Analysis of the pulmonary inflammatory phenotype of the CF mutant mouse.

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Presented for the degree of Ph.D.
University of Edinburgh
1998
Declaration.

I declare that

a) this thesis has been composed by myself
b) that the work is my own, except where otherwise stated

Gillian Morrison
December 1998
Acknowledgements.

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Love and thanks go to mam and dad who have always been there for me, throughout the course of my education and who I can always rely on for support and encouragement.

Finally I would like to dedicate this thesis to Dougie, for his encouragement and advice on my work and for being my favourite distraction from it.
Abstract.

Recent evidence suggests that the profile of pro-inflammatory cytokines in the cystic fibrosis lung differs from that of the normal lung before there is any clinical evidence of lung inflammation due to infection. This raises the question of whether bacterial pathogens cause the excessive local inflammation or if they simply aggravate an underlying defect in the host defence system resulting from the CFTR mutation.

The Edinburgh CF knockout mouse has previously been shown to develop a lung disease with aspects similar to human CF patients following repeated exposure to bacterial pathogens. It therefore provides a good model system in which to study this topic whilst being able to control many of the factors which complicate human studies of inflammation.

In order to establish whether the inflammatory response of the \( C_{ftr}^{tm1Hgu} \) mouse differs to that of its control litter mates, the pulmonary host defence system was examined under specified pathogen free and conventional housing conditions and also following instillation of the inflammatory stimulus of both lipopolysaccharide (LPS) and the CF relevant pathogens \( Staphylococcus aureus \) and \( Pseudomonas aeruginosa \). Bronchoalveolar lavage fluid was assayed for inflammatory cells and relevant inflammatory markers. No significant differences were found between wild type mice and \( C_{ftr}^{tm1Hgu} \) mutant mice following a single challenge with \( S. aureus \) or \( P. aeruginosa \). TNF-\( \alpha \) levels were found to be significantly different between wild type mice and \( C_{ftr}^{tm1Hgu} \) mutant mice in the conventional housing conditions in the absence of any detectable pulmonary infection. Conversely, levels of TNF-\( \alpha \) were not significantly different in SPF housed mice or following intratracheal instillation of LPS. These results and additional studies using isolated alveolar macrophages, led to the hypothesis that the pulmonary inflammatory cells of the \( C_{ftr}^{tm1Hgu} \) mouse function normally when given a direct inflammatory stimulus and that another aspect of the host defence system is not functioning adequately to deal with repeated exposure to low level non-specific bacteria.

Recent studies on the human airway defensin molecule, hBD-1, reveal that its bactericidal properties are inactivated by high salt concentrations. It is possible that
the airway surface fluid of CF patients has a raised NaCl concentration and relates to a defective host defence system caused by the CFTR mutation. This thesis describes the identification and characterisation of a mouse beta defensin gene, Defb1, sometimes referred to as mBD-1, which was shown to be homologous to the human airway beta defensin hBD-1. It was found that Defb1 was expressed in a variety of tissues including the airways and like hBD-1 is not upregulated by LPS. A genomic targeting vector was constructed and Defb1 successfully knocked out in the mouse. This study also led to the isolation of a 150 kb BAC contig which was shown to contain members of both the alpha and beta defensin gene families. Using low stringency hybridisation a novel murine beta defensin gene was identified which is not highly expressed in the airways under normal conditions although it appears to be weakly upregulated by LPS. A second human beta defensin gene, hBD-2, was recently identified which was shown to be upregulated in the airways by inflammatory stimuli, thus this novel murine gene is similar to hBD-2 both in structure and function. It is hoped that the study of the murine beta defensin gene family will lead to a clearer understanding of the normal function of defensins as well as of the consequences of their dysfunction in CF.
**Abbreviations used.**

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<tr>
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<tr>
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<td>asialoganglioside 1</td>
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<td>airway surface fluid</td>
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<td>adenosine triphosphate</td>
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<td>BAC</td>
<td>bacterial artificial chromosome</td>
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<td>bronchoalveolar lavage</td>
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<tr>
<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
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<td><em>Burkholderia cepacia</em></td>
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<td>basic local alignment search tool</td>
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<td>CFTR</td>
<td>cystic fibrosis transmembrane conductance regulator</td>
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<td>Cftr</td>
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<td>CITB</td>
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DEPC  diethyl pyrocarbonate
dGTP  deoxyguanosine triphosphate
dH2O  distilled water
DNA  deoxyribonucleic acid
dNTP  deoxynucleotide triphosphate
DTT  dithiothreitol
dTTP  deoxythymidine triphosphate
ECL  enhanced chemiluminescence
E. coli  *Escherichia coli*
EDTA  ethylenediamine tetra-acetic acid disodium salt
ELISA  enzyme linked immunoadsorbent assay
ENaC  epithelial sodium channel
ES  embryonic stem
EST  expressed sequence tag
FISH  fluorescent *in situ* hybridisation
FMLP  N-formyl-met-leu-phe
g  gram
G  guanine or gauge
Gal-1  gallinacin-1
GET  glucose/Tris/EDTA solution
hBD-1  human beta defensin-1 gene
HBD1  human beta defensin 1
hBD-2  human beta defensin-2 gene
HBD2  human beta defensin 2
HBSS  Hanks buffered salt solution
*H. influenzae*  *Haemophilus influenzae*
HIV  human immunodeficiency virus
HNK-1  human neutrophil peptide-1
Hprt  murine hypoxanthine phosphoribosyltransferase gene
hr  hour
ICAM-1  intercellular adhesion molecule-1
IL-(1β,2-8,10,12)  interleukin-(1β, 2-8, 10, 12)
iNOS  inducible nitric oxide synthase
JAK  Janus kinase
kb  kilobase
kDa  kiloDalton
kV  kilovolts
l  litre
LAP  lingual antimicrobial peptide
L-B  Luria-Bertani
LPS  lipopolysaccharide
M  molar
MEP  mucoid exopolysaccharide
mFd  milliFaraday
mg  milligram
Mg2+  magnesium ion
MgCl2  magnesium chloride
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<td>soluble intercellular adhesion molecule-1</td>
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<td>SLPI</td>
<td>secretory leucocyte protease inhibitor</td>
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<td>Abbreviation</td>
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<td>SPF</td>
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<td>Streptococcus pneumoniae</td>
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<td>SSC</td>
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<td>signal transducers and activation of transcription</td>
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<tr>
<td>Ste</td>
<td>murine steroid sulfotransferase e</td>
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<td>Salmonella typhi</td>
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<td>TBE</td>
<td>Tris-borate/EDTA solution</td>
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<td>turkey heterophil peptide-1</td>
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<td>tumour necrosis factor-alpha</td>
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<td>units or uracil</td>
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<td>ultra violet</td>
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<td>w/v</td>
<td>weight:volume</td>
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Chapter 5 Treatment with S. aureus and P. aeruginosa

5.1 Introduction
Chapter 1

Introduction
1. Introduction.

1.1 The history of cystic fibrosis.

It was only after the work of Anderson et al. (1938) that the group of clinical conditions we now associate with cystic fibrosis (CF) was assigned as a single disease. CF is now recognised as one of the most common single-gene disorders affecting approximately 1 in 2500 children of European descent and over 50,000 people world-wide.

This disorder once led to death in infancy and whilst recent advances in the understanding of the microbiology of CF have led to the use of antibiotic based therapies which improve the length and quality of life of CF sufferers, no treatment currently in use can correct the molecular defect and patients die at an average age of 29 years (Davis et al., 1996).

It was discovered that in CF patients, the epithelia lining the surface of the ducts of the sweat glands failed to take up chloride efficiently from the lumen of the glands (Quinton et al., 1983). In another study it was found that chloride movement from the epithelial tissue into the airway lumen was diminished and sodium uptake by the epithelium was enhanced (Knowles et al., 1981). These observations suggested that the protein product of the gene whose mutation led to CF was involved with chloride transport across cell membranes and hence, when the gene responsible was discovered in 1989, following the cumulative efforts of a large team of collaborators (Riordan et al., 1989), it was named the cystic fibrosis transmembrane conductance regulator (CFTR).

1.2 The cystic fibrosis transmembrane conductance regulator.

The CFTR gene, localised on the long arm of chromosome 7, represents approximately 250 kb of genomic DNA made up of 27 exons which result in a 6.5 kb
mRNA. It is highly expressed in the epithelial cells of the sweat ducts, the pancreatic ducts, the digestive tract (particularly in the crypts of the small intestine and Brunner’s glands), the biliary ducts, the salivary glands, the reproductive organs and the lungs, particularly the serous cells of the submucosal glands of the respiratory epithelial cells (Davis et al, 1996). The CFTR mRNA encodes a 1480 amino acid protein whose sequence was similar to that of a family of proteins known as ATP-hydrolysing transporter proteins (Higgins, 1989). This family includes the transporter protein, p-glycoprotein which confers multi-drug resistance on cells and STE6 which transports a polypeptide pheromone out of yeast cells. The similarity of CFTR to these proteins again suggested a role for CFTR in transportation.

1.3 CFTR and chloride ion conductance.

Several hypotheses were offered as to how CFTR functioned in chloride conductance including one that CFTR might serve directly as a chloride channel, although the characteristics of CFTR did not fit with those of any ion channel recognised at that time. Figure 1.1 shows the structure of CFTR as predicted by the amino acid sequence. It was theoretically possible that the two membrane spanning domains of CFTR could form a pore through which chloride could pass through the membrane and confirmation of this came when CFTR proteins were expressed in Xenopus oocytes which led to chloride ion transport (Bear et al, 1991). This demonstration of CFTR ion transport function and many subsequent studies resulted in the following model for CFTR as a low conductance chloride-specific pore. Parts of the transmembrane domains form the pore and the regulatory (R) domain functions as a channel inhibitor until it is phosphorylated by protein kinase A (PKA) and undergoes a conformational change to make the pore accessible to chloride ions. Binding and hydrolysis of ATP then takes place at the first nucleotide binding domain (NBD) and the channel opens. Binding and hydrolysis of ATP at the second NBD closes the channel and this closed state is secured by dephosphorylation of the R domain. (Gadsby et al, 1994, Ma et al, 1997).
The complex structure of CFTR suggests that it may function in other ways than purely as a chloride pore and CF cells have been shown to have abnormalities other than in chloride conductance. These include diminished sialylation of cell surface glycoproteins and defective chloride conductance of non-CFTR channels (Wine 1995). It has recently been shown that CFTR can inhibit the amiloride sensitive epithelial sodium channel (ENaC) by a chloride-independent mechanism (Stutts et al, 1995). Although this report would support the observation that sodium channel activity is increased in the CF airways (Welsh et al, 1995), no further studies have confirmed this finding. The outwardly rectifying chloride channel (ORCC) has been shown to be indirectly activated by CFTR via purinergic receptors (Schwiebert et al, 1995). There is also evidence that CFTR is involved in the transport of water (Hasegawa et al, 1992) and in other ion transport processes. For instance, it has been shown that calcium regulated chloride conductance is raised in human and mouse CF
though the involvement of CFTR in the transport of ATP is controversial, present studies suggest there is a definite relationship between CFTR and ATP, even if it remains unclear exactly what that relationship is. However, these studies have demonstrated quite conclusively that CFTR can have functional relevance in systems other than basic chloride conductance and this should be considered when trying to interpret the phenotype of CF. Interestingly, recent evidence suggests that CFTR may also have an antimicrobial role by acting as a specific receptor for the clearance of P. aeruginosa from the epithelial cell surface (Pier et al, 1996). This particular aspect will be discussed in more detail later in section 1.10.3.

1.5 Mutational analysis of CFTR.

The CFTR gene has been subjected to in-depth analysis by the CF Genetics Analysis Consortium amongst others and to date, more than 600 different mutations have been described that affect various aspects of CFTR function. The most common CFTR mutation, accounting for approximately 70% of CF cases, arises from a 3 bp deletion resulting in the removal of a single amino acid, phenylalanine, from the protein. This mutation, commonly known as ΔF508, belongs to a class of mutations that disrupt the processing of CFTR such that it fails to reach the apical membrane of the cell. Another class of CFTR mutations are those which result in the correct localisation of the CFTR protein but disrupt the ability of CFTR to act as a chloride channel. The third group affects the production of the protein and include nonsense mutations that result in a truncated and non-functional CFTR (Delaney et al, 1996b). All three classes of mutation result in insufficient levels of functional CFTR at the apical membranes of epithelial cells, resulting in the clinical defects associated with cystic fibrosis.
1.6 Phenotype genotype.

No clear relationship between phenotype and genotype has been established at the level of the whole organism in CF but it has become possible to correlate the level of CFTR function with phenotype in an organ specific manner (Davis et al, 1996). Figure 1.2 clearly demonstrates that there are distinct differences between tissues in their sensitivity to loss of CF. One possible explanation for this is that different levels of alternative splicing of the CFTR pre-mRNA occur in different tissue systems and this might affect the amount of functional protein present in any particular tissue.

For none of the CF genotypes studied to date can predictions be made about the severity of pulmonary disease (The cystic fibrosis genotype-phenotype consortium 1993, Hamosh et al, 1993, Kerem et al, 1996). Variation in CF pulmonary disease severity exists amongst patients with the same CFTR genotype and also occurs amongst patients of the same family which indicates that modifiers of CF disease severity must exist. This is discussed further in section 1.7.

![Tissue affected vs CFTR activity](image_url)

**Fig. 1.2 CFTR activity levels and tissues affected.**
1.7. Genetic modifiers of CF.

The identification of genetic modifiers in humans would be an extremely difficult and complicated process due to the high degree of genetic heterogeneity and the low sample numbers. However, a CF twin and sibling study has recently been conducted using clinical data from 120 twins and 844 CF siblings from 158 European CF centres (Bronsveld et al, 1998). Surprisingly, residual chloride secretion was detected in 76% of CF patients with the ΔF508 mutation and in 66% of cases this was found to be due to the activity of an alternative chloride channel, indicating the importance of other genes for the overall level of chloride secretion. Although the origin of the residual chloride secretion was found to be the same in homozygous patient pairs, the level of the residual chloride secretion differed amongst different tissues and within patient pairs.

An alternative strategy to twin or sibling studies was to use a CF mouse model. Rozmahel et al. (Rozmahel et al, 1996) generated a CF knockout mouse which they carried to several different inbred strains and by assessing intestinal phenotype and carrying out a whole genome scan, successfully identified a major modifier locus. Several candidate genes were identified in the syntenic region of the human chromosome including regions coding for a subunit of protein kinase C, a subunit of a Na+/K+ exchanging ATPase and the sodium channel, type 1, b polypeptide. As has already been mentioned, CFTR is capable of modulating other channels and therefore alterations in channels such as those identified in the modifier locus may be relevant to CF intestinal disease severity. It has since been found that the basis of the intestinal disease variability among the CF mice was influenced by at least two major modifiers which map to chromosome 5 and 7. The steroid sulfotransferase genes, Std and Ste, map to these locations. The discovery that the expression of these genes is elevated in CF mice with mild intestinal disease relative to severe disease and that the substrate for these gene products has been shown to exacerbate intestinal lesions in CF mice strongly suggests these genes have a role in CF disease genotype (Rozmahel et al, 1998). The region of human chromosome 19 syntenic to the cystic
fibrosis modifier found on mouse chromosome seven has been finely mapped and two candidate genes identified (Zielenski et al, 1998). One of which is STD, the human homologue of murine Std, mentioned above and the other is CALM3 (calmodulin). Their association with CF intestinal disease is currently being investigated.

Another murine study is currently being undertaken to examine modifier genes in the CF mouse with relevance to lung disease but, to date, no further major modifier loci have been identified (Innes et al, 1998).

An alternative approach to identify genes which may be involved in the phenotypic variation of CF is to study candidate genes such as those already known to play a role in the pathology of the disease. Mucin genes contain tandem repeats (TR) which are highly polymorphic and it has been proposed that the biophysical and biochemical properties of the mucin molecule are affected by TR length. Therefore it was hypothesised that mucin genes may be candidates for CF modifiers. In this case there was no correlation found between TR length of the MUC6 gene and disease status in CF, however, further studies are required to investigate other mucin genes (Harris et al, 1998).

1.8 Lung disease in CF patients.

The clinical defects associated with cystic fibrosis are a reflection of its tissue and cell specific expression mentioned previously and therefore many organs are affected by this disease. However, for the purpose of this introduction only the pulmonary disorder shall be considered.

In the UK, 85 % of deaths in adult CF patients are caused by lung disease. In contrast to other organs affected by CF, there has been no evidence of histological lung abnormalities of the CF lung in utero or in new borns (Bedrossian et al, 1998) apart from possible submucosal gland hypertrophy or mild dilation of the acini of tracheal submucosal glands (Sturgess et al, 1982). In early childhood many CF patients acquire lung infection that results in inflammation which is slow to recede and
infection becomes established with a distinctive range of bacteria which shall be discussed in more detail below. Pulmonary infection causes additional airway obstructions inducing a bronchiolar inflammatory response. A persistent microbial colonisation results in a vicious cycle of infection consisting of intermittent episodes of debilitating and ultimately fatal infection.

1.9 Bacterial infection in CF patients.

The predominant bacterial species in CF are *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Susceptibility to these pathogens, which are usually regarded as being of low virulence and would not usually cause chronic infections in normal airways, tends to be age-dependent with *S. aureus* being predominant in the first two years of life followed by *H. influenzae*, after which *P. aeruginosa* predominates. Recently, multiresistant, transmissible strains of *B. cepacia* have emerged as fatal pathogens in CF and have presented a formidable new challenge to the clearance of pathogens in the CF lung (Gilligan, 1991). However, *P. aeruginosa* remains the most frequently reported respiratory pathogen with evidence of infection in a third of CF infants by 5 years of age (Grimwood et al, 1997) and 93.2 % of CF patients between the age of 18 and 24 years (Cystic Fibrosis Foundation, 1995). It is possible to eradicate the colonisation of non-mucoid strains of *P. aeruginosa*, however, in most cases the strains persist and alter their phenotype to the alginate-producing mucoid variant. Once the mucoid variant has been established they are seldom eradicated and therefore is recognised as a poor prognostic indicator.

1.10 How does the loss of CFTR lead to lung disease?

Though it is still not clear precisely how the CFTR defect leads to persistent lung infection and inflammation, research conducted in recent years has given rise to some interesting hypotheses which are summarised in figure 1.3 and discussed below.
1.10.1 The defective mucociliary clearance theory.

It has been suggested that the unusual viscosity of the host mucus as a result of altered electrolyte and water movement across the surface epithelium leads to dehydration of mucus, inspissation of secretions and a consequent reduction in the efficiency of mucociliary clearance of pathogens in the mucoid blanket (Koch et al, 1993). However, another theory states that the thickened mucus represents debris composed of cellular DNA and cytoskeletal products following an inflammatory response (Goldman et al, 1997). Several studies have examined mucociliary clearance in the trachea of CF patients but to date these have proved to be inconclusive (Regnis et al, 1994). Primary ciliary dyskinesia syndrome (PCD) is a disorder of mucociliary clearance cause by immotile cilia (Afzelius, 1976) and if it were to be proposed that a defect in mucociliary clearance was the primary defect in CF that leads to lung disease then it may be supposed that patients with PCD and CF would display similar profiles of lung infection. Recurrent respiratory tract infections are a clinical feature of PCD, but in most cases it is H. influenzae and S. pneumoniae that colonise the respiratory tract and not predominantly P. aeruginosa as with CF (Narayan et al, 1994). It is important to note, however, that this does not rule out defective mucociliary clearance as a contributing factor to CF lung disease but it does not appear sufficient to explain the specificity of the pathogens found to colonise the CF lung. Mucin gene expression and its relevance to CF lung disease will be discussed in section 1.11.3.

1.10.2 The adherence theory.

An alternative theory proposed by Imundo et al. (1995) was that there is increased adherence of P. aeruginosa and S. aureus to the respiratory epithelial cells in the CF lung compared with normal epithelium thought to be due to the asialation of glycolipids which generates receptors for adhesins. It was suggested that the CFTR
dysfunction, by limiting chloride and hydrogen ion transport into the trans-Golgi complex, may result in decreased sialotransferase activity and production of a greater proportion of asialated surface glycolipids. Saiman et al. (1993) reported that *P. aeruginosa* adhered significantly better to CF epithelium than to normal epithelium and that an asialoganglioside 1 (aGM1) was present at higher levels in the CF epithelium. Many other bacterial pathogens use adherence as their method of colonisation including *Bordetella pertussis*, the causal agent of Whooping cough, which uses its adhesins to adhere to lung epithelial cells. However, adhesion of *P. aeruginosa* to CF bronchial epithelial cells has only been demonstrated in *in vitro* cell culture and these results do not agree with *post mortem* data on CF patients in which *P. aeruginosa* is not observed adhering to epithelial cells.

### 1.10.3 The internalisation theory.

In 1996, Pier et al. proposed that airway epithelial cells expressing a mutant form of CFTR are defective in the internalisation of *P. aeruginosa* and that the emergence of variants of *P. aeruginosa* during chronic infection further impairs their internalisation. Additional studies of *in vitro* cell cultures treated with blocking antibodies led to the conclusion that *P. aeruginosa* interacts specifically with the first extracellular domain of CFTR via the oligosaccharide core of lipopolysaccharide (LPS) on its outer cell wall. The *P. aeruginosa* is then internalised which leads to an upregulation in the presentation of CFTR at the epithelial cell surface and then the epithelial cells containing the ingested bacteria are shed (Pier et al, 1997). However, this theory has not been confirmed by histopathology and the *in vivo* relevance of the *P. aeruginosa* internalisation experiment has not been deduced since the cell lines used were producing higher levels of CFTR protein than would be found in human bronchial epithelial cells. Interestingly, it has recently been reported that *Salmonella typhi* uses CFTR to enter intestinal epithelial cells (Pier et al, 1997). In this case a decrease in the amount of CFTR would actually be a benefit since *S. typhi* is an intracellular pathogen and its uptake by cells leads to colonisation and typhoid fever.
in the host. In addition to providing further evidence that CFTR may act as a receptor for bacterial uptake into the cell it also offers an explanation for the high incidence of mutant CFTR alleles in the population since resistance to typhoid fever could provide a heterozygote advantage. However, there is no evidence that CF patients or carriers are more resistant to S. typhi infection and since wild-type mice are entirely resistant to S. typhi the CF mouse model cannot be used to test this hypothesis in vivo (Prince, 1998).

1.10.4. Reduced antimicrobial activity of airway surface fluid (ASF) theory.

Finally, Smith et al. (1996) provided a direct link between CFTR dysfunction and lung disease by demonstrating that the airway surface fluid (ASF) from cultured CF epithelial cells had a reduced bactericidal activity and this could be restored to normal by decreasing its salt concentration. The measurement of sodium and chloride concentrations in the airway surface fluid of CF patients and normal controls has been complicated by the difficulty of sampling such a small volume of liquid but the most recent experiments have shown the airway surface fluid from CF patients has a salt concentration higher than normal controls (Zabner et al., 1998). It has since been shown that several antimicrobial peptides exist in the airway surface fluid whose activity is decreased by increased salt concentrations including the human beta defensins hBD-1 and hBD-2 (Goldman et al., 1997, Morrison et al., 1998, Bals et al., 1998b). The relevance of airway defensins in the pathogenesis of CF lung disease will be discussed in greater detail later.

Recent evidence, such as that detailed above, suggests there are numerous factors specific to the CF lung that eventually lead to colonisation with a specific range of pathogens. The mucociliary clearance defect and reduced antimicrobial activity of the ASF may lead to a general reduced ability to kill pathogens whereas the reduced receptor mediated uptake appears to be specific to P. aeruginosa. It is likely there are other consequences of loss of CFTR function that are specifically advantageous to
the other CF-relevant pathogens, since for instance, *B. cepacia* has been shown to be resistant to the actions of defensins (Hancock, 1997, Morrison *et al*, 1998, Steen *et al*, 1998) (see section 1.14.4). However, another possibility is that the initial reduced ability to kill pathogens initiates the overactive inflammatory response, possibly resulting in conditions within the CF lung advantageous for a specific range of pathogens.

Although the reduced antimicrobial activity of the CF ASF offers a tempting explanation for the initial stages of the excessive inflammatory response in the lung, the question of whether bacterial pathogens cause the initial inflammatory response or just aggravate an underlying problem of the host defence system caused by the CFTR defect has not been settled. The immune system in CF patients was once considered to be functionally normal and the hyperimmune response leading to neutrophil dominated lung inflammation was thought to be simply a result of a persistent infection by bacterial pathogens. However, recent studies suggest that it is possible that inflammation may be initiated by a defective host defence system prior to any infective episode.

1.11 The pulmonary defence system.

Since the lungs (in common with other mucous surfaces) are unusual in having a large surface area in close association with the external environment it has been necessary to develop a range of rapid acting and efficient defence mechanisms to cope with the potential for external assault. The inflammatory response in the lung comprises both the lymphoid system and the myeloid system. Normally, these are extremely efficient at clearing short-term infections and the lung is left undamaged. In CF, however there appears to be to an excessive response, especially by the myeloid system which results in neutrophil-dominated inflammation of the lung associated with progressive damage of the lung tissue by oxygen radicals and lysosomal and granule protease enzymes released by the neutrophils.
Fig. 1.3 Possible factors involved in the pathogenesis of CF lung disease.
Recent evidence of direct links between the CFTR defect and initiation of CF lung disease are detailed in section 1.11. Once bacterial colonisation is established a vicious cycle of infection and inflammation occurs ultimately leading to severe damage of the lungs.
What follows is a brief introduction to the components of the pulmonary host system which may have some relevance to CF lung pathogenesis. This project primarily focuses on the study of cytokines, cell adhesion molecules and defensins and these shall be considered in some detail. Several other factors have been subject to investigation in the context of CF lung disease and these are discussed briefly below.

1.1.1 Proteases and protease inhibitors.

A massive influx of neutrophils into the lungs is a characteristic symptom of CF. The neutrophils accumulate and decay but are not ingested by alveolar macrophages and as a consequence huge amounts of harmful substances including oxidants and proteases are released into the lung environment. The neutrophil proteases released include elastase, cathepsin G and proteinase 3 which have a diverse range of actions on the host cells, all of them harmful. Neutrophil elastase can cleave immunoglobulins, cell receptors and complement components and as a consequence opsonophagocytosis and killing of *P. aeruginosa* and other CF relevant pathogens is markedly reduced (Doring, 1997). Neutrophil elastase also stimulates airway gland secretion and along with the other proteinases can damage epithelial cells by cleaving fibronectin, cilia and elastin which may increase the survival of *P. aeruginosa* in the lung by enhancing its adhesion to epithelial cells (Doring et al, 1995). There are several endogenous proteinase inhibitors present in the airways including the elastase inhibitors α1-protease inhibitor and elafin and the secretory leucocyte protease inhibitor (SLPI). However it is estimated that about 90 % of these inhibitors are inactivated following elastase cleavage. This process does not occur unless the elastase to inhibitor ratio is greater than one, which unfortunately is the case in the CF lung (Goldstein et al, 1986).
1.11.2 Surfactant.

The pulmonary surfactant system is a complex of lipids and proteins produced by epithelial cells that reduces the surface tension in the alveoli thus keeping the work of breathing to a minimum and keeping the alveoli and small conducting airways open during expiration. There is mounting evidence that the surfactant proteins SP-A and SP-D play a role in the host defence system in the lung. SP-A and SP-D may act as potent neutrophil chemoattractants and enhance neutrophils’ respiratory burst in response to pathogens and it has also been demonstrated that the killing of *Aspergillus fumigatus* spores by alveolar macrophages is enhanced by SP-A and SP-D. Interestingly, SP-A has been shown to bind to *S. aureus* and *P. aeruginosa* and it has recently been reported that the concentration of SP-A is significantly reduced in CF patients (Griese et al., 1997). A SP-A knockout mouse has been generated (Korfhagen et al., 1996) and LeVine et al. (1998) studied the susceptibility of SP-A deficient mice to infection with mucoid *P. aeruginosa*. Interestingly, it was found that the SP-A deficient mice were less able to clear *P. aeruginosa* from the airways and they displayed an exaggerated inflammatory response in terms of proinflammatory cytokine release and nitrite production. These murine studies suggest SP-A does play a role in host defence in the airways and if indeed the levels of SP-A are reduced in CF patients then this could be contributary to CF lung disease.

1.11.3 Mucins.

Mucins are large glycoproteins which make up the major structural component of mucus. Mucin accumulation is abnormally high in CF which contributes to obstruction of the small airways following lung infection, intestinal obstruction and blocked pancreatic ducts. Nine mucin genes have been identified, *MUC1*, 2, 3, 4, 5AC, 5b, 6, 7 and 8 and at least six of these are known to be expressed in human
airway epithelial cells. MUC2 has been shown to be expressed at three to four fold higher levels in CF than non-CF nasal mucosa (Li et al, 1997a). Many factors have been shown to upregulate MUC2 gene expression including bacterial products such as P. aeruginosa LPS (Li et al, 1997b) and products of inflammatory cells including the proinflammatory cytokines TNF-α (Levine et al, 1995), IL-1β and IL-6. The MUC5 gene has also been shown to be upregulated by the products of P. aeruginosa (Dohrman et al, 1998). All of these mucin inducing factors have been shown to be at increased levels in the CF lung and could therefore go towards an explanation of the hypersecretion of mucins in the airways of CF patients. However it was not known whether there was a mucin gene abnormality preceding infection and, in an attempt to answer this, the levels of mucins have been compared in CF knockout mice and wild-type littermates. Interestingly, there was a six fold increase in Muc1 protein levels in the colon of the CF mouse which was thought to be of importance in the obstruction observed in the gastrointestinal tract of these mice (Parmley et al, 1998). It has yet to be established how the CFTR defect leads to this rise in mucin gene expression or whether alterations in the expression of mucin genes also exists in the lungs prior to bacterial infection.

