection of type I epithelial cells was apparent although these cells were more difficult to visualise due to their physical shape and size.

6.3.7. Utility of CaP in optimising protocols for Ad-o-elafin administration *in vivo*.

The data from the last experiment prompted the investigation of the instillation of Ad-o-elafin into the ovine lung in order to subsequently examine the potential for elafin to modulate the lung's inflammatory response.

3 sheep were instilled endo-bronchially, in separate lung segments with $1x10^7$, $5x10^7$ and $1x10^8$ plaque forming units (pfu) of Ad-o-elafin after incubation with CaP for 20 minutes (chapter 2 section 2.3.6). Other segments received PBS alone or PBS+CaP, or were not treated (naïve). 48 hours after virus administration the sheep were killed and bronchoalveolar lavage (BALF) collected from each segment. The cellular and protein content of the BALF samples was assessed and compared to pre-experimental samples taken 1 week prior to virus administration.

Neutrophil numbers in BALF increased significantly in the high dose Ad-o-elafin+CaP segment when compared to the naïve segment ($P<0.05$). No significant change occurred in relation to the pre-experimental BALF samples (figs. 15A and B). No other cell type was affected as a consequence of treatment.

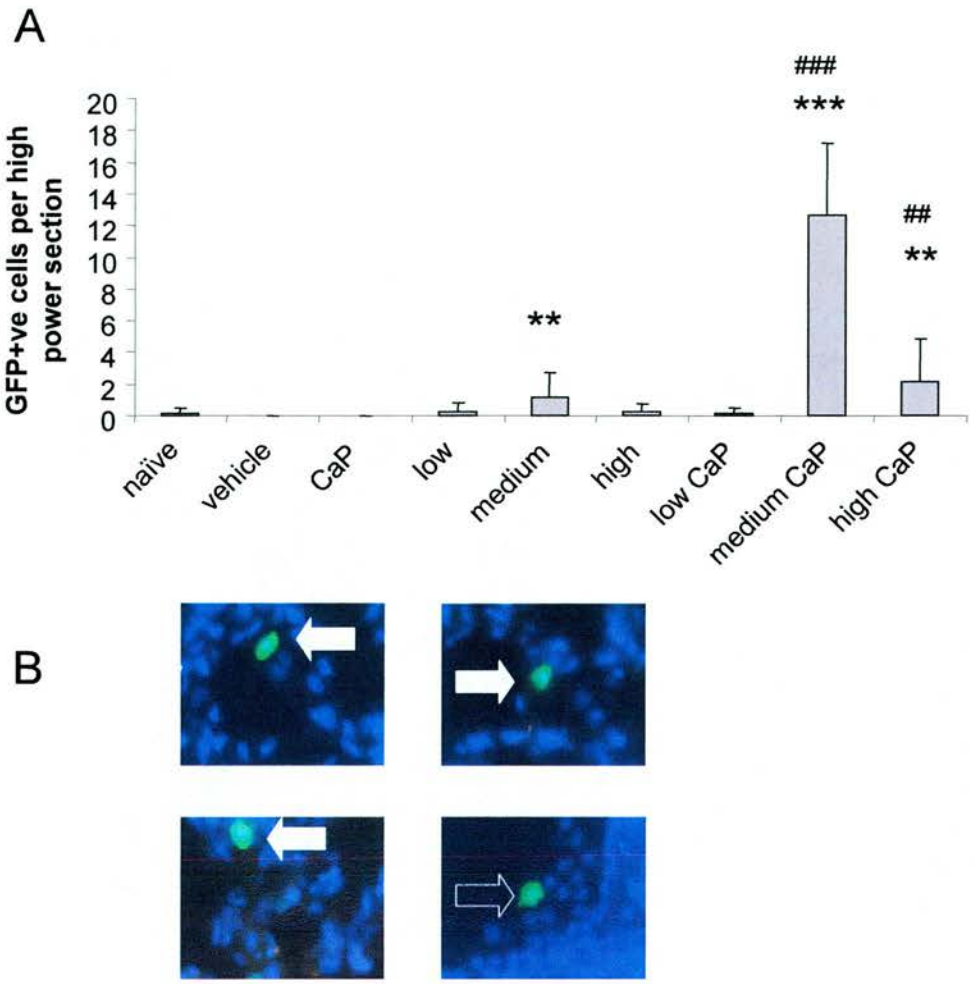

Fig. 14. The *in vivo* infection of ovine lung with Ad-GFP/CaP co-precipitates.

Cryo-sections were prepared from lung tissue 48 hours after instillation of vehicle (PBS), vehicle and CaP, $1x10^7$, $5x10^7$ and $1x10^8$ pfu Ad-GFP either in PBS or after pre-incubation with calcium phosphate. 24 random fields were counted under high power using UV light and the number of GFP+ve cells recorded.

A. The number of GFP+ve cells per high power field is presented here as the mean with error bars indicating standard deviation. ** and *** indicate $P<0.005$ and $P<0.0005$ respectively compared to naïve; ## and ### indicate $P<0.005$ and $P<0.0005$ respectively when compared to the same dose of virus minus CaP co-precipitates using a T Test.
B. Representative fields from the high dose/CaP segment showing infection of Type II epithelial cells (filled arrow) and an alveolar macrophage in an airway (open arrow). Counterstaining of nuclei is with DAPI.

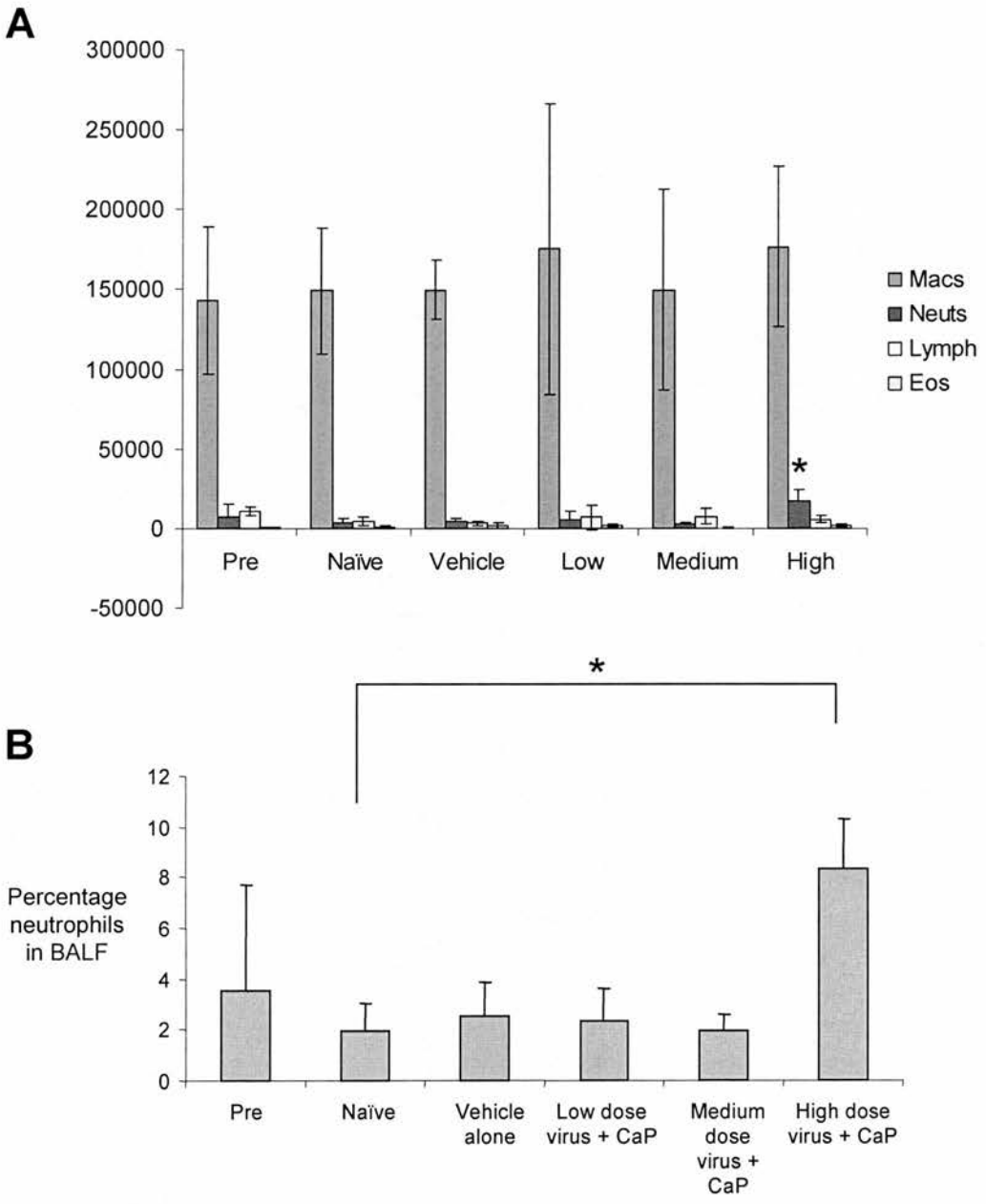

Fig. 15. The effects of Ad-o-elafin/CaP instillation on cell number and percentage neutrophils in bronchoalveolar lavage (BALF).

BALF was collected 48 hours after instillation of vehicle (PBS) and CaP, $1x10^7$, $5x10^7$ and $1x10^8$ pfu Ad-ovine elafin after pre-incubation with calcium phosphate, and the differential cell counts (A) and the percentage neutrophils in the BALF (B) shown. Data is shown as mean (n=3) and error bars represent the standard deviation. * represents $P<0.05$ when compared to naïve segment counts when analysed by T test.

BALF total protein increased significantly in the high dose Ad-o-elafin+CaP segment when compared to the naïve segment ($P<0.05$). No significant change occurred in relation to the pre-experimental BALF samples (figs. 16A and B).

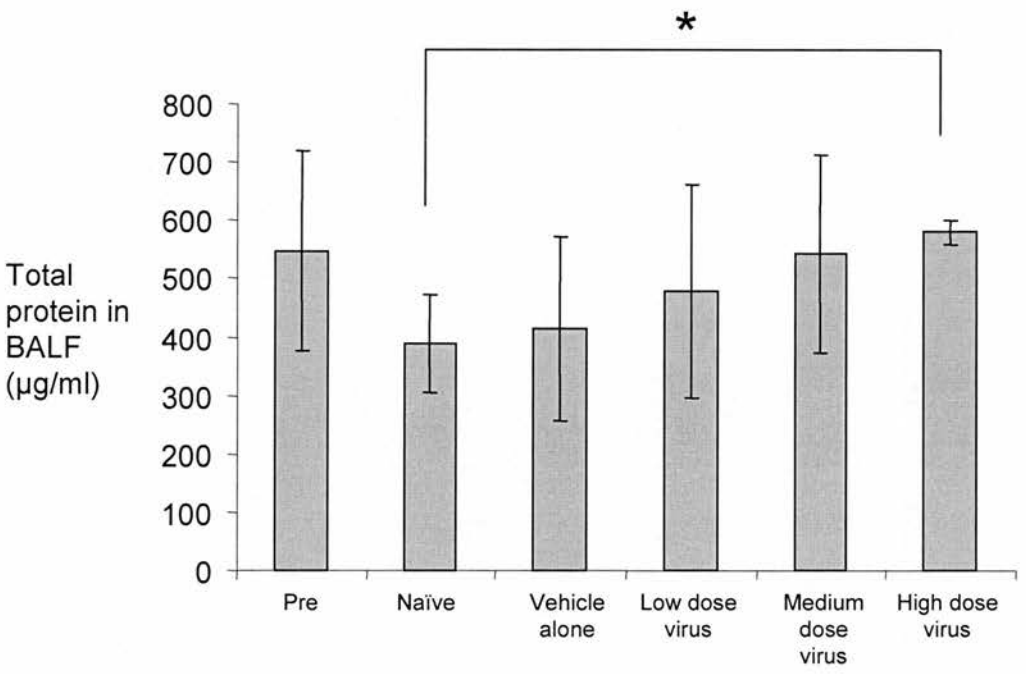

Fig. 16. The effects of Ad-o-elafin/CaP instillation on total protein content of broncho-alveolar lavage (BALF).

BALF was collected 48 hours after instillation of vehicle (PBS and CaP), or $1x10^7$, $5x10^7$ and $1x10^8$ pfu Ad-o-elafin after pre-incubation with calcium phosphate, and the total protein content of the BALF shown. Data is presented as means (n=3) and error bars represent standard deviation. * indicates $P<0.05$ compared to naïve segment when data analysed by T test.

6.4. DISCUSSION

6.4.1. The construction of Ad-o-elafin.

As discussed in depth in chapter 5 ovine elafin was chosen in this study from the two newly characterised ovine anti-proteases detailed in chapters 3 and 4, as the more likely to have beneficial modulatory activity in models of inflammation. This chapter detailed the use of the ovine elafin cDNA to produce a replication deficient Ad vector in order to further study the effects of elafin delivery *in vivo*.

The ligation of the ovine elafin cDNA into the shuttle vector pDC516 as discussed above was relatively undemanding and as such warrants little in the way of discussion. However the site-specific recombination between genomic plasmid and shuttle plasmid in 293 cells was more complex and carried the potential for error introduced by the recombination process. Hence the reason for assessing the 'banding' pattern of the potentially positive cultures either at the stage of CPE in 293 cells in normal culture conditions, or at the latter stage of plaque formation under agarose. Analysis of the same digested DNA at the CPE stage by Southern Blot confirmed the presence of the ovine elafin cDNA.

Positive samples showed positive bands at approximately 3.5 and 4.5kbps. Fig. 2C shows the map of the resultant Ad Ad-o-elafin. The predicted size of the portion of DNA after Hind III digestion which contains the ovine elafin cDNA is 3617bps. This band is likely to correspond to the band seen at approximately 3.5kbps on the Southern blot (fig. 2) and the Hind III digested DNA post re-plaquing (fig. 3). The source of the band seen at approximately 4.5kbps is likely to be due to partial digestion of the Hind III site at the extreme 5' end of the ovine elafin cDNA (see chapter 3, fig. 10 base pair 607). The partial digestion at this site would lead to a Hind III product of 4645bps (fig. 2).

Western blot analysis demonstrated that only the supernatants from A549 cells 'infected' with the lysate from the plaques which were positive for the band at approximately 3.5kbps by Hind III

digestion (plaques 4 to 6) showed production of secreted ovine elafin-FLAG (fig. 4). A 40kDa secreted protein was detectable by Trab-2O (detecting the repeat PVKGQD motifs) and the anti-FLAG monoclonal antibody. This, taken together the Western blot analysis, restriction analysis and Southern blot analysis, suggested that plaques 4, 5 and 6 contained adenovirus which could successfully infect A549 cells leading to the correct transcription, translation and processing of ovine elafin-FLAG.

Large amounts of adenovirus were produced by further infection of 293N3S cells in a spinner culture system using a cell lysate from the positive plaque. The infected cells in the spinner culture were then subject to further purification involving ultra-centrifugation in a caesium chloride gradient to allow the isolation of a concentrated solution of adenoviral particles. This solution was then de-salted in an ion exchange column before the final elution of 'banded' Ad for use in subsequent studies. This final eluent was aliquoted to avoid freeze-thawing virus stocks, and stored at -70°C. All the stages in the purification of Ad subsequent to the identification of a positive plaque were performed in the Rayne Laboratory Adenovirus Facility by Alison Harris using techniques developed in McMaster University, Ontario (Graham and Prevec, 1995).

The final outcome of the bulking up of the Ad constructed within this chapter was a virus stock of $1.26x10^{11}$ plaque forming units (pfu) per ml ($1.25x10^{13}$ particles per ml) of Ad-o-elafin for use in the latter part of this chapter looking at the adenoviral infection of respiratory epithelium and alveolar macrophages both *in vitro* and *in vivo*.

6.4.2. The Investigation of methods to up-regulate adenoviral infection efficiency *in vitro* and *in vivo*.

The first experiments focused on investigating the possibility of up-regulation of transgene expression after a defined, and low, number of adenoviral particles were used to infect epithelial cells in culture. To this end, we used both an Ad expressing GFP (Ad-GFP) and also the potentially therapeutic Ad (as described earlier in this chapter) coding for ovine elafin (Ad-o-elafin). The main goal of the experimental work presented within this part of this chapter was to develop a protocol for the efficient infection of the ovine lung with an adenovirus encoding ovine elafin (Ad-o-elafin) in order that subsequent work could investigate the potential benefits elafin may have (as discussed in chapter 5) in the inflamed lung (see chapter 7).

Efforts were directed at maximising the protein output from recombinant Ad infected cells *in vitro*. It is pertinent to review the scientific literature which prompted this work.

Adenovirus particles normally enter cells in culture by the interaction of the Ad fiber with cellular receptors (Bergelson at al., 1997; Mayr at al., 1997) and the binding of the penton base with α-β integrins (Wickham et al., 1993). Further work by Walters et al. (1999) showed that the adenoviral receptors CAR and MHC class I were located baso-laterally in the differentiated human airway epithelial cultures used in their work. Both the prevalence of these receptors, and also their accessibility, are crucial to the efficacy of this process. Indeed, Zabner et al. (1997) showed that ciliated airway epithelium was resistant to Ad infection (unless a long incubation period was used – Zabner et al., 1996) due to the lack of such receptors on the apical surface of differentiated respiratory epithelium. Fasbender et al. (1997) and Arcasoy et al. (1997) both used complexes of Ad and cationic compounds to try to circumvent this receptor-based viral entry pathway with good success. Both groups found that complexing the Ad with either poly-L-lysine (PLL) or cationic lipids increased the efficiency of Ad infection due to the cationic compound forming non-covalent bonds with the virus and these positively charged moieties interacting with the negatively charged cell surface membrane. However, the use of cationic lipids can cause significant cyto-toxicity *in vitro* (Fasbender et al., 1995; Cortesi et al., 1996) and also can cause inflammation *in vivo* as shown by Scheule et al. (1997). Fasbender et al. (1998) postulated that the incorporation of Ad with a cationic lipid may result in an

exaggerated immune response to the virus with the lipid acting as an adjuvant. Hunter et al. (1981) showed an adjuvant activity in surface active non-ionic surfactants potentially due to their lipophilic nature allowing close contact between surfactant coated antigens and the hydrophobic cell wall. In contrast, however, subsequent work has shown that coating of Ad particles shields them from the host immune system. Chillon et al. (1998) demonstrated that coating of Ad with polyethylene glycol and lipid necessitates a 50x increase in amount of neutralizing serum in order to abrogate infection. In another example, Dodds et al. (1999) showed a similar phenomenon when infecting mouse myotubules with Ad-lacZ complexed with polycations.

Ad access to the specific cellular receptors (CAR and MHC class I) is of fundamental importance in allowing primary attachment of the virus to the cell surface before integrin modulated internalisation can begin. Specifically the number and localisation of these receptors are important predictors of the 'sensitivity' of a tissue or epithelial layer to adenoviral infection. In the respiratory tract, with the Ad-specific receptors found baso-laterally on the columnar cells making up the airway wall, virus access to the receptors is limited, hence there is a low level of infectability (Zabner et al., 1997). One way that has been used to increase access to these receptors is to simply damage the intercellular attachments, the 'junctional complex' made up of tight junctions, adherens junctions and desmosomes. This has been investigated primarily by the use of chelators of bivalent cations exploiting the fact that all 3 components of the junctional complex depend on Ca^{2+} for their correct functioning. Specifically, the Ca^{2+} chelator ethylene-glycol-bis-(beta-aminoethyl ether)-*N,N,N',N'*-tetra-acetic acid (EGTA) has been the focus of much work both *in vitro* and *in vivo.* Wang et al. (2000) demonstrated that EGTA in a hypotonic buffer caused an effective and rapid disruption of the epithelial junction complex in human differentiated epithelial cells in culture allowing viral access to the intercellular spaces. This group also showed that *in vivo* the same formulation could markedly increase the magnitude of transgene-driven protein production in the rabbit trachea. A similar approach albeit this time using an isotonic formulation of EGTA was used by Chu et al. (2001) where up to a 50 fold increase in transgene derived protein was seen both *in vitro* and *in vivo* (murine trachea using both standard instillation and aerosolisation). Of note is the more recent use of sodium caprate (another epithelial junction disruptor) both *in vitro* and *in vivo* where this compound's efficacy in up-

regulating adenoviral infection was associated with fewer side effects (Gregory et al., 2003; Johnson et al., 2003). Of significant importance to projected *in vivo* use is the time required for the chelator to be in contact with the target tissue prior to vector administration. For example, Wang et al. (2000) instilled hypotonic EGTA solution into rabbit tracheas for 45 minutes before removing this solution and applying the virus vector. In contrast, cationic substances can be instilled as a complex with Ad i.e. no pre-treatment of the tissue is required.

Prior literature indicated the potential of using inorganic salt complexes *in vivo* in the same way that calcium phosphate had long been used to increase the transfection efficiency of plasmid DNA into cells in culture (Chen et al., 1987 and 1988). Fasbender et al. (1998) were the first to show that the incorporation of Ad into a calcium phosphate precipitate markedly enhanced the efficiency of infection into cells that were low in fiber receptors. In addition, however, cells with sufficient fiber receptors were also infected more efficiently with the use of the calcium phosphate co-precipitate technique. This methodology was therefore permitting infection of the cells by a non-fiber/fiber receptor mediated route. Furthermore the success of this method indicated that α-β integrins were not necessary due to their paucity on the apical surface of airway epithelial cells (Zabner et al., 1997; Goldman and Wilson, 1995). Fasbender et al. (1998) also investigated the *in vivo* infection of the murine lung and found a similar up-regulation of transgene expression. Interestingly the increase in efficiency seemed to be localized to the murine airway with no real change in infection efficiency in the parenchyma. Subsequent work (Walters and Welsh, 1999) has shed light on the mechanism by which calcium phosphate co-precipitate enhances gene delivery and has demonstrated that this mechanism is independent of fiber receptors and integrins.

The above work concentrated on the modification of the interaction between Ad and host cell either by facilitating access to viral receptors or allowing viral infection to bypass the usual mechanisms of cell entry. Once the virus has entered the cell the protein output of that cell depends primarily on the activity of the transgene within the recombinant virus. This, in turn, relies on the activity of the promoter which in the case of the Ad constructed in this chapter contains a promoter derived from the murine cytomegalovirus (MCMV). This promoter was used after previous work showed a higher

transgene activity from constructs using this promoter when used in human alveolar epithelial cells (A549 cells as used here) and human and rat primary fibroblasts (Sallenave et al., 1998) when compared to the human cytomegalovirus (HCMV) promoter and the Ad major late promoter. Similarly to the HCMV promoter, the MCMV promoter also contains regulatory elements sensitive to NF-κB. This leads to the promoter being sensitive to LPS stimulation via the activation of NF-κB (Löser et al., 1998; Sallenave et al., 1998; Simpson et al., 2001B), hence the possibility of up-regulating transgene expression by exogenous LPS. Löser et al. (1998) showed the re-activation by LPS of HCMV promoter driven transgene expression in murine hepatocytes, which was dependent on NF-κB activation. In addition, the same group showed activation of the HCMV promoter in HCMV-lacZ transgenic mice after the administration of a recombinant Ad, hence the Ad itself up-regulates factors which have a positive feedback on the expression of transgene contained in that virus. Simpson et al. (2001B) showed marked up-regulation of MCMV driven transgene production after *in vivo* administration of recombinant Ad and bacterial LPS. These data indicate the usefulness of a highly active promoter in a recombinant Ad which is up-regulated by the very inflammation (here exemplified by LPS) which the Ad induces. Because of this a low dose of virus can be used with the addition of LPS to up-regulate transgene expression.

This background inspired the investigation of these techniques to up-regulate the transgene activity for a given, low dosage of administered recombinant adenovirus. The data indicated that the incorporation of Ad into a calcium phosphate precipitate was highly effective in increasing the infection efficiency of the virus. Further, the data indicated that the 20 minute incubation time was optimal.

With the physiological goal of achieving efficient transgene expression *in vivo*, a delivery and assay system modeled on endo-bronchial administration (Emerson et al., 2003) of Ad was selected. With such a system the target cell populations consist of those cells lining the bronchioles, alveolar ducts and sacs, and the mobile cell population therein. This last population is made up in the quiescent ovine lung almost entirely of alveolar macrophages (Collie et al., 1999). As alveolar macrophages are easily sampled by bronchoalveolar lavage and play a pivotal role in the initiation and progression of the innate immune response they were considered suitable candidates to follow the progression of events

ex vivo following exposure to Ad. Alveolar macrophages are believed to be relatively resistant to Ad infection due primarily to a low level of expression of the CAR receptor (Kaner et al., 1999). This group, however, showed that human alveolar macrophages (AMs) could be infected by recombinant Ad at high MOIs by a fiber dependent (and hence CAR independent) mechanism. Conron et al. (2001) showed that alveolar macrophages from human sarcoid sufferers were permissive for Ad infection and that this was due to an increase in surface expression of both CAR and the internalisation integrins $\alpha v\beta_3$ and $\alpha v\beta_5$. The change in the surface expression of the receptors and integrins was attributed to the alveolar macrophages being in contact with an inflamed milieu. It was hypothesised that the use of Ad in conjunction with calcium phosphate may potentially bypass this 'requirement' for inflammation by allowing infection of the alveolar macrophages by the endocytosis based pathway previously referred to.

Initial experiments with Ad-GFP demonstrated the beneficial effect of co-precipitation on the infection efficiency and further experiments with Ad-o-elafin confirmed those benefits in relation to elafin secretion.

The issue of calcium phosphate induced cyto-toxicity was addressed for both alveolar macrophages and an alveolar epithelial cell line (A549 cells). A significant and marked increase in cell death due to the use of Ad+CaP relative to Ad alone was apparent in A549 cells (as measured by trypan blue exclusion) (fig. 9) and a similar situation was seen in alveolar macrophages (with cytotoxicity assayed by propidium iodide staining) (fig. 11). Although different methods render accurate comparisons impossible some information can be gained from this work. Data from the scientific literature examining calcium phosphate mediated cell death is limited currently but it can be fairly assumed that at least some of the cytotoxicity observed here is due to an increase in the viral burden seen by the cells infected due to the fact that the co-precipitation with calcium phosphate is increasing the infection efficiency of the cells. Additionally, some workers have reported a cytotoxic effect attributed directly to GFP itself (Liu et al., 1999). Obviously the level of cytotoxicity seen in these experiments (medians of 40% and 50% for A549s and alveolar macrophages respectively) cannot be described as negligible in itself. However, this experiment was designed to investigate primarily the effect of calcium phosphate co-precipitation on the infection efficiency of Ad-GFP and as such has showed that there is a great benefit over Ad-GFP alone with respect to the number of cells infected and to the GFP

contained within each cell. Additionally the alveolar macrophages infected by such a protocol using Ad-o-elafin secrete much increased amounts of ovine elafin into the culture supernatant compared to Ad-o-elafin alone (fig. 10C). It is this beneficial effect on transgene mediated protein production that proved persuasive and prompted the decision to use adenovirus co-precipitated with calcium phosphate for the subsequent *in vivo* work detailed below.

6.4.3. Preliminary investigation of the instillation of recombinant adenoviruses segmentally to the ovine lung.

With the benefits of calcium phosphate co-precipitation established *in vitro* using A549 cells and *ex vivo* using primary cultures of alveolar macrophages, the foundation for progressing towards *in vivo* studies was established. Initial experiments examined the expression of Ad-GFP as a 'reporter construct' in the lung. As described, one sheep was used to look at the local effects of 3 different dosages of instilled Ad-GFP ($1x10^7$, $5x10^7$ and $1x10^8$ pfu) with or without pre-incubation with calcium phosphate to form a co-precipitate. These dosages of Ad are referred to as low, medium and high respectively. However, it must be stressed that these terms are meant only as comparisons to each other. In other words, a dosage of $1x10^8$ pfu of adenovirus is not very high when used in an *in vivo* context. Dosages of adenovirus used in animal models of lung-directed gene therapy vary from $3x10^7$ pfu (Simpson et al., 2001A and B – murine model) to $4.6x10^{10}$ pfu (Weiss et al., 2002 – macaque model) and although in the experiments described here the total pfu instilled per animal was $1.6x10^8$ there was little evidence locally in the BALF of any major inflammatory response to the vector at 48 hours post-instillation. In the highest dose of Ad with the calcium phosphate co-precipitate the percentage of neutrophils increased from zero to approximately 9% neutrophils at 48 hours. Although the practical relevance of an inflammatory response of this magnitude is difficult to predict it should be considered in the context of inflammatory responses to other non-viral vectors in sheep where neutrophilic inflammation predominates (Emerson et al., 2003). Indeed the lack of change in the protein content of the BALF (fig. 13) is indicative of an intact alveolar epithelial barrier.

The information collected from this preliminary experiment informed the design of a further progression whereby the dose-response relationship of Ad-o-elafin precipitated with calcium phosphate was assessed in the segmental model. Here the only significant difference between any of the cell counts was noted when the neutrophil counts in the high dose (10^8 pfu) segment were compared with those in the naïve segment. However, this difference was not seen when neutrophil counts in the high dose (10^8 pfu) segment were compared to the pre-experimental values in this segment. The absolute neutrophil counts for this experiment are shown in Appendix 1 and they serve to indicate that values for one animal were unexpectedly high in the pre-experimental analysis. It is likely that this data is the source of this anomaly.

It is clear that the significant, but mild, inflammatory response that develops 48 hours following instillation of 10^8 pfu (high dose) of Ad co-precipitated with calcium phosphate does not occur when 10^7 or $5x10^7$ pfu (low and medium doses) are instilled.

6.5. CONCLUSIONS

This work prompted the selection of a dosage of $1x10^8$ pfu of Ad co-precipitated with calcium phosphate, as an appropriate dose for future *in vivo* instillations. This dosage causes a small amount of inflammation in the segment as assessed by BALF neutrophil counts and total protein but of equal interest is the fact that there is no evidence that the calcium phosphate alone has any pro-inflammatory effect itself on the lung. Other workers using Ad/calcium phosphate co-precipitates in the lung have reported minimal 'toxicity' after intra-pulmonary deposition (Fasbender et al., 1998). Further, Fasbender states the potential usefulness of Ad co-precipitated with calcium phosphate, in murine models, in that the Ad now 'targets' the airways rather than the parenchyma (Fasbender et al., 1998). This is seen to be an advantage in the pursuit of therapeutic strategies for airway related diseases such as cystic fibrosis. The data presented here does not support this 'targeting' in the ovine lung - relatively few airway cells were infected, but multiple sections demonstrated infection of alveolar epithelial cells and macrophages (fig. 14). This is a potential major difference between the two models (murine and ovine) in looking at adenoviral gene therapy in the lung and potentially reflects a difference in the physical access of instilled vector to the alveolar structures between the two species. Equally, comparisons between the results seen by Fasbender at al. and those seen in this chapter must be approached with caution as the promoter in the adenoviral vectors used in the two pieces of work are different. In the murine model (Fasbender et al., 1998) the human cytomegalovirus promoter (HCMV) was used whereas in this work the murine cytomegalovirus (MCMV) promoter was used due to the latter showing higher activity in the murine lung (Sallenave et al., 1998).

The potential use of adenoviral gene transfer to the ovine lung in the modulation of inflammation is investigated in the next chapter in addition to studies examining the effects of repeated instillations of recombinant adenovirus.

CHAPTER 7

THE *IN VIVO* EFFECTS OF REPEATED INSTILLATION OF RECOMBINANT ADENOVIRUS AND CALCIUM PHOSPHATE CO-PRECIPITATES AND THE MODULATION OF THE LUNG'S RESPONSE TO LPS

7.1. INTRODUCTION

Chapter 6 detailed the construction of a replication-deficient adenovirus coding for ovine elafin (Ad-o-elafin) and its optimisation both *in vitro* and *in vivo*.

There exists good background data to suggest the therapeutic potential for manipulating the lung's innate immune response through the up-regulation of ovine elafin (Simpson et al., 2001A and B). To investigate, and expand upon this, experiments were performed to examine the effect that Ad-o-elafin/CaP delivery would have on the lung's response to endo-bronchial instillation of lipopolysaccharide. The ovine model used allows the further investigation not only of the LPS response in the segment treated with Ad-o-elafin but also in a distant non-Ad treated segment. Additionally, the circulating response to LPS can be examined in this model.

Below is a review of the current scientific knowledge detailing the response of the lung to bacterial lipopolysaccharide.

The recognition and effects of LPS in the lung.

This thesis examines the modulation of the lung's response to lipopolysaccharide (LPS) derived from *Mannheimia haemolytica*. *Mannheimia haemolytica* (previously known as *Pasteurella haemolytica*) is a weakly haemolytic, gram-negative coccobacillus that is an opportunistic pathogen of cattle, sheep and other ruminants. Pneumonic pasteurellosis is a common disease of sheep, where outbreaks are associated with management practices such as overcrowding and transport. The ovine lung demonstrates a characteristic cellular inflammatory response to LPS derived from this pathogen (Brogden et al., 1984). The response which is neutrophil-dominated conforms to that seen in other species, including man in response to LPS (Michel et al., 1997; Nightingale et al., 1998).

LPS itself is the archetypal Gram negative bacterial 'alarm signal' and comprehensive literature surrounds the recognition and effects of LPS in the lung. LPS interacts with specific pattern-recognition receptors leading to a tightly regulated sequence of events. LPS itself is made up of a lipid A core attached to a chain of oligosaccharides which gives the LPS its antigenic properties and also varies between bacterial species. LPS naturally forms micelles which do not favour interaction with the cell surface. However, these micelles are disrupted by the presence of LPS-binding protein (LBP)

which binds to LPS molecules with a 1:1 stoichiometry and transfers the LPS to cell surfaces and to high density lipoproteins (HDL). LPS bound to HDL is inactive and this appears to be one mechanism by which LPS is rendered non-toxic *in vivo* (Wurfel et al., 1994; Ulevitch et al., 1981; Baumberger et al., 1991). The receptor for LPS/LBP was originally identified as CD14 (Wright et al., 1990). CD14, which is membrane bound on macrophages, also exists as a soluble form in the plasma and interaction of LPS/LBP with soluble CD14 allows activation of some CD14 negative cells (Pugin et al., 1993). The LPS-induced stimulation of CD14 positive cells is not a direct activity of the CD14 itself and in man the Toll-like receptor family of proteins (TLRs) play an essential role in this regard. More than 10 members of this family have been characterised now conferring the ability to respond to a whole range of bacterial products. The member of importance with respect to LPS-stimulation is TLR-4 (Lien et al., 2000). TLR-4 was originally thought to be present only on myeloid cells (Muzio et al., 2000) but has since been shown to be present intra-cellularly in intestinal epithelial cells (Hornef et al., 2002) and in human alveolar epithelial cells and bronchial epithelial cells (Guillot et al., 2004).

In the lung, LPS first encounters the epithelial lining fluid which lines the air-spaces. This is a lipid rich fluid containing surfactant proteins as well as soluble CD14 and LBP. The two main constituents in BALF that can interact with LPS are surfactant protein A (SP-A) and LBP. In the absence of inflammation, the concentration of SP-A is far in excess of that of LBP (250-500μg/ml compared to 50-100ng/ml respectively – Greene et al., 1999; Martin et al., 1997). SP-A, which is produced by type II alveolar epithelial cells and non-ciliated bronchial cells (McCormack and Whitsett, 2002), may serve a purpose in binding LPS and rendering it less active – a hypothesis supported by *in vitro* evidence (Kalina et al., 1995). In the inflamed lung, however, the ratio of SP-A to LBP is reversed with the SP-A decreasing to approximately 25μg/ml and the LB-P rising to approximately 5-10μg/ml (Gregory et al., 1991; Greene et al., 1999). The potential for LPS to interact with LBP is increased with anticipated effects on macrophage activation via CD14. Originally LBP was thought to be solely produced in the liver however several lines of evidence point to the lungs as an additional source of this protein. LBP RNA is increased in rat lungs undergoing acute inflammation and this increase in gene expression is associated with increased levels of this protein in BALF (Su et al., 1994). Human LBP has been shown to be produced at the level of RNA and protein by A549 cells, a cell line derived

from a pulmonary adenocarcinoma (Dentener et al., 2000). This group also showed production of murine LBP by a murine alveolar epithelial cell line and also that human LBP RNA was found in primary isolates of human type II epithelial cells. Crucially, the A549 production of LBP was shown to be up-regulated by IL-1β, IL-6 and TNF, these cytokines being part of the acute inflammatory process in the lung.

The major source of the soluble CD14 seen in BALF is likely to be the circulation itself and the increase seen in inflammatory conditions in the lung probably relates to a change in the permeability of the lung vasculature and alveolar epithelium. Alveolar macrophages release soluble CD14 *in vitro* (Hasday et al., 1997) and extra-myeloid sources of CD14 have also been shown *in vivo* (Fearns et al., 1995; Fearns and Ulevitch 1998).

The interaction between LPS and other proteins already present in the lung lining fluid likely depends upon the relative concentrations of LBP and SP-A which themselves depend on the inflammatory status of the lung. As previously noted, IL-1β and TNF also promote the up-regulation of the anti-proteinase elafin which acts as an 'alarm antiprotease' in the lung (Sallenave, 2000). The intriguing possibility of elafin acting as a 'surrogate' LBP-type molecule has arisen from prior work in this laboratory (J. McMichael et al., submitted manuscript) in which the incubation of synthetic elafin peptides with bacterial LPS up-regulates the production of TNF by murine macrophages. The balance between activation of target cells by LPS and the detoxifying effects of other BALF components is complicated by the distinction between the epithelial cells' response to LPS compared to that of the alveolar macrophages. Hamann et al. (2002) have shown that moderately increasing the concentration of LBP in culture conditions stimulates rat alveolar macrophages to increase LPS uptake and show a greater activation in response to the LPS. In contrast, highly elevated concentrations of LBP in the same situation inhibit both the response and the LPS uptake by the alveolar macrophages. The same group also looked at an A549 alveolar epithelial cell line under the same conditions. Interestingly, the very high LBP concentrations enhance LPS uptake into these cells but without any signs of activation. A549 cells themselves have very low levels of TLR-4 expression (Monick et al., 2003) which may have a bearing on their response to LPS and LBP.

However, dissecting alveolar macrophage and epithelial cell responses *in vitro* is of questionable relevance to the *in vivo* situation where these cells are in very close contact. Indeed, recent work has shown that the epithelial cells may have a 'memory' of LPS activation and may activate the alveolar macrophages in contact with them via epithelial surface ICAM-1 expression (Lee et al., 2004). The authors postulate that this inter-dependence may be one reason why strategies aimed at neutralising LPS have led to disappointing results in the clinic.

The modulation of the lung's response to LPS by the localised up-regulation of the anti-protease ovine elafin prompted the experimental work presented within this chapter.

7.2. AIMS

1. The data presented in this chapter concerns an analysis of the effects of repeated administration of adenoviruses (either Ad-o-elafin or Ad-GFP) on both transgene activity and local and systemic responses to administration.

2. Additionally, the potential role of ovine elafin as a 'defensin-like' molecule (see chapter 5) is explored in relation to the innate immune response to bacterial lipopolysaccharide.

7.3. RESULTS

7.3.1. The effects of repeated administration of Adenovirus/calcium phosphate co-precipitates on bronchoalveolar lavage cytology.

A flow diagram outlining the experimental protocols employed is shown in fig. 1 and a more detailed representation is shown in fig. 2. Bronchoalveolar lavage fluid (BALF) was collected from 8 animals pre-experimentally (d-7) and 3 days (d3), 7 days (d7) and 10 days (d10) after the administration of 10^8 pfu of either Ad-GFP (n=4) or Ad-o-elafin (n=4) (CaP co-precipitated) to each of three selected lung segments. A second dose of the same adenovirus was administered to those segments on day 14 and again BALF collected after a further 3, 7 and 10 days (left hand branch fig. 1). In a further eight animals, the initial process of delivery and assessment was as described (n=5 for Ad-o-elafin and n=3 for Ad-GFP), however 10 days after the initial administration of Ad, LPS was delivered to both a naïve segment and a segment pre-treated with Ad and the innate immune response assessed in those segments and a further naïve segment at day 14 (right hand branch fig. 1).

The total cell counts per ml BALF in naïve segments are shown in fig. 3A. There are no significant differences in total cell count per ml BALF for the Ad-o-elafin group of animals. However, in the Ad-GFP group there is a significant increase in total cell count per ml BALF at both day 21 and day 24 compared to pre-experimental cell counts ($P<0.05$). There are no significant differences between the groups treated with the different viruses at any time point.
When the absolute cell counts are converted to percentage of pre-experimental values (fig. 3B) there are no significant differences between the two Ad groups.

Fig. 4A shows the total cell counts per ml BALF for the Ad treated segments. The Ad-o-elafin group show a significant increase in total cells per ml BALF at days 17, 21 and 24 compared to pre-experimental values ($P<0.005$, $P<0.005$ and $P<0.05$ respectively). The Ad-GFP group show a

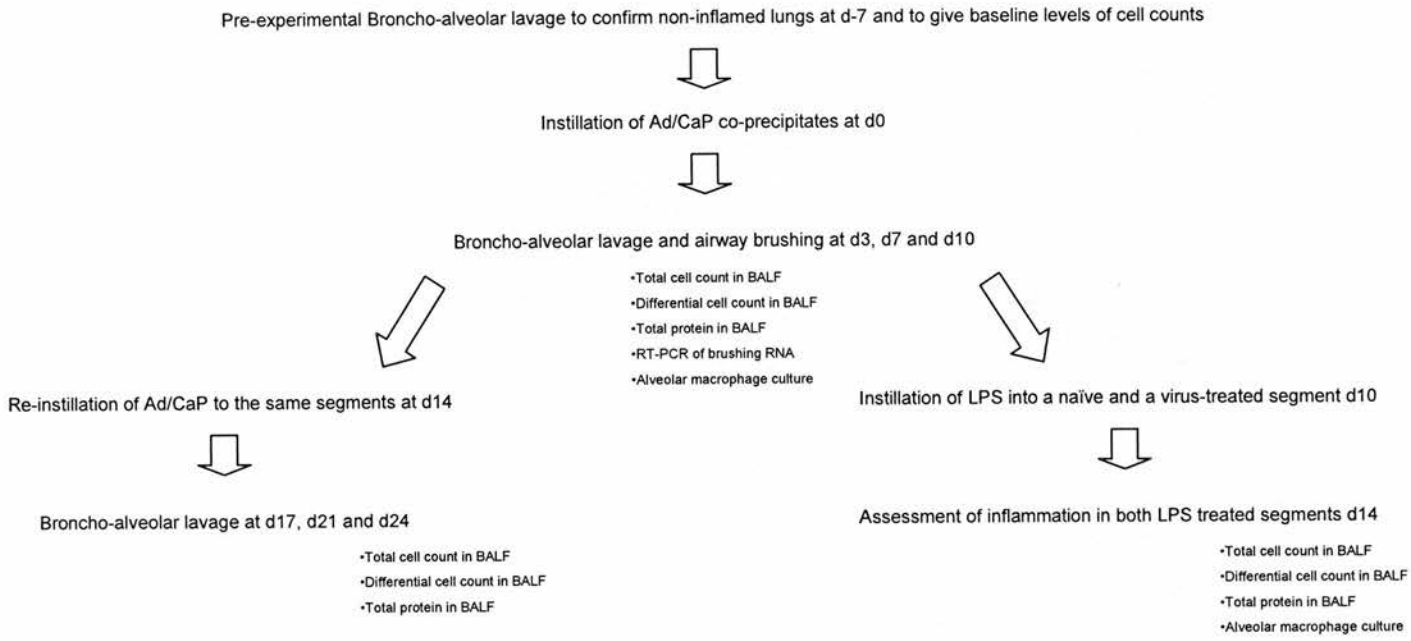

Fig. 1. Flow diagram of the administration of adenovirus/CaP co-precipitates to the ovine lung and the modulation of the response to locally administered LPS.

The diagram shows the time-course of the administration of either Ad-o-elafin or Ad-GFP to the ovine lung and subsequent sampling by broncho-alveolar lavage and airway brushing. The left hand branch of the diagram (A) represents the repeated administration of the viruses over a period of 24 days. The right hand branch (B) shows a single administration of each virus and the modulation of the response to LPS after 10 days.

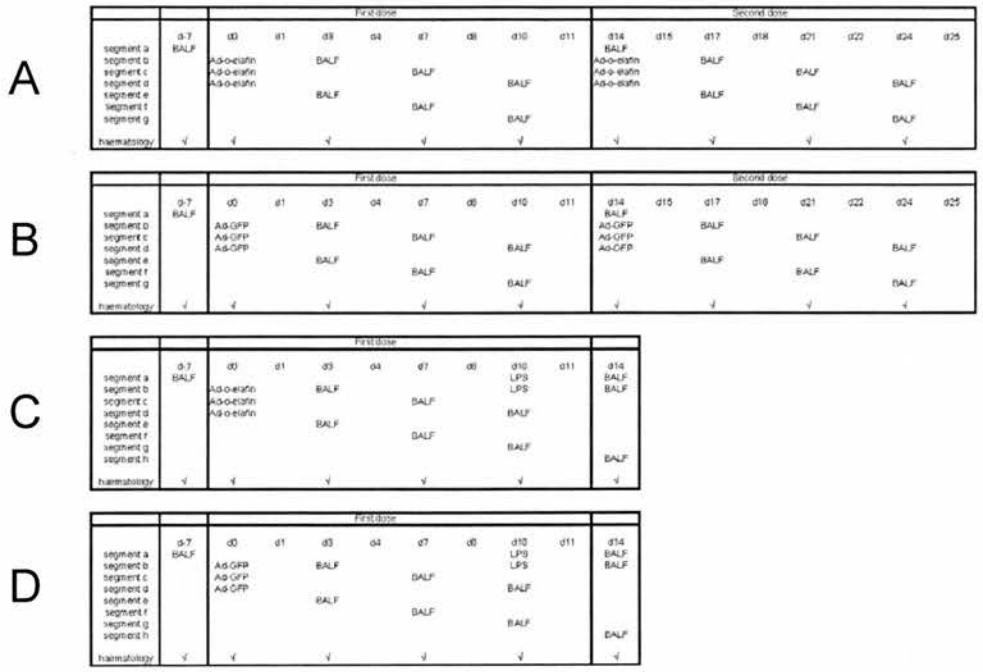

Fig. 2. Protocol for the repeated administration of adenovirus and also the modulation of the LPS response in the ovine lung.

The diagram shows the times of the administration of either Ad-o-elafin or Ad-GFP to the ovine lung and subsequent sampling by broncho-alveolar lavage and airway brushing. A represents the protocol for animals 188-192; B the protocol for animals 193-196; C the protocol for animals 453, 468, 809, 897 and 909; D the protocol for animals 472, 478, and 920.
(In protocols C and D, examining the effects of Ad-o-elafin and Ad-GFP on the response to bacterial LPS within the lung and also systemically, segment a represents Ad-/LPS+ segments, segment b represents Ad+/LPS+ segments and segment h represents Ad-/LPS- segments as discussed in section 7.3.5).

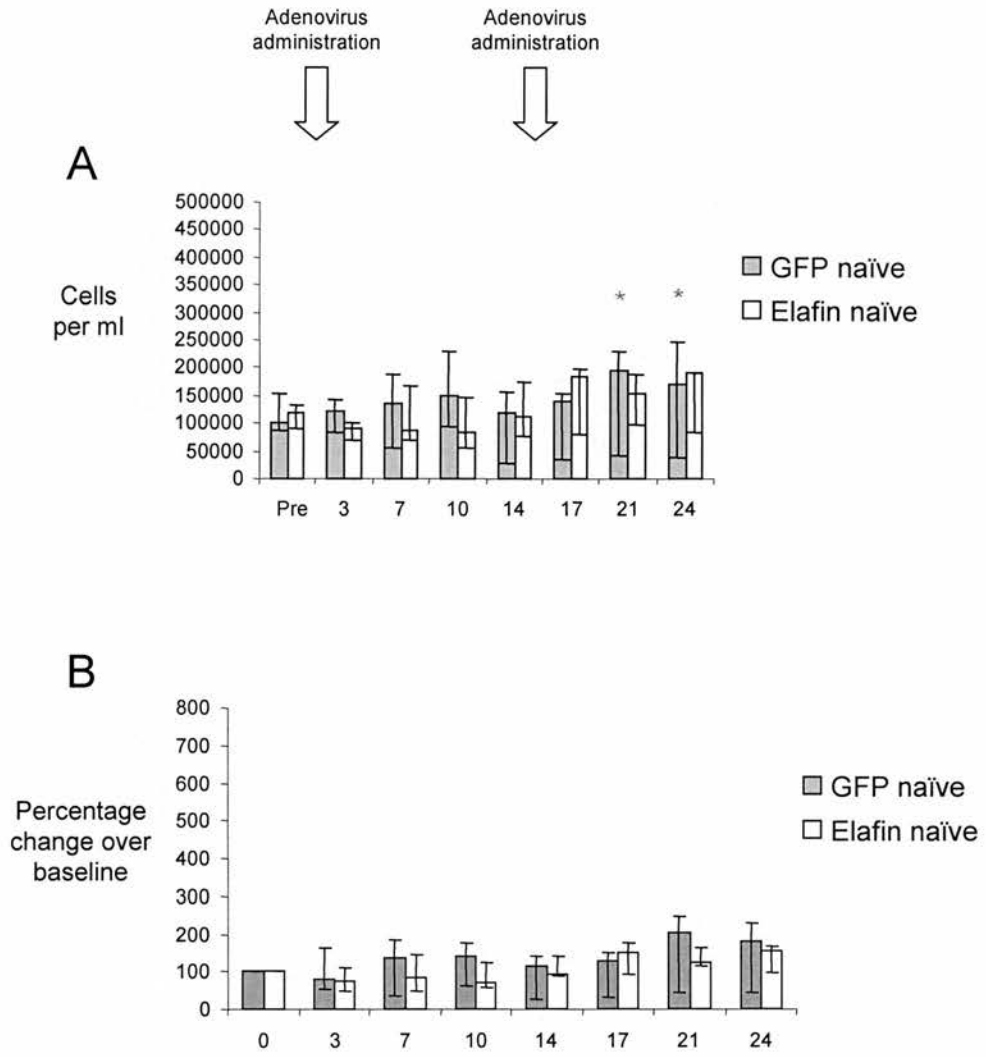

Fig. 3. The effect of repeated adenovirus administration on the total cell count per ml of BALF in naïve segments.

A. Graph showing the total cell count in the BALF of naïve segments (i.e. segments which did not receive recombinant adenovirus) pre-experimentally and also after adenovirus administration at day 0 and day 14 in separate treated segments (either Ad-o-elafin or Ad-GFP).

B. Graph showing the above data presented as percent change in total cell count compared to pre-experimental values.

All data is shown as median and inter-quartile range. Statistical significance calculated by Mann-Whitney analysis.* represents $P<0.05$ comparing values at the points indicated with the pre-experimental values.

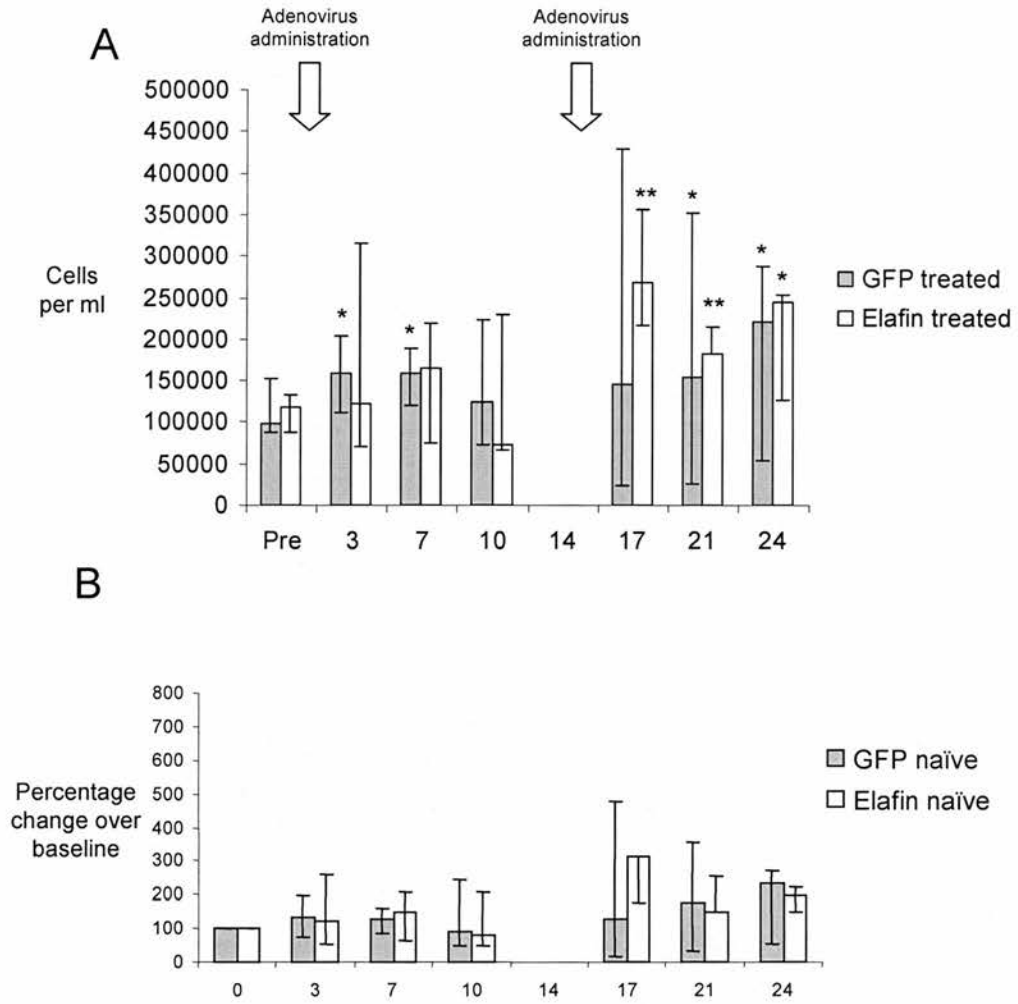

Fig. 4. The effect of repeated adenovirus administration on the total cell count per ml of BALF in treated segments.

A. Graph showing the total cell count in the BALF pre-experimentally and also after adenovirus administration at day 0 and day 14 in treated segments (either Ad-o-elafin or Ad-GFP).

B. Graph showing the above data presented as percent change in total cell count compared to pre-experimental values.

All data is shown as median and inter-quartile range. Statistical significance calculated by Mann-Whitney analysis.* and ** represent $P<0.05$ and $P<0.005$ respectively when comparing values at the points indicated with the pre-experimental values.

significant increase in total cells per ml BALF at 3, 7, 21 and 24 days compared to pre-experimental values ($P<0.05$). There are no significant differences between the 2 groups at any time point.
Again when the counts are converted to percentage of the pre-experimental values, there are no significant differences between the groups (fig. 4B).

Fig. 5A shows the absolute neutrophil counts per ml BALF n the naïve segments at each time point. As shown, there are no significant differences at any time point when naïve segment counts are compared to pre-experimental values. When the absolute numbers are converted to percentage of pre-experimental values (Fig. 5B), the only significant difference when comparing the two adenovirus groups is at day 3 with the elafin group showing a higher percentage change compared to the GFP group ($P<0.05$).

In fig. 6A the absolute neutrophil counts per ml BALF are compared with pre-experimental values in the virus treated segments. Here there are significant increases in BALF neutrophils per ml at day 3 and 17 for Ad-o-elafin ($P<0.05$) and at day 17 alone for Ad-GFP ($P<0.05$). There are no significant differences between the two adenovirus groups at any time point. When the numbers are converted to percentage of pre-experimental values (fig. 6B) there are no significant differences between the two adenovirus groups at any time point.

Fig. 7A shows the absolute lymphocyte counts per ml BALF in the naïve segments for each time point. There are significantly fewer lymphocytes per ml BALF in the elafin treated animals at day 3 and 24 compared with pre-experimental values ($P<0.005$) and significantly fewer than the GFP treated animals at day 24 ($P<0.05$). When the values are converted to percentages of pre-experimental values there are significant differences between the two Ad treated groups at days 3, 17 and 24 with the elafin treated group showing a lower percentage change compared to the GFP treated group at days 3 and 24 and a higher percentage change at day 17 ($P<0.05$) (fig. 7B).

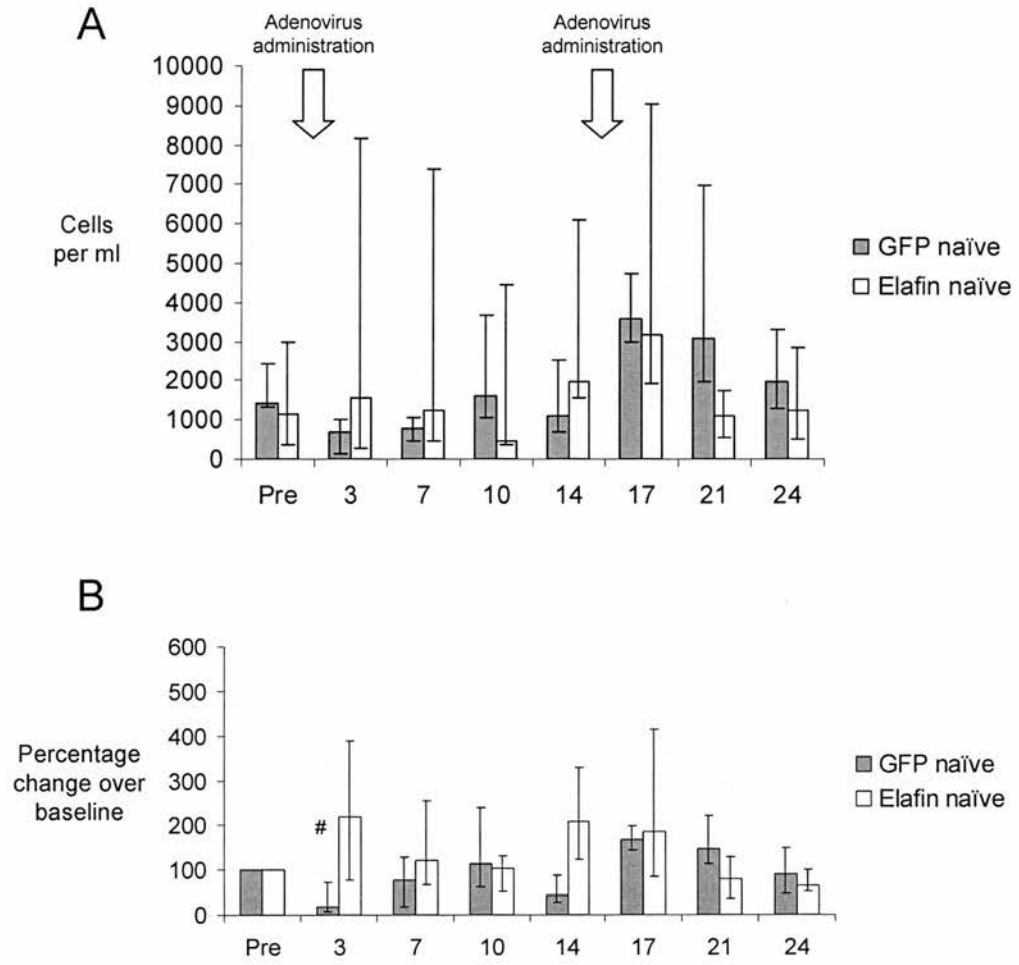

Fig. 5. The effect of repeated adenovirus administration on the PMN count per ml of BALF in naïve segments.