1.11.4 Nitric Oxide.

Nitric oxide (NO) is a simple gas which is involved in numerous pulmonary functions including the host inflammatory response where its cytotoxic and cytostatic activities have been shown to act on microbes, parasites and viruses (Liew et al, 1991). Nitric oxide is also capable of damaging the host tissue especially when it is released in large amounts and it may also interact with reactive oxygen intermediates to produce peroxynitrite, an extremely reactive compound which leads to tissue damage. The main producers of nitric oxide are inflammatory cells principally macrophages and neutrophils but it is also produced constitutively in the airway epithelium under the control of constitutive nitric oxide synthase (cNOS). Another form of nitric oxide synthase exists (iNOS) can be induced by inflammatory stimuli,
such as proinflammatory cytokines and bacterial products, and leads to the production of large amounts of nitric oxide. The expression of iNOS from airway epithelial cells is increased in inflammatory diseases such as asthma (Hamid et al, 1993) and it was expected that high expression of iNOS and subsequently high levels of NO would also be found in patients with CF. Many studies have been carried out to examine nitric oxide and its possible involvement with CF lung disease. To date, the results of these studies have been somewhat contradictory. In contrast to the asthma studies, the concentration of exhaled NO has been found to be decreased in cystic fibrosis (Grasemann et al, 1997). However, nitric oxide synthase activity was reported to be elevated in lung samples from patients with CF (Belvisi et al, 1995) and in accordance with this result it has recently been reported that total sputum nitrate and nitrite (products of NO) are elevated in CF patients during acute pulmonary infection. Studies by Meng et al. (1998) have demonstrated a reduced expression of iNOS in CF bronchial epithelium compared to normal or inflamed lungs which they offer as a possible explanation for the reduced levels of NO in the exhaled breath of CF patients and their susceptibility to pulmonary bacterial infection. However, the significance of the lack of production of iNOS from bronchial epithelial cells is unclear since the overall level of NO in the CF lung is raised. Further studies on NO are required before a clear understanding of the possible role this substance may play in the CF lung disease is reached.

1.12 Cytokines.

1.12.1 Cytokine function.

Cytokines represent an expanding group of proteins which function, as ligands through a related set of receptors, as autocrine, paracrine or endocrine intercellular signals. They are implicated in numerous aspects of lymphoid and myeloid cell function and thus have importance in the control of the immune and inflammatory response to infection in the host. They were originally thought to be produced exclusively by cells of the immune system but it has been shown that other cell types
such as bronchial epithelium have the ability to secrete cytokines (Abdelaziz et al., 1995) and therefore probably have a role in the regulation of pulmonary inflammation.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Cellular Source</th>
<th>Effects Relevant to Lung Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>Macrophages, epithelium, T and B cells</td>
<td>Direct tissue injury, increases expression of IL-1α, IL-1β, IL-6, IL-8, increases NO production</td>
</tr>
<tr>
<td>IL-1β</td>
<td>macrophages, epithelium, antigen presenting cells, neutrophils</td>
<td>Activation of T and B cells, increases expression of IL-2, IL-6, IL-8</td>
</tr>
<tr>
<td>IL-2</td>
<td>T cells</td>
<td>Expansion of T cells, augmentation of neutrophil and macrophage activity</td>
</tr>
<tr>
<td>IL-4</td>
<td>T cells</td>
<td>Inhibits cytokine production by macrophages</td>
</tr>
<tr>
<td>IL-6</td>
<td>T cells, epithelium</td>
<td>Increases expression of TNF-α, IL-1β and IL-2 receptor, stimulates B cells to secrete IgG</td>
</tr>
<tr>
<td>IL-8</td>
<td>macrophages, fibroblasts, epithelium, neutrophils</td>
<td>Chemotactic activity for neutrophils, activates neutrophils to release lysosomal enzymes, induces adhesion of neutrophils to endothelium</td>
</tr>
<tr>
<td>IL-10</td>
<td>macrophages, epithelium</td>
<td>Reduces expression of TNF-α, IL-1β, IL-6, IL-8, increases expression of IL-1 receptor antagonist</td>
</tr>
<tr>
<td>IL-12</td>
<td>macrophages</td>
<td>Mediates differentiation of Th1 cells, stimulates activity of NK cells</td>
</tr>
</tbody>
</table>

Table 1.1 Cytokines relevant to CF lung inflammation.

Cytokines could be involved with CF-associated lung disease at several levels, including the regulation of the local inflammatory response to infection, the regulation of the antibody response to infection, direct injury to the airways and indirect injury to the host. Figure 1.4 illustrates some of the functions of a few of the cytokines involved. It is important to emphasise that this figure, in common with many others portraying cytokine networks, has been displayed in a grossly oversimplified fashion. The precise components and the relative importance of the numerous cytokines involved in the regulation of lung inflammation remain unclear.
For the purpose of this introductory discussion, only the cytokines which are suspected to be the most relevant to CF lung inflammation are considered.

TNF-α and IL-1β are often considered to be the primary pro-inflammatory cytokines as they are secreted early in response to infection and influence the pattern of later cytokine secretion in an inflammatory response. IL-6 has been shown to regulate the expression of many other pro-inflammatory cytokines and IL-8 is also regarded as important since it is a potent chemoattractant for neutrophils, which are the dominant cell type recruited into the lung during inflammation. Thus, much of the work regarding lung inflammation has concentrated on these cytokines.

1.12.2 Control of cytokine expression.

The cytokine network is a very complex system of pro and anti-inflammatory signals which must be tightly regulated to avoid inappropriate host responses to bacterial or viral challenge.

Cytokines themselves play a major role in the control of the cytokine based inflammatory process, either in a self-regulatory manner or by controlling the expression of other cytokines. In addition to this, several components of *P. aeruginosa* have been shown to be capable of inducing the release of high levels of pro-inflammatory cytokines from human monocytes. These included *P. aeruginosa* LPS, MEP, toxin A, porins and haemolytic phospholipase C (Cusumano *et al.*, 1997, König *et al.*, 1997). *B. cepacia*, another CF relevant pathogen, has also been shown to be capable of upregulating cytokines in the airways with *B. cepacia* LPS producing a higher inflammatory response in the airways than *P. aeruginosa* LPS in terms of TNF-α release from human leucocytes (Shaw *et al.*, 1995). An additional study by Palfreyman *et al.* (1997) demonstrated the ability of an extracellular factor from *B. cepacia*, unrelated to LPS, to release IL-8 from human monocytes.
CFTR defect
dysregulated cytokine expression?

chronic bacterial colonisation
release of bacterial products

alveolar macrophage activation

IL-1
TNF-α

T-lymphocyte activation

IL-8
IL-2, 3, 4, 5, 6

IL-2R
IL-2

IL-3, 4, 6

neutrophil chemotaxis and activation

release of proteases and oxygen radicals

LUNG DAMAGE

Fig. 1.4 Cytokines involved in the process of CF lung inflammation.
It is likely that components of *P. aeruginosa* and *B. cepacia* are involved in the overactive inflammatory response observed in CF patients colonised with these bacteria, although this alone is not sufficient to explain all the features of CF lung disease since these bacteria do not generally colonise the lung until after the first few years of life of the CF patient and several studies have shown signs of excessive inflammation in the airways prior to this.

### 1.12.3 Cytokine signalling.

Cytokines signal through transcription factors which then go on to activate the transcription of other genes involved in the inflammatory process. TNF-α and IL-1β mediate their downstream signals via the nuclear transcriptional factor NF-κB. TNF-α signalling has been studied in some detail in an attempt to understand the molecular basis of the pathway. When TNF-α binds to its receptor this leads to the recruitment of proteins to the TNF-α receptor tail which leads to the activation of NF-κB inducing kinase (NIK) and IκB-specific kinases (IKKα and IKKβ). IKKα and IKKβ then phosphorylate IκBα which leads to IκBα degradation which liberates the NF-κB complex, allowing it to translocate to the nucleus where it can modulate the transcription of various NF-κB-responsive target genes (Lin *et al*, 1998). IL-1β binds to a different receptor but the downstream signalling mechanisms are very similar to that of TNF-α (Muzio *et al*, 1997). NF-κB can also be activated by many other agents, including LPS (Baeuerle *et al*, 1994) and adherent *P. aeruginosa* (DiMango *et al*, 1998) to induce acute phase responses.

Many other cytokines including IL-6 transmit their signal via the JAK/STAT pathway. Several Janus kinases (JAKs) are activated by IL-6 binding to its receptors and these JAKs then activate the signal transducers and activators of transcription (STATs) which allows them to dimerise and leave the cytoplasm for the nucleus where they can bind to DNA and activate transcription. Most cytokine signalling is transient and therefore mechanisms to attenuate transcription must also be activated.
However, at present, little is known about the identity or regulation of factors that inactivate transcriptional factors though it is likely many of these will be phosphatases (Hill et al, 1995).

If there is dysregulation of the cytokine pathway in CF, it is possible that it arises from the CFTR defect interfering with the signalling mechanisms of cytokines, although at this point in time there is no evidence to support this theory.

1.12.4 Cystic Fibrosis and cytokine expression.

Over recent years many studies have found elevated levels of inflammatory cytokines in the bronchoalveolar lavage fluid (BALF), BAL macrophages or sputum from patients with cystic fibrosis. The main findings of this research will be summarised below.

In a study by Bonfield et al. (1995b) increased levels of the proinflammatory cytokines TNF-α, IL-8, IL-1β and IL-6 were detected in the BAL fluid of CF patients compared to those of healthy controls. In contrast, levels of IL-10 were found to be lower in CF patients compared to controls which may be relevant to the pathogenesis of CF since IL-10 is considered to play a regulatory role in lung inflammation by inhibiting immune complex-induced injury (Mulligan et al, 1997) and reducing the release of TNF-α (Gerard et al, 1993). It has since been shown that normal bronchial epithelial cells constitutively produce IL-10 and this appears to be downregulated in bronchial epithelium taken from CF patients (Bonfield et al, 1995a). Schuster et al. (1995) conducted a study measuring cytokine levels in the sputum of CF patients and also reported significantly increased levels of TNF-α and IL-8 in CF sputum versus normal controls and, in addition, found increased levels of IL-1β in CF sputum. A more detailed study was carried out by Salva et al. (1996) to investigate whether sputum concentrations of TNF-α and IL-8 differed in patients in a stable condition or experiencing a clinical exacerbation. In what first appears to be quite an unexpected result they report elevated levels of TNF-α and IL-8 concentrations in CF patients versus normal controls but no difference between stable and acutely ill CF patients.
However, it is possible that the presence of an acute-on-chronic response may not allow for further upregulation of TNF-α and IL-8 production as it may already be maximal.

CF has been described as a chronic lung disorder characterised by benign bacterial deposition in the airways at an early age punctuated at a later age by more frequent bouts of respiratory tract exacerbations which ultimately lead to lung damage and death. However the presence of bacteria in the airway at an early age may not be benign as it has been found to be associated with a sustained inflammatory response at a much earlier age than expected (Cantin, 1995). In a study of 27 CF children, some as young as 1 year, it was found that all had BALF cultures which were positive for pathogens and most had active neutrophil elastase, indicating that an inflammatory response had been generated in response to these pathogens (Birrer et al., 1994). A similar study by Khan et al. (1995) showed that in CF patients with an average age of less than 12 months and very mild lung disease, BAL neutrophils were increased more than 100-fold over controls and again, neutrophil elastase was detectable. These results indicate that the vicious circle of infection can be present in children within the first year of life.

1.12.5 Cytokine profiles in CF prior to lung infection.

It has been known for some time that inflammation and mild ventilatory defects occur before heavy lung infection is obvious. Doring et al. (Doring 1988) suggested there may be defects in the immune system, for instance in the regulation of cytokine production, which lead to an excessive immune response. Several groups of investigators therefore became interested in assessing the cytokine profile in the CF lung before there is any clinical evidence of lung inflammation due to infection. The results of these studies were intriguing and suggest that there may be a proinflammatory immune defect present in CF patients which is independent of infection.
Raised serum concentrations of the soluble IL-2 receptor (sIL-2r) found in CF patients in the absence of lung disease and *P. aeruginosa* colonisation suggested a role for cytokines in the earliest stages of the development of lung damage in CF (Dagli et al, 1992). Further evidence came from an investigation of CF lung inflammation in children with a mean age of less than 6 months (Khan et al, 1995). Reflecting the previously mentioned study of Birrer (1994) (see section 1.12.4) the CF children had marked elevations in BAL neutrophils and active neutrophil elastase, and in addition, raised levels of IL-8 were also detected. Seven of the sixteen infants used in this study had no detectable bacterial pathogens in the airways. These results may be interpreted to suggest that a CFTR defect could contribute directly to an exaggerated inflammatory response through its actions on proinflammatory cytokines. Even though stringent criteria were used in the pathogen screens, it is possible that the 7 infants termed pathogen-free were false negatives. Alternatively, an earlier infection may have occurred which was cleared completely by the immune system leaving only the remaining inflammatory response, though this is unlikely since it is very rare for pathogens to be completely cleared from the CF lung.

Recently, another study was performed on CF children: all had elevated levels of IL-1β, IL-8 and TNF-α in their BALF, but only 4 of the 14 children studied had detectable airway pathogens (Balough et al, 1995). Conflicting data were presented recently by Armstrong et al. in 1997 when it was reported that CF infants without any detectable respiratory pathogens had inflammatory cell numbers, IL-8 and free neutrophil elastase levels comparable to control subjects. In addition it was demonstrated that if an infection was present and then eradicated in CF infants, by the use of antibiotics, then the inflammatory component of CF lung disease was reversed suggesting that it is infection that is involved in initiating and maintaining airway inflammation. This is in stark contrast to data from Sharma et al. (1995) who demonstrated that the eradication of pathogens from the lung is not sufficient to halt the progression of pulmonary disease. However in the same year Noah et al. (1997) presented data which came to the same conclusions as Armstrong et al. (1997), that increased airway inflammation in uninfected CF infants represent the persistent sequelae of recent infection. However, it is not possible for either group to rule out
the possibility that the increased inflammatory reaction, previously reported by several independent groups, is due to a constitutive inflammatory process present in the respiratory system preceding bacterial infection.

1.12.6 Cytokine expression from cultured epithelial cells.

*In vitro* methods have also been utilised in the search for immunological abnormalities in the CF lung. Nakamura *et al.* (1992) studied the effect of culturing a normal human bronchial epithelial cell line (BET-1A) with ASF taken from individuals with CF. The CF ASF, which contained high levels of neutrophil elastase, was found to induce the bronchial epithelium to secrete IL-8. This result led to the suggestion that a self-perpetuating inflammatory response exists whereby neutrophil elastase induces the secretion of IL-8 from the bronchial epithelium which results in additional neutrophils migrating to the lung.

Ruef *et al.* (1993) demonstrated that a transformed airway epithelial cell line (JME/CF15) containing a ΔF508 homozygous mutation showed increased levels of secreted IL-6 and IL-8 following stimulation with IL-1β or TNF-α compared with a normal airway epithelial cell line. Additionally, the CF cell line in an unstimulated condition secreted more IL-6 and IL-8 than the normal cell line. This is an intriguing observation which may also be interpreted to be the direct result of the CFTR mutation, however, whether this is a true difference between CF and non-CF epithelial cells or just due to variation between the cell lines has not been established.

CFTR is expressed in macrophages and neutrophils, albeit at low levels (Yoshimura *et al.*, 1991) and has also been detected in T lymphocytes (Dong *et al.*, 1995) and it is now known that epithelial cells are capable of cytokine expression so it is theoretically possible that a CFTR defect in these inflammatory cells may somehow lead to dysregulated expression of cytokines.

In summary, the general theme that has emerged from recent literature is that cells involved in the regulation of inflammation within the airways appear to have altered
patterns of cytokine secretion in the respiratory systems of CF patients compared to controls. Whether this is dependent on bacterial colonisation is a controversial point due to the contradictory experimental evidence which has been described in the last few sections.

Regardless of whether inflammation is initiated by abnormal cytokine expression or by pathogens, the study of these molecules in CF lung disease is very relevant and merits further investigation since cytokine based therapy may be a useful complement to anti-microbial strategies.

1.13 Cell adhesion molecules.

1.13.1 Cell adhesion molecule function.

Cell adhesion molecules may also be considered relevant in CF lung disease since they are involved in the recruitment of leucocytes from the blood to the site of injury. This migration of cells across the endothelial barrier is a tightly regulated event which requires selective leucocyte-endothelial cell recognition. The basic molecular mechanisms for this cell trafficking have been studied in some detail over recent years and are thought to comprise a cascade of events brought about by the sequential activation of different types of cell adhesion molecules. Since most of these studies have been centred around neutrophil migration and this is the most relevant aspect of leucocyte trafficking as regards CF lung disease, this section will deal primarily with these cells.

Neutrophil-endothelial recognition is an active process which requires at least 3 main steps as shown in figure 1.5. The first requires the binding of constitutively functional neutrophil adhesion molecules to the endothelial counter-receptors. This initial step is transient and reversible, often referred to as rolling. In vitro studies suggest that neutrophil L-selectin contributes to this rolling action by presenting neutrophil carbohydrate ligands, called sialyl Lewis X, to the endothelial E and P selectins (Picker et al, 1991).
<table>
<thead>
<tr>
<th>Cell Adhesion Molecule</th>
<th>Ligands</th>
<th>Cell Expression</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM-1</td>
<td>LFA-1, Mac-1</td>
<td>endothelium, epithelium, monocytes, T and B cells</td>
<td>leukocyte adhesion to endothelium</td>
</tr>
<tr>
<td>P-selectin</td>
<td>sialyl Lewis X</td>
<td>platelets, endothelium</td>
<td>neutrophil adhesion to endothelium</td>
</tr>
<tr>
<td>E-selectin</td>
<td>sialyl Lewis X</td>
<td>endothelium</td>
<td>initial binding of neutrophil to endothelium</td>
</tr>
<tr>
<td>L-selectin</td>
<td>sialyl Lewis X</td>
<td>neutrophils, monocytes, eosinophils, basophils, B and T cells</td>
<td>mediates lymphocyte rolling</td>
</tr>
<tr>
<td>Mac-1 (α_3β_2)</td>
<td>ICAM-1</td>
<td>monocytes, macrophages, NK cells, neutrophils</td>
<td>adherence of neutrophil to endothelium</td>
</tr>
<tr>
<td>LFA-1 (α_4β_2)</td>
<td>ICAM-1</td>
<td>lymphocytes, neutrophils, monocytes, macrophages</td>
<td>adherence of neutrophil to endothelium</td>
</tr>
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</table>

Table 1.2 Cell adhesion molecules relevant to CF lung inflammation.

The next stage is thought to involve cellular arrest on the endothelium which has been reported to involve shedding of L-selectin from neutrophils and the rapid upregulation of Mac-1 (α_3β_2) (Kishimoto et al., 1989). There is also evidence that receptor clustering and cell spreading are involved (Stewart et al., 1995). The molecular details of how neutrophils or the endothelium could become activated are not yet clear but it has been proposed that macrophages in the airways release IL-1β and TNF-α in response to bacterial LPS and these cytokines stimulate the release of IL-8 by epithelial cells. This in turn then creates a gradient across the mucosa which leads to neutrophil migration across the endothelium (McEver, 1994).

Once activation has taken place the neutrophil must firmly adhere to the endothelium. This is believed to take place via the neutrophil β2 integrins LFA-1 and Mac-1 which attach to endothelium ICAM-1. The neutrophil then flattens and transmigrates the endothelium via its ligation with ICAM-1, a process called diapedesis. Once the neutrophil has navigated through the endothelium it becomes further activated by chemoattractants in the intracellular space including IL-8, the complement component C5a, LPS derived from the cell wall of gram negative bacteria and the product of bacterial metabolism FMLP.
To date, little research has been carried out to investigate the relevance of cell adhesion molecules to CF lung inflammation compared to that of cytokines, however several interesting reports have been published. It is assumed that endothelial molecules such as ICAM-1 and E-selectin will be upregulated to allow the mass migration of neutrophils into the lung and it was therefore surprising that sICAM-1 levels were reported to be lower in CF patients during acute exacerbations than in stable CF patients (Salva et al, 1996, De Rose et al, 1998). Increased levels of sICAM-1 have been observed in several other inflammatory diseases, however it may be that in CF sICAM-1 is binding to cell membranes, immune complexes or proteins or that it is being broken down by the high levels of proteases present. Recently
Russell et al. (1998) reported that, when stimulated, neutrophils from both stable and acutely infected CF patients shed significantly less L-selectin than those stable or acutely infected non-CF bronchiectasis patients. The exact physiological consequence of this is not clear but shedding less L-selectin may prolong lymphocyte rolling and allow more neutrophils to enter the airways.

1.14 Defensins.

1.14.1 Classification and structure.

Recent investigations have indicated airway beta defensins as having relevance in the initiation of CF lung disease.

Defensins are small, 3-4 kDa, antimicrobial cationic peptides that exist in a wide range of organisms including plants, insects and vertebrates. The secondary structure of defensins has been examined by nuclear magnetic resonance spectroscopy and by X-ray crystallography. Both techniques showed defensins to have a secondary structure characterised by a beta-sheet element stabilised by intramolecular cysteine disulphide bridges (Hill et al, 1991) as shown in figure 1.6A. The secondary structure of novel defensins can now also be predicted using computer analysis.

The vertebrate defensin family consists of two branches, designated alpha and beta defensins. These two groups differ with respect to the nature of their precursors, the sites of expression and the spacing and connectivity of their six conserved cysteine residues although they have identical folds in their secondary structure (see figure 1.6B). Figure 1.7 shows a list of the beta defensins identified to date.
Fig. 1.6 Tertiary structure of mature defensin peptides.
(A) Simplified cartoon of predicted tertiary structure of mature defensin peptides. The beta sheets are shown in blue, the alpha helix in red and the three disulphide bridges in black. (B) Spacing and connectivity of cysteine residues in human alpha and beta defensins. The mature peptide regions of alpha defensin HNP-1 and beta defensin HBD-1 are shown with the conserved cysteine residues shown in red. The characteristic connections between the cysteine residues of the alpha and beta defensins are also shown.
Fig. 1.7 Peptide sequences of beta defensins.

Dashes were used to optimise the alignment and the conserved cysteine residues are shown in red. The pre-pro sequences of Gal-1 and THP-1 are unknown. There are 13 bovine neutrophil beta defensins of which only one, BNBD4 is shown above. Abbreviations: hBD, human beta defensin; rhBD, rhesus monkey beta defensin; Def, mouse beta defensin; Rbd, rat beta defensin; TAP, tongue antimicrobial peptide (bovine); LAP, lingual antimicrobial peptide (bovine); BNBD4, bovine neutrophil beta defensin; SBD, sheep beta defensin; pBD, porcine beta defensin; Gal-1, gallinacin-1 (chicken); THP-1, turkey heterophil peptide-1.
1.14.2 Evolution of defensins.

The existence of related antimicrobial gene products in insects, mammals and birds suggests that the defensin gene family evolved as a very ancient means of host defence. Although it is not clear what the precise mechanism of evolution was for this gene family it is likely that the mammalian alpha and beta defensin gene families arose as a result of a gene duplication event before mammalian radiation. The close chromosomal location of the human alpha and beta gene families also strongly suggests these 2 gene families arose from a common ancestor (Liu et al, 1997).

The presence of highly homologous alpha defensin genes in several mammalian species and the close chromosomal locations of these genes suggests that repeated gene duplication occurred independently in the separate species during the evolution of the defensin multi-gene family. It has been suggested that after gene duplication, certain pairs of newly duplicated defensin genes were subject to positive Darwinian selection favouring diversification of the mature defensin at the amino acid level (Hughes et al, 1997). A factor that may account for these changes in the amino acid sequence of the defensin would be a change in the microbial species to which the host is exposed. The effects of defensin diversity on the host-pathogen response has yet to be investigated, however it is hoped that the study of mouse and human beta defensins and the range of pathogens against which they are active may shed some light on this interesting hypothesis of gene divergence. The number of beta defensins found in mammals to date has been considerably less than that of the alpha defensins and therefore no studies to date on the evolution of this branch of the defensin family, similar to that of the alpha family mentioned above, have been made.

1.14.3 Biosynthesis.

All defensins are initially synthesised as prepropeptides, consisting of an amino terminal signal sequence, a propiece and a mature peptide at the carboxyl- terminus as shown in figure 1.8. The function of the signal sequence is well understood and, in
both alpha and beta defensins, is to target the prepropeptide for insertion into the endoplasmic reticulum. The signal sequence is then cleaved very rapidly, probably co-translationally, to yield the propeptide. By studying propiece deletions, Liu and Ganz (1995) demonstrated that the propiece of the alpha defensin, human neutrophil peptide -1 (HNP-1), contained a 12 amino acid segment which appeared to be essential for correct subcellular sorting. They proposed that the folded mature peptide contains motifs that would retain the peptide in the endoplasmic reticulum or Golgi and the propiece serves to mask these motifs either directly or via a chaperone molecule. Alternatively, or perhaps additionally, it has been shown that the HNP-1 peptide is inactive until it has undergone propiece cleavage and therefore it was proposed that the propiece inhibits the cytotoxicity of HNP-1 (Valore et al, 1996). The alpha defensins are stored in granules and therefore it would be essential to suppress the cytotoxic activity of the mature peptide whilst in storage to prevent damage to the host.

The propiece of beta defensins is shorter and less acidic than that of the alpha defensins and several recent studies conducted on HBD-1 suggest that the propiece in this beta defensin does not function in an equivalent fashion to HNP-1. HBD-1 was originally isolated as a 36 amino acid peptide from hemodialysate fluid which was considered to be the mature form of the peptide. However, when attempts to isolate this peptide were made in urine, kidney, blood plasma and vaginal lavage, multiple forms of the HBD-1 peptide ranging in length from 36 to 47 amino acids were found, reflecting variable amino terminal proteolytic processing (Valore et al, 1998). All forms of HBD-1 were found to exhibit antimicrobial activity against E. coli indicating that no inhibitory proregion was present. However, they did show different profiles of activity when tested under varying conditions indicating the proregion did have some role in regulating the antimicrobial activity. In the light of recent studies into the salt-sensitivity of defensins and its possible relevance to CF, it is interesting that the activity of only some of the forms of HBD-1 found were affected by altering the salt concentration. A similar study was conducted by Zucht et al. (1998). Again, several N-terminally truncated variants of HBD-1 were identified from urine with the main form being 44 amino acids in length. This peptide was shown to have a
weak, salt-sensitive antimicrobial activity but one which was significantly more potent against *Bacillus subtilis, Bacillus megaterium, Staphylococcus carnosus* and *Escherichia coli* than the 36 amino acid peptide. Further investigation of the variant forms of HBD-1 will be necessary to determine if different variants of HBD-1 exhibit antimicrobial activity on different spectrums of pathogens or if the lengths of the peptide are controlled in a tissue specific manner.

**Fig. 1.8 Biosynthesis of defensin peptides.**

The main action of defensins is in the eradication of bacteria from the host. They are effective against a wide range of both gram negative and gram positive bacteria and are also capable of killing yeast, fungi and viruses.

Again, most of the work in this area has concentrated on the alpha defensins HNP-1 - 3 but it is thought that beta defensins act in a similar fashion. The main site of defensin action is the cytoplasmic membrane where the peptides assemble to form channels. For gram negative bacteria an additional outer cell wall must be crossed by the defensins and it is thought this occurs by a process of self-promoted uptake. LPS, a component of the outer membrane of gram negative bacteria, possesses divalent cation binding sites. Defensins however, have a higher affinity for LPS than the native divalent cations, such as Mg$^{2+}$, and are thus able to competitively displace these ions on the cell wall. This disrupts the outer membrane causing it to crack and allowing the defensins to enter the cell. This action of "opening up" the cell also explains the synergistic relationship between defensins and conventional antibiotics. The defensins then form channels in the bacterial cytoplasmic membrane and their cationic nature allows them to interact with the negatively charged membranes and enter the membrane under the influence of the large electrical potential of the bacterial cytoplasmic membrane. The necessity of the high membrane electrical potential was demonstrated by Kagan et al. (Kagan et al., 1990) when defensins were shown to be unable to enter phospholipid membranes until an electromotive force was applied. It is now thought that it is not simply the permeabilisation of the cytoplasmic membrane that leads to bacterial cell death since cells may be rescued after they have become permeabilised. Treatment of cells with defensins leads to activation of poly (ADP-ribose) polymerase, a DNA repair enzyme and it has also been shown that if DNA repair mechanisms are inhibited then defensin-induced cell lysis is enhanced (Lehrer et al., 1993). This led to the proposal that a second, irreversible phase of defensin-mediated toxicity occurs which causes DNA injury to the cell. This proposed mechanism of defensin killing is shown in figure 1.9.
Interestingly, *B. cepacia* has been found to be naturally resistant to the action of defensins (Hancock, 1997, Morrison *et al*., 1998). It has been suggested that this may be by virtue of having a non-interactive outer membrane. Screens are currently being conducted on mutant strains of *B. cepacia* which are not resistant to defensins in an attempt to isolate genetic factors which may be responsible for its resistant nature. Preliminary results have suggested methyl transferase and glucosyl transferase-like genes as candidates but further complementation and characterisation studies are required (Steen *et al*., 1998).

Fig. 1.9 Proposed mechanism of bactericidal activity of defensins.
1.14.5 Effects of defensins on eukaryotic cells.

Eukaryotic cells have low membrane potentials, high levels of cholesterol and low amounts of anionic lipids which offers some explanation as to the bacterial specificity of defensin molecules. However, defensins have been shown capable of having cytotoxic effects on mammalian cells (Zucht et al, 1998). HBD-1 was demonstrated to have a strong cytotoxic effect on the mouse NIH-3T3 cells in a dose and time dependent manner. It is unlikely that this response reflects a true physiological occurrence since it was inhibited by the presence of serum, although if HBD-1 was functioning at mucosal surfaces, which is devoid of serum, this may not be a relevant point. However, the concentrations of HBD-1 required to exert a toxic effect on the cells are much higher than the concentrations found in these areas under normal conditions.

An additional activity of defensin molecules is their ability to inhibit the production of corticosterone by rat adrenal cells, known as the corticostatic effect. It is thought that the defensin exerts this effect by interfering with receptor binding (Zhu et al, 1992). This effect has only been described for rabbit defensin NP-3A and it is not known whether other defensins can exert a similar effect or if any participate in receptor mediated inhibition of other systems.

It has recently been shown that the murine intestinal alpha defensins, cryptidin 2 and 3, can form pores in epithelial cells which are then capable of transporting chloride (Lencer et al, 1997). When cryptidins 2 or 3 were applied to apical membranes of intestinal epithelial cells in culture, chloride secretion was stimulated. This response was found to be reversible and dose dependent and suggested that the defensins may function in paracrine signalling mechanisms through the formation of ion conductive channels in the crypts of the intestine. It is not known if airway defensins are capable of forming chloride-transporting pores or what relevance this may have to the action of defensins in CF lung disease.

Defensins have a similar structure to the C1q binding domain of the transmembrane protein gp41 of HIV and it has recently been demonstrated that defensins, HNP-1, 2
and 3 can also bind the C1q subcomponent of the complement system and trigger the complement activation cascade through the classical pathway (Prohaszka et al, 1997). It is possible that this is a feature shared by the beta defensins since the C1q binding domain of HIV is structurally similar to all defensins composed of a beta-sheet stabilised by disulphide bonds.