A. Graph showing the PMN count in the BALF of naïve segments pre-experimentally and also after adenovirus administration at day 0 and day 14 in separate treated segments (either Ad-o-elafin or Ad-GFP).

B. Graph showing the above data presented as percent change in PMN count compared to pre-experimental values.

All data is shown as median and inter-quartile range. Statistical significance calculated by Mann-Whitney analysis. # indicates $P<0.05$ when the 2 virus treatments are compared at the time point indicated.

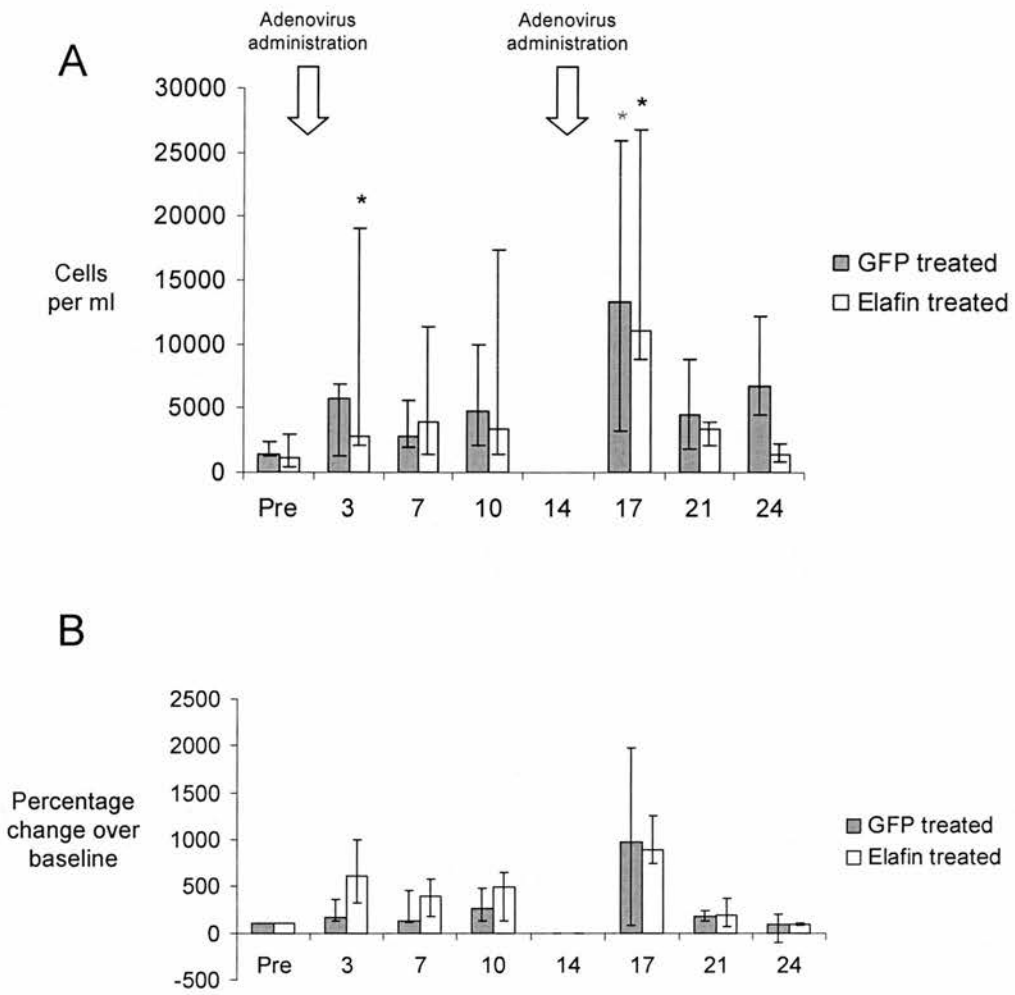

Fig. 6. The effect of repeated adenovirus administration on the PMN count per ml of BALF in treated segments.

A. Graph showing the PMN count in the BALF of treated segments pre-experimentally and also after adenovirus administration at day 0 and day 14 (either Ad-o-elafin or Ad-GFP).

B. Graph showing the above data presented as percent change in PMN count compared to pre-experimental values.

All data is shown as median and inter-quartile range. Statistical significance calculated by Mann-Whitney analysis.* represents $P<0.05$ comparing values at the points indicated with the pre-experimental values.

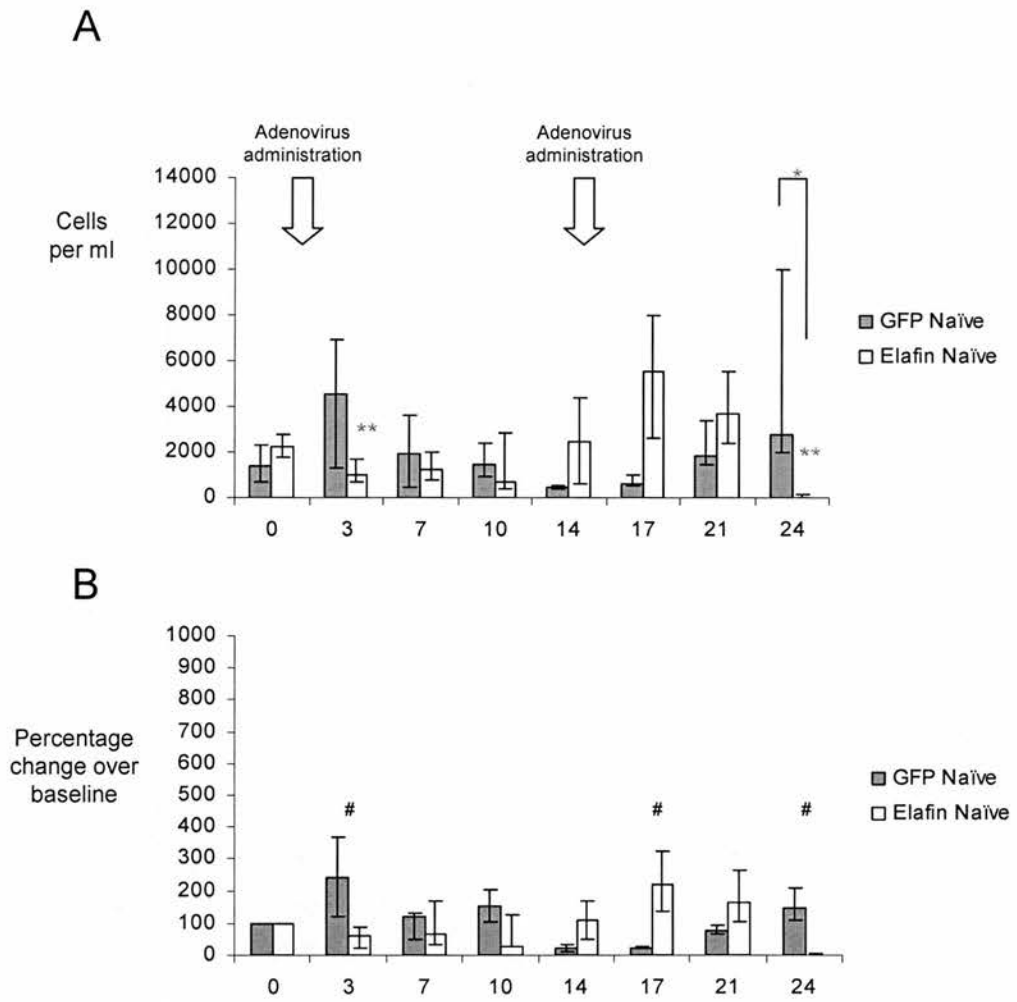

Fig. 7. The effect of repeated adenovirus administration on the Lymphocyte count per ml of BALF in naïve segments.

A. Graph showing the lymphocyte count in the BALF of naïve segments pre-experimentally and also after adenovirus administration at day 0 and day 14 in separate treated segments (either Ad-o-elafin or Ad-GFP).

B. Graph showing the above data presented as percent change in lymphocyte count compared to pre-experimental values.

All data is shown as median and inter-quartile range. Statistical significance calculated by Mann-Whitney analysis. * and ** indicate $P<0.05$ and $P<0.005$ respectively comparing data to pre-experimental values at the time-point indicated or between treatment groups. # indicates $P<0.05$ when the 2 virus treatments are compared at the time point indicated.

Fig. 8A shows the absolute lymphocyte counts per ml BALF in the Ad treated segments. The GFP treated group showed a significant increase in BALF lymphocyte concentration at day 24 ($P<0.05$) and the elafin treated group showed a significant increase at days 17 and 21 ($P<0.005$). The GFP treated group had significantly higher lymphocyte counts per ml BALF than the elafin treated group at day 24 ($P<0.05$).

When converted to percentage change over baseline (fig. 8B) then the only significant difference between the two Ad treatment groups is at day 24 with the GFP treated animals showing a higher percentage change compared to the elafin treated animals ($P<0.05$).

7.3.2. The effects of repeated administration of Adenovirus/calcium phosphate co-precipitates on the circulating leukocyte profile.

Fig. 9A shows the total cell counts per ml of blood up to 24 days after the start of the experiment when the first Ad instillation was performed. There are no significant changes in the circulating white cell number per ml when any of the time points are compared to the pre-experimental values. There are also no significant differences between the two adenovirus groups at any time point.

There are also no significant differences between the two adenovirus groups when the total cell counts are converted to percentage of pre-experimental values as shown in fig. 9B.

Circulating lymphocyte counts are shown in fig. 10A. There are no significant differences between the pre-experimental values and the values at any of the time-points shown for the Ad-GFP group. There is, however, a significant decrease at day seven compared to pre-experimental values in the Ad-o-elafin group.

When the values are converted to percentage of pre-experimental values as shown in fig. 10B, there are significantly lower values at both days 7 and 14 in the Ad-o-elafin group when compared to the Ad-GFP group.

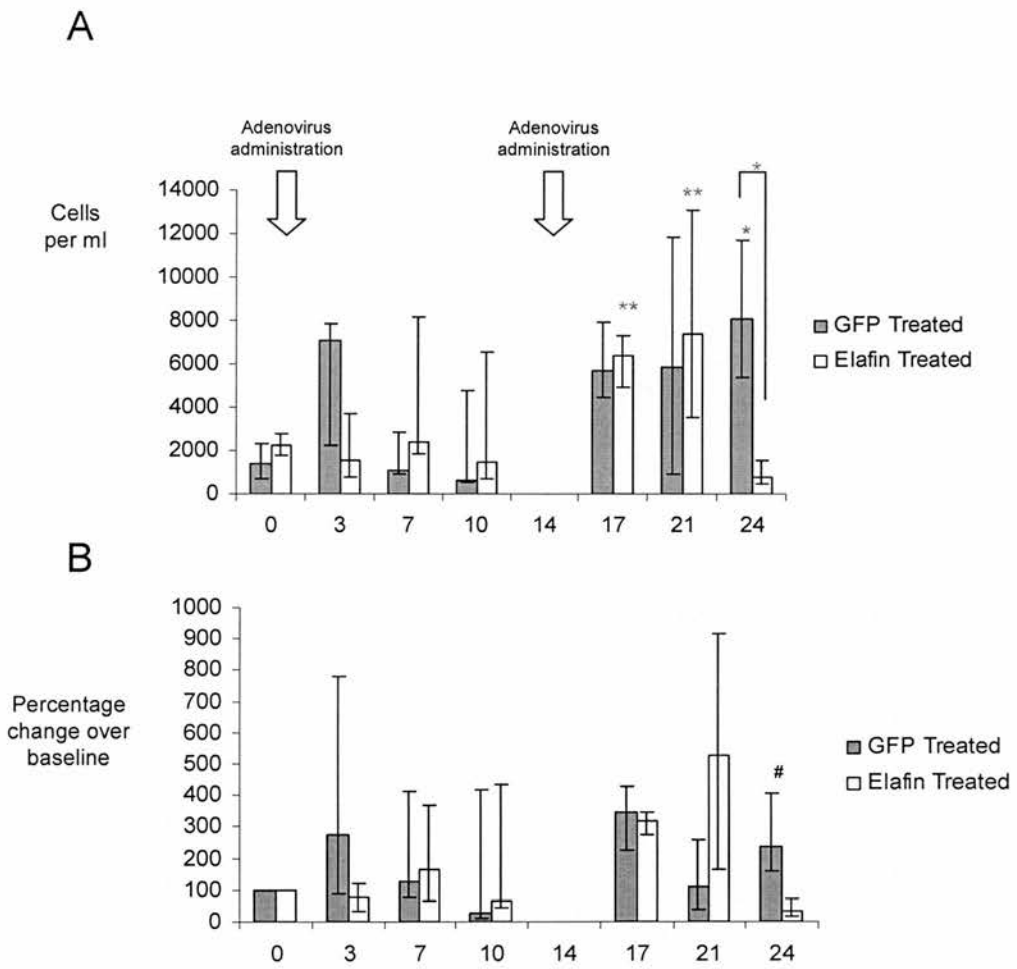

Fig. 8. The effect of repeated adenovirus administration on the Lymphocyte count per ml of BALF in treated segments.

A. Graph showing the lymphocyte count in the BALF of treated segments pre-experimentally and also after adenovirus administration at day 0 and day 14 in separate treated segments (either Ad-o-elafin or Ad-GFP).

B. Graph showing the above data presented as percent change in lymphocyte count compared to pre-experimental values.

All data is shown as median and inter-quartile range. Statistical significance calculated by Mann-Whitney analysis. * and ** indicate $P<0.05$ and $P<0.005$ respectively comparing data to pre-experimental values at the time-point indicated or between treatment groups. # indicates $P<0.05$ when the 2 virus treatments are compared at the time point indicated.

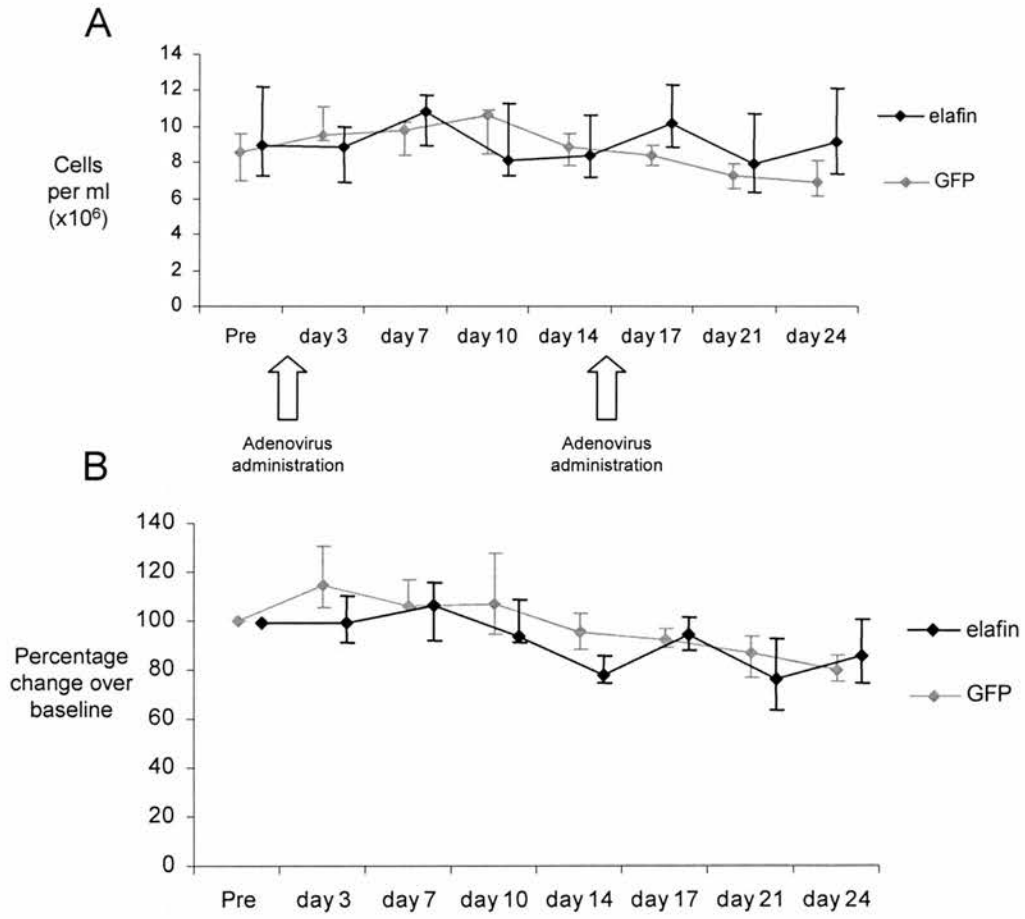

Fig. 9. Total circulating white cell count over 24 days with 2 administrations of Adenovirus.

A. Graph showing the total circulating white cell count over 24 days with the administration of adenovirus (either Ad-GFP or Ad-o-elafin) at days 0 and 14.

B. Graph showing the total circulating white cell count as a percentage of the pre-experimental cell count.

All data is shown as median and inter-quartile range. Statistical significance was calculated by Mann-Whitney analysis. No significant differences between pre-experimental values and the other values in A or between the 2 Adenovirus groups in B

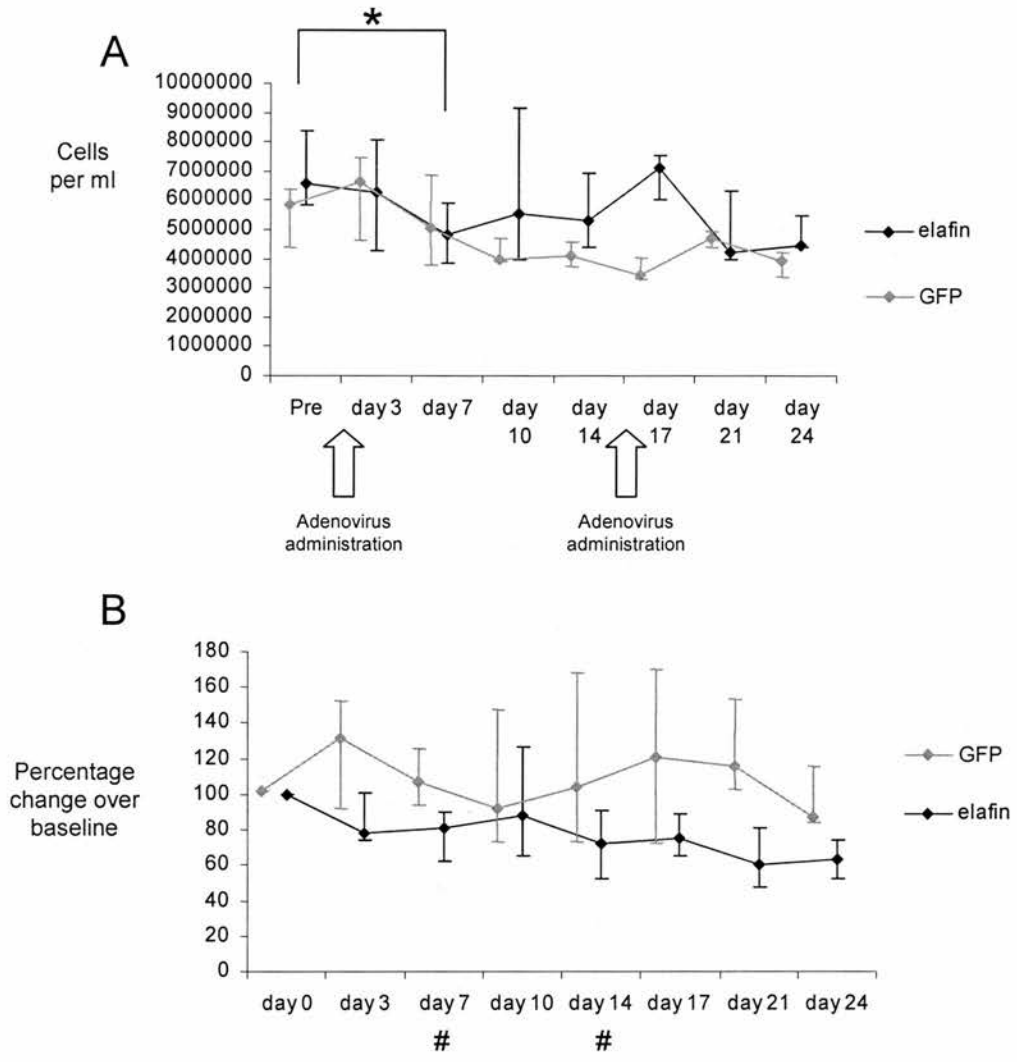

Fig. 10. Total circulating lymphocyte count over 24 days with 2 administrations of Adenovirus.

A. Graph showing the total circulating lymphocyte count over 24 days with the administration of adenovirus (either Ad-GFP or Ad-o-elafin) at days 0 and 14.

B. Graph showing the total circulating lymphocyte count as a percentage of the pre-experimental lymphocyte count.

All data is shown as median and inter-quartile range. Statistical significance was calculated by Mann-Whitney analysis. * indicates $P<0.05$ comparing the data at points indicated. # indicates $P<0.05$ between the 2 Adenovirus groups at the time-point indicated.

Finally, in fig. 11A are shown the absolute circulating neutrophil counts over the 24 days for the two virus groups. There are no significant changes in PMN counts compared to pre-experimental values in the Ad-GFP group, but there is a significant increase in absolute PMN counts compared to pre-experimental values at day 7 in the Ad-o-elafin group ($P<0.05$).

When the values are converted to percentage of pre-experimental values there are no significant differences between the two virus groups (fig. 11B).

7.3.3. The duration of transgene expression after the repeated administration of adenovirus to the ovine lung.

Fig. 12 shows the percentage of GFP+ve alveolar macrophages (AMs) in the Ad-GFP administered animals after the cellular content of the BALF sampled at each time point was cultured for 24 hours. There is a rapid decrease from the day 3 median of 6.061% to 0.472% at day 7 and to 0.002% at day 10. After day 10 no GFP+ve cells were isolated even after a second dose of Ad-GFP at day 14.

Fig. 13 shows the Western blots obtained from culture supernatants of AMs collected at 3, 7 and 10 days after the instillation of Ad-o-elafin or Ad-GFP. There is increased secretion of ovine elafin by AMs collected from the animals that were instilled with Ad-o-elafin compared to Ad-GFP. The secretion is most marked 7 days after the instillation of virus and continues for up to at least 10 days. AM supernatants were not examined for secretion of ovine elafin after the second virus instillation.

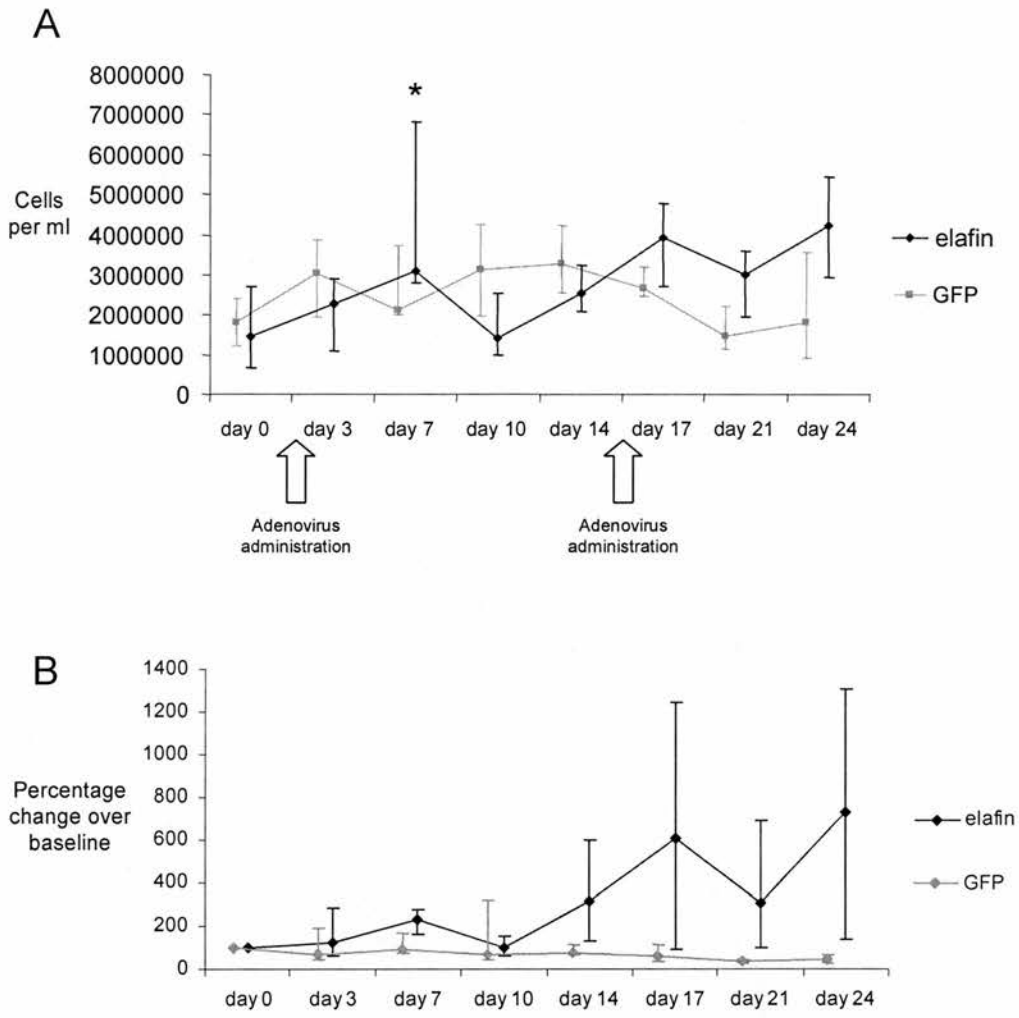

Fig. 11. Circulating neutrophil counts over 24 days with 2 administrations of Adenovirus.

A. Graph showing the circulating neutrophil count over 24 days with the administration of adenovirus (either Ad-GFP or Ad-o-elafin) at days 0 and 14.

B. Graph showing the circulating neutrophil count as a percentage of the pre-experimental cell count.

All data is shown as median and inter-quartile range. Statistical significance was calculated by Mann-Whitney analysis. * represents $P<0.05$ when comparing the values at the indicated time point with the pre-experimental values.

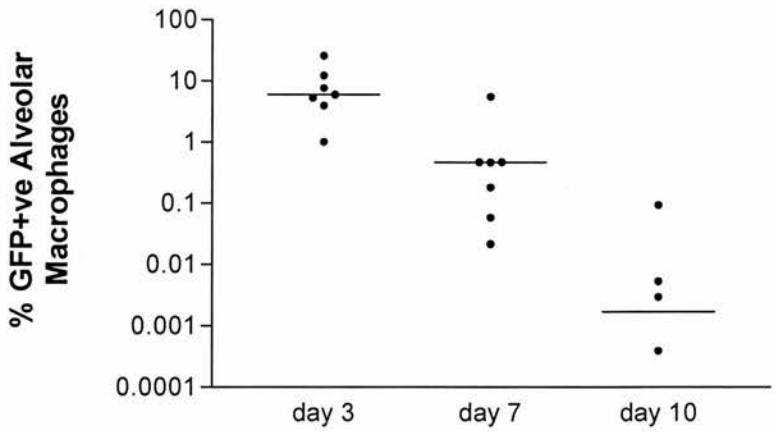

Fig. 12. Decrease in GFP+ve alveolar macrophages in BALF after administration of Ad-GFP.

Graph showing the percentage of alveolar macrophages in BALF after administration of Ad-GFP at day 0. Lines represent the median. NB. Zero values are not shown due to the use of a log scale.
After day 10 no GFP+ve cells were lavaged from any of the Ad-GFP animals even after the second dose of Ad-GFP at day 14. None of the Ad-o-elafin administered animals yielded any GFP+ve cells at any time-point.

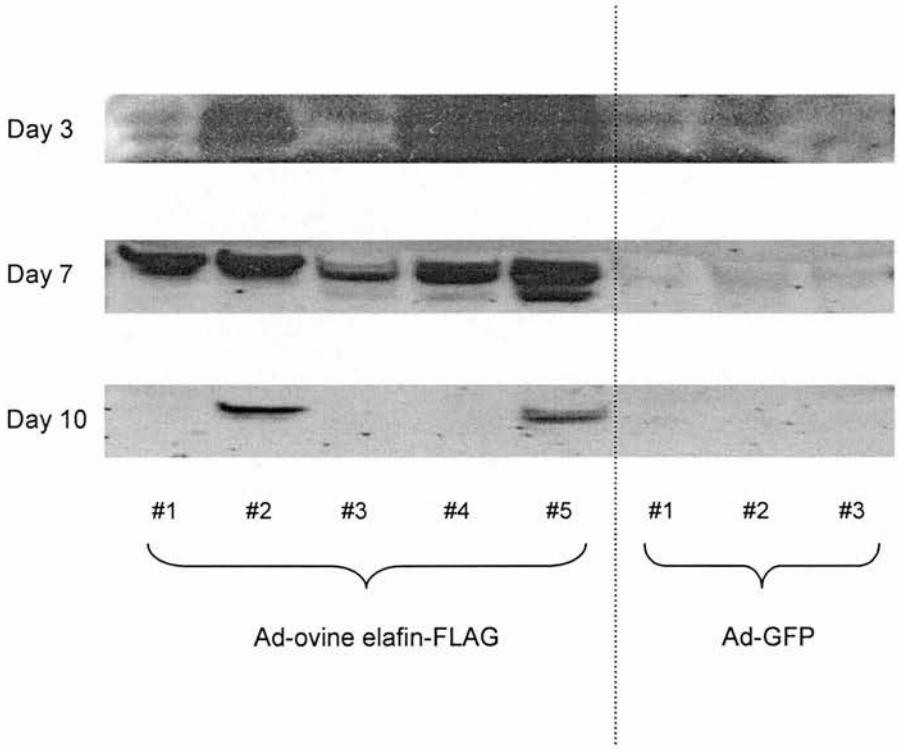

Fig. 13. Western blot analysis of alveolar macrophage supernatant after collection by broncho-alveolar lavage 3, 7 and 10 days after recombinant adenovirus instillation.

This figure shows Western blots of the alveolar macrophage supernatants after 1 weeks culture following harvesting by broncho-alveolar lavage of virus treated segments. The blots show macrophage supernatants from 5 Ad-ovine elafin-FLAG treated animals and 3 Ad-GFP treated animals.
The blots were probed with Trab-2O monoclonal antibody which detects the endogenous ovine elafin and also ovine elafin-FLAG.

7.3.4. The effects of the repeated instillation of adenovirus on circulating anti-adenovirus antibody activity.

Fig. 14 shows the percentage change in serum anti-adenovirus activity, after complement inactivation, in the serum of the animals that received two instillations of either Ad-o-elafin or Ad-GFP. The elafin treated animals show a higher relative activity at each time point compared to the GFP animals and this difference is significant at days 10, 17, 21 and 24 ($P<0.05$).

7.3.5. The effects of Ad-o-elafin and Ad-GFP on the local lung response to bacterial lipopolysaccharide.

After a single administration of each Ad as shown in fig. 2, bacterial lipopolysaccharide (LPS) was instilled topically into previously naïve segments (fig. 2, protocol C and D, segment 'a') and also into segments previously instilled with the Ad/CaP (fig. 2, protocol C and D, segment 'b') (n=5 for Ad-o-elafin, n=3 Ad-GFP). 4 days after the LPS instillation BALF was collected and analysed for total cell counts per ml and also neutrophil concentration. These values are graphed alongside pre-experimental values and the values for a naïve segment at day 14 (fig. 2, protocol C and D, segment 'h').

In order to simplify the discussion of the results from this experiment segment 'a' will be referred to as Ad-/LPS+, segment 'b' as Ad+/LPS+ and segment 'h' as Ad-/LPS-.

Fig. 15A shows the absolute total cell counts at day 14 (4 days post-LPS administration) for the two Ad groups. In the Ad-o-elafin treated animals there was a significant increase in total cell count in both the Ad-/LPS+ segments and the Ad+/LPS+ segments when compared to both pre-experimental values ($P<0.005$) and also compared to Ad-/LPS- segments at day 14 ($P<0.05$). In the Ad-GFP treated animals there is a small but significant increase in total cell count at day 14 in the Ad-/LPS+ segments compared to the pre-experimental values ($P<0.05$) but also in the Ad-/LPS- segments at day 14 ($P<0.05$). However, in these animals the total cell counts in the Ad+/LPS+ segments are not

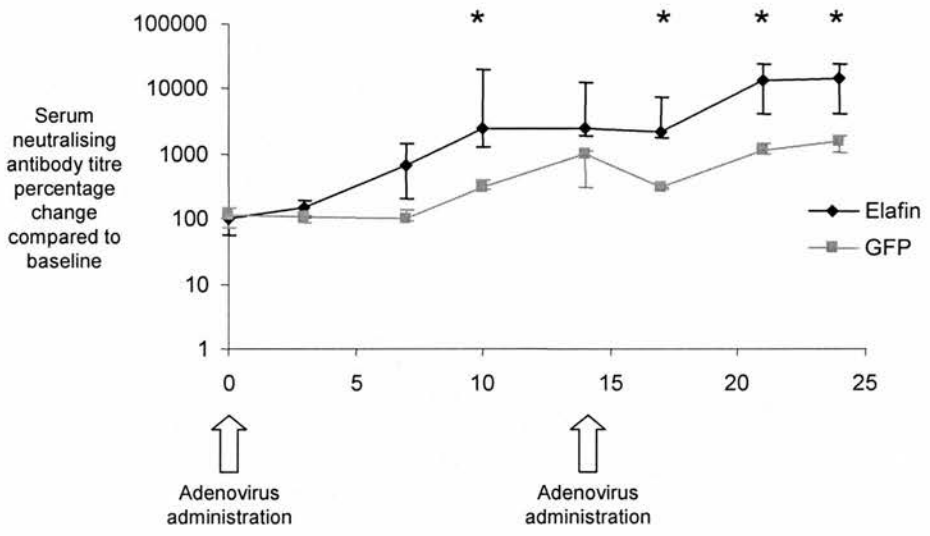

Fig. 14. Circulating Anti-Adenovirus antibody activity after 2 doses of adenovirus.

Graph showing the neutralising antibody titre of the serum after 2 doses of either Ad-GFP or Ad-ovine elafin FLAG at day 0 and day 14. Data is shown as percentage of titre at day 0.

All data is shown as median and inter-quartile range. Statistical significance calculated by Mann-Whitney analysis.* represents $P<0.05$ comparing the 2 virus groups at the points indicated.

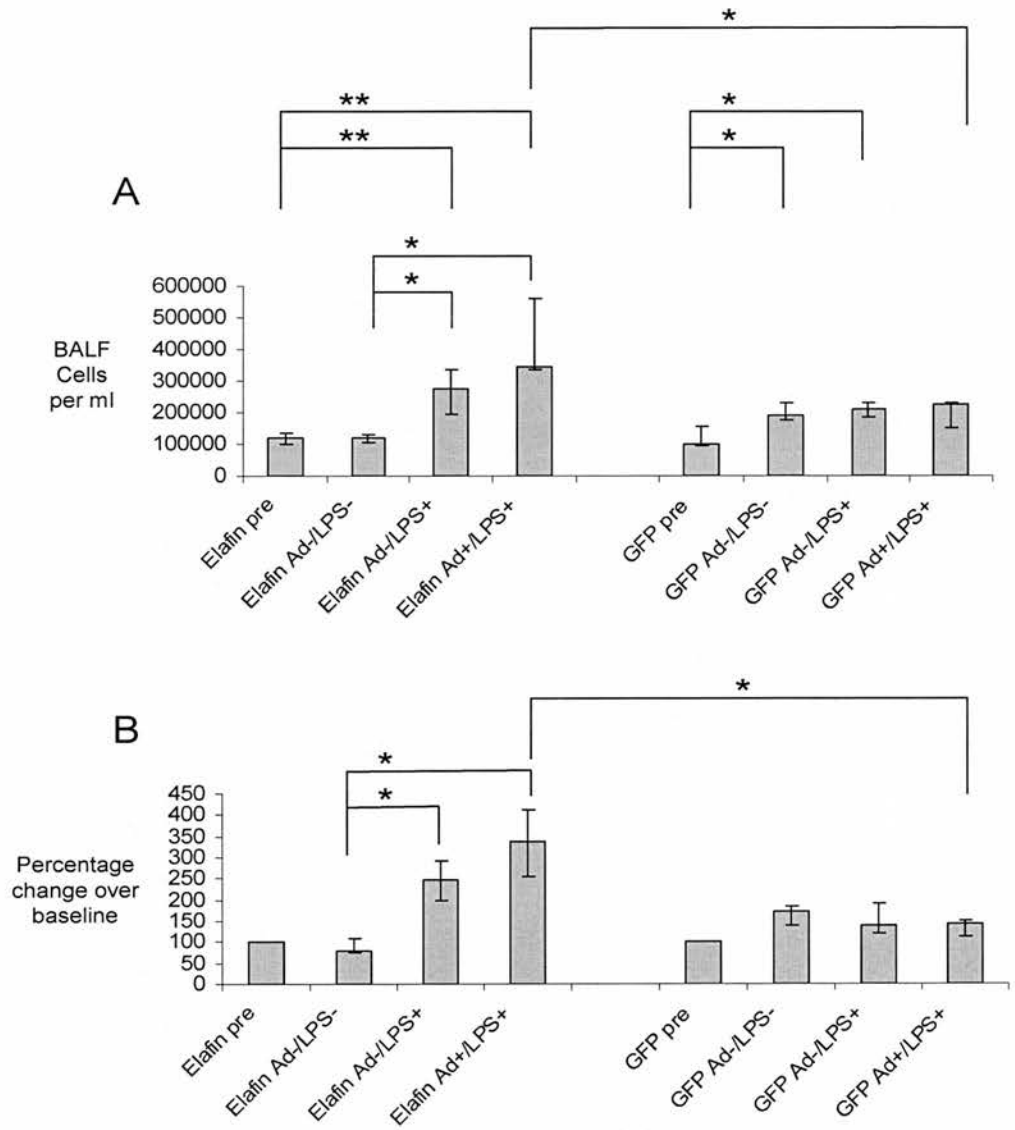

Fig. 15. The effect of local LPS administration on the total cell count per ml BALF after adenovirus administration.

A. Graph showing the total cell count in the BALF pre-experimentally and also post-LPS administration. Post-LPS BALF was collected on day 14 from naïve segments (Ad-/LPS-), LPS treated segments (Ad-/LPS+), and also LPS treated segments that had also been treated at day 0 with adenovirus (either Ad-o-elafin or Ad-GFP) (Ad+/LPS+).

B. Graph showing the above data presented as percent change in total cell count compared to pre-experimental values.

All data is shown as median and inter-quartile range. Statistical significance calculated by Mann-Whitney analysis.* and ** represent $P<0.05$ and $P<0.005$ respectively comparing values at the points indicated.

significantly different to the pre-experimental values or from the Ad-/LPS- segments at day 14. Additionally, in the Ad+/LPS+ segments the Ad-o-elafin treated animals have a significantly increased total cell count compared to the Ad-GFP treated animals.

Fig. 15B shows the percentage change over pre-experimental values for the total cell count and the effects of local LPS instillation. Again we see a significant increase in the Ad-o-elafin animals in the Ad-/LPS+ and Ad+/LPS+ segments compared to Ad-/LPS- segments at day 14 ($P<0.05$). This increase is not seen in the Ad-GFP animals. Equally, there is a significant increase in the Ad-o-elafin group in the Ad+/LPS+ segments compared to the Ad-GFP group ($P<0.05$).

Fig. 16A shows the absolute BALF neutrophil concentration at day 14 (post LPS) along with the pre-experimental values. There is a significant increase in the BALF neutrophil concentration in the Ad-o-elafin animals in the Ad-/LPS+ and Ad+/LPS+ treated segments compared to both pre-experimental values and the Ad-/LPS- segments at day 14 ($P<0.05$). In the Ad-GFP group there is a significant decrease in neutrophils per ml BALF in the Ad-/LPS- segments at day 14 compared to pre-experimental values ($P<0.05$) and also a small but significant increase in neutrophils per ml in the Ad+/LPS+ segments compared to pre-experimental values ($P<0.05$).

Fig. 16B shows that there is a significant increase in the neutrophil percentages in the Ad-/LPS+ and Ad+/LPS+ segments compared to the Ad-/LPS- segments at day 14 in the Ad-o-elafin treated animals. This difference is not seen in the Ad-GFP treated animals.

Fig. 17A shows the absolute lymphocyte counts per ml BALF at day 14 (4 days post LPS) compared with pre-experimental values. There is a significant increase in BALF lymphocyte concentration at day 14 in the Ad-/LPS+ segments ($P<0.005$) and a significant decrease in the Ad-/LPS- segments ($P<0.05$) compared to pre-experimental values. There is also a non-significant increase in the Ad+/LPS+ segments compared to pre-experimental values. There is also a significant increase in lymphocyte concentration in the Ad-/LPS+ segments compared to the Ad-/LPS- segment s ($P<0.05$) and an increase in the Ad+/LPS+ segments compared to the Ad-/LPS- segments which approaches

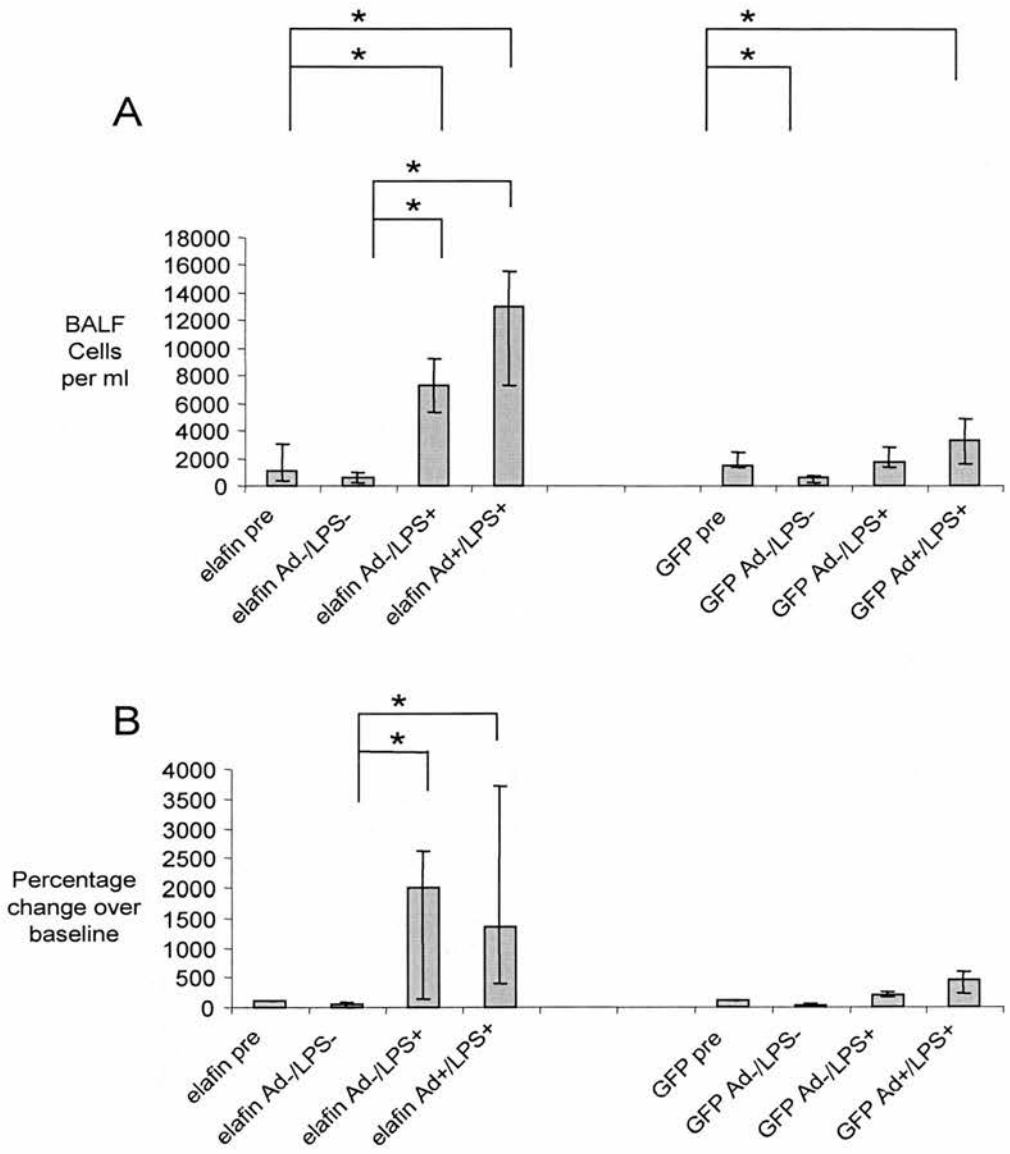

Fig. 16. The effect of local LPS administration on the BALF PMN concentration after adenovirus administration.

A. Graph showing the PMN count per ml of BALF pre-experimentally and also post-LPS administration. Post-LPS BALF was collected in naïve segments (Ad-/LPS-), LPS treated segments (Ad-/LPS+), and also LPS treated segments that had also been treated at day 0 with adenovirus (either Ad-o-elafin or Ad-GFP) (Ad+/LPS+).

B. Graph showing the above data presented as percent change in PMN count compared to pre-experimental values.
All data is shown as median and inter-quartile range. Statistical significance calculated by Mann-Whitney analysis.* and ** represent $P<0.05$ and $P<0.005$ respectively comparing values at the points indicated.

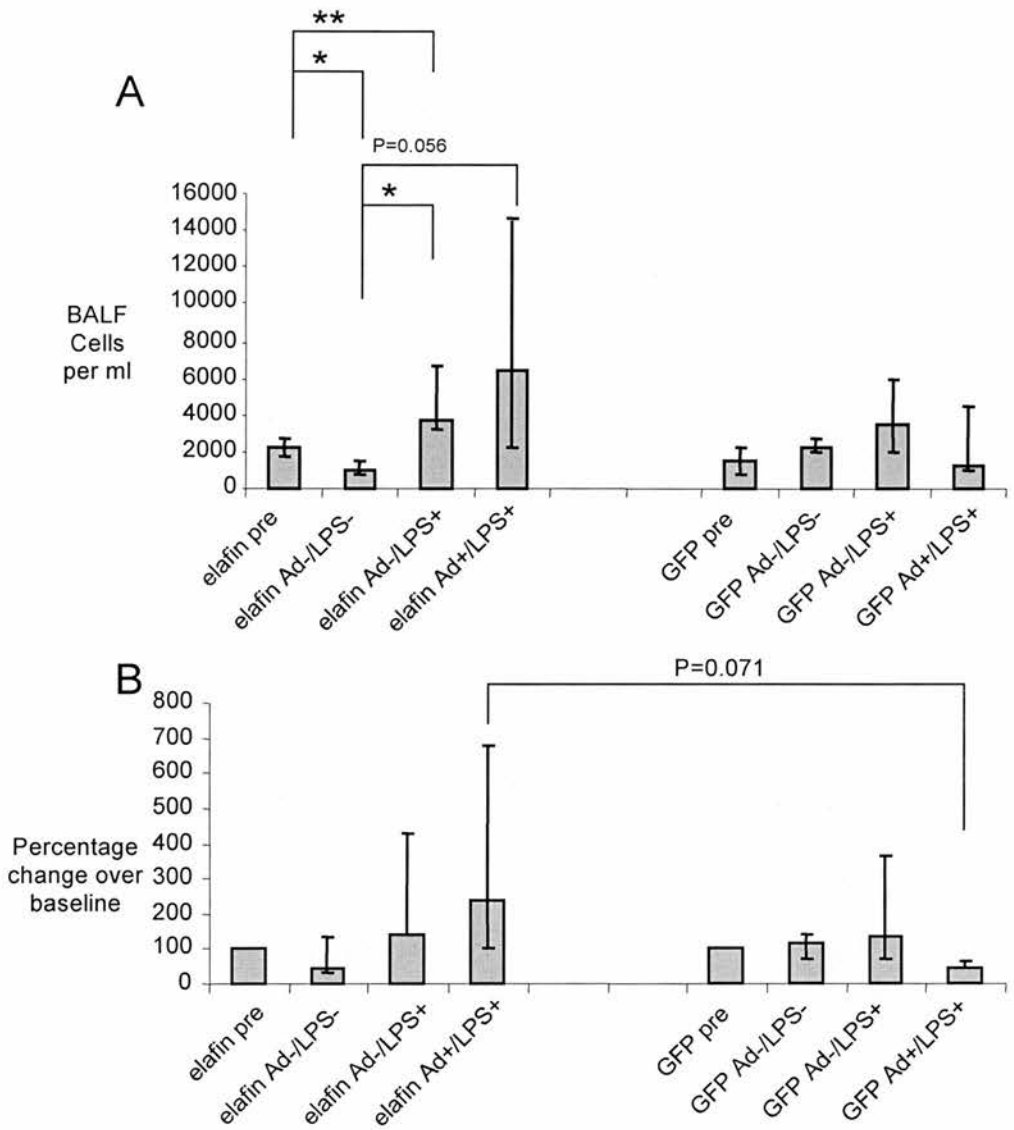

Fig. 17. The effect of local LPS administration on the BALF lymphocyte concentration after adenovirus administration.

A. Graph showing the lymphocyte count per ml of BALF pre-experimentally and also post-LPS administration. Post-LPS BALF was collected in naïve segments (Ad-/LPS-), LPS treated segments (Ad-/LPS+), and also LPS treated segments that had also been treated at day 0 with adenovirus (either Ad-o-elafin or Ad-GFP) (Ad+/LPS+).

B. Graph showing the above data presented as percent change in lymphocyte count compared to pre-experimental values.
All data is shown as median and inter-quartile range. Statistical significance calculated by Mann-Whitney analysis.* and ** represent $P<0.05$ and $P<0.005$ respectively comparing values at the points indicated.

significance (P=0.056). In the Ad-GFP treated group there were no significant changes from the pre-experimental values or from the Ad-/LPS- segments at day 14.

When these results are converted to percentage change over pre-experimental values (fig. 17B) then the only result of note is that the elafin treated animals showed a non-significantly higher percentage change than the GFP treated animals in the Ad+/LPS+ segments (P=0.071).

The myeloperoxidase (MPO) activity in the BALF is shown in fig. 18. Fig. 18A shows the absolute MPO activity at day 14 in the segments instilled with LPS (both Ad-/LPS+ and Ad+/LPS+ segments). There is no significant difference in any of the segments though there seems to be trend towards higher MPO levels in the Ad+/LPS+ segments in the o-elafin treated animals compared to the GFP treated animals.

The MPO activity per cell present in the BALF is shown in fig. 18B. There is no significant difference between any of the segments.

7.3.6. The effects of Ad-o-elafin and Ad-GFP on the systemic response to bacterial lipopolysaccharide.

Fig. 19A shows the total circulating white cell counts per ml for the two Ad treatment groups from pre-experimental samples through to day 14 after receiving LPS at day 10. In the Ad-GFP treated group there is a significant increase in the total circulating white cell counts at day 14 compared to pre-experimental values ($P<0.05$). There is a significant difference between the Ad-o-elafin and the Ad-GFP group at day 14 ($P<0.05$). When the values are converted to percentages of the pre-experimental values as shown in fig. 19B, there are no significant differences between the two virus treatment groups at any of the time points although post-LPS at day 14 the difference approaches significance (P=0.071).

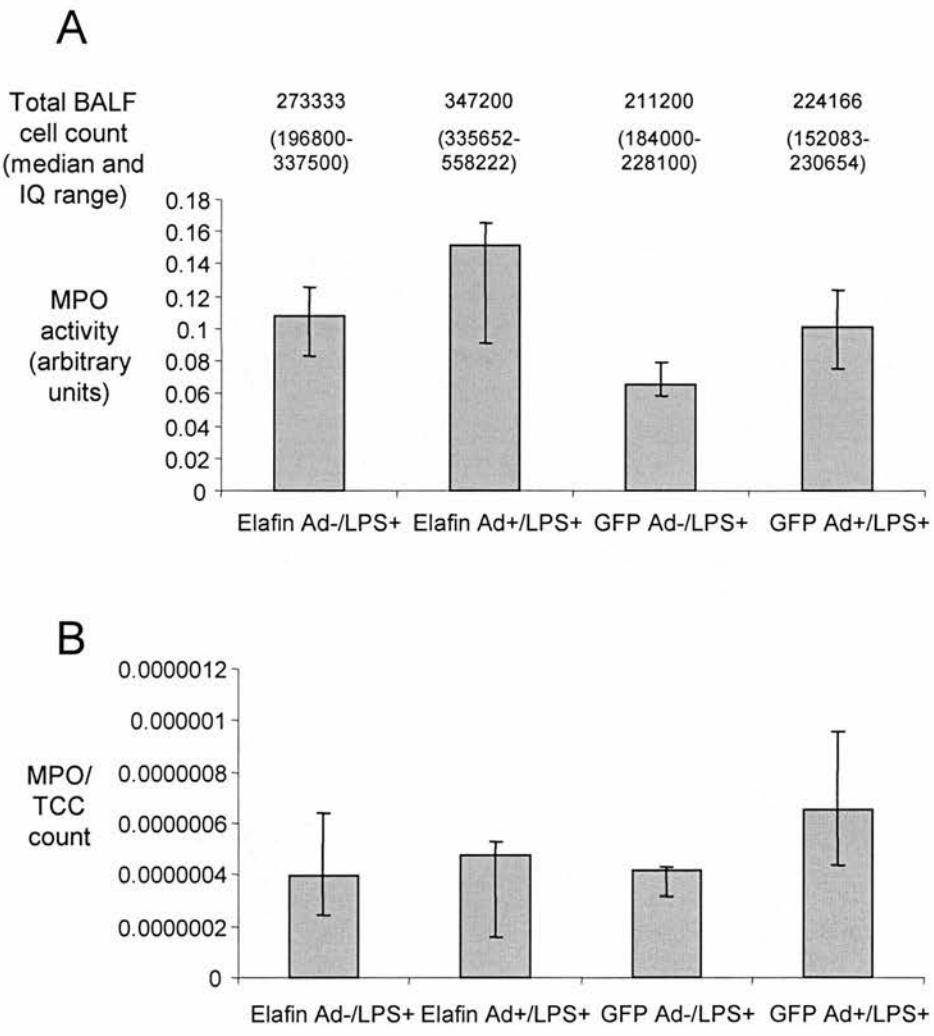

Fig. 18. Myeloperoxidase activity in broncho-alveolar lavage does not correlate with BALF cellularity.

A. Graph showing the total myeloperoxidase (MPO) levels in the BALF collected at day 14 after LPS was instilled at day 10 into both a naïve segment (Ad-/LPS+) and a segment previously exposed to adenovirus (Ad+/LPS+). The figures above each segments data indicate the total cell counts in the BALF (median and IQ range).

B. Graph showing the MPO activity divided by the total cell count in the BALF.

No statistical significance was seen.