Interestingly it has been suggested that defensins are capable of stimulating the release of the cytokine IL-8 by airway epithelial cells (Van Wetering et al, 1997). When primary cultures of bronchial epithelial cells were treated with HNP-1,2 and 3, which had been isolated from stimulated neutrophils, it resulted in a 12 fold increase of IL-8 transcription and a 16 fold increase in neutrophil chemoattraction. This induction is higher than that observed following TNF-α stimulation. It was thought that the concentrations of defensin used were relevant in vivo and it was concluded that defensins could play a role in the influx of neutrophils into the airways during an inflammatory response. The mechanism of IL-8 induction by neutrophil defensins has not been ascertained and it has also not been reported whether this function also exists in beta defensins or whether this process is salt sensitive. It may be the case that this aspect of defensin action is not affected in the CF environment of the lung and therefore it is possible that the action of defensins promotes lung disease in CF not only through the loss of its bactericidal activity but also through stimulating the release of a chemoattractant, drawing more neutrophils into the lung and contributing to the excessive inflammatory response.

1.14.6 Regulation of defensin genes.

The factors that govern the regulation of expression of defensins are not well understood at present, however it is anticipated that the continued discovery of novel defensins and the identification of regulatory elements in the untranslated regions of the genes will soon remedy this.

There is tissue specificity of defensin expression and there is also control over the time of expression. Some defensins are expressed at the time of birth (Ouellette et al,
1989) whilst it appears others are synthesised in increasing amounts following host exposure to microbial substances (Diamond et al, 1993). The regulatory factors governing the expression of defensins is not known but many alpha defensins contain highly conserved domains in their 5’ untranslated regions (UTR) and it has been proposed that these elements may be involved in the developmental and tissue specific aspects of defensin expression. Studies have revealed that the expression of some defensins can be induced at the sites of inflammation, supporting a role for these antimicrobial peptides as integral components of the inflammatory response. The bovine airway beta defensins LAP and TAP have both been shown to be upregulated at sites of inflammation (Schonwetter et al, 1995, Russell et al, 1996). The 5’ UTR of TAP has been studied in some detail and several regulatory elements have been found including a consensus sequence for the nuclear transcription factors NF-κB (Diamond et al, 1993). Several insect defensins are responsive to LPS and the signal transduction pathway found to mediate the response involves dif which is a homologue of NF-κB (Ip et al, 1993) thus providing an evolutionary link between LPS regulation of defensin genes and NF-κB. Several putative binding sites for another transcription factor, NF-IL6 (also known as C/EBP), have also been found in the 5’ UTR of TAP. Recent studies suggest CD14, a well-characterised LPS-binding protein, may be involved in the LPS mediated upregulation of defensin genes. Bovine tracheal epithelia cells treated with LPS resulted in a 13 fold induction of TAP and this induction could be blocked by the addition of a monoclonal antibody to CD14 (Diamond et al, 1996). The CD14 protein was found to be of epithelial cell origin and in situ hybridisation experiments have detected CD14 mRNA in multiple epithelial cell types including bronchial epithelial cells of the mouse (Fearns et al, 1995) suggesting the possibility that mouse defensin genes may be upregulated in the same manner. It has been shown that LPS stimulation activates NF-κB and Lee et al. (1992) demonstrated that CD14 expressing cells activate significantly more NF-κB than non-CD14 expressing cells. Therefore defensin genes could be upregulated by binding NF-κB which has been activated by CD14 binding LPS.

The cytokine TNF-α is also capable of inducing both TAP and LAP (Russell et al, 1996) and as mentioned in section 1.12.3, TNF-α signals through the transcriptional
factor NF-κB. Hence it is possible that the activation of NF-κB by TNF-α leads to NF-κB binding to the upstream region of the defensin genes and upregulating their transcription.

The human airway beta defensin, hBD-2, unlike hBD-1, has been shown to be upregulated in the airways in response to an inflammatory challenge. This defensin was initially isolated in the skin and hBD-2 expression in keratinocytes was found to be upregulated in response to TNF-α, and a range of bacteria including *P. aeruginosa*, *E. coli* and *S. aureus*. Preliminary reports on hBD-2 expression in the airways indicate that treatment with pro-inflammatory cytokines TNF-α and IL-1β and LPS can induce the expression of hBD-2 (Singh et al, 1998, Verghese et al, 1998) supporting the relevance of this defensin in pulmonary inflammatory responses.

### 1.14.7 Cystic Fibrosis and defensins.

The possible relevance of human beta defensins to CF arises from their expression in airway epithelial cells, their existence in the ASF and their salt sensitive antimicrobial activity (see section 1.10.4).

Smith *et al.* (1996) demonstrated that the ASF covering the apical surface of primary cultures of airway epithelial cell had antimicrobial activity, however the ASF from epithelial cells from CF individuals had a markedly reduced antimicrobial activity. When the salt concentration of the normal ASF was increased, the antimicrobial activity of the fluid decreased which agreed with the previous reports that the ASF from CF individuals had elevated concentrations of Cl\(^-\) and Na\(^+\) compared to normal. This conclusion however, remains contentious and shall be discussed in more detail in section 6.8. hBD-1 was the first human beta defensin found to be expressed in the airways (Goldman *et al.*, 1997, McCray *et al.*, 1997) and an HBD-1 synthetic peptide was shown to have broad spectrum bactericidal activity which was markedly reduced in salt concentrations similar to those reported for CF ASF. As mentioned in section 1.10.4, there has been a second human airway beta defensin found, hBD-2 which has
also been shown to have a salt sensitive antimicrobial activity and to be induced by inflammatory stimuli. It will be interesting to learn more about their antimicrobial function, possible synergistic relationships, influence on the expression of pro-inflammatory cytokines and ultimately their relevance to the initiation of CF lung disease.

The discovery of a mouse beta defensin expressed in the airways, Defb1, has recently been described (Morrison et al, 1998). Since part of this project was concerned with the cloning and characterisation of this gene, a more detailed description of Defb1 will be made in chapter 6.

1.15 The Cystic Fibrosis mouse models.

1.15.1 Creation and characterisation.

CF has been a particularly complex disorder to study in humans due to genetic heterogeneity, inadequate sample sizes and the lack of a controlled environment. In vitro experimentation can overcome some of these problems, though the study of cells in isolation from the rest of the system can lead to misleading results. Investigators therefore looked to an alternative method of studying CF, the animal model.

No natural animal model of CF had been identified, thus it was necessary to create mutations in the laboratory mouse. Soon after the human CFTR gene was cloned the murine homologue was identified (Tata et al, 1991). This was found to have 78% overall identity at the amino acid level with the human gene, and, encouragingly, most of the human CFTR mutations occurred at positions of conserved residues, suggesting conservation of the function across species. Additionally wild-type mice were found to have bioelectric properties in the respiratory and intestinal tracts, consistent with a common mechanism of cAMP-dependent chloride ion conductance (Smith et al, 1992).
Within a few years of CFTR being cloned, homologous recombination had been used to create several Cftr knockout mice in independent laboratories (Snouwaert et al, 1992 (Cftr<sup>tm1Unc</sup>), Dorin et al, 1992 (Cftr<sup>tm1Hgu</sup>), Colledge et al, 1992 (Cftr<sup>tm1Cam</sup>), O'Neal et al, 1993 (Cftr<sup>tm1Bay</sup>)).

<table>
<thead>
<tr>
<th>Mutant Allele</th>
<th>Type of Mutation</th>
<th>Genomic Site</th>
<th>Residual Cftr Function</th>
<th>Survival to Adulthood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cftr&lt;sup&gt;tm1Hgu&lt;/sup&gt;</td>
<td>Insertion</td>
<td>Exon 10</td>
<td>&lt;10% of normal</td>
<td>&gt;90%</td>
</tr>
<tr>
<td>Cftr&lt;sup&gt;tm1Cam&lt;/sup&gt;</td>
<td>Replacement</td>
<td>Exon 10</td>
<td>None detected</td>
<td>&lt;10%</td>
</tr>
<tr>
<td>Cftr&lt;sup&gt;tm1Bay&lt;/sup&gt;</td>
<td>Insertion</td>
<td>Exon 3</td>
<td>None detected</td>
<td>&lt;50%</td>
</tr>
<tr>
<td>Cftr&lt;sup&gt;tm1Unc&lt;/sup&gt;</td>
<td>Replacement</td>
<td>Exon 10</td>
<td>None detected</td>
<td>&lt;5%</td>
</tr>
</tbody>
</table>

Table 1.3 The Cftr knockout mouse models.

It is not possible to detect Cftr mRNA in 3 of the 4 knockout mice, indicating these mice were absolute nulls. However, in the Cftr<sup>tm1Hgu</sup> homozygous mice, a low level of Cftr mRNA, <10 % of normal level, can be detected which is thought to result from aberrant splicing and exon skipping (Dorin et al, 1994). The detected message is not the expected mutant mRNA but one with a novel 200 bp exon derived from plasmid sequence which is incorporated between exons 9 and 11 by the utilisation of a cryptic splice site. It has been proposed that these mice would be similar to CF patients carrying a leaky splice site in intron 19 (Highsmith et al, 1994) and those which carry missense mutations in the first transmembrane domain (Sheppard et al, 1993), retaining ~8 % and ~15 % of normal CFTR transcript, respectively.

As an alternative to a knockout model, several groups independently created a ΔF508 mutation in the mouse Cftr gene (Colledge et al, 1995, van Doorninck et al, 1995, Zeiher et al, 1995). Since then several other specific mutations have been created in the mouse by single step gene replacement including the G551D mutation (Delaney et al, 1996a) and the G480C mutation (P. Dickinson, personal communication). Rozmahel et al. (1996) created a new CF mouse, Cftr<sup>tm1Hsc</sup>, by disrupting exon 1 of
Cftr, which has been previously mentioned with regard to the genetic modifier studies.

The electrophysiology and histopathology of the different cystic fibrosis mice has been studied in some detail and the most salient points, to date, are summarised in the table below.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>cAMP-mediated chloride conductance of lower respiratory tract</th>
<th>Death due to intestinal disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cftr&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>decreased (in vitro only)</td>
<td>~90%</td>
</tr>
<tr>
<td>Cftr&lt;sup&gt;−/−/Cftr&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>decreased (after TBHQ treatment)</td>
<td>~90%</td>
</tr>
<tr>
<td>Cftr&lt;sup&gt;−/−/Homo&lt;/sup&gt;</td>
<td>decreased (in vitro only)</td>
<td>~50%</td>
</tr>
<tr>
<td>Cftr&lt;sup&gt;−/−/Higro&lt;/sup&gt;</td>
<td>decreased (in vivo)</td>
<td>~7%</td>
</tr>
</tbody>
</table>

Table 1.4 Chloride conductance and intestinal disease in CF mice.

1.15.2 CF mouse lung disease.

Due to the high rate of mortality caused by lung disease in CF patients, the relevant importance of the mouse model depends upon how well it mimics this aspect of the disease. Initially it was feared that the mouse would serve as a poor model due to its lack of submucosal glands, which highly express CFTR in humans. However recent studies have demonstrated the presence of Cftr expressing submucosal glands in the proximal region of mouse trachea. Although fewer in number than in humans they exhibit a similar cell morphology, structure and neonatal pattern (Borthwick et al, 1999). It has been observed, however, that pulmonary disorders in human CF sufferers begin initially in an area of the lung which lack submucosal glands (Dorin et al, 1994) and therefore it may not be of relevance that the mouse contains fewer submucosal glands than humans.
CF null mice failed to exhibit any of the gross pathological abnormalities present in the human disease but they did have severe intestinal pathology. Clarke et al. (1994) attempted to explain this difference by demonstrating the presence of a calcium activated chloride channel in mouse lung epithelium, but not in the intestine, which had no functional disruption in the CF mice. It was proposed that this alternative channel could go some way to correcting the chloride ion defect in the lungs of these mice. This upregulation of a calcium activated chloride channel is not observed in the Cfrtm1Hgu mice and it is thought this may be due to the presence of low levels of residual Cfr in these mice (Smith et al, 1995). There was also no signs of CF-like lung diseases in the Cfrtm1Hgu mice but significant differences were found in lung pathology between Cfrtm11Hgu mice and their wild-type littermates following exposure to pathogens as shall be discussed in more detail in the next section.

1.15.3 CF mouse and response to CF relevant pathogens.

Cfrtm1Hgu mice challenged transiently with aerosols containing the CF associated pathogens, S. aureus or B. cepacia had a reduced capacity to clear the inhaled organisms compared with normal littermates. Following exposure there was also a significant difference between Cfrtm1Hgu mice and their normal littermates in the incidence of goblet cell hyperplasia, pneumonia, mucus retention, bronchiolitis and severe bronchopneumonia (Davidson et al, 1995).

However, the clearance data and histology of mice with the same genotype displayed a high degree of variability. This may have arisen through differing degrees of Cfr mutation “leakiness” between mice or alternatively due to the outbred background of the mice.

Heeckeren et al. (1997) recently described the excessive inflammatory response observed in Cfrtm1Unc mice following P. aeruginosa challenge. Agarose beads were used to retain P. aeruginosa in the airways to test the hypothesis of whether abnormal retention of bacteria in itself accounts for CF lung disease. Following instillation of the P. aeruginosa beads it was found that equal numbers of P.
*P. aeruginosa* colony forming units could be recovered from wild-type or CF mice but that the CF mice had an excessive inflammatory response compared to wild type littermates. 82 % of CF mice died within 10 days of infection compared to 23 % of wild-type littermates. It was concluded that the CF genotype is associated with an excessive inflammatory response and that abnormal retention of *P. aeruginosa* in the airways does not in itself account for this. This result is in support of the mouse being a suitable model in which to study CF lung disease, but several aspects of this study have not been taken into account. It has not been stated what caused the high mortality of the CF mice. Significant differences between CF and wild-type littermates were only found in TNF-α and the neutrophil chemoattractants, MIP-2 and KC after 3 days but no differences were found in other cytokines, histology or inflammatory cell numbers. It would have been more informative to use higher numbers of mice and to analyse the pulmonary inflammatory status of the animals at more timepoints following treatment. It is assumed by the authors that the heightened mortality of the CF mice was due to the excessive pulmonary inflammation but there is no experimental evidence to prove this. Also no consideration of the effects of background strain of the mouse was made in the interpretation of the results. This had been shown to be a very relevant factor as has previously been discussed in section 1.7 and the existence of genetic factors influencing the lung phenotype was also demonstrated by Kent *et al.* (1997) who showed that the lung phenotype of the *Cftr<sup>tm1Unc</sup> mouse on a mixed genetic background differed from that of the *Cftr<sup>tm1Unc</sup> mouse on an inbred C57Bl/6 background. However, there is doubt over whether the results presented following this study support the conclusion made by the authors that these CF mice on the inbred background develop progressive lung disease which is significantly different from control mice.

### 1.15.4 CF mouse and mucociliary clearance.

A decreased level of mucociliary clearance has been reported for *Cftr<sup>tm1Hgu</sup> mice* (Zahm *et al.*, 1997). Mucociliary transport velocity was found to be significantly
higher in control mice compared to CF mice but there was no difference observed in the mean mucociliary beat frequency. An increased thickness of the mucus lining the surface epithelium and an increased number of inflammatory cells in the lamina propria were also reported although there was no bacterial infection obvious. However, a high degree of variability in results within groups was observed, again thought to be due to the use of an outbred strain background. Another study examining mucociliary clearance in the CF mouse was recently reported. Cowley et al. (1997b) used \textit{Cftr}^{im1/Unc} mice on a C57Bl/6 background and also found differences in mucociliary clearance between CF mice and controls. The results produced were similar to the other study in that the CF mice differed from the controls in their particle transport rate but not in their ciliary beat frequency. Although it is unclear how the CFTR mutation uncouples the normal relationship between particle transport and ciliary beat frequency it has been suggested that this could occur indirectly by alterations to the salt concentration of the ASF (Cowley et al, 1997b) although, to date, there is no evidence to support this hypothesis.

1.15.5 CF mouse and surfactant

Surfactant was examined in the \textit{Cftr}^{im1/Igu} mouse in an attempt to answer the question of whether surfactant alterations in the CF lung are present before bacterial infection or whether it is secondary to this event (Bernhard et al, 1997). In uninfected mice, there was no difference found in the amounts of surfactant protein A or the composition of phospholipid and phosphatidylcholine molecular species in the BALF and lung tissue. Although the composition was not changed, there was a higher amount of phospholipids found in the CF mice compared to wild-type mice. It was concluded that surfactant homeostasis is perturbed in the CF mouse. It is of interest to note that the results obtained from the CF mouse were in contrast to those obtained from CF patients where the phospholipid concentration was found to be significantly reduced (Griese et al, 1997). Possible reasons for the discordance between results obtained from the CF mouse and human CF patients will be discussed later.
Although not conclusive, these studies display differences in the airways between CF mice and wild-type littermates. It is hoped that future studies using these CF mouse models, which mimic the human disorder in many aspects, will allow further studies into the many putative factors which influence the initial development of lung disease and its progression.

1.16 Murine homologues of cytokines and cell adhesion molecules relevant to CF.

All of the murine homologues to the cytokines and cell adhesion molecules described in tables 1.1 and 1.2 have been cloned apart from IL-8, though several other neutrophil chemoattractant factors have been identified. These are KC (Bozic et al, 1995), MIP-2 (Wolpe et al, 1989) and Cp-10 (Lackmann et al, 1992). Cp-10 may prove to be particularly interesting as it has 59 % amino acid identity with the human heterodimer MRP-8/14, also known as the CF Antigen (CFAg). The CFAg was once considered a candidate for the CF gene mainly due to its presence in serum of CF patients, absence in normal healthy individuals and intermediate expression in the serum of carriers (Dorin et al, 1987). The murine pro-inflammatory cytokines and cell adhesion molecules have been found to function in a very similar fashion to their human counterparts and therefore it was considered relevant to study these molecules in CF mice and wild-type littermates.
1.18 Aims of this thesis.

1.18.1 The study of proinflammatory molecules.

The studies mentioned previously on young CF patients (see section 1.12.5) indicated the possible existence of a dysregulated immune system in CF which was responsible for the initiation of lung disease. Therefore, in an attempt to establish whether the presence of bacteria or the bacterial load has any relevance on the initiation of inflammation and lung disease in the CF mouse, the first aim of this thesis was the analysis of the pulmonary inflammatory phenotype of the $Cftr^{m11/lgu}$ mouse under specified pathogen free (SPF) conditions and conventional housing conditions. The results of this study led to the decision to also analyse the proinflammatory profiles of the CF and wild-type mice following a general inflammatory stimulus of LPS and a bacterial challenge with $S. aureus$ and $P. aeruginosa$.

1.18.2 The study of murine airway beta defensins.

Following the discovery that a human airway beta defensin, HBD-1, may be of relevance to the initiation of CF lung disease, a second aim of this thesis was to identify and characterise a murine airway beta defensin molecule homologous to $hBD-1$. Following the successful discovery of a homologue it was decided to attempt to create a mouse with the murine homologue of $hBD-1$, $Defb1$, knocked out to allow future studies into the effects of the loss of this peptide on the bactericidal activity in the lungs.

A second human airway beta defensin was discovered and therefore the final aim of this work was to identify any additional murine beta defensin molecules and assess their relevance to pulmonary defence.
Chapter 2

Materials and Methods
Chapter 2 Materials and Methods.

2.1 Materials.

Restriction enzymes were obtained from Boehringer Mannheim, Lewes, East Sussex; New England Biolabs, CP Labs, Herts. or from GibcoBRL, Paisley. Proteinase K, RNase A, DNase 1, Polynucleotide kinase, T4 DNA ligase, T4 DNA polymerase, High Prime and Calf intestinal phosphatase were obtained from Boehringer Mannheim and Taq polymerase from Perkin Elmer Applied Biosystems, Roche Molecular Systems Inc, New Jersey, U.S.A..

All radioisotopes came from Amersham International P.L.C., Amersham, Bucks.

DNA size markers \( V/Hind \) III, \( fX174/Hae \) III, DNA molecular weight marker XV and RNA molecular weight marker III came from Boehringer Mannheim. The 20 bp ladder came from Invitrogen BV, Leek, Netherlands and the 1 kb ladder was from GibcoBRL.

Serum, media and supplements for cell isolation were obtained from GibcoBRL. Plastic-ware was supplied by Nunc Intermed, Roskilde, Denmark and Corning Costar, High Wycombe, Bucks.

Mice were supplied from Charles River or experimental stocks of \( Cftr^{m1Hgu} \) mice on an MFI background and littermates were used. Mice were aged between 8 to 12 weeks and of mixed sex.

Secondary antibodies and blocking sera were obtained from Vector Laboratories Ltd, Peterborough. Rat monoclonal antibody to mouse ICAM-1 was from Serotec. Cp-10 antibody was a gift from C. Geczy, Heart Research Institute, Camperdown, NSW. Slides and coverslips were from Chance propper Ltd, Warley.
Bacto-tryptone, bacto-agar and yeast extract were from DIFCO laboratories, Detroit, USA and agarose was from Sigma, Sigma-Aldrich company Ltd, Dorset. Phenol was from Rathburn Chemicals Ltd, Peebleshire. Xylene was from Fisher Scientific U.K. Ltd, Loughborough. All other chemicals were obtained from Sigma, Sigma-Aldrich Company Ltd, Poole, BDH Laboratory supplies, Poole or Pharmacia Biotech AB, Uppsala, Sweden.

Plasmids pMC1NeoPolyA and pBluescript II SK, were obtained from Stratagene Ltd, Cambridge.

2.2 Bacterial and LPS challenge.

2.2.1 Intratracheal instillations.

Adult mice were anaesthetised with 0.2-0.3 ml/10 g body weight of avertin (2.5 g tribromo-ethanol; 5 ml 2-methyl-2-butanol; 200 ml dH2O) intraperitoneally. Induction of anaesthesia was within 1 min and duration was approximately 45 min. The mice were then instilled with 8 µg, 40 µg or 80 µg lipopolysaccharide (LPS) (E. coli 055:B5, Sigma), 1 x 10^6 cfu or 1 x 10^5 cfu S. aureus (C1705 clinical isolate, supplied by J. Govan) or sterile PBS as a control. All samples were in a total volume of 20 µl. The mice were instilled using a non-surgical, intratracheal instillation method of Ho and Furst (Ho et al, 1973). Briefly, the anaesthetised mouse was supported in a frame so the pharynx, larynx and trachea were arranged in a vertical straight line. The airway was illuminated externally by a lamp, the tongue moved to one side and a blunted Terumo spinal needle (Surgicon) passed down the pharynx. The tip of the needle was inserted between the vocal folds at the base of the larynx and 20 µl of the solution instilled into the trachea. The animal was maintained in an upright position 2 min after instillation to allow the fluid to drain into the respiratory tree (see figure 2.1).
Fig. 2.1 Intratracheal instillation technique.
Adapted from an original drawing from Ho et al., 1973.

2.2.2 Nebulisation.

Mice were maintained in HEPA air filtered isolator cabinets for the duration of the experiment. Groups of Cfr<sup>−/−</sup>gu mice and wild-type littermates were exposed in parallel. Aerosolisation was carried out using a restraining apparatus in which the mice were placed in parallel tubes open to a vented cone, using a sidestream nebuliser with the addition of a baffle system (System-22 Optimist<sup>®</sup>, Medic-Aid) to reduce the air particle size and aid delivery to the small airways. The <i>P. aeruginosa</i> cultures (J1385 clinical isolate, supplied by J. Govan) (either 3 ml of 1 x 10<sup>9</sup> cfu/ml or 3 ml of 1 x 10<sup>8</sup> cfu/ml) were delivered with a carrier gas mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub> at a rate of 12 l/min over a period of 10 min. It was estimated that each mouse would receive approximately 5 x 10<sup>5</sup> cfu from the 1 x 10<sup>9</sup> cfu/ml starting culture and 5 x 10<sup>4</sup> cfu from the 1 x 10<sup>8</sup> cfu/ml starting culture. Aerosol was vented from the delivery chamber and purged through disinfectant. Treated animals were returned to standard cages within the isolator after the exposure.
2.2.3 LPS treatment of isolated alveolar macrophages.

Various concentrations of LPS (10 ng/ml-100 μg/ml) were added directly to isolated alveolar macrophages in 96-well plates (see section 2.4.2). After 16 hr the cell medium was drained off and used for ELISA (see section 2.6) either undiluted or diluted 1:10 with RPMI solution.

2.3 Bronchoalveolar lavage (BAL).

Non-instilled mice or instilled mice at various time points after challenge were killed by intraperitoneal injection of 20 mg sodium pentobarbitone (Euthatal, Rhone Merieux). The trachea was exposed by opening the skin and a blunted 21 G hypodermic needle was inserted, as a cannula, through a small incision in the upper trachea and tied in place with suture thread. Lavage was performed by introducing 1 ml sterile PBS into the lungs via the tracheal cannula and then after a few seconds, withdrawing the fluid. This was repeated 2 more times with fresh PBS. The lavage fluid was placed in a tube on ice and the total recovery volume per mouse was approximately 2.75 ml. The BAL fluid was centrifuged at 1200 rpm for 10 min, the clear supernatant removed and the cell pellet resuspended in 200 μl PBS. Total cells were counted in a Neubauer haemocytometer chamber and an air-dried preparation made of each sample (Cytospin, Shandon Scientific) which were then stained with Diff-Quik® stain (Dade®) and a differential cell count performed. The proteinase inhibitor phenylmethylsulfonyl fluoride (PMSF) was added to the supernatant to a final concentration of 100 mg/ml and 0.5 ml aliquots stored at -70°C.

2.4 Cell isolation

2.4.1 Neutrophil isolation.

Separation of neutrophils from the peritoneal cavity was based on a method from Rot (1991). Adult mice were injected intraperitoneally with 0.5 ml 9 % (w/v) casein
solution. 24 hr later the mice were injected with a second dose of casein and 3 hr later killed by cervical dislocation. The peritoneal cavity was lavaged with 5 ml DMEM/0.5mM EDTA and the collected fluid placed on ice. 16 ml ice cold 0.83 % (w/v) ammonium chloride (pH 7.4) were added to the lavage and incubated at room temperature for 15 min then 30 ml 1 x Hanks Buffered Salt Solution (HBSS) were added and the sample centrifuged for 10 min at 1450 rpm. The supernatant was discarded and 10 ml HBSS added to the cells and centrifuged at 775 rpm for 10 min after which the supernatant was discarded and the cells resuspended in 5 ml HBSS and centrifuged at 775 rpm for 5 min. The resulting pellet was resuspended in 500 μl PBS. The total cell count was performed using a Neubauer haemocytometer chamber and the percentage of neutrophils estimated from a differentially stained cytospin (Cytospin, Shandon Scientific). Samples of over 90 % neutrophils were used for the chemotaxis assay and the cells were resuspended in DMEM/0.5 mM EDTA at a concentration of 4 x 10⁶/ml.

2.4.2 Alveolar macrophage isolation.

The isolation of alveolar macrophages was base on a method by Denis and Bisson (1995). Bronchoalveolar lavage of non-challenged adult mice was performed as described in section 2.3. The collected BAL was centrifuged at 1500 rpm for 10 min at 4°C and the supernatant removed. The cells were washed in RPMI solution (356 ml RPMI 1640; 4 ml L-glutamine; 40 ml foetal calf serum) centrifuged at 1500 rpm for 10 min, resuspended in 200 μl RPMI solution and then counted using a Neubauer haemocytometer chamber. The cells were then resuspended in RPMI solution at 2.5 x 10⁵/ml. 200 μl of the cell suspension (5 x 10⁴ cells) were added to each well of a 96 well plate (Costar) and incubated in a humid, 5 % CO₂ incubator at 37°C for 2 hr which allowed the macrophages to adhere to the plastic. The non-adherent cells were washed off with RPMI solution warmed to 37°C and then fresh RPMI solution was added to the wells and incubated at 37°C for a further 2 hr. The macrophages were then ready to be treated with E. coli LPS (see section 2.2.3). To check the purity of the cell isolate, several wells from each plate were stained with Diff-Quik® (Dade®).
Only plates with greater than 95% macrophages were used in subsequent experiments.

2.5 Chemotaxis assay.

30 µl of BAL fluid or the control neutrophil chemoattractant, N-formyl-met-leu-phe (FMLP) (1 x 10^-7 M) or PBS as a negative control, which had been preheated to 37°C and vortexed, were added to the bottom chamber of a Neuro Probe Standard 48 Well Chemotaxis Chamber (Neuro Probe, Inc). All samples were assayed in triplicate. A Nuclepore Polycarbonate membrane (Costar) was placed on top of the bottom chamber followed by the silicon gasket and then the top chamber to which 50 µl of the neutrophil suspension were added to each well. The chamber was incubated in a humid 5% CO₂ incubator at 37°C for 1 hr after which the chamber was inverted and the filter removed (migrated cells side up). The side of the filter with non-migrating neutrophils was washed in PBS and scraped across a blade to remove these cells. The filter was then fixed and stained using Diff-Quik® (Dade®), placed on a 76 mm x 52 mm microscope slide (Marienfeld Laboratory Glassware) and the number of neutrophils migrated in each well counted.

2.6 Enzyme Linked Immunoabsorbent Assay (ELISA).

ELISA was used to estimate the concentration of specific cytokines present in the BAL fluid. Murine TNF-α, IL-6 and IL-1β were measured using commercially available ELISA kits (Genzyme) according to the manufacturers instructions. Undiluted BAL fluid in addition to a 1:10 dilution of BAL fluid was assayed in duplicate. The sample absorbance was read at 450 nm on a Multiskan MS plate reader (Labsystems).
2.7 Nitric Oxide Assay.

This method based on an assay designed by Francoeur and Denis (1995) is used to measure nitric oxide synthase (NOS) activity by converting the unstable product of NOS, nitric oxide, into a stable nitrite whose amount can then be measured by the Greiss assay. 100 µl of BAL fluid were placed in a 1.5 ml tube and α-Nicotinamide Adenine Dinucleotide Phosphate (NADPH) (Sigma) and nitrate reductase (Sigma) added to final concentrations of 100 mM and 0.1U/ml respectively. The samples were incubated overnight at room temperature in darkness. Then 200 µl of Greiss reagent (1 % (w/v) sulfanilamide in 30 % (v/v) acetic acid : 0.1 % (w/v) N-(1-naphtyl)ethylenediamine dihydrochloride in 60 % (v/v) acetic acid) were added to each sample and 200 µl aliquots of each sample added to a flat bottomed 96 well plate (Costar). The absorbance of the samples was read using a Multiskan MS plate reader at a wavelength of 595 nm. A standard curve of absorbance values was produced for each experiment with known concentrations of sodium nitrate and control samples with no nitrate reductase were also assayed.