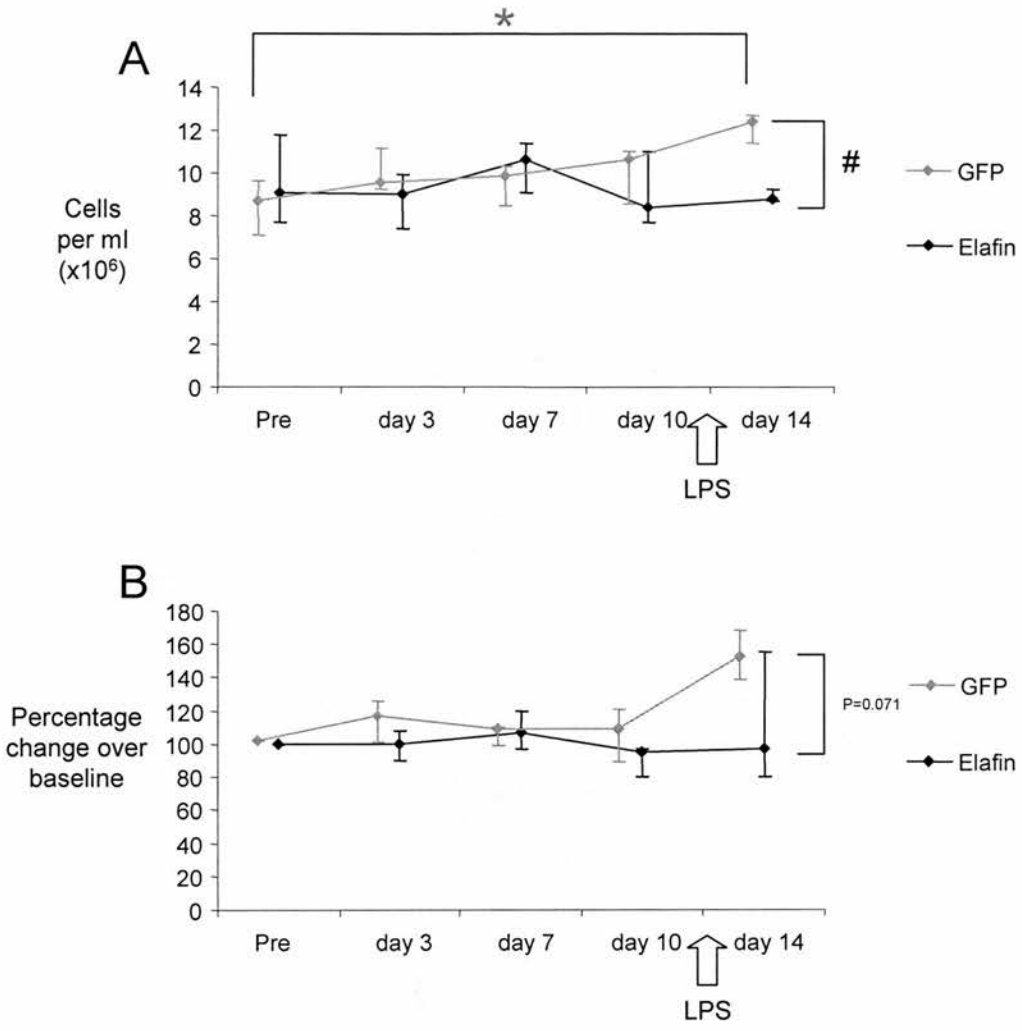

Fig. 19. Total circulating white cell count after one administration of adenovirus and one administration of LPS.

A. Graph showing the total circulating white cell count over 14 days with the administration of adenovirus (either Ad-GFP or Ad-o-elafin) at days 0 and intra-pulmonary LPS administration at day 10.

B. Graph showing the total circulating white cell count as a percentage of the pre-experimental cell count.

All data is shown as median and inter-quartile range. Statistical significance calculated by Mann-Whitney analysis.* represents $P<0.05$ comparing values at the points indicated. # indicates $P<0.05$ comparing the Ad-GFP and Ad-o-elafin groups.

The circulating lymphocyte counts after the first administration of either Ad at day 0 followed by intra-pulmonary instillation of LPS at day 10 are shown in fig. 20A. In the Ad-o-elafin group there is a significant decrease in circulating lymphocytes at day 7 compared to pre-experimentally ($P<0.05$). In the Ad-GFP group there is a significant increase in the lymphocyte count after administration of LPS ($P<0.05$). When the figures are converted to percentages of pre-experimental values (fig. 20B) there are no significant differences between the two adenovirus groups except at day 7 with the elafin group having lower counts compared to the GFP group ($P<0.05$).

The circulating neutrophil counts are shown in fig. 21A over 14 days with either Ad-o-elafin or Ad-GFP given at day 0 and LPS given at day 10. There is a significant increase in the circulating neutrophils per ml at day 7 compared to pre-experimentally in the Ad-o-elafin animals ($P<0.005$). There are no significant changes in the Ad-GFP group. When the numbers are converted to percentages of the pre-experimental values there are no significant differences between the two virus groups at any time point (fig. 21B).

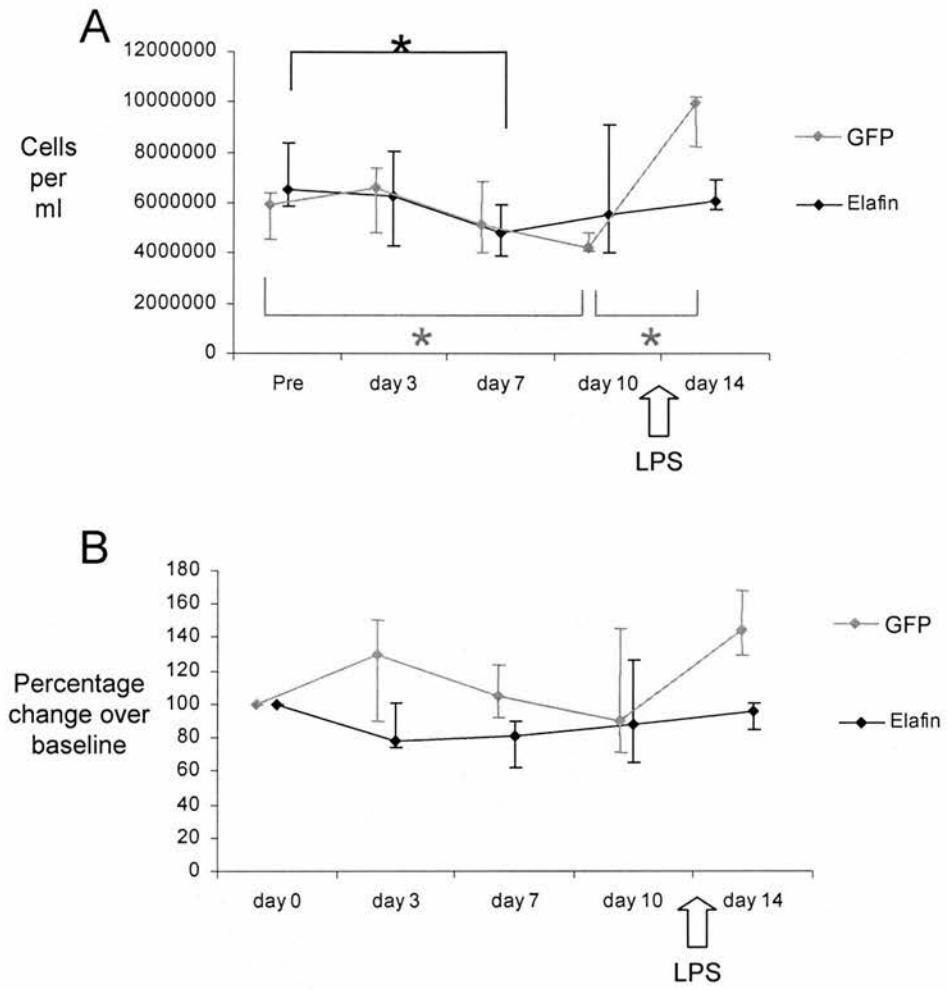

Fig. 20. Circulating lymphocyte counts after one administration of adenovirus and one administration of LPS.

A. Graph showing the total lymphocyte count over 14 days with the administration of adenovirus (either Ad-GFP or Ad-o-elafin) at day 0 and intra-pulmonary LPS administration at day 10.

B. Graph showing the lymphocyte count as a percentage of the pre-experimental lymphocyte count.

All data is shown as median and inter-quartile range. Statistical significance calculated by Mann-Whitney analysis.* represents $P<0.05$ comparing values at the points indicated.

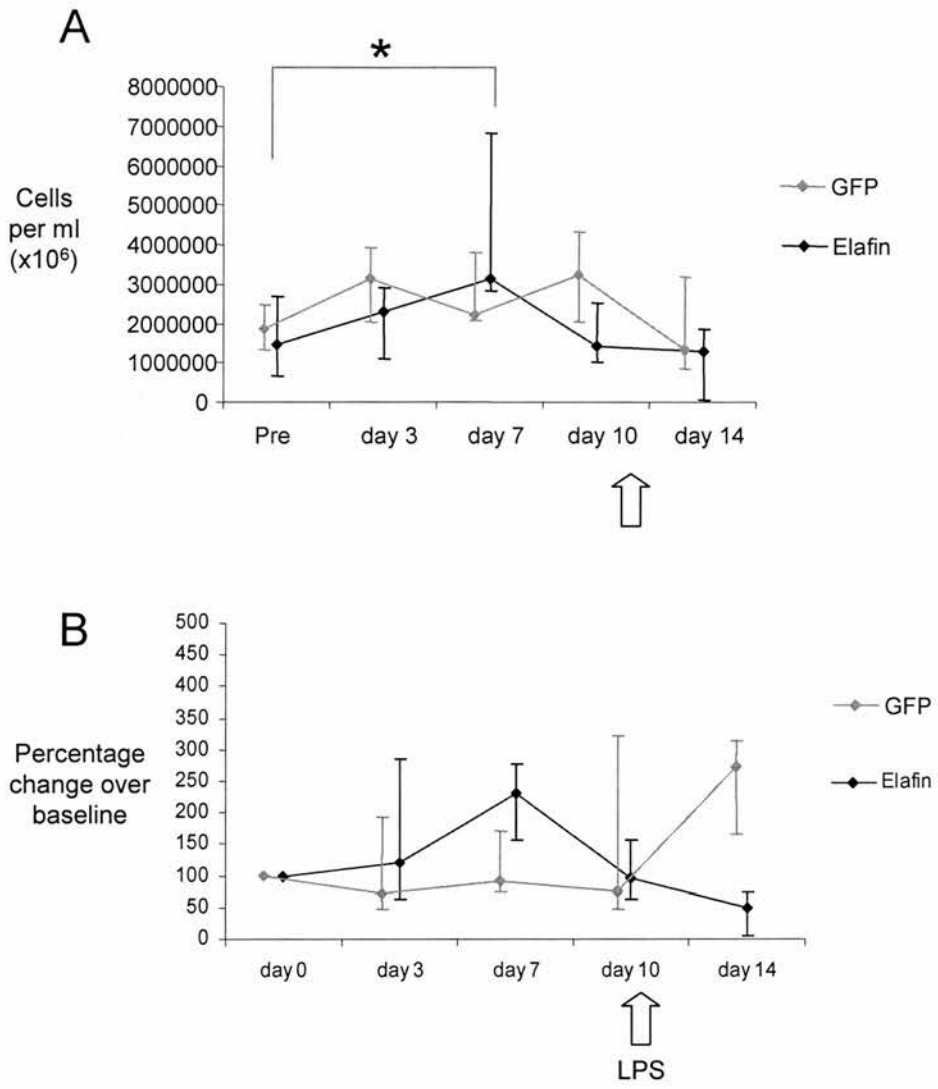

Fig. 21. Total circulating neutrophil count after one administration of adenovirus and one administration of LPS.

A. Graph showing the total circulating neutrophil count over 14 days with the administration of adenovirus (either Ad-GFP or Ad-o-elafin) at days 0 and intra-pulmonary LPS administration at day 10.

B. Graph showing the total circulating neutrophil count as a percentage of the pre-experimental count.

All data is shown as median and inter-quartile range. Statistical significance calculated by Mann-Whitney analysis.** represents $P<0.005$ comparing values at the points indicated.

7.4. DISCUSSION

7.4.1. Controls used in the *in vivo* experiments.

Selection of Ad-GFP as a control virus was governed by practical issues rather than an idealised concept of what properties the control virus should possess. Indeed selection of a transgene encoding for a small, secreted protein or even a genetically modified form of the ovine elafin gene would potentially have offered particular advantages in interpreting effects of this antiprotease. However, time constraints and the perceived need to visualise the target cell population largely dictated the selection of Ad-GFP as a 'reporter' construct.

Despite the widespread use of this reporter it should be borne in mind that GFP itself may induce toxicity (Liu et al., 1999).

7.4.2. The time-course of the response both locally and systemically to the instillation of one or two doses of recombinant adenoviruses.

The experiments performed in the previous chapter provided the background information necessary to investigate the time-course of the inflammatory response both locally in the lung and also systemically. In this experiment the instillation of Ad-o-elafin was compared directly to the instillation of Ad-GFP (this time acting as a virus control). Figs. 1 and 2 show a flow diagram of the experiment performed and a more detailed outline of the animal groups used (more details are found in chapter 2).

There was no change in the total cell count per ml BALF in the untreated naïve segments after the initial Ad instillation (day 0) in either Ad-o-elafin or Ad-GFP treated animals. After the second instillation, there is an increase in cellularity which becomes significant in the Ad-GFP treated group

at days 21 and 24 (fig. 3A). Hence there is a need for caution in the interpretation of results at these time-points and the assumption of segmental interdependence, at the phenotypic level (a requirement if one is to use the naïve segments as internal controls), is seen to be invalid. The neutrophil counts in the BALF of the naïve segments do not change significantly through the whole experiment (fig. 5).

Examination of the BALF cell counts from the Ad instilled segments indicates that Ad-o-elafin treatment limits the total cellular influx at days 3 and 7 (fig. 4) but promotes a more marked neutrophil influx (fig. 6). The lymphocyte data from Ad treated segments (fig. 8) is difficult to assess due to the wide variation and low absolute counts within the BALF.

Human elafin has been shown to act as a chemo-attractant for neutrophils on its own *in vitro* and increases the influx of neutrophils into the lung in response to LPS *in vivo* (Simpson et al., 2001B; Sallenave et al., 2003).

Ad-o-elafin instillation at day 0 causes both a significant lymphopenia (fig. 10) and a significant neutrophilia (fig. 11) at day 7 which is not seen in the Ad-GFP treated animals.

7.4.3. The duration of transgene expression after the segmental instillation of recombinant adenovirus in the sheep.

The lack of any evidence of GFP expression after the second administration likely indicates the onset of a rapid anti-viral response presumably comprising cell-mediated and humoral components. No macrophages from the naïve segments were seen to be GFP+ve. Whilst not conclusive, this latter observation adds weight to the assumption that there is negligible transfer of material between segments either at the point of instillation or subsequently.

Western blot analysis of the supernatant collected after culture of alveolar macrophages (AMs) collected from virus treated segments after the 1st dose of either Ad-o-elafin or Ad-GFP confirmed

the infection of these cells in the Ad-o-elafin treated segments and the subsequent secretion of ovine elafin (fig. 13). The secretion of elafin appeared to extend beyond the time course suggested by GFP expression profiles. Whether this reflects the sensitivity of the respective assays or other factors including potential cytotoxicity of GFP must remain speculative.

7.4.4. Serum neutralising anti-adenovirus antibody activity after one and two instillations of adenovirus.

The BALF cytology and circulating white cell counts examined above (section 7.3.2) demonstrated an inflammatory response to the local instillation of Ad vectors. This prompted the investigation of the serum anti-Ad neutralising capacity. To examine the circulating anti-adenovirus immunoglobulin levels the neutralising activity of the serum from each animal at each time-point through the repeated virus administrations for the two virus groups was assayed. The elafin treated animals show higher anti-adenovirus antibody activities compared to the GFP group when the titers at each point are compared to pre-experimental titers ($P<0.05$ at days 10, 17, 21 and 24) indicating that the elafin treated animals mounted an increased anti-adenovirus immune response (fig. 14). If we accept the fact that the increase in titer in the GFP group is indicative of the animals mounting an anti-adenovirus response sufficient to inhibit infection of at least the alveolar macrophages in the instilled segment then we can expect that the far more marked response in the elafin group will mean that the AMs in these animals will not be infected either after a repeated instillation. This, however, is perhaps an over-simplification as we have so far examined the percentage change over baseline and not absolute titers. For example the absolute titers for the GFP group and the elafin group at day 10 are 1:18.27 and 1:18.40 respectively. Harvey et al. (1999) showed that there was no transgene activity observed after infection of sub-segmental bronchi of cystic fibrosis patients with an adenovirus expressing human cystic fibrosis trans-membrane conductance regulator (CFTR) if their circulating anti-adenovirus titer was greater than 1:80. Obviously methodology has a major effect on calculation of titer and in the

work presented here the titer is a measure of the maximum dilution of serum which will still inhibit 100% of virus activity. On the other hand Harvey et al. (1999) use a measure of dilution until 50% inhibition of adenovirus infection is achieved. In fact it is entirely plausible that individual titers are only usefully compared to titers in the same animal at a different time point. If this is the case, however, it is useful to observe that the absolute titers in the two Ad groups here were similar at day 10 and so again if there were no GFP+ve AMs seen after the second instillation then it is highly unlikely that there will be elafin secreting AMs after a second instillation.

Of note is the fact that although not examined directly in this work, there is a possibility that the incorporation of adenovirus into calcium phosphate precipitates may have an effect on the antigenicity of the virus itself. Others with an interest in the repeated administration of adenovirus have used techniques to covalently modify the capsid proteins by conjugation of polyethylene glycol (PEG) to free lysine groups on the adenovirus (Croyle et al., 2001). This group showed a diminution of the humoral antibody response of instilled animals directed towards these 'PEGylated' adenoviruses. However if the same PEGylated virus was administered twice then the usual antibody and cell mediated immunity was seen to neutralise the second instillation markedly. The authors postulated that the PEGylated virus was directed away from antigen presenting cells (APCs) and hence there was a decrease in antigen presentation. Due to the co-precipitation of adenovirus with calcium phosphate the infection of alveolar macrophages is facilitated as shown in chapter 6. As the protocol used in this chapter is directed at infecting the APCs preferentially there is the possibility that an increased antigenicity will be seen.

7.4.5. Ad-o-elafin and its role in modulating the innate immune response to local lung instillation of bacterial LPS.

The instillation of bacterial LPS into a segment of ovine lung which had 10 days previously been instilled with Ad-o-elafin, caused an exaggerated cellular response relative to that seen following a similar challenge in a segment with a prior history of exposure to a control adenovirus (figs. 15 to 17). This observation warrants a consideration of the potential influence of ovine elafin on the response of the lung to bacterial LPS:

What is the mechanism whereby elafin up-regulates the lung's cellular response to LPS?

When bacterial LPS is instilled locally into the lung it acts predominantly on the mobile cell population contained therein specifically the alveolar macrophages (Koay et al., 2002). LPS responsive cells express a variety of 'pattern-recognition molecules' which are involved in the recognition of micro-organisms. Predominantly with regards the LPS response are the Toll-like receptors (specifically TLR-4) and CD-14. The currently accepted mechanism for LPS stimulation of macrophages is shown in fig. 22. Briefly, LPS derived from Gram negative bacterial cell walls is present in micelles until these make contact with LPS-binding protein (LBP). LBP catalyses the transfer of individual LPS moieties to the 'membrane receptor complex' made up of CD14, Toll-like receptor 4 (TLR4) and the myeloid differentiation protein 2 (MD-2). Downstream signalling then occurs via MyD88, TRAF6, NIK and IKK ending in the nuclear translocation of NFκB and hence the up-regulation of pro-inflammatory cytokines such as TNFα, IL-1β, IL-6 and IL-8 (Martin, 2000; Fujihara et al., 2003). In the lung instilled or inhaled LPS first comes into contact with the fluid lining the respiratory tract (the epithelial lining fluid or ELF). This fluid is rich in lipids and also contains soluble CD14 and LBP. Acute lung injury leads to a marked increase in the concentrations of these two proteins in the ELF as assessed by bronchoalveolar lavage (BALF) (Martin et al., 1997). ELF also contains large amounts of surfactant protein A (SP-A). This protein binds LPS via its lipid A portion (Kalina et al., 1995) reducing its biological activity. In acutely inflamed lungs the concentration of SP-A is markedly reduced leaving more LPS to associate with LBP contained within the epithelial lining fluid and increasing the potential for macrophage activation.

It is quite plausible that secreted elafin acts as a 'surrogate' lipopolysaccharide binding protein. In the normal lung the bronchoalveolar fluid contains low levels of lipopolysaccharide binding protein (LBP) (0.05-0.1μg/ml – Martin, 2000). In this situation the majority of inhaled or instilled LPS binds

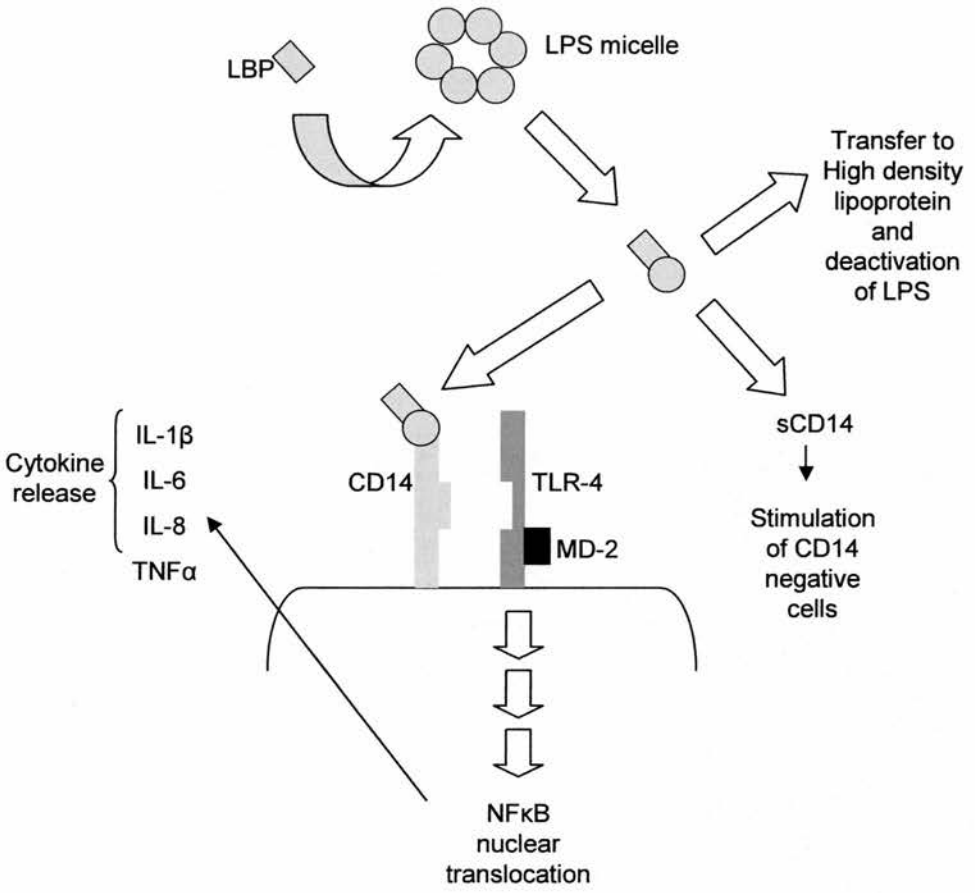

Fig. 22. Schematic representation of the stimulation of macrophages by LPS in the presence of LBP.

This figure shows the currently accepted pathway by which LPS stimulates macrophages. Firstly, LPS micelles are transferred as monomers to the complex of proteins in the macrophage membrane made up of CD14, TLR-4 and MD-2. The binding of LPS to this complex sets in motion a series of downstream reactions ending in the nuclear translocation of NFκB and the activation of genes encoding for IL-1β, IL-6, IL-8 and TNFα.

to surfactant protein A (SP-A) and is inactivated. If secreted proteins can act as a 'surrogate' LBP then there is the possibility of increasing the activation of the macrophage population contained in the air-spaces. There is little in the scientific literature to confirm the presence of LBP-type substances in vivo but human elafin does interact with certain LPS types. Synthetic human elafin peptides bind to E. coli LPS and significantly increase TNFα secretion from murine RAW cells in culture conditions deplete in LBP (i.e. serum-free conditions) (McMichael et al., submitted manuscript). If secreted ovine elafin functions in this manner in the LPS-induced inflammation model used in this chapter then the BALF would indeed contain more activated macrophages which would in turn attract more macrophages and neutrophils into the airspaces. A subsequent increase in LBP concentration due to leakage from the serum would propagate the LPS induced inflammation. The cell counts in the BALF would tend to substantiate this hypothesis and indeed the total protein content of the BALF in the Ad-o-elafin treated segments after LPS had a median of 602μg/ml (range 435-1198) compared to the Ad-GFP segments post LPS of 282 μg/ml (range 281-596) (see Appendix I) although this difference is not statistically significant. Simpson et al. (2001B) showed an increase in BALF MIP-2 in LPS treated mice that had previously received Ad-human elafin, when compared to LPS treatment of mice that had received vehicle alone or Ad-lac Z. There were also non-significant increases in TNF-α and MIP-1α in the Ad-elafin animals. This situation reflects that expected by the up-regulation of the LPS response in the elafin-rich lung. The MIP-2 increase could explain the increase in BALF neutrophil counts due to its chemo-attractant properties. MIP-1α is released by LPS stimulated macrophages and is a known chemo-attractant for neutrophils and macrophages (VanOtteren et al., 1994; Standiford et al., 1995).

Further, although data exists to suggest that intra-cellular elafin down-regulates NFκB activation and hence the inflammatory response to LPS (Henriksen et al., 2004), the converse of the observed situation in the ovine model, there is some evidence for a link between the down-regulation of the nuclear translocation of NFκB and an increase in lung cellularity seen after in vivo LPS administration. Aoki et al. (2002) showed that dexamethasone prior to intraperitoneal LPS suppressed NFκB activation but enhanced MIP-2 and neutrophil counts in the lung. The authors speculate that

this enhancement of neutrophil influx by dexamethasone is one of this steroid's important anti-inflammatory properties.

It is of particular note that LPS instillation into a naïve segment in an animal which had received Ad-o-elafin in the contra-lateral lung (ten days prior to LPS) caused a significant increase in BALF total cell counts and neutrophil counts compared to a totally naïve (i.e. un-touched) segment at day 14 (figs. 15 and 16). This situation was not seen in the animals instilled with Ad-GFP. Elafin appears therefore to up-regulate the inflammatory response at sites distant to the site of instillation. This situation has been reported in other gene transfer experiments, most notably in models of arthritis and kidney inflammation, and has led to the term 'contra-lateral effect' implying an effect being manifest at a site distant to the primary site of gene transfer. It is pertinent to briefly review the work in this area that has led to this term.

Ghivizzani et al. (1998) were the first to report a contra-lateral effect after adenoviral transfer of IL-1 and TNF-α soluble receptors to the stifle joint of rabbits conferred a beneficial effect to the opposite 'control' stifle in an antigen-induced arthritis model. In this model transgene expressing cells were found in the instilled joint and also in the drainage lymph node and the opposite joint synovium, although not in the contra-lateral lymph node, and no transgene product could be detected out-with the injected joint. Further work by the same group (Lechman et al., 1999) showed a similar effect in the same model using an adenovirus expressing viral IL-10. This paper also demonstrates the migration of a specific morphologically discrete population of adenovirus infected cells to the contra-lateral joint. The authors postulate that these cells may be immature dendritic cells and macrophages (Lechman unpublished data). Kim et al. (2002) also demonstrated a contra-lateral effect after intra-articular injection of ex vivo modified synovial fibroblasts expressing soluble IL-1 and TNF-α receptors. However, as these cells don't migrate from their site of injection the contra-lateral effect is considered to be less likely related to the migration of the transfected cell population. Whalen et al. (2001) showed that Ad-viral IL-10 injection in mice could lead to the generation of antigen-presenting cells (APCs) capable of inhibiting both local and distant delayed type hypersensitivity (DTH) reactions

following adoptive transfer. However, this transfer was only effective when the donor mice were sensitised to the same antigen used to illicit the DTH response in the recipients indicating an antigen dependence. Subsequent elegant work by Lechman et al. (2003) demonstrated that Ad-viral IL-10 will only ameliorate arthritis in the contra-lateral limb to the joint instilled if the same antigen is used in both joints as the causative factor. Kluth et al. (2001) demonstrated a contra-lateral effect in rat kidneys using macrophages infected ex vivo with Ad-IL-4. The localised expression of IL-4 led to a marked reduction in proteinuria and a decrease in macrophage infiltration both in the injected kidney and the contra-lateral kidney. Although systemic IL-4 was not detected a small number of infected macrophages did arrive in the contra-lateral kidney and cannot be ruled out as the source of the contra-lateral effect.

The current body of evidence, therefore, suggests that exposure of APCs to a milieu rich in a specific mediator may modify the future biological activity of that APC leading to a contra-lateral, or perhaps more precisely a 'distant' effect.