2.8 Histology.

2.8.1 Preparation of Tissues for Paraffin Wax Sectioning.

Tissues which required paraffin wax sectioning were dissected from the animal and placed in 10 % Neutral Buffered Formalin (50 ml formaldehyde solution; 9.3 g NaH₂PO₄; 2.1 g sodium hydroxide; 450 ml dH₂O) and left at room temperature for at least 4 hr to fix. The fixed tissues were then placed in a Tissue-Tek embedding machine and put through a programme that consisted of the following steps:

1. PBS - 1 hr at 40°C
2. 30% (v/v) ethanol - 1 hr at 40°C
3. 50% (v/v) ethanol - 1 hr at 40°C
4. 70% (v/v) ethanol - 1 hr at 40°C
5. 85% (v/v) ethanol - 1 hr at 40°C
6. 95% (v/v) ethanol - 1 hr at 40°C
7. 100% (v/v) ethanol - 1 hr at 40°C
8. 100% (v/v) ethanol - 1 hr at 40°C
9. xylene - 1 hr at 40°C
10. xylene - 1 hr at 40°C
11. wax - 1 hr at 60°C
12. wax - 1 hr at 60°C
13. wax - 1 hr at 60°C
14. wax - 1 hr at 60°C

The tissue was then placed in a mould and covered with molten wax. The wax was allowed to cool and solidify on ice and the wax block containing the tissue was removed from the mould ready for sectioning. Wax blocks were sectioned on a Reichert-Jung 2030 microtome using disposable blades. 5 µm sections were cut from the embedded tissue and floated out on dH₂O at 42°C. The sections were then floated onto Vectabond™ (Vector Laboratories Ltd.) coated glass slides (see section 2.8.3) and left to dry at room temperature. Sections which were not going to be used immediately were stored at room temperature in a slide box (Kartell®).

2.8.2 Cryostat tissue sectioning.

Tissues to be used for cryostat sectioning were placed into a Universal then placed directly into liquid nitrogen. The lobes of the lung were filled with O.C.T. embedding solution (Raymond A. Lamb) via a cannula preceding dissection. The temperature controls on the cryostat (Leica) were set as follows: chamber -40°C; cutting head -25°C. The tissue was removed from the liquid nitrogen and placed in the cryostat chamber for at least an hour allowing the tissue temperature to equilibrate. To mount the tissue for sectioning a drop of O.C.T. was placed on a chuck and just before solidification the tissue was placed on top. O.C.T. was then used to completely cover the tissue on the chuck which was mounted onto the cutting head and fixed in
position. 10 μm sections were cut from mouse lung which were adhered to Vectabond™ (Vector Laboratories Ltd.) coated glass slides and stored at -70°C until required.

2.8.3 Coating of glass slides with Vectabond™.

Sections were placed onto Vectabond™ coated glass slides to ensure the tissue did not dislodge during further procedures. This process was carried out using the manufacturer’s instructions. Briefly, slides were immersed in acetone for 5 min, VECTABOND™ Reagent (Vector laboratories) solution for 5 min and then dH₂O for 5 x 10 sec. Slides were air dried and stored at room temperature.

2.8.4 Rehydration of paraffin wax embedded tissue sections.

Wax tissue section slides were incubated at 60°C overnight to melt the wax and then processed through the following steps: xylene 2 x 5 min; 100 % (v/v) ethanol 2 x 5 min; 90 % (v/v) ethanol 1 x 5 min; 70 % (v/v) ethanol 1 x 5 min; 50 % (v/v) ethanol 1 x 5 min; 30 % (v/v) ethanol 1 x 5 min; dH₂O 1 x 5 min.

2.8.5 Dehydrating and mounting tissue sections.

Following staining, tissue section slides were dehydrated as follows: 30 % (v/v) ethanol 1 x 2 min; 50 % (v/v) ethanol 1 x 2 min; 70 % (v/v) ethanol 1 x 2 min; 90 % (v/v) ethanol 1 x 2 min; 100 % (v/v) ethanol 2 x 5 min; xylene 2 x 5 min. Once the xylene had evaporated a small amount of DPX mountant (BDH laboratory supplies) was added to the slide and a coverslip gently placed over the tissue section ensuring no air bubbles were trapped. The slides were left to air dry overnight.
2.8.6 Haematoxylin and eosin staining of tissue sections.

This staining technique was used to examine basic tissue morphology. Tissue section slides were rehydrated through graded alcohol to dH₂O (see section 2.8.4) and then stained by immersion in the following solutions: Haematoxylin (Sigma) 5 min; running H₂O 2 min; acid alcohol (1 % (v/v) HCl in 70 % (v/v) ethanol) 10 sec; running H₂O 2 min; saturated lithium carbonate 10 sec; running H₂O 5 min; Eosin (3 parts 1 % (v/v) aqueous eosin; 1 part 1 % (v/v) ethanol/0.05 % (v/v) acetic acid) 2 min; H₂O 30 sec; 100 % (v/v) ethanol 15 sec; 100 % (v/v) ethanol 1 min. The slides were then dehydrated and mounted as described in section 2.8.5.

2.8.7 Periodic acid Schiff staining of tissue sections.

This staining technique is used to visualise mucin containing cells which stain as bright pink. Tissue section slides were rehydrated through graded alcohol to dH₂O (see section 2.8.4) and then stained by immersion in the following solutions: 1 % (v/v) Periodic Acid (Sigma) 5 min; running H₂O 2 min; Schiff reagent (Sigma) 10 min; running H₂O 10 min. Slides were then counterstained with Haematoxylin as in section 2.8.6 with the omission of the Eosin staining step.

2.8.8 Microscopy and imaging.

Brightfield imaging of tissue sections and cells was performed on an Axioplan 2 microscope (Zeiss, Germany). Images were captured using a CCD (charged coupled device) Micro-imager 1400 camera (Xillix, Vancouver, Canada) and SmartCapture software (Digital Scientific, Cambridge). Adobe photoshop and Adobe illustrator software (Adobe Systems Incorporated, Mountain View, CA, USA) were used for the annotation of the images.
2.9 Immunohistochemistry.

2.9.1 Antibody staining of tissue sections.

Tissue section slides were rehydrated through graded alcohol to dH2O (see section 2.8.4). Endogenous peroxide activity was quenched by incubating the sections in 0.3 % (v/v) hydrogen peroxide in methanol at -20°C for 30 min and washing in PBS for 5 min. To avoid non-specific staining, sections were incubated with diluted normal blocking serum for 20 min, prepared from the species in which the secondary antibody was made. Non-specific biotin and avidin binding was blocked using the Biotin/Avidin Blocking Kit (Vector Laboratories) following the manufacturer’s instructions. Sections were incubated with the primary antibody, diluted to the appropriate concentration in PBS, for 30-60 min and washed in PBS for 5 min. The sections were then incubated with the biotinylated secondary antibody for 30 min and washed as above. Sections were incubated with VECTASTAIN® Elite ABC Reagent (Vector Laboratories) for 30 min and washed in PBS for 5 min. The antibody signal was detected by incubation with peroxidase substrate solution (3,3'-Diaminobenzidine tetrahydrochloride (1 mg/ml) (Sigma)) for 2-10 min. Excess substrate was washed off with H2O and then the slides were counter stained with Haemotoxylin and Eosin as described in section 2.8.6.

2.9.2 Western blot transfer of proteins.

The gel plates were assembled in a AE-6220 Dual Slab Chamber (Atto Corporation, Tokyo, Japan) as detailed in the manufacturer’s instructions. 10 ml of 12 % separating gel were prepared (2.5 ml 1.5 M Tris-HCl (pH 8.8); 100 µl 10 % (w/v) SDS; 3 ml 40 % (v/v) Acrylamide/Bis; 50 µl 10 % (w/v) ammonium persulphate; 5 µl TEMED and 4.35 ml dH2O) and poured into the gel cast to a height of 2 cm below the gel comb teeth. 10 ml 4 % stacking gel were then prepared (2.5 ml 0.5 M Tris-HCl (pH 6.8); 100 µl 10 % (w/v) SDS; 975 µl 40 % (v/v) Acrylamide/Bis; 50 µl 10 % (w/v) ammonium persulphate; 10 µl TEMED; 6.425 ml dH2O) and added on top
of the separating gel to the top of the gel comb. BAL samples were diluted 1:4 with sample buffer (1 µl 0.5 M Tris-HCl (pH 6.8); 800 µl glycerol; 1.6 µl 10 % (w/v) SDS; 400 µl 2-b-mercaptoethanol; 200 µl 0.05% (w/v) bromophenol blue; 4 ml H₂O) and denatured at 95°C for 4 min. The gel apparatus was placed in the electrophoresis unit with 1 x running buffer (5 x stock: 9 g Tris; 43.2 g glycine; 3 g SDS in total volume of 600 ml with dH₂O). The BAL samples were loaded using a Hamilton syringe and the gel electrophoresed at 200 V, constant voltage setting for 45 min. The gel was removed from the electrophoresis apparatus and soaked in transfer buffer (25 mM Tris, 190 mM glycine, 20 % (v/v) methanol) for 1 hr. The nitrocellulose filter (Schleicher and Schuell) and 3 mm filter paper were pre-soaked in transfer buffer for 5 min and then placed in a Mini Trans-Blot® Electrophoretic Transfer Cell (BioRad) along with the gel and blotted at 0.5 A for 3 hr at 4°C. The cassette was then dismantled and the nitrocellulose filter removed, dried and stained for the presence of protein using Ponceau S solution (Sigma) which was then rinsed off with H₂O.

2.9.3 Antibody staining of Western blots.

The filter was pre-soaked in PBS for 5 min and then in a 5 % dried milk solution at 37°C for 2 hr to block non specific antibody binding sites. The filter was incubated with the primary antibody for 1 hr at room temperature in a 5 % (w/v) dried milk/0.3 % (v/v) Tween20/PBS solution and then washed 3 x 5 min with a 0.3 % (v/v) Tween20/PBS solution (PBST). The secondary antibody in PBST was added and incubated for 1 hr at RT and then the filter was washed 3 x 5 min with PBST. The filter was incubated with VECTASTAIN® ABC Elite reagent (Vector Laboratories) for 30 min at room temperature and then washed 6 x 5 min with PBST. The filter was then incubated for 1 min with ECL™ (enhanced chemiluminescence) detection solution (Amersham), wrapped in cling film and exposed to film for 30 sec to 60 min depending on the strength of the signal.
2.10 General molecular biology techniques.

2.10.1 Restriction enzyme digestion of DNA.

Plasmid and BAC DNA was digested with 5-10 units enzyme/μg DNA for 1-5 hr depending on the suppliers instructions. Genomic DNA was digested overnight with 10 units/μg DNA. Digestions were analysed for completeness by gel electrophoresis.

2.10.2 Agarose gel electrophoresis.

Agarose gel electrophoresis of DNA was performed using gel apparatus (Biosciences Services) with agarose concentrations between 0.5-2.0 % (w/v) depending on the desired fragment resolution. The agarose was dissolved and cast in TBE buffer (0.09 M Tris-borate; 0.002 M EDTA (pH 8.0)) which was also used as the running buffer. Ethidium bromide (5 μg/100 ml) was added directly to the molten agarose. Gels consisting of 4 % (w/v) NuSIEVE agarose gel (FMC BioProducts) were used for resolution of DNA fragments below 200 bp.

2.10.3 Purification of DNA fragments from agarose gels.

Using UV illumination the fragment of interest was excised with the minimum amount of agarose. Purification was carried out using QIAquick Gel Extraction Kit (Qiagen) following the manufacturer’s instructions and eluting the purified DNA in 30 μl dH2O.

2.11 Preparation of DNA.

2.11.1 Rapid small scale plasmid preparation.

1.5 ml of L-Broth, containing the appropriate antibiotic, usually 100 μg/ml ampicillin, were inoculated with a single bacterial colony using a sterile pipette tip. The cultures were incubated overnight at 37°C. 1.5 ml of the cell cultures were
centrifuged and the supernatant resuspended in 100 μl of STET buffer (8 % (w/v) sucrose; 0.5 % (v/v) Triton-X100; 50 mM EDTA (pH 8.0); 10 mM Tris-Cl (pH 8.0)). 100 μl of lysozyme (2 mg/ml) in STET buffer were added to each tube, mixed and left at room temperature for 5 min. The samples were then boiled for 1 min and centrifuged at 15 000 rpm for 10 min. The resulting pellet was removed with a toothpick and the DNA precipitated with 30 μl 4 M ammonium acetate and 250 μl isopropanol, mixed and centrifuged at 15 000 rpm for 10 min. The pellet was washed with 70 % (v/v) ethanol, dried and resuspended in 50 μl TE (pH 8.0). RNase A (100 μg/ml) was included at the time of restriction enzyme analysis.

2.11.2 Small scale preparation of high quality plasmid DNA.

High quality DNA suitable for sequencing was prepared using the QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer’s instructions. The DNA was eluted in 50 μl of dH2O and the concentration assessed by spectrophotometry.

2.11.3 Large scale preparation of high quality DNA.

A 5 ml starter culture was prepared overnight. This was then added to 500 ml of L-Broth containing the appropriate antibiotic (usually ampicillin at 100 μg/ml) and incubated for 16 hr at 37°C. The culture was then centrifuged in 2 x 250 ml Sorvall bottles at 8000 rpm for 5 min at 4°C. Both pellets were resuspended in a total of 20 ml of GET solution (0.05 M glucose; 0.025 M Tris, 0.01 M EDTA (pH 8.0)) and a small amount of lysozyme added and left on ice for 5 min. 40 ml of freshly prepared 0.2 M sodium hydroxide/1 % (w/v) SDS were added, mixed by inversion and incubated on ice for 10 min. Then 30 ml of potassium acetate buffer (60 ml 5M potassium acetate; 11.5 ml glacial acetic acid and 28.5 ml dH2O) were added, mixed by inversion and incubated on ice for 10 min. The samples were then centrifuged at 12 000 rpm for 20 min and the resulting supernatant decanted to a fresh bottle by straining through muslin where the DNA was precipitated by the addition of 0.6 volumes of isopropanol, mixed and then centrifuged at 12 000 rpm for 10 min. The
pellet was washed with 70 % (v/v) ethanol, dried and then resuspended in 2.5 ml TE (pH 8.0) and transferred to a universal containing 2.75 g caesium chloride. 1 mg of ethidium bromide was added and the solution transferred to an ultracentrifuge tube. The tubes were heat sealed and centrifuged at 80 000 rpm overnight. The DNA, which was present as an ethidium bromide stained band, was removed using a 21 G needle, transferred to a universal tube and the ethidium bromide washed out with H2O-saturated butanol. The DNA was then precipitated by the addition of 10 ml 100% (v/v) ethanol and centrifuged at 5 000 rpm for 15 min. The pellet was washed in 70 % (v/v) ethanol, dried and then resuspended in the desired volume of TE or dH2O.

2.11.4 Preparation of BAC DNA.

A single BAC colony was inoculated into 5 ml L-Broth culture containing 12.5 μg/ml chloramphenicol and grown overnight. 100 ml L-Broth containing 12.5 μg/ml chloramphenicol were inoculated with 0.5 ml of the starter culture and grown at 37°C for 14 hr. The cells were divided into 2 x 50 ml Sorval tubes, harvested by centrifugation for 20 min at 4500 rpm and then the QIAGEN Plasmid Midi Kit protocol was followed. To ensure complete lysis, the volume of buffers P1, P2 and P3 was increased to 10 ml of each per tube. The cleared lysates were subsequently pooled and processed on a single QIAGEN-tip 100 column. The DNA was eluted from the column by adding 5 aliquots of 1 ml Elution Buffer, QF, pre-warmed to 65°C. The DNA was then precipitated by adding 3.5 ml of isopropanol and centrifuged at 15 000 rpm for 30 min. The pellet was washed with 70 % (v/v) ethanol and then resuspended in 50 μl TE (pH 8.0) by shaking overnight at 4°C.

2.11.5 Preparation of total genomic DNA from tail tips.

Tail tips were incubated overnight at 50°C in 500 μl of quick lysis buffer containing 5 μg of proteinase K. The samples were then vigorously vortexed and then centrifuged for 10 min at 15 000 rpm. The resultant supernatants were added to 0.5
ml of 100 % (v/v) ethanol and the precipitated DNA spooled onto glass rods, washed with 70 % (v/v) ethanol and then air-dried for 5 min. The DNA was then resuspended in 50 μl of TE.

2.12 Preparation of RNA

2.12.1 Preparation of RNA from tissues.

All RNA preparations were performed in a double HEPA filter hood (Medical Air Technology, Manchester) using RNase, DNase free plugged tips. Tissue samples were removed from liquid N₂ or -70°C storage and 1 ml RNAzol B (Biogenesis Ltd) added and the samples homogenised using an Omni portable homogeniser (Camlab limited). When the tissue samples were completely lysed they were transferred to a fresh tube containing 100 μl chloroform and vortexed for 15 sec followed by a 5 min incubation on ice. The samples were then centrifuged at 15000 rpm for 15 min at 4°C and the top layer transferred to a fresh tube on ice. The RNA was precipitated by the addition of 4 μl glycogen and 450 μl isopropanol and incubation at 4°C for at least 15 min. The samples were then centrifuged at 15000 rpm for 15 min and the resulting RNA pellet washed with 75 % (v/v) ethanol and dried. The RNA was resuspended in 50 μl DEPC treated H₂O and the quantity and quality of the preparation assessed by spectrophotometry.

2.12.2 DNase treatment of RNA.

RNA samples were treated with DNase1 to remove any DNA present. The RNA samples were treated in a total volume of 50 μl containing 5.8 μl of 10 x DNase1 buffer; 10 units of DNase 1 (Rnase free) and 40 units of RNase inhibitor. The samples were incubated at 37°C for 60 min and then extracted using an equal volume of phenol:chloroform by vortex and then centrifugation for 15 min at 15 000 rpm. The top layer was transferred to a fresh tube and an equal volume of chloroform was added. The samples were then vortexed and centrifuged for 15 min at 15 000 rpm. The top layer was then transferred to a fresh tube on ice and the sample precipitated
with 0.1 volumes of 3 M sodium acetate (pH 5.6) and 2 volumes of ethanol and incubated at -70°C for 30 min. The sample was then centrifuged at 15 000 rpm for 15 min and the resulting pellet washed with 70 % (v/v) ethanol. The pellet was dried and resuspended in the desired volume of DEPC treated H$_2$O and stored at -70°C.

2.13 General cloning techniques.
2.13.1 Removal of 5' Phosphate groups from DNA.

DNA was dephosphorylated with calf intestinal alkaline phosphatase (CIP) to minimise the recircularisation of plasmid during ligation reactions. 10-20 μg of plasmid DNA were digested to completion and extracted with an equal volume phenol:chloroform. The DNA was then precipitated with 2.5 volumes of ethanol for 20 min at 4°C and recovered by centrifugation at 12 000 rpm for 30 min. The DNA was then resuspended in 90 μl TE buffer (pH 8.0). 10 μl of 10 x CIP dephosphorylation buffer were then added to the sample along with 2 units of CIP enzyme. This was incubated at 37°C for 30 min and then another 2 units of CIP were added and the incubation continued for another 30 min. EDTA was added to a final concentration of 5 μM and the reaction heated to 85°C for 15 min to inactivate the enzyme. The DNA was then extracted with phenol:chloroform as before and precipitated by adding 0.1 volumes of 3 M sodium acetate (pH 7.2) and 2.5 volumes of ethanol. The DNA was recovered by centrifugation at 12 000 rpm and washed in 70 % ethanol, dried in a speedivac and resuspended in TE buffer (pH 8.0) or dH$_2$O.

2.13.2 Blunt ending DNA fragments.

DNA fragments which had a 5'overhang were treated with T4 DNA polymerase to create blunt ends for further cloning procedures. The purified DNA fragments were treated in a total volume of 50 μl containing 10 μl of 5 x T4 DNA polymerase buffer; 1 μl of 100 x BSA; 100 mM dNTPs and 1 unit of T4 DNA polymerase. The samples were incubated at 11°C for 20 min followed by extraction with an equal volume of phenol:chloroform by vortex and centrifugation at 15 000 rpm for 3 min. The
samples were then ethanol precipitated as in section 2.13.1. The dried pellet was resuspended in the desired volume of dH2O, ready for use in the ligation reaction.

2.13.3 Ligation of fragments.

Fragments with sticky complementary ends were ligated overnight at 16°C, using 1 unit of T4 DNA ligase (Boehringer Mannheim) whereas blunt-ended ligations were carried out overnight at room temperature using 5 units high concentration ligase (Gibco BRL). A molar ratio of 2:1 to 3:1 between insert and linearised vector was used with the amount of vector at approximately 50 ng. Commercially supplied 10 x ligation buffer was used and the volume of the reaction kept to a minimum, usually 10 µl.

2.14 Bacterial transformation.

2.14.1 Electroporation.

Electrocomp™ TOP10F’ cells (Invitrogen), were used for electroporation and a Bio-Rad Gene Pulser™ and Bio-Rad Pulse Controller were used for the electroporation procedure with capacitance set at 25 mFD, volts at 2.5 kV and resistance at 200 Ohms. 2 µl of ligation reaction were added to 40 µl of cells and incubated on ice for 1 min. The sample was then transferred to an electroporation cuvette, placed in the electroporation chamber and the electric charge discharged. 450 µl of room temperature L-Broth were added immediately after electroporation and the sample transferred to a 15 ml polypropylene tube. Samples were incubated with shaking at 37°C for 60 min. 10-100 µl of sample were spread onto LB plates (1.5 % (w/v) bacto-agar in L-broth) supplemented with the appropriate antibiotic. The plates were incubated overnight at 37°C.

TOP10F’ One Shot™ Competent Cells (Invitrogen) were used for heat shock transformation. 2 μl of ligation reaction were added to 40 μl of cells and incubated on ice for 30 min. The sample was heat shocked at 42°C for 30 sec, placed on ice for 2 min and then 250 μl of L-Broth added. The tubes were shaken at 37°C for 1 hr and then spread onto plates as above.

2.15 Direct cloning of PCR products.

PCR products generated by Taq polymerase have 3' deoxyadenosine overhangs and use of vectors containing single 3' deoxythymidine residues allows PCR inserts to ligate efficiently with the vector. Direct PCR cloning was achieved using the pCR2.1 vector (Stratagene). Briefly, 1-3 μl of fresh PCR product (less then 24 hr old) was incubated in a total volume of 10 μl containing 1 μl 10 x T4 DNA ligation buffer; 50 ng pCR2.1 and 4 units of T4 DNA ligase. The reaction was incubated overnight at 16°C and then used directly for transformation (see section 2.14).

2.16 Amplification of DNA and RNA by the polymerase chain reaction (PCR).

All PCR reactions were performed using plugged RNase/DNase free tips (Rainin Instrument Co. Inc.).

2.16.1 Amplification from DNA.

DNA was amplified according to Saiki et al. (Saiki et al, 1988). A 50 μl reaction containing up to 1 μg of genomic DNA or 1 ng of plasmid or BAC DNA, 5 μl 10 x PCR buffer (P), 5 μl 25 mM MgCl₂, 1 μl of 50 x dNTP mix (10 mM of each nucleotide) and 100 nm of each amplimer was prepared in a 0.5 ml thin-walled PCR
tube (Advanced Biotechnologies). 2.5 units of thermostable DNA polymerase from Thermus aquaticus (Taq polymerase), were added to the mixture which was then overlayed with 50 µl mineral oil (Sigma). Thermal cycling was controlled by a programmable heating block (OmniGene, Hybaid). Three-step temperature cycling conditions varied, depending on the amplimers used and length of amplification.

2.16.2 Amplification of large DNA fragments.

Amplification of large DNA fragments was achieved using the Expand™ Long Template PCR System (Boehringer Mannheim). 50 ng of BAC DNA were amplified in a total volume of 50 µl containing 1.75 mM of PCR buffer 1, 2 or 3 with 17.5 mM MgCl₂, 22.5 mM MgCl₂ or 22.5 mM MgCl₂ and detergents, respectively; 500 mM of dATP, dCTP, dGTP, dTTP; 300 nM of each amplimer and 2.5 units of enzyme mix (thermostable Taq and Pwo polymerases) in 0.2 ml thin-walled PCR tubes (Advanced Biotechnologies) using the following cycling conditions: denaturation 92°C for 2 min; 30 cycles of 92°C for 10 sec, 65°C for 30 sec and 68°C for 15 min, final extension of 68°C for 7 min. The amplified products were then analysed on a 0.8 % (w/v) agarose gel by electrophoresis.

2.16.3 Amplification from RNA.

Amplification of RNA was carried out according to the method of Sambrook et al. (1989). 1st strand cDNA synthesis was accomplished using a 1st Strand cDNA Synthesis Kit for RT-PCR (Boehringer Mannheim). Briefly, duplicate samples of 1 µg of total RNA in a total volume of 7.8 µl in DEPC treated H₂O were denatured at 65°C for 15 min. To one set of samples were added 2 µl 10 x PCR buffer, 4 µl MgCl₂, 2 µl 10 mM dNTPs, 0.4 µl gelatin, 2 µl of random primer p(dN)6, 40 units Rnase Inhibitor, 0.8 µl AMV reverse transcriptase. The other was set up identically except that AMV reverse transcriptase was replaced with diethyl pyrocarbonate (DEPC)-treated H₂O to verify for RNA amplification. A minus RNA control was
also included to assay for contamination. The samples were incubated at room temperature for 10 min to allow the primers to anneal and then placed at 42°C for 1 hr followed by 5 min at 95°C to inactivate the enzyme. After the tubes had been cooled on ice, 5 µl of each reaction were used in a standard PCR reaction as described in section 2.16.1.

2.16.4 Sequence and annealing temp of amplimers.

Amplimers used for screening for the presence of the *Defb1* gene

def5'    5' CACTCTGGACCCTGGCTGCC 3'
def1     CCAGCTGCCCACATCTAATACC
def2     AATCCATCGCTCGTCTTTA

Amplimers used for screening for the presence of the *Defb2* gene

DR5' (5'UTR)  5' TCTGGAGTCTGAGTGCCCTT 3'
DR5' (exon2)  TCAGAGCCATTTGTCCCTCCT
DR3' (3'UTR)  TGACTTCCCATGTGCTTCTCTTC
DR5' (exon2)  TGACTTCCCATGTGCTTCTCTTC
DR5'new      GCCATGAGGACTCTCTGCTC
DR3'new      TGTCACCTTGACTTCCATGTGC

Amplimers used for RACE

R1        5' TGACTTCCATGTGCTTCTCTTC 3'
R1 (a)    CATTTCATGTACTTGCAACAG
R2        GGTGTTGAGCTCTCTCTAGATGGTGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG

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Amplimers used for screening for the presence of Hprt gene

Hprt 5' 5' CTGTAGATTTTATCAGACTGAAGAG 3'
Hprt 3' GTCAAGGGCATATCCAAACAAAA

Amplimers used for sequencing

M13F 5' GTAAAACGACGGCCAGT 3'
M13R GGAAACAGCTATGACCATG
Mbdseq1 CCATTCGTGTGACCTTC
Mbdseq2 GCCATCTAATACCAAACACTAC
DR5 CATAGCCCTCCAAACATTG
DR5'2 CCACCCAAACCTATAATTG
DR5'3 GGAGGACAAATGGCTCTG
DR5'4 GGCACGGTTGAGCAGCAAG
DR3 GACTTCCATGTGCTTCCTTC
DR3'1 GGCATTATAGCTATGACTC
DR3'2 CTTGCTGCTCACCCCTTGCC

2.17 Transfer of DNA and RNA.

2.17.1 Southern blot transfer of DNA.

DNA fragments were separated by agarose gel electrophoresis and the resulting gel was photographed under UV illumination and then soaked in denaturing buffer
(0.5 M sodium hydroxide; 1.5 M sodium chloride) for 30 min and then in neutralisation buffer (0.5 M Tris; 1.5 M sodium chloride) for 10 min. The gel was then transferred onto a MSI Magna nylon transfer membrane (Micron Separations) by capillary transfer overnight in 20 x SSC (3 M sodium chloride; 0.3 M sodium citrate). The membrane was fixed by baking at 80°C for 20 min and then UV stratalinked (Stratalinker 1800, Stratagene). The membrane was then hybridised as described in section 2.18.3 or 2.18.4.

2.17.2 Northern blot transfer of RNA.

10-20 μg of total RNA were resuspended in a total volume of 20 μl sample buffer (50 % (v/v) formamide, 2.2 M formaldehyde, 1 x MOPS (20 mM MOPS; 5 mM sodium acetate; 1 mM EDTA)) and incubated at 55°C for 10 min then placed on ice. 5 μl of 6 x formaldehyde gel-loading buffer (50 % (v/v) glycerol, 1 mM EDTA (pH 8.0), 0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF) were added to each sample and then loaded immediately onto the formaldehyde gel (1 % (w/v) agarose; 1 x MOPS; and 18 % (v/v) formaldehyde). RNA molecular weight marker II (Boehringer Mannheim) was also loaded. The gel was run in 1 x MOPS at 50-80 V for 4-8 hr with recirculating buffer. Following electrophoresis the gel was washed 3 x 20 min in dH2O and the gel blotted onto MSI nitrocellulose membrane (Micron separations) as described in section 2.17.1. The portion of the membrane containing the RNA ladder was excised and stained in 0.5 M sodium acetate (pH 5.2); 0.04 % (w/v) methylene blue to assess the success of the RNA transfer and measure autorad bands. Hybridisation was performed as described in section 2.18.3.

2.17.3 Construction of bacterial colony hybridisation filters.

Nitrocellulose filters (0.45 μm Schleicher and Schuell) were placed on LB agar plates and marked for orientation. Individual bacterial colonies were then streaked onto the filters within the gridded boxes marked on the filter and the plates incubated overnight at 37°C. The filters were removed from the plate and floated in 10 % (w/v)
SDS (colony side up) for 1 min to lyse the cells and then left to dry on filter paper for 1 min. The filters were submerged in denaturing solution (0.5 M sodium hydroxide; 1.5 M sodium chloride) for 5 min and then neutralisation buffer (0.5 M Tris; 1.5 M sodium chloride) for 5 min followed by 2 x SSC for 30 sec and left to dry on 3 mm filter paper for 30 min. The DNA was fixed onto the membrane by baking at 80°C under vacuum which was then stored until used in hybridisation procedures.

2.18 Radioactive hybridisation.

2.18.1 Preparation of radioactively labelled DNA probes.

Labelled probes from double stranded DNA were made by the random priming method (Feinberg et al, 1983). Approximately 50 ng of purified DNA in a total volume of 12 µl were boiled for 10 min to ensure denaturation and then labelled using 4 µl of High Prime (Boehringer Mannheim) and 30 mCi [α-32P] dCTP. The reaction was incubated at 37°C for 30 min and incorporation estimated by washing a small amount of the probe with trichloroacetic to remove unincorporated nucleotides. The probe was stripped by boiling for 10 min with 1 mg sonicated salmon sperm and 1 mg mouse genomic DNA and then incubating at 68°C for 45 min. The probe was then added to the prehybridised filters.

2.18.2 Preparation of radioactively labelled oligonucleotide probes.

Labelled probes from oligonucleotides 15-25 bp in length were made by end-labelling. Approximately 50 ng of oligonucleotide were labelled in a total volume of 20 µl containing 2 µl of 10 x PNK buffer, 10 units of polynucleotide kinase and 30 mCi [γ-32P] dATP. The reaction was incubated at 37°C for 1 hr and then added directly to the prehybridised filters.
2.18.3 Hybridisation of radioactive DNA probes.

Filters were pre-hybridised at 68°C for a minimum of 1 hr in rotating hybridisation bottles (Hybaid) with 20-50 ml hybridisation solution (6 x SSC; 10 % (w/v) Dextran sulphate; 0.5 % (w/v) SDS; 0.1 % (w/v) BSA; 0.1 % (w/v) Ficoll; 0.1 % (w/v) polyvinylpyrrolidone; 0.1 % (w/v) disodium pyrophosphate). After pre-hybridisation, radiolabelled probe (see section 2.18.1) was added directly to the solution and the blots hybridised overnight at 68°C. Filters were washed with 2 x SSC/0.1 % (w/v) SDS at 68°C for 15 min then 0.2 x SSC/0.1 % (w/v) SDS at 68°C for 15 min with monitoring of background radiation with a Geiger counter. The filters were exposed to Kodak X-OMAT AR film at -70°C in cassettes containing intensifying screens.

2.18.4 Hybridisation of radioactive oligonucleotide probes.

Filters were pre-hybridised at 45°C for a minimum of 1 hr in rotating hybridisation bottles with 20-50 ml oligo hybridisation buffer (6 x SSC; 0.5 % (w/v) SDS; 0.1 % (w/v) BSA; 0.1 % (w/v) Ficoll; 0.1 % (w/v) polyvinylpyrrolidone; 0.1 % (w/v) disodium pyrophosphate) and 1 mg sonicated salmon sperm. After pre-hybridisation, radiolabelled oligo probe (see section 2.18.2) was added directly to the solution and the blots hybridised for 4 hr at a temperature of 7-10°C lower than the oligo Tm. Filters were then washed with 4 x SSC/0.1 % (w/v) SDS for 15 min followed by a second wash of 2 x SSC/0.1 % (w/v) SDS if required. The filters were then exposed to Kodak X-OMAT AR film at -70°C in cassettes containing intensifying screens.

2.18.5 Sequence and hybridisation temperature of oligonucleotides.

<table>
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<th>3'</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
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<td></td>
</tr>
<tr>
<td>Defb1 3'</td>
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<td>50°C</td>
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<tr>
<td>neo oligo</td>
<td>CTTGGCGGCGAATGGGCTGA</td>
<td>48°C</td>
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</tbody>
</table>
2.19 Screening BAC Libraries.

This technique is shown in figure 6.4. BAC clones containing the desired genomic sequences were isolated by screening Mouse BAC DNA pools by PCR and Southern hybridisation of colony mouse BAC membranes. The CITB (Californian Institute of Technology) Mouse BAC DNA Pools (Research Genetics) were derived from the cell line, CJ7^5, from mouse strain 129SV. The inserts, cloned into pBeloBAC11 were an average of 100-150 kb. The pools consist of 72 superpools and the corresponding 576 plate pools. Two rounds of PCR (see section 2.16.1) were required to obtain the plate location of the individual clones. Once individual plates were identified they were obtained as colony mouse BAC membranes (Research Genetics) which were then screened for the presence of the desired sequence by hybridisation using the appropriate radio labelled probe (see sections 2.18.1 and 2.18.3). Individual clones, available as stab cultures in LB agar were then obtained (Research Genetics).

2.20 Rapid Amplification of cDNA ends (RACE).

This technique is shown in figure 8.5. Identification of the 5' region of cDNA was achieved by RACE based on a method by Townley et al. (1997). Total RNA was prepared from adult mouse kidney as described in section 2.12.1. 1st strand cDNA synthesis was performed by incubating 5 µg RNA in a total volume of 13 µl containing 10 ng of primer R1 at 70°C for 5 min. The sample was then placed on ice and 4 µl 5x Superscript II buffer; 2 µl 0.1 M DTT and 1 µl 10 mM dNTPs added and
incubated for 2 min at 37°C after which 1 μl of Superscript II (Gibco BRL) was added followed by incubation at 37°C for a further hour. The enzyme was inactivated by the addition of 2.2 μl 1 M sodium hydroxide with incubation at 65°C for 20 min then adding 2.2 μl of 1 M hydrochloric acid. cDNA products were size selected by microdialysis on a 0.025 μm filter against TE (pH 8.0) for 4 hr. Poly-A addition was then performed by adding 6 μl 5 x terminal deoxynucleotidyl transferase (TdT) buffer and 2 μl 2 mM dATP to the 1st strand reaction, incubating at 37°C for 2 min and then adding 15 units recombinant TdT and incubating for a further 5 min at 37°C and 2 min at 70°C. 2nd strand cDNA synthesis was accomplished using 15 μl of the tailed cDNA and adding 2 μl 10 x restriction buffer M (Boehringer Mannheim); 1 μl 10 mM dNTPs; 10 ng of amplimer R2 and 2 units of Klenow enzyme with incubation at room temperature for 30 min followed by 30 min at 37°C and 5 min at 70°C. The products were microdialysed on a 0.1 μm filter against TE for 4 hr, collecting the sample in 37 μl of dH2O. PCR amplification was performed using all of the collected products in a total volume of 50 μl as described in section 2.16.1 using primers R3 and R4 and the following conditions: 30 cycles of 94°C for 1.5 min, 60°C for 1.5 min and 72°C for 3 min. PCR products were then microdialysed on a 0.1 μm filter against TE for 4 hr and collected in a total volume of 40 μl. A nested PCR was then performed using 5 μl of the dialysate and primers R4 and R5 with cycling conditions as above. PCR products were analysed on a 1 % agarose gel by electrophoresis and cloned directly into pCR2.1 (see section 2.15). Cloned products were analysed by hybridisation with an exon-specific oligo and an intron-specific oligo (see sections 2.17.1, 2.18.2 and 2.18.4) Clones positive for only the exon-specific oligo were then sequenced as in section 2.21.

2.21 DNA sequencing.

Sequencing of double strand DNA was performed using the ABI Prism™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). 500 ng of double stranded DNA were amplified in a total volume of 20 μl containing 18 ng or 3.2 pmol of amplimer and 8 μl of Terminator Ready Reaction
Mix in 0.2 ml Micro-Tubes (Advanced Biotechnologies Ltd) on a Gene Amp® PCR system 9700 (Perkin Elmer Applied Biosystems) with the following cycling conditions: 25 cycles of 96°C for 30 sec; 50°C for 15 sec; 60°C for 4 min. The resulting products were then precipitated by the addition of 2 μl 3 M sodium acetate and 50 μl 95 % (v/v) ethanol and centrifuged at 15 000 rpm for 30 min. The resulting pellet was washed with 70 % (v/v) ethanol and dried in a speedivac. Samples were electrophoresed on a ABI 377 sequencing machine (Perkin Elmer Applied Biosystems) and the results analysed using Sequencing analysis software ImageQuant™ (Molecular Dynamics).

2.22 Statistical analysis.

Calculations of means, standard deviations and Student’s T test (paired and unpaired) were performed using MultiStat 1.12 software (BIOSOFT, Cambridge, UK).
Chapter 3

Inflammatory profile of conventionally and SPF housed CF mice
Chapter 3 Inflammatory profile of conventionally and SPF housed CF mice.

3.1 Introduction.

There have been many reports published (see section 1.13.4-6) which have analysed the profile of inflammatory molecules in CF patients and although the results from these have been instrumental in bringing proinflammatory cytokines to the attention of the CF research field there exist some obvious experimental problems. Sampling in human subjects must be as non-invasive as possible and therefore cytokines have been measured from accessible locations such as the circulation or from sputum (Schuster et al, 1995, Salva et al, 1996). However, it is unclear whether changes in the levels of inflammatory mediators at these locations will accurately reflect those in the lung and therefore studies on cytokine profiles were usually performed on BALF (Bonfield et al, 1995b, Cantin, 1995, Birrer et al, 1994, Khan et al, 1995, Balough et al, 1995, Armstrong et al, 1997, Noah et al, 1997). Only a small area of the lung can be lavaged in CF patients and the recent report that inflammation varies significantly among geographically distinct areas of the lung (Meyer et al, 1997) indicates that the BALF retrieved and subjected to cytokine analysis may not accurately represent the inflammatory situation in the lung. This could also contribute to the high degree of variability observed in the above studies, as would the heterogeneity of the human CF patient population. A further problem in human CF studies, is the great deal of variation in CF lung disease, not only arising from the different CFTR mutations but also from modifiers of CF disease severity (Kerem et al, 1996). Increasing the size of the sample population can help reduce the degree of variability but, although CF is the most common single gene disorder in Europe, obtaining high numbers of patients for studies on lung inflammation is difficult, especially when non repeatable techniques, such as lavage, are used. Additional problems are encountered in studies concerned with the assessment of the inflammatory state of the CF lung prior to any infective episode (Dagli et al, 1992, Balough et al, 1995, Armstrong et al, 1997, Jaenisch et al, 1983, Noah et al, 1997). BALF samples are assessed for particular
pathogens, usually those associated with CF such as *S. aureus* and *P. aeruginosa* but not all bacteria can be assayed for. There is no way of telling whether an infective episode has already occurred which has resulted in the eradication of the pathogen before lavage but which may result in a prolonged inflammatory response. Finally, there is no control over the CF patients environment and therefore it is impossible to make any definite statement about the overall bacterial status of the lung.

The use of an animal model that reflects human CF lung disease, would overcome many of these problems. As previously mentioned, the *Cftr*<sup>tm1Hgu</sup> mouse develops lung disease following administration of CF-relevant pathogens (Davidson *et al*, 1995) and therefore it was considered to be a suitable model for the study of pulmonary inflammatory mediators. Many in vivo procedures not suitable for CF patients can be performed on mice and high numbers of mice in each experimental group can be attained. The environment of the mice can be controlled to a certain extent by housing them in sterile conditions (an isolator) or specified pathogen free (SPF) facilities. Although not entirely bacteria-free, SPF conditions are considerably more clean than the conventional housing conditions and therefore the comparison of mice housed in these two conditions could give some interesting information on the relevance of bacterial load to the profile of inflammatory mediators in the airways of the CF mice. The mice used in the following series of investigations were of an outbred, MF1 strain, background which undoubtedly led to some variation in results. However, the use of highly related groups of mice would give substantially less variation than that observed in studies with CF patients.

### 3.2 Bronchoalveolar lavage.

When performing bronchoalveolar lavage (see section 2.3 for details) it is important to lavage the largest area of the lung possible to obtain an accurate representation of the cell types and inflammatory molecules present. Several different volumes were used to lavage the mouse lungs and following cell counts and histological examination it was concluded that 3 volumes of 1 ml PBS retrieved the maximum amount of cells from the lavage with minimal damage to the lung. The lavage
procedure was standardised to give a collected lavage volume of 2.75 ml. Any collected lavage samples of a different volume or containing more than 5% epithelial cells were excluded from further studies.

3.3 Lung histology.

The left lungs of the mice were embedded in paraffin and sectioned for histological examination. Following PAS staining no gross differences were observed in lung histology between the CF mice and wild-type littermates of the conventionally or SPF housed mice as shown in figure 3.1. In general the lungs were found to be free of infection, with no goblet cell hyperplasia or signs of inflammation. Any slight physical damage observed was presumed to be due to the lavage or sectioning procedures. Very occasionally abnormalities of the lung were observed, with an increase of inflammatory cell types present within the airways and some visible tissue damage or infection. However these cases of inflammation, probably caused by an infection from opportunistic pathogens, were found to be random and bore no relationship to the genotype. It was noted that the worst cases of infection, judged by the inflammatory cell counts from BALF and gross histology, occurred in the conventionally housed mice, however raised levels of neutrophils were also occasionally found in the SPF housed mice. The pathogens for which the SPF facility has been specified not to contain is listed in the appendix. Since the SPF facility is not sterile it is possible that random lung infections can occur in the mice housed under those conditions and the low percentage of mice from the SPF facility which were found to contain raised levels of BALF neutrophils may be a reflection of this.

3.4 BALF cell numbers and differential cell counts.

As can be seen in figures 3.2 and 3.3A, there were no differences found in the total cell counts of CF and wild-type littermates in the conventional and SPF housing conditions. The differential cell counts were found to be similar in SPF and
Fig. 3.1 PAS stained lung sections of CF mice and wild-type littermates housed in conventional and SPF conditions. Images of the stained sections were captured at 200 X magnification. See section 2.8 for details on histology and microscopy.
Fig. 3.2 Cytospins of BALF from CF mice and wild-type littermates housed in conventional and SPF conditions. Cytospins were stained using Diff-Quik (Dade) as described in section 2.4 and images captured at 400 X magnification.
Fig.3.3 Cell analysis of BALF from CF mice and wild-type littermates.

(A) Total cell counts. (B) Differential cell counts. Cells were harvested from the airways by lavage and the total and differential cell counts performed as described in section 2.4. Values shown represent the total amount of cells retrieved by lavage and standard deviation bars have been applied to all values.
conventionally housed mice and the cells retrieved by lavage were found to consist almost exclusively of alveolar macrophages as seen in figures 3.2 and 3.3B.

3.5 ICAM-1 immunohistochemistry.

There was no difference observed in ICAM-1 levels between the wax or frozen sections and since the former presented better morphology these were used for subsequent antibody staining. Similar levels of ICAM-1 protein were found in CF and wild-type littermates from both the conventional and SPF housing conditions, as can be seen in figure 3.4A. Particularly strong staining was observed on the alveolar epithelium and the endothelium lining the blood vessels. It is possible that the antibody staining was not sensitive enough to detect small but potentially significant changes in ICAM-1 concentration, and reducing the concentration of primary antibody used in the staining procedure did not alter this. The concentration of the secondary antibody was also altered but again this did not lead to a more quantitative result. Control slides which contained either no primary antibody or no secondary antibody gave no staining as can be seen in figure 3.4B. Vector red detection agent was used as an alternative to the DAB substrate but gave the same intense staining pattern. It was concluded that since constitutive levels of ICAM-1 were high in the mouse alveolar epithelium and the endothelium lining the pulmonary blood vessels, it would be difficult to quantify any changes in levels of this cell adhesion molecule using antibody staining. A soluble form of ICAM-1 (s-ICAM-1) circulates in the blood and has been found to be increased in inflammatory disease including bronchopulmonary dysplasia (Kojima et al, 1993) and asthma (Kobayashi et al, 1994). A murine sICAM-1 ELISA is available and since this assay is likely to be more quantitative, serum samples were collected from conventionally housed CF and wild-type mice that had been exposed to S. aureus over a period of 28 days, exposed to pathogens once or not exposed and the ELISA performed. The results displayed in figure 3.5 show that there was no difference in levels of sICAM-1 between mice which have been repeatedly exposed to pathogens, those which have had a single
Fig. 3.4 ICAM-1 immunohistochemistry on lung sections of CF and wild-type littermates.
(A) ICAM-1 stained lung sections of CF mice and wild-type littermates housed in conventional and SPF conditions. (B) Control sections with the omission of rat monoclonal antibody to mouse ICAM-1 (no 1° antibody) or the rabbit anti-rat secondary antibody (no 2° antibody). Images were captured at 200 X magnification.
Fig. 3.5 Serum levels of soluble ICAM-1 from CF mice and wild-type littermates.
Mice were either exposed to *S. aureus* once (1), exposed once daily for 28 days (28) or not exposed (0). sICAM-1 was measured from serum by ELISA as described in section 2.6. Standard deviation bars have been applied to all values.
exposure and those which have not been exposed. There was also no difference found between CF and wild-type mice.

3.6 Western analysis of Cp-10.

Western blots were produced from lavage samples which were used either unconcentrated or vacuum dried and resuspended in a tenth of their original volume. Ponceau S staining indicated that protein transfer onto the blots had been successful. Lavage samples, from CF or wild type mice in the conventional and SPF housing conditions failed to give a detectable signal following antibody staining with anti-Cp-10 antibody. The sensitivity of immunoblotting is lower than an ELISA and in most cases a protein will not be detected if its concentration falls below 1ng/sample (Sambrook et al, 1989). The loading capacity of the SDS-polyacrylamide gel is approximately 150 μg, and therefore a protein antigen must be present at 1 part in 150,000. Cp-10 is one of the most potent chemotactic factors described to date with optimal activity for murine neutrophils at $10^{12}$ to $10^{13}$ M (Devery et al, 1994) and is therefore unlikely to be present at detectable levels in the BALF considering the high dilution factor of the lavage. In fact, if Cp-10 was present in high enough concentrations to be detected by Western blot analysis, there would be expected to be a massive neutrophil influx into the lung. Such an influx was not observed for any of the samples tested.

CFAg, which is a human homologue of Cp-10, is released from neutrophils in humans and is present at high levels in the serum of CF patients, decreased levels in heterozygotes and undetectable in normal, healthy individuals (Dorin et al, 1987). To test if the expression of Cp-10 protein was similarly elevated in the CF mouse neutrophils, large amounts of neutrophils were elicited for harvest by inducing peritonitis in wild-type, heterozygotes and CF mutant mice by a single interperitoneal injection of a 9 % solution of casein. The peritoneum was lavaged 24 hours later and the supernatant separated from the cells. Samples of both the supernatant and the cell suspension were analysed for the presence of Cp-10 by Western analysis.
**Fig. 3.6 Western analysis of Cp-10 from activated neutrophils.**

The Western blot was probed with anti-Cp-10 antibody. Lanes contain supernatant or cell suspension (neutrophils) from lavaged mouse peritoneum following intraperitoneal treatment with an inflammatory stimuli. A band of 10 kDa, corresponding to Cp-10 protein, can be seen in several lanes. Control samples (no 1° antibody) are also shown.
Figure 3.6 shows that Cp-10, represented by a band of 10 kDa, was present in the cell suspension and appeared to be at a much lower level or absent from the cell supernatant samples. A faint band of the correct size is present in the CF supernatant sample which may suggest that Cp-10 is secreted at heightened levels from CF neutrophils in comparison to wild-types. However it is possible that this band has originated from the sample in the next lane which has over-ran the well during loading. These results suggest that Cp-10 is produced by neutrophils during peritonitis and is present in the cytoplasm or in granules but does not appear to be secreted in a similar fashion to CF-Ag in this experimental situation. Cp-10 was detected in both the CF and wild type cell suspension samples at approximately the same level but was present at a lower level in the heterozygote cell suspension. The Ponceau S staining (see section 2.9.2) indicated approximately equal amounts of protein for each sample, however, this indicator is not very sensitive and the difference in Cp-10 may have arisen due to unequal loading of the samples. A control blot prepared with no primary Cp-10 antibody showed no bands relating to Cp-10. Cp-10 was originally isolated from activated CD4+ lymphocytes and has been demonstrated to be expressed by macrophages following LPS treatment (Hu et al., 1996). This experiment demonstrates that Cp-10 is also present in neutrophils following an inflammatory stimulus but does not seem to be secreted. However, unlike CF-Ag in humans, Cp-10 was not present in increased levels in the neutrophils of CF mutant or heterozygote mice compared to wild-type littermates.

3.7 Neutrophil chemotaxis assay

This assay demonstrates whether a sample contains substances that are capable of chemoattracting neutrophils and unlike ELISA or Western analysis does not require pre-knowledge of what those substances might be. Although this assay may detect the action of these factors at much lower concentrations than would be required for detection by Western analysis it is still limited by the dilution factor of the BALF. The neutrophil chemoattractant FMLP was used as a control at a concentration of $10^{-7}$ M which resulted in a high level of neutrophil chemotaxis through the chamber. No
<table>
<thead>
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<td>3</td>
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<td>7 - 16</td>
<td>w.t. conventional</td>
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<td>17 - 26</td>
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</tr>
<tr>
<td>37 - 46</td>
<td>CF SPF</td>
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</table>

Fig. 3.7 Neutrophil chemotaxis assay on BALF from CF mice and wild-type littermates.
(A) Filter from the chemotaxis chamber stained with Diff-Quik. (B) Schematic representation of the chemotaxis chamber with numbered wells. (C) Results of the chemotaxis assay (w.t. = wild-type).
neutrophil migration above that of the PBS controls was observed for any of the BALF samples assayed, as shown in figure 3.7.

3.8 Nitric oxide assay.

Figure 3.8 shows there were no significant differences found between conventionally housed wild-type and CF mice in levels of nitric oxide. The levels found were no higher than 10 μM which is around the limit of detection of this assay. The levels of nitric oxide present in the CF and wild-type SPF housed mice were below the minimum detectable level.

3.9 TNF-α and IL-1β ELISA

The mean level of IL-1β found in CF mice that were conventionally housed was 4 fold higher than that of their wild-type littermates, though due to the high variation found within each group this result was not found to be statistically significant (figure 3.9). The levels of IL-1β in SPF mice were too low to be detected by ELISA. The result observed in the conventionally housed mice may be a reflection of the random infections that occur in these housing conditions. It is possible that when a low level infection does occur it leads to a higher release of IL-1β in the CF mice compared to their wild type littermates.

TNF-α levels were found to be significantly higher in conventionally housed CF mice than their wild type littermates (p = 0.02). This difference was not observed in SPF housed mice where the overall levels of TNF-α were much lower as shown in figure 3.9.
Fig. 3.8 Nitric oxide analysis of BALF from CF mice and wild-type littermates.
The levels of nitric oxide in BALF samples from CF and wild-type mice from the SPF housing were too low to be detected by this assay which is described in section 2.7. Standard deviation bars have been applied to all values.
Fig. 3.9 Cytokine analysis of BALF from CF mice and wild-type littermates.

Cytokines were measured by ELISA as described in section 2.6. The values shown are for BALF (not ASF) and standard deviation bars have been applied to all values. * indicates that these two values are significantly different.
### Table 3.1 Summary of inflammatory phenotype results.

<table>
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**3.10 Discussion.**

A major drawback to these studies is the level of variation observed within experimental groups. A contributing factor to this variation may have been the use of the outbred strain of mice, MF1, for these experiments. When this project was initiated the only source of *Cftr<sup>tm1Hgu</sup>* mice available were those on an MF1 background but since then, breeding programs have successfully produced these CF mutant mice of several different congenic strains. However recent evidence suggest that MF1 may have advantages over inbred strains in terms of accurate modelling of the human disease. It has been demonstrated that MF1 mice have ion transport properties similar to those of normal human subjects and the *Cftr<sup>tm1Hgu</sup>* mutant mice on this background have electrophysiological characteristics that resemble those of CF subjects (Smith *et al.*, 1995). It has become clear that there is considerable variability in short circuit current (Isc) across the tracheal epithelium in response to forskolin between inbred strains of mice. Isc was measured in 6 strains of inbred mice and BALB/c mice were the only inbred strain to show a forskolin response similar to that of the MF1 strain (Farley *et al.*, 1998). This was significantly greater than all other strains which produced a response no greater than that of the *Cftr<sup>tm1Unc</sup>*
null mice suggesting that the function of the Cftr channel had been modified in these strains. It has also been found that there is variability in the susceptibility to *P. aeruginosa* infection and silicosis in mice of different inbred strains (Morissette *et al*, 1995, Ohtsuka *et al*, 1995) and although this variability can be used beneficially in the search for modifier genes it can also be a significant complicating factor in phenotypic analysis. Thus, although an outbred strain of mouse was used for these experiments, it was considered more important to have a phenotype in the CF mouse that represents the human condition. The ideal situation may have been the use of the BALB/c strain, however the *Cftr*<sup>tm11Hgu</sup> mouse was not available on this background.

Another factor which may have contributed to variation within groups is unequal sampling of the airway surface fluid. To minimise this it would have been helpful to have an experimental procedure to directly quantify the volume of airway surface fluid recovered by lavage by measuring the dilution factor of components such as urea, which diffuses freely throughout the body (Crystal *et al*, 1986). Several methods were used in an attempt to quantify the dilution factor of the lavage fluid however none were successful in the mouse and all gave inconsistent results (Donald Davidson, personal communication). This is most likely to be due to the small volume of airway surface fluid present in the mouse lung and the high dilution factor which would lead to molecules such as urea or glucose falling below a detectable level. It may be argued that these assays may have worked if the dilution factor of the ASF had not been so high and a smaller volume of fluid was used for the lavage. However, a series of experiments were conducted in which various volumes of fluid were used for lavage (see section 3.2) and it was found that if the volume was decreased then the lungs were not inflated across their entire area. As has already been mentioned, regional variation in inflammation exists in the lungs of CF patients (Meyer *et al*, 1997) and therefore it was considered important to use a volume of fluid which would lavage as large an area of the mouse lungs as possible.

The sensitivity of the assays employed in the inflammatory analysis is another factor which can lead to variability in results. In many cases the substance being detected probably fell below the limit of sensitivity as previously discussed for Cp-10. An alternative would be to use RT-PCR although this is not very efficient as a
quantitative assay and it may be hard to distinguish the levels of transcript production between samples from the PCR products produced. There may be additional problems to using RT-PCR to study the level of inflammatory mediators, as an increase in transcript of a gene does not always lead to an increase in protein level. For example, TNF-α has been shown to be tightly regulated at both the transcriptional and post-transcriptional level (Sherry et al., 1988). The post-transcriptional regulation of TNF-α is thought to be through a regulatory element in the 3’ UTR which controls the half life of the mRNA and the efficiency with which they are translated. It is also possible that an inflammatory stimulus may lead to increased levels of protein processing or protein secretion whilst the mRNA levels remain the same.

ICAM-1 was shown to be constitutively expressed at a high level in the mouse alveolar epithelium and endothelium. It has previously been shown, using immunogold labelled ICAM-1, that constitutive expression of ICAM-1 on murine alveolar epithelial cells is 22-fold greater than on capillary endothelium (Burns et al., 1994). This was not observed for these experiments, however, it is possible that the immunohistochemistry used may be less sensitive than the immunogold labelling and transmission electron microscopy that Burns et al. employed.

No difference was found in the levels of sICAM-1 in CF mice and wild-type littermates even after exposure to S. aureus. These results reflect the previously described study of Salva et al. (1996) where sICAM-1 was not found to be increased in CF patients during an inflammatory response. However, they do contrast with a recent study by De Rose et al. (1998) who found that sICAM-1 was elevated in the serum of CF patients even when the patients are clinically stable. It is feasible that increases in murine sICAM-1 may not be detected due to the formation of complexes or its breakdown by proteases. However, the possibility that there is no difference in levels of sICAM-1 following an inflammatory stimulus cannot be ruled out.

The constitutive expression of ICAM-1 may suggest that it is the regulation of another molecule during an inflammatory response that leads to an increased neutrophil infiltration into the lung. As has already been mentioned in section 1.13.2, L-selectin shedding may be significant in addition to the concentration gradient of IL-
8 (or the equivalent murine chemoattractant) across the epithelium. Transient increases in endothelial adhesion have been reported to be largely attributable to P-selectin (Geng et al, 1997). Western analysis of BALF with mouse P-selectin and E-selectin antibodies was attempted without success.

As can be seen from table 3.1, apart from TNF-α, no other inflammatory molecule was found to be present at significantly differing levels in CF and wild-type littermates in the conventional housing conditions. This result may be considered as surprising considering that TNF-α is a primary cytokine which has a regulatory role in the function of many downstream pro-inflammatory molecules. It is possible that other proteins are upregulated but their concentration still falls below the limit of sensitivity of the detection assay since they may only need to be present at extremely low concentrations to have a physiological effect in the mouse. For example concentrations of $10^{-14}$ to $10^{-12}$ M IL-1β are sufficient to obtain a maximal cellular response (Bona et al, 1996) which may help to explain why such low levels of IL-1β were detected by ELISA. However, it may not just be simply the levels of cytokine that are relevant to pulmonary inflammation but also the time of production and the local environment into which they are released.

IL-1β levels were found to be higher, though not significantly so in CF mice and TNF-α levels were significantly higher. It is suggested here that this was due to random cases of infection from conventionally housed mice, which gave rise to a higher inflammatory response in the CF mice. Following routine inspection for bacteria in the conventional and SPF housing it was found that much higher levels of bacteria were found in the former. This would increase the chance of a mouse becoming infected in the conventional housing and may explain why differences in TNF-α levels were only found between CF and wild-type littermates when they were kept under these conditions. No differences were found in the other inflammatory molecules assayed for but as mentioned earlier this could be due to the level of sensitivity of the detection assay used.

There are several possible explanations as to why the CF mice release statistically higher amounts of TNF-α when housed in conventional conditions:
1. the housing conditions of the CF mice had higher levels of pathogens than those of the wild-type mice, however this is unlikely to be the case since efforts were made to ensure that CF mice were compared to wild-type littermates which would have shared the same environmental conditions.

2. There is a defect in the pulmonary inflammatory cells of the CF mouse that leads to a higher release of TNF-α after encountering low level environmental bacteria and this defect is caused directly by the CFTR mutation. In humans alveolar macrophages, T-lymphocytes, neutrophils and epithelial cells express proinflammatory cytokines and all have been shown to express CFTR (Yoshimura et al, 1991, Dong et al, 1995), albeit, in some cases, at very low levels. Therefore it may be possible, especially considering the ever increasing range of actions of which CFTR has shown to be capable, that the CFTR defect in these cells may be responsible for abnormal cytokine production and this is what is also observed in the mouse model. It seems unlikely that there are higher levels of inflammatory molecules present in the CF mouse lung preceding a bacterial encounter since no difference in the level of TNF-α or any other molecule tested was found in SPF mice, however, this may be due to the sensitivity limitations of the assays used in these experiments.

3. The increased release of inflammatory mediators in the CF mice may reflect a mechanism to compensate for the inhibition of the function of another arm of the host pulmonary defence system, caused by the Cfr mutation.

The experiments described in the following two chapters were conducted with the aim of resolving which of the above, if any, were responsible for the heightened inflammatory response observed in the conventionally housed CF mice.
Chapter 4

LPS induced inflammation in the CF mouse
Chapter 4 LPS induced inflammation in the CF mouse.

4.1 Introduction.

In an attempt to resolve whether the difference in TNF-α levels was caused by an inflammatory cell defect, CF mice and wild-type littermates were treated intratracheally with LPS derived from *E. coli*.