In considering possible mechanisms underlying the contra-lateral effect in this model a working hypothesis emerges in relation to possible events following local lung instillation of Ad-o-elafin. Fig. 23 depicts the instillation of adenovirus to one segment in our model system. The open arrows signify the presence of 'free' (i.e. not cell associated or phagocytosed) adenovirus/calcium phosphate co-precipitates. This precipitate can infect both type I and type II alveolar epithelial cells, airway cells and alveolar macrophages. Infection of these cells with Ad-o-elafin will result in the secretion of ovine elafin into the epithelial lining fluid. Infected alveolar macrophages will then migrate to local drainage lymph nodes via the tissue and local lymphatics (Harmsen et al., 1985). Kato et al. (2003) demonstrated that alveolar epithelial cells could phagocytose latex beads and transfer them by a process of transcytosis to monocytes in local capillaries. This may be a further route by which the adenovirus may reach a draining lymph node. Finally, Harmsen at al. (1987) showed that pulmonary neutrophils may also translocate particulate matter from the airspaces to draining lymph nodes. Thus

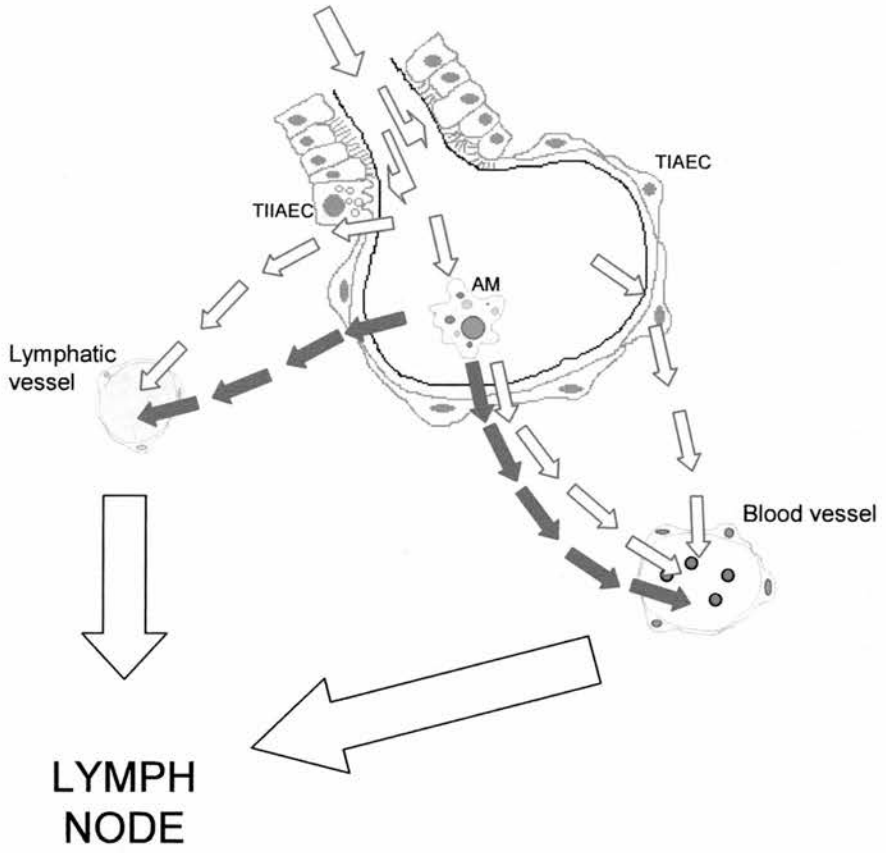

Fig. 23. Diagrammatic representation of the infection of the lung with adenovirus.

The diagram shows a schematic of the lungs anatomy in relation to instilled adenovirus. Free adenovirus/calcium phosphate is represented by open blue arrows. This has been shown in the sheep to infect type I and type II alveolar epithelial cells (TIAEC and TIIAEC) and also alveolar macrophages (AM). Free adenovirus may also be able to migrate to lymphatics in the lung and to blood vessels, and by either route arrive at local drainage lymph nodes. In addition, the infection of the alveolar epithelial cells may enable transcytosis of adenovirus to local blood vessels. Finally, adenovirus may arrive at local lymph nodes via mobile cells specifically the alveolar macrophages, but also possibly by air-space neutrophils (closed blue arrows).

there are a variety of routes through which locally administered adenovirus could reach the lymph node, either in a free form whereby it can infect permissive cells present there, or in infected cells which may continue to secrete elafin. Either would potentially result in a lymph node environment with a higher than normal concentration of elafin. As was discussed in the introduction and chapter 5 human elafin has been shown to be a substrate for transglutaminase and as such is cross linked to various proteins (Molhuizen et al., 1993; Nara et al., 1994; Steinert and Marekov, 1995) and in an in vivo setting elafin has been shown to be cross-linked to heavier and as yet unidentified proteins present in human BALF (Tremblay et al., 1996; Nara et al., 1994; Sallenave et al., 1999). The lack of data in this model in relation to elafin concentrations, whether in serum or BALF, is disappointing. Much effort was directed towards identifying secreted elafin in the BALF samples collected during this work but this task proved unrewarding as a consequence of limitations wth respect to the available antibodies and possible cross-linking of the secreted elafin with BALF or tissue components. However, given the likely initial sequence of events following Ad-o-elafin administration further consideration can be given to the mechanism of the contra-lateral effect in this model.

It is possible that elafin-rich epithelial lining fluid may have moved from the instilled segment to the naïve segment or that such fluid raised systemic levels of elafin by leakage. However, although unpublished data from intra-nasal Ad-human elafin administration to mice at up to 10^9 pfu elicited no detectable elafin in the serum of instilled animals both at five and nine days after administration (Simpson, 2002), intra-tracheal administration of $5x10^{10}$ pfu of an adenovirus encoding for human LL-37 in mice led to detection of LL-37 in the serum after 5 days which decreased to background again at 14 days whereas lung levels remained elevated until at least 21 days (Bals et al., 1999). Although dosage of virus in these respective models is of critical importance the notion that elafin protein may have translocated to other segments via the epithelial lining fluid or systemically, cannot be discounted.

It is potentially more plausible however that the modulation of the response is taking place either in the draining lymph nodes common to the experimental lung segments used or at the level of the

nervous system. Background data involving the modulation of dendritic cell function and lymphocyte phenotypic modulation are detailed in Appendix II but an examination of the specific mechanisms at play in this model system are naturally beyond the scope of this thesis.

7.4.6. The effect of local instillation of Ad-o-elafin on the neutrophil activation within the lung and on the systemic response to bacterial LPS.

The inflammatory profile seen in the cell counts was not reflected by the changes in myeloperoxidase (MPO) activity. The major source of MPO in the lung is the neutrophil (Schmekel, 1990). In the study presented here the MPO activity was not expressed per neutrophil in the BALF as some of the segments contained no neutrophils. When expressed in relation to the total cell count there was the suggestion that the cellular infiltrate in the elafin treated animals was less activated than that seen in the GFP animals.

The local stimulation of the LPS response (as assessed by BALF cellularity) in segments in animals that were treated with Ad-o-elafin (Ad+/LPS+ and Ad-/LPS+ segments) was not, however, reflected in the circulating leukocyte counts (figs. 19 and 20) which show a more marked response post-LPS in the GFP animals compared to the elafin animals.

The instillation of Ad-o-elafin into the ovine lung has a dual effect on the response to the segmental administration of bacterial LPS, an up-regulation of the local response to LPS and a dampening down of the circulating leukocytosis caused by the administration of LPS. Conversely, the systemic anti-adenovirus antibody levels are increased in the elafin group compared to the GFP group.

The reason for the dampening down of the systemic response to intra-pulmonary LPS in the sheep which were instilled with Ad-o-elafin compared to those which received Ad-GFP (see figs. 19-21) remains at this point unresolved. The only other model system in which this scenario has been

replicated is in mice transgenic for human elafin (Sallenave et al., 2003). These animals showed an increase in LPS-induced white cell influx into the lung but also showed reduced serum TNF-α levels after intra-peritoneal LPS. The authors suggest that these results are due to elafin having an effect on the cytokine gradient between systemic circulation and the lung itself. Again, it is an attractive mechanism to explain the ovine model results but since cytokine data are lacking this must remain purely speculation.

7.5. CONCLUSIONS

The work presented in this chapter utilised the advantages inherent in the ovine segmental lung model in order to expand our current knowledge with regards adenoviral gene therapy in the lung. Specifically this work demonstrated a systemic response to adenoviral vectors which limits repeated administration. The low doses administered in this chapter caused relatively mild inflammation within the lung itself but the up-regulation of ovine elafin does have a significant effect on the response to bacterial LPS both within the lung itself and in the systemic white cell counts. This result adds to our knowledge of the functions of elafin from rodent model systems (both models of inflammation and transgenic animals) and is important for the future development of adenoviral delivery of elafin to regulate the inflammatory response with benefits to the recipient animal.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The data presented within this thesis can readily be sub-divided into three sections.

In the first, data is presented in relation to the identification and characterization of the ovine orthologs of the anti-proteases elafin and secretory leukoprotease inhibitor (SLPI) at the gene, cDNA and protein levels. While both ovine elafin and SLPI have features characteristic of their respective families, the ovine form of elafin has been shown to exist in allelic forms, a situation not previously demonstrated within the trappin family. Both ovine elafin and SLPI are shown to display characteristics of potential value in the context of modulating inflammation in the lung.

The second section details the construction of an adenoviral vector encoding one of the ovine elafin alleles (Ad-o-elafin) and the preliminary *in vitro* investigation of methods to maximise the infection efficiency of adenoviral vectors. The section culminates in the demonstration of local up-regulation of ovine elafin following lung instillation in the sheep. The efficiency of this system was augmented by co-precipitating the vector with calcium phosphate. The target cell population consisted primarily of alveolar macrophages.

The final section examines the influence that local administration of Ad-o-elafin has on the local and systemic innate immune response to LPS. The data suggests a role whereby this anti-protease mediates effects specific to tissue and systemic compartments, namely an up-regulation of the LPS response within the Ad-o-elafin treated lung concurrent with a down-regulation of the LPS response systemically. Additionally, the *in vivo* experimentation identified a 'contra-lateral' effect not previously documented within the field of pulmonary gene therapy whereby the up-regulation of ovine elafin within one lung segment influences the innate response within a separate, discrete segment. This observation is potentially of major interest with regards the modulation of inflammation at sites distant to that exposed to gene transfer agent. Fig. 1 shows a summary of the results of this work whereby segmental administration of Ad-o-elafin enhances the LPS-induced leukocyte influx within the same segment (segment B) and also to a distant segment within the opposite lung (segment C), in addition to decreasing the systemic response to intra-pulmonary LPS. The potential mechanisms for this action have been discussed in chapter 7 and indeed further in Appendix 2. Briefly, it is

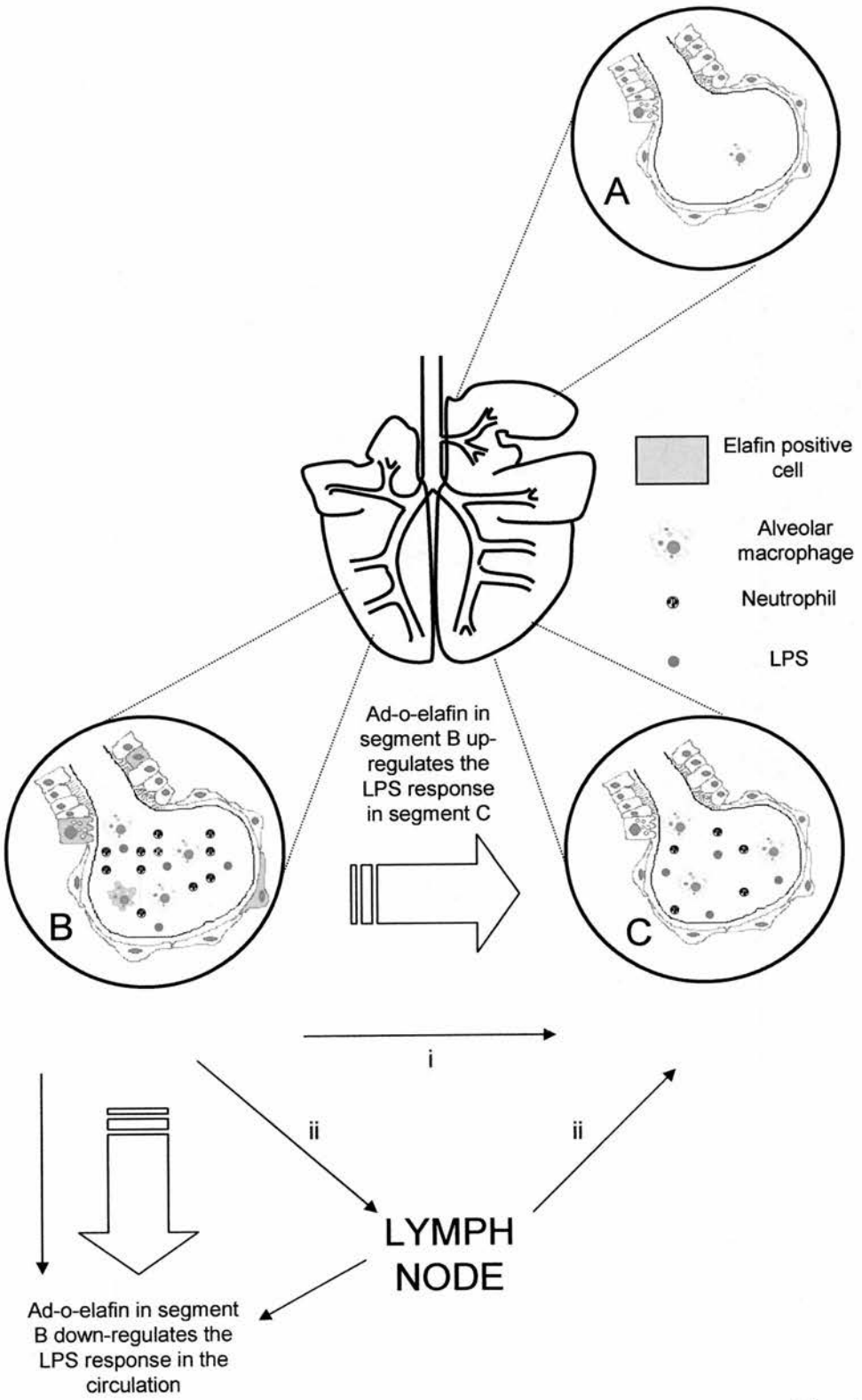
A
B
C
Elafin positive cell
Alveolar macrophage
Neutrophil
LPS
Ad-o-elafin in segment B up-regulates the LPS response in segment C
i
ii
ii
LYMPH NODE
Ad-o-elafin in segment B down-regulates the LPS response in the circulation

Fig. 1...

Fig. 1. Diagrammatic summary of the modulation of the LPS response by local administration of Ad-o-elafin.

The administration of Ad-o-elafin to segment B augments the LPS-induced leukocyte infiltration in both the virus instilled segment (segment B) and a distant segment which received LPS alone (segment C), but has no effect on the BALF cellularity in a naïve segment which receives neither Ad-o-elafin nor LPS (segment A).
Additionally, the administration of Ad-o-elafin to segment B causes a down-regulation of the LPS induced circulating leukocytosis.
The most likely mechanism for this response (as discussed within the text) is a migration of a distinct cellular population from the treated region to other sites of inflammation (signified by the line arrows i and ii).

considered that the most probable explanation involves the movement of a specific cell population away from the site of adenoviral instillation to directly or indirectly influence responses elsewhere within the same animal. Although this could involve infected cells (dendritic cells, alveolar macrophages) it is equally possible that the high local concentrations of elafin might influence migration and/or differentiation of an alternative cell population. As discussed previously the lymph system within the lung is a potential 'bridge' between adenoviral treated and non-treated segments either via the lymphatic network in the lung or via the blood stream. Work within this thesis has demonstrated the efficient infection of alveolar macrophages within the treated segments with no apparent migration of this cell type to naive segments. However, it is possible that lymphatic migration might indeed occur and lead to immune modulation at the level of the draining lymph node itself.

A natural progression of the work presented in this thesis would be aimed at defining the anatomical and immunological mechanisms behind the actions of elafin. A preliminary experiment of great interest would be the *ex vivo* infection of alveolar macrophages with recombinant adenovirus (again comparing Ad-o-elafin with Ad-GFP) followed by adoptive transfer to recipient animals and subsequent analysis of the response to intra-pulmonary LPS. This would yield information as to whether the alveolar macrophages are the pivotal cell population underlying the observed local and systemic effects and indeed whether the infection of these cells with Ad-o-elafin is the reason behind the marked increase in circulating neutralising antibody levels.

Equally important would be tracing any presumed migration of these cells to facilitate investigation of the mechanisms underlying both the systemic and 'contra-lateral' effect within the lung. Labelling of alveolar macrophages infected with Ad-o-elafin *ex vivo* would potentially add to our knowledge regarding the interactions of this cell type with other cell populations after leaving the bronchoalveolar milieu. Knowledge of local lung cytokine regulation as a consequence of elafin expression would be of value in relation to both defining the specific nature of the innate immune response in this model and relating such amongst the comparative species where such data exists. In parallel to the work within this thesis, other investigators in Edinburgh have demonstrated the utility of real time PCR on

RNA samples isolated from ovine bronchial wall brushings and also from the BALF cell pellet and have developed repeatable assays for a panel of cytokines which were previously unavailable.

The identification of cellular migration is obviously time dependant and as such an analysis of the time-course of transgene activity would be of benefit both with regards the localised secretion into the BALF (by the development of an o-elafin specific ELISA) and the sensitive detection of o-elafin containing cells which have left the bronchoalveolar compartment (by immunohistochemistry). The presence of detectable o-elafin within the bronchoalveolar lavage fluid (BALF) itself would prompt analysis of transglutaminase activity (in addition to metabolites of this important enzyme) within the BALF as this may have an influence on the inflammatory milieu as discussed within Appendix II.

Several avenues of investigation would benefit from the identification of the ovine cytokines mentioned previously, Indeed, the consequence of intra-pulmonary elafin secretion on remote organ responses would be of direct relevance to the pathogenesis of ARDS as a consequence of sepsis. In this regard, tracking LPS responses in the skin as well as in the lung after elafin up-regulation would offer further insight into the wider potential benefits of local gene therapy protocols.

Additionally, the segmental up-regulation of ovine elafin by the use of Ad-o-elafin may have beneficial effects with regards the lung injury seen after systemic LPS administration. Indeed the systemic administration of bacterial LPS to sheep is a well characterised model of the acute respiratory distress syndrome (ARDS) and has been used to examine the effects of potential therapies. As discussed in Chapter 1, interest has focused on the compartmental effects of Il-10 on the lung's immune response. (see 1.3.2) The ovine model used within this thesis presents an ideal opportunity to examine the situation with regards the localised expression of elafin in similar situations. Equally, the converse situation is of potential interest whereby the effects of systemic administration of Ad-o-elafin on the pulmonary response to LPS could be examined (see 1.3.3).

These different strategies aimed at examining the local and systemic modulation of inflammatory processes by the application of compartmentalised adenoviral gene transfer will be of major benefit with regards the future development of relevant therapies and the implications in terms of safety and delivery strategies in compromised patients are obvious.

Finally, the presence of o-elafin at sites of continuous bacterial contamination as discussed in chapters 3 and 5 of this thesis acts as a reminder of the potential for this protein, and indeed o-SLPI, as a local

modulator of inflammation with a broad range of actions. So far there has been limited progress in relation to examining the potential interactions between these anti-proteases and other areas of the immunological response. Indeed, it would be of particular interest to examine the relationship of these anti-proteases to the γδ subset of T lymphocytes which have been recognised as important both in the primary immunological defense of the body and also in the pathogenesis of acute lung injury and its sequelae. Given that local expertise exists with regards the identification of ovine lymphocyte subsets and the identification of these in the ovine models utilised here, further investigation is warranted.

In summary, the experimental data presented has expanded upon our current knowledge of the use of adenoviral vectors in the lung, the sheep as a model for respiratory gene transfer, and the functions of elafin in localised inflammatory responses. As anticipated the work poses new questions of direct relevance to gene therapy and the modulation of inflammation in the lung.

APPENDICES

APPENDIX I

BALF Neutrophil counts in animals receiving three instillations of Ad-o-elafin (see 6.4.3).

	Animal 1	Animal 2	Animal 3	TT cf pre	TT cf naïve
Pre-experimental	500	16830	3363	1	0.54
Naïve	1554	6573	2056	0.54	1
Vehicle alone	6006	4874	1390	0.62	0.76
1x10^7 pfu Ad-o-elafin	2445	11606	1801	0.80	0.62
5x10^7 pfu Ad-o-elafin	3651	3212	1946	0.48	0.80
1x10^8 pfu Ad-o-elafin	19791	22576	9190	0.19	0.03

The table shows the absolute neutrophil counts per ml BALF pre-experimentally (day -7) and 48 hours after the instillation of PBS+CaP, 10^7 pfu Ad-o-elafin/CaP, $5x10^7$ pfu Ad-o-elafin/CaP, and 10^8 pfu Ad-o-ealfin/CaP. An additional naïve segment was lavaged after 48 hours. Student's T Test probability values are shown comparing the data from the treated segments either with the pre-experimental results or the naïve results at 48 hours.

Total protein levels in segments post-LPS treatment.

A

	Elafin Pre-experimental	Naïve day 14	LPS day 14	LPS/V day 14
	586.1	448.7	616.3	673.4
	370.1	359.8	432.9	435.2
	517.0	309.0	332.8	1198.3
	725.9	725.1	617.9	602.8
	849.7	461.4	712.3	579.0
	577.4			
	373.3			
	645.7			
median	581.7	448.7	616.3	602.8
Q1	481.1	359.8	432.9	579.0
Q3	665.7	461.4	617.9	673.4

B

	GFP Pre-experimental	Naïve day 14	LPS day 14	LPS/V day 14
	586.1	351.9	220.8	282.0
	370.1	679.8	367.0	596.4
	517.0	364.6	790.2	281.2
	725.9			
	849.7			
	577.4			
	373.3			
	645.7			
median	581.7	364.6	367.0	282.0
Q1	481.1	358.2	293.9	281.6
Q3	665.7	522.2	578.6	439.2

Tables A and B show the total protein content of the BALF pre-experimentally (day -7) and at day 14 for naïve segments, segments which received LPS at day 10 and segments which were instilled with adenovirus at day 0 and then LPS day 10. Table A shows the total protein for the animals which were instilled with Ad-o-elafin and table B the animals which were instilled with Ad-GFP. All data shows individual animal results along with the median, upper quartile (Q1), and lower quartile (Q3).

None of the medians are significantly different from each other when compared by Mann-Whitney analysis.

APPENDIX II

Possible mechanisms for ovine elafin mediated modulation of dendritic cell function.

The effects that the potentially defensin-like molecule ovine elafin may have on dendritic cell function could possibly have functional relevance with respect the 'pro-inflammatory' effects seen within the lung after instillation with Ad-o-elafin and then bacterial LPS (see Results section chapter 7). There are many possible mechanisms to explain this up-regulation which is seen not only in the virus instilled segments but also in 'naïve' segments in the contra-lateral lung and these were discussed briefly in the Discussion section of chapter 7. It is of interest to discuss potential dendritic cell modulation here as the effects of human elafin on murine dendritic cell functions are being investigated within this laboratory currently. Unfortunately the mechanism in the sheep remains pure conjecture at this moment.

The basis behind the hypothesis presented here is that the development of dendritic cells in a specific milieu alters the subsequent phenotype of the dendritic cells. This is the fundamental principle behind the work of Davidson et al. (2004) who has shown that the *ex vivo* culture of murine monocytes in the presence of the human cationic peptide LL-37 along with factors to direct them towards a dendritic cell phenotype has important effects on the functioning of the resultant immature dendritic cells (iDCs). For example, LL-37 derived iDCs show an altered degree of interaction with antigen as predicted by an alteration in surface receptor expression. Additionally, LL-37 derived iDCs showed a characteristic Th-1 inducing cytokine response after maturation with LPS and stimulated increased proliferation of T cells secreting IFN-γ. This work is of fundamental interest with regards the data presented in this chapter as LL-37 shows many similarities to elafin. LL-37 is the cleavage product of the human cathelicidin hCAP-18 (Lehrer and Ganz, 2002) and as such is a cationic secreted protein produced by many inflammatory white cells and epithelial cells. Of particular interest is its presence in BALF and indeed its up-regulation in states of inflammation (Schaller-Bals et al., 2002). Ovine elafin is up-regulated during acute pulmonary inflammation induced by bacterial LPS (chapter 5) and other work in this laboratory supports this finding (Sallenave, 2000). Therefore, in an inflamed lung there are potentially high elafin levels in the epithelial lining fluid. Following up-regulation using an

adenoviral vector, we can expect even higher local concentrations compared to a viral control. Such elevated concentrations of elafin could potentially have modulatory effects on tissue dendritic cells in the lung in a similar way to LL-37, and have an influence on the subsequent response after migration of the mature dendritic cell to the local drainage lymph node.

In addition, the secreted protein murine β-defensin-2 has been shown to have a direct maturing effect on DCs via a TLR-4 dependant mechanism (Biragyn et al., 2002) which is not seen in the LL-37 work discussed above. The β-defensins are also cationic proteins (Lehrer and Ganz, 2002) and so this maturation may also be a possible consequence of increased elafin levels locally. Interestingly, the prostaglandin PGE_2 has been shown to increase iDC maturation and promotes a Th-2 response (Kalinski et al., 1998) while PGE_2 blockade induces IL-12 release from DCs (Harizi et al., 2001). PGE_2 is a product of phospholipase A2 (PLA2) activity on membrane phospholipids and as such may be influenced by transglutaminase inhibition as seen in a guinea pig model of conjunctivitis (Sohn et al., 2003). In guinea pig models of LPS induced acute lung injury alveolar macrophages are one of the major contributors of secretory PLA2 in the BALF (Arbibe et al., 1997). The secretion of PLA2 *ex vivo* induced by LPS was shown to be TNFα dependant and so the alveolar macrophages may be regulating secretion in an autocrine fashion. This increase in secretory PLA2 has also been shown to increase the levels of fatty acid and lyso-phosphatidylcholine levels in the BALF post LPS, substances which are injurious to the lung cellular membranes in acute lung injury (Arbibe et al., 1998). So the PGE_2 may have direct effects on DC function but also the enzymatic cascade results in direct lung injury.

A diagrammatic representation of the possible interactions between elafin and dendritic cell functioning along with inhibition of transglutaminase mediated phospholipase A2 stimulation are shown in fig. 1 (App.).

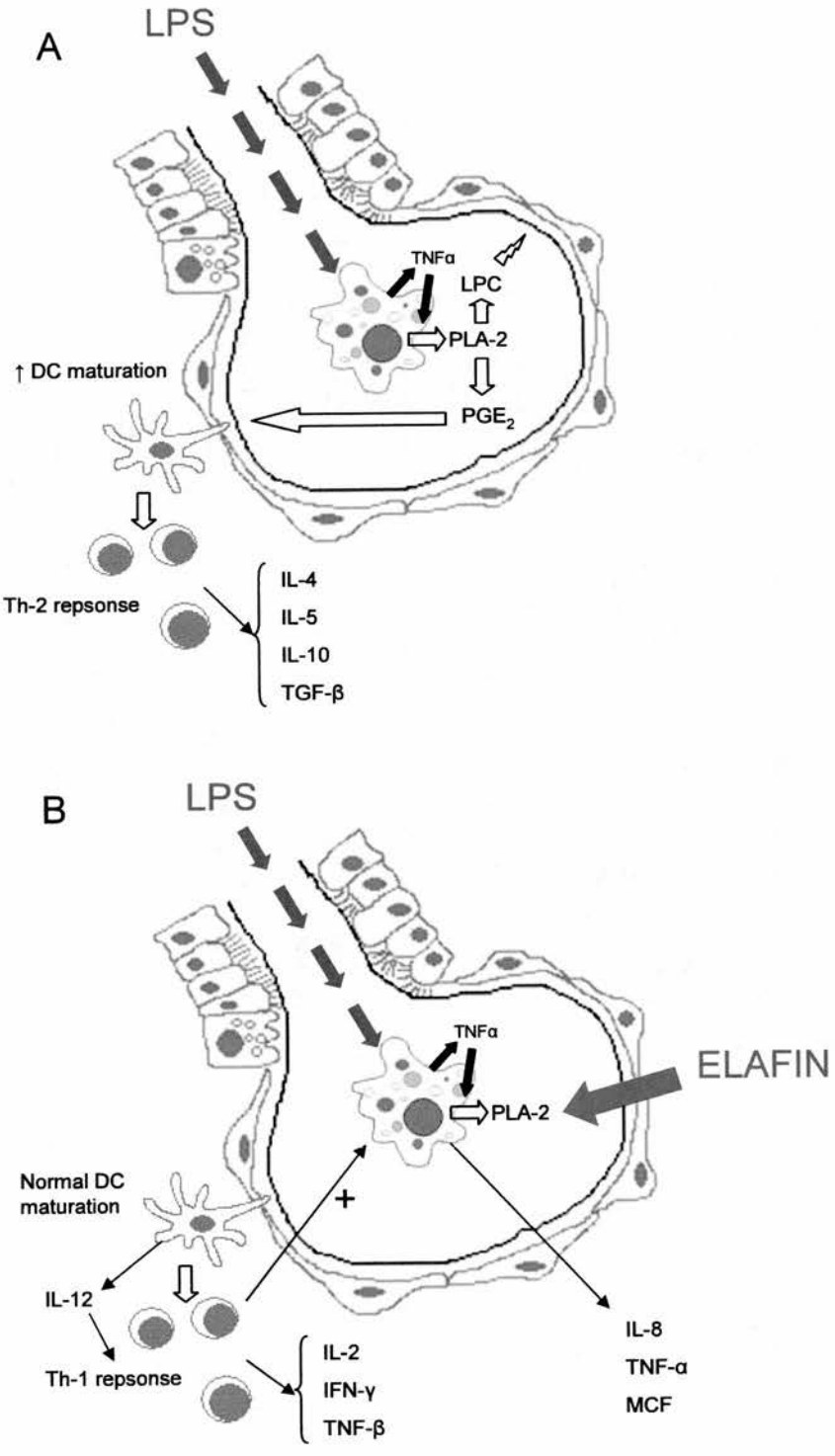
A
LPS
TNFα
LPC
PLA-2
PGE2
↑ DC maturation
Th-2 repsonse
IL-4
IL-5
IL-10
TGF-β
B
LPS
TNFα
ELAFIN
PLA-2
Normal DC maturation
+
IL-12
Th-1 repsonse
IL-2
IFN-γ
TNF-β
IL-8
TNF-α
MCF

Fig. 1 (App.)...