LPS is an integral component of the outer membrane of gram-negative bacteria and a major contributing factor in the initiation of a generalised inflammatory process. As its name suggests it is composed of a lipid component, lipid A, and a polysaccharide component. The polysaccharide is made up of two parts: the core oligosaccharide which is connected to the lipid and the longer O-specific chain which projects from the core and is the outermost part of the endotoxin. The O-specific chain is the most variable segment and usually differs from one bacterial species to the next. LPS has been shown to stimulate macrophages to release many inflammatory mediators including proinflammatory cytokines, oxygen free radicals and lipids such as prostaglandin and platelet-activating factor (Rietschel *et al*, 1992). It has already been mentioned that the LPS receptor, CD14, is found on airway epithelial cells and therefore LPS may also stimulate epithelial cells to release inflammatory mediators (Fears *et al*, 1995).

If there is a defect in the CF inflammatory cells, then under normal conditions the consequences may be subtle and therefore, if this was the case, it was hoped that exposure of these cells to a potent inflammatory stimulus such as LPS may lead to the detection of the defect in terms of the release of proinflammatory cytokines or other inflammatory mediators.

The potent inflammatory capabilities of the LPS from CF-relevant pathogens including *P. aeruginosa* and *B. cepacia* may be of relevance in the later stages of CF lung inflammation (see section 1.13.2) but since no *P. aeruginosa* or *B. cepacia* was found in the conventional housing following routine inspections it is unlikely that this led to the increased release of TNF-α observed in the CF mice (section 3.9). Therefore, the LPS from these bacteria was not used since it was the basic response
of the inflammatory cells to a general stimulus that was the aim of the study. The use of an LPS which may have an increased inflammatory capacity but which is not thought to be relevant to the initial stages of CF lung disease could complicate the interpretation of results.

Alveolar macrophages which had been isolated from CF and wild type lungs were also stimulated with *E. coli* LPS to ascertain whether these cells were directly affected by the loss of CFTR function.

### 4.2 Administration of LPS.

A non-invasive method of administering LPS to the airways was chosen as unlike a surgical procedure, the risk of damage to the trachea or of an infection is decreased (see section 2.2.1 for details). The effectiveness of this procedure had previously been tested and the administered fluid was distributed equally throughout the airways draining right down to the arterioles (Donald Davidson, personal communication).

An initial experiment tested two different doses of LPS, one of 8 μg and another of 80 μg. After intratracheal instillation of the high dose of LPS 50 % of the mice (n = 16) displayed signs of endotoxic shock with ruffled coats and discharge from the eyes, nose and mouth which in most cases led to death. However, no signs of endotoxic shock were observed for the mice given the lower dose of LPS and the treated mice recovered fully from the procedure. Therefore all subsequent experiments used the low dose. Mice were killed 1 hr, 2 hr, 4 hr, 24 hr and 48 hr after LPS instillation.

### 4.3 Lung histology.

At 24 and 48 hr following LPS administration tissue damage was evident in the lung in the form of pneumonia and haemorrhage. There would likely have been infiltration of inflammatory cells into the lung before these timepoints but presumably these have mostly been removed by the lavage process. No goblet cell hyperplasia was
Fig. 4.1 PAS stained lung sections of CF mice and wild-type littermates following LPS treatment. Images were captured at 200 X magnification.
Fig. 4.2 Cytospins of BALF from CF mice and wild-type littermates following LPS treatment.
Images were captured at 400 X magnification. The BALF samples collected 48 hr after LPS treatment were diluted 1:10 for the cytospin.
Fig. 4.3 Total cell counts from BALF of CF mice and wild-type littermates following LPS treatment. Standard deviation bars have been applied to all values.
Fig. 4.4 Differential cell counts from BALF of CF mice and wild-type littermates following LPS treatment. Standard deviation bars have been applied to all values.
Fig. 4.5 Analysis of BALF from CF mice and wild-type littermates following treatment with PBS.

(A) Cytospin of BALF following PBS treatment. (B) Differential cell count following PBS treatment.
observed at any timepoint and there was no difference in histology observed between CF and wild-type littermates as seen in figure 4.1.

**4.4 BALF cell numbers and differential cell counts.**

Cytospin results and the total and differential cell counts following LPS administration are shown in figures 4.2 - 4.4). The total amount of cells present within the airways was seen to increase between 2 and 4 hr after instillation of LPS followed by an even greater increase between 4 and 24 hr. After 24 hr the total cell count began to decrease slightly. The differential cell counts at all the timepoints tested indicates that the cell number increase was mostly due to a massive influx of neutrophils into the airways. The total number of macrophages, T-cells and epithelial cells present in the BALF did not alter greatly at the different timepoints except at 48 hr when the macrophage number increased. This massive influx of neutrophils into the airways is typical of an acute inflammatory response towards LPS. A control experiment was included in which sterile PBS was instilled into the airways instead of LPS and lavage performed 24 hr after instillation. This also resulted in an inflammatory response with the total cells retrieved from the lungs being increased in number and an influx of neutrophils as seen in figure 4.5. However the percentage of neutrophils present in the BALF was only 20 % in the PBS treated samples compared to 90 % in the 24 hr LPS treated lungs.

No difference was observed between CF and wild-type littermates in either the total or differential cell count of the BALF.

**4.5 ICAM-1 immunohistochemistry**

There was no difference observed between CF and wild-type littermates in terms of ICAM-1 expression. Figure 4.6 shows there was no upregulation of ICAM-1 at any timepoint following LPS instillation and as mentioned earlier this may be due to the sensitivity limitations of the assay employed.
Fig. 4.6 ICAM-1 immunohistochemistry of lung sections from CF and wild-type littermates following LPS treatment. All images were captured at 200 X magnification except for CF 4 hr LPS which was captured at 100 X magnification.
4.6 Western Analysis of Cp-10.

Cp-10 protein was detectable in the BALF of both wild-type and CF mice at 24 and 48 hr following LPS instillation as shown in figure 4.7. Cp-10 protein has a molecular weight of 10 kDa and a band of this size was seen in addition to another smaller band. It is not known what this additional band represents. It may be a breakdown product of the Cp-10 protein or it may be a related protein. Neutrophils were present in increased numbers in the airways before 24 hr as seen in figure 4.2 so it is likely that the levels of Cp-10 were raised before this time if this molecule is relevant to neutrophil chemotaxis. It is possible that the Western blot analysis was not sensitive enough to pick this up. Alternatively, another chemoattractant may be more important for neutrophil transmigration immediately following an inflammatory stimulus and this may then have a role in the upregulation of Cp-10. A previous experiment demonstrated Cp-10 to be present intracellularly in neutrophils and it was not tested whether this was also the case for this experiment. However a possible model may be proposed in which Cp-10 is initially secreted from the activated pulmonary macrophages resulting in an influx of neutrophils into the airways. If the neutrophils are then subject to an inflammatory stimulus they may be stimulated to produce Cp-10 which would be released when the neutrophils degranulate attracting more neutrophils into the airways until the inflammatory stimulus on the neutrophils ceased.

4.7 Neutrophil chemotaxis assay.

No neutrophil migration was observed for any of the BALF samples tested however neutrophils had migrated in the FMLP positive controls indicating that the neutrophils were viable. It is not clear why there was no migration observed especially for the samples taken from mice that had massive influxes of neutrophils into the airways. It is possible that the dilution of the chemoattractant factor by the lavage procedure has left it at a concentration too low to elicit its chemoattractant activity as previously mentioned in section 3.7. Lavage samples were concentrated by
Fig. 4.7 Western analysis of Cp-10 from BALF of CF mice and wild-type littermates following LPS treatment.
The Western blot was probed with anti-Cp-10 antibody. Lanes contain BALF samples from wild-type (+/+) and CF mice at various timepoints following intratracheal treatment with LPS.
vacuum drying but there was still no chemoattractant activity detected. The concentration of the molecules may have remained too low or it is possible that the drying procedure promoted the destruction of the chemoattractant molecules by proteolysis. Alternatively, the molecules may have formed complexes that quenched their chemoattractant ability. The neutrophils used in these experiments had been harvested from the peritoneum and it is possible that these migratory neutrophils, unlike neutrophils from the blood, cannot respond to pulmonary chemoattractants present in the BALF. However, this seems unlikely since these neutrophils could respond to FMLP.

4.8 Nitric oxide assay.

No nitric oxide was detected in any of the BALF samples tested. In each case a standard curve was successfully produced indicating that the reagents used in this assay had not lost their activity. An increase in the concentration of nitric oxide compared to that of the conventionally housed mice was expected since LPS has been shown to induce the release of nitric oxide from alveolar macrophages, however this was not observed. It is possible that the storage conditions of the BALF led to a breakdown of nitric oxide or its intermediates, however this is unlikely since samples were stored at -70°C. The low sensitivity of this assay has already been mentioned in section 3.8 and therefore it is possible that nitric oxide was increased in the airways following LPS treatment but it was not detected using this assay.

4.9 TNF-α, IL-1β and IL-6 ELISA.

IL-1β, TNF-α and IL-6 levels in BALF were measured by ELISA. Figure 4.8 shows that there were no significant differences found between CF and wild-type mice for IL-6 and TNF-α. No IL-1β was detected at any of the timepoints following LPS instillation. TNF-α is known to be upregulated very quickly following an inflammatory stimulus which was the case in this experiment with TNF-α at its maximum level after 2 hr. IL-6 is activated downstream of TNF-α in the
Fig. 4.8 Cytokine analysis of BALF from CF mice and wild-type littermates following LPS treatment. Standard deviation bars have been applied to all values.
Fig. 4.9 Cytokine levels following LPS administration to alveolar macrophages.
Macrophages were isolated and treated with LPS as described in sections 2.4.2 and 2.2.3. The cell supernatants were assayed by ELISA. Standard deviation bars have been applied to all values.
inflammatory pathway and this was confirmed following LPS instillation when the maximum level was reached at 24 hr. A TNF-α ELISA was also performed on the BALF collected from mice which had survived the high dose instillation of LPS. The overall levels of TNF-α were similar to those found at the low dose levels and there were no differences between CF and wild-type mice.

Isolated alveolar macrophages were treated with various concentrations of LPS and the cell medium removed after 24 hr. Figure 4.9 shows there were no significant differences found between CF and wild type alveolar secretions of TNF-α and IL-1β. In both cases the response was maximal with 1 μg of LPS though it is possible that higher concentrations of LPS may have had a toxic effect on the macrophages. There are no reports of LPS being intrinsically cytotoxic but it is possible that the high release of reactive oxygen intermediates by macrophages in response to LPS may subsequently damage the cell itself, however the macrophages were not analysed after treatment to check for signs of cell death to assess this. Alternatively, at higher concentrations of LPS the inflammatory response may have been more acute with higher amounts of the cytokines being released at an earlier time point and by 24 hr the levels may have decreased due to a self regulatory feedback mechanism.

4.10 Discussion.

These results present evidence that the pulmonary inflammatory cells of the CF mouse function normally when given a direct inflammatory stimulus such as LPS. There were no significant differences observed from the BALF or isolated alveolar macrophages. It was surprising that there was no change in levels of IL-1β following LPS administration to the lungs since IL-1β is often referred to as a primary cytokine in the establishment of inflammatory responses. An IL-1β knockout mouse has been created (Asano et al., personal communication) and these mice were shown to be as sensitive to LPS as wild-type mice. TNF-α knockout mice, on the other hand, were shown to be more resistant to LPS-induced inflammation than wild-type mice (Asano et al., personal communication). These results indicate that IL-1β is not involved in LPS-induced inflammatory responses and this may explain why an IL-1β response
was not observed for mice treated intratracheally with LPS here. However, IL-1β production was observed for isolated macrophages treated with LPS. This may indicate that IL-1β in the airways is upregulated in response to LPS but the concentrations are still too low to be detected following lavage or that significant differences in results may be obtained depending on whether in vivo or in vitro methods are employed. This is discussed in more detail below.

There were no differences detected between CF and non CF macrophages harvested by lavage, in terms of the release of TNF-α and IL-1β, following treatment with LPS. These macrophages gave lower levels of TNF-α than might have been expected if these cells were primarily responsible for TNF-α secretion in the lung. It may be that cell types other than alveolar macrophages, such as epithelial cells, are also important for TNF-α induction either by releasing TNF-α themselves or by producing other molecules that have a regulatory role on the production of proinflammatory cytokines by alveolar macrophages. Conversely IL-1β was found to be present at a higher level from the alveolar macrophages than the BALF. In this case the increased level may also reflect the reduced dilution factor of the macrophage sample compared to that of the BALF. Interestingly, a study on hamsters revealed the apparent existence of a population of airway macrophages that resisted being harvested by lavage. These macrophages appeared to be morphologically different to those removed by lavage and it was proposed that they may also be functionally distinct (Geiser et al, 1995). Therefore the inflammatory response of macrophages harvested from the lung by lavage may not be representative of the in vivo situation. This study by Geiser et al. (1995) highlights the possible problems of studying one cell type in isolation.

Due to problems of assay sensitivity, variability of results and the overall complexity of the cytokine network as well as all the possible candidate molecules involved it cannot be stated with total certainty that there is no difference in the reaction of CF and non CF cells to a general inflammatory stimulus. If the pulmonary inflammatory cells from CF mice do act normally when given a direct inflammatory stimulus, as these results suggest, then it is possible that some other component of the host defence mechanism is functioning abnormally when faced with a pathogen challenge and this leads to the rise in TNF-α secretion to compensate for this. Figure 4.10
Fig. 4.10 Proposed model for heightened inflammatory response in conventionally housed CF mice.

- CF or wild-type inflammatory cell
  - no stimulus (SPF conditions) → Basal levels of TNF-α in CF and wild-type mice.
  - direct chemical stimulus → Similar response in CF and wild-type mice.
  - wild-type inflammatory cell → Host defence system eradicates most of the inflammatory agents
  - low "background levels" of inflammatory stimuli
    - CF inflammatory cell → An element of the innate host defence is lost as a result of the Cft mutation.
    - A higher compensatory inflammatory response occurs in CF mice.
Chapter 5

Treatment with *S. aureus* and *P. aeruginosa*
Chapter 5 Treatment with *S. aureus* and *P. aeruginosa*.

5.1 Introduction.

In an attempt to test further the proposed model for excessive inflammatory response in CF mice shown in figure 4.10, CF and wild-type mice were instilled with a low dose of bacteria and their airways analysed 16 hr later. The CF-relevant pathogens *S. aureus* and *P. aeruginosa* were used for these experiments (see section 2.2.1 and 2.2.2 for specifics on the strains of bacteria used).

Three different doses of *S. aureus* were administered to CF and wild-type mice by intratracheal instillation whereas two doses of *P. aeruginosa* were administered to CF and wild-type mice by nebulisation. After the *S. aureus* results had been analysed it was decided to change the administration method of *P. aeruginosa* to nebulisation in an attempt to reduce the variability of the inflammatory response observed between mice of the same experimental group. Nebulisation should, in theory, ensure that each mouse receives the same number of bacteria. Previous experiments have shown that mice produce a more acute response to *P. aeruginosa* than to *S. aureus* (Donald Davidson, personal communication) therefore the colony forming units (cfu) of *P. aeruginosa* administered to mice was reduced to a level which would not prove fatal.

5.2 Lung histology.

5.2.1 *S. aureus*.

When $5 \times 10^4$ cfu of *S. aureus* were instilled intratracheally, similar histology was observed in the CF and wild-type mice as seen in figure 5.1. Both had goblet cell hyperplasia and an increase in the numbers of inflammatory cells present in the airways most of which were removed by the lavage process. Administration of the higher concentrations led to increased levels of goblet cell hyperplasia and at the
Fig. 5.1 PAS stained lung sections of CF mice and wild-type littermates following *S. aureus* treatment. Images were captured at 200 X magnification. Goblet cells are stained bright red and can be seen on the bronchiolar epithelium.
Fig. 5.2 PAS stained lung sections of CF mice and wild-type littermates following *P. aeruginosa* treatment. Images were captured at 200 X magnification.
highest dose of $1 \times 10^6$ cfu there was a marked increase in the number of neutrophils infiltrating into the airways.

5.2.2 *P. aeruginosa*.

Figure 5.2 shows that goblet cell hyperplasia was present in CF and wild-type mice at both concentrations of *P. aeruginosa* administered. A notable increase in the number of inflammatory cells present in the airways was detected for both CF and wild-type mice following nebulisation with $5 \times 10^5$ cfu *P. aeruginosa*.

5.3 BALF cell numbers and differential cell counts.

5.3.1 *S. aureus*.

Figure 5.3A shows the total cell count after *S. aureus* instillation does not increase greatly from the number present in the uninfected lung except at the highest dose of $1 \times 10^6$ cfu where the number of inflammatory cells retrieved by lavage rises to approximately twice normal. The differential count and cytospin results shown in figures 5.3B and 5.4 indicated this was due to the large influx of neutrophils. Following instillation of $5 \times 10^4$ cfu *S. aureus* the total cell number and the number of neutrophils in the CF mice is higher than that of the wild-type littermates, but due to the variability in the numbers within the experimental groups this result cannot be considered statistically significant.

5.3.2 *P. aeruginosa*.

At the low dose of *P. aeruginosa* the total cell count in BALF had increased several fold from the untreated mice, as seen in figure 5.5A and surprisingly the percentages of neutrophils and macrophages lavaged from the lung showed a significant difference ($p = 0.0052$) between wild-type and CF mice (see figure 5.5B and 5.6). Although the total cell numbers were similar, they comprised 70 % neutrophils in
Fig.5.3 Cell analysis of BALF from CF mice and wild-type littermates following *S. aureus* treatment.

(A) Total cell counts. (B) Differential cell counts. Standard deviation bars have been applied to all values.
Fig. 5.4 Cytospins of BALF from CF mice and wild-type littermates following S. aureus treatment. Images were captured at 400 X magnification.
A.  

Fig. 5.5 Cell analysis of BALF from CF mice and wild-type littermates following *P. aeruginosa* treatment.  
(A) Total cell counts. (B) Differential cell counts. Standard deviation bars have been applied to all values.
Fig. 5.6 Cytospins of BALF from CF mice and wild-type littermates following *P. aeruginosa* treatment.
Images were captured at 400 X magnification. The BALF samples from mice treated with $5 \times 10^5$ *P. aeruginosa* were diluted 1:10 for the cytospin.
the wild-type mice compared to only 23% in the CF mice. Similarly the macrophage percentages were significantly different with the CF BALF containing 75% macrophages whereas BALF from the wild-type littermates consisted of only 29% macrophages ($p = 0.0054$). At a nebulised dose of $5 \times 10^5$ cfu the total cell number was greatly increased and this was shown to consist of approximately 90% neutrophils for both CF mice and their wild-type littermates (see figures 5.5 and 5.6).

5.4 ICAM-1 immunohistochemistry.

No difference in levels of ICAM-1 protein on the epithelium and endothelium after treatment with *S. aureus* and *P. aeruginosa* were observed between these samples and those of untreated mice and there was also no difference detected between CF and wild-type littermates with either of the two pathogens at all concentrations tested.

5.5 TNF-α, IL-1β and IL-6 ELISA.

5.5.1 *S. aureus*.

Figure 5.7 shows the mean levels of TNF-α and IL-6 in CF mice to be higher than their wild-type littermates at the $5 \times 10^4$ and $1 \times 10^6$ doses of *S. aureus*. However these results were not found to be statistically significant due to the high level of variability within the experimental groups. IL-1β was below the minimum detectable level for all mice at all concentrations of *S. aureus*.

5.5.2 *P. aeruginosa*.

There were no significant differences found between the CF and wild type mouse in levels of TNF-α, IL-6 or IL-1β following *P. aeruginosa* nebulisation as can be seen in figure 5.8. The levels of TNF-α and IL-6 following nebulisation of $5 \times 10^4$ cfu were comparable to the levels obtained with *S. aureus*. However, at the higher dose
Fig. 5.7 Cytokine analysis of BALF from CF mice and wild-type littermates following *S. aureus* treatment. Standard deviation bars have been applied to all values.
Fig. 5.8 Cytokine analysis of BALF from CF mice and wild-type littermates following *P. aeruginosa* treatment. Standard deviation bars have been applied to all values.
of *P. aeruginosa* the levels were greatly increased probably indicating a much more severe inflammatory response in the murine airways towards this pathogen.

### 5.6 Discussion.

Treatment with low levels of *S. aureus* resulted in a higher mean level of TNF-α and IL-6 and a higher percentage of neutrophils present in the BALF of CF mice compared to wild-type littermates. None of these results could be deemed significant however and the main reason for this is the high degree of variability within experimental groups. As discussed previously, one source of this variability may be due to the use of an outbred strain of mice and therefore increasing the numbers of mice used in these experiments may have improved the results. Another source of variability in results may have been due to the unequal administration of pathogens to the lung via intratracheal instillation. Although control experiments have shown the instilled material does disperse throughout the lung there is always the risk that some of it travels back up the trachea and is swallowed or left behind in the cannula. It would therefore be advantageous to assess the actual amount of pathogens that have been placed in the lung of every mouse. One way of doing this would be to plate out a homogenate of the lung and count the colonies which are recovered. However, this was not a feasible option in this case, since the lung tissue had already been lavaged prior to treatment for immunohistochemistry thus removing most of the pathogens. It was decided to plate out some of the recovered BALF from each mouse treated with *S. aureus*, in order to give some indication of how many bacteria were present in the lung and then relate this to the extent of the inflammatory response observed in that mouse. Unfortunately, no colony forming units were observed for any of the samples plated out which was probably due to the bacteria being present in the cell pellet following the centrifugation of the BALF sample and not in the supernatant. It was found that nebulisation of bacteria also led to variability in results within experimental groups. Although genetic variation would have been a factor it is thought that some of the variability must have arisen from mice receiving unequal
loads of bacteria and this could arise, for example, through differences in the respiratory rate of the mice during treatment.

The significant difference observed in the BALF differential cell count between CF mice and wild-type littermates treated with $5 \times 10^4$ *P. aeruginosa* could indicate a difference in the kinetics of the inflammatory response. It may be that the CF mice have a delayed reaction, in terms of neutrophil influx compared to their wild-type littermates. Interestingly, there was an increase in the number of macrophages retrieved from the airways of the CF mice compared to wild-type littermates and it is possible that this is also a reflection of a difference in kinetics of the inflammatory response. An increase in macrophage number may lead to a greater secretion of proinflammatory cytokines and a heightened inflammatory response in the CF mice.

It would have been of interest to study the response to *P. aeruginosa* at timepoints exceeding 16 hr to examine whether the CF mice had an inflammatory response greater than that observed for the wild-type littermates. It is not clear if this difference between CF mice and wild-type littermates in response to *P. aeruginosa* is specific to this pathogen or why it appears to be dose-dependent.

Although a single treatment with both pathogens resulted in a significant dose-dependent increase in the basal production of pro-inflammatory cytokines, it is possible that a single dose is not sufficient to generate any differences between the CF mice and wild-type littermates. The mice housed in the conventional conditions are likely to have had many encounters with low levels of bacteria before their proinflammatory cytokine levels were assessed and these multiple encounters may have been necessary to generate the increased levels of TNF-α that were found (see section 3.9). The differences observed in the mice treated with low dose *S. aureus* mentioned above suggests that the CF mice do have an initial increased inflammatory response and although not shown to be statistically significant this may well be significant at the local level in the lung. Davidson et al. (1995) found that, in general, mice had to be nebulised with *S. aureus* more than once to generate any significant differences between CF mice and their wild-type littermates in terms of bacterial clearance or lung histology. Although it may be that any excessive cytokine response would manifest itself prior to these other indicators.
The non-mucoid CF isolate of *P. aeruginosa* used in these experiments had been shown capable of conversion to mucoidy since an isogenic mucoid variant of this strain was detected in the CF patient 3 months after the isolation of the non-mucoid isolate. The conversion from a non-mucoid strain to an alginate-producing mucoid strain does not occur in the normal lung, probably due to the clearance of *P. aeruginosa* before this can happen. There is evidence that alginate production inhibits opsonic and non-opsonic phagocytosis, suppresses the oxidative burst of neutrophils, quenches reactive oxygen intermediates and induce proinflammatory cytokines, therefore rendering the pathogen more resistant to the host defence mechanisms (Deretic, 1996). This action of alginate is not thought to be CF specific and would also occur in the normal lung. A recent study by Yu *et al.* (1998) supported this hypothesis by demonstrating that a mucoid strain of *P. aeruginosa* was cleared less efficiently from the lung of normal mice than the non-mucoid parental strain. It is highly unlikely that the 16 hr timecourse of these experiments was sufficient to allow conversion of a non-mucoid strain to a mucoid variant since this event takes an average of 3 months to occur in CF patients. If it is the conversion to mucoidy that is the essential difference between CF and non-CF in terms of *P. aeruginosa* infection then this may explain why no significant differences were observed between the CF mice and the wild-type littermates. The study by Heekeren *et al.* (1997) mentioned previously (see section 1.17.3) is not in agreement with this theory since their results indicated a more severe inflammatory phenotype in the CF mice compared with wild-types when a mucoid strain of *P. aeruginosa* was retained in the lung. However, the inadequacies of these experiments have already been discussed and therefore, additional studies are required to validate fully this theory.

It would have been of interest to use a pathogen for these experiments which is not associated with CF to ascertain whether there is a basic defect, caused by the *Cftr* mutation, in host defence relevant to an initial response to all pathogens and which results in mild inflammation. This would support a two stage model of CF lung disease in which the inflammation then progresses when the animal encounters a CF-relevant pathogen which may have the additional advantages of novel receptor
binding and internalisation such as previously described for *P. aeruginosa* (see sections 1.11.2 and 1.11.3).

It is also possible that no significant differences were found in the inflammatory profile of CF mice and wild-type littermates because the host defence systems of mice and humans are different and therefore the CFTR mediated defects of the CF patients lung defence may not be relevant in the CF mouse. It is feasible that the mouse has a more effective defence against the pathogens tested and therefore the loss of an element(s) of the host defence system caused by the loss of Cftr may not lead to a heightened compensatory release of inflammatory molecules. Alternatively, the mouse may have a less effective defence against respiratory pathogens which leads to an acute inflammatory response after treatment in which subtle differences caused by the loss of CFTR are not noticed. In addition there are differences in the cellular composition of the respiratory tract of the mouse compared to humans which may be of relevance to CF lung disease. Mice have fewer submucosal glands (the major site of expression of CFTR in the airways) and an increased number of Clara cells in the tracheal epithelium (Dorin *et al.*, 1996). The existence of a non-CFTR mediated calcium-dependent chloride ion channel present in the airways of mice has already been mentioned (Clarke *et al.*, 1994) but since then a similar channel has been found in human airways (Smith *et al.*, 1995) and therefore the relevance of this channel to lung phenotype is unclear since it obviously does not protect CF patients from lung disease. However, it has been proposed that this channel is upregulated in airway epithelium of the CF “null” mouse and this buffers the severity of airway disease (Grubb *et al.*, 1994). There may be additional factors worth considering when comparing human CF lung disease to that of the CF mouse such as the difference in life span. CF mice do not normally acquire lung disease unless deliberately treated with pathogens but this could just be a consequence of not being exposed to “environmental” pathogens for as long a period of time as humans or that they don’t encounter as many pathogen-carrying subjects.

In summary, these studies have demonstrated that the *Cfr^pm/Hgu* mice release higher levels of TNF-α than their wild-type littermates when housed in conventional
conditions. This was proposed to be due to a higher inflammatory response to the “background” pathogens that are found in these housing conditions. There was no difference found in the SPF housed mice suggesting that the increased inflammatory response does not occur prior to bacterial encounters, or prior to the bacterial load reaching a certain level. No difference in the inflammatory profile of CF mice and wild-type littermates was found following treatment with LPS suggesting the inflammatory cells could function as normal when given a basic inflammatory stimulus. This led to the model in figure 4.10 being proposed. Unfortunately, no statistically significant differences were found between CF mice and their wild-type littermates following treatment with various doses of S. aureus or P. aeruginosa. This may be a reflection of giving the mice a single dose of pathogens and it is possible that multiple encounters of bacteria, such as would happen in the conventional housing, is necessary before any significant differences in the inflammatory profile of CF mice and their wild-type littermates can be detected with the assays used in these experiments.

The studies presented here support the possibility that there is a basic fault in the host defence system caused by a mutant CFTR. Recent studies have indicated the activity of airway beta defensins may be decreased in the CF lung environment. The decreased activity of these antimicrobial peptides in the CF ASF could lead to an upregulation of proinflammatory mediators in the airways, such as TNF-α, in an attempt by the host defence mechanism to overcome bacterial infection. Therefore, the raised levels of TNF-α found in the airways of conventionally housed CF mice is supportive of this theory.
Chapter 6

Identification of *Defb1*, a murine homologue of *hBD-1*.
Chapter 6 Identification of Defb1, a murine homologue of hBD-1.

6.1 Introduction.

A study conducted by Goldman et al. (1997) indicated that the human beta defensin HBD-1 is involved in host defence at the pulmonary epithelial cell surface and the impaired function of HBD-1, by the heightened salt content of the CF ASF, plays a prominent role in the initiation of lung disease (see section 1.15.7).

It has been hypothesised that the loss of the action of beta defensins would deprive the host of an effective defence mechanism against a wide range of pathogens, however this has only been tested in *in vitro* experiments using primary airway epithelial cell cultures. The identification of a murine homologue would therefore aid the study of the involvement of beta defensins in the onset of CF lung disease since *in vivo* experiments could be performed. The regulation of expression could be examined in the whole animal which may have advantages over the cell culture system since it is possible that signalling mechanisms from other cell types present in the airway could be involved in defensin expression or indeed, that cell types other than epithelial cells are capable of expressing the defensin which may be under cell-specific control.

In addition, the development of a transgenic mouse model defective in the function of the murine homologue of hBD-1 would facilitate the study of defensin activity and its involvement in host defence and CF lung disease. This shall be discussed in more detail in the next chapter.

6.2 Identification of murine sequences homologous to hBD-1.

Seven near identical cDNA sequences from mouse heart, kidney, embryonic carcinoma and macrophage cDNA libraries were retrieved from an EST database following a BLAST search using the hBD-1 sequence (GenBank #AA104376,
AA065510, AA071757, AA105324, AA107538, AA107977, AA108061). The nucleotide sequence of this murine gene named Defb1 was found to have 61% identity to the sequence of hBD-1 and the peptide translation of these sequences was found to be 57% similar to HBD-1. Fig 6.1 shows the nucleotide and peptide comparisons between Defb1 and hBD-1.

The cDNA clones found in the database search were ordered from the IMAGE consortium group and DNA was prepared. Sequencing of the clones demonstrated that all contained two cDNA inserts which had co-ligated into the polylinker site, one of which was Defb1 and the other sequences were found not to be related to Defb1 and did not hybridise to the same chromosomal region of the mouse genome. Several of the clones contained the same double insert indicating that contamination of individual clones had occurred which is a common problem encountered with these cDNA resources.

6.3 Expression of Defb1.

In order to detect the expression pattern of Defb1, amplimers def1 and def2 were designed to the sequence of the GenBank cDNAs. Expression by RT-PCR was detected in kidney, lung, heart, gut and purified alveolar macrophages as seen in figure 6.2A. Restriction enzyme analysis was used to confirm the identity of the PCR product coming from Defb1 sequence. The expression of Defb1 was also examined by Northern blot hybridisation using Defb1 PCR product (as described above) as the probe. Figure 6.2D shows that hybridisation signals were only detected in the kidney samples where two bands were observed, one of the expected size of approximately 400 bp and another of 800 bp. It was initially thought that the larger fragment corresponded to pre-mRNA, however, long range PCR analysis of Defb1 revealed an intron of approximately 16 kb (see section 6.5) and therefore it is unlikely that this band represents the unspliced transcript. The 800 bp band may represent an alternatively spliced form of Defb1 although if this was the case, an additional PCR product would have been expected in the PCR analysis shown in figure 6.3.
A.

Defbl
CCACCACTATGAAAATCTCATTACCTTCTCTGGATGATATGTTTTGTTCTTCACATGGAGCC
AGTGTGGCAATGCTGGGCTTTTGACATACATCGGCTCTTGACATGCTGACAGTATGCCTG
hBD-1
CAGGGCTTTTGCCAGAGAAAAATCTCGTTGAGCCTTGTCAGTCTGCTGCTGCTGCTGCTGCTG

B.

--- signal piece --- pro-piece --- mature peptide ---

exon 1 exon 2

Defbl
MKTHYFLLMICFLFSQMEPGVGILTSLGRRTDQYKCLQHGGFCLRSSCPSNTKLQCKPDIPCK
hBD-1
MRTSYLLFLTLCLLSMASSGFLTGLHRSDYNYCQSSACPIFTKIQQTCYRGAAKCK

C.

<table>
<thead>
<tr>
<th>nucleotide identity</th>
<th>amino acid identity</th>
<th>amino acid similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>hBD-1 vs Defbl</td>
<td>61 %</td>
<td>51 %</td>
</tr>
</tbody>
</table>

Fig. 6.1 Defbl sequence and comparisons to hBD-1.
(A) Comparison of Defbl and hBD-1 nucleotide sequences. The start codon, stop codon and polyA signal are highlighted in bold. (B) Comparison of the Defbl amino acid translation to HBD-1. The positions of the signal piece, the pro-piece and the mature piece of the peptide are shown as are the positions of the two exons. The mature peptides are shown in bold and the conserved cysteine residues are shown in red. (C) Table of percentage identity and similarity between Defbl and hBD-1.
**Fig 6.2 Tissue expression of Debf1.**

(A) RT-PCR analysis of Debf1 expression from various tissues. (B) Alu1 restricted RT-PCR products. Digestion with Alu1 gave products of 67 bp and 45 bp as expected. (C) Partial nucleotide sequence of Debf1. The 5' and 3' primers are underlined and the Alu1 restriction site is shown in red. (D) Northern blot of RNA from various tissues probed with Debf1 PCR product.
Alternatively, it is possible that the additional band may be a related mouse gene.

To examine the response of Defb1 expression to an inflammatory stimulus, mice were treated intratracheally with E. coli LPS and sacrificed at various time points after treatment. RNA was isolated separately from the trachea and lower airways and expression of Defb1 detected by RT-PCR using oligos def5' and def2 which spanned the predicted intron exon boundary (see section 6.5). Figure 6.3 shows there was no Defb1 expression detected in the lower airways at any time point after LPS treatment. Defb1 was expressed in the trachea and there was no evidence of gross upregulation of Defb1 at any time point tested following administration directly to the mouse lung (see figure 6.3). These results indicate that Defb1 expression is restricted to the trachea and that it is not upregulated in response to an inflammatory stimulus.

6.4 Genomic characterisation of the Defb1 gene.

Two genomic clones of Defb1, I8 from plate 421 and I20 from plate 524, were isolated following the BAC library screen described in figure 6.4 and section 2.19. Defb1 PCR product (see section 6.3) was used as the probe in the hybridisation stage of this screen. As an alternative method of isolating genomic clones a mouse genomic lambda GET library, also derived from a 129SV mouse strain, was screened by Fiona Kilanowski which resulted in the isolation of 2 clones. These were converted into plasmids and shown to be identical; only one was used in subsequent analysis and was termed pDef1. PCR confirmed the presence of Defb1 in the clones isolated from the BAC and λ. GET libraries as seen in figure 6.5.

Restriction analysis of the genomic clones and mouse genomic DNA was performed with a range of 6 bp recognition sequence restriction enzymes. The resulting gels were blotted and set up in hybridisation reactions with full-length Defb1 cDNA as the probe which resulted in the detection of multiple hybridising bands for most of the digestions. The blots were also hybridised with oligos designed either to the 5’ or the 3’ end of the Defb1 cDNA. The autoradiograph in figure 6.6 shows that following hybridisation, in most cases, the 5’ and 3’ oligos did not hybridise to the same BAC
Fig. 6.3 *Defb1* expression following LPS treatment.
RT-PCR analysis of *Defb1* and *Hprt* expression was carried out separately on trachea and lung (lower airway) tissue samples following intratracheal instillation of LPS. A plus (+) reverse transcriptase (RT) and minus (-) RT PCR reaction is shown for each sample. Bands of the expected size were produced.
PCR for *Defb1* on BAC DNA superpools. (Each superpool consists of 8 BAC plate pools)

↓

PCR for *Defb1* on BAC plate pools 1-8 of positive BAC superpool. (Each plate pool consists of 384 individual BAC clones)

↓

BAC filter corresponding to the positive BAC plate pool ordered and probed with *Defb1*.

↓

Positive BAC clone 120 ordered and plated out. BAC DNA prepared and screened for presence of *Defb1* by PCR.

Fig.6.4 Procedure for screening BAC libraries. Results of the BAC screen are shown for clone 120. BAC clone 18 was also isolated from this BAC screen.
Fig. 6.5 PCR analysis of *Defb1* genomic clones.

*Defb1* PCR on clones I8 and I20 isolated from a BAC library and pDef1 clones isolated from a λ GET library. For a control reaction, *Defb1* PCR was also carried out on BAC clone H20 which was not positive for *Defb1* following the BAC screen.
fragment. Since the sequence of the Defb1 cDNA did not contain any of the restriction enzymes used to restrict the BACs it was concluded that this gene contained an intron. It was also estimated that this was a large intron due to the high number of 6 bp recognition sequence restriction sites it contained. Restriction digests and hybridisations were also performed on pDef1 which demonstrated that it contained the 3' fragment of Defb1, later identified as exon 2, approximately 4 kb of intronic sequence and 4 kb of sequence 3' to exon 2. Oligos derived from the vector backbone of pDef1 were used to give orientation to the positions of restriction sites. pDef1 was also used as a probe onto the BAC filters to give information on restriction sites within the intron. Information collected from these hybridisation experiments and additional restriction analysis with enzymes known to have restriction sites within the Defb1 cDNA sequence led to the construction of a restriction map of the Defb1 gene in its genomic context as shown in figure 6.7.

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**Fig. 6.7** Restriction map of Defb1.
Fig. 6.6 Hybridisation of BACs I20 and I8 with *Defb1* cDNA.

Autoradiographs of BAC filters digested with a range of enzymes and probed with the entire *Defb1* cDNA. A similar pattern of bands was produced from genomic DNA (not shown). The BAC filters were then probed with oligos (*Defb15* and *Defb13*) corresponding to either the 5' or the 3' end of *Defb1* cDNA and these results are indicated on the autoradiographs above. A scale has been included showing size in kb.

★ indicates that the sample was not digested efficiently by the restriction enzyme.
6.5 Long Range PCR of Defb1.

It was hypothesised that Defb1 may have the same intron-exon structure as hBD-1 so amplimers def5' and def 2 were used in a standard PCR reaction since they had been designed to regions in the mouse sequence that correlated to exons 1 and 2 in the human sequence. A PCR product of the expected size was produced when Defb1 cDNA was used as a template but no product was produced using genomic or BAC I8 and I20 DNA under the same conditions suggesting that the two exons may be separated by a large intervening sequence (see figures 6.8A + B). Long range PCR (described in section 2.16.2) confirmed the presence of a single intron of approximately 16 kb as seen in figure 6.8C. An oligo which corresponded to Defb1 cDNA sequence located between the amplimers designed for the long range PCR was used as an internal oligo in a hybridisation reaction with a blot created from the PCR products. The autoradiograph in figure 6.8D shows that this internal oligo hybridised to the long range PCR product. It was a concern that this hybridisation may have taken place just because of the large amounts of DNA present on the blot. However when this blot was probed with a radiolabelled sequence which was not designed to the Defb1 gene, no signal was obtained. Sequence information obtained from this PCR product showed the exact position of the exon to intron boundary, as seen in figure 6.9 and confirmed that the Defb1 gene consists of 2 exons separated by 1 intron.

(A) GTTTTCTTTTCTCCCAGATGGAGCCAGttaagctttgggaacttgttgatggctttgtttgggactgggg
(B) agttacagataaaagtttaaacatatttgttctccccggttagGTGTTGGCATTTCACACAGGTCTTGGAC

(C) exon 1 exon 2
GTTTTCTTTTCTCCCAGATGGAGCCAG|GTGTTGGCATTTCACACAGGTCTTGGAC

Fig. 6.9 Sequence from the long range Defb1 PCR.
(A) Sequence from 5' end of the Defb1 PCR product. (B) Sequence from the 3' end of the Defb1 PCR product. (C) Partial Defb1 cDNA sequence indicating the exon boundary. Exonic sequence is shown in upper case letters and intronic sequence is shown in lower case lettering.
Fig. 6.8 Exonic and Intronic Defb1 PCR.
(A) PCR for Defb1 using primers designed to exon 2 sequence on BAC I8, I20 and pDef1 DNA with Defb1 cDNA as a positive control. (B) PCR using amplimers which hybridise to either side of the predicted intron of Defb1. (C) The same PCR as B using Expand Taq and 3 different buffers as described in section 2.16.2. (D) Southern blot hybridisation of the PCR products from C with a Defb1 internal probe.
6.6 Chromosomal location of Defb1 by fluorescent in situ hybridisation (FISH).

FISH analyses using BACs I8 and I20 and pDef1 as probes were performed by Fiona Kilanowski. When the BACs were hybridised together on interphase cells it was possible to see a region of shared signal between the BACs and also areas to either side to which only one BAC hybridised (see figure 6.10A). This confirmed the restriction analysis data that these BACs contained a considerable region of overlap of sequence and each one also contained unique sequence information to either side of that sequence. Unfortunately it was not possible to assign chromosomal orientation to the BACs. Dapi stained chromosome spreads indicated that the probes hybridised to chromosome 8 and this was confirmed by the use of a mouse chromosome specific paint as seen in figure 6.10B. The hybridisation signals in figure 6.10C show that BAC I8 localised to band A4 of mouse chromosome 8 and this analyses was repeated for BAC I20 and pDef1 which were found to hybridise to the same chromosomal band.

6.7 Identification of alpha defensin sequence on BAC I20.

Southern blot analysis was performed on BACs I8 and I20 using 2 distinct radiolabelled oligos as probes which had been based on the alpha defensin consensus regions (Ouellette et al, 1994) αdefF and αdefR (see section 2.18.5 for amplimer sequences). This resulted in a positive signal only in BAC I20 as can be seen in figure 6.11A. These hybridising bands did not correspond to those resulting from the hybridisation with Defb1 cDNA indicating that there was no cross hybridisation between the alpha oligos and Defb1 sequence. It was concluded that BAC I20 extended into the region of chromosome 8 where the alpha defensin gene family is located and therefore, considering the average size of a BAC genomic insert to be 100 kb, it could be estimated that the alpha family of genes and Defb1 in the mouse are separated by less than 100 kb (see figure 6.11B). This is of interest because the human α and β defensin genes have also been shown to be located in
Fig. 6.10 Chromosomal localisation of *Defb1*.

(A) BAC clones I8 (red) and I20 (blue) used as probes in combination in 2 colour FISH on mouse ES cells. A region of overlap (yellow) can clearly be seen between the two BAC probes. (B) Hybridisation of BAC I8 probe (red) to mouse Chr 8 on mouse ES cells which have been treated with a mouse Chr 8 specific paint (green). (C) Mouse Chr 8 probed with BAC I8 and stained with Dapi to reveal chromosomal banding patterns and a diagrammatic representation of mouse Chr 8 showing a summary of the FISH mapping results. Each dot represents an individual FISH image.
Fig. 6.11 BAC I20 hybridisation to alpha defensin sequences.

(A) Southern blot of BAC I20 DNA digested with a range of restriction enzymes and hybridised with αdefF, a probe specific for a mouse alpha defensin consensus region. No hybridisation signals were detected for BAC I8. (B) Diagrammatic representation of the genomic localisation of BACs I8 and I20 in relation to mouse alpha (α) defensins and Defb1.
close proximity to each other on a conserved syntenic region of human chromosome 8 (Liu et al, 1997).

6.8 Discussion.

The data described above present strong evidence that Defb1 is a murine homologue of hBD-1. The expression pattern of Defb1 is similar to that of hBD-1 with no gross induction of expression following stimulation with LPS. Using backcross analysis Huttner et al. (1997) mapped the Defb1 locus to the proximal region of mouse chromosome 8 and this position was more precisely defined here, by FISH analysis, to the chromosome region A4. The murine alpha defensins have previously been mapped to this region (Ouellette et al, 1989) and Southern analysis of BAC I20 physically shows that the alpha and beta gene cluster lie within 100 kb of each other. It has recently been shown in humans that alpha and beta defensins map to the same region of chromosome 8 (Liu et al, 1997), a region syntenic with mouse chromosome 8. The close proximity of the alpha and beta gene families in man and mouse and the similarity in beta genes between species compared to the low level of similarity between alpha and beta genes within a species strongly suggests that gene duplication of a common ancestral defensin gene occurred before man/mouse species divergence. Functional studies have been conducted to assess the antimicrobial activity of a synthetic peptide corresponding to the mature region of the mouse peptide (Morrison et al, 1998) referred to here as Defb1. It was shown that Defb1 was active against both a lab strain and a CF clinical strain of P. aeruginosa and this activity was shown to be extremely salt sensitive dropping from 85 % killing to 35 % as the NaCl concentration rose from 0 mM to 125 mM. Defb1 was found to be less effective than HBD-1 at killing P. aeruginosa. This may reflect a functional difference between the mouse and human peptides, however further studies are required to rule out the possibility that this occurred simply through differences in the success of the peptide synthesis. Similar to HBD-1, Defb1 had no antimicrobial effect against B. cepacia. This suggests that some other component of pulmonary defence must be impaired in CF to allow the survival and colonisation of B. cepacia. An additional study by Bals
et al. (1998a), using lysates from cells transfected with Defb1 cDNA as their source of Defb1 peptide, produced similar results. These peptides were found to have antimicrobial activity against *P. aeruginosa* in addition to *S. aureus* and *E. coli* and the activity against *E. coli* was shown to be salt sensitive.

A key feature in the proposal that airway beta defensins are involved in CF lung disease is the assumption that CF ASF has an abnormally high salt (NaCl) content compared to non-CF airways. However, the results produced from studies estimating the ion concentration of ASF in CF patients and controls have been widely disparate. Several groups have found the ASF to have a heightened salt content in CF patients, (Gilljam *et al*, 1989, Joris *et al*, 1993, Zabner *et al*, 1998) whilst others have found there to be no difference (Knowles *et al*, 1997). A major factor contributing to this variability in results is the technical difficulty of measuring the ion content of such a small volume of liquid, estimated to be at a depth of only 20 µm on the epithelial cell surface (Zabner *et al*, 1998). Several different techniques have been employed to collect the ASF including the use of filter paper, lavage, and drainage from excised tracheas. Various methods have also been used to measure the ion content of the ASF including capillary electrophoresis, X-ray microanalysis and the latest publication uses a radiotracer to measure radiolabeled sodium and chloride in primary cultures of human epithelial cells (Zabner *et al*, 1998). Using this technique it was found that the sodium concentration was 50 mM and 100 mM in non-CF and CF ASF respectively and the chloride concentration was 37 mM and 85 mM in non-CF and CF ASF respectively. The raised salt content found in the CF ASF falls within a concentration range which has been shown to reduce the activity of HBD-1 (Goldman *et al*, 1997), Morrison *et al*, 1998). In order to understand the effect of loss of CFTR activity on salt and fluid absorption across the airway epithelium, these processes must be first understood in the normal situation. Two theories on this subject have emerged, which are depicted in figure 6.12. Theory one proposes that the ASF is normally hypotonic (lower salt than plasma) and the loss of CFTR function leads to the ASF having a raised salt content. Chloride ions follow the actively transported sodium ions through the cell to maintain electroneutrality and are transported through the apical membrane via CFTR. It has been found that when the CFTR channel is blocked that transport of
chloride ions across the apical membranes is greatly reduced (Yamaya et al, 1992) which is in support of CFTR being the major route of chloride ions into the cell. Transcellular movement of chloride as opposed to chloride flowing between the cells, also requires the existence of chloride ion channels on the basolateral membrane of the epithelial cells. Widdecombe et al. (1997) have identified inwardly rectified cAMP activated channels that may mediate the net outward flux of chloride across the basolateral membrane. This absorption would lead to a decreased concentration of salt in the ASF compared to plasma, which would also create an osmotic pressure. The epithelial cells of the sweat duct are impermeable to water so there is no water absorption from the lumen and therefore its salt content is decreased with no alteration to its fluid content. However, airway epithelial cells are permeable to water (Folkesson et al, 1996) and therefore it would be expected that any difference in salt concentration gradients would be corrected by osmosis. It has been suggested that the osmotic pressure, is overcome by capillary pressure (Widdicombe et al, 1995) which could be created by cilia and microvilli generating a large surface tension when the fluid level of the ASF is decreased by water from the ASF being absorbed. Thus, chloride and sodium ion transport can occur but fluid transport is overcome and a hypotonic ASF is produced. When CFTR is absent, chloride ions cannot cross the apical membrane of the cell and sodium ions are also retained in the ASF since there is no permeant counterion for its movement. This leads to a heightened salt concentration of the ASF in the CF airways.

The alternative, theory 2, proposes that normal ASF is isotonic (has the same salt concentration as plasma) since chloride ions can also diffuse through tight junctions between the cells and water rapidly follows creating an osmotic equilibrium. When CFTR is absent, chloride ions can still be absorbed and in fact it is predicted that sodium and chloride ion movement would be increased since it has been shown that the sodium channel activity is increased in CF cells (Welsh et al, 1995). This would result in a decreased volume of isotonic ASF in the CF airways.
theory 1

normal airway epithelial cell

CF airway epithelial cell

osmotic disequilibrium

No movement of chloride or sodium ions across the apical membrane
ASF = raised NaCl concentration

theory 2

increased chloride transport via tight junctions

increased Na\(^+\) absorption
increased fluid absorption from ASF to create osmotic equilibrium
ASF = isotonic

chloride ions diffuse freely through tight junctions

osmotic equilibrium

ASF = isotonic

Fig. 6.12 Theories of ion transport across the apical epithelium.
These theories have been based on results which remain inconclusive and therefore it is not clear whether one or the other of these theories is correct. It is hoped that future experiments will lead to accurate measurements of ASF being made in vivo which should help to clarify the role of the ionic composition of ASF in CF lung disease.

Preliminary experiments have indicated that normal mouse ASF appears to be hypotonic (Cowley et al, 1997a). Studies to examine the ionic composition of the CF mouse ASF compared to that of wild-type littermates are presently ongoing. Preliminary results indicate that ion concentration is significantly raised in the ASF of Cfr<sup>tm1Hgs</sup> mice compared to wild-type littermates, with chloride and sodium ion concentrations of 37 mM and 52 mM in the CF mice and 8.5 mM and 12 mM in the wild-type mice respectively (Jean-Marie Zahm and Edith Puchelle, personal communication). Interestingly, these values found for the Cfr<sup>tm1Hgs</sup> mice are of a salt concentration which has been found to decrease the antimicrobial activity of Defb1 synthetic peptides (Morrison et al, 1998).

The similarity of this antimicrobial profile of Defb1 to HBD-1 suggests that Defb1 is part of an equivalent pulmonary defence system in the mouse and supports the use of this peptide in the mouse as a model for further functional studies.
Chapter 7

Creation of a *Defb1* knockout mouse
Chapter 7 Creation of a \textit{Defb1} knockout mouse.

7.1 Introduction.

The results described in the previous chapter led to the conclusion that functional conservation exists between \textit{Defb1} and HBD-1 and therefore it would be valuable to create a mouse model defective in \textit{Defb1} function. A \textit{Defb1} knockout mouse would give information as to the role of this defensin in the innate pulmonary defence system and if there was a distinct inflammatory phenotype found it would be of interest to make comparisons between the \textit{Defb1} knockout inflammatory profile and that of the human CF lung. In addition, studies could be performed comparing the phenotype of the CF mice and the \textit{Defb1} knockout mice and the creation of compound heterozygote mice for the \textit{Cftr} and \textit{Defb1} mutant allele could help to determine the similarities or dissimilarities of the two systems. It is hoped this will give an indication as to the role this defensin plays in the development of lung disease in the CF mice.

7.2 Construction of the \textit{Defb1} genomic targeting vector.

Figure 7.1 summarises the strategy for constructing this targeting vector. Restriction analysis and hybridisation experiments conducted previously had shown that exon 2 of \textit{Defb1} was completely contained within a 1.3 kb Eco RV fragment (see figure 6.7). pDefl was found to contain approximately 4 kb of DNA to either side of the \textit{Eco} RV fragment which would make it a suitable vector for homologous recombination. The strategy used for constructing a \textit{Defb1} targeting vector is summarised in figure 7.1. Briefly, pDefl was treated with the restriction enzyme \textit{Eco} RV and the resultant 12.2 kb fragment purified and treated with calf intestinal phosphatase to prevent recircularisation during the ligation reaction. The pMC1NeoPolyA plasmid contains the neomycin resistance gene with a polyA tail under the control of the TK promoter and this construct was excised from the vector backbone by a \textit{Xho I Sal I} double digest. The 1.13 kb fragment containing the neomycin gene and its control elements
Fig. 7.1 Construction of the *Defh1* targeting vector.
Fig. 7.2 Analysis of *neo* targeting clones.
Nine ampicillin resistant clones were digested with *Bam* HI and the products hybridised with an oligo designed to a region of the neo gene 5' to the *Bam* HI restriction site. This allowed the orientation of the inserted neo gene to be ascertained and also indicated if multiple copies of the gene had inserted into the pDef1 plasmid.
was purified from an agarose gel and blunt ended to allow ligation into the Eco RV site of the pDefl purified fragment. A ligation reaction was set up and the products electroporated into E. coli. Ampicillin resistant clones were digested with Bam HI since the neo gene fragment contains a BamHI site at its 3’ end. A hybridisation reaction was carried out on the Bam HI restricted products with an oligo designed from a region of the neomycin gene which was 5’ to its BamHI site. The results of the digest and hybridisation are shown in figure 7.2.

Clone 3 was chosen for the targeting experiment since it contained a single copy of the neo gene in the same orientation as Defbl. A large scale preparation of high quality DNA was made and the entire quantity was digested with Asp 718 to linearise the construct. 80 µg of the linearised DNA was electroporated into 10^7 ES cells of the line E14IV derived from the 129 strain of mouse and selection of G418 resistant clones (indicating the presence of neomycin) was performed by Fiona Kilanowski.

7.3 Preparation of the external probe.

For identification of a correct genomic targeting event it is appropriate to use a probe which is derived from a sequence of DNA that is external to the region used in the targeting construct to ensure the replacement sequence has inserted in the correct genomic location. The procedure used to isolate a suitable probe is summarised in figure 7.3. Briefly, the restriction map of Defbl showed a 6 kb Bam HI fragment which contained DNA 5’ to the region used for homology in the targeting construct. This fragment was cloned from BAC 18 into pBluescript II and analysed by restriction enzyme digestion followed by hybridisation using pDefl as a probe. pDefl contains sequence from pBluescript so hybridisation of the probe to the pBluescript II sequence present in the clone also occurred but this could be easily distinguished from the Bam HI inserted sequence by its size. The autoradiograph in figure 7.3 shows a 1.3 kb Bam HI Pst I fragment which did not hybridise to the pDefl probe indicating that it was sequence external to the targeted region. This probe, named D1, was tested on genomic DNA which had been digested with Bam HI, Eco RV and Bsm I which resulted in hybridisation signals of the expected size as seen in figure
BAC Bam HI digest and isolate 6 kb fragment which hybridises to pDef1

digest 6 kb fragment and identify fragments which do not hybridise to pDef1

isolate 1.3 kb Bam HI/Pst I fragment and clone into pBluescript II cloned fragment named D1

test out D1 on mouse genomic DNA (1-3) digested with Bam HI (H), Eco RV (E) and Bsm I (B).

bands of the expected size produced.

Fig. 7.3 Isolation of D1: a probe for the identification of correctly targeted alleles.
7.3. Two Eco RV restriction sites are lost in the targeting construct therefore Eco RV could be used to identify correctly targeted clones. Hybridisation with the D1 probe would result in a 21 kb band if the genomic region had been successfully targeted and a 15 kb band if it had integrated into an alternative site as shown in figure 7.4.

7.4 Identification of Defb1 targeted clones.

Two hundred and ten G418 resistant ES cell clones were individually expanded to confluence on 2 wells of a 96 well plate, cells from one well were cryopreserved at -70°C and cells from the other well were expanded to confluence on a 24 well plate and DNA prepared. The DNA samples were digested with Eco RV, the resultant gels containing the digested products blotted and Southern blot analysis performed with the D1 probe. Targeting had been successful in 9 of the 210 clones, seven of which are shown in figure 7.4B. Two of these targeted ES cell lines with normal karyotypes, 3.15 and 6.2, were chosen for blastocyst injections.

7.5 Production of chimeras and germline transmission of the targeted Defb1 allele.

Injections of the targeted ES cell lines into C57Bl/6N blastocysts was performed as a service by Jan Ure at the Centre for Genome Research, Edinburgh. Injections with cell line 3.15 resulted in 8 male chimeras whereas 6.2 resulted in 1 male chimera. The single 6.2 chimera and 3 of the 3.15 chimeras were mated with wild-type C57Bl/6N females. The agouti coat colour of all the pups, shown in figure 7.5 and Southern analysis of their DNA (see figure 7.6A), indicated that germline transmission was 100% successful from both the 6.2 chimera and 2 of the 3.15 chimeras. Figure 7.6B shows the Southern analysis of the offspring from matings with the heterozygote mice indicated in figure 7.6A. Heterozygote and homozygote mice for the targeted Defb1 allele and wild-type mice were generated from these matings at a ratio of 5:2:4 respectively. The homozygous mice, currently housed in an isolator, appeared normal apart from one mouse being of a smaller size than
Fig. 7.4 ES cells targeted at the *Defb1* locus.
(A) Diagrammatic representation of wild-type and targeted *Defb1* alleles. (B) Southern blot hybridisation of DNA prepared from G418 resistant ES cell clones digested with *Eco RV* and using D1 as a probe. Clone 2.18 also had a 15 kb and 21 kb band present which could be seen after a longer exposure of the autoradiograph.
Defb1 targeted ES cells from lines 3.15 and 6.2 injected into C57Bl/6N blastocysts

3.15 = 8 chimeras
6.2 = 1 chimera

chimeras bred onto C57Bl/6N females

100% germline transmission from both cell lines

Fig. 7.5 Germline transmission of the targeted cell lines.
Fig. 7.6 Southern analysis of mouse tail-tip DNA.

(A) DNA analysis of offspring from male chimera (produced from 3.15 targeted ES cell line) and wild-type C57Bl/6N female. Mice 1, 6, 7 and 8 were heterozygous for the targeted Defb1 allele, 1 was male and 6, 7 and 8 were female. (B) DNA analysis of offspring from matings of heterozygous mice 1 and 7 (1, 5, 6), 1 and 8 (2, 3, 4) and 1 and 6 (7, 8, 9, 10, 11). Mice 6 and 7 (both female) were found to be homozygous for the Defb1 targeted allele.
average. To date, twenty seven heterozygote mice have been born and it is anticipated that, in the near future, multiple homozygote mice will be born from heterozygote matings.

7.6 Discussion.

A gene targeting strategy of replacing a segment of the *Defb1* gene with the neomycin gene was chosen for this experiment since the creation of the targeting vector was a relatively simple procedure which could be performed quickly and efficiently. It may have been more informative to construct a targeting vector which incorporated a reporter gene, such as *LacZ*, whose expression, under the control of the endogenous *Defb1* promoter, could be detected in the mouse knockout. This type of system which would give information as to the cell types which express *Defb1* may be helpful in correlating regions of loss of *Defb1* expression to any observed phenotype. This type of strategy would be dependent on the ES cells used for the targeting procedure expressing *Defb1* to allow for selection of correctly targeted clones but experiments conducted to assess this have not been conclusive. It can occasionally be an advantage to use a conditional targeting strategy to avoid the problem of the loss of the targeted gene causing embryonic lethality which, although informative about the developmental role of the gene, would not be helpful in the study of pulmonary inflammatory phenotypes. Human enteric defensins are present in the small intestine during gestation but at very low levels compared to the full-term newborns or adults and there is no evidence of their involvement in developmental processes (Mallow et al, 1996). The sheep beta defensins SBD-1 and SBD-2 (Huttner et al, 1998) are also expressed in the intestine during gastrulation (Huttner et al, 1998) but again, there is no evidence presently available which links this expression to any developmental process. In contrast, there has been no prenatal expression of the bovine beta defensins, LAP and TAP detected (Diamond et al, 1993, Schonwetter et al, 1995). This may suggest that the mouse beta defensins will not be expressed to any significant level prenatally and are therefore unlikely to play a role in development, however, no data is currently available on prenatal beta defensin

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expression in mouse to answer this definitely.