Fig. 1 (App.). Diagrammatic representation of the possible effects of elafin as an inhibitor of PLA2 in the lung.

The diagram shows a schematic of the lungs anatomy in relation to instilled LPS and the modulation of the subsequent actions of the secreted PLA2 by the presence of increased amounts of ovine elafin.

A. This part of the diagram is the sequence of events with regards PLA2 activity in the normal lung. LPS activates alveolar macrophages (AM) to secrete TNFα which in turn stimulates the AMs to secrete PLA2 which then leads to the production of harmful lyso-phosphatidyl choline (LPC) and also PGE_2. PGE_2 stimulates the maturation of local dendritic cells (DC) and favours the clonal expansion of Th-1 lymphocytes which secrete IL-4, IL-5, IL-10 and TGF-β.

B. In the presence of up-regulated amounts of ovine elafin, the PLA2 activity of the lining fluid will be inhibited and so harmful products such as LPC will not be formed. In addition the DCs will mature normally and a Th-1 phenotype favoured with the lymphocytes producing IL-2, IFN-γ and TNF-β. These Th-1 lymphocytes can then further activate the macrophages to produce increased levels of TNFα, IL-8 and MCF, the latter of which may lead to increased chemo-attraction of neutrophils and macrophages into the air-spaces.

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PUBLICATIONS ARISING FROM THIS THESIS

One publication has arisen from the work presented within this thesis and an additional manuscript has been submitted for review.

Physiol Genomics 19: 11–21, 2004.
First published August 3, 2004; doi:10.1152/physiolgenomics.00113.2004.

CALL FOR PAPERS | *Comparative Genomics*

Trappin ovine molecule (TOM), the ovine ortholog of elafin, is an acute phase reactant in the lung

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Submitted 12 May 2004; accepted in final form 16 July 2004

Brown, Thomas I., Rohit Mistry, D. David Collie, Steven Tate, and Jean-Michel Sallenave. Trappin ovine molecule (TOM), the ovine ortholog of elafin, is an acute phase reactant in the lung. *Physiol Genomics* 19: 11–21, 2004. First published August 3, 2004; doi: 10.1152/physiolgenomics.00113.2004.—As large animal models continue to play an important role in translating lung-directed therapeutic strategies from laboratory animals to humans, there is an increasing interest in the analysis of endogenous regulators of inflammation at both a genomic and a therapeutic level. To this end, we have sought to characterize the ovine ortholog of elafin, an important regulator of inflammation in humans. We have isolated both the elafin cDNA and gene, which have a similar structure to other species' orthologs. Interestingly, we have isolated two alleles for ovine elafin, which contain a very high number of transglutamination repeats, thought to be important in binding elafin to the interstitium. The mainly mucosal mRNA distribution for ovine elafin suggests that ovine elafin may, like its human ortholog, have functions in innate immunity. This is supported by analysis of elafin and the related protein secretory leukocyte protease inhibitor (SLPI) in ovine bronchoalveolar fluid in response to locally administered lipopolysaccharide and confirmation of them acting as "alarm" antiproteases. We have also cloned the ovine elafin cDNA into an adenoviral vector and have demonstrated correct processing of the secreted protein as well as biological activity. Overexpression of ovine elafin in a lung-derived epithelial cell line has a protective effect against the enzymes human neutrophil and porcine pancreatic elastase. The identification of the ovine elafin gene and its translated protein are important in developing practical strategies aimed at regulating inflammation in the large mammalian lung.

antiprotease; elastase; sheep

THE TRAPPINS [acronym for "transglutaminase substrate and WAP (whey acidic protein) domain containing protein"; Ref. 27] are a group of proteins characterized by the presence of a NH_2-terminal transglutaminase substrate ("cementoin") domain and a C-disulfide core domain, also called WAP domain, which contains, in some members, an antiprotease active site. Current knowledge suggests that the two domains may have evolved from exon shuffling and gene duplication between rapidly evolving seminal vesicle transcribed (REST) genes and the WAP domains of ancestral genes (3). Indeed, in humans, colocalization of the human trappin-2 gene (also known as elafin/elastase-specific-inhibitor/SKALP) and the secretory leukocyte protease inhibitor (SLPI) gene (which contains two WAP domains) in the same region of chromosome 20 is in agreement with this hypothesis (8, 12). Our group is particularly interested in the study of elafin, which was identified as an antielastase inhibitor in the early 1990s (19, 21, 26, 35). The closely related protein SLPI also has important functions as an inhibitor of neutrophil elastase (Ref. 20 and references therein), and both molecules have been found to be present in humans at mucosal sites and also have been shown to be synthesized by epithelial cells and alveolar macrophages. In addition, it has recently been demonstrated that both elafin and SLPI have antimicrobial functions and may be able to prime the innate immune system (20).

More than 15 trappins and many more WAP-containing proteins have been identified to date, throughout the animal kingdom. Although the sequence in the WAP domain predicts that a number of trappin species orthologs are antielastase molecules, this has only formally been demonstrated for human and porcine elafin (19, 21, 26, 32, 35).

Because of our interest in the modulation of lung inflammation in ovine models (2) and the known modulatory activity of antiproteases (20), we set out to isolate the ovine elafin cDNA and gene ortholog and to study its properties.

We identified in this study two allelic forms of the ovine elafin gene (named TOM-1 and TOM-2, for "trappin ovine molecule" 1 and 2) and analyzed their mRNA distribution in a variety of tissues. Furthermore, we have demonstrated that secreted ovine elafin can protect a lung-derived epithelial cell line from proteolytic damage due to both human neutrophil elastase (HNE) and porcine pancreatic elastase (PPE). The distribution, antielastase activities, and kinetics of expression in vivo of ovine elafin predict that it may also have mucosal protective functions in inflammation. This should prove a valuable tool in developing therapeutic strategies in a large animal model of lung inflammation, currently being developed in our laboratories.

EXPERIMENTAL PROCEDURES

Molecular biology reagents. Primers were synthesized and supplied by MWG-Biotech (Milton Keynes, UK). Primer design for 5′-rapid amplification of cDNA ends (RACE) (GSP1) was based on preliminary ovine elafin sequence (R. Mistry, unpublished data). Subsequent primers were based on the newly determined ovine elafin

Article published online before print. See web site for date of publication (http://physiolgenomics.physiology.org).

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sequence: elafin gene-specific reverse primer (GSP1) 5′-CAT GGC GCA CCG GAT CA-3′; elafin gene-specific forward primer (GSP2) 5′-ATG AAG ACC AGA AGC TTC TTG GTC C-3′, full-length reverse primer (r-el) 5′-CTG GGG ATC CAT ACA GGT CTT-3′; *Bgl*II forward elafin primer (Bgl-f-el) 5′-GCC GGA GAT CTG **ACA ACA** TGA AGA CCA GAA GCT TCT TGG TC-3′ (*Bgl*II site is shown with underscore; Kozak sequence in bold); *Sac*I elafin reverse primer (Sac-r-el) 5′- TAC GTG AGC TCT CAT CAC TGG GGA TCC ATA CAG GTC TT-3′ (*Sac*I site is shown with underscore); forward bacterial artificial chromosome (BAC) primer (fBAC) 5′-TGA AAA GGG TCC AAT CAA CG-3′; reverse BAC primer (rBAC) 5′-GAG CCA CGC TTA GTG AGG AG-3′.

Taq DNA polymerase, 25 mM magnesium chloride, DNA polymerase buffer, dNTPs, oligo (dT) primer, *Eco*R1, Buffer H for *Eco*R1, *Bgl*I, Buffer D for *Bgl*II, *Sac*I, Buffer J for *Sac*I, MMLV reverse transcriptase (MMLV RT), and 5× MMLV RT buffer were supplied by Promega UK (Southampton, UK).

Shuttle vector pDC516 was obtained from Microbix Biosystems (Toronto, Ontario, Canada). Shuttle vector pIRES-GFP3 was kindly donated by Dr. Mary Hitt (Dept. of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada).

RNA preparation and RACE. Ovine tissues were obtained locally from a selection of 5-mo-old crossbred lambs (Moredun Research Institute, Edinburgh, Scotland, UK). The tissue samples obtained from sheep organs were treated as follows. Samples of each tissue were removed at autopsy and frozen at −80°C. Approximately 1 g of each of these was then homogenized, and the RNA was extracted using TRIzol (Invitrogen, Paisley, UK). RNA quality was assessed by running 10 μl on a 1% agarose gel. Quantification of RNA in samples was by dual-wavelength spectrophotometry.

RACE-PCR for ovine elafin was performed using ovine tracheal mucosal RNA. First, to obtain 5′ information, we used a primer specific for ovine elafin, GSP1 (derived from partial sequence already obtained from preliminary experiments, not shown), and the 5′ forward GeneRacer Primer. This was followed by a nested PCR using GSP1 and nested 5′ forward GeneRacer Primer. Cycling parameters were as follows: initial incubation of 92°C for 5 min, followed by 92°C for 1 min, 55°C for 1 min, and 72°C for 1 min (30 cycles followed by a final amplification step of 72°C for 5 min). Products were run out on a 1% agarose gel. These conditions were used for both the original and nested PCRs.

To perform the 3′-RACE, a slightly different strategy was used. Reverse transcription of the original RNA sample was performed using the GeneRacer oligo-dT supplied in the GeneRacer kit and MMLV RT (Promega). PCR was then performed using the forward primer GSP2 and the reverse GeneRacer primer to obtain the full-length cDNA sequence. GSP2 was designed from the sequence derived from the 5′-RACE procedure. PCR conditions were similar to those above except for a magnesium chloride concentration of 2.2 mM. This was followed by a nested PCR using GSP2 and nested reverse GeneRacer primer.

DNA preparation and sequence analysis. Genomic DNA was extracted from lung and liver samples of the above animals by proteinase K digestion in NTES (50 mM Tris, 50 mM EDTA, 100 mM NaCl, 1% SDS, pH 8), followed by phenol/chloroform extraction and washing with 100% isopropanol followed by 70% ethanol. The DNA was resuspended in water and kept at −20°C.

DNA sequence analysis was done both in house and by MWG-Biotech, and the sequence data were analyzed with the online computer software packages NCBI Open Reading frame finder [National Center for Biotechnology Information (NCBI)], NCBI Blast (NCBI), and BCM Multiple Sequence Alignment tools (BCM).

Extraction of PCR products and cloning reactions. All PCR products were run on 1% agarose gels, and bands of interest were cut out and purified using the QIAQuick gel extraction kit (Qiagen, Crawley, W. Sussex).

Cloning of the extracted RACE cDNA fragments was achieved by using the TOPO TA cloning kit for sequencing included with the GeneRacer kit. The cDNAs were ligated into pCR 4-TOPO plasmid followed by transformation into TOP10 *Escherichia coli* cells (Invitrogen). Plasmid DNA was purified with the Promega SV Miniprep system.

BAC library screening and subcloning. An ovine BAC library was screened using primers specific for elafin by Dr. F. Piumi at Institut National de la Recherche Agronomique (INRA), Laboratoire mixte INRA-CEA de Radiobiologie et d'Etude du Genome (LREG), Jouy-en-Josas, France. One positive clone was isolated and DNA was prepared with Qiagen Plasmid Maxi kit (Qiagen, Crawley, UK). One microgram of the BAC-positive clone was digested with *Eco*R1, run out on a 1% agarose gel, and blotted onto GeneScreen membranes [PerkinElmer Life Sciences (UK), Cambridge, UK]. This was probed for 1 h with [α-^{32}P]dCTP-labeled full-length cDNA coding for ovine elafin, using ExpressHyb (Clontech, Palo Alto, CA) following the manufacturer's protocol. Positive bands were identified with a Storm PhosphorImager system (Amersham Biosciences UK, Little Chalfont, UK) and further subcloned into plasmid PDC516 for sequencing using various internal primers.

Tissue screening of ovine elafin by Northern blot and RT-PCR analysis. Twenty micrograms of each tissue RNA sample was denatured in 50% formamide, 16% formaldehyde, 20 mM MOPS, pH 7.0, 5 mM sodium acetate, and 1 mM EDTA, for 15 min at 65°C, electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde, and transferred to a GeneScreen membrane. The membrane was hybridized with [α-^{32}P]dCTP-labeled full-length cDNA coding for ovine elafin in ExpressHyb following the manufacturer's recommendations. The membrane was then visualized on a Storm PhosphorImager system.

One microgram of each tissue RNA sample was used in a reverse transcriptase reaction using MMLV RT (Promega). Product from this reaction was split into two PCRs, using either forward and reverse β-actin primers or the full-length elafin primers GSP2 and r-el. Conditions were as for the RACE elafin PCR except for there being 35 cycles for elafin and 28 cycles for the β-actin.

Comparison of ovine elafin products synthesized by PCR from positive BAC clone, genomic DNA, and RT-PCR synthesized cDNA. To compare the elafin cDNA, identified by the RACE procedure, with the gene sequences, PCR was performed using as template purified elafin BAC DNA and ovine liver DNA alongside cDNA synthesized from ovine skin and pIRES-GFP-ovine elafin, using the non-intron-spanning primers fBAC and rBAC. PCR parameters were as for the RACE elafin PCR. Products from the genomic DNA PCR were ligated into pCR 4-TOPO plasmid, which was transformed into TOP10 *E. coli* cells. Plasmid was extracted with Promega SV Miniprep system, and elafin sequences were sequenced.

An additional PCR was performed on ovine genomic DNA using the primers GSP2 and r-el. Products from this PCR were also cloned into pCR 4-TOPO as above and sequenced.

Phylogenetic tree analysis of WAP domains of the elafin and SLPI family members. To investigate the phylogenetic relationships of the ovine forms of elafin, we constructed a phylogenetic tree by the neighbor-joining method (18) using data from multiple alignment of the amino acid sequences of the whey acidic protein (WAP) domains of members of the trappin family, the known SLPI sequences (human, mouse, rat, pig, and sheep), the murine elafin-like proteins 1 and 2 (5), and the human vasopressin molecule, which contains a rudimentary WAP motif and can be assumed to be evolutionarily related to the elafin and SLPI families of proteins.

Lipopolysaccharide-induced upregulation of ovine elafin and SLPI in bronchoalveolar lavage fluid. Bronchoalveolar lavage fluid (BALF) was collected to assess the regulation of ovine elafin and SLPI following local lung challenge with LPS. Briefly, 12 crossbred sheep (10 female and 2 male) were anesthetized with 20 mg/kg intravenous thiopentone (Intraval sodium; Merial Animal Health, Harlow, Essex,

UK), intubated, and maintained on 2–3% halothane in oxygen and nitrous oxide. Lipopolysaccharide (LPS), 1 mg, from *E. coli* O26:B6 diluted in 5 ml distilled water was instilled via bronchoscope into random segments. All experimental animal procedures were approved by the Ethical Review Committee of the University of Edinburgh. Bronchoalveolar lavages from these sheep were collected a week prior to the experiment and 6, 24, and 168 h after the administration of LPS. The lavages were spun at 400 *g* for 7 min, and 200 μl of the resultant supernatant was used in a dot blot onto GeneScreen membrane and blocked with 5% milk powder in PBS + 0.1% Tween-20 (TPBS). The membrane was then probed with either polyclonal antihuman SLPI antibody (24) or monoclonal TRAB2O antielafin antibody (HyCult Biotechnology, Uden, the Netherlands). Resultant films after exposure to X-Omat radiograph-quality film were scanned, and densitometry measurements taken using a white-light transilluminator (UVP, Upland, CA). Control membranes probed with secondary antibody alone showed no detectable protein (not shown).

Construction of adenoviral vector expressing ovine elafin. Full-length ovine elafin cDNA in pCR 4-TOPO (see above) was used as a template for two consecutive PCRs with first Bgl-f-el and FLAG-r-el, followed by Bgl-f-el and Sac-r-FLAG-el (to yield *Bgl*I-elafin-FLAG-*Sac*I). PCR conditions were as for the RACE PCR for elafin. This product was ligated into shuttle vector pDC516 [which contains the murine cytomegalovirus (MCMV) promoter] using standard techniques.

This plasmid was used to create a replication-deficient adenovirus (Ad) by cotransfection of type 293 cells with pBHGfrt(del)E1,3FLP (a kind gift from Dr. M. Hitt, McMaster University). Ad-ovine elafin was obtained through homologous recombination between the two plasmids using the FLP recombinase/frt mechanism (15).

The presence of the correctly inserted ovine elafin cDNA into the viral genome was confirmed by *Hin*dIII digestion followed by Southern blot analysis (not shown). Infection of A549 cells with this virus confirmed correct processing of the ovine elafin protein (not shown).

Adenovirus-mediated protection of A549 cells against proteolytic enzyme damage. A549 cells (11) were plated out in 48-well plates at 50,000 cells/well and kept in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), penicillin G (final concentration 100 U/ml), streptomycin sulfate (final concentration 100 μg/ml), and L-glutamine (final concentration 2 μM) overnight at 37°C. The following day the cells were washed with fresh medium and incubated for 45 min with Ad-ovine elafin, Ad-human elafin (25), Ad-murine SLPI (6), or Ad-murine eotaxin (6) in fresh medium. The cells were then washed with serum-free DMEM and medium replaced with DMEM containing 2% USG serum substitute (GIBCO-BRL). After 72 h the medium was removed, the cells were washed twice with PBS, and medium was replaced with fresh DMEM with no serum or USG and containing 1 μg/ml HNE (Elastin Products, Owensville, MO) or PPE (Sigma-Aldrich, Dorset, UK). Plates were incubated overnight and photomicrographs taken.

To quantify elastase-mediated injury, medium containing detached cells was spun at 13,000 rpm for 5 min. The cell pellet was then washed in sterile PBS twice and resuspended in distilled water, followed by two successive freeze/thaw rounds. The suspension was further centrifuged (13,000 rpm, 5 min), and the supernatant was assessed for protein content (indirect readout of cell detachment) using the bicinchoninic acid method (Pierce) using purified albumin (Pierce) as standards.

Residual HNE and PPE activity in the supernatants was assayed in 96-well microtiter plates. Ten microliters of supernatant diluted with 40 μl assay buffer (50 mM Tris, 0.1% Triton, 0.5 M NaCl, pH 8.0) was incubated at 37°C with the chromogenic substrates *N*-methoxy-succinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (for HNE activity) or *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide (for PPE activity) diluted to 1 μg/ml in assay buffer. Both chromogenic substrates were supplied by Sigma-Aldrich, Dorset, UK. Enzyme activity was shown by a change in absorbance measured at 405 nm over 90 min.

RESULTS

Isolation and sequence analysis of ovine elafin cDNA. Using ovine tracheal mucosal RNA, and PCR primers annealing to a portion of the ovine elafin sequence already available (R. Mistry, unpublished observations), with 5′-RACE PCR we produced a product of 612 bp, which was cloned into pCR 4-TOPO plasmid for sequencing. This PCR product contained the sequence corresponding to the GeneRacer oligo ligated to the 5′ terminus of the mRNA followed by 18 bp of 5′-untranslated region (UTR) and the ovine elafin cDNA sequence through to the priming site for the gene-specific primer. This information allowed 3′-RACE to proceed. This yielded a product of 887 bp, which was also cloned into pCR 4-TOPO plasmid for sequencing and was shown to contain sequence 3′ of the stop codon corresponding to 3′-UTR through to the polyadenylation signal. This product terminated with the sequence corresponding to the GeneRacer oligo-dT sequence used in the reverse transcription step of the RACE procedure. Altogether, the 5′- and 3′-RACE procedure allowed us to identify an open reading frame for a protein of 220 amino acids which showed homology with human, simian, porcine, and bovine elafin (see Fig. 1) and will be referred to as ovine elafin. The protein consists of a putative hydrophobic signal sequence of 23 amino acids at the NH_2 terminus followed by a distinct region of hexapeptide repeats of consensus sequence PVKGQD, which corresponds to a potential transglutaminase substrate domain (27), and a COOH-terminal portion containing a presumed disulfide core region with eight cysteine residues in positions corresponding to those in the human elafin protein WAP domain.

Sequence analysis of the ovine elafin gene. Screening of an ovine BAC library was performed at INRA using probes generated by PCR (see EXPERIMENTAL PROCEDURES). This screening identified one clone for elafin. Southern blot analysis using 1 μg of BAC clone DNA allowed identification of a positive *Eco*R1 band of ~10 kb from the elafin clone. This band was purified and subcloned into PDC516 for sequencing with sequential internal primers through to the polyadenylation signal at the 3′ end and proximally through toward the promoter region. This approach only enabled us to obtain full 3′ sequence information (up to the poly-A signal) and no 5′ information. Indeed, because the BAC library was generated by partial *Hin*dIII digestion, and since the elafin 5′ promoter region contains a *Hin*dIII site, we were unable to obtain 5′ information using this methodology and only obtained sequence from this restriction site forward. The elafin promoter region sequence was obtained by using PCR (with ovine genomic DNA as template obtained locally; see EXPERIMENTAL PROCEDURES) with a primer designed to anneal to the 5′ end of the human elafin promoter along with a reverse primer annealing to a portion of the first intron of the ovine elafin gene. This PCR product was cloned into pCR 4-TOPO and sequenced. This allowed us to show that the elafin gene spans ~2.5 kb and is split into three exons and two introns (Fig. 2, GenBank accession no. AY344541).

Tissue distribution of ovine elafin. Total RNA was extracted from multiple ovine tissues and analyzed by Northern blot. A 750-bp ovine elafin RNA species was detected in tongue, trachea, small intestine, large intestine (strongest signal), and skin (see Fig. 3*A*).

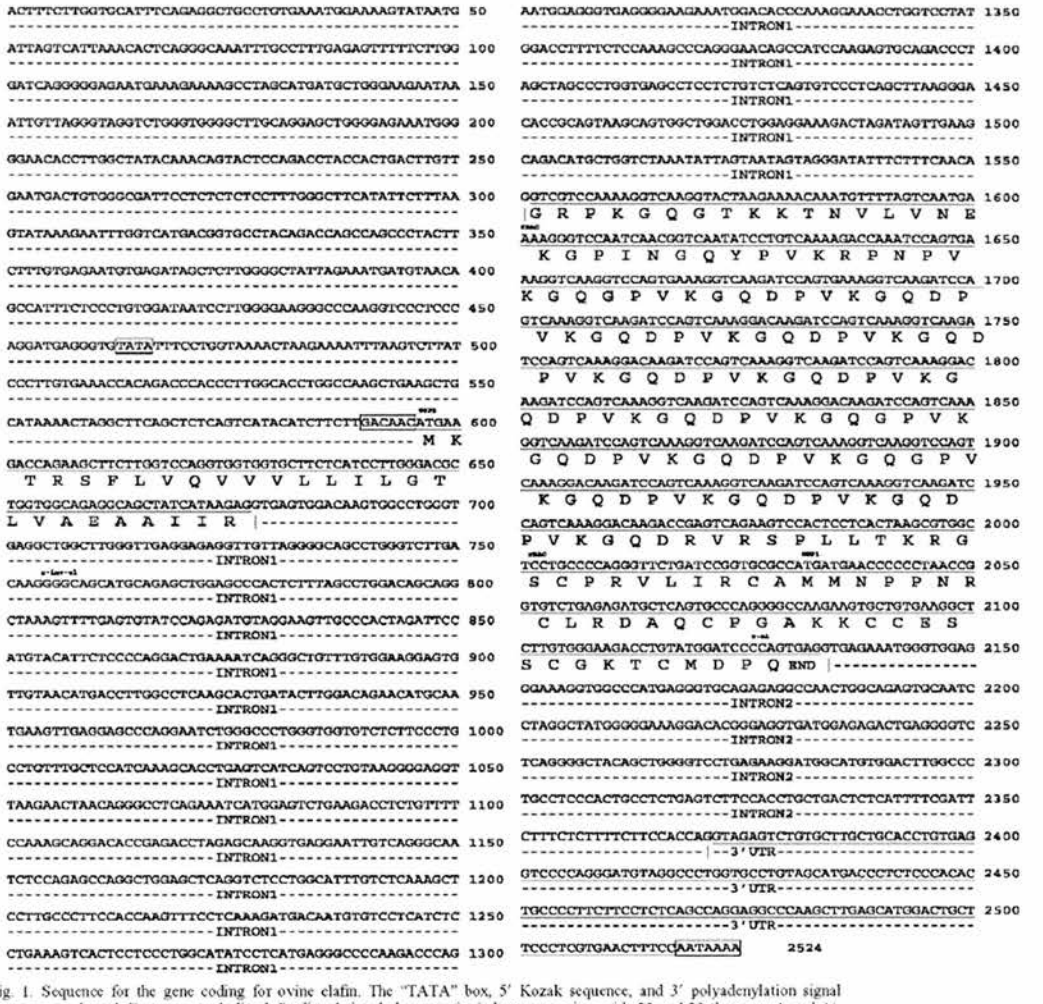

Fig. 1. Sequence for the gene coding for ovine elafin. The "TATA" box, 5′ Kozak sequence, and 3′ polyadenylation signal sequence are boxed. Exons are underlined. Predicted signal cleavage site is between amino acids 23 and 23 (between A and A). Annealing sites for the primers GSP1, GSP2, r-el (used in RACE-PCR), fBAC, rBAC (used in PCR screening of BAC library), and r-inv-el (used to amplify the promoter region) are indicated.

RT-PCR reaction using primers GSP2 and r-el for ovine elafin was performed using the same RNA samples (see Fig. 3*B*). Consistent with the Northern blot analysis, we found RNA to be present in tongue, trachea, small intestine, large intestine, and skin. Interestingly, products of two different lengths were obtained, ~660 bp (the expected size as predicted from the gene sequence) and 800 bp.

Genomic PCR for ovine elafin using a combination of non-intron-spanning and intron-spanning primers. On account of isolating two different ovine elafin mRNA products by RT-PCR (see Fig. 3*B*), although only one gene was isolated from the BAC library screening, we performed additional PCR reactions using non-intron-spanning primers and a variety of templates to determine whether these RNA products are splice variants from a single gene or products of different/allelic genes. Figure 4*A* shows the PCR products when fBAC and rBAC primers are used with various templates: genomic ovine DNA (obtained locally), cDNA synthesized by RT-PCR from ovine skin RNA (shown to be positive for elafin expression; see Fig. 3), DNA extracted from the elafin-positive BAC clone, and finally DNA extracted from the expression plasmid pIRES-GFP3 containing the cDNA for ovine elafin isolated by RACE-

```
        1           15 16          30 31          45 46          60
simian  ---------VVVFLI AGMLVVEAA------ --------------- ---------------
human   MRASSFLI-VVVFLI AGTLVLEAA------ --------------- ---------------
porcine MRSRSFLVLVVVFLI CGTLVAQAAGRIRRP KGKGTKKILALV--- ---------------
bovine  --------------- --------------- --------------- ---------------
ovine   MKTRSFLVQVVVLLI LGTLVAEAAIIRGRP KGQGTKKTNVLVNEK GPINGQYPVKRPNPV

        61          75 76          90 91          105 106         120
simian  -----------VTGV -PVKGQDTVKGRVPF NGQDPVKGQVSVKGQ DRVKGRGPVKG----
human   -----------VTGV -PVKGQDTVKGRVPF NGQDPVKGQVSVKGQ DKVKAQEPVKG----
porcine KGQGPVRGKDQVKGQ GPVKGQDLGKSQDPV KAQLPDKGQDLGKGE DSVKGQDPFKAQ---
bovine  --QEPVKGQDPVKGQ DPVKGQDPVKGQDPV KDQNPVRGQEPVKGQ DPVKGQDPVKGQDPV
ovine   IGQGPVKGQDPVKGQ DPVKGQDPVKGQDPV KGQDPVKGQDPVKGQ DPVKGQDPVKGQDPV

        121         135 136         150 151         165 166         180
simian  --------------- --------------- --------------- -----PVSTKPGSCP
human   --------------- --------------- --------------- -----PVSTKPGSCP
porcine --------------- ------------LPD KLQDPVKAQPAIK-- ---RLILLTKPGSCP
bovine  KGQ------------ ------------EPV KGQDPVKGQDPVKRQ GRIGGPLLTKPGSCP
ovine   KGQGPVKGQDPVKGQ DPVKGQGPVKGQDPV KGQDPVKGQDPVKGQ DRVRSPLLTKRGSCP
                                                                *

        181         195 196         210 211
simian  NILIRCAMLNPPNRC LKDTD---------- ----------     89
human   IILIRCAMLNPPNRC LKDTDCPGIKKCCEG SCGMACFVPQ    117
porcine RILIRCLMVNPPNRC LSDAQCPGLKKCCEG FCGKACMDPK    167
bovine  RVLIRCAMMNPPNRC LRDAQCPGVKKCCEG SCGKTCMDPQ    134
ovine   RVLIRCAMMNPPNRC LRDAQCPGAKKCCES SCGKTCMDPQ    220
          *       *       *     **    *   *
```

Fig. 2. Comparison of the known elafin protein sequence between species. The shaded area corresponds to the region of hexapeptide repeats with the consensus sequence PVKGQD. The eight conserved cysteine residues within the whey acidic protein domain (WAP) are indicated by asterisks. Where there is sequence overlap (i.e., ignoring the size difference between the proteins), the ovine elafin sequence shows 70% homology at the amino acid level with the simian, 68% with the human, 49% with the porcine, and 80% with the bovine elafin sequence.