It is possible that the Defb1 homozygote mutants may develop a severe inflammatory phenotype when not housed in an isolator or following treatment with pathogens since the studies on the synthetic peptide of Defb1 indicated that if the ASF of the CF mouse has a heightened salt content then Defb1 activity is reduced but not completely absent. If this is a relevant factor to the development of lung disease in the CF mice after repeated exposure to pathogens, then the complete absence of Defb1 may render the mice highly susceptible to bacterial infections. It is also possible that the knockout mice will have abnormalities in other organs where Defb1 is normally expressed. Defb1 was found to be most highly expressed in the kidneys and therefore it is conceivable that its loss may lead to urinary tract or kidney infections. The loss of Defb1 in the heart and liver may also lead to an abnormal phenotype in these organs. It has previously been mentioned that defensins are thought to have other functions in addition to their bactericidal activity (see section 1.15.6) and if Defb1 has a role in cytokine regulation or complement activation this may compound the effects of a reduced ability of the host to rid bacteria from the lungs. An excessive inflammatory response towards pathogens in these mice may also indicate the existence of synergistic relationships between Defb1 and other antimicrobial factors and these possible relationships shall be discussed in more detail in section 8.8.

It will be interesting to observe if Defb1 mutant mice have an increased susceptibility to a wide or narrow range of pathogens and if this includes the CF-specific bacteria. It is possible that Defb1 has a different target range of pathogens than HBD-1 but there may be difficulties in making comparisons between the mutant Defb1 mouse and in vitro studies using HBD-1 due to the limitations experienced using in vitro experimentation mentioned previously in section 6.8. However if differences are noted between Defb1 and HBD-1 in the in vitro, synthetic peptide experiments and these are supported by the phenotype of the Defb1 mutant mice then this may reveal some information in regard of how the amino acid content of the defensin may influence the range of pathogens against which it is active.

It may be the case that no defect in the host pulmonary defence mechanism will be
found in the *Defb1* mutant mice since it is possible that the activity of Defb1 in the lung is redundant. The discovery of a second mouse beta defensin whose expression can be induced in the airways, as described in the next chapter, may support this theory. However, it could also be the case that Defb1 and the novel murine defensin have a synergistic relationship and individually are very pathogen specific. It will also be of interest to examine what effect, if any, the loss of Defb1 activity has on the expression or activity of other antimicrobial molecules in the mouse airways.
Chapter 8

Identification of *Defb2*: a novel mouse beta defensin gene

8.1 Introduction.

A second beta defensin, hBD-2, expressed in the human airways has been identified (Harder et al, 1997a). This defensin has also been shown to be salt sensitive (Bals et al, 1998b) and interestingly, like the bovine airway beta defensins, is upregulated in response to LPS (Harder et al, 1997a). The differences between the human and mouse beta defensins found to date, suggests that expansion of the human beta defensins may have taken place after man/mouse species divergence and therefore it cannot be assumed that there will also be additional airway beta defensins present in the mouse. However, the discovery that more than one airway beta defensin is present in cows (Diamond et al, 1991, Schonwetter et al, 1995), sheep (Huttner et al, 1998) and rat (Jia et al, 1998) strongly suggests the existence of multiple airway beta defensins in the mouse.

Chapter 6 described the isolation of 2 mouse BACs which were demonstrated to cover a region of genomic DNA containing both alpha and beta defensins (see section 6.7). If additional mouse beta defensins were present in the mouse it was thought that they would lie within the same chromosomal region and this is supported by the close chromosomal location of hBD-1 and hBD-2 (Harder et al, 1997b). Therefore this BAC resource was used for the search of additional mouse beta defensins.

8.2 Reduced stringency hybridisation of BACs with Defb1.

Southern blot analysis was performed on the blots of the digested products of BACs 18 and 120 using Defb1 cDNA as a probe at a reduced hybridisation temperature of 55°C. This resulted in very strong hybridisation signals from the bands already identified as representing Defb1 in addition to the presence of weaker hybridising
bands which were not present following Defb1 hybridisations at 68°C as can be seen in figure 8.1. It was interesting to note that these novel bands were only present on BAC 18, which had previously been shown to contain no alpha defensin sequences. The novel bands present in BAC 18 corresponded to restriction fragments which were not present following restriction of 120 and therefore it is likely that the novel bands represented sequence which was only present on BAC 18.

8.3 Sequencing and analysis of Defb1 hybridising DNA.

A 5.5 kb Bam HI fragment from BAC 18 which represented a novel band following the low stringency hybridisation was isolated and cloned into pBluescript II. A series of restriction enzyme digestions was performed on the cloned fragment and the resulting gels blotted and hybridised at low stringency with Defb1 cDNA in an attempt to reduce the size of the hybridising fragment. The Defb1 cDNA used as a probe had been cloned into pBluescript II and since the novel Bam HI fragment had also been cloned into pBluescript II the probe hybridised to the pBluescript II derived sequences in addition to sequence of interest. However, the size of the vector backbone was known and could be easily distinguished from the cloned insert. A Bam HI/Bgl II fragment of approximately 2 kb was identified which appeared to contain the entire hybridising region, as indicated in figure 8.2, and was subsequently cloned into pBluescript II and sequenced, the result of which is shown in figure 8.3A. The top hits following a BLAST search of the new sequence were the seven mouse sequences corresponding to Defb1 and hBD-1 was also identified as having similarity to this sequence. At the amino acid level Defb1 and part of the translated 2 kb sequence had 38 % identity and 58 % similarity and importantly the characteristic 6 cysteine residues were present in the novel sequence in the correct positions (see figure 8.3B). Therefore it was concluded that this piece of genomic DNA isolated from BAC 18 did contain sequence which was related to Defb1. It was only the exon 2 portion of that matched up to the 2 kb novel sequence and therefore it was suspected that the rest of the 2 kb sequence corresponded to an intron, thus
Fig. 8.1 Low and high stringency *Defb1* hybridisations on BAC 18. BAC 18 DNA digested with various restriction enzymes and probed with *Defb1* cDNA at 55°C (low stringency) or 68°C (high stringency). Novel bands present after the low stringency hybridisation are indicated by a yellow box. BAC 120 DNA digests did not produce these novel bands following low stringency *Defb1* hybridisation.
<table>
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<th>Bam HI/Asp 718</th>
<th>Bam HI/Bgl II</th>
<th>Bam HI/Eco RI</th>
<th>Bam HI/Eco RV</th>
<th>Bam HI/Hind III</th>
<th>Bam HI/Pst I</th>
<th>Bam HI/Sma I</th>
<th>Bam HI/Stu I</th>
<th>Bam HI/Xho I</th>
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</tr>
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**Fig. 8.2 Digestion and Southern blot hybridisation of the 5.5 kb fragment.**

The 5.5 kb *Bam* HI BAC I8 fragment was digested with a range of restriction enzymes and then probed with *Defb1* at 60°C to isolate smaller fragments containing the *Defb1* related sequence. A 2 kb band from a *Bam* HI/Bgl II digest can be seen to hybridise to the *Defb1* probe. Cross hybridisation occurred with the vector backbone but this could be distinguished from insert sequence by size.
A.

Sequence from BAC 18
cttacttcaa gcoccttcac tctatgctat tggatgttttt tgcttctgtg ctgatgccat actgtttctg
tatacttcaac tctcgctctgt agttagtataat ggaaaggata ccttctgtgac cctctattctg
tctacatc ggagcttctt gcctggcctct attggagaag gcttcatctt aacatgcagct
tgatctgttga gcgtgcttttgt gattatgctt tattattgtaact
tagaactttg ccttctgtgt tgcagtttttt cccttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
explaining why there were no matches found to the exon 1 sequence of Defb1. The new sequence was entered into an exon/intron boundary predictor program, Geneparser, and an intron/exon boundary site was identified in a position corresponding to the intron/exon boundary of Defb1 (see figure 8.7A). Amplimers DR5' (exon 2) and DR3' (exon2) were designed to the new sequence and RT-PCR performed on a variety of tissues. Although the RNA isolated from the tissues had been DNase treated, PCR products were also present in the minus reverse transcriptase (-RT) samples. This indicated DNA contamination was present in the RNA samples and confirmed that no intron was present within this sequence. Reducing the number of cycles of amplification reduced the “background” DNA derived bands and at 30 cycles a band could only be seen in the +RT sample from mouse kidney as shown in figure 8.4. This indicated that the gene was expressed, suggesting it was not a pseudogene, and that it was most highly abundant in the kidney. It was anticipated that a better RT-PCR assay, such as one that crossed an intron and therefore excluded the amplification of DNA derived products, would lead to a better characterisation of this genes expression profile.

8.4 Rapid amplification of cDNA ends (RACE).

RACE was used in an attempt to isolate the 5' end of this potential novel defensin sequence. An overview of this technique is described in section 2.20 and shown in figure 8.5. The initial attempt resulted in the isolation of an 800 bp fragment of DNA. This product was sequenced and it was found that these primers had amplified a different cDNA, monocyte chemotactic protein 3 precursor, that had 2 regions of sequence which showed high similarity to primer 4 which had been designed to be random. The sequence also hybridised to the exon specific oligo and regions of homology to this oligo could also be seen in this PCR product. New amplimers were designed to alternative areas of the known sequence and another random sequence was used to make primer 4. RACE was repeated using the new primers and several amplified products, all of approximately 300 bp were shown to hybridise to the exon specific probe and not the intron specific probe, as shown in
Fig. 8.4 Expression of *Defb2* (exonic RT-PCR analysis).

RT-PCR analysis for *Defb2* expression was performed on various tissues using amplimers designed towards the partial sequence isolated from BAC 18. To minimise the amplification of genomic DNA the PCR cycle number was reduced to 30 and a band of the expected size is visible only in the kidney sample. BAC 18 DNA was used as a +ve control for the *Defb2* PCR. A minus (-) reverse transcriptase control is shown for each sample and RT-PCR was also carried out for *Hprt* to control for the amount of RNA used for each *Defb2* reaction.
Fig. 8.5 Rapid amplification of cDNA ends (RACE).
A diagrammatic representation of the method used for RACE. The primer sequences are detailed in section 2.16.4.
figure 8.6A + B. These amplified products were sequenced and it was found that they were of the same sequence which matched the exon 2 sequence already found 5' to primer 5a. The sequence then began to diverge from that of the 2 kb fragment at the predicted exon/intron boundary (see figure 8.6C). The cDNA sequence of this novel gene named Defb2 is shown in figure 8.7A and comparisons of this gene at the nucleotide and amino acid level are shown in figure 8.7A-C. Defb2 was shown to have 60 % sequence identity to Defb1 and the predicted translation was found to be 50 % similar to Defb1. A summary table of the identities and similarities of mammalian beta defensin peptides is shown in table 8.1.

Primers DR5' and DR3' were designed to the 5' and 3' regions of the cDNA sequence and RT-PCR performed on kidney RNA. Surprisingly multiple bands were produced from this reaction and when the most prominent band was sequenced it had a 100 % match to a region of the rat mRNA for inward rectifier 9, an inward rectifier potassium channel which has no relationship to beta defensins. It was found that both the 5' and the 3' amplimers had homology to the 5' region of this gene and this explained why it had been amplified. New amplimers, DRS'new and DR3'new were designed to a different region of the Defb2 sequence and the RT-PCR repeated. On this attempt a single band was produced which, upon sequencing, was found to be Defb2.

8.5 Expression profile of Defb2.

Various tissues were analysed for the expression of Defb2 by RT-PCR. Expression was detectable only in the kidney, uterus and, at a much lower level, the heart. An internal oligo R5 was used as a probe in a hybridisation reaction to confirm the identity of the PCR product as shown in figure 8.8A. To examine if this gene's expression was upregulated in response to an inflammatory stimulus mice were treated intratracheally with LPS and the trachea and lower airways collected at various timepoints after instillation. RNA was isolated from the tissues and RT-PCR performed to examine possible changes in expression levels.
Fig. 8.6 *Defb2* sequence from RACE.

(A) RACE clone digests. Bands of approximately 300 bp were seen for clones 2, 4, 5 and 6. (B) Southern blot analysis of RACE clone digests probed with a *Defb2* internal oligo. Strong hybridisation signals are present for clones 4, 5 and 6. (C) Sequence of clone 5. The RACE amplimers are highlighted in red and *Defb2* exon 2 sequence is indicated in blue.
A.

**Defb2**

ctctctgtggagcttgcccttttcttaaacagccatgagctctctgctctctgctgctgatatgctgcctccttttctcatata

**Defb1**

exon 1 | exon 2

cactctgcgtctctggaccctggctgccaccactatgaaaactcattactttctcctggtgatgatatgttttcttttctcccaga

tggagcttcgttggcaactccaaagcgttttccagagaaggtaatcttaccggatatgagaataatctagttttcaggtcatg

ggaagcacatgagaagctcaatgccttcaacatggaggattctgtctccagccatttgtcctccttctgccaggcgtcctgggagctgtttcccagagaagaacccctgttgcaagtacatgaaatgattagaa
ggaagcacatgagaagctcaatgccttcaacatggaggattctgtctccagccatttgtcctccttctgccaggcgtcctgggagctgtttcccagagaagaacccctgttgcaagtacatgaaatgattagaa

gccacattgtgctctttctggccagctctggttcccaagagaaggaaacccctggtaaggctgaagatcagac

B.

**Defb2**

1 MRTLCSLILLICCLFSYTTPAVGLKSLIGAYEELDHCHTNGGVFRAICTPSARRPGCPPEKKNPSCKYMK 71

1 MKTHYFLLVMICFLFSQMEFVGILTSGLRRTDQYKCLQHGPCLRSSCPNKLQGTCFDPKPCNSCXK 69

**Defb1**

C.

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<td>41 %</td>
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**Fig 8.7 Defb1 and Defb2 comparisons.**

(A) Comparison of **Defb2** and **Defb1** nucleotide sequence. The start codons, stop codons and polyA signal are highlighted in bold. The boundary between exon 1 and 2 for both sequences is also shown. (B) Comparison of the predicted amino acid sequence of Defb2 and Defb1. Conserved cysteine residues are highlighted in red. (C) Table of percentage identity and similarity between **Defb1** and **Defb2**.
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Table 8.1 Beta defensin identities and similarities.
Amino acid similarities are shown in blue whilst identities are shown in black. The symbol * indicates that the peptides were not compared over their entire length and therefore a value higher than the actual percentage of similarity/identity has been produced.
Although *Defb2* expression could not be detected in the lungs or trachea under normal conditions, expression was detected in the trachea at 24 and 48 hr after LPS administration. However, the expression was very low and could barely be detected following agarose gel electrophoresis of a 5 μl sample of the PCR reaction. When the PCR products were blotted and hybridised with an oligo internal to the sequence of *Defb2* used for the RT-PCR amplimers, the amplified products were clearly present as can be seen in figure 8.8B.

**8.6 Long range PCR of *Defb2*.**

The sequence of the 2 kb cloned fragment containing exon 2 of *Defb2*, indicated there was at least 1.5 kb of an intron between exon 1 and exon 2. No products were produced from genomic or BAC I8 DNA following a standard PCR reaction using amplimers that spanned the intron, suggesting that *Defb2*, similar to *Defb1*, contained a large intron. Long range PCR on BAC I8 and total genomic DNA produced 2 bands of approximately 3 kb and 4 kb as seen on figure 8.9. The PCR products were blotted and a hybridisation reaction performed with an oligo which corresponded to sequence from the *Defb2* cDNA internal to the PCR amplimers. The internal oligo only hybridised to the 4 kb band (see figure 8.9) indicating that this PCR product represented the *Defb2* intron and that exon 1 and exon 2 were separated by 4 kb. The production of an additional band which did not hybridise to the internal oligo may be the result of the amplimers hybridising non-specifically to an unrelated sequence of genomic DNA. However, it is also possible that the PCR product represents another beta defensin that has sequence similarity to *Defb2* at the sites to which the PCR amplimers were designed but not to the sequence to which the hybridisation oligo was designed. Further investigations are required to determine if this is the case.
Fig. 8.8 Expression of *Defb2*.
(A) RT-PCR of *Hprt* and *Defb2* from various mouse tissue RNA. (B) RT-PCR of *Hprt* and *Defb2* from tracheal RNA following LPS instillation. The hours shown represent the time after LPS instillation when the mice were sacrificed. A plus (+) and minus (-) reverse transcriptase reaction is shown for each sample. For both (A) and (B) the *Defb2* RT-PCR samples have been hybridised with a *Defb2* internal oligo.
**Fig. 8.9 Long range Defb2 PCR.**

PCR across the Defb2 intron using BAC 18 or mouse total genomic DNA. PCR products of approximately 3 and 4 kb can be seen in lanes 1 and 2 of the BAC 18 samples and lane 1 of the genomic DNA samples. Only the 4 kb band hybridises to an internal Defb2 oligo probe. 1, 2 and 3 refers to the buffers used in the PCR reaction as described in section 2.16.2.
8.7 Discussion.

The characteristic amino acid sequence, chromosomal localisation and expression data of *Defb2* indicate it to be a member of the murine beta defensin family. To date, this is only the second murine beta defensin identified that is expressed in the airways. The fact that its expression, unlike *Defb1*, was not found to be constitutive in the lung but that it is induced following an inflammatory stimuli suggests that this defensin may be an important component of the host response to pathogens. Functional studies have not been performed on this novel defensin but, due to its predicted structural similarities to the other defensins with conserved cysteine domains capable of forming disulphide bridges, it is anticipated that it will be antimicrobial. It is hoped that future experiments studying the function of this peptide may also reveal the potency of its antimicrobial action and whether it is salt sensitive. The mechanisms which lead to the upregulation of *Defb2* in response to LPS are unknown. Studies on the bovine airway beta defensin TAP, have revealed the presence of NF-κB and NF-IL6 binding sites in the 5' untranslated region (UTR) of the gene (Diamond *et al*, 1993) and the relevance of this has already been discussed in section 1.15.7. Therefore, it will be of interest to investigate whether there are any similar regulatory control elements present in the 5'UTR of *Defb2*. CD14 is known to be released from mouse bronchial epithelial cells (Fears *et al*, 1995) and therefore it is also possible that this LPS binding protein is involved in the induction of *Defb2*. RT-PCR analysis indicated that after LPS stimulation the gene was expressed in the trachea but at a low level and it is not clear whether this is a reflection of the genes' expression profile or a problem with the PCR reaction perhaps caused by substandard amplimers or inappropriate cycling conditions. However, a different set of amplimers has been used and a very similar result was produced. Estimates of protein level, using an antibody made against Defb2 may be more informative.

Figure 8.8 shows that *Defb2* is most similar to *Defb1* at the amino acid level and although it may be functionally similar to hBD-2 in regard of its ability to be induced by LPS, it is unlikely that this defensin is the orthologue of hBD-2. As mentioned previously, the beta defensin families may have expanded independently in the
separate species following species divergence and therefore there may not be a murine orthologue of \textit{hBD-2}. However, the nucleotide sequence identity of 60\% between \textit{Defb1} and \textit{Defb2} suggests that these two genes diverged from each other a long time ago, possibly before mammalian radiation and therefore it is not clear whether this branch of the defensin family expanded after mouse man species divergence as appears to be the case for the alpha defensins. It is possible that accelerated evolution by positive Darwinian selection occurred (see section 1.15.2) and therefore the two mouse beta defensin genes discovered to date have diverged from each other more than would have been expected.

Two rat beta defensins have been identified, named \textit{RBD-1} and \textit{RBD-2}, and \textit{RBD-2} shows much greater similarity to \textit{hBD-2} than \textit{Defb2} (Jia et al, 1998) (P. McCray, personal communication) as do both the bovine and sheep defensins. This suggests that there may be a murine peptide more closely related to \textit{hBD-2} than \textit{Defb2} and it may also suggest that, unlike the alpha defensin branch, there is some degree of conservation of beta defensins between the separate species. The identification of additional members of the beta defensin family in mouse and man may help to clarify the evolution of these peptides. It will also be of interest to discover if there exists a human beta defensin which is more similar to \textit{Defb2} and work is currently underway to explore this possibility.

Several studies have been conducted on assessing the amount of HBD-1 and HBD-2 in the airways of CF patients and controls. Ganz (1998) reported that the levels of HBD-1 and HBD-2 fell below the concentration found to be required for a detectable microbicidal effect whereas another antimicrobial agent, lysozyme, was present in much greater quantities. This may suggest that HBD-1 and HBD-2 are not the principal antimicrobial agents in the ASF, and that their loss in CF may not be relevant to the decreased antimicrobial activity observed. However, several assumptions have been made in this study. The defensins were detected from BALF and, as has previously been mentioned with regard to cytokine studies, the high dilution factor of the ASF in BALF decreases the sensitivity of the detection assays. Also the dilution factor of the lavage fluid was only an estimation and therefore the calculations of the actual amounts of defensins present in the ASF may have been
highly inaccurate. In addition, the antimicrobial activity assay of HBD-2 was conducted *in vitro* using peptides produced from baculovirus infected insect cells and the activity of these peptides may not accurately reflect the actions of the peptides *in vivo*. A study examining defensin levels in lavage from non-CF patients found that the neutrophil alpha defensins HD1-3 were the most prominent antimicrobial factors in the BALF samples (Schnapp *et al.*, 1998). This result is quite surprising since patients in these studies did not suffer from persistent lung infection or have obvious signs of inflammation and therefore large numbers of neutrophils capable of releasing HD1-3 would not be thought to be present, however no assessment of neutrophil numbers in the lung was made. No HBD-1 or HBD-2 were found in any of the BALF samples assayed in this study. Soong *et al.* (1997) found CF sputum to have levels of HD1-3 so high they were capable of being toxic to cultured tracheal epithelial cells, suggesting that the excessive amounts of neutrophil defensins present in the CF lung may contribute directly to CF lung disease. In addition, neutrophil defensins have also been shown to be salt sensitive (Shimoda *et al.*, 1995) so in the environment of the CF lung they may lose their antimicrobial activity but retain their toxicity for epithelial cells.

These studies have shown other antimicrobial factors to be present at higher concentrations in the airway which may raise the question of why beta defensins would be considered relevant to CF lung disease. When the pulmonary system first encounters pathogens there are no neutrophils present in the lung and therefore high amounts of neutrophil defensins would not be present to destroy the pathogen. The antimicrobial factors in the ASF which would include HBD-1 and HBD-2 would be the first line of defence against inhaled bacteria and if these were not functioning effectively it is easy to conceive how this could initiate an excessive inflammatory response. This response could then be exacerbated by additional factors, such as has been described previously, that favour the persistent colonisation of a narrow range of pathogens which would eventually lead to lung disease. Alternatively, it has been suggested that lysozyme, an antimicrobial agent which is present at high concentrations in the ASF, acts on the incoming bacteria, leading to bacteriostasis and it is at this point that the antimicrobial activity of defensins kill the bacteria by
the mechanisms described in section 1.15.4 (Ganz, personal communication). This theory is supported by the recent report that the antimicrobial activity of HBD-2 has a synergistic relationship with lysozyme (Bals et al, 1998b). It has also been found that a form of HBD-1 extracted from urine had its antimicrobial activity increased when urine was added to the test cultures (Valore et al, 1998) indicating the existence of other factors in the urine which may act synergistically with HBD-1. This may offer an explanation as to why the inactivation of a peptide at relatively low amounts in the ASF could lead to a compromised host defence system.

A disorder of neutrophil defensin synthesis exists in humans called specific granule deficiency (Ambruso et al, 1984). This rare congenital disorder results in neutrophil defensin concentrations of 10% of normal due to the absence of myelocyte granules in neutrophils (Johnston et al, 1992). Patients with this disorder have a defective bactericidal activity and suffer from frequent infections caused by common bacteria (Ambruso et al, 1984). No disorders of beta defensin synthesis have been reported but there is a condition called Pseudohypoaldosteronism type 1, caused by the dysfunction of the sodium channel ENaC, which leads to elevated levels of sodium and chloride in the sweat similar to that reported in CF (Hanukoglu et al, 1994). The systemic form of this disorder also affects the respiratory system and the airway surface fluid has a raised salt content. However there is also a chronic secretion of clear fluid into the respiratory system which is distinctive from the purulent secretions present in CF. Several studies have examined the type of respiratory pathogens to which pseudohypoaldosteronism patients are susceptible. Kerem et al. (1997) found several instances of *H. influenzae* and *S. aureus* infection with high neutrophil counts and high levels of IL-8 in these patients. Frequent pulmonary infections caused by *P. aeruginosa* have also been reported for a patient with pseudohypoaldosteronism (Marthinsen et al, 1998). These studies indicate that this defect characterised by high salt secretion also leads to a compromised host pulmonary defence system characterised by infections with pathogens associated with CF. However, it is not clear if this is due to the high salt affecting the activity of defensin molecules or if it is another effect of the phenotype of this disorder that has reduced the host’s bactericidal capabilities.
Further studies are required to fully understand the relevance of airway beta defensin molecules in CF lung disease. It is hoped that the study of the Defb1 mutant mouse will help clarify some of these issues.
Chapter 9

Summary and future prospects
Chapter 9 Summary and future prospects.

This study has demonstrated the presence of a cytokine abnormality, in terms of an increased in TNF-α levels in conventionally housed CF mice. Treatment with LPS did not give rise to any excessive inflammatory response in the CF mice compared to their wild-type littermates and there was also no difference found in the inflammatory response of isolated alveolar macrophages. Therefore it was proposed that the inflammatory cells of the CF mouse could function as normal and that the rise in TNF-α in conventionally housed CF mice was the result of the host defence system compensating for the loss of another aspect of the innate pulmonary defence system which was caused directly by a defective CFTR, as was shown in figure 4.10. No statistically significant differences were found in the cytokine levels of the CF mice and their wild-type littermates following a single instillation of S. aureus and P. aeruginosa. However, after treatment with the low dose of P. aeruginosa a significant difference was observed between CF mice and wild-type littermates in the percentages of inflammatory cell types retrieved from the airways by lavage. It was thought that the differences between the neutrophil and macrophage cell numbers may represent a difference in the kinetics of the inflammatory response towards this pathogen in the CF mice and wild-type littermates. However, further studies would be required to confirm this hypothesis.

It was thought that multiple instillations of bacteria may be required to manifest a difference in the profile of inflammatory cytokines of the CF mice and wild-type littermates following treatment with S. aureus and P. aeruginosa.

The airway beta defensin, HBD-1, was thought to be a suitable candidate for this “lost” aspect of host defence since a dysfunctional CFTR could lead to an environment in which these antimicrobial molecules can no longer function efficiently. The activity of these molecules in vivo is hard to analyse and it was thought that the creation of a mouse model with its homologous beta defensin knocked out would overcome many of these problems. The murine homologue of hBD-1, Defb1, was identified and mice heterozygous for the loss of this gene have been created.
A second mouse beta defensin was identified, *Defb2*, which is upregulated in the airways by inflammatory stimuli and work is currently ongoing to assess the functional significance of this gene’s product in the pulmonary defence system. There is much uncertainty about the role of defensins in CF lung disease, most of which has arisen due to the difficulty in obtaining accurate measurements of these peptides concentrations in the lung, their antimicrobial activity and whether the ASF has alterations of its ion content in the CF individual. Accurate measurements of these factors will be essential if the role of defensins in CF is to be fully understood. The development of primary cell culture systems will be helpful for these studies but it is possible that the study of isolated airway epithelium may give misleading results. The development of techniques capable of making accurate measurements of peptide and salt concentrations *in vivo* will be important in the evaluation of the role played by defensins in CF lung disease.

It is also hoped that the *Defb1* knockout mouse will shed some light on the role of defensins in pulmonary defence by studying the consequence of the loss of *Defb1* on the bactericidal capability of the mouse lung. It has already been mentioned that the activity of *Defb1* may be redundant in the lung and therefore it may be necessary to knockout the functions of the other airway beta defensins in the mouse before a phenotype is observed. The technology is now available to make large deletions in the mouse genome and therefore it may be possible to delete the entire region of mouse chromosome 8 that contains the beta defensins. So far, only one other airway beta defensin *Defb2*, described here, has been found, and if there are other beta defensins present, it is likely that they will have evolved through gene duplication and therefore will also be present in the same chromosomal region. More information will be required on the size of the beta defensin family in the mouse and the chromosomal locations of the genes before such a targeting experiment can be conducted. Alternatively, the functions of the other antimicrobial agents in the ASF may be of more relevance to CF lung disease than the defensins or they may act together in a synergistic fashion. It is anticipated that studies on other antimicrobial factors such as lysozyme and lactoferrin will lead to a clearer understanding of how
these molecules function in the lung and what effect the CF environment has on their function.

It has become obvious from the research conducted over recent years that multiple factors contribute to CF lung disease. It is tempting to imagine that the dysfunction of beta defensins by the loss of CFTR function is the initiating factor of disease in the airways but more research is required to prove this is the case. It is anticipated that the study of genetic modifiers in CF mice will reveal additional genes that may be of relevance to the onset of lung disease.

The fact the *P. aeruginosa* does not usually colonise the lung of CF patients until later life suggests it is not an initiating factor in the disease but since the presence of the mucoid variant of this pathogen is recognised as a poor prognostic indicator the continued study of this pathogen is of paramount importance in CF research. It will be essential to find out which aspects of the CF lung environment allows the colonisation of *P. aeruginosa*, and other CF-relevant pathogens such as *B. cepacia*. Preliminary reports proposing that *P. aeruginosa* has immunoevasive capabilities promoted by a dysfunctional CFTR are very interesting but require additional studies preferably in an *in vivo* system.

It is hoped that much information will be gained over the next few years regarding the initiation of CF lung disease through the use of techniques such as cell culture systems, knockout mouse models and genetic modifier studies. It is possible that the use of molecular techniques may identify genes whose expression is directly affected by the loss of CFTR. And it is hoped that new technologies such as cDNA microarray analysis will be used with the aim of identifying genes relevant to CF lung disease.

It is almost ten years since the discovery of the gene responsible for CF and although the precise mechanism of the development of CF lung disease is still not known it is anticipated that the continual development of new technologies and the collaborative efforts of many research groups will lead to a better understanding of this disease and ultimately a better quality of life for CF patients.
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Appendix
List of pathogens for which the SPF unit is screened regularly.

**Viral**

- Minute virus of mice
- Mouse hepatitis virus
- Orphan parvovirus
- Pneumonia virus of mice
- Reovirus 3
- Sendai virus
- Theiler’s encephalomyelitis virus

**Bacteriology**

- *Bordetella bronchiseptica*
- *Citrobacter freundii*
- *Corynebacterium kutscheri*
- *Mycolplasma spp*
- *Pasteurella spp*
- *Pseudomonas aeruginosa*
- *Salmonellae*
- *Staphylococcus aureus*
- *Streptobacillus moniliformis*
- *β-haemolytic streptococci*
- *Streptococcus pneumoniae*
- Tyzzer’s disease

**Mycology**

- Dermatophores

**Parasitology**

- Intestinal protozoa
- Encephalitozoon cuniculi
- Helminths
- Arthropods