PCR (see above). Two bands were seen with the first two templates (~400 bp and 525 bp). The low-molecular-weight band corresponded to the ovine elafin cDNA product isolated by RACE-PCR (coding for a 220 amino acid protein) and present in the pIRES-GFP3 plasmid containing the ovine elafin cDNA. The higher-molecular-weight band was of unknown origin. The two products obtained with the genomic DNA template were isolated and cloned into pCR 4-TOPO and sequenced. As predicted, the low-molecular-weight product sequence matched exactly the sequence from the ovine elafin original sequence, whereas the high-molecular-weight product sequence showed an increase in number in PVKGQD repeats

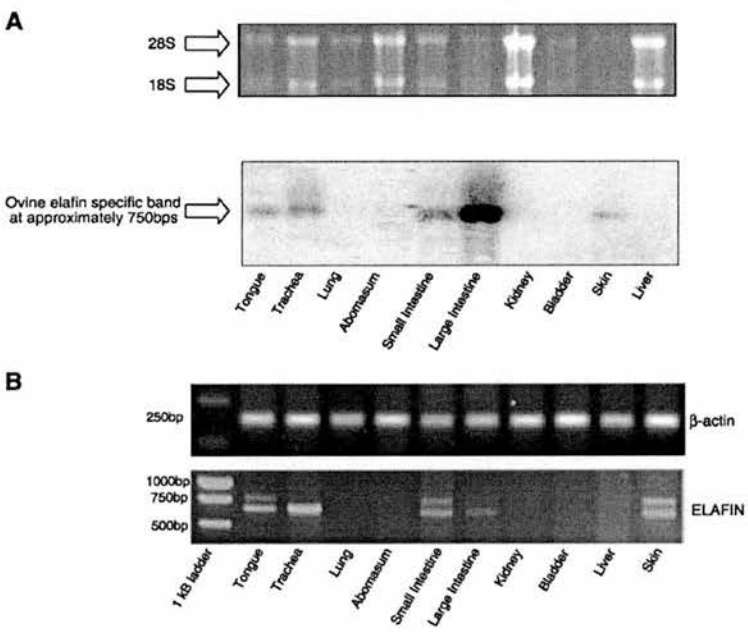

Fig. 3. Tissue distribution of ovine elafin RNA as assessed by Northern blot and RT-PCR. *A*: for Northern blot, 20 μg of total RNA was loaded for each tissue. Ribosomal 18S and 28S RNA subunits are indicated by arrows. The membrane was probed with the full-length cDNA (660 bp) corresponding to the coding portion of the gene for ovine elafin. *B*: RT-PCR for β-actin and ovine elafin from RNA extracted from the various organs indicated. The position of molecular weight markers is indicated (1-kb ladder). Primers used for amplification of ovine elafin were GSP2 and r-el, yielding two products of ~660 bp and 800 bp.

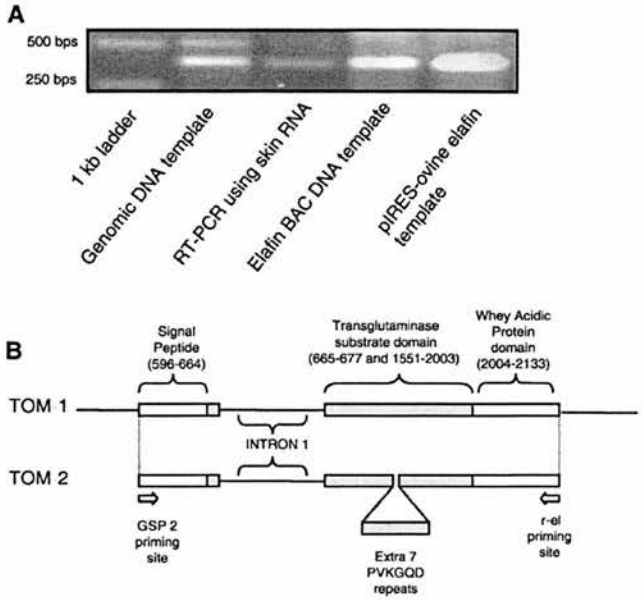

Fig. 4. The relationship between the two ovine elafin alleles (trappin ovine molecules TOM-1 and TOM-2) coding for proteins of 220 amino acids and 262 amino acids, respectively. *A*: PCR was performed using the non-intron-spanning primers fBAC and rBAC as detailed in the text. Templates used were ovine genomic DNA, cDNA synthesized by reverse transcription of RNA isolated from ovine skin, DNA isolated from an ovine BAC clone positive by PCR for the elafin gene, and the expression plasmid pIRES-GFP3 containing the cDNA for ovine elafin (220 amino acid form). The lower-molecular-weight product of ~400 bp is due to amplification of exon 2 of the ovine elafin gene or cDNA (and also inserted into pIRES-GFP3); the longer product of ~525 bp corresponds to amplification of a longer form of ovine elafin. *B*: the promoter sequence and 3′ sequence is known for the TOM-1 gene only. There is 100% homology between the known portion of TOM-2 and the corresponding portion of TOM-1, except for the fact that TOM-2 contains seven extra repeats with the consensus PVKGQD as shown. Numbers below the representation of TOM-1 denote base pairs from the start of the gene sequence. TOM-2 has been sequenced after PCR of ovine genomic DNA using the primers GSP2 and r-el as shown.

(26 instead of 19). The existence of two different genomic forms was further confirmed using a different set of PCR primers (the intron-spanning primers GSP2 and r-el), yielding two distinct products of ~1,500 and 1,600 bp (not shown). These products were cloned into pCR 4-TOPO and sequenced and confirmed the presence of a second, heavier form of the ovine elafin otherwise identical to the original gene isolated by BAC library screening. The original form of the gene described will hereafter be referred to as TOM-1, and the higher-molecular-weight form, coding for the longer form of ovine elafin of 262 amino acids, will be referred to as TOM-2. Alignment of TOM-1 and the known region of TOM-2 are shown in a diagrammatic representation in Fig. 4*B*.

Southern blot analysis of ovine genomic DNA (from the same animal as the RNA used in the RT-PCR; Fig. 3*B*) is shown in Fig. 5 and indicates the presence of only one ovine elafin gene, showing that indeed TOM-1 and TOM-2 are two allelic forms of the same gene.

In vivo upregulation of ovine SLPI and elafin in response to bacterial LPS. Bronchoalveolar lavage samples were collected 1 wk prior to and 6, 24, and 168 h after LPS administration. Dot blots were performed using these lavages and probed with either a polyclonal antibody against human SLPI or the monoclonal antibody TRAB2O to detect ovine elafin (Fig. 6). Although both SLPI and elafin concentrations in the BALF from LPS-treated sheep increase with time compared with preexperiment values, the kinetics and degree of elafin and SLPI upregulation are different. SLPI increases significantly (1.5-fold) at 6 h ($P < 0.0005$) and at 24 h ($P < 0.005$), whereas the increase in elafin levels is much more dramatic (8-fold) ($P < 0.005$ at 24 h).

Protection of A549 cells against proteolytic damage. To show potential antielastase activity attributable to the protein encoded for by the ovine elafin cDNA, A549 cells were infected with Ad-ovine elafin. To compare its activity with other known antiproteases, cells were also infected with Ad-human elafin and Ad-murine SLPI (Fig. 7). Ad-murine eotaxin

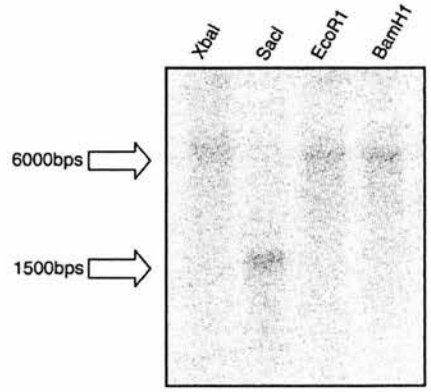

Fig. 5. Southern blot of ovine genomic DNA probed for ovine elafin. Genomic ovine DNA, 20 μg, was digested with restriction enzymes (*Xba*1, *Sac*I, *Eco*R1, and *Bam*H1), fractionated on a 0.8% agarose gel, then blotted onto a nylon membrane. The membrane was probed with radiolabeled full-length cDNA corresponding to the coding sequence of TOM-1. The sizes of the bands seen are indicated.

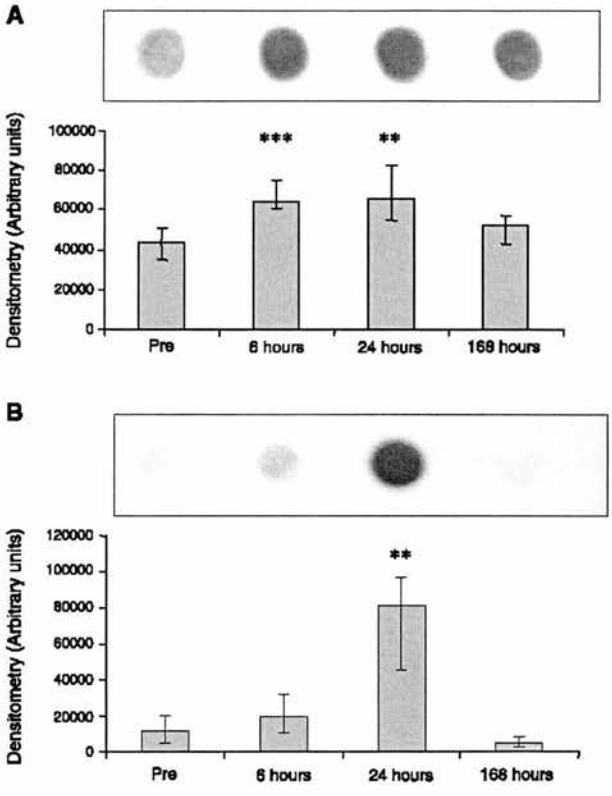

Fig. 6. Secretion of secretory leukocyte protease inhibitor (SLPI) and elafin in bronchoalveolar lavage collected from sheep lung segments exposed to lipopolysaccharide (LPS). *E. coli* LPS, 1 mg, was administered to discrete lung segments of 12 sheep. Bronchoalveolar lavage was collected a week prior to and 6, 24, and 168 h after administration of the LPS. Then, 200 μl of bronchoalveolar lavage from each animal at each time point was used in a dot blot and probed with either human anti-SLPI polyclonal antibody (*A*) or TRAB2O monoclonal antibody detecting ovine elafin (*B*). Results are shown as median arbitrary densitometry units against time after dosage of LPS, with error bars showing inter-quartile ranges. Also shown are representative dot blot results for each protein. $^{**}P < 0.005$ and $^{***}P < 0.0005$ compared with preexperimental values when Mann-Whitney analysis was performed. All results are based on $n = 12$.

was used as a negative control. Ad-ovine elafin and Ad-human elafin conferred protection of the A549 cells, irrespective of the enzyme used. Ad-murine SLPI protected the cells against HNE only, whereas, as anticipated, the negative control Ad-murine eotaxin was ineffective against either enzyme.

This array of results was obtained using qualitative (fig. 7*A*) and quantitative (fig. 7*B*) methods. In agreement with the above, the protection by Ad constructs against HNE and PPE activity was paralleled by measuring residual enzymatic activity in the supernatants (fig. 7*C*).

DISCUSSION

Neutrophil elastase inhibitors have been shown to be important endogenous molecules involved in liniting the extent of neutrophil-induced inflammation in humans, as well as in a variety of animal models (4, 10, 17, 30). Among these inhibitors, our group is particularly interested in the study of elafin, a member of the trappin multigene family (27). The "trappin" name has been coined by Schalkwijk and collaborators (27) as an acronym for "transglutaminase substrate and WAP domain containing protein," to illustrate its property of being "trapped" in a tissue (at least in the skin; Refs. 13 and 29) and therefore potentially acting as an anchored protein. Trappins consist of an NH_2-terminal transglutaminase substrate domain with hexapeptide repeats (PVKGQD) and a COOH-terminal four-disulfide core, containing, in some members, the antielastase activity (27). Members of this family share the WAP domain with another elastase inhibitor, SLPI. Trappin members have been isolated from a number of animal species, including humans, but, until now, no studies had identified the ovine ortholog of elafin.

We have in the present study isolated both the genes and cDNA coding for this protein and have analyzed the antielastase activity of the translated product.

We have isolated from a trachea sample a cDNA encoding for ovine elafin (o-elafin) which is composed of 220 amino acids, inclusive of a 23 amino acid signal peptide indicating that the protein is, like other species orthologs, a secreted protein (Fig. 1 and 2). Following this signal peptide, we identified a region containing 19 hexapeptide repeats of the consensus sequence PVKGQD. A very similar region of repeats has been termed "cementoin" (14) in the trappin family of proteins (27), to which the elafin members belong (see above). This glutamine- and lysine-rich region is thought to be

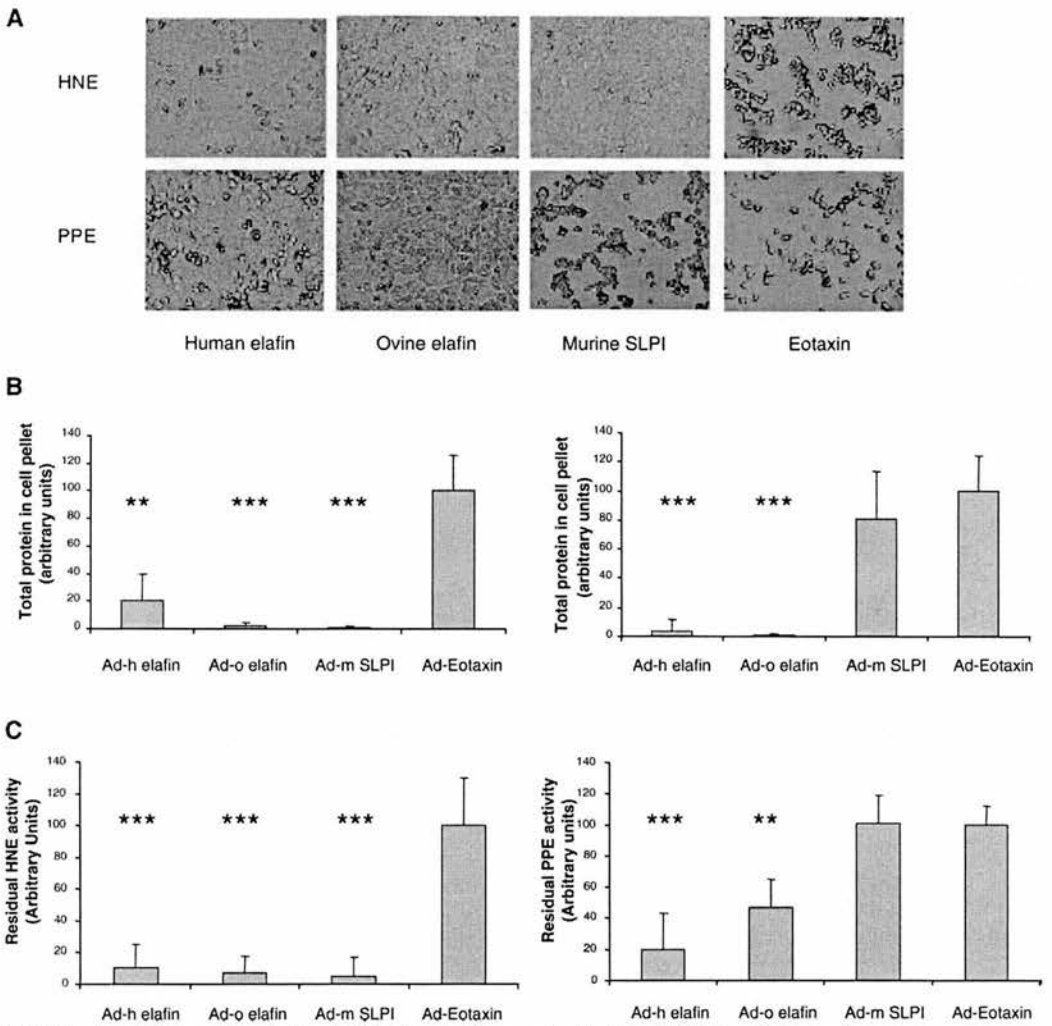

Fig. 7. Adenovirus (Ad)-ovine elafin protection of A549 cells against human neutrophil elastase (HNE) and porcine pancreatic elastase (PPE): correlation with inhibition of enzymatic activity. Ad-ovine elafin was shown to protect A549 cells against HNE and PPE (1 μg/ml) (*A*). By contrast, the negative control Ad-murine eotaxin provided no protection against either enzyme. Ad-human (Ad-h) elafin protected cells against both HNE and PPE, whereas Ad-murine (Ad-m) SLPI was efficient only against HNE (*A*). These data were confirmed quantitatively, when protein concentration was assessed as a surrogate marker of cell damage (*B*; and EXPERIMENTAL PROCEDURES). The protection (or lack of) conferred (*A* and *B*) by the Ad constructs against proteolytic enzymes was paralleled by the residual enzymatic activity recovered in cell culture supernatant (*C*). $^{**}P < 0.005$ and $^{***}P < 0.0005$ compared with Ad-murine eotaxin with Mann-Whitney analysis; $n = 5$ (HNE) or 6 (PPE).

involved in a transglutaminase reaction resulting in ε-(γ-glutamyl)lysine covalent bonds between the repeat region and matrix proteins such as loricrin and keratin-1, to promote the anchorage of the molecule to the interstitium, at least in the skin (13, 29). The second domain of o-elafin is composed of a WAP domain (27), which has strong homology with o-SLPI, which we sequenced (accession no. AY346135), and based on the sequence contains the antielastase site (see below).

Using both the ovine genomic BAC library described above and genomic DNA obtained locally, we isolated the o-elafin gene TOM-1 (Fig. 1, accession no. AY344541), the sequence of which predicted exactly the cDNA sequence obtained from

tracheal RNA. The gene structure is similar to that for human elafin (23) in that the gene contains 3 exons and 2 introns with similar intron/exon boundaries.

The promoter region shown contains a "TATA" box at bp 463 but no sequence identifiable as a "CAAT" box. An ATG initiation codon was detected, directly 3′ of a consensus Kozak sequence (9). The o-elafin leader sequence coding for the signal peptide was found to be very similar to other species' orthologs.

RT-PCR and Northern blot analysis using RNA from various ovine tissues showed o-elafin to be present in tongue, trachea, small intestine, large intestine, and skin. This is similar to porcine elafin's distribution, where elafin has been shown by in situ hybridization to be secreted by goblet cells in the tracheal mucosa and large intestinal crypts (31), and to human elafin, which has been detected in bronchial mucus (19) and psoriatic skin (26, 35) at the protein level and in the epiglottis, pharynx, vocal fold, and psoriatic skin at the mRNA level (16). Of note, higher levels of expression of ovine elafin were seen in the large intestine when analyzed by Northern blot analysis compared with RT-PCR. This is possibly due to degradation of the RNA, which would limit RT-PCR yield but may not have such a marked effect on Northern blot analysis.

To summarize tissue distribution of mRNA, o-elafin is transcribed in the upper respiratory tract, oral cavity, skin, and gastrointestinal tract. The mainly mucosal tissue distribution of o-elafin is consistent with it being, like its human orthologs, an antimicrobial "defensin-like" molecule (20). Indeed, evidence is forthcoming that, in addition to their antiprotease activity, SLPI and elafin have important functions in innate immune responses (20).

Interestingly, we obtained either one or two o-elafin cDNA products, depending on the tissues (Fig. 3). The origin of the two bands has been shown to be two allelic forms of the same gene (TOM-1 and TOM-2) (Fig. 4). The reason for the varied expression of TOM-1 and TOM-2 in different tissues is unexplained at present, although it is most likely due to differential allele-specific expression, a phenomenon recognized in other systems. The reason why we only obtained TOM-1 from the BAC library analysis while both TOM-1 and TOM-2 were obtained from genomic DNA probably lies with the origin of the samples: indeed, the BAC library used (INRA, France) was derived from a different ovine breed (Romanov; Ref. 34) from the one used for the RNA and subsequent RACE analysis (Suffolk crosses from the UK). Our results suggest that the Romanov breed animal used to construct the BAC library only has TOM-1, whereas the Suffolk breed has both TOM-1 and TOM-2. It is therefore possible that the two allelic forms of o-elafin may be caused by the interbreeding of the commercial Suffolk lowland flock.

The large transglutaminase substrate domain present within the o-elafin molecule is of interest with regard to its potential interaction with other tissue components. As mentioned above, this region is thought to be involved in cross-linking to extracellular matrix proteins at least in the skin. Human elafin has been shown to be cross linked in this way to the cornified envelope of keratinocytes (13, 29). Human elafin contains five repeats loosely based on the consensus sequence PVKGQD. The o elafin orthologs discussed here (TOM-1 and TOM 2) contain 19 or 26 repeats with far higher conservation of the consensus sequence, suggesting, potentially, higher levels of transglutaminase-mediated cross-linking. Interestingly, recent work has implicated transglutaminase repeats to be of importance in competitively inhibiting tissue transglutaminase and in turn phospholipase A_2, hence leading to a downregulation of proinflammatory mediators in a hypersensitivity model (28). This link between members of the trappin family and downregulation of phospholipase A_2 leads to the fascinating possibility that the extensive transglutaminase substrate region of the o-elafin proteins may provide a particularly high anti-inflammatory activity to the secreted protein compared with those already studied. The transglutaminase substrate domain has also been identified as one of the *cis*-acting elements implicated in the gene multiplication seen in the members of the trappin family [Furutani et al. (3) identify a correlation between number of repeats and number of trappin family members, i.e., the fewest repeats are seen in human and peccary, in each of which only one trappin family member has been described]. Surprisingly, given the high number of repeats identified here, we only isolated one trappin, ovine elafin, which was shown to exist in two allelic forms. This is the first time a trappin family member gene has been shown to be present in more than one allelic form.

The phylogenetic tree analysis (Fig. 8) shows the close relationship between the WAP domain of TOM-1 and TOM-2, and the other trappin family members, with the second WAP domain of the SLPI orthologs. This suggests a divergence in evolution of the trappin WAP motifs and the second SLPI WAP motif from a common ancestral protein subsequent to the divergence of the first and second WAP motifs of the SLPI family from each other. Interestingly, the hominid trappins (human elafin and simian elafin) have diverged from the rest of the known trappins (Bovidae, Hippopotamidae, Tayassuidae, and Suidae). The two o-elafin proteins (TOM-1 and TOM-2) are most closely linked with bovine elafin and bovine trappins 4 and 5.

To ascertain whether the ovine elafin protein is behaving like a local acute phase reactant, we assessed the levels of ovine elafin in a model of lung inflammation triggered by local bronchoscopic administration of LPS. The dramatically increased level of elafin in inflammatory conditions such as described here finds an echo in some of our previous in vitro and in vivo human work. We showed (33) that elafin levels were sharply increased in BALF from human patients with farmer's lung disease. By contrast, in the same patients, and in the present study, SLPI levels increased less dramatically. This may be due to the increased sensitivity of elafin to proinflammatory cytokines such as IL-1 and TNF (22). The time courses of elafin and SLPI expression presented here add further to that concept, in agreement with murine SLPI in vivo studies (1, 7). Western blot analysis of BALF for ovine elafin and SLPI using the antibodies utilized in the dot blot analysis above was unsuccessful at detecting protein, presumably because of the low concentration of these antiproteases in the BALF.

In summary, the identification and cloning of the gene encoding for the ovine orthologs of human elafin have allowed us to postulate important and exciting functions of this protein in addition to the antiprotease functions. Furthermore, the in vivo demonstration that ovine elafin is an acute inflammatory reactant in the lung indicates that it can be considered as an "alarm inhibitor" in that organ. Its efficiency as a neutrophil elastase inhibitor makes it an attractive candidate for use in adenovirus-mediated gene transfer in ovine models of neutro-

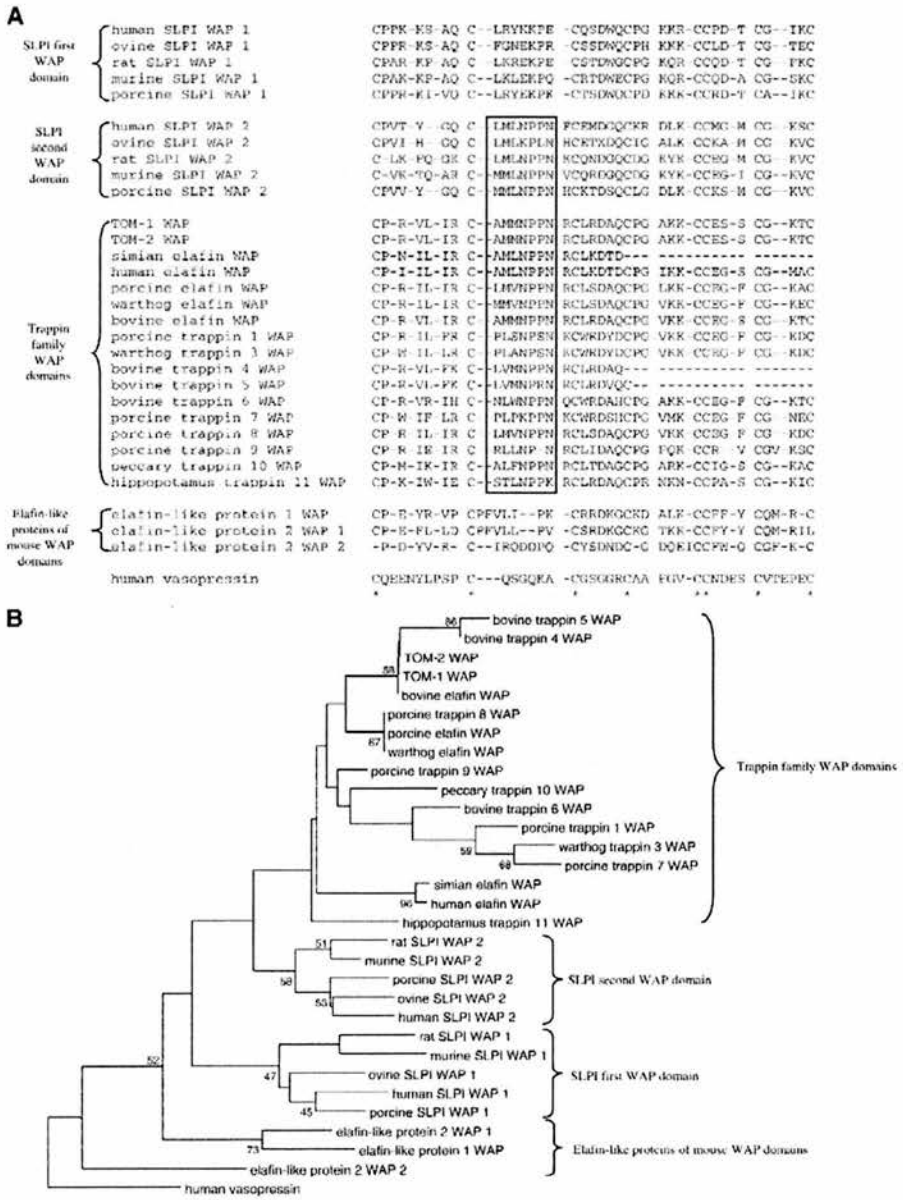

Fig. 8. Whey acid protein (WAP) comparison (SLPI, trappins, mouse elafin-like proteins, vasopressin). *A*: alignment of the 1st and 2nd WAP domains of the currently known SLPI proteins, along with the WAP domains for a variety of trappins including the ovine forms of elafin derived from the alleles TOM-1 and TOM-2, and including the WAP domains of the murine elafin-like proteins 1 and 2 (Hagiwara et al., Ref. 5) and human vasopressin. The WAP motifs for all the proteins were aligned by using ClustalW software available online. Conserved cysteine residues are indicated by asterisks. The boxed region indicates the putative active antiprotease active site in the second WAP domains of the SLPI family and in the WAP domain of the trappin family. *B*: phylogenetic tree constructed using a neighbor-joining technique with Poisson corrected data from the above alignment. Bootstrap values above 40 are indicated. Branch length is proportional to evolutionary distance.

phils-driven lung injury, currently being explored in our laboratories. Additionally, specific and potentially very important effects of the transglutaminase substrate domain in this protein warrant further investigation.

ACKNOWLEDGMENTS

We are grateful to M. Marsden, A. Baker, and P. Tennent for technical assistance.

We also thank Prof. K. J. Johnson (Molecular Genetics, Anderson College, Institute of Biomedical Sciences, University of Glasgow, UK) and Dr. T. D. Tetley (Lung Cell Biology, National Heart and Lung Institute, Imperial College, London, UK) for input in this project.

GRANTS

We acknowledge the Wellcome Trust for funding this work (Clinical Research Training Award in Veterinary Medicine for T. Brown) and the MRC for funding the LPS administration experiments.

